AN INVESTIGATION OF DISEASE RESERVOIRS IN COMPLEX ECOSYSTEMS: RABIES AND CANINE DISTEMPER IN THE SERENGETI

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Declaration

I declare that this thesis was composed by myself, that the work contained herein is my own except where explicitly stated otherwise in the text, and that this work has not been submitted for any other degree or professional qualification except as specified.

Tiziana Lembo

ABSTRACT

Rabies virus (RABV) and canine distemper virus (CDV) have been implicated as the cause of dramatic declines in wildlife populations worldwide and rabies is considered a growing public health problem throughout much of the developing world. In Tanzania, recent severe epidemics of both diseases in Serengeti carnivores have been associated with the large population of domestic dogs (*Canis familiaris*) living in proximity to the protected areas, but many questions remain about their epidemiology, particularly with respect to the understanding of reservoir infection dynamics and patterns of viral maintenance and transmission in multi-host communities. This study examines all the available evidence for reservoirs of rabies in the Serengeti and presents data on the temporal and spatial dynamics of CDV in domestic and wild carnivores (dogs and lions [*Panthera leo*]) to help understand long-term patterns of infection in the two populations.

Practical difficulties in detecting rabies in areas such as the Serengeti, where surveillance and laboratory confirmation of disease are severely constrained, limit the collection of epidemiological data, a critical step in identifying reservoirs of infection. A novel direct rapid immunohistochemical test (dRIT) as a field test for rabies surveillance was therefore investigated. Preliminary evaluation on frozen and glycerolated field brain samples under field and laboratory conditions showed a sensitivity and specificity equivalent to those of the direct fluorescent antibody (DFA) test, the gold standard in rabies diagnosis. Examination by molecular phylogenies of the genetic characteristics of RABVs isolated from a range of species revealed one single major variant belonging to the group of southern Africa canid-associated viruses (Africa 1b), a high degree of genetic relatedness among viruses with no evidence for distinct virus-host associations, and patterns consistent with temporal direction of evolutionary change from dogs to other species. Overall, these analyses point to the domestic dog being responsible for supporting the cycle of a single virus variant in the ecosystem. Rabies incidence data available from the Serengeti (1991-2005) and data on the genetic characteristics of the virus were then used to draw conclusions on reservoirs of infection: domestic dog populations occurring at high densities were the only population essential for persistence, whereas other carnivores contributed to the reservoir as non-maintenance

components. Serological and case morbidity/mortality data on CDV indicated that infection patterns in unvaccinated dog and lion populations were consistent with periodic re-introductions with no evidence for persistent infection, re-introduction of infection in vaccinated dog populations coincided with declines in vaccination coverage, but patterns of exposure were not different in vaccinated and unvaccinated populations, and circulation in lions did not appear to occur in the absence of infection in dogs, suggesting that dogs may be the only source of infection. The lack of evidence for long-term persistence in any of the populations suggests that no single population may be capable of independent maintenance, but a network of populations may constitute a maintenance community.

Finally, the overall results are discussed in relation to the surveillance of rabies and the design of appropriate control strategies for rabies and CDV for the Serengeti ecosystem and areas of sub-Saharan Africa where both diseases occur. The qualities of the dRIT for rabies surveillance in field conditions and countries with limited diagnostic infrastructures and the potential benefits of its wider application in developing country settings are highlighted. Efforts directed at controlling infection in domestic dogs through mass vaccination programmes are expected to have the most significant impact on reducing or eliminating disease in all the other species. Elimination of CDV in dog populations would also provide definite insights into their role in disease persistence within a potentially complex reservoir system but questions remain about the cost-effectiveness of such an approach as a long-term management strategy for African wild carnivore populations.

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DEDICATION

A mio padre,
a mia madre

"Vorrei essere un condor per volare libero nel cielo e nei prati.

Vorrei avere le ali per vedere la gente da sopra e andare dove mi pare.

Vorrei volare per sfuggire ai cacciatori, per fare una passeggiata, per allevare i piccoli, per insegnargli a volare e per sgranchirmi le ali nell'aria.

Vorrei volare per visitare altri popoli e persone diverse: inglesi, eschimesi, spagnoli, africani."

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CHAPTER 1: GENERAL INTRODUCTION

Throughout the world, infectious diseases are becoming an emerging problem in human and veterinary medicine and a growing concern in wildlife conservation (Schrag and Wiener, 1995; Daszak *et al.*, 2000; Dobson and Foufopoulos, 2001; Funk *et al.*, 2001; Woodroffe *et al.*, 2004). Pathogens that pose the greatest threat to human health and biodiversity are those that infect a wide range of species, including humans (i.e. zoonotic) and those that are viruses (Taylor *et al.*, 2001; Cleaveland *et al.*, 2001). In particular, it is estimated that of over 1,400 known pathogens, more than half are zoonotic (Taylor *et al.*, 2001; Woolhouse and Gowtage-Sequeira, 2005) and generalist pathogens are very prevalent amongst human and domestic animal pathogens (Cleaveland *et al.*, 2001). Pathogens considered emerging and re-emerging (i.e. 'pathogens whose incidence is increasing following its appearance into a new host population or those whose incidence is increasing in an existing host population as a result of long-term changes in its underlying epidemiology': Woolhouse and Dye, 2001) are more likely to be zoonotic and the probability of emergence is higher for viruses, particularly RNA viruses, than for other pathogens (i.e. helminths and fungi) (Taylor *et al.*, 2001; Cleaveland *et al.*, 2001).

It has been suggested that the predilection for RNA viruses to emerge may be due to their ability to evolve rapidly (as a result of extremely high substitution rates, short generation times and immense intra-host population sizes), which would increase the chances for host switches (Burke, 1998; Woolhouse *et al.*, 2005). RNA viruses are indeed the fastest-evolving organisms with mutation rates on average six orders of magnitude higher than those in eukaryotes and DNA viruses (Holland *et al.*, 1982; Drake *et al.*, 1998; Jenkins *et al.*, 2002). Notably, pathogens that are thought to have emerged following a species jump and that have caused many of the most dramatic human and animal disease epidemics are RNA viruses. Important examples include influenza A viruses (H1N1 and H3N2), human immunodeficiency virus (HIV), and *Morbilliviruses* such as rinderpest virus (RV) and canine/phocine distemper virus (CDV and PDV) (for a review see Burke, 1998 and Woolhouse *et al.*, 2005). Adaptation to a new host species may result from mutation or recombination/reassortment. Although high rates of mutation may render RNA viruses more prone to successful host switches, evolutionary models indicate that the rate and progress of virus adaptation are not only influenced by the overall rate at

which mutations occur, but also by their fitness (Kuiken *et al.*, 2006). For instance, it is known that not all the mutations that arise within a given viral population are advantageous, but a large proportion of them are deleterious (Sanjuán *et al.*, 2004a) and there is growing evidence that both advantageous and deleterious mutations show complex epistatic interactions (Sanjuán *et al.*, 2004b). As large numbers of advantageous mutations may be required to allow viral adaptation to a new host, successful host switches may occur very infrequently. However, viruses with an ability to recombine or reassort, may acquire many of the key mutations required for inter-species transmission in one single step, which will accelerate successful adaptation to new hosts.

Rabies virus (RABV) and CDV (which are both RNA viruses) typify these pathogens. Because of their implication in some of the most dramatic population declines in wildlife worldwide (Table 1.1), they are now recognised amongst the diseases of greatest concern to biodiversity. Human rabies is also considered a growing problem throughout much of the developing world (Cleaveland, 1998; Cleaveland *et al.*, 2002).

In the Serengeti ecosystem over the past 15 years, rabies has been responsible for devastating epidemics that decimated endangered canid populations of African wild dogs (*Lycaon pictus*) (Gascoyne *et al.*, 1993a; Kat *et al.*, 1995; Woodroffe, 2001) and canine distemper (CD) caused high mortality in lions (*Panthera leo*) and a range of wild carnivore species (Roelke-Parker *et al.*, 1996). In the region rabies is not only a concern for the conservation of wildlife populations but also for the substantial public health burden it causes to the local communities (Cleaveland *et al.*, 2002). Although both diseases have been associated with the large population of domestic dogs (*Canis familiaris*) bordering the Serengeti National Park (SNP) (Cleaveland and Dye, 1995; Cleaveland, 1996; Cleaveland *et al.*, 2000), many questions remain about their epidemiology, particularly with respect to the understanding of reservoir infection dynamics and patterns of viral maintenance and transmission in multi-host communities.

The objective of this chapter is to review the literature focusing on specific aspects relevant to this study and on the work which has led to the project, with an emphasis

on the key issues that still remain unresolved. A statement of the overall and specific aims of the study and an outline of the thesis are also given.

Table 1.1. Severe major declines in wildlife populations caused or thought to have been caused by rabies virus (RABV) and canine distemper virus (CDV). US = United States.

Pathogen	Species	Area	Year/s	Estimated mortality	Reference
CDV	Black-footed ferret (*Mustela nigripes*)	Wyoming, US	1985	70%	Thorne and Williams, 1988
RABV	Ethiopian wolf (*Canis simensis*)	Bale Mountains, Ethiopia	1990	52%	Sillero-Zubiri *et al.*, 1996
			1991-1992	77%	Sillero-Zubiri *et al.*, 1996
			2003	76%	Randall *et al.*, 2004
RABV	African wild dog (*Lycaon pictus*)	Serengeti-Mara ecosystem, Tanzania/Kenya	1985-1992	extinction by 1992	Gascoyne *et al.*, 1993a; Kat *et al.*, 1995; Woodroffe, 2001
CDV	African wild dog	Chobe National Park, Botswana	1994	disappearance of one study pack	Alexander *et al.*, 1996
		Mkomazi, Tanzania	2000	94%	van de Bildt *et al.*, 2002
CDV	Lion (*Panthera leo*)	Serengeti-Mara ecosystem, Tanzania/Kenya	1994	30%	Roelke-Parker *et al.*, 1996
CDV	Island fox (*Urocyon littoralis*)	Santa Catalina Island, California, US	1999	decline by 90%	Timm *et al.*, 2000
CDV	Crab-eater seal (*Lobodon carcinophagus*)	Antartica	1950s	thousands of deaths	Bengston *et al.*, 1991
CDV	Lake Baikal seal (*Phoca siberica*)	Lake Baikal, Siberia	1987-1988	high mortality	Grachev *et al.*, 1989
CDV	Caspian seal (*Phoca caspica*)	Caspian sea	1997 and 2000	> 10,000	Kennedy *et al.*, 2000

1.1 Disease persistence and identifying reservoirs of infection

It has long been recognised that pathogens of human and animal diseases persist in reservoir host(s) (Daszak *et al.*, 2000), knowledge of which is required for the design of effective disease control measures. Single-host pathogens must, by definition, be able to persist in their host species. In contrast, generalist pathogens can infect more than one host species, but not all hosts are able to act as reservoir(s). Host species in which the pathogen does not persist can be occasionally affected through 'spill-over' infection from the reservoir(s). Since generalist pathogens can be encountered in many hosts, elucidating the reservoir(s) of infection may be problematic but of critical importance. A lack of understanding of reservoirs of multi-host pathogens has hampered control of many diseases of economic and zoonotic importance, for example bovine tuberculosis in the United Kingdom (UK) and rabies in Africa (Krebs *et al.*, 1998; Bingham *et al.*, 1999a,b; Donnelly *et al.*, 2003; Macdonald *et al.*, 2006).

The existence of many conflicting, and often incomplete definitions of reservoirs (for a review, see Haydon *et al.*, 2002a) prompted some authors to redefine a reservoir of infection (Ashford, 1997; Haydon *et al.*, 2002a; Ashford, 2003). They suggested that reservoirs may comprise an ecologic system (i.e. a range of epidemiologically connected populations or environments) in which an infectious agent survives indefinitely. This complexity particularly applies to multi-host systems where various susceptible hosts can contribute to the reservoir, as essential (to maintenance of the pathogen) or nonessential components (Haydon *et al.*, 2002a). Haydon *et al.* (2002a) propose a framework for defining reservoirs, which will be adopted throughout this thesis. First, reservoirs can only be understood in relation to defined target populations, i.e. the populations of concern to us (for example humans for rabies or endangered wildlife populations for rabies and CD). Second, all susceptible host populations that can transmit infection directly (source populations) or indirectly to the target populations can constitute all or part of the reservoir (nontarget populations). Third, overall, reservoirs are capable of permanent maintenance. Figure 1.1, adapted from Haydon *et al.* (2002a), illustrates examples of simple and more complex target-reservoir systems.

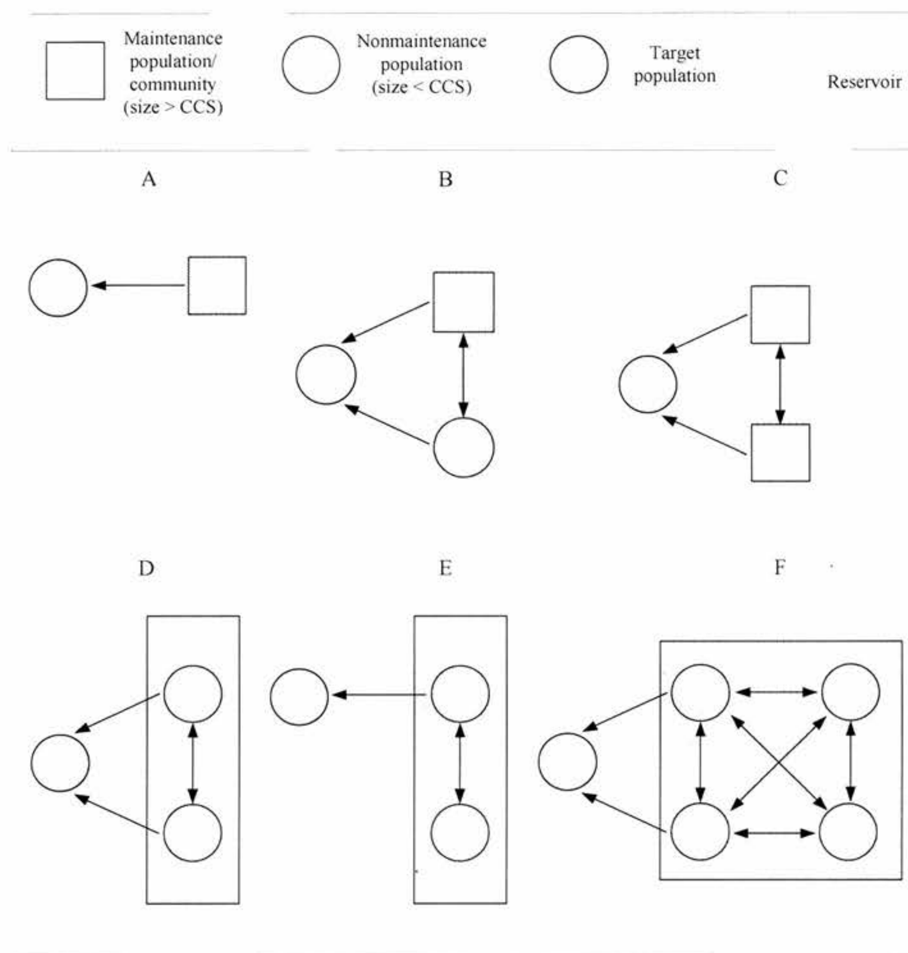


Figure 1.1. Examples of simple and more complex target-reservoir systems (adapted from Haydon *et al.*, 2002a). (A) A maintenance population (the reservoir) transmits infection to the population of concern (target population). (B) The reservoir comprises a maintenance and a non-maintenance population or two maintenance populations (C). (D) Neither of the populations constituting the reservoir, which are both source populations for the target, are capable of independent maintenance, but they constitute a maintenance community. (E) The same as D, but only one population is the source. (F) A complex maintenance community comprising a range of epidemiologically connected populations. CCS = critical community size.

Host population thresholds for invasion or persistence are central tenets of reservoir concepts. Epidemiological theory indicates that invasion can succeed when the density of susceptible hosts ensures a basic reproductive number (R_0), defined as the number of expected secondary cases caused by the first infectious individual in a susceptible population, above 1 (Anderson and May, 1991). After a disease has successfully invaded a population, it can persist or go extinct for variable periods of time. Diseases that are persistent and almost never go locally extinct are termed endemic. In contrast, non-endemic (i.e. epidemic) diseases are characterised by

episodic outbreaks and may disappear from the host population for extended periods (local extinction or fade-out), as the chain of transmission is broken. A substantial body of epidemiological research has explored the issue of persistence for a number of disease systems (examples include Bartlett, 1957, 1960; Keeling, 1997; Keeling and Grenfell, 1997, 1998; Swinton *et al.*, 1998; Keeling and Gilligan, 2000a,b; Keeling and Grenfell, 2002; Broutin *et al.*, 2004a). In particular, extensive work on childhood infections, especially measles, for which highly detailed data records are available over many generations for developed countries (e.g. Grenfell and Anderson, 1989; Cliff *et al.*, 1993; Grenfell and Harwood, 1997), has provided important insights into infectious disease dynamics with implications for the design of successful immunisation strategies.

Population size is a critical determinant of disease persistence. The notion of a critical community size (CCS), below which disease cannot persist during the epidemic troughs, arose from studies of measles (Bartlett, 1957, 1960). Measles is a respiratory disease caused by a highly infectious single-stranded RNA virus belonging to the *Morbillivirus* group, measles virus (MV), mostly contracted by children. It is transmitted by means of aerosol particles. Infection produces lifelong immunity after recovery, which is the norm in immunocompetent and healthy individuals. Death of the host may occur in immunocompromised individuals. Case fatality in developed countries is very low, whereas, in the developing world, measles is still a major cause of mortality (McLean and Anderson, 1988). In the UK, the spatiotemporal dynamics of measles exhibits irregularities and non-stationary patterns with the disease shifting from regular and spatially synchronised cycles over most cities across England and Wales before mass immunisation (1944-1966) to irregular and spatially uncorrelated cycles in the vaccine era (Bolker and Grenfell, 1996; Grenfell and Harwood, 1997; Earn *et al.*, 1998; Bjørnstad *et al.*, 2002). Bartlett (1957) defined three levels of dynamics based on the large spatiotemporal pre-vaccination dataset of measles incidence in England and Wales: (i) Type I dynamics observed in large centres (above the CCS-e.g. population of 3.4 million) where measles was endemic and did not go extinct in time; (ii) Type II dynamics observed in medium-sized centres (below the CCS-e.g. population of 300,000) with regular biennial epidemics and fade-outs in the troughs; and (iii) Type III dynamics observed

in small centres (e.g. population of 11,000) with irregular epidemics and long fade-outs between them ended by reintroduction of infection from outside. The CCS estimated for other communities, including cities in the United States (US) (Bartlett, 1960; Bolker and Grenfell, 1996) and islands (Black, 1966), was remarkably similar to the figures based on the UK dataset. Extensive data analysis and modelling have been carried out in an attempt to capture the observed level of the CCS and the mechanisms driving the complex dynamics (both endemic and epidemic) of measles before and after the vaccination era (examples include Olsen and Schaffer, 1990; Rand and Wilson, 1991; Bolker and Grenfell, 1993; Grenfell *et al.*, 1994, 1995; Bolker and Grenfell, 1995; Keeling, 1997; Keeling and Grenfell, 1997; Earn *et al.*, 1998; Keeling and Grenfell, 2002; Grenfell *et al.*, 2002; Bjørnstad *et al.*, 2002). Beside the view of a persistence threshold, recent studies emphasise the importance of demographic (i.e. factors acting on host birth and death rates), temporal (e.g. seasonality and synchrony), and spatial (e.g. social grouping and mixing) heterogeneities as key determinants of disease persistence, and the need to incorporate them into epidemiological models to adequately describe observed disease dynamics (Grenfell *et al.*, 1995; Lloyd and May, 1996; Keeling, 1997, 2000; Hagenaars *et al.*, 2004; Keeling *et al.*, 2004; Conlan and Grenfell, 2007).

In the case of measles, the most important driving force in repeated outbreaks is the seasonal forcing in the contact rate corresponding to aggregation during school terms (Schenzle, 1984; Finkenstädt and Grenfell, 1998; Bjørnstad *et al.*, 2002; Grenfell *et al.*, 2002). Host demography, particularly birth and vaccination rates, has also a dramatic influence on epidemic dynamics. Due to the prolonged immunity following infection, births are necessary to increase the susceptible densities before another outbreak can occur. The effects of variations in birth rate on cycle periods have been highlighted (McLean and Anderson, 1988; Finkenstädt *et al.*, 1998; Finkenstädt and Grenfell, 2000; Earn *et al.*, 2000; Grenfell *et al.*, 2002; Conlan and Grenfell, 2007). In the pre-vaccination era, disease epidemics in England and Wales exhibited predominantly biennial cycles. During times of higher birth rates (e.g. at the time of the baby boom in the late 1940s and 1960s) a change from two-year cycles to one-year cycles occurred (Finkenstädt and Grenfell, 2000). Similar patterns were observed in cities characterised by permanent high birth rates such as Liverpool

(Finkenstädt *et al.*, 1998; Earn *et al.*, 2000; Grenfell *et al.*, 2002). In New York and Baltimore epidemics were irregular during the Great Depression (when birth rates were relatively low). Increased birth rates after World War II lead to more regular cycles, biennial (New York) or annual (Baltimore) (Earn *et al.*, 2000). Finally, high birth rates in developing countries drive measles dynamics to annual cycles (McLean and Anderson, 1988; Earn *et al.*, 2000). Vaccination as well as birth rate influence the susceptible recruitment rate. In particular, with vaccination the pool of susceptibles is greatly reduced. This explains the transitions in temporal dynamics (from regular to irregular cycles) after the introduction of mass vaccination in England and Wales (Bolker and Grenfell, 1996; Earn *et al.*, 2000).

Although seasonal variations in contact rate and longer-term variations in birth rate describe sufficiently well measles dynamics in large cities (above the CCS), the spatial nature of the epidemics needs to be taken into account to fully understand disease dynamics in smaller centres (below the CCS) (Grenfell *et al.*, 2002). Metapopulations have been used by ecologists and epidemiologists as a means for understanding the dynamics of spatially subdivided populations (Gilpin and Hanski, 1991; Hanski and Gilpin, 1997; Grenfell and Harwood, 1997; Hanski and Gaggiotti, 2004). Using a metapopulation approach the community is viewed as an assemblage of discrete or relatively discrete entities (local populations or habitat patches), each with its own independent dynamics, but coupled by some degree of migration (Levins, 1970). In the study of infectious diseases habitat patches correspond to host organisms containing a local population of the microparasites (viruses and bacteria) (for a review, see Grenfell and Harwood, 1997 and Keeling *et al.*, 2004). A metapopulation persists in a balance between local extinctions ('deaths') and re-colonisation ('births') of the different sub-populations by dispersing individuals from surviving sub-populations. For infectious diseases colonisation corresponds to the establishment of infection in an uninfected patch (colonisers are therefore infected individuals) and local extinction corresponds to death or recovery of the host. Only infections that do not result in life-long immunity (e.g. many sexually transmitted diseases) exist as true metapopulations at the individual level (with each individual host forming a patch of resources for its microparasite), as once infected individuals recover, they are again susceptible to infection/re-colonisation. On the contrary,

immunising or acute fatal infections (e.g. childhood diseases such as measles, infections caused by other *Morbilliviruses*, such as CDV and PDV, and rabies) render the host-patch indefinitely unsuitable for re-colonisation. However, the spatial and/or social aggregation of host individuals into units (for example for human communities families, neighbourhoods, schools, villages, towns, cities, countries or continents) form heterogeneous patches of favourable habitat for the pathogen. Persistence properties of acute or immunising infections can therefore be strongly dependent on the host spatial organisation and the mixing patterns that arise from a patchy host population.

The key process for the persistence or extinction of disease metapopulations is the degree of coupling between local populations, which generally arises because of host movements, for example commuter movements (Bolker and Grenfell, 1995; Grenfell *et al*., 1995; Lloyd and May, 1996; Keeling, 1997, 2000; Keeling *et al*., 2001, 2004). If the coupling is very low, the dynamics in each patch are independent and the disease dies out because of absence of 'rescue effect' (i.e. recolonisation from another patch). Similarly, high levels of coupling induce synchrony of epidemics (i.e. local populations are in the same dynamical state simultaneously and spatial heterogeneity is lost), which may lead the entire metapopulation to extinction. As the patch dynamics become desynchronised, extinction of some patches is balanced by recolonisation from other patches, enhancing the overall chances of persistence. There is therefore an intermediate level of coupling for which disease persistence is maximised and extinctions are minimised (Grenfell, 1992; Tidd *et al*., 1993; Grenfell *et al*., 1994; Bolker and Grenfell, 1995; Grenfell and Harwood, 1997; Keeling, 2000; Keeling *et al*., 2004).

Spatial coupling in metapopulations consisting of a geographic mosaic of cities and villages (Anderson and May, 1991; Grenfell and Bolker, 1998) has represented a difficult question and work to address this question is still in progress. Before the onset of vaccination, regular temporal dynamics of measles were accompanied by strong spatial synchronisation across the UK from big to small cities suggesting that metapopulation dynamics in small centres may be dependent on the hierarchy of coupling to larger centres (Cliff *et al*., 1993; Finkenstädt and Grenfell, 1998; Grenfell and Bolker, 1998). A wavelet phase analysis used to reconstruct the observed spatial

patterns showed conspicuous hierarchical waves of infection moving regionally from large cities to small towns indicating that coupling to the large population is a main synchronising force across the whole metapopulation (Grenfell *et al.*, 2001). A mechanistic stochastic model suggests that the waves-spread can be understood as a 'core-satellite' metapopulation (Grenfell and Harwood, 1997; Grenfell *et al.*, 2001). Given two epidemiologically coupled towns, one above the CCS and one below the CCS, after a large epidemic, infection persists in the large town, but goes locally extinct in the small town. As soon as the critical number of susceptibles is reached again in the large town (R_0 above 1), a new epidemic occurs. By contrast, in the small ('satellite') town, an infective 'spark' originating in a larger ('core') community is necessary to trigger a new epidemic. This spark may come through individuals moving between towns. This implies that large centres act as reservoirs of infection for the disease metapopulation and indicates that identification of these populations could be used to target control efforts (Grenfell and Bolker, 1998). Results from studies carried out on a much finer spatial scale were also indicative of urban-rural hierarchies in pertussis epidemics in Senegal (Broutin *et al.*, 2004b).

For effective control, knowledge of how infections persist in reservoir host(s) and are transmitted from reservoir host(s) to populations of concern is required (Haydon *et al.*, 2002a). For understanding persistence of zoonotic diseases (where humans are the population of concern) it is critical to consider the disease dynamics within the reservoir host(s), as well as within the human population. Work on bubonic plague, a vector-borne zoonotic disease generally transmitted to humans (dead-end hosts) from rats via fleas, has highlighted the importance of focussing on the interaction among rats, fleas and humans (Keeling and Gilligan, 2000a,b), and not solely on the human dynamics (Noble, 1974; Scott *et al.*, 1996), to fully understand and predict disease outbreaks in humans. Historical data show long durations of 'disease-free' periods followed by periodic pathogen resurgence even in communities with tight quarantine controls. Using a stochastic, spatial metapopulation model encompassing the disease dynamics in both the human and animal populations, Keeling and Gilligan (2000a,b) show that outbreaks in the human population are driven by changes in the epidemiology of infection in the rat population. They describe two sets of dynamics in the rat population, short-lived epidemics and persistent endemics, which are

dependent on the proportion of susceptible rats. If the level of susceptibles is high, large epidemics occur which may drive the rat population to very low levels. Infectious fleas are therefore forced to feed on alternative hosts such as humans and human epidemics occur. When the level of susceptibles is low, infection leads to an endemic persistence in the rat population and rare human cases. The global persistence of the disease is dependent on few local populations of rats that are in the endemic state and from which infection spreads to other sub-populations creating waves of short-lived epidemics. This suggests that control by eradication of rats when human cases have already arisen can dramatically exacerbate the epidemic by releasing many infected fleas seeking alternative hosts.

A key priority in the context of emerging diseases is to clarify the epidemiological and evolutionary dynamics that determine the establishment of a pathogen in an alternative host after being transmitted from a host in which it already persists (for a review, see Woolhouse *et al.*, 2005 and Kuiken *et al.*, 2006). One important example is the highly pathogenic H5N1 influenza A virus, a zoonotic pathogen transmitted from animal reservoir species (avian populations) to humans (Kuiken *et al.*, 2005). The virus represents a serious pandemic threat due to the potential for reassortment of avian and human viruses in co-infected individuals following cross-species transmission, resulting in a novel variant capable of direct human-to-human transmission (Webby and Webster, 2001). Kuiken *et al.* (2006) review the interaction of factors that limit the transmission and subsequent establishment of a pathogen into a novel host species (see also Woolhouse *et al.*, 2005). First, contact between the new ('recipient') host species and the 'donor' species must be sufficient to allow exposure of the recipient host species to the pathogen (host-host inter-specific interactions). Factors that influence contact rates are therefore of critical importance, with ecological factors (e.g. urbanisation, climate change, habitat degradation, and human encroachment into wildlife areas), behavioural factors, and movements (e.g. global travel) resulting in increased transmission of disease between populations (Rogers and Randolph, 2000; Daszak *et al.*, 2000; May *et al.*, 2001; Kovats *et al.*, 2001; Randolph, 2001; McMichael, 2004; Peiris and Guan, 2004). Second, there must be enough compatibility between the pathogen and the new host species to allow replication and shedding to infect other individuals of the same species (host-

pathogen interactions). For viruses for instance, crucial to infection are cell receptors on the host cells that allow virus entry. The ability of certain viral strains (e.g. avian influenza viruses) to replicate in a new host (e.g. humans) may be limited by lack/insufficiency of receptors that bind these viral strains (Suzuki, 2005; Ibricevic *et al.*, 2006). Third, the pathogen must be sufficiently transmissible between individuals within the recipient host species (host-host intra-specific interactions). This relates to the value of R_0 in the new host population (Anderson and May, 1991; Woolhouse *et al.*, 2005). If R_0 is < 1, a large proportion of infections will be acquired directly from the donor species (Woolhouse *et al.*, 2005). If R_0 is > 1, most infections will be acquired from within the new host population (i.e. self-sustaining transmission), with potential for a major epidemic to occur (Woolhouse *et al.*, 2005). Hence, for a highly pathogenic avian influenza virus to cause a human pandemic, human-to-human transmission must occur above self-sustaining levels (Ferguson *et al.*, 2004). Despite the uncertainty in surveillance data and the impossibility to predict the R_0 of any future pandemic strain, estimates of R_0 are below the $R_0 = 1$ threshold, indicating that H5N1 is currently incapable of sustained transmission in humans (Ferguson *et al.*, 2004). Every individual co-infected with human and avian strains will then represent a possibility that in that one person a reassortment event will occur. The risk of a reassortment event is therefore proportional to the number of co-infected individuals. Ferguson *et al.* (2004) estimate that 600 human infections would be required for a 50% chance of reassortment, and around 45 for a 5% chance. Beside the fact that reassortment is a rare outcome of co-infection, other processes (i.e. ecological and immunological) reduce the chance of co-infection (Ferguson *et al.*, 2003). Considering the possibility of a new transmissible ($R_0 > 1$) pandemic strain arising, models predict that a prepared response with targeted antiviral prophylaxis and 'social distance measures' (i.e. quarantine or other measures to reduce contact rates within the population) could contain an outbreak, provided that R_0 is below 2 (Ferguson *et al.*, 2005; Longini *et al.*, 2005).

Given a reservoir that may comprise a range of epidemiologically connected populations or environments (Haydon *et al.*, 2002a), difficulties may arise in identifying all its components. However, an understanding of maintenance (i.e. populations larger than the CCS) and nonmaintenance (i.e. populations smaller than

the CCS) constituent populations (Figure 1.1) is crucial to the design of disease control measures, since directing control efforts within the nonmaintenance components with no control in the maintenance components would not successfully control or eliminate infection.

Haydon *et al.* (2002a) recognise many of the problems associated with identifying reservoirs of infection in the field and propose a number of 'practical indicators' that may be used:

(i) Epidemiological evidence of association (e.g. through observations or identification of risk factors that may suggest links between nontarget and target populations and analysis of temporal/spatial patterns of disease/infection).

(ii) Evidence of natural infection in nontarget and target populations by identifying previous (e.g. antibody detection) or current (e.g. demonstration of the pathogen within the host) infection.

(iii) Evidence of pathogen persistence in nontarget populations (e.g. through longitudinal studies to assess long-term temporal and spatial patterns of infection).

(iv) Evidence of direct or indirect nontarget-to-target transmission: for instance genetic/antigenic characteristics of pathogens isolated from different populations may elucidate transmission links within and between host populations (e.g. nontarget-to-target transmission).

(v) Intervention trials (e.g. vaccination, treatment, barriers) that may be directed at controlling infection within the putative reservoir or maintenance component(s). The success of such interventions (i.e. clearance of infection from all populations, including the target) will provide confirmation of the original assumptions concerning the identity of the reservoir or its maintenance constituents.

None of the above approaches may independently allow unequivocal identification of the reservoir. However, a combination of approaches may lead to reasonable evidence of its existence and identity.

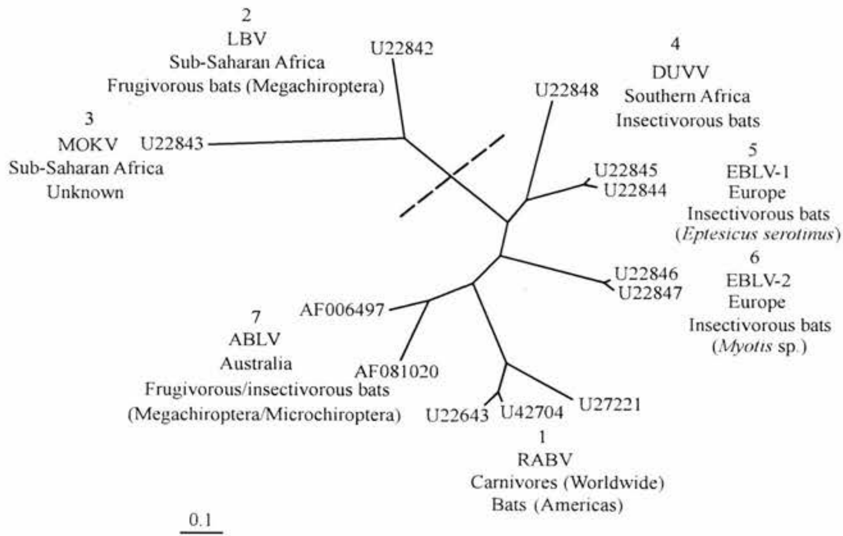
1.2 Rabies

Rabies is an acute, progressive encephalitis caused by a neurotropic RNA virus to which all mammals are more or less susceptible. The disease has been the subject of intense research and many studies and extensive overviews of the existing literature have recently been published in the form of reviews and texts (examples include Rupprecht *et al.*, 2002; Hemachudha *et al.*, 2002; Jackson and Wunner, 2002; Jackson, 2003; King *et al.*, 2004). Only aspects of the disease most relevant to this work are highlighted below.

1.2.1 Rabies virus

RABV is the prototype member of the *Lyssavirus* genus (*Lyssa*: rage) in the family *Rhabdoviridae* (*Rhabdos*: rod). Seven genotypes have so far been delineated within the genus (Bourhy *et al.*, 1992, 1993a,b; Kissi *et al.*, 1995; Gould *et al.*, 1998). RABV (genotype 1, serotype 1) is distributed throughout most of the world. In contrast, the rabies-related viruses are restricted in their geographical distribution to Africa (Lagos bat virus [LBV], genotype 2, serotype 2; Mokola virus [MOKV], genotype 3, serotype 3 and Duvenhage virus [DUVV], genotype 4, serotype 4), Europe (European bat lyssaviruses type 1 [EBLV-1], genotype 5 and type 2 [EBLV-2], genotype 6), and Australia (Australian bat lyssavirus [ABLV], genotype 7) (Figure 1.2A: the phylogeny was reconstructed using *Lyssavirus* nucleotide sequences available from GenBank). Four additional lyssaviruses have been isolated from bats in Eurasia and have been proposed as new members of the genus: Aravan virus (ARAV), Khujand virus (KHUV), Irkut virus (IRKV) and West Caucasian bat virus (WCBV) (Kuzmin *et al.*, 1992; Botvinkin *et al.*, 1996; Kuzmin *et al.*, 2001, 2003; Arai *et al.*, 2003; Botvinkin *et al.*, 2003; Kuzmin *et al.*, 2005) (Figure 1.2B: the phylogeny was reconstructed using *Lyssavirus* nucleotide sequences available from GenBank).

A



B

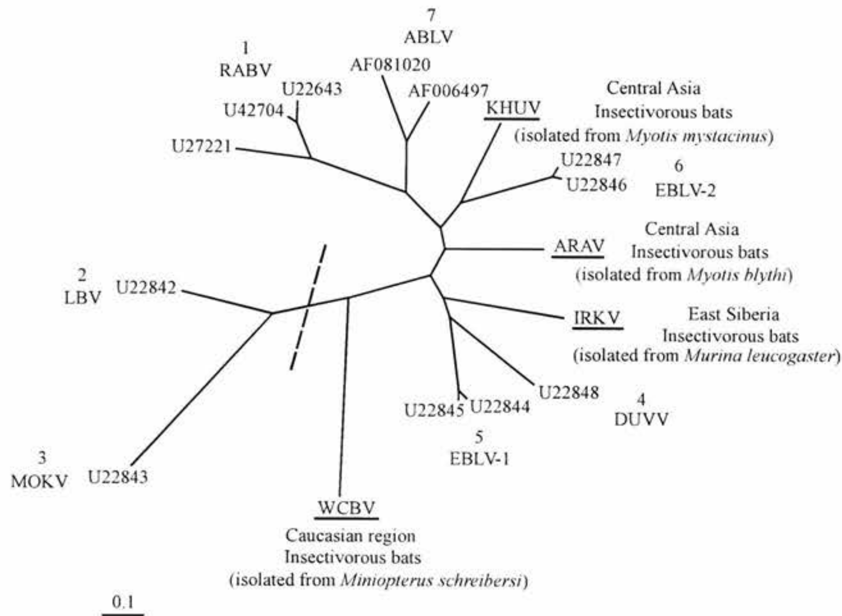


Figure 1.2. (A) Phylogenetic relationships between representative isolates of the genus *Lyssavirus* based on the complete nucleoprotein (N) gene sequences. (B) Lyssavirus phylogeny including the newly-described Eurasian viruses which may be characterised as new genotypes (underlined). The isolates are designated by GenBank accession numbers or strain names (Bourhy *et al.*, 1992, 1993a, 1999; Kissi *et al.*, 1995; Nadin-Davis *et al.*, 1996; Gould *et al.*, 1998, 2002; Kuzmin *et al.*, 2003, 2005). The relationships are presented as unrooted phylograms based on Bayesian Markov chain Monte Carlo (MCMC) analysis. The scale indicates branch-length expressed as the expected number of substitutions per site. The dashed line shows the separation of phylogroups 1 (genotypes 1, 4, 5, 6 and 7) and 2 (genotypes 2 and 3) proposed by Badrane *et al.* (2001). RABV = rabies virus; LBV = Lagos bat virus; MOKV = Mokola virus; DUVV = Duvenhage virus; EBLV = European bat lyssavirus; ABLV = Australian bat lyssavirus; ARAV = Aravan virus; KHUV = Khujand virus; IRKV = Irkut virus; WCBV = West Caucasian bat virus.

1.2.2 Lyssavirus genome

The *Lyssavirus* genome is a single strand of negative sense RNA (~12 kilo base pairs) comprising five structural genes encoding the nucleoprotein (N), phosphoprotein (P), matrix or membrane protein (M), glycoprotein (G) and RNA transcriptase (L), and the vestigial non-coding pseudogene (Ψ) (Tordo *et al.*, 1986a,b; Tordo *et al.*, 1988; Conzelmann *et al.*, 1990). The N, P, and L form the ribonucleoprotein (RNP) core that in association with the M is condensed into the typical bullet-shaped particle characteristic of rhabdoviruses. The RNP-M structure is surrounded by a bilayered lipoprotein envelope in which the surface trimeric G spikes are anchored (Wunner *et al.*, 1988). The N is involved in the regulation of transcription and replication, the P is important in transcription and replication and for interactions with cellular protein components during axoplasmic transport, the L is responsible for the majority of enzymatic activities involved in transcription and replication, the M is a multi-functional protein playing a crucial role in virus assembly and budding, and the G reacts with host cell receptors and is important in determining pathogenicity (Dietzschold *et al.*, 1983; Emerson, 1987; Tuffereau *et al.*, 1989; Tordo and Kouknetzoff, 1993; Tuffereau *et al.*, 1998; Mebatsion *et al.*, 1999; Gaudin *et al.*, 1999; Poisson *et al.*, 2001). The level of conservation of the proteins is highly variable, with the N being the most conserved followed by the M, G and P (Bourhy *et al.*, 1993a). The degree of conservation also varies within specific regions. For instance, a relatively high degree of genetic diversity within the amino- and carboxy-terminal domains of the N within and between genotypes has been described (Smith *et al.*, 1992; Kissi *et al.*, 1995; Velasco-Villa *et al.*, 2005). Similarly, the G consists of a well-conserved ectodomain and a more variable transmembrane region and endodomain (Tordo and Kouknetzoff, 1993), and a highly variable central region of the P gene has recently been identified (Nadin-Davis *et al.*, 2002). The choice of the genomic region for typing and analysis is determined by the purpose of the investigation as discussed in section 1.2.5.

1.2.3 Rabies transmission

The bite route is still considered the most important mode of transmission leading to infection (McKendrick, 1941). The significance of non-bite transmission (e.g. oral exposure or aerosol inhalation) in natural rabies of animals and humans remains

uncertain, although it has been occasionally reported. For instance, oral exposure, which may occur naturally by consumption of carcasses of rabid animals, might elicit fatal or immunising infections, depending on dose and host susceptibility (Baer *et al.*, 1971; Ramsden and Johnston, 1975; Wandeler, 1993). In kudu (*Tragelaphus strepsiceros*), field observations and transmission experiments involving transfer of infective saliva to the oral mucosa of susceptible animals indicated that oral transmission of virus may also occur by mouth-licking and grooming (Barnard and Hassel, 1981; Hübschle, 1988). Exposure of this kind has also been suggested for wild carnivores (Gascoyne *et al.*, 1993a; Maas, 1993; Kat *et al.*, 1995; East *et al.*, 2001; Nel *et al.*, 2005). Aerosol transmission of rabies has been reported to occur rarely under particular circumstances (i.e. in caves containing densely packed aggregations of bats or in laboratory accidents; Constantine, 1962; Winkler *et al.*, 1973; Tillotson *et al.*, 1977).

1.2.4 Rabies epidemiology

Rabies occurs on all continents except Antarctica. RABV has been isolated from nearly all mammalian orders, but not all mammals are capable of independent maintenance. Typically, the virus forms two kinds of association with its host species.

In the first, within a given geographic area, distinct virus variants within a genotype tend to establish sustained transmission in a particular mammalian species (the reservoir host) that is responsible for supporting the virus cycle (Carey, 1985; Smith, 1989; Rupprecht *et al.*, 1991; Bourhy *et al.*, 1993b; King *et al.*, 1994; Wandeler *et al.*, 1994). This concept is known as compartmentalisation and is well-documented in some parts of the world (Smith *et al.*, 1995) (Figure 1.3).

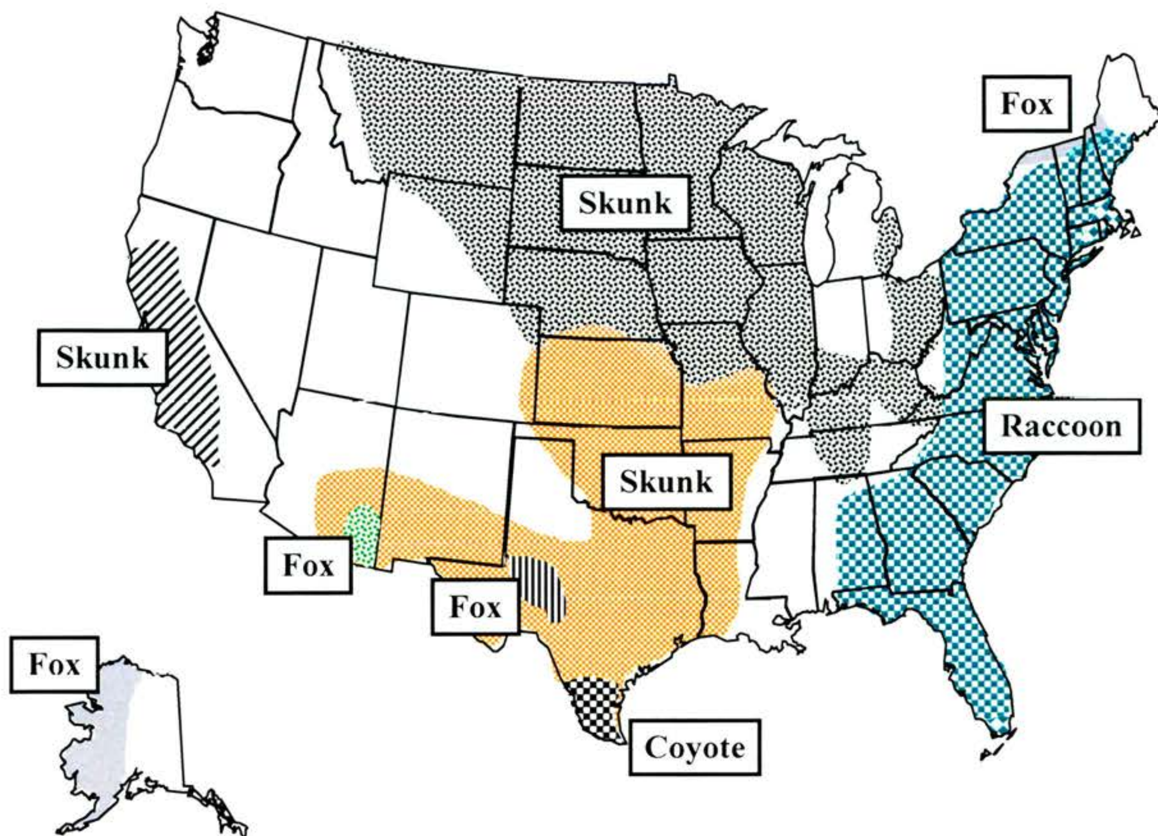


Figure 1.3. Geographical distribution of rabies virus variants associated with major terrestrial wildlife reservoirs of rabies in the United States (US). Source: Centers for Disease Control and Prevention (CDC), Atlanta, US.

Adaptation between virus and host has been proposed to explain this phenomenon. There is indeed evidence that susceptibility varies between virus variants and hosts (Sikes, 1962, 1970), although the mechanisms responsible for species differences in susceptibility and the link between host susceptibility and specific properties of virus variants are not well understood. For example, foxes (*Vulpes vulpes*) are highly susceptible to fox viruses and show a greater degree of salivary shedding, hence potential to transmit to conspecifics, than foxes infected with canine, bat or raccoon dog (*Procyon lotor*) viruses (Blancou, 1988). The virus generally produces infection in its host with onset of clinical disease and fatal outcome. The high mortality caused by the virus means that beside virus adaptation other factors are required for a host to be capable of independent maintenance. With the exception of bat rabies, infections of this type are most notably observed in species in the order *Carnivora* (Table 1.2), which have some common demographic and ecological attributes, allowing a

continuous supply of susceptible animals to maintain cycles of infection. They are small to medium sized omnivores, with opportunistic foraging behaviour that allows them to inhabit a variety of habitats and reach high population densities close to human settlements (Wandeler, 1991; Wandeler *et al.*, 1994). They are also characterised by high birth and death rates and hence high turn-over rates.

Table 1.2. Carnivore species considered capable of independent maintenance of rabies worldwide.

Species	Area	Reference
Canidae		
Domestic dog (*Canis familiaris*)	Middle East, Africa, Asia, Latin America	WHO, 1999
Red fox (*Vulpes vulpes*)	North America, Eurasia	Tierkel *et al*., 1958; Tabel *et al*., 1974; Rosatte, 1988; Blancou *et al*., 1991; Bourhy *et al*., 1999
Arctic fox (*Alopex lagopus*)	Arctic region	Tabel *et al*., 1974; Crandell, 1991
Gray fox (*Urocyon cinereoargenteus*)	North America	Tierkel *et al*., 1958; Carey *et al*., 1978
Bat-eared fox (*Otocyon megalotis*)	southern Africa (South Africa)	Thomson and Meredith, 1993
Raccoon dog (*Nyctereutes procyonoides*)	Eurasia	Cherkasskiy, 1988; Bourhy *et al*., 1999
Coyote (*Canis latrans*)	North America	Clark *et al*., 1994
Black-backed jackal (*Canis mesomelas*)	southern Africa (Zimbabwe)	Bingham *et al*., 1999a
Side-striped jackal (*Canis adustus*)	southern Africa (Zimbabwe)	Bingham *et al*., 1999a
Herpestidae		
Small Indian mongoose (*Herpestes auropunctatus*)	Caribbean	Everard and Everard, 1985; Smith *et al*., 1992
Yellow mongoose (*Cynictis penicillata*)	southern Africa (South Africa)	Swanepoel *et al*., 1993; Taylor, 1993
Slender mongoose (*Herpestes* [*Galerella*] *sanguinea*)	southern Africa (Zimbabwe)	Foggin, 1988; Bingham *et al*., 2001
Mephitidae		
Skunk (*Mephitis mephitis*)	North America	Charlton *et al*., 1991
Procyonidae		
Raccoon (*Procyon lotor*)	North America	Winkler and Jenkins, 1991

The second kind of virus-host interaction occurs when other species acquire infection from the major host. Such cross-species transmission events usually result in dead-end 'spill-over' infections (e.g. humans), but can occasionally lead to the establishment of stable infection cycles into a new host species, depending on favourable ecological (e.g. demographic changes), genetic (e.g. involving host susceptibility to infection or viral infectiousness) or behavioural factors (e.g. naturally aggressive biting behaviour). Important examples of such successful host switches involved the cross-species transfer of the virus from the domestic dog to the red fox and from the red fox (or the domestic dog) to the raccoon dog (*Nyctereutes procyonoides*) during the 20th century in Europe (Anderson *et al.*, 1981; Bourhy *et al.*, 1999).

The typical traits of RABV (i.e. short infectious cycles, high pathogenicity and mortality rates) prohibit its persistence in small-sized populations of endangered wild carnivores for instance, as infection will repeatedly fade out due to the depletion of susceptible individuals following an epidemic (Anderson and May, 1991). These populations are however threatened by infection following 'spill-over' from more abundant reservoir hosts. Population viability analyses have shown that highly pathogenic viruses, such as RABV, pose an immediate extiction risk to these populations (Woodroffe, 1999; Haydon *et al.*, 2002b). 'Spill-over' transmission from domestic animals is a common feature of diseases that threaten wildlife (Woodroffe, 1999) and most of the disease-related extinctions and major population declines in wild canids have followed this pattern (e.g. the African wild dog and the Ethiopian wolf [*Canis simensis*]: Gascoyne *et al.*, 1993a; Kat *et al.*, 1995; Sillero-Zubiri *et al.*, 1996; Woodroffe, 2001; Randall *et al.*, 2004).

An alternative mechanism proposed to account for rabies maintenance in carnivores includes an infectious healthy carrier state, where animals actively shed virus in the saliva for prolonged periods, but remain clinically normal (Andral, 1964; Fekadu, 1991). Cleaveland and Dye (1995) showed theoretically that carrier dogs would have a dramatic impact on rabies dynamics and persistence but, to date, experimental or field observations supporting such a state in domestic or wild animal populations are scanty and its epidemiological significance is believed to be limited. In rare instances, naturally infected dogs have been documented to excrete virus in the saliva

(Fekadu, 1972; Aghomo *et al.*, 1989) and a dog excreted virus for months after recovery from experimental rabies (Fekadu *et al.*, 1981), but there was no evidence for a carrier state in large-scale studies of healthy dogs in Ethiopia and Nigeria (T. Mebatsion, unpublished data). One report exists of non-lethal rabies infection in a wild-living carnivore, the Serengeti spotted hyaena (*Crocuta crocuta*) (East *et al.*, 2001). Although in this study healthy hyaenas were demonstrated to be saliva-positive for RABV by reverse transcriptase-polymerase chain reaction (RT-PCR), virus isolation was not successful.

Throughout much of the developing world, rabies is most often reported and confirmed in dogs, which are considered to play a critical role in disease maintenance and transmission to humans, accounting for over 90% of human rabies cases (WHO, 1999). Knobel *et al.* (2005) report an estimated annual human mortality from endemic canine rabies of 55,000 deaths in Africa and Asia alone, with 56% and 44% of the deaths estimated to occur in Asia and Africa respectively. Rabies poses the major threat in areas where dog and human populations reach high densities. Even in parts of the world where dog rabies has declined and wildlife rabies predominates, domestic dogs may serve as links between wildlife and humans and remain therefore an important risk for transmitting rabies to humans.

In addition to the domestic dog, wildlife hosts in parts of southern Africa are considered capable of sustaining independent rabies cycles. Two variants ('biotypes') of rabies are recognised, the mongoose biotype and the canid biotype (Swanepoel *et al.*, 1993; Nel *et al.*, 1993; King *et al.*, 1993, 1994; von Teichman *et al.*, 1995; Johnson *et al.*, 2004a; Sabeta *et al.*, 2003; Nel *et al.*, 2005). The yellow mongoose (*Cynictis penicillata*) and the slender mongoose (*Herpestes* [*Galerella*] *sanguinea*) are considered maintenance hosts of the mongoose virus (Swanepoel *et al.*, 1993; Taylor, 1993; Foggin, 1988; Bingham *et al.*, 2001), which is thought to be well adapted to these small carnivores. Canid viruses infect dogs, jackal species (*Canis adustus*, *C. mesomelas*) and the bat-eared fox (*Otocyon megalotis*) and, although wild canids have been implicated as independent maintenance hosts in some areas (Thomson and Meredith, 1993; Bingham *et al.*, 1999a), their role has been debated (Cleaveland and Dye, 1995; Rhodes *et al.*, 1998; Bingham *et al.*, 1999b). In particular, the controversy has centered on whether jackal species in

Zimbabwe may sustain infection independently of dogs. Bingham *et al.* (1999a) reported that during the period 1950-1996 jackals accounted for > 25% of all confirmed cases, second only to domestic dogs and spatial and temporal trends suggested independent cycles of infection. However, Cleaveland and Dye (1995) showed that jackal cases followed, rather than preceded, dog cases with a lag of one year indicating that epidemics in jackals were driven by epidemics in dogs. Similarly, mathematical models suggested that the *C. adustus* population may be unable to support rabies infection without frequent re-introductions (Rhodes *et al.*, 1998).

1.2.5 Molecular epidemiology and phylogeny of rabies virus in Africa

Beginning in the early 1990s, genetic typing has been applied as a method to investigate the molecular epidemiology of rabies at global, national and regional levels. According to the purpose of the investigation (e.g. global or highly detailed regional and local epidemiological studies), partial or complete sequences of the N gene (Smith *et al.*, 1992; Nadin-Davis *et al.*, 1994; Smith *et al.*, 1995; Kissi *et al.*, 1995; de Mattos *et al.*, 1996, 1999; Bourhy *et al.*, 1999; Ito *et al.*, 1999; Johnson *et al.*, 2004a,b; Velasco-Villa *et al.*, 2005), G gene (Bourhy *et al.*, 1999; Nadin-Davis *et al.*, 1999; Real *et al.*, 2005a) and neighbouring G-L intergenic region (Sacramento *et al.*, 1991, 1992; von Teichman *et al.*, 1995; Sabeta *et al.*, 2003; Nel *et al.*, 2005), and P gene (Nadin-Davis *et al.*, 1997, 2002) have been employed. For example, sequence analyses of more conserved regions of the genome (e.g. the N gene) have made it possible to reconstruct the historical events leading to the introduction of rabies into an area (Smith *et al.*, 1992), whereas analyses of more variable regions (e.g. the G gene) have allowed reconstructing more recent chains of transmission (Nadin-Davis *et al.*, 1999).

In Africa, two studies have investigated the phylogeny of rabies on a continental scale. The study of Smith *et al.* (1992), based on partial sequences of the N gene, was the first to describe clustering of genetically linked RABVs originating from around the world, the 'cosmopolitan lineage', which is believed to have arisen initially in Europe in the 17th century before spreading worldwide due to human activity through dog importation. This lineage includes isolates from Europe, the Middle East, Africa, Asia, and the Americas and, although it appears to be maintained mainly in dogs

(Africa, Asia and Latin America), it has also 'adapted' to wildlife species (e.g. skunks [*Mephitis mephitis*], gray foxes [*Urocyon cinereoargenteus*] and coyotes [*Canis latrans*] in North America) (Figure 1.4: the phylogeny was reconstructed using RABV nucleotide sequences available from GenBank).

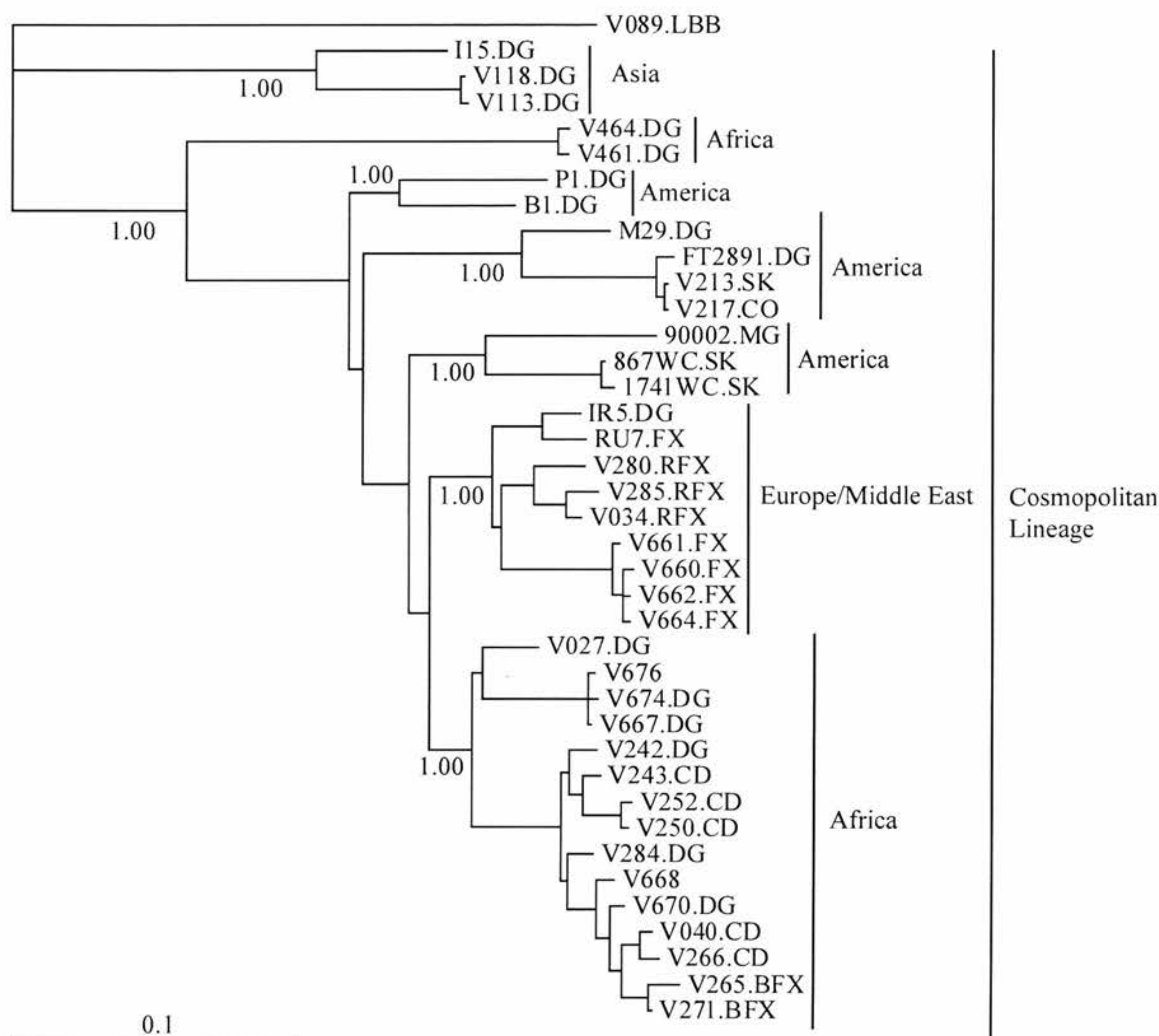


Figure 1.4. Majority-rule consensus tree of phosphoprotein (P) gene complete sequences for previously described rabies virus isolates of the cosmopolitan lineage (Nadin-Davis *et al.*, 1997, 2002) based on Bayesian Markov chain Monte Carlo (MCMC) analysis. The isolates are designated by the specimen name followed by a suffix indicating the species of origin, where known (LBB, little brown bat; DG, dog; SK, skunk; CO, coyote; MG, mongoose; FX, fox of undefined species; RFX, red fox; CD, canid; BFX, bat-eared fox), except for an isolate (M29.DG) recovered from a cat, for which the suffix indicates the presumed host reservoir. The tree is rooted with isolate V089.LBB, defined as outgroup. The scale indicates branch-length expressed as the expected number of substitutions per site. Numbers on branches indicate Bayesian bootstrap values.

A more detailed phylogenetic investigation of African RABVs was carried out by Kissi *et al.* (1995) who used the entire N gene coding region and analysed a large number of African isolates. Distinct African lineages were identified (Figure 1.5: the phylogeny was reconstructed using RABV nucleotide sequences available from GenBank):

(i) Africa 1, associated with canids and subdivided into 2 subgroups: (1) Africa 1a, broadly distributed in north-east Africa and (2) Africa 1b, broadly distributed in south-east Africa.

(ii) Africa 2, associated with canids and broadly distributed in western Africa.

(iii) Africa 3, associated with herpestid (mongoose) isolates from South Africa.

(iv) putative Africa 4, defined by a single isolate from Egypt that segregated distinctly.

Viruses of lineages Africa 1a and 1b and viruses from Europe and the Middle East showed a high degree of genetic relatedness supporting the view of their emergence from a common ancestor and the hypothesis of the introduction of the cosmopolitan lineage into Africa via importation of rabies-infected companion animals during European colonisation in the 19th century. By contrast, Africa 2 and 3 are believed to have arisen independently from different progenitor viruses and to have been present in Africa before importation of the cosmopolitan variant. They would therefore represent older lineages.

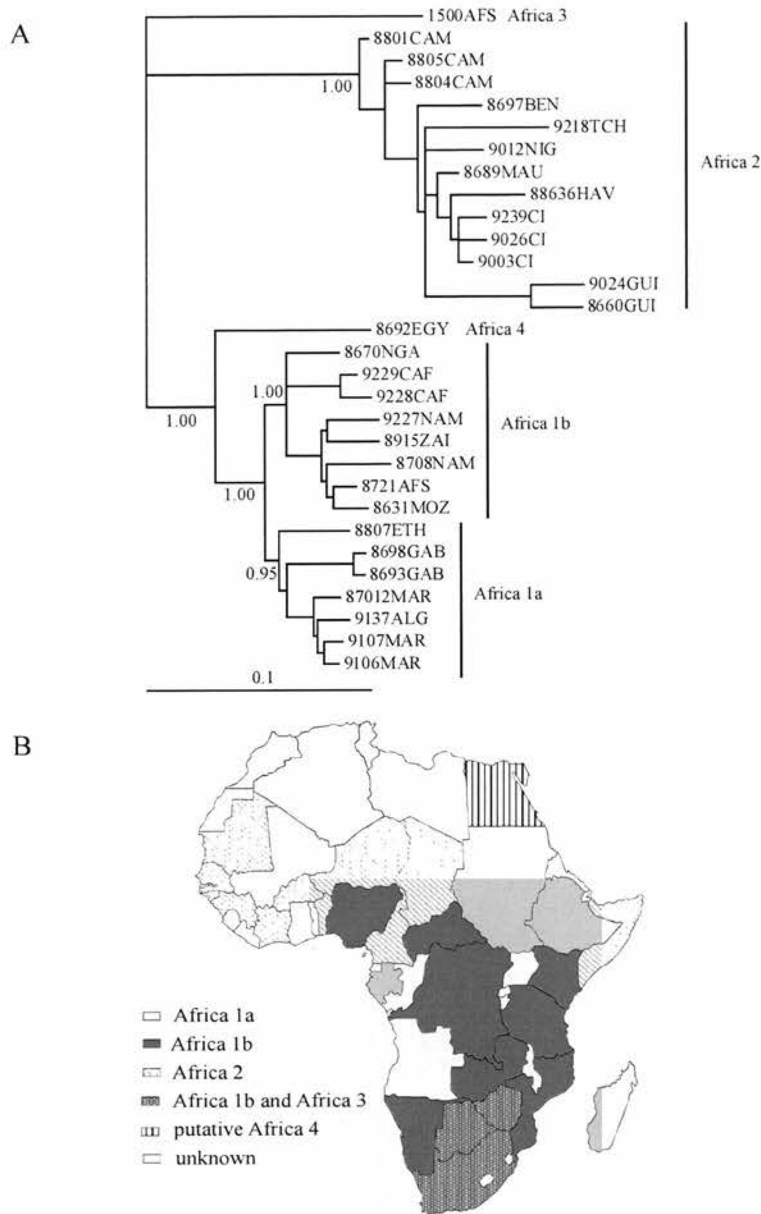


Figure 1.5. (A) Majority-rule consensus tree of nucleoprotein (N) gene complete sequences for previously described rabies virus (RABV) isolates representative of the African lineages (Kissi *et al.*, 1995) based on Bayesian Markov chain Monte Carlo (MCMC) analysis. The isolates are designated by the strain names. The scale indicates branch-length expressed as the expected number of substitutions per site. Numbers on branches indicate Bayesian bootstrap values. (B) Their geographical distribution. Data for Sudan, Botswana and Zimbabwe were obtained from Johnson *et al.* (2004a and b) and Nel *et al.* (2005). AFS = South Africa; ALG = Algeria; BEN = Benin; CAF = Central African Republic; CAM = Cameroon; CI = Ivory Coast; EGY = Egypt; ETH = Ethiopia; GAB = Gabon; GUI = Guinea; HAV = Burkina Faso; MAR = Morocco; MAU = Mauritania; MOZ = Mozambico; NAM = Namibia; NGA = Nigeria; NIG = Niger; TCH = Tchad; ZAI = Zaire.

Further studies in southern Africa confirmed the existence of two phylogenetically distinct groups, one associated with canid viruses and belonging to the cosmopolitan lineage, and one associated with mongoose viruses and appearing to be unique to the African subcontinent (Nel *et al.*, 1993; von Teichman *et al.*, 1995; Sabeta *et al.*, 2003; Johnson *et al.*, 2004a; Nel *et al.*, 2005). The higher antigenic and genetic diversity of the latter indicated a more ancient origin.

1.2.6 Rabies surveillance and diagnosis

In many parts of the less-developed world, the surveillance and diagnosis of disease in domestic and wild animals are hampered by a number of logistical, practical and technical constraints, making it difficult to detect trends in disease incidence and to implement effective strategies for treatment, control or prevention of disease. Wildlife disease monitoring in tropical areas is particularly problematic as the abundance of scavengers and high ambient temperatures make it difficult to collect fresh diagnostic samples from carcasses. When diagnostic centres or specific laboratory tests are unavailable locally, samples have to be shipped to reference laboratories outside the country, with the associated problems of delayed diagnosis and management decisions, high transport costs, the risk of sample mishandling, cumbersome exportation and importation procedures and non-availability of appropriate shipping material (e.g. dry ice). To overcome these problems, alternative preservation techniques and reliable in-country diagnostic testing procedures are required.

One of the most important examples is rabies, where optimal conditions for surveillance are met in only a few countries, with an obvious dichotomy between the more and less developed world. Two key outcomes emerge. First, ineffective surveillance mechanisms and difficulties in obtaining diagnostic results from field material have led to widespread under-reporting of disease. As a result, the true public health impact of the disease in much of the developing world has been greatly under-estimated (Dodet *et al.*, 2001; Cleaveland *et al.*, 2002; Coleman *et al.*, 2004; Knobel *et al.*, 2005) and there has been a low level of political commitment generated for rabies control efforts. Second, the absence of a confirmatory diagnostic test can result in the inappropriate management of animal bite injuries, with human mortality a potential consequence of delays in rabies post-exposure prophylaxis

(PEP) and unnecessary administration of PEP. The latter is a particular concern in countries where the use of antirabies biological agents is limited by their high cost.

A number of rabies diagnostic methods have been described and thoroughly reviewed (Meslin *et al.*, 1996; Trimarchi and Smith, 2002). The vast majority of these techniques are of limited availability or impractical in developing countries. The fluorescent antibody (FA) technique, which is recommended by both the World Health Organization (WHO) and the Office International des Epizooties (OIE), remains the gold standard rabies diagnostic test since it is rapid, reliable, accurate, economical, sensitive and highly specific (Dean *et al.*, 1996; Trimarchi and Smith, 2002). The test is carried out on brain touch impressions (Figure 1.6) or smears by application of fluorescent-labelled anti-rabies antibodies. Examination of the smears under ultra-violet microscopy reveals fluorescence associated with particulate antigen located in the cytoplasm of infected neurons (bright apple green) (Figure 1.7). A good quality fluorescence microscope is critical to the highest sensitivity and specificity of the FA test. Proper microscope function requires a high standard of maintenance. These factors, a lack of trained and experienced operators, and the frequent unavailability of anti-rabies conjugates limit the use of this technique in many developing country settings (Dodet *et al.*, 2001).

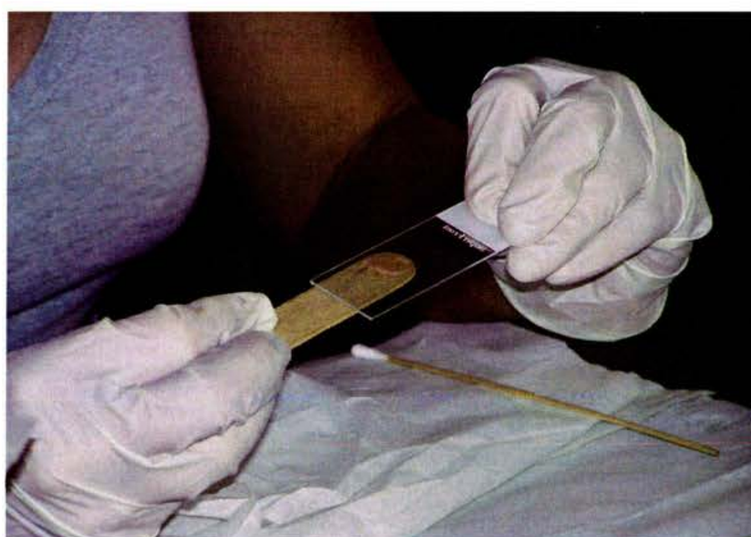


Figure 1.6. Preparation of a brain touch impression.

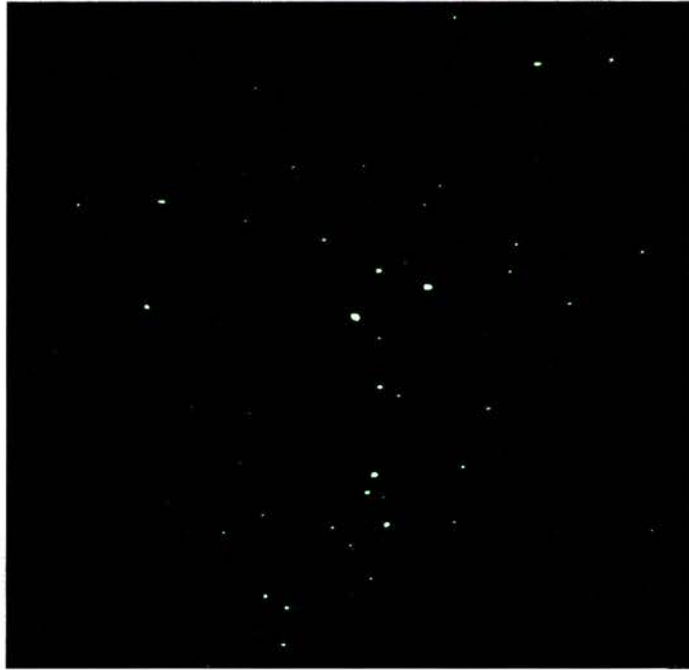


Figure 1.7. Immunofluorescent apple-green viral inclusions in a rabies-positive Tanzanian domestic dog brain processed in the fluorescent antibody (FA) test. Magnification, X200.

WHO has addressed the issues of increasing in-country capabilities for infectious diseases surveillance and diagnosis, including rabies infection. Attempts have been made by WHO collaborating centres for reference and research on rabies to develop simple and cheap techniques for sample preservation and rapid post-mortem diagnosis that have been proposed for laboratories with limited storage and/or diagnostic resources. Such techniques will be discussed below with emphasis on a novel immunohistochemical test, the evaluation of which was one of the objectives of this study. The potential value of this technique for developing country settings is highlighted.

1.2.6.1 Sample preservation

For the routine diagnosis of rabies, glycerol saline has proved to be a convenient preservative in situations where refrigeration or freezing facilities are not promptly available (Barrat and Blancou, 1988; Barrat, 1996). The medium does not inactivate the virus and current rabies diagnostic techniques such as the FA test (Barrat, 1996), *in vivo* and *in vitro* virus isolation tests (Barrat *et al.*, 1988) and molecular methods for detection of viral RNA (Aguilar-Setien *et al.*, 2003) may be used. However, when

performing the FA test, reduction in immunofluorescence intensity has been observed even with extensive washing (Lennette *et al.*, 1965).

Molecular methods may be successfully applied to brain tissue dried on filter paper stored at room temperature for relatively long periods of time (Wacharapluesadee *et al.*, 2003). Experimental infectivity studies showed that such preservation enables safe transportation of infected material (A.R. Fooks, unpublished data).

1.2.6.2 Rabies diagnostic methods suitable for developing countries

An enzyme-linked immunosorbent assay (ELISA), the rabies rapid enzyme immunodiagnosis (RREID), has been developed for the detection of rabies antigen by means of a specific antigen-antibody reaction tagged with a visible label and used for many years in some laboratories (Perrin *et al.*, 1986; Bourhy *et al.*, 1989). Rabies anti-nucleocapsid (N) antibody is used to bind rabies nucleocapsid in positive brain samples. The bound viral antigen is quantified with the same anti-N antibody conjugated to peroxidase. Manual or automated (by using a spectrophotometer) readings are then made. Results can be achieved within 4 hours. Evaluation of RREID in rabies laboratories in Europe, North America and in developing countries showed a sensitivity (Se) and specificity (Sp) in the range of 95.0%-96.7% and 96.4%-99.8% respectively (Perrin and Sureau, 1987; Bourhy *et al.*, 1989), although a higher Se (100.0%) was reported by other workers (Miranda and Robles, 1991). The technique is sensitive in detecting antigen in decomposed samples, which are not suitable for immunofluorescence (Saxena *et al.*, 1989). RREID has been proposed as a confirmatory test for the FA technique, for large epidemiological studies and laboratories that are not equipped for performing the FA test. However, because of its lower Se, it has been emphasised that the technique should not replace the FA test where the latter is routinely performed (Perrin and Sureau, 1987; Bourhy *et al.*, 1989). Although the test is currently not commercially available, previously produced commercial kits were well-suited for testing large numbers of samples, but not to test a small number of specimens at one time. Another drawback of the technique is that it does not appear to perform well on glycerol-preserved samples. Saxena *et al.* (1989) observed 93.0% correlation in the case of fresh brains and 71.0% correlation in samples preserved in glycerol solution. Finally, the 'routine'

version of the test is not sensitive to rabies-related viruses, but only detects genotype 1 lyssaviruses. Therefore, a modified RREID (RREID-lyssa) has been developed (Perrin *et al.*, 1992; Oelofsen and Smith, 1993).

Other simple and rapid techniques have been described to successfully detect viral antigen, including a latex agglutination (LA) test (Se = 95.2% and Sp = 98.7%: Kasempimolporn *et al.*, 2000), an enzyme immuno-assay (EIA) (98.4% concordance with FA test; Vasanth *et al.*, 2004) and a dot blot enzyme immunoassay (DIA) (Se = 97.4% and Sp = 100.0%: Jayakumar *et al.*, 1995; Madhusudana *et al.*, 2004). All of the above have the potential to be easily applied in the field and further evaluation studies are in progress.

Immunohistochemistry (IHC) for rabies antigen detection has been applied to formalin-fixed, paraffin-embedded brain sections (Atanasiu *et al.*, 1971; Fekadu *et al.*, 1988; Feiden *et al.*, 1988; Warner *et al.*, 1999) with recent modifications to achieve greater sensitivity (Hamir *et al.*, 1995, 1996). The Rabies Section of the Centers for Disease Control and Prevention (CDC), Atlanta, US has developed and optimised a rapid immunohistochemical test (RIT) that applies IHC to brain impressions (Figure 1.6). RIT was initially developed as an indirect test (Figure 1.8A) using a cocktail of rabies anti-N monoclonal antibodies (MAbs) produced in mouse ascites fluid (Niezgoda and Rupprecht, 1999). The technique was compared with the FA test in a collaborative study involving five diagnostic laboratories in the US and over 98.0% agreement was obtained (Niezgoda *et al.*, 2002). In order to further increase the test Se and Sp and minimise the staining time, the test has been subsequently modified over the period 2001-2004 with three important improvements: (i) replacement of MAbs produced *in vivo* with a cocktail of highly purified and concentrated MAbs produced *in vitro*; (ii) direct labelling of anti-N MAbs with biotin that has led to a direct test (dRIT) (Figure 1.8B), allowing a diagnosis to be made in less than one hour; and (iii) expansion of the concept using the indirect RIT and a panel of antirabies MAbs to permit antigenic typing (Niezgoda *et al.*, 2004).

The test has been designed for potential use in confirmation of the FA test, according to the US national standard operating procedure for the diagnosis of rabies in animals

(http://www.cdc.gov/ncidod/dvrd/rabies/Professional/publications/DFA_diagnosis/DFA_protocol-b.htm). Furthermore, several characteristics of the technique underline its potential to be used worldwide in laboratories with limited diagnostic infrastructures and in field conditions:

(i) Being an immunohistochemical colorimetric method, the product of the reaction can be analysed with an ordinary light microscope, with RABV antigen appearing as magenta inclusions against the blue neuronal background (Figure 1.9).

(ii) When performing the FA test, acetone is used as a fixative. Formalin, the gold standard fixative in IHC, allows for greater biosafety (i.e. deaths occurred in mice inoculated intracerebrally with swab suspensions taken from brain impressions after fixation in acetone for 10 minutes, but not from formalin-fixed slides: M. Niezgoda, personal communication). Furthermore, an explosion proof -20°C freezer is required for acetone fixation of impression slides and storage of acetone, whereas formalin fixation is performed at ambient temperature.

(iii) The cocktail of high-avidity MAbs recognises antigens from a global spectrum of lyssaviruses, including different genotype 1 RABV variants associated with terrestrial wildlife species and insectivorous bats in the US (Table 1.3), all representative rabies-related lyssaviruses and the newly-described bat lyssaviruses (Fig 1.9C) (Niezgoda and Rupprecht, 1999).

(iv) RIT can be successfully performed on poorly-preserved samples (Figure 1.9D), unlike the FA test where interpretation can be difficult in the presence of non-specific fluorescence.

(v) All the reagents necessary to perform the test can be stored at room temperature, apart from two that require refrigeration, namely the cocktail of MAbs and the streptavidin-peroxidase.

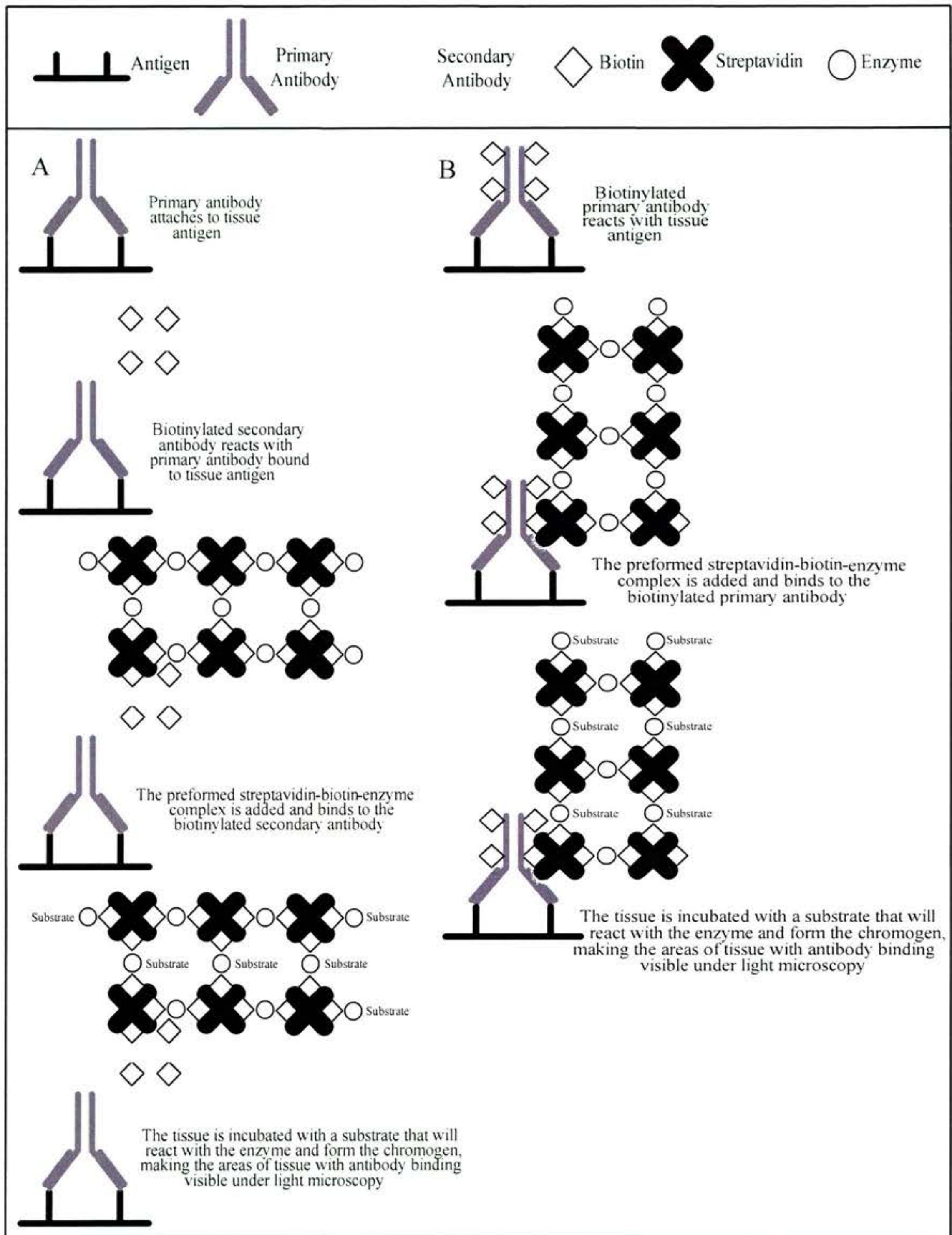


Figure 1.8. Schematic representation of the indirect (A) and direct (B) rapid immunohistochemical methods for rabies antigen detection in brain impressions. Immunohistochemical staining is performed using the avidin-biotin complex (ABC) technique whereby, after application of a biotinylated secondary (A) or primary (B) antibody, a preformed complex between avidin or streptavidin and a biotinylated enzyme is added. The tissue antigen is then visualised by incubation with a substrate for the enzyme.

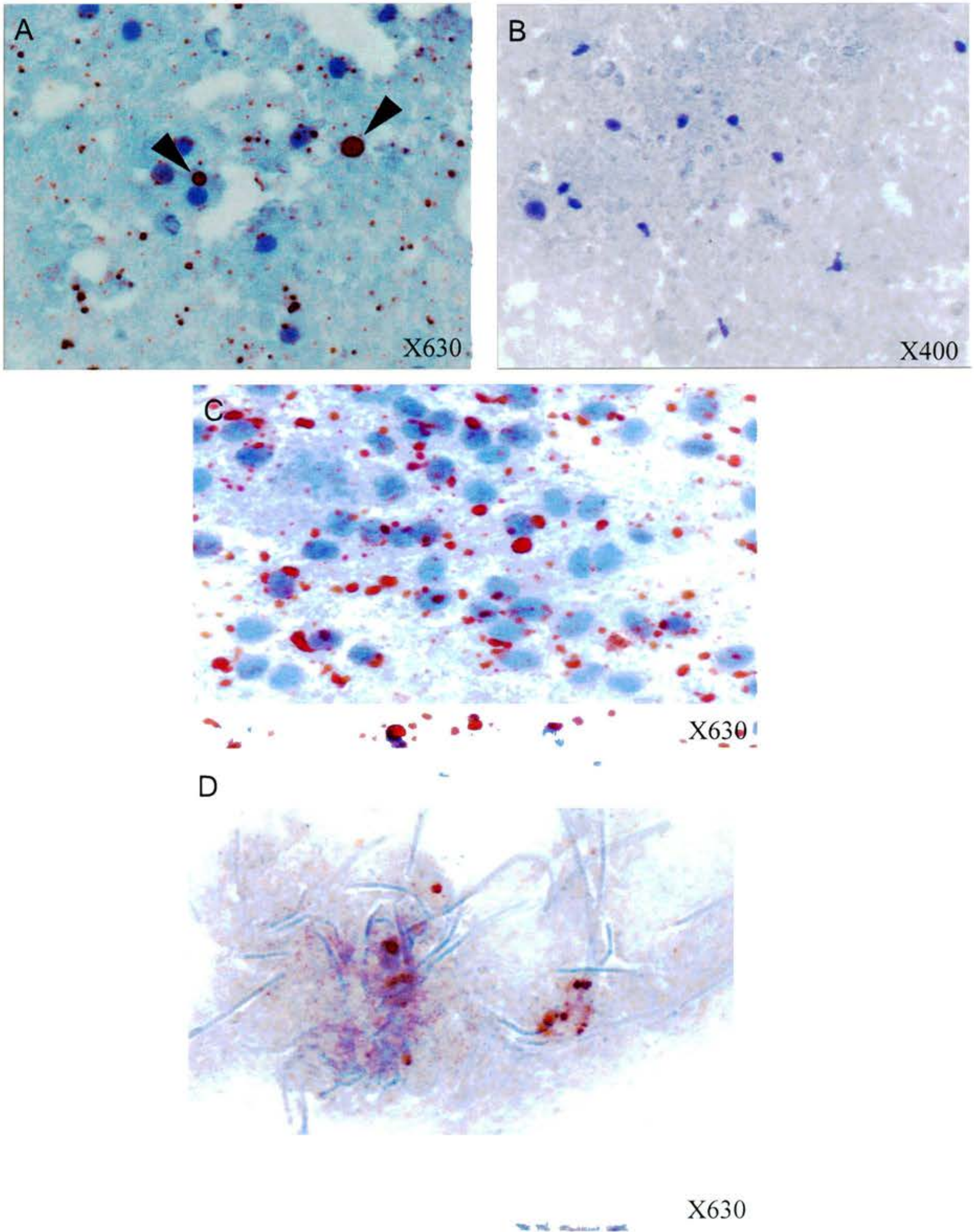


Figure 1.9. (A) Touch impression of a rabies-positive Tanzanian domestic dog brain stained by direct rapid immunohistochemical test (dRIT): rabies virus antigen appears as magenta inclusions (arrowheads) against the blue neuronal background. (B) Touch impression of a rabies-negative Tanzanian bat-eared fox (*Otocyon megalotis*) brain stained by dRIT. (C) Touch impression of an Aravan virus-positive mouse brain stained by dRIT (virus courtesy of I. V. Kuzmin, picture courtesy of M. Niezgoda). (D) Touch impression of a decomposed domestic cat brain with rabies (virus and picture courtesy of M. Niezgoda).

Table 1.3. Genotype 1 rabies virus variants associated with carnivore species and insectivorous bats in the United States (US) recognised by the cocktail of anti-nucleocapsid (N) monoclonal antibodies (MAbs) used to perform the rapid immunohistochemical test (RIT) (Source: M. Niezgoda, unpublished data).

Species host	Region
Carnivores	
Red fox (*Vulpes vulpes*)/Arctic fox (*Alopex lagopus*)	Arctic, New York
Raccoon (*Procyon lotor*)	Eastern US
Skunk (*Mephitis mephitis*)	North Central, South Central, California
Gray fox (*Urocyon cinereoargenteus*)	Texas, Arizona
Coyote (*Canis latrans*)/domestic dog (*Canis familiaris*)	Texas
Sonora dog	Mexico/Texas
Insectivorous bats	
Silver-haired (*Lasionycteris noctivagans*)	
Red (*Lasiurus borealis*)	
Big brown (*Epesticus fuscus*)	
Mexican free-tailed (*Tadarida brasiliensis*)	
Hoary (*Lasiurus cinereus*)	
Eastern pipistrelle (*Pipistrellus subflavus*)	

1.2.7 Rabies control

Due to the multi-host nature of rabies, questions arise as to the most appropriate population for directing control measures. Given a target-reservoir system (Figure 1.1), several approaches to control may be considered: (i) target control, (ii) blocking tactics and (iii) reservoir control (Haydon *et al.*, 2002a). The first two approaches focus on protecting the target population, which is also the ultimate goal of reservoir control. For infection to be eliminated however, control measures must target the reservoir.

For target control, knowledge of the reservoir is not required as efforts are directed within the target population (e.g. vaccination of humans or endangered canids). Human rabies vaccines currently recommended by WHO are cell-culture vaccines (high- or low-quality), which, with the exception of some developing countries, have widely replaced the nerve tissue-derived vaccines, renowned for the severe pain and neuroparalytic complications associated with their administration (for a review, see Briggs *et al.*, 2002 and Briggs, 2002). Despite being safe and highly effective, cell-culture vaccines are costly and not promptly available in many parts of the world. Direct and indirect costs associated with post-exposure treatments (PETs) in Africa and Asia, for example, exert a substantial economic burden ($40 and $49/treatment in Africa and Asia respectively: Knobel *et al.*, 2005). Safe and effective parenteral or oral vaccines are also available for immunisation of animals. Even though modified live virus vaccines are still in use in some areas, the use of safer inactived (killed) cell-culture vaccines is increasing worldwide. Vaccination of endangered canids has therefore been proposed as a conservation tool to respond to acute disease outbreaks threatening the survival of critical populations and used successfully on a number of occasions (Hall and Harwood, 1990; Woodroffe, 1999; Hofmeyr *et al.*, 2004). However, under other circumstances, its use has been the subject of considerable debate (Gascoyne *et al.*, 1993b; Burrows *et al.*, 1994, 1995).

Blocking tactics aim to block transmission between the source and target populations and therefore require knowledge of source populations within the reservoir, but not a complete understanding of reservoir infection dynamics. Movement/contact restriction (confinement, leashing, muzzling) of dogs 'so that nobody could be bitten' constituted one of the so-called 'classical' measures that led to the elimination of

canine rabies in Europe in the 19th and first half of the 20th century (for a review, see Bögel, 2002).

For reservoir control, an exhaustive identification of all constituent populations of the reservoir or its maintenance component/s is necessary. Rabies control in a reservoir may be achieved by: (i) culling programmes, (ii) vaccination programmes and (iii) animal birth control. For centuries culling of rabid, bitten and 'stray' dogs has constitued an important 'classical' measure (for a review, see Bögel, 2002). Until relatively recently this approach was widely adopted throughout the world and it is still promoted by local and national authorities in many developing country settings. The earliest attempts to control fox rabies in Europe were also based on drastic decimation of the population (for a review, see Pastoret *et al.*, 2004). The limits of host population removal have been emphasised by failure in controlling the disease in both domestic and wild reservoir hosts mainly due to a lack of understanding of the ecology and dynamics of the host and local cultural attitudes towards dogs. Examples from a number of developing countries have indicated that these measures are ineffective, counter-productive and unpopular: they reduce the overall herd immunity as the communities respond to them by acquiring new unvaccinated dogs (WHO, 1988) and they are unacceptable to local communities (WHO, 1992). Culling of 'stray' dogs for example has been shown to strengthen the overall population: in these campaigns predominantly weak, sick and less productive animals are captured, so that more shelter and food are left to the productive population segment and health risks are reduced (WHO-WSPA, 1990). Fox elimination programmes had similar effects in terms of population disruption, such as faster growth through density-dependent increases in survival/fecundity and increased dispersal, hence increased opportunities for intraspecific transmission and spread over wider areas (Macdonald and Bacon, 1982; Aubert, 1994).

Since the second part of the 20th century large-scale vaccination of reservoir host populations has been successfully used to eliminate or control rabies in dog and wildlife populations both in the developed (Europe and north America) and developing world. Important examples of countries where successful dog rabies mass immunisation programmes were achieved include: Japan, Hungary, Tennessee (USA), Malaysia, Hong Kong, Taiwan, Philippines, Brazil, Tunisia, Peru, Mexico

and the Caribbean (Tierkel, 1950; Wells, 1954; Manninger, 1968; Beran *et al.*, 1972; WHO, 1987; Belotto, 1988; Ben Osman and Haddad, 1988; Chomel *et al.*, 1988a, b; WHO, 2004; Belotto *et al.*, 2005). As for wildlife rabies control, the development of oral immunisation techniques has made the elimination of rabies in reservoir hosts a realistic goal. Rabies control through implementation of oral immunisation of red foxes, for example, has led to disease elimination in a number of European countries (e.g. Switzerland, Italy, France, Belgium, Luxembourg, the Netherlands and Finland) or a dramatic reduction in cases in others (reviewed by Pastoret *et al.*, 2004). Rabies wildlife control in north America is also centred around immunisation of reservoir hosts (for a review, see Johnston and Tinline, 2002). Evidence from countries where mass vaccination of rabies reservoirs has been extremely successful in controlling the disease indicates that the same approach is likely to be effective in countries where rabies still remains uncontrolled, such as parts of sub-Saharan Africa and Asia (Cleaveland, 1998; WHO, 2002). Studies in northern Tanzania, for example, indicated that vaccination of 60-70% of dogs (the target considered necessary to prevent outbreaks of dog rabies: Coleman and Dye, 1996) was sufficient to control dog rabies in this area and to reduce bite-injuries from suspected rabid dogs (Cleaveland *et al.*, 2003). Together with benefits for human health and wildlife conservation, canine vaccination would also have an impact on public health economics by reducing the demand for PEP, as demonstrated for countries of Asia and suggested by preliminary estimates from Tanzania (reviewed by Cleaveland *et al.*, 2006a). In addition to vaccination coverage however, the sustainability of campaigns (in terms of economic and human resources), which still remains an enduring problem in Africa and Asia, would be crucial to effective rabies control by this means (Bögel and Meslin, 1990; Meslin *et al.*, 1994; Cleaveland, 1998; Kitala *et al.*, 2002; Kayali *et al.*, 2003, 2006).

1.2.8 Rabies in Serengeti: background information and unresolved issues

The Serengeti is located in the Mara Region of northwestern Tanzania (Figure 1.10), where canine rabies was first reported in 1932 (Rweyemamu *et al.*, 1973). In 1947 the disease was contained through quarantine and stray dog destruction. It reappeared in the region in 1955. As a result of vaccination and culling of the dog population,

rabies was apparently absent from the Serengeti between 1958 and 1977 (Rweyemamu *et al.*, 1973; Magembe, 1985a). In the late 1970s, an outbreak of rabies was recorded where domestic dogs were the predominant species involved, with occasional reports in livestock and wild carnivores (mainly jackals and hyaenas). Destruction of dogs was brought into effect, but it failed to control the disease and dog cases have been reported since then (Magembe, 1985b). In the late 1980s, rabies was responsible for high mortality in Serengeti bat-eared foxes (Maas, 1993) and was identified as the cause of mortality in a pack of African wild dogs in the Maasai Mara National Reserve in Kenya, the northern extension of the Serengeti ecosystem (Kat *et al.*, 1995, 1996). In 1990 rabies infection was confirmed in an African wild dog carcass in the Serengeti region of Tanzania and, together with CD, was suggested to have caused the virtual disappearance of this species from the Serengeti-Mara region in 1991 (Macdonald *et al.*, 1992; Gascoyne *et al.*, 1993a; Alexander and Appel, 1994; Ginsberg *et al.*, 1995; Woodroffe, 2001). The population of domestic dogs surrounding the Serengeti National Park was believed to be the most likely source of infection. Characteristics of dog populations living adjacent to the park were examined and shown to differ significantly, with dog populations in agro-pastoralist areas to the west occurring at much higher densities ($>5/km^2$) than those in pastoralist areas to the east ($<1/km^2$) (Figure 1.10) (Cleaveland and Dye, 1995; Cleaveland, 1996).

The role of domestic and wild carnivores in the epidemiology of rabies in the ecosystem was also investigated and several lines of evidence suggested that rabies occurred only sporadically in lower-density dog and wild carnivore populations, which appeared unable to maintain infection, but persisted as endemic infection in higher-density dog populations, which were the likely reservoir (Cleaveland and Dye, 1995). One finding supporting the view of dogs as the sole reservoir was that antigenic and genetic characteristics of viruses isolated from wild (one African wild dog) and domestic (one domestic dog) carnivores pointed to a single canid-associated variant (Africa 1b) affecting multiple hosts (King, 1991; Bourhy *et al.*, 1993b; Kissi *et al.*, 1995). Further complexity in the epidemiology of the disease in the Serengeti arose from the study by East *et al.* (2001) which proposed the potential involvement of wild carnivores (i.e. the spotted hyaena) in maintaining infection independently of

dogs through an atypical pattern (i.e. an infectious carrier state - see page 22). In particular, this species was reported to maintain a genetically distinct virus variant with the following characteristics: (i) reduced or no virulence to hyaenas; (ii) no evidence of spill-over transmission to other species within the ecosystem; and (iii) close phylogenetic similarity to European and Middle Eastern rather than African isolates of RABV.

Many questions still remain about the role of domestic and wild carnivores in maintaining and transmitting rabies in the Serengeti. The study by Cleaveland and Dye (1995) was based on limited data and did not take into account multi-host aspects of reservoirs. A complex reservoir system including domestic and wild carnivores (Haydon *et al.*, 2002a) is a realistic possibility in a highly diverse ecosystem like the Serengeti where species implicated as carnivore hosts for rabies elsewhere in Africa (Thomson and Meredith, 1993; Bingham *et al.*, 1999a) co-exist. The questions as to whether species other than the domestic dog may have a role as maintenance components, and the extent to which nonessential (to maintenance) constituents may contribute to infection of essential (to maintenance) hosts, are critical if rabies is to be eliminated from the system. One reason to believe that patterns of dog and wildlife rabies might have changed is that, over the past 10 years, the human and associated domestic dog populations to the west and east are believed to have grown considerably. Thus, dog populations previously too small to sustain rabies cycles may now be large enough for independent maintenance (e.g. pastoralist dogs). Populations of some wild carnivores, especially opportunistic foragers in the proximity to human settlements (e.g. jackals or hyaenas), may also have attained higher densities, and contact rates between sympatric wild and domestic carnivores might have changed (Cleaveland, 1998) with one of two possible consequences: (i) wild carnivore populations might now be capable of maintaining rabies independently of dogs; or (ii) wild carnivore populations may be part of a maintenance community (e.g. pastoralist dog and wild carnivore populations together). The possibility also remains that wild carnivores may still be unable of independent maintenance. However, patterns of wildlife rabies remain difficult to interpret while rabies still circulates in dogs living to the west of the park (hence dog-to-wildlife transmission may occur). Finally, the 'hyaena' variant described by East

et al. (2001) does not appear to infect any other species (within the limits of current knowledge), hence the inter-specific transmission requirement is not met, indicating that the relevance of this species (infected by this variant) in potentially complex reservoir infection dynamics in the Serengeti may be limited. However, a fundamental question relates to the possible contribution of multiple hosts in the maintenance of potentially distinct variants within the ecosystem or of multiple hosts to the maintenance of a single variant.

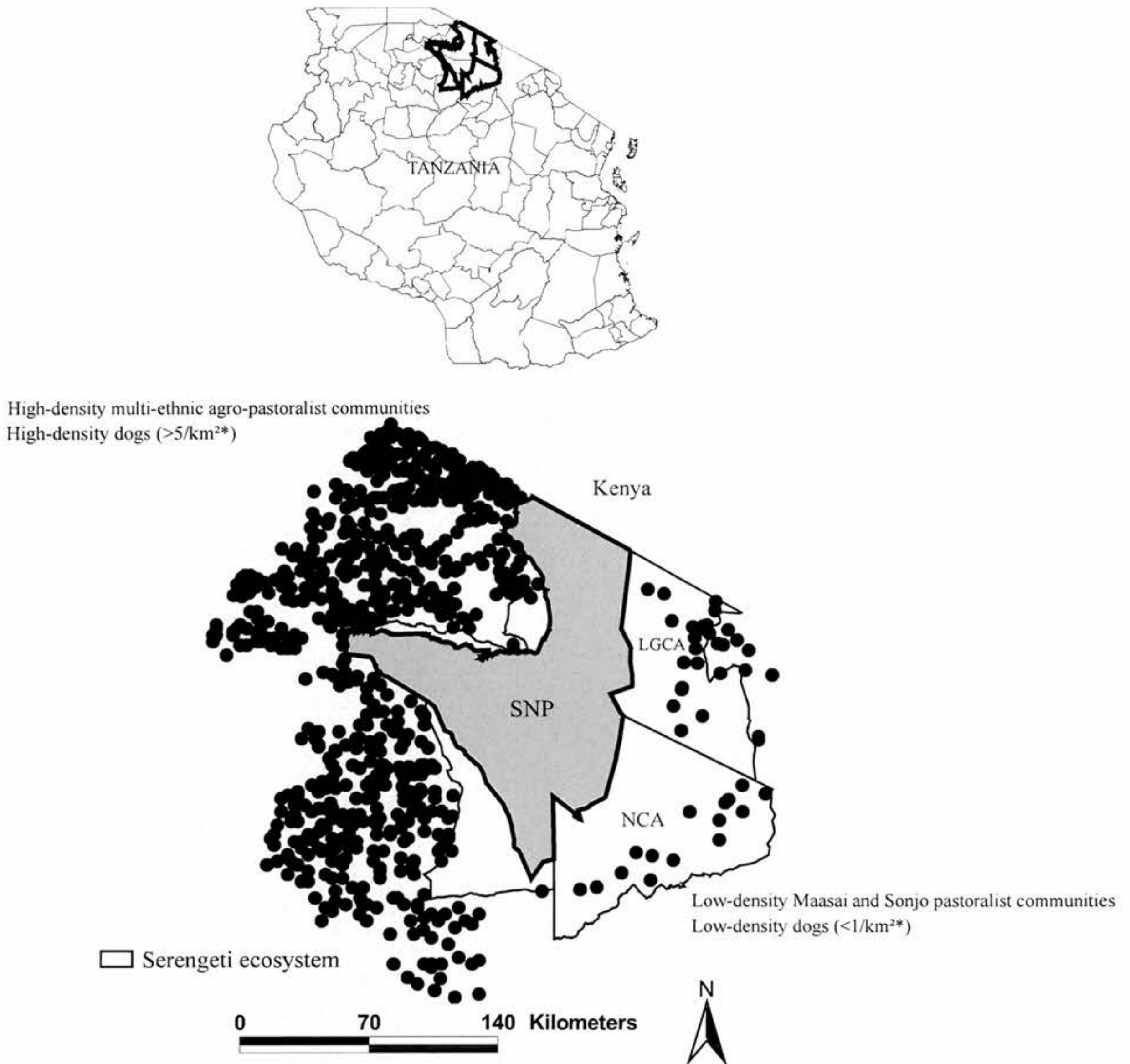


Figure 1.10. Map of the Serengeti ecological region of northwestern Tanzania showing the characteristics of human and dog populations living adjacent to and within the Serengeti ecosystem. Human settlements are indicated as solid black circles. SNP = Serengeti National Park; LGCA = Loliondo Game Control Area; NCA = Ngorongoro Conservation Area. *Cleaveland and Dye, 1995.

1.3 Canine distemper

CD is a severe highly contagious viral disease with a worldwide distribution and affecting many different host species. CDV, a single-stranded RNA virus with a lipoprotein envelope, is a member of the *Morbillivirus* genus in the family *Paramyxoviridae*. This genus comprises pathogens that have had a huge impact on both humans and animals: measles virus, rinderpest virus, peste des petits ruminants virus (PPRV), and the recently discovered phocid distemper virus and cetacean morbillivirus (CMV).

1.3.1 An expanding host range: canine distemper in Serengeti lions

CDV infection or exposure has been reported in a wide and expanding variety of terrestrial carnivore species (Table 1.4). CD has also become established as a disease of the aquatic environment, and all pinnipeds might be at serious risk from CDV infection (Osterhaus *et al.*, 1988; Grachev *et al.*, 1989; Kennedy *et al.*, 2000; Jensen *et al.*, 2002; Müller *et al.*, 2002).

In the 1990s, outbreaks among large captive felids in American zoos drew attention to CDV, which was previously thought not to be pathogenic in cats, as a potential threat to *Felidae* (Appel *et al.*, 1994). The best-studied example of CDV infection in free-ranging large cats comes from the Serengeti where, from late 1993 to 1994, a severe epidemic was recorded in the lion population (Roelke-Parker *et al.*, 1996). Thirty per cent of lions (approximately 1,000 individuals) within a well-known study population of the SNP died within six months, showing encephalitis and pneumonia, and over 85% of individuals were exposed. CD was also confirmed in spotted hyaenas and bat-eared foxes (Roelke-Parker *et al.*, 1996; Haas *et al.*, 1996). The epidemic spread north to the Maasai Mara and infected the lion population, probably due to the movement of nomadic lions and/or commuting hyaenas following the wildebeest migration from Tanzania to Kenya (Kock *et al.*, 1998), and the spotted hyaena population (Harrison *et al.*, 2004). No association with other factors such as co-infection with other viral pathogens could be found (Roelke-Parker *et al.*, 1996). It was first thought that a specially adapted feline variant of CDV had emerged causing high mortality in captive and free-ranging large cats in different continents. However, viruses recovered from Serengeti lions, hyaenas and bat-eared foxes were antigenically and genetically indistinguishable from one particular isolate from a

domestic dog adjacent to the SNP, and the Serengeti CDV was distinctive from CDV isolates from other parts of the world (Harder *et al.*, 1995; Roelke-Parker *et al.*, 1996; Cleaveland, 1996; Haas *et al.*, 1996; Carpenter *et al.*, 1998), indicating that local variants rather than a new cat-adapted variant were responsible for the epidemics. The role of domestic dogs in the epidemic was investigated retrospectively by serological and demographic analyses (Cleaveland, 1996; Cleaveland *et al.*, 2000). The study revealed that prior to the lion epidemic CD persisted as a relatively stable infection in higher-density dog populations to the northwest of the park (exposed between 1992 and 1994), whereas it occurred only sporadically in lower-density dog populations to the east of the park (exposed in late 1991 and late 1994, but not in 1992 and 1993), suggesting that the former were the most likely source of infection for wildlife. Subsequently, the infection in wildlife spread north to the Maasai Mara and south through the park, before occurring as an epidemic in lower-density dog populations in late 1994 and in higher-density dog populations to the southwest of the park early in 1995, although the latter observation was based on anecdotal evidence (Figure 1.11). Definitive conclusions about the role of dogs and wild carnivores in disease maintenance and transmission could not be drawn, but data from lion and hyaena populations in the ecosystem suggested sporadic exposure rather than persistent infection, and indirect rather than direct contact between dogs and lions through chains of transmission in other species (e.g. hyaenas or jackals) was believed to be likely (Alexander *et al.*, 1995; Roelke-Parker *et al.*, 1996; Cleaveland, 1996; Cleaveland *et al.*, 2000).

Table 1.4. Terrestrial wild carnivore species and sub-species susceptible to canine distemper virus (CDV). Free-living species that have been affected by dramatic CDV epidemics are indicated by an asterisk. RT-PCR = reverse transcriptase-polymerase chain reaction.

Species	Area	Evidence for exposure/infection	Reference
Ailuridae			
Lesser panda (*Ailurus fulgens*)	-	Vaccine-induced	Erken and Jacobi, 1972; Bush *et al*., 1976; Itakura *et al*., 1979
Red panda (*Ailurus fulgens*)	-	Vaccine-induced	Bush and Roberts, 1977
Ailuropodidae			
Giant panda (*Ailuropoda melanoleuca*)	China	Serological survey	Mainka *et al*., 1994
Canidae			
African wild dog (*Lycaon pictus*)*	Mara, Kenya; Bushmanland, Namibia; Selous, Tanzania	Serological survey	Alexander *et al*., 1993; Laurenson *et al*., 1997; Creel *et al*., 1997
	Chobe, Botswana; Mkomazi, Tanzania	Confirmed mortality	Alexander *et al*., 1996; van de Bildt *et al*., 2002
Raccoon dog (*Nyctereutes procyonoides*)*	Japan	Histopathology, immunocytochemistry	Machida *et al*., 1993; Aoyagi *et al*., 2000
	Japan	Serological survey	Neagari *et al*., 1998
Coyote (*Canis latrans*)	US	Serological survey	Guo *et al*., 1986; Gese *et al*., 1991, 1997, 2004; Cypher *et al*., 1998; Grinder and Krausman, 2001; Arjo *et al*., 2003; Bischof and Rogers, 2005
Jackal (*Canis mesomelas, C. aureus, C. adustus*)	Mara, Kenya; Israel; Zimbabwe; Namibia	Serological survey	Alexander *et al*., 1994; Spencer *et al*., 1999; Shamir *et al*., 2001; Gowtage-Sequeira, 2005

Table 1.4. Continued.

Species	Area	Evidence for exposure/infection	Reference
Australian dingo (*Canis dingo*)	Zoological park, Australia		Armstrong and Anthony, 1942
Red fox (*Vulpes vulpes*)	Southeastern US	Histopathology	Little *et al*., 1998
	Wisconsin, US; Germany; Luxembourg	Serological survey	Amundson and Yuill, 1981; Hentschke, 1995; Truyen *et al*., 1998; Frölich *et al*., 2000; Damien *et al*., 2002
	Spain	Immunohistochemistry	Lopez-Pena *et al*., 1994
	Germany	RT-PCR	Frölich *et al*., 2000
Gray fox (*Urocyon cinereoargenteus*)	Florida, US	Histopathology	Jakowski and Wyand, 1971
	Southeastern US	Confirmed morbidity and mortality	Hoff *et al*., 1974; Davidson *et al*., 1992; Black *et al*., 1996
Bat-eared fox (*Otocyon megalotis*)	Tanzania	Confirmed mortality	Roelke-Parker *at al*., 1996
Swift fox (*Vulpes velox*)	Western US	Serological survey	Miller *et al*., 2000; Gese *et al*., 2004
Kit fox (*Vulpes macrotis*)	Western US	Serological survey	Miller *et al*., 2000
San Joaquin kit fox (*Vulpes macrotis mutica*)	California, US	Serological survey	McCue and O'Farrel, 1988
Grey fox (*Dusicyon griseus*)	Argentina	Serological survey	Martino *et al*., 2004
Culpeo fox (*Dusicyon culpaeus*)	Argentina	Serological survey	Martino *et al*., 2004
Island fox (*Urocyon littoralis*)*	Santa Catalina Island, California, US	Confirmed mortality	Timm *et al*., 2000, 2002

Table 1.4. Continued.

Species	Area	Evidence for exposure/infection	Reference
Fennec fox (*Fennecus zerda*)	-	Vaccine-induced	Montali *et al.*, 1987
Wolf (*Canis lupus*)	US; Canada	Serological survey	Choquette and Kuyt, 1974; Stephenson *et al.*, 1982; Zarnke and Ballard, 1987; Johnson *et al.*, 1994; Zarnke *et al.*, 2004
Maned wolf (*Chrysocyon brachyurus*)	Brazilian zoos	Serological survey	Maia and Gouveia, 2001
Mexican wolf (*Canis lupus baileyi*)	Arizona and New Mexico, US	Confirmed mortality	Hedrick *et al.*, 2003
Ethiopian wolf (*Canis simensis*)	Bale Mountains, Ethiopia	Serological survey	Laurenson *et al.*, 1998
Felidae			
Lion (*Panthera leo*)*	Serengeti, Tanzania; North American zoos	Confirmed mortality	Appel *et al.*, 1994; Wood *et al.*, 1995; Roelke-Parker *at al.*, 1996
	Serengeti, Tanzania; Mara, Kenya; Japanese zoos	Serological survey	Kock *et al.*, 1998; Packer *et al.*, 1999; Endo *et al.*, 2004
	Swiss zoo	Immunohistochemistry	Myers *et al.*, 1997
Mountain lion (*Felis concolor*)	North American zoos	Serological survey	Appel *et al.*, 1994
Siberian tiger (*Panthera tigris altaica*)	North American zoos	Confirmed mortality	Appel *et al.*, 1994
	-	Confirmed encephalitis	Gould and Fenner, 1983

Table 1.4. Continued.

Species	Area	Evidence for exposure/infection	Reference
Bengal tiger (*Panthera tigris tigris*)	-	Histopathology and serology	Blythe *et al.*, 1983
	North American zoos	Confirmed mortality and serological survey	Appel *et al.*, 1994
	Swiss zoo	Immunohistochemistry	Myers *et al.*, 1997
Jaguar (*Panthera onca*)	North American zoos	Confirmed mortality and serological survey	Appel *et al.*, 1994
Leopard (*Panthera pardus*)	North American zoos	Confirmed mortality and serological survey	Appel *et al.*, 1994
Chinese leopard (*Panthera pardus japonensis*)	North American zoos	Confirmed mortality	Appel *et al.*, 1994
Snow leopard (*Panthera uncia*)	North American zoos	Antigen detection and serology	Fix *et al.*, 1989
Cheetah (*Acinonyx jubatus*)	Namibia	Serological survey	Munson *et al.*, 2004
Canada lynx (*Lynx canadensis*)	western North America	Serological survey	Biek *et al.*, 2002
Bobcat (*Lynx rufus*)	Canada	Confirmed mortality	Woodford, 1995
Hyaenidae			
Spotted hyaena (*Crocuta crocuta*)	Serengeti, Tanzania	Confirmed mortality	Roelke-Parker *at al.*, 1996; Haas *et al.*, 1996
	Mara, Kenya	Serological survey	Alexander *et al.*, 1995; Harrison *et al.*, 2004

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Table 1.4. Continued.

Species	Area	Evidence for exposure/infection	Reference
Mustelidae			
Black-footed ferret* (*Mustela nigripes*)	Wyoming, US	Confirmed mortality	Williams *et al*., 1988
Striped skunk (*Mephitis mephitis*)	Illinois, US	Histopathology, immunocytochemistry	Diters and Nielsen, 1978; Woolf *et al*., 1986
Stone marten (*Martes foina*)	Switzerland; Germany	Confirmed mortality	Ulbrich, 1972; Palmer *et al*., 1983; van Moll *et al*., 1995
	Germany	Immunohistochemistry	Steinhagen and Nebel, 1985
Stone marten	Germany	Serological survey, RT-PCR	Frölich *et al*., 2000
Polecat (*Mustela putorius*)	Germany	Confirmed mortality	Hewicker *et al*., 1990; van Moll *et al*., 1995
Badger (*Meles meles*)	Germany; Denmark	Confirmed mortality	Kolbl *et al*., 1990; van Moll *et al*., 1995; Hammer *et al*., 2004
	Germany	RT-PCR	Frölich *et al*., 2000
American badger (*Taxidea taxus*)	US	Reported	Armstrong, 1942
Weasel (*Mustela* sp.)	Germany	Confirmed mortality	van Moll *et al*., 1995
Wolverine (*Gulo gulo*)	Alaska	Serological survey	Dalerum *et al*., 2005
Otter (*Lutra lutra*)		Reported	Geisel, 1979
North American river otters (*Lontra canadensis*)	northern and easter New York State, US	Serological survey	Kimber *et al*., 2000

Table 1.4. Continued.

Species	Area	Evidence for exposure/infection	Reference
Mink (*Mustela lutreola*, *M. vison*)	-	Vaccine-induced	Sutherland-Smith *et al*., 1997; Ek-Kommonen *et al*., 2003
Procyonidae			
Raccoon (*Procyon lotor*)*	New Jersey, US	Confirmed mortality	Hoff *et al*., 1974; Roscoe, 1993; Appel *et al*., 1994; Lednicky *et al*., 2004
	US	Co-infection with other pathogens	Wojcinski and Barker, 1986; Hamir and Rupprecht, 1990; Stoffregen and Dubey, 1991; Thulin *et al*., 1992; Hamir *et al*., 1998
	US	Serological survey	Jamison *et al*., 1973; Hoff *et al*., 1974; Mitchell *et al*., 1999; Bischof and Rogers, 2005
Pygmy raccoon (*Procyon pygmaeus*)	Cozumel Island, Mexico	Serological survey	McFadden *et al*., 2005
Kinkajou (*Potos flavus*)	-	Vaccine-induced	Kazacos *et al*., 1981
Viverridae			
Binturong (*Arctictis binturong*)	Zoological gardens, Korea	Confirmed mortality	Hur *et al*., 1999
	US		Chandra *et al*., 2000
Masked palm civet (*Paguma larvata*)	Japan	Confirmed mortality	Machida *et al*., 1992
Genet (*Genetta genetta*)	northwestern Spain	Confirmed mortality	Lopez-Pena *et al*., 2001

Table 1.4. Continued.

Species	Area	Evidence for exposure/infection	Reference
Ursidae			
Polar bear (*Ursus maritimus*)	-	Clinical disease	Schonbauer *et al*., 1984
	Alaska; Russia; Canadian arctic; Norway	Serological survey	Follmann *et al*., 1996; Garner *et al*., 2000; Cattet *et al*., 2004; Tryland *et al*., 2005
Florida black bear (*Ursus americanus floridanus*)	Florida, US	Serological survey	Dunbar *et al*., 1998
Marsican brown bear (*Ursus arctos marsicanus*)	Abruzzo, Italy	Serological survey	Marsilio *et al*., 1997
Spectacled bear (*Tremarctos ornatus*)	-	Clinical disease	Schonbauer *et al*., 1984
Grizzly bear (*Ursus arctos horribilis*)	Alaska	Serological survey	Chomel *et al*., 1998

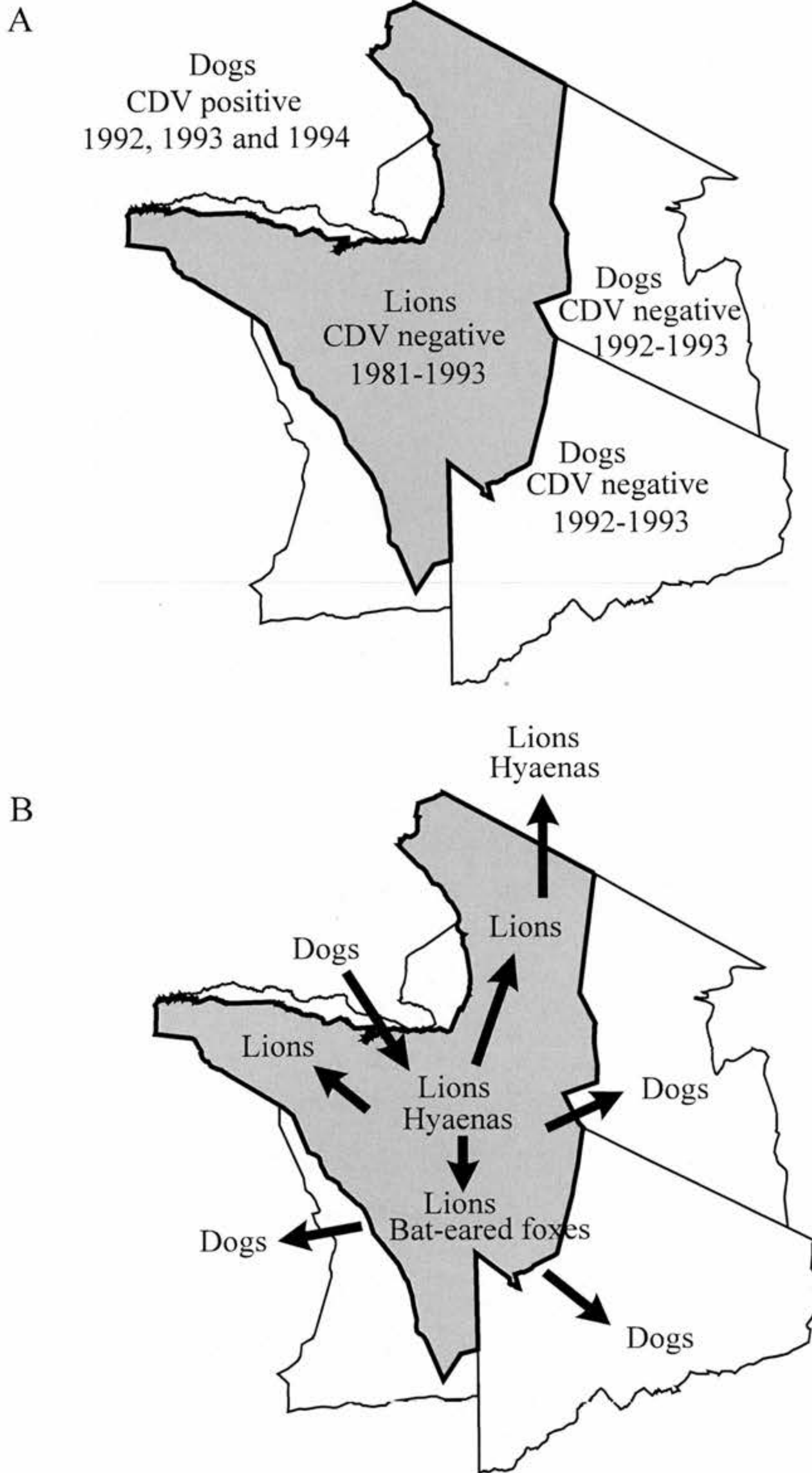


Figure 1.11. (A) Status of canine distemper virus (CDV) before the 1994 epidemic in Serengeti lions as reconstructed using serological evidence and (B) its spread during the epidemic as reconstructed using case-surveillance data and serology (Roelke-Parker *et al.*, 1996; Kock *et al.*, 1998; Packer *et al.*, 1999; Cleaveland *et al.*, 2000; Harrison *et al.*, 2004).

1.3.2 Infection and immunity

The most important means of CDV transmission is through aerosolisation of respiratory exudates containing virus (Gorham, 1966). After initial multiplication in lymphatic tissue of the respiratory tract, the virus spreads throughout the body and may induce an acute/sub-acute multisystemic infection with variable involvement of the respiratory, gastro-intestinal, integumentary and central nervous systems, although it is estimated that up to 70% of infections in domestic dogs are sub-clinical (Greene, 1984; Appel, 1987; Greene and Appel, 1990). Outcome and severity of infection are in part determined by the host immune response, with serum antibody response varying inversely with the severity of the infection (Appel, 1967). By 2 weeks after infection, dogs with an adequate cellular and humoral response clear the infection, generally without developing clinical signs. Intermediate immune responsiveness usually results in sub-acute forms that may resolve as antibody levels increase, whereas in poor responders pan-systemic viral dissemination with development of acute disease occurs. Infection generally results in life-long immunity in recovered hosts.

1.3.3 Canine distemper control

Assuming a target-reservoir system, the same principles illustrated for rabies control applies to the control of CDV depending on whether protection of the target population (i.e. endangered canids) or disease elimination is the goal (see section 1.2.7).

Target control through immunisation of threatened host species however presents complications. Currently available CDV vaccines are modified live vaccines derived from either avian cell or canine cell culture adaptations (Appel, 1987). They are very effective in inducing immunity in susceptible dogs (up to 100% of dogs generally become immune following immunisation with canine cell-adapted strains), are generally safe (particularly the avian cell-adapted strains), and provide immunity of long duration (three years). Commercially live vaccines have also shown their safety and effectiveness when used in natural populations (e.g. lions; Kock *et al.*, 1998). However, any modified live vaccines carry a risk of virulence and earlier vaccines were pathogenic and caused mortality in certain wildlife and zoo animals (e.g. minks [*Mustela lutreola*, *M. vison*], ferrets [*M. nigripes*], foxes [*Urocyon*

cinereoargenteus], lesser pandas [*Ailurus fulgens*], African wild dogs: Erken and Jacobi, 1972; Bush *et al.*, 1976; Carpenter *et al.*, 1976; Itakura *et al.*, 1979; McCormick, 1983; Durchfeld *et al.*, 1990; Sutherland-Smith *et al.*, 1997; Ek-Kommonen *et al.*, 2003). Inactivated vaccines, which carry a lower risk, induce a limited immunity (Appel *et al.*, 1984) and are no longer commercially available. To address these difficulties, attempts have been made to produce new recombinant vaccines. Canary-pox vectored CDV recombinant vaccines with hemagglutinin (H) and fusion (F) inserts are now commercially available and have been successfully used on dogs (Pardo *et al.*, 1997), ferrets (Stephenson *et al.*, 1997; Welter *et al.*, 1999), island foxes (*Urocyon littoralis*) (Timm *et al.*, 2000) and Siberian polecats (*Mustela eversmanni*) (Wimsatt *et al.*, 2003).

When reservoirs cannot be identified, control efforts may be directed at blocking transmission between source and target populations, which clearly requires identifying source populations. Once the source has been identified controlling infection within the source (e.g. through vaccination) may lead to effective control. For CDV, blocking tactics such as confinement of sick individuals may be also important in blocking transmission due to the high contagiousness of the virus which is shed in all body excretions during the acute systemic disease (hence direct animal-to-animal contact appears to be the main route of spread).

For controlling infection within the reservoir, a more or less complete understanding of its structure (i.e. the maintenance component/s) is required. Although CDV infection/exposure has been demonstrated in a wide range of species (Table 1.4), surprisingly little is known about reservoirs of infection and patterns of maintenance in wildlife populations throughout the world. Control measures in developed nations have mainly targeted dog populations and widespread active immunisation with modified live virus vaccines has kept the disease under control in theses populations. Moreover, as the persistence of maternally derived antibodies (MDAs) interfere with immunisation in puppies (dogs younger than 3 months), vaccination with modified live MV or a combination of both MV and CDV vaccines have been used, despite induction of only partial protection in the presence of MDAs. However, high-titre CDV vaccines developed in the early 1990s protect pups against challenge infection more effectively than MV (Chalmers and Baxendale, 1994). Given the high birth rate

of rural African dog populations, including puppies in vaccination programmes is likely to be important to maintain the required temporal herd immunity for both rabies and CD control (Perry *et al.*, 1995; Cleaveland, 1996; Coleman, 1999). For rabies, although some laboratory trials indicate that vaccine-induced active immunity is likely to be affected by the presence of MDAs (Prècausta *et al.*, 1985), other studies show that puppies are capable of responding to rabies vaccination without any significant interference by MDAs (Chappuis, 1998; Seghaier *et al.*, 1999).

1.3.4 Canine distemper in Serengeti: unresolved issues

The fact that high-density dog populations were the likely source of infection in the 1994 Serengeti epidemic (see section 1.3.1), and that infection appeared to persist in these populations for a number of years, does not provide unequivocal evidence for a CD reservoir comprising solely dogs in the ecosystem. By definition, being a source only guarantees reservoir membership (Haydon *et al.*, 2002a). Indeed, the hypothesis of a complex reservoir system comprising an intricate network of populations also applies to CD and one of the key issues would be to clarify the role of each component in disease maintenance. Many questions also remain about the factors responsible for the observed variable degree of morbidity and mortality inflicted by CDV on Serengeti lions as retrospective age-seroprevalence data showed that CDV was not a new disease: earlier exposure in the early-1980s had no impact in terms of morbidity and mortality, in contrast to the 1994 epidemic, which was much more severe and caused a sharp decline in the population (Packer *et al.*, 1999). Current interpretations include the following factors that may be important, alone or combined, in determining pathogenicity of the virus in the population:

(i) Virus factors such as strain variation of the virus.

(ii) Host factors such as co-infection with other pathogens as CDV, like all strains of *Morbillivirus*, is a significant cause of immune suppression (Arneborn and Biberfeld, 1983; Krakowka *et al.*, 1987; Wohlsein *et al.*, 1995; Heaney *et al.*, 2002).

(iii) Ecological factors such as drought which may increase possibility of intra- and inter-species contact (e.g. at water-holes). This interpretation is

supported by the view that the 1994 die-off in Serengeti lions coincided with a severe drought (Cleaveland *et al.*, 2000; Kissui and Packer, 2004).

1.4 Aims and outline of this thesis

1.4.1 Overall aims

(i) To obtain definitive evidence for the identification of rabies reservoirs in multi-host communities of the Serengeti through an integrated epidemiological study bringing together a number of 'practical indicators'.

(ii) To achieve a better understanding of CDV infection dynamics in Serengeti dog and lion populations through serological surveys.

1.4.2 Specific aims

(i) To investigate and validate an alternative diagnostic technique (dRIT) as a field test for rabies surveillance in order to enhance capacity for local screening and rabies diagnosis in the region, and ultimately in countries with a lack of capacity for rabies surveillance. Specific objectives were: (1) to improve surveillance in order to determine trends in disease incidence in domestic and wild animal populations with reasonable accuracy, and (2) to provide a tool to facilitate future evaluation of the impact of dog rabies control programmes (i.e. vaccination) on disease incidence.

(ii) To evaluate changes in population densities and describe long-term dog and wildlife rabies incidence patterns, and to use the data to provide evidence for the relative role of domestic and wild carnivore populations in disease maintenance.

(iii) To examine the genetic characteristics of RABVs isolated from a range of species to test the hypothesis of the existence of distinct virus-host associations.

(iv) To explore the power of genetic data to elucidate patterns of intra- and inter-specific rabies viral transmission and uncover transmission pathways.

(v) To provide a description of post-epidemic spatio-temporal changes in the exposure of domestic dog and lion populations to CD.

1.4.3 Outline of the thesis

Chapter 2 describes the activities carried out during the study period and provide documentation of other data sources.

In Chapter 3 a preliminary study to evaluate the dRIT under field and laboratory conditions is described.

In Chapter 4 the genetic characteristics of Serengeti RABVs are described.

Chapter 5 focuses on the question of reservoirs of rabies in the Serengeti and draws on the long-term surveillance data from the Serengeti carnivore populations (1991-2005).

Chapter 6 focuses on CDV and draws on long-term serological studies in Serengeti domestic dog and lion populations.

The overall results are discussed in Chapter 7 and future lines of research proposed.

CHAPTER 2: GENERAL METHODOLOGIES AND OTHER DATA SOURCES

In order to be able to provide as complete and accurate a picture as possible of the epidemiology of rabies and canine distemper (CD) in the Serengeti, this study draws on the long-term surveillance data from the Serengeti carnivore populations as well as data obtained as a result of surveillance operations established in the course of this study. The aim of this chapter is to describe the activities carried out during the study period and provide documentation of other data sources. For the sake of clarity, details of the samples collected and households visited for questionnaire surveys as well as specific methods are included in the relevant chapters.

2.1 Project activities

The project involved: (i) design and implementation of field studies (Serengeti ecological region, northwestern Tanzania); (ii) laboratory (diagnostic, molecular and serological) analyses (Rabies Section of the Centers for Disease Control and Prevention [CDC], Atlanta, United States [US] and Intervet, United Kingdom [UK]); and (iii) analyses of epidemiological data (Wildlife and Emerging Diseases Section, Centre for Tropical Veterinary Medicine, University of Edinburgh, UK). The timeline of these activities, which are described in detail below, is given in Table 2.1.

2.1.1 Field studies

This work was part of a large scale research project, the Viral Transmission Dynamics Project, that has implemented mass domestic dog vaccination trials as a tool to investigate the infection dynamics of rabies, CD and canine parvovirosis in the Serengeti ecological region. Dog vaccinations have been introduced around the Serengeti National Park (SNP) since October 1996. The Viral Transmission Dynamics Project has extended the previous zone of dog vaccination, which included only one district (Serengeti) to the west of SNP, to encompass all villages within a 10 km zone bordering the western boundaries of the park (six districts: Tarime, Serengeti and Bunda in the Mara Region, Magu in the Mwanza Region, and Bariadi and Meatu in the Shinyanga Region) and all villages to the east of the park (one district: Ngorongoro in the Arusha Region). The three regions to the west, inhabited by large multi ethnic-communities, are predominantly agro-pastoralist. Ngorongoro District to the east is a multiple-use controlled wildlife area inhabited by low-density Maasai and Sonjo communities with production systems based on traditional pastoralism and limited cultivation. Figure 2.1 shows the location of villages

included in previous and current vaccination campaigns. The present study exploited the opportunity provided by the Viral Transmission Dynamics Project to undertake field studies. Field activities involved the implementation of surveillance programmes in domestic dog and wildlife populations both outside and inside SNP to obtain case incidence data, post-mortem samples for diagnosis and virus characterisation and dog and wildlife blood samples. Moreover, questionnaire surveys were conducted outside SNP to obtain demographic data from domestic dog populations.

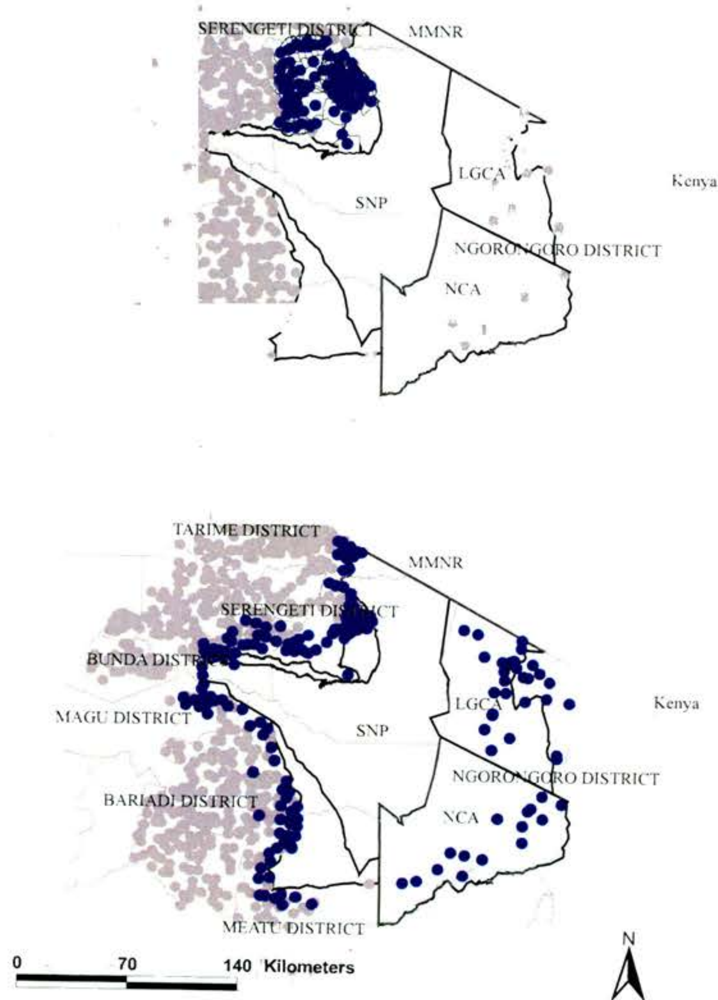


Figure 2.1. Map of the Serengeti ecological region of northwestern Tanzania showing the location of human settlements (each village is represented by solid gray circles) to the west and east of the Serengeti National Park (SNP) and of villages included in the previous (top) and current (bottom) domestic dog vaccination campaigns (represented by solid blue circles). LGCA = Loliondo Game Control Area; NCA = Ngorongoro Conservation Area; MMNR = Maasai Mara National Reserve.

2.1.1.1 Disease surveillance in domestic dog and wildlife populations outside SNP

2.1.1.1.1 Selection of study villages

Study villages were selected at random in each district at different distances from the park boundaries for intensive study. In the vaccination zone to the west of SNP two study villages were selected from each of the six districts within 0 to 10 km from the park boundaries. For five districts, unvaccinated (control) villages were selected outside the 10 km zone, five within 10 and 20 km and five not farther than 40 km from the park boundaries. Since the 2004 vaccination campaign in Serengeti District comprised all the villages within the district, the two unvaccinated villages for this district were selected in Musoma District, to the west of Serengeti District. Nine villages were randomly selected within the Loliondo Game Control Area of Ngorongoro District to the east. Figure 2.2 shows the location of the study villages.

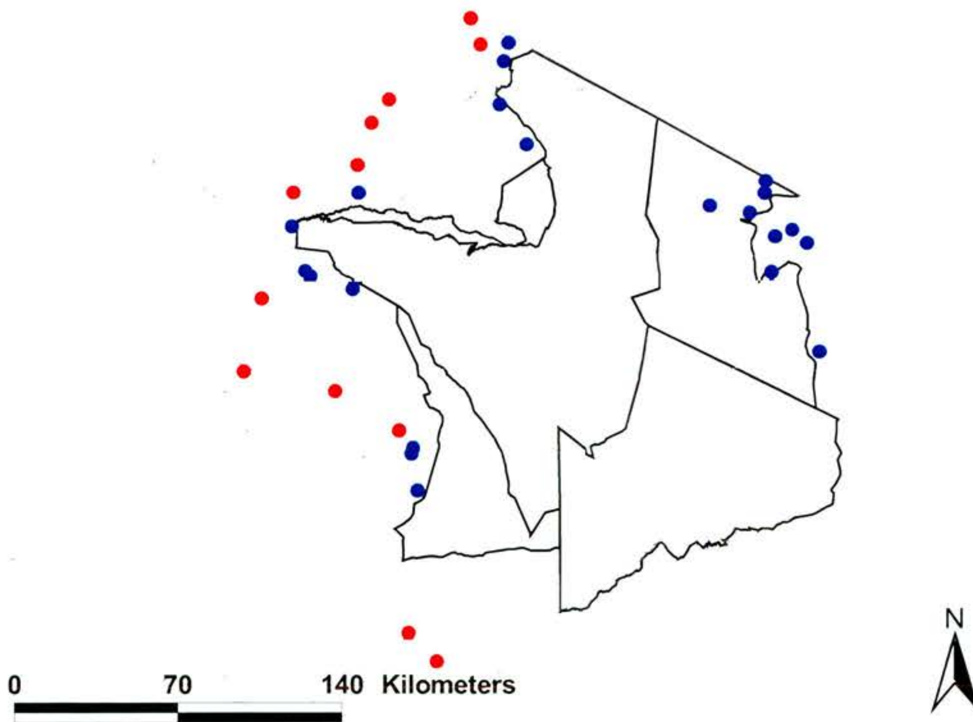


Figure 2.2. Map of the Serengeti ecological region of northwestern Tanzania showing the location of study villages in the vaccination (solid blue circles) and control (solid red circles) zones.

2.1.1.1.2 Rabies case-incidence data

To obtain rabies case incidence data outside SNP, community-based active surveillance measures were implemented in each of the study villages to the west within the vaccination and control zones based on previous studies in rural Kenya (Kitala *et al.*, 2000) and Tanzania (Cleaveland *et al.*, 2003). Livestock officers stationed in each of the villages were responsible for monthly collection of standardised information from village leaders, school teachers, medical dispensary staff and local healers on any suspect rabies cases in the village. In Serengeti and Ngorongoro Districts rabies detection also relied upon contact tracing activities implemented and coordinated by Katie Hampson, Princeton University, using case reports from active surveillance activities and animal-bite injury data from hospitals and medical dispensaries as primary sources. Due to logistical, financial and time constraints, contact tracing measures could only be adopted in these two districts. As part of the surveillance operations, financial incentives were offered to livestock officers and basic training provided for collection of brain stem samples from carcasses. Finally, post-mortem examination of any carcasses and sample collection were conducted during regular visits to the study area.

2.1.1.1.3 Serological surveillance

To obtain canine distemper virus (CDV) seroprevalence data, serological surveillance of domestic dogs was carried out in all the study villages. In the vaccination zone to the west and east of SNP, systematic sampling of dogs of a range of age classes was conducted during vaccination campaigns. In the unvaccinated zone, dogs were sampled in households randomly selected within each study village for questionnaire surveys (see section 2.1.1.4). The sampling methodology of households was based on random selection of ten-cell units (*balozis*) within each subvillage with sampling of all households within the ten-cell unit as described in section 2.1.1.4. Figure 2.3 shows the administrative units used for selection of households for serological surveillance in the unvaccinated areas and questionnaire surveys in both the vaccinated and unvaccinated areas. All dogs in the household were bled. Data were recorded on the name of the owner, name of the dog, age, sex and, in vaccination areas, on the previous vaccination history. Specific methods for dog sampling are described in detail in Chapter 6 (section 6.3.2).

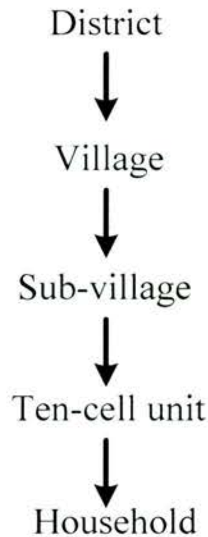


Figure 2.3. Administrative units used for selection of households for serological surveillance in the unvaccinated areas and questionnaire surveys in both the vaccinated and unvaccinated areas.

2.1.1.2 Disease surveillance in wildlife populations inside SNP

2.1.1.2.1 Rabies case-incidence data

Rabies surveillance in wildlife populations inside SNP mainly relied on the existing infrastructures of opportunistic surveillance established by Tanzania National Parks (TANAPA) and Tanzania Wildlife Research Institute (TAWIRI) that include a network of veterinarians, scientists, rangers, tourists and tour operators. With the establishment of the Viral Transmission Dynamics Project surveillance operations were reinforced by holding introductory workshops and meetings involving TANAPA veterinarians and rangers and TAWIRI veterinarians and scientists. Moreover, each of the 17 ranger posts within the SNP (Figure 2.4) was visited and practical sessions on animal disease surveillance and general post-mortem procedures were held. Post-mortem examination and sampling of any carnivore carcasses found constituted a major component of the disease surveillance inside the park and it was conducted whatever the apparent cause of death.

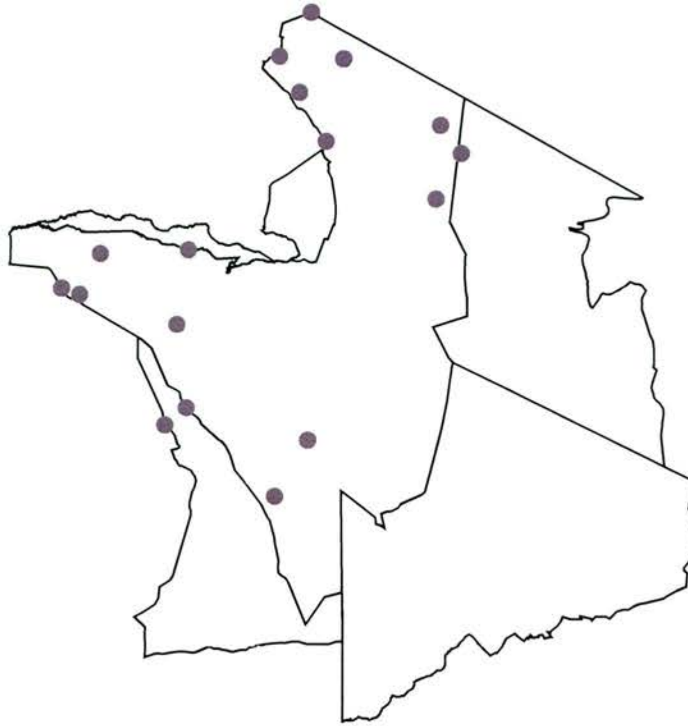


Figure 2.4. Location of ranger posts within the Serengeti National Park.

2.1.1.2.2 Serological surveillance

Serological surveillance in SNP focused on the lion population monitored by the Serengeti Lion Project, as several hundreds individuals on the western sectors of the park are individually recognised from natural markings (Packer and Pusey, 1993) and most have been regularly observed since birth (Pusey and Packer, 1994), therefore their precise ages are known. Moreover, base-line CDV serological data are available since the 1980s (Packer *et al.*, 1999). Systematic sampling of lions was conducted in collaboration with the TANAPA and TAWIRI Veterinary Departments. Specific methods of lion sampling are described in detail in Chapter 6 (section 6.3.3).

2.1.1.3 Field evaluation of a direct rapid immunohistochemical test in diagnostic rabies

As part of the surveillance operations implemented in this study, a direct rapid immunohistochemical test (dRIT) recently developed and optimised at the Rabies Section of the CDC was tested and validated in the field, as described in Chapter 3. The opportunity for the work only arose during the second year of the project after the first visit to the CDC, where the necessary training was provided.

2.1.1.4 Household surveys

Questionnaire surveys were conducted to collect information on dog demography, as described in Chapter 5 (section 5.3.2). Although the surveys included all study villages within the vaccination and control zones, only data for Serengeti and Ngorongoro Districts are presented in this study. Questionnaires were administered in randomly selected households within each study village. In areas to the west, the sampling methodology of households was based on random selection of ten-cell units (*balozis*) within each subvillage with sampling of all the households (10-15 per unit) within the selected units (Figure 2.3). In areas to the east, the household unit is the *boma*, a circular enclosure typically comprising 5-20 huts, occupied by one Maasai elder with one or more wives and children. Due to fewer and higher dispersion of Maasai *bomas*, the use of ten-cell units was not logistically feasible in these communities. Therefore, 10% of bomas in a village were randomly selected. The head of the household or, in his absence, any adult (above 18 years) was interviewed. Questionnaires were conducted in a language understandable to respondents (mainly Swahili or local languages whenever necessary). For this reason, a well-trained team of local project members accompanied the author during house-to-house interviews.

2.1.2 Laboratory analyses

A range of laboratory analyses were undertaken in the present study, including diagnostic, molecular (for rabies) and serological (for CDV) analyses, as described in the relevant chapters.

Rabies diagnostic work on the material obtained in the present study up to 2004 was conducted at the Rabies Section of the CDC and in Tanzania using the dRIT, with confirmatory testing carried out at CDC by a different operator (see Chapters 3-5, sections 3.3.3, 4.3.2 and 5.3.3.3). The opportunity for performing the dRIT in the field only arose during the second year of the project, as described in section 2.1.1.3. Diagnostic tests on brains collected in 2005 was carried out at CDC by a different operator. Genetic analyses of archived isolates and newly obtained virus samples described in Chapters 4 and 5 (sections 4.3.1 and 5.3.4) was conducted at CDC. The methods employed included RNA extraction, reverse transcriptase-polymerase chain reaction (RT-PCR) and nucleotide sequencing (see Chapter 4, section 4.3.3).

Serological analyses for CDV were performed at Intervet, UK using a microneutralisation test (see Chapter 6, section 6.3.4). The analyses were performed on all the more recent dog and lion serum samples obtained in this study and on some of the archived sera, as described in Chapter 6, section 6.3.1.

2.1.3 Data analyses

Data analyses, which are described in detail in the relevant chapters (3-6), included evaluation of diagnostic test performance, phylogenetic analyses of sequence data by using a range of phylogenetic reconstruction methods, analyses of demographic and case-surveillance data, and analyses of age-seroprevalence patterns.

Table 2.1. Timeline of activities carried out during the study period (gray = time spent in Edinburgh; green = fieldwork in Tanzania; blue = laboratory work at CDC and Intervet). SNP = Serengeti National Park; TANAPA = Tanzania National Parks; TAWIRI = Tanzania Wildlife Research Institute; US = United States; UK = United Kingdom; CDC = Centres for Disease Control and Prevention; dRIT = direct rapid immunohistochemical test; SEARG = Southern and Eastern Africa Rabies Group. *Because of time constraints serological assays of these samples could not be completed and the results are not presented in this study. **Rabies diagnosis on these brains was carried out by CDC operators.

Period	Activities
Oct-02	Registration for full-time PhD programme and organisation of field equipment.
Nov-02	Research and residency permits.
Dec-02/Apr-03	Launch of the Viral Transmission Dynamics Project and organisation of field station in SNP.
	Reinforcement of surveillance operations in SNP (TANAPA, TAWIRI).
	Establishment of field protocols for post-mortem examination and sample collection in SNP.
	Coordination of and active post-mortem sample collection in SNP.
	Lion serological surveillance in SNP in collaboration with TANAPA and TAWIRI.
	Introductory meetings in study areas outside SNP (Government Veterinary and Medical Officers).
	Selection of study villages outside SNP.
May/Jul-03	With the start of vaccination campaigns to the west of SNP:
	1. Implementation of community-based active surveillance measures in vaccinated and unvaccinated study villages: training and distribution of brain sample collection kits.
	2. Dog serological surveys in vaccinated study villages during vaccination campaigns.
	3. Questionnaire surveys in study villages in vaccination zone.
	Processing of export/import permits and shipment of brains and sera (US and UK).
Aug-03	First-year report write-up.
Sep-03	CDC: training in the dRIT method and rabies diagnostic work.
Oct-03	First-year report write-up and assessment.
Nov-03	Intervet: training in the serological assay methods.

Table 2.1. Continued.

Period	Activities
Nov-03	Organisation of field equipment.
Dec-03/Mar-04	Rabies surveillance operations follow-up both inside and outside SNP and sample collection.
	Implementation of dRIT in the field and processing of samples.
	With the start of vaccination campaigns to the east of SNP:
	1. Dog serological surveys in study villages.
	2. Questionnaire surveys in study villages.
	Dog serological surveys in study villages in unvaccinated zone to the west of SNP.
Apr/May-04	Intervet: serological assays.
Jun/Jul-04	Rabies surveillance operations follow-up both inside and outside SNP and sample collection.
	Processing of samples by dRIT.
	Dog serological surveys in vaccinated study villages to the west of SNP*
	Lion serological surveillance in SNP.
	Processing of export/import permits and shipment of brains and sera (US and UK).
Aug/Dec-04	CDC: dRIT diagnostic work and genetic analyses.
Jan/Apr-05	Rabies surveillance operations follow-up both inside and outside SNP and sample collection.
	Dog serological surveys in unvaccinated study villages to the west of SNP*.
	Dog serological surveys in vaccinated study villages to the east of SNP*.
	Lion serological surveillance*.
	Data entry.
	Processing of export/import permits and shipment of dog and lion sera (US and UK).
May/Jun-05	Data entry, dRIT manuscript write-up and submission to journal, and sequence editing.
Jul/Sep-05	Intervet: serological assays.
Oct/Nov-05	Sequence editing, data analysis and thesis write-up.
Dec-05/Jan-06	Rabies surveillance operations follow-up both inside and outside SNP and sample collection.
	Processing of export/import permits and shipment of brains (US)**.
	Oral presentations at TAWIRI conference (Tanzania) and SEARG meeting (Namibia).
Feb/Sep-06	Data analysis, phylogeny manuscript write-up and submission to journal, and thesis write-up.

2.2 Summaries of all data sources

Data sources for the rabies and CD studies described in this thesis are summarised in Table 2.2 and 2.3 respectively.

Table 2.2. Sources of data for the rabies study. SNP = Serengeti National Park; SC = Sarah Cleaveland; TL = Tiziana Lembo; KH = Katie Hampson; AFSSA = Agence Française de Sécurité Sanitaire des Aliments; CDC = Centres for Disease Control and Prevention; LFO = Livestock officer. †In collaboration with Tanzania National Parks and Tanzania Wildlife Research Institute. *With the exception of three previously typed viruses (Kissi *et al.*, 1995).

Periods	Zone	Type	Study design	Sampling	Diagnosis	Virus characterisation	Data analysis
1991-1996	Outside and inside† SNP	Passive	SC	SC	AFSSA	TL (CDC)*	TL
1997-1999	Outside SNP	Active (LFOs)	SC	SC	AFSSA	TL (CDC)	TL
	Inside† SNP	Passive	SC	SC	AFSSA	TL (CDC)	TL
2000-2001	Outside and inside† SNP	Passive	SC	SC	AFSSA	TL (CDC)	TL
2002	Outside SNP	Active (contact tracing)	KH	SC/TL	TL (CDC)	TL (CDC)	TL
	Inside† SNP	Passive	SC	SC/TL	TL (CDC)	TL (CDC)	TL
2003-2005	Outside SNP	Active (LFOs)	TL	TL	TL (CDC)	TL (CDC)	TL
		Active (contact tracing)	KH	TL	TL (CDC)	TL (CDC)	TL
	Inside† SNP	Passive	TL	TL	TL (CDC)	TL (CDC)	TL

Table 2.3. Sources of data for the canine distemper study. SC = Sarah Cleaveland; TL = Tiziana Lembo. †In collaboration with Tanzania National Parks, Tanzania Wildlife Research Institute and Serengeti Lion Project.

Periods	Study design	Sampling	Testing	Data analysis
Dog sampling				
1992-1994	SC	SC	SC (Intervet)/Cornell	SC
1996-1997	SC	SC	SC (Intervet)/Cornell	TL
1998-2000	SC	SC	SC/TL (Intervet)	TL
2001	SC	SC	Cornell	TL
2003-2004	TL	TL	TL (Intervet)	TL
Lion sampling				
1997-2001	SC†	SC†	SC (Intervet)/Cornell	TL
2002	SC†	SC†	TL (Intervet)/Cornell	TL
2003-2004	TL†	TL†	TL (Intervet)/Cornell	TL

CHAPTER 3: EVALUATION OF A DIRECT RAPID IMMUNOHISTOCHEMICAL TEST FOR RABIES DIAGNOSIS: A PRELIMINARY STUDY

Lembo, T., Niezgoda, M., Velasco-Villa, A., Cleaveland, S., Ernest, E. and Rupprecht, C.E. 2006. Evaluation of a direct, rapid immunohistochemical test for rabies diagnosis. *Emerging Infectious Diseases*, **12**: 310-313.

3.1 Abstract

A direct rapid immunohistochemical test (dRIT), recently developed at the Rabies Section of the Centers for Disease Control and Prevention (CDC), Atlanta, United States (US) for detection of rabies virus antigen using biotinylated anti-nucleocapsid monoclonal antibodies, was compared to the direct fluorescent antibody (DFA) test with 159 field brain samples from different animal species collected in northwestern Tanzania. The test was evaluated on frozen and glycerol preserved samples under both field and laboratory conditions. There was 100% agreement between results of the dRIT and DFA test regardless of whether the dRIT was performed in field or in laboratory conditions. Preservation in glycerol solution did not influence the test sensitivity and specificity. Rabies antigen was successfully detected in samples preserved in glycerol solution for up to 15 months prior to testing. Because the dRIT is rapid, simple, sensitive, specific, can be performed on glycerolated samples and does not require equipment other than an ordinary light microscope, it is believed that the use of this technique may be of value in countries with limited diagnostic infrastructures and in tropical settings.

3.2 Introduction

In much of the developing world, rabies surveillance and diagnosis in domestic and wild animals are severely constrained. High ambient temperatures hinder the collection and preservation of fresh specimens. The use of the direct fluorescent antibody (DFA) test, the gold standard in rabies diagnosis (Dean *et al.*, 1996; Trimarchi and Smith, 2002), is limited by the costs of acquiring and maintaining a fluorescent microscope. Difficulties in obtaining diagnostic results from field material have led to widespread under-reporting of disease. Consequently, the true public health impact of rabies has been greatly under-estimated, and political commitment for its control has been lacking (Dodet *et al.*, 2001; Cleaveland *et al.*, 2002; Coleman *et al.*, 2004). Moreover, the absence of a confirmatory test can result in the inappropriate management of animal bite injuries, with human mortality a potential consequence of delays in rabies post-exposure prophylaxis (PEP) and unnecessary administration of PEP. The latter is a particular concern given the scarcity and costs of human rabies vaccines and immunoglobulin in many parts of the world.

A rapid immunohistochemical test (RIT) for the detection of rabies virus (RABV) antigen has been developed at the Rabies Section of the Centers for Disease Control and Prevention (CDC), Atlanta, United States (US) by incorporating various components of existing immunoperoxidase techniques (Niezgoda and Rupprecht, 2006) (see also section 1.2.6.2). Like the DFA test, the RIT is performed on brain touch impressions (Figure 1.6) and is based on antibody recognition of rabies antigen. However, the product of the immunoperoxidase reaction can be observed by light microscopy and RABV antigen appears as magenta inclusions against a blue neuronal background (Figure 1.9). The test has shown its utility as a confirmatory test for the DFA test with the potential to be used as a field test for rabies diagnosis. The RIT recognises all genotype 1 variants of RABV examined to date and all representative *Lyssaviruses* (Niezgoda and Rupprecht, 1999). Modifications of a former indirect test have led to a direct test (dRIT) (Figure 1.8) that uses a cocktail of highly concentrated and purified biotinylated anti-nucleocapsid (N) monoclonal antibodies (MAbs) produced *in vitro* in a direct staining approach and allows a

diagnosis to be made in less than 1 hour (Niezgoda and Rupprecht, 1999; Niezgoda *et al*., 2002).

For the routine diagnosis of rabies, glycerol saline has proved to be a convenient preservative in situations where refrigeration or freezing facilities are not promptly available (Barrat and Blancou, 1988; Barrat, 1996) (see also section 1.2.6.1).

In this chapter findings are reported of a preliminary study to evaluate the dRIT, comparing results of the dRIT and DFA test performed under laboratory conditions at CDC and results of the dRIT carried out under field conditions in Tanzania with the dRIT and DFA test performed at CDC. The objectives were: (i) to evaluate the dRIT on frozen and glycerol preserved field brain samples (under both field and laboratory conditions) and (ii) to validate the dRIT as a field test for rabies surveillance.

3.3 Materials and methods

3.3.1 Sample collection

Samples used in this study were obtained between December 2002 and September 2004 as a result of passive and active rabies surveillance operations established in the Mara, Mwanza, Shinyanga, and Arusha Regions of northwestern Tanzania. In particular, the study area included the Serengeti National Park (SNP), four districts to the northwest of the park (Musoma, Tarime, Serengeti and Bunda), three districts to the southwest (Magu, Bariadi and Meatu), and one district to the east (Ngorongoro) (Figure 3.1).

Sample collection in wildlife populations inside SNP relied mainly on the existing infrastructures of opportunistic surveillance established by Tanzania National Parks (TANAPA) and Tanzania Wildlife Research Institute (TAWIRI) veterinarians and scientists. Post-mortem examination was performed and brain stem samples collected from any suspect and non-suspect carcasses encountered by chance or reported by park veterinarians, rangers, scientists, tourists or tour operators, whatever the suspected cause of death.

To obtain samples from domestic and wild animals outside SNP, community-based active surveillance measures were implemented based on previous studies in rural Kenya (Kitala *et al.*, 2000) and Tanzania (Cleaveland *et al.*, 2003). Livestock field officers stationed in randomly selected study villages (see sections 2.1.1.1.1 and 2.1.1.1.2 and Figure 2.2) were offered financial incentives for collection of samples from any suspect and non-suspect domestic and wild animals. Whenever necessary, basic training on sample collection techniques was provided.

Sample collection was coordinated from two field stations where refrigeration or freezing facilities were available, a field laboratory of the TANAPA Wildlife Veterinary Unit located in SNP and the district veterinary office in Mugumu in Serengeti District (Figure 3.1). Field officers stationed in villages located in other districts were visited regularly to collect any samples collected.

Fifteen archived glycerolated specimens obtained in previous investigations (between 1999 and 2002) and stored at -20°C were also analysed to evaluate test sensitivity with samples stored for longer periods of time.

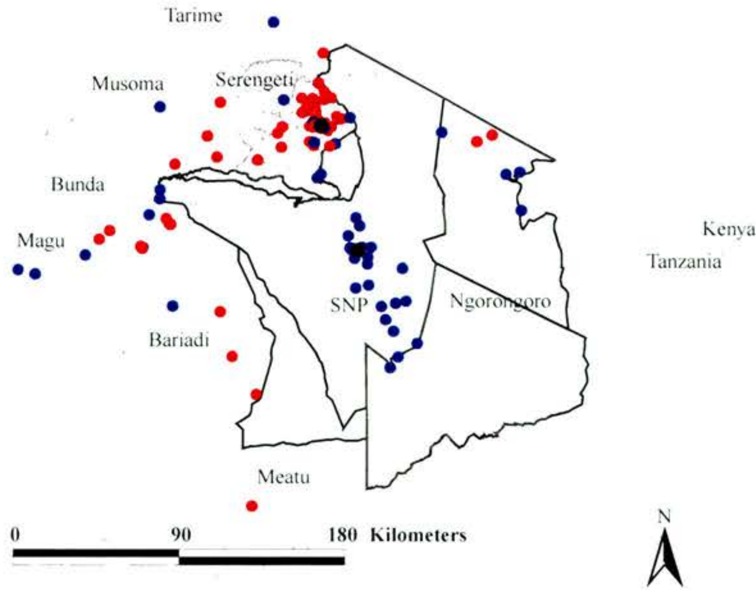


Figure 3.1. A map of the study area showing the geographical distribution of samples used in this study for which accurate locations were available. Samples recovered from domestic species are indicated as red dots and those recovered from wildlife species as blue dots. Black dots indicate the locations of the two field stations, in Serengeti National Park (SNP) and Mugumu (Serengeti District) from which sample collection was coordinated.

The number of samples by year and area is given in Table 3.1.

Table 3.1. The number of brain samples from domestic and wild animals tested in this study. SNP = Serengeti National Park.

	SNP	Outside SNP		
Year of collection	Wildlife	Domestic	Wildlife	Total
Archived				
1999	-	10	-	10
2001	2	-	-	2
2002	3	-	-	3
This study				
2002 (December)	4	1	-	5
2003	23	37	11	71
2004	8	47	13	68
Total	40	95	24	159

The technique of choice for sample collection was removal via the occipital foramen (*foramen magnum*) by inserting a drinking-straw (Figure 3.2), according to World Health Organisation (WHO) recommendations (Barrat, 1996). For this purpose, the

field officers were provided with ready-to-use collection kits prepared following the WHO guidelines. Occasionally samples were collected by opening the skull (Figure 3.3).

Figure 3.2. Occipital foramen (*foramen magnum*) route brain sampling.

Figure 3.3. Brain sampling by opening the skull.

3.3.2 Sample preservation and handling

Sample preservation varied based on logistical constraints related to the collection and transport of samples and the availability of refrigeration or freezing facilities. Some specimens were frozen just after collection (-20°C). The other samples were placed into a phosphate-buffered 50% glycerol solution at the time of collection without being extracted from the straw and stored either in kerosene refrigerators (0 - +4°C) or in an electric freezer (-20 - -10°C) or, whenever refrigeration or freezing

facilities were not promptly available, kept at room temperature (25 ± 5°C) for between a few hours and 4 months prior to refrigeration or freezing.

The reagents necessary to perform the dRIT became available in the field in 2004, as described in section 2.1.1.3. Therefore, 105 samples were processed by the dRIT and DFA test at CDC only, and 54 samples were tested by dRIT in the field, and re-tested at CDC by both dRIT and DFA test or DFA test only if the aliquot available was not sufficient to perform both tests.

According to the method of preservation and whether the samples were tested in the field and at the CDC laboratory, or at CDC only, samples fell into 4 groups (Table 3.2). Group A samples were kept in glycerol solution for <1-15 months and washed in phosphate buffered saline (PBS) before testing by dRIT in the field. They were then stored at -20°C for <1-5 months and re-transferred into fresh glycerol for shipment. At CDC, the samples were kept in glycerol for <1-2 months at +4°C and re-washed in PBS before re-testing by both dRIT and the DFA test or the DFA test only. Group B samples were stored frozen for <1-6 months, processed by dRIT in the field and placed into glycerol solution for shipment to CDC where they were stored for 2 months at +4°C before being washed in PBS and re-tested. Group C samples were preserved in glycerol solution for <1-60 months, shipped and processed at CDC by the dRIT and DFA test without previous testing in the field. These samples were washed in PBS just before testing. Group D samples were stored at -20°C in the field for 2-24 months, shipped frozen and tested at CDC by the dRIT and DFA test. The 15 archived samples fell into group C.

Frozen samples were transported to the CDC laboratory on dry ice. Samples shipped in glycerol were preserved either cold (ice packs or dry ice) or at room temperature during transit to the CDC laboratory.

Table 3.2. Methods of sample preservation and number of samples processed by each test. PBS = phosphate-buffered saline; dRIT = direct rapid immunohistochemical test; CDC = Centers for Disease Control and Prevention; DFA = direct fluorescent antibody assay. *For 5 samples, no aliquots were available to perform the dRIT at CDC. These samples were therefore re-tested at CDC by DFA test only.

Preservation	No. washes in PBS	No. of samples tested by each test		
		dRIT field	dRIT CDC	DFA test CDC
Group A. Glycerol saline/frozen/glycerol saline	2	44	39*	44
Group B. Frozen/glycerol saline	1	10	10	10
Group C. Glycerol saline	1	0	89	89
Group D. Frozen	0	0	16	16

3.3.3 dRIT procedure

A qualitative assessment of the samples was made prior to testing.

The protocol used to perform the dRIT in the field and CDC laboratory was the same and it is summarised in Box 3.1. What varied were the storage conditions of the reagents that require refrigeration, namely the CDC cocktail of anti-rabies biotinylated MAbs and streptavidin-peroxidase (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD, US). The reagents were transported to the field station in a cooler containing ice packs and stored in a kerosene refrigerator at an average daily temperature of 0 ± 2°C instead of +4°C as indicated in the CDC protocol and manufacturer's instructions.

Glycerolated tissue specimens were soaked in PBS for a few minutes, shaken vigorously and rinsed in PBS one or two more times. This treatment was repeated in the CDC laboratory for samples re-transferred into glycerol solution for shipment.

A small amount of brain was placed on a wooden tongue-depressor or blotting-paper and a touch impression made by touching a glass microscope slide against the sample (Figure 1.6) and blotting the excess of sample and glycerol onto clean blotting-paper. The slides were allowed to air-dry for 2 minutes.

Five specimens at a time were stained by dRIT at ambient temperature as described below. The slides were fixed in 10% buffered formalin for 10 minutes, dip-rinsed in wash buffer PBS with 1% Tween 80 (TPBS), immersed in 3% hydrogen peroxide for 10 minutes and dip-rinsed in fresh TPBS. After dipping, the excess buffer was shaken from the slides and blotted from the edges surrounding the impression. This treatment was repeated after each rinsing step. The slides were incubated in a 'humidity chamber' (a cover on a moistened paper towel on an even surface) (Figure 3.4) with the MAb cocktail for 10 minutes, dip-rinsed in TPBS and incubated as described before with streptavidin-peroxidase complex for 10 minutes. The slides were then dip-rinsed using the same wash buffer used in the previous step. A 3-amino-9-ethylcarbazole (AEC) stock solution was prepared by dissolving one 20 mg tablet of AEC (Sigma-Aldrich Corp., St Louis, MO, US) into 4 ml of N,N-dimethylformamide (Fisher Scientific International, Inc., Pittsburgh, PA, US) and stored at +4°C. A working dilution was prepared by adding 1 ml of the AEC stock solution to 14 ml of 0.1 M acetate buffer (Polyscientific, Bay Shore, NY, US) and 0.15 ml of 3% hydrogen peroxide. The mixture was filtered just prior to use (0.45 μm filter). The slides were incubated with the AEC peroxidase substrate for 10 minutes and dip-rinsed in distilled water. They were then counterstained with Gill 2 hematoxylin (Fisher Scientific International) diluted 1:2 with distilled water and filtered to 0.45 μm in a humidity chamber for two minutes. Two dip-rinses in distilled water followed, the first of which was made in the same distilled water as used in the previous step. Finally, the slides were mounted with a water-soluble mounting medium (BioMeda Corp., Foster City, CA, US) and examined by light microscopy (Leica Microsystems AG, Wetzlar, Germany in Tanzania and Axioplan 2, Carl Zeiss AG, Göttingen, Germany at CDC) at magnifications of x200 to x400. The slides were stained and read by the same operator (the author) in the field and at CDC. However, at CDC identification numbers unknown to the operator were assigned. The DFA test (FITC Anti-Rabies Monoclonal Globulin, Fujerebio Diagnostic Inc., Malvern, PA, US) was performed in a blind manner by another operator following the minimum standard protocol for rabies diagnosis (http://www.cdc.gov/ncidod/dvrd/rabies/Professional/publications/DFA_diagnosis/DFA_protocol-b.htm).

Confidence intervals (CIs) for the sensitivity (Se) and specificity (Sp) of the dRIT compared against the gold standard test (DFA test) were calculated using the exact binomial distribution (S-Plus, Insightful Corp., Seattle, WA, US).

Box 3.1. Direct rapid immunohistochemical test (dRIT) general procedure. PBS = phosphate-buffered saline. MAb = monoclonal antibody.

1. Soak tissue samples in PBS for a few minutes (only glycerolated samples).
2. Make brain touch impressions on miscroscope slides (include standard positive and negative controls).
3. Air-dry slides for 2 minutes.
4. Fixation in 10% buffered formalin for 10 minutes.
5. Dip-rinse slides in fresh wash buffer TPBS (PBS with 1% Tween 80).
6. Immerse slides in 3% hydrogen peroxide for 10 minutes.
7. Dip-rinse slides in fresh wash buffer TPBS.
8. Incubate slides with biotinylated anti-rabies MAb cocktail in a humidity chamber for 10 minutes.
9. Dip-rinse slides in fresh wash buffer TPBS (can use this same wash buffer through step 11).
10. Incubate slides with streptavidin-peroxidase complex in a humidity chamber for 10 minutes.
11. Dip-rinse slides in wash buffer TPBS.
12. Incubate slides with peroxidase substrate (3-amino-9-ethylcarbazole, AEC) for 10 minutes (prepare the working dilution just prior to use).
13. Dip-rinse slides in fresh deionised/distilled water (can re-use through step 15).
14. Counterstain (Gills Hematoxylin diluted 1:2 with deionised/distilled water) for 2 minutes.
15. Dip-rinse in deionised/distilled water.
16. Dip-rinse in fresh deionised/distilled water.
17. Mount slides with water-soluble mounting medium.
18. View slides by light microscopy.

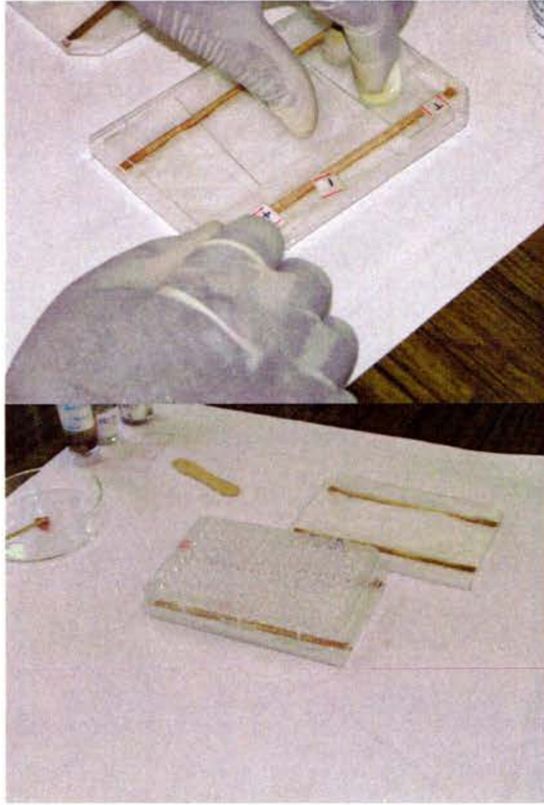


Figure 3.4. Humidity chamber.

3.4 Results

Fifty-nine specimens (37.1%) were rabies positive and 100 (62.9%) negative by dRIT, with 100% agreement between the dRIT and the DFA test regardeless of whether the dRIT was performed in field or laboratory conditions. Table 3.3 shows the proportion of positive samples in domestic and wildlife species according to the period of sampling and whether the samples were tested in the field and at CDC or at CDC only. The proportion of positive samples in the various animal species is given in Table 3.4.

Table 3.3. Proportion of brain samples that tested positive by direct rapid immunohistochemical test (dRIT) and direct fluorescent antibody (DFA) test in domestic and wildlife species. Sample sizes are shown in brackets. *see section 3.3.2 and Table 3.2.

Tests performed	Period of sample collection	Domestic % positive	Wildlife % positive	Total % positive
Groups A and B* (dRIT field and DFA test CDC)	2003-2004 (this study)	91.4 (35)	15.8 (19)	64.8 (54)
Groups C and D* (dRIT and DFA test CDC)	1999-2002 (archived)	0.0 (10)	0.0 (5)	0.0 (15)
	2002-2004 (this study)	46.0 (50)	2.5 (40)	26.6 (90)
Total		57. 9 (95)	6.2 (64)	37.1 (159)

Table 3.4. Proportion of brain samples that tested positive by direct rapid immunohistochemical test (dRIT) and direct fluorescent antibody (DFA) test for different animal species. Sample sizes are given in brackets. *see section 3.3.2 and Table 3.2. †Species not definitively identified.

	Tests performed and period of sample collection			
	Groups A and B* (dRIT field and DFA test CDC)	Groups C and D* (dRIT and DFA test CDC)		
	2003-2004 (this study)	1999-2002 (archived)	2002-2004 (this study)	Total
Species	% positive	% positive	% positive	% positive
Domestic dog (*Canis familiaris*)	92.3 (26)	0.0 (10)	40.5 (37)	53.4 (73)
Domestic cat (*Felis catus*)	50.0 (2)	-	40.0 (5)	42.8 (7)
Cow (*Bos taurus*)	100.0 (4)	-	75.0 (4)	87.5 (8)
Goat (*Capra hircus*)	100.0 (3)	-	66.6 (3)	83.3 (6)
Livestock†	-	-	100.0 (1)	100.0 (1)
Aardwolf (*Proteles cristatus*)	0.0 (1)	-	-	0.0 (1)
African civet (*Civettictis civetta*)	0.0 (1)	-	0.0 (1)	0.0 (2)
Banded mongoose (*Mungos mungo*)	-	-	0.0 (2)	0.0 (2)
Slender mongoose (*Herpestes sanguineus*)	-	0.0 (1)	0.0 (2)	0.0 (3)
Dwarf mongoose (*Helogale parvula*)	-	0.0 (1)	0.0 (1)	0.0 (2)
White-tailed mongoose (*Ichneumia albicauda*)	50.0 (2)	-	0.0 (6)	12.5 (8)
Mongoose†	-	-	0.0 (2)	0.0 (2)
Black-backed jackal (*Canis mesomelas*)	0.0 (1)	-	0.0 (2)	0.0 (3)
Bat-eared fox (*Otocyon megalotis*)	0.0 (3)	-	0.0 (5)	0.0 (8)
Black-backed jackal/bat-eared fox†	50.0 (2)	-	-	50.0 (2)
Cheetah (*Acinonyx jubatus*)	-	0.0 (2)	0.0 (1)	0.0 (3)
Small-spotted genet (*Genetta genetta*)	0.0 (1)	0.0 (1)	20.0 (5)	14.3 (7)

Table 3.4. Continued.

	Tests performed and period of sample collection			
	Groups A and B* (dRIT field and DFA test CDC)	Groups C and D* (dRIT and DFA test CDC)		
	2003-2004 (this study)	1999-2002 (archived)	2002-2004 (this study)	Total
Species	% positive	% positive	% positive	% positive
Lion (*Panthera leo*)	0.0 (2)	-	0.0 (4)	0.0 (6)
Serval (*Felis serval*)	-	-	0.0 (1)	0.0 (1)
Spotted hyaena (*Crocuta crocuta*)	16.6 (6)	-	0.0 (6)	8.3 (12)
Striped hyaena (*Hyaena hyaena*)	-	-	0.0 (1)	0.0 (1)
Zorilla (*Ictonyx striatus*)	-	-	0.0 (1)	0.0 (1)
Total domestic	91.4 (35)	0.0 (10)	46.0 (50)	57.9 (95)
Total wildlife	15.8 (19)	0.0 (5)	2.5 (40)	6.2 (64)
Total	64.8 (54)	0.0 (15)	26.6 (90)	37.1 (159)

Overall, assuming that the DFA test was 100% sensitive and specific, the dRIT was 100% sensitive (95% CI: 93.9-100.0) and 100% specific (95% CI: 96.3-100.0). The Se and Sp of the dRIT compared against the gold standard test are reported in Tables 3.5A-C for samples tested in the field and at CDC (groups A and B) and Tables 3.6A-C for samples tested at CDC only (groups C and D), excluding the 15 archived samples which were all negative (Table 3.3).

Table 3.5. Correlation between results obtained by the direct rapid immunohistochemical test (dRIT) performed in the field and the direct fluorescent antibody (DFA) test performed in the laboratory on 54 field brain specimens: (A) overall, (B) on domestic animal samples, and (C) on wildlife samples.

A

	DFA result		
dRIT result	Positive	Negative	Total
Positive	35	0	35
Negative	0	19	19
Total	35	19	54

Sensitivity = 100.0% (95% CI: 89.9-100.0)
Specificity = 100.0% (95% CI:82.3-100.0)

B

	DFA result		
dRIT result	Positive	Negative	Total
Positive	32	0	32
Negative	0	3	3
Total	32	3	35

Sensitivity = 100.0% (95% CI: 89.1-100.0)
Specificity = 100.0% (95% CI: 29.2-100.0)

C

	DFA result		
dRIT result	Positive	Negative	Total
Positive	3	0	3
Negative	0	16	16
Total	3	16	19

Sensitivity = 100.0% (95% CI: 29.2-100.0)
Specificity = 100.0% (95% CI: 79.4-100.0)

Table 3.6. Correlation between results obtained by the direct rapid immunohistochemical test (dRIT) and the direct fluorescent antibody (DFA) test performed in the laboratory on 90 field brain specimens: (A) overall, (B) on domestic animal samples, and (C) on wildlife samples.

A

	DFA result		
dRIT result	Positive	Negative	Total
Positive	24	0	24
Negative	0	66	66
Total	24	66	90

Sensitivity = 100.0% (95% CI: 85.7-100.0)
Specificity = 100.0% (95% CI: 94.5-100.0)

B

	DFA result		
dRIT result	Positive	Negative	Total
Positive	23	0	23
Negative	0	27	27
Total	23	27	50

Sensitivity = 100.0% (95% CI: 85.2-100.0)
Specificity = 100.0% (95% CI: 87.2-100.0)

C

	DFA result		
dRIT result	Positive	Negative	Total
Positive	1	0	1
Negative	0	39	39
Total	1	39	40

Sensitivity = 100.0% (95% CI: 2.5-100.0)
Specificity = 100.0% (95% CI: 90.9-100.0)

The sensitivities of the dRIT and DFA test were comparable regardless of the method of preservation. There is no evidence that storage times affected positivity as 34 of 44 (77.2%) samples stored in glycerol solution for up to 10 months before being tested in the field and re-tested at CDC after an interval of up to 6 months remained positive. Furthermore, RABV antigen was successfully detected in the sample that

had been preserved in glycerol for the longest duration (15 months) before performing the dRIT in the field, stored frozen for 3 months before shipment to CDC and kept in glycerol for 2 months before being re-tested (Figures 3.5A and B). Similarly, viral inclusions were detected in a sample stored frozen for 24 months, although the antigen distribution was sparse with both tests. These data do not provide any unequivocal conclusions on test sensitivity with samples preserved in glycerol solution for over 15 months as all the 15 archived brains were negative. For these samples, the presence of antigen at the time of collection cannot be excluded.

Four of 10 (40.0%) deteriorated specimens were positive (Figures 3.6A and B). Among the 6 negative brains only one was a rabies suspect specimen. The negative finding might have been due to inadequate preservation since the sample had been stored in glycerol solution at ambient temperature for up to 4 months prior to refrigeration.

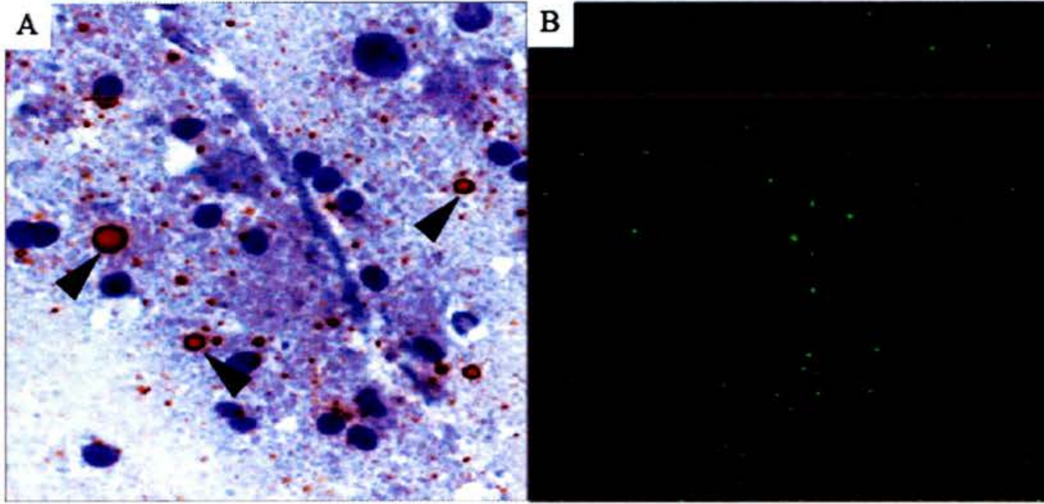


Figure 3.5. Touch impression of a rabies-positive Tanzanian domestic dog (*Canis familiaris*) brain preserved in 50% glycerol saline solution for 15 months prior to testing by direct rapid immunohistochemical test (dRIT) and re-tested by direct fluorescent antibody (DFA) test after an interval of 5 months. (A) Brain stained by dRIT: rabies virus antigen appears as magenta inclusions (arrowheads) against the blue neuronal background. Magnification, x630. (B) Immunofluorescent apple-green viral inclusions in the same brain processed by DFA test. Magnification, x200.

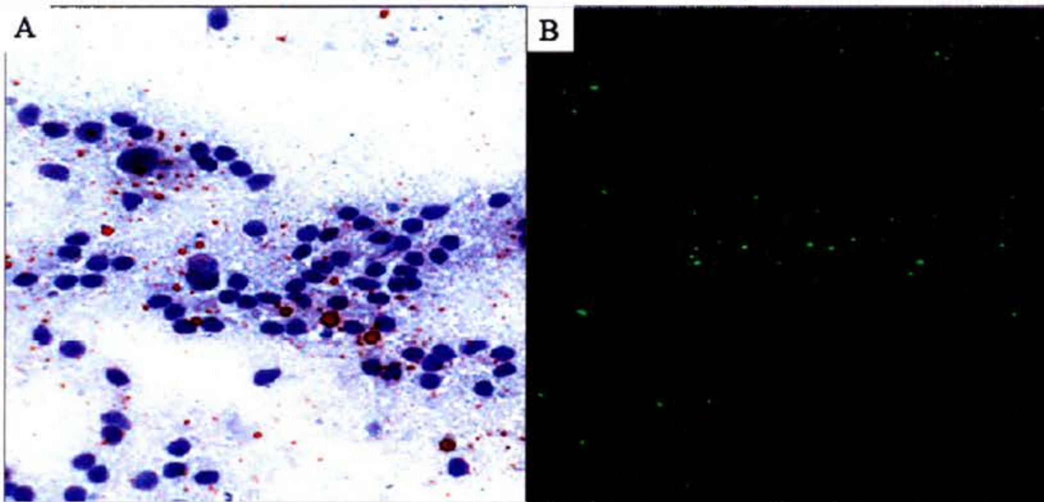


Figure 3.6. Touch impression of a deteriorated glycerolated brain from a Tanzanian spotted hyaena (*Crocuta crocuta*) with rabies. (A) Brain processed by direct rapid immunohistochemical test (dRIT). Magnification, x630. (B) DFA staining procedure on the same brain. Magnification, x200.

3.5 Discussion

The aim of this investigation was to explore and validate the use of a novel rabies immunohistochemical diagnostic test (dRIT) for epidemiological studies of rabies in countries with limited facilities for collection and storage of diagnostic material and poor infrastructures for rabies diagnosis. The Se and Sp of the dRIT were evaluated by comparing it with the gold standard rabies diagnostic technique, the DFA test. The dRIT was 100% sensitive and specific regardless of the method of preservation and whether the dRIT was performed in field and laboratory conditions or laboratory conditions only. Although limited, this study provides valuable data about the performance and potential of this test in developing country settings.

Throughout much of the developing world, the submission of brain material from suspected and non-suspected rabid animals is hampered by difficulties in sample collection, storage and transport, especially in very remote areas. Simple methods for sample collection and preservation that can easily be carried out by a range of operators (e.g. field assistants, public health inspectors, livestock and veterinary officers) have therefore been developed by WHO collaborating centres (Barrat and Blancou, 1988; Barrat, 1996). In particular, glycerol saline is the preservative of choice for samples stored under field conditions (i.e. without prompt refrigeration/freezing facilities) (Barrat and Blancou, 1988; Barrat, 1996). Although current rabies diagnostic tests such as the DFA test, virus isolation tests and molecular methods for detection of viral RNA may be used on glycerol-preserved samples (Barrat *et al.*, 1988; Barrat, 1996; Aguilar-Setien *et al.*, 2003), not all laboratories in developing countries have the necessary equipment to perform these tests and none of these techniques can be used in field conditions. The rabies rapid enzyme immunodiagnosis (RREID), a potential diagnostic method for local laboratories in developing countries (Perrin *et al.*, 1986; Bourhy *et al.*, 1989) (see also section 1.2.6.2), does not appear to perform well on glycerol-preserved samples: Saxena *et al.* (1989) reported 93% correlation between RREID and FA test in the case of fresh brains and 71% correlation in glycerol-preserved samples. Before the present study the dRIT had not yet been used on glycerolated field brain material. In this study the test was successfully performed on samples preserved in glycerol solution for 15 months and on frozen samples for 24 months with 100% correlation

between dRIT and DFA test, although definitive conclusions on samples preserved in glycerol solution for longer periods could not be drawn. These results are promising and highlight the potential value of the dRIT for enhancing rabies surveillance in areas where glycerol saline is the only feasible means of sample preservation. Further evaluation of test performance on samples preserved in this medium should therefore be conducted.

Over the past 40 years the DFA test has been the global standard for rabies diagnosis because it is rapid, reliable, accurate, sensitive and highly specific (Dean *et al.*, 1996). However, the test has practical drawbacks that have limited its use in developing countries and field conditions (Dodet *et al.*, 2001). One is the requirement of specialised laboratory facilities, i.e. the fluorescence microscope equipment, which is costly and necessitates high standards of maintenance. Second, considerable expertise is essential to perform the test and interpret the results (Trimarchi, 2000). Finally, the antirabies conjugate necessary to carry out the test is not regularly available. Before the development of the dRIT, no assays suitable for laboratories with reduced diagnostic infrastructures (e.g. RREID, latex agglutination (LA) test, enzyme immuno-assay (EIA), and dot blot enzyme immunoassay (DIA): Perrin *et al.*, 1986; Bourhy *et al.*, 1989; Jayakumar *et al.*, 1995; Kasempimolporn *et al.*, 2000; Vasanth *et al.*, 2004; Madhusudana *et al.*, 2004) had showed characteristics equivalent to those of the DFA test (see also section 1.2.6.2) so to be considered as a possible replacement for the DFA test. The present study indicates that such a test is now available with a significant improvement on the gold standard test in that it can be performed using only a light microscope, a piece of equipment widely available in clinics throughout the developing world. Other qualities make the dRIT ideal for testing in field situations and tropical settings, such as enhanced biosafety (due to the use of formalin as a fixative), fixation at ambient temperature, and sensitivity in detecting antigen in decomposed material (see also section 1.2.6.2). The test is simple and can be performed by a range of operators if appropriate training is provided. The dRIT clearly offers the hope of revolutionising rabies diagnostics in developing countries. The present study was the first application of the dRIT in African settings. The results are encouraging, although they were based on relatively small sample sizes, which is clearly a limitation in this study. Overall, 95%

CIs for the Se and Sp of the dRIT compared against the gold standard test were relatively narrow. However, wider intervals were obtained when the results were broken down according to whether the samples were tested in the field (dRIT) with confirmatory testing at the CDC laboratory (DFA test) or at CDC only (both dRIT and DFA test), and by species (i.e. domestic or wildlife). For instance, the small number of negative samples in domestic species (as reported in Table 3.5B) and positive samples in wildlife species (as reported in Tables 3.5C and 3.6C) makes inferences about test Sp and Se respectively somewhat unreliable as indicated in the wide CIs reported in the above-mentioned tables. Sample sizes should be increased before definitive conclusions can be drawn. Further laboratory and field evaluation are therefore required and, because of the enormous potential of the method, should be regarded as a priority.

In the present study the standardised protocol for post-mortem diagnosis of rabies in animals by DFA test (http://www.cdc.gov/ncidod/dvrd/rabies/Professional/publications/DFA_diagnosis/DFA_protocol-b.htm) was adopted to validate the results of the dRIT by comparison. The protocol has been developed by the Rabies Diagnostic Committee, US (including the Rabies Section of the CDC) to ensure a test of highest Se and Sp. The DFA test remains the standard against which newly established diagnostic approaches are compared on condition that the test is performed by properly trained technologists and the results are interpreted by experienced microscopists (Trimarchi and Smith, 2002). Although the data presented in this study may include false positive (lack of Sp) and false negative (lack of Se) results, DFA testing was performed by a competent CDC operator following the recommendations included in the standardised protocol which should provide a Se and Sp approaching 100%. It is therefore believed that for the purpose of this study there was no more precise estimation of the prevalence of rabies in the samples tested than the proportion of positive DFA results.

The proportion of positives that are correctly diagnosed by the test (true positives) is the positive predictive value (PPV) of the test. A major factor influencing the PPV is the disease prevalence. The higher the prevalence in the samples submitted for diagnosis, the greater the probability that a positive result will be correct (i.e. the PPV increases). Conversely, as the prevalence increases, the negative predictive

value, NPV, (i.e. the proportion of negatives that are correctly diagnosed by the test) decreases. In case of human exposure to a bite by a suspect animal, the consequences of false-negative results can be expected to include human mortality. The NPV is therefore critical. The PPV is also important, since false-positive results can lead to unnecessary initiation of PEP. In the present study it was found that rabies prevalence in samples tested both in the field and at CDC was higher than in samples tested at CDC only (64.8 vs 26.6%, as reported in Table 3.3). Furthermore, within each group prevalence in domestic samples was higher than in wildlife samples (91.4 vs 15.8% in samples tested in the field and at CDC, and 46.0 vs 2.5% in samples tested at CDC only, as reported in Table 3.3). Assuming a gold standard test of lower Se and Sp (e.g. 99 or 98%) and given the prevalence of rabies in domestic samples tested in field conditions (91.4%), the PPV would remain high (>99%), whereas the decrease in NPV would be more marked (e.g. >82% if Se and Sp of the DFA test were 98%). Considering the disease prevalence in wildlife samples tested in the field (15.8%) and domestic and wildlife samples tested at CDC only (46.0% and 2.5% respectively), decreases in Se and Sp would have more influence on the PPV than the NPV. For example, the NPV of the test, with a Se and Sp of 98% and given a rabies prevalence of 2.5%, would be only >55%.

One quality of the dRIT is its ability to recognise antigens from a global spectrum of *Lyssaviruses*. A single test can therefore be used to detect genotype 1 RABV variants and rabies-related viruses (Niezgoda and Rupprecht, 1999), unlike the RREID for example whose 'routine' version is not sensitive to rabies-related viruses (Perrin *et al.*, 1986). Rabies-related viruses such as Mokola, Lagos bat, and Duvenhage viruses have not yet been confirmed in Tanzania. However, their geographical distribution, which includes southern, central, western and eastern Africa, indicates that they may also occur in Tanzania. With the current version of the dRIT cross-reactivity to other *Lyssavirus* genotypes cannot be ruled out. However, attempts have been made by the Rabies Section of the CDC to replace the cocktail of MAbs used to perform the dRIT with panels of MAbs with strain discriminatory reactivities (Niezgoda *et al.*, 2004).

The dRIT could increase in-country capabilities for rabies surveillance and improve disease management in both animal and human populations. First, the application of this technique could greatly enhance epidemiological surveillance in areas where

rabies incidence data are difficult to obtain because of their remoteness, as occurs throughout much of rural Africa, providing a better indication of the magnitude of the rabies problem and trends in disease incidence. This is of particular importance given that WHO is evolving various strategies to control and eliminate animal rabies in developing countries. Local capacity for rabies surveillance is clearly an essential component of any rabies control/elimination programme, with confirmation of cases by laboratory diagnosis needed to determine disease incidence at the start of the programme and evaluate the impact of the intervention. Second, the dRIT could improve the ability to respond to epidemics with effective management decisions. The importance of a prompt diagnosis leading to a timely response to rabies outbreaks threatening endangered wildlife for instance cannot be overemphasised (Randall *et al.*, 2004). Third, the dRIT could be extremely valuable in guiding decisions regarding rational use of rabies PEP. Rabies currently poses a major economic burden on public health budgets in developing countries as a results of the high cost of PEP (Meslin *et al.*, 1994; WHO, 1997). The current high rate of administration in Tanzania, as well as in much of the developing world, arises largely because, in the absence of laboratory confirmed diagnosis, any person bitten by an animal is invariably prescribed PEP. Tangible economic benefits could therefore accrue from the establishment of local capacity for accurate rabies diagnosis.

In order to enhance capacity for local screening and rabies diagnosis in developing countries using dRIT, technical support and training would be required. Although there are as yet no commercial kits, all the reagents required for the dRIT are commercially available, apart from the cocktail of MAbs, which is currently only produced by the Rabies Section of the CDC. As a WHO reference laboratory with a commitment to supporting rabies surveillance in the developing world the CDC offers both training and technology transfer in the use of the new biological. It is therefore expected that the cocktail will ultimately become widely available.

CHAPTER 4: MOLECULAR EPIDEMIOLOGY IDENTIFIES DOMESTIC DOGS AS RESERVOIR OF A SINGLE RABIES VIRUS VARIANT IN COMPLEX CARNIVORE COMMUNITIES OF THE SERENGETI

The sequences of rabies viruses produced in this study have been deposited in the GenBank database (accession nos. DQ900547-DQ900579).

Lembo, T., Haydon, D.T., Velasco-Villa, A., Rupprecht, C.E., Packer, C., Brandão, P.E., Kuzmin, I.V., Fooks, A.R., Barrat, J. and Cleaveland, S. 2007. Molecular epidemiology identifies only a single rabies virus variant circulating in complex carnivore communities of the Serengeti. *Proceedings of the Royal Society of London. Series B, Biological Sciences* (in press).

4.1 Abstract

The role of wildlife communities in rabies maintenance in highly diverse African ecosystems is poorly understood. Yet, the extent to which multiple hosts contribute to maintenance of rabies virus variants in a single ecological system is fundamental for effective disease control. Here molecular phylogenetics is used to test whether distinct virus-host associations might occur in the species-rich carnivore community of the Serengeti ecosystem (northwestern Tanzania). The analysis identifies a single major variant belonging to the group of southern Africa canid-associated viruses (Africa 1b) to be circulating within the ecosystem with a high degree of genetic relatedness among viruses isolated from a range of hosts and no evidence for species-specific grouping. A statistical parsimony analysis of nucleoprotein and glycoprotein gene sequence data supports within- and between-species linkages suggesting intra- and inter-specific transmission. Coalescent theory allows the inference of roots that are consistently placed at nodes representing domestic dog sequences suggesting temporal direction of evolutionary change from domestic dogs to other species. This study emphasises the value of the analysis of genetic data for revealing elusive aspects of virus transmission. The results are consistent with dogs comprising the reservoir of rabies and highlight the importance of applying control efforts in dog populations.

4.2 Introduction

Rabies virus (RABV), prototype member of the genus *Lyssavirus*, family *Rhabdoviridae*, is a multi-host pathogen capable of infecting a wide range of species. The paradigm of rabies epidemiology is the compartmentalisation of the circulating virus by species and geographic area leading to the evolution of distinct virus variants that establish sustained transmission networks in a single species, the reservoir host (Rupprecht *et al*., 1991; Smith *et al*., 1995; Real *et al*., 2005b). However, this paradigm largely applies to areas with relatively low species diversity and it has been suggested that in some areas, particularly in species-rich communities, multiple variants of the virus may circulate in different host species (East *et al*., 2001) or multiple host species may independently maintain infection of a single variant (Thomson and Meredith, 1993; Bingham *et al*., 1999a,b).

It is generally considered that, as a result of the fatal outcome of the disease, maintenance host populations can only maintain the virus if they have specific demographic and ecological characteristics. For instance, species that are terrestrial rabies reservoirs tend to have high birth rates that allow rapid population recovery from rabies-induced mortality (Wandeler, 1991; Wandeler *et al*., 1994). Host-virus adaptation has also been proposed as a mechanism for increased efficiency of transmission in maintenance hosts, for example, through high rates of salivary virus excretion (Blancou, 1988). Conversely transmission to non-adapted 'spill-over' hosts typically results in short-lived chains of transmission. Occasionally cross-species transfers may lead to sustained transmission when a virus variant gains access to a novel host species with favourable ecological, genetic and behavioural characteristics (e.g. species jump from dogs, *Canis familiaris*, to the European red fox, *Vulpes vulpes*, in the 20th century; Anderson *et al*., 1981; Bourhy *et al*., 1999).

Evidence from epidemiological studies coupled with the isolation of a typically canid-associated African variant (Africa 1b) from the domestic dog, African wild dog (*Lycaon pictus*), bat-eared fox (*Otocyon megalotis*) and white-tailed mongoose (*Ichneumia albicauda*) (Kissi *et al*., 1995; Cleaveland and Dye, 1995; East *et al*., 2001) have suggested that domestic dogs may be the sole maintenance host of rabies in the Serengeti ecosystem. However, these conclusions were drawn from a limited range of epidemiological data and several alternative hypotheses have been proposed

for the maintenance of rabies in multi-host communities in Africa (Thomson and Meredith, 1993; Bingham *et al.*, 1999a,b; East *et al.*, 2001). The question is important because multiple variants in distinct hosts would prevent effective disease control by targeting a single host population.

An atypical pattern of infection proposed to account for rabies maintenance involves an infectious healthy carrier state where animals actively shed virus in the saliva for prolonged periods, but remain clinically normal. In rare instances, naturally infected healthy dogs have been documented to excrete virus in saliva (Fekadu, 1972; Aghomo *et al.*, 1989), and non-lethal rabies infection has been suggested to occur in spotted hyaenas (*Crocuta crocuta*) in the Serengeti (East *et al.*, 2001). In East *et al.*'s study (2001), hyaenas were deduced to maintain an avirulent variant based on detection of viral RNA in saliva of healthy animals by reverse transcriptase-polymerase chain reaction (RT-PCR). Sequence analysis of these PCR products indicated that the presumed hyaena variant was phylogenetically more closely related to European and Middle Eastern RABVs than to African isolates.

Bingham *et al.* (1999a,b) suggested that a single variant may be maintained by multiple canine species (i.e. dogs and jackals [*Canis mesomelas* and *C. adustus*]) in southern Africa through independent cycles, although other studies have indicated that jackals are unlikely to support infection independently of dogs (Cleaveland and Dye, 1995; Rhodes *et al.*, 1998). Similarly, bat-eared foxes, which are also infected by this variant (von Teichman *et al.*, 1995; Sabeta *et al.*, 2003), have been implicated as maintenance hosts in the Western Cape (Thomson and Meredith, 1993).

High species diversity of wild carnivores in the 27,000 km^2 Serengeti ecosystem and the lack of fencing between wildlife-protected areas and human settlements provide an ideal interface for testing the paradigm of compartmentalisation of RABVs in a multi-host community. Compartmentalisation has never been tested in a system with co-existing species that have been implicated elsewhere as maintenance hosts of rabies, such as jackals and bat-eared foxes (Thomson and Meredith, 1993; Bingham *et al.*, 1999a).

With additional samples and epidemiological data available from the Serengeti, and the application of phylogenetic analyses, these alternative hypotheses can now be

rigorously examined. RABVs isolated from a wide range of species from the Serengeti and surrounding areas were characterised genetically to determine the phylogeographical relationships among Serengeti viruses and RABVs recovered elsewhere (i.e. Europe, Middle East and Africa) and identify viral variants that might signify distinct virus-host associations. In a second analysis, the genealogic relationships among Serengeti viruses were examined to infer directionality of mutational changes and identify transmission routes. A parsimony-based network construction procedure (Templeton *et al.*, 1992) was employed which has proven useful in hypothesis testing of intra- and inter-specific transmission of human immunodeficiency virus (HIV) and human and simian T-cell leukemia/lymphoma virus type I (HTLV-I/STLV-I) (Crandall, 1995, 1996). The application of this method to rabies virus sequence data illustrates how genetic analysis can reveal elusive aspects of virus transmission in a complex ecosystem.

4.3 Materials and methods

4.3.1 Study samples and sequence data

Twenty-two virus specimens obtained from a range of animal species in the Serengeti ecological region of northwestern Tanzania between 1994 and 2004 were included in this study. Two other specimens originated from the Tarangire ecosystem, to the south-east of Serengeti. Archived isolates obtained between 1994 and 2001 (n = 18) were provided by Dr. S. Cleaveland and sequenced in the present study. The remaining six brain samples were collected and sequenced in the present study. The location from which the isolates of the study originated is shown in Figure 4.1. The species from which the viruses were obtained, the date of sample collection and geographical origin are summarised in Table 4.1. Details concerning previously published nucleotide sequence data compared with the sequences produced in this study are given in Tablc 4.2.

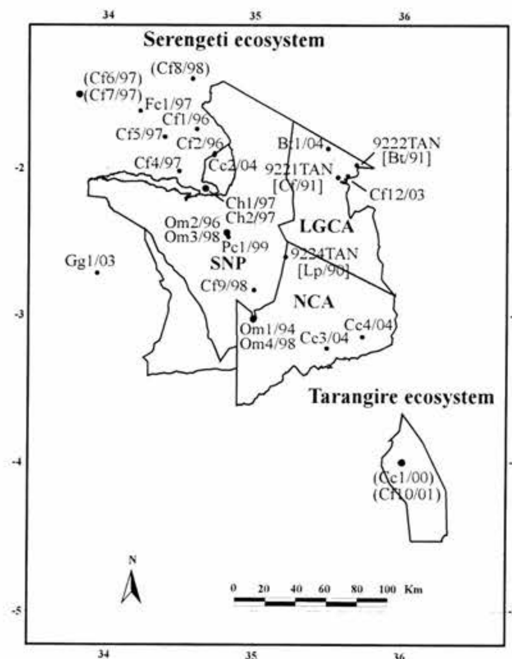


Figure 4.1. Map of the Serengeti and Tarangire ecosystems showing the location where the field isolates originated (including three previously described viruses: 9221TAN, 9222TAN, and 9224TAN; Kissi *et al.*, 1995). The precise sampling location of the isolates in round brackets is not known. The isolates are designated by a prefix indicating the species of origin (Bt, *Bos taurus*; Cc, *Crocuta crocuta*; Cf, *Canis familiaris*; Ch, *Capra hircus*; Fc, *Felis catus*; Gg, *Genetta genetta*; Om, *Otocyon megalotis*; Pc, *Proteles cristatus*), the isolate number and the year of collection. For isolates 9221TAN, 9222TAN and 9224TAN, the species of origin and year of collection are indicated within square brackets (Lp, *Lycaon pictus*). SNP, Serengeti National Park; LGCA, Loliondo Game Control Area; NCA, Ngorongoro Conservation Area.

Table 4.1. The Tanzanian rabies virus nucleoprotein (N) and glycoprotein (G) gene sequences produced in this study. SNP, Serengeti National Park; LGCA, Loliondo Game Control Area; NCA, Ngorongoro Conservation Area.

					GenBank accession no.	
Lab #	Isolate	Species isolated from	Date of sampling	Geographical origin	N	G
78	Cf1/96	*Canis familiaris*	Nov 1996	Rung'abure, Serengeti	DQ900547	DQ900571
82	Cf2/96	*Canis familiaris*	Dec 1996	Romchanga, Serengeti	DQ900548	DQ900572
RV1032	Cf4/97	*Canis familiaris*	Feb 1997	Mbiso, Serengeti	DQ900549	DQ900571
RV756	Cf5/97	*Canis familiaris*	Apr 1997	Kemgesi, Serengeti	DQ900550	DQ900571
RV758	Cf6/97	*Canis familiaris*	Oct 1997	Butiama, Musoma	DQ900551	DQ900573
RV759	Cf7/97	*Canis familiaris*	Dec 1997	Makoko, Musoma	DQ900552	
RV761	Cf8/98	*Canis familiaris*	Feb 1998	Kenyamanyori, Tarime	DQ900553	DQ900574
RV762	Cf9/98	*Canis familiaris*	Apr 1998	Naabi, SNP	DQ900554	
RV1013	Cf10/01	*Canis familiaris*	Jan 2001	Tarangire, Simanjiro	DQ900555	
A04-4995	Cf12/03	*Canis familiaris*	Jun 2003	Loliondo, LGCA	DQ900556	DQ900575
RV757	Fc1/97	*Felis catus*	Apr 1997	Wegero, Musoma	DQ900557	DQ900571
A04-5098	Bt1/04	*Bos Taurus*	Apr 2004	Soitsambu, LGCA	DQ900558	
90	Ch1/97	*Capra hircus*	Jan 1997	Guruneti, Serengeti	DQ900559	DQ900571

Table 4.1. Continued.

Lab #	Isolate	Species isolated from	Date of sampling	Geographical origin	GenBank accession no.	
					N	G
91	Ch2/97	*Capra hircus*	Jan 1997	Guruneti, Serengeti	DQ900560	DQ900571
39	Om1/94	*Otocyon megalotis*	Jul 1994	Ndutu, SNP	DQ900561	
74	Om2/96	*Otocyon megalotis*	Aug 1996	Seronera, SNP	DQ900562	DQ900576
RV763	Om3/98	*Otocyon megalotis*	Aug 1998	Seronera, SNP	DQ900563	DQ900576
RV764	Om4/98	*Otocyon megalotis*	Nov 1998	Ndutu, SNP	DQ900564	DQ900577
311	Pc1/99	*Proteles cristatus*	Feb 1999	Serengeti Plains, SNP	DQ900565	DQ900578
RV853	Cc1/00	*Crocuta crocuta*	Jan 2000	Tarangire, Simanjiro	DQ900566	
A04-5091	Cc2/04	*Crocuta crocuta*	Mar 2004	Misseke, Serengeti	DQ900567	
	Cc3/04	*Crocuta crocuta*	Oct 2004	Simba campsite, NCA	DQ900568	
	Cc4/04	*Crocuta crocuta*	Nov 2004	Irkeepus, NCA	DQ900569	
A04-4981	Gg1/03	*Genetta genetta*	Apr 2003	Nyaumata, Bariadi	DQ900570	DQ900579

Table 4.2. The previously published rabies virus nucleoprotein (N) gene sequences included in the phylogenetic analysis for comparison.

Country	Virus name	Species isolated from	Year	Reference	GenBank accession no.
Europe					
Estonia	9142EST	*Nyctereutes procyonoides*	1985	Kissi *et al*., 1995	U22476
	9342EST	*Nyctereutes procyonoides*	1991	Bourhy *et al*., 1999	U43432
France	8903FRA	*Vulpes vulpes*	1989	Bourhy *et al*., 1999	U42606
	9223FRA	*Vulpes vulpes*	1974	Bourhy *et al*., 1999	U43433
	9244FRA	*Vulpes vulpes*	1992	Bourhy *et al*., 1999	U42607
Germany	9202ALL	*Vulpes vulpes*	1991	Bourhy *et al*., 1999	U42701
	9212ALL	*Vulpes vulpes*	1991	Kissi *et al*., 1995	U22475
	9213ALL	*Vulpes vulpes*	1991	Bourhy *et al*., 1999	U42702
Yugoslavia	86107YOU	*Vulpes vulpes*	1976	Bourhy *et al*., 1999	U42703
	86111YOU	*Vulpes vulpes*	1986	Bourhy *et al*., 1999	U42706
Middle-East					
Iran	8681IRA	*Canis familiaris*	1986	Kissi *et al*., 1995	U22482
Africa					
Algeria	9137ALG	*Canis familiaris*	1982	Kissi *et al*., 1995	U22643
Burkina Fasso	8636HAV	*Canis familiaris*	1986	Kissi *et al*., 1995	U22486
Central Africa Republic	9228CAF	*Canis familiaris*	1992	Kissi *et al*., 1995	U22650
	9229CAF	*Canis familiaris*	1992	Kissi *et al*., 1995	U22651
Egypt	8692EGY	*Homo sapiens*	1979	Kissi *et al*., 1995	U22627

Table 4.2. Continued.

Country	Virus name	Species isolated from	Year	Reference	GenBank accession no.
Ethiopia	8807ETH	Hyaena sp.	1987	Kissi *et al.*, 1995	U22637
	ETH2003	*Canis simensis*	2003	Randall *et al.*, 2004	AY500827
Gabon	8693GAB	*Canis familiaris*	1986	Kissi *et al.*, 1995	U22629
	8698GAB	*Canis familiaris*	1986	Kissi *et al.*, 1995	U22630
Guinea	8660GUI	*Canis familiaris*	1986	Kissi *et al.*, 1995	U22487
Morocco	87012MAR	*Canis familiaris*	1986	Kissi *et al.*, 1995	U22631
Namibia	9227NAM	Jackal sp.	1992	Kissi *et al.*, 1995	U22649
South Africa	1500AFS	*Cynictis penicillata*	1987	Kissi *et al.*, 1995	U22628
Tanzania	9221TAN	*Canis familiaris*	1992	Kissi *et al.*, 1995	U22645
	9222TAN	*Bos taurus*	1992	Kissi *et al.*, 1995	U22647
	9224TAN	*Lycaon pictus*	1992	Kissi *et al.*, 1995	U22648
	X125	*Otocyon megalotis*	?	East *et al.*, 2001	AY034157
	A540	*Crocuta crocuta*	?	East *et al.*, 2001	AY034166
	S186	*Crocuta crocuta*	?	East *et al.*, 2001	AY034167
	S226	*Crocuta crocuta*	?	East *et al.*, 2001	AY034168
	S83	*Crocuta crocuta*	?	East *et al.*, 2001	AY034169
	X518	*Crocuta crocuta*	?	East *et al.*, 2001	AY034170
	X542	*Crocuta crocuta*	?	East *et al.*, 2001	AY034171
	631	*Ichneumia albicauda*	?	East *et al.*, 2001	AY034173
Zaire	8915ZAI	*Canis familiaris*	1989	Kissi *et al.*, 1995	U22638

4.3.2 Rabies diagnosis

Rabies was confirmed by laboratory diagnosis in each case. Diagnostic tests on brains collected between 1994 and 2001 and virus isolation were carried out at the Agence Française de Sécurité Sanitaire des Aliments (AFSSA), Malzéville, France using the fluorescent antibody test (FAT; Dean *et al.*, 1996), inoculation of murine neuroblastoma cells and mouse inoculation (Barrat *et al.*, 1988). Rabies diagnosis on more recent brain tissues was conducted where possible in the field by direct rapid immunohistochemical test (dRIT; Lembo *et al.*, 2006; Niezgoda and Rupprecht, 2006), and at the Rabies Section of the Centers for Disease Control and Prevention (CDC), Atlanta, United States (US) by dRIT and FAT (http://www.cdc.gov/ncidod/dvrd/rabies/Professional/publications/DFA_diagnosis/DFA_protocol-b.htm).

4.3.3 RNA extraction, RT-PCR and nucleotide sequencing

Total RNA was extracted from infected brain material using the TRIzol™ method (Invitrogen, San Diego, CA, US) according to the manufacturer's recommendations. Reverse transcription of 11 isolates was performed at the Veterinary Laboratory Agency (VLA), Weybridge, Addlestone, Surrey, United Kingdom (UK) following methods of Heaton *et al.* (1997). RT-PCR of the other isolates and direct sequencing were performed at CDC using previously described methods (Sacramento *et al.*, 1991; Kuzmin *et al.*, 2003) with primer sets for the nucleoprotein (N) region and the region encoding the central part of the ectodomain of the glycoprotein (G) published earlier (Smith, 2002). In brief, complementary DNA (cDNA) was obtained during reverse transcription with a sense primer (90 minutes at 42°C) in the presence of deoxynucleotide triphosphates (dNTPs) and AMV (avian myeloblastosis virus) RT (Roche Diagnostics Corp., Mannheim, Germany) and subjected to 41 PCR cycles: 30 seconds at 94°C, 30 seconds at 37°C and 90 seconds at 72°C, supplemented by a final extension for 10 minutes at 70°C in the presence of both sense and anti-sense primers and Taq polymerase (Roche Molecular Systems Inc., Branchburg, NJ, US). PCR products were purified by using Wizard PCR Preps DNA Purification Systems (Promega Corp., Madison, WI, US) following the manufacturer's instructions and sequenced (25 cycles: 10 seconds at 96°C, 5 seconds at 50°C and 4 seconds at 60°C) using Big Dye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, Foster City, CA, US) with either sense or anti-sense primer. The sequencing product

was purified using Centrisep Spin Columns (Princeton Separations, Adelphia, NJ, US) and processed on the ABI Prism™ 377 DNA Sequencer (Applied Biosystems, Foster City, CA, US).

4.3.4 Phylogenetic analysis and network estimation

A phylogenetic analysis consists of distinct stages (for a review, see Holder and Lewis, 2003), which are summarised in Box 4.1 and Figure 4.2 in order to better understand the methodology adopted in this study and described in detail below.

Box 4.1. Stages of a phylogenetic analysis and some commonly used methods.

1. Generation of sequences (laboratory work) and sequence editing.
2. Generation of multiple sequence alignments.
3. Selection of the evolutionary model and model parameters that best fit the data using criteria such as maximum likelihood (ML) through hierarchical likelihood ratio tests (hLRTs) or an Akaike information criterion (AIC) or a Bayesian information criterion - particularly important in the context of ML and Bayesian phylogenetic estimation.
4. Choice of method for phylogenetic reconstruction. Commonly used approaches:
* 'Traditional':
- Algorithmic methods (e.g. neighbour-joining) - fast, easy to implement, they almost always produce an unambiguous answer (a single tree), but they do not allow to evaluate competing hypotheses.
- Methods based on optimality criteria to choose among the set of all possible trees (e.g. parsimony and ML):
+ Parsimony - the best tree is the one that requires the smallest amount of evolutionary change to explain the actual data.
+ ML - attempts to estimate the actual amount of change according to the evolutionary model in place (the probability of seeing the observed data given that the model is true), hence the evolutionary model and model parameters need to be specified (see model selection). Statistically robust.
* Bayesian using Markov chain Monte Carlo (MCMC) - strong connection to the ML method, but attempts to estimate that a certain model is correct given the observed data. The evolutionary model needs to be specified (see model selection), whereas the model parameters can be either fixed prior to analysis (informative priors) or estimated from the data assuming no prior knowledge about their values (uninformative, flat or vague priors).
5. Confidence assessment (e.g. bootstrapping and posterior probabilities):
* Bootstrapping - support needs to be assessed separately as all methods but Bayesian produce only point estimates of the phylogeny, i.e. a single phylogenetic tree.
* Posterior probabilities (for Bayesian approaches) - assessment of confidence performed at the same time as estimating the tree.

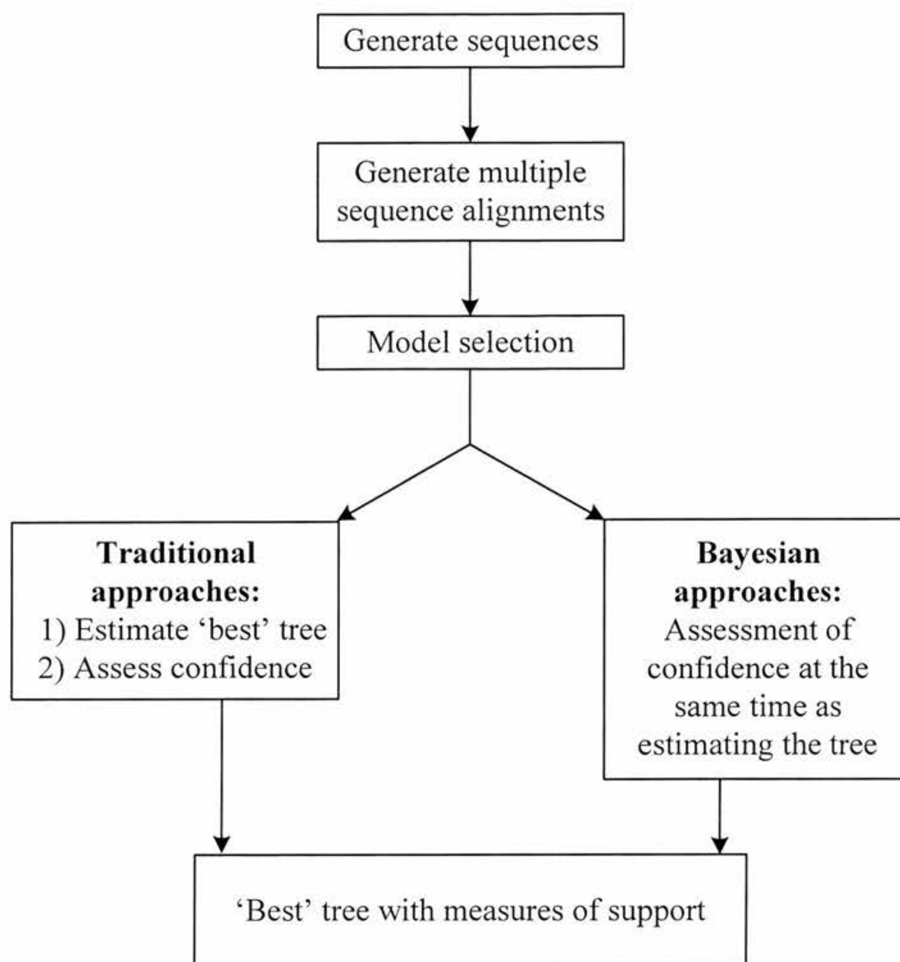


Figure 4.2. Schematic representation of the phylogenetic inference process.

Sequence editing and translation to amino acid sequences were performed using BioEdit software 7.0.0 (Hall, 1999). All edited sequences were deposited in GenBank under the accession numbers provided in Table 4.1. Multiple alignments were generated using the Clustal X package version 1.83 (Jeanmougin *et al.*, 1998) and sequence alignments were trimmed to include only complete non-stop codons.

Phylogenetic relationships among Tanzanian isolates and selected representatives of African and European/Middle Eastern lineages of RABVs (Table 4.2) were estimated using Bayesian Markov chain Monte Carlo (MCMC) methods. The N gene was chosen because the N sequence of isolates is available for all four African lineages (Kissi *et al.*, 1995). Two N gene data sets were constructed for analysis. The first data set contained 24 partial sequences produced from this study (1,158 bp, 386

deduced amino acids, nucleotide positions 263-1,420 on the SAD B19 genome; Conzelmann *et al.*, 1990) (Table 4.1) and 28 previously published sequences, including three recovered from an African wild dog (virus 9224TAN), a domestic dog (virus 9221TAN) and a cow (virus 9222TAN) adjacent to the Serengeti National Park (Kissi *et al.*, 1995; Bourhy *et al.*, 1999; Randall *et al.*, 2004) (Table 4.2). The second dataset was constructed to allow the comparison of viruses obtained from rabid spotted hyaenas sequenced in the present study (n = 4) with the previously published sequence data generated from apparently healthy hyaenas (n = 6) (East *et al.*, 2001) of which only short fragments have been deposited in the GenBank database. This dataset therefore contained shorter fragments of a number of sequences used in the first analysis (222 bp, 74 deduced amino acids, nucleotide positions 1,199-1,420 on the SAD B19 genome; Conzelmann *et al.*, 1990) and the hyaena sequence data reported earlier (East *et al.*, 2001).

Bayesian reconstructions were conducted in MrBayes 3.0b4 (Ronquist and Huelsenbeck, 2003). Two analyses were performed to check for any substantial sensitivity associated with fixing model parameters prior to analysis rather than estimating them as per MrBayes default settings. The first analysis specified the model of evolution and estimates of all parameters identified with the joint use of the programmes PAUP* version 4.0b10 (Swofford, 2002) and ModelTest version 3.7 (Posada and Crandall, 1998) using Akaike Information Criterion (AIC; Sakamoto *et al.*, 1986). ModelTest compares models of DNA substitution by using log likelihood scores computed by PAUP* under 56 different models of evolution to calculate hierarchical likelihood ratio tests (hLRTs; Frati *et al.*, 1997; Huelsenbeck and Crandall, 1997; Posada and Crandall, 1998) and AIC. The second analysis was performed using the general-time reversible (GTR) model with a proportion of invariable sites and a gamma-shaped distribution of rates across sites (GTR + I + Γ; Yang *et al.*, 1994) treating values for model parameters as unknown variables with uniform priors to be estimated in each analysis. Analyses were conducted with two independent runs initiated with random starting trees without constraints. Four simultaneous MCMC chains, three heated and one cold as per the programme's defaults, were run for 1×10^7 generations with trees sampled every 100th generation, resulting in 1×10^5 sampled trees. To ensure that the chains reached stationarity, log-

likelihood values for sampling points were plotted against generation time and the convergence diagnostic was examined. The first 25,000 trees were discarded as the burn-in phase and the remaining trees were used to estimate consensus phylograms and Bayesian posterior probabilities. Posterior probability values of 0.95 or greater were considered significant. A graphical representation of the trees was generated with the programme TreeView v. 1.6.6 (Page, 1996).

The phylogenetic intra- and inter-specific relationships among the Tanzanian N gene sequences (n = 27, 1,158 bp) included in the first Bayesian analysis (including the three previously described viruses: 9221TAN, 9222TAN, and 9224TAN; Kissi *et al.*, 1995) were estimated using statistical parsimony (SP) networks constructed using the TCS software (version 1.20; Clement *et al.*, 2000). A SP analysis was also performed on G gene data available for 15 Tanzanian isolates (398 bp, 132 deduced amino acids, nucleotide positions 3,761-4,158 on the SAD B19 genome; Conzelmann *et al.*, 1990) (Table 4.1), a number of which were identical over the 398 bp region. The TCS software implements the procedure of SP developed by Templeton *et al.* (1992), a population based method for reconstructing historical relationships among gene sequences. The SP approach, based on the parsimony criterion as defined by Templeton *et al.* (1992), first defines the maximum number of mutational steps among sequences as a result of single substitutions at a particular site with a 95% statistical confidence ('parsimony limit'). Then, all connections are established starting with the smallest distances until all the sequences are connected into a single network or the distance corresponding to the parsimony limit has been reached. On the basis of coalescence theory, the programme also implements an algorithm to determine root probabilities by calculating 'outgroup weights' using the position of each candidate root sequence within the network, its number of connections and frequency (Donnelly and Tavaré, 1986; Castelloe and Templeton, 1994). While it is unlikely that the precise ancestral genotype is contained within the small set of sampled viruses included in this study, the root probabilities do provide an indication of where the root is likely to be close to, and therefore the most likely temporal direction of evolutionary change.

4.4 Results

For the data set including 52 partial N gene sequences (1,158 bp) of RABVs originating from Tanzania (Serengeti and Tarangire ecosystems), other African countries, Europe and the Middle East, the AIC identified GTR + I + Γ as the best-fit model (-ln*L* = 6486.5688). The estimated parameters were as follows: nucleotide frequencies A = 0.2911, C = 0.2132, G = 0.2396, T = 0.2561; nucleotide substitution rates of the GTR rate matrix A ↔ C 1.4665, A ↔ G 6.5059, A ↔ T 0.7601, C ↔ G 0.1703, C ↔ T 10.8510, G ↔ T 1.0000; proportion of invariable sites = 0.3530 and shape parameter of the gamma distribution of rate variation = 0.7587. The majority-rule consensus tree obtained after defining model and model parameters is shown in Figure 4.3. The same topology was obtained when reconstruction was performed by Bayesian analysis with vague priors. The phylogeny revealed clear phylogeographic structure. The group of viruses isolated from red foxes and raccoon dogs (*Nyctereutes procyonoides*) in Europe and one domestic dog in Iran was supported by a posterior probability of 1.00, so were isolates 8638HAV and 8660GUI, representatives of Africa type 2 lineage. The Africa 1a group, which included viruses associated with domestic dogs from northern, western and eastern Africa, received a posterior probability of 0.98. All Tanzanian isolates grouped together (1.00) and fell into the Africa 1b group of canid-associated viruses (1.00). Within the Tanzanian group two major lineages were identified. One group (B) corresponded to viruses isolated from domestic and wild species from the Serengeti ecosystem and adjacent areas to the west during a 13-year period. The other group (A) included Serengeti and Tarangire viruses. Within the two lineages, a number of smaller groups was evident that roughly corresponded to viruses recovered from outbreaks linked in time and space.

The Tanzanian isolates showed between 0.1 and 3.3% (average 1.6%) nucleotide and between 0.0 and 2.6% (average 0.7%) amino acid sequence divergence. Maximum nucleotide diversity was between the virus recovered from *Lycaon pictus* (9224TAN) in 1990, the oldest Serengeti isolate, and a virus recovered from a spotted hyaena in 2004 (nucleotide and amino acid divergences 3.3% and 2.1% respectively).

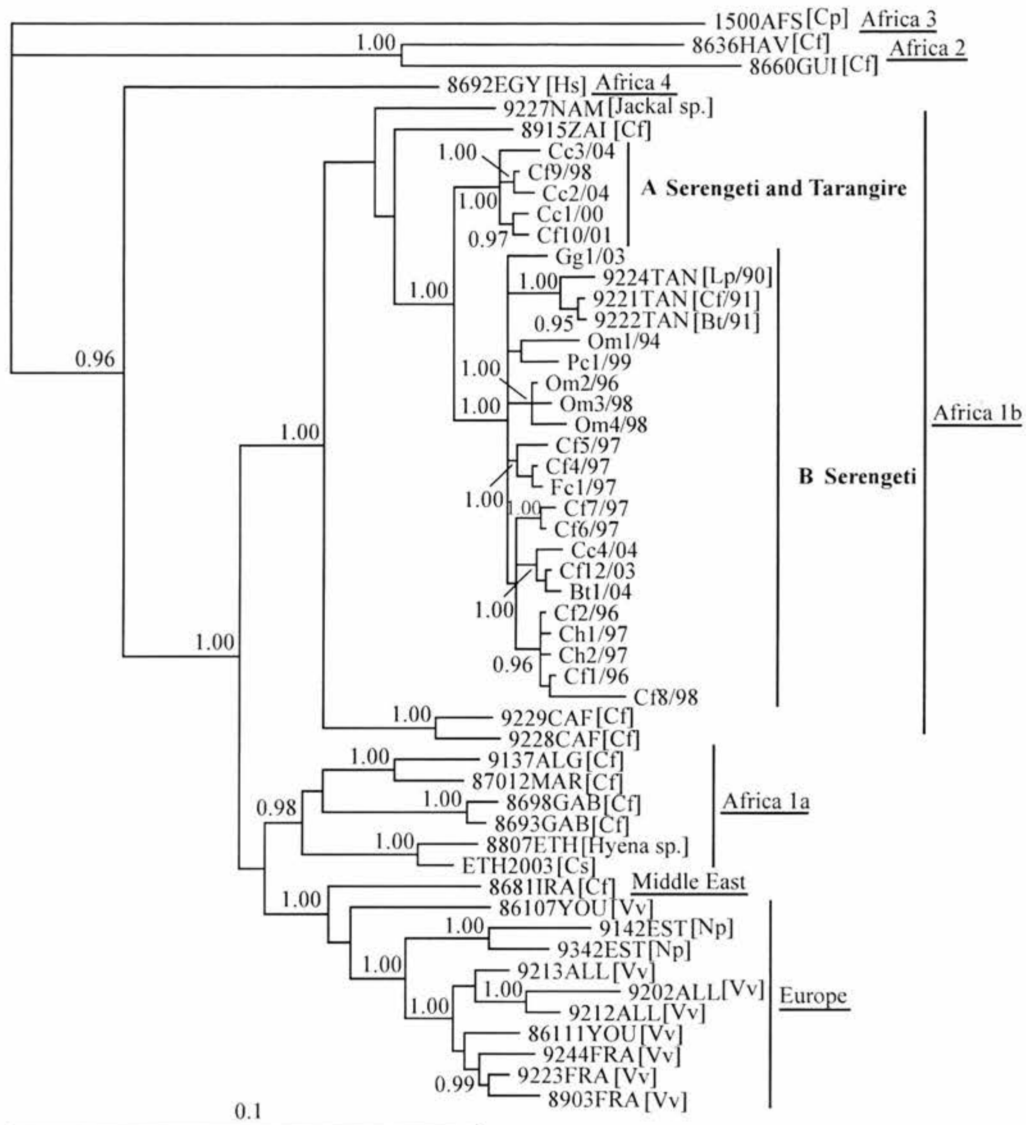


Figure 4.3. Majority-rule consensus tree of nucleoprotein gene sequences (1,158 bp) for rabies viruses from Tanzania (Serengeti and Tarangire ecosystems) compared with isolates from other areas of Africa, Europe and the Middle East recovered with Bayesian phylogenetics under the general-time reversible (GTR) plus invariant sites (I) plus gamma shape (Γ) model of evolution (Yang *et al.*, 1994) (details of model parameters are given in the text). The tree is rooted with isolate 1500AFS defined as the outgroup, representative of the lineage Africa 3 (Kissi *et al.*, 1995). Isolates described in this study are designated by a prefix indicating the species from which virus was recovered (Bt, *Bos taurus*; Cc, *Crocuta crocuta*; Cf, *Canis familiaris*; Ch, *Capra hircus*; Fc, *Felis catus*; Gg, *Genetta genetta*; Om, *Otocyon megalotis*; Pc, *Proteles cristatus*), the isolate number and the year of collection. Strain names are given for published isolates (Bourhy *et al.*, 1999; Kissi *et al.*, 1995; Randall *et al.*, 2004) and the species of origin is indicated within square brackets (Cp, *Cynictis penicillata*; Cs, *Canis simensis*; Hs, *Homo sapiens*; Lp, *Lycaon pictus*; Np, *Nyctereutes procyonoides*; Vv, *Vulpes vulpes*). Numbers on branches indicate Bayesian bootstrap values: only values ≥ 0.95 are shown. The scale indicates branch-length expressed as the expected number of substitutions per site.

For the data set containing also previously published partial N gene sequence data (222 bp) obtained from the saliva of Serengeti healthy hyaenas, the AIC favored the transitional model (TIM; Posada and Crandall, 1998) with a gamma shaped distribution of rates across sites (TIM + Γ) (-ln*L* = 1191.8756). The model parameters were as follows: nucleotide frequencies A = 0.3119, C = 0.2286, G = 0.2173, T = 0.2422; nucleotide substitution rates A ↔ C 1.0000, A ↔ G 2.8824, A ↔ T 0.1225, C ↔ G 0.1225, C ↔ T 6.1412, G ↔ T 1.0000 and shape parameter of the gamma distribution of rate variation = 0.2836. The results of this analysis are presented in Figure 4.4. The same tree topology was obtained when reconstructions were performed by Bayesian analysis with vague priors. Geographically defined clusters, as depicted in the first tree, were also evident in this phylogeny. The sequences representing the newly described Serengeti hyaena variant fell into the Europe/Middle East lineage, whereas the other Tanzanian isolates, including viruses obtained from rabid hyaenas, clustered together and fell into the Africa 1b lineage. Over the 222 bp region, a number of European and Tanzanian viruses were identical and there was little resolution within the Tanzanian group. Viruses from rabid hyaenas were identical to isolates recovered from Tanzanian dogs, cats (*Felis catus*), bat-eared foxes, white-tailed mongooses and goats (*Capra hircus*), whilst, within the Europe/Middle East group, a previously published Serengeti hyaena sequence and 3 red fox isolates showed 100% sequence identity.

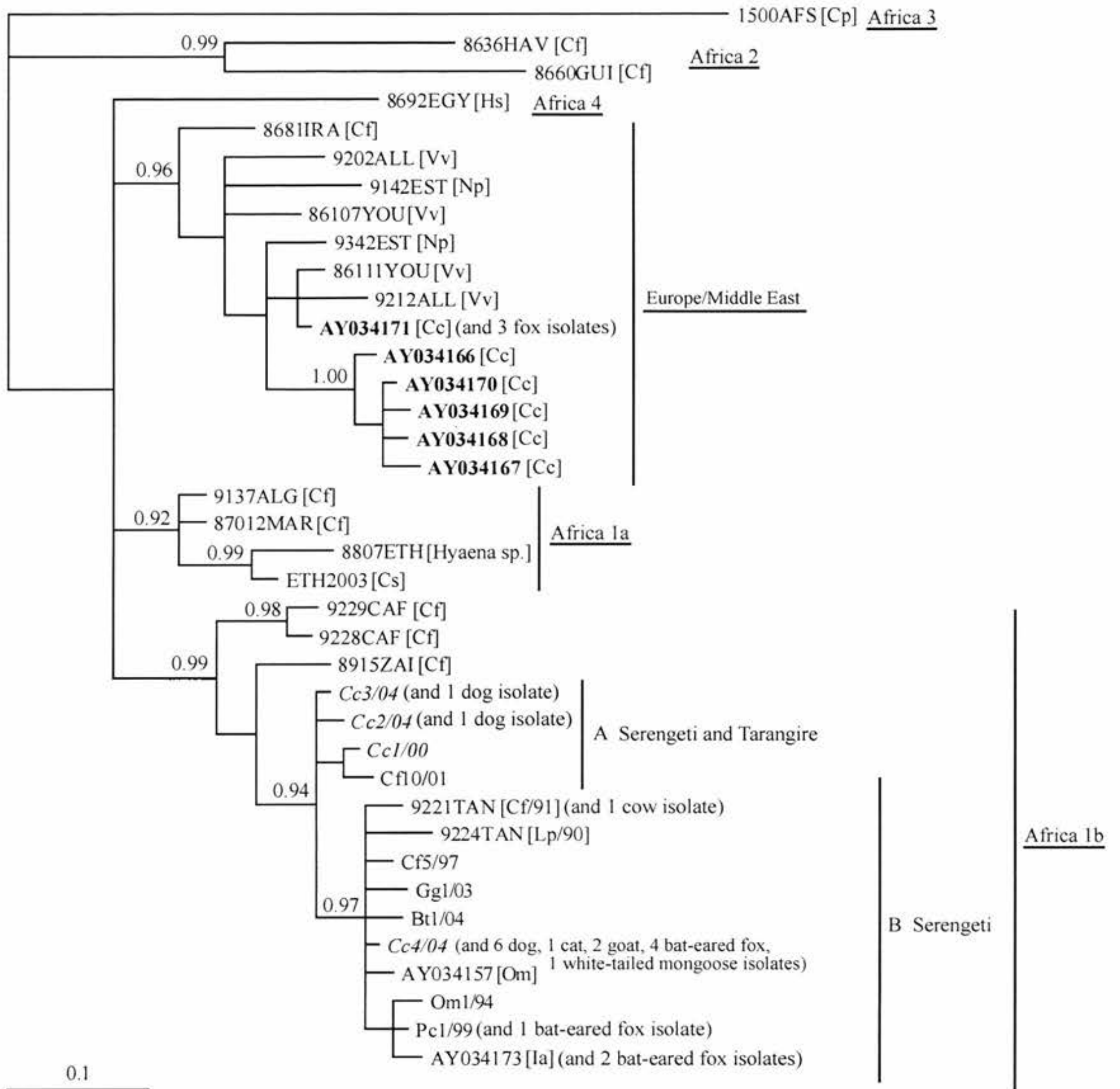


Figure 4.4. Phylogenetic tree of nucleoprotein gene sequences (222 bp) for a number of rabies viruses used in the analysis shown in Figure 4.3 (this study; Kissi *et al.*, 1995; Bourhy *et al.*, 1999; Randall *et al.*, 2004) and sequences described in the study by East *et al.* (2001) resulting from Bayesian reconstruction under the transitional model (TIM) of nucleotide evolution with a gamma-shaped distribution of rates across sites (Γ) (Posada and Crandall, 1998) (details of model parameters are given in the text). The tree is rooted with isolate 1500AFS, defined as out-group, representative of the lineage Africa 3 (Kissi *et al.*, 1995). Isolates described in this study are designated by a prefix indicating the species from which virus was recovered (Bt, *Bos taurus*; Cc, *Crocuta crocuta*; Cf, *Canis familiaris*; Gg, *Genetta genetta*; Om, *Otocyon megalotis*; Pc, *Proteles cristatus*), the isolate number and the year of collection. Strain names or GenBank accession numbers are given for published sequences and the species of origin is indicated within square brackets (Cp, *Cynictis penicillata*; Cs, *Canis simensis*; Hs, *Homo sapiens*; Ia, *Ichneumia albicauda*; Lp, *Lycaon pictus*; Np, *Nyctereutes procyonoides*; Vv, *Vulpes vulpes*). Numbers on branches indicate Bayesian bootstrap values: only values over 0.90 are shown. The scale indicates branch-length expressed as the expected number of substitutions per site. The viruses indicated in italics were obtained from Tanzanian clinically rabid hyaenas (this study). Previously published sequences obtained from the saliva of apparently healthy hyaenas from the Serengeti (East *et al.*, 2001) are emboldened.

Excluding the identical sequences, the nucleotide divergence among the Tanzanian isolates was in the range of 0.5-4.1% (average 1.8%) and the amino acid identity in the range of 0.0-2.7% (average 1.2%). Nucleotide divergence among isolates of rabid hyaenas ranged from 0.5 and 1.8% and among sequences obtained from healthy hyaenas was in the range of 0.5-3.2%, with divergence among the former and the latter in the range of 6.8-9.0% (Table 4.3).

Table 4.3. Nucleotide (upper diagonal) and amino acid (lower diagonal) p-distance values for partial (222 bp) nucleoprotein gene sequences of rabies viruses obtained from rabid hyaenas (this study) and apparently healthy hyaenas (East *et al.*, 2001). ID = identical.

	1	2	3	4	5	6	7	8	9	10
1 AY034169		0.009	0.009	0.014	0.032	0.005	0.072	0.081	0.086	0.086
2 AY034168	ID		0.009	0.014	0.023	0.005	0.072	0.081	0.086	0.086
3 AY034166	ID	ID		0.014	0.023	0.005	0.072	0.081	0.086	0.086
4 AY034167	ID	ID	ID		0.027	0.009	0.068	0.077	0.081	0.081
5 AY034171	0.014	0.014	0.014	0.014		0.027	0.068	0.072	0.077	0.077
6 AY034170	ID	ID	ID	ID	0.014		0.077	0.086	0.090	0.090
7 Cc4/04	0.014	0.014	0.014	0.014	0.027	0.014		0.014	0.018	0.018
8 Cc3/04	0.014	0.014	0.014	0.014	0.027	0.014	0.014		0.005	0.005
9 Cc2/04	0.014	0.014	0.014	0.014	0.027	0.014	0.014	ID		0.009
10 Cc1/00	0.014	0.014	0.014	0.014	0.027	0.014	0.014	ID	ID	

For the N gene data set, parsimonious connections were justified ($P \geq 0.95$) among sequences differing by as many as 14 nucleotide substitutions. These sequences were connected into a single parsimony network (Figure 4.5, network I and 4.6), whereas other sequences formed independent networks (Figure 4.5, networks II and III and Figure 4.7). For network I, which included viruses obtained between November 1996 and November 2004, the isolate Cf2/96 from a dog host sampled in December 1996 had the highest root probability (> 0.23). The remaining viruses located in the interior and on the tips of the network were obtained from dogs, livestock (a cow [*Bos taurus*] and goats), a cat, bat-eared foxes, a small-spotted genet (*Genetta genetta*), an aardwolf (*Proteles cristatus*), and a spotted hyaena. In network II, which included isolates recovered from a dog, a cow and an African wild dog between August 1990 and November 1991, the dog isolate had the highest root probability (0.66). Network III corresponded to group A in the Bayesian phylogeny. The dog isolate Cf9/98, recovered in April 1998, was identified as the sequence most closely related to the common ancestor (root probability = 0.33), with the isolates terminating the chains recovered from a dog and spotted hyaenas between January 2000 and October 2004. Isolate Om1/94, recovered from a bat-eared fox in July 1994, could not be connected to any network.

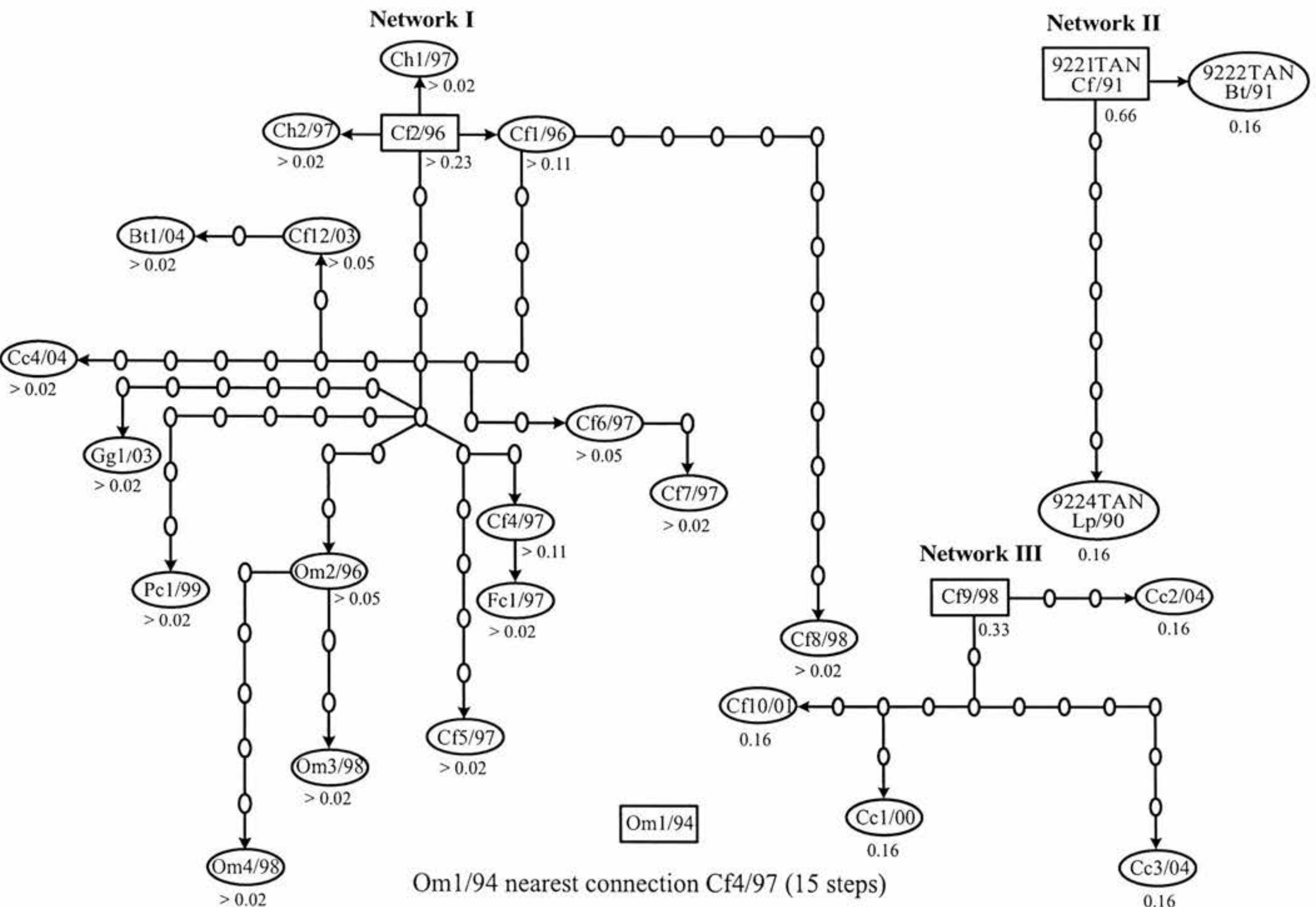


Figure 4.5. Statistical parsimony networks connecting the Tanzanian nucleoprotein gene sequences described in Figure 4.3 (see figure legend for virus designations). Each branch represents a single mutational step (nucleotide substitution). The lengths of the connecting lines are not significant. Ovals represent sequences. Zeros indicate nodes in the tree that represent intermediate sequences not found in the sample. Rectangles identify the sequence with the highest root probabilities. The arrows indicate temporal direction of evolutionary change based on the root probability estimation. Numbers below ovals and rectangles indicate outgroup weights.

Figure 4.6. Nucleotide and amino acid substitutions relative to the sequence of Cf2/96, identified as the sequence most closely related to the common ancestor for network I (see Figure 4.5 and legend of Figure 4.3 for virus designations). Transversions are indicated in italics and amino acid changes are embolded. Numbers indicate nucleotide and amino acid positions on the nucleoprotein gene of the SAD B19 genome (Conzelmann *et al.*, 1990).

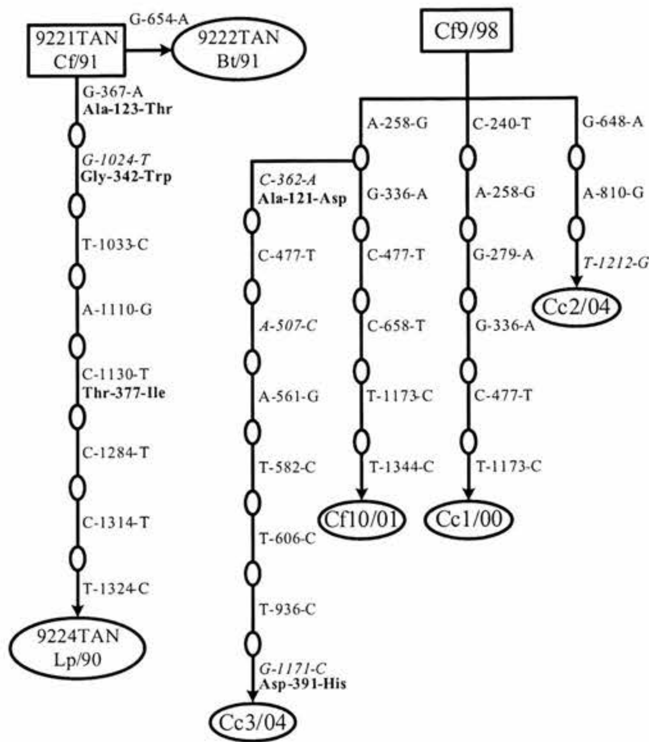


Figure 4.7. Nucleotide and amino acid substitutions relative to the sequences with the highest root probability for network II (left) and III (right) (see Figure 4.5 and legend of Figure 4.3 for virus designations). Transversions are indicated in italics and amino acid changes are embolded. Numbers indicate nucleotide and amino acid positions on the nucleoprotein gene of the SAD B19 genome (Conzelmann *et al.*, 1990).

For the G gene data available for 15 Tanzanian isolates, a number of which were identical over a 398 bp region, the SP procedure justified connections among sequences that differed by 8 or fewer nucleotide substitutions. The resulting network is shown in Figure 4.8. Isolate Cf1/96 had the highest root probability (> 0.43).

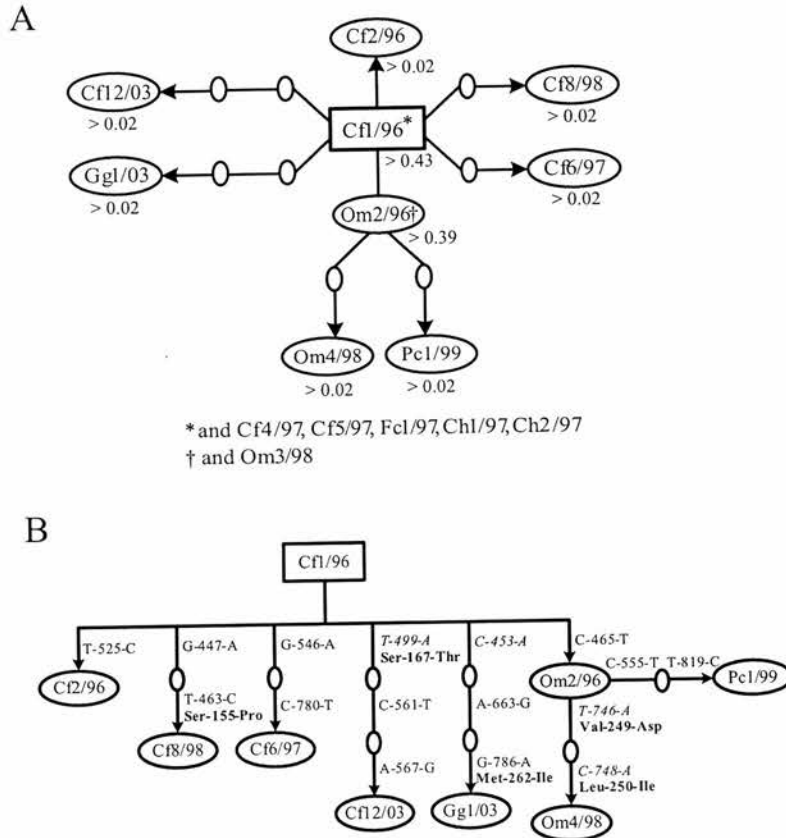


Figure 4.8. (A) Network of statistically supported relationships for the glycoprotein (G) sequence data (398 bp) available for 15 isolates described in Figure 4.3 (see figure legend for virus designation) inferred using a statistical parsimony approach. Ovals represent sequences in the sample. Lines connecting sequences, whose length is not significant, are equivalent to one mutational difference, with empty nodes representing unobserved intermediate sequences. The rectangle represents the sequence with the highest root probability and the arrows the temporal direction of evolutionary change. Numbers below the rectangle and ovals indicate outgroup weights. Asterisks indicate that identical genotypes were recovered from multiple animals. (B) Nucleotide and amino acid substitutions relative to the sequence with the highest root probability (Cf1/96). Transversions are indicated in italics and amino acid changes are embolded. Numbers indicate nucleotide and amino acid positions on the G gene of the SAD B19 genome (Conzelmann *et al.*, 1990).

4.5 Discussion

In order to elucidate patterns of rabies transmission within and between host populations and to test the hypothesis of co-circulation of multiple virus variants associated with distinct hosts in the carnivore species-rich community of the Serengeti, the phylogenetic relationships among viruses isolated from different populations were characterised using nucleotide sequences. The analyses strongly suggest that only a single Africa 1b virus variant circulates among Serengeti's domestic and wild mammal species and cross-species transmission is a frequent event. These observations are consistent with the hypothesis that domestic dogs maintain the virus in this ecosystem, as suggested by previous epidemiological studies and genetic analyses (Cleaveland and Dye, 1995; Kissi *et al.*, 1995), and more recent data (see Chapter 5). These findings raise interesting questions about why highly species-diverse communities only support a single virus variant.

Overall, the Bayesian phylogenies revealed site-specific rather than species-specific grouping, and the Tanzanian viruses clustered in a lineage associated primarily with domestic dogs throughout southern and eastern Africa (Kissi *et al.*, 1995). The partial N gene sequences of RABVs identified two lineages. One lineage included only Serengeti viruses (group B), whereas the other comprised viruses originating from the Serengeti and Tarangire ecosystems (group A). One possible explanation for the distribution of group A is the seasonal migration of nomadic Maasai pastoralists and their dogs from Tarangire to the Crater Highlands each year. The phylogeny did not reveal any host-distinguishable variants and domestic dog isolates were present in both clusters. Divergences among viruses were low, consistent with previous analyses of Tanzanian viruses (Kissi *et al.*, 1995; East *et al.*, 2001) and southern Africa canid viruses (von Teichman *et al.*, 1995; Sabeta *et al.*, 2003; Johnson *et al.*, 2004a,b; Mansfield *et al.*, 2006), suggesting that a single dog-introduced lineage can infect a range of hosts (e.g. dogs, jackals and bat-eared foxes). Although bat-eared fox viruses appear to be more distinct in South Africa (Sabeta *et al.*, 2003), definitive virus-host associations have not yet been identified among canid species in this geographic area.

A high degree of genetic similarity between African viruses, specifically of the lineages 1a and 1b, and European and Middle Eastern viruses has been documented

and, combined with historical data, attributed to introduction of rabies into Africa via importation of rabid dogs during European colonisation in the 19th century (Smith *et al.*, 1992; Kissi *et al.*, 1995). Lineages Africa 1a and 1b would have then arisen from the European progenitor. The Bayesian phylogenies obtained in this study support the view of the emergence of current European/Middle Eastern and Africa 1a and 1b variants from a hypothetical common ancestor.

The results of the Bayesian analyses suggest cross-species transmission of a single variant among a range of domestic and wild species, since viruses recovered from different hosts cluster together. The SP approach shows strong support for one *Canidae*-associated virus variant circulating within the Serengeti carnivore community. The estimation procedure applied to the N gene sequences connects all the viruses assigned to group B into a single network except for older viruses (Om1/94, 9221TAN, 9222TAN and 9224TAN). The remaining viruses (group A) form an independent network. When the SP method is applied to the G gene sequences of a number of viruses used to estimate the N gene main network (Figure 4.5, network I), the Serengeti viruses are again connected into a single parsimony network, which corroborates the results of the analysis of the N gene dataset. In addition, estimation of outgroup weights identifies sequences recovered from the domestic dog as most closely related to the root sequences of these networks with other species located at the end of transmission chains. The sparse and opportunistic nature of the sampling process required of this sort of study prohibits a definitive inference regarding the identity of the reservoir host, but the genealogical pattern repeatedly identified in these results is most consistent with a direction of transmission from the domestic dog to other species, suggesting that the reservoir for rabies is the domestic dog.

These findings suggest that, even in highly species-rich areas, the paradigm of maintenance of a single virus variant by a single host species (see section 4.2) holds true. Despite the abundance of other mammalian hosts, the domestic dog appears to act as the principal host of a typical canid variant. Similar characteristics of viruses isolated from a range of other species indicate that this variant is freely able to jump species boundaries, but the transmission networks suggest that wildlife species cannot establish stable infection cycles independently of dogs. The domestic dog

population surrounding the Serengeti is rapidly expanding and is well suited to serve as a rabies reservoir, with high turn-over rates generating large numbers of susceptible hosts. Several Serengeti species with attributes consistent with reservoir hosts (Wandeler, 1991; Wandeler *et al.*, 1994) have been diagnosed with the disease (e.g. the bat-eared fox, the white-tailed mongoose, the small-spotted genet) and the limited sample sizes available for this study do not permit definitive rejection of these species as part of a reservoir system. However, with the possible exception of the bat-eared fox, the available evidence indicates that these species are all associated with sporadic, short-lived epidemics with no evidence for species-specific virus-host associations.

What are the factors preventing the establishment of sustained cycles in a new host in the ecosystem? First, no single Serengeti wild carnivore population may be large enough or reach high enough densities to support independent cycles of a host-adapted virus. Although the Serengeti is renowned for the abundance of its carnivore populations, the high diversity of species co-existing within the park may prevent any single species reaching high enough densities to maintain infection. For example, population densities of jackals in less diverse farmland in Zimbabwe far exceed those recorded in the Serengeti (Cleaveland and Dye, 1995), and is one explanation for the suggestion that dogs and jackals are both able to maintain rabies in Zimbabwe (Bingham *et al.*, 1999a,b). Second, in general, there are no biogeographic barriers around the Serengeti to impede animal movement (as emphasised by the lack of genetic isolation of virus variants) that might promote localised viral evolution in specialised host niches (Bourhy *et al.*, 1999). Third, while high species diversity might be expected to provide many opportunities for host-viral adaptation, such adaptation presumably requires successive generations of infection within the same species and may be inhibited by high levels of interference between generalist carnivores that afford frequent opportunities for cross-species transmission.

In contrast with the observations of a single species supporting the virus cycle in the ecosystem, East *et al.* (2001) suggested that healthy carrier hyaenas maintain a genetically distinct non-pathogenic variant on the basis of viral RNA detected in hyaena saliva by RT-PCR. This variant shows characteristics consistent with RABVs circulating in Europe and the Middle East, primarily among foxes and distinct from

hyaena viruses in this study (Figure 4.4 and Table 4.3). Typically, fox RABVs cause rabies clinical signs and inevitable death in foxes (George *et al.*, 1980) and are known to be pathogenic to a range of other species in which no evidence of survival has been documented (Charlton *et al.*, 1988; Blancou, 1988). The finding of this variant in healthy Serengeti hyaenas, without evidence for clinical disease, is difficult to explain. In this study (see Chapter 5), diagnostic material was obtained from 41 hyaenas. Of these, 4 were confirmed rabies positive and Africa 1b RABVs were recovered. Clinical signs of rabies in hyaenas infected with this variant are quite typical, with signs of altered behaviour, increased aggression (attacking humans and animals), ataxia and death. Rabies morbidity and mortality in hyaenas have previously been reported in Tanzania and elsewhere in Africa (Rweyemamu *et al.*, 1973; Röttcher and Sawchuk, 1978; Barnard, 1979; Mills, 1990; Mebatsion *et al.*, 1992; Swanepoel *et al.*, 1993; Edelsten, 1995). There is no doubt that Serengeti hyaenas can die when infected with dog rabies and that rabid hyaenas pose a severe risk to humans and other mammals. The claim by East *et al.* (2001) is exceptional in several aspects and a number of questions arise. How do we explain the occurrence of two very dissimilar evolutionary processes in carnivore communities within the same ecosystem? How do we explain the circulation of a typically virulent fox variant in healthy Serengeti hyaenas? Why does a variant that is capable of inter-specific transmission not appear to circulate within other Serengeti carnivore species? Which genetic changes, if any, might have enabled the 'adaptation' of such a variant to the spotted hyaena and be responsible for reduced virulence? Experimental infectivity studies and sequence analysis of other gene regions where key mutations might reside are necessary to provide answers to these questions. However, lack of isolates of infectious virus and currently available sequence data limit the possibility of conducting the above-mentioned studies, confirming the carrier state in Serengeti hyaenas and ensuring that such a result in not an artefact of laboratory-based contamination. Finally, while carriers remain rare aberrant (see section 1.2.4) and it is doubtful whether they have any epidemiological significance, rabid hyeanas infected with a virus capable of crossing species barriers to infect multiple hosts may play a significant role in rabies epidemiology. With their intra- and inter-specific kleptoparasitic behaviour (Kruuk, 1972), wide-ranging

'commuting' outside the protected areas (Hofer and East, 1995), scavenging in agricultural areas (Kruuk, 1972) and predation on domestic dogs (Butler *et al.*, 2004; S. Cleaveland, personal observation), hyaenas likely constitute a critical link in disease transmission between domestic and wild carnivore populations in the Serengeti and elsewhere (Cleaveland *et al.*, 2000; Butler *et al.*, 2004). Attempts to elucidate patterns of carnivore movements and dog-wildlife contacts are underway in the Serengeti region and are likely to provide insights into the role played by wild canids in rabies transmission dynamics in multi-host communities.

Viral generalist pathogens pose a grave threat to biodiversity and human health (Taylor *et al.*, 2001; Cleaveland *et al.*, 2001). The impact of rabies on African wild canids can be substantial, as documented following rabies outbreaks in the African wild dog and the Ethiopian wolf (*Canis simensis*; Gascoyne *et al.*, 1993a; Sillero-Zubiri *et al.*, 1996; Randall *et al.*, 2004). The disease also inflicts a considerable public health burden in many parts of the world (Knobel *et al.*, 2005). This study is consistent with the view that, in the Serengeti, domestic dogs maintain a single major virus variant belonging to the Africa 1b group with spill-over cases occurring in other species and does not provide evidence for the co-circulation of multiple variants associated with distinct hosts. Efforts directed at controlling infection in dogs through mass vaccination can therefore be expected to eliminate rabies in all other species with benefits for both human health and wildlife conservation.

CHAPTER 5: IDENTIFYING RESERVOIRS OF INFECTION: A CASE STUDY OF RABIES IN THE SERENGETI ECOSYSTEM

Authors of paper in preparation: Lembo, T., Hampson, K., Haydon, D.T., Craft, M., Dobson, A., Dushoff, J., Ernest, E., Hoare, R., Kaare, M., Mlengeya, T., Mentzel, C. and Cleaveland, S.

5.1 Abstract

Knowledge of infection reservoirs is critical for effective disease control. For multi-host pathogens, identifying reservoirs is a conceptual and practical challenge, with unsuccessful control a potential consequence of incomplete understanding and inappropriate management of complex reservoir systems. Although no single approach may independently allow unequivocal identification of reservoirs, a combined approach may lead to reasonable evidence of its existence and identity. Here, bringing together old and new data, several lines of evidence are synthesised to identify key components of rabies reservoirs in complex carnivore communities of the Serengeti, northwest Tanzania, where the disease has been confirmed in twelve carnivore species. Changes in population densities of domestic dogs in agro-pastoralist and pastoralist areas adjacent to the Serengeti National Park were evaluated. Differences between the two areas were observed (> 11 dogs/km² and < 5 dogs/km² respectively) and, compared with earlier estimates, a considerable growth was detected. Long-term surveillance data suggested that rabies occurred continuously in high-density domestic dog populations and only sporadically in other populations, including lower density dog and wild carnivore populations. Genetic data showed that a single rabies virus variant belonging to the group of southern Africa canid-associated viruses (Africa 1b) circulated in a range of species with no evidence of species-specific virus-host associations. Spatio-temporal analysis of rabies case detection data identified clusters of dog rabies cases with or without other carnivore cases, suggesting that cases in wild carnivores and domestic cats were spatially and temporally linked with domestic dog outbreaks. There was no temporal correlation between dog rabies in high- and low-density populations. The most plausible explanation for the observed epidemiological patterns is that intra-specific transmission in dogs predominates with infrequent spill-over from dogs into other carnivores. The balance of evidence suggests that the reservoir of rabies in the Serengeti ecosystem is a complex multi-host community where dogs are the only population essential for persistence and main source population of rabies for humans. Other carnivores contribute to the reservoir as non-maintenance populations. Control programmes targeting dog populations should therefore eventually eradicate rabies from all other species. However, spill-over and transient chains of infection in other

species may prolong persistence by increasing the effective size of the susceptible population and act as a source of re-infection for dog populations, which is likely to increase the vaccination coverage required to control rabies above that predicted for dog populations alone. This study provides a framework for exploring the reservoir dynamics of multi-host pathogens that can be applied to any disease system.

5.2 Introduction

Pathogens that infect multiple host species comprise a group of pathogens that are: (i) economically important, (ii) major threats to human health, (iii) risks factors for endangered wildlife populations and (iv) a cause of emerging human and livestock diseases (Daszak *et al.*, 2000; Cleaveland *et al.*, 2001; Dobson and Foufopoulos, 2001; Taylor *et al.*, 2001). To control such pathogens effectively it is important to identify and appropriately manage the reservoir(s) of infection. However, identification of disease reservoirs often remains a fundamental problem (Krebs *et al.*, 1998; Bingham *et al.*, 1999a,b; Haydon *et al.*, 2002a; Donnelly *et al.*, 2003; Macdonald *et al.*, 2006; Hall *et al.*, 2006; Clifford *et al.*, 2006), particularly where multiple host species have the potential to act as reservoirs.

Although there has been a tendency to think of reservoirs in terms of single-host systems, reservoirs may include a wide range of populations and/or environments (Ashford, 1997; Swinton *et al.*, 1998; Swinton *et al.*, 2001; Haydon *et al.*, 2002a). To aid identification of disease reservoirs Haydon *et al.* (2002a) proposed that: (i) reservoirs can only be understood in relation to a particular target population defined as the population of concern that requires protection; (ii) susceptible host populations that can transmit infection directly (source populations) or indirectly to the target population can constitute all, or part of the reservoir; and (iii) reservoirs are capable of permanently maintaining infections of the pathogen.

A key threshold in ecology is that of a critical community size (CCS) which defines the persistence of infections like measles as a function of population size (Bartlett, 1957, 1960; see also section 1.1). Pathogens can persist in populations larger than a certain threshold size (maintenance populations), whereas smaller populations cannot maintain a pathogen independently (nonmaintenance populations), but together with other maintenance or nonmaintenance populations can constitute part of a reservoir. However, persistence is not only determined by a threshold population size. In recent years demographic heterogeneities, and temporal and spatial scales have received considerable attention as key determinants in evaluating disease persistence (Grenfell *et al.*, 1995; Lloyd and May, 1996; Keeling, 1997, 2000; Hagenaars *et al.*, 2004; Keeling *et al.*, 2004; Conlan and Grenfell, 2007; see section 1.1).

Rabies virus (RABV) is a classic example of a multi-host pathogen for which the identification of reservoirs has proven challenging (Nel, 1993; Cleaveland and Dye, 1995; Bingham *et al.*, 1999a,b; Johnson *et al.*, 2003; Bernardi *et al.*, 2005). Although domestic dog (*Canis familiaris*) rabies predominates among reported and confirmed cases and dogs are the source of infection for over 90% of human cases (WHO, 1999), it has been argued that this may reflect surveillance bias rather than the actual distribution of rabies among carnivore species (Swanepoel *et al.*, 1993; Wandeler *et al.*, 1994). Rabies surveillance is lacking across most of Africa and wildlife disease monitoring is particularly problematic as abundant scavengers and high ambient temperatures hinder diagnostic sample collection. Wildlife hosts in parts of southern Africa are considered capable of maintaining infection independently of dogs: for example the yellow mongoose (*Cynictis penicillata*) and the slender mongoose (*Herpestes [Galerella] sanguinea*) (Foggin, 1988; Swanepoel *et al.*, 1993; Taylor, 1993; King *et al.*, 1993; Bingham *et al.*, 2001; Nel *et al.*, 2005). Other carnivores such as bat-eared foxes (*Otocyon megalotis*), side-striped jackals and black-backed jackals (*Canis adustus*, *C. mesomelas*) appear to be able to sustain rabies cycles in some ecosystems (Thomson and Meredith, 1993; Bingham *et al.*, 1999a) but their role as independent maintenance hosts and reservoirs of infection is debated (Cleaveland and Dye, 1995; Rhodes *et al.*, 1998; Bingham *et al.*, 1999b).

Given the potential complexity of rabies reservoir systems in communities comprising multiple hosts, it may not be possible to reach an exhaustive understanding of their structure. However, identifying populations that act as source of infection for the target population(s) and maintenance hosts has important practical applications. Several 'spill-over' hosts can be considered target populations of concern in the context of rabies transmission, including humans (Knobel *et al.*, 2005), endangered wildlife populations (Gascoyne *et al.*, 1993a; Sillero-Zubiri *et al.*, 1996; Randall *et al.*, 2004), and livestock with economic (and subsistence) implications. One approach to rabies control would be through interventions within the target populations, however there are long-term economic benefits to controlling infection in the source and/or maintenance populations, particularly in countries with limited resources for disease control. While human post-exposure treatments (PETs) are extremely effective, they are costly and not widely available in many parts of the

world. Furthermore, routine vaccination of livestock is rarely economically feasible; and vaccination of endangered canids has been considered controversial (Gascoyne *et al.*, 1993b; Burrows *et al.*, 1994, 1995; Morell, 1995).

Most of the challenges likely to be encountered when working with a complex reservoir system are encapsulated in the Serengeti where rabies infects multiple host species. Twenty-six wild carnivore species are found in the ecosystem (Sinclair and Arcese, 1995) including Canidae and Herpestidae species implicated as independent maintenance hosts of rabies in parts of southern Africa (i.e. bat-eared foxes, side-striped and black-backed jackals, and slender mongooses). No definitive barriers separate wildlife-protected areas and human settlements and therefore frequent interactions between wild carnivores and domestic dogs can occur. Cleaveland and Dye (1995) found that rabies appears to persist as an endemic infection in high-density dog populations (> 5/km^2) to the west of Serengeti National Park (SNP), whereas in lower-density dog populations (< 1/km^2) to the east of SNP and wild carnivore populations within the study area, infection occurred only sporadically. They concluded that domestic dog populations were likely to be the sole reservoir in the ecosystem, however, their study considered only the 'one host-one virus' paradigm for rabies maintenance (i.e. the view of a principal host maintaining a single RABV variant within a given geographic area), and was based on limited data. With the rapid growth of human populations, associated domestic dog populations might have also increased and it is now possible that dog populations which previously could not support rabies cycles (e.g. dog populations to the east of SNP) may be large enough to maintain infection. Rabies in the Serengeti is therefore a good model situation for addressing questions relating to the structure and dynamics of potentially complex reservoir systems.

Cleaveland and Dye's preliminary hypotheses were that either domestic dogs (Figure 5.1A) or wild carnivores (Figure 5.1B) were reservoirs of infection, acting as independent maintenance and source populations of rabies for humans (the target population) and other species. However, two additional hypotheses should be considered: (i) that dogs and wild carnivores may together constitute a maintenance community, neither of them being capable of independent maintenance (non-maintenance populations) (Figure 5.1C) or (ii) that dogs and wild carnivores may

both be maintenance populations and together constitute the reservoir (Figure 5.1D). Approaches to rabies control will depend upon which of the above propositions is correct. If dogs are the sole maintenance population, or neither dogs nor wild carnivores can maintain rabies independently of the other (Figure 5.1A, C), control measures targeted at eliminating dog rabies should in theory successfully eliminate human and animal rabies from the ecosystem. However, if wild carnivores are the sole maintenance population (Figure 5.1B), strategies will need to target wildlife or if dog and wild carnivore populations each maintain rabies independently (Figure 5.1D), strategies will only be successful if infection in both populations is controlled.

In this chapter, several lines of evidence are presented to identify rabies maintenance and source populations (for humans) in multi-host carnivore communities of the Serengeti. The objectives were: (i) to evaluate changes in population densities of domestic dogs adjacent to the SNP, (ii) to describe domestic dog and wildlife rabies incidence patterns by synthesising data from long-term epidemiological records, (iii) to examine by phylogenetic analysis the genetic characteristic of virus samples from a range of domestic and wild species, and (iv) to analyse spatial and temporal patterns of rabies incidence.

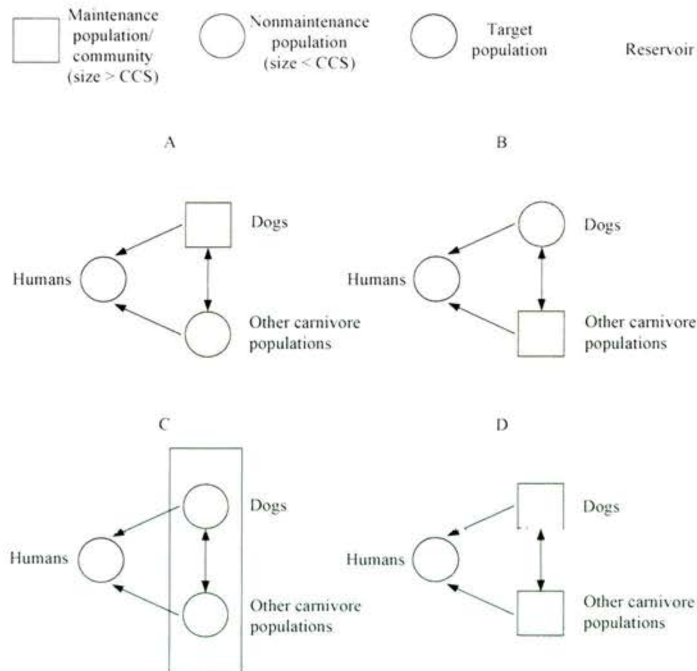


Figure 5.1. Potential reservoir systems in the Serengeti. CCS = critical community size.

5.3 Materials and methods

5.3.1 Study area

The study area in the Serengeti ecological region of northwest Tanzania was divided into 3 zones based on the characteristics of dog and wildlife populations (Figure 5.2). The first zone, the SNP, comprises diverse wildlife communities, with domestic dogs found extremely rarely. The two other zones are districts adjacent to the park, Serengeti District (SD) to the west and Ngorongoro District (ND) to the east, comprising the Loliondo Game Control Area (LGCA) and Ngorongoro Conservation Area (NCA). SD is inhabited by multi ethnic, agro-pastoralist communities and higher-density dog populations (Cleaveland and Dye, 1995). ND is a multiple-use controlled wildlife area, inhabited by low-density pastoralist communities, predominantly Maasai and Sonjo, and lower-density dog populations (Cleaveland and Dye, 1995). A greater range of wildlife species is documented in ND than SD.

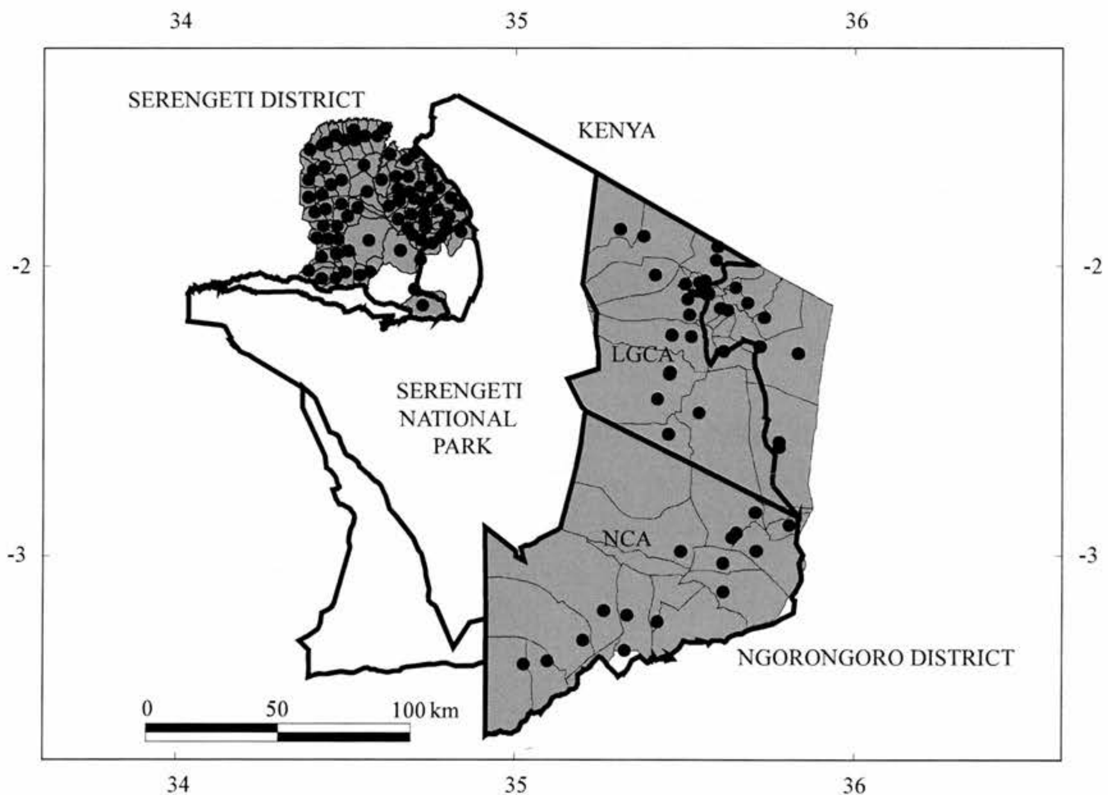


Figure 5.2. Map of the Serengeti ecological region (northwestern Tanzania) illustrating the three study regions: Serengeti National Park, Serengeti District to the west of the park and Ngorongoro District to the east. Village centres are indicated as solid black circles. LGCA = Loliondo Game Control Area; NCA = Ngorongoro Conservation Area.

5.3.2 Household questionnaire surveys and dog population density estimates

Dog population sizes were estimated from human:dog ratios (Wandeler *et al.*, 1988; Brooks, 1990; Cleaveland, 1996) obtained from household questionnaire surveys conducted in agro-pastoralist communities in 2003 and in pastoralist communities (in LGCA only) in 2004. The surveys were conducted during vaccination campaigns implemented in agro-pastoralist communities within a 10 km zone adjacent to the border of SNP and in pastoralist communities across the district (see section 5.3.3.1). In SD two villages were randomly selected from the list of all villages included in the vaccination campaign (within 0 to 10 km from the park boundaries) (see section 2.1.1.1.1). The sampling methodology was based on random selection of ten-cell units (known locally as *balozis*) within each subvillage. All households within units were sampled (see section 2.1.1.4). A total of 135 households were interviewed. In pastoralist communities the surveys were conducted in nine villages randomly selected across the LGCA (see section 2.1.1.1.1). Due to fewer and higher dispersion of Maasai households, the use of ten cell units was not logistically feasible in this community. Therefore 10% of traditional Maasai homesteads (known locally as *bomas*) in a village were selected at random and each *boma* was treated as a single household (see section 2.1.1.4). A total of 323 households were sampled. Human population sizes were obtained from 2002 human census data with projected population growth rates of 2.6% per annum in SD and 3.8% per annum in ND (Tanzania National Bureau of Statistics, 2005). Bootstrap confidence intervals (CIs) were calculated based on bias-corrected and adjusted (Bca) percentiles at 5 and 95%, respectively (Crawley, 2003).

5.3.3 Dog and wildlife rabies incidence

5.3.3.1 Interventions in the study populations

Dog vaccination programmes against rabies were implemented in SD in 1997. Relatively high vaccination coverage (65%) was attained between 1997 and 1999 (Cleaveland *et al.*, 2003), but coverage was lower (35-40%) and more patchy from 2000 to 2003. An expanded programme was initiated in 2003 within a 10 km zone adjacent to the western border of SNP with coverage ranging from 43 to 83% from 2003 to 2005 (Kaare *et al.*, under review). In 2004, the Tanzanian Government also

conducted campaigns in villages beyond the 10 km zone which resulted in 55% coverage across the rest of the district.

From 1997-2003, dog vaccination in ND was restricted to small-scale localised campaigns in the one urban centre (Loliondo-Wasso). Widespread vaccination across the district was implemented from 2004 to 2006, with overall coverage exceeding 80%.

No vaccination of wildlife has been conducted inside or outside the park during the period of this study.

5.3.3.2 Monitoring and disease surveillance

Data presented in this study were the result of disease surveillance operations in the study area between 1991 and 2005 (Table 5.1). Before 1991 records were fragmentary. Disease surveillance operations were reinforced in 2003 with the establishment of a large scale research project investigating the infection dynamics of viral diseases in the Serengeti (the Viral Transmission Dynamics Project). The research presented in this study was carried out as part of this larger study.

Detection of rabies in wildlife in SNP was based upon passive surveillance: sightings of sick and dead carnivores were reported through a network of veterinarians from Tanzania National Parks (TANAPA) and Tanzania Wildlife Research Institute (TAWIRI), rangers, scientists, tourists and tour operators. Surveillance cases were defined cases reported as snared, injured/wounded from other or unknown causes, animals observed with signs of disease and those found or reported dead with unknown history.

To obtain case incidence data outside SNP, passive and active surveillance operations were employed. Passive surveillance data were available through Veterinary Office records (Government offices, TAWIRI, TANAPA and NCA Authority). Community-based active surveillance measures, based on previous studies in rural Kenya (Kitala *et al.*, 2000) and Tanzania (Cleaveland *et al.*, 2003), were implemented in SD, using livestock field officers stationed in randomly selected study villages to collect information on rabies cases and animal bite-injuries from key informants (village leaders, school teachers, medical dispensary staff, local healers). During the period 1996-1999, active surveillance measures were

implemented and coordinated by Dr. S. Cleaveland, whereas the author established and supervised active surveillance operations starting from 2003 (see also Chapter 2). Finally, contact tracing, which was implemented and coordinated by Katie Hampson, Princeton University, was used to collect data on spatial and temporal patterns of disease. Home visits were made to every incident involving suspect rabid animals, using animal-bite injury data from hospitals and medical dispensaries, and case reports from livestock offices and community-based surveillance activities as primary sources. Each case was mapped and interviews conducted to evaluate the status of bite victims, determine their case history, and identify the source of exposure and resulting cases. The same procedure was followed for all resulting exposures and preceding cases, where identified.

As part of the surveillance operations, wherever possible, post-mortem examination was performed and brain stem samples collected from suspect rabies cases or from any carnivore carcasses, whatever the apparent cause of death. Sample collection was carried out by a range of operators, including research personnel, and veterinary and livestock field officers. The preferred technique for sample collection was removal via the occipital foramen (*foramen magnum*) by inserting a drinking straw, according to World Health Organization (WHO) recommendations (Barrat, 1996). Occasionally samples were collected by opening the skull. Some specimens were frozen (-20°C) immediately after collection. The other specimens were placed into a phosphate-buffered 50% glycerol solution at the time of collection without being extracted from the straw and preserved either cold (-20°C - +4°C) or at room temperature (25±5°C) where refrigeration or freezing facilities were not promptly available.

Table 5.1. Timeline of surveillance operations in each zone in quarterly units. TAWIRI = Tanzania Wildlife Research Institute; TANAPA = Tanzania National Parks; LFO = Livestock Officer; NCAA = Ngorongoro Conservation Area Authority.

YEAR: 1991 | 1992 | 1993 | 1994 | 1995 | 1996 | 1997 | 1998 | 1999 | 2000 | 2001 | 2002 | 2003 | 2004 | 2005

QUARTERS: 1 2 3 4 (for each year)

ZONE	Operation
SNP	TANAPA Veterinary Unit
	TAWIRI Wildlife Veterinary Programme
	Viral Transmission Dynamics Project*
SD	Passive (Veterinary records, village reports, hospital records, opportunistic carcass sampling)
	Active (LFOs)
	Active (LFOs)
	Contact tracing
ND	Passive (Veterinary and hospital records, village reports, opportunistic carcass sampling, TAWIRI, TANAPA and NCAA records)
	Contact tracing

*A large scale research project investigating the infection dynamics of viral diseases.

5.3.3.3 Rabies diagnosis

Diagnostic tests on brains collected up to 2001 and virus isolation were carried out at the Agence Française de Sécurité Sanitaire des Aliments (AFSSA), Malzéville, France using the fluorescent antibody test (FAT; Dean *et al.*, 1996), inoculation of murine neuroblastoma cells and mouse inoculation (Barrat *et al.*, 1988). Rabies diagnosis on more recent brain tissues was conducted where possible in the field by direct rapid immunohistochemical test (dRIT, Lembo *et al.*, 2006; Niezgoda and Rupprecht, 2006; see also Chapter 3), and at the Rabies Section of the Centers for Disease Control and Prevention (CDC), Atlanta, US by dRIT and FAT (http://www.cdc.gov/ncidod/dvrd/rabies/Professional/publications/DFA_diagnosis/DFA_protocol-b.htm).

When brain tissues were not available for laboratory confirmation, diagnosis was based on epidemiological (history of exposure) and clinical criteria. Where applicable, clinical evaluation was based on the six criteria proposed by Tepsumethanon *et al.* (2005) for a presumptive rabies diagnosis in living dogs: (1) the age of the dog at the time of the bite, (2) the state of health of the dog during observation, (3)-(6) various signs and the sequence with which they presented. It is noteworthy that, unlike the above-mentioned study, clinical signs were mainly reported (by villagers and livestock field officers) and rarely directly observed. In brief, the factors that were considered in determining whether a case was suspect for rabies were:

(i) For owned dogs: history of a bite, clinical signs (Tepsumethanon *et al.*, 2005), disappearance or death within 10 days.

(ii) For dogs of unknown origin: clinical signs (as above) and disappearance or death.

(iii) For wild carnivores: clinical signs (as above), including tameness, loss of fear of humans, diurnal activity (for nocturnal species), and unprovoked biting of objects and animals without eating.

Sensitivity (Se), specificity (Sp) and positive predictive value (PPV) of clinical rabies diagnosis (i.e. recognition of rabies by villagers, livestock officers and research personnel) against the gold standard test (FAT), which was assumed to be

100% sensitive and specific, were calculated with Win Episcope 2.0 (Thrusfield *et al.*, 2001).

5.3.4 Virus characterisation and phylogenetic analysis

Virus samples were typed at CDC in the present study. As described in section 4.3.3, RNA was extracted, reverse transcribed, amplified by polymerase chain reaction and nucleoprotein (N) gene sequences generated using standard methods (Sacramento *et al.*, 1991; Smith *et al.*, 1995; Smith, 2002; Kuzmin *et al.*, 2003).

The Tanzanian N gene dataset constructed for analysis comprised 57 partial sequences (282 bp, 94 deduced amino acids, nucleotide positions 1,139-1,420 on the SAD B19 genome; Conzelmann *et al.*, 1990), including shorter fragments of 17 sequences from the study area (SD, ND and SNP) described in Chapter 4 (Table 4.1). Twelve of these sequences were from viruses obtained by Dr. S. Cleaveland during the period 1994-1999 and 5 from more recent viruses obtained in the present study (see section 4.3.1). Seven sequences newly described in this chapter were generated from archived isolates (1992-2000) provided by Dr. S. Cleaveland, whereas 30 were from viruses recovered in the present study (2003-2005). Three previously published Serengeti sequences (Kissi *et al.*, 1995) were also included (see section 4.3.4). The location from which the virus samples originated is shown in Figure 5.3. Over the 282 bp region a number of sequences were identical as shown in Table 5.2, which also gives details concerning the species from which the viruses were obtained, the year of collection and the zone within the study area (SD, ND or SNP). Details concerning sequence data for RABVs from other African countries compared with the Tanzanian sequences are given in Table 4.2 and 5.3.

As described in detail in section 4.3.4, nucleotide sequences were edited using BioEdit software 7.0.0 (Hall, 1999) and multiple alignments generated by using the Clustal X package v. 1.83 (Jeanmougin *et al.*, 1998). Phylogenetic relationships were estimated using Bayesian Markov chain Monte Carlo (MCMC) methods implemented in MrBayes 3.0b4 (Ronquist and Huelsenbeck, 2003). The analysis was conducted under selected model and model parameters. Model testing was performed with the programme ModelTest v. 3.7 (Posada and Crandall, 1998) using Akaike Information Criterion (AIC). Analysis was conducted with two independent runs

initiated with random starting trees without constraints. Four MCMC chains, three heated and one cold, were run for 1 x 10^7 generations with trees sampled every 100th generation, resulting in 1 x 10^5 sampled trees. The first 25,000 trees were discarded as the burn-in phase and the remaining trees were used to generate 50% majority rule consensus trees and Bayesian posterior probabilities. Posterior probabilities of 0.95 or greater were considered significant. The programme TreeView v. 1.6.6 (Page, 1996) was used to generate the graphic output.

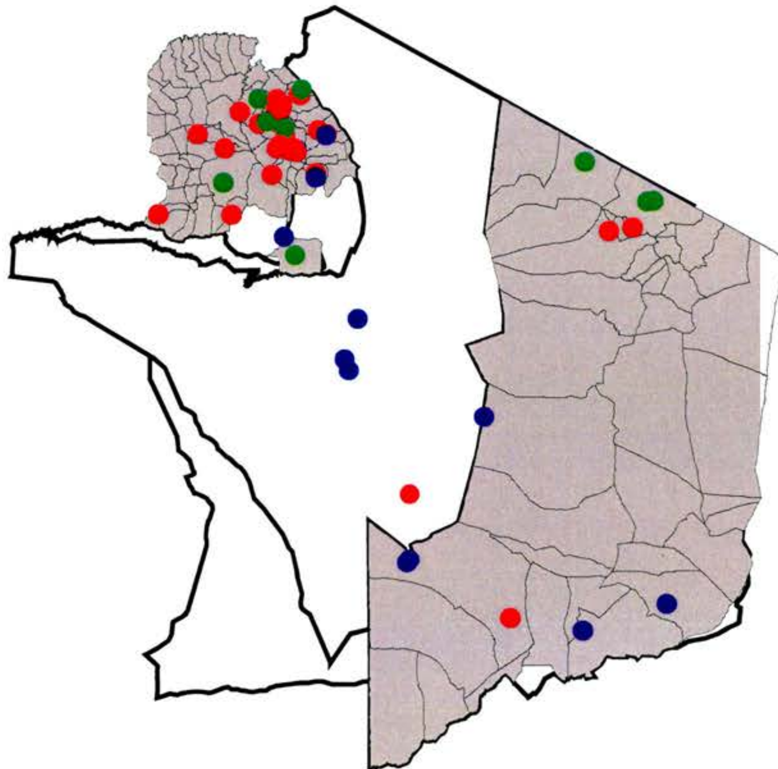


Figure 5.3. Map of the study area showing the location where the virus samples included in the phylogenetic analysis originated. Viruses recovered from domestic carnivores are indicated as red dots, those obtained from wild carnivores as blue dots and those recovered from livestock as green dots.

Table 5.2. Virus samples from the study area compared in phylogenetic analysis. Numbers in brackets indicate numbers of individuals with identical sequences. SNP = Serengeti National Park; SD = Serengeti District; ND = Ngorongoro District. *Species not definitively identified.

Species recovered from	Years of isolation	Zone within study area	Reference/Source	GenBank accession no.
African wild dog (*Lycaon pictus*)	1990	SNP	Kissi *et al.*, 1995	U22648
Domestic dog (*Canis familiaris*), cow (*Bos taurus*)	1991, 1992	ND	Kissi *et al.*, 1995	U22645
Cow, domestic dog	1992, 2004	ND, SD	This study	
Domestic dog (2)	1993, 1998	ND, SNP	This study	DQ900554
Bat-eared fox (*Otocyon megalotis*)	1994	SNP	This study	DQ900561
Bat-eared fox (4), domestic dog	1996, 1997, 1998	SNP, SD	This study	DQ900549
Domestic dog (3), goat (2) (*Capra hircus*), spotted hyaena (*Crocuta crocuta*)	1996, 1997, 2003, 2004	SD, ND	This study	DQ900547
Domestic dog	1996	SD	This study	
Domestic dog	1997	SD	This study	DQ900550
Aardwolf (*Proteles cristatus*), African wild cat (*Felis lybica*)	1999	SNP, SD	This study	DQ900565
Domestic dog (10), cow (2), livestock, spotted hyaena	2000, 2003, 2004	SD	This study	DQ900567
Domestic dog (2)	2003, 2004	SD	This study	
Domestic dog (2)	2004	SD	This study	
Domestic dog (2), goat, domestic cat (*Felis catus*), donkey (*Equus asinus*), white-tailed mongoose (*Ichneumia albicuda*)	2003, 2004	SD	This study	

Table 5.2. Continued.

Species recovered from	Years of isolation	Zone within study area	Reference/Source	GenBank accession no.
Domestic dog	2004	SD	This study	
Domestic dog	2004	SD	This study	
Domestic dog	2004	SD	This study	
Domestic cat	2004	SD	This study	
Cow	2004	ND	This study	
Spotted hyaena	2004	ND	This study	DQ900568
Leopard (*Panthera pardus*)	2004	SNP	This study	
Small-spotted genet (*Genetta genetta*)	2005	SD	This study	
White-tailed mongoose	2005	SD	This study	
Cow	2005	SD	This study	

Table 5.3. The rabies virus nucleoprotein (N) gene sequences (n = 11) included in the phylogenetic analysis for comparison. Details of other 11 previously published sequences included in the analysis are given in Table 4.2.

Country	Virus name	Species isolated from	Year	Reference	GenBank accession no.
Benin	8697BEN	Cat (*Felis catus*)	1986	Kissi *et al*., 1995	U22485
Cameroon	8801CAM	Dog (*Canis familiaris*)	1987	Kissi *et al*., 1995	U22634
	8804CAM	Cat	1988	Kissi *et al*., 1995	U22635
Ivory Coast	9003CI	Dog	1989	Kissi *et al*., 1995	U22639
Mauritania	8689MAU	Camel sp.	1986	Kissi *et al*., 1995	U22489
Mozambique	8631MOZ	Dog	1986	Kissi *et al*., 1995	U22484
Namibia	8708NAM	Kudu (*Tragelaphus strepsiceros*)	1987	Kissi *et al*., 1995	U22632
Niger	9012NIG	Dog	1990	Kissi *et al*., 1995	U22640
Nigeria	8670NGA	Human (*Homo sapiens*)	1983	Kissi *et al*., 1995	U22488
South Africa	8721AFS	Human	1981	Kissi *et al*., 1995	U22633
Tchad	9218TCH	Dog	1992	Kissi *et al*., 1995	U22644

5.3.5 Spatio-temporal analyses

5.3.5.1 Temporal relationship between domestic dog rabies cases in high-density and low-density populations

The temporal relationship between rabies cases in high-density and low-density dog populations was investigated with no lag and a one-year lag using the higher-resolution data from contact tracing (2002-2005). The Spearman's rank correlation coefficient (r_s) was used to test the strength of the relationship. The level of significance was set at $p < 0.05$.

5.3.5.2 Detection and identification of clusters of infection

To locate and determine the statistical significance of spatio-temporal clusters among reported cases of rabies in SD and ND a methodology using space-time permutation scan statistics (Kulldorff *et al.*, 2005) implemented in the Sa TScan programme version 7.0 (Kulldorff and Information Management Services, Inc., 2007) was employed. In the scan statistic there is a scanning window that moves across space, time or space-time. The space-time permutation scan statistic utilises overlapping cylinders to define the scanning window with a circular (or elliptic) base representing space and the height representing time. The window begins as a point at the smallest scale defined in the study at each point in space, time or space-time. The size of the window increases until it reaches the next recorded point in space, time or space-time. For each location and size of the window, the number of observed cases within the window and expected cases (if cases are randomly distributed in space) is counted. A generalised likelihood ratio (GLR) is calculated for each window comparing the ratio 'observed cases over expected'. The window with the maximum GLR constitutes the most probable space-time cluster of cases. Statistical significance of the detected clusters is evaluated using Monte Carlo simulations, generating random replications of the dataset under the appropriate null hypothesis.

Scan statistics were performed for each individual year using case detections from contact tracing (2002-2005) as accurate geographic coordinates were not available for the earlier period. The unit of space was defined by the coordinates of each case and the unit of time was one month. To estimate the significance levels of the clusters 9999 Monte Carlo replications were performed. The null hypothesis of no clusters was rejected when the simulated p-value was less than 0.05.

5.4 Results

5.4.1 Dog population densities

Overall dog densities in SD and LGCA exceeded 11/km² and 4/km² respectively (Table 5.4).

Table 5.4. Domestic dog population densities in Serengeti (SD) and Ngorongoro Districts (ND – Loliondo Game Control Area [LGCA] only). Percentile-based confidence intervals are indicated in brackets.

Area	Area (km²)	Dog: Human ratio	Average human density (/km²) at the village level	Average domestic dog density (/km²) at the village level
SD urban (Mugumu town)	18.8	6.6	619.8	93.9
SD rural	3128.0	6.6	67.8 (61.4-75.8)	10.1 (9.2-11.4)
SD overall	3146.8	6.6	75.2 (65.3-98.3)	11.4 (9.8-14.4)
LGCA urban (Loliondo and Sakala)	14.1	6.7	113.1 (49.5-172.3)	16.8 (7.3-25.6)
LGCA rural	8852.4	6.7	15.4 (11.4-20.3)	2.2 (1.7-3.0)
LGCA overall	8866.5	6.7	28.1 (17.5-55.5)	4.1 (2.6-7.9)

5.4.2 Rabies incidence

5.4.2.1 Rabies recognition probability

Table 5.5 shows the Se, Sp and PPV of clinical rabies diagnosis compared against the gold standard test, FAT. As a result of the relatively high rabies recognition probability (> 74% of animals reported as suspect rabies cases were confirmed rabies positive) and the small number of confirmed cases, the analyses presented include all suspect rabies cases unless otherwise stated.

Table 5.5. Table showing the sensitivity, specificity and positive predictive value of clinical rabies diagnosis compared against the fluorescent antibody test. Data include only cases for which complete history was available (2002-2005).

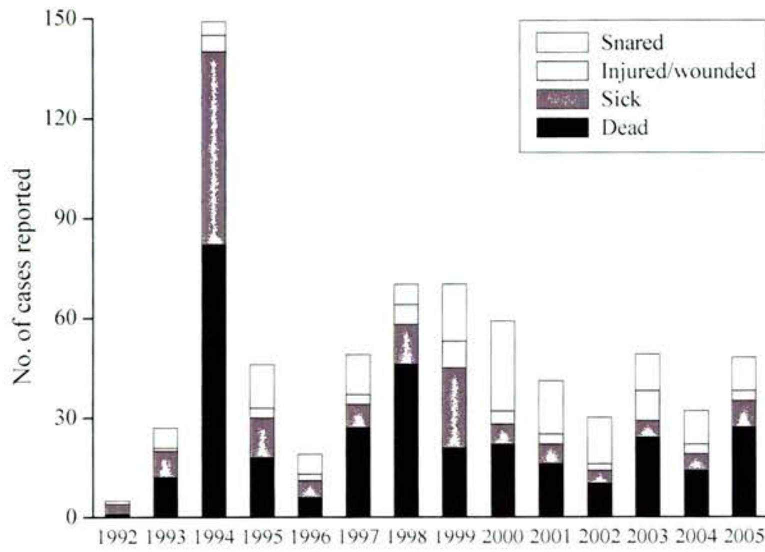
Clinical diagnosis	Laboratory diagnosis		Total
	Positive	Negative	
Positive	55	19	74
Negative	0	33	33
Total	55	52	107

Sensitivity = 100.0%
Specificity = 63.5% (95% CI: 50.4-76.55)
Positive predictive value = 74.3% (95% CI: 64.4-84.3)

5.4.2.2 Rabies incidence inside the park

Surveillance efforts in SNP remained generally stable during the study period since the establishment of Park Veterinary Units and programmes in late 1992 (TAWIRI) and mid-1996 (TANAPA) (Table 5.1, Figure 5.4A). A peak in carnivores reported dead and sick coincided with a canine distemper outbreak in lions in late 1993 and 1994 (Roelke-Parker *et al.*, 1996). Most rabies cases were reported and confirmed in 1998 and 1999, with only sporadic detection of the disease in other years (Figure 5.4B, Table 5.6). Low numbers of snared animals were reported in 1998 suggesting that the increase in carnivore carcasses recorded that year (Figure 5.4A) was not due to increased surveillance effort.

A



B

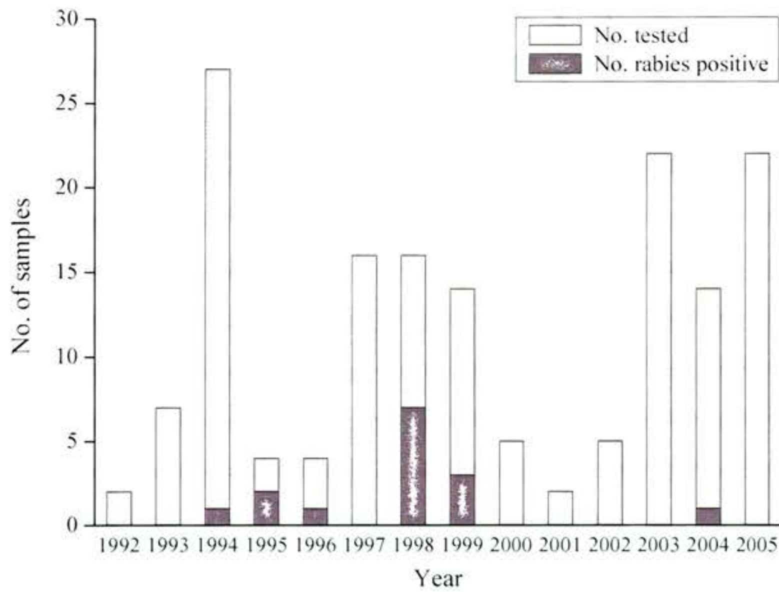


Figure 5.4. Wildlife surveillance effort in Serengeti National Park (A) based on reports of carnivore cases and (B) from samples retrieved for rabies diagnosis. The peak in 1994 coincided with a canine distemper outbreak in lions (Roelke-Parker *et al.*, 1996).

Table 5.6. Details of all wildlife confirmed and suspect cases in Serengeti National Park.

Year/s	Species	No. confirmed	No. unconfirmed
1986-1989	Bat-eared fox (*Otocyon megalotis*)	3	91
1990	African wild dog (*Lycaon pictus*)	1	4
1994	Bat-eared fox	1	-
1995	Bat-eared fox	1	-
	Spotted hyaena (*Crocuta crocuta*)	-	1
	Jackal sp.	1	-
1996	Bat-eared fox	1	-
1997	Bat-eared fox	-	1
1998	Bat-eared fox	5	2
	African civet (*Civettictis civetta*)	1	-
	Mongoose sp.	-	1
	Jackal sp.	1	1
1999	Bat-eared fox	1	1
	Aardwolf (*Proteles cristatus*)	1	-
	Spotted hyaena	-	4
	Mongoose sp.	-	3
	Jackal sp.	1	2
2000	Bat-eared fox	-	1
2001	Bat-eared fox	-	1
2001	Mongoose sp.	-	1
2004	Leopard (*Panthera pardus*)	1	-

5.4.2.3 Rabies incidence outside the park

Rabies cases were reported continuously throughout the study period in high-density dog populations to the west of the ecosystem (Figure 5.5, 5.6A), although incidence was affected by the variable vaccination coverage. A decline was recorded during periods when coverage was high (1997-1999). A peak in reported incidence was consistent with an epidemic beginning in 2003, followed by a decline reflecting re-implementation of dog vaccination programmes. In ND dog rabies cases occurred sporadically. A peak in incidence coincided with an epidemic beginning in 2003 and a decline following dog vaccination campaigns (Figure 5.5, 5.6B). In both districts, cases in wildlife were sporadic and when reported coincided with outbreaks of dog rabies (Figure 5.6).

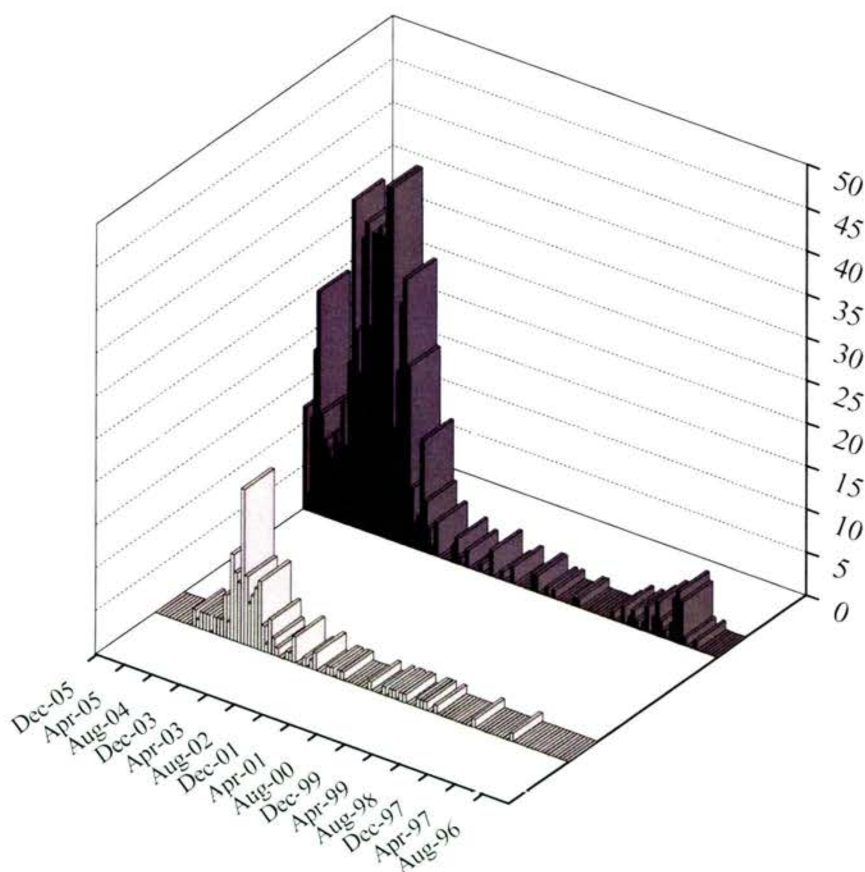
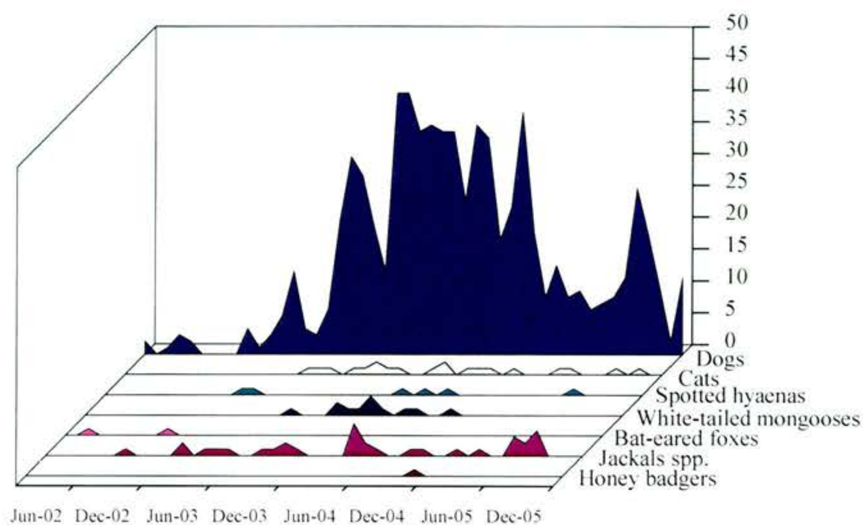


Figure 5.5. Suspect dog rabies cases in Serengeti District (dark gray) and Ngorongoro District (light gray). Note that cases from January 2002 to December 2005 were monitored by contact tracing.

A



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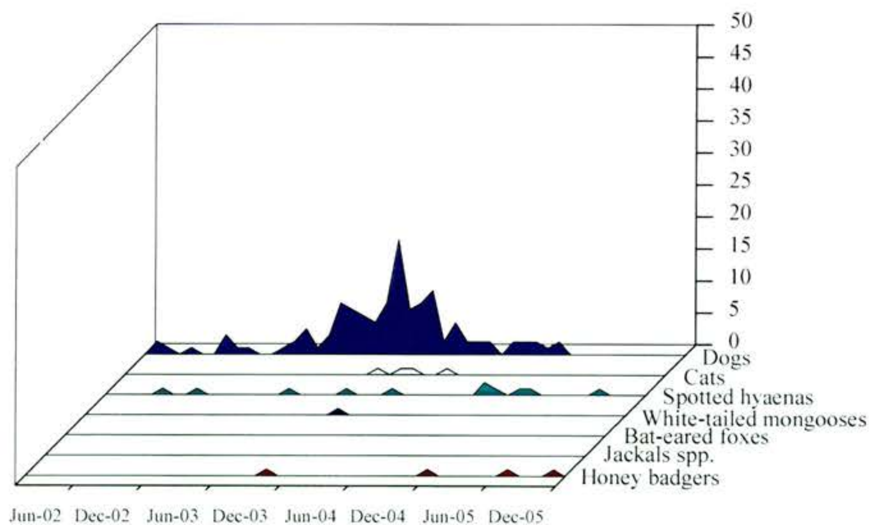


Figure 5.6. Suspect rabies cases amongst carnivore species in (A) Serengeti District and (B) Ngorongoro District monitored by contact tracing from January 2001 to January 2006.

Human exposures by suspect rabid carnivores in SD and ND during the period 2002-2005 are shown in Table 5.7. Dogs were the origin of the highest number of human exposures, followed by cats, jackal spp., hyaenas and other wildlife species.

Table 5.7. Human exposures by suspect rabid animals in the Serengeti ecosystem from January 2002 to January 2006.

Species	Number of human exposures
Domestic dog (*Canis familiaris*)	442
Cat (*Felis catus*)	29
Jackal spp.	22
Spotted hyaena (*Crocuta crocuta*)	16
Honey badger (*Mellivora capensis*)	8
Bat-eared fox (*Otocyon megalotis*)	2
Leopard (*Panthera pardus*)	2
White-tailed mongoose (*Ichneumia albicauda*)	2
African wild cat (*Felis lybica*)	2
Small-spotted genet (*Genetta genetta*)	1

5.4.3 The virus: inter- and intra-specific transmission

The 57 rabies viruses recovered from a range of species from the study area showed characteristics consistent with southern Africa canid-associated viruses (Africa 1b) and the phylogeny did not reveal any clustering by host species (Figure 5.7). The genomic sequences showed between 0.0 and 4.6% (average 1.5%) nucleotide and between 0.0 and 4.3% (average 1.2%) amino acid divergence. The evident intermingling of viral lineages from dogs and wildlife are indicative of frequent inter-specific transmission. More detailed analyses of a subset of Serengeti viruses are described in Chapter 4.

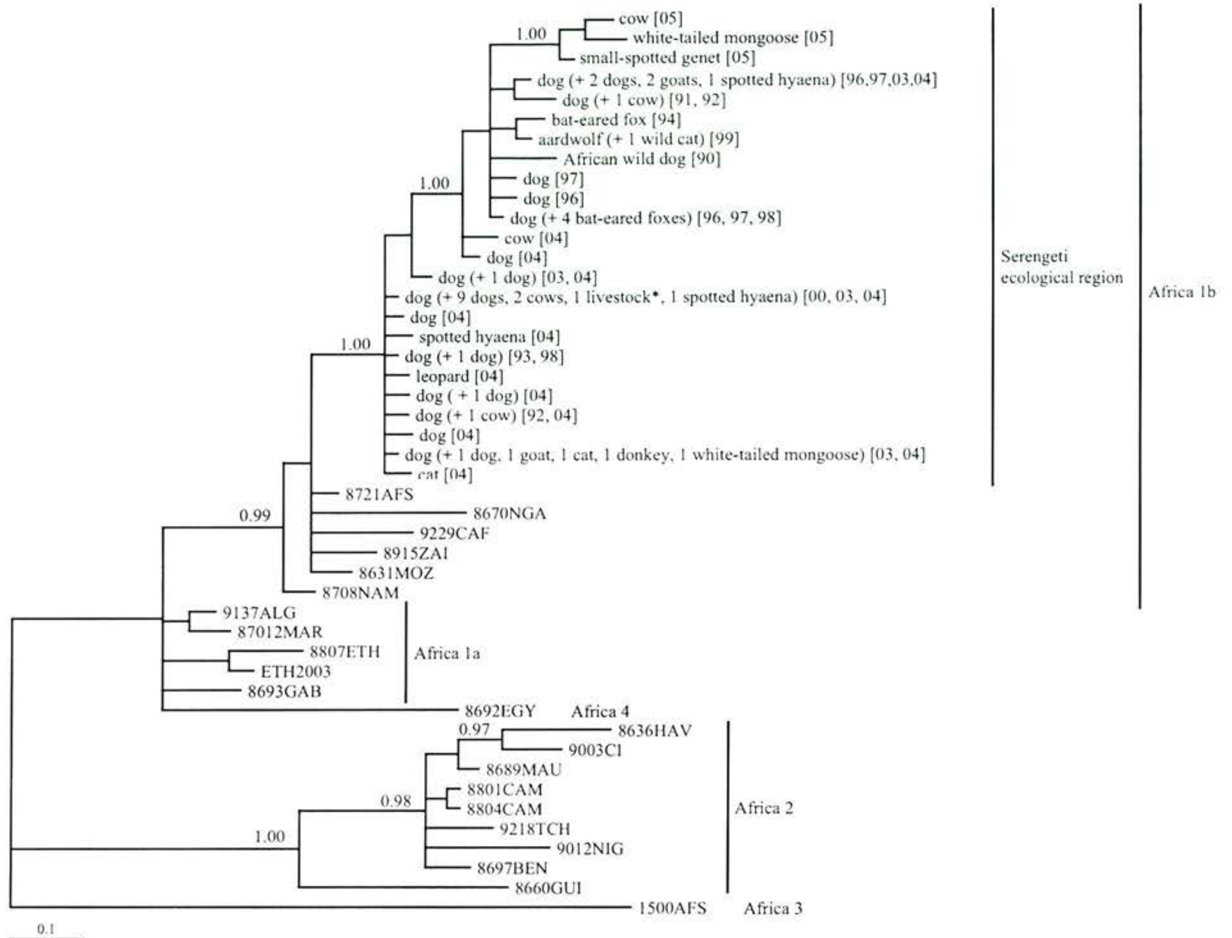


Figure 5.7. Phylogenetic tree of nucleoprotein (N) gene sequences (282 bp, 94 deduced amino acids, nucleotide positions 1,139-1,420 on the SAD B19 genome, Conzelmann *et al.*, 1990) for rabies virus samples from the study area (n = 57) compared with isolates recovered from other areas of Africa (n = 22). The tree is constructed using Bayesian phylogenetics under the transitional model of nucleotide evolution with a gamma-shaped distribution of rates across sites (TIM + Γ) (Posada and Crandall, 1998) (base frequencies = 0.3253, 0.2134, 0.2360, 0.2253; nucleotide substitution rates = 1.0000, 3.6723, 0.4393, 0.4393, 8.1773, 1.0000; Γ = 0.3390). For samples from the study area the year of collection is indicated within square brackets. Previously published sequences are designated by the strain names (Kissi *et al.*, 1995; Randall *et al.*, 2004). The tree is rooted with isolate 1500AFS, defined as outgroup, representative of the lineage Africa 3. Nodal posterior probabilities > 0.95 are shown. The scale indicates branch-length expressed as the expected number of substitutions per site.*Species not definitively identified.

5.4.4 Spatial and temporal patterns

5.4.4.1 Temporal relationship between domestic dog rabies cases in high-density and low-density populations

Number of dog cases in high-density populations showed no correlation with dog cases in low-density populations: no lag (Figure 5.8A), Spearman's rank correlation, $r_s = 0.6$, $p = 0.4167$ and one year-lag (Figure 5.8B), Spearman's rank correlation, $r_s = -1$, $p = 0.3333$.

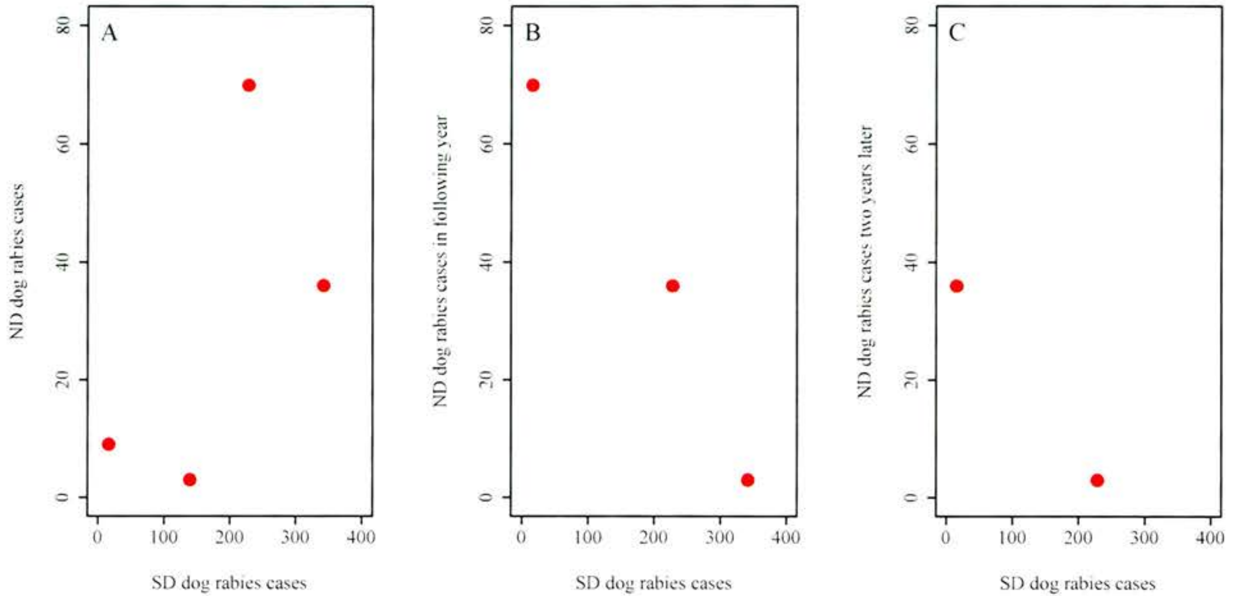


Figure 5.8. Correlation between dog rabies cases in high- and low-density populations with no lag (A), a one-year lag (B), and a two-year lag (C). SD = Serengeti District; ND = Ngorongoro District.

5.4.4.2 Space-time clusters

The spatial distribution of cases detected in 2002 in SD and ND is shown in Figure 5.9. No statistically significant high rate clusters at the 5% level were identified by the spatial scan statistic (p = 0.7036 for SD and p = 0.6579 for ND).

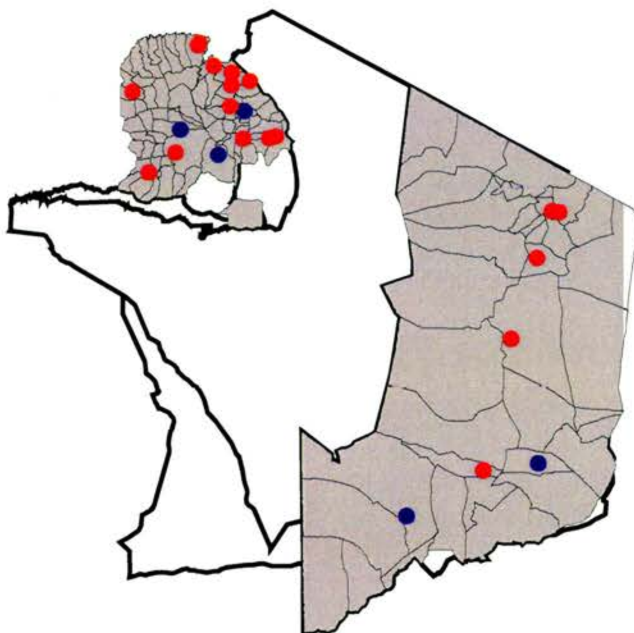


Figure 5.9. Spatial distribution of carnivore rabies cases recorded in Serengeti and Ngorongoro Districts in 2002. Domestic dog cases are indicated as red dots and wild carnivore cases as blue dots.

Details of significant primary (I) and secondary (II) space-time clusters of rabies incidence detected during the period 2003-2005 are given in Table 5.8. These analyses included all cases detected. There was no loss of significance when the analyses were performed including only dog cases (p = 0.0001 and 0.0005 for SD and ND respectively in 2003; p = 0.0307 for ND in 2004 and p = 0.0104 for SD in 2005) with the exception of the SD 2004 dataset (p = 0.0986). In contrast, no significant clusters were identified when wildlife and cat cases only were scanned.

Table 5.8. Significant high rate primary (I) and secondary (II) space-time rabies clusters in Serengeti District (SD) and Ngorongoro District (ND) during 2002-2005, detected by retrospective space-time analysis.

Year	Type of cluster	Area	Date of cluster	No. of cases in cluster	Rate ratio (Obs/Exp)	p-value
2003	I	SD	Feb-03	11	15.231 (11/0.72)	0.0001
	II	SD	May-03 – Aug-03	63	2.250 (63/28)	0.0001
	II	SD	Dec-03	16	3.840 (16/4.17)	0.0005
	I	ND	Oct-03 – Nov-03	18	2.385 (18/7.55)	0.0046
2004	I	SD	Oct-04 – Nov-04	36	1.988 (36/18.11)	0.0494
	I	ND	Mar-04 – Apr-04	4	6.200 (4/0.65)	0.0244
2005	I	SD	Nov-05 – Dec-05	7	5.608 (7/1.25)	0.0363

The spatial distribution of cases and the location of the clusters identified in 2003 in SD and ND is shown in Figure 5.10. Two of the clusters detected in SD (I and II) included only dog cases, and one dog, wildlife and cat cases. The cluster identified in ND included both dog and wildlife cases.

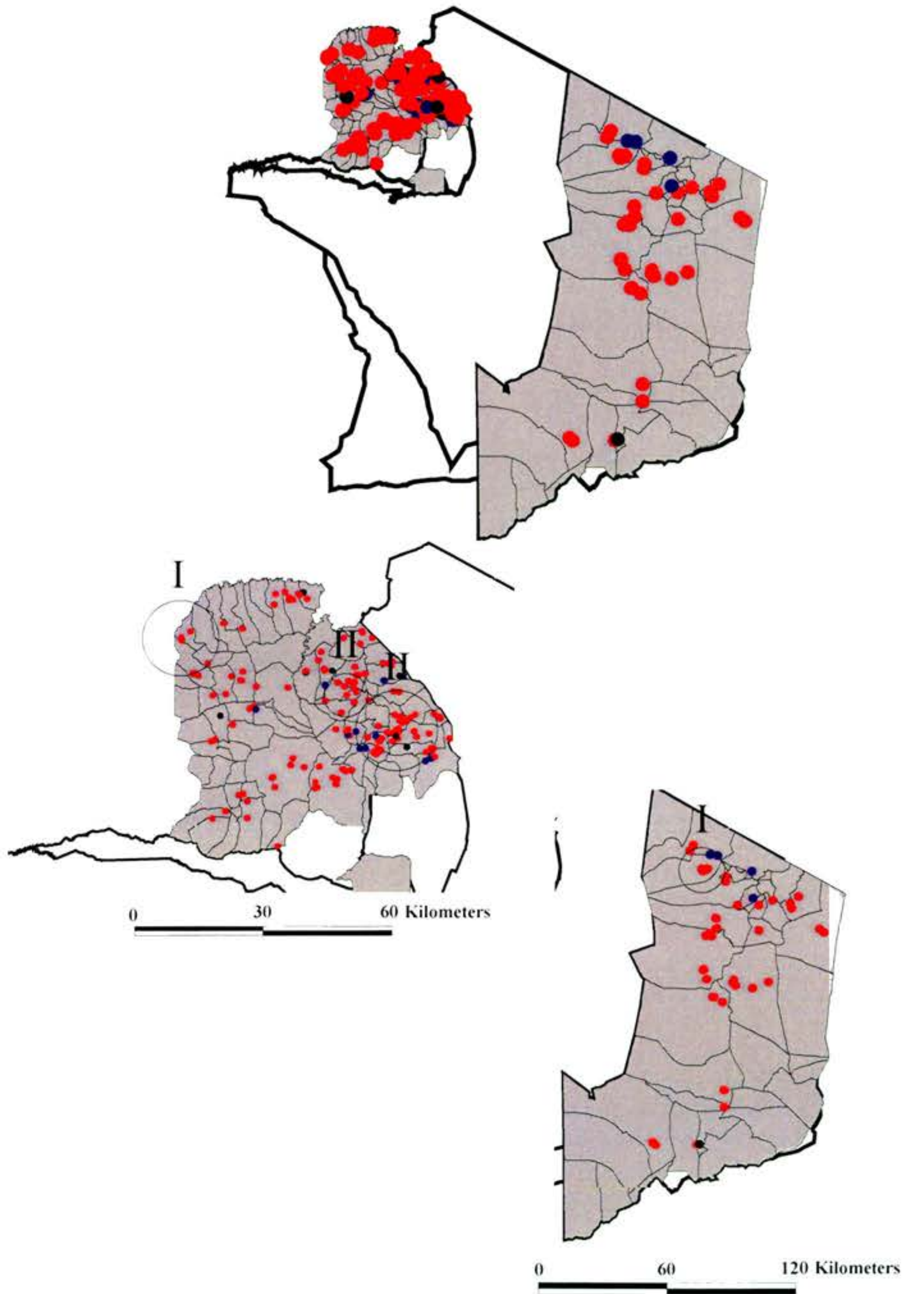


Figure 5.10. Spatial distribution of rabies cases recorded in Serengeti and Ngorongoro Districts in 2003 and primary (I) and secondary (II) space-time clusters detected by retrospective space-time analysis. Domestic dog cases are indicated as red dots, wild carnivore cases as blue dots and domestic cat cases as black dots.

Significant primary space-time clusters of rabies incidence detected in the study area in 2004 are shown in Figure 5.11. The cluster in SD included dog, wildlife and cat cases.

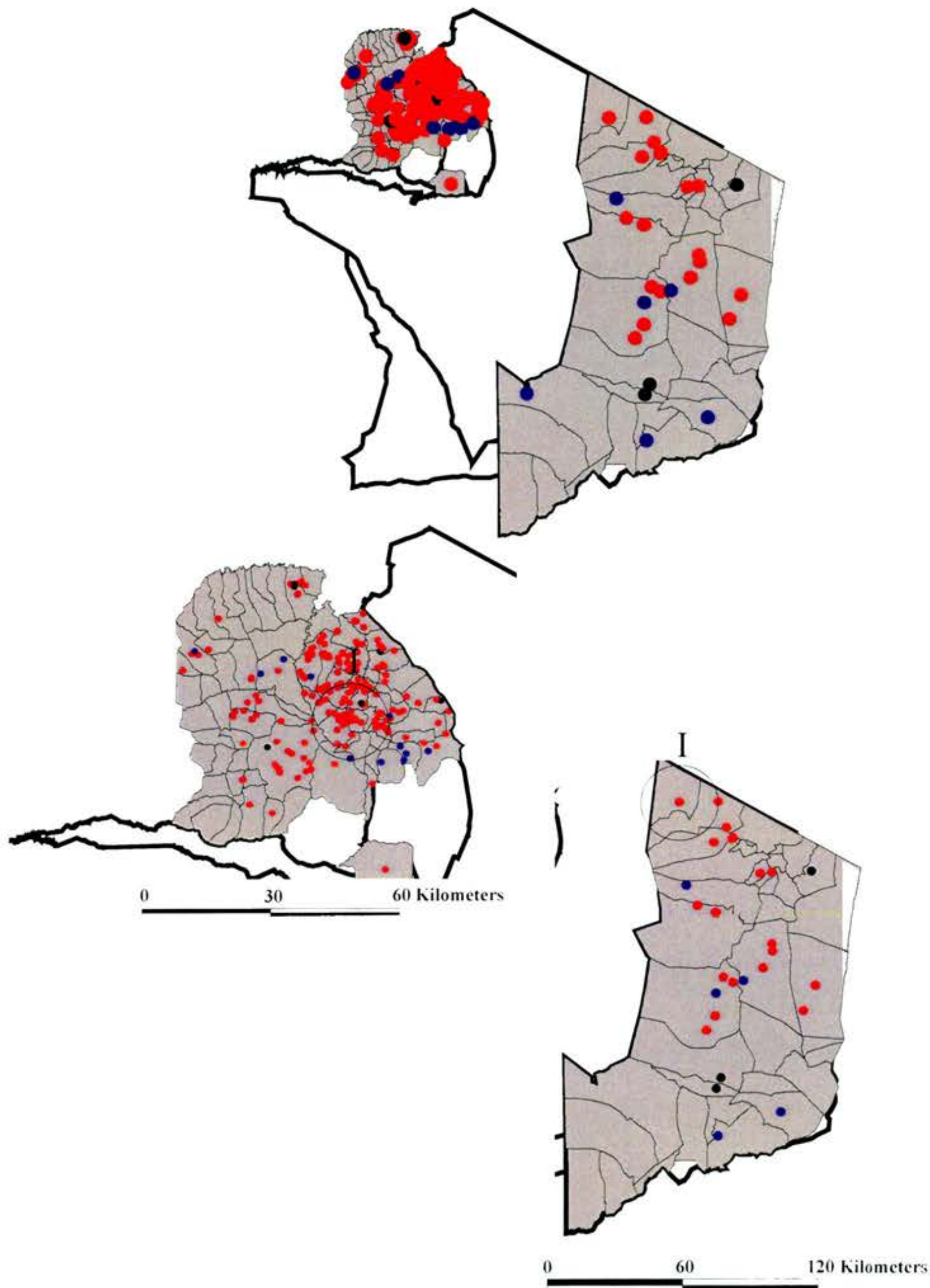


Figure 5.11. Spatial distribution of rabies cases recorded in Serengeti and Ngorongoro Districts in 2004 and primary space-time clusters detected by retrospective space-time analysis. Domestic dog cases are indicated as red dots, wild carnivore cases as blue dots and domestic cat cases as black dots.

No statistically significant high rate clusters were identified in ND in 2005 (see Table 5.8). The cluster detected in SD, which included only dog cases, is shown in Figure 5.12.

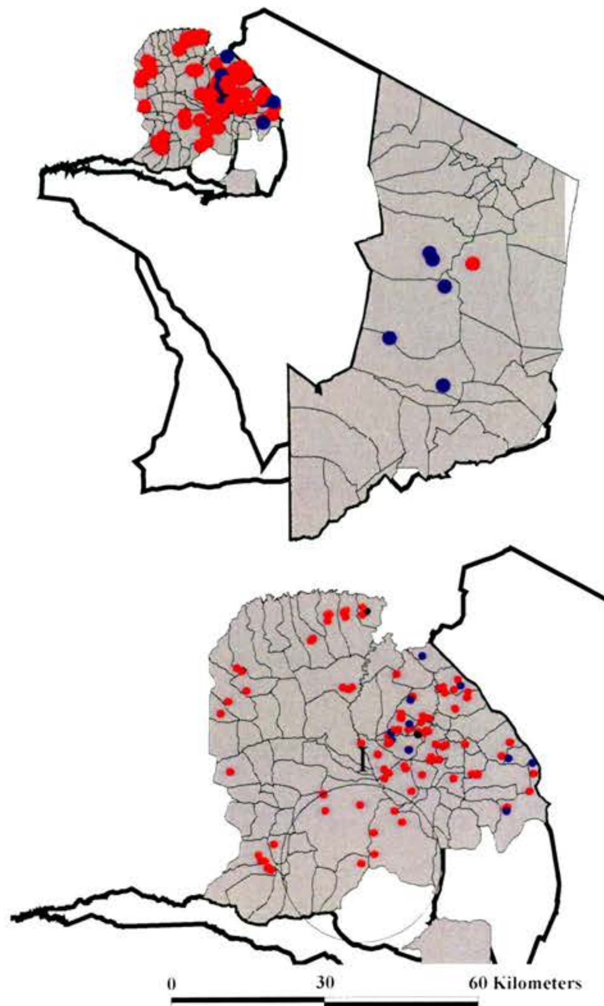


Figure 5.12. Spatial distribution of rabies cases recorded in Serengeti and Ngorongoro Districts in 2005 and primary space-time clusters detected by retrospective space-time analysis. Domestic dog cases are indicated as red dots, wild carnivore cases as blue dots and domestic cat cases as black dots.

5.5 Discussion

Few studies have attempted to identify reservoirs of infection in complex multi-host systems. Definitive identification of reservoirs is rarely possible because single lines of evidence are often refutable and the most incontrovertible tool, intervention to prevent transmission from reservoir to target, is costly, difficult to implement perfectly and must be maintained for long periods to eliminate residual infections. In order to achieve a better understanding of the structure of a potentially complex rabies reservoir system in the Serengeti, in this study multiple, complementary approaches were employed using data from a 15 year study of Serengeti carnivores. The results suggest domestic dogs to be an essential component of the reservoir as maintenance hosts and the main source population of rabies for humans. Other carnivores are non essential to maintenance, but because they transmit disease to target populations, also constitute the reservoir.

The demographic characteristics of hosts have a profound influence on the dynamics of microparasite infections. Specific demographic attributes (high densities and/or turnover rates) are thought to be required for sustained rabies cycles and prolonged transmission. A threshold density for persistence of > 5 dogs/km² has previously been identified for rabies in dog populations of SD (Cleaveland and Dye, 1995) and other African settings (Foggin, 1988; Brooks, 1990; Kitala *et al.*, 2001). In recent years, human populations to the west and east of SNP have grown considerably (Tanzania National Bureau of Statistics, 2005) and the present study indicated that dog populations in both areas have also increased substantially: > 11 vs > 5/km² reported by Cleaveland and Dye (1995) for SD and < 5 vs < 1/ km² reported by Cleaveland and Dye (1995) for ND. Notably, in the lower density populations (ND), previously considered too small to sustain rabies infection, the average dog density now approaches the threshold value for persistence. Although during the early years of the study rabies was detected sporadically in these populations, in 2003 population sizes were sufficient for a substantial epidemic to occur. Recent rabies control measures make it hard to determine whether current dog densities in ND are high enough for infection to persist, but, in the absence of vaccination, they may now be close. This finding has important implications not only for public health but also for

conservation as a great range of wildlife species including threatened populations of African wild dogs are documented in this area.

The first steps towards identifying constituent populations of a reservoir are demonstrating natural infection in the hosts that may be included in the reservoir and their ability to transmit the pathogen to the target populations (Haydon *et al.*, 2002a). Among the range of species in which rabies has been confirmed in the Serengeti region, domestic dogs and cats, bat-eared foxes, jackals, white-tailed mongooses and genets each have the characteristics of rabies reservoir hosts and all the canid species have been implicated as independent maintenance hosts elsewhere in Africa (Swanepoel *et al.*, 1993; Thomson and Meredith, 1993; Rhodes *et al.*, 1998; Bingham *et al.*, 1999a,b). These species have all been recorded as biting humans qualifying them as components of a target-reservoir system (Table 5.7).

Confirmed presence of the disease and transmission to the target population does not however demonstrate a species ability to maintain rabies transmission. In the Serengeti region domestic dogs living at high densities (SD) are the only species in which rabies has been recorded continuously throughout the study period with incidence affected by implementation of dog vaccination programmes. This contrasts with only sporadic detection of rabies in other species (both inside and outside the protected areas). It is believed this is not an artifact of surveillance, which will only ever detect a proportion of cases in wildlife. Animal health has become a subject of increasing importance for ecosystem management in the Serengeti (Gascoyne *et al.*, 1993a; Dobson, 1995; Kat *et al.*, 1996; Roelke-Parker *et al.*, 1996). Consequently, surveillance levels have improved progressively since the 1990s (Table 5.1). Given that rabies was detected during periods when surveillance levels were lower (e.g. 1996), it seems likely that infection in wildlife populations, had it been present continuously, would have been detected subsequently by the improved surveillance effort. This provides circumstantial evidence for rabies persistence in domestic dog populations and implies a lack of persistence in other carnivore populations. Explosive epidemics such as those reported in bat-eared foxes in 1987 and 1988 each lasting less than two months (Maas, 1993), are consistent with short-lived chains of transmission that fade out. Incidence patterns in wild carnivores that are believed to maintain rabies through independent cycles in other parts of Africa are very different.

For example, rabies was recorded sporadically in bat-eared fox populations in South Africa from the 1950s to 1970, then continuously until 1992, suggesting a shift to persistent infection (Thomson and Meredith, 1993).

Approaches applied to genetic and spatial and temporal incidence data can be used to draw independent inferences about how rabies is circulating, the direction and quantity of spill-over events if they occur and whether rabies transmission is being maintained. Genetic characterisation of pathogens isolated from different populations provides a powerful tool for identifying key components of reservoirs even in sparsely sampled populations, whereas active surveillance allows more detailed evaluation of epidemiological trends. However, conclusions drawn from incidence data are contingent on the assumption of reliable case detection. Despite intensive effort to obtain diagnostic samples, most cases reported here were suspected rather than confirmed. Clinical evaluation based on the 'six-step' method (Tepsumethanon *et al.*, 2005) proved a valuable aid for a presumptive diagnosis. Unlike the above-mentioned study, in the present study this approach was mainly applied indirectly in retrospective interviews with eye-witnesses. Assuming a gold standard test (FAT) of highest Se and Sp, the rabies recognition probability was > 74%. This was lower than that of > 89% extrapolated from the study by Tepsumethanon *et al.* (2005), but higher than other authors obtained using active surveillance measures (Kitala *et al.*, 2000). The lower positive predictive value might have resulted from: long intervals between sample collection and testing, during which samples were preserved in variable conditions and underwent repeated freeze-thaw cycles; rare availability of composite samples of each brain to achieve the highest test reliability; and inaccurate rabies recognition by local communities, although this is thought to be rare. Even given these caveats, it is believed the use of suspect rabies cases for evaluating epidemiological trends is both justified and reliable. Furthermore, the relatively accurate rabies recognition by local communities indicates that a systematic approach to the diagnosis of rabies based on the case history and careful observation of clinical signs may be of great value in areas where diagnostic material is difficult to obtain and/or infrastructures for rabies diagnosis are limited.

In this study, the detection of one canid-associated variant (Africa 1b) circulating among a range of species, with no evidence for species-specific virus-host

associations points to a principal host, the domestic dog, responsible for maintaining the virus in the ecosystem. A high degree of genetic relatedness between virus samples from different hosts suggests transmissibility of the variant between domestic dogs and other species, indicating that cross-species transmission events occur frequently. The transmission networks described in Chapter 4 also support within- and between-species linkages consistent with intra- and inter-specific transmission and suggest temporal direction of evolutionary change from domestic dogs to other species.

Analysis of temporal patterns using timelagged regression models, which were performed by K. Hampson, Princeton University and are presented in the Appendix, detected robust relationships between dog and livestock cases: rabies in livestock was predicted by rabies in dogs with a one month time-lag corresponding to the characteristic incubation period before the onset of clinical signs. When the regression models were applied to carnivore species with the potential to act as reservoir hosts, the relationships were less robust, probably due to the lower abundance of carnivores compared to cattle and therefore reduced likelihood of spill-over and case detection. Time-lags, if detected, were small, in contrast to one year lags recorded in Zimbabwe between domestic dog and jackal epidemics (Cleaveland and Dye, 1995; Bingham *et al.*, 1999a), suggestive of spill-over from dogs, rather than independently maintained co-circulating epidemics.

Similarly, space-time clusters of dog rabies cases were identified in both SD and ND with or without other carnivore cases, whereas no independent clusters of wildlife or domestic cat cases were detected. Despite the potential bias due to the smaller sample size of other carnivore cases compared to dog cases, the results indicate that cases in wildlife and domestic cats appear to be temporally and spatially linked with outbreaks of dog rabies. Taken together these analyses strongly suggest spill-over occurs from epidemics in domestic dog populations. When the temporal relationship between dog rabies in high- and low-density populations was investigated, there was no correlation suggesting that dog rabies epidemics in low-density populations are not temporally linked with epidemics in high-density populations.

The evidence presented here points to the domestic dogs as the principal host supporting the rabies virus cycle, with other species acquiring infection from the principal host, but not sustaining transmission (see also Chapter 4). This is consistent with broad patterns observed elsewhere (i.e. a single maintenance host species) (Smith, 1989; Wandeler, 1991; Rupprecht *et al.*, 1991; Smith *et al.*, 1995), but this is the first time it has been documented in such a diverse community with potential for transmission between a wide range of host species. The distribution of rabies cases amongst wildlife did not appear to be associated with species abundance. For example, most wildlife cases in SD were recorded in jackals (Figure 5.6A), although during night transects jackals were observed much less frequently than other carnivores (K. Hampson, unpublished data). Similarly, in ND no cases were detected in bat-eared foxes, the most abundant carnivore, but hyaenas predominate among rabies cases (Figure 5.6B). This indicates that abundance is not the only determinant of rabies maintenance (Wandeler, 1991; Wandeler *et al.*, 1994) and that other factors, potentially related to host ecology, behaviour and co-evolution of the virus lineage, may also play a role. Therefore, it appears that rabies dynamics in the Serengeti are driven by domestic dogs, despite the availability of a range of potentially independent maintenance hosts, implying that they are better demographically configured, distributed, and behaviourally suited to maintain rabies.

In conclusion, the balance of evidence implicates domestic dogs as the only maintenance population of the rabies reservoir in the Serengeti, with other carnivores constituting part of the reservoir as nonessential hosts (Figure 5.13A). The results have important implications for the design of appropriate rabies control strategies for the ecosystem and more generally for protected areas across Africa where rabies poses a threat to endangered wildlife. Efforts directed at controlling infection in domestic dogs are expected to have the most significant impact on reducing or eliminating rabies in all other species, which most importantly include the target populations of concern: humans and endangered wildlife. Epidemiological theory indicates that to prevent epidemic and eliminate endemic infection, the number of susceptible hosts needs to be reduced to maintain the reproductive ratio, R_0 (the expected number of secondary cases caused by an average infectious individual in an entirely susceptible population) below 1 (Anderson and May, 1991). Reducing the

number of susceptible dogs through vaccination, for instance, should lead to a situation where the dog population cannot sustain rabies cycles ($R_0 < 1$) and the reservoir will no longer exist (Figure 5.13C). However, if insufficient numbers are vaccinated, it is possible that susceptible dogs together with wild carnivores could comprise a maintenance community ($R_0 > 1$) (Figure 5.13B). Questions therefore remain as to the impact of alternative hosts on long-term control efforts (e.g. could they be responsible for reintroduction of infection into the dog population during inter-campaign intervals), which will be addressed in future research. However, a vaccination coverage of 65-70% (the target considered necessary to bring R_0 below 1 for dog rabies; Coleman and Dye, 1996) has been found empirically to be sufficient to control dog rabies even in the presence of alternative hosts (Cleaveland *et al.*, 2003) and this study indicates that to reduce the burden of rabies, at least in the Serengeti ecosystem, control efforts should focus on domestic dog populations.

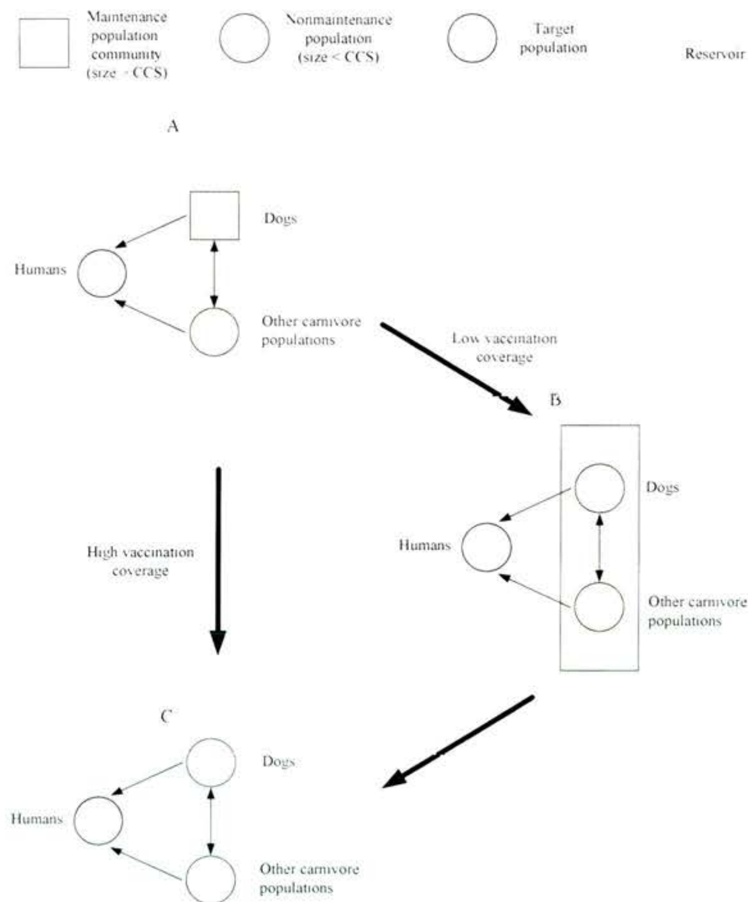


Figure 5.13. Reservoir and stages of control.

CHAPTER 6: DESCRIPTIVE EPIDEMIOLOGY OF CANINE DISTEMPER IN THE SERENGETI

6.1 Abstract

Canine distemper (CD) is considered one of the greatest threats to wild carnivores. From late 1993 to 1994 an epidemic severely affected the Serengeti-Mara lion (*Panthera leo*) population. The outbreak was also recorded in other wild carnivores and domestic dogs (*Canis familiaris*), with a single virus variant recovered from all the species affected. High-density domestic dog populations bordering the Serengeti National Park (SNP) to the west were implicated as the likely source of infection for wildlife, but many questions remain on the long-term patterns of viral maintenance, transmission dynamics and role played by different species within a potentially complex reservoir system. This study draws on long-term serological studies in Serengeti unvaccinated and vaccinated dog populations and lion populations to evaluate spatio-temporal changes in the exposure of the two populations to CD. Several important conclusions can be drawn that allow formulating hypotheses for CD reservoirs in the ecosystem. Patterns of infection in unvaccinated high-density and low-density dog populations were consistent with fade-out of infection after the 1994 epidemic with no evidence for persistence in any of the populations. Re-introductions of infection occurred at roughly 2- to 3- year intervals in high-density dog populations and 2- to 6-year intervals in low-density dog populations. Re-introduction of infection coincided with declines in vaccination coverage, but patterns of exposure did not differ in vaccinated and unvaccinated populations. Canine distemper virus (CDV) infection in Serengeti lions occurred in sporadic episodes and, in contrast to 1994, there was no evidence for any morbidity and mortality associated with infection. Temporal and spatial patterns appeared to follow those observed in domestic dog populations with no infection in lions in the absence of infection in dogs. This suggests that, although the dog populations adjacent to the park do not maintain virus for prolonged periods, they may still be the principal source of infection for lions. The lack of evidence for long-term persistence in any of the district-level populations suggests that none of these populations may be capable of independent maintenance but their assemblage may constitute a maintenance community or infection may need to be re-introduced from outside sources (e.g. another maintenance community). Clarifying whether the maintenance community would persist without the contribution of dogs is important, but cannot be definitely

addressed while CDV still circulates in unvaccinated dog populations. Elimination of infection in dog populations would therefore provide insights into their role in disease persistence within the reservoir system. Given that outbreaks in dogs currently fade out naturally, CDV infection is not invariably pathogenic in lions, and CDV vaccines extremely costly, questions are raised about the cost-effectiveness of mass CDV vaccination of dogs for the conservation management of lions within the SNP. However, the impact of CDV vaccination of dogs for protection of other species, such as African wild dogs (*Lycaon pictus*), remains to be evaluated.

6.2 Introduction

Generalist pathogens capable of infecting a wide range of hosts are of particular concern in the conservation of valuable wildlife resources (Daszak *et al.*, 2000; Dobson and Foufopoulos, 2001; Cleaveland *et al.*, 2001). Understanding patterns of maintenance and transmission processes within reservoirs of these pathogens is critical for effective and sustainable control. However, distinguishing whether different host species maintain or contribute to the maintenance of the pathogen in the reservoir as nonmaintenance hosts or are only occasionally infected as a result of 'spill-over' from the reservoir (Haydon *et al.*, 2002a) may be problematic. Particular difficulties arise when attempting to address these questions in species-rich areas where interactions between a range of populations are frequent and multiple hosts have the potential to play a role in the reservoir. The lack of detailed epidemiological data, particularly in wildlife populations, or the availability of data for some populations, but not others co-existing within the same ecosystem are also important constraints. Only long-term studies of particular animal populations for instance can help determine critical issues such as that of persistence of infection in the reservoir.

Pathogen persistence is a central tenet of reservoir concepts, yet understanding the mechanisms underlying persistence of infectious agents that are highly pathogenic or induce life-long host immunity in survivors remain a fundamental problem (Anderson and May, 1991). Canine distemper virus (CDV) and other *Morbilliviruses* typify these pathogens. They have short incubation and infectious periods, are highly contagious and cause high mortality in infected individuals or life-long immunity in individuals that survive primary infection (Anderson and May, 1991). Because of these traits, the infection dynamics of such pathogens show marked epidemic behaviour with inter-epidemic intervals necessary to replenish the pool of susceptibles (Anderson and May, 1991). For example, epidemic measles models predict complex recurrent epidemics (generally biennial) in large unvaccinated urban communities (Fine and Clarkson, 1982; Anderson and May, 1991; Grenfell *et al.*, 2001, 2002; Bjørnstad *et al.*, 2002), as described in detail in Chapter 1 (section 1.1). Recurrent introductions of infection have also been reported for a number of *Morbilliviruses* in a range of wild animal populations: e.g. phocine distemper virus (PDV) in harbour seals (*Phoca vitulina*) (Kennedy *et al.*, 1988; Osterhaus and

Vedder, 1988; Jensen *et al*., 2002) and CDV in African lions (*Panthera leo*) (Packer *et al*., 1999) and spotted hyaenas (*Crocuta crocuta*) (Alexander *et al*., 1995; Harrison *et al*., 2004). Periodic epidemics of CDV have also been reported in Kenyan domestic dog (*Canis familiaris*) populations (Bwangamoi *et al*., 1989). Even in animal populations considered capable of independent CDV maintenance, such as raccoons (*Procyon lotor*) in north America, infection appears to be maintained through shifting, localised cycles occurring at 4-year intervals in any given location (Roscoe, 1993).

The availability of a rich data set and the infection's simple life history have made measles the prototypical system for understanding factors determining persistence of *Morbilliviruses* and other infectious agents (Bartlett, 1957, 1960; Anderson and May, 1991; Cliff *et al*., 1993; Grenfell and Harwood, 1997; Keeling and Grenfell, 1997; Grenfell *et al*., 2001, 2002; Bjørnstad *et al*., 2002; Conlan and Grenfell, 2007), as described in section 1.1. In his now seminal work Bartlett (1957, 1960) identified a critical community size (CCS) for measles below which infection becomes extinct. Demographic processes such as host birth, death and migration play a critical role in the dynamics of measles in small communities (McLean and Anderson, 1988; Grenfell and Harwood, 1997; Finkenstädt and Grenfell, 2000; Earn *et al*., 2000; Keeling *et al*., 2004; Conlan and Grenfell, 2007). For instance, Conlan and Grenfell (2007) showed a strong positive relationship between birth rate and CCS, with a doubling of the birth rate leading to a fivefold reduction in the CCS. For small population sizes, an increase in birth rate always led to increased persistence. Measles dynamics are also strongly influenced by temporal heterogeneities with seasonal drivers such as schooling in developed countries (Schenzle, 1984; Finkenstädt and Grenfell, 1998; Grenfell *et al*., 2002) and the agricultural cycle (and associated droughts and famines) in developing countries (Duncan *et al*., 1997) playing a critical role. Finally, spatial heterogeneities at different scales (e.g. social grouping and mixing), which can be incorporated into models using metapopulation models (Gilpin and Hanski, 1991; Hanski and Gilpin, 1997; Grenfell and Harwood, 1997; Hanski and Gaggiotti, 2004), have been considered as major factors in the greater persistence of measles (Grenfell and Harwood, 1997; Keeling, 1997; Grenfell and Bolker, 1998; Keeling, 2000; Keeling *et al*., 2004).

Age-stratified seroprevalence data obtained in cross-sectional and longitudinal surveys have been extensively used to investigate the epidemiology of a wide variety of infections and measure the effectiveness of control programmes. For childhood diseases, infections are broadly dependent on age. Studies of measles attempted to develop methods to measure the variation in infection rate with age in the United Kingdom (UK) and United States (US) using age-specific serological data (Grenfell and Anderson, 1985). A consistent pattern of change with age in the force of infection (FOI - i.e. the *per capita* rate at which susceptible individuals acquire infection) was observed in both the UK and US, and unvaccinated and vaccinated populations. Rates of infection showed a rise up to around 10 years of age followed by a decline in the older age-classes. Age-stratified cross-sectional and longitudinal serological data have been widely used in the study of dog and wildlife leishmaniasis both in Europe (Dye *et al.*, 1992; Hasibeder *et al.*, 1992) and Latin America (Courtenay *et al.*, 1994; Quinnell *et al.*, 1997; Courtenay *et al.*, 2002). The interesting study by Courtenay *et al.* (2002) examined the epidemiological role of the crab-eating fox (*Cerdocyon thous*) in parasite transmission in Amazon Brazil. Although the parasite prevalence and incidence in foxes was similar to those of sympatric dogs, the possible contribution of foxes to transmission was estimated to be 9% compared to 91% by sympatric domestic dogs and the basic case reproduction number (i.e. the expected number of secondary cases caused by an average infectious individual in an entirely susceptible population) was below the threshold condition for pathogen persistence, suggesting that the fox population is unable to maintain transmission cycles independently of domestic dogs. The utility of age-specific seroprevalence rates and parasite prevalence data in distinguishing between long- and short-term patterns of disease transmission was explored in studies on malaria in hyper- and hypo-endemic areas of Tanzania (Drakeley *et al.*, 2005). In particular, the relationship between altitude, parasite prevalence and seroprevalence in individuals of different ages was examined: antibody prevalence in young children mirrored the relationship between altitude and point-prevalence parasitemia, and the correlation between seroprevalence and parasite prevalence decreased with increasing age. This suggests that parasite prevalence provides information about recent malaria infection (within the previous 12-24 months), whereas seroprevalence reflects longer-term

transmission trends (periods of several years). Serological data can also be of value in assessing the effectiveness of control measures, particularly for chronic diseases characterised by delayed clinical manifestations and that are difficult to diagnose. For instance, in order to measure the impact of control programmes in areas of Venezuela highly endemic for *Trypanosoma cruzi*, retrospective age-specific seroprevalence data from cross-sectional surveys were analysed using maximum likelihood methods (Williams and Dye, 1994) to estimate the FOI, allowing it to vary independently in each study period (Feliciangeli *et al.*, 2003). Transmission rates showed little tendency to decrease until the implementation of a national control programme in the 1960s that determined a dramatic drop in FOI with no further decrease in subsequent years. This suggests that, although the programme has been successful in greatly reducing transmission, the goal of interrupting it has yet to be achieved.

Given a reservoir system that may comprise a network of populations, an exhaustive understanding of all its components may be difficult. Questions therefore arise as to the most appropriate population for directing control measures. If the goal is to protect the population of interest to us (the 'target population' *sensu* Haydon *et al.*, 2002a) rather than eliminate infection, identifying the source populations and controlling infection within them should lead to effective control.

Amongst generalist viral pathogens, CDV is considered one of the most serious threats to wild carnivores, especially to critical populations that may be at risk of extinction through the introduction of infection (Woodroffe, 1999; Haydon *et al.*, 2002b). Between late 1993 and 1994, CDV was responsible for a severe epidemic that killed approximately 30% of the Serengeti lions (Roelke-Parker *et al.*, 1996) and also affected spotted hyaenas and bat-eared foxes (*Otocyon megalotis*) within the Serengeti and the lion population of the Maasai Mara National Reserve (MMNR) (Kock *et al.*, 1998). The close similarity of viruses recovered from Serengeti lions, bat-eared foxes, spotted hyaenas and domestic dogs (Harder *et al.*, 1995; Roelke-Parker *et al.*, 1996; Cleaveland, 1996; Haas *et al.*, 1996; Carpenter *et al.*, 1998) indicated that a single variant was responsible for the die-off. Serological, demographic and case-surveillance data pointed to the large population of domestic dogs to the west of the Serengeti National Park (SNP) as the most likely source of

infection for wildlife (Cleaveland *et al.*, 2000). In particular, prolonged viral circulation (1992-1994) was documented in higher-density populations to the northwest of the park (Serengeti District; Figure 6.1), whereas exposure was more sporadic in lower-density dog populations to the east (Ngorongoro District; Figure 6.1) (exposed in late 1991 and 1994, but not in 1992 and 1993). This suggested that only the former had been exposed to CDV prior to the lion epidemic, with the latter being infected after its subsequent spread throughout the ecosystem in wild carnivores. The exact routes of transmission were unknown, but indirect transmission between dogs and lions through chains in other species (e.g. jackal spp. and hyaenas) was suggested.

Although the study by Cleaveland *et al.* (2000) provided insights into the role of domestic dogs in the 1994 epidemic, it raised many questions about the mechanisms of long-term maintenance of CDV infection in the ecosystem, transmission dynamics and reservoirs of infection. There were similarities in infection patterns between wildlife (lions and hyaenas) and low-density dog populations, with serological evidence of sporadic exposure rather than persistent infection (Alexander *et al.*, 1995; Packer *et al.*, 1999; Cleaveland *et al.*, 2000; Harrison *et al.*, 2004). On the contrary, CDV appeared to be maintained as a more stable infection in high-density dog populations generating the hypothesis of CDV persistence only in this population (Cleaveland, 1996; Cleaveland *et al.*, 2000). However, the study was based on limited epidemiological data collected over a 3-year period. Long-term data, which are rarely available in African animal populations (especially wildlife), are required to evaluate temporal changes in the exposure of a given population to a certain pathogen and establish whether infection is endemic or epidemic in that population (Packer *et al.*, 1999; Drakeley *et al.*, 2005). Since 1994, dog populations surrounding the Serengeti ecosystem and lion populations within the ecosystem have been studied continuously in order to help understand long-term patterns of infection in the two populations.

In this study, data are presented from cross-sectional studies of high-density (unvaccinated and vaccinated) and low-density dog populations living adjacent to the park and lion populations. All available serological data between 1992-2004 from dog populations and 1997-2004 from lion populations combined with information on

ages at the time of sampling and locations of sampled individuals/villages (where available) are used to evaluate temporal and spatial changes in the exposure of the two populations to CDV. Hypotheses for CDV reservoirs in the Serengeti are formulated.

6.3 Materials and methods

6.3.1 Study area, serological surveys and interventions

The study area was the Serengeti ecological region of northwestern Tanzania including SNP and areas surrounding the park (Figure 6.1). Areas to the west of the park are inhabited by large multi-ethnic communities (predominantly Sukuma and Kurya, but also Jita, Luo, Ikizu and Zanaki) with agro-pastoralist production systems. They are characterised by high-density dog populations (see Chapter 5) unlike the pastoralist areas to the east where dog populations occur at lower densities (see Chapter 5). The latter is inhabited by low-density Maasai and Sonjo pastoralist communities.

Cross-sectional serological surveys have been conducted in dog populations adjacent to SNP since 1992. Previous investigations by Dr. S. Cleaveland focused on two districts: Serengeti District (SD), to the northwest and Ngorongoro District (ND), comprising the Loliondo Game Control Area (LGCA) and Ngorongoro Conservation Area (NCA), to the east (Figure 6.1). Methodology and analysis for the period 1992-1994 are described in Cleaveland *et al.* (2000). A mass dog vaccination programme against rabies, canine distemper and parvovirus was initiated as an intervention trial in dog populations of SD in October 1996, with unvaccinated (control) villages comprising a proportion of villages within Musoma District (MD), to the west of SD (Figure 6.1). In both the vaccinated (SD) and unvaccinated (MD and ND) areas, study villages were selected at random for intensive study as described (Cleaveland *et al.*, 2003). Systematic blood sampling was conducted from dogs brought to vaccination stations during vaccination campaigns in the vaccination area and at the household level, with households selected at random from a list of households provided by the village leaders, in the unvaccinated areas. Data were recorded on the name of the owner, name of the dog, age, sex and, in vaccination areas, on the previous vaccination history. During 1998-1999, cross-sectional serological surveys were also undertaken in three districts (Bariadi, Meatu and Magu) to the south-west of SNP (Figure 6.1).

The zone of dog vaccination was extended in 2003 to encompass all villages within a 10 km zone adjacent to the western boundaries of SNP, including six districts, Tarime, Serengeti, Bunda, Magu, Bariadi and Meatu (Figure 6.1), as described in

Chapter 2 (section 2.1.1, see Figure 2.1). In 2004 widespread vaccination was also implemented in all villages within ND (section 2.1.1, see Figure 2.1). The present study exploited the opportunity provided by the vaccination trials to implement serological surveillance during 2003-2004 as described in Chapter 2 which gives details of the study design for this period (sections 2.1.1.1.1, 2.1.1.1.3 and 2.1.1.4). The sampling carried out in 2003 to the west of SNP and in 2004 to the east included all study villages within the current vaccination area, whereas the sampling carried out in 2004 included all study villages within the current unvaccinated area. The former was carried out during vaccination campaigns at the start of the vaccination programme (pre-vaccination sera), the latter at the household level (sections 2.1.1.1.3 and 2.1.1.4).

The total number of dogs sampled by year and area is given in Table 6.1, which also provides documentation of samples obtained and analysed in previous investigations.

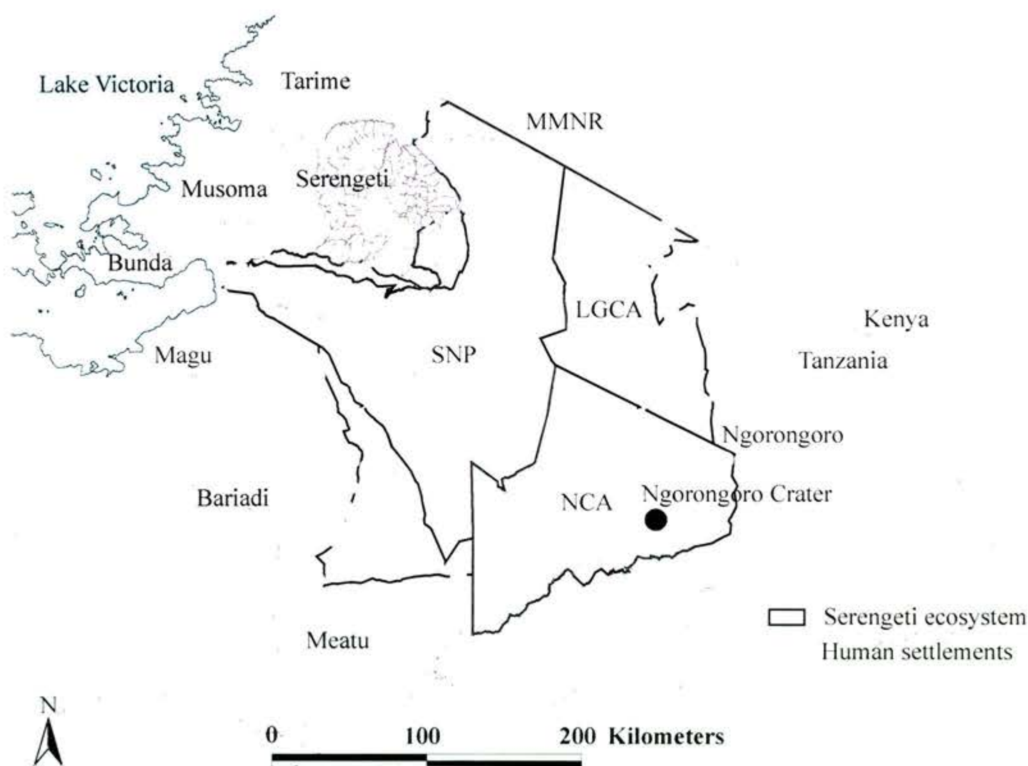


Figure 6.1. Map of the Serengeti National Park (SNP) and surrounding districts to the west and east. MMNR = Maasai Mara National Reserve; LGCA = Loliondo Game Control Area; NCA = Ngorongoro Conservation Area.

Table 6.1. Number of serum samples from dogs sampled each year in areas surrounding the Serengeti National Park available for analysis. Serologica data for the period 1992-2002 were provided by Dr. S. Cleaveland (in italics), except for samples indicated in normal font which were tested in the present study. Collection and serological assays of samples indicated in bold and italics were carried out in the present study. NCA = Ngorongoro Conservation Area; LGCA = Loliondo Game Control Area.

	West							East	
Year	Serengeti	Musoma	Bariadi	Meatu	Magu	Bunda	Tarime	NCA	LGCA
1992	*103*	-	-	-	-	-	-	*133*	
1993	*101*	-	-	-	-	-	-	*99*	
1994	*134*	-	-	-	-	-	-	*141*	
1995	-	-	-	-	-	-	-	-	-
1996	*235*	-	-	-	-	-	-	-	-
1997	*428*	*265*	-	-	-	-	-	79	-
1998	*502*	*443*	73	61	-	-	-	-	40
1999	*31*	*86*	183	50	107	-	-	-	31
2000	*109*	-	-	-	-	-	-	-	-
2001	-	-	-	-	-	-	-	118	-
2002	-	-	-	-	-	-	-	-	-
2003	***52***	-	***140***	***89***	***79***	***81***	***147***	-	-
2004	-	***41***	***49***	***66***	***19***	***85***	***78***	-	***155***

The lions of the SNP and the Ngorongoro Crater (Figure 6.1) have been continuously monitored since the 1960s. The Serengeti study area includes the southeastern quarter of the park (2,000 km^2). Individual lions of both populations are recognised from natural markings and whisker-spot patterns (Pennycuick and Rudnai, 1970; Packer and Pusey, 1993) and most have been observed since birth (Pusey and Packer, 1994), therefore their precise age is known. Serological surveillance of lions during the period 1997-2002 was conducted in previous investigations, whereas the collection of samples for the period 2003-2004 was undertaken in the present study. Sampling of lions was conducted in collaboration with veterinarians from the Tanzania National Parks (TANAPA) and Tanzania Wildlife Research Institute (TAWIRI) Veterinary Departments and the Serengeti Lion Project (see also section

2.1.1.2.2). Lions were sampled for disease surveillance, focussing on lions of known identity and age and from a range of prides, and opportunistically as part of park management or research interventions (for example wound treatment, fitting or removal of radio-collars). No vaccination of lions was conducted inside or outside the park during the period of this study. Annual sample sizes are given in Table 6.2.

Table 6.2. Number of serum samples from the Serengeti and Ngorongoro lion study populations available for analysis. Serologica data for the period 1997-2002 were provided by Dr. S. Cleaveland (in italics), except for samples indicated in normal font which were tested in the present study. Collection and serological assays of samples indicated in bold and italics were carried out in the present study.

Year	Serengeti	Ngorongoro Crater
1997	*7*	-
1998	*14*	-
1999	*4*	-
2000	*5*	-
2001	*3*	*11*
2002	7	-
2003	***19***	-
2004	***12***	-

6.3.2 Dogs: serum sampling

Dogs were manually restrained, muzzled using a simple tape muzzle and blood samples without anticoagulant collected by cephalic venipuncture. Blood samples were either allowed to clot overnight or centrifuged within 24 hours of collection. After separation, serum was extracted and stored at minus 20°C in electric or kerosene freezers. Whenever possible, the location of each household or village was recorded using handheld Global Positioning System (GPS) units. All the samples were heat inactivated at 56°C for 30 minutes before exportation to the UK for testing.

6.3.3 Lions: serum sampling

Immobilisation was performed using techniques established by TANAPA and TAWIRI park veterinarians. The animals were anesthetised with 2 or 3 ml disposable

darts containing Telazol or Zoletil (Zolazepam-HCl plus Tiletamine-HCl). Dosage used was as follows: 300-400 mg for female lions and 400-750 mg for male lions. Blood samples without anticoagulant were collected by cephalic or tarsal venipuncture, allowed to clot at room temperature and the separated serum samples were stored at minus 20°C in electric or kerosene freezers. Sampling locations were recorded using handheld GPS units. All the samples were heat inactivated at 56°C for 30 minutes before exportation to the UK or US for testing.

6.3.4 Serum neutralisation test

Lion and dog serum samples were analysed at Intervet (UK) for serum neutralising antibodies to CDV using a virus neutralisation test (Chalmers and Baxendale, 1994). Before testing, the sera were heat inactivated at 56°C for 30 minutes to destroy heat labile non-specific virus inhibitory substances. Four-fold dilutions of test sera and positive and negative controls were prepared (from an initial dilution of 1:4 to 1:256) in tissue culture medium. A suspension containing 100-300 $TCID_{50}$/ml antigen (CDV-Bussel strain) was also prepared in tissue culture medium and an equal volume of the suspension was added to each serum dilution to give final dilutions of 1:8 to 1:512. The serum/virus mixtures were incubated in a humidified 5% CO_2 tissue culture incubator at 37°C for one hour to allow neutralisation to occur. Monolayers of a suspension of freshly-seeded Vero cells in 96-well microtitre plates were inoculated with each aliquot of serum/virus mixture and the plates incubated at 37°C for 3-5 days. The plates were then examined microscopically (using an inverted tissue culture microscope) for viral infectivity of the Vero cell monolayer: infected wells were identified by the presence of cytopathic effect (CPE). Virus titres (expressed as $\log_{10}$ $TCID_{50}$/ml) were calculated according to the method of Reed and Muench (Reed and Muench, 1938) using the Intervet International Computer program and expressed in ratio form. Two cut-off points were applied: a cut-off point of $\geq$ 1:16 ($\log_{10}$ 1.2) (Cleaveland *et al.*, 2000) and a more stringent cut-off point of $\geq$ 1:32 ($\log_{10}$ 1.5).

Lion serum samples were also analysed by the New York State Animal Health Diagnostic Laboratory, Cornell University, Ithaca, NY, USA using a serum neutralisation test (Appel and Robson, 1973). Serum samples from dogs sampled in NCA in 2001 were analysed at Cornell only. In the Cornell test, the CDV-

Onderstepoort strain was used as neutralising antigen. The two cut-off points described above were adopted also for this assay.

Exact binomial 95% confidence intervals for the seroprevalences were calculated using the program EpiInfo 6 (Centers for Disease Control and Prevention, Atlanta, US).

6.3.5 Vaccination coverage

Vaccination coverage for Serengeti District from 1996-1998 was determined from household questionnaire data collected previously (Cleaveland *et al.*, 2003). From 1999-2003, vaccination was patchy and CDV vaccine not available for most of the period, therefore it was not possible to estimate coverages for the whole zone based on household questionnaire data. For this period, vaccination coverage for CDV was estimated using a projected decline in coverage from 1998 levels, assuming the population age-structure, proportion vaccinated in each class, and age-specific mortality rates from the Serengeti District population (Cleaveland *et al.*, 2003 and Kaare, 2006). In 2001, the coverage estimates incorporated 3,459 new dogs vaccinated during a campaign at the end of 2000. The dog population size for each year was determined from extrapolated human populations sizes, using a 2.9% growth rate and human:dog ratio of 6, as described in Cleaveland *et al.* (2003).

6.4 Results

6.4.1 High-density domestic dog populations to the northwest of SNP

6.4.1.1 Serengeti District domestic dogs

Cross-sectional age-seroprevalences of Serengeti District (Figure 6.1) high-density dogs sampled over the period 1992-2003 can be seen in Figure 6.2A-H. Dog vaccination was conducted in this area as described below.

Age-seroprevalence curves for the period 1992-1994 (Figures 6.2A-C), which were adapted from Cleaveland *et al.* (2000), are described for completeness. These curves showed a similar pattern with seropositivity detected in all age classes in each of the three years which is consistent with prolonged viral circulation, as previously described (Cleaveland *et al.*, 2000).

In 1996 (Figure 6.2D) the CDV serostatus of dogs sampled prior to the implementation of dog vaccination programmes across the district indicated natural disappearance of infection as seropositivity was detected only in dogs ≥ 24 months of age.

Vaccination programmes were implemented in October 1996, as described in section 6.3.1 and 6.3.5. Relatively high coverage was attained in 1997 and 1998 (64.5 and 61.1% respectively). During 1997-1999 there was no evidence for new CDV exposure: all dogs born after previous vaccination campaigns (> 3 months and < 12 months of age) were seronegative (Figures 6.2E,F). Note that age-seroprevalences of dogs sampled in 1999 are not shown in Figure 6.2 as all dogs (n = 31) were seronegative.

From 1999-2003, CDV vaccine was not available for most of the period and vaccination was patchy. As a result coverage was lower: 28.8, 43.5, 12.0 and 0.1% for the years 1999, 2000, 2001 and 2002, respectively. Note that in 2001, the coverage estimates incorporated new dogs vaccinated during a campaign at the end of 2000 (see section 6.3.5). Case-surveillance and serological data are consistent with CDV being re-introduced into the area, but the exact timing is unclear. In late 2000 dogs showing signs of lacrimation, blindness, coughing, respiratory distress, salivation, anorexia, vomiting, diarrhoea, emaciation, incoordination, circling and death were reported by dog owners, local veterinary and livestock officers. Age-

seroprevalence patterns however were indicative of CDV being re-introduced into the area in 1999, as seropositive young dogs were detected between May and November 2000 (Figure 6.2G). In 2003, seropositivity was detected in animals ≥ 36 months of age, which is also consistent with exposure in 1999 (Figure 6.2H).

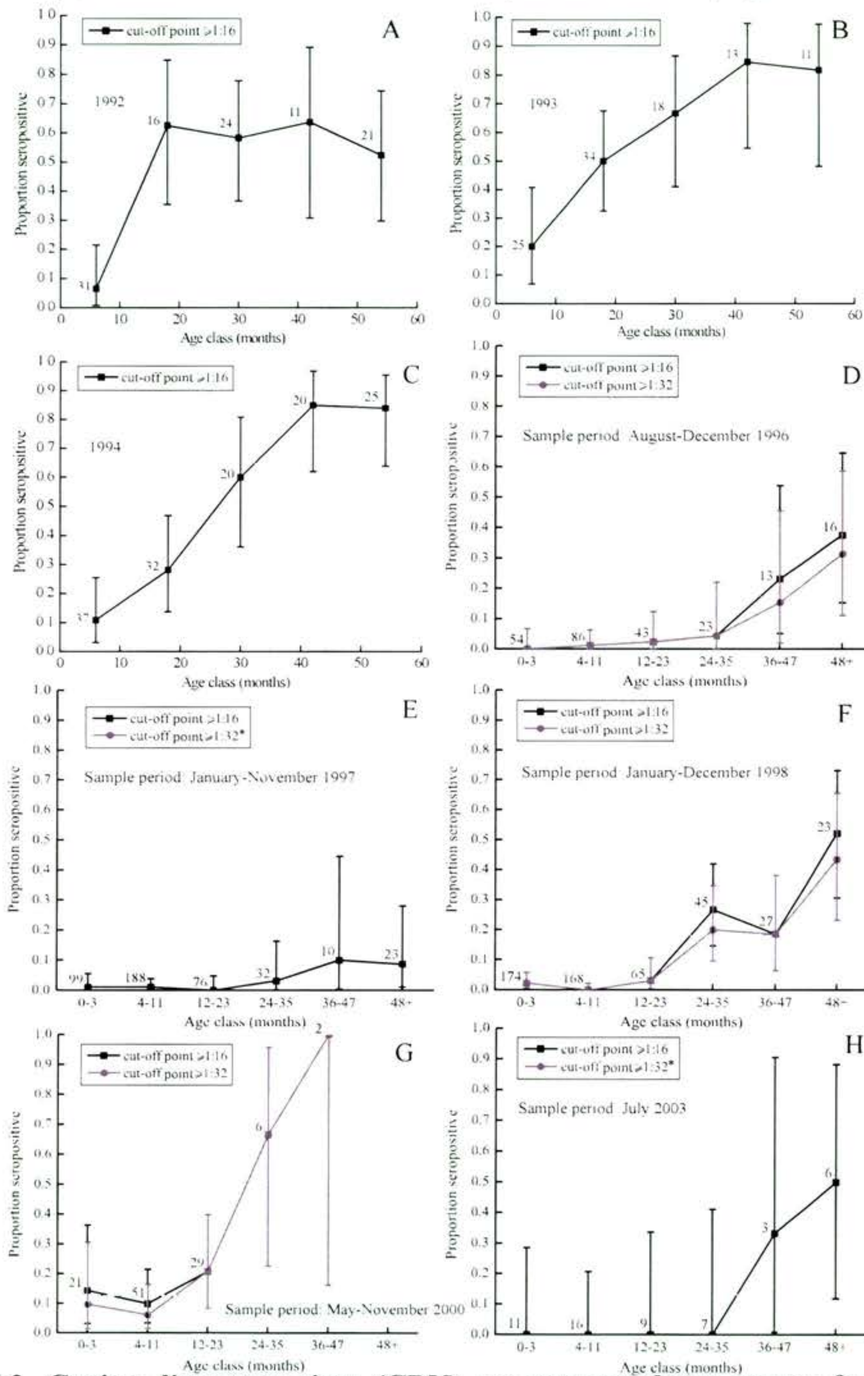


Figure 6.2. Canine distemper virus (CDV) age-seroprevalence curves for Serengeti District domestic dogs. Numbers above each point of the curve indicate sample sizes. Bars show exact binomial 95% confidence intervals. Curves for the period 1992-1994 were adapted from Cleaveland *et al.* (2000). *No changes in seroprevalence were detected using two different cut-off points (≥ 1:16 and ≥ 1:32).

6.4.1.2 Musoma District domestic dogs

Cross-sectional age-seroprevalence curves of Musoma District (to the west of Serengeti District; Figure 6.1) dogs are shown in Figure 6.3A-C.

CDV antibodies were present in older dogs sampled in 1997 suggesting exposure in previous years (Figure 6.3A). There was no evidence for new exposure in subsequent years (1998 and 1999) as seropositivity was detected only in dogs ≥ 36 months (Figure 6.3B) and 48 months (Figure 6.3C) and all dogs sampled in 2004 (n = 41) were negative.

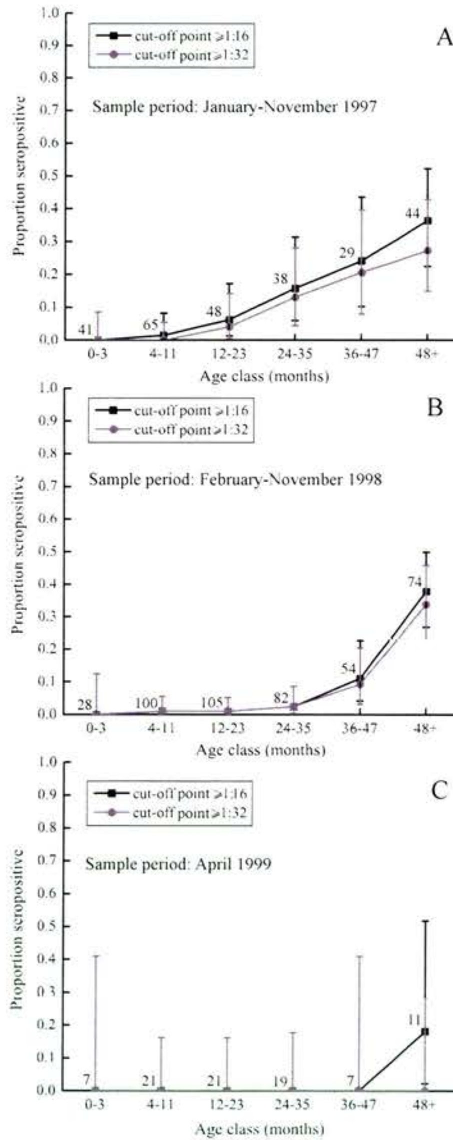


Figure 6.3. Canine distemper virus (CDV) age-seroprevalence curves for Musoma District domestic dogs. Numbers above each point of the curve indicate sample sizes. Bars show exact binomial 95% confidence intervals.

6.4.1.3 Tarime and Bunda District domestic dogs

No age-seroprevalence data were available for earlier years for Tarime and Bunda (Figure 6.1) dog populations. Age-seroprevalence patterns in dogs sampled in 2003 and 2004 were consistent with viral circulation at some time in 2000 and 2001 as seropositive dogs were found only among those animals that were $\geq$ 24 months of age (Figures 6.4 and 6.5).

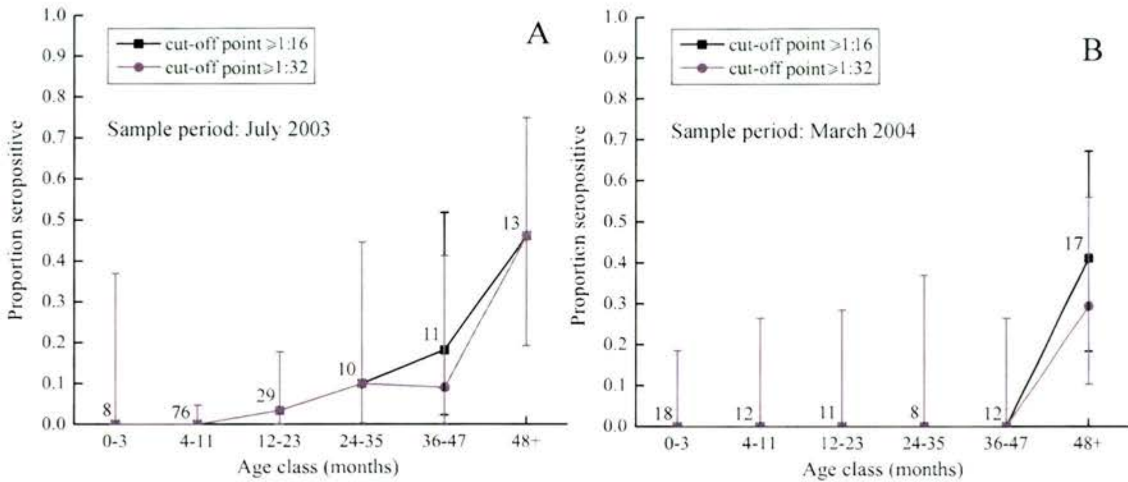


Figure 6.4. Canine distemper virus (CDV) serostatus of Tarime District dogs sampled in 2003 (A) and 2004 (B) plotted according to their age class. Number above each point give sample sizes. Bars show exact binomial 95% confidence intervals.

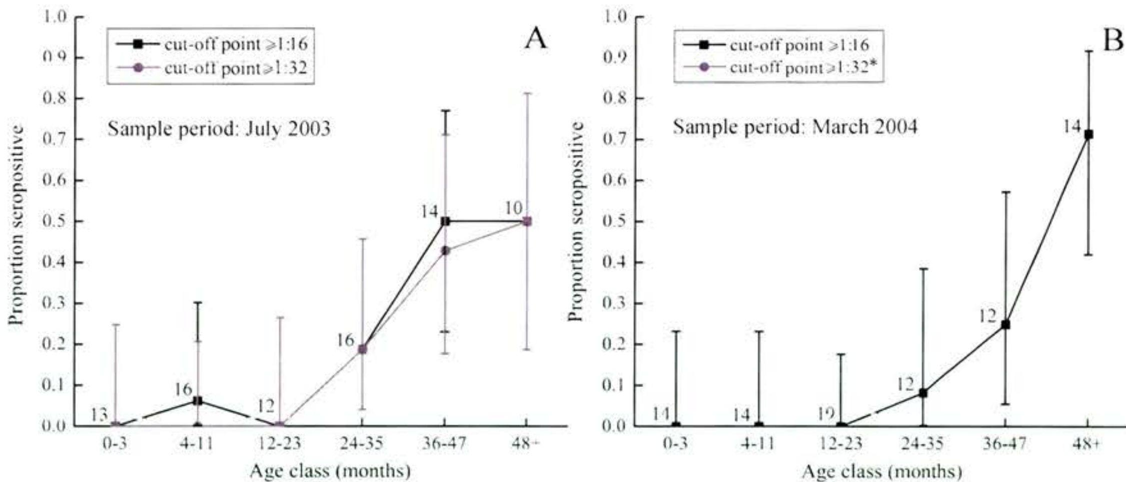


Figure 6.5. Canine distemper virus (CDV) serostatus of Bunda District dogs sampled in 2003 (A) and 2004 (B) plotted against their age class. Numbers above each point give sample sizes. Bars show exact binomial 95% confidence intervals. *No changes in seroprevalence in dogs sampled in 2004 were detected using two different cut-off points (≥1:16 and ≥1:32).

6.4.2 High-density domestic dog populations to the southwest of SNP

In 1997 and 1998, age-seroprevalence data from unvaccinated dog populations to the southwest of SNP (Bariadi and to a lesser extent Magu Districts) were indicative of recent exposure as detectable antibodies to CDV were found in dogs of all age classes (Figure 6.6A). The serological pattern coincided with an episode of morbidity (with lacrimation, anorexia and residual muscle twitching) and mortality reported by dog owners in the same area, although no systematic data were collected (S. Cleaveland, personal communication). When the serological pattern was examined in individual villages, the detection of seropositivity only in dogs ≥ 24 months old (Figure 6.6B) in villages situated to the northeast bled in August 1999 suggested the presence of circulating virus in these areas sometime in 1997. In villages located further south, CDV antibodies were present in dogs of all age groups sampled in late 1998-early 1999 (Figure 6.6C), suggesting later exposure.

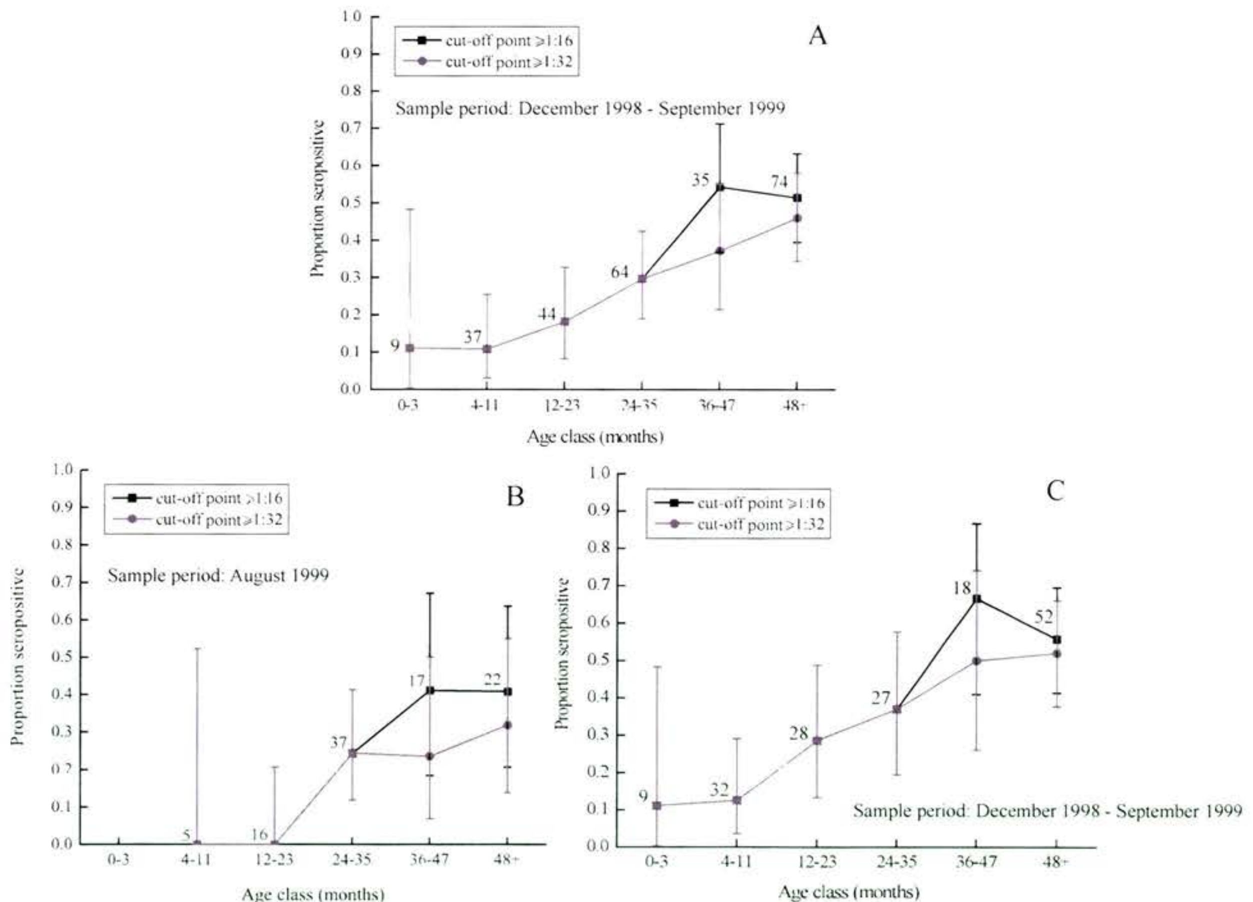


Figure 6.6. (A) Bariadi and Magu District dogs exposed to canine distemper virus (CDV) in late 1997-1998. Dogs sampled in the northest (B) and in villages located further south (C).

There was no evidence of new episodes of infection in dogs sampled further north (Magu District; Figure 6.7A) and in southern villages (Meatu District; Figure 6.7B).

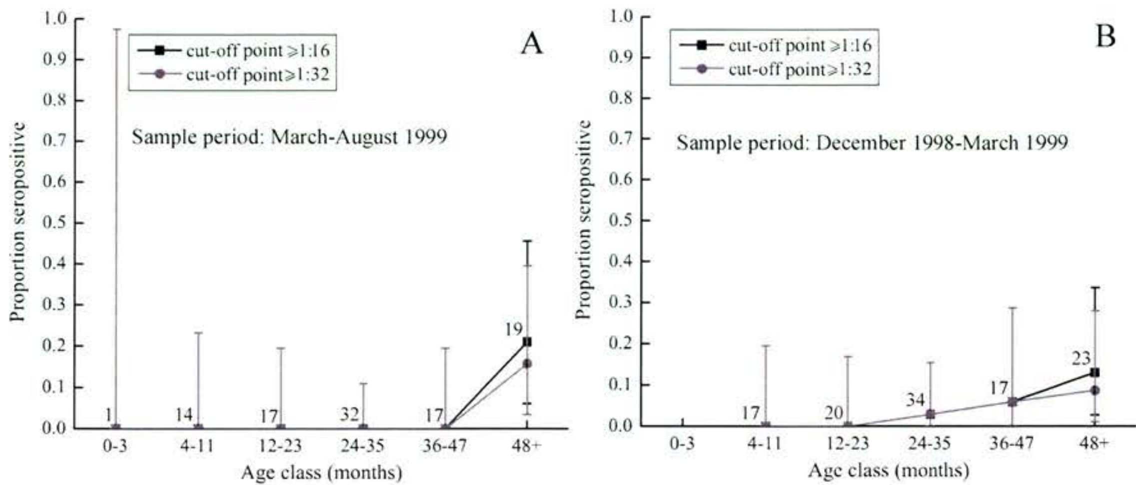


Figure 6.7. Canine distemper virus (CDV) serostatus of Magu (A) and (B) Meatu District dogs sampled in 1998-9.

Cross-sectional age-seroprevalences of dogs sampled in 2003 and 2004 in Meatu District, which are shown in Figure 6.8, indicated exposure sometime in 2000 and 2001 (seropositive dogs were found only among older dogs), with no evidence for more recent episodes.

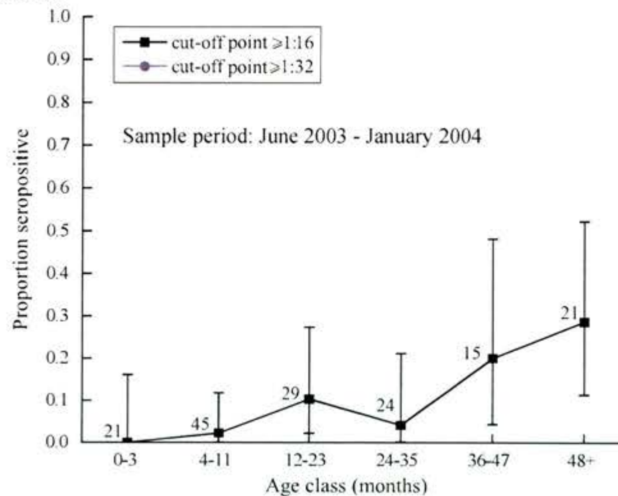


Figure 6.8. Canine distemper virus (CDV) serostatus of Meatu District dogs sampled in 2003-4. *No changes in seroprevalence in dogs sampled in 2004 were detected using two different cut-off points (≥1:16 and ≥1:32).

On the contrary, case-surveillance data and age-seroprevalence patterns in dogs sampled in 2003 and 2004 in northern districts (Bariadi, Figure 6.9A,B and Magu, Figure 6.10) were consistent with CDV being re-introduced into the area. In early 2003 an outbreak of disease amongst dogs was reported by local veterinary officers in Bariadi District and attributed to dogs contacting at a local market occurring in Bariadi town during that month. The following signs were described and observed: ocular and nasal discharge, coughing, vomiting, diarrhoea, emaciation, anorexia, myoclonus, hind-limb ataxia and death. Serological patterns confirmed recent exposure to CDV in some areas, with detectable antibodies to CDV found in dogs of all age classes (Figure 6.9A), but not others within the district where seropositivity was detected only in dogs $\geq$ 24 months (Figure 6.9B). Similarly, there was serological evidence for new infections in some areas within Magu District, with seropositive dogs in all age classes (Figure 6.10), but not others sampled over the same period (n = 19 dogs with no detectable antibodies).

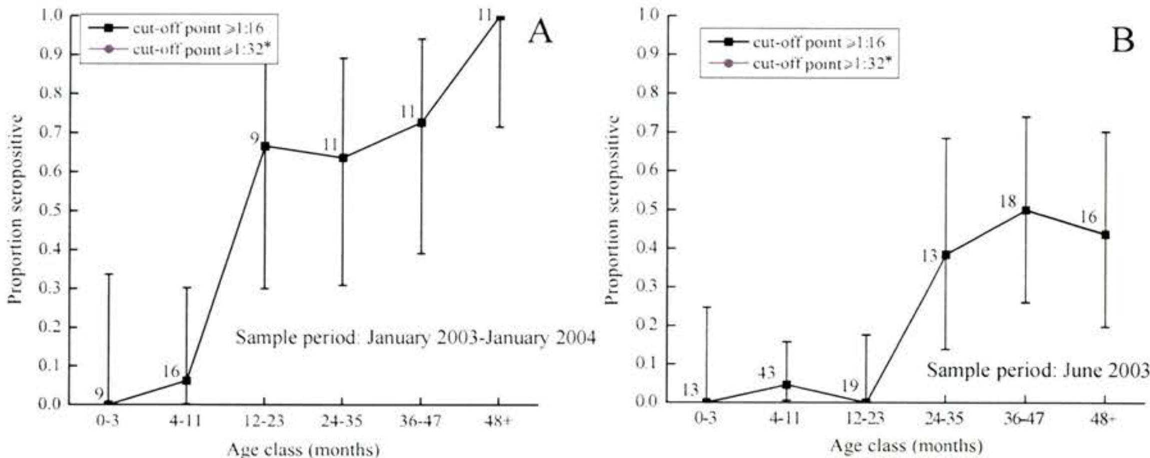


Figure 6.9. (A) Age-seroprevalence curve for Bariadi District dogs from areas of active infection and (B) areas were no recent exposure was detected. *No changes in seroprevalence in dogs sampled in 2004 were detected using two different cut-off points (≥1:16 and ≥1:32).

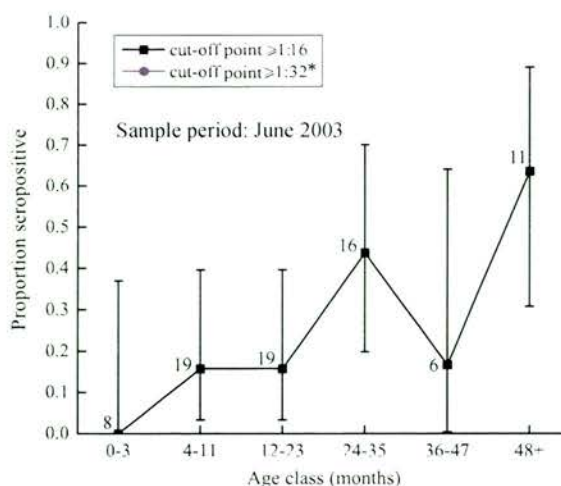


Figure 6.10. Age-seroprevalence curve for Magu District dogs from areas of active infection. *No changes in seroprevalence in dogs sampled in 2004 were detected using two different cut-off points (≥1:16 and ≥1:32).

6.4.3 Low-density domestic dog populations to the east of SNP: Ngorongoro District

CDV cross-sectional age-seroprevalence curves for low-density dog populations of Ngorogoro District (Figure 6.1) are shown in Figure 6.11A-F. No dog vaccination was conducted in this area throughout the study period.

For the period 1992-1994 (seroprevalence curves adapted from Cleaveland *et al.*, 2000 and described for completeness: Figures 6.11A-C), age-seroprevalence patterns were consistent with exposure in 1991 and 1994, but not in 1992 and 1993 as seropositivity was detected in pups in 1994 only, as previously described (Cleaveland *et al.*, 2000).

Cross-sectional age-seroprevalences of dogs sampled from 1997 to 1999 showed that since 1994 CDV had disappeared naturally from these dog populations: seropositive dogs were ≥ 12 months of age in 1997 (Figure 6.11D) and all dogs sampled in 1998 (n = 40) and 1999 (n = 31) were seronegative.

Infection re-emerged in these dog populations in late 2000 as indicated by age-seroprevalences of dogs sampled in late 2001 with seropositivity detected in dogs ≥ 12 months (Figure 6.11E). The ages of dogs with detectable antibodies to CDV sampled in 2004 were suggestive of infection dying out after the 2000 outbreak as seropositive dogs were ≥ 36 months (Figure 6.11F).

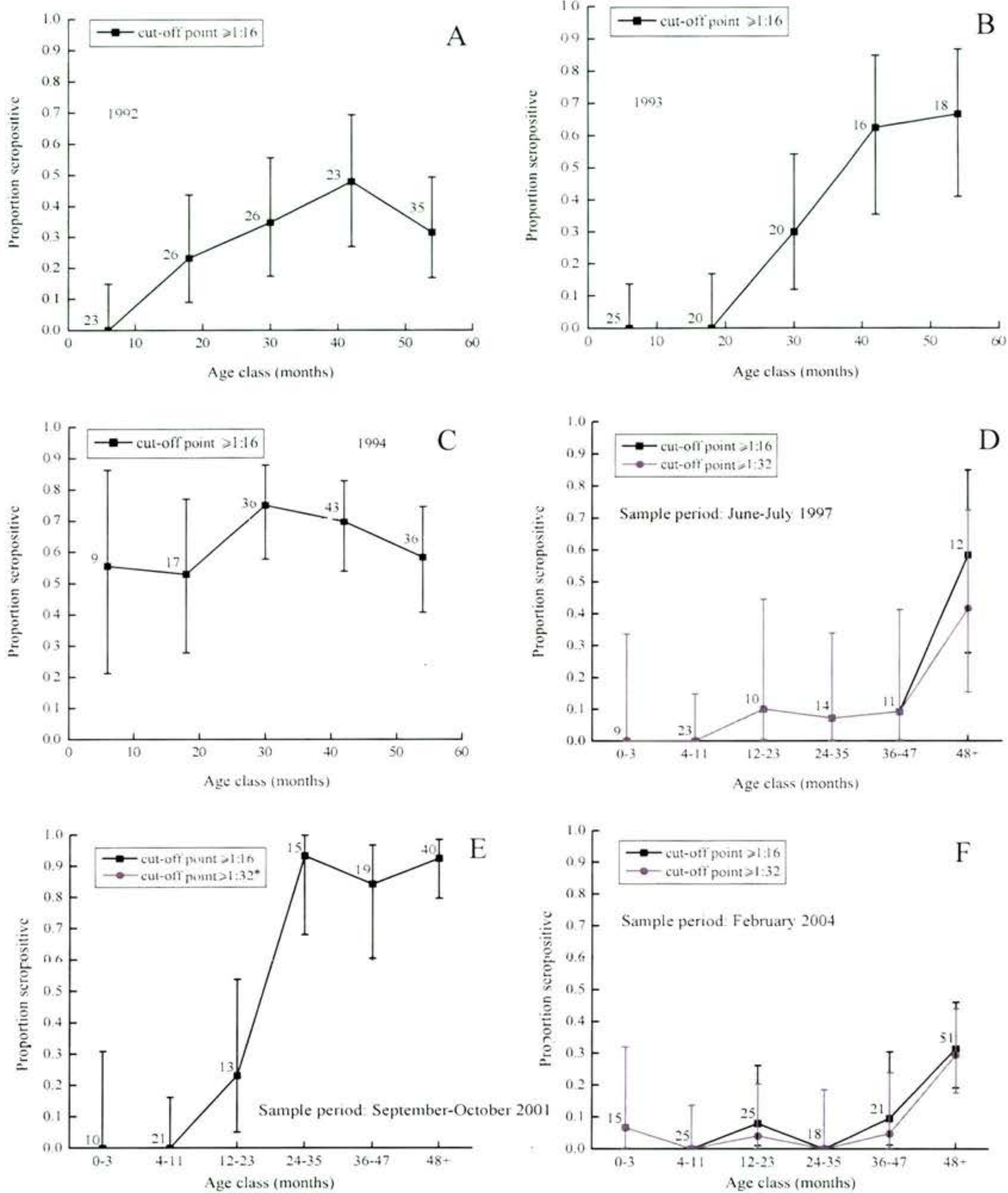


Figure 6.11. Canine distemper virus (CDV) age-seroprevalence curves for Ngorongoro District domestic dogs. Numbers above each point of the curve indicate sample sizes. Bars show exact binomial 95% confidence intervals. Curves for the period 1992-1994 were adapted from Cleaveland *et al.* (2000). *No changes in seroprevalence were detected using two different cut-off points (≥ 1:16 and ≥ 1:32).

6.4.4 Lion populations

6.4.4.1 Serengeti lion population

No lion sampling was carried out in 1996 and of the 7 Serengeti lions sampled in 1997, the only lion that had been born after the 1994 epidemic (in July 1996) had a borderline titre of 1:16 by Intervet neutralisation test (applying a cut-off point of ≥ 1:16). No aliquot was available for testing by Cornell methods. Distemper antibodies were detected in all of the remaining 6 lions by both assays. In 1998, there was serological evidence for exposure in two young lions from the same Pride (Plains) born in 1996 and sampled in 1998 within the western sectors of the lion study area: the youngest seropositive had been born in August 1996 and was sampled in June 1998. The absence of disease-associated morbidity and mortality in these individuals did not allow the exact timing of exposure to be determined. 'Immatures' (≤ 4 years of age: Packer *et al.*, 1999) from other prides sampled on the eastern sectors of the park had no antibodies to CDV. Seroprevalence for Serengeti lions sampled during the period 1997-1998 plotted according to their year of birth is shown in Figure 6.12.

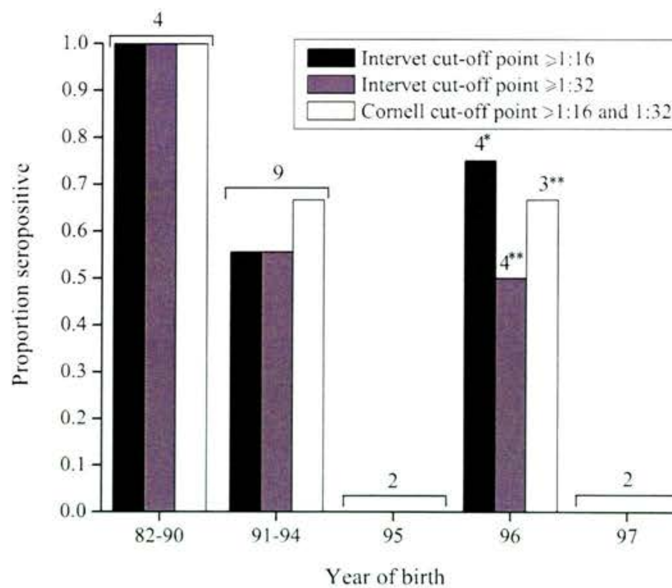


Figure 6.12 Seroprevalence of antibodies to canine distemper virus (CDV) in Serengeti lions sampled in 1997 and 1998 plotted according to their year of birth. The number of individuals sampled is noted above the bars. **Two lions from the Plains pride sampled in 1998 had high titres of antibodies by both the Intervet and Cornell assays (1:861, 1:512 and 1:256, 1:384, respectively). *A borderline lion (applying a cut-off point of ≥ 1:16) sampled in 1997 is included.

The only young Serengeti lion sampled in 1999 (born in 1996) was seronegative.

Data from lions sampled between 1999 and 2004 suggested a further and more widespread episode of exposure after 1998 not associated with obvious morbidity and mortality: antibodies were detected in lions born in 1999 (Figure 6.13). The youngest seropositive animal had been born in August 1999 and sampled in Janury 2002, and a wider range of prides was affected both on the western and eastern sectors of the lion study area. None of the lions that had been born after 1999 had detectable CDV antibodies.

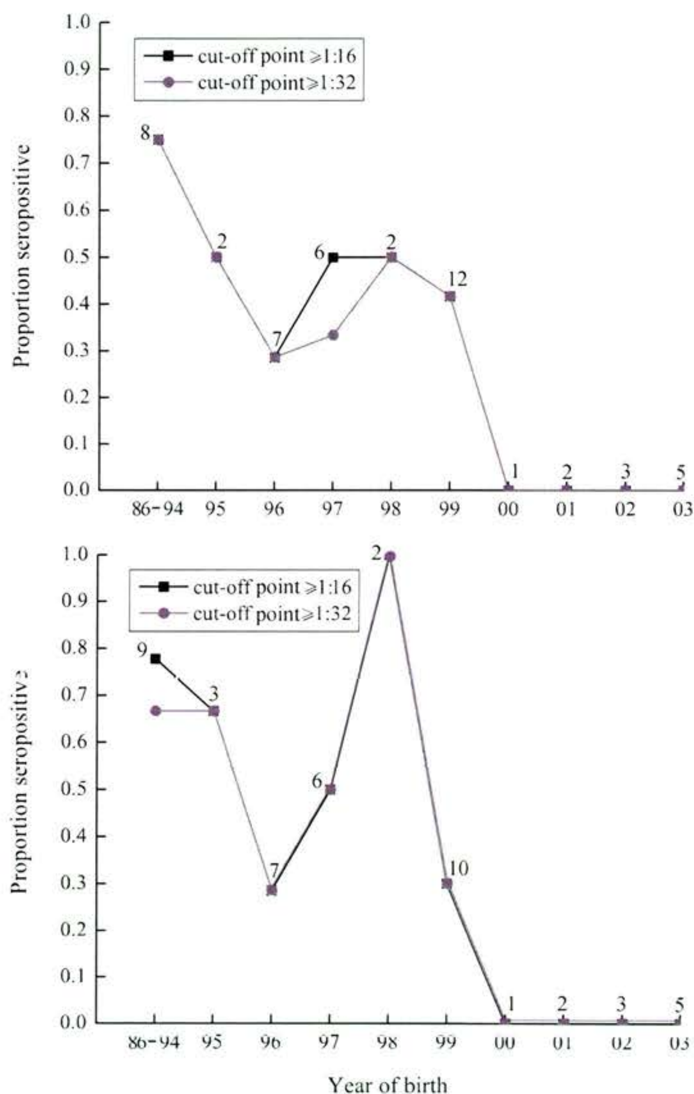


Figure 6.13. Seroprevalence of canine distemper virus (CDV) in Serengeti lions sampled between 1999 and 2004 plotted according to their year of birth obtained by Intervet (top) and Cornell (bottom) assays. The number of individuals sampled is noted above each point. Note that sera from two individuals born in 1993 and 1995 respectively were available for Cornell assay only and sera from two individuals born in 1999 were available for Intervet assay only.

6.4.4.2 Crater lion population

A period of high lion mortality was recorded in early 2001 (Kissui and Packer, 2004). The following signs were reported by park veterinarians and scientists: poor body conditions, lethargy, weakness, skin lesions (e.g. ulceration) and pale mucosae. High levels of tick/fly infestation were observed. Only one carcass was recovered after the main lion die-off (a cub < 5 months of age) and there was no evidence of active CDV infection in that lion (i.e. absence of CDV lesions and negative immunohistochemistry results; L. Munson, personal communication), although CDV antibodies were present in lions sampled during the die-off. With no changes when applying two cut-off points (≥ 1:16 and ≥ 1:32) by both the Intervet and Cornell assays, seropositive lions were detected in all of the three prides and all age groups > 12 months sampled: 1 to 2 years of age (1 of 4 tested by Intervet assay and 1 of 3 tested by Cornell assay), 2 to 3 years (1 of 1 tested by both assays), 4 to 5 years (1 of 1 tested by both assays) and > 6 years (2 of 3 tested by both assays). The two youngest lions sampled (< 5 months of age) had borderline titres (1:16), one by both the Intervet and Cornell assays and the other by the Cornell assay only, the Intervet titre being < 1:16.

6.4.5 Summary and hypotheses for CDV spread

6.4.5.1 1992-1994: the outbreak in Serengeti lions

Earlier age-seroprevalence data indicated that neither the Ngorongoro low-density dog nor the Serengeti lion populations were exposed to infection in the years prior to the 1994 outbreak in Serengeti lions (Roelke-Parker *et al.*, 1996; Packer *et al.*, 1999; Cleaveland *et al.*, 2000). In contrast, the high-density dogs in Serengeti District showed evidence of circulating virus in 1992 and 1993 (Cleaveland *et al.*, 2000), suggesting that they were the source of the 1994 epidemic. The spatial and temporal pattern of cases indicated that CDV subsequently spread throughout the ecosystem in wild carnivores, re-emerging in domestic dogs in Ngorongoro in late 1994 and, based on anecdotal evidence, Shinyanga Region (that includes Bariadi and Meatu Districts) to the southwest of SNP in 1995 (Figure 6.14).

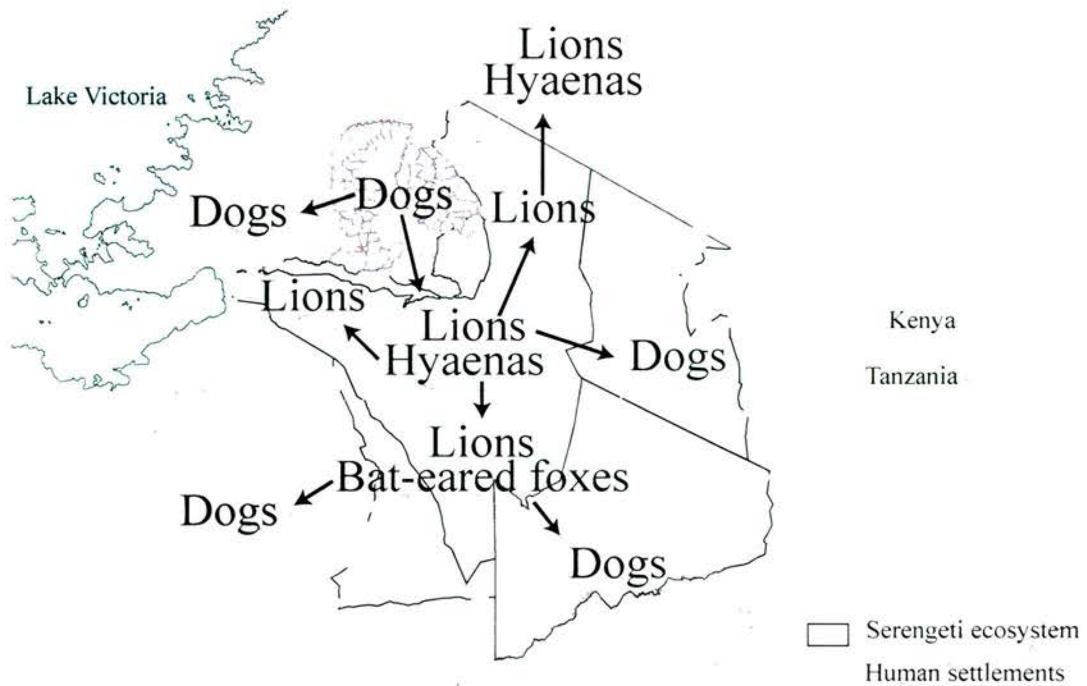


Figure 6.14. Canine distemper virus (CDV) spread during the 1994 outbreak in Serengeti lions as reconstructed using case-surveillance and serological data (Roelke-Parker *et al.*, 1996; Kock *et al.*, 1998; Packer *et al.*, 1999; Cleaveland *et al.*, 2000; Harrison *et al.*, 2004.

6.4.5.2 1995-1997: post-epidemic fadeout

Cross-sectional age-seroprevalences of Serengeti, Musoma and Ngorongoro dogs indicated that since 1994 CDV had disappeared naturally from both high- and low-density dog populations to the northwest and east of the park (sections 6.4.1.1, 6.4.1.2 and 6.4.3).

6.4.5.3 1997-1998: infections in unvaccinated dog populations to the southwest of SNP and new infections in lions

In 1997 and 1998, data from dog populations to the southwest were consistent with CDV being re-introduced into the Shinyanga Region to the southwest of the park (section 6.4.2). CDV exposure in dogs coincided with serological evidence for exposure in lions of a particular pride in the west of the lion study area (Figure 6.15) with no overt disease and mortality observed (section 6.4.4.1). There was no evidence for new exposure of vaccinated dog populations to the northwest and unvaccinated dogs to the northwest and east of SNP (sections 6.4.1.1, 6.4.1.2 and 6.4.3).

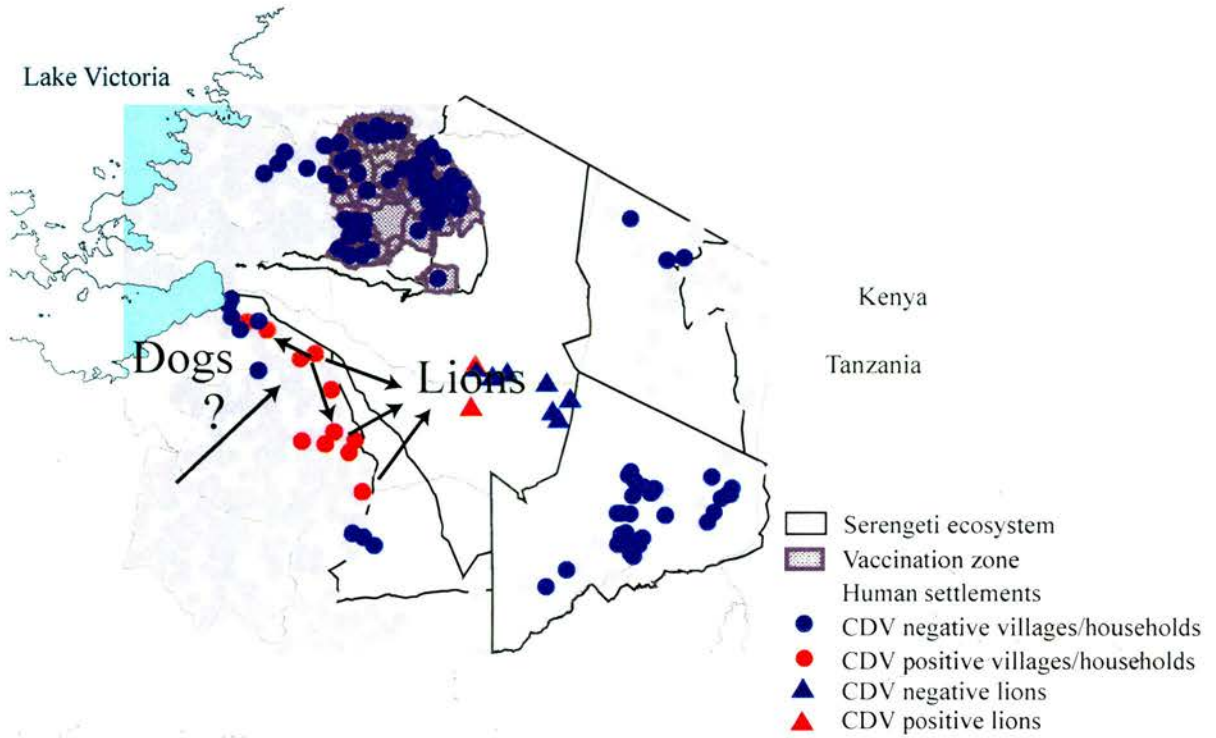


Figure 6.15. Status of canine distemper virus (CDV) in 1997-8 as reconstructed using serological evidence (sections 6.4.1.1, 6.4.1.2, 6.4.2, 6.4.3 and 6.4.4.1) and hypotheses for spread.

6.4.5.4 1999-2001: wide-spread viral circulation in all dog populations and new infections in lions

A much more wide-spread viral circulation was documented in the period 1999-2001 (Figure 6.16). Declines in coverage from 1998 coincided with re-introduction of infection in vaccinated dog populations of Serengeti District (section 6.4.1.1). Age-seroprevalence patterns in unvaccinated high-density dogs sampled in subsequent years (2003 and 2004) were also consistent with circulation sometime in 2000 and 2001 (sections 6.4.1.3 and 6.4.2). Data from Serengeti lions suggested a further episode of exposure affecting a wider range of prides, both on the western and eastern sectors of the lion study area, with no signs of morbidity/mortality (section 6.4.4.1). Infection re-emerged also in low-density dog populations in Maasai areas east of the park in late 2000 (section 6.4.3). The re-establishment of the virus in the Ngorongoro area coincided with a period of high lion mortality (early 2001) with evidence for CDV exposure (section 6.4.4.2). Studies in the Mara, indicated that juvenile Mara hyaenas sampled in 2000 and 2001 also had antibodies to CDV, but no overt clinical disease and mortality were observed (Harrison *et al.*, 2004).

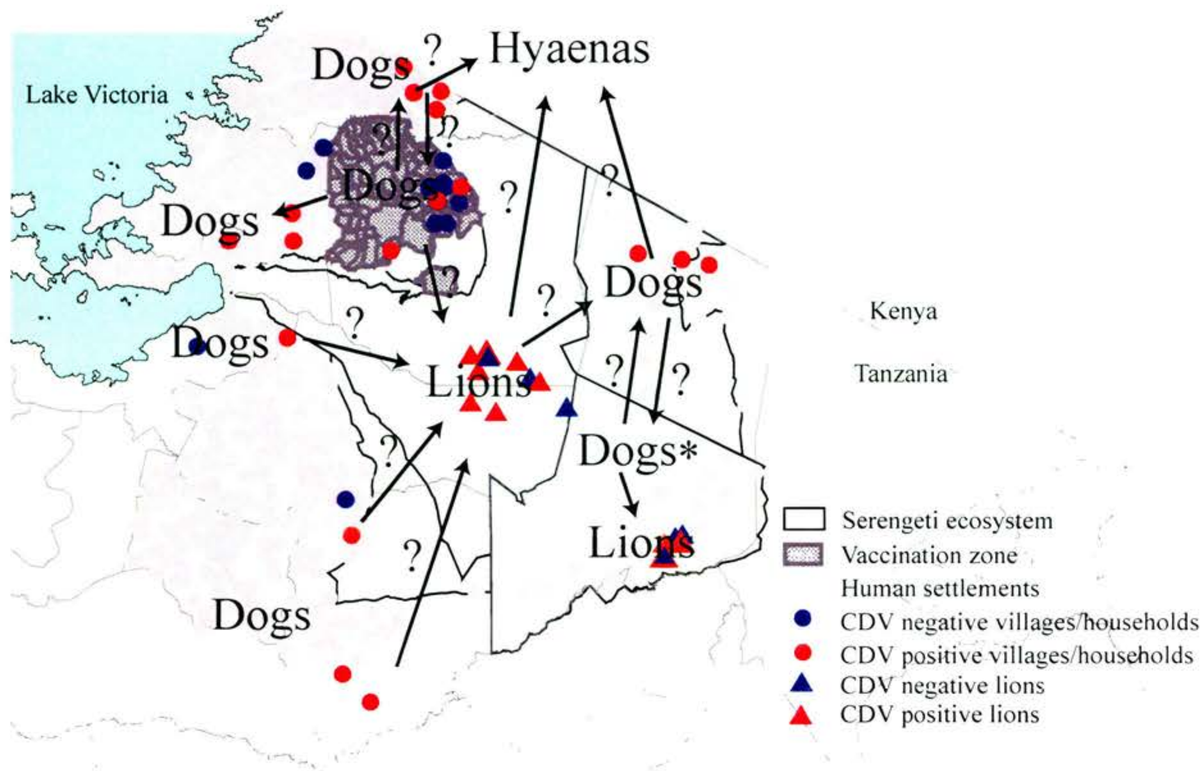


Figure 6.16. Status of canine distemper virus (CDV) in 1999-2001 as reconstructed using serological evidence (sections 6.4.1.1, 6.4.1.3, 6.4.2, 6.4.3, 6.4.4.1, 6.4.4.2 and Harrison *et al.*, 2004) and hypotheses for spread. *No village locations were available for NCA (Ngorongoro Conservation Area) dogs sampled in early 2001.

6.4.5.5 2003-pre-vaccination period: new infections in dog populations to the southwest of SNP with no evidence of new infections in lions

Localised outbreaks were documented to the southwest of SNP (section 6.4.2). Data from other areas adjacent to the park indicated absence of new infections (sections 6.4.1.1, 6.4.1.2, 6.4.1.3, 6.4.2 and 6.4.3). None of the young lions sampled between 2002 and 2004 showed evidence for exposure (section 6.4.4.1) (Figure 6.17).

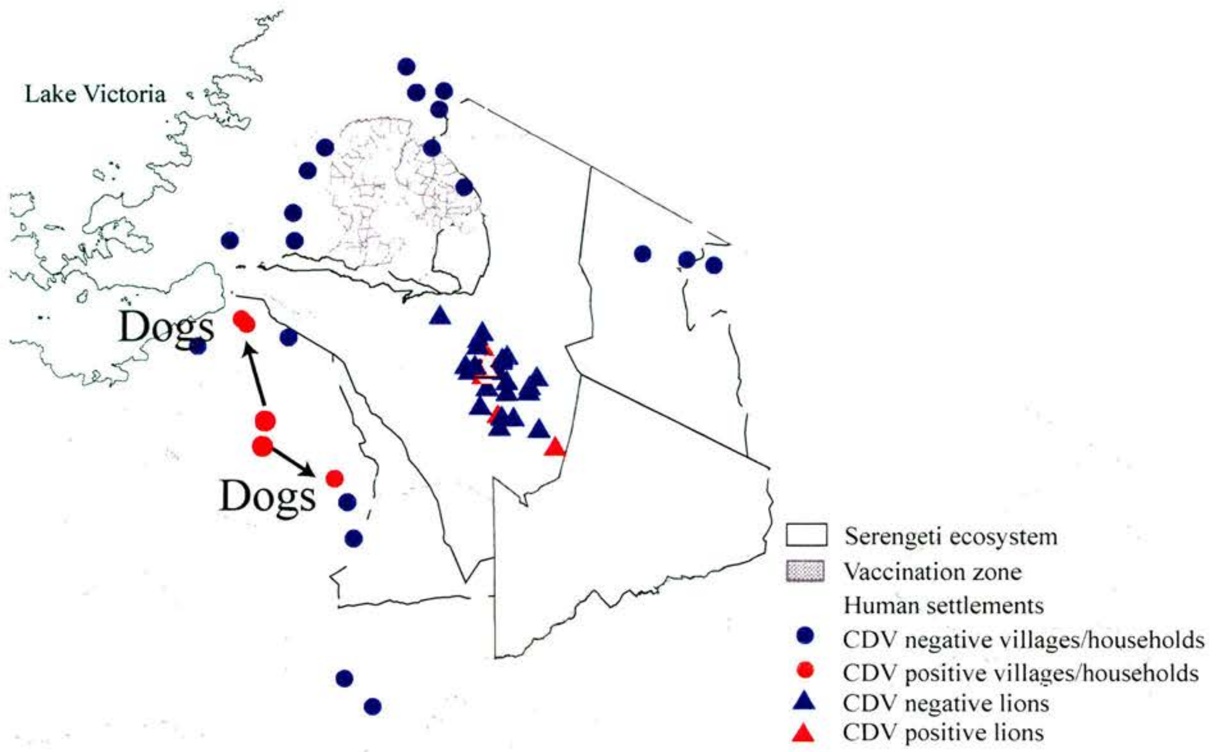


Figure 6.17. Status of canine distemper virus (CDV) in 2003 as reconstructed using serological evidence (sections 6.4.1.1, 6.4.1.2, 6.4.1.3, 6.4.2, 6.4.3 and 6.4.4.1) and hypotheses for spread.

6.5 Discussion

6.5.1 Infection patterns in unvaccinated dog populations: no evidence of persistence at the district level

In this study, there was no evidence for patterns of stable endemicity in any of the district-level dog populations examined, including high- and low-density unvaccinated populations and high-density vaccinated populations.

Previous studies indicated that, between 1992 and 1994, CDV appeared to persist in high-density Serengeti District dog populations (Cleaveland *et al.*, 2000), as age-seroprevalence patterns did not vary across years. Different patterns were however observed post-1994. By 1996 seropositivity was detected only in dogs ≥ 2 years which is in line with the view of disappearance of infection. One possible explanation for viral circulation over three years is that of a prolonged epidemic. CDV epidemics lasting up to two years have been reported in both domestic and wildlife animal populations, for example domestic dogs (Blixenkrone-Møller *et al.*, 1993), raccoons and gray foxes (*Urocyon cinereoargenteus*) (Hoff *et al.*, 1974). However, observations of a prolonged epidemic for a pathogen such as CDV are generally difficult to explain, given the infection characteristics of the virus.

With respect to the Ngorongoro District dogs, the results of this study compare well with previous investigations in that they support the view of a lack of persistence in this lower-density population (Cleaveland *et al.*, 2000). Serological data suggest that CDV infected these dogs in 1991 and 1994, with a decline after 1994. Exposure to the virus was then detected in late 2000, with no evidence of new infections during the periods 1996-1999 and 2001-2003.

The ages of dogs with detectable antibodies to CDV sampled in Musoma District in 1997 (≥ 2 years), 1998 (≥ 3 years) and 1999 (≥ 4 years), Magu and Bariadi Districts in 1999 (≥ 4 years), and Meatu District in 1998-9 (≥ 3 years) also indicate natural disappearance of infection after the outbreak documented in 1994. They also suggest that during the epidemic infection had spread to dog populations to the west of Serengeti District (Musoma District) and give support to the interpretation (based on anecdotal evidence) that domestic dogs in Shinyanga Region had been infected sometime in early 1995 (Cleaveland *et al.*, 2000).

Infection patterns in high-density and low-density dog populations were consistent with re-introductions at variable intervals of time. Data from the Shinyanga region for instance show that the dog population encountered the virus at least four times over the study period, at 2- to 3-year intervals: in early 1995, in 1997-1998, in 2001 and 2003. Similarly after 1994, infection was re-established in Serengeti District dogs in 1999-2000. In late 2000 CDV was also re-introduced in low-density dog populations, with 2- to 6-year inter-epidemic intervals (Cleaveland *et al.*, 2000; this study). An interesting exception to the interpretation of periodic re-introductions is that of Musoma District dogs. Despite being infected by CDV in 1995, no new infections were recorded during the following 9 years, even in periods of wide-spread infection, such as 2000-2001. The observation of recurrent outbreaks with only short-term maintenance followed by clearance of infection well fits theoretical predictions for highly transmissible microparasites (Anderson and May, 1991) and is consistent with data from other African dog populations (e.g. in Kenyan dog populations CDV outbreaks were reported at 10-year intervals: Bwangamoi *et al.*, 1989) and north American raccoon populations (4-year intervals; Hoff *et al.*, 1974; Roscoe, 1993).

Another feature of patterns of CDV infection in domestic dog populations that emerged from this study is that of patchy spatial patterns of infection (e.g. infection was detected in some villages, but not in nearby villages sampled over the same period), although in other periods infection appeared to be more widespread (e.g. in 2000-2001). These patterns raise interesting questions about the contribution of movements of infected dogs to the spread of CDV over more or less wide areas. The number of ownerless dogs within villages is limited (Cleaveland *et al.*, 2003; Kaare, 2006) which suggests their contribution to CDV spread may be relatively insignificant. On the contrary, movements of owned dogs associated with mobilisation of people are probably common. The CDV outbreak limited to some areas within Bariadi District in 2003 was anecdotally associated with dogs accompanying people to the market. Movements may also occur when dogs accompany their owners and livestock to grazing areas, which may be located within the village or may be relatively distant (e.g. during the dry season). Pastoralist Maasai communities are semi-nomadic, and move with their herds and dogs over

long distances during seasonal migrations. Long-distance transportation of dogs may also be due to human resettlement (e.g. in another village, town or district).

6.5.2 Infection patterns in vaccinated dog populations

Due to the absence of infection in dogs at the start of vaccination programmes in Serengeti District, definitive conclusions cannot be drawn on the impact of these trials on these populations. Although high vaccination coverage may have prevented re-introduction of infection from 1997-1999, this may also be explained by a general absence of infection in the region, as there was no evidence for circulating virus in unvaccinated populations.

However, progressive declines in coverage from 1998 coincided with new episodes of infection in 1999, observations of clinical cases in 2000, and widespread infection in 2001. Further analyses of samples collected from subsequent years (2001-2002) will help clarify this interpretation.

6.5.3 Infection patterns in wildlife populations: no evidence of persistence and evidence for dogs as likely source of infection

Earlier age-seroprevalence data from Serengeti lion populations indicated that the population experienced at least two episodes of infection (in the 1980s and in 1994), with no evidence for viral maintenance between them (Packer *et al.*, 1999). Only the 1994 episode was harmful to the population and was attributed to spill-over infection from domestic dog populations to the northwest of SNP (Cleaveland *et al.*, 2000). In the present study, the detection of antibodies in lions that had been born after the 1994 epidemic indicated subsequent re-introduction of infection, on two separate occasions, with no evidence for maintenance between episodes and afterwards, which is consistent with a lack of evidence for persistence. The absence of any overt clinical disease and mortality and the unavailability of serum samples from individuals < 1.5 years of age did not allow establishing the exact timing of exposure. However, temporal and spatial patterns of infection in lions appeared to follow or coincided with those observed in domestic dog populations, with no evidence of circulation of virus in lions without concurrent infection present in dogs. Infection in 1996-8 was documented only in lions in the west of the park, and can be linked with exposure of dogs living on the southwestern boundaries of the park

(Bariadi and Magu Districts), which showed evidence of infection from 1997 onwards.

A much more wide-spread viral circulation was documented in dog populations to the west and east of the park in the period 1999-2001 and there was evidence of a new episode of exposure in Serengeti lions as individuals born in 1999 (after the previous episode) had detectable antibodies. The new episode appeared to affect a wider range of prides both on the western and eastern sectors of the lion study population. Although no animal born later than 1999 had been infected, which may suggest that infection could have entered the lion population in 1999 rather than later, definitive conclusions cannot be drawn due to the small number of sampled lions which had been born in 2000 and 2001. This is an important limitation of the sampling carried out over that period and it is hoped that increasing the sample size by sampling more individuals born in 2000 and 2001 will aid interpretation.

The 2003 outbreak in Bariadi dogs seemed to have been limited geographically and restricted only to dog populations, with no evidence for infection in any of the young lions sampled between 2002 and 2004. Similarly, all lions sampled in 2004 were seronegative. An expanded vaccination programme was initiated in 2003 in a 10 km zone adjacent to SNP (in all of the 6 districts: Tarime, Serengeti, Bunda, Magu, Bariadi and Meatu) with coverage > 80% attained in 2003 (Kaare *et al.*, under review), which might have blocked the spread of infection into the park.

Data from the Crater lion population are much scantier, but there is evidence that this population had also encountered the virus sporadically. An earlier episode of exposure was determined from age-seroprevalence patterns and timed sometime in 1980 with no evidence of new infections until 1991 (Packer *et al.*, 1999). Two population declines were recorded in 1994 and 1997 and thought to have been caused by disease outbreaks (Kissui and Packer, 2004), but no diagnostic data were available for confirmation. The re-introduction of CDV into the Ngorongoro dog population in late 2000 coincided with a period of high lion mortality. Although no high titres were detected in the two lion cubs sampled in 2001, which would have allowed confirming the exact timing of exposure, seropositivity was detected in all age groups and prides sampled.

Serological data from other wild species in the ecosystem are in line with the interpretation of lack of persistence in wildlife. Unstable patterns of infection have been described for the Mara hyaena population with evidence for exposure on at least three occasions, in the early 1980s (1980-1982: Alexander *et al.*, 1995), in 1994-1995 and 2000-2001 (Harrison *et al.*, 2004). On each occasion, seroconversion of Mara hyenas mirrored the seroconversion of Serengeti lions, and on at least two occasions of dogs, although no earlier serological data are available for Tanzanian dogs to draw conclusions on the episode in the 1980s. No distemper antibodies were detected in juvenile hyaenas during the 1997-1998 episode reported in this study, reinforcing the interpretation that this episode was restricted to the southwestern areas of the Serengeti. On the contrary, exposure of hyaenas in 2000-2001 coincided with more widespread infection throughout the ecosystem. Interestingly, Harrison *et al.* (2004) reported that hyaenas from the interior of the MMNR showed a significantly higher level of exposure than hyaenas sampled in close proximity to human habitation (to the north of the MMNR), suggesting that Mara dogs were an unlikely source of infection on that occasion. Our data suggest that infection may have been re-introduced into the Mara from Tanzania.

Analysis of age-seroprevalence data from other Serengeti species, including hyaenas or jackals could provide additional insights into patterns of CDV infection in wildlife populations. It would also indicate whether infection in dogs and lions in 1997-1998 and 1999-2001 was limited to only these two populations or affected other species, for example Serengeti hyaenas, which would help clarify the potential role of these species in disease transmission. Direct dog-to-lion contact is believed to be rare, whereas indirect infection through chains of transmission in other species is considered more likely. Unfortunately, no samples from hyaenas of known age are available for those two periods.

6.5.4 Patterns of morbidity and mortality

Many questions also remain about the factors responsible for the observed variable degree of morbidity and mortality inflicted by CDV on wildlife. Despite the evidence for several episodes of infection, Serengeti and Ngorongoro lions suffered significant mortality only in 1994 and 2001, respectively. Similarly, although hyeana morbidity and mortality due to CDV infection were recorded during the 1994 outbreak,

exposure of Mara hyaenas in 2000-2001 was not associated with mortality (Harrison *et al.*, 2004).

Variable mortality rates from CDV has been reported in other species (e.g. raccoons and island foxes [*Urocyon littoralis*]: Lednicky *et al.*, 2004; Clifford *et al.*, 2006) and attributed to factors such as the genetic resistance of the host, co-infection with other pathogens or strain variation of the virus (Lednicky *et al.*, 2004). Ecological factors have also been suggested (Cleaveland *et al.*, 2000; Kissui and Packer, 2004). For example, the 1994 epidemic in Serengeti lions and the 2001 die-off in the Crater population followed two periods of sever drought, which increased the probability of contact between dog and wildlife (e.g. at water-holes).

The hypothesis of concurrent infections (i.e. tick-borne infections) affecting host responses to CDV is currently being tested in lions and in both Serengeti and Ngorongoro populations high mortality appeared to be associated with high levels of *Babesia* infection (L. Munson, K. Terio and C. Packer, unpublished data). For instance, although piroplasmosis, not CDV infection, was diagnosed as the proximate cause of death in Crater lions in 2001, the level of tissue damage was abnormally high (L. Munson, personal communication), which gave rise to the interpretation that concurrent CDV infection had been responsible for the high mortality rates observed (as CDV is known to be immunosuppressive). Many pathogens have been reported to occur concurrent with distemper in other wild species, for example gray foxes (e.g. *Toxoplasma gondii*, rabies, *Yersinia pseudotuberculosis*, *Cryptosporidium* sp., *Listeria monocytogenes*; Hoff *et al.*, 1974; Davidson et al., 1992; Black *et al.*, 1996; Kelly and Sleeman, 2003) and raccoons (e.g. rabies; Hamir and Rupprecht, 1990), but, to date, little is known about the interactions between these pathogens.

The study should be expanded to include domestic dogs as little is known about patterns of morbidity and mortality in this population. There is evidence that CDV infection in dogs was associated with morbidity and mortality during the 1994 epidemic (Cleaveland, 1996; Cleaveland *et al.*, 2000), in late 1998 in the Shinyanga region (reported), in late 2000 in Serengeti District (reported) and in early 2003 in Bariadi District (reported and observed). But, it is not known whether exposure of

dogs to CDV may occur in absence of any morbidity and mortality and if so whether interacting co-factors may be involved in disease-associated episodes.

A variable degree of virulence of CDV variants has been reported (Lednicky *et al.*, 2004). In the study by Lednicky *et al.* (2004) different mortality rates and severity of lesions were observed in raccoons in two distinct outbreaks, which were found to have been caused by two genetically distant virus lineages. To date, the only CDV isolates available from the Serengeti are those obtained during the 1994 epidemic, which does not render it possible to test this intriguing hypothesis. Enormous efforts are required to obtain CDV isolates in field conditions especially in tropical areas. This is due to the fragility of the virus which results in very limited survival in the environment. Appropriate facilities (i.e. liquid nitrogen for long-term storage and dry shippers for transport of samples from infected areas) are required to preserve viable virus. In the Serengeti such facilities were not available until mid-2003, but they have now been established and it is hoped that this may make it possible to obtain isolates should further disease outbreaks occur in dog and/or wildlife populations.

6.5.5 The value of long-term studies and sampling issues

No long-term serological datasets are available for animal populations within the ecosystem other than for the Serengeti dog and lion populations and Mara hyaenas. For this reason, definitive conclusions cannot be drawn on the role of other wildlife species (e.g. critical species such as hyaenas and jackals) in CDV transmission/maintenance in the ecosystem.

Single cross-sectional surveys have limitations: they only give an indication of whether the population has or has not been exposed to the pathogen and of seroprevalence at a certain point in time. On the contrary, cross-sectional surveys focussing on the same populations sampled regularly (e.g. every year) over prolonged periods of time are valuable (Grenfell and Anderson, 1985; Feliciangeli *et al.*, 2003) and do allow addressing important questions such as that of persistence. Another important benefit of such surveys in African dogs is that CDV infections in these populations are not generally reported and recorded by veterinary and livestock officers. Serological surveys allow detecting episodes of exposure without prior knowledge. In addition, analysis of age-seroprevalence data renders it possible to

time the episodes with relative accuracy. In this study information on the exact age of individuals sampled was available for lion populations, although one limitation is that immobilisation of very young individuals is not allowed in the Serengeti. The lack of data from individuals younger than 1.5 years of age and the absence of morbidity and mortality associated with infection limited the possibility to time exposure in lions accurately. Ageing of dogs relied on the information provided by dog owners. Because of possible misclassification of age, the data will inevitably include both false positives and false negatives. However, patterns of exposure in different dog populations appeared relatively consistent and clear trends emerged.

With respect to sampling strategies for CDV surveillance in African domestic dog populations, this study raises a number of issues. The patchiness of infection indicates that a larger number of villages may need to be sampled in order to be able to detect the appearance and spread of CDV within any given area. However, increased sampling of villages is likely to be offset by smaller sample sizes within villages, given the resources available for serological testing (even with the samples collected in this study not all have yet been analysed). Further exploration of an optimum sampling strategy is clearly warranted.

6.5.6 Conclusions: hypotheses for CDV reservoirs in the Serengeti

The question of reservoirs of CDV in the Serengeti remains unresolved. By definition reservoirs are capable of permanent maintenance (Haydon *et al.*, 2002a). Based on the apparently more stable patterns of infection observed in high-density Serengeti dog populations over the period 1992-1994, it had been suggested that this population was the likely reservoir (Cleaveland, 1996). The present study however demonstrated subsequent natural disappearance of infection in the Serengeti population and in general did not provide evidence for long-term persistence in any of the other populations examined. This suggests that no district-level population appears capable of independent maintenance in the ecosystem. However, the 'Serengeti ecosystem metapopulation' may constitute a maintenance community, hence be the reservoir of infection (Figure 6.18A). Alternatively, infection may need to be re-introduced from outside sources (e.g. the 'Lake zone metapopulation'), which may constitute a maintenance community, possibly comprising a network of populations (Figure 6.18B). If the 'Serengeti ecosystem metapopulation' did

constitute a maintenance community, it would not be possible to address the question as to whether the maintenance community would persist without the contribution of dogs as a nonmaintenance component while CDV still circulates in unvaccinated dog populations and transmission from dogs to wildlife remains a possibility. The expanded vaccination programme currently implemented is hoped to provide definitive insights into the role played by dog populations in disease transmission and maintenance within a potentially complex reservoir system comprising an assemblage of metapopulations. However, it is important to ask whether in the Serengeti CD should be controlled through dog vaccination. Although it is still clear that dogs are an important source of CDV infection for wildlife, in these populations there is currently no evidence for any substantial impact in terms of morbidity and mortality in the absence of interacting co-factors. At the district level, disease outbreaks occur sporadically in dog populations and appear to die out naturally without intervention. However, vaccination may be important to prevent the periodic re-introduction of new infections to wildlife. This may be of critical importance for threatened populations, such as the African wild dog (*Lycaon pictus*), which have become re-established in the eastern Serengeti plains and which are highly vulnerable to the potentially devastating impacts of CDV introduction (van de Bildt *et al.*, 2002). Given the high costs of CDV vaccines (relative to rabies vaccines), further examination of cost-effectiveness is needed to determine the value of mass CDV vaccination of dogs for the conservation management of wildlife in the Serengeti.

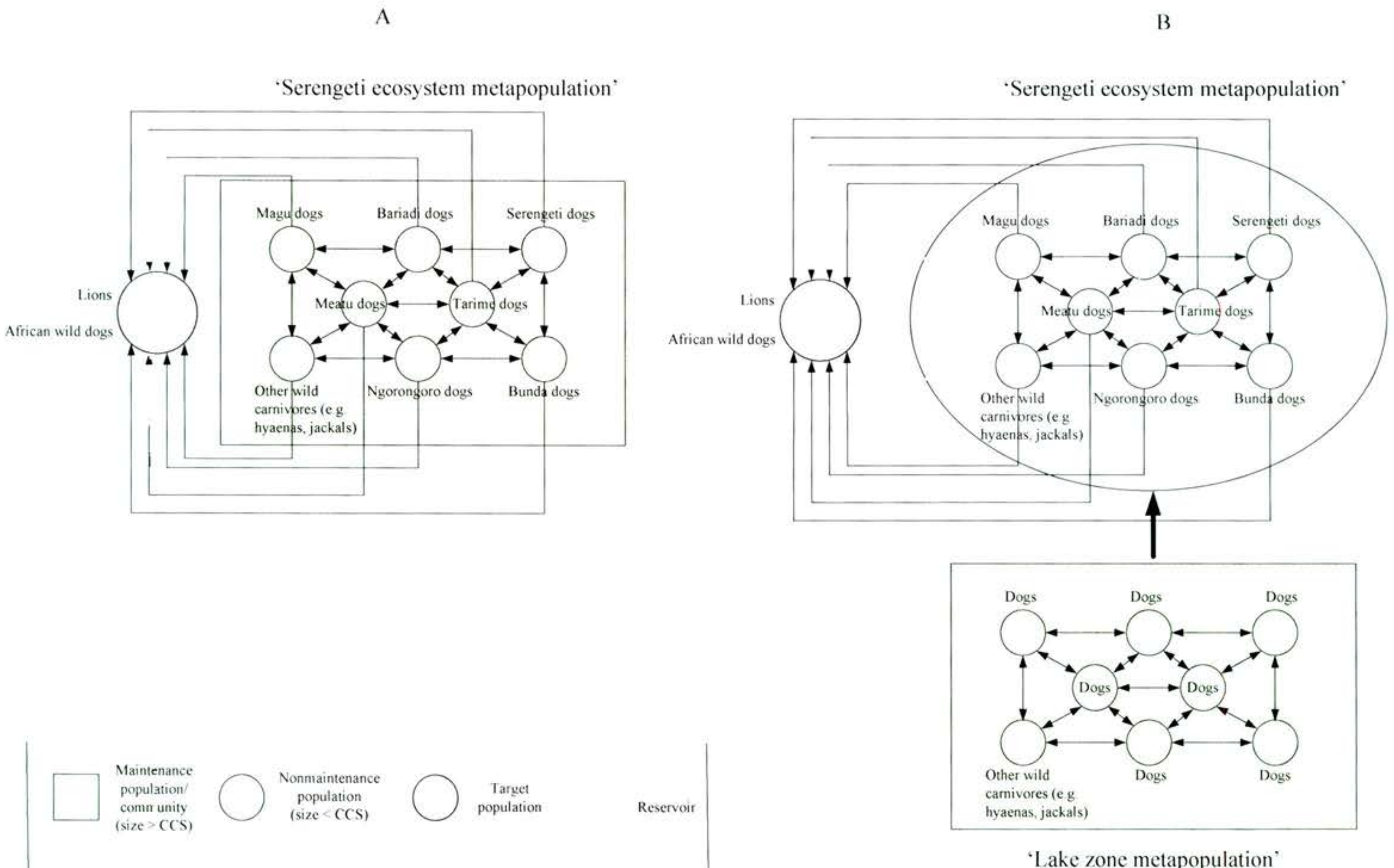


Figure 6.18. Proposed canine distemper reservoir systems in the Serengeti. CCS = critical community size.

CHAPTER 7: GENERAL DISCUSSION

This study explored the infection dynamics of two viral diseases, rabies and canine distemper (CD), that pose a substantial health risk to human and animal populations in the Serengeti ecosystem. The ultimate objective was to achieve a better understanding of their epidemiology that would aid the development of appropriate control strategies to minimise their threats. Chapter 3 investigated a novel direct rapid immunohistochemical test (dRIT) as a field test for rabies surveillance. Chapter 4 examined the genetic characteristics of rabies viruses (RABVs) recovered from a range of species and explored the power of genetic data to establish patterns of disease transmission within and between host populations. Chapter 5 focused on the question of rabies reservoirs in the ecosystem by assessing the evidence for maintenance within domestic dog and wild carnivore populations. Chapter 6 presented data on spatio-temporal changes in the exposure of domestic dog and lion (*Panthera leo*) populations to CD. In the present chapter, the overall results are discussed in relation to the surveillance and control of rabies and CD in the Serengeti ecosystem and areas of sub-Saharan Africa where both diseases pose a threat to human health and wildlife conservation. Future lines of research are also proposed.

7.1 Disease surveillance

Disease surveillance is an essential pre-requisite for evaluating epidemiological trends in human and animal populations, determining the burden of disease, enabling informed policy decision regarding management of disease threats (treatment, control and prevention), and assessing the impact of control programmes (e.g. Leendertz *et al.*, 2006).

7.1.1 Rabies surveillance

The enduring problem of rabies surveillance throughout much of the developing world has constrained all of the above with the consequence that the disease still remains widely under-reported and uncontrolled.

Practical difficulties in rabies surveillance encountered in previous epidemiological investigations in the Serengeti ecological region (Cleaveland and Dye, 1995; Cleaveland, 1996; Cleaveland *et al.*, 2003) prompted a study of the use of a novel diagnostic technique, the dRIT, developed by the Rabies Section of the Centers for Disease Control and Prevention (CDC), Atlanta. This is the first time such a method

has been adopted in African settings and on field brain material preserved in glycerol saline, the preservative of choice for samples stored under field conditions (Barrat and Blancou, 1988; Barrat, 1996). In Chapter 3, a preliminary evaluation on frozen and glycerol-preserved brains showed that the dRIT had a sensitivity and specificity equivalent to those of the global standard rabies diagnostic technique, the direct fluorescent antibody (DFA) test, demonstrating its potential value as an epidemiological tool in countries with a lack of capacity for rabies surveillance, as discussed in section 3.5.

Although further evaluation of the test (both in laboratory and field conditions) is necessary, this is the first assay suitable for laboratories with reduced diagnostic infrastructures that has shown characteristics equivalent to those of the DFA test (see also section 1.2.6.2 for a review). This indicates that the dRIT could be considered as a possible replacement for the DFA test in these laboratories.

Over the past years, many rabies laboratories in developing nations, especially in Africa, have expressed interest in the technique. One way forward would be to provide technical support and training to carry out dRIT rabies diagnosis at central reference laboratories (where light microscopes are widely available) using the DFA test for quality assurance (where the test is routinely performed) with further confirmatory tests at the Rabies Section of the CDC.

In the Serengeti ecological region future studies will exploit the infrastructure and collaborations developed during the course of this project and as a result of previous investigations in the region to establish local capacity for rabies diagnosis using the dRIT. In the area rabies diagnostic facilities are currently available only at a single facility, the Veterinary Investigation Centre in the city of Mwanza, located approximately 400 km to the southwest of the Serengeti National Park (SNP), although the fluorescence microscope is not routinely functioning and the conjugate not regularly available. Laboratory technicians have been provided with some preliminary training to perform the dRIT, but additional technical support, monitoring and validation of the results obtained will be critical. In terms of wildlife surveillance, it is aimed to establish technical capacity at the Tanzania Wildlife Research Institute (TAWIRI) veterinary laboratory of the SNP. Depending on

fund/time availability and government commitments to enhancing veterinary public health capacity for rabies control, surveillance and diagnosis, a wider establishment of the dRIT in Tanzania would be the ultimate goal.

A lack of capacity for rabies diagnosis is however not the only constraint to effective rabies surveillance. In Tanzania, as in much of Africa, diagnostic material is not routinely obtained by veterinary and livestock officers as a result of difficulties associated with the collection, storage and transportation of samples (especially in remote areas). To overcome these problems, in previous investigations (Cleaveland *et al.*, 2003) and in this study community-based active surveillance measures were implemented with training on simple sample collection and preservation techniques and resources (collection kits and financial incentives for reports of rabies-suspect cases and collection and transport of samples) provided. Although in both studies active surveillance proved a valuable aid to obtain information on cases and samples for rabies diagnosis, and increase detection rates, these measures were costly and required intensive supervision, which raises doubts about their sustainability.

As part of the active surveillance measures, contact tracing implemented and coordinated by Katie Hampson, Princeton University, proved highly effective in detecting rabies in domestic and wild animal populations and allowed more accurate evaluation of epidemiological trends. Although potential benefits could accrue from implementing these measures in countries with limited capacity for rabies surveillance, these measures were operationally difficult and consuming in time, labour and capital and are thus unlikely to be of use unless implemented in conjunction with specific research objectives.

7.1.2 Canine distemper surveillance

Serological approaches have been used as a key method to assess exposure of wildlife populations to CDV (see Table 1.4), although most populations have been studied via single cross-sectional serological surveys. As highlighted in Chapter 6 (section 6.5.5) long-term serological data for Serengeti dog and lion populations provided a unique opportunity to evaluate the spatial and temporal dynamics of CDV in the ecosystem. In African dog populations, while it is clear that these surveys are a valuable and cost-effective (especially if carried out in conjunction with vaccination

campaigns) tool for disease surveillance and determining spatial and temporal trends (Cleaveland *et al.*, 2006b), there is a clear need for optimising strategies taking into account availability of resources for sample collection and testing. A number of limitations in this study constrained the interpretation of patterns of infection in some populations and highlighted the importance of exploring an optimum strategy for future studies. One limitation was the interval between sampling, serological testing and data analysis, which determined the late detection of some critical trends. Earlier detection would have aided decision-making concerning subsequent sampling and testing strategies. For instance, analyses of Serengeti District dog samples collected during 1999-2002 and sampling of further lions born in 2000 and 2001 would have provided a clearer picture of patterns of infection in both populations over that period. The patchy spatial patterns of CDV infection in dogs from the study area, which are likely to be encountered in other African dog populations, raise the question as to whether increased sampling of villages rather than dogs/village would be required. Resolving these issues would be advantageous for future research in the Serengeti and other African settings and one approach could be the application of analytical methods (e.g. Bayesian statistics) to explore the implications of incomplete sampling and incomplete knowledge of a contact network structure in interpretation of patterns of infection.

7.2 Identifying reservoirs of infection: relevance to rabies and canine distemper control

A key issue in the epidemiology of infectious diseases is the difficulty in identifying reservoirs of infection. Although identification of reservoirs can aid disease management, it presents considerable difficulties, especially in multi-host systems such as the Serengeti. Identifying reservoirs or components of the reservoir essential for the permanent maintenance of the pathogen will ensure effective control and ultimately elimination of infection. When reservoirs cannot be definitely identified, control efforts can be directed at protecting populations of concern, in which case identifying source populations is critical.

An exhaustive identification of all or essential components of pathogen reservoirs may be possible only through detailed/long-term investigations and using a range of 'practical indicators', particularly in complex disease systems. Critical issues such as

that of persistence of infection in the reservoir can only be determined through continuous studies of the same population/s. In this study, it would not have been possible to tackle the question of rabies and CD reservoirs without drawing on the long-term surveillance data available from the Serengeti carnivore community, although definitive conclusions on the reservoir of CD could not be reached. Availability of baseline information and of resources (in terms of time and capital) should be rigorously considered when embarking on investigations of this kind.

In Chapters 4 and 5 several approaches were employed to address the question of rabies reservoirs in the Serengeti. Epidemiological data and data on the genetic characteristics of the virus supported the view that domestic dogs (occurring at high-densities) were the only maintenance population of the rabies reservoir in the ecosystem, with wild carnivore populations contributing to the reservoir as nonessential components. Genetic characterisation of RABVs from a range of populations proved a powerful tool for identifying reservoirs. The statistical parsimony approach described in Chapter 4 was particularly valuable in elucidating patterns of intra- and inter-specific transmission and uncovering transmission pathways. This methodology could prove useful in addressing similar questions in other disease systems and should be explored further. The possibility of applying this method to the analysis of CDV genetic data is limited by the lack of isolates (except the 1994 ones, as highlighted in Chapter 6, section 6.5.4). During the course of this study, more rigorous surveillance measures and appropriate facilities (i.e. liquid nitrogen cylinders) were established in SNP and they are still in place, which may render it possible to obtain further isolates, provided that disease outbreaks are detected timely. As discussed in Chapter 6, the availability of CDV isolates would also render it possible to test the hypothesis as to whether strain variation of the virus may be responsible for the variable degree of morbidity and mortality observed in lions.

Results of this study indicate that to protect human populations (the target population) rabies control programmes in the Serengeti should target dog populations. Large-scale mass dog vaccination trials encompassing all agropastoral villages within a 10 km zone bordering the western boundaries of the SNP are currently underway in the Serengeti region and may provide definitive evidence for

the level of vaccination coverage needed. Although in earlier studies coverage of 65-70% was effective in controlling dog rabies (Cleaveland *et al.*, 2003), questions remain as to the role of alternative hosts in long-term control efforts (e.g. reintroduction of infection in inter-campaign intervals), which should be the subject of future research. A critical question is also that of sustainability of dog vaccination programmes in terms of long-term strategies for funding and human resources, which will be crucial to effective control and ultimately rabies elimination. An integrated approach involving public health, veterinary, wildlife conservation and animal welfare agencies is seen as the way forward and the strong infrastructure already in place in the region raises hope of the feasibility of this approach.

In Chapter 5, it is shown that dog populations in pastoralist regions to the east of SNP, previously considered too small to sustain infection (< 1/km^2: Cleaveland and Dye, 1995), have grown considerably (< 5/km^2) and they are now approaching the threshold for persistence. Besides the implications for public health, with the re-establishment of two new packs of African wild dogs (*Lycaon pictus*) in the eastern Serengeti plains, in grazing areas utilised by Maasai and Sonjo communities, Maasai dogs have become an immediate threat for the survival of this critical population. Dog vaccination campaigns have therefore been extended to cover all villages in Ngorongoro District and vaccination strategies appropriate for pastoralist communities explored (Kaare *et al.*, under review). Addressing the question as to whether wild carnivore populations may act as a source of re-infection for dog populations, complicating predictions of vaccination coverage levels necessary to eliminate infection, would be even more crucial in these areas where wild carnivore populations are more abundant (K. Hampson, unpublished data).

This study provided no evidence to support the hypothesis that CD persists in any of the district-level domestic dog populations living in proximity to the protected areas, but the results suggest that dogs may still be considered the principal source of infection for wildlife, raising concerns about the vulnerability of critical populations such as African wild dogs. Furthermore, although there is evidence that CDV infection is not invariably pathogenic in lion populations, mortality rates can be substantial in the presence of interacting co-factors. Although efforts at controlling infection in dogs through mass vaccination may be important in preventing the

spread of infection into the protected areas, there are concerns about their cost-effectiveness, hence sustainability. Evaluation of this approach to disease management in Serengeti wildlife conservation is clearly warranted. Data obtained from mass dog vaccination programmes currently implemented in the region will be of value to address this issue. Alternative approaches should also be considered, such as localised vaccination of dogs in areas of concern (e.g. Ngorongoro District) that could be undertaken by local authorities. Previous studies identified dog rabies vaccination strategies likely to be effective in pastoralist communities (i.e. combined approaches using central-point and either house–to-house vaccination or trained community-based animal health workers that yielded coverage > 70%: Kaare *et al.*, under review; see also Coleman, 1999), which however proved costly (> US$6 and 4/dog, respectively). Given the high costs of CDV vaccines, relative to rabies, there remains an important question of cost-effectiveness of such an approach also in these areas, which needs to be examined. Finally, although there has been much debate about the benefits of direct vaccination of threatened carnivores, in recent years profound progress has been made on the development of safe and efficacious vaccines (i.e. canary-pox vectored CDV vaccine). Such vaccines have shown to be safe for use in other carnivores (e.g. the endangered island fox [*Urocyon littoralis*]: Timm *et al.*, 2000) and may be considered as an alternative disease management strategy also for threatened African canids. For instance, mathematical models suggest that vaccination of a 'core' (i.e. 30-40%) of individuals within African wild dog populations against rabies, using vaccines that provide two years of immunity, would be sufficient to ensure persistence of small populations and even lower coverage levels may be necessary in larger populations (Vial *et al.*, 2006). A policy of core vaccination strategies against CD could also be a feasible and more cost-effective disease management strategy.

This study did not allow drawing definitive conclusions on CDV reservoirs in the Serengeti. Many questions remain about mechanisms of CDV persistence and patterns of infection in the 'Serengeti ecosystem metapopulation' and other populations that were not examined in this study. If the hypothesis that other populations (e.g. the 'Lake zone metapopulation') may constitute a maintenance community and be responsible for re-introduction of infection into the 'Serengeti

ecosystem metapopulation' is correct, one approach could be to explore patterns of infection in these populations. Although it is unlikely that other wild carnivore species (e.g. hyaenas and jackals) may be capable of independent maintenance in the ecosystem, monitoring of these populations may also provide some insights into their role in the transmission dynamics of CDV. However, to obtain definitive evidence for reservoirs of CDV in the Serengeti theoretical studies may be the only way forward and in general may be relevant for a better understanding of mechanisms of maintenance and transmission of *Morbillivirus* infections in animal populations.

APPENDIX

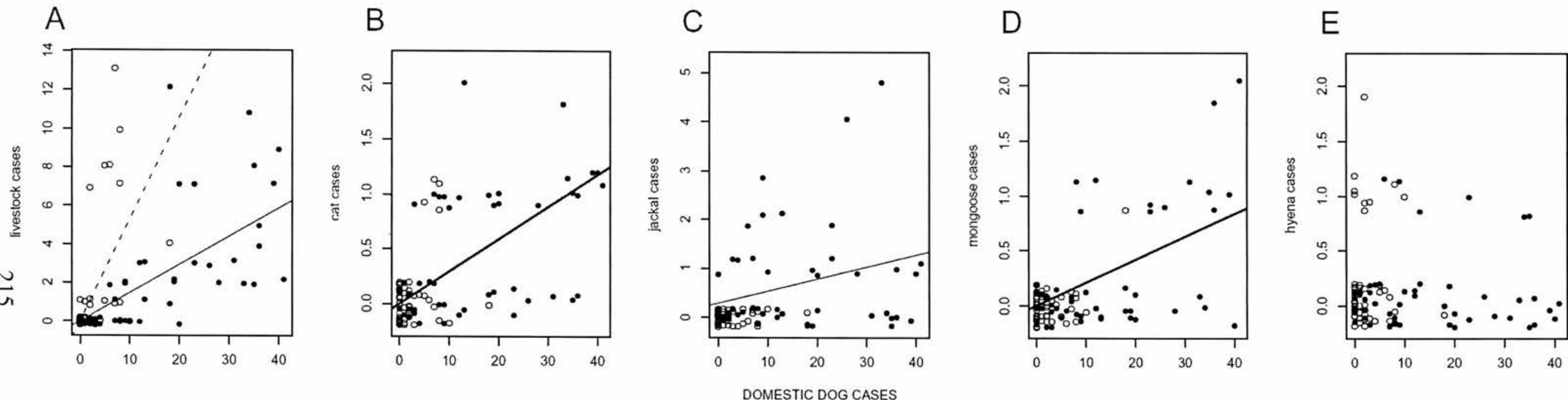
This appendix describes and presents the results of the analysis conducted by Katie Hampson, Princeton University, discussed in Chapter 5, section 5.5.

Statistical analysis

Temporal and spatial patterns in rabies incidence were examined using high resolution data from contact tracing (see section 5.3.3.2). Given sufficient spillover, rabies incidence in reservoir populations should *a priori* predict incidence in non-maintenance populations with a time lag corresponding to the incubation period. Poisson regression models were fitted across a range of time-lags using maximum likelihood to determine whether cases in domestic dogs (*Canis familiaris*) predict cases in other species or vice versa. Models were fitted that constrained the intercept to zero and that simultaneously fitted the intercept and slope. Akaike Information Criteria (AIC) were compared (truncating datasets when comparing across lags) and confidence intervals calculated to evaluate the significance of fitted coefficients to determine the most appropriate model.

Results

The best fitting models for dog rabies cases predicting cases in other species in each district are presented in the figure below. Rabies cases in dogs were significant predictors of cases in livestock in both districts (A). This relationship was most significant and best met model assumptions in Serengeti District (SD) with a one month time-lag. While the relationship was also significant with a one month time-lag in Ngorongoro District (ND), the best model had no lag. More rabies cases in livestock were predicted per dog case in ND. Domestic cat (*Felis catus*) and white-tailed mongoose (*Ichneumia albicauda*) cases were each predicted by dog cases in both districts, but because there were no significant differences between the species specific relationships in either district the data were pooled. The best model for cat cases predicted by dog cases had a lag of one month, whereas a model with no time lag proved slightly better for white-tailed mongoose cases (B and D). Jackal (not identified to species) cases in SD (C) were best predicted by dog cases with no time-lags. There were no jackal cases in ND. Hyaena (*Crocuta crocuta*) cases were not predicted by domestic dog cases in either district (E).



Relationships between rabies cases in domestic dogs (*Canis familiaris*) and cases in other species: (A) livestock, (B) domestic cats (*Felis catus*), (C) jackals (not identified to species), (D) white-tailed mongooses (*Ichneumia albicauda*), and (E) spotted hyaenas (*Crocuta crocuta*). Filled cases correspond to cases in Serengeti District (SD) and open circles are those in Ngorongoro District (ND). The best time-lagged model as determined from Akaike Information Criterion values is presented, showing rabies cases in dogs plotted against rabies cases in other species according to the appropriate lag. Significant relationships are drawn as solid thin lines (SD), dashed lines (ND), or solid thick lines (combined data for SD and ND). Random jitter (0.02) is added to y coordinates for clearer visibility of overlapping points. Higher incidence of livestock rabies predicted per domestic dog case in ND versus SD is presumably due to the higher ratio of cattle per head in the district. Source: Katie Hampson.

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Evaluation of a Direct, Rapid Immuno-histochemical Test for Rabies Diagnosis

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A direct rapid immunohistochemical test (dRIT) was evaluated under field and laboratory conditions to detect rabies virus antigen in frozen and glycerol-preserved field brain samples from northwestern Tanzania. Compared to the direct fluorescent antibody test, the traditional standard in rabies diagnosis, the dRIT was 100% sensitive and specific.

In much of the developing world, rabies surveillance and diagnosis in domestic and wild animals are severely constrained. High ambient temperatures hinder the collection and preservation of fresh specimens. The use of the direct fluorescent-antibody assay (DFA), the traditional standard in rabies diagnosis (*1,2*), is limited by the costs of acquiring and maintaining a fluorescent microscope. Difficulties in obtaining diagnostic results from field material have led to widespread underreporting of disease.

Consequently, the true public health impact of rabies has been greatly underestimated (*3–5*), and political commitment for its control has been lacking. Moreover, the absence of a confirmatory test can result in the inappropriate management of animal bite injuries, with human deaths a potential consequence of delays in rabies postexposure prophylaxis (PEP) and unnecessary administration of PEP. The latter is a particular concern, given the scarcity and costs of human rabies vaccines and immunoglobulin in many parts of the world.

A rapid immunohistochemical test (RIT) to detect rabies virus (RABV) antigen has been developed in the Rabies Section of the Centers for Disease Control and Prevention (CDC) by incorporating various components of existing immunoperoxidase techniques (*6*). Like the DFA, the RIT is performed on brain touch impressions, but the product of the reaction can be observed by light microscopy, and RABV antigen appears as magenta inclusions against a blue neuronal background. The test recognizes all genotype 1 variants of RABV examined to date and all representative lyssaviruses. Modifications of a former indirect test have led to a direct test (dRIT) that uses a cocktail of highly concentrated and purified biotinylated anti-nucleocapsid monoclonal antibodies produced in vitro in a direct staining approach and allows a diagnosis to be made in <1 hour. For the routine diagnosis of rabies, glycerol saline is a convenient preservative in situations in which refrigeration or freezing facilities are not promptly available (*7*).

We report findings of a preliminary study to evaluate the dRIT, comparing results of the dRIT carried out under field conditions in Tanzania with the dRIT and DFA performed at CDC. The objectives were to validate the dRIT as a field test for rabies surveillance and evaluate the dRIT on glycerol-preserved field samples.

The Study

Brain stem samples from various animal species were obtained from December 2002 to September 2004 as a result of rabies surveillance operations established in the Mara, Mwanza, and Shinyanga regions of northwestern Tanzania. Some archived glycerolated specimens were also analyzed. Samples were collected by inserting a drinking-straw through the occipital foramen, according to World Health Organization recommendations (*7*) or by opening the skull.

Some specimens were frozen (–20°C). Other samples inside straws were placed into a solution of phosphate-buffered 50% glycerol and stored either at +4°C or at –20°C or kept at room temperature (25°C ± 5°C) for up to 4 months before refrigeration or freezing.

Samples were allocated to 4 groups, according to the method of preservation and whether the samples were tested in the field and at the CDC laboratory or at CDC only (Table 1). Group A samples were kept in glycerol solution for ≤15 months and washed in phosphate-buffered saline (PBS) before testing by dRIT in the field. They were then stored at –20°C for ≤5 months and retransferred into fresh glycerol for shipment. At CDC, the samples were kept in glycerol for ≤2 months and rewashed in PBS before retesting by both dRIT and DFA or DFA only. Group B samples were stored frozen for ≤6 months, processed by dRIT in the field, and placed into glycerol solution for shipment to CDC, where they were stored for 2 months before being washed in PBS and retested. Group C samples were preserved in glycerol solution for ≤60 months, shipped, and processed at CDC by dRIT and DFA without previous testing in the field. These samples were washed in PBS just before testing. Group D samples were stored at –20°C in

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Table 1. Methods of sample preservation and number of samples processed*

Preservation	No. washes in PBS	No. samples tested		
		dRIT field	dRIT CDC	DFA test CDC
Group A. glycerol saline/frozen/glycerol saline	2	44	39	44
Group B. frozen/glycerol saline	1	10	10	10
Group C. glycerol saline	1	0	89	89
Group D. frozen	0	0	16	16

*dRIT, direct rapid immunohistochemical test; PBS, phosphate-buffered saline; DFA, direct fluorescent-antibody assay; CDC, Centers for Disease Control and Prevention.

the field for 2 to 24 months, shipped frozen, and tested at CDC by dRIT and DFA

A qualitative assessment of the samples was made before testing. Five specimens at a time were stained by dRIT at ambient temperature as described below. Touch impressions were made on glass microscope slides as described (*8*). The slides were air-dried, fixed in 10% buffered formalin for 10 min, dip-rinsed in wash buffer PBS with 1% Tween 80 (TPBS), immersed in 3% hydrogen peroxide for 10 min, and dip-rinsed in fresh TPBS. After dipping, the excess buffer was shaken from the slides and blotted from the edges surrounding the impression. This treatment was repeated after each rinsing step. The slides were incubated in a humidity chamber (a cover on a moistened paper towel on an even surface) with the MAb cocktail for 10 min, dip-rinsed in TPBS, incubated with streptavidin-peroxidase complex (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD, USA) for 10 minutes and dipped in TPBS. A 3-amino-9-ethylcarbazole (AEC) stock solution was prepared by dissolving one 20-mg tablet AEC (Sigma-Aldrich Corp, St Louis, MO, USA) in 4 mL N,N-dimethylformamide (Fisher Scientific International, Inc., Pittsburgh, PA, USA) and stored at 4°C. A working dilution was prepared by adding 1 mL AEC stock solution to 14 mL 0.1 mol/L acetate buffer (Polyscientific, Bay Shore, NY, USA) and 0.15 mL 3% hydrogen peroxide. The slides were incubated with the AEC peroxidase substrate for 10 min and dip-rinsed in distilled water. They were then counterstained with Gill's formulation #2 hematoxylin (Fisher Scientific International) diluted 1:2 with distilled water for 2 min and dip-rinsed in distilled water. Finally, they were mounted with a water-soluble mounting medium (BioMeda Corp., Foster City, CA, USA) and examined by light microscopy (Leica Microsystems AG, Wetzlar, Germany) in Tanzania and Axioplan 2 (Carl Zeiss AG, Göttingen, Germany) at CDC at magnifications of ×200 to ×400. The same operator performed the dRIT in the field and at CDC. However, at CDC, identification numbers unknown to the operator were assigned. The DFA (FITC Anti-Rabies Monoclonal Globulin, Fujerebio Diagnostic Inc., Malvern, PA, USA) was performed in a blind manner by another operator as described (*8*) and read by fluoresent microscopy (Axioplan 2).

Confidence intervals for the sensitivity and specificity were calculated by using the exact binomial distribution (S-Plus, Insightful Corp., Seattle, WA, USA). Of 159 total samples tested, 59 specimens (37.1%) were positive for RABV antigen, and 100 (62.9%) were negative by dRIT, with 100% agreement between the tests, whether dRIT was performed in field conditions only, both in field and laboratory conditions, or in laboratory conditions only. Assuming that the DFA was 100% sensitive and specific, the dRIT was 100% sensitive (95% confidence interval [CI] 93.9%–100.0%) and 100% specific (95% CI 96.3%–100.0%). Table 2 shows the distribution of positive samples in the various animal species.

Table 2. Number of Tanzanian brain samples processed by dRIT and DFA for different animal species*

Species	No. brains examined†
Domestic dog	73 (39)
Domestic cat	7 (3)
Cow	8 (7)
Goat	6 (5)
Livestock‡	1 (1)
Aardwolf (Proteles cristatus)	1
African civet (Civettictis civetta)	2
Banded mongoose (*Mungos mungo*)	2
Slender mongoose (Herpestes sanguineus)	3
Dwarf mongoose (*Helogale parvula*)	2
White-tailed mongoose (*Ichneumia albicauda*)	8 (1)
Mongoose‡	2
Black-backed jackal (*Canis mesomelas*)	3
Bat-eared fox (Otocyon megalotis)	8
Black-backed jackal/bat-eared fox‡	2 (1)
Cheetah (Acinonyx jubatus)	3
Small-spotted genet (*Genetta genetta*)	7 (1)
Lion (Panthera leo)	6
Serval (Felis serval)	1
Spotted hyena (*Crocuta crocuta*)	12 (1)
Striped hyena (*Hyaena hyaena*)	1
Zorilla (Ictonyx striatus)	1
Total domestic	95 (55)
Total wildlife	64 (4)
Total	159 (59)

*dRIT, direct immunohistochemical test; DFA, direct fluorescent-antibody assay.
†The number of rabies-positive samples is shown in brackets.
‡Species not definitively identified.

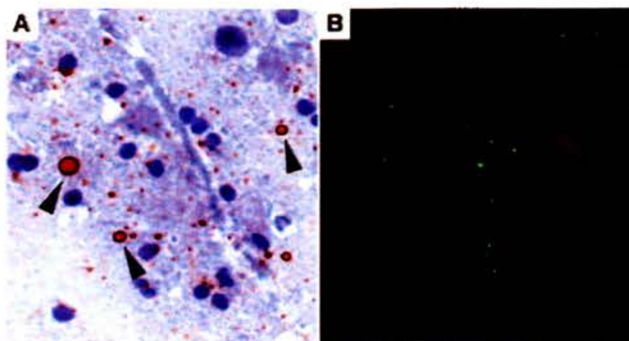

Figure 1. Touch impression of a rabies-positive Tanzanian domestic dog brain preserved in 50% glycerol saline solution for 15 months before testing by direct rapid immunohistochemical test (dRIT) and retested by direct fluorescent-antibody assay (DFA) after 5 months. A) Brain stained by dRIT: rabies virus antigen appears as magenta inclusions (arrowheads) against the blue neuronal hematoxylin counterstain. Magnification, ×630. B) Immunofluorescent apple-green viral inclusions in the same brain processed by DFA. Magnification, ×200.

The sensitivities of the dRIT and DFA were comparable, regardless of the method of preservation. We have no evidence that storage times affected positivity because 34 (77.2%) of 44 samples stored in glycerol solution remained positive for up to 10 months before being tested in the field and retested at CDC after an interval of up to 6 months. Furthermore, RABV antigen was successfully detected in the sample that had been preserved in glycerol for the longest duration (15 months) before dRIT in the field, stored frozen for 3 months before shipment to CDC, and kept in glycerol for 2 months before being retested (Figure 1). Similarly, viral inclusions were detected in a sample stored frozen for 24 months, although the antigen distribution was sparse with both tests. Our data do not provide any unequivocal conclusions on test sensitivity with samples preserved in glycerol solution for >15 months because results from all 15 archived brains were negative. For these samples, the presence of antigen at the time of collection cannot be excluded.

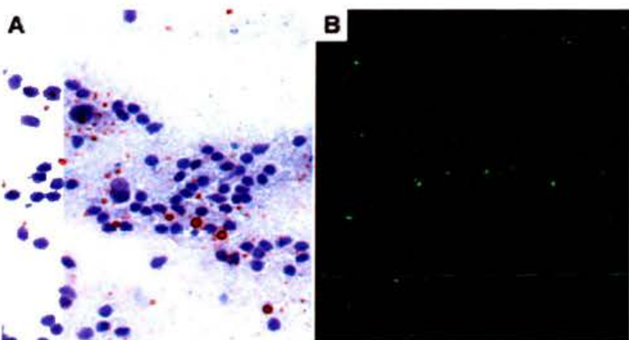

Figure 2. Touch impression of a deteriorated glycerolated brain from a Tanzanian spotted hyena (*Crocuta crocuta*) with rabies. A) Brain processed by direct rapid immunohistochemical test (dRIT). Magnification, ×400. B) DFA staining procedure on the same brain. Magnification, ×200.

Four of 10 (40.0%) deteriorated specimens were positive (Figure 2). Among the 6 brains with negative results, only 1 was suspected of containing rabies. The negative finding might have been caused by inadequate preservation, since the sample had been stored in glycerol solution at ambient temperature for up to 4 months before being refrigerated.

Conclusions

The dRIT showed a sensitivity and specificity equivalent to those of the DFA. The test is simple, requires no specialized equipment or infrastructure, and can be successfully performed on samples preserved in glycerol solution for 15 months or frozen for 24 months and in variable conditions of preservation. These qualities make it ideal for testing under field conditions and in developing countries. Although further laboratory and field evaluations are required, our results are promising and highlight the potential value of the dRIT for countries with limited diagnostic resources. First, this technique could greatly enhance epidemiologic surveillance in remote areas where rabies incidence data are difficult to obtain. Second, the test could improve the ability to respond to outbreaks with effective management decisions. Third, it could be extremely valuable in guiding decisions regarding rational use of rabies PEP.

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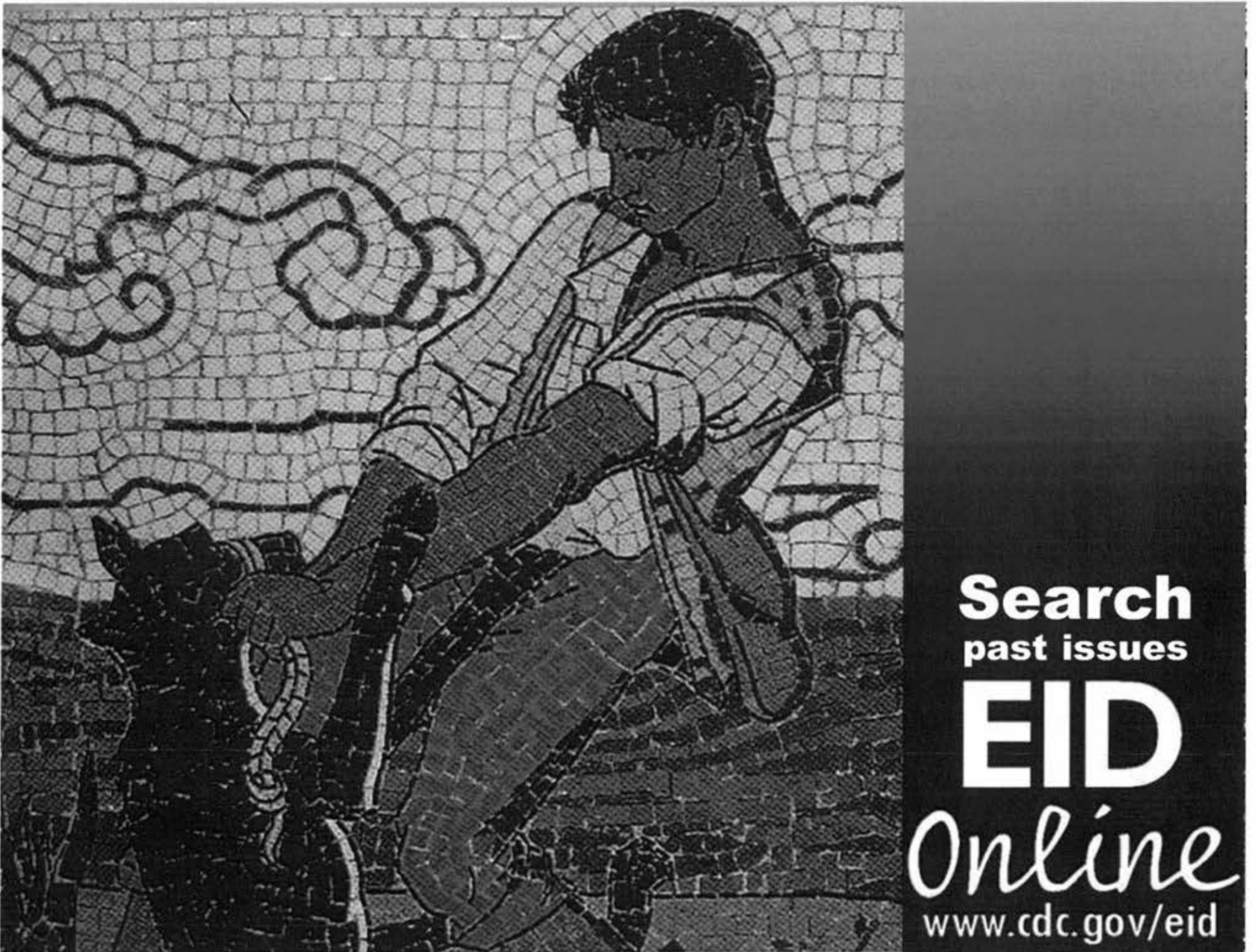

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Molecular epidemiology identifies only a single rabies virus variant circulating in complex carnivore communities of the Serengeti

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Understanding the transmission dynamics of generalist pathogens able to infect multiple host species is an essential prerequisite for their effective control. Only by identifying those host populations that are critical to the permanent maintenance of the pathogen, as opposed to populations in which outbreaks are the result of 'spillover' infections, can control measures be appropriately directed. Rabies virus is capable of infecting a wide range of host species, but in many ecosystems, particular variants circulate among only a limited range of potential host populations. The Serengeti ecosystem (northwestern Tanzania) supports a complex community of wild carnivores that are threatened by generalist pathogens that also circulate in domestic dog populations surrounding the park boundaries. While the combined assemblage of host species appears capable of permanently maintaining rabies in the ecosystem, little is known about the patterns of circulation within and between these host populations. Here we use molecular phylogenetics to test whether distinct virus–host associations occur in this species-rich carnivore community. Our analysis identifies a single major variant belonging to the group of southern Africa canid-associated viruses (Africa 1b) to be circulating within this ecosystem, and no evidence for species-specific grouping. A statistical parsimony analysis of nucleoprotein and glycoprotein gene sequence data is consistent with both within- and between-species transmission events. While likely differential sampling effort between host species precludes a definitive inference, the results are most consistent with dogs comprising the reservoir of rabies and emphasize the importance of applying control efforts in dog populations.

Keywords: rabies; evolution; statistical parsimony; Serengeti

1. INTRODUCTION

Rabies virus (RABV), prototype member of the genus *Lyssavirus*, family Rhabdoviridae, is a multi-host pathogen capable of infecting a wide range of species. The paradigm of rabies epidemiology is the compartmentalization of the circulating virus by species and geographical area leading to the evolution of distinct virus variants that establish sustained transmission networks in a single species, the reservoir host (Rupprecht *et al.* 1991). However, this paradigm largely applies to areas with relatively low species diversity, and it has been suggested that in some areas, particularly in species-rich communities, multiple variants of the virus may circulate in different host species (East *et al.* 2001) or multiple host species may independently maintain infection of a single variant (Thomson & Meredith 1993; Bingham *et al.* 1999*a*,*b*).

It is generally considered that, as a result of the fatal outcome of the disease, maintenance host populations can only maintain the virus if they have specific demographic and ecological characteristics. For instance, species that are terrestrial rabies reservoirs tend to have high birth rates which allow rapid population recovery from rabies-induced mortality (Wandeler *et al.* 1994). Host–virus adaptation has also been proposed as a mechanism for increased efficiency of transmission in maintenance hosts, for example, through high rates of salivary virus excretion (Blancou 1988*a*). Conversely, transmission to non-adapted 'spillover' hosts typically results in short-lived

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Data deposition. The sequences of RABVs produced in this study have been deposited in the GenBank database (accession nos. DQ900547–DQ900579).
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chains of transmission. Occasionally, cross-species transfers may lead to sustained transmission when a virus variant gains access to a novel host species with favourable ecological, genetic and behavioural characteristics (e.g. the species jump from dogs, *Canis familiaris*, to the European red fox, *Vulpes vulpes*, in the twentieth century; Anderson *et al.* 1981; Bourhy *et al.* 1999).

Evidence from epidemiological studies coupled with the isolation of a typically canid-associated African variant (Africa 1b) from the domestic dog, African wild dog (*Lycaon pictus*), bat-eared fox (*Otocyon megalotis*) and white-tailed mongoose (*Ichneumia albicauda*; Cleaveland & Dye 1995; Kissi *et al.* 1995; East *et al.* 2001) have suggested that domestic dogs may be the sole maintenance host of rabies in the Serengeti ecosystem. However, these conclusions were drawn from a limited range of epidemiological data and several alternative hypotheses have been proposed for the maintenance of rabies in multi-host communities in Africa (Thomson & Meredith 1993; Bingham *et al.* 1999*a*,*b*; East *et al.* 2001). The question is important because multiple variants in distinct hosts would prevent effective disease control by targeting a single host population.

An atypical pattern of infection proposed to account for rabies maintenance involves an infectious healthy carrier state where animals actively shed virus in the saliva for prolonged periods, but remain clinically normal. In rare instances, naturally infected healthy dogs have been documented to excrete virus in saliva (Fekadu 1972; Aghomo *et al.* 1989), and non-lethal rabies infection has been suggested to occur in spotted hyaenas (*Crocuta crocuta*) in the Serengeti (East *et al.* 2001). In East *et al.*'s study (2001), hyaenas were deduced to maintain an avirulent variant based on detection of viral RNA in saliva of healthy animals by reverse transcriptase-polymerase chain reaction (RT-PCR). Sequence analysis of these PCR products indicated that the presumed hyaena variant was phylogenetically more closely related to European and Middle Eastern RABVs than to African isolates.

Bingham *et al.* (1999*a*,*b*) suggested that a single variant may be maintained by multiple canine species (i.e. dogs and jackals (*Canis mesomelas* and *Canis adustus*)) in southern Africa through independent cycles, although other studies have indicated that jackals are unlikely to support infection independently of dogs (Cleaveland & Dye 1995; Rhodes *et al.* 1998). Similarly, bat-eared foxes, which are also infected by this variant (von Teichman *et al.* 1995; Sabeta *et al.* 2003), have been implicated as maintenance hosts in the Western Cape (Thomson & Meredith 1993).

High species diversity of wild carnivores in the 27 000 km^2 Serengeti ecosystem and the lack of fencing between wildlife-protected areas and human settlements provide an ideal interface for testing the paradigm of compartmentalization of RABVs in a multi-host community. Compartmentalization has never been tested in a system with coexisting species that have been implicated elsewhere as maintenance hosts of rabies, such as bat-eared foxes and jackals (Thomson & Meredith 1993; Bingham *et al.* 1999*b*).

With additional samples and epidemiological data available from the Serengeti, and the application of phylogenetic analyses, we are now in a position to examine these alternative hypotheses more rigorously. We characterized genetically RABVs isolated from a range of species from the Serengeti and the surrounding areas to determine

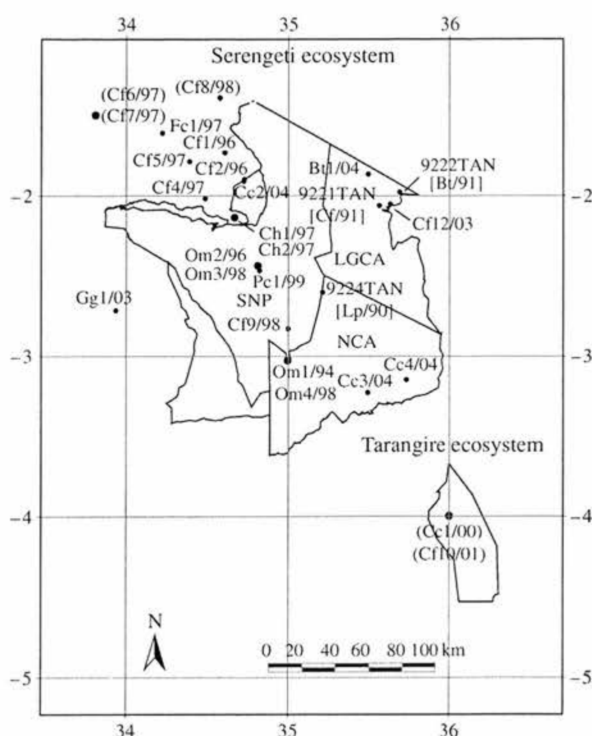

Figure 1. Map of the Serengeti and Tarangire ecosystems showing the location where the field isolates originated (including three previously described viruses: 9221TAN; 9222TAN; and 9224TAN; Kissi *et al.* 1995). The precise sampling location of the isolates in round brackets is not known. The isolates are designated by a prefix indicating the species of origin (Bt, *Bos taurus*; Cc, *Crocuta crocuta*; Cf, *Canis familiaris*; Ch, *Capra hircus*; Fc, *Felis catus*; Gg, *Genetta genetta*; Om, *Otocyon megalotis*; Pc, *Proteles cristatus*), the isolate number and the year of collection. For isolates 9221TAN, 9222TAN and 9224TAN, the species of origin and the year of collection are indicated within square brackets (Lp, *Lycaon pictus*). SNP, Serengeti National Park; LGCA, Loliondo Game Control Area; NCA, Ngorongoro Conservation Area.

the phylogeographic relationships among Serengeti viruses and RABVs recovered elsewhere (i.e. Europe, Middle East and Africa) and identify viral variants that might signify distinct virus–host associations. In a second analysis, we examined the genealogic relationships among Serengeti viruses to infer and identify transmission routes. We employed a parsimony-based network construction procedure (Templeton *et al.* 1992) which has proven useful in hypothesis testing of intra- and interspecific transmission of HIV and human and simian T-cell leukaemia/lymphoma virus type I (Crandall 1995, 1996). The application of this method to RABV sequence data illustrates how genetic analysis can reveal elusive aspects of virus transmission in a complex ecosystem.

2. MATERIAL AND METHODS

(a) *Study samples and rabies diagnosis*

Twenty-four viruses obtained from a range of animal species in the Serengeti ecological region of northwestern Tanzania and Tarangire ecosystem (to the southeast of Serengeti) were included in this study (figure 1; for sample details see electronic supplementary material, S1). All the viruses were from animals diagnosed rabies positive. For brains collected

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257 between 1994 and 2001, diagnostic tests and viral isolations
258 were carried out at the Agence Française de Sécurité Sanitaire
259 des Aliments (AFSSA), Malzéville, France using the
260 fluorescent antibody test (FAT; Dean *et al.* 1996), inoculation
261 of murine neuroblastoma cells and mouse inoculation (Barrat
262 *et al.* 1988). Rabies diagnosis on more recent brain tissues was
263 conducted at the Rabies Section of the Centers for Disease
264 Control and Prevention (CDC), Atlanta, USA by FAT
265 (Dean *et al.* 1996).
266
267 **(b) *RNA extraction, RT-PCR and nucleotide***
268 ***sequencing***
269 Total RNA was extracted from infected brain material using
270 the TRIzol method (Invitrogen, San Diego, CA) according to
271 the manufacturer's recommendations. Reverse transcription
272 of 11 isolates was performed at the Veterinary Laboratory
273 Agency (VLA), Weybridge, Addlestone, Surrey, UK
274 following methods of Heaton *et al.* (1997). RT-PCR of the
275 other isolates and direct sequencing were performed at CDC
276 using previously described methods (Sacramento *et al.* 1991)
277 with primer sets for the regions encoding the nucleoprotein
278 (N) and the central part of the ectodomain of the
279 glycoprotein (G) published earlier (Smith 2002).
280
281 **(c) *Phylogenetic analyses***
282 Sequence editing and translation to amino acid sequences were
283 performed using BioEdit software v. 7.0.0 (Hall 1999).
284 Multiple alignments were generated using the ClustalX
285 package v. 1.83 (Jeanmougin *et al.* 1998), and sequence
286 alignments were trimmed to include only complete non-stop
287 codons. Prior to proceeding with phylogenetic analysis, we
288 examined the alignments for the presence of recombination
289 events using Worobey's informative sites test (Worobey 2001).
290 No significant evidence of recombination was detected.
291 The evolutionary relationships among the Tanzanian
292 isolates newly described in this article and selected representa-
293 tives of African and European/Middle Eastern lineages of
294 RABV (Kissi *et al.* 1995; Bourhy *et al.* 1999; Randall *et al.* 2004)
295 were determined using Bayesian Markov chain Monte Carlo
296 (MCMC) methods. The N gene was chosen because the N
297 sequence of isolates is available for all four African lineages
298 (Kissi *et al.* 1995). Bayesian reconstructions were conducted in
299 MrBayes v. 3.0b4 (Ronquist & Huelsenbeck 2003). Two
300 analyses were performed to check for any substantial sensitivity
301 associated with fixing model parameters prior to analysis rather
302 than estimating them as per MrBayes default settings. The first
303 analysis specified the model of evolution and estimated
304 parameters identified by the program ModelTest v. 3.7
305 (Posada & Crandall 1998) using Akaike Information Criterion
306 (Sakamoto *et al.* 1986). The second analysis used the general
307 time reversible (GTR) model with a proportion of invariable
308 sites and a gamma-shaped distribution of rates across sites
309 (GTR+I+Γ; Yang *et al.* 1994) treating model parameters as
310 unknown variables with uniform priors to be estimated in the
311 analysis. Four MCMC chains with initial random starting trees
312 without constraints were run for 1×10^7 generations with trees
313 sampled every 100th generation, resulting in 1×10^5 sampled
314 trees. To ensure that the chains had reached stationarity, log-
315 likelihood values for sampling points were plotted against
316 generation time and the convergence diagnostic was examined.
317 The first 25 000 trees were discarded as the burn-in phase
318 and the remaining trees were used to estimate consensus
319 phylograms and Bayesian posterior probabilities. Posterior
320 probability values of 0.95 or greater were considered significant.

Graphical representations of the trees were generated with the 321
program TreeView v. 1.6.6 (Page 1996). 322
In order to generate the highest possible degree of resolution 323
for the Tanzanian sequence set, a phylogenetic tree was 324
constructed using a Bayesian MCMC algorithm implemented 325
Q5 in the BEAST program v. 1.4.1 (Drummond & Rambaut 2006, 326
available from http://beast.bio.ed.ac.uk/) that permitted the 327
year of virus isolation to be explicitly incorporated into the 328
analysis. Analysis was performed assuming a constant 329
viral population size and a relaxed molecular clock model 330
(Drummond *et al.* 2002), which allows rates to vary over 331
branches in an exponentially autocorrelated fashion. MCMC 332
analysis chains were run for 1×10^7 generations with trees 333
sampled every 1000th generation using the SRD06 substitution 334
model (Shapiro *et al.* 2006). The pre-burn-in was set at 10 000 335
steps. BEAST output was assessed using the Tracer program 336
(Drummond & Rambaut 2006). 337
Statistical parsimony (SP) networks were constructed to 338
estimate the genealogical intra- and interspecific relationships 339
among the Tanzanian N gene sequences included in the 340
previously described analyses. An SP analysis was also 341
performed on G gene data available for 15 Tanzanian isolates, 342
a number of which were identical over a 398 bp region. 343
Analyses were performed using the TCS software v. 1.20 344
(Clement *et al.* 2000), which implements the procedure of SP 345
developed by Templeton *et al.* (1992), a population-based 346
method for reconstructing historical relationships among 347
gene sequences. The SP approach is based on the parsimony 348
criterion as defined by Templeton *et al.* (1992) with a 349
statistical procedure to evaluate the limits of the parsimony 350
assumption, i.e. the probability that a nucleotide difference 351
between two variant sequences is caused by a single 352
mutational event (the parsimonious state) and not by 353
multiple mutational events at a single site (the non- 354
parsimonious state). An absolute distance matrix is calculated 355
for all pairwise comparisons of sequences. The probability of 356
parsimony is calculated for pairwise differences until the 357
probability exceeds 95% using the model developed by 358
Templeton *et al.* (1992), eqns (6)–(8). The number of 359
mutational differences just before this 95% cut-off point 360
represents the maximum number of mutational steps between 361
pairs of sequences justified by the parsimony criterion. The 362
TCS program then connects the sequences into networks 363
with the number of mutational steps connecting two 364
sequences indicated by the lines connecting sequences. 365
366
367
3. RESULTS 368
The majority-rule consensus tree of partial N gene 369
sequences for RABVs from Tanzania compared with 370
isolates recovered from other locations obtained after 371
selecting and fitting an appropriate nucleotide substitution 372
model (Posada & Crandall 1998) is shown in figure 2. The 373
same topology was obtained when reconstruction was 374
performed by Bayesian analysis with vague priors. The 375
phylogeny revealed clear phylogeographic structure with 376
all of the major clades supported by posterior probabilities 377
greater than 0.95. All Tanzanian isolates grouped together 378
and fell into the Africa 1b group of canid-associated 379
viruses. Within the Tanzanian group (figure 2, inset), 380
which included viruses isolated from domestic and wild 381
species from the Serengeti and Tarangire ecosystems, 382
there was little resolution as reflected in the low posterior 383
probabilities (note that most of the nodes have no 384

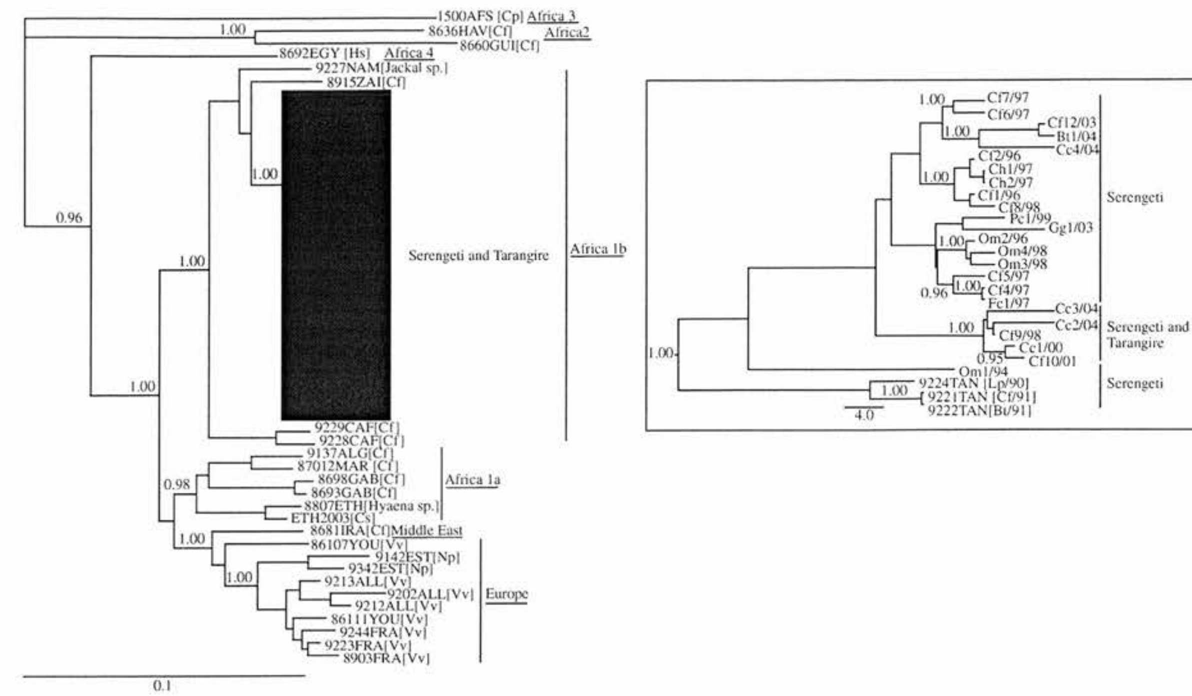

Figure 2. Majority-rule consensus tree of nucleoprotein gene sequences (1158 bp, 386 deduced amino acids, nucleotide positions 263–1420 on the SAD B19 genome; Conzelmann *et al.* 1990) for RABVs from Tanzania (Serengeti and Tarangire ecosystems) compared with isolates from other areas of Africa, Europe and the Middle East recovered with Bayesian phylogenetics under the GTR + invariant sites (I) + gamma shape (Γ) model of evolution (Yang *et al.* 1994; base frequencies = 0.2911, 0.2132, 0.2396 and 0.2561; nucleotide substitution rates of the GTR rate matrix = 1.4665, 6.5059, 0.7601, 0.1703 and 10.8510; $I = 0.3530$; and $\Gamma = 0.7587$). The tree is rooted with isolate 1500AFS defined as the out-group, representative of the lineage Africa 3 (Kissi *et al.* 1995). Numbers on branches indicate Bayesian bootstrap values and are shown next to key nodes only. For a detailed phylogenetic tree of the Tanzanian viruses, see inset (and methods described in main text). Only posterior probabilities greater than or equal to 0.95 are shown. The scales indicate branch length expressed as the expected number of substitutions per site. Isolates described in this study are designated by a prefix indicating the species from which virus was recovered (Bt, *Bos taurus*; Cc, *Crocuta crocuta*; Cf, *Canis familiaris*; Ch, *Capra hircus*; Fc, *Felis catus*; Gg, *Genetta genetta*; Om, *Otocyon megalotis*; Pc, *Proteles cristatus*), the isolate number and the year of collection. Strain names are given for published isolates (Kissi *et al.* 1995; Bourhy *et al.* 1999; Randall *et al.* 2004) and the species of origin is indicated within square brackets (Cp, *Cynictis penicillata*; Cs, *Canis simensis*; Hs, *Homo sapiens*; Lp, *Lycaon pictus*; Np, *Nyctereutes procyonoides*; Vv, *Vulpes vulpes*).

posterior probabilities associated with them as only nodes with values greater than or equal to 0.95 are labelled). However, a number of smaller groups (posterior probabilities ≥ 0.95) were evident that corresponded to viruses recovered from outbreaks linked in time.

The Tanzanian isolates showed between 0.1 and 3.3% (average 1.6%) nucleotide and between 0.0 and 2.6% (average 0.7%) amino acid sequence divergence. Maximum nucleotide diversity was between a virus recovered from an African wild dog (9224TAN) in 1990, the oldest Serengeti isolate, and a virus recovered from a spotted hyaena in 2004 (nucleotide and amino acid divergences were 3.3 and 2.1%, respectively). The BEAST analysis generated an estimated rate of change for the molecular clock of 0.0013 nucleotide substitutions per year (95% CIs 0.0005–0.0021) for the nucleoprotein gene, and dated the most recent common ancestor to the sampled sequences to be from 1976 (95% CIs 1953–1989).

For the N gene dataset, parsimonious connections were justified ($p \geq 0.95$) among sequences differing by as many as 14 nucleotide substitutions. These sequences were connected into a single parsimony network (figure 3, network I and electronic supplementary material, S2), whereas other sequences formed independent networks (figure 3, networks II and III and electronic supplementary material, S3). Isolate Om1/94 could not be connected to any network.

For the G gene dataset, the SP procedure justified connections among sequences that differed by eight or fewer nucleotide substitutions. The resulting network is shown in figure 4 (see also electronic supplementary material, S4).

4. DISCUSSION

Our analysis strongly suggests that only a single Africa 1b virus variant circulates among Serengeti's domestic and wild mammal species, and cross-species transmission is a frequent event. These findings raise interesting questions about why highly species-diverse communities only support a single virus variant.

Overall, our phylogeny revealed site-specific rather than species-specific grouping, and the Tanzanian viruses clustered in a lineage associated primarily with domestic dogs throughout southern and eastern Africa (Kissi *et al.* 1995). No host-distinguishable variants were identified, and domestic dog isolates were present in all clusters. Divergences among viruses were low, consistent with previous analyses of Tanzanian viruses (Kissi *et al.* 1995; East *et al.* 2001) and southern Africa canid viruses

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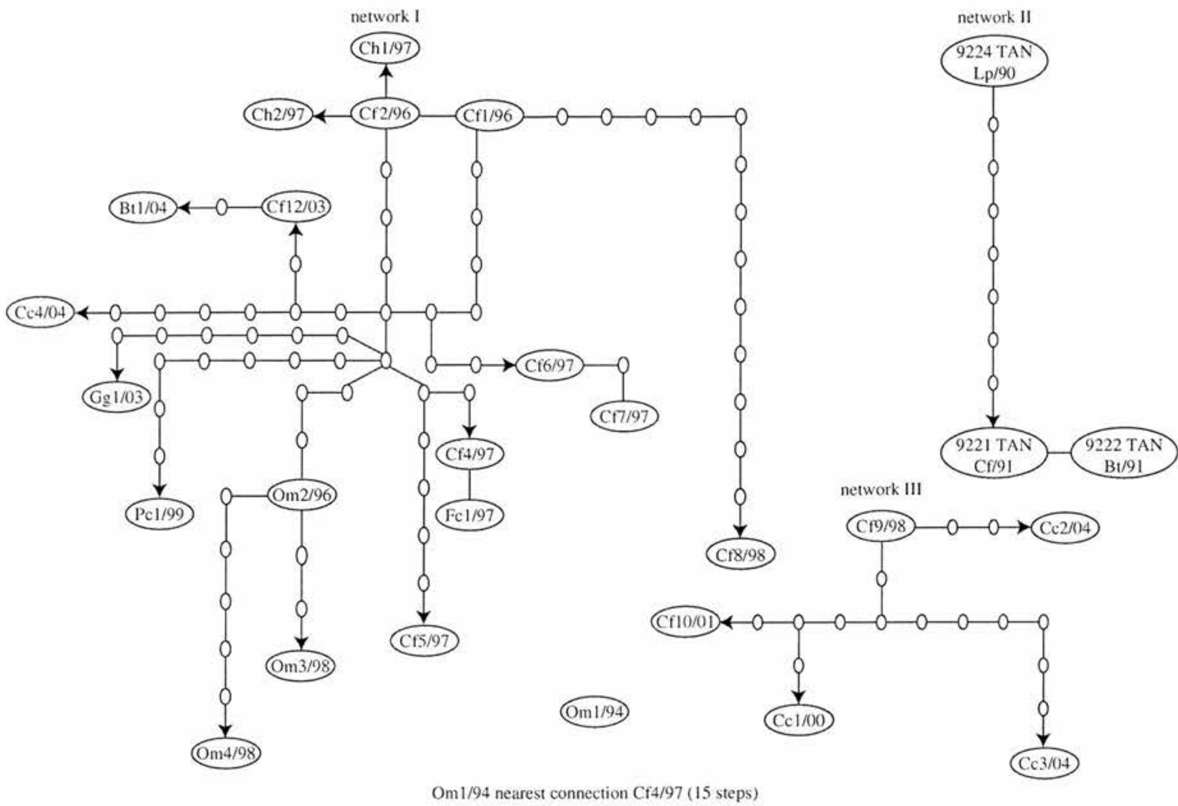

Figure 3. SP networks connecting the Tanzanian nucleoprotein gene sequences described in figure 2 (virus designations are the same as given in figure legend 2). Each branch represents a single mutational step (nucleotide substitution). The lengths of the connecting lines are not significant. Large ovals represent sequences, smaller ovals indicate nodes in the tree, which represent intermediate sequences not present in the sample. The arrows indicate temporal direction of evolutionary change.

(von Teichman *et al.* 1995; Sabeta *et al.* 2003; Johnson *et al.* 2004), suggesting that a single dog-introduced lineage can infect a range of hosts (e.g. dogs, jackals and bat-eared foxes). Although bat-eared fox viruses appear to be more distinct in South Africa (Sabeta *et al.* 2003), definitive virus–host associations have not yet been identified among canid species in this geographical area.

A number of viruses originating from the Serengeti and Tarangire ecosystems grouped together. One possible explanation for this is the seasonal migration of nomadic Maasai pastoralists and their dogs from Tarangire to the Crater Highlands each year.

The results of the Bayesian analyses suggest cross-species transmission of a single variant among a range of domestic and wild species, since viruses recovered from different hosts cluster together. The SP approach shows strong support for one Canidae-associated virus variant circulating within the Serengeti carnivore community. The estimation procedure applied to both the N and G gene sequences connects viruses recovered from a range of hosts into parsimony networks with domestic dog viruses present in all the networks. The sparse and necessarily opportunistic nature of the sampling process required of this sort of study introduces biases in the proportion of domestic and wild animal hosts represented in the dataset over space and time (which are not reflective of any obvious changes in the distribution or movement of host species). This sparse sampling process prohibits a definitive inference regarding the identity of the reservoir host, but the genealogic pattern repeatedly identified in these results is most consistent with the domestic dog comprising the reservoir of rabies.

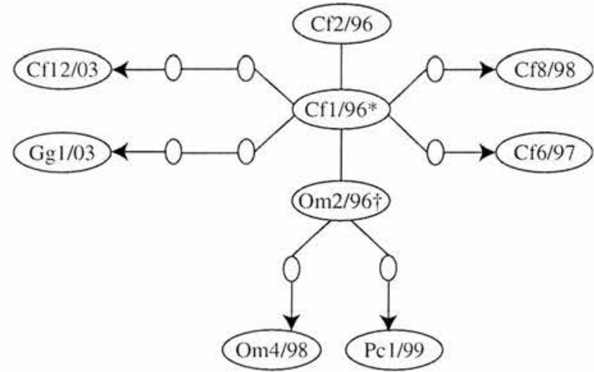

Figure 4. Network of statistically supported relationships for the glycoprotein sequence data (398 bp, 132 deduced amino acids, nucleotide positions 3761–4158 on the SAD B19 genome; Conzelmann *et al.* 1990) available for 15 isolates described in figure 2 (virus designations are the same as given in figure legend 2) inferred using a SP approach. Asterisks indicate that identical genotypes were recovered from multiple animals.

Our findings suggest that, even in highly species-rich areas, the paradigm of maintenance of a single virus variant by a single host species holds true. Despite the

abundance of other mammalian hosts, the domestic dog appears to act as the principal host of a typical canid variant. Similar characteristics of viruses isolated from a range of other species indicate that this variant is freely able to jump species boundaries, but the transmission networks suggest that wildlife species cannot establish stable infection cycles independently of dogs. The domestic dog population surrounding the Serengeti is rapidly expanding and is well suited to serve as a rabies reservoir, with high turnover rates generating large numbers of susceptible hosts. Several Serengeti species with attributes consistent with reservoir hosts (Wandeler *et al.* 1994) have been diagnosed with the disease (e.g. the bat-eared fox, the white-tailed mongoose, the small-spotted genet), and the limited sample sizes available for this study do not permit definitive rejection of these species as part of a reservoir system. However, with the possible exception of the bat-eared fox, the available evidence indicates that these species are all associated with sporadic, short-lived epidemics with no evidence for species-specific virus–host associations.

What are the factors preventing the establishment of sustained cycles in a new host in the ecosystem? First, no single Serengeti wild carnivore population may be large enough or reach high enough densities to support independent cycles of a host-adapted virus. Although the Serengeti is renowned for the abundance of its carnivore populations, the high diversity of species coexisting within the park may prevent any single species reaching high enough densities to maintain infection. For example, population densities of jackals in less diverse farmland in Zimbabwe far exceed those recorded in the Serengeti (Cleaveland & Dye 1995), and this is an explanation for the suggestion that dogs and jackals are both able to maintain rabies in Zimbabwe (Bingham *et al.* 1999*a*,*b*). Second, in general, there are no biogeographic barriers around the Serengeti to impede animal movement (as emphasized by the lack of genetic isolation of virus variants) that might promote localized viral evolution in specialized host niches (Bourhy *et al.* 1999). Third, while high species diversity might be expected to provide many opportunities for host-viral adaptation, such adaptation presumably requires successive generations of infection within the same species and may be inhibited by high levels of interference between generalist carnivores that afford frequent opportunities for cross-species transmission.

In contrast with our observations of a single species supporting the virus cycle in the ecosystem, East *et al.* (2001) suggested that healthy carrier hyaenas maintain a genetically distinct non-pathogenic variant on the basis of viral RNA detected in hyaena saliva by RT-PCR. East *et al.* (2001) provide evidence indicating that this variant is genetically most closely related to RABVs circulating in Europe and the Middle East, primarily among foxes, and distinct from hyaena viruses in our study (see electronic supplementary material, S5). Typically, fox RABVs cause rabies clinical signs and inevitable death in foxes (George *et al.* 1980) and are known to be pathogenic to a range of other species in which no evidence of survival has been documented (Blancou 1988*b*; Charlton *et al.* 1988). We consider the finding of this variant in healthy Serengeti hyaenas, without evidence for clinical disease, difficult to explain. In this study, diagnostic material was obtained from 41 hyaenas. Of these, four were confirmed rabies positive and Africa 1b RABVs were recovered. Clinical signs of rabies in hyaenas infected with this variant are quite typical, with signs of altered behaviour, increased aggression (attacking humans and animals), ataxia and death. Rabies morbidity and mortality in hyaenas have been reported elsewhere in Africa (Mills 1990; Swanepoel *et al.* 1993). There is no doubt that Serengeti hyaenas can die when infected with dog rabies and that rabid hyaenas pose a severe risk to humans and other mammals. With their intra- and interspecific kleptoparasitic behaviour (Kruuk 1972), wide-ranging 'commuting' outside the protected areas (Hofer & East 1995), scavenging in agricultural areas (Kruuk 1972) and predation on domestic dogs (Butler *et al.* 2004; S. Cleaveland, personal Q6 observation), hyaenas probably constitute a critical link in disease transmission between domestic and wild carnivore populations in the Serengeti and elsewhere (Cleaveland *et al.* 2000; Butler *et al.* 2004).

Viral generalist pathogens pose a grave threat to biodiversity and human health (Cleaveland *et al.* 2001). The impact of rabies on African wild canids can be substantial, as documented following rabies outbreaks in the African wild dog and the Ethiopian wolf (*Canis simensis*: Gascoyne *et al.* 1993; Randall *et al.* 2004; Haydon *et al.* 2006). The disease also inflicts a considerable public health burden in many parts of the world (Knobel *et al.* 2005). Our study is consistent with the view that, in the Serengeti, domestic dogs maintain a single major virus variant belonging to the Africa 1b group with spillover cases occurring in other species, and does not provide evidence for the co-circulation of multiple variants associated with distinct hosts. Efforts directed at controlling infection in dogs through mass vaccination can therefore be expected to eliminate rabies in all other species with benefits for both human health and wildlife conservation.

Q7

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