Animal sentinel surveillance: Evaluating domestic dogs as sentinels for zoonotic pathogen surveillance

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PhD

2010

THE UNIVERSITY OF EDINBURGH

Declaration of Authorship

I declare that this thesis is my own composition and that the research described within this thesis is my own work. The work presented in this thesis has not been submitted for any other degree or professional qualification.

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Abstract

The capacity of zoonotic pathogens to infect multiple hosts creates surveillance challenges but also provides opportunities to gather data from animal species that can be used to understand risks to human health. This thesis presents a conceptual and practical assessment of the utility of domestic dog serosurveillance for the detection and surveillance of two pathogens, influenza A and *Leptospira* spp. The first chapter gives a theoretical framework that can be used to explore the attributes of animal sentinels and assess their utility in different contexts. In subsequent chapters, this framework is applied in a practical assessment of the utility of a domestic dog serosurveillance approach for the detection and surveillance influenza A and *Leptospira* spp. at two sites in Africa.

Two cross-sectional surveys of the avian and mammal populations at a site in Northern Cameroon were conducted in early 2006 to determine if H5N1 influenza A viruses had circulated in this area and in which species that presence could be detected. Serological and molecular evidence of extensive H5 virus circulation in the domestic duck population was identified. 47% of domestic ducks at the Maga site were cELISA positive for anti-influenza A antibodies and 20% were HI test positive against an H5N1 antigen. There was also evidence of exposure to H5 subtype viruses in the local dog and pig populations.

At the Kibera site in Nairobi, a cohort study was established to carry out surveillance of influenza A and *Leptospira* spp. in the domestic dog population and cross-sectional surveys of the domestic poultry and rodent populations were completed. There was no indication of influenza A circulation in any of the animal species surveyed, indicating low risk of zoonotic influenza A infection in the human population of Kibera. In contrast, there was extensive molecular and serological evidence of the presence of *Leptospira* spp. in both the rodent and dog populations. 18% of 236 trapped rodents were PCR positive for kidney carriage of pathogenic leptospires and the estimated seroprevalence of anti-*Leptospira* antibodies in the dog population ranged from 5-36% during the course of the study, indicating high potential risk of leptospirosis infection in the human population.

The results indicate that dog serosurveillance can be used as useful tool for the determination of broad-scale patterns of pathogen presence and relative levels of population exposure. However, there are limitations of the data that can be gathered from animal sentinels and the complexities introduced particularly by incomplete understanding of diagnostic test performance must be recognized. Animal sentinel surveillance may be of most use for addressing fundamental questions of what pathogens are present where. In the developing world particularly where disease burden data are still lacking, dog sentinel serosurveillance can provide essential baseline data that can be used to target future research and resource allocation.

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Abbreviations

AFT	**A**ccelerated **F**ailure **T**ime
AGID	**A**gar **G**el Immuno**d**iffusion
AIC	**A**kaike **I**nformation **C**riterion
BSA	**B**ovine **S**erum **A**lbumin
CAAT	**C**ross-**A**gglutination **A**bsorption **T**est
CDC	**C**enters for **D**isease **C**ontrol and Prevention
CCC	**C**oncordance **C**orrelation **C**oefficient
cELISA	**c**ompetitive **E**nzyme **L**inked **I**mmuno**s**orbent **A**ssay
DNA	**D**eoxyribo**n**ucleic **A**cid
DKHH	**D**og **K**eeping **H**ouse**h**olds
DOHH	**D**og **O**wning **H**ouse**h**olds
DSHH	**D**og **S**upervising **H**ouse**h**olds
EDTA	**E**thylene**d**iamine**t**etraacetic **A**cid
ELISA	**E**nzyme **L**inked **I**mmuno**S**orbent **A**ssay
EMA	**E**uropean - **M**iddle Eastern - **A**frican
EMJH	**E**llinghausen & **M**cCullough Medium (**J**ohnson and **H**arris modification)
GABA	**g**amma-**A**mino**b**utyric **a**cid
GLM	**G**eneralised **L**inear **M**odel
HI test	**H**aemagglutination **I**nhibition test
HPA	**H**ealth **P**rotection **A**gency
HPAI	**H**igh **P**athogenicity **A**vian **I**nfluenza
IEIP	**I**nternational **E**merging **I**nfections **P**rogramme

IHA	**I**ndirect **H**aemagglutination **A**ssay
ILI	**I**nfluenza **L**ike **I**llness
KEMRI	**K**enya **M**edical **R**esearch **I**nstitute
LBM	**L**ive **B**ird **M**arket
LPAI	**L**ow **P**athogenicity **A**vian **I**nfluenza
LRT	**L**ikelihood **R**atio **T**est
LRU	**L**eptospirosis **R**eference **U**nit
MCMC	**M**arkov **C**hain **M**onte **C**arlo
MLST	**M**ulti-**L**ocus **S**equence **T**yping
MOSS	**Mo**nitoring and **S**urveillance **S**ystem
NMK	**N**ational **M**useums of **K**enya
OD	**O**ptical **D**ensity
ODA	**A**verage **O**ptical **D**ensity
ODN	**N**ormalised **O**ptical **D**ensity
OIE	**O**ffice **I**nternational des **E**pizooties
	World Organisation for Animal Health
OR	**O**dds **R**atio
PBS	**P**hosphate **B**uffered **S**aline
PCR	**P**olymerase **C**hain **R**eaction
PFGE	**P**ulse **F**ield **G**el **E**lectrophoresis
RBC	**R**ed **B**lood **C**ell
RDE	**R**eceptor **D**estroying **E**nzyme
RHDV	**R**abbit **H**aemorrhagic **D**isease **V**irus
RNA	**R**ibonucleic **A**cid
RT-PCR	**R**everse **T**ranscriptase - **P**olymerase **C**hain **R**eaction
rRT-PCR	**r**eal-time **R**everse **T**ranscriptase - **P**olymerase **C**hain **R**eaction
ROC	**R**eceiver **O**perating **C**haracteristic analysis
SA	**S**ialic **A**cid
SAT	**S**lide **A**gglutination **T**est
SSS	**S**yndromic **S**urveillance **S**tudy

SVA   **S**tatens **V**eterinärmedicinska **A**nstalt

                National Veterinary Institute of Sweden

TAE   **T**ris **A**cetate **E**DTA

TBE   **T**ris **B**orate **E**DTA

TR   **T**ime **R**atio

UV   **U**ltra**v**iolet

VLA   **V**eterinary **L**aboratories **A**gency

WHO   **W**orld **H**ealth **O**rganization

WNV   **W**est **N**ile **V**irus

Chapter 1

Evaluating Animals as Sentinels for Infectious Disease Surveillance

A copy of the original article is included in Appendix C

1.1 Abstract

Infectious disease host ranges, host responses to pathogens and the relationships between hosts are heterogeneous. This heterogeneity poses challenges but also opportunities for effective pathogen surveillance. Animal sentinels can be used to address many surveillance questions but they may currently be underused as a surveillance tool and there is a need for improved interdisciplinary collaboration and communication in order to fully explore the potential of animal sentinels. In different contexts, different animal hosts will themselves vary in their capacity to provide useful information. This chapter describes a conceptual framework within which the characteristics of different host populations and their potential value as sentinels can be evaluated in a broad

range of settings. The particular attributes of carnivores and domestic dogs specifically that may enhance their utility as sentinels for global zoonotic disease surveillance are described.

1.2 Introduction

The dynamics of infectious disease systems are inherently variable. The outcome of any infection depends on multiple factors relating to pathogen characteristics, host suscep- tibility, infecting dose, and routes of transmission, all of which can vary widely for any particular infectious organism. Many of the major diseases of medical, veterinary and conservation importance are caused by pathogens with wide host ranges (Woolhouse and Gowtage-Sequeria, 2005), which introduces further complexity. While the complex epidemiology of multi-host pathogens presents considerable challenges for understand- ing infection dynamics and implementing disease control, heterogeneities in host range and infection outcome also provide opportunities for disease surveillance. This chapter discusses the surveillance of zoonotic pathogens and presents a conceptual framework that can be applied to examine those characteristics of host populations that influence their potential value as sentinels for disease surveillance in different ecological and epi- demiological settings. In the following chapters of this thesis, a practical assessment of the use of domestic dogs as sentinels for the surveillance of influenza A and leptospirosis at two field sites in Africa is presented and discussed.

1.2.1 Emerging and Re-emerging Pathogens

Emerging or re-emerging diseases are those that have recently appeared for the first time, are increasing in incidence, or are spreading into new areas (Cleaveland et al., 2001). As compared to non-emerging pathogens, comparative surveys reveal that emerging pathogens are more likely to be viruses (Cleaveland et al., 2001; Taylor et al., 2001; Woolhouse and Gowtage-Sequeria, 2005), or bacterial/rickettsial pathogens (Jones et al., 2008), depending on the classification of drug-resistant microbes. Emerg- ing diseases are more likely to be zoonotic (Taylor et al., 2001; Woolhouse and Gowtage- Sequeria, 2005; Jones et al., 2008), and are also more likely to have broad host ranges

(Cleaveland et al., 2001). An understanding of these characteristics, as well as recognition of the huge diversity that exists within emerging and re-emerging diseases (Woolhouse and Gowtage-Sequeria, 2005) is essential for the design and effective implication of surveillance and control strategies for emerging pathogens.

Zoonotic pathogens are defined as those diseases and infections which are naturally transmitted between vertebrate animals and humans (WHO, 1959). The majority of all human pathogens are zoonotic (Taylor et al., 2001), and zoonotic pathogens show a higher probability of emerging than non-zoonotic pathogens (Taylor et al., 2001; Woolhouse and Gowtage-Sequeria, 2005). Zoonoses with wildlife origins specifically have been identified as the most significant emerging disease threat to global health (Jones et al., 2008).

The likelihood of pathogen emergence or re-emergence is significantly associated with the host range of the pathogen, such that pathogens that have broader host ranges, and specifically those that can infect hosts of more than one taxonomic order, are more likely to be defined as emerging or re-emerging (Cleaveland et al., 2001; Woolhouse and Gowtage-Sequeria, 2005). This capacity to infect multiple hosts is associated with zoonotic potential and also has implications for surveillance. Multihost pathogens have complex ecology that involves interactions between multiple species and numerous transmission opportunities. Effective surveillance of these pathogens requires the development of new strategies that recognize this complexity and should utilize the range of potential sentinel species that is an obvious consequence of this generalist capacity (Cleaveland et al., 2006).

1.2.2 Drivers of Pathogen Emergence

The factors associated with the emergence and re-emergence of pathogens can generally be categorized into: 1) genetic and biological factors, 2) environmental factors, 3) ecological factors and 4) demographic factors. The majority of these are directly attributable to human activity (Morse, 1995; King et al., 2004). Together, these factors combine to increase contact between humans and pathogens or their animal hosts, helping to create novel niches that can be exploited by pathogens and increasing the

likelihood of interspecies transmission (Morse, 1995; King et al., 2004). Human population density has been identified as a consistent predictor of disease emergence events (Jones et al., 2008). However, this observation may reflect patterns in the spatial distribution of the detections of disease emergence, the locations of which may differ from those of the emergence events themselves.

1.2.3 Pathogen Surveillance

Surveillance is defined by the World Health Organization (WHO) as "the ongoing systematic collection, collation, analysis and interpretation of data and the dissemination of information to those who need to know in order for action to be taken"(WHO, 2001). A critical element of surveillance is that an identified response is made on the basis of the surveillance data generated, to allow appropriate action to be taken. Monitoring and surveillance systems (MOSS) (Doherr and Audigé, 2001) can be designed to address a number of specific questions, including detecting the presence of pathogens, identifying changes in the prevalence of a pathogen over time, determining the rates and direction of pathogen spread, testing specific hypotheses about the ecology of a pathogen and evaluating the efficacy of potential disease control interventions (McCluskey, 2003).

1.2.4 Surveillance of Emerging Zoonoses

The increasing recognition of the threat posed by emerging and re-emerging zoonoses and the awareness that this threat will persist and probably increase in the future, has prompted a reappraisal of the existing networks and systems of infectious disease surveillance. The importance of interactions between human, wildlife and domestic animal populations, the potential for the rapid global spread of emergent pathogens and appreciation of the need to carry out surveillance for as yet unknown pathogens demonstrates the necessity for novel, interdisciplinary surveillance strategies that are both more comprehensive and more flexible than any that have existed previously.

Integration between human and animal surveillance is repeatedly identified as key to the successful surveillance of emerging infectious diseases (Morse, 1995; Murphy, 1998;

Cleaveland et al., 2001; Woolhouse, 2002; Kuiken et al., 2005; Woolhouse and Gowtage-Sequeria, 2005; Kahn, 2006). In order to effectively monitor pathogens and characterize their potential to cross into and spread throughout human populations, surveillance must include both human populations and the animal populations from which such pathogens may emerge (Woolhouse and Gowtage-Sequeria, 2005). The collection of accurate and comprehensive field data is the essential foundation of the required integrated approach to emerging zoonosis research, surveillance, assessment and control (Chomel, 2003; King et al., 2004), which must prioritize international and interdisciplinary exchange of existing data and techniques as well as the utilization of novel sources of information, in order to rapidly detect and respond to emerging disease threats (Chomel, 2003; King et al., 2004; Hoinville et al., 2009).

The effective integration of the medical and veterinary disciplines may be particularly important in the developing world, where a basic lack of infrastructure often means that human disease surveillance networks are poorly developed and that resources are scarce (Shears, 2000b). In many regions, veterinary personnel may more numerous, and animal health surveillance better established, than in the equivalent human health profession (Shears, 2000a).

The developing world has been identified as at particular disadvantage in terms of existing capacity to cope with the threat posed by emerging diseases (Shears, 2000a; Breiman et al., 2007). High profile pathogen outbreaks in Africa such as viral haemorrhagic fevers and the spread of H5N1 influenza A in 2006-2007 have revealed the current shortcomings in surveillance capacity and prompted the proposal of strategies to develop core capacities vital to the surveillance of all potential disease threats (Shears, 2000a; Breiman et al., 2007). These practical proposals fit well with the argument that surveillance efforts should be concentrated on areas of the world identified as more likely locations of pathogen emergence, which include tropical Africa, Latin America and Asia (Jones et al., 2008).

1.2.5   Surveillance Approaches

The above definition of surveillance encompasses a broad range of activities and purposes. Different approaches to surveillance are appropriate in different circumstances, which may be defined by the purpose of the surveillance activity as well as the context in which it is conducted and the resources available. These approaches and the associated terminology have recently been described and compiled in an effort to clarify and unify the language used and work towards a standardized system for the description of surveillance activities (Hoinville et al., 2009).

The term general surveillance is used to describe surveillance that is not focused on a particular disease or health issue and can detect any disease or pathogen (Hoinville et al., 2009). Such an approach can be used flexibly to detect any disease or pathogen through the collection of non-specific diagnostic data such as clinical information and indirect indicators (Hoinville et al., 2009). At the other end of the spectrum in terms of precision, targeted surveillance is designed to address specific questions about the occurrence or epidemiological features of a defined disease or condition (Hoinville et al., 2009).

Hoinville et al. (2009) note that the very resource limitations that are frequently identified as constraints to effective disease surveillance in the developing world can also prompt the development of innovative 'outside the box' surveillance approaches such as risk-based and sentinel approaches. The core concept of risk-based surveillance is that issues that present higher risks merit higher priority for surveillance resources and its goal is to achieve a higher benefit-cost ratio than conventional approaches with existing or even reduced resources (Stärk et al., 2006). Hoinville et al. (2009) define risk-based surveillance as a surveillance activity in which efficiency is increased by including sample units that are more likely to: 1) be infected with the disease of interest, 2) be detected as infected with the disease of interest, 3) become infected with the disease of interest or 4) transmit the disease of interest to other units in the population (Hoinville et al., 2009). This definition overlaps with the definition of sentinel surveillance provided by these authors and also with the definition used in this thesis (See Section 1.2.6). Hoinville et al. (2009) define sentinel surveillance as the regular collection of information from selected sites about the occurrence of infection, disease

or the health of a specified population. When the sentinel unit is selected on the basis of likely increased risk then these two approaches to surveillance overlap.

1.2.6 Animal Sentinels

Sentinel surveillance is a form of surveillance in which activities focus on specific sub-populations to enhance detection of disease and/or improve the cost-effectiveness of surveillance (McCluskey, 2003). The aim of sentinel surveillance is to obtain timely information in a relatively inexpensive manner rather than to derive precise estimates of prevalence or incidence in the general population (CDC, 2008). It has long been recognized that animal populations have the potential to act as sentinels for environmental health hazards (CAMEH, 1991), but, given the importance of domestic and wild animal hosts in emerging human diseases, it is clear that surveillance in animals is also critical for understanding and managing emerging disease threats (Kuiken et al., 2005; Woolhouse and Gowtage-Sequeria, 2005; Kahn, 2006). Animal sentinels almost certainly represent an important but under-used surveillance tool (Rabinowitz et al., 2005) that may be capable of accommodating and capitalizing upon the variability that exists in infectious disease processes.

The term sentinel is widely used in both epidemiological and veterinary clinical literature, and is implicitly understood but rarely defined. While all uses invoke the common concept of standing guard or keeping watch, existing definitions tend to be context-specific. The classic example of an animal sentinel is that of the coal-miner's canary. In this case an individual animal of a different species is deliberately selected and placed in a situation where it can provide evidence of increased risk to the human population on the basis of its greater sensitivity and obvious observable response to the presence of carbon monoxide. Since the mid-twentieth century, it has been recognized that animals can act as important sentinels for a wide range of environmental health hazards (CAMEH, 1991). The term proxy has also been used to describe such uses of animal sentinels, when a proxy species or population is identified and selected - usually on the basis of higher susceptibility to the hazard - and observed in place of the target population (Hoinville et al., 2009). The sentinel or proxy population may not play an important role in the epidemiology of the pathogen of interest, instead the rationale for

Table 1.1: Summary of applications of animal sentinels for environmental and infectious hazards

Type of sentinel	Example	Reference
Individual animal	Coal-miner's canary used to detect the presence of carbon monoxide	(Burrell and Seibert, 1916) (Schwabe, 1984)
Herd/population	Sentinel cattle herds and chicken flocks used to monitor the distribution of arboviruses and their vectors in Australia and the USA	(NAMP, 2008) (Loftin et al., 2006)
Same species	Unvaccinated chickens placed within vaccinated flock to detect HPAI	(Suarez, 2005)
Different, more susceptible species	Feral pigs released into New Zealand to detect the presence of bovine TB	(Nugent et al., 2002)
	Coal-miner's canary (as above)	(Burrell and Seibert, 1916)

Sentinel application	Example	Reference
Deliberately placed (experimental)	Standard laboratory mice sentinel programmes using outbred mice, sacrificed and tested to detect presence of a panel of rodent pathogens in the core experimental or breeding colony	(ILAR, 1991)
	Use of sentinel chickens to evaluate the effectiveness of cleaning and disinfection procedures for eradication of Newcastle disease	(McCluskey et al., 2006)
In natural habitat (observational)	Evaluation of white-tailed deer as natural sentinels for *Anaplasma phagocytophilum*, the cause of human granulocytic anaplasmosis	(Dugan et al., 2006)
	Mesothelioma in pet dogs associated with exposure of their owners to asbestos	(Glickman et al., 1983)
	Amphibian population declines as indicators of environmental stresses including habitat destruction, pollution, increased UV-B radiation or climate change	(Halliday, 2000)
Sentinel unit	Equine premises used to investigate presence of vesicular stomatitis in Colorado	(McCluskey et al., 2002)

its selection as the unit of surveillance is based solely on its capacity to provide data on the presence of a pathogen.

Sentinels can vary from individual animals to herds or larger populations, from animals of the same species to different, more susceptible, more expendable or more accessible species, and from animals deliberately placed or introduced to those already existing in a particular location. The sentinel concept can also refer to a physical location, such as a farm, abattoir, veterinary practice or laboratory (the "sentinel unit") which is selected to monitor a particular hazard (Table 1.1).

For many people, the term animal sentinel relates particularly to situations in which a signal observed in an animal population can herald or give advance warning of a risk to human health (Yale Occupational and Environmental Medicine Program, 2010). The general principal of using data gathered from an animal population to inform understanding of risks to other species (most commonly humans) can however be extended to cover a broader range of scenarios. In the context of pathogen surveillance, animal sentinels may be used to address a range of questions, including:

- detecting a pathogen in a new area;
- detecting changes in the prevalence or incidence of a pathogen or disease over time;

- determining the rates and direction of pathogen spread;
- testing specific hypotheses about the ecology of a pathogen and
- evaluating the efficacy of potential disease control interventions (McCluskey, 2003).

Throughout this thesis, 'animal sentinels' is used as an umbrella term for the general use of data from animal populations to inform understanding of health risks in other populations. The term 'sentinel population' is used to refer to the unit of observation in a particular case.

Animal sentinels appear under-utilized, particularly in the context of infectious disease surveillance (Rabinowitz et al., 2005, 2008), and their value has been discussed primarily in the context of environmental health (CAMEH, 1991). A basic lack of integration between disciplines, most noticeably between human and veterinary medicine but also between different branches within these fields is likely to have contributed to this under-use of animal sentinels and the persistence of critical knowledge gaps (Rabinowitz et al., 2005, 2008). There are currently no standard criteria which are applied for the evaluation of animal sentinels (although (Hoinville et al., 2009) have recently proposed mechanisms to resolve this for surveillance activities more generally) limiting the ease with which data can be transferred between disciplines (Rabinowitz et al., 2005). The infectious disease literature regarding animal sentinels consists largely of descriptive studies that have generated hypotheses regarding animal sentinel use (Rabinowitz et al., 2005; Scotch et al., 2009; Yale Occupational and Environmental Medicine Program, 2010), but as yet includes few studies that were purposefully designed to evaluate their potential. A lack of quantitative methods for linking animal and human health surveillance data and a tendency amongst researchers to underestimate the degrees of association between human and animal health, may both play a role in this shortfall (Rabinowitz et al., 2008; Scotch et al., 2009). One major exception is the extensive research that has been carried out into the use of animal sentinels in the surveillance of West Nile virus (WNV) in North America, which is discussed in Section 1.5.1.

1.2.7   Chapter Objectives

The principal aim in this chapter, is to develop and discuss a conceptual framework that can be used to evaluate animal sentinels of infectious diseases. The attributes of carnivores, and domestic dogs specifically, that may enhance their utility as animal sentinels for a range of epidemiological questions are explored.

1.3   Identifying and Assessing Animal Sentinels

For any population to be useful for disease surveillance it must be under observation and must be capable of developing a detectable response to a particular pathogen. Sentinel populations are distinguished from other populations by having attributes that enhance detection of the disease or of the etiological agent and/or improve the cost-effectiveness of surveillance (McCluskey, 2003). In most cases, this means that the sentinel population is more likely to be exposed to, or to respond to the pathogen than other populations. This sentinel concept encompasses the variety of uses described above and can refer to any level of grouping from an individual to a larger unit, such as a herd or even a species.

Various authors have compiled lists of attributes of an 'ideal' sentinel (CAMEH, 1991; Komar, 2001) but these have invariably been created with a particular sentinel application in mind and there exists little or no consensus about the common characteristics or defining features of 'the sentinel'. This ambiguity of course reflects the fact that there is no innate quality of sentinel suitability that particular species or populations have. Instead, the criteria against which the usefulness of a given sentinel population is assessed are influenced by the aim of surveillance and the context in which the sentinel would be used.

1.4   The Sentinel Framework

Within any pathogen surveillance context the sentinel population must always interact with both the pathogen and the target population and it is essential to consider and
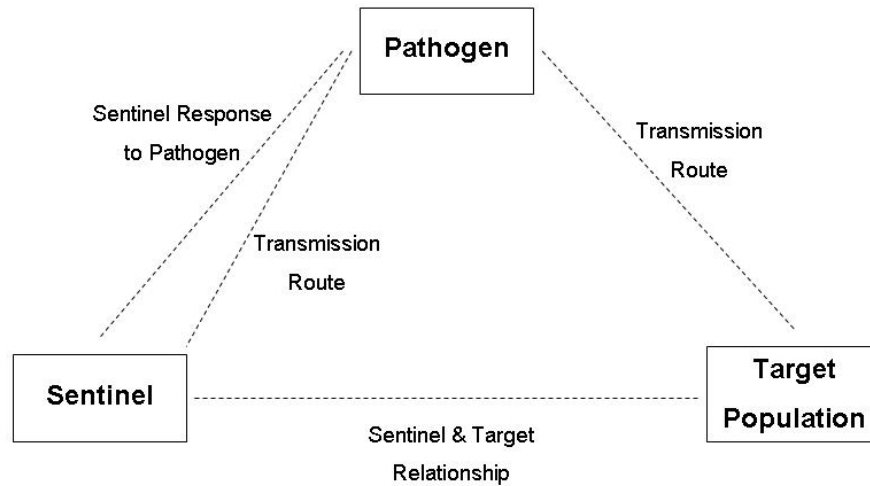
Figure 1.1: Key components and attributes of the sentinel framework

describe the interactions between these fundamental components (Figure 1.1). The three components of the sentinel framework are:

Pathogen The pathogen that is under surveillance;

Target population The population of concern, to which information gathered from the sentinel is applied;

Sentinel population

This framework is not intended to represent the transmission dynamics of a pathogen, but rather the ways in which the components of the sentinel framework are associated. Three critical attributes of this system must be considered in order to assess the utility of a potential sentinel for a particular surveillance aim and in any given ecological context: 1) the sentinel response to the pathogen, 2) the relationship between sentinel and target populations and 3) routes of transmission to both target and sentinel populations. This framework is discussed with reference to the surveillance of WNV in North America (Section 1.5.1).

1.4.1 Sentinel Response to Pathogen

The sentinel response to a pathogen can range from the production of antibody in an otherwise healthy individual, through morbidity and ultimately to mortality. It may also be possible to detect the presence of the pathogen in a sentinel population before other responses develop and sentinel responses can therefore include:

Current infection or presence of pathogen

Seroconversion

Morbidity

Mortality

There is a clear intuitive distinction between sentinel populations that develop high levels of morbidity or mortality in response to pathogen exposure and those that remain healthy. Sick or dying sentinels show an obvious and dramatic response to a pathogen and provide a readily appreciable signal of the presence of a pathogen within an ecosystem (See the discussion of crow mortality as a sentinel of WNV presence in Section 1.5.1). At the other end of the spectrum, apparently healthy sentinels that develop a subclinical response are often more useful for investigating the maintenance patterns and transmission dynamics of a pathogen within the sentinel and target populations. Following the consumption of prey infected with rabbit haemorrhagic disease virus (RHDV), foxes in northern Germany developed antibody responses that declined after just two weeks. Serosurveillance of this fox population therefore reveals the proportion of the population that has been exposed in the 1-2 weeks prior to testing. These serological data can therefore provide a good indication of the incidence patterns of RHDV in the corresponding rabbit population (Frölich et al., 1998). In cases in which healthy sentinels are used, it may be desirable to re-sample the same individuals or populations over time. It is also important that the observation and sampling of the sentinel population, and perhaps also the sentinel response itself, has minimal impact upon the study system.

This example also demonstrates the influence of the temporal characteristics of the sentinel response to a pathogen upon the choice and application of sentinel populations.

Sentinel populations which respond to a pathogen prior to the exposure of the target population may be useful for those surveillance programmes that aim to prevent the exposure of the target population. For other sentinel uses, the rapid development of a response may not be required. The duration of the potential sentinel's response can also influence the types of question it can usefully be used to address. An equivalent sentinel population (to that of the foxes) that developed a longer lasting antibody response in the above RHDV example would be of limited use for investigating the incidence of disease in the rabbit population on this immediate timescale.

The sentinel response can be viewed as a test for the presence of the pathogen within the target population and as such has properties that are analogous to diagnostic test sensitivity and specificity:

Sentinel Sensitivity The sensitivity of the sentinel refers to its capacity to respond to the presence of the pathogen in the target population and effectively translates as susceptibility to infection. An insensitive sentinel population would be unlikely to display evidence of infection with the pathogen even if it were present in the target population and would therefore be poorly suited for use as a sentinel.

Sentinel Specificity The specificity of the sentinel response relates to the ease with which a sentinel response can be interpreted and attributed to a particular pathogen. Specificity is thus closely linked to the response type and the methods used to detect the response. Morbidity and mortality are generally less specific indicators of the presence of a particular pathogen than molecular responses that are observed using a diagnostic test or assay unique to the pathogen in question.

Whatever type of response a particular sentinel population mounts to a pathogen, it is important that the individual members of that population are consistent in the development of the response. Excessive variation within a sentinel population would greatly complicate the interpretation of surveillance findings and it may therefore be important to ensure that members of the sentinel population are of similar age, sex, or other relevant characteristics, depending upon the type of response measured.

1.4.2 Relationship between Sentinel and Target Populations

The relationship that exists between the sentinel and target populations may include behavioural, epidemiological or spatial aspects or any other form of ecological association. Detailed understanding of the associations between the sentinel and target populations is not required to address all questions. However, a comprehensive understanding of the relationship between a sentinel and a target population will allow for the investigation of more complex epidemiological questions and better informed interpretation of the data collected through surveillance of that sentinel. The minimum association that must exist between a sentinel and a target population is a spatial association. This need not imply spatial overlap however. If the pathogen is spreading on a wave-front, or emanating from a focal source, then a sentinel population may be selected on the basis of its closer proximity to the focus than the target population (NAMP, 2008).

At the other extreme, the sentinel population may consist of a specific subset of the target population, ensuring a very close relationship between the two populations. A sub-population that experiences high transmission risk, or is particularly sensitive to infection with a particular pathogen, may serve as a sentinel for the wider population and can clearly provide a more accurate assessment of risk to the target than a population occupying a dissimilar ecological niche and consequently experiencing a very different pattern of exposure to the pathogen (e.g. unvaccinated sentinel birds are used to detect the presence of highly pathogenic avian influenza (HPAI) viruses within the otherwise vaccinated flock (Suarez, 2005)). The sentinel and target population may also be epidemiologically linked such that the sentinel may act as a source of infection for the target population, as is often the case with arthropod vector surveillance.

1.4.3 Transmission Route

This attribute is essentially a component of the relationship between the sentinel and target populations that explicitly considers the route or routes through which the two populations can become infected with the pathogen. In circumstances where the target and sentinel are exposed to infection via the same route, the relative intensity and patterns of exposure of the two populations to the source of infection are important

(Estrada-Franco et al., 2006). It may be desirable to select a sentinel that has higher levels of exposure and which is therefore more likely to show evidence of a pathogen if it is present than to directly survey the target population itself. For pathogens that are transmitted by a vector or vectors, the feeding preferences of the vector(s) can therefore be important in informing sentinel selection. Domestic dogs are the preferred source of blood meals for *Triatoma infestans*, one of the main vectors of *Trypanosoma cruzi* in Mexico. A comparative serosurvey revealed overall anti-*T. cruzi* IgG prevalence of 16% in dogs, compared with a 2% prevalence in humans, and a strong positive correlation between human and dog seropositivity within the study area. These data suggest that the feeding preferences of this vector make the domestic dog population a good sentinel for identifying areas of human seropositivity and monitoring prevalence in this context (Estrada-Franco et al., 2006). There are also circumstances in which the route of exposure of the sentinel and target population may differ (See the discussion of exposure through the consumption of infected material in Section 1.7). An understanding of the predator-prey relationships between the target population and potential sentinels may prove useful in sentinel selection. The principal transmission route of bluetongue virus, which infects wild and domestic ruminants across East Africa, is via *Culicoides* midge vectors. Serosurveillance of free-ranging African carnivores revealed that both the seroprevalence and the virus serotype identified varied dramatically across carnivore species (Alexander et al., 1994). This study suggested that the most likely route of infection of carnivores with bluetongue was via consumption of infected prey, and that the variation seen between species was attributable to dietary differences. Different carnivore species may therefore vary in their utility as sentinels for the presence of bluetongue virus in different ruminant species.

1.5 Placing the Sentinel Framework in Context

The sentinel response can be viewed as the output of the sentinel framework. The nature of this response, in combination with other sentinel host factors and practical influences which depend upon the context in which surveillance is conducted, determine the overall detectability of the sentinel response (Figure 1.2). Unlike the attributes which operate within the sentinel framework, detectability is a quality of the interaction between the
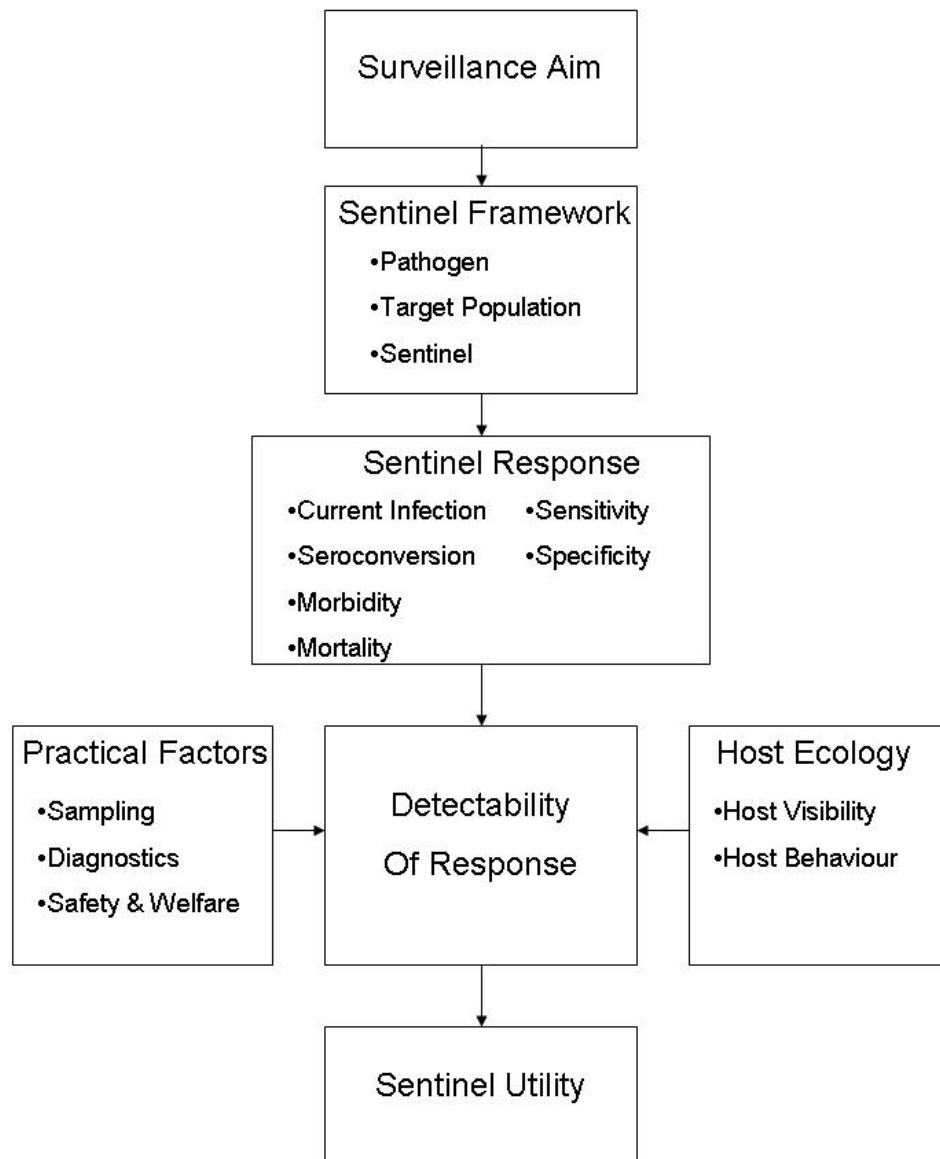
Figure 1.2: The sentinel framework in context

sentinel and the observer. The overall utility of any potential sentinel can only be assessed by considering both the sentinel framework and the influences of the context in which it would be applied (Figure 1.2 and Figure 1.3).

The visibility of any animal population is determined by the morphology, behaviour, distribution and abundance of the individual animals of which it is comprised. The detectability of the sentinel response includes both the visibility of the animal and of its response to a pathogen. The type of response that an animal mounts will directly affect the ease with which it is detected by the observer. Widespread morbidity or mortality within a sentinel population are often more readily appreciable than seroconversion or current infection/presence of pathogen, which can only be detected by the observer after first sampling the sentinel population and then conducting laboratory analyses. In the case of overt sentinel responses such as mortality, the existence of a reliable network of 'observers' and of a mechanism through which data are reported are crucial. It is equally important to consider the available capacity to detect any less overt responses including the existence of a reliable sampling protocol and a diagnostic test (McCluskey, 2003). The majority of diagnostic tests for human and livestock pathogens have not been validated for use in non-target species and the sensitivity and specificity of tests can vary hugely between species (Greiner and Gardner, 2000). The existence of a suitable negative control population and recognition of the time required to identify and validate diagnostic tests must be considered in any proposed sentinel surveillance programme.

The practical difficulties involved in sampling any potential sentinel population must also be evaluated and it may sometimes be difficult to reconcile the use of a theoretically ideal sentinel with such practicalities. For a sentinel population to be useful it must be both logistically feasible and safe to sample sufficient numbers of the population (CAMEH, 1991). Because sentinels are often selected on the basis of increased likelihood of exposure to a pathogen, sentinel surveillance can enable targeting of resources and often has improved cost-effectiveness as compared, for example to more comprehensive surveys (McCluskey, 2003). In addition to consideration of time and cost, the potential risks to research personnel and the public that are associated with the desired sampling strategy must be evaluated, as well as the effects of sampling upon

the sentinel population itself in the context of animal welfare and conservation status (CAMEH, 1991).

1.5.1 West Nile Virus Surveillance in North America: Animal Sentinel Case Study

WNV is an arbovirus of the genus *Flavivirus*, that is maintained in a mosquito–bird–mosquito cycle primarily involving *Culex* spp. mosquitoes (Campbell et al., 2002). Humans and other mammal species are incidental dead-end hosts. The majority of human infections with WNV are asymptomatic or result in transient febrile illness but in a small proportion of cases, meningoencephalitis can occur (Mostashari et al., 2001). The geographical range of WNV has historically included Africa, Europe, Asia and Australia (Campbell et al., 2002). In 1999, the first North American cases of WNV were reported in New York and since then the virus has spread across the continental United States and into Canada, Latin America and the Caribbean (Hayes and Gubler, 2006). The surveillance of WNV in North America has included investigation of the utility of different animal sentinels. Some of the findings of these studies are described below with reference to the sentinel framework.

Sentinel response to pathogen A number of North American bird species including corvids, house sparrows, house finches and grackles are competent reservoirs for mosquito infection with WNV (Komar et al., 2003). Among these potential sentinel species, corvids and specifically American crows, *Corvus brachyrhynchos*, are particularly susceptible to infection with WNV and have a high mortality rate (McLean et al., 2001; Komar et al., 2003; Yaremych et al., 2004). In 2000, it was established that dead crow reports preceded both the confirmation of viral activity (through laboratory analysis) and the onset of human cases by several months (Eidson et al., 2001b). Subsequent spatial analyses using data collected in New York have identified a positive association between the risk of human disease caused by WNV and elevated local dead crow reports in the previous one to two weeks (Mostashari et al., 2003; Eidson et al., 2005; Johnson et al., 2006).

The thorough characterization of this temporal association ensures that the observation of crow deaths can be acted upon immediately without the need for time-consuming laboratory analyses. The observation of clusters with high crow mortality can therefore be used to predict human risk early enough to implement targeted mosquito control and personal protection warnings (Mostashari et al., 2003; Eidson et al., 2005; Johnson et al., 2006).

Relationship between sentinel and target populations Domestic dogs have also been evaluated as sentinels of WNV presence (Komar, 2001; Kile et al., 2005). This sentinel choice is informed by the particular relationship that domestic dogs have with humans, which means that they are well suited to act as indicators of the infectious disease risks that their owners are likely to encounter. North American domestic dogs consistently show higher seroprevalence of anti-WNV antibodies than humans (Komar, 2001; Kile et al., 2005) and one analysis revealed that outdoor dogs were nearly 19 times more likely to have seroconverted to WNV than indoor-only pet dogs (Kile et al., 2005). The pattern of human exposure to the arthropod vectors of WNV is likely to be more similar to that of indoor only dogs, but within the context of broad spatial association with humans; this divergence from the human niche means that outdoor-only dogs are more sensitive sentinels of WNV presence and human risk than indoor-only dogs (Kile et al., 2005).

Transmission route The role played by different mosquito species (predominantly of the genus *Culex*) in the transmission of WNV between birds and to humans is quite variable (Kilpatrick et al., 2005; Molaei et al., 2006). In one study conducted in Maryland and Washington DC, over 90% of all Culex mosquitoes identified were of the species *Culex pipiens* (Kilpatrick et al., 2006). At this site, the rise in human WNV cases that occurs in late summer and early autumn is apparently caused by a shift in the feeding preferences of this vector species from birds to humans (Kilpatrick et al., 2006) that is associated with the dispersal of a preferred host, the American robin *Turdus migratorius*. This temporal variation in vector feeding preferences means that the transmission of WNV to bird hosts (including corvids) occurs earlier in the season than transmission to humans and explains the capacity for bird die-offs to provide an early warning of human risk. A similar

shift in feeding patterns associated with a rise in human cases is also seen in *Culex tarsalis* mosquitoes in Colorado and California (Kilpatrick et al., 2006).

Detectability Although the pathogenicity of WNV to birds including crows has been demonstrated within the historical geographical range of WNV (Work et al., 1955), the very high mortality seen in American corvid populations is unusual (Eidson et al., 2001a). Clearly, this difference may limit the application of corvids as useful sentinels of WNV to contexts within the Americas. Even within North America, there is variation in the suitability of corvids to act as a sentinel for WNV activity related to the density of human populations. A study using decoy crows revealed that both detection and reporting rates were lower in rural areas compared with urban areas (Ward et al., 2006). Spatial analyses have also identified reduced capacity of dead crow density measures to forecast human infections in rural areas (Eidson et al., 2005). These effects are seen because the capacity of crows to act as useful sentinels depends upon the likelihood that bird deaths are observed and reported by people. The power of dead crow sentinel surveillance to predict human risk is greatly reduced in rural areas as a consequence of a reduced detectability of the sentinel response.

1.6   Applications of Animal Sentinels

Many of the questions addressed through the use of animal sentinels, such as the assessment of pathogen control efforts, the monitoring of prevalence fluctuations over time, and the demonstration of the absence of a pathogen, require only the basic qualities of a sentinel as defined above. While the more specific requirements of any particular sentinel are unique to the context and aim to which it is applied, there are some general qualities and subtypes of sentinels that correspond to major applications of animal sentinels. For example, only sentinels in which the response to a pathogen and the detection of that response, occur prior to exposure or cases in the target population can provide early warning of pathogen presence. Early warning sentinels are used to provide a predictive signal of risk to the target population. Sentinels that are exposed and which respond to a pathogen before the exposure of the target population may

provide an opportunity to implement pre-emptive control measures and to prevent the infection of the target population (See discussion of WNV surveillance in Section 1.5.1). Other early warning sentinels may respond to the pathogen more rapidly than the target population but not necessarily before the target's exposure (e.g. the coal-miner's canary). In such cases, data collected from the sentinel cannot be used to prevent cases in the target population altogether. However, the information they supply can provide advance warning of cases, enabling the prioritization of resources for treatment and the prevention of additional cases. In most cases early warning sentinels are highly visible and develop a very obvious response to the pathogen. Data provided by sentinels with these qualities can be more rapidly processed, analysed and acted upon than data from apparently healthy sentinels for which the potentially lengthy processes of sample collection and laboratory analyses must be carried out before any data are available. Ideally, the response of early warning sentinels should also be very specific, to minimize the likelihood of false positive responses and consequently improve confidence in decision-making based on the sentinel response alone.

Sentinels can also be used retrospectively to provide evidence of the timing of pathogen introduction and spread through a target population. In situations where a number of populations or locations are sampled, this information can be combined to reveal the spatial and temporal pattern of pathogen spread. Following the widespread rinderpest outbreak that occurred in Kenya in 1993-97, the retrospective serosurveillance of buffalo herds and analysis of age-seroprevalence patterns allowed the estimation of the time of infection in different herds, the identification of the probable point of entry of the pathogen into the wildlife population and the elucidation of where the pathogen had been, how it had spread and where it was likely to move to (Kock et al., 1999). In this case, buffalo herds were selected as sentinels on the basis of the increased susceptibility of the species to this virus (Rossiter, 1994), and served as sentinels for the larger livestock population in the affected areas. In such circumstances the appropriate sentinel population must develop a response to the pathogen that persists and is detectable a long time after exposure. When used retrospectively it is also important that individuals of the sentinel population can be reliably aged.

Figure 1.3: Application of the framework to H5N1 Influenza A

Surveillance aim

To establish if H5N1 viruses have been introduced into a country with underdeveloped disease surveillance and reporting structure. Should sentinels be used?

- Cross-sectional survey may be expensive and time consuming
- Sentinel surveillance potentially cost-effective alternative

Sentinel framework

Pathogen HPAI H5N1 virus
Target population the national poultry population
Potential sentinels .

- Backyard chicken populations in areas of perceived high risk of virus introduction, e.g. close to areas of wild bird congregation or to livestock markets
- Backyard ducks in similar locations
- Wild bird populations
- Domestic cats
- Domestic dogs

Other potential sentinels are excluded altogether on the basis of a lack of response to the pathogen or of any type of meaningful relationship with the target population.

Relationship between sentinel and target populations

Chickens

- Subset of target population

Ducks

- Occupy a very similar niche to target population
- May act as silent carrier of viruses (Hulse-Post et al., 2005)

Wild birds

- May act as source of infection for domestic species
- May not occupy the same geographical areas as the target population (especially true for large congregations of migratory birds)

Cats & dogs

- Spatial correspondence with target population
- Cats and dogs may prey upon the target population

Transmission routes

Chickens, ducks & wild birds

- Bird–bird transmission
- Environmental contamination

Cats & dogs

- Consumption of infected birds (Keawcharoen et al., 2004, Kuiken et al., 2004)
- Horizontal transmission in cats (Rimmelzwaan et al., 2006)

Sentinel response

Chickens

- Consistent, rapid and widespread mortality
- Die-offs provide a prompt indication of virus presence

Ducks

- Variable pathogenicity and thus mortality (Sturm-Ramirez et al., 2005)
- Isolation of virus from healthy birds (Hulse-Post et al., 2005)

Wild birds

- Variable pathogenicity (Ellis et al., 2004)
- Isolation of virus from healthy birds (Chen et al., 2006a)

Cats

- Experimental evidence of mortality response (Rimmelzwaan et al., 2006)
- Mortality reports associated with bird die-offs (Butler, 2006a, Songserm et al., 2006b, Yingst et al., 2006)
- High seroconversion rates (Butler, 2006b)
- Subclinical infections (Leschnik et al., 2007)

Dogs

- High seroconversion rates (Butler, 2006b)
- Mortality report associated with bird infection (Songserm et al., 2006c)

$\boxed{\text{Sensitivity \& specificity of responses}}$

Chickens

- $\checkmark\checkmark\checkmark$ Highly sensitive but specificity of mortality response is low, as
- XXX chicken die-offs not necessarily unusual where poultry are not routinely vaccinated against other pathogens, e.g. Newcastle disease virus
- $\checkmark\checkmark\checkmark$ High specificity of laboratory tests

Ducks

- $\checkmark/X$ Variable mortality response limits sensitivity
- $\checkmark\checkmark\checkmark$ High specificity of laboratory tests

Wild birds

- XXX Very low prevalence in healthy birds limits sensitivity (Chen et al., 2006a)
- $\checkmark/X$ Unknown or variable sensitivity & specificity of laboratory tests

Cats & Dogs

- $\checkmark/X$ Serological analyses non-specific for distinguishing high- and low-pathogenicity viruses

$\boxed{\text{Host ecology}}$

- $\checkmark\checkmark\checkmark$ Domestic species are all highly observable as a consequence of their close association with humans
- XXX Wild birds are considerably less visible and may occupy relatively remote and inaccessible areas

$\boxed{\text{Practical factors}}$

Risk to sampling personnel must be considered as a priority when developing all sampling protocols.

- $\checkmark\checkmark\checkmark$ Domestic species approachable and handleable
- $\checkmark/X$ Distribution of cats and dogs relatively to poultry may vary according to factors such as urbanization
- XXX Considerable investment of money, time and expertise required to sample sufficient numbers of wild birds
- $\checkmark\checkmark\checkmark$ For the identification of virus presence, standard test protocols include RT-PCR and virus isolation (OIE, 2005) which are generally adaptable across species
- $\checkmark/X$ Serological analyses may not be well developed for wild birds, cats or dogs

$\boxed{\text{Detectability}}$

Chickens

- $\checkmark\checkmark\checkmark$ Mortality response easily appreciated
- $\checkmark\checkmark\checkmark$ High visibility within human communities
- XXX Low specificity of mortality limits detectability

Ducks

- XXX Mortality response variable
- $\checkmark\checkmark\checkmark$ Additional responses detectable through laboratory tests
- $\checkmark\checkmark\checkmark$ High visibility within human communities

Wild birds

- XXX Mortality response variable
- XXX Low visibility compared with domestic species
- Logistically complex and time-consuming sampling required

Cats & Dogs

- $\checkmark\checkmark\checkmark$ High visibility within human communities
- $\checkmark\checkmark\checkmark$ Sudden and widespread morbidity or mortality uncommon
- $\checkmark/X$ Non-mortality responses less detectable

In all cases, a comprehensive network of observers is vital and it may be necessary to develop education programmes aimed at improving reporting levels.

$\boxed{\text{Utility}}$

- Domestic chicken and ducks sentinels are likely to provide the most rapid and dramatic response to HPAI H5N1 virus within a country. However, in this context in which mortality in domestic birds is not unusual, this mortality may not be reported and the detectability of the response in the context of this surveillance aim may be very low.
- To best address this surveillance aim, the specificity of the chicken mortality response to HPAI H5N1 presence could be enhanced by using a combination of sentinels such that priority was given to the investigation of chicken die-offs that were accompanied by morbidity or mortality in cats or dogs (Yingst et al., 2006).
- Retrospective analysis of sera collected from ducks, cats and dogs could also be used to identify those areas in which an H5N1 virus had been present.

1.7 Carnivores as Sentinels

Almost half (43%) of recently surveyed zoonoses are known to have a carnivore host (Cleaveland et al., 2001). Carnivores constitute a relatively small proportion of host species, suggesting a disproportionate concentration of pathogens, and zoonoses particularly, in carnivores. The most likely explanation for this imbalance considers the apical position of carnivores within ecosystems and recognizes their potential for exposure to pathogens of numerous species through ingestion. A review of infectious diseases of large carnivores identified 52 diseases, 31 (60%) of which could be transmitted by either ingestion or inhalation of infected material (Murray et al., 1999) and there is a growing literature indicating that a number of emerging zoonoses, including WNV and HPAI H5N1 viruses can be transmitted via this route (Komar et al., 2003; Austgen et al., 2004; Rimmelzwaan et al., 2006; Thiry et al., 2007; Giese et al., 2008; Reperant et al., 2008; VanDalen et al., 2009).

Carnivore and scavenger species that are exposed through consumption of infected prey may prove useful sentinels for a wide range of pathogens, specifically because of this additional route of exposure that is not shared with the target population (Cleaveland et al., 2006). A single predator or scavenger typically consumes material from multiple individuals, increasing the probability of exposure to pathogens circulating within the prey population. Predators and scavengers can effectively sample from the prey population, leading to a 'bio-accumulation' effect whereby pathogens present at relatively low prevalence in the prey population may be detected at higher prevalence in the predator/scavenger species (Cleaveland et al., 2006) (Figure 1.4).

The 'bio-accumulation' concept suggests that either the pathogen itself, or evidence of exposure to it, may effectively accumulate within carnivore populations (Cleaveland et al., 2006). The detection of the presence of a pathogen within a particular area could therefore be achieved by sampling relatively few carnivore sentinels, as compared to an exhaustive and costly survey of the prey population within which the pathogen may circulate at very low prevalence, providing a relatively rapid and inexpensive surveillance option (Frölich et al., 1998; Leighton et al., 2001; Csángó et al., 2004; Cleaveland et al., 2006).
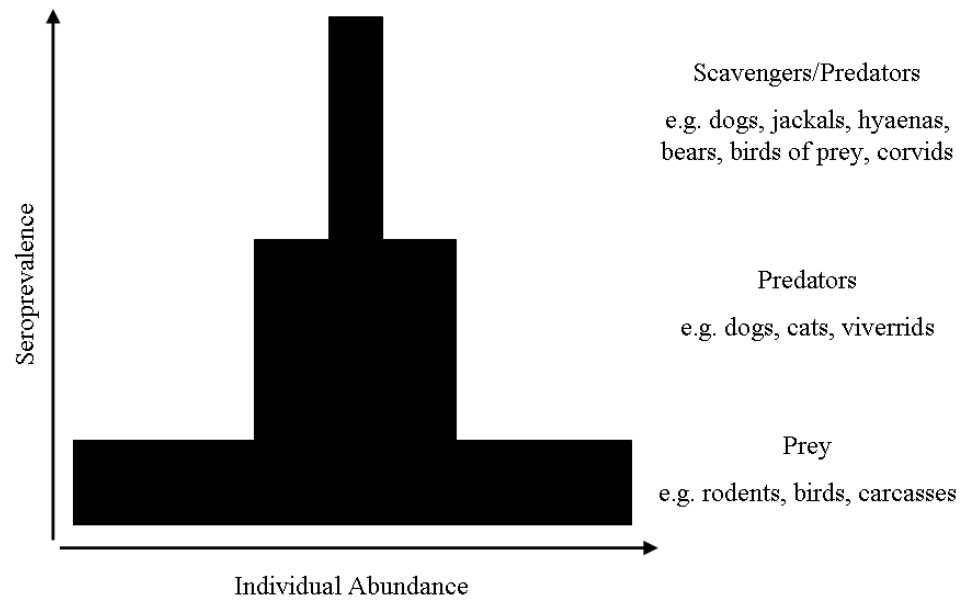
Figure 1.4: Summary of the bio-accumulation effect. Adapted from a presentation by Dr Sarah Cleaveland

1.8   Domestic Dogs as Sentinels

Domestic dogs are unique amongst carnivores in the position that they occupy within human communities, and have been identified as a suitable sentinel species for assessments of human risk because of their close association with people. Dogs share a broadly similar environment with their human owners and consequently share a wide range of potential exposure routes to environmental hazards such as chemical contamination and pathogens (Backer et al., 2001). Dogs can therefore act as indicators of the pathogens present within a local human community.

The niche occupied by domestic dogs in many communities across the world gives them a number of attributes that are desirable in a sentinel:

- Dogs are widely distributed and almost ubiquitous (e.g. mean human:dog ratios of 7.4 and 21.2 in rural and urban areas of Africa respectively (Knobel et al., 2005))
- Dogs live in close proximity to people
- Dogs are broadly accessible for sampling and can be safely handled

- Dogs are readily identifiable by both physical markings and name and owner details

- Dogs can be repeatedly located and recruited into follow-up studies

Across large parts of Africa and Asia, domestic dogs are effectively free-ranging scavengers, feeding on a wide and varied range of species and sources in the locality of human settlements. The bio-accumulation effect described above could explain the surprisingly high seroprevalences seen in global domestic dog populations to a number of pathogens (Cleaveland et al., 2006). A summary of some of these findings is given in Table 1.2.

Table 1.2: Selection of pathogens for which a high seroprevalence has been demonstrated in domestic dogs

Pathogen	Dog Population	Prevalence	Reference
Avian influenza H5N1	Village dogs Central Thailand	25%	(Butler, 2006c)
Trypanosoma cruzi	Domestic dogs NW Argentina	65%	(Castañera et al., 1998)
Francisella tularensis	Rural dogs SW Canada	14%	(Leighton et al., 2001)
Yersinia pestis	Rural dogs SW Canada	10%	(Leighton et al., 2001)
Ebola	Village dogs Ebola endemic area, Gabon	25%	(Allela et al., 2005)
African Horse Sickness	Domestic dogs Moremi, Botswana	11%	(Alexander et al., 1995)

With understanding of the pathogen-specific time-course of antibody responses in dogs, serological data can be used in both the short and longer terms, to detect the presence of a pathogen and also as part of integrated monitoring systems designed to retrospectively track the spread of a pathogen. Sentinel dog sampling could also be a very comprehensive and cost-effective surveillance method if combined with a rabies vaccination campaign. Rabies vaccination provides a strong incentive for dog owners to participate and facilitates the sampling of several hundred dogs per day at a cost of just $1-2 per dog vaccinated (Cleaveland et al., 2006).

1.9 Discussion

The objective of this chapter has been to provide a consistent and inclusive framework that clarifies our understanding of the role of animal sentinels and their potential value in the surveillance of human and animal infectious diseases, as well as providing a conceptual tool that can be applied to assess and characterize potential sentinels in the future. At present, surveillance of many pathogens involves the target population alone, however, the broad host-range of many important human and animal diseases provides opportunities for exploiting a wide range of species for surveillance purposes. The variability of host responses to a pathogen, the heterogeneities in pathogen exposure in different populations and the differing relationships between sentinel and target populations indicate that different animal hosts will themselves vary in their ability to act as effective sentinels in different circumstances.

Animal sentinels may not serve as a useful surveillance tool in all contexts. The generic framework developed in this chapter describes the attributes of host species that need to be considered to identify appropriate sentinel populations for different surveillance purposes. This same framework should also be used to identify characteristics of potential sentinels that perhaps make them unsuitable in a particular circumstance. For example, sentinels must by definition be intentionally observed. This classification distinguishes the use of animal sentinels from scenarios in which responses of animal populations to novel pathogens are 'noticed'. For this reason, animal sentinels cannot really provide the solution to the question of how to carry out surveillance for pathogens that are currently unknown. However, as a consequence of greater awareness of the potential of animal sentinels and improved observation of animal populations, instances of unusual morbidity and mortality in animal populations that result from the emergence of novel pathogens would perhaps be more likely to be noticed and their potential significance to other species recognized.

To date, there has been limited appreciation of the data resource that different animal hosts represent for disease surveillance. This chapter has highlighted the variety of surveillance functions for which animal sentinels may be used, the range of animal host species that may usefully be exploited (particularly for human disease surveillance),

and the potential benefits of animal sentinels for enhanced pathogen detection and improved cost-effectiveness of surveillance. The potential value of animal sentinels in disease prevention and control can only be realized with close integration and effective communication between and within human and animal health sectors; information generated from sentinel populations must be disseminated to those who need to take action, and appropriate responses must be generated as a result of this information to mitigate disease risk.

The use of domestic dogs as sentinels represents a broadly applicable epidemiological tool that may be applied globally for the detection of a vast range of multi-host and zoonotic pathogens. By sampling sentinel species such as domestic dogs it may be possible to observe the dynamics of zoonotic pathogens within animal populations and use this information to assess the risk posed to human populations. The following chapters explore these concepts with reference to influenza A and leptospirosis surveillance using domestic dogs sampled in Cameroon and Kenya.

Chapter 2

Influenza A H5N1 Surveillance in Cameroon

2.1 Abstract

Opportunistic sampling of wild and domestic avian and mammal populations was conducted in early 2006 in Northern Cameroon to determine if influenza A viruses including H5N1 were present, which species were exposed, and to evaluate the use of a range of serological and molecular diagnostic tests for sentinel surveillance of H5N1 using domestic animal species. The influenza A M gene was detected by rRT-PCR in swabs collected from domestic ducks, dogs, a Spur-winged goose and waterholes at sites of wild waterfowl congregation. Two M gene positive swabs collected from ducks at the Maga dam were also positive for the H5 gene. There was serological evidence of extensive exposure to H5N1 in the domestic duck populations sampled near Maga. Two cELISA tests yielded anti-influenza A seroprevalence estimates of 47% (ID VET cELISA) and 61% (BioChek cELISA). The sera were also tested by HI test using a range of antigen subtypes and 20% of the ducks sampled in April 2006 at Maga were positive in the H5N1 HI test, whilst largely negative against other influenza A subtypes. There was also evidence of exposure to influenza A in the domestic mammal populations at the same sites but the data gathered from a range of tests applied to these populations was less clear. Three different cELISA protocols were used, yielding seroprevalence estimates ranging

from 8-40% for pigs and 11-75% for dogs at the Maga site and the HI data from these species was inconsistent. The molecular and serological data gathered are consistent with the circulation of an H5N1 subtype virus in the domestic duck population at the Maga dam site. There is also evidence of exposure in the domestic mammals at this site but the diagnostic test results are contradictory. These data support the use of domestic ducks as sentinels of influenza A virus presence but reveal that the equivalent data gathered from domestic mammal populations using currently available diagnostic tests cannot be readily interpreted for similar surveillance purposes.

2.2   Introduction

2.2.1   Influenza A H5N1 in Africa

The first outbreak of H5N1 in Africa was reported from a commercial poultry farm in Nigeria and confirmed on February 6th 2006 (ISID, 2006c). Samples collected in January 2006 have also now been identified as positive cases (De Benedictis et al., 2007; Aiki-Raji et al., 2008). Since these first Nigerian cases, H5N1 was confirmed in poultry and wild birds in ten other African countries (Benin, Burkina Faso, Cameroon, Cote d'Ivoire, Djibouti, Egypt, Ghana, Niger, Sudan and Togo) (OIE, 2010) and human cases were confirmed in three of these countries (Djibouti, Egypt and Nigeria) (WHO, 2009a).

All African influenza A H5N1 sequences cluster together with viruses from Europe and the Middle East to form a phylogenetic clade (EMA or 2.2) that is distinct from other H5N1 isolates collected in Asia. The relatedness of viruses within this clade suggests a single ancestral source from which all viruses within this EMA clade are descended. Phylogenetic comparison suggests that this source virus may have come from Russia or Qinghai, China (Salzberg et al., 2007). Within Africa, the sequence data is most consistent with a scenario of three introduction events, which probably occurred in early 2006, followed by the spread within Africa of strains belonging to three sublineages (EMA A, B, and C or 2.2 II, IV and I respectively) (Ducatez et al., 2006, 2007a; Monne et al., 2008; Cattoli et al., 2009). More recently, it is proposed that

a further introduction of a clade 2.2 sublineage III virus has occurred some time prior to its first detection in Nigeria in July 2008 (Fusaro et al., 2009).

The capacity for the surveillance and control of pandemic influenza A in Africa is greatly limited as compared to other global regions. Existing veterinary and human disease surveillance networks are weak and control of animal movements within and between nations is limited (Fasina et al., 2007). Partly as a consequence of these data limitations, the relative importance of wild bird movements and trade in the introduction and spread of influenza A viruses in Africa remains unresolved (Cattoli et al., 2009). The speed with which the virus has spread within Africa implicates the movement of poultry and poultry products as the vehicle of spread and suggests that the biosecurity and control measures that have been imposed are insufficient to contain outbreaks (Breiman et al., 2007; Fasina et al., 2007; Salzberg et al., 2007). It is considered that H5N1 viruses are now endemic in countries such as Nigeria (Owoade et al., 2008) where the co-circulation of different viral sublineages and the emergence of reassortant viruses has been documented (Monne et al., 2008; Owoade et al., 2008). The documentation of reassortant viruses is a further indication that existing biosecurity measures are inadequate to contain these viruses (Monne et al., 2008).

Wild waterbird surveillance conducted in mid January - early March 2006 (the period in which H5N1 outbreaks were first reported in African countries) identified avian influenza viruses in wild birds sampled in eight (Chad, Ethiopia, Mali, Mauritania, Morocco, Niger, Senegal and Tunisia) of the fourteen sampled African countries . Sampled species were selected from avian families recognized as major influenza A reservoirs (principally Anseriformes and Charadriiformes) and both live caught and shot birds were sampled (shot birds were provided by hunters except in Ethiopia, Burkina Faso and Niger in which special hunting permits were obtained for sample collection) (Gaidet et al., 2007). The overall prevalence of influenza A in the 4553 cloacal and faecal samples collected was 3.5%. Eleven H5 positive samples were detected but none were H5N1 and no other highly pathogenic viruses were detected (Gaidet et al., 2007). Continuing surveillance in Northern Nigeria in 2007 has however identified H5N2 viruses with some genetic characteristics of high pathogenicity viruses in apparently healthy wild ducks and geese (Gaidet et al., 2008). The sequences of the Nigerian H5N2 viruses were most

closely related to low pathogenicity influenza strains found in wild and domestic duck species in Southern and Central Europe and South Africa but were found in species that do not migrate out of Africa (Gaidet et al., 2008). The mechanism through which these viruses reached wild birds in Nigeria and the potential for onward spread to migratory species and domestic poultry is unknown. H5N1 viruses were identified in wild hooded vultures showing neurological and respiratory disease signs in Ouagadougou, Burkina Faso in early 2006. The observation of illness in these wild birds was associated with an H5N1 outbreak that occurred in a nearby intensive poultry farm at the same time and from which very closely related H5N1 isolates were collected (Ducatez et al., 2007b). This case is consistent with a spillback scenario rather than independent maintenance of the virus in wild birds and suggests a possible role for vultures as indicators of H5N1 presence (Ducatez et al., 2007b).

A consequence of the common ancestry of African isolates is a broad similarity in some key molecular properties. To date, all African strains retain HA gene sequences associated with a binding preference for $\alpha2,3$ avian-type cell surface receptor linkages (Cattoli et al., 2009) rather than the $\alpha2,6$ preference more commonly observed in human-adapted influenza A viruses. Concerningly though, the majority of EMA (clade 2.2) viruses and all African sequences, possess the E627K mutation in the PB2 gene which is associated with enhanced cold tolerance and replication in mammals (Shinya et al., 2004; Hatta et al., 2007; Cattoli et al., 2009). Perhaps as a consequence of this mutation, the spread of H5N1 within the EMA region has been associated with an increase in the number of mammalian cases (Salzberg et al., 2007), including human cases in Turkey, Egypt, Iraq and Djibouti (Salzberg et al., 2007) and cat infections in Turkey, Iraq and Germany (Yingst et al., 2006; Salzberg et al., 2007; Thiry et al., 2007).

2.2.2 Influenza A H5N1 in Cameroon

The first case of H5N1 in Cameroon was reported on the 11th March 2006, following the identification of infected domestic ducks from Maroua, in the Extreme North province of the country (ISID, 2006a). This case was detected on the 21st February 2006 and was closely followed by two further cases which were confirmed at the Pasteur Institute, Paris (Njouom et al., 2008). The second case, an infection in a dead wild duck found

on Lake Malape near the Nigerian border (40km west of Garoua), occurred on March 9th, and the third case took place on a domestic duck farm in Vélé, in the Far North Province (Njouom et al., 2008). The locations of these confirmed cases are given in Figure 2.1.
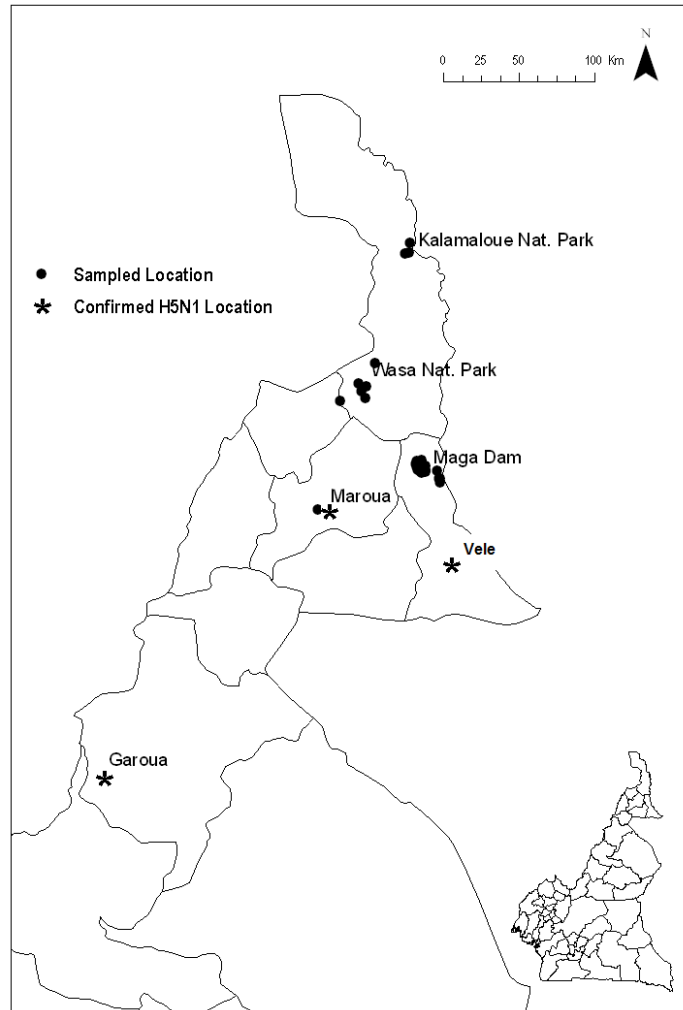
Figure 2.1: Map of Northern Cameroon showing sampled locations and sites of confirmed H5N1 avian cases

2.2.3   Influenza A H5N1 in Dogs and Pigs

There is relatively little published material on H5N1 infection in dogs. Anecdotal reports of infected dogs in Azerbaijan and Thailand have been published on ProMED (ISID, 2004, 2006b) and there is one confirmed fatal dog infection reported from Thailand (Songserm et al., 2006b). A serosurvey conducted in central Thailand identified antibodies against H5N1 in 160/629 (25%) village dogs and virus was isolated from at least one dog (Butler, 2006c). This high seroprevalence indicates that a proportion of dogs were surviving exposure to H5N1 virus. However, this early report of such high seroprevalence which was published at the time of the outbreak has not since been confirmed or validated and published in a peer-reviewed format. In support of the indication that dogs can survive infection with H5N1 viruses though, an experimental infection study using a German H5N1 isolate that possessed the 627K mutation showed that infected dogs developed only mild symptoms. Viral RNA was detected by PCR in the pharyngeal swabs of 3 of 4 dogs 2 days post infection but no live virus was isolated, and antibody was later detected in the 3 dogs that shed viral RNA (Giese et al., 2008).

Pigs are relatively frequently involved in interspecies transmission of influenza A viruses (Webster et al., 1992; Castrucci et al., 1993) and it has been suggested that they may play an important role in influenza A epidemiology as 'mixing vessels' in which co-infection with avian and human viruses may result in the generation of reassortant viruses with pandemic potential (Webster et al., 1992). A study conducted in areas of Korea in which H5N1 and H7 viruses circulate in birds found no seroepidemiological evidence of avian H5N1 infection in pigs (Jung et al., 2007). Experimental infections have shown that pigs can be infected with H5N1 viruses but that they are not readily transmissible between pigs (Choi et al., 2005).

2.2.4   Influenza A Surveillance using Domestic Animals

The global spread of H5N1 and H1N1 influenza A viruses have provided a clear and immediate demonstration of the capacity of influenza A viruses to transmit between species and these recent crises have contributed to a recognition of the need to conduct influenza A surveillance at sites of interface between human and animal populations

(WHO, 2006) and to explore the use of domestic species for influenza A surveillance. Particularly in countries and situations where surveillance resources are limited, it is hoped that surveillance of influenza A viruses in animal populations may provide new understanding and consequently new opportunities for the prevention and control of human and animal influenza outbreaks (Fouchier et al., 2003).

Influenza A surveillance in domestic animals is traditional concentrated upon commercial poultry flocks. H5N1 subtype HPAI viruses have consistently caused widespread mortality amongst domestic chicken populations around the world, and the extremely high levels of mortality caused often provides a clear and dramatic indication of virus presence. In countries where poultry are not routinely vaccinated against other pathogens such as Newcastle Disease, non-H5N1 related poultry die-offs may occur relatively frequently however, significantly reducing the specificity of high bird mortality as an indication of HPAI and effectively reducing the detectability of H5N1 HPAI presence. In such circumstances, additional surveillance options and targets may prove useful.

In parallel with the global spread of avian H5N1 infections, there have been numerous reports of illness and death in cats and dogs associated with simultaneous outbreaks in birds (Butler, 2006a,c; Songserm et al., 2006a,b; Yingst et al., 2006). Whilst poultry die-offs alone may not be recognized/considered unusual and trigger a targeted surveillance response, the concurrent occurrence of infection indications in cat and dog populations could be used to provide additional support for the targeting of limited surveillance resources. The potential for domestic carnivores to act as sentinels of H5N1 presence, within either domestic populations in which infection may not be readily appreciated, or in local wild bird populations in which prevalence may be very low and which may not develop clinical symptoms, has yet to be fully explored.

The recent H1N1 'swine flu' pandemic has reaffirmed the potentially pivotal role that domestic pig populations can play in the epidemiology of influenza A viruses. Pig trachea contains both $\alpha 2,3$ and $\alpha 2,6$ sialic acid receptors, facilitating pig infection with both avian and mammal adapted viruses and raising concerns about the role that pigs can play in the epidemiology of influenza A viruses, either through direct reassortment of avian and mammal adapted viruses in the pig host or through a process in which avian origin viruses may become mammal adapted in the pig host and acquire the capacity

for efficient spread within human populations (Webster et al., 1992; Kida et al., 1994; Ito et al., 1998; Zhou et al., 1999; Cohen, 2009). The propensity for influenza A viruses to infect multiple species makes it both desirable and necessary to conduct influenza A surveillance in both wild and domestic animal species and this should include but not be restricted to domestic poultry.

2.2.5   Laboratory Diagnosis of Influenza A

There are a variety of molecular and serological methodologies available for the detection of influenza A infections. In most cases these tests have been developed and extensively validated for use in domestic poultry but their suitability for application to other species and populations is considerably less well defined (Cattoli and Capua, 2007; VanDalen et al., 2009).

2.2.5.1   Molecular Tests and Isolation

The diagnostic gold standard test for any influenza A infection is virus culture via inoculation into chicken eggs or into cell cultures (Cattoli and Capua, 2007). Isolation can be used on many types of specimens and is the only method that can determine the presence of viable virus. Isolates can be fully characterized, sub-typed and potentially sequenced. However, isolation is a time consuming assay that can only be conducted in laboratories with the appropriate equipment, expertise and containment facilities (Cattoli and Capua, 2007). It also depends upon the collection and appropriate storage of viable live virus which is often difficult in field settings and can therefore lead to false-negative results (Cattoli and Capua, 2007).

Increasingly, PCR based molecular techniques such as reverse transcriptase-PCR (RT-PCR) are used to detect and characterize influenza A virus RNA. RT-PCR can be used to detect sequences common to all influenza A viruses such as matrix and nucleoprotein gene targets but can also be used to sub-type detected viruses. RT-PCR techniques can be very sensitive and specific and high throughput methodologies enable the rapid testing of large numbers of samples (Cattoli and Capua, 2007). The interpretation of RT-PCR test results can be complicated by false-negative results due to inefficient

RNA extraction and/or the presence of contaminants that can inhibit the PCR reaction (Cattoli and Capua, 2007). The evaluation of the significance of such potential problems is though complicated by the fact that in many cases, there exists no appropriate control target that can be used to evaluate the success of extraction from for example swab samples. It has also been demonstrated that some RT-PCR tests for influenza A perform poorly with wild bird samples (Xing et al., 2008) indicating that these assays may not be as readily transferable across species as initially hoped.

In real-time RT-PCR (rRT-PCR), the PCR product can be visualized with a fluorogenic probe that binds to a specific sequence of the product. As the product is synthesized, a fluorescent signal is produced that is directly correlated to the amplicon concentration in the log phase of amplification (Spackman and Suarez, 2008). The fluorescence signal is monitored in every amplification cycle, yielding a quantitative measure of the amount of product in real time as the reaction progresses (Spackman and Suarez, 2008). The results of rRT-PCR tests are reported as cycle threshold (Ct) values. A threshold fluorescence value, the level at which a reaction reaches a fluorescent intensity above background, is set in the exponential phase of the amplification of a positive standard (Applied Biosystems, Accessed 2010). The Ct value of a given sample is the first real-time amplification cycle in which the signal from amplified target material is detectable at this given threshold value. A low Ct score indicates that more copies of the target sequence were present in the test sample as compared to a sample with a large Ct value. Because both the primers and probe used in rRT-PCR reactions are specific to target sequences, these assays have very high specificity (Spackman and Suarez, 2008).

Both isolation and RT-PCR depend on the collection of virus at the time of sampling and can thus only be used to detect active infections. Birds infected with influenza A viruses can shed virus in both respiratory and digestive tract excretions. Shedding of H5N1 viruses following both natural and experimental of ducks has been recorded for periods of between one week and 11 days post infection (Hulse-Post et al., 2005; Chen et al., 2006a; Songserm et al., 2006c). Dogs infected with an H3N2 virus were shown to shed virus in nasal discharge for up to 6 days post infection (Song et al., 2008), whereas viral RNA was only detected at 2 days post infection (and not at 4 or 6 days) in pharyngeal swabs collected from dogs experimentally infected with an

H5N1 virus (Giese et al., 2008). These relatively short periods of virus shedding mean that there is only a brief window of opportunity in which influenza A infections can be detected using these molecular techniques. Neither isolation nor RT-PCR assays can provide any indication of previous exposure to influenza A virus and for population based surveillance particularly, that is not targeted at potential or probable cases, these testing approaches can therefore require a huge investment in sampling effort that may lead to the detection of very few or no active infections.

2.2.5.2 Serological Tests

Serological tests detect antibodies against influenza A viruses and all therefore reveal evidence of previous exposure rather than acute infection. They cannot be used to distinguish between high and low pathogenicity infections but are widely used for sero-prevalence evaluations and also for antigenic characterization of viruses and assessment of vaccine immunogenecity (Stephenson et al., 2007)

The agar gel immunodiffusion (AGID) is the traditional screening tool for detecting antibodies to influenza A in poultry populations. The test uses concentrations of antigen common to all influenza A viruses and is therefore not able to provide an indication of virus subtype. It is also the case that waterfowl rarely produce precipitins and although the AGID is the standard test for screening commercial flocks it has not been validated for use with other species (Cattoli and Capua, 2007; VanDalen et al., 2009).

A variety of enzyme linked immunosorbent assays (ELISAs) have been developed for influenza A testing, including both pan - influenza A and subtype specific tests. ELISAs are particularly useful in screening contexts as they are relatively cheap, simple to perform and can be completed within a matter of hours. Many influenza A ELISA formats traditionally use an anti-chicken secondary antibody conjugates and have not been validated for use in populations other than commercial chicken and turkeys (Cattoli and Capua, 2007). However, competitive ELISA (cELISA) test formats have now been developed, which do not employ any species specific reagents and can therefore be used to test sera from a range of species. Two different commercially available cELISA kits were used to test the bird and mammal sera collected in Cameroon. The ID Screen

Influenza A Antibody Competition ELISA (ID VET Montpelier, France) (ID VET cELISA) and the Avian Influenza Multispecies ELISA (BioChek UK Ltd) (BioChek cELISA) are both designed to detect antibodies against the influenza A nucleocapsid protein which is conserved across all H and N influenza A subtypes.

The manufacturers of the ID VET cELISA kit provide data on validation testing conducted by themselves and other laboratories. For the testing of bird sera, the specificity of the test as determined by the manufacturer using 600 European bird sera (predominantly chickens) was determined as 100% (IDVET, Distributed 2008a). The manufacturer also determined that the cELISA was more sensitive than the AGID and HI tests for detecting antibodies in vaccinated birds (subtypes H5 and H7). 15 of 15 sera from birds naturally infected with a variety of strains (including H5 and H3 subtypes) were also positive in the cELISA test. For testing swine sera, the manufacturers report that the cELISA has 100% specificity for influenza A based on the testing of 88 sera from influenza free herds and 75 sera from specific pathogen free animals (IDVET, Distributed 2008b). Using sera from 120 animals with unknown influenza A infection status and 25 sera from vaccinated individuals, the cELISA test had better diagnostic sensitivity than the HI, neutralization tests and indirect ELISA tests used for comparison (IDVET, Distributed 2008b).

Independent validation testing conducted by the OIE-FAO and National Reference Laboratory for Newcastle Disease and Avian Influenza determined that the sensitivity and specificity of the ID VET cELISA for chicken sera were 98.7% and 98.7% respectively, and for duck sera testing the sensitivity and specificity of the test were 89.0% and 88.8% as compared to AGID and HI test results (Terregino, Distributed 2010). The cELISA also correctly classified hyper-immune reference sera against strains of all sixteen influenza HA subtypes as positive (Terregino, Distributed 2010).

Data provided by the manufacturer of the BioChek cELISA reports that the cELISA has been validated using sera positive against fifteen influenza A subtypes (not H16) and that the cELISA has >95% sensitivity and >99% specificity when compared to agar gel precipitin test and HI (BioChek, 2008). Sera positive for a range of other avian pathogens were all negative in the BioChek test and the test showed 99.5% and 100% specificity when used to screen field flocks of 376 broilers and 123 Peking ducks

respectively that were screened regularly by indirect ELISA to confirm negative status (BioChek, 2008). In a population of zoo birds vaccinated using an H5N2 strain, the BioChek cELISA showed sensitivity of 81% and the HI test had sensitivity of 87% in the same population (BioChek, 2008).

These cELISAs are not subtype specific and HI tests are the principal serological test used to determine the infecting virus subtype. The haemagglutination inhibition (HI) test utilizes the capacity of influenza A viruses to cause the agglutination of red blood cells (RBCs). Preparations of RBCs will typically sediment, but in the presence of influenza virus or viral antigen, the RBCs are bound and will fail to sediment. In the presence of antibodies against the particular subtype of influenza virus or antigen preparation used the RBCs will not be agglutinated and will sediment. These reactions can be visually distinguished to identify the presence or absence and titre of antibody in test sera.

The HI test is a standard serological test for the detection of subtype specific antibody in avian species and has the advantage of not utilizing any strictly species-specific reagents. However, there are species specific influences upon test performance. The sera of some species contain non-specific haemagglutinins that can interfere with the HI test and lead to false negative results. There are though two common sera pre-treatment protocols that can be used to mitigate this problem (WHO, 2002). A number of studies have identified a problem of limited sensitivity of this traditional test when used to detect antibody against avian influenza viruses in mammalian sera including human sera (Lu et al., 1982; Beare and Webster, 1991; Rowe et al., 1999). Different influenza A viruses have variable capacity to bind to different SA linkages and this binding specificity also influences HI tests. Traditionally, HI tests utilize either chicken or turkey RBCs which are large and settle clearly. Both chicken and turkey RBCs express predominantly $\alpha 2,3$ SA linkages but also some $\alpha 2,6$ SA linkages. Horse RBCs express $\alpha 2,3$ linkages exclusively and the use of horse RBCs in the HI test can lead to a marked increase in the sensitivity of the HI test as compared to the standard protocol using turkey RBCs (Stephenson et al., 2004). The horse RBC adapted HI test has now been used in a number of studies and has been shown to increase test sensitivity for H5 viruses

particularly (Stephenson et al., 2003; Meijer et al., 2006; WHO, 2006; Jia et al., 2008; Kayali et al., 2008).

Finally, the virus neutralization (VN) test, which is often considered the gold-standard serological test, uses live infectious virus and detects functional antibody directed mostly against the viral HA molecule (Stephenson et al., 2007). The VN assay can be rapidly developed for all subtypes without the need for the purification of antigens that is required for the HI test (Stephenson et al., 2007). However, the VN takes 3 to 4 days to complete and because of the requirement for live virus, can only be conducted in specialist facilities (WHO, 2006). The microneutralization (MN) test is a variation of this VN test that can be performed in microtitre plates and can yield results on a next-day basis (VanDalen et al., 2009). In a recent study of inter-laboratory test result comparisons, the results of both HI and VN tests were found to vary between laboratories, with the VN results showing significantly more variability than the HI (Stephenson et al., 2007). In comparison to the VN, the HI test is easy to perform and is the most widely used test for the detection of subtype specific antibody populations (Stephenson et al., 2007).

2.2.6 Chapter Objectives

This chapter describes two opportunistic cross-sectional surveys conducted in Northern Cameroon in early 2006 with two principle objectives. First, to determine the presence of influenza A H5N1 infection in different avian and mammal populations using both serological and molecular tests and second, to evaluate the performance of different molecular and serological tests for detecting influenza A viruses for sentinel surveillance in these different animal populations.

2.3   Methodology

2.3.1   Sampling Locations

Cameroon is located at the cross-over point of major migratory bird flyways - the East Atlantic, Black Sea/Mediterranean and East African West Asian flyways and the Northern Province area, on the edge of Lake Chad is a major over-wintering site for large numbers of birds including ducks and waders. The Northern Province contains irrigated rice schemes at which wild and domestic birds, pigs and people and other domestic animals interact. Areas of interface between wildlife, livestock and humans such as this may be important for the transmission of avian viruses from the wild bird reservoir population to other species, and ultimately for the evolution of pandemic strains of influenza (Fouchier et al., 2003; Gilbert et al., 2006a,b).

Opportunistic sampling of wild and domestic species was carried out in the period February-April 2006 and covered three major areas (Figure 2.1). The Maga dam, in the Extreme North province of Cameroon was visited twice, in February and April 2006. The Maga site was selected because the large dam and wetland area attracts wild waterfowl to an area at which domestic species are also present and it is also the closest large water body to the Maroua site at which the first H5N1 cases in Cameroon were reported. This site included ten villages clustered around a rice scheme that is irrigated from the dam. The villages around the rice scheme were all visited to sample domestic animal species, including chickens, ducks, pigs and dogs. Local paravet assistants visited the villages on days prior to sample collection to inform residents that the project would visit their village for sampling and questionnaire surveys. On the day of visits, a small number of key points were set-up in the villages and residents were invited to bring their animals for sampling. All domestic animal owners provided verbal consent for participation in the sampling exercise prior to sample collection. Animal owners were asked about recent animal mortality on an *ad hoc* basis.

Samples were also collected from wild birds (predominantly wild geese) shot at a hunting lodge near the Maga dam on one day of sampling in February 2006. The shot species

and individuals were selected by the hunters rather than the surveillance project and samples were collected from the birds within hours of their collection.

The urban site of the confirmed H5N1 case in Maroua was visited in April 2006. Local assistants identified households that had reported bird die-offs in March 2006 and additional bird owning households proximate (within 100m) to these locations. Domestic birds in the area had been depopulated in the period between the confirmed case in March and this sampling visit. Any chickens and ducks present at households that had reported die-offs previously and at proximate households were sampled.

In the period between February and April 2006, wild bird sampling was conducted at sites across the Extreme North of Cameroon including the Maga dam area, Wasa National Park and Kalamaloue National Park (Figure 2.1) all of which are hosts to large populations of wild migratory birds, including ducks. Live wild birds were trapped using mist netting techniques targeting wild duck species and environmental faecal and water samples were collected to supplement wild bird sampling. Watering hole and trapping sites within these areas were selected on the basis of sightings of large congregations of wild waterbirds and logistic constraints.

2.3.2 Sample Collection

Tracheal and cloacal swabs were collected from all wild and domestic birds. Swabs were moistened before sample collection by dipping them in viral transport media and after collection the tip of each swab was broken off and stored individually in viral transport media. The transport media used was brain heart infusion broth supplemented with antibiotics (provided by the Veterinary Laboratories Agency (VLA), Weybridge, UK). Blood samples were collected from domestic birds and shot birds but not live caught wild birds. From domestic birds, blood samples were collected from the brachial vein using a sterile vacutainer (4ml red topped plain vacutainers) and 23 gauge needle. Up to 4ml of blood was collected from each adult domestic bird, ensuring that the total collected volume was less than 1% of body mass (FAO, 2007). Post-mortem blood samples from shot birds were collected from the heart chambers and major blood vessels using a syringe and transferred into a vacutainer (FAO, 2007).

Sampling of domestic dogs and pigs included collection of nasal and faecal swabs and a blood sample. Nasal and faecal swabs were collected using the equipment and media described for bird tracheal and cloacal swabs. Dog blood samples of maximum 10ml were taken from the cephalic vein. Blood was collected using 21G S-Monovette needles and S-Monovette collection tubes (Sarstedt AG & Co.). Pig blood samples of up to 10ml were collected from the anterior vena cava into the S-Monovette tubes.

For environmental sample collection, the predominant species present at a waterhole was recorded before collecting water itself or swabbing fresh faeces from visibly contaminated areas. Swabs were handled as described above using the same media. All swabs and water samples were frozen in liquid nitrogen within 24 hours of collection. Blood samples were centrifuged on the day of collection to separate sera which was refrigerated for up to five days before transfer to liquid nitrogen. All samples were shipped in a liquid nitrogen dry shipper and stored in the UK at -80℃ for swabs and -20℃ for sera.

2.3.3 Molecular Laboratory Analysis

All swabs were tested by rRT-PCR for the influenza A M and H5 genes (OIE, 2006) at the VLA and the Ct value recorded. All samples in which either the M or H5 genes were detected through rRT-PCR were inoculated into specific pathogen-free embryonated chicken eggs.

2.3.4 Competitive ELISAs

82 mammal sera collected from dogs (41 sera) and pigs (41 sera) at Maga and Maroua in April were screened at a dilution of 1:10 at the National Veterinary Institute, Sweden (Statens Veterinärmedicinska Anstalt (SVA)) using the ID VET cELISA. Bird and mammal sera with sufficient material were also tested at the University of Edinburgh using the ID VET cELISA and the BioChek cELISA. The ID VET cELISA was used to re-test those mammal sera already tested at the SVA because of a change in manufacturers recommendations regarding test dilutions for dog and pig sera.

163 sera (82 duck sera, 40 pig sera and 41 dog sera) were tested with the ID VET cELISA at Edinburgh (Table 2.3). Duck sera were tested at 1:20, dog sera at 1:20 and pig sera at 1:41 as recommended by the manufacturer (specific recommendations for the dilution of dog sera are not included as standard in the kit and were made by the manufacturer directly). For all species, the recommended cut-off of ⩽45% competition as compared to kit negative control was used to define positive sera.

All sera were tested in duplicate and an average optical density (OD) recorded for each sample. Results from the ID VET cELISA are reported as percentage competition (Equation 2.1).

$$competition\ \% = \frac{sample\ OD}{negative\ control\ OD} \times 100 \qquad (2.1)$$

Sample values less than 100% competition indicate that antibody present in the test sample bound to the antigen on the plate and inhibited the binding of the labelled test antibody. Values closer to zero indicate a greater concentration of antibody present in the test sample. Values greater than 100 indicate that the test sample bound to the test plate less effectively than the negative control.

153 sera (79 duck sera, 35 pig sera and 39 dog sera) were also tested with the BioChek cELISA. Sera from all species were tested at 1:50 as recommended by the manufacturer. Results from the BioChek cELISA are reported as sample to negative ratios (Equation 2.2). This measure is analogous to the % competition used in the ID VET test but is recorded on a 0 to 1 scale. The recommended cut-off of a sample/negative control ratio of ⩽0.6 was used to define positive sera.

$$sample/negative\ ratio = \frac{sample\ OD}{negative\ control\ OD} \qquad (2.2)$$

2.3.5 Haemagglutination Inhibition Tests

A range of haemagglutination inhibition (HI) tests were run for both avian and mammal sera. A summary of the HI tests conducted at different laboratories and the details

of the antigens used is given in Table 5.1. All HI tests were run following standard protocols (WHO, 2002; OIE, 2005), except as described below.

The first HI tests were conducted at the VLA with the principal aim of identifying antibodies against highly pathogenic (H5 or H7) influenza A viruses. All bird sera were tested at a starting dilution of 1:2. Mammal sera were pre-treated with four volumes of receptor-destroying enzyme and incubated overnight at 37℃. The enzyme was inactivated by incubation at 56℃ for 30 minutes prior to testing at a starting sera dilution of 1:7.5. All bird and mammal sera were tested against the VLA H5N1 antigen (Table 5.1). Those mammal sera that gave a non-zero titre in the first test were re-tested against H5N1 and H5N2 antigens with a starting dilution of 1:5. All domestic duck and mammal sera collected in Maga and Maroua in April were tested using a Newcastle Disease Virus (NDV) antigen and the duck sera were also tested against H5N2, H7N7 and H9N9 influenza A antigens (Table 5.1). All HI tests conducted at the VLA were run using a 1% suspension of chicken red blood cells and positive and negative controls were run with each batch of test samples. The HI titre was read and defined as the reciprocal of the last dilution of serum that completely inhibited hemagglutination.

All mammal sera collected at Maga and Maroua were later tested at the SVA against H5N1, H5N2, H1N1 and H3N2 antigens. This additional HI testing was conducted to allow comparison of the results obtained using horse RBCs at the SVA as compared to the chicken RBCs used at the VLA, and to include the non-H5N1 N1 and H3N2 antigens in the test panel. The additional N1 antigen was used to assess the potential that the antibodies detected in the H5N1 assay at the VLA were 'against' the N1 rather than H5 epitopes of this antigen and the H3N2 antigen was included as viruses of this subtype are commonly associated with swine populations.

All tests run at the SVA apart from the H1N1 assay were run using a modified HI test protocol utilizing horse erythrocytes. The H1N1 antigen did not agglutinate horse erythrocytes and this test was run using chicken erythrocytes as in standard tests. The protocol for HI tests using horse erythrocytes was adapted from Jia et al. (2008). Whole equine blood was centrifuged and the supernatant discarded. The erythrocytes were washed three times in phosphate buffered saline (PBS) then reconstituted at 1% in PBS with 0.75% bovine serum albumin (BSA). For all HI tests at the SVA, sera

Table 2.1: Summary of haemagglutination inhibition tests

Testing laboratory	Virus	Antigen	Virus subtype	Erythrocytes	n Sera tested Avian	Mammal
VLA	Influenza A	A/turkey/Turkiye/05	H5N1	Chicken	166	84
		A/ostrich/Denmark/72420/96	H5N2	Chicken	114	12
		A/turkey/Englang/647/77	H7N7	Chicken	113	0
		A/knot/England/SV4/02	H9N9	Chicken	114	0
	Newcastle Disease	Ulster 2C	NDV	Chicken	114	84
SVA	Influenza A	A/ostrich/Denmark/72420/96	H5N2	Horse	0	84
		A/chicken/Scotland/59	H5N1	Horse	0	84
		A/swine/Beligium/1/98	H1N1	Chicken	0	84
		A/swine/Flanders/1/98	H3N2	Horse	0	84
Edinburgh	Influenza A	A/turkey/England/69	H3N2	Chicken	70	0

were pre-treated with a trypsin-heat-periodate protocol (WHO, 2002) and diluted to a starting dilution of 1:8. Tests using horse erythrocytes were incubated for 1 hour at room temperature before the HI titre was read and defined as the reciprocal of the last dilution of serum that completely inhibited hemagglutination. Sera and antigen controls as well as positive and negative control sera were run in each batch of tests with each antigen.

Finally, those avian samples with sufficient sera remaining were tested by HI test using an H3N2 antigen (Table 5.1). Sera were pre-treated using the same protocol as described above for the SVA HI tests and tested using a 1% suspension of chicken erythrocytes.

2.3.6 Statistical Analyses

The diagnostic tests used have been extensively validated for use with bird sera. However, their use for the analysis of sera from mammals is less well described and for this reason, lab data from bird and mammal serological tests were analysed separately. Receiver operating characteristic (ROC) analysis was used to evaluate the performance of cELISA tests as compared to HI test outcomes in both groups. Area under the curve (AUC) values were calculated for all tests. AUC values give the probability that a randomly selected true positive has a lower (more positive) cELISA score than a randomly selected true negative sample (Dohoo et al., 2003).

For the bird samples, the widely used cut-off of $\geq 1:16$ was used to define positive sera in the H5N1 HI test. For the mammal sera, in which reduced test sensitivity

was anticipated, all sera with a titre $\geq$1:7.5 were considered positive and the modelled outcome was a response (titre of $\geq$1:7.5) vs. no response (titre of <1:7.5) in the VLA H5N1 HI test. Logistic regression modelling was carried out to examine the influence of species on the H5N1 HI test status of mammal sera. The three ELISA test scores (ID VET cELISA using species specific sera dilution, BioChek cELISA and ID VET cELISA using 1:10 dilution) were also considered in the model as continuous predictors of HI status. Generalized linear models (GLMs) were compared using likelihood ratio tests. Variables were added to the intercept only model in order of decreasing significance until no further variables were significant at $p \leq 0.05$. At each step all variables were checked to ensure that they maintained significance at $p \leq 0.05$. The overall goodness of fit of the model was assessed using the unweighted sum of squares test (Hosmer et al., 1997) and diagnostic plots of fitted values, residuals and influence measures (Hosmer et al., 1997, 2008). All statistical analysis was carried out using R (R Development Core Team, 2009). The unweighted sum of squares test was calculated using the *Design* package (Harrell, 2009).

2.4 Results

A total of 75 wild birds (predominantly Spur-winged geese *(Plectropterus gambensis)* and Ruff *(Philomachus pugnax)*), 161 domestic Muscovy ducks *(Cairina moschata)*, 39 chickens *(Gallus domesticus)*, 43 dogs *(Canis familiaris)* and 41 pigs *(Sus domesticus)* were sampled. Eighty-eight environmental faecal swabs and eight water samples were collected (Tables 2.2 and 2.3).

2.4.1 Real Time RT-PCR and Virus Isolation

The influenza A M gene was detected by real time RT-PCR in twelve domestic duck swabs (6 tracheal and 6 cloacal), one Spur-winged goose cloacal swab, two environmental faecal swabs collected from waterholes visited by Spur-winged geese and two domestic dog nasal swabs. Two of the M gene positive duck tracheal swabs collected at Maga in February were also positive for the H5 gene (Table 2.2). All real time RT-PCR

Table 2.2: Summary of real time RT-PCR results for all swabs collected

Sampling Location and Dates	Species/Origin	Swabs tested					M Gene Positive	H5 Gene Positive
		E	Tr	N	Cl	F		
Maga 21-23 Feb	Spur-winged goose	-	31	-	31	-	1*Cl	0
	Knob-billed goose	-	9	-	9	-	0	0
	Frankolin	-	2	-	2	-	0	0
	Spur-winged plover	-	4	-	4	-	0	0
	Domestic duck	-	43		43	-	3*Tr & 3*Cl	2*Tr
Wasa N.P. 24th Feb	Env - Faeces	65	-	-	-	-	2	0
	Env - Water	7	-	-	-	-	0	0
Wasa and Kalamaloue N.P. 24 Mar-7 Apr	Little ringed plover	-	3	-	3	-	0	0
	Wood sandpiper	-	9	-	9	-	0	0
	Spur-winged plover	-	2	-	2	-	0	0
	Ruff	-	17	-	17	-	0	0
	Common sandpiper	-	1	-	1	-	0	0
	Moorhen	-	1	-	1	-	0	0
	Temmineck's stint	-	1	-	1	-	0	0
	Environmental	23	-	-	-	-	0	0
Maga 3-6 April	Domestic duck	-	95	-	95	-	3*Tr & 3*Cl	0
	Domestic chicken	-	21	-	21	-	0	0
	Domestic pig	-	-	41	-	41	0	0
	Domestic dog	-	-	40	-	40	2*N	0
Maroua 7th April	Domestic duck	-	23	-	23	-	0	0
	Domestic dog	-	-	3	-	3	0	0

E = Environmental, Tr = Tracheal, N = Nasal, Cl = Cloacal, F = Faecal swab

Ct scores were borderline positive and fell near the VLA's recommended cut-off point of 35Ct. Samples with a positive PCR result completed two passages in embryonated eggs. No haemagglutinating viruses were isolated.

2.4.2 Competitive ELISAs

Antibodies against influenza A were detected in sera from ducks, pigs and dogs sampled in April 2006 using all three competitive ELISA protocols. All prevalence estimates are reported with exact binomial confidence intervals. In total 28/60 (47%, 95% CI: 33-60%) duck sera, 3/40 (8%, 95% CI: 1.5-20%) pig sera and 4/38 (11%, 95% CI: 3-25%) dog sera from Maga were classified positive by the ID VET cELISA at the species specific recommended sera dilutions. The mammal sera were also tested at a dilution of 1:10 using the ID VET cELISA and 12/35 (34%, 95% CI: 19-52%) pig sera and 21/37 (57%, 95% CI: 39-73%) dogs sera were classified as positive. None of the sera collected at Maroua were classified as positive by this test at either test dilution. The BioChek

cELISA classified 35/57 (61%, 95% CI: 48-74%) duck sera from Maga, 2/22 (9%, 95% CI: 1-29%) duck sera from Maroua, 14/35 (40%, 95% CI: 24-58%) pig sera from Maga, 27/36 (75%, 95% CI: 58-88%) dog sera from Maga and 2/3 (67%, 95% CI: 9-99%) dog sera from Maroua as positive for anti-influenza A antibodies (Table 2.3).

2.4.3 Haemagglutination Inhibition Tests

No antibodies were detected in the wild bird sera using the HI tests. One of 37 domestic ducks sampled at Maga in February was HI positive for neutralizing antibodies to H5N1 (Table 2.3). Eighteen of the 89 (20%, 95% CI: 12-30%) domestic ducks sampled at Maga in April but none of 23 ducks sampled at Maroua were positive in the H5N1 test (0%, 95% CI: 0-15%). All duck samples were negative in the H5N2 HI test but seven ducks gave non zero titres (2 at 1:2 and 5 and 1:4). All seven of these samples had a titre of ⩾1:16 in the H5N1 HI test. All bird sera were negative in the HI tests using H7N7 and H3N2 antigens. One duck was positive at a titre of 1:16 against the H9N9 antigen. 21 of 90 (23%, 95% CI: 15-33%) ducks from Maga and eight of 24 (33%, 95% CI: 16-55%) from Maroua tested positive for neutralizing antibodies against NDV.

Some low titre inhibition of haemagglutination was recorded in the H5N1 HI tests conducted at the VLA for three of 41 (7%, 95% CI: 2-19%) pig sera and eight of 40 (20%, 95% CI: 9-36%) dog sera collected at Maga in April, and for one of the three dog sera collected at Maroua. The 12 mammalian sera that showed HI activity (non-zero titres recorded) in the first H5N1 test were re-tested against H5N1 and H5N2 antigens at a starting dilution of 1:5. Eleven of these 12 sera also gave a non-zero titre in the second H5N1 test including 3 sera with a titre of 1:20. Only one sample showed any HI activity (at 1:10) against the H5N2 antigen. In the H5N1 and H5N2 HI tests run at the SVA using horse erythrocytes, all of the mammal sera tested were negative and no non-zero titres were observed.

There was some evidence of H3N2 exposure in the mammal sera collected at Maga. Three sera (1 dog and 2 pig sera) gave non-zero titres of 1:8, 1:128 and 1:128, with repeat tires of 1:16, 1:128 and 1:256 respectively. No antibodies against NDV were detected in any of the pig or dog sera tested.

Table 2.3: Summary of influenza A serology results for the H5N1 HI conducted at the VLA and the competitive ELISA tests. Data for mammal HI test refer to the first screen at a starting dilution of 1:7.5

Location and Dates	Species	H5N1 HI n Tested	HI Positive Number	HI Positive Titre	HI Activity Number	HI Activity Titre	ID VET cELISA Species specific dilutions n Tested	n Positive	ID VET cELISA All 1:10 n Tested	n Positive	BioChek cELISA All 1:50 n Tested	n Positive
Maga Dam 21-23 Feb	Spur-winged goose	30	0	-	0	-	0	-	0	-	0	-
	Knob-billed goose	8	0	-	0	-	0	-	0	-	0	-
	Francolin	1	0	-	0	-	0	-	0	-	0	-
	Domestic duck	37	1	1 (1:32)	0	-	0	-	0	-	0	-
	Domestic chicken	17	0	0	0	-	0	-	0	-	0	-
Maga Dam 3-6 April	Domestic duck	89	18	9(1:16) 4(1:32) 4(1:64) 1(1:128)	27	2(1:2) 12(1:4) 13(1:8)	60	28	0	-	57	35
	Domestic pig	41	0	-	3	1(1:7.5) 2(1:15)	40	3	35	12	35	14
	Domestic dog	40	0	-	8	6(1:7.5) 2(1:15)	38	4	37	21	36	27
Maroua 7th April	Domestic duck	23	0	-	0	0	22	0	0	-	22	2
	Domestic dog	3	0	-	1	1(1:7.5)	3	0	2	0	3	2

2.4.4 Test Comparisons

The relationships between the two cELISAs and the VLA H5N1 HI data for 78 duck samples tested by all three tests are shown in Figure 2.2. The ROC curves for the two cELISAs as compared to the H5N1 HI results are shown in Figure 2.3. The AUC values for the two tests are 0.897 and 0.899 for the ID VET and BioChek tests respectively.
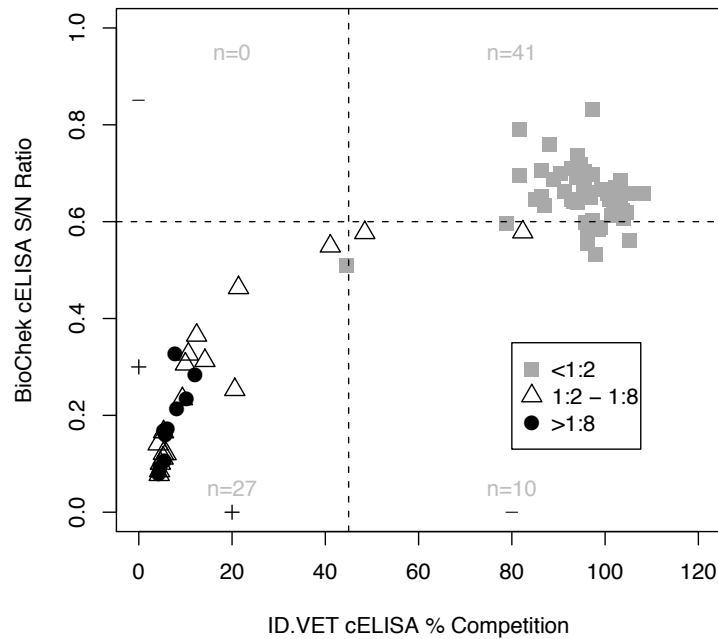
Figure 2.2: Correspondence between the two cELISA scores and the VLA H5N1 titre for the Cameroon duck samples. Grey squares indicate samples with a negative H5N1 HI titre of <1:2, black triangles indicate samples with a non-zero H5N1 HI titre below 1:16 and black points indicate samples with a positive H5N1 HI titre of ⩾1:16. Dotted lines show the recommended cut-offs for the cELISA tests and positive and negative signs indicate the classification of scores by each test. Text in grey indicates the number of samples in each section of the graph as defined by the recommended cELISA cut-offs.

The relationships between the ID VET cELISA scores (at recommended test dilution), the BioChek cELISA scores and the VLA H5N1 HI titres for the mammal sera are shown in Figure 2.4. The ROC curves for the three cELISAs as compared to the H5N1 HI non-zero H5N1 HI titre classifier of the mammal sera are shown in Figure 2.5. The
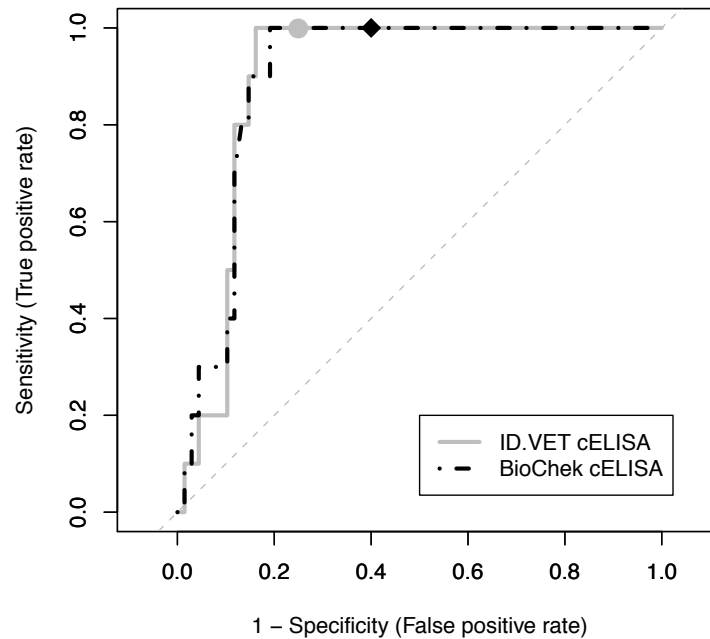
Figure 2.3: ROC curves for the two cELISA scores for discriminating between H5N1 HI test positive and negative duck sera ($\geqslant$1:16 cut-off). The grey diagonal line indicates the performance of a random classifier. The grey circle and black diamond indicate the recommended cut-offs for the ID VET and BioChek tests respectively.

AUC values for the three cELISAs are (in descending order): ID VET at recommended dilutions - 0.914, BioChek - 0.899 and ID VET at 1:10 - 0.867.

GLM model building for the mammal samples considered the H5N1 HI response status (response vs no-response) of samples as the outcome variable and included the three cELISA scores and species as candidate covariates. Models were fitted using data from 72 individuals tested with all four tests (ELISA and HI data). The three cELISA scores are strongly correlated and all are negatively associated with the log odds of a non-zero H5N1 HI titre. The relative improvements of model fit for the univariate models as compared to the intercept only model given with the three cELISA scores in descending order were ID VET 1:10 (LRT=16.87, df=1, p$\leqslant$0.001), ID VET at recommended dilutions (LRT=14.66, df=1, p$\leqslant$0.001) and BioChek (LRT=8.50, df=1, p$\leqslant$0.01). The addition of the species factor also significantly improved model fit and indicated that
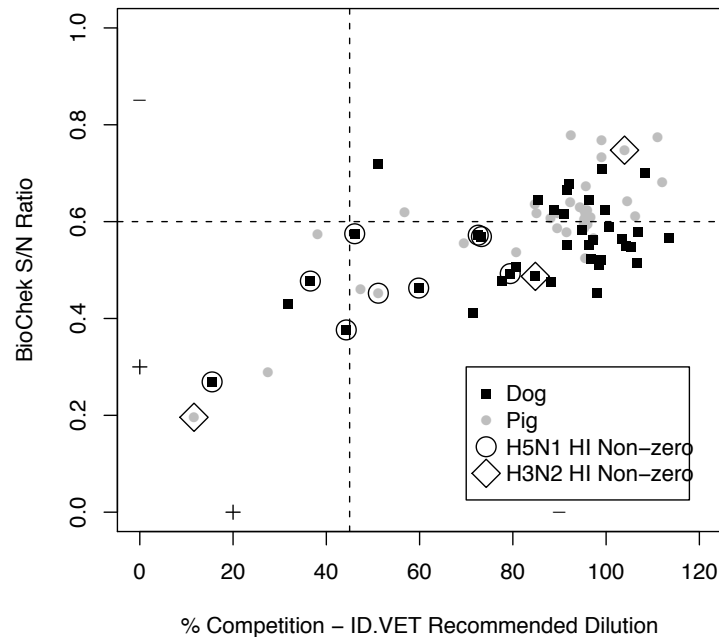
Figure 2.4: Correspondence between test scores for the mammal samples tested with the ID VET cELISA at recommended dilution, the BioChek cELISA, the H5N1 (VLA) HI and the and H3N2 (SVA) HI titres. Black squares indicate sera from dogs, and grey points indicate sera from pigs. Black circles indicate samples with an H5N1 HI titre of $\geqslant$1:2. Black diamonds indicate samples with an H3N2 HI titre of $\geqslant$1:8. Dotted lines show the recommended cut-offs for the cELISA tests and positive and negative signs indicate the classification of scores by each test

sera from pigs were less likely to give a non-zero H5N1 HI titre as compared to dog sera. The final model is given in Table 2.4. The unweighted sum of squares test had a test statistic value of 3.90 and a p value of 0.95 providing no indication of poor model fit (Hosmer et al., 1997).

2.5 Discussion

The molecular and serological data presented are consistent with the circulation of an H5N1 virus in the domestic duck populations around the Maga dam and there are also indications of H5N1 exposure in the domestic mammal populations at this site.
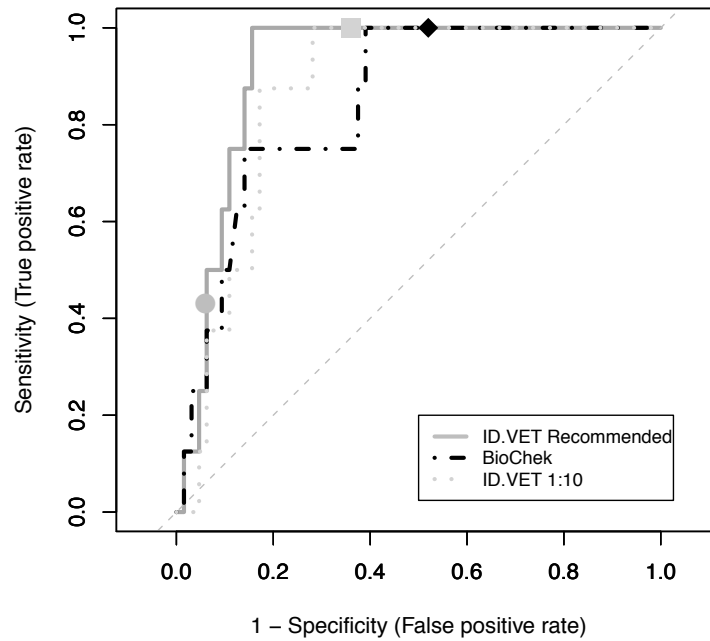
Figure 2.5: ROC curves for the three cELISA scores for discriminating between VLA H5N1 HI test responding and completely negative mammal sera. The grey diagonal line indicates the performance of a random classifier. The grey circle, black diamond and light grey square indicate the recommended cutoffs for the ID VET recommended dilutions, BioChek and ID VET 1:10 tests respectively.

Table 2.4: GLM of mammal sera VLA H5N1 HI reaction status

Variable	Level	Coef.	s.e.	z	p	OR	95% CI	n Observations
Intercept		2.84	1.60	1.77	<0.01			
ID VET cELISA 1:10		-0.26	0.13	-2.02	<0.05	0.78	0.59:0.99	72
Species	Dog	ref	–	–	–	–	–	37
	Pig	-3.38	1.38	-2.45	<0.05	0.03	0.002:0.51	35

Null deviance = 50.23, df=71. Residual deviance = 24.67, df=69.
OR = Odds Ratio

Although H5N1 presence had not been previously recorded at Maga, this finding is in keeping with data from nearby areas. The Maga dam is linked by direct road routes to both Vélé (approximately 45km) and Maroua (approximately 75km) (Figure 2.1) at which H5N1 virus presence has been confirmed during the same period as this study (Njouom et al., 2008).

The weight of evidence for H5N1 circulation at the study sites comes from data collected from domestic ducks. The majority of M gene positive swabs and both of those that were also H5 positive were collected from domestic ducks. The serological diagnostic tests all performed well with the duck sera samples. In Maroua where the confirmed H5N1 outbreak occurred, none of the sampled ducks were positive in the HI test. However, this is unsurprising as birds in the area were depopulated in response to the outbreak prior to sampling for this study. At the Maga site, 20% of the ducks sampled were positive in the H5N1 HI test and both cELISA tests classified the H5N1 HI status of the ducks very well (Figure 2.3). The cELISAs are relatively simple to perform and read and can both be used as screening tests prior to further characterization using subtype specific HI tests. In this screening context, the sensitivity of the test would be prioritized over its specificity. At the recommended cut-offs for the two cELISAs, 100% sensitivity as compared to the HI results is maintained in both cases and the ID VET achieves better specificity than the BioChek kit. Figure 2.2 shows that these recommended cut-offs are relatively lenient and that in addition to correctly classifying the HI positive samples, they also classify almost all sera with non-zero H5N1 HI titres as cELISA test positive whilst maintaining good specificity regarding the sera with HI titres of <1:2. When comparing the cELISA and HI results it is important to recognize that the cELISA tests are designed to detect antibodies against all influenza A viruses whilst the HI tests are subtype specific. If we assume that the cELISA tests have equal sensitivity for antibodies against all influenza A subtypes, the high specificity of both cELISA tests as compared to the H5N1 HI data for these duck sera, indicate that an H5N1 subtype virus was the predominant if not only influenza A virus circulating in this population at the time of sampling.

The survival of ducks infected with H5N1 viruses has been documented previously

(Hulse-Post et al., 2005; Chen et al., 2006b). Whilst raising concerns about the un-detected spread of viruses, the capacity of ducks to survive H5N1 and influenza A infections more generally, makes them suitable for use as *in situ* monitors of virus presence and circulation. Sentinel ducks have been used in this way to monitor influenza A viruses circulating amongst wild bird populations in Europe and the approach was found to be relatively efficient as compared to wild bird monitoring (Globig et al., 2009). The number of influenza A RT-PCR positive swabs identified through wild bird sampling in this study corresponds with the low prevalence of influenza A detected by RT-PCR in wild birds that has been reported in other studies (Gaidet et al., 2007; Globig et al., 2009). Wild birds, particularly ducks and geese, are the principle reservoir of influenza A viruses and are therefore a desirable target for surveillance. However wild bird surveillance programs face considerable problems with the detectability of responses (Xing et al., 2008). Comprehensive surveys of wild bird populations are a massive logistical undertaking and can also be subject to problems of sampling bias as the success of capture methods varies across species (Fouchier and Munster, 2009; Globig et al., 2009). Few diagnostic tests are validated for use with samples from wild species (Gardner et al., 1996) and some influenza A tests have been shown to perform poorly with wild bird samples (Xing et al., 2008). In this study, insufficient sera from wild birds was available for cELISA testing and none of the sera collected from wild birds were positive in the HI tests. However, the appropriate interpretation of these negative results from wild bird samples is not clear and further work is required before serological data from wild species can be usefully interpreted (Cattoli and Capua, 2007). Overall, in this study as in others, the 'yield' of data from the domestic duck population is considerably greater than that from the wild bird surveillance and the investment required for the wild bird sampling was considerably greater. In Europe, sentinel ducks have been actively placed amongst wild bird populations to monitor the circulation of viruses in this reservoir population (Globig et al., 2009). At the Maga site, where owned and wild birds mix, the owned domestic ducks serve the same function passively, as a consequence of the ecology of the domestic and wild bird populations in this area. In contrast to the wild bird population, the ducks are easy to catch and handle, and the appropriate diagnostic tests have been well validated and give readily interpretable results.

A small number of domestic chickens were sampled at Maga in February 2006 but none were sampled during the second visit in April and no evidence of H5N1 presence was detected in chickens. The surveillance of HPAI viruses using domestic chickens in the context of the backyard poultry keeping seen within the study area is complicated by two key factors. Firstly, chickens are likely to die rapidly upon exposure to HPAI viruses, leaving a very short time window for the collection of diagnostic samples. In the absence of well developed veterinary surveillance infrastructure, laboratory diagnosis of an influenza A infection in chickens is therefore unlikely. The 'signal' provided by the occurrence of poultry mortality could though provide a useful indication of pathogen presence. A systematic questionnaire survey was not conducted as part of this study but *ad hoc* records of poultry die-offs were made. Reports of extensive mortality amongst poultry were recorded at six of the ten villages around the Maga dam covering the period from September 2005 to March 2006 (data not shown). These reports are consistent with the spread of a highly pathogenic influenza A virus, such as H5N1. However, the circulation of other viruses such as NDV in this region complicate the interpretation of die-off reports. The pathogenesis and reported symptoms associated with these two viruses are very similar and in areas such as this, where viruses including NDV are circulating, high mortality amongst bird populations is not uncommon. The 'signal' given by poultry die-offs is therefore less likely to be reported and detected by any surveillance system and is also difficult to interpret. Ducks that were positive for antibodies against NDV were identified at seven of the villages at Maga, including four of the six villages at which poultry die-offs were reported. In this context therefore, it is not possible to use chicken mortality reports as a specific indicator of the possible presence of highly pathogenic influenza viruses such as H5N1 (WHO, 2005).

In contrast to the data from domestic ducks, the evidence of influenza A infections in the mammals sampled at Maga is less clear. The influenza A M gene was detected in two dog nasal swabs collected at Maga but the subtype of the influenza A virus could not be determined. In contrast, none of the pig swabs were RT-PCR positive for influenza A.

When considered as dichotomous classifiers of VLA H5N1 HI response, the performance of the ELISA tests was variable using the recommended cut-off values. Figure 2.5 shows

that this apparent variation between the tests is largely a consequence of the choice of the test cut-offs, with the cut-off for the ID VET cELISA at the recommended test dilutions particularly poorly placed for this sample. The ROC curves for the two sets of tests using the ID VET kit support the manufacturers recommendation to test the mammal sera at more dilute concentrations. At all levels of sensitivity, the specificity of the test run at the recommended test dilutions is better than or equal to the test using a 1:10 dilution for all samples, suggesting that the additional dilution of sera helps to reduce the influence of non-specific factors present in these mammal sera that bind the antigen on the ELISA plate.

When assessed as continuous classifiers in comparison to the H5N1 HI test data the cELISAs all performed relatively well and relatively consistently. The data presented here on the sensitivity and specificity of the cELISA tests relative to the H5N1 HI provide information on the relative performance of these diagnostic tests with these samples, rather than the absolute performance of the tests relative to true disease status. The analysis also considered both the dog and pig data and the summary measures relate to this combined population. The GLM analysis indicates that pigs were less likely to be HI positive than dogs with similar ELISA scores indicating that the tests performed quite differently for these two species. More data from these populations would be required to assess this fully. The ROC curves show that the cELISAs categorize the samples with and without an H5N1 HI non-zero titre relatively well (Figure 2.5) but that there is some loss of specificity. The HI test with the standard protocol using chicken erythrocytes is known to have relatively poor sensitivity for the detection of antibodies to avian influenza A viruses in mammal sera (Stephenson et al., 2003) and we might therefore expect the cELISAs to show relatively poor specificity in comparison. The apparently poor specificity of the cELISAs could also be explained by the presence in these sera of antibodies against other influenza A subtypes which would not be detected by the H5 subtype specific HI test. The pig serum sample with the most positive score in both cELISA tests for example was negative in the H5N1 HI test but strongly positive at a titre of 1:128 against the H3N2 antigen in the HI test.

The GLM modelling of the H5N1 HI test outcome revealed that pigs were significantly less likely to have a non-zero response in this test as compared to dogs (Table 2.4).

However, Figure 2.4 shows that there are a number of samples from pigs with cELISA scores in the same range as those from the dog sera with non-zero H5N1 HI titres. H3N2 and H1N1 influenza subtypes are the influenza A subtypes that are classically associated with swine populations. All of the mammal sera were HI tested against antigens of these subtypes but apart from three individuals with observable non-zero titres against the H3N2 antigen (Figure 2.4), all of the sera were negative. Concerningly, the three samples with detectable H3N2 HI responses have widely variable cELISA scores. The sera were not tested against an exhaustive panel of HI antigens so the circulation of other virus subtypes cannot be ruled out.

The mammal sera were re-tested at the SVA using an H5N1 antigen and a modified HI protocol using horse RBCs, to evaluate the influence of using horse RBCs on the sensitivity of the HI assay. Surprisingly, in these tests that would be expected to have shown greater sensitivity (Stephenson et al., 2003; Meijer et al., 2006; WHO, 2006; Jia et al., 2008; Kayali et al., 2008), no inhibition was detected in any of the mammal sera tested using H5N1, H5N2, or H1N1 antigens at the SVA. This lack of correspondence between the results of the H5N1 HI tests conducted at the two labs is concerning. Both the absolute number of individuals with non-zero titres in the VLA tests and the antibody titres recorded in dog and pig sera were low. However, the re-testing of those sera that gave non-zero titres indicated reasonable reproducibility of these findings at the VLA (data not shown). Previous studies have documented relatively poor reproducibility of HI test titres between laboratories (Stephenson et al., 2007) and given the low titres recorded at the VLA this may have contributed to the difference in results from the two labs. However, the protocol used at the SVA should have had greater sensitivity as compared to that run at the VLA. It is possible that the non-zero titres observed at the VLA were due to non-specific inhibitors present in the dog sera, but, the sera were pre-treated with RDE to minimize this risk (WHO, 2002) and using exactly the same sera and protocols no similar inhibition of haemagglutination was observed using other antigens. This same cELISA test has been used to test sera from 6,859 Italian dogs and in these tests only 2 samples (0.03%) were positive and the two positive sera were also positive by HI and immunofluorescence assays against H3 strains. Although we cannot make direct comparisons between these Cameroonian and Italian dog results, the Italian study provides no indication of poor specificity of this

cELISA when used to test dog samples (De Benedictis et al., 2009). Exploration of the application of standard influenza A diagnostic tests for testing mammal species have consistently identified potential problems with false-negative rather than false-positive findings (WHO, 2006; VanDalen et al., 2009). In addition to the erythrocytes used, the HI tests conducted at the VLA and SVA also differed in the sera pre-treatment protocol and actual test antigen used, both of which could have contributed to the difference in findings observed.

2.5.1 Conclusions

The data gathered from the domestic duck population at Maga provides clear evidence of the circulation of an H5N1 subtype influenza A virus in an area in which it had not been previously recorded. However, the utility of the other animal populations assessed as potential sentinels of H5N1 presence is affected by a number of factors relating to the detectability of responses. The logistical problems associated with comprehensive surveillance of influenza A in wild bird populations have been documented previously and in the ecological context of this study, chicken die-off surveillance would provide a very poor specificity signal of HPAI presence. Previous studies have indicated that the extent of subclinical infections of avian influenza A viruses in mammals may be greater than is generally recognized (Butler, 2006c; Meijer et al., 2006; Kayali et al., 2008) and the data presented here indicate that dogs particularly may show serological evidence of exposure to H5N1 that is associated with infections in local bird populations. However, the sample sizes in this study are small and for the data from serosurveys of mammals to be more useful, further evaluation of the available diagnostic tests is required.

The impact of influenza A in the developing world, where underlying malnutrition, chronic disease conditions and HIV all increase susceptibility to flu complications is generally under-estimated (Schoub et al., 2002) and the economic and nutritional impact of mortality in poultry is also considerable (Breiman et al., 2007). H5N1 is now endemic in a number of African countries (Ortu et al., 2008; Owoade et al., 2008), as of February 2010, 33 African countries have reported confirmed cases of pandemic H1N1 infections (WHO, 2009b), and seasonal influenza is grossly under-reported (Fasina et al., 2007). Given the limited resources and capacities available, and the need to balance

the allocation of resources for influenza A surveillance with continued support for other ongoing health threats in Africa (Breiman et al., 2007), there is an argument for making more efficient use of the basic tools that are currently available for influenza A surveillance. Given the very low level of the baseline information available, and the limited diagnostic laboratory capacities, the surveillance of influenza A in Africa should perhaps be aimed towards technologically simple but comprehensive baseline surveys. The serosurveillance of domestic bird and mammal species should offer a relatively cheap and logistically simple method of collecting essential data concerning the influenza A viruses (including H5N1, H1N1 and non-H5N1 subtypes) that circulate in different species and different regions within Africa, particularly if concentrated at areas of interaction between multiple species including wild waterbirds, domestic animal species and humans. The work presented in this chapter demonstrates the potential value of serosurveillance in a range of domestic animal species for the cost-effective identification of previously unrecognized influenza A circulation. However, it also demonstrates the limitations placed on such a surveillance approach by the current lack of suitable diagnostic tools.

Chapter 3

The Kibera Study Site

3.1 Abstract

The urban slum environment is home to an ever-increasing proportion of the global human population and yet this environment and the disease status of its human and animal occupants are relatively poorly understood. Key characteristics of the urban slum are described and some of the implications of these characteristics for pathogen emergence, transmission and maintenance are explored. Human populations in slums have poor health as determined using a variety of outcome measures and urban livestock-keeping in particular creates an increased risk of zoonotic pathogen transmission in this context. The Kibera study site is representative of an increasingly relevant ecological setting in which the linked surveillance of animal and human health can provide useful data on zoonotic pathogen presence, transmission and risk.

3.2 Introduction

The proportion of the world population that lives in slum areas is projected to rise dramatically within the next 30 years but the human health risks associated with this increasingly important environment are poorly described (Riley et al., 2007). Many of the defining characteristics of slums predispose their occupants to infectious disease

risks including zoonotic diseases. The following chapters describe the zoonotic disease surveillance project established in the Kibera slum in Nairobi, Kenya. This chapter describes the Kibera study site and the need for surveillance of zoonotic diseases in the urban slum context.

3.2.1 The Urban Slum Context

UN Habitat calculate that the current global population living in urban slums is approximately one billion, and that if current trends continue, this will reach 1.4 billion people by 2020 (UN-HABITAT, 2006). In sub-Saharan Africa specifically, the rate of slum growth, 4.53% per year, is higher than that seen in any other global region (UN-HABITAT, 2006). 36% of the 700 million total population in 2003 lived in cities and towns, and this urban population is projected to increase by 300 million before 2030, by which time the majority of sub-Saharan Africans will live in cities and towns (Giddings, 2007). Within many of these sub-Saharan cities and towns, less than 10% of the population live in formal sector housing (Giddings, 2007).

An operational definition for a slum was provided by The United Nations Expert Group in 2002 (UN-HABITAT, 2003). This defines a slum as an area of human settlement that combines, to various extents, the following characteristics: inadequate access to safe water; inadequate access to sanitation and other infrastructure; poor structural quality of housing; overcrowding; and insecure residential status (UN-HABITAT, 2003).

3.2.2 Health and Disease Surveillance in Slums

Most if not all of these defining slum characteristics have intuitive implications for the health and disease risks faced by residents (Unger and Riley, 2007). A survey of urban and slum characteristics or indicators, across developing world cities, revealed direct correlations between high child mortality and indicators including inadequate sanitation, structure durability, overcrowding and lack of access to safe water (Martínez et al., 2008).

Water-borne diseases such as diarrhoeal diseases, cholera, typhoid and hepatitis are commonly associated with poor sanitation and inaccessibility of clean water (Unger and Riley, 2007; Bhunia et al., 2009). A study conducted in the slums of five Indonesian cities found that the household use of inexpensive drinking water purchased from street vendors was positively associated with mortality in under-fives and with diarrhoea in children (Semba et al., 2009), highlighting the risks associated with the poor water and sanitation infrastructure within slums. Overcrowding also contributes to the maintenance and transmission of communicable diseases such as tuberculosis, acute respiratory infections and meningitis (Sclar et al., 2005; Unger and Riley, 2007).

Slum residents are also at high risk of infection with vector borne pathogens. Spatial analysis of Dengue seroprevalence patterns in Rio de Janeiro, Brazil found higher seropositivity in slum areas than in a suburban and central urban locations. The highest seroprevalence occurred in the slum site despite the fact that mosquito abundance was lowest there and is thought to be explained by the fact that the household conditions in slums were best for promoting contact between hosts and vectors (Honório et al., 2009). Areas of high human movements were also identified as seroprevalence hotspots (Honório et al., 2009).

The susceptibility of slum dwellers to communicable disease risks is also higher than many other populations. HIV/AIDS rates are high in urban populations in developing countries (Sclar et al., 2005; Unger and Riley, 2007). Limited access to health care facilities contributes to reduced vaccination rates in slums as compared to other urban environments and poor management of chronic conditions (Sclar et al., 2005). Malnutrition, chronic stress and depression can also all contribute to enhanced transmission of communicable diseases in slum communities (Sclar et al., 2005).

Cities in sub-Saharan Africa compare poorly to other global regions in terms of both access to safe water and improved sanitation (UN-HABITAT, 2006; Martínez et al., 2008) and this corresponds to poor health indicators. In Kenya specifically, a demographic and health survey covering the period 1995-2003 revealed that the percentages of children with both diarrhoea and acute respiratory infections were higher in slum areas as compared to non-slum areas (diarrhoea - 16.5% vs 10.7%, acute respiratory

infections - 19.2% vs. 10.8% for slum and non-slum areas respectively) (Martínez et al., 2008).

A burden of disease study conducted in two slums in Nairobi found that the highest mortality burden fell among children under five and that 77% of all mortality was caused by communicable diseases, maternal, perinatal and nutritional causes (Kyobutungi et al., 2008). Specifically, pneumonia, diarrhoea and stillbirths were the leading contributors to mortality, accounting for nearly 60% of deaths in under fives. In the population aged five years and over, AIDS and tuberculosis combined accounted for nearly 50% of mortality (Kyobutungi et al., 2008). The study indicated that Nairobi slum residents had been affected by the HIV/AIDS epidemic more severely than other sub-Saharan sub-populations, that the overall mortality burden in Nairobi slums was higher than that in a comparable Tanzanian coastal site and that this mortality burden fell disproportionately on the youngest members of the population (Kyobutungi et al., 2008).

Despite these studies that provide clear indications of the scale of the urban slum health problem, the extent of the human health problems associated with the urban slum context are still relatively infrequently assessed and poorly understood. Partly as a function of their inherently informal nature, access to health services is poor across most of the world's slums (Riley et al., 2007). In many cases, the morbidity and mortality data that exist concerning slum areas are gathered at end point facilities such as clinics, hospitals and mortality registers. Data from these sources are likely to underestimate the true burden of disease morbidity, and this in turn contributes to inadequate provision of health service resources (Riley et al., 2007).

3.2.3 Urban Livestock Keeping and Zoonotic Risks

In addition to conditions of high human population density, slum inhabitants often also live in close association with animal populations. The urban poor, who are increasingly concentrated within slums, engage in urban agriculture, including livestock keeping, in response to limited livelihood options and poor food security (Guendel, Accessed 2010). For the individual household, livestock keeping can provide a mechanism for

diversifying household livelihood, enhancing food security and improving dietary diversity (Branckaert and Guéye, 1993; Maxwell et al., 1998; Richards and Godfrey, 2003; RUAF, Accessed 2010). It also has wider societal benefits, enhancing urban food security, contributing to local urban economic development, alleviating poverty, enhancing social inclusion of the urban poor and providing a mechanism for the re-use of urban waste (RUAF, Accessed 2010).

Although keeping animals has the potential to improve urban livelihoods, it also has a number of associated hazards and is considered by some to be indicative of a population in crisis (Richards and Godfrey, 2003). With limited or non-existent zoonosis surveillance or food safety capacity, urban livestock keeping leads to an increased risk of zoonotic pathogen transmission, particularly in rapidly growing urban centers of resource limited countries (Zinsstag et al., 2007).

Growing urban populations create increasing demand for meat and milk products, providing a strong economic incentive for livestock keeping and increases in urban and peri-urban livestock keeping. Animals are moved into urban areas from surrounding rural areas, facilitating the exchange of pathogens and creating hubs of onward pathogen transmission (Fèvre et al., 2006; Acosta-Jamett et al., 2010). Urban livestock are often left to graze and roam freely. This enables both the access of roaming animals to diverse human and animal waste products that may be utilized as food, and in turn the widespread contamination of the immediate environment with the faeces of the livestock themselves (Mantovani, 2001). Urban livestock are often slaughtered close to human dwellings, independent of any existing systems for meat inspection and disease surveillance (Acosta-Jamett et al., 2010; Mantovani, 2001). This creates immediate risks of exposure to zoonotic pathogens in those people carrying out the slaughter and the waste products are often disposed of inappropriately and are thus accessible to other scavenging animals (Acosta-Jamett et al., 2010; Mantovani, 2001).

In the urban setting, and particularly in slums which are characterized by poor water security and inadequate sanitation, close proximity between people, livestock, domestic pet species and wildlife populations has the potential to create considerable risks of zoonotic disease transmission (Richards and Godfrey, 2003). In addition to increased

risks of transmission, urban slum populations are subject to causes of immunosuppression such as malnutrition and high rates of HIV/AIDS which also increase the vulnerability of these populations to zoonoses (Mantovani, 2001).

3.2.4 Linked Disease Surveillance in Animal and Human Populations

The research presented in this thesis from the Kibera study site was carried out within the framework of a larger study designed to investigate the relationships between patterns of pathogen infection in domestic animals and diseases that cause major human morbidity syndromes. The aim of this full study is to examine the relationships between human and animal health, focusing on zoonoses in Africa that are either an emerging disease threat or which exert a substantial disease burden.

The opportunity for conducting this integrated human and animal study was provided by the establishment of a human population-based study by the Kenya Medical Research Institute (KEMRI) and Centers for Disease Control and Prevention (CDC). The KEMRI/CDC human syndromic surveillance study (SSS) is conducted at two sites, the Kibera urban site and a rural site at Asembo bay in the Lake Victoria basin. Together, these sites reflect two ecological systems that are likely to be important in the emergence of human pathogens. The Lake Victoria basin is one of the most densely-populated rural areas in Africa, with extensive transport networks and rapid land-use changes that are likely to facilitate disease emergence. The informal settlement of Kibera in Nairobi, typifies the expansion of urban slums and the population of the urban poor.

The SSS involves biweekly visits to all recruited households during which, data is collected on symptoms and signs of recent illness for all household members. Recently or currently ill individuals showing signs of pneumonia, diarrhoea, jaundice and/or fever are referred to the designated clinic for free clinical evaluation, treatment and specimen collection. In Kibera, the SSS comprises a cohort of approximately 25,000 people from roughly 6,000 households in the Kibera site. The total population of the site is approximately 30,000 people within 8,000 households (CDC unpublished data).

In this thesis the Kibera animal surveillance components of this larger study are presented. The full study is ongoing and all of the animal data discussed were collected

with a view to additional research and analysis to enable linkage of these data with parallel data collected from the human population.

3.2.5   Chapter Objectives

The aims of this chapter are to describe and characterize the Kibera study site, to consider the implications of certain key attributes of the urban slum environment for the maintenance and surveillance of zoonotic pathogens, to provide an overview of the animal study established at this site and to outline the importance of the urban slum environment as a target for zoonotic disease surveillance.

3.3   The Kibera Study Site

3.3.1   The Kibera Slum

By 1995, roughly 60% of the total population of Nairobi occupied informal settlements (UN-HABITAT, 2003; da Cruz et al., 2006). Natural growth and rural-to-urban migration have continued to increase the size of Nairobi's slum population, with 75% of all urban population growth absorbed by slum areas (da Cruz et al., 2006). Across Nairobi's slum areas as a whole, the majority of households occupy single rooms and residents typically earn low incomes and have limited assets (UN-HABITAT, 2003). Only 22% of Nairobi's slum households have water connections (da Cruz et al., 2006).

Kibera is frequently described as the largest informal settlement area in sub-Saharan Africa (Richards and Godfrey, 2003; da Cruz et al., 2006). Estimates for the total population of Kibera now range from 600,000 to 1,000,000 within a total area of approximately 2.56km^2 (Candiracci and Syrjänen, 2007). There is no access to formal urban sanitation services within Kibera and household waste is dumped within the slum (Gulis et al., 2004). The river and railway lines which form the upper and lower boundaries of the study area (Figure 3.1) are used as principle dumping grounds (Richards and Godfrey, 2003). Most residents use communal pit latrines, communal areas or plastic

bags for human and domestic waste disposal (Candiracci and Syrjänen, 2007) and a single pit latrine may serve up to 150 people (Richards and Godfrey, 2003).

3.3.2 Study Area Description

The Kibera animal study site includes the three adjoining villages of Gatwikira, Soweto and Kisumundogo, located at the western end of the Kibera informal settlement in Nairobi. The study site has a total area of 0.53km^2 and is bounded by a railway line along the northern edge, a river along the southern edge and a major path along the western edge of the site (Figure 3.1).

Figure 3.1: Outline map of Kenya with Nairobi indicated and satellite image of Kibera with outline of study area indicated

The study site for the animal project included but exceeded that of the KEMRI/CDC SSS which was ongoing throughout the period of this project in the Gatwikira and Soweto villages. The combined area of these two villages is 0.39km^2.

A recent survey of animal ownership practices within the Kibera study site revealed that the most commonly kept livestock species was chicken, with 8.2% (95% CI: 7.5-9.0%) of the 5549 households surveyed keeping chickens. Large livestock species keeping was

however rare, with less that ten households keeping each of cattle, sheep, pigs and goats. Approximately 50% of households reported keeping a cat whereas 2.1% of households kept one or more dogs (CDC unpublished data).

3.3.3 Household Identifiers

All households with the KEMRI/CDC SSS study site are allocated a unique household identifier code. This code describes the hierarchical classification of each household within a cluster and structure. The SSS site is divided into 10 clusters based on existing geographical boundaries such as streams and major paths (Figure 3.2). Within each cluster, every structure has a three digit identifier and within each structure, each household (defined as those people using a common cooking site) has a two digit household number. Every household within the KEMRI/CDC SSS site therefore has a unique identifier in the format 01/001/01. This system of household identification was adopted by this animal study to ensure correspondence with the KEMRI/CDC SSS at the household level.
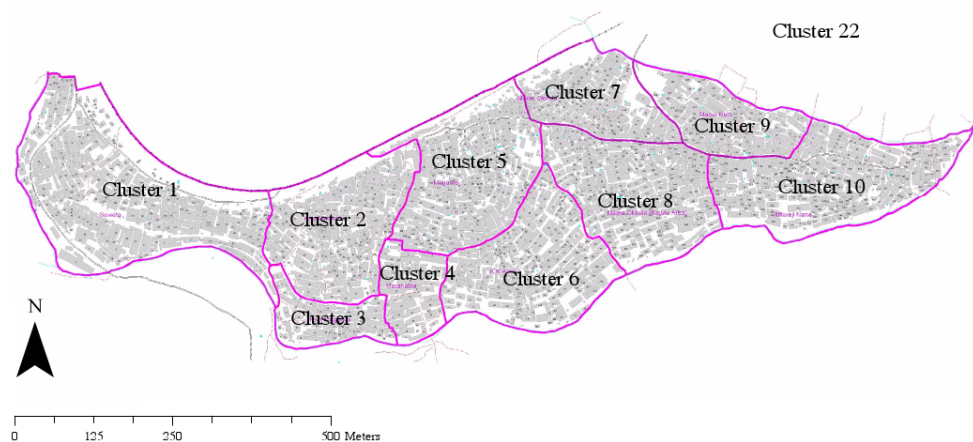
Figure 3.2: Map illustrating the distribution of structures and clusters within the Kibera SSS study area and the location of the adjacent Kisumundogo village, coded as Cluster 22. Grey polygons show the location of structures. Pink lines mark the boundaries of each cluster. Map adapted from original created by the Kenya Bureau of Statistics

At the beginning of the study, the project local representative and data handler spent two days with a member of the KEMRI/CDC project training in the use of this numbering system. Ongoing correspondence was maintained through the use of reference maps drawn up by KEMRI/CDC and the physical numbering of structures and households with painted reference numbers.

3.4 Animal Study Overview

The field component of the Kibera animal surveillance study was conducted in two principal sampling phases (Figure 3.3). The first phase started in July 2007 and ended in December 2007. Six sampling visits (A-F) were made to households enrolled in the dog cohort survey and two cross-sectional surveys of the domestic bird population were conducted. At the beginning of 2008 the field study was interrupted by the political unrest that occurred in Kenya following the national elections held in December 2007. The disturbances that occurred within Kibera at this time prevented the continuation of field sampling until roughly one year after the start of the study. The second phase of sampling took place in September and October 2008. During this period, one follow-up check of the dog cohort population (08F) and two further sampling visits (G and H) were conducted, and a cross-sectional survey of the rodent population was conducted. Questionnaire surveys relevant to all three animal populations took place in October and November 2008. The methodology for the surveys of the dog population are described in the next chapter (See Chapter 4). Details of rodent and bird population surveys are provided in Chapters 6 and 5 respectively.

3.5 Discussion

The global countryside has already reached its maximum population size. After 2020 the rural portion of the global population will begin to shrink and virtually all future human population growth will occur within cities (Davis, 2006). 95% of urban growth will occur in developing world countries and in contrast to the developed world, in
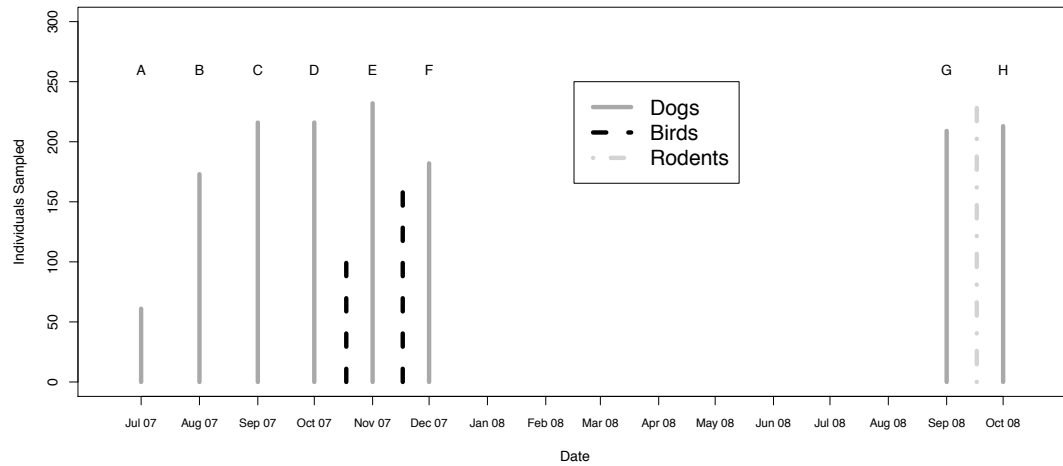
Figure 3.3: Timeline illustrating the two phases of sample collection and number of individuals of each type sampled. The letters at the top of the figure indicate the dog sampling visit ID.

which urbanization is consistently associated with economic growth and industrialization, urbanization in the developing world often continues despite economic stagnation or contraction, leading to a huge global growth of slums (Davis, 2006). In Africa, slums now grow at twice the speed of 'cities' and by 2020 sub-Saharan Africa will have 393 million slum-dwellers, a number that will double every fifteen years (Davis, 2006; UN-HABITAT, 2006).

The data on human and animal health in urban slum settings are sparse but those that exist create a concerning picture. On many health measures, slum residents have worse outcomes than occupants of more developed urban areas or rural sites (Sclar et al., 2005; Unger and Riley, 2007). The defining characteristics of urban slums: high human population density, limited availability of clean water and poor sanitation create ecological conditions in which numerous well described infectious diseases can pose considerable threats. High human population density is also of concern with respect to 'new' pathogen emergence as it has been identified as a correlate of pathogen emergence or re-emergence (Jones et al., 2008) and the modern urban slum environment is a clear example of a novel environment that provides opportunities for pathogens to exploit new ecological niches (Morse, 1995; King et al., 2004).

We know that the urban slum will be the home of an increasing proportion of the human population in the future, that the health of slum residents is poor, that the presence of livestock and wildlife animal species creates risks of zoonotic pathogen transfer and that the slum environment is also of concern as a likely site of future pathogen emergence. We also know that we currently have only limited understanding of the ecology and epidemiology of the urban slum and that considerable research and surveillance is required to address the challenges of slum population health. The following chapters present data on the surveillance of two key zoonotic pathogens, influenza A and *Leptospira* spp. in the Kibera urban slum.

Chapter 4

The Kibera Dog Cohort

4.1 Abstract

A domestic dog cohort of 637 individuals was recruited to carry out longitudinal pathogen surveillance at the Kibera study site. In addition to the collection of samples for diagnostic testing, data describing the demography of the dog population and the ecological interactions of this population were collected. The data were analysed to assess some of the practical and ecological factors that might influence the overall utility of the Kibera dog sentinel population for pathogen surveillance. The human and dog population density are both very high at the Kibera site. Dogs are rarely confined and they obtain food from multiple sources, providing frequent and varied opportunities for interaction with other species and exposure to a range of pathogens. There is considerable puppy mortality in the Kibera dog population but the survival hazard declines with age and the population that survives the first few months of life is relatively stable. Dogs were readily accessible for sampling, they could be reliably identified at repeat visits and they could be accurately aged. The overall proportion of sampling attempts that were successful was 80% and no major practical obstacles to the successful use of dogs as sentinels were identified.

4.2   Introduction

Most of the data from the Kibera study site were gathered from the domestic dog cohort population. In addition to collecting samples for diagnostic testing, the cohort study was also designed to collect and use demographic and descriptive data about the sampled individuals and the households at which they live. The data gathered in Cameroon (See Chapter 2) demonstrated the potential for using dog sentinel serosurveillance to detect the presence of influenza A viruses. These and other data presented later in this thesis address the first key hypothesis related to the use of dogs as sentinels - that a response to the pathogen of interest is detectable in the sentinel population. There are though, additional questions that could and should be addressed to use and evaluate data collected form dog sentinel populations, and additional data are required to do this.

4.2.1   Demographic Data and Sentinel Assessment

Demographic data can provide direct evidence on the relationship between contextual factors and the levels, patterns, and causes of death and other indicators of pathogen presence (Chandramohan et al., 2008). With reference to the framework presented in Chapter 1 (See Figure 1.2), many of the analyses described in this chapter refer to the demographic characteristics of the dog sentinel population such as age and sex that may well impact upon the response that a given individual mounts to the presence of a pathogen and on the repeatability of that response across the population as a whole. In the following chapters (particularly Chapter 7), the contextual data described here are used to assess the degree to which the sentinel population varies in its response to the pathogen of interest and to look at the correlations between observed sentinel responses and contextual variables.

The data examined in this chapter also relate to the practical and ecological factors that might influence the detectability of dog sentinel responses and the ultimate utility of dog as sentinels of pathogen presence in the Kibera context. The proposed attributes of dogs that make them suitable for use as sentinels include being broadly accessible for handling, being safe to handle, being readily identifiable and being relocatable (See

Section 1.8). Several of the analyses presented in this chapter address these attributes and the following key questions: Are the dogs in Kibera accessible? Can they be located, reliably identified and sampled? It is also important to describe the dog population in order to be able to assess 1) the degree to which the sample is representative of the total population and 2) the external validity of this population.

4.2.2 Longitudinal Patterns and Dog Age

The longitudinal nature of the cohort study design also allows assessment of temporal patterns in sentinel responses and for the study to address particular surveillance questions. To interpret serological data particularly, it is useful to know how old individual dogs are and how long the measured response can persist for. Good quality age data can be used to look at variation in seroprevalence across age groups and also at different times to make inferences about the epidemiology of a given pathogen. Previous studies have used age seroprevalence data to address a range of different questions: to look at changes in the epidemiology of pathogens over time (Ades and Nokes, 1993); to make inferences about the timing of epidemics (Kock et al., 1999; Lembo et al., In Prep.); to investigate patterns of acquired immunity (Welburn et al., 2008) and to evaluate the success of disease control programmes (Letaief et al., 2005).

To address all of these questions you need accurate data on the age of sampled individuals. Studies looking at domestic dog populations have used owner-reported ages to make inferences about the planning of rabies and population control programmes (Butler and Bingham, 2000; Ratsitorahina et al., 2009). Such studies also often use broad descriptive age classes such as mature/young/juvenile but the difficulty of such an approach and the impact of this uncertainty upon inferences made has been noted (Ratsitorahina et al., 2009). Despite its wide use, the accuracy of owner-reported dog ages has not been validated or assessed previously. In this chapter we assess the consistency and accuracy of owner-reported ages (See Section 4.4.2).

4.2.3 Chapter Objectives

The overall aim of this chapter is to describe the domestic dog cohort study established at the Kibera site and examine the effect of practical and ecological influences upon the potential use of the Kibera domestic dog population for sentinel surveillance of zoonotic pathogens at this site. Particular aims are to describe the demography of the dog population, the relationships and interactions between the human and dog populations, the accessibility of the dog population for successful sampling/surveillance, the degrees to which the enrolled and sampled dog populations are representative of the total dog population at this site and to assess the influence of these factors upon dog sentinel utility.

4.3 Methodology

An open, prospective, population-based cohort study of the Kibera domestic dog population was conducted. The outcomes of interest were dog mortality and dog exposure to *Leptospira* spp. and influenza A. The influenza A data are discussed in Chapter 5. The data relating specifically to *Leptospira* spp. are discussed in Chapters 6, 7 and 8. Dogs were enrolled and visited at the households at which they were resident. Households included in the cohort study were visited for repeat sampling and descriptive data collection as well as a single questionnaire survey conducted at the end of the study period. In addition, transect surveys were conducted to estimate the size of the total dog population within the study area. The detailed methods for each of these components of the study are given in the following sections.

4.3.1 Defining Dog Ownership

The majority of dogs encountered and enrolled by this study were clearly 'owned' by a single household. However, some dogs did not belong to any one household but were communally owned or supervised by a number of households or individuals. Dog households were therefore defined under one of two ownership definitions (adapted from Knobel et al. (2008)) at the time of enrolment:

Dog-owning households (DOHH) where residents of a single household claimed ownership of the dog(s)

Dog-supervising households (DSHH) where dogs were not owned or claimed by any identifiable individual or household but were consistently resident at a specific location.

In the case of DSHH, residents around the area of dog residence were consulted to identify an individual who was considered most responsible for the dog(s) and consent for sampling was sought from that individual. This person was questioned to clarify that they were at least as responsible for the dog(s) (if not more) as any other members of the local community. The term dog-keeping household (DKHH), includes both DOHH and DSHH and is used as an umbrella term to describe all households with which dogs were associated.

4.3.2   Sampling Frame Identification

Two different protocols were used to identify DKHH. In the villages of Gatwikira and Soweto, the sampling frame of DKHH was generated through a cross-sectional household survey integrated within the KEMRI/CDC SSS household visit protocol. For one round of sampling visits conducted from the 16th to 27th April 2007, all households visited through the KEMRI/CDC SSS (n=6103) were asked additional questions regarding dog-keeping by the KEMRI/CDC project Community Interviewers. Household respondents were first asked if the household kept any dogs, and at those households that responded yes, the name of the head of household and the numbers of dogs and puppies (aged $\leqslant 6$ months) were recorded. The household identifiers of all responding and non-responding households were recorded. At the time of this survey, dog-keeping was essentially defined by the interpretation of the respondent.

The data collected through this survey constituted a sampling frame for the recruitment of DKHH and a rough census of the kept dog population within the villages of Gatwikira and Soweto (as the survey was only conducted at households participating in the SSS, dogs kept at households not enrolled in the SSS would have been omitted).

In Kisumundogo, which is not included within the SSS study area, DKHH were identified through a combination of techniques including key reporters (local representatives with previous knowledge of DKHH), word of mouth, and by simply walking throughout the village looking for dogs on the streets and attempting to identify where they were resident. DKHH were allocated a Household ID of identical format to that used within the KEMRI/CDC SSS study area.

4.3.3 Household and Dog Recruitment and Exclusion

Initial sample size calculations were made using data from the household survey of dog ownership conducted by the SSS team in which 1.2% (95% CI: 0.9-1.6%) of responding households were found to keep one or more dogs. Extrapolating this proportion of households that keep dogs to estimate the total number of DKHH within the 8000 households in the study area (to include non-responding households and households not enrolled in the CDC study) yielded an estimate of 99 dog-owning households within the SSS study area (95% CI: 75-129). The mean number of adult dogs (>6 months) kept at the DKHH was 1.9. Assuming that this estimate was representative for all DKHH (See Section 4.4.1 for assessment of this assumption), the size of the total adult dog population within the SSS study area was estimated at 189 dogs (95% CI based on CI for number of DKHH: 143-246). Allowing for a total population at the top end of this confidence interval (250 dogs), the sample size required to allow the detection of the presence of a pathogen with 2.5% prevalence in a total population of 250 animals with 95% confidence was calculated (Dohoo et al., 2003). This prevalence level was not selected on the basis of the expected prevalence of either of the two study pathogens, but to generate sample size estimates sufficient for the detection of any pathogen present at only low prevalence in the population. This yielded a required sample size of 95 dogs. All households identified as keeping one or more dogs and/or puppies by the CDC household survey were approached for recruitment into the study. Additional households were recruited after identification through the house-to-house and transect surveys.

All DKHH at which informed consent for participation in the study was provided (See Section 4.3.4) were enrolled, and all dogs resident at enrolled households were recruited.

The cohort was open to new dogs throughout the course of the study. During 2007, records were kept of dogs observed within the study site that were not known to be enrolled in the study. The locations of these *ad hoc* sightings were recorded and added to a continuously maintained list of non-enrolled dogs. During 2007, new households identified through this methodology were recruited into the study at the next sampling visit as permitted by logistical and time constraints. The method of identification (CDC survey, house-to-house survey or transect) was recorded for all study households. New dogs were recruited at enrolled households as they were acquired by or born at the households.

The cohort study was interrupted by the political unrest that occurred in Kenya around the time of the national elections held in December 2007. The continuation of cohort sampling at the beginning of 2008 was prevented and the cohort was instead re-visited roughly one year after the start of the study for follow-up. During the 2008 follow-up no additional DKHH were recruited but new dogs present at previously enrolled households were recruited and sampled.

Individual dogs left the cohort when they died or moved out of the study site. In addition, dogs that could not be handled at three successive household visits were excluded from the study. The reason for loss from the cohort was recorded for all individuals. Households were lost from the study if there were no enrolled dogs remaining at the household.

The sample size calculations described above were based on the assumption that the mean number of adult dogs calculated from the CDC survey of only those households that were enrolled in the SSS was also representative of the mean number of adult dogs present at DKHH that were not enrolled in the SSS. Although data on participation in the SSS was not collected directly, both the village in which households were located and the method of household identification are likely to be strongly associated with SSS enrolment. Only those households that were enrolled in the SSS were recruited through the CDC ownership survey and although some SSS enrolled households may have been enrolled through house-to-house and transect identification in Gatwikira and Soweto, none of the DKHH in Kisumndogo were enrolled in the SSS. To check the validity of this assumption and the impact of these study design parameters upon the data recorded,

the data on dog numbers collected during the household questionnaire were compiled and analysed to assess the influence of ownership type, method of identification and village upon dog numbers at the household. These effects were assessed by fitting a generalized linear model with poisson errors to the number of dogs at each household. The influence of these variables as predictors of dog number were assessed by comparing the intercept only null model to univariate models using likelihood ratio tests (Bolker, 2008).

4.3.4 Informed Consent

Written informed consent for participation in the dog cohort study was obtained from all households at the time of enrolment. A copy of the consent form used is given in Appendix A. The consent form for participation in the dog cohort study described the types of samples that would be collected from the dogs and the vaccination and treatments that would be offered by the study (See Section 4.3.6.3). The risks and benefits associated with sampling and treatments were described in the consent form. It was made clear to the household representative that the provision of vaccination was not dependent upon the provision of consent for the sampling components of the project. It was also made clear that refusal of consent for any or all components of the study would not have any negative effect upon the members of the household (other than non-provision of dog anti-helminthics which could be provided at follow-up visits) and that households could withdraw from the study at any time. The content of the consent forms were approved by the Kenya Medical Research Institute Scientific Steering Committee and Ethical Review Committee.

In addition, supplementary consent was later obtained from the majority of participating households to link the data obtained through the cohort project with that obtained from the same households by the KEMRI/CDC SSS study and with the data obtained through questionnaire surveys of sampled households. Consent documents enabling the linking of data collected through this study with data obtained from the same households by the KEMRI/CDC SSS study were also approved by the CDC Internal Review Board.

4.3.4.1 Consent Procedure

The head of household or other adult household member (aged 18 years or older) was identified at each household approached to take part in the study and the project explained to them. The consent form was read to the respondent in full by the project vet or data handler. Consent documents were administered in English, or using Dholuo or Kiswahili translations, as appropriate according to the preference of the respondent. Following the provision of consent, two copies of the consent form were signed by the respondent or witness. Witness signatures were obtained at those households at which the identified respondent was unable to sign the form him/herself. In these cases the forms were signed by the witness who testified that he/she had witnessed the consent procedure in full and confirmed the provision of consent by the named respondent. One copy of the signed consent form was retained by the project whilst the second signed copy was retained by the respondent. In addition to the signature of the respondent or witness, the name of the respondent (and witness where appropriate) and the unique household identifier code were recorded on the project copy of each consent.

4.3.4.2 Consent at Follow-Up Household Visits

At follow-up visits to enrolled households, verbal consent from a responsible representative of the household was obtained before any animal handling, sampling or treatment was conducted. Animal handling and sampling was therefore dependent upon the presence of a responsible household member at the time of the household visit. Information about the risks associated with anti-helminthic treatments for dogs were explained to the household member by the project vet and verbal consent provided before anti-helminthics were administered at every visit.

4.3.5 Animal Handling, Safety Measures and Waste Procedures

Training was given to the sampling team in the safe handling of animals and all domestic animals were manually restrained for sample collection by a member of the project team. Dogs were muzzled as necessary and any animals which could not be safely restrained

were not handled. This event was extremely rare. Pre-exposure vaccination against rabies was offered to all members of the field teams involved in handling dogs and paid for by the project. All field personnel were provided with a protective overall or lab coat and gum boots for all animal handling and sampling visits. Disposable examination gloves were worn for animal and sampling handling and changed between households. Alcohol handwash, clean water and a basic first aid kit were carried by the field team. All clinical waste generated during sampling was collected by the sampling team and transported at the end of each day to the KEMRI/CDC laboratories for disposal by incineration. All used needles were discarded into a sharps container carried by the sampling team which were also incinerated at KEMRI/CDC when full.

4.3.6 Dog Household Visits

All currently enrolled households were sought at every study visit (A-H) (Figure 3.3), with the exception of 18 households that could not be visited before the end of Visit F in 2007. Each round of household sampling visits lasted for 17 days and within the two phases of data collection, visits were conducted at intervals of 28 days. The order in which households were sought within each round of visits was determined by their location and the days of the week on which the respondents were likely to be present. Households that were located near each other were likely to be visited on the same or consecutive days, with the exception of households that could only be visited on Saturdays which were more widely distributed across the study area. The order in which households were sought was maintained as far as possible across all visits so the interval between visits at any given household was 28 days. When a household respondent could not be identified on the day on which they were sought, the household was revisited on subsequent days within the same sampling visit where possible, in an attempt to include the household in that sampling round. The GPS coordinates of all enrolled households were recorded and the unique household identifier recorded on every occasion that data was collected.

4.3.6.1 Dog Identification

Dogs enrolled in the study were allocated a unique identification code based on the village in which they were located and a number reflecting the order of recruitment. The name, age, sex and physical description of the dog was recorded for each individual at the time of enrolment. Digital photographs of each dog were taken at the time of enrolment and at all subsequent visits at which any significant change in the appearance of the individual was observed. Most puppies under approximately six months were photographed at every visit because of their rapid growth rate. Two photos were taken of each individual (one front view and one side view) and all photos also included an identification board labelled with the household and dog identifier codes. Additional confirmation of dog enrolment was provided through the use of identification collars (Figure 4.1). Adult dogs were fitted with a bright collar at the time of enrolment and re-fitted at follow-up visits when the existing collar was either damaged or missing. Collars were made using polypropylene strapping tape and closed metal seals that were covered with insulating tape prior to sealing. These collars were not fitted to puppies that were still growing rapidly due to their semi-permanent and inflexible nature.

Figure 4.1: Example photographs of an individual dog taken at the time of enrolment. Digits on the board record the household and dog identifiers. Highly visible green identification collars were fitted at the time of enrolment.

4.3.6.2 Dog Data Collection and Sampling

All dogs enrolled at each household were sought at the time of each household visit. The form used for dog data collection at each household visit is provided in Appendix B. The descriptive identification data recorded for all dogs were carried by the data handler at all subsequent visits. These data were checked and updated as necessary at each visit. When new dogs were present and when a dog found at a study household could not be matched with confidence to a previously enrolled individual, a new unique ID was allocated at the time of data collection and identification photos taken. These photos and details were then checked against records and photos for enrolled individuals and either matched to an existing dog ID or confirmed as a new individual. Additional data recorded at each visit included the daytime and night-time confinement status of each dog as reported by the respondent. At visit F, the observed confinement status of every dog at the time of the household visit was also recorded in order to assess the accuracy of the owner reported measure. The presence/absence of an existing collar and fitting of new or replacement collars was recorded at every visit, as was the collection or provision of the samples and treatments described below. Enrolled dogs that were reported as still present but that were not observed at the household during a household visit were recorded as 'not present' and the data fields requiring the observation or handling of the dog were completed as 'not done'.

Blood samples of maximum 10ml were taken from the cephalic vein. The area of skin above the vein was swabbed with surgical spirit and the hair clipped as necessary. Blood was collected using 21 gauge S Monovette needles and S-Monovette collection tubes (Sarstedt AG & Co.). Sample volumes were appropriate for the size of the individual animal (i.e. no more than 0.9% body weight for adults). Blood samples were collected from puppies when considered safe by the project vet according to the mass and condition of the individual, and samples were rarely collected from puppies less than 3 months old. All blood sample tubes were labelled at the time of collection with the household ID, a unique animal/sample identifier code and the date of sample collection.

Immediately after collection, all samples were stored in a cool box with ice packs carried by the sampling team. At the end of daily sampling, the samples were taken to the

labs at KEMRI/CDC for processing and storage. Blood samples were processed on the afternoon of collection or stored upright at 4℃ overnight before processing the following day. Whole blood was centrifuged at 2000 x g for 10 minutes at room temperature in the collection tube to separate serum. Duplicate serum aliquots were transferred into two sterile cryovials using a sterile disposable transfer pipette. All handling of untreated sera was conducted in a microbiological safety cabinet. Serum samples were labelled with the full household and individual ID from the sample tube as well as the date of collection, visit number and aliquot number. The blood clot remaining in the collection tube and the sera aliquots were listed and stored at -20℃.

The collection or non-collection of each sample (distinguishing failed attempts at collection and non-attempts) was recorded for each dog in the dog record sheet completed for every attempted dog handling visit (Appendix B). Data from these paper forms was manually entered into a sample database and matched to the sample lists created upon return to the lab to identify any errors in sample labelling, data entry and/or missing samples.

4.3.6.3   Incentives and Veterinary Interventions

Dog rabies vaccination was provided free of charge at all DKHH at which verbal consent for vaccination was obtained. The vaccination did not constitute a key part of the epidemiological research project but it was considered ethically responsible to vaccinate dogs that were handled during the course of the study. The provision of vaccines was also important in building a relationship within the community. All dogs older than three months that were present at study households and could be safely restrained were vaccinated against rabies (1ml Rabisin (Merial Ltd.) administered subcutaneously). A vaccination certificate was provided for the dog owner. In addition, vaccine was administered to cats presented to the field team during house-to-house visits, and to non-enrolled dogs encountered in and around the study site upon provision of verbal consent from the owner.

The project also provided anti-helminthic treatments for enrolled dogs. Ivermectin treatment was provided to all dogs older than six months that were present at the

household, could be safely restrained and for which owner consent was provided. Ivermectin can cause adverse reactions in a very small number of dogs (principally in specific breeds not encountered at the study site) (Merck & Co., Inc, 2005). These risks were explained and verbal consent confirmed prior to the administration of ivermectin at each visit. Ivermectin (Ivomec, Merial Ltd.) was administered subcutaneously using a 1% ivermectin preparation at a dosage of 0.1ml per 5kg of body weight. Antihistamine was carried by the team for use in the event of an allergic reaction to ivermectin administration in any of the dogs treated. Dogs with any characteristics of those breeds at higher risk of adverse reaction with ivermectin and all puppies were instead treated with Canex Multi Spectrum All Wormer (Pfizer. Active ingredients: Pyrantel embonate, Oxantel embonate and Praziquantel) administered by the project vet. Canex was provided at a dosage of one tablet per 10kg of animal weight. When requested by owners, Canex was also provided for the cats present at the household.

Ivermectin is a GABA agonist that acts to cause paralysis in susceptible arthropods and nematodes, leading to the starvation and expulsion of parasites (Merck & Co., Inc, 2005). It is used extensively to control a variety of parasitic nematodes in domestic animal species. In addition to this parasite-specific mode of action, there is some evidence that ivermectin can also influence the immune system in lab animals and humans. The studies conducted thus far have yielded variable indications of immunomodulatory effects, with some observing enhancement of antibody production against antigens unrelated to the parasite target of treatment, some observing a reduction in antibody responses at some timepoints and others reporting no influence at all (Sajid et al., 2006). The assessment of the influence of ivermectin and Canex administration upon the antibody responses measured in the Kibera dog cohort is discussed in Chapters 5 and 7.

4.3.7 Analysis of Dog Ages

Dog age was not requested or provided at every visit, instead the age of most dogs was recorded at the time of enrolment and again at one of the follow-up visits in 2008. For those individuals where any numeric age was provided, an age in months at each visit was generated through extrapolation from the first reported age for each individual.

This extrapolated age variable was calculated using the first numeric age provided in months and the number of days since the provision of that age. A month was defined as 30.4375 days (=365/12) (Hosmer et al., 2008). The calculated age variable was used to assess the validity of additional owner reported ages using 398 additional numeric ages provided at later visits. A linear regression model was used to assess the relationship between the calculated and additional owner reported ages.

For some individuals, no numeric age was provided and instead, qualitative responses such as "Adult" or "Old" were given. To allow consideration of this qualitative data, the calculated age variable was categorized and an additional "Adult Unknown" class created to accommodate the non-numeric ages provided.

4.3.8 Evaluating Sampling Success

The potential influences of household ownership type, village and method of identification upon respondent presence at the time of household visits were assessed using Fisher's exact test or chi-squared tests as appropriate.

Because samples were collected at repeat visits to enrolled dogs, the influence of potential covariates upon sampling success was modelled at the dog level. The modelled response variable for each dog was the proportion of sampling attempts that were successful, weighted by the number of attempts. A sampling attempt was defined as a visit to an enrolled dog where the household respondent was present and the dog was aged >3 months. A success was defined as the collection of a blood sample. The binomial mixed model was fitted with the household ID as the random effect. The covariates considered in the model were the household ownership type, method of identification and village as well as the sex of the dog and categorized dog age at recruitment. Models were fitted with a quasibinomial error distribution to accommodate overdispersion and compared using F tests (Crawley, 2002) as well as AIC and BIC values.

4.3.9 Questionnaire Survey

A questionnaire survey of DKHH enrolled in the study was conducted between 27th October 2008 and 11th November 2008 at the end of the cohort study. Consent for questionnaire administration and linkage of the data collected with the existing sample data was obtained as described in Section 4.3.4, and the head of household or an adult relative was interviewed. Interviews were conducted in English or DhoLuo as appropriate according to the preference of the respondent. Household-level questionnaire data included information regarding the ownership of different animal species, the motivation for dog ownership, details of those people at the household who were principally engaged with caring for the dogs and details of any dogs that had left the household within the previous 12 months. Data collected for each dog present at surveyed households included information on the dog's origin and date of acquisition, sickness in the previous 12 months, previous vaccination, feeding practices and a reproductive history for all adult females. The body condition score of all adult dogs present at the household at the time of the questionnaire interview was also assessed and recorded using a five state scale adapted from GFAH (2007). A copy of the questionnaire used is provided in Appendix B.

4.3.10 Dog Survival Analysis

Survival analysis was conducted to evaluate the impact of key demographic and study design parameters upon the mortality of dogs enrolled in the cohort study. The analysis covers the 16 month period between the enrolment of the first dog at the end of June 2007 and the end of the study at the end of October 2008. The survival status of each dog was recorded for each attempted household visit. Dogs were recorded as alive when seen at the household visit or reported to be still alive by the household respondent. At household visits where no respondent could be identified, dog survival status was recorded as unknown. When dogs were reported absent, the fate of the dog was recorded. In this analysis, the event of interest was the death of an individual dog. Dogs were considered to have died if their reported fate was one of: died, disappeared, or fate unknown presumed dead.

Because of the periodic nature of dog status assessments (including the long interval between visit F in 2007 and follow-up in 2008), these data were modelled on the time scale of visits using techniques for asynchronous interval censored data (Radke, 2003). Dogs that died during the period of study were handled as interval-censored observations, where the exact time of death was unknown but had occurred in the interval between the visit at which the dog was last known to be alive and the visit at which the death of the dog was reported. Dogs that survived for the duration of the study, and those that were excluded for reasons other than death (recorded fates of stolen, given away, sold, taken/moved out of the study site or excluded for logistical reasons) were handled as right censored observations, where the exact time of death of the dog was unknown but must have been after the time at which the individual was last known to be alive. Survival time was modelled on the scale of study visits, which represent 28 day intervals. The interval between visit F in 2007 and the follow-up in 2008 was fitted to the same scale so that visit G occurred 10 'visits' after visit F.

The influence of the following potential covariates upon dog survival time were assessed - dog sex (M or F), age at enrolment ($\leqslant$3mo or >3mo), the household ownership type (owned or supervised), method of household identification for recruitment (CDC survey, house-to-house or transect) and the village (Gatwikira and Soweto or Kisumundogo). No time-dependent covariates were included. Age was handled as a factor, considering the categorized age of the individual at the time of enrolment. Covariate effects were initially explored using non-parametric Kaplan-Meier estimations of survival functions. This technique does not allow the consideration of interval censored data and for these exploratory analyses, the survival time for individuals that died was approximated by setting exact times of event at the mid-point of their censoring interval. The use of approximations such as this in models of survival time can lead to underestimation of both effect size and variance (Radke, 2003). For this reason, correct coding of the censoring interval was used for the regression analysis of survival time. Data were analysed using accelerated failure time (AFT) parametric regression models with interval and right censoring, using a Weibull distribution of survival times.

$$ln(t) = \beta_0 + \beta_1 x + \sigma \times ln(\epsilon) \qquad (4.1)$$

Equation 4.1 gives the general formula for the Weibull model, where $ln(t)$ is the natural log of the survival time, β_0 is the intercept, $\beta_1 x$ is a linear combination of the covariates and $\sigma \times ln(\epsilon)$ is the error term distribution. In the case of a Weibull model, $1/\sigma$ is the shape parameter from the Weibull distribution of survival times. When $1/\sigma$ is greater than one, the hazard (the instantaneous risk of death (Crawley, 2002)) increases over time, when $1/\sigma$ is less than one, the hazard decreases over time. If σ is equal to 1, the Weibull model can be simplified to an exponential model (Dohoo et al., 2003; Hosmer et al., 2008).

All analyses were conducted in R (R Development Core Team, 2009) using the *survfit* and *survreg* functions of the *survival* package (Therneau and Lumley, 2009). A step-wise method was used for model building and survival models were compared using likelihood-ratio tests using a chi-square test of significance and a significance cut-off of $p \leqslant 0.05$. Two-sided Wald tests using a cut-off of $p \leqslant 0.05$ were used to describe the risk factors included in the multivariate model. The suitability of the Weibull distribution was assessed by plotting a cumulative hazard plot of the observed data (Dohoo et al., 2003; Klein and Moeschberger, 2003). The fit of the final multivariate model was assessed using plots of deviance and residuals against the parameters estimated in the model (including the shape parameter) and the survival times predicted by the model. The overall fit of the model was also assessed using a plot of log cumulative hazard against log time for each group as defined by the covariates in the final model (Klein and Moeschberger, 2003).

4.3.11 Dog Population Estimation

A capture-mark-recapture approach was used to estimate the size of the total dog population within the animal study area. The enrolment of dogs into the study constituted the initial capture and mark. Transect surveys at which dogs were recaptured were conducted at intervals throughout the study period to generate approximate population

estimates and to identify non-enrolled dogs. A variety of different routes were defined and used during the entire study but only a subset of the data generated were used to estimate the population size presented here to avoid bias in these estimates introduced by the fact that some dogs were recruited after identification on transect paths. The data used to calculate these population estimates were gathered in November 2007 and then in September and October 2008. The transect routes used were defined for the population estimates and had not been used previously.

Transect paths were defined by selecting points at opposite sides of the study area and by setting a bearing through the site from one fixed point to another on the opposite side of the site. Project personnel then walked through the study area guided by a GPS unit programmed to set a course to the point on the opposite side of the village. Given the density of housing and infrastructure within the study site, deviation from the bearing was expected but was minimized as far as possible. Three sets of transect surveys were conducted in November 2007, September 2008 and October 2008. Two independent transect lines were defined for these surveys (Figure 4.2). The East-West line (E-W), which was 1.6km long, was established in November 2007 and repeated in the 2008 surveys. The North-South (N-S) composite line, which was 1.7km long in total, was first used in September 2008 and repeated in October 2008. The number of passages of each transect route during each survey is given in Table 4.4.

Transect lines were walked by a recorder and local guide. The recorder and guide collaborated to record dog sightings made by either or both people. All dogs seen within 50m of the transect line were recorded, however, within the Kibera site built structures are very densely distributed and the open ground on either side of the transect paths in which dogs might be seen was often as little as 1m in width. Only dogs that were directly visible from the transect path were recorded. As a consequence, dogs that were for example behind a closed door/gate and invisible from the transect line would not have been recorded even if they were within 50m of the line. Photographs were taken and a brief physical description recorded for all dogs sighted during transects. Using these data, each dog sighted during transect surveys was classified as known or unknown to the study. Known dogs were those that could be matched to an enrolled dog using photographs and physical descriptions. A conservative classification was used

and dogs that were suspected to be enrolled but could not be matched to a specific dog ID were classified as unknown. All sighted dogs were also classified as puppies or adults using a cut-off age of approximately three months.

The probability that an encountered dog was known was estimated using a simple binomial Bayesian recapture model (Bolker, 2008). Equation 4.2 describes the binomial model where k represents the number of known dogs encountered during the transect survey (binomial successes), n represents the total number of dogs encountered (binomial trials) and p represents the probability that an encountered dog is known (binomial probability of success) (Bolker, 2008). It was assumed that the probability of trial success was equal over all transect line passages within each of the three surveys.

$$k \sim binom \ (n \ , \ p) \tag{4.2}$$

$$prob(p) \sim beta \ (a \ , \ b) \tag{4.3}$$

$$prob(p \mid k) \sim beta \ (a + k, \ n - k + b) \tag{4.4}$$

The prior for the probability of trial success was set with a beta (1,1) prior, reflecting the lack of previous data on the coverage achieved by the study (Equation 4.3). The posterior distribution of the probability that an encountered dog was known was calculated using Equation 4.4 (Bolker, 2008). Under the assumption that the probability of encounter in the transect surveys was equal for enrolled and non-enrolled dogs this estimated probability was then used to calculate the mean, median and 95% quantiles of the total population size, given the recorded size of the enrolled (known) population at the time of each transect survey. This was defined as the number of dogs that had been enrolled in the study prior to the date of the transect survey and were excluded on or after the date of the survey. In addition to calculating the size of the total dog population, the analysis was repeated to calculate the estimated size of the adult population, where puppies ⩽3 months were excluded from the analysis. The analysis was also run using data from Gatwikira/Soweto only to allow comparisons between the SSS study area and the Kisumundogo village. Finally, the mean estimate of the total population and of the adult population were used to calculate the proportion of the estimated total

population that was enrolled in the study at the time of each survey and the proportion of the estimated total adult population that had been blood sampled at the visit just prior to each survey.

4.4 Results

4.4.1 Dog Cohort Recruitment

In the period 16th - 27th June 2007 the SSS community interviewers visited 6103 households within Gatwikira and Soweto to ask about dog ownership. A respondent was identified at 4292 of these households and 52 (1.2%, 95% CI: 0.9-1.6%) were identified as dog keeping (unless otherwise stated, all point estimates for proportions are reported with exact binomial confidence intervals). A cohort of 637 dogs was recruited from 133 households over the course of the cohort study - defined as starting on the 29th June 2007 when the first dog was enrolled and ending on the 20th October 2008, the date of the last household sampling visit. 49 of these households were identified through the CDC survey, 19 through preliminary transect surveys and 65 through house-to-house enquiries. All of the 38 households enrolled in the Kisumundogo village were recruited through house-to-house enquiries.

A map showing the locations of the enrolled DKHH is given in Figure 4.2. Throughout the study only two DKHH were omitted from the study as a result of consent refusal. Recruitment of dogs at three additional households/locations failed when no respondent could be identified to provide consent for unowned/unsupervised dogs at these locations. Of the 133 households enrolled during the course of the study, 15 (11.3%, 95% CI: 6.5-17.9%) were classified as dog-supervising rather than dog-owning. The status of two additional households changed during the course of the study from dog-owning to dog-supervising when the original owners moved away and responsibility for the remaining dogs was taken by a neighbour.

The distributions of the number of dogs (>3 months) and puppies ($\leqslant$3 months) present at enrolled DKHH at each study visit are shown in Figure 4.3. The mean number of adults present at enrolled households ranged between 2.14 (Visit B) and 2.88 (Visit

Figure 4.2: Map of all DKHH enrolled during the dog cohort study and the routes of the transects used for dog population size estimates. The black outline defines the total study area. The dashed black line indicates the border between Kisumundogo and Gatwikira villages. Dark grey filled squares and circles indicate the E-W and N-S transect lines respectively. Open black points indicate the location of enrolled dog-keeping households.

H) at the different sampling visits, whilst the mean number of dogs in total (including adults and puppies) ranged between 3.19 (Visit C) and 4.23 (Visit G). These mean adult dog numbers per household are larger than those generated from the initial ownership survey conducted by the CDC (See Section 4.3.2) in which a mean number of 1.9 adults was recorded. This discrepancy most likely reflects the fact that the definition of adult used in the initial ownership survey was $\geqslant 6$ months whereas the adult numbers tabulated from the data collected at sampling visits was based on a definition in which all dogs $\geqslant 3$ months were considered adult.

The number of adult dogs present at enrolled households (as recorded in the household questionnaire) was not significantly associated with the ownership type, the method of household identification or the village. These effects were assessed by fitting a generalized linear model with poisson errors to the number of dogs at each household. The influence of these variables as predictors of dog number were assessed using GLM models of dog number fitted with poisson errors and by comparing the intercept only null model to univariate models using likelihood ratio tests. In each case, the addition of the variable to the model made no significant improvement to the overall fit of the

model: ownership type (LRT: statistic = 0.408, df = 1, p>0.5), method of identification (LRT: statistic = 0.403, df = 3, p>0.3), village (LRT: statistic = 1.077, df = 1, p>0.25).
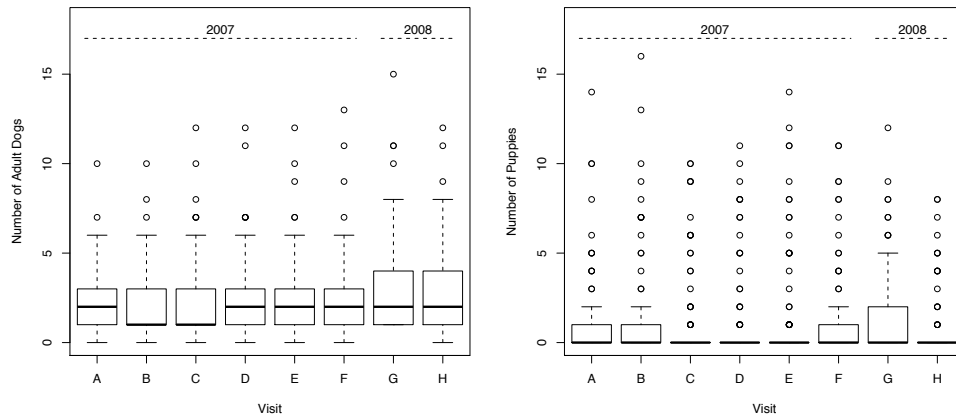
Figure 4.3: Number of adults and puppies present at study households across sampling visits. Bold horizontal lines indicate the median number of adults/puppies present at each visit. The top and bottom lines of each box show the 25 and 75 percentiles respectively. Horizontal lines joined by the dotted lines approximate the 95% confidence interval for the median. Outliers are drawn as individual points. The dashed lines and text at the top of the figure illustrate the year in which the visit occurred.

Of the 637 dogs enrolled during the course of the study, 288 (45%, 95% CI: 41-49%) were still present and enrolled at the end of the study. Of the 349 dogs that were lost from the cohort during the course of the study, the majority - 184 individuals - died (53%, 95% CI: 47-58%). 80 individuals disappeared and had unknown fate (23%, 95% CI: 19-28%), 23 were reported stolen (7%, 95% CI: 4-10%) and 49 were given away, sold or taken out of the study area by their owner (14%, 95% CI: 11-18%). A small number of individuals remained in the study area but were excluded from the study for one of two logistical reasons. Eight dogs were excluded when they could not be located or handled at three consecutive attempted visits, and five individuals were excluded as a consequence of household non-compliance. During the entire study, fifteen dogs that could not be matched to an existing dog ID were issued with a 'new' unique ID at the time of sampling. All 15 were later matched to the correct original ID using the dog description and photographs.

4.4.2 Cohort Age and Sex Structure

The relationship between calculated and owner-reported age for those individual dogs for which more than one age measure was provided during the study, and the linear regression model used to assess the relationship between these age measures are represented in Figure 4.4. The model predicts the reported ages (excluding those used to calculate ages) using the calculated age variable (the extrapolation from the first reported age) and has a coefficient of 0.994 and s.e value of 0.015. The mean interval between the provision of the first and additional ages was 225.1 days (approx 7.5 months) but the distribution of these intervals between age reports was clearly bimodal as a consequence of the break in the study at the beginning of 2007. In total, 49.4% (95%CI: 45.0-53.9%) of the owner-reported additional ages considered in the model were provided 9 or more months after the first age report.

The age and sex distribution of the dogs enrolled in the study at the time of each visit using the categorized age variable is shown in Figure 4.5. There was a predominance of individuals in the less than 3 month and Adult Unknown age classes. The proportion of the total cohort that was aged 3 months or younger ranged from 19 to 30% at each sampling visit, and the proportion aged 1 year or younger ranged from 28 to 46%. The Adult Unknown class is likely to contain individuals with actual ages of 2 years and above. The cohort population overall comprised 320 males, 315 females and 2 dogs where sex was unknown (2 puppies that died before they were handled). The sex distribution in the different age classes did not deviate consistently from an equal sex ratio in any age class (Figure 4.5). The 95% exact binomial confidence intervals for the proportion of males in each age/visit group included 50% in all but three cases. At visit C, 60.4% (95% CI: 50.6-70.0%) of puppies aged < 3 months were male. At visits C and D, the proportion of dogs in the Adult Unknown class that were male was 38.2% (95% CI: 29.4-47.8%) and 39.7% (95% CI: 30.7-49.2%) respectively.

4.4.3 Cohort Confinement and Collar Survival

Confinement of dogs as reported by respondents was relatively infrequent. The percentage of dogs reported to be confined during the day ranged between 10 - 22% over
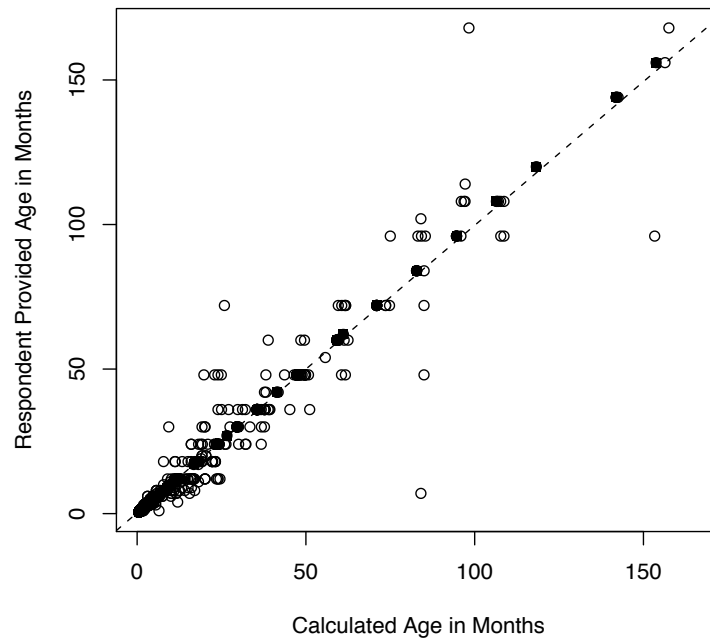
Figure 4.4: Plot of the calculated age variable based upon extrapolation from the first age reported against additional numeric ages reported. Filled squares indicate the first ages provided and used as the basis of extrapolation. Open circles indicate additional provided ages as compared to the calculated age. The dotted line represents a linear model of additional reported ages as predicted by the calculated age variable.

all visits. Night-time confinement was lower, with a range of 1-10% of dogs over the different study visits. The reliability of owner-reported confinement measures was assessed during visit F by recording both the reported and observed confinement of each dog at the time of the household visit. The two measures agreed extremely well with a Cohen's kappa value of 0.90.

The fitting and presence of collars was recorded at every household visit, allowing assessment of collar survival. There were 342 pairs of visits to individual dogs at which a new collar was fitted at the first visit and its presence/absence recorded at the follow-up visit. The analysis only considered those visit pairs in which the second visit occurred within 28±7 days. In fact, the mean inter-visit interval for the pairs used in the analysis was 28 days with an interquartile range of 28-29 days. For 252/342 of
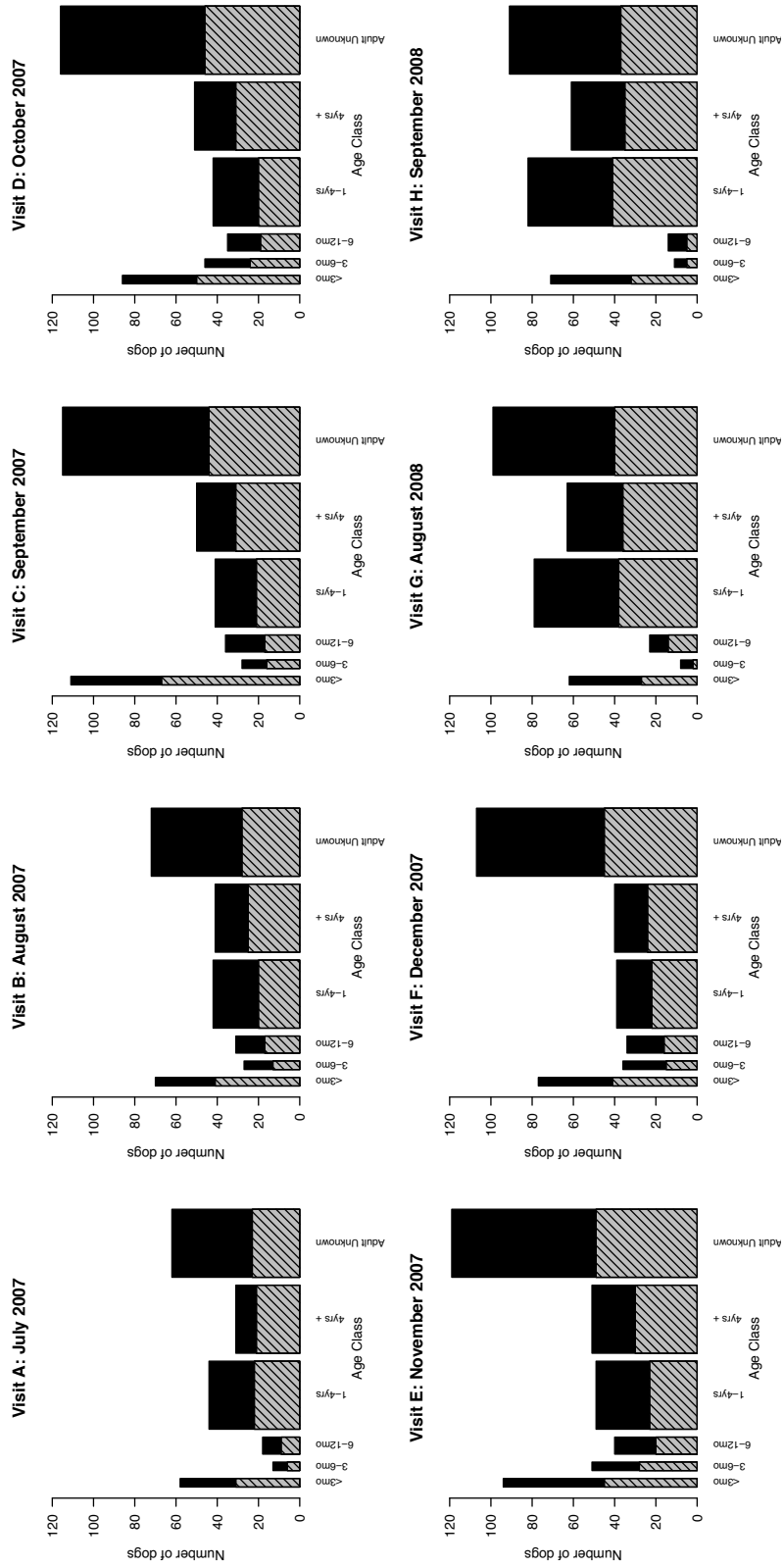
Figure 4.5: The age and sex structure of the enrolled dog population at each sampling visit. Black areas indicate female dogs and hatched grey areas indicate males. Bar widths approximate the width of the age classes

Table 4.1: Summary of dog sera samples collected during the study. Letters at the top of columns indicate the sampling visit.

	A	B	C	D	E	F	G	H	Total
Kisumundogo	61	67	61	59	74	29	60	60	471
Gatwikira & Soweto	–	106	155	157	158	153	149	153	1031
Total	61	173	216	216	232	182	209	213	1502

these visit pairs, the collar was still present at the following visit, yielding a crude collar survival measure of 74% (95% CI: 69-78%).

4.4.4   Cohort Sampling Success

A total of 721 household sampling visits were attempted during the dog cohort study. On 35 of these occasions, (5% , 95% CI: 3-7%), no respondent was present at the household and dog handling and sampling could not be attempted. None of the considered variables - household ownership type, village or method of household identification - were significantly associated with respondent presence as assessed using Fisher's exact test or chi-squared tests as appropriate.

Over the course of the study, a total of 1502 blood samples were collected (Table 4.1) and blood was successfully collected at 80% of all sampling attempts (95% CI: 78-82%). Figure 4.6 gives a mosaic plot that illustrates the relationship between the number of visits at which sampling was attempted (the number of trials) and the number of samples collected (number of successes) from each dog. Mosaic plots illustrate the relationships between categorical variables. They start with a square with length one, which is divided into vertical columns whose widths are proportional to the probabilities associated with the first categorical variable - in this case, the number of samples attempted from each dog. Each vertical column is then split horizontally into sections, the height of which is proportional to the conditional probability of the second categorical variable - in this case, the number of samples that were successfully collected. Dashed lines indicate empty areas corresponding to combinations of the two variables in which no observations were recorded. In this case, the number of samples collected can

Table 4.2: Summary of quasibinomial mixed model of sampling success

Variable	Level	Coefficient	s.e.	95% CI		n observations
				Lower	Upper	
Intercept		2.532	0.031	2.473	2.592	
Age Enrolled	$\leqslant$3 months	-1.156	0.034	-1.223	-1.090	75
	3-6 months	-0.102	0.052	-0.203	2.4×10^{-5}	33
	6-12 months	0.592	0.047	0.500	0.683	45
	1-4 years	0.028	0.028	-0.052	0.108	46
	4 + years	0.116	0.045	0.028	0.204	46
	Adult Unknown	ref	–	–	–	132
Ownership Type	Owned	ref	–	–	–	342
	Supervised	-1.208	0.070	-1.344	-1.072	35

377 observations. Random effect levels: 126 households
Variance components: Household 0.035 (65%), Dog/Residual 0.019 (35%)

never be larger than the number of samples attempted, leading to the tapered group of dashed lines at the bottom of the figure.

The number of samples that were attempted for a given dog is essentially a measure of the number of sampling visits for which it was enrolled in the study. The column representing dogs from which 8 samples were attempted is narrow because only a relatively small number of dogs were recruited and sampled in the first visit and then attempted at every subsequent visit. Many dogs were initially recruited and sampled in visit B and were then present throughout the study, corresponding with the fact that the column representing dogs from which 7 samples were attempted has the greatest width. The plot is shaded so that all areas representing the same number of samples achieved are the same shade. In each column, the section with the largest area represents dogs from which the number of samples collected is equal to the number attempted, indicating that 100% sampling success was the most probable outcome whatever the number of attempts.

The final model of sampling success is summarized in Table 4.2. A considerable portion of the variance in the data (65%) was attributable to differences between households. The fixed effects of the model indicate that sampling success was reduced in the youngest age class (dogs enrolled aged $\leqslant$3 months) and in supervised dogs as compared to owned dogs.
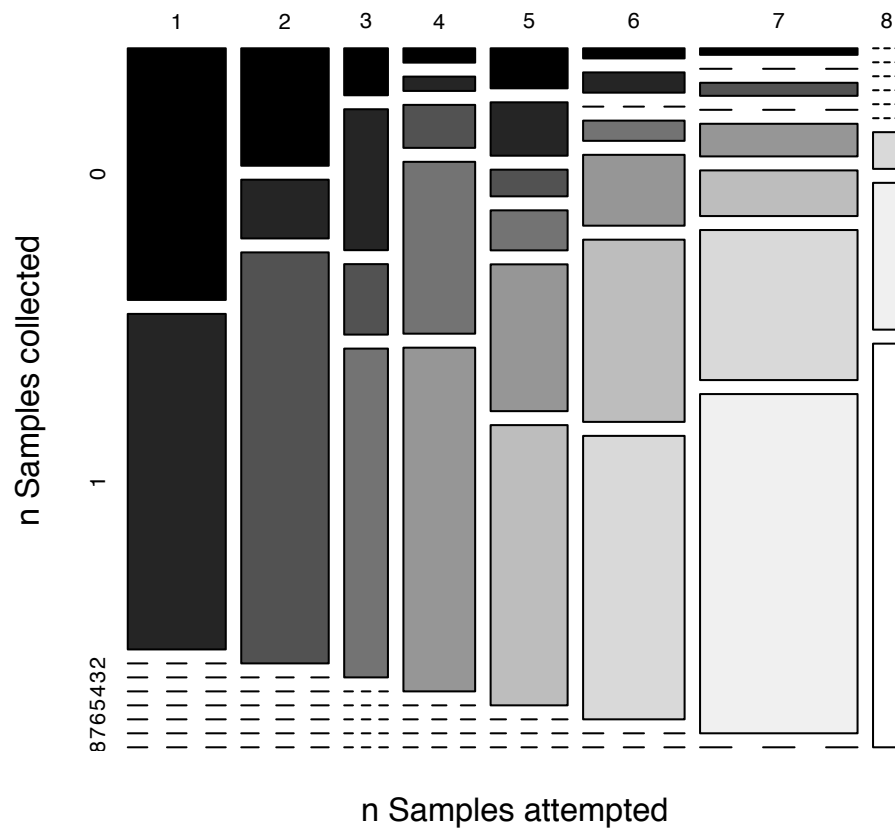
Figure 4.6: Mosaicplot of dog sampling success. The figure represents the relationship between the number of samples attempted and the number achieved for every dog in the cohort. Column widths represent the probabilities associated with each number of samples attempted. The height of each section within columns represents the conditional probability of achieving each potential number of samples given the number of attempts. All areas representing the same number of samples achieved are shaded the same. Black areas indicate zero samples collected and white indicate eight samples collected.

4.4.5 Questionnaire Survey

The questionnaire survey of DKHH included 95 households. This number constituted 92 of the 96 households enrolled in the study at the final sampling visit (H) and three additional households at which dogs were present but that had been previously excluded from the sampling survey on the basis of inability to capture the dogs. At the majority of surveyed households 76% (95% CI: 66-84%), dogs were the only animals kept by the household at the time of the questionnaire survey. 18% (95% CI: 11-27%) of dog households also owned one or more cats and 12% (95% CI: 6-20%) households also kept poultry (6 households with ducks, 6 with chickens and 1 household with both).

The household questionnaire survey included 9 households (10% , 95% CI: 4-17%) that were dog-supervising rather than dog-owning at the time of the survey, a similar proportion to that observed in the population as a whole (11%, 95% CI: 7-18%). The primary reported reason for owning dogs at 93 of 95 (98% , 95% CI: 93-100%) interviewed households was to guard the household against human intruders. This was consistent across dog-owning (85/86 households, 99% , 95% CI: 94-100%), and dog-supervising households (8/9 households, 89% , 95% CI: 52-100%). Additional secondary motivations for dog ownership reported at five or more households were, to dispose of household waste (86%, 95% CI: 78-93%), to provide companionship (40%, 95% CI: 30-50%) and to breed puppies for sale (6%, 95% CI: 2-13%).

The majority of dogs included in the questionnaire survey were born or acquired within the study area. 66% (95% CI: 61-71%) of the 345 individuals included in the questionnaire survey were born at the household at which they were found. 17% (95% CI: 13-21%) were acquired as a gift from within Kibera and 6% (95% CI: 4-9%) were described as having been adopted off the street. 51% (95% CI: 47-55%) of all dogs enrolled throughout the cohort study were aged three months or less at the time of enrolment, indicating that they were born within the study site.

65 of the 95 households interviewed (68%, 95% CI: 58-78%) reported the loss of one or more dogs from the household within the previous year. The majority of these losses were due to the death of the dog (41%, 95% CI: 37-45%) or to its disappearance with unknown fate - presumed dead (36%, 95% CI: 38-48%). A smaller proportion of

individuals were 'lost' from these households when they were given away (12%, 95% CI: 10-15%), reported killed by someone outside the household (4%, 95% CI: 2-6%), abandoned (1%, 95% CI: 0-3%) or reported stolen (6%, 95% CI: 4-8%). The mean number of dogs that had left these 65 households in the previous year was 8.5. The median was 6.5 with an interquartile range of 4.8-12. The median age at the time that dogs left these households was less than 3 months in all cases except for those individuals that were reported killed, indicating that most of the dogs that were lost from study households were puppies that died.

Most of the individual dogs present at the time of the household questionnaire were fed leftovers or household scraps (85%, 95% CI: 80-88%). Nearly all dogs (83%, 95% CI: 76-87%) were fed daily and the reported sources of feed (allowing multiple sources) were the household itself (85%, 95% CI: 80-88%), other households and hotels (food kiosks) (63%, 95% CI: 58-68%) and local butcheries (48%, 95% CI: 42-53%). 71% (95% CI: 66-76%) of dogs were also reported to feed at other houses, and 57% were reported to feed at rubbish dumps (95% CI: 52-63%).

None of the dogs observed at the time of questionnaire administration were classified as obese and only a single dog was classified as overweight by body condition scoring. 30% (95% CI: 25-36%) of the individuals observed were classified as ideal condition whilst the majority were classified as either, thin (16%, 95% CI: 12-20%) or underweight (54%, 95% CI: 48-60%). Only one of the 345 individuals included in the questionnaire survey was reported to have been previously vaccinated against any pathogens, the dog having received a previous rabies vaccination. Reporting of sickness in dogs within the previous twelve months was relatively rare with only 7% (95% CI: 5-10%) of dogs reported as having had any illness in this period. There were no consistent syndromes in the signs of illness reported in these cases. Owners reported that nine dogs had mange, four had an illness characterized by vomiting and loss of appetite and five had received injuries as a consequent of fights with other dogs or after being beaten by someone else.

Table 4.3: Summary of accelerated failure time model of dog survival fitted with a
Weibull distribution of survival times

Variable	Level	Coef.	s.e.	z	p	TR	95% CI	n Dogs
Intercept	-	4.538	0.222	20.5	<0.001			–
Age at Enrolment	⩽3 months	-2.494	0.202	-12.3	<0.001	0.08	0.06 : 0.12	317
	>3 months	ref	–	–	–	–	–	297
Ownership Type	Owned	ref	–	–	–	–	–	563
	Supervised	-0.808	0.287	-2.81	<0.01	0.45	0.25 : 0.78	51
Identification Method	CDC Survey	ref	–	–	–	–	–	207
	House-to-house	-0.456	0.189	-2.42	<0.05	0.63	0.44 : 0.92	295
	Transect	-0.316	0.239	-1.32		0.73	0.46 : 1.17	112
ln(1/shape)		0.252	0.053	4.67	<0.001	–	0.15 : 0.36	–

n=614 observations, n=253 events. Log likelihood=-644, LRT chi-sq=211.5, df=4, p<0.001
TR = Time Ratio

4.4.6 Dog Survival Analysis

The survival analysis included 614 of the 637 individuals enrolled in the study, for which full survival time and covariate data were available. 253 individuals died during the course of the study and were interval censored. 280 individuals that survived throughout the course of the study and 81 individuals that were excluded for other reasons were right censored. A summary of the multivariate model of dog survival is given in Table 4.3 and Kaplan-Meier plots of estimated survival functions for the three covariates included in the final model are given in Figure 4.7.

Accelerated failure time (AFT) models are fitted on the scale of ln(survival time) and the coefficients can be thought of as acceleration factors, which have an additive effect upon ln(survival time). The exponentiated coefficients constitute time ratios (TR) which have a multiplicative effect upon survival time (Dohoo et al., 2003; Hosmer et al., 2008). TRs with values greater than one increase expected survival time and TRs less than one decrease expected survival time. The TRs presented in Table 4.3 indicate that the expected survival time of dogs aged ⩽3 months at the time of recruitment is 0.08 times, or 8% of, the expected survival time for dogs aged more than three months at enrolment. The model-fitted survival times had median values of 4.90 visits (approximately 4.5 months) for puppies enrolled at ⩽ 3 months and 68.20 visits (approximately 5.2 years) for dogs enrolled at > 3 months.

This effect of age at enrolment is shown by the initially steep drop in the estimated survival probability of dogs aged ⩽3 months shown in Figure 4.7(a). The age of dogs at enrolment was initially modelled as a multi-level factor but simplified to a binary factor during model building. When modelled as a multilevel factor the coefficients for all levels apart from ⩽3mo were very similar and these levels were combined to give a two level factor comparing dogs that were enrolled at ages ⩽3mo to those enrolled at ages >3mo. The analysis revealed no significant influence of dog sex upon survival.

The TR for dog ownership type indicates that the expected survival time of supervised dogs is approximately 0.45 times that of owned dogs (Figure 4.7(b)). The TR values for the levels of the household identification method variable show that dogs at households identified through the survey of CDC households had better survival than dogs at households identified through house-to-house or transect observations (Figure 4.7(c)). This household identification variable is strongly correlated with the village in which households were located as all households enrolled in Kisumundogo were identified through house-to-house surveys. In the univariate analysis both the village and identification variables improved model fit and showed reduced survival for dogs in Kisumundogo as compared to Gatwikira/Soweto and for dogs identified through house-to-house and transect methods as compared to the CDC survey respectively. The identification method variable also showed similar coefficient values when run using only the Gatwikira/Soweto data, indicating that the significance of this variable is not entirely an artefact of the village influence. In the multivariate analysis, the village variable was not significant and was not included in the model. The relative importance of village characteristics and the characteristics of households identified through the different methods cannot be disentangled here but it is likely that the significance of the coefficient for house-to-house identification is influenced by properties of the Kisumundogo households that differed from those of the Gatwikira/Soweto households.

The plot of log cumulative hazard against log time described a straight line, indicating that the Weibull distribution is appropriate for these data. In addition, the confidence interval for the shape parameter estimated by the model ($\ln(1/\text{shape}) = 0.252$ (95% CI: 0.15-0.36) does not include zero. This translates to a value for the Weibull shape parameter of 0.77 (95% CI: 0.70-0.86), further supporting the Weibull model. The value
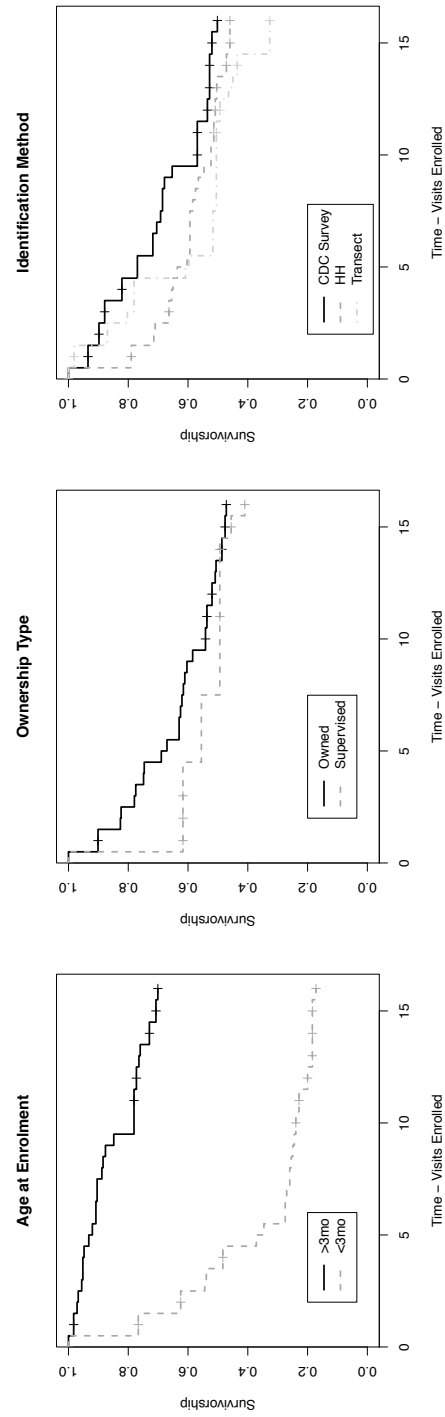
Figure 4.7: Kaplan-Meier plots of dog survivorship. Survival times for dogs that died were set as exact survival times at the mid-point of the censored interval for plotting. Individual plots show the univariate relationships between dog survival and a) Age at enrolment, b) Ownership type and c) Method of household identification.

Table 4.4: Transect survey summaries. k = number of known dogs encountered on the transect, n = total number of dogs encountered on the transect. Data are summarized for the entire study site and for the Gatwikira/Soweto village only as well as for all dogs and adults only.

| | n passages | | Total study area | | | | Gatwikira/Soweto Only | | | |
| | | | Total | | Adults | | Total | | Adults | |
Month of survey	E-W	N-S	k	n	k	n	k	n	k	n
November 2007	4	0	77	117	76	110	64	104	63	97
September 2008	7	4	242	284	205	237	201	238	175	204
October 2008	6	6	200	289	200	270	233	252	169	233

of the shape parameter is <1, indicating that the hazard declines over time (Dohoo et al., 2003), which is consistent with the evidence of high mortality in the youngest individuals ($\leqslant$3 months) obtained from the questionnaire survey.

4.4.7 Dog Population Estimates

The data recorded during the three transect surveys are summarized in Table 4.4 and the population estimates generated from these data using the binomial recapture model and the estimates of study coverage measures are given in Table 4.5.

The size of the total study site dog population estimated using the binomial model fell from 552 (95% CI: 491-637) in November 2007 to mean values of 375 and 417 in September and October 2008 respectively (Table 4.5). The estimated all dogs and adult only population sizes in the Gatwikira/Soweto village consistently constitute approximately 75% of the equivalent total value for the entire study area. This value was consistent with the proportion of the enrolled cohort that came from Gatwikira/Soweto at the time of each transect survey, which ranged from 70 to 74%. Similarly, in both the total study site and Gatwikira/Soweto only, the estimated size of the adult (>3mo) population constituted between 75 and 80% of the total estimated population. The equivalent proportions of adults in the enrolled cohort population ranged between 78 and 85%.

The proportion of the estimated total population that was enrolled in the study cohort ranged from 66% (61-69%) in November 2007 to 85% (81-89%) in September 2008. The

Table 4.5: Estimates of the total dog population and coverage achieved by the cohort study

Month of survey		Total Popn. Mean	Total Popn. 95%	Adult Popn. Mean	Adult Popn. 95%	Total Enrolled %	Total Enrolled	Adults Sampled %	Adults Sampled
		Estimated populations				Coverage measures			
November 2007	All	552	(491-637)	413	(369-474)	66	(61-69)	56	(51-61)
	Gat/Sow	411	(358-485)	308	(270-362)	61	(56-66)	51	(45-56)
September 2008	All	375	(359-396)	298	(285-315)	85	(81-89)	67	(62-73)
	Gat/Sow	280	(267-298)	217	(206-230)	84	(79-88)	65	(59-72)
October 2008	All	417	(388-452)	334	(313-360)	69	(64-73)	62	(56-67)
	Gat/Sow	305	(281-334)	242	(225-263)	67	(61-72)	60	(54-67)

Table 4.6: Estimates of dog population density and dog:human ratios. Figures in brackets are 95% confidence intervals for estimates

Survey	Total Study Area Dogs/km^2		Gatwikira/Soweto Dogs/km^2		Dog:Human Ratio	
November 2007	1059	(942-1222)	1086	(946-1281)	1:73	(61-84)
September 2008	719	(689-760)	740	(705-787)	1:107	(101-112)
October 2008	800	(744-867)	806	(742-882)	1:98	(90-107)

proportion of the estimated adult population that was sampled at the time of each survey ranged from 56% (51-61%) in November 2007 to 67% (62-73%) in September 2008. Within each survey, the estimated coverage measures calculated for Gatwikira/Soweto only were very similar to those calculated for the total study site and there was a slight increase in the coverage achieved by the study in 2008 as compared to 2007.

The dog densities calculated from the dog population estimates for the total site and Gatwikira/Soweto only are given in Table 4.6. In addition, the dog:human ratios calculated for the Gatwikira/Soweto village are reported, based on an approximate total human population of 30,000 at all survey times. This figure of 30,000 is the CDC estimate of the total human population within the SSS study area (Gatwikira and Soweto; CDC unpublished data).

4.5 Discussion

This field study was designed with the aim of recruiting and sampling a dog cohort population that was representative of the Kibera dog population as a whole. The data presented above show that a high level of sampling success was achieved throughout the study and indicate that a large and consistent proportion of the total population was enrolled in and sampled by the cohort study. The range of household identification methods used avoided the potential bias that would have been introduced by enrolling only owned dogs in the cohort. Amongst enrolled dogs there was some variation in sampling success, with dogs in the youngest age class and dogs that were supervised rather than owned having a lower probability of successful sample collection. However even in these classes, sampling success was still quite high and it was not the case that individuals of these classes were not represented in the sample collected.

Even at households at which dogs were owned (DOHH), dog confinement during both the day and night was relatively rare in the Kibera population and as a consequence the size of the unowned and potentially inaccessible dog population can be over-estimated. In fact, only 10% of enrolled households were classified as dog-supervising rather than dog-owning and this proportion is similar to the proportions of dog populations that are un-owned estimated by studies conducted in urban Antananarivo (Ratsitorahina et al., 2009), and N'Djaména (Kayali et al., 2003). Although there was a reduction in sampling success in the supervised population as compared to owned, it is certainly not the case that unowned dogs were entirely inaccessible for sampling. By determining the ownership status on a dog-by-dog basis and by visiting dogs at their households, we encountered only 5 households/locations at which identified dogs could not be enrolled in the study. Consent for participation in the study was refused at 2 households and we did have to omit three locations at which dogs were identified because no responsible person could be identified. This excluded population is unlikely to have introduced considerable bias to the cohort.

The dog population density observed at the Kibera study site (719-1059 dogs/km^2; See Table 4.6) is comparable to that reported for other urban African dog populations (e.g. 460-1952 dogs/km^2 in N'Djaména, Chad (Kayali et al., 2003)), but considerably higher

than the dog densities recorded in rural (6-21 dogs/km^2) and peri-urban sites (110 dogs/km^2) in Kenya (Kitala et al., 2001). The human population density is so high at this site though that the dog:human ratios calculated for Kibera (1:73 - 1:107) are considerably higher than both those reported in rural and peri-urban sites in Kenya - overall ratio 1:7.7 (Kitala et al., 2001), for urban sites in Tanzania - overall ratio 1:18.0 (Knobel et al., 2008), and for urban African populations overall (mean dog:human ratio of 1:21.2) (Knobel et al., 2005). The human population size figures that these densities are based upon are an approximation, but even allowing for this source of error in the estimates it is clear that the Kibera dog:human ratios are on a different scale to other previously recorded figures. Previous studies have found that the dog:human ratios observed at many different urban and rural sites and studies are consistent, allowing extrapolation of rough dog population estimates from human population figures (Knobel et al., 2008). The results from Kibera indicate that in the particular case of urban slum environments, the application of a 'general' urban dog:human ratio may lead to an over-estimation of the size of the dog population. In terms of the interactions between human and dog populations and almost certainly in other qualities, the urban slum context is likely to have its own unique ecology that warrants greater study, particularly in light of its importance as the home of an increasing proportion of the human population (Giddings, 2007). The results of this study refer to this urban slum context.

The analysis of the transect data used to generate population size estimates and dog density measures made three key assumptions. Firstly, that the probability of encounter on a transect line was equal for enrolled and un-enrolled dogs. Although transect surveys had been used previously to identify dog households for recruitment, the potential for bias was removed by defining new transect routes specifically for the purpose of population estimation that were not the same as those routes used previously to identify dog households. The principal determinant of dog encounter probability on a transect is likely to be dog confinement and it can be assumed that un-owned/supervised dogs are less likely to be confined than owned dogs (Matter et al., 2000). In this study, both owned and supervised dogs were enrolled and the overall level of daytime confinement was low. Because of the variety of household identification methods used, it is unlikely that this assumption of equal encounter probability has contributed to significant bias. Any bias that were introduced would lead to an under-estimation of the enrolled dog

population and consequently the coverage measures generated here may be slightly conservative.

The population size estimates are also based on the assumptions that the population was closed between the time of capture and recapture and that dog status (known or unknown) was accurately recorded for all encountered dogs. The three transect surveys were all completed within 1 week of the end of a sampling visit, but because sampling visits lasted 17 days, a dog recruited and captured on the first day of a sampling visit could have been recaptured up to 24 days later, on the last day of a transect survey. Within this period the most likely changes in the size of the dog population would be due to births, which would not have influenced the population estimates considerably as young puppies are essentially immobile and very unlikely to be encountered on a transect line (Matter et al., 2000), particularly in the Kibera setting as the effective coverage of the transect line was low. There may have been small changes in the size of the adult population between capture and recapture but these are unlikely to have had a systematic effect upon the size of the estimated population. The potential length of time between capture and recapture may have resulted in dog status misclassification if using collars or another temporary identification method. Indeed, after an interval of one sampling visit, approximately 26% of fitted collars had been lost. Although the interval between collaring and the transect survey was less than 28 days, these data on collar survival indicate that a considerable proportion of the recaptured population could have been misclassified as unknown if using collars as the identification method, leading to an over-estimation of the total dog population and under-estimation of the coverage achieved by the study. However, because dog status was confirmed using photographs and significant changes in dog appearance over a period of a few weeks are unlikely, the risk of misclassification was small.

This cohort was created to carry out surveillance for zoonotic diseases and a number of characteristics of the dog population may influence the levels of exposure to different zoonoses. Dog confinement levels were low and most of the Kibera dog population is effectively free-ranging. Most dogs are fed on household scraps but many were provided with food from a variety of additional sources and although most are fed daily at their household, the majority of the population were also reported to feed at other households

and to scavenge at rubbish sites. As a consequence, this population is effectively open to exposure to zoonotic pathogens from a wide variety of sources. The majority of enrolled dogs were also born in Kibera, reducing the potential influence of evidence of exposure to pathogens seen in dogs that were introduced from other areas.

Survival analysis and questionnaire data on dogs that left the household both indicate that there is considerable mortality amongst puppies aged $\leqslant 3$ months. 77% (95% CI: 72-82%) of puppies enrolled at $\leqslant 3$ months were excluded before the end of the study. In comparison, just 34% (95% CI: 29-39%) of dogs enrolled at > 3 months were lost over the entire 14 months period of the study which included a period of unrest in which approximately a quarter of the human population left the study site (most temporarily). The survival analysis indicates that the survival hazard declines over time, and these data suggest that the population of dogs that survives the first few months of life is then relatively stable. In terms of using this population for sentinel serosurveillance, this demographic pattern is quite desirable. Puppies aged < 3 months were not blood sampled, principally because the interpretation of any serological data collected from these young individuals would be complicated by the possible presence of maternal antibodies. The success of blood sampling was reduced in dogs that were aged < 3 months at recruitment as compared to older classes and in supervised as compared to owned dogs but was high overall. The dog population that is most readily sampled therefore represents the portion of the population that is most stable, facilitating the repeat sampling and follow-up required for longitudinal surveillance.

The longitudinal nature of this study allowed an assessment of the accuracy and consistency of owner-reported dog ages over time and the analysis of these data demonstrate that when numeric ages are provided, owner-reported age is actually very consistent and accurate. Approximately half of the additional owner-reported ages considered in the analysis were provided in 2008 for dogs that were recruited in 2007 and therefore assess the consistency of age reports over a period of 9 months or greater. The two age measures were not strictly independent as the calculated ages were based on the first owner-reported age, and the model comparing the two age indicators therefore effectively evaluates the repeat consistency of owner reported ages. However, because so many dogs were recruited soon after birth and the first reported ages were unlikely to

err by more than a couple of weeks, this consistency of reporting equates to accuracy in the reporting of 'true' age. It is also more likely that consistent age reports reflect the true age than that respondents were consistent in the provision of incorrect ages. The comparison of owner-reported and calculated ages was made on the continuous scale of age in months. In practice, for surveillance purposes, dog ages would often be recorded on a categorical scale, which requires even less accurate recall of dog age than is seen in these analyses. One of the most useful applications of dog age data in the surveillance context is likely to be in the interpretation of patterns of exposure in different age classes in order to determine the timing of previous infections in a population (Kock et al., 1999; Lembo et al., In Prep.). These data indicate that inferences of this type based on owner-reported dog ages can be made with some confidence.

The analysis of reported age consistency did not however include dogs for which no numeric age was provided. Figure 4.5 shows that the proportion of the dog population that fell into this "Adult Unknown" age class in 2008 (Visits G and H) was smaller than that seen in 2007, whilst the relative proportion of the population in the 1–4 years and > 4 years classes was greater in 2008. This pattern almost certainly reflects the fact that owner-reported ages are more likely to be more accurate and to be given as numeric ages for younger individuals. In 2008, the age of many of the dogs in the 1–4 years and > 4 years classes would probably have been reported as "Adult Unknown", but the more accurate classification of ages was possible because of the ages provided a year earlier. In order to gather accurate age data for older dogs (2 years and above) it may be necessary to develop and validate techniques such as the use of key events to establish the year of birth for older dogs.

4.5.1 Conclusion

The dog cohort study at the Kibera site was established to practically assess the feasibility of disease surveillance using domestic dogs and overall the study design proved very successful. The Kibera dog and human populations live in extremely close proximity, with very high densities of both populations within the study site. Although most of the Kibera dog population was largely unconfined it was perhaps more accessible for surveillance and sampling than anticipated. The vast majority of households

approached to participate in the study were enrolled, enrolled dogs were consistently re-located at their households at repeat visits, individuals were clearly identifiable and the sampling success rate was high. Further assessment of the utility of dog sampling for the surveillance of specific pathogens is discussed in the following chapters. In this chapter, no practical obstacles to the successful use of dogs as sentinels were identified.

Chapter 5

Influenza A Surveillance in Kibera

5.1 Abstract

Influenza A viruses (subtypes H3N2 and H1N1) have been detected in the human population at the Kibera study site but the epidemiology of influenza A viruses in the animal populations is undescribed. There is considerable movement of live birds into Kibera, creating the risk of influenza A virus introduction and potential zoonotic transmission at this very densely populated site. A serosurvey of the Kibera dog and poultry populations was conducted to assess whether or not similar or entirely distinct influenza A viruses could be detected in the Kibera animal populations. Dog samples from five rounds of cohort sampling (n=689) and bird samples from two cross-sectional surveys (n=212 chicken and n=64 duck sera) were tested for antibodies against influenza A viruses using the ID VET cELISA and a panel of HI assays. A single dog serum sample was positive in the cELISA test. No other dog or bird samples were positive in the cELISA or HI tests. Questionnaire survey data was collected to determine the source of all sampled birds and examine interactions between the human and poultry populations. At the time of the two cross-sectional surveys, 60% and 47% of sampled birds were born within Kibera, revealing considerable immigration of live birds into Kibera. The questionnaire data also reveal potentially risky interactions between the human and bird populations within Kibera that could facilitate zoonotic influenza A transmission. These data provide no evidence of influenza A virus presence in the

Kibera poultry or dog populations. Using the same serological tests, the surveillance conducted in Cameroon and Kibera has revealed very different pictures of influenza A epidemiology. At both sites, the data gathered through serosurveillance of the dog population is consistent with data obtained through the more conventional surveillance of the avian populations present, demonstrating that dog serosurveillance can provide useful data on broad-scale patterns of zoonotic influenza A risk.

5.2 Introduction

In Chapter 2, the potential use of domestic mammal serosurveillance as a tool for baseline influenza A surveillance and for the detection of previously unrecognized flu circulation was proposed and discussed in the context of the Cameroon surveillance site. In this chapter, similar serological survey and testing tools are applied at the Kibera study site (See Chapter 2, Section 2.2.5 for discussion of influenza A diagnostics). The Cameroon and Kibera sites represent two very different ecological contexts but both are valid targets for influenza A virus surveillance. There is considerable movement of poultry into the Kibera study area through trade in live birds. This creates the potential for the introduction and exchange of viruses between the bird, dog and human populations at this very densely populated site. The SSS study has identified subtype H3N2 and H1N1 viruses in the human population at this site but the status of influenza A viruses in the animal populations is unknown.

5.2.1 Avian Influenza A Transmission and Zoonotic Risks

Trade in live birds, eggs, poultry products and the migration of wild birds have all been implicated in the international spread of H5N1 and other influenza A viruses (Olsen et al., 2006; Cattoli et al., 2009; van den Berg, 2009). In the case of low-pathogenicity influenza A viruses, the weight of evidence does implicate wild birds as the primary source of infections in poultry populations (Capua and Alexander, 2008). It is though very difficult to determine the source of primary infections retrospectively, particularly when limited surveillance resources are available or when data on the scale

and dynamics of legal trade, illegal trade and potentially also wild bird movements are limited (van den Berg, 2009). In cases of secondary spread to domestic poultry the main sources of infection appear to be man-made (Capua and Alexander, 2008). In Africa, there is very little documentation of the trade and movement of poultry and poultry products, which complicates assessment. Data from Nigeria indicates that after the initial introduction of H5N1 virus(es), spread within the country occurred at local/regional scales consistent with the geographical scales over which the trade of poultry occurs (Cattoli et al., 2009) and that the distribution of cases (farm infections) is consistent with epidemic spread via the major highway network in Nigeria (Rivas et al., 2010).

The recent H5N1 avian pandemic has been associated with over 440 human cases (WHO, 2009a). There is also increasing data on the transmission of non-H5N1 avian influenza A viruses to humans and indications that the poor sensitivity of standard influenza A diagnostic tests when applied to detecting evidence of avian influenza infections in human sera (Lu et al. 1982; Beare and Webster 1991; Rowe et al. 1999 and see Section 2.2.5) may have contributed to an under-estimation of avian-to-human influenza transmission (Meijer et al., 2006; WHO, 2006; VanDalen et al., 2009). Direct avian-to-human transmission of H7 viruses has been reported on multiple occasions with human cases documented in Canada, Italy, the Netherlands, UK and USA (Belser et al., 2009). The number of human infections associated with the 2003 H7N7 avian outbreak in the Netherlands (during which one human fatality occurred (Fouchier et al., 2004)) was apparently much larger than originally determined using standard tests. Using horse RBCs in a modified HI test, approximately 50% of individuals exposed to infected poultry showed an antibody response to H7N7 (Bosman et al., 2005; Meijer et al., 2006). As a consequence of the enhanced surveillance prompted by the H5N1 outbreak that started in 1997 cases of H9N2 infection in humans have also been identified in China and Hong Kong (Peiris et al., 1999).

The zoonotic transmission risks posed by the movement of animals into urban areas were discussed in Section 3.2.3. Live bird markets (LBMs), which are typically urban, can serve as hubs in the transmission networks of influenza A viruses (Cardona et al., 2009). In the United States, influenza A viruses are endemic in many LBM systems

and both H5 and H7 viruses have emerged in association with LBMs (Webster, 2004; Cardona et al., 2009). Multiple H5N1 viruses have been detected in LBMs in Thailand (Amonsin et al., 2008) and the movements of birds to and from live bird markets has been associated with H5N1 outbreaks on Hong Kong farms (Kung et al., 2007) and with the repeat introductions of H5N1 that occurred in Vietnam (Magalhães et al., 2010). In a central China LBM, isolates of the subtypes H3N6, H9N2, H2N9, H3N3, H4N6, H1N1 and H3N2 were all detected over a 16 month period (Liu et al., 2003) indicating the diversity of sources that can contribute virus at these key transmission hubs. As well as enabling onwards transmission to other bird populations, LBMs can also act as a source of infection for human influenza A cases (Webster, 2004; Wang et al., 2006). Direct or indirect contact with infected poultry is a recurring correlate of human cases (Chotpitayasunondh et al., 2005; Sandrock and Kelly, 2007; Anderson et al., 2010; Fasina et al., 2010). Transmission routes that play a role in human-to-human transmission of influenza A include contact spread, aerosol spread and droplet exposure (Anderson et al., 2010). Additional potential routes of transmission from avian to human hosts are likely to include contact with the mucous membranes or conjunctiva of infected birds, inhalation of contaminated fomites generated through normal husbandry and slaughter practices and inhalation of droplets generated during slaughter (Anderson et al., 2010).

In addition to areas of interface between wild and domestic bird species, influenza A surveillance should also focus upon poultry trading networks and points at which there is considerable turnover of birds or bird products. There are no formal LBMs in Kibera but the key factors that mediate the risk of influenza introduction and transmission into and from LBMs: the combination of a continuous supply of animals; multiple species in close contact; and multiple sources of birds from far-flung suppliers (Cardona et al., 2009); are all relevant at the Kibera site.

5.2.2   Human Seasonal Flu Patterns

Influenza is maintained in human populations through direct person-to-person spread but the mechanisms of global persistence and spread and the factors that influence antigenic changes and variant emergence are still relatively poorly understood (Cox

and Subbarao, 2000). The H3N2 and H1N1 subtypes that dominate human seasonal flu have co-circulated for over 30 years (Cox and Subbarao, 2000; Barr et al., 2010). In the temperate regions of both the northern and southern hemispheres, influenza A cases typically peak annually in the colder months of the year (Simonsen, 1999; Cox and Subbarao, 2000). Within tropical and sub-tropical regions though, influenza cases occur year-round without distinct seasonality or twice a year with increased incidence during the two rainy seasons (Simonsen, 1999; Cox and Subbarao, 2000; Nicholson et al., 2003; Viboud et al., 2006; Gordon et al., 2009). It has been suggested that the tropics as a region, might act as a reservoir or source of new influenza A viruses, from which, viruses spread out annually towards the poles (Viboud et al., 2006; Finkelman et al., 2007). This hypothesis is supported by the findings of a recent study of incidence data from 19 temperate region countries for the period 1997 to 2005 which showed a positive correlation between increased distance from the equator and later occurrence of epidemics (Finkelman et al., 2007). More recently, antigenic and genetic studies of isolates from 2002-2007 have indicated that new strains of H3N2 influenza A emerge from East and Southeast Asia specifically and then spread out across the globe (Russell et al., 2008). More data from tropical regions are required to elucidate these patterns of spread. In both of these recent analyses, the lack of data from Africa specifically has been highlighted (Finkelman et al., 2007; Russell et al., 2008).

5.2.3   Human Influenza A Surveillance in Africa

Influenza A surveillance effort and capacity within Africa has increased considerably in response to the H5N1 pandemic but remains very limited on the global scale (Schoub et al., 2002; Finkelman et al., 2007; Bulimo et al., 2008). South Africa and Senegal have operated active monitoring programmes for several years (Schoub et al., 2002), but the recent focus on global influenza epidemiology has highlighted the fact that there remains very little data from Africa as a whole (Yazdanbakhsh and Kremsner, 2009). The studies that have been conducted within Africa have identified a number of outbreaks caused by H3N2 viruses, in Madagascar and the Democratic Republic of Congo in 2002 (Nicholson et al., 2003), in South Africa in 2003 (Besselaar et al., 2004) and evidence of significant previous H3N2 circulation in Gabon prior to 2007 (van Riet

et al., 2007). The burden of influenza in Africa has traditionally been considered to be negligible (Yazdanbakhsh and Kremsner, 2009) but this may in part be a consequence of the complexity of its assessment and the shortage of good quality demographic and cause of death data. The more severe outcomes of influenza infection are often caused by secondary bacterial infections or exacerbation of existing chronic conditions and the primary influenza infection may not be recorded (Simonsen, 1999; Cox and Subbarao, 2000; Viboud et al., 2006). In temperate regions, the mortality burden of influenza is assessed by quantifying the 'excess' deaths that occur during influenza seasons, over and above the mortality levels expected in the absence of influenza (Simonsen, 1999). In tropical areas where there are no distinct influenza seasons, this methodology is less suitable (Simonsen, 1999). Although limited, the sporadic reports from Africa and studies conducted in other tropical regions suggest that influenza is in fact prevalent in tropical regions and Africa specifically and may have a significant impact upon human health (Gordon et al., 2009; Yazdanbakhsh and Kremsner, 2009). It is also argued that per-head income explains a large proportion of the variance in the mortality attributable to influenza (Murray et al., 2006). When the pandemic mortality patterns observed in nations with good quality data are extrapolated to the global scale, it is predicted that the majority of deaths caused by pandemic influenza will occur in poor countries (Murray et al., 2006).

5.2.4 Human Influenza A in Kenya

In Kenya, a human influenza sentinel centre surveillance system was established in 2006, through a collaboration including KEMRI, CDC, Kenya Ministry of Health and US Army Research Unit - Kenya, as part of the World Health Organization's global influenza surveillance programme (Schnabel et al., 2008). In the first year of operation of this surveillance system, nine H3N2 viruses were isolated in 2006-2007 from a total of 1014 nasopharyngeal swabs collected (Bulimo et al., 2008). In 2007-2008, the sampling period that coincides with the animal surveillance presented in this chapter, over 12,000 swabs were tested and over 940 influenza A positive samples were detected as well as over 300 influenza B cases (Gikundi, 2009). Amongst 310 influenza A positives collected from eight sentinel sites across the country, 35% of isolates were subtype H1N1 and 65%

were H3N2. In Nairobi specifically, 34 influenza A positive samples were detected in 2007-2008. Approximately 80% of these cases were H3N2 viruses, and the remaining 20% were H1N1 viruses (Gikundi, 2009).

This increased surveillance effort has provided evidence that, as in temperate regions, influenza A viruses of subtypes H3N2 and H1N1 have dominated in Kenya over the past few years. The Kenyan H3N2 and H1N1 isolates described for the 2006-2007 and 2007-2008 seasons have all been closely related to the recommended vaccine strains used for the respective Southern hemisphere seasons (Bulimo et al., 2008; Gikundi, 2009; Barr et al., 2010). At the Kibera study site, the SSS study collects data on the number of individuals reporting influenza-like-illness (ILI) at every household visit, where ILI is defined as fever and cough or difficulty breathing. This definition of ILI can successfully predict influenza infection in as many as 79% of adults and 83% of children during influenza seasons (Ohmit and Monto, 2006). However, when applied over longer periods the predictive value of ILI can be lower (Ohmit and Monto, 2006) and these symptoms can also be caused by a large number of other pathogens including malaria and a range of other respiratory viruses (Monto, 2002). Over the period between July and December 2007, when the animal survey was conducted, the monthly totals for the number of individuals reporting ILI at SSS household interviews peaked at 705 in September and declined to 307 in December (CDC/KEMRI unpublished data). These reported case numbers translate to incidence rates of between 1.9 and 7.8 ILI cases per 100 people per year within the period in which animal surveillance was conducted (CDC/KEMRI unpublished data). Only a proportion of the individuals reporting disease in the household survey present at the project run clinics for assessment. 63 nasopharyngeal/oropharyngeal swabs collected during this six month period (July–December 2007) were tested by RT-PCR influenza A and 8 of these were positive for influenza A (CDC/KEMRI unpublished data).

5.2.5 Influenza A in Dogs

Influenza A viruses of subtype H3 are the most broadly distributed in mammals, including both humans and dogs (Payungporn et al., 2008) and evidence of canine influenza A infections has grown in the past few years. In 2004 an H3N8 virus was identified as

the causative agent of an outbreak of respiratory disease in greyhounds at a Florida racetrack. Phylogenetic and serological analyses indicate the occurrence of a single transmission event at which an entire equine virus had been transmitted into the dog population, followed by the subsequent persistence and spread of the virus throughout the canine population (Crawford et al., 2005; Payungporn et al., 2008). In a similar case, an H3N8 virus was recovered from the tissues of dogs that died during an outbreak of severe respiratory disease in English foxhounds in 2002 and retrospective analyses identified an H3N8 equine influenza A virus as the cause of the outbreak (Daly et al., 2008).

More recently, an avian origin H3N2 virus was isolated from Korean dogs with severe respiratory disease (Song et al., 2008). Experimental infection studies using the Korean canine H3N2 virus have demonstrated direct dog-to-dog transmission of virus associated with viral RNA detected in nasal swabs and seroconversion (Song et al., 2009), and a serosurveillance study of farmed and pet dogs in Korea found a high seroprevalence in dogs from farms at which outbreaks of respiratory disease had occurred, but very low seroprevalence in other populations, indicating sporadic transmission of the virus (Lee et al., 2009). α2,3Gal linkages have been identified at all levels of the canine respiratory tract (Daly et al., 2008; Song et al., 2008), indicating the potential for the direct transmission of avian adapted influenza viruses from birds to dogs (Song et al., 2008). In the UK H3N8 canine influenza outbreak, the affected pack had been fed meat from two recently euthanized horses, suggesting that the virus may have been acquired through the consumption of contaminated meat (Daly et al., 2008). Viral antigen expression was confined to the respiratory tract but it is considered possible that the consumption of infected material could lead to respiratory infection through the inhalation of viral particles (Daly et al., 2008).

These demonstrations of the transmission of both equine and avian adapted viruses into dog populations with subsequent onwards transmission have raised questions about the potential role played by dogs in the inter-species transmission of influenza A viruses and suggests the potential for detecting influenza A virus presence through sentinel surveillance using dogs (Payungporn et al., 2008; Song et al., 2008).

5.2.6 Chapter Objectives

This chapter presents data from a serosurvey of the Kibera domestic dog and poultry populations. The study was conducted to determine if the Kibera animal populations had been exposed to the same (or entirely different) influenza A subtypes that circulated in the human population and which animal species were affected. Data were also collected on the interactions between the human and domestic bird populations to assess the potential for zoonotic flu transmission at this site.

5.3 Sampling Methodology

5.3.1 Dog Samples

The collection protocols for the dog serum samples used in this analysis are described in full in Chapter 3. This influenza A analysis was restricted to the Gatwikira/Soweto village as the bird sampling frame was established through a survey of KEMRI/CDC SSS study households and no equivalent survey mechanism was possible in Kisumundogo. A total of 689 dog sera samples collected in Gatwikira/Soweto were tested for antibodies against influenza A. These samples were collected during dog sampling visits B to F which covered the period from August to December 2007 (Table 5.2).

5.3.2 Bird-Owning Household Identification

Two independent surveys of bird ownership were conducted in July and October 2007. During two rounds of sampling visits to households enrolled in the KEMRI/CDC SSS, additional questions regarding bird ownership were asked by the SSS Community Interviewer. Respondents were asked if any members of the household owned any birds that were present at the household in Kibera. At bird-owning households, the total number of birds (adults and chicks combined) was recorded for those species that were present. A zero was recorded for those species not present. A copy of the data collection sheet used at household visits is given in Appendix B.

The data collected identified those households within the SSS cohort that were 're-sponders' during each respective round of household visits and that kept one or more bird(s) in Kibera at the time of that survey. These data were used as the sampling frames for two cross-sectional surveys of the domestic bird populations within the study site conducted in October and November 2007 respectively.
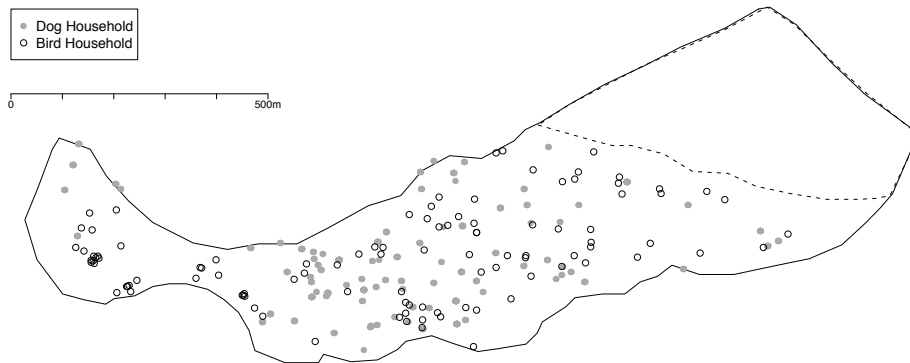
Figure 5.1: Map of dog and bird owning households sampled during influenza A surveil-lance. The black line gives the outline of the animal study site. The dashed line surrounds the Kisumundogo village which was excluded from this anal-ysis.

5.3.3 Bird Household Sampling Visits

A pilot sampling visit to 10 households was conducted in July 2007, followed by the first cross-sectional survey in October 2007 and the second in November 2007 (Table 5.2 and Figure 3.3). All households identified as bird-owning in the CDC household surveys were approached for sampling during the subsequent cross-sectional survey. At all visited households at which a respondent was present and birds were still kept, consent for participation in the study and bird sampling was sought. Copies of the consent forms and data collection sheets used are provided in Appendix A and B. The procedure for obtaining informed consent was as described in Section 4.3.4. At all households at which study consent was obtained, the number and species of birds present at the household was recorded.

At houses with 5 or fewer birds, all individuals were sampled. At houses where there were more birds, the age groups of birds was assessed and every second bird (processing birds of the same species and age class consecutively) was sampled. Bird age groups were based on the presence of adult feathers and the similar size of individuals from the same batch in younger birds. Samples were obtained from both chickens (*Gallus gallus domesticus*) and Muscovy ducks (*Cairina moschata*). All birds were manually restrained for sample collection. Blood samples were taken from the brachial (wing) vein using a sterile vacutainer (4ml red topped plain vacutainers) and 23 or 21 gauge needle. Up to 4ml of blood was collected from each bird, ensuring that the total collected volume was less than 1% of body mass (FAO, 2007). Data on the source of all sampled birds was recorded.

Free vaccination against Newcastle Disease Virus and Infectious Bronchitis (Hipraviar-B1/H120, Laboratorios Hipra, Spain) was offered at all bird-owning households visited. Subject to the provision of consent from the household representative, vaccine was administered to all birds older than approximately 5 days. The vaccine was administered via the nare at a dosage of 1 drop (approx 0.03ml: live Newcastle virus B1 $10^{6.5}$ EID_{50} and live infectious bronchitis virus Mass H120 strain 10^3 EID_{50}) to each individual at no cost to the owner.

5.3.4 Bird Household Questionnaire Survey

All of the households sampled during the two cross-sectional surveys were approached during the questionnaire survey in October 2008. The questionnaire was administered at 72 households at which a member of the originally sampled household was present and consent was provided. The questionnaire collected data on bird management practices and was completed with reference to all birds owned at the household rather than the sampled birds specifically. A copy of the questionnaire is provided in Appendix B.

5.4 Laboratory Methods

5.4.1 Competitive ELISA

The ID.VET cELISA (ID.VET, Montpelier, France) was used was used to test the sera of all birds and dogs. This cELISA test was the same as that used to test dog and bird samples from Cameroon (See Chapter 2). Sera from different species were tested at serum dilutions as recommended by the manufacturer: 1:20 for dogs and ducks and 1:10 for chickens. For all species, the recommended cut-off of $\leqslant 45\%$ competition as compared to kit control was used to define positive sera.

5.4.2 Haemagglutination Inhibition Tests

All 159 bird sera from the second cross-sectional survey in November 2007 and a sample of 100 dog sera from across the sampling visits were tested by haemagglutination inhibition tests. The sample of 100 dog sera consisted of the 10 lowest cELISA scoring samples, the 10 highest scorers and 80 samples selected at random. All of the bird and dog sera were tested using an H3N2 A/turkey/England/69 antigen. The 100 dog sera were also tested against the other antigens listed in Table 5.1 at the National Veterinary Institute, Sweden (SVA). Antigens were selected to include a range of H3 and H1 virus antigens in the test panel. The A/turkey/England/1969 H3N2 strain is recommended as a prototype avian H3N2 antigen and the A/Mallard/Sweden/S90462/2005 is a more recent avian H3N2 strain isolated from a healthy duck. Two mammal origin H3 viruses were included, A/swine/Flanders/1/1998 and A/equine/New Market/1/1993 which is antigenically similar to American canine influenza strains. Two swine H1 antigens were included, A/swine/Belgium/WVLI/1979 H1N1 which is typical of swine H1 viruses and A/swine/Gent/7625/1999 H1N2 which is a recombinant virus with an H1 sequence similar to human H1 viruses. Finally, the A/ost/Denmark/72420/1996 H5N2 strain was included to test for the presence of antigens against H5 viruses.

Prior to HI testing, all sera were pre-treated to reduce non-specific reactions and to dilute to a starting dilution of 1:8 (protocol adapted from WHO (2002)). Each serum sample was mixed with an equal volume 0.4% trypsin solution and incubated at 56℃

Table 5.1: Summary of haemagglutination inhibition tests

Virus	Antigen	Virus subtype	n Sera tested		n Sera Positive	
			Avian	Dog	Avian	Dog
Influenza A	A/turkey/England/1969	H3N2	159	100	0	0
	A/Mallard/Sweden/S90462/2005	H3N8	0	100	–	0
	A/equine/New Market/1/1993	H3N8	0	100	–	0
	A/swine/Flanders/1/98	H3N2	0	100	–	0
	A/swine/Belgium/WVLI/1979	H1N1	0	100	–	0
	A/swine/Gent/7625/1999	H1N2	0	100	–	0
	A/ost/Denmark/72420/1996	H5N2	0	100	–	0

for 30 minutes. This mixture was treated with 3 volumes of 0.011M metapotassium periodate (KIO_4) and incubated at 56℃ for 15 minutes. Finally, 3 volumes of 1% glycerol saline were added to give a final dilution of sera at 1:8. All sera were two-fold serially diluted in phosphate buffered saline (pH 7.2) (PBS) for testing using 4 haemagglutinating (HA) units of viral antigen in V-shaped 96 well plates. All sera were tested using 1% suspension of chicken erythrocytes. The HI titre was read and defined as the reciprocal of the last dilution of serum that completely inhibited hemagglutination. Antigen controls as well as positive and negative control sera were run in each batch of tests.

5.5 Results

5.5.1 Bird Ownership and Sampling

During the surveys conducted by the SSS interviewers, the bird ownership status of 4411 and 4260 households respectively was established and 2.7% (95% CI: 2.3-3.3%) and 3.1% (95% CI: 2.7-3.7%) of responding households owned birds. The great majority of birds recorded at these households were chickens, with 88% (95% CI: 80-93%) and 93% (95% CI: 88-97%) of bird owners respectively keeping one or more chicken(s) at the household. One household was reported to keep turkeys in the July 2007 survey but all of the other owned birds reported were domestic ducks.

The mean number of birds (chickens and ducks) kept at all bird owning households was 4.71 and 4.67 birds respectively for the two surveys. These mean values are influenced by a small proportion of households that kept relatively large number of birds (maximum numbers of 23 and 32 birds at the time of each visit) and the median number of birds per household was 2 at each survey. The proportion of surveyed bird owners that had just one or two birds was 48% (95% CI: 39-58%) and 52% (95% CI: 43-61%) at each survey respectively.

The first cross-sectional sampling survey was conducted approximately three months after the collection of household ownership data and just 40 of the 121 (33%, 95% CI: 25-42%) households visited still had birds at the time of sample collection. However, even after an interval of less than one month between the ownership survey and sampling, just 53% (95% CI: 44-62%) of households identified in the second ownership survey still had birds, indicating that bird ownership in Kibera is quite transient.

The mean number of birds at the sampled households was marginally larger than that recorded for the total bird-owning population. The mean number of birds kept at sampled households was 5.3 birds as compared to 4.7 at all households visited at the first survey visit. Similar mean bird numbers of 6.3 at sampled households vs 4.67 at all households were recorded in the second survey. The proportion of sampled households that kept chickens (as opposed to ducks) was slightly lower than recorded for the bird owning population as a whole. 75% (95% CI: 59-87%) and 83% (95% CI: 73-91%) of sampled households kept chickens, whilst 18% (95% CI: 7-33%) and 10% (95% CI: 4-19%) of households kept ducks. Only 3/40 and 4/72 households respectively kept both species. It is likely that the households with slightly larger numbers of birds and perhaps also duck keepers have longer-term and more stable ownership and were thus more likely to still have birds at the household when visited for sampling.

In total, serum samples from 212 chickens and 64 ducks were collected and tested for influenza A antibodies (Table 5.2). The origin of all sampled birds was recorded. Most sampled birds were born within Kibera with approximately a quarter of the population brought in from other regions. During the first survey, 60% of sampled birds had been born within Kibera, whilst 13% were obtained outside Kibera and 22% were obtained within Kibera with unknown ultimate origin. One month later, the proportion of

sampled birds that were born within the study area was 47%, the proportion obtained outside the site was 15% and the proportion obtained within Kibera was 38%. The sampled birds that were sourced outside Kibera came originally from locations within 5 of Kenya's 8 provinces. The majority (66%) of sampled birds for which a source was provided came from the West of the country (Western and Nyanza provinces).

5.5.2 Competitive ELISA

A total of 689 dog sera were cELISA tested. The sera consisted of repeat samples from a total of 205 dogs sampled at 87 study households across the sampling visits in 2007 (Table 5.2 and Figure 5.1). 212 chicken sera and 64 duck sera from both cross-sectional surveys were tested using the ID.VET cELISA (Table 5.2). The cELISA test scores for all dog, chicken and duck sera tested are shown in Figure 5.2.

Only one dog serum sample and none of the bird sera gave positive scores in the cELISA test. The positive and negative controls on all test plates performed as expected and were within the range recommended by the manufacturer for plate validation. The positive dog sample was from a single individual sampled at visits D and E. The sample from visit D was negative whilst that for visit E was positive with an average percentage inhibition score of 35%.

The 689 tested dog sera included 268 samples collected from dogs that had been treated with ivermectin at the visit immediately prior to sample collection, and 19 dogs that had been treated previously with Canex. The Mann-Whitney test was used to test the hypothesis that the distribution of scores from dogs that had been treated with ivermectin at the previous visit differed from the distribution of scores from dogs that had not been treated with ivermectin at the previous visit. The results of this test (W=56717, p=0.7809) and visual inspection of the data provided no indication of any association between the previous provision of ivermectin and the magnitude of the cELISA score.
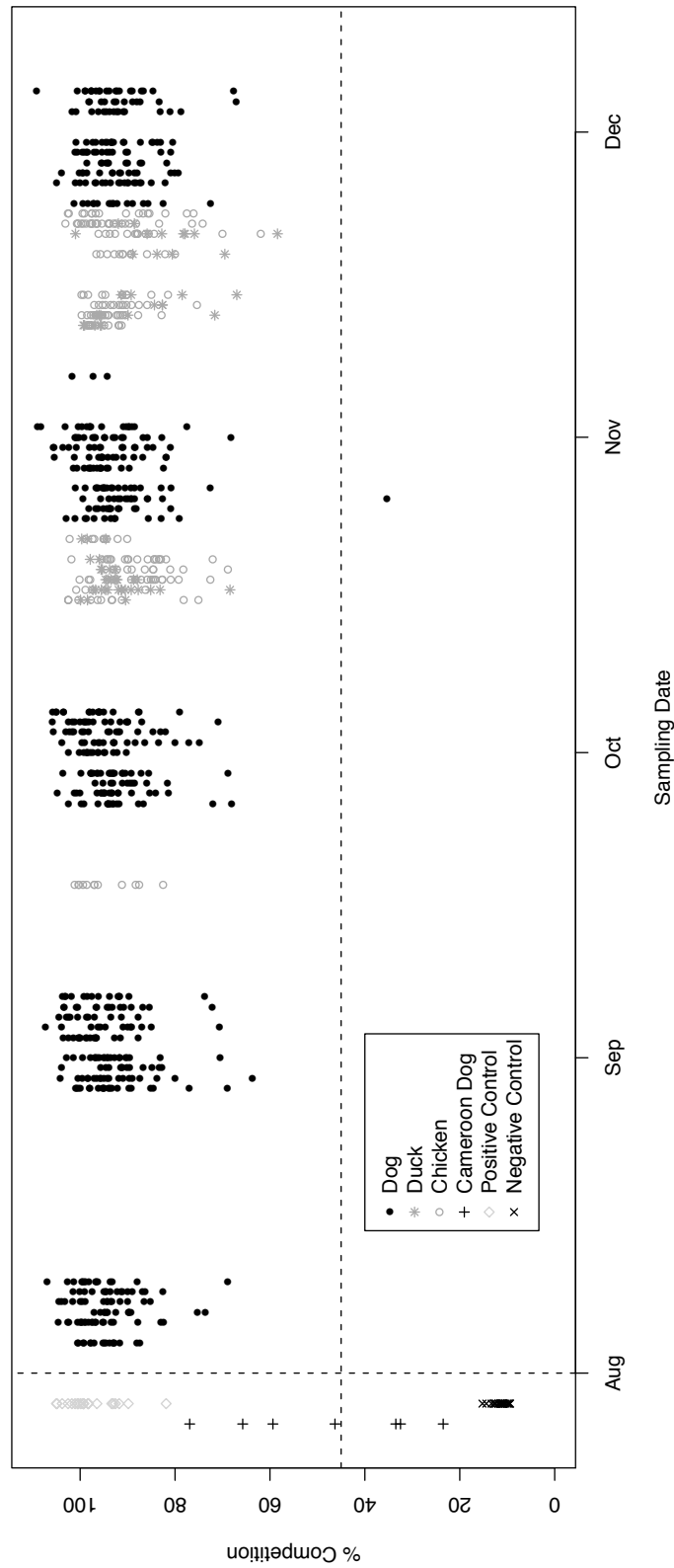
Figure 5.2: ID.VET cELISA results for Kibera dogs and birds sampled in 2007. The horizontal dashed line illustrates the recommended test cut-off. The vertical dashed line to the left of the figure separates Kibera test samples on the right from control samples plotted on the left of the figure for comparison. Black crosses indicate the range of scores obtained with the same test for a sample of Cameroonian dog sera (See Chapter 2)

Table 5.2: Influenza A sera samples

Visit		Dog B	Dog C	Bird Pilot	Dog D	Bird 1	Dog E	Bird 2	Dog F
Sera	Dogs	98	144	–	153	–	144	–	150
	Chicken	–	–	12	–	71	–	129	–
	Ducks	–	–	0	–	34	–	30	–

5.5.3 Haemagglutination Inhibition Tests

None of the bird or dog sera yielded positive HI results against any of the test antigens and in all tests samples were scored with a titre of <1:8. All positive and negative controls yielded the appropriate results.

5.5.4 Questionnaire Data

Amongst the households at which the questionnaire was administered, 65% (95% CI: 53-76%) had birds at the time of the questionnaire, which was conducted roughly a year after sampling. All of the household respondents reported confining birds during the nighttime but birds from 70% of households (95% CI: 58-81%) roamed outside the compound during the day. At the majority of households (77% , 95% CI: 66-87%), birds were fed daily but no feed was provided at the remaining 23% of households. None of the household respondents reported having vaccinated the birds against any disease prior to the study sampling visit.

38% of household respondents (95% CI: 26-50%) reported sickness in their birds in the six months preceding the survey but there was no consistent timing of the illness reported. Only one household reported any sickness in ducks, with the remainder referring to chickens. Common symptoms included diarrhoea (15/27 households), green faeces (15/27 households), dozing/drowsiness (7/27 households) and swelling of the head (8/27 households). The median numbers of birds that were reported to have died or been sick (and recovered) at these households were 4.5 and 0 with equivalent mean values of 16.19 and 1.115 respectively. The mean value for the number of birds that died was strongly influenced by the report of 160 birds that died at a single household.

61/72 households reported slaughtering their birds at the household (85%, 95% CI: 75-92%) and essentially all of the households that slaughtered birds at the household reported simply throwing the bird carcass and entrails away. Commonly reported locations for discard were pit latrines and drains near the house as well as the river. The handling of birds was done predominantly by female members of the household - 70% (95% CI: 58-81) of people reported as being responsible for looking after birds were female and 83% of people (95% CI: 71-92%) who slaughtered birds were women.

5.6 Discussion

The aim of this study was to use serosurveillance to determine if any influenza A subtypes circulate in the Kibera domestic animal populations and if present, whether or not the virus(es) that these populations were exposed to were the same as those identified in the human population. Apart from a single dog serum sample that yielded a positive cELISA score, the cELISA and HI testing of the Kibera dog and bird populations provides no indication of exposure to influenza A viruses in these animal populations. The single dog serum sample that was positive in the cELISA was negative in all of the HI tests and given the overall picture from the population it is quite likely that this single dog serum represents a false-positive result. If truly positive for antibodies against influenza A this sample is nevertheless unusual within this population. The data obtained from the animal population contrasts with that from the human population at this site, within which there is evidence for the circulation of influenza A viruses. However, the H3N2 and H1N1 virus subtypes characterized from the human population are typical of seasonal strains that are maintained through human-to-human transmission and there is no evidence from the human population surveillance of inter-species influenza A transmission at this site. In simple terms, the results from the animal population surveys are consistent with the data from the human population. All of the data indicate that there is no circulation of influenza A viruses in Kibera that might pose a zoonotic risk and we can therefore argue that this surveillance of the bird and dog sentinel species has provided useful information about the risk of zoonotic influenza A to the human population.

The overall aim of this study is to assess the utility of animal sentinel surveillance and this assessment must include discussion of both the strengths and potential limitations of this approach and of the data obtained. The influence of some strengths and limitations of this study upon the capacity of the animal surveillance described to detect influenza A virus presence if it were there are discussed below.

If influenza A viruses had circulated within the Kibera bird population prior to the sampling study we would expect the presence of this pathogen to be detectable through surveillance of the bird population. Either as a consequence of very high bird mortality in the case of a highly pathogenic strain or through the detection of antibodies in birds that had survived exposure to a low pathogenicity strain. A high pathogenicity influenza A strain would cause considerable mortality amongst chickens (if not also ducks) and there would therefore be very few chickens present. However, approximately 90% of the households that kept birds had chickens and although a significant proportion were brought in from around the country, roughly half of the sampled birds were born within Kibera and must have been present for at least several months to have grown to a size at which a blood sample could be safely obtained. It is therefore very unlikely that a highly pathogenic influenza A virus had circulated within Kibera in the several months preceding the ownership and sampling study. There were reports of disease with sometimes quite considerable mortality in the poultry kept in Kibera after the sampling surveys were conducted and the possibility of influenza A transmission after the sampling survey cannot be excluded. However, the morbidity and mortality reported was not consistent with a widespread synchronous die-off and was also not reported as unusual. The pattern of mortality and symptoms reported are most consistent with the circulation of Newcastle Disease Virus which is known to kill an average of 70-80% of unvaccinated village hens in developing countries (Branckaert and Guéye, 1993) but other avian pathogens cannot be ruled out. Anecdotally, it was the case that bird owners interviewed in the questionnaire survey reported very favourable improvements to the health of their flocks following the provision of Newcastle Disease vaccine in association with sample collection. This finding provides more support for the argument (discussed in Chapter 2) that bird morbidity and mortality reporting can be a very non-specific tool for the surveillance of influenza A viruses, in the developing world particularly.

If a low pathogenicity influenza A virus circulated in the Kibera bird population we would expect much lower mortality and that a proportion of the sampled bird population would have antibodies against influenza A. In chickens, antibodies against influenza A can last for longer than 35 weeks (Swayne and Halvorson, 2003). The results of the cELISA provide no evidence of exposure to influenza A in the sampled chicken and duck populations. The bird samples were not as extensively HI tested for the reason that in contrast to the dog sera for which the cELISA has not been validated we can have greater confidence in the validity of the cELISA test results from this bird population (Greiner and Gardner, 2000; VanDalen et al., 2009; IDVET, Distributed 2008a; Terregino, Distributed 2010).

Partly as a consequence of the transient nature of bird keeping in Kibera, the coverage achieved by the sampling study was quite low and the sample sizes for each species at each visit are relatively small. In the case of the duck population, it was also the case that the small sample size reflects the small overall size of the population. The chicken sample sizes allow quite good confidence in the estimated zero prevalence of infection detected but the number of ducks sampled was small and we do have to recognize that whilst the observed data from the duck population yield 0% prevalence estimates the exact binomial confidence intervals for these estimates do allow for low prevalence virus circulation (upper bounds of 95% CI of 10.3 and 11.6% at the first and second visits respectively).

The bird slaughter and carcass disposal practices recorded in the questionnaire survey provide ample opportunity for transmission of any bird influenza viruses to dogs that scavenge throughout Kibera and are extremely likely to consume discarded carcasses. Seroconversion of dogs to influenza A viruses following the consumption of infected bird carcasses has been documented (Songserm et al., 2006a; Daly et al., 2008; Giese et al., 2008) and we might therefore expect that surveillance of the domestic dog population could give an indication of the influenza A subtypes present in the local bird population (See Chapter 2). In agreement with the results obtained through direct survey of the bird populations, there is essentially no indication of exposure to influenza A viruses in the Kibera dog population. As discussed in Chapter 2 the cELISA test used in this study has not been validated for use with dog sera but in the Cameroon population

there was quite good correspondence between the dog cELISA and the VLA H5N1 HI results. In the Kibera population it is also the case that the cELISA and HI data agree. The HI assays used to test the dog sera were conducted using the standard chicken erythrocytes rather than horse erythrocytes which can improve test sensitivity for detecting antibodies against avian adapted viruses in mammalian sera (Stephenson et al., 2003; Meijer et al., 2006; Jia et al., 2008; Kayali et al., 2008). However, the previously documented exposure of dogs to an avian origin H3N2 virus was demonstrated using a commercial cELISA that detects antibodies against the nucleoprotein (similar to the test used in this study) and standard HI tests utilizing chicken red blood cells (Song et al., 2008; Lee et al., 2009; Song et al., 2009).

The cELISA and HI results from the Kibera dog population also indicate that the dogs are not exposed either to canine maintained influenza viruses or to the influenza A viruses that circulate in the human population. Although there is evidence from older studies that dogs mount detectable antibody responses to experimental infection with human influenza A viruses (Todd and Cohen, 1968; Kilbourne and Kehoe, 1975), recent serological studies conducted in Korea and Italy indicate that when not associated with an outbreak in either the local poultry population or with a respiratory disease outbreak in the dog population itself there is little evidence of influenza A exposure in dog populations (De Benedictis et al., 2009; Lee et al., 2009). There are problems of sensitivity reduction with the HI test, related to the detection of antibodies against avian adapted viruses in mammal sera (Beare and Webster, 1991; Kida et al., 1994; Stephenson et al., 2003) but similar problems have not been described with the cELISA tests used in this study and previously (De Benedictis et al., 2009). The application of these tests for this kind of dog surveillance is novel and we must therefore recognize the potential for poor test performance (Greiner and Gardner, 2000; VanDalen et al., 2009). However, the data from Kibera, in which human adapted viruses are known to circulate, are consistent with serosurveillance of dogs in other areas and indicate that dog populations do not show detectable antibody responses to 'normal' seasonal flu circulation. When assessed alongside the data from Cameroon (See Chapter 2) and from other studies (Crawford et al., 2005; Daly et al., 2008; Payungporn et al., 2008; Song et al., 2008), these findings indicate that the antibody responses detectable in dog populations to influenza A may be specific to the kinds of unusual inter-species

flu transmission events that are of the greatest surveillance concern. The factors that determine inter-species transmission of influenza A viruses are numerous and complex however (Kuiken et al., 2005) and further work will be required to explore this pattern further.

Domestic backyard poultry flocks are often identified as at high risk of influenza A introduction (Gilbert et al., 2006a; Songserm et al., 2006a; Bavinck et al., 2009; Zheng et al., 2010) and are thought to act as the epidemiological link between the wild bird reservoirs of influenza A viruses and intensively reared poultry (Terregino et al., 2007). In Thailand, the distribution of H5N1 outbreaks has been most closely associated with the distribution of free-grazing ducks (Gilbert et al., 2006a), nearly 300 H5N1 isolates have been collected from backyard and small scale farm poultry in Nigeria (Joannis et al., 2008), and in Northern Italy, 27 influenza A viruses were isolated through surveillance of backyard flocks over a two year period (Terregino et al., 2007). The data regarding the epidemiological role of backyard flocks is not entirely consistent though and backyard poultry flocks seem to have played a minor role in the Netherlands H7N7 epidemic that occurred in 2003 (Bavinck et al., 2009). The characteristics of flocks described as backyard will vary considerably in different areas and the probability of infection of any flock will be influenced by numerous factors including the number/density and species of birds, the number and type of contacts between flocks, with markets, and with wild birds and the housing and sanitary conditions in which birds are kept (Alexander and Capua, 2008; Bavinck et al., 2009; Magalhães et al., 2010).

Apart from outbreak associated surveillance, only a small number of influenza A sero-surveillance studies have been conducted in small backyard poultry flocks. In keeping with this survey, those studies that have been conducted in similar populations have reported little or no evidence of exposure to influenza A viruses in non-outbreak associated populations (Hernandez-Divers et al., 2006; Buscaglia et al., 2007; Hernandez-Divers et al., 2008; Zheng et al., 2010). However, one survey of backyard birds conducted in Mali using the ID VET cELISA classified 13.7% of sera as positive for anti-influenza A antibodies (Molia et al., 2010). This overall seroprevalence (generated from a convenience rather than random sample) translated to 18.3% positive ducks and 8.9% positive chickens. The sampled birds were also swabbed and rRT-PCR testing revealed

that 3.6% of the birds had at least one influenza A positive swab. None of the swabs were positive for H5 or H7 subtypes, indicating the circulation of a low pathogenicity influenza A strain in this Malian poultry population (Molia et al., 2010).

The source of primary introduction of influenza A viruses into poultry populations is most commonly wild birds, whereas human activity is then the key determinant of secondary spread (Capua and Alexander, 2008). Up to a quarter of the sampled Kibera bird population was brought in from towns and villages located across Kenya. Studies of H5N1 influenza epidemiology within Africa have suggested that once introduced into an area, viruses have evolved independently with limited exchange between populations (Cattoli et al., 2009), consistent with a predominantly local scale of trade (Sonaiya and Swan, 2004; Cattoli et al., 2009). The transportation of birds from rural areas into Nairobi, provides the potential for an introduction of influenza A viruses which could then spread within the Kibera population. The relatively low levels of bird confinement within Kibera and the poor biosecurity and sanitation of household level bird-keeping also create opportunities for the onwards transmission of virus following the initial introduction. Indeed, the apparent impact of Newcastle Disease in this population demonstrates the capacity for the effective transmission within this bird population of a viral pathogen with similar morbidity and mortality patterns to influenza A. The questionnaire data collected indicate that the bird handling and slaughter practices adopted in Kibera create circumstances in which onwards transmission to both the human and dog population could occur. Infected birds can shed virus in both respiratory and digestive tract excretions and surfaces and water supplies can all become contaminated and act as a source of infections (Alexander and Capua, 2008; Anderson et al., 2010). With the very close association between people and birds at the Kibera site, where household birds live adjacent to and sometimes within human habitations, there exist very few barriers to prevent the exposure of people to any viruses present in the bird population. This study has not identified any evidence of influenza A viruses in the Kibera bird population that might pose a zoonotic threat but the recent spread of H5N1 influenza in poultry populations in other African countries (Ducatez et al., 2006, 2007a; Monne et al., 2008; Cattoli et al., 2009) highlights the potential risks to the human population associated with this kind of bird husbandry.

5.6.1 Kibera Site Conclusions

There is laboratory confirmed evidence of influenza A circulation in the human population at this site and the virus subtypes seen are typical of H3N2 and H1N1 seasonal flu viruses that are maintained through transmission within human populations (Gikundi, 2009). The absence of cELISA positive results, the lack of haemagglutination inhibition responses at the 1:8 dilution against any of the antigens used and the consistency of results from the two tests indicate that the animal sera from Kibera are truly negative for antibodies against these influenza antigens. The results from the animal and human population surveillance conducted at this site are therefore both consistent with the absence of zoonotic influenza transmission. The questionnaire data reveal that characteristics of bird management at this site might allow for the introduction of viruses, secondary spread within the bird population and also onwards transmission to both the human and other animal populations. The potential for the transmission of zoonotic influenza at this site does exist but this study has identified no evidence that this situation has in fact arisen.

5.6.2 Influenza A Surveillance Conclusions

Comparison of the data on influenza A presence obtained using similar techniques in the Cameroon and Kibera surveys reveals very different epidemiological pictures. In both the Cameroon and Kibera populations the data from the dog population agrees with that seen from the corresponding poultry population and with other data available from these respective sites. In itself, this clear difference between sites provides support for the use of animal, and specifically dog, serosurveillance for influenza A and suggests that it could be applied on relatively broad spatial scales in order for example to conduct village level assessments of the potential risk of zoonotic influenza A. At the Kibera site, there is no evidence of exposure of the dog population to the H3N2 and H1N1 influenza A subtypes that are known to circulate in the human population. This suggests that when evidence of influenza A exposure is detected in the dog population (as in Cameroon - See Chapter 2), this signal of pathogen presence is not complicated by 'non-specific' serological reactions in the dog population to human maintained seasonal

flu viruses. The negative findings in the Kibera population reinforce the significance of the positive results from Cameroon and support the data indicating that the positive results observed in the dog and pig populations at Maga were specific to the H5N1 viruses that apparently circulated in the avian population at this site.

However, the limitations of the available diagnostic tests identified by this and other studies impact upon the confidence that we can have in these kinds of data. This kind of sentinel surveillance approach would make sense only if an easily conducted dog sampling survey could be rapidly and cheaply translated into reliable data on pathogen presence. The sampling surveys are themselves quick, easy and relatively cheap to conduct and we have good reasons to expect that dog serosurveillance particularly should reveal the presence of influenza A viruses circulating in bird populations. The problem arises at the level of the diagnostic tests that are currently available. The cELISA test used in this research is easy to use, provides quick and relatively cheap data and has yielded results from both sites that are consistent with the much of the other available data. However, we don't yet know enough about the performance of this test in different populations and for the detection of different influenza A subtypes to rely on this test alone. In this study, additional HI tests have been conducted in order to confirm and validate the cELISA results. On balance the cELISA and HI results from each population have agreed reasonably well. However, in Chapter 2, the two sets of H5N1 HI test results from two different labs were very different and although the sample size was small there is some indication also from these data that the test performance varied for different influenza A subtypes (H5 and H3). The considerable doubts raised in other studies about the performance of standard HI tests particularly for testing mammalian sera (Kida et al., 1994; Stephenson et al., 2003; Meijer et al., 2006) raise the concern that the application of existing tests alone may lead to widespread under-reporting of influenza virus exposure (VanDalen et al., 2009).

The sentinel framework described in Chapter 1 is illustrated in Figure 1.3 with reference to a scenario of determining if influenza A H5N1 had been introduced into a country with underdeveloped disease surveillance capacity. The data presented here and in Chapter 2 provide two practical examinations of the issues and questions outlined in Figure 1.3 and they reveal that the factors that currently limit the utility of animal

and specifically dog serosurveillance for influenza A viruses in general, relate primarily to the available diagnostic tests and the detectability of sentinel responses. The domestic species sampled in these studies were consistently accessible for sampling and the serological tests used did provide a relatively cheap, rapid and transferable tool for the assessment of these populations. However, recognizable problems with the HI tests used to evaluate the performance of the practical screening cELISA test mean that we don't have good test validation data for these and similar populations. There are other gold standard tests such as neutralization assays that could be used for the validation of the cELISA performance. However, conducting these tests requires handling live virus, is generally restricted to specialist laboratories and was outside the scope of this project. This study was not designed with the aim of conducting extensive diagnostic test validation but of assessing how useful the data that we can currently obtain from animal serosurveillance and specifically from domestic dogs are for evaluating the risks posed by zoonotic pathogens. In the case of influenza A the utility of the animal sentinel serosurveillance approach is currently limited by the lack of confidence that we can have in the diagnostic test data that we can currently obtain. This kind of approach relies on the use of quick and cheap screening tests. The cELISA test used in this research may well be performing extremely well but until we can confirm this, the utility of the data obtained from this and similar tests that are currently available will remain limited.

Chapter 6

Surveillance of Leptospirosis in Animal Populations: Infection Patterns in Kibera Rodents

6.1 Abstract

Leptospirosis is a widespread but under-diagnosed zoonosis that is of increasing importance as a cause of morbidity in the urban slum context. A cross-sectional survey was conducted to determine the presence and prevalence of pathogenic *Leptospira* in the Kibera rodent population. A questionnaire survey was administered to investigate contacts between the human and rodent populations and potential risk factors for human infections. A total of 237 individuals (195 *Mus* and 42 *Rattus*) were trapped within households with an overall trap success of 25% although there were significant differences in trap success in the five trapping zones within the Kibera site. The presence of pathogenic leptospires was detected in the kidneys of 18% of the trapped rodents by PCR. There was a significant difference in the log odds of leptospire detection in the kidneys of *Rattus* individuals as compared to *Mus* (OR=0.05, 95% CI:0.005-0.41). Antibodies against *Leptospira* were detected in the sera of <10% of 162 individuals by

ELISA and MAT and there was no correspondence between the PCR and ELISA results at the individual level. 60% of respondents in the questionnaire survey reported seeing 5 or more rodents in their house on a daily basis. These data provide molecular and serological evidence of the circulation of pathogenic leptospires in the Kibera rodent population and of frequent potentially risky interactions between the human and rodent populations, indicating that leptospirosis may impose a considerable health burden upon the human population in Kibera.

6.2   Introduction

Leptospirosis is thought to be the most widespread zoonosis in the world (WHO, 1999; Levett, 2001) and has been described as a paradigm for an urban health problem that has emerged as a consequence of the growth of slums (Reis et al., 2008). Leptospirosis causes a variety of clinical syndromes in people, most commonly an undifferentiated febrile illness and cases are often not reported or are misdiagnosed. Partly as a consequence of this clinical picture and the lack of knowledge about the zoonotic threat posed by leptospirosis it is under-diagnosed in tropical countries particularly (WHO, 1999). Infrastructural deficiencies such as open sewers and refuse deposits have been demonstrated to serve as transmission sources for leptospirosis in the slum environment (Reis et al., 2008). Human leptospirosis is often acquired through indirect contact with the urine of infected hosts and in the slum environment high densities of both rodents and dogs and poor water sanitation contribute to conditions that facilitate widespread environmental contamination with leptospires. Figure 6.1 illustrates the condition of the water, sewage and refuse infrastructure within the Kibera study site.

Many previously identified risk factors for human leptospirosis infection are present at the Kibera site, indicating that leptospirosis may cause considerable disease in this population but the human disease burden of leptospirosis is unknown. The *Leptospira* status of the potential animal reservoir populations at this site are also unknown and leptospirosis in Kibera clearly warrants surveillance investment. The transmission of leptospirosis depends on interactions between humans, mammalian reservoir hosts and the environment (Vinetz, 2001) and leptospirosis therefore provides a good case study

Figure 6.1: Photographs of the Kibera study site illustrating the accumulation of refuse and proximity of open drainage systems to housing

for the assessment of the animal sentinel approach. In this and the following two chapters, the surveillance of leptospirosis in Kibera is considered with reference to the animal sentinel framework developed in Chapter 1. Figure 6.2 summarizes the application of the framework in this context. The target population in this case is the human population of Kibera, the Kibera domestic dog population is considered as the potential sentinel population and the pathogen in question is pathogenic *Leptospira* spp. The ecological context of this analysis and the key characteristics of the Kibera human, rodent and dog populations and their relation to each other are described in this chapter and in Chapters 3 and 7. In this chapter, which focuses on the rodent population, and the following chapter which considers data gathered from the dog population, the presence of pathogenic leptospires in the Kibera rodent and dog populations are explored. The inferences that can be made from these data to understand human risk are discussed further in Chapter 8.
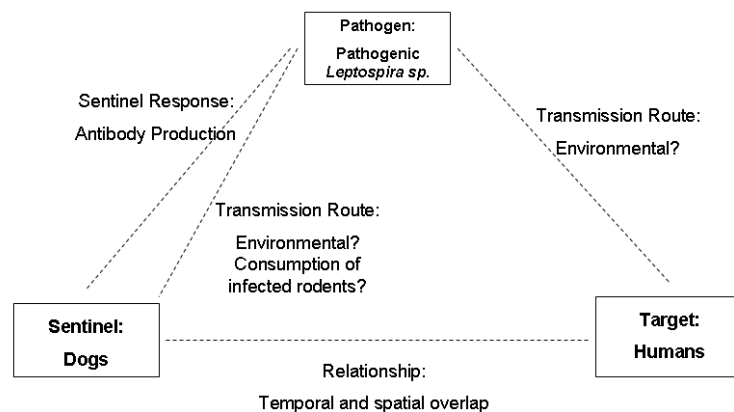
Figure 6.2: Framework for evaluating dogs as sentinels of human leptospirosis in Kibera

6.2.1 Classification of Leptospira

The genus leptospira includes 17 currently recognized species (Bharti et al., 2003), classified on the basis of their genetic relatedness. The genus includes both pathogenic and non-pathogenic species as well as a small number of species containing leptospires of as yet undetermined pathogenicity (Smythe et al., 2002). This taxonomic system has replaced the older classification in which all pathogenic leptospires were classified

as *L. interrogans* whilst all saprophytic strains were classified as *L. biflexa*. Under the current classification, different species can include both pathogenic and non-pathogenic leptospires (Levett, 2001).

Leptospires are also classified phenotypically on the basis of their serological characteristics. The genus is divided into over 200 serovars which are grouped together into serogroups (Bharti et al., 2003). Serological and genetic classifications are still used, as correspondence between these classifications is poor. Component members of an individual serovar can represent multiple genetically defined species and both genetic and serological groupings may contain both pathogenic and non-pathogenic strains (Levett, 2001). In this and subsequent chapters the genetic classification at the species level is used and the serovar or serogroup is also given.

6.2.2 Laboratory Diagnosis of Leptospirosis

Numerous techniques are available for the detection of leptospires and diagnosis of leptospirosis. The great variety of symptoms that can be observed in clinical cases necessitates accurate laboratory testing for accurate diagnosis of infection. The classical gold standard tests are the culture of live leptospires from clinical material and the microscopic agglutination test (MAT) for the detection of serogroup specific antibodies. Both of these techniques are labour intensive, require access to a panel of live cultures of reference strains and have low sensitivity. Increasingly, molecular detection and typing techniques play an important role in the detection of leptospires and understanding of their epidemiology (Levett, 2007) and alternative serological tools are being developed.

6.2.2.1 Culture, Isolation and Typing

The isolation of live leptospires through culture provides the opportunity for analysis and characterization through the full range of diagnostic tests and typing systems. The time required for detection of leptospires after inoculation varies according to the serovar and the number of viable leptospires present in the inoculating tissue (OIE, 2006). Some serovars may be positive by culture in 7-10 days but cultures should be incubated for up to 26 weeks to allow a conclusive negative test result (OIE, 2006). Isolated

leptospires in culture can be typed using both serological (cross-agglutination absorption test (CAAT)) and molecular techniques (sequencing, pulse-field gel electrophoresis (PFGE), DNA-DNA hybridization and multi-locus sequence typing (MLST)). Genetic methods are now enabling more accurate typing of infectious organisms than serological techniques ever allowed (Ahmed et al., 2006; Levett, 2007). In a recent study a single MLST clone was identified as responsible for 76% of human cases during an outbreak and was also identified in the 7/8 bandicoot rats (Thaipadungpanit et al., 2007). The kind of diagnostic accuracy that these genetic typing techniques allow can greatly assist the design of targeted control efforts and inform our understanding of the epidemiology and evolution of leptospires (Ahmed et al., 2006) but they are dependent upon a good supply of epidemiologically relevant isolates before they can be usefully applied (Levett, 2007). Successful culture of organisms provides a great deal of information but the process is extremely labour intensive and time-consuming. In addition the technique has very low sensitivity in comparison to other available tests, particularly PCR (Gravekamp et al., 1993; Levett, 2001; de Faria et al., 2008) and it is not therefore, a viable option for large scale routine surveillance.

6.2.2.2 Serology

Most cases of human leptospirosis are diagnosed by serology (Levett, 2001). Antibodies against leptospires are detectable in many species within days of illness (5-7 days after the onset of symptoms in humans (Levett, 2001)). Serological tests are therefore limited by low sensitivity during the first week of illness (Bajani et al., 2003; Ooteman et al., 2006; McBride et al., 2007). The microscopic agglutination test (MAT) is the reference method for serological diagnosis of leptospirosis (Levett, 2001). The test utilizes live or formalinized preparations of cultured leptospires which are mixed with a dilution of test sera. After incubation, the antigen and serum mixtures are examined by dark-field microscopy for evidence of agglutination of the leptospires, indicative of the presence of antibodies in the test sera. Sera are usually MAT tested in serial dilution and an end-titre (usually the greatest dilution at which 50% of free leptospires are agglutinated) reported. A MAT titre of $\geqslant 1{:}100$ is widely recognized as indicative of exposure in a single sample test (Levett, 2001; WHO, 2003; OIE, 2008) although different authors

have suggested the use of alternative cut-offs in different epidemiological circumstances (Zochowski et al., 2001; Bajani et al., 2003; Maciel et al., 2008; Reis et al., 2008).

The MAT is a flexible test that detects all antibody subgroups and can be used with sera from all species. Unlike most other serological tests which are genus specific, the MAT can also be used to indicate the identity of the infecting serovar/serogroup (Ooteman et al., 2006). However, antibodies against leptospires can be highly cross-reactive to strains from multiple serogroups, particularly in the acute phase of infection. In addition, 'paradoxical' reactions can be observed in the MAT test, in which the highest antibody titres are observed to antigens of a serogroup that differs from the infecting serogroup (Levett, 2001). As a consequence, the MAT is considered a serogroup rather than serovar specific assay and " at best, the MAT can give a general impression about which serogroups are present within a population"(Levett, 2001).

The key drawbacks of the MAT are that it is time and labour intensive and is a complex test to control, perform, and interpret (Levett, 2001). The MAT requires that a panel of live stock cultures is maintained (Cole et al., 1973). The panel of test antigens should include enough serogroups and serovars to provide adequate coverage of the antigenic diversity present in a given area and the sensitivity of the test can be increased by including locally representative test antigens (Levett, 2001). The capacity of the test in different contexts is therefore determined by the availability of isolates representative of those expected in the test samples, which in turn depends on their being previous knowledge of the expected serovars and ideally isolates from the same site. The requirement for live antigen also generates inherent safety risks of handling cultures of live leptospiral organisms (Smythe et al., 2002) and as a consequence the MAT is often only available at specialist laboratories (Brandão et al., 1998; Smythe et al., 2002).

These limitations of the MAT have to the development of numerous, largely genus-specific serological tests, including a range of ELISAs, indirect haemagglutination assays (IHA) and slide agglutination tests (SAT). In comparison to the MAT, ELISAs particularly offer reliable, safe, and reproducible tests (Ribotta et al., 2000; Jimenez-Coello et al., 2008) that are useful for the rapid diagnosis of cases (Blacksell et al., 2006) and for screening large numbers of sera in epidemiological surveys. ELISA tests validated in both humans and dogs have demonstrated similar or improved sensitivity

of IgM ELISA tests as compared to the MAT, particularly for the early detection of IgM antibodies in acute cases (Brandão et al., 1998; Ribotta et al., 2000; Jimenez-Coello et al., 2008) and non-MAT tests can detect antibodies as early as 2 days after the onset of illness (McBride et al., 2007). Two in-house ELISAs used at the Leptospirosis Reference Unit (LRU) of the Health Protection Agency (HPA) that were developed using a very similar protocol to that used in this study (See Section 6.4.4) showed similar sensitivity and specificity as compared to the MAT (Zochowski et al., 2001). However, some rapid format test may be subject to false positive reactions and require confirmation of these results by the MAT (Zochowski et al., 2001; Smythe et al., 2002; Bajani et al., 2003).

The long persistence of high MAT detectable titres (sometimes for many years) necessitates the collection of paired specimens for a definitive serological diagnosis of an acute case (Brandão et al., 1998; Bajani et al., 2003). A fourfold or greater rise in MAT titer in the setting of an appropriate clinical syndrome is the standard case definition (Bajani et al., 2003). The requirement for testing of paired samples particularly, makes the MAT an impractical test for clinical decision making (Bajani et al., 2003; McBride et al., 2007). ELISA detectable antibodies appear to decline more rapidly but still persist for many months and even years (Brandão et al., 1998; Johnson et al., 2004).

The MAT detects antibodies against only those leptospiral surface antigens involved in agglutination, whereas the ELISA can detect antibodies against other immunogenic components of the leptospire cell wall and internal proteins (Lupidi et al., 1991). The ELISA is therefore considerably less-specific to individual serovars and is considered a genus-specific test (Lupidi et al., 1991; Levett, 2001). Findings regarding the sensitivity and specificity of non-MAT serological tests can be geographically variable (Blacksell et al., 2006; McBride et al., 2007). An assessment of four commercial serological assays alongside the MAT, that used sera from a range of geographic areas, reported good performance of the ELISA and dipstick format tests particularly (Bajani et al., 2003). However, a recent study conducted in Laos, where leptospirosis in endemic, found poor diagnostic sensitivity using two commercial assays for the testing of acute phase human sera (Blacksell et al., 2006). In contrast, a study based in urban Brazil, evaluated 4

commercial tests and found that all had similarly high specificities and overall sensitivities ranging from 72-88%, indicating that ELISA and rapid formats may be a useful alternative to the MAT for diagnosing urban leptospirosis (McBride et al., 2007).

The interpretation of all serological test results are complicated by the fact that the duration, magnitude and specificity (to the infecting serovar) of antibody responses to leptospires are variable and in chronically infected animals, antibodies may fall after an initial response to undetectable levels whilst the individual remains a chronic carrier (OIE, 2006). It is proposed that variation in the utility of different tests in different contexts may be explained by factors including the time of sera collection after the onset of illness, the proportion of patients experiencing primary and secondary infections, and the serovars responsible for infections (McBride et al., 2007). As with any diagnostic test, variation in the performance of rapid serological tests for leptospirosis that are applied in a novel population or setting might be expected and should be evaluated (Greiner and Gardner, 2000).

6.2.2.3 PCR

The development of PCR diagnostic tests has been prompted largely by the limited sensitivity of serological tests in the first week of illness, before a detectable antibody response develops. The efficacy of antibiotic treatment in people is greatest in the first five days of illness (WHO, 2003) and non-serological tests are therefore particularly relevant to the clinical management of acute suspected cases (Blacksell et al., 2006; Ooteman et al., 2006).

A number of gene targets have been described for the detection by PCR of leptospires as a whole and pathogenic leptospires specifically and several protocols designed to distinguish pathogenic and saprophytic leptospires have now been described (Gravekamp et al., 1993; Murgia et al., 1997; Smythe et al., 2002; Kositanont et al., 2007). Because of the poor correspondence between genetic and serological classifications of leptospires, PCR-based diagnoses of infections are largely unable to identify the infecting serovar (Levett, 2001). However, in comparison to culture, PCR assays for the detection of leptospires are very sensitive and very quick (Smythe et al., 2002; Ooteman et al., 2006).

They also show high specificity and many of the available protocols have been validated using a range of other bacterial pathogens (Gravekamp et al., 1993; Smythe et al., 2002; Kositanont et al., 2007). PCR assays are rapid and accurate and can potentially be applied to a wide range of sample types. However, they can be vulnerable to inhibitory effects of a variety of substances that may be present in clinical samples including urine (Bal et al., 1994; Smythe et al., 2002).

The test used in this present study is one of the more extensively validated protocols (Levett, 2001). The G1/G2 primer pair designed by Gravekamp et al. (1993) targets a secY sequence which encodes the pre-protein translocase SecY protein and has been shown to distinguish pathogenic and non-pathogenic leptospires consistently, with the exception of one pathogenic species (Gravekamp et al., 1993). Gravekamp et al. (1993) determined that the G1/G2 primer pair does not amplify products with strains belonging to the species *L. kirshneri* and to overcome this limitation a second primer pair (B64I/B64II) which targets a sequence seen in *L. kirshneri* is included in the same assay as the G1/G2 primers to allow the detection of all pathogenic species (Gravekamp et al., 1993). The protocol described by Gravekamp et al. (1993) was developed for use with sera from suspected human cases of acute leptospirosis. Adaptations of this protocol have also been used to test a range of additional tissue types, including human urine (Bal et al., 1994), blood (de Abreu Fonseca et al., 2006; Ooteman et al., 2006), isolates from rats (Machang'u et al., 2004), rodent urine (de Faria et al., 2008) and mammal kidneys (Bunnell et al., 2000).

6.2.3 Leptospirosis Epidemiology and Surveillance

Leptospirosis is maintained through chronic renal infection of carrier animals, and the principal source of human infection is contact (direct or indirect) with the urine of an infected animal (Levett, 2001, 2003). Small mammals, specifically rodents, are considered to be the most important maintenance hosts or reservoirs for a variety of pathogenic serovars (Levett, 2001). There are a number of host-serovar associations that appear to be essentially universal, such as serovar icterohaemorrhagiae maintained in *Rattus* species, serogroup Ballum serovars in *Mus* and serovar canicola in dogs (Bharti et al., 2003). However, these relationships between leptospires and hosts are flexible. A

single species can carry different serovars in different populations and a single serovar may have a range of different hosts in different contexts (Levett, 2001; Bharti et al., 2003).

Human infections most commonly involve entry of the pathogen through cuts or abrasions in the skin or via the conjunctiva but water-borne transmission has also been described in the context of several large outbreaks (Levett, 2001). Human infections have traditionally been associated with occupational exposures and activities such as farming, veterinary practice, mining and sewer maintenance (Levett, 2001; Bharti et al., 2003). Reliable data on the morbidity and mortality associated with leptospirosis are lacking though and the disease is generally under-reported in tropical countries (WHO, 1999; Levett, 2001; Bharti et al., 2003). As a consequence, leptospirosis is often only reported when specifically looked for (Matthias et al., 2008). Despite this under-reporting it is clear that the incidence of leptospirosis is higher in tropical regions of the world. This pattern is attributed to the differential survival of leptospires in different environments and enhanced survival in warm, humid conditions (Levett, 2001). It is also argued that many tropical countries are also developing countries in which interactions between human and animal populations are more common and the potential for zoonotic transmission is thus greater (Levett, 2001; Holt et al., 2006). Finally, there is a strong seasonality in leptospirosis incidence, associated with climatic influences upon leptospire survival in the environment such as rainfall patterns and flooding risk (Levett, 2001; Bharti et al., 2003; Holt et al., 2006). There is increasing recognition of the burden of disease cause by leptospirosis that is acquired through normal day-to-day activities in tropical areas, where a number of factors combine to enhance the contamination of the wider environment with viable leptospires (Levett, 2001; Bharti et al., 2003)

The symptoms of infection in humans are highly variable and can mimic the symptoms of a variety of other diseases (Levett, 2001; Bharti et al., 2003). The great majority of infections are subclinical (Levett, 2001; Bharti et al., 2003; Johnson et al., 2004). In the proportion of cases that experience symptoms, most patients develop anicteric leptospirosis and present with a febrile illness of sudden onset, accompanied by a variety of other non-specific symptoms such as headache, chills and myalgia (Levett, 2001).

Anicteric leptospirosis usually lasts for roughly one week and resolves with the production of antibody (Levett, 2001). In the Iquitos region of Peru, 20-30% of patients presenting at health posts with acute undifferentiated fever have serological evidence of acute leptospirosis (Bharti et al., 2003). However, between 5 and 10% of all patients with leptospirosis develop icteric disease. Icteric leptospirosis is a much more severe disease that may progress very rapidly, can result in multi-systemic disease and causes death in 5-15% of cases (Levett, 2001).

6.2.3.1 Urban Leptospirosis

The informal and un-planned nature of slums, and the poor sanitation that exists in these environments often create conditions that favour the presence of large numbers of rodents and consequently the maintenance of leptospirosis within slum communities (Reis et al., 2008; Oliviera et al., 2009). In urban environments the major potential reservoir mammals are rats and dogs (Bharti et al., 2003). Studies conducted in slum communities in Brazil indicate that within the slum environment as a whole, there is heterogeneity in leptospirosis risk and that urban leptospirosis disproportionately affects poor communities that lack adequate sewage and refuse collection facilities (Reis et al., 2008). Risk of infection appears to cluster at the household level (Maciel et al., 2008) and the proximity of households to open sewers, low elevation as a proxy for seasonal flooding risk and rat sightings at the household have been identified as strong risk factors for exposure to leptospires (Sarkar et al., 2002; Reis et al., 2008). The serovar and host combination most commonly associated with urban leptospirosis is serovar Copenhageni maintained in a *Rattus norvegicus* host (Ko et al., 1999; Ooteman et al., 2006; McBride et al., 2007; de Faria et al., 2008).

6.2.3.2 Leptospirosis in Africa

The similarity of some symptoms of leptospirosis to those of many better known febrile illness syndromes of importance in Africa including malaria, Rift valley fever and brucellosis, mean that it is frequently misdiagnosed in humans and its impact on African communities is largely undocumented (Levett, 2003; Machang'u et al., 2004; Holt et al.,

2006; Mgode et al., 2006). Leptospirosis is only rarely described in Africa, but those studies that have focused on this disease have demonstrated extensive exposure to *Leptospira* in a range of human populations (Bertherat et al., 1999; Schoonman and Swai, 2009).

The diagnostic difficulties posed by leptospirosis, combined with the fundamental lack of surveillance capacity and lack of knowledge regarding the zoonotic risk posed by leptospirosis also contribute to a lack of data on the presence of leptospires in African animal populations (Machang'u et al., 2004; Mgode et al., 2005). Leptospirosis in cattle has been recorded in a number of African countries (Feresu et al., 1995, 1998; Scolamacchia et al., 2010), and in Tanzania, a collection of studies have demonstrated the presence of pathogenic leptospires in a range of species. Pathogenic leptospires have been identified in rodents (Machang'u et al., 2004; Mgode et al., 2005), cattle (Mgode et al., 2006) and pigs (Kessy et al., 2010) at different sites. In Kenya, a survey of leptospirosis in animals conducted in 1963 cultured leptospires from the kidneys of trapped gerbils and demonstrated antibodies against leptospires in a range of rodent and domestic animal species (Ball, 1966). There has though been little data published on leptospirosis in Kenya in recent years although an outbreak of this disease occurred in Bungoma district (Western Kenya) in 2004 with 151 suspected cases including 6 deaths (WHO, 2004).

6.2.4   Chapter Objectives

This chapter presents a cross-sectional survey of the Kibera rodent population conducted with the principal aim of determining if pathogenic *Leptospira* were present in this population. Further aims were: to determine the prevalence of leptospirosis in the rodent population; to characterize the rodent population itself and to the examine the interactions between the rodent and human populations that may represent rodent related risk factors for human infection with leptospirosis at this site.

6.3   Rodent Cross-Sectional Survey

A cross-sectional survey of the rodent population within the study site was conducted in collaboration with staff from the National Museums of Kenya (NMK). The Kibera study site that corresponded with the SSS study area (Soweto and Gatwikira villages) was divided into five zones (A to E) on the basis of existing administrative boundaries (Figure 6.3). Within each zone a 50m X 50m trapping area was defined using a map of all built structures (provided by the SSS), and placing each sampling grid with one side relating to an existing path or building edge.
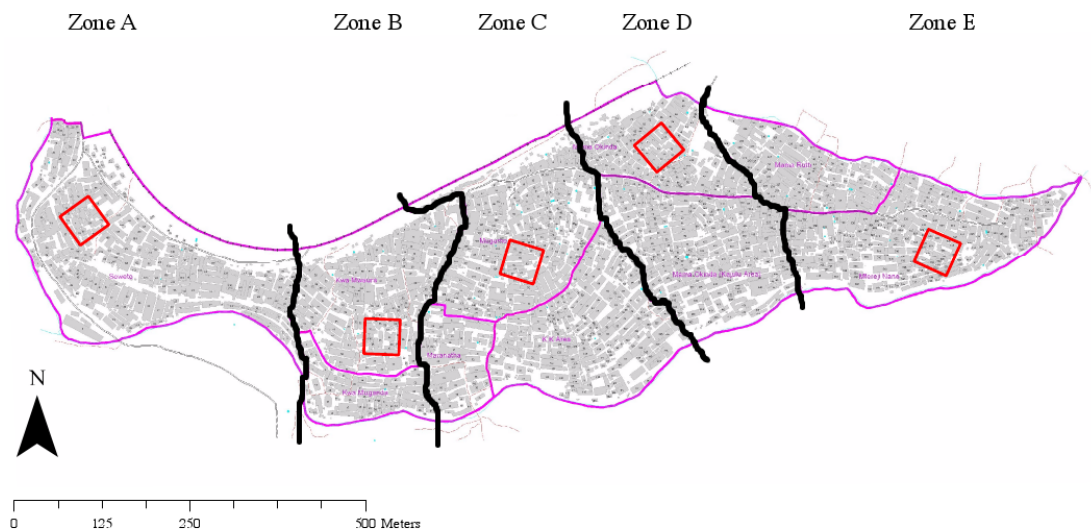
Figure 6.3: Map illustrating the location of the 5 rodent trapping zones within the Kibera SSS study area. Red squares illustrate the approximate location of the 50m X 50m trapping areas inside each zone

6.3.1   Household Identification and Consent

Approximately every other household within each trapping area was approached for participation in the study. The frequency of non-responding households was moderate as households were recruited during the daytime when many potential respondents were at work. At responding households, informed consent for participation was obtained from household representatives prior to the placement of traps. The consent process was as described previously in Section 4.3.4. Where possible, traps were placed in

the same households over all nights of trapping in each area but *ad hoc* additions of new households were made as and when traps could not be replaced into previously consented households to maintain a target trap number of 50 traps placed per night. A copy of the consent form used is provided in Appendix A.

6.3.2 Trap Placement, Checking and Collection

Within each of the five trapping areas, Sherman traps were placed for a minimum of two consecutive nights and a maximum of six nights with the aim of trapping approximately 50 rodents per zone. All traps were placed indoors and trap locations were therefore determined by the location of structures and households within the pre-defined trapping area, as well as the provision of consent at the households approached. Traps were baited with dried fish and placed on the floor against walls and under furniture. Members of study households were asked not to move or handle traps. In the majority of cases, traps were placed in the early afternoon and checked again the following morning. In two trapping areas (A and C) daytime checking of traps was conducted but this was not continued in other trapping areas for logistical reasons. The status of traps at follow-up was classified and recorded as one of: open, closed and empty, closed and full or missing. The bait and trap mechanism of open and empty traps were checked before replacing the trap. Traps that contained rodents were transported to a central processing site and a new clean trap placed in the same location within the same day. Missing traps were replaced by recruiting additional households and identifying new trap locations within the same grid for the next trap period.

6.3.3 Rodent Handling and Sample Collection

Full traps were transported to a central sample processing site. Trapped rodents were assigned a unique individual ID and euthanized by Halothane overdose. Blood was collected through cardiocentesis after swabbing the thorax and abdomen with alcohol. Immediately after collection, whole blood was transferred into a sterile 2ml cryovial and stored on ice. On the afternoon of collection, whole blood samples were centrifuged at 300rpm for ten minutes to separate serum which was removed to a sterile cryovial using

sterile pipettes in a microbiological safety cabinet. The clot and serum were transferred for storage at -80℃. Prior to necropsy, conducted using sterile blunt-end scissors and forceps, the ventral surface was cleaned again with alcohol. Both kidneys were removed from each individual, stored in separate sterile cryovials and placed immediately on ice. All tissues were then transferred to storage at -80℃ at the end of each sampling day. The genus of each individual was recorded at the time of collection as well as the sex, total length, tail length, right hind foot length and ear length.

6.3.4   Questionnaire Survey

The primary aim of the questionnaire survey was to determine the nature and frequency of contacts between the rodent and human populations. Questionnaires were administered at 20 households within each trapping area. In each area, households were selected at random from those that had already been recruited for rodent sampling. Interviews were conducted in English or DhoLuo as appropriate according to the preference of the respondent. The questionnaire focused on the frequency and location of rodent sightings, exposures to rodents and rodent faeces and rodent control practices. A copy of the questionnaire is given in Appendix B.

6.4   Laboratory Methodology

6.4.1   Kidney Sample Processing

Rodent kidney tissues remained frozen at -80℃ or -196℃ (during shipping on liquid nitrogen) until processing. All handling of untreated tissues was carried out in a biological safety cabinet at the laboratories of the University of East London. Individual kidneys were dissected using a new sterile scalpel blade and petri dish (working surface) for each kidney. For inoculation into culture media half of the central portion of each kidney was excised and transferred immediately into a sterile sample pot containing approximately 5ml culture media and transferred to a 30℃ incubator. The other half of the centre of each kidney was transferred to a sterile sample tube for DNA extraction on the same day (Section 6.4.3).

6.4.2 Culture

Kidney tissue sections were initially inoculated into commercial Ellinghausen and Mc-Cullough Medium (Johnson and Harris modification)(EMJH) - *Leptospira* Medium Base EMJH (Difco) supplemented with *Leptospira* Enrichment EMJH (Difco). Subsequent sub-cultures were maintained in EMJH prepared at the University of Edinburgh. A total of 236 kidney portions from 236 individuals were inoculated for culture at the University of East London and transferred for ongoing culture at the University of Edinburgh. The 236 initial cultures formed batch A which were first checked 5 to 11 days after inoculation. All cultures at this and subsequent checks were checked using dark-field microscopy. At every check, the status of the culture with respect to contaminants and the presence of leptospires was recorded. Contamination with non-leptospire organisms was scored on a scale of 1-4. A score of one indicates no contamination or a very small number of non-leptospire motile organisms visible and a score of four indicates very heavy visible contamination. After each culture check, individual cultures with a contamination score greater than two were filtered again through a 0.2μm filter. All batch A cultures were filtered and sub-cultured to create batch B to remove contaminating bacteria visible in a large proportion of batch A. For each culture, 1ml of the batch A culture was removed using a sterile 2ml syringe. This volume was then passed through a 0.2μm filter into the fresh media to create the batch B sub-culture for each sample. Weekly or biweekly checks of the latest sub-cultures and sub-culturing continued into early January 2009 by which time 6 parallel batches (B-G) had been created and were maintained at 30℃. In January 2009, further routine sub-culturing of all cultures was suspended, and checks of the existing batches continued at approximately weekly-biweekly intervals. The final culture checks were made in April 2009. All cultures in batches C and F were checked as well as a randomly selected sample of 50 cultures from each of batches D, E and G. At the time of creation of every batch two or more negative media controls were created and cultured alongside the samples. Positive control cultures of the 18 strains listed in Table 6.9 were provided by the LRU and maintained using the same media and culture methods as described above.

6.4.3 PCR

To evaluate the success of the sample preparation and DNA extraction, kidney extracts were tested using primers targeting the β-actin gene which encodes a cytoskeletal actin present in most eukaryotic cells. The samples were also tested with primers targeting gene sequences common to pathogenic leptospires. All PCR assays were adapted from previously published protocols and conducted at the laboratories of the University of East London.

6.4.3.1 Tissue Processing and DNA Preparation

One half of the central portion dissected from each kidney was processed to extract total DNA. The tissue was added to 180μl Qiagen buffer ATL and 20μl Qiagen Proteinase K, mixed thoroughly by vortexing and incubated overnight at 56℃ to achieve complete tissue digestion and lysis. The digested tissue was then heat treated at 80℃ for 25 minutes to inactivate any pathogens present in the tissue as well as the proteinase K. Total DNA was extracted from 100μl of this digested and heat inactivated kidney tissue. Extraction was carried out manually or using a QIAcube robot. In all cases, extractions were performed using the Qiagen DNeasy Blood and Tissue Kit, following the manufacturers protocol for Purification of Total DNA from Animal Tissues (Spin-Column Protocol) (QIAGEN, 2006). Briefly, 96-100% ethanol and the provided AL buffer were added to each sample, mixed and added to a QIAamp spin column. The columns were washed twice with the provided buffers AW1 and AW2, before the DNA bound to the spin columns was eluted in 200μl Qiagen AE buffer (using two elution steps of 100μl volume each). The eluted DNA was stored at -20℃. In two cases, the extraction process failed and there was insufficient tissue remaining to repeat the extraction. A total of 234 sample kidneys were processed to yield extracted DNA.

6.4.3.2 PCR Control Preparation

Positive control samples for PCR tests were created by extracting DNA from cultures of known leptospire serovars and through the use of spiked samples. Four pathogenic

strains (*L. interrogans* serovar Hardjo, *L. interrogans* serovar Canicola, *L. kirshneri* serovar Cynopteri, and *L. noguchi* serovar Panama) and one saprophytic strain (*L. biflexa* serovar Patoc) were used as PCR controls. All reference cultures were provided by the HPA LRU. Cultures between four and ten days of growth were used. Culture strength was checked using dark-field microscopy and assessed using a qualitative scale:

- 0 - no leptospires visible;
- 1 - very few leptospires visible (<1per 40X field);
- 2 - some leptospires visible (>1 per 40X field);
- 3 - abundant leptospires (100s per field) and
- 4 - prolific leptospires (many 100s per field).

For DNA extraction, only cultures scored 4 were used. A volume of 500μl of each culture was centrifuged at 12,000g for 10 minutes and 450μl of supernatant removed to leave the pellet in 50μl of media. This volume was vortexed and 20μl of this concentrated culture was treated with 20μl proteinase K, incubated at 56℃ for 1 hour and re-suspended in 180μl Qiagen ATL buffer, following the Qiagen protocol for Pretreatment for Gram-Negative Bacteria (QIAGEN, 2006).

The kidney tissues used for spiked controls were humanely harvested from two Sprague Dawley rats and two C57 Black mice maintained in laboratory conditions at the Roslin Institute, Edinburgh. These tissues were stored on dry ice immediately after harvest, transferred to storage at -80℃ on the day of collection and transported to the University of East London on dry ice. The control tissues were processed as described in Section 6.4.3.1 except that these tissues were not inoculated into culture media. Replicate sections from each individual were created for kidney positive/leptospire negative controls and spiked kidney positive/leptospire positive controls. Leptospire positive control tissues were spiked with a 10μl volume of the *Leptospira* culture extract. Spiked and unspiked kidney tissues were all replicated to allow examination of the effects of the 80℃ heat treatment of tissue samples upon the results of the PCR tests.

Table 6.1: β-actin PCR reagents

Reagent	Reaction Volume	Reaction Concentration	Supplier
Template	1μl	-	-
10X Buffer	2.5μl	1X	Invitrogen
dNTPs	2.5μl	200nM each	Invitrogen
$MgCl_2$	1.75μl	3.5mM	Invitrogen
Forward	2.5μl	300nM	Sigma Genosys
Reverse	2.5μl	300nM	Sigma Genosys
Taq	0.2μl	1U	Invitrogen
H_2O	12.05μl	-	Sigma
Total	25μl	-	-

6.4.3.3 β-actin PCR

The conventional β-actin PCR test protocol was adapted from a quantitative real-time protocol developed by Pahl et al. (1999). DNA extracted from kidney samples was tested using 1μl of template in a total reaction volume of 25μl. The master mix reagents and concentrations used are given in Table 6.1. All preparation of master mix reagents was conducted in an ultra-violet (UV) radiation cabinet. The primer pair used in this test target the murine β-actin gene (Table 6.2) and yield an expected 347bp product (Pahl et al., 1999). Amplification was performed in a Bio-Rad MyCycler under the following conditions: 95℃ for 10 minutes and 55 cycles of 95℃ for 15 seconds, 60℃ for 60 seconds and 72℃ for 60 seconds before a final elongation step of 72℃ for 10 minutes. Amplification products were separated by electrophoresis on a 2% agarose gel in Tris acetate EDTA (TAE) and visualized using ethidium bromide (5μg per 100ml agarose gel). To explore the presence and effect of any potential inhibitory factors in the DNA extracts from kidney tissues, three test samples (1 *Rattus* and 2 *Mus* samples) were tested in serial dilution. Following this optimization, a total of 234 sample extracts were screened in a 25μl reaction volume using 1μl of sample DNA diluted 1:10 in sterile water prior to testing.

Table 6.2: β-actin PCR oligonucleotides

Name	Oligonucleotide
Forward β-actin	5' - TCACCCACACTGTGCCCATCTACGA - 3'
Reverse β-actin	5' - GGATGCCACAGGATTCCATACCCA - 3'

6.4.3.4 Pathogenic Leptospire PCR

A conventional PCR assay was conducted to detect two gene targets present in pathogenic leptospires. The assay was adapted from Gravekamp et al. (1993) and Bal et al. (1994). The G1/G2 primer pair yields a 285bp product with all pathogenic leptospires apart from *L. kirshneri*. The B64I/B64II primer pair used in this study is one of two primer pairs published by Gravekamp et al. (1993) that identify pathogenic leptospires belonging to this species. The B64I/B64II pair used in this study yield an expected 352bp product (Cai et al., 2002). All preparation of master mix reagents was conducted in a UV radiation cabinet. The master mix reagents and concentrations (for a template volume of 8μl) are given in Table 6.3 and the primer sequences are given in Table 6.4.

Both primer pairs (G1/G2 and B64I/B64II) were used in the same reactions. Amplification was performed in a BIORAD MyCycler under the following conditions: 94℃ for 10 minutes and 40 cycles of 94℃ for 90 seconds, 55℃ for 60 seconds and 72℃ for 120 seconds before a final elongation step of 72℃ for 10 minutes. Amplification products were separated by electrophoresis on a 2% agarose gel in Tris-Borate-EDTA (TBE) using GelRed Nucleic Acid Gel Stain (Biotium, 10μl per 100ml agarose gel) and imaged using a Bio-Rad Molecular Imager FX.

The PCR was optimized using a panel of 16 controls and samples and a series of template dilutions. The published protocol uses a template volume of 40μl in the 50μl reaction volume (Gravekamp et al., 1993). Each sample in the control panel was tested four times using a decreasing volume of sample DNA extract - 40, 20, 8 and 4μl of sample. All other components of the reaction master mix were maintained at the same reaction concentration and the total volume of 50μl per reaction was maintained by adjusting the volume of sterile water added to the master mix. A total of 233 DNA extracts from kidney samples were screened using 8μl of template in a total reaction

Table 6.3: Leptospire PCR reagents for screening test

Reagent	Reaction Volume	Reaction Concentration	Supplier
Template	8μl	-	-
10X Buffer	5μl	1X	Invitrogen
dNTPs	0.5μl	0.25mM each	Invitrogen
$MgCl_2$	2μl	2mM	Invitrogen
G1	5μl	1μM	Sigma Genosys
G2	5μl	1μM	Sigma Genosys
B64 I	5μl	1μM	Sigma Genosys
B64 II	5μl	1μM	Sigma Genosys
Taq	0.1μl	0.5U	Invitrogen
H_2O	14.4μl	-	Sigma
Total	50μl	-	-

Table 6.4: Leptospire PCR oligonucleotides

Name	Oligonucleotide
G1	5' - CTGAATCGCTGTATAAAAGT - 3'
G2	5' - GGAAAACAAATGGTCGGAAG - 3'
B64 I	5' - CTGAATTCTCATCTCAACTC - 3'
B64 II	5' - GCAGAAATCAGATGGACGAT - 3'

volume of 50μl. Extracts were considered positive for pathogenic leptospires if a band of the appropriate size for one or both of the primer pairs was visible in the gel.

6.4.4 ELISA

The indirect ELISA used in this study was developed at Edinburgh University. The test was designed to allow detection of antibodies against *Leptospira* in the sera of a range of species. It utilizes protein A and protein G conjugates which in combination allow detection of most IgG classes and IgM (Inganäs, 1981; Björck and Kronvall, 1984; Akerström et al., 1985; Invitrogen, Accessed 2010) in all of the tested species. The antigen used in the ELISA was created through the extraction and purification of total protein from cultures of 15 different pathogenic leptospire cultures (Table 6.9). Proteins were extracted and purified as follows. 100ml of each culture grown up to

a strength of 4 (See Section 6.4.3.2) in EMJH media was formalinized. This volume was centrifuged for 30 minutes at 4000g and 4℃. The supernatant was discarded and the pellet washed with 1ml phosphate buffered saline (PBS) and centrifuged for 30 minutes at 15000g and 4℃. This 15000g spin step was repeated three times with the supernatant discarded and fresh PBS added in each cycle. After these purification steps the pellet was treated with 1ml 8M Urea buffer supplemented with 2% CHAPS (detergent), 0.4% DTT (reducing agent) and Roche complete mini EDTA-free protease inhibitor. The 1ml volume for each culture was then transferred to a Fast Prep bead beater and mixed for two 40 second periods, the remaining volume was centrifuged for 30 minutes at 15000g and 4℃ and the supernatant containing the extracted proteins transferred to a clean eppendorf and stored at 4℃. The proteins from each culture were purified using a Waters HPLC System. A bicinchoninic acid (BCA) protein estimation assay (Pierce, Rockford, IL) was conducted to quantify the concentration of protein in each preparation. The fifteen antigens were mixed in PBS in appropriate volume to give a concentration of $0.1\mu g/ml$ of each protein mixture in the ELISA coating antigen.

The buffers used in the ELISA test are given in Table 6.5. Serum samples were diluted to 1:50 in dilution buffer and tested in duplicate. Twelve human positive control sera (positive against 12 different serovars) provided by the HPA LRU were tested at a dilution of 1:50. Controls run on every sample test plate included four blank wells and two human positive control sera. The test conjugate consisted of horseradish peroxidase labelled purified protein A (1mg/ml, CALBIOCHEM) and purified protein G (1mg/ml, Molecular Probes) diluted at 1:10000 and 1:2000 respectively in dilution buffer. The substrate used was SureBlue TMB Microwell Peroxidase Substrate (1-Component)(KPL), and 0.18mM H_2SO_4 was used to stop substrate colour development immediately before the optical densities were read at 450nm. The ELISA test procedure is given in Table 6.6. A total of 162 rodent sera were tested using this ELISA.

6.4.5 Microscopic Agglutination Tests

The MAT test was used to characterize a sample of seventeen Kibera rodent sera selected on the basis of their scores in the ELISA test. Thirteen sera with the highest

Table 6.5: Buffers used in the *Leptospira* ELISA

Reagent	Composition
Dilution and Blocking Buffer	
	Purite with 2mM TRIS/HCl pH 7.5, 15mM NaCl, 0.05% Tween 80 and 4% BSA
Wash Buffer	
	Distilled water with 250mM NaCl and 0.05% Tween 20
Carbonate Buffer	
	0.1M NaHCO$_3$ titrated to pH 9.6 with 0.1M Na$_2$CO$_3$

BSA = Bovine Serum Albumin

Table 6.6: Summary of *Leptospira* ELISA test procedure

Process	Details	Incubation
Antigen coating		
	Add 50μl coating antigen to every well	
	Cover or wrap plate	Overnight @ 4℃
Wash		
	Shake off antigen	
	Add approximately 200μl wash buffer to every well and shake off	
	Repeat wash *6	
	Blot plate on absorbent material	
Blocking		
	Add 100μl blocking buffer to every well	
	Cover or wrap plate	30 minutes @ RT
Remove block		
	Shake off blocking buffer	
	Blot plate on absorbent material	
Sample addition		
	Add 50μl dilution buffer to each of 8 wells in the first column	
	Add 50μl of positive and negative controls to 2 adjacent wells each	
	Add 50μl diluted sample to 2 adjacent wells each	
	Cover or wrap plate	1hour @ RT
Wash		
	Shake off samples	
	Add approximately 200μl wash buffer to every well and shake off	
	Repeat wash *6	
	Blot plate on absorbent material	
Conjugate addition		
	Add 50μl diluted conjugate to every well	
	Cover or wrap plate	1hour @ RT
Wash		
	Shake off conjugate	
	Add approximately 200μl wash buffer to every well and shake off	
	Repeat wash *6	
	Blot plate on absorbent material	
Substrate addition		
	Add 50μl SureBlue substrate to every well	5 minutes @ RT
Stop addition		
	Add 50μl H$_2$SO$_4$ (0.18mM) to every well	
Plate reading		
	Read the plate at 450nm	

RT = Room temperature

scores in the ELISA and four with lower scores were tested with re-coded sample identifiers to blind the MAT testing with respect to ELISA scores. These MAT tests were run at Edinburgh using the 18 *Leptospira* cultures listed in Table 6.9. The panel includes at least one representative serovar from most of the serogroups previously documented in the area and that are most likely to have been present in this population. Individual cultures between 4 and 10 days growth were used as test antigens. Each antigen was checked prior to use to ensure growth up to strength of 4 (See Section 6.4.3.2) and filtered using a 0.8μm filter to remove clumps of antigens. Sera were heat treated at 56℃ for 30 minutes prior to testing and each sample was tested at dilutions of 1:50 to 1:800 in phosphate buffered water (PBW). A control well containing only PBW and leptospires was also set up for each serum sample and serovar. Test plates were incubated at 37℃ for 2 hours and read in the microplate under dark-field microscopy at 40X magnification. Test wells were scored for patterns of agglutination as illustrated in Cole et al. (1973) and the endpoint for each positive sample was the highest dilution in which at least 50% of the leptospires were agglutinated. Positive control sera were available for twelve of the serovars used and were run alongside test samples in the appropriate tests.

6.5   Data Analysis

6.5.1   Leptospire PCR

The variables that influenced the *Leptospira* PCR status of the rodent kidney extracts were explored using logistic regression modelling. The modelled outcome was the positive/negative status of each kidney DNA extract in the PCR for pathogenic leptospires. Covariates included in the model were trapping area, individual genus, mass, total length, and sex. Generalized linear models (GLMs) were compared using likelihood ratio tests (LRT) (Bolker, 2008). Variables were added to the intercept only model in order of decreasing significance until no further variables were significant at $p \leqslant 0.05$. At each step all variables were checked to ensure that they maintained significance at $p \leqslant 0.05$. Statistical analyses were carried out using R (R Development Core Team, 2009) using the *glm* and *anova* functions in the *stats* package (R Development Core Team,

2009). The fit of the model was assessed using diagnostic plots of fitted values and residuals (Hosmer et al., 1997, 2008).

6.5.2 ELISA Score Standardization

The repeatability of sample ELISA scores within test plates was assessed using the concordance correlation coefficient (CCC) (Lin, 1989) which evaluates the degree to which score pairs fall on the 45°line (Lin, 1989; Sanchez et al., 2002; Dohoo et al., 2003). A CCC value of 1 indicates perfect concordance. CCC values were calculated in R (R Development Core Team, 2009) using the *epi.ccc* function in the package *epiR* (Stevenson, 2009). The average OD from the duplicate readings in each test was calculated to give the reported score (ODA) for each sample and the ODA scores were normalized for comparison across plates using equation 7.1. The ODA scores for the positive human control serum sample and blank negative control wells on each plate were used as the standards on each plate.

$$Normalized\ OD\ (ODN) = \frac{sample\ ODA - blank\ ODA}{positive\ control\ ODA\ -\ blank\ ODA} \tag{6.1}$$

6.6 Results

6.6.1 Rodent Trapping

A total of 237 rodents were trapped from 948 trap placements in 269 households. A summary of the trapping in the five areas is given in Table 6.7. The overall trap success was 25% when defining full traps as a success. Trap success (full traps only) and consequently the number of trap nights required to achieve the target of roughly 50 individuals from each trapping area was highly variable across the five trapping areas (Table 6.7). The vast majority of trapped rodents (195/237 (82.4%)) were of the genus *Mus* rather than *Rattus* but this percentage also varied between areas (Table 6.7). Approximately half of the trapped population for which the sex was determined was female (108/194 (55%)).

Table 6.7: Rodent trapping summary. The trap success figures reported are based on the number of rodents caught.

Trapping Zone	Trap Nights/Days	Individuals Caught			Trap Success
		n. *Rattus* spp.	n. *Mus* spp.	Percentage *Mus* spp.	
A	152 Nights	4	72	94.7%	50%
	30 Days	0	7	100%	23.3%
B	98 Nights	6	40	87%	46.9%
C	224 Nights	17	17	50%	15.5%
	44 Days	0	2	100%	5.12%
D	149 Nights	2	37	94.9%	25.5%
E	250 Nights	13	20	60%	13.6%
Total	947	42	195	82.4%	25%

6.6.2 Kidney Culture and PCR Tests

At the end of the period of culture checks no cultures from any of the 236 starting tissues were positive for pathogenic leptospires by dark-field microscopy. All negative and control cultures were negative and positive as appropriate.

In the β-actin PCR test, the panel of control samples yielded products for all kidney preparations and there was no apparent influence of heat treatment (80℃ incubation for 25 minutes) upon the size of products observed. Samples from individuals of both *Rattus* spp. and *Mus* spp. yielded bands in this assay at the expected 349bp product size. In addition, samples from mice, but not rats, also gave a second fainter band of product of approximately 450bp. Control and test samples diluted at 1:10 prior to testing yielded clearer bands than those observed for the undiluted templates. All 234 test sample DNA extracts yielded observable products at the 1:10 dilution.

42/228 (18%, 95% CI: 14-24%) test samples yielded products in the PCR test for pathogenic leptospires. The results for 6 extracts could not be read clearly. The panel of 16 control and test samples tested using a range of template volumes revealed clear evidence of inhibition at the more concentrate template dilutions. The clearest products were observed using template volumes of 8μl and 4μl. The 8μl template volume was chosen for sample testing to minimize the loss of test sensitivity caused by this dilution of the target DNA. The positive and negative controls in each test batch performed as expected. Positive control extracts from the cultures of *L. interrogans* serovar Hardjo, *L. interrogans* serovar Canicola and *L. noguchi* serovar Panama all give clear bands

Table 6.8: Logistic regression model of rodent pathogenic leptospire PCR status

Variable	Level	Coefficient	s.e.	z	p	Odds Ratio	95% CI	n observations
Intercept		-0.66	0.24	-2.72	<0.001			
Trapping Area	A	ref						78
	B	-1.83	0.65	-2.83	<0.001	0.16	0.04 : 0.16	43
	C	0.99	0.52	1.91	<0.1	2.70	0.98 : 7.49	34
	E	-2.32	1.05	-2.20	<0.05	0.10	0.02 : 0.77	33
Genus	*Mus*	ref						154
	Rattus	-3.08	1.11		<0.001	0.05	0.005 : 0.41	34

Null deviance =199.72, df = 187. Residual deviance = 160.15, df = 183.

at approximately 290bp as expected with the G1/G2 primer pair. The extract from *L. kirshneri* serovar Cynopteri also gave a clear product but at approximately 352bp, corresponding to the expected product of the second primer pair (B64I/B64II). The spiked kidney controls also yielded products at 290bp or 352bp as expected for their spike. There was an influence of the 80℃ heating step on the strength of bands seen in this test such that those control tissues that were heated at 80℃ consistently gave stronger and clearer product bands than their non-heat treated equivalents. Many test samples yielded double bands, with some product at the band sizes corresponding to the products of both primer pairs. Purification and sequencing of the products from test samples was attempted but was unsuccessful.

Logistic regression modelling revealed that the log odds of a rodent kidney sample being positive in the pathogenic leptospire PCR were significantly associated with both the trapping zone and the genus of the individual. The fitted model and odds ratios (OR) are summarized in Table 6.8. In summary, individuals trapped in zones A and C were significantly more likely to be positive by PCR than individuals trapped in zones B and E. Zone D was not included in the model as no positive individuals were trapped in this zone and the standard error of the estimated coefficient was severely inflated. Individuals of the genus *Rattus* were less likely to give a PCR positive test result as compared to the genus *Mus* (OR=0.05, 95% CI: 0.005-0.41) The raw prevalence of kidney infections was 21% (41/191 samples, 95% CI: 16-28%) in *Mus* and 3% (1/37 samples, 95% CI: 0-14%) in *Rattus*. After controlling for genus, the sex and size (mass and length measurements) of the sampled rodents were not significantly associated with the PCR result.

6.6.3 Serological Data

The within plate repeatability for the ELISA test was very good. The calculated CCC value for the 162 rodent samples and 27 controls considered together was 0.998 (95% CI: 0.997-0.998). Figure 6.4 shows the range of standardized ELISA scores (ODN) observed from the rodent samples. The selection of samples that were MAT tested at Edinburgh and a summary result for these tests are indicated on the plot.
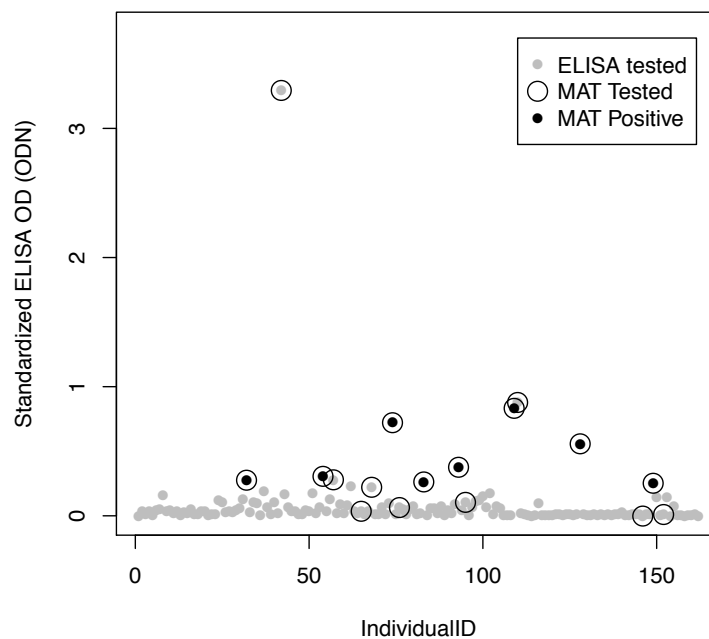
Figure 6.4: Rodent sera standardized ELISA scores. Circled points indicate samples selected for MAT testing and black points identify samples that had a MAT score of $\geqslant$1:100 against one or more serovars.

The standardized ELISA scores indicate that a low number of tested rodent sera had a detectable level of anti-leptospire antibodies. A formal quantitative cut-off was not defined for these data as the test has not previously been used to test a similar population of samples and we therefore have no prior data on which to base the decision on the position of a justifiable cut-off. The small number of samples from this population that have yielded scores that are qualitatively above background also limit the options

for the calculation of a robust cut-off using these data alone. However, there is an apparent qualitative distinction between samples above and below a threshold ODN value of approximately 0.2 (Figure 6.4). Less than 10% of samples tested in the ELISA had a standardized score exceeding 0.2 and 8/12 of these higher scorers were positive at 1:100 in at least one MAT test.

The results of the microscopic agglutination tests are given in Table 6.9, which gives a summary of the number of sera positive in each test using increasingly stringent cut-offs of 1:50, 1:100 and 1:200. In total, 8/17 MAT tested samples (8/13 ELISA high scorers and 0/4 low scorers) gave a titre of $\geqslant$1:100 in one or more MAT.

6.6.4 ELISA and PCR Correspondence

The serological data indicate a lower prevalence of infection in the rodent population than the molecular data. 155 rodents were tested using both the ELISA serology test and the kidney PCR test. Within this sample, there is no evidence of correspondence between the ELISA score and the status of the kidney PCR result (Wilcoxon rank sum test. W=1681.5, p=0.382).

6.6.5 Questionnaire Survey

One hundred questionnaires were completed. The respondent at 88% of households reported seeing rodents in their village and the majority of respondents reported daily sightings of both rodents and their excreta in and around households. The majority of respondents reported evidence of biting by rodents and rodent excreta on a range of household items including food, cooking utensils and bedding. In addition, 20% of households reported that a member of the household had been bitten by a rodent and at 8% of all households a household member had been bitten within the preceding six months. A summary of the results from the questionnaire is given in Table 6.10.

Table 6.9: MAT details and results for rodent sera

Species	Serogroup	Serovar	Strain	ELISA Antigen	MAT Results n positive at each dilution out of 17 tested		
					≥1:50	≥1:100	≥1:200
Leptospira biflexa	Semaranga	Patoc	Patoc 1	✓	2	0	0
Leptospira borgpetersenii	Ballum	Castellonis	Castellon 3	✓	8	5	0
Leptospira borgpetersenii	Javanica	Poi	Poi	✓	6	1	0
Leptospira borgpetersenii	Tarassovi	Tarassovi	Perepelicin	✓	2	0	0
Leptospira interrogans	Australis	Bratislava	Jez Bratislava	✓	5	0	0
Leptospira interrogans	Autumnalis	Autumnalis	Akiyami A	✓	2	0	0
Leptospira interrogans	Bataviae	Bataviae	van Tienen	✓	8	3	0
Leptospira interrogans	Canicola	Canicola	Hond Utrecht IV	✓	7	2	0
Leptospira interrogans	Grippotyphosa	Valbuzzi	Valbuzzi	✓	9	7	0
Leptospira interrogans	Hebdomadis	Hebdomadis	Hebdomadis	✓	3	1	0
Leptospira interrogans	Icterohaemorrhagiae	Copenhageni			9	4	0
Leptospira interrogans	Icterohaemorrhagiae	Icterohaemorrhagiae	Ictero 1	✓	9	3	0
Leptospira interrogans	Icterohaemorrhagiae	Icterohaemorrhagiae	RGA	✓	3	0	0
Leptospira interrogans	Pomona	Pomona	Pomona	✓	10	5	0
Leptospira interrogans	Pyrogenes	Pyrogenes	Salinem	✓	8	1	0
Leptospira interrogans	Sejroe	Hardjo	Hardjoprajitno	✓	7	0	0
Leptospira kirshneri	Cynopteri	Cynopteri	3522 C	✓	3	1	0
Leptospira santarosai	Mini	Georgia	Georgia	✓	10	4	1

Table 6.10: Summary of selected rodent questionnaire results

Rodents seen in the village	% households			
	Yes	No		
	88	12		
Frequency of sightings	Daily	Weekly	Monthly	Never
In the house				
< 5 rodents	69	16	2	13
⩾ 5 rodents	60	11	1	28
rodent excreta	53	13	0	34
Around the house				
< 5 rodents	70	8	5	16
⩾ 5 rodents	57	9	4	30
rodent excreta	48	11	2	39
Contact with household items	Yes			
Rodents ever reported biting				
Food	78			
Clothes	58			
Kitchen Utensils	63			
Bedding	56			
Rodent excrement ever observed on				
Food	69			
Clothes	67			
Kitchen Utensils	65			
Bedding	63			
Floor	80			
Bites of household members by rodents	Yes			
Any household member ever bitten?	20			
At this household in the past 6 months?	8			

6.7 Discussion

The results of both the molecular and serological tests indicate that pathogenic *Leptospira* are present in the Kibera rodent population. Both *Rattus* and *Mus* species are known to act as reservoirs for a variety of *Leptospira* serovars. The prevalence of kidney infection was much higher in *Mus* as compared to *Rattus*, suggesting that mice may be of greater relevance than rats in the epidemiology of leptospirosis at this site.

In the PCR test of rodent kidneys, the G1/G2 and the B64I/B64II primer pairs were included in the same reactions. The G1/G2 primer pair should generate products with all pathogenic leptospires other than *L. kirshneri*, whilst the B64I/B64II pair should only generate products with *L. kirshneri* (Gravekamp et al., 1993). However, bands of the sizes expected with both primer pairs were observed for a number of samples. This pattern of imperfect discrimination by the two primer sets has been reported previously. Ooteman et al. (2006) used the G1/G2 primer pair to test a range of reference strains and serum from Brazilian patients with suspect leptospirosis. They observed different product sizes with different reference serovars, reported some products with *L. kirshneri* strains and also observed two fragments in the gel for some test samples (Ooteman et al., 2006). The B64I/B64II primer pair used here has previously been observed to yield a 352bp product with both *L. kirshneri* serovar Grippotyphosa and *L. borgpetersenii* serovar Sejroe (Cai et al., 2002) and other studies using these primers have shown that the distinction between different pathogenic species (*L. kirshneri* or not) with these two sets of primers is not perfect (Parma et al., 1997; Ooteman et al., 2006). The PCR results from the Kibera rodent kidneys reveal a high prevalence (18%) of renal carriage of pathogenic leptospires in this population. The identification of the leptospires present in this population as *L. kirshneri*, non-*L. kirshneri* or potentially a mixture of different pathogenic *Leptospira* strains is not however possible from these data. Further molecular speciation tests and sequencing would be required to conclusively identify the serovar or serovars present in this population. Re-amplification of PCR products and sequencing was attempted but as in previous studies (Hartskeerl, *pers.comm.*) this approach was unsuccessful.

The overall rodent kidney colonization prevalence of 18% detected in this study is in the range observed in a number of similar studies (Bunnell et al., 2000; Johnson et al., 2004; Mgode et al., 2005; Agudelo-Flórez et al., 2009) but kidney carriage rates as high as 83.9% have been detected amongst rats sampled in an urban setting (de Faria et al., 2008). The pathogenic leptospire PCR method adapted for use in this study was originally published with an additional southern blotting step which increases sensitivity (Gravekamp et al., 1993; de Abreu Fonseca et al., 2006). In addition, the kidney extracts were tested at a 1:5 dilution as compared to the published protocol to minimize the influence of inhibitors that affected this assay. It is therefore probable that the positive

samples detected with the PCR in this study represent an under-estimation of the true number of positive rodent kidneys. The likely loss of sensitivity and the apparent presence of inhibitors in the kidney extracts may also have contributed to the failure of attempts to sequence the products obtained in the test.

18% of the rodent kidneys tested were positive for pathogenic leptospires in the PCR assay, yet none yielded a positive culture. Leptospires are particularly fastidious and different leptospires have variable growth rates and different optimal culture conditions (Levett, 2001). The culture protocol used in this study, in which parallel batches were maintained, was designed to accommodate the varied requirements of different leptospires that can be encouraged or inhibited by sub-culturing. However, it is probable that the initial transfer from commercial to 'home-made' media and the large proportion of batch A cultures that were contaminated and required filtering may have contributed to the failure of culture at this early stage. Culture of leptospires is a relatively insensitive technique (Gravekamp et al., 1993; Levett, 2001; de Faria et al., 2008) and successful culture from these tissues would possibly have required the use of a variety of different media and methodological experience available only at a specialist reference laboratory.

In comparison to ELISA tests similar to that used in this study, the MAT has been shown to have relatively low sensitivity, particularly for acute phase antibodies (predominantly IgM) (Brandão et al., 1998; Ribotta et al., 2000; McBride et al., 2007). This reduced sensitivity in the acute phase may explain the negative MAT results observed for some of the samples with the highest standardized ELISA scores (Figure 6.4). The use of a longer substrate incubation step in the ELISA (as used in the following chapter) may have increased the sensitivity of the ELISA and allowed better discrimination between week responding and background scores. Repeat testing of these samples was not possible due to the limited sera volume available.

The MAT data given in Table 6.9 indicate that the majority of the rodent sera with higher scores in the ELISA do have agglutinating antibodies against leptospires. However, these data do not provide a clear indication of the infecting serovar or even serogroup. In previous studies conducted in urban settings, human and rodent sero-surveys have revealed a clear predominance of single serovars, such as *L. interrogans*

serovar Copenhageni amongst people (Reis et al., 2008) and rats (de Faria et al., 2008) in Salvador, Brazil. In these data, no similar pattern is observed. The highest single MAT titre was observed against *L.santarosai* serovar Georgia but similar proportions of positive samples at the 1:100 cut-off were seen against the serogroups Ballum, Grippotyphosa, Pomona and Copenhageni. 7/8 of the samples positive at a titre of $\geqslant$1:100 had this titre against >1 serovar. Of these observed serogroups, Ballum, Grippotyphosa and Copenhageni are all frequently associated with rodent reservoirs.

The interpretation of MAT data is complicated by a number of factors (Levett, 2003). Cross-reacting antibodies are often the first to appear, whereas homologous antibodies appear later in the course of infection but persist much longer (WHO, 2003). A survey of *R. norvegicus* carried out at an urban market in Colombia identified a similar pattern of broadly cross-reactive MAT results in that rodent population (Agudelo-Flórez et al., 2009). The authors of that study suggested that the cross-reactivity observed may be attributable to exposure of the rodent population to a variety of leptospires carried by different animal species and transmitted via sewer systems and floodwater (Agudelo-Flórez et al., 2009). The pattern of low-level broadly reactive antibodies would also be consistent with the presence in these samples of antibodies that are specific to a leptospire that is not well represented in this panel of test antigens and there may also be more than one serovar circulating in this population. The over-interpretation of MAT data in the absence of knowledge of locally prevalent serovars can limit its utility (Levett, 2003) and it has been demonstrated that the utility of the MAT test for the identification of the infecting serovar is highly variable with a specificity as low as 0% for some serovars (Katz et al., 2003; Levett, 2003). In this case we have MAT data from a small number of samples and we have little or no data from other sources on which serovar or serovars may be circulating in this area. Using these data, we can say that there is serological evidence of the circulation of one or more leptospires in the Kibera rodent population but the successful isolation of leptospires from this site will be required to determine the identity of the leptospire or leptospires present in this population. The interpretation of the overall epidemiological picture revealed by these data and methods for their analysis are discussed further in Chapter 8.

There was no evidence of correspondence at the individual level between the ELISA

data and the kidney PCR results. Numerous previous studies have reported a similar lack of correspondence between data generated using these two laboratory approaches (Agudelo-Flórez et al., 2009). This is largely due to the fact that these techniques are really being used to address different questions. The PCR detects the DNA of the pathogen and when used to test kidney tissues is most useful for assessing the proportion of a population that is currently carrying the pathogen and that may be chronically infected and therefore potentially infectious. Serological tests provide evidence of recent exposure to leptospires but the data from these tests give a poor indication of carrier status (Priya et al., 2007). Many studies report higher rates of seropositivity in incidental hosts than in carrier species in which chronic shedding of leptospires can occur in the absence of an ongoing antibody response (Levett, 2001; Priya et al., 2007; de Faria et al., 2008).

Interestingly, both the serological and molecular data indicate that individuals of the genus *Mus* rather than *Rattus* may be more important in the epidemiology of leptospirosis at the Kibera site. The logistic regression analysis of the PCR data revealed that PCR positives were more likely in the *Mus* samples as compared to *Rattus* and in fact only 1 of 37 PCR tested kidneys from *Rattus* were PCR positive. It was also the case that all of the higher scoring sera in the ELISA test (standardized ELISA scores above 0.2) were from *Mus* rather than *Rattus*. It is possible in the case of the ELISA data that there was a difference in the binding properties of the secondary antibodies for sera from each genus. However, all of the *Rattus* sera were also tested using an anti-*Rattus* specific conjugate antibody (data not shown) and no reactions were observed in this test.

The source of human leptospirosis infection is usually either direct or indirect contact with the urine of an infected animal (Levett, 2001) and the questionnaire data gathered in this study reveal evidence of multiple frequent interactions between the human and rodent populations. In more than half of the surveyed households (53%), rodent excreta were encountered on a daily basis within the household. A previous study conducted in a Brazilian slum environment identified a positive correlation between the number of rats sighted by residents and their risk of acquiring *Leptospira* (Reis et al., 2008). This

questionnaire study included questions about rodent sightings overall rather than specific rat and mouse sightings and 60% of respondents reported seeing 5 or more rodents in their household on a daily basis. In combination with the PCR and serological data which indicate that the Kibera rodent population does carry pathogenic *Leptospira*, these data suggest that the residents of Kibera may face considerable risk of exposure to *Leptospira* in the household environment.

6.7.1 Conclusion

The data presented in this chapter provide molecular and serological evidence of the circulation of pathogenic *Leptospira* within the Kibera rodent population, and frequent potentially risky interactions between the human and rodent populations. It is quite possible that human cases are of leptospirosis are under-reported at this site. The data presented here from the rodent population indicate that studies of the human population in Kibera are warranted, to both raise awareness of leptospirosis as a potential cause of illness and to determine if leptospirosis is in fact an important cause of morbidity at this site.

Chapter 7

Surveillance of Leptospirosis in Animal Populations: Infection Patterns in Kibera Dogs

7.1 Abstract

An indirect ELISA test utilizing mixed antigens from 15 pathogenic *Leptospira* serovars was used to examine the exposure of the Kibera dog population to *Leptospira*. Over 1400 sera collected from the Kibera cohort during 8 sampling visits in 2007 and 2008 were tested. The within and between plate repeatability of the ELISA was assessed and standardized scores were generated to allow comparison of scores from the whole population. The distribution of ELISA values within the population was examined using multilevel models to assess the impact of key demographic and study design variables upon ELISA results. Samples of 141 and 52 dog sera were also tested using the MAT at the VLA and at Edinburgh respectively to try to validate the ELISA test and characterize the *Leptospira* serogroup(s) against which antibodies were observed. The ELISA test showed good within (CCC=0.9936) and between plate (CCC=0.8792) repeatability of standardized OD values. Multilevel modelling identified temporal fluctuations in the distribution of ELISA scores and influences of dog age, the presence of puppies at the

household and prior treatment with ivermectin upon the magnitude of ELISA scores. The results of the MAT tests conducted at the VLA provided little evidence of exposure to leptospirosis in this population. In contrast, the MAT tests conducted at Edinburgh suggested that just under 50% of the dog samples with the highest ELISA scores were MAT positive at a titre of 1:100 or greater against one or more pathogenic serovars. In most MAT positive samples, positive titres against multiple serovars were observed and there was no clear predominance of a likely infecting serovar(s). The disparity between the results of different diagnostic tests complicates assessment of leptospirosis in this population. On balance though these data indicate a pattern of extensive exposure of the Kibera dog population to pathogenic *Leptospira* consistent with endemic pathogen presence.

7.2 Introduction

Domestic dogs can become incidentally infected with *Leptospira* serovars maintained in other animals including rodents (Adesiyun et al., 2006) but there are also pathogenic serovars that are relatively commonly maintained through the renal colonization of dogs. In tropical countries particularly, dogs are considered an important reservoir for human infections and potential source of infection for human outbreaks (Levett, 2001). *Leptospira interrogans* serovars Canicola and Icterohaemhorrhagiae are classically associated with dogs (Adin and Cowgill, 2000; Aslantaş et al., 2005; Adesiyun et al., 2006) but the importance of different serovars in canine infections is variable across countries and apparently also through time as the predominant serovars in a given area can change (Cai et al., 2002; Ward, 2002; Aslantaş et al., 2005; Adesiyun et al., 2006; Jimenez-Coello et al., 2008).

In Figure 6.2, environmental exposure and the consumption of infected rodents are proposed as the key potential transmission routes through which dogs may become exposed to pathogenic leptospires in the Kibera context. Previous studies have identified significant influences of factors such as dog age (Aslantaş et al., 2005; Adesiyun et al., 2006) and type (stray/hunting/farm dogs) (Adesiyun et al., 2006) upon dog exposure to *Leptospira*. The likelihood that the Kibera dogs were exposed to leptospires through

these potential routes may be influenced by factors such age, sex and other demographic variables such as the number of other dogs present at the household. Equally, these and other factors could impact upon the way in which a dog responds to pathogen exposure. The identification and description of any effects of such variables upon ELISA test results may inform our understanding of the routes of dog exposure and would also be important in the context of sentinel evaluation as it might well be desirable to select dogs of a particular class (e.g. young male dogs) that are most likely to be exposed to a pathogen as the sentinel population. The influence of a range of household, dog and visit level variables upon dog ELISA result are examined in this chapter and the evaluation of dogs as sentinels for leptospirosis is also discussed further in the next chapter.

The interpretation of serological data on *Leptospira* exposure in dogs can be complicated by the presence of antibodies due to previous vaccination (Harkin et al., 2003; Adesiyun et al., 2006; Iwamoto et al., 2009). At the Kibera study site, however, the rates of vaccination against leptospirosis are extremely low. Only one of 345 dogs included in the household questionnaire survey was reported to have received any previous vaccination and this was against rabies. Throughout the course of the study there were no reports of previous leptospirosis vaccination for any of the dogs at this site and it is therefore very unlikely that any of the apparently positive results obtained are attributable to vaccine responses rather than natural exposures.

In this chapter, the infection patterns of leptospirosis in the Kibera dog population are examined using the serosurveillance laboratory techniques described in the previous chapter. Multilevel models that accommodate the repeat measures and hierarchical structure in these data are used to examine the distribution of ELISA values on the continuous scale. In the following chapter, the dog population data presented here and data from the previous chapter on rodents are compared and considered together to address the assessment of animal sentinels for leptospirosis at this site.

7.2.1 Chapter Objectives

This chapter presents a serological survey of leptospirosis in the Kibera dog population conducted with three key aims. First, to determine if there was evidence of exposure to *Leptospira* in the dog population and second, to characterize the serovar or serovars responsible for infection in the dog population. Finally, the second part of the chapter examines the distribution of ELISA scores within the dog population.

7.3 Laboratory Methodology

7.3.1 ELISA

A total of 1456 tests of dog sera samples collected during repeat visits to dogs enrolled in the cohort across all of the sampling visits A to H were conducted using the leptospirosis ELISA. The protocol for the ELISA testing of dog sera was essentially as described for the rodent samples in the previous chapter (Section 6.4.4) with the following modifications. The substrate was incubated for 15 minutes prior to the addition of the stop solution to allow better discrimination between scores. Additional controls included on test plates for dog samples were a positive control dog sera tested in four wells and two negative control dog sera. The dog serum sample used as a dog positive control was originally obtained from Autogen Bioclear (UK). The positive status of the sample was determined through repeat MAT testing as described below. Two consistently low scoring Kibera samples were used as representative negative controls on all dog sample test plates.

ELISA plates made up using two antigen batches were used. The samples collected in visits A to F in 2007 were tested using one batch of antigen whilst the samples collected at visits G and H in 2008 were tested using a second batch. The protocol for the preparation of these two batches was exactly the same as described in the previous chapter (Section 6.4.4). The correspondence of scores from the two antigen batches was checked using the full panel of twelve positive control sera. Repeat samples from individual dogs taken during visits A-F were tested together on the same ELISA plate to

minimize the influence of inter-plate variation on the assessment of within-dog variance in ELISA scores for the repeat samples from 2007.

7.3.2 MAT

Sera were MAT tested at two labs (the VLA and Edinburgh) and the tested sera were selected for one of two reasons (Figure 7.1 gives an overview of sample selection and testing). First, 100 sera collected in Kibera in November 2007 (Visit E) were tested at the VLA. These sera constitute a representative sample of approximately 45% of the sera collected during a single cross-sectional visit and were tested at the VLA to look at the correspondence between the ELISA and the VLA MAT across the range of ELISA scores. These sera were not MAT tested at Edinburgh. Secondly, a smaller sample of Kibera dog sera from all visits were selected on the basis of their ELISA scores. 46 'high scoring' dog sera were selected from those with the highest standardized ELISA scores (ODN$\geqslant$1.5) and six samples with intermediate or low ELISA scores were also included in this panel. These high scoring sera were tested in order to characterize the apparently ELISA positive sera. All 52 of these samples were MAT tested at Edinburgh but there was only sufficient volume to test 43 of these (40 high and 3 intermediate/low) at the VLA as well. Four blinded replicates of the dog control sera used as the ELISA dog positive standard were also tested in the VLA pools test and this sample was included on every plate in the Edinburgh protocol.

The sera sent to the VLA were tested using their standard *Leptospira* Pool Test in which a panel of 19 *Leptospira* strains are grouped into six antigen pools. The antigen pools consist of mixtures of between two and four different serovars and are created by mixing equal volumes of the composite serovars together (See Table 7.2 for details of the antigen pools). Each sample was tested at a starting dilution of 1:50 (effective test dilution of 1:100) against all six antigen pools. Any sample for which any degree of agglutination was observed against any of the pooled antigens was later tested against the individual constituent serovars in that pool with a starting sera dilution of 1:25 or 1:100 depending on the date of testing. The MAT tests at Edinburgh were run using the 18 *Leptospira* cultures listed in Table 7.3 with the same test procedure as described in the previous chapter (Section 6.4.5).
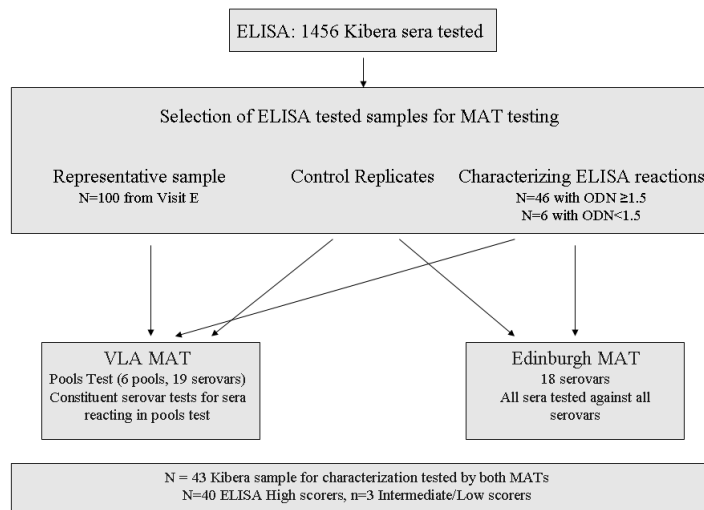
Figure 7.1: Overview of leptospirosis serology indicating the testing location and rationale for sample selection

7.4 Data Analysis

7.4.1 ELISA Score Standardization

The repeatability of dog sample ELISA scores within and between plates were assessed using the concordance correlation coefficient (CCC) (Lin, 1989; Sanchez et al., 2002; Dohoo et al., 2003). Within plate repeatability for all plates was assessed using the raw duplicate OD readings for every run of the test and control samples. The average OD (ODA) from the duplicate readings in each test was calculated for each sample. For assessment of between plate repeatability, standardized scores (ODN) were calculated using equation 7.1. The ODA scores for the positive dog serum sample and blank negative control wells on each plate were used as the positive control and blank standards. The other control samples (two human positive controls and two dog negative control samples) that were run on every plate were used in the assessment of between plate repeatability. CCC values were calculated for each of the 37 test plates to assess the ODA and ODN score repeatability. The ODA and ODN scores for each plate were compared to the set of ODA and ODN average scores derived from all plates (Sanchez et al., 2002). Finally, the correspondence of ELISA scores generated using the two

antigen batches was also assessed using the ODA scores from 47 samples tested using both batches. All CCC values were calculated in R using the *epi.ccc* function in the package *epiR* (Stevenson, 2009).

$$Normalised\ OD\ (ODN) = \frac{sample\ ODA - blank\ ODA}{positive\ control\ ODA\ -\ blank\ ODA} \qquad (7.1)$$

7.4.2 Multilevel Modelling

The associations between ELISA scores and a range of potential covariates were examined using regression models. In previous studies that have looked at serological responses to leptospirosis in dog populations, the factors associated with dog ELISA score and the test performance are assessed by first defining a cut-off for the ELISA test and then evaluating the association of this binary outcome with other variables and with MAT results from the same samples (Ribotta et al., 2000; Jimenez-Coello et al., 2008). The way in which a cut-off is defined can have considerable influence over the findings generated using a diagnostic test and the definition of a cut-off for these dog ELISA data is discussed further in Chapter 8. In this analysis, the ELISA scores are considered on the numeric scale to avoid the loss of data associated with the imposition of a cut-off and to consider the population of scores as a whole. The ELISA scores represent repeat measures from individual dogs that are themselves grouped within households. This hierarchical structure and pseudoreplication of these data was accommodated by using mixed models in which the household ID and dog ID were included as random effects (Paterson and Lello, 2003). A total of 1410 sample records were considered in the multilevel modelling. The distribution of these samples across the different visits of the study is given in Table 7.1.

The ELISA ODN scores were log transformed to linearize the data and improve the variance and residuals observed during modelling (Dohoo et al., 2003). Initially, an intercept only model was fitted to the log transformed standardized ELISA scores (lODN) with the dog ID and household ID fitted as nested random effects. Variance components analysis was used to describe the proportion of the overall variance in the ELISA data

Table 7.1: Summary of dog sera samples tested by ELISA and considered in the multilevel modelling analysis

	A	B	C	D	E	F	G	H	Total
Kisumundogo	61	66	56	57	74	29	56	53	452
Gatwikira & Soweto	–	98	144	153	146	146	133	138	958
Total	61	164	200	210	220	175	189	191	1410

that is attributable at each level of the hierarchy i.e. the household level, the individual dog level or at the within dog/residual level (Rasbash et al., 2009).

Potential covariates explored in this analysis included household level factors - the method of household identification (CDC survey, house-to-house or transect) and dog ownership type - dog level factors - dog sex, origin and condition - and visit level factors, including the visit identifier, categorized dog age at the time of sampling, number of dogs present at the household, presence or absence of puppies (<3mo) at the household and daytime and night-time confinement. The 1410 samples considered in the analysis included 577 samples collected from dogs that had been treated with ivermectin at the visit immediately prior to sample collection and 37 samples from dogs previously treated with Canex (not including dogs sampled at visit G that had been treated at visit F - in the previous year). Both ivermectin and Canex treatment at the previous visit were also included as potential covariates in the regression models. Models were fitted with maximum likelihood methods using the *lme* function of the *nlme* package (Pinheiro et al., 2009) in R (R Development Core Team, 2009). Models were compared using likelihood ratio tests and AIC values (Pinheiro and Bates, 2000; Bolker, 2008). For the multivariate analysis, all variables with a p value below 0.4 in the univariate analysis likelihood ratio test were included in model building. The provision of ivermectin at the previous visit was a significant predictor of log-transformed ODN in the univariate analysis, such that the provision of ivermectin was associated with larger log-transformed ODN scores. As a consequence this variable was forced into the multivariate model to accommodate this apparent treatment effect. For all other variables, forward and backwards variable selection approaches were used with an inclusion rule of $p \leqslant 0.05$.

Finally, the autocorrelation structure of the within group residuals was checked. Multilevel models assume that the within group residuals are independent. In longitudinal studies this assumption is often violated because it is likely that observations temporally close to each other (e.g. ELISA scores from the same dog at consecutive visits) will be more strongly related than observations that are temporally far apart (Bliese, 2009). Model criticism was conducted using comparative plots of predicted values and residuals (Bolker, 2008).

7.5 Results

7.5.1 ELISA and MAT Scores

The overall CCC value for within plate repeatability of the leptospirosis ELISA (1456 test and 301 control results) had a value of 0.9936 (95% CI: 0.9939-0.9942) indicating extremely good reproducibility of scores on the same plate. For between plate repeatability, the mean CCC values using scores from all 37 plates were 0.8374 with quartile range:0.7884-0.9051 using the ODA scores and 0.8792, quartile range: 0.8334-0.9320 using the ODN standardized scores. The CCC value generated from samples tested using both antigen batches was 0.9780 (95% CI: 0.96109-0.9876) indicating little influence of antigen batch upon ELISA scores.

The ODN standardization of scores was used for all subsequent analyses in preference to the ODA scores on the basis of the improved between plate repeatability seen using this standardization. The correspondence between the replicate standardized ELISA scores (ODN) for all samples and controls is shown in Figure 7.2. The figure also illustrates the distribution of test sample scores relative to those of the dog sera controls run on all test plates and the twelve different human positive control sera.

There was some variation in ELISA scores for the human reference positive controls. 10 of the 12 human positive control sera gave ELISA ODN scores at the very top end of the distribution of all scores, but two sera which were the positive controls for serovars Pyrogenes and Hebdomadis gave lower scores at ODN=0.46 and ODN=0.75 respectively (Figure 7.2). In the MAT tests against their homologous serovars these
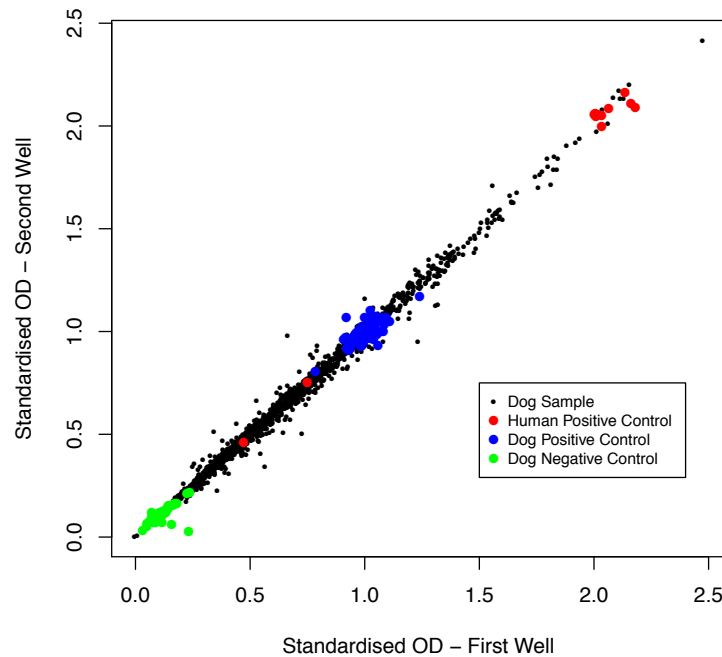
Figure 7.2: ELISA repeatability and controls. The figure shows the replicate scores
for each sample tested in the leptospirosis ELISA. Black points represent
Kibera test sera. The repeat scores of the dog positive control serum and
dog negative control serum tested on every plate are shown in blue and
green respectively. Red points indicate the scores from individual tests of
each of the twelve human positive control serum.

sera both gave titres of $\geqslant$1:800 and the relatively low ELISA scores could indicate

that the ELISA has relatively reduced sensitivity for antibodies against these serovars

particularly.

Fourteen of the 141 Kibera dog sera screened in the VLA pools MAT showed a degree of

agglutination against one or more of the six antigen pools, but in the subsequent single

serovar tests only four Kibera sera showed any agglutination of the leptospires (See

Figure 7.3(a)). The sera varied in the pools against which agglutination was observed

and only low-titre reactions were seen (Table 7.2). Four replicate samples of the dog

sera used as the positive standard in the ELISA test were independently tested at the

VLA. In the four tests of this serum, the sample was classified as positive at a dilution

of 1:100 or 1:200 against the serogroups Grippotyphosa and in two tests it was also

classified as positive at 1:100 against Copenhageni and Autumnalis (Table 7.2).

In contrast to the tests conducted at the VLA, the MAT tests conducted at Edinburgh revealed that 24/52 of the high scoring dog sera tested (ELISA ODN⩾1.5, See Figure 7.1) had a MAT titre of 1:100 or greater in at least one serogroup specific MAT test (Table 7.2). The positive standard dog serum was tested in duplicate against all sixteen leptospire serovars used at Edinburgh and was positive at 1:100 against the strains of serogroups Javanica, Autumnalis and Pomona as well as titres at 1:50 against 5 additional serogroups including Grippotyphosa and Copenhageni.

43 of the sera tested at Edinburgh were also tested at the VLA. 19 of these 43 samples gave a maximum MAT titre of 1:100 or greater in at least one MAT test conducted at Edinburgh but only 5 of these samples was positive at 1:100 against any pool at the VLA and only one of these yielded any positive reaction in the serovar specific tests.

The number of positive samples seen in the MAT tests conducted at Edinburgh ranged from 0/51 to 14/52 with the different serovars tested (Table 7.3) and as for the data from the rodent samples (See Chapter 6), a high degree of cross-reactivity was observed. Ten or more samples were positive at a titre of 1:100 or greater against serovars Tarassovi, Bratislava, Autumnalis, Bataviae and Icterohaemorrhagiae RGA. The cross-reactivity in these results and the interpretation of the MAT data are discussed further in the following chapter. The correspondence of the two sets of microscopic agglutination test data (conducted at the VLA and at Edinburgh) with the ELISA data are shown in Figure 7.3. The positive dog sera used as the standard in the ELISA tests is indicated in both figures.

The results from the MAT tests conducted in the VLA indicate little evidence of exposure to leptospires in the Kibera dog population. A small number of samples (n=14) were positive in one or more MAT using the pooled antigens and n=4 of these subsequently also gave a non-zero titre in the serovar specific tests. The comparison with the ELISA data (Figure 7.3(a)) shows that the samples with both pool and serovar specific reactions had a wide range of ELISA scores. In contrast, the MAT tests conducted at Edinburgh indicate that a relatively large proportion of the tested samples with high ELISA scores (ODN⩾1.5) were also positive in the MAT test (24/52 overall).

Table 7.2: VLA MAT screen results. - indicates that the serovar test was not run as the relevant pool test was negative. ND indicates that the serovar test was not run due to insufficient sera volume

Pool	Serovar	ELISA Dog Standard				SampleID													
		1	2	3	4	G69-2	G225-3	G267-4	G218-5	G275-5	K132-5	K70-5	K331-5	G502-5	K07-5	G266-9	K132-8	G52-8	G404-9
1	Canicola	<1:100	<1:100	<1:100	-	-	-	-	-	-	-	-	<1:25	-	<1:25	-	-	-	-
1	Icterohaemorrhagiae	<1:100	<1:100	<1:100	-	-	-	-	-	-	-	-	<1:25	-	<1:25	-	-	-	-
1	Ballum	<1:100	<1:100	<1:100	-	-	-	-	-	-	-	-	ND	-	<1:25	-	-	-	-
1	Copenhageni	1:100	1:100	1:100	-	-	-	-	-	-	-	-	ND	-	<1:25	-	-	-	-
2	Pomona	<1:100	<1:100	<1:100	<1:100	<1:100	-	-	<1:25	-	<1:25	-	<1:25	-	-	<1:100	-	-	-
2	Grippotyphosa	1:200	1:100	1:100	1:100	1:100	-	-	<1:25	-	1:25	-	<1:25	-	-	<1:100	-	-	-
2	Tarrassovi	<1:100	<1:100	<1:100	<1:100	<1:100	-	-	<1:25	-	<1:25	-	<1:25	-	-	<1:100	-	-	-
2	Mozdok	<1:100	<1:100	<1:100	<1:100	<1:100	-	-	<1:25	-	<1:25	-	<1:25	-	-	<1:100	-	-	-
3	Australis	<1:100	<1:100	<1:100	<1:100	-	<1:100	<1:100	-	<1:25	-	-	-	<1:25	-	<1:100	<1:100	<1:100	<1:100
3	Bratislava	<1:100	<1:100	<1:100	<1:100	-	<1:100	<1:100	-	1:25	-	-	-	<1:25	-	<1:100	<1:100	<1:100	<1:100
3	Autumnalis	1:200	1:100	<1:100	<1:100	-	<1:100	<1:100	-	1:25	-	-	-	<1:25	-	<1:100	<1:100	1:100	<1:100
4	Sejroe	-	-	-	-	-	-	-	<1:25	-	-	<1:25	-	-	-	-	-	-	-
4	Mini	-	-	-	-	-	-	-	<1:25	-	-	<1:25	-	-	-	-	-	-	-
4	Hebdomadis	-	-	-	-	-	-	-	<1:25	-	-	1:25	-	-	-	-	-	-	-
5	Bataviae	-	-	-	-	-	-	-	<1:25	-	-	-	-	-	-	-	-	-	-
5	Zanoni	-	-	-	-	-	-	-	<1:25	-	-	-	-	-	-	-	-	-	-
5	Javanica	-	-	-	-	-	-	-	<1:25	-	-	-	-	-	-	-	-	-	-
6	Hardjo Prajitno	-	-	-	-	-	-	-	<1:25	-	-	<1:25	-	-	-	-	-	-	-
6	Hardjo Bovis	-	-	-	-	-	-	-	<1:25	-	-	1:25	-	-	-	-	-	-	-

Table 7.3: Edinburgh MAT details and results. Details of strains used for the MAT and ELISA tests are given and the MAT results for the Kibera dog samples are summarized

Species	Serogroup	Serovar	Strain	ELISA Antigen	MAT Results n positive at ≥1:100/ n tested
Leptospira biflexa	Semaranga	Patoc	Patoc 1		5/40
Leptospira borgpetersenii	Ballum	Castellonis	Castellon 3	✓	7/51
Leptospira borgpetersenii	Javanica	Poi	Poi	✓	6/52
Leptospira borgpetersenii	Tarassovi	Tarassovi	Perepelicin	✓	10/52
Leptospira interrogans	Australis	Bratislava	Jez Bratislava	✓	13/52
Leptospira interrogans	Autumnalis	Autumnalis	Akiyami A	✓	12/52
Leptospira interrogans	Bataviae	Bataviae	van Tienen	✓	10/52
Leptospira interrogans	Canicola	Canicola	Hond Utrecht IV	✓	9/51
Leptospira interrogans	Grippotyphosa	Valbuzzi	Valbuzzi	✓	9/51
Leptospira interrogans	Hebdomadis	Hebdomadis	Hebdomadis	✓	0/51
Leptospira interrogans	Icterohaemorrhagiae	Copenhageni			7/50
Leptospira interrogans	Icterohaemorrhagiae	Icterohaemorrhagiae	Ictero 1	✓	5/51
Leptospira interrogans	Icterohaemorrhagiae	Icterohaemorrhagiae	RGA	✓	14/52
Leptospira interrogans	Pomona	Pomona	Pomona	✓	4/51
Leptospira interrogans	Pyrogenes	Pyrogenes	Salinem	✓	2/51
Leptospira interrogans	Sejroe	Hardjo	Hardjoprajitno	✓	2/51
Leptospira kirshneri	Cynopteri	Cynopteri		✓	0/51
Leptospira santarosai	Mini	Georgia	3522 C		4/52

7.5.2 Multilevel Modelling

The variance component analysis of the intercept only random effects model reveals that very little (2%) of the variance in ELISA scores was attributable to differences between households. Most of the variance in the ELISA scores is attributable to the differences between dogs (53%), with almost as much variance in scores (45%) attributable to residual/within dog differences.

The final multivariate model of log-transformed dog ELISA scores is summarized in Table 7.4. The model reveals influences of the sampling visit and the age of the dog at the time of sampling and the presence of one or more puppies at the household at the time of sampling. The administration of ivermectin at the previous visit has a positive coefficient in the model indicating that the provision of ivermectin did enhance

(a) ELISA and VLA MAT Comparison

(b) ELISA and Edinburgh MAT Comparison
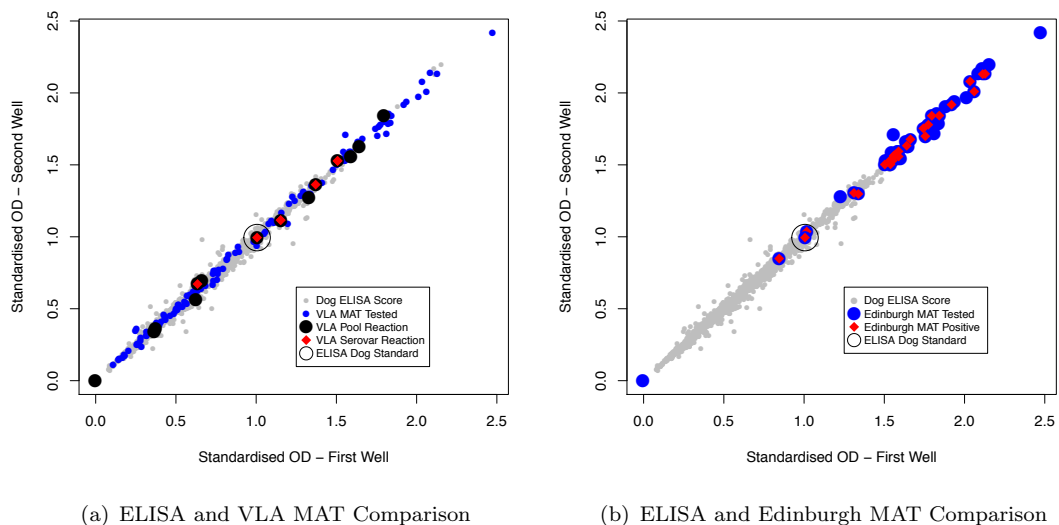
Figure 7.3: ELISA repeatability and correspondence with MAT results. Grey points indicate the ELISA scores for all dog samples in both figures and a single point is plotted for the positive dog standard sample at the mean standardized ODN values from all ELISA tests. a) Larger blue points indicate those samples that were MAT tested at the VLA. Black circles show the VLA tested samples that had any non-zero reaction in a pools test and red diamonds show those samples that had a non-zero titre in a subsequent serovar specific test. b) Larger blue points indicate those samples that were MAT tested at Edinburgh. Red diamonds show those samples that had a titre of 1:100 or greater in one or more serovar specific tests.

Table 7.4: Multilevel model of log dog ELISA scores

Variable	Level	Coefficient	s.e.	95% CI		n observations
				Low	High	
Intercept		-0.614	0.062	-0.736	: -0.493	1316
Ivermectin	Yes	0.131	0.032	0.067	: 0.195	531
	No	ref	–	–	–	785
Visit	A	0.038	0.064	-0.087	: 0.163	53
	B	0.107	0.040	0.029	: 0.186	163
	C	ref	–	–	–	173
	D	0.053	0.039	-0.022	: 0.128	188
	E	0.027	0.042	-0.055	: 0.110	211
	F	0.011	0.047	-0.082	: 0.102	162
	G	-0.248	0.044	-0.335	: -0.162	183
	H	-0.276	0.048	-0.370	: -0.181	183
Age	<3 mo	-0.127	0.112	-0.346	: 0.091	27
	3-6 mo	-0.090	0.080	-0.247	: 0.066	82
	6-12 mo	0.062	0.070	-0.075	: 0.199	170
	1-4 yrs	0.163	0.060	0.047	: 0.280	263
	4 yrs +	ref	–	–	–	271
	Adult Unknown	-0.048	0.069	-0.182	: 0.087	503
Puppies at household	Yes	0.080	0.028	0.026	: 0.135	531
	No	ref	–	–	–	785

1316 observations. Random effect levels: 124 households, 347 dogs
Variance components: Household <0.0002 (<1%), Dog 0.403 (51%), Within dog 0.383 (49%)

log-transformed ELISA scores. Figure 7.4 displays the coefficient values of the visit and age variables. Visit C was selected as the reference level in the model as this was the first visit with a full sampling of the entire cohort. The clearest influence of the visit identifier upon ELISA score is that the scores in both of the visits conducted in 2008 are likely to be significantly reduced as compared to the 2007 (Visit C) reference level and indeed, lower than the scores observed at any visit in 2007.

Figure 7.4(b) shows the relationship between dog age and ELISA score. The age categorization used in this model is as described in Section 4.4.2. The age class for individuals aged 4 years and greater was the reference level for this factor. The coefficients plotted in Figure 7.4(b) show a clear pattern of increase in scores during the first four years of dog age followed by a decline in scores in the oldest age categories. The presence of puppies aged <3 months at the household at the time of sampling was a significant risk factor associated with higher scores. This model was reached using forward stepwise

(a) Sampling visit level coefficients

(b) Dog age category coefficients
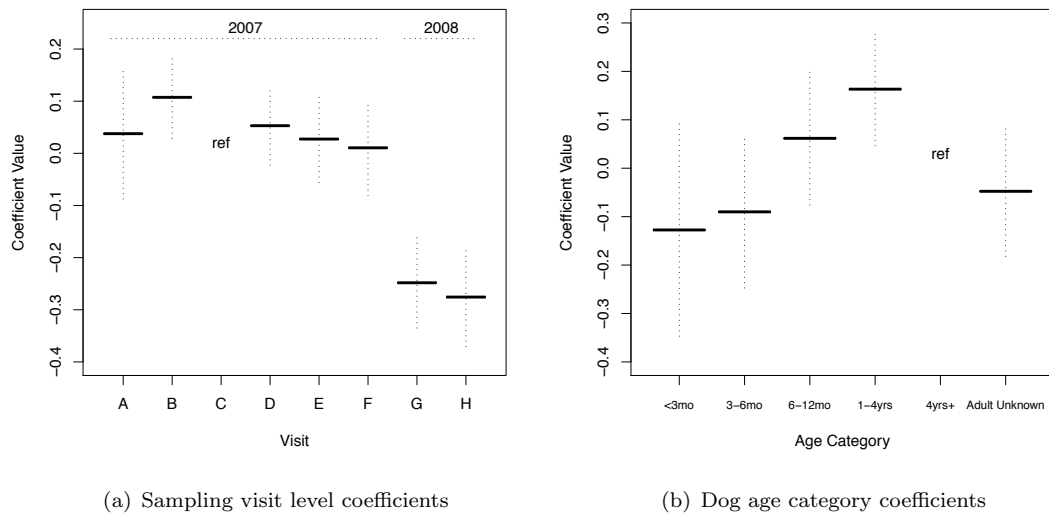
Figure 7.4: Influence of visit number and dog age upon ELISA scores. Variable level coefficient values and 95% confidence intervals are plotted to illustrate the relative effects of factor levels within these variables

variable selection and the multivariate model was fitted with a first order autocorrelation structure for the within group residuals. The final model had a autocorrelation coefficient of 0.207 with an approximate 95% confidence interval of 0.111-0.299, indicating only limited correlation between samples collected at sequential visits. The patterns of dog scores observed over the different visits of the study are discussed further in Chapter 8. The 95% confidence intervals for model coefficients were calculated using the *intervals* function in the package *nlme* (Pinheiro et al., 2009).

7.6 Discussion

7.6.1 Diagnostic Test Data

The first aim of this chapter was to determine if there was evidence of exposure to *Leptospira* in the Kibera dog population. The ELISA and two sets of MAT data were used to address this question. The ELISA and the MATs conducted at Edinburgh indicate quite considerable exposure to *Leptospira* in the Kibera dog population. However, the

interpretation of these data is complicated by the results of the MATs conducted at the VLA which provide considerably weaker evidence of *Leptospira* exposure in this population.

The relative advantages and disadvantages of the different diagnostic tests for leptospirosis are summarized in Chapter 6, Section 6.2.2. In this study the ELISA was the best practical serological assay for screening this large number of samples. The ELISA test showed good within and between plate reproducibility and gives a plausible distribution of sample scores relative to the controls used. In addition, the MAT tests at both the VLA and Edinburgh consistently classified the dog serum used as the positive standard in the ELISA test as positive. The direct comparison of ELISA scores for samples from different populations should be made cautiously as test performance can vary across populations (Greiner and Gardner, 2000). However, it is the case that a considerable proportion of the Kibera dog samples yielded ELISA test scores greater than that seen for this positive control, with some Kibera samples showing effectively double the degree of antigen binding as shown by the positive control.

The MAT tests at the VLA were conducted in order to validate the ELISA and confirm the antibody positive and negative status of ELISA high and low scorers respectively. However, in the VLA MAT test, the population of samples from Kibera are almost all negative and suggest relatively little exposure of the Kibera dog population to *Leptospira*. The MAT tests conducted at Edinburgh were conducted to characterize the ELISA high scorers only. In contrast to the findings of the VLA MAT, the Edinburgh MAT titres indicate that the sera with high scores in the ELISA do have agglutinating antibodies against leptospires.

There are some methodological differences between the MAT protocols at Edinburgh and the VLA that may have contributed to the differences seen in these two sets of data. In the VLA test protocol, the panel of 19 strains were grouped into six pools, presumably on the basis of antigenic relatedness of these particular strains. Individual pools consist of mixtures of equal volumes of between two and four contributing strains. Although antigenic cross-reactivity between similar strains would be expected, this is likely to be incomplete. The pooling of strains for the screening test may thus lead to a reduction in the effective concentration of each strain (and of the surface antigens

characteristic of that strain) and may consequently lead to a loss of test sensitivity with this protocol. In addition, the starting serum dilution used in the pools test is 1:100 which is widely recognized as an accepted titre indicative of previous exposure (Levett, 2001; WHO, 2003; OIE, 2008). A sample with a true positive titre of 1:100 against some but not all of the constituents of a test pool could therefore give a 'false' negative result in this screening test and no additional serovar specific tests would be conducted.

The sensitivity and specificity desirable in a diagnostic test depend considerably upon the application of that test and relative implications of false positive or negative results (Somoza et al., 1989; Dohoo et al., 2003). The test protocol used at the VLA is employed principally to define suspected clinical cases of leptospirosis in animals in which high titres may be expected and for which the predominantly UK/European panel of serovars included in the test may be very well representative of the likely infecting serovar. In such cases the potential reduction in sensitivity created by the initial pooling step is unlikely to lead to mis-classification of samples. In this study however, the pools test was applied to the surveillance of a population in which evidence of previous exposure is just as important a result as detection of clinical cases and in which any circulating leptospires are likely to be quite dissimilar to those used in the test panel. In the Edinburgh tests, the leptospire strains were not diluted into pools and a more concentrated starting serum dilution of 1:50 was used. The Edinburgh tests may therefore have had better sensitivity when applying the test for the screening of the Kibera dog population.

The alternative interpretation of these data is that the VLA MAT data give an accurate representation of dog exposure to *Leptospira* in the Kibera dog population, and that the putative positive results seen in the Edinburgh ELISA and MAT tests are false positives. However, the MAT is considered a very specific diagnostic test and although a proficiency testing study that compared MAT results from a range of participating laboratories did identify variation in the titres and status reported for a panel of control sera, the mis-classification problems detected in this study were problems of incorrect serovar identification and false-negative status determination rather than false-positives (Chappel et al., 2004). In addition, previous serosurveys of canine leptospirosis conducted in Canada (Ribotta et al., 2000) and Mexico (Jimenez-Coello et al., 2008) have

used ELISA tests developed using very similar antigen preparation and sample testing procedures as used in this study. In both cases, the ELISA tests were found to have very high sensitivity (100% and 98.6% respectively) and specificity (95.6 and 95.8% respectively) as compared to the MAT (Ribotta et al., 2000; Jimenez-Coello et al., 2008). In an urban context in which leptospirosis was endemic, an evaluation of four serological tests (ELISA and rapid formats) demonstrated good sensitivity and specificity of these tests in comparison to the MAT for the detection of human acute cases (McBride et al., 2007) and no non-specific cross-reactivity was observed in the evaluation of an ELISA for human testing using a panel of sera positive to a range of non-*Leptospira* pathogens (Zochowski et al., 2001). Although not determined directly in this study, there is no indication from the previous literature that the ELISA test used in this study would be expected to have poor specificity.

It is also the case that the epidemiological picture of *Leptospira* dog exposure indicated by the ELISA and Edinburgh MAT data are entirely plausible and in keeping with the findings of previous studies. Serovars Canicola and Icterohaemmorrhagiae are classically associated with dog reservoir hosts and 18 and 27% of the MAT tested dog samples that had high ELISA scores were positive against these serovars respectively. A similarly high number of dog samples were positive in the MATs against serogroups Tarassovi, Bratislava, Autumnalis, Bataviae and Grippotyphosa (Table 7.3). This pattern of reactivity against a number of serogroups may be attributable to cross-reactivity (as seen previously in similar dog serosurveys (Aslantaş et al., 2005; Adesiyun et al., 2006)) but most of the serogroups against which a high number of dogs were positive have been described in dogs previously. Serological responses against serogroups Grippotyphosa and Bratislava have been described in dog populations sampled in Turkey (Aslantaş et al., 2005), the United States (Adin and Cowgill, 2000; Harkin et al., 2003; Ward et al., 2004) and Trinidad (Adesiyun et al., 2006). Antibodies against Autumnalis were also observed in the Trinidadian study (Adesiyun et al., 2006).

The cross-reactivity seen in the dog serology results could be explained by diagnostic test cross-reactivity or by the exposure of the dog population (an indeed individual dogs) to multiple different serovars. The pattern of human exposure to leptospirosis at the Kibera site is not known and we cannot therefore determine the correspondence between

the responses seen in the dog and human populations. However, cross-reactivity was also seen in the rodent serological results and the results of leptospirosis surveillance currently underway in a Tanzanian human population also have attributes in common with the serological patterns seen here in the Kibera dog population. In the Tanzanian morbidity study, leptospirosis has been identified as a common cause of febrile illness in the human population and a diverse pattern of serovar reactivity was observed (Biggs & Crump *pers.comm.*). Previous serosurveys have reported the predominance of single serovars within some animal and human populations (de Faria et al., 2008; Reis et al., 2008) when using the same serological techniques, the MAT, as used here and in the Tanzanian study. This suggests that the cross-reactivity seen in the results of this and other studies may either reflect the presence of diverse leptospires within a community or the serological patterns that might be expected if the 'true' infecting serovar is perhaps novel and therefore not well represented in standard MAT serovar panels.

7.6.2 Patterns of Dog Exposure

The final aim of this chapter was to describe the patterns of exposure to *Leptospira* in the Kibera dog population. The multilevel model summarized in Table 7.4 shows that there are temporal fluctuations in the leptospirosis ELISA scores of the Kibera dog population and that dog age, the presence of puppies at the household and the previous provision of ivermectin all influence dog ELISA scores. This analysis was conducted to evaluate the consistency of the sentinel response and to determine if key demographic and study design variables influenced the sentinel response measured in this case - the leptospirosis ELISA ODN.

The variance components analysis and multilevel model of ELISA scores identified co-variates associated with ELISA scores and also clear partitioning of the variance in these data at different levels of the data hierarchy. The variance components analysis revealed that less than 2% of the variance in the ELISA score data is attributable to properties of the household at which the dogs were sampled, indicating that household level variables are not critically important in determining the infection status and therefore ELISA score of individuals. In keeping with this, the household level covariates

considered in the modelling, which included the geographic coordinates of the house-hold, the method of identification and the ownership type, were all non-significant in the multilevel model analysis.

Most of the variance in these ELISA data (53%) is attributable to differences between individual dogs. However, none of the dog level covariates considered in this analysis made a significant improvement to the fit of the multilevel model, indicating that the factors associated with this dog-level variation in scores were not considered in this study. Previous studies have identified influences of dog factors such as sex, breed and type (e.g. hunting, stray, farm dogs) upon leptospirosis exposure and disease risk (Ward, 2002; Adesiyun et al., 2006). At the Kibera site, the sex of the dog had no apparent influence upon ELISA scores (univariate LRT statistic = 0.11, d.f =1, p=0.739). The vast majority of dogs in Kibera are mixed-breed mongrels and the potential influence of breed effects were not assessed in this study. It is proposed that these previously identified dog level factors all relate to leptospirosis exposure through their influence upon dog ranging behaviour and consequent variation in environmental exposures to leptospires (Ward, 2002; Adesiyun et al., 2006). The ranging behaviour of the Kibera dog population has not been described but as shown in Chapter 4 (See Section 4.4.3), the vast majority of individuals included in this study were free to roam during the day, night or both . A number of other potential covariates that may relate to dog ranging behaviour were included in this analysis. In addition to the sex and level of confinement of the dog, these included the ownership type of the dog, and the feeding behaviour reported in the household questionnaire (See Section 4.3.9). Considered variables included feeding frequency at the household, observed feeding at other households and observed feeding at rubbish dumps. None of these covariates were significantly associated with the log-transformed ELISA ODN values. Dog ranging behaviour and the spatial patterns in these ELISA scores are considered further in the following chapter.

The distribution of ELISA scores varies over the different sampling visits of the study (Figure 7.4(a)) and the significant variables identified during multilevel modelling all operate at the within dog/repeat visit level (Table 7.4). The clearest comparison is between visits from the two years of sampling. Visits G and H which were conducted

in September and October 2008 respectively both showed markedly reduced ELISA scores in comparison to the data from September 2007 (Visit C) which was used as the reference level. Figure 7.4(a) shows that the confidence intervals for most of the visit level coefficients within 2007 overlap considerably and that only the confidence interval of the positive Visit B coefficient does not include zero. In contrast, the coefficients for the two visits in 2008 are negative and the confidence intervals for these two estimates do not span zero. The sera collected in 2007 and 2008 were tested using different antigen test batches and an influence of this element of the study upon the score difference observed for 2007 and 2008 samples cannot be entirely ruled out. However, the CCC for the comparison of 47 samples tested using both batches was very high indicating that the different antigen batches actually generated entirely comparable results. All of the serovar specific positive control sera were tested using both batches and the lack of any marked difference in scores for any of these sera indicates that the as expected given the antigen preparation procedure (in which the concentration of each constituent antigen was standardized), the representation of antigens from the different serovars used was consistent for these two batches. The significance of the visit identifier in the multilevel model indicates that there are temporal fluctuations in the ELISA scores seen in the Kibera dog population. The interpretation of these changes is discussed further in the following chapter.

The second visit level variable associated with ELISA scores is the categorized age of the dog at the time of sample collection. The influence of age upon ELISA score shown in these data (Figure 7.4(b)) is consistent with similar patterns seen in previous serosurveys of stray dog populations (Aslantaş et al., 2005). It is also similar to that seen in human serosurveillance studies conducted in areas where leptospirosis in endemic. In these human studies, this pattern is interpreted as evidence that continuous exposure throughout life may result in an age-dependent increase in leptospiral seropositivity (Sehgal et al., 1999; Johnson et al., 2004). In this study there is a decline in ELISA scores in the oldest age classes (4 years and above and adult unknown). This decline in antibodies against *Leptospira* in the oldest age classes has also been seen in both dog (Aslantaş et al., 2005) and human populations (Sehgal et al., 1999) and may be associated with changes in behaviour that reduce environmental exposures in this age class or possibly with the phenomenon of immunosenescence. In humans, alterations

of the humoral immune system and antibody responses are observed during ageing, leading to poor response to vaccines and greater susceptibility of elderly subjects to infectious diseases (Ginaldi et al., 2001). It is conceivable that similar changes occur in dogs, influencing the capacity of older dogs to mount effective antibody responses to pathogens including *Leptospira*. The interpretation of this age effect is complicated by the scarcity of data concerning the duration of anti-*Leptospira* antibodies in dogs. The patterns of age-seroprevalence seen in a given population will be determined by the relative likelihood of exposure in each age class, the relative capacity of individuals in each age class to mount antibody responses and the duration of those responses. The interpretation of the temporal and age-related patterns of exposure seen in this population and the relevance of these patterns to sentinel evaluation are discussed further in the following chapter.

A previous study of leptospirosis in dogs identified increased risk of acute infections in the youngest (<5 months) and oldest (>3 years) age classes of a mixed dog population (Adesiyun et al., 2006). This increased risk of acute cases would be consistent with the patterns of antibody scores observed in this study and with the presence of puppies factor that is included in the multilevel model. The presence of puppies (one or more puppies aged <3 months) at the household at the time of the sampling visit was identified as a significant risk factor for higher log ELISA ODN scores. Young post-weaning animals are particularly susceptible to acute leptospirosis (Adesiyun et al., 2006) and transplacental infections can occur (McDonough, 2001). In a population in which leptospirosis were endemic, young puppies may become infected early in life, start shedding leptospires, and therefore pose an exposure risk to other dogs present at the household.

The multilevel model presented in Table 7.4 also includes the factor variable indicating whether or not a dog was treated with ivermectin at the visit prior to sample collection (approximately 28 days earlier). This variable was forced into the model to accommodate the influence of this treatment upon scores. During the modelling exercise the ivermectin variable was also considered alongside the other candidate variables without forcing its inclusion. The final model generated through this procedure was the same as the model described here and the inclusion of the ivermectin variable does provide

a significant improvement to model fit. The provision of ivermectin has a positive co-efficient indicating that ivermectin treatment at the visit prior to sample collection is associated with increased log ELISA ODN values. The capacity of ivermectin treatment to enhance the production of antibody against antigens other than the target of treatment has been described previously (Sajid et al., 2006). Whilst it is of course not desirable for the way in which a study is conducted to influence or interfere with the system that is being observed, the provision of a useful treatment at every sampling visit during this study was a very important element in the relationship between the project and the community. By recording the administration of ivermectin and other treatments, the influence of ivermectin upon the ELISA ODN values was detected and could be controlled for in this multilevel model.

7.7 Conclusions

This study was conducted principally to determine whether or not the presence of *Leptospira* could be detected through the serosurveillance of the Kibera dog population. On balance, the ELISA and MAT data presented in this chapter indicate that there is considerable exposure of the Kibera dog population to *Leptospira*. However, the poor correspondence of results obtained with the different tests conducted reduces the confidence that we can have in these findings. The data obtained from the ELISA and MAT tests also give little indication of the characteristics of the serovar or serovars to which this population is exposed. The ELISA is a pan-*Leptospira* test but the MAT test can often be used to determine the likely infecting serogroup, if not the serovar (Levett, 2001). However, the MAT data that we have from this population are not consistent. The data from the tests conducted at the VLA indicate little evidence of exposure whilst the data from the Edinburgh MAT tests show a pattern of positive cross-reactivity rather than the predominance of any particular serovar(s). The multilevel model of leptospirosis ELISA scores reveals temporal variation in the exposure of this population to *Leptospira*, and age and demographic patterns consistent with endemic pathogen presence and persistent exposure. Extensive testing of these dog sera indicate that there is considerable exposure of this population to *Leptospira*,

but provides little indication on the characteristics of the *Leptospira* strain or strains circulating at this site.

Chapter 8

Kibera Dogs as Leptospirosis Sentinels? Addressing other Surveillance Questions?

8.1 Abstract

Analyses presented in previous chapters (6 and 7) have demonstrated evidence of exposure to *Leptospira* in both the peri-domestic rodent and domestic dog populations in Kibera. To assess the degree to which dog serology data can be used to answer further questions about the epidemiology of leptospirosis in Kibera three main analyses were conducted. First, Bayesian mixture models were employed to estimate the seroprevalence of anti-*Leptospira* antibodies in the dog population and to examine temporal patterns in the dog population response. Second, the spatial distribution of seropositive and seronegative dogs within the study site was examined using tests of random labelling. Finally, the specificity of the dog sentinel population response to *Leptospira* was characterized by comparing the patterns of exposure seen in the rodent and dog populations to different pathogenic serovars. The estimated seroprevalence of anti-*Leptospira* antibodies in the Kibera dog population ranged from 27-36% during 2007 but fell considerably to just 7 and 5% at the sampling visits conducted in 2008. There

was no evidence of a non-random distribution of seropositive vs. seronegative dogs within the spatial pattern of the Kibera dog sampling locations at any sampling visit. Hierarchical cluster analysis of the rodent and dog MAT data indicates that although there is cross-reactivity in the responses of both populations, there are different patterns of exposure to *Leptospira* in the dog and rodent populations. These data are consistent with the circulation of more than one population of pathogenic *Leptospira* in the Kibera animal populations and suggest that dogs may show evidence of exposure to the same serovars as are seen in the rodent population but that the same pattern is not seen in reverse. Addressing complex and specific surveillance questions through the use of dog serology data is complicated by the variation in dog responses observed and the scarcity of data required to inform interpretation of these serological data. However, the data gathered from the Kibera sentinel dog population provides useful evidence of the circulation of leptospires at the Kibera site and can be used to prompt further targeted research into the human risk of leptospirosis at this site.

8.2 Introduction

The data presented in the two preceding chapters provide evidence of the presence of *Leptospira* in the rodent and dog populations in Kibera. In this chapter, these data are examined to assess the degree to which the dog serology data particularly may be used to address additional questions about the epidemiology of *Leptospira* at this site. These analyses are made with reference to the framework proposed in Chapter 1 and applied to leptospirosis in Kibera in Chapter 6 (Figure 6.2). In this chapter, the serological data from the dog and rodent populations are analysed to assess the evidence for temporal and spatial variation in the prevalence of leptospirosis at this site. The nature of the sentinel response to leptospirosis presence and its detectability are also discussed.

8.2.1 Chapter Objectives

This chapter considers the serological data gathered from the surveillance of leptospirosis in the Kibera rodent and dog populations in order assess the utility of leptospirosis

surveillance in Kibera using the domestic dog population. Specifically, the chapter aims to address three questions: does the estimated anti-*Leptospira* seroprevalence in the dog population fluctuate over time?, is there evidence of a non-random spatial distribution of seropositive vs seronegative dogs? and how specific is the sentinel population response at the serovar/serogroup level?

8.3 Data Analysis

8.3.1 Estimating Seroprevalence

The objective of this first analysis was to obtain estimates of the anti-*Leptospira* seroprevalence in the Kibera dog population at each sampling visit during the course of the study. The results of the multilevel modelling analysis described in the previous chapter reveal that the distribution of ODN values changes over the different visits of the study but does not help us to define the proportion of the population that can be considered antibody positive or antibody negative at any visit. The determination of seroprevalence requires that the continuous score for each serum sample generated by the ELISA be classified as either positive of negative for antibodies against *Leptospira*. The interpretation of diagnostic test data produced by reducing continuous data to a dichotomous variable through the application of a cut-off is obviously very sensitivity to the cut-off selected (Greiner et al., 1994). Test cut-offs are often defined by testing a reference population of known negative samples and setting the cut-off at a value of the population mean plus two or three standard deviations. Using this approach, the selection of a representative reference population is critical and is often particularly difficult when screening for tropical infectious diseases (Greiner et al., 1994), such as leptospirosis in the Kibera dog population. Sera from an appropriate reference population were not available in this case. Cut-offs can also be defined relative to a known gold-standard test. The MAT is the traditional gold-standard serological test for leptospirosis but as discussed in the previous chapter the MAT data that we have from this population are not suitable for the estimation of a reliable ELISA cut-off.

Seroprevalence estimates were made using mixture models of the standardized ELISA results (ODN) which allowed for the estimation of seroprevalence from the ELISA data themselves rather than through the application of a poorly informed or arbitrary cut-off. Finite mixture models with two components representing antibody positive and antibody negative dogs were fitted, adopting a modelling approach that has been used previously for the estimation of diagnostic test cut-offs and seroprevalence estimates (Franke et al., 1994; Greiner et al., 1997; Cohen and Haas, 1999; Nielsen et al., 2007). Two slightly different models were run, both considering all 1410 sample records. The first - Full Model - only included the sampling visit variable. The second - Full Model + ivermectin - was run with the addition of the ivermectin treatment variable to assess this influence upon seroprevalence estimates and generate adjusted seroprevalence estimates that excluded the influence of ivermectin treatment. Finally, the Full Model was also run considering the data from each year of sampling separately. These models are summarized below as 2007 Model and 2008 Model.

Conditioned on the exposure/antibody status of the sampled dogs (Z - antibody positive or negative), the models assumed that the leptospirosis ELISA ODN values followed a Normal distribution, where the mean and variance were determined by the exposure status (Z). The exposure status (Z), which was either antibody positive or antibody negative, was Bernoulli distributed with the probability that a given ODN value was from a truly exposure/antibody positive individual represented by p. The probability p was influenced by the sampling visit in both models and also by the previous provision of ivermectin in the model that included this variable. The mixture model with two mixture components that included the ivermectin variable was specified:

$$ODN_i | Z_i \sim N(\mu_{Z_i}, \sigma_{Z_i})$$
$$Z_i \sim Bern(p_i)$$
$$logit(p_i) = \beta_{visit_i} + \alpha_{ivermectin_i}$$

where ODN_i is the observed ODN value for the ith sample, which, conditional on the Bernoulli distributed exposure status (Z_i) follows a normal distribution with mean μ and standard deviation σ. p_i is the probability that the ith sample is from an exposed dog. $\beta\mathrm{visit}_i$ is the effect of the sampling visit upon the probability that the ith sample is from an exposed dog. $\alpha\mathrm{ivermectin}_i$ is the effect of the previous treatment with ivermectin upon the probability that the ith sample is from an exposed dog. The $\alpha\mathrm{ivermectin}_i$ variable was not included in the Full Model, 2007 Model or 2008 Model.

The visit and ivermectin variables were coded as binary dummy variables and no intercept was fitted. As a consequence, the values of β estimated from the models that do not include the ivermectin variable are analogous (when inverse logit back transformed) to the mean probability that a sample collected at a given visit is from an exposed dog and are thus effectively seroprevalence estimates for each sampling visit. In the model that includes the ivermectin variable, the back transformed values of β give adjusted seroprevalence estimates excluding the effect of ivermectin upon the probability that a given sample is from an exposed dog.

The analysis was run in R (R Development Core Team, 2009) using the *rjags* package (Plummer, 2009) for Bayesian analysis and Markov Chain Monte Carlo (MCMC) based estimation methods. Vague priors for the estimated parameters were given, reflecting the lack of previous data and uncertainty of these estimates. The means of the two estimated normal distributions and the coefficients of the logistic model were set with vague normal priors (mean = 0, precision = 0.001, where precision = 1/standard deviation^2). The standard deviations of the two distributions were set with gamma priors (0.001, 0.001). The first 2500 iterations of each model were discarded as adaptation, the next 2500 were discarded as burn-in and the following 30,000 iterations were recorded for posterior inference. The convergence of the three chains used to estimate the parameters of each model was checked using visual inspection of chain traces, kernel-density plots and Gelman-Rubin diagnostics (Bolker, 2008). The assumption that the ODN data were a mixture of two normal distributions was checked by comparing the moments of the observed data distribution (mean, variance, kurtosis and skew) to distributions of these summary measures generated from 90,000 datasets simulated from each iteration of each model. The mixture model that included the ivermectin

variable model was also used to simulate a dataset used to determine a cut-off for the ELISA test at which the sensitivity and the specificity of the ELISA test for predicting sample status as defined by the mixture model was maximized (Dohoo et al., 2003).

8.3.2 Spatial Patterns

The seroprevalence estimates consider the response to the presence of *Leptospira* provided by the Kibera dog population as a whole. For this analysis, the status of each individual sample was defined using the population cut-off derived from the final mixture model described above (the model including all 1410 records and the ivermectin variable) and the spatial distribution of positive and negative samples was examined. This analysis considered the status (ELISA positive or negative) or label, of each sample given the existing spatial pattern of all points in space. For each full sampling visit (C to H) the null hypothesis that the samples were randomly labelled was tested (Goreaud and Pélissier, 2003; Baddeley, 2008). Coordinates were recorded for all sampled households and the locations for each sample were generated by jittering the points around the household coordinates. Jittered values for sample coordinates were generated by adding or subtracting a small value generated at random from a uniform distribution to the coordinates of the household.

This analysis was conducted in R using the *spatstat* package (Baddeley and Turner, 2005). The test statistic was based on the univariate ($K(r)$) and i-to-any ($K_{i\bullet}(r)$) intertype K-functions (Baddeley, 2008). The univariate K function computes the expected number of points within a given radius (r) of a typical point and characterizes the spatial structure of a univariate pattern at different scales (r). In a bivariate pattern, where points may be labelled either i or j (negative or positive in this case), the i-to-any K-function ($K_{i\bullet}(r)$ or Kdot(r)) computes the expected number of points of any type within a given radius (r) of a typical point of type i (Baddeley, 2008). Under an assumption of random labelling $K_{i\bullet}(r)$ is equal to $K(r)$ if $K(r)$ is calculated for all points irrespective of label. The value of $K_{i\bullet}(r)$-$K(r)$ was used as the test statistic to assess the status of samples within the spatial patterns obtained at each full sampling visit (C to H) (Baddeley, 2008). Both $K(r)$ and $K_{i\bullet}(r)$ were corrected for edge effects using Ripley's isotropic correction (Baddeley, 2008). For each visit, the test statistic for the

observed data was compared to the distribution of the statistic generated from 1000 simulated datasets generated by fixing the spatial point pattern observed and randomly re-labelling the points through permutation of the observed values using the *rlabel* and *simulate* functions in the *spatstat* package (Baddeley and Turner, 2005). The upper and lower critical envelopes for the test statistic were calculated pointwise at each value of r by sorting the test statistic values from the simulated datasets and selecting the high and low values consistent with the desired test significance level. In all cases the point-wise envelopes were calculated for a test with significance level 0.05 using the *envelope* function in the *spatstat* package (Baddeley and Turner, 2005; Baddeley, 2008).

8.3.3 Response Specificity

The specificity of a sentinel response is defined in Chapter 1 as the degree to which a response observed in the sentinel can be attributed to a particular pathogen of interest. In this current analysis the pathogen of interest is defined as pathogenic *Leptospira*. In the absence of previous knowledge about the particular serovar or serovars that may pose a threat to the target human population this pathogen definition is appropriate in this case. To address more refined questions about the use of dog serology for the surveillance of leptospirosis in other contexts, the specificity of dog responses for the identification of particular *Leptospira* serovars is also considered. Both of the diagnostic techniques used in the surveillance of the Kibera dog population detect antibodies against *Leptospira* but the tests vary in the antibody populations that they can detect. As a consequence, the ELISA which can detect antibodies against components of the leptospire wall and internal proteins that can be conserved across serovars is considered a genus-specific test (Lupidi et al., 1991), whereas the MAT, which only detects antibodies against surface proteins is specific to different serogroups (Levett, 2001).

To explore the likely serovar or serogroup specificity of the dog population responses to *Leptospira* exposure, the correspondence between the MAT data from the rodent and dog populations was compared. The results of the microscopic agglutination tests are represented using heatmaps plotted in R (R Development Core Team, 2009) using the *gplots* package (Warnes, 2009). The data were arranged in a matrix in which each row represented the MAT results for a given sample and each column represented a

MAT run with a particular serovar. The data recorded in the cells of the matrix were the maximum serum titre at which 50% agglutination was observed for each sample and test combination. The ordering of the columns was fixed to arrange the different leptospires by species. The initial ordering of rows in the matrix was determined by a randomly selected numeric identifier generated to ensure that the results of the analysis were not influenced by the order of data entry.

The distance or dissimilarity between each row of the matrix was then calculated (Everitt, 2005). All columns of the matrix contained data measured on the same scale (maximum serum titre at which 50% agglutination was observed) and the Euclidean distances (square root of the sum of squared differences between the elements of a pair of rows) were calculated for all sample comparisons (Everitt, 2005). These measures of dissimilarity were then used to reorder samples using hierarchical clustering methods. The algorithm used starts by assigning each sample to a cluster and then iteratively combines the most similar pairs into a cluster. The clustering algorithm combined pairs using a complete clustering or furthest neighbour method (Everitt, 2005) whereby the intercluster distances are defined as the maximum distance between elements of two clusters. At each step, the distance measures between clusters are updated and the most similar or closest pair of clusters are fused. The algorithm continues until all samples are grouped together in a single cluster. Finally the data are plotted using the arrangement of samples defined by the clustering algorithm. The colours in the heatmap are coded to represent the test scores (See Figure 8.6).

8.4 Results

8.4.1 Seroprevalence Modelling

The full Bayesian mixture models were fitted to a total of 1410 ODN values collected from 348 dogs throughout the entire period of the study. The 2007 Model and 2008 Model were fitted to 1030 and 380 records respectively. A histogram of the observed ELISA ODN values with the estimated posterior mean ODN values for the antibody positive and antibody negative populations and their 95% credible intervals as estimated

from the Full Model + ivermectin is given in Figure 8.1. The Full Model + ivermectin defined the seronegative distribution with a posterior mean ODN value of 0.50 and standard deviation of 0.21, whereas the seropositive distribution had a mean value of 1.03 and a standard deviation of 0.39. The Full Model and 2007 Model that did not include the ivermectin variable gave very similar estimates for the parameters of the two normal distributions. The parameter values and credible intervals estimated using all four models are given in Table 8.1 for comparison. The seroprevalence estimates for each sampling visit generated from the Full Model and Full Model + ivermectin are also shown for comparison in Figure 8.2.

The Full Model and Full Model + ivermectin yield similar seroprevalence patterns (comparing seroprevalences estimates from the Full Model with the adjusted seroprevalence estimates from the Full Model + ivermectin) except that many of the estimates from the Full Model + ivermectin are moderately reduced in comparison, especially the estimates for Visits D-F and H. The coefficient for the ivermectin variable influence of + 0.68 on the probability on a sample being positive is quite considerable and the results from the Full Model + ivermectin therefore agree with the findings of the multilevel model that revealed a significant positive influence of previous ivermectin treatment on ELISA ODN value. The pattern of adjusted seroprevalence estimates estimated from the Full Model + ivermectin and the pattern of differences between these estimates and those from the Full Model are consistent with the greatest influence of previous ivermectin treatment upon ODN values seen at these visits after widespread treatment at the previous visit.

The kernel density plots for the estimated parameters of the Full Model + ivermectin are shown in Figure 8.3. Both full models and the 2007 Model had multivariate potential scale reduction factors (psrf) of less than 1.02 indicating that there were no problems with chain convergence after 30,000 monitored iterations (Bolker, 2008). The 2008 Model had a psrf value of 1.06 after 30,000 iterations which is still well below the value of 1.2 considered indicative of a convergence problem (Bolker, 2008). In all cases, the values of the moments of the observed data distribution (mean, variance, skew and kurtosis) fell within the 95% range of the distributions of these measures from 90,000

Table 8.1: Mixture model summaries

Model Component	Estimated Parameter	Full Model Mean	Full Model 2.5%	Full Model 97.5%	2007 Model Mean	2007 Model 2.5%	2007 Model 97.5%	2008 Model Mean	2008 Model 2.5%	2008 Model 97.5%	Full Model + ivermectin Mean	Full Model + ivermectin 2.5%	Full Model + ivermectin 97.5%
Seronegative distribution	μ, Z=negative	0.51	0.49	0.53	0.52	0.49	0.54	0.50	0.45	0.54	0.50	0.48	0.53
	σ, Z=negative	0.21	0.19	0.22	0.20	0.18	0.22	0.26	0.20	0.27	0.21	0.19	0.22
Seropositive distribution	μ, Z=positive	1.03	0.95	1.13	1.03	0.94	1.14	1.38	0.71	2.42	1.03	0.94	1.11
	σ, Z=positive	0.39	0.35	0.43	0.39	0.35	0.43	0.54	0.15	1.53	0.39	0.36	0.43
Visit	β Visit = A	0.26	0.10	0.45	0.27	0.11	0.47	–	–	–	0.27	0.11	0.46
	β Visit = B	0.36	0.24	0.50	0.36	0.23	0.51	–	–	–	0.37	0.24	0.50
	β Visit = C	0.32	0.21	0.43	0.32	0.21	0.44	–	–	–	0.30	0.20	0.41
	β Visit = D	0.41	0.31	0.53	0.42	0.30	0.54	–	–	–	0.36	0.25	0.48
	β Visit = E	0.39	0.29	0.50	0.39	0.28	0.51	–	–	–	0.31	0.21	0.43
	β Visit = F	0.35	0.24	0.48	0.36	0.24	0.49	–	–	–	0.27	0.27	0.40
	β Visit = G	0.07	0.02	0.14	–	–	–	0.05	0.003	0.22	0.07	0.02	0.15
	β Visit = H	0.08	0.02	0.15	–	–	–	0.05	<0.001	0.25	0.05	0.01	0.11
Ivermectin	α	–	–	–	–	–	–	–	–	–	0.64	0.54	0.74

Visit and ivermectin parameter estimates are reported on the probability scale
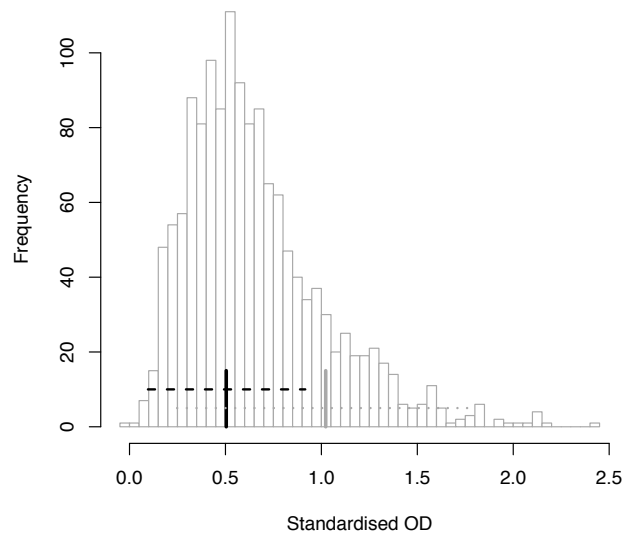
Figure 8.1: Histogram of observed ELISA ODN scores. The estimated mean posterior ODN and the 95% credible interval for the antibody positive population (grey vertical bar and grey dotted horizontal line) and the antibody negative population (black vertical bar and black dashed horizontal line) estimated using the Full Model + including the ivermectin variable are superimposed.

simulated datasets generated from each model, indicating that the assumption that the mixture consisted of two normal distributions is not invalid.

The ELISA cut-off value generated from the Full Model + ivermectin at which the sensitivity and specificity of the ELISA relative to the model was maximized was an ODN value of 0.76. At this value the ELISA had a sensitivity of 0.75 and a specificity of 0.89. The equivalent cut-off value estimated from the Full Model (not including the ivermectin variable) was an ODN value of 0.78.

8.4.2 Spatial Patterns

At each of the visits tested (C to H) the value of the test statistic $(K_{i\bullet}(r)\text{-}K(r))$ generated from the observed distribution of positive and negative dog samples was within the critical envelope generated from 1000 randomly labelled simulated datasets at all values

of r. The observed spatial distribution of positive and negative samples at visit C is shown in Figure 8.4 and the plot of the $K_{i\bullet}$(r)-K(r) statistic calculated from these data is shown in Figure 8.5. The test statistics generated from the observed data at visits C - H all fall within the critical envelopes for all values of r. As a consequence we cannot reject the null hypothesis that given the spatial distribution of samples observed, the distribution of sample status at each visit (ELISA positive or negative) was random (Baddeley, 2008).

8.4.3 Response Specificity

The heatmap that combines the MAT results from the tests conducted at Edinburgh for both the dog and rodent samples is shown in Figure 8.6. The heatmap shows that as discussed in chapters 6 and 7, there is no clear predominance of a particular serovar
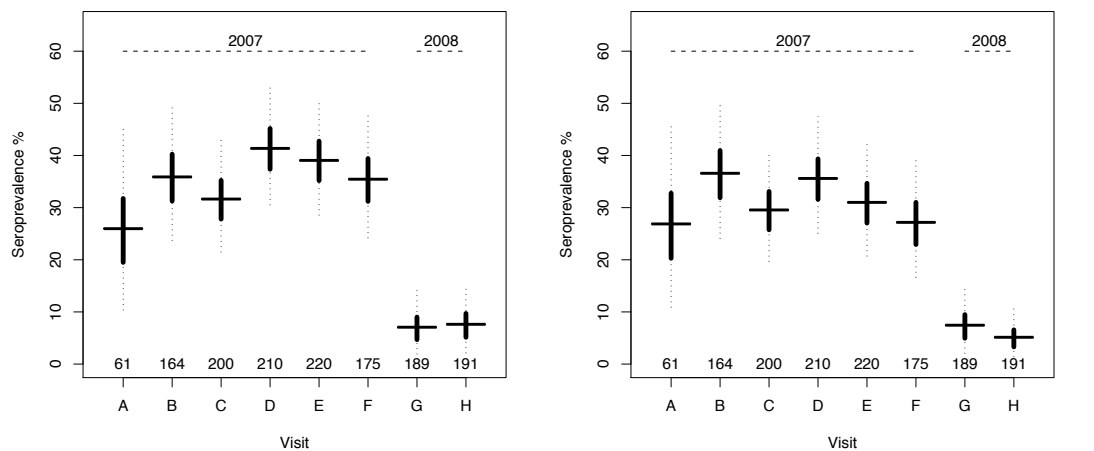
(a) Seroprevalence estimates from Full Model

(b) Seroprevalence estimates from Full Model + ivermectin

Figure 8.2: Posterior seroprevalence estimates generated from the full mixture models. Seroprevalence estimates from the Full Model + ivermectin are adjusted estimates that exclude the influence of ivermectin upon ODN scores. Bold horizontal lines indicate the mean of the posterior distribution. Dashed vertical lines show the 95% credible interval around the mean estimates. Figures at the bottom of each plot indicate the number of samples from each visit included in the model.
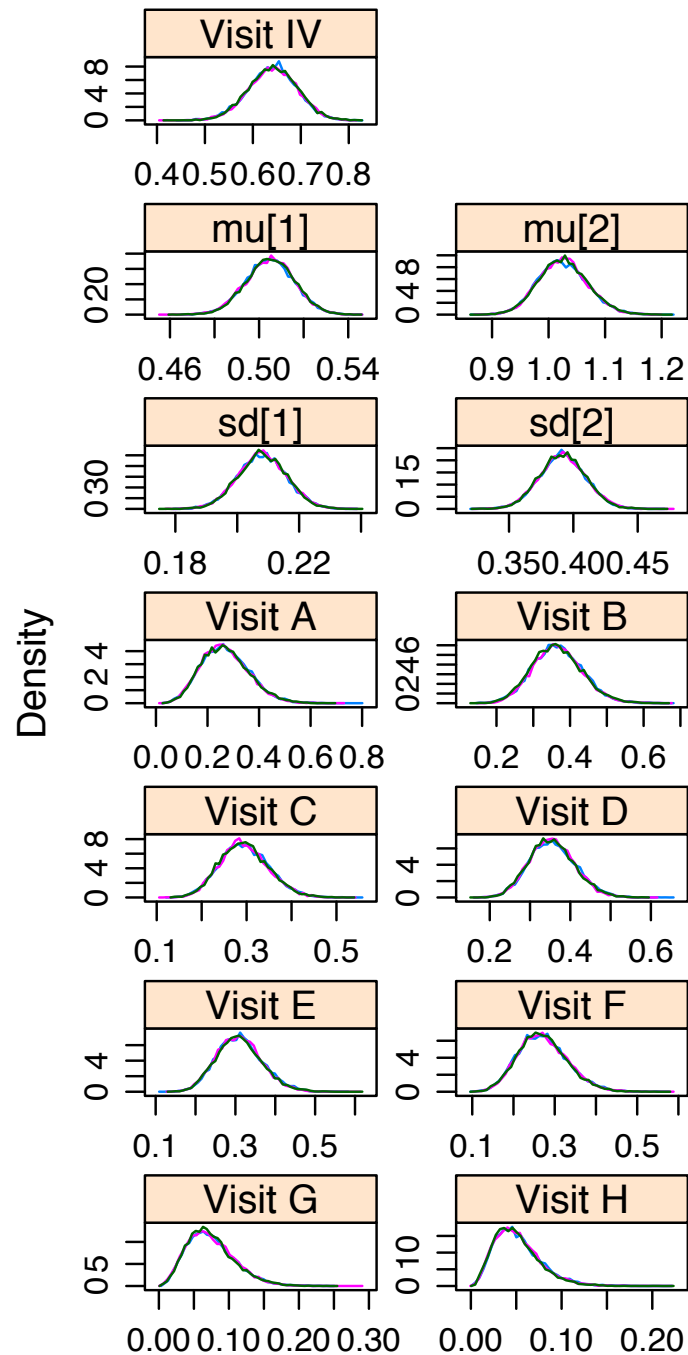
Figure 8.3: Kernel density plots for posterior parameter estimates from the Full Model + ivermectin. The top panel shows the posterior estimate for the distribution of the ivermectin parameter. The next four panels (Mu and sd plots) indicate the estimates for the mean and standard deviation of the seronegative [1] and seropositive [2] distributions respectively. The lower 8 plots show the posterior estimates of seroprevalence at each visit. The seroprevalence and ivermectin parameter values are back-transformed to the probability scale
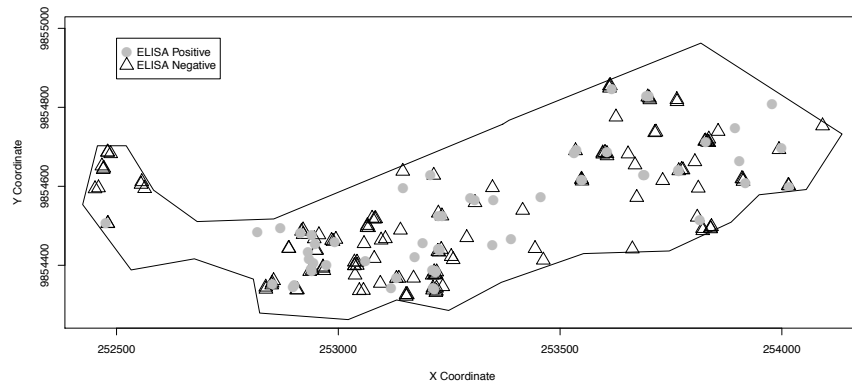
Figure 8.4: Spatial distribution of ELISA positive and negative samples showing all samples from Visit C. Points representing individual samples are jittered around the shared household locations. The outline shows the polygon used as the perimeter for spatial analysis.
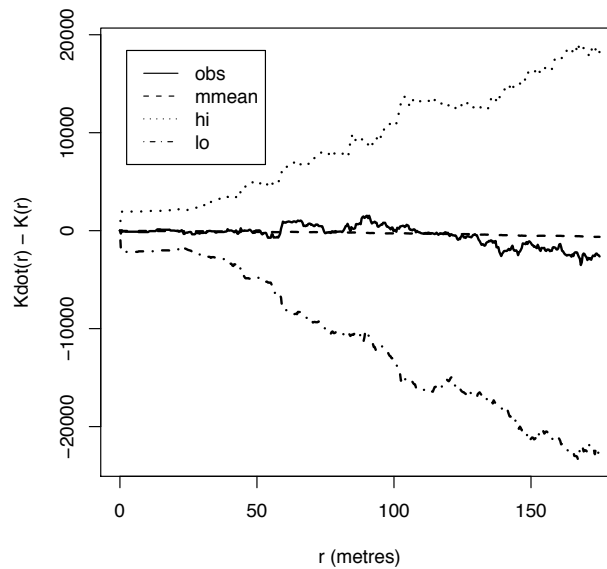
Figure 8.5: Test of random labelling at dog sampling visit C. The values of the test statistic calculated from the observed data are represented by the continuous black line (obs). The upper (hi) and lower (lo) values of the credible envelope at each value of r are represented by the dotted and dashed lines respectively. The dashed line (mmean) represents the mean value of the test statistic for the simulated datasets.

or serovars in either the dog or rodent populations, and that there is a high degree of cross-reactivity within the results of individual samples.

The results of the clustering algorithm are represented by the tree to the left of the figure. The algorithm essentially divides the samples into four generalized groups. The centre of the figure is dominated by dog and rodent samples that were largely or entirely negative in the serovar tests. The black and light green bar to the left of the figure indicates rodent samples in light green and dog samples in black. The highest scoring dog sera are grouped together at the top of the figure, with a second grouping of dogs with positive titres against some serovars located at the bottom of the figure. The positioning of these two groups of dog samples at the top and bottom of the figure should not be interpreted as maximal separation by the clustering algorithm. At each step in the clustering process the algorithm orders the subtree so that the tighter cluster is on the left (top) of the tree.

The higher scoring rodent samples are all grouped together below the high scoring dog samples at the top of the figure. At the start of the clustering analysis used to generate the heatmap, the samples (rows) are arranged in a random order and it is therefore interesting to note the complete separation of higher scoring dog and rodent samples generated by the clustering algorithm which indicates that there are differences in the patterns of exposure (or in the patterns of detectable responses) to *Leptospira* in the rodent and dog populations. Although these data should be interpreted cautiously it appears that there is evidence of dog exposure to the serovars against which most rodent positive reactions are observed (serogroups Ballum, Grippotyphosa and Pomona) but that the same is not true in reverse and there is little indication of rodent exposure to the serovars that dominate the dog positive responses (serogroups Icterohaemorrhagiae RGA, Bataviae, Autumnalis, Australis and Tarassovi). Analysis of MAT data is often limited to reporting the number or proportion of samples that yield a MAT titre greater than a selected cut-off. A titre of ⩾1:100 is commonly used but there is evidence that different cut-offs are appropriate in different circumstances (Reis et al., 2008). It is also common to report only the serovar against which the highest MAT titre was observed. The heatmap representation of MAT data used here is complex but does provide an honest representation of the cross-reactivity and patterns of response observed in these

samples. This hierarchical cluster analysis of these data indicates that the dog and rodent populations in Kibera may be exposed to different populations of leptospires.

8.5 Discussion

8.5.1 Temporal Variation

The seroprevalence estimates shown in Figure 8.2 indicate that less than half of the sampled dogs were seropositive at any sampling visit and that whilst the mean of the estimates range from 27% to 36% seropositivity in 2007, they fall to 7 and 5% in 2008. These figures are reported from the Full Model + ivermectin but very similar values were estimated from the Full Model. The comparison of the two mixture models reveals that previous treatment with ivermectin did influence the ODN scores of individual dogs but that at the population level, the overall pattern seen in these data is similar using both models.

The seroprevalence estimates for the 2007 visits generated from the two full models and from the 2007 Model show a slight increase from visits A to C/D (July to September/October) and then decline again in the later visits D to F (October to December). The credible intervals on all of these estimates are broad and overlap considerably. In contrast, in the estimates made with both full models, visits G and H (September and October 2008) show markedly reduced seroprevalence in comparison to all of the 2007 visits. The 2007 Model and 2008 Model were run to include each year of data separately to further check that the antigen batch used to test these subsets of sera was not unduly influential upon the seroprevalence estimates obtained. The mixture models run with each year of data separately (Model 2007 and Model 2008 , See Table 8.1) give similar seroprevalence estimates as compared to the full models. The 2008 Model considered much less data than any of the other three models and consequently all of the parameter estimates from this model have wider credible intervals than are seen with the other models. However, when the 2008 data are considered alone the seroprevalence estimates obtained are still very low. The two antigen batches used to test the 2007 and 2008 samples respectively show very good concordance (Section 7.5.1)
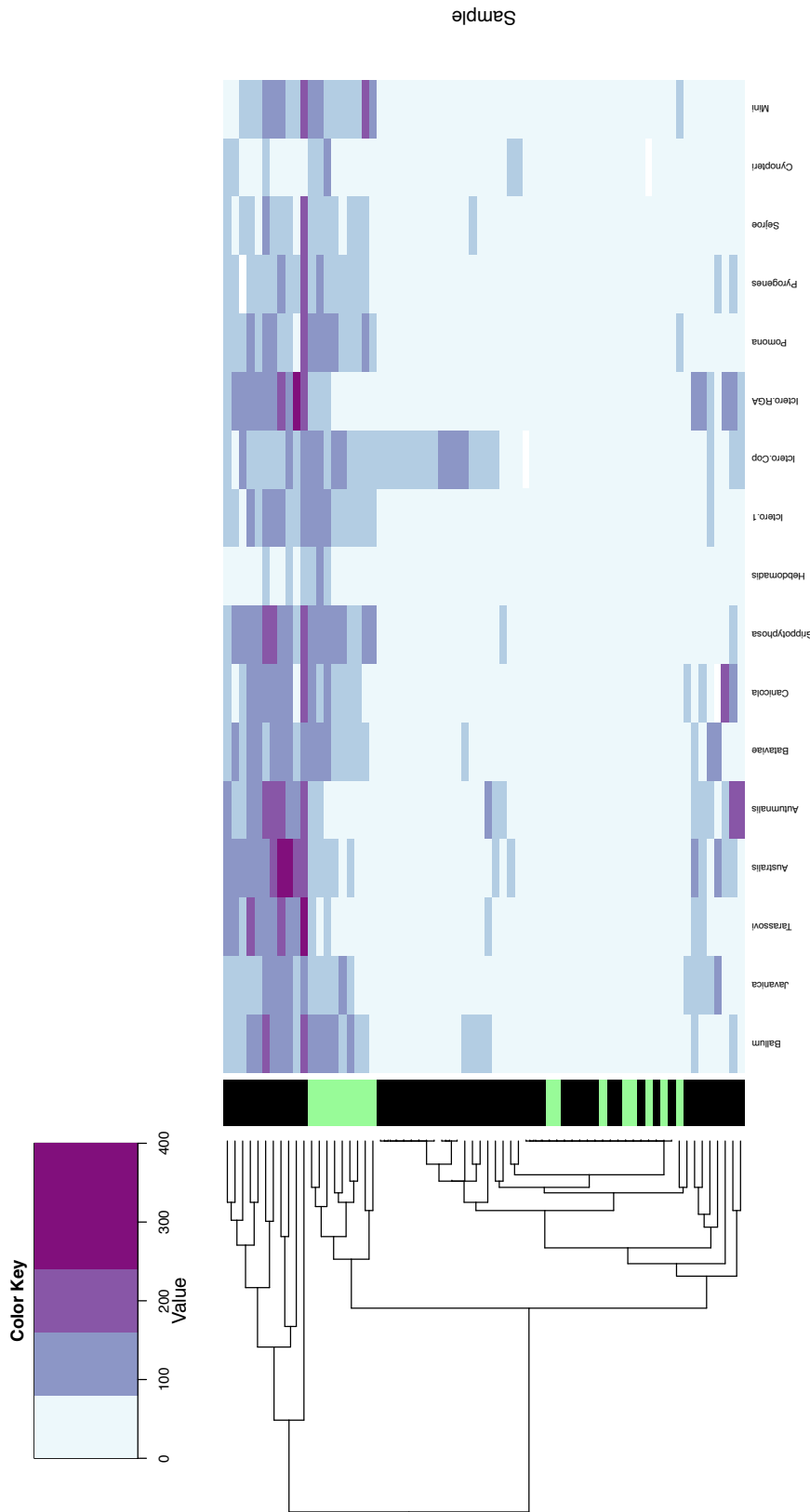
Figure 8.6: Heatmap illustrating the dog and rodent MAT data. Rows show the MAT scores for each sample tested. Columns show the MAT scores observed with these samples for each of the serovars tested at Edinburgh. The colour in each cell within the figure indicates the value of the maximum MAT titre observed. Columns are labelled with the serogroup (and serovar for Icterohaemorrhagiae) names as corresponding to Tables 6.9 and 7.3. The tree to the left of the figure shows the hierarchical clustering of samples on the basis of test score similarity. White cells indicate missing values. The black and light green bar between the tree and figure identifies the rodent samples (indicated in light green) and the dog samples (indicated in black).

and the data generated form these mixture models generate similar seroprevalence estimates when considering the data from each year separately as when considering all of the data together. These data all indicate that the reduction in seroprevalence estimates for the 2008 visits are a consequence of a real difference in seroprevalence, rather than an artefact of the lab testing procedure.

Previous surveys of stray dogs in Mexico (Jimenez-Coello et al., 2008) and Turkey (Aslantaş et al., 2005) have reported anti-*Leptospira* seroprevalences of 35% and 43.96% respectively and whilst slightly reduced, the estimated seroprevalences for the Kibera population in 2007 are within a similar range. The seroprevalence analysis was conducted to determine if there was evidence of change in the prevalence of pathogenic *Leptospira* over time at this site. We do not have enough data on the performance of this ELISA and on the nature of Kibera dog responses to *Leptospira* exposure to assess how good these estimates are for determining the 'true' seroprevalence in this population. However, because the same diagnostic and analytical techniques are applied to the data from each visit, we can look at the relative patterns of exposure over time. The results indicate that the dog seroprevalence estimates do vary over time, particularly between the two years of sampling. In order to assess how this evidence of change in dog seroprevalence can be interpreted and perhaps used to make inferences about temporal changes in human risk, a number of other factors need to be considered.

The first of these is the nature of the relationship between the target human population and the sentinel dog population and most importantly the routes of transmission of pathogenic *Leptospira* that are important in these two populations. Leptospirosis has been described as a disease of the environment (Vinetz, 2001). The poor sanitation and housing infrastructure at the Kibera site create and maintain numerous potential sources of human infection both inside the household (See Section 6.6.5) and in the immediate environment (See Figure 6.1). These same routes of exposure also apply to the sentinel dog population. The outdoor nature of dogs at this site and their foraging and scavenging behaviour probably increases their rates of contact with for example contaminated water sources in comparison to humans and the dog population may also acquire infection through consumption of infected rodents (Millán et al., 2009).

The epidemiology of human leptospirosis is markedly seasonal, and in warmer countries incidence is often highest during rainy seasons when conditions for the survival of leptospires in the environment are optimal (Levett, 2001; McBride et al., 2005). Seasonal patterns in human leptospirosis are also often influenced by the population dynamics of rodent hosts (Holt et al., 2006) but in urban environments there is relatively little seasonal variation in rodent abundance as compared to rural areas (Castillo et al., 2003; Holt et al., 2006). In such environments, seasonal variation in the number of rodents infected with leptospires is driven by seasonal variation in leptospire survival in the environment (Holt et al., 2006). The risk of both human and dog exposure to leptospires via the most likely routes is therefore dependent upon the survival of leptospires in the environment which is turn is largely determined by rainfall patterns. Unfortunately as a result of the interruption to this study during the political disturbances in early 2008 we don't really have the data to look at seasonal patterns in the dog exposure. If as seems likely, both dog and human exposure risk are determined by seasonally variable environmental factors, then the evaluation and monitoring of these causal determinants of risk to both populations may be a more cost-effective and timely method of evaluating human risk than sampling dogs which are likely to become infected at the same time as people.

The second key factor that should be considered when evaluating the temporal variation in dog seroprevalence is our understanding of the temporal dynamics of the sentinel response. In dogs, acute infection with *Leptospira* and vaccination both lead to the production of IgM antibodies (Hartman et al., 1984a, 1986). The IgM response is relatively short-lived and is followed by a longer lasting IgG response (Hartman et al., 1986; OIE, 2008). The ELISA used in this study, is not specific to either IgM or IgG antibody classes and therefore cannot distinguish between antibody titres due to acute infections or previous infections (Hartman et al., 1984b). In humans, MAT detectable titres can persist for years after infection (Lupidi et al., 1991; Brandão et al., 1998). ELISA detectable antibodies appear to decline more rapidly but still persist for many months and even years (Brandão et al., 1998; Johnson et al., 2004). In areas where leptospirosis is endemic, repeat exposure to both pathogenic and non-pathogenic leptospires may occur and contribute to the persistence of antibodies (Blacksell et al., 2006). It is also the case that at the level of the individual, there is considerable

patient-to-patient variability in the timecourse and magnitude of human MAT and ELISA detectable responses to known *Leptospira* infection (Lupidi et al., 1991; Levett, 2001). It is likely that dog antibody responses to *Leptospira* are similarly complex and variable. Figure 8.7 shows a sample of timelines of ELISA scores from eight individual dogs with repeat samples from 2007. This figure represents a small sample of dogs but illustrates that there is no consistent pattern of response over time in this population and that there is considerable variation in ELISA scores both within and between individual dogs sampled at this site.

The period of time over which antibody responses to *Leptospira* can persist and are detectable by tests such as the ELISA used in this study (which is not specific to a particular Ig class) is greater than the interval between sampling visits in 2007. It is therefore unsurprising that the seroprevalence estimates for different visits are similar over the monthly intervals within each year. More time-specific data about recent exposures to *Leptospira* should in theory be obtainable by using diagnostic tests that detect only IgM antibodies produced in the acute phase of infections. Such tests do exist, but human studies have shown that even IgM titres can persist for long periods and that ELISA tests based on IgM detection can have limited diagnostic accuracy, particularly in areas where leptospirosis is endemic and repeat exposure may occur (Blacksell et al., 2006).

The longitudinal nature of this study allows the analysis of changes in ELISA score between visits. The ELISA tested Kibera dog samples make up a total of 827 pairs of samples collected from the same individual over consecutive visits (approximate 28 day interval). Using an increase of $\geqslant 100\%$ or more (a doubling or more of ELISA score between visits) as a rough indicator of a biologically meaningful increase in ELISA score, a total of 44 sample pairs were identified, with score increases of 100-720%. A simple chi-squared analysis of these sample pairs reveals no significant departure from the expected number of pairs with large score increases (vs. smaller increase or decrease in scores between visits) over the different visits of the study (χ^2=6.8352, df=5, p=0.2332). Overall, the results of the analyses conducted and the sample of traces seen in 8.7 are consistent with the endemic circulation of *Leptospira* within the Kibera dog population, with different individuals showing increases and decreases in
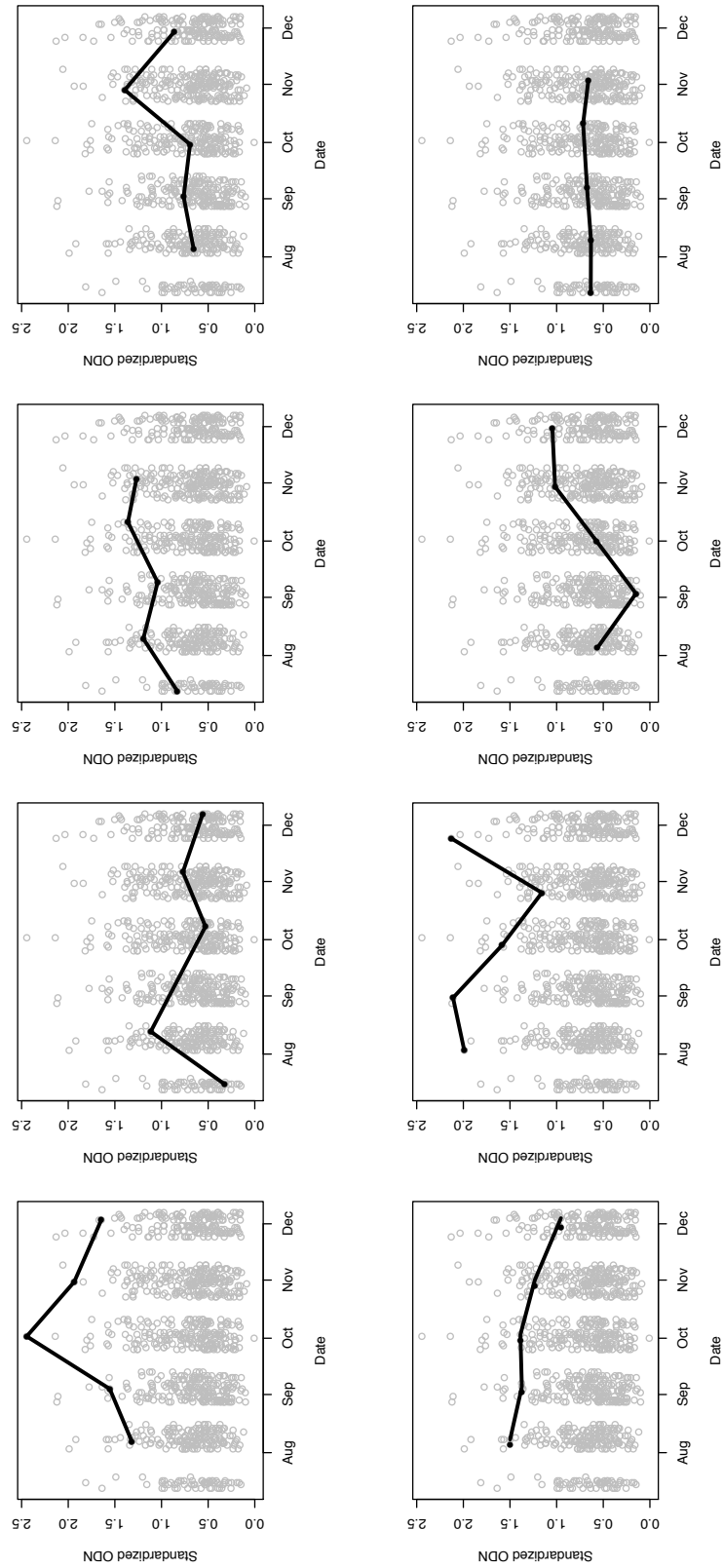
Figure 8.7: Sample of dog *Leptospira* ELISA scores over time (2007). Grey dots represent all dog ELISA scores plotted against the date of collection. In each panel, lines connect the points representing repeat samples from a single individual.

score throughout the sampling periods of this study. Some individuals with repeat samples show ELISA scores greater than the cut-off ODN value calculated using the mixture model (ODN = 0.78) for all visits in 2007, indicating that as described in other populations, positive titres may persist in the Kibera dog population for at least 5-6 months and potentially much longer. This duration of response has to be considered when interpreting the seroprevalence estimates.

8.5.2 Spatial Variation

The spatial analyses of the distribution of dogs with positive/negative ELISA scores, indicate that the response of the sentinel population to *Leptospira* is relatively homogeneous throughout the study site. This should not however, be interpreted as an indication that the sources of *Leptospira* exposure for dogs and potentially also the target human population are also homogeneously distributed. Instead it is important to consider the effective resolution of the response of the sentinel population to pathogen presence. The entire Kibera study site has an approximate area of 0.5km^2. The ranging behaviour of the Kibera dogs specifically is not known but previous studies of urban dogs indicate that the home range of individuals could include 10-50% of this total area (Pal et al., 1998; Vanak and Gompper, 2009).

For the purposes of this spatial analysis the location of individual dogs and samples was fixed at the household location and although individuals were found to have very high fidelity to the household location when sought for sampling, this point location is not necessarily an accurate representation of the spatial location(s) at which dogs may become exposed to *Leptospira*. Studies of human leptospirosis in urban slum environments have identified risk factors such as proximity to open sewers, proximity to refuse sites and household location flood-risk as strong indicators of human exposure to *Leptospira* (Reis et al., 2008; Oliviera et al., 2009). At the Kibera site, the home range of essentially all of the dogs included in the study will include open sewers and accumulations of refuse. The effective resolution of the response of an individual dog to *Leptospira* exposure is probably too coarse to identify variation in exposure risk on such a fine spatial scale. Comparisons made between different dog populations on

a spatial scale considerably greater than the scale on which dogs range may though identify variation in the risk of *Leptospira* exposure at for example the village level.

8.5.3 The Sentinel Response

The sensitivity of the sentinel response is defined in Chapter 1 as the capacity of the sentinel population to respond to the presence of the pathogen. The seroprevalence estimates calculated above indicate that the Kibera dog population as a whole responds quite clearly to the apparent presence of leptospires. However, the variance components analysis of the dog ELISA scores (Chapter 7) indicates considerable variation in scores that is attributable to dog level factors. Ideally, different members of a sentinel population would be consistent in their response to pathogen presence. The response to the presence of *Leptospira* that we detect with any serological test in this sentinel dog population will be influenced both by variation in dog exposure which is entirely unknown and also by dog level variation in the responses mounted to any exposure. The responses of exposed individuals will likely depend upon numerous factors that may include the previous exposure history of that dog to *Leptospira*, the nutritional and overall health status of the individual and possibly also on the exposure status of that individual to other pathogens. This variation in individual responses complicates their interpretation and suggests that these data are most reliably interpreted at the population rather than the individual level.

It is difficult to assess the relative sensitivity of the Kibera dog population to the presence of pathogenic *Leptospira* at this site in the absence of similar data from other populations, specifically the target human population. We do though have data from both the dog and rodent populations. In this comparison we are not using these test data to estimate the true infection prevalence in these two populations. Instead we are asking how readily we can detect the presence of the pathogen at this site by surveying the two populations and using the diagnostic tests that we have. We have not tried to analyse the ELISA scores for the rodent and dog populations on the same scale or imposed the same cut-offs on the two sets of data because it is likely that the test performance differs considerably in the two populations (Greiner and Gardner, 2000). In fact, no cut-off was defined for the ELISA test when applied to the rodent population.

Given the small number of rodent sera that gave scores that are qualitatively above background, these data indicate that the anti-*Leptospira* seroprevalence in the rodent population was certainly less than the estimates observed in the dog population, in 2007 at least.

The kidneys of 18% of rodents sampled in September/October 2008 were PCR positive for pathogenic *Leptospira*. At the time of this survey, the dog seroprevalences indicated by the Full Model + ivermectin were 7 and 5% in the visits conducted around the time of this survey. This comparison considers two entirely different populations assessed using different tests. For the purposes of identifying evidence of *Leptospira* presence at the Kibera study site, the data from 2008 indicate that PCR testing of the rodent population may prove an equally or more sensitive technique than serological surveillance of the dog population. This comparison also illustrates the influence of response detectability and practical factors upon sentinel selection. The sampling and testing of the dog population using the ELISA test is considerably easier and cheaper than sampling and testing an equivalent number of rodents.

Most studies of leptospirosis in urban contexts have implicated rodents as the maintenance reservoirs of the *Leptospira* serovars that cause observed human disease (Ko et al., 1999; Sarkar et al., 2002; Johnson et al., 2004). However, it is known that dogs can act as a source of infection for human leptospirosis (Levett, 2001), and argued that the possible role of dogs and other potential reservoirs in the epidemiology of urban leptospirosis should not be discounted (Vinetz, 2001). Because the epidemiology of leptospirosis can vary considerably depending on the serovar responsible for disease, it is possible that the utility of animal sentinels for the surveillance of different serovars may also vary. The heat map representation and hierarchical cluster analysis of the MAT data (Figure 8.6) indicates that dogs and rodents at the Kibera site show differences in the serovars against which antibodies are produced. Interestingly, this analysis indicates that dogs are exposed to the serovars present in the rodent population but this is not true in reverse. These inferences are based on complex MAT data from a small number of individuals but would be consistent with the maintenance of two parallel populations of *Leptospira* in the dog and rodent populations and with the exposure of the dog population to both *Leptospira* populations. If this is the case, it suggests that

dog serological responses can provide an indication of exposure to both dog and rodent maintained serovars, presumably because of the exposure of dogs through both environmental sources of infection and potentially also through the consumption of infected reservoir hosts (Millán et al., 2009).

8.5.4 Conclusion

In recent years the epidemiology of leptospirosis has changed dramatically. Human disease in traditionally associated with rural areas and occupational exposures (Levett, 2001). As the global human population has moved into urban areas, leptospirosis has moved too and is now a considerable urban problem (McBride et al., 2005). An expert in the field has written that "it seems likely that leptospirosis is endemic in other urban environments where surveillance in not being carried out, and where rat and stray dog populations are prominent "(Vinetz, 2001).

This description is true of Kibera where the human disease burden attributable to leptospirosis is unknown. The data presented in the previous two chapters revealed that pathogenic *Leptospira* are present in the Kibera animal populations. In this chapter the degree to which dog serosurveillance data could be used to address more specific questions was addressed and several limitations have been identified. Assessing the evidence of change in seroprevalence over time is complicated by lack of data on the performance of this diagnostic tests used and of the duration and variability of the sentinel response to exposure. There is no indication of spatial variation in dog exposure within the Kibera study site but this is unsurprising given the mismatch in the spatial resolution of the question that we are addressing and of the response that we can observe in the dog population. The MAT data analysis is not conclusive but does indicate that serosurveillance of the dog population may detect the circulation of both rodent and dog maintained serovars, both of which can pose a zoonotic threat to people.

The ultimate aim of this surveillance has been to assess human risk. At the Kibera site we had no prior data on the presence of pathogenic *Leptospira*. Through the surveillance of the dog and rodent populations we have gathered evidence that this pathogen is present at the site. When we try to address more specific surveillance

questions, the interpretation of the data we have is complicated in some respects by its 'noisiness' but it does provide a clear indication of the circulation of pathogenic leptospires at this site that we didn't have before.

The utility of these data is evident as they have already been used to prompt additional surveillance of the human population at the Kibera site for leptospirosis as part of the ongoing KEMRI/CDC SSS. In many ways, this is exactly the kind of context in which animal sentinel surveillance makes most sense. The data collected indicate that the risk to human health could be considerable and can be used to prompt direct surveillance of the human population. This baseline data collection is what dog sentinel serosurveillance is most suitable for and in the first instance, we don't really need more specific data than this surveillance can provide. Additional surveillance would be required to determine which serovars may cause disease in the human population and to identify the reservoir host and this knowledge is of course relevant to understanding the epidemiology. However, in the Kibera environment, the greatest contributions to the reduction of human morbidity caused by leptospirosis would initially be achieved by raising awareness in the human population of the disease and its transmission routes and by improving the sanitation infrastructure at the Kibera site. The data generated through this sentinel surveillance do not answer all of the questions but do provide the data needed to prompt these measures.

Chapter 9

Discussion

Over the past decade, a number of international zoonotic disease transmission events have drawn attention to the limitations of existing global disease surveillance capacity, particularly in the developing world. Considerable knowledge gaps concerning what pathogens are present where and what the 'normal' disease burdens imposed by different pathogens are, have been identified. In response, there has been a call for novel and interdisciplinary surveillance approaches that can help to address these capacity and knowledge deficiencies.

Successful surveillance of zoonoses must include both human and animal populations. It is argued that the separation of human and animal health disciplines is perpetuated by an 'us vs. them paradigm' in which animals are viewed as a potential threat rather than a key to the understanding of probably shared risks (Rabinowitz et al., 2008). Zoonotic pathogens don't recognize a distinction between humans as opposed to other animals and researchers and disciplines hoping to understand and ultimately control these pathogens need to conduct surveillance and research that incorporates both the target human population and the other species in which the pathogen circulates and is maintained. Greater integration between human and animal surveillance and disciplines can also enhance the cost-effectiveness of limited surveillance resources (Shears, 2000a; Zinsstag et al., 2005).

Novel and interdisciplinary surveillance approaches do not necessarily need to be technologically complex (Butler, 2006b). The continuing relevance and importance of traditional surveillance techniques such as serosurveys has been demonstrated recently by the surveillance of pandemic H1N1 influenza A in the UK human population. Clinical surveillance provided a good indication of "the overall temporal and geographical unfolding of the pandemic" but these techniques detect only the most clinically severe cases (HPA, 2010). Serosurveillance has demonstrated widespread asymptomatic infection, with approximately 15% of children in England apparently infected during the first wave (HPA, 2010). This knowledge, that could only have been obtained through serosurveillance, updates the understanding of risk associated with infection with this virus and has considerable implications for the control measures taken.

In the developing world particularly, the surveillance priority is for the development of flexible approaches and techniques that can be applied to generate comprehensive baseline data on a local scale (Shears, 2000a). Simple and cheap techniques are most likely to be applied comprehensively and domestic dog populations have a number of attributes that suggest that they may be used as useful indicators of the pathogens that are present within the local human community. In this study, the capacity of domestic dog serosurveillance specifically to provide a flexible and yet comprehensive and cost-effective tool for zoonotic diseases surveillance has been explored.

For the fundamental surveillance question of "is this pathogen present?", the dog serosurveillance described in this thesis has certainly yielded useful information. At the Maga dam site in Cameroon, there was evidence of exposure to H5N1 subtype viruses in the dog population at a site at which viruses of this subtype had not previously been identified and the data obtained form the dog population were in agreement with the results of surveillance of the duck population at the same site. In contrast, there was no indication of the circulation of zoonotic influenza A viruses in the Kibera dog population, a finding that was again in agreement with the findings of parallel surveillance of the domestic bird and human populations at this site. At the Kibera site there was also evidence of extensive exposure to *Leptospira* in the dog population, consistent with evidence of pathogenic *Leptospira* maintenance in the rodent population. In these

cases, the serosurveillance of the dog populations has yielded data on pathogen presence that is consistent with that obtained through other more conventional surveillance techniques and can be used to inform understanding of human risk.

This study has also demonstrated that domestic dog sentinel surveillance is practically feasible. At both the Maga dam and Kibera study sites, the domestic dog population was readily accessible for sampling. At the Kibera site, there were no significant problems with the recruitment of households or sampling of dogs and the overall sampling success rate was very high. Many other studies have reported that high levels of coverage of domestic dog populations can be achieved through vaccination campaigns conducted around the world. Dogs that can be handled for vaccination can also be blood sampled, and in combination with the previous studies looking at vaccination coverage, this research suggests that domestic dog populations are broadly accessible for sampling and therefore for use as sentinels for disease surveillance.

We do however, have to recognize some of the limitations of this surveillance approach. This thesis was conceived as an evaluation of the use of animal sentinels and in practice it consists largely of the presentation and discussion of diagnostic test data. Rather than the tests themselves, the key limitation at present is the lack of knowledge that we have about the performance of the tests that we currently have. The dog serosurveillance approach will invariably involve the adaptation of existing tests for application to a population for which they were not designed, and this can often lead to considerable changes in test performance (Greiner and Gardner, 2000). We also don't have basic data that can help us interpret the results obtained. How long do the antibody responses that we can detect with these tests persist? and consequently what time-frame does a positive result refer to? Do the influenza A tests perform equally well for all subtypes? Can even the *Leptospira* MAT test tell us much about likely infecting serovars? Are there locally representative *Leptospira* strains for all of the regions in which surveillance would be useful?

The difficulties of properly assessing and interpreting diagnostic test performance are not unique to tests conducted as part of sentinel surveillance. However, the nature of sentinel surveillance is such that the observed sentinel population is used as a proxy, from which information about the target population is inferred. This approach and

the collection of data at a step removed from the target population, will inevitably introduce noise and uncertainty around the true picture of pathogen presence in the target population. Additional noise and uncertainty introduced through application of any diagnostic test will likely amplify this problem.

In Chapter 8 we saw that as soon as we ask even slightly more complicated surveillance questions than "Is there evidence of pathogen presence ?", the prior knowledge required and the accuracy of the data that we need to obtain grows considerably. Dog serosurveillance is not very well suited to looking at subtle changes or teasing out the complex details of disease transmission networks. However, these are unlikely to be the situations in which sentinel surveillance makes most sense anyway. The fundamental aim of sentinel surveillance is to enhance the detection of disease and/or improve the cost-effectiveness of surveillance (McCluskey, 2003). Given that the sentinel population is not the ultimate target of surveillance it is questionable whether or not the research investment required to address complex epidemiological questions through sentinel surveillance would not be better targeted to direct surveillance of the target population. When using a different species as the sentinel, the degree of removal of the observed sentinel population from the target population and imperfect diagnostic test performance means that sentinel surveillance will always provide a relatively 'noisy' or 'blurry' picture of the underlying situation in the target population. As a consequence it is best suited to describing broad-scale patterns in pathogen distribution.

The data gathered through this research indicates that when *Leptospira* and influenza A are circulating within a animal community, you can see this through surveillance of the domestic dog population. Generating this kind of data is useful only if it is acted upon. Dog serosurveillance can be used to provide baseline data that can then be used to target resources for education and more comprehensive surveillance. Animal sentinel surveillance in this context could be used in the first step of multi-stage surveillance processes to collect baseline data. Following an indication of pathogen presence obtained through sentinel surveillance it is then important to look at the target population and assess how important the pathogen actually is. The data on *Leptospira* in Kibera, illustrate this issue. Surveillance for leptospirosis at this site has started with this animal serosurveillance and the data obtained are very helpful for identifying the

potential risk to the human population. From the dog and rodent surveillance we have a picture of the circulation of pathogenic leptospires in Kibera. However, we cannot tell from the animal surveillance which if any of the pathogenic leptospires identified may actually cause illness in the human population. The next step (which is already underway) will be to conduct a survey of the human population and to characterize any strains that causes illness in the human population. Finally, it would be important to then go back to the animal populations to look for this human pathogen specifically and address questions about its epidemiology that could inform pathogen control efforts.

This kind of multistage surveillance approach requires significant resource investment and in practice, the most useful application of data obtained from animal sentinel surveillance may be the targeting of public health education resources. At the Kibera site for example, between 11% and 21% of the human population enrolled in the SSS study reported symptoms consistent with the syndromic surveillance case definition for fever in each of the six months at the end of 2007. Studies conducted in South America have determined that significant proportions of patients presenting with fever have signs of acute leptospirosis (Bharti et al., 2003) and in an urban slum context, over a quarter of the human population was seropositive for leptospirosis (Johnson et al., 2004). At the Kibera site, the SSS study provides good health care facilities for this population. Education programmes to inform Kibera residents of the symptoms of leptospirosis and of its routes of transmission could make residents more aware that their fever symptoms may be due to leptospirosis and to present for diagnosis, facilitating a more accurate assessment of the burden of leptospirosis at this site. The data gathered here could then be used to inform surveillance and intervention measures that could also be applied in other populations.

Animal sentinel surveillance is probably not a sufficiently precise surveillance tool to address subtle epidemiological questions. However, in many places the really basic questions about what pathogens are where haven't been addressed. Sentinel surveillance using dogs particularly may provide a very cheap and efficient mechanism for generating these broad-scale pictures of what pathogens pose potential problems for the human population where and the data obtained can serve as a useful trigger for further investigation in the target population.

Appendix A

Consent Documents

Dog Sampling Consent Form

Bird Sampling Consent Form

Questionnaire Survey Consent Form

Rodent Sampling Consent Form

Dog Sampling Consent Form

We are visiting your household as part of a long-term research project to investigate the links between human and animal health. This project is administered by the Kenya Medical Research Institute (KEMRI) and the Centers for Disease Control and Prevention (CDC), Nairobi together with the University of Edinburgh, UK, Kenya and the University of Nairobi. The goal of this project is to look at the diseases that can be transmitted from animals to humans and to design new ways of carrying out surveillance and control of infectious diseases in this part of Africa.

What we would like to do:
If you agree for your house to be part of this project we would like to re-visit your household once a month to repeat the collection of samples. The project will continue for up to 1 year. We would like to visit your household to collect some samples of blood, stool and material from the nostrils and throat from any dogs that are owned by this household as well as ticks from your dog. Only a small amount of blood (roughly 1 tablespoon) will be taken from each dog using sterile equipment. We will take a photo of your dog and provide a free collar so that we can identify it and we would also like to ask some questions about the characteristics of your dogs and how you keep them. The samples that we collect will be stored and tested for diseases that the dogs may currently have or have had in the past which could also affect human health.

We would like to ask your permission to link the results of the tests on the animal samples with the facts that are collected about the members of your household every two weeks in the CDC/KEMRI IEIP project, and with any results of tests done on samples that may have been collected from you or your family at the KEMRI/CDC clinics. This will be used to see whether things about your household are related to your chances of getting ill with these diseases.

As before, the facts about you and your family from this study will be kept private as much as allowed by law. No names will be used on any of the study reports.

Benefit from being in this study:
People who agree to be in this study will get free advice on management and veterinary care of their animals, including for those illnesses which are diagnosed in this study. Any information obtained from these tests that might be important for your family's health, or for your animals' health and welfare, will be communicated to you through project staff. Identifying what diseases your animals have will help improve their health and welfare, and that of your family

As part of the project, we are offering a rabies vaccination for all dogs and cats at the household. If you would like your dogs/cats to be vaccinated, this vaccine will be provided free of charge but this is your choice. Vaccination will help reduce the number of cases of animal rabies in the community and the number of people that are bitten by animals with rabies. The vaccine is well tested and has been safely used on thousands of dogs in East Africa. A clean needle will be used to vaccinate each animal. You will be provided with a vaccination certificate free-of-charge to demonstrate the vaccination status of your dog/cat. When we visit your household for follow-up visits we will provide a free treatment for your dogs for worms and mange (a skin disease of dogs). This will help to improve the health and condition of your dog.

1/2

Risks from being in the study:
Handling and restraining animals for sample collection can be slightly stressful for the dogs and for people from the household who are participating. Every care will be taken to minimize this stress. Drawing blood, taking swabs and removing ticks can cause brief pain to the dogs, and may result in brief bleeding. Having someone visit your house every month may be a bother to your family. Sampling the dogs may take some time, as will answering the questions about the dogs. We may ask you or some of your family to assist us in handling and restraining the dogs. This may expose you or your family members to risk of injury from the dogs: all measures will be taken to ensure that dogs are handled properly for their own safety and for the safety of people assisting.

There is a small risk that a dog may have a bad reaction to the treatment that we will offer for worms and mange. In some cases, a dog may have an allergic reaction to the treatment. There is a small risk that a dog that has this reaction may die. This treatment is very common and it is very rare that this type of reaction occurs. We will not be able to provide any compensation if a dog dies as a result of the treatment provided during this study. If you would prefer for us not to provide this treatment that is not a problem.

If you do not want to take part in this project, this decision will have no negative effects upon you or your family. We will provide the rabies vaccine for your dogs and cats even if you do not want to take part in the rest of the project. You can also take part in the study if you do not want us to vaccinate your animals. Your participation in this project is voluntary and you may withdraw at any time. If you have any questions or concerns about the study and your rights as a participant, or if you feel that you or your animal has been harmed as a result of this study please contact one of the project representatives: Jo Halliday (##### #####) or Project Vet (as appropriate at time of administration).

The consent form has been explained to me and I agree for my family and animals to take part in the study. I have been told that I am free to choose not to take part in this study at any time and that saying "NO" will have no effect on my family or me.

Head of family	Name:	Signature:	date ☐☐/☐/☐☐
Witness*	Name:	Signature:	date ☐☐/☐/☐☐

* Subject may sign or provide verbal consent in the presence of a witness. The witness (by his/her signature) verifies that the consent form has been accurately translated to the subject and this is the subject's signature or that he/she has provided verbal consent.

BIRD SAMPLING CLIENT CONSENT FORM

We are visiting your household as part of a scientific project to investigate the links between human and animal health. This project is administered by the University of Edinburgh, UK together with the University of Nairobi and Kenyan Medical Research Institute (KEMRI). The goal of this project is to look at the diseases that can be transmitted from animals to humans and to design new ways of carrying out surveillance and control of infectious diseases in this part of Africa.

As part of the project, we would like to visit your household to collect some samples of blood, stool and material from the nostrils and throat from any domestic birds (including chickens, ducks, turkeys and geese) that are owned by this household. Only a small amount of blood (roughly 1 tablespoon) will be taken from each bird using sterile equipment and this will not affect the animal's health.

The samples that we collect will be stored and tested for diseases that the birds may currently have or have had in the past which could also affect human health. For any positive test results, advice will be provided through the local veterinary officer as to any preventive measures that you should take, if any, to reduce potential health risks that these animal diseases may pose to you or your family.

If you do not want to take part in this project, this decision will have no negative effects upon you or your family. Your participation in this project is voluntary and you may withdraw at any time. If you have any questions or concerns, please contact your local veterinary office. You can also contact the project representatives, Jo Halliday (##### #####).

The consent form has been explained to me and I agree for this household to take part in the project. I understand that I am free to chose not to take part in this project at any time and that saying 'NO' will have no effect on me or my family.

Client Name (Head of Household or Proxy)   _____

Client signature _____   Date_____

If client is unable to sign, complete below section.

I confirm that the client has given informed consent and that I have been present as witness during the whole consent process.

Name of witness (not involved in project)   _____

Signature of witness   _____

Sampling team representative   _____

Questionnaire consent form

We are visiting your household as part of a research project to investigate the links between human and animal health. This project is administered by the Kenya Medical Research Institute (KEMRI) and the Centers for Disease Control and Prevention (CDC), Nairobi together with the University of Edinburgh, UK, Kenya and the University of Nairobi. The goal of this project is to look at the diseases that can be transmitted from animals to humans and to design new ways of carrying out surveillance and control of infectious diseases in this part of Africa.

What we would like to do:
If you agree for your house to be part of this project we would like to ask you some questions about the animals that you keep and see around your household. All this will take about one hour, depending on the number of animals that you have.

We would like to ask your permission to link the data that we collect with this questionnaire with the results of the laboratory tests done on the samples that we have collected from your animals in the past, with the facts that are collected about the members of your household every two weeks in the CDC/KEMRI IEIP project, and with any results of tests done on samples that may have been collected from you or your family at the KEMRI/CDC clinics. This will be used to see whether things about your household are related to your chances of getting ill with these diseases.

As before, the facts about you and your family from this study will be kept private as much as allowed by law. No names will be used on any of the study reports.

Benefit from being in this study:
This study is being done in order to better understand the health risks that are involved in keeping animals. Any information obtained from the data that we collect that might be important for your family's health, or for your animals' health and welfare, will be communicated to you through project staff.

Risks from being in the study:
Answering the questions about your animals may take some time.

If you do not want to take part in this project, this decision will have no negative effects upon you or your family. Your participation in this project is voluntary and you may withdraw at any time. If you have any questions or concerns about the study and your rights as a participant, or if you feel that you or your animal has been harmed as a result of this study please contact one of the project representatives: Jo Halliday (#### #####) or Project Vet (as appropriate at time of administration).

> The consent form has been explained to me and I agree for my family and animals to take part in the study. I have been told that I am free to choose not to take part in this study at any time and that saying "NO" will have no effect on my family or me.

Head of family	Name:...............................	Signature:	date☐☐/☐☐/☐☐
Witness*	Name:...............................	Signature:	date☐☐/☐☐/☐☐

* Subject may sign or provide verbal consent in the presence of a witness. The witness (by his/her signature) verifies that the consent form has been accurately translated to the subject and this is the subject's signature or that he/she has provided verbal consent.

Rodent sampling consent form

We are visiting your household as part of a research project to investigate the links between human and animal health. This project is administered by the Kenya Medical Research Institute (KEMRI) and the Centers for Disease Control and Prevention (CDC), Nairobi together with the University of Edinburgh, UK, Kenya and the University of Nairobi. The goal of this project is to look at the diseases that can be transmitted from animals to humans and to design new ways of carrying out surveillance and control of infectious diseases in this part of Africa.

What we would like to do:
If you agree for your house to be part of this project we would like to visit your household to place some traps to catch rats, mice and other small mammals in and around your household. We will collect the traps and take away the trapped animals. We do not want you or any members of your household to handle any traps or small mammals as part of this project. The trapped animals will be taken away and killed humanely and will not be returned to the area. We would also like to ask you some questions about the types of contact that you and your family have with rats, mice and other small mammals in and around your household. The questions that we would like to ask will take about one hour and the trapping will be completed within 2 weeks. The samples that we collect from the trapped animals will be stored and tested for diseases that the animals may currently have or have had in the past which could also affect human health.

We would like to ask your permission to link the results of the tests on the animal samples with the facts that are collected about the members of your household every two weeks in the CDC/KEMRI IEIP project, and with any results of tests done on samples that may have been collected from you or your family at the KEMRI/CDC clinics. This will be used to see whether things about your household are related to your chances of getting ill with these diseases.

As before, the facts about you and your family from this study will be kept private as much as allowed by law. No names will be used on any of the study reports.

Benefit from being in this study:
Any information obtained from these tests that might be important for your family's health, or for your animals' health and welfare, will be communicated to you through project staff. Identifying what diseases are present in animals in this area can help to improve the health and welfare of your family

Risks from being in the study:
Having someone visit your house for two weeks and placing the traps in and around your house may be a bother to your family. Collecting and removing the animals may take some time, as will answering the questions about the animals.

If you do not want to take part in this project, this decision will have no negative effects upon you or your family. Your participation in this project is voluntary and you may withdraw at any time. If you have any questions or concerns about the study and your rights as a participant, or if you feel that you or your animal has been harmed as a result of this study please contact one of the project representatives: Jo Halliday (#### ####) or Project Vet (as appropriate at time of administration).

> The consent form has been explained to me and I agree for my family and animals to take part in the study. I have been told that I am free to choose not to take part in this study at any time and that saying "NO" will have no effect on my family or me.

Head of family	Name:	Signature:	date☐☐/☐☐/☐☐
Witness*	Name:	Signature:	date☐☐/☐☐/☐☐

* Subject may sign or provide verbal consent in the presence of a witness. The witness (by his/her signature) verifies that the consent form has been accurately translated to the subject and this is the subject's signature or that he/she has provided verbal consent.

Appendix B

Data Collection Sheets & Questionnaires

Dog sampling data collection sheet

Bird sampling data collection sheet

Household questionnaires:

- Dog owning households

- Bird owning households

- Rodent sampled households

Kibera Sentinel Project
Dog Sampling Visits

Household Level Data

1: Household ID Code: __ __ / __ __ __ / __ __

2: Visit Date: _____ **3**: Interviewer: _____

4: Visit Number: _____ **5**: Village: _____

6: GPS South: _____ **7**: GPS East: _____

8: Total number of dogs and puppies at household **a**: Dogs (3mo and older) _____
b: Puppies (less than 3mo) _____

Dog Register Data

Dog A

A1: Dog ID: _____ **A2**: Dog Name _____ **A3**: Dog Age _____

A4: Dog Sex _____ **A5**: Dog Colour _____

A6: Dog Features _____

A7: Conf Day ☐ **A8**: Conf Night ☐ **A9**: Collar Fitted ☐ **A10**: Collar Present ☐ ☐**A11**: Photo ☐

A12: Vacc ☐ **A13**: Ivermectin ☐ **A14**: Canex ☐ **A15**: Ticks Present ☐

A16: Blood ☐ **A17**: Nasal Swab ☐ **A18**: Faecal Swab ☐ **A19**: Ticks Collected ☐

Dog B

B1: Dog ID: _____ **B2**: Dog Name _____ **B3**: Dog Age _____

B4: Dog Sex _____ **B5**: Dog Colour _____

B6: Dog Features _____

B7: Conf Day ☐ **B8**: Conf Night ☐ **B9**: Collar Fitted ☐ **B10**: Collar Present ☐ ☐**B11**: Photo ☐

B12: Vacc ☐ **B13**: Ivermectin ☐ **B14**: Canex ☐ **B15**: Ticks Present ☐

B16: Blood ☐ **B17**: Nasal Swab ☐ **B18**: Faecal Swab ☐ **B19**: Ticks Collected ☐

Dog C

C1: Dog ID: _____ **C2**: Dog Name _____ **C3**: Dog Age _____

C4: Dog Sex _____ **C5**: Dog Colour _____

C6: Dog Features _____

C7: Conf Day ☐ **C8**: Conf Night ☐ **C9**: Collar Fitted ☐ **C10**: Collar Present ☐ ☐**C11**: Photo ☐

C12: Vacc ☐ **C13**: Ivermectin ☐ **C14**: Canex ☐ **C15**: Ticks Present ☐

C16: Blood ☐ **C17**: Nasal Swab ☐ **C18**: Faecal Swab ☐ **C19**: Ticks Collected ☐

Sentinel Project Bird Sampling Sheet

<table>
<tr><td colspan="2">Kibera Sentinel Project
Bird Sampling Visits</td></tr>
</table>

1: Household ID Code: __ __ / __ __ __ / __ __

2: Visit Date: _____ **3**: Interviewer: _____

4: Visit Number: _____ **5**: Village: _____

6: GPS South: _____ **7**: GPS East: _____

8: How many of the following birds belong to this household?
Please ask for each species in turn and record 0 if no animals of a given age and species are present.

	Chickens	Ducks	Turkeys	Geese	Other
Adults *(birds with adult feathers)*					
Young *(birds with juvenile feathers)*					

If other what species? _____

<table>
<tr><td>Bird Sample Collection Record</td></tr>
</table>

	Species	Bird ID (ABC/AB/123)	Origin (see code)	Treatment given?	Bl	Tr	Cl
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
14							
15							

(Samples columns: Bl, Tr, Cl)

Codes for source of individuals:
1   Chick of own bird/ born at household
2   Bought within Kibera   (Give name of Market if from market)
3   Bought outside Kibera   (Give name of Market if from market)
4   Gift from within Kibera
5   Gift from outside Kibera
6   Adopted off the street
7   Other (specify)
8   Unknown

1/1

Q1 – Dog-Owning Household Data

Gatwikira/Soweto Sentinel Project
Dog Owning Household Questionnaire

Household Level Data

#: Household ID Code: __ __ / __ __ __ / __ __

#: Interview Date: _____   **#**: Interviewer: _____

#: GPS South: _____   **#**: GPS East: _____

#: Head of Household Name: _____

#: Respondent Name: _____
Please write "As above" if respondent is the head of household

#: How many of the following animals are present at this household today?
Please ask for each species in turn and record 0 if no animals of a given age and species are present.

	Sheep	**Goats**	**Chickens**	**Ducks**	**Dogs**	**Cats**	**Other**
Adults *(birds with adult feathers + others older than 3mo)*							
Young *(birds with juvenile feathers + others 3mo and younger)*							

If other what species?   _____

#: Have any of the animals (<u>apart from dogs</u>) that belong to this household been sick in the past 1 year? This includes animals that were sick and then recovered as well as animals that were sick and died.

No Other Animals   ☐

Yes   ☐   No   ☐   Don't Know   ☐

If Yes complete table below. If No Other Animals, No or DK go to question #.

#: Species	**#**: Number Died	**#**: Number Sick	**#**: Clinical Signs/Details	**#**: Sickness When?
1=Sheep *2=Goats* *3=Dogs* *4=Cats* *5=Chickens* *6=Ducks* *7=Turkeys* *8=Geese* *9=Other (Specify)*				*Please record weeks or months since event*

1

Q1 – Dog-Owning Household Data

#: Have any new animals (<u>apart from dogs</u>) been born at the household or bought into the household in the past 1 year?

Yes ☐   No ☐   Don't Know ☐

If Yes complete table below. If No or DK go to question #.

#: Species	**#:** Number Born/ Brought In	**#:** Source	**#:** Born/ Brought In When?
1=Sheep *2=Goats* *3=Dogs* *4=Cats* *5=Chickens* *6=Ducks* *7=Turkeys* *8=Geese* *9=Other (Specify)*		*1=Bought inside Kibera* *2=Bought outside Kibera* *3=Gift inside Kibera* *4=Gift outside Kibera* *5=Adopted off the street* *6=Market (specify name)* *7=Born at this Household* *8=Other (specify)*	*Record number of weeks since born or acquired*

Dog Ownership Data

#: What is the primary reason why the household owns a dog/dogs?

Please ask the respondent the question above to obtain the unprompted primary reasons for ownership. Select only one of the following options & mark that option under the 'Primary' column. Please then ask specifically about the other ownership reasons and mark these under the 'Secondary' column:

	Primary:	Secondary:
To guard the household against human intruders	☐	☐
To protect livestock against predators	☐	☐
To chase pests away from crops	☐	☐
To dispose of household waste	☐	☐
For hunting purposes	☐	☐
To provide companionship	☐	☐

Other reasons _____

#: Which person/people in the household is/are responsible for looking after the dog/dogs (for example, feeding them)?

Please record the name, age and gender of the person or people as well as their relationship to the head of household e.g. head of household, wife, son/daughter. If no-one takes responsibility for the dog/dogs write 'no-one'.

#: Name	**#:** Age Class	**#:** Sex	**#:** Relationship to Head of Household
	1=0-5 years *2=6-19 years* *3=20-49 years* *4=50 years and older*	*M= Male* *F= Female* *DK= Don't Know*	

Q1 – Dog-Owning Household Data

#: Over the past 1 year, did any dogs that the household owned die or leave the household?

Yes ☐ No ☐ Don't Know ☐

If Yes complete table below. If No or DK go to question #.

For adults please record one record for each individual. For puppies with no individual names, please record one record for each sex and record'Puppies' and the number on the 'Name' column.

#: Name	#: Sex	#: Fate		#: Age at Event	#: When?
	M= Male *F= Female* *DK= Don't Know*	*1= Sold* *2= Given Away* *3= Killed by owner* *4= Killed by authorities* *5= Killed by someone else* *6= Died in accident*	*7= Died of disease/parasite* *8= Died through starvation* *9= Died other* *10= Disappeared* *11= Abandoned* *12= Stolen* *13=Unknown*	*Age in years (or months if <12mo old)*	*Please record weeks or months since event*

Gatwikira/Soweto Sentinel Project **Bird Owning Household Questionnaire**

Household Level Data

#: Household ID Code: __ __ / __ __ __ / __ __

#: Interview Date: _____   **#**: Interviewer: _____

#: GPS South: _____   **#**: GPS East: _____

#: Head of Household Name: _____

#: Respondent Name: _____
Please write "As above" if respondent is the head of household

#: How many of the following animals belong to this household?
Please ask for each species in turn and record 0 if no animals of a given age and species are present.

	Sheep	**Goats**	**Dogs**	**Cats**	**Other**
Adults *(older than 3mo)*					
Young *(3mo and younger)*					

If other what species?   _____

#: How many of the following birds are present at this household today?
Please ask for each species in turn and record 0 if no birds of a given age and species are present

	Ducks	**Chickens**	**Turkeys**	**Geese**
Adults *(birds with adult feathers)*				
Young *(birds with juvenile feathers)*				

If any birds of these species are present, complete questions # to # below. If no birds of these species are present go to the end of the questionnaire.

Q4 – Bird Sampling

#: Have any of the animals that belong to this household been sick in the past 6 months? This includes animals that were sick and then recovered as well as animals that were sick and died.

Yes ☐   No ☐   Don't Know ☐

If Yes complete table below. If No or DK go to question #.

#: Species	**#:** Number Died	**#:** Number Sick	**#:** Clinical Signs/Details	**#:** Sickness When?
1=Sheep *2=Goats* *3=Dogs* *4=Cats* *5=Chickens* *6=Ducks* *7=Turkeys* *8=Geese* *9=Other (Specify)*				*Please record weeks or months since event*

Q4 – Bird Sampling

Bird Data

#: How are your birds restrained?

Tick one box for day and one for night

	During the day? (during daytime hours – daylight)	During the night? (during night time hours – darkness)
In a coup		
Restricted to household/compound (within walls, secure fence)		
Free, but stay at home/close		
Free, roaming away from home		
Free, unknown		

#: How often do you feed your birds?

Never	☐	Once/a/few times a week	☐
One/more times a day	☐	Once/a few times a month	☐
Other	☐	_____	

#: What do you feed your birds?

Commercial Feed	☐	Home-made	☐
Leftovers	☐	None	☐
Other	☐	_____	

#: Have your birds been vaccinated against any diseases?

Yes ☐   No ☐   Don't Know ☐

If Yes, what disease or diseases?
_____

#: Which person/people in the household is/are responsible for looking after the birds (for example, feeding them)?

Please record the name, age and gender of the person or people as well as their relationship to the head of household e.g. head of household, wife, son/daughter. If no-one takes responsibility for the birds write 'no-one'.

#: Name	**#**: Age Class	**#**: Sex	**#**: Relationship to Head of Household
	1=0-5 years *2=6-19 years* *3=20-49 years* *4=50 years and older*	*M= Male* *F= Female* *DK= Don't Know*	

Q4 – Bird Sampling

#: Are your birds slaughtered here at the house?

Yes ☐ No ☐ Don't Know ☐

If Yes, go to questions # & #. If No, where are the birds slaughtered?
_____

#:.Which person/people in the household slaughter your birds?
Please record the name, age and gender of the person or people as well as their relationship to the head of household e.g. head of household, wife, son/daughter.

#: Name	**#**: Age Class	**#**: Sex	**#**: Relationship to Head of Household
	1=0-5 years *2=6-19 years* *3=20-49 years* *4=50 years and older*	*M= Male* *F= Female* *DK= Don't Know*	

#: Where do you dispose of the bird carcass and entrails when you slaughter birds at the house?

_____

_____

Thank you for your time and cooperation. Do you have any questions or comments?
Record any questions or additional information provided by the respondent.

Q5 – Rodent Sampling

Gatwikira/Soweto Sentinel Project
Rodent Questionnaire

Household Level Data

#: Household ID Code: __ __ / __ __ __ / __ __

#: Interview Date: _____ **#**: Interviewer: _____

#: GPS South: _____ **#**: GPS East: _____

#: Head of Household Name: _____

#: Respondent Name: _____
Please write "As above" if respondent is the head of household

#: How many of the following animals belong to this household?
Please ask for each species in turn and record 0 if no animals of a given age and species are present.

	Sheep	**Goats**	**Chickens**	**Ducks**	**Dogs**	**Cats**	**Other**
Adults *(birds with adult feathers + others older than 3mo)*							
Young *(birds with juvenile feathers + others 3mo and younger)*							

If other what species? _____

Rodent Data

#: Do you see rats and/or mice in your village?
Yes ☐   No ☐   Don't Know ☐
If Yes complete questions # to # below. If No or DK go to question end of questionnaire.

Where and how frequently do you see rodents and fresh rodent excreta in your village (including sightings during both the day and night)?
Please tick the most appropriate frequency box for each location and type of sighting .

	Fewer than 5 rodents at a time				Groups of 5 or more rodents				Fresh rodent excreta (faeces/urine)			
	Once/a few times a day (24hrs)	Once/a few times a week	Once/a few times a month	Never	Once/a few times a day (24hrs)	Once/a few times a week	Once/a few times a month	Never	Once/a few times a day (24hrs)	Once/a few times a week	Once/a few times a month	Never
#: In the house												
#: Around the house (compound and alleys)												
Other areas (specify)												

Q5 – Rodent Sampling

#: Do rodents ever attack or bite the following items in your house:

Food:	Yes	☐	No	☐	DK	☐
Clothes:	Yes	☐	No	☐	DK	☐
Kitchen Utensils:	Yes	☐	No	☐	DK	☐
Bedding:	Yes	☐	No	☐	DK	☐

If Yes regarding Food go to question # Otherwise go to question #

#: What do you do with food that has been attacked or bitten by rodents?

Discard completely:	Yes	☐	No	☐	DK	☐
Discard affected portion:	Yes	☐	No	☐	DK	☐
Consume as normal:	Yes	☐	No	☐	DK	☐
Other :	Yes	☐	No	☐	DK	☐

If other please specify? _____
_____

#: Do you ever see rodent excrement (faeces or urine) on the following items in your house:

Food:	Yes	☐	No	☐	DK	☐
Clothes:	Yes	☐	No	☐	DK	☐
Kitchen Utensils:	Yes	☐	No	☐	DK	☐
Bedding:	Yes	☐	No	☐	DK	☐
Floor:	Yes	☐	No	☐	DK	☐

#: Has any member of your household ever been bitten by a rodent?
Yes ☐   No ☐   Don't Know ☐
If Yes go to question #. If No or DK go to question #.

#: Has any member of your household been bitten by a rodent within the past 6 months whilst present at this household?
Yes ☐   No ☐   Don't Know ☐
If Yes go to question #. If No or DK go to question #.

#: Which person/people within the household have been bitten by a rodent within the past 6 months whilst present at this household?

Please record the name, age and gender of the person or people as well as their relationship to the head of household e.g. head of household, wife, son/daughter. If no-one has been bitten in the past 6 months write 'no-one'

#: Name	#: Age Class	#: Sex	#: Relationship to Head of Household
	1=0-5 years 2=6-19 years 3=20-49 years 4=50 years and older	M= Male F= Female DK= Don't Know	

#: Do any members of this household carry out rodent control?
Yes ☐   No ☐   Don't Know ☐
If Yes go to Question #. If No or DK go to question #.

#: What type of rodent control do you use?
The respondent may indicate Yes for any or all of these options.

#	Mechanical (e.g. traps)	Yes	☐	No	☐	DK	☐
#	Chemical (e.g. poisons)	Yes	☐	No	☐	DK	☐
#	Biological (e.g. keeping predators)	Yes	☐	No	☐	DK	☐
#	Other	Yes	☐	No	☐	DK	☐

If other plase specify _____

Q5 – Rodent Sampling

#: Do any of your close neighbours carry out rodent control?

      Yes ☐   No ☐   Don't Know ☐

If Yes go to Question #. If No or DK go to question #.

#: What type of rodent control do your neighbours use?
The respondent may indicate Yes for any or all of these options.

#	Mechanical (e.g. traps)	Yes ☐	No ☐	DK ☐
#	Chemical (e.g. poisons)	Yes ☐	No ☐	DK ☐
#	Biological (e.g. keeping predators)	Yes ☐	No ☐	DK ☐
#	Other	Yes ☐	No ☐	DK ☐

If other please specify _____

#: Please tick one box (Many, Few, None or DK) for each mon th of the year to indicate the numbers of rodents that you see in each month.

Tick DK across table if unknown

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Many												
Few												
None												
DK												

Thank you for your time and cooperation. Do you have any questions or comments?
Record any questions or additional information provided by the respondent.

Appendix C

Halliday et al. 2007 - Publication of Introduction

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Halliday, J.E.B., Meredith, A.L., Knobel, D.K., Shaw, D.J., Bronsvoort, B.M. de C. & Cleaveland, S. (2007). A framework for evaluating animals as sentinels for infectious disease surveillance. *Journal of the Royal Society Interface* 4:973-984.

A framework for evaluating animals as sentinels for infectious disease surveillance

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The dynamics of infectious diseases are highly variable. Host ranges, host responses to pathogens and the relationships between hosts are heterogeneous. Here, we argue that the use of animal sentinels has the potential to use this variation and enable the exploitation of a wide range of pathogen hosts for surveillance purposes. Animal sentinels may be used to address many surveillance questions, but they may currently be underused as a surveillance tool and there is a need for improved interdisciplinary collaboration and communication in order to fully explore the potential of animal sentinels. In different contexts, different animal hosts will themselves vary in their capacity to provide useful information. We describe a conceptual framework within which the characteristics of different host populations and their potential value as sentinels can be evaluated in a broad range of settings.

Keywords: sentinel; surveillance; infectious diseases; epidemiology

1. INTRODUCTION

The dynamics of infectious disease systems are inherently variable. The outcome of any infection depends on multiple factors relating to pathogen characteristics, host susceptibility, infecting dose and routes of transmission, all of which can vary widely for any particular infectious organism. Many of the major diseases of medical, veterinary and conservation importance (such as highly pathogenic avian influenza (HPAI), foot-and-mouth disease, bluetongue and rabies) are caused by pathogens with wide host ranges (Woolhouse & Gowtage-Sequeria 2005), which introduces further complexity.

While the complex epidemiology of multi-host pathogens presents considerable challenges for understanding infection dynamics and implementing disease control, heterogeneities in host range and infection outcome also provide opportunities for disease surveillance. In this paper, we develop a conceptual framework that can be applied to examine those characteristics of host populations that influence their potential value as sentinels for disease surveillance in different ecological and epidemiological settings.

Surveillance is defined by the World Health Organization as 'the ongoing systematic collection, collation, analysis and interpretation of data and the dissemination of information to those who need to know in order for action to be taken' (World Health Organization 2001). The aim of disease surveillance is to identify changes in the infection and/or health

status of animal and human populations and is essential to provide rigorous evidence of the absence of disease or to determine the prevalence of a pathogen when present (Salman 2003). A critical element of surveillance is that an identified response is made on the basis of the surveillance data generated to allow appropriate action to be taken. Sentinel surveillance is one form of surveillance in which activities focus on specific subpopulations to enhance detection of disease and/or improve the cost-effectiveness of surveillance (McCluskey 2003). The aim of the sentinel surveillance is to obtain timely information in a relatively inexpensive manner rather than to derive precise estimates of prevalence or incidence in the general population (Centers for Disease Control and Prevention 2002). It has long been recognized that animal populations have the potential to act as sentinels for environmental health hazards (CAMEH 1991), but, given the importance of domestic and wild animal hosts in emerging human diseases, it is clear that surveillance in animals is also critical for understanding and managing emerging disease threats (Kuiken *et al.* 2005; Woolhouse & Gowtage-Sequeria 2005; Kahn 2006). Animal sentinels almost certainly represent an important but underused surveillance tool (Rabinowitz *et al.* 2005) that may be capable of accommodating and capitalizing on the variability that exists in infectious disease processes.

Animal sentinels may potentially be used to address a range of surveillance questions including (i) detection of a pathogen in a new area, (ii) detection of changes in the prevalence or incidence of a pathogen or disease over time, (iii) determining the rates and direction of

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Table 1. Summary of applications of animal sentinels for environmental and infectious hazards.

type of sentinel	example	reference
individual animal	coal miner's canary used to detect the presence of carbon monoxide	Burrell & Seibert (1916); Schwabe (1984)
herd/population	sentinel cattle herds and chicken flocks used to monitor the distribution of arboviruses and their vectors in Australia and the USA	National Arbovirus Monitoring Program (2003–2004); Loftin *et al.* (2006)
same species	unvaccinated chickens placed within vaccinated flock to detect HPAI	Suarez (2005)
different, more susceptible species	feral pigs released into New Zealand to detect the presence of bovine TB—more susceptible than possums; coal miner's canary (as above)	Nugent *et al.* (2002)

sentinel application	example	reference
deliberately placed (experimental)	standard laboratory mice sentinel programmes using outbred mice, sacrificed and tested to detect presence of a panel of rodent pathogens in the core experimental or breeding colony	Institute of Laboratory Animal Resources (US). Committee on Infectious Diseases of Mice and Rats (1991)
	use of sentinel chickens to evaluate the effectiveness of cleaning and disinfection procedures for eradication of Newcastle disease	McCluskey *et al.* (2006)
in natural habitat (observational)	wildlife as detectors of DDT and PCB toxicity	CAMEH (1991)
	evaluation of white-tailed deer as natural sentinels for *Anaplasma phagocytophilum*, the cause of human granulocytic anaplasmosis	Dugan *et al.* (2006)
	mesothelioma in pet dogs associated with exposure of their owners to asbestos	Glickman *et al.* (1983)
sentinel unit	equine premises used to investigate presence of vesicular stomatitis in Colorado	McCluskey *et al.* (2002)

pathogen spread, (iv) testing specific hypotheses about the ecology of a pathogen, and (v) evaluating the efficacy of potential disease control interventions (McCluskey 2003). The appropriate use of animal sentinels can facilitate the early detection and identification of outbreaks that is of critical importance for the success of control and prevention efforts (Chomel 2003; Kahn 2006) and reducing the magnitude of subsequent outbreaks (Ferguson *et al.* 2005). However, the potential of animal sentinel surveillance can only be realized if the information provided from animal populations is acted upon. For example, in an Ebola outbreak in central Africa, few preventive health measures were taken despite warnings of an imminent human outbreak being provided from monitoring of Ebola deaths in primate sentinels (Rouquet *et al.* 2005).

The term 'sentinel' is widely used in both epidemiological and veterinary clinical literature and is implicitly understood but rarely defined. While all uses invoke the common concept of standing guard or keeping watch, existing definitions tend to be context-specific. The classic example of an animal sentinel is that of the coal miner's canary. In this case, an individual animal of a different species is deliberately selected and placed in a situation where it can provide evidence of increased risk to the human population on the basis of its greater sensitivity and obvious observable response to the presence of carbon monoxide. Since the mid-twentieth century, it has been recognized that animals can act as important sentinels for a wide range of environmental health hazards (CAMEH 1991). For example, domestic dogs and the tumours they develop may facilitate identification of environmental carcinogens that affect humans (Thrusfield 2005). Sentinels can vary from individual animals to herds or larger populations, from animals of the same species to different, more susceptible, more expendable or more accessible species, and from animals deliberately placed or introduced to those already existing in a particular location. The sentinel concept can also refer to a physical location, such as a farm, abattoir, veterinary practice or laboratory (the 'sentinel unit') which is selected to monitor a particular disease (table 1). Throughout this paper, we use 'animal sentinels' as an umbrella term for the topic in general and 'sentinel population' to refer to the unit of observation in a particular case.

Despite the apparent potential for animal sentinels to inform decisions about risk to both human and animal populations, animal sentinels appear underutilized, particularly in the context of infectious disease surveillance (Rabinowitz *et al.* 2005), and their value has been discussed primarily in the context of environmental health (CAMEH 1991). A basic lack of integration between disciplines, most noticeably between human and veterinary medicine and also between different branches within these fields, is likely to have contributed to this underuse of animal sentinels (Rabinowitz *et al.* 2005). There are currently no standard criteria which are applied for the evaluation of animal sentinels, limiting the ease with which data can be transferred between disciplines (Rabinowitz *et al.* 2005). The existing infectious disease literature regarding animal sentinels consists largely of descriptive studies that have generated hypotheses regarding animal sentinel use (Rabinowitz *et al.* 2005), but as yet includes few studies that were

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Box 1. West Nile virus surveillance in North America: animal sentinel case study.

West Nile virus (WNV), an arbovirus of the genus *Flaviviridae*, is maintained in a mosquito–bird–mosquito cycle primarily involving *Culex* sp. mosquitoes (Campbell *et al.* 2002). Humans and other mammal species are incidental dead-end hosts. The majority of human infections with WNV are asymptomatic or result in transient febrile illness but in a small proportion of cases, meningoencephalitis can occur (Mostashari *et al.* 2001). The geographical range of WNV has historically included Africa, Europe, Asia and Australia (Campbell *et al.* 2002). In 1999, the first North American cases of WNV were reported in New York and since then the virus has spread across the continental United States and into Canada, Latin America and the Caribbean (Hayes & Gubler 2006). The surveillance of WNV in North America has included investigation of the utility of different animal sentinels. Some of the findings of these studies are described below with reference to the sentinel framework.

Sentinel response to pathogen

A number of North American bird species including corvids, house sparrows, house finches and grackles are competent reservoirs for mosquito infection with WNV (Komar *et al.* 2003). Among these potential sentinel species, corvids and specifically American crows (*Corvus brachyrhynchos*) are particularly susceptible to infection with WNV and have a high mortality rate (McLean *et al.* 2001; Komar *et al.* 2003; Yaremych *et al.* 2004). In 2000, it was established that dead crow reports preceded both the confirmation of viral activity (through laboratory analysis) and the onset of human cases by several months (Eidson *et al.* 2001*b*). Subsequent spatial analyses using data collected in New York have identified a positive association between the risk of human disease caused by WNV and elevated local dead crow reports in the previous one to two weeks (Mostashari *et al.* 2003; Eidson *et al.* 2005; Johnson *et al.* 2006). The thorough characterization of this temporal association ensures that the observation of crow deaths can be acted upon immediately without the need for time-consuming laboratory analyses. The observation of clusters of high crow mortality can therefore be used to predict human risk early enough to implement targeted mosquito control and personal protection warnings (Mostashari *et al.* 2003; Eidson *et al.* 2005; Johnson *et al.* 2006).

Relationship between sentinel and target populations

Domestic dogs have also been evaluated as sentinels of WNV presence (Komar *et al.* 2001; Kile *et al.* 2005). This sentinel choice is informed by the particular relationship that domestic dogs have with humans, which means that they are well suited to act as indicators of the infectious disease risks that their owners are likely to encounter. North American domestic dogs consistently show higher seroprevalence of anti-WNV antibodies than humans (Komar *et al.* 2001; Kile *et al.* 2005) and one analysis revealed that outdoor dogs were nearly 19 times more likely to have seroconverted to WNV than indoor-only pet dogs (Kile *et al.* 2005). The pattern of human exposure to the arthropod vectors of WNV is likely to be more similar to that of indoor-only dogs, but within the context of broad spatial association with humans; this divergence from the human niche means that outdoor-only dogs are more sensitive sentinels of WNV presence and human risk than indoor-only dogs (Kile *et al.* 2005).

Transmission route

The role played by different mosquito species (predominantly of the genus *Culex*) in the transmission of WNV between birds and to humans is apparently quite variable (Kilpatrick *et al.* 2005; Molaei *et al.* 2006). At one study site in Maryland and Washington DC, over 90% of all *Culex* mosquitoes identified were of the species *Culex pipiens* (Kilpatrick *et al.* 2006). At this site, the rise in human WNV cases that occurs in late summer and early autumn is apparently caused by a marked shift in the feeding preferences of this vector species from birds to humans (Kilpatrick *et al.* 2006) that is associated with the dispersal of a preferred host, the America robin (*Turdus migratorius*). This temporal variation in vector feeding preferences means that the transmission of WNV to bird hosts (including corvids) occurs earlier in the season than transmission to humans and explains the capacity for bird die-offs to provide an early warning of human risk. A similar shift in feeding patterns associated with a rise in human cases is also seen in *Culex tarsalis* mosquitoes in Colorado and California (Kilpatrick *et al.* 2006).

Detectability

Although the pathogenicity of WNV to birds including crows has been demonstrated within the historical geographical range of WNV (Work *et al.* 1955), bird die-offs are not typically associated with human WNV outbreaks within this historical geographical range and the very high mortality seen in American corvid populations is apparently unusual (Eidson *et al.* 2001*a*). Clearly, this difference may limit the application of corvids as useful sentinels of WNV to contexts within the Americas. Even within North America, there is variation in the suitability of corvids to act as a sentinel for WNV activity according to the density of human populations. A study using decoy crows revealed that both detection and reporting rates were lower in rural areas compared with urban areas (Ward *et al.* 2006). Spatial analyses have also identified reduced capacity of dead crow density measures to forecast human infections in rural areas (Eidson *et al.* 2005). These effects are seen because the capacity of crows to act as useful sentinels depends upon the likelihood that bird deaths are observed and reported by people. The power of dead crow sentinel surveillance to predict human risk is greatly reduced in rural areas as a consequence of a reduced detectability of the sentinel response.

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purposefully designed to evaluate their potential. The major exception is the extensive research that has been carried out into the use of animal sentinels in the surveillance of West Nile virus (WNV) in North America (see box 1). The utility of different animal sentinel populations varies enormously according to both the ecological context and the aim of the surveillance programme, and in many cases animal sentinels may not prove a useful surveillance tool.

2. IDENTIFYING AND ASSESSING ANIMAL SENTINELS

For any population to be useful for surveillance, it must be under observation and must be capable of developing a detectable response to a particular pathogen. Sentinel populations are distinguished from other populations by having attributes that enhance detection of the disease or of the etiological agent and/or improve the
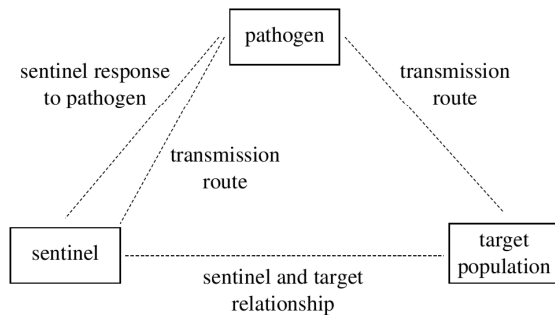
Figure 1. Key components and attributes of the sentinel framework.

cost-effectiveness of surveillance (McCluskey 2003). In most cases, this means that the sentinel population is more likely to be exposed to, or to respond to the pathogen than other populations. This sentinel concept encompasses the variety of uses described above and can refer to any level of grouping from an individual to a larger unit, such as a herd or even a species.

Various authors have compiled lists of attributes of an 'ideal' sentinel (CAMEH 1991; Komar 2001), but these have invariably been created with a particular sentinel application in mind and there exists little or no consensus about the common characteristics or defining features of 'the sentinel'. This ambiguity, of course, reflects the fact that there is no innate quality of sentinel suitability that particular species or populations have. Instead, the criteria against which the usefulness of a given sentinel population is assessed are influenced by the aim of surveillance and the context in which the sentinel would be used. We describe a conceptual framework which we believe can be used to evaluate potential sentinel populations for any combination of surveillance aim and ecological context (figures 1 and 2).

3. THE SENTINEL FRAMEWORK

Within any surveillance context, the sentinel population must always interact with both the pathogen and the target population and it is essential to consider and describe the interactions between these fundamental components (figure 1). The following are the three components of the sentinel framework.

— *Pathogen.* The pathogen that is under surveillance.
— *Target population.* The population of concern to which information gathered from the sentinel is applied.
— *Sentinel population.*

This framework is not intended to represent the transmission dynamics of a pathogen, but rather the ways in which the components are associated. Three critical attributes of this system must be considered in order to assess the utility of a potential sentinel for a particular surveillance aim and in any given ecological context: (i) the sentinel response to the pathogen, (ii) the relationship between sentinel and target populations, and (iii) routes of transmission to both target and sentinel populations. The conceptual issues raised

are discussed with reference to the surveillance of WNV in North America (box 1).

3.1. Sentinel response to pathogen

The sentinel response to a pathogen can range from the production of antibody in an otherwise healthy individual, through morbidity and ultimately to mortality. It may also be possible to detect the presence of the pathogen in a sentinel population before other responses develop and sentinel responses can therefore include the following.

— Current infection/presence of pathogen.
— Seroconversion.
— Morbidity.
— Mortality.

There is a clear intuitive distinction between sentinel populations that develop high levels of morbidity or mortality in response to pathogen exposure and those that remain healthy. Sick or dying sentinels show an obvious and dramatic response to a pathogen and provide a readily appreciable signal of the presence of a pathogen within an ecosystem (see the discussion of crow mortality as a sentinel of WNV presence in box 1). At the other end of the spectrum, apparently healthy sentinels that develop a subclinical response are often more useful for investigating the maintenance patterns and transmission dynamics of a pathogen within the sentinel and target populations. Following the consumption of prey infected with rabbit haemorrhagic disease virus (RHDV), foxes in northern Germany developed antibody responses that declined after just two weeks. Serosurveillance of this fox population therefore reveals the proportion of the population that has been exposed in the one to two weeks prior to testing. These serological data can provide a good indication of the incidence patterns of RHDV in the sympatric rabbit population (Frölich *et al.* 1998). In cases in which healthy sentinels are used, it may be desirable to resample the same individuals or populations over time. It is also important that the observation and sampling of the sentinel population, and perhaps also the sentinel response itself, has minimal impact upon the study system.

This example also demonstrates the influence of the temporal characteristics of the sentinel response to a pathogen upon the choice and application of sentinel populations. Sentinel populations which respond to a pathogen prior to the exposure of the target population may be useful for those surveillance programmes that aim to prevent the exposure of the target population. For other sentinel uses, the rapid development of a response may not be required. The duration of the potential sentinel's response can also influence the types of question which can be usefully addressed. An equivalent sentinel population (to that of the foxes) that developed a longer lasting antibody response in the above RHDV example would be of limited use for investigating the incidence of disease in the rabbit population on this immediate time-scale.

The sentinel response can be viewed as a test for the presence of the pathogen within the target population

and as such has properties that are analogous to test sensitivity and specificity.

— *Sentinel sensitivity.* The sensitivity of the sentinel refers to its capacity to respond to the presence of the pathogen in the target population and effectively translates as susceptibility to infection. An insensitive population would be unlikely to display evidence of infection with the pathogen even if it were present in the target population and would therefore be poorly suited for use as a sentinel.
— *Sentinel specificity.* The specificity of the sentinel response relates to the ease with which a sentinel response can be interpreted and attributed to a particular pathogen. Specificity is thus closely linked to the response type. Morbidity and mortality are generally less specific indicators of the presence of a particular pathogen than molecular responses that are observed using a test or assay unique to the pathogen in question. Across parts of rural Africa and Asia, for example, bird die-offs due to pathogens other than H5N1 avian influenza virus can be relatively common occurrences, reducing the specificity of bird mortality as an indicator of H5N1 presence (World Health Organization 2005).

Whatever the type of response a particular sentinel population mounts to a pathogen, it is important that the individual members of that population are consistent in the development of the response. Excessive variation within a sentinel population would greatly complicate the interpretation of surveillance findings and it may therefore be important to ensure that members of the sentinel population are of similar age, sex or other relevant characteristics, depending upon the type of response measured.

3.2. Relationship between sentinel and target populations

The relationship that exists between the sentinel and target populations may include behavioural, epidemiological or spatial aspects or any other form of ecological association. Detailed understanding of the associations between the sentinel and target populations is not required to address all questions. However, a comprehensive understanding of the relationship between a sentinel and a target population will allow for the investigation of more complex epidemiological questions and better-informed interpretation of the data collected through surveillance of that sentinel. The minimum association that must exist between a sentinel and a target population is a spatial association. This need not imply spatial overlap however. If the pathogen is spreading on a wavefront or emanating from a focal source, then a sentinel population may be selected on the basis of its closer proximity to the focus at the target population.

At the other extreme, the sentinel population may consist of a specific subset of the target population, ensuring a very close relationship between the two populations. A subpopulation that experiences high-transmission risk, or is particularly sensitive to infection with a particular pathogen, may serve as a sentinel for the wider population and can clearly provide a more accurate assessment of risk to the target than a population occupying a dissimilar ecological niche and consequently experiencing a very different pattern of exposure to the pathogen (e.g. unvaccinated sentinel birds are used to detect the presence of HPAI viruses within the otherwise vaccinated flock; Suarez 2005). The sentinel and target population may also be epidemiologically linked such that the sentinel may act as a source of infection for the target population, as is the case with arthropod vector surveillance.

3.3. Transmission routes

This attribute is essentially a component of the relationship between the sentinel and target populations that explicitly considers the route or routes through which the two populations can become infected with the pathogen. In circumstances where the target and sentinel are exposed to infection via the same route, the relative intensity and patterns of exposure of the two populations to the source of infection are important (Estrada-Franco *et al.* 2006). It may be desirable to select a sentinel that has higher levels of exposure and which is therefore more likely to show evidence of a pathogen if it is present than to directly survey the target population itself. For pathogens that are transmitted by a vector or vectors, the feeding preferences of the vector(s) can therefore be important in informing sentinel selection. Domestic dogs are the preferred source of blood meals for *Triatoma infestans*, one of the main vectors of *Trypanosoma cruzi* in Mexico. A comparative serosurvey revealed overall anti-*T. cruzi* IgG prevalence of 16% in dogs compared with a 2% prevalence in humans, and a strong positive correlation between human and dog seropositivity within the study area. These data suggest that the feeding preferences of this vector make the domestic dog population a good sentinel for identifying areas of human seropositivity and monitoring prevalence in this context (Estrada-Franco *et al.* 2006).

There are also circumstances in which the route of exposure of the sentinel and target population may differ. A number of emerging zoonoses, including WNV and HPAI H5N1 viruses can be transmitted through the ingestion of infected material (Komar *et al.* 2003; Austgen *et al.* 2004; Rimmelzwaan *et al.* 2006). Carnivore and scavenger species that are exposed through consumption of infected prey may prove useful sentinels for a wide range of pathogens, specifically because of this additional route of exposure that is not shared with the target population (Cleaveland *et al.* 2006). A single predator or scavenger typically consumes material from multiple individuals, increasing the probability of exposure to pathogens circulating within the prey population. Predators and scavengers can effectively sample from the prey population, leading to a 'bioaccumulation' effect whereby pathogens present at relatively low prevalence in the prey population may be detected at higher prevalence in the predator/scavenger species (Cleaveland *et al.* 2006). An understanding of the predator–prey relationships between the target population and potential sentinels may prove useful in sentinel selection. The principal
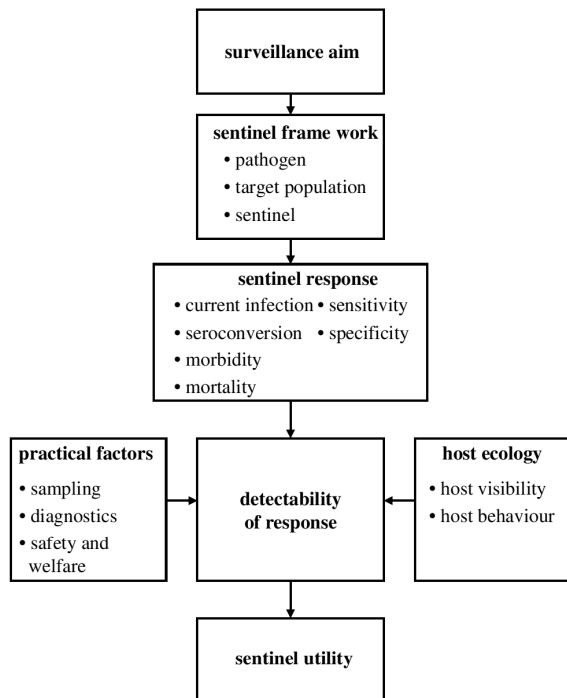
Figure 2. The sentinel framework in context.

transmission route of bluetongue virus, which infects wild and domestic ruminants across East Africa, is via *Culicoides* midge vectors. Serosurveillance of free-ranging African carnivores revealed that both the seroprevalence and the virus serotype identified varied dramatically across carnivore species (Alexander *et al.* 1994). This study suggested that the most probable route of infection of carnivores with bluetongue was via consumption of infected prey, and that the variation seen between species was attributable to dietary differences. Different carnivore species may therefore vary in their utility as sentinels for the presence of bluetongue virus in different ruminant species.

4. PLACING THE SENTINEL FRAMEWORK IN CONTEXT

The sentinel response can be viewed as the output of the sentinel framework. The nature of this response, in combination with other sentinel host factors and practical influences which depend upon the context in which surveillance is conducted, determines the overall detectability of the sentinel response (figure 2). Unlike the attributes which operate within the sentinel framework, detectability is a quality of the interaction between the sentinel and the observer. The overall utility of any potential sentinel can only be assessed by considering both the sentinel framework and the influences of the context in which it would be applied (figure 2; box 2).

The visibility of any animal population is determined by the morphology, behaviour, distribution and abundance of the individual animals of which it is comprised. The detectability of the

sentinel response includes both the visibility of the animal and its response to a pathogen. The type of response that an animal mounts will directly affect the ease with which it is detected by the observer. For example, lions are being used as sentinels for canine distemper in the Serengeti National Park in Tanzania, as a result of their high visibility to observers and the dramatic manifestations of clinical disease, which include grand mal seizures (Roelke-Parker *et al.* 1996). Information from lion sentinels would be used to increase disease detection efforts within other wild carnivore populations of the park to establish the extent and impact of any epidemic and initiate a risk–benefit assessment for possible interventions (such as vaccination) for protecting threatened wildlife populations. A wide range of other carnivore species such as hyaenas, bat-eared foxes and leopards are known to be susceptible to canine distemper (Roelke-Parker *et al.* 1996), but are less suitable as sentinels for disease in the Serengeti owing to ecological and behavioural factors that reduce visibility (e.g. nocturnal behaviour, small body size, den-living characteristics, lower levels of tourist observation). Widespread morbidity or mortality within a sentinel population are more readily appreciable than seroconversion or current infection/presence of pathogen, which can only be detected by the observer after first sampling the sentinel population and then conducting laboratory analysis. In the case of overt sentinel responses such as mortality, the existence of a reliable network of 'observers' and a mechanism through which data are reported are crucial. It is equally important to consider the available capacity to detect any less overt responses including the existence of a reliable sampling protocol and a diagnostic test (McCluskey 2003). The majority of diagnostic tests for human and livestock pathogens have not been validated for use in non-target species and the sensitivity and specificity of tests can vary hugely between species (Greiner & Gardner 2000). The existence of a suitable negative control population and recognition of the time required to identify and validate diagnostic tests must be considered in any proposed sentinel surveillance programme.

The practical difficulties involved in sampling any potential sentinel population must also be evaluated and it may often be difficult to reconcile the use of a theoretically ideal sentinel with such practicalities. For a sentinel population to be useful, it must be both logistically feasible and safe to sample sufficient numbers of the population (CAMEH 1991). Since sentinels are often selected on the basis of increased likelihood of exposure to a pathogen, sentinel surveillance can enable targeting of resources and often has improved cost-effectiveness as compared, for example, with more comprehensive cross-sectional surveys (McCluskey *et al.* 2003). The bioaccumulation effect discussed above suggests that evidence of exposure to a pathogen may effectively accumulate within carnivore populations (Cleaveland *et al.* 2006). The identification of the presence of a pathogen within a particular area can therefore be achieved by sampling relatively few carnivore sentinels, as compared to an exhaustive and costly survey of the prey population

Box 2. Simplified application of the conceptual framework represented in figures 1 and 2 to the evaluation of potential sentinel populations for the surveillance of HPAI H5N1.

Surveillance aim
To establish if H5N1 viruses have been introduced into a country with underdeveloped disease surveillance and reporting structure.

Should sentinels be used?
— Cross-sectional survey–may be expensive and time consuming.
— Sentinel surveillance–potentially cost-effective alternative

↓
Sentinel framework
pathogen = HPAI H5N1 virus.
target population = the national poultry population.
Potential sentinels
    — Backyard chicken populations in areas of perceived high risk of virus introduction, e.g. close to areas of wild bird congregation or to livestock markets.
    — Backyard ducks in similar locations.
    — Wild bird populations.
    — Domestic cats.
    — Domestic dogs.

Other potential sentinels are excluded altogether on the basis of a lack of response to the pathogen or of any type of meaningful relationship with the target population.

Relationship between sentinel and target populations
Chickens
    — Subset of target population.
Ducks
    — Occupy a very similar niche to target population.
    — May act as silent carrier of viruses (Hulse-Post *et al.* 2005).
Wild birds
    — May act as source of infection for domestic species.
    — May not occupy the same geographical areas as the target population (especially true for large congregations of migratory birds).
Cats and dogs
    — Spatial correspondence with target population.
    — Cats and dogs may prey upon the target population.

Transmission routes
Chickens, ducks and wild birds
    — Bird–bird transmission.
    — Environmental contamination.
Cats and dogs
    — Consumption of infected birds (Keawcharoen *et al.* 2004; Kuiken *et al.* 2004).
    — Horizontal transmission in cats (Rimmelzwaan *et al.* 2006).

↓
Sentinel response
Chickens
    — Consistent, rapid and widespread mortality.
    — Die-offs provide a prompt indication of virus presence.
Ducks
    — Variable pathogenicity and thus mortality (Sturm-Ramirez *et al.* 2005).
    — Isolation of virus from healthy birds (Hulse-Post *et al.* 2005).
Wild birds
    — Variable pathogenicity (Ellis *et al.* 2004).
    — Isolation of virus from healthy birds (Chen *et al.* 2006).
Cats
    — Experimental evidence of mortality response (Rimmelzwaan *et al.* 2006).
    — Mortality reports associated with bird die-offs (Butler 2006*a*, Songserm *et al.* 2006*a*, Yingst *et al.* 2006).
    — High seroconversion rates (Butler 2006*b*).
    — Subclinical infections (Leschnik *et al.* 2007).
Dogs
    — High seroconversion rates (Butler 2006*b*).
    — Mortality report associated with bird infection (Songserm *et al.* 2006*b*).

Sensitivity and specificity of responses
Chickens
    — ✓✓✓ Highly sensitive but specificity of mortality response is low, as
    — ✗✗✗ chicken die-offs not necessarily unusual where poultry are not routinely vaccinated against other pathogens,

(*Continued.*)

Box 2. (*Continued.*)

      e.g. Newcastle disease virus.
    — ✓✓✓ High specificity of laboratory analyses.
Ducks
    — ✓/✗ Variable mortality response limits sensitivity.
    — ✓✓✓ High specificity of laboratory analyses.
Wild birds
    — ✓/✗ Variable mortality response limits sensitivity.
    — ✗✗✗ Very low prevalence in healthy birds limits sensitivity (Chen *et al.* 2006).
    — ✓✓✓ High specificity of laboratory analyses.
Cats and Dogs
    — ✓/✗ Serological analyses non-specific for distinguishing high- and low-pathogenicity viruses.

<div align="center">+</div>

Host ecology

    — ✓✓✓ Domestic species are all highly observable as a consequence of their close association with humans.
    — ✗✗✗ Wild birds are considerably less visible and may occupy relatively remote and inaccessible areas.

<div align="center">+</div>

Practical factors

    — Risk to sampling personnel must be considered as a priority when developing all sampling protocols.
    — ✓✓✓ Domestic species approachable and handleable.
    — ✓/✗ Distribution of cats and dogs relatively to poultry may vary according to factors such as urbanization and religion.
    — ✗✗✗ Considerable investment of money, time and expertise required to sample sufficient numbers of wild birds.
    — ✓✓✓ For the identification of virus presence, standard test protocols include RT-PCR and virus isolation (World Organization for Animal Health 2005) which are generally adaptable across species.
    — ✓/✗ Serological analyses may not be well developed for wild birds, cats or dogs.

<div align="center">↓</div>

Detectability

Chickens
    — ✓✓✓ Mortality response easily appreciated.
    — ✓✓✓ High visibility within human communities.
    — ✗✗✗ Low specificity of mortality limits detectability.
Ducks
    — ✗✗✗ Mortality response variable.
    — ✓✓✓ Additional responses detectable through laboratory analysis.
    — ✓✓✓ High visibility within human communities.
Wild birds
    — ✗✗✗ Mortality response variable.
    — ✗✗✗ Low visibility compared with domestic species.
    — Logistically complex and time-consuming sampling required.
Cats and Dogs
    — ✓✓✓ High visibility within human communities.
    — ✓✓✓ Sudden and widespread morbidity or mortality uncommon.
    — ✓/✗ Non-mortality responses less detectable.

    In all cases, a comprehensive network of observers is vital and it may be necessary to develop education programmes aimed at improving reporting levels.

<div align="center">↓</div>

Utility

    — Domestic chicken and ducks sentinels are likely to provide the most rapid and dramatic response to HPAI H5N1 virus within a country. However, in this context in which mortality in domestic birds is not unusual, this mortality may not be reported and the detectability of the response in the context of this surveillance aim may be very low.
    — To best address this surveillance aim, the specificity of the chicken mortality response to HPAI H5N1 presence could be enhanced by using a combination of sentinels such that priority was given to the investigation of chicken die-offs that were accompanied by morbidity or mortality in cats or dogs (Yingst *et al.* 2006).
    — Retrospective analysis of sera collected from ducks, cats and dogs could also be used to identify those areas in which an H5N1 virus had been present.

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within which the pathogen may circulate at very low prevalence, thereby providing a relatively rapid and inexpensive surveillance option (Frölich *et al.* 1998; Leighton *et al.* 2001; Csángó *et al.* 2004; Cleaveland *et al.* 2006). In addition to consideration of time and cost, the potential risks to research personnel and the public that are associated with the desired sampling strategy must be evaluated, as well as the effects of sampling upon the sentinel population itself in the context of animal welfare and conservation status (CAMEH 1991).

5. APPLICATIONS OF ANIMAL SENTINELS

Many of the questions addressed through the use of animal sentinels, such as the assessment of pathogen

control efforts, the monitoring of prevalence fluctuations over time and the demonstration of the absence of a pathogen, require only the basic qualities of a sentinel as defined above. While the more specific requirements of any particular sentinel are unique to the context and aim to which it is applied, there are some general qualities and subtypes of sentinels that correspond to major applications of animal sentinels. For example, sentinels in which the response to a pathogen and the detection of that response occur prior to exposure, or cases in the target population, can provide early warning of pathogen presence. Early warning sentinels are used to provide a predictive signal of risk to the target population. Sentinels that are exposed and which respond to a pathogen before the exposure of the target population may provide an opportunity to implement pre-emptive control measures and to prevent the infection of the target population (see discussion of WNV surveillance in box 1). Other early warning sentinels may respond to the pathogen more rapidly than the target population but not necessarily before the target's exposure (e.g. the coal miner's canary). In such cases, data collected from the sentinel cannot be used to prevent cases in the target population altogether. However, the information they supply can provide advance warning of cases, enabling the prioritization of resources for treatment and the prevention of additional cases. In most cases, early warning sentinels are highly visible and develop a very obvious response to the pathogen. Data provided by sentinels with these qualities can be more rapidly processed, analysed and acted upon than the data from apparently healthy sentinels for which the potentially lengthy processes of sample collection and laboratory analyses must be carried out before any data are available. Ideally, the response of early warning sentinels should also be very specific to minimize the likelihood of false positive responses and consequently improve confidence in decision making based on the sentinel response alone.

Sentinels can also be used retrospectively to provide evidence of the timing of pathogen introduction and spread through a target population. In situations where a number of populations or locations are sampled, this information can be combined to reveal the spatial and temporal pattern of pathogen spread. Following the widespread rinderpest outbreak that occurred in Kenya in 1993–1997, the retrospective serosurveillance of buffalo herds and analysis of age-seroprevalence patterns allowed the estimation of the time of infection in different herds, the identification of the probable point of entry of the pathogen into the wildlife population and the elucidation of where the pathogen had been, how it had spread and where it was likely to move to (Kock *et al.* 1999). In this case, buffalo herds were selected as sentinels on the basis of the increased susceptibility of the species to this virus (Rossiter 1994), and served as sentinels for the larger livestock population in the affected areas. In such circumstances, the appropriate sentinel population must develop a response to the

pathogen that persists and is detectable a long time after exposure. When used retrospectively, it is also important that individuals of the sentinel population can be reliably aged.

6. CONCLUSION

The objective of this paper has been to provide a consistent and inclusive framework that clarifies our understanding of the role of animal sentinels and their potential value in the surveillance of human and animal infectious diseases, as well as providing a conceptual tool that can be applied to assess and characterize potential sentinels in the future. At present, surveillance of many pathogens involves the target population alone; however, the broad host range of many important human and animal diseases provides opportunities for exploiting a wide range of species for surveillance purposes. The variability of host responses to a pathogen, the heterogeneities in pathogen exposure in different populations and the differing relationships between sentinel and target populations indicate that different animal hosts will themselves vary in their ability to act as effective sentinels in different circumstances.

Animal sentinels may not serve as a useful surveillance tool in all contexts. The generic framework that we have developed in this paper describes the attributes of host species that need to be considered to identify appropriate sentinel populations for different surveillance purposes. This same framework should also be used to identify characteristics of potential sentinels that perhaps make them unsuitable in a particular circumstance. For example, sentinels must by definition be intentionally observed. This classification distinguishes the use of animal sentinels from scenarios in which responses of animal populations to novel pathogens are 'noticed'. For this reason, animal sentinels cannot provide the solution to the question of how to carry out surveillance for pathogens that are currently unknown. However, as a consequence of greater awareness of the potential of animal sentinels and improved observation of animal populations, instances of unusual morbidity and mortality in animal populations that result from the emergence of novel pathogens would perhaps be more likely to be noticed and their potential significance to other species recognized.

To date, there has been limited appreciation of the data resource that different animal hosts represent for disease surveillance. This paper aims to highlight the variety of surveillance functions for which animal sentinels may be used, the range of animal host species that may usefully be exploited (particularly for human disease surveillance) and the potential benefits of animal sentinels for enhanced pathogen detection and improved cost-effectiveness of surveillance. The potential value of animal sentinels in disease prevention and control can only be realized with close integration and effective communication between and within human and animal health sectors; information generated from sentinel populations must be disseminated to those who need to

take action, and appropriate responses must be generated as a result of this information to mitigate disease risk.

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