A serological survey of *Bacillus anthracis* reveals widespread exposure to the pathogen in free-range and captive lions in Zimbabwe.

Running title: Widespread exposure to *B. anthacis* in African lions.

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Summary

Numerous unknown factors influence anthrax epidemiology in multi-host systems, especially at wildlife/livestock/human interfaces. Serology tests for anthrax in carnivores is one tool which can be useful in identifying the presence or absence of *Bacillus anthracis* in a range, and it was employed in this study to ascertain if the disease pattern followed the recognized high and low risk anthrax zonation in Zimbabwe, and also to establish if anthrax was absent from Hwange National Park in which there has been no reported outbreaks. African lions (*Panthera leo)* (n= 114) drawn from free-range protected areas and captive game parks located in recognized high and low risk zones across Zimbabwe were tested for antibodies to anthrax PA antigen. Overall 21.9% (25/114) of the lions tested positive for antibodies to anthrax. Seropositivity was recorded in all the study

areas and there was no significant difference (p= 0.852) in seropositivity between lions in high and low risk anthrax zones. Results of this study indicate that anthrax could be more widespread than realized in Zimbabwe, and present in recognized high and low risk zones, including where it has not been reported in over 20 years such as Hwange National Park. The research results point to a need for revisiting the currently recognized anthrax risk zones in Zimbabwe. This should be based on improved surveillance of the disease in both wild and domestic animals for better understanding and control of the disease. Vigilance in feeding of captive carnivores with disease-free meat diets cannot be overemphasized.

Key words

African lions (*Panthera leo*), anthrax (*Bacillus anthracis),* anthrax protective antibody antigen, , protected areas, captive game parks

Introduction

Anthrax, caused by *Bacillus anthracis* bacterium, is a zoonotic disease of warm-blooded animals that can be fatal for livestock, wildlife and humans (Hirsch & Biberstein, 2004; OIE, 2012). There are numerous unknown factors which influence the epidemiology of anthrax in multi-host systems, especially at wildlife/livestock/human interfaces. Anthrax induces fatal acute to peracute syndromes with no or little protective antibody immunity in herbivores. When present, this protective antibody immunity often lasts less than a year in herbivores (Turnbull *et al*,. 1992; de Vos and Turnbull, 2004). However, the duration of antibody reactivity to anthrax has been found to be much longer in surviving carnivores, and indefinite in humans (Beyer and Turnbull, 2009*)*. In Namibia, results of serological reactions to *B. anthracis* in wild carnivores

were related to the occurrence of anthrax in wild herbivores in Etosha National Park, thus constituting an epidemiological tool for monitoring anthrax distribution (Turnbull *et al.*, 1992).

Despite anthrax being considered endemic in some parts of Zimbabwe (Chikerema *et al.*, 2012; Mukarati *et al.*, 2018), its epidemiology in wildlife is poorly understood due to suboptimal surveillance and outbreak investigations. Over the past 20 years, no overt anthrax outbreaks have been reported in wildlife in Hwange National Park (HNP) despite sporadic outbreaks of the disease in livestock in adjacent communal areas of Tsholotsho District to the South-East of the park (Mukarati *et al.*, 2018). In this study, we hypothesized that low risk areas for anthrax such as HNP with no reported wildlife anthrax outbreaks for 20 years would result in significantly low seropositivity to anthrax in wild carnivores compared to high risk areas.

Materials and methods

African lion (*Panthera leo*) sera samples (n = 114) were obtained from serum banks from wild carnivore conservation projects in protected areas and recreational game parks in Zimbabwe. Each sample was allocated to high or low risk areas for anthrax (Figure 1, Table 1) based on a previous classification (Chikerema *et al.*, 2013). The samples were collected from lions immobilized for various reasons over a period of 20 years (1996 – 2016) and stored frozen at -20^{o}C at the Wildlife Unit of the Department of Veterinary Services, Ministry of Lands, Agriculture and Rural Resettlement, Zimbabwe.

The sera samples were tested for antibodies to *B. anthracis* capsule (PA) antigen as detailed in Mukarati *et al.* (2018). A conventional PA ELISA was used to analyze samples for specific immunoglobulins according to Hahn *et al.* (2004) and modified by Ndumnego *et al.*

(2013). For determination of the cut-off value for positive lion sera in the multi-species ELISA a similar approach depicted previously by Mukarati *et al* (2018) was adopted. Previously identified negative and positive control sera were sourced from a domestic cat presenting for an unrelated condition at the Onderstepoort Veterinary Academic Hospital and from a vaccinated goat (Ndumnego, Koehler, Crafford, Beyer, & van Heerden, 2018) respectively. Each ELISA plate contained duplicate wells of the known negative and positive control sera. Also six blank wells containing only the blocking solution (skimmed milk powder) were provided for each plate and background OD values from these wells were subtracted from the test sera wells.Seroprevalence estimates of anthrax with 95% confidence intervals were computed using Stata Version SE/11. The primary sampling strata were the recognized high and low anthrax risk zones. Wildlife management systems (protected areas vs recreational game parks), represented the secondary strata while the individual animals were the sampling units. Data analysis was done in Stata Version SE/11 for Windows (Stata Corp., College Station, TX, USA) at a 95% confidence interval, and difference between strata noted.

Results

Overall, 21.9% (25/114) of African lions in this study tested positive for antibodies to anthrax PA antigen. Seropositivity was recorded in almost all the study areas except for Antelope Game Park (Figure 1). A total of 5 lions (22.7%, n= 22) from high risk zones and 20 lions (21.7%, n = 92) from low risk zones respectively were positive for antibodies to anthrax PA antigen. However, the difference in seropositivity between the anthrax high and low risk zones was not significant (p= 0.852) (Table 1). Again seropositivity between free-range and captive lions was not significantly different (p = 0.951) respectively at 22.6% (21/93) and 19.1% (4/21).

Figure 1. Map of Zimbabwe showing seroprevalence of anthrax in African lions sampled across the country. Anthrax risk zones adapted from Chikerema *et al.* (2013*)* with minor modifications.

With respect to lions in Hwange National Park which formed the bulk of the animals under this study, the sample distribution was biased towards the northern half of the park reflecting the spatial emphasis of the ongoing wild carnivore research projects from which the samples were drawn (Fig. 2).

Figure 2. Map of Hwange National Park (Zimbabwe) showing distribution of anthrax positive lions in sampled areas.

Seropositive lions were concentrated to the northeast of the park in areas which are adjacent to northern Tsholotsho (Ngamo) and Hwange Communal Lands although there were also positive lions far inland of HNP at about 90 km from the nearest park boundary. This distance was well outside the lions' home range of about 20km radius (Loveridge et al., 2009) indicating unlikely influence of diseases between the lions and domestic livestock. No outbreaks of anthrax were reported in HNP and Hwange Communal Lands during this period under review. On the other hand, outbreaks of anthrax were reported in Tsholotsho Communal Lands including Ngamo area adjacent to southeastern HNP (Fig. 2) (Mukarati *et al*., 2018).

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Discussion

The anthrax protective antigen (PA), in addition to the edema and lethal factors (EF and LF), make up the tripartite anthrax toxin complex (Schwartz 2009). These anthrax toxins are encoded by the anthrax-specific virulence plasmid, pXO1, with PA combining with LF to form

126 the lethal toxin (LT). To date, no cross reacting antigens to PA are known and available data
127 indicate anti-PA antibodies as being the most diagnostically stable (Turnbull, Doganay, Aygen,
128 Lindeque, & Mclaughlin, 1992) ***Leppla, Robbins et al, 2002)***. The use of PA as the sole antigen
129 in diagnostic immunoassays has been validated in humans (Ghosh et al., 2015; Quinn et al.,
130 2002; Semenova et al., 2012), horses (Caldwell, Hathcock, & Brock, 2017) and goats
131 (***Ndumnego, Crafford et al, 2013***). In the latter, Ndumnego *et al*. (2013) compared and observed
132 that the use of skimmed milk powder gives a lower background reading compared to the use of
133 foetal calf serum. While there may be the risk of lion IgG detecting milk or any other ruminant
134 proteins, there is no evidence of this in studies using the PA-ELISA to monitor *Bacillus*
135 *anthracis* exposure in lions or other carnivore species (Hampson et al., 2011; Turnbull et al.,
136 1992) ***Bagamian, Alexander et al, 2013; Switzer, Munson et al, 2016)***. Thus the presence of
137 anti-PA antibodies in animals indicates that non-lethal systemic infection has taken place (Hugh-
138 Jones & Blackburn, 2009; Turnbull et al., 1992) ***(Ndumnego, Crafford et al, 2013***. Therefore
139 based on these serological assays in African lions, there were indications that *B. anthracis*
140 pathogen was present and distributed widely in Zimbabwe.

141 In the case of HNP where anthrax outbreaks have not been reported in over 20 years, this
142 represents the first confirmation of the presence and wide exposure of *B. anthracis* to lions in the
143 park. There is a possibility that some lions from HNP could have been exposed to anthrax from
144 consumption of livestock carcasses in adjacent anthrax endemic Tsholotsho Communal areas
145 (Chikerema *et al*., 2012) as the two areas share an unfenced porous interface. However, more
146 seropositive lions were far removed from this community (more than 90 km away), a distance
147 larger than an average lion home range in the area of about 20 km in diameter (A. Loveridge,
148 person. comm.). It is most likely that such seropositive lions were exposed to other sources of *B.*

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anthracis in HNP. Although this was not strictly a cross-sectional survey in that the lions were sampled over 20 years, it nonetheless provides useful information. That lions sampled over this period were positive for *B. anthracis* in the absence of reported disease outbreaks suggests either occurrence of undetected outbreaks of the disease or sublethal infections in wildlife in HNP (Hugh-Jones and Blackburn, 2009; Cizauskas *et al.*, 2014). Thus, the hitherto general belief that HNP was free of anthrax based on no reported cases is uncertain. The presence of antibodies to anthrax in lions in Mana Pools NP, Save Valley Conservancy and Malilangwe Wildlife Reserve indicates that *B. anthracis* was already circulating in the area long before the disease outbreaks occurred (Mukarati *et al.*, 2018; Clegg *et al.*, 2007; OIE, 1997). However, there was no correlation between anthrax outbreaks and seropositivity in lions from this dataset. Improved surveillance and additional studies on possible environmental and soil geochemical factors possibly influencing *B. anthracis* distribution in protected areas are necessary (Hugh-Jones and Blackburn, 2009; Griffin *et al.*, 2014).

There were no known anthrax outbreaks that could account for exposure and seroconversion in captive lions in this study. According to de Vos and Turnbull (2004), anthrax outbreaks affecting wildlife in captivity are limited to consumption of infected meat. Carnivores on small game parks are managed essentially as in zoological gardens and thus are presumably similarly exposed to anthrax. An anthrax outbreak occurred in lions and cheetahs in 1997 at Lion and Cheetah Park, Harare, when they were fed infected bovine meat donated by a farm (OIE, 1997*)*. On the other hand, wild carnivores in captivity maybe exposed to *B. anthracis* from infected meat without necessarily developing clinical disease either due to their relative resistance or exposure to sublethal anthrax doses (Beyer and Turnbull, 2009; Hugh-Jones and Blackburn, 2009; Cizauskas *et al*., 2014).

A pertinent question which arises is the specificity of the serological assay used in this survey, given that there are few reports of atypical *B. cereus* strains causing similar disease in humans and wild primates (Marston *et al.*, 2016). Indeed, rare cases of anthrax-like illness in humans and wild chimpanzees caused by a *B. cereus* strain possessing the anthrax toxin genes have been reported in the US and West Africa (Hoffmaster *et al.*, 2004, 2006; Avashia *et al*., 2007; Klee *et al*., 2006). However, to date there are no documented cases of outbreaks caused by this strain of *B. cereus* in wild or domestic ruminants, or the carnivores who predate on these animals. While not ruling out the possible exposure to environmental *B. cereus* in grazing animals, the presence of the rare toxin-producing *B. cereus* strains have not been reported in Southern Africa. Thus, the seropositivity of lions in this study is assumed to be attributable to exposure to *B. anthracis* strains.

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The finding of no significant difference in seroprevalence between lions located in currently recognized high and low risk zones for anthrax ($p = 0.852$, Table 1) tally with earlier findings in domestic dogs (Mukarati *et al.*, 2018). A much wider range of anthrax endemic areas in Zimbabwe can be hypothesized similar to other endemic regions of the world. This suggests that the categorization of areas in Zimbabwe into high and low risk zones may not represent the true status of anthrax risks across the country. This needs review based on improved surveillance and epizootiological investigations. Anthrax serology in resident wild and/or domestic carnivores could serve as sentinel and indicator of anthrax circulation in given areas and thus can be useful epidemiological tools.

The widespread presence of anthrax antibodies in lions in protected areas, irrespective of absence of reported disease outbreaks in wild or domestic ungulates or humans, confirms a much larger circulation of *Bacillus anthracis* in Zimbabwe. These results raise new questions on the

epidemiology of anthrax in endemic regions. There is need to investigate local factors that could be associated with anthrax outbreaks apart from presence of the pathogen. On the other hand, there could be small outbreaks of anthrax occurring but going unnoticed in HNP similar to what has been noted elsewhere (Cizaukas *et al.,* 2014). Overall improved surveillance of anthrax in all animals could shed more light on whether outbreaks were indeed taking place but being missed because of inadequate surveillance and may also give pointers on risk factors.

Author contributions

Norman Leo Mukarati was the main researcher who conceived the ideas and designed the methodology as well as carrying out the field work and partly the laboratory work;

Okechukwu C. Ndumnego and ***Henriette van Heerden*** designed, carried out the serological testing of the samples for *B. anthracis* PA antigen antibodies and revised final manuscript.

Andrew Loveridge and ***Tapiwa G. Hanyire*** collected the blood samples from lions used in this study.

Davies M. Pfukenyi and ***Gift Matope*** carried out data analysis and redrafting of the manuscript especially epidemiological aspects.

Alexandre Caron and ***Michel de Garine-Wichatitsky*** –critiqued the manuscript and contributed to its redrafting.

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Conflict of interest

None.

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Table 1**:** Seroprevalence of anthrax in lions sampled in protected areas and captive recreational game parks in Zimbabwe.

Wildlife Management Area	District	*Anthrax risk zone	Total tested	Total positive	% Seroprevalence (95% CI)†	Year (s) of sample collection	Year (s) of reported anthrax outbreak (s) in the area
Hwange National Park	Hwange	L	73	17	23.3 (14.5-34.9)	2001-2015	Nil
Mana Pools National Park	Hurungwe	L	11	2	18.2 (3.2-52.3)	2001, 2002 & 2004	2011 (*7*)
Save Valley Conservancy	Bikita/ Chiredzi	H	6	1	16.7 [0.9 – 63.5]	2003	2004-2005 (*13*)
Malilangwe Wildlife Reserve	Chiredzi	L	3	1	33.3 [1.8 - 87.5]	1999, 2003 & 2004	2004-2005 (*13*)
Overall protected areas			***93***	***21***	***22.6[a] [14.8 - 32.7]***		
Lion & Cheetah Park	Norton	H	6	1	16.7 [0.9 - 63.5]	1996, 1997, 2002, 2003 & 2004	1997 Lion & Cheetah Park (*14*)
Chengeta Game Park	Chegutu	H	4	1	25.0 [1.3 - 78.1]	2002, 2016, 2017	Nil
Antelope Game Park	Gweru	L	5	0	0.0 [1.9 - 53.7]	1996, 1997	Nil
Bally Vaughan Game Park	Shamva	H	6	2	33.3 [6.0 - 75.9]	2002, 2004	Nil
Overall captive recreational parks			***21***	***4***	***19.1[a] [6.3 - 42.6]***		
Grand Total			**114**	**25**	**21.9 [15.0 - 30.9]**		

*Risk zone: H = high risk for anthrax, L = low risk for anthrax.

†There was no significant difference (p= 0.852) in anthrax seropositivity between lions in high and those in low anthrax risk zones, and between lions in protected areas and those in captive game parks (p = 0.951).