Detection of tick-borne pathogen co-infections and co-exposures to foot-and-mouth disease, brucellosis and Q fever in selected wildlife from Kruger National Park, South Africa, and Etosha National Park, Namibia

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ABSTRACT

Background: Although the rate of emerging infectious diseases that originate in wildlife has been increasing globally in recent decades, there is currently a lack of epidemiological data from wild animals.

Methodology: We used serology to determine prior exposure to foot-and-mouth disease virus (FMDV), *Brucella* spp., and *Coxiella burnetii,* and used genetic testing to detect blood-borne parasitic infections in the genera *Ehrlichia, Anaplasma, Theileria* and *Babesia* from wildlife in two national parks, Kruger National Park (KNP), South Africa and Etosha National Park (ENP), Namibia. Serum and whole blood samples were obtained from free-roaming plains zebra (*Equus quagga*), greater kudu (*Tragelaphus strepsiceros*), impala (*Aepyceros melampus*) and blue wildebeest (*Connachaetes taurinus*). Risk factors (host species, sex, sampling park) of infection for each pathogen were assessed, as well as the prevalence and distribution of co-occurring infections.

Results: In KNP, none of the 13/29 (45%; CI: 26-64%) kudu tested positive for FMD. For brucellosis, seropositive results were obtained for 3/29 (10%; CI: 2-27%) kudu samples. Antibodies against *C. burnetii* were detected in 6/29 (21%; CI: 8-40%) kudu, 14/21 (67%; CI: 43-85%) impala and 18/39 (46%; CI: 30-63%) zebra. A total of 28/28 kudu tested positive for *Theileria* spp. (100%; CI: 88-100%) and 27/28 to *Anaplasma/Ehrlichia* spp. (96%; CI: 82-100%) whereas 12/19 impalas (63%) and 2/39 zebra (5%) tested positive for *Anaplasma centrale*. In ENP, only 1/29 (3%; CI: 0-18%) wildebeest samples tested positive for FMD. None of the samples tested positive for brucellosis while *C. burnetii* antibodies were detected in 26/30 wildebeest (87%; CI: 69-96%), 16/40 kudu (40%; CI: 25-57%) and 26/26 plains zebra (100%; CI: 87-100%). A total of 60% *Anaplasma/Ehrlichia* spp. and 35% *Theileria/Babesia* spp. in kudu; 37% wildebeest tested positive to *Theileria* sp. (sable), 30% to *Babesia occultans,* 3-7%

to *Anaplasma spp.* The seroprevalence of Q fever was significantly higher in ENP, while *Brucella* spp., *Anaplasma, Ehrlichia, Theileria* and *Babesia* species were significantly higher in KNP. Significant co-infections were also identified.

Conclusion: This work provided baseline serological and molecular data on 40+ pathogens in four wildlife species from two national parks in southern Africa.

Keywords: brucellosis, foot-and-mouth disease, wildlife disease, tick-borne disease, Q fever, epidemiology, zoonosis.

INTRODUCTION

Wildlife are often linked with emerging infectious diseases relevant to human and animal health, and are considered to be the source of 70% of zoonoses worldwide (1,2, USGS 2024). Several studies have highlighted the wide range of pathogens that wild animals may carry without necessarily showing overt clinical signs (3–9). Multiple endemic diseases (*i.e.* bovine tuberculosis, brucellosis, rabies, Ebola, leptospirosis) have been associated with a wildlife source, and their management imposes serious challenges at the wildlife/human interface (8–15). As a result of increased mortality, reduced productivity, costs related to disease control, loss in trade, decreased market value, and food insecurity, wildlife-emerging diseases constitute an additional and important threat to the economy of the livestock industry (16,17). Moreover, many wildlife diseases have caused important decrease in endangered animal populations, affecting their conservation status (18). Most infectious diseases are still largely neglected in wildlife, especially those that are endemically persistent and do not cause obvious clinical signs or have long incubation period.

In this study, we investigated the exposure to Foot-and-Mouth Disease Virus (FMDV), *Brucella* spp. and *Coxiella burnetii*, as well as infection with several tick-borne pathogens (*Anaplasma,*

73 *Ehrlichia, Theileria* and *Babesia* spp.) in greater kudu (*Tragelaphus strepsiceros*), plains zebra
74 (*Equus quagga*), impala (*Aepyceros melampus*) and blue wildebeest (*Connachaetes taurinus*)
75 from two national parks namely Kruger National Park, South Africa, and Etosha National Park,
76 Namibia.
77 FMDV causes Foot-and-Mouth Disease (FMD), a World Organization for Animal Health listed
78 disease that has been reported from more than 70 wildlife species (6, 19). FMD is endemic in
79 various African countries (*e.g.* South Africa, Mozambique, Zimbabwe) and has a negative
80 impact on the national economy of a disease-endemic setting, also having the potential to spread
81 across boundaries (20). The circulation of FMDV in wildlife represents a significant burden on
82 wildlife management and conservation of endangered species (21,22). In livestock animals,
83 FMD primarily occurs in an acute form with fever, lameness, inappetence, and the formation
84 of vesicles in and around the mouth and on the feet. Clinical signs are often severe in pigs,
85 obvious in cattle and mild in sheep and goats (23). Clinical FMD in wildlife seems to be a rare
86 event, but it can occasionally be devastating to some species of antelope as has been
87 documented in South Africa in impala (*Aepyceros melampus*) (24) and in mountain gazelles
88 (*Gazella gazella*) in Israel (25).

89 Important subsets of infectious diseases that are neglected in wildlife include intracellular
90 bacterial pathogens. Inter alia, *Brucella* spp. and *C. burnetii* cause important veterinary and
91 zoonotic diseases worldwide. Brucellosis is a disease of great economic importance, especially
92 for the livestock industry, causing significant production losses and impediments to trade and
93 exportation (26). Brucellosis has been recorded in a wide range of African wildlife, but the
94 effect of the disease in sylvatic settings has been largely ignored and understudied. The
95 circulation of the pathogen in wildlife raises challenges for disease control and management.
96 For instance, France was bovine brucellosis free since 2005 but experienced bovine and human

97 cases due to *B. melitensis* in 2012 in French Alps. The investigation identified spillover from
98 wild Alpine ibex (*Capra ibex*) to domestic ruminants (27). Few serological tests have been
99 validated for use in wild animal species. The standard indirect enzyme-linked immunosorbent
100 assays (ELISAs) are designed to be specific to livestock species and thus limited for wildlife
101 testing. As none of the serological tests are 100% sensitive and specific (28), the criteria for
102 seropositive brucellosis diagnosis require two positive test results in series.
103 Q fever is an emerging disease caused by bacterium *C. burnetii* which has a high impact on
104 public health, animal health and economy. It is listed by WOAH as a multi-species disease of
105 concern for its high zoonotic potential, worldwide distribution, airborne spread, persistent
106 infection (potentially lifelong) and direct production losses for the dairy industry (abortions,
107 dead or weak offspring, infertility, metritis). *Coxiella burnetii* is severely under-reported and
108 under-appreciated throughout Africa (29,30), even though wildlife have been demonstrated to
109 play an important role in Europe and elsewhere (31–33).

110 Among the emergent threats, tick-borne pathogens (TBPs) have a great impact on animal and
111 human health throughout the African continent (29,34). The epidemiology of ticks and TBPs is
112 complex and multimodal such that environmental variables and contact among wildlife,
113 livestock, and humans participate in the transmission dynamics of TBPs. Therefore, wildlife
114 loss and climate changes may result in the increase of disease risk (35). *Anaplasmataceae* and
115 *Piroplasmida* are two major taxa of obligate intracellular pathogens transmitted by blood-
116 sucking arthropods (especially ticks). Members of the family *Anaplasmataceae* are frequently
117 reported in African wildlife, especially African buffalo (*Syncerus caffer*) and several antelope
118 species (36–39). The most important tick-borne diseases affecting livestock in Africa are
119 *Theileria parva* (East Coast fever, January disease and corridor disease), *Ehrlichia ruminantium*
120 (heartwater), *Anaplasma marginale* (gallsickness), *Theileria annulata* (tropical theileriosis),

121 *Babesia bovis* and *Babesia bigemina* (Asiatic and African redwater, respectively) (40).
122 Anaplasmosis, heartwater, theileriosis and babesiosis are known to cause 18% of reported cattle
123 mortalities in South Africa (41).

124 Kruger National Park (KNP) in South Africa is classified as an endemic zone for FMD and an
125 infected zone for brucellosis and corridor disease, where sporadic outbreaks are reported
126 (19,42). In contrast, Etosha National Park (ENP) is a protected, non-infected FMD zone with
127 no brucellosis detected in wildlife. According to the systematic review performed by Simpson
128 et al. (7), three prevalence studies have been conducted on *Brucella* spp. in Namibian wildlife,
129 all of them reporting negative results although with small sample sizes *i.e.* 0/23 white rhinoceros
130 (*Ceratotherium simum*) and 0/9 black rhinoceros (*Diceros bicornis*) from Waterberg National
131 Park (43), 0/27 impala from ENP (44) and 0/122 farmed springbok (*Antidorcas marsupialis*)
132 and gemsbok (*Oryx gazella*) (45). Only one publication investigated and reported the presence
133 of *C. burnetii* in KNP wildlife *i.e.* in vervet monkeys (*Chlorocebus pygerythrus*) (46) with no
134 investigations or reports on *C. burnetii* available from ENP, highlighting the lack of research
135 on these diseases in South African wildlife. The two parks differ in many aspects with the main
136 difference that might play a significant role in diseases is the presence of African buffalo in
137 KNP.
138 The objectives of this study were to (1) assess the presence/absence and estimate the prevalence
139 of infection or serological prevalence of selected pathogens in four free-ranging wild animal
140 species in KNP and ENP, (2) evaluate risk factors for infection, including animal species, sex,
141 and sampling park, and (3) assess significance of co-infections and/or co-exposure to multiple
142 pathogens.

MATERIALS AND METHODS

Study design

Whole blood and serum samples were collected during May 2018 to September 2019 from two national Parks, KNP and ENP, and the host species targeted in this study included free roaming greater kudu (n=72; 32 from KNP and 40 from ENP), plains zebra (n = 65; 39 from KNP and 26 from ENP), impala (n=21 from KNP) and blue wildebeest (n=30 from ENP) (Figure **1**). These samples were originally tested for the presence of antibodies against *Bacillus anthracis* (causal agent of anthrax) (47). The sample size was small due to budget constraints as the animals were chemically immobilized and collared to monitor their movement and exposure to *B. anthracis* in KNP and ENP (48). In the framework of the present work, the same samples were also screened using serology to detect FMDV, *Brucella* spp. and *C. burnetii* and DNA from blood using molecular reverse line blot method (RLB) to detect *Anaplasma, Ehrlichia, Theileria* and *Babesia* spp.

Each animal was selected randomly from different herds. When working with wildlife, it is often infeasible to count each individual of the reference population so as to select a random sample, so we cannot exclude a selection bias. All animals were adults or sub-adults as was required for the collaring study. Each sample was assigned a unique identification number. Supplementary data on sampling date and GPS location were recorded.

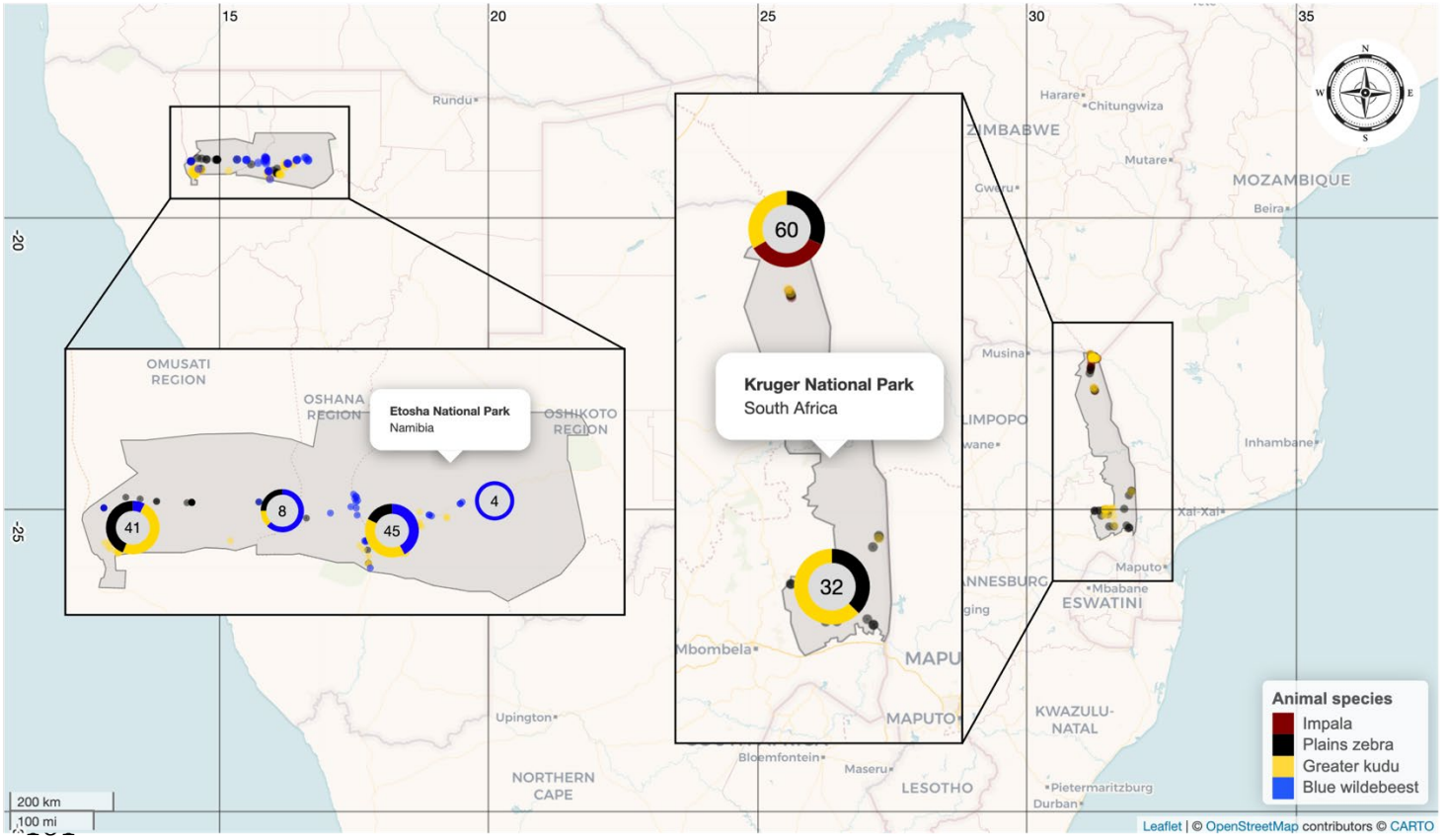


Figure 1: Spatial distribution of serum and EDTA blood samples collected in Kruger National Park (on the right) and Etosha National Park (on the left). Color legend stratifies samples per animal species, including impala (*Aepyceros melampus*), plains zebra (*Equus quagga*), greater kudu (*Tragelaphus strepsiceros*) and blue wildebeest (*Connachaetes taurinus*).

Study area

Kruger National Park (KNP) is situated in the Limpopo and Mpumalanga provinces of South Africa. It is regarded as one of the largest and most important National Parks in Africa, hosting a total of 148 wild mammal species, including the big five (*i.e.* lion, leopard, elephant, rhino and buffalo), in a 19,485 km^2 fenced conservation area situated in the FMD infected zone (49). Population estimates for the selected wildlife species in KNP include: 11,200-17,300 greater kudu, 132,300-176,400 impala, and 23,700-35,300 plains zebra (https://www.sanparks.org).

Etosha National Park (ENP), also situated in the FMD protected zone (50), is an almost 23,000 km^2 wildlife reserve located in northern Namibia. ENP is home to 114 mammal species but it

is not considered a big five reserve as African buffaloes are not present in the park (51). Aerial estimates of selected wildlife include: 2,822-5,592 blue wildebeest, 11,338-17,126 plains zebra (51) and 394-580 greater kudu (52).

Laboratory protocols

Blood-borne parasite detection

Pure-Link DNA extraction kit (Invitrogen, Germany) was used to extract DNA from 200 µl of each blood sample according to the manufacturer's instructions and eluted in 100 µl of elution buffer. The RLB hybridization assay was performed as previously described (53–56) to detect *Theileria, Babesia, Ehrlichia* and *Anaplasma* species. Negative and known positive controls were included for each pathogen species. The probes included in the RLB membrane are listed in Table S1.

Serological tests

For serological screening, we employed commercially available ELISA kits produced by ID-VET. The ID Screen FMD is a non-structural protein competitive ELISA (NSPCE) and was used for the detection of antibodies against the 3ABC proteins of FMDV. Similarly, the ID Screen Brucellosis Serum Indirect Multi-species ELISA was used to detect antibodies against the lipopolysaccharide (LPS) of smooth *Brucella* spp., while the ID Screen Q Fever Indirect Multi-species ELISA was used in the detection of antibodies against *C. burnetii* antigenic phases I and II. All the serum samples were run in duplicates and the coefficient of variation (%CV) was ensured to be less than 20% for all duplicates and less than 10% overall. FMDV SAT serotyping of NSPCE positive sera were tested by Agricultural Research Council – Onderstepoort Veterinary Institute (ARC-OVI), South Africa, and Central Veterinary

Laboratory in Namibia for all serotypes with a solid-phase cELISA (SPCE). SPCE is the official screening test in South Africa and Namibia.

For *Brucella* spp., serum was first screened using Rose Bengal Test (RBT) obtained from Onderstepoort Biological Products (OBP) as per manufacturer's instruction with the *Brucella* positive serum from OBP. Sera were analyzed using ID-VET Multi-species iELISA as per the manufacturer's instructions. Negative RBT sera were tested with iELISA in pools of 10 animals grouped animal species. If positive reactions were obtained in the pools, the samples were re-tested individually. Animals were confirmed seropositive only if positive to both RBT and iELISA due to the well documented problem of extensive serological cross-reactions with other bacteria (57).

Data analysis and reporting

Data were analyzed in R programming language (version 4.2.1) using the R studio IDE software (RStudio Team, 2021). To account for our small sample sizes, confidence intervals and hypothesis testing were estimated employing exact/non-parametric methods, and the results were interpreted with great caution. The 95% confidence intervals (CI) were calculated to measure variability and error of our estimated point prevalences by species. Because of small sample sizes, we opted for the more conservative Clopper Pearson method (58) using the R function "exactci" from the "PropCIs" package.

To determine which infections were most likely to co-occur in hosts, we used the Spearman's correlation coefficient (r_s) using function "cor" (with method = "Spearman") from package "stats" in R. Coefficient (r_s) values from 0 to 0.25 or from 0 to -0.25 indicate absence of correlation, whereas values from 0.25 to 0.50 or from -0.25 to -0.50 point to poor correlation between variables; values ranging from 0.50 to 0.75 or -0.50 to -0.75 are regarded as moderate

220 to good correlation, and r values from 0.75 to 1 or from -0.75 to -1 indicate very good to
221 excellent correlation between variables (59). This correlation was considered significant if the
222 t Test for Spearman Rank Correlation indicated a p-value <0.05 under the null hypothesis of no
223 correlation (58). When performing multiple comparisons, the family-wise error rate increases
224 hence the probability of finding at least one false positive (Type I error) (60). To yield
225 conservative results, p-values were adjusted using the Bonferroni correction in which the p-
226 values are multiplied by the number of comparisons (61). This was achieved by applying
227 function "p.adjust" (method "bonferroni") from package "stats".

228 To assess correlation between prevalence and independent variables (*i.e.* animal species, sex
229 and sampling park), we employed the Chi-squared test. An alternative when the conditions for
230 a chi squared test are not met (*i.e.* no cells with expected values < 1, and no more than 20% of
231 cells with values < 5), is a Monte Carlo simulation (62) performed with the option
232 "simulate.p.value = TRUE" in the function "chisq.test". We set the number of replicates in the
233 simulation of B = 2000. Again, p-values were adjusted using the Bonferroni correction and
234 statistical level was set at $\alpha = 0.05$.

235 RESULTS

236 A summary of the laboratory diagnostic results, including estimates and errors (95% confidence
237 intervals) of prevalences in each animal species and park are reported in Table 1.

238 **Table 1:** Seroprevalence of Foot-and-Mouth Disease Virus (FMDV), *Brucella* spp., and *Coxiella burnetii*, and prevalence of infection of
239 *Anaplasma, Ehrlichia, Theileria* and *Babesia* species in blue wildebeest (*Connachaetes taurinus*), kudu (*Tragelaphus strepsiceros*), impala
240 (*Antidorcas marsupialis*) and zebra (*Equus quagga*) from Kruger National Park, South Africa, and Etosha National Park, Namibia. Pathogens that
241 have not been detected in any of the wildlife species are not included here.

Pathogen species (Diagnostic)	**Positive/Tested = Prevalence [95% confidence interval]**					
	Blue wildebeest	**Greater kudu**		**Impala**	**Plains zebra**	
	Etosha National Park	**Etosha National Park**	**Kruger National Park**	**Kruger National Park**	**Etosha National Park**	**Kruger National Park**
Anaplasma/Ehrlichia spp. (RLB)	18/30 = **60%** [41-77%]	24/40 = **60%** [43-75%]	27/28 = **96%** [82-100%]	19/19 = **100%** [82-100%]	4/17 = **24%** [7-50%]	28/39 = **72%** [55-85%]
Anaplasma bovis (RLB)	1/30 = **3%** [0-17%]	0/40 = **0%** [0-9%]	6/28 = **21%** [8-41%]	0/19 = **0%** [0-18%]	0/17 = **0%** [0-20%]	0/39 = **0%** [0-9%]
Anaplasma centrale (RLB)	2/30 = **7%** [1-22%]	0/40 = **0%** [0-9%]	0/28 = **0%** [0-12%]	12/19 = **63%** [38-84%]	0/17 = **0%** [0-20%]	2/39 = **5%** [1-17%]
Anaplasma platys (RLB)	0/30 = **0%** [0-12%]	0/40 = **0%** [0-9%]	3/28 = **11%** [2-28%]	1/19 = **5%** [0-26%]	0/17 = **0%** [0-20%]	0/39 = **0%** [0-9%]
Anaplasma sp. (Omatjenne) (RLB)	1/30 = **3%** [0-17%]	0/40 = **0%** [0-9%]	11/28 = **39%** [22-59%]	5/19 = **26%** [9-51%]	0/17 = **0%** [0-20%]	0/39 = **0%** [0-9%]
Ehrlichia ruminantium (RLB)	0/30 = **0%** [0-12%]	0/40 = **0%** [0-9%]	0/28 = **0%** [0-12%]	0/19 = **0%** [0-18%]	0/17 = **0%** [0-20%]	2/39 = **5%** [1-17%]

Pathogen species (Diagnostic)	Positive/Tested = Prevalence [95% confidence interval]					
	Blue wildebeest	**Greater kudu**		**Impala**	**Plains zebra**	
	Etosha National Park	**Etosha National Park**	**Kruger National Park**	**Kruger National Park**	**Etosha National Park**	**Kruger National Park**
Theileria/Babesia spp. (RLB)	15/30 = **50%** [31-69%]	14/40 = **35%** [21-52%]	28/28 = **100%** [88-100%]	19/19 = **100%** [82-100%]	14/17 = **82%** [57-96%]	38/39 = **97%** [87-100%]
Babesia spp. (1) (RLB)	1/30 = **3%** [0-17%]	0/40 = **0%** [0-9%]	0/28 = **0%** [0-12%]	0/19 = **0%** [0-18%]	8/17 = **47%** [23-72%]	37/39 = **95%** [83-99%]
Babesia occultans (RLB)	9/30 = **30%** [15-49%]	0/40 = **0%** [0-9%]	0/28 = **0%** [0-12%]	0/19 = **0%** [0-18%]	0/17 = **0%** [0-20%]	0/39 = **0%** [0-9%]
Brucella spp. (RBT and iELISA)	0/29 = **0%** [0-12%]	0/40 = **0%** [0-9%]	3/29 = **10%** [2-27%]	0/21 = **0%** [0-16%]	0/25 = **0%** [0-14%]	0/35 = **0%** [0-10%]
Coxiella burnetii (iELISA)	26/30 = **87%** [69-96%]	16/40 = **40%** [25-57%]	6/29 = **21%** [8-40%]	14/21 = **67%** [43-85%]	26/26 = **100%** [87-100%]	18/39 = **46%** [30-63%]
Foot-and-mouth disease virus (NSPCE)	1/29 = **3%*** [0-18%]	0/40 = **0%** [0-9%]	13/29 = **45%*** [26-64%]	0/21 = **0%** [0-16%]	Not tested	Not tested
Theileria spp. (RLB)	10/30 = **33%** [17-53%]	0/40 = **0%** [0-9%]	27/28 = **96%** [82-100%]	19/19 = **100%** [82-100%]	7/17 = **41%** [18-67%]	33/39 = **85%** [69-94%]
Theileria bicornis (RLB)	0/30 = **0%** [0-12%]	0/40 = **0%** [0-9%]	27/28 = **96%** [82-100%]	19/19 = **100%** [82-100%]	0/17 = **0%** [0-20%]	1/39 = **3%** [0-13%]
Theileria buffeli (RLB)	0/30 = **0%** [0-12%]	0/40 = **0%** [0-9%]	27/28 = **96%** [82-100%]	19/19 = **100%** [82-100%]	0/17 = **0%** [0-20%]	1/39 = **3%** [0-13%]

Pathogen species (Diagnostic)	Positive/Tested = Prevalence [95% confidence interval]					
	Blue wildebeest	Greater kudu		Impala	Plains zebra	
	Etosha National Park	Etosha National Park	Kruger National Park	Kruger National Park	Etosha National Park	Kruger National Park
Theileria equi (RLB)	0/30 = **0%** [0-12%]	0/40 = **0%** [0-9%]	0/28 = **0%** [0-12%]	0/19 = **0%** [0-18%]	1/17 = **6%** [0-29%]	1/39 = **3%** [0-13%]
Theileria sp. (kudu) (RLB)	0/30 = **0%** [0-12%]	0/40 = **0%** [0-9%]	27/28 = **96%** [82-100%]	0/19 = **0%** [0-18%]	0/17 = **0%** [0-20%]	0/39 = **0%** [0-9%]
Theileria sp. (sable) (RLB)	11/30 = **37%** [20-56%]	0/40 = **0%** [0-9%]	25/28 = **89%** [72-98%]	5/19 = **26%** [9-51%]	0/17 = **0%** [0-20%]	0/39 = **0%** [0-9%]
Theileria taurotragi (RLB)	0/30 = **0%** [0-12%]	0/40 = **0%** [0-9%]	27/28 = **96%** [82-100%]	0/19 = **0%** [0-18%]	0/17 = **0%** [0-20%]	0/39 = **0%** [0-9%]

RLB = Reverse Line Blot; RBT = Rose Bengal Test; iELISA = indirect ELISA; NSPCE = non-structural protein competitive ELISA. * A subset of samples positive for Foot-and-Mouth Disease Virus (FMDV) based on NSPCE were tested for confirmation based on structural protein competitive ELISA (SPCE). All of these were negative by SPCE, including the wildebeest in Etosha and 4 kudu from KNP.

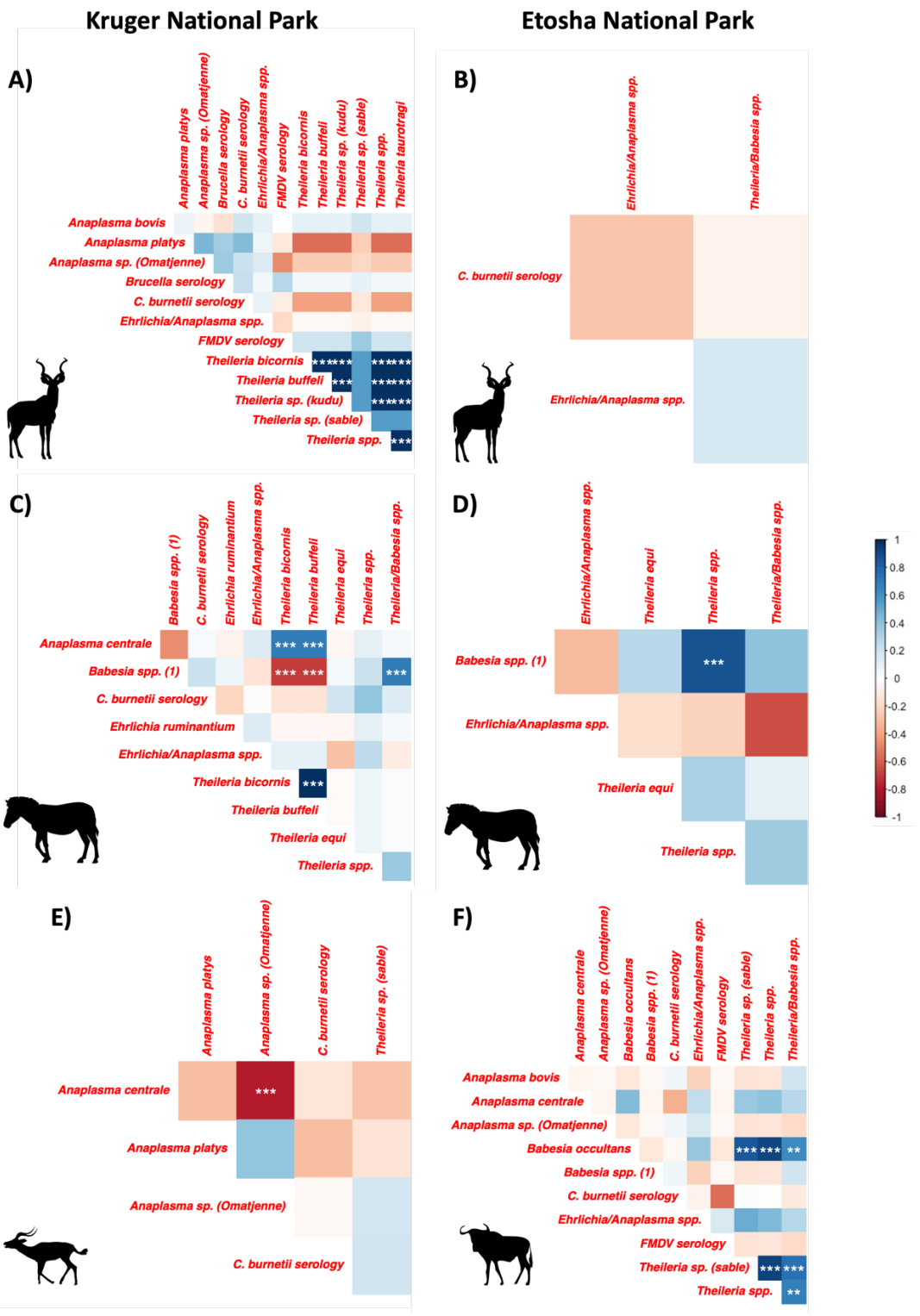
245 The NSP-cELISA for FMDV detected antibody in the sera of 13 greater kudu samples (40.6%;
246 13/32) from KNP, 12 of which had high titres (*i.e.* 10 < SN < 30; Supplementary Figure S1;
247 Table **1**). These animals were sampled during October 2018, mostly in the northern area of
248 KNP. Only four of the 12 FMDV positive kudu samples were tested with SPCE ELISA due to
249 financial constraints, none of which could be serotyped and thus interpreted as negative by
250 SPCE. In ENP, only one blue wildebeest (3.3%; 1/30), sampled near Ozonjuitji m'Bbari
251 (Central ENP) in July 2018, tested weakly positive using the NSP-cELISA for FMDV, but
252 tested negative using SPCE.

253 For *Brucella* spp., the first serological screening with RBT indicated four clear positive sera
254 (three kudu and one zebra from KNP), and an additional five (two kudu and one zebra from
255 KNP, and two wildebeest from ENP) were regarded as suspect due to a faint positive reaction.
256 At the second testing with the commercial *Brucella* spp. iELISA seven animals tested positive
257 and one suspect. From KNP, 3/29 kudu (10%) tested positive using both serological techniques
258 and were thus considered as confirmed seropositive. Additionally, eight greater kudu (28%;
259 8/29), one impala (5%; 1/21) and three plains zebra (9%; 3/35) tested positive using either the
260 RBT or iELISA assay and were regarded as negative results. The brucellosis positive animals
261 originate from KNP and were sampled mostly in the northern part of KNP. No animals in ENP
262 were positive for *Brucella* spp.

263 A summary of *C. burnetii* serology is reported in Table **1**. As a general trend, the prevalence of
264 antibodies against *C. burnetii* in all samples collected from ENP (71%) was much higher than
265 those collected from KNP (43%). We also report the presence of several strong reactions *i.e.*
266 high iELISA titers in most individuals (Supplementary Figure **S1**).

267 We investigated co-infection and co-exposure to the different pathogens (Figure **Figure 2**). We
268 highlight that in kudu from KNP, *T. buffeli, T. bicornis, Theileria* sp. (sable) and *Theileria* sp.

(kudu) occurred almost always together. In zebra from KNP, *T. bicornis* and *T. buffeli* occurred always together and were positively correlated with *A. centrale* ($p < 0.001$; $r_s = 0.7$) but negatively correlated to *Babesia* spp ($p < 0.001$; $r_s = -0.7$). On the other hand, in zebra from ENP positivity to the *Theileria* spp. probe was positively correlated to the *Babesia* spp. (1) probe (($p < 0.001$; $r_s = 0.87$). In impala from KNP, infection with *A. centrale* was negatively correlated to infection with *Anaplasma* sp. (Omatjenne) ($p < 0.001$; $r_s = -0.78$). In wildebeest from ENP, *B. occultans* infected animals were almost always co-infected with *Theileria* sp. (sable). Interestingly, one kudu from KNP (ID: TS-E-10, female, adult, sampled in KNP) bore most infections/exposures at the same time, as it was seropositive to FMDV, *Brucella* spp. and *C. burnetii,* and co-infected with *A. platys, Anaplasma* sp. (Omatjenne), *T. bicornis, T. buffeli, Theileria* sp. (kudu), *Theileria* sp. (sable) and *T. taurotragi.* According to the Pearson's Chi-squared test (with Monte-Carlo replicates), the variables "Sampling Park" and "Animal species" were the most associated with pathogen prevalence and seroprevalence (Table **2**).



282

283 **Figure 2:** Correlation matrix representing correlation coefficients for concurrence of pathogen
284 infection/exposure in kudu (*Tragelaphus strepsiceros*) (A-B), zebra (*Equus quagga*) (C-D),
285 impala (*Antidorcas marsupialis*) (E) and wildebeest (*Connachaetes taurinus*) (F) from Kruger
286 National Park (left panel) and Etosha National Park (right panel). Blue squares indicate positive
287 correlation, red squares indicate negative correlation. Color intensity indicates strength of

correlation. Asterisks indicate significant correlation: *** = p-value <0.001; ** = p-value < 0.01; * = p-value < 0.05. P-values were adjusted with Bonferroni correction.

Table 2: Sample sizes, Bonferroni corrected p-values and X^2 values of Pearson's Chi-squared test with Monte Carlo simulation where prevalence has been used as outcome variable. Significant *p*-values are displayed in bold.

Pathogen (sample size)	**Bonferroni corrected p-values (X^2 values)**		
	Animal species	**Sex**	**Sampling Park**
Anaplasma bovis (173)	1 (7.2)	1 (1)	1 (3.8)
Anaplasma centrale (173)	**< 0.001 (75.1)**	1 (0.6)	0.152 (10.1)
Anaplasma platys (173)	1 (4.1)	1 (0)	1 (4.1)
Anaplasma sp. (Omatjenne) (173)	0.076 (16.5)	1 (0)	**< 0.001 (14.9)**
Babesia occultans (173)	**< 0.001 (45.3)**	1 (0.7)	0.38 (9.4)
Babesia spp. (1) (173)	**< 0.001 (122.8)**	1 (1.5)	**< 0.001 (23.7)**
Ehrlichia ruminantium (173)	1 (4.2)	1 (2.3)	1 (2)
Ehrlichia/Anaplasma spp. (173)	0.304 (14.6)	1 (0.4)	**< 0.001 (22.4)**
Theileria bicornis (173)	**< 0.001 (85.8)**	1 (0.1)	**< 0.001 (65.3)**
Theileria buffeli (173)	**< 0.001 (85.8)**	1 (0.1)	**< 0.001 (65.3)**
Theileria equi (173)	1 (4.2)	1 (0)	1 (0)
Theileria sp. (kudu) (173)	**< 0.001 (49.4)**	1 (0)	**< 0.001 (32.4)**
Theileria sp. (sable) (173)	**< 0.001 (26.7)**	1 (0)	0.076 (11.8)
Theileria spp. (173)	**< 0.001 (33.8)**	1 (1.5)	**< 0.001 (91.6)**
Theileria taurotragi (173)	**< 0.001 (49.4)**	1 (0)	**< 0.001 (32.4)**
Theileria/Babesia spp. (173)	**< 0.001 (31.3)**	1 (1.9)	**< 0.001 (54.9)**
Brucella spp. (179)	1 (4.9)	1 (0.3)	1 (3.4)
Coxiella burnetii (183)	**< 0.001 (32.4)**	1 (1.7)	0.076 (14.9)
Foot-and-Mouth Disease Virus (111)	1 (8.1)	1 (0.3)	**< 0.001 (16.8)**

DISCUSSION

This study established baseline data of infection with tick borne diseases as well as exposure to FMD, coxiellosis and brucellosis in four wild animal species in two national parks. Laboratory analysis revealed very high prevalence (70-100%) of *Theileria/Babesia* and *Anaplasma/Ehrlichia* spp. infection in kudu, impala and zebra from KNP. Moreover, most or even all of the zebra and wildebeest sampled in ENP were seropositive for Q fever. Indeed, the seroprevalence of Q fever was found to be significantly higher in ENP while *Brucella* spp., *Anaplasma, Ehrlichia, Theileria* and *Babesia* species were significantly higher in KNP.

Anaplasma/Ehrlichia and *Theileria/Babesia* prevalences are higher in KNP compared to ENP

As highlighted by the comparison of the 95% CI and the chi-square statistics, infection prevalences of *Anaplasma/Ehrlichia* and *Theileria/Babesia* genera were significantly higher in KNP compared to ENP in both kudu and zebra. This may be due to the relative diversity and abundance of ticks inhabiting the parks. Indeed, the prevalence of tick infestation in ENP wildlife is reportedly well below those reported in other parts of southern Africa (63–65). Tick distribution and ultimately the survival of pathogens in ticks and animal hosts are, in turn, affected by abiotic factors. Indeed, hot dry conditions and desiccating winds adversely affect the population of questing ticks by imposing mortality on unfed ticks (66). Moisture-related indices significantly affect the presence of ticks and TBDs, with wetter conditions almost always beneficial (66). ENP is located in a semi-arid region of Namibia characterized by a large salt pan, which may be dry for extended periods of the year, especially during the dry season (67). On the other hand, KNP is situated in northeastern South Africa and has a more diverse climate with a greater availability of water throughout the year compared to ENP. Overall, ENP is considerably drier than KNP and therefore a less suitable region than KNP for tick

proliferation, infestation and transmission of TBDs. For instance, *Amblyomma hebraeum, Amblyomma variegatum* (vectors of *Ehrlichia ruminantium*), *Rhipicephalus decoloratus* (vector of *Babesia bigemina* and *Anaplasma marginale*), *Rhipicephalus appendiculatus* (vector of *Theileria parva* and *Anaplasma bovis*) are present mainly or only in KNP, whereas *Hyalomma rufipes* (vector of *Babesia occultans*), *Hyalomma truncatum* (vector of several *Anaplasma/Ehrlichia* spp.) and *Rhipicephalus evertsi* (vector of *T. equi* and *B.caballi*) are found in both parks (68–70).

High prevalence and co-infection of *Theileria* spp. in kudu and impala from KNP

In the present study, we report extremely high prevalence of *T. buffeli* and *T. bicornis* in 27/28 kudu (96%; CI: 82-100%) and 19/19 impala (100%; CI: 82-100%) from KNP. In addition, in KNP kudu, there was high prevalence (90-100%) and significantly associated co-infections of pathogens from the genera *Theileria*, including *T. taurotragi, T. buffeli, Theileria* sp. (kudu) and *Theileria* sp. (sable) (Table **1**). *Theileria* spp. (sable) was also detected in 5/19 impala (26%; CI: 9-51%) from KNP. None of the 40 kudu from ENP tested positive for any of the tested *Theileria* species.

Theileria taurotragi and *T. buffeli* are "schizont non transforming" *Theileria* spp. and therefore classified as benign parasites, with rare clinical signs that mainly occur due to piroplasm-induced acute hemolytic anemia (71). Indeed, *T. taurotragi* caused bovine cerebral theileriosis in young African shorthorn cattle (71) and theileriosis in eland (*Tragelaphus oryx*) (71). *Theileria* sp. (sable) and *Theileria* sp. (kudu) (56) are regarded as pathogenic species in African wild artiodactyls. Mortalities in roan antelope (*Hippotragus equinus*) due to *Theileria* sp. (Sable) have been reported after translocation (56). Infection with *Theileria* sp. (sable)

negatively affects attempts to establish breeding herds and reintroduction efforts into the wild due to calf mortalities (72). *Theileria bicornis* has not been found to cause mortality but has been reported in free-ranging white and black rhinoceroses in South Africa and Kenya (55,73,74), as well as from apparently healthy nyalas (*Tragelaphus angasii*), (75), impalas, eland (*Taurotragus oryx*) and sable antelope (*Hippotragus niger*) in South Africa (76). The very high *T. bicornis* prevalences obtained in this study in kudu and impala from KNP (Table **1**) might raise concerns for the rhino populations as they are already suffering from poaching and stress induced by unavoidable translocations (77,78).

Further studies may assist in determinating the health effects of the above-mentioned *Theileria* infections in wildlife species. Co-infections may alter virulence of pathogens and subsequent disease outcomes in the hosts (79–81). As a general rule, co-infections may lead to worse health outcomes for hosts and increase within host pathogen titers, altering transmission ecologies. Nevertheless, the impact on animal fitness due to coinfections between pathogenic and benign *Theileria* species appears to be intricate. For instance, apathogenic *T. mutans* and *T. velifera* seem to protect cattle from the detrimental consequences of *T. parva* infection (82). This could also be our case, with the benign *T. taurotragi, T. bicornis, T. buffeli* protecting wild antelopes from the adverse effects of pathogenic *Theileria* sp. (sable) and *Theileria* sp. (kudu), but this hypothesis needs further investigation. The occurrence and effects of co-infection of multiple pathogen species within wildlife populations remains largely unknown. Indeed, understanding dynamics of co-infection or co-exposure to different pathogens are useful in improving our knowledge of pathogen epidemiology in wildlife and in the development of risk models for diseases in various epidemiological contexts.

Anaplasma centrale in impala and zebra from KNP and wildebeest from ENP

Anaplasma centrale and *A. marginale* are closely related species that cause bovine anaplasmosis in cattle (83). *Anaplasma centrale* is known to be less pathogenic than *A. marginale* in domestic animals as it induces a low degree of anaemia, with rare clinical outbreaks (84) but it confers immunity against infection by *A. marginale*. Nonetheless, a clinical case of bovine anaplasmosis caused by *A. centrale* was reported in Europe in 2008 (85). *Anaplasma centrale* seems to be largely subclinical in wildlife (38) where it occurs with moderate prevalences (10 to 30%), especially in African buffalo, impala, eland, waterbuck (*Kobus ellipsiprymnus*), blue and black wildebeest (*Connachaetes gnou*) (37–39,76,86). These wild animal species may be able to maintain *A. centrale* much more efficiently than tick vectors. In fact, although experimental transmission of *A. centrale* by ticks (*e.g. Rhipicephalus simus, Dermacentor andersoni*) has been proven (87,88), secretion of this pathogen into tick saliva occurs at a much lower rate than *A. marginale* and, hence, transmission is achieved only when tick numbers are dramatically increased to compensate for the low pathogen load (88). In addition, *A. centrale* prevalence in ticks is very low in all tick species considered (89), making them an inefficient reservoir for *A. centrale*. In support of this hypothesis, we report infection with *A. centrale* in 12 impalas (63%; 12/19) and two zebra (5%; 2/39) from KNP, and in two wildebeest (7%; 2/30) from ENP. The occurrence of *A. centrale* in impala from KNP is not surprising as the pathogen was already reported in the same species and in buffalo, black wildebeest, common eland and waterbuck from South Africa (37–39,76,86), while the occurrence of *A. centrale* in zebra from KNP and wildebeest from ENP is a new finding that sheds light on the geographic and host range of the pathogen.

Anaplasma platys in kudu and impala from KNP

Anaplasma platys is the etiologic agent of thrombocytic anaplasmosis in dogs and is the only recognized *Rickettsiales* species known to infect platelets (90). After the first description, *A. platys* has been reported worldwide, including the Americas, Eurasia, Africa, and Australia, mainly in tropical and subtropical areas (91–93). For a long time, *A. platys* was considered only a canine pathogen, but a wider host tropism for *A. platys* has been demonstrated in recent decades. Cases of *A. platys* infection have been reported in cats, goats, cattle, Bactrian camels (*Camelus bactrianus*), red deer (*Cervus elaphus*), sika deer (*Cervus nippon*) and sable antelope (94–101). Occurrences in atypical hosts have been attributed to *A. platys*-like bacteria (102,103). However, *A. platys*-like species cannot be distinguished from *A. platys* based on 16S rRNA as they are very closely related. These *A. platys*-like species in atypical hosts are considered the probable cause of human infections (104), with clinical signs varying from chronic and nonspecific, including headaches and muscle pains (105) to migraines and seizures due to mixed *A. platys*, *Bartonella henselae*, and "*Candidatus* Mycoplasma haematoparvum" infection (106).

Rhipicephalus sanguineus is considered the primary vector for *A. platys* (98,107,108) which rarely infests impala and kudu. The agent has also been detected in *Haemaphysalis longicornis* and *Ixodes persulcatus* in Korea, *Rhipicephalus turanicus* in Israel, and *Rhipicephalus* spp. in China (98,109–111).

Here, we found three kudu (11%; 3/28) and one impala (5%; 1/19) positive to *A. platys* by means of RLB hybridization. Given the limited information available on *A. platys* infections in Africa, it is of particular interest to understand the sylvatic cycle of *A. platys* in kudu and impala and which tick vector (if any) is involved in pathogen transmission.

Babesia occultans in wildebeest from ENP

Babesia occultans is considered less pathogenic than other *Babesia* species (112). Observable clinical signs due to infection with *B. occultans* in cows include anorexia, weakness, fever (≤ 40 °C), anaemia, and pale mucous membranes. However, unlike *B. bigemina, B. bovis*, and *B. divergens* infections, no jaundice, hemoglobinuria, gastrointestinal disorders, and nervous symptoms have been found in cows infected with *B. occultans* (113,114). In this study, we identified nine *B. occultans* positive wildebeest (30%; 9/30). Since its clinical signs are nearly identical to those of piroplasm infections, it is important for local animal health officers and veterinarians to acknowledge the presence of the pathogen and consider it in diagnoses and treatment strategies.

Ehrlichia ruminantium in KNP zebra

Reports of *E. ruminantium* in African non-ruminant wildlife are rare and controversial. For instance, *E. ruminantium*-like colonies were detected in brain endothelial cells of a Nigerian African elephant (*Loxodonta africana*) that reportedly died of anthrax (115). This report requires verification due to the unusual nature of the case and the possible presence of pathogens similar to *E. ruminantium*. Black and white rhinoceroses from Zimbabwe tested serologically positive to *E. ruminantium* using a MAP1 competitive ELISA (116). However, this technique is known to cross-react with other *Anaplasmataceae* (117) and, therefore, no confirmation can be drawn from these findings.

In our study, two plains zebra from KNP tested positive to *E. ruminantium* with RLB. The occurrence of the pathogen in a wild equid could be most likely incidental, but it may still be of epidemiological importance to understand the source of infection and transmission

dynamics, for which further molecular characterization of the pathogen may provide significant insights.

Seropositivity to FMDV in greater kudu in KNP

A total of 13 greater kudu (41%; 13/32) from KNP sampled in October 2018, South Africa, were found seropositive to FMD by means of NSPCE. While natural infection with FMD has already been reported in greater kudu from Botswana by means of reverse-transcriptase PCR (118,119), the present study represents the first report of FMD based on NSPCE in greater kudu in South Africa using serology. This test has not been validated for wildlife. Risk factor analysis (Table **2**) indicates that greater kudu has significantly higher prevalence of FMD among the affected animal species investigated. The location (sampling park) was a significant predictor of infection. Antibodies against 3ABC complex of FMDV can be detected in a window of between 1 week to 6 months after exposure to the pathogen (120). These observations point to circulation of FMD in kudu population from the northern area of KNP that were exposed to the pathogen anytime during April-October 2018. Interestingly, this event might have occurred in proximity and just a few months before the January 2019 outbreak in Vhembe district, Limpopo, South Africa in cattle. Greater kudu has been reported to shed the virus up to 160 days after experimental infection, more than any other African non-buffalo bovid ("antelope"), and clinical signs have been reported from this species without mortality (118,119,121). Nonetheless, the role of kudu in maintaining and spreading FMDV is still to be investigated and clarified. This report underscores the importance of further investigation into the role of kudu in the epidemiology of FMD in Kruger National Park and validation of FMD serological tests for wildlife. The lack of seropositive kudu from ENP- – where buffalo populations are absent – may indicate that the source of infection for kudu in KNP was most likely the contact with FMD-infected buffaloes. As highlighted by Thomson et al. (19) and Hargreaves et al.

455 (122), antelope species (like kudu and impala) infected through contact with buffalo herds
456 within the park, have the potential to jump over the fences and transmit the virus to the cattle
457 living in adjacent communal farms. SPCE is the official screening test in South Africa and
458 Namibia for livestock, which is not validated for wildlife. In this study, the SPCE for SAT-1, 2
459 and 3 was negative in KNP and all serotypes in ENP. However, to our knowledge this work
460 represents the first attempt of FMD SAT serotyping in African non-buffalo species by SPCE
461 (6); hence the sensitivity of the technique in these animals is not known as there has been no
462 report, to our knowledge of SPCE for SAT in kudu and wildebeest. SPCE is serotype specific
463 meaning that it targets the structural proteins whose aminoacidic variability is per definition the
464 highest among all viral proteins (119). Antigenic variation is considered more common in wild
465 animal populations, due to repeated exposure and immune selective pressure of a highly diverse
466 population of infected host species (120,121). The strains of the serotypes (SAT1-2-3) coated
467 to the plate of the SPCE may be significantly different than the ones circulating in KNP wildlife,
468 as the SPCE is validated for livestock animals. Hence the sensitivity of the SPCE might be
469 mildly to markedly lower than the NSPCE, which on the other hand targets a highly conserved
470 component of the FMDV capsid *i.e.* the 3ABC complex. Alternatively, positive reactions in
471 kudu by NSPCE might be considered as false positive results, although this is very unlikely due
472 to the high specificity of the test (>99%) which does not depend on a species-specific conjugate
473 (being a competitive ELISA), and also due to the high titres observed in 12 kudu from KNP
474 (38%; 12/32). Additional research and characterization (using VNT or other tests) are strongly
475 expected to shed light on this phenomenon and could be investigated in the future using
476 available samples.

Seropositivity to FMDV in a blue wildebeest from Etosha National Park

One blue wildebeest (CT05, male, adult; 3%; 1/30) from Etosha National Park, Namibia, was found seropositive for FMD by means of NSPCE but seronegative using SPCE. This finding has to be interpreted cautiously because: the positive sample had a S/N percentage close to the ELISA cutoff (Figure **S1**); all the other animals (kudu and wildebeest) from the same park, area and sampling period, tested negative by the assay; buffalo, considered the main maintenance host for FMD in wildlife, are not present in ENP (67). FMD infection in blue wildebeest from Tanzania, Botswana and Kenya has been reported by means of RT-PCR with serotypes O, A, SAT-1 and SAT-2 (121,123). Blue wildebeest may also suffer the clinical disease, developing oral and foot lesions associated with lameness, fever and inappetence (123). However, the NSPCE results were not confirmed with SPCE and thus require further investigation using a larger samples size and alternative techniques such as RT-PCR on oropharyngeal lymph nodes.

Confirmed *Brucella* exposure in KNP kudu, questionable for plains zebra, blue wildebeest and impala

Three kudu (10%; 3/29) in KNP could be considered seropositive for *Brucella* spp. These animals reacted to two serological tests and an additional five kudu were positive to only one serological technique. Numerous studies conducted in southern Africa could not find any serological response in greater kudu, although sample sizes were often small (<30) and used serological tests validated for livestock (124–128). In this study, seropositivity means that kudu were exposed to *Brucella* spp. and it remains unknown whether they are incidental hosts or part of the maintenance host community for *Brucella* spp. in wildlife. Three plains zebra from KNP (9%; 3/35) tested positive either with RBT (two animals) or iELISA (one animal) and were regarded as suspect cases. This is an area for additional research as agglutination reaction to

Brucella spp. in zebra has been reported by a previous study (129). The domestic horse, which is evolutionarily related to zebra, has been demonstrated to harbor different *Brucella* spp. (*i.e. B. abortus* and *B. suis* under natural circumstances and *B. canis* after experimental challenge), and may eventually experience clinical signs (fistulous withers, abortion and other reproductive problems) (130). Moreover, a study from Nigeria conducted by Bertu et al. (131), isolated *B. abortus* from asymptomatic horses living in a multispecies farm in Nigeria. However, the risk of transmission of brucellosis from equids is still to be clarified as horses have been indicated as dead-end host (132).

Widespread exposure to *Coxiella burnetii* in KNP and ENP

In this study, a remarkably high number of individuals (57%; 106/185) across all evaluated wild animal species (44/65 zebra, 22/69 kudu, 14/21 impala, 26/30 wildebeest) tested positive to the *C. burnetii* iELISA (Table 1). We also obtained many strong positive reactions (19%; 35/185) in any species considered (33/65 zebra, 9/69 kudu, 9/21 impala, 18/30 blue wildebeest). Finally, our seroprevalence estimates were significantly different than those reported by Gakuya et al. (133), where similar wildlife species were investigated in Kenya using the same serological technique (iELISA). These findings led us to assume that *C. burnetii* is ubiquitous in both KNP and ENP and might have a predilection for southern Africa's ecosystems and/or soils. A significantly higher seroprevalence was registered in animals from ENP. Coxiellosis seroprevalence was especially higher in blue wildebeest, plains zebra and impala. However, the multispecies *C. burnetii* iELISA has only been validated for use in domestic animals and not wildlife and has not been validated for wildlife species as iELISA tests are designed to be host specific. Use of inaccurate tests could overestimate the prevalence of disease. In multiple species iELISA assays, IgG-binding proteins (such as protein A, protein G and protein A/G) are suggested and used as conjugates (134–137) but it is not known how these react with every

524 wildlife host species. According to Kelly et al. (135) and Stobel et al. (137), impala, wildebeest,
525 greater kudu and zebra react weakly with protein A and strongly with protein A/G, while
526 binding affinity with protein G varies; for impala and wildebeest, reactivity is weak, whereas
527 for kudu it is moderate and for zebra is strong. The binding affinity with protein A/G is
528 particularly strong for kudu (135). The Q fever iELISA kit employed in this study used protein
529 A/G. Considering all the facts discussed above, additional investigation may determine if kudu
530 is less affected/exposed to *C. burnetii* than the other species.

531 Further testing on tissues of wild animals matched with investigation in feeding ticks, may
532 provide important details for the clarification of Q fever epidemiology in African wildlife. Also,
533 the expansion of *C. burnetii* investigations in predator animals may provide further information
534 on the sylvatic cycle of the pathogen.

535 Limitations of the study and suggestions

536 We could detect reactions to nonspecific probes for *Anaplasma/Ehrlichia* and
537 *Theileria*/*Babesia* in ENP, but not too many of the species-specific probes investigated. This
538 suggests that the strains present in ENP may not be detectable by the probes which were
539 designed for strains occurring in South Africa due to the presence of local SNPs that do not
540 allow binding with RLB probes. Sequencing data could characterize *Anaplasma/Ehrlichia* and
541 *Theileria*/*Babesia* species occurring in ENP wildlife and thus design probes that can hybridize
542 reliably also with these strains. It may also indicate the occurrence of new species not reported
543 in literature.

544 RLB probes cross-reactions are not infrequent and a subset of positive samples should be
545 sequenced to confirm specificity of the RLB probes. However, due to funding constraints, we
546 could not sequence nor characterize any positive RLB occurrences. As a future study, it would

547 be particularly interesting to sequence and confirm the occurrence of *A. platys* in kudu and
548 impala and *E. ruminantium* in zebra from KNP, given their relevance for human and animal
549 health.
550 For serology, there is lack of known positive reference material from wild animals. Multispecies
551 ELISA make use of conjugates that react with multispecies with cutoffs that are not animal
552 species-specific. It is ideal to develop and validate ELISA assays specifically tailored for
553 detecting FMDV, brucellosis, and coxiellosis across a range of wildlife species.

554 Our prevalence estimates have wide confidence intervals due to small sample sizes and need to
555 be interpreted cautiously. Interpretations and interventions are conducted by considering both
556 the point estimate/prevalence as well as the entire confidence interval, that is where the true
557 population lies with 95% confidence.

558 Samples used in this study were part of another project that aimed to unravel differences in
559 exposure to anthrax in endemic and non-endemic locations. Although randomization was
560 introduced as much as possible when selecting sampling units, a moderate-high selection bias
561 has to be considered as it is not possible to extract a proper random sample from wildlife.
562 Moreover, due to prior use in other research, the total number of available samples was reduced
563 leading to a slight discrepancy in the number of animals tested for certain pathogens. For
564 instance, out of the total 32 kudu samples collected from KNP, we had only 28 sera and 29
565 DNA samples available for testing. This depletion meant that for four of the 32 kudu, we had
566 only one of the two sample types available (either DNA or sera, but not both).

567 CONCLUSION

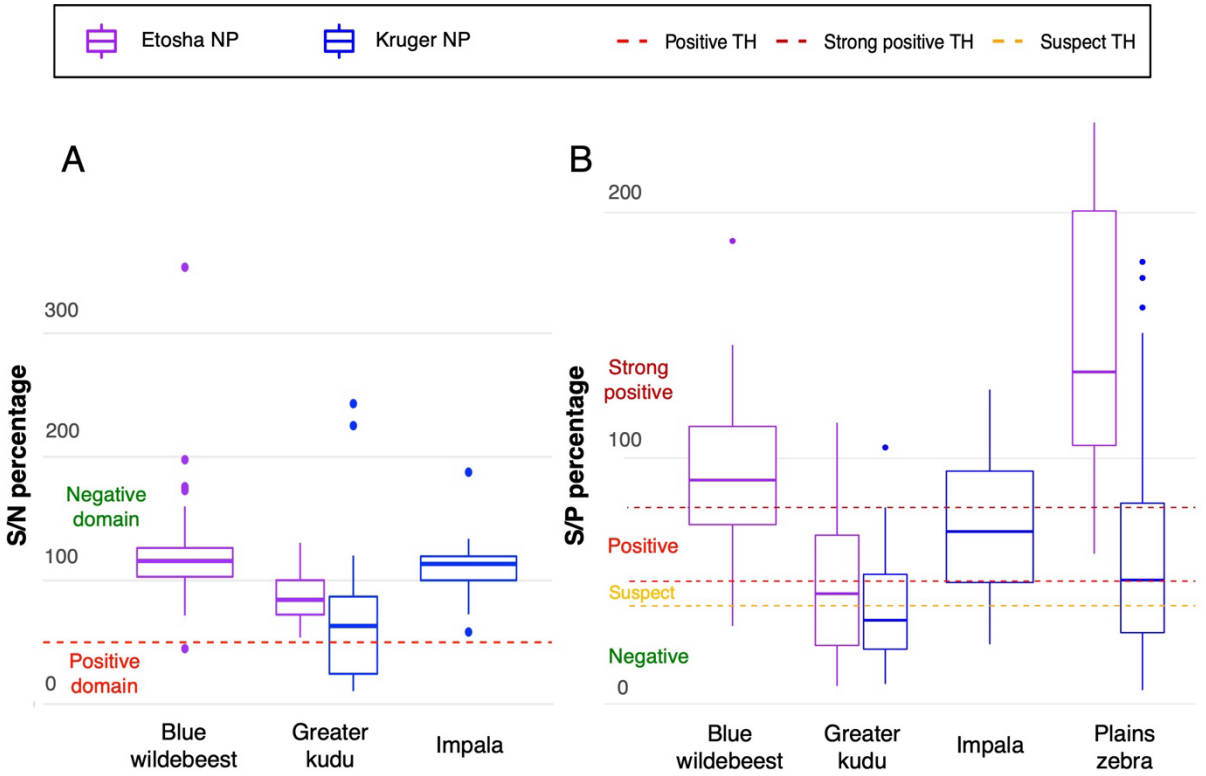
568 With the present study, we report infections and exposure to several pathogens in wild animal
569 species. We provided evidence-based information that increased the knowledge of

570 pathogen/disease epidemiology in natural settings. This work constitutes a baseline of data
571 useful for implementation and improvement of surveillance and monitoring tools, which are
572 highly valuable for public and animal health stakeholders (*i.a.* farmers, communities,
573 governments), and lay the foundations for considerable research advancement.

574 SUPPORTING MATERIAL

575 Table S1: Oligonucleotide probes fixed on the RLB membrane for the detection of *Anaplasma*,
576 *Ehrlichia*, *Theileria* and *Babesia* spp. DNA.; References (53–56, 138–151) are here cited.

		(Nagore2004?)



578

579 Figure S1: Boxplots of A) ELISA S/N percentages for foot and mouth disease virus (FMDV)
580 and B) ELISA S/P percentages for *Coxiella burnetii*. TH = Threshold. Boxplot for *Brucella*
581 spp. iELISA S/P percentages are not shown since some of the samples were tested in pools.

582 ## ETHICS STATEMENT

583 The project received research and animal ethics permits under reference number REC047-22
584 and section 20 approval under the Animal Disease Act 35 of 1984 that allows serological testing
585 at Hans Hoheisen Wildlife Centre laboratory in South Africa. The standard operating
586 procedures of the laboratory were followed with all the safety precautions as stipulated.

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AUTHORS CONTRIBUTION (CRediT taxonomy)

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All authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

Raw data are publicly available on Mendeley Data: https://data.mendeley.com/preview/ssf29pytwf?a=47d91a5e-2b3b-4764-8308-a3583af567bc. Questions about the data may be directed to the corresponding author.

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CONFLICT OF INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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