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Seroepidemiological study of anthrax among cattle in Zambia

(ザンビアにおけるウシの炭疽に関する血清疫学研究)

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Abbreviations

AGID	Agar gel immunodiffusion
CBB	Coomassie brilliant blue
CBPP	Contagious bovine pleuropneumonia
CV	Coefficient of variation
CVRI	Central Veterinary Research Institute
DTT	Dithiothreitol
EDTA	Ethylenediaminetetracetic acid
EF	Edema factor
ELISA	Enzyme linked immunosorbent assay
EU_a	Endotoxin units
EU_b	Elisa units
FMD	Foot and mouth disease
GST	Glutathione S transferase
HS	Haemorrhagic septicaemia
IgG	Immunoglobulin G
IMHA	Indirect microhaemagglutination
IPTG	Isopropyl β -D-thiogalactopyranoside
LAL	Limulus amebocyte lysate
LB	Lysogeny broth
LF	Lethal factor
LSD	Lumpy skin disease

Mw	Molecular weight
NaCl	Sodium chloride
NALEIC	National Livestock Epidemiology and Information Center
NC	Negative control
OD	Optical density
PA	Protective antigen
PBS	Phosphate buffered saline
PC	Positive control
PCR	Polymerase chain reaction
PMSF	Phenylmethanesulfonyl fluoride
rPA-D1	Recombinant protective antigen domain 1
rpm	Rotations per minute
RT	Room temperature
SDS	Sodium dodecyl sulfate
PAGE	Polyacrylamide gel electrophoresis
TMB	3,3', 5,5' tetramethylbenzidine
TB	Terrific broth
WB	Western blotting
WHO	World Health Organization
OIE	World Organization for Animal Health

Unit measures abbreviations

%	percent
g	gram
kDa	kilodalton
km	kilometer
L	liters
ml	milliliters
mM	millimolar
ng	nanogram
N	normality
μg	microgram
μl	microliter
°C	degree celsius

Notes

The contents of Chapter I and Chapter II have been published in *PLOS ONE*.

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Preface

Bacillus anthracis causes anthrax, a disease that mainly affects herbivorous animals and wildlife (Bales et al. 2002; Turnbull 2008). In domesticated herbivorous animals such as cattle, sheep, and goats infection occurs primarily through the ingestion of a high dose of spores from contaminated grazing pastures or contaminated soil (Beyer and Turnbull 2009). The disease usually occurs as either the acute or peracute form and is often characterized by edema, necrosis, lymphadenopathy, subcutaneous hemorrhage and lack of rigor mortis on the carcass (Hugh-Jones and de Vos 2002; Beyer and Turnbull 2009). Humans acquire the infection through the ingestion or handling of contaminated meat and meat products. In humans, anthrax may occur as the pulmonary, intestinal or cutaneous form (Swartz 2001; Sirisanthana and Brown 2002; Azarkar and Bidaki 2016; Hu et al. 2016). Recently, the injectional form of anthrax has been identified in heroin users (Hicks et al. 2012). Anthrax is an important disease because *B. anthracis* spores can persist in the environment (soil) for a long period of time and they have the potential for use in biological warfare. In areas where the disease occurs naturally, outbreaks cause massive mortalities in wildlife in national parks, game management areas and wildlife conservation centers (Turnbull et al. 1991; Hugh-Jones and de Vos 2002; Clegg et al. 2007; Hoffmann et al. 2017). In regions where livestock is an important part of the agricultural industry, livestock anthrax causes significant economic losses and is a constant threat to public health (Odontsetseg et al. 2007; Chikerema et al. 2012; Goel 2015).

Virulent strains of *B. anthracis* possess both the pXO1 and pXO2 plasmid (Uchida et al. 1986). Genes encoding the three anthrax toxin proteins *pagA* (protective

antigen [PA]), *lef* (lethal factor [LF]) and *cya* (edema factor [EF]) and regulatory genes *atxA* and *pagR* are located on the pXO1 plasmid (Mikesell et al. 1983; Koehler et al. 1994; Dai et al. 1995; Okinaka et al. 1999). PA combines with either EF to form the edema toxin or with LF to form the lethal toxin (Leppla et al. 1995). EF is an adenylate cyclase (Leppla 1982) and LF is a zinc metalloprotease (Klimpel et al. 1994) which disrupt cellular function and cause mortality. The genes encoding for the antiphagocytic poly- γ -D-glutamic acid capsule *capB*, *capC*, and *capA* and cap gene regulators *acpA* (Vietri et al. 1995) and *acpB* (Drysdale et al. 2004) are located on the pXO2 plasmid (Makino et al. 1989). PA binds to cellular receptors and facilitates the entry of LF and EF into the cytoplasm (Sellman et al. 2001). PA has 4 domains: domains 1, 2, 3 and 4 (Petosa et al. 1997). Each of these domains is immunogenic and plays a role in the host cell intoxication process. Domain 1 (residues 1–258) contains the furin cleavage site which is essential in mediating toxin entry into the cell. It also possesses the calcium binding sites that provide stability to PA (Gupta et al. 2003). Domain 2 (residues 259–487) plays a part in heptamerization process (Lacy et al. 2004). Domain 3 (residues 488–595) is involved in the oligomerization process (Mogridge et al. 2001) and Domain 4 (residues 596–735) is involved in host cell receptor binding (Singh et al. 1991; Little et al. 1996).

The main assays that have been used in anthrax serology include antigen detection tests such as immunochromatographic assays and Ascoli test. The former is more useful as an antemortem test and the latter is not specific. Tests detecting antibodies to *B. anthracis* toxin components such as the agar gel immunodiffusion (AGID) are insensitive and time consuming, while the indirect microhemagglutination (IMHA) test lacks rigor and its reagents have a short shelf life (Buchanan et al. 1971).

Compared to the above methods enzyme-linked immunosorbent assay (ELISA) based on *B. anthracis* PA is more specific, sensitive and rapid (Johnson-Winegar 1984). Although the domains of PA are immunogenic, development of an ELISA based on a domain of PA is yet to be demonstrated.

Anthrax outbreaks have been reported in several countries (Heyworth et al. 1975; Turner et al. 1999b; Woods et al. 2004; Clegg et al. 2007; Odontsetseg et al. 2007). In Zambia, sporadic cases of anthrax have been reported since the 1970's and from 1990 outbreaks have been reported on an almost annual basis (Bbalo 1996). Recently, anthrax outbreaks were reported in wildlife: hippopotamuses (*Hippopotamus amphibious*), elephants (*Loxodonta africana*) and buffalo (*Syncerus caffer*) in game management areas in the Eastern Province (Hang'ombe et al. 2012; Ohnishi et al. 2014). In livestock, anthrax outbreaks were reported in cattle in the Western Province (Fig 1). Human anthrax outbreaks usually occur after an outbreak in either wildlife or cattle (Lehman et al. 2017; Sitali et al. 2017). Despite its long history in the country, national anthrax targeted surveillance programs in place: mass vaccination campaigns, decontamination and disinfection, and livestock movement control, the persistence and frequency of anthrax outbreaks among cattle still remains an issue of great concern.

Cattle in Zambia are reared under the commercial and traditional farming sectors. Although the commercial sector rears exotic breeds (Friesian and Jersey), the traditional sector is characterized by subsistence farming, the management of indigenous breeds (Barotse and Angoni), and communal grazing. There are an estimated 3.0 million cattle in Zambia (MAL 2010), with more than 80% present in the traditional farming sector. Cattle are the main source of income and food security in

Zambia's rural communities where they are used in traditional ceremonies for cultural purposes and in agriculture as draft oxen and to provide manure. Despite the important role of cattle in Zambia's agricultural and livestock sectors, cattle productivity is hampered by diseases such as foot and mouth disease (FMD), contagious bovine pleuropneumonia (CBPP), haemorrhagic septicaemia (HS), lumpy skin disease (LSD) and anthrax. These diseases cause mortality, slow growth and lower cattle productivity (Muma et al. 2007). For anthrax, this situation coupled with the under reporting of cases, has led to the underestimation of the impact of anthrax in livestock and continued to generate less public health and veterinary attention.

In the Western Province of Zambia, the incidence, serology and impact of the disease remains unknown. In addition to the retrospective surveillance systems in place, information on anthrax serology in cattle is necessary for the control of anthrax outbreaks. Considering this situation, it would be important to determine the geographical extent of anthrax in endemic areas, and evaluate the current seroepidemiological situation. In Chapter I, to establish a method for the serological surveillance of cattle anthrax in Zambia, an ELISA based on *B. anthracis* recombinant protective antigen domain 1 (rPA-D1) was developed. The rPA-D1 ELISA could detect seroconversion in cattle post anthrax vaccination. In Chapter II, a field study was conducted to demonstrate the utility of the rPA-D1 ELISA and to determine immune status of cattle during the anthrax outbreak period. The study provided essential serological data that will be useful to inform policy and the re-evaluation of anthrax control programs in Zambia. In Chapter III, to gain more insight on the current epidemiological situation, the seroprevalence of anti-PA antibodies at district level and the geographical distribution of cattle anthrax outbreaks were determined. The studies

identify the factors that precipitate anthrax outbreak recurrence and highlight areas that need to be prioritized in anthrax control in Zambia.

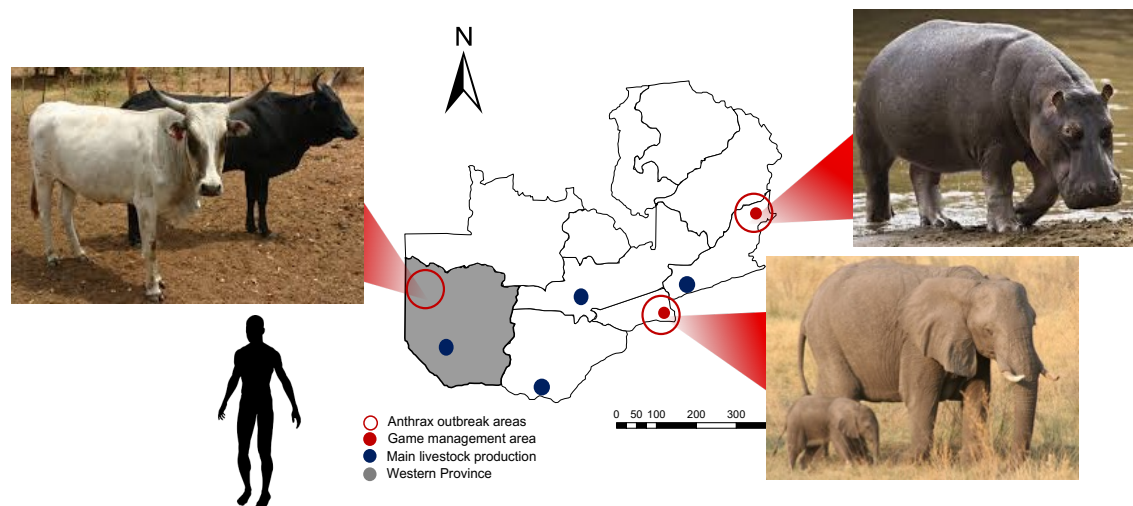


Figure 1. Anthrax in Zambia.

Location of the main livestock production areas, game management areas (anthrax outbreak areas in wildlife) and the anthrax endemic area Western Province. In the Eastern Province, outbreaks occur among wildlife, in the Western Province outbreaks occur among cattle. Human anthrax outbreaks occur after outbreaks in livestock and wildlife.

CHAPTER I:

Development of a *Bacillus anthracis* protective antigen domain-1 in-house ELISA for the detection of anti-protective antigen antibodies in cattle in Zambia

Summary

In Zambia, anthrax outbreaks among cattle are reported on nearly an annual basis. Presently, there is a lack of serological assays and information to develop an anthrax management and control strategy. In this study, an indirect ELISA based on rPA-D1 of *B. anthracis* was developed and used to detect anti-PA antibodies in cattle in Zambia. An antigen coating of 10 ng/well and a serum dilution of 1:100 were determined to be the optimal rPA-D1 ELISA titration conditions. The intra- and inter-assay % coefficients of variation were less than 10% and 15%, respectively. The rPA-D1 ELISA could detect seroconversion in cattle 1 month after anthrax vaccination. ELISA cut-off value was determined as mean +3 standard deviations of the negative control cattle sera. The use of lyophilized antigen in the rPA-D1 ELISA eliminates the need for cold chain facilities for storage of precoated ELISA plates and rPA-D1 antigen. The rPA-D1 ELISA would be a useful and valuable tool not only in anthrax seroepidemiological studies but also in monitoring the effectiveness of anthrax vaccination programs in Zambia.

Introduction

Anthrax affects a wide range of species causing substantial morbidity and mortality among susceptible wildlife and livestock populations (Bales et al. 2002). Anthrax continues to pose a serious burden on the human health, economic and agriculture sectors of several developing countries, particularly those with poor public health systems and where livestock farming is the main source of income (Turnbull 2008). Anthrax is endemic in the Western Province of Zambia. Presently, there is a lack of serological assays for the seroepidemiological investigations of anthrax in cattle in Zambia.

At present, Zambia has a passive surveillance system that is mostly functional following the reports of suspected anthrax cases. The reporting system in Western Province comprises three administrative levels: veterinary camp, district levels and provincial levels. After a suspected case of anthrax has been identified, samples are collected and sent to the provincial veterinary office where Giemsa staining is performed; thereafter, the positive samples are sent to Lusaka (Lusaka Province) for confirmatory tests. In Lusaka, bacterial culture and isolation are performed. It is sometimes difficult to confirm the diagnosis through the sample cultures from suspected anthrax cases that are undergoing antibiotic treatment. Since antibiotics do not eliminate the anthrax toxin components or other components of the bacteria, using a serological assay to detect antibodies to toxin components would complement the present methods being used in anthrax surveillance.

The virulence of *B. anthracis* is caused by the anthrax toxin and antiphagocytic capsule. Genes encoding the anthrax toxin are located on the pXO1 plasmid (Uchida et

al. 1993; Okinaka et al. 1999) and the cap region which is important for encapsulation is located on the pXO2 plasmid (Keppie et al. 1963; Green et al. 1985; Makino et al. 1989). Anthrax toxin comprises three proteins namely: PA, EF and LF. Because PA is the superior antigen in the natural and vaccine-induced immunity to anthrax infection (Reuveny et al. 2001; Marcus et al. 2004), the PA-based ELISA is presently the most effective method for detecting antibodies against *B. anthracis* in serum, plasma or other biological fluids. It is sensitive and enables hundreds to thousands of samples to be screened in a short period of time. Certain advances have been made in the ELISA mainly based on full-length PA, and the ELISA has been applied to human anthrax vaccine evaluation and diagnosis (Iacono-Connors et al. 1994; Quinn et al. 2002) ; however, it still has certain drawbacks such as false positives, lack of robustness and lack of assay standardization. Few studies have assessed the development and use of ELISAs in the detection of anti-PA antibodies in livestock populations (Hassan et al. 2015; Turnbull et al. 1992; Kingston et al. 2015). In these studies, the commercial ELISA kits were primarily used in developed or nonendemic countries for the evaluation of an antibody response after the anthrax vaccination in livestock under commercial settings. Additionally, the commercial assays are unsuitable for use in resource-limited settings, where it is difficult to maintain cold chain facilities for the storage of antigens or ELISA precoated plates. Thus, a sensitive, specific and robust ELISA for detecting antibodies against *B. anthracis* PA in cattle in resource-limited settings is necessary.

PA has four domains: domains 1, 2, 3 and 4 (Petosa et al. 1997). Each domain has a specific function in the host cell intoxication (Varshney and Goel 2016). Protective antigen domain 1 (PA-D1) contains the furin cleavage site RKKR and

proteolytic cleavage is required for the activation of anthrax toxin (Klimpel et al. 1992). PA-D1 also contains the Ca^{2+} binding sites that provide structural stability to PA (Gupta et al. 2003). PA-D1 is immunogenic and its qualitative immune response has been shown to be equivalent to that elicited by whole PA (Flick-Smith et al. 2002; Abboud and Casadevall 2008). Immunoassays based on PA or its domain for the detection of anti-PA antibodies in the serum of livestock in endemic areas may provide useful information on the anthrax exposure patterns and the effectiveness of anthrax vaccination programs. Previous studies have suggested the potential use of PA domains in the development of serodiagnostic assays for anthrax (Varshney et al. 2016); however, an ELISA based on a subunit of PA has not yet been developed. To overcome the above limitations and to demonstrate the utility of a subunit of PA, I developed a qualitative bovine ELISA using rPA-D1 as an ELISA antigen.

Materials and methods

Plasmid construction

PA-D1 is composed of residues 1–258. The following primers were designed and then synthesized by Sigma Aldrich: PA-D1 forward primer (5'-ATG GAA GTT AAA CAG GAG AAC CGG-3') and PA-D1 reverse primer (5'-TGC CAC AAG GGG GTG TCT TG-3'). The gene encoding PA-D1 was amplified from *B. anthracis* CZC5 (accession number: AP018443) by TaKaRa Ex Taq. The amplified PCR products were analyzed on 1% agarose gel and extracted using the QIAquick Gel Extraction Kit (Qiagen). The purified fragments were then subjected to cloning experiments with the pGEM-T vector system (Promega) and *Escherichia coli* DH5 α . Plasmid DNA was purified by a QIAprep Spin Miniprep Kit (Qiagen). The pGEMT-D1 and pGEX6p2 plasmids were then digested with the restriction enzymes BamHI and NotI (New England BioLabs). The PA-D1 gene was ligated into the BamHI and NotI sites of the expression vector pGEX6p2 (pGEX6p2-PA-D1).

Expression and purification of rPA-D1

E. coli BL21 cells were transformed with the expression vector pGEX6p2-PA-D1. The transformed *E. coli* BL21 cells were grown at 37°C for 15 h at 180 rpm in 3 ml of Lysogeny broth supplemented with 100 μ g/ml of ampicillin. The culture was then added to 70 ml of Terrific Broth (TB) and the cells were grown at 37°C for an additional 2 h at 180 rpm. After reaching an OD₆₀₀ of 1.8, the culture was diluted until it reached an of OD₆₀₀ 0.05 in 1 L of TB. The cells in the 1 L of culture were grown at 37°C for 90 min at 80 rpm and then at 15°C for 30 min. When the OD₆₀₀ reached 0.8, protein expression was induced by adding isopropyl β -D-thiogalactopyranoside (IPTG) to

reach a final concentration of 1 mM; thereafter, the cells were grown at 18°C for 20 h. The cell culture was centrifuged at 4,000 rpm for 15 min and the supernatant was extracted. The resulting cell pellet was suspended in lysis buffer [phosphate buffered saline (PBS) containing 0.5% Triton X-100, 0.1 mM benzamidine, 1 mM PMSF, pH 7.5]. The resuspended cell pellet was centrifuged for 15 min at 4,000 rpm, supernatant removed and stored at –80°C. The supernatant fraction (containing the soluble fusion protein) was extracted from the frozen *E. coli* cell pellet. Briefly, the cell pellet was resuspended in lysis buffer and whole cells were lysed using a pressure cell homogenizer (Stansted Fluid Power). The resulting suspension was centrifuged at 4°C for 30 min at 15,000 rpm. The supernatant was treated with 50% Glutathione Sepharose 4 beads (GE Healthcare) using the manufacturer's protocol for batch purification of GST-tagged proteins. The GST tag of the protein bound to the resin was cleaved by a PreScission Protease (GE Healthcare) reaction for 16 h at 4°C. The recombinant protein was eluted using elution buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.5). The eluted fraction was then filtered using a 0.22-µm filter unit (Corning) and loaded onto a HiPrep 26/10 desalting column (GE Healthcare) for buffer exchange. After the desalting process, the collected fraction was refiltered and loaded onto a Resource-S column (GE Healthcare) equilibrated with 25 mM MES, pH 6.5. A NaCl gradient was used for the elution. The purified rPA-D1 protein was stored in aliquots at –80°C. Fractions at all stages of the expression and purification process were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 12%) and Western blotting. For the Western blot analyses of the rPA-D1 protein, GST Mouse Monoclonal IgG (Santa Cruz) was used as a primary antibody and anti-mouse IgG-HRP (GE Healthcare UK Limited) was used as a secondary antibody. The rPA-D1 was further treated with PAb anti-*B. anthracis* protective antigen (Norvus) and anti-

Rabbit IgG (GE Healthcare UK Limited). All steps of the purification process were performed at 4°C.

Limulus amebocyte lysate assay

The purified rPA-D1 protein was checked for residual lipopolysaccharide (LPS) content using the Limulus amebocyte lysate (LAL) chromogenic assay (Endospecy ES-50M Kit; Seikagaku) according to the manufacturer's instructions.

rPA-D1 ELISA

An indirect ELISA format was used. Titration tests were performed to determine the optimal concentrations of the reagents. Briefly, 96-well flat-bottomed microtiter plates (Corning) were coated with 100 µl per well of diluted rPA-D1 at a concentration of 100 ng/ml in (PBS, pH 7.4) overnight at 4°C. The coated plates were manually washed thrice (300 µl per well) with a wash buffer (PBS containing 0.05% Tween-20) and were blotted dry on paper towels. The wells were then blocked with 100 µl of blocking buffer (5% skim milk in PBS containing 0.05% Tween-20, pH 7.4). After washing, the serum samples were diluted to 1:100 in blocking buffer and then 100 µl of the diluted solution was added to wells in triplicate and incubated for 1 h at room temperature (RT). After another washing step, the Rabbit anti-Bovine IgG-HRP (Invitrogen) was diluted to 1:12,000 in blocking buffer, and 100 µl of the diluted antibody solution was added to each well and incubated for 1 h at RT. After the final washing step, the bound secondary antibody was detected by adding 100 µl of TMB substrate (KPL) per well. Color development was observed after 20 min of incubation at RT. Color development was stopped by the addition of 50 µl of 1 N sulfuric acid per well. Absorbance values were read using a microtiter plate reader (Thermoscientific)

at a wavelength of 450 nm (OD₄₅₀). OD₄₅₀ values of the test sera were expressed in ELISA units (EUa). Each plate contained the negative control (NC) and positive control (PC) sera. The mean OD₄₅₀ of the three blank wells was subtracted from each OD₄₅₀ value of the test serum sample and the result expressed as a proportion of the PC.

Cut-off value determination

The rPA-D1 ELISA cut-off value at OD₄₅₀ was determined as follows: the mean of the NC group ($n = 53$) + 3 standard deviations (mean + 3 SD). Samples with OD₄₅₀ values greater than the cut-off were considered positive. Coefficients of variation (CVs) within an assay plate and between different assay plates were used to assess the reproducibility of the rPA-D1 ELISA. To evaluate the intra-assay variation, replicates of the control sera were run within a plate; to evaluate the interassay variation samples were run on different occasions. The CV was calculated using the following formula: $CV = SD/mean \times 100\%$.

Cattle serum samples

A total of 80 cattle serum samples were used in this study. NC group ($n = 55$): The NC group included a total of 29 serum samples that were obtained from Lusaka from cattle with no known history of anthrax vaccination and 26 serum samples that were obtained from unvaccinated calves in herds with a history of anthrax vaccination in Lusaka. Of the 55 samples, 53 were used to determine the ELISA cut-off value. PC group ($n = 25$): The PC group included serum samples ($n = 24$) that were obtained from cattle one month after the anthrax vaccination and one pooled serum sample that was obtained from cattle with naturally acquired anthrax infection.

Results

Antigen preparation

PA-D1, one of the four domains of PA, is crucial in mediating the anthrax toxin entry into the host cell. To prepare rPA-D1 (Fig 2A) as an ELISA antigen, rPA-D1 was expressed as a GST fusion protein in *E. coli* and was clarified in the supernatant fraction of the cell lysate. Before and after the GST tag cleavage, a GST-rPA-D1 band of approximately 54 kDa and rPA-D1 of 28 kDa were detected (Fig 2B). Following the affinity purification process, rPA-D1 was subjected to further purification via ion exchange chromatography. Coomassie brilliant blue staining and Western blot analysis were used to confirm the purity of the collected protein fractions (Fig 2C and 2D). From 1 L of culture, approximately 0.8 mg of rPA-D1 was obtained with a purity of more than 90%. The LPS content of the rPA-D1 protein used in the ELISA was determined as 0.02 endotoxin units (EUB) /ml using the LAL assay.

Evaluation of antigen stability

To determine rPA-D1 stability and use in the absence of cold chain facilities, I compared the use of rPA-D1-precoated ELISA plates with lyophilized rPA-D1 stored at RT. After 3 weeks of storage, I found that the use of freshly prepared plates using lyophilized rPA-D1 was superior to that of the rPA-D1-precoated plates (Fig 3A and 3B)

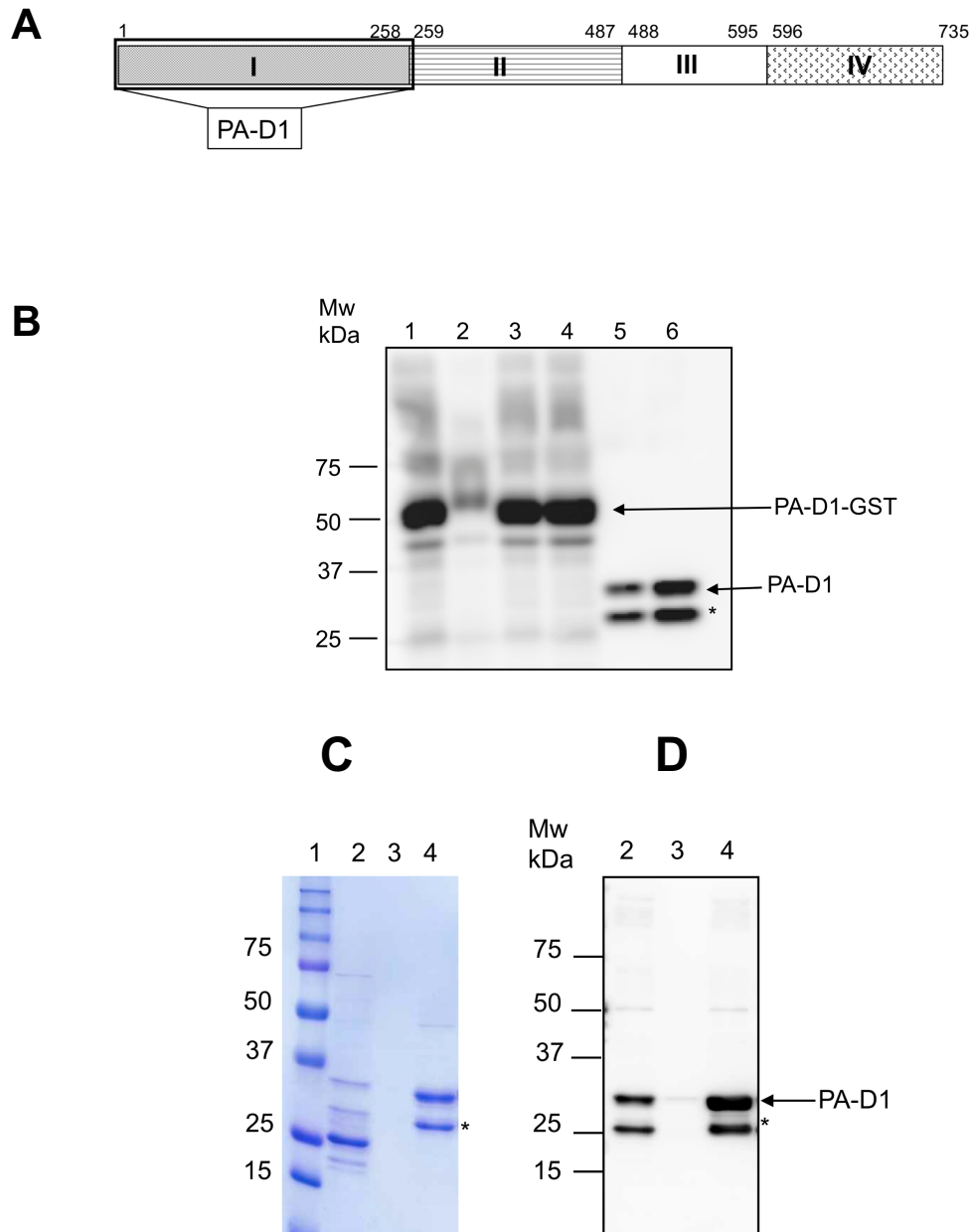


Figure 2. Production and purification of rPA-D1.

(A) Cartoon of full-length PA revealing the PA-D1 gene used in antigen preparation. (B) Western blot analysis of the affinity purification and desalting process: lane 1, total cell lysate; lane 2, pellet fraction; lane 3, supernatant fraction (PA-D1-GST: 54 kDa); lane 4, beads bound; lane 5, elute after treatment with PreScission Protease (PA-D1: 28 kDa); lane 6, desalting fraction. (C and D) Coomassie brilliant blue staining and Western blot analysis of the fractions collected during the cation exchange process. Lane 1, molecular weight marker; lane 2, sample loaded onto the cation exchange chromatography column; lane 3, flow through; lane 4, Resource-S fraction. Mw; molecular weight marker (in kDa), *: truncated product.

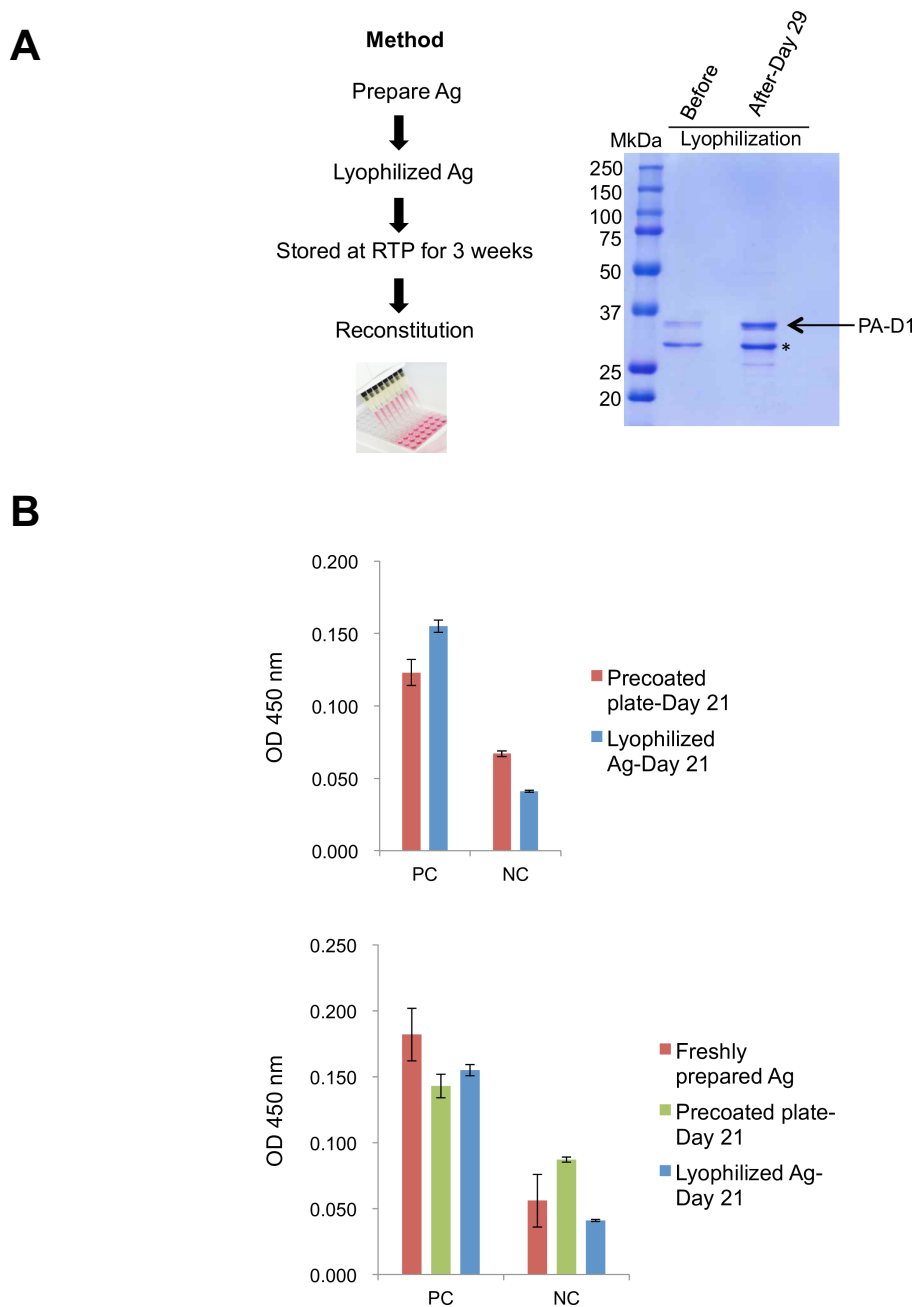


Figure 3. Comparison of rPA-D1 precoated and freshly prepared ELISA plates.

(A) Strategy for the evaluation of antigen (Ag) integrity after the lyophilization and storage process. Coomassie brilliant blue staining of lyophilized antigen. (B) Mean OD₄₅₀ of serum ($n = 2$, in triplicates) showing a comparison of the use of a freshly prepared ELISA plate using rPA-D1 stored at -80°C to that of a rPA-D1 precoated ELISA plate stored at RT and freshly prepared ELISA plate using lyophilized rPA-D1 stored at RT. Each PC and NC sample was measured in triplicate. Error bars represent the standard deviation of the mean. *: truncated product. RT: Room temperature, PC: Positive control, NC: Negative control cattle serum.

Development of rPA-D1 ELISA

To determine the optimal ELISA conditions and concentrations of reagents, checkerboard titrations were performed. An antigen concentration of 100 ng/ml, a serum dilution of 1:100 and a secondary antibody dilution of 1:12,000 were determined as the optimal titration conditions (Fig 4A). Moreover, I compared the sera from herds in the NC group (unvaccinated cattle with no history of vaccination versus unvaccinated calves from a herd with a history of vaccination) used to determine the cut-off value and found no significant difference between the groups (Mann–Whitney U test, $p > 0.05$). The rPA-D1 ELISA cut-off value was determined as the mean of the NC group + 3 SD. The OD₄₅₀ values of the NC group ($n = 53$) ranged from 0.03 to 0.68. The mean of the NC group was 0.249 and the SD was 0.143; and the cut-off value was thus determined to be 0.678 (Fig 4B). To validate the repeatability of the rPA-D1 bovine ELISA, I calculated the intra- and interassay CVs of the NC and PC serum samples. The intra-assay CV of PC was 5.5% ($n = 10$ replicates, 0.3%–13.2%) and that of NC was 4.5% ($n = 9$ replicates, 0.3%–12.7%). The interassay CV of PC was 4.13% ($n = 11$ replicates, 0.58%–10.4%) and that of NC was 8.1% ($n = 10$ replicates, 4%–16.4%). The CVs were within the reference ranges of $\leq 10\%$ and $\leq 15\%$, respectively.

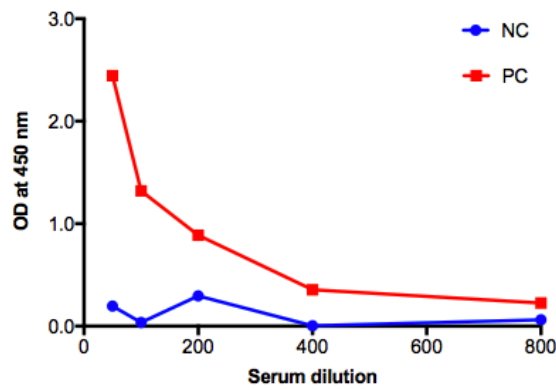
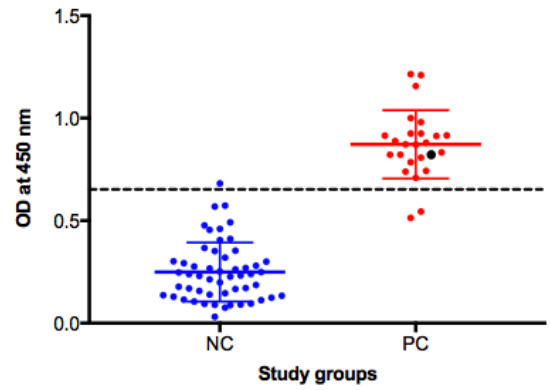
A**B**

Figure 4. Optimization of rPA-D1 ELISA.

(A) By using the checkerboard titrations, I determined the optimal concentrations of the antigen, the antibody and serum dilutions as follows: antigen, 10 ng/well; serum dilution, 1:100; second antibody dilution, 1:12,000. (B) Analysis of control serum samples using rPA-D1 ELISA under optimal conditions. NC: negative control group, ($n = 53$). PC: positive control group ($n = 25$). Red dot: pooled sample from cattle with exposure to natural infection. Error bars represent the mean and standard deviation. Horizontal dotted line: cut-off value of 0.67.

Comparison of in-house ELISA with a Commercial ELISA

I compared the performance of the in-house rPA-D1 ELISA and a commercial ELISA (bovine anthrax protective antigen-antibody APA-IgG ELISA kit) using 9 randomly selected negative serum samples. These samples were tested in the rPA-D1 ELISA and also evaluated using the APA-IgG ELISA. The Bland-Altman bias plot (Fig 5) including computation of bias and 95% limits of agreement was used to analyze data obtained from both assays. The mean bias $\pm$ 1.96 standard deviations were between 0.310 and -0.185, respectively.

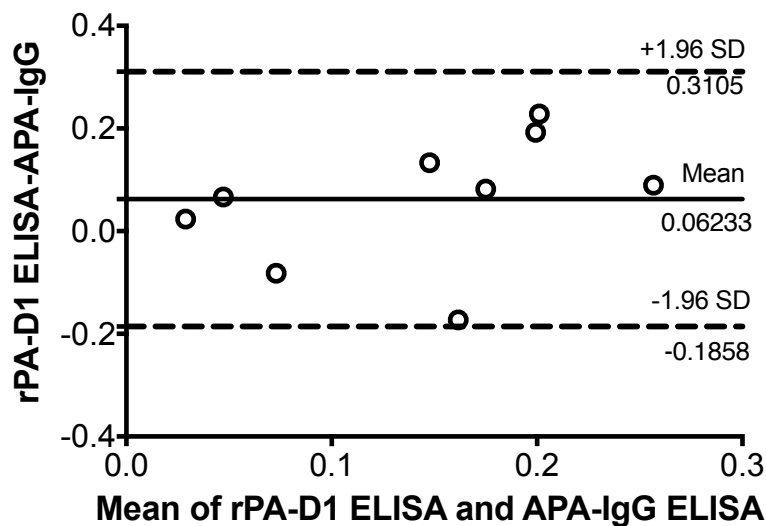


Figure 5. Blant-Altman plot comparing rPA-D1 ELISA and Commercial ELISA.

Measurement of the presence of anti-PA antibodies. The mean OD₄₅₀ values of anti-PA antibodies from both assays plotted against the difference of rPA-D1 ELISA and APA-IgG ELISA. Bias between the assays is indicated by the horizontal solid line. Dashed lines represent the 95% confidence limits above and below the mean. SD is the standard deviation.

Discussion

Serological assays for anthrax epidemiological investigations are currently unavailable in Zambia. In this study, I developed an indirect qualitative bovine ELISA using rPA-D1 of *B. anthracis* as an antigen.

In accordance with the previous observations, rPA-D1 was prone to degradation during the purification process; however, that had no effect on the ELISA setup and optimization (Flick-Smith et al. 2002). The residual content of LPS in the rPA-D1 protein used for ELISA plate coating was less than 0.05 EUb/ml according to the LAL assay. Although a low background noise due to the rPA-D1 protein and other bacterial contaminants during the assay optimization and sample analyses was observed, there is a possibility that this LPS content may have influenced some of the results.

The rPA-D1 ELISA revealed high levels of reproducibility with CVs of less than 10% and 15%, respectively. The ELISA was able to detect seroconversion in cattle 1-month postvaccination. Some serum samples in the NC group had high OD₄₅₀ values. This may be attributed to passive immunity, since some serum samples were collected from the unvaccinated calves in a herd that was previously exposed to anthrax vaccination. To determine whether the sera from this group were increasing the mean OD₄₅₀ readings of the NC group, I compared the two groups (unvaccinated cattle with no history of vaccination versus unvaccinated calves from a herd with a history of vaccination) used to determine the cut-off value; however, I found no significant difference between the groups ($p > 0.05$). The group containing sera from the unvaccinated calves did not increase the mean OD₄₅₀ of the NC group. Conversely, of the 25 positive samples, 2 had OD₄₅₀ values below the cut-off value. This finding

occurred because these cattle may have developed insufficient immunity after anthrax vaccination. These results further substantiate the previous findings from which it was concluded that there is a variation in seroconversion in the individual animals and that time is essential to develop adequate immunity after vaccination; for example, following an anthrax outbreak in cattle in Australia, 132 cases of anthrax occurred 10 days after vaccination and 3 cases occurred 25–30 days after vaccination (Van Ness 1971; Turner et al. 1999a). Since a single cut-off value based on the NC group sera was assigned to the rPA-D1 ELISA, there may be some degree of false positive and negative results, in particular considering the history of the control group. In future works, the use of control sera obtained from herds with no history of exposure to vaccination and anthrax should be used to estimate the sensitivity and specificity of this assay.

Although the performance characteristics of most ELISAs used in the serological studies of anthrax in livestock and wildlife have not been reported, I noted a few variations between these assays and the rPA-D1 ELISA, including differences in antigen preparation, antigen purity and the ELISA plate preparation process. For example, Turnbull (Turnbull et al. 2004) used 20 µg/ml of whole PA purified from cell-free filtrates of the Sterne strain of *B. anthracis* and used precoated ELISA plates. Conversely, I used 100 ng/ml of rPA-D1 and found that freshly prepared plates using lyophilized rPA-D1 stored at RT were superior to the precoated plates stored at RT. In addition, rPA-D1 ELISA eliminates the risk of having nonreactive wells, likely due to the denaturation of precoated capture antibody antigen complexes by using freshly prepared ELISA plates. Moreover, the use of a lyophilized antigen is ideal because it is difficult to maintain cold chain facilities in Zambia. The rPA-D1 antigen is safe and can be readily prepared. I also compared the performance of the in-house rPA-D1

ELISA with a commercial ELISA. Results indicated that the 2 assays are highly comparable. Therefore, rPA-D1 ELISA can be used for determination anti-anthrax antibodies in bovine serum samples. In conclusion, the rPA-D1 ELISA could be used for detection of anti-PA antibodies in bovine serum after *B. anthracis* exposure or vaccination.

CHAPTER II:

Application of a *Bacillus anthracis* protective antigen domain-1 in-house ELISA: Herd level detection of anti-protective antigen antibodies in cattle in Western Zambia

Summary

In a cross-sectional study conducted in the Western Province of Zambia, 187 serum samples from 8 herds of cattle were screened for anti-PA antibodies using the rPA-D1 ELISA. The seropositive rate of the serum samples was 8%, and the mean anti-PA antibody was 0.358 ELISA units. Additionally, I screened 131 cattle serum samples from Lusaka, which is a nonendemic area, and found no significant association between the antibody levels and sampling area (endemic versus nonendemic area). Conversely, significant differences were observed between the anti-PA antibody levels and herds, anti-PA antibody levels and vaccination status and anti-PA antibody levels and vaccination timing. Collectively, these findings suggest that the rPA-D1 ELISA is a useful tool for the detection of anti-PA antibodies in cattle in Zambia.

Introduction

Anthrax continues to pose a serious public health and socioeconomic threat in several developing Asian and African countries. Occasionally, sporadic cases occur in the nonendemic areas and developed countries (Mongoh et al. 2008; Lewerin et al. 2010).

In Zambia, frequent anthrax outbreaks occur in cattle in Western Province and occasionally in wildlife in the game management areas (Turnbull et al. 1991; Tuchili et al. 1993; Hang'ombe et al. 2012). These outbreaks cause significant economic losses because a majority of the population is dependent on livestock for their livelihood and wildlife is a source of revenue for the government. In 2016, five anthrax outbreaks occurred in cattle in Western Province, Zambia and one anthrax outbreak occurred in wildlife in the Chama District, Zambia (Mwambi et al. 2017). Over 60 cases in cattle and 18 cases in wildlife were reported. Furthermore, more than 100 suspected human cases were caused by exposure from the handling and consumption of the contaminated meat of infected cattle and wildlife (Kasese-Chanda et al. 2017; Mwambi et al. 2017).

Previous studies have demonstrated that the ecology of *B. anthracis* is influenced by the climatic and environmental factors (Hugh-Jones and de Vos 2002). In Zambia, the recurrence of a livestock anthrax outbreak has been closely linked to seasonal variations and human dependency on the Barotse Floodplain (Siamudaala et al. 2006a; Munang'andu et al. 2012b). Despite the available information on the epidemiology of anthrax in Zambia, it remains difficult to evaluate the susceptibility of cattle and vaccination coverage due to under-reporting and the remoteness of the affected areas. Furthermore, much of what is known about serology of anthrax in the

endemic areas elsewhere is largely based on the studies in wildlife (Turnbull et al. 1992; Lembo et al. 2011; Cizauskas et al. 2014), and the serology of anthrax in cattle has thus been a neglected area of anthrax surveillance. In this regard, the detection of antibodies against *B. anthracis* in cattle in endemic areas is important to understand the present seroepidemiologic situation and its contribution to anthrax outbreak recurrence.

In this study, to demonstrate the usefulness of the in-house rPA-D1 ELISA developed in Chapter I, serum samples collected from herds of cattle in anthrax endemic and nonendemic areas were analysed. To understand the actual anthrax management and control practices from the livestock farmer's perspective, I collected information on the history of anthrax outbreaks and anthrax management practices at herd level.

Materials and methods

Study area

Blood samples were collected from the Kaunga Lueti and Luampa veterinary camps which cover two herds in the Nalolo District ($n = 42$, $16^{\circ} 01.417'S$, $22^{\circ} 55.976'E$ and $16^{\circ} 01.252'S$, $22^{\circ} 56.124'E$), and six herds in the Luampa District ($n = 145$, $15^{\circ} 18.354'S$, $24^{\circ} 11.331'E$, $15^{\circ} 15.961'S$, $24^{\circ} 18.281'E$ and $15^{\circ} 37.229'S$, $24^{\circ} 46.901'E$) of Western Province, Zambia (Fig 6 and 7). Farmers in these areas practice the transhumance type of livestock farming which is characterized by the seasonal movement of cattle and communities out of the floodplain to the upland areas. The plains are important because they provide grazing pasture for the cattle and influence the livelihood and culture of its inhabitants. The Western Province sampling sites were chosen based on the history of anthrax outbreaks obtained from the district veterinarians. In this study, Western Province is referred to as an endemic area because anthrax outbreaks are reported almost annually here. Samples were collected in Lusaka Province ($n = 131$, $15^{\circ}25'S$, $29^{\circ}0'E$). The nonendemic area, Lusaka, refers to an area with no anthrax outbreak reports for more than 20 years. An anthrax case was suspected when there was a sudden death and the epidemiological history indicated reports of previous outbreaks in that location. In the cases in which the carcasses were opened, extravasation of blood from orifices and an enlarged spleen were observed.

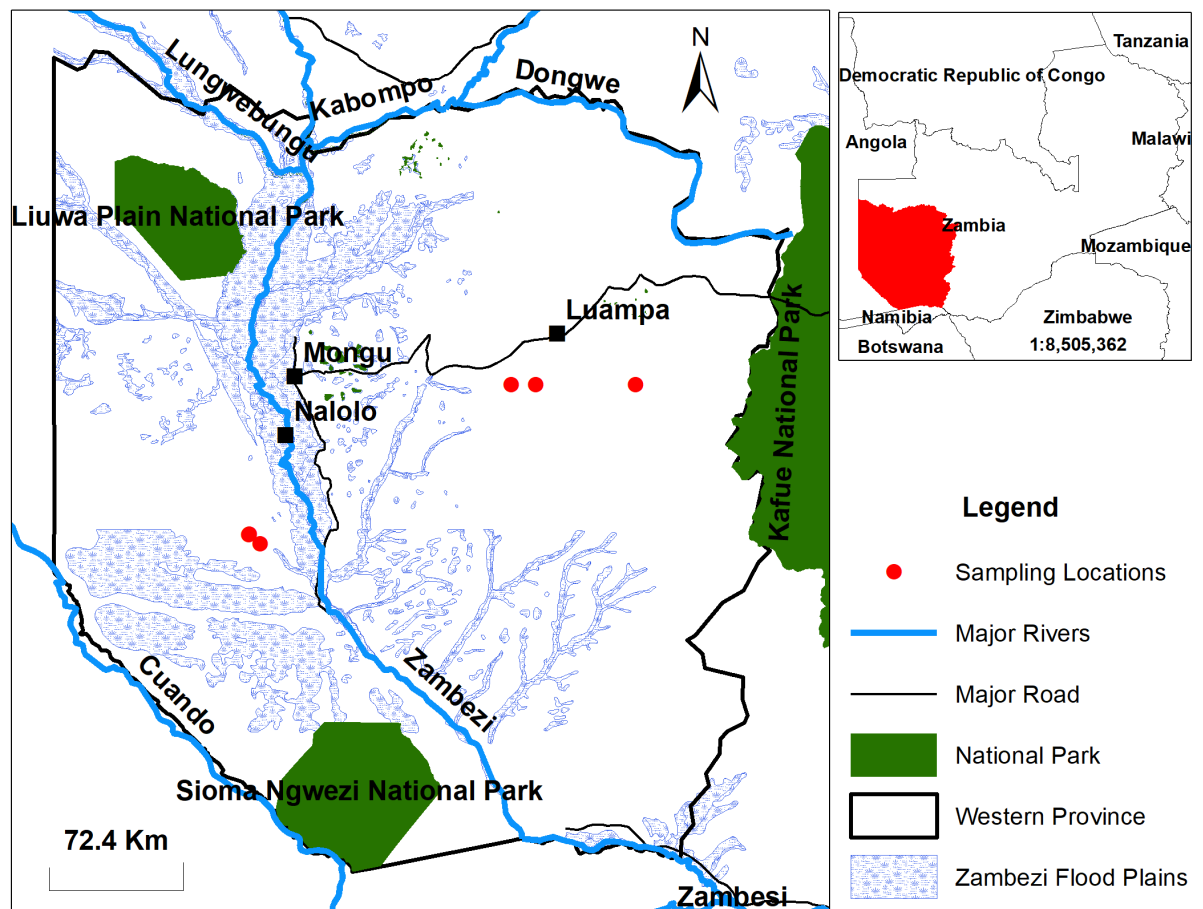


Figure 6. Sampling areas in Western Province, Zambia.

The blood samples used in this study were collected from eight herds of cattle in the Nalolo District and Luampa Districts.

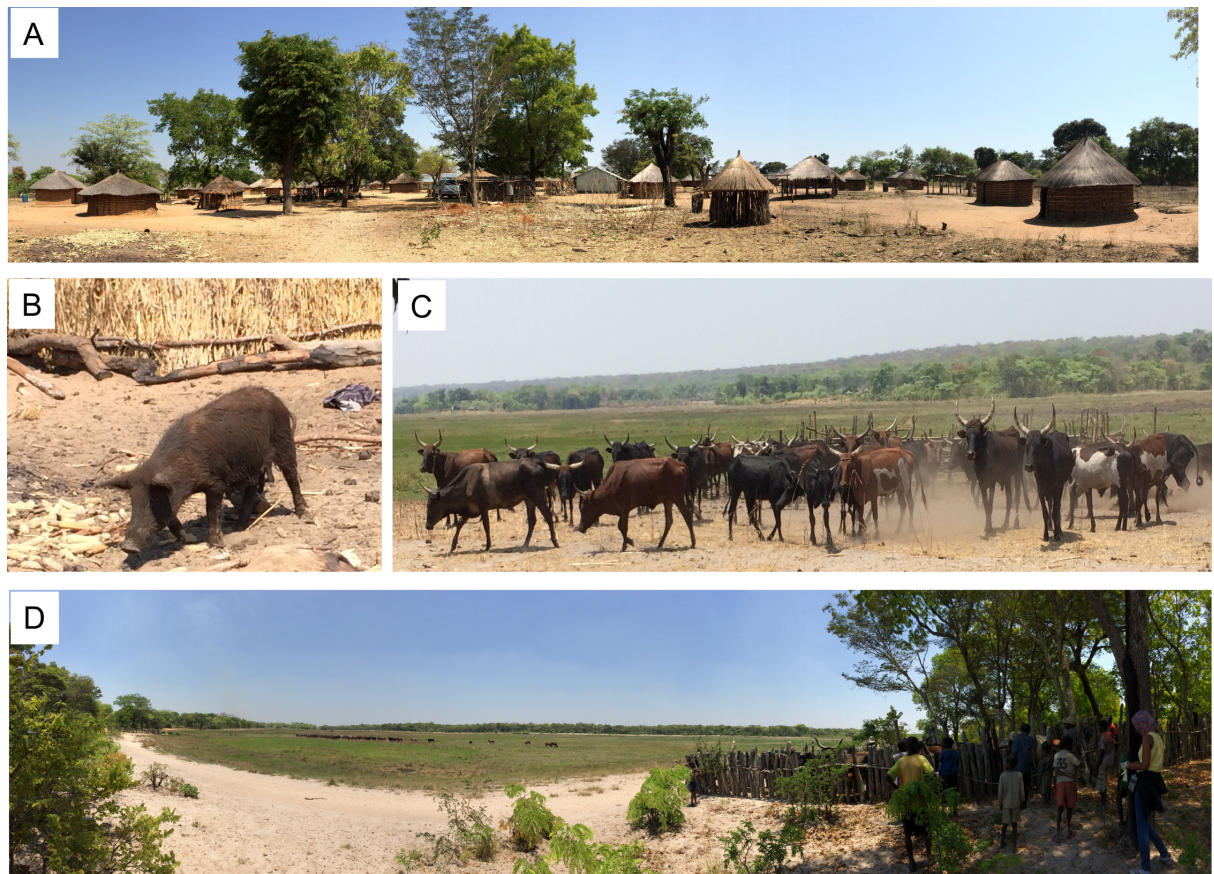


Figure 7. Cattle rearing at herd level in Luampa, Western Province, Zambia.

(A) Household. **(B)** Pig scavenging around the household. **(C)** Herd of Barotse cattle. **(D)** Left: Cattle grazing, Right: Kraal.

Cattle test sera

Approximately 5 ml of blood was collected in sterile plain blood collection tubes and stored at 4°C until serum sample preparation. The serum samples were prepared by allowing the collected blood samples to stand at RT for 1 h. The samples were then centrifuged at 25°C for 15 min at 3,000 rpm. The serum samples were aliquoted into clean microcentrifuge tubes and were stored at –20°C. The samples were then analyzed at the Hokudai Center for Zoonosis Control, School of Veterinary Medicine in Zambia.

Endemic area ($n = 187$): The endemic area group included serum samples that were obtained from eight herds of cattle in Western Province. Nonendemic area ($n = 131$): The nonendemic area included serum samples that were obtained from cattle in the Lusaka Province.

Statistical analysis

The symptom-based prevalence was calculated by dividing the number of suspected anthrax cases in a herd by the number of cattle in that herd. The mortality rate was calculated by dividing the number of deaths in a herd by the number of cattle in that herd. For the statistical analysis, I initially tested whether the data followed a normal distribution using the Shapiro–Wilk test. Comparisons of the means of anti-PA antibodies of the herds were performed using the Kruskal–Wallis test. To test the significance of the association between anti-PA antibodies and various states (vaccination timing and epidemic status), the Wilcoxon rank sum test was performed. Graphs were generated using computer graphics software (GraphPad Prism, version 7.0b; GraphPad, San Diego, CA) and R software version 3.3.3 (R Development Core Team 2013). A p value <0.05 was considered as statistically significant.

Ethical statement

Ethical clearance was obtained from the ERES Converge Review Board, Lusaka (Ref. No. 2017-Apr-006). Permission to conduct sampling was obtained from the Ministry of Fisheries and Livestock (MFL), Zambia. Before collecting the blood samples from various cattle herds, meetings were held with the farmers and community leaders to explain the purpose of the study, to obtain a brief history of anthrax vaccination and to obtain informed verbal consent. Blood samples were collected in accordance with the regulations for animal husbandry in Zambia. The ERES Converge Review Board, Lusaka, approved both the data collection from cattle as and the data collection through interviews/discussions with farmers and community leaders. No formal documentation was required when obtaining oral consent. Obtaining written consent was not a requirement as per the Ethics Committee that approved this study.

Results

Herd history

Information on the history of the herds was obtained through meetings with district veterinarians, livestock farmers and community leaders (Fig 8). I found that between November 2013 and January 2014, there was an anthrax outbreak that affected herds 1–8 in the Nalolo District and Luampa Districts (Table 1). In response to these outbreaks, the district veterinarians performed vaccinations, burying and decontamination with lime. In some villages, the carcasses were consumed. At the time of sample collection in October 2016, the cattle had not been revaccinated since the 2013–2014 anthrax outbreak. I calculated the symptom-based prevalence and mortality rates in eight herds of cattle based on the number of suspected cases and mortalities recorded during the outbreak. The symptom-based prevalence among herds ranged from 0% to 36.4% with an average of 15.3%. The average herd symptom-based prevalences in the Nalolo District and Luampa Districts were 21.7% and 13.9%, respectively, and the mortality rates in the two districts were 15.2% and 5.1%, respectively. The average herd mortality rate was 6.9% (Table 1).

Fascioliasis and Black quarter are the common illnesses that usually affect cattle. Livestock owners generally administer broad spectrum antibiotics (oxytetracycline and penicillin) to treat any sick animals in the herds.



Figure 8. Collection of herd history.

(A) Meeting with community members to explain the purpose study. **(B)** Obtaining informed verbal consent and information on herd history.

Table 1. Herd history.

Herd ID	Camp	Crush pen	Village	Herd size	2013–2014 anthrax outbreak				
					Date of outbreak ^a	Vaccination status prior to outbreak	Symptom-based prevalence (%)	Mortality (%)	Control measures taken
1	Luampa	Mbundu	Muzangabantu	13	Nov-2013	Not vaccinated	0 (0.0)	0 (0.0)	Vaccination
2	Luampa	Mbundu	Muzangabantu	80	Nov-2013	Not vaccinated	20 (25.0)	4 (5.0)	Vaccination
3	Luampa	Mbundu	Muzangabantu	37	Nov-2013	Not vaccinated	0 (0.0)	0 (0.0)	Vaccination
4	Luampa	Pulobende	Mwembwe	32	Nov-2013	Not vaccinated	4 (12.5)	2 (6.3)	Burying and vaccination
5	Luampa	Nakayembe	Sikabilima	37	Nov-2013	Not vaccinated	4 (10.8)	2 (5.4)	Burying and vaccination
6	Luampa	Pulobende	Mwembwe	17	Nov-2013	Not vaccinated	2 (11.7)	3 (17.6)	Vaccination
7	Kaunga lueti	Malombe	Kaonga	35	Jan-2014	Not vaccinated	6 (17.1)	5 (14.2)	Burying
8	Kaunga lueti	Malombe	Kaonga	11	Jan-2014	Not vaccinated	4 (36.4)	2 (18.1)	Burying and vaccination

^aDates; month-year.

Serology

A total of 318 serum samples were screened for anti-PA antibodies using rPA-D1 ELISA. Of the 187 samples from the endemic area, only 8% (95% confidence interval (CI): 4.1–11.9%) were positive (Table 2). Herd 8 revealed majority of positive samples (22.2%), whereas no seropositive samples were detected in Herd 3 and 5. The seropositivity of the samples from the nonendemic area was 18.3% (95% CI: 11.7–24.9). Seropositivity ranged from 0% to 22.2% in the endemic area and it ranged from 5.7% to 42.8% in the nonendemic area. The mean OD₄₅₀ of the serum samples from the endemic and nonendemic area were 0.358 (95% CI: 0.328–0.388) and 0.452 (95% CI: 0.406–0.499), respectively (Table 2).

Table 2. Proportion of seropositive cattle in the herds sampled.

Sampling area	Herd ID	When last known anthrax case was detected	When last vaccinated before sampling ^a	Date of sample collection ^a	Mean (SD) anti-PA Ab (EUa)	No. of samples tested	No. positive	% positive (95% CI)
Western Province	1	No cases	Dec-2013	Oct-2016	0.428 (0.292)	11	2	18.2 (−4.6, 41.0)
	2	Nov-2013	Dec-2013	Oct-2016	0.277 (0.181)	39	1	2.6 (−2.4, 7.5)
	3	No cases	Dec-2013	Oct-2016	0.270 (0.138)	32	0	0 (0.0, 0.0)
	4	Nov-2013	Jan-2014	Oct-2016	0.454 (0.201)	22	3	13.6 (−0.7, 28.0)
	5	Nov-2013	Jan-2014	Oct-2016	0.319 (0.150)	25	0	0 (0.0, 0.0)
	6	Nov-2013	Jan-2014	Oct-2016	0.396 (0.212)	16	2	12.5 (−3.7, 28.7)
	7	Jan-2014	May-2015	Oct-2016	0.427 (0.217)	33	5	15.2 (2.9, 27.4)
	8	Jan-2014	May-2015	Oct-2016	0.495 (0.259)	9	2	22.2 (−4.9, 49.4)
Total					0.358 (0.207)	187	15	8 (4.1, 11.9)
Lusaka Province	9	No cases	NA	Oct-2016	0.744 (0.268)	21	9	42.8 (21.7, 64.0)
	10	No cases	Nov-2015	Apr-2017	0.430 (0.196)	28	4	14.2 (1.3, 27.3)
	11	No cases	Oct-2014	Oct-2016	0.254 (0.173)	35	2	5.7 (−2.0, 13.4)
	12	No cases	Nov-2015	Sep-2016	0.636 (0.209)	23	7	30.4 (11.6, 49.2)
	13	No cases	Oct-2014	Oct-2016	0.335 (0.167)	24	2	8.3 (−2.7, 19.4)
Total					0.452 (0.268)	131	24	18.3 (11.7, 24.9)

^aDates; month-year.

NA; not available.

The Kruskal-Wallis test was used to compare the means of anti-PA antibodies in different herds and found a significant difference in the means of anti-PA antibodies of herds ($p < 0.05$) Table 3. Since most of the herds in this study were previously exposed to vaccination, I compared the distribution and residual levels of anti-PA antibodies according to the epidemic status. I found no significant difference between the anti-PA antibodies of cattle in the endemic area and the nonendemic area ($p > 0.05$), specifically between herd 7 vs herd 10, herd 8 vs herd 10, herd 2 vs herd 11 and herd 6 vs herd 13. The overall residual level of anti-PA antibodies in herds in the endemic area was comparable to that in herds in the nonendemic area (Fig 9A). I then analyzed the association between the antibodies and vaccination timing using the Wilcoxon rank sum test. I found significant differences between the unvaccinated and vaccinated herds. A significant difference was observed between the anti-PA antibodies and vaccination timing, specifically 1 month ago displayed significantly higher anti-PA antibodies compared to >1 year ago, 1 year ago displayed significantly higher anti-PA antibodies compared to 2 years ago ($p < 0.05$). One month and >1 year ago displayed significantly higher anti-PA antibodies compared to the unvaccinated group ($p < 0.05$). Conversely, >2 years ago was not significantly different from the unvaccinated group ($p > 0.05$; Fig 9B).

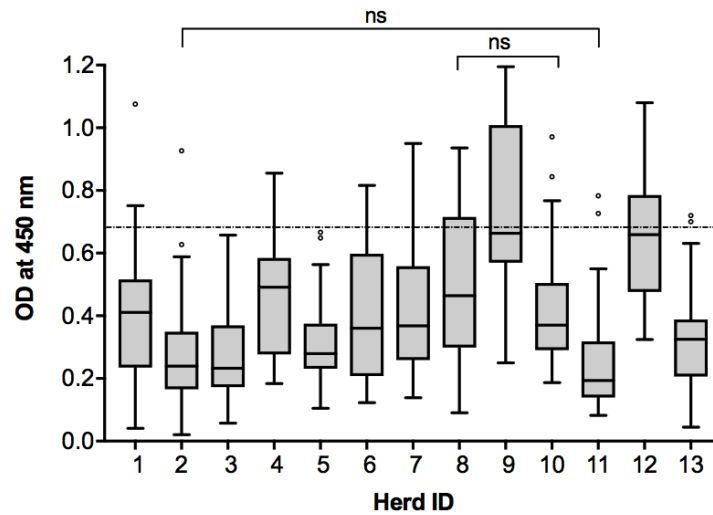
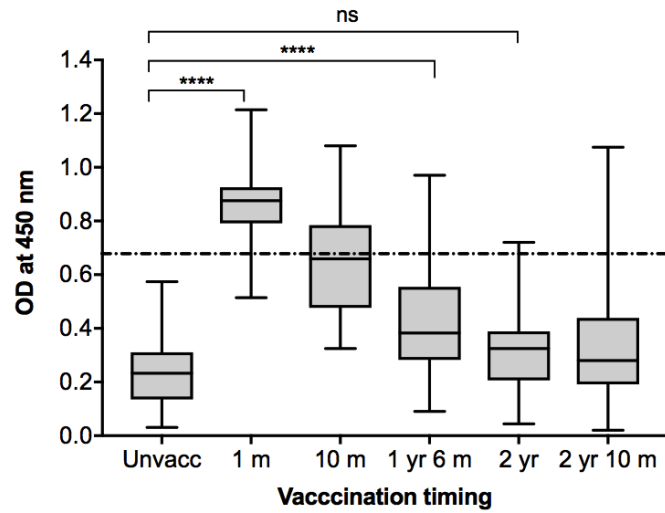
A**B**

Figure 9. Association between the anti-PA antibodies and state.

Box plots for the distribution of anti-PA antibodies. **(A)** Herds 1–8 in the endemic area and 9–13 in the nonendemic area. **(B)** Vaccination timing. The boxes indicate the interquartile range, the horizontal lines indicate the median, and the lower and upper hinges indicate the minimum and maximum values, respectively (outliers not included). Horizontal dotted line: cut-off value of 0.67. yr: year, m: month. ns: not significant, ****: $p < 0.0001$.

Table 3. Multiple comparison of herds.

Herd ID		1	2	3	4	5	6	7	8	9	10	11	12	13
2	Mean rank diff.	60.8												
	Sig Diff.	ns												
3	Mean rank diff.	60.4	-0.414											
	Sig Diff.	ns	ns											
4	Mean rank diff.	-20.2	-81	-80.6										
	Sig Diff.	ns	ns	ns										
5	Mean rank diff.	35	-25.9	-25.4	55.2									
	Sig Diff.	ns	ns	ns	ns									
6	Mean rank diff.	6.81	-54	-53.6	27	-28.1								
	Sig Diff.	ns	ns	ns	ns	ns								
7	Mean rank diff.	-6.94	-67.8	-67.3	13.3	-41.9	-13.8							
	Sig Diff.	ns	ns	ns	ns	ns	ns							
8	Mean rank diff.	-29.2	-90	-89.6	-8.99	-64.2	-36	-22.3						
	Sig Diff.	ns	ns	ns	ns	ns	ns	ns						
9	Mean rank diff.	-95.1	-156	-156	-74.9	-130	-102	-88.2	-65.9					
	Sig Diff.	ns	****	****	ns	***	ns	*	ns					
10	Mean rank diff.	-14.5	-75.3	-74.9	5.73	-49.5	-21.3	-7.56	14.7	80.6				
	Sig Diff.	ns	ns	ns	ns	ns	ns	ns	ns	ns				
11	Mean rank diff.	76	15.2	15.6	96.3	41.1	69.2	83	105	171	90.5			
	Sig Diff.	ns	ns	ns	**	ns	ns	*	ns	****	**			
12	Mean rank diff.	-82	-143	-142	-61.7	-117	-88.8	-75	-52.7	13.1	-67.5	-158		
	Sig Diff.	ns	****	****	ns	***	ns	ns	ns	ns	ns	****		
13	Mean rank diff.	25.6	-35.2	-34.8	45.9	-9.34	18.8	32.6	54.8	121	40.1	-50.4	108	
	Sig Diff.	ns	ns	ns	ns	ns	ns	ns	ns	***	ns	ns	**	

Dunn's multiple comparison test results for Kruskal-Wallis analysis of variance on ranks. Asterisks indicate significant differences, ns not significant. ****: $p < 0.0001$, ***: $p < 0.0002$, **: $p < 0.0021$, *: $p < 0.0332$

Discussion

Findings regarding herd history corroborate with the results of epidemiological studies conducted in Western Province, Zambia (Munang'andu et al. 2012b; Sitali et al. 2017). Previously, people usually resorted to culling very sick cattle in the herd and consuming the meat. Additionally, the meat from infected carcasses or animals that died of unknown causes was consumed due to the assumed low risk of acquiring anthrax infection among the people in rural communities. Notably, Fascioliasis and Clostridial infections were still among the most common conditions that affected the animal health (Munang'andu et al. 1996), and farmers reported that they administered broad spectrum antibiotics to treat sick animals. In this study, the average herd mortality rate was 7%, with a range from 0% to 18%. A similar herd mortality rate was reported during an anthrax outbreak in the Sesheke District (Munang'andu et al. 2012a), suggesting that the impact of anthrax does not necessarily depend on the proximity to parts of the Barotse Floodplain along Zambezi River, which are considered to be the stable natural anthrax foci.

Results regarding anthrax outbreak management and control during the November 2013 to January 2014 anthrax outbreak are consistent with measures taken in past outbreaks both in Zambia and elsewhere (Turnbull et al. 1999; Mongoh et al. 2008). Herds 1–6 experienced their first anthrax outbreak in November 2013. Prior to this outbreak, these cattle herds had no history of anthrax vaccination. Furthermore, during blood sampling in October 2016, I found that the cattle had not been revaccinated since the 2013 outbreak. These findings suggest that there was a spread of infection from active anthrax foci. This is supported by the serological data (Table 2)

and a previous study that reported that anthrax outbreaks were limited to the Barotse Floodplain (Munang'andu et al. 2012b).

To demonstrate the usefulness of the rPA-D1 ELISA, I screened the cattle sera collected from the anthrax endemic and nonendemic areas in Zambia. Although there was no ongoing outbreak in Western Province at the time of sampling in October 2016, sampling was performed in October because the livestock anthrax outbreaks usually peak during the dry months of September–November. I expected that cattle in the endemic area may be exposed and that I would detect a high proportion of seropositive individuals. In contrast, I found that the overall proportion of seropositive sera from cattle in Western Province was 8% (15/187). Since these herds were once exposed to anthrax vaccination, presumably I detected the residual antibodies from anthrax vaccinations. Collectively, with herd history, the results suggest that there is inadequate vaccination of cattle against anthrax in the study area.

Naturally acquired antibodies against anthrax have been reported in wildlife (Good et al. 2008). In serological studies of anthrax in wildlife, animals and humans, it was found that naturally acquired antibodies against anthrax were rarely found in herbivores, and that animals in the endemic areas have a high residual level of antibodies (Turnbull et al. 1992). Conversely, in this study, I found an insignificant difference between the anti-PA antibody levels of cattle in the endemic and nonendemic area. Overall, the residual level of anti-PA antibodies in cattle in the endemic area was similar to that in cattle in the nonendemic area. Although most of the herds revealed low seropositive rates, I was able to detect a significant difference between the vaccinated and unvaccinated cattle and vaccination timings; I also observed a decrease

of anti-PA antibodies over time (Fig 9B). These findings further validate the effectiveness of the rPA-D1 ELISA. Interestingly, I found no significant differences between cattle that were last vaccinated >2 years ago and the unvaccinated group. The detection of higher numbers of seropositive individuals in herds that were last vaccinated >2 years ago may indicate exposure to natural infection. I could not rule out the exposure of cattle to natural infection in this study; therefore, I equally found it difficult to make inferences concerning the exposure to natural infection because samples were collected at one-time point in the absence of an outbreak and the herds were once exposed to vaccination. During sampling, I did identify some herds with no history of vaccination; however, I was unable to collect samples from those herds because the livestock owners did not give consent. Further serological studies in Western Province in herds without a history of anthrax vaccination or anthrax outbreaks would provide more insights on anthrax exposure in Barotse cattle.

Anthrax outbreaks in the endemic systems have been linked to the ecological factors, environmental, or reproductive stress factors (Turner et al. 2013). My results provide evidence that, in addition to the aforementioned factors, the outbreak occurrence is mainly due to inadequate vaccination. Although protection conferred by a single dose of livestock anthrax vaccine has been reported to be sufficient for one year (Kaufmann et al. 1973), anthrax outbreaks have been reported in the vaccinated herds (Turner et al. 1999a). In this study, I detected a few seropositive sera from cattle in a herd in a nonendemic area 9 months after vaccination. The low seropositivity could be caused by factors such as batch quality in vaccine production and variations in the maintenance of cold chain facilities, although I cannot exclude the possibility that this finding is due to variability in the efficacy of anthrax vaccine (34F2 Sterne strain) in

the field. Hence, further work is necessary to evaluate the present immunization schedule and duration of immunity conferred by the anthrax vaccines used in Zambia.

This study provides evidence that due to inadequate vaccination, the low proportion of seropositive cattle may be a factor contributing to anthrax outbreak recurrence in Western Province, Zambia. The rPA-D1 ELISA will be a useful tool not only in surveillance but also in monitoring the effectiveness of anthrax vaccination programs in Zambia.

CHAPTER III:

Seroepidemiology and geographic distribution of anthrax among cattle in Western Province, Zambia

Summary

Cattle anthrax outbreaks have been reported in Western Province, Zambia since the 1970s. Although several efforts have been made to control these outbreaks, their persistent occurrence remains a major problem. To understand the current seroepidemiological situation, the seroprevalence of antibodies against *B. anthracis* PA in cattle sera obtained from Western Province was determined. Of the 1619 cattle serum samples analyzed, 24.9% (403/1619) were positive and seropositivity varied among districts. Mwandi District had the highest seropositive rate of 44.4% (35/79) and Senanga District had the lowest seropositive rate of 15.8% (24/154). This suggests that there is need to reinforce anthrax vaccination programs in Zambia. To understand the current epidemiological situation, I analyzed the geographical distributions of cattle anthrax in Zambia from 2000 to 2016. Results indicate a decrease in the annual number of reported cases and a change in geographical distribution of outbreaks. Although areas along the Barotse Floodplain had the highest number of outbreak recurrences, outbreaks have also been reported in areas that previously had no reports of anthrax, suggesting that cattle anthrax outbreaks are no longer limited to the floodplain.

Introduction

In Zambia, anthrax outbreaks usually occur in May–January and peak toward the end of the dry season (between October and November). The species that are frequently affected include cattle and goats. Cattle anthrax outbreaks invariably lead to the occurrence of the disease in humans (Siamudaala et al. 2006b). Lack of awareness, poor practices of animal husbandry, and inadequate vaccination of livestock are some factors contributing to the persistence of anthrax in endemic areas (Mongoh et al. 2008; Biswas et al. 2012). The occurrence of cattle anthrax has usually been reported in remote rural communities of the Western Province of Zambia, particularly those along the Barotse Floodplain (Fig 6). The Barotse Floodplain has an estimated area of >5,000 km² extending from Lukulu in the north to Senanga in the south (Timberlake et al. 1998). The onset and duration of inundation vary greatly, influencing various anthropogenic activities in the area as well as the time spent by cattle in the floodplain (Turpie et al. 1999). Flooding plays a significant role in agriculture as it is a source of grassland irrigation, which is essential for cattle in traditional farming sector. Farmers in this area practice the transhumance type of farming, in which cattle spend most of the time in the floodplains but moving out of the floodplains when flooding peaks.

Regular cattle anthrax outbreaks in the Western Province of Zambia have been reported since the 1970s (Bbalo 1996). An average of 232 and 135 cattle anthrax cases were annually reported between 1989 and 1995, and between 1999 and 2007, respectively (Munang'andu et al. 2012b). Throughout the years, the number of reported cattle anthrax cases has decreased; however, the frequency and persistence of such outbreaks are still not under control. Although livestock anthrax is the leading cause of

anthrax infections in humans in endemic areas of several developing countries (Turnbull 2008), the geographical distribution of cattle anthrax outbreaks remains unclear. In Zambia, the lack of this information for managing and controlling cattle anthrax outbreaks has resulted in the underestimation of the impact of anthrax on veterinary medicine, public health, politics, and economics.

In Zambia, anthrax is a management and notifiable disease. As a management disease, farmers have a responsibility to ensure that their animals are vaccinated against anthrax every year. As a notifiable disease, any suspected cases must be reported to government veterinary officials. Intervention measures carried out by the veterinary department during outbreaks include mass vaccination campaigns, disposal of carcasses, disinfection and decontamination, and control of livestock movement. Even though notifications and anthrax-targeted surveillance following outbreaks are important, they provide little information on outbreak severity and geographic extent, the susceptibility of the cattle population, and effectiveness of anthrax vaccination campaigns.

In order to understand the current situation of anthrax vaccination at district level, 1619 cattle serum samples were screened for anti-PA antibodies using an rPA-D1 ELISA. To understand the geographic distribution of cattle anthrax cases in the Western Province, retrospective data on cattle anthrax cases reported from 2000 to 2016 was analyzed.

Materials and methods

Study area

The study was carried out in the following administrative districts of the Western Province of Zambia: Mongu District, Sesheke District, Limulunga District, Senanga District, Mulobezi District, Mitete District and Mwandi District (Fig 10).

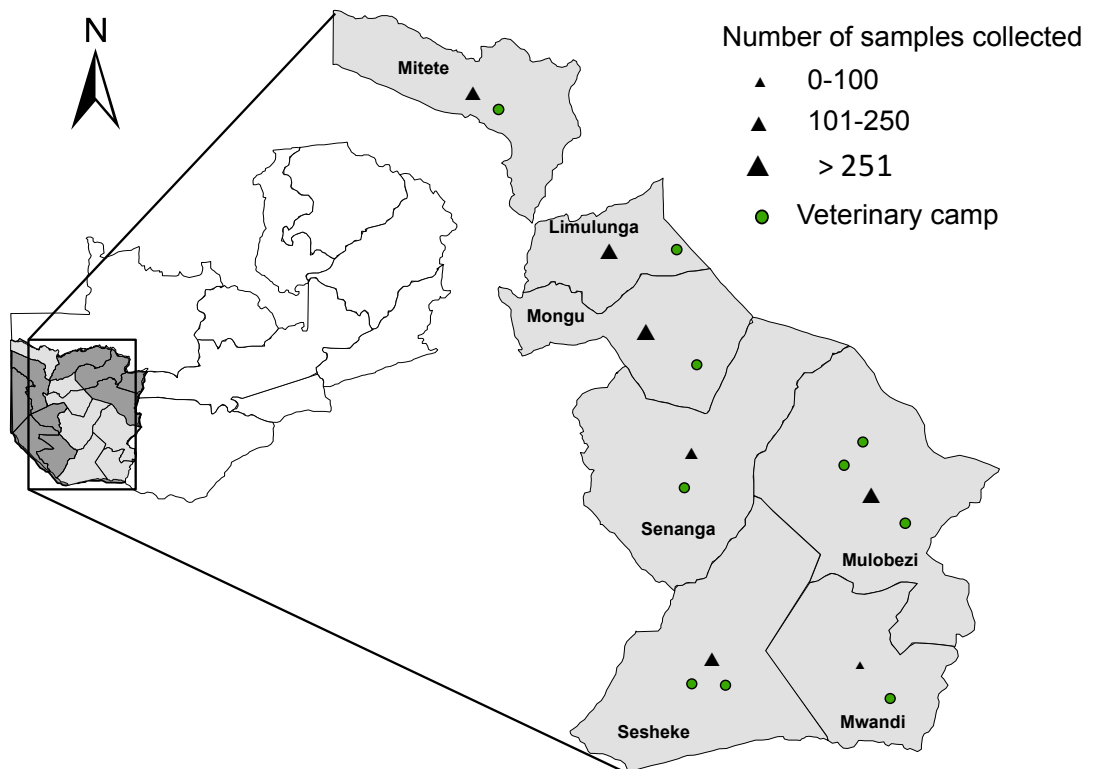


Figure 10. Study area and number of samples collected in each district.

Districts of the Western Province of Zambia. Number of samples collected. Mulobezi, 295; Senanga, 154; Mongu, 320; Sesheke, 230; Mwandi, 79; Limulunga, 306; Mitete, 235.

Sample collection and analysis

A total of 1619 cattle serum samples were collected from 10 veterinary camps in the Western Province of Zambia. Samples were collected from December 2016 to February 2017 as part of the national surveillance programme on vaccine-preventable diseases and were stored in the serum bank at the CVRI. Samples were collected from herds with a history of exposure to anthrax vaccination. Serum samples were screened for anti-PA antibodies using an in-house indirect bovine PA-D1 ELISA (Simbotwe et al. 2018). Serum samples obtained from cattle with no known history of exposure to anthrax vaccination or natural infection were used as negative controls and serum samples obtained from vaccinated cattle were used as positive controls. Rabbit anti-bovine IgG-HRP (Invitrogen) was used as a secondary antibody. Plates were read at a wavelength of 450 nm using a microtiter plate reader (Thermoscientific). The results of the test sera at OD₄₅₀ were normalized by subtracting the OD₄₅₀ of non-specific antigen coated wells from that of the rPA-D1 coated wells. The adjusted OD₄₅₀ values were then divided by the OD₄₅₀ of positive control samples in each run. Anti-PA antibodies in sera of NC samples were determined in the rPA-D1 ELISA just like test serum samples. Samples were considered positive if the sample OD₄₅₀ value was greater than the mean OD₄₅₀ of the NC samples plus 3 SD.

Data collection

Data on the cattle anthrax outbreaks from 2013 to 2016 was obtained from NALEIC. Data included, the number of cattle anthrax cases, deaths, population at risk, cattle treated, cattle vaccinated, dipped and cattle quarantined. The number of cattle anthrax cases reported from 1999 to 2007 was obtained from reviews of previously published literature (Munang'andu et al. 2012b). Data in the literature for cattle anthrax

cases were obtained from annual reports of the provincial veterinary office in Western Province. In this study, anthrax outbreak refers to the occurrence of one or more suspected anthrax cases that have been confirmed via culture. Suspected anthrax cases refer to the sudden death of animals with an unknown cause or accompanied by blood oozing from natural orifices. The geographic distribution of cattle anthrax cases was mapped using ArcGIS version 10.4.1 (ESRI, California).

Statistical analysis

Raw ELISA data were entered into Microsoft Excel and normalized. Statistical analyses were performed using GraphPad Prism, version 7.0b (GraphPad, San Diego, CA). The Kruskal Wallis test was used to determine the significance of association between districts. $p < 0.05$ was considered statistically significant.

Ethical considerations

The study proposal to conduct this work was approved by the ERES Converge Ethics Review Board, Lusaka. Permission was also obtained from the Ministry of Livestock and Fisheries, Lusaka, Zambia. Permission to obtain and use national livestock surveillance data for anthrax in Western Province was obtained from NALEIC, Ministry of Livestock and Fisheries, Lusaka, Zambia. Approval to use cattle serum samples collected from Western Province, Zambia was obtained from CVRI, Ministry of Livestock and Fisheries, Lusaka, Zambia.

Results

Seroprevalence

To determine the seroprevalence of anti-PA antibodies in Barotse cattle, I analyzed 1619 cattle serum samples. Of the 1619 cattle serum samples screened for anti-PA antibodies, 24.9% (95% CI: 22.7–27.0) were positive (Table 4). Seropositive rate was highest in Mwandi District 44.3% (95% CI: 33.3–55.3) and lowest in Senanga District 15.8% (95% CI: 10.0–21.5). There was a significant difference in seropositive rates among the districts (Kruskal Wallis test, $p < 0.05$) and between veterinary camps in Sesheke District and Mulobezi District.

Geographic Distribution

A total of 1,134 cattle anthrax cases were reported from 2000–2016, with an annual mean of 66.71 [95% confidence interval (CI): 24.88–108.5] cases. Overall, a decreasing trend was observed for the number of cattle anthrax cases reported during this period, with the highest number being reported in 2002 and 2003 ($n = 234$ per year) and the lowest in 2011 ($n = 1$) (Fig 11). Cattle anthrax cases reported between 2013 and 2016 were almost invariably fatal, with the case fatality rate ranging from 0% to 100% (Table 5).

Next, I analyzed the geographical distribution of cattle anthrax cases from 2000 to 2007 and 2013 to 2016. From 2008 to 2012, data on cattle anthrax outbreak locations were unavailable. From 2000 to 2007, >90% of cattle anthrax outbreaks were reported in areas along the Barotse Floodplain. The affected districts were Lukulu, Mongu,

Senanga, Sesheke, Kaoma, and Kalabo. Mongu, followed by Senanga, had the highest rate of cattle anthrax outbreak recurrence (Fig 12).

From 2013 to 2016, cattle anthrax outbreaks were reported in Lukulu, Mongu, Kalabo, Limulunga, Sioma, Nalolo, Shangombo, Luampa, and Mwandi. The Kalabo district had the highest number of cattle anthrax outbreak recurrences, followed by Sioma, Limulunga, and Nalolo. Subsequently, the annual distribution of cattle anthrax cases from 2013 to 2016 was analyzed. In 2013, cattle anthrax outbreaks occurred in districts lying northwestern to the floodplain, and in 2014, 2015, and 2016, these outbreaks occurred in the northeastern, southern, and southwestern and northwestern parts, respectively (Fig 13). Notably, districts that previously had no reports of outbreaks such as Shangombo reported cattle anthrax cases in 2015 and 2016. The majority of the affected districts were adjacent to districts that experienced such outbreaks in the previous years.

Table 4. Seropositivity of cattle sera from districts sampled.

District	Number of samples tested	Number of samples positive	Number of samples positive %	95% CI
Mwandi	79	35	44.3	33.3-55.3
Limulunga	306	76	24.8	19.9-29.6
Mitete	235	45	19.1	14.0-24.1
Senanga	154	24	15.6	10.0-21.5
Mongu	320	81	25.3	20.5-30.0
Sesheke				
Camp 1	75	10	13.3	5.6-20.9
Camp 2	155	42	27.1	20.8-34.9
Sub total	230	52	22.6	17.2-28.0
Mulobezi				
Camp 1	77	2	2.5	0.0-5.9
Camp 2	75	10	13.3	5.6-20.9
Camp 3	143	78	54.5	46.3-62.7
Sub total	295	90	30.5	25.2-35.7
Total	1619	403	24.9	22.7-27.0

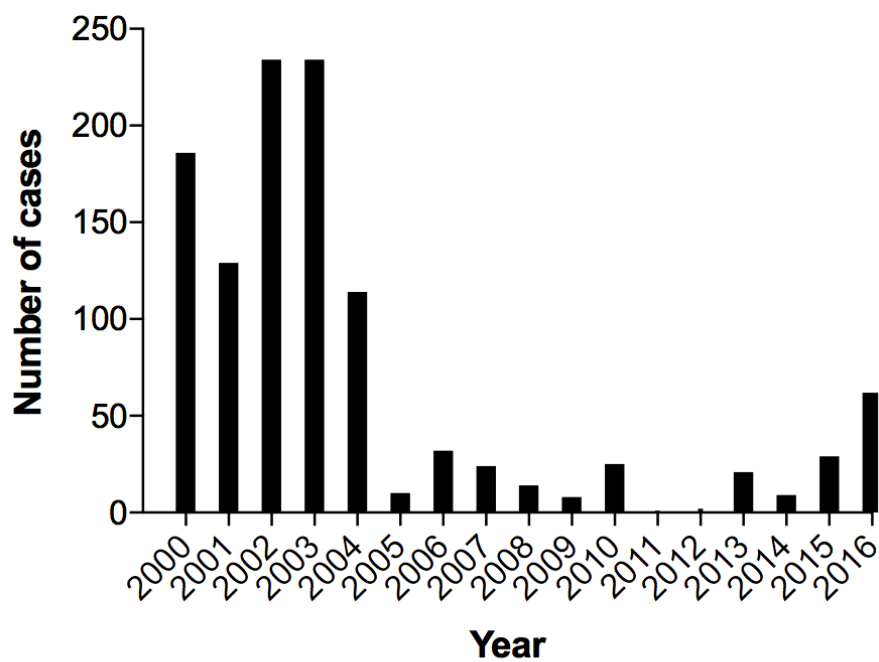


Figure 11. Annual distribution of cattle anthrax cases from 2000 to 2016.

Number of cattle anthrax cases reported from 2000 to 2007 (Munang'andu et al., 2012).

Table 5. Cattle anthrax cases and deaths from 2013-2016.

Year	District	Herd size	Cases	Mortality	Case fatality rate %	Population at risk
2013	Kalabo	54	1	1	100	54
	Mongu	230	20	20	100	330
2014	Kalabo	81	1	1	100	1300
	Luampa	210	4	4	100	210
	Lukulu	50	3	3	100	100
	Nalolo	80	1	1	100	80
2015	Limulunga	378	10	9	90	2210
	Sioma	60	7	7	100	500
	Mwandi	ND	11	0	0	ND
	Senanga	ND	1	0	0	ND
2016	Kalabo	1600	40	25	63	5900
	Limulunga	ND	4	4	100	12000
	Nalolo	ND	6	6	100	12000
	Shangombo	ND	4	4	100	11000
	Sioma	ND	8	8	100	6000

ND: not determined

A

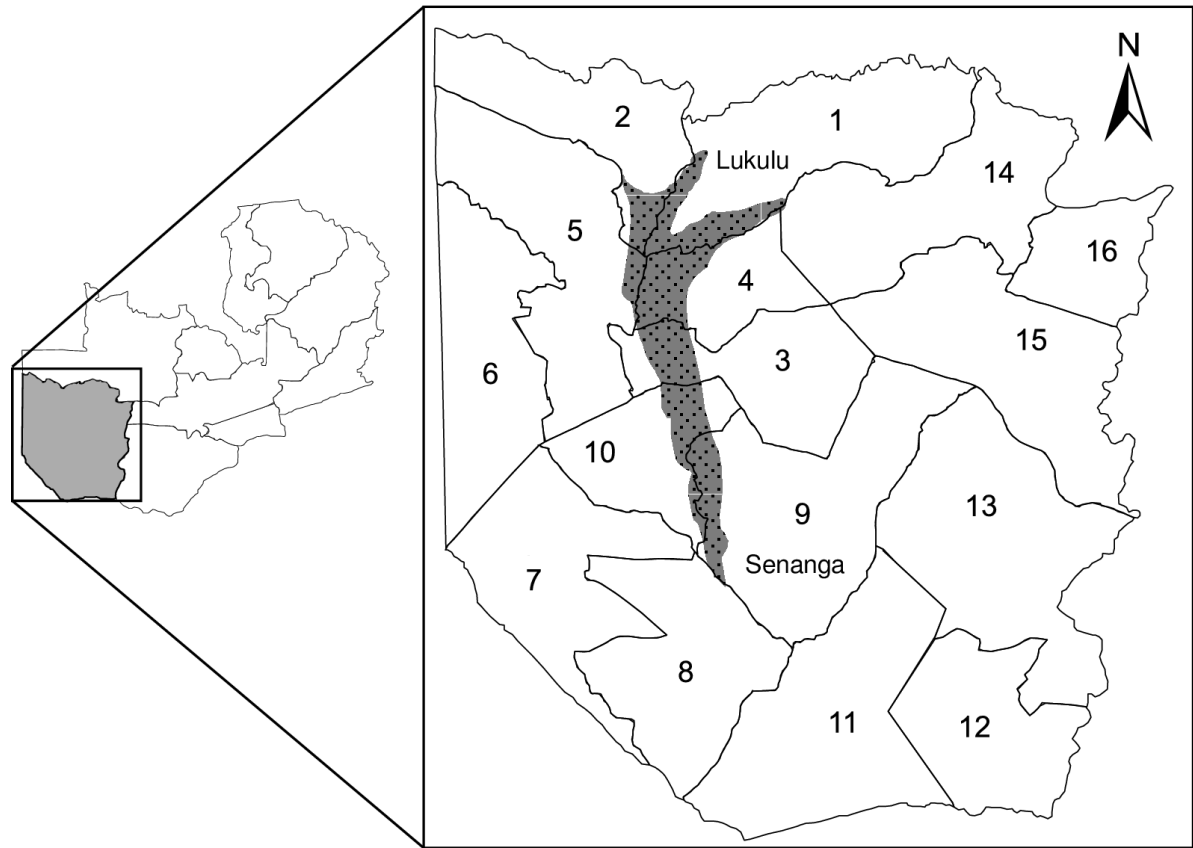


Figure 12A. Incidence and distribution of livestock anthrax cases from 2000-2016 in Western Province, Zambia.

1. Lukulu, 2. Mitete, 3. Mongu, 4. Limulunga, 5. Kalabo, 6. Sikongo, 7. Shangombo, 8. Sioma, 9. Senanga, 10. Nalolo, 11. Sesheke, 12. Mwandi, 13. Mulobezi, 14. Kaoma, 15. Luampa, and 16. Nkeyema districts. Gray area: Outline of the Barotse Floodplain, tributaries not included.

B

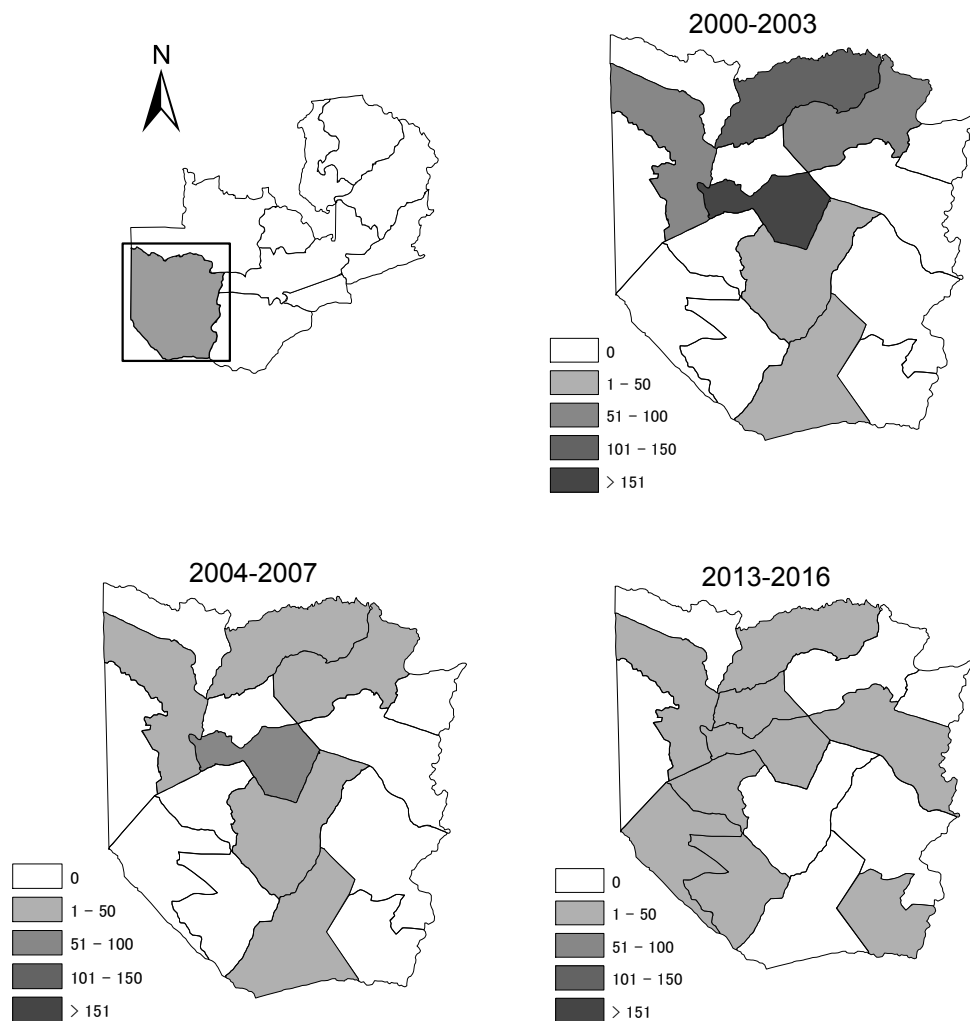


Figure 12B. Incidence and distribution of livestock anthrax cases from 2000-2016 in Western Province, Zambia.

(B) The number of cases reported were 783 from 2000 to 2003, 180 from 2004 to 2007, and 113 from 2013 to 2016. There was a decrease in disease incidence over the study period.

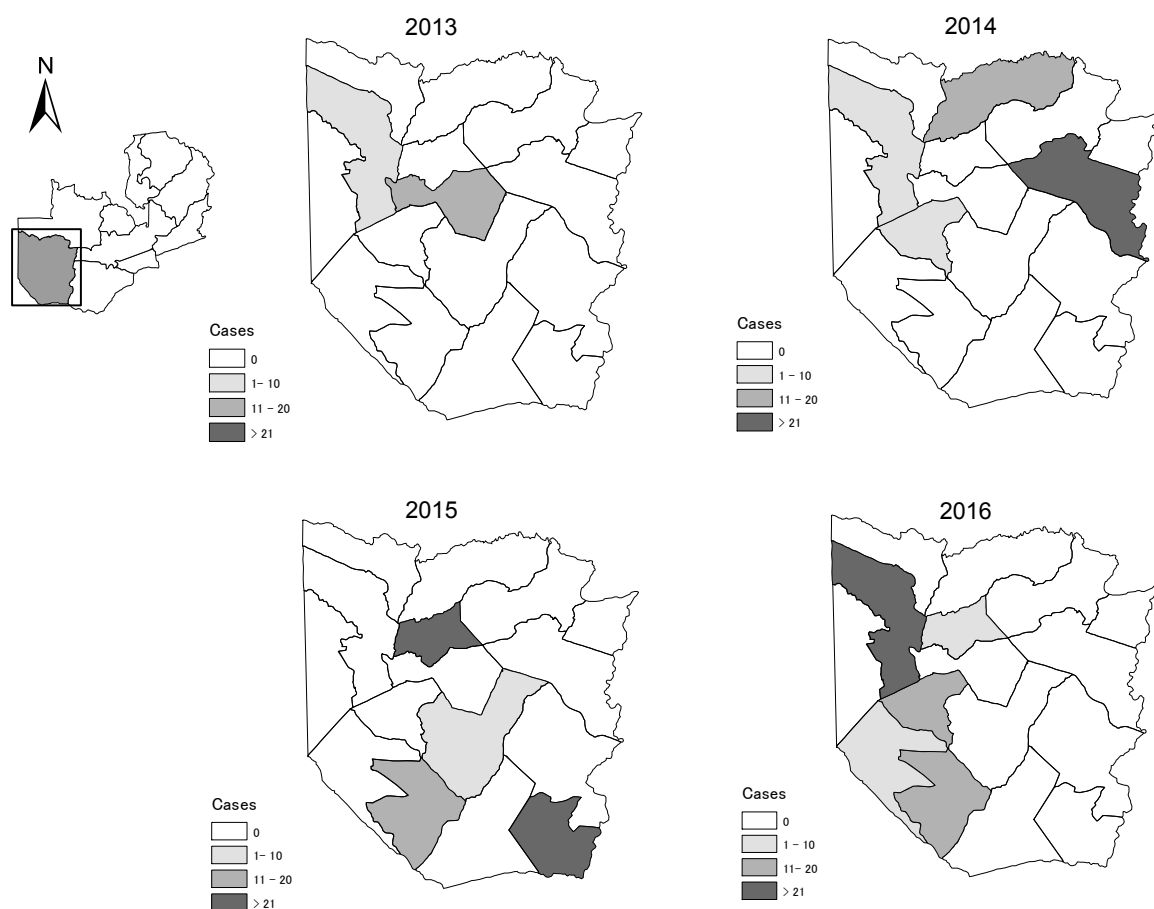


Figure 13. Annual distribution of cattle anthrax cases from 2013 to 2016 in Western Province, Zambia.

Most of the newly affected districts were adjacent to districts with previously reported cattle anthrax outbreaks.

Discussion

The overall seropositive rate detected may indicate inadequate vaccination and the variation in seropositive rates among districts could be a reflection of the differences in the proportion of cattle that were exposed to vaccination in each district. My results differ from previous results reported in the literature. Compared with the seropositive rate of 24.9%, in my previous study I found a seropositive rate of 8% (15/187) at 18 months after an anthrax outbreak and retrospective vaccination (Simbotwe et al. 2018).

During the period between 2000 and 2016, I was only able to collect serum samples from December 2016. The results on the seropositive rate from the period between December 2016 and February 2017 suggest that the outbreak frequency during this period might be the result of insufficient cattle anthrax vaccinations. Combined with the geographic distribution data showing that districts adjacent to the outbreak areas reported cattle anthrax cases in the subsequent years, it is possible that cattle anthrax vaccinations were mainly administered in the affected areas after an outbreak occurrence. Therefore, efforts must be made to strengthen and promote serosurveillance programs that will identify specific areas with poor vaccination practices and will educate livestock farmers on the benefits of anthrax vaccination in the Western Province.

The finding regarding the highest number of cattle anthrax outbreak recurrences in the Mongu and Senanga districts in this study is consistent with that in previous studies (Siamudaala et al. 2006b; Bbalo 1996). Moreover, it was found that Kalabo and Sioma districts were among those with the highest number of such outbreak occurrences from 2013 to 2016. The present analysis showed that cattle anthrax cases were also reported in rarely flooded areas, apart from the floodplain. Considering the incubation period of anthrax (approximately 3–5 days) (Beyer and Turnbull 2009), I hypothesized that cattle acquired the infection in the floodplain, resulting in subsequent death in the adjacent upland areas, establishing new foci of infection. In this study, mapping at the district level was performed because I was unable to obtain georeference data on the locations of cattle herds. Mapping at the herd or veterinary-camp level may be more useful in identifying high-risk areas and the actual geographical extent of cattle anthrax outbreak occurrence.

In the Western Province of Zambia, anthrax vaccination is provided for a part of the population-at-risk following an anthrax outbreak. In areas where vaccination coverage was >80%, there was a reduction in prevalence (Bbalo 1996). On the other hand, during the 2016 anthrax outbreak in the Kalabo district, 31.3% (1,846/5,900 animals) of the cattle population-at-risk were vaccinated (NALEIC 2016). These data are consistent with those of previous reports indicating inadequate anthrax vaccination of cattle in Zambia and in anthrax endemic areas elsewhere (Dietvorst 1996; Kracalik et al. 2017).

The transhumance type of farming and inadequate resources render the provision of veterinary services (i.e., herd health management, vaccination, and farmer education) challenging. Until 2012, the Western Province was divided into seven districts, which may have had a negative impact on the detection and reporting of cattle anthrax cases. District veterinarians and veterinary assistants had to travel long distances (>100 km) to rural communities and the duration of time between case detection and intervention would vary from a day to a month (Bbalo 1996). Currently, the province comprises 16 administrative districts, increasing the ability to detect and report cattle anthrax cases. Considering these recent changes, the findings of this study may be useful in formulating and implementing strategies for controlling anthrax in Zambia. Future studies focusing on the improvement of detecting and reporting systems are warranted to determine high-risk areas and to optimize the distribution of resources.

General conclusion

B. anthracis is the etiological agent of anthrax, a zoonotic disease that is endemic in the Western Province of Zambia. Anthrax outbreaks among cattle in the Western Province are reported on nearly an annual basis and cause significant economic losses because the majority of the rural population is dependent on livestock for their livelihood. Despite the persistence and frequency of cattle anthrax outbreaks, the present seroepidemiologic situation and its contribution to anthrax outbreak recurrence remains poorly understood. Surveillance of cattle anthrax is crucial for determining the impact, high risk areas and the effectiveness of intervention measures. In Zambia, serological tools and seroepidemiological data are necessary to complement the current retrospective anthrax targeted surveillance system.

In Chapter I, an indirect ELISA based on *B. anthracis* rPA-D1 was developed. The study demonstrated the utility of a domain of PA. In addition to the assay specificity and precision, stability of lyophilized rPA-D1 antigen when stored at room temperature is an added asset that will be useful in resource-limited settings. The rPA-D1 ELISA was able to detect seroconversion in cattle following anthrax vaccination.

In Chapter II, the usefulness of the rPA-D1 ELISA was demonstrated. For the first time, in a cross sectional study conducted in the Western Province of Zambia, serum samples collected from eight herds of Barotse cattle were screened for anti-PA antibodies using the rPA-D1 ELISA. Analysis of the association between antibodies and vaccination timing

showed significant differences in the level of the detectable antibodies over time, suggesting that the rPA-D1 ELISA is a useful tool for anthrax surveillance and for the detection of anti-PA antibodies in cattle in Zambia. The study also provides essential epidemiological information on herd health and anthrax vaccination practices in the Western Province of Zambia.

In Chapter III, to understand the current seroepidemiological situation in anthrax outbreak areas, the incidence, geographic distribution of cattle anthrax outbreaks and the seroprevalence of anti-PA antibodies in 1619 Barotse cattle was assessed. The study indicated that, although the number of reported anthrax cases was decreasing, there was a change in the distribution of outbreaks, suggesting that anthrax outbreaks were no longer limited to the Barotse Floodplain. The study detected a low seropositive rate of anti-PA antibodies among the cattle sampled indicating that in addition to other epidemiological factors, inadequate cattle anthrax vaccination may be a contributing factor to anthrax outbreak recurrence.

These findings have important implications on the epidemiology of cattle anthrax and also point out areas in disease control that may require prioritization for delivery of veterinary services. Other findings add substantially to our understanding and knowledge of anthrax serology among Barotse cattle in the Western Province of Zambia.

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