Epidemiological Analysis of Host Populations with Widespread Sub-Patent Infections

African Trypanosomiasis

by

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Abstract

The epidemiological study of pathogens largely depends on three technologies, serology, microscopy and the polymerase chain reaction (PCR). Serological methods are unable to differentiate between current and past infections. Microscopy has historically been the mainstay of epidemiological study. In recent times the use of microscopy has been in decline, as it has been shown to have an inherent lack of sensitivity and specificity and produces many false negative results. PCR is now the method of choice for screening samples for the presence or absence of pathogens. Although PCR is widely regarded as an extremely sensitive technique, the fact that it assays a very small volume of sample is often overlooked. If the target pathogen is not present in the tiny aliquot of sample from an infected host, then a false negative results will occur. In endemic situations were the pathogen is present at low infection intensities, then the potential for false negatives results of this type is high. This intensity related false negative effect can lead to serious underestimation of diagnosed prevalence and incidence with consequent misinterpretation of the resulting data. This phenomenon has been reported in the literature for a range of pathogens and especially for epidemiological study of schistosomiasis. The extensive occurrence of false negatives during study of schistosomiasis samples was such an obstacle to epidemiological study it prompted the world health organisation to repeatedly call for quantitative methods to be employed to combat the problem.

The main objectives of this thesis are to rationalise and simplify the methods of diagnosing African trypanosomes in epidemiological studies and to investigate the consequences of, and methods of dealing with infection intensity related false negative results that occur as a result of widespread sub-patent infections in the study population

A new PCR assay was developed that was capable of analysing whole blood placed onto treated filter paper. The PCR assay was capable of differentiating between all the important African trypanosome species, producing a unique size of amplicon for each species of trypanosome. Initial results from repeated screening of human and cattle samples known to be parasitologically positive indicated that many false negative results occur. A more extensive analysis of thirty five bovine blood samples randomly chosen from a collection of field samples revealed that false negative results occurred regularly. The prevalence

of infection after a single screening was 14.3% whereas the cumulative prevalence after over 100 repeated screenings rose to 85.7%. This showed that a severe underestimation of prevalence occurs from a single screening of the samples.

In order to investigate the consequences of, and develop methods of dealing with this problem, computer based simulations were used to model the dynamics of screening samples with sub-patent infections. In order to construct the model the data obtained from repeat screening of the thirty-five bovine blood samples was fitted to a number of mathematical distributions. A negative binomial distribution best described the distribution of trypanosomes across the hosts. Exploration of the phenomenon with the resulting model showed the extensive underestimation of true prevalence that is possible. The simulations also showed that it is possible for populations with very different patterns of infection and true prevalence to all have the same diagnosed prevalence from a single screening per sample. Statistical comparison of these very different populations by diagnosed prevalence alone would conclude there was no significant difference between the populations. It was therefore concluded that the diagnosed prevalence from a single (or even multiple) screenings is an inadequate and potentially misleading measure of both infected hosts and parasite numbers.

In order to deal with these problems new methods were evaluated for use in epidemiological studies. A simple method of producing quantitative measures of infection was advocated. The insensitivity of existing screening methods in detecting significant difference between populations was highlighted and a greatly improved methodology was shown. Finally, a method for inferring the true population prevalence from the data obtained from repeat screening of samples was suggested. Although some of these new methodologies have limitations, they represent a great improvement on the use of a single diagnostic test for each host. The work presented in this thesis highlights a serious potential limitation to our understanding of the epidemiology of pathogens that exist at sub-patent levels, and develops some possible methods of overcoming these limitations.

Declaration

This is to certify that the thesis comprises only my original work towards the PhD. Due acknowledgement has been made in the text to all other material used. This work has not been submitted for any other degree or professional qualification.

It has in part been published in the following scientific publication

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Signed……

Dedication

This thesis is dedicated to my parents, whose constant support, encouragement and advice through this study and throughout my life has always been one of my greatest assets.

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First of all I would like to thank my supervisors, who I regard as both mentors and friends. Collectively they have provided me with a unique and complimentary mix of influences and assets. Prf. Sue Welburn for enthusiasm, an instinct for distinguishing the wheat from the chafe and for support with the personal difficulties we all encounter over such periods of time. Prf. Geoff Hide, for his phenomenal ability to turn problems into opportunities, insight and vision blended with the most pragmatic of approaches. Dr Aimee Tilley, for her ability to discuss and explore complex ideas, constant encouragement and her ability to see the humorous side of life in academia and research. I would also like to thank Dr Paul Coleman of the London School of Hygiene and Tropical Medicine and Dr Darren Shaw of the University of Edinburgh for brief but important consultations on the mathematical and statistical aspects of the early part of my work.

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Definitions

Detection Threshold

The detection threshold of a diagnostic technique is the minimum number of parasites (or fraction of a single parasite) that can be detected in the volume of sample analysed by the diagnostic technique. For whole parasites the practical lower limit of this threshold is one. It differs from the concept of sensitivity in that it accounts for the volume of sample analysed. It refers to and is a property of the diagnostic technique and does not infer information about the infection intensities within the host.

Diagnosed Prevalence

The prevalence achieved from a screening of samples with a diagnostic test. This may not be equivalent to the population prevalence.

Diagnostic Sensitivity

The diagnostic sensitivity of a test is the test's ability to detect hosts with the condition of interest in a population or group and is expressed as a proportion or percentage: the number of persons who have both the condition and a positive test result divided by the number of persons who have the condition. Diagnostic sensitivity often has more to do with the ability to obtain the target substance in a processed sample from a host who has the condition than with the ability to detect very low concentrations of a substance. If the target substance is not in the processed sample because of vagaries of sampling or processing, an assay with perfect analytical sensitivity still fails to give a positive result (Saah et al, 1997).

Infection Intensity

This represents the number of pathogens / parasites present within an infected host. More specifically, in the context of this work it refers to the number of parasites per volume of blood. Typically, parasites / pathogens per millilitre, although occasionally per microlitre.

IRFN

Intensity Related False Negatives refers to negative diagnostic results obtained from infected hosts. This is a direct result of failing to obtain sufficient quantities of the parasite, in the aliquot drawn for a diagnostic test, to give a positive diagnostic test result. This will be intrinsically related to the infection intensity present in the infected hosts.

Overdispersion

Describes the distribution of a pathogen / parasite across a host population or sub-population. If more parasites were found to occur in fewer hosts than would be expected assuming a random distribution of parasites then the parasites would be said to be overdispersed or aggregated. In many cases most hosts have low numbers of parasites or no parasites, whilst a few hosts harbor a large number. In this situation the parasites are said to be overdispersed. Such a distribution of parasites is frequently well described by a negative binomial distribution. The negative binomial distribution is described by the mean and overdispersion value K. In this case overdispersion (K) is a direct measure of overdispersion.

Patent Infection

A patent infection is usually defined as an infection that is present at an intensity that is detectable by the diagnostic technique. Because of stochastic effects in detection of the parasites (see the definition of sub-patent infection), for this work the definition will be modified. A patent infection is defined as infection with a parasite that has a high enough infection intensity to provide consistently repeatable positive results (no false negatives).

Population Mean Infection Intensity (or Mean infection intensity of the population)

For a given population, the mean infection intensity is the sum of all the infection intensities of all infected hosts divided by the total number of individuals within the population of interest.

Population Prevalence

The population prevalence is the 'true' prevalence present in the population. In reality this value is unknown; ascertaining this value is the object of many types of epidemiological study.

Repeatability

Is defined as the degree of repeatability of a positive diagnostic results. For example a repeatability of 0.6 would infer that if a diagnostic test on an infected individual were to be repeated ten times only six tests would be positive, the remaining four would be false negatives. The false negative rate is equivalent to one minus the repeatability.

Sub-patent Infection

The more accepted understanding of a sub-patent infection is an infection that is present at too low an intensity to be detected by the diagnostic technique. This definition is somewhat imprecise, because infections are diagnosed on a stochastic basis not a definitive one. Even an infection that is present as one parasite per ten litres of blood has a (remote) probability of being detected. If a diagnostic technique is capable of detecting a single parasite and assays a single microlitre of sample, then a parasite which is present with an infection intensity of one parasite per two microlitres of blood only has a mean probability of detection in a single assay of 0.5. Therefore in this work the definition of sub-patent infection will be modified. A sub-patent infection is defined as infection with a parasite that causes false negative results in repeated diagnostic rests, this being due to the low infection intensity present in the host.

Chapter 1: Introduction

1.1. African trypanosomiasis: An introduction

The African trypanosomiases comprise a group of economically important animal diseases and medically important zoonotic diseases that affect much of sub Saharan Africa. The causative organisms are a few species and sub species of a heteroxenous parasite of the genus Trypanosoma, which are capable of infecting a wide range of mammalian and some reptilian species. (See table 1.1.) The African trypanosomes are transmitted by tsetse flies (*Glossina* spp.); a large biting fly of the order dipteral. The geographical range of African trypanosomiasis largely coincides with the range of the tsetse, some 8.7 million square kilometres, between the latitudes 15^{o}N to 25^{o}S. Most of the areas affected are rural and relatively remote; this isolation compounds the effects of the disease. The diseases affect rural communities in two ways; firstly all species of African trypanosome affect livestock. Infection of cattle and other domestic livestock with *Trypanosoma congolense* or *Trypanosoma vivax* causes a serious disease locally known as Nagana. *T. brucei* s.l. is by contrast considered relatively non pathogenic in livestock and wildlife. Secondly, two sub species of *Trypanosoma brucei*; *T.b.rhodesiense* and *T.b.gambiense* are zoonotic and also infect humans, in which they are fatal. *T. brucei* infection in humans is called sleeping sickness and can be acute (*T.b.rhodesiense*) or chronic (*T.b.*gambiense) (Welburn *et al*, 2001). In many respects Nagana is of equal importance to the human form of the disease, susceptibility of domestic livestock to trypanosomiasis is responsible for livestock losses and poor productivity and prevents farmers from improving cattle stock. Therefore this disease is a major factor that holds back the further development of the sub Saharan African.

1.2. The importance of human African trypanosomiasis

Current estimates suggest that 60 million people are at risk of infection, this number is spread over 36 different countries in sub Saharan Africa (WHO, 2001). In seven countries the status is classed as highly endemic, four countries are classed as endemic; twelve are classed as moderately endemic and in thirteen countries the epidemiology is poorly understood. In some areas such as Angola, The Democratic Republic of Congo and Southern Sudan prevalence may reach as high as twenty to fifty percent (Moore *et al.*, 1999); in these areas sleeping sickness may be the first or second highest cause of mortality after

HIV/AIDS (WHO, 2001). It is estimated that there are around 50,000 deaths per annum.

Table 1.1. African trypanosomiasis: Important species and sub species

Species	Important Hosts	Pathogenicity
Trypanosoma brucei brucei	Wide range of mammals birds and lizards	Regarded as non pathogenic – not human infective
Trypanosoma brucei gambiense	Domestic livestock particularly cattle. Humans	Human infective causing the chronic form of the disease
Trypanosoma brucei rhodesiense	Domestic livestock particularly cattle. Humans	Human infective causing the acute form of the disease
Trypanosoma congolense	Domestic livestock	Pathogenic to cattle causing Nagana
Trypanosoma vivax	Domestic livestock	Pathogenic to cattle causing Nagana

Table showing the species and sub species of the genus *Trypanosoma* that are responsible for human sleeping sickness and the disease known as Nagana in livestock. The table also lists important hosts of the particular trypanosome and gives a few details of pathogenicity.

However, because most cases are in remote areas and therefore go undiagnosed, the world health organisation estimates the true number of deaths to be ten times this amount, 500,000 deaths per anum (Odiit *et al.*, 2005). The cost in disability adjusted life years is estimated at 1,585,000.

1.3. The importance of animal African trypanosomiasis (Nagana)

African trypanosomiasis has been described by the World Health Organisation as “One of the most important if not the most important constraints to livestock and mixed crop-livestock farming in tropical Africa” (WHO, 2001). This in spite of the fact that much of the land affected by tsetse borne trypanosomiasis is capable of supporting a much larger population of cattle, this land is prevented from being as productive as it might be by the presence of the tsetse and

trypanosomiasis. The disease affects millions of cattle, goats, sheep, donkeys, camels and horses, and makes it very difficult for essentially rural communities to make any kind of effective living from farming. On a national scale the disease generally holds back much of the benefits of efficient nutrient cycling, and animal traction for the farmers of a particular nation or area (Kristjanson *et al.*, 1999). If farmers are forced to work the land by hand then it is impossible, in economic terms, to make the natural progression from hand working of the land to animal traction power and from there on to machine power; with the accompanying increases in productivity and gross domestic product that are associated with these transitions. The incomes to individual farmers from meat, milk and other livestock derived products are also reduced. It can be seen therefore that African trypanosomiasis reduces the amount of land that can be farmed, the efficiency with which a given area of land can be farmed, reduces the available workforce and the efficiency of that workforce.

There are many socio-economic factors that compound these basic problems. Many parts of sub Saharan Africa suffer from civil unrest and war; the incidence of this disease is often increased following such social upheaval. In addition, government funded provision for veterinary healthcare in contemporary Africa is in decline (Eisler *et al.*, 2003). Most of the communities affected are essentially rural and relatively remote, and are not likely to have access to medical or veterinary facilities. In addition the loss of potential production of crops, meat and milk reduces the nutritional quality of the diets of the inhabitants of these areas.

1.4. Classification

African Trypanosomes are protozoan parasites of the sub phylum Kinetoplasta. This sub phylum is divided into three families: - Bodonidae, Cryptobiidae and the family to which trypanosomes belong; Trypanosomatidae. Members of the genus Trypanosoma are all heteroxenous (except *Trypanosoma equiperdum*). Differentiation of trypanosomes at the genus level is difficult in many cases, many methods have been useful for differentiation, but none have proven universal. Members of the genus Trypanosoma have been divided into two groups, the stercoraria and the salivaria (Hoare, 1966). See Table 1.2.

The names of the two groups reflect the site within the vector where trypanosome development takes place. The stercorarian trypanosomes complete development in the posterior section, are present in the faeces and require the contamination of an open wound with the faeces of the vector for transmission. One of the most important trypanosomes in this group is *Trypanosoma cruzi* from the subgenus *Schizotrypanum*, an important pathogenic trypanosome of South America and *Trypanosoma theileri* from the subgenus *megatrypanum*, a large and ubiquitous trypanosome of cattle and other species that is thought to be largely non pathogenic (Rodrigues *et al*, 2003). The salivarian trypanosomes complete development in the anterior section (salivary system) of the vector, they are directly transmitted by the bite of the vector. This group is divided into four subgenuses, almost all of which are transmitted by the tsetse fly (*Glossina* spp.), except *T.equiperdum* which is venerally transmitted amongst horses. Other species such as *Trypanosoma evansi* and *Trypanosoma equinum* are transmitted by tabanid flies. This group contains a number of important pathogenic trypanosomes of a variety of mammals including humans. Most important are *Trypanosoma vivax* of the *Dutonella* subgroup that causes Nagana in cattle, sheep, equines, goats and dogs. *Trypanosoma congolense* from the subgroup *Nannomonas* also cause Nagana in cattle, sheep and equines. *Trypanosoma brucei* of the *Trypanozoon* group, is divided into three subspecies; *Trypanosoma brucei brucei* infecting a wide variety of mammals and is regarded as only mildly pathogenic in domestic cattle (Taylor & Authie, 2004); *Trypanosoma brucei gambiense* causing the chronic form of human sleeping sickness and *Trypanosoma brucei rhodesiense* causing the acute form of human sleeping sickness (Welburn *et al*, 2001).

The distinction between *T.brucei brucei*, *T.brucei gambiense* and *T.brucei rhodesiense* has long been problematic, all subspecies being morphologically indistinguishable from each other. Differentiation has been based largely on the differences in virulence in hosts and rodents, clinical course of disease, host infectivity and geographical range. *T.brucei brucei* is present across much of the range of the tsetse in sub Saharan Africa and is not infective to humans. *T.brucei gambiense* is human infective causing a chronic form of the disease and is present to the west of the Rift valley; whilst *T.brucei rhodesiense* causes an acute form of the disease and is present to the east of the Rift Valley (Welburn *et al.*, 2001). See Figure 1.1

Table 1.2. Classification and characteristics of the genus Trypanosoma

(Hoare, 1956; 1964; Hoare, 1966)

The type species of each subgenus is cited first.

A. STERCORARIA

Free flagellum present; kinetoplast large, not terminal; posterior end of body pointed; multiplication in mammal discontinuous, typically in crithidial or leishmanial stages; typically non-pathogenic; development in vector in posterior station, transmission contaminative.

Subgenus *Megatrypanum*

Large species; kinetoplast typically near nucleus, far from posterior end of body; includes *T. (M.) theileri, Iragchphi, ingens, melophagium* and others

Subgenus *Herpetosoma* Medium-sized species; kinetoplast subterminal; includes *T.* (*H.*) *lewisi, duttoni, nabiasi,* and others

Subgenus *Schizotrypamun* Small species, typically curved; kinetoplast voluminous, close to posterior end of body; includes *T. (S.) cruzi, vespetilionis, pipistrelli* and others

Subgenus *Endotrypanum* Endoglobular crithidial and trypanosome forms; includes only *T. (E.) schaudinni*

B. SALIVARIA

Free flagellum present or absent; kinetoplast terminal or subterminal; posterior end of body usually blunt; multiplication in mammal continuous in Trypanosomal stage; typically pathogenic; development in vector in anterior station and transmission inoculative; includes also some atypical species transmitted non-cyclically by arthropod vectors, or by coitus

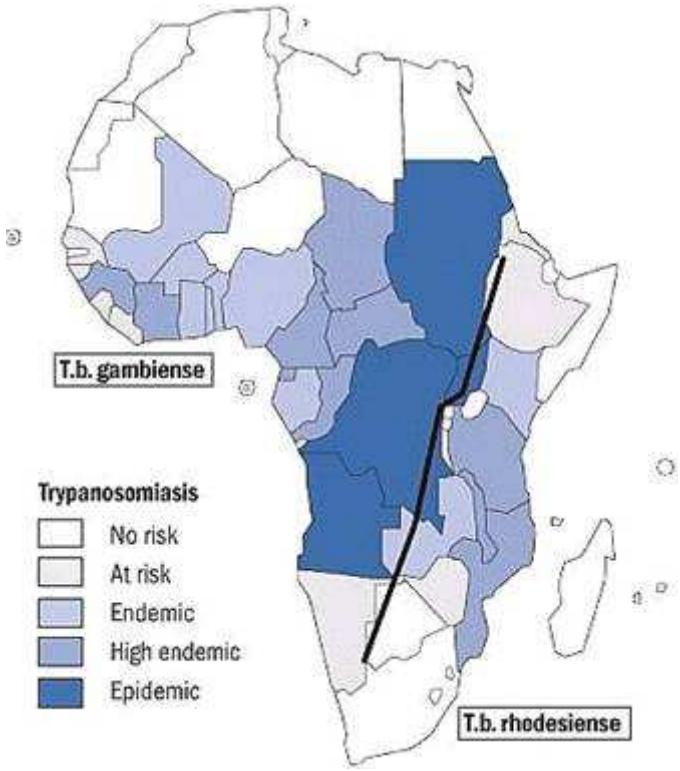
Subgenus *Duttonella* Monomorphic species; posterior end of body rounded; kinetoplast large, terminal; free flagellum present; development in vector *(Glossina)* in proboscis only; includes *T.* (*D.*) *vivax, uniforme*

Subgenus *Nannomonas* Small species; monomorphic or polymorphic; kinetoplast of medium size, typically marginal; free flagellum usually absent; development in vector *(Glossina)* in midgut and proboscis; includes *T.* (*N.*) *congolense, dimorphon, simiae*

Subgenus *Pycnomonas* Short, stout species; monomorphic; kinetoplast small, subterminal; free flagellum short; development in vector *(Glossina)* in midgut and salivary glands; includes *T. (P.) suis*

Subgenus *Trypanozoon* Typically polymorphic species with small subterminal kinetoplast; development in vector (*Glossina*) in mid-gut and salivary glands; includes some aberrant species transmitted non-cyclically or by coitus; includes *T. brucei, gambiense, rhodesiense, evansi, equinum, equiperdum*

Figure 1.1. Map of Africa showing the relative ranges of east (*T.b.rhodesiense*) and west (*T.b.gambiense*) African human trypanosomiasis



The line shows the approximate position of the rift valley. *T.b.brucei* is present across much of both ranges. (Map From World Health Organisation)

1.5. Lifecycle

Type example *Trypanosoma brucei.*

The trypanosomes causing sleeping sickness and Nagana are heteroxenous parasites; their lifecycle alternates between the insect vectors and mammalian hosts. The insect vector is *Glossina* spp (tsetse fly); for *Trypanosoma brucei* the main species involved are *Glossina mortisans, Glossina pallidipes* and *Glossina swynnertoni*. The mammalian host can be cattle, sheep, equines, pigs, antelopes, waterbuck, camels, zebras, human or others depending on the species of trypanosome involved. *Trypanosoma brucei brucei* is also occasionally found in lizards (Njagu *et al*, 1999). Around 90% of tsetse flies are refractive to infection, it is not yet clear why this is the case (Welburn & Maudlin, 1999). The lifecycle therefore begins when a trypanosome is ingested by a susceptible, teneral fly (a fly taking its first blood meal) with a blood meal. Once ingested the trypanosome locates in the posterior section of the insects midgut where it multiplies in number, at this stage in the lifecycle the trypanosome is in the amastigote form. After

about ten days the slender forms migrate towards the anterior section of the gut (foregut). Between twelve to twenty days a further forward migration takes place; finally the trypanosomes end up in the salivary glands. Once in the salivary glands the trypanosomes transform into the epimastigote form and either are free in the lumen or attach themselves to the host cells, multiplication continues asexually. After several generations the trypanosomes transform into the metacyclic trypanomastigote form, this is the only stage in the vector that is infective to the vertebrate host. When the fly feeds as many as several thousand trypanosomes may be inoculated into the host. The entire lifecycle within the fly may be completed within fifteen to thirty five days.

Once inoculated into the vertebrate host the trypanosomes enter the lymph and blood where they undergo intense multiplication by binary fission with a doubling time of around six hours (Vickerman, 1985). The level of parasitaemia present in the blood fluctuates; as the old antigenic type that is beginning to be recognized by the immune system is replaced by a new antigenic type that is not recognized. Early on in the infection slender forms dominate and later on stumpy forms dominate via an intermediate type. During the final stage of infection the human infective forms invade the central nervous system and can be found in the cerebro-spinal fluid. This stage of the infection gives rise to the classic sleeping sickness symptoms, disrupted sleeping patterns, disrupted speech, behavioral and motor functions and a general dementia, the patient eventually lapses into a coma.

1.6. Epidemiology

Elements of the epidemiology of both human and animal African trypanosomiasis have already been discussed. The epidemiology of both human and animal trypanosomiasis is extremely complex and subject to a myriad of interacting factors, some known, some suspected and some awaiting discovery.
The classical tsetse – vertebrate transmission cycle is complicated by the number of vertebrate species that act as a reservoir for infection. An additional reservoir of infection is considered to be more important for spread of *Trypanosoma brucei brucei* and *Trypanosoma brucei rhodesiense* than in *Trypanosoma brucei gambiense.*

Reservoirs found for *Trypanosoma brucei gambiense* include hartebeest, kob, waterbuck, buffalo and significantly pigs and dogs (Gibson *et al.*, 1978; Truc *et al.*, 1997). How these animal reservoirs affect disease transmission depends upon the proximity of livestock and humans to the wild reservoir species. Clearly

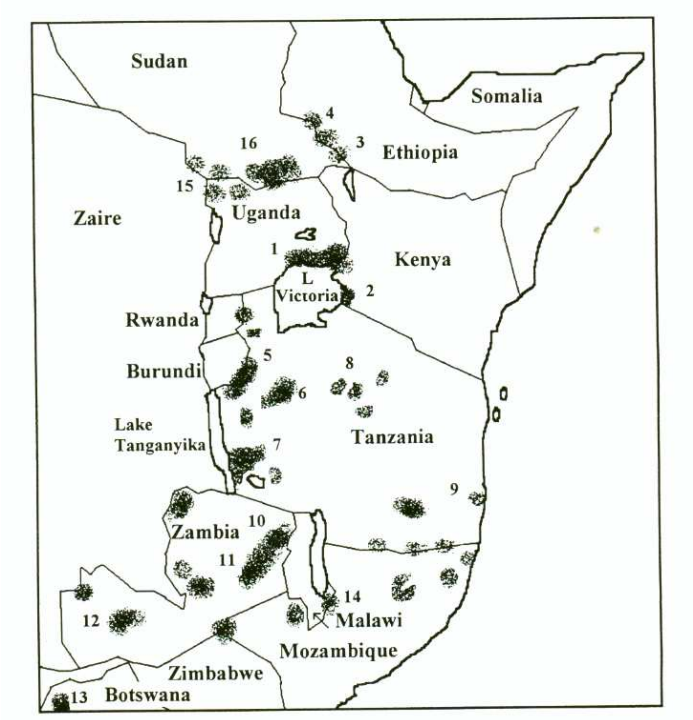
pigs and dogs are more likely to be in closer proximity to humans and other livestock than the wild animal reservoirs. Disease transmission may also be affected by tsetse feeding preferences. However an animal reservoir is not considered to be as important in the gambiense form of the disease (Welburn *et al,* 2004) as this form of sleeping sickness is chronic in nature and may be asymptomatic for a long period, in this case human-tsetse-human is considered to be the most important transmission route (WHO, 1986).

In *Trypanosoma brucei rhodesiense* the role of non-human reservoirs is considered to be much more important. Originally bushbuck were identified as a reservoir for *Trypanosoma brucei rhodesiense* (Heisch *et al.*, 1958), since that time domestic cattle are now thought to play the most important role in the transmission of human rhodesiense sleeping sickness (Hide *et al.*, 1996). In fact, study suggests that between 21% and 33% of *Trypanosoma brucei* infected animals (especially cattle) can be infected with human infective trypanosomes, at least during an epidemic and that a fly infected with *T.b.rhodesiense* is five times more likely to have picked up the infection from domestic cattle than from a human (Welburn *et al.*, 2001). This confirms the findings that cattle are the most important reservoir in rhodesiense sleeping sickness (Fevre *et al*, 2001). It was originally thought that *T.b.rhodesiense* was generally carried by *Glossina mortisans* and *Glossina pallidipes* (mortisans group) and that *T.b.gambiense* was carried by *Glossina palpalis* and *Glossina tachinoides* (palpalis group). However this distinction is now unclear, as exceptions have been found (Gibson, 1986).

In addition, the geographical distribution of *T.b.rhodesiense* sleeping sickness cases is characteristic; rhodesiense sleeping sickness is characterized by long periods of endemicity punctuated by epidemics, in well-established endemic 'foci' (See Figure 1.2.). For example the focus to the north of Lake Victoria in Uganda has seen four epidemics since 1900 interspersed by long periods of low endemicity (Hide, 1999).

For *Trypanosoma brucei brucei* the picture is more confused, although this species is not human infective or pathogenic to domestic livestock, it is still of importance. This species is found in a very wide range of wild and domestic animals, most wild game, lions and other animals (Onyango *et al*, 1966; Heisch *et al*, 1958; Ashcroft *et al*, 1959; Baker *et al*, 1967; Sachs *et al*, 1967). For species such as *Trypanosoma vivax* and *Trypanosoma congolense* much less research has been carried out so the picture is much less clear.

Figure 1.2. Foci of *Trypanosoma brucei rhodesiense* sleeping sickness in east Africa



(Hide, 1999)

1.7. Diagnostic methods

1.7.1. Microscopy

Perhaps the most widely used and simplest of diagnostic techniques are those relying on direct observation of the parasites either in blood, lymph or cerebrospinal fluid. There are many different microscopic techniques; each has its own merits. The simplest is perhaps the wet blood film method; a drop of blood is placed on a microscope slide and the slide is then examined at x40 (with x10 objective lens). This method is often used in conjunction with the preparation of a thick blood film; both are examined for diagnosis. Whilst this method is undoubtedly one of the simplest available, it is also the least sensitive. Thick blood films are slightly more sensitive as more blood is observed. These types of slides are often stained with giemsa.

A number of techniques improve on this simple observation of parasites in blood. The haematocrit centrifugation method (Woo, 1970) is widely used; is relatively simple and is considered to be more sensitive than the methods

previously described (Paris &McOdimba, 1982). However a centrifuge that can accommodate the microhaematoctrit tubes is required. Blood is mixed with sodium citrate (anticoagulant) and is drawn into the microhaematocrit tube, which are then sealed at one end. The tubes are spun in the centrifuge and then examined under the microscope; the trypanosomes will be concentrated in the area between the red blood cells and the plasma; known as`the buffy coat'. An alternative method of concentration is to filter the trypanosomes from the blood by a technique known as mini anion exchange centrifugation (m-AECT) (Lumsden *et al*, 1979). Red blood cells are filtered out as they are held within a filtration matrix by virtue of the negative charge on their surface. The trypanosomes are less negatively charged so pass through the matrix, the resultant filtrate is then centrifuged to concentrate the trypanosomes and can be detected by microscopic examination (Buscher & Lejon, 2004).

Other body fluids that are used for the diagnosis of trypanosomiasis are lymph aspirate (especially from glands draining the chancre), bone marrow (aspiration useful for early stage infection) and cerebro-spinal fluid obtained from lumbar puncture; this is widely used to confirm late stage sleeping sickness. However, some of these techniques require a high degree of skill and experience.

A range of methods exist for microscopic diagnosis of trypanosomiasis, ranging from the very simple and cost effective, to technically involved and requiring equipment that may not be practical in the field. These techniques are something of a trade off between sensitivity and complication. As trypanosomiasis is largely a problem of rural and remote areas it may not be possible to carry out techniques like mini ion exchange centrifugation in the field.

An additional problem for methods relying on direct observation is that *Trypanosoma brucei brucei, Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense* are morphologically indistinguishable; the identification of other species also requires a good deal of experience and careful observation. It is thought that microscopy techniques widely used in the field miss a large proportion of infections present, at least in the case of animal infections, which are often at lower infection intensities (Picozzi *et al.*, 2002).

1.7.2. Serodiagnostic methods

Card Agglutination Test for Trypanosomiasis (CATT)

Indirect methods rely on the detection of antibodies to a current infection. The Card Agglutination Test for Trypanosomiasis (CATT) (Magnus *et al.*, 1978) is a widely used indirect method for detection of *Trypanosoma brucei gambiense* infections only. Most mass screening programs rely on CATT, when tested on blood-impregnated filter papers a sensitivity of 91% was determined and reproducibility was found to be good (Chappuis *et al.*, 2002). Whilst it is simple to use and reasonably cost effective it does not discriminate between current and previous infections (Kanmogne *et al.*, 1996). The technique has undoubtedly proven useful but has limitations; a variable percentage of screened populations show as CATT positive with no clinical signs of infection or without visible confirmation of parasites. Limited sensitivity has also been cited as a problem; therefore some positives are missed. In addition cured patients can remain positive for up to three years (Radwanska *et al.*, 2002).

Enzyme Linked Immunosorbent Assay (ELISA)

Although there have been some protocols for detection of a wider species range of trypanosomes than for the card agglutination test (Nantulya *et al*, 1992), although initial protocols were criticized as unreliable (Rebeski *et al.*, 1997; Eisler *et al.*, 1998). Efforts have been made since to improve the repeatability and sensitivity of the methods (Lejon *et al.*, 2003b).

1.7.3. Polymerase Chain Reaction

Species diagnostic techniques for molecular epidemiological analysis are somewhat different from medical diagnostic techniques. This is because for epidemiological analysis the timeframe from sample collection to test result can be much longer. Previously analysed sample sets may be screened retrospectively with new techniques. In addition, more complex and technology intensive techniques can be employed, as much of the work is carried out in well-equipped laboratories away from the field. For medical diagnostic purposes the results of screening must be available quickly, and as a result they must be conducted in the field or as close to the field as possible. This places practical limits on the complexity of the diagnostic technique.

Since the introduction of the polymerase chain reaction (PCR) the ability to both detect and differentiate species has improved considerably. Utilizing this

technique, species-specific primers can be designed for each species of interest so that a range of species diagnostic PCR's can be carried out on a sample set.

Table 1.3. PCR primers used for species screening

Adapted from (Masiga *et al.*, 1992; Desquesnes & Dávila, 2002)

Name	Primer Sequence	Size (bp)	Target	Reference
KIN	GCGTTCAAAGATTGGGCAAT CGCCCGAAAGTTCACC	Var	Kinetoplasta	(McLaughlin *et al.*, 1996)
NRP	CGA ATG AAT ATT AAA CAA TGC GCA GT AGA ACC ATT TAT TAG CTT TGT TGC	177	*Trypanozoon*	(Moser *et al.*, 1989)
TBR	GAATATTAAACAATGCGCAG CCATTTATTAGCTTTGTTGC	164	*Trypanozoon*	(Masiga *et al.*, 1992)
Trypan	CACAATGGCACCTCGTTCCC TTAGAATGCGGCAACGAGA	300 - 400	*Trypanozoon*	(Artama *et al.*, 1992)
Trypan	TAGCGTTAGTTGAAAGC TATTATTAGA ACAGTTTCTGTAC	1350	*Trypanozoon*	(Artama *et al.*, 1992)
ILO342 & ILO343	GATCCGCAGCCGGGCCTG CCGCGGTGGCTCCTTCCC	1500	*Trypanozoon*	(Majiwa *et al.*, 1994)
MP	CAACGACAAAGAGTCAGT ACGTGT TTT GTG TATGGT	994	*T.evansi*	(Artama *et al.*, 1992)
TP	GAATCAGTGTCTTTTGAGGG AACCGTGTGTGTATTACA	500	*T.evansi*	(Diall *et al.*, 1992)
TVW	CTGAGTGCTCCATGTGCCAC CCACCAGAACACCAACCTGA	150	*T.vivax*	(Masiga *et al.*, 1992)
Il01264 & 1265	CAGCTCGCCGAAGGCCACTTGGCTGGG TCGCTACCACAGTCGCAATCGTCGTCTCAA GG	400	*T.vivax*	(Masake *et al.*, 1997b)
TV80 & TV322.24	CAGTGCTCCCGCTCGTACACGGAC GCACGCCACATAGCCGGGGAACAG	266	*T.vivax*	(Clausen *et al.*, 1998)
TSM1 TSN2	CCGGTCAAAAACGCATT AGTCGCCCGGAGTCGAT	437	*T.simiae*	(Masiga *et al.*, 1992)
TCF1 TCF2	GGACACGCCAGAAGGTACTT GTTCTCGCACCAAATCCAAC	350	*T.congolense forest*	(Masiga *et al.*, 1992)
TCS1 TCS2	CGAGAACGGGCACTTTGCGA GGACAAACAAATCCCGCACA	316	*T.congolense savannah*	(Masiga *et al.*, 1992)
TCK1 TCK2	GTGCCCAAATTTGAAGTGAT ACTCAAAATCGTGCACCTCG	294	*T.congolense Kenya Coast*	(Masiga *et al.*, 1992)
TVW1 TVW2	CTGAGTGCTCCATGTGCCAC CCACCAGAACACCAACCTGA	150	*T.vivax West Africa*	(Masiga *et al.*, 1992)

Previously the only method for achieving this was to examine the live trypanosomes microscopically. At present separate species specific techniques are used to screen for the various trypanosomes which are thought to be present, each involves an individual set of primers and an individual PCR protocol (See table 1.3.).

1.8. Diagnostic techniques and false negative results

In 1970-1973 the world health organisation conducted an extensive project designed to investigate the epidemiology and control of malaria in the Sudan Savannah of West Africa. As a quality control 20% (n = 8427) of the blood slides were examined for 400 instead of the standard 200 fields. The 20% sample was representative of the wider study with respect to age, sex, time and geographical location. In the two groups of blood films obtained for the whole of the baseline data, it was found that doubling of the standard volume of blood examined produces a relative increase in diagnosed prevalence of 10% for *Plasmodium falciparum*, 24% for *Plasmodium malariae* and 21% for *Plasmodium ovale* (Molineaux & Gramiccia, 1980). Despite an extensive 307 page report the matter was allocated only perhaps about ten lines of text and a table of results. This is however to the credit of the authors; as such matters are rarely reported in the literature. These results echoed an earlier finding, where it was noted that an increase in malaria positivity in a group of semi-immune adults resulted from prolonging the time for which blood slides were examined. It was shown that the observed parasite rate (38%) in routine 100 field thick film examinations was doubled when the examination time (and hence number of fields examined) was extended. This was due to the prevalence of scanty parasitaemias in this group of adults, which escaped detection in routine study (Dowling & Shute, 1966). Similar findings have been reported elsewhere (Bottius *et al.*, 1996; Ohrt *et al.*, 2002).

Using PCR, another study investigating the epidemiology of malaria in 369 samples from miners in an endemic area of the Brazilian Amazon reported the existence of false negative results, mainly in subjects with sub-clinical parasitaemia (Scopel *et al.*, 2004). The authors further reported that the mean infection intensity of samples positive at the first screening as 598 parasites per microlitre of blood, whilst that of the samples found falsely negative at the first

screening to have a mean infection intensity of 12 parasites per microlitre. This indicated that the false negative results are due to low intensity infections.

Direct statements of the occurrence of false negatives results for PCR tests are rare in the literature. Instead there is a wealth of 'indirect' evidence that points at the occurrence of false negative results and consequent under-detection of infection. This 'indirect' evidence largely comes from comparison of diagnostic techniques which report disagreement on which samples are infected. For a range of different pathogens, many find that PCR has given a negative result where another diagnostic technique has shown the same sample to be positive. Some examples in the literature are; malaria (Barker *et al.*, 1994; Masake *et al.*, 1997a; Gonzales *et al.*, 2006); leishmania (de Brujin *et al.*, 1993) and trypanosomiasis (LeFrancois *et al.*, 1999; Garcia *et al.*, 2000; Penchenier *et al.*, 2000; Solano *et al.*, 2002). A number of authors openly state that detection of low infection intensity samples is intermittent or difficult by PCR or any other currently available technique (Truc *et al.*, 1994; Masake *et al.*, 1997a; Garcia *et al.*, 2000; Kyambadde *et al.*, 2000; Ohrt *et al.*, 2002; Solano *et al.*, 2002; Lejon *et al.*, 2003a; Scopel *et al.*, 2004; Van den Bossche *et al.*, 2004b; Koffi *et al.*, 2006). It appears that we are happy to report and base epidemiological conclusions on a diagnosed prevalence from 200 microscopy fields, or a single PCR assay, despite the knowledge that the prevalence is dependent on the number of fields or PCR tests examined. It may be argued that as long as the diagnosed prevalence is based on a standard number of fields, or PCR assays are standardised then valid comparisons can be made. There are however problems with this argument, in comparing underestimated results, important trends and differences in the data are also underestimated. The work presented here will highlight some of these difficulties.

Information for control programmes and epidemiological studies of schistosomiasis is based on the detection and quantification of faecal egg counts (Katz *et al.*, 1972; Mott & Cline, 1980). As has been the case for other pathogens, repeated examination of samples has shown that a significant number of light (low intensity infections) may be missed (false negatives), leading to an underestimation of prevalence (Jordan *et al.*, 1975; Barreto *et al.*, 1978; Ruiz-Tiben *et al.*, 1979; Mott & Cline, 1980; Sleigh *et al.*, 1982; Polderman *et al.*, 1985; da Cunha *et al.*, 1987; Barreto *et al.*, 1990; Gryseels *et al.*, 1991). The underestimation of prevalence, even after repeated examination of samples

may be surprisingly large (de Vlas & Gryseels, 1992). These undiagnosed infections have been found to be responsible for the maintenance of transmission after chemotherapy regimes (Goddard, 1977; da Cunha *et al.*, 1987). For this reason the World Health Organisation has repeatedly advocated the use of quantitative methods in all aspects of the epidemiological study of schistosomiasis (WHO, 1967;1980). There is little in the reasoning of this recommendation by the WHO that does not potentially apply to other pathogens. Quantitative techniques have measurable variability and therefore their inherent differences in one application to another can be taken into account. This increases the accuracy of epidemiological measurements and improves the confidence and validity of conclusions derived from these data. Additionally, the use of quantitative measures improves the understanding between morbidity and intensity of infection (Mott & Cline, 1980). The use of a quantitative measure adds a further dimension to the data, rather than just measuring infected / uninfected hosts in a population, quantitative measures allow data to express how infected and record information on the distribution of the parasite. This extra dimension allows a more precise comparison of epidemiological situations, and allows the possibility to infer to what extent prevalence is being underestimated.

1.9. The sensitivity of diagnostic techniques

Examples of false negative results are perhaps not surprising, in that few doubt that whilst microscopy is an extremely practical and useful diagnostic technique, it is relatively ‘insensitive’ and misses a proportion of infections. Many studies evaluating the usefulness of microscopy have reached such conclusions (Paris *et al.*, 1982; Brown *et al.*, 1992; Snounou *et al.*, 1993; Urdaneta *et al.*, 1998; Tham *et al.*, 1999; Cavasini *et al.*, 2000; Craig *et al.*, 2002; Owusu-Agyei *et al.*, 2002; Picozzi *et al.*, 2002). All of the studies, which made comparisons between microscopy and PCR, conclude that PCR is a more sensitive technique and support adopting PCR for diagnostic testing and screening of samples.

In this case the scientific community recognised a problem in the currently used technique (lack of sensitivity of microscopy), advocated the use of a more sensitive technique (PCR) and moved on. It is worth considering the nature of sensitivity in this context. Analytical sensitivity is defined as the ability to register small physical amounts, concentrations or differences in a target. Whereas, the diagnostic sensitivity of a test is the test's ability to detect hosts with the

condition of interest in a population or group and is expressed as a proportion or percentage: the number of persons who have both the condition and a positive test result divided by the number of persons who have the condition. Diagnostic sensitivity often has more to do with the ability to obtain the target substance in a processed sample from a host who has the condition than with the ability to detect very low concentrations of a substance. If the target substance is not in the processed sample because of the vagaries of sampling or processing, an assay with perfect analytical sensitivity may still give a false negative result (Saah et al,1997). This is an important distinction, and diagnostic sensitivity, which is the more relevant to detection of parasites, deserves further consideration. For the detection of parasites by microscopy and PCR we have a minimum unit, a single parasite. Microscopy is certainly capable of detecting a single parasite if the parasite happens to be present in the field of view. The ability of PCR to detect a single pathogen is also dependent upon the pathogen being present in the analysed sample volume. Somewhat ironically, not all PCR protocols are capable of detecting a single pathogen even if that pathogen is within the sample assayed by the PCR (Njiru *et al*, 2005; Li *et al*, 2007).

Some PCR protocols make claims to be able to detect fractional parts of a parasite. For example, a species specific real time PCR assay for detection of *T. brucei* (Becker *et al.*, 2004) claims to be able to detect 0.1 of a trypanosome. This claim is of course a result of using extracted DNA in solution and detecting a few copies of the many repeat copies forming the target sequence. Such claims are complicated by the fact that the target sequences are present on the genome in groups or clusters, and we have little knowledge of how they may 'break up' in solution. For practical purposes the detection limit of both PCR and microscopy is a single parasite. The question of sensitivity is then not being able to detect ever decreasing fractions of a parasite – analytical sensitivity, but the capturing of a single parasite in the volume of blood or sample analysed by the diagnostic technique – diagnostic sensitivity. For small assay volumes and low infection intensities there is a large degree of chance that can effect the probability of obtaining a positive diagnostic result from an infected sample. Diagnostic sensitivity is therefore a stochastic process, doubling the assay volume doubles the probability of capturing a parasite for any particular level of infection intensity. The reason why microscopy is less diagnostically sensitive than PCR is that it assays a smaller volume of blood or tissue, so there is less

probability of obtaining the parasite in the volume of sample analysed. This gives rise to a further complication, the probability of obtaining the parasite in the analysed volume is also affected by the infection intensity in the host, or mean intensity present in the host population. Since this may differ in different epidemiological settings (endemic versus epidemic for example) the diagnostic sensitivity may likewise differ in different epidemiological settings. This phenomenon is known as 'spectrum bias' (Ransoff & Feinstein, 1978).

The introduction of the technically complex PCR screening protocol has given an increase in sensitivity by increasing the volume of sample analysed. Microscopy is capable of analysing perhaps about 0.13 microlitres for a thin film and 0.32 microlitres for a thick film (Dowling & Shute, 1966). PCR is capable of analysing, typically, one microlitre (Cox *et al.*, 2005). The issue of sensitivity is then perhaps a misnomer, the real issue is the volume of sample assayed. A single microlitre still represents a very tiny proportion of the total blood volume of a typical host. In the light of this information an important question arises; Has the original problem as previously described for the 'sensitivity' of microscopy really been solved or just shifted?

1.10. Dealing with false negative results – previous work

In spite of the wide reporting of underestimation of prevalence for many different pathogens (See previous discussion), remarkably few attempts have been made to deal with the problem. Only in the field of schistosomiasis has any serious attempt been made to address the problem of underestimation. For example, on the basis of data obtained from a study of eight communities in St Lucia, the prevalence obtained from a single screening was related mathematically to the cumulative prevalence obtained from three screenings of the samples (Jordan *et al.*, 1975). In this way an improved estimation of true prevalence could be gained from applying a formula describing the relationship between diagnosed prevalence from a single screening to cumulative prevalence from three screenings. However, this method does not take into account the fact that the prevalence from three repeated screenings may still also underestimate the population prevalence by an unknown amount. Additionally, the formula describing the relationship is likely only to apply to that particular population and screening methodology – spectrum bias. Whilst this method represented an improvement, clearly a more sophisticated methodology is required. It has been shown that the relationship between the diagnostic sensitivity and prevalence

(Jordan *et al.*, 1975), is in fact due to the relationship between prevalence and infection intensity (Goddard, 1977). Goddard and co-workers modelled the probability of false negative outcome as a negative exponential function of the prevalence. A system of re-examination of a proportion of the negative samples in order to determine the false negative rate (FNR) has also been introduced to large studies (Jordan *et al.*, 1975; Molineaux & Gramiccia, 1980). Again all these methods still significantly underestimate the true prevalence.

A more sophisticated and interesting approach to solving the problem was attempted by de Vlas and co-workers (1992a), they developed a stochastic model incorporating inter and intra individual variation in schistosoma egg counts (based on a negative binomial distribution) to predict the true prevalence of infection from single egg counts per host. The parameters for the model were derived from a number of field studies. The model was later successfully validated on data obtained from another field study (de Vlas *et al.*, 1992b). Although certain parameters of the model were found to be independent of the endemic situation, others have to be re-estimated for each particular setting. In order to address this inconvenience the authors constructed a reference chart that makes possible a projection of the true prevalence from any observed data set (de Vlas *et al.*, 1993), although the data must be stratified into discrete age classes. Whilst this model is undoubtedly useful for schistomiasis, it is based on estimation of parameters from a number of studies examining the intra and inter individual variation in egg counts. Such detailed data is not available for many other parasites, and the model for schistosomiasis does not have the added complication that the parasites replicate within the host, as is the case for protozoans.

1.11. Quantitative measures of infection intensity

For blood borne parasites there is some difficulty in obtaining quantitative measures of infection intensity. Real time PCR may seem to be ideally suited to quantifying the pathogen load in a blood samples. However, real time PCR is essentially a PCR reaction, and suffers from the same problems as non-quantitative PCR in detecting low infection intensities. It is therefore only useful to apply to samples where there exists prior knowledge that the repeatability of the sample is good (indicating a high infection intensity), that is samples where false negatives do not occur. A second problem with real time PCR, at least for trypanosomiasis where there are multiple coinfecting species to consider, is that

there is currently only one published protocol (for detecting *T.brucei*) (Becker *et al.*, 2004). There also appears to be a degree of over-optimism regarding the abilities of real time PCR technology. There is a tendency to be more likely to overlook problematic aspects in new high technology solutions than with older tried and tested lower technology methods. For example, Becker *et al* (2004) developed a real time PCR assay for *T. brucei* and claimed to be able to detect 100 parasites per millilitre. This claim gave the false impression that an infection intensity of this level could be reliably quantified. In actual fact, on scrutiny of the results, at parasite dilutions of 100 parasites per millilitre the cycle threshold (CT) value obtained was not in the linear range of the standard curve. This meant that the detection limit of the RT-PCR was indeed 100 parasites per millilitre, as a positive result was evident. However, the parasite intensity could not be reliably quantified at this level. In fact the limit of quantification was tenfold higher at 1000 parasites per millilitre. In fact when the RT-PCR was tested on 13 samples from parasitologically confirmed *T. brucei gambiense* patients, all patients were detected as positive, but could not be quantitatively assessed because the CT values fell outside the linear range of the standard curve. This, even though the positive samples used had been found positive by a, supposedly, less sensitive technique - microscopy. Furthermore, this protocol analysed 4 microlitres of template DNA, adapting an ordinary PCR assay, with the ability to detect a single parasite, such as ITS-PCR (Cox et al, 2005), to analyse this volume of sample would in principle increase the theoretical detection limit to 250 parasites per millilitre. This real time PCR assay is therefore still unable to accurately quantify the levels of infection intensity needed to assess sub-patent infections.

Quantification of infection intensity with microscopy.

Microscopy has been the method of choice for study of micro-parasites for many years, in more recent times it has been criticised because of a lack of sensitivity and specificity (Picozzi et al., 2002). However, microscopy can readily provide quantitative data. Although less sensitive than PCR; the quantitative data provided by microscopy may still be useful in determining the distribution of parasites with the host population. The accuracy of inferring the distribution of parasites within a host population by use of microscopy needs empirical support. Yet, should this prove practical, microscopy may be extremely effective. The difficulty with microscopy is the small volume of blood analysed, typically

0.3 microlitre per 200 fields of a thick film (Dowling & Shute, 1966). The scope for increasing this analysed volume is limited as 200 fields of view per sample is perhaps the limit of human endurance for a large epidemiological study. Nevertheless, some of the problems previously described for real time PCR also apply to the use of microscopy.

1.12. Appropriateness of the negative binomial distribution in describing the distribution of trypanosomes amongst the host population

Before directly discussing the appropriateness of the negative binomial distribution to modelling the distribution of trypanosomes (or other pathogens) in a host population, a general outline of the negative binomial may be worthwhile. Parasitic organisms tend to be aggregated within or upon their hosts, with most hosts having low level infections with or no infection at all and a few hosts harbouring high levels of the pathogen in question. For example it is typical for 80% of the population of helminth parasites of humans to be present in only 15% of individuals (Gregory & Woolhouse, 1993). This phenomenon is variously termed ‘togetherness’, ‘aggregation’, ‘patchiness’, ‘contagion’ or ‘over-dispersion’ (the later term will be used here). Such ‘overdispersed’ populations tend to have a variance which is greater than its mean, and indeed one measure of over-dispersion is the variance to mean ratio. When the pathogen population is not distributed randomly throughout the host population and tends toward aggregation, the frequency distribution of the different levels of abundance of the pathogen are best modelled with a negative binomial distribution. The negative binomial distribution is described by the mean and the dispersion factor k (another commonly used measure of over-dispersion). The dispersion factor k is an inverse measure of over-dispersion, as k approaches a value of zero the population is said to be over-dispersed, as k approaches infinity the population is said to be randomly distributed, in practice populations with values of k below eight are said to be over-dispersed (Elliot, 1977).

Aggregation within the host population may be due to heterogeneity within the host species, this may be due to age or differential susceptibility to the pathogen, heterogeneity may also be due to the physical distribution of the pathogen in the environment or vector. The nature of this over-dispersion is of paramount importance for the transmission dynamics of the parasites in question. Often the tail of a negative binomial distribution, those hosts having

the highest infection intensities, play an important role in the transmission and persistence of infection (Medley & Anderson, 1985), and represent an important target for control strategies. It has been shown that an over-dispersed distribution of parasites amongst their hosts can reduce the levels of competition between species of parasites inhabiting the same hosts or vectors (Pacala & Dobson, 1988) and influence the regulation of host parasite communities (Anderson, 1982).

Over-dispersion is an important characteristic of many populations, the negative binomial distribution has been shown to be the most appropriate empirical distribution describing populations of benthic macro-invertebrates (Orroth *et al.*, 2003), weed species (Gonzalez-Andujar & Saavedra, 2003), cotton bollworms (Beyo *et al.*, 2004), mosquito's (Zhou *et al.*, 2004), ticks (Barrett *et al.*, 1997; Tyre *et al.*, 2003). Parasitic helminth populations have been most widely investigated, and it is remarkable how universally over-dispersed are the populations of various, filarial worms (*Wucheria bancrofti, Brugia pharangi* & *Ochocerca spp*.) (Srividya *et al.*, 1991; Vivas-Martinez *et al.*, 2000; Snow & Michael, 2002), nematodes (*Ascaris* spp) (Guyatt & Bundy, 1993), schistosomes (*Schistosoma* spp.) (Eppert *et al.*, 2002; Feng *et al.*, 2004) and tapeworms (*Taeniaeformis* spp.) (Theis & Schwab, 1992). Much less work has been conducted for microparasites, in this case the importance of overdipersion is less well researched. However, over-dispersion has been shown in the protozoan *Cryptosporidium molnari* (Sitja-Bobadilla *et al.*, 2005), *Borreliae* spp in the tick (Hubalek *et al.*, 1998), *Babesia* spp in engorged female ticks (Guglielmone *et al.*, 1997), *Theileria* spp from cattle (Flach *et al.*, 1993) *Trypanosoma cruzi* (Pecora *et al.*, 1980) and in the distribution of *Plasmodium.*

Chapter 2: A PCR based assay for detection and differentiation of African trypanosome species in blood.

2.1. Introduction

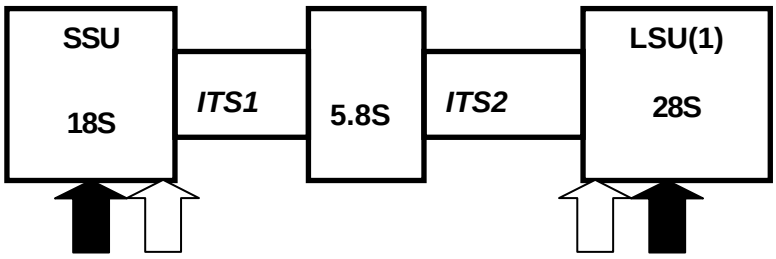
Effective control and management of African trypanosomiasis depends heavily upon knowledge of the epidemiology of the disease, which in turn relies upon methods that incorporate screening of both animal and human populations (Hutchinson et al., 2003). Current methods of epidemiological screening include direct parasite examination using traditional dark ground microscopy, examination of buffy-coat and more recently molecular methodologies based on the polymerase chain reaction (PCR). Microscopy is labour intensive and can lack sensitivity under field conditions due to routinely low peripheral parasitaemia in infected livestock (Picozzi et al., 2002). It is believed that PCR based diagnostic methods have largely overcome difficulties associated with sensitivity and specificity. A number of methods have been developed for the following species and subspecies of Trypanosoma – Trypanozoon (Artama et al., 1992, Kabiri et al., 1999), *Trypanosoma congolense* (Riverine/Forest) (Masiga et al., 1992), *Trypanosoma congolense* (Kilifi) (Masiga et al., 1992), *Trypanosoma congolense* (Savannah) (Masiga et al., 1992), *Trypanosoma vivax* (Masake et al., 1994, Masake et al., 1997), *Trypanosoma simiae* (Masiga et al., 1992), *Trypanosoma evansi* (Artama et al., 1992), *Trypanosoma congolense* (Kenya Coast) (Masiga et al., 1992), *Trypanosoma theileri* (Rodrigues et al., 2003). Using these approaches accurate species / sub species differentiation requires up to eight different PCR reactions per sample, which increases the costs and impacts on the practical application of the technique for large-scale epidemiological studies (Table 1.3). Furthermore, many of the PCR techniques developed in recent years are based on complex protocols requiring samples to be mouse passaged, and therefore mouse adapted, a process which some trypanosome isolates do not survive (Hoare, 1972, Masiga et al., 1992) resulting in loss of species or strains, selection and sampling bias (Welburn and Coleman, 2004).

Recent developments in matrices for sample collection and archive, which permit direct PCR identification from tissue / fluids may overcome such bias. Simplified protocols incorporating these improved sample collection techniques, together with rapid PCR-based screening methodologies for the direct analysis of field samples are therefore required. The internal transcribed spacers (ITS) located within the ribosomal RNA genes (Figure 2.1) have been used to establish relationships and differentiate species in an extremely wide range of organisms (Wesson et al., 1992, Schlotterer et al., 1994, Mai and Coleman, 1997, Samuel, 1998). A high copy number combined with inter-species length variation makes the ITS region a useful

marker for species differentiation in trypanosomes, as has been recently demonstrated (McLaughlin et al., 1996, Desquesnes et al., 2001, Njiru et al., 2004). However, this technique was shown to be relatively insensitive and in some cases was problematic for detection of *Trypanosoma vivax* (the principal pathogenic species in cattle) in either concentrated genomic DNA or DNA extracted from field samples. In addition these techniques have not previously been evaluated for use directly on samples of whole blood. Here we test the technique developed by Desquesness *et al* (2001) on samples of whole blood and report the development of a simple nested PCR method, which detects the inter-specific length variation of the ITS regions of ribosomal genes and thereby producing a unique size of PCR product for each species of trypanosome. The technique is able to detect the following African trypanosome species. (Trypanozoon , *Trypanosoma congolense* (River/Forest) , *Trypanosoma congolense* (Kilifi) , Trypanosoma congolense (Savannah) , *Trypanosoma vivax* , *Trypanosoma simiae*, *Trypanosoma evansi*, *Trypanosoma congolense* (Kenya Coast) and *Trypanosoma theileri*). It is able to detect a single trypanosome per analysed blood volume and has been optimised for PCR amplification of blood applied to filter paper (Whatman FTA™) permitting direct PCR analysis of field material.

The work presented in this chapter was published in a peer reviewed journal (Cox *et al*, 2005)

Figure 2.1. The structure of part of the ribosomal RNA gene locus



Ribosomal genes are present in tandem arrays of around 100 – 200 copies per trypanosome. Each gene consists of a number of conserved coding regions and non-coding spacer regions. Large boxes represent conserved coding regions (SSU = Small sub-unit, LSU = Large subunit) and small boxes represent spacer regions. The two spacers, internal transcribed spacers (ITS) 1 and 2 are known to vary in size between species and occasionally sub species. A set of nested primers designed to the conserved regions are represented by black arrows (outer primers) ITS1 & ITS2 and white arrows (inner primers) ITS3 & ITS4.

2.2. Materials & Methods

2.2.1. Samples

Field samples consisted of two hundred and forty five samples of bovine blood taken from two villages in the Soroti & Tororo districts of Uganda and collected on Whatman FTA™ cards. Genomic DNA stocks are as detailed in Table2.1.

Table 2.1. Details and origin of trypanosome genomic DNA used in the development of the ITS-PCR protocol

Species	Stock Code	Origin
Trypanosoma brucei brucei	BUTEBA135	Tororo, SE Uganda, Cow, 1990
Trypanosoma brucei rhodesiense	BUG H2	Kamuli, Uganda, Human, 2000
Trypanosoma brucei rhodesiense	DO	Katerema, Uganda Human,1990
Trypanosoma congolense (Savannah)	IL1180 (ILNat3.1)	Serengeti, Tanzania
Trypanosoma congolense (Forest)	IL3900	Burkina Faso
Trypanosoma congolense (Kalifi)	IL45.1	Kilifi, Kenya
Trypanosoma vivax	ILDatt1.2	Kenya
Trypanosoma brucei	OBUR C19	Soroti, Uganda, Cow, 2000
Trypanosoma congolense (Forest)	TSW103	Liberia, Pig
Trypanosoma simiae	TV008	Unknown

2.2.2. Sample storage

Samples were either stored as extracted liquid DNA which was stored at -20^{o}C for long term storage or 4^{o}C for short term storage. When in use the DNA samples were kept on ice. Alternatively the samples were stored (dry) on treated filter paper of one of two types, Whatman FTA Cards (Whatman Biosciences, Cambridge, UK) or IsoCode (Schleicher & Schuell Bioscience, Inc). Samples were stored at room temperature in foil pouches with silica desiccant in each pouch to protect against strong light and moisture.

2.2.3. FTA cards

Preparation of samples with Whatman FTA cards

Blood & other samples were applied to the cards in accordance with recommendations of Whatman Biosciences. Samples were applied in a spiral

pattern with care not to over saturate the filter paper. Once applied the cards were allowed to dry for a minimum of ninety minutes at room temperature.

FTA purification protocol

A 3mm punch was removed from the sample, transferred to a suitable eppendorf tube, to which 200µl of FTA purification reagent was added (Whatman Biosciences, Cambridge, UK). The solution was then mixed by pippetting the solution up and down twice. The solution was then incubated for 5 minutes at room temperature. This process was repeated for a total of three times. After the final wash the purification reagent was carefully removed and 200µl of TE buffer (Sigma Aldrich, Dorset, UK) was added to the tube containing the washed punch, this was then incubated for 5 minutes. This step was then repeated one further time. The remaining TE buffer was discarded and the punches carefully transferred to a clean PCR tube. The 'wet' punch was then allowed to dry at room temperature for 90 minutes before a PCR reaction was performed.

2.2.4. Polymerase Chain Reaction protocols

Re-suspension of stock primers

Primers were re-suspended in TE buffer to a working concentration of 100µM and stored frozen at –20^{o}C. The working stock was then diluted to a concentration of 10µM before use and stored at 4^{o}C.

ITS PCR

Although the development of ITS-PCR is described in this chapter, the full protocol is given here for ease of reference. ITS PCR is targeted to the ribosomal genes of African trypanosomes. The expected band size for a positive result will vary dependent upon the species of trypanosme (Table 2.4) The PCR was carried out using a nested method as two separate consecutive reactions. The primer sequences were - outer primers ITS1 (5' – GAT TAC GTC CCT GCC ATT TG – 3'), ITS2 (5' – TTG TTC GCT ATC GGT CTT CC – 3') and inner primers ITS3 (5' – GGA AGC AAA AGT CGT AAC AAG G – 3'), ITS4 (5' – TGT TTT CTT TTC CTC CGC TG – 3'). All primers were synthesised by MWG Biotech. Each PCR was carried out using 25ul volumes containing the following components. 10mM Tris-HCl pH 9.0, 1.5mM MgCl2, 50mM KCl, 0.1% TritonX-100, 0.01% (w/v) stabilizer (Purchased as a 10X SuperTaq PCR buffer from HT biotechnologies, Cambridge), 2µM of each primer, 1mM total dNTP's and 1.25 Units of Biotaq (Bioline Ltd, London) and 1µl of extracted template DNA or a single 3mm diameter washed and dried punch from the blood sample applied to a Whatman FTA card. The reaction conditions were as

follows; one cycle of 95°C for seven minutes, followed by 35 cycles of 94°C for 1 minute, 54°C for 1 minute and 72°C for 2 minutes. The thermal cycling was carried out on a Stratogene – Robocycler. All PCR conditions were optimised using modified 'Taguchi' methods (Cobb & Clarkson, 1994).

Trypanosoma brucei specific PCR

Samples were screened for the presence of *Trypanosoma brucei* using primers targeted to a 177bp satellite DNA repeat present in the mini chromosomes (Moser *et al.*, 1989). The expected amplicon size for a positive result is 164bp.

The PCR amplification was carried out using the following primer sequences - TBR-1 (5' – GAA TAT TAA ACAATG CGC AG– 3') & TBR-2 (5' – CCA TTT ATT AGC TTT GTT GC– 3'). All primers were synthesised by MWG Biotech. Each PCR was carried out using 25µl volumes containing the following components. 10mM Tris-HCl pH 8.3, 1.5mM $MgCl_2$, 50mM KCl, 0.01% gelatin, 200µM of each of the four dNTP's, 10µM of each oligonucleotide primer, 1.25 Units of Taq (*Thermus aquaticus*) DNA polymerase (Perkin Elmer Cetus, Norwalk, Connecticut) and 1µl of extracted template DNA or a single 3mm diameter washed and dried punch from the blood sample applied to a Whatman FTA card. The reaction conditions were as follows; one cycle of 94°C for seven minutes, followed by 27 cycles of 94°C for 30 seconds, 55°C for 60 seconds, followed by 72°C for 30 seconds.

Trypanosoma congolense (Forest) Specific PCR

Samples were screened for the presence of *Trypanosoma congolense* (Forest) using primers targeted to a satellite DNA repeat present in the mini chromosomes (Masiga *et al*, 1992). The expected amplicon size for a positive result is 350bp.

The PCR amplification was carried out using the following primer sequences; TCF-1 (5' – GGA CAC GCC AGA AGG TAC TT– 3') & TCF-2 (5' – GTT CTC GCA CCA CCA AC – 3') all primers were synthesised by MWG Biotech. Each PCR was carried out using 25µl volumes containing the following components; 10mM Tris-HCl pH 8.3, 1.5mM $MgCl_2$, 50mM KCl, 200µM of each of the four dNTP's, 1µM of each oligonucleotide primer, 2.5 Units of Biotaq (Bioline Ltd, London) and 1µl of extracted template DNA or a single 3mm diameter washed and dried punch from the blood sample applied to a Whatman FTA card. The reaction conditions were as follows: one cycle of 94°C for three minutes, 30 cycles of 94°C for 1 minute 60°C for 2 minute and 74°C for 30 seconds. The thermal cycling was carried out on a Peltier Thermal Cycler DYAD DNA Engine (MJ Research, Waltham, MA, USA).

Trypanosoma congolense (Savannah) Specific PCR

Samples were screened for the presence of *Trypanosoma congolense* (Savannah) using primers targeted to a satellite DNA repeat present in the mini chromosomes (Masiga *et al*, 1992). The expected amplicon size for a positive result is 316bp.

The PCR amplification was carried out using the following primer sequences; TCS-1 (5' – CGA GAA CGG GCA CTT TGC GA– 3') & TCS-2 (5 CCC GCA CA – 3') all primers were synthesised by MWG Biotech. Each PCR was carried out using 25µl volumes containing the following components: 10mM Tris-HCl pH 8.3, 1.5mM $MgCl_2$, 50mM KCl, 0.01% gelatin, 200µM of each of the four dNTP's and 1µM of each oligonucleotide primer and 1.25 Units of Taq (*Thermus aquaticus*) DNA ploymerase (Perkin Elmer Cetus, Norwalk, Connecticut). 1µl of extracted template DNA or a single 3mm diameter washed and dried punch from the blood sample applied to Whatman FTA cards. The reaction conditions were as follows: one cycle of 94^{o}C for seven minutes once followed by 27 cycles of 94^{o}C for 30 seconds, 55^{o}C for 60 seconds and 72^{o}C for 30 seconds. The thermal cycling was carried out on a Peltier Thermal Cycler DYAD DNA Engine (MJ Research, Waltham, MA, USA).

Trypanosoma congolense (Kilifi) Specific PCR

Samples were screened for the presence of *Trypanosoma congolense* (Kilifi) using primers targeted to a satellite DNA repeat present in the mini chromosomes (Masiga *et al*, 1992). The expected amplicon size for a positive result is 294bp.

The PCR amplification was carried out using the following primer sequences TCK-1 (5' – GTG CCC AAA TTT GAA GTG AT– 3') and TCK-2 (5' CCA TT ATT AGC TTT GTT GC – 3') all primers were synthesised by MWG Biotech. Each PCR was carried out using 25µl volumes containing the following components: 10mM Tris-HCl pH 8.3, 1.5mM $MgCl_2$, 50mM KCl, 200µM of each of the four dNTP's, 1µM of each oligonucleotide primer, 2.5 Units of Biotaq (Bioline Ltd, London) and 1µl of extracted template DNA or a single 3mm diameter washed and dried punch from the blood sample stored on a Whatman FTA card. The reaction conditions were as follows, one cycle of 94^{o}C for three minutes, 30 cycles of 94^{o}C for 1 minute, 60^{o}C for 2 minutes, 74^{o}C for 30 seconds. The thermal cycling was carried out on a Peltier Thermal Cycler DYAD DNA Engine (MJ Research, Waltham, MA, USA).

Trypanosoma vivax Specific PCR

Samples were screened for the presence of *Trypanosoma vivax* using primers targeted to a satellite DNA repeat present in the mini chromosomes (Masiga *et al*, 1992). The expected amplicon size for a positive result is 150bp.

The PCR amplification was carried out using the following primer sequences TVW-1 (5' – CTG AGT GCT CCA TGT GCC AC– 3') & TVW-2 (5' –CCA CCA GAA CAC CAA CCT GA – 3') all primers were synthesised by MWG Biotech. Each PCR was carried out using 25µl volumes containing the following components: 10mM Tris-HCl pH 8.3, 1.5mM $MgCl_2$, 50mM KCl, 200µM of each of the four dNTP's, 1µM of each oligonucleotide primer, 2.5 Units of Biotaq (Bioline Ltd, London) and 1µl of extracted template DNA or a single 3mm diameter washed and dried punch from the blood sample stored on a Whatman FTA card. The reaction conditions were as follows; one cycle of 94^{o}C for three minutes, followed by 30 cycles of 94^{o}C for 1 minute, 60^{o}C for 2 minutes, 74^{o}C for 30 seconds. The thermal cycling was carried out on a Peltier Thermal Cycler DYAD DNA Engine (MJ Research, Waltham, MA, USA).

Mammalian Specific Tubulin PCR

This PCR method is targeted to mammalian tubulin genes and is as described by Terry *et al* (2001). Tubulin genes are present in thousands of copies throughout the mammalian genome and the sequences of the primers are specific to mammalian tubulin only. This protocol is often used to verify the integrity of DNA samples.The PCR was carried out using the following primer sequences;

MtubF (5'-CGTGAGTGCATCTCCATCCAT-3'), & MtubR (GCCCTCACCCACATACCAGTG-3') all primers were synthesised by MWG Biotech. Each PCR was carried out using 25µl volumes containing the following components, 10mM Tris-HCl pH 9.0, 1.5mM MgCl2, 50mM KCl, 0.1% TritonX-100, 0.01% (w/v) stabiliser (Purchased as a 10X SuperTaq PCR buffer from HT biotechnologies, Cambridge), 2uM of each primer, 1mM total dNTP's ,1.25 Units of Biotaq (Bioline Ltd, London) and 1µl of extracted template DNA or a single 3mm diameter washed and dried punch from the blood sample stored on a Whatman FTA card. The reaction conditions were as follows; one cycle of 94^{o}C for five minutes, 40 cycles of 94^{o}C for 50 seconds followed by 55^{o}C for 1 minute, 72^{o}C for 1 minute 30 seconds with a final step of 72 ^{o}C for 10 minutes.

2.2.5. DNA extraction

DNA was extracted from blood samples by use of a QIAamp DNA Blood Mini Kit (Qiagen GmbH, Germany).The protocol was as per the manufacturer's instructions. Briefly the samples and AE Buffer were allowed to equilibrate at room temperature (15-25^{o}C). 20µl of protease were pipetted into the 1.5ml microcentrifuge tube provided. To this 200µl of sample were added, then 200µl of buffer AL was added. The resulting mix was then vortexed for 15 seconds. The sample was then

incubated at 56^{o}C for 10 minutes. The sample was then subject to centrifugation in order to remove drops from the inside of the tube. 200µl ethanol (96-100%) was added to the tube and the mix was again subject to pulse vortexing for 15 seconds. The tube was again centrifuged briefly. The mixture was then carefully added to the QIAamp spin column, which was placed in a 2ml collection tube, this was centrifuged at 6000xg (8000rpm) for 1 minute. The spin column was then placed in a clean 2ml collection tube and the previous filtrate was discarded. 500µl of buffer AW1 was added to the spin column and the sample was subject to centrifugation at 6000xg (8000rpm) for 1 minute. Again the spin column was placed in a clean 2ml collection tube and the previous filtrate discarded. 500µl of Buffer AW2 was added to the spin column, the sample was then centrifuged at 20,000xg (14000rpm) for 3 minutes. The spin column was placed in a fresh collection tube and the previous filtrate was discarded. 200µl of buffer AE was added and the sample was incubated at room temperature for 1 minute, the sample was then centrifuged for the final time at 6000xg (8000rpm) for 1 minute. The resulting liquid contains the extracted DNA.

2.2.6. Extraction of DNA from agarose gels

PCR product was extracted from agarose gels using a QIAquick Gel Extraction Kit. (Qiagen GmbH, Germany). The protocol was as the manufacturers instructions. Briefly the band of interest was excised from the gel taking care to leave as little excess gel as possible and using clean DNA free blades for each excision. The slice was weighed and three volumes of buffer QG were added to 1 volume of gel (100µl buffer for100mg weight of gel). This was incubated at 50^{o}C for 10 minutes, until the gel slice had dissolved. One volume of isopropanol alcohol was then added to the mix. The mixture was placed in a QIAquick spin column which in turn was placed in a 2ml collection tube. This was then centrifuged for one minute. The spin column was then placed in a fresh collection tube and 0.5ml of buffer QG was added. The previously collected filtrate was discarded. The column was then centrifuged for one minute. The resulting filtrate was discarded and the column placed in a fresh collection tube and centrifuged for an additional minute at 13000xg (~13900rpm). The spin column was then placed in a clean collection tube and 50µl of buffer EB (10mM Tris-Cl, pH 8.5) added. This was then centrifuged for 1 minute. The resulting eluate contains the extracted PCR product (DNA).

2.2.7. Sequencing of PCR products

In order to sequence the PCR products the amplicons were eluted from the gel as described in Section 2.2.6. The resulting eluate was sent for sequencing to Lark

Technologies Inc. (Takely, Essex,UK) as per recommendations. Sequences received from Lark Technologies were analysed using Bioedit sequence alignment editor version 5.0.9 (Hall, 1999) available from http://www.mbio.ncsu.edu/BioEdit/bioedit.html and MegAlign version 4.0 (DNA Star Inc, Madison, WI, USA).

2.2.8. Gel loading buffer

The loading buffer used in electrophoresis of the PCR products consisted of the following 15% Ficoll, 10mM Tris pH8.0, 10mM EDTA, 0.1% SDS, 0.1% Bromophenol Blue in distilled water.

2.2.9. Electrophoresis & gel visualisation

In all cases electrophoresis was carried out with a 30cm by 20cm 1.5% agarose gel. The gel was prepared by adding 10ml of Tris-Borate-EDTA 10x concentrate (Sigma Aldrich, Dorset, UK) to 90ml of distilled water and then adding Ethidium Bromide to the buffer at 0.5g ml^{-1}. To this buffer 1.5g of general purpose agarose (Sigma Aldrich, Dorset, UK) was added. The mixture was heated until the agarose was disolved, poured into the tray and allowed to cool until set. 10µl of PCR product was mixed with an equal quantity of loading buffer, this was loaded into the wells.10µl of Superladder-Mid 100bp Ladder (Abgene, Epsom, UK) size reference marker was loaded, at each end of the row, on each gel. The electrophoresis was then conducted at 100 volts for around one hour. The resulting gel was then visualised on a Biorad electrophoresis gel visualiser (Milan, Italy) and bands were sized using Biorad Gel Doc, Quantity One v4.1.0 (Biorad, Hercules, CA, USA).

2.2.10. Investigation of PCR performance on different dilution series types

In order to evaluate existing protocols targeted to ribosomal genes for use directly on whole blood samples, a set of different types of dilution series were constructed. The aim of this was to measure the relative performance of the PCR (KIN primers) as described by McLaughlin *et al* (1996). The dilution series were constructed to provide a progression from genomic DNA suspended in water (on which the protocols performed well) to whole trypanosomes in blood (on which the tested protocol did not work) by adding stepwise one element (filter paper sample media, genomic DNA, whole trypanosomes, blood serum, red blood cells, white blood cells) at a time. In this way the cause of any inhibition of the PCR could be ascertained. The details of construction of each of the different types of dilution series are outlined below.

Dilution series 1: Genomic DNA in liquid form

A stock of *T.brucei* Buteba135 genomic DNA at a concentration of 80µg ml^{-1} was diluted with sterile water in tenfold dilutions, the dilutions used ranged from neat genomic DNA to a dilution of $1:10^6$; calculated to be an equivalent of a single trypanosome genome per microlitre.

Dilution series 2: Genomic DNA placed on treated filter paper

The stock of *T.brucei* Buteba135 genomic DNA was diluted with sterile water in tenfold dilution, the dilutions used ranged from neat genomic DNA to a dilution of $1:10^6$. The dilutions were then placed on to Whatman FTA cards and allowed to dry before PCR analysis.

Dilution series 3: Trypanosomes placed on treated filter paper

T.brucei Buteba135 cultured metacyclic trypanosomes at an original concentration of 10^9 trypanosomes per millilitre were diluted in phosphate buffered saline. The tenfold dilution series ranged from 10^6 trypanosomes per millilitre to a $1:10^8$ dilution of the original stock. The dilutions were then placed on to Whatman FTA cards and allowed to dry before PCR analysis.

Dilution series 4: Trypanosomes and blood placed on treated filter paper

T.brucei Buteba135 cultured procyclic trypanosomes were diluted in bovine blood. The tenfold dilution series ranged from 10^6 trypanosomes per millilitre to a $1:10^8$ dilution of the original stock. The dilutions were then placed on to Whatman FTA cards and allowed to dry before PCR analysis.

Dilution series 5: Red blood cells with trypanosomes placed on treated filter paper

White blood cells were removed from uninfected bovine blood by extraction of the buffy coat. The red blood cell suspension was then spun and the plasma removed in order to concentrate the red blood cells. The enriched red blood cell suspension was then subject to tenfold dilution with phosphate buffered saline to a final dilution of $1:10^8$. To each dilution a volume of *T.brucei* Buteba135 procyclic trypanosomes were added so that the final number of trypanosomes for each of the dilutions was 10^3 trypanosomes per millilitre. The dilutions were then placed on to Whatman FTA cards and allowed to dry before PCR analysis.

Dilution series 6: White blood cells with trypanosomes placed on treated filter paper

Red blood cells were removed from uninfected bovine blood by extraction of the buffy coat. The white blood cell suspension was then spun and the plasma removed

in order to concentrate the red blood cells. The enriched white blood cell suspension was the subject to tenfold dilution with phosphate buffered saline to a final dilution of $1:10^8$. To each dilution a volume of *T.brucei* Buteba135 metacyclic trypanosomes were added so that the final number of trypanosomes for each of the dilutions was 10^3 trypanosomes per millilitre. The dilutions were then placed on to Whatman FTA cards and allowed to dry before PCR analysis.

Dilution series 7: Plasma with trypanosomes placed on treated filter paper

The plasma was extracted from a sample of uninfected bovine blood by centrifugation. The plasma was then subject to tenfold dilutions using phosphate buffered saline to a final dilution of $1:10^8$. To each dilution a volume of *T.brucei* Buteba135 metacyclic trypanosomes were added so that the final number of trypanosomes for each of the dilutions was 10^3 trypanosomes per millilitre. The dilutions were then placed on to Whatman FTA cards and allowed to dry before PCR analysis.

2.2.11. Measurement of trypanosome and DNA concentrations

All cell counts were made using a haemocytometer; each count was calculated from an average of separate counts from five aliquots from the samples. In the case of trypanosome counts these were also verified by an independent count by another colleague. The cell counts were made on the initial dilution and subsequent concentrations were calculated according to the resulting dilution

The concentration of all extracted genomic DNA was calculated by measurement of optical density at 260nm using a Hitachi U-2001 spectrophotometer. According to the manufacturers manual at this wavelength one OD unit is equivalent to 50µg ml^{-1} of double stranded DNA.

Calculation of equivalent numbers of trypanosomes for genomic DNA samples

For conversion of concentration of genomic DNA to equivalent number of trypanosome genomes the following parameters were used.

Size of haploid Trypanosome genome 3.5×10^7bp (Sanger Centre)

GC content of genome assumed to be 50%

Molecular weight of GC basepair = 650 Da

Molecular weight of AT basepair =649 Da

Molecular weight subtracted for each base pairing due to loss of water in condensation reaction = 36 DA

Calculated weight of trypanosome haploid genome = 0.0713 pg

The figure given by Desquesnes and Davila (2002) is 0.1pg of DNA per single trypanosome, in view of this difference the more conservative figure (0.0713pg), which would estimate greater trypanosome numbers and so over estimate sensitivity in comparison to the published figure was used.

2.2.12. Primer Design

Sixteen trypanosome ribosomal DNA sequences were selected from the NCBI database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide). *T.brucei* AF306771, AF306772, AF306773, AF306774, AF306775 AF306776, AF306777, X05862; *T.congolense* U22315; *T.congolense* (Kilifi) U22316; *T.congolense* (River/Forest) U22317; *T.congolense* (Tsavo) U22318; *T.vivax* U22319, *T.simiae* U22320. Two additional sequences (*Trypanosoma cruzi* AY362826 & *Trypanosoma rangeli* AY230240) were selected for comparison as out-groups to ensure optimal specificity of the primers. Sequences were aligned with CLUSTALX software (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/) (Thompson *et al*., 1997) and viewed using the Bioedit programme (Hall, 1999). A set of nested primers targeting the ribosomal gene locus was selected using PRIMER3 web primer selection software (http://www.broad.mit.edu/cgi-bin/primer/primer3_www.cgi).

Primers were evaluated using NETPRIMER software available at (http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html).The specificity of the primers was evaluated using a BLAST search against human and mouse genomes (http://www.ncbi.nlm.nih.gov/BLAST/). The outer primer sequences were ITS1 (5’ – GAT TAC GTC CCT GCC ATT TG – 3’), and ITS2 (5’ – TTG TTC GCT ATC GGT CTT CC – 3’(MWG Biotech), and inner primer sequences ITS3 (5’ – GGA AGC AAA AGT CGT AAC AAG G – 3’), and ITS4 (5’ – TGT TTT CTT TTC CTC CGC TG – 3’) (MWG Biotech). All PCR conditions were optimised using modified ‘Taguchi’ methods (Cobb and Clarkson, 1994). Expected band sizes were calculated from the distance between the primer locations as determined from the sequences for each trypanosome species present in bioinformatic databases. The expected band sizes are shown in Table 2.4.

During design of the outer primers the highest design emphasis was given to specificity (as few blast hits with human and mouse genomes as possible) at the

expense of stringency in primer design, so that the outer primers are best suited to cope with high amounts of background DNA present in blood samples. For design of the inner primers emphasis was given to primer design parameters such as hairpins, cross dimers, melting temperatures etc. at the expense of BLAST hits on human and mouse genomes. As during the second round the amount of background DNA is greatly reduced.

2.2.13. Bioinformatics and DNA sequences

The details of all sequences used obtained from bioinformatics databases are shown in Table 2.2. The alignment of the four primers against all trypanosome DNA sequences is shown in Figure 2.2.

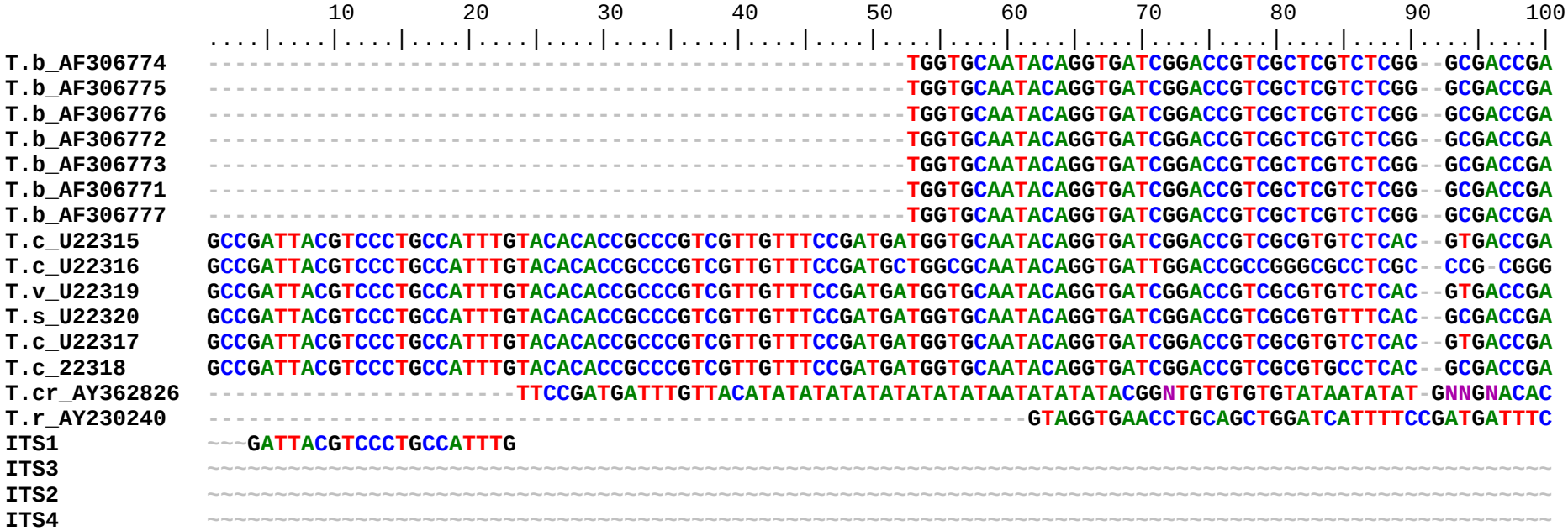
Table 2.2. Details of sequences contained in bioinformatic databases

Accession Number	Strain Reference	Species / Sub-species
AF306771	H3	*T.brucei*
AF306772	STIB215	*T.brucei*
AF306773	B8-18	*T.brucei*
AF306774	KP2	*T.brucei*
AF306775	DA1972	*T.brucei*
AF306776	SUZENA	*T.brucei*
AF306777	NW2	*T.brucei*
X05682		*T.brucei*
U22315		*T.congolense*
U22316		*T.congolense* (Kilifi)
U22317		*T.congolense* (River/Forest)
U22318		*T.congolense* (Tsavo)
U22319		*T.vivax*
U22320		*T.simiae*
AY230240		*T.ranglei*
AY362826		*T.cruzi*

The table above lists all the sequences for complete and partial ribosomal subunits used in this work. The details of accession number and strain type (Where appropriate) are shown. The sequences for *Trypanosoma ranglei* and *Trypanosoma cruzi* were used as outgroups.

Figure 2.2. Alignment of Trypanosome small ribosomal subunits in relation to the ITS PCR inner and outer primers

(The central portion of the sequences are omitted in order to save space)



```
                         110       120       130       140       150       160       170       180       190       200
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
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T.b_AF306776    AAGTTCACCGATATTGCTTCAATAGAGGAAGCAAAAGTCGTAAC--AAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCTGATATCCAT-TATAC
T.b_AF306772    AAGTTCACCGATATTGCTTCAATAGAGGAAGCAAAAGTCGTAAC--AAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCTGATATCCAT-TATAC
T.b_AF306773    AAGTTCACCGATATTGCTTCAATAGAGGAAGCAAAAGTCGTAAC--AAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCTGATATCCAT-TATAC
T.b_AF306771    A-GTTCACCGATATTGCTTCAATAGAGGAAGCAAAAGTCGTAAC--AAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCTGATATCCAT-TATAC
T.b_AF306777    AAGTTCACCGATATTGCTTCAATAGAGGAAGCAAAAGTCGTAAC--AAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCTGATATCCAT-TATAC
T.c_U22315      AAGTTCACCGATATTGCTTCAATAGAGGAAGCAAAAGTCGTAAC--AAGGTAGCTGTAGGTGAACCTGCAGCTGGCTCATTTTCCGATGATAATATATAT
T.c_U22316      AAGTTCACCGATATTGCCTCATTAGAGGAAGCAAAAGTCGTAAC--AAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCCGA-------------
T.v_U22319      AAGTTCACCGATATTGCTTCAATAGAGGAAGCAAAAGTCGTAAC--AAGGTAGCTGTAGGTGAACCTGCAGCTGGCTCATTTTCCGATGATAAAA---AA
T.s_U22320      AAGTTCACCGATATTGCTTCAATAGAGGAAGCAAAAGTCGTAAC--AAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCCGATA------------
T.c_U22317      AAGTTCACCGATATTGCTTCAATAGAGGAAGCAAAAGTCGTAAC--AAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCCGATGATACGATCCAA
T.c_22318       AAGTTCACCGATATTGCTTCAATAGAGGAAGCAAAAGTCGTAAC--AAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCCGATA------------
T.cr_AY362826   ACACAATCAGGCAACAAAACTCTGGCGTGTATATATATTACTAC-TATGCTACTAATATAATATACTCTGTGCTGNGTGTGTTGNTGTTGCCGCGCGGGA
T.r_AY230240    ATAATACCCTATAATACATGTGTG-CGTATATATATATATATATATG-TGCGCGTACA------TGCATGCGAGAGGAACAACTGTGATGACTCCACA
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ITS3            ~~~~~~~~~~~~~~~~~~~~~~~~~~GGAAGCAAAAGTCGTAAC~~AAGG
ITS2            ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
ITS4            ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
```

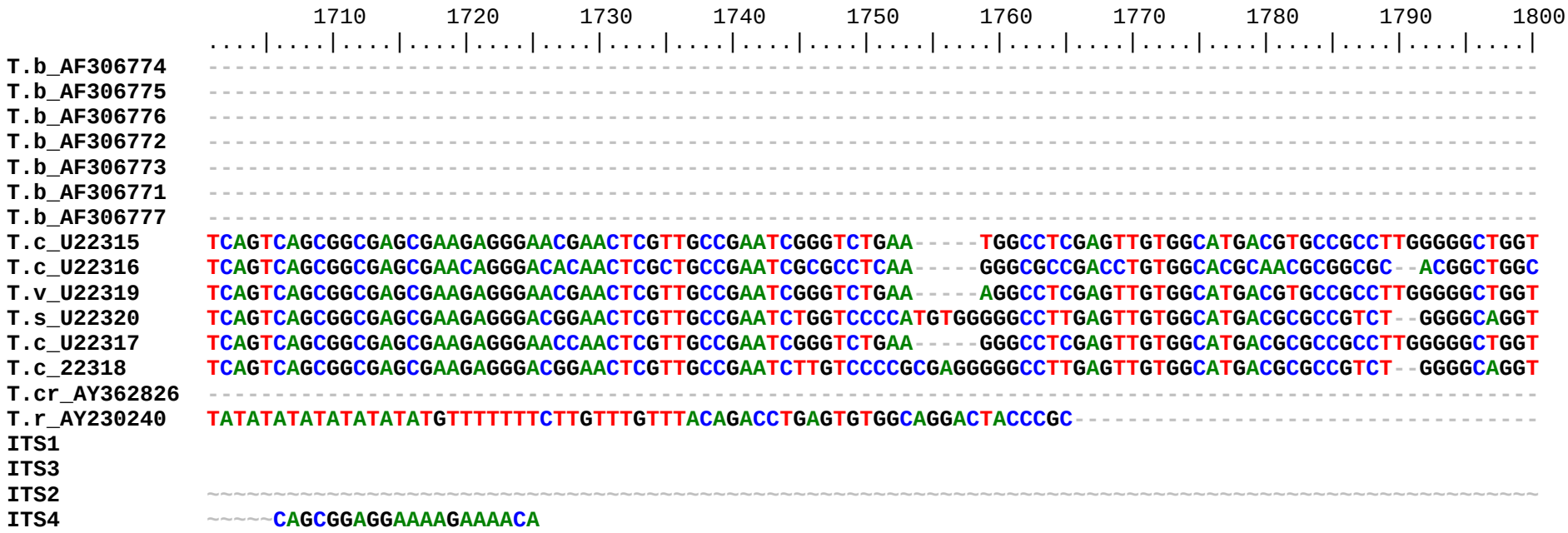
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T.v_U22319 TCAGTCAGCGGCGAGCGAAGAGGGAACGAACTCGTTGCCGAATCGGGTCTGAA-----AGGCCTCGAGTTGTGGCATGACGTGCCGCCTTGGGGGCTGGT
T.s_U22320 TCAGTCAGCGGCGAGCGAAGAGGGACGGAACTCGTTGCCGAATCTGGTCCCCATGTGGGGGCCTTGAGTTGTGGCATGACGCGCCGTCT--GGGGCAGGT
T.c_U22317 TCAGTCAGCGGCGAGCGAAGAGGGAACCAACTCGTTGCCGAATCGGGTCTGAA-----GGGCCTCGAGTTGTGGCATGACGCGCCGCCTTGGGGGCTGGT
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T.r_AY230240 TATATATATATATATATATGTTTTTTTCTTGTTTGTTTACAGACCTGAGTGTGGCAGGACTACCCGC
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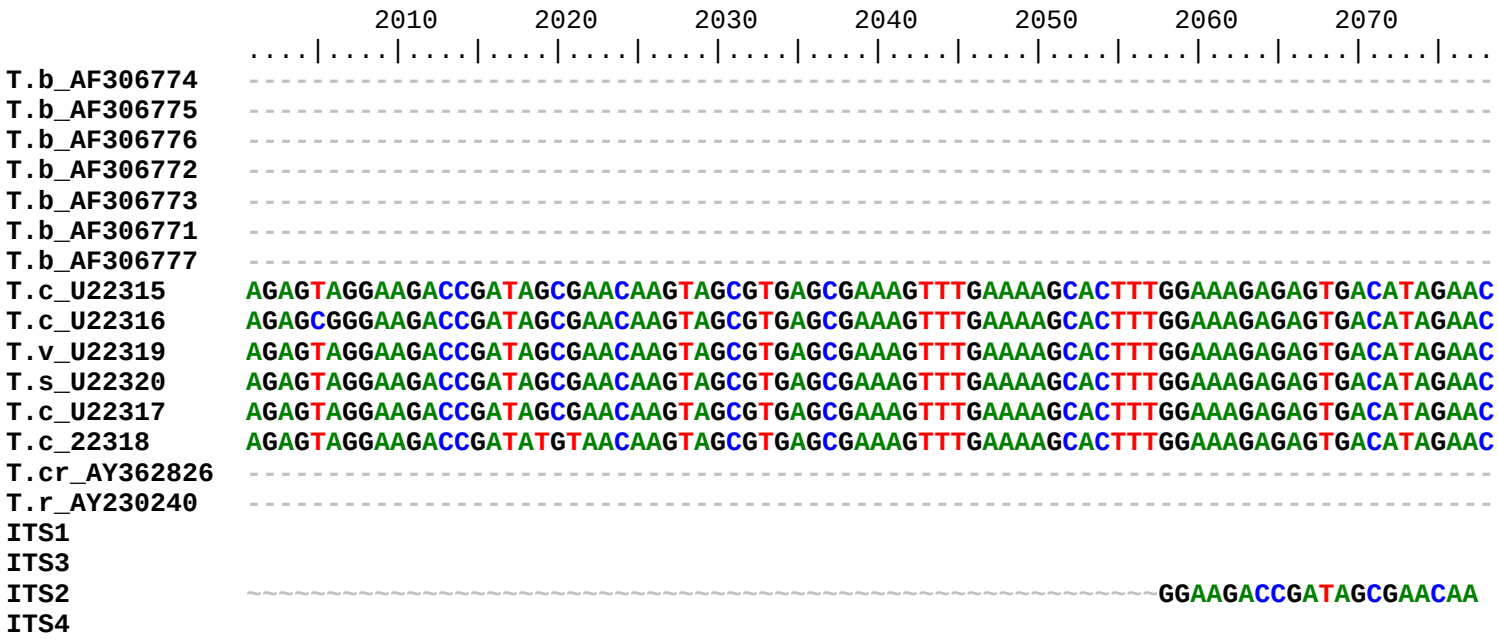
ITS3
ITS2
ITS4 ~~~~~CAGCGGAGGAAAAGAAAACA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2050
2060
2070
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T.c_U22315 AGAGTAGGAAGACCGATAGCGAACAAGTAGCGTGAGCGAAAGTTTGAAAAGCACTTTGGAAAGAGAGTGACATAGAAC
T.c_U22316 AGAGCGGGAAGACCGATAGCGAACAAGTAGCGTGAGCGAAAGTTTGAAAAGCACTTTGGAAAGAGAGTGACATAGAAC
T.v_U22319 AGAGTAGGAAGACCGATAGCGAACAAGTAGCGTGAGCGAAAGTTTGAAAAGCACTTTGGAAAGAGAGTGACATAGAAC
T.s_U22320 AGAGTAGGAAGACCGATAGCGAACAAGTAGCGTGAGCGAAAGTTTGAAAAGCACTTTGGAAAGAGAGTGACATAGAAC
T.c_U22317 AGAGTAGGAAGACCGATAGCGAACAAGTAGCGTGAGCGAAAGTTTGAAAAGCACTTTGGAAAGAGAGTGACATAGAAC
T.c_22318 AGAGTAGGAAGACCGATATGTAACAAGTAGCGTGAGCGAAAGTTTGAAAAGCACTTTGGAAAGAGAGTGACATAGAAC
T.cr_AY362826
T.r_AY230240
ITS1
ITS3
ITS2 GGAAGACCGATAGCGAACAA
ITS4

2.3. Results

In order to differentiate important species (and some sub species) of African trypanosome a nested PCR reaction was developed which amplified the variable ITS region of the ribosomal gene locus, using primers designed to the conserved flanking sequences (Figure 2.1.).

2.3.1. Evaluation of PCR techniques on different sample media.

Application of the PCR technique described by Desquesnes (2001) to blood samples stored on FTA cards failed to produce any results. In order to ascertain the reason(s) for this, the same technique was applied to different types of dilution series to ascertain the limit of sensitivity of the technique for each dilution series type (See Table 2.3.). The results obtained showed that there was no difference in the sensitivity of the technique (Desquesnes, 2001) between the different types of dilution series that did not contain blood. The sensitivity achieved for the dilution series that contained blood was between ten to one hundred times less than that on other dilution series types. When the technique was applied to dilutions of different components of blood (red blood cells, white blood cells and plasma) with a constant level of trypanosome DNA only the dilution series of red blood cells showed significant inhibition of the PCR as the number of red blood cells increased. The original IRT PCR technique (Desquesnes, 2001) did not provide any positive results on these types of dilutions.

Application of the new ITS PCR technique described in this paper to the different types of dilution series showed that the PCR was able to detect a single trypanosome across all types of dilution series. No inhibition of PCR was noted when blood was present in the sample tested.

2.3.2. Specificity

Amplification of genomic DNA from trypanosome stocks resulted in a specific size band for each species, which was within the bounds of measurement error and was in complete agreement with the expected band sizes (Table 2.4.). Control DNA samples were not available for some trypanosome species (e.g. *T. theileri*), therefore when unexpected band sizes appeared in field samples the

Table 2.3. Performance of Polymerase Chain Reaction protocols on different types of dilution series

Dilution Series Type	Limit of Detection (trypanosomes per µl)	
	IRT PCR (McLaughlin *et al*, 1996)	ITS PCR
Genomic DNA diluted with water	>70	1
Genomic DNA diluted with water on filter cards	>70	1
Whole trypanosomes diluted with water on filter cards	>70	1
Whole trypanosomes diluted with bovine blood on filter cards	350 ~ 3000	1
Dilution series of elements of blood with constant target DNA concentration		
10^3 trypanosomes per ML with dilutions of rbc's	All Negative	PCR Inhibition at 10x rbc's per ml
10^3 trypanosomes per Ml with dilutions of wbc's	All Negative	All positive
10^3 trypanosomes per Ml with dilutions of Plasma	All Negative	All positive

Shows the mean limit of sensitivity of the previously published single round IRT PCR (McLaughlin *et al*, 1996) and ITS PCR as measured against different types of dilution series of the sample and other components present in the sample. The different types of dilution series are designed so as to highlight the cause of any PCR inhibition that might be attributable to the component that is present in that dilution series but not in the other dilution series.

Table 2.4. Observed and expected amplicon sizes

Species	Expected band size from NCBI Database	Band sizes obtained
T.congolense (Forest)	1513bp	1501bp
T.congolense (Kilifi)	1422bp	1430bp
T.congolense (Savannah)	1413bp	1408bp
T.congolense (Tsavo)	954bp	951bp
T.brucei	1207~1224bp	1215bp
T.simiae	850bp	847bp
T.vivax	611bp	620bp
T.theileri	988bp	998bp

bands were cut out, sequenced and compared with database sequences to confirm species identity (Data not shown). The specificity of the primers was further tested by PCR amplification with host DNA (human, cow and mouse), which produced no visible bands.

2.3.3. Sensitivity

To investigate the sensitivity of the nested PCR, the technique was tested on a dilution series of whole trypanosomes (diluted in phosphate buffered saline) on Whatman FTA™ cards and a dilution series of genomic DNA (diluted in water) in liquid form and also applied to Whatman FTA™ cards. In the two dilutions of genomic DNA positive amplification was detected at a DNA concentration of 49pg ml^{-1} (or less than a single trypanosome equivalent). To investigate the efficacy of the technique on samples containing host material, trypanosomes were diluted in bovine blood (UK origin) and applied to Whatman FTA cards to mimic field samples. Positive amplification was detected at DNA a concentration of 55pg ml^{-1}, which is again equivalent to less than a single trypanosome.

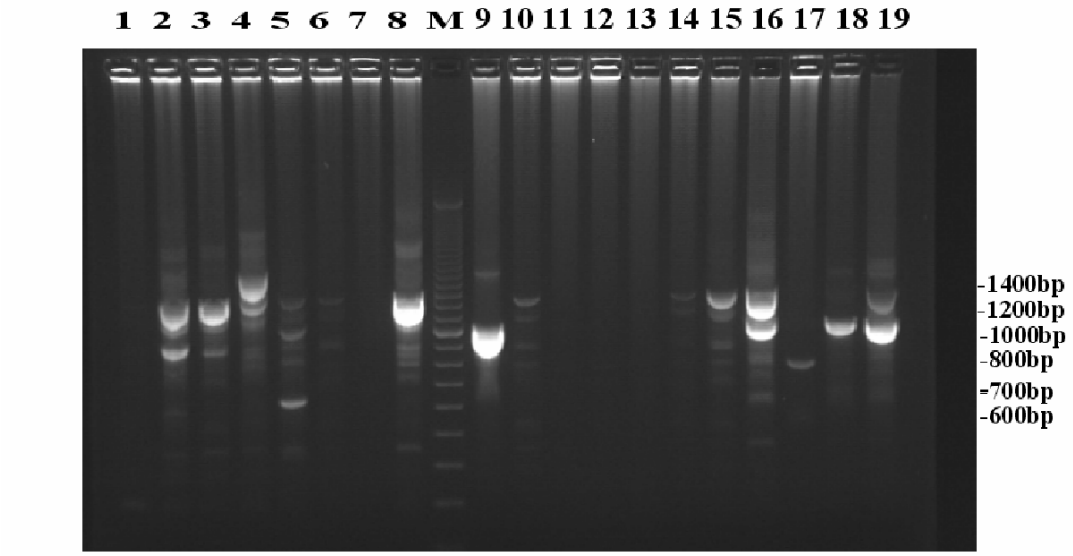
2.3.4. Application to field samples

Application of the nested ITS primers to two hundred and forty five samples of bovine blood taken from the Soroti &Tororo districts of Uganda and collected on Whatman FTA™ cards resulted in successful amplification of the target ITS region as shown by species specific band sizes. This technique was also able to show samples infected with multiple species (Figure 2.3; lanes 2, 16 and 19), as shown by the presence of multiple bands.

2.3.5. Technique evaluation

The efficacy of the technique was tested against the most widely used screening method; individual species specific PCR's (Artama et al., 1992, Masiga et al., 1992, Majiwa et al., 1994, Masake et al., 1997, Clausen et al., 1998), using samples collected from two different villages in Uganda (Cow blood applied to Whatman FTA™ cards). Analysis of the two hundred and forty five samples using the individual species-specific PCR screening method demonstrated a low prevalence of trypanosomes in cows from the first village and a high prevalence of trypanosomes in cows from the second village (Data courtesy of Jenna Fyfe and Francis McOdimba, CTVM, University of Edinburgh). Application of the ITS-PCR method showed that a comparable prevalence and greater number of species were detected in each case (Table 2.5).

Figure 2.3. Representative gel showing bands obtained from PCR amplification (using nested ITS primers) of 19 blood samples (on Whatman FTA cards) taken from cattle in the Tororo district of Uganda



Samples 2, 3, 8, 16 & 19 are all positive for *T.brucei*, Samples 2, 9, 16 ,18 & 19 are positive for *T.theileri*. Sample 17 is positive for *T.simiae*, sample 5 is positive for *T.vivax* and samples 1, 6, 7, 10, 11, 12, 13, 14 are negative. Lane M represents a marker graduated in 100bp intervals (band sizes illustrated). Mixed species infections were found in lanes 2,3,4,5,16 & 17.

Table 2.5. Evaluation of the detection of trypanosome DNA from Whatman FTA™ cards

Species	Low prevalence village Prevalence (%)		High prevalence village Prevalence (%)	
	Species specific PCR method	ITS- PCR	Species specific PCR method	ITS-PCR
T. brucei	5	7	32	33
T. theileri	ND	3	ND	47
T. congolense	0	1	1	5
T. vivax	1	1	8	5
T. simiae	ND	0	ND	2
	ND = Not Done	(N=101)	ND = Not Done	(N=144)

The filter paper cards contained bovine blood samples from cattle in villages in the Tororo and Soroti districts of Uganda, the two groups of samples had been previously screened with individual species specific primers (Artama *et al.*, 1992; Masiga *et al.*, 1992; Majiwa *et al.*, 1994; Masake *et al.*, 1997b; Clausen *et al.*, 1998). The prevalence from the two sample sets were therefore known prior to the second screening with ITS-PCR. This second screening was conducted without knowledge of the prevalence of the sample set (blind). Data from species specific techniques courtesy of Jenna Fyfe and Francis McOdimba, CTVM, University of Edinburgh)

2.4. Discussion

Existing methods for screening samples for detection and differentiation of trypanosomes are not suited to large-scale epidemiological study. This work addressed the requirement for improved techniques that simplify the sample analysis process but maintain the diagnostic sensitivity and specificity required for directly analysing field samples.

Initial tests of a single round PCR targeted to the ribosomal gene subunit internal transcribed spacer (McLaughlin *et al*, 1996) were successful on genomic DNA extracted after mouse passage. However the same PCR when applied to blood samples stored on treated filter paper failed to produce any results. In order to determine the reasons for this the limit of sensitivity of the PCR was tested on a number of different types of dilution series. Each dilution series was designed so as to represent a gradual transition from genomic DNA in liquid form (on which the PCR was known to work) to trypanosomes in blood stored on treated filter paper cards (on which the PCR did not work). Any differences in the limit of sensitivity (the lowest dilution that consistently produced a positive result) between types of dilution series would then highlight the probable cause of the inhibition. There was no difference in the sensitivity of the PCR between the liquid genomic DNA dilution series and the same dilution series when placed on the treated filter paper cards, the highest dilution detected for both was equivalent to greater than 70 trypanosomes. From this we could conclude that the storage on the filter paper cards did not cause any inhibition of the PCR. An identical minimum level of detection was consistently achieved for the next type of dilution series; that of whole trypanosomes diluted with sterile water placed onto the treated filter paper cards. From this we could conclude that the adequate presence of whole trypanosomes instead of genomic DNA did not inhibit the PCR. In the next dilution series whole trypanosomes were diluted in uninfected cow blood (U.K. origin) and the dilutions placed on the filter cards. PCR on this type of sample showed that the limit of detection was consistently between five to fifty times less than with all the other dilution series (300 ~ 3000 trypanosomes detected). From this result it was evident that the addition of blood to the sample had an inhibitory effect on the PCR technique used.

In order to identify the specific component of blood that was the cause of the inhibition further sets of dilutions were prepared. In these dilutions the numbers of trypanosomes were kept at a stable level and the various components of

blood (red blood cells, white blood cells and plasma) were diluted in tenfold series. In order to achieve a useful dilution series the red and white blood cell components were enriched prior to the preparation of the dilution series, so that the starting dilutions were much higher than would be found in a blood sample. Results from PCR (with the ITS PCR technique) of these dilution series showed that red blood cells to be the major PCR inhibitory factor present in blood. This is in line with other work that identified heme, present in the red blood cells, to be one of the major PCR inhibitory components of blood (Akane et al, 1994). White blood cells showed only a minor inhibitory effect, probably due to the fact that they increase the amount of background DNA present.

In addition to the inhibitory effect of blood on the PCR the dilution series also showed that the single round IRT PCR (Desquesnes, 2001) was not sensitive enough for use on field samples. The levels that might be expected to be found in infected blood are likely to be very much lower than the maximum detection limit for this technique of around seventy parasites (disregarding the inhibition shown due to the presence of blood). As blood samples for screening are likely to be from any stage of infection the number of parasites present in the sample will vary considerably. For practical purposes the only sensible detection level for a PCR designed to detect infection is a single parasite cell. This is the target detection limit (in the presence of inhibitory factors from blood) that was set for the design of the subsequent ITS-PCR. In order to achieve this target it was clear that different strategy was required. It was decided that a nested PCR technique should be used, a nested PCR is two separate PCR reactions; the second carried out on the product of the first. The second set of primers is designed to amplify inside the first round amplicon. This makes the technique very much more sensitive, and in this application a two round amplification strategy has a second desirable effect. In the second round of the PCR the amount of the target is increased, whilst the amount of potentially inhibitory factors are greatly diluted.

In light of the evaluation of existing techniques we therefore developed, a new nested PCR targeted to include both internal transcribed spacers of the ribosomal RNA genes (ITS PCR), that was capable of detecting trypanosomes in the presence of host DNA and the PCR inhibitors present in blood (Heme, Lactoferrin, IgG and non-target DNA). This nested technique was found to be sensitive enough for detection of a single parasite in blood samples and has been shown to be able to differentiate all important African trypanosome species and some sub species. In order to simplify the sample collection and

processing methodology, we investigated the storage of samples on treated filter paper cards, which make possible the direct analysis of biological samples, in addition to circumventing the requirement for mouse passage. When the nested technique was evaluated against the current single PCR per species screening method, using a complete sample set containing positive and negative samples, it was found to have a similar level of detection, but was capable of detecting a greater number of species in both high and low prevalence sample sets.

The epidemiology of African trypanosomiasis is complex and poorly understood and requires large-scale field based investigation. This newly developed technique has greatly simplified epidemiological studies involving sample screening. As a result the costs and time involved in screening samples for the eight major species/sub-species of trypanosome have been reduced by a factor of four (conservative estimate). This nested PCR technique can be used to screen large numbers of biological samples directly, quickly and accurately, making it a simple, cost effective, robust and reliable tool for investigating the complex epidemiology of African trypanosomiasis.

Chapter 3: Mixed species trypanosoma infections in African zebu cattle.

3.1. Introduction

The extent to which African trypanosome species co-exist within the same host has been studied most extensively in tsetse. Far less work has been carried out to investigate the frequency of mixed trypanosome species infections in cattle and other non-domestic animals. Although mixed species infections have been widely documented in tsetse, the reported prevalence varies considerably, for example, dissection of 9306 tsetse from Zimbabwe showed that mixed species infections accounted for only 6.2% of the positives observed (Woolhouse *et al.*, 1996). In contrast, dissection in of 688 tsetse from Cote d'Ivoire showed that mixed species infections accounted for 64% of the positives observed (McNamara *et al.*, 1995). In addition, it has been shown that it is possible for tsetse to acquire mixed infections experimentally. Of 140 tsetse fed on cattle which were experimentally infected with both *Trypanosoma congolense* and *Trypanosoma brucei*, 33% (46) were found to have picked up an infection. Of these, 63% were diagnosed as single *Trypanosoma congolense* infections, 8.7% were diagnosed as single *Trypanosoma brucei* infections and 28% were diagnosed as mixed species infections (Van den Bossche *et al.*, 2004a). Similar findings have been reported elsewhere (Moloo, 1982). Furthermore, it has been demonstrated experimentally that tsetse can acquire mixed species infections via sequential in vitro feeds with single species infections (Gibson & Ferris, 1992).

Whilst there have been fewer investigations into the existence of mixed species infections in cattle, the picture is similar to that for tsetse. Reported proportions of mixed species infections amongst trypanosome positive cattle varies from 5.4% in a study of 1617 cattle from Tanzania (Connor & Halliwell, 1987) to 47.8% in a study of 422 cattle in Ghana (Kayang *et al.*, 1997). As with tsetse it has also been shown that it is possible for cattle to acquire mixed infections under experimental conditions, from tsetse infected with mixed species and from sequential feeds of single species infected tsetse (Masake *et al.*, 1984; Kayang *et al.*, 1997; Mattioli *et al.*, 1999).

Whilst there is evidence that mixed trypanosome species infections exist within the vector and host, there are a number of important questions that remain unclear, most notably 'How widespread are mixed species infections in the host and vector?' and 'Do the different species of trypanosome interact in mixed infections?' questions which are directly related to the epidemiology of the disease and its transmission dynamics.

In this study we set out to investigate the extent and composition of mixed species trypanosome infections in a group of African zebu cattle using a single nested PCR targeted to the intergenic spacers of the small ribosomal sub-unit genes (Cox *et al.*, 2005). The method is capable of detecting all important African trypanosome species – including *Trypanosoma theileri* (a species largely ignored in other studies) and provides a unique band size for each of the species of interest. *Trypanosoma theileri* is commonly found in cattle worldwide (Ogassawara *et al.*, 1981; Nunes *et al.*, 1983; Samad & Shahidullah, 1985; Kennedy, 1988; Farrar & Klei, 1990; Tarimo-Nesbitt *et al.*, 1999; Greco *et al.*, 2000; Verloo *et al.*, 2000) and is generally regarded as largely non-pathogenic (Schafler, 1979; Hussain *et al.*, 1985; Doherty, 1993; Seifi, 1995). Despite the widespread distribution of *T. theileri,* its presence is seldom reported in the literature. This may be due, in part, to its supposed non-pathogenicity and to the fact that until recently it has not been possible to detect this species by PCR (Rodrigues *et al.*, 2003), although it is easily identified by microscopy. The importance of this species in the context of coinfection with other trypanosome species has not yet been established.

The direct analysis of the blood samples used in this study, combined with repeated PCR analysis of the same samples (allowing analyses of up to 114 microlitres of blood) constitutes what is the most sensitive and in depth analysis of blood samples yet reported in this field.

3.2. Materials & Methods

3.2.1. Sample description

Thirty-five blood samples were randomly selected from a large set of samples collected from the ears of 35 Zebu cattle in the village of Ojilai, Tororo in Uganda in June 2001. Approximately 200µl of blood from the ear vein of each cow was applied to Whatman FTA™ cards and allowed to dry for a minimum of twenty-four hours at room temperature.

3.2.2. Mapping of PCR results

Each blood sample present on the FTA card was subject to between 92 and 114 separate PCR assays (depending on the amount of blood available on the sample) and the position of each sample punch taken from the FTA card was recorded so that a positive result could be related back to the position on the card from which the sample punch was taken The total number of trypanosome positive and negative

samples was recorded for each sample. The number of positives for each species was also recorded.

3.2.3. Controls

Uninfected bovine blood (UK origin) was used as a negative control to ensure that the results were not biased by false positives during repeated PCR assays. A positive control sample was constructed with known numbers of trypanosomes (procyclic *Trypanosoma brucei*) diluted in cow blood (UK origin). The resultant concentration of trypanosomes was calculated with allowance for the dilution factor, at 508 trypanosomes per millilitre using a mean of thirty readings from a Neubauer haemocytometer. The positive and negative controls were subjected to the same treatment as the other samples. For the positive control the total number of PCR assays conducted and the total number of positives obtained were recorded.

3.2.4. Comparison of observed and expected frequencies

The prevalence of each species was used as the probability of detecting that particular species in a calculation of the expected frequency of each permutation of the species present. A monte-carlo simulation (@Risk V4.1, Palisade Corp) was set up to repeat the calculation over 10,000 iterations, thus simulating the variability inherent in probabilistic associations of elements. Over the 10,000 iterations of the calculation, the mean and 95% confidence intervals of the expected frequencies were calculated, this would be equivalent to the range of results that would be found if the species associated randomly. If the observed results fall outside these confidence intervals there would be a significant association between the relevant species, which may be either negative or positive.

3.3. Results

In this study we investigated the extent and composition of mixed infections in a group of African Zebu cattle. A total of 3602 PCR reactions were carried out on 35 filter paper cards containing whole blood samples taken from African zebu cattle selected randomly from a herd in Ojilai, Uganda. The diagnostic results and position on the filter paper card of each result was recorded (Figure 3.1. shows examples of the results from a high, medium and low intensity infection samples). Table 3.1. shows the data obtained for the thirty-five cattle, only five cattle remained uninfected after repeated PCR screenings. The negative control samples remained negative throughout repeated PCR assays. Table 3.2. shows the cumulative prevalence of each trypanosome species or combinations of species in this sample of the herd.

The proportions of single and mixed species infections are shown in Table 3.3. The majority 18/30 (60%) of positive animals contained mixed species infections, All single species infections contained *Trypanosoma theileri*. Furthermore, the mixed species infections all involved *T. theileri*.

The comparison of the observed frequencies of the mixed infections to those expected assuming random association between species showed the frequency of all the combinations of species observed in this sample set (including the occurrence of single species infections) were within the 95% confidence intervals. This indicated that for this sample set, the occurrence of species combinations was no different from that expected with a random association between species (Table 3.4).

Table 3.1. Results obtained from multiple PCR of thirty five blood samples from zebu cattle

Sample No.	*T.theileri*	*T.brucei*	*T.congolense*	*T.vivax*	Negative/ False Negative
1	12	0	0	0	80
2	2	0	0	0	101
3	6	3	7	0	85
4	1	1	0	0	98
5	0	0	0	0	109
6	2	2	4	2	88
7	4	0	0	0	100
8	8	7	4	0	91
9	8	0	0	13	83
10	7	0	2	0	92
11	3	0	0	1	106
12	10	2	0	3	87
13	0	0	0	0	110
14	2	1	3	0	100
15	21	10	6	0	65
16	6	0	3	0	96
17	12	0	0	0	90
18	1	0	1	0	98
19	3	0	3	2	94
20	3	0	0	0	100
21	19	0	0	0	85
22	18	1	2	0	78
23	0	0	0	0	102
24	0	0	0	0	107
25	3	0	3	0	95
26	4	1	2	0	101
27	0	0	0	0	98
28	4	0	0	0	97
29	1	1	0	0	100
30	1	0	3	3	95
31	1	1	0	1	100
32	15	0	0	0	99
33	2	0	0	0	100
34	9	3	14	14	73
35	2	0	1	0	99
Negative control	0	0	0	0	107
Positive control	0	45	0	0	56

The frequency of positive results for *T.theileri, T. brucei, T. congolense, T. vivax* and of negative results is recorded in columns 2 to 6 respectively.

Table 3.2. Prevalence of trypanosome species in African zebu cattle

Species	Cumulative Prevalence (%)	Exact Binomial 95% CI	
		Upper (%)	Lower (%)
T.theileri	85.7	69.7	95.2
T.brucei	34.3	19.1	52.2
T.congolense	42.9	26.3	60.6
T.vivax	22.9	10.4	40.1
Any trypanosome	85.7	69.7	95.2
Mixed Infections			
Prevalence	60	42.1	76.1
Proportion of positives	70		

Table showing the cumulative prevalence of the different species of trypanosome and the prevalence of mixed infections detected in thirty-five blood samples collected from Zebu cattle together with the exact binomial confidence intervals. These samples were subject to repeated PCR samplings until the sample was exhausted, between 92 to 114 ten times each.

Table 3.3. Proportion of mixed species infections present in positives samples

No. of Species Present	Proportion of Positives (%)	Species Combination	Proportion of Positives (%)
Single Species Infections	30	*T.t*	30
Two Species Infection	30	T.t, T.b	6.7
		T.t, T.v	6.7
		T.t, T.c	16.7
Three Species Infection	33	T.t, T.b, T.c	20
		T.t, T.b, T.v	6.7
		T.t, Tc, T.v	6.7
Four Species Infection	6.7	T.t, T.b, T.c, T.v	6.7

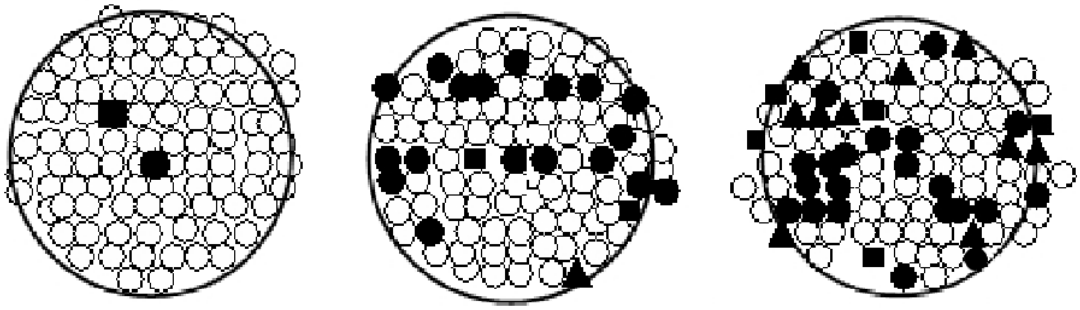
The table above shows proportion of the positive samples found to be single, two or three species infections. Details of the specific species combinations found are also given on the right of the table. T.t, *Trypanosoma theileri;* T.b, *Trypanosoma brucei;* T.v, *Trypanosoma vivax*; T. c, *Trypanosoma congolense.*

Table 3.4. Results of Monte Carlo simulations showing the observed and expected number of observations of each combination of species

Species Combination	Observed (N)	Expected (Mean N)	95% CI
Tt, Tb	2	4.54	(2 ~ 8)
Tt, Tc	5	6.50	(3 ~ 10)
Tt,Tv	2	2.58	(0 ~ 5)
Tb, Tc	0	0.56	(0 ~ 2)
Tb, Tv	0	0.22	(0 ~ 1)
Tc, Tv	0	0.32	(0 ~ 1)
Tt, Tb, Tc	6	0.39	(1 ~ 6)
Tt, Tb, Tv	2	1.36	(0 ~ 3)
Tt, Tc, Tv	2	1.94	(0 ~ 4)
Tb, Tc, Tv	0	0.17	(0 ~ 1)
Tt, Tb, Tc, Tv	2	1.01	(0 ~ 3)
Tt Only	9	8.66	(5 ~ 13)
Tb Only	0	0.75	(0 ~ 2)
Tc Only	0	1.10	(0 ~ 3)
Tv Only	0	0.43	(0 ~ 2)
Negative	5	10.12	(5 ~ 15)

For each combination of species the table details the observed numbers of each particular species combination present The numbers expected if each species were distributed at random is shown for comparison The expected frequencies and their respective 95% confidence intervals were determined from 10,000 iterations of a Monte Carlo simulation. The observed and expected frequencies are for those combinations exactly, for instance the combination Tt, Tb does not include three species combinations with Tt, Tb.

Figure 3.1. Three examples of the sample maps produced from the repeated PCR of blood samples stored on filter paper



The figure shows three diagrammatic representations of the repeated PCR of blood samples from Zebu cattle. The large circles represent the area on the filter paper card where the blood sample was applied. Each small circle or shape represents a punch (or aliquot) taken for PCR analysis. The positions of each punch were recorded and the results for that PCR were related back to the position on the original sample Examples of a low (OJ20), medium (OJ25) and high (OJ18) infection intensity results are shown..

Key; O, negative PCR result; ●, T. *theileri*; ▲, *T.brucei*; ■, *T.congolense*.

3.4. Discussion

Most field based studies of African trypanosomiasis approach the analysis of the samples in a similar way; a large number of samples are collected and subject to a single diagnostic test for presence or absence of a particular species of trypanosome for each sample (Connor & Halliwell, 1987; Waiswa & Katunguka-Rwakishaya, 2004; Magona *et al.*, 2005). From the resulting data prevalence for each species is then calculated the raw data may also be used for statistical analysis.

In terms of detection of mixed infections, there are several inherent problems with this approach to large scale sampling. The use of genomic DNA extracted from blood samples makes study of mixed species infections difficult to interpret as the target DNA (often present in multiple copies throughout the genome) is free to disperse in the liquid media. Positive results by PCR may therefore result from target DNA representing less than a single trypanosome, this makes accurate interpretation of the proportions of species present difficult. In addition, extraction of genomic DNA from blood samples involves a complex processing protocol, in which a proportion of the DNA present in the sample may be lost or degraded.

An additional problem associated with conventional studies of this type is that individual species specific PCR reactions are utilised for each of the species of interest, this approach immediately excludes from the analysis any species not targeted by the specific PCR reaction being used, and may also give biased results if the sensitivities of each technique differ. The detection of *Trypanosoma theileri* in this study illustrates this point; conventional studies have excluded this prevalent trypanosome.

It is clear from these results that PCR or microscopy, used in a conventional manner would have detected few if any of the mixed infections, as the number of trypanosomes in the 200µl blood samples was very low. This is not due to the sensitivity of the techniques as such, but due to the probabilistic effect of acquiring a trypanosome in the aliquot of blood taken for analysis when infection intensity is low.

In contrast this study attempted a more 'in depth' look at the information contained in a smaller number of samples. In order to analyse a large amount of blood, up to 114 PCR reactions were conducted on each blood sample, each single PCR reaction being capable of detecting and differentiating a range of trypanosome species (Cox *et al.*, 2005). This approach combined with the application of the blood samples to Whatman FTA filter paper allowed a unique unbiased assessment of the ratios of different species present in mixed Trypanosoma species infections. This was possible because DNA from individual trypanosomes is captured in situ on the matrix of the filter paper and therefore the pattern of positive results obtained is representative of the distribution of parasites in the blood sample. This is evident from the maps of PCR results plotted for all samples; a selection of which are shown in Figure 3.1. The advantage of analysing blood samples from filter paper, apart from cost and simplicity, is that the results obtained are a more accurate representation of the natural parasite population present in the blood sample.

Very little is known about the occurrence of mixed species trypanosome infections in African Zebu cattle, although mixed infections have been reported, they have been found to be present in only a low percentage of cattle (Connor & Halliwell, 1987; Kidanemariam *et al.*, 2002; Magona *et al.*, 2003). This study clearly shows that mixed species infections are present at low parasitaemia in most of the infected cattle. In fact mixed species infections were present in 21 of 35 samples (60%) this is much higher than has been noted before. Two, three and four species mixed

infections involving various combinations of species were all detected in this study and all involved *T.theileri*.

Although the results obtained indicated that the frequency of the combinations of trypanosomes involved in mixed infections was no different to that which would be expected from a random association of species with respect to the measured prevalence, this by no means rules out the possibility that there is some element of competition or association between the species present, as only a relatively small number of samples were analysed.

The high prevalence of *T.theileri* found in this study is in line with prevalence's reported elsewhere. In this set of samples *T.theileri* appeared be the most dominant species. It is most interesting to note that all single infections were *T.theileri,* suggesting a reduction in the ability of other species to establish a single infection when *T. theileri* is present (Although not found to be significant in the Monte-Carlo analysis). Should some form of competition exist between the trypanosome species when infecting a common host, then *T.theileri* cannot be excluded from the epidemiology of what have been regarded as the more important African trypanosome species? It is at least a possibility that exclusion of this 'dominant' parasite from the cattle by blanket drug treatment regimes combined with reduction in the tabanid population, the main vector of this species (Rodrigues *et al.*, 2003), could eventually lead to a much greater prevalence in the cattle of the other, pathogenic, tsetse transmitted species.

None of the cattle examined in this study showed any clinical signs of trypanosomiasis when examined in the field by experienced veterinarians. The fact that almost all of the randomly chosen cattle samples examined were found to be positive for trypanosomes after repeated analysis is perhaps one of the most important findings of this study. The data suggests that the majority of infections are asymptomatic and that a high proportion of the animals act as parasite carriers. If this were true in the wider population, it would have important consequences for our understanding of the epidemiology of trypanosomiasis and how the disease may be diagnosed and controlled. This observation is particularly important for human sleeping sickness; the cumulative prevalence for *T. brucei* was 34.3%, this prevalence is much higher than is usually reported, given that it has been reported that *Trypanosoma brucei rhodesiense,* causing the acute form of human sleeping sickness, exists in *T.brucei* populations at a proportion of around 33% (Welburn &

Coleman, 2004), then many of these animals may be acting as carriers of the human infective sub species.

The results presented here provide novel information on the presence of mixed infections and *Trypanosoma theileri* as well as showing that in this study group asymptomatic infection was present at a high prevalence. Whilst this extensive and in depth analysis of a few samples is not practical for widespread screening it has provided an insight that can form a basis for further investigations. The most important question raised in this work is how these results might apply to the wider population. Future work will focus on extending this study to determine if these findings are also repeated in other study groups in different locations.

Chapter 4: An empirical investigation into the occurrence of false negative results in populations with sub-patent infections.

4.1. Introduction

One of the key results obtained from the repeat screening of the 35 samples shown in the previous chapter was that the cumulative prevalence appeared to increase with every repeat screening of the samples. This result may have very important implications for epidemiological studies and diagnosis of infection, the remaining work is devoted to investigating this phenomenon.

Prevalence and incidence are perhaps two of the most basic epidemiological measures. Prevalence can be defined as “the amount of infection in a known population, at a designated time, without distinction between old and new cases”. Similarly, incidence is defined as “the expression of the number of new infections that occur in a known population over a period of time” (both definitions are adapted from Thrusfield, 1986). In practice, both measures are based on achieving accurate quantitative measurement of the numbers of infected and uninfected subjects. The implication of this is that for prevalence and incidence to be correct a high degree of confidence in the infected state or aetiological agent is important. In the case of the thirty-five samples examined in the previous chapter, diagnosed prevalence for all trypanosome species of 14.3% (8.5% for all species except *T. theileri*) was indicated from the first round of screening of these samples (one diagnostic test per sample). The mean diagnosed prevalence for all screenings was 9.7% whilst the cumulative diagnosed prevalence from over 100 screenings was much higher at 85.7% (60% for all species except *T. theileri*). If this type of situation is widespread in field based epidemiological studies, then underestimation of prevalence and incidence will also be widespread.

Further problems are also evident from the examination of the sample maps, examples of which are presented in Figure 3.1. It is apparent that the diagnostic result obtained depends on which portion of the blood sample is selected for analysis. To be more specific, for the three infected samples shown, both the diagnosis as infected and the species of parasite diagnosed are all probabilistic in nature. This probabilistic diagnosis would appear to be dependent on the infection intensity of the parasite within the blood, in that there is a greater probability of obtaining a positive diagnostic result from the sample with the higher infection intensity. Although all the cattle from which these samples were taken were infected with trypanosomes, there are a large number of false negative results. It follows that prevalence obtained from a single screening may be completely representative of the population if infection intensities are generally very high, of may be a severe

underestimate of the population prevalence if infection intensities are generally very low in the population. Epidemiological screening methods, as currently applied, do not take a quantitative view of the parasite population and instead deal with infected hosts rather than parasite numbers.

The probabilistic nature of diagnosing low intensity infections as positive leads to false negative results. The false negative results in turn lead to underestimation of the prevalence and incidence. In this work I have termed this effect as infection 'Intensity Related False Negatives' (IRFN) to distinguish them from other types of false negative results that may occur for other reasons (failure or inhibition of the diagnostic technique) and the objective of the remaining chapters is to explore this phenomenon.

Perhaps most important is the question of how widespread are the low infection intensities that are capable of producing the false negative results (sub-patent infections). If false negative results are frequently obtained from other sample sets then IRFN become more important. For example if IRFN is frequently found elsewhere in other sample sets, populations and for other pathogens then it is potentially of great importance.

Although the cause of IRFN would, at first, appear to be infection intensity, it is important to establish if any other factors may have an caused the phenomenon, observed here; perhaps overdispersion of the parasites within the population, factors relating to the sensitivity of the diagnostic technique may also play a role. These key questions need to be investigated not only with samples stored on filter paper cards (as in this study), but in other sample media such as extracted DNA in solution. Underestimation of true prevalence is one obvious consequence of IRFN. However there may be other serious consequences? For example, how might IRFN effect different types of epidemiological study (cross sectional, longitudinal, comparisons between populations). A further important issue is the role of low intensity infection in transmission dynamics. Although IRFN may result in underestimation of prevalence, do animals with low intensity infections make a significant contribution to transmission?

Widely used methods for screening populations by PCR are generally not quantitative and therefore view the epidemiology of the parasite from the standpoint of infected hosts. A further important question is how we might begin to deal with IRFN in a cost effective and practical way. Whilst IRFN would at first seem to present a difficult problem, it also presents an opportunity to move the focus of

epidemiological studies away from the dynamics of infected and uninfected hosts towards the dynamics of the parasite population.

The work presented in the following chapter(s) attempts to address some of these questions by looking at how widespread the problem is, investigating the relationship between the different parameters involved, exploring the consequences of the phenomenon and attempting to develop some practical methods of dealing with the problem of IRFN.

4.2. Materials and Methods

4.2.1. Samples

Samples of human blood known to be infected with *T. brucei rhodesianse* were obtained from the University of Salford, Centre for molecular Epidemiology. Samples were of whole blood stored on Whatman FTA cards obtained from Angola in 2000.

Samples of bovine blood, other than those mentioned in the previous chapter, were chosen randomly from a large set of samples obtained from a longitudinal study carried out by the University of Edinburgh, Centre for Tropical Veterinary Medicine. The samples were of whole blood stored on Whatman FTA cards obtained from Sitengo, Uganda in 2002.

Sample Preparation

Sample preparation was as described in section 2.2.3

4.2.2. Diagnostic techniques

ITS - PCR

The protocol and primer sequences are as described by Cox *et al* (2005) and in Section 2.2.4.

Mammalian tubulin specific PCR

For further details of this protocol see Section 2.2.4.

Electrophoresis

Electrophoresis is as described in Section 2.2.9.

4.2.3. Calculated infection intensity

The estimated infection intensity was calculated by counting the number of positive and negative results obtained for each sample and calculating the volume of blood analysed by the PCR assays given that a single 2mm punch taken from the card contained a single microlitre of blood. This calculation makes the assumption that at

low infection intensities (below 1000 parasites per millilitre) positive results generally represent the presence of a single trypanosome on the sampled 2mm disc. The accuracy of this method of assessing parasitaemia was tested using uninfected blood spiked with known numbers of trypanosomes (the positive control).

4.2.4. Fitting negative binomial and Poisson distributions to the data

An empirical distribution (either Negative Binomial or Poisson) was selected with parameters that maximised the probability of obtaining the given data set, this was carried out using maximum likelihood methods. Tests of goodness of fit to this theoretical distribution were carried out using a Chi square test. Values of k, the dispersion parameter for the negative binomial distribution, were calculated first as an estimate using a corrected moment estimate (Elliot, 1977) and more accurately by maximum likelihood methods (Pacala & Dobson, 1988).

4.2.5. Simulation: Screening of four populations

In order to theoretically demonstrate the effects of screening samples from populations with different patterns of infection, four models were set up using the mathematical programming language of R version 1.8.1 (Ikaha & Gentleman, 1986). The sequence of the model is as follows: A vector containing $1x10^6$ data points, representing a population of hosts is generated, each data point is assigned an infection intensity according to the distribution assumption of the simulation. A value of zero denotes an uninfected host. The distribution of infection intensities throughout the simulated population are assigned in one of two ways.

Population infection patterns generated from a negative binomial distribution

Firstly, the infection intensities are randomly chosen from a negative binomial distribution having the properties 'mean intensity' – denoting the mean infection intensity of the population (theoretically, the total number of parasites present in the population, divided by the number of hosts), and the dispersion factor 'K' – describing how the parasite population is distributed among the host population. The dispersion factor k is an inverse measure of over-dispersion, as k approaches a value of zero the population is said to be over-dispersed, where relatively few hosts harbour the majority of all parasites in a population. As K approaches infinity the population is said to be randomly distributed, in practice populations with values of k below eight are said to be over-dispersed (Elliot, 1977). The negative binomial distribution was chosen on the basis of an analysis which fitted a Poisson and negative binomial distribution to the data collected from field samples (See this chapter Figure 4.6 and Table 4.4).

Population infection patterns not conforming to a distribution.

In this population the data points are allowed to take two values for infection intensity, zero for uninfected or an infection intensity of 10,000 (parasites per millilitre) for infected hosts. This represents a high infection intensity that will give fully repeatable results with the diagnostic test, for the purposes of the model the fact that each infected host possesses the same infection intensity is unimportant for high infection intensity values. The number of infected hosts within the population vector was assigned according to the desired population prevalence, in this case 14.5%.

Each model is independent of other models, once the population is generated the remaining calculations are the same in all cases. Once the population vectors have been constructed the population prevalence is calculated and stored in a data frame ready for output.

Number of samples drawn for analysis

A number of samples are drawn from the population vector for analysis to simulate the sampling of a host population in epidemiological studies. The number of samples drawn for analysis was calculated for a population survey or descriptive study using random (non-cluster) sampling. The expected frequency was 50% (as this is the 'worst case' level assumed in epidemiological studies which have no prior expectation of the likely prevalence) in a population of $1x10^6$ individuals. A 95% confidence interval with a confidence level of 5% gave an estimated sample size of 382. The calculation was performed after the method of Bristol (1989), the calculation is described below.

$$n = \frac{Z * p * (1 - p)}{C}$$

Correction for finite population.

$$n_c = \frac{n}{1 + \frac{n}{P}}$$

Where:

n = Sample size

Z = Z value (e.g. 1.96 for 95% confidence interval)

p = Expected frequency

C = Confidence level (expressed as a decimal e.g. 0.05 = +/- 5%)

n_c = Corrected sample size

P = Population size

The vector of infection intensities is then converted to the number of parasites per microlitre by dividing by 1000. This is because the values given to infected hosts within the population are in parasites per millilitre, the diagnostic technique modelled is assumed to be capable of detecting a single parasite in one microlitre of blood (one microlitre is a typical volume analysed in PCR assays). Hence dividing the infection intensity by 1000 gives the number of parasites per microlitre, the analysed volume. This value describes the mean number of parasites per analysed volume of sample. In practice parasites are not uniformly distributed in the blood or sample and so the number will be subject to stochastic variation. In this case the probability of obtaining any give count of the parasite can be modelled as a Poisson distribution with 'mean per microlitre' as the mean of a Poisson distribution for that blood sample. This allows for the probabilistic effects of obtaining a parasite in the analysed volume due to both the infection intensity and the random distribution of parasites throughout the sample. The number of parasites obtained in that diagnostic test is then determined from the Poisson distribution modelled for each sample. If the sample contains a count of greater than or equal to one parasite then that is determined as a positive diagnostic test. If no parasites are present in the analysed volume then that is determined as a negative result. Having determined the result of the diagnostic test (infected or uninfected) for each sample a diagnosed prevalence is then calculated and the data is then stored.

Monte Carlo simulations

Because the model is essentially stochastic in nature there will be variation in the results obtained for each iteration of the model. In order to allow for this variation, the model was repeated for many iterations (typically 1,000 or 10.000), the results of each iteration are then stored in an output data frame. After completion of the iterations, the mean and 95% confidence intervals of the population prevalence and diagnosed prevalence are calculated.

4.3. Results

A blood sample from a human patient known to be infected with *Trypanosoma brucei gambiense* was screened using a trypanosome specific PCR protocol targeting the small ribosomal subunit (ITS-PCR). The diagnostic test was repeated

six times on the same sample (Figure 4.1. (A)), three of the six samples were positive for *Trypanosoma brucei* the other three samples were false negatives. In order to determine if the lack of repeatability was due to inhibition of the PCR or deterioration of the sample, a PCR targeted to the host DNA (specifically mammalian tubulin) was used to repeat screen the same sample (Figure 4.1. (B)). In this case no false negative results were obtained indicating that the lack of repeatability observed in the first screening was not due to sample degradation or inhibition of the PCR reaction.

A sample of blood from an African zebu cow known to be infected with *Trypanosoma brucei* was repeat screened thirty three times using ITS-PCR (Figure 4.2.). In this case only thirteen of the thirty three (39.4%) of the samples were positive for trypanosomes. Twelve of the samples were positive for *T. brucei* and one was positive *Trypanosoma congolense*. These result showed that 20 of the tests gave false negative results and for the positive results different species can be diagnosed in different assays.

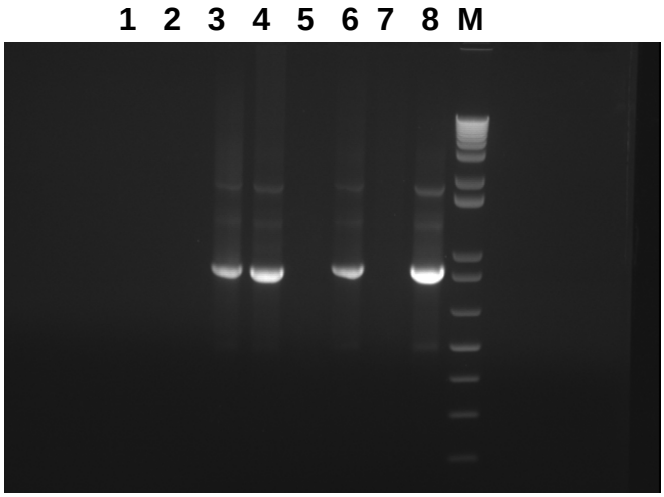
False negative results have been found to occur in other parasite species (Data and pictures courtesy of Olga Tosas-Auguet, University of Edinburgh), during screening of a large number of samples of DNA (in solution) extracted from tick salivary glands for infection with *Theileria parva* the same intermittent positive results have been observed (Figure 4.3). To investigate the cause of the false negatives a dilution series was produced from a sample known to be positive for *T. parva*, each dilution was screened five times. The results showed that at high concentration all repeated tests are positive, whilst as the DNA becomes more diluted false negatives begin to occur. When there is less than one copy of the target gene per microlitre positive results are rarely obtained.

The previous results indicated that the intermittent positive results could be due to low infection intensities. To examine the effect of a range of different infection intensities, a sample of uninfected bovine blood (U.K. origin) was spiked with known numbers of cultured *Trypanosoma brucei*. By diluting aliquots of this with further quantities of uninfected bovine blood a dilution series was constructed. Each dilution was screened eight times (Figure 4.4). At a dilution of $1:10^{-5}$ all samples were positive, indicating complete repeatability of the diagnostic test. Performing repeat diagnostic tests on the next dilution $1:10^{-6}$ demonstrated false negative results with only two of the eight samples. Repeat analysis of the $1:10^{-7}$ sample demonstrated

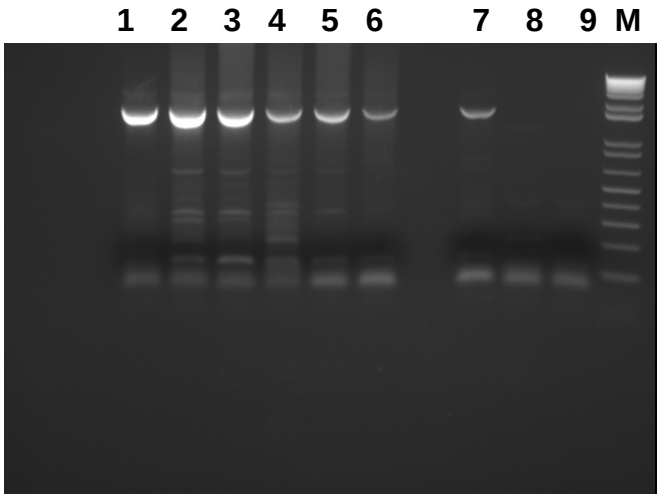
that repeatability had deteriorated further with only one sample (and one very weak sample) testing positive.

Figure 4.1. False negative results obtained from a human blood sample parasitologically positive for *T.b.gambiense*

(a)

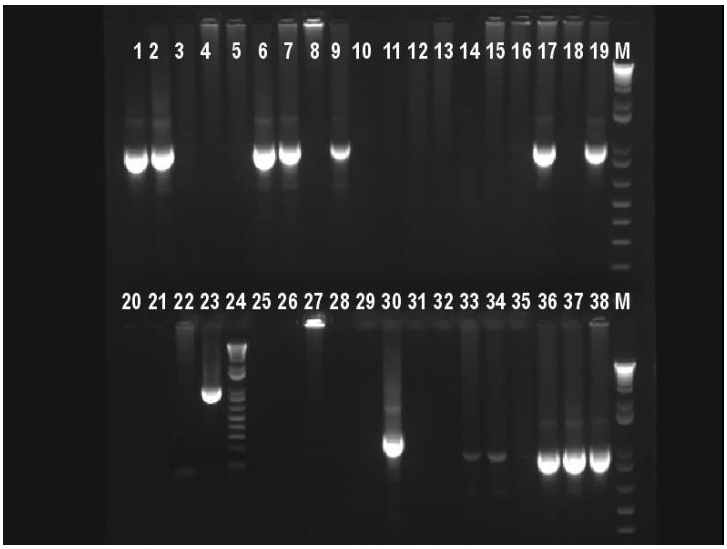


(b)



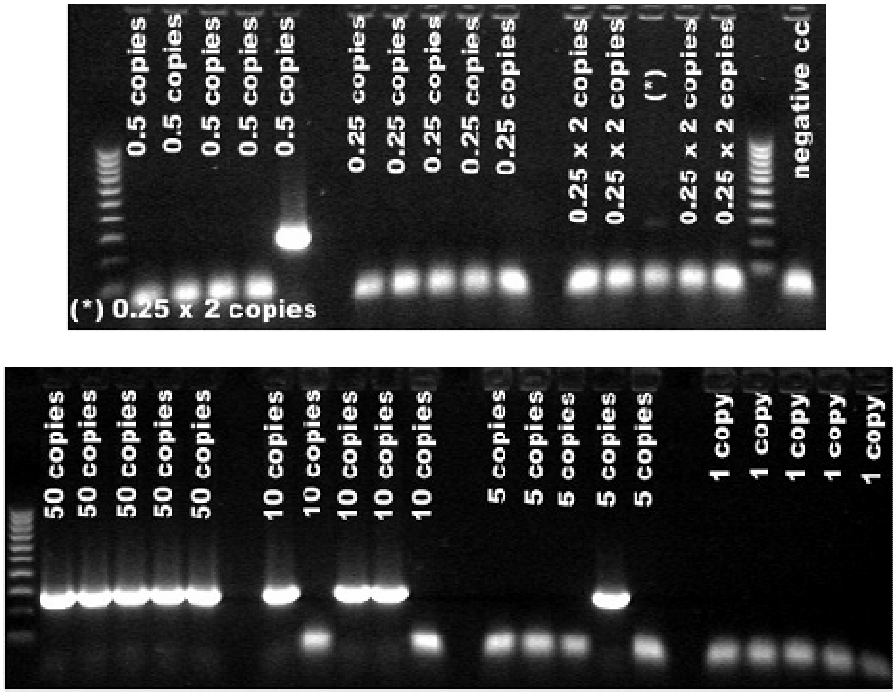
The figure shows a repeated PCR screening of blood from a human patient known to be infected with *T.brucei gambiense.* There are a number of false negative results. Lanes 1, 2 & 5 are negative, 3,4 & 6 are positive, 7 is a negative control, 8 is a positive control and M is a DNA size marker. (B) In order to confirm that the results are not related to PCR inhibition the same sample was also screened with a PCR specific for mammalian tubulin, all the results for this screening are positive. Lanes 1 – 6 are positive, lane 7 is a positive control, 8 & 9 are negative controls and M is a DNA size marker.

Figure 4.2. False negative results in a sample of bovine blood from a cow known to be infected with *T.b.brucei*



The figure shows a repeated screening of the same blood sample taken from an African zebu cow. There are many false negative results and in some cases different species are detected in different PCR screenings. Lanes 1,2,6,7,9,17,36,37,38 are all positive for *T. brucei,* whilst lane 30 is positive for *T. congolense*. Lane 19 is a positive control, lane 18 is a negative control. Lanes 22, 23 & 24 are reruns of positive samples and unrelated to this experiment.

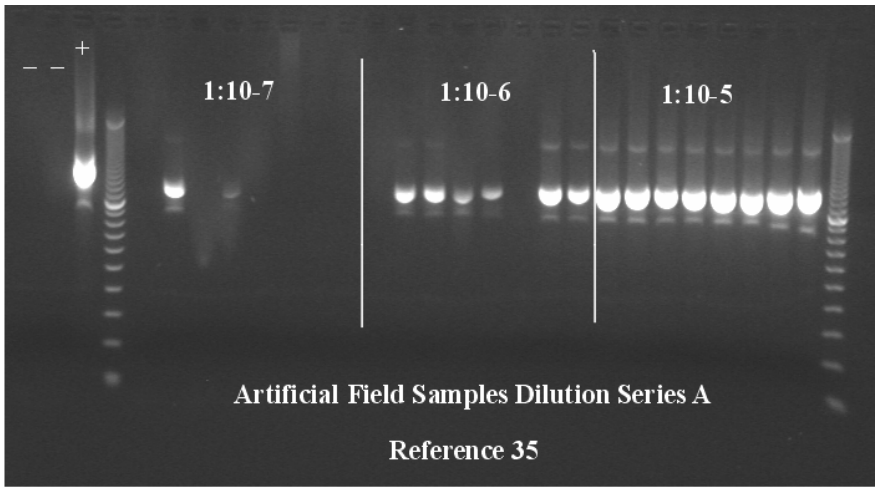
Figure 4.3. False negative results in dilutions of *Theileria parva* DNA



The figure shows serial dilutions of genomic DNA from a *T.parva* stock. As the dilution of the DNA increases the number of positive results declines. The estimated number of copies of target sequences in the extracted DNA is shown.

(Results courtesy of Olga Tosas-Auguet, University of Edinburgh)

Figure 4.4. False negative results in serial dilutions of *T.brucei brucei* in blood



A quantity of cultured *T.brucei brucei* was diluted in cow blood and placed onto Whatman FTA cards. The results shown here show that as the dilution of the *T.brucei brucei* increases the number of false negatives results increases.

The false negative rate was further investigated exploring the data obtained from repeat screening of thirty five randomly chosen blood samples taken from African Zebu cattle of unknown infection status. Each sample was repeatedly screened until the sample was exhausted. The results of conducting 3621 PCR based diagnostic tests on the thirty five samples are shown in Table 4.1. The mean number of diagnostic tests performed per sample was 103, but the actual number was dependent on the volume of blood available. The IRFN rate ranged from 64 false negative results for every 100 tests, to as high as 98 IRFN results for every 100 diagnostic tests, with a mean of 89 IRFN results per hundred tests. From the results of these screenings, estimated infection intensity was calculated for each sample (see materials and methods – 'calculated infection intensity'), the results are shown in Table 4.2

The prevalence at each round of screening of the thirty five samples was recorded. Table 4.3. shows the diagnosed prevalence at the first round and the cumulative prevalence after repeated screenings. The total prevalence for any trypanosome species obtained from the first round of screening each sample only once was 14.3% (8.5% excluding *T. theileri*). Each sample was screened repeatedly, and the cumulative prevalence was recorded, after 92 rounds of screening the cumulative prevalence for any trypanosome species risen to 85.7%. The mean prevalence for any trypanosome species across all repeat screenings was 9.7%. Figure 4.5 shows the cumulative prevalence, mean prevalence and the cross sectional prevalence for any trypanosome species obtained at each round of screening. Up to fifteen rounds of screening the prevalence rises sharply and almost continuously with each additional screening. After fifteen rounds of screening the increase in cumulative prevalence begins to decline.

Table 4.1. Results obtained from multiple PCR of thirty five blood samples from zebu cattle (Standardised to positives per 100 repeat tests)

Sample No.	*T. theileri*	*T. brucei*	*T. congolense*	*T.vivax*	All	Not *T. theileri*
OJ01	13	0	0	0	13	0
OJ02	2	0	0	0	2	0
OJ03	6	3	7	0	16	10
OJ04	1	1	0	0	2	1
OJ06	0	0	0	0	0	0
OJ07	2	2	4	2	10	8
OJ08	4	0	0	0	4	0
OJ09	7	6	4	0	17	10
OJ10	8	0	0	13	20	13
OJ13	7	0	2	0	9	2
OJ14	3	0	0	1	4	1
OJ15	10	2	0	3	15	5
OJ16	0	0	0	0	0	0
OJ17	2	1	3	0	6	4
OJ18	21	10	6	0	36	16
OJ19	6	0	3	0	9	3
OJ20	12	0	0	0	12	0
OJ21	1	0	1	0	2	1
OJ22	3	0	3	2	8	5
OJ23	3	0	0	0	3	0
OJ24	18	0	0	0	18	0
OJ25	18	1	2	0	21	3
OJ26	0	0	0	0	0	0
OJ27	0	0	0	0	0	0
OJ28	3	0	3	0	6	3
OJ33	4	1	2	0	6	3
OJ34	0	0	0	0	0	0
OJ36	4	0	0	0	4	0
OJ38	1	1	0	0	2	1
OJ39	1	0	3	3	7	6
OJ40	1	1	0	1	3	2
OJ45	13	0	0	0	13	0
OJ46	2	0	0	0	2	0
OJ47	8	3	12	12	35	27
OJ49	2	0	1	0	3	1
Negative Control	0	0	0	0	0	0
Positive Control	0	44	0	0	0	44

The frequency of positive results for *T.theileri, T. brucei, T. congolense, T. vivax* all species and all species except *T. theileri* is shown in the table. The number of positive results has been standardised so that the number of positive results per 100 repeat tests is shown. This data is used to fit negative binomial and Poisson distributions in the following sections.

Table 4.2. Estimated infection intensity calculated for each sample and each species

Sample No.	*T.theileri*	*T.brucei*	*T.congolense*	*T.vivax*	Not *T.theileri*	All
OJ01	130	0	0	0	0	130
OJ02	19	0	0	0	0	19
OJ03	59	30	69	0	99	158
OJ04	10	10	0	0	10	20
OJ06	0	0	0	0	0	0
OJ07	20	20	41	20	82	102
OJ08	39	0	0	0	0	39
OJ09	73	64	36	0	100	173
OJ10	77	0	0	125	125	202
OJ13	69	0	20	0	20	89
OJ14	27	0	0	9	9	36
OJ15	98	20	0	29	49	147
OJ16	0	0	0	0	0	0
OJ17	19	9	28	0	38	57
OJ18	206	98	59	0	157	363
OJ19	57	0	29	0	29	86
OJ20	118	0	0	0	0	118
OJ21	10	0	10	0	10	20
OJ22	29	0	29	20	49	78
OJ23	29	0	0	0	0	29
OJ24	183	0	0	0	0	183
OJ25	182	10	20	0	30	212
OJ26	0	0	0	0	0	0
OJ27	0	0	0	0	0	0
OJ28	30	0	30	0	30	59
OJ33	37	9	19	0	28	65
OJ34	0	0	0	0	0	0
OJ36	40	0	0	0	0	40
OJ38	10	10	0	0	10	20
OJ39	10	0	29	29	59	69
OJ40	10	10	0	10	19	29
OJ45	132	0	0	0	0	132
OJ46	20	0	0	0	0	20
OJ47	80	27	124	124	274	354
OJ49	20	0	10	0	10	29
Negative control	0	0	0	0	0	0
Positive control	0	437	0	0	437	437

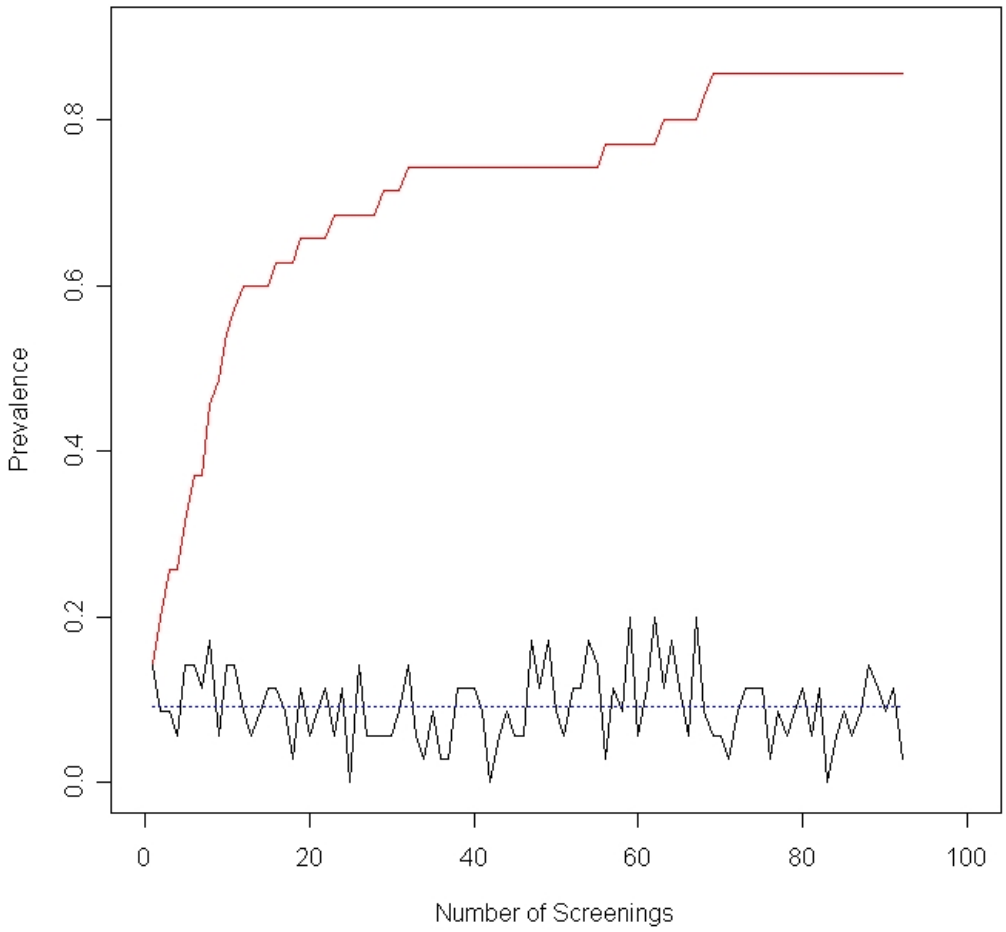
The figure shows the estimated infection intensity by species for each sample. The estimated intensity was calculated from the number of positive results obtained from each sample. The column entitled 'Not *T.theileri*' is the total estimated intensity of *T.brucei*, *T.congolense* and *T.vivax*. The final column shows the infection intensities for all trypanosomes combined. For details of how the parasitaemia was calculated refer to materials and methods section 4.2.3.

Table 4.3. Prevalence of trypanosome species in African zebu cattle at the first round of screening and after repeated screening

Species	Single PCR per Sample Prevalence (%)	Multiple PCR per Sample Prevalence (%)
T. theileri	*5.7**	*85.7*
T. brucei	2.9*	34.3
T. congolense	5.7*	42.9
T vivax	0*	22.9
All trypanosomes	14.3*	85.71
Mixed Infections (All Samples)	0*	60
Mixed Infections (Positive Samples Only)	0*	70

Table showing (In the first column) the prevalence of the different species of trypanosomes and the prevalence of mixed infections detected in thirty-five blood samples collected from zebu cattle. In the second column the cumulative prevalence of the different species of trypanosomes and the prevalence of mixed infections is shown. These samples were subject to repeated PCR samplings until the sample was exhausted, between 92 to 110 times each. * denotes prevalence obtained from the first round of screening, all samples tested once. This would have been the prevalence assumed in an epidemiological study.The mean diagnosed prevalence of any trypanosome species for all repeat screenings was 9.7%.

Figure 4.5. Cumulative prevalence achieved at each round of screening of blood samples taken from thirty five African zebu cattle

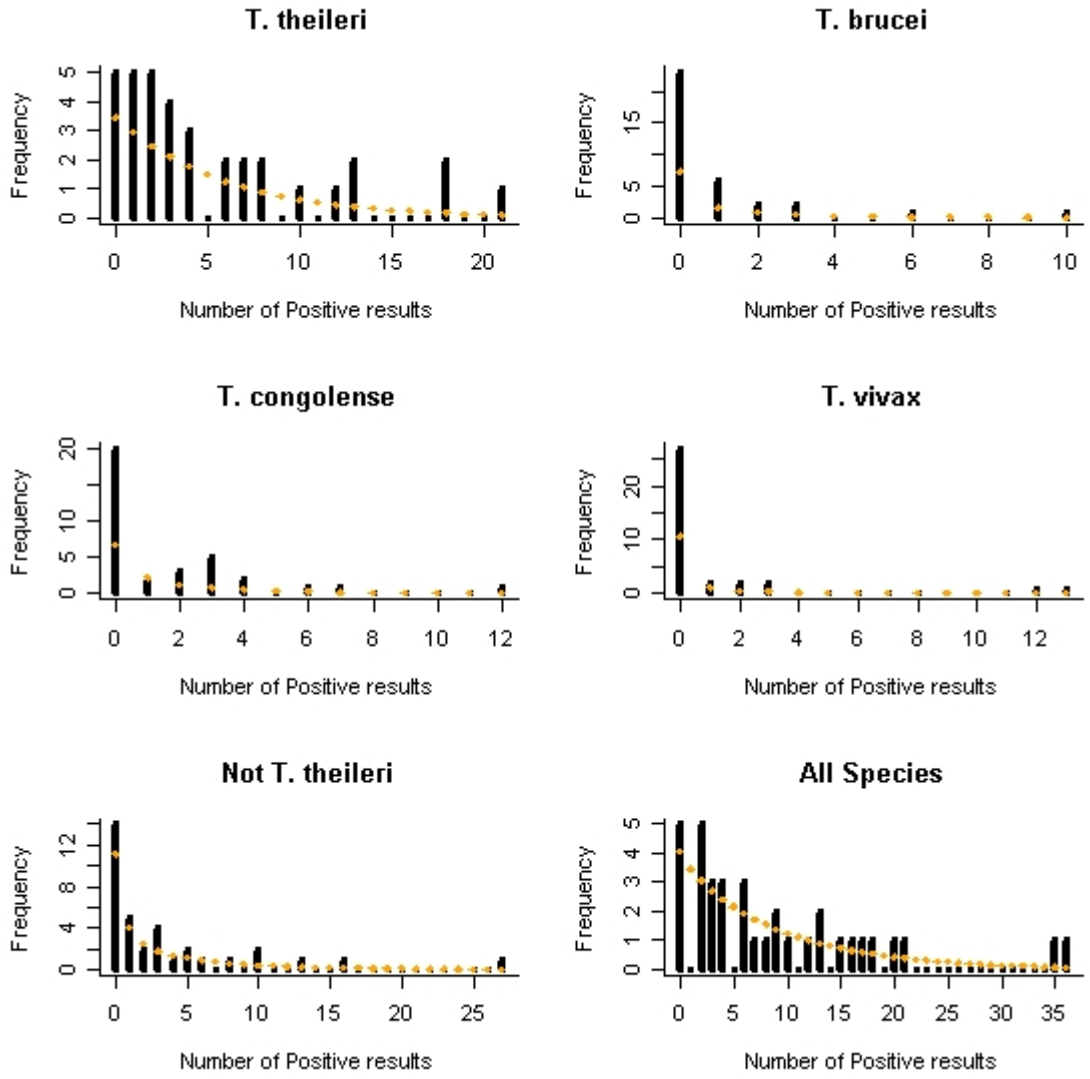


The figure above shows the plot of the cumulative prevalence (red) for any species of trypanosome at each round of screening of the thirty five blood samples. As the number of screenings increases the cumulative prevalence also continues to increase as new samples are found positive. The cross sectional prevalence at each round of screening is also shown (black). The mean cross sectional prevalence across all screenings is shown by the dotted line (9.7%).

The frequency distributions of each species individually and all species are shown in Figure 4.6. The distributions for *T.brucei*, *T.congolense*, and *T.vivax* contained few positive results in comparison to the number of negative results. The frequency distribution of *T.theileri* alone and all species appeared to be consistent with what might be expected from an empirical distribution.

The data obtained from analysis of the thirty five blood samples were fitted to either a Poisson or a negative binomial model in order to determine which, if any, empirical distribution provided the best fit to the data. In addition, the variance to mean ratio and K were calculated as measures of over-dispersion. However, K was only calculated for those datasets that provided a good fit to the negative binomial distribution as it is only appropriate in those cases. Tests of goodness of fit were carried out using a Chi square test against an empirical distribution with parameters that maximise the probability of obtaining the given data set (determined using maximum likelihood methods) the results are shown in Table 4.4. No data set provided a fit to a Poisson distribution, indicating a significant departure from randomness. Only *T.theileri* and all species combined provided fits to a negative binomial distribution. The values of K for these samples showed a high level of over-dispersion as in each case the value of K was below one. All the variance to mean ratios were much greater that one, indicating that all the species were very overdispersed.

Figure 4.6. The frequency distributions of positive results per 100 repeat screenings for the various trypanosome species present



The x axis shows the number of positives per 100 repeat tests obtained from the samples (n = 35) the y axis shows the frequency of observations within given ranges of infection intensity. Observed values are shown by the black bars, whilst the values expected for a negative binomial distribution with mean value (P) and overdispersion (K) estimated by maximum likelihood methods is shown by the yellow dots.

Table 4.4. Results obtained from fitting a negative binomial distribution (NBD) and a Poisson distribution to the data obtained from the samples

Species	NBD	Poisson	K^	μ	Mean/Var
T.theileri	-df	-df	0.99	5.43	6.21
T.brucei	-df	-df	0.27	0.94	4.68
T.congolense	-df	-df	0.35	1.66	4.90
T.vivax	-df	-df	0.11	1.11	9.23
Not *T.theileri*	0.339 (0.913)	<0.001 (84.5)	0.39	3.71	10.60
All	0.437 (1.654)	<0.001 (96.7)	0.91	9.14	10.34

The values shown for the fit are the p values and Chi-square test value in brackets. P values of greater than 0.05 indicate no significant difference to the empirical distribution under comparison. Where the data was found to be well described by a NBD the value of 'K^' is shown (Dispersion parameter for the NBD) as calculated by maximum likelihood methods and corrected moment estimate in brackets. The mean to variance ratio is also shown for all data. (-df) indicates that there were not enough degrees of freedom left to perform the Chi-Square test, more specifically to few bins with observed values of greater than or equal to five were obtained to make a valid statistical test .

Simulation of the consequences of false negative results in comparison of four different populations

The outputs from four models simulating diagnostic screening of samples randomly drawn from four populations with different patterns of infection are shown in Table 4.5. The details of how the infection patterns of each population of one million hosts differs is also shown in Figure 4.7. In population (A) 14.5% of the population are infected and have very high infection intensities of greater than 10^4 parasites per millilitre of blood. In population (B) 28% of the population are infected with a range of different high, low and medium infection intensities. (C) 65.5% of the population are infected most of which have infection intensities below 10^3 parasites per millilitre of blood. Finally in population (E) the entire population is infected with very low level infection intensities less than 10^3 parasites per millilitre of blood. The results of Monte Carlo simulations of each of the models are shown in Table 4.5., despite the obvious differences in the population prevalence and patterns of infection of each simulated population, the mean diagnosed prevalence from a single screening of 382 samples was around 14.5% in all cases. Clearly the levels of infection intensity present within the population have an important influence on the diagnosed prevalence.

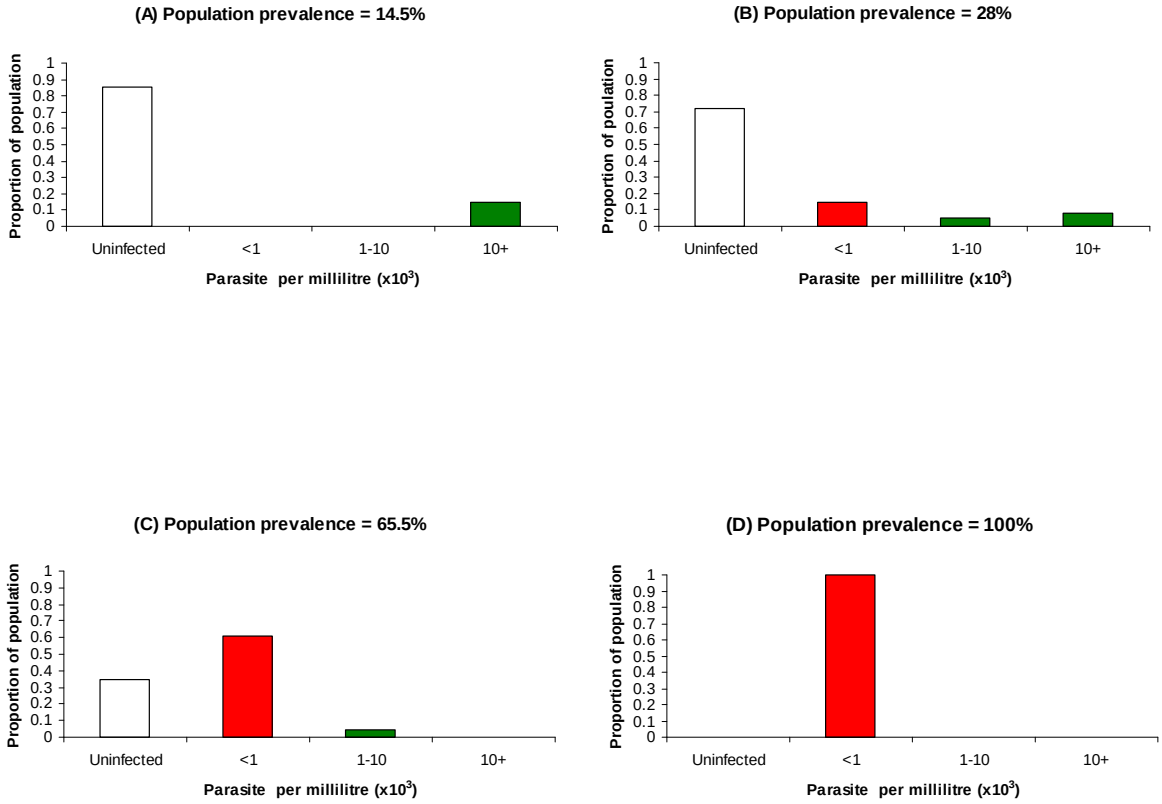
Table 4.5. Characteristic parameters of differing patterns of parasite infection in four simulated populations of 1x 10^6 hosts

Population Prevalence	Mean Intensity (Parasites per ml)	Overdispersion (k)	Mean Diagnosed Prevalence (%)	95% C.I.
14.5	3000	N/A[1]	14.58	11.26 / 18.12
26.7	6000	0.027	14.5	11.52 / 17.55
66	200	0.15	14.77	10.99 / 18.32
100	150	8	14.69	10.72 / 18.20

[1] – This population was not modelled with a distribution. In this model 14.5% of the hosts were assigned infection intensities greater than 10,000 parasites per ml, whilst the rest were uninfected.

The diagnosed prevalence and associated 95% confidence intervals obtained from a Monte Carlo simulation are shown (1000 iterations). The model was designed to simulate screening 382 samples from each of the populations with a diagnostic technique capable of detecting a single parasite per microlitre. Although the true population prevalence varies greatly the diagnosed prevalence for each population is remarkably similar. The mean diagnosed prevalence obtained from the simulations and associated 95% confidence intervals are also shown.

Figure 4.7. Theoretical demonstration of how differing patterns of infection in four host populations can result in a similar diagnosed prevalence



Each histogram represents the pattern of parasite infection within each of four different host populations. The proportion of hosts is shown on the Y axis. The population has been divided into four categories on the X axis. Firstly, the proportion of uninfected hosts (no fill). The next category shows the proportion of hosts with infection intensities below 1,000 parasites per millilitre and which will show a high false negative rate. The next bin shows infection intensities of between 1,000 to 10,000 parasites per millilitre, these hosts will exhibit a lower false negative rate with the diagnostic technique but may not present with overt clinical signs. The next category shows those hosts with the highest infection intensities (greater then 10,000 per millilitre) and correspond to the most clinically ill hosts which are likely to present with clinical signs and no false negative results. The infection intensities selected for these last two categories are arbitrary, and have been selected in order to demonstrate a point, and are not based on current knowledge. The true prevalence of infection in each population is shown above each histogram. Each of the four histograms represents data used for computer simulations of an epidemiological screening of a population of $1x10^6$ hosts. 382 samples (calculated as an appropriate sample size) were drawn at random from each of the four populations and each sample was screened once with a (simulated) diagnostic technique capable of detecting a single parasite per microlitre, the probability of detection was directly related to the infection intensity of each host. The mean diagnosed prevalence achieved from the four simulations was as follows: (A) 14.58% (B) 14.5% (C) 14.77% (D) 14.69%. Further details of these results are given in Table 4.5. The inaccuracy of these results derives from the proportion of animals with low infection intensities which result in false negatives by the diagnostic technique (Red) [sub-patent infections].

4.4. Discussion

The work conducted in the previous chapter investigated the data obtained from repeated screening of thirty-five blood samples and showed a high proportion of mixed infections within those samples. However, there are additional aspects consequent from the results obtained from this repeat test study. It is apparent from these results that not all aliquots drawn from clearly infected blood samples provide positive diagnostic results (the existence of many false negative results). A second aspect is that the prevalence achieved from a single round of screening is very different from the cumulative prevalence achieved after multiple rounds of screening. These issues are clearly of great potential importance for epidemiological studies in that there is a clear underestimation of prevalence in this case. The work presented in this chapter was designed to investigate these aspects in greater detail, and in particular to assess the potential consequences and importance of this phenomenon. In order to achieve this it was necessary to determine if this effect occurred in other species and geographical areas or was restricted to only this sample set. Possible causes for the occurrence of false negative results in these samples were then investigated. A theoretical distribution was fitted to the infection intensity data in order to provide a means of modelling the problem, this allowed further investigation and assessment of the consequences of the phenomenon.

Initial investigation indicated that the occurrence of false negatives was not restricted to the set of samples examined in the previous chapter. Repeated testing of a number of blood samples from patients diagnosed with the chronic form of sleeping sickness (*Trypanosoma brucei gambiense*) also showed false negative results (Figure 4.1.a). False negative results were also found in samples from other cattle known to be positive for *Trypanosoma brucei brucei* (Figure 4.2.) and has also been shown in other samples taken from cattle from different geographical regions which were infected with *T.brucei brucei, T. congolense, T. vivax* and *T. theileri* (data not shown). These results provide strong evidence that the phenomena is more widespread and is not restricted to the thirty five samples subject to repeat testing in the previous chapter.

It was therefore postulated that the effect could either be due to inhibition of the PCR technique by elements within the blood sample or by the low intensity of the parasites within the blood. The *T. brucei gambiense* infected human blood samples which had previously been repeat tested were subject to a further repeat screening

with a PCR protocol targeted to mammalian tubulin genes. As the target of this PCR was present in the white blood cells of the patient, repeated testing of the samples should not produce false negative results unless the PCR reaction was under inhibition. The samples were tested six times each and all results were positive (Figure 5.3.1.b.), this indicated that the false negative results are unlikely to have been related to the inhibition of PCR by elements within the blood or by excess DNA present in the sample.

To determine if low infection intensity could be the cause of the intermittent nature of positive results a sample of uninfected bovine blood was spiked with a known concentration of cultured trypanosomes, the spiked blood was then used to produce a dilution series which was subject to the same repeat testing as the field samples. At a dilution of $1:10^5$ all repeated tests were positive, at a further dilution of $1:10^6$ three false negative results occurred. At a dilution of $1:10^7$ seven false negative results were obtained from eight repeated diagnostic tests (Figure 4.4.). These findings are augmented by other work which showed that dilution of a known concentration of DNA extracted from tick salivary glands infected with *Theileria parva* (the causative organism of East Coast Fever in Cattle) produced the same effect when aliquots from a dilution series were examined (Figure 4.3. [results courtesy of Dr Olga Tosas-Auguet, University of Edinburgh]). Such false negative results have also occurred in field samples of genomic DNA extracted from the blood cattle infected with *Theileria parva* (personal communication; Dr Olga Tosas-Auguet, University of Edinburgh). This latter work was also important in that it provided evidence that IRFN results could also be obtained from extracted genomic DNA in liquid form in addition to blood applied to filter paper.

These results provide very strong evidence that the occurrence of false negative results is related to the infection intensity of the parasites. Hence if the diagnostic target is not present in the aliquot drawn from the sample then the test will be negative despite the fact that the host may be infected with the diagnostic target (in this case the parasite). A similar situation occurs with the use of microscopy in that the parasite will not always be present in every field of view examined, and even examination of 200 fields does not definitively denote an uninfected host.

For the repeated testing of the dilution series of the spiked blood sample (Figure 4.4.) the number of positive and negative repeat tests obtained from each dilution was approximately consistent with the number of positives that would be expected

to be obtained from the concentration of parasites per millilitre for each dilution in the series. The initial concentration of trypanosomes in the spiked blood sample was measured at 1.0 x 10^9 trypanosomes per millilitre, when this sample was diluted by 1:10^5 the resultant infection intensity in the sample would be expected to be 10^4 parasites per millilitre. This infection intensity would rarely be expected to give false negative results as the infection intensity is high, and this proved to be the case. A further ten-fold dilution would give an intensity of 10^3 parasites per millilitre, enough give a moderate number of false negative results. The final ten-fold dilution resulted in a parasite intensity of 10^2 parasites per millilitre, which would be expected to produce many false negative results; again the results obtained were consistent with one positive from eight repeated tests. During the more extensive repeated screening of the 35 samples and controls shown in the previous chapter a control sample with estimated infection intensity after serial dilution of 508 parasites per millilitre was also subject to the same repeat screening. This sample gave 44 positive results from 100 repeated tests. With the assumption that when the positive results are widely spaced on the filter paper containing the blood sample (infection intensities are low) that one positive result generally equates to the presence of a single parasite in the microlitre of blood taken for that particular test, the estimated parasitaemia from the number of positives obtained would work out at approximately 437 parasites per millilitre. This estimated intensity is remarkably close to the measured value. It is evident therefore that the number of positive results obtained from repeated screening has an approximate relationship to the infection intensity in the blood sample. This is provided that the parasites are randomly distributed in the blood and that the spatial distribution of the parasites is preserved when the blood is applied to the filter paper.

During screening and mapping of the thirty five blood samples it was evident that the mean prevalence from repeated screening of the samples (9% for all trypanosome species) was very different to the cumulative prevalence obtained after more than 100 repeated tests (85.71% for all trypanosome species) see Table 4.3. The cumulative prevalence of trypanosomes increased with the number of repeated tests. The cumulative prevalence at each round of screening is shown in Figure 4.5., initially the increase in cumulative prevalence was rapid, although after approximately 20 repeated tests the increase after each round was considerably less. Since the prevalence of many epidemiological studies is determined from a single PCR test of each sample, or screening of 200 fields by microscopy, the

findings presented here raise important questions for the use of prevalence as an epidemiological measure; in that, in such cases as described here it is not clear what the prevalence of infection obtained from a single screening of samples actually represents. Additionally, it is evident that for populations with low intensity infections the samples detected as positive in the first round of screening were not necessarily those detected as positive in the second round of screening, as is evidenced by the increasing cumulative prevalence. This too has important implications for the diagnosis of infected hosts and measurement of agreement between diagnostic techniques.

Using the infection intensity estimated from the number of positive results obtained from each of the thirty-five samples, the frequency distributions for different ranges of infection intensity were plotted for each species separately and for all species combined (Figure 4.6.). The shape of these distributions and the degree of overdispersion indicated that the distribution of infection intensities in the samples may be modelled with a negative binomial distribution. In order to test this hypothesis the data was fitted to both a negative binomial distribution and a Poisson distribution, and the goodness of fit was tested (Table 4.4.). The data was tested for each species individually, for all species and for all species except *T. theileri*. None of the data was found to provide an acceptable fit with a Poisson distribution, whereas the data for *T. theileri*, the most prevalent trypanosome, and for all species combined was not found to be significantly different from that of a negative binomial distribution. For the other species it was not possible to conduct a 'goodness of fit' test as the low number of positives for these species failed to provide observations of greater than five in many of the bins in the frequency distribution. The Chi square test requires at least five observations to be valid. Importantly, this does not mean that the data did not fit a negative binomial distribution. These findings are useful to the wider objectives of this and subsequent work, in that it provides an important, if somewhat tentative, model for the distribution of infection intensities within the host population. This model can be used to investigate the theoretical effects and importance of IRFN results.

In order to demonstrate the possible consequences of IRFN results the NBD was then used in simulations of sample screening using conventional methods (a single screening to obtain prevalence). Exploration of the phenomenon in this way revealed a further consequence of infection IRFN. A simulation was constructed to

model the conventional screening of four populations with very different patterns of distribution of infection intensities and population prevalence (Figure 4.7 and Table 4.5.). The results obtained from the simulation showed that even though the 'true' population prevalence varied between the four populations (from 14.5% to 100%,), the mean diagnosed prevalence (from 1,000 iterations of the model) from a single screening of samples drawn from each population was around 14.6% in all four cases. In the first case (population A) 75 of the 500 hosts sampled were infected with intensities of greater than 10^3 parasites per millilitre. At this intensity level repeated diagnostic testing of samples produced no false negatives, and all of the infected hosts were diagnosed as positive with a single testing of the samples. This situation equates to a conventional understanding of how prevalence is produced in epidemiological studies, the diagnosed prevalence accurately represents the population prevalence. In population B the infection intensities were distributed according to a negative binomial distribution with a mean infection intensity of 6000 parasites per millilitre and very high overdispersion (K = 0.027). The population prevalence was 26.6%, yet because a number of the hosts in the population present with low infection intensities which are only stochastically detectable, some of the infected hosts in the sample were diagnosed with false negative results, this leads to an underestimation of the 'true' population prevalence, as a result the diagnosed mean prevalence was 14.5%. For population C, the infection intensities are again distributed according to a negative binomial distribution with a mean infection intensity of 200 parasites per millilitre and overdispersion of K = 0.15, this population would approximate that found in the thirty five samples which were subject to repeated PCR analysis in this study. Here the population prevalence was 66%, yet because many of the hosts presented with low intensity infections, which are again only detectable on a stochastic basis, many of the infections were falsely diagnosed as negative. The resulting diagnosed mean prevalence was 14.9%. In the final population all of the hosts are infected with very low level infections. There is no overdispersion in the data and the distribution approximates that of a Poisson. In spite of 100% true prevalence in the population the single screening produced a mean diagnosed prevalence of 14.97%. Clearly, on the basis of diagnosed prevalence from a single screening of these samples, there is no difference between the four populations, all the four cases produced a diagnosed prevalence of infection of around 14.6%. This is clearly not the case despite the large sample size used (n = 500), it is difficult to imagine four more different populations. Furthermore, this

point does not depend upon the aggregation pattern of parasites within the host population fitting that of a negative binomial distribution, whilst another population approximated a Poisson distribution. Standard epidemiological methodology is clearly lacking in three of these cases, statistical methodologies would also be found to be in error in the latter three populations. Methods for calculating sample size, power, confidence intervals and agreement between different diagnostic techniques would all give highly misleading results in three of the four cases. Although the population infection patterns were chosen in this case for demonstration of the point, the question arises as to how plausible are these types of infection patterns?

It can be argued that all of these types of infection patterns are likely to exist. In situations of acute infection with a virulent pathogen and little resistance in the host population a number of animals may present with very high infection intensities, approximating population A. In chronic disease with a high level of resistance in the population infections may be widespread but well controlled in the population, this 'endemic' situation may equate to that of population D. Clearly all degrees are possible within these two extremes making the likelihood of these patterns of distribution being present in field situations very high. Furthermore, it is likely that this is applicable to a wide range of pathogens. Additionally, it is evident that information regarding the patterns of distribution of parasites within the hosts and samples are in highly informative with regard to the epidemiology of the parasite in question.

The conventional approach of obtaining a simple prevalence from a single screening of the samples does not discriminate among these patterns of infection within the host population and can lead to serious misinterpretation of the situation. A revised method is required to determine both if a conventional approach is applicable, and to provide information on the distribution of parasites within hosts. An approach that only determines infection or no infection can be highly misleading, a quantitative assessment of infection in each infected host / sample must be included in any future approaches designed to account for this problem. Additionally, the applicability of statistical and epidemiological methods such as the calculation of confidence intervals, sample size and power calculations, methods of statistical testing and measurement of agreement between diagnostic techniques must also be re-evaluated.

Chapter 5: An exploration into the effect of different variables on the measurement of prevalence.

5.1. Introduction

Work in the previous chapter highlighted a number of important problems related to the epidemiological screening of blood samples for trypanosomiasis. Under certain conditions positive results from a diagnostic test are not repeatable, and this leads to a serious underestimation of the population prevalence. Furthermore, use of a simulation indicated that it is possible for populations with markedly different true prevalence and patterns of infection to have the same diagnosed prevalence. For epidemiological studies, the object of screening samples for the presence of a particular haemoparasite is to estimate the prevalence of infection within a population of interest. The presence of sub-patent infections within these populations can lead to serious misinterpretation of the prevalence, incidence and therefore the epidemiology.

In order to investigate this phenomenon further, it is necessary to understand the relationship between each of the parameters which may influence the measurement of prevalence. There are clearly five parameters directly related to the infection of haemoparasites within a population. Definitions of each of the parameters are given after the definitions on page xiii

As can be seen from the previous chapter, initial data from repeated screening of thirty-five blood samples suggested that a negative binomial distribution would best describe the distribution of parasites within the host population. For a negative binomial distribution, which has been used frequently to describe the distribution of parasites in their hosts (Snow & Michael, 2002, Guyatt *et al*, 1990, Eppert *et al*, 2002, Theis & Schwab, 1992, Sitja-Bobadilla *et al*, 2005, Flach *et al*, 1993, Pecora *et al*, 1980), the defining parameters are the mean and the dispersion parameter ‘K’. The mean in this case is taken as the mean infection intensity within the population.

If the infection intensity in the host is responsible for the occurrence of false negative results it is evident that there are two aspects to this phenomenon, firstly whether the parasite is at such allow intensity it cannot be present in every aliquot of sample drawn for diagnostic screening. If this were the only factor then once the parasite reached an infection intensity of one per aliquot, then false negatives would cease to occur. However, this assumes that the parasite is uniformly distributed in the sample. It is important to note that the parasite will be randomly distributed in the host and because of this non-uniform distribution false negatives will still occur at infection intensities well above the theoretical threshold of one parasite per

diagnostic aliquot. Such a random distribution of parasite counts per blood volume can be modelled with a Poisson distribution. It is therefore important to understand at what infection intensity results in individual hosts become consistently repeatable, and more specifically to describe the relationship between the infection intensity in a single host and the occurrence of false negatives [= 1 – repeatability of positive result]). Secondly, it is also important to understand that assuming the parasite is distributed according to a negative binomial distribution at what mean infection intensity in the population positive diagnostic results become consistently repeatable across the population and therefore diagnosed prevalence is equivalent to the population prevalence.

The aims of this chapter are therefore; To explore the relationships between mean infection intensity of the population, overdispersion, population prevalence and diagnosed prevalence. Additionally, for clarity, underestimation of true prevalence will also be included. To establish the relationship between infection intensity in a single host and the probability of false negative results. To establish the relationship between mean infection intensity of the population and the probability of false negative results.

5.2. Materials and Methods

5.2.1. Simulation to produce a data frame of values for all five variables

In order to explore the relationship between each of the five variables, a simple model was constructed to simulate a large population of hosts from which samples are drawn and analysed by a diagnostic technique. For particular levels of population prevalence, mean infection intensity and overdispersion, the simulation calculated the diagnosed prevalence and extent of underestimation of true prevalence obtained from screening a set of samples, given the errors associated with IRFN. The process is repeated in a Monte Carlo simulation up to 10^4 times for different levels of population prevalence, mean infection intensity and overdispersion. The levels of all parameters are stored in a database; this database is then used to examine the relationship between the variables. The basic structure of the simulation is described by the flowchart in Appendix figure 5.1. The simulation was written in the language of ‘R’ version 2.3.0 (Ikaha & Gentleman, 1999).The structure for this simple simulation forms the basis of all subsequent simulations presented in later chapters.

Initially, the simulation randomly selects the overdispersion (K) and mean intensity (P) parameters of a negative binomial distribution. Where overdispersion (K) is allowed to vary in the range of 0 ~ 0.4 and mean intensity (P) is allowed to vary in the range of 100 ~ 2000 parasites per millilitre. Next, a population of hosts / samples ($N=10^5$) is generated within the simulation. Each host /sample is assigned a value for infection intensity, where a value of zero denotes an uninfected host and a value of greater than zero denotes an infected host / sample. The infection intensities are assigned to the samples / hosts from a negative binomial distribution. Once infection intensity values have been assigned according to the distribution parameters selected for this iteration of the simulation, the population prevalence can be calculated. This is achieved by dividing the number of hosts / samples with an assigned infection intensity value of greater than zero by the total size of the population ($N = 10^5$).

The infection intensity for each host, in number of parasites per millilitre, is then converted to the mean number of parasites per microlitre. For example, a host or sample with an infection intensity of 500 parasites per millilitre would have a mean count of parasites per microlitre (the analysed volume of sample) of 0.5 (infection intensity / 1000 = 0.5).

To simulate the epidemiological screening of the population of hosts created in the simulation, a number of samples are randomly selected from the population (n = 382 [see Section 4.2.5]). A set of diagnostic results are generated from each sample by determining the count of parasites that would be obtained in the analysed sample volume. This is determined from a Poisson distribution for each sample with the mean count of parasites per analysed volume previously calculated. A count of greater than or equal to one parasite in the analysed sample volume is recorded as a positive result. The diagnosed prevalence is then calculated by summing all the positive results and dividing by the total number of samples screened. The underestimation is then determined by simply taking the difference between the population prevalence and the diagnosed prevalence. The levels of overdispersion (K), Mean Intensity (P), population prevalence, diagnosed prevalence and underestimation are then stored. The entire process is then repeated 10^4 times storing the values of each of the variables for each iteration of the simulation. The stored results are then output in the form of a data frame (.csv file) which is used to analyse the relationship between the variables (see Figures 5.1. to 5.3). Repeating

the simulation in this way captures the variability inherent in what is, for samples with sub-patent infection intensities, a stochastic process

5.2.2. Determination of the relationship between the infection intensity in an individual host and the probability of false negative results

In order to determine the relationship between infection intensity in a single host and the probability of obtaining false negative results from a single host, a simple simulation was constructed. The simulation assumed that the parasite was randomly distributed in the blood volume, so that counts of the parasite per blood volume were Poisson distributed. The mean count of parasites per blood volume was used as the mean of the Poisson distribution and was calculated from the infection intensity at that iteration of the simulation. For example, for an infection intensity of 1,000 parasites per millilitre a mean count per analysed volume of sample of one was used for the Poisson distribution. At this infection intensity there would on average be one parasite per each microlitre of blood.

In the simulation the distribution of the parasite in 1 litre of blood was modelled for each microlitre of that volume using a Poisson distribution. The simulation then drew one sample from this volume and determined a diagnostic result as positive or negative dependent on if a parasite appeared in the sample of blood drawn. The simulation assumed that the diagnostic technique was capable of detecting a single parasite. This was repeated 100 times and the number of positive results counted to give the probability of obtaining repeated positive results from that positive sample. This value was then subtracted from one to give the probability of obtaining a false negative for this level of infection intensity. This process was then repeated a further hundred times for the same level of infection intensity. The mean probability and associated 95% confidence intervals were then calculated from this data. This process was then repeated for different infection intensities from 100 parasites per millilitre to 10,000 parasites per millilitre and the results recorded for each level.

5.2.3. Determination of the relationship between the mean infection intensity of a population and the probability of false negative results

The code for the simulation is included in Appendix Figure 5.8. Briefly, for each level of mean infection intensity the simulation was conducted as follows:

The level of overdispersion was randomly chosen from possible values between k = 0.1 to 0.4. A population of hosts was generated where infection intensities were spread through the population according to a negative binomial distribution with

parameters k (overdispersion) and P (mean infection intensity). 382 samples were then drawn from the population to simulate random selection of samples from a population in an epidemiological study. A positive or negative screening result was then generated for each sample according to the infection intensity assigned to each sample. This process was repeated five times for each sample and the repeatability for each infected sample was then calculated by dividing the number of positive results obtained by the number of times the samples were screened. The mean probability of obtaining a false negative result for all infected samples was then calculated and the results stored. The process was then repeated for different levels of overdispersion 1000 times (for the same level of mean infection intensity of the population) in order to allow for variation in results due to stochastic effects, and the results stored. The mean and 95% confidence intervals were then calculated for the resulting data. This process was repeated for each level of mean infection intensity of the population.

5.3. Results

5.3.1. Exploration of the relationship between the five variables

Using a simple simulation, a data frame containing 10^4 data points for values of overdispersion (K), mean infection intensity (P), population prevalence, diagnosed prevalence and underestimation of population prevalence was generated. An extract from the data frame is shown in Table 5.1. The relationship between the variables was then explored using a series of contour plots. For all the contour plots the variable of interest is shown in terms of the mean infection intensity of the population (P) and overdispersion of the population (K).

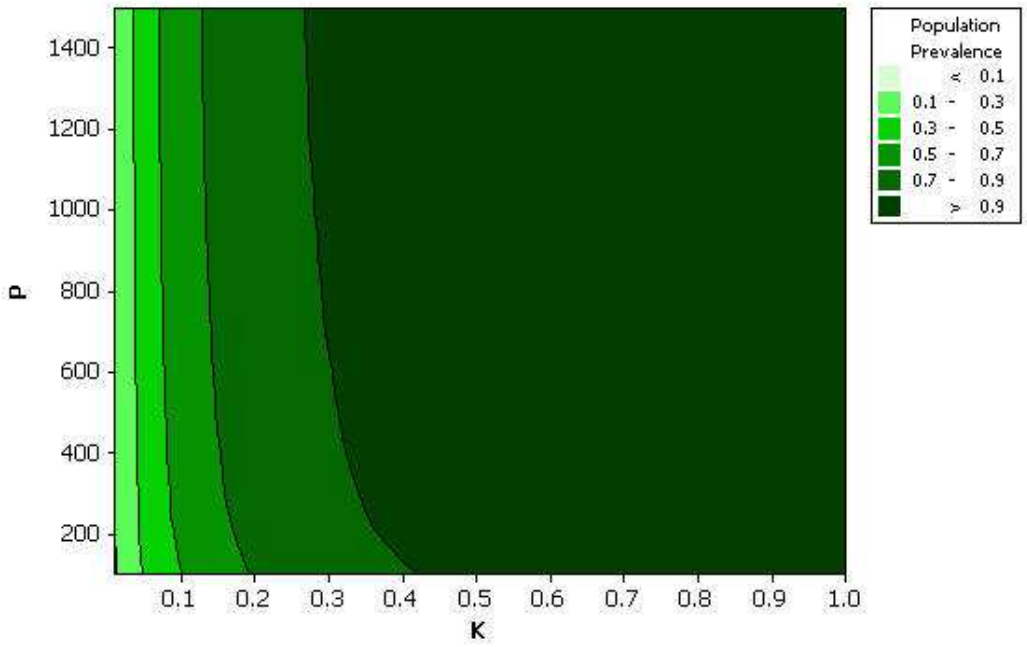
Table 5.1. Extract from the data frame used to explore the relation ship between the five variables

Number	Overdispersion (K)	Mean Infection Intensity (P)	Population Prevalence	Diagnosed Prevalence	Underestimation
1	0.18	327	0.7375	0.206806283	0.530693717
2	0.19	301	0.75	0.217277487	0.532722513
3	0.35	338	0.9132	0.238219895	0.674980105
4	0.3	150	0.8478	0.154450262	0.693349738
5	0.22	1838	0.8652	0.426701571	0.438498429
6	0.07	939	0.4851	0.17539267	0.30970733
↓	↓	↓	↓	↓	↓
9996	0.4	1644	0.963	0.557591623	0.405408377
9997	0.02	1038	0.1921	0.081151832	0.110948168

These two variables are the parameters of the population of interest and have no relation to each other as each is chosen randomly in the simulations to give unique populations. The contour plot showing the relationship of population prevalence to the population parameters K and P is shown in Figure 5.1. There is a simple relation with overdispersion in that as overdispersion increases so does the population prevalence. After an overdispersion value if approximately K = 4, the population prevalence reached 100%. There appears to be no strong relationship between mean infection intensity (P) and population prevalence.

The contour plot describing the relationship of diagnosed prevalence to overdispersion and mean infection intensity is shown in Figure 5.2. Here diagnosed prevalence seems to be equally influenced by both the population parameters, only reaching its highest values when mean infection intensity of the population is high and overdispersion is high.

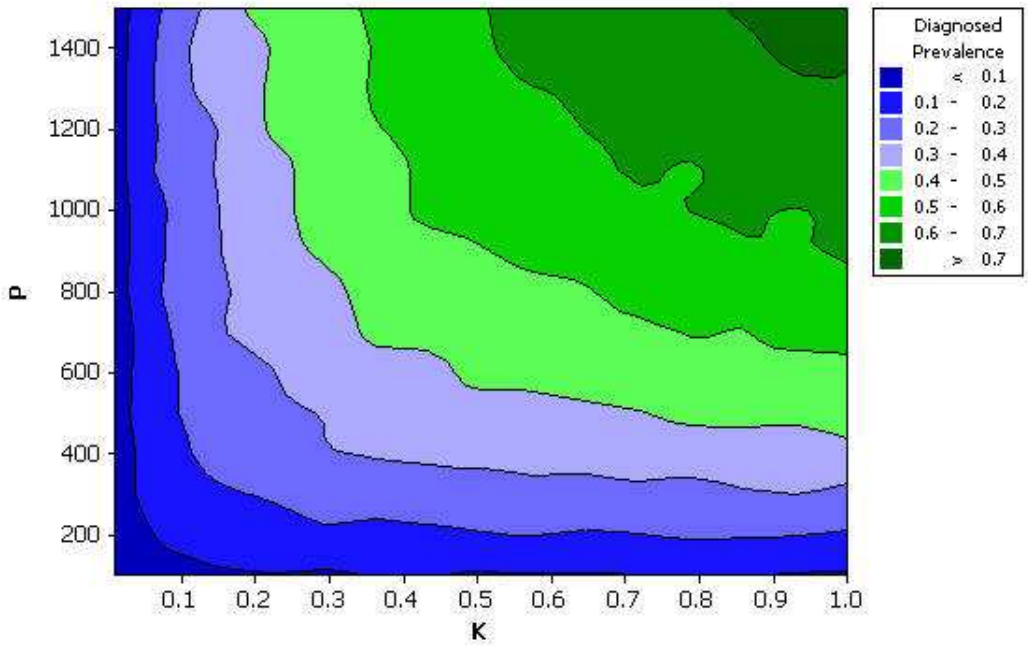
Figure 5.1. Contour plot giving an overview of the relationship between overdispersion (K), mean infection Intensity (P) and population prevalence



The contour plot shown above gives an overview of the relationship between overdispersion (K), the mean infection intensity of the population (P) in parasites per millilitre, and the population prevalence. Different levels of the population prevalence are shown as colour coded contours in the plot area. Details of the colour codings and bands used are shown in the legend to the right of the plot.

The contour plot describing the relationship between underestimation of prevalence and the population parameters K and P is shown in Figure 5.3. Here the relationship is more complex. Underestimation is lowest for the lowest values of overdispersion (K), initially as overdispersion increases so does the underestimation of prevalence. However above an overdispersion value of approximately 0.2, the degree of underestimation begins to be influenced by the mean infection intensity of the population with the lowest levels of underestimation being obtained only at the highest values for P.

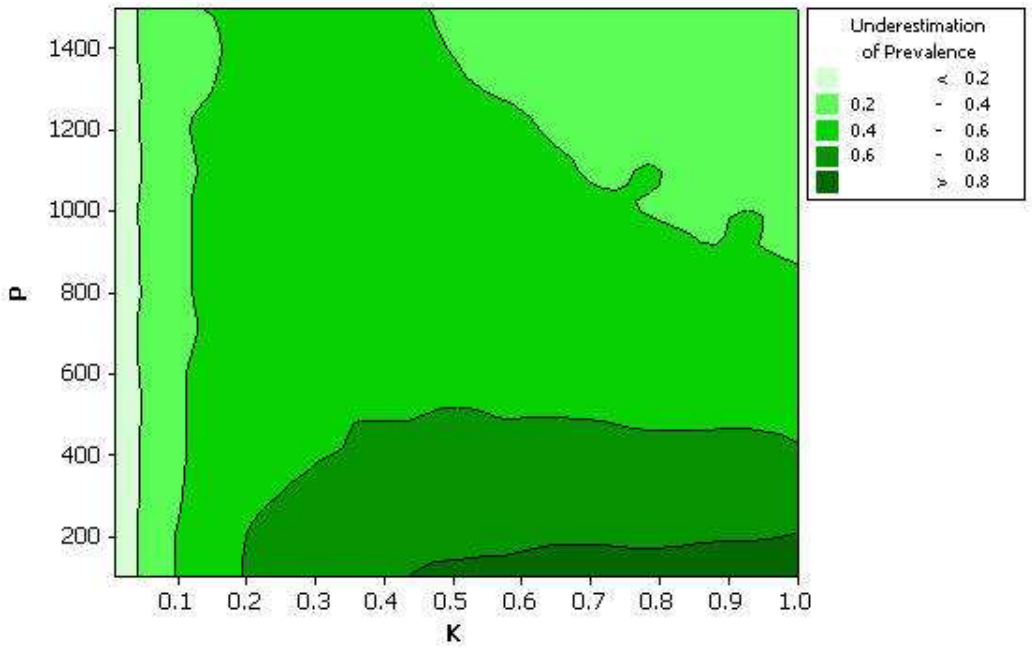
Figure 5.2. Contour plot giving an overview of the relationship between overdispersion (K), mean infection Intensity (P) and diagnosed prevalence



The contour plot shown above gives an overview of the relationship between overdispersion (K), the mean infection intensity of the population (P) in parasites per millilitre, and the diagnosed prevalence obtained from a single screening of 382 samples from the population. Different levels of the population prevalence are shown as colour coded contours in the plot area. Details of the colour codings and bands used are shown in the legend to the right of the plot.

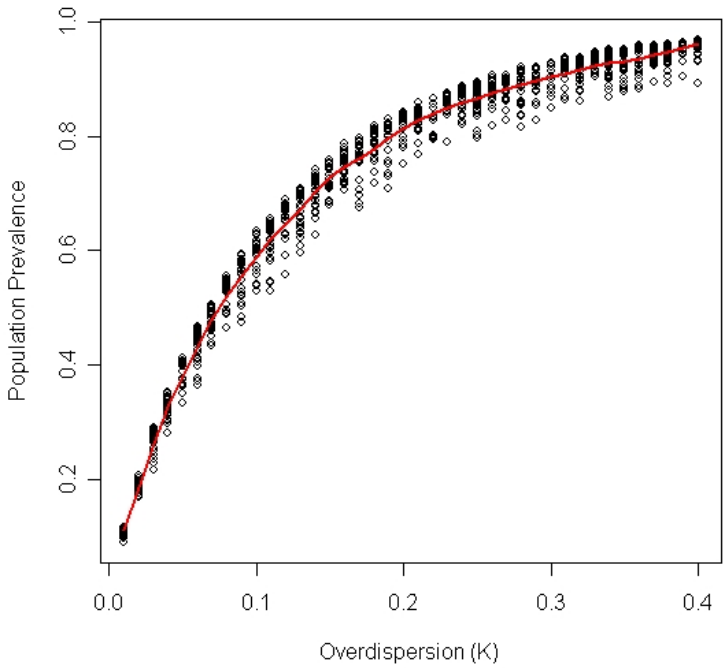
A more detailed representation of the relationship between population prevalence and overdispersion (K) can be shown with a two dimensional scatter plot (Figure 5.4), as there is no relationship between either of these variables and mean infection intensity of the population. The data suggests that the relationship is that of an asymptotic exponential. Clearly, overdispersion appears to be closely related to population prevalence.

Figure 5.3. Contour plot giving an overview of the relationship between overdispersion (K), Mean infection Intensity (P) and underestimation of prevalence



The contour plot shown above gives an overview of the relationship between overdispersion (K), the mean infection intensity of the population (P) in parasites per millilitre, and the underestimation of prevalence obtained from a single screening of 382 samples from the population. Different levels of the population prevalence are shown as colour coded contours in the plot area. Details of the colour codings and bands used are shown in the legend to the right of the plot.

Figure 5.4. Scatter-plot showing the relationship between overdispersion and population prevalence

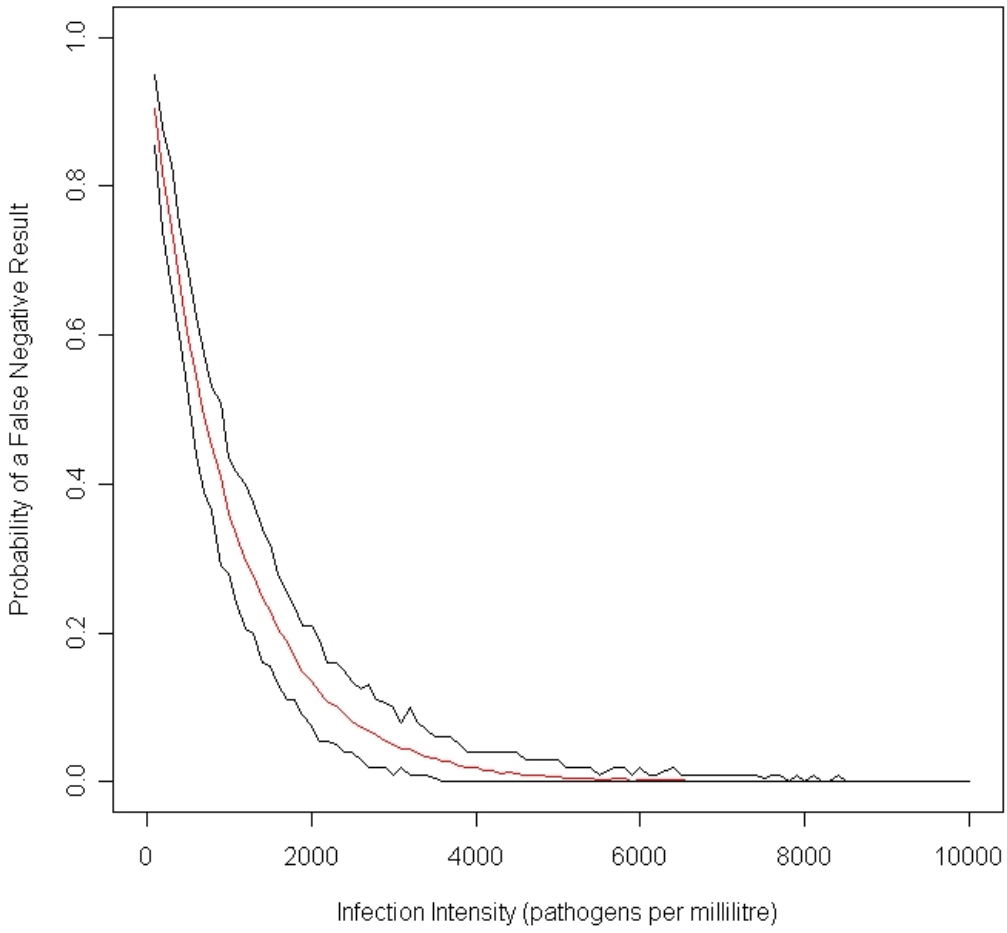


The scatter-plot shows a more detailed view of the relationship between the population prevalence and the level of overdispersion. A smoothed curve fitted to the data is shown in red.

5.3.2. Determination of the relationship between the infection intensity in an individual host and the probability of false negative results

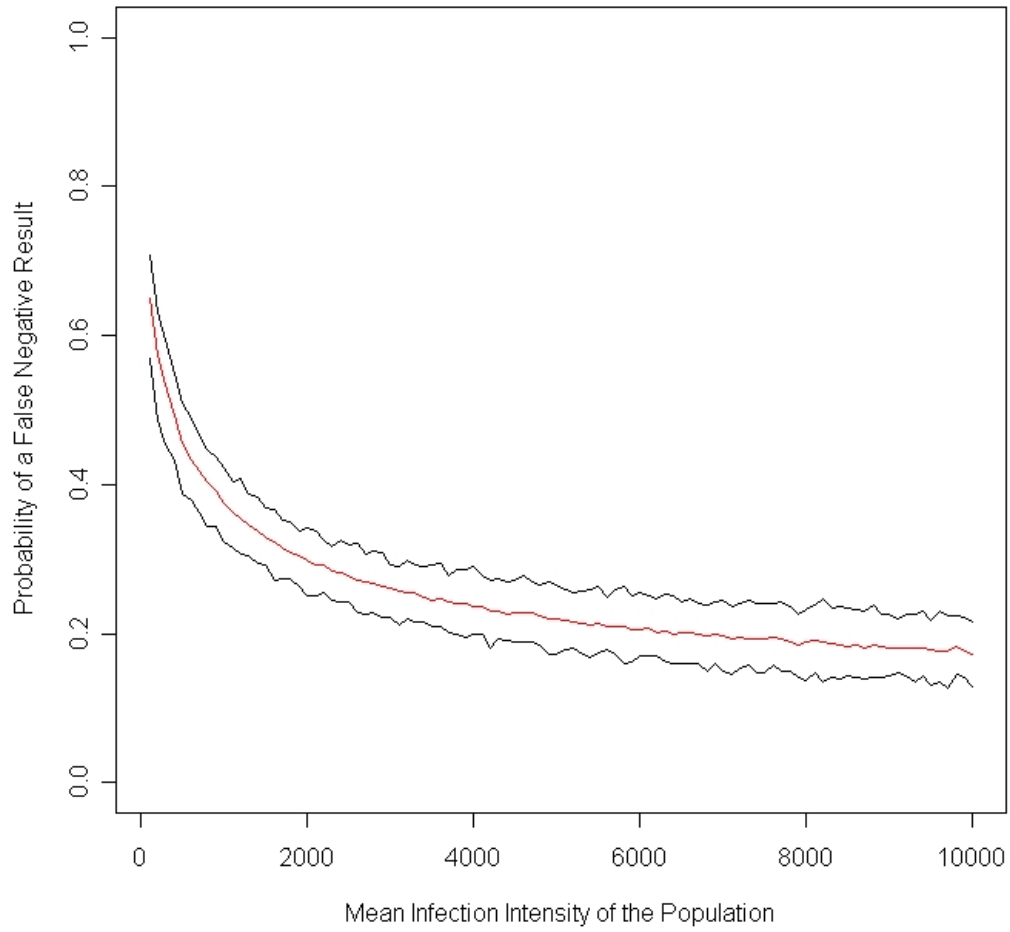
The results (Figure 5.5) show that the mean probability of obtaining a false negative result does not reach zero until the host infection intensity reaches 8,750 parasites per millilitre. Whilst at 800 parasites per millilitre the probability of obtaining a false negative result is 0.5. At the theoretical detection threshold of the technique, 1,000 parasites per millilitre (a mean of one parasite for every microlitre of blood) false negative results were obtained with a probability of 0.4.

Figure 5.5. The relationship between infection Intensity in an individual host and the probability of obtaining a false negative diagnostic result from a sample taken from the infected host



The scatter plot shows the results of a simulation to determine the relationship between infection Intensity in a host and the probability of obtaining a false negative test result from a sample taken from a host with a given infection intensity . The mean probability (red line) and the associated 95% confidence intervals (black lines) are shown. The results shown apply to a diagnostic technique with an assay volume of 1 microlitre which is capable of detecting a single parasite in that volume.

Figure 5.6. The relationship between the mean infection intensity of the sampled population and probability of obtaining false negative diagnostic results



The figure shows the results of a simulation to determine the mean probability of obtaining a false negative result from a population for different levels of mean infection intensity of the population. The simulation assumes the parasite distribution across the population conforms to a negative binomial. The Monte-Carlo simulations for each level of mean infection intensity were conducted for all possible levels of population prevalence. The associated 95% confidence intervals are shown by the black lines. The results shown apply to a diagnostic technique with an assay volume of 1 microlitre which is capable of detecting a single parasite in that volume.

5.3.3. Determination of the relationship between the mean infection intensity of the population and the probability of false negative results

For all levels of mean infection intensity of the population there exists a high level of occurrence of false negative results. Even at the highest level of mean infection intensity of the population (10,000 parasites per millilitre) false negative results still

occurred with a mean probability of 0.19. The greatest variability in the levels of false negative results occurred at mean infection intensities of less than 1,000 parasites per millilitre.

5.4. Discussion

In the previous chapter repeated screening of a range of samples revealed a number of problems associated with current methods of screening for infection status. Many false negative results occurred, leading to a potentially large underestimation of population prevalence. In addition, it was shown that this phenomenon can lead to serious misinterpretation of the epidemiological state of a pathogen. Empirical results suggested that the underestimation was related to infection intensity (IRFN). In order to fully understand this problem it is necessary to understand the relationship between five parameters namely; population prevalence, mean infection intensity, overdispersion, diagnosed prevalence and underestimation of prevalence. A detailed study is clearly not possible with field samples as these population parameters are essentially unknown and work with artificially constructed samples could not provide the volume of information necessary to examine the relationship fully. Furthermore, experimental studies of this kind are time consuming and expensive. As the process of screening samples from an infected population with a diagnostic technique is essentially a simple one and because the lack of repeatability and underestimation of prevalence is related to a stochastic phenomenon, the problem lends itself well to mathematical modelling.

In order to investigate the problem a simple computer simulation was constructed to randomly select different values for the population parameters and to calculate the diagnostic results of screening a number of samples from that population. The resulting data was then examined to determine the relationship between the variables and determine the nature of the causes of this lack of repeatability and underestimation of population prevalence.

There is an important, but logical, correlation that should be understood before the data presented in this chapter can be interpreted correctly. As the population prevalence decreases, clearly the diagnosed prevalence will decrease and so the potential for underestimation of prevalence will also decrease. At very low population prevalence's there is no potential for high levels of either diagnosed prevalence or underestimation of prevalence.

The strongest relationship is that between population prevalence and the overdispersion of the population (Figures 5.1 and 5.4). There is a clear correlation between the two parameters; this is perhaps not surprising as the overdispersion factor of the negative binomial distribution is heavily influenced by the frequency of zero values (uninfected hosts). In this case the overdispersion parameter (K) is related to the number of uninfected hosts within the population, which in turn determines the prevalence of infected cases. A more detailed representation of this potentially important relationship is shown in Figure 5.4. A trend line superimposed on the data suggests that an asymptotic exponential may describe the relationship. This may be useful, in that a good estimation of overdispersion (K) may allow calculation of the 'true' population prevalence with some accuracy, thereby circumventing the underestimation problem previously described. However, an estimation of overdispersion can only be made if the samples are assessed in a quantitative manner.

Additionally the strong relationship between overdispersion and population prevalence explains how underestimation of prevalence and diagnosed prevalence are influenced by overdispersion (K). Clearly, as population prevalence and therefore overdispersion decrease so does the potential for underestimation of prevalence. This shows that overdispersion limits the underestimation but is not the direct cause of that underestimation, the cause of underestimation must therefore be the mean infection intensity of the population (P).

It is interesting to note that for the ranges of population parameters and distribution assumptions investigated in this model there is clearly always an underestimation of the true prevalence, except in some cases where the population prevalence is close to zero (See Figure 5.3).

Probability of obtaining false negative results from an individual host

Assuming a uniform distribution of parasites in the blood volume would give consistently repeatable positive results at an infection intensity of 1,000 parasites per millilitre (assuming the diagnostic technique is capable of detecting a single parasite and assays one microlitre of blood). However, the parasites will not be uniformly distributed; the distribution is more likely to be random (Poisson) in nature. Modelling a Poisson distribution of the counts of parasites within each microlitre volume of the blood showed that the probability of obtaining false negative results did not reach zero until the parasite was present with an infection intensity of 8,750

parasites per millilitre. This is a surprisingly high level of infection intensity and it quite probable that the host would be seriously affected if it were able to sustain this high intensity of infection. At 1,000 parasites per millilitre the probability of obtaining false negative results was 0.4. These results show that the occurrence of false negative results is likely to be present in cases were the parasite is present at intensities below 5,500 parasites per millilitre. If the parasites are overdispersed in the blood volume then the frequency of false negative results is likely to increase still further.

Determination of the relationship between the mean infection intensity of the population and the probability of false negative results

Since the underestimation of prevalence when screening samples is related to the frequency of false negative results and therefore the infection intensity, then a measure of the probability of obtaining false negative results becomes an important index of the potential for underestimation of prevalence in a sampled population. The relationship described in Figure 5.6 is both interesting and important. It is important to note that the frequency of occurrence of false negative results at mean population infection intensities of 10,000 parasites per millilitre still remains relatively high (P = 0.19), implying that even at this level of mean infection intensity the population prevalence will still be underestimated. Extrapolation of the relationship beyond infection intensities of this level shows that complete repeatability is never likely to be reached and it is doubtful that hosts could survive for any length of time with infection intensities of above 10,000 parasites per millilitre. These results assume that the parasite is distributed throughout the population according to a negative binomial distribution, and some evidence has been presented for this (see Section 4.3.). However, not all infected hosts within the population will have high infection intensities and therefore some hosts will present with low levels of infection and some with high as a result of variations in susceptibility, time since infection, age, co-infections and previous exposure. Even if the NBD were not an appropriate description of the distribution of infection intensities within the host population, these kind of results would still be relevant as long as there is heterogeneity in the Population.

Chapter 6: Methods for epidemiological screening and statistical analysis of populations with widespread sub-patent infections

6.1. Introduction

The work presented to this point has established that for populations where infection with haemoparasites is widespread and present at low intensities, the prevalence obtained from a single screening of each sample can seriously underestimate population prevalence. The cause of this underestimation in prevalence has been shown to be due to the levels of infection intensity present in the population and hence the phenomenon has been termed 'Intensity Related False Negatives' (IRFN). Furthermore, a single diagnostic test per sample approach to screening is not capable of differentiating between populations with 'sub-patent' infections (which produce an underestimation of the true prevalence) and populations with patent infections (where diagnosed prevalence is representative of population prevalence).

Having explored the consequences and importance of the IRFN effect, it is necessary to develop tools which can help to overcome the problems previously described. Any techniques which are aimed at overcoming these problems should in the first instance be able to differentiate when the IRFN effect is occurring and when it is not occurring and be applicable in both instances. Secondly, it is evident that methods aimed at dealing with this problem should take a quantitative or at least a semi-quantitative approach to analysing the samples. The methodology should not only determine the infection status of the host but give some indication of the intensity of infection within the host. This is an essential prerequisite for any method attempting to deal with IRFN.

An obvious method for quantitatively assessing the level of infection intensity within each host would be to process the samples using real time PCR (RT-PCR). Whilst RT-PCR can provide a quantitative evaluation for samples with complete repeatability, i.e. patent infections, the technique is not useful for quantifying for sub-patent infections of the type that produce the IRFN effect. This is because the basis of RT-PCR is the polymerase chain reaction, it therefore is affected by IRFN in the same way as other diagnostic screening techniques, some alternate approach is required. A semi-quantitative measure of infection intensity can be obtained by assessing the degree of repeatability of results from each sample. For example a sample screened five times and found positive three times would have a repeatability value of 0.6 (3 divided by 5).

For repeat testing of samples the number of samples tested (n) by the number of repeat tests on each sample (r) can be regarded as a sampling strategy (n x r). In order to develop methods that use repeat testing of samples, it is necessary to determine the relative importance of number of samples analysed (n) and the

number of repeat tests per sample (r), the relative efficiency of each sampling strategy should be assessed.

Routine epidemiological calculations such as those for sample size and power and for determination of confidence intervals are not applicable where IRFN is present. Therefore, new methods of calculating these important epidemiological parameters for studies based on the repeatability of the samples should be developed.

The objectives of this chapter are therefore to (i) Determine the relative accuracy of different sampling strategies (e.g. number of samples and number of repeat tests on each sample) in estimating the population overdispersion (K) and population mean infection intensity (P). (ii) To assess the relative performance of different approaches (combinations of statistical and sampling methodologies) in detecting significant difference between populations. (iii) To develop a method for estimating the true population prevalence in populations with sub-patent infection intensities.

6.2. Materials and Methods – A general overview

The simulations used in this chapter are all similar, with only minor differences depending on the purpose of the analysis. The basis of the code for all simulations is shown in Figure Appendix 6.4. Simulations were written in the mathematical programming language of 'R Version 2.3.0. Each parameter in the simulations has been given a symbol. The key to the symbols is given below (Table 6.1). More detailed methods for specific simulations are described separately in each results section of this chapter. Each simulation is described with its own flowchart when necessary (See Appendix).

6.3. Specific Methods & Results

6.3.1. Assessment of the accuracy of different sampling strategies in estimating overdispersion (K) and mean infection intensity (P) of the population

Description of 'Simulation 1'

The objective of this simulation was to determine which sampling strategy (n samples by r repeats) most accurately estimates the overdispersion (K) and mean infection intensity (P) of the population. The error of the estimates for each sampling strategy is calculated as $K_a=K-K_e$ for overdispersion, and $P_a=P-P_e$ for the mean infection intensity.

The uncertain variables in this simulation were the mean infection intensity of the population (P) the overdispersion (K) of the population and the screening strategy (n x r). The simulation was repeated 1.0×10^5 times to produce a data frame of estimate errors for different sampling strategies, different population mean infection

intensities and overdispersion values. An extract from the data frame is shown in Table 6.2.

Table 6.1. Key to the parameters used in the simulations

Variables

I_i	Infection intensity of the i^{th} sample (i.e. parasites per millilitre)
ρ_i	Probability of determining the i^{th} sample as positive
k_e	Estimate of overdispersion (K)
P_e	Estimate of mean infection intensity (P)
k_a	Accuracy of estimate of overdispersion (K)
P_a	Accuracy of estimate of mean infection intensity (P)
r	Number of repeated screens on each sample
Θ_r	Test result of screening the r^{th} repeat of sample i
C_u	Upper 95% Confidence Interval
C_l	Lower 95% Confidence interval
Φ	Accuracy of calculated confidence intervals
ω_p	Population prevalence
ω_e	Estimated prevalence
ω_k	Estimated prevalence based on assessment of overdispersion
ω_s	Standard estimate of prevalence
ω_c	Cumulative estimate of prevalence
Randomly chosen parameters	
P	Mean infection intensity of the Negative Binomial Distribution. Chosen from the range P = 100 to 1000 parasites per millilitre
K	Overdispersion of the Negative Binomial Distribution Chosen from the range K = 0.1 to 0.4
n	Number of samples to screen Chosen from n = 30 to 100
Constants	
N	Total population size = 1.0×10^5 samples
S_e	Sensitivity of screening techniques = 1000 parasites per millilitre

Table 6.2. Extract from the dataset obtained from '*Simulation 1*' to determine the accuracy of estimates of overdispersion and mean infection intensity of the population for different sampling strategies

			Population Values		*Estimates*	
Iteration	**Population Prevalence**	**Repetitions**	**Infection Intensity (P)**	**Overdispersion (K)**	k_e	P_e
1	0.60	30	410	0.11	0.06	1015
2	0.66	46	1000	0.11	0.19	2467
3	0.64	32	200	0.14	0.15	485
4	0.84	35	930	0.22	0.27	2256
5	0.60	74	1000	0.10	0.07	2422
6	0.59	32	400	0.11	0.14	966
7	0.61	36	940	0.10	0.19	2265
↓	↓	↓	↓	↓	↓	↓
9,994	0.72	50	770	0.15	0.20	1832
9,995	0.26	33	750	0.05	0.10	775
9,996	0.59	33	500	0.10	0.182	1176
9,997	0.67	32	680	0.12	0.15	1595
9,998	0.74	43	880	0.15	0.20	2052
9,999	0.65	61	420	0.12	0.17	971
10,000	0.74	33	150	0.20	0.52	346

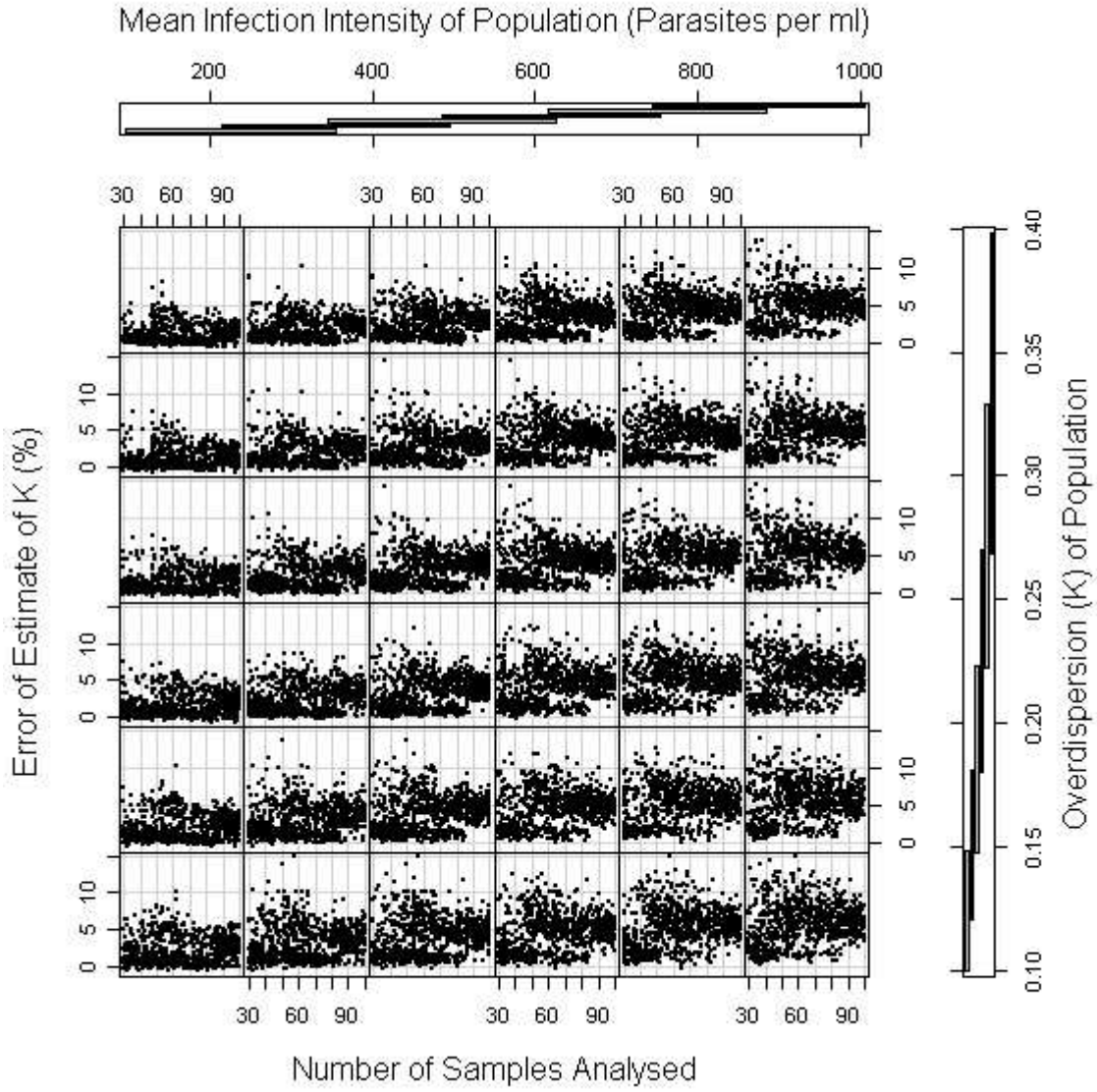
The construction of the model is shown in the form of a flowchart in Figure Appendix 6.5. The simulation first determines the parameters of a negative binomial distribution (NBD) that describes the distribution of infection intensities within the population. The values of mean infection intensity (P) and overdispersion are chosen randomly from a vector of possible values (for P = 100 to 1000 parasites per millilitre and K = from 0.1 to 0.4). The maximum value of 0.4 for overdispersion was chosen as this gives a population prevalence of 100% [see Section 6.3.3.]. A population of 1.0×10^5 samples is then generated, each with an infection intensity value (I_i). When $I_i = 0$ this denotes an uninfected individual or sample. The probability of obtaining a positive diagnostic result from each sample in the population is as described for previous simulations (See Section 4.2.5).

The simulation then determines the sampling strategy. All sampling strategies (n x r) use a maximum of 300 total screenings (diagnostic tests). This figure was chosen for reasons of practicality, economy and convenience in conducting the required number of diagnostic tests. The total number of samples to be screened (n) is chosen randomly from a vector of values (from n = 30 to 100). The number of repeat screenings is then determined by dividing the total number of screenings allowed (300) by the number of samples to be screened and rounding to the nearest integer. The samples for screening (n) are then randomly selected from the population and a positive or negative screening result (θ_i) is generated according to the probability of

finding each sample positive. This process is then repeated for the number of times the samples are to be repeat screened (r). The estimates of overdispersion (k_e) and mean infection intensity (P_e) are then calculated and the accuracy of each estimate determined by comparison with the corresponding population values. The process is then repeated for 1.0×10^5 iterations to obtain results across the range of the uncertain variables. The data obtained from '*Simulation 1*' is then output in the form of a data frame which can be used to determine the distribution of errors for each sampling strategy, as shown in Table 6.2.

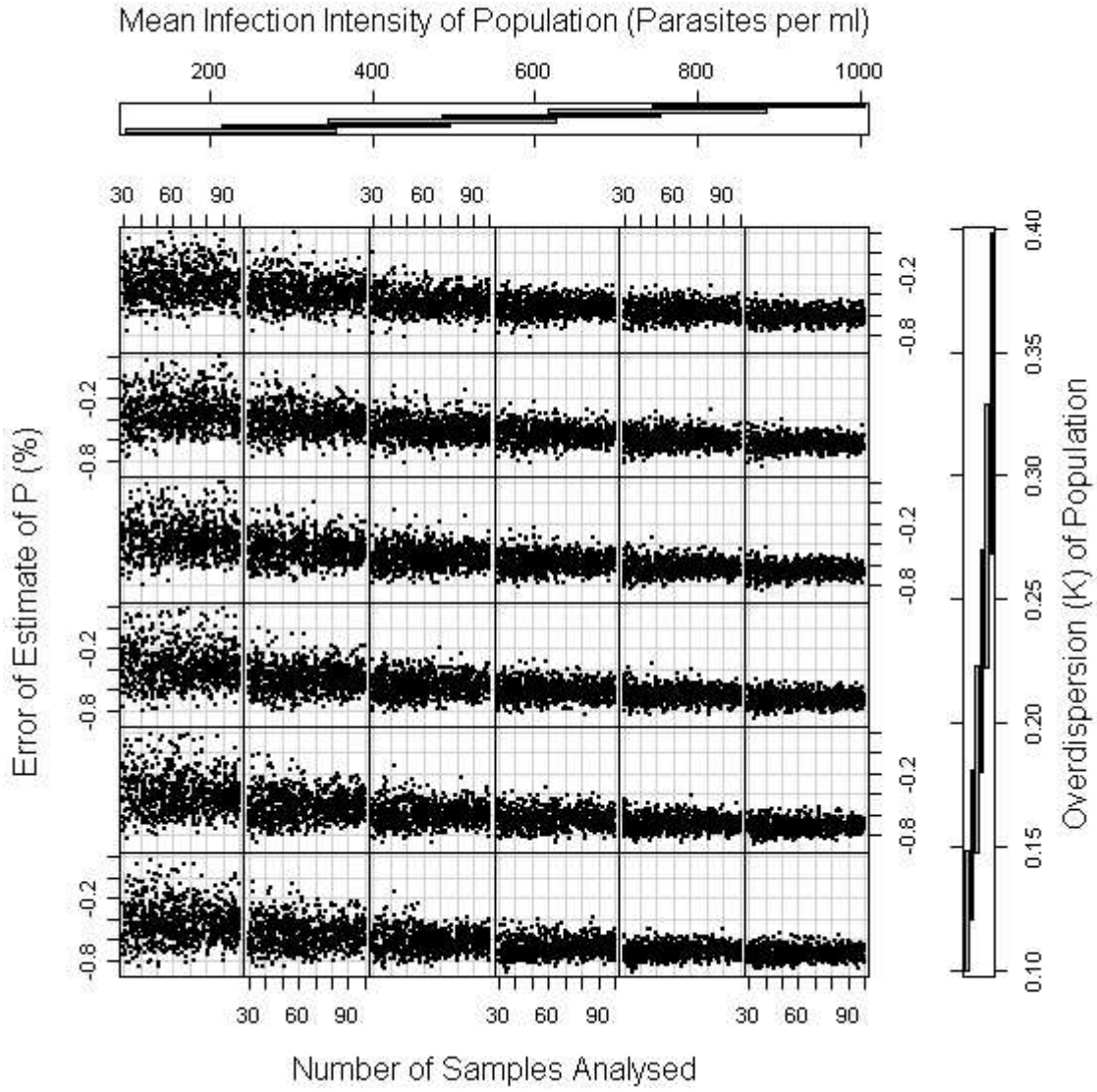
The data created from '*Simulation 1*' is shown in the form of double conditioning plots in Figures 6.1a and 6.1b. The distribution of the data across the lattice of conditioning plots consistently shows a decreasing level of error toward sampling strategies that analyse greater numbers of samples with fewer repeat samplings of the same sample. The most accurate strategy of those tested would appear to be the maximum number of samples tested (n = 100) with three repeats for each sample (r = 3), as this produces the lowest and least dispersed errors in the estimated parameters. For estimations of overdispersion (Figure 6.1a) estimates always tend to overestimate the population value and the dispersion of the errors increases as the mean infection intensity of the population increases. As the mean infection intensity of the population decreases the error of the estimates become less. Estimation of mean infection intensity (Figure 6.1b) always tends to underestimate the population mean infection intensity. Whilst the underestimate is reduces the dispersion of estimates increases as the mean infection intensity of the population decreases. The error is marginally reduced as overdispersion increases.

Figure 6.1a. Conditioning plot showing the accuracy of the estimate of overdispersion for different sampling strategies



The co-plot shows the range of results obtained from '*Simulation 1*' designed to determine the accuracy of different sampling strategies using a fixed number of tests. The sampling strategies comprised different combinations of number of samples tested by number of repeat tests on each sample (n x r). The x axis shows the number of samples tested (n), as 300 tests were used in total, the number of repeat tests (r) in each case is 300 divided by the number of samples tested. For example; 50 samples were tested six times, 100 samples were tested three times. The y axis shows the accuracy of the estimate of the overdispersion. The secondary x and y axis are designed to show the effects of mean infection intensity of the population and the overdispersion of the population on the accuracy. In the co-plot the results are dived into a lattice of 36 segments, columns from right to left show the effect of increasing mean infection intensity on the results. Each column represents the range of infection intensities indicated by the six bars in the top box. Similarly the rows show the affects of decreasing overdispersion for ranges of overdispersion indicated by the bars in the box to the left of the co-plot. In this way the minimum range infection intensity and overdispersion is shown in the bottom left plot of the lattice, whilst the maximum infection intensity and overdispersion range is shown in the top right box. The plot shows there is a trend toward less error with increasing number of samples analysed (as opposed to increasing the number of repetitions on fewer samples), and decreasing error with higher infection intensities. 50 estimates (outliers) were removed from data for clarity of presentation.

Figure 6.1b. Conditioning plot showing the accuracy of the estimate of mean infection intensity for different sampling strategies



The co-plot shows the range of results obtained from '*Simulation 1*' designed to determine the accuracy of different sampling strategies using a fixed number of tests. The sampling strategies comprised different combinations of number of samples tested by number of repeat tests on each sample (n x r). The x axis shows the number of samples tested (n), as 300 tests were used in total, the number of repeat tests (r) in each case is 300 divided by the number of samples tested. For example; 50 samples were tested six times, 100 samples were tested three times. The y axis shows the accuracy of the estimate of mean infection intensity The secondary x and y axis are designed to show the effects of mean infection intensity of the population and the overdispersion of the population on the accuracy. In the co-plot the results are dived into a lattice of 36 segments, columns from right to left show the effect of increasing mean infection intensity on the results. Each column represents the range of infection intensities indicated by the six bars in the top box. Similarly the rows show the affects of decreasing overdispersion for ranges of overdispersion indicated by the bars in the box to the left of the co-plot. In this way the minimum range infection intensity and overdispersion is shown in the bottom left plot of the lattice, whilst the maximum infection intensity and overdispersion range is shown in the top right box. The plot shows there is a trend toward less error with increasing number of samples analysed (as opposed to increasing the number of repetitions on fewer samples), and decreasing error with higher infection intensities. 50 estimates (outliers) were removed from data for clarity of presentation.

6.3.2. Comparative efficiency of different methods of detecting significant difference in patterns of infection when comparing host populations

A Monte Carlo simulation ('*Simulation 2*') was developed using the basic code described in Figure Appendix 6.4. in order to assess the efficiency of different methods in determining significant difference of infection between two populations. The tests were conducted over a range of assumptions for overdispersion of infections and mean infection intensity in the populations. The tests were also assessed for ranges of values where the mean infection intensities were low and overdispersion was high (low K values) in order to determine how the methods performed under a scenario where the populations might present with widespread sub-patent infections.

The different methods comprised different sampling strategies and statistical methodologies, a method equivalent to the way samples are currently screened (prevalence based method) and compared statistically and a method based on the number of positive results obtained from repeat screenings of the samples (repeatability based method). The compared populations were selected so that there was always a difference of prevalence of at least 10% and a difference in mean infection intensity of at least 100 parasites per millilitre. This minimum difference was selected so that the performance of the techniques could be compared, but was not intended to be a definitive measure of true significant difference in populations.

'*Simulation 2*' determines a mean figure for the probability of finding a significant difference between populations given different sampling strategies and statistical methodologies. Associated 95% confidence intervals are generated for each mean probability.

6.3.2.1. The different methods of sample analysis

Prevalence based method for 100 and 500 samples per population

Each sample was tested once, and a prevalence of infection was then calculated for each population. The Chi square test statistic was used to compare number of infected samples and number of samples tested for each population. This method was conducted, using 100 and then 500 samples. Single screening of samples (i.e. one diagnostic test per sample) is currently the conventional method of assessing differences in prevalence.

Repeatability method for 3 and 5 repeat tests per sample

Each sample was repeatedly tested, and the result of each test recorded for each sample. Data obtained for each sample were arranged in a data frame containing columns for 1/. Sample number, 2/. Number of positive tests and 3/. Population label

(A or B). Generalised linear modelling with Poisson errors was used to compare infection prevalence between populations. This method was conducted twice, using 3 and 5 repeated tests for n = 100 samples. The residual scaled deviance 'a' of the GLM model should be approximately equal to the model's residual degrees of freedom 'b'. In cases where the ratio a/b is >1, then the assumption that the dispersion (or scale) parameter of the model equals one, does not hold. In such cases correction for overdispersion required specifying a different error structure (i.e. "family=quasipoisson") and the use of the F test rather than the Chi square test statistic was employed.

Description of 'Simulation 2'

In this simulation the variable of interest was the mean probability of detecting differences in prevalence and mean infection intensity between populations where a minimum difference in prevalence of 10%, and a minimum difference in mean infection intensity of 100 parasites per millilitre exists. The construction of '*Simulation 2*' is shown in the form of a flowchart in Figure Appendix 6.6.

'*Simulation 2*' is similar to '*Simulation 1*' except for the following modifications. The methods were assessed using three separate simulations. Each simulation uses different ranges of possible values for the distribution parameters describing infection intensity and overdispersion in two separate populations (See Table 6.4a and 6.4b).

In '*Simulation 2*' the mean infection intensity and overdispersion parameters of the two populations are first selected randomly from the range of values for that particular analysis. The prevalence (ω) and the mean infection intensity in each population is then determined and stored. Only those pairs of populations, with differences greater than or equal to the benchmark values, are selected and compared using the eight sample analysis methodologies described earlier. For each method, a p-value resulting from the corresponding test statistics is obtained at each iteration. A p-value of < 0.05 is recorded as a statistically significant result and assigned a value of 1. A p-value ≥ 0.05 is recorded as a not statistically significant result and coded as 0. The process is repeated for 100 iterations, each time taking different samples from each population. On completion, the mean probability of obtaining a statistically significant result for each method is recorded. These results are then stored in a vector.

For each sample analysis method, the whole process is conducted for 1000 population pairs. This results in 1000 values of 'probability of a statistically significant result' which are then stored in a vector. Upon completion of the

simulation, the values are used to calculate the mean probability (and 95% confidence intervals) of determining a statistically significant result for each method.

For all sample analysis methods, the mean probability of a significant result is lowest when the range of infection intensities for the populations is lowest. The mean probability of a significant result increases as the range of infection intensities for the populations increases. The prevalence based method simulates a standard approach to screening samples for haemoparasites, in that each sample is subjected to a single diagnostic test to determine infection status. Using this method to compare populations with low mean infection intensities and high overdispersion (low K value), results in a very low probability of detecting a significant difference between populations (P = 0.3). Under any assumption for the range of the mean infection intensity and overdispersion, this method always presents with the lowest probability for obtaining a significant difference as compared to the other sample analysis technique.

Using repeat samplings (Repeatability Method) proves to be the most reliable method (that with the highest probability of detecting a significant difference). In cases where mean infection intensities are low, the probability of obtaining a statistically significant result is 0.56 for three repeated samplings and 0.65 for five repeated samplings. In methods involving repeated testing of samples, five repeated tests resulted in an increase in the probability of obtaining a statistically significant result as compared to three repeated tests only for those populations with the lowest range of mean infection intensities. See Table 6.4a.

Table 6.3. Summary of the different approaches to screening samples used in the comparison of methods

Method	Number of sample screened (n)	Number of repeat screenings (r)	Method
Prevalence Method	100	1	Chi-square test of prevalence results obtained from a single screening of each population. A standard approach
	500	1	As above except with 500 samples
Repeatability Method	100	3	Test of results of number of positives obtained and number of repeat screenings for each population using a Generalised Linear Model with Poisson errors and an F-test
	100	5	As above except with five repeat screenings

Table 6.4a. Probability of detecting a significant difference of greater than a minimum level in two populations under different ranges of infection intensity patterns for two different methods

Range		Prevalence Method - Single Test per Sample	
K	P	100 Samples	500 Samples
0.01 to 0.6	100 to 300	0.30 (0.22 ~ 0.38)	0.42 (0.34 ~ 0.50)
0.01 to 0.6	300 to 1000	0.49 (0.36 ~ 0.58)	0.56 (0.49 ~ 0.64)
0.01 to 0.6	1000 to 10,000	0.63 (0.56 ~ 0. 70)	0.70 (0.60 ~ 0.80)
		Repeatability Method	
		Three Repeat tests on 100 Samples	**Five Repeat Tests on 100 Samples**
0.01 to 0.6	100 to 300	0.56 (0.47 ~ 0.64)	0.65 (0.58 ~ 0.73)
0.01 to 0.6	300 to 1000	0.66 (0.57 ~ 0.75)	0.73 (0.65 ~ 0.80)
0.01 to 0.6	1000 to 10,000	0.67 (0.56 ~ 0.75)	0.74 (0.64 ~ 0.81)

The table shows the results of a simulation and analysis of resulting data to determine the efficiency of eight different approaches to sampling and statistically testing for significant difference between two populations. The far left columns show the ranges of distribution parameters from which the corresponding values for each of the populations was selected. The results and associated 95% confidence intervals indicate the probability of that particular method of determining a P-value of less than 0.05, indicating a significant difference in two populations with differences greater than a minimum value (minimum levels: Prevalence >10% difference and / or mean infection intensity difference of > 100 parasites per millilitre). A brief description of the methods used is given in table 6.3. Methods using the cumulative prevalence and using data on infection intensity were also tested (data not shown) both methods were found to be better than testing prevalence from a single screening but not as efficient as the repeatability method.

Table 6.4b. Probability of detecting a significant difference of greater than a minimum level in two populations under different ranges of overdispersion for two different methods

Range		Prevalence Method - Single Test per Sample	
K	P	100 Samples	500 Samples
0.01 to 0.2	100 to 10,000	0.55 (0.43 ~ 0.68)	0.64 (0.55 ~ 0.73
0.2 to 0.4	100 to 10,000	0.41 (0.31 ~ 0.52)	0.44 (0.38 ~ 0.46)
0.4 to 1	100 to 10,000	0.49 (0.39 ~ 0.61)	0.61 (0.50 ~ 0.69)
		Repeatability Method	
		Three Repeat tests on 100 Samples	**Five Repeat Tests on 100 Samples**
0.01 to 0.2	100 to 10,000	0.70 (0.62 ~ 0.77)	0.74 (0.65 ~ 0.86)
0.2 to 0.4	100 to 10,000	0.46 (0.40 ~ 0.55)	0.56 (0.50 ~ 0.65)
0.4 to 1	100 to 10,000	0.42 (0.32 ~ 0.49)	0.51 (0.42 ~ 0.58)

The table shows the results of a simulation and analysis to determine the efficiency of eight different approaches to sampling and statistically testing for significant difference between two populations. The far left columns show the ranges of distribution parameters from which the corresponding values for each of the populations was selected. The results and associated 95% confidence intervals indicate the probability of that particular method of determining a P-value of less than 0.05, indicating a significant difference between populations with differences greater than a minimum value (minimum levels: Prevalence >10% difference and / or mean infection intensity difference of > 100 parasites per millilitre). A brief description of the eight methods used is given in table 6.3. Methods using the cumulative prevalence and using data on infection intensity were also tested (data not shown) both methods were found to be better than testing prevalence from a single screening but not as efficient as the repeatability method.

6.3.3. Development of a method for estimating the true population prevalence in populations with sub-patent infection intensities

Results presented in chapter five showed a strong relationship between the overdispersion parameter of a negative binomial distribution and population prevalence. See Figure 5.4.

This relationship may be useful in predicting the true level of prevalence in populations with widespread sub-patent infection, when diagnosed prevalence is unreliable. However, in order for the overdispersion to be useful in this way there must also exist a strong relationship between the population prevalence and an estimate of overdispersion derived from samples drawn from the population. In order to explore the potential of this relationship a simulation was constructed which estimated the overdispersion parameter (K) from screening a set of samples drawn at random from a population and subject to repeat screening.

Description of simulation 3

The purpose of this model was to provide data to allow analysis of the relationship between the estimate of overdispersion (K_e) and the population prevalence (ω_p). In this simulation the variable of interest was the estimate of overdispersion. The uncertain variables in this case were the mean infection intensity of the population (P) and the overdispersion (K) of the population. The construction of the model is shown in the form of a flowchart in Figure Appendix 6.7.. The simulation first determines the parameters of a negative binomial distribution (NBD) that describes the distribution of infection intensities across the population. The values of mean infection intensity and overdispersion are chosen randomly from a vector of possible values (for P = from 100 to 1500 parasites per millilitre and K = from 0.1 to 1). A population of 1.0×10^5 samples is then generated each with an infection intensity value (I_i), where the distribution of infection intensity values conforms to that of the distribution assumption selected in the previous step and where a value of zero denotes an uninfected individual or sample. The probability of obtaining a positive diagnostic result from each sample in the population is as described in Section 4.2.5. The simulation then determines the prevalence of infected samples within the population and stores the result. Samples are then selected at random from the population (n = 382) and positive or negative results are generated for each sample (θ_i) according to the probability of finding the sample positive (ρ_i). This process is repeated three times (r = 3) and the results of each round are stored.

The estimated prevalence from the first screening and the cumulative prevalence for all three repeat screenings are then calculated and stored. The repeatability of each

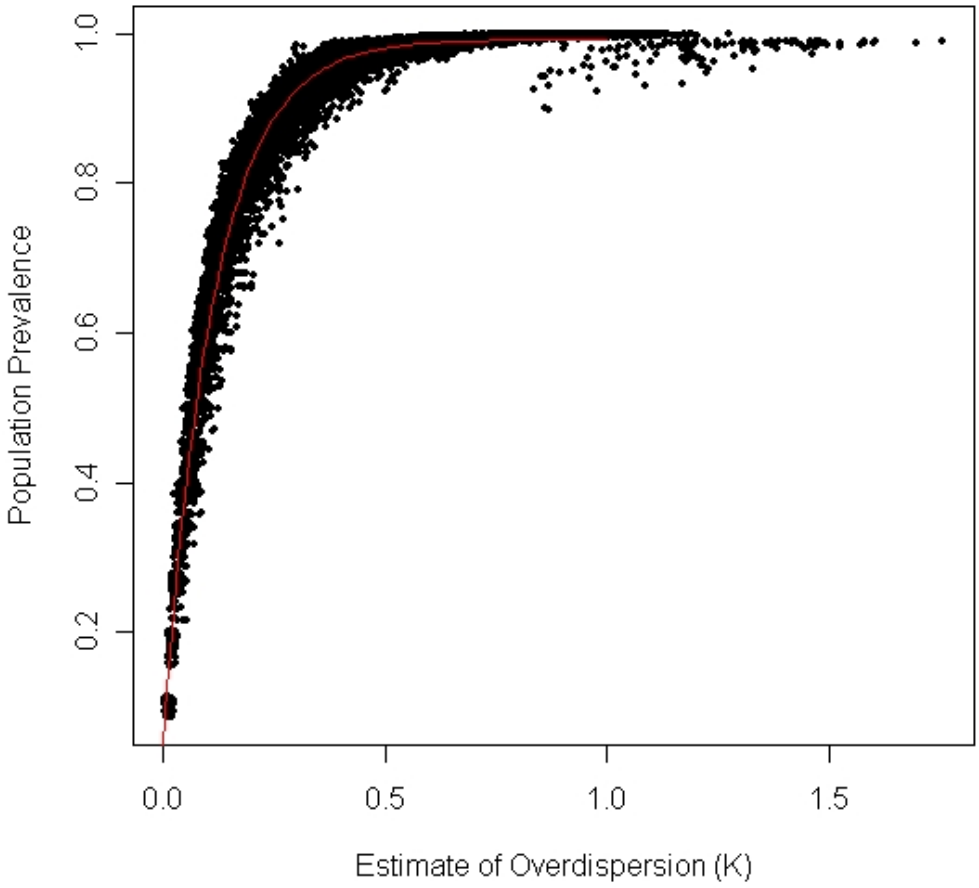
sample is then calculated by dividing the number of positives obtained by the number of repeat screenings. The overdispersion of the proportion of positive results for the samples is then estimated using maximum likelihood methods and the result is stored. The entire process is then repeated 1.0×10^5 times to produce a large data frame for analysis.

Table 6.5. Extract of the data frame obtained from the simulation for determining the estimate of overdispersion from repeated screening results

Iteration Number	P	K	Population Prevalence	Estimate of K	Cumulative Prevalence Estimate	Standard Prevalence Estimate
1	700	0.79	0.99495	0.791914	0.727749	0.520942
2	1500	0.21	0.84522	0.21436	0.424084	0.442408
3	1000	0.46	0.97135	0.431379	0.557592	0.510471
4	1200	0.47	0.97559	0.460965	0.612565	0.615183
5	300	0.27	0.85103	0.235826	0.36911	0.232984
6	100	0.08	0.43293	0.066666	0.10733	0.078534
7	200	0.8	0.98816	0.57938	0.494764	0.227749
8	200	0.6	0.96855	0.560322	0.468586	0.212042
9	1500	0.77	0.99693	0.712168	0.748691	0.672775
10	700	0.69	0.99173	0.58444	0.63089	0.492147
11	1500	0.86	0.99803	0.833981	0.743455	0.701571
↓	↓	↓	↓	↓	↓	↓
99,996	400	0.76	0.99152	0.648253	0.625654	0.366492
99,997	800	0.32	0.9183	0.297177	0.458115	0.403141
99,998	800	0.31	0.91173	0.316702	0.494764	0.421466
99,999	400	0.57	0.97573	0.474913	0.539267	0.361257
100,000	1500	0.91	0.99899	1.045518	0.780105	0.772251

A plot of the relationship between estimated overdispersion and population prevalence obtained from a second simulation is shown in Figure 6.2. Although the relationship is not as strong as the relationship between population prevalence and overdispersion parameter K, there is still potential for using the estimate of K to predict population prevalence.

Figure 6.2. Relationship between estimates of overdispersion and population prevalence



The scatter-plot shows the relationship between estimates of overdispersion and the population prevalence. The relationship is strong although slightly more dispersed than the relationship between population overdispersion and population prevalence (Figure 5.4.). A prediction of the asymptotic exponential function derived from the non-linear regression analysis is shown in red

Non-linear regression analysis of data

The data obtained from the simulation was used to determine the relationship between the estimate of overdispersion (K_e) and population prevalence (ω_p) in a non linear regression analysis. The two variables were plotted on a scatter-plot and after visual inspection of the data a three parameter asymptotic exponential curve of the form $y = a - be^{-cx}$ and a two parameter asymptotic exponential curve of the form $y = a(1 - e^{-cx})$ were both fitted to the data.

The results of the non-linear regression analysis of the relationship between estimated overdispersion and population prevalence are shown in Table 6.6. For the three parameter model all terms were found to be significant in deletion tests so the

initial model was accepted as the minimal model. Comparison of this model to a two term asymptotic exponential model of the form $y = a (1 – e^{-cx})$ by ANOVA showed that the residual standard error (RSE) for the two term model was slightly higher (RSE = 0.03201 as opposed to RSE = 0.03099 for the three parameter model) and that the two models were significantly different (df = 1, F-value = 67.574, P-value = <0.001). In this case the three term model was accepted as the most appropriate model. The minimum model for determination of population prevalence from estimates of overdispersion is shown in Table 6.8., a plot of the predictions of the model is shown superimposed on the data in Figure 6.2.

Table 6.6. Results of the non-linear regression analysis of data showing populations prevalence values for estimated overdispersion values

Minimum Model: $\omega_e = a - be^{-ck_e}$

N = 1.0×10^5

Model Term	Estimate	Df	F-value	P-value
a	0.993331	997	20,719	<0.001
b	0.938248	997	91.626	<0.001
c	8.466109	997	13,305	<0.001

The table shows the results of the non-linear regression analysis to determine the relationship between the population prevalence and the estimates of overdispersion. The data was fitted to a asymptotic exponential model of the form $y = a – be^{-cx}$. The estimate is the value of each of the constants in the fitted model predicted by the regression analysis. The P-value indicates whether a model not containing each term was significantly different form a model containing that term. All three terms were found to be significant in deletion tests and so the initial model was accepted as the minimal model. Comparison of this model to a two term asymptotic exponential model using ANOVA showed that the models were significantly different and that the three term model had a slightly smaller residual standard error. The minimum three term model was therefore accepted as the most appropriate description of the relationship.

6.3.3.1. Determining the confidence intervals of estimated prevalence.

Description of simulation 4

In order to provide a means of calculating 95% confidence intervals for estimated prevalence (ω_e) derived from estimation of overdispersion (K), a new data frame was created by adapting simulation 2. The simulations were identical except the new simulation provided and estimate of the population prevalence based on the estimate of overdispersion using the function derived from the previous non-linear regression analysis.

Data extraction

To provide a means of calculating 95% confidence intervals for estimates of population prevalence derived from an estimation of overdispersion (K), a data frame of 1.0×10^5 data points each containing the population prevalence and the estimated prevalence derived from the estimate of overdispersion. For each level of estimated prevalence (0 to 1 in 0.01 increments) the population prevalence values associated with each level were extracted from the data frame and the 95% confidence intervals of the population prevalence data were calculated. This produced a new data frame listing the confidence intervals for each level of estimated prevalence, the relationship between these variables was then assessed with a non-linear regression analysis.

Non-linear regression analysis of the data

The plots of the data for the upper and lower confidence intervals suggested a quadratic relationship, a function of the form $y = ax^2 + bx + c$ was fitted to both upper and lower confidence interval data.

The results of a non-linear regression analysis of the data frame detailing distributions of population prevalence for each level of estimated prevalence is shown in Table 6.7. A quadratic model successfully described the relationship between the confidence limits and the estimated prevalence in both cases. For the upper confidence limit all three parameters in the model were found to be significant, so the initial model was accepted as the minimal model. For the lower confidence interval a model without the term c was found not to be significantly different from the initial model and the reduced model resulted in a slight decrease in residual standard error (RSE = 0.0187 as opposed to RSE = 0.01999 for the initial model), so the reduced model was accepted as the minimum model. A summary of the minimum models for the estimation of population prevalence and the upper and lower confidence intervals is shown in Table 6.8.

Table 6.7. Results of the non-linear regression analysis and deletion tests to determine the relationship between the confidence intervals of population prevalence and the estimate of prevalence based on overdispersion

Upper Confidence Interval (C_u)

Minimum Model: $C_u = a\ \omega_e^2 + b\ \omega_e + c$

N = 10,000

Model Term	Estimate	Df	F-value	P-value
a	-0.42826	996	525.89	<0.001
b	1.41088	996	34.162	<0.001
c	0.02912	996	2.993	0.1271

Lower Confidence Interval (C_u)

Minimum Model: $C_l = a\ \omega_e^2 + b\ \omega_e$

N = 10,000

Model Term	Estimate	Df	F-value	P-value
a	0.439110	997	143.16	<0.001
b	0.507743	997	160.81	<0.001
c	0.001574	997	0.0045	0.9485

The table shows the results of the non-linear regression analysis to determine the relationship between the upper and lower confidence intervals of population prevalence results associated with each level of estimated prevalence. For both models quadratic functions of the form y = ax2 + bx + c where fitted to the data. The estimate is the value of each of the constants in the fitted model predicted by the regression analysis. The P-value indicates whether a model not containing each term was significantly different from a model containing that term. For the upper confidence interval all three terms in the model were significant so the initial model was accepted as the minimum model. During model simplification the lower confidence interval the term c was found not to be significant, and a two term model without c was found not to be significantly different from the initial model. In addition the reduced model provided a slightly smaller residual standard error (initial model RSE = 0.01999, reduced model RSE = 0.0187) so the reduced model was accepted as the minimal model as all other terms were found to be significant.

Table 6.8. Summary of the functions derived from the non-linear regression analysis of data produced from the simulations

$$\omega_e = 0.993331 - 0.938248e^{-8.466109K_e}$$

$$C_u = -0.42826\omega_e^{\,2} + 1.41088\omega_e + 0.02912$$

$$C_u = 0.43437\omega_e^{\,2} + 0.51372\omega_e$$

Where:

ω_e = Estimated prevalence

C_u = Upper 95% confidence interval

C_l = Lower 95% confidence interval

K_e = Estimated overdispersion.

6.3.3.2. Accuracy of the functions describing the confidence intervals of the estimated prevalence

Description of simulation 5

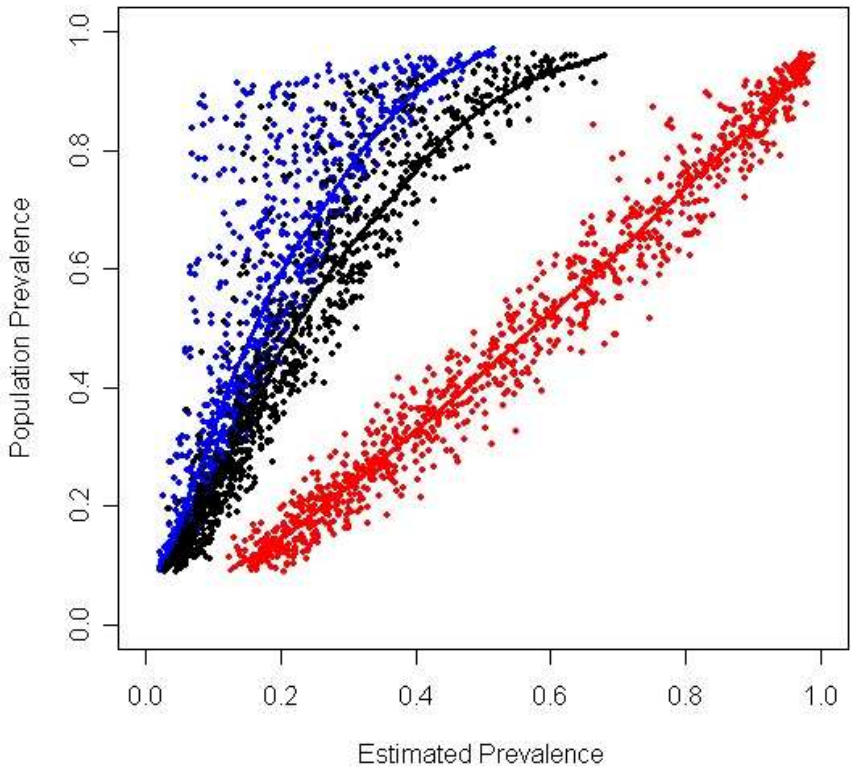
To test the accuracy of the confidence intervals, simulation 3 was adapted to calculate confidence intervals using the functions derived from the non-linear regression analysis. The model was set up to test if the population prevalence fell within the calculated confidence intervals, giving a value of one for a true evaluation and zero for a false evaluation. This data was collected for 1.0 x 10^4 iterations of the model, the results were summed and divided by the number of iterations to produce a figure for the accuracy of the confidence intervals that ranged between zero for complete inaccuracy and one for total accuracy.

Results

The results of the test showed that the population prevalence fell within the calculated confidence intervals 8440 times out of 1.0 x 10^4 simulations, achieving an accuracy of 0.844. These results show that under all conditions of overdispersion, mean infection intensity and population prevalence the functions derived from the non-linear regression analysis are can reliably predict population prevalence in close to 85% of cases. A plot of the estimated prevalence of three techniques is shown for comparison in Figure 6.3. The data obtained from 1.0 x 10^4 iterations of the model (simulation 5) shows prevalence estimates using a standard sample screening

technique (n samples screened only once), a cumulative prevalence estimate from three repeated screenings of the samples and an estimate of prevalence derived using the estimate of overdispersion. The results clearly show the greatly improved accuracy of the newly developed method and the poor performance of more conventional approaches to obtaining prevalence estimates. It should be noted that the methodology described for calculating estimates of population prevalence based on an estimate of overdispersion only apply to cases where there exists a distribution of infection intensities in the host population that can be described by the negative binomial distribution.

Figure 6.3. Estimates of population prevalence obtained from three different methods



The figure shows the accuracy of estimates of population prevalence obtained by three different methods over a range of different population mean infection intensities, overdispersion and population prevalence. The estimates obtained from a conventional method of screening the samples once are shown in blue. The cumulative prevalence estimates obtained from screening the samples five times are shown in black. The estimates obtained from the newly described method based on assessment of overdispersion in the samples are shown in red. The method based on cumulative prevalence and estimation of overdispersion are obtained from screening three 382 samples three times each. Trend lines for each estimate type are shown by the solid lines. The results show that the overdispersion (K) based method is capable of accurately assessing the population prevalence, whilst more conventional methods are inaccurate. These data apply to populations where the infection intensities present within the hosts can be described by a negative binomial distribution.

6.4. Discussion

Work in previous chapters has highlighted the importance and consequences of intensity related false negatives (IRFN). The relationship among each of the five variables involved has also been explored. The work in this chapter attempted to develop methods of dealing with the underestimation of prevalence that arises as a result of IRFN. This work also attempted to develop methods that can overcome the effects of IRFN in addition to highlighting the benefits of taking a quantitative or semi-quantitative measure of the parasite population.

Quantifying the infection intensity in sub-patent samples is complicated by the effects of IRFN, for samples that have complete repeatability and where there is no IRFN effect, RT-PCR can be used to determine accurate quantitative information. However, without repeat testing of the samples it is not possible to know from any single diagnostic result if IRFN is present. Hence, samples must still be assessed for IRFN and if repeatability is not complete, samples measured by RT-PCR must still be averaged over repeated tests. Since the IRFN effect is caused by low infection intensity of the parasite, then it follows the number of positive results obtained from r repeated screenings of a sample is therefore a semi-quantitative representation of infection intensity, the more repeated samplings the greater the resolution of the measure. The estimated repeatability is important at two levels, firstly the repeatability of an individual sample is a measure of the level of infection intensity within that host / sample. Secondly, the repeatability values for all samples represents the distribution of parasites in the host population, this is important if the epidemiology of the parasite is to be fully understood.

With a single diagnostic test per sample the power of the screening is directly related to the sample size (n). However, with the repeat screening the relationship between the number of samples, the number of repeat tests per sample and the statistical power of the screening is not clear. In order to determine the relative importance of the number of samples and the number of repeat screenings the accuracy of different sampling strategies was assessed with a simulation designed to obtained estimates of population overdispersion (K) and mean infection intensity (P) by using a range of different sampling strategies. In order to have comparable results the total number of diagnostic tests was fixed at 300. The accuracy of the different strategies was measured by the percentage difference of each estimate from the population value. The results showed that, over a range of values for mean infection intensity and overdispersion of the population, the error was minimal when the maximum number of samples was analysed as opposed to an increased number

of repeat tests on each sample. In the simulation, with a fixed amount of tests (300) the most reliable sampling strategy was that of 100 samples tested three times each. The results presented here indicate that given a limited number of diagnostic tests available, which is likely to be the case in practice, it is more efficient to maximise the number of samples with a minimum of three repeat tests per sample.

It is proposed that by obtaining semi quantitative measure of infection intensity results from repeat screening of samples (i.e. assessing the diagnostic repeatability of each sample) that the effects of IRFN can be mitigated. However, it is necessary to determine the relative efficiency of such semi-quantitative methods in comparison to the more standard method of assessing prevalence from a single screening. To test the performance of the two methods (prevalence based and repeatability based) in detecting significant difference between two populations, a simulation was constructed that collected and analysed the data from screening a randomly selected set of samples from two different populations. In this way the performance of each method in detecting significant difference between two populations with known minimum difference, could be compared for each of the two methodologies.

The results of these comparative tests are presented in Tables 6.1a and 6.1b in the form of a probability of the detection of a significant difference. The most striking feature of the results is the very poor performance of prevalence based method (single screening of the samples and Chi square test of prevalence). When comparing populations with low mean infection intensities (100 to 300 parasites per millilitre) and using 100 samples, a statistical test of diagnosed prevalence from each population, using a Chi-Square test, was only able to detect significant difference with a probability of 0.30. In other words on 70% of occasions when there was a known minimum difference between the compared populations this method was not able to detect a significant difference. Furthermore, increasing the number of samples screened in this manner only increases the probability to 0.42, still a very inefficient level of detection. Considering, that obtaining prevalence from a single diagnostic test per sample is the most widely used method for comparing infection in two populations these results suggest that it performs very poorly on populations with sub patent infections.

The performance of this method improves as the mean infection intensity increases, where for populations with mean infection intensities between 1,000 and 10,000 parasites per millilitre the probability of detection rises to 0.63 for 100 samples and 0.70 for 500 samples, still surprisingly less efficient than might be expected.

In contrast, the probability of significant difference using an estimate of repeatability for each sample (instead of diagnosed prevalence) in populations with the lowest mean infection intensities was 0.56 for three repeat tests on 100 samples, a significant improvement on using the diagnosed prevalence from 100 samples (+ 0.26). It is notable that even though this repeat screening method utilised 300 diagnostic tests in total it was still more efficient than estimating from a single test per sample prevalence of 500 samples. The probability of detecting significant difference rose by 0.09 when 100 samples were screened with five repeat tests. Again the probability rose as the mean infection intensity of the populations increased. However, for the comparisons between populations, where mean infection intensity was highest, the difference between the different methods of screening the samples was minimal. These results indicate that the more standard methodology of assessing diagnosed prevalence is only efficient, for populations with high mean infection intensities. Methods using the cumulative prevalence and using data on infection intensity (with a Wilcoxen test) were also tested (data not shown), both these methods were found to be better than testing prevalence from a single screening but not as efficient as the repeatability method.

Comparison of the results obtained using three and five repeated tests showed that as might be expected, five repeated tests increases the mean probability of detecting significant difference of between 0.7 to 0.9. Whether three or five repeat tests are used on a sample set will depend on whether the extra investment of conducting the extra tests is feasible. The comparison made here gives valuable information that can help inform such decisions.

Repeating this test for different ranges of overdispersion (K) (as opposed to different ranges of mean infection intensity), showed that the probability did not vary greatly for the different ranges. The lack of variability when tested with different ranges of overdispersion (K) provides further evidence that the main cause of underestimation of prevalence and therefore the lack of sensitivity in these tests is due to low infection intensities.

The comparative analysis just described undoubtedly shows, that for populations with sub-patent infections, a semi-quantitative assessment of the infection intensity of each sample (by means of repeat screening of samples) improves the probability of detecting significant difference between populations. However there are a number of important potential limitations. The results of the simulations presented here all rely on the assumption of the distribution of the parasite fitting that of a negative binomial. There is no obvious reason why these findings should not also apply to

patterns of parasite distribution other than the negative binomial; although the comparative efficiency may change under different distribution assumptions. It is important that testing should be conducted to extend this work to other assumptions for the distribution pattern of the parasites. Whilst the use of repeatability is an undoubted improvement over the use of prevalence, a probability of detecting a significant difference in populations that are known to have a minimum level of difference of 0.56 is still a relatively insensitive level of detection. This highlights the need for further improvement in the diagnostic techniques, data collection and data analysis.

The comparative analysis presented in Tables 6.1a and 6.1b. highlight the inadequacies of methods associated with diagnosed prevalence in detecting significant difference. The table provides valuable information that comparatively quantifies the improvements that might be made by adopting different approaches. This information is important in deciding the approach to screening a population especially if the prior information on the potential mean infection intensity of the target populations is known.

The overdispersion parameter K of the negative binomial distribution is strongly influenced by the number of zero values present, which equates in this case to the number of uninfected hosts. This gives K a very strong relationship with population prevalence, as shown in Figure 5.4. As diagnosed prevalence underestimates population prevalence in populations with sub-patent infections, fitting a distribution to the data obtained from repeat screening of each sample allows an estimation of K. By use of the strong relationship between K and population prevalence, an estimate of the population prevalence that is less affected by IRFN can be calculated. Testing this method of estimating population prevalence using a simulation showed that it was many times more accurate than using either the prevalence from a single screening or the cumulative prevalence from repeated testing of the samples (see Figure 6.3.). The results obtained from the diagnosed prevalence method (the standard methodology) severely underestimated the population prevalence. The estimates derived from the estimation of the overdispersion parameter K spread around the actual population prevalence values, this allowed development of a method for calculating confidence intervals. This method for estimating population prevalence further increases the usefulness of using repeatability data from repeated screening of samples rather than simple diagnosed prevalence. The method described in this simulation utilised 382 samples repeat screened three times each. However, it would depend on the distribution of

parasites fitting a negative binomial and it is possible that this may not be the case. It may be possible to adapt the methods for inference of population prevalence for other distribution assumptions.

In summary, the results presented in this chapter show that for populations where the infections are present at high mean intensity, the use of diagnosed prevalence from a single screening for both representing the number of infected hosts and for comparing differences between populations is an appropriate method. However, if the populations have a significant proportion of sub-patent infections, then the use of diagnosed prevalence is inadequate in both representing the numbers of infected hosts and in determining differences between populations. For these types of infection patterns within populations the use of repeat testing of samples provides a significant improvement in detecting differences between populations. The inference of population prevalence from the repeat test data via an estimate of the overdispersion parameter (K) provides much more accurate estimates than the use of diagnosed prevalence. Additionally, the use of data from repeat screening of samples provides quantitative information on the distribution and numbers of the parasite population that is lacking when diagnosed prevalence from a single screening is used.

Chapter 7: General Discussion

Discussion of the results obtained from empirical and theoretical work

Although there have been vast improvements in the techniques used to screen field samples for the presence of African trypanosomes, epidemiological research in this area is still difficult. Current protocols require the use of a species specific PCR protocol for each species of trypanosome that may be present. The use of separate screening protocols is both time consuming and makes inefficient use of valuable research funds. In addition, each separate protocol may have different diagnostic sensitivities and protocols must be selected according to what species of trypanosome are expected to be present in the samples. These limitations are not conducive to acquiring high quality and representative epidemiological data.

In the first part of this work (Chapter 2) a new PCR based screening technique was developed which allowed field samples to be screened for all important African trypanosome species with a single protocol. The technique was developed to function on whole blood applied to filter paper cards. This development allowed major simplification of the entire sample processing protocol whilst at the same time allowing the same diagnostic sensitivity to be applied in the detection of all Important African trypanosome species. This rationalisation of the sample screening methodology overcomes many of the problems with the current methodology, and significantly reduces the cost and time involved in sample processing. Creating a more efficient protocol in this way allows valuable research funds to be targeted elsewhere.

Assessing the infection intensity of a parasite quantitatively presents a number of problems. Firstly, it is not possible to definitively count even macro-parasites, such a procedure would require a post-mortem examination, and this is certainly neither practical nor ethical. For micro-parasites the situation is even more complicated, as the small size of the parasites makes them much more difficult to enumerate. A definitive enumeration of parasite load would require examination of the entire blood or tissue volume of the sample of hosts under study. Instead quantitative measures must be inferred from samples in the same manner as prevalence is inferred from a population by selecting a sufficient number of samples. To represent the number of parasites within a host, according to statistical reasoning, would require the enumeration of parasite load in a statistically significant number of aliquots of blood from the host. However there are still many problems, the parasites may not be distributed evenly in the blood, the parasite may exhibit cyclical patterns of migration (e.g. from peripheral blood to venous blood). Without knowledge of these aspects quantification is difficult. Never the less, the benefits of a quantitative assessment

may well outweigh the difficulties. This approach was adopted with a number of randomly chosen blood samples taken from apparently healthy African zebu cattle of unknown infection status (n = 35). Repeated PCR assays were conducted on each sample, so that the results of up to 114 diagnostic tests for each host were available. Relating the spatial position of these results back to the position of the punch / aliquot taken from the filter paper card showed the 'sparse' distribution of trypanosomes within the blood samples. Whilst this approach is clearly not practical for large numbers of samples, it was hoped that it would provide a unique insight into the distribution of each species of trypanosome in a relatively small number of hosts (Chapter 3).

Such an 'in depth' analysis of field samples has never been attempted before. The results revealed that most (85.7%; n=30) of the cattle were infected with trypanosomes and many harboured mixed species infections (60%). Analysis of the resulting data failed to reveal any evidence of interaction between the infecting species, although some of the species combinations were at levels close to the 95% confidence intervals of the expected values, suggesting that a larger study may reveal important interactions between the trypanosome species.

In addition, the data obtained from repeat screening of these thirty five field samples revealed that although the mean prevalence for any trypanosome species after many rounds of screening was 9.8%, the cumulative prevalence reached 85.7%. These results indicated that diagnosed prevalence from a single screening of samples seriously underestimates the true level of population prevalence. The underestimation was shown to be due to a large number of false negative results obtained from infected samples. The occurrence of these false negative results was found to be due to the intensity of infection present in the host (Chapter 4). Repeated examination of a range of other samples from different hosts and geographical areas revealed that false negative diagnostic results occurred at a high level in all the infected samples examined. Because the false negative diagnostic results where shown to be caused by low infection intensities present in the host population (sub-patent infection) the phenomenon was termed 'Intensity Related False Negatives' (IRFN). The occurrence of IRFN results in a range of randomly chosen samples is of great importance to epidemiological study of trypanosomiasis. Evidence in the literature of the occurrence of false negative results in epidemiological studies was presented (Chapter 1), making the occurrence of IRFN of potential importance to a wider range of pathogens.

In order to describe the distribution of trypanosomes across the host population the data obtained from repeated screening of the 35 cattle samples was fitted to negative binomial and Poisson distributions. The data was significantly different from that of a Poisson. Although there was too little data to allow fitting of all the individual species of trypanosome, except for *T. theileri*, the frequency distributions of all species were overdispersed and visually suggestive of a negative binomial distribution (NBD). The data obtained for all species combined and *T. theileri* separately, proved to be not significantly different from a negative binomial distribution. This is the first case where evidence has been presented for African trypanosomes being distributed according to a NBD. This kind of information regarding the distribution of the parasites is of importance in modelling and in determining the transmission dynamics of the parasite. In this work the NBD was subsequently utilised as a model for the distribution of trypanosome species across the host population. Although other distribution assumptions could equally have been used.

Stochastic mathematical modelling techniques were employed to demonstrate the consequences of IRFN. The results from these simulations demonstrated that, due to the effects of IRFN, it is possible to obtain the same diagnosed prevalence from populations with very different levels of true population prevalence and patterns of infection (Section 4.3). Data obtained from further simulations showed that for individual hosts with infection intensities of below 6,000 to 8,000 parasites per millilitre and a diagnostic technique analysing a volume of one microlitre of sample (the typical assayed volume of a PCR protocol), false negative results are certain to occur, albeit at an initially low level, 1% to 2% (mean probability = 0.01 ~0.02). Whilst for a host with an infection intensity of 1,000 parasites per millilitre false negative results will occur with a mean probability of 0.4. For populations of hosts with parasites distributed according to a negative binomial distribution with a mean infection intensity in the population of 10,000 parasites per millilitre false negative results will occur with a mean probability of 0.19. Given that field infections are likely to occur with lower levels of mean intensity than this, these results strongly suggest that false negative results are likely to occur in most epidemiological situations.

In chapter 6, quantitative methods of sample collection were advocated to counteract the effects of IRFN results. The poor performance of current approaches to statistical comparison of different populations was highlighted in further simulation studies. Statistical comparisons of prevalence obtained from single screenings of two populations only identified difference between compared populations with a probability of 0.3 (Tables 6.4a and 6.4b) when a large difference in prevalence and

infection pattern was present. The work carried out in this chapter demonstrated that improvement in epidemiological analysis can be achieved by using a quantitative method of sample analysis. A method for statistical comparison between different populations was also tested and found to offer improvements over currently used techniques (Section 6.3.2) in situations where IRFN is present. Furthermore, a method of calculating the true level of prevalence in a population via estimation of the parameters of a negative binomial distribution was shown to give estimates which were, theoretically, an order of magnitude more accurate than conventional methods. However, this method of estimation of population prevalence should be supported with empirical work.

The work presented throughout this thesis has attempted to demonstrate that IRFN results are very likely to occur under field conditions. And that sole reliance on assessment of prevalence can lead to serious misinterpretation of the epidemiological state of the pathogen in question. Adopting a quantitative approach to screening field samples can help to both identify and overcome this problem. In addition assessment of the distribution of pathogens across host populations provides an additional dimension to the available data for epidemiological investigation.

The implications of failing to account for the presence of IRFN

The occurrence of IRFN is of great concern as many epidemiological studies depend upon reporting diagnosed prevalence or on comparing diagnosed prevalence between two or more populations. Important results may therefore be easily missed or misinterpreted. In fact, although African trypanosomiasis has been closely studied for more than 100 years, there are many aspects of the epidemiology that still remain unclear. It is possible, that an over reliance on prevalence information and a failure to ascertain quantitative data on the parasite has been a major factor hindering epidemiological discovery. The existence of sub-patent infection intensities in a host population (and therefore IRFN in epidemiological study) has a number of serious consequences.

Treatment regimes and control strategies

Studies or control programmes that rely on targeted treatment of infected individuals can fail to diagnose a large number of the infected hosts. Failure to treat many infected hosts in this type of programme may lead to the failure of the control effort. Untreated infected hosts can provide a significant reservoir of infection and the transmission from these sub-patent hosts may be more important than the

transmission contributed by the hosts diagnosed as infected. Persistence of transmission of schistosomiasis after control programmes has been noted on a number of occasions (Polderman & de Caluwe, 1989; Webbe & el Hak, 1990; Butterworth *et al.*, 1991; Gryseels *et al.*, 1991) and ascribed to significant numbers of hosts with low level infection being missed by the screening methodology.

Misleading data supplied to transmission models.

Transmission models based on epidemiological data derived from populations where widespread sub-patent, low intensity infections are present may seriously misrepresent the true epidemiological situation. A majority of undiagnosed low intensity infection may contribute the majority of the transmission.

Statistical measures and reasoning

Epidemiological measures that depend upon accurate and repeatable identification of infected hosts, such as diagnosed prevalence, incidence, diagnostic sensitivity, diagnostic agreement between techniques, sample size and power calculations all have to be re-evaluated in the cases where IRFN are a significant factor. This work has shown the basic assumption of accurate and repeatable identification of infected hosts may not be reliable under conditions were sub patent infections are present. For diagnostic sensitivity, diagnostic agreement between techniques, sample size and power calculations it follows that applicability of these measures is dependent upon the levels, and variations of infection intensity present in the population under analysis, and that they must therefore be assessed for every study population. This effect is known as spectrum bias (Ransoff & Feinstein, 1978). In cases where sub-patent infections are not present these measures may be reliable, but a failure to assess the possibility of sub-patent infections will result in a lack of confidence in the results and conclusions of such studies.

Diagnosed prevalence

The underestimation of population prevalence resulting from the existence of low intensity infections in a population together with the fact that the same diagnosed prevalence can be obtained from populations with a true population prevalence of 14% or 100% raises an interesting question; what is the meaning of diagnosed prevalence? The prevalence obtained from a single diagnostic test per sample may or may not be representative of the true prevalence. Without knowledge of the distribution of parasites the difficulty is that, for current approaches, it is not possible to calculate what proportion of the true prevalence the diagnosed prevalence represents. Although multiple tests per sample can give an increased estimation of

prevalence (cumulative prevalence), as has been demonstrated here and elsewhere (de Vlas *et al.*, 1992b), the situation remains essentially unchanged, in that the cumulative prevalence from n samples screened r times may still represent only an unknown proportion of the true prevalence. Simply increasing the number of tests per sample or the sensitivity of a technique does not solve the problem. Similarly, increasing the volume of blood analysed will increase the diagnosed prevalence in the same manner as repeat testing, and again the true prevalence remains unknown. Therefore increasing the sensitivity of diagnostic tests or improving the sensitivity by extracting DNA in solution will increase the proportion of hosts detected as infected but will still not ensure an accurate estimation of the true prevalence or give any confidence in the results. The true prevalence must therefore be inferred from the diagnostic test data, a quantitative assessment of the parasite load and distribution of parasites within the samples and host population.

Conversely, should an endemic parasite be present in most hosts in the population then the question arises as to what a prevalence of 90% or 100% means. If all hosts in the population are infected, what is the use is prevalence as an epidemiological measure? If all or most hosts are infected, then variations in the transmission and epidemiology of the disease must be based on quantitative changes in parasite load within individual hosts not on changes in prevalence. The epidemiological importance of infection status is therefore transferred from an infected / uninfected paradigm, to a how infected paradigm.

It seems that in some situations the diagnosed prevalence is an unreliable, misleading and inadequate epidemiological measure. The only way to counteract this is to base epidemiological study not on prevalence but on the distribution of parasite load within populations.

Limitations of this work

Whilst the demonstration of the existence of IRFN in African trypanosomiasis presented in this study is based on empirical data, the worked concerned with demonstrating its effects and methods of dealing with the phenomenon are based on modelling and are therefore theoretical. Further empirical work should be conducted in order to validate the conclusions and findings of this study. The work presented in this thesis represents the early stages of investigation into the existence of this phenomenon and into methods of dealing with IRFN, additional work will undoubtedly further improve on the methods described.

Diurnal patterns, the negative binomial distribution and apparent sub-patent infections

For parasites that replicate within the host the apparent distribution of parasites within the host population may be influenced or even be a result of fluctuating levels of parasite numbers due to either diurnal variation in parasite numbers present in the site from which the blood sample was drawn (venous or peripheral blood). In field studies, the collection of blood from a large number of hosts is spread over time, should there be diurnal variation in parasite numbers then the difference in time over which the samples were drawn could create an overdispersed distribution of infection intensities within the host population, and the appearance of the existence of widespread sub-patent infection. For example, supposing the parasites under study are present in low numbers in the peripheral blood early in the morning and gradually increase in numbers during the day reaching a peak around midday. An epidemiological study that collects samples from peripheral blood would show low infection intensities in those samples collected in the morning, as the sample collection process begins, and high infection intensities in those samples collected around midday. It is therefore possible that the distribution of infection intensities within the hosts are an artefact of the diurnal variation and the fact that sample collection is spread over time. However, this assumes that a more homogenous distribution of parasites within the host population underlies the diagnostic results. Given the number of potential factors that are capable of producing biological variation in the infection intensity of any host (age, previous exposure, genetic factor, behavioural factors etc.), this is unlikely to be the case. Whilst this does not affect any of the conclusions of this study, it would imply that caution and careful consideration are used before reaching any epidemiological conclusions. Therefore, the existence of any diurnal patterns of variation in infection intensity should be determined by empirical study as a matter of some importance.

Future work will focus on obtaining empirical support for the findings contained in this thesis. In addition the relevance of this work to incidence as an epidemiological measure will also be assessed.

Conclusions

The main conclusions of this study are as follows:

- The diagnosed prevalence (and incidence) determined from a single screening per sample can be an unreliable and misleading measure of infected hosts and gives little or no information on parasite distribution. Where sub-patent infections are present, prevalence can be severely underestimated. Where sub-patent infections are not present prevalence may be reliable, however, without a specific assessment of the existence of the rate of occurrence of intensity related false negatives (IFRN) there is no way of establishing confidence in the prevalence value obtained.

- In order to mitigate the effects of IRFN quantitative or semi quantitative measures should be used in any epidemiological study.

- The existence of IRFN should be assessed before or during any epidemiological study.

- It is possible to mitigate the effects IRFN, the work presented here offers improved sensitivity of statistical detection of significant difference between populations and a method for estimating true populations prevalence. This work can be developed further.

- IRFN may be applicable to a wide range of pathogens.

- IRFN can be a serious restriction to gaining an accurate understanding of the epidemiology of a pathogen. If the widespread occurrence of this problem in epidemiological study of schistosomiasis was enough to prompt the World Health Organisation to recommend the use quantitative measures (WHO, 1967; 1980), then the problem should not be underestimated.

- The occurrence of IRFN results in epidemiological studies may result in incorrect information on which transmission models and control programmes may subsequently be based. This incorrect information may be enough to cause the failure of a control programme.

8. References

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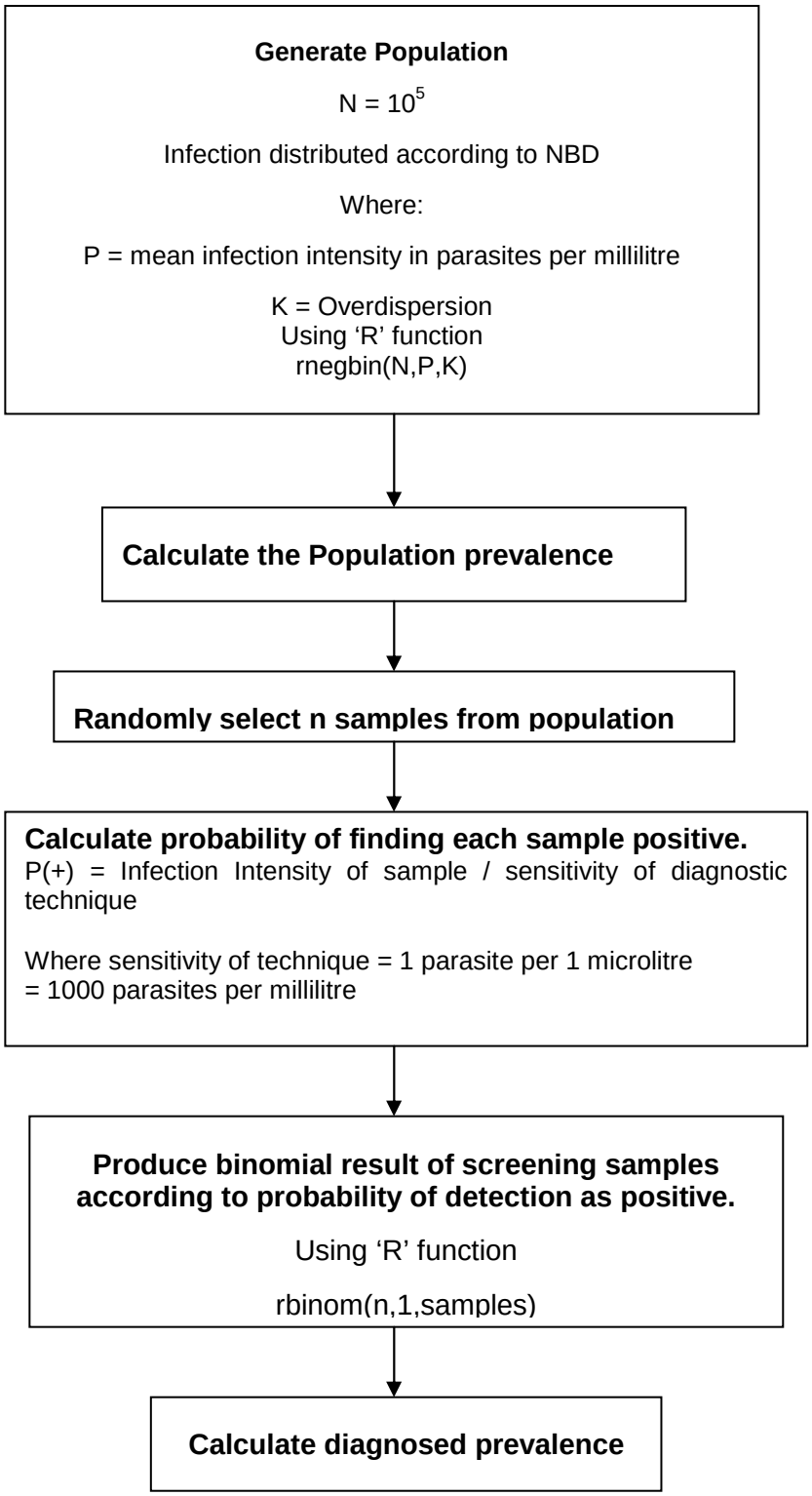
Appendices

Appendix Figure 5.1. Flowchart showing the basic premise of the simulations



Appendix Figure 5.2. Code for basic simulation to produce data frame of variables

```
#Simulation to create a data frame of results of screening a
#population with a standard screening approach
#Load appropriate package
library(MASS)
#Set simulation parameters
N<-10000 #Size of the population
n<-382#Number of samples drawn from population for study
se<-1000 #Sensitivity of the technique
i<-1000#Number of iterations (data points)
#Create empty data collection vectors
K1<-c()
P1<-c()
popprev1<-c()
estprev1<-c()
diff1<-c()
##Determine range of values from which the population parameters
#will be selected
pvect<-(100:2000)
kvect<-seq(0.01,0.4,0.01)
##Start iterations
for(j in 1:i){
#Select population parameters
P<-sample(pvect,1)#Mean intensity of the population
K<-sample(kvect,1)#Overdispersion of the population
#Create the population
pop<-rnegbin(N,P,K)
##Calculate the population prevalence
popprev<-subset(pop,pop>0)
popprev<-length(popprev)
popprev<-popprev/N
#Screen n samples once
samp1<-sample(pop,n,replace = FALSE)
samp2<-samp1/se
samp3<-replace(samp2,samp2>1,1)
sampres<-rbinom(n,1,samp3)
#Calculate diagnosed prevalence
estprev<-sum(sampres)
estprev<-estprev/n
#Calculate the underestimation
diff<-popprev-estprev
#Collect data from each iteration
K1[j]<-K
P1[j]<-P
popprev1[j]<-popprev
estprev1[j]<-estprev
diff1[j]<-diff
#End iterations
}
#Create data frame
data1<-data.frame(K1,P1,popprev1,estprev1,diff1)
#Output data frame
write.table(data1, file = "C://rdata//x.csv", sep = ",", col.names = NA, na="NA", qmethod = "double")
#Rename column labels of data frame
labs<-c("Overdispersion (K)","Mean Intensity (P)","Population Prevalence","Diagnosed Prevalence","Underestimation")
colnames(data1)<-labs
```

Appendix Figure 5.6 Code for determining the probability of obtaining false negative results from a single host with a given infection intensity

```
#Code for determining the repeatability threshold in a host
# and obtaining 95% ci for each level of infection intensity
#set parameters
ii<-100
res5<-numeric(100)
ii1<-numeric(100)
res3<-numeric(100)
mean1<-numeric(100)
lowerci1<-numeric(100)
upperci1<-numeric(100)
#Start iterations
for(k in 1:100){
for(j in 1:100){
#Create volume of blood with infection
iip<-ii/1000
blood<-rpois(1000000,iip)
for(i in 1:100){
#Screen blood
res1<-sample(blood,1)
res2<-replace(res1,res1>1,1)
res3[i]<-res2
}
res4<-sum(res3)/100
res5[j]<-res4
}
#Calculate mean and confidence intervals
mean<-mean(res5)
mean<-1-mean
lowerci<-quantile(res5,0.025,names=FALSE)
lowerci<-1-lowerci
upperci<-quantile(res5,0.975,names=FALSE)
upperci<-1-upperci
mean1[k]<-mean
upperci1[k]<-upperci
lowerci1[k]<-lowerci
ii1[k]<-ii
ii<-ii+100
}
#Output data frame
out<-data.frame(P1,levelmean1,lowerci1,upperci1)
```

Appendix Figure 6.4. General 'R' code for all simulations in this chapter

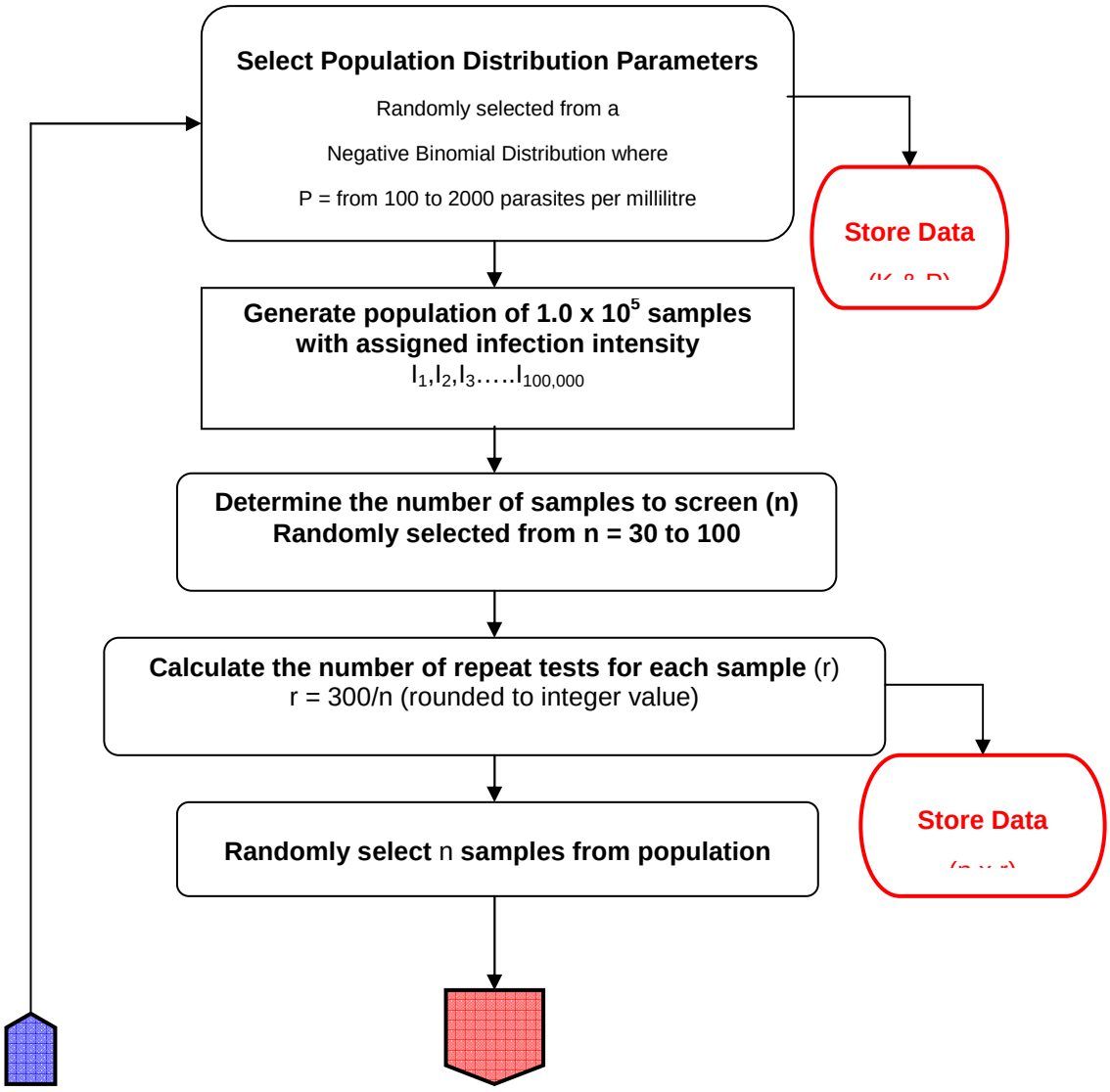
All simulations described in following sections were adapted from this code.

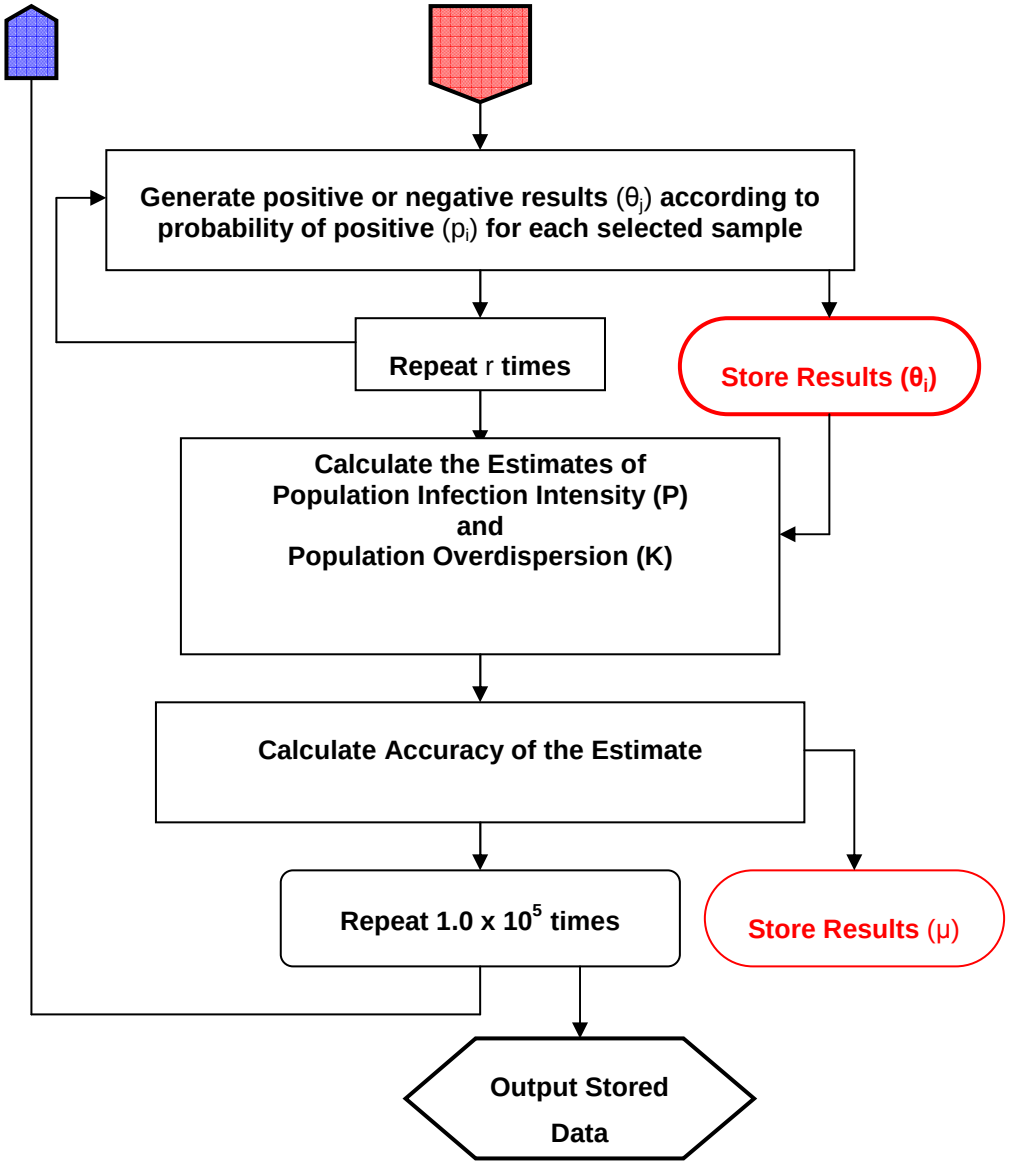
```
#Output a dataset with 'standard', 'k based' and 'cumulative' estimate of population
#prevalence

#Load Library

library(MASS)
#Initialise Parameters
thresh<-1
it<-1000
N<-100000
kp2<-seq(0.01,1,0.01)
kp3<-seq(1,10,1)
kp1<-c(kp2)
p1<-seq(100,1500,100)
n<-382
se<-1000
nxsamp<-5
bins<-10
kest1<-c()
kp2<-c()
it1<-it
p2<-c()
cumprevest<-c()
popprev1<-c()
kprev1<-c()
stdprev<-c()
test2<-c()
uci1<-c()
lci1<-c()
#Start Iterations
repeat{
#Select parameters of population
kp<-sample(kp1,1,replace=TRUE)
p<-sample(p1,1,replace=TRUE)
#Generate population
pop<-rnegbin(N,p,kp)
#Calculate the population prevalence (i.e. true prevalence of the population)
pop2<-subset(pop,pop>0)
pop3<-length(pop2)
popprev<-pop3/N
#Take samples from the population
samp<-sample(pop,n,replace=FALSE)
sampprob2<-samp/se
sampprob1<-replace(sampprob2,sampprob2>1,1)
#Estimate prevalence by 'standard' method
std1<-rbinom(n,1,sampprob1)
std2<-subset(std1,std1>0)
std3<-length(std2)
stdprev1<-std3/n
#Estimate prevalence by 'estimation of k' method
#Collect in a vector those samples that give constant positive (+) results
constpos<-subset(sampprob2,sampprob2>=thresh)
#Collect in a vector those samples that give inconsistent (+/-) results
```

```
onoff<-subset(sampprob2,sampprob2<thresh)
n1<-length(onoff)
res1<-rep(0,n1)
nxsamp1<-nxsamp
repeat{
res2<-rbinom(n1,1,onoff)
res1<-res2+res1
nxsamp1<-nxsamp1-1
if(nxsamp1<=0)break
}
res3<-res1/nxsamp
res4<-c(res3,constpos)
#Calculate the 'cumulative' prevalence
prev1<-subset(res4,res1>0)
prev2<-length(prev1)
prevest1<-prev2/n
# Prepare a vector for recording the frequency of infection intensities in samples
res5<-res4*bins
res5<-round(res5,0)
res5<-res5+1
res6<-max(res5)
#Count the frequency of infection intensities
freq <- c()
max1 <- res6
j <- c()
for(i in 1:max1){
freq[i] <- length(res5[res5==i])
}
#Estimate 'k' by maximum likelihood method
lhs<-numeric()
rhs<-numeric()
y<-0:(length(freq)-1)
j<-0:(length(freq)-2)
m<-sum(freq*y)/(sum(freq))
s2<-(sum(freq*y^2)-sum(freq*y)^2/sum(freq))/(sum(freq)-1)
k1<-m^2/(s2-m)
a<-numeric(length(freq)-1)
for(i in 1:(length(freq)-1)) a[i]<-sum(freq[-c(1:i)])
i<-0
for(k in seq(k1/1.2,2*k1,0.001)){
i<-i+1
lhs[i]<-sum(freq)*log(1+m/k)
rhs[i]<-sum(a/(k+j))
}
k<-seq(k1/1.2,2*k1,0.001)
d<-min(abs(lhs-rhs))
sdd<-which(abs(lhs-rhs)==d)
kest<-k[sdd]
#Calculate the prevalence based on the 'k' estimate
kprev<-0.993331-0.938248*exp(-8.466109*kest)
#Calculate confidence intervals of the 'k based' estimate of prevalence
uci<--0.42826*(kprev^2)+1.41088*kprev+0.02912
lci<-0.43437*(kprev^2)+0.51372*kprev
one<-0
two<-0
three<-0
test1<-0
if(popprev<uci)one<-1
```

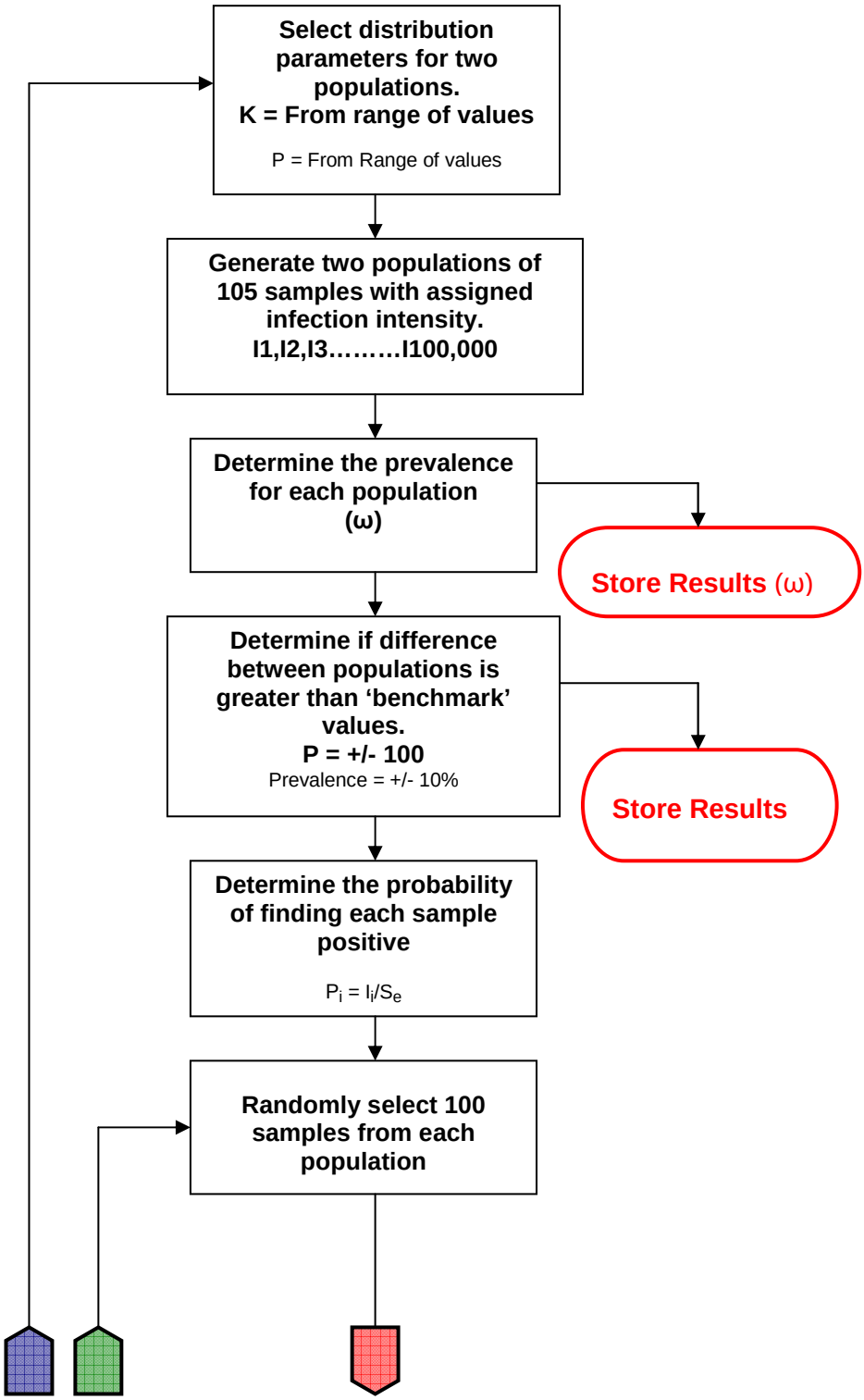
```
if(popprev>lci)two<-1
three<-one+two
if(three==2)test1<-1
test2<-c(test2,test1)
uci1<-c(uci1,uci)
lci1<-c(lci1,lci)
kprev1<-c(kprev1,kprev)
kest1<-c(kest1,kest)
kp2<-c(kp2,kp)
p2<-c(p2,p)
cumprevest<-c(cumprevest,prevest1)
popprev1<-c(popprev1,popprev)
stdprev<-c(stdprev,stdprev1)
it1<-it1-1
if(it1<=0)break
}
estrep<-mean(res4)
sum(test2)/it
outx<-cbind(p2,kp2,popprev1,lci1,uci1,kest1,kprev1,cumprevest,stdprev,test1)
write.table(outx, file = "C://rdata//t3.csv", sep = ",", col.names = NA, na="NA", qmethod = "double")
```

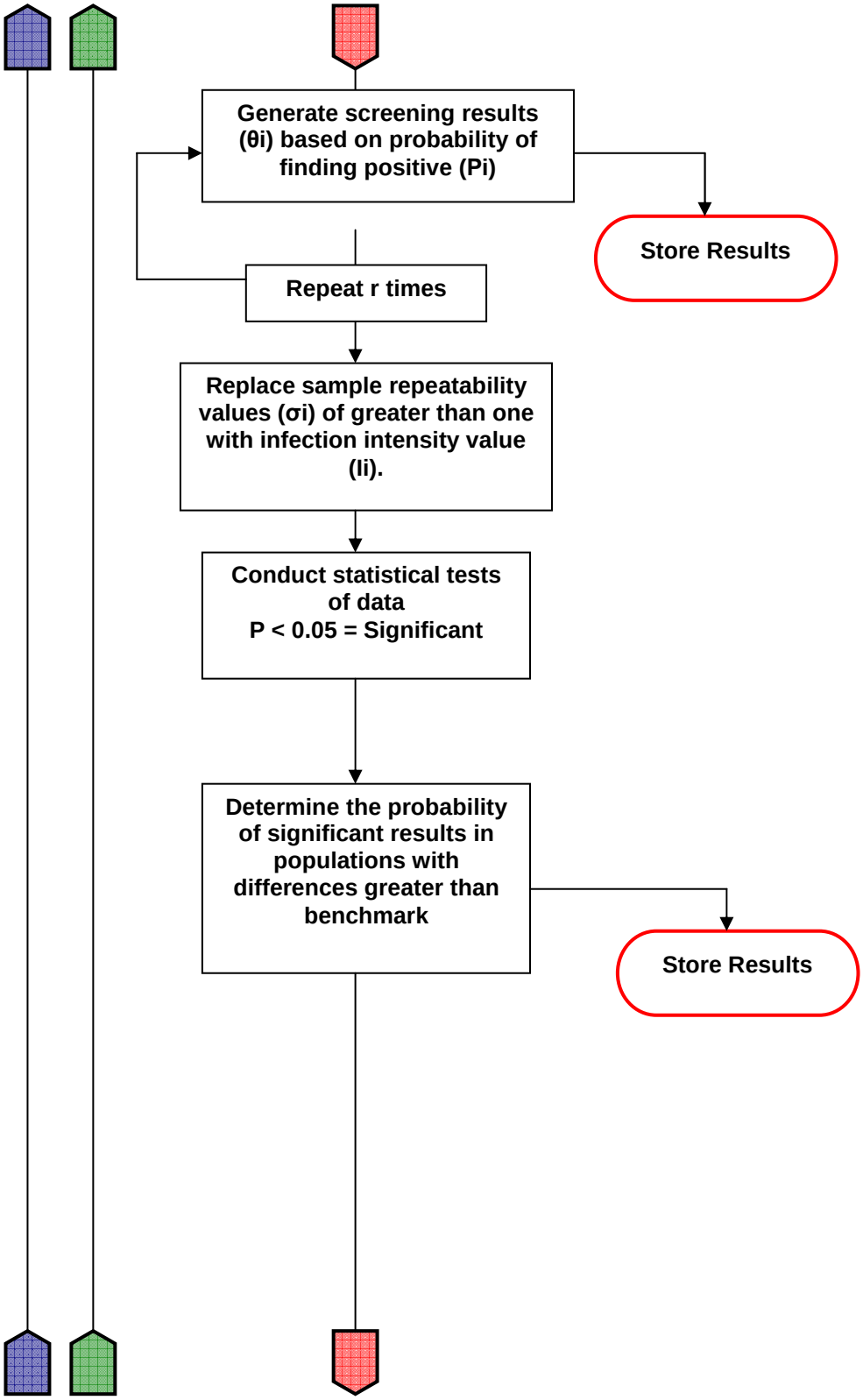
Appendix Figure 6.5. Simulation 1: Flowchart describing the design of a simulation to determine the accuracy in estimating the population overdispersion (K) and mean infection intensity (P) using different sampling strategies

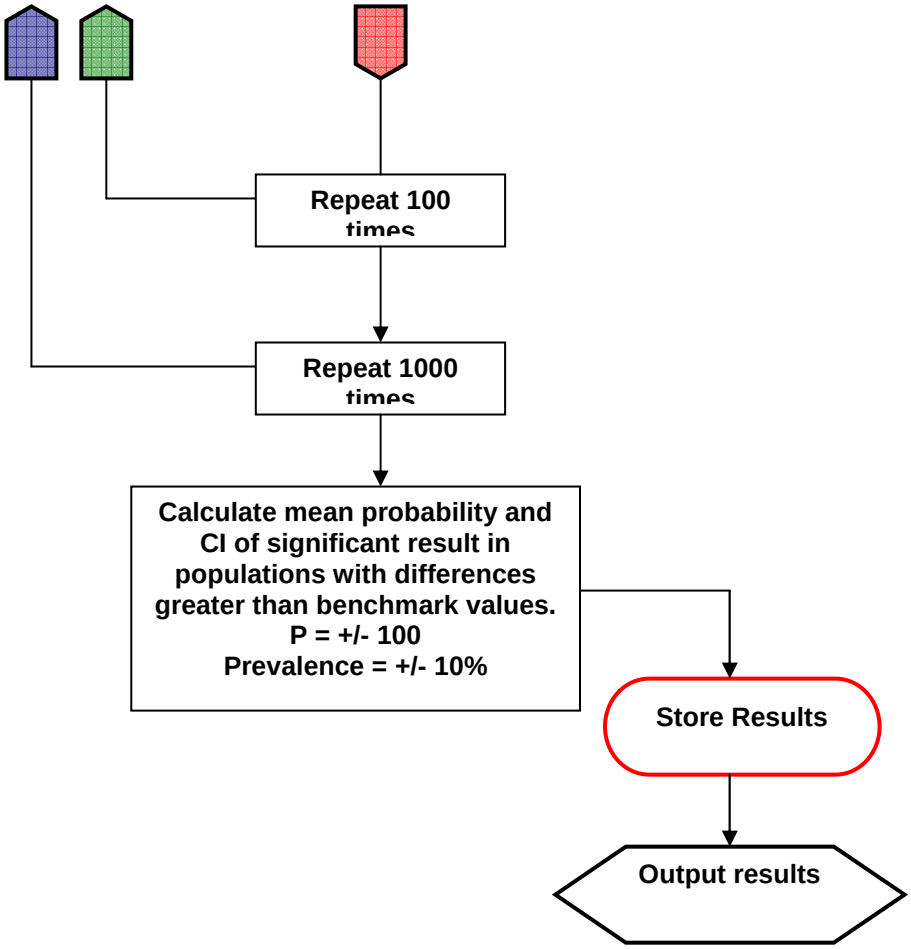



Generate positive or negative results (θ_j) according to probability of positive (p_i) for each selected sample
Repeat r times
Store Results (θ_i)
Calculate the Estimates of Population Infection Intensity (P) and Population Overdispersion (K)
Calculate Accuracy of the Estimate
Repeat 1.0 x 10^5 times
Store Results (μ)
Output Stored Data

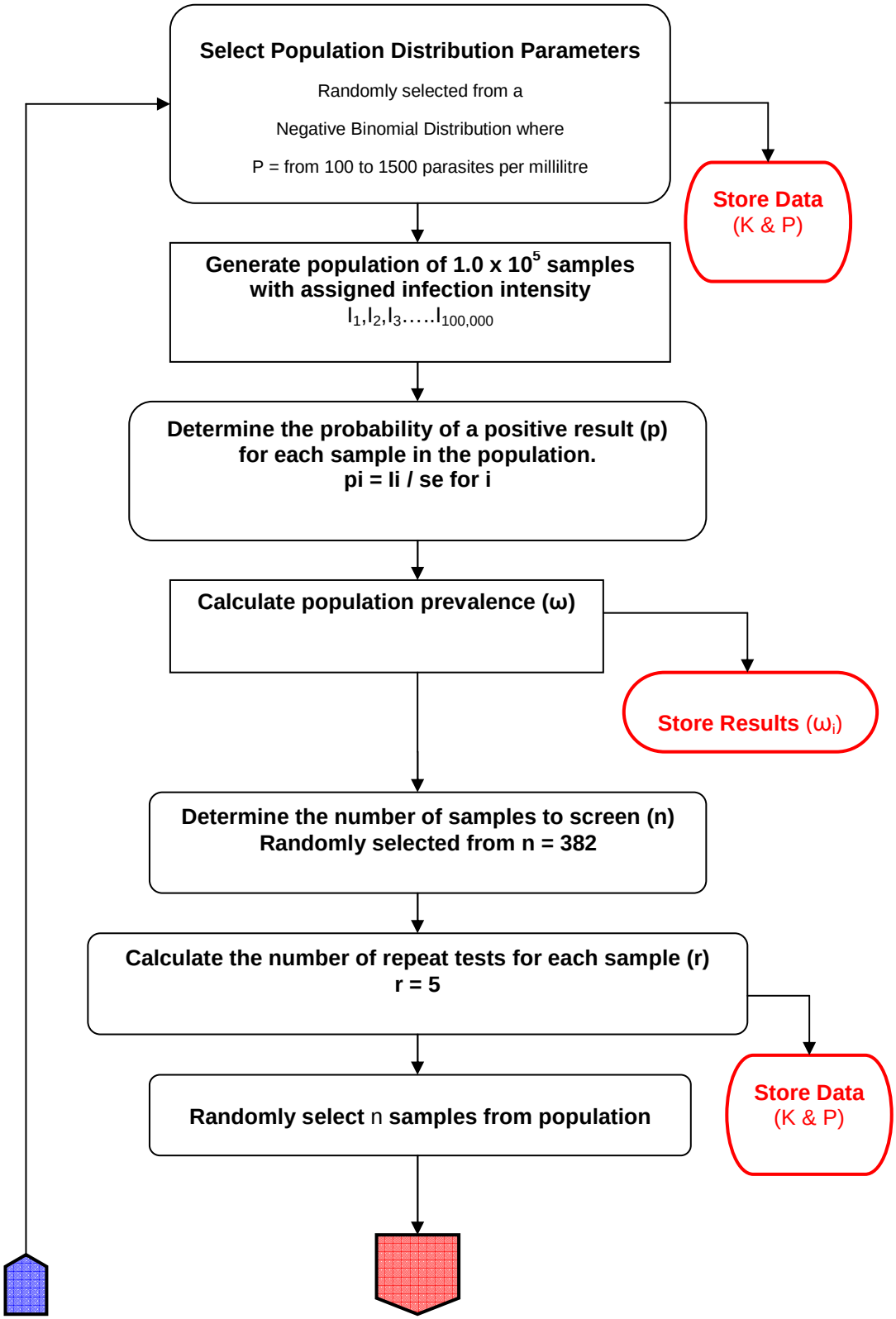
Appendix Figure 6.6. Simulation 2: Flowchart describing the design of a simulation to determine the probability of detecting significant difference in the infection patterns of two populations for ten different methods

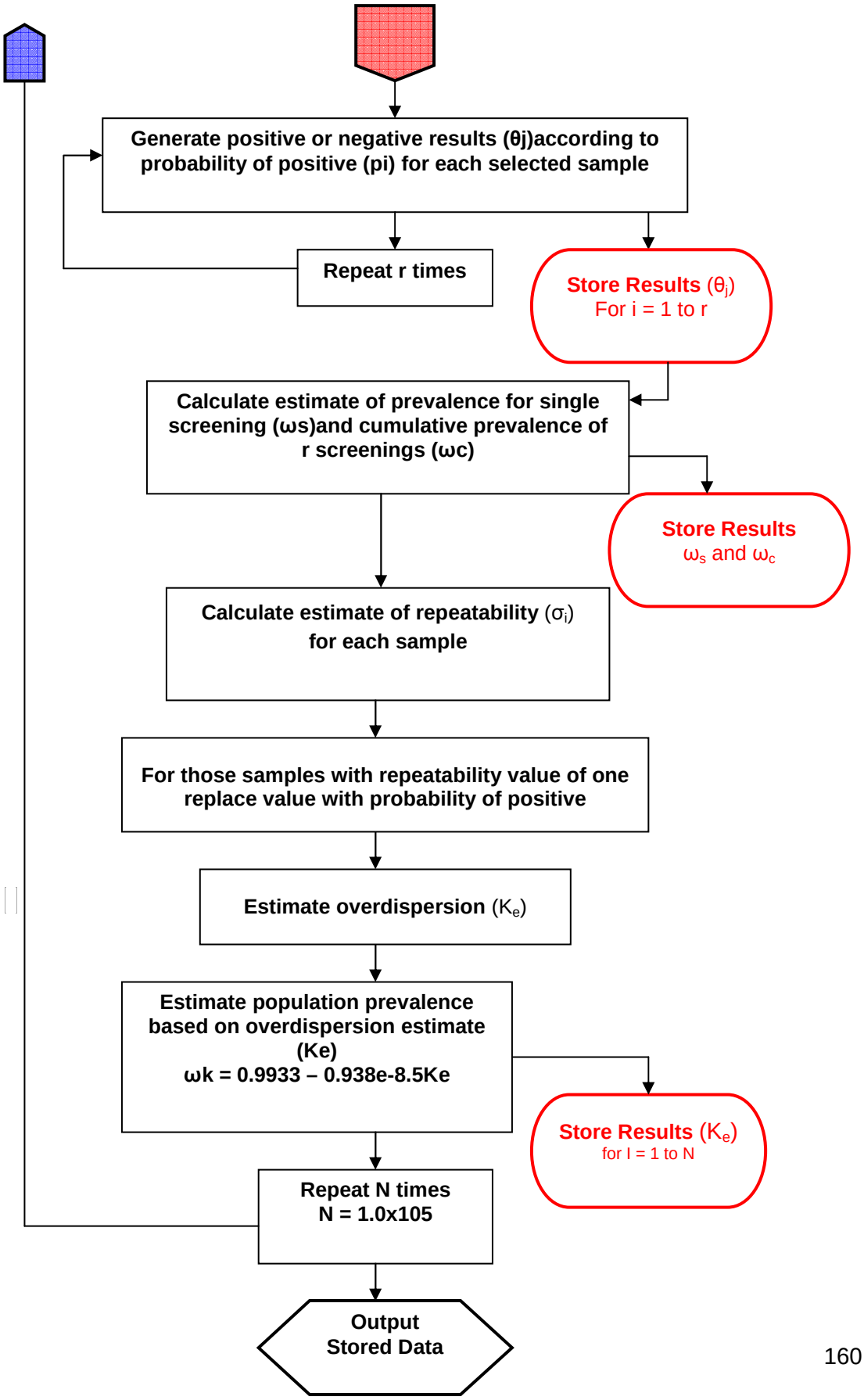



Generate screening results (θi) based on probability of finding positive (Pi)
Store Results
Repeat r times
Replace sample repeatability values (σi) of greater than one with infection intensity value (Ii).
Conduct statistical tests of data
P < 0.05 = Significant
Determine the probability of significant results in populations with differences greater than benchmark
Store Results


Repeat 100 times
Repeat 1000 times
Calculate mean probability and CI of significant result in populations with differences greater than benchmark values.
P = +/- 100
Prevalence = +/- 10%
Store Results
Output results

Appendix Figure 6.7. Simulation 3: Flowchart describing the design of a simulation to produce a data frame of estimates of population prevalence based on estimating overdispersion (K)




Generate positive or negative results (θj)according to probability of positive (pi) for each selected sample
Repeat r times
Store Results (θj) For i = 1 to r
Calculate estimate of prevalence for single screening (ωs)and cumulative prevalence of r screenings (ωc)
Store Results ωs and ωc
Calculate estimate of repeatability (σi) for each sample
For those samples with repeatability value of one replace value with probability of positive
Estimate overdispersion (Ke)
Estimate population prevalence based on overdispersion estimate (Ke) ωk = 0.9933 – 0.938e-8.5Ke
Store Results (Ke) for I = 1 to N
Repeat N times N = 1.0x105
Output Stored Data