Enhancing surveillance and quantifying impacts to improve our understanding of endemic anthrax in low resource settings

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BSc (Hons), MSc

Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy (PhD)

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February 2020

Abstract

Anthrax is endemic in many parts of Africa where livelihoods are dependent on animals, yet our understanding of the impact of the disease on people and livestock is limited. Advocating for the prioritization of anthrax requires such evidence. Surveillance – including case detection and diagnosis and subsequent disease reporting – is poor in endemic areas; as such, the incidence of anthrax is largely unknown. Resources are often limited in these affected areas, prompting the need for practical but efficient mechanisms for control. Control interventions leading to a significant reduction in the impacts of the disease will depend on strategies that target the determinants and drivers of anthrax in humans and livestock, many of which are unknown in endemic areas.

This multidisciplinary study was carried out in the Ngorongoro Conservation Area of northern Tanzania, an area hyperendemic for anthrax, with the following objectives: 1) Improve our understanding of the impact of anthrax by quantifying the occurrence of the disease in people and livestock and determining the economic losses due to livestock deaths through anthrax incident investigations and a cross-sectional questionnaire-based study in 209 households. Findings show that confirmed anthrax led to losses of over $28,000 in 36 households in six months, and upwards of $88,000 in reported livestock deaths from suspected anthrax over 2 years. Overall 42% and 23% of households living in perceived high-risk and low-risk areas respectively had experienced the disease in animals, with the highest number of those living in Ngoile, Olbalbal and Endulen administrative wards affected. Human anthrax had also been experienced by 19% and 16% of households in these high- and low-risk areas respectively. Communities had a high awareness of anthrax; however, under-reporting was pervasive and animal vaccination rates were poor, with several associated barriers identified. 2) Investigate community experiences and knowledge of anthrax and its management in livestock and people, in addition to the practices driving the risk of anthrax transmission, carried out through the household surveys and additional focus group discussions. Practices that drive the risk of contracting anthrax were related to the handling of suspected carcasses and the movement of livestock and their products. 3) Improve surveillance through animal anthrax confirmation in the field. To this end, the performance of a newly proposed

azure B stain for the rapid detection of *Bacillus anthracis'* capsule with smear stain microscopy was assessed on samples collected from suspect carcasses. The sensitivity and specificity of azure B was compared with those of polychrome methylene blue (the recommended standard for *B. anthracis*), Giemsa and Rapi-Diff stains – stains commonly used in veterinary laboratories – as well as polymerase chain reaction (PCR) on blood smear samples using Bayesian latent class analysis. The unavailability of blood smear samples led to a study to investigate the use of other sample materials, namely blood swabs, skin tissues, insects and whole blood for anthrax confirmation with PCR following storage at ambient temperature. The sensitivity and specificity of microscopy using azure B (91% and 100%) was comparable to PMB (92% and 100%). However, Giemsa and Rapi-Diff performed poorly in detecting *B. anthracis* capsule. Among the various samples tested, skin tissues were available for most carcasses, producing the second highest sensitivity and specificity with PCR after blood smears. In field conditions, the collection of blood smears (when available for microscopy and PCR) and tissue samples (for PCR) for the detection of *B. anthracis* can greatly improve the surveillance of anthrax in livestock. 4) Understand areas where livestock are at increased risk of contracting anthrax. Participatory mapping was conducted, taking advantage of local disease knowledge, and combined with environmental data in GIS to quantify the environmental conditions favourable to the persistence and transmission of anthrax in these areas. Findings show that perceived high-risk areas are closer to water sources and are characterised by lower organic carbon content compared to low-risk areas. High-risk areas identified occupied central locations within the NCA, increasing the likelihood of animals contracting anthrax during seasonal long-distance movements. Overall, the findings of this study are useful for improving the surveillance and control of anthrax in endemic areas.

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Acknowledgements

This PhD scholarship would not have been possible without the contribution of many individuals and organisations, to whom I owe gratitude. To begin, I want to thank my supervisors Dr Tiziana Lembo, Prof Ruth Zadoks, Dr Roman Biek, Dr Taya Forde and Dr Gabriel Shirima for their exceptional mentorship and support, throughout the duration of my study. I am especially fortunate to have this team of supervisors who through their tremendous experience and continued interest in my work and academic development provided the supervision required to achieve this thesis. Immense appreciation goes to the Bill and Melinda Gates Foundation for providing the funding for my scholarship through the Program for Enhancing the Health and Productivity of Livestock (PEHPL). This award not only provided funds for my fees, research and stipends, but it provided the opportunity to obtain relevant training that was valuable to my study and will be useful for my overall career progression.

I have received unwavering support here at the University of Glasgow and want to thank Profs Sarah Cleaveland and Neil Metcalfe, and Dr Jo Halliday for their useful comments, advice and suggestions on improving my overall study. To Dr Paul Johnson and Dr Luca Nelli, thank you for providing expertise with statistical problems. I am grateful to Dr Will de Glanville for introducing me to Open Data Kit and providing me with advice on implementing it, and to Dr Alicia Davies for her useful discussions on qualitative research analysis. I would also like to thank Mr Mike Shand, Honorary Research Fellow at the School of Geography and Earth Sciences for his help with producing the maps used in the field. I am grateful to Dr John Claxton for his help with managing the project here at Glasgow and for always providing help with administrative issues. Many thanks to Prof. Dan Haydon for your open-door policy and for always welcoming my random questions in need of authoritative answers. I am inspired by your leadership style.

Immense appreciation to Dr Matt Denwood of the University of Copenhagen, for his contributions to developing the Bayesian latent class model detailed in Chapters 4 and 5. His interest and enthusiasm to contribute his expertise was

unmistakable throughout the iterative analytical process. I am grateful to Prof Louise Matthews and Dr Richard Reeve for brokering this collaboration.

This thesis would not have been possible without the support of collaborators, individuals and organisations in Tanzania. I extend enormous gratitude to Prof Blandina Mmbaga, Dr Irene Kiwelu and all the great people at the Kilimanjaro Clinical Research Institute where all the laboratory work was carried out, for the support enjoyed throughout the study. At the Nelson Mandela African Institution of Science and Technology (NM-AIST) Tanzania where PEPHL was managed, I would like to thank Prof Joram Buza, Ms Rose Mosha, and Ms Dassa Nkini, for their great help with administrative and logistic matters relating to the project and fieldwork. I am lucky to be part of a cohort of 16 early career researchers funded through PEHPL to contribute to improving the health and productivity of livestock in Tanzania. Our yearly interactions at NM-AIST have been beneficial.

I am indebted to the field team in Tanzania including Mr Deogratius Mshanga, Mr Godwin Mshumba, Mr Sabore Ole Moko, Mr Sironga Nanjicho, and Mr Paulo Makutian for making my fieldwork a success. Without their sacrifice, patience and resilience, the work reported in this thesis would not have been possible. *Ashe naleng* (many thanks) to all the participants involved in the study for contributing to the valuable data that generated new information to improve the understanding of endemic anthrax. I also acknowledge the Ngorongoro Conservation Area Authority, Dr Nestory Mkenda and other medical personnel at Endulen Hospital, and Dr Victor Kakengi at Tanzania Wildlife Research Institute for supporting the research activities that led to this thesis.

I am grateful to Drs Suzanna Lewis and Andrew Simpson at Public Health England and Drs Henriette Van Heerden and Ayesha Hassim of the University of Pretoria for providing specialised advice, resources and training for anthrax diagnostics.

I am grateful for having friends who have encouraged me and provided help in many ways too numerous to mention. Special thanks to Rhoda Ocholi and Becca Bodenham. My family has been of tremendous influence. I appreciate the support and sacrifices my husband Godwin and son Barnabas (my encourager/*drama king*) have shown. They both have been my rock all through

this journey. I am especially grateful to my parents - Charles and Ruth Aminu - for laying a great foundation for me. Their encouragements, advice and prayers have tremendously benefited me. This rewarding journey would not have been possible without the help of the Almighty God. Having him as my Father (as simple as it may seem) is my most important achievement.

Author's Declaration

I declare that this thesis is my own composition and the work described in it is my own except where otherwise stated. Here and throughout the thesis, the relative contributions of others to the research are indicated where relevant.

Chapter 2: The two costs of neglect: evaluating the health and economic impacts of anthrax in an endemic area of northern Tanzania: Initial concept developed by O. R. Aminu, T. Lembo and T. Forde. Data collection was carried out by O. R. Aminu and D. Mshanga. Analysis was conducted by O. R Aminu with advice from R. Biek. The chapter was written and revised by O. R. Aminu, R. Biek, R. Zadoks, T. Lembo, and T. Forde reviewed and provided feedback on the chapter drafts.

Chapter 3: Understanding anthrax and factors associated with risks to livestock and humans in endemic settings: Initial concept developed by O. R. Aminu and T. Lembo. Data collection was carried out by O. R. Aminu and D. Mshanga. Analysis was conducted by O. R. Aminu with advice from P. Johnson, and A. Davies. The chapter was written by O. R. Aminu with T. Lembo and T. Forde reviewing and provided feedback on the chapter drafts.

Chapter 4: Assessing a modified staining technique for the detection of *Bacillus anthracis* in field samples from endemic areas: The study was designed by O. R. Aminu, T. Forde, R. Biek, R. Zadoks, and T. Lembo. Samples were collected by O.R. Aminu and a field team. Laboratory work was conducted by O. R. Aminu with supervision from T. Forde. The analysis was carried out by O. R. Aminu but the latent class model was co-developed with M. Denwood. O. R. Aminu wrote the chapter, which was reviewed by R. Zadoks, M. Denwood, T. Lembo, and T. Forde.

Chapter 5: Optimal animal sample materials to improve *Bacillus anthracis* surveillance in anthrax endemic areas: The initial concept of the study was developed by O. R. Aminu, T. Forde, and R. Zadoks provided technical advice on improving the study. Samples were collected by the field team and O.R. Aminu. Laboratory analysis was conducted by T. Forde and O. R. Aminu. The chapter

was written by O.R. Aminu and revised by R. Biek, R. Zadoks, T. Lembo, and T. Forde.

Chapter 6: Environmental predictors of anthrax in livestock in community-defined high-risk areas: The study was designed by O. R. Aminu, T. Lembo, and R. Biek. Data were collected by O.R. Aminu. Analysis was conducted by O.R. Aminu with advice from R. Biek, L. Nelli and P. Johnson. O. R Aminu wrote the chapter, which was reviewed by R. Biek, T. Lembo, and T. Forde.

The work described in this thesis has not been submitted at the University of Glasgow or elsewhere for the award of any other degree or qualification.

Definitions/Abbreviations

AFSIS	Africa Soil and Information Service
AIC	Akaike information criterion
AlHV-1	*Alcelaphine herpesvirus I*
ASSURED	Affordable, specific, sensitive, user-friendly, rapid and robust, equipment free, and deliverable
ATL buffer	Qiagen tissue lysis buffer
AUC	Area under the curve
B. anthracis	*Bacillus anthracis*
CAHW	Community animal health worker
CEC	Cation exchange capacity
COSTECH	Tanzanian Commission for Science and Technology
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
DOWS	Distance to inland water bodies
ELISA	Enzyme-linked immunosorbent assay
EVI	Average enhanced vegetation index
FAO	Food and Agricultural Organisation
FG	Focus groups
GAM	Generalised additive model
GDP	Gross domestic product
GIS	Geographic information system
GLM	Generalised linear model
HIV/AIDS	Human immunodeficiency virus/Acquired immunodeficiency syndrome
IDW	Inverse distance weighting
KCRI	Kilimanjaro Clinical Research Institute
LCA	Latent class analysis
LCM	Latent class model
LFO	Livestock field officer
LINKS	Livestock Information Network Knowledge System
LR	Likelihood ratio
LSTD	Average day time land surface temperature
MCF	Malignant catarrhal fever
MCMC	Markov chain Monte Carlo
NBS	Tanzania National Bureau of Statistics
NCA	Ngorongoro Conservation Area
NIMR	National Institute for Medical Research

NPV	Negative predictive value
NTC	No-template controls
NTD	Neglected tropical diseases
NZD	Neglected zoonotic diseases
ODK	Open Data Kit
OIE	World Organisation for Animal Health
OR	Odds ratio
PABAK	Prevalence- and bias-adjusted Kappa
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pH	Measure of alkalinity or acidity
PHE	Public Health England
PMB	Polychrome methylene blue
PPE	Personal protective equipment
ppm	Parts per million
PPV	Positive predictive value
QGIS	Quantum geographic information system
qPCR	Quantitative polymerase chain reaction
ROC	Receiver-operating characteristic
SOC	Predicted topsoil organic carbon content
TVLA	Tanzania Veterinary Laboratory Agency
TZS	Tanzanian shillings
URT	United Republic of Tanzania
USD	United States Dollar
UTM	Universal Transverse Mercator coordinate system
VIF	Variance inflation factor
WHO	World Health Organization
WHOCC	World Health Organization Collaborating Center for Remote Sensing and Geographic Information Systems for Public Health

Chapter 1

Chapter 1 General introduction

1.1 Neglected zoonotic diseases

Neglected tropical diseases (NTDs) is the term used to describe a group of diseases that are particular to marginalised and disadvantaged populations (Hotez and Kamath, 2009). These diseases have a higher prevalence in areas characterised by extreme poverty. In much of rural Africa and Asia, NTDs continue to impact on the health, wellbeing and economies of households and communities. Among the NTDs are a particular group of diseases that can be transmitted between people and animals, which are termed neglected zoonotic diseases (NZDs). This group of diseases has broad impacts because of their dual burden on the health of people and animals, as well as on livelihoods due to the deaths or decreased productivity of livestock these diseases cause (Molyneux, Hallaj and Keusch, 2011). In many places where NZDs are prevalent, people depend on animals for their livelihoods. Although NTDs have received greater attention over the last two decades (WHO, 2006; World Health Organization, 2010; World Bank, 2012), much progress can still be made towards directly achieving a number of the sustainable development goals by addressing diseases that affect people and their livestock, including the goals of 'no poverty', 'good health and wellbeing', and 'quality education' (Sachs, 2012). Studies have demonstrated the wider benefits that improved livestock health can have on poor households (Marsh *et al.*, 2016).

One such important neglected zoonosis is anthrax. In endemic areas such as in many parts of Africa, anthrax is often responsible for widespread mortality among a range of wildlife and livestock species (Muoria *et al.*, 2007; Gombe *et al.*, 2010; Lembo *et al.*, 2011; Hoffmann *et al.*, 2017). As a typical NZD, anthrax affects humans, both directly as a cause of morbidity and mortality, and indirectly through impacts on the health and productivity of their livestock. The latter economic losses are particularly important for communities and families that depend on livestock keeping or products for their livelihood, as in most of rural Africa (Food and Agricultural Organisation (FAO), 2019). Because of these losses, the World Health Organization (WHO) recognises the relationship

between anthrax and poverty (WHO, 2011), but estimates of the impacts of the disease on people's health and livelihoods in endemic areas are very limited.

1.1.1 Anthrax in animals and humans

The etiological agent of anthrax is the Gram-positive bacterium *Bacillus anthracis*. This rod-shaped, non-motile, spore-forming facultative anaerobe primarily affects herbivorous animals (Figure 1.1), although other warm-blooded species including carnivores and humans are susceptible to infection (Turnbull, 1998; Lembo *et al.*, 2011). Anthrax was historically one of the most important causes of mortality of livestock and wildlife worldwide, and it continues to have impacts on animal health in many parts of Africa, Asia and the middle East where it is still endemic (WHO, 2008; Gombe *et al.*, 2010). In addition, sporadic cases occur occasionally in parts of Europe, North America and Australia (Turnbull, 1998; Hugh-Jones, 1999; Anaraki *et al.*, 2008; Tran, 2015). Humans can become infected from direct contact with infected animal carcasses or indirectly through contact with contaminated animal products such as ingesting contaminated meat or handling contaminated animal skins (Turnbull, 1998; Mock and Fouet, 2001; Gombe *et al.*, 2010; Sitali *et al.*, 2017).

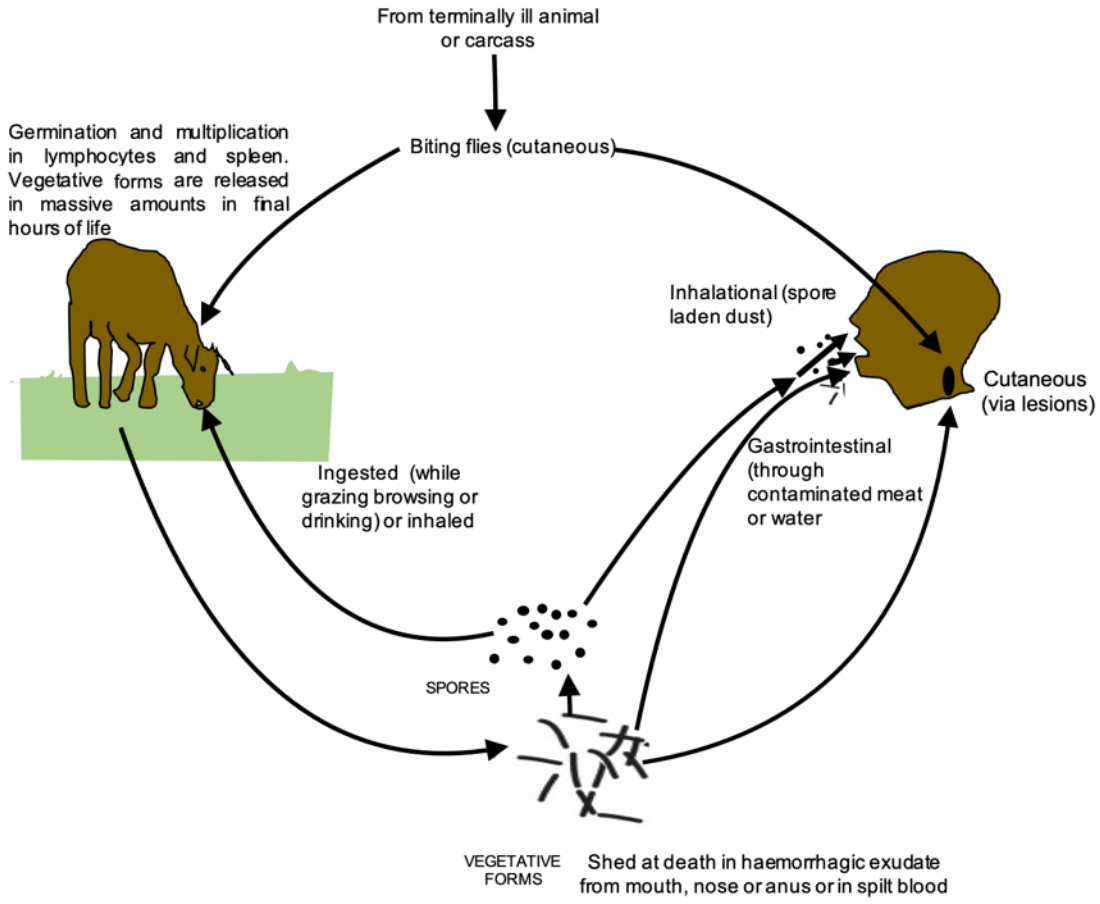


Figure 1.1: Anthrax transmission in humans and animals. Infection primarily occurs through the uptake of spores; however, vegetative forms may also play a role, for example in transmission through infected meat. Adapted with permission from Fig. 1 in Turnbull *et al.*, (2008).

Anthrax usually occurs once spores of *B. anthracis* are taken up internally by a suitable animal host, after which an immediate immune response is mounted. Macrophages engulf the spores and transport them to the lymphatic system. However, germination commences within the macrophages and infection progresses as they reach the lymphatic system, multiplying rapidly (Hanna and Ireland, 1999). Vegetative *B. anthracis* cells have a systematic ability to evade immune responses enabled by the bacterial capsule, allowing the pathogen to survive. Apart from the capsule which aids the pathogenicity of *B. anthracis*, the bacteria produce virulence toxins, namely lethal and oedema factors, which cause host cell damage and death (Mock and Fouet, 2001). Detection of the capsule and toxins of *B. anthracis* are among the primary bases for molecular and microscopy diagnostic testing. Eventual lysis of macrophages releases the vegetative bacteria into the blood stream where they continue to multiply,

expressing their virulence factors and leading to fatal septicaemia and toxaemia (Mock and Fouet, 2001; WHO, 2008). Observations suggest that a massive build-up of the pathogen in the bloodstream occurs as the disease progresses naturally to the final stages (Turnbull, 1998). This eventually promotes the release of potentially sporulating bacilli into the environment upon death of the animal, maintaining the cycle of persistence and infection. Vegetative forms of the pathogen are thought to contribute to infection, for example when transmission to humans occurs through the ingestion of contaminated meat (WHO, 2008). In humans, internal uptake of spores can result in gastrointestinal or inhalational anthrax, depending on the route of infection. Cutaneous anthrax develops differently as it is believed that spores are usually acquired through lesions on the skin, resulting in localised infection characterised by a black eschar and swelling. While gastrointestinal and inhalational anthrax are often fatal if undetected promptly, cutaneous anthrax usually resolves in several weeks. However, a proportion of cases can develop life-threatening sepsis or meningitis. Antibiotic treatment is generally effective in treating anthrax cases if administered early, and may also prevent the development of systemic complications (Turnbull, 1998).

Inhalational anthrax occurs when anthrax spores are breathed into the respiratory system, while gastrointestinal anthrax typically results from the ingestion of meat contaminated by anthrax spores. While more severe, these two forms of anthrax are less prevalent than cutaneous anthrax. Together they account for <5% of anthrax cases and have historical mortality rates of 89%-96% and >50% respectively (Swartz, 2001; Inglesby *et al.*, 2002). A study in Tanzania detected a 50% case-fatality in gastrointestinal anthrax patients (Lembo *et al.*, 2011). Cutaneous anthrax has a fatality rate of up to 20% if untreated, and accounts for >95% of reported cases (Swartz, 2001). With timely medical intervention, case-fatality rates decrease significantly; thus, the prevalence and case-fatality rates of anthrax might differ across settings. The three forms of anthrax described above (inhalational, gastrointestinal and cutaneous anthrax) are the usual forms of the disease in humans, (WHO, 2008), although a new form of the disease – injectional anthrax – has recently been described in drug users in Europe (Ringertz *et al.*, 2000; Hope *et al.*, 2012).

The higher prevalence of cutaneous anthrax may in fact be the product of underreporting of gastrointestinal and inhalational forms due to the nonspecific nature of their symptoms. Studies have shown that in an outbreak, more severe and fatal gastrointestinal anthrax cases may outnumber the cutaneous form of the disease. For example, outbreak investigations in Uganda and Thailand highlighted that gastrointestinal anthrax outnumbered cutaneous cases, accounting for 74% of confirmed cases (Sirisanthana and Brown, 2002). Underreporting is a particular problem in developing countries (Lembo *et al.*, 2011).

1.1.2 Prevention and treatment of anthrax

Anthrax is generally a preventable disease. In animals, vaccination against anthrax provides immunity against the disease for 8 – 12 months when administered in a timely manner (i.e. allowing sufficient time for immunity to develop) (Turner *et al.*, 1999; Ndiva Mongoh *et al.*, 2008). However, immunity is short-lived, and regular (yearly) vaccination is recommended. The WHO recommends vaccination for the whole livestock herd in the event of a case of anthrax occurring in an endemic area where vaccinations have not been routinely carried out (WHO, 2008). Although vaccination against anthrax is plagued with challenges – including the cost associated with yearly vaccination, mistrust related to adverse reactions, and the failure to develop immunity (Turner *et al.*, 1999) – it is regarded as the most effective strategy to control the disease in endemic areas. Although human vaccines have been developed, they are not available in most developing countries. Prevention of anthrax in humans thus depends on control in animals, as well as prevention of contact with suspected or confirmed cases and treatment when the disease occurs.

On a broader scale, public health promotion principles applied to the control of anthrax have the potential to yield effective results. One of the main principles of health promotion is enabling people to take control of their health or circumstances (WHO, 1986). People and communities affected by anthrax can be enabled to contribute to the control of anthrax through public interventions that empower them with information and services related to anthrax prevention and

control. For example, interventions that ensure vaccine availability, accessibility, and affordability as part of efforts to improve the willingness and ability to vaccinate animals could be developed and implemented. Others might aim to empower livestock keepers with knowledge of the actions to take in the event of an anthrax incidence or outbreak. However, in order to plan and implement such interventions, an understanding of local context of the disease, including existing gaps in surveillance and control, is required.

Understanding the reasons why diseases occur, including socio-cultural and environmental drivers, is one of the first steps to their control. Studies that aim to assess risk factors and experiences of a disease may improve our understanding of how optimal interventions might be developed. Many studies have been conducted in order to understand risk factors for anthrax occurrence in African countries (Opare *et al.*, 2000; Siamudaala *et al.*, 2006; Gombe *et al.*, 2010; Munang'andu *et al.*, 2012). However, few studies have investigated risk factors for anthrax occurrence in Tanzania. A recent study carried out in northern Tanzania (Mwakapeje, Høgset, Softic, *et al.*, 2018) found that practices such as laying on animal skins, and butchering or skinning infected carcasses were associated with infection in humans. However, the study did not investigate the underlying social determinants of these practices. These studies suggest educational and behavioural intervention approaches, but these are likely to be ineffective without addressing the underlying causes of risk factors. For example, telling people to vaccinate their animals without making vaccines available and accessible is unlikely to yield positive results. Strategies to reduce the occurrence of anthrax in animals and people (from surveillance to the implementation of control measures) will depend on the co-operation of communities most affected by the disease. Engaging with communities is crucial to identifying the factors that contribute to disease occurrence and persistence. It is also important for identifying sustainable control strategies (Halliday *et al.*, 2017).

If the prevention of anthrax is unsuccessful and the disease occurs, treatment of anthrax involves administering antibiotics. *B. anthracis* is susceptible to readily available broad-spectrum antibiotics such as penicillin and ciprofloxacin, and

natural resistance of the pathogen to antibiotics has rarely been documented (Turnbull *et al.*, 2004; Hang'ombe *et al.*, 2012). As the disease can be rapidly fatal, early treatment in animals showing symptoms of the disease, especially following an index case is recommended (WHO, 2008). Although most cases of cutaneous anthrax in humans are self-limiting, early treatment should be given to people suspected of having the disease to help prevent the development of potentially life-threatening complications. Timely treatment is particularly important in gastrointestinal and inhalational anthrax cases because infection can rapidly become systemic and fatal (WHO, 2008).

1.1.3 The ecology of Bacillus anthracis

The ecology of anthrax revolves around a rather complex relationship between the pathogen, its host(s) and the environment. Although infection occurs primarily through the uptake of spores (Figure 1.1), both forms are essential for the lifecycle of the pathogen and are highly dependent on prevailing environmental conditions (Hugh-Jones and Blackburn, 2009). The vegetative form of *B. anthracis* undergoes growth and reproduction usually in a suitable host or when conditions for growth are artificially induced. When conditions are unconducive to growth, sporulation occurs. In contrast to sensitive vegetative bacteria, *B. anthracis* spores are highly resistant to most environmental conditions, including ultraviolet radiation, and are able to persist for decades until conditions become favourable for growth, usually when taken up by a suitable host (Dragon and Rennie, 1995; Sinclair and Boone, 2008) (Figure 1.1).

B. anthracis has a well-studied relationship with the environment. While ecological aspects of anthrax are probably among the best studied components of the disease, several knowledge gaps remain, particularly related to its ecology in many endemic areas. Most ecological studies have been carried out in southern Africa (Smith *et al.*, 1999, 2000; Cizauskas *et al.*, 2014; Steenkamp, Van Heerden and van Schalkwyk, 2018), however, it is unclear whether their findings can be generalised to other endemic areas in Africa. Limited data on anthrax ecology exist for Nigeria, Chad, Cameroon and Tanzania (Hampson *et al.*, 2011; Blackburn *et al.*, 2015), and none for Kenya, Zambia, Zimbabwe,

Ghana, and Ethiopia where anthrax is considered endemic (Carlson *et al.*, 2019). *B. anthracis* spores persist in environmental conditions that favour spore survival, concentration and transmission. Alkaline and calcium rich soil, the action of wind, and the presence of insect vectors can influence the persistence and transmission of the disease (Van Ness, 1971; Dragon and Rennie, 1995; Hugh-Jones and De Vos, 2002; Hampson *et al.*, 2011). The 'incubator area' theory postulated by Van Ness argues "that under environmental conditions of high pH (alkalinity), high moisture, and the presence of organic matter, *B. anthracis* may undergo cycles of vegetative cell growth outside of a host, involving spore germination and then resporulation, which can cause an increase in spore numbers potentially leading to new anthrax outbreaks" (Van Ness, 1971). However, this hypothesis of spore cycling in the environment is controversial and little evidence has been found to support it. Experimental evidence demonstrates that *B. anthracis'* vegetative form requires specific physiological and nutrient conditions and survives poorly outside a suitable host or artificial media (Dragon and Rennie, 1995). The conditions, rather than promote spore cycling, more likely maintain high concentrations of viable spores readily available to infect a host (Hugh-Jones and Blackburn, 2009). Although a recent laboratory-based study shows that *B. anthracis* spores can germinate and replicate in amoebas (Dey *et al.*, 2012), in the natural environment, this would depend on the ecological suitability for the presence and growth of the amoeba species.

1.1.4 Global distribution of anthrax

Surveillance of anthrax is poor globally, particularly in endemic areas (WHO, 2008) and therefore the incidence of the disease is largely unknown. The map below (Figure 1.2) – produced by the World Health Organization Collaborating Center for Remote Sensing and Geographic Information Systems for Public Health (WHOCC) – illustrates the relative importance of anthrax by country. However, it was last updated more than fifteen years ago (in September 2003), and is based on very incomplete data. Anthrax is endemic in many places in Africa, Asia and the Middle East. However, sporadic cases occur in North America and Europe. It is estimated that over 63 million livestock keepers in poor and rural regions live

in areas with environmental conditions suitable for anthrax persistence (Carlson *et al.*, 2019).

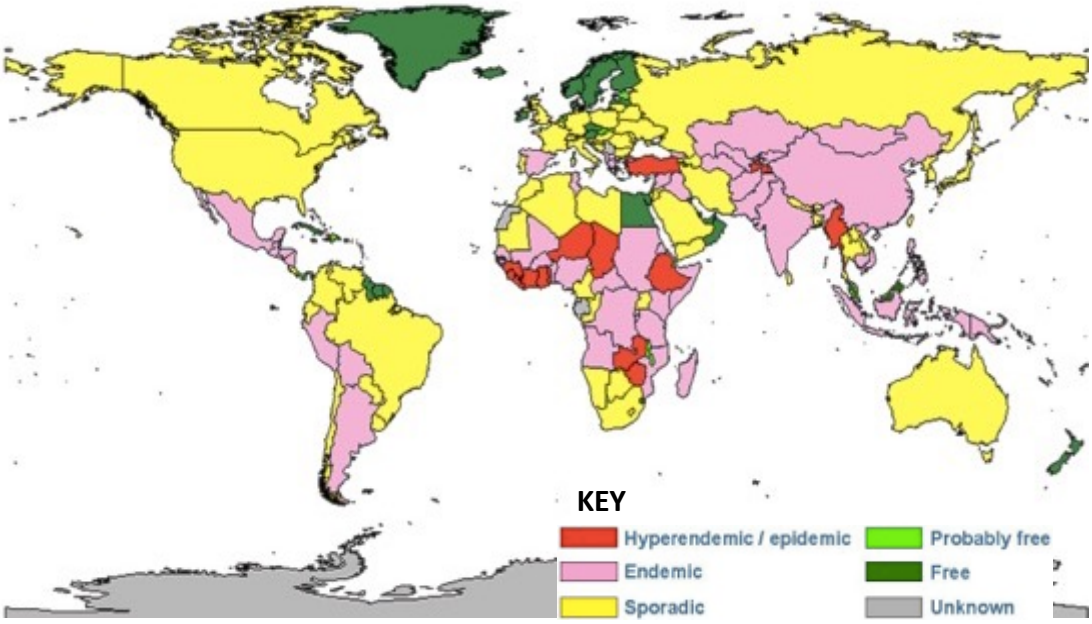


Figure 1.2: Global geographic distribution of anthrax. Reproduced from WHOCC.

1.2 Anthrax in endemic areas: the impacts and data challenge

Anthrax is one of the earliest diseases known to man. Notwithstanding the early identification of the disease, it is still poorly studied, understood and controlled, especially in many currently endemic areas. This means that the burden and impact of the disease is largely unknown. Except for the milder cutaneous form of the disease in humans, anthrax is often a fatal disease. Thus, associated mortality and morbidity may present a significant burden in endemic areas. In animals, death usually occurs suddenly (WHO, 2008). For poor people that depend on livestock for their livelihood, anthrax may affect both physical health directly and psychosocial health indirectly (Galaty, 1982).

Documents of the impact of anthrax in the 20th century, indicates that the disease has been responsible for devastating economic losses, for example the disease caused the death of an estimated one million sheep in Iran in 1945 (Goel, 2015). The disease is likely responsible for much greater economic losses than is publicly reported. Livestock is an important contributor to the GDP of many countries in Africa. Most households in Africa (80%) possess livestock either for income or subsistence purposes (Krätli *et al.*, 2013). In Tanzania, animal agriculture contributes significantly to the country's GDP, having the highest population of livestock in Africa after Sudan and Ethiopia (United Republic of Tanzania, 2015). Since anthrax is often a fatal disease in animals, livestock-owning households in highly affected areas are likely to suffer significant losses to their livelihoods. Although many studies have shown that anthrax commonly occurs in Africa (Opare *et al.*, 2000; Gombe *et al.*, 2010; Lembo *et al.*, 2011; Hang'ombe *et al.*, 2012), research studies have hardly quantified the impact of the disease on humans and livestock. These types of studies are needed to improve our understanding of the disease in highly affected areas.

1.3 Anthrax in endemic areas: the surveillance and control challenge

Anthrax control involves deliberate action to halt the natural occurrence of the disease. Effective surveillance is crucial to the control of anthrax. Surveillance can be defined as an ongoing systematic collection, collation and analysis of data and the dissemination of the information to those responsible for taking action to control the disease (Turnbull, 1998). Control of anthrax in endemic areas is impeded by ineffective core surveillance mechanisms like disease reporting, record keeping and diagnostic systems. In Tanzania for example, the current official surveillance system is passive and involves reporting of carcasses or suspected cases to health professionals in government offices, with, wherever possible, follow up blood sample collection and stained blood smear microscopy to detect the bacilli (Lembo *et al.*, 2011). However, case underreporting is considered widespread, especially in remote areas, due to limited communication between affected communities and the responsible professionals, among other factors (Lembo *et al.*, 2011). Underreporting limits epidemiological understanding of anthrax globally. For example, Carlson *et al.* (2019) observed that the paucity of data on anthrax incidence in areas considered highly endemic limited the ability to investigate the environmental suitability of *B. anthracis* in these areas.

The lack of data on anthrax cases contributes to its neglect, as understanding the occurrence of disease is important for planning and implementing management and control mechanisms. As with any disease, the control of anthrax requires cooperation among stakeholders responsible for animal health including affected livestock keepers, health professionals and policymakers. A lack of cooperation among these stakeholders can impede the implementation and effectiveness of control measures. For instance, in Africa - despite anthrax being a notifiable disease in many countries - underreporting is widespread (Allport *et al.*, 2005; Gombe *et al.*, 2010; Hang'ombe *et al.*, 2012). Underreporting, and by extension the lack of prioritization of anthrax, may also be due to the lack of awareness of disease reporting responsibility on the part of

stakeholders (including livestock keepers and health professionals), as it may be due to the lack of policy and control framework.

In Tanzania, most surveillance data on anthrax incidence in both humans and animals is based on clinical presentation (i.e. syndromic surveillance). In a study published in 2018, 80% of the data used to describe the epidemiology of anthrax in northern Tanzania was based on syndromic surveillance (Mwakapeje, Høgset, Fyumagwa, *et al.*, 2018). Anthrax case confirmation is dependent on the availability of appropriate diagnostic tests. Bacterial culture, polymerase chain reaction (PCR) and stain microscopy are established methods of detecting *B. anthracis* (WHO, 2008). Of these diagnostic options, stain microscopy is a rapid, simple and cheap technique, making it practicable in resource-poor areas. Thus, in most developing countries, anthrax can be rapidly confirmed by microscopic examination of a blood smear from a suspected animal carcass.

In the early 20th century M'Fadyean established stain microscopy for *B. anthracis* detection using polychrome methylene blue (PMB) (M'Fadyean, 1903b). This method was useful at the time for many countries including those that have now successfully controlled the disease. With anthrax brought under control in many developed countries, commercial quality-controlled PMB became difficult to obtain in many areas where the disease is still endemic (Owen *et al.*, 2013). Many laboratories in endemic areas may rely on poor quality formulations of the stain that can affect the ability to detect the pathogen accurately. Another challenge with the use of PMB is that the stain requires about 12 months to develop the biological property that makes it specific for *B. anthracis*. These drawbacks hamper the use of PMB in areas where anthrax is encountered frequently. Azure B has shown potential as an alternative stain (Owen *et al.*, 2013), however, until now it has not been tested on animal samples obtained directly from the field.

B. anthracis can also be detected by growing the pathogen in suitable media in the laboratory. However, diagnostic testing that does not necessitate multiplying the pathogen is desirable for several reasons. First, the pathogen is considered very dangerous and culture is required to be performed at laboratories higher

than Containment Level 2. Such facilities require extensive resources to set up and may not be available in low-income areas. Second, the pathogen is also classified as having the potential for misuse as a bioweapon (Sinclair and Boone, 2008). Not having to culture the pathogen minimises the risk of misuse. Thus, testing for *B. anthracis* using culture-free methods like microscopy or PCR is preferential.

PCR tests are generally thought to better (i.e. more sensitive and specific) for the detection of pathogens, including *B. anthracis,* compared to microscopy (Berg *et al.*, 2006). PCR techniques detect and amplify targeted fragments of DNA, such as genes specific for *B. anthracis*. The technique can also be useful as a second-line confirmation of anthrax or for detailed epidemiological studies including those that require bacterial typing. However, PCR is comparatively expensive and the necessary equipment may not be available in many endemic areas; appropriate facilities may only be available in a select number of laboratories in-country. In Tanzania for example, the closest laboratory to endemic areas in the Ngorongoro Conservation Area where anthrax has been shown to occur frequently (Lembo *et al.*, 2011) is not equipped for carrying out PCR, however, the facility exists in a laboratory at the Kilimanjaro Clinical Research Institute situated in a different region. Thus, although logistic and time constraints may mean that molecular diagnostics cannot be applied for the rapid confirmation of cases in order to make timely treatment/vaccination decisions in the event of an incident or outbreak, they may aid long term epidemiological understanding of the disease dynamics.

Because anthrax typically occurs in rural and challenging areas with limited infrastructure (for instance, lack of well-equipped laboratories, good network of roads), cold chain storage of samples prior to testing may present difficulties. Methods for detecting or studying *B. anthracis* that enable the use of samples without the requirement for cold chain storage will benefit the surveillance of the disease in these areas. Until now, no research has been conducted to test whether and what animal samples collected in field conditions and stored at ambient temperature can be used to improve the surveillance of anthrax.

In resource-poor settings, it is advantageous to prioritize disease control efforts to provide maximum value for the limited funds available. Although anthrax is believed to be a localised disease, often confined to specific geographical areas and having little potential for spread, studies in Africa have reported the spread of the disease to new regions previously free of the disease (Siamudaala *et al.*, 2006). As anthrax is soil borne and difficult to eradicate from the environment, factors that contribute to creating new disease foci could be addressed, to limit escalating the already existing impact of the disease. This type of spread is often facilitated by activities such as trade and nomadism. In addition to preventing the spread of anthrax, another advantage of identifying environmental risk factors is that areas of high-risk unknown due to underreporting, can be identified. Again, understanding the factors favouring anthrax occurrence and persistence, including human behaviour and environment conditions, will enable the design and implementation of relevant public health interventions.

1.4 The study area

This study was carried out in the Ngorongoro Conservation Area (NCA) of northern Tanzania (Figure 1.3). The NCA covers an area of 8,292km^2 and was inhabited by 70,084 people in 2012. It has an estimated population growth rate of 2.7% (National Bureau of Statistics (NBS), 2013). For administrative purposes, the NCA is also referred to as Ngorongoro division which is one of the three divisions in the Ngorongoro district council. The Ngorongoro district council and 6 other district councils make up the Arusha region.

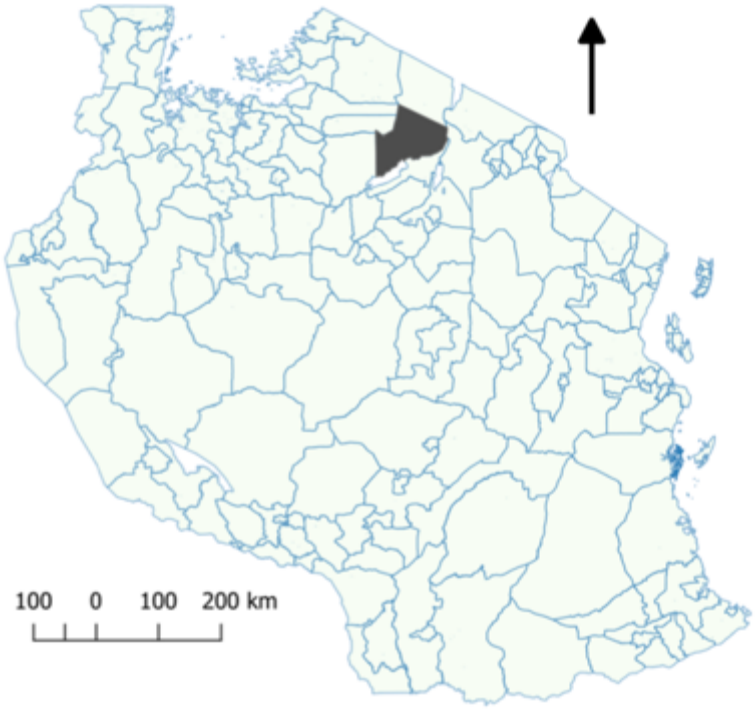


Figure 1.3: The study area within Tanzania, the Ngorongoro Conservation Area, shown in grey. It is a multiple land use area where people, livestock and wildlife live in close proximity, increasing the risk of anthrax transmission.

The NCA is a multiple-use area where people, wildlife and livestock co-exist. The major ethnic group in the study area is the Maasai who practise pastoralism with nomadism. Pastoralism is the practice of extensively rearing livestock. In the NCA, pastoralism is practiced with nomadic transhumance, which is characterised by seasonal long-distance movement of livestock in search of pastures and water (Galaty, 1982). The occurrence and persistence of zoonotic

diseases like anthrax depends on interactions between people and animals (World Health Organization, 2010), such as those that can be found in pastoralist communities.

The NCA typifies many rural settings in Africa and the risks and challenges to the control of neglected diseases for such areas. Some of the common characteristics of these settings include the remoteness of communities, the unavailability of well-developed infrastructure, and the co-existence of people and animals. Anthrax has been identified as a problem affecting communities in the NCA, however, this was based on limited opportunistic data (Lembo *et al.*, 2011).

1.5 Thesis outline

This is a thesis of interdisciplinary research work (Figure 1.4) carried out with the aim of improving the understanding and surveillance of endemic anthrax, specifically in the NCA. It presents unique data and new knowledge on the impact and context of the disease in the NCA, assesses the potential of a new diagnostic method for surveillance in the area, and identifies areas at high-risk of anthrax. The thesis has been presented in seven chapters, with original research and findings reported in Chapters 2-6.

In Chapter 1 the thesis is introduced, and a literature review of the subject area is presented. The objective of the work detailed in Chapter 2 was to assess the impact of anthrax in terms of livestock losses due to the disease on affected households in order to provide evidence for the need to prioritise the disease. In Chapter 3, a mixed-methods approach using both qualitative and quantitative methods was implemented to assess knowledge, perception and experiences of human and animal anthrax. This study also identified practices that may put people and livestock at risk of contracting anthrax.

In order to improve the surveillance of anthrax, a newly described staining technique (azure B) was assessed for use on samples obtained directly from the field and stained smears were assessed for their use in the molecular detection of *B. anthracis* using PCR (Chapter 4). In this study, the sensitivity and specificity of azure B along with other commonly used stains for the diagnosis of bacterial infections (polychrome methylene blue (PMB), Giemsa, Rapi-Diff) were quantified using a no-gold standard approach. For the study described in Chapter 5, the performance of various sample materials (i.e. skin tissues, whole blood, insects, blood swabs and smears), collected and stored under field conditions (i.e. ambient temperature) were assessed by quantifying the sensitivity and specificity of PCR for the different sample types. In Chapter 6, a participatory mapping approach combined with geographic information system (GIS) was applied to understand the environmental conditions suitable for the persistence of anthrax in high-risk areas of the NCA. The thesis is discussed and concluded in

Chapter 7 and recommendations for the improvement of anthrax control and for future studies are made.

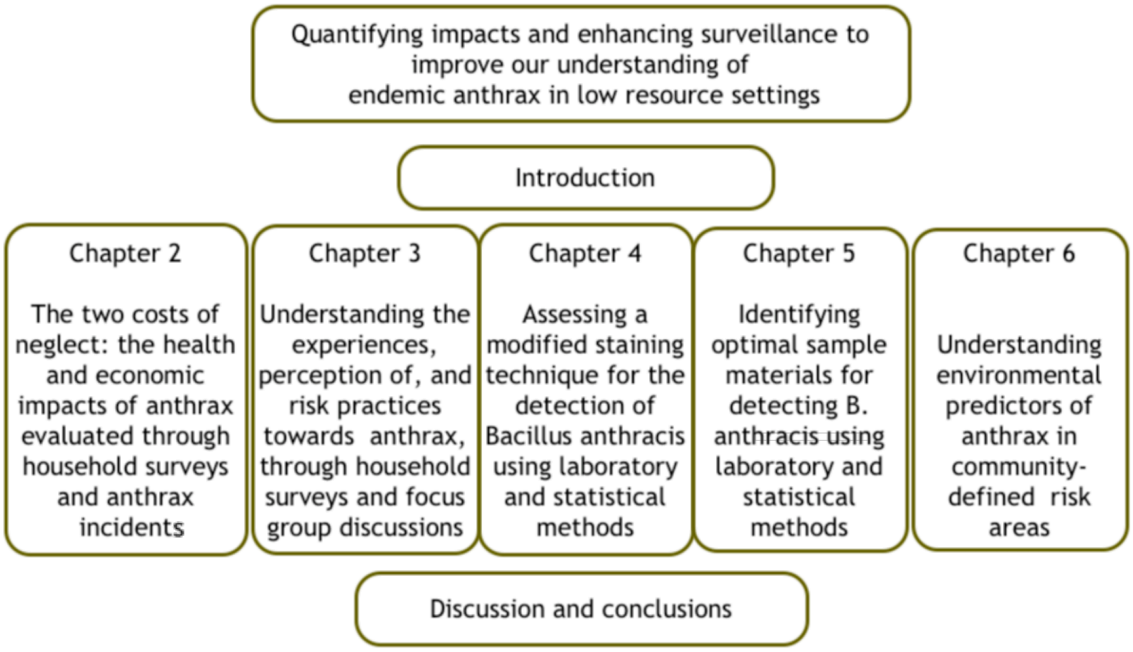


Figure 1.4: A summary outline of the thesis

Chapter 2

Chapter 2 The two costs of neglect: evaluating the health and economic impacts of anthrax in an endemic area of northern Tanzania

2.1 Introduction

Anthrax causes a serious disease in humans and animals. The primary hosts are herbivorous mammals, including livestock and wildlife. Humans are affected mainly as a result of spill-over infections from animals. In the global north anthrax occurs sporadically, producing a misconception of a low-priority disease requiring action mostly towards avoiding intentional release of the causative agent (Check, 2003). In the past decade, drug-related injectional anthrax cases in the UK has raised concerns about this new form of the disease in the developed world (Hanczaruk *et al.*, 2014). However, attention is largely away from the endemic nature of anthrax in Africa and other resource-poor settings because quality data and evidence demonstrating the scale of the impact of the disease in these contexts are lacking. Many of these areas are remote, with little or no infrastructure for health care, communication, etc. Many scientific studies conducted in Africa focus on ecological processes and are targeted towards the conservation of wildlife (Smith *et al.*, 1999; Leendertz *et al.*, 2006; Cizauskas *et al.*, 2014). Much less effort has been placed on understanding the disease in areas where humans and their livestock live in close proximity, as in most of rural Africa. In such areas, anthrax might be far from sporadic and can be responsible for widespread mortality and morbidity among a range of species (Hugh-Jones, 1999; Lembo *et al.*, 2011).

2.1.1 Anthrax: the impact of a neglected zoonotic disease

Anthrax produces indirect negative impacts on people's lives by affecting the health and productivity of their livestock. The latter economic losses are likely to be particularly important for communities and families that depend on livestock keeping or products for their livelihood. The occurrence of livestock diseases can make the difference between access to basic life necessities like education and healthcare. For example, a recent study in Kenya demonstrated

that control of East Coast Fever enabled access to education for girls (Marsh *et al.*, 2016). Disease impacts are unnecessarily borne by these communities because anthrax is preventable (WHO, 2008). However, without adequate data on the impacts of the disease, prioritization of the disease by relevant stakeholders – including policy makers as well as affected communities – may not be achieved for successful control strategies. The impact that NZDs like anthrax might have on the health and livelihoods of people solely depending on livestock remains largely unquantified, while contributing to perpetuate a circle of poverty for the people who bear the brunt.

Anthrax is known to be endemic in many areas in Africa, and reports of disease occurrence have been made in Kenya, Tanzania, Zimbabwe, Ghana, Namibia, South Africa, Nigeria, Chad, Cameroon and Ethiopia (Carlson *et al.*, 2019). However, studies showing the impact of the disease in these areas are limited. In Kenya, a study found that livestock keepers considered anthrax more important than foot and mouth disease, African animal trypanosomiasis, contagious bovine pleuropneumonia, and east coast fever (Nthiwa *et al.*, 2019), but no studies were found that quantified the cost associated with disease in livestock and people.

It is estimated that 80% of the population of humans in Africa own livestock (Krätli *et al.*, 2013). Tanzania has the third largest population of livestock in Africa after Ethiopia and Sudan. However, livestock contributes only 6% to its national gross domestic product (GDP) (Engida, *et al.*, 2012). This low contribution has been attributed to poor livestock growth and reproductive rates, as well as high mortality, among other factors. Apart from contribution to GDP, other benefits of livestock include contribution to food security, and income not accounted for in GDP estimates (United Republic of Tanzania, 2006). These benefits are particularly important for pastoralists, most of whom live in difficult environments and whose livelihoods depend on livestock. Since livestock is key to people's livelihoods, and a number of factors including disease can affect the health and productivity of livestock, it is important to understand the relative impact of such factors. Most of the livestock in the country is concentrated in northern Tanzania, and the Ngorongoro Conservation Area (NCA)

is a key area (Chapter 1, section 1.4). Evidence that anthrax occurs frequently in the NCA has been shown (Lembo *et al.*, 2011), however, no studies have quantified the occurrence of the disease in livestock and humans and the losses associated with it. Understanding the importance and impact of the disease may support proactive measures to prioritize and control the disease.

2.1.2 Objectives

The study described in this chapter was carried out to understand the occurrence of anthrax in people and animals and the losses associated with the disease in pastoralist communities where the disease is endemic in the NCA of northern Tanzania. Specific objectives were to

(1) Quantify the occurrence of anthrax in people and animals in the NCA

(2) Estimate the economic losses due to livestock mortality from anthrax

(3) Determine the implications for the livelihoods of the affected communities.

These objectives were achieved by carrying out a cross-sectional household survey and conducting investigations into suspected anthrax cases.

2.2 Methods

Participatory epidemiology approaches were combined with short-term data (obtained over 6 months) on livestock mortality incidences due to anthrax, for the following reasons. 1) Challenges associated with surveillance in resource-poor settings means that data from which mortality and associated impacts can be estimated is lacking. 2) When data is lacking, incidence studies are useful, however they are expensive and involve longitudinal approaches (Pearce, 2012) that may not be possible for a short term project. Participatory approaches in epidemiology enable affected populations to contribute to the understanding and appraisal of the importance and impact of disease, as well as to the design and implementation of appropriate control strategies (Catley *et al.*, 2012).

2.2.1 Data sources

Two sources of data are used in this chapter. The first set of data were obtained through a questionnaire-based household survey, administered using a cross-sectional design. The second set of data were derived from active investigations carried out to detect and confirm anthrax cases, including those not officially recorded. More details about the data sources are provided below. Data analyses were carried out in R 0.99.484 Software (RStudio Team, 2016) and maps produced in the QGIS 2.18.2-Las Palmas software (Petrella *et al.*, 2012) using data from NBS Tanzania (NBS, 2013).

Questionnaire-based household survey

To calculate the number of households to survey in order to identify the proportion of households affected by anthrax in the NCA at 95% confidence level with a maximum margin of error of 5%, an appropriate sample size was determined based on the number of households in the NCA. Based on information from the 2012 census in Tanzania, with a population of 70,084 and average household size of 4.8, 14 601 households live in the NCA. To estimate a household level prevalence (i.e. proportion of households with a history of anthrax) of 15% with 95% confidence

and 5% error, a sample size of 194 or more households would be required (Greiner and Sergeant, 2016).

To select wards and sub-villages from which households would be sampled, data obtained from 10 focus group discussions were utilized.

Focus groups: Focus group discussions with between 10 and 13 participants and including animal health professionals, community leaders and village or ward executive officers, were held at ward level – a ward is the largest administrative subunit in the NCA. Participants were identified in collaboration with village or ward executive officers who acted as gatekeepers and knew community members highly Informed about livestock matters. The animal health professionals are also known as extension officers and includes both livestock field officers (LFOs) and community animal health workers (CAHWs).

These officers engage in multiple roles but mainly function to provide livestock health services to the resident communities. This includes examining sick animals and administering treatments, vaccination, and advice, usually for a fee. Each ward had at least one extension officer, but the number varied from ward to ward. For instance, in Endulen, one LFO and two CAHWs served the area. All extension officers in each ward were invited to participate in the focus groups. Ward and village executive officers act as intermediary between the government and the communities and work with community leaders. Community leaders from every village were represented in the focus groups. Since participants were recruited based on their position in the community and were those considered to hold the most knowledge regarding animal health, more men than women were recruited. There were 113 participants in total with only 6% being women.

The focus group activities were attended by participants from all 11 administrative wards of the NCA (one exercise was held for two wards (previously one ward and only recently split into two in 2015), hence 10 focus groups), and wards across the area were ranked according to perceived anthrax risk.

In each ward, participants were asked to list the sub-villages comprising the ward and rank them according to their perceived level of risk. Participants discussed among themselves, agreed and gave a score to sub-villages in each ward depending on perceived level of risk. A Likert scale with scores from one to five (where 1 = very high risk, 2 = high risk, 3 = intermediate risk, 4 = low risk and 5= very low risk) was employed. Unanimous scores were recorded for each sub-village. This provided a representation of wards and sub-villages (the smallest administrative subunit in the NCA) at variable degrees of risk from which households were selected. Other data obtained during the focus groups include those on anthrax risk practices and maps of anthrax risk areas. These are reported in chapters 3 and 6. The five categories were reduced to 3 groups; very high- and high-risk sub-villages were combined into one category (high-risk), and very low- and low-risk sub-villages were combined into one group (low-risk). High-, intermediate- and low-risk sub-villages remained for further selection and a purposive sample of wards were formed that represented areas thought to be at high, intermediate and low risk for anthrax.

Selection of households: A purposive sampling strategy was applied to select the wards in which surveys would be held. Wards having only high- or low- risk sub-villages, as well as those that contained a mixture of high-, low- and intermediate-risk sub-villages were selected, resulting in the inclusion of seven wards (Figure 2.1).

A purposive selection of wards as opposed to random selection enabled the sample to be at least a quasi-representation of both high and low risk areas. In some cases, a purposive sample may be representative of the population if carried out in a systematic way as is done in this study (Van Hoeven *et al.*, 2015). This sampling strategy was carried out in order to obtain the most information within the constraints of time and the challenging environment.

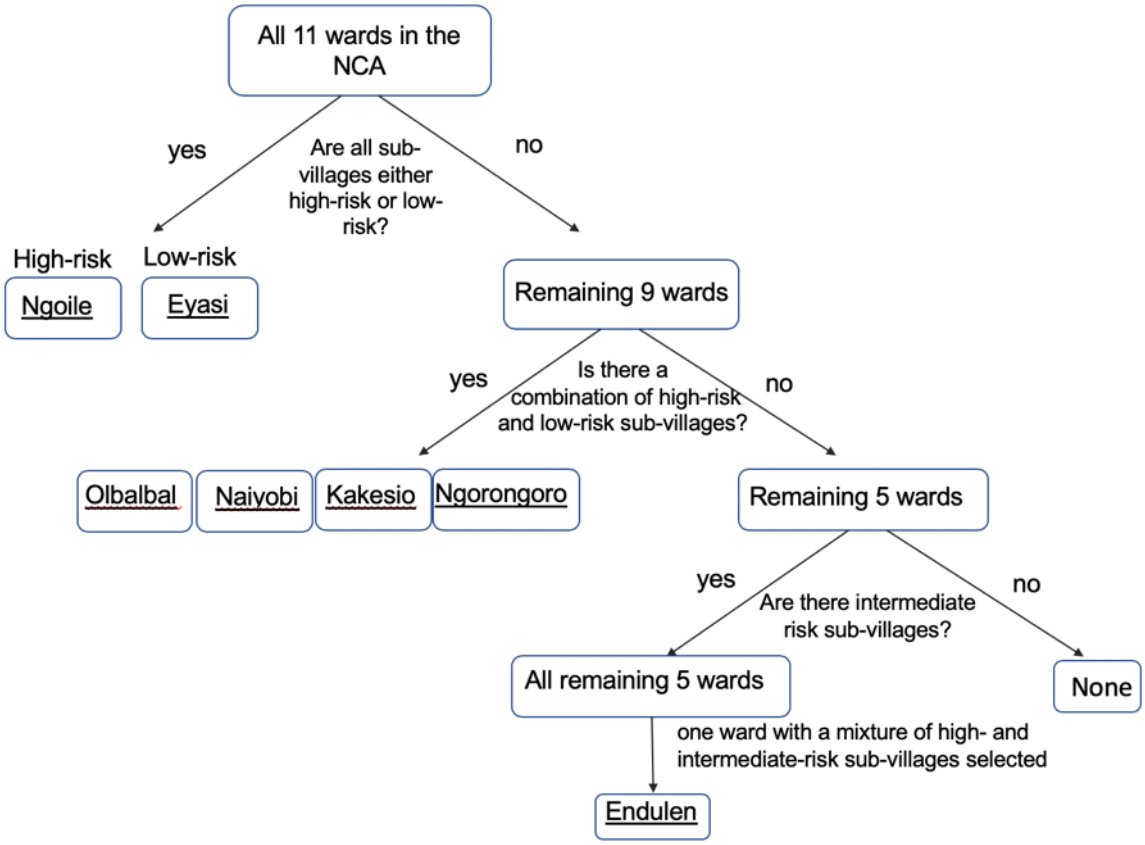


Figure 2.1: Wards (underlined) selected for inclusion in the household-level survey. Seven out of the eleven wards in the NCA were included.

A total of 22 sub-villages were selected from these seven wards (Figure 2.2). In wards that had a mixture of risk categories, high risk sub-villages were selected and matched one-to-one with lower risk sub-villages to capture information from both high and low risk areas. However, surveys were not possible in one sub-village due to remoteness and inaccessibility of the area, bringing the total to 21 sub-villages from seven wards of the eleven in the NCA. No other sub-village with the same risk-level (i.e. high) existed within the same ward, as such an intermediate-risk sub-village was chosen to replace its matched sub-village. Overall, ten high-risk, three intermediate-risk and eight low risk sub-villages were selected (n = 21). For each selected sub-village, lists of households were obtained from ward offices, village offices and sub-village chairmen depending on where the list was available. Households (n = 10) for administration of questionnaires were then selected randomly from these lists and a total of 210 households were selected. These stratifications, matching and random selection

processes were carried out to ensure that the sampled households provided data representative of different anthrax situations in the NCA. A household was defined as a social unit consisting of a head of the household, and all dependents who live together in the same dwelling. Culturally, in these pastoralist communities, this usually consists of a man, his wife/wives and their children. I piloted the questionnaire with a convenience sample of two households to test the feasibility of the study and the time required, as well as interpretation and cultural appropriateness of the questions in order to identify problems that might affect the implementation of the study.

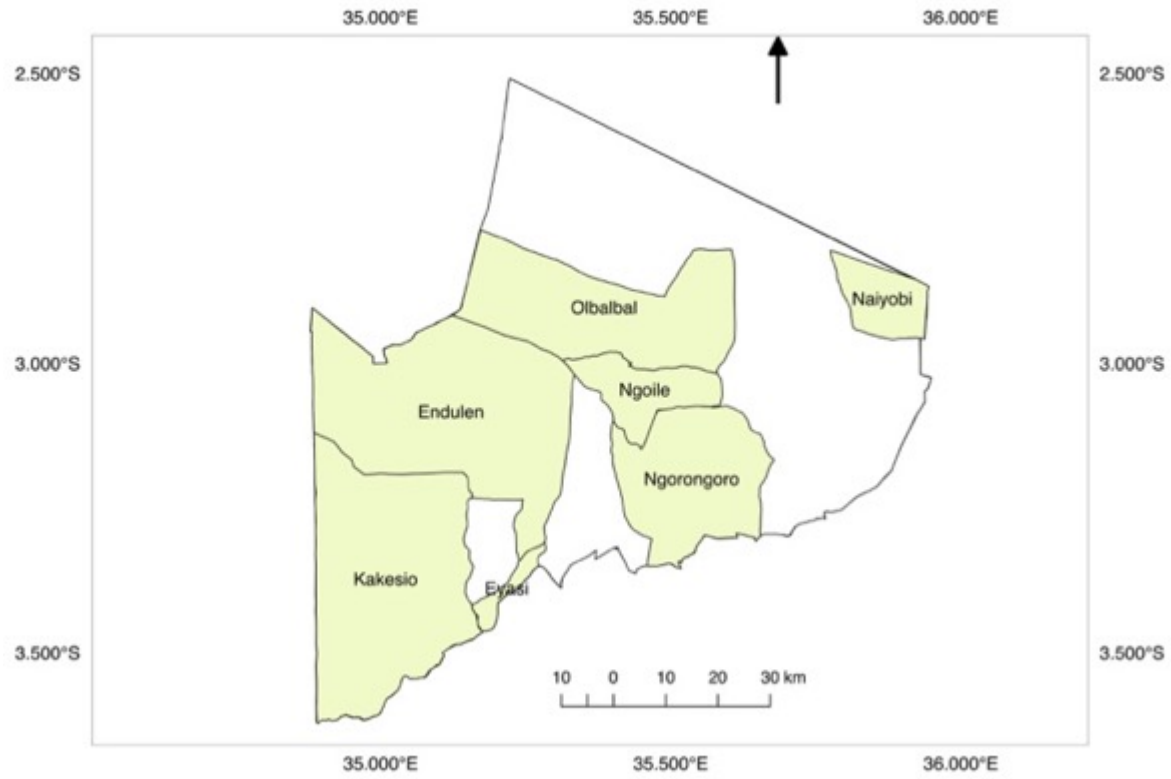


Figure 2.2: Parts of the Ngorongoro Conservation Area where the survey was carried out. The seven wards included in the survey which was conducted in 2016 are shown in yellow.

To understand the disease situation in the area, closed- and open-ended questions (see Appendix 2 for the full questionnaire) were asked about livestock (this included cattle, sheep and goats) management practices, including causes of mortality, perceived importance of livestock diseases, knowledge of anthrax and prevention of the disease, anthrax reporting and response, herd-level morbidity and mortality, and history of anthrax in the household/herd/village

with associated losses. More generic questions about household characteristics were also asked including household and livestock demographics, livestock use for subsistence and income generation, and other household income. The household surveys were initially targeted at respondents most knowledgeable about the livestock herd, and at an equal number of male and female respondents in order to eliminate gender-related bias in the responses while obtaining accurate and complete information (Fisher *et al.*, 2010). However, cultural factors meant heads of households were the choice of respondents in most households selected. Cultural norms prevalent in the NCA confers on heads of household (who are most often male) the power to make decisions relating to the household, and other members of the household are often very careful or cautious about sharing household information.

Data was collected on an electronic platform, Open Data Kit (ODK) (Hartung *et al.*, 2010). The form was built in Excel and converted to an XML format for the smart phone client, which uses the mobile platform ODK Collect to render the built form and enable user interaction. The storage server was used to upload, download, store and transfer data. Briefly, questions developed for the surveys (see Appendix 2) were transferred to the ODK platform Collect on a mobile device. The survey responses were entered onto the mobile electronic device and responses were then exported from the mobile devices in a comma separated file format for analysis. The surveys were conducted in a face to face interview either in Swahili or Maa language. I worked with a translator who was fluent in Swahili, Maa and English languages to record responses in the English. In all, 209 survey responses were obtained.

Figure 2.3: The survey process. Random selection of households in each sub-village (a), working with community leaders to identify households (b), locating very remote households (c) and interviewing a respondent (d).

Outbreak investigations

A total of 25 local animal health professionals including community animal health workers (CAHWs) and livestock field officers (LFOs) were trained to respond to reports of anthrax cases and outbreaks in the NCA and to collect samples, if available, for confirmation (see Figure 2.4). LFOs and CAHWs are paraprofessionals who deliver animal health services in rural areas. They can act as an interface between communities and official health and disease surveillance systems. Suspected cases of anthrax in animals were defined as the occurrence of sudden deaths in previously healthy-looking animals, with associated signs such as blood oozing from the natural orifices and the rapid decomposition of carcasses, characterised by swelling of the carcass. This training activity was part of strategies to improve the local capacity for surveillance of the disease. The identification of suspected anthrax cases in animals by the households and animal health professionals was based on the definition above. The officers received sample kits containing materials for sample collection and personal protective equipment (PPE). Each kit contained primary containers (plastic

tubes) for tissues, soil, insects, swabs, and blood, secondary containers (Ziplock bags), in addition to slides for blood smear samples, a disposable scalpel and a pair of forceps. The PPE included two sets of gloves, a face mask, over sleeves and cover boots, and chlorine release tablets (to be dissolved in water to obtain approximately 10000ppm chlorine solution) for decontamination.

In addition to sample collection, animal health professionals collected epidemiological metadata associated with the samples by filling an outbreak form (Appendix 4). The data collected included the location (GPS address) of the household affected, the species and number of animals affected, date, the number of animals in the herd (susceptible animals), symptoms associated with case(s), fate of the carcasses, information on any associated human cases, and information on previous suspected cases of anthrax in the past two years. Livestock officers were required to report back to the research team, and send samples and data collected to the local veterinary diagnostic lab in the NCA for storage prior to processing and analysis. The samples were tested, and details of the testing and the results have been documented in Chapters 4 and 5.

Figure 2.4: An animal health worker responding to a report of suspected anthrax in a sheep, fills out a form with epidemiological information and collects samples for testing.

Reports of suspected anthrax cases were obtained disproportionally from particular wards of the NCA. The active surveillance was maintained through

weekly follow-up phone call to the health professionals who had not reported any cases for follow-up. Only livestock officers in the most affected areas were actively responding to cases, specifically in the wards Olbalbal and Endulen. The livestock officer stationed in Olbalbal served both Ngoile and Olbalbal, as both wards are located in close proximity. The two wards were combined in the participatory mapping exercise as they had only recently been split (in 2015). Due to resources and logistical constraints such as the remoteness and difficult accessibility of many locations in the area, the investigations focused on wards that reported the highest cases of suspected anthrax. In order to improve response within the areas where cases occurred - as there were many more cases than the two livestock officers could attend to - a senior veterinary officer toured the NCA for a week every month to respond to cases occurring throughout the NCA. The investigations were carried out between August 2016 and March 2017 in response to reports of suspected anthrax cases for a total of 54 case reports.

Samples collected were tested in the laboratory at Kilimanjaro Clinical Research Institute (KCRI). Confirmation of an anthrax incident depended on it meeting one or both of the following criteria: 1- available sample testing positive for anthrax either by microscopy and/or quantitative polymerase chain reaction (qPCR) OR 2- the presence of at least one human pathognomonic case linked to the outbreak. For this purpose, a human case was defined as a person with a history of having come into contact with or consumed a suspected anthrax carcass and showing signs and symptoms of cutaneous anthrax lesions (Figure 2.5). Suspected human cases developing gastrointestinal or inhalational anthrax were not included in the definition, as the symptoms for these forms of the disease are non-specific. Sample processing methods for PCR and microscopy have been described elsewhere in this thesis in Chapters 4 and 5.

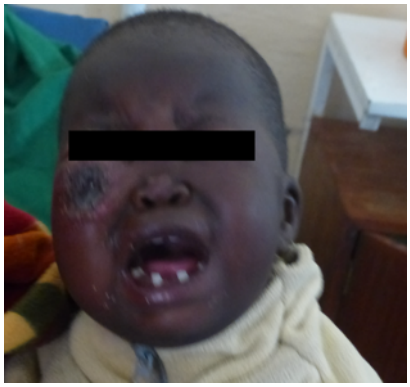


Figure 2.5: Cutaneous anthrax in a child. Photo is used with permission from the Clinical Officer in Endulen Hospital.

2.2.2 Estimating livestock losses

To estimate the minimum cost to livelihoods associated with anthrax, direct costs of livestock losses that survey respondents attributed to this disease were calculated, using information on livestock prices from the Livestock Information Network Knowledge System (LINKS) for Tanzania (http://www.lmistz.net/Pages/Public/Home.aspx) (Appendix 5). Where data were available (for current cases in the outbreak surveys), calculations were based on the local prices (i.e. LINKS) of the species affected. When species information could not be recalled, e.g. when households had lost a large number of animals over a two-year period, the average price of sheep was used. Sheep are considered the species most affected by anthrax and are the livestock of lowest value in the area (Chapter 3). This ensured that the estimates were conservative rather than overstated. The estimates of losses were computed in United States dollars (USD) using the exchange rate (average between 2015-2016) of 1USD to 2,157.75 Tanzanian shillings (TZS) (https://www.oanda.com/).

Logistic regression was used to test whether the probability of a household being affected by anthrax was associated with socio-economic characteristics obtained through the household surveys (Appendix 2). These characteristics include gender and age of the head of household, income, savings, and education. This was carried out in the R statistical program (RStudio Team, 2016).

2.2.3 Ethical considerations

The study received approval from the National Institute for Medical Research (NIMR), Tanzania, with Reference Number NIMRJHQ/R.8a/Vol. IX/2660; Tanzanian Commission for Science and Technology (COSTECH) number 2016-94-NA-2016-88; Kilimanjaro Clinical Research Institute; and the University of Glasgow College of Medical Veterinary and Life Sciences ethics committee (application number 200150152). Approval and permission to access communities and participants were also obtained from relevant local authorities. Verbal and/or written informed consent was obtained from all participants involved in the study (Chapter 1Appendix 1). Ethical approval obtained for the study included/permitted verbal consent in lieu of written consent where participants were unable to write. All data collected were analysed anonymously, ensuring the confidentiality of participants. The results of this study will be shared with relevant authorities including the NCA authority and NIMR who might utilise them to inform selection of diseases for prioritization of control, in line with the ethical principle of beneficence.

2.3 Results

2.3.1 Household demography and characteristics

A total of 209 out of 210 households participated in the surveys. One household was inaccessible. Overall, 98 households were interviewed in high-risk, 81 households in low-risk and 30 households in intermediate-risk areas. The characteristics of these households are summarized in Table 2.1 and Table 2.2.

Table 2.1: Characteristics of households surveyed

Variable	N=209	
Gender	**Head of Household**	**Respondent**
F	18 (8.6%)	65 (31.1%)
M	191 (91.4%)	144 (68.9%)
Age (years)	**Head of Household**	**Respondent**
19-34	38 (18.2%)	59 (28.2%)
35-54	99 (47.4%)	92 (44.0%)
55 and above	72 (34.5%)	58 (27.8%)
Education	**Head of household**	**Most educated member**
No formal education	129 (69.7%)	35 (16.8%)
Some primary school	12 (5.7%)	41 (19.6%)
Completed primary school	58 (27.8%)	99 (47.4%)
Completed secondary school	7 (3.4%)	26 (12.4%)
Completed post-secondary	3 (1.4%)	5 (2.4%)
Completed university	0 (0.0%)	3 (1.4%)
Tribe		
Datoga	18 (8.6%)	
Maasai	191 (91.4%)	
Main source of income		
Business	4 (1.9%)	
Sale of livestock	195 (93.3%)	
Sale of livestock products	2 (1.0%)	
Sale of crops	1 (0.5%)	
Employment	5 (2.4%)	
Remittances	2 (1.0%)	
Median household size (people)	**9**	
Median household number per compound	**2**	
Median number of persons per compound	**16**	

Table 2.2: Animal ownership of the households

Animal ownership	Number (percentage) of households with animals	Mean number per household (standard deviation)
Cattle	190 (91%)	25 (36)
Goats	196 (94%)	42 (47)
Sheep	171 (82%)	40 (77)
Compound animal ownership		
Cattle	190 (91%)	42 (47)
Goats	196 (94%)	43 (49)
Sheep	171 (82%)	75 (130)
Donkeys	179 (86%)	5 (5)
Dogs	186 (89%)	3 (2)
Cats	66 (32%)	2 (2)
Pigs	0 (0%)	0
Chickens	27 (13%)	10 (11)
Camels	1	8

2.3.2 Causes of mortality in livestock

Unexpected deaths among livestock had been experienced by many participating households. Overall, 70.8%, 81.3% and 68.9% of households reported cattle, goat and sheep deaths, respectively, in the 12 months preceding the survey. The causes of these deaths are outlined in Table 2.3 below.

Table 2.3: Perceived causes of mortality in livestock in the Ngorongoro Conservation Area in the 12 months preceding the study. Data were obtained from surveys conducted in 209 households in the area.

	Cause of mortality			
	Disease	Drought	Predation	Others[Y]
Mean number of cattle deaths per household (total number of affected households)	8 (n=119)	14 (n=50)	3 (n=33)	12 (n=7)
Mean number of goat deaths per household (total number of affected households)	14 (n=140)	7 (n=14)	7 (n=49)	7 (n=3)
Mean number sheep deaths per household (total number of affected households)	15 (n=110)	4 (n=13)	6 (n=34)	3 (n=3)

[Y] –From poisoning, flooding and trauma

2.3.3 The perception of anthrax importance

Perceived importance of anthrax was assessed relative to five other selected diseases common in livestock in northern Tanzania (Matthew, *et al.*, 2016) and in the study area, namely black quarter, brucellosis, East Coast Fever, foot-and-mouth disease, and Rift Valley fever. Participants (n=134) listed the diseases according to their perceived importance (see Figure 2.6) and stated the reasons for their choices. In high-risk areas, 58.6% (41/70) of households considered anthrax the most important disease for livestock. This proportion was 61.5% (8/13) in intermediate risk areas and 31.4% (16/50) in low-risk areas. Overall, 48.5% of the households able to assesses the importance of anthrax (n=134) considered it the most important disease for livestock out of the six diseases. The most common reasons for their considering anthrax important were the number of livestock affected in the household herd and in other herds in the area (i.e. the number of livestock deaths caused) (24.6%) and the perceived threat posed to human health by the disease (9.7%). This was followed by the lack of availability of treatment options in the event of disease occurrence (5.2%).

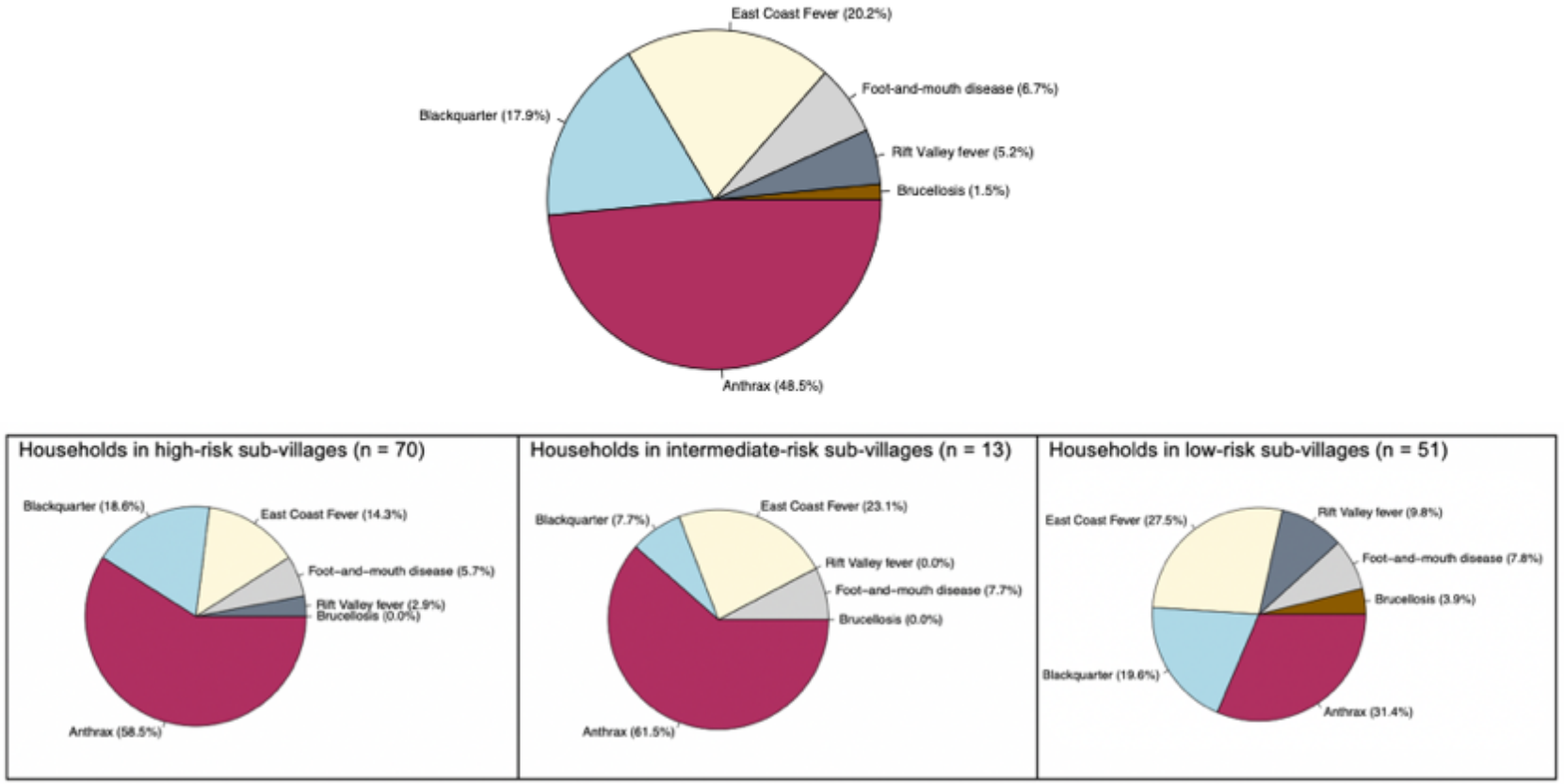


Figure 2.6: Perceived level of importance of six selected livestock diseases in the Ngorongoro Conservation Area, northern Tanzania

The importance of different diseases varied across ward administrative divisions, with anthrax considered very important in certain locations compared to others. Figure 2.7 illustrates the extent of geographic variability in the proportion of people interviewed who considered anthrax the most important disease for livestock. For example, it was never reported as the most important disease by people living in Eyasi, whereas 81% of respondents living in Olbalbal ranked it as the livestock disease of greatest importance.

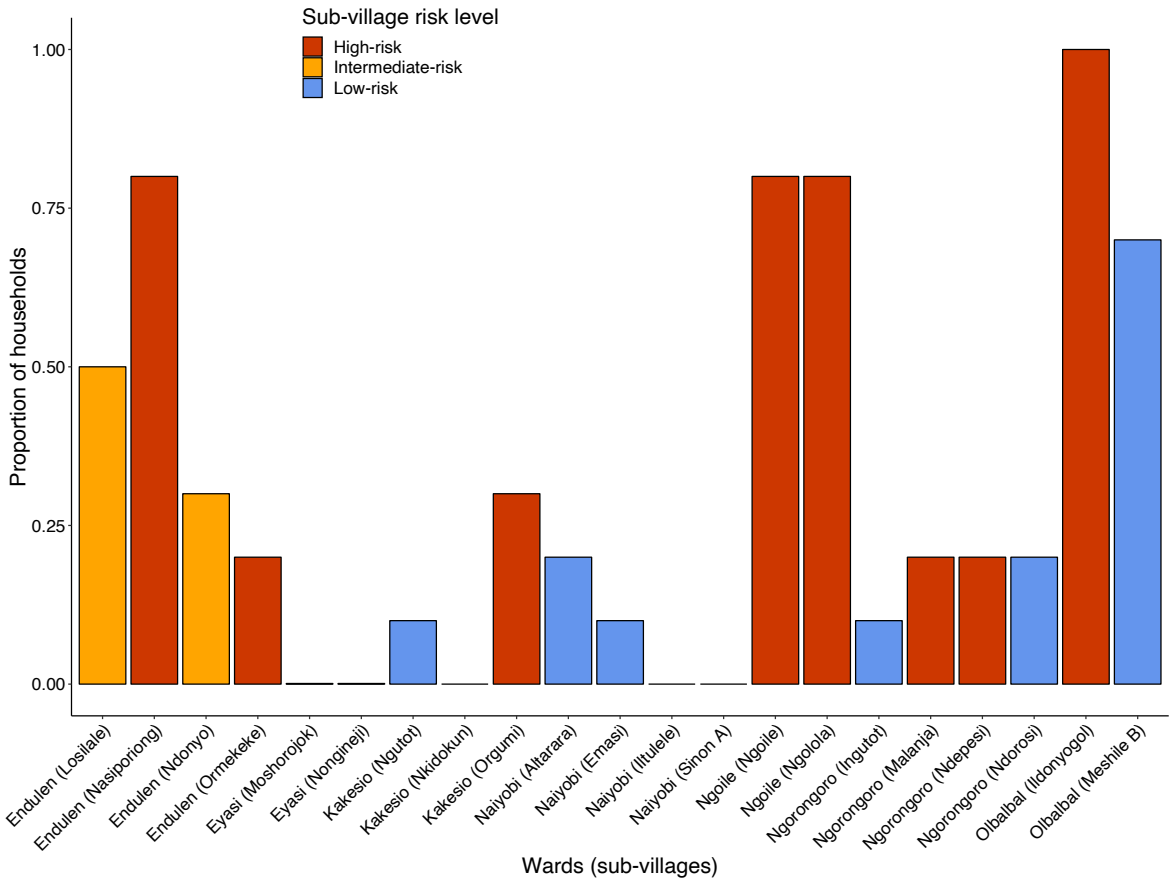


Figure 2.7: Proportion of households in each ward (and sub-villages) who ranked anthrax as the most important disease for livestock. All households interviewed in Eyasi lived in low-risk areas, while all those in Ngoile lived in high-risk areas. However, households living in low-risk areas in other wards also considered anthrax an important disease.

2.3.4 The occurrence of anthrax in livestock and people

Households that were aware of the disease reported cases of suspected anthrax in their herd and in family members based on the syndromic presentation described in 2.2.1.

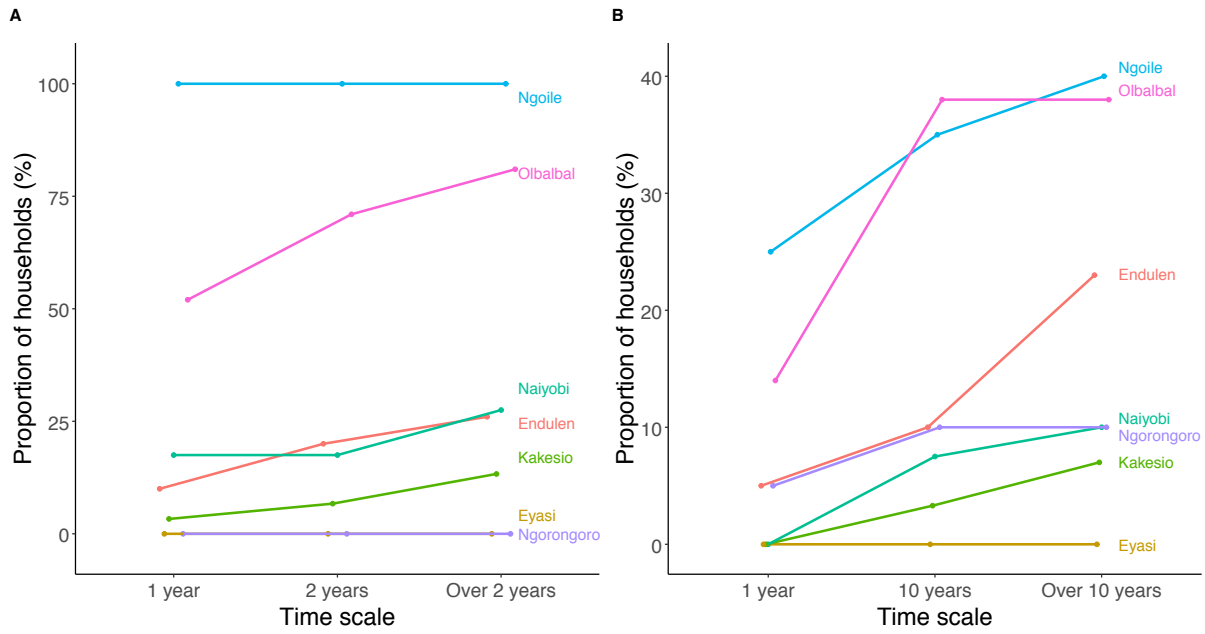


Figure 2.8: Proportion of households in the Ngorongoro Conservation Area, northern Tanzania, reporting cases of suspected anthrax in their (A) herds and (B) family members across different timescales in different wards. Data were obtained from a cross-sectional survey of 209 households in 2016.

The proportion of households reporting previous cases of anthrax in their livestock was 30.4% ($CI_{95\%}$ [24.2%, 36.7%]). About 25% ($CI_{95\%}$ [11.3%, 38.4%]) of households reported experiencing animal anthrax in the 2 years prior to the study and 20.6% ($CI_{95\%}$ [7.9%, 33.3%]) in the 12-month period preceding the study. The proportion of households with experience of the disease ranged from 0% to 100% if estimation was carried out for each ward separately. Proportions of households reporting anthrax in each category of risk are detailed in Table 2.4.

Overall, 100% of households in Ngoile, 85.7% in Olbalbal and 25.6% in Endulen wards reported past cases of anthrax in their livestock. In contrast, no households in Eyasi and Ngorongoro reported the disease in their livestock (Figure 2.8). Anthrax in humans was also common, with 16.7% ($CI_{95\%}$ [11.7%, 21.9%]), of households reporting at least one case of anthrax among family members. In the 12 months preceding the study, 5.7% ($CI_{95\%}$ [2.5%, 8.9%]) of households had experienced human anthrax. The proportion of households

reporting a history of anthrax in people was as high as 40.0% ($CI_{95\%}$ [18.5%, 61.5%]) in certain wards (Figure 2.8), with 3.8% reporting at least 2 cases and 1% reporting at least 3 cases. Four households interviewed (1.9%) reported the death of a family member due to anthrax.

Table 2.4: Proportion of households in high-and low-risk areas reporting previous anthrax incidents

	Risk category	**Time Scale**		
		12months	**2 years**	**Over 2 years**
Animals	High-risk areas	34.7% ($CI_{95\%}$ [25.3%, 44.1%])	36.7% ($CI_{95\%}$ [27.2%, 46.3%])	41.8% ($CI_{95\%}$ [32.1%, 51.6%])
	Low-risk areas	8.6% ($CI_{95\%}$ [2.5%, 14.8%])	16.0% ($CI_{95\%}$ [8.0%, 24.1%])	22.5% ($CI_{95\%}$ [13.3%, 31.7%])
		12months	**10 years**	**Over 10 years**
People	High-risk areas	8.2% ($CI_{95\%}$ [2.7%, 13.6%])	16.3% ($CI_{95\%}$ [9.0%, 23.6%])	19.1% ($CI_{95\%}$ [11.6%, 27.2%])
	Low-risk areas	3.7% ($CI_{95\%}$ [0.0%, 7.8%])	13.6% ($CI_{95\%}$ [6.1%, 21.1%])	16.0% ($CI_{95\%}$ [8.0%, 24.1%])

2.3.5 The cost of livestock losses due to anthrax

Household income and subsistence

The surveys showed that there is a high reliance on livestock for livelihoods in the NCA. Food produced by livestock or purchased using income generated from the sale of livestock and/or their products, form the basis of nourishment for these communities. In addition, many household products and goods required for daily living are sourced from animals (Figure 2.9). For example, cow, goat and

sheep hides are used as mattresses and mats for sitting and lying; hides are locally used to make clothing, shoes and belts; animal fat is used for cooking and making products for personal hygiene such as pomades; and houses are built from hides, mud and animal dung.

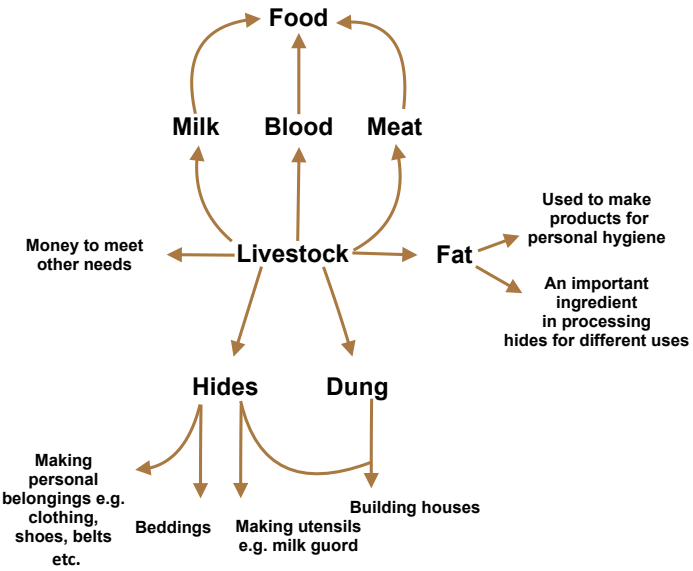


Figure 2.9: The multiple uses of livestock and their products in communities of the Ngorongoro Conservation Area. The figure is based on responses obtained from household surveys investigating anthrax in livestock and humans in 2016.

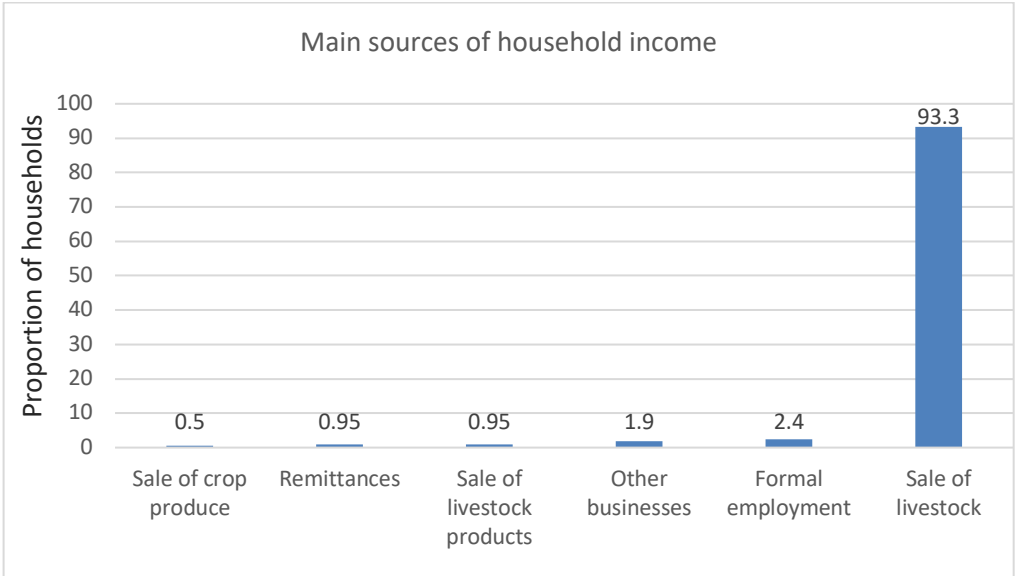


Figure 2.10: Major sources of household income. The plot shows the proportion of households reporting the main sources of income for household subsistence. Data were obtained from 209 households, through a

cross-sectional study carried out between July and October 2016 in the Ngorongoro Conservation Area.

Apart from the many different uses of livestock products in these communities, 94.3% of households reported that their main source of income for other purposes, such as for healthcare and education, was the sale of livestock or their products (Figure 2.10), illustrating the level of dependence on livestock in the area. Only 17.7% of respondents were unaware of the amount of income generated for household use in a month. The 82.3% (n=172) of respondents knowledgeable about household income reported a monthly income of 400,000 TZS or less, equivalent to 185 USD.

Anthrax-associated losses estimated from household surveys

Anthrax occurrence in animals in some wards (i.e. Ngoile and Olbalbal) was so commonplace that it was difficult for certain households to keep track of the affected species or the number of animals dying from the disease in a 12- or 24-month period. The estimates reported in the following paragraph are based on information from 55 households, which represents 86% of the households reporting a history of anthrax in the herd. The remaining 14% of households had difficulty recalling the losses experienced.

Households experiencing the highest disease incidence reported that there were up to three suspected cases of anthrax every week in the household herd during the dry season which is the peak season of the disease. Over the 2-year period, from mid-2014 to mid-2016, households affected by anthrax reported a median number of 10.5 animals lost to suspected anthrax. Moreover, 27.4% of households (15/55) reported the loss of over 100 animals due to the disease and 9.1% (5/55) reported a loss of over 200 animals (Figure 2.11A).

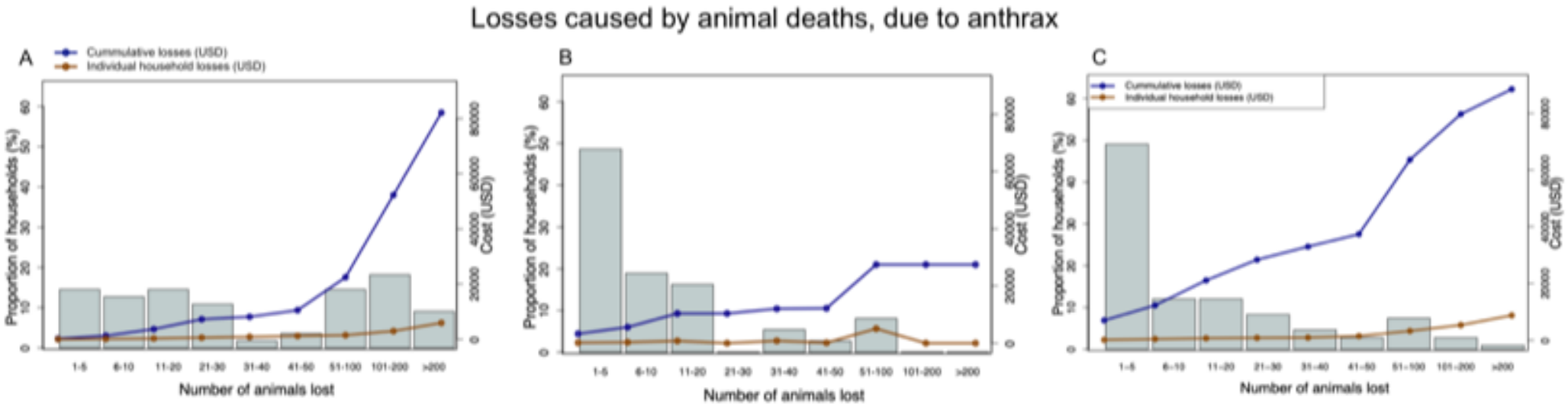


Figure 2.11: Losses caused by suspected and confirmed anthrax in the Ngorongoro Conservation Area; Losses were estimated from A) reported deaths in high risk areas between 2014 and 2016, data was obtained from 55 households through a survey and were based on the price of sheep; B) confirmed anthrax deaths and C) reported and confirmed anthrax deaths. Data for B and C are based on confirmed animal anthrax incidents in 36 households, investigated between August 2016 and March 2017, however, C includes reported but unconfirmed past deaths from the same herds occurring during the two years prior to the incidents. Estimates are based on the local prices of each species affected, when the data is available. For cases in which species information is unavailable, estimates are based on the price of sheep (29 USD). The individual household losses are the average losses per household in each category and the cumulative losses are losses in each category and those in prior categories. Cost estimates are based an exchange rate (average between 2016 and 2017) of 1USD to 2249.54 TZS.

In 2015 and 2016, the average price of a sheep was TZS 64,348.42, the equivalent of USD 29.82. The estimated minimum cost of losing livestock to anthrax ranged between USD 29.82 and USD 5,964 per household. The total losses estimated from reported deaths due to anthrax for households recalling at least one suspected case in the preceding two years (n = 55), amounted to USD 82,094.

Anthrax-associated losses estimated from case and outbreak investigations

Fifty-four investigations were conducted between August 2016 and March 2017 in response to reports of suspected anthrax cases. The 54 investigations were carried out mostly in response to reports from the two wards where investigations were focused (Endulen, n = 24 and Olbabal, n = 17). Limited investigations were also carried out in three additional wards (Esere, n = 3; Kaksesio, n = 2; and Ngoile, n = 8).

Additional data were collected from 104 past incidents through the outbreak investigation surveys. A total of 2455 cattle, sheep and goats were reportedly lost in these 158 incidents (current and past incidents), each lasting between a day and four months. In total, 44.4% of the investigations were carried out in Endulen, 31.5% in Olbalbal, 14.5% in Ngoile, 5.6% in Esere, and 3.7% in Kakesio. Of these outbreaks, confirmation of anthrax as the cause, was made for 37 of the 54 investigations (69% of current cases). Confirmation was based on molecular diagnosis using PCR (n=25), microscopy (n = 1), or the occurrence of at least one associated pathognomonic human case (n = 11). Only 18 samples from the 54 investigations tested negative by PCR and microscopy and were not associated with any human case. A total of 506 livestock were lost in the 37 confirmed outbreaks (Table 2.5).

Table 2.5: Cost (in USD) of livestock losses associated with 37 confirmed anthrax incidents between August 2016 and March 2017 in the Ngorongoro Conservation Area.

Livestock species (number of outbreaks involved)	Number of animals lost	Median number of livestock lost per outbreak (range)	Median proportion of herd lost per outbreak (range)	Local unit value (USD)	Total value (USD)
Cattle (n=9)	58	2(1-35)	1.3% (0.2 - 11.7%)	250	15,022
Sheep (n= 30)	411	6 (1-80)	1.6% (0.2 – 28.0%)	29	11,919
Goats (n= 7)	37	2 (1-20)	4% (0.5 - 23.3%)	31	1,147

Cattle losses occurred in nine incidents/outbreaks, while goats and sheep losses occurred in seven and 30 incidents respectively (Table 2.6).

Table 2.6: Species affected in confirmed anthrax incidents. In 15 of the 37 incidents, humans were affected and in one incident, only a human was affected with no associated incidents noted in livestock

Species involved	Number of incidents/outbreaks
Cattle only	6
Sheep only	21
Goats only	0
Cattle and sheep	2
Sheep and goats	6
Cattle, sheep and goats	1
Cattle and goats	0
Humans only	1
Humans and animals	14

The total number of sheep lost was more than 7-fold that of cattle and goats. The median loss experienced by a household was 250 USD per outbreak. The middle 50% of losses (25 -75 percentile) fell between 125 USD and 509 USD. The maximum loss experienced by a household was 9910 USD. The total losses

experienced by households in the confirmed incidents were 28,100 USD. When adding reported losses in the same herds during past incidents between 2015 and 2017, the total amount was 88,500 USD.

Table 2.7: Estimates of the losses caused by anthrax

Data source	Time period	Estimated total losses (USD)
Confirmed cases and outbreaks (n=37 households)	8 months	28,100
Confirmed cases and outbreaks with reported cases from the same herd two years prior to the study (n=37 households)	2 years	88,500
Household survey (n=55 households)	2 years	82,094

No significant difference was found in any of the socioeconomic characteristics assessed (Table 2.8).

Table 2.8: Results of simple logistic regression to identify whether households with reported anthrax have different socio-economic characteristics compared to those without anthrax.

Variable		Odds ratio (95% confidence interval)
Gender (head of household)	Female	1
	Male	0.98 (0.35 - 2.76)
Age (head of household)	<35 year	1
	35-54 years	1.53 (0.67 - 3.52)
	Over 54	1.76 (0.74 - 4.19)
Household income	≤185 USD	1.85 (0.96 - 3.57)
	>185 USD	1
Savings	Households with savings	1.57 (0.73 - 3.38)
	Households without savings	1
Education (head of household)	No formal education	1.29 (0.71 - 2.34)
	Some formal education	1

2.3.6 The control of anthrax

Reporting and response

Only 20.6% of respondents indicated that they would communicate suspected anthrax cases if they occurred (27.5% and 16.0% of people in high- and low-risk areas respectively). Only 10.5% would communicate to an animal health professional, and others would communicate to the community leader. For households that had experienced suspected anthrax in people and/or animals (n=72), 12.5% would always report suspected cases while 47.2% did not report suspected cases. However, 40.3% would consider reporting in certain circumstances, which included when anthrax affected a large number of animals (33.3%), when the household lacked veterinary drugs to treat the animals themselves (8.3%), and when people were affected (8.3%).

Many participants (47.2%) noted difficulties and disincentives to reporting suspected cases. They cited not knowing that they had to report such cases, or whom to report to, as reasons for not reporting (16.7%). Others mentioned a lack of response from animal health professionals as the reason for the unwillingness to report (17%), while 8.3% mentioned difficulties in physically accessing veterinary services and the lack of such services within their village, as the reason for not reporting suspected cases.

Vaccination

Although 8.1% of respondents mentioned that vaccination prevents anthrax in animals, this was not reflected in anthrax vaccination rates. In the 12-month period preceding the survey, only 2.9% (CI $_{95\%}$ [0.6%, 5.1%]) of households had vaccinated their livestock against anthrax, compared to an already low proportion of households (12.4%) that had vaccinated against any livestock disease in the same period.

Local strategies to control anthrax.

Only 13.9% of participants mentioned that they did something to prevent their animals from getting anthrax. Prevention strategies include the treatment of animals with antibiotics before moving to a known risk location (1.9%), and the restriction of animal movements (3.3%).

2.4 Discussion

Although the WHO has advocated for studies to enhance the availability of data and to control neglected diseases such as anthrax, evidence of activity to support an increased effort to prioritize these diseases remains slim. This study adds to this body of evidence by investigating the importance and impact of anthrax in an endemic area of northern Tanzania, while simultaneously improving the capacity for local surveillance through stakeholder engagement and participation. The results presented in this chapter show that anthrax is an important disease for human and animal health in the area. The disease also impacts considerably on the livelihoods of households in the affected areas. The results of this study demonstrate the impacts of anthrax on the health and livelihoods of people and livestock in the NCA. These data reveal important gaps in the surveillance of the disease and may help advocate for action to control this NZD in the area and similar settings.

Every household surveyed in the NCA possessed livestock. Sheep, cattle and goats are the predominant livestock in the area. Livestock ownership has long been identified as critical to the health, wellbeing and survival of the poor in many developing countries (Randolph *et al.*, 2007). In the NCA, livestock provides the basic needs of human existence such as food, shelter, clothing, etc. (Figure 2.9) as well as the main source of income (Table 2.1) This reliance on livestock indicates that negative impacts on the health and productivity of these animals is likely to have far-reaching negative consequence on livelihoods in those communities. For example, a study showed that protecting the health of livestock by vaccinating them against East Coast Fever improved family income and gender equality and enabled access to education (Marsh *et al.*, 2016)

The dependence on livestock by these communities and the close proximity of animals and humans indicate that human and animal health are inter-connected and diseases such as anthrax may have a host of implications. A large proportion of survey respondents indicated anthrax as the most important disease for livestock among the six other diseases mentioned to them. Both households in high- and low-risk areas considered the disease important although the

proportion of people who considered the disease important was greater in high-risk areas. As with the perception of importance of the disease, both households in high- and low- risk sub-villages had reported experiencing the disease in both livestock and people. The proportion and location of households reporting and experiencing suspected anthrax was a validation for the results showing geographical variability in anthrax perception as the most important disease for livestock (Figure 2.8). These results therefore show that low-risk areas are not an indication of the absence of disease, but that cases occur at a lower incidence in comparison to high-risk areas. Most of the respondents experiencing anthrax and indicating it as the most important disease for livestock live in Ngoile, Olbalbal and Endulen (Figure 2.7). This variability indicates a geographical component to the disease as observed in other studies (Hoffmann *et al.*, 2017; Muturi *et al.*, 2018). Anthrax is often localised and confined within specific geographical locations in many endemic areas, and the pathogen is not invasive - i.e. does not spread rapidly - (WHO, 2008). This makes prevention and control of the disease easier to achieve compared to an invasive disease.

Case and outbreak investigation activity centred mainly around areas indicated as high-risk by the communities and where most reports initially originated. The focus on these wards and the logistical challenges that prevented all the trained animal health professionals from participating in active surveillance may produce slightly conservative estimates of the occurrence of the disease and its impacts as it does not take into account cases occurring elsewhere. This also makes it difficult to generalise the results of the outbreak investigation to the wider population as the overall occurrence may have been overestimated in high-risk areas and underestimated in low-risk areas. The focus on these areas enabled us to obtain more information on household losses due to anthrax, within the limits of the logistical constraints and available resources. However, data from the questionnaire-based survey showed that anthrax is also experienced in areas considered to be low-risk.

The period of the year that the studies were conducted coincided with seasons associated with anthrax; The questionnaire-based surveying being conducted in the dry season – although the data collected included losses in 24 months

including for periods of high and low incidence - and outbreak investigations overlapping both dry and rainy seasons, the estimates of loses obtained likely represents periods of both high and low disease incidence. Although a high proportion (69%) of the cases and outbreaks were confirmed through diagnostic testing, it is likely that a higher proportion were truly anthrax cases, as the tests carried out have nearly perfect specificity, but do not have perfect sensitivity (Chapters 4 and 5). It is not clear how much the storage conditions of the samples (i.e. at ambient temperature) impacted on the quality of DNA, and thus the sensitivity of qPCR. However, it is believed any impact was negligible, as the samples were from carcasses with terminal bacteraemia and thus high concentrations of *B. anthracis*. This high proportion of confirmed cases demonstrates that the capability of community members to recognise anthrax is high, and that the majority of sudden deaths in livestock in the NCA can confidently be attributed to anthrax. This diagnostic confirmation greatly strengthens the reported data on losses due to suspected anthrax obtained during the survey.

Anthrax is a severe disease and is often fatal in animals, thus it is not unexpected that the disease can cause significant losses of livestock; however, the economic losses are staggering in the context of the circumstances and living standards of communities in the NCA. Both reported and confirmed losses were substantial (Figure 2.11 and Table 2.7), particularly when considered in terms of the average household income. Most households in the NCA earn less than 185 USD per month, which, for a median family size of 9, is equivalent to 69 cents per person per day. While these losses are substantial in comparison to household income, the losses might be even more substantial when the traditional value of livestock is considered. Livestock is a dominant aspect of the culture of the people living in the NCA, who are mostly Maasai. Their identity, customs and social behaviour are directly linked to livestock ownership. A high value is placed on livestock, as they determine the social status of a household, create and maintain family relationships, and mark significant events in a person's life history (Galaty, 1982; Hauff, 2003). This cultural value placed on livestock might mean that households only consider trading a small proportion of their livestock to support their subsistence. Thus, losses due to deaths from

anthrax are more likely to represent livestock used for subsistence purposes compared to those that represent the household's cultural and social status. For communities like these that depend entirely on livestock for their livelihood and identity, the need to control livestock diseases such as anthrax that result in considerable losses and that could be transmitted to humans cannot be overstated.

It is expected that the two sources of data informing this analysis improves the reliability of the estimates of the magnitude of the losses. For example, the magnitude of reported losses in a 2 year period obtained from both the surveys and the incident investigations are comparable. Therefore, estimates of the annual loss due to anthrax may be obtained front the data presented (Table 2.7). However, I recognise that there are potential weaknesses to the study. Some differences exist between reported losses estimated from the surveys and those obtained as part of case investigations, for instance more households reported the death of a larger number of animals in the surveys compared to the incidence investigations (Figure 2.11). This difference may be due to a number of reasons. 1) That the former is based on unconfirmed reports from 55 households and the latter is associated with confirmed incidents from 36 households. 2) Random occurrence: the sample sizes at 55 and 36 respectively may be insufficient to provide an accurate and representative distribution of the number of animals lost due to anthrax in the NCA. 3) Parts of the estimates have relied on reported deaths, which may be prone to recall bias and dependent on the ability of the livestock owner to recognise the disease. For instance, estimates that included incident investigation and confirmation showed a higher proportion of households reporting fewer animals compared to estimates from the questionnaire-based surveys where livestock owners had to recall losses over a two-year period (Figure 2.11). This difference may be explained by household being more likely to remember outbreaks in which a larger number of animals are affected. Thus, it appears that larger outbreaks may have been captured more through reported cases over two years, compared to intermittent cases. For the estimates obtained through confirmed incident, recall bias is much less likely and more likely to capture data from both large and intermittent outbreaks. However, the Maasai are known for good record keeping on matters

relating to livestock and this limitation means that the losses are more likely to have been underestimated rather than overestimated. Furthermore, as the price of sheep was used for the entries where recall of species affected by the disease was impossible, the risk of overestimating the losses was minimised. Since the losses occurring as a result of confirmed anthrax (Figure 2.11B) are also substantial, the reported losses are thought to be reliable.

Since data obtained from both sources covers periods of high and low incidence, estimates of the annual losses per household may be calculated. This may be done in two ways; 1) reported losses can be obtained by dividing the total losses by the total number of households from which the data were obtained and dividing by two. The annual loss that would be obtained per household is USD 927 and is the equivalent of five months of income for majority of households in the study area. 2) Annual losses per households from confirmed cases can be obtained by extrapolating from losses estimated from confirmed cases. The annual loss of USD 1139 is substantial and is the equivalent of 6 months of household income. Due to the limitations described above, these estimated values should be regarded as a guide and are not intended to be exact measurement of the losses attributable to animal anthrax deaths. It is also important to note that the estimates refer only to losses due to animal deaths and does not include the full cost of the anthrax incidents such as the cost of mitigating further incidents or treatment and preventing further cases (Bennett *et al.*, 1999). Data for these were not obtained but substantial treatment and mitigation costs may be incurred by a livestock keeper following an anthrax incidence, which may further increase the burden of the disease.

No statistically significant differences were found among the socio-economic characteristics of those households affected by anthrax and those not affected. However, the results suggest that households dominated by women; those with a leader over 54 years old and without formal education; those with monthly income of <185 USD and having some savings may be more likely to experience anthrax. The lack of statistical significance may be due to a number of reasons, including that no large differences exist because the population is quite homogeneous, and that anthrax losses do not significantly translate into lower

socioeconomic status or that other factors such as productivity of grazing lands or having a large number of livestock compensate for the associated losses.

A number of challenges and limitations of the study exist. First, a total of 209 survey responses were obtained. These represent 95.5% of the sub-villages initially selected. However, the minimum target sample size of 194 was achieved. Second, equal numbers of responses from men and women were initially expected from the surveys, however this was not possible due to cultural and gender circumstances in the communities that confer on men more authority and influence on livestock matters. Thus, a certain degree of gender bias may be expected in the survey responses. However, I do not consider this to have a significant impact on the quality of the data as the choice of respondents were those with good knowledge of livestock management in the households. Third the sampling design is purposive, and results may not be generalisable.

Infectious disease surveillance presents challenges that are unique to each setting. For instance, the 2014 Ebola outbreak had unique challenges brought about by the remote location where the outbreak occurred, in addition to the challenge of an already weak health system available in the affected country (Hayden, 2015). Similarly, challenges such as these also represent obstacles to the control of anthrax in the study area of the NCA. Poor vaccination rates and under-reporting are main challenges. Other challenges that relate to knowledge, behaviour and practices of the affected communities are presented in Chapter 3.

Successful mitigation strategies for anthrax have been implemented in many developed countries, including the identification and barring of infection sources; proper handling, decontamination and disposal of infected carcasses and materials; and vaccination of susceptible animals (WHO, 2008). However, infrastructure and resources to carry out these procedures in an African context are often limited, leading to persistence of infection. This is especially true for rural communities in remote areas, where the burden of anthrax is likely highest. These areas may lack adequate basic infrastructure such as access to roads, electricity, and health and veterinary services that could be harnessed to boost the control of diseases like anthrax. Further constraints to effective

disease prevention may be rooted in local traditions and practices around handling infected carcasses and their products. Such practices are likely to contribute to continuous infection risks and need to be fully understood in order to identify malleable targets for intervention.

The losses due to anthrax described in this chapter should be seen as an impetus for the control of anthrax to be prioritised. The successful control of anthrax will promote the health of people and animals, improve the wellbeing and happiness of farmers, and make the environment safe for people and animals for future generations. I hope that these results will stimulate stakeholder dialogue, encourage further research, and create the visibility that is required for the prioritisation of anthrax control in the NCA and other endemic settings in Tanzania and Africa. Further study may look into assessing the economic benefits of control strategies such as vaccination, compared to the cost of implementing such strategies.

2.5 Conclusions

Anthrax in the NCA is an important neglected zoonotic disease for animal health, and human health and livelihoods. Disease caused the death of livestock in more households than other factors such as draught and predation. Anthrax is considered important and is reported and experienced more in certain locations than others. The disease in animals had been experienced by as many as 30% of households in the NCA, but with geographical variability. All (100%) households in Ngoile, 85.7% in Olbalbal and 25% in Endulen wards had reported past cases of anthrax in their livestock. The deaths caused by these cases and outbreaks produced substantial monetary losses relative to reported household income. Human disease was reported by 1 in 6 households, highlighting the high zoonotic risk in addition to loss of livestock. Disease reporting and vaccination, which are important surveillance and control strategies for anthrax, were sparsely carried out at the time of the study. The challenges to surveillance in the NCA are typical of endemic areas where limited resources and infrastructure compromise the ability to effectively control diseases. Decisions, policies and actions on the control of diseases depend on the availability of data quantifying the importance and impact a disease has on society. This study has added to the body of evidence needed to justify improved efforts to address and control anthrax in endemic areas.

Chapter 3

Chapter 3 Understanding anthrax and factors associated with risks to livestock and humans in endemic settings

3.1 Introduction

Disease occurs as a result of a complex interaction of many components, among which socio-cultural factors cannot be ignored (Reidpath *et al.*, 2011). For example, the causative agents of many diseases of animals and humans are well established, however, social, cultural and behavioural factors may play an important role in predisposing individuals to the risk of infection. Health governing bodies, policy makers and public health practitioners often rely on an understanding of the biomedical components of disease to inform management strategies, while neglecting the social and cultural phenomena that drive disease occurrence and persistence (Reidpath *et al.*, 2011; Parkhurst and Abeysinghe, 2016). However, considering the complex interactions between biological and social processes is critical to the development of prevention and control strategies tailored to any given context. For example, reductions in HIV/AIDS incidence around the world are linked to an active effort to address the social causes of disease transmission, alongside those that focus on diagnostic and treatment improvements (Crepaz *et al.*, 2006).

Understanding the context in which disease occurs, and more specifically the experiences, knowledge and practices around a given disease problem, can provide insights into the factors that contribute to the risk of occurrence and its persistence, and highlight potential awareness inadequacies that may affect control. This is not a new concept in the social and epidemiological sciences. The branch of epidemiology that concerns itself with understanding and addressing issues related to the social, cultural, and economic determinants of health (and disease) is termed social epidemiology (Krieger, 1994; Link and Phelan, 1995) and can be traced back to the 19th century. John Snow's work to solve the mystery behind the London cholera outbreak in the 19th century (Snow, 1857; Paneth, 2004) was one of the earliest proofs of the relevance of this approach to the control of infectious disease. Snow's work determined that

water supply and the absence of sanitary conditions were determinants of the cholera outbreak. His findings were generated through social science research methods targeting over 600 households to establish the source of infection (Paneth, 2004). In the context of endemic anthrax, an understanding of the social epidemiology of the disease – particularly in relation to the experiences of affected households, possible knowledge gaps, and practices that put people and animals at risk of the disease – is important in order to identify strategies to reduce the incidence and mitigate the impact of the disease in this particular and other similar systems.

A useful approach towards understanding socio-epidemiological dynamics involves a mixed methods approach to data collection and analyses (Johnson and Onwuegbuzie, 2004). Incorporating both qualitative and quantitative data in these studies offers unique advantages in terms of improving depth of knowledge towards answering complex research questions that cannot be obtained from qualitative or qualitative approaches alone. In-depth exploration of the social determinants of health-related issues enabled by a qualitative approach in particular leads to an appreciation of the factors that might contribute to disease occurrence and persistence in local populations (Marmot *et al.*, 2008; Glanz and Bishop, 2010). It may also provide insights into sustainable, informed and community-relevant control strategies. The complex factors associated with health problems may be difficult to quantify (Livingood *et al.*, 2011). Qualitative approaches help bridge this gap (Baum, 1995). Although they are stand-alone approaches with separate epistemological underpinnings, they can also help to make meaning from data that is obtained and analysed using quantitative methods (Bryman, 2006).

As for many other pathogens, social and cultural factors may be associated with the likelihood of *Bacillus anthracis* encountering a host and causing disease as well as the spread and persistence of the pathogen in the environment. Broadly this may be the result of traditional livestock management practices in most areas where the disease is common. These involve close co-existence with animals or their products and movements of livestock for grazing and watering (WHO, 2008). For instance, it is speculated that cattle movements are

associated with the introduction of anthrax into Zambia (Siamudaala *et al.*, 2006). The complete dependency of livestock owners on animals and their products for their subsistence and livelihoods, as well as a lack of insurance or compensation mechanisms, may also give rise to risky behaviours aimed at reducing loss and wastage (such as the sale or consumption of infected carcasses). More specifically, cultural views and practices related to the management of potentially infected animals or their products, combined with limited awareness of their transmission potential, may enhance risks. For example, a study conducted in Ghana implicated a lack of knowledge about anthrax sources and subsequent practices around the disposal of anthrax-carcasses as important risk factors for the disease in people (Opare *et al.*, 2000). Similarly, in endemic areas of Zambia, infected meat is usually consumed and this has been implicated in infection risk (Sitali *et al.*, 2017). Given these complex interactions, for more effective management, an understanding of biological processes needs to be integrated with that of the social and cultural context which perpetuates the disease in question.

In this study, I therefore combined qualitative and quantitative data to obtain a comprehensive understanding of local knowledge of anthrax in humans and animals as well as practices, locations and periods of the year that pose particular risks. The participatory nature of the approaches used facilitated open discussions and could be exploited further to involve the communities themselves in the development of locally-specific solutions (Catley *et al.*, 2012).

3.1.1 Objectives

The study was aimed at exploring local understanding of anthrax and drivers of risk in affected communities. Specific objectives were:

1. To investigate communities' understanding, experiences and knowledge of anthrax and its management in animals and people.

2. To identify practices that put people and animals at risk of contracting anthrax, as well as determinants of risk.

3.2 Methods

The study used qualitative and quantitative data from two sources: 1) ten focus group activities that were held in 10 out of the 11 administrative wards of the NCA (which generated qualitative data only) and 2) household-level cross-sectional surveys in which 209 respondents were interviewed (which generated both quantitative and qualitative data) as described in chapter 2.

3.2.1 Data collection

Focus groups

Focus group discussions were hosted in each of the administrative wards of the NCA except for one ward which was merged with another from which it had recently been split (Chapter 2). Each focus group had between 10 and 13 participants selected to represent members of the community concerned with animal health, and believed to knowledgeable about animal health and disease, specifically anthrax. These participants included livestock officers, community leaders and village or ward executive officers. Informed and written consent was obtained from all participants.

The questions used in the focus group discussions were open ended and were meant to elicit information on: (1) perceived high-risk areas and locations where anthrax suspect carcasses are buried and their characteristics (e.g. type of vegetation present in these areas, proximity to temporary or permanent water sources, and use of the areas by livestock for grazing); and (2) use of hides within the household/village and practices around their processing (including waste disposal) given that this is a well-recognised risk practice (Anaraki *et al.*, 2008). A full list of questions used to guide the discussions is provided in Appendix 13.

The focus group activities were conducted in Swahili language. The responses obtained in Swahili were translated into English and hand transcribed immediately into field notes. Hand-transcribed responses were then typewritten for analysis by the researcher.

Household interviews

Interviews were conducted in households selected based on the process described in Chapter 2. The survey included closed- and open-ended questions that generated data both for quantitative and qualitative analyses. The data were collected electronically using ODK and downloaded for analysis in an .csv format (section 2.2.1). Questions were designed to capture information on both human and animal anthrax focusing on local knowledge of the disease and its prevention, susceptible hosts, seasonality, sources of infection, concerns around the disease and practices that put people and animals at risk. Data on previous cases of anthrax in livestock and people were also obtained. Chapter 2 and Appendix 2 provide more details of the methodology of the household survey and the specific questions asked, respectively.

3.2.2 Qualitative analysis

Based on the methods of Braun and Clarke (2006), a thematic approach was used to identify themes relevant to the areas under investigation. Thematic analysis is an approach that enables the identification of meaning from a dataset that explains the phenomenon of interest. As the term thematic analysis suggests, this approach can be used to reveal evident and latent meanings and patterns in the dataset. Thematic analysis has been applied extensively in psychological studies, but has also proven valuable in the study of risk practices and social drivers of disease (Friedman and Shepeard, 2007; Pérez-Guerra *et al.*, 2009; Garforth *et al.*, 2013).

The analysis followed a step by step process that included 1) familiarisation with the data, 2) coding to identify instances in the data that were relevant to the research aims and 3) aggregating the codes into patterns or themes. An inductive (data-driven) approach was applied to the analysis and the themes identified were strongly linked to the research aims and questions. This is in contrast to a theoretical approach, which is more theory and analyst-driven, and may focus on a particular aspect of the data that supports (or contradicts) a given theory (Braun and Clarke, 2006). An inductive approach was particularly useful for this study as it enabled the exploration of meanings relating to the

subject of study as expressed within the data. It helped to reduce subjectivity and limit the researcher's ability to influence outcomes through preconceived ideas, as the analysis was carried out free of any pre-existing theoretical framework.

Familiarisation of the data was carried out whilst the data were being transcribed and thereafter by re-reading the transcript. Coding was done in MAXQDA12 Analytics Pro (VERBI software, 2016) software for qualitative data analysis. Codes were initially generated by identifying instances across the datasets relevant to the research objectives. The codes were verified by reiterating the coding process. Generated codes were then aggregated to form themes which described the results. Validation of the themes was carried out by reiteration and reference to the dataset to ensure themes were truly representative of the dataset.

3.2.3 Quantitative analyses

Quantitative analysis was carried out on the household survey interview data (n=209 respondents). Descriptive statistics were compiled and simple and multiple logistic regression analyses were performed. Specifically, the outcome variables of interest were the probability of a household having experienced a previous case of suspected anthrax 1) in their livestock and 2) in household members. These outcome variables were defined as households who had answered yes to one or more of the questions shown below:

1. Previous suspected case(s) of anthrax in livestock:

    a. Have you had any cases of anthrax in your animals in the last 12 months? Yes/No

    b. Have you had any cases of anthrax in your animals in the last 2 years? Yes/No

    c. Have you ever had any cases of anthrax in your animals? Yes/No

2. Previous case(s) of anthrax in household members:

    a. Has anyone in the household become sick with anthrax in the past 12 months? Yes/No

    b. Has anyone in the household become sick with anthrax in the past 10 years? Yes/No

    c. Has anyone in the household ever become sick with anthrax? Yes/No

The independent variables of interest included the location of the household at ward administrative level, total number of livestock (including cattle, sheep and goats), total number of cattle, total number of sheep, total number of goats, movement to seasonal grazing locations, vaccination against anthrax, vaccination against other diseases, consumption of any animal carcasses, use of animal blood, use of animal hides, source of animal hides, and wildlife presence (Table 3.1). Livestock numbers were modelled as continuous variable, while the other predictors were categorical variables. Livestock, cattle, sheep and goat numbers were tested for normality and were log transformed due to positive skewness (Bland and Altman, 1996). Variables that were significantly associated with households having previous case(s) of anthrax were selected for multivariable modelling.

Table 3.1: Classes of variables used in the statistical models.

Predictor	Class	Range/Percentage
Household location	Nominal categorical variable with 6 levels	-
Number of livestock	Continuous variable	5 – 1200 livestock
Number of cattle	Continuous variable	1 – 300 cattle
Number of sheep	Continuous variable	1 – 700 sheep
Number of goats	Continuous variable	1 – 400 goats
Movement to seasonal grazing locations	Nominal categorical variable with binary response (yes and no)	Yes- 88.9 % No – 11.1 %
Percentage of households who vaccinated animals against anthrax	Nominal categorical variable with binary response (yes and no)	Yes- 2.9 % No – 97.1 %
Percentage of households who vaccinated animals against other disease	Nominal categorical variable with binary response (yes and no)	Yes- 12.5 % No – 87.5 %
Percentage of households who consumed animal carcasses	Nominal categorical variable with binary response (yes and no)	Yes- 90.1 % No – 9.9 %
Percentage of households who use animal blood for food	Nominal categorical variable with binary response (yes and no)	Yes- 87.5 % No – 12.5 %
Percentage of households who use animal hides	Nominal categorical variable with binary response (yes and no)	Yes- 92.3 % No – 7.7 %
Source of animal hides	Nominal categorical variable with 2 levels (livestock and livestock and wildlife)	Livestock - 95.5 % Livestock and wildlife – 4.5 %
Percentage of households who encounter wildlife	Nominal categorical variable with binary response (yes and no)	Yes- 99.0 % No – 1.0 %

Multivariable logistic regression models

The outcome (response) variables with binary responses yes and no were converted to 1 and 0 representing the probability of a household with a previous case of anthrax (p = 1) and a household without a previous case of anthrax (p = 0), respectively. Two generalised linear models (GLMs) with all the predictor variables that were significantly associated with households having a previous

case(s) of animal and human anthrax respectively (Table 3.6) were built. A backward step-wise elimination method was used to select the predictors with greatest effect on the outcomes. Following this, predictors without effect were removed from the model. Details for each of the two models are given in the sections below. All quantitative analyses were carried out in the R 0.99.484 software for statistical analysis (RStudio Team, 2016).

Modelling past cases of animal anthrax

Predictors significantly contributing to the probability of a household having a past case(s) of anthrax in their livestock included the location of the household (ward), number of livestock owned, history of vaccinating the herd, using dead animals for food, the consumption of blood and the source of hides (either from livestock or wildlife) used by the household. I ran a multivariable model incorporating the different livestock species (cattle, sheep and goats), as the only explanatory variables to reveal the species with effect on the risk of anthrax. Sheep were identified as the only livestock species with a significant effect, thus the modelling excluded the number of cattle and goats, and included only sheep numbers to predict the effect of livestock numbers on the probability of experiencing anthrax. Following this a multivariable model (model1) with these predictors - household location (ward), log number of sheep, history of vaccinating the herd, using dead animals for food, consumption of blood, and source of hides - was built. A stepwise procedure based on backward elimination (Mundry and Nunn, 2009) was used to produce a simpler model and eliminate predictors with least effect on the model. This procedure eliminated the use of blood and the consumption of dead animals, producing an update of model1 (model2).

Modelling past cases of human anthrax

Simple logistic regression identified these predictors – a history of anthrax in the livestock herd, the location of the household (ward), total number of sheep and the source of hides – as the significant predictors of a household having a previous human anthrax case. The predictors were modelled against the probability of a previous human anthrax case in a household (model 3). With backward elimination, a history of anthrax in the household's livestock herd was

the significant contributor to the model. Model3 was thus updated eliminating location, log number of sheep and source of hides to produce model 4.

3.3 Results

3.3.1 Themes identified in the qualitative data

The themes that emerged from the analysis are reported in Table 3.2. Direct anonymised quotations are shown in the subsequent sections where appropriate in order to illustrate key themes. The quotations presented herein have been translated from either Swahili or Maa into the English language by a local translator and have been paraphrased in some instances.

Table 3.2: Themes and sub-themes identified through analyses of qualitative data gathered in focus group discussions and household surveys.

Research objectives	Themes	Subthemes (where applicable)
Experiences, perceptions and knowledge of anthrax	Knowledge of anthrax	-
	Seasonality	-
	Perceived sources of infection	-
	Concerns about anthrax	Livestock and livelihood losses
		Human illness
		Frustrations about the difficulty of early detection
Practices that put people and livestock at risk	Carcass and hide handling	-
	Practices around livestock movement	Movement of animals
		Movement of animal products
Knowledge and practices around anthrax prevention and treatment in animals and people	Prevention	
	Treatment	

3.3.1.1 Knowledge of anthrax

Anthrax in animals

Overall in both focus group discussions and household surveys, participants and respondents showed familiarity with the disease and described it as having a strong presence in the area:

"Basically the disease is all over here" (Participant, focus group [FG]).

Nearly two thirds (60.7%) of the 209 survey respondents knew anthrax and were aware that the disease affects animals. While 34.4% of participants had knowledge of animal anthrax only in livestock, 26.3% knew that the disease affected wildlife. Amongst wildlife, herbivorous species such as zebras, wildebeest and antelopes were mentioned as examples of species that can contract anthrax as described in this statement:

> *"The communities live in close proximity to wildlife; there are lots of cases also in zebras and impalas" (Participant, FG).*

Anthrax was depicted as a disease more frequently affecting livestock, especially small ruminants, compared to humans and wildlife. Overall, 57%, 54% and 41% of respondents knew anthrax as a disease of sheep, goats and cattle respectively. The disease was perceived to manifest more often in goats and sheep than in cattle and donkeys:

> *"Anthrax affects sheep and goats much more than cattle" (Respondent 002).*

Participants had good knowledge of the clinical signs and presentation of anthrax in animals (Table 3.3). The most common sign mentioned was sudden death and was reflected in accounts like "*anthrax kills animals when they are very healthy*". One key informant mentioned that anthrax "*affected healthy animals*" and wondered if "*fat or the heat produced as a result of fat*" contributed to the increased risk of anthrax in animals.

> *"Many times we see no signs, other times the animal suddenly stops grazing, has raised hair at the forehead, and then it jumps up about three or four times and it is dead" (Respondent 031).*

"Upon local post-mortem, the lungs are attached to the ribs and the internal organs are blackish with blood accumulation, the blood is black too" (Participant FG).

Table 3.3: Signs of anthrax in animals listed by household respondents.

Clinical signs in animals	Proportion of respondents aware of the disease and listing associated signs
No signs prior to death	22.0%
Sudden death	39.4%
Excitement	5.5%
Blood oozing from natural orifices	7.9%
Swelling/bloating	23.6%
Raised/ruffled fur	11.0%

Anthrax in people

Fifty-two percent of participants were aware that anthrax is a zoonotic disease. The experience of the disease in humans was one consideration for judging the disease important and was clearly depicted by participants, including the different forms of the disease:

"There are two types of the disease in humans, the one that goes through the stomach and comes out of the skin and the one that goes through the skin" (Participant, FG).

"The type that affects the stomach is very bad and kills much more than the type that affects the skin" (Participant, FG).

In people, the most commonly listed sign was the characteristic lesion associated with cutaneous anthrax (Table 3.4). Other signs mentioned were fever, swelling or bloating.

"(…) When it [the infection] comes out of the body through the skin you see a black wound" (Respondent 059).

Table 3.4: Signs of anthrax in people listed by participants. Others include pain, dehydration, headache.

Clinical signs in people	Proportion of respondents aware of anthrax
Swelling/bloating	51.2%
Fever	24.4%
Eschar/wound/bruise/sore/boil/rashes	55.1%
Others	11.0%

3.3.1.2 Seasonality

Focus groups indicated that in the NCA anthrax is strongly linked with the dry season, which is associated with elevated temperatures, or the hottest hours of the day. In fact, the word for animal anthrax in the local language (Maa) literally means 'hot disease'. Participants described anthrax as a disease that occurs during the "*dry and hot season*" typically from June to October (long dry season) and January to March (short dry season), but patterns and timings are continuously changing.

> *"This disease happens in the warm periods especially in the afternoon during grazing and watering" (Participant, FG).*

While more cases are reported during the dry season, based on participants narratives, in the most affected areas anthrax cases may occur year-round:

> *"The disease is more common in the dry season, there are minimal cases in the wet season" (Participant, FG).*

Participants reported that anthrax tends to occur when the grasses begin to sprout at the beginning of the wet season and livestock start recovering from a shortage of pasture during the dry season.

3.3.1.3 Perceived sources of infection

Sources of infection for animals

In the NCA, sources of infection for animals are recognised to be pasture, water, mineral licks and dust as suggested by the following quotes:

> *"A poisonous grass causes anthrax" (Respondent 042).*
>
> *"Raising and sniffing dust causes anthrax" (Respondent 079).*
>
> *"Salts (mineral licks) causes anthrax" (Respondent 151).*
>
> *"Stagnant dirty water causes anthrax" (Respondent 036).*
>
> *"Animals are affected when they drink water accumulating in holes" (Participant, FG).*

Many (44.1%, n= 127) of the survey respondents aware of anthrax believed animals get infected from grazing on pasture and 17.3% from soil or dust, while 22.8% believed the disease can be contracted from contaminated water sources. However, 16.6% of respondents aware of the disease did not know how animals contract the disease.

Sources of infection for people

For humans, the most common mode of transmission referred to was contact with infected animals. Participants of the focus groups mostly associated human infection with eating affected carcasses, and in certain instances believed that eating these carcasses was the way by which people also got cutaneous anthrax.

Of the household-level respondents aware of the disease in people (n=119), 91% mentioned that people got infected from consuming an infected animal carcass. However, others believed that any contact with infected animal carcasses caused infection in people. Some 10.2% of respondents mentioned contact with soil and 2.5% believed that people become infected when they drink from the same water source as animals. When asked how people contracted anthrax, respondents replied:

"Eating the meat of infected animals, touching the blood, skin and soil" (Respondent 020).

"Animals spread the disease to people through eating [infected carcasses] and contact with blood and skin" (Respondent 023).

Most (78%) households with previous cases of human anthrax (n=46) believed the disease was contracted through the consumption of infected carcasses, 9% through contact with parts of the carcass and 4% through contact with soil.

3.3.1.4 Concerns about anthrax

Anthrax is generally a cause of concern among NCA residents because:

1. It affects and kills large numbers of animals. Deaths can occur concurrently and/or continuously over a period of time, causing severe losses to livelihood.

2. It affects humans, with most human infections occurring as a result of animal infections.

3. It causes sudden deaths in animals and it is therefore difficult to diagnose early to allow for treatment.

These sub-themes are expanded upon in the following section.

Livestock and livelihood losses

All households experiencing anthrax reported death of livestock as the outcome of the disease.

"We see deaths [from anthrax] almost on a daily basis" (Respondent 024).
"I lose animals always, about twenty animals die monthly" (Respondent 033).
"No month passes without cases [of anthrax]" (Respondent 070).

Many participants described the disease as one that ***"steals"*** a large number of livestock suddenly, either concurrently or continuously, or both, for instance

causing deaths in more than 10 animals a day or over two weeks. When asked how many times he had recorded incidents of anthrax in his herd in the past two years, a respondent replied *"almost every day in our boma in Olduvai"*. Other respondents indicated that livestock deaths caused by the disease are commonplace. Most often these deaths occur in the dry season and when the animals are not being directly monitored, such as during the night, or when they had just returned from grazing or watering.

> *"Cases happen every week. If I go without a case in a week, then the following week there are four or more deaths" (Respondent 071).*

> *"Last week [in] sub-village B, 6-10 animals died per day from one boma" (Participant FG).*

> *"When an animal dies [of anthrax] deaths usually continue for three to four days" (Participant FG).*

While many participants could recall considerable losses from anthrax and could give details about the losses experienced (Chapter 2), a few were less willing and appeared to avoid recalling their experiences:

> *"When my animal(s) dies, I don't keep it in my head, I forget about it" (Respondent 038).*

Human illness

The fact that anthrax is transmitted to humans from animals was another reason the disease was considered important among the household respondents interviewed. Seventy-one percent (71%) of respondents with knowledge of the zoonotic nature of anthrax (n= 109) indicated people usually got anthrax around the same periods that animals are infected with the disease. Seventeen percent of households surveyed had reported at least one previous case of human anthrax. There were 46 previous cases in 35 households, with age group of the affected persons ranging from infants (less than 1 year old) to the elderly (77 years).

Frustrations about detection

Respondents expressed concern about the sudden death caused by anthrax which prevents them from treating sick animals and avoiding losses. Some farmers believe that anthrax cannot be treated:

> *"Anthrax kills animals without notice" (Respondent 041).*

> *"This disease does not have treatment" (...) (Respondent 037).*

3.3.1.5 Risk practices for anthrax in people and livestock

Carcass and hide handling

Respondents described the frequency of slaughtering animals for food as *"occasional"*, usually only when there is a festivity or ritual, for example when a woman has just had a baby in the family. Between these periods, only animals that die naturally are used as a source of food. Participants generally believed that meat should always have some form of use, possibly due to a culture of waste avoidance. The general belief is that if it is not fit for human consumption then domestic dogs or wild scavengers and carrion eaters should feed on it:

> *"No way [to prevent anthrax]. Nothing [can be done to prevent anthrax], because if my animal dies I must eat it. That is what I depend on to survive" (Respondent 037).*

Although the vast majority (91%) of respondents interviewed in households would prepare livestock carcasses for food unless they were visibly unfit for consumption (such as a decomposing carcass), a few households described being selective about the carcasses to consume. For example, 43% of respondents would not consume a carcass if the animal had died of unknown causes. Focus group participants explained that the suitability of a carcass for human consumption may be determined after some form of internal and external assessments. External examination involves assessing the physical state of the carcass, for example the state of decomposition of the carcass, which is determined based on the stench emanating from it. Internal examination involves assessing the internal organs of the carcass by tearing open the abdominal and thoracic cavity in a vertical line and checking organs such as the spleen, liver, lungs, heart, intestines etc.

> *"A Maasai never leaves a carcass intact. If we cannot eat it, we open it to discover what killed it and so that the hyaenas can smell blood and come to take it away" (Participant FG).*

As participants were aware of anthrax risks posed by consuming suspect carcasses, so they were of the fact that cutaneous anthrax can be contracted from hides:

> *"We get it even when we touch the skin [of an animal dead of suspected anthrax](…)" (Respondent 020).*

However, they did not appear to avoid this practice. For example, households that reported avoiding consuming anthrax-suspect carcasses would skin the animal and preserve the hide for use while throwing out the rest of the carcass for carrion eaters.

Participants described that after an animal is skinned, the hide is usually stretched out and dried in the sun during the day, and left in a cool place at night for a few days until completely dry. Following this, the hair is removed with a sharp bone, and some form of fat (usually sheep fat) is then rubbed in on both sides making it ready for use. It might be important to note that there was no existing mechanism for the trade of livestock hides in the area at the time of data collection, as reported by the participants. Instead, excess hides not needed for immediate use were thrown out into the environment.

In contrast to the perception of risk from the consumption of infected carcasses and the use of associated products, a handful of participants believed that, by consuming a carcass suspected to have died of anthrax, they are lowering their risk of contracting the disease.

There was no practice of burning or burying an anthrax-suspect carcass reported by the study communities. On the contrary, livestock owners reported exhuming animals diagnosed with anthrax and buried by livestock officers because *'they couldn't waste such healthy-looking animals'*.

Information on the practices around handling a suspect carcass or its parts generated in this study is summarised in Figure 3.1. The figure also illustrates

how these practices may result in transmission to humans and animals, or environmental contamination.

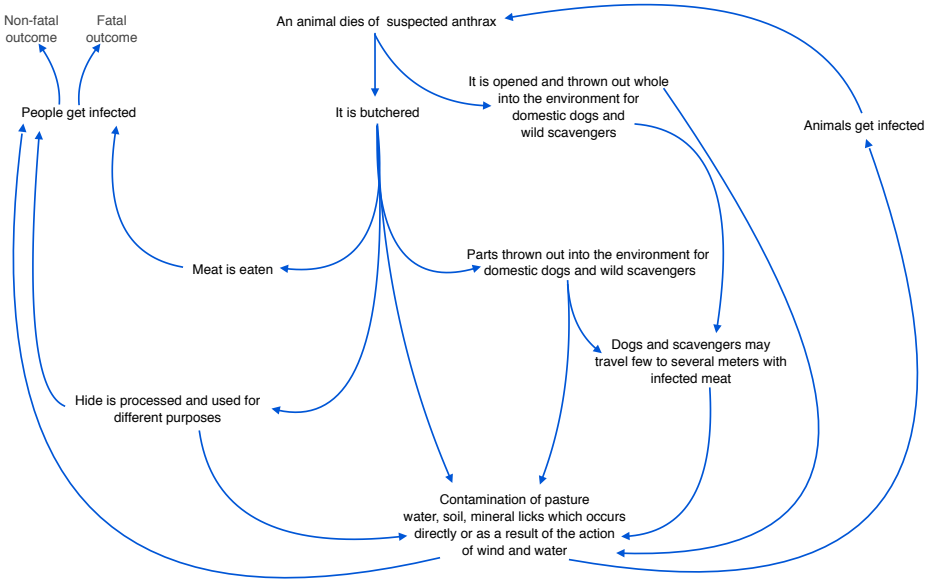


Figure 3.1: A depiction of the practices around handling an anthrax carcass and the possible risk to people and animals. These events may not be spatially (e.g. an animal may be butchered and eaten miles away from the place of death) or temporally linked (e.g. animals may graze on pasture contaminated a year or more ago).

Practices around livestock movement

Findings revealed that local communities move their livestock in search for resources, for controlling diseases, as well as for cultural exchanges, gifting, and trading purposes. Livestock movements carried out for cultural and trading purposes are excluded here because this practice causes livestock to move between owners. Disease history was therefore difficult to trace by the new owner. Some 25% of respondents mentioned that they had animals kept elsewhere that may return to the household. Daily livestock movements in search for pasture and water ranged from 2 minutes to 8 hours minutes in the dry season and between 5 minutes and 6 hours in the wet season (see Table 3.5).

Table 3.5: Amount of time livestock owners spend moving their livestock each day in search for pasture and water.

Livestock	Median time to grazing location in the dry season (wet season) as reported by household respondents	Median time to watering location (wet season) as reported by household respondents
Cattle	120 (30) minutes	60 (20) minutes
Goats and sheep	60 (30) minutes	60 (30) minutes

Furthermore, 80% of respondents reported moving animals to temporary grazing camps during certain periods of the year. In many cases, these temporary camps are located outside the villages (44% of respondents), wards (24%) or district (1.4%) where the household permanently resides.

When determining the drivers of movements, mineral salts were considered as the most important factor in deciding where to move livestock to. This was followed by water and pasture availability. Movements to access mineral salts where implicated in anthrax transmission to animals as illustrated by the quotes below.

> *"Many cases happen (...) when animals move to Olduvai at hot salty areas" (Respondent 157).*

> *"Grazing on salty soil or drinking salty water causes anthrax" (Participant, FG).*

The types of and reasons for livestock movements are summarised in Figure 3.2

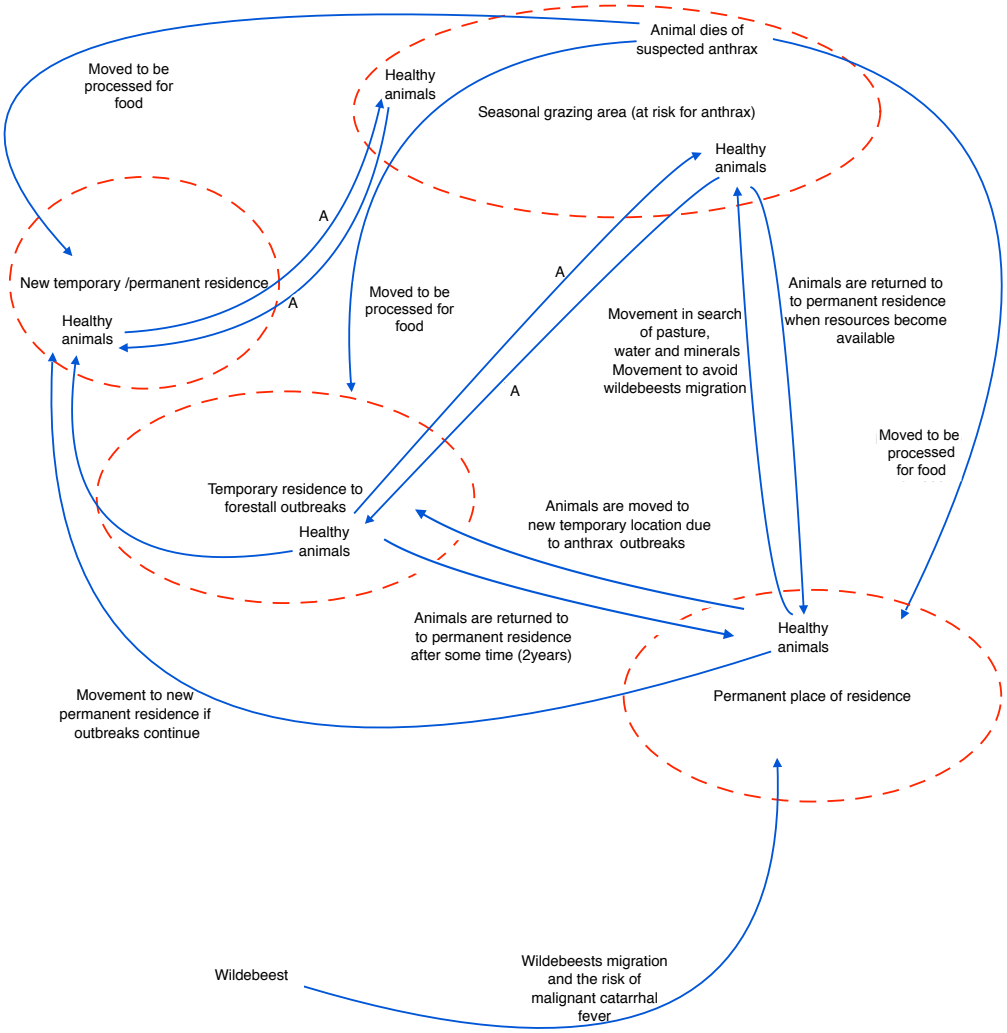


Figure 3.2: A schematic representation of livestock movements. The dotted red lines depict geographical areas and the blue lines represent movement patterns. "A" represents movements in search for pasture, water and minerals as well as those to avoid the wildebeest migration.

Practices around movements of animal products

If an animal has died in the field due to suspected anthrax or any other causes, sometimes the entire carcass or only the meat are transported to the household to be used by the family for food (Figure 3.2). When prompted for details about an anthrax case reported by a participant, the following paraphrased conversation ensued.

Participant: "*Our cow died in Olduvai of anthrax (...)*"

Interviewer: *"What did you do with it?"*

> Participant: *"We put the meat on a donkey and brought it to Endulen [which is 17.6 great circle miles from Olduvai] for the household to eat, 2 days later the donkey died (...) and one person was affected but recovered (...)."*
>
> Interviewer: *"What did you do with the donkey?"*
>
> Participant: *"We threw it out for the hyenas."* *(Respondent 145)*

In addition to this, although wild animals are not often consumed in the area, participants reported bringing home parts of dead animals they found in the field for dogs to feed on or for other uses, for example for making handheld fans.

3.3.1.6 Knowledge and practices around anthrax prevention and treatment

Prevention and treatment in animals

Although 80% of respondents mentioned that they did nothing to prevent anthrax in their herd, treatment with veterinary antibiotics was very common. About 96% of respondents report using antibiotics to treat animals even when the cause of illness is not clear. In certain cases, antibiotics will be used as prophylaxis before moving animals to areas of high anthrax risk.

> *"Before moving to a risky place I inject my animals with penni-strep (penicillin-streptomycin)" (Respondent 37)*

A typical resilience mechanism against anthrax is the movement away from possible sources of infection. This theme emerged both in relation to anthrax and another disease of local concern - malignant catarrhal fever (MCF). For example, one of the control strategies employed by farmers experiencing anthrax is to move the herd to a different location, thought to be free of the disease (Figure 3.2).

> *"When we notice this disease in our boma, we try to move to another location (...) but the disease continues" (Respondent 035).*

Many families abandon their permanent places of residence also to protect their livestock from contracting MCF from seasonally migrating wildebeests. When

asked why livestock are moved away even from areas where resources are abundant, livestock owners responded:

> *"We only try to avoid co-habiting with wildebeest because of MCF. MCF kills more [animals] than anthrax and no medications are available" (Participant FG).*

> *"We try to avoid the wildebeest migration (…). When there is increased competition for pasture between livestock and wildlife, diseases such as MCF result" (Participant FG).*

Livestock keepers also reported using local remedies for anthrax like "*bleeding the ears*" of animals.

Prevention and treatment in people

The majority (78%) of participants indicated that they were unsure as to how to prevent anthrax in people. Some (9.5%) participants mentioned that stopping the consumption of dead animals was a way of preventing anthrax in humans. However, as reported earlier (see 3.3.1.5), most participants indicated that using an animal for food that had died of known or unknown causes was common in their households.

Despite the recognised potential risk of contracting anthrax from suspected carcasses, living in the NCA, where no cultivation is allowed (some participants - especially women - lamented their inability to use land to cultivate) means that local communities depend solely on meat and the acquisition of food crops from external sources. The study participants therefore considered it logical to weigh the risk of hunger above that of contracting anthrax. Another reported reason for exposing themselves to risks was previous experience suggesting differential risk:

> *"Why do ten people eat an anthrax carcass and only two get sick?" (Participant FG).*

In many cases, when the suspected reason for animal death is clearly anthrax, locals have devised strategies of escaping infection, as one participant indicated:

> *"We boil the meat the first time and throw the stock away and then we boil a second and third time, each time throwing the stock away. After this we boil the final time and eat" (Participant FG).*

While anthrax could be prevented in people, the majority of participants (78%) mentioned that they did nothing to prevent the disease. Belief that anthrax is not preventable exists and captures the experiences of some people contracting the disease without any known contact with an infected carcass or contaminated products. Therefore changing behaviours around the consumption of suspected carcasses was considered unnecessary. In such cases, treatment when the disease occurs was preferred over prevention.

The participants indicated that a plethora of conventional and traditional remedies are available to them for self-treatment of anthrax despite the limited access to health care facilities. These include veterinary antibiotics (for example oxytetracyclines) which are widely available in these communities and administered to livestock by the pastoralists themselves. Veterinary drugs have been observed to work for humans with suspected anthrax and are sometimes taken prophylactically before consuming an anthrax carcass.

> *"No prevention, when a person is affected we inject [them with] oxytetracycline and prevent them from sleeping before taking them to hospital" (Respondent 003).*
> *"We treat them at home with OTC (oxytetracycline) injection" (Respondent 045).*
> *"I was injected with penicillin by Maasai doctors" (Respondent 094).*

Traditional strategies for treating and managing anthrax in people include "*applying sheep fat on carbuncle*", "*drinking and applying honey to carbuncle*", "*ritual practice of tying a rope or muscle around the middle finger*", "*drinking herbal mixtures*".

While self-treatment and the use of local remedies are common in these communities, the need to seek medical attention in case of a worsening situation is well recognised. Many participants indicated that anthrax is a very serious disease in humans, "*especially the internal form*", needing urgent intervention, either with traditional or conventional medicine. In many cases, self-medication and the use of local remedies was the first line of action before

seeking the intervention of a medical facility to avert casualties. Reasons given for self-treatment/use of traditional medicine included the great distance from good dispensaries and medical centres, especially from remote areas where the burden of disease was reported as being the highest.

3.3.2 Determinants of risk

Animal anthrax

Univariate analysis revealed a number of practices and household characteristics that were significantly associated with households having past cases of anthrax in humans and animals (Table 3.6).

Multivariable modelling revealed that only the household location (ward) and number of sheep were significantly associated with anthrax in livestock. Specifically, the odds of having a past case of anthrax for households in Olbalbal and Ngoile wards were very high (45.9) compared to those in Kakesio, while the odds for households in Ngorongoro and Eyasi were very low with very wide confidence intervals (Table 3.7). The odds for households in Endulen was 0.7. The odds for those in Olbalbal and Ngoile were significantly different compared to other wards.

Table 3.6: Results of univariate logistic regression analysis of household characteristics and risk factors for anthrax.

Model	Dependent variables	Independent Variable(s)	Log likelihood of model	Relationship, *P*-value
Intercept models	Any previous anthrax case in animals	-	-127.2 (df=1)	NA, <0.001 ***
	Any previous anthrax case in people	-	-94.25 (df=1)	NA, <0.001 ***
Univariate models	Human cases	Cases in animals	-80.65(df=2)	Positive, <0.001 ***
	Animal cases	Location (ward)	-68.24 (df=6)	Positive, <0.001 ***
	Human cases	Location (ward)		Not significant (NS)
	Animal cases	Log of total livestock	-103.15(df=2)	Positive, <0.001 ***
	Human cases	Log of total livestock	180.8 (df=2)	Positive, 0.008 **
	Animal cases	Log number of cattle	-104.47 (df=2)	Positive, <0.001 ***
	Human cases	Log number of cattle	-84.16(df=2)	Positive, 0.034 *
	Animal cases	Log number of goats	-92.99(df=2)	Positive, <0.001 ***
	Human cases	Log number of goats	-78.47(df=2)	Positive, 0.012 *
	Animal cases	Log number of sheep	-71.29(df=2)	Positive, <0.001 ***
	Human cases	Log number of sheep	-61.55(df=2)	Positive, <0.001 ***
	Animal cases	Seasonal movement		NS
	Human cases	Seasonal movement		NS
	Animal cases	Vaccination	-122.85(df=2)	Positive, 0.005 **
	Human cases	Consumption of carcasses		NS
	Animal cases	Consumption of carcasses	-123.11(df= 2)	Positive, 0.0358 *
	Animal cases	Carcass use (unknown cause of death)		NS
	Human cases	Carcass use (unknown cause of death)		NS
	Animal cases	Herbivorous wildlife presence		NS
	Human cases	Herbivorous wildlife presence		NS
	Human cases	Blood use		NS
	Animal cases	Blood use	-123.87(df=2)	Positive, 0.034 *
	Human cases	Use of wildlife hide	-87.47(df=2)	Positive, 0.047 *
	Animal cases	Use of wildlife hide	-113.93(df=2)	Positive, 0.007 **

The history of vaccinating the herd and the source of hides were not significant in multivariable analysis. We investigated a non-linear relationship of the number of sheep to the probability of experiencing animal anthrax. Four categories were created using the median and interquartile ranges (minimum, 1st quartile, median, 3rd quartile, maximum) of sheep numbers, and modelled in place of the log number of sheep. The results indicated that having more than 21 sheep increases the odds of having animal anthrax by 3.8 fold.

Table 3.7: The odds of experiencing animal anthrax.

Predictor		**Odds ratio ($CI_{95\%}$)**	**Odds**	***P*-value**
Location (ward-level)	Kakesio	1	0	-
	Olbalbal/Ngoile	46.9 (7.3, 298.9)	45.9	4.72e-05
	Endulen	1.7 (0.4, 6.9)	0.7	-
	Naiyobi	3.8 (0.9, 16.2)	2.8	-
	Ngorongoro	2.2e-08 (0.00, inf)	-1	-
	Eyasi	2.7e-08 (0.00, inf)	-1	-
Number of sheep		3.1 (1.4, 6.7)	2.1	0.0037

Human anthrax

For human anthrax, univariate analysis indicated that log number of sheep, previous cases of anthrax in the animal herd, the household location, and the source of hides were significantly associated with the probability of households experiencing at least one human case of anthrax (Table 3.6). The results of the multivariable generalised linear modelling carried out on the data shows that there is evidence that the probability of a household having experienced human anthrax is associated with a history of having anthrax in the herd. The odds ratios for households with past case(s) of anthrax in their herds were 7.1 $CI_{95\%}$ (3.1, 16.1), thus these households were 6 times more likely to have experienced human anthrax compared to those with no animal cases.

3.4 Discussion

This study used qualitative and quantitative data to assess local knowledge of anthrax in the NCA, and risk practices and determinants. The study found a high level of anthrax awareness and ability of communities to identify infection signs and sources in both animals and humans. The study also identified key practices around carcass consumption and handling, and management of livestock and their products that explain high exposure rates, particularly in some locations. Finally, the findings demonstrate that communities are largely aware of these high-risk practices and have developed their own risk management strategies within the constraints that resource and logistic limitations impose in the area.

This study shows a high level of knowledge and awareness of anthrax within affected communities, which is similar to studies conducted in other African contexts (Opare *et al.*, 2000; Gombe *et al.*, 2010; Mebratu, Hailu and Weldearegay, 2015; Sitali *et al.*, 2017). Respondents interviewed in the NCA were able to recognise the common signs and forms of the disease in animals and people, strengthening the belief that lay people hold a wealth of knowledge about health issues affecting them (Wallerstein and Duran, 2006; Lewin *et al.*, 2013). This is especially true for pastoralists around topics related to the management of the health of their livestock (Caudell *et al.*, 2017). For instance, respondents mentioned that animals with suspected anthrax usually die suddenly or show no signs at all before death, which are typical signs of anthrax infection. The low proportion of respondents reporting blood discharge from natural openings is consistent with observations that this clinical manifestation of the disease is not always present (WHO, 2008), but might also indicate that this sign occurs less than previously thought. The signs of swelling and bloating, and excitement reported by study participants are also consistent with other observations (Turnbull, 1998).

In addition to knowledge about the signs of anthrax in animals, there was a clear understanding of the zoonotic nature of the disease. Although there are three forms of anthrax in people – cutaneous, gastrointestinal and inhalational anthrax – the latter two forms do not present with specific symptoms and signs and may

be difficult to detect. However, individuals with a recent history of having consumed a suspected anthrax carcass may attribute symptoms such as fever, diarrhoea, swelling and bloating to the disease. Over 50% of respondents who were aware of anthrax as a human disease mentioned the characteristic skin lesion. This sign is pathognomonic for the cutaneous form of the disease that occurs in over 95% of human anthrax cases (WHO, 2008).

Local knowledge of common sources of infection in both people and animals was also demonstrated in this study and was generally consistent with scientific knowledge of how the pathogen persists. For animals, anthrax exposure was associated with the environment, for example pasture, water, soil and dust. Contact with infected carcasses was the most commonly observed route of infection in humans, although direct contact with soil and water was also mentioned.

Anthrax affects animals in the NCA regardless of the season of the year, although most cases are experienced in the dry seasons. This indicates a hyper-endemic situation, which is in contrast to the occasional outbreaks experienced in many parts of the developed world where the disease has been controlled. Increased anthrax incidents have been reported during dry periods in several locations in eastern and southern Africa (Hampson *et al.*, 2011), as well as in North America and Australia (Turner *et al.*, 1999; Parkinson *et al.*, 2003)). This pattern has been associated with short abrasive grasses that may cause injury to the oral cavity, which in turn may favour entry of spores.

While seasonal fluctuations in anthrax incidence have been noted across several locations, the patterns of seasonality vary, with particular areas typically experiencing peaks in anthrax in either very wet or dry periods (Hampson *et al.*, 2011; Mullins *et al.*, 2015). The occurrence of anthrax in the NCA at the start of the wet season may support the spore storage area theory (Dragon and Rennie, 1995), where new rains facilitate the concentration of spores from areas that have been contaminated by anthrax carcasses in the preceding dry season. Although anthrax in the NCA is more likely to occur in the dry season in specific months of the year, it is important to note that that the patterns of seasonality

in East Africa are rapidly changing. A progressive decline in rainfall has been noted in recent years with corresponding increases in dry and drought periods, and changes in the associated periods (or months) of the year when the dry seasons would normally be expected (Rowell *et al.*, 2015). This trend may have implications for anthrax occurrence and periods of the year at increased risk.

Local communities in the NCA associate anthrax with the environment, which is consistent with scientific evidence of the source of natural infection for animals (Hugh-Jones and De Vos, 2002). The claim that "anthrax is caused by a poisonous grass" could be explained by participant observations that the disease is contracted from pastures. *B. anthracis* spores persist in the soil (Hugh-Jones and Blackburn, 2009) and grazing on contaminated pasture is one of the most common sources of infection in animals. Such pastures are likely to be more attractive to animals compared to other pastures as nutrients from the organic matter of infected carcasses deposited on soil may lead to richer pasture (Ganz *et al.*, 2014). Infections that arise from contaminated pastures may explain the observation of the pasture being "poisonous", particularly since sudden deaths could also occur as a result of poisoning. Inhaling, sniffing and snorting dust or soil laden with spores have been implicated in anthrax transmission (Ganz *et al.*, 2014), which is consistent with observations by study participants. They also associated the ingestion of salts and minerals with the disease. Natural mineral licks are the main source of salts and minerals for livestock in the NCA, and could be a potential source of infection if they are contaminated with spores. Participants described animals getting anthrax when they "graze on salty soil" or "drink salty water". It is not clear what mineral deposits exist in these areas and what their effect would be on the transmission of anthrax. However, anthrax spores have been shown to thrive in soils rich in certain minerals such as calcium (Dragon and Rennie, 1995; Himsworth, 2008). There is also a local perception that stagnant dirty water causes anthrax infection in animals. During periods of excessive rainfall, spores could be transported along with run-off and deposited in low lying areas (Hugh-Jones and Blackburn, 2009). This could be the underlying basis for the association of anthrax with pooled stagnant water.

Heat was the factor study participants most commonly associated with anthrax. The local term for anthrax, “hot disease”, likely derives from this widespread belief. A reason for this association may be that most infections occur during periods of elevated environmental temperatures as discussed previously. Furthermore, participants reported that certain locations within the NCA, especially those at high elevation with cooler weather, usually have no anthrax cases. Consistent with these observations, this study found a statistically significant difference in reported anthrax in Olbalbal and Ngoile wards compared to other locations. Spatial heterogeneities and localised risk are further supported by a previous study in the NCA that found a high prevalence of the disease in these wards, with location being a significant predictor of seropositivity (Lembo *et al.*, 2011). The NCA comprises a range of environmental conditions across its landscape (Galvin *et al.*, 2004), which community members well recognise and associate to specific disease risks.

Although heat and resulting stress may directly alter immune response to infection (Das *et al.*, 2016), it may also mark the onset of resource scarcity and nutrient deficiency which may also affect resistance to disease. Furthermore, elevated temperatures and resource scarcity may result into changes to livestock management practices, such as movements in search for alternative resources, which may also increase infection risks. The complex interplay between environmental conditions and factors related to host and pathogen biology remains an important area for further study as it would enable us to predict locations of risk and target control accordingly. This study contributes further evidence of the areas most affected in the NCA which should receive priority for control.

Anthrax impacts on livestock owners are multifaceted, encompassing animal and human morbidity and mortality, and the loss of livelihoods (World Health Organization, 2010). Anthrax almost always results in the sudden death of livestock, as ruminant animals are very susceptible to the disease. Human infection may have devastating consequences and can lead to morbidity and mortality depending on the route of infection, with gastrointestinal and inhalational anthrax resulting in the highest fatality rates compared to the

cutaneous form of the disease (Turnbull, 1998). Consistent with this, communities in the NCA believe that the gastrointestinal form of the disease is more severe than the cutaneous one. Apart from the direct ill-health caused by infection with *B. anthracis*, and the tangible livestock losses experienced, findings suggest that the outcomes of disease may present psychological health issues to livestock keepers. The depiction of anthrax as a 'thief' that comes unexpectedly portrays the helplessness felt by livestock owners at preventing the deaths. A coping mechanism identified was a denial of the significance of these losses.

Sudden death in animals compromises the ability to recognise and treat infection, which leads to frustrations. Although the disease is very responsive to treatment with antibiotics (WHO, 2008), available treatment options have limited usefulness if the disease cannot be detected early. Indeed, a more viable approach for anthrax control in the NCA would be vaccination. Vaccinating against anthrax in livestock has enabled the control of the disease in countries where it was previously endemic (WHO, 2008). Although there was a low uptake, farmers in the NCA understand the importance of vaccination to prevent anthrax. Understanding the gaps in vaccine availability and accessibility, as well as demand and use, will be critical in order to improve the uptake of vaccination against anthrax.

Practices such as butchering and consuming infected animals pose a risk of anthrax infection (Woods *et al.*, 2004). These are practices that have also emerged in the present study. Animals that are suspected to have died of anthrax in the NCA are not usually disposed of in the manner recommended by local authorities (i.e. by burning or burying) and are processed for food. Consuming such carcasses appears to be an ingrained practice that is difficult to abandon. Food security was cited as an important consideration in deciding the fate of any livestock carcass independently of the suspected cause of death. The practices of handling a carcass for food preparation or opening it to investigate the likely cause of death do not only pose health risks, but also contribute to anthrax persistence by enabling the bacteria to sporulate and contaminate the environment (WHO, 2008).

The risk of contracting anthrax is present even before a carcass is eaten. It might be argued that eating an infected carcass is not as risky as the physical handling of carcasses. Avoiding the consumption of anthrax-suspect carcasses without proper disposal of the whole carcass is unlikely to eliminate the risk of infection. The handling of any animal part contaminated with anthrax spores is also an immediate or potential risk. In particular, the processing of hides may contribute to transmission to an extent that depends on the handler, the process and the prevailing weather conditions (e.g. windy conditions and the risk of inhaling contaminated dust particles).

Interestingly the belief that consuming anthrax-suspect carcasses has a protective effect is widespread in the study area. This is similar to beliefs identified in Zambia (Sitali *et al.*, 2017), but contrasts those in Zimbabwe (Gombe *et al.*, 2010). There is a lack of direct scientific evidence that cooking renders an anthrax carcass safe for consumption. However, Woods *et al.* (2004) found that eating cooked infected meat was not significantly associated with human disease and consuming boiled meat was linked to lower risk of cutaneous anthrax. Such findings can be explained by the fact that *B. anthracis* in an infected host is in the vegetative form which is readily destroyed by heat. The spores, which are hardy and resistant to heat, are only formed with exposure to oxygen and a scarcity of resources. The initiation of spore development can take between 5 to 36 hours to complete (Liu *et al.*, 2004; Baweja *et al.*, 2008). Thus, the local strategy in the NCA of cooking meat multiple times and discarding the stock might decontaminate the meat or reduce the infectious dose and indeed make meat relatively safe for consumption. This hypothesis could be tested using an experimental approach through laboratory confirmation of the absence of viable bacteria. An explanation for the belief of a protective effect of consuming low doses of live bacteria (and numerous inactivated bacteria brought about by cooking) is that this might trigger some degree of immunity, which may reduce the likelihood of infection from further exposure. The possible decontamination of meat from cooking may explain why cutaneous anthrax is a more common form of infection in the area, compared to the gastrointestinal form of the disease. In addition, the infectious dose for gastrointestinal anthrax

is significantly higher than that for cutaneous anthrax which in theory requires only one spore (A.Simpson and S.Lewis, oral communication, August 2017).

The misconception of low risks posed by the handling and consumption of carcasses may contribute to the persistence of these practices and of the disease itself. While cooking may have an effect on potential infection through ingestion, cutaneous anthrax remains a high risk for a person also involved in the butchering of the animal, due to exposure to blood and other infectious parts of the carcass. In addition, during the butchering process, blood is hardly contained, and *B. anthracis* could be introduced into the environment, left to sporulate and await the next suitable host to continue the transmission chain. There is a need to enable and incentivise these communities to properly dispose of whole carcasses suspected to have died of anthrax. However, remoteness of sites where cases mostly occur and lack of resources are likely to compromise the effectiveness of any potential interventions.

Livestock movements are potential contributors to the spread of anthrax. The incubation period for anthrax in animals is between a few hours to three weeks (WHO, 2008). During this period, an infected animal reared in a pastoral livestock system may travel few to several miles away from the source of infection, succumbing to the disease in an entirely different location. Resources that drive the movement of livestock include minerals (obtained from mineral licks), water and grazing pasture, with mineral salts being the most important reason provided by study participants. This might be because pasture and water are readily accessible at least during the wet season, compared to minerals that are not readily available throughout the year. This implies that movements are made in search for mineral salts to areas that are likely contaminated by anthrax spores regardless of the season. Livestock owners in the area associate anthrax risk to congregation of livestock, for instance at water sources or salt licks during the dry season. This may be due to animals drinking water that had been contaminated by runoff during the wet season with spores concentrating over time. Some participants believed that anthrax might also be transmitted through direct contact with an infected live animal, although there is no evidence that this occurs.

It is unclear how much seasonality and environmental conditions drive infections as opposed to changing livestock management practices brought about by altering seasonal conditions. However, the belief that areas rich in minerals are contaminated may align with scientific knowledge of *B. anthracis* persistence and survival in soils rich in calcium (Dragon and Rennie, 1995; Hugh-Jones and Blackburn, 2009). I expand upon environmental drivers of anthrax persistence in Chapter 6.

As disease risks are usually the result of complex interactions among many different factors, multivariable analysis of household characteristics and risk practices was carried out to enable an understanding of the relative contributions of the selected variables to the outcome – the probability of a household having experienced a previous case of anthrax in people and/or animals. This analysis did not aim to establish the causes of anthrax, but the relationship or association that the different factors might have to the outcomes. The results provide evidence that households possessing more sheep and those living in Ngoile or Olbalbal wards were more likely to experience anthrax in their livestock. The qualitative data also suggested that sheep were the most affected livestock species. The modelling was carried out with the number of sheep as a continuous variable, thus a linear relationship was forced on the predictor. However, we envisaged a non-linear relationship and fit an additional model with sheep number as a categorical variable, with results indicating that the probability of a household having animal anthrax becomes significant with more than 20 sheep. With reference to the study setting, this is quite a small number of sheep to be associated with increased anthrax, as the average number of sheep owned by households was 75 (Chapter 2).

The result that sheep are mostly affected by anthrax in the NCA differs from reports indicating a predominance of cattle cases in other areas (Hugh-Jones, 1999; Siamudaala *et al.*, 2006; Chakraborty *et al.*, 2012). These differences may be explained by geography or distinct management practices for different species. It is also possible that more valuable livestock like cattle feature more prominently in disease reports. As this study did not rely on formal records of reported anthrax, which may be biased towards more valuable livestock, we

expect this finding to be a true representation of the situation in the NCA. Discrepancies in disease reports for different species might additionally be explained by variability in the spatial distribution of different species. For example, sheep are the dominant livestock population in the study area and as such might be much more affected by anthrax compared to cattle and goats. With uncertainties in climatic conditions, specifically declining rainfall in East Africa, there has been a recent shift in livestock keeping practices, favouring greater stocking of small ruminants compared to cattle (McCabe, 2003).

Behaviour may also explain the more frequent occurrence of anthrax in sheep. Sheep graze very close to the soil surface where they may be more likely to ingest anthrax spores. Anthrax cases in dry periods have been significantly associated with animals that have predominantly grazing as opposed to browsing behaviours (Hampson *et al.*, 2011). In addition, sheep are known for their sniffing behaviour, which in the arid and dusty risk areas of the NCA may increase sheep's risks of contracting the disease.

Our data from the household surveys did not indicate statistically significant contributions of a number of household characteristics and risk practices to the probability of a household having experienced previous cases of anthrax. Cross-sectional studies are useful for identifying risk factors for infectious disease conditions (Woods *et al.*, 2004; Klun *et al.*, 2006). However, the lack of statistical significance may be due to a number of reasons relating to the limitations of the study or the absence of significant effect of the predictors. One limitation of the study is that the probability of a household having experienced suspected anthrax was based on reports of the disease which is subject to recall or detection bias. The former is more likely as there is evidence that these communities have a very high ability to recognise the disease, as demonstrated by diagnostic confirmation of reported cases (Chapter 4). Recall bias may have resulted in a low proportion of households indicating past cases, thus underestimating the number of affected households. Lack of statistical significance can arise from large variability in the data collected (response variable), leading to a lack of statistical power to detect differences. The lack of effect of many predictors is however plausible, with the risk of contracting

anthrax more dependent on other (e.g. environmental) factors as demonstrated by a significant association with the location of the household.

Although studies may lack statistical significance, they may possess practical significance which considers effect sizes (Nakagawa and Cuthill, 2007). In the case of this study, the odds of experiencing anthrax based on the location of the household and the number of sheep owned provides valuable information on the magnitude of differences in households with previous cases of anthrax, belonging to the groups of predictors explored.

With actions taken towards anthrax prevention hardly existent in these areas (i.e. animal vaccination and proper disposal of carcasses), communities cope with anthrax in ways that may be ineffective or create additional challenges, like the misuse of antimicrobial agents. Antimicrobial agents (e.g. antibiotics) are readily available over-the-counter in Tanzania, and recent studies have shown that antimicrobial use and resistance is highly prevalent in Maasai communities (Caudell, Quinlan, Subbiah, *et al.*, 2017; Caudell *et al.*, 2018). Restricting the use of antibiotics would be much more difficult to achieve when disease incidence is high and access to healthcare and veterinary services is poor. Interventions that prevent anthrax from occurring in the first place will benefit both the control of anthrax and antimicrobial resistance.

Moving to a different location as a coping mechanism during an anthrax outbreak might offer some protection from the disease especially if the corral (or the household location) is the source of infection. However, if any proportion of the herd is incubating (up to 3 weeks) at the time of movement, anthrax could be introduced to the new area (Figure 3.2). During the wet season, when resources that are necessary to keep livestock healthy are available, no (or very limited) livestock movements occur. However, the risk of other diseases such as MCF pushes farmers to move livestock long distances even to known anthrax-risk areas. MCF is caused by Alcelaphine herpesvirus I (AlHV-1) and is associated with wildebeest, despite no clinical disease occurring in this species. In cattle, however, the virus causes a serious and lethal disease, and is a particular problem for pastoralists in East Africa living in close proximity to wildlife

(Lankester *et al.*, 2015). When it comes to choosing between the risk of anthrax and MCF, pastoralists tend to take a risk with anthrax. Another important factor that could be associated with the spread of anthrax and create new foci of the disease is the practice of transporting carcasses and animal products for use in a different location. Depending on the cause of death of these animals, this may contribute to the spread of infections to previously disease-free areas, with implications for a variety of other zoonotic diseases, e.g. Q-Fever.

3.5 Conclusions

This study has examined the local understanding and practices that contribute to anthrax occurrence in people and animals in the NCA. The disease occurs more frequently in certain locations and is most commonly experienced in the dry season, however, cases occur all year round. Communities that are affected most by the disease have a good knowledge of its presentation in animals and humans. Animals are believed to contract the disease from the environment, while humans through contact with an infected carcass as well as from the environment. The two forms of the human disease recognised by the communities are cutaneous and gastrointestinal anthrax. Farmers are often helpless as anthrax can become fatal suddenly when animals are not vaccinated.

This study shows that anthrax is considered an important disease because of its nature, frequency of occurrence and the impact it has on livestock keepers. This further emphasises the conclusions of the analyses presented in Chapter 2, demonstrating the substantial financial loses and perceptions of the importance anthrax. This study presents important findings that may be considered with regards to the dependence on livestock for food and income.

Although the disease has considerable impact on the study communities, local practices such as animal movements to known risk areas, as well as the handling and consumption of carcasses, contribute to an increased risk. Encouraging communities to alter these practices would however come at considerable costs. Negative consequences include the risk of livestock death from starvation, or MCF infections, and food insecurity.

Communities engage in a range of risk-coping strategies, such as the use of conventional and traditional treatment administered by affected households themselves. These strategies may create additional challenges brought about by the misuse of antimicrobial agents. In addition, while the majority of households would consume an animal independently of the cause of death, some participants indicated selectivity in the consumption of anthrax-suspect carcasses. However, given that parts are often kept for other uses, this selectivity might not necessarily preclude the risk of contracting anthrax. Since carcasses are rarely buried, burned or left intact (for decomposition to destroy the bacteria and prevent spores from forming), and might be transported to a different location intact or in parts, these various practices could contribute to the environmental reservoir of *B. anthracis*. Given local reliance on movements of live and dead animals, and their products, a more feasible control strategy would be livestock vaccination. However, only a few households had vaccinated their livestock against the disease when the study was carried out. Several reasons may influence the ability and willingness of communities to vaccinate against anthrax, which would be valuable to explore in a further study.

Chapter 4

Chapter 4 Assessing a modified staining technique for the detection of *Bacillus anthracis* in field samples from endemic areas

4.1 Introduction

For most diseases, including anthrax, detection and confirmation of the presence of the causative agent are essential for case and outbreak management, epidemiological investigations, and for implementing effective surveillance and control measures. Poor diagnostic tests can lead to misdiagnosis and incorrect management of disease cases, with potentially negative consequences. For example, a failure to confirm anthrax in livestock can result in a missed opportunity to take preventative measures – like vaccination and prophylactic treatment – and lead to an outbreak of the disease in animals and humans.

Recognition of the importance of accurate diagnosis, as well as implementation of practices to promote it, are still evolving in many parts of the developing world. Misdiagnosis (and often non-diagnosis) is widespread in developing countries where the diagnosis of many infectious diseases is syndromic (Petti and Polage, 2006). Accurate diagnosis here refers to the detection of the specific causative pathogen. Diagnoses may be correct with regards to disease syndrome, but incorrect with regards to the causative agent. Syndromic (symptomatic) diagnosis is made on the basis of the physical presentation of a disease and treatment options will normally target the possible causes of the syndromes. Syndromic diagnosis differs from etiological diagnosis in which the causative agent of the disease is identified in the laboratory. For instance, febrile illness (syndromic diagnosis) in humans is most often diagnosed as malaria (presumed etiological diagnosis) even though many infectious agents produce febrile illness (Crump and Morrissey, 2013; Prasad *et al.*, 2015). This may result, for example, in incorrect treatment with antimalarial compounds when the actual etiological diagnosis may be bacterial or viral (Maze *et al.*, 2018).

From an epidemiological standpoint, the distinction among different etiological agents causing similar disease presentations is essential for determining the relative contributions of different pathogens to a given syndrome (e.g. sudden deaths in livestock). For zoonotic diseases, detection in animals can serve as an indicator of potential spread to humans due to increased infection risk (Gourdon *et al.*, 1999). With anthrax, infected animal carcasses are an actual source of infection for humans. However, underdiagnosis is likely even more prevalent in animals than in human cases (Kuiken *et al.*, 2005). In many parts of the developing world, such as in Africa, underdiagnosis and misdiagnosis are important reasons for limited availability of data on the prevalence, incidence and impact of many diseases (Maudlin *et al.*, 2009; Molyneux *et al.*, 2011; WHO, 2011). This dearth of data leads to a lack of impetus towards prioritisation of control of these diseases. As a result, some of these diseases are neglected.

Disease underdiagnosis in resource-poor settings is compounded by factors extending beyond resource limitation, to a predominant underemphasis on the importance of accurate diagnosis, limited skilled personnel, poor opportunities for training, lack of binding standards and guidelines, and political indifference (Petti and Polage, 2006). These factors may act either separately or synergistically to hinder surveillance efforts. There remains a critical challenge to improve awareness of the importance of etiological diagnosis, to develop sustainable and practicable field and diagnostic infrastructure and procedures, as well as to train personnel to enable disease detection and confirmation in areas where the need exists.

4.1.1 Diagnostic methods for anthrax confirmation

Confirmation of anthrax is required for the improved control of this neglected disease. Reliable diagnostic tests already exist for the detection of *B. anthracis* and include techniques that employ traditional microbiological methods such as microscopy on stained smears, bacterial culture, and immunological methods such as serology (which can detect *B. anthracis* antigens or antibodies raised against this bacterium (Edwards *et al.*, 2006). In addition to these, molecular

approaches are increasingly relevant to the diagnosis of anthrax (Schwarz *et al.*, 2015).

Culture, polymerase chain reaction (PCR) and microscopy are all recommended for the confirmation of *B. anthracis*, however culture is considered the gold standard by the (WHO, 2008). Culture is particularly useful in isolating *B. anthracis* from aged samples, where fresh blood for smearing cannot be obtained and spores have had the chance to form (Turnbull, 1998). Suboptimal storage of samples caused by difficult field conditions or a lack of storage facilities may also compromise sensitivity of culture. This is especially the case if conditions have not favoured the formation of spores, which are resistant to harsh environmental conditions (Berg *et al.*, 2006; WHO, 2008). Culturing is also useful for detailed biochemical and molecular characterisation of the pathogen. It enables deoxyribonucleic acid (DNA) of sufficient quantity and quality for whole genome sequencing to be generated. Culture must be carried out in laboratory facilities equipped at biosafety level 2+ or ideally level 3, which are commonly lacking in areas where anthrax is endemic. The sensitivity of culture of *B. anthracis* may decrease with the age of the carcass as the bacteria can become non-viable, before sampling is carried out.

PCR has been implemented since the late 20th century and is a sensitive and specific method to detect various pathogens including *B. anthracis*. PCR is achieved by amplifying specific DNA sequences in the *B. anthracis* genome (Makino *et al.*, 1993; Jackson *et al.*, 1998; Berg *et al.*, 2006). Since PCR is based on DNA detection, viable and non-viable bacteria can be identified, therefore, the method can detect pathogens in samples where culture might not be successful. While the possibility exists that the specificity of anthrax PCR may be affected by the high degree of genetic homology *B. anthracis* shares with other species in the *B. cereus* group (Helgason *et al.*, 2000), certain chromosomal targets have shown to be highly specific to *B. anthracis* (Ågren *et al.*, 2013). Molecular methods, however, are difficult to perform in many endemic areas, as few laboratories in developing countries have access to the infrastructure and consumables required for these methods.

In contrast to *B. anthracis* culture and PCR, both of which require sophisticated facilities and equipment, smear stain microscopy is a rapid and simple method requiring limited equipment. It therefore holds substantial value as a field-friendly diagnostic method in resource limited settings. The ideal diagnostic test for any disease in resource limited setting would meet WHO's 'ASSURED' criteria. This term describes the ideal test as Affordable, Specific, Sensitive, User-friendly, Rapid and robust, Equipment free, and Deliverable to those who need it (Mabey *et al.*, 2004). An ASSURED test should meet both scientific criteria (e.g. sensitivity and specificity) – which may be prioritised in a reference laboratory – and convenience criteria (e.g. user-friendly, equipment-free and affordable), prioritised in field conditions. Developing an 'ASSURED test' may be difficult in practice, as one characteristic might come at the expense of another, and it is best to weigh the importance of a given characteristic for the confirmation of a particular disease in a particular situation. A common example is compromising on either the sensitivity or the specificity of a test in order to improve the other (Ruxton and Colegrave, 2010). An 'ASSURED test' may not necessarily be a gold standard but can be used in place of the gold standard if it is more available or practical (4.1.3).

4.1.2 Microscopy for *B. anthracis* detection

Detection of *B. anthracis* by microscopic examination of a stained smear is a rapid, inexpensive and simple technique (WHO, 2008). Microscopy is applied to a range of samples and is a commonly used method in resource-limited regions for detection of microbial pathogens and anthrax confirmation. Many laboratories in anthrax endemic areas may rely only on the morphological characteristics of *B. anthracis*, revealed through stain microscopy, to make a diagnosis, using a variety of easily available stains such as Gram stain, Field stain, methylene blue and other Romanowsky-type stains (WHO, 2008; Horobin, 2011; Lembo *et al.*, 2011). Smear samples revealing characteristic short chains of two or three bacilli with square ends may be classified suspect for anthrax without the presence of visible capsular material surrounding the cells (WHO, 2008). For example, Gram staining is used as a presumptive identification method in many conventional laboratories (Swartz, 2001; Begier *et al.*, 2005), however, it may not

differentiate *B. anthracis* from numerous other Gram-positive organisms such as *Clostridium chauvoei* or *B. thuringensis*, especially because it is a non-capsule staining method. The WHO recommends polychrome methylene blue (PMB) and Giemsa-type stains for anthrax diagnostic purposes even though Giemsa stain produces variable results in relation to capsule identification (WHO, 2008).

Chemistry of the staining procedure for *B. anthracis* capsule identification

In 1903, M'Fadyean established capsule staining with PMB as a reliable technique to detect *B. anthracis* and confirm anthrax (M'Fadyean, 1903b). The capsule is a key component of *B. anthracis'* complex surface structure and contributes to the pathogenicity and virulence of the bacterium (Mock and Fouet, 2001). The capsule is a specific feature of *B. anthracis* and is not usually produced by closely related bacteria in the same genus such as *B. cereus* and *B. thuringensis* (Vilas-Bôas, *et al.*, 2007). Although a capsule may be present in other bacterial species, this is most commonly found in Gram negative bacteria (Cross, 1990). Gram-positive bacteria possessing capsules – such as some species of *Streptococcus* – are usually morphologically different from *B. anthracis*. For example, *Streptococcus pneumoniae* is coccus-shaped. Thus, identifying the capsule of Gram-positive *B. anthracis* in relation to its distinct blunt-ended rod shape provides a highly specific detection of the pathogen.

Staining with PMB remained a highly reliable rapid diagnostic tool for decades at a time when anthrax was endemic throughout the world. This technique contributed to the control of the disease in developed countries when implemented in combination with measures like vaccination (Turnbull, 1998; Owen *et al.*, 2013). Quality-controlled commercially available PMB became hard to obtain as anthrax became rare in developed countries. As a result, tests were increasingly conducted with alternative methylene blue-based stains, leading to diagnostic inaccuracies and confusion (Forshaw *et al.*, 1996) and portrayal of the M'Fadyean smear staining technique as unreliable (Owen *et al.*, 2013). Indeed, as far back as 1903, M'Fadyean had noticed variability in the ability of methylene blue stains to detect *B. anthracis* (M'Fadyean, 1903a, 1904). Forshaw *et al.* (1996) also described this difference, which has been attributed to the variable presence of products formed naturally with time from the

demethylation and oxidation of methylene blue (Figure 4.1). These products include azures A, B and C (Turnbull, 1998; Owen *et al.*, 2013). These derivatives, formed from the oxidation and demethylation of methylene blue, and azure B in particular, are credited with conferring the stain its metachromatic capsule staining property (Owen *et al.*, 2013). The metachromatic property of a biological material is its ability to retain a colour different from that of the stain. In the case of *B. anthracis*, the capsule usually takes on a pink colour different from the blue/purple colour of the stain taken up by the bacterial cell. The concept also applies to stains, and is the ability of the stains to change their colour when in contact with certain biological materials (Walton and Ricketts, 1954).

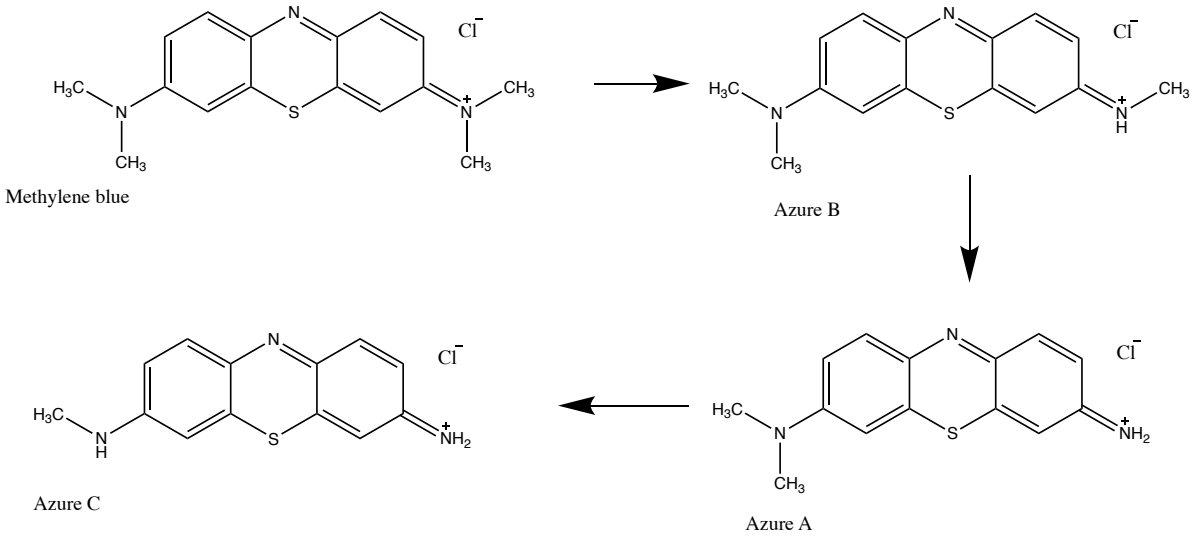


Figure 4.1: Oxidative demethylation of methylene blue and the formation of azures A, B and C. Adapted from Owen *et al.* (2013). Image has been produced in ChemDraw Prime version 16.0.1.4

Oxidative demethylation, and thus development of capsule staining ability, requires up to 12 months or more to occur, a process known as 'ageing'. This, in addition to the unavailability of quality-controlled PMB, is a limitation for the rapid confirmation of anthrax in the field. The requirement for ageing also makes it difficult for laboratories to initiate testing for *B. anthracis* using microscopy and makes protocol standardization difficult. Replacing PMB, which needs to be converted into metachromatic compounds, with azure B, which is a metachromatic reagent, might improve the ability to detect *B. anthracis* using stain microscopy for anthrax confirmation. This replacement may reduce the

limitations associated with obtaining quality-controlled PMB and decrease the time required for its capsule staining property to develop. The staining procedure utilising azure B in place of PMB has been assessed on smears prepared from bacterial culture of *B. anthracis* (Owen *et al.*, 2013), but not on blood smear samples obtained directly from suspected anthrax carcasses. It is therefore unknown what the performance of this technique is for *B. anthracis* confirmation in field samples that have not undergone bacterial propagation. This study therefore aimed at validating this technique on field samples. In this case, the validity of a test is the ability of the test to distinguish between *B. anthracis* positive and negative samples. The term validity can be used synonymously with accuracy (Greiner and Gardner, 2000) and both are indicators of test performance.

4.1.3 Evaluating the performance of diagnostic tests

The value of diagnostic tests in improving disease management can be evaluated based on a number of criteria that can be broadly classified into scientific and convenience criteria (Reitsma *et al.*, 2005). Convenience criteria relate to the practical aspects of test implementation such as capacity to conduct the tests, costs, and turn-around time. Scientific criteria on the other hand, encompass the diagnostic performance of the test. This can be measured in a number of ways, including its sensitivity and specificity, likelihood ratios, repeatability (i.e. inter-rater agreement), etc. (Florkowski, 2008). Convenience and scientific criteria are key to the choice of a test in any setting (Reitsma *et al.*, 2005).

The core aim of diagnostic testing is to ascertain the presence or absence of a disease condition or its causative agent. To be high performing, a diagnostic test must possess the ability to accurately and precisely detect a disease or pathogen. Sensitivity and specificity are the most common parameters used to characterize the performance of diagnostic tests (Hui and Zhou, 2014). Sensitivity refers to the proportion of true positive samples that are test positive ("the ability of the test to recognize disease or pathogen presence"), while specificity is the proportion of true negatives that test negative ("the ability of the test to recognize health or pathogen absence"). High sensitivity means that

there are few false negative results, high specificity means that there are few false positive results. Which feature is more important is context dependent. For detection of anthrax in humans, false negative diagnoses must be avoided so that no patient goes untreated. This requires high sensitivity. However, for other diseases or situations, especially those where a false positive diagnosis might result in culling of animals or major investment in vaccination campaigns to control transmission, specificity might be more important.

Inter-rater agreement measures the concordance between two or more observations and is useful in understanding if agreement or disagreements in test results are a function of chance (Viera and Garrett, 2005). This measure of test performance is particularly useful in tests where subjectivity is involved e.g. microscopy.

Estimating sensitivity and specificity

The conventional approach to estimating test sensitivity and specificity requires a knowledge of the true state of a case. For example, the performance of a test used to confirm anthrax can be evaluated by identifying true positive and true negative animals using a gold standard test, and then estimating the sensitivity of the test from positive animals and the specificity of the test from negative animals (Toft *et al.*, 2005). The challenge with this approach, however, is that true gold standard tests rarely exist, and so-called gold standards may therefore not determine presence of a disease or pathogen with absolute certainty. By definition, gold standard tests do not necessarily imply perfect performance, but are the best available tests for a given condition or setting (Reitsma *et al.*, 2009). They may therefore have limitations in their estimates of the disease or infection status or other status of interest as the case may be. The term 'reference standard' has been proposed as a more appropriate term (Reitsma *et al.*, 2009), however the limitations remain. Thus, assessment of a diagnostic test should be (and is) achievable without a reference standard. Latent class analysis (LCA) is an approach that can be applied to the assessment of diagnostic tests in the absence of a reference standard.

LCA is a statistical approach widely applied to estimating sensitivity and specificity, and thus the performance of a diagnostic test without a reference standard. The phrase “latent class” is used because the true status in the absence of a perfect reference standard is latent and will be unknown (Rindskopf and Rindskopf, 1986; Enøe *et al.*, 2000). This is important for identifying test limitations since observed measures or results obtained from non-gold standard tests are inaccurate measures of the true status.

In order to establish the true but latent status of a sample (which will in turn enable evaluation of the performance of a given test) in the absence of a reference standard using LCA, multiple diagnostic tests are required. One of the earliest LCA approaches based on the Hui and Walter paradigm (Hui and Zhou, 2014) requires certain conditions to be fulfilled. These include that for comparison between two tests, 1) the tests are conditionally independent given the disease status (or condition) measured; 2) the cases tested can be divided into two or more populations and 3) the test properties (i.e. sensitivity and specificity) are constant in the populations (Toft *et al.*, 2005). The assumption of conditional independence implies that the probability of a test result is unaffected by the knowledge of another test result on the same subject (Hui and Zhou, 2014). In many cases, this implies that tests are based on different criteria, e.g. the detection of DNA and the direct detection of viable pathogen. The two-population condition is a result of problems with model identifiability caused by the need to estimate more test parameters than the degrees of freedom in the data (Joseph *et al.*, 1995). For example, to assess two tests using data from one population, five parameters need to be estimated. These include the sensitivity and specificity of both tests and the prevalence of the condition in the population; however, the data contains three degrees of freedom. When represented in a 2X2 table with four classes (namely, true positives, true negatives, false positives and false negatives) knowledge of the total sample size and data in any three of the four cells fixes the data in the fourth cell. In a two-population situation, six parameters are being estimated but with six degrees of freedom available in the dataset.

Nowadays, Bayesian approaches to LCA are preferred over the original Hui and Walter frequentist approach, as they provide a better framework to account for conditional dependence and improve model identifiability and the precision of estimates through the use of prior information (Toft *et al.*, 2005). LCA approaches have been employed for estimating the performance of diagnostic tests for a variety of diseases including brucellosis (Muma *et al.*, 2007), melioidosis (Limmathurotsakul *et al.*, 2010) and leptospirosis (Limmathurotsakul *et al.*, 2012). The work described in this thesis chapter focused on microscopy and PCR to detect *B. anthracis* and estimate the true status of the samples and thus the performance of the tests.

4.1.4 PCR for *B. anthracis* detection and surveillance of anthrax

When compared to bacterial culture and microscopy, PCR is a relatively new technique. PCR detects genetic material in a sample, including bacterial DNA. The infrastructure required for PCR may only be available in a select number of facilities nationally. This is especially the case in low-income countries, meaning that PCR is not routinely used as a diagnostic tool. In Tanzania, for example, district veterinary laboratories such as the Ngorongoro Conservation Area laboratory, situated in close proximity to areas affected by anthrax, are those most likely to receive anthrax-suspect samples but are not equipped to carry out PCR. However, this type of infrastructure exists in larger regional or zonal laboratories, such as the Kilimanjaro Clinical Research Institute (KCRI). Molecular methods are considered to be more sensitive than microscopy and can be valuable for confirming negative results obtained by microscopy, especially when samples are collected from older carcasses (Berg *et al.*, 2006). In addition, they are useful for molecular typing and characterisation of *B. anthracis* (Okinaka *et al.*, 2008). Therefore, strategies to enable the use of molecular techniques for at least a second-line diagnostic tool might improve surveillance and understanding of the disease in these areas.

Laboratories in low-resource settings may possess archived blood smear samples used for diagnosis based on microscopy techniques, kept either as references or for re-testing and further analysis in the future, when resources and

infrastructure become available. In such situations, it is worth understanding if stained smears on slides already examined by microscopy are useful for the detection of *B. anthracis* using PCR.

If PCR is to be used on stained smears, it is important to evaluate the sensitivity of the test on stained material because the process and chemical reactions involved in the preparation of smear specimens could affect the DNA quality of the sample, thus reducing the sensitivity of the technique. For example, fixing blood smears with formalin has been shown to affect the ability to detect pathogens using PCR, as formalin crosslinks and damages DNA (Douglas and Rogers, 1998). The effect of histological stains on PCR in the context of bacterial pathogens has been poorly studied. It is therefore unknown whether histological stains may damage DNA or affect its integrity or quality (e.g. by intercalating between the genetic material) (Murase *et al.*, 2000; Horobin, 2011).

In addition to the advantage of achieving retrospective testing of archived stained smear samples using molecular methods, the ability to utilise the same sample material for multiple tests is valuable. It is not only useful for maximising the value of samples collected during surveillance efforts, particularly in areas where sampling and storage facilities are limited due to resource constraints, but might also aid in situations in which limited sample collection is encouraged, as is the case for dangerous pathogens.

4.1.5 Objectives

The objectives of carrying out this work were to

1. Assess the performance (sensitivity, specificity, inter-observer agreement, and stain deterioration over time) of azure B, a recently proposed alternative stain for the identification of *B. anthracis* by microscopy, and compare its performance to PMB, Giemsa and Rapi-Diff II - stains commonly used to diagnose bacterial infections.

2. Assess the potential usefulness of stained and unstained blood smears for detection of *B. anthracis* by means of PCR.

The relationship between different testing methods and objectives is summarised in Figure 4.2.

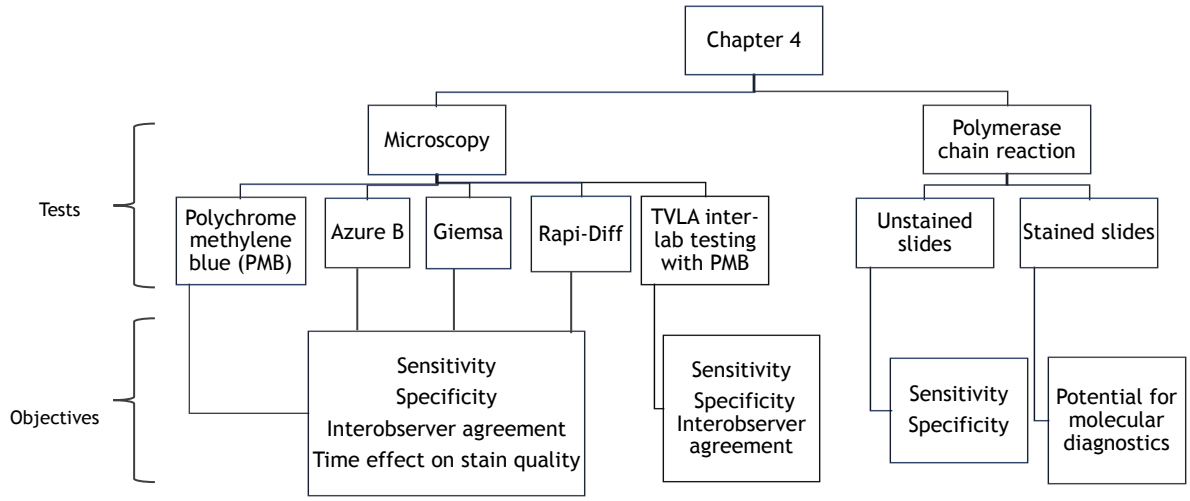


Figure 4.2: Schematic representation of the work carried out in this study

4.2 Methods

This study employed the field-based active surveillance system that was set up within the NCA – as detailed in Chapter 2 – to follow up on deaths in livestock suspected to be caused by anthrax and to obtain samples for confirmation of the disease based on identification of *B. anthracis*. Blood smear samples were tested for *B. anthracis* using azure B, PMB, Giemsa and Rapi-Diff stain microscopy techniques and by qPCR as detailed in subsequent sections. The performance of azure B and the other techniques were assessed using qPCR as the reference standard because culture could not be performed with the available infrastructure. Polymerase chain reaction is considered superior to stain microscopy and has been used as reference standard in several studies (Morgan *et al.*, 1998; Berg *et al.*, 2006; Niloofa *et al.*, 2015). The sensitivity and specificity of the tests were also determined by conducting LCA assuming no reference standard. Both analyses were carried out in order to compare results and understand the implications of assuming an imperfect test as the reference standard, and of using the LCA approach. Inter-rater comparisons were conducted for the microscopy tests. In addition, the effect of time on the ability to visualise the capsule was evaluated. Finally, the molecular diagnostic value of stained blood smears was also assessed in this study. The methodology for this study is described in detail below.

4.2.1 Sample collection

Blood smear samples were obtained from carcasses of animals suspected to have died of anthrax in the field (Figure 4.3). The clinical presentation of anthrax is characterised by sudden death of an apparently healthy animal, and often the presence of blood oozing out of the natural orifices of the carcass, as well as rapid decomposition of the carcass, etc. (Beyer and Turnbull, 2009). It was not possible for the investigating field team to obtain blood smear samples from all cases investigated, as in many cases, only dry animal skin was available (see Figure 4.7). Smear samples were collected from carcasses less than a week after death. In the field at the site of the carcass, a drop of blood obtained with a syringe was smeared onto a slide using a second slide. Per carcass, multiple slides were prepared. Smears were air dried, and slides were carefully wrapped

in paper towel and sealed in primary and secondary Ziplock bags for transport to the laboratory.

Figure 4.3: Sampling suspected anthrax carcasses in the Ngorongoro Conservation Area (NCA). An animal suspected to have died of the disease and a community animal health worker ready for sampling (a). Blood smears are made (b) and samples are packed into primary and secondary bags (c). Samples are then stored in a locked cabinet in the NCA laboratory (d) prior to transporting them to the Kilimanjaro Clinical Research Institute laboratory

The doctoral researcher trained livestock officers to safely collect and store samples, and participated in initial sample collection (10% of samples). Most samples were therefore collected by the field team. Smears were stored at

ambient temperature at the NCA laboratory prior to transporting them to KCRI in Moshi, Tanzania. Smeared slides were opened for further processing in a class 2 biosafety cabinet within the biosafety level 3 laboratory facility at KCRI. Metadata associated with these cases were collected along with the samples (Appendix 3 for the sample collection forms). Data were collected on species, age and sex of the animal, the location, and body condition before death.

4.2.2 Microscopy

Staining procedures

After receipt of slides in the laboratory, they were organised into slide boxes in preparation for staining. Each of the multiple smeared slides prepared per carcass were stained with a single stain, allowing diagnostic testing of a single carcass with multiple stains. Three staining techniques utilizing azure B, Giemsa and Rapi-Diff II stains were applied to each of multiple blood smear samples from individual carcasses (n=152) sampled between June 2016 and November 2017. M'Fadyean PMB staining was carried out when four smear samples were available from a carcass (n=102). One positive control slide obtained from the Rare and Imported Pathogens Laboratory, Public Health England (PHE), was included in each staining batch of up to 12 slides. The control slides consisted of smears of *B. anthracis* isolated from pure culture and served as control for the staining method without providing information about the impact of smear technique, sample storage or sample transportation. The control smears were fixed in formalin and heat inactivated as standard procedure carried out by PHE. The staining procedures were as follows.

Azure B: Azure B staining solution was prepared according to the method of Owen *et al*. (2013). Briefly, 0.23% azure B (VWR, United Kingdom) was obtained by dissolving 0.03g of azure B powder in 3ml of 95% ethanol. After gentle swirling for about 30 seconds, 10ml of 0.01% potassium hydroxide solution was added. Air-dried smears were fixed in 99% ethanol for 1 minute and allowed to air-dry. The fixed smears were stained by spreading a drop of azure B solution using a Pasteur pipette and leaving it in contact for approximately 5 minutes. The stain was washed off with water into chlorine solution greater than

10,000ppm, as part of biosafety measures and to ensure inactivation of any viable *B. anthracis*.

Giemsa: Giemsa stock solution (0.72% (w/v)) was prepared by dissolving 0.5g Giemsa stain (Sigma-Aldrich, Germany) in 27ml glycerol (Sigma-Aldrich, Germany). The solution was heated to 60 °C for 2 hours and then cooled to room temperature, after which 42 ml of methanol was added. The stain was kept in a dark area for more than 3 months to mature. Working solution was prepared by diluting the stock solution 1:20 in phosphate-buffered saline (PBS). Air-dried smears were fixed in absolute methanol for five to seven minutes and left to air dry. The smear was stained for 60 minutes. Stain was washed off with deionised water into chlorine solution greater than 10,000ppm.

PMB: Polychrome methylene blue stain (BDH Chemicals, United States of America) was obtained from Public Health England, having aged for more than 12 months and up to 10 years. Staining was carried out according to WHO guidelines (WHO, 2008). Smears were fixed by dipping in 99% ethanol for 1 minute. Fixed smears were allowed to air dry before adding a drop of PMB stain, which was spread to cover the smear completely. The stain was left on the smear for 30 to 60 seconds and then washed off with water from a wash bottle into chlorine solution greater than 10,000ppm.

Rapi-Diff: Rapi-Diff II stain kit (Vetlab Supplies, United Kingdom) was used as obtained directly from the manufacturer. Reagents included in the kit were methanol-based fixative solution (solution A), eosin Y dye in phosphate buffer (solution B), and polychrome methylene blue in phosphate buffer (solution C). Aliquots of each solution were dispensed into staining containers. Air-dried smears were fixed by dipping slides into solution A for approximately five seconds. The slides were then removed and transferred immediately into solution B, dipping and withdrawing the slides every two seconds for a total of five times. Excess stain was drained with paper towel and the stain was completely rinsed off using PBS from a wash bottle into chlorine solution. Slides were transferred into solution C, dipping and withdrawing the slides five times

with two second-intervals in between each dip. Solution C was washed off with PBS into chlorine solution greater than 10,000ppm and the slide allowed to dry.

Smear examination

Stained slides were numbered, packed arbitrarily into slide boxes and examined using a light microscope (magnification X1000). The slides were examined following no particular order and smears were considered positive if blue or purple square-ended rods were observed surrounded by a pink or pinkish-red capsule or 'shadon', a remnant of capsular material (Turnbull, 1998), using a modified protocol to define the quality and strength of capsule presence based on metachromatic property of the stains and the ability to clearly demarcate the capsule from the cells (Owen *et al.*, 2013). Scores were assigned to each slide based on the chart in Figure 4.4. All samples processed with the different stains and examined by microscopy were classified as positive only if the presence of bacilli with characteristic square ends and capsular material surrounding the cells were observed. Thus, samples with a capsule score of +/-, 1+, 2+ and 3+ (Figure 4.4) were classified as positive for anthrax. For all these possible scores, there was clear evidence of the presence of a capsule.

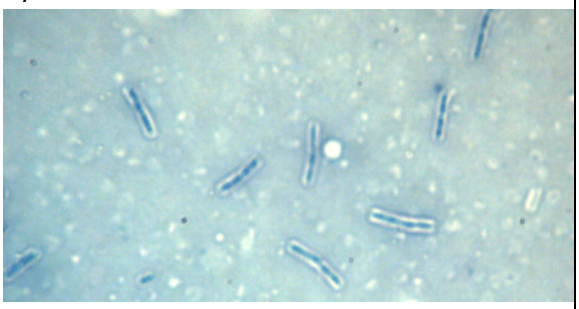
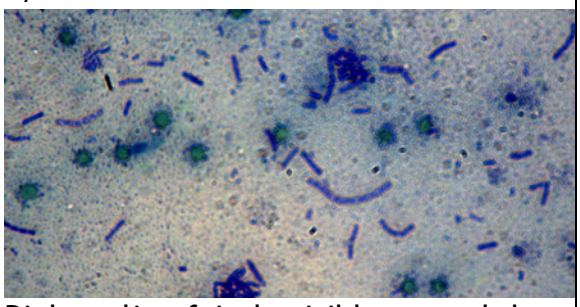
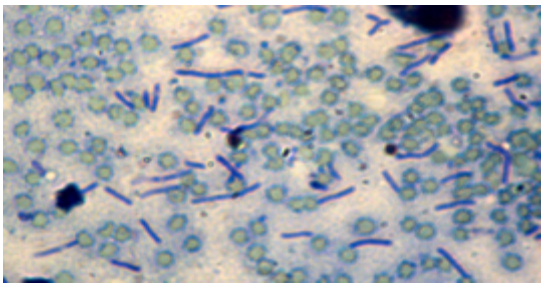
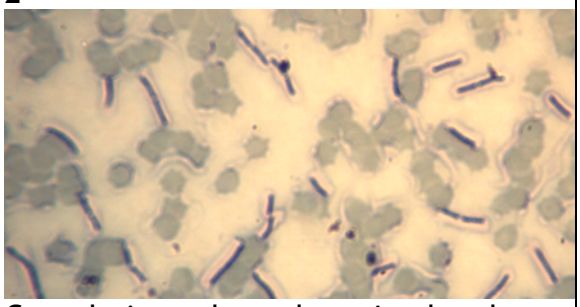
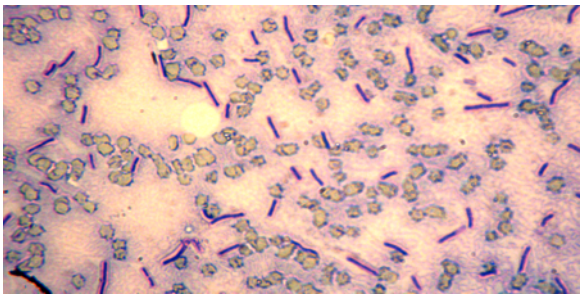
<table>
<tr><td>+/-
 
Presence of capsule not metachromatic.</td><td>+/-
 
Pink outline faintly visible around the bacilli, but not demarcated around the bacilli.</td></tr>
<tr><td>1+
 
Capsule is visible as a faint pink weakly demarcated around the bacilli.</td><td>2+
 
Capsule is moderately stained and well demarcated.</td></tr>
<tr><td colspan="2">3+
 
Capsule is strongly stained and well demarcated.</td></tr>
</table>

Figure 4.4: Chart used to establish presence and strength of *Bacillus anthracis* capsule material. The strength of the capsule is based on the presence of a demarcated capsule or a 'shadon' surrounding the cell and the metachromatic property of the capsule (Owen et al., 2013). The chart is interpreted subjectively, and all categories are interpreted as *B. anthracis* positive as they show evidence of the presence of a capsule. Samples with a +/- score are so indicated due to the absence of either a demarcated or metachromatic capsule. Images were obtained from pictures of slides examined in the study.

Inter-rater agreement

Two experiments were carried out to assess the agreement between two observers. The first involved the same batch of slides stained by one observer

and viewed by two observers. This was carried out on slides that were used to assess the performance of the four stains. The second procedure involved the second observer independently fixing and staining a different set of slides made from the same animal cases and assessing them independently. This second approach was carried out using only the azure B stain to further assess the reproducibility of results obtained from this new technique. In assessing the agreement (or disagreement) between different observers, we used Kappa statistics (Viera and Garrett, 2005) to measure inter–rater agreement and to quantify the consistency of observed agreement.

Cohen's Kappa was used to assess inter-rater agreement in the irr package (Gamer *et al.*, 2012b) in R, based on an equation used for two observers or two observations:

$$\mathcal{K} = \frac{P(a) - P(e)}{1 - P(e)} \quad (1)$$

In the equation $P(a)$ is the proportion of observed agreement by two observers, and $P(e)$ is the probability of agreement due to chance. As Kappa values may be affected by the prevalence of anthrax in the samples tested and bias between observers expressed in the proportion of samples determined as positive and negative (Byrt *et al.*, 1993; French *et al.*, 2016), prevalence- and bias-adjusted Kappa (PABAK) values were computed using formula 2

$$\mathcal{K}_{PABAK} = 2P(a) - 1 \quad (2)$$

Guidelines for the assessments of the statistic indicate that estimates of Kappa greater than 0 represent some degree of agreement above what could be observed by chance. Generally, a range of 0.01 to 0.20 indicates slight agreement, 0.21-0.40 indicates fair agreement, 0.41-0.60, moderate agreement, 0.61-0.80, substantial agreement and 0.81-0.99 nearly perfect agreement. A score of 1 indicates perfect agreement (Cohen, 1960; Viera and Garrett, 2005). Although used to rule out the possibility of test agreement between two observers occurring due to chance, the Kappa estimate itself may be obtained

due to chance. To avoid this, a *P* value was obtained to determine whether the Kappa statistic is significantly different from what would be expected as the result of chance (Viera and Garrett, 2005).

Effect of time on the quality of stained smears

A second microscopic examination was carried out on stained blood smears 6 months after staining in order to evaluate the degree of variability in repeated assessments, i.e. the deterioration of stain quality on smears over time. The first and second examination were carried out by a single observer. Cohen's Kappa statistics described above were applied to measure the agreement between the first and second examination.

Between-laboratory diagnostic test assessment

A subset of blood smear samples (n=66) was assessed independently by the Tanzania Veterinary Laboratory Agency (TVLA) zonal veterinary centre in Arusha, which is responsible for veterinary diagnostic services within the study region. Here, smears were processed by laboratory personnel following their routinely used protocol with PMB stain, prepared in TVLA and aged for 4 years. This procedure is hereafter referred to as the TVLA technique. The sensitivity and specificity of their protocol were calculated using the true status of the carcasses determined by the LCA (detailed in 4.2.4). The agreement with the results of the azure B and PMB techniques carried out as part of this study was determined using the Kappa statistic described above.

4.2.3 Quantitative polymerase chain reaction (qPCR)

Assessment of qPCR methodology for blood smears

An experiment was carried out to determine if the quantity of blood present in a smear would be sufficient for testing by qPCR. The experiment was aimed at assessing if and what amount of smear scrapings may yield detectable DNA upon extraction and amplification of target *B. anthracis* sequences. One new, unstained blood smear from each of 5 carcasses identified as positive by microscopy testing was selected. Two weights of scrapings were obtained from each slide. One scraping contained all material from the thinnest half of the smear and a second scraping comprised a ~1cm diameter section of the thicker

part of the smear (Figure 4.5). The weights of the sample used to determine the minimum amount of scraping required for detecting *B. anthracis* ranged from 0.2mg to 3.5mg (Appendix 6).

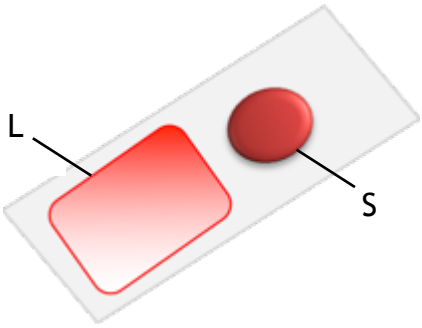


Figure 4.5: A depiction of a slide showing sections from where scrapings were obtained to determine the ability to detect *B. anthracis* DNA blood smear scrapings using qPCR. Scrapings were obtained from one half of the slide (L) and from a portion of thick smear (S)

DNA extraction

Extraction was conducted with a modified protocol of the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Germany). Smear scrapings were collected in a 1.5ml microcentrifuge tube. After this, 200µl PBS and 20µl of 20mg/ml proteinase K were added to the tubes. Lysis and spin-column protocols were carried out according to manufacturer's instructions. No-template controls (NTCs) were included by performing extraction with no smear scraping (i.e. taking only reagents through the extraction process.

qPCR

Taqman probe-based assays were carried out on the Rotor-Gene Q platform, targeting one chromosomal sequence (*PLF3*) (Wielinga *et al.*, 2011) and two plasmid targets, *cap* (pXO2) and *lef* (pXO1) sequences. Primer and probe sequences for the plasmid targets were obtained from protocols provided by Rare and Imported Pathogens Laboratory, Public Health England, as shown in Table 4.1.

Table 4.1: Primer and probe sequences used in the qPCR reactions targeting two plasmids and one chromosomal sequence in the *B. anthracis* genome

Name of primer/probe	Sequence	Target	Reaction concentrations
BA cap fwd* TM	GAA GCA GTA GCA CCA GTA AAA CAT C	pXO2 plasmid	300 nM (0.6 µl of 10µM)
BA cap rev$^{\delta}$ TM	CTT TTA CGT GAC GTC CCA TCA	pXO2 plasmid	900 nM (1.8 µl of 10µM)
BA cap prb$^{\gamma}$ TM	FAM TTG ACG ATG ACG ATG GTT GGT GAC A BHQ1	pXO2 plasmid	250 nM (0.5 µl of 10µM)
BA lef fwd* TM	GGA ACA AAA TAG CAA TGA GGT ACA AGA	pXO1 plasmid	900 nM (1.8 µl of 10µM)
BA lef rev$^{\delta}$ TM	TTC CGG TGC ATA AAG CTG TAA AAC	pXO1 plasmid	600 nM (1.2 µl of 10µM)
BA lef prb$^{\gamma}$ TM	FAM TTG CAT ATT ATA TCG AGC CAC AGC ATC GTG BHQ1	pXO1 plasmid	250 nM (0.5 µl of 10µM)
PLF3_f*	AAAGCTACAAACTCTGA AATTTGTAAATTG	Chromosomal sequence	250 nM (0.5 µl of 10µM)
PLF3_r δ	CAACGATGATTGGAGA TAGAGTATTCTTT	Chromosomal sequence	250 nM (0.5 µl of 10µM)
Tqpro_PL3$^{\gamma}$	FAMAACAGTACGTTTCA CTGGAGCAAAATCAAB HQ1	Chromosomal sequence	150 nM (0.3 µl of 10µM)

* Forward primer
δ Reverse primer
γ Probe

Lyophilised primers and probes were resuspended using nuclease free water to produce 1mM stock solution and then working solutions of 10µM were made by diluting the stock solution 1:100. Reagents were stored at -20°C when not in use. Master mixes sufficient for the number of sample extracts to be tested including NTCs and qPCR controls (18µl per reaction) were prepared as follows: 2X PrimeTime® Gene Expression Master Mix and 10µM primers and probes were thawed, vortexed and centrifuged. For each reaction, primers and probes were added to 10µl of PrimeTime® Gene Expression Master Mix according to Table 4.1 and made up to 18µl with nuclease free water. The mixture was vortexed and centrifuged briefly.

In the template room, 18µl of the master mix was added to each qPCR spin tube and 2 µl of the template DNA was added to make a reaction volume of 20 µl, with one sample's DNA extract per tube. Negative and positive qPCR controls were included in each run and for each sequence target to check the quality of the process. DNA extracted from a previously confirmed *B. anthracis* positive sample was used as positive control. The master mix was used as template for the negative control. The cycling conditions for each run were as follows: (1) activation/denaturation at 95 °C for 3 minutes, and (2) amplification, using 40 cycles of 60 °C for 35 seconds and 95 °C for 5 seconds.

Application of qPCR methodology: testing for *B. anthracis* on all blood smear samples

Following the observation that smear scrapings yielded detectable quantity of *B. anthracis* DNA (see 4.3.4), testing was carried out on all smear samples available (n = 152). One dried blood smear from each carcass sampled was selected. Blood smears were scraped off from the entire slides into microcentrifuge tubes, after establishing that the quantity of blood on a slide is sufficient for DNA extraction and qPCR. DNA extraction and qPCR were conducted according to the methodology described above. Extraction was carried out in batches of up to 23 scrapings. No-template controls (NTCs) were included in each extraction batch by performing one or two extraction(s) with no smear scraping. The number of NTCs included per batch (one or two) was chosen so as to give an even number of extractions for balancing of the centrifuge.

PLF, *cap*, and *lef* sequences were targeted in each sample. At the time of the qPCR testing, cut-off Ct values were determined based on the results of the NTCs. An arbitrary cut-off value was set at 36 for batches in which no amplification was observed for the NTCs. This cut-off was applied across all three targets in order to account for problems with the qPCR, specifically, amplification artefacts such as may occur from cross-contamination or the degradation of probes (Caraguel *et al.*, 2011). Those samples with Ct-values less than or equal to 36 were called positive for the respective target, while those with Ct values above 36, as well as those with no amplification were negative. It was intended to repeat the PCRs or adjust the cut-off value if any amplification

of NTCs occurred. Samples in which all three targets were amplified below the cut-off were considered positive for *B. anthracis*.

Evaluating the diagnostic value of blood smear samples for molecular detection of *B. anthracis*: stained slides

A subset of blood smear samples from carcasses testing positive for *B. anthracis* by both qPCR of unstained slides and microscopy (n=15), and PHE control slides (n=2) were used to determine the usefulness of stained slides for molecular analysis. Four blood smears, each stained with one of the four stains described in 4.2.2, namely PMB, azure B, Giemsa and Rapi-Diff, as well as one unstained slide, were selected for each of the 15 carcasses and the two PHE controls, bringing the total number of stained and unstained smears assessed in this to 68 and 17, respectively. The smear from each slide was scraped off and the DNA was extracted. qPCR was carried out on the extracted DNA using the process described above. Primers and probes for the chromosomal target were used in the qPCR reaction. The cycle threshold (Ct) values for stained smear samples were compared to values for unstained samples.

4.2.4 Statistical analysis

Results were collated in Excel version 15.39 (Microsoft Office, 2016) and analyses conducted in R version 1.1.419 (R core Team, 2017). The relationship between the weight of the input smear scraping and the resulting Ct value was explored using a linear model.

Sensitivity and specificity of the tests were calculated in comparison to qPCR as a reference standard (using equations 3 and 4), and by using a Bayesian latent class model (LCM). Inter-rater agreements and deterioration of stain quality over time were assessed for the different tests using Kappa statistics in R with the irr package (Gamer *et al.*, 2012a).

$$Sensitivity\ (se) = \frac{Test\ positives}{Reference\ positives} = \frac{Test\ positives}{True\ positives + False\ negatives} \quad (3)$$

$$Specificity(sp) = \frac{Test\ negatives}{Reference\ negatives} = \frac{Test\ negatives}{True\ negatives + False\ positives} \quad (4)$$

Latent class modelling

To assess how well the tests identify true anthrax cases in the absence of a reference standard, we assumed two latent classes (or hidden states) for each of the cases studied – anthrax true positive and anthrax true negative carcasses. However, these two classes may comprise further sub-divisions such as anthrax-positive cases with co-infections – e.g. with *Clostridia* spp.

In order to estimate the sensitivity and specificity for each test in the absence of a gold standard, a LCM was applied. The model is equivalent to an extension of the standard Hui Walter model, but the formulation is more similar to that of a state space model where there is a formal separation of the observation layer (consisting of the imperfect tests conditional on the sample statuses), and the underlying process layer (consisting of the presence of *B. anthracis* DNA and detection of bacilli with capsule in the samples conditional on the anthrax disease status of the carcass). These types of models are also known as latent process (or latent class) models and can be described as a generalised form of a mixture model in which the latent classes are related to each other in some way rather than being independent (Ghahramani and Jordan, 1997). This analysis used diagnostic test results described here and in Chapter 5, section 5.2.1. A breakdown of the data informing the LCM is shown in Figure 4.6.

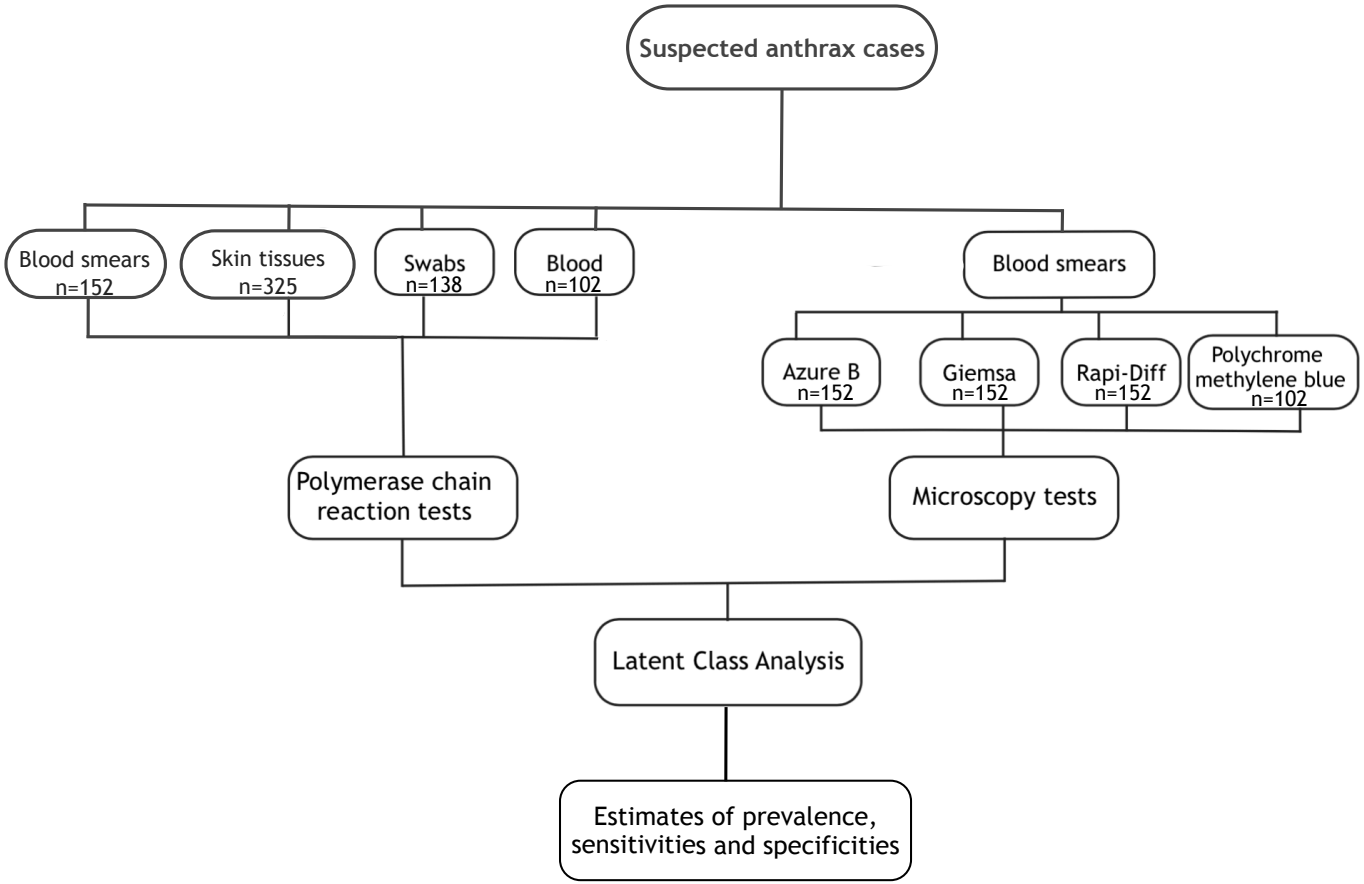


Figure 4.6: Data informing the latent class model used to estimate the sensitivities and specificities of the tests

Microscopy results as well as qPCR data were treated as binary data, with 0 representing negative results and 1 representing positive results, and were modelled as discrete variables. Results of NTCs (negative for *B. anthracis*) were also included in the model, as they provide a form of prior information for the model.

The latent class model estimated the true but latent disease status of each sampled animal as a Bernoulli distribution based on the prevalence of *B. anthracis* within the population of carcasses. This depended on the underlying process (i.e. the presence of capsule or DNA within a sample), which was modelled as a second latent process conditional on the true disease status of the corresponding animal, and the observation process (i.e. the test ability to detect the capsules or DNA in the sample as observed in the PCR and microscopy test results). Minimally informative priors were imposed on the model for the prevalence of *B. anthracis* as no published studies on the prevalence of anthrax in livestock in Tanzania are available. The prior for prevalence was a Beta (1, 1) distribution. Other minimally informative priors used include the probability of observing a capsule if present independent of the stain used, the sensitivities of the four staining techniques, the probability of detecting DNA if present and the sensitivity of the PCR test. Each of those priors was set to Beta (1, 1).

Much more informative priors for the specificities of the four staining techniques were imposed on the model. This assumed that observing a capsule (of the correct colour and morphology) on a bacillus or chain of bacilli in a sample from a suspected anthrax case is very specific for *B. anthracis* and thus the specificities of the test based on this criterion is high. Thus, the same prior (Beta (371, 1)) indicating specificities between 99% to 100% was used for all four staining techniques. For the specificity of PCR, a prior indicating specificity between 92% to 100% (Beta (50, 1)) was applied to the model.

The model was fitted using Markov chain Monte Carlo (MCMC) methods implemented using JAGS (Plummer, 2003), called from the R statistical package (R Core Team, 2017) and using the runjags package (Denwood, 2016) as an interface. For the model, two MCMC chains each with 20,000 iterations were

run. Convergence in the models was assessed visually from the plots generated, as well as from the potential scale reduction factor (psrf) of the Gelman Rubin statistic. Adequate sample size was assessed using the effective sample size of the resulting chains. The LCM was co-developed with Dr Matt Denwood of the University of Copenhagen, Denmark.

The sensitivity and specificity of qPCR were obtained in two ways. Firstly, they were obtained directly from the model which uses a Ct cut-off value of 36, the value used when qPCR was considered reference standard. Secondly, sensitivity and specificity were estimated by optimising the Ct cut-off. Optimising the balance between sensitivity and specificity yields a threshold for which the total highest sensitivity and specificity is obtained (Meredith *et al.*, 2015). Receiver-operating characteristic (ROC) analysis is useful for assessing diagnostic tests for which a threshold value differentiating between positive and negative test results is required. All possible combinations of the sensitivity and specificity of a test at possible threshold values can be obtained with the ROC curve (Greiner and Gardner, 2000). The ROC curve enables the selection of a threshold that gives the needed and obtainable sensitivity and specificity of a test. This will be further illustrated in Chapter 5.

4.3 Results

4.3.1 Case investigations and availability of smear samples

Three hundred and sixty-seven suspected anthrax cases were investigated between June 2016 and November 2017. Of this number, smear samples were collected from 152 (41%) cases for test comparisons.

a)

Photo: Rhoda Aminu

b)

Photo: Sabore Ole Moko

Figure 4.7: An example of a suspected anthrax case from which blood smear was obtainable and one from which a blood smear could not be obtained (b)

4.3.2 Description of livestock demography

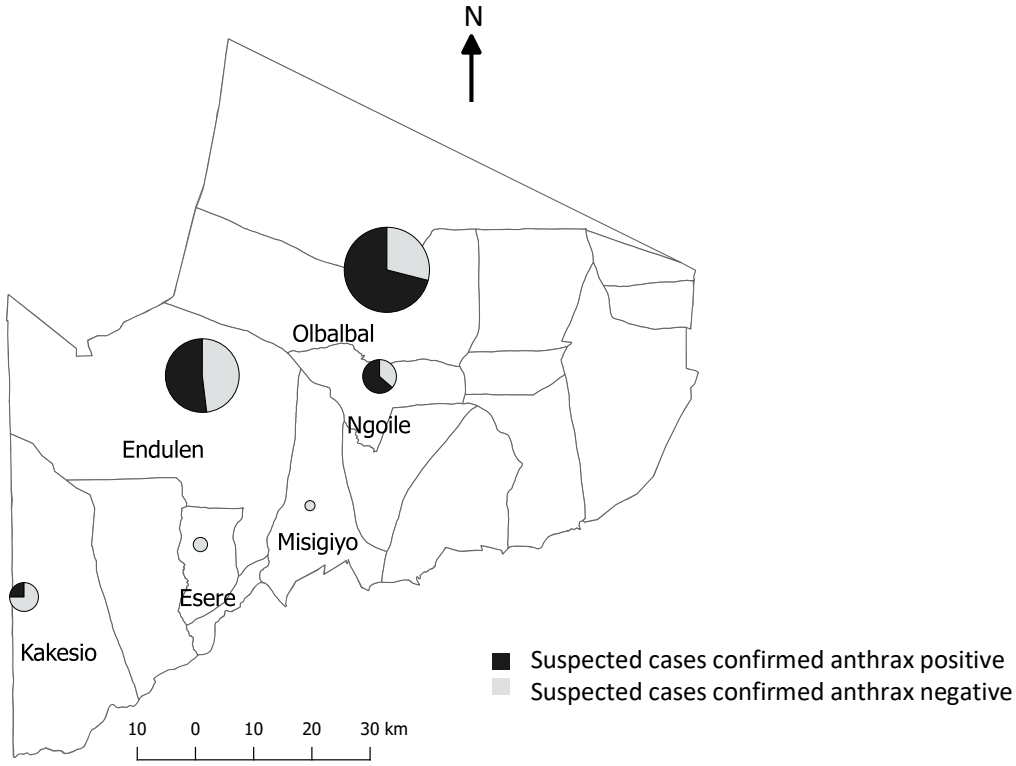
The majority (65.8%) of the smear samples were collected less than 24 hours after the death of the animal, while only 1.3% were collected more than a week after the animal had died. The remaining 32.9% cases were estimated to have died between 1 and 7 days prior to sampling. Data is incomplete for some cases, but these cases have been included in this descriptive summary and indicated accordingly in Table 4.2.

Table 4.2: Characteristics of the animal carcasses suspected to have died from anthrax, from which smear samples were collected

Variable	Number (%) of samples with data	Number of samples (%) with missing values
Species	**148 (97.4)**	**4 (2.6)**
Cattle	17 (11.2)	
Goat	12 (7.9)	
Sheep	104 (68.4)	
Donkey	10 (6.6)	
Giraffe	1 (0.6)	
Antelope	1 (0.6)	
Wildebeest	1 (0.6)	
Zebra	1 (0.6)	
Elephant	1 (0.6)	
Age	**146 (96.1)**	**6 (3.9)**
Juvenile	9 (5.9)	
Sub-adult	37 (24.3)	
Adult	100 (65.7)	
Sex	**95 (62.5)**	**57 (37.5)**
Female	65 (42.8)	
Male	30 (19.7)	
Location (ward)	**149(98.0)**	**3 (2.0)**
Endulen	58 (38.7)	
Esere	2 (1.3)	
Kakesio	8 (5.3)	
Misigiyo	1 (0.6)	
Ngoile	11 (7.3)	
Olbalbal	69 (46.0)	
Body condition prior to death	**140 (92.1)**	**12 (7.9)**
Fat	74 (48.7)	
Normal	64 (42.1)	
Thin	2 (1.3)	
Intactness of carcasses	**136 (89.5)**	**16 (10.5)**
Intact carcass	19 (12.5)	
Open carcass	117 (87.5)	

The majority of smear samples that were positive by PCR originated from Olbalbal (33% of total samples) and Endulen (20%) wards (Figure 4.8). In Olbalbal, 71% (n=69) of the samples collected were positive for *B. anthracis*, whereas 64% (n=11) of samples in Ngoile and 52% (n=58) of samples in Endulen were positive. Misigiyo and Esere had no confirmed cases out of the one and two samples collected, respectively, while 25% (n=8) of samples obtained from Kakesio were positive.

Figure 4.8: Map of the Ngorongoro Conservation Area, showing locations of carcasses from which smear samples were obtained (n = 152) by ward, and the proportion of cases that tested positive for anthrax by qPCR. The size of the circle is proportionate to the number of samples originating from the respective ward, the black and grey segments of the circle represent positive and negative samples respectively.



4.3.3 Microscopy

The highest number of positive results were obtained with qPCR testing. PMB and azure B stained smears also gave very high numbers of positive results while Giemsa and Rapi-Diff gave lower numbers of positive results (Table 4.3). Since

testing using PMB was carried out on only a subset of samples (n=102), comparisons among the tests were also carried out on this subset of samples.

Table 4.3 Summary of results using qPCR and staining techniques for the detection of *Bacillus anthracis* from blood smears.

Technique	Number of positive samples out of the subset of 102 samples tested (enabling direct comparison with PMB (%)	Number of positive samples out of 152 tested with respective method (%)
qPCR (n=152)	69 (67.6%)	90 (59.2%)
PMB (n=102)	62 (60.8%)	Not applicable
Azure B (n=152)	62 (60.8%)	81 (53.3%)
Giemsa (n=152)	11 (10.8%)	14 (9.9%)
Rapi-Diff (n=152)	12 (11.8%)	15 (9.9%)

Inter-rater agreement for interpretation of stained smears

The results for the assessment of agreement in test outcomes of smears stained by one observer and microscopically examined by two observers showed a nearly perfect inter-observer agreement for azure B and PMB, with Kappa scores of 0.94 and 0.95, respectively. However, agreement for Giemsa or Rapid Diff stain was moderate with scores of 0.51 and 0.41 respectively (Table 4.4). When adjusted for prevalence and bias indices, Kappa values for Giemsa and Rapi-Diff were 0.80 and 0.86 respectively. Two by two tables showing agreement between the two observers (inter-rater agreement) for the different stains are shown in Appendix 9.

Table 4.4: Inter-rater agreement for the interpretation of smears stained with different techniques

Technique (number of observations)	Cohen's Kappa	Prevalence-bias-adjusted Kappa	z statistic, *P*-value
Azure B (144)	0.94	0.94	11.3, 0.00
PMB (84)	0.95	0.95	8.71, 0.00
Giemsa (140)	0.51	0.80	6.01, 1.91e-09
Rapi-Diff (143)	0.41	0.86	5.03, 4.79e-07

Inter-rater agreement between two observers on smear samples from the same carcass that were stained separately by the observers using the azure B technique also yielded high agreement, with a Kappa score of 0.94 (z= 7.93, *P*= 2.22e-15, PABAK = 0.94). See Appendix 10 for more details of the two by two table.

Effect of time on the quality of stained smears

The agreement between two observations carried out six months apart by a single observer. To evaluate the effect of time on the quality of stained smears, was high for smears stained with PMB and azure B (0.89 and 0.82 respectively). There was moderate agreement for smears stained with Giemsa and Rapi-Diff (Table 4.5). Two by two tables showing agreements between the two observations are shown in Appendix 8.

Table 4.5: Effect of time on the quality of stained smears. Assessment of the agreement of test results carried out immediately after staining slides, and after 6 months.

Technique (number of observations)	Cohen's Kappa	Prevalence-bias-adjusted Kappa	z statistic, Agreement between observations over time *P*-value
Azure B (61)	0.89	0.90	7.01, 2.36e-12
PMB (12)	0.82	0.83	2.90, 3.75e-03
Giemsa (61)	0.50	0.84	3.93, 8.45e-05
Rapi-Diff (61)	0.65	0.93	5.07, 3.93e-07

The *P* value for all the Kappa estimates obtained in this study (see Table 4.4 and Table 4.5) indicate that they were statistically significantly different from estimates that may have been obtained due to chance.

Between laboratory diagnostic test agreement

The kappa (PABAK) scores for the agreement between results obtained with the TVLA testing (as conducted by TVLA independently of the testing carried out by the PhD student) and azure B stain testing of microscope slides carried out by

the PhD student was 0.79 (z = 6.54, *P* >0.05). The agreement with PMB was 0.73, (z = 6.03, *P* >0.05). Both scores indicate substantial agreement.

4.3.4 Quantitative polymerase chain reaction

Assessment of qPCR methodology

The DNA of *B. anthracis* was detected in all 10 samples tested. The Ct values of qPCR testing for the chromosomal target *PLF3* on dried unstained blood smears ranged from 19 to 27, for sample weights ranging from 0.2mg to 3.5mg. Larger scrapings from a sample did not necessarily produce lower Ct values compared to the smaller ones. A regression model produced a weak and non-significant indication that the variation in the Ct values is correlated with the weight of the scrapings, explaining only 27% of the variation in Ct values (R^2 =0.27, F (1,8)=2.29, P > 0.1) (Figure 4.9).

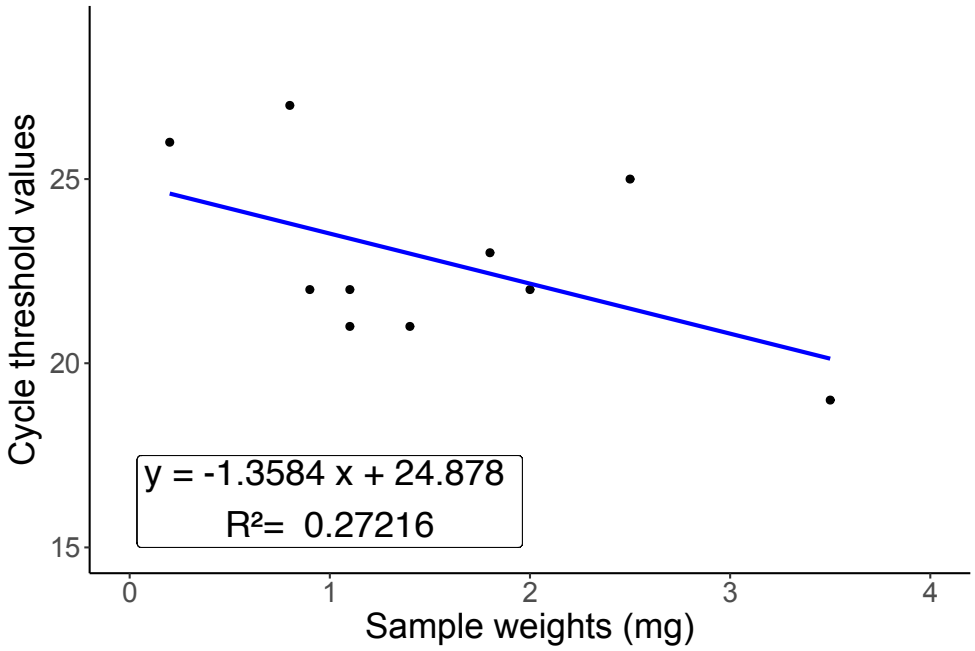


Figure 4.9: The relationship between cycle threshold values and weight of smear scraping. Results are obtained from qPCR targeting a chromosomal sequence (*PLF3*) of *Bacillus anthracis*

Quantitative PCR results of the smear samples

Quantitative PCR targeting chromosomal *PLF3* DNA sequence and plasmid DNA sequences *cap* and *lef* carried out on unstained blood smears for the 152 cases indicated that 67% of these samples (n = 102) were positive for chromosomal and plasmid *B. anthracis* DNA. Samples were only considered positive if amplification of all three targets occurred. At the time of the qPCR testing, nine NTCs were

included in the six extractions carried out and amplification of only one target sequence (*cap*) occurred in one of the nine NTCs at cycle 37. For this batch, the cut-off value was set at 35 and samples with Ct of 36 were considered negative. Only two samples were consequently affected. For the remaining five extraction batches with no amplification of the NTCs included, Ct cut-off was set at 36 and samples with Ct values above 36 were considered negative.

Molecular diagnostic value of stained smears

Average Ct values for stained smears were 2.90 (SD=2.10) higher than for unstained smears ($p<0.02$). Only four out of 68 stained smears showed no amplification below the maximum of 40 cycles in a qPCR run. These four smears were PHE controls each stained with azure B, Giemsa, Rapi-Diff and PMB. The average Ct values for unstained and stained slides can be found in Table 4.6.

Table 4.6: Average Ct values across the different stains in comparison with unstained smears.

Stain type	Average Ct value of stained smear
Unstained smears (n=17)	23.76 (SD=4.85)
Azure B (n=17)	25.82 (SD=4.45)
Giemsa (n=17)	28.50 (SD=5.45)
Rapi-Diff (n=17)	26.64 (SD=5.01)
PMB (n=17)	26.71 (SD=4.81)

The four stained smears with no amplification had Ct values of 36 on the unstained smear, allowing a slim window for amplification before the maximum of 40 cycles in the qPCR run, since staining increased Ct values by 2.90 cycles on average (Figure 4.10).

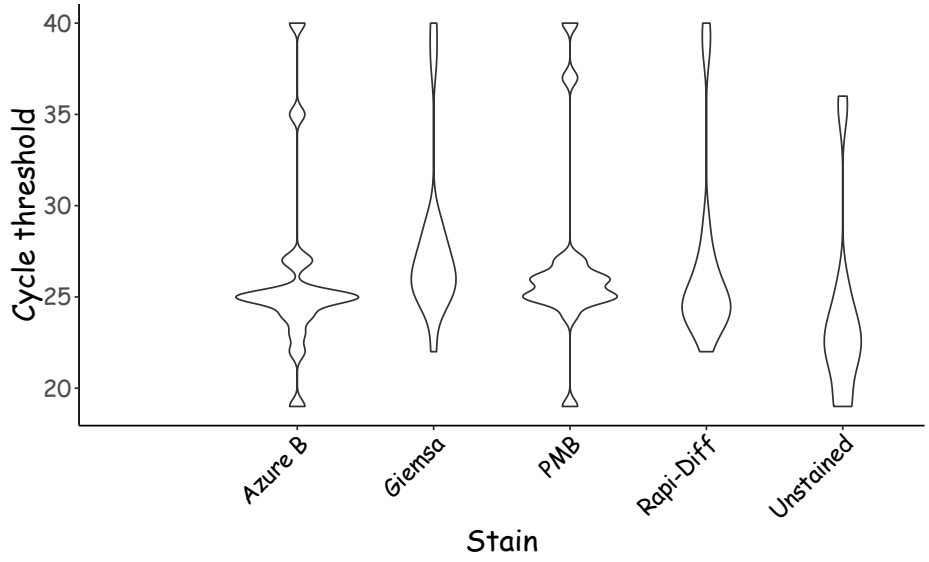


Figure 4.10: Plot showing the cycle threshold values of qPCR testing on stained and unstained slides. Florescence was detected early for unstained slides compared to stained slides

4.3.5 Sensitivities and specificities of the microscopic tests assuming qPCR as the reference standard

The calculated sensitivities and specificities for the different staining techniques while using qPCR as the reference standard are shown in Table 4.7. Based on the criterion of detecting the capsule of *B. anthracis*, azure B and PMB had high sensitivity while Giemsa and Rapi-Diff had poor sensitivity. All four techniques showed optimal specificity (100%).

Table 4.7: Sensitivity of microscopy techniques for detection of *Bacillus anthracis* in blood smears, using qPCR as the reference standard. The specificities of all the tests were 100%.

Stain	Sensitivity
Azure B (n=152)	90%
Giemsa (n=152)	16%
Rapi-Diff (n=152)	17%
PMB (n=102)	90%

4.3.6 Sensitivity and specificity of the tests as determined by the LCM

The LCM allowed the estimation of the sensitivity and specificity of qPCR in addition to the staining techniques. The performance of qPCR was estimated in two ways. The first used a Ct cut-off value of 36 (the initial cut-off set if qPCR was considered the gold standard) and the second optimised the sensitivity and specificity of qPCR based on the model outcomes. Upon visual assessment of the model plots, convergence between and within the chains was observed and the Gelman Rubin statistic of all parameters was close to 1 indicating that the model had explored the full posterior distribution (Appendix 11). The overall prevalence of *B. anthracis* in the samples was 68% (62-73%). The overall probability of detecting a capsule if present, independent of the staining technique used was 96% (91- 99%), while the probability of not detecting a capsule if absent was 99% (99-100%).The sensitivity and specificity of qPCR, PMB and azure B techniques were estimated to be very high compared to those of Giemsa and Rapi-Diff. Azure B was comparable to PMB – the recognised reference standard for the detection of *B. anthracis* by microscopy. Based on LCM, all microscopy tests had slightly higher specificity than qPCR. The estimates (Table 4.8) take into account the probability that *B. anthracis* capsule or DNA in a sample indicates that the associated animal had anthrax.

Table 4.8: Latent class model estimates of diagnostic test sensitivity and specificity, and their posterior 95% confidence intervals (CI) for the detection

of *B. anthracis* from blood smear samples. The sensitivity estimates take into account the probability of detecting a capsule (or DNA where applicable) if present and the specificity estimates take into account the probability of not finding a capsule if absent, independent of the test used.

Test	Sensitivity (CI)	Specificity (CI)
Azure B	91% (84-97%)	100% (99-100%)
Giemsa	16% (9-24%)	100% (99-100%)
Rapi-Diff	18% (10-26%)	100% (99-100%)
PMB	92% (85-97%)	100% (99-100%)
qPCR on smears (cut-off of 36)	97% (93-100%)	96% (92-99%)

Between laboratory diagnostic test assessment

The sensitivity and specificity of the TVLA technique were 77% (62-89%) and 100% (85-100%) respectively. Compared to azure B and PMB, the PMB technique carried out at TVLA had lower sensitivity with wider confidence intervals.

4.4 Discussion

To our knowledge, this is the first study to assess the performance of the azure B capsule staining technique – an alternative to PMB – for the rapid detection of *B. anthracis* based on microscopic analysis of blood smear samples collected from animals suspected to have died from anthrax. We assessed the potential for molecular confirmation of anthrax, based on detection of *B. anthracis* DNA in stained smears. To summarise the findings, azure B was equivalent to PMB on scientific criteria (sensitivity, specificity, and inter-observer agreement) and superior in convenience criteria, whereas the sensitivities of Giemsa and Rapi-Diff for detecting the capsule of *B. anthracis* were very poor. In addition, inter-rater agreement for Giemsa and Rapi-Diff was much lower than for azure B and PMB, and results were less consistent when reassessing slides 6 months after staining. The latent class model estimated the sensitivity of qPCR to be higher than the staining techniques.

The majority (94.1%) of the samples were obtained from livestock, as the study focused mainly on the disease in livestock species with a few opportunistic wildlife samples (3.3%). Most livestock samples were obtained from sheep (68%) with 78% of positive samples from this this species. This agrees with results in Chapters 2 and 3, which found that reports of the disease in sheep are more frequent than for cattle and goats in the NCA. More adult and female animals were affected and may reflect the composition of the animal population rather than a higher risk of the disease in adult and female animals. Maasai livestock keepers have been shown to favour herd structures with a greater female to male ratio (King *et al.*, 1984). Only a few carcasses were intact; the majority (87.5%) had already been opened before sampling was carried out. A fresh unopened carcass infected with *B. anthracis* contains the vegetative forms of the pathogen that can be readily destroyed. Once an infected carcass is opened, direct transmission of the pathogen to people and animals through contact can occur readily. In addition, the vegetative form of the pathogen then has access to oxygen that promotes sporulation, making the pathogen resistant to destruction (WHO, 2008). Handling carcasses from animals suspected to have

died of anthrax is common practice in the NCA and has implications for the spread and persistence of the disease as discussed extensively in Chapter 3.

Microscopy is a simple and rapid method for the detection of anthrax in the field (WHO, 2008), it is particularly useful in resource-limited locations where infrastructure for bacterial culture and/or molecular diagnostics is not in place. When qPCR is used as the reference standard technique, the sensitivity of the azure B technique is high and similar to that obtained for PMB. Conversely, Giemsa and Rapi-Diff have comparably lower sensitivity. These results suggest that azure B could be used as a replacement for PMB for the detection of *B. anthracis* in the field. The advantage of azure B is commercial availability and the ability to apply the stain immediately once prepared.

Re-examination of slides after six months showed a decrease in the number of positive smears, indicating that storage of stained smears decreases the ability to visualise the capsule and reduces the sensitivity of microscopy. In the current study, the entire smear was harvested for qPCR, so re-evaluation of qPCR from smear scrapings after 6 months was not possible. Harvesting of half of a smear and prolonged storage of the other half would allow for assessment of the impact of time on qPCR sensitivity.

The performance of a diagnostic test is partly a function of the agreement in the test outcomes produced by different test operators. This is particularly important in testing where subjectivity is involved, such as in the detection of anthrax using microscopy, based on assessing the morphological characteristics of *B. anthracis*. Tests need to take into account this subjectivity by measuring the agreement between the interpretations of results obtained from different observers. The Kappa statistic is commonly used to quantify inter-rater agreement (Viera and Garrett, 2005). However, the robustness of this method has been widely criticized because the score can be obtained due to chance (Viera and Garrett, 2005). Another limitation of the Kappa statistic is the influence that the prevalence of the disease has. Essentially, agreement is penalised in assessments where the prevalence of a disease condition is very low or very high (Walter and Irwig, 1988). Since the prevalence observed for azure B

and PMB were closer to 0.5 compared to Giemsa and Rapi-Diff, the Kappa scores were unaffected. The results of the study show that azure B compares well to PMB for detection of *B. anthracis* in animal blood smear samples, a finding that is robust across multiple observers, indicating the reliability of the method (Viera and Garrett, 2005).

Stain microscopy using azure B fulfils most of the ASSURED criteria when compared to other methods for the detection of *B. anthracis*. The reasons for this are that it is much more affordable, user-friendly, rapid and deliverable compared to culture and qPCR. Microscopy can be carried out and results obtained in less than 30 minutes with just a battery powered microscope, whereas qPCR requires multiple pieces of equipment and much more substantial effort and resources to set up in the field. This study demonstrated that microscopy using azure B is also robust (i.e. it does not require samples to be refrigerated), sensitive and specific, thus fulfilling the scientific criteria of a good diagnostic test. Although microscopy utilising azure B may not fulfil all the criteria for an ASSURED test, largely due to the requirement for a microscope, it holds huge potential for improving anthrax surveillance in the NCA and similar anthrax-endemic settings, as most basic diagnostic laboratories will have access to a microscope.

The lower sensitivity of azure B compared to qPCR (91% vs. 96%) should not have a serious negative impact on the ability of this technique to improve anthrax surveillance, because in an endemic situation, detecting all cases may not be as important as in elimination stages. Different trade-offs may be employed at different stages of disease surveillance and control and the desired balance between sensitivity and specificity may differ depending on the situation (Houe *et al.*, 2006). For example, when eliminating or eradicating a disease, it is important to find every last case, i.e. test sensitivity needs to be very high (often at the expense of specificity, which may create the need for confirmatory testing with a highly specific test). When a disease is still endemic, reducing its incidence and prevalence is more important than finding every case, and detecting most cases would be an improvement over not having any case confirmation. However, resources are limited, and control may have negative

consequences (e.g. animal culling, side effects of vaccination). Thus, azure B with its very high specificity and slightly lower sensitivity (100% and 91% respectively) is suitable for surveillance of anthrax.

The availability of a sufficient quantity of good quality DNA is essential for molecular techniques. Given that blood smears are often made from a very small volume of blood – about 0.05 mL or less – it was important to evaluate the ability to detect *B. anthracis* DNA on dried blood smears. We did not formally assess the minimum genome copy number, bacterial counts, or sample weights that are the lower limit for detection, but our results show DNA of sufficient quality and quantity can be obtained from dried blood scraped from smears even with weights as low as 0.2mg. The weights of the scrapings were not significantly associated with the Ct values obtained, which provides an indication of the pathogen concentration in the sample. This may be due to varying infection intensity in the different animals, however the small sample size impedes any analysis to investigate variability in Ct values between species. We would expect different concentrations (w/v) of pathogen in blood, since studies have shown that species respond differently to *B. anthracis*. Some species are more susceptible to the toxins produced by *B. anthracis* and will succumb to the disease even with lower bacteraemia (Beyer and Turnbull, 2009). It is possible that the efficiency of the extraction process could decrease if too much sample is added, for example, if extraction is unable to remove all inhibitory substances found in blood (Schrader *et al.*, 2012). However, considering the small volume of blood used to make smears, this is unlikely a concern. Thus, these results show that smear sample weight is not a practical constraint on the feasibility of testing for *B. anthracis* with qPCR.

The ability to utilize the same sample material for multiple testing may be important when working with pathogens requiring high containment and when infrastructure for sample storage is lacking. Thus, the ability to use the same smear for the detection of *B. anthracis* using microscopy and qPCR could be advantageous. This study found qPCR works on stained smears, although staining reduces the sensitivity of the qPCR approach, as there was a statistically significant increase in the Ct values of the stained smears compared with

unstained smears. Staining may interfere with the quality of DNA (Horobin, 2011) and thus affect the sensitivity of techniques to detect DNA from stained materials.

The majority of the stained smear samples that originated from cases that were confirmed as anthrax positive by qPCR on unstained smear samples showed detectable amplification of the target DNA before the end of the qPCR run. Since Ct values of stained slides were on average 3 cycles greater than for unstained smears, it follows that positive anthrax cases with lower bacterial loads may be missed in theory. However, in practice this may rarely occur. The mean Ct values of unstained smears positive for *B. anthracis* was 24 cycles. This gives a large interval for increase of the Ct value of most stained smears before the end of the qPCR run (40 cycles). Molecular detection of *B anthracis* on stained blood smears may be safer as bacteria present in smears may already be inactivated by drying and fixation in alcohol (although alcohol fixation is not effective against spores) (Vardaxis *et al.*, 1997), potentially reducing the risk of exposure to the pathogen. It is also useful after microscopic examination has been conducted or if they are the only samples available. The only stained samples that did not show detectable amplification of DNA were PHE controls which had been fixed with formalin and inactivated. Fixation in formalin damages DNA, which explains negative PCR results (Douglas and Rogers, 1998).

The LCM enabled the estimation of the true but latent status of the samples, and the parameters of sensitivity and specificity of the five techniques, as well as the prevalence of anthrax in the sampled population. This modelling was carried out with the assumption of no reference standard. The estimates of specificity for the staining techniques obtained from the LCM were near perfect and similar to those obtained from the calculations using qPCR as the reference standard. This indicates that the information from the data (likelihood) are consistent with our prior belief of the high specificity of the capsule visualisation for *B. anthracis* detection. Additional information on confidence intervals obtained from the LCM gives credence to the high specificity of these tests. Although PCR is believed to be highly reliable for detecting anthrax (Berg *et al.*, 2006), it may not be considered a perfect reference standard because of *B.*

anthracis' genetic similarity with the *B. cereus* group (Wielinga *et al.*, 2011), which could decrease its specificity. However, this was accounted for in this study by the inclusion of the chromosomal target- *PLF3*, which is believed to be *B. anthracis* specific (Ågren *et al.*, 2013). While the specificity of the PCR assays has been based on laboratory experiments using *B. anthracis* isolates, there is no evidence to suggest that the sequences are less specific to field samples. Other factors that may reduce PCR specificity include amplification of non-specific genetic sequences, for example in primer-dimer amplification (D'aquila *et al.*, 1991), although this would not be detected in probe-based qPCR techniques. Cross-contamination may also occur during DNA extraction, although the inclusion of no-template controls helps to observe and avoid this. Alternatively, the specificity of qPCR in the LCM could be penalised by the occurrence of some false negative results during microscopy testing. The specificity of qPCR is very high at the lower Ct cut-off of 32 as optimised by the latent class model, however, at a higher cut-off it is slightly lower than that obtained for the staining techniques. This indicates some probability of false positive results with a higher cut-off. This Ct cut-off of 32 is quite conservative and the estimates obtained using a cut-off of 36 are still very good for a diagnostic test.

One of the advantages of using a Bayesian approach is that the estimation of the latent status can make use of prior beliefs (Joseph *et al.*, 1995) such as of the prevalence of the condition and the performance of the diagnostic tests. Without informative priors, the model may consider all tests equal before considering information in the data, whereas there may be some prior evidence or belief that one or more tests perform better than others and should carry more weight. Usually this prior belief should be supported by scientific evidence. When no published evidence is available, it is difficult to define a very informative prior based only on beliefs. Priors also help to improve model identifiability. In the model, less informative priors were used for the specificity of qPCR as there were no scientific data to support a more informative belief that qPCR is more specific compared to the stain microscopy tests. By contrast, PCR has been described as more sensitive than microscopy (Berg *et al.*, 2006). A model with a more informative prior for the sensitivity of PCR (92-100%) produced the same results as the one using minimally informative priors (2-98%).

I am therefore confident that the priors used for the specificity of the microscopy test did not dominate the estimates, as when the same priors (specificity of 92-100%) were used for both microscopy and PCR, the specificity of the microscopy tests were higher notwithstanding.

One assumption of the LCA approach is the conditional independence of the test given the disease status. This is a difficult assumption to meet when several tests are being considered (Toft et al., 2005). In such situations, the dependence structure must be specified. For the stain tests, the dependence structure was specified in the model by making the results conditionally independent given that the bacilli are encapsulated. This indicates that the stain tests are more correlated to each other than with the PCR test. The model also assumed that the sensitivities rather than the specificities of the stain tests are correlated since there is no evidence that detecting encapsulated bacilli with the morphology of *B. anthracis* is possible in a truly negative sample.

With the latent class modelling, the sensitivity and specificity of azure B and their associated CI are comparable to PMB. This indicates that azure B with all its advantages (i.e. commercial availability, useable immediately after preparation) would provide a better alternative for PMB for the confirmation of anthrax in the field. We are confident that azure B results obtained from a sample retrieved from an anthrax-suspect carcass are likely to represent the true state of the sample. The high sensitivity and specificity of azure B was observed in samples collected mostly from goats, sheep and cattle that are very susceptible and present with high bacteraemia easily detected by microscopy. It is important that these findings be interpreted as such. In species that are more resistant to anthrax, the sensitivity of the stain may be lower, as these animals are more likely to have low terminal bacteraemia, although it is not expected that the specificity will be reduced.

Both microscopy (with azure and PMB) and PCR tests produced very high sensitivity and specificity. Nevertheless, the interpretation of these test properties should consider the following issues. First, the carcasses from which samples were collected were anthrax-suspect based on clinical presentation.

This screening of carcases based on clinical presentation before diagnostic testing could be regarded as form of serial testing. In serial testing, diagnostic procedures are performed sequentially, with subsequent testing dependent on the result of the initial test (Gordis, 2013). Serial testing leads to improved specificity and may have especially aided the specificity of the microscopy tests; for example, such high specificity might not have been expected/estimated if all carcasses were screened with this method, as opposed to just those of animals with a history of sudden death. Second, samples were obtained from animals with terminal bacteraemia, which is characterised by high quantity of the bacteria. This improves sensitivity of the tests but especially for microscopy tests since in PCR, DNA will be amplified prior to detection. Third, the criteria for classifying microscopy tests as positive was based on observing capsule on bacilli with blunt ends, which is highly specific to *B. anthracis* (WHO, 2008). To achieve the high specificity obtained for azure B microscopy testing, detecting the capsule is paramount.

While the clinical presentation of cutaneous anthrax, representing approximately 95% of cases in humans, is considered pathognomonic, clinical signs in animals are not as specific. Sudden deaths in ruminant livestock in anthrax-endemic areas - particularly where bleeding from natural orifices does not occur - are suggestive but not specific of the disease. In many cases, these signs are lacking. Syndromic diagnosis may lack both sensitivity and specificity as such, diagnostic testing is important for case confirmation to properly assess the incidence of anthrax in animals and to direct specific action for disease control. Furthermore, as animals are useful sentinels for human anthrax (WHO, 2008), and more accessible for sampling compared to humans, surveillance of the disease in animals could benefit human health. Theoretically, stain microscopy should be easy to implement in areas like the NCA area because it is a simple, reliable and rapid method with little need for equipment and infrastructure and we have successfully used the azure B test in the field for rapid diagnosis. However, certain factors might hinder the implementation and the ability of this technique to improve surveillance. One of these is the paradox of sample unavailability. Our data suggested that blood smear sample retrieval from over half of the suspected cases reported during the period of investigations was not

possible as many of the carcasses were no longer intact or had been consumed. Although other parts of the animal, such as the hides, may still be available, blood smear stain microscopy, the quickest and simplest method for the detection of anthrax, requires fresh blood as sample material and is therefore not an option if the carcass has been consumed or if the carcass is difficult to access in a timely manner.

Results reported in Chapters 2 and 3 indicate that the disease is underreported. Therefore, any significant improvement to surveillance that may occur as a result of enhanced detection of anthrax based on improved stain techniques must be implemented together with other strategies to improve reporting and the proper handling of suspect anthrax cases. Increased disease reporting could be achieved through incentivising communities with the improved diagnostics that azure B offers. Without diagnostics, there may be no incentive to report anthrax, as livestock keepers are often interested in a diagnosis which may enable them to take proactive measures to control the disease or lobby health authorities for improved anthrax control. Implementing azure B microscopy may thus serve as incentive to more frequent and timely reporting. To address the problem of sample availability, further studies described in Chapter 5 assess the potential of other sample materials for the surveillance of anthrax.

4.5 Conclusions

This study was aimed at enhancing the surveillance of anthrax in endemic areas through improving the detection of *B. anthracis* and confirming the disease in animals. Techniques that can be implemented in areas with limited resources and without access to infrastructure for classic detection methods such as qPCR and bacterial culture were assessed. The results show that azure B is a reliable replacement for PMB for the rapid detection of *B. anthracis* by microscopy. It possessed high and similar sensitivity and specificity, and inter-rater agreement scores to PMB. The sensitivity of Giemsa and Rapi-Diff techniques were poor, and thus these methods would not be recommended for the confirmation of anthrax. Azure B overcomes the challenges of availability that make PMB less suitable in endemic and resource-poor areas. Microscopy is a simple, inexpensive and quick detection method, although staining reduces the sensitivity of qPCR, molecular detection of *B. anthracis* can still be achieved on stained smears. There is a slight deterioration in the visualisation of the capsule and thus the ability to detect *B. anthracis* by microscopic examination after six months of storage for stained smears, but sensitivity and specificity of azure B stain microscopy were still good. This could be useful, for example, for periodical re-evaluation of slides and re-training of staff in field laboratories. However, it is not clear what effect this deterioration will have on the molecular detection of the bacteria. Further study would be useful to assess the potential of stained smears for molecular detection after storage for prolonged time periods (up to a decade), and to explore the feasibility of using stained smears for molecular strain typing. This may be useful for long term surveillance of the disease when conditions, resources, infrastructure and best practices allow only the storage of limited sample materials.

Chapter 5

Chapter 5 Optimal animal sample materials to improve *Bacillus anthracis* surveillance in anthrax endemic areas

5.1 Introduction

Neglected zoonotic diseases often occur in remote, hard-to-reach and resource-limited locations presenting challenges for their surveillance and control. One of the first steps in controlling a disease is demonstrating its presence. Confirmation of anthrax is possible by detecting the causative agent – *Bacillus anthracis* – in animal samples (Chapter 4). Work described in Chapter 4 demonstrated that stain microscopy using azure B is a highly sensitive and specific method for the detection of *B. anthracis* in blood smears, and it is also a quick and simple technique that fulfils many of the convenience criteria as described in the ASSURED guidelines for diagnostic tests in low-resource settings (Mabey *et al.*, 2004). In Chapter 4, I also established that quantitative polymerase chain reaction (qPCR) on blood smears is possible, and highly sensitive and specific as well. However, blood smear samples could only be obtained for less than half of anthrax-suspect cases. This creates the need to explore the use of other sample types that are more readily available, although this may mean compromising on the ASSURED criteria to find a trade-off between simpler and cheaper diagnostic methods such as microscopy, and methods that require more expensive infrastructure such as PCR that could be implemented for a wider range of sample types.

Diagnostic tests described for the detection of *B. anthracis* include those based on microbial culture and polymerase chain reaction (PCR) (Berg *et al.*, 2006). Bacterial culture is often impractical for *B. anthracis*, given the infrastructure required. At the time of conducting the study, culture could not be performed anywhere in Tanzania. On the other hand, the infrastructure for PCR is available in-country and might provide a solution when culture and microscopy testing cannot be carried out due to the lack of infrastructure and available blood smear samples, respectively. Molecular detection of *B. anthracis* using PCR is a highly sensitive and specific method to identify the pathogen (Berg *et al.*, 2006; Wielinga *et al.*, 2011). However, there have been no systematic studies to assess

the sensitivity or specificity of PCR on different sample types, especially those that may be collected under typical field conditions in endemic areas where cold-chain storage for biological materials is unavailable. For animal anthrax, PCR has more commonly been applied to *B. anthracis* isolates (Turnbull, 1998; Ellerbrok *et al.*, 2002; Wielinga *et al.*, 2011), than directly to field samples. Very little is known about the optimal and practical methods of sampling for detecting *B. anthracis* using PCR in resource-poor field conditions.

Detection of *B. anthracis* from blood smears using PCR was possible and highly sensitive and specific. However, evidence from the study of other pathogens, e.g. *Mycobacterium tuberculosis* (Da Cruz *et al.*, 2011), shows that PCR may be more sensitive for one sample type (i.e. blood) and less sensitive for others (i.e. sputum). Certain sample materials may be more suitable for detection of a particular pathogen and the type of disease they cause. For example, the kidneys are the preferred tissue for the detection of *Leptospira* spp. (Bharti *et al.*, 2003). For human cutaneous anthrax, samples from skin lesions may yield more detectable *B. anthracis* than blood samples (Ringertz *et al.*, 2000). The ideal diagnostic sample would be one that is easy and minimally invasive to collect, but where pathogen concentration is likely to be high. It is thus important to understand what sample materials are readily obtainable from endemic and resource-poor areas and that yield the best results for the surveillance of anthrax.

5.1.1 Thresholds for pathogen identification and quantitative PCR

Quantitative PCR (also known as real-time PCR or qPCR) is an improvement on conventional PCR that allows the quantification of the initial concentration of DNA in a sample (Kubista *et al.*, 2006). Detection and visualisation of DNA amplification is achieved by the use of either intercalating dyes or labelled probes that give off a measurable florescent signal. Intercalating dyes bind non-specifically to double-stranded DNA, whereas probes (aka reporters) bind specifically to the DNA fragment being amplified, conferring an extra degree of specificity. The number of DNA amplification cycles needed to generate a detectable signal depends on the presence and concentration of template DNA.

The PCR cycle at which florescence is detected is called the fluorescence detection threshold, or more commonly 'cycle threshold' (Ct) (Kubista *et al.*, 2006). To distinguish between positive and negative results, a cut-off threshold is usually set for the Ct value. For example, a cut-off threshold of 36 means that samples with Ct of 36 and lower are considered positive and those above 36 are negative. In this study, this threshold is referred to as the Ct cut-off value to differentiate it from the florescence detection threshold. Ct values are also influenced by amplification efficiency (Svec *et al.*, 2015). Both the concentration of pathogen DNA and the amplification efficiency may differ between sample types and affect the sensitivity and specificity of detection. Biological materials may contain complex intrinsic compounds such as haemoglobin or immunoglobulin with the ability to inhibit PCR efficiency (Al-Soud and Rådström, 2001). In addition, depending on the material, extrinsic factors may be present in samples; for example, organic compounds in soil or materials that may have come into contact with soil (Tsai and Olson, 1992) and sample additives such as heparin (Al-Soud and Rådström, 2001). There are variable reports on the effect of storage conditions, such as storage time and temperature on PCR efficiency (Roberts *et al.*, 1997; Färnert *et al.*, 1999; Jung *et al.*, 2003). The presence of inhibitory substances in a sample material or suboptimal storage conditions may therefore reduce the sensitivity and specificity of PCR.

Many tests produce outcomes with continuous measures, where a threshold differentiating between positive and negative samples is required. Determination of a cut-off is commonly reported for serological techniques such as enzyme-linked immunosorbent assay (ELISA) (Jacobson, 1998), but can be done for qPCR since results are obtained as continuous measures (Caraguel *et al.*, 2011). A threshold is usually set to optimise all or some measures of test performance in order to meet certain criteria required under specific circumstances. A threshold may be set to maximise sensitivity, even though there is a cost in terms of decreased specificity. For example, in developed countries where anthrax is rare, a threshold that favours a highly sensitive test may be preferred over that favouring a highly specific test, if the cost of a false negative test (i.e. risk of spread or damage to trade) is considered higher than

the cost of a false positive result (i.e. cost of culling and decontamination). Other thresholds may optimise both sensitivity and specificity and yield the highest possible accuracy with the test. Receiver-operating characteristic (ROC) analysis illustrates how sensitivity and specificity vary over different threshold values and enables the selection of an optimal threshold (section 4.2.4).

5.1.2 Objectives

This chapter was aimed at

1. Assessing the usefulness of various sample types for the detection of *B. anthracis* under field conditions. It assesses availability of different sample types, and the sensitivity and specificity of qPCR for *B. anthracis* detection in those sample types using latent class analysis in the absence of a perfect reference standard method.

2. Establishing the optimal qPCR Ct cut-offs for different sample types.

5.2 Methods

5.2.1 Sample collection

Samples were collected from or around animals suspected to have died of anthrax in the field, using the field-based surveillance system detailed in the method sections of Chapters 2 and 3. Briefly, animals suspected to have died of anthrax were sampled. Blood smear, skin tissue, whole blood, blood swabs, and insects were collected from the carcass when available. After collection in the primary container, all sample types were packaged in secondary Ziplock bags and stored at ambient temperature prior to transportation to the KCRI laboratory. Samples were stored for up to six months before testing.

5.2.2 DNA extraction and molecular testing

Blood samples

Blood available from carcasses was collected into sterile blood tubes with no additives. DNA extraction was done using the DNeasy Blood & Tissue Kit (Qiagen, Germany) protocol for blood with nonnucleated erythrocytes. Briefly, 20 µl proteinase K was pipetted into a 1.5mL microcentrifuge tube. A 100µl aliquot of the blood sample was transferred into the tube containing proteinase K. The solution was adjusted to 220µl by adding 100µl of phosphate buffered saline (PBS). DNA extraction was completed following the manufacturer's spin column protocol as described in Chapter 4.

Swab samples

Swab samples were taken by inserting a cotton swab into available blood from a carcass, then packaged into 30 ml Sterilin tubes (primary containers) and secondary Ziplock bags. In the laboratory, the sampled end of the swab was cut off and placed into a 1.5mL microcentrifuge tube and soaked in 200µl PBS with 20µl proteinase K. The mixture was incubated at ambient temperature for at least one hour, vortexing the tubes mid-way and after incubation. Following this, the spin column protocol of the DNeasy Blood and Tissue kit was carried out according to the manufacturer's instructions to complete the extraction process.

Insect samples

Flies on and around the carcasses and areas where the animals have been butchered or scavenged were collected into 30ml Sterilin tubes. For each carcass, about 100mg of the insects were transferred into a 2 ml MagNA Lyser bead tube (Roche, United Kingdom) and 360 µl of PBS was added. The sample was bead beaten twice at 5000rpm for 18 seconds in a Precellys tissue homogeniser (Bertin, France). Exactly 200 µl of the homogenised sample was then transferred into a microcentrifuge tube with 20 µl of proteinase K added. The manufacturer's protocol for the DNeasy Blood & Tissue Kit was continued from step 2 (see Appendix 7) to complete DNA extraction.

Skin tissue samples

Depending on the state of the animal remains, tissue samples were collected from the tip of the ear (if carcass was still intact) or other available pieces of skin (if they had been opened and/or butchered; see Chapter 4 Figure 4.7). Using disposable scalpel and forceps, skin tissues were collected into 30 ml Sterilin tubes in the field. In the lab, again using a disposable scalpel, 50 mg of tissue was cut into small pieces in a petri dish and transferred into a 2 ml MagNA Lyser bead tube. Following this, 360µl tissue lysis buffer (ATL buffer, included in the Qiagen kit) was added to each tube and bead beaten twice at 5000rpm for 18 seconds in a Precellys tissue homogeniser (Bertin, France). Proteinase K (40 µl) was added to the mixture and left to incubate at 56°C for 6-8 hours or overnight until complete tissue lysis was achieved. Exactly 220 µl of the supernatant was transferred to a new microcentrifuge tube and the DNeasy Blood & Tissue Kit spin column protocol was completed according to the manufacturer's protocol.

Smear samples

Blood smear samples and their PCR test results as described in Chapter 4 were included in this study. The procedure for DNA extraction and qPCR was as described in Chapter 4.

All procedures related to sample aliquoting and DNA extraction were carried out in a class 2 biosafety cabinet. Sterile filter pipette tips were used throughout all

extractions. All DNA extracts were stored at -20°C prior to use in PCR. Quantitative PCR was carried out on all DNA extracts as described in Chapter 4, section 4.2.3.

5.2.3 Analysis

The latent class model (LCM) described in Chapter 4 forms the basis for this section and the results reported in this chapter are based on this model. The results of microscopy (using azure B, Giemsa, Rapi-Diff, and PMB) and molecular testing on blood smears, whole blood, blood swabs and skin tissue defined the model (Figure 4.2 Chapter 4). All of these data were combined in order to increase the power of the model. Insect samples were not included in the data informing the model because of the small number of samples available.

To summarise how the model was built, blood smears, whole blood, skin tissue, and swab samples were considered positive when the Ct values for the amplification of each of the three genetic targets *(cap, lef* and *PLF3*) equalled or were below the cut-off (Chapter 4, section 4.2.4). For the model the PCR results were treated as a binary variable along with results of microscopy testing (Chapter 4). The true states of the carcasses were modelled as a Bernoulli distribution based on the prevalence of *B. anthracis* in the samples, which depends on the presence of *B. anthracis* capsule or DNA detected by the tests. Worthy of note is that the prevalence reported in this study is not the true prevalence of anthrax in the NCA, but the prevalence of anthrax in the sampled population or in other words, the proportion of carcasses sampled that were truly infected with *B. anthracis*. The model is based on the assumption that given that *B. anthracis* DNA is present within a carcass, qPCR tests on the different sample materials obtained from that carcass are conditionally independent. This assumption also applied to the microscopy tests (i.e. given that the capsule is present, the tests are conditionally independent). The probabilities of the animals being infected with *B. anthracis* were calculated from the posterior distribution of the LCM. Monte Carlo approximation (Papadopoulos and Yeung, 2001) was carried out with 10,000 iterations for each case. The mean of the 10,000 probabilities obtained for each case represented the true probability of the case having been infected. Probabilities greater than

or equal to 0.5 were interpreted as true positives while those below 0.5 were considered true negatives. The sensitivity and specificity of qPCR with the sample materials at different Ct cut-off values were then obtained over Ct values from 15 to 50 along with uncertainties (confidence intervals) in the estimates, using Monte Carlo approximation. The sensitivity and specificity of qPCR with insect samples were calculated from the true disease state of the carcasses as a reference standard.

The range of possible cut-off values (15 to 50) was chosen with prior belief that an appropriate threshold would fall within this range. It enables observation of changes in the sensitivity and specificity of the tests over this wide range of cut-offs. The cut-off range extends to 50 (Burns and Valdivia, 2008), which is above the 40 cycles in a qPCR run, to better observe tests that may have very poor sensitivity or specificity. Receiver operating characteristic (ROC) curve was created for each sample type in order to visualise their sensitivities and specificities at different cut-off values. An ROC curve allows the visualisation of the performance of a diagnostic test using different thresholds for interpretation by plotting the true positive rate of a test (sensitivity) on the Y axis and the false positive rate (1-specificity) on the X axis (Opsteegh *et al.*, 2010; Xiao *et al.*, 2016). Thresholds closer to the upper left part of the plot are preferred as they optimise sensitivity and specificity.

5.3 Results

In this study, 367 suspected anthrax cases were investigated from which 747 samples were collected (see Flowchart in Figure 4.6). Blood smears were available from 152 of these carcasses. Other sample types for qPCR testing were variably available for collection from these carcasses: blood (n = 102), swabs (n = 138) and insects (n = 30). Tissue samples were collected from 320 carcasses, more than twice the number than for other sample types.

Detectable DNA amplification of at least one target was observed in 591 out of 747 samples. The majority (83.4%) of samples in which at least one target was detected showed amplification of the other two targets before 40 PCR cycles (Table 5.1). Table 5.1 shows details of samples for which detection was observed in one, two, or three DNA targets.

Table 5.1: Number and proportion of samples with detection of only one, two or all three DNA targets before the end of PCR run of 40 cycles.

Sample type	Number of targets amplified			
	0	1	2	3
Blood smear (n=152)	33 (21.7)	8 (5.3)	9 (6.9)	102 (67.1)
Blood (n= 102)	27(26.5)	11(10.7)	1 (0.1)	63 (61.8)
Swab (n= 138)	34 (24.6)	12 (8.7)	7 (5.1)	85 (61.6)
Skin tissue (n= 325)	47 (14.5)	24 (7.4)	18 (5.5)	233 (72.6)
Insect (n=30)	16 (53.3)	5 (16.7)	3 (10)	6 (20.0)
Total samples (n= 747)	157 (21.0)	60 (8.0)	38 (5.1)	492(65.9)

The mean (and median) Ct values for those samples with only one target amplified were 37 (37) for *cap*, *lef* and *PLF3* targets. For samples with only two targets amplified, the mean (and median) Ct values were 37 (37) for *cap* and *lef,* and 37(38) for *PLF3*.

The area under the ROC curve for all samples except insect samples shows that qPCR provides the ability to differentiate between *B. anthracis* negative and positive cases much greater than what might be observed by an un-informative test or one that randomly classifies samples as positive and negative (Figure

5.1). An uninformative test will produce a ROC curve close to the diagonal lines. With insect samples, the test is only slightly informative, as shown in Figure 5.1 (panel 5).

For all sample types except insects (i.e. blood, swabs, skin tissue, and smears) the sensitivity and specificity of qPCR were very high at the optimal sample-specific threshold (Table 5.2).

Table 5.2: Optimal cycle threshold (Ct) cut-off value for detecting *B. anthracis* with quantitative polymerase chain reaction (qPCR) in sample materials from the field and the associated sensitivity and specificity

Sample material	Number of samples available	Optimal threshold	Sensitivity	Specificity
Blood smear	152	32	96.3% (90.7-99.2%)	98.5% (93.1-99.9%)
Blood	102	39	93.7% (85.9-98.0%)	86.9% (74.5-95.0%)
Swab	138	37	89.5% (81.6-94.8%)	91.8% (81.4-97.8%)
Tissue	325	37	95.2% (90.7-98.1%)	94.3% (85.2-98.8%)
Insect	30	36	19.5% (5.2-41.9%)	93.7% (74.8-99.8%)

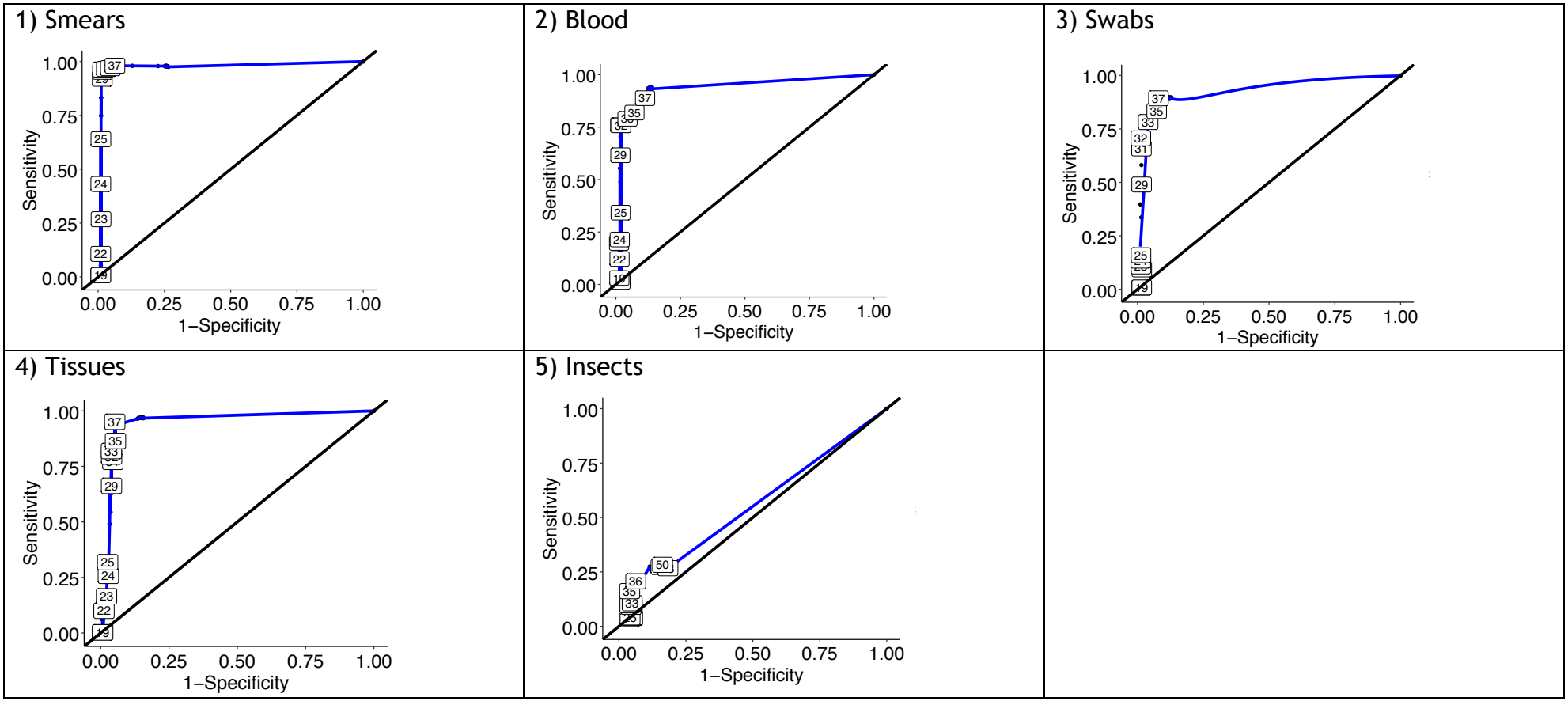


Figure 5.1: Area under the receiver operating characteristic (ROC) curve for the different sample materials, showing the sensitivity and specificity of qPCR at varying thresholds. The blue lines represent the curve for the respective sample material and the diagonal line represents chance classification of samples. Ct values are shown in the rectangles along the curves.

5.4 Discussion

The primary objective of the study reported in this chapter was to identify sample types that fulfil both convenience and scientific criteria for qPCR test performance, with the aim of improving the surveillance of anthrax in endemic, hard-to-reach areas. The convenience criteria we aimed to maximize were those of sample availability, while assessing the suitability of sample storage at ambient temperature to overcome the lack of cold-chain storage infrastructure in field conditions characteristic of endemic and remote communities in low income countries. The scientific criteria considered were those of sensitivity and specificity of qPCR for the molecular detection of *B. anthracis* in various sample types. Skin tissue was available for sampling from twice as many suspected anthrax cases as blood smears, and testing tissues by qPCR provided comparable sensitivity and specificity to those obtained by testing blood smears, even following storage at ambient temperature for several months prior to DNA extraction. Use of qPCR on tissue could dramatically improve the ability to detect *B. anthracis* in endemic settings, particularly in remote and challenging areas that typify areas of highest anthrax burden.

The results of this study show that different sample materials (i.e. blood smears, whole blood, blood swabs, skin tissues and insects) have different optimal Ct cut-off thresholds, sensitivity and specificity of qPCR for detecting *B. anthracis*. Sensitivity and specificity of qPCR from all sample types except insects were very high. The best chance of observing the true *B. anthracis* infection status of the suspected case (highest combined sensitivity and specificity) was obtained from smear samples, followed closely by tissue samples. However, the convenience criterion of sample availability was lacking for smear, blood and swab samples compared to tissue samples, since these samples could be obtained from fewer than half of the total carcasses investigated.

Endemic anthrax usually occurs in rural and remote areas in developing countries, making surveillance difficult (Turnbull, 1998). In many affected areas in Africa and Asia, anthrax carcasses are consumed by the local population (Gombe *et al.*, 2010; Biswas *et al.*, 2012; Sitali *et al.*, 2017) or by scavengers

(Dragon *et al.*, 2005; Beyer and Turnbull, 2009), limiting the availability of fresh samples for diagnostic testing. When disease surveillance is carried out at remote locations, the ability to use available sample materials for confirmation of a suspected anthrax case or outbreak is important. Out of the total number of suspected cases investigated, tissue samples could be obtained for most (90%). In cases where skin samples were not available, the options were to collect anecdotal reports with the possibility of obtaining soil and/or insects from the site where the animal died and had been butchered or scavenged. Soil samples were not included in the testing carried out as they require extensive modification to the protocols, requiring more expensive consumables, significantly increasing the cost of testing, if the method were implemented in a low-resource setting. This modification includes an enrichment step that enables multiplication of *B. anthracis* cells (Gulledge *et al.*, 2010). Although it is advantageous when low concentration of the pathogen is present, it is a disadvantage when considering culture-free methods. Blood smears and swabs were obtained from less than half of suspected cases, while blood in tubes could be obtained from less than a third of them. This shows that the use of tissue samples can transform anthrax surveillance in remote and endemic communities.

Detection of *B. anthracis* DNA was possible in the samples which were all stored at ambient temperature, enabling the surveillance of anthrax in areas where infrastructure for cold chain storage of samples is lacking. In low-resource areas, it is advantageous to use approaches for sample collection and storage that do not require expensive equipment that may also be difficult to maintain or run. It is also important that the storage conditions do not impact negatively on the ability to detect the pathogen. It is possible to detect *B. anthracis* from samples stored at ambient temperature for up to six months, but that the pathogen can be detected with high specificity and sensitivity. *B. anthracis'* ability to form spores may make storage of samples at ambient temperature for molecular diagnostic purposes more feasible, since the DNA sequestered in spores is protected from damage.

One of the limitations of this study is that no comparisons were made between the outcomes (i.e. sensitivity and specificity) of samples stored at ambient temperature and those stored using cold chain. Notwithstanding, samples stored at ambient temperature produced good sensitivity and specificity and any difference may likely be negligible. The findings were consistent with results obtained from stain microscopy which is based on a different method of pathogen detection and strengthens confidence in the inference that sample storage at ambient temperature is unlikely to have a major impact on the detection of *B. anthracis*.

Although tissue samples were readily available, and qPCR testing yielded very high sensitivity and specificity with them, the performance of qPCR using smear samples was greater than those of tissues and other sample types at any Ct cut-off value between 32 and 37. This suggests that in terms of scientific criteria (Chapter 4 section 4.1.3), smear samples provide the best chance of detecting *B. anthracis* among these sample types. Smear samples are also advantageous as they are easy to store, and confirmation of anthrax can be achieved quickly by stain microscopy. Moreover, stained smears can be subsequently scraped off for qPCR (Chapter 4). Although blood and swab materials had lower performance compared to smears and tissues, they may still be considered good sample materials for the sensitive and specific detection of *B. anthracis*. However, since they are not as readily available, they are not recommended as the primary sample type for routine surveillance.

The poor sensitivity of qPCR for detecting *B. anthracis* from insect samples indicates that they are not an optimal diagnostic material. The analysis of insect samples can, however, provide insights into the epidemiology of anthrax in areas where carcasses are even more challenging to find and sample. Hoffmann *et al.* (2017) detected the DNA of anthrax-causing *B. cereus* from fly samples, which allowed these authors to better define the geographical distribution of this pathogen in dense jungle settings in West Africa. DNA was detected in only 5% of the 784 insect samples tested by these authors; given the low sensitivity of qPCR

with insect samples we found in this study, the true prevalence of anthrax related to *B. cereus* in West Africa could be much higher than what Hoffmann *et al.* (2017) reported.

Results of this study were based on the amplification of all three DNA targets; samples in which only one or two sequences were amplified were considered negative. These samples could either be truly positive and at the limit of the analytical sensitivity of qPCR, or be false positives caused by non-specific amplification of other bacteria or – more likely – cross-contamination during extraction. Our choice of classifying these samples as negative could result in a slight overestimation of specificity and underestimation of sensitivity

5.5 Conclusion

Detection of *B. anthracis* by qPCR is possible using a variety of field samples collected in remote endemic areas with poor sample collection and storage infrastructure. Smear samples are particularly useful as they can be used to confirm the presence of the pathogen using stain microscopy with subsequent analysis of the same stained smear by qPCR. However, tissues also provide a good alternative, and may be more readily available for collection in areas where carcasses are likely to be opened and butchered before it is possible for blood smears to be taken. This study showed that in the field conditions of the NCA, collecting skin tissue samples increased the likelihood of animal anthrax case confirmation by more than 2-fold compared to only collecting smear samples. Moreover, qPCR testing of tissue samples remains highly sensitive and specific for samples stored at ambient temperature for up to several months. Thus, tissue samples hold great promise for improving the surveillance of anthrax when facilities for PCR are available in-country.

Chapter 6

Chapter 6 Environmental predictors of anthrax in livestock in community-defined high-risk areas

6.1 Introduction

Disease mapping is the concept of identifying geographical variability in rates of disease occurrence (Hay *et al.*, 2013). In addition to providing information about the distribution of diseases, it enables an understanding of the interaction between a pathogen and the environment (Hugh-Jones and Blackburn, 2009). The interaction of a pathogen with the environment may create a spatial structure with implications for the persistence or transmission of the pathogen. Understanding the factors related to the distribution of a disease is an important epidemiological underpinning (Bhopal, 2009) and it is essential for disease surveillance and control, for which resources are generally limited. Understanding the distribution of disease is also key to generating hypotheses to enable an understanding of risk factors and determinants of disease occurrence (Lawson *et al.*, 2000).

6.1.1 The biology of anthrax persistence and the relationship of the pathogen with the environment.

Anthrax is primarily a disease of animals, but it is zoonotic in nature and humans can get infected with the bacterium that causes the disease. The causative agent of anthrax, *Bacillus anthracis*, can exist either in a vegetative or a spore form. Key to the environmental persistence of *B. anthracis* is its ability to form metabolically inactive spores that are very resistant to destruction in the environment (Dragon *et al.*, 2001), where they can persist for long periods of time, even up to a century (Dragon and Rennie, 1995). Most infection with the pathogen is likely to occur through contact with spores (WHO, 2008). Once ingested, the favourable conditions in the host enable the spores to germinate into vegetative forms capable of replicating and producing the toxins that eventually cause the death of the host. Upon death of the host, the vegetative forms of the pathogen sporulate if exposed to oxygen and deprived of essential nutrients. This process can take from six to thirty-six hours to complete under

laboratory conditions (Baweja *et al.*, 2008). The initiation of the process and the time required to complete it depend on a number of factors including temperature, pH, nutrient and oxygen availability (Dragon and Rennie, 1995).

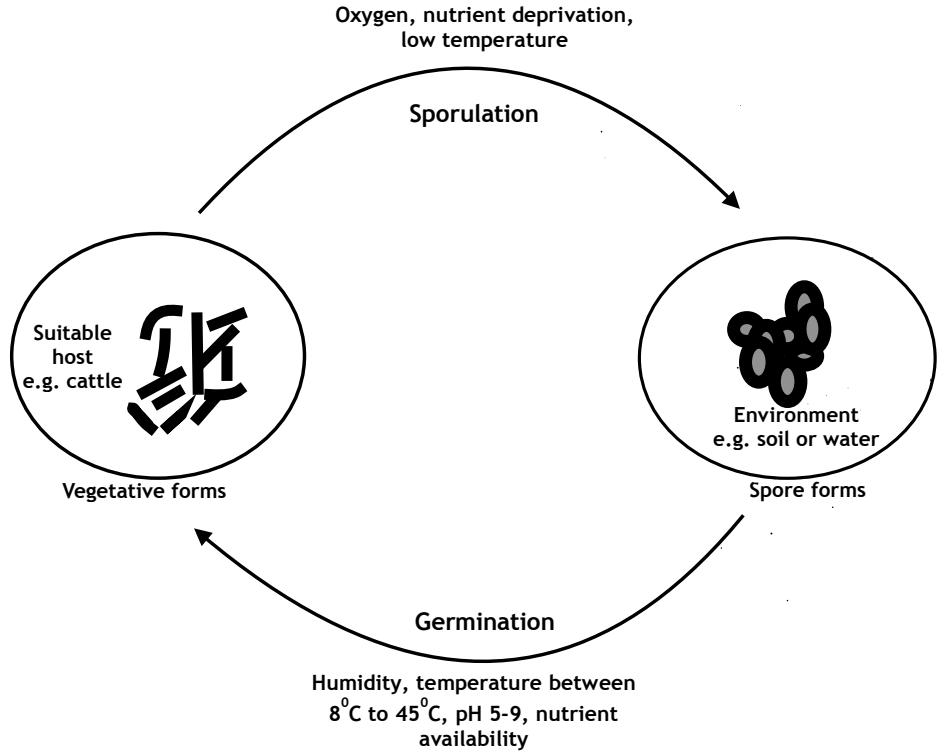


Figure 6.1:The different forms of *Bacillus anthracis* and the conditions that favour germination and sporulation. The spores can survive in the environment for decades before encountering a suitable host, in which they germinate and cause disease.

The spores have several surface layers that interact with the environment to ensure the bacterium's survival (Mock and Fouet, 2001). The exosporium – which is the outermost part of the spore – surrounds the spore coat. These two structures contribute to dormancy and are responsible for the hydrophobicity of the spore and impede the entry of harmful chemical agents. The exosporium, which is negatively charged, also plays a role in spore concentration by enabling the spores to adhere to soil particles once they are formed, thus maintaining the concentration of spores at a site of contamination (Hugh-Jones and Blackburn, 2009). Many hypotheses of *B. anthracis* persistence in the environment, and interactions and factors that promote this, have been put forward. These factors are similar to those that promote sporulation, and include temperature,

precipitation and moisture, organic matter, inorganic calcium ion, pH, average vegetation index and soil topography (Hugh-Jones and Blackburn, 2009). In many areas where anthrax is endemic, data are lacking on the environmental conditions suitable for the persistence of *B. anthracis* (Carlson *et al.*, 2019). In sub-Saharan Africa, much of the research aimed at understanding the relationship between *B. anthracis* and the environment has focused on southern Africa (Chikerema *et al.*, 2012; Steenkamp *et al.*, 2018). Little is known about the environmental factors that contribute to the risk of anthrax in Tanzania. Hampson *et al.* (2011) showed that anthrax seropositivity in domestic dogs and wildlife in the Serengeti and Ngorongoro national parks were associated with alkaline soil pH, close proximity to inland water bodies, and cumulative extremes in annual rainfall. In this larger ecosystem, varied environmental conditions exist that may influence the transmission and persistence of anthrax (Galvin *et al.*, 2004). Since the disease is often localised, geographical areas affected by the disease may be characterised by distinguishing environmental conditions, but these conditions are not very well established. In this study, factors that promote the persistence of *B. anthracis* refer to those conditions that are associated with the survival of the pathogen in the environment and enable it to come in contact with a suitable host. Both factors that promote spore survival and those that facilitate exposure of animals may promote the persistence of the disease. For instance, while dry conditions (e.g. caused by lack of rainfall) and alkaline soil promote spore formation and survival, high rainfall and close proximity to water bodies may facilitate transmission to animals by the action of water unearthing spores and washing them into water bodies. Arguably, spore survival and transmission are both critical, however it is unclear whether under certain situations, one may drive the risk of anthrax more or less than the other.

6.1.2 Approaches to anthrax mapping in the context of the NCA

Many methods may be employed to map the patterns of anthrax in space, including those that utilise incidence data from passive surveillance, active surveillance and surveys (Blackburn *et al.*, 2007; Lembo *et al.*, 2011; Bagamian *et al.*, 2014; Chen *et al.*, 2016; Carlson *et al.*, 2019). Anthrax endemic areas are

often located in remote and challenging areas with limited infrastructure like roads. This makes it difficult and expensive to conduct surveillance or large-scale field and environmental surveys or experiments to identify areas that are contaminated by anthrax spores and that are particularly risky for animals and humans. The difficulty of conducting environmental surveys, like collecting soil specimens for testing, is further compounded by the lack of specific information indicating anthrax carcass burial sites (see section 3.3.1.5). Because of these challenges, identifying areas that pose the highest risks to people and their livestock is important and would allow a more targeted approach to managing the risk of anthrax.

One approach that has not yet been applied to anthrax mapping is the use of participatory mapping methods. Participatory mapping can be defined as approaches that utilise indigenous spatial knowledge. It is a form of participatory research and places emphasis on the generation of research data through consultations with relevant local communities (Maman *et al.*, 2009). Participatory mapping tools are increasingly being used for disease surveillance. The approach has been applied to disease vector control, for example mosquitoes and malaria (Dickin *et al.*, 2014), as well as to the surveillance of West Nile Virus (Shuai *et al.*, 2006). Participatory mapping is highly suitable for understanding the geographical distribution of anthrax in the NCA for a number of reasons. First, pastoralists have been shown to have a very good indigenous ecological knowledge and an understanding of their environment and are able to recall incidents relating to their livestock health and productivity even over long periods of time (Berkes *et al.*, 2000; Mapinduzi *et al.*, 2003). Second, pastoralists typically move their livestock long distances in search for resources. This means that the place where an animal becomes infected may differ from that where it succumbs to the disease. The incubation period for animal anthrax can range between a few hours to 21 days (WHO, 2008). Since animals are typically moved long distances, an infected animal may travel many miles before symptoms appear or death occurs. Thus, case locations recorded in surveillance databases are likely to reflect the current residence of the farmer rather than the specific location where the animal contracted the disease.

Participatory mapping approaches can be integrated with geographic information systems (GIS) to inform knowledge of disease risk. For instance, some malarial control programs use GIS and participatory mapping to target areas for vector control (Dongus *et al.*, 2007). Although participatory mapping is still useful without the added value of georeferencing (Dickin *et al.*, 2014), integrating data from participatory mapping and GIS can enhance the value of the information obtained (Smith, *et al.*, 2000) and it may be used to identify environmental predictors of risk over larger spatial scales, as has been previously demonstrated with anthrax incidence data (Carlson *et al.*, 2019). However, the use of participatory mapping integrated with GIS to understand the environmental risks of anthrax is novel, since the spatial risk of anthrax has never before been studied using participatory mapping methods.

6.1.3 Objectives

The study was conducted to

1. Map animal anthrax-risk areas as defined by livestock-keeping communities and to identify environmental characteristics that might explain why such areas are likely to be favourable to anthrax persistence, in terms of spore survival and transmission to animals.

6.2 Methods

This section describes methods used to map defined high-risk areas for anthrax in the NCA and to investigate the environmental conditions that explain anthrax persistence. Participants in ten mapping groups were asked to draw areas associated with livestock contracting anthrax during grazing, watering or salt licking on geo-referenced maps. Defined high-risk areas were digitised in Quantum GIS, with random points generated within and outside the defined areas to represent high-risk and low-risk areas respectively. Using secondary environmental data, these spatial points where characterised in terms of pH, cation exchange capacity, distance to inland water bodies, topsoil organic carbon content, daytime land surface temperature, and enhanced vegetation index. Regression analysis was employed to identify associations between these environmental variables and the probability of a random point occurring within a defined risk area. The predicted probability of being anthrax high-risk area was determined and represented spatially on a map using a new dataset generated as described above.

6.2.1 Study area

The study was conducted in the Ngorongoro Conservation Area (NCA) in northern Tanzania where anthrax is endemic (Chapter 1). The NCA comprises eleven administrative wards: Alailelai, Endulen, Eyasi, Laitole, Kakesio, Misigiyo, Ngorongoro, Naiyobi, Nainokanoka, Ngoile, and Olbalbal (Figure 6.2).

6.2.2 Participatory mapping

The participatory mapping exercises were designed based on previous research conducted to map natural resources in arid areas in Kenya and Tanzania (Rowley, 2010). Georeferenced maps of the NCA were produced by the University of Glasgow, Department of Geography using data from Google and DigitalGlobe (2016). The maps used datum Arc 1960 /UTM zone 36S and grid intervals of 1000km. The maps were produced at 1:10,000 and 1:50,000 scales, in order that participants be provided with a choice of a suitable scale. Ten participatory mapping focus group were held at ward administrative level

(Figure 6.2) in order to identify areas in the NCA that communities perceive as posing a high risk of anthrax. One mapping exercise was held in each ward. Ngoile and Olbalbal wards were covered at the same time as they had only recently (in 2015) been formed from one ward (Olbalbal). Each exercise had between ten and thirteen participants and consisted of village and ward administrators, animal health professionals, community leaders, and selected pastoralist community members. These groups of participants were recruited because they represent members of the community concerned with animal health and believed to hold most of the knowledge relating to community experience of animal health and disease, specifically anthrax. Participants were recruited by consulting with animal health professionals and village and ward administrators.

Figure 6.2: Map of the Ngorongoro Conservation Area showing wards and locations where mapping exercises took place (red dots).

The mapping was conducted in Swahili and translated to English by an interpreter. During these exercises, participants identified areas that they believed posed a high risk for animals of contracting anthrax. Participants'

knowledge of the maps and locations were verified by testing whether they could correctly identify locations such as health centres, places of worship, markets and schools. Participants then used maps to identify areas of risk for livestock (Figure 6.3). The focus groups where conducted using the schedule in Appendix 13, participants discussed among themselves and came to a consensus about areas that were at high risk of anthrax and these were drawn on the maps provided. In order to improve the fidelity of the data, participants defined risk areas in relation to their own locality (ward) and locations where their animals access for resources. Therefore, the areas were not defined by administrative boundaries as communities may access locations outside of their wards for resources. The resulting maps were scanned, digitised and analysed as detailed in the following sections.

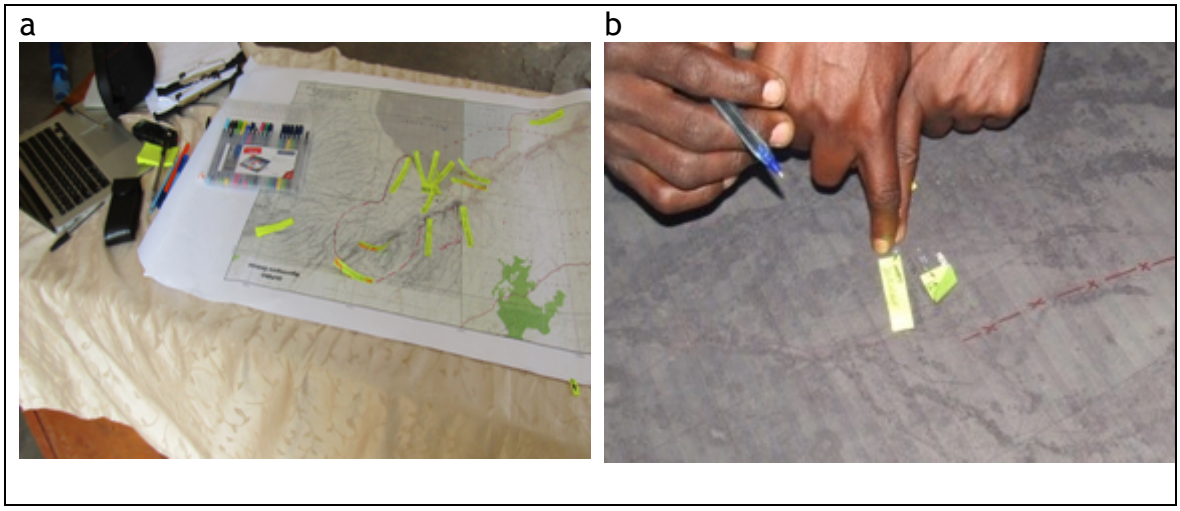


Figure 6.3: Participatory mapping sessions of anthrax risk areas in the Ngorongoro Conservation Area. Images show (a) the set-up of a mapping session and (b) participants engaged during a session.

6.2.3 Digitisation of maps and generation of random points

Scanned maps were obtained as .pdf files and converted to very high quality .tiff files for digitisation in QGIS 2.18.2-Las Palmas software (QGIS_Development_Team, 2017). All the maps were georeferenced with geographical coordinates during production and reference points were available to enable fixing of the maps to their precise locations. The digitization was carried out using the QGIS digitizing tools and by creating polygon layers of the

defined risk areas with attribute data on the wards, corresponding to locations where mapping was done.

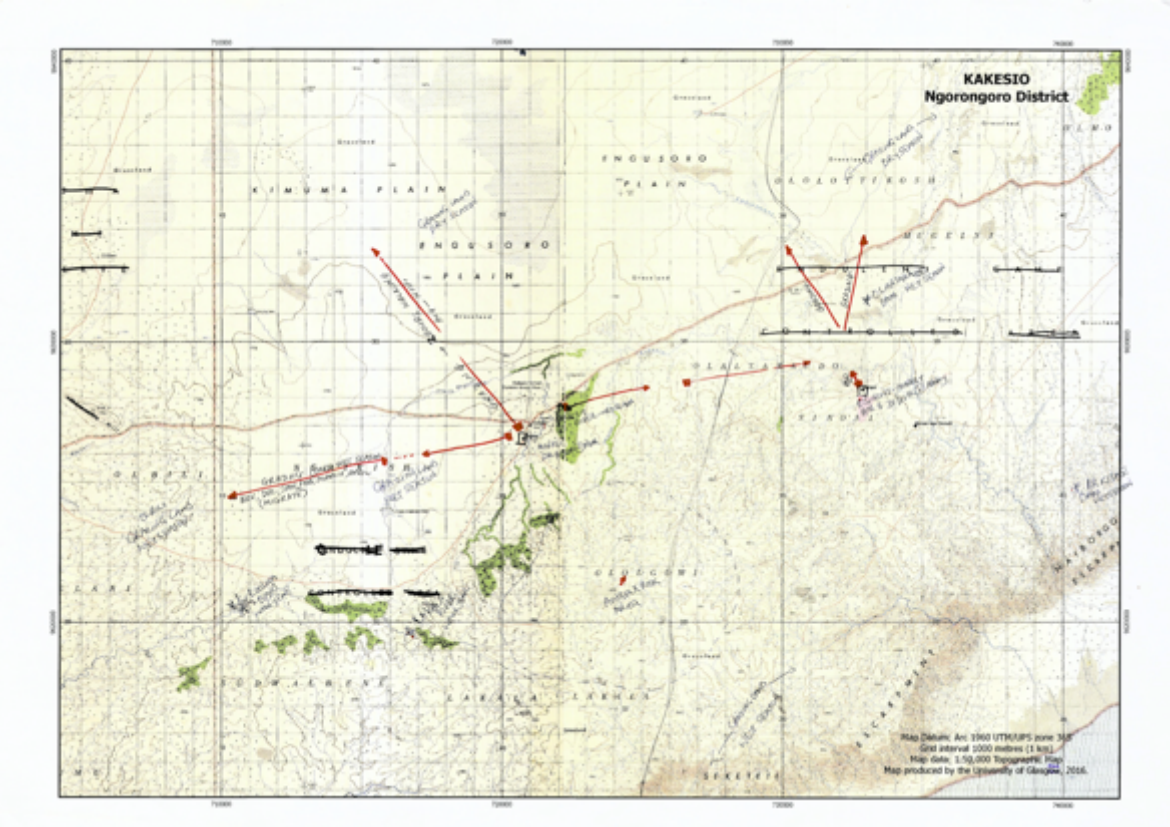


Figure 6.4: An example of maps annotated by communities in the Ngorongoro Conservation Area, in order to identify anthrax high risk areas.

6.2.4 Sourcing data on the environmental predictors of anthrax

Secondary soil and environmental data for Tanzania were obtained from the Africa Soil and Information Service (AFSIS) (http://africasoils.net/). Variables that had been shown to contribute to the risk of anthrax or those that may explain the risk of anthrax based on the biology and ecology of *B. anthracis* were identified and selected from the available datasets (Table 6.1):

Cation exchange capacity (CEC)

Measured in cmol/kg, CEC is the total capacity of the soil to retain exchangeable cations such as Ca^{2+}, Mg^{2+} etc. It is an inherent soil characteristic and is difficult to alter significantly. It influences the soil's ability to hold onto essential nutrients and provides a buffer against soil acidification (Bache, 1976).

In this study it is used as an indirect measure of the calcium content of the soil (i.e. a higher CEC means a higher calcium content). A positive relationship between CEC and the risk of anthrax is expected.

Predicted topsoil pH (pH)

pH is a measure of acidity or alkalinity of a substance. Soil pH below 6.0 (acidic soil) is thought to inhibit the viability of spores (Hugh-Jones and Blackburn, 2009) thus a positive effect of pH on the risk of anthrax is expected. Soil matter in suitable places for the persistence of spores is positively charged with neutral to slightly alkaline pH (up to 8). It has been suggested that this positive charge attracts the negative charge on the bacterial exospore and enables the attachment of the spore to soil particles, thus maintaining viability (Hugh-Jones and Blackburn, 2009; Chen *et al.*, 2010).

Distance to inland water bodies (DOWS)

Distance to water indicates the degree to which an area is dry/arid. Anthrax outbreaks have been shown to occur in areas with very dry conditions (Hugh-Jones and Blackburn, 2009). Low moisture content of soils may promote spore formation and viability and limit the potential for spore germination (Dragon and Rennie, 1995). Conversely, a closer distance to water bodies may indicate a higher level of risk of exposure to *B. anthracis*. Hampson *et al.* (2011) reported that anthrax occurred close to water sources in the Serengeti, Tanzania and Steenkamp *et al.* (2018) found that close proximity to water bodies was key to the survival of *B. anthracis* in the Kruger National Park, South Africa. Water is an important resource for livestock and a large number of animals may congregate at water sources during dry seasons. The close proximity of a water source to a risk area may increase the chance of infection. With these contrasting effects, it is unclear what effect DOWS will have on the risk of anthrax in the NCA.

Average enhanced vegetation index (EVI)

Vegetation density may influence the likelihood of an animal coming into contact with soil that may be contaminated with spores. Grazing animals are more likely to encounter bacteria in soil with low vegetation density (Hampson *et al.*, 2011), although there is a possibility that spores can be washed onto

higher vegetation by the action of water (Hugh-Jones and Blackburn, 2009). Vegetation index may also be an indication of the moisture content of soil. Arid/dry conditions favour the formation and resistance of spores in the environment, thus lower vegetation may be associated with *B. anthracis* persistence. It is unclear what the effect of EVI on the risk of anthrax will be

Average day time land surface temperature (LSTD)

Anthrax-risk areas may be associated with elevated temperatures. The disease has been commonly reported to occur in regions with elevated temperatures worldwide (Hugh-Jones and Blackburn, 2009). It is hypothesized that the stress caused by heat alters immune responses of animals making them more susceptible to succumbing to infection. Thus a lower ID50 (infectious dose) and LD50 (lethal dose) is sufficient to cause infection and death respectively (Hugh-Jones and Blackburn, 2009). A positive effect of LSTD on the risk of anthrax is expected.

Slope

Slope may contribute to the risk of anthrax by retaining spores or causing them to disseminate more rapidly. Spores of *B. anthracis* are hypothesized to persist more easily in steppe landscapes that are characterised by shallow slopes (Hugh-Jones and Blackburn, 2009). Therefore, a negative relationship between slope and the risk of anthrax is expected.

Predicted topsoil organic carbon content (SOC)

Organic matter (g/kg) may aid spore persistence by providing mechanical support. The negatively charged exosporium of spores is attracted to the positive charges on hummus-rich soil, thus anthrax is thought to persist in soil rich with organic matter (Dragon and Rennie, 1995). Based on available evidence, a positive effect is expected on the risk of anthrax.

These environmental data for Tanzania had been compiled by Dr Markus Walsh of AFSIS and were available for use. The data were obtained in the raster data format and their characteristics have been summarised in Table 6.1

Table 6.1: Environmental factors with potential to influence anthrax persistence.

Variable	Dates	Source (website)	Research evidence associated with variable	Predicted association with high-risk areas
Cation exchange capacity (CEC)	NA	SoilGrids (https://soilgrids.org)	Hugh-Jones and Blackburn, 2009, Steenkamp *et al.*, 2018	Positive. CEC is used as a proxy for soil calcium. High calcium promotes anthrax persistence.
pH	NA	SoilGrids (https://soilgrids.org)	Hugh-Jones and Blackburn, 2009	Positive. *B. anthracis* spores have been shown to survive best in alkaline soils (pH >6).
Distance to inland water bodies (DOWS)	NA	SurfaceWater (https://global-surface-water.appspot.com/)	Hampson *et al.*, 2011 , Dragon and Rennie, 1995, Steenkamp *et al.*, 2018	Unknown. Anthrax has been shown to occur in dry areas but has also been reported to occur near water sources.
Average enhanced vegetation index (EVI)	2000-2016	Africa Soil Information Service (africagrids.net)	Hampson *et al.*, 2011, Hugh-Jones and Blackburn, 2009	Unknown. Higher or lower EVI may promote the risk of anthrax.
Average day time land surface temperature (LSTD)	2001-2015	Africa Soil Information Service (africagrids.net)	Hugh-Jones and Blackburn, 2009	Positive. Anthrax occurrence is associated with places having elevated temperature.
Slope	NA	Open Topography (http://opentopo.sdsc.edu)	Hugh-Jones and Blackburn, 2009	Negative. Anthrax has been more often observed in flat topography.
Predicted topsoil organic carbon content (SOC)	NA	SoilGrids (https://soilgrids.org)	(Dragon and Rennie, 1995)	Positive. Soils with high organic matter may retain spores more readily.

6.2.5 Creating the dataset

After digitization, 10,000 random points were generated as recommended by Barbet-Massin *et al.* (2012) to cover the 8,292km^2 area of the NCA, from which 5000 random points were selected. These random processes were carried out to ensure that the points were representative of the defined areas and reduce issues with auto correlation. Auto correlation occurs when locations exhibit similar characteristics due to their close spatial proximity. This can bias findings, as patterns observed from the data may be dominated by auto-correlation (Dormann *et al.*, 2007). Points falling within the defined risk areas (n= 413) were selected to represent risk areas while those falling outside represented low-risk areas (n= 4587). QGIS 'add Rasta data to points' was used to obtain the measures of the environmental characteristics associated with individual points.

The points within the defined risk-areas represent presence data (i.e. anthrax is present). Usually, validation of a disease risk area can easily be carried out with both presence and absence (no-/low-risk area or anthrax is absent) data available. When presence only data exists (as in this case, where areas thought to be free or least affected by anthrax were not specifically defined), quasi-absence data can be used (Stevens and Pfeiffer, 2011). Thus, areas and points falling outside of the defined risk areas were used as absence data.

In order to exclude areas that may not pose risk of anthrax to communities due to their inaccessibility, only points within a certain range of distance from settlements (Figure 6.5) were retained for analysis. On average, livestock are moved 4.26km away from settlements for grazing and watering during the day (data obtained from household survey as described in Chapter 2 and 3). Thus, only points falling within 4.26km of settlements were selected. Data on locations of settlements were obtained from satellite imagery and include temporary and well as permanent settlements. These data, which were collated from the Center for International Earth Science Information Network (CIESIN), were obtained along with the other environmental variables assessed in this study from Dr Markus Walsh.

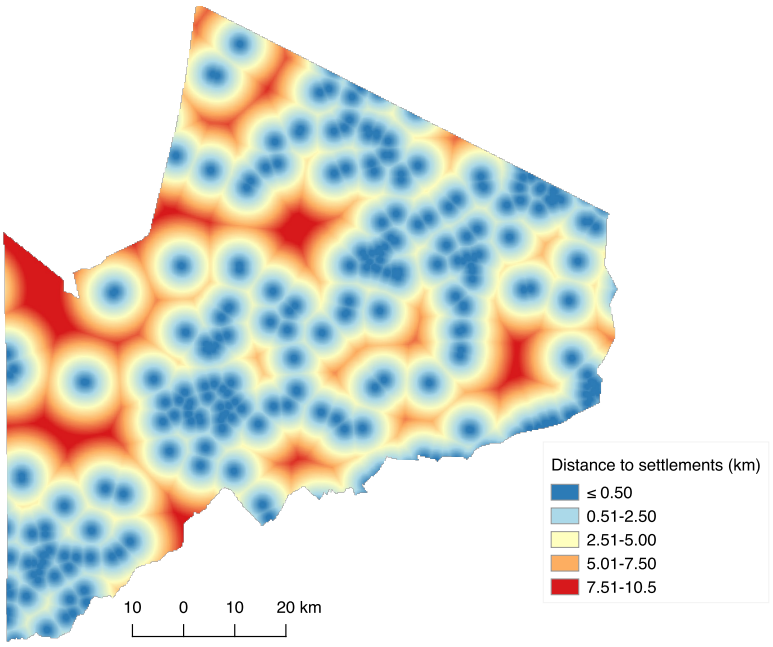


Figure 6.5: Ngorongoro Conservation Area map showing distance to settlements.

The annotated (Figure 6.4) and digitised maps yielded polygons of high risk areas within the NCA (Figure 6.6), with a total area of 695.27km^2. After adjusting for accessibility of resource locations using the average distance moved by livestock, and removing duplicate entries, 2173 points were included in the analysis. The proportion of points falling within high-risk areas was 11.00% (n=239) while that of low-risk areas was 89.00% (n =1934).

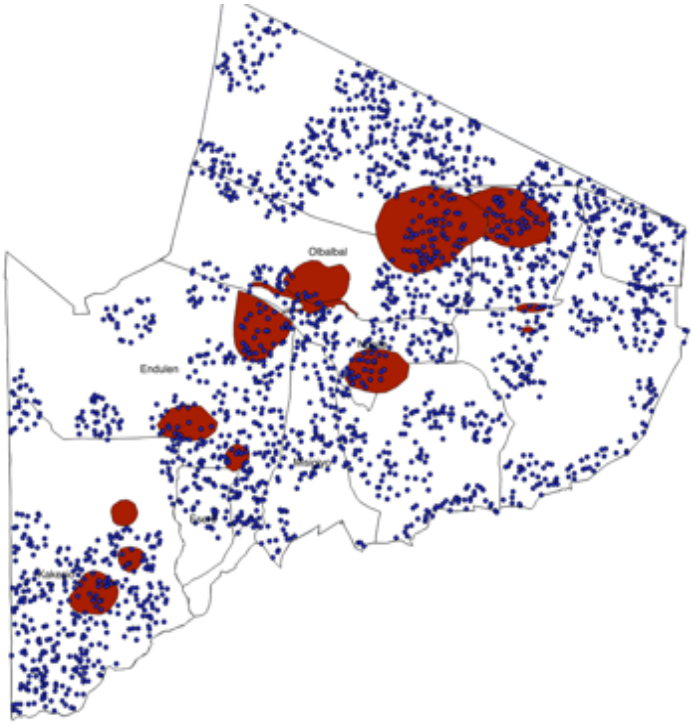


Figure 6.6: Anthrax-risk areas (in red) generated from participatory mapping and the boundaries of the Ngorongoro Conservation Area highlighted in black. Random points (purple) were generated throughout the area, and points falling within 4.26 km of human settlements were retained for analysis.

6.2.6 Data analysis

Statistical analyses were carried out in R (v 0.99.484) within the R Studio environment (RStudio Team, 2016). Logistic regression approaches were employed in order to identify what environmental factors were different between high-risk and low-risk areas.

Simple logistic regression

All covariates (Table 6.1) were continuous variables. Their potential effects were first assessed individually using simple generalised linear modelling with binomial error structure (logistic regression) and 'high-risk'/'low-risk' as the binary response variable.

Multiple regression analysis

A logistic regression model was built that combined all seven independent variables (CEC, pH, DOWS, LSTD, EVI, SOC and slope) and tested for collinearity

among the explanatory variables (i.e. the relationships between the explanatory variables that may bias their estimates). Collinearity of explanatory variables inflates the standard errors of the model coefficients and underestimates their effects on the response variable (Dormann *et al.*, 2013). The variance inflation factor (VIF) was computed for the explanatory variables. Briefly, the VIF is a measure of the increase in the variance of the coefficients when collinearity exists (Dormann *et al.*, 2013). Variables with a VIF greater than 4 (LSTD and EVI) were removed from the analysis (Hair *et al.*, 2010).

A generalised additive model (GAM) was built with this reduced set of variables in the mgcv package in R (Hebebrand, 2010). GAM approach is useful when non-linear relationships exist between response and explanatory variables, also to account for spatial autocorrelation in the data. It is a recognised regression method for modelling presence/absence data and accounting for spatial autocorrelation (Wood, 2006; Barbet-Massin *et al.*, 2012). GAMs are semi-parametric generalised forms of linear regressions in which fewer restrictions are imposed on the underlying distribution of the data (Wood, 2006). Although points had been generated randomly, they may be autocorrelated and not independent since they were created within the bounds of spatially defined areas. Non-independence may overestimate the effect of the covariates, leading to biased estimates of the significance of the variables (Dormann, 2007). Although GAM with spline regression on the geographical coordinates of location data does not completely remove autocorrelation, it does account for it. Accounting for spatial trends, as opposed to removing them, might prevent the risk of losing meaningful variation along with autocorrelation (Beale *et al.*, 2010). The GAM was built mixing both smooth and parametric terms, essentially by adding location as a two-dimensional tensor product (te) smoothed on longitude and latitude (Wood, 2006), while retaining other co-variates as parametric terms.

The model fit (i.e. how well the model uses the explanatory variable(s) to predict the response variable) was assessed using the R^2 statistic, deviance explained and visual inspection of the residual plots. The multivariable GAM was compared with a model that accounts only for spatial autocorrelation without

including any of the explanatory variables, using Chi test and Akaike information criterion (AIC). The predicted probability of being anthrax high-risk area was determined using a different dataset generated randomly as detailed in 6.2.5 and depicted on a map of the NCA. Inverse distance weighting (IDW) interpolation was used to compute the probability of risk for unsampled points to produce a heat map in QGIS (Figure 6.10). IDW is a deterministic approach that enables the generation of an attribute value for unsampled areas, based on the weighted average of values in neighbouring areas that adjusts for the distance between predicted points and that for which a value is being determined (Lu and Wong, 2008).

In addition, the predictive performance of the model (i.e. the ability of the model to be generalised to locations other than those from which the data was obtained) was assessed. This was carried out by dividing the dataset into parts for training and testing the model. Briefly, data were divided into four using spatial sectors corresponding to the north-east, north-west, south-west and south-east of the NCA. Four rounds of training were carried out with data points from three sectors while testing (prediction) was carried out on the remaining part of the data using the predict function in R. The predicted response variable (probability of risk) was dichotomised into high-risk and low-risk points using a cut-off value of 0.5. Visual analysis of the area under the curve (AUC) of receiver operating characteristic (ROC) plot was used to access the predictive accuracy of the model in the R Metrics package (McPherson, *et al.*, 2004; Hamner, *et al.*, 2018).

6.3 Results

Simple logistic regression revealed that all candidate predictors (CEC, pH, DOWS, EVI, LSTD, slope, and SOC) were significantly associated with high risk areas. Details for each of the predictors are presented below. The mean (Figure 6.7) and standard deviation (SD) of the variables are presented and results of simple regression using GLMs showing the pattern of relationship between these seven variables and the anthrax-risk areas are presented below.

CEC: CEC ranged from 9.75 to 47.25 cmol/kg. Points with higher CEC had a small but increased odds of falling into high-risk areas (OR 1.06, 95% CI: 1.04-1.08; $P < 0.001$), with a mean (and SD) CEC of 31.99 (7.26) cmol/kg compared to 29.69 (6.39) cmol/kg in low-risk areas (Figure 6.7a and Figure 6.8a).

pH

The measure of hydrogen ion concentration in the soil ranged from 5.32 to 8.70. There was a significantly higher odds of being an anthrax risk area for points with higher pH (OR 2.21, 95% CI: 1.84-2.66; $P < 0.001$). The mean (and SD) pH for points falling in high risk and low-risk locations were 7.45 (0.62) and 6.95 (0.84), respectively (Figure 6.7b and Figure 6.8b).

Distance to inland water bodies:

The distance of points to water bodies ranged from 0.25 to 48.84km. Points at greater distance from water bodies had increased odds of falling into high risk areas (OR 1.04, 95% CI: 1.02-1.05; $P < 0.001$), with a mean (and SD) DOWS of 19.26 (6.92) km compared to 15.41 (9.97) km in low-risk areas (Figure 6.7c and Figure 6.8c).

Average enhanced vegetation index

The EVI ranged from 934 to 6261. Points with lower EVI had increased odds of falling into high-risk areas (OR 0.9987, 95% CI: 0.9984-0.9989; $P < 0.001$), with a mean (and SD) EVI of 1873.23 (561.03) compared to 2766.05 (1169.85) in low-risk areas (Figure 6.7d and Figure 6.8d).

Average daytime land surface temperature

Average daytime temperature in the NCA, between 2001 and 2015, ranged from 14.94 to 46.79. Points with higher temperatures had an increased odds of falling in high-risk areas compared to low-risk areas (OR 1.19, 95% CI: 1.16-1.23; $P < 0.001$). The mean (and SD) temperature (Figure 6.7e and Figure 6.8e) was 40.62 °C (4.62) for points falling high risk areas and 34.16 °C (7.60) for those in low-risk areas.

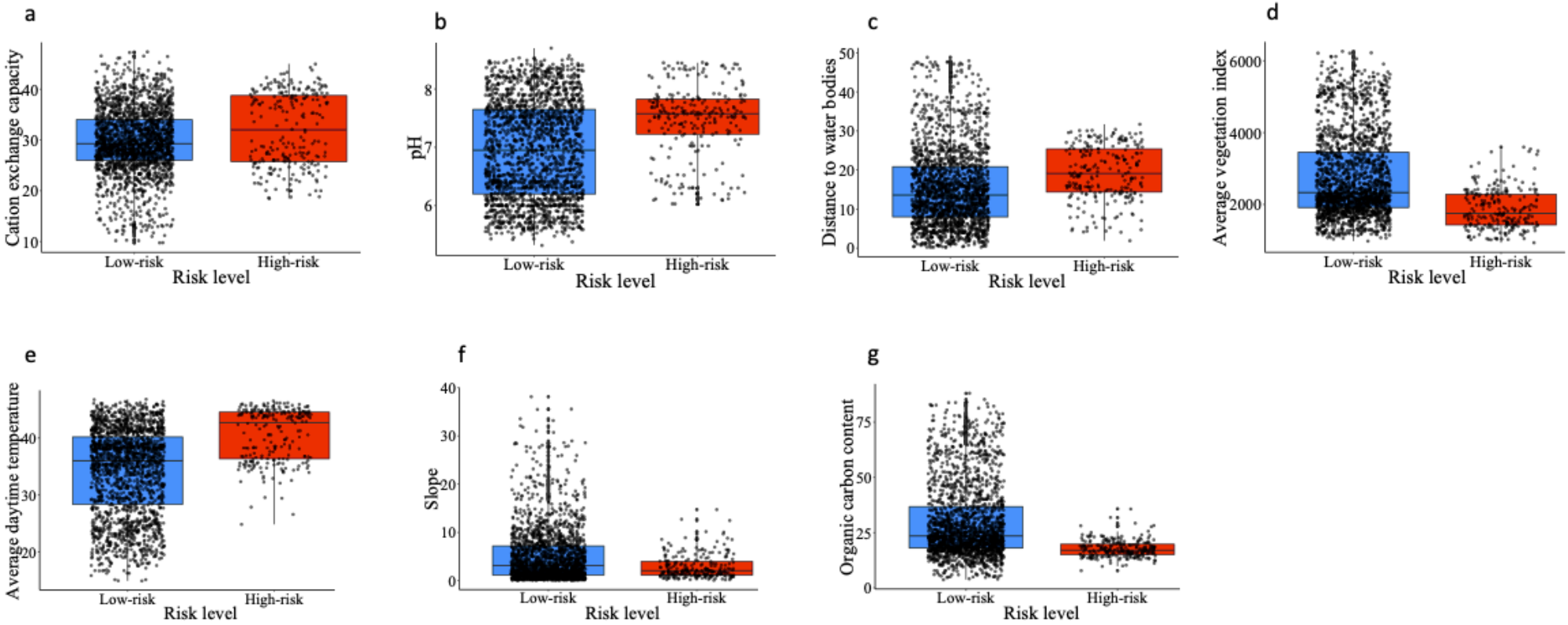


Figure 6.7: Boxplots of environmental variables showing data values, mean and standard deviation for points falling in both anthrax high- (red) and low-risk (blue) areas as defined through participatory mapping in the Ngorongoro Conservation Area. Plots are shown for cation exchange capacity (a), pH (b), distance to inland water bodies (c), average enhanced vegetation index (d), average daytime land surface temperature (e), slope (f), and predicted topsoil organic carbon content (g).

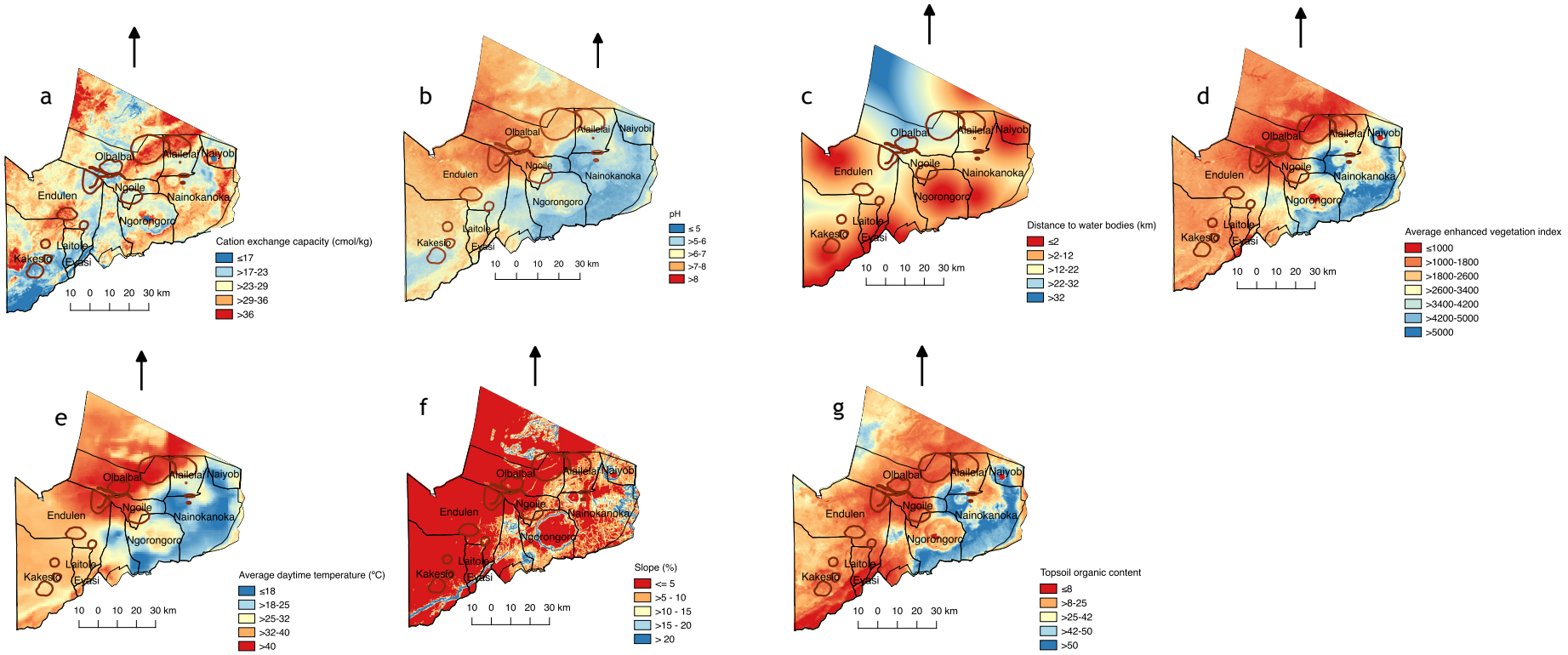


Figure 6.8: Maps of the Ngorongoro Conservation Area showing environmental predictors of anthrax, namely a) cation exchange capacity, b) pH, c) distance to water bodies, d) enhanced vegetation index, e) daytime temperature, f) slope, and g) topsoil organic carbon content. Based on available scientific evidence, areas with warm colours (red, orange and yellow) have environmental conditions favourable to a high risk of anthrax, while areas with cool colours (blue and navy) have conditions associated with a low-risk. Perceived risk areas are shown in circles.

Slope

The slope in the NCA ranged from 0.04 % to 38.12 %. Points with low slopes had an increased odds of falling into high-risk areas (OR 0.88, 95% CI: 0.85-0.92; $P < 0.001$) with a mean (and SD) of 2.91 (2.49) % compared to 5.05 (5.55) % in low-risk areas (Figure 6.7f and Figure 6.8f).

Predicted topsoil organic carbon content

Topsoil organic carbon content ranged from 3.75 to 88.00 g/kg. Points with low SOC had an increased odds of falling into high-risk areas (OR 0.90, 95% CI: 0.88-0.92; $P < 0.001$), with a mean (and SD) of 17.68 (3.82) g/kg compared to 29.63 (16.87) g/kg in low-risk areas (Figure 6.7g and Figure 6.8g).

6.3.1 Regression analysis

In all cases the smooth terms were statistically significant indicating that the points were substantially autocorrelated. Therefore, the results of regression analysis using GAM are preferred and reported. Results of univariable GAM accounting for spatial autocorrelation are presented in Table 6.2, and indicate that DOWS, slope, SOC, EVI, and LSTD for points falling in high-risk areas were significantly different from those falling in low-risk areas. However, CEC and pH for those areas were not significantly different.

While all variables were significantly different between high- and low-risk areas in the GLM, pH and CEC were not statistically significant in the GAM analysis and the direction of relationship of DOWS with high-risk areas was reversed from a positive association to a negative one. However, given the significance of the smooth term accounting for spatial autocorrelation, the results of the GAM are preferred.

Table 6.2: Results of simple regression accounting for spatial autocorrelation , using generalised additive models. The odds of points falling into a high-risk area with each environmental variable are shown.

Variable	GAM regression analysis	
	Odds ratio (95% confidence interval)	*P*-value
CEC	0.98 (0.93 - 1.04)	0.543
pH	1.58 (0.79 - 3.18)	0.199
DOWS	0.69 (0.59 - 0.81)	<0.001
slope	0.92 (0.84 - 1.00)	0.005
SOC	0.86 (0.81 - 0.91)	<0.001
EVI	0.998 (0.997 - 0.999)	<0.001
LSTD	1.43 (1.25 - 1.63)	<0.001

6.3.2 Multivariable analysis

There was strong statistical support for the multivariable model compared to the model that accounts only for spatial autocorrelation without including any of the explanatory variables (Δ AIC:50). The variability in the predicted and actual response variable (explained deviance) for the multivariate model was 61.60% and it had an adjusted R^2 (how much of the variability in the observed response variable is predicted by the explanatory variables) of 58.30%. Visual inspection of the binned residual plot indicated no problem with model fit (Figure 6.9).

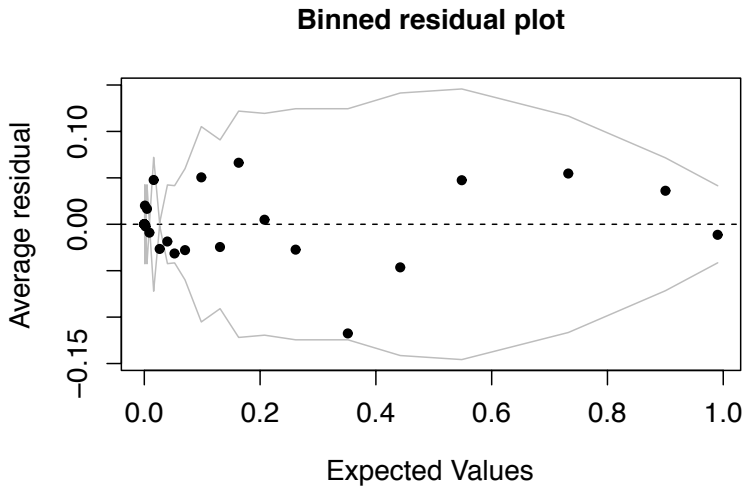


Figure 6.9: Binned residual plot of multivariable generalised additive model explaining perceived high-risk areas. The residuals which all lie within the confidence limits are centred around zero, and no distinctive pattern can be observed, indicating a good fit.

Results of the multivariable GAM shows that the odds ratio of points with greater pH and CEC (1.84 and 1.03 respectively) falling in high-risk areas were not significantly higher from those falling in low-risk areas (Table 6.3). Similarly, for slope, the odds ratio (0.93) of points with lower values falling in high-risk areas were not significantly different from those falling in low-risk areas. However, points closer to water bodies (DOWS) and with a low organic carbon content (0.64 and 0.86 respectively) had significantly greater odds of falling in high-risk areas compared to low-risk areas (Table 6.3). The maximum likelihood and AIC of the model with these five variables were better than resulting models of a stepwise backward elimination method. This indicates that removal of any of these variables is unjustified and will result in a considerable loss of fitness. The smoothing term included to account for autocorrelation was significant (P <0.001) and the estimated degrees of freedom was 24.

The level of risk predicted by the participatory mapping data indicated that Ngoile and Obalbal areas extending into Endulen and Alailelai are the most affected by anthrax (Figure 6.10).

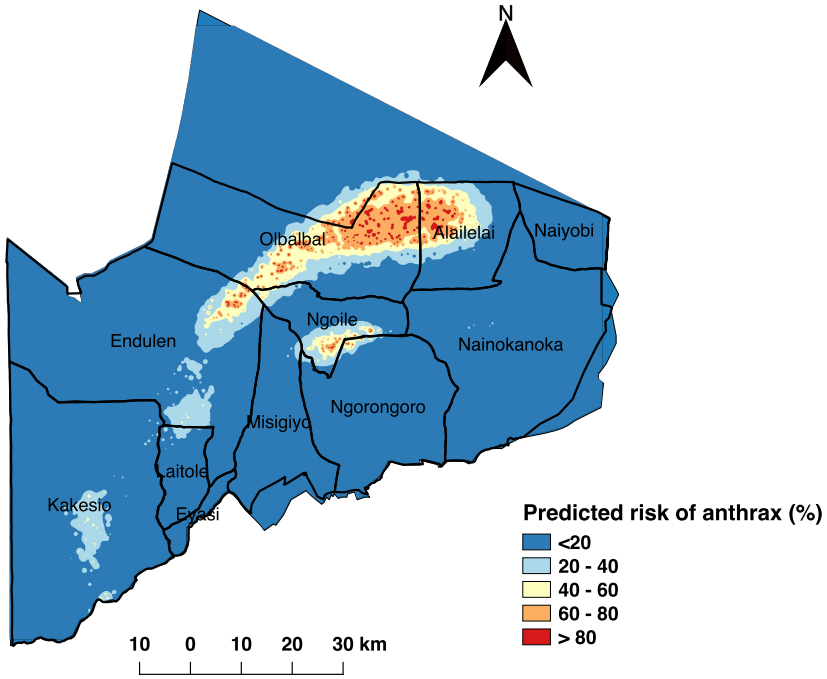


Figure 6.10: Predicted probability of being an anthrax-risk area in the Ngorongoro Conservation Area

Results of the predictive performance of the model indicate a low ability of the model to be generalised to other locations. Visual inspection of the ROC plots shows that the model did not consistently predict the test data better than can be achieved by chance alone The accuracy of the models in predicting the observed data ranged from 18% to 92%, with sensitivity and specificity ranging from 0% to 96% and 14% to 100% respectively (Appendix 12).

Table 6.3: Results of multiple and simple regression GAM analysis showing the odds of anthrax risk explained by environmental conditions in the Ngorongoro Conservation Area.

Data source	Variable	Odds ratio for multiple regression (95% confidence interval)	Odds ratio for simple regression (95% confidence interval
Participatory mapping (Uses GAM to account for spatial autocorrelation)	CEC	1.031 (0.964 - 1.102)	0.982 (0.927 - 1.041)
	pH	1.841 (0.720 - 4.706)	1.581 (0.785 - 3.184)
	DOWS	0.641 (0.529 - 0.789) *	0.690(0.585 - 0.814) *
	Slope	0.926 (0.844- 1.016)	0.9158 (0.837 - 1.001) *
	SOC	0.8640 (0.810 - 0.921) *	0.858 (0.812 - 0.909) *
	EVI	-	0.998 (0.997 - 0.998) *
	LSTD	-	1.431 (1.253 - 1.634) *

* $P<0.05$

6.4 Discussion

This study has shown that participatory mapping approaches can yield valuable information about disease risk. When combined with GIS technologies, this also enabled an understanding of the underlying environmental factors associated with areas of perceived high anthrax-risk in the NCA. The study was conducted with previous knowledge of environmental factors related to anthrax risk. The spores of *B. anthracis* are known to interact with components of the environment through their outer coating in order to persist and to come into contact with a suitable host.

Findings show substantial autocorrelation in the data justifying the use of a GAM that accounts for and avoids the pitfalls of autocorrelation. Autocorrelation may underestimate the variance in the model leading to type-1 error (Dormann *et al.*, 2007) in which a significant difference is found in the variables for points falling into high-risk areas compared to low-risk areas, where this difference does not truly exist. Univariable GAM analysis showed clearly that high-risk areas were closer to permanent water bodies, had lower organic carbon content, lower slope, less vegetation density, and higher daytime temperatures. Results for pH and CEC were ambiguous, as the 95% confidence interval of their odds ratio included the value of one, albeit point estimates shows that pH was higher in high-risk areas.

Similar to the simple regression, the multiple regression approach using GAM, showed that the odds ratio of being a high-risk area was significantly greater in places with smaller DOWS and lower SOC. The results for the odds ratios of CEC, pH and slope were ambiguous as their 95% confidence intervals crossed the value of one. However, point estimates of their odds ratio indicated that pH and CEC were higher in high-risk areas, and the slope lower in those areas. When interpreting odds ratios, a value of one indicates that the variable is not associated with the risk of anthrax, while values below or above 1 indicates that the variable is associated with a lower or higher risk of anthrax respectively (Szumilas, 2010). This lack of a clear result may not mean the absence of an association of these variables but may be caused by insufficient evidence to

conclude on the effect of pH, CEC and slope based on data from this participatory mapping study.

Soils with high organic content have previously been associated with anthrax spore persistence (Van Ness, 1971; Hugh-Jones and Blackburn, 2009). However, this study shows that high-risk areas within the NCA are more likely to have lower SOC compared to low-risk areas. SOC is usually determined by the composition of organic matter obtained from plant and animal tissue residues as well as microbial biomass (Lal, 2018). Organic matter is thought to promote anthrax risk as shown by the following two hypotheses. The disputed "incubator area" hypothesis by Van Ness postulates that *B. anthracis* undergoes cycles of germination and sporulation in soils rich with organic matter (Van Ness, 1971; Turnbull *et al.*, 1989). According to a second hypothesis, organic matter may attract and sequester spores, shielding them from environmental damage (Hugh-Jones and Blackburn, 2009). This may create high concentration of spores (infectious doses) in particular locations. This study shows, however, that presence of high organic matter is not very important for the risk of anthrax in the NCA. Since SOC is contributed to by plant biomass (Lal, 2018), it is likely to be related to soil vegetation index, and low SOC might mean low vegetation in high-risk areas. In areas where vegetation is low, animals may ingest spores from soil more easily (Hugh-Jones and De Vos, 2002), thus, this could explain how low SOC increases the risk of anthrax in the NCA.

While the simple logistic regression initially suggested a higher risk associated with points further from inland water bodies (DOWS), results based on the GAM analysis indicated that perceived high-risk areas were closer to water sources, a finding consistent with Lembo *et al.* (2011) and Steenkamp *et al.* (2018). Areas close to water sources may be contaminated through the death of infected animals usually seeking water during the late stages of the disease (WHO, 2008), although this may not be the case for livestock, as their movement is largely controlled by humans. However, both livestock and wildlife presence contribute to anthrax dynamics in the NCA, as such, the results may suggest small but salient distinction in the ecology of anthrax in areas dominated by wildlife in

contrast to areas dominated by livestock or areas at the wildlife-livestock interface, which could be investigated.

High-risk areas located closer to water bodies (DOWS) creates implications for greater risk of anthrax transmission to animals. The results suggest that animals are more likely to contract the disease during or close to the time of watering. This corresponds to findings from the mixed methods study reported in Chapter 3 where participants believed anthrax is usually contracted from water points. Observations of anthrax outbreaks occurring close to water sources have been documented extensively (Clegg *et al.*, 2007; Wafula *et al.*, 2007; Hampson *et al.*, 2011; Steenkamp *et al.*, 2018). In dry conditions, animals may be forced to graze around water sources where spores have accumulated from previous outbreaks, increasing the risk of infection. In addition, when animal deaths due to anthrax occur closer to water bodies, the higher risk of anthrax created for aquatic animals (e.g. hippopotamus (Wafula, Patrick and Charles, 2007)), may further increase the probability of areas in and around water bodies being high-risk.

CEC has been used as a proxy for calcium in this study, and higher levels of CEC and pH have been associated with high-risk areas in both in the NCA (Hampson *et al.*, 2011) and elsewhere (Van Ness, 1971; Smith *et al.*, 2000). For instance, geographical locations with calcium levels greater than 150 milliequivalents per litre and pH above 7 in South Africa have been found to have many more wildlife deaths from anthrax compared to locations with lower values (Hugh-Jones and Blackburn, 2009). Alkaline pH and calcium are important for maintaining spore dormancy. While calcium plays a role in stabilizing enzymes and genetic materials within the cell, alkaline pH creates a high osmotic pressure within the spores that aids drying (Dragon and Rennie, 1995).

The cortex layer of the spore is sensitive to changes in the ionic strength of the spore and in response to increased pH may undergo pressure changes that pull water out of the spore (Gould, 1977). The decrease in the water content of the spore improves its buoyant density and increases its ability to be disseminated and transported by environmental agents (such as wind or water) with sufficient

force. Reduced water content also protects spores from the effect of heat and ultraviolet radiation (Dragon and Rennie, 1995). Although the spore is largely impermeable and resistant to many chemical substances, small molecular weight compounds may still pass through and the innermost core of the spore is able to interact with the environment to aid germination when conditions are favourable (Dragon and Rennie, 1995). This study did not clearly show a higher CEC or pH in high-risk areas, which may be due to 1) the use of CEC as a proxy for calcium, and/or 2) limitations with the methods used to define high-risk areas.

Although results of slope were ambiguous, high-risk areas may have lower slope compared to low-risk areas. The topography of an area may affect anthrax persistence and thus increase the risk of contracting the disease. Anthrax usually occurs in steppe areas (Hugh-Jones and Blackburn, 2009) which are characterised by large arid and flat grasslands. Lands with flat topography may retain spores more readily because of resistance to the action of wind or water, that may otherwise disperse spores more easily along areas with a higher gradient.

The modelled high-risk areas (Figure 6.10) appear to create a form of barrier across the NCA. Depending on the direction of movements, this makes it less likely to transverse the NCA without encountering a high-risk area, with particular implications for seasonal north to south movements in search for pasture and water. The predictors accounted for only part (58%) of the variation in high-risk areas compared to low-risk areas, indicating some unexplained variation that may be due to limitations of the qualitative approach used to identify high-risk areas, leading to the inability to clearly quantify existing associations. The variation unaccounted for may also be the result of other factors not investigated or a limited understanding by communities of more recently established high risk areas

Overall, uncertain results (i.e. wider confidence intervals) were obtained through the methodology used in this study. While it is believed that this method is advantageous in the context where the study was carried out, some

limitations relating to the subjective nature of data collection may exist. To improve on this limitation, mapping may be conducted a number of times and data combined to reduce the effect of subjectivity and/or to have a better understanding on the scale of uncertainty expected. Another limitation relates to the use of settlement data to exclude certain locations from the dataset. This exclusion of areas out with a certain distance from settlements may not produce a true representation of areas which may be inaccessible to livestock for the following reason; The distance used was an average of daily livestock movement for both dry and wet seasons. Livestock typically move longer distances during the dry season; therefore, the average distance will underestimate the areas assessible to livestock. To avoid this, a different strategy may use other criteria such as elevation. However, it may be difficult to define a threshold which excludes animals. In addition, the strategy may not take into account other criteria, for example inaccessible forested areas at 'assessible' elevations, or areas with assessible elevation but made inaccessible by surrounding areas.

The quality of the data obtained using participatory mapping was useful for providing descriptive as well as inferential information about the pattern and dynamics of environment factors present in high-risk areas. However, its use for more advanced inferential analysis such as predictive risk in other locations was not demonstrated in this study. The inability to demonstrate predictive capacity of the model may not be due, solely, to the quality of the data. The smaller scale on which the study was carried out (i.e. a focus on the NCA only) may mean less variation in the environmental variables considered, thereby leading to non-significant results. In addition, varying climatic conditions may prevail in different locations affected by the same disease. This has been demonstrated for anthrax, which for example occurs in rainforest ecosystems in West Africa compared to arid ones (Leendertz *et al.*, 2004, 2006; Hugh-Jones and Blackburn, 2009). On a smaller geographical scale (in northern Tanzania), the study by Hampson *et al.* (2011), found that anthrax can be frequently observed in a number of habitats. Across the NCA, a range of climatic conditions can be observed at a given timepoint (Galvin *et al.*, 2004). This may make it difficult to generalise findings across different locations and may explain why some of the variables are not statistically different between high- and low-risk areas. The

results of the ROC analysis indicate poor predictive performance of the model, which may be due to limited power of the testing dataset, caused by the small sample size of points falling in defined risk areas (see Appendix 12 for results of the ROC analysis).

Further research may consider a larger spatial scale and look into delineating the variability in the ecosystems that support anthrax infection and understanding the environmental conditions associated with them. For further re-analysis into delineating the variability in the probability of risk in these areas, a stratified random sampling of points could be employed. This may employ stratification based on a number of variables e.g. elevation, vegetation. This would create a representative sample and also increase statistical power, since variability within each strata will be lower in comparison to that obtained from a simple randomly selected points (Theobald *et al.*, 2007).

Participatory research including its use in mapping areas affected by disease is a useful approach in its remit (Dongus *et al.*, 2007; Rowley, 2010; Dickin *et al.*, 2014). The validation of findings obtained using statistical methods should be considered an added gain when this is possible. The results of this work can help guide the selection of geographical locations for prioritization of anthrax control in the NCA. For example, targeted interventions could include livestock vaccination, public health promotion messages, provision of water and mineral salts, as well as reservation of pasture in low-risk areas to avoid seasonal movements to high-risk locations. Vaccination well before the time of the year when anthrax is expected has shown to be effective against the disease (Turner *et al.*, 1999). Although no example could be found in the literature for control of anthrax through interventions to move livestock away from high-risk areas, animal movement restriction is recommended as an effective disease control strategy (Turnbull, 1998; OIE, 2014).

6.5 Conclusions

This chapter demonstrates the value of participatory mapping techniques for the understanding of disease distribution and the identification of priority areas for disease control. In developing countries, where systems for disease monitoring and for managing data are often lacking or expensive, identifying alternative solutions for mapping risk becomes valuable. In many cases, communities affected live in rural areas without access or links to health or veterinary services, making disease reporting and response challenging. In such situations, targeted disease control is needed. Participatory mapping approaches provide a quick means of understanding the distribution of disease for targeted control in such areas. This approach, applied to understand anthrax risk areas in the NCA, was particularly useful due to the pastoralist livestock movement practices prevalent in the area. Using locations where animals have died or samples were collected in these settings is not likely to be a true representation of high-risk areas, due to the nature and extent of livestock movement practised.

GIS technologies are increasingly becoming affordable in developing countries and can be combined with participatory approaches to generate rich data. A combination of GIS tools and participatory approaches yielded information about anthrax-risk areas in the NCA and the environmental conditions associated with those areas. The study investigated the relationship between anthrax-risk areas and known environmental factors for the persistence of *B. anthracis* in the environment. Findings show that anthrax occurs mostly in areas characterised as plains lands with low organic matter, and around water bodies. These areas may have flat topography, high soil PH and CEC. The findings also show that high risk areas occupy central locations in the NCA, and animals moving long distances across the NCA may encounter areas of higher infection risk.

Chapter 7

Chapter 7 General discussion

This chapter discusses the main findings of the data chapters. The strengths and weaknesses of the thesis are discussed, as well as the generalisability of research findings and directions for further studies. Finally, recommendations based on the findings reported in the thesis are provided. These recommendations – aimed at stakeholders involved in disease control (including affected communities, health authorities and policy makers) – are expected to benefit the control of anthrax in northern Tanzania and may be applicable to other similar endemic areas.

Anthrax is a disease that is known to affect poor and disadvantaged communities, but control is impeded by the lack of data demonstrating its impacts. In sub-Saharan Africa, anthrax contributes to the burden of zoonotic diseases with health, social and economic significance (World Bank, 2012; Vieira *et al.*, 2017). The burden of anthrax is underestimated as it has hardly been quantified for people and livestock. The health impacts of human anthrax – which is most often associated with infections in livestock – is ignored in efforts to estimate disease burden. The World Health Organization having previously considered anthrax as "not a major public-health problem in the world today" (Maudlin *et al.*, 2009), now calls for studies to estimate the burden of neglected zoonoses including anthrax (World Health Organization, 2010). In addition to the health impacts of human anthrax is the consequence of the disease on animal health and productivity. Anthrax most commonly results in sudden animal deaths leading to loss of livelihoods in livestock dependent households and communities (WHO, 2006).

Livestock is critical to the livelihoods of individuals and communities in sub-Saharan Africa. As many as 80% of households in Africa live with and depend on livestock for sustenance (Krätli *et al.*, 2013). Consequently, zoonotic diseases such as anthrax that cause important livestock losses will not only affect livestock and human health but will perpetuate a cycle of poverty. This makes the control of anthrax particularly important for poor rural households who depend almost entirely on livestock for their livelihoods. The control of anthrax

in endemic areas is vital for achieving the sustainable development goals of no poverty, zero hunger and good health and wellbeing (Swaminathan and Kesavan, 2016), as well as indirectly contributing to the goals of quality education and decent work and economic growth. This multidisciplinary thesis was carried out to simultaneously improve our understanding of anthrax in endemic settings and to identify strategies for improved control.

This thesis has provided data on the occurrence of anthrax in the NCA of northern Tanzania where humans live in close proximity to animals. The results of the study outlined in Chapter 2 demonstrate that in most households, diseases are responsible for the deaths of more livestock than either drought or predation. This correlates with findings of a recent study in northern Tanzania, which showed that disease accounted for more than twice the number of livestock deaths as compared to drought (Ahmed *et al.*, 2019). Anthrax, which contributes to disease-related deaths in the NCA, was reported in both livestock and humans. Small stock, particularly sheep, were most affected by anthrax in the NCA. Evidence shows that animal behaviour is linked to the risk of anthrax (Ganz *et al.*, 2014) and sheep may contract *B. anthracis* spores from soil more easily by grazing on very low pasture. Unpredictable climatic conditions mean that livestock keepers are favouring small stock over cattle because of their resistance to drought (McCabe, 2003). This suggests that diseases that may predominantly affect small stock in the area need to be prioritised. Given the higher susceptibility of sheep to anthrax due to their ethology and the trend towards small-ruminant keeping among pastoralists, an increased incidence in anthrax could be anticipated.

Anthrax is considered important by many livestock-keeping households, both in areas considered high- and low-risk, which is justified when the dependence on livestock and losses due to anthrax in monetary terms are considered. Most of the animals affected by the disease are female in their productive age; this is likely associated with further losses that are difficult to quantify. In Tanzania, agriculture - including livestock and crop production - accounts for over a quarter of GDP (United Republic of Tanzania (URT), 2012). However, in the NCA, crop production is prohibited (Goldman, 2011) meaning that communities have

limited alternatives to good nutrition other than that produced by their livestock, making it critical to promote livestock health. Deaths of livestock associated with anthrax resulted in large financial losses - money that might otherwise have contributed to improving the socio-economic status of households by increasing nutrition, as well as spending on health, housing, and education. For example, losses due to confirmed anthrax in 36 households amounted to more than 20,000 USD over a six-month period. The control of anthrax thus has the potential to reduce poverty, increase income and promote health, as well as provide indirect benefits such as improved spending on education (Randolph *et al.*, 2007; Marsh *et al.*, 2016). While naturally-occurring anthrax may not appear to be a major global health problem, largely because it has been brought under control in developed nations, the losses we have documented in association with anthrax indicate a substantial problem for the vulnerable communities in affected areas.

Anthrax was perceived to be important by people living in areas considered high- and low-risk. This perception of importance, in addition to the proportion of suspected cases confirmed positive as outlined in Chapters 2, 3 & 4, indicates a hyper-endemic situation, a familiarity with the disease and an understanding of the associated negative impacts. The impact of the disease on human health includes not only ill-health associated with infection, but an impact on the psychological health of farmers. The Maasai culture places a significant value on livestock ownership (Galaty, 1982) and the psychological effect of livestock loss further contributes to the burden of the disease. The full burden (i.e. disability-adjusted life year) of anthrax was not quantified in this study and the full extent of human mortality and morbidity remain unknown. The occurrence and losses associated with anthrax calls for improvements to the surveillance and control of the disease.

Low income countries often experience challenges across many developmental sectors, and usually have limited resources to address those challenges. Thus, disease control prioritization is very important. One way in which prioritization can be achieved is identifying locations where the burden of disease is highest. Although this study demonstrated that anthrax is experienced by people and

animals living in areas considered both high-risk and low-risk, it appears that most infections are contracted from geographically defined areas where control could be targeted. Through participatory mapping (Chapter 6), specific geographical locations where animals are believed to most often contract the disease were identified. These areas corresponded with ward locations where the odds of reporting anthrax were greatest (as determined through the household surveys), and where highest disease incidence occurs. Identifying these areas makes the prioritization and the allocation of resources for more efficient anthrax control easier to achieve. Places with high probability of anthrax contamination can be targeted for control in a risk-based surveillance approach (Stärk *et al.*, 2006) that maximises resources while achieving vital statistics to inform control.

Once locations where diseases can be prioritised are identified, surveillance and control may then focus on those areas. The surveillance platform which was set up as part of this study was aimed at simultaneously collecting data as well as improving local surveillance of the disease. We set up mechanisms for communities to report suspected anthrax cases and for diagnostic samples to be collected for case confirmation. The setup highlighted concerns with anthrax surveillance in rural and challenging areas, including the problem of underreporting and the logistical challenges associated with accessing locations where anthrax incidence occurs. Improving anthrax surveillance in endemic and resource-poor areas is possible by using practical methods that overcome the challenges identified in the field. The way in which this study achieved this was 1) to focus surveillance on areas most affected by anthrax, 2) to assess a field friendly diagnostic tool for the detection of *B. anthracis* and 3) to identify practical sampling methods for areas in which cold chain storage of samples is not possible or is difficult to attain.

An integral part of anthrax surveillance is detecting the causative pathogen. For anthrax, as with many zoonotic diseases, animal sentinel surveillance can provide useful information about transmission to humans (Lembo *et al.*, 2011). In most cases, reports of anthrax in livestock in endemic areas are associated with human cases and there is evidence to show that transmission occurs almost

exclusively from animals to humans (Hugh-Jones, 1999; Munang'andu *et al.*, 2012; Fasanella *et al.*, 2013). Surveillance in animals may enable anthrax to be detected before transmission to humans occurs. In Tanzania, anthrax diagnostic capacity is inadequate, especially in areas where the burden of the disease in highest (Mwakapeje, Høgset, Fyumagwa, *et al.*, 2018). While communities involved showed a high ability for syndromic detection of anthrax, etiological detection of *B. anthracis* is still important to limit the uncertainties around syndromic detection. Although anthrax should be considered in the event of sudden deaths in livestock (WHO, 2008), this syndrome is not definitive for the disease (Abd El-Moez *et al.*, 2013) and the characteristic oozing of blood from the natural orifices of animals after death is not always present as previously reported (WHO, 2008; Chapter 3) and as confirmed in this study (Chapter 2).

Smear stain microscopy, which is rapid, cheap and easy to implement can provide confirmation. The current recommended stain microscopy test is based on M'Fadyean's 1903 (M'Fadyean, 1903b) protocol and uses polychrome methylene blue (PMB) that is difficult to obtain (Owen *et al.*, 2013). The study outlined in Chapter 4 demonstrated that stain microscopy utilizing azure B has high and similar sensitivity and specificity to that using PMB on blood smears from the field. Staining with azure B also proved robust to inter-observer variability. These findings provide a great step forward in terms of simplifying the detection of *B. anthracis*, as this is the first study to assess the stain directly on blood smear samples obtained from the field. Previously, azure B had only been tested on a limited number of samples from smears made from laboratory isolates of *B. anthracis* (Owen *et al.*, 2013). The similarity in the sensitivity and specificity of azure B and PMB for detecting *B. anthracis* in clinical samples is an important finding with the potential to improve the surveillance of anthrax in endemic situations where testing is likely to be carried out frequently and in large volumes.

Smear microscopy with azure B meets most of WHO's ASSURED criteria (Mabey *et al.*, 2004). Specifically, it meets the convenience criteria of affordability, being user-friendly, rapid, and deliverable to those who need it since it can be implemented in the field with relative ease. While it does require the use of a

microscope, these are one of the basic pieces of equipment found in most laboratories. They are thus readily available and can be powered by batteries or solar energy in field conditions where electricity is lacking (Figure 7.1). Thus, this method of detection is deliverable to areas where the burden of anthrax is greatest. Moreover, diagnostic tests that can be carried out without the need to culture *B. anthracis,* such as stain microscopy with azure B, are preferred because of the associated biosafety and biosecurity concerns. The United States Center for Disease Control classifies *B. anthracis* as a category A select agent because of the health, social and economic impact that mis-use of the pathogen can cause (Sinclair and Boone, 2008). Culture-free diagnostic methods that avoid the risks of accidental infection or misuse associated with pathogen propagation are important.

Figure 7.1: A microscope powered by a car battery being used in the field to confirm anthrax cases.

Although this study showed that azure B stain microscopy with its ASSURED characteristics can help to improve detection of *B. anthracis* in poor and endemic settings, its utility may have certain limitations within the context of the study area. The ability to obtain smear samples was limited, as they were unavailable from more than half of the carcasses investigated. Results of the mixed methods study discussed in Chapter 3 provided insights into the reasons

for the lack of blood smear availability, which is largely attributed to the social practice of consuming a carcass suspected to have died of anthrax. To ensure that surveillance of anthrax is not impeded by the lack of samples for testing, it is important that other types of samples can be tested. Thus, work was carried out to understand if other sample types could be used to detect *B. anthracis*, thereby improving the surveillance of anthrax. Microscopy, although simple, cheap and rapid, cannot be applied to other samples such as dry skin (see Figure 4.7), which is often all that is left of a carcass by the time a response to a report of an anthrax incident is made (e.g. carcasses will be butchered in less than 24 hours). Tissue samples were thus more readily available compared to blood smears, whole blood, blood swabs and insects because they are not usually consumed along with a carcass.

While polymerase chain reaction (PCR) does not meet as many of the ASSURED test criteria as microscopy (i.e. it is more expensive and requires more advanced infrastructure), it is a diagnostic test that can make use of the available tissue samples, and represents another culture-free diagnostic method. Since facilities and capacity for PCR may only be available in few laboratories in developing countries, there are likely to be delays between sample collection and testing related to lengthy transport of samples from remote locations to the laboratory. On route to the laboratory, sample storage may present challenges as the infrastructure for cold chain storage is often lacking. In Chapter 5, I showed that qPCR on tissue samples that have been stored at ambient temperature for several months can still be used for the molecular detection of *B. anthracis* with high sensitivity and specificity. Overall, in endemic settings it is thus recommended to collect blood smear samples when available for rapid confirmation of anthrax by microscopy, with tissue samples representing a valuable alternative for molecular confirmation. Combined, these samples and tests would enable the confirmation of the majority of anthrax cases in livestock.

When blood smear samples are available, the findings of this thesis suggest that they are useful for multiple diagnostic testing. First *B. anthracis* can rapidly be detected by smear stain microscopy and then by PCR on the stained smear.

Alternatively, multiple smear samples can be collected, and testing done separately. Blood smears are easy to collect and store. Although the sensitivity of qPCR was reduced slightly with stained smears, this is still advantageous for settings with limited infrastructure for sample collection and storage. Apart from testing samples from anthrax cases as they occur, PCR on stained or unstained smears can be useful for research and epidemiology in laboratories with archived samples not yet characterised.

The findings in this study show that the prevalence of anthrax within the population of suspected cases sampled is high, warranting proactive control. While much can be said in terms of improving the prioritization of anthrax by policy makers and enhancing surveillance by improving diagnostic capacity, much more may be achieved when surveillance is a carried out simultaneously with anthrax control measures. Key reasons for conducting surveillance are to inform and evaluate control strategies (World Health Organization, 2010). The control of anthrax can be achieved by taking proactive rather than the reactive measures that currently characterise the control of zoonotic diseases in endemic areas (Shadomy *et al.*, 2016). These proactive measures involve focusing on the prevention of anthrax in animals and halting transmission of the disease from animals to humans, for which adopting public health promotion principles that enable affected communities to contribute to these strategies will be relevant.

Current evidence shows that anthrax can effectively be controlled by vaccination (Hugh-Jones, 1999; WHO, 2008; Beyer and Turnbull, 2009; Fasanella *et al.*, 2010). In endemic situations, regular vaccination should be maintained (WHO, 2008) and may even be needed in certain situations when the disease becomes sporadic (Hugh-Jones, 1999). It is not clear what factors hinder consistent vaccination coverage against anthrax in the NCA since a vaccine is available and is manufactured in Tanzania. This may be an area for further study. Anecdotal reports have indicated issues with ineffectiveness of vaccines, high costs and inaccessibility by livestock keepers that could be investigated. Routine vaccination has declined worldwide and currently, vaccination in endemic areas in Africa is mostly carried out as a reactive measure to outbreaks (Wafula *et al.*, 2007; Muturi *et al.*, 2018). Reactive vaccination against anthrax

is unlikely to yield substantial benefits for control. Verbal communication reveals that vaccination campaigns are usually organised by the NCA authorities for livestock, but it not clear how effective, timely, or regular these are. Data from this study taken at only one time point suggests irregularity, but a longitudinal study or a retrospective assessment of records if they exist may provide clearer insights. An understanding of the process will reveal areas where improvements can be made. To improve vaccination against anthrax, mechanisms and infrastructure already in place for other diseases can be co-opted, for instance, a system that synergises livestock vaccinations for multiple diseases.

Livestock movement to areas free from anthrax is another recognised anthrax control strategy (Turnbull, 1998). In Chapter 6, it was shown that locations of high-risk were closer to water bodies compared to those of low-risk, which is consistent with previous findings (Lembo *et al.*, 2011). In dry season conditions, animals may congregate at water sources leading to a large number of animals being infected from the same source. The disease was also shown to occur in plains lands which is preferred for grazing by livestock keepers, further increasing the risk of infection. Ideally measures could be taken to avoid these high-risk areas where animals are more likely to contract disease. However, nomadic livestock management involves seasonal livestock movement in search of grazing, water and salting resources. Therefore, strategies to control anthrax through modified movement practices may be challenging. Since it may be difficult to intervene in terms of restricting animal movement for grazing and watering, vaccinations are advantageous because animals will develop immunity that provides protection even when *B. anthracis* spores are ingested.

Modifying socio-cultural practices for anthrax prevention may be difficult to achieve. Findings show that the practice of risk behaviours is not solely a result of the lack of knowledge and awareness, but is due to a host of wider determinants. Key examples are the consumption of infected carcasses due to food insecurity rather than a lack of knowledge about the risk of anthrax transmission to humans, or the movement of animals to locations with high risk of anthrax in order to avoid wildebeest and associated malignant catarrhal

fever. Preventing the disease first by vaccinating animals may prove most effective, when modifying human behaviour and other wider determinants of the behaviour are challenging.

Other gaps in the control of anthrax in the NCA can be filled by targeting socio-cultural factors. Cultural and social practices implicated in the transmission and persistence of anthrax in the NCA are centred around carcass handling and livestock movement. At the time of conducting the study, burying or burning carcasses suspected to have died of anthrax was not carried out despite these being the methods prescribed by health authorities for wildlife (Mlengeya and Mlengeya, 2000). The findings of the study showed that the majority of carcasses are opened for a variety of reasons, notably for human consumption. Across Africa, reports of the consumption of anthrax carcases by local communities have been made (Opare *et al.*, 2000; Gombe *et al.*, 2010). Carcasses will also be opened even without the intention to consume them as food. Although my study did not reveal whether this practice is associated with any stringent customary law, a study conducted in an area with similar culture found that burying of intact carcasses due to disease (in this case Rift Valley Fever) is taboo (Mutua *et al.*, 2017). These types of customary beliefs present enormous challenges for disease control as they undermine regulations for proper disposal of anthrax carcasses and disease control as a whole.

Other practices contributing to poor anthrax surveillance and control, including under-reporting and late reporting, need to be improved. Although disease underreporting and a lack of communication are difficult to measure objectively, they are recognised as significant contributors to the lack of appropriate response to infectious diseases (World Bank, 2010; World Health Organisation, 2010; Brabazon *et al.*, 2015). Disease reporting and response is crucial to an effective surveillance system. Under-reporting is an important reason for limited data on and consequently an underestimation of the burden of zoonotic diseases (Molyneux, Hallaj and Keusch, 2011), and anthrax represents a key example. Underreporting is usually caused by an unwillingness and/or the inability to communicate disease occurrence (Figure 7.2). Understanding the contextual reasons for underreporting is key to addressing local issues in relation to anthrax

surveillance. The studies outlined in Chapters 2 and 3 showed that the inability to confirm anthrax, a lack of awareness, indifferent attitudes, and a lack of response are all contributing factors to underreporting of anthrax in the NCA. Implementing the field-friendly azure B staining technique within the communities using already existing infrastructure and personnel (i.e. veterinary centres and community animal health workers) will enable anthrax confirmation as evidence to accompany reports. Disease reporting structures and pathways that incentivise people through appropriate response to anthrax incidents and outbreaks could be created. These structures should also take into consideration the barriers to disease reporting and engage communities in the decision-making processes aimed at improving reports. Communities should not just be made aware of the importance of reporting, but also understand and experience incentives to reporting. For instance, a livestock keeper will highly consider reporting anthrax if an appropriate response will prevent the disease from affecting a larger proportion of the herd.

Many communities in the NCA are remote and located in areas not accessible by roads. As the NCA is a wildlife conservation area, the infrastructural situation is unlikely to change significantly in the future. Reaching resident communities involves walking on foot for long periods, effectively making untargeted routine surveillance laborious and expensive. A synergy between passive surveillance (i.e. communities reporting anthrax) and active surveillance (i.e. health authorities and professionals requesting information about anthrax incidence in communities), would help to document the occurrence of anthrax in endemic areas, contributing to the much-needed impetus for control. Creating effective surveillance systems for endemic anthrax will not only be advantageous for the control of the disease, but in the long run may strengthen overall disease surveillance. A focus on effective and adaptable systems for endemic diseases could have great benefits, for example in the timely identification and control of emerging diseases, that may otherwise cause devastating consequences before detection occurs with existing weak systems (Halliday *et al.*, 2017).

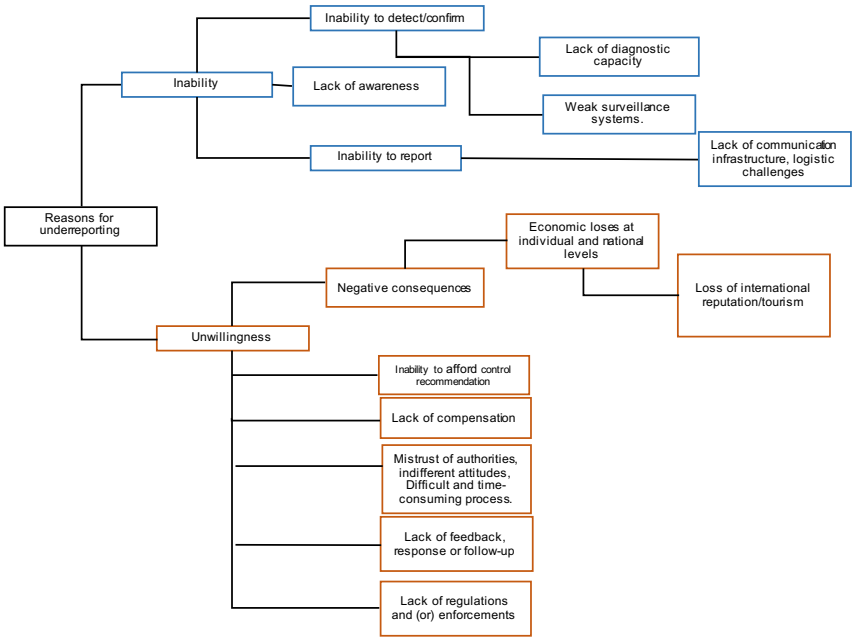


Figure 7.2: A schematic illustration of the possible reasons for underreporting in the NCA. Adapted from World Bank (2010) and Halliday *et al.* (2012)

The overall study indicates the potential for surveillance of anthrax to be improved with community participation and engagement. First, case investigations could be carried out by trained members of the community. Second, the proportion of confirmed cases through diagnostic testing was high, meaning livestock owners have a very good ability to recognise the disease. This finding supports the results of the household surveys, which would have otherwise been based only on reported accounts. Third, engaging with the affected communities enabled an understanding the concepts, experiences and the gaps relating to the prevention of the disease. The communities involved demonstrated good knowledge and enthusiasm that could be leveraged for interventions that target the disease. In order for interventions targeting the control of anthrax to be effective, adequate and appropriate engagement with communities is important. A literature search revealed no reports of the implementation and evaluation of health interventions for endemic anthrax control in people and livestock in Africa. However, studies on other diseases have shown that engagement is critical in such settings (Schelling *et al.*, 2007; Bouyer *et al.*, 2011).

Appropriate control of anthrax will limit the need for livestock owners to carry out local practices that they believe will control the disease but may in contrast be contributing to its spread and to new infection foci (Chapter 3). In addition, the control of anthrax may have broader positive impacts. For instance, indiscriminate treatment of anthrax in both people and animals with antibiotics may be contributing to antimicrobial resistance, with negative implications for diseases caused by other pathogens. Caudell *et al*. (2018) found a higher proportion of antimicrobial resistant bacteria carried by the Maasai – the major tribe in our study area - compared to other tribes, indicating that this is an already existing problem. By prioritizing anthrax prevention, the need to treat animals and humans with antibiotics will be minimised.

7.1 Generalisability of findings

Anthrax is endemic in many parts of Africa, and the characteristics of the human and livestock populations living in the NCA are typical of many areas where anthrax has been reported. For example, the disease in humans and livestock has commonly been reported in areas where people live in close proximity to wildlife (Siamudaala *et al.*, 2006; Hang'ombe *et al.*, 2012). These areas are often remote with limited basic infrastructure like roads, electricity, human health and veterinary services. A dependence on livestock for sustenance can also be observed in these areas. Thus, the risk of anthrax and challenges for the control of the disease may be similar for such areas. This means that many of the results of this study can be applied across areas where anthrax is endemic. These include the implementation of surveillance schemes using members of the community trained for response to anthrax reports, capitalising on existing field diagnostic centres for the rapid confirmation of anthrax using azure B microscopy testing on blood smears, and the collection of tissue samples that can be stored at ambient temperature prior to molecular testing.

Although the NCA typifies many areas where anthrax is endemic, it is a unique area in many respects, with implications for recommendations related to our findings and their generalisability. These features include 1) the NCA is a government-controlled conservation area, which means a special interest to protect wildlife health. Since wildlife are susceptible to anthrax, the prospect of wildlife protection through the control of anthrax in humans and livestock may be used to lobby for prioritization of the disease. This enthusiasm may be lacking in other areas where anthrax is endemic. 2) In contrast, since the NCA is a protected area, where minimal human activities are wanted (Catherine *et al.*, 2015), it is unclear the extent to which interventions to control anthrax that may lead to economic prosperity for people and negate conservation strategies, will be permitted. However, authorities in the NCA have shown great interest in controlling diseases both in wildlife and livestock and have supported the research activities leading to this thesis. This potential reservation would be irrelevant in other endemic areas where wildlife conservation is of less concern.

In terms of the generalisability to other locations, an important consideration would be whether the socio-cultural practices that drive anthrax differ in other endemic areas. Studies have shown that the consumption of suspect carcasses is common in many endemic areas (Opare *et al.*, 2000; Gombe *et al.*, 2010). It is also not clear if livestock keepers in endemic areas practice nomadism or sedentism. Strategies aimed at modifying behavioural practices for anthrax control will require an understanding of the local practices related to risk. In addition to these limitations, the participatory mapping of high-risk areas was based on the Maasai's in-depth understanding of their environment and ability to recollect events relating to their livestock. This may differ in other locations.

In Tanzania, it is reported that only one laboratory is designated and equipped for *B. anthracis* detection using PCR (Mwakapeje, Høgset, Fyumagwa, *et al.*, 2018). This laboratory is based in Dar Es Salaam, situated hundreds of miles away from endemic areas mostly in northern Tanzania. However, the qPCR testing conducted for this study was carried out in a different laboratory situated in northern Tanzania (at the Kilimanjaro Clinical Research Institute). Setting up this testing did not require the procurement of any equipment but made use of available bio-safety category >2 facility. This indicates that enabling *B. anthracis* detection in higher-containment laboratories already equipped to carry out PCR would not require significant investment. Much of the work needed to enable more laboratories to conduct PCR for *B. anthracis* detection will be centred around training of lab personnel, particularly with respect to biosafety, and the purchase of consumables.

7.2 Future research directions

This thesis has resulted in new knowledge and strategies for the surveillance and control of anthrax, however, areas for future research were also identified. Detailed below, the questions generated could help direct future studies to further improve the understanding of anthrax in endemic areas.

7.2.1 Understanding the true burden of anthrax in Tanzania

In Tanzania, the true incidence of anthrax in livestock and humans is unknown. This study focused only on the NCA, while Mwakapeje *et al*. (2018) provided insights into the occurrence of human anthrax in Arusha and Kilimanjaro regions of northern Tanzania. However, anthrax has been reported in other parts of the country, both in the North (e.g. Manyara region) and South (e.g. Songwe, Iringa, Dar es Salaam regions), as well as on Zanzibar (Shirima *et al.*, 2003; proMED-mail, 2004; ProMED-mail, 2019a, 2019b). Apart from understanding the occurrence of anthrax, studies are also needed to understand the full economic and health burden of anthrax throughout the country, including impacts related to mortality and morbidity, loss of earnings, and spending on illness and hospitalization.

7.2.2 Investigating the use and effectiveness of vaccines against anthrax in the field

A vaccine against animal anthrax is produced and available in Tanzania. However, the results of this study show that vaccination coverage against anthrax is poor. Anecdotal accounts have indicated vaccine inaccessibility and ineffectiveness. Studies are therefore needed to 1) understand the barriers to vaccination of livestock against anthrax and 2) test the effectiveness of vaccines in the field. To understand the barriers to vaccination, quantitative and qualitative methods through household surveys, focus group discussions and one-on-one interviews may be conducted in order to generate data on vaccine value chain and accessibility. In addition, understanding the barriers and drivers of vaccine uptake is also important. The effectiveness of the vaccines in the field can be investigated through quasi-experimental methods in randomised field

trials (Orenstein *et al.*, 1985). Results of such studies may generate further questions about the efficacy of the vaccine, which might depend the quality of manufacture and storage, as well as its match to circulating *B. anthracis* strains.

7.2.3 What behavioural interventions will be effective for the control of anthrax in people and livestock?

Behavioural interventions are needed in order to control anthrax, by deterring the practice of risky behaviours and promoting healthy and safe practices. Using or modifying a behavioural theory (Glanz and Bishop, 2010), interventions designed with participation from communities in high-risk areas could be implement and evaluated.

7.3 Policy recommendations

This research was carried out with the intention to influence public policy and action on the control of anthrax in animals and humans alike. During the course of the study, the Tanzanian government adopted anthrax among five other zoonotic diseases for prioritization of control (One Health Coordination Unit et al., 2017). This section will thus provide recommendations on how the findings in this study can be used to support Tanzania's national plan to control anthrax. The recommended strategies are listed below in no particular order.

1. The passive surveillance platform (that motivated communities to report anthrax suspect-cases, and trained community animal health workers to respond to those reports) set up through this study may be utilized by health authorities for response to anthrax cases and outbreaks in livestock. With further training and the provision of logistical support (i.e. transport to remote locations), surveillance can be greatly improved.

2. Improved surveillance through diagnostic testing can be achieved by creating centres for testing samples for anthrax confirmation using already available infrastructure (i.e. veterinary centres within wards and community animal health workers/livestock field officers). The studies presented in Chapter 4 and 5 provide specific recommendations on what sample materials are suitable to collect and tests that are practical to conduct.

3. Once a mechanism for response to anthrax incidents has been created (1 and 2 above) policies to improve the willingness to report cases can be enacted. For example, compensation, subsidised veterinary services, imposing fines etc.

4. Targeted control (e.g. yearly vaccination campaigns) aimed at identified high-risk areas can be implemented to maximise resources. In the NCA, interventions may focus first on Olbalbal, Ngoile, Alailelai, Endulen and Kakesio wards. In addition, the participatory mapping approach can be applied to identify high-risk areas in other parts of the country or to

validate suspected high-risk areas identified though environmental suitability modelling.

Appendices

Appendix 1 Participant information sheets and consent forms

Anthrax project in northern Tanzania

Participant information sheet - Household survey

INTRODUCTION

You are being invited to take part in a research study. But before you decide if you want to take part, it is important for you to understand why the research is being done and what it will involve. Please take the time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Please take time to decide whether or not you wish to take part.

PURPOSE OF THE STUDY

Anthrax is primarily a disease of livestock. It mainly affects goats, sheep, and cattle, but anthrax can also affect wildlife. This disease often leads to quick and sudden death in animals causing great losses to the livestock keeper. Anthrax also affects humans when the pathogen is breathed in, ingested or settles on open skin. Breathing in or ingesting anthrax spores often leads to more severe disease that can cause sudden death. For animals, anthrax is usually contracted when grazing on land that is contaminated with anthrax spores: these are usually areas where animals that have died of anthrax have been buried or left to decompose. For humans, eating or touching the skin of an animal that has died from anthrax or of unknown causes is the most usual way anthrax is contracted. We have identified that the disease affects some communities within this district, and seems to be causing ongoing losses. For this reason, our study aims to understand how much anthrax affects you and your community, and we want to establish community surveillance and detection systems that ensure rapid response in the event of an anthrax outbreak.

WHY HAVE I BEEN CHOSEN?

You have been asked to take part in this study because you are a livestock keeper and a member of this community in the Ngorongoro Conservation Area where we know that anthrax is a problem. We think you have valuable information that will help achieve the aims of the study. We will be asking other community animal health workers, community leaders, household leaders and other members of the community to take part. You may also be asked to take part in the study if you have presented to the hospital with a suspected anthrax infection.

DO I HAVE TO TAKE PART?

You have the right to decide whether you want to take part in the study or not. Participation is voluntary. If you do decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. You can withdraw at any point if you feel you no longer want to participate in the study.

WHAT WILL HAPPEN TO ME IF I TAKE PART?

You will be asked to fill in a questionnaire and/or participate in a participatory mapping activity. You may also be asked to take part in workshops and meetings. If you present to the hospital having a suspected anthrax infection, we will request your sample from the hospital. For suspected cutaneous anthrax, a sample from the lesion will be taken. For suspected gastrointestinal and inhalational anthrax, a blood sample will be requested. Taking part in this study is voluntary. All information which is collected about you, or responses that you provide, during the course of the research will be kept strictly confidential.

WHAT ARE THE POSSIBLE DISADVANTAGES AND RISKS OF TAKING PART?

There is no anticipated risk to taking part in this study other than what is usually encountered in daily life. We will ask to take some of your time. We envisage that undertaking a full questionnaire is expected to take 90 minutes. The information you provide will be kept confidential. The risk of compromising

your confidentiality will be mitigated by encoding your personal identifying information. If blood or lesion samples are being taken by a clinician because you present to the hospital with a suspected anthrax infection, taking a sample could take 5 to 10 minutes. You may experience a little discomfort, bruising, and swelling at the site where the sample is being taken. You can indicate your wish to discontinue in any activity at any point during the study even if you have consented to taking part at the beginning.

BENEFITS OF TAKING PART IN THE STUDY

This study will help increase your knowledge of anthrax prevention and control in animals and people. You will learn about ways to protect yourself, household and livestock from anthrax. You will also be contributing to an important study that will enable policy decisions to be taken, that will promote your health and that of your livestock and community. This study ultimately aims to improve surveillance, diagnosis and control of anthrax in Tanzania, although you are likely to see these benefits only after completion of the study.

WILL MY TAKING PART IN THIS STUDY BE KEPT CONFIDENTIAL?

All information which is collected about you, or responses that you provide, during the course of the research will be kept strictly confidential. You will be ultimately identified by an ID number, and any information about you will have your name and address removed so that you cannot be recognised from it. Please note that assurances on confidentiality will be strictly adhered to.

WHAT WILL HAPPEN TO THE RESULTS OF THE RESEARCH STUDY?

The results of this study will be available to you and the public after they have been analysed. We will make sure to feedback the results of the current study to the communities and individuals involved. Please note that any results reaching the public domain will contain no personal identifying information. The information and knowledge obtained from the study is expected to contribute to scientific knowledge and inform policy making, surveillance and control of anthrax in this and similar areas affected by this disease.

WHO IS ORGANISING AND FUNDING THE RESEARCH?

This study is funded by The Gates Foundation and is being carried out in collaboration with the Nelson Mandela African Institution of Science and Technology, Arusha.

WHO HAS REVIEWED THE STUDY?

This study has been reviewed by the Tanzania Commission for Science, and Technology, Kilimanjaro Clinical Research Institute, National Institute for Medical Research and the University of Glasgow ethics committee

Thank you for taking the time to read this, we look forward to your collaborations on this project.

ANY OTHER INFORMATION: If you have any questions or require any information not included in this sheet, or you will like to withdraw from this study, please contact the addresses below:

Mr Deo Gratius Mshanga, Tanzania Veterianary Laboratory Agency, Arusha
email address:

University of Glasgow

Nelson Mandela African Institute of Science and Technology
Arusha
Academia for Society and Industry

CONSENT FORM

Title of Project: Development of surveillance and typing schemes for anthrax epidemiological studies in endemic areas.

Please initial box

I confirm that I have read and understand the information sheet dated 2016 (version1.1) for the above study and have had the opportunity to ask questions. ☐

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my rights being affected. ☐

I agree to take part in the above study. ☐

Name of subject	Date	Signature
Name of Person taking consent	Date	Signature
Researcher	Date	Signature

Contact information:
Project:

Mr. Deo Gratius Mshenga, email address:
Dr. Tiziana Lembo, email address:
Dr. Taya Forde, email address:
Ms. Rhoda Aminu, email address:

NatHREC:

3 Barack Obama Drive, P. O. Box 9653, 11101 Dar es Salaam, Tanzania
email address:

Appendix 2 Household questionnaire

ANTHRAX PROJECT – QUESTIONNAIRE

Please provide as much details as possible. If the answer to a question is unknown simply record DK

SECTION 1: Household details

1.1 Head of household (HoH)

_____

1.2 Date of interview (dd/mm/yyyy)

____/_____/_____

1.3 Interviewer name

_____

1.4 Language

◯ English ◯ Swahili ◯ Maasai

1.51 GPS E/W Coordinate

_____/_____

1.52 GPS N/S Coordinates

_____/_____

1.53 Altitude (metres)

_____

1.54 Waypoint ID

_____

1.6 GPS Accuracy <=(10m)

◯ Yes ◯ No

1.7 Accuracy (metres)

_____

2.1 Region

◯Arusha

2.2 District

◯Ngorongoro

2.3 Ward

_____

2.4 Village/Street name

_____

2.5 Sub-village name (leave blank if none)

_____

Respondent details

1.81 What is your name?

1.82 What is your tribe? ◯Arusha ◯Barabaig ◯Chagga ◯Iraqw ◯Maasai ◯Pare ◯Sambaa ◯Other (please specify) _____

+ Other Tribes: - Temi - Sonja - Jar – Kurya – Meru – Ikon - Taturu - Ngerem- Nyamwezi

1.83 What is your age? _____

1.84 What is your Gender? ◯Female ◯Male

1.85 What is your phone number_____

Household, education and income details

We have defined a household as all members of a household unit who have been resident at the household for at least 12 months (including children less than 12 months), and whose members share ownership of a herd. Therefore, if members of a boma herd their livestock differently and make decisions separately, such a boma may not be referred to as a household for the purpose of this study.

3.1 Family size

How many people live in this household?	Men	Women	Children (ages 0-16 years)	Total
Number of people				

Please answer the next question if more than one household live in this boma.

How many households live in this boma?_____

Total number of people living in this boma	Men	Women	Children (ages 0-16 years)	Total
Number of people				

Is this abode the permanent place of residence for this household? ◯Yes ◯No

If No, when did you arrive here? _____

At what times of the year do you live here?_____

When will you leave this location? _____

Where else does the household live at (ward, village and subvillage)?_____

_____

3.2 Education: Head of household highest level of education?

Please indicate a number ☐

Most educated person in the family ☐

Key:
1-no formal schooling
2-some primary schooling
3-primary complete
4-some secondary or intermediate school
5-secondary complete
6-post secondary qualifications, other than university
7-some university
8- Other (specify)

3.3 Is anyone in the household a:

Livestock dealer or trader ◯Yes ◯No
butcher ◯Yes ◯No
slaughterhouse worker ◯Yes ◯No

3.4 We will ask questions about the livelihood activities that you or members of your household are engaged in.

Please answer questions about your household's income in a typical year?

SOURCES	Which of these is your primary income (select one)	Are any of these secondary sources of income?
a. Crop sales	◯	◯
b. Sale of livestock	◯	◯
c. Sale of livestock products	◯	◯
d. Work on others' farms	◯	◯
e. Non-farm employment?	◯	◯
f. Sales of natural products, including charcoal?	◯	◯
g. Income from businesses	◯	◯
h. Other income sources Please specify_____	◯	◯

3.5 What is the total household wage income per month on average from livestock?
Enter a number.

3.7 Does any household member maintain a savings account?

◯Yes ◯No

☐0
☐1- 25,000
☐25,000-100,000
☐100,000-200,000
☐200,000-400,000
☐400,000-700,000
☐700,000-1,000,000
☐ Over 1 million
☐ Does not want to answer
☐ Does not know

3.6 What is the total household wage income per month on average from labour, INCLUDING WAGE, SALARY, SMALL BUSINESS and NOT from livestock or crops?
Enter a number.

☐0
☐1- 25,000
☐25,000-100,000
☐100,000-200,000
☐200,000-400,000
☐400,000-700,000
☐700,000-1,000,000
☐ Over 1 million
☐ Does not want to answer
☐ Does not know

If yes, please specify

☐Bank
☐mpesa ☐Saccos / cooperative
☐Others
☐None

3.8 What is the current household savings balance? (SKIP IF NO BANKING ACCOUNT)
Enter a number

☐0
☐1- 25,000
☐25,000-100,000
☐100,000-200,000
☐200,000-400,000
☐400,000-700,000
☐700,000-1,000,000
☐ Over 1 million
☐ Does not want to answer
☐ Does not know
☐Skipped

Land

4.1 What is the status of the land on which this compound is located (choose only one)

◯Commonland
◯Rented from others
◯Owned by the members of the compound
◯Other, Please specify _____

4.2 Does the household own land?
◯Yes ◯No

if yes, how much_____

Crops

5.1Does your household cultivate crops?
◯ Yes
◯ No

5.2 Do you usually sell any of your crops?
◯ Yes
◯ No

5.3 During the last harvest, what percentage of your crops did you sell?
◯0%
◯25%
◯50%
◯75%
◯100%

SECTION 2: Livestock

6.11 How many animals are managed together in the same household / compound (epi unit)?					How many does the household own in total here and elsewhere?	
	Juvenile	Adult M	Adult F	Total	Here	Elsewhere
Cattle						
Sheep						
Goat						

6.12 How many are managed together in the same household / compound?
Pigs
Donkeys
Domestic birds (chickens, ducks, etc)
Dogs
Cats
Others, please specify_____

6.21 Do you graze your cattle, sheep and goats together? (choose only one)

◯Cattle, sheep, goats together
◯Cattle and sheep together
◯Cattle and goats together
◯Sheep and goats together
◯Species herded separately
◯NA (only 1 species owned, only 1 grazed, or none grazed)

6.22 Do you confine your cattle, sheep and goats together (choose only one)?

◯Cattle, sheep, goats together
◯Cattle and sheep together
◯Cattle and goats together
◯Sheep and goats together
◯Species confined separately
◯NA (only one species owned/confined)

6.3 Where are your adult livestock normally kept during the night in the dry/wet seasons? (choose one for each species and season)

	Cattle		Sheep		Goats	
	Dry	Wet	Dry	Wet	Dry	We
Confined at the compound within 10m of household location	◯	◯	◯	◯	◯	◯
Confined at the compound >10m of household location	◯	◯	◯	◯	◯	◯
Not confined	◯	◯	◯	◯	◯	◯
Confined elsewhere outside the compound, in seasonal camp.	◯	◯	◯	◯	◯	◯
Other_____	◯	◯	◯	◯	◯	◯

6.41 Are animals slaughtered in this household? ◯Yes ◯No

if yes who slaughters the animals?

_____

6.42 How frequently are animals slaughtered in this household?

◯Every week
◯Every two weeks
◯Every month
◯Other, please specify_____

6.43 Do you slaughter animals when they are sick and are going to die? ◯Yes ◯No

6.44 Do you prepare animals that are found dead, for food?
Livestock ◯Yes ◯No
Wildlife ◯Yes ◯No
If yes how often do you prepare animals found dead, for food?

◯Every week
◯Every two weeks
◯Every month
◯Other, please specify_____

6.51 Do you process animal hides from livestock in this household? ◯Yes ◯No

6.52 Do you process animal hides from wildlife in this household? ◯Yes ◯No

6.53 If you answered yes 6.51 or 6.52, what are the hides used for_____

6.54 Who does the processing?

_____

6.55 Please briefly describe the processing of hides_____

6.56 How do you process and prepare meat for food?

_____

6.57 Who processes and prepares the meat for food?

_____

6.58 Do you make use of blood from all slaughtered animals? ◯Yes ◯No
if yes, what is the blood used for? _____

_____

6.59 Do you milk small ruminants (goats and sheep) in this household? ◯Yes ◯No
If yes, what is the milk used for_____

_____

6.6 Grazing and watering livestock

6.61 Who in the household looks after the livestock?

	Cattle	Sheep	Goats	Donkeys
Person looking after livestock				

6.62 Where have you grazed and watered your herds in the last 12 months
Key: No distance, < 1hour, >= 1hour.

		Cattle [NA?]	Sheep [NA?]	Goats [NA?]
Dry season	Watering place distance			
	Optional location			
	Grazing place distance			
	Optional location			
Short rains	Watering place distance			
	Optional location			
	Grazing place distance			
	Optional location			
Long rains	Watering place distance			
	Optional location			
	Grazing place distance			
	Optional location			

6.7 Do any of your livestock ever come into contact with livestock from other herds and/or wildlife while grazing or watering? ◯DK ◯No ◯NA ◯Yes

DK=don't know, NA=not applicable

If respondent does not know about contacts while grazing, mark DK and skip to 6.8 If no animals graze, mark NA and skip to 6.8. If animals do graze but do not come into contact with other animals, mark No and skip to 6.8. If any animals graze and come into contact with other animals, mark Yes and fill in table.

If yes, indicate how frequently 1=daily, 2=weekly, 3=monthly, 4=less than monthly. 0=never.

	Cattle		**Sheep**		**Goats**	
	◯ NA	◯ NA	◯ NA	◯ NA	◯ NA	◯ NA
	Dry	**Wet**	**Dry**	**Wet**	**Dry**	**Wet**
Cattle						
Sheep						
Goats						
Buffalo						
Wildebeest						
Zebra						
Others, please specify						

6.8 Acquisitions, sales, etc.

6.81 Do you remember the last time you went to market to buy or sell animals? ◯Yes ◯No

If yes, when_____

Was a pink slip associated with this transaction? ◯Yes ◯ No. If yes, what was written on the pink slip?

_____

Where did the animals come from?	Where did the animals go to?	Number of cattle	Number of sheep	Number of goats

6.82 Generally, when you go to a market to buy or sell animals, what location would you put on the pink slip as where you are? (possibly multiple depending on market?)

Location on pink slip

6.83 Animals acquired in the last 12 months

		Cattle [NA?]	Sheep [NA?]	Goat [NA?]
Born	Number / No / DNK			
From relatives in village	Number / No / DNK			
From relatives outside village	Number / No / DNK Where?			
From friends / age mates	Number / No / DNK Where?			
From livestock traders in village (not via market)	Number / No / DNK Who?			
From livestock traders outside village (not via market)	Number / No / DNK Who? Where?			
From market	Number / No / DNK Which market?			
Others	Number / No / DNK Where from? How did you get them?			

6.84 Animals sold and left herd in the last 12 months

		Cattle	Sheep	Goat
To relatives in village	Number / No / Do not know (DNK)			
To relatives outside village	Number / No / DNK Where?			
To friends/ age mates	Number / No / DNK Where?			
To livestock traders in village (not via market)	Number / No / DNK Who?			
	Number / No / DNK			

To livestock traders outside village (not via market)	Who? Where?
To market	Number / No / DNK Which market?
Direct to slaughter house or butcher	Number / No / DNK Which? Where?
Died of disease	Number / No / DNK Which disease (if diagnosed or suspected)?
Killed by predators	Number / No / DNK Where?
Other	Number / No / DNK Where to? How?

SECTION 3: DISEASE

7.1 Mortality and morbidity

Have any livestock (cattle, sheep and goats) become sick/ died (from disease, predation, draught, snake bite, accident or others) in the past four months?
◯Yes ◯ No If yes, how many_____
Have any livestock died from unknown causes in the past 12 months? ◯Yes ◯ No
If yes, how many_____
What do you do to an animal that has died of unknown causes?
_____
What do you do to an animal that is sick of unknown causes?_____

Do you know of any disease that can cause sudden death in livestock? ◯Yes ◯ No
Can you tell us their names and describe these diseases?
Name of disease Signs and symptoms

7.2 Anthrax

Have you heard of the disease anthrax?
◯Yes ◯ No
If yes, can you tell us if anthrax affects animals?
◯Yes ◯No
Can you tell us what animals anthrax affects?
_____
_____
How do animals get anthrax?
_____
_____

Can you tell us if anthrax affects people?
◯Yes ◯No
Can you tell us how people get anthrax?
_____
_____
What are the signs you see in animals with anthrax?
_____
_____
What are the signs you see in people with anthrax?
_____
_____

Please rank these seven diseases in order of importance. (1= the most important amongst these seven and 7= the least important amongst these seven)

Brucellosis ☐
Black quarter ☐
Foot and mouth disease ☐
Anthrax ☐
Rift valley fever ☐
East coast fever ☐
Q-fever ☐

For each disease can you tell us how important you consider it. Place a number in the box next to the disease corresponding to the order of importance e.g., From 1 to 5 how serious do you consider anthrax in animals (1 = the most serious disease, 2 = very serious, 3 = serious, 4 = not very serious, 5 = not serious at all

Brucellosis ☐
Black quarter ☐
Foot and mouth disease ☐
Anthrax ☐
Rift valley fever ☐
East coast fever ☐
Q-fever ☐
Why have you ranked it this way?
_____

7.3 Anthrax occurrence

Can you tell us if there are specific periods when animals get sick with anthrax? ◯Yes ◯No
If yes, what periods are these? -
_____
_____

Are there specific periods when people get sick with anthrax? ◯Yes ◯No
If yes, what periods are these? -
_____
_____

Are there specific areas or locations where animals get anthrax? ◯Yes ◯No
If yes, what area/locations are these? -
_____
_____

Are there specific areas or locations where people get anthrax? ◯Yes ◯No
If yes, what area/locations are these (Please indicate on the map provided or describe the location)? -
_____

7.4 Anthrax prevention

Can you tell us if you do anything to prevent anthrax in animals? ◯Yes ◯No
If yes, what do you do to prevent anthrax in animals?
_____
_____

Can you tell us how to prevent anthrax in people?
◯Yes ◯No
If yes, what do you do to prevent anthrax in people?
_____
_____

Do you inform anyone when you suspect anthrax?
◯Yes ◯No
If yes, who do you inform (select all applicable options).
◯Neighbours
◯Other livestock owners
◯Community leader
◯Community animal health worker
◯Livestock field officer
◯Other_____
_

How do you inform them?
◯Word of mouth
◯Phone call
◯Other, please specify_____

Who do you inform first?
◯Neighbours
◯Other livestock owners
◯Community leader
◯Community animal health worker
◯Livestock field officer
◯Other_____
_

The following section aims to identify difficulties that people face in reporting anthrax to relevant authorities. The answers provided are for research purposes and are not intended for direct use by authorities.

Do you always report suspected cases of anthrax to authorities?
◯Yes ◯No
If no, do you sometimes report suspected cases of anthrax to authorities?
◯Yes ◯No
If yes, how do you decide what cases to report and cases not to report?
_____

If you do report cases, who do you report to?
◯Community leader
◯Community animal health worker
◯Livestock field officer
◯Other_____

How do you inform them?
_____
◯Word of mouth
◯Phone call
◯Other, please specify_____

At what point in time do you report to authorities?
◯Immediately ◯Within a few days
◯After a week ◯Within 24 hours

Are there any difficulties you face communicating after you suspect an anthrax case? ◯Yes ◯No
_____

What action is taken by the person/ people you report to?
_____

What additional measures would you like them to take?
_____

Have you vaccinated your livestock against anthrax in the past 12 months? ◯Yes ◯No
If yes, how many animals did you vaccinate?
_____

Which species did you vaccinate?

_____

Which vaccine did you use?
_____

Did you pay for the vaccine? ◯Yes ◯No
If yes, how much did you pay per
Cow_____
Sheep_____
Goat_____

Who administered the vaccine?
_____

How did you get the vaccine?
◯From local supplier
◯From community animal health worker
◯Through vaccination programs
◯Veterinary officer
◯Livestock field officer
◯Other, please specify_____
_

What other vaccinations do have you given your animals in the past 12 months?

Species	Vaccine given
Cattle	
Sheep	
Goats	

7.5 History of anthrax in the herd

Have you ever had any cases of anthrax in your animals? ◯Yes ◯No
Have you had any cases of anthrax in your animals in the last 2 years? ◯Yes ◯No
Have you had any cases of anthrax in last 12 months? ◯Yes ◯No
If you answered yes to any of the above questions, please fill out the following table. Record information on different outbreaks by date.

Date	Species affected	Number of animals affected	How many animals were pregnant (P)or lactating (L)	Details of how the animals contracted anthrax?	What happened to the animal(s) after contracting anthrax?	Did you do anything to prevent the disease from affecting other animals? What did you do?
			P_____ L_____ P_____ L_____ P_____ L_____			
			P_____ L_____ P_____ L_____ P_____ L_____			

Please provide details of the last outbreak

Species affected	Age	Sex	Number of animals	Clinical signs shown	Please indicate the number of animals that were pregnant (P) or lactating (L)
					P_____ L_____
					P_____ L_____
					P_____ L_____
					P_____ L_____

Did you know the source of the last outbreak? ◯ Yes ◯No
If yes, please specify_____

7.6 History of anthrax in the household

Has anyone in the household ever gotten sick with anthrax? ◯Yes ◯No
Has anyone in the household gotten sick with anthrax in the past 10 years? ◯Yes ◯No.
Has anyone in the household gotten sick with anthrax in the past 12 months? ◯Yes ◯No.
If you answered yes to any of the above questions, please fill out the table below

Date	Names of the people affected	Age	Information on how they contracted anthrax	What clinical signs did they show?	What treatment was given?	Where were they treated (e.g. at home, in hospital/dispensary or both)?	Did they make full recovery? If no, why?

7.7 History of anthrax in animals in the area

Have you ever heard of any cases of anthrax in animals in this community? ◯Yes ◯No
Have you heard of any cases of anthrax in animals in this community in the past 2 years? ◯Yes ◯No.
Have you heard of any cases of anthrax in this community in the past 12 months? ◯Yes ◯No
If you answered yes to any of the above questions, please fill out the table below.

Dates	Location	Species	Number of animals affected	How did the animals get anthrax?

7.8 History of anthrax in people in the area

Have you ever heard of any cases of anthrax in people in this area? ◯Yes ◯No
Have you heard of any cases of anthrax in people in this area in the past 10 years? ◯Yes ◯No
Have you heard of any anthrax cases in people in this area in the past 12 months? ◯Yes ◯No
If yes, please fill out the table below

Dates	Location	Names of people affected	How did the people get sick from anthrax?	Were they treated? If yes, Where did they get treated?	Did they make full recovery (if known)?

Appendix 3 Sample collection sheet

Sample Collection Sheet KIT ID # _____.

Please complete one sample kit / collection sheet per carcass sampled

BACKGROUND INFORMATION				
Sample collector's name:				
Sample collector's phone #:				
Date of collection (year-month-day):				
Location of collection: • Nearest village/sub-village • Rough directions to location (GPS coordinates if possible) • *If livestock:* Farm or pastoralist grazing? Number of animals in group Name/contact # of owner				
Number of animals dead / Species dead:				
Species being sampled:				
Age (approximate):	Juvenile	Sub-adult	Adult	Unknown
Sex:	Male	Female	Unknown	
Approximate time of death:	< 24 hours	24-48 hours	less than a week	more than a week

PRE-SAMPLING CHECKLIST	
☐	*Livestock:* Provide information about the study to animal's owner and request permission to collect samples.
☐	If possible, take a picture of the carcass site.
☐	Conduct external examination. • Is the carcass intact (circle one)? YES NO If no, please describe (ex: evidence of predation, abdomen perforated, etc.) • What is the body condition? FAT NORMAL THIN • Are there insects on/around the carcass? LOTS A FEW NONE Type of insect(s) if present: • Is there evidence of struggling (ex: soil around hooves disturbed by thrashing)? YES NO • Describe the location of the carcass (ex: near a body of water? What species are present in the area? Area with previous known anthrax cases?) • What has the recent weather been like? (temperature, amount of rain, etc.)
☐	Ensure all PPE and sampling materials are available and prepared, including disinfectant (2 Haztabs in 500 ml water/full water bottle, or 1 Haztab in 250 ml water/half water bottle). Put on PPE.
☐	Lay out your sampling materials to minimize contamination of containers.

SAMPLING CHECKLIST	
☐	Collect insects on/near the carcass if applicable. • *Use sterile forceps. Place insects in labeled plastic container.* ***Keep forceps for next step.***
☐	Collect a piece of tissue. • *Cut a small (2-3 cm) piece from the tip of the ear using forceps and scalpel.* • *Using forceps, place tissue in labeled plastic container.* • *Carefully replace lid on scalpel and discard in sharps container (if available) or waste bag.* • *If collecting different tissue (carcass already open), please name type of tissue collected:* _____.
☐	Make blood smears (6). • *Collect a drop of blood from the cut ear (or use syringe to collect other available blood pooled in carcass) onto the edge of a labeled microscope slide. Make smear using second microscope slide.* • *Make a total of 6 slides (ideally some thick and some thin). Allow them to air dry then attach with rubber band and place in envelope provided. Place the 7th slide used for making smears in the same envelope.* • ***Keep syringe for next 2 steps.***
☐	Collect blood swabs (4) • *Swab one end of each of 4 swabs in blood. This can be the same as the incision made for the smear or from other available pooled blood in carcass. Moisten cotton completely.* • *Place swab back in labeled transport container.*
☐	Collect tube of blood if pooled blood is accessible. • *Use the syringe (may have been used in prior steps) to collect blood from any accessible pooled blood. Transfer to labeled blood tube.* • ***Wrap blood tube in paper towel****.*
☐	Collect soil around the carcass that is stained with blood (preferable) or other body fluids. If there does not appear to be any fluid-soaked soil around the carcass, collect a sample close to the animal's mouth. • *Use a sterile spoon to collect soil into a labeled plastic container.* How would you describe the soil collected? **Soaked with blood** **Soaked with fluids other than blood** **Not fluid-soaked, collected near mouth**
☐	Disinfect and compile samples collected: • *Remove outer gloves and discard in waste bag.* • *Disinfect the outside of all primary plastic containers (swabs, insects, soil) with Haz-tab solution.* • *Make sure that all primary containers are tightly sealed.* • *Place primary containers (including blood tube wrapped in paper towel and envelope containing slides) in whirl-pak / Ziploc bags (secondary contains).* • *Place all secondary containers* ***except slides*** *inside labeled inner sample collection bag. Seal well.*
☐	Personal decontamination. • *Disinfect rubber boots.* • *Remove disposable coat and place in waste bag.* • *Remove and disinfect goggles.* • *Remove mask and place in waste bag.* • *Remove inner gloves.* • *Thoroughly wash hands with soap and water. If not available, use hand sanitizer.*
☐	Complete sample collection log. Place inside labeled outer sample collection bag with slides and waste bag. Seal well.
☐	Bring samples to the nearest laboratory when convenient. **Samples do not need to be refrigerated, and can be kept at ambient temperature for days to weeks.**

Appendix 4 Case and outbreak investigation form

ANTHRAX OUTBREAK INVESTIGATION FORM 1-HOUSEHOLD

BASIC INFORMATION		
Today's Date:	Ward:	Village:
Sub-village:	GPS location:	Name of investigator:
Head of household's name and phone number:	Respondent's name and phone number:	Name of Balozi:

EPIDEMIOLOGICAL INFORMATION

When was the first case seen?..... In what species?.....

Have any animals died in the current outbreak so far? ○ Yes ○ No.
if Yes, how many_____. Please fill out the following section with the number of animals dead in this outbreak

Cattle	Sheep	Goats	Others (please specify)
Juvenile_____	Juvenile_____	Juvenile_____	Juvenile_____
Adult male_____	Adult male_____	Adult male_____	
Adult female_____	Adult female_____	Adult female_____	Adult _____
Pregnant_____	Pregnant_____	Pregnant_____	
Lactating_____	Lactating_____	Lactating_____	

Juvenile animals are those less than 12 months old.

What signs and symptoms did the animals show?

☐ Swelling of the body	☐Sudden death	☐Difficulty breathing
☐Increased body temperature	☐Excitement	☐Depression
☐Blood oozing from nose, mouth and/or anus	☐Rapid decomposition of carcass	☐Diarrhoea
☐Uncoordinated movements/convulsions	☐Anorexia	☐Other_____

What was the total number of animals in the herd before the outbreak? _____

Cattle	Sheep	Goats
Juvenile_____	Juvenile_____	Juvenile_____
Adult male_____	Adult male_____	Adult male_____
Adult female_____	Adult female_____	Adult female_____
Pregnant_____	Pregnant_____	Pregnant_____
Lactating_____	Lactating_____	Lactating_____

What measures are already being taken against the outbreak?

Please provide more details about this specific outbreak in the form below. Details of each carcass should be be recorded in a row

Species	Age	Sex	Was this animal pregnant (P) or lactating (L)?	Carcass presentation	Approx. distance from nearest carcass if any (in metres).	What has been done with this carcass?	Sample kit number (if samples are collected)

Has anyone in the household reported any of these signs since the outbreak began?

Diarrhea	◯ Yes ◯ No		Cough	◯ Yes ◯ No
Bloody stools	◯ Yes ◯ No		Runny nose	◯ Yes ◯ No
Vomiting	◯ Yes ◯ No		Sore throat	◯ Yes ◯ No
Nausea	◯ Yes ◯ No		Rash	◯ Yes ◯ No
Abdominal cramps	◯ Yes ◯ No		Itching	◯ Yes ◯ No
Fever	◯ Yes ◯ No		Other, please specify	◯ Yes ◯ No
Chills	◯ Yes ◯ No			

If you answered yes to any of these signs, please fill out the table below.

Age	Sex	Does the person still have this signs?

Have you heard of other herds affected at this time? ◯ Yes ◯ No
If yes, please fill out the table below.

Where(village and sub village)	Name of owner	When	Species affected	Description

Have you had any other cases of anthrax disease in the past 12 months? ◯ Yes ◯ No. If no,
Have you had any cases of anthrax disease in the past two years? ◯ Yes ◯ No.
If you have had any cases of anthrax in the past two years, please fill up the table below:

Species (e.g. cattle, goat, sheep)	Year	Month	Number of animals affected	Suspected origin of outbreak (e.g. water source or grazing land)

Have you heard of any cases of anthrax disease in animals in this area in the past 12 months? ◯ Yes ◯ No.
If yes, please fill in the table below:

Where(village and subvillage)	Name of owner (if known)	When	Species affected	Description

Appendix 5 Additional information- Cost of livestock

Average livestock prices in 2015 and 2016.

Species	Average price (TZS)	Average price (USD)
Cattle	497804.16 (SD = 84168.96)	230.70 (SD = 39.01)
Sheep	64348.42 (SD = 4135.51)	29.82 (SD = 5.01)
Goats	67075.75 (SD = 10820.97)	31.09 (SD = 1.92)

Average livestock prices in 2016 and 2017.

Species	Average price (TZS)	Average price (USD)
Cattle	557320.6 (SD = 207432.91)	249.96 (SD = 3.33)
Sheep	64151.5 (SD = 16124.42746)	28.77 (SD = 7.23)
Goats	68745.05 (SD = 13646.66343)	30.83 (SD = 6.12)

Appendix 6 Assessing PCR methodology: the quantity of blood smear scraping sufficient for PCR

Impact of the smear scraping technique used on the starting sample weight and resulting cycle threshold (Ct) values. Dried blood was either scraped from half the slide (L) or from ~1cm diameter of a section of thick smear (S) (Figure 4.5).

Working ID	Weight of sample (g)	Ct value
19SM S	0.0008	27
19SM L	0.0002	26
21SM S	0.0020	22
21SM L	0.0025	25
25SM S	0.0014	21
25SM L	0.0035	19
36SM S	0.0011	21
36SM L	0.0009	22
39SM S	0.0011	22
39SM L	0.0018	23

Appendix 7 Qiagen DNA extraction protocol

Quick-Start Protocol April 2016

DNeasy® Blood & Tissue Kit

The DNeasy Blood & Tissue Kit (cat. nos. 69504 and 69506) can be stored at room temperature (15–25°C) for up to 1 year if not otherwise stated on label.

Further information

- *DNeasy Blood & Tissue Handbook*: **www.qiagen.com/HB-2061**
- Safety Data Sheets: **www.qiagen.com/safety**
- Technical assistance: **support.qiagen.com**

Notes before starting

- Perform all centrifugation steps at room temperature (15–25°C).
- Redissolve any precipitates in Buffer AL and Buffer ATL.
- Add ethanol to Buffer AW1 and Buffer AW2 concentrates.
- Equilibrate frozen tissue or cell pellets to room temperature.
- Preheat an incubator to 56°C.
- Refer to the handbook for pretreatment of fixed tissue, insect, bacterial or other material.

1a. **Tissue**: Cut tissue (≤10 mg spleen or ≤25 mg other tissue) into small pieces, and place in a 1.5 ml microcentrifuge tube. For rodent tails, use 1 (rat) or 2 (mouse) 0.4–0.6 cm lengths of tail. Add 180 µl Buffer ATL. Add 20 µl proteinase K, mix by vortexing and incubate at 56°C until completely lysed. Vortex occasionally during incubation. Vortex 15 s directly before proceeding to step 2.

1b. **Nonnucleated blood**: Pipet 20 µl proteinase K into a 1.5 ml or 2 ml microcentrifuge tube. Add 50–100 µl anticoagulant-treated blood. Adjust volume to 220 µl with PBS. Proceed to step 2.

1c. **Nucleated blood**: Pipet 20 µl proteinase K into a 1.5 ml or 2 ml microcentrifuge tube. Add 5–10 µl anticoagulant-treated blood. Adjust volume to 220 µl with PBS. Proceed to step 2.

1d. **Cultured cells**: Centrifuge a maximum of 5 x 10^6 cells for 5 min at 300 x *g* (190 rpm). Resuspend in 200 µl PBS. Add 20 µl proteinase K. Proceed to step 2.

2. Add 200 µl Buffer AL. Mix thoroughly by vortexing. Incubate blood samples at 56°C for 10 min.
3. Add 200 µl ethanol (96–100%). Mix thoroughly by vortexing.
4. Pipet the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge at ≥6000 x *g* (8000 rpm) for 1 min. Discard the flow-through and collection tube.
5. Place the spin column in a new 2 ml collection tube. Add 500 µl Buffer AW1. Centrifuge for 1 min at ≥6000 x *g*. Discard the flow-through and collection tube.
6. Place the spin column in a new 2 ml collection tube, add 500 µl Buffer AW2 and centrifuge for 3 min at 20,000 x *g* (14,000 rpm). Discard the flow-through and collection tube.
7. Transfer the spin column to a new 1.5 ml or 2 ml microcentrifuge tube.
8. Elute the DNA by adding 200 µl Buffer AE to the center of the spin column membrane. Incubate for 1 min at room temperature (15–25°C). Centrifuge for 1 min at ≥6000 x *g*.
9. **Optional**: Repeat step 8 for increased DNA yield.

Scan QR code for handbook.

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual.

Appendix 8 Results of agreement for tests performed on stained smears read 6 months apart (assessing the effect of time on stained smear quality)

Agreement for slides stained with Azure B read 6 months apart

1st rating	2nd rating		
	Positive	Negative	Total
Positive	20	3	23
Negative	0	38	38
Total	20	41	61

Azure B: Cohen's Kappa for 2 Ratings (Weights: squared), Subjects = 61, Raters = 2. Kappa = 0.893, z = 7.01, *p*-value = 2.36e-12

Agreement for slides stained with PMB read 6 months apart

1 st rating	2nd rating		
	Positive	Negative	Total
Positive	4	1	5
Negative	0	7	7
Total	4	8	12

PMB: Cohen's Kappa for 2 Ratings (Weights: squared), Subjects = 12, Raters = 2. Kappa = 0.824, z = 2.9, *p*-value = 0.00375

Agreement for slides stained with Giemsa read 6 months apart

1st rating	2nd rating		
	Positive	Negative	Total
Positive	3	3	6
Negative	2	53	55
Total	5	56	61

Giemsa: Cohen's Kappa for 2 Raters (Weights: squared), Subjects = 61, Raters = 2. Kappa = 0.501, z = 3.93, *p*-value = 8.45e-05

Agreement for slides stained with Rapi-Diff read 6 months apart

1st rating	2nd rating		
	Positive	Negative	Total
Positive	2	1	3
Negative	1	57	58
Total	3	58	61

Rapi-Diff: Cohen's Kappa for 2 Ratings (Weights: squared), Subjects = 61, Raters = 2. Kappa = 0.649, z = 5.07, *p*-value = 3.93e-07

Appendix 9 Results of inter-rater agreement for stained smear tests examined by two persons

Inter-rater agreement for Azure B

Rater 1	Rater 2		
	Positive	Negative	Total
Positive	71	3	74
Negative	1	69	70
Total	72	72	144

Azure B: Cohen's Kappa for 2 Raters (Weights: squared), Subjects = 144, Raters = 2. Kappa = 0.944 , z = 11.3, *p*-value = 0

Inter-rater agreement for PMB

Rater 1	Rater 2		
	Positive	Negative	Total
Positive	49	1	50
Negative	1	33	34
Total	50	34	84

PMB: Cohen's Kappa for 2 Raters (Weights: squared), Subjects = 84, Raters = 2. Kappa = 0.951, z = 8.71, *p*-value = 0

Inter-rater agreement for Giemsa

Rater 1	Rater 2		
	Positive	Negative	Total
Positive	9	8	17
Negative	6	117	123
Total	15	125	140

Giemsa: Cohen's Kappa for 2 Raters (Weights: squared), Subjects = 140, Raters = 2. Kappa = 0.506, z = 6.01, *p*-value = 1.91e-09

Inter-rater agreement for Rapi-Diff

Rater 1	Rater 2		
	Positive	Negative	Total
Positive	4	3	7
Negative	7	129	136
Total	11	132	143

Rapi-Diff: Cohen's Kappa for 2 Raters (Weights: squared), Subjects = 143, Raters = 2. Kappa = 0.409, z = 5.03, *p*-value = 4.79e-07

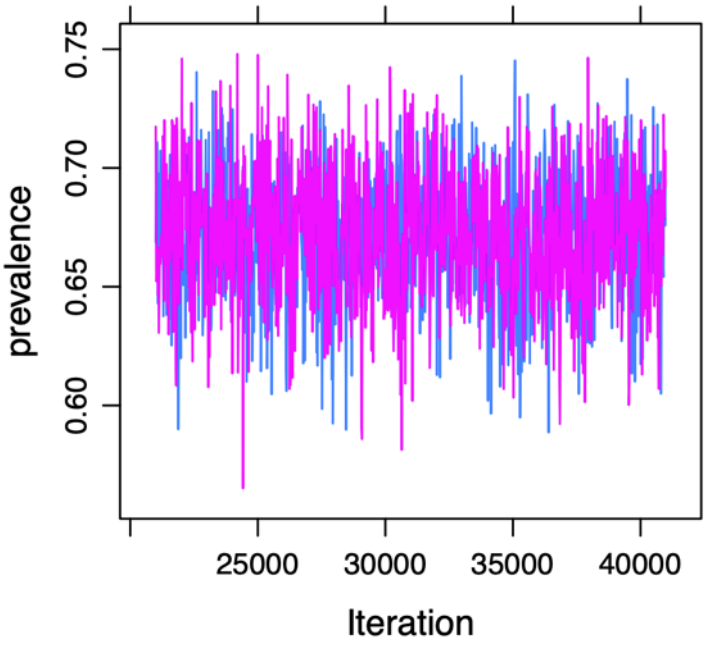
Appendix 10 Results of inter-rater agreement for smears stained and examined independently by two persons

Inter-rater agreement for azure B on samples from the same cases, processed separately from staining through to observation.

Rater 1	Rater 2		
	Positive	Negative	Total
Positive	42	1	43
Negative	1	27	28
Total	43	28	71

Cohen's Kappa for 2 Raters (Weights: squared), Subjects = 71, Raters = 2. Kappa = 0.941, z = 7.93, *p*-value = 2.22e-15

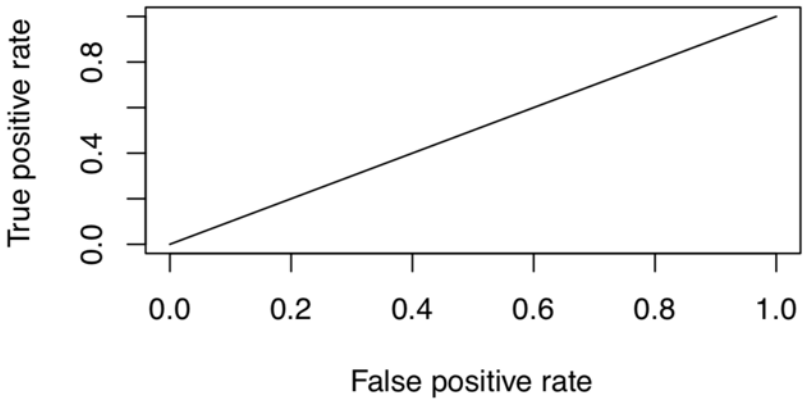
Appendix 11 Trace plot of the prevalence of anthrax in the sampled population showing convergence of the latent class model



Appendix 12 ROC curves showing the accuracy of prediction models to explain the environmental risk of anthrax

Plots showing the Area Under the Curve (AUC) Receiver Operating Characteristics (ROC) for the accuracy of the prediction model

Plot 1(tested on data from north-east of study area)



Observed and predicted frequencies for point within and without defined risk areas

	Observed	
Predicted	Point within high-risk areas	Point out-with high-risk areas
Point within high-risk areas	0	0
Point out-with high-risk areas	25	300

Sensitivity: 0 %, Specificity: 100%, Accuracy : 92%

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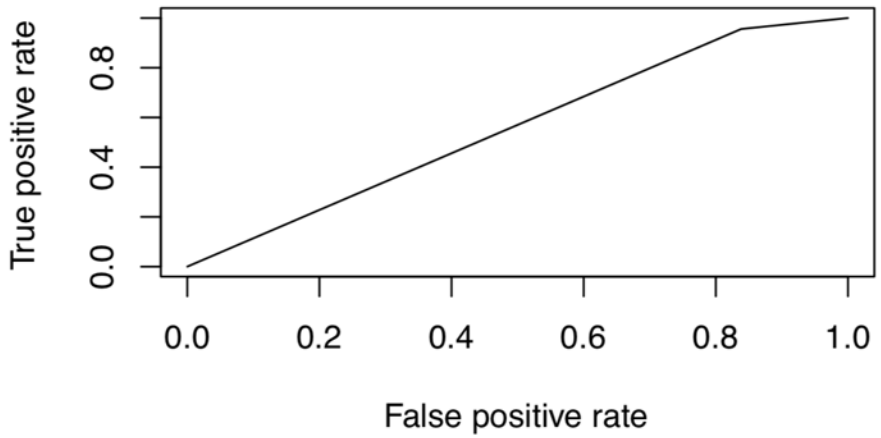
<td>Plot 2(tested on data from north-west of study area)</td>
<td> 
</td>
<td>Observed and predicted frequencies for point within and without defined risk areas
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<tr><td></td><td colspan="2">Observed</td></tr>
<tr><td>Predicted</td><td>Point within high-risk areas</td><td>Point out-with high-risk areas</td></tr>
<tr><td>Point within high-risk areas</td><td>65</td><td>581</td></tr>
<tr><td>Point out-with high-risk areas</td><td>3</td><td>112</td></tr>
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Sensitivity: 95 %, Specificity: 16%, Accuracy : 23%</td>
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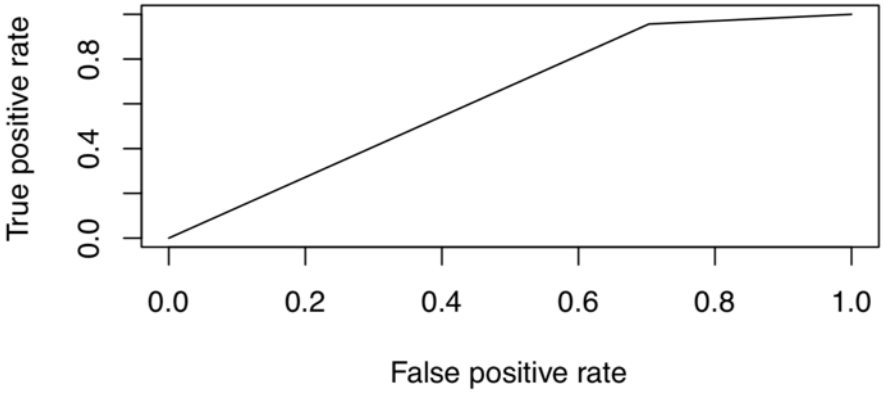
<tr>
<td>Plot 3(tested on data from south-east of study area)</td>
<td> 
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<td>Observed and predicted frequencies for point within and without defined risk areas
<table>
<tr><td></td><td colspan="2">Observed</td></tr>
<tr><td>Predicted</td><td>Point within high-risk areas</td><td>Point out-with high-risk areas</td></tr>
<tr><td>Point within high-risk areas</td><td>110</td><td>581</td></tr>
<tr><td>Point out-with high-risk areas</td><td>5</td><td>455</td></tr>
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Sensitivity: 96 %, Specificity: 30%, Accuracy : 40%</td>
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<table>
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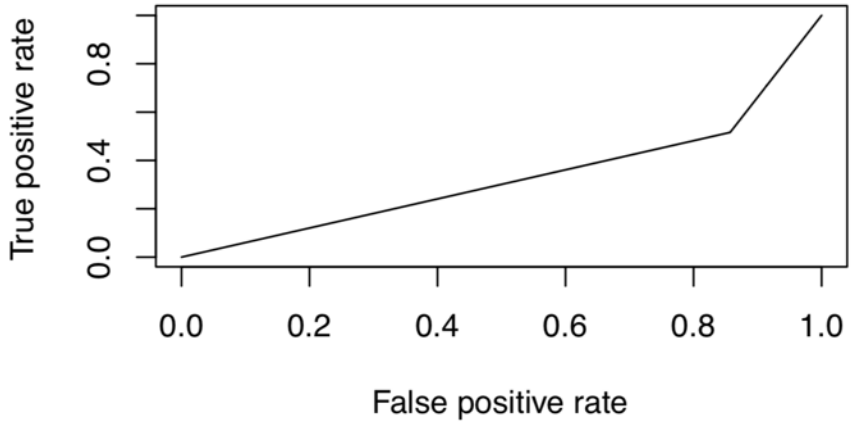
<td>Plot 4(tested on data from south-west of study area)</td>
<td> 
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<td>Observed and predicted frequencies for point within and without defined risk areas
<table>
<tr><td></td><td colspan="2">Observed</td></tr>
<tr><td>Predicted</td><td>Point within high-risk areas</td><td>Point out-with high-risk areas</td></tr>
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<tr><td>Point out-with high-risk areas</td><td>15</td><td>42</td></tr>
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Sensitivity: 52 %, Specificity: 14%, Accuracy : 18%</td>
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Appendix 13 Focus group schedule for mapping exercise

1. What geographical locations are perceived as anthrax risk areas (and locations not perceived as risk areas?)

2. Where are the locations of natural resources suitable for livestock grazing and watering?

3. During dry seasons, does the number of these areas decrease?

4. What areas did your animals graze within the last year? What water sources did they use?

5. Are these areas permanent grazing areas? Or would you change them next year?

6. What areas do you prefer to graze your animals? Score all the identified areas from question i. according to the most preferred areas.

7. Why do you prefer certain areas to others?

8. What makes certain areas preferable to other?

9. Do you consider the attractiveness of a grazing area as well as the risk of livestock grazing on those areas? What risks are involved?

10. Does wildlife share these grazing areas with your livestock?

11. What wildlife have you come across while grazing animals?

12. Where is the market where livestock is traded located? Are there other markets? Where are they located?

13. What areas within this community do you think have been contaminated with anthrax, and that animals get sick from grazing on these areas? (what areas do you think are free from anthrax spores?)

14. What exact locations have outbreaks occurred in the past? When?

15. What exact locations have carcasses suspected to have died of anthrax/ unknown causes been buried in the past?

16. What is the nature of vegetation present in these areas where carcasses have been buried? Are they near temporary or permanent water bodies?

17. Do livestock graze on these areas or do they have alternate areas to graze?

18. How far do your animals move? Do you select specifically where they graze?

19. Over the course of a year, are there specific routes that you move along?

20. During the dry season What locations does your livestock graze on?

21. Are animal skins processed in this village? If yes where are they processed and waste discarded? Where do the processed hides go? Do you sell them in the local market?

22. Please list the uses of processed hides?

23. Are drums made of animal skins made locally in this community? Where do the drums go?

24. Do you make use of drums made of animal skins in this community? Where do the drums come from?

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