The Application of Immunological Biomarkers and Enhanced Pathogen Detection for the Epidemiological Characterisation of Bovine Tuberculosis in African Rhinoceros

by
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Declaration

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This dissertation consists of 7 chapters which includes 3 original publications in peer-reviewed journals (Chapters 2-4), one manuscript submitted to a peer-reviewed journal (Chapter 5), and 3 chapters that include the general introduction (Chapter 1), the general discussion (Chapter 6) and the conclusion (Chapter 7). The development and writing of the papers (published and unpublished) were the principal responsibility of myself and for each of the cases where this is not the case, a declaration is included in the dissertation indicating the nature and extent of the contributions of co-authors.

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Summary

African rhinoceros, specifically the black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceros, are iconic species that are under threat due to poaching for their horns, range/habitat loss, unbalanced genetic/demographic structure, climate change, and infectious diseases, including tuberculosis (TB). *Mycobacterium bovis* (*M. bovis*) infection, a cause of TB, has been identified in African rhinoceros populations in Kruger National Park (KNP), South Africa. An interferon-gamma release assay (IGRA) is routinely applied for testing of individuals earmarked for translocation out of the park, and for general surveillance purposes. However, relatively little is understood about the overall susceptibility and pathogenesis of TB in these species, and its impact on affected populations.

This study had four broad aims: i.) to collate information on the epidemiology of *Mycobacterium tuberculosis* complex (MTBC) infections in African rhinoceros, ii.) to determine prevalence and risk factors for *M. bovis* infection in KNP rhinoceros, iii.) to assess the impact of refrigeration and delayed stimulation of rhinoceros whole blood on mitogen stimulated interferon-gamma (IFN-γ) production, to increase flexibility in implementation of testing, and iv.) to determine whether MTBC can be detected in nasal swabs from rhinoceros with immunological evidence of infection, as an indication of potential infectiousness.

Drawing from existing literature on MTBC infections in other species and contexts, a foundational understanding of TB epidemiology in rhinoceros species was developed. In other species, demographic risk factors include sex and age, with males and adults generally being at higher risk than females and younger individuals. Review of limited historical information reflected similar age- and sex-associated patterns for TB in captive African rhinoceros, with more reports of TB disease in black rhinoceros than white rhinoceros. Intra-species transmission of MTBC in rhinoceros was also considered to be a potential source of infection. Free-ranging rhinoceros in bovine TB (bTB) endemic areas may be exposed to MTBC, likely shed by maintenance hosts in KNP such as African buffaloes (*Syncerus caffer*), greater kudus (*Tragelaphus strepsiceros*), or warthogs (*Phacochoerus africanus*), through shared environmental niches, and resources.

Based on previous reports, hypotheses were generated then investigated in a population-based study of *M. bovis* infection in 437 African rhinoceros in KNP. We determined an estimated overall infection prevalence of 15.4% (95% CI: 10.4-21.0%) based on mycobacterial culture and IGRA results for animals sampled between 2016-2020. Notably, a significant spatial

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clustering of cases was detected near the southwestern park border, although infection was widely distributed. Multivariable logistic regression models, including demographic and spatiotemporal variables, showed a significant, increasing probability of *M. bovis* infection in white rhinoceros based on increased numbers of African buffalo herds in the vicinity of the rhinoceros sampling location. Spillover of infection from African buffaloes to white rhinoceros sharing the environment was suspected. There was also a significantly higher proportion of *M. bovis* infection in black rhinoceros in the early years of the study (2016-2018) than in 2019 and 2020, which coincided with periods of intense drought, although other temporal factors could be implicated. Species of rhinoceros, age, and sex were not identified as risk factors for *M. bovis* infection.

Ante-mortem surveillance for *M. bovis* infection in the Kruger National Park (KNP) rhinoceros population currently relies on results from (QFT)-Mabtech equine interferon-gamma (IFN-γ) release assay (IGRA). However, the requirement for same-day processing of rhinoceros blood samples for the IGRA is a logistical challenge to performing this test, particularly in remote locations. A pilot study showed that relative concentrations of IFN-γ (based on optical density values) in mitogen stimulated whole blood plasma decreased significantly with increased time blood was stored post-collection and prior to QFT stimulation. These findings support a need for same-day processing of rhinoceros blood samples for QFT-IGRA testing, as per the current practice to ensure optimal test performance.

It was previously unknown whether *M. bovis*-infected rhinoceros could shed mycobacteria in respiratory secretions. Previous studies suggested that subclinically *M. bovis*-infected rhinoceros may pose minimal transmission risk. However, recent advances that have improved detection of MTBC members in paucibacillary samples prompted further investigation of respiratory secretions from rhinoceros with immunological evidence of infection, to elucidate the potential for mycobacterial shedding. A pilot study detected *M. bovis* in 14/64 (22%; 95% CI: 13-33%) of the IGRA positive rhinoceros, and none in the IGRA negative rhinoceros (n = 11) studied, suggesting that *M. bovis*-infected rhinoceros may be a source of infection for other susceptible animals sharing the environment.

Overall, these studies address important knowledge gaps related to surveillance and epidemiology of TB in African rhinoceros, specifically, the free-ranging populations in KNP. This has created awareness of the potential threat of this pathogen to the conservation of these species and highlighted important areas for future research that will contribute to understanding the multi-host TB ecosystem in KNP and other complex systems.

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Opsomming

Afrikaanse renosters, spesifiek die swart (*Diceros bicornis*) en wit (*Ceratotherium simum*) renosters, is ikoniese spesie wat bedreig word deur stropery vir hul horings, verlies van habitat, ongebalanseerde genetiese en demografiese struktuur, klimaatverandering, en aansteeklike siektes, insluitend tuberkulose (TB). *Mycobacterium bovis* (*M. bovis*) infeksie, 'n oorsaak van TB, is geïdentifiseer in Afrika-renosters in die Kruger Nasionale Park (KNP), Suid-Afrika. 'n Interferon-gamma vrystellingstoets (IGRA) word gereeld gebruik vir toetsing van individue wat vir verplasing uit die park gemerk is, en vir algemene toesig. Tog is daar relatief min begrip van die algemene vatbaarheid en ontwikkeling van TB in hierdie spesies, en die impak daarvan op die geaffekteerde bevolkings.

Hierdie studie het vier breë doelwitte gehad: i) om inligting oor die epidemiologie van *Mycobacterium tuberculosis* kompleks (MTBC) infeksies in Afrika-renosters bymekaar te bring, ii) om die voorkoms en risikofaktore vir *M. bovis* infeksie in KNP-renosters te bepaal, iii) om die impak van verkoeling en vertraagde stimulasie van renosters se volbloed op mitogeen gestimuleerde IFN-γ produksie te beoordeel, vir meer vryheid in toetse, en iv) om vas te stel of MTBC in neus deppers van renosters met immunologiese bewyse van infeksie opgespoor kan word, as bewys van potensiële aansteeklikheid.

Met behulp van bestaande literatuur oor MTBC-infeksies in ander spesies en kontekste, is ‘n grondliggende begrip van TB-epidemiologie in renosterspesies ontwikkel. In ander spesies is die volgende demografiese risikofaktore van belang. Geslag en ouderdom, waar manlike en volwassenes algemeen 'n hoër risiko dra as vroulike en jonger individue. Beperkte historiese inligting het soortgelyke ouderdoms- en geslagsverwante patrone vir TB in gekoërdineerde Afrika-renosters weerspieël, met meer verslae van TB-siekte in swart renosters wanneer dit versgelyk word met wit renosters. Intra-spesie oordrag van MTBC in vrylopende renosters kon as 'n moontlikheid beskou word.

Gebaseer op vorige verslae was hipotese geskep en word verder geondersoek in 'n bevolkingsgebaseerde studie van *M. bovis* in 437 Afrika-renosters in KNP. Ons het 'n geskatte algehele infeksie voorkoms van 15.4% (95% CI: 10.4-21.0%) gerapporteer en hierdie data was gebaseer op mikrobakteriële kultuur- en IGRA-resultate tussen 2016-2020. 'n Beduidende ruimtelike groepering van gevalle is naby die suidwestelike parkgrens opgespoor, alhoewel infeksie wye verspreiding getoon het.

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Meerveranderlike logistiese regressiemodelle, insluitend demografiese en tydruimtelike veranderlikes, het 'n beduidende, toenemende waarskynlikheid van *M. bovis* infeksie in wit renosters getoon. Hierdie was gebaseer op ‘n verhoogde aantal Afrikaanse buffeltroppe in die nabyheid van die renosters se monsterneming pleak. Daar is vermoed dat besmetting van Afrikaanse-buffels na witrenosters wat die omgewing deel, oorgeloop het. Aangesien Afrikaanse buffels belangrike onderhoudshospers vir *M. bovis* in KNP is, word besmettingsoorskryding van hierdie gasheer-spesie na wit renosters wat die omgewing deel, vermoed.

Daar was ook 'n beduidende hoër proporsie *M. bovis* infeksie in swart renosters in die vroeë jare van die studie (2016-2018) in vergelyking met 2019 en 2020, wat saamgeval het met periodes van intens droogte, alhoewel ander temporele faktore geïmpliseer kan word. Spesies van renoster, ouderdom, en geslag was nie as risikofaktore vir *M. bovis* infeksie geïdentifiseer nie.

Voor-doodse ondersoek vir *M. bovis* infeksie in die KNP se renosterbevolking steun tans op resultate van die QuantiFERON-TB Gold (In-Tube) Plus (QFT)-Mabtech perde interferon-gamma (IFN-γ) vrystellingstoets (IGRA). Maar die vereiste vir dieselfde-dag verwerking van renoster bloedmonsters vir die IGRA bly steeds 'n logistieke uitdaging om hierdie toets uit te voer, veral in afgeleë gebiede. 'n Loodsstudie het getoon dat relatiewe konsentrasies van IFN-γ (gebaseer op optiese digtheidswaardes) in mitogeen gestimuleerde volbloedplasma beduidend afgeneem het met toenemende tyd wat bloed na versameling; en voor QFT stimulasie gestoor is. Hierdie bevindinge ondersteun 'n behoefte aan dieselfde-dag prosessering van renoster bloedmonsters vir QFT-IGRA toetsing om voort te gaan soos die huidige praktyk, vir optimale toets prestasie.

Dit was voorheen onbekend of *M. bovis*-besmette renosters mikro-organismes in respiratoriese afskeidings kon afskei. Vorige studies het voorgestel dat subklinies *M. bovis*-besmette renosters kan minimale oordragrisiko inhou.. Onlangse vooruitgang wat die opsporing van MTBC lede in paucibacillêre monsters verbeter het, het gelei na verdere ondersoek van respiratoriese afskeidings van renosters met immunologiese bewyse van infeksie. Hierdie study is om die potensiaal vir mikrobakteriese afskeiding toe te lig. 'n Loodsstudie het *M. bovis* in 14/64 (22%; 95% CI: 13-33%) van die IGRA-positiewe renosters gevind, en geen in die IGRA-negatiewe renosters (n = 11) wat ondersoek is nie, wat aandui dat *M. bovis*-besmette renosters 'n bron van infeksie vir ander vatbare diere wat die omgewing deel, mag wees.

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Oor die algemeen spreek hierdie studies belangrike kennisleemtes aan rakende toesig en epidemiologie van TB in Afrika-renosters, spesifiek die vrylewende bevolkings in KNP. Dit het bewustheid van die potensiële bedreiging van hierdie patogeen geskep om die bewaring van hierdie spesies en belangrike areas vir toekomstige navorsing uitgelig wat sal bydra tot die begrip van die multi-gasheer TB-stelsel in KNP en ander komplekse ekosisteme.

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https://scholar.sun.ac.za

Table of Contents

Declaration ... i

Summary ... ii

Opsomming ... iv

Acknowledgements ... vii

Table of Contents ... ix

List of Figures ... xi

List of Tables ... xiv

List of Abbreviations ... xv

Chapter 1 ... 1

Introduction ... 1

Chapter 2 ... 45

Epidemiology of Tuberculosis in Multi-Host Systems: Implications for Black (*Diceros bicornis*) and White (*Ceratotherium simum*) Rhinoceros ... 45

Chapter 3 ... 86

Epidemiology of *Mycobacterium bovis* Infection in Free-Ranging Rhinoceros in Kruger National Park, South Africa ... 86

Chapter 4 ... 122

Reduced Capability of Refrigerated White Rhinoceros Whole Blood to Produce Interferon-Gamma upon Mitogen Stimulation ... 122

Chapter 5 ... 133

Ante-Mortem Detection of *Mycobacterium bovis* in Nasal Swabs from African Rhinoceros ... 133

Chapter 6 ... 157

Discussion ... 157

Chapter 7 ... 185

Conclusion ... 185

https://scholar.sun.ac.za

Addendum A ... 187

Supplementary material: Chapter 2 ... 187

Addendum B ... 195

Supplementary material: Chapter 3 ... 195

Addendum C ... 204

Supplementary material: Chapter 4 ... 204

Addendum D ... 205

Published manuscripts (Chapters 2-4) ... 205

https://scholar.sun.ac.za

List of Figures

Figure 2.1. Possible (theoretical) outcomes of *M. bovis* infection in African rhinoceros. After initial exposure, MTBC may be eliminated by the host's immune response, persist as a subclinical or latent infection, or progress to active infection/disease. Following the establishment of subclinical or latent infection, the host's immune response may clear the MTBC, or infection may persist in this form, either naturally progressing in a slow or rapid fashion to active tuberculosis, or cycling through subclinical and latent states, before development into symptomatic disease or eventual eradication of the infection by the host's adaptive immune response. *Rising disease burden implies an increase in abundance of MTBC biomarkers, immunological changes characteristic of stage of infection, and increasing pathology, with a declining recovery prognosis. ˜Although controversial, there is evidence that latent infection of animals with *M. bovis* may occur. More research is required to determine whether latent infection can occur in animals, including rhinoceros. 49

Figure 2.2. Summary of globally recorded historical cases of tuberculosis in black rhinoceros (*D. bicornis*) and white rhinoceros (*C. simum)* (1. Hofmeyr, 1956; 2. Keep and Basson, 1973; 3. Mann et al., 1981; 4. Dalovisio et al., 1992; 5. Barbiers, 1994; 6. Stetter et al., 1995; 7. Valandikar and Raju, 1996; 8. Rookmaaker, 1998; 9. Oh et al., 2002; 10. Duncan et al., 2009; 11. Espie et al., 2009; 12. Morar et al., 2013; 13. Miller et al., 2015; 14. Miller et al., 2017, 15. Miller et al., 2018; 16. Witte pers.comm., 2020)*. *These data were located in the Rhinoceros Resource literature database, or through extensive web searches with Miller et al. (2017) as a guide. 52

Figure 3.1. Prevalence (%) of *M. bovis* infection in rhinoceros in Kruger National Park, South Africa, 2016-2020 (n = 420). Prevalence estimates are reported in the legend in descending order. Areas with insufficient data (n < 10 sampled animals) are shown in white. Frame A shows the prevalence of *M. bovis* in the study population according to ranger management area (Kloppers and Bornman, 2005). No significant differences in *M. bovis* prevalence according to ranger area were identified (Fisher's exact p > 0.05 for all comparisons). A single, statistically significant cluster of radius 6.5 km is depicted by a hatched yellow circle based on Kulldorff's spatial scan statistic (Kulldorff, 1997); twelve cases of *M. bovis* were identified in this cluster, whereas the model predicted only 4 (relative risk = 3.5, p = 0.036). Frame B shows the prevalence of *M. bovis* according to ecozone (Gertenbach, 1983). Significant differences in *M. bovis* infection prevalence were detected between the ecozones with the same superscript letter. Prevalence in the Pretoriuskop Sourveld ecozone was significantly different than in

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Sabie/Crocodile Thorn Thickets ($p < 0.001$); Mixed Bushwillow Woodlands ($p = 0.02$); and Lebombo Mountain Bushveld ($p = 0.01$). Fisher's exact $p > 0.05$ for all other area comparisons. 101

Figure 3.2. Forest plots depicting species-specific multivariable models of factors associated with M. bovis infection African rhinoceros in Kruger National Park, South Africa (2016-2020). Parameters for both models, including coefficients, standard errors, and fit statistics are reported in detail in Addendum B (Table S3.4.1). 105

Figure 4.1. Mean optical density (OD) results for plasma harvested from QuantiFERON-TB Gold (In-Tube) Plus (QFT) mitogen tubes and measured in the equine interferon-gamma (IFN-γ) ELISA are shown. A fixed plasma dilution factor for each of seven white rhinoceros (represented by different symbols) was selected and OD values shown for each whole blood storage time point (0, 24, 48 h at 4°C prior to stimulation). Error bars indicate standard deviation across two replicates for each mean OD result. Note: for one individual, there was insufficient plasma volume to complete the IGRA on the Nil and Mit samples from the 24 h test point; hence, only the 0 and 48 h timepoint samples could be tested for this animal. 126

Figure 5.1. Flow chart for identifying the study population and methods pipeline for detection of MTBC in nasal swabs from African rhinoceros. KNP, Kruger National Park; b, black rhinoceros; w, white rhinoceros; IGRA, interferon-gamma release assay; Ag, antigen; IFN-γ, interferon-gamma; cMGIT, conventional BACTEC™ MGIT™ Mycobacterial Growth Indicator Tube culture method; TiKa – MGIT, TiKa decontamination and growth supplement enhanced-MGIT culture; NCBI BLASTn, National Centre for Biotechnology Information Basic Local Alignment Search Tool (nucleotide); MTBC, *Mycobacterium tuberculosis* complex; *M. bovis*, *Mycobacterium bovis*; RD4, (genetic) Region of Difference 4. ~A rhinoceros was classified as IGRA positive or negative, according to previously defined cutoffs (Chileshe et al., 2019; Dwyer et al., 2022). An animal was considered IGRA negative if it had a TB Ag-specific IFN-γ response ≤ 21 pg/mL, a mitogen IFN-γ response ≥ 21 pg/mL, and a Nil IFN-γ response ≤ 21 pg/mL. It was classified as IGRA positive if it had a TB Ag-specific IFN-γ response >21 pg/ml. Individuals who could not be defined as IGRA positive or negative according to the described case definitions were considered inconclusive and excluded. [$]IGRA positive rhinoceros were included in the study subset for examination of nasal swabs based on the magnitude of their Ag-specific IFN-γ response. Of the 93 IGRA positive individuals, the 64 with the highest Ag-specific IFN-γ responses (ultimately, all with Ag-specific [IFN-γ] > 40 pg/ml) were included in the study subset. The remaining 29 IGRA positive individuals with

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lower Ag-specific IFN-γ (ultimately, all with Ag-specific [IFN-γ] < 40 pg/ml) were excluded from the study subset. *A small selection of IGRA negative rhinoceros (n = 11) were included for comparison. [%]For a consensus sequence to be considered reliable for further analysis, the sequence length had to fall within 30 bp of the expected target length, and the forward and reverse Sanger sequences had to match at a minimum of 80%. Any sequences that did not meet this criteria were not included for further analysis. 139

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List of Tables

Table 3.1. Mycobacterial QFT-IGRA[i] test and culture[ii] results for 475 African rhinoceros in Kruger National Park, South Africa (2016-2020) 99

Table 3.2. Frequency distributions and univariate analyses of potential risk factors for *M. bovis* infection in African rhinoceroses in Kruger National Park, South Africa, 2016-2020 (n=437) 102

Table 4.1. Linear mixed model of the relationship between mean optical density (OD) values of plasma from QuantiFERON-TB Gold (In-Tube) Plus (QFT) mitogen-stimulated rhinoceros whole blood screened with an equine interferon-gamma (IFN-γ) ELISA, and the time blood was stored at 4ºC prior to stimulation (n = 17 observations). Random effects for intercept were included for individual animals. 127

Table 5.1. Summary of demographic characteristics of rhinoceros selected as study population from which nasal swabs were tested for direct detection of MTBC (n = 75). Antigen-specific interferon-gamma (IFN-ɣ) concentrations were measured in QuantiFERON TB Gold Plus stimulated heparinised whole blood plasma using the Mabtech Equine IFN-ɣ ELISA, and those with antigen-specific interferon-gamma concentrations < 21 pg/ml, mitogen interferon-gamma concentration > 21 pg/ml, and Nil interferon-gamma concentration < 21 pg/ml were considered IGRA negative; IGRA positive rhinoceros included in this cohort had antigen-specific IFN-ɣ concentrations ≥ 40 pg/ml. 143

Table 5.2. Demographic characteristics and direct detection test results of nasal swabs for 14 interferon-gamma release assay positive rhinoceros in which *Mycobacterium tuberculosis* complex/*Mycobacterium bovis* was detected by at least one of the applied methods. 145

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List of Abbreviations

%	percent
°C	degrees Celsius
95% CI	95% confidence interval
xg	times gravity
µl	microlitre
K	kappa
ACU	Animal Care and Use
AIC	Akaike's Information Criterion
APHIS	Animal and Plant Health Inspection Service
BALF	bronchoalveolar lavage fluid
BD	Becton Dickinson
BLASTn	Nucleotide Basic Local Alignment Search Tool
BMTA	biomaterial transfer agreement
BSL3	biosafety level 3
bTB	bovine tuberculosis
bp	base pair
C. simum	*Ceratotherium simum*
CAF	Central Analytical Facility
CFP-10	culture filtrate protein 10
cMGIT	Conventional MGIT
CMI	cell-mediated immunity
COVID-19	coronavirus disease of 2019
CRA	cytokine release assay
CRP	C-reactive protein
CXCL-9	chemokine (C-X-C motif) ligand 9 encoding gene
CXCL-10	chemokine (C-X-C motif) ligand 10 encoding gene
D. bicornis	*Diceros bicornis*
DALRRD	Department of Agriculture, Land Reform and Rural Development
DEA	Department of Environmental Affairs
DMU	deer management unit
DNA	deoxyribonucleic acid
DNPWC	Department of National Parks and Wildlife Conservation

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DPP	dual path platform
DSI-NRF	Department of Science and Innovation-National Research Foundation
e.g.	exempli gratia; for example
EEC	European Economic Community
ELISA	enzyme-linked immunosorbent assay
ESAT-6	early secreted antigenic target 6
GEA	gene expression assay
GIS	Geographic Information Systems
GPS	Global Positioning System
h	Hours
HiP	Hluhluwe iMfolozi Park
IFNG	interferon-gamma encoding gene
IFN-γ	interferon-gamma
IGRA	interferon-gamma release assay
IL	Interleukin
IP-10	IFN-γ-induced protein 10
IQR	interquartile range
IS1081	insertion sequence 1081
IS6110	insertion sequence 6110
IUCN	International Union for Conservation of Nature
km	kilometre
KNP	Kruger National Park
KZN	KwaZulu Natal
LOQ	limit of quantification
M. africanum	*Mycobacterium africanum*
M. bovis	*Mycobacterium bovis*
M. canetti	*Mycobacterium canetti*
M. caprae	*Mycobacterium caprae*
M. microti	*Mycobacterium microti*
M. mungi	*Mycobacterium mungi*
M. orygis	*Mycobacterium orygis*
M. pinnipedii	*Mycobacterium pinnipedii*
M. suricattae	*Mycobacterium suricattae*

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M. tuberculosis	*Mycobacterium tuberculosis*
MAZ	Modified Accredited Zone
MGIT	Mycobacterial Growth Indicator Tube
MIRU-VNTR	Mycobacterial Interspersed Repetitive Units-Variable Number Tandem Repeats
Mit	mitogen
ml	millilitre
MPB83	*M. bovis* protein 83
MTBC	*Mycobacterium tuberculosis* complex
NAATs	nucleic acid amplification tests
NCBI	National Centre for Biotechnology Information
NTM	non-tuberculous mycobacteria
NY	New York
OD	optical density
OR	odds ratio
OTF	officially tuberculosis free
pg/mL	picograms per millilitre
PPD	purified protein derivative
QFT+	QuantiFERON® TB Gold (In-Tube) +
qPCR	quantitative polymerase chain reaction
RD-PCR	region of difference polymerase chain reaction
SA	South Africa
SANParks	South African National Parks
SD	standard deviation
SE	standard error
Se	sensitivity
SICTT	Single Intradermal Comparative Tuberculin Test
Sp	specificity
TB	tuberculosis
Th1	T- helper 1
Th2	T- helper 2
TST	tuberculin skin test
UK	United Kingdom

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Ultra	GeneXpert MTB/RIF Ultra
USA	United States of America
USDA	United States Department of Agriculture
VNTR	Variable Number Tandem Repeat
vs.	Versus
WAHIS	World Animal Health Information System
WGS	whole genome sequencing
WOAH	World Organisation for Animal Health
y	Years
Zoo	zoological garden

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Chapter 1

Introduction

Tuberculosis: a Multi-Host Disease

Tuberculosis (TB) is a chronic infectious disease that is known to impact a broad range of host species (Bernitz et al., 2021; Fitzgerald and Kaneene, 2012; Pesciaroli et al., 2014). It is caused by infection with members of a closely related group of pathogenic mycobacteria known as the *Mycobacterium tuberculosis* complex (MTBC) (Fogel, 2015; Malama et al., 2013; Meiring et al., 2018). These species include *Mycobacterium tuberculosis, M. bovis*, *M. africanum*, *M. caprae*, *M. microti*, *M. canetti*, *M. orygis*, *M. pinnipedii*, *M. suricattae*, *M. mungi* and the "Dassie" bacillus (Shamputa et al., 2015).

In humans (*Homo sapiens*) and cattle (*Bos taurus*), TB is typically transmitted via inhalation of tubercle bacilli-containing respiratory aerosols produced by infectious individuals (Domingo et al., 2014; Shamputa et al., 2015). Other known modes of transmission include ingestion, inoculation, and rarely, via the placenta. Infection begins when bacteria are phagocytosed by host macrophages; surviving bacilli may begin replicating inside the macrophages and infiltrate local epithelial cells. Bacilli contained in macrophages can also spread to lymph nodes through the lymphatic system, and into the bloodstream (Domingo et al., 2014; Shamputa et al., 2015).

Mycobacterial infection triggers an inflammatory response resulting in the migration of additional immune cells (e.g., neutrophils) to the site of infection (Domingo et al., 2014; Shamputa et al., 2015). Eventually, if the infection is not cleared or controlled by the host's immune responses, it may progress to formation of granulomatous lesions, or Ghon foci, which are characteristic of TB in humans (Donald et al., 2020; Delgado and Bajaj, 2023). Bacilli may continue to multiply, resulting in ongoing inflammation and adaptive immune responses, which increase granuloma size and ultimately lead to necrosis, potential cavitation in the lungs and disease spread. Bacilli can spread from the primary site of infection via haematogenous or lymphatic routes, resulting in extrapulmonary and miliary TB (Domingo et al., 2014; Shamputa et al., 2015). These general pathological features of TB are shared by many affected species, although there may be host, pathogen, or exposure-related differences in the types and distributions of TB lesions (Baykan et al., 2022; Domingo et al., 2014; Shamputa et al., 2015).

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While many of the MTBC members are known to infect multiple host species, different co-evolutionary scenarios have resulted in varying degrees of host adaptation (Brites and Gagneux, 2015; Gordon and Behr, 2015). For example, *M. tuberculosis* and *M. africanum* are considered to be human-adapted pathogens. Although other species including Asian (*Elephas maximus*) and African (*Loxodonta africana*) elephants (Miller et al., 2019a; Sternberg Lewerin et al., 2005; Zlot et al., 2016), cattle (Hlokwe et al., 2017), and domestic dogs (*Canis lupus familiaris*) (Marfil et al., 2022) are known to be susceptible to infection, humans and some non-human primate species are the primary hosts in which infection with these pathogens occurs efficiently, and infection cycles are sustainably maintained (Brites and Gagneux, 2015; Flynn et al., 2015; Scanga and Flynn, 2014).

Animal-adapted MTBC species have varying host preferences; for example, *M. suricattae*, *M. mungi*, and the dassie bacillus exclusively infect meerkats (*Suricata suricatta*), banded mongooses (*Mungos mungo*), and hyraxes, respectively (Clarke et al., 2016). Some MTBC have zoonotic potential, but have non-human host preferences; for example, *M. microti* is most commonly found in voles, wood mice (*Apodemus sylvaticus)* and shrews (Smith et al., 2009), *M. pinnipedii,* in sea lions and seals (Roe et al., 2019). Both *M. caprae* and *M. bovis* primarily infect livestock but are known to cause disease in multiple domestic and wildlife species (Aranaz, 2015; Buddle et al., 2015; Chambers et al., 2015; Gortázar et al., 2015; Lyashchenko and Miller, 2015; Michel et al., 2015; Mikota et al., 2015; Palmer et al., 2015; Waters et al., 2015). *Mycobacterium orygis* has been isolated in oryxes and gazelles (van Soolingen et al., 1994), as well as deer, antelopes, and waterbucks (*Kobus ellipsiprymnus*) (Smith et al., 2006); although its exact host range remains unclear (van Ingen et al., 2012), it has also been identified in humans (Duffy et al., 2020). Inter-species transmission of MTBC from animals to humans (and vice versa) has been increasingly recognised and constitutes a public health concern (Adesokan et al., 2019; Davies, 2006; Lombard et al., 2021; Moyo, 2021).

TB in Humans: the Role of Zoonotic Infection

While most TB cases in humans are caused by *M. tuberculosis* infection, an under recognised proportion of cases are caused by infection with *M. bovis.* In 2018, the World Health Organisation reported an estimated 1.8% of TB cases globally, and up to 30.2% of TB cases in varying regions of Africa, were caused by *M. bovis* infection (World Health Organisation, 2019). Humans can be exposed to *M. bovis* through respiratory aerosols during contact with infected animals, or via alimentary routes including consumption of contaminated animal

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products (such as unpasteurised milk or cheese) (Adesokan et al., 2019; Davies; 2006; Zinsstag et al., 1998). Human-to-human transmission of *M. bovis*, although rare, has occurred (Evans et al., 2007).

Human disease caused by *M. tuberculosis* and *M. bovis* infection is often clinically indistinguishable, and unless mycobacterial culture and molecular speciation are performed, misdiagnosis may occur (Ayele et al., 2004; Bilal et al., 2010; Carruth, 2016). It is therefore likely that the global burden of TB resulting from *M. bovis* infection is underestimated (Ayalew et al., 2023; Torres-Gonzalez et al., 2016). This is especially concerning due to the inherent resistance of *M. bovis* to pyrazinamide (a drug commonly included in human TB treatment), which may render routine regimens ineffective in zoonotic TB cases, thereby increasing risk of spread and exacerbating the global TB burden (Zinsstag et al., 2011). Moreover, although *M. bovis* is widely accepted to be the predominating cause of zoonotic TB, there is increasing evidence for a substantial contribution of *M. orygis* to the global zoonotic TB burden (Duffy et al., 2020; Kock et al., 2021).

Animal TB: a Global Perspective

Animal TB, especially cases caused by *M. bovis* infection, is a disease with multi-sectoral impact; in addition to being a threat to public health (for zoonotic MTBC species), it can have substantial socio-economic costs. In developed countries, where (with some exceptions) animal TB prevalence is typically low, these costs are primarily related to trade barriers in place for live animals and animal products, and the financial cost of compliance with compulsory bTB eradication programs; in particular, this is related to funding of required veterinary testing services and compensation for culling (Barnes et al., 2023; Caminiti, 2019; Caminiti et al., 2017). Other negative impacts, such as loss of consumer trust and adverse global and local market reactions, are rarely assessed but may also influence indirect costs of animal TB to society. Conversely, in many developing countries, there is often a high prevalence of animal TB, associated with inadequate preventative measures (such as the lack of food safety and quality control protocols), limited resources and logistics of control programs, and the presence of wildlife reservoirs (Arnot and Michel, 2020; Malama et al., 2013; Pokam et al., 2019). In these contexts, the socio-economic cost of animal TB is predominantly related to the resulting livestock production losses, including increased mortality and decreased milk and meat production, as well as impact on rural farmer livelihoods (Azami and Zinsstag, 2018; Caminiti, 2019).

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A bulletin released by the World Organisation for Animal Health (WOAH) in 2019 revealed that between January 2017 and June 2018, 82 (44%) of 188 participating countries reported animal TB (associated with *M. bovis* infection) via the World Animal Health Information System (WAHIS), demonstrating the widespread distribution of the disease (Murai et al., 2019). Animal TB was most commonly reported in livestock. Among 82 affected countries, 29 (35.4%) reported presence of TB in livestock and wildlife, and an additional 51 (62.2%) indicated that only livestock were affected (Murai et al., 2019). This is a concern since studies have demonstrated that a significant proportion of cattle with bovine TB (animal TB in bovines) excrete *M. bovis* (Collins et al., 2022; Kao et al., 2007; Neill et al., 1988). Therefore, infected livestock and wildlife should be considered potential infection sources for other domestic animals or susceptible species with which they associate, including humans.

The application of effective disease control measures is considered critical for preventing and controlling animal TB at its source to prevent animal-animal and animal-human transmission (Murai et al., 2019; Verteramo Chiu et al., 2019). Important measures include active and ongoing surveillance (usually accomplished through tuberculin skin testing of herds) (Verteramo Chiu et al., 2019), complete or partial eradication of diseased animals/herds (e.g., through test-and-slaughter programs (De Garine-Wichatitsky et al., 2013; le Roex et al., 2016; Verteramo Chiu et al., 2019)) and movement control (quarantine, abattoir meat inspection, etc.) (Gilbert et al., 2005; Murai et al., 2019; Verteramo Chiu et al., 2019). However, the success of these strategies is often contingent on the participation of all stakeholders in the livestock industry, and the absence of other reservoirs of infection (especially wildlife) that may result in spillback of the disease into livestock (Murai et al., 2019; Verteramo Chiu et al., 2019).

The same WOAH bulletin reported on the implementation of prevention and control strategies by the 188 participating countries (Murai et al., 2019). This showed that in animal TB-affected countries, 23% had implemented all relevant control strategies, most (62%) reported implementing at least one control strategy, and 3% had not implemented any control strategies. Even most TB-unaffected participating countries (82%) had implemented at least one animal TB control strategy (Murai et al., 2019). These data demonstrate that there is generally a high global awareness and participation in control efforts for animal TB.

Animal TB control efforts are considered necessary to maintain trade and global economic stability (Azami and Zinsstag., 2018), as well as to mitigate the spread of zoonotic tuberculosis. The Roadmap for Zoonotic Tuberculosis (World Health Organisation et al., 2017), focuses on adequate, complete data collection and reporting of animal TB, proposing that human

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tuberculosis cannot be adequately controlled unless animal TB is controlled. Therefore, it is vital that international animal TB surveillance and reporting is maintained and continues to improve.

Although some similarities exist between countries affected by animal TB, the epidemiology and impact of this disease in different ecosystems is uniquely influenced by the susceptible host species and interfaces present, current regulatory implementation of disease management interventions, roles and involvement of stakeholders, anthropogenic changes to the landscape (e.g., encroachment, agricultural intensification), and climate change (Allen et al., 2018; Mikota et al., 2015; O'Brien et al., 2023). Countries which have addressed TB in wildlife reservoirs have incorporated holistic approaches to design effective TB control or eradication strategies for livestock and other animals (Department of Agriculture, Food and the Marine, 2021; DNPWC, 2011; Mikota et al., 2015; Smith and Budgey, 2021). The following sections will describe some examples of multi-host animal TB scenarios, and the complexity and challenges associated with implementing effective control strategies.

Animal TB (*M. bovis*) in European Union, Ireland, and the United Kingdom

In Europe, *M. bovis* infection in livestock is of major economic importance due to its significant impact on trade (Allen et al., 2018). The European Economic Community (EEC) took the first legal initiative in Europe to combat the disease in 1964, introducing the concept of "officially tuberculosis free" (OTF) countries (Reviriego Gordejo and Vermeersch, 2006). This term referred to a status granted to nations where cattle herd prevalence is less than 0.1% for six consecutive years. This was followed by mandating that EEC member countries finance and facilitate test-and-slaughter programs to eradicate bTB (Allen et al., 2018). The success of this approach was demonstrated in 2009, when Denmark, The Netherlands, Germany, Luxembourg, Austria, France, Belgium, Finland, Sweden, Czech Republic, Poland, and Slovenia had achieved OTF status (European Food Safety Authority and European Centre for Disease Prevention and Control, 2011). However, despite initial progress, England, Wales, and Northern Ireland have seen a rise in animal TB incidence since the late 1980s (European Food Safety Authority and European Centre for Disease Prevention and Control, 2011).

One of the key factors contributing to the disparity in success of animal TB control between countries is the presence of wildlife reservoirs (Palmer, 2013). Spillback of *M. bovis* from wildlife can lead to reinfection of cattle herds or other livestock. In Ireland and the UK, the European badger (*Meles meles*) is a recognised *M. bovis* reservoir host and has been implicated

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in the epidemiology of animal TB in these countries (Bourne et al., 2007; Corner et al., 2011; Donnelly et al., 2007; Donnelly and Hone, 2010). The difficulty in eradicating TB in livestock in these countries has, in part, been attributed to the high badger density and *M. bovis* prevalence (Allen et al., 2018). Control programs for bovine TB in the UK have therefore increased their focus on the role of badger TB in cattle, leading to advances in diagnostic tests, surveillance, knowledge of TB epidemiology, and potential vaccination strategies to address this risk (Department of Agriculture, Food and the Marine, 2021; Smith and Budgey, 2021) .

Animal TB (*M. bovis*) in Michigan, USA

In North America, DNA sequencing of fossilised remains containing tuberculous-like lesions suggest that MTBC was present from as early as the Pleistocene era (Rothschild et al., 2001). Others theorise that the ancestral pathogens were carried over the Bering strait by bovids later in the Pleistocene era or accompanied settlers upon their arrival on the continent (Palmer and Waters, 2011). In 1900, TB was the leading cause of human mortality in the USA (Crimmins and Condran, 1983). Importantly, an estimated 10% of human tuberculosis cases were attributed to exposure to tuberculous cattle or cattle products (Olmstead and Rhode, 2004). In children, approximately 25% of tuberculosis cases were caused by *M. bovis* infection (Roswurm and Ranney, 1973). Over the following decades, the public health threat posed by zoonotic TB was recognised and led to the development of the National Tuberculosis Eradication Program (Kiernan et al., 1919; Palmer and Waters, 2011). This program is administered by the U.S. Department of Agriculture's (USDA) Animal and Plant Health Inspection Service (APHIS), in partnership with state animal health agencies, accredited veterinarians, and U.S. livestock producers. By 1941, the program had nearly eradicated bovine TB from the country's livestock population (Hastings, 1942). However, TB is still sporadically detected in livestock herds today, and the burden in U.S. cattle herds may be on the rise again (O'Brien et al., 2023).

At present, the USDA uses five bTB status zoning levels that states (or regions within states) are classified according to the presence of *M. bovis* infection in cattle; these range from no apparent prevalence of bTB in cattle or bison (zone 1), to an unknown or $\geq$ 0.5% herd prevalence (zone 5) (VerCauteren et al., 2018). The implementation of this zoning strategy allows stakeholders to tailor approaches for surveillance and disease management according to regional disease prevalence and potential risk of spread.

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The state of Michigan is an accredited (TB) free zone (VerCauteren et al., 2018), except for a four-county area, which is classified as a Modified Accredited Zone (MAZ; zone 3). Within a MAZ, cattle producers are allowed to participate in national and international markets but movement of cattle from these zones is regulated (VerCauteren et al., 2018). The four-county MAZ area, also referred to as the Deer Management Unit (DMU), is endemic for *M. bovis* infection in the free-ranging white-tailed deer (*Odocoileus virginianus*) population (Michigan Department of Agriculture and Rural Development, 2020). This presence of this infected wildlife reservoir increases the risk of possible spillback of *M. bovis* to livestock, which is reflected by the rise in bTB incidence in cattle in this area in recent years (O'Brien et al., 2023). The consequences of continued infection include loss of the four-county MAZ status and statewide TB-free status, with detrimental impacts to the livelihoods of over 13,000 cattle producers in Michigan (VerCauteren et al., 2018). Increased disease mitigation efforts in this area have expanded focus on wildlife TB by introducing deer management strategies. These include the employment of professional hunters to reduce deer numbers, regulations to eliminate human-related food sources to prevent increased deer densities and congregation, thus reducing transmission risk (VerCauteren et al., 2018).

Animal TB (*M. tuberculosis*) in Nepal

The world's extant elephant population currently comprises only three species – the Asian elephant, the African bush elephant and the African forest elephant (*Loxodonta cyclotis*) (DNPWC, 2011; IUCN, 2022). The first two species are classified by the International Union for the Conservation of Nature (IUCN) as endangered, and the latter, critically endangered (IUCN, 2022).

The remaining Asian elephant population is primarily distributed across 13 countries, including Nepal (DNPWC, 2011; Shrestha and Shrestha, 2021). In Nepal, elephants were historically kept for various purposes, including for royal hunting safaris, as a means of transportation, and even as status symbols. Since the introduction of Nepalese wildlife protection areas, elephants have been utilised for patrolling, wildlife capture and translocation, work animals in the logging sector, transportation, and wildlife tourism (DNPWC, 2011; Shrestha and Shrestha, 2021). Today, they are mostly confined to protected areas, although free-ranging elephants from India's Bengal State are occasionally found in the forest areas of the eastern lowland.

Elephants are prone to multiple infectious diseases, including tuberculosis, which threatens captive and wild populations in different areas of the world (Ghielmetti et al., 2017; Kerr et al.,

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2019; Mikota et al., 2015; Miller et al., 2021; Rajbhandari et al., 2022; Zlot et al., 2016). At the start of the 21st century, a third of the world's estimated 45,000 remaining Asian elephants were kept in captivity; their frequent close contact with humans, often in range countries with high human TB burden, places them at greater risk for TB.

Elephant TB due to *M. tuberculosis* infection has resulted in morbidity and mortality in captive Asian elephants in the USA and Europe (Ghielmetti et al., 2017; Zlot et al., 2016). In Nepal, TB was first detected in working elephants in 2002 (DNPWC, 2011; Gairhe, 2002). Later, when TB-like lesions were identified post-mortem in several valuable patrol elephants, concerns about the possibly under recognised threat of TB in this population increased. In 2006, comprehensive surveillance using four serological screening tools and mycobacterial culture was initiated. In an initial survey of 120 captive elephants, 26 (22%) individuals tested positive for TB on one or more of the diagnostic tests applied, highlighting a substantial infection prevalence (DNPWC, 2011; Mikota et al., 2015).

Motivated by the desire of wildlife authorities in Nepal to protect captive elephants, mitigate TB at the human-elephant and captive-wild elephant interfaces, and preserve tourism, the Nepalese government instituted the Nepal Elephant Tuberculosis Control and Management Action Plan (DNPWC, 2011; Mikota et al., 2015). This comprises a practical, holistic approach to preventing and managing elephant TB and protecting public health and economic welfare in this unique context (DNPWC, 2011).

Detection of MTBC Infection and Animal TB

An important universal aspect of animal TB surveillance, control and eradication strategies involves the development and application of diagnostic tools to identify infected hosts. Available detection methods for MTBC infection in animals can be broadly categorised into two main types; direct detection methods, which are focused on identifying the presence of MTBC bacteria in the host, or indirect detection methods, which are based on the measurement of pathogen-specific host immune responses (Bernitz et al., 2021; Sakamuri et al., 2015).

Direct Detection of MTBC Organisms

A presumptive diagnosis of mycobacterial infection is typically based on histopathological changes consistent with TB, and the identification of acid-fast bacilli in sample material by Ziehl-Neelsen staining and microscopic examination (Bernitz et al., 2021; Larenas-Muñoz et al., 2022). However, the absence of acid-fast bacteria is insufficient to rule out infection, which

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may be paucibacillary (Krishna and Gole, 2017). Conversely, there are species of non-tuberculous mycobacteria (NTMs), and other bacteria, including Actinobacteria such as *Rhodococcus* sp., or *Nocardia* sp. (Pujic et al., 2015), that also stain acid-fast and may lead to a presumptive false positive diagnosis. A definitive diagnosis is therefore dependent on methods that can differentiate between NTMs and MTBC, and can speciate within the MTBC (Bernitz et al., 2021).

Mycobacterial culture and species determination of isolates, using molecular tools such as targeted PCR, are considered the gold standard diagnostic test for MTBC infection. However, the application of these methods for MTBC (particularly *M. bovis*) detection in animals is often challenging due to both the slow growth of mycobacteria in culture, as well as the paucity of bacilli in most ante-mortem samples (de la Rua-Domenech et al., 2006). Additionally, some MTBC organisms, including *M. bovis,* require the inclusion of pyruvate in culture medium (Keating et al., 2005); this can complicate the application of standard protocols designed for direct detection of *M. tuberculosis*. In recent years, the use of different media to improve mycobacterial culture techniques has been investigated. One of the longest-standing methods for conventional mycobacterial culture relies on the use of Jensen's modification of the Lowenstein medium, combined with trisodium phosphate as a mucous digestion and decontamination agent (Levin et al., 1950; Sakamuri et al., 2015). However, a major limitation of this test is its turnaround time, which can be up to 8 weeks or more (Sakamuri et al., 2015). The introduction of agar-based Middlebrook medium decreased the average time for a result to approximately 2 to 3 weeks; however, a 6-week-long incubation is still a requirement for optimal sensitivity (Sakamuri et al., 2015).

The development of automated culture systems, such as the BACTEC 460TB™ and subsequently the BACTEC 960™ (Becton Dickinson (BD), Sparks, Maryland, USA), has improved the rapid, efficient, and standardised detection of mycobacteria (Aggarwal et al., 2008; Cutler et al., 1994; Sewell et al., 1993). A newer system, the TiKa-MGIT culture (TiKa) method (TiKa-Diagnostics, London, United Kingdom), has incorporated the BACTEC MGIT conventional culture method, along with a less harsh decontamination step, and has recently shown promise for detection of MTBC in animal samples (e.g., nasal and oral swabs, faecal samples, bronchoalveolar lavage fluids (BALFs) and tissues) (Bull et al., 2017; Goosen et al., 2021). This method has increased sensitivity and decreased time-to-positivity for detection of MTBC compared to the conventional BACTEC MGIT™ system.

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Although culture and speciation are the gold standard method, the fiscal cost of culture and system setup renders it inaccessible for many developing countries or remote locations. Additionally, the global rise in prevalence of multi-drug resistant TB has meant that Biosafety Level 3 (BSL3) containment is increasingly becoming a requirement for processing and culturing samples suspected to contain MTBC organisms (Sakamuri et al., 2015). In addition, BSL3 laboratories are relatively scarce since they are expensive to maintain and require personnel with extensive training and experience (Sakamuri et al., 2015). Therefore, there has been increasing focus on the development and validation of culture-independent methods for directly detecting MTBC infection, using techniques such as targeted quantitative polymerase chain reactions (qPCRs) (Goosen et al., 2020a, 2020b) and immunohistochemical techniques (Nourani and Ashouri, 2022; Phom et al., 2016).

One of the most widely used qPCR methods for human TB diagnosis is the GeneXpert MTB/RIF Ultra assay (Ultra; Cepheid, Sunnyvale, California, USA), which is recommended by the World Health Organisation for use with sputum samples (Dorman et al., 2018; Sakamuri et al., 2015). The assay is based on the detection of a single copy target, the *rpoB* gene, as well as two different multi-copy targets, the MTBC specific *IS6110* and *IS1081* genes (Boyle et al., 2014; Cepheid, 2023). More recently, Ultra has shown utility for direct detection of MTBC in samples from livestock and wildlife (Goosen et al., 2020b; Hlokwe and Mogano, 2020; Kerr et al., 2020), including post-mortem tissue homogenates (Hlokwe and Mogano, 2020; Kerr et al., 2020), and ante-mortem samples such as bronchoalveolar lavage samples, trunk wash fluids, and oronasal swabs from elephants, rhinoceros, and African buffaloes (*Syncerus caffer*) (Clarke et al., 2022; Goosen et al., 2020b). The Ultra is an attractive method for application in animals due to its suitability for use in field settings (i.e., rapid, automated, simple-to-use, with a small, portable cartridge) and operator friendliness (Bernitz et al., 2021; Clarke et al., 2022; Goosen et al., 2020b; Kerr et al., 2020). However, culture-independent detection methods based solely on Ultra are limited to detection of MTBC DNA, and do not provide information on the viability of MTBC bacilli present.

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Indirect Detection of MTBC Organisms

Indirect diagnostic methods rely on assays that measure adaptive host immune responses (i.e., humoral responses associated with antibodies in sera or cell-mediated immune (CMI) responses to *M. bovis* antigens) in different species. The advantages of using indirect diagnostic tests in domestic and wild animal species include potential for field use, access to commonly available samples such as blood, and improved sensitivity compared to direct detection by mycobacterial culture (Bernitz et al., 2021). However, these methods are limited by the lack of species-specific reagents, especially for wildlife. Furthermore, they may not be able to distinguish between subclinical infection and active disease (Dwyer et al., 2020; Drain et al., 2018; Jakob-Hoff, 2014).

Humoral response-based assays typically use serum samples; these are usually more readily available than fresh whole blood samples required for in vitro CMI assays. Serological assays can measure circulating antigen-specific antibodies, which may be used to differentiate *M. bovis*-infected and uninfected individuals, especially in those species with robust humoral responses or later in the course of disease (Sakamuri et al., 2015). Examples of serological tests that have been used for diagnosis of *M. bovis* infection in cattle include the IDEXX *Mycobacterium bovis* Ab test (IDEXX, 2015; McCallan et al., 2021; Waters et al., 2011), and the Enfer multiplex antibody assay (McCallan et al., 2021; O'Brien et al., 2023; World Organisation for Animal Health, 2019). In cattle, these tests are highly specific, but generally lack sensitivity (McCallan et al., 2021; Moens et al., 2023). Therefore, they are recommended for use as ancillary tests, along with other methods for surveillance and management of tuberculosis (IDEXX, 2015; World Organisation for Animal Health, 2019).

Serological tests based on humoral responses have had variable success in TB diagnosis. These tools are useful in species in which there are Th2 type immune responses to MTBC infection, which often occurs as disease progresses (McCallan et al., 2021). However, in some animal species, a Th1 type immune response predominates in early MTBC infection, thus serological tests are not sufficiently sensitive for use in screening and diagnosis (McCallan et al., 2021). Serological assays have been useful for TB diagnosis in suids, elephants, camelids, cervids, and felids (Infantes-Lorenzo et al., 2018; Kerr et al., 2019; Lyashchenko et al., 2013; Miller et al., 2019b; Olivier et al., 2017; Roos et al, 2016;). For example, in warthogs (*Phacochoerus africanus*), assays based on detection of *M. bovis*-specific antibodies, such as the purified protein derivative (PPD)-ELISA (Vacunek, Bizkaia, Spain) and the TB ELISA-VK® kit, have

been reported to have high specificity and sensitivity (Roos et al., 2016). However, serological assays are limited by the requirement for species-specific reagents. Species non-specific platforms have been successfully used in wildlife; for example, the Dual Path Platform Vet TB Assay (DPP® Vet TB) for Cervids (Chembio Diagnostic Systems, Medford, New York, USA) has been used to differentiate between *M. bovis*-infected and uninfected warthogs (Roos et al., 2016), elephants, (Kerr et al., 2019) and lions (*Panthera leo*) (Miller et al., 2019b; Olivier et al., 2017). Conversely, the low sensitivity of serological assays in cattle, was also observed with the IDEXX *Mycobacterium bovis* antibody test (van Der Heijden et al., 2020), as well as rapid lateral flow assays (Lyashchenko et al., 2020), in African buffaloes. In many species, CMI response-based assays have shown greater sensitivity for detecting MTBC infection than humoral response-based assays (Maas et al., 2013; Smith et al., 2021). This suggests that serological testing is suboptimal for individual diagnosis of TB in certain species and emphasises the need to understand comparative immune responses to MTBC infection.

Animal TB diagnosis, based on CMI responses, can include both in vivo and in vitro tests (Bernitz et al., 2021; Sakamuri et al., 2015). The Single Intradermal Comparative Tuberculin Test (SICTT) is an in vivo CMI-based test that measures the delayed type hypersensitivity reaction of the host (indicative of an immunological memory response) to intradermal injection of bovine and avian purified protein derivatives (PPDs) (Srinivasan et al., 2019). This is currently the standard method for diagnosis of bTB in livestock, recommended by the WOAH (World Organisation for Animal Health, 2018). This method has been used in various species of wildlife, although it is rarely validated for each species; this includes African lions (Miller et al., 2019b), warthogs (Roos et al., 2018), and African buffaloes (Bernitz et al., 2018; Smith et al., 2021). In some species, notably rhinoceros and elephants, tuberculin skin tests are considered unreliable and not recommended (Lyashchenko et al., 2006; Miller et al., 2017b). However, studies using MTBC specific antigens to replace PPDs may be an option to improve performance of skin tests in these species (Flores-Villalva et al., 2012; Subramanian et al., 2022).

Although the SICTT has shown utility in some wildlife species, it is accompanied by logistical challenges, such as requiring repeat immobilisation and housing or capture twice within 72 hours, and substantial fiscal cost associated with these requirements (Bernitz et al., 2021). Therefore, there is a need to develop and validate in vitro blood-based assays that only require

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a single handling event, such as antigen-specific cytokine release assays (CRAs) and gene expression assays (GEAs).

Both CRAs and GEAs measure CMI responses of antigen-stimulated circulating immune cells by quantifying the change in cytokine production, or transcription of cytokine genes, respectively (Smith et al., 2021). Both bovine and avian PPDs, as well as MTBC specific (peptide) antigens, such as early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10), have been used in stimulation platforms for these assays. Use of peptide antigens appeared to improve specificity for discrimination of MTBC infected from uninfected or non-pathogenic mycobacteria-exposed animals (Jenkins et al., 2018). The cytokine biomarker commonly used for quantification of MTBC specific CMI responses in humans, livestock, and wildlife is interferon-gamma (IFN-γ) (Chegou et al., 2014; Palmer et al., 2020); however, alternative cytokine biomarkers have also been used in other species. For example, the *M. bovis* antigen stimulated interferon-γ-induced protein 10 (IP-10) is a useful biomarker for TB diagnosis in cattle (Parsons et al., 2016), warthogs (Roos et al., 2018), and African buffaloes (Goosen et al., 2015).

Gene expression assays are based on quantification of antigen-specific changes in transcription of cytokine genes, indicative of an immune response (Smith et al., 2021). Some examples of cytokine gene targets that have been used in GEAs for *M. bovis* diagnosis in wildlife include *CXCL9* in lions and cheetahs (*Acinonyx jubatus*) (Kerr et al., 2020; Olivier et al., 2017), *IFN-γ* in buffaloes (Parsons et al., 2012), and *CXCL10* in warthogs (Roos et al., 2019) and white rhinoceros (*Ceratotherium simum*) (Chileshe et al., 2021). An advantage of using GEAs compared to CRAs is that they do not require the production of unique species-specific reagents, other than PCR primers. The requirement to produce antibodies to species-specific cytokines can be both difficult and costly. Instead, published cytokine gene primer sequences in the same or related species can facilitate implementation of these assays in different laboratories globally, without the need for access to non-commercially available reagents (Chileshe et al., 2021; de Waal et al., 2021; Higgitt et al., 2017; Olivier et al., 2017; Parsons et al., 2012; Roos et al., 2019).

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Application of Diagnostic Tests for the Investigation of the Epidemiology of Animal Tuberculosis in Multi-Host Systems

The development and implementation of effective, targeted TB control interventions has benefited from the availability of various bacterial genotyping systems. Until recently, the techniques applied for *M. bovis* surveillance and control have relied mainly on targeted PCR assays for a small number of genetic biomarkers (Collins, 2011; Downs et al., 2008; Durr et al., 2000; El-Sayed et al., 2016). Today, these widely accessible tools are invaluable for high throughput, population-level screening and surveillance of MTBC infection, and specifically, *M. bovis* infection. Some of the commonly used tools include region of difference (RD) analysis (Warren et al., 2006), spacer oligonucleotide typing, or "spoligotyping" (Haddad et al., 2004), and variable number tandem repeat (VNTR) typing (Musoke et al., 2015; Roring Solvig et al., 2002).

As *M. bovis* whole genome sequencing (WGS) technology advances and becomes more affordable, it is increasingly the preferred technique for animal TB surveillance. In addition to pathogen identification, WGS can be utilised to understand the source of TB outbreaks, and to study spatiotemporal transmission patterns and pathogen evolution (Bryant et al., 2012; Dippenaar et al., 2017; Gilchrist et al., 2015; Guimaraes and Zimpel, 2020). It has also been extensively employed for detecting and monitoring antimicrobial resistance (Didelot et al., 2012). Whole genome sequencing has improved the resolution of *M. bovis* epidemiologic investigations, scrutinizing transmission patterns and persistence of the pathogen at livestock-wildlife interfaces, and enabling identification of critical focus points for effective public health and disease control interventions (Crispell et al., 2019; Reis et al., 2021; Rossi et al., 2023).

Bovine TB in South Africa

Introduction of *Mycobacterium bovis* to livestock in southern Africa occurred in the early 1800s, by transmission from imported European cattle (Arnot and Michel, 2020; Henning, 1949; Myers, 1969). Importation of large numbers of untested cattle from Europe, Australia, and South America likely contributed to the increased prevalence of tuberculous disease in cattle identified in South African abattoirs (Arnot and Michel, 2020; Cousins et al., 2004). From as early as 1911, TB has been recognised as a major livestock disease in South Africa (Arnot and Michel, 2020).

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South African control programs for *M. bovis* infection in livestock, implemented in the 1970s, aimed to eradicate the disease in commercial cattle, and establish an official herd accreditation scheme (Michel et al., 2019). These efforts were highly successful; TB prevalence in the commercial cattle farming sector decreased markedly from 11.8% in 1971 to 0.39% in 1995 (Michel et al., 2008). In subsequent years, however, the subsidiarity of the State Veterinary Services, the introduction of budgetary constraints, and the reprioritisation of resources for disease control efforts, were a substantial hindrance to bovine TB control; the number of cattle tested countrywide on an annual basis decreased drastically from the late 1990s onwards (Arnot and Michel, 2020; Cloete, 2015). This and other difficulties with bTB control have led to the gradual re-emergence of the disease in commercial cattle production across the country (Arnot and Michel, 2020; Michel et al., 2008, 2019). Bovine TB also affects the communal farming sector; recent studies have shown that communal cattle herd prevalence is highly variable, ranging from < 0.5% to > 15% (Musoke et al., 2015; Sichewo et al., 2019) in different regions of South Africa.

At present, the onus is on provincial veterinary services to perform TB surveillance of cattle herds in their province (Department of Agriculture, Forestry and Fisheries, 2016). Varying funding allocations and available resources dictate the extent of surveillance performed across provinces (Davey, 2023). Should cattle owners wish to test their livestock, they may do so at personal expense. Some commercial farms undergo routine testing to be able to participate in trade of products. Therefore, only some of the national herds are tested for TB (Department of Agriculture, Forestry and Fisheries, 2016). However, any suspected or confirmed (through meat inspection or ante-mortem testing) outbreaks of TB must be reported, and control measures implemented according to the Bovine Tuberculosis Scheme (Department of Agriculture, Forestry and Fisheries, 2016). Infected farms are placed under quarantine; a test-and-slaughter or depopulation plan is then devised. If depopulation is not performed, cattle herds are required to undergo testing, using the tuberculin skin test at 90-day intervals, and slaughter of test positive individuals. Testing is repeated until the entire herd has three consecutive negative tests. This is often an expensive approach since subclinical infection in the herd may take years to eradicate (Department of Agriculture, Forestry and Fisheries, 2016). In addition, the absence of a compensation component of the plan often results in owners refusing to cull cattle, especially in rural communities where it is not feasible to implement quarantine due to communal grazing practices (Davey, 2023).

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M. bovis Infection in South African Wildlife

Mycobacterium bovis infection is widespread in South African wildlife, having been detected in 24 wildlife species to date (Bernitz et al., 2021), with the country's two largest wildlife reserves, Kruger National Park (KNP) and Hluhluwe iMfolozi Park (HiP) having been declared endemic for the disease (Hlokwe et al., 2016; Michel et al., 2006). These parks are bordered by communal lands, where livestock graze freely (Hlokwe et al., 2014; De Garine-Wichatitsky et al., 2013) and have been implicated in transmission of *M. bovis* to wildlife in the parks, especially prior to the construction of adequate boundary fencing (Hlokwe et al., 2011; Wadge, 2007). Infected wildlife species in these parks may now also pose a risk of spillback to neighbouring livestock, other wildlife, and people.

In KNP, *M. bovis* was first isolated in 1990 from a buffalo found near the south-western boundary with signs of poor health (de Vos et al, 2001). Further investigations revealed that spillover of the pathogen into various other species in the park, including chacma baboon (*Papio ursinus*), lion, cheetah, greater kudu (*Tragelaphus strepsiceros*), and leopard (*Panthera pardus*), had already occurred. Compelling circumstantial evidence indicated that *M. bovis* was likely introduced into the southern area of KNP circa 1960 (de Vos et al., 2001; Renwick et al., 2007). The African buffalo is now widely recognised as a TB reservoir host in KNP and implicated in the spillover of *M. bovis* to other susceptible hosts in KNP and surrounding areas (de Vos et al., 2001; Musoke et al., 2015; Renwick et al., 2007; Sichewo et al., 2020). As of 2021, a total of 16 species found in KNP have been confirmed with *M. bovis* infection, including black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceros (Chileshe et al., 2019; Dwyer et al., 2022; Miller et al., 2017a, 2017b, 2018).

Overall, the endemic presence of *M. bovis* in South African livestock and wildlife has several important potential consequences. From a wildlife conservation perspective, the gradually evolving impact of TB on population dynamics of affected social animal species, and on survival of affected endangered species, are of critical concern (Michel et al., 2006; Miller et al., 2017b). The risk of spillover at human-livestock-wildlife interfaces (e.g., through communal livestock) could have a substantial impact on public health, particularly in rural communities, like those surrounding KNP, HiP and other national parks, that may have limited access to healthcare (Meiring et al., 2018; Michel et al., 2006). Finally, trade and movement restrictions placed on animals and animal products in TB affected areas could be a major

impediment to economic and food stability of the country, which is largely dependent on livestock and wildlife tourism industries (Azami and Zinsstag., 2018).

TB in KNP Rhinoceros

In 2016, a black rhinoceros with *M. bovis* infection was discovered in KNP (Miller et al., 2017a). Rangers had reported that the adult female rhinoceros appeared weak and emaciated and had been stationary for 36 hours since it was initially spotted in the southern area of the park. Following euthanasia due to poor prognosis, a necropsy revealed the presence of multiple lung lesions, most of which had a fibrous capsule and contained creamy necro-caseous material (Miller et al., 2017a). Numerous acid-fast bacilli were observed in impression smears of lung lesions. Additionally, MTBC antigen-specific release of IFN-γ in stimulated whole blood collected prior to euthanasia was consistent with immune sensitisation to *M. bovis* or *M. tuberculosis* (Miller et al., 2017a). Mycobacterial culture and speciation confirmed infection with *M. bovis*.

A surveillance program was initiated to screen rhinoceros carcasses in KNP for TB; between June 2016 and October 2017, 35 white and 5 black rhinoceros carcasses were macroscopically examined, and samples were collected for histopathologic studies and mycobacterial culture (Miller et al., 2018). *Mycobacterium bovis* infection was confirmed in six white rhinoceros (Miller et al., 2018). Macroscopic lesions were observed in the retropharyngeal or tracheobronchial lymph nodes and lungs; on histological examination, granulomatous inflammation in lung and lymph node sections was noted, and acid-fast organisms cultured from some granulomas were spoligotyped as *M. bovis* SB0121, a strain commonly isolated in KNP (Dippenaar et al., 2017; Miller et al., 2018).

The discovery of *M. bovis* infection in KNP rhinoceros has resulted in regulatory restrictions that prevents movement of animals out of the park, without quarantine and approved testing (P. Buss, pers. comm., 2020). This is a hindrance to conservation program activities that include translocation of rhinoceros from *M. bovis*-endemic (e.g., KNP) to TB-free areas. In response to these actions, there was an urgent requirement for an ante-mortem diagnostic test that could identify *M. bovis*-infected rhinoceros, prior to their translocation. The plan would ensure compliance with *M. bovis* control measures while allowing for continued movement of rhinoceros out of the park, a critical component of the conservation program. Thus, the QFT-IGRA test was developed and partially validated for detection of *M. bovis* infection in white

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rhinoceros (Chileshe et al., 2019), and has been similarly applied in black rhinoceros (Dwyer et al., 2022). This CRA employs the QuantiFERON-TB Gold (In-Tube) Plus (Qiagen, 2023) MTBC antigen stimulation platform on rhinoceros whole blood, along with the anti-equine IFN-γ ELISA^{PRO} kit (Mabtech Ab, Nacka Strand, Sweden) to measure IFN-γ production (Chileshe et al., 2019). Subsequently, the use of this assay was conditionally approved by the South African Department of Agriculture, Land Reform and Rural Development as part of the South African National Parks Rhinoceros Tuberculosis Management Plan (Buss et al., 2017).

Justification for Study

Although TB has been confirmed in the KNP rhinoceros population, there are knowledge gaps regarding factors affecting susceptibility, routes and sources of infection, and progression of disease. Furthermore, in the KNP population, which is one of the largest free-ranging populations of African rhinoceros remaining today, relatively little is understood about the prevalence and pathogen transmission dynamics of *M. bovis,* and the role that these species play in this multi-host TB endemic system.

Similar to the concerns associated with TB in Asian elephants in Nepal, the unknown impact of this pathogen on rhinoceros health and conservation in KNP, as well as other free-ranging populations globally, is critically concerning. Evidence from other multi-host animal TB systems (e.g., white-tailed deer in Michigan (VerCauteren et al., 2018), European badgers in Ireland (Allen et al., 2018)) indicates that certain affected wildlife species may be important contributors to the persistence of the pathogen in the system. Given the frequent, mandated translocation of rhinoceros for conservation reasons, it is important to understand the role these species may play in the persistence of *M. bovis* in KNP, and whether they could present a risk for spillover to naïve populations post-translocation.

Overall, a critical step in addressing the concern of TB in African rhinoceros is the development of tools to conduct TB surveillance to provide information for effective control strategies, as well as support conservation plans. It is crucial to identify drivers of MTBC infection and disease in African rhinoceros and assess the potential risk they pose to rhinoceros and other susceptible hosts. In this study, the QFT-IGRA and improved direct detection tools were used to investigate the epidemiology of rhinoceros TB. Findings will contribute to further development of surveillance programs and effective intervention strategies, both for the

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conservation of these important species, and TB management of affected rhinoceros populations and ecosystems.

Study Research Questions, Aims, and Objectives

Research Question 1: What are the existing knowledge gaps regarding detection, transmission, and risk factors for MTBC infection in African rhinoceros?

Aim 1. To collate information on the epidemiology of MTBC infections in African rhinoceros.

Objectives:

1.1. To describe cases in the scientific literature and identify knowledge gaps surrounding detection, transmission, and risk factors for MTBC infection in African rhinoceros.

1.2. To generate hypotheses on potential risk factors for *M. bovis* infection in African rhinoceros.

Research Question 2: What are the risk factors for *M. bovis* infection in rhinoceros living in an *M. bovis* endemic ecosystem?

Aim 2. To determine prevalence and risk factors for *M. bovis* infection in KNP rhinoceros.

Objectives:

2.1. To screen KNP rhinoceros for *M. bovis* infection using the QFT-IGRA and conventional mycobacterial culture.

2.2. To assemble and describe demographic and spatial characteristics for *M. bovis*-infected and uninfected rhinoceros from KNP.

2.3. To determine overall and area-specific prevalence of *M. bovis* infection in KNP rhinoceros.

2.4. To develop a statistical model to identify risk factors associated with *M. bovis* infection in the KNP population of black and white rhinoceros.

Research Question 3: How do storage conditions, commonly encountered in remote locations, affect results of the rhinoceros QFT-IGRA?

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Aim 3. To assess the impact of refrigeration and delayed stimulation of rhinoceros whole blood on mitogen stimulated IFN-γ production.

Objectives:

3.1. To collect and stimulate replicate samples of whole blood from African rhinoceros using the QFT Nil and Mit tubes within 6 hours of collection, and after 24 and 48 hours stored at 4˚C.

3.2. To use the anti-equine IFN-γ ELISA^{PRO} kit (Mabtech Ab, Nacka Strand, Sweden) to measure IFN-γ concentrations in plasma from stimulated whole blood samples (objective 3.1).

3.3. To compare the concentrations of IFN-γ in plasma from Nil and Mit stimulated whole blood samples, respectively, for the three different storage conditions to quantify effects on test results.

Research Question 4: Can MTBC be detected in nasal swabs from QFT-IGRA positive rhinoceros?

Aim 4. To determine whether MTBC can be detected in nasal swabs from rhinoceros with immunological evidence of infection.

Objectives:

4.1. To screen rhinoceros in KNP for MTBC infection using the QFT-IGRA.

4.2. To determine presence of MTBC in nasal swabs from IGRA positive rhinoceros using conventional and novel mycobacterial culture methods along with mycobacterial species determination by PCR.

4.3. To screen samples (from objective 4.2) directly with the Cepheid GeneXpert MTB/RIF Ultra qPCR assay to detect MTBC DNA.

Ethics and Other Permits

Ethical approval for this project was granted by the Stellenbosch University Animal Care and Use Committee for black and white rhinoceros (ACU-2020-19019), and the Stellenbosch University Biological and Environmental (REC:BES) Research Ethics Committee (SU-BEE-2021-22561). Section 20 approval was granted by the Department of Agriculture, Land Reform and Rural Development (DALRRD; 12/11/1/7/2; 12/11/1/7/2A(JD)). Additionally,

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Biomaterial Transfer Agreements (BMTAs) were approved by South African National Parks (SANParks) Veterinary Wildlife Services to obtain and use rhinoceros samples (BMTA 005/22, 011/19, 006/16); this includes evaluation by their Animal Care and Use Committee. An approved research agreement (Research Agreement: SS228) was obtained from SANParks Scientific Services for spatial data access permission. A Threatened or Protected Species (TOPS) permit was obtained through the Department of Environmental Affairs (DEA Standing Permit S02556; S65805 and DEA Registration Certificate 29416; 02256).

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Chapter 2

Epidemiology of Tuberculosis in Multi-Host Systems: Implications for Black (*Diceros bicornis*) and White (*Ceratotherium simum*) Rhinoceros

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Abstract

Cases of tuberculosis (TB) resulting from infection with *Mycobacterium tuberculosis* complex (MTBC) have been recorded in captive white (*Ceratotherium simum*) and black (*Diceros bicornis*) rhinoceros. More recently, cases have been documented in free-ranging populations of both species in bovine tuberculosis (bTB) endemic areas of South Africa. There is limited information on risk factors and transmission patterns for MTBC infections in African rhinoceros, however, extrapolation from literature on MTBC infections in other species and multi-host systems provides a foundation for understanding TB epidemiology in rhinoceros species. Current diagnostic tests include blood-based immunoassays but distinguishing between subclinical and active infections remains challenging due to the lack of diagnostic techniques. In other species, demographic risk factors for MTBC infection include sex and age, where males and adults are generally at higher risk than females and younger individuals. Limited available historical information reflects similar age- and sex-associated patterns for TB in captive black and white rhinoceros, with more reports of MTBC associated disease in black rhinoceros than in white rhinoceros. The degree of MTBC exposure in susceptible wildlife depends on their level of interaction, either directly with other infected individuals or indirectly through MTBC contaminated environments, which is dependent on the presence and abundance of infected reservoir hosts and the amount of MTBC shed in their excreta. Captive African rhinoceros have shown evidence of MTBC shedding, and although infection levels are low in free-ranging rhinoceros, there is a risk for intraspecies transmission. Free-ranging

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rhinoceros in bTB endemic areas may be exposed to MTBC from other infected host species, such as the African buffalo (*Syncerus caffer*) and greater kudu (*Tragelaphus strepsiceros*), through shared environmental niches, and resource co-utilisation. This review describes current knowledge and information gaps regarding the epidemiology of TB in African rhinoceros.

Introduction

Tuberculosis (TB) is a chronic infectious disease that affects a broad range of host species (World Organisation for Animal Health, 2019). It is caused by members of a group of closely related pathogenic mycobacteria known as the *Mycobacterium tuberculosis* complex (MTBC) (World Organisation for Animal Health, 2019). Bovine tuberculosis (bTB) is caused by *Mycobacterium bovis* (*M. bovis*), which is known to infect livestock as well as captive and free-ranging wildlife species (Corner, 2006; Michel et al., 2006). For most wildlife, however, little is known about susceptibility to MTBC infection, pathogenesis, and its impact on affected populations.

Rhinoceros are iconic species which are under threat due to habitat destruction and heavy poaching pressure. Cases of TB resulting from infection with *M. tuberculosis* and *M. bovis* have been recorded in captive, semi-captive (maintained in private reserves and more intensively managed), and free-ranging rhinoceros worldwide (Hofmeyr, 1956; Keep and Basson, 1973; Mann et al., 1981; Dalovisio et al., 1992; Barbiers, 1994; Stetter et al., 1995; Valandikar and Raju, 1996; Rookmaaker, 1998; Oh et al., 2002; Espie et al., 2009; Duncan et al., 2009; Miller et al., 2017, 2018; Witte pers. comm., 2020); TB was implicated as the cause of death in some of these cases.

Kruger National Park (KNP) and Hluhluwe–iMfolozi Park (HiP) are home to large populations of free-ranging black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceros in South Africa. As of 2016, HiP housed ~1,500 white rhinoceros and 360 black rhinoceros (KZN Nature Conservation Board, 2017). In 2017, the Kruger National Park contained ~5,150 white rhinoceros and 500 black rhinoceros (SANParks Scientific Services, 2011), at which time the global populations totalled 20,300 white rhinoceros and 5,200 black rhinoceros (International Rhino Foundation, 2017). The HiP and KNP populations have been and continue to be central to the “Integrated Strategic Management of Rhinoceros” plan, introduced by the South African Department of Environmental Affairs (Department of Environment, Forestry and Fisheries, 2014; Department of Environmental Affairs (DEA), 2018). Part of this strategy relies on the translocation of rhinoceros from the feeder populations in these parks to newly developing

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rhinoceros safeguarding strongholds around the country. However, KNP and HiP are endemic for bTB, and these rhinoceros populations share habitat ranges and various resources with *M. bovis*–infected wildlife (over 20 species in KNP), including important bTB maintenance hosts such as African buffaloes (*Syncerus caffer*) (Michel et al., 2006), and greater kudu (*Tragelaphus strepsiceros*) (Bengis et al., 2001; Renwick et al., 2007; Michel et al., 2009). The identification of disease in free-ranging wildlife is often challenging due to limited resources and access to these populations for diagnostic testing, and it was only with the increase in poaching and associated veterinary interventions that evidence of MTBC infection in white and black rhinoceros in KNP was discovered (Miller et al., 2017, 2018).

Although *M. bovis* and *M. tuberculosis* have not been considered an immediate threat to the world's African rhinoceros populations, the potential impact of these pathogens on their health and conservation is largely unknown. Because bTB is a World Organisation for Animal Health (WOAH) and nationally notifiable disease (World Organisation for Animal Health, 2019), animals with *M. bovis* infection are subject to regulatory requirements which limit their movements between populations; this hampers conservation efforts that are reliant on translocation of rhinoceros from bTB-endemic to bTB-free areas.

Since the discovery of *M. bovis* infection in the free-ranging rhinoceros populations in KNP (Department of Environment, Forestry and Fisheries, 2014; Miller et al., 2017), knowledge gaps regarding the risk of MTBC infection, intra- and inter-species transmission, and disease progression in these species have become apparent. This review describes the current knowledge regarding TB in African rhinoceros and provides information on epidemiological aspects of this disease in other relevant species, especially free-ranging populations, to improve understanding of the disease and inform management strategies.

Assessment of Infection Risk in African Rhinoceros

With any infectious disease, the risk of becoming infected is based on an individual's susceptibility when exposed to an infectious dose of the etiological agent, as well as the likelihood of exposure to the pathogen (Jakob-Hoff, 2014). These factors provide a foundation for investigating infection risks and will be discussed as they pertain to *M. tuberculosis* and *M. bovis* infection in black and white rhinoceros.

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Outcomes and Detection of MTBC Infections: Epidemiological Implications

MTBC infections are typically chronic and once disease occurs, usually progressive. In various human and animal hosts, infections have been observed to move between various stages in a dynamic host-pathogen interaction network, where clinical manifestations of the infection vary between stages (Domingo et al., 2014; Flynn et al., 2015; Drain et al., 2018; Palmer et al., 2020). Following infection, the host's innate immune system may eliminate the mycobacteria. If the mycobacteria are not eliminated, T helper type-1 cell-mediated immune (CMI) responses develop and are followed by the T helper type-2 humoral immune response through B-lymphocyte activation and an increase in circulating antibodies (Mogues et al., 2001; Pollock and Neill, 2002; Myllymäki et al., 2016). Immunological responses of the host play a key role in determining the outcome of infection, and in humans, these include latent, incipient and subclinical stages (Drain et al., 2018). These stages have not been clearly defined in animal hosts, but subclinical MTBC infections have been reported in a variety of species (Gallagher et al., 1998; Gill et al., 2009; Diedrich et al., 2010; The Centre for Food Security and Public Health, 2019). Although controversial, there is speculation that latent infection of animals with *M. bovis* may also occur (Pollock and Neill, 2002; Sabio y García et al., 2020). These complex and dynamic interactions between host and pathogen can lead to elimination of infection, or an asymptomatic stage in which the mycobacteria are either dormant or result in localised disease, or progression to active disease, which has been observed in a multitude of species (Domingo et al., 2014; Cadena et al., 2017; Drain et al., 2018; Vallejo et al., 2018).

Although there is a paucity of information on outcomes of MTBC infection in rhinoceros, a hypothesised scenario is shown in Figure 2.1; however, further investigation is required to verify these stages. Most cases of TB in zoos have only been detected after disease is sufficiently advanced to detect clinical signs, resulting in the death of the rhinoceros either due to euthanasia or disease complications (Addendum A, Table S2.1) (Hofmeyr, 1956; Keep and Basson, 1973; Rookmaaker, 1998; Espie et al., 2009). However, a study involving three experimentally *M. bovis*-infected white rhinoceros, monitored serially over 2 years, suggests that although immunological responses could be detected, the animals appeared to contain, and potentially eliminate, the infection (Michel et al., 2017; Parsons et al., 2017). Similarly, in the limited cases of natural *M. bovis* infection in white rhinoceros, pathological lesions were localised in lymph nodes or other tissues (Miller et al., 2018). The outcome of infection appears to be more complex than simply a progression to disease, based on these observations as well as reports of immunological responses (without evidence of disease) in rhinoceros that have

been exposed to other known TB cases (Mann et al., 1981; Stetter et al., 1995; Duncan et al., 2009). In addition, decreases in immunocompetence as a result of comorbidities, drought, capture/transport–induced stress, increased age, or other factors may be associated with greater susceptibility to disease as sequelae of acute infection or activation of subclinical infection in rhinoceros, although tools to identify these stages need to be developed.

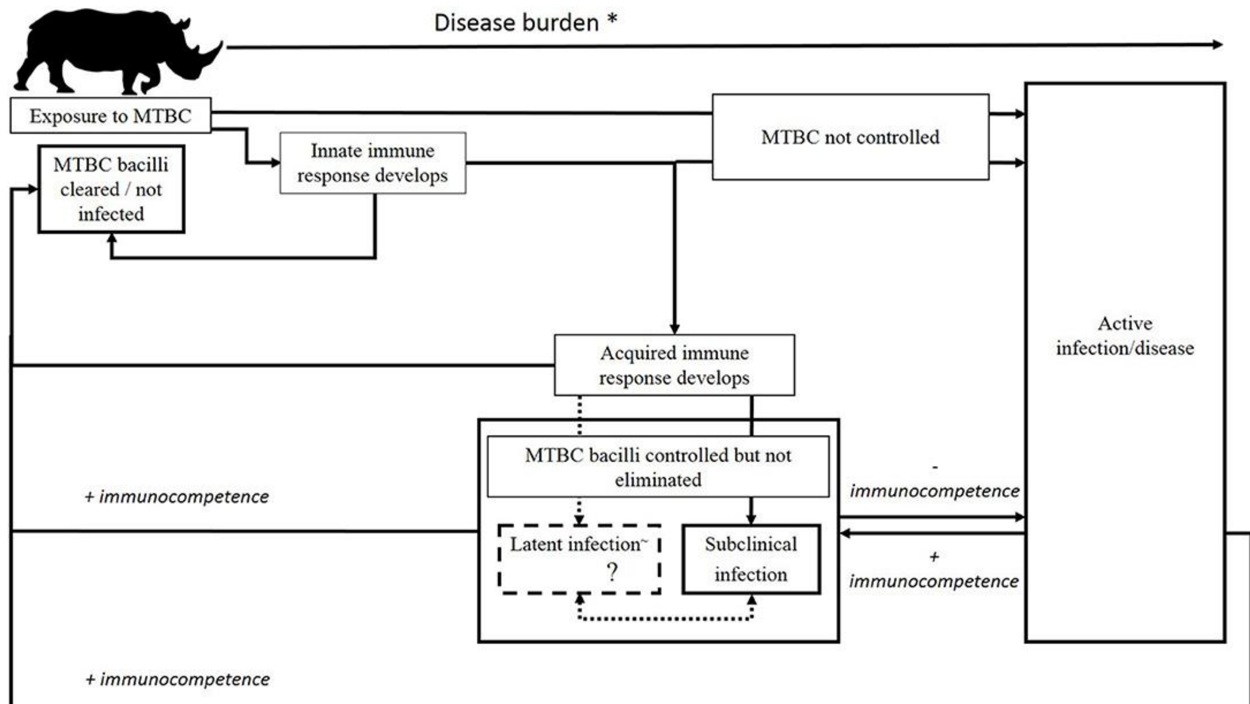

Figure 2.1. Possible (theoretical) outcomes of *M. bovis* infection in African rhinoceros. After initial exposure, MTBC may be eliminated by the host's immune response, persist as a subclinical or latent infection, or progress to active infection/disease. Following the establishment of subclinical or latent infection, the host's immune response may clear the MTBC, or infection may persist in this form, either naturally progressing in a slow or rapid fashion to active tuberculosis, or cycling through subclinical and latent states, before development into symptomatic disease or eventual eradication of the infection by the host's adaptive immune response. *Rising disease burden implies an increase in abundance of MTBC biomarkers, immunological changes characteristic of stage of infection, and increasing pathology, with a declining recovery prognosis. ~Although controversial, there is evidence that latent infection of animals with *M. bovis* may occur. More research is required to determine whether latent infection can occur in animals, including rhinoceros.

In order to characterise the epidemiology of TB in a population, it is important to have accurate diagnostic methods that can distinguish between various stages of infection and disease, since individuals in different stages of infection may present with altered levels of transmission risk (Walzl et al., 2018). Risk factors associated with acquiring an infection may be different from those that increase the likelihood of disease progression, or the maintenance of a subclinical or latent infection (Jakob-Hoff, 2014; Drain et al., 2018). This important distinction may have

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implications for understanding the epidemiology of TB and could impact subsequent management decisions.

In vivo and *in vitro* indirect detection methods for early MTBC infection primarily rely on the detection of TB-specific adaptive immune responses of the host, including the tuberculin skin test (TST) and MTBC antigen stimulated cytokine assays (Pollock et al., 2005; Chambers, 2009). In addition, serological assays for the detection of host-specific antibodies to MTBC antigens have been useful for diagnosis of TB in certain animal species (Miller, 2008; Roos et al., 2018; Kerr et al., 2019), although they are considered unreliable for TB diagnosis in humans (World Health Organisation, 2011). In rhinoceros, the TST is unreliable due to cross-reactivity with environmental mycobacteria (Mann et al., 1981; Godfrey et al., 1990; Stetter et al., 1995); therefore, a white rhinoceros whole blood MTBC antigen-specific interferon-gamma release assay (IGRA) for *M. bovis* infection has recently been developed (Parsons et al., 2017; Chileshe et al., 2019). Serological assays for the detection of antigen-specific antibodies have also been shown to be useful for the diagnosis of MTBC infection in rhinoceros (Duncan et al., 2009; Miller et al., 2015). However, the use of these indirect immunological diagnostic assays alone may not distinguish between recent infection, latent/incipient/subclinical infection, and active disease.

One way to overcome the challenges posed by these indirect tests is to directly detect the pathogen by mycobacterial culture and nucleic acid amplification tests (NAATs) (Po-Liang et al., 2011; Maas et al., 2013). Mycobacterial culture and species determination are useful as both ante- and post-mortem diagnostic tests for MTBC infection. Ante-mortem samples obtained for culture include bronchoalveolar, tracheal and gastric lavages, as well as nasal and faecal swabs, although culture of tissue obtained during necropsy may be more sensitive for detection of bacilli. Although this method is highly specific, culture of ante-mortem samples has low sensitivity, which may be related to the site of infection and whether the individual is shedding at the time of sampling (Drewe et al., 2010). For example, in a study evaluating shedding in three experimentally *M. bovis*-infected rhinoceros, only one of 36 tracheal lavage samples collected monthly over a 2-year period was *M. bovis* culture positive (Michel et al., 2017). In humans and recently in wildlife, mycobacterial culture has been supplemented with NAATs including the automated GeneXpert MTB/RIF Ultra qPCR assay (Ultra) (Goosen et al., 2020; Hlokwe and Mogano, 2020). This rapid ancillary test may enable the direct detection of MTBC DNA in some tissues (Kerr et al., 2020), as well as animal respiratory samples (Goosen et al., 2020). Regardless, the direct detection of MTBC organisms alone also does not

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provide information on the host's stage of infection or disease. The presence and classification of lesions detected by macroscopic and microscopic examination provides important information for staging disease, although this is primarily used post-mortem (Gupta et al., 2016; Michel et al., 2017).

Molecular methods such as spoligotyping, mycobacterial interspersed repetitive unit-variable number of tandem repeat (MIRU-VNTR) genotyping, or whole genome sequencing of *M. bovis* isolates may be useful not just for diagnosis, but also for tracing the origin of MTBC infection in multi-host systems (Michel et al., 2009; Cunha et al., 2012; Dippenaar et al., 2017; Price-Carter et al., 2018; Guimaraes and Zimpel, 2020). The application of these techniques in rhinoceros and other free-ranging wildlife, though useful, is challenging due to limited samples. Nonetheless, these techniques have been employed to investigate the distribution and transmission of MTBC strains in some wildlife multi-host systems (Price-Carter et al., 2018), including between brushtail-possums (*Trichosurus volpecula*) (Crispell et al., 2017), badgers (Biek et al., 2012; Crispell et al., 2017), deer (Crispell et al., 2020), or African buffaloes (Sichewo et al., 2020) and cattle at the livestock/wildlife interface (Biek et al., 2012; Crispell et al., 2017; Sichewo et al., 2020; Crispell et al., 2020). Therefore, use of both direct and indirect detection methods should be included in investigations of transmission in rhinoceros.

Investigating Susceptibility of Black and White Rhinoceros to Infection with *M. tuberculosis* and *M. bovis*

As a result of the popularity of rhinoceros for zoological exhibition, they have historically been globally distributed through importation. Reports of TB in captive rhinoceros in zoological gardens worldwide date back to the late 1800's. Historical cases of bTB and TB in black and white rhinoceros are summarised in Figure 2.2. While TB is still considered a rare occurrence in domestic perissodactyls (Pesciaroli et al., 2014), these cases provide evidence for susceptibility of black and white rhinoceros.

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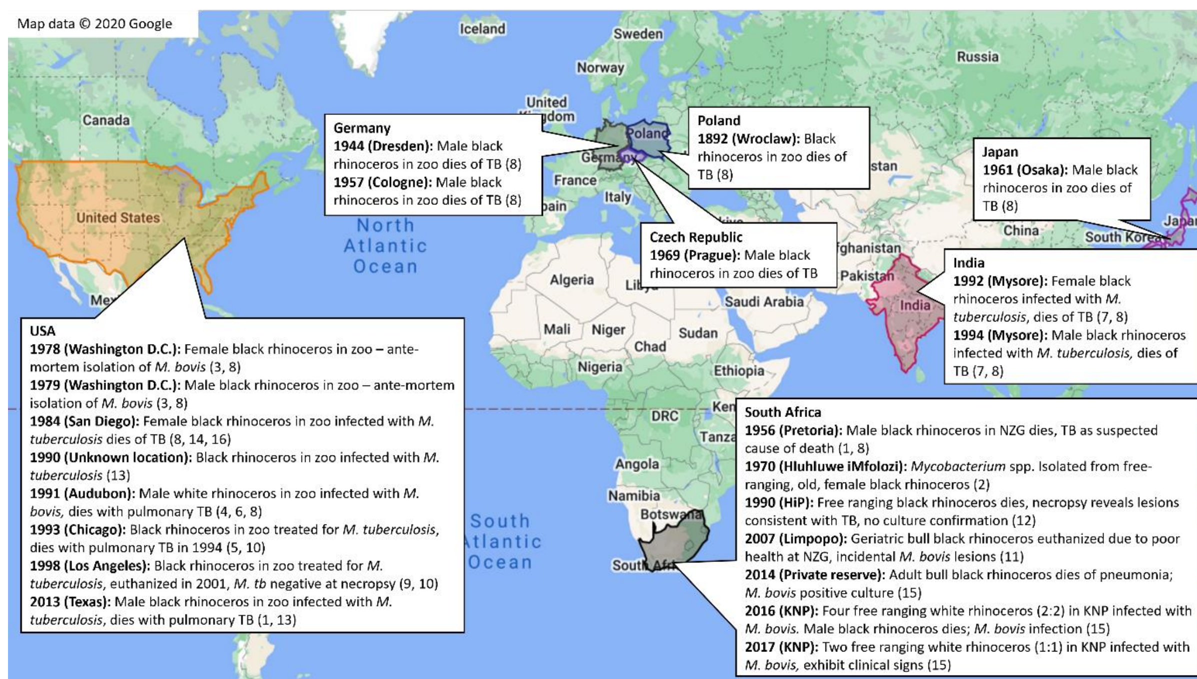

Figure 2.2. Summary of globally recorded historical cases of tuberculosis in black rhinoceros (*D. bicornis)* and white rhinoceros (*C. simum*) (1. Hofmeyr, 1956; 2. Keep and Basson, 1973; 3. Mann et al., 1981; 4. Dalovisio et al., 1992; 5. Barbiers, 1994; 6. Stetter et al., 1995; 7. Valandikar and Raju, 1996; 8. Rookmaaker, 1998; 9. Oh et al., 2002; 10. Duncan et al., 2009; 11. Espie et al., 2009; 12. Morar et al., 2013; 13. Miller et al., 2015; 14. Miller et al., 2017, 15. Miller et al., 2018; 16. Witte pers.comm., 2020)*. *These data were located in the Rhinoceros Resource literature database, or through extensive web searches with Miller et al. (2017) as a guide.

According to limited information, most TB cases have been recorded in black rhinoceros, with an apparent paucity of cases identified in white rhinoceros (Hofmeyr, 1956; Keep and Basson, 1973; Mann et al., 1981; Dalovisio et al., 1992; Barbiers, 1994; Stetter et al., 1995; Valandikar and Raju, 1996; Rookmaaker, 1998; Oh et al., 2002; Duncan et al., 2009; Espie et al., 2009; Morar et al., 2013; Miller et al., 2017, 2018; Witte pers.comm., 2020). This observation may be related to differences in numbers, demographics, housing or management of these species in zoological collections, exposure to other infected animals, employees or visitors, differences in species-specific susceptibility to the different pathogens, or the impact of individual or species-specific co-morbidities on immunocompetence and TB susceptibility. Black rhinoceros in captivity are known to suffer from a variety of syndromes (Dennis et al., 2007); afflicted individuals may have compromised immunity that increases their susceptibility to TB, which could explain the apparent higher prevalence in this species compared to white rhinoceros.

Most reports of TB in captive rhinoceros have been caused by infection with *M. tuberculosis*, with only a few caused by *M. bovis*. This is hypothesised to be a result of a high level of

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exposure of captive animals to *M. tuberculosis* through direct or indirect interactions with humans (especially in high TB burden countries), while in captivity or during exportation (Rookmaaker, 1998) (Addendum A, Table S2.1). Another explanation might be differences in virulence of *M. tuberculosis* and *M. bovis* in rhinoceros, although limited studies suggest that *M. bovis* has greater virulence in other species, such as mice and goats, compared to *M. tuberculosis* (Bezos et al., 2015; Dong et al., 2017).

The susceptibility of white rhinoceros to *M. bovis* was studied in experimentally infected animals (Michel et al., 2017; Parsons et al., 2017). The results confirmed susceptibility to infection and potential to shed bacilli, albeit based on the detection of viable *M. bovis* in only one of 36 tracheal lavage samples collected over the course of the study. None of the individuals developed clinical signs or evidence of disease based on gross and histological examination, although *M. bovis* DNA was detected by PCR in lung tissues of two animals at necropsy (Michel et al., 2017). The immune response kinetics and pathological findings suggested that the rhinoceros were able to contain and possibly clear *M. bovis* infection (Parsons et al., 2017). This observation is consistent with the historical lack of TB cases in white rhinoceros, which may be due to the ability to contain and clear the infection before the onset of disease. However, it should be noted that the response of rhinoceros to experimental inoculation in this study may not reflect natural infection, which could occur through one or more exposure events over time, each with variable numbers of MTBC bacilli. Additionally, the individuals in this study were not subject to stressful conditions and were young (4–7 years old); all individuals had adapted to living in managed care by the time of initial infection and were not exposed to the seasonal variations in food availability that they might have been if free-ranging. It is unknown whether the lack of disease development in these white rhinoceros was a consequence of low susceptibility to disease resulting from *M. bovis* infection, or the conditions associated with the experimental infection. In contrast, a naturally *M. bovis* infected 29-year-old white rhinoceros in a zoo developed weight loss, cough and nasal discharge and succumbed to the infection (Stetter et al., 1995), demonstrating that this species can develop disease.

In contrast to white rhinoceros, evidence of TB disease in black rhinoceros has been reported more frequently. An elderly (estimated 35–40 years old) black rhinoceros, euthanised due to loss of condition, had small non-encapsulated pulmonary granulomas associated with *M. bovis* infection (Espie et al., 2009). Similarly, the free-ranging black rhinoceros in KNP, infected with *M. bovis*, had evidence of significant pulmonary pathological changes (Miller et al., 2015).

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Interestingly, the first case was in an elderly animal and the second rhinoceros case was discovered after a prolonged period of drought. However, cases of *M. bovis*-associated disease in zoo black rhinoceros have been reported in animals that were ~20–25 years old (Mann et al., 1981). In addition, post-mortem pulmonary changes consistent with *M. tuberculosis* disease were observed in multiple zoo black rhinoceros aged 13–33 years old (Valandikar and Raju, 1996; Duncan et al., 2009; Miller et al., 2015). The reports of clinical signs and presence of changes post-mortem in black rhinoceros infected with *M. bovis* or *M. tuberculosis* suggest this species may be more prone to TB disease.

Assessing Risk Factors for Infection and Transmission Patterns of MTBC in African Rhinoceros Populations

Demographic Risk Factors

There is limited literature that characterises risk factors of MTBC exposure and transmission patterns in free-ranging black and white rhinoceros populations, likely a result of the logistical and technical difficulties associated with disease surveillance and sporadic cases in these animals. Therefore, extrapolation from literature on TB in other species and multi-host systems may aid in understanding the epidemiology of MTBC in African rhinoceros populations. Demographic risk factors for MTBC infection, such as sex and age, have been described in humans, mice, cattle, and limited species of wildlife (Uplekar et al., 1999; O'Brien et al., 2002; Zanella et al., 2008; Santos et al., 2009; Di Marco et al., 2012; Brooks-Pollock et al., 2013; Graham et al., 2013; Byng-Maddick and Noursadeghi, 2016; Madeira et al., 2017; Roos et al., 2018; Kerr et al., 2019; World Health Organisation, 2019). Results from these studies may inform hypotheses regarding demographic patterns of MTBC infection in rhinoceros.

In many TB-susceptible species, sex is considered a risk factor for infection (Uplekar et al., 1999; O'Brien et al., 2002; Zanella et al., 2008; Di Marco et al., 2012; Graham et al., 2013; Roos et al., 2018; Kerr et al., 2019; World Health Organisation, 2019). In humans, the global male to female ratio for individuals that develop TB is 2:1 (World Health Organisation, 2019). While this has partially been due to socioeconomic and cultural barriers in access to healthcare (Uplekar et al., 1999), inherent biological characteristics are also implicated. Sex-based differences in susceptibility are usually observed in adults, but not in children or adolescents (Neyrolles and Quintana-Murci, 2009). This suggests that the relative male:female difference in susceptibility is related to the effect of steroid sex hormones and their regulatory activities on immune cells (Bini et al., 2014).

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Both testosterone and progesterone are immunosuppressive. These hormones impair macrophage activation and may increase TB susceptibility (D'Agostino et al., 1999; Rook et al., 2004). In contrast, estrogen is a pro-inflammatory mediator that stimulates the production of tumour necrosis factor alpha (TNF-α) (Zuckerman et al., 1995), and interacts with the IFN-γ promoter (Fox et al., 1991). In mice, increased susceptibility to TB has been observed in post-adolescent males relative to post-adolescent females, with this difference partially mitigated by castration (Bini et al., 2014).

In various wildlife species, studies have shown a higher frequency of MTBC infection in males, which could be linked to hormonal differences, but behavioural differences may also play a role. In a cohort of free-ranging African elephants (*Loxodonta africana*) tested in KNP, overall TB seroprevalence was higher in males than in females (Kerr et al., 2019). Another study reported a higher risk for both bTB infection and disease in male badgers (*Meles meles*) than in females (Graham et al., 2013). An epidemiological study of white-tailed deer (*Odocoileus virginianus*) in Michigan also reported a higher odds of being bTB test-positive in males compared to females (O'Brien et al., 2002). This study also reported a dramatic effect of sex on the association of increasing age with positive TB test status. In fawns and yearlings, no significant difference in TB incidence between males and females was found; however, in age groups of 2 years and above, males were increasingly more likely to test positive for TB than females of the same age class. This is a similar trend to what is observed in humans and may be due either to sex-based hormonal differences, or to the contrasting social/reproductive behaviour between mature males and females (O'Brien et al., 2002; Vanderwaal et al., 2016). Therefore, it may be difficult to separate risk factors associated with hormonal and behavioural differences in adults.

Most wildlife epidemiological studies report a higher risk of bTB in males than in females; however, this association does not strictly hold true for all species. Studies of different populations of wild boar, for example, have yielded conflicting results with respect to the association between sex and bTB risk. One study on wild boars in Portugal (Madeira et al., 2017) reported a significantly higher bTB incidence in females than males in all age groups. In a different wild boar population in Spain (Santos et al., 2009), studies showed a significantly higher bTB prevalence in males. Studies of wild boar populations in France (Zanella et al., 2008) and Italy (Di Marco et al., 2012) reported no significant association between sex and TB risk. Variable findings from different populations of the same species illustrate the complexity of determining sex-associated bTB risk. Hormone-derived TB susceptibility of a species may

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be largely conserved between different multi-host systems; however, sex may also be a mediator of pathogen exposure due to sex-related differences in social, reproductive, and territorial behaviour as well as movement patterns. The degree to which such sex-related factors alter rhinoceros' exposure to MTBC may depend on the unique characteristics of dispersal and transmission in that particular host system (Vanderwaal et al., 2016).

Most historical reports of TB in captive rhinoceros have occurred in black rhinoceros males (Figure 2.2 and Addendum A, Table S2.1). It is unknown whether this observation represents a true species and sex predilection for TB, or whether reports are biased for other reasons, such as the skewed natal sex ratios in captive black rhinoceros (Dennis et al., 2007), or a disproportionate number of black rhinoceros and/or males kept in zoos. However, available records show a preference for importation and exhibition of female white rhinoceros over males, and no substantial preferential importation of male vs. female black rhinoceros for exhibition during the twentieth century (Rookmaaker, 1998). Records for the USA, Mexico, and Australia show a near-equivalent sex ratio of black rhinoceros currently kept in captivity (Ferrie, 2020a, 2020b), and the sex ratio of white rhinoceros in captivity in Canada, the USA, Mexico, Chile, and Singapore is substantially skewed toward females (Capiro, 2020). These records also indicate that a higher number of white rhinoceros are kept in captivity compared to black rhinoceros; there are currently 278 white rhinoceros in captivity in Canada, the USA, Mexico, Chile, and Singapore (Capiro, 2020), compared to 96 black rhinoceros in the USA, Mexico, and Australia (Ferrie, 2020a, 2020b). These data do not support an apparent bias toward males or black rhinoceros in captivity, which suggests that reports (Figure 2.2 and Addendum A, Table S2.1) may reflect a true increased risk for TB in these groups. While the absolute historical numbers of rhinoceros housed in captivity globally are unknown, and therefore cannot be used to draw conclusions on TB risk in rhinoceros, these observations provide avenues for further investigation into species and sex-specific susceptibility.

In humans, TB occurs in individuals of all ages, although the highest burden is in men past adolescence (≥15 years old) (World Health Organisation, 2019). Human susceptibility to TB shows an increase with age, which may be due to age-related effects on the immune system or possibly the outcome of multiple exposures over time (Byng-Maddick and Noursadeghi, 2016). A similar age-related bTB trend has been observed in cattle, with a peak in incidence after 12 months of age (Brooks-Pollock et al., 2013). Adult warthogs (*Phacochoerus africanus*) and African elephants (>25 years old) in bTB endemic regions also showed a higher seroprevalence than their younger counterparts (Roos et al., 2018; Kerr et al., 2019). Increasing age had the

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greatest effect on TB disease risk in a meerkat population in South Africa (Patterson et al., 2017).

While most studies in wildlife show increasing bTB prevalence with age, studies conducted in the Iberian Peninsula report higher prevalence in juvenile wild boar than adults in high-prevalence multi-host systems (Gortázar et al., 2008; Santos et al., 2009). This could be due to higher susceptibility in juveniles compared to fully-grown adults in this species, possibly related to immunological maturity, or age-related changes in behaviour that result in increased exposure to the pathogen. Interestingly, historically reported cases of TB in captive African rhinoceros appear to have occurred exclusively in adults (Figure 2.2 and Addendum A, Table S2.1). These observations suggest that infection can take years to manifest in these species (Miller et al., 2015), or that there is an increase in susceptibility and/or repeat exposures with age. Based on other species, it is likely that both sex and age are risk factors for MTBC exposure and infection in black and white rhinoceros.

Transmission of *M. bovis* in Multi-Host Systems

Investigation of TB transmission has been limited in free-ranging rhinoceros until recently because of a lack of diagnostic assays and paucity of samples. Therefore, characterisation of transmission depends largely on extrapolation using patterns observed in other multi-host systems. Some of the predictors for persistence and transmission of a pathogen within a multi-host system appear to be related to patterns of movement, migration, and different modes of interactions between host species (Rogers et al., 1998; Vicente et al., 2007; Jakob-Hoff, 2014). Importantly, the presence of an infected reservoir species in the system has been shown to increase the risk of spillover to other susceptible hosts. Wildlife reservoir hosts for bTB are present globally, including African buffaloes in South Africa (Renwick et al., 2007) [and possibly other areas in Africa where the species occurs (Kalema-Zikusoka et al., 2005; Clifford et al., 2013)], greater kudu in South Africa (Renwick et al., 2007), brush-tailed possums (*T. volpecula*) in New Zealand (Caley et al., 1999), European wild boar (*Sus scrofa*), red deer and fallow deer (*Dama dama*) in Spain (Gortázar et al., 2008), white-tailed deer (*O. virginianus*) in the USA (Schmitt et al., 1997), elk (*Cervus canadensis*) (Lees et al., 2003) and American bison (*Bison bison*) (Nishi et al., 2006) in Canada, and European badgers (*M. meles*) in the United Kingdom (Vicente et al., 2007). In wildlife populations with *M. bovis*, there are numerous examples of intra- and inter-species transmission (Michel et al., 2007; Renwick et al., 2007; Santos et al., 2009; Musoke et al., 2015; Barasona et al., 2017). Direct intra-species *M. bovis* transmission can occur through respiratory droplets in social species like African

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buffaloes (Michel et al., 2007) or through antagonistic or territorial behaviours like those that occur between white-tailed deer (O'Brien et al., 2002).

The mechanism of inter-species *M. bovis* transmission to herbivores is largely unknown but has been attributed to indirect interactions through contamination of pastures, feed, or browse with MTBC shed by infected hosts. Various studies have demonstrated that infected hosts shed *M. bovis* into the environment (Neill et al., 1988; Santos et al., 2015). In one study, intranasal administration of *M. bovis* to calves resulted in intermittent shedding for up to 38 weeks (Neill et al., 1988). A European study of infected wild boar and red deer demonstrated shedding by oronasal, bronchial-alveolar, faecal and urinary routes (Santos et al., 2015). In that study, 83% of wild ungulates with bTB had mycobacteria isolated in at least one type of excretion, which suggests a high level of shedding into the environment. In a study in Spain, interactions between four different species (cattle, domestic pigs, red deer, and wild boar) in a bTB endemic system found that although there was a low percentage of direct interactions between these species, there was a high percentage of indirect interactions over the 3-day time frame investigated, suggesting a high risk of indirect transmission (Cowie et al., 2016). A similar study in France detected a high frequency of indirect interactions between badgers, wild boar, and red deer at waterholes and baited locations (Payne et al., 2017). Therefore, environmental contamination may present risks for transmission to susceptible hosts sharing the same resources as infected individuals. However, in addition to the presence of an infected host that is shedding, the pathogen must remain viable in the environment for enough time to encounter the susceptible host.

Routes of Transmission of *M. bovis* to Rhinoceros in Sub-Saharan Africa

Predicting routes of transmission of MTBC requires an understanding of patterns of shedding, movement patterns, social behaviour, and resource utilisation of susceptible hosts in relation to infected hosts, and the persistence of the pathogen in a contaminated environment.

Wildlife Maintenance Hosts as a Source of *M. bovis* Infection in African Rhinoceros in Sub-Saharan Africa

Domestic livestock (such as cattle) are implicated as bTB maintenance hosts where spillover into wildlife occurs (Bengis et al., 2001; De Garine-Wichatitsky et al., 2013). However, since KNP and HiP have perimeter fencing in place to prevent disease interactions between wildlife and cattle, transmission from livestock is unlikely to be a major mode of *M. bovis* infection acquisition in rhinoceros in these areas. In South Africa, the African buffalo is a recognised

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bTB reservoir host that is implicated in the spillover of *M. bovis* to other susceptible hosts, both directly, and indirectly through shedding into the environment (de Vos et al., 2001; Renwick et al., 2007; Musoke et al., 2015; Sichewo et al., 2020). There is evidence that greater kudu can also be maintenance hosts in KNP and possibly in other bTB endemic areas where the species occurs (Renwick et al., 2007). Since these large herbivorous hosts are often found in similar ranges and utilise the same resources as white and black rhinoceros, interactions between these species are likely to occur. These interactions may be a potential route for transmission of *M. bovis* to African rhinoceros.

According to recent biodiversity statistics, KNP African rhinoceros share the park with an estimated 37,130 African buffaloes (SANParks Scientific Services, 2011). Similarly, HiP has a buffalo population of ~3,500 (Hlokwe et al., 2011). African buffaloes are socially organised into herds, which can be as large as 1,000 individuals (de Vos et al., 2001; Hughes et al., 2017). A study that investigated seasonal movements and habitat use by these animals revealed home ranges varying between 73 and 601 km^2 (Roug et al., 2020). Due to the size of their home ranges, interactions with other species (including rhinoceros), particularly at aggregation points such as water sources or shared feeding areas, are likely to occur at a relatively regular frequency. Dispersal events, though less frequent in adult females, occur in adults of both sexes of buffalo (Olff et al., 2002). Natal dispersal events occur at least once in most adult male buffaloes and can be driven in both sexes by seasonal (water and nutrient) or social resource limitations (Spaan et al., 2019). Additionally, bTB disease may influence individual health and body condition in buffaloes, which could indirectly impact dispersal events (Caron et al., 2003). The resulting frequency of dispersal events may influence the probability of pathogen exposure opportunities resulting in spillover from buffaloes to other susceptible species, including white and black rhinoceros in bTB endemic areas.

Investigation of preferred vegetation and habitat of buffaloes showed the strongest association with open to closed herbaceous vegetation on temporarily flooded land, closed shrubs, open shrubs or with 40–65% crown cover (Roug et al., 2020). The white rhinoceros, like the African buffalo, is a grazing species (Pienaar, 1994; Roche, 2001). Their vegetation preference closely mirrors that of buffaloes. In wet months, white rhinoceros may concentrate their grazing in the short grass-dominated grasslands, while in the dry seasons, they move to tall grass grasslands, with a general preference for shaded grasses. Thickets are generally rejected in favour of open grassland vegetation. The black rhinoceros is a browsing species, and their vegetation preference has less in common with that of African buffaloes. They tend to associate closely

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with thickets (closed shrubland or low forest areas) for access to food (Roche, 2001). For this reason, pathogen exposure interactions with buffaloes due to aggregation at shared feeding areas may be more likely to occur in white rhinoceros than in black rhinoceros.

Available statistics indicate that there are between 11,200 and 17,300 greater kudu in KNP (SANParks Scientific Services, 2011). For HiP, a recent estimate of the greater kudu population was not found. The greater kudu is a browsing species of antelope that is socially organised into small bachelor, cow, or mixed herds, typically of fewer than ten individuals (Owen-Smith, 1988; du Toit, 1990; Perrin, 1999). The home ranges of these herds are typically small and stable, and male home ranges often overlap; the greater kudu social system appears to be based on absolute social dominance (according to age) and territoriality is not evident in this species. Due to their small and stable home ranges, interactions with other species (even indirect) are likely to be less frequent than those observed in buffaloes, who range more widely. However, black rhinoceros share their vegetation preference of thickets or more woody, covered vegetation with this species (Mukinya, 1973); as a result, indirect interactions with infected greater kudu (e.g., via shedding of *M. bovis* through fistulated lymph nodes in kudu leading to contamination of vegetation during browsing) may be an important mode of bTB transmission to black rhinoceros. Buffaloes, greater kudu, black and white rhinoceros share water pans, which may also increase the frequency of interactions within and between these species (Owen-Smith, 1988; Hutchins and Kreger, 2006; Valeix et al., 2008; Bennitt et al., 2015).

Overall, the wide ranges traversed by buffaloes on a seasonal basis and the potential for contamination of browse by infected greater kudu, coupled with evidence supporting their integral roles as maintenance hosts for bTB, support the potential for *M. bovis* transmission to white and black rhinoceros (Caron et al., 2016; Roug et al., 2020). While overlapping vegetation preference, mud wallow usage, and ranges may be important infection predictors, indirect transmission of *M. bovis* from maintenance hosts may not be the only risk factor for infection of rhinoceros. Risk factors for infection should be considered as part of a multi-dimensional network, with the potential for transmission from other infected species, or possibly intra-species transmission.

Intra-Species Transmission of MTBC in Rhinoceros

Initial *M. bovis* infection in free-ranging rhinoceros in KNP was likely a result of spread from African buffaloes or other infected wildlife species, since the strains of *M. bovis* isolated from rhinoceros cases were the same as those identified in other KNP wildlife, based on comparison

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of spoligotypes in different studies (Dippenaar et al., 2017; Miller et al., 2018). However, it is unclear whether these infections were the immediate result of inter- or intra-species transmission in rhinoceros. Although rhinoceros have been translocated extensively, it is interesting that the only reported cases of bTB in rhinoceros in South Africa are in animals that originated from or spent time in parks with *M. bovis*-infected reservoir hosts (Espie et al., 2009; Michel et al., 2017; Miller et al., 2018). Therefore, further investigation is needed to determine if there is a risk of intra-species spread in rhinoceros.

As with inter-species transmission, the risk of intra-species transmission is dependent on whether the infected host is shedding MTBC, and the frequency of interactions between shedding and susceptible individuals, either directly or indirectly through utilisation of shared resources. Evidence suggests that rhinoceros can shed MTBC into their environment in respiratory secretions, or at least that mycobacteria are present in the respiratory system of infected individuals (Lewerin et al., 2005; Michel et al., 2017). Necropsies of two black rhinoceros in an Indian zoo revealed the presence of acid-fast organisms and large volumes of purulent material in the lungs (Valandikar and Raju, 1996). One of these rhinoceroses was sneezing and had a yellow muco-purulent nasal discharge in the days before its death. *M. tuberculosis* has also been isolated in nasal secretions from an infected black rhinoceros in a zoo which had diagnosed TB in several different species of animals (Oh et al., 2002). In addition, *M. tuberculosis* was isolated from a gastric lavage sample of a captive black rhinoceros with pulmonary disease, which suggests that like humans, infected material may be coughed up and swallowed, leading to potential shedding of mycobacteria in faeces (Barbiers, 1994; Duncan et al., 2009). These observations suggest the possibility that infected rhinoceros may transmit MTBC in secretions, presenting a risk for spread to other animals, and possibly humans, that are in close prolonged contact, such as in a zoo setting.

In free-ranging African rhinoceros, characteristics of social organisation may inform the frequency of interactions between shedding and susceptible individuals. Adult female rhinoceros tend to occupy home ranges of up to 70 km^2, whereas adult bulls are often territorial, and occupy ranges up to 40 km^2 with little to no overlap, although young bulls may share their territory (Adcock et al., 1998; Roche, 2000). Young adult females generally range more widely than males, then settle into a similar, smaller home range to have their first calf (Adcock et al., 1998). Home ranges are typically based on permanent water sources and food availability. African rhinoceros may move beyond their usual home ranges during dry periods and peak mating months (Mukinya, 1973; Owen-Smith, 1975). Although there are some cases in which

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social groups have been observed in black rhinoceros (Buss pers. comm., 2020), they tend to be more solitary. In general, cohesive social groups of white rhinoceros are mostly pairs; these can be adolescent-adolescent, cow-adolescent, cow-cow, and cow-calf pairs (Roche, 2000). However, social groups of up to ten individuals have been observed in white rhinoceros in the KNP (Buss pers. comm., 2020). As density of white rhinoceros in a home range increases, the range occupied by individual cows or territorial bulls decreases. Groups of up to four adult cows with their offspring generally have smaller home ranges than solitary cows (Roche, 2000).

Behavioural characteristics of rhinoceros may also influence the risk of intra-species transmission of pathogens. Territorial behaviour is prominent in adult bulls, with frequent olfactory territorial marking, or urine "spraying." Additionally, both defecation and urination are ritualised in territorial bulls, using specific locations, called "middens," scattered around the territory (Owen-Smith, 1975; Adcock et al., 1998; Seidel et al., 2019). Therefore, if *M. bovis* is excreted in faeces, like in wild boar and red deer (Santos et al., 2015), middens might serve as a contaminated site where MTBC bacteria persist. In both black and white rhinoceros, mud wallowing is a behaviour practiced more frequently during summer, and in the heat of the day, but can occur at any time (Owen-Smith, 1975). Therefore, shared use of wallows may be a potential source of intra-species transmission of *M. bovis* excreted in respiratory secretions or faeces.

In summary, both direct and indirect interactions occur between individual rhinoceros, with direct contacts likely occurring at a higher frequency in white rhinoceros. Social exchanges, as well as overlap of ranges, and shared utilisation of water sources, middens, and mud wallows are likely to result in indirect interfaces that carry potential for pathogen transmission. However, the apparent low prevalence and lack of disseminated disease in affected free-ranging populations of rhinoceros suggest that the risk of intra-species transmission of MTBC is lower than in captive rhinoceros (Miller et al., 2018).

Environmental Contamination as a Route for Indirect Transmission of *M. bovis*

For indirect transmission of bTB to occur, an area, shared by the recipient host, must be contaminated with MTBC by an infected individual (Cowie et al., 2016). Several studies have successfully isolated pathogenic mycobacterial DNA from various environmental substrates, including water, soil, sediments, and grass; this finding supports the possibility of a *M. bovis*-contaminated environment as a source of exposure for rhinoceros (Adams et al., 2013).

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Recipient host exposure risk likely increases with an increase in the mycobacterial load shed by infected hosts into the environment.

Factors affecting persistence of *M. bovis* in the environment have been investigated but are still poorly understood. Because of the low sensitivity associated with culture of MTBC from the environment, qPCR assays specific for MTBC DNA have been adapted as a supplementary quantification method (Adams et al., 2013; Barbier et al., 2017), and 16S rRNA has also been used as a proxy for viable MTBC (Young et al., 2005). In addition, the type of samples collected within a system also appears to influence detection of *M. bovis*. For example, using PCR, MTBC DNA has been more frequently recovered in sediments around waterholes than in water in environments with *M. bovis*-infected hosts (Santos et al., 2015).

Seasonal changes in environmental conditions appear to influence persistence of *M. bovis* in the environment. Both air and soil temperatures affect detection of MTBC DNA in environmental samples (Adams et al., 2013; Santos et al., 2015; Barbier et al., 2017). Soil concentrations of *M. bovis* DNA were higher in spring compared to all other seasons in the Iberian Peninsula (Santos et al., 2015). Regardless of soil type, *M. bovis* DNA concentrations were higher when air and soil temperatures were moderate (averaging ~15°C and 17°C, respectively), and with greater soil moisture content (~50%) in spring, compared to higher air and soil temperatures (maximum averages 32.6 and 26.4°C, respectively) and lower soil moisture content (~2%) in summer in this region (Santos et al., 2015). A study in Michigan (USA) showed that the persistence of *M. bovis* (measured by PCR and culture) in contaminated environmental substrates, which varied between 4 weeks and 6 months, was shortened by exposure to high ambient temperatures, increased intensity of solar radiation, and higher loss of substrate moisture through evapotranspiration (Adams et al., 2013). In addition, when soil was spiked with *M. bovis*, persistence was longer at 4°C than at 22°C (Barbier et al., 2017). This was in agreement with field studies that showed that *M. bovis* persisted longer in soil in autumn/winter than in summer in Michigan and New Zealand (Jackson et al., 1995; Fine et al., 2011), though in these cases it is not clear whether this was only correlated to temperature, or a combination of variables associated with certain seasons. One previous study done under controlled conditions presented contradictory findings related to the correlation of *M. bovis* persistence with temperature; *M. bovis* persisted longer in spiked soils at 37°C than at 4°C (Young et al., 2005), highlighting some of the continued knowledge gaps related to persistence of *M. bovis* in the environment. Physico-chemical properties in soil, such as the proportional contribution of clay, silt, sand and organic matter to overall composition, as well as pH, and

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mineral content (Barbier et al., 2017), may also affect *M. bovis* persistence in the environment. Presence of shade has also been associated with the persistence of environmental *M. bovis*, likely due to maintenance of higher water content and moderate temperatures of the soil and vegetation (Rodríguez-Hernández et al., 2015). Additionally, reduced ultraviolet radiation in shade results in less cell stress and fewer genetic mutations, improving bacterial survival (Rodríguez-Hernández et al., 2015). These areas may play a role in exposure to *M. bovis* because resting rhinoceros and other species often occupy shady areas during the hottest times of the day, which may promote concentration of bacteria shed in secretions.

In addition to abiotic factors, there are other biological reservoirs that are ubiquitous in the environment and may play a vital role in environmental persistence and subsequent transmission of MTBC. MTBC bacilli have been isolated from free-living amoeba, found frequently in soil (Sanchez-Hidalgo et al., 2017). It has also been discovered that earthworms (*Lumbricus terrestris*) can disseminate *M. bovis* from contaminated animal faeces to the surrounding soil through casting egestion (Barbier et al., 2016). These worms can shed bacteria for up to 4 days after initial ingestion of contaminated faeces. The presence of these organisms in areas where grazing by *M. bovis*-infected and susceptible hosts occurs may promote exposure through indirect interactions.

It is hypothesised that, due to the influence of environmental variables on the ability of *M. bovis* to persist in the environment, there may be a seasonal variation in bTB transmission risk. Environmental persistence of *M. bovis* under cold and wet conditions, as well as seasonal changes in the presence of shade and vegetation, may influence *M. bovis* exposure risk in rhinoceros; however further studies are needed to determine pathogen persistence in the natural habitats of these species.

Discussion

The Importance of MTBC Infections in African Rhinoceros

Today, the largest free-ranging populations of African rhinoceros in South Africa are located in bTB endemic areas. While poaching of African rhinoceros for their horns is currently the most substantial threat to their conservation [Department of Environment, Forestry and Fisheries, 2014; Department of Environmental Affairs (DEA), 2018], the presence of bTB in rhinoceros presents a considerable barrier to conservation due to the inability to translocate animals from bTB endemic areas to safeguarding areas that are bTB free. Without the tools to screen rhinoceros in endemic areas for *M. bovis* infection, regulations prevent translocation to

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bTB-free areas. Individuals that cannot be moved for safeguarding purposes are then exposed to risk of mortality resulting from poaching incidents. Although bTB is not currently recognised as a major cause of morbidity, mortality, or a threat to rhinoceros population health, understanding the epidemiology and pathogenesis of this disease in rhinoceros will provide a foundation for studying the impact of bTB on these species. African rhinoceros populations in zoos around the world may also be at risk of MTBC infection, as individuals in these populations have exhibited morbidity and mortality (Hofmeyr, 1956; Keep and Basson, 1973; Mann et al., 1981; Dalovisio et al., 1992; Barbiers, 1994; Stetter et al., 1995; Valandikar and Raju, 1996; Rookmaaker, 1998; Oh et al., 2002; Duncan et al., 2009; Espie et al., 2009; Morar et al., 2013; Miller et al., 2017, 2018; Witte pers. comm., 2020). Based on limited case reports, both *M. tuberculosis* and *M. bovis* infect black and white rhinoceros, although the epidemiology and sources of these infections may differ. In addition to the impact on individual rhinoceros health, these infections may also result in spread to other animals, as well as humans, in the zoo environment (Dalovisio et al., 1992; Stetter et al., 1995; Lewerin et al., 2005). Therefore, it is essential to investigate the epidemiology of MTBC infections in rhinoceros in various settings to inform the most appropriate disease management and control strategies.

Hypothesised TB Risk Factors

As in other species, it is expected that the risk of MTBC infection in rhinoceros will be based on factors influencing susceptibility of the individual and exposure to the MTBC (Jakob-Hoff, 2014).

M. tuberculosis vs. *M. bovis*

Most reported cases of TB in captive rhinoceros resulted from infection with *M. tuberculosis*, with only a few cases caused by *M. bovis*. This is likely due to differing levels of exposure to each pathogen according to its prevalence, as well as the likelihood of interaction with an infected host, including both other animals and humans. Tuberculosis affects human populations worldwide, and most human cases are caused by infection with *M. tuberculosis* (Heemskerk et al., 2015). Captive rhinoceros may therefore have a higher likelihood of exposure to *M. tuberculosis* than *M. bovis* through their prolonged close contact with infected human caregivers. In these cases, transmission may occur through aerosols or through a contaminated environment, and possibly both.

While *M. bovis* can cause TB in humans, the pathogen is less efficient than *M. tuberculosis* at propagating through human hosts (Behr and Gagneux, 2011). This animal-adapted MTBC

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species predominantly occurs in livestock and wildlife populations and is maintained in certain endemic areas by susceptible host populations (Corner, 2006). Free-ranging rhinoceros in bTB endemic areas may be exposed to *M. bovis* through infected hosts or a contaminated environment; the latter is likely to occur more frequently, especially for inter-species transmission, as free-ranging animals are less likely to have close prolonged contact with other infected host species than they are to share aggregation points in their environments (e.g., water sources) with these species. Exposure to *M. tuberculosis* is less likely in free-ranging populations than in captive rhinoceros, as they have little to no interaction with humans, which are most affected by this pathogen.

Species-Specific Susceptibility

The more frequently reported occurrence of TB disease in captive black rhinoceros than in white rhinoceros, from the case series outlined in Figure 2.2 and Addendum A, Table S2.1), lends credence to the hypothesis that black rhinoceros are more prone to TB disease than white rhinoceros. However, this observation may be due to other confounding factors, including inapparent differences in numbers of black and white rhinoceros in captivity, and differences in *M. tuberculosis* or *M. bovis* exposure. Disease surveillance and reporting biases also exist because not all institutions that house rhinoceros conduct post-mortem TB surveillance or have equal diagnostic capabilities, not all cases of TB in rhinoceros are recorded in the literature, and non-cases are often not reported (Lécu and Ball, 2011). Nevertheless, the data presented provide foundational hypotheses for further evaluation of TB risk in black and white rhinoceros.

Sex as a Risk factor for MTBC Infection and TB

Limited available data from scientific reports on rhinoceros show more cases of TB in males compared to females (Figure 2.2 and Addendum A, Table S2.1). It is unknown whether this observation represents a true sex predilection for TB, or whether reports are biased toward males for other reasons. That said, a proposed hypothesis is that African rhinoceros males have a higher susceptibility to TB than females, and this is further supported by males in other species tending to have higher rates of TB compared to females (refer to section on Demographic Risk Factors).

In free-ranging African rhinoceros populations in South Africa, it is hypothesised that females have a higher exposure to *M. bovis* due to their wider home range, and more consistent association in cohesive groups than males, and it is unknown how this may contribute to the

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overall TB risk of males and females in these populations. More controlled investigation in free-ranging populations is required to test these hypotheses.

Age as a Risk Factor for MTBC infection and TB

All recorded TB and bTB cases (Figure 2.2 and Addendum A, Table S2.1) have occurred in adults. One explanation for this observation is the chronic and recurring nature of this disease. It is possible that young individuals are just as susceptible to MTBC infection as older individuals, but that the disease takes extended time to clinically manifest. An alternative explanation is that diminishing immunocompetence with age (as occurs with age-based hormonal changes in humans as well as the immune effects of old age) could render adults more susceptible to infection and onset of disease than young rhinoceros. It is possible that this observation is attributed not only to age-based changes in susceptibility, but is also due to age-related changes in exposure to either *M. tuberculosis* and *M. bovis* due to translocation of captive rhinoceros for management and breeding (Figure 2.2 and Addendum A, Table S2.1) (Rookmaaker, 1998). Based on this limited information, and age-related TB risk trends observed in most other species, it is hypothesised that susceptibility to MTBC infection and disease progression with exposure increases with age in black and white rhinoceros species.

In free-ranging populations of African rhinoceros in South Africa, incidence of *M. bovis* is expected to increase with age, due to more intense, and consistent exposure of adults in their smaller, more "settled" home ranges compared to that of the more nomadic young, and the accumulation of repeat exposures over their lifetime. The prevalence of *M. bovis* may also be higher in older animals, reflecting the chronic nature of disease in these long-lived species. The impact of increasing age on the overall TB risk of these populations is still unknown and requires more investigation.

Environmental and Seasonal Factors Affecting Exposure to and Transmission of *M. bovis* in Free-Ranging Rhinoceros in South Africa

The characteristic closer association of black rhinoceros with closed, shady environments than white rhinoceros may increase exposure to *M. bovis* in this species; this is expected to occur as a result of their sharing of this habitat with the suspected bTB maintenance host, the greater kudu, as well as the longer persistence of *M. bovis* in shady (vs. irradiated) conditions. Conversely, the more frequent association of white rhinoceros with soil-associated bTB reservoirs and African buffaloes (a prominent bTB maintenance host) with whom they share their environment and food source, is likely to increase their *M. bovis* exposure. Additionally,

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because the white rhinoceros is considered a more social species than the black rhinoceros, it is hypothesised that *M. bovis* risk resulting from intra-species interactions will be comparatively higher in white rhinoceros. Overall, because of the higher population of buffaloes (the maintenance host with which white rhinoceros is expected to more frequently associate) than greater kudus (with which the black rhinoceros is expected to more frequently associate), as well as the expected occurrence of more intra-species interactions in white rhinoceros, it is hypothesised that there will be a higher risk of *M. bovis* exposure in white rhinoceros populations than in black rhinoceros populations in KNP and HiP.

In free-ranging African rhinoceros populations in South Africa, it is hypothesised that there is an association between seasonal fluctuations in environmental conditions, rhinoceros spatial patterns, and *M. bovis* infection risk. During hot or dry periods, the *M. bovis* exposure of rhinoceros (and other species) may increase due to increased aggregation of *M. bovis*-infected and susceptible hosts at water sources. During hotter periods, specifically, the aggregation of infected and susceptible hosts in shady areas and/or mud wallows may be associated with increased *M. bovis* exposure. Seasonal fluctuations in soil-associated bTB reservoirs such as free-living *M. bovis* in the soil, as well as earthworms and amoeba, may also be associated with seasonal changes in incidence of *M. bovis* infection in African rhinoceros and other animals.

Future Research

The most pressing concern related to TB in African rhinoceros is the acquisition of knowledge and the development of tools to inform surveillance and control strategies for the disease, as well as conservation plans. The development of sophisticated diagnostic tools may allow for early detection of infection; this would enable earlier interventions that could improve the prognosis of infected individuals and mitigate the spread of infection in captive and free-ranging systems. Of particular consideration is logistical feasibility and fitness-for-purpose of a test. Capture and immobilisation of rhinoceros, especially in free-ranging populations, is extremely costly and is a source of stress for the animal. Therefore, the use of a test like the TST, in addition to being unreliable in this species, would be ill-advised, as it involves immobilisation for both administration and interpretation of the test on separate occasions, increasing the cost and stress for the rhinoceros undergoing testing. Development of blood-based cytokine release assays for bTB in African rhinoceros is currently ongoing; these tests require a single capture and immobilisation, and once validated, may be reliable, cost-effective diagnostic methods for bTB in rhinoceros.

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Coordinated studies in captive populations may help to clarify demographic factors (e.g., age, sex, species) as risks for *M. bovis* infection and disease progression in African rhinoceros. This would involve ante-mortem monitoring for MTBC infection, as well as thorough post-mortem exams that include histopathology and ancillary diagnostic tests. Because zoological facilities tend to keep curated medical records, retrospective, longitudinal data may already be available to address these knowledge gaps. Careful, standardised data curation across institutions could inform and enumerate a study population and be used to identify cases and non-cases. In any such study, attention to confounding factors such as differences in exposure to MTBC based on animal origin, movement history, and TB prevalence in human populations should be considered.

In South Africa, population-based epidemiological studies of bTB in free-ranging African rhinoceros populations are currently ongoing. Findings from such studies could help identify major drivers of bTB infection in free-ranging populations as well as identify low risk individuals, which would have immediate benefit to current conservation and translocation efforts. This knowledge could be applied to inform management decisions for these populations, e.g., to minimise the probability of moving a false test-negative infected animal out of bTB endemic areas and inadvertently spreading bTB to other areas. Such studies may also aid in identifying important bTB risk mitigation opportunities aimed to decrease continued spread of bTB in black and white rhinoceros living in these fragile ecosystems.

Conclusion

This review has focused on available literature that could help to characterise the risk posed by MTBC (including *M. bovis* and *M. tuberculosis*) to African rhinoceros species. It has also drawn attention to major knowledge gaps pertaining to TB in rhinoceros. By identifying and systematically addressing each of these knowledge gaps, advances will inform management decisions for conservation of African rhinoceros, and South African biodiversity.

Supplementary Material

Supplementary Table S.2.1 for this chapter can be found in Addendum A.

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Chapter 3

Epidemiology of *Mycobacterium bovis* Infection in Free-Ranging Rhinoceros in Kruger National Park, South Africa

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Significance

African rhinoceros survival is threatened by poaching, habitat loss, and climate effects. The presence of *Mycobacterium bovis* in wild populations creates an additional potential threat to health and conservation programs. This study reports a large survey of *M. bovis* infection in free-ranging rhinoceros. Our findings confirm a widespread, high infection burden in the rhinoceros population of Kruger National Park, South Africa and identify risk factors for infection. These findings provide a foundation for understanding the spread of bovine tuberculosis in complex

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ecosystems. This study reflects the complexity of investigating a multi-host pathogen in a previously naïve system. It provides an opportunity to increase awareness of the global impact that tuberculosis can have on animal populations, food security, and conservation.

Key words: bovine tuberculosis, epidemiology, prevalence, rhinoceros, risk

Abstract

Mycobacterium bovis (*M. bovis*) infection, which is a prominent cause of bovine tuberculosis, has been confirmed by mycobacterial culture in African rhinoceros species in Kruger National Park (KNP), South Africa. In this population-based study of the epidemiology of *M. bovis* in 437 African rhinoceros (*Diceros bicornis, Ceratotherium simum*), we report an estimated prevalence of 15.4% (95% CI: 10.4 to 21.0%), based on results from mycobacterial culture and an antigen-specific interferon-gamma release assay from animals sampled between 2016 and 2020. A significant spatial cluster of cases was detected near the southwestern park border, although infection was widely distributed. Multivariable logistic regression models, including demographic and spatiotemporal variables, showed a significant, increasing probability of *M. bovis* infection in white rhinoceros based on increased numbers of African buffalo (*Syncerus caffer*) herds in the vicinity of the rhinoceros sampling location. Since African buffaloes are important maintenance hosts for *M. bovis* in KNP, spillover of infection from these hosts to white rhinoceros sharing the environment is suspected. There was also a significantly higher proportion of *M. bovis* infection in black rhinoceros in the early years of the study (2016–2018) than in 2019 and 2020, which coincided with periods of intense drought, although other temporal factors could be implicated. Species of rhinoceros, age, and sex were not identified as risk factors for *M. bovis* infection. These study findings provide a foundation for further epidemiological investigation of *M. bovis*, a multi-host pathogen, in a complex ecosystem that includes susceptible species that are threatened and endangered.

Introduction

African rhinoceros (*Diceros bicornis, Ceratotherium simum*) are currently under threat due to poaching activity and habitat destruction, as well as the underrecognised threat of infectious diseases (Dwyer et al., 2020; Miller et al., 2017). *Mycobacterium bovis* infection has been confirmed in the African rhinoceros population in Kruger National Park (KNP), South Africa (Chileshe et al., 2019a, 2019b; Goosen et al., 2021; Miller et al., 2018). The discovery of *M. bovis*

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infection in this population has led to a quarantine of rhinoceros intended for translocation from the park to other protected areas, which has significant conservation consequences (Dwyer et al., 2020). The paucity of knowledge regarding the epidemiology and risk of transmission from infected rhinoceros has become evident when assessing impact on the KNP population and potential for spread to other populations (Dwyer et al., 2020).

Because *M. bovis* infection is chronic and may not cause clinical signs of disease for months to years, its presence in an ecosystem with multiple susceptible host species may not be recognised for decades, as has been documented in several bovine tuberculosis (bTB) afflicted wildlife populations worldwide (Fitzgerald and Kaneene, 2012; Hlokwe et al., 2014; Michel et al., 2006). The KNP is considered endemic for bTB, with African buffaloes (*Syncerus caffer*) being the key maintenance hosts (de Vos et al., 2001; Hlokwe et al., 2014; Michel et al., 2006). Historically, *M. bovis* is believed to have originated from infected cattle adjacent to the park boundaries in the 1960s and 1980s but was not detected until the 1990s in infected buffalo herds (Bengis, 1999). Since then, 15 additional wildlife species in KNP have been documented with infection (Bernitz et al., 2021), including rhinoceros, in which infection was confirmed using mycobacterial culture and *M. bovis* species confirmation (Chileshe et al., 2019a, 2019b; Goosen et al., 2021; Miller et al., 2018).

The epidemiology of bTB in a complex system that contains multiple hosts with varying susceptibility results in an array of opportunities for infection spread. Black and white rhinoceros in KNP share environmental resources (including browse/grazing, and water sources) with potentially *M. bovis*–infected African buffaloes (de Vos et al., 2001; Hlokwe et al., 2014; Michel et al., 2006), greater kudu (*Tragelaphus strepsiceros*) (Bengis et al., 2001; Renwick et al., 2007), warthogs (*Phacochoerus africanus*) (Roos et al., 2019), and other species (Bernitz et al., 2021). A recent review of potential scenarios for inter-species transmission has suggested that rhinoceros may become infected with *M. bovis* in ecosystems containing other infected hosts (Dwyer et al., 2020). Inter-species spread has been demonstrated in other systems, including badgers and cattle in the United Kingdom (Allen et al., 2018), wild boars, deer, and cattle in Spain (Santos et al., 2015), and deer and cattle in the United States (O'Brien et al., 2002). Mechanisms of transmission between herbivores are poorly understood but have been attributed to indirect interaction through shared resources such as pastures, feed, or water holes that are contaminated by *M. bovis*–shedding hosts (Cowie et al., 2016; Payne et al., 2017; Santos et al., 2015). The potential for intraspecies

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transmission between rhinoceros is also plausible based on ante-mortem detection of mycobacteria in respiratory secretions (Dwyer et al., 2020; Michel et al., 2017).

For species (like white and black rhinoceros) that are considered threatened or endangered, the presence of a controlled infectious disease can significantly hamper conservation efforts and potentially impact population health and survival (Gortázar et al., 2008; Meiring et al., 2021; Thapa et al., 2017). Regulations imposed by the Department of Agriculture, Land Reform and Rural Development in KNP, due to the diagnosis of *M. bovis* in rhinoceros and other species, are an additional barrier to the movement of rhinoceros from the park to other national or private reserves. This can have a significant impact on conservation of the species, as KNP has historically been an important source population of rhinoceros for other conservation strongholds in South Africa and other African countries. In order for a captured rhinoceros to be moved out of KNP, it must first be placed in a quarantine facility for three months and must test negative for *M. bovis* infection during repeated testing events.

It is therefore critical to be able to assess infection status in these populations. Recent advances in the development of diagnostic tests (Bernitz et al., 2021; Smith et al., 2021) for *M. bovis* infection in wildlife beyond conventional mycobacterial culture and *M. bovis* species confirmation has allowed for ante-mortem testing. The Quanti-FERON TB Gold In-Tube Plus (QFT) (Qiagen, Venlo, The Netherlands)-interferon-gamma release assay (QFT–IGRA) was recently validated for use in white rhinoceros (Chileshe et al., 2019a), and has been used for testing KNP rhinoceros for *M. bovis* infection. These results provide the opportunity to generate an understanding of epidemiological determinants and risk factors for infection and disease transmission within the rhinoceros population.

Here, we report a population-based study on the epidemiology of *M. bovis* infection in free-ranging African rhinoceros. We investigated the distribution of *M. bovis* infection in rhinoceros over the KNP landscape and identified the demographic, spatial, and temporal factors that may drive infection in this population (Dwyer et al., 2020). Our findings begin to uncover the complex epidemiology of bTB for rhinoceros in a multi-host system where bTB is endemic. Results from this study emphasise the importance of disease surveillance in managed wildlife systems and support current quarantine and testing requirements for rhinoceros in KNP. These findings are important for preventing the spread of *M. bovis* infection to other rhinoceros populations (Miller

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et al., 2017, 2018). In a broader sense, this study reflects the complexity of investigating a multi-host pathogen that has been introduced into a previously naïve system. It provides an opportunity to increase awareness of the global impact that TB and other zoonotic pathogens can have on domestic and wild animal populations, food security, and conservation of species and ecosystems.

Materials and Methods

Source Population and Data Collection

Black and white rhinoceros populations in the KNP were sampled opportunistically during post-mortem examinations (n = 9) or immobilisations performed as part of management and veterinary activities conducted in 2016–2020. In total, 528 rhinoceros (130 black rhinoceros and 398 white rhinoceros) were sampled and considered for inclusion in this study. Data collected for individual rhinoceros included date of sample collection, GPS coordinates for capture locations, demographic characteristics (sex, species, and age class), and general health status of the animals prior to immobilisation.

During immobilisation, whole blood was collected from the auricular or radial vein of the rhinoceros in lithium heparinised vacutainer tubes (BD Biosciences, Johannesburg, South Africa), as previously described (Miller et al., 2018). Postmortem tissue samples were collected from nine white rhinoceros during necropsy. These samples included submandibular, retropharyngeal, cervical, prescapular, axillary, inguinal, mediastinal, tracheobronchial, and mesenteric lymph nodes and lung, which were frozen at -20 °C for transport to Stellenbosch University for further laboratory testing under biosafety level 3 conditions, as previously described (Miller et al., 2018).

All living animals (n = 519) were immobilised by wildlife veterinarians for management, or for other approved procedures according to KNP's Wildlife Veterinary Services' standard operating procedures for the capture, transportation, and maintenance in holding facilities of wildlife (South African National Parks). Ethical approval for this project was granted by the Stellenbosch University Animal Care and Use Committee (ACU-2020-19019), and a section 20 research permit was issued by the Department of Agriculture, Land Reform and Rural Development (DALRRD; 12/11/1/7/2).

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Study Design and Study Population

A cross-sectional retrospective study design was used to identify factors associated with *M. bovis* infection in rhinoceros from KNP. An individual rhinoceros from the sampled source population (described above) was included in this study if 1) a blood sample was obtained from the individual during immobilisation, or tissues were sampled from the individual at necropsy, and 2) it was sampled while free-ranging in KNP or within 2 months [this is the expected length of time after infection with *M. bovis* within which an immune response is detectable using QFT-IGRA (Parsons et al., 2017) after translocation out of the park to a quarantine location. A total of 475 out of the 528 rhinoceros (90%) from the source population met these inclusion criteria.

Determination of *M. bovis* Infection Status

The *M. bovis* infection status of individual rhinoceros was determined using one of the previously described methods: 1) QFT-IGRA (Chileshe et al., 2019a, 2019b) (sensitivity = 78%; 95% CI: 52.3 to 93.5%: specificity = 92%; 95% CI: 63.9 to 99.8%) or 2) mycobacterial culture for *M. bovis* isolation from a (postmortem) tissue (Tortoli et al., 1999; Miller et al., 2018) with Region of Difference (RD)-PCR for *M. bovis* species confirmation (Warren et al., 2006). The QFT-IGRA is a standard method for diagnosis of active or latent *M. tuberculosis* infection in humans (European Centre for Disease Prevention and Control, 2011; Walzl et al., 2018), and for *Mycobacterium tuberculosis* complex (MTBC) infection in animals, including rhinoceros (Chileshe et al., 2019a, 2019b; Michel et al., 2017; Parsons et al., 2017). A rhinoceros was classified as *M. bovis* infected if it had a positive IGRA result (antigen-specific TB response $\geq$ 21 pg/mL) or positive BACTEC Mycobacterial Growth Indicator Tube (MGIT; BD Biosciences) culture result with subsequent PCR identification of *M. bovis*, as previously described (Miller et al., 2018; Warren et al., 2006). An individual was assigned a negative infection status if it had a negative IGRA result (antigen-specific TB response $\leq$ 21 pg/mL, mitogen response $\geq$ 21 pg/mL, Nil response $\leq$ 21 pg/mL) and, if conducted, any MGIT culture result with subsequent PCR that did not identify the presence of *M. bovis* (Chileshe et al., 2019b). Individuals for whom the *M. bovis* status could not be defined according to these criteria were classified as "unknown" infection status.

A small number of rhinoceros (n = 38) had multiple immobilisations and QFT-IGRA results during the study period. For these individuals, a negative infection status was assigned if all test results (QFT-IGRA and mycobacterial culture, if applicable) were negative. A positive infection status

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was assigned if any of the tests (QFT-IGRA, and/or mycobacterial culture with RD-PCR confirmation of *M. bovis*) were positive. Rhinoceros were considered positive for *M. bovis* infection on the date of the first positive test result. Importantly, data assigned to each of the individuals in this subset were associated with a single GPS point location at which the animal was sampled - either the point of capture at which an *M. bovis*-infected individual first tested positive for *M. bovis*, or, for individuals that consistently tested negative for *M. bovis*, a randomly selected point from their multiple capture locations.

Evaluated Risk Factors

Risk factors hypothesised to be associated with *M. bovis* infection in black and white rhinoceros populations from KNP (Dwyer et al., 2020) were evaluated in this study based on availability of data at each individual's sampling event. For *M. bovis* positive rhinoceros that were sampled multiple times, the immobilisation date (and corresponding data) associated with the first positive sample was included. For *M. bovis* negative rhinoceros that were sampled multiple times, a single date (and corresponding data) was randomly selected from among all of that individual's capture dates. Evaluated factors are further described below and include the following 13 variables: species, sex, age class, orphan status, health status at time of sampling, sampling year, sampling season, nearest permanent water source type, distance to nearest permanent water source, distance to the nearest *M. bovis*-infected rhinoceros, number of nearby kudu herds, number of nearby buffalo herds, and buffalo density.

Demographic, Health, and Temporal Risk Factors

Demographic factors selected and evaluated in this study included species (white or black rhinoceros), sex (male, female), and age. Age was estimated by field veterinary staff and categorised as follows: adult (>7 y), subadult (>2 y to 7 y), and calf (0 y to 2 y). Calves were further classified as orphaned or with their mother at the time of sample collection. Health status at the time of sampling was assessed by veterinary staff as normal or abnormal. Examples of conditions associated with abnormal health status included poor body condition, visible injuries, or any treatment undergone for illness or injury at the time of sampling. The health status variable was used to determine whether there was an association between *M. bovis* infection and health status. Temporal factors like year of sampling (2016-2020) together with rainfall season, that is, dry (March-August) or wet (September-February), were evaluated.

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Spatial Risk Factors

Spatial risk factors were based on a single GPS point for each rhinoceros' immobilisation location plotted onto a map using Geographic Information System (GIS) software (ArcGIS Pro, version 2.8; Environmental Systems Research Institute, Redlands, California, USA). Spatial data were further processed and evaluated in the GIS software. Rhinoceros that did not have a GPS point recorded (n = 4) were omitted from the spatial analyses.

Spatial data describing the distance between the rhinoceros capture point and the nearest water source, or other *M. bovis*-infected rhinoceros were summarised. Risk of *M. bovis* infection was then evaluated as a function of distance (continuous predictor).

A circular polygon buffer was placed around each rhinoceros capture location to approximate a crude home range, in which exposure to African buffalo and greater kudu, which are known bTB maintenance hosts (Renwick et al., 2007), could occur. The radius of the home range was derived from a subset of rhinoceros (n = 38) with GPS coordinates from multiple immobilisation events occurring within a maximum of 3 years of each other. The distribution of distances between pairs of capture points for the same individual (n = 70 total pairs of capture points for the 38 rhinoceros individuals; 4 individuals had 4 immobilisation events, 7 individuals had 3 immobilisation events, and 27 individuals had 2 immobilisation events) was examined, and 95% of pairwise distance observations occurred within 23 km of each other. This distance served as an approximation of the upper limit of the distance that a rhinoceros would travel; however, it is assumed that most of the movement probably occurs within a smaller core area (Miller et al., 2018; Roche, 2000; Thompson et al., 2016) of unknown size. Therefore, the size of the circular home ranges was varied to include radii at 75%, 50%, and 25% of the maximum, corresponding to 17.25, 11.5, and 5.75 km, respectively. Variables characterizing relative exposure to African buffalo and greater kudu (further described below) were summarised for each of the four circular home range sizes.

African Buffaloes in Home Range

Two different spatial data layers were available to estimate the presence and density of African buffaloes in each rhinoceros' assigned home range. The first dataset was a zero-inflated Poisson model generated by Hughes et al. (2017) for prediction of buffalo density per square kilometre across KNP. The predicted buffalo density map was overlaid with rhinoceros home ranges to derive an estimated buffalo density (per square kilometre) value for each rhinoceros as a proxy for

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M. bovis exposure due to the presence of these maintenance hosts (Caron et al., 2003; de Vos et al., 2001; Michel et al., 2017; Renwick et al., 2007).

The second dataset was obtained with permission from SANParks GIS Scientific Services and contained buffalo census data (describing distribution of herds and individuals) that were collected across KNP in 2015 and 2017 using aerial line transect sampling and distance analysis methods, as previously described (Kruger et al., 2008). The census data for the two years were combined into a single mapped data layer, which was applied as a crude estimate of buffalo distribution in KNP. The combined census data were then overlaid with the rhinoceros' home ranges to estimate the number of buffalo individuals and herds within the rhinoceros home range.

Greater Kudu in Home Range

Overlap of rhinoceros distribution with kudu was evaluated because greater kudu are considered *M. bovis* maintenance hosts (Renwick et al., 2007). Census data on greater kudu were collected in three separate years (2014, 2016, and 2017), using aerial line transect sampling and distance analysis methods as previously described (Kruger et al., 2008), and made available by SANParks GIS Scientific Services.

The three datasets were combined into a single mapped data layer, providing a crude measure of kudu distribution in KNP. The combined kudu census data were then overlaid with each rhinoceros' home range to estimate the number of kudu individuals and herds that each rhinoceros may have been exposed to.

Proximity to Water Source(s)

Mapped datasets of the rivers and water holes in KNP were provided by SANParks GIS Scientific Services. The river dataset described main and secondary rivers and was compiled in 2018 using older data sources in combination with National Geo-Spatial Information aerial imagery (Department of Rural Development and Land Reform, 2013). The water hole dataset described the location of available water holes in KNP, updated through June 2016.

The distance (kilometres) between each rhinoceros' capture location and the nearest water source (river or water hole) was determined in GIS; this value was used to represent the proximity of each rhinoceros to water. This variable was tested based on the hypothesis that aggregation of infected hosts at available water sources may result in increased pathogen exposure of susceptible hosts

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living in close proximity to these water sources, either through direct interactions with infected hosts or due to mycobacterial loads shed into the environment. The nearest water source type (river or water hole) was also evaluated as an independent risk factor.

M. bovis Status of Nearby Rhinoceros

A continuous variable was created to evaluate whether the risk of *M. bovis* infection was a function of the infection status of other nearby rhinoceros. All rhinoceros capture location points were plotted in ArcGIS, and the infection status of each animal was determined. The distance (kilometres) from each study subject to the nearest *M. bovis* positive rhinoceros was then determined with ArcGIS and ascribed to the study subject.

Data Analyses

Apparent *M. bovis* infection prevalence was estimated for the full KNP study population (number of test-positive rhinoceros/total number of study rhinoceros), as well as within different ranger sections (Kloppers and Bornman, 2005) and different ecozones (number of test-positive rhinoceros in specific area/total number sampled in specific area) (Gertenbach, 1983). Within ranger sections and ecozones, prevalence calculations were limited to the areas where >10 animals were sampled. These apparent prevalence values were then adjusted to account for the sensitivity and specificity of the IGRA (sensitivity = 78%; 95% CI: 52.3 to 93.5%: specificity = 92%; 95% CI: 63.9 to 99.8%), thereby estimating true prevalence, using the following equation: estimated true prevalence = (apparent prevalence + IGRA specificity – 1)/(IGRA sensitivity + IGRA specificity – 1) (based on formulas implemented by Stevenson et al., 2022). Prevalence values were compared across ranger areas and ecozones using Fisher's exact tests.

Differences in geographical distribution of *M. bovis* infection were further explored using Kulldorff's spatial scan statistic (Kulldorff, 1997). The statistic was applied using a Bernoulli based model and SatScan software (version 10.0). SatScan is a trademark of Martin Kulldorff and developed under the joint auspices of Martin Kulldorff, the National Cancer Institute, and Farzad Mostashari of the New York City Department of Health and Mental Hygiene (Kulldorff and Information Management Services, Inc., 2009). The methods identify significant case clustering by moving a circular window over the geographic area; the maximum spatial cluster size was set to half of the population. For this statistic, the null hypothesis assumed that the relative risk of *M. bovis* infection is the same inside the geographic area compared to outside. Significance was

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determined by comparing likelihood ratio tests from 999 iterations of a Monte Carlo simulation. We performed this evaluation among all rhinoceros, and then among black and white rhinoceros separately.

Univariate logistic regression was used to screen for associations between each factor and *M. bovis* infection. Crude odds ratios (ORs), 95% confidence intervals (CIs), and type III Wald's p values were estimated. Evaluation of the association between *M. bovis* infection status and orphan status was completed only within the subset of calves. Functional forms of continuous variables were determined by fitting a logit-transformed Loess curve for single-variable models. Natural log transformations were used for covariates that were not normally distributed. If there was evidence of nonlinearity in the logit, then the variable was categorised into quartiles. Important demographic covariates, potential effect modifiers, and associations with $p \leq 0.2$ were further examined in multivariable analyses.

The three factors derived from rhinoceros areas of exposure (i.e., number of kudu herds, number of buffalo herds, buffalo density) were further evaluated to determine the optimal spatial scale for each variable separately. For each variable, a single radius distance for area of rhinoceros exposure was selected for further evaluation in the multivariable model. The chosen distance was based on the strongest association and the best-fitting single-variable logistic regression using the Akaike information criterion (AIC) statistic (Akaike, 1974).

Multivariable logistic regression analyses evaluated associations between multiple factors and *M. bovis* infection. A backward stepwise approach was used to fit models that included species and demographic factors (age and sex) as well as those that met inclusion criteria ($p \leq 0.2$). Effect modification was evaluated by including an interaction term between plausible effect modifiers (species, age, and sex) and the other factors in the model. It was also examined whether water type was an effect modifier of the association between distance to the nearest water source and *M. bovis* infection. Competing models with similar predictors were chosen based on the AIC statistic. The final model included covariates (species, age, and sex) as well as other significant risk factors. Since significant interaction between species and year of sampling with *M. bovis* infection was identified in the final multivariable model with all rhinoceros, the same model was further explored for black and white rhinoceros separately, to describe potential differences in risk factors between species.

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Spatial data processing and analyses were performed in ArcGIS (version 2.8), except for tests of spatial clustering performed with SatScan, as described above. Statistical analyses were performed in R (version 4.0; R Core Team, Indianapolis, Indiana, USA); true prevalence was calculated with the package EpiR with the function epi.prev (Stevenson et al., 2022), and univariate and multivariable models were fit with the glm function (Marschner, 2011). Associations with $p < 0.05$ were considered statistically significant.

Results

Prevalence of *M. bovis* Infection and Spatial Clustering

The study population consisted of 475 free-ranging African rhinoceros that were opportunistically sampled in KNP from 2016 to 2020, as described in Materials and Methods. The *M. bovis* infection status could be determined for 437 (92%) of 475 rhinoceros, largely based on ante-mortem test results from IGRA (n = 428) (Chileshe et al., 2019a, 2019b), with a few individuals' case statuses confirmed using (post-mortem) mycobacterial culture of tissues with *M. bovis* species confirmation using a rapid diagnostic multiplex PCR (RD-PCR) (n = 9) (Miller et al., 2018; Warren et al., 2006) (Table 3.1). Fifty-eight of the 437 study population individuals with a known infection status were tested after recent translocation out of the park to quarantine areas. Of these, only five were *M. bovis* positive according to the IGRA result. All five of these individuals were sampled for testing within hours of their translocation out of the park; therefore, their positive status reflects infection acquired in KNP.

The apparent *M. bovis* prevalence was estimated and adjusted according to the sensitivity and specificity of the IGRA assay, as described in Materials and Methods (Stevenson et al., 2022). The overall adjusted prevalence (based on the IGRA) during the study period was 15.4% (82/437; 95% CI: 10.4 to 21.0%). The majority of *M. bovis* positive cases were considered clinically normal (68/82; 83%), based on veterinary clinical assessment at the time of immobilisation. Species-specific prevalence was 17.0% (63/317; 95% CI: 11.0 to 23.9%) for white rhinoceros and 11.2% (19/ 120; 95% CI: 3.1 to 22.2%) for black rhinoceros and were not statistically different (Fisher's exact p = 0.41). The 38 rhinoceros that were classified as having an "unknown" infection status were excluded from prevalence calculations and further analyses. Further description of demographic characteristics of the study population, according to *M. bovis* status, species, sex, and age, is shown in Addendum B, Table S3.1.1.

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Prevalence of *M. bovis* infection (adjusted based on IGRA sensitivity and specificity) in rhinoceros according to ranger area and ecozone is shown in Figure 3.1. In total, 420 individuals were included in prevalence calculations across the different areas. For ranger area, the highest *M. bovis* prevalence was in Pretoriuskop (28.1%), and the lowest was in Tshokwane (9.7%; Figure 3.1A); however, no significant differences in prevalence across ranger areas were identified (Fisher's exact $p > 0.05$ for all comparisons). Similarly, the highest prevalence by ecozone was in the Pretoriuskop Sourveld (36.4%), and the lowest prevalence was in the Lebombo Mountain Bushveld (13.3%; Figure 3.1B). The Pretoriuskop Sourveld ecozone had a significantly higher prevalence of *M. bovis* infection than the Sabie/Crocodile Thorn Thickets ($p < 0.001$), the Mixed Bushwillow Woodlands ($p = 0.02$), and the Lebombo Mountain Bushveld ($p = 0.01$) ecozones. No other significant differences were detected (Fisher's exact $p > 0.05$ for all other area comparisons). Ecozone and ranger area were not evaluated in the univariate and multivariable analyses, due to the numerous categories and the potential for missing covariate patterns.

Further exploration of differences in geographical distribution of *M. bovis* infection using Kulldorff's spatial scan statistic (Kulldorff, 1997) showed significant spatial clustering of infection in white rhinoceros. A single, statistically significant cluster of radius 6.5 km was detected toward the northern border of the Pretoriuskop ranger area (Figure 3.1A). Twelve cases of *M. bovis* were identified in this cluster, whereas the model predicted only four (relative risk = 3.5, $p = 0.036$). No other significant spatial clustering was detected

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Table 3.1. Mycobacterial QFT-IGRA[i] test and culture[ii] results for 475 African rhinoceros in Kruger National Park, South Africa (2016-2020)

M. bovis status	Test result for IGRA[i] and culture[ii]	White rhinoceros	Black rhinoceros	Total
M. bovis positive (n=82)	IGRA+/no culture performed	55	19	74
	IGRA+/culture+	3	0	3
	IGRA-/culture+	5	0	5
M. bovis negative (n=355)	IGRA-/no culture performed	253	101	354
	IGRA-/culture-	1	0	1
Unknown (n=38)	IGRA status undetermined/no culture performed	30	8	38
	Total	347	128	475

[i]Interferon-Gamma Release Assays (IGRAs) were completed on plasma from QuantiFERON-stimulated rhinoceros whole blood (Chileshe et al., 2019a, 2019b) to detect immune-sensitisation to mycobacterial antigens. Rhinoceros were classified as IGRA positive, negative, or unknown for *M. bovis* infection based on criteria outlined in Materials and Methods section on Determination of *M. bovis* infection status.

[ii]*M. bovis* was isolated in tissues obtained at necropsy from 8 of 9 white rhinoceroses through conventional mycobacterial culture using BACTEC™ MGIT™, with *M. bovis* species confirmation using region of difference (RD)-PCR (Warren et al., 2006). These culture positive animals included: 3 adult males (one necropsied in 2016, two in 2018), 1 subadult male (necropsied in 2016), 2 adult females (necropsied in 2016 and 2018) and 2 subadult females (necropsied in 2016 and 2018).

Univariate Analyses

Since environmental risk factors were hypothesised to be similar for black and white rhinoceros due to sharing of resources, the initial univariate analyses were performed with data from the two rhinoceros species (black rhinoceros, or *D. bicornis*, and white rhinoceros, or *C. simum*) combined. In total, 13 variables were evaluated in the univariate analysis using logistic regression (as outlined in Table 3.2). This included three spatial variables: number of buffalo herds, number of kudu herds, and buffalo density. Because the spatial scale for these variables was unknown, we used our data from repeated captures to estimate a plausible spatial scale over which rhinoceros in the study

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population could potentially move. We then evaluated the three spatial variables across different potential circular home ranges, with radii of 5.75, 11.5, 17.25, and 23 km. For each variable, a single home range size was ultimately chosen for further statistical evaluation based on the best-fitting single-variable logistic regression model. Chosen home ranges included 17.25 km for number of buffalo herds and 5.75 km for both number of kudu herds and buffalo density (Table 3.2). Addendum B, Tables S3.2.1. and S3.2.2. show all results from evaluations using home ranges with various distances; methodology is described in detail in Materials and Methods.

Four of the 13 variables met screening criteria ($p < 0.2$; Table 3.2). Two of these factors were statistically significant ($p < 0.05$) in the univariate analyses, including sampling year and number of buffalo herds within the rhinoceros home range (circular buffer; radius = 17.25 km). Rhinoceros species, age group, sex, and nearest permanent water source type were also considered important demographic covariates or plausible effect modifiers and were evaluated in the final multivariable model(s).

Even though 82 of the 437 study individuals tested positive for *M. bovis* infection, no significant association between *M. bovis* infection and (apparent) clinical health status was detected. The majority of the test-positive individuals appeared clinically normal (83% of infected animals were clinically normal, 68/82), and 12 of the 14 *M. bovis* positive individuals with recorded clinical abnormalities had afflictions that were associated with poaching or fighting injuries, rather than evidence of infection.

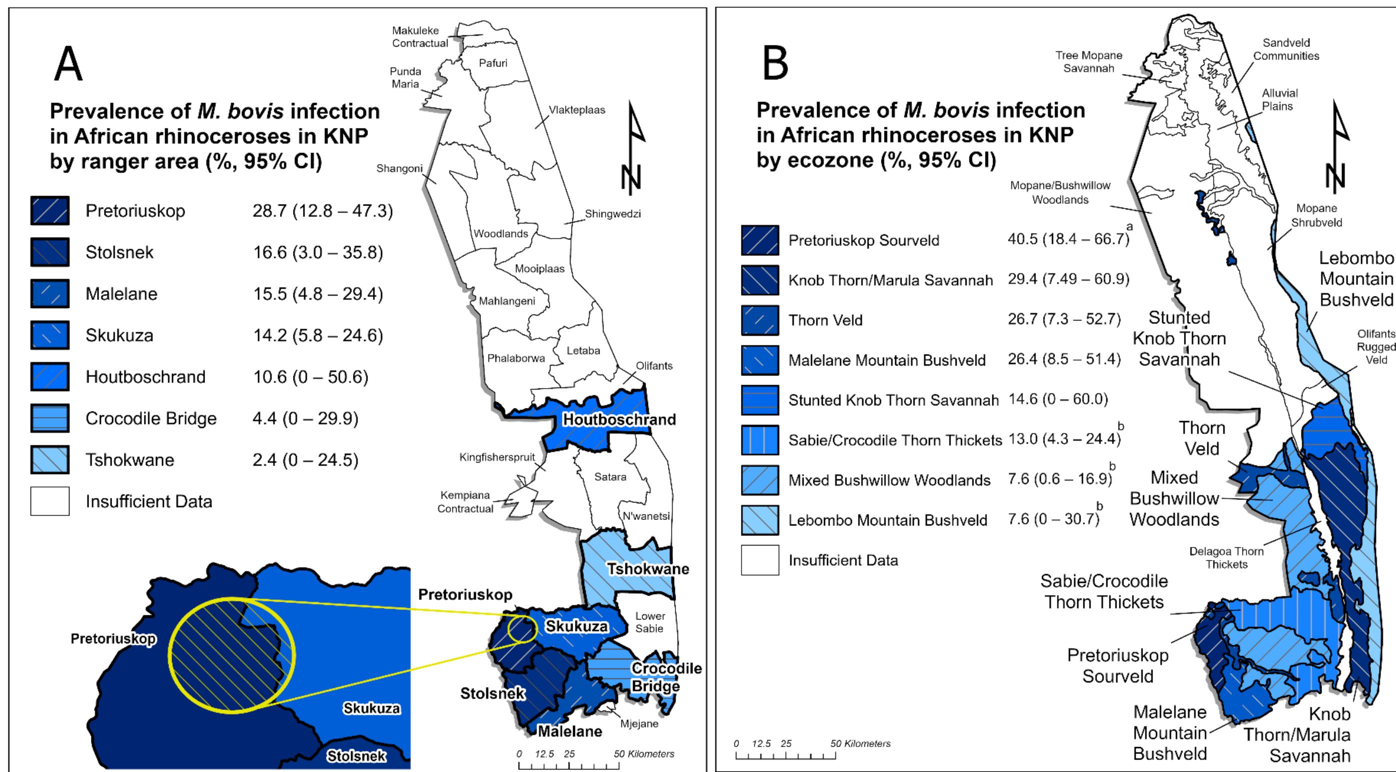

Figure 3.1. Prevalence (%) of *M. bovis* infection in rhinoceros in Kruger National Park, South Africa, 2016-2020 (n = 420). Prevalence estimates are reported in the legend in descending order. Areas with insufficient data (n < 10 sampled animals) are shown in white. Frame A shows the prevalence of *M. bovis* in the study population according to ranger management area (Kloppers and Bornman, 2005). No significant differences in *M. bovis* prevalence according to ranger area were identified (Fisher's exact $p > 0.05$ for all comparisons). A single, statistically significant cluster of radius 6.5 km is depicted by a hatched yellow circle based on Kulldorff's spatial scan statistic (Kulldorff, 1997); twelve cases of *M. bovis* were identified in this cluster, whereas the model predicted only 4 (relative risk = 3.5, $p = 0.036$). Frame B shows the prevalence of *M. bovis* according to ecozone (Gertenbach, 1983). Significant differences in *M. bovis* infection prevalence were detected between the ecozones with the same superscript letter. Prevalence in the Pretoriuskop Sourveld ecozone was significantly different than in Sabie/Crocodile Thorn Thickets ($p < 0.001$); Mixed Bushwillow Woodlands ($p = 0.02$); and Lebombo Mountain Bushveld ($p = 0.01$). Fisher's exact $p > 0.05$ for all other area comparisons.

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Table 3.2. Frequency distributions and univariate analyses of potential risk factors for *M. bovis* infection in African rhinoceroses in Kruger National Park, South Africa, 2016-2020 (n=437)

Risk factor	**Number of *M. bovis* positive rhinoceros n = 82 (% of total in category)**	**Number of *M. bovis* negative rhinoceros n = 355 (% of total in category)**	**Odds Ratio (95% CI)**	**p**
Species				0.33[vii]
White rhinoceros (*Ceratotherium simum)*	63 (77)	254 (72)	1.3 (0.8 – 2.3)	
Black rhinoceros (*Diceros bicornis*)	19 (23)	101 (28)	Reference	
Sex				0.82[vii]
Female	48 (59)	203 (57)	1.1 (0.6 – 1.7)	
Male	34 (41)	152 (43)	Reference	
Age				0.27[vii]
Adult	52 (63)	192 (54)	1.7 (0.8 – 3.6)	
Subadult	21 (26)	107 (30)	1.2 (0.5 – 2.8)	
Calf	9 (11)	56 (16)	Reference	
Orphan status (calves only, n=65)				0.61
Orphaned	2 (22)	17 (30)	0.7 (0.1 – 3.5)	
With mother	7 (78)	39 (70)	Reference	
Health status				0.38
Injured/abnormal health	14 (17)	47 (13)	1.3 (0.7 – 2.6)	
Appear healthy	68 (83)	307 (87)	Reference	
Sampling year				0.02[vii,viii]
2016	10 (12)	19 (5)	4.5 (1.7 - 12.1)	
2017	15 (18)	42 (12)	3.1 (1.3 – 7.2)	
2018	13 (16)	59 (16)	1.9 (0.8 – 4.5)	
2019	33 (40)	141 (40)	2.0 (1.0 – 4.2)	
2020	11 (13)	94 (26)	Reference	
Season				0.61

Dry	43 (52)	197 (55)	0.9 (0.5 – 1.4)	
Wet	39 (48)	158 (45)	Reference	
Nearest permanent water source type[i]				0.93[vii]
Waterhole	36 (44)	156 (44)	1.0 (0.6 – 1.6)	
River	46 (56)	195 (56)	Reference	
Number of kudu herds nearby[i,ii,iii]				0.30
8 – 14	24 (29)	95 (27)	1.1 (0.5 – 2.3)	
5 – 7	23 (28)	132 (37)	0.7 (0.4 – 1.6)	
3 – 4	21 (26)	64 (18)	1.4 (0.7 – 3.0)	
0 – 2	14 (17)	60 (17)	Reference	
Continuous variables	**Median (IQR)**	**Median (IQR)**	**Odds Ratio (95% CI)**	**p**
Distance to nearest water source (km)[i]	2.34 (1.33 – 3.62)	2.41 (1.32 – 4.08)	0.9 (0.8 – 1.0)	0.15[vii,viii]
Distance to nearest *M. bovis* infected rhinoceros (km)[i,iv,v]	2.76 (1.00 – 4.81)	2.54 (1.35 – 4.89)	0.8 (0.6 – 1.1)	0.11[vii,viii]
Number of buffalo herds nearby[i,iv,vi]	86 (46 – 111)	64 (41 – 102)	1.8 (1.2 – 2.8)	0.006[vii,viii]
Surrounding buffalo density (estimated buffalo per km^2)[i,ii,iv,v]	1.21 (0.55 – 2.30)	1.13 (0.67 – 2.60)	0.9 (0.6 – 1.3)	0.41

SD: Standard deviation; IQR: Interquartile range; [i]n = 433, excludes individuals with unknown capture locations; [ii]within home range of 5.75 km radius; [iii]categories created according to quartile distribution of measure over the study population; [iv]odds ratio and confidence interval (CI) calculated with a log transformation of the associated variable; [v]added 1 before log transformation of the measured variable for the study population as variable is equal to 0 for at least one of the included individuals) [vi]within home range of 17.25 km radius; [vii]selected for inclusion in multivariable model; [viii]met screening criteria

Multivariable Analyses

The final, combined-species model with all rhinoceros included 433 individuals, and consisted of five variables, namely, species, sex, age, sampling year, and number of buffalo herds within a 17.25 km radius of the capture location (Addendum B, Tables S3.3.1.–S3.3.3).

This model indicated that the year of sampling and the number of buffalo herds within a 17.25 km radius of the capture location were significantly associated with *M. bovis* infection in rhinoceros. Specifically, rhinoceros sampled in years 2016 (odds ratio [OR] = 4.4; 95% CI: 1.6

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to 12.3), 2017 (OR = 3.4; 95% CI: 1.4 to 8.1), and 2019 (OR = 2.2; 95% CI: 1.0 to 4.6) had higher odds of infection compared to the reference year 2020 (p = 0.01). Additionally, for each additional log-transformed buffalo herd in the rhinoceros home range, the odds of M. bovis infection increased by 75% (OR = 1.75; 95% CI: 1.1 to 2.8). However, there was significant effect modification identified across the sampling year by species (Addendum B, Table S3.3.3). Therefore, we also constructed species specific models with the same variables. The final species-specific models are reported in Figure 3.2, and in greater detail (including model fit parameters) in Addendum B, Table S3.4.1.

Additional effect modification by species or other factors was not identified, and other variable combinations did not improve the fit of the model or indicate additional sources of confounding.

Species-Specific Models

Variables found to be significantly associated with *M. bovis* infection differed between white and black rhinoceros. For the white rhinoceros, each additional log transformed buffalo herd in the home range resulted in an increase in odds of infection by 77% (OR = 1.77; 95% CI: 1.07 to 2.92, p = 0.02; Figure 3.2 and Addendum B, Table S3.4.1). This corresponded to a probability of *M. bovis* infection of 0.057 when the number of buffalo herds in the white rhinoceros home range was at the minimum (number of buffalo herds = 6) and all other factors in the model were held at their mean. This probability increased to 0.192 when the number of buffalo herds was at the median (number of buffalo herds = 66) and all other factors were held at their mean.

Importantly, the numbers of buffalo herds should be considered a relative, rather than an absolute, measure of exposure to buffaloes, since we did not have a precise measure of exposure to buffalo herds for each rhinoceros; however, our data support the hypothesis that white rhinoceros in areas with more buffalo herds were at an increased risk of *M. bovis* infection compared to those in areas with fewer buffalo herds, while controlling for other factors. Sampling year was not significantly associated (p = 0.15) with *M. bovis* infection in white rhinoceros.

Conversely, for black rhinoceros, the year of sampling was significantly associated with *M. bovis* infection (p = 0.01; Figure 3.2 and Addendum B, Table 3.4.1), while controlling for other factors in the model; individuals sampled in years 2016 (OR = 18.11; 95% CI: 2.09 to 157.15), 2017 (OR = 4.36; 95% CI: 0.98 to 19.41), and 2018 (OR = 4.60; 95% CI: 1.11 to 19.05),

compared to years 2019 and 2020 (note that the years 2019 and 2020 were combined for black rhinoceros, due to small numbers of animals in those categories). However, the number of buffalo herds nearby (within 17.25 km) was not significantly associated (p = 0.68) with *M. bovis* infection in black rhinoceros (Figure 3.2 and Addendum B, Table S3.4.1).

The ORs from final models reporting adjusted associations for *M. bovis* infection among white rhinoceros and black rhinoceros, separately, are reported graphically in Figure 3.2. Species specific model estimates, including coefficients and standard errors (SEs), are included in Addendum B, Table S3.4.1.

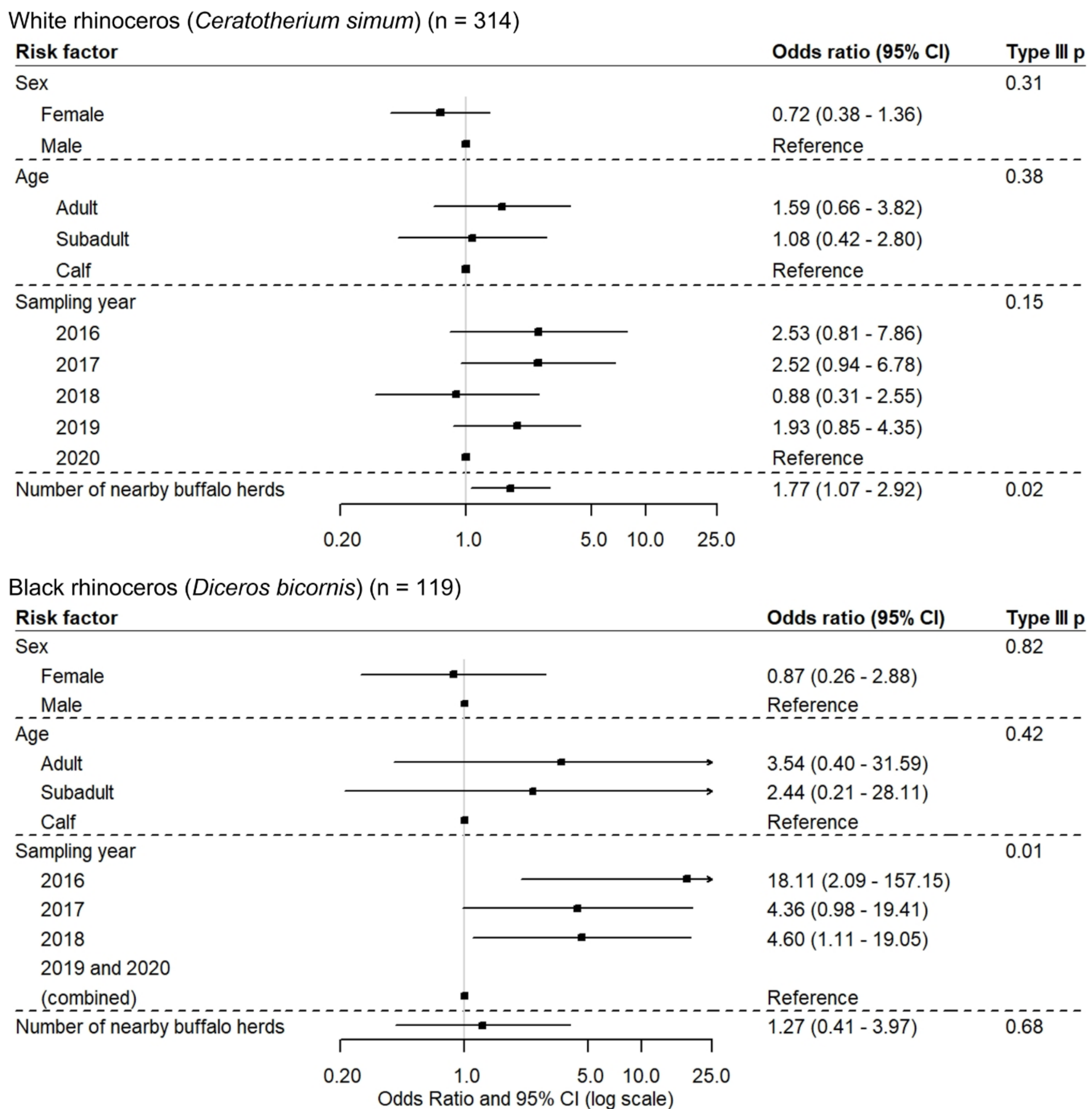

Figure 3.2. Forest plots depicting species-specific multivariable models of factors associated with M. bovis infection African rhinoceros in Kruger National Park, South Africa (2016-2020). Parameters for both models, including coefficients, standard errors, and fit statistics are reported in detail in Addendum B (Table S3.4.1).

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Discussion

A considerable and widespread *M. bovis* infection burden was reported for the KNP rhinoceros population (15.4%), with similar rates of infection found in males and females of all age groups and in both black and white rhinoceros. Although demographic factors were not associated with risk, an increasing number of buffalo herds in the white rhinoceros home range, and year of sampling in black rhinoceros, increased the risk of *M. bovis* infection in this population. The KNP rhinoceros are central to the "Integrated Strategic Management of Rhinoceros" plan introduced by the South African Department of Environmental Affairs (Department of Environment, Forestry and Fisheries, 2014; Department of Environmental Affairs (DEA), 2018). This strategy relies, in part, on translocation of individuals from the KNP population to newly developed safeguarding strongholds around the country. Therefore, the findings in this study support the decision to impose quarantine (Department of Agriculture, Forestry and Fisheries, 2016) on all rhinoceros (regardless of demographics) prior to translocation, in order to mitigate the risk for inadvertent *M. bovis* spread to other ecosystems outside KNP.

The distribution of *M. bovis* infection in KNP rhinoceros is similar to that reported for other species in the park. For example, a 1991–1992 survey of bTB in 1122 African buffaloes in KNP showed widespread bTB in the central and southern regions of the park (including Houtboschrand and regions to the south of it), with individual herd bTB prevalence up to 67% (de Vos et al., 2001). A later study showed spread of *M. bovis* infection to African lions (n = 70) sampled in 2012/2013 in the same areas of KNP, with an overall infection prevalence of 44% (Sylvester et al., 2017). Such extensive infection is increasingly observed in additional species in KNP, including warthogs (Roos et al., 2018), African wild dogs (*Lycaon pictus*) (Meiring et al., 2021), and African elephants (Kerr et al., 2019), with cases identified in more than 15 species in the park, to date (Bernitz et al., 2021). Taken together, these findings suggest that spillover of bTB is not a new occurrence and support the need for ongoing bTB surveillance across species to continuously assess disease risk and conservation impact, and to better understand transmission within and from the multi-host system in KNP.

The detection of a single statistically significant *M. bovis* infection cluster, with a 6.5-km radius, in white rhinoceros toward the northern border of the Pretoriuskop ranger area (Figure 3.1A) is in concordance with the higher infection prevalence in the Pretoriuskop Sourveld ecozone, compared to other areas in the east and the north (Figure 3.1B). The identified cluster is in close proximity to the KNP border with the surrounding Mpumalanga province. Importantly, the region outside of the southern KNP borders is primarily farmland and home

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to livestock herds, specifically, cattle. Livestock in areas around the southern border of the park have historically been implicated in spillover of *M. bovis* to wildlife in KNP, including African buffaloes (Arnot and Michel, 2020; de Vos et al., 2001). Now recognised as maintenance hosts for *M. bovis* in KNP (Arnot and Michel, 2020; de Vos et al., 2001; Dippenaar et al., 2017; Rodwell et al., 2001), African buffaloes share similar vegetation preferences to that of white rhinoceros. Interestingly, favoured vegetation is abundant in the Pretoriuskop Sourveld ecozone where the highest *M. bovis* prevalence in rhinoceros, as well as the only significant case cluster, occurred.

Results from this study suggest a role for buffalo in *M. bovis* infection, specifically, in white rhinoceros. Adjusted associations showed an increasing risk of *M. bovis* infection in white rhinoceros with increasing numbers of nearby buffalo herds. This suggests that the African buffalo in KNP may serve as a potential source for spillover of *M. bovis* infection into white rhinoceros, as observed in other species (de Vos et al., 2001; Dwyer et al., 2020; Musoke et al., 2015; Renwick et al., 2007; Sichewo et al., 2020). The importance of buffalo as a predictor of infection in white rhinoceros as compared to black rhinoceros was expected, as the landscape and preferred vegetation of white rhinoceros closely mirror that of buffaloes (Pienaar, 1994; Roche, 2000); both are grazing species, and, therefore, white rhinoceros are more likely to share habitat with buffaloes than are black rhinoceros.

Interestingly, the association was only significant when examining buffalo herds; the number of individual buffaloes nearby and buffalo density were not significantly associated with *M. bovis* infection. Importantly, the crude nature of the available data makes it difficult to define the exact relationship of the buffalo variable with *M. bovis* infection. It is possible that the identification of a buffalo "herd" is a more precise measure compared to estimating total number of buffalo in a herd from aerial surveys. Alternatively, this finding could simply be due to variation in individual versus herd-level prevalence in KNP buffaloes (Rodwell et al., 2001). Our findings could also suggest that effective contact rates between infected buffalo and susceptible rhinoceros that led to transmission did not depend on buffalo herd size.

Presence of infected buffalo herds was likely to increase environmental contamination with *M. bovis* due to shedding, consequently increasing exposure of rhinoceros to the pathogen. This phenomenon has been observed in other bTB multi-host systems, which involve wild boars, red deer, and cattle populations, across continental Europe (Cowie et al., 2016; Payne et al., 2017; Santos et al., 2015). Mechanisms of transmission between herbivores are unclear but have been attributed to indirect interaction through shared resources such as pastures, feed, or

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water holes that are contaminated by *M. bovis*-shedding hosts (Cowie et al., 2016; Payne et al., 2017). In the current study, we did not find any associations with distance to nearby water source or water type. Prospective evaluations with refined measures of frequency of individuals at particular water sources may further elucidate exposure to contaminated environments.

Potential transmission of TB between rhinoceros was also considered plausible (Dwyer et al., 2020; Michel et al., 2017; Stetter et al., 1995), but we did not find an association in adjusted models when evaluating distance to a nearby infected rhinoceros. For our initial exploratory analyses, our models included only crude evaluations of landscape-level effects and did not include the potential for infection in more than one nearby rhinoceros. We also did not have information on infection status of all (unsampled) individuals within a rhinoceros group, which may further lead to misclassification of exposure that could mask associations (Dohoo et al., 2009). More refined, individual-level measures that include longitudinal social network effects may improve understanding of the potential for intraspecies transmission.

Our findings also show a strong temporal association with risk of *M. bovis* infection, especially among black rhinoceros, albeit data were sparse for this group. Prevalence and odds of *M. bovis* infection were significantly higher in the earlier years of the study compared to 2019 and 2020. The magnitude of the adjusted OR was substantial in 2016 (OR = 18.1) and high in 2017 and 2018 (OR = 4.4 and OR = 4.6, respectively), compared to the more recent years of 2019 and 2020 (combined). It is unclear whether the observed decreasing prevalence over the study period was a result of changes in infection incidence or infection clearance rates, changes in contact patterns, an artifact of sampling bias, or another unobserved cause. All of these scenarios could be considered plausible.

Importantly, severe drought occurred in the year preceding and the first year of this study (2015–2016) (Ferreira et al., 2019). Associations between drought and *M. bovis* infection or disease have been detected in other multi-host systems. In a large study of Mediterranean wild boars (n = 3 923), which are known bTB reservoirs, the occurrence of drought and increasing drought severity were significantly associated with increased occurrence of TB-like lesions (Abrantes et al., 2021). Another study investigating risk factors for bTB in cattle in Great Britain showed a positive association between atmospheric dryness and areas of high risk for *M. bovis* infection (Wint et al., 2002). Drought may impact rhinoceros body condition, with adverse consequences for immune responses (Field et al., 2002; Schaible and Kaufmann, 2007), and susceptibility to TB (Biratu et al., 2014; Downs et al., 2008; van Crevel et al., 2002). Alternatively, reduced availability of water sources during periods of drought could have led

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to greater congregation of animals at the limited available drinking or wallowing sites. This could increase the risk of transmission through indirect interaction via *M. bovis*-contaminated water sources or surrounding resources, as is posited to occur in other multi-host systems (Cowie et al., 2016; Payne et al., 2017). Multiyear, longitudinal investigations may elucidate potential interactions between disease, climate change, and other factors that vary over time, on rhinoceros' susceptibility and exposure to *M. bovis* infection.

Of the 18.8% of rhinoceros that tested positive for *M. bovis* infection, most appeared clinically normal (83% of infected individuals were clinically normal, 68/82, and only 2/14 *M. bovis*–positive individuals with clinical abnormalities showed possible evidence of infection); this is consistent with reports that *M. bovis* infection does not always lead to clinical disease in rhinoceros. Findings from a study of experimentally infected white rhinoceros suggest that individuals that are healthy may be able to contain and clear the infection before developing active or overt disease (Michel et al., 2017; Parsons et al., 2017). However, it was difficult to confirm that there was no association between *M. bovis* infection and clinical disease or death in KNP rhinoceros; if present, this association could be obscured by the currently high mortality rate due to poaching (Ferreira et al., 2015).

Whether or not *M. bovis* infection is likely to progress to disease in KNP rhinoceros, the substantial infection burden in this population should raise concern that changes in (unknown) factors impacting disease progression could lead to increased TB-related morbidity and mortality in this population. This could have further negative consequences for survival of this population, which is already experiencing pressures associated with habitat loss, climate change, and poaching (Ferreira et al., 2015, 2017, 2019). Overall, more research that utilises sensitive indicators of rhinoceros clinical health is required to improve understanding of *M. bovis* infection and pathogenesis.

In recent years, there have been numerous important diagnostic advances for the detection and characterisation of *M. bovis* infection and disease in wildlife species, including free-ranging populations (Bernitz et al., 2021). These tools have been used in bTB surveillance efforts in wildlife species and, importantly, provide platforms to investigate bTB at the population level. Mycobacterial culture of tissue or secretions, followed by *M. bovis* confirmation using PCR, is a highly specific, gold standard diagnostic method, which can confirm true infection. However, this technique is most accurate when applied post-mortem, and tissues from mortalities in wildlife populations are often unavailable. For this reason, most of our study population was defined according to IGRA (Chileshe et al., 2019b) results, which were used to classify

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rhinoceros infection status ante-mortem. Importantly, there is a potential for misclassification of *M. bovis* infection status, due to the sole reliance on this single available diagnostic platform. The IGRA is a standard method for diagnosis of active and latent *Mycobacterium tuberculosis* infection in humans (Lewinsohn et al., 2017; Walzl et al., 2018), and for active *M. tuberculosis* complex (MTBC) infection in animals (Palmer et al., 2020; Smith et al., 2021), including rhinoceros (Chileshe et al., 2019a, 2019b; Michel et al., 2017; Parsons et al., 2017). A limitation of this test is that it may not always detect cases of recently acquired infection (Parsons et al., 2017). A study using *M. bovis* experimentally infected rhinoceros showed that an immune response (detected by IGRA) was measurable within 1-2 months after infection by airway inoculation, decreasing gradually between 5- and 12-months post-infection and reverting to negative results between 12- and 16-months post-infection, indicating clearance of active infection (Parsons et al., 2017). In general, the chronic nature of *M. bovis* infection makes it difficult to reliably estimate infection incidence, although the presence of a positive IGRA result signifies current infection and, therefore, can be used to estimate prevalence. To minimise misclassification of infection status, 38 individuals that could not be defined as positive or negative were removed (refer to section in Materials and Methods on Determination of *M. bovis* infection status). Efforts focused on improving *M. bovis* diagnosis in rhinoceros are currently ongoing and may lead to more accurate and reliable case classification in future studies.

The spatial analyses in this study relied on capture location data for the study population as proxy for the location or area where each rhinoceros may have been exposed during the study period, since information on the individual ranges was not available. Our rationale for using our own location data for individuals captured multiple times over the study was to provide contemporaneous and contextual estimates of the spatial scale in KNP over which study individuals could move (and therefore be exposed to spatial factors). The chosen sizes for potential home ranges around each individual's capture location were approximated from our data based on the distances that rhinoceros travelled, as indicated by repeated captures, with 95% of point-to-point distances travelled falling within 23 km. These were consistent with limited reports of rhinoceros movement within home ranges, typically reported to have sizes between 5 and 65 km^2, in the published literature (Adcock et al., 1998; Conway and Goodman, 1989; Joubert and Eloff, 1971; Lent and Fike, 2003; Morgan-Davies, 1996; Owen-Smith, 1975; Pienaar et al., 1993; Plotz et al., 2016; Rachlow et al., 1999; Roche, 2000; Thompson et al., 2016; van Gyseghem, 1984). However, the single time point capture locations do not reflect

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the movement patterns of rhinoceros and provide only a crude representation of the area that the rhinoceros regularly inhabited. Similarly, movements of other rhinoceros, and potential maintenance hosts (buffalo and greater kudu), were based on single time point location data for a cross-sectional sample of individuals, and the true rhinoceros home ranges are likely to vary over time according to the landscape and resource availability, and by individual (Adcock et al., 1998; Conway and Goodman, 1989; Lent and Fike, 2003; Morgan-Davies, 1996; Pienaar, 1994; Plotz et al., 2016; Rachlow et al., 1999; Roche, 2000; Thompson et al., 2016). Additionally, information on the infection status of these potential maintenance hosts was unavailable; therefore, the applied model assumed a uniform risk over space and time, which is unlikely to be the case. Lack of precision in these measures would be expected to misclassify exposure variables, often biasing associations toward the null (Dohoo et al., 2009). The fact that we detected associations between numbers of nearby buffalo herds and risk of *M. bovis* infection in rhinoceros may suggest that the true magnitude of the association is higher than our estimate. Future studies could circumvent these challenges by tracking each rhinoceros over the course of the study, using satellite trackers to record their movement over time and space.

Conclusion

This study examines the epidemiology of *M. bovis* in a free-ranging population of rhinoceros and includes a large sample population from what is historically the world's largest population (free-ranging rhinoceros in KNP). We detected evidence of widespread *M. bovis* infection in African rhinoceros in KNP, with a substantial infection burden (*M. bovis* prevalence was 17.0% [63/317; 95% CI: 11.0 to 23.9%] for white rhinoceros and 11.2% [19/120; 95% CI: 3.1 to 22.2%] for black rhinoceros), the extent of which was previously unknown. This emphasises the importance of cross-species surveillance in bTB-afflicted multi-host systems. Since bTB can affect wildlife, domestic animals, and humans, its spread to different areas could have serious consequences for human and animal health and, consequently, the agriculture and tourism industries in southern Africa. For rhinoceros specifically, translocation to other populations is an integral part of conservation strategies but may be accompanied by the risk of introducing novel pathogens, including *M. bovis*, into other ecosystems. Due to the presence and widespread impact of *M. bovis* in KNP rhinoceros, imposed quarantine and testing requirements prior to translocation are warranted across both rhinoceros species and all age groups.

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Results from this study also highlight the potential role of different factors in infection risk for each species. In black rhinoceros, we found temporal associations, with a higher risk of infection in individuals sampled in the early years of the study compared to the final years (2019-2020). This may suggest the involvement of drought and changing climatic conditions in infection risk in black rhinoceros. In white rhinoceros, we found an association with distribution of buffalo in the individual's surrounding vicinity. This highlights the potential role of buffalo, a recognised bTB maintenance host, in infection of white rhinoceros. Further study of *M. bovis* risk in these populations is warranted.

Future related work should focus on the development of diagnostic tools that may improve surveillance in these species. These techniques would also enhance the ability to classify cases and improve resolution to understand the epidemiology of bTB in complex systems. In particular, a targeted cohort study that tracks individual rhinoceros longitudinally and measures their resource usage in relation to their infection status could aid in further developing the understanding of infection and transmission risk. Characterizing threats to the survival of these species in the KNP ecosystem is vitally important for conservation and protecting other vulnerable populations.

Data Availability

Summarised data are included in the manuscript and supplementary information. Rhinoceros data are highly sensitive due to the ongoing crisis of poaching for rhinoceros horn and data restrictions apply.

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Chapter 4

Reduced Capability of Refrigerated White Rhinoceros Whole Blood to Produce Interferon-Gamma upon Mitogen Stimulation

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Abstract

Ante-mortem surveillance for *Mycobacterium bovis* (*M. bovis*) infection in the Kruger National Park (KNP) rhinoceros population currently relies on results from the QuantiFERON-TB Gold (In-Tube) Plus (QFT) interferon-gamma (IFN-γ) release assay (IGRA). However, same-day processing of rhinoceros blood samples for this test is a logistical challenge. Therefore, a pilot study was performed to compare mitogen-stimulated and unstimulated IFN-γ concentrations in plasma from rhinoceros whole blood processed within 6 h of collection or stored at 4°C for 24 and 48 h prior to incubation in QFT tubes. Replicate samples of heparinised whole blood from seven subadult male white rhinoceros were used. Results showed no change in IFN-γ levels in unstimulated samples, however the relative concentrations of IFN-γ (based on optical density values) in mitogen plasma decreased significantly with increased time blood was stored post-collection and prior to QFT stimulation. These findings support a need for same-day processing of rhinoceros blood samples for QFT-IGRA testing as per the current practice. Further investigation using TB-antigen stimulated samples is warranted to properly assess the impact of blood storage on TB test results in rhinoceros.

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Introduction

The survival of white rhinoceros (*Ceratotherium simum*) populations is threatened by poaching, habitat loss, drought, and potentially infectious diseases, such as bovine tuberculosis (Dwyer et al., 2020; Miller et al., 2018). *Mycobacterium bovis* infection, which is the primary cause of bovine tuberculosis (TB), is present in African rhinoceros in Kruger National Park (KNP), South Africa (Goosen et al., 2021; Miller et al., 2018). Ante-mortem surveillance of *M. bovis* infection in this population currently relies on an in vitro cytokine release assay called the QuantiFERON-TB Gold (In-Tube) Plus (QFT) interferon-gamma release assay (IGRA), which has been recently validated for white rhinoceros (Chileshe et al., 2019a, 2019b). This process requires that heparinised whole blood samples collected from immobilised rhinoceros are transported to laboratories and processed the same day to perform whole blood incubation in QFT tubes. This requirement is a logistical challenge for using the QFT-IGRA for TB detection. Rhinoceros in national parks and game reserves are often located in remote areas, without easy access to laboratory facilities or sample transport options, or with inconsistent availability of personnel to process these samples timeously upon their arrival at laboratories. Therefore, it is important to investigate the impact of extended whole blood storage (at 4°C to mimic transport conditions/extended delays) on the ability of immune cells to produce interferon-γ (IFN-γ) when stimulated in vitro. The goal of this pilot study was to measure and compare mitogen-stimulated IFN-γ production in rhinoceros whole blood processed within 6 h of collection, to that in replicate samples stored at 4°C for 24 and 48 h prior to stimulation. These results will inform whether transport/processing delays could impact QFT-IGRA results in rhinoceros.

Materials and Methods

4.1. Animals

Whole blood samples were opportunistically collected from the metacarpal vein of seven clinically normal subadult male white rhinoceros that were chemically immobilised by experienced wildlife veterinarians in Kruger National Park (KNP), South Africa, for dehorning procedures. Immobilisations were performed according to the Standard Operating Procedures for the Capture, Transportation and Maintenance in Holding Facilities of Wildlife (South African National Parks). Ethical approval for this project was granted by the Stellenbosch Animal Care and Use Committee (SU-2020–19019), and a section 20 research permit was

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issued by the Department of Agriculture, Land Reform and Rural Development (DALRRD;12/11/1/7/2).

4.2. Whole blood stimulation

Rhinoceros whole blood was collected in 9 mL lithium heparin vacutainers (BD Biosciences, Franklin Lakes, New Jersey, USA), as described by Chileshe et al., 2019a, 2019b. Samples were transported at ambient temperature (approximately 18-23°C) in a Styrofoam container to the Veterinary Wildlife Services laboratory within 6 h of collection. A 2 mL aliquot of blood was removed (t = 0 h) after arrival at the laboratory and 1 mL added to each of the QFT (Qiagen, Venlo, Limburg, The Netherlands) Nil (containing saline), and mitogen (containing phytohaemagglutinin) tubes. To ensure sufficient stimulation, additional pokeweed mitogen (Sigma Aldrich, St. Louis, Missouri, USA) was added to the QFT mitogen tube (10 µL) at a final concentration of 10 µg/mL. Tubes were thoroughly inverted 10 times then transferred to a 37°C incubator for 24 h. The remaining heparinised whole blood was refrigerated for 24 h at 4°C, then allowed to warm to room temperature, prior to adding aliquots to a set of QFT tubes, as described above. For the three rhinoceros with sufficient remaining volume of heparinised whole blood, an additional aliquot was stored at 4°C for 48 h prior to adding the sample to a set of QFT tubes. All QFT tube sets were incubated at 37°C for 24 h. After incubation, plasma was harvested following centrifugation at 800 x *g* for 10 min, transferred to a 2 mL microcentrifuge tube, and frozen immediately at -80°C until testing (completed within 1 month).

4.3. Interferon-Gamma (IFN-γ) ELISA

Interferon-gamma detection in the Nil and mitogen plasma samples was performed using the anti-equine IFN-γ ELISAPRO kit (Mabtech Ab, Nacka Strand, Sweden; custom precoated plate using reagent product 3117-1 H-6). The procedure, as previously described (Chileshe et al., 2019a, 2019b), was conducted with slight modifications; all mitogen plasma samples were serially diluted 1:10, 1:100, 1:1000 and 1:10,000, and Nil plasma samples were diluted 1:2, in ELISA sample diluent prior to adding each sample to duplicate wells. The remainder of the assay steps were followed as previously reported (Chileshe et al., 2019b).

4.4. Data Analysis

The mean optical density (OD) values for each sample were calculated after subtracting OD_{630} value from OD_{450} value for each well. The mean OD values of the serial dilutions for each rhinoceros were then compared to the working range of OD values of the standard curve in

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order to select the appropriate dilution for comparison of IFN-γ concentrations in the replicate samples at each time point. This step was performed by using empirical OD values for comparison rather than extrapolating IFN-γ concentrations in the mitogen samples, which were expected to be outside the linear range of the assay. A linear mixed effects model (fit with R statistical software (R Core Team, 2020) using packages lme4 (Bates et al., 2015) and lmertest (Kuznetsova et al., 2017) was then used to compare mean OD values in mitogen plasma from whole blood stored at 4°C for 0, 24, and 48 h. The overall model outcome was the mean OD value at the selected dilution, and the main fixed effect was storage time. Random effects for intercept were included for each animal to account for repeated measures on the same individual. Likelihood ratio tests compared models with and without the fixed effect of storage time to generate a p-value. A p-value < 0.05 was considered statistically significant.

Results and Discussion

The mean OD results for unstimulated (Nil) rhinoceros samples, diluted 1:2, were all near zero (OD < 0.01), which was below the assay's limit of quantification based on IFN-γ concentration (LOQ = 7.8 pg/mL; Chileshe et al., 2019b). These results were similar at all three storage time points for all individual rhinoceros. Since these results indicated that there was no production of IFN-γ in the unstimulated rhinoceros samples, no further analyses were undertaken. Therefore, refrigerated storage of rhinoceros whole blood for up to 48 h did not appear to affect background levels of IFN-γ.

The mitogen sample mean OD values were all significantly higher than mean OD values for the corresponding Nil samples (Addendum C, Figure S4.1.), which suggested stimulation of IFN-γ production in blood processed after all storage time points. To compare empirical measurements of IFN-γ production in mitogen samples across time points, the dilution factor resulting in an OD value in the range of 0.07–1.00 was selected for each set of samples from an individual rhinoceros. The dilution factor chosen was 1:100 plasma dilution for two rhinoceros and 1:1000 plasma dilution for five rhinoceros. Since the dilution factor was kept constant for a given individual rhinoceros, selecting different dilution factors for different rhinoceros did not affect the comparisons. Mean OD values for each set of mitogen samples from an individual rhinoceros decreased as whole blood storage time increased (Figure 4.1). The results from a linear mixed model, that included storage time as the fixed effect and individual rhinoceros as a random effect, are shown in Table 4.1. These findings confirmed that there was a significant decrease in mean mitogen sample OD values when whole blood was stored at 4°C for 24 and 48 h prior to stimulation (-2 log likelihood of the reduced model

containing the intercept only compared to the full model with time as a fixed effect = 13.63; $\chi 2$ = 12.75, p = 0.002). Therefore, these pilot data suggest that storage of rhinoceros whole blood at 4°C for 24-48 h may impact the ability of immune cells to produce IFN-γ when stimulated in vitro.

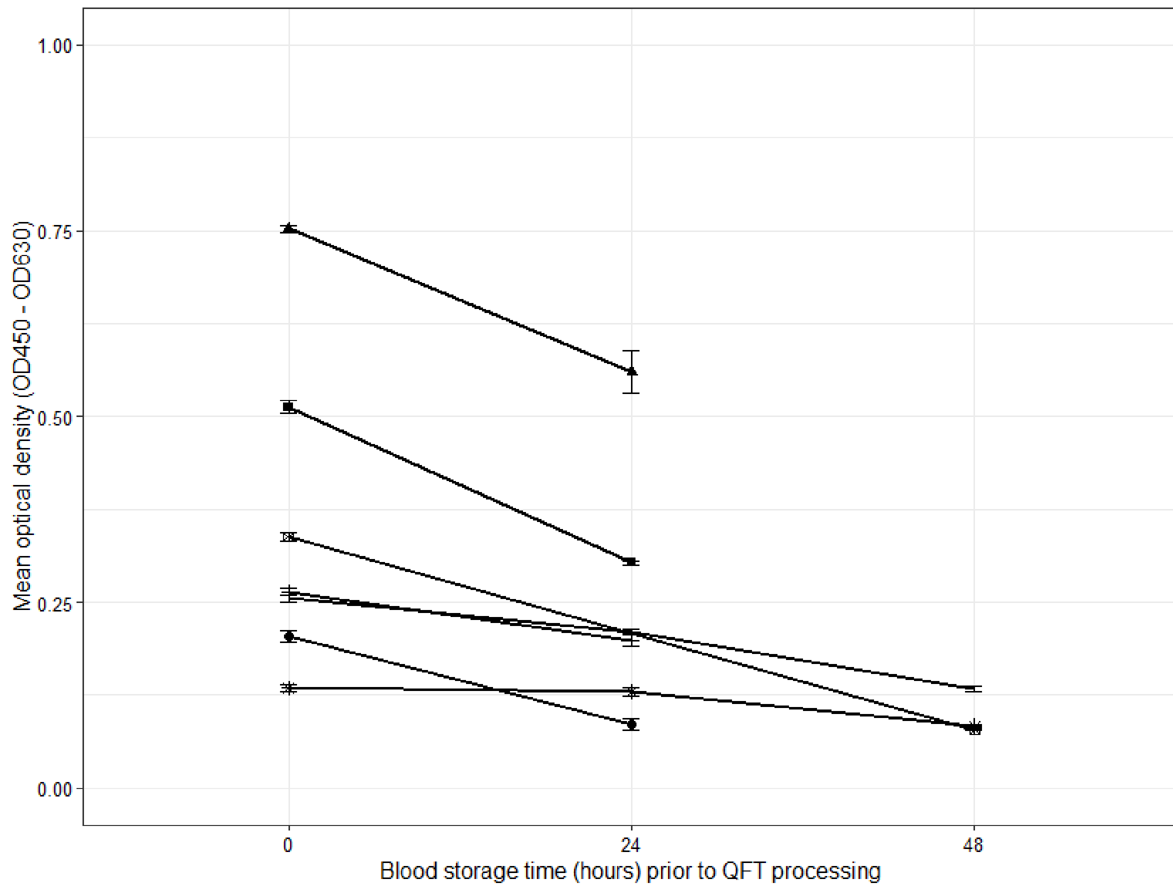

Figure 4.1. Mean optical density (OD) results for plasma harvested from QuantiFERON-TB Gold (In-Tube) Plus (QFT) mitogen tubes and measured in the equine interferon-gamma (IFN-γ) ELISA are shown. A fixed plasma dilution factor for each of seven white rhinoceros (represented by different symbols) was selected and OD values shown for each whole blood storage time point (0, 24, 48 h at 4°C prior to stimulation). Error bars indicate standard deviation across two replicates for each mean OD result. Note: for one individual, there was insufficient plasma volume to complete the IGRA on the Nil and Mit samples from the 24 h test point; hence, only the 0 and 48 h timepoint samples could be tested for this animal.

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Table 4.1. Linear mixed model of the relationship between mean optical density (OD) values of plasma from QuantiFERON-TB Gold (In-Tube) Plus (QFT) mitogen-stimulated rhinoceros whole blood screened with an equine interferon-gamma (IFN-γ) ELISA, and the time blood was stored at 4ºC prior to stimulation (n = 17 observations). Random effects for intercept were included for individual animals.

	Coefficient	Standard Error	df$	p%	-2 log likelihood*
Intercept	0.35	0.065	8		7.251
24 hours storage vs. 0 hours	-0.11	0.029	9	0.004	
48 hours of storage vs. 0 hours	-0.17	0.039	9	0.002	

$df – degrees of freedom;

%p – p-value for fixed effect;

*Storage time was a significant indicator of mean OD value in the uniformly diluted mitogen plasma samples, as determined by comparing the -2 log likelihood statistic from this model to that of a reduced model containing the intercept and random effects only (-2 log likelihood of the reduced model = 13.63; $\chi^2 = 12.75$, p = 0.002).

A diagnostic cut-off value for mitogen induced interferon-gamma responses in rhinoceros has been previously determined (Chileshe et al., 2019a, 2019b) to be > 84 pg/mL, although IFN-γ concentrations are typically greater than 400 pg/mL. All specimens in this study met this criterion at each time point, indicating that processed blood retained a sufficient, albeit reduced, ability to produce IFN-γ upon mitogen stimulation. Antigen-specific IFN-γ concentrations in *M. bovis* sensitised rhinoceros have ranged between 22 and 700 pg/mL (Chileshe et al., 2019b; Dwyer et al., 2022), and were usually lower than Mit-stimulated IFN-γ concentrations. One of the limitations of this study was the inability to select known *M. bovis* sensitised rhinoceros at the time of sample collection since the animals were free-ranging. Therefore, data are unavailable to determine whether delays in processing decreases IFN-γ production enough to change the test positive to negative classification; this would require further investigation to confirm.

The rationale for this pilot study was based on the limited resources available to veterinary staff collecting and transporting samples in the context of Kruger National Park and other areas in South Africa, as well as incorporating the manufacturer's guidelines for use of the QFT system with human blood (Qiagen, 2019) for IGRA. These state that human blood samples should be kept at 17–25°C, for a maximum of 12 h, or transferred to storage at 2–8°C within 3 h of collection for a maximum of 48 h prior to processing. However, limited studies conducted in humans and other animal species have shown that delays in processing of blood samples (kept

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at ambient temperature) for antigen and mitogen stimulation can result in compromised viability of cells, and therefore, a reduced capacity for cytokine production in the stimulated sample (Doherty et al., 2005; Gormley et al., 2006; Smith et al., 2009). For example, a study conducted on human blood found that the number of spot-forming cells (T-cells) detected using the ELISPOT assay was significantly reduced in samples where processing delays of 4 h (at ambient temperature) occurred, compared to immediately processed aliquots (Smith et al., 2009). A similar study showed that 2-hour delays in processing human blood samples (held at ambient temperature) had a negative impact on the production of IL-4 and IFN-γ following immune stimulation (Doherty et al., 2005).

Various other studies in humans and animals have reported similar negative impacts on the sensitivity of cytokine release assays with delayed sample processing (Duvigneau et al., 2003; Smith et al., 2009; Whipple et al., 2001). For example, one study showed that delays in sample processing resulted in a significant increase in the expression of several cytokines (e.g., IL-1, IL-6, and IL-8) measured in unstimulated blood (Duvigneau et al., 2003). In this case, both the delay in processing (h) and the temperature at which samples were stored prior to processing (4°C or ambient temperature) had an impact on the measured cytokine expression. Interestingly, the effects differed according to the cytokine measured.

Conversely, other studies have shown no impact of delays in sample processing on sensitivity of blood-based biomarker assays, sometimes depending on the analyte being measured (Ryan et al., 2000; Verberk et al., 2022). Since the effect of storage of blood at different temperatures on cytokine concentration appears to differ according to the host context, a pilot study using rhinoceros whole blood was necessary to determine how sample processing delays would affect IFN-γ production using conditions relevant to the rhinoceros QFT-IGRA. Due to the variability of ambient temperatures under field collections and transport, a single practical temperature condition was used, after receiving samples at the laboratory. In the current study, whole blood samples were refrigerated at 4°C for 0-48 h prior to processing in accordance with the QFT manufacturer guidelines for human blood. Specifically, this study was designed to mimic conditions in which blood is collected in the field, transported to the clinic at ambient temperature within 6 h, and then placed on ice packs for shipment to a laboratory for further processing. In South Africa, many wildlife veterinarians would not have access to equipment required for initial sample processing and therefore, would need to rely on a courier or other service to get the samples to a laboratory.

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The comparison of IFN-γ production in mitogen-stimulated rhinoceros whole blood in the present study used mean OD values rather than IFN-γ concentrations extrapolated from the standard curve. This approach was selected because the mitogen samples contained high levels of IFN-γ that were beyond the linear range of the assay, which would lead to the extrapolated concentrations having greater variability and potential inaccuracy. Such variability could bias the results towards not showing a change in assay performance with blood storage time when there truly was one.

To minimise this effect and use empirical values, the mitogen samples were serially diluted. The dilution factor for each rhinoceros, for comparison of replicate samples over storage time points, was selected based on a mean OD value in the working range of the assay. Therefore, empirical changes in OD values (rather than extrapolated concentrations) were compared between time points within an individual rhinoceros sample set, which created a more sensitive approach for detecting change in relative IFN-γ concentrations.

A limitation of this study was that samples were used from rhinoceros with unknown *M. bovis* infection status, and therefore, the impact of blood storage on antigen-specific IFN-γ results could not be evaluated. Since the concentrations of antigen-specific IFN-γ are typically lower than that of the mitogen-stimulated samples (Chileshe et al., 2019b), the decrease associated with blood storage may lead to a false negative result in *M. bovis* sensitised rhinoceros, and reduced assay sensitivity. Therefore, future studies should investigate QFT-IGRA results using stored blood from a cohort of known *M. bovis* sensitised rhinoceros. Based on the preliminary findings of this study, it is recommended that blood samples from white rhinoceros are processed the same day as collection and kept at room temperature prior to stimulations in the QFT platform, to maintain sensitivity of the QFT-IGRA. Delayed processing should be avoided as this could have negative consequences for test interpretation and confidence in reporting results.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chapter 5

Ante-Mortem Detection of *Mycobacterium bovis* in Nasal Swabs from African Rhinoceros

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Abstract

Mycobacterium bovis (*M. bovis*) infection has been identified in black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceros populations in Kruger National Park, South Africa. However, it is unknown whether *M. bovis*-infected rhinoceros, like humans and cattle, can shed mycobacteria in respiratory secretions. Limited studies have suggested that rhinoceros with subclinical *M. bovis* infection may present minimal risk for transmission. However, recent advances that have improved detection of *Mycobacterium tuberculosis* complex (MTBC) members in paucibacillary samples warranted further investigation of rhinoceros secretions. In this pilot study, nasal swab samples from 75 rhinoceros with defined infection status based on *M. bovis* antigen-specific interferon-gamma release assay (IGRA) results were analysed by GeneXpert MTB/RIF Ultra, BACTEC™ MGIT™ and TiKa – MGIT culture. Following

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culture, species determination was done using targeted PCRs followed by Sanger sequencing for mycobacterial species identification, and a region of difference (RD) 4 PCR. Using these techniques, MTBC was detected in secretions from 14/64 IGRA positive rhinoceros, with viable *M. bovis* isolated from 11 animals and no detection in any of the IGRA negative rhinoceros (n = 11). This finding suggests the possibility that MTBC/*M. bovis*-infected rhinoceros may be a source of infection for other susceptible animals sharing the environment.

Introduction

Mammalian tuberculosis (bTB) is a chronic progressive disease that is primarily caused by infection with *Mycobacterium bovis* (*M. bovis*). Between 2016 and 2019, *M. bovis* infection was discovered in black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceros in Kruger National Park (KNP), South Africa (Chileshe et al., 2019; Miller et al., 2017b, 2018). These findings were followed by a population-wide study in KNP (of 437 rhinoceros sampled between 2016 and 2020), which revealed that 15.4% (95% CI; 10.4 – 21.0%) of rhinoceros were infected with *M. bovis,* based on a combination of mycobacterial culture and antigen-specific interferon-gamma release assay (IGRA) results (Dwyer et al., 2022). The relatively high prevalence suggests that rhinoceros in KNP are regularly infected with *M. bovis*.

Excretion of *M. bovis* from infected cattle and wildlife has been associated with respiratory tract disease (Palmer, 2013; Phillips et al., 2003). In *M. bovis*-infected rhinoceros diagnosed post-mortem, lesions and *M. bovis* isolation were primarily associated with the respiratory tract (Michel et al., 2017; Miller et al., 2018; 2017a; Stetter et al., 1995; Valandikar and Raju, 1996). However, it is unknown whether infected rhinoceros, like humans and cattle, can shed mycobacteria in respiratory secretions (Lombard et al., 2021; Zhang et al., 2022).

Although mycobacterial culture is the gold standard for diagnosing *M. bovis* infection, it is widely recognised that ante-mortem detection can be insensitive, due to intermittent shedding, inability to obtain appropriate samples, and harsh decontamination processes, resulting in false negative diagnosis of infected individuals (de la Rua-Domenech et al., 2006; Goosen et al., 2014). Therefore, diagnostic tests based on mycobacterial antigen-specific immunological responses, such as the delayed hypersensitivity response in the tuberculin skin test or in vitro cytokine release assays, are commonly used to identify *M. bovis*-infected hosts (Bernitz et al., 2021; de la Rua-Domenech et al., 2006). Currently, the only available ante-mortem test that has been validated for detection of *M. bovis* infection in rhinoceros is the QuantiFERON TB Gold Plus Mabtech equine interferon-gamma release assay (IGRA) (Buss et al., 2017; Chileshe

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et al., 2019). Although tests based on host responses are useful for screening and surveillance, direct detection of *M. bovis* in secretions, such as respiratory samples, is crucial for understanding the epidemiology of bTB and determining risk of spread from infected animals (Clarke et al., 2022a; Goosen et al., 2020; Meiring et al., 2021; Santos et al., 2015).

Some studies have suggested that subclinically infected rhinoceros are unlikely to shed *M. bovis*, and, therefore, may present minimal risk for transmission (Michel et al., 2017; Parsons et al., 2017). Conventional mycobacterial culture methods, applied to bronchoalveolar lavage samples, recovered *M. bovis* in only 1 out of 60 samples collected over 20 months from three experimentally-infected white rhinoceros (Michel et al., 2017). It is unknown whether the low recovery rate was due to the true absence of *M. bovis* in lavage samples, or low sensitivity of culture methods (Michel et al., 2017). However, recent advances have improved detection of MTBC in paucibacillary samples and warrant further investigation of rhinoceros secretions (Dwyer et al., 2022, 2020; Stetter et al., 1995).

Applications of novel enhanced mycobacterial culture techniques and improved PCR-based species determination have led to increased *M. bovis* detection in wildlife (Bernitz et al., 2021; Bull et al., 2017; Clarke et al., 2022a, 2022b; Goosen et al., 2020, 2021, 2022). Use of cationic D-enantiomer cationic peptide supplementation and modified decontamination methods (TiKa-MGIT) have facilitated MTBC isolation from paucibacillary tissue and respiratory samples (Bull et al., 2017; Goosen et al., 2021). Culture-independent direct detection, using Cepheid's GeneXpert MTB/RIF Ultra qPCR assay (Ultra), supports same day MTBC DNA detection in a variety of animal specimens (Clarke et al., 2021, 2022a; Goosen et al., 2020, 2022; Hlokwe and Mogano, 2020; Kerr et al., 2020). Development of PCRs, targeting highly conserved genomic region flanking areas within the *rpoB* and *hsp65* genes, provides new methods for detection and species determination of *Mycobacteria* spp. (Adékambi et al., 2003; Clarke et al., 2022b; Goosen et al., 2022; Telenti et al., 1993). The value of applying these techniques has already been demonstrated in studies of wildlife TB (Goosen et al., 2022). These may also be valuable for detecting *M. bovis*, especially in ante-mortem samples, from suspected infected rhinoceros, and supporting future epidemiological studies. Therefore, in this study, the overall aims were 1) to determine whether *M. bovis* (DNA and viable bacilli) could be detected using novel direct detection approaches for nasal swabs from suspected *M. bovis*-infected (IGRA positive) rhinoceros in KNP, and 2) to calculate the proportion of IGRA positive rhinoceros with *M. bovis* present in nasal secretions to elucidate the potential for mycobacterial shedding.

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Materials and Methods

Study Population

Black (n = 39) and white (n = 472) rhinoceros in KNP were opportunistically sampled during immobilisations performed as part of management and veterinary activities between January 2020 and April 2022. Demographic characteristics were recorded, including species, sex, and age class, which was estimated by veterinary staff and summarised as follows: calf (0-2 years); subadult (>2 to7 years); adult (>7 years).

Routinely collected samples included heparinised whole blood and nasal swabs, which were processed as described below, and in Figure 5.1. A subset of nasal swab samples was chosen based on rhinoceros interferon gamma release assay (IGRA) results. The IGRA was applied for measurement of antigen-specific interferon-gamma (IFNg) release in whole blood that had been stimulated using the QuantiFERON Gold Plus (QFT) platform (Qiagen, Venlo, Limburg, Netherlands) and an anti-equine interferon-gamma ELISA (Mabtech Ab, Nacka Strand, Sweden) (Chileshe et al., 2019; Dwyer et al., 2022). Specifically, antigen-specific IFNg concentrations were determined by subtracting the concentrations in the nil tubes from the TB2 antigen tubes, as recommended by the manufacturer. This study subset consisted of 75 total rhinoceros: 64 that were IGRA positive (presumed *M. bovis*-infected) and 11 that were IGRA negative (presumed *M. bovis*-uninfected). The number of IGRA negative animals included in the study subset was determined by available resources for testing, and was randomly selected from all IGRA negative rhinoceros. A detailed explanation of the selection criteria for this subset is provided in Figure 5.1.

Nasal Swab Sample Collection and Processing

A single nasal swab (FLOQswab, Copan Diagnostics, Murrieta, California, USA) was collected from each rhinoceros at the time of blood collection. The swab was immediately transferred into 1 ml of sterile saline and frozen at -80°C, then transported to Stellenbosch University. Nasal swabs were thawed and further processed for direct detection of MTBC, using the Ultra qPCR assay and two different mycobacterial culture methods, followed by molecular identification of mycobacterial DNA, as shown in Figure 5.1.

Conventional and Modified Mycobacterial Cultures

Frozen nasal swab samples were thawed and 2 ml sterile phosphate (PO_4) buffer was added and mixed in the BSL-3 laboratory. Each sample was split into three equal aliquots (Figure 5.1). The first aliquot (1 ml supernatant) was stored at -80°C and was not used further for this

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study. The second aliquot (nasal swab tip and 1 ml supernatant) was decontaminated with N-acetyl L-cysteine sodium hydroxide (NALC-NaOH) and processed for culture using the BACTEC™ Mycobacteria Growth Indicator Tube (MGIT) 960™ TB System (Becton Dickinson, Franklin Lakes, New Jersey, USA), as previously described (Clarke et al., 2022b; Goosen et al., 2014, 2021). The third 1 ml aliquot was processed for culture using a modified version of the conventional MGIT (cMGIT) system, TiKa-MGIT (TiKa Diagnostics, London, United Kingdom), as previously described (Goosen et al., 2021). Briefly, samples were transferred to 30 ml sterile tubes containing 10 ml TiKa-Kic decontamination agent (TiKa Diagnostics) and incubated overnight (for a minimum of 20 hours) at 37ºC. Thereafter, samples were centrifuged at 3000xg for 20 min and the supernatant discarded. The cell pellets were resuspended in 1.6 ml PO_4 buffer and thoroughly mixed before 1 ml of each sample was removed for testing with the GeneXpert MTB/RIF Ultra qPCR assay (Cepheid, Sunnyvale, California, USA). The remaining 600 µl was used to inoculate MGIT tubes containing 800 µl BD BACTEC™ MGIT™ 960 Supplement Kit (Becton Dickinson) and 8.5 µl TiKa growth supplement B (TiKa Diagnostics). All culture samples were incubated in the BACTEC™ MGIT™ 960 TB System incubator at 37˚C for a minimum of 56 days. Samples with no growth after 56 days were regarded as culture negative, and no further downstream analysis was performed. One ml aliquots of culture growth positive samples were removed from the bottom of the MGIT tube, where bacterial growth had settled as seen by visible turbidity, boiled for 30 min at 99˚C and then removed from the BSL-3 facility for downstream testing.

Molecular Detection of MTBC DNA using PCR and Amplicon Sequencing

Specialised PCRs, using primers for the highly conserved genomic regions within the *hsp65* and *rpoB* genes (Adékambi et al., 2003; Telenti et al., 1993), were performed to screen boiled culture aliquots for the presence of *Mycobacteria spp*., as previously described (Clarke et al., 2022b; Goosen et al., 2022). Presence of amplified products with the correct target sizes (*hsp65*: ± 439 bp and *rpoB*: ± 764 bp) was confirmed using 1% agarose gel electrophoresis, followed by gel imaging using the ChemiDoc M.D. Universal Hood III Gel Documentation System (Bio-Rad Laboratories, Hercules, California, USA). Amplicons were sent to the Central Analytical Facility (CAF), Stellenbosch University, for Sanger sequencing. Sequence pairwise alignments were performed using A plasmid Editor (ApE; Version 3.1.3) (Davis and Jorgensen, 2022). For consensus target sequences to be considered reliable for further analysis, the sequence length had to fall within 30 bp of the expected target length, and the forward and reverse Sanger sequences had to match at a minimum of 80%. Any sequences that did not meet

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these criteria were not included for further analysis (Figure 5.1). Generated consensus sequences were analysed using the National Centre for Biotechnology Information (NCBI) nucleotide Basic Local Alignment Search Tool (BLASTn) (Altschul et al., 1990) to find sequence alignment matches in the NCBI database (Sayers et al., 2022).

Culture isolates that produced consensus target sequences with ≥ 95% shared MTBC reference sequence identity were selected for further investigation. Additional 1 ml aliquots of these cultures (which had remained in incubation with regular supplementation every 8-10 weeks for 4 months) were boiled at 99˚C for 30 min and removed from the BSL-3 facility. Total DNA was extracted from each sample aliquot using the QIAGEN DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany), as previously described. The *hsp65* PCR was repeated, using extracted DNA instead of boiled culture suspension as the template, to confirm mycobacterial identification. Presence of the *hsp65* amplicon was confirmed using 1% agarose gel electrophoresis, and amplicons were sent to CAF for Sanger sequencing. Sequence pairwise alignments and analyses using the NCBI database were completed as described above. Presence of MTBC in the extracted DNA samples was based on a threshold of 95% similarity between the query (sample DNA) amplicon sequence, and the MTBC reference target sequence. Extracted DNA samples identified as MTBC positive underwent an additional PCR targeting the genetic region of difference 4 (*RD4*) to confirm the presence of *M. bovis,* as previously described (Warren et al., 2006). A 25 µl reaction contained 12.5 µl Q5® High-Fidelity 2X Master Mix (New England Biolabs, Massachusetts, USA), 0.5µl of each 50µM primer stock solution, 6 µl sterile, nuclease free water, and 5 µl extracted DNA. The PCR cycling conditions were as follows: 1 cycle initial denaturation at 98˚C for 15 min, followed by 40 cycles of denaturation (98˚C for 30 s), annealing (62˚C for 1 min) and elongation (72˚C for 1 min). Final elongation took place at 72˚C for 2 min. Presence of the amplified products was confirmed by 1% agarose gel electrophoresis, followed by gel imaging.

GeneXpert MTB/RIF Ultra qPCR Assay

The Ultra qPCR assay (Cepheid) was used for direct detection of MTBC DNA in the remaining 1 ml sample aliquots processed with overnight TiKa decontamination (Figure 5.1). One ml of GeneXpert sample reagent was added to the 1 ml sample aliquot. The mixture was incubated for 15 min at room temperature, followed by vortexing for 10 s, and incubated for another 5 min before a final vortex for 10 s. The total volume (2 ml) of lysed sample was transferred to the Ultra cartridge sample chamber, loaded into the GeneXpert® instrument and PCR performed, according to manufacturer's guidelines. Possible result outputs included MTB not

detected, MTB detected high/medium/low/very low/trace. Detection of any MTB (trace amounts or greater) was interpreted as an Ultra positive result.

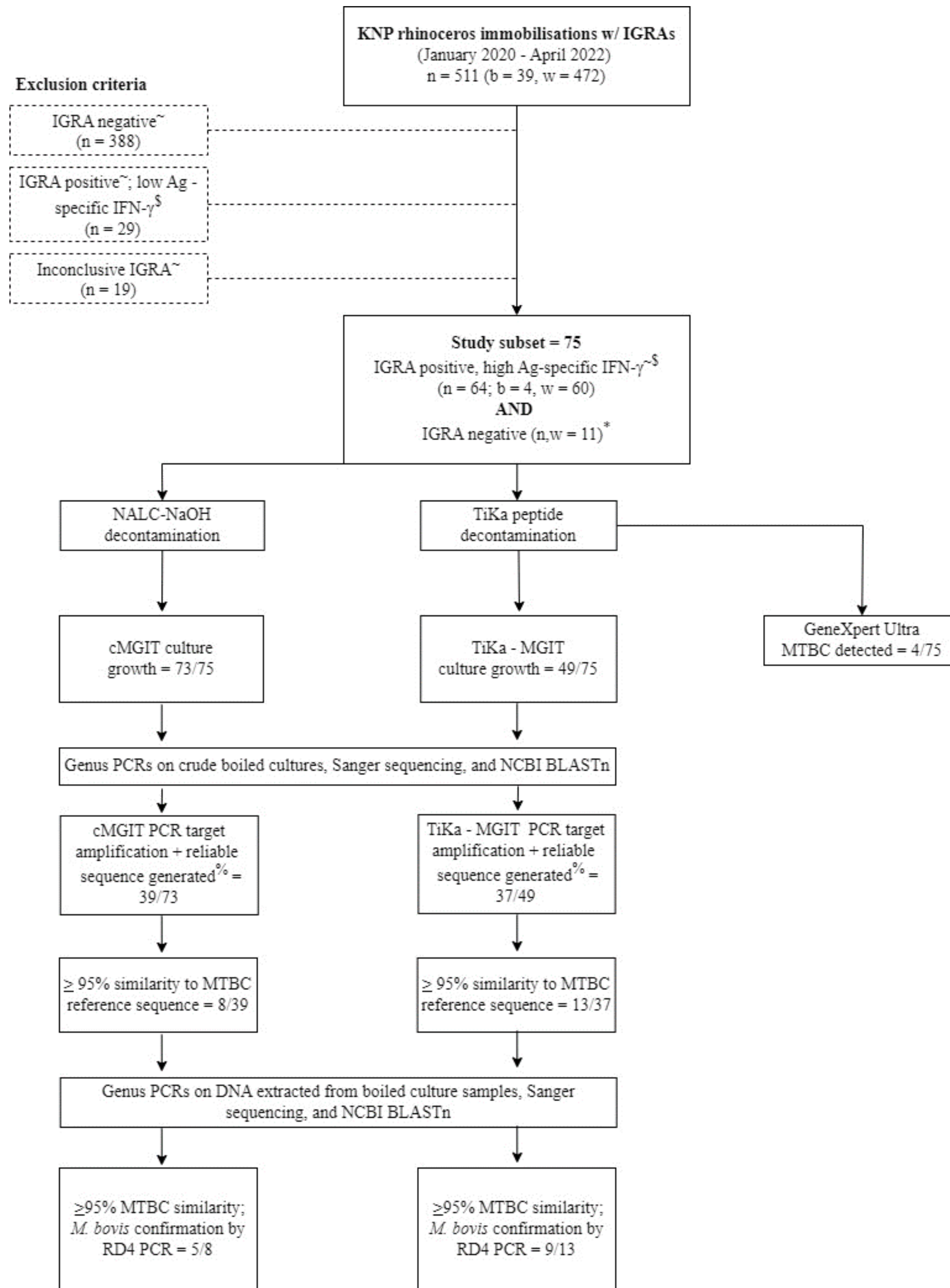

Figure 5.1. Flow chart for identifying the study population and methods pipeline for detection of MTBC in nasal swabs from African rhinoceros. KNP, Kruger National Park; b, black rhinoceros; w, white rhinoceros; IGRA, interferon-gamma release assay; Ag, antigen; IFN-γ, interferon-gamma; cMGIT, conventional BACTEC™ MGIT™ Mycobacterial Growth Indicator Tube culture method; TiKa – MGIT, TiKa decontamination and growth supplement enhanced-MGIT culture; NCBI BLASTn, National Centre for Biotechnology Information Basic Local Alignment Search Tool (nucleotide); MTBC, *Mycobacterium tuberculosis* complex; *M. bovis*, *Mycobacterium bovis*; RD4, (genetic) Region of Difference 4. ~A rhinoceros was classified as IGRA positive or negative, according to previously defined cutoffs

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(Chileshe et al., 2019; Dwyer et al., 2022). An animal was considered IGRA negative if it had a TB Ag-specific IFN-γ response ≤ 21 pg/mL, a mitogen IFN-γ response ≥ 21 pg/mL, and a Nil IFN-γ response ≤ 21 pg/mL. It was classified as IGRA positive if it had a TB Ag-specific IFN-γ response >21 pg/ml. Individuals who could not be defined as IGRA positive or negative according to the described case definitions were considered inconclusive and excluded. [$]IGRA positive rhinoceros were included in the study subset for examination of nasal swabs based on the magnitude of their Ag-specific IFN-γ response. Of the 93 IGRA positive individuals, the 64 with the highest Ag-specific IFN-γ responses (ultimately, all with Ag-specific [IFN-γ] > 40 pg/ml) were included in the study subset. The remaining 29 IGRA positive individuals with lower Ag-specific IFN-γ (ultimately, all with Ag-specific [IFN-γ] < 40 pg/ml) were excluded from the study subset. *A small selection of IGRA negative rhinoceros (n = 11) were included for comparison. [%]For a consensus sequence to be considered reliable for further analysis, the sequence length had to fall within 30 bp of the expected target length, and the forward and reverse Sanger sequences had to match at a minimum of 80%. Any sequences that did not meet this criteria were not included for further analysis

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Data Analyses

The proportion of individuals that were IGRA positive was determined for the entire study population. Demographic characteristics of the rhinoceros subset selected for further investigation were summarised. Frequency distributions of MTBC detection in nasal swabs from IGRA positive and negative groups were determined across each of the three different detection methods (Ultra qPCR, cMGIT culture/PCR, TiKa-MGIT culture/PCR). In addition, parallel interpretation was used to estimate the proportion of IGRA positive, as well as IGRA negative, rhinoceros with MTBC detected in nasal swabs using any of the three methods; 95% confidence intervals were calculated using the Agresti-Coull method (Whitlock and Schluter, 2020). Test agreement of these three methods within the IGRA positive group was evaluated using a Cohen's kappa statistic, and qualitatively interpreted (Cohen, 1960). All statistical calculations were performed in R (version 4.3.0, R Core Team).

Ethics

All procedures including immobilisation of animals and blood and swab collection were undertaken by South African Veterinary Council – registered wildlife veterinarians for management or other veterinary procedures unrelated to this study. Procedures were carried out according to the SANParks Standard Operating Procedures for the Capture, Transportation and Maintenance in Holding Facilities of Wildlife. Ethical approval for this project was granted by the Stellenbosch University Animal Care and Use Committee (ACU-2020-0966, ACU-2020-19019, ACU-2021-19019, ACU-2022-19019) and the Stellenbosch University Biological and Environmental Safety Research Ethics Committee (SU-BEE-202122561; SU-BES-202322561). Section 20 approval was issued by the South African Department of Agriculture, Land Reform and Rural Development (DALRRD; 12/11/1/7/2, 12/11/1/7/2A (JD)).

An approved Biomaterial Transfer Agreement (BMTA 005/22; 011/19) was obtained from South African National Parks (SANParks) which included evaluation by their Animal Care and Use Committee. A Threatened or Protected Species (TOPS) permit was obtained through the South African Department of Environmental Affairs (DEA Standing Permit S02556; S65805 and DEA Registration Certificate 29416; 02256).

All procedures involving potentially infectious material (e.g., mycobacterial culture) were performed in a Biosafety Level 3 (BSL3) facility that is certified for compliance under the Directorate of Animal Health (DAH) of DALRRD.

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Results

The *M. bovis* infection status, based on IGRA results, was determined for 492 of the 511 (472 white, 39 black) sampled rhinoceros. A total of 93 out of 492 individuals (19%; 95% CI: 16 - 23%) were IGRA positive, which included 87 white rhinoceros and 6 black rhinoceros. Demographic characteristics of the rhinoceros included in the study subset (64 IGRA positive and 11 IGRA negative) are outlined in Table 5.1.

Table 5.1. Summary of demographic characteristics of selected study rhinoceros for collection of nasal swabs and direct detection of MTBC (n = 75). Antigen-specific interferon-gamma (IFN-ɣ) concentrations were measured in QuantiFERON TB Gold Plus stimulated heparinised whole blood plasma using the Mabtech Equine IFN-ɣ ELISA. Those with antigen-specific IFN-ɣ concentrations < 21 pg/ml, mitogen IFN-ɣ concentrations > 21 pg/ml, and Nil IFN-ɣ concentrations < 21 pg/ml were considered IGRA negative; IGRA positive rhinoceros had antigen-specific IFN-ɣ concentrations ≥ 40 pg/ml.

Demographic characteristics	**Number of IGRA positive rhinoceros (% of total in category) n = 64**	**Number of IGRA negative rhinoceros (% of total in category) n = 11**
Species		
White rhinoceros (*C. simum*)	60 (94%)	11 (100%)
Black rhinoceros (*D. bicornis*)	4 (6%)	0 (0%)
Sex		
Male	33 (52%)	4 (36%)
Female	31 (48%)	7 (64%)
Age		
Calf (0 to 2 years)	9 (14%)	3 (27%)
Subadult (> 2 to 7 years)	9 (14%)	3 (27%)
Adult (> 7 years)	46 (72%)	5 (46%)

A total of 14 of the 64 (22%; 95% CI: 13 – 34%) study rhinoceros that tested IGRA positive had MTBC detected in their nasal swab by at least one of the methods (parallel interpretation), with viable *M. bovis* isolated in 11/64 (17%; 95% CI: 10 – 28%) cases. Individual test results are shown in Table 5.2. In summary, were 4/64 (6%) swabs were *M. bovis* positive by Ultra, 5/64 (8%) swabs were *M. bovis* positive by cMGIT, and 9/64 (14%) were *M. bovis* positive using TiKa-MGIT. None of the swab samples had MTBC detected using all three methods; however, four samples had MTBC detected by two methods- specifically, three swabs were *M. bovis* positive by cMGIT/PCR and TiKa-MGIT/PCR, and one swab was MTBC positive by Ultra and TiKa-MGIT/PCR (with confirmation of *M. bovis* in the latter). None of the nasal swabs from IGRA negative rhinoceros had MTBC detected by any of the methods. Importantly, none of the *rpoB* target amplicon sequences from any of the samples had ≥95% similarity to the MTBC reference sequence in the NCBI BLASTn database; therefore, identification of possible MTBC presence in the samples was determined solely based on a ≥95% similarity of

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the sample *hsp65* target amplicon sequences to the MTBC reference *hsp65* sequence in the NCBI BLASTn database. In the IGRA positive group (n = 64), there was fair test agreement between MTBC detection by cMGIT and TiKa-MGIT culture methods ($\kappa = 0.36$, $p = 0.04$), and no test agreement between cMGIT culture and Ultra ($\kappa = -0.0746$, $p = 0.003$) and Tika culture and Ultra ($\kappa = 0.07$, $p = 0.6$), respectively.

Mycobacteria other than MTBC were present in the nasal swab samples, based on DNA amplification of conserved regions of *hsp65* and *rpoB* in initial PCRs. Although Sanger sequencing and sequence alignment matches in the NCBI database identified NTM species, no further NTM characterisation was performed in this study.

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Table 5.2. Demographic characteristics and direct detection test results of nasal swabs for 14 interferon-gamma release assay positive rhinoceros in which *Mycobacterium tuberculosis* complex/*Mycobacterium bovis* was detected by at least one of the applied methods

Rhinoceros ID	**Species**	**Sex**	**Age**	**TB2 – Nil [IFN-γ] (pg/ml**	**Ultra result** (+/-)*	***M. bovis* present in culture sample, determined by *hsp65* PCR followed by *RD4* PCR confirmation using extracted DNA**	
						Conventional MGIT	**TiKa-MGIT**
1	White rhinoceros	Female	Adult	80	-	Yes	Yes
2	White rhinoceros	Male	Adult	128	-	Yes	Yes
3	White rhinoceros	Male	Adult	146	-	Yes	Yes
4	Black rhinoceros	Male	Adult	163	-	Yes	No
5	White rhinoceros	Female	Adult	57	-	Yes	No
6	Black rhinoceros	Male	Calf	40	-	No	Yes
7	White rhinoceros	Female	Adult	95	-	No	Yes
8	White rhinoceros	Female	Subadult	40	-	No	Yes
9	White rhinoceros	Male	Adult	87	+	No	Yes
10	White rhinoceros	Male	Subadult	63	-	No	Yes
11	White rhinoceros	Male	Subadult	470	-	No	Yes
12	White rhinoceros	Male	Adult	66	+	No	No
13	White rhinoceros	Male	Adult	89	+	No	No
14	White rhinoceros	Female	Adult	412	+	No	No
Total MTBC positive by method					4	5	10

*Positive Ultra results in this study were all trace

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Discussion

Mycobacterium tuberculosis complex organisms were detected in nasal swabs using direct detection methods from 14/64 (22%, 95% CI: 13-34%) of the IGRA positive rhinoceros tested, with viable *M. bovis* isolated in 11/64 (17%; 95% CI: 10-28%) cases. Similarly, MTBC has been directly detected in oronasal samples from various species with confirmed infection. For example, in a selected group (n = 12) of culture-confirmed *M. bovis*-infected African buffaloes (*Syncerus caffer*), MTBC DNA was detected in 5 of 12 (41%) nasal swabs (Clarke et al., 2022a). In a separate study of a human population with culture-confirmed *M. tuberculosis* infection (n = 80), *M. tuberculosis* DNA was detected in oral swabs from 29 (36.3%) individuals (Molina-Moya et al., 2020). In KNP, *M. bovis* was recovered by conventional mycobacterial culture in 4 oronasal samples from African wild dogs (*Lycaon pictus*) with immunological sensitisation to *M. bovis,* determined using IGRA (n = 136) (Meiring et al., 2021). However, to our knowledge, this is the first report of *M. bovis* detection in nasal (swab) samples from African rhinoceros. These findings demonstrate that MTBC/*M. bovis* can be sporadically detected in nasal cavities of infected African rhinoceros.

There are several possible scenarios to explain the presence of *M. bovis* in rhinoceros nasal secretions. Since respiratory samples were obtained from free-ranging rhinoceros in a *M. bovis*-endemic area (de Garine-Wichatitsky et al., 2013; Michel et al., 2006), the presence of *M. bovis* in these samples may be due to environmental contamination. Alternatively, *M. bovis* might have been shed by infected rhinoceros. In this study, MTBC DNA and/or viable *M. bovis* were only detected in nasal secretions from study individuals with immunological evidence of infection (IGRA positive), and not from any IGRA negative individuals. This supports the hypothesis that at least some of the IGRA positive individuals are truly infected and may be excreting mycobacteria. This raises concern that *M. bovis*-infected rhinoceros may be a source of exposure for other susceptible animals sharing the environment (Barasona et al., 2017; Meiring et al., 2021; Santos et al., 2015). Inclusion of a larger sample size of IGRA negative rhinoceros in future studies is needed to confirm this hypothesis.

This study applied multiple techniques for direct detection of MTBC, including the culture-independent GeneXpert MTB/RIF Ultra qPCR assay, followed by two mycobacterial culture methods, including cMGIT and TiKa-MGIT cultures, along with mycobacterial genus PCRs and Sanger sequencing to identify presence of MTBC DNA. The cMGIT and TiKa-MGIT cultures only agreed 36% of the time (p = 0.04), which reflects the substantially higher recovery of MTBC from TiKa-MGIT culture (n = 9 positive) compared to cMGIT culture (n = 5

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positive). This is concordant with findings from other comparative studies investigating the use of TiKa agents to enhance culture recovery from paucibacillary specimens (Goosen et al., 2021, 2022).

Both culture methods identified more MTBC positive samples than the Ultra, and there was no agreement between the Ultra and the two culture methods, respectively. In Ultra positive, culture-negative samples (Table 5.2), this could reflect the presence of non-viable bacilli, or numbers of viable bacilli below the culture limit of detection, which were detected using Ultra, but not culture. Conversely, there were several samples in which *M. bovis* was isolated from cultures but had Ultra negative results (Table 5.2). This may reflect the ability of culture (and to a greater extent, TiKa-MGIT culture) to select for and enhance viable *M. bovis* to detectable levels, even in the presence of other environmental microorganisms (Goosen et al., 2021). Alternative explanations for the culture positive, but Ultra negative results include the presence of PCR inhibitors, or low numbers of bacilli that fall below the limit of detection for the Ultra, but are sufficient to grow in culture. A previous study has shown that the Ultra's limit of detection for *M. tuberculosis* was 2 colony forming units (CFU) per ml, versus 30 CFU/ml for *M. bovis* (Goosen et al., 2020).

While both *rpoB* and *hsp65* PCRs were initially applied post-culture in this study, evidence suggested that the *rpoB* target selected was less specific to MTBC. Since the *rpoB* primers targeted highly conserved genomic regions in *Mycobacteria* spp., the PCR would have amplified this target from both NTMs and MTBC DNA. However, low levels of MTBC may have been obscured in the Sanger sequencing alignment matches if there was a high abundance of NTMs with *rpoB* target sequences (Adékambi et al., 2003). The *hsp65* target appeared to be more robust and better suited for detection of MTBC, with MTBC only detected in culture isolates based on this marker in combination with Sanger sequencing and a NCBI BLASTn database search, followed by species confirmation of *M. bovis* using the RD4 PCR. However, since the RD4 PCR was developed for use with tissues (higher mycobacterial load) compared to paucibacillary respiratory samples (Warren et al., 2006), it is recommended that future studies explore the combinational use of different specialised PCRs for MTBC detection, rather than a single target, to increase confidence in results.

A limitation of this study was that rhinoceros were selected based on IGRA results. The presence of false IGRA positive rhinoceros, due to cross-reactive host immune response to NTMs, could not be ruled out. A previous study in African buffaloes has reported the high diversity of NTMs present in respiratory samples (Clarke et al., 2022). A study investigating

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M. bovis shedding in African wild dogs in KNP described a low percentage of *M. bovis* positive cultures (3.1 – 3.5%) of respiratory samples from IGRA positive individuals (Meiring et al., 2021), like findings in the current study. However, Parsons et al. (2017) described waning QFT-IGRA responses in three experimentally *M. bovis*- infected white rhinoceros over time, with only a low IFNg in vitro response to purified protein derivative of *M. avium* (Parsons et al., 2017). This suggests that a positive QFT-IGRA response in rhinoceros is more likely due to infection with MTBC than NTMs.

Since only a single nasal swab was obtained from each study individual, there was a limited sample volume available for examination using direct detection methods. Furthermore, examination of a single swab sample per individual provides only a single time point representation of the possible shedding status. Sampling of a rhinoceros cohort at multiple time points may provide a better indication of the potential for mycobacterial shedding by rhinoceros over the course of *M. bovis* infection.

An additional limitation was the small number of IGRA negative (suspect uninfected) individuals included in this study for comparison. These samples were chosen randomly from the larger IGRA negative cohort, and the number was based on available resources for testing the samples. To increase confidence that *M. bovis* detection in rhinoceros nasal samples is indicative of infection and shedding, a larger subset of IGRA negative individuals should be examined.

Finally, while nasal swabs are easier and cheaper to obtain from rhinoceros than bronchoalveolar lavage samples, they are also more likely to contain environmental contaminates. These could include environmental MTBC, which may lead to a false positive result (Meiring et al., 2021). Alternatively, contamination by NTMs or other environmental microorganisms may obscure detection of paucibacillary MTBC, resulting in misclassification as MTBC negative (Liang et al., 2020). Examination of respiratory samples from the lower airways, using the described techniques, may increase confidence in detection of *M. bovis* excretion by infected rhinoceros.

Conclusion

This study showed that viable *M. bovis* could be detected in nasal secretions from rhinoceros with immunological evidence of infection. The KNP rhinoceros population has an unknown role in persistence and transmission of *M. bovis* in this system. Larger scale studies of ante-mortem respiratory samples of KNP rhinoceros, employing direct detection techniques with

additional MTBC biomarkers, and sequencing techniques with higher depth of coverage, may be warranted. In addition, comparison of *M. bovis* whole genome sequences isolated from rhinoceros and other reservoir hosts, such as African buffaloes, would provide valuable insight into the epidemiology of TB in the KNP system. Future research would enable better characterisation of possible shedding patterns, presence of other MTBC members, contribution of the KNP rhinoceros population to persistence of *M. bovis* in the ecosystem, and the possible transmission risk associated with the required translocation of KNP rhinoceros for management and conservation purposes.

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Author Contributions

Conceptualization, R.D., R.W., W.J.G., C.W., P.B., M.M.; methodology, R.D, R.W., W.J.G, C.W, M.M.; software, R.D; formal analysis, R.D., C.W; investigation, R.D.; resources, P.B., W.J.G., M.M.; data curation, R.D; writing—original draft preparation, R.D.; writing—review and editing, R.D., R.W., P.B., C.W., M.M., W.J.G; visualization, R.D., C.W.; supervision, C.W., M.M., W.J.G.; project administration, W.J.G, M.M.; funding acquisition, W.J.G., M.M. All authors have revised the manuscript.

Data Availability

Summarised data are included in the manuscript. The reference DNA sequence dataset analysed during the current study is available in the NCBI BLASTn database [https://blast.ncbi.nlm.nih.gov/Blast.cgi]. Rhinoceros-specific data are highly sensitive due to the ongoing crisis of poaching for rhinoceros horn and data restrictions apply.

Additional Information

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Chapter 6

Discussion

This chapter will summarise and integrate the different study findings from the previous chapters and will provide insight into their broader significance as it pertains to the management of animal TB and its impact on conservation of African rhinoceros. Limitations of the study will also be addressed in this chapter and conclusions in the next chapter.

Executive Summary

The research findings in this dissertation have addressed several knowledge gaps related to the diagnosis, surveillance, and epidemiology of tuberculosis in African rhinoceros, particularly the population in KNP. The project began by extrapolating literature on MTBC infections in other species and systems, to generate hypotheses regarding potential risk factors that would be investigated as *M. bovis* infection drivers in the KNP African rhinoceros population. The prevalence of *M. bovis* infection was estimated (15.4%), and significant risk factors for infection in the KNP population were identified, in a population-wide study. These included the number of buffalo herds present in white rhinoceros' home range and year of sampling for black rhinoceros.

During the population-wide epidemiologic study, and concurrently with ongoing *M. bovis* surveillance, additional knowledge gaps were identified. One of these was related to the use and limitations of the only available ante-mortem diagnostic test for *M. bovis* infection in rhinoceros, the QFT-IGRA. Except for a few individual animals with available mycobacterial culture results from post-mortem tissues, the QFT-IGRA was the primary screening tool used for the population-level study. The QFT-IGRA required same-day processing of fresh blood samples, which could be logistically challenging. Therefore, the impact of extended refrigerated storage of blood prior to QFT stimulation on assay results was evaluated. Findings indicated that refrigerated storage of heparinised whole blood for 24 and 48 hours prior to mitogen stimulation resulted in significantly reduced IFN-γ concentrations compared to samples processed within 6 hours of collection (and transported at room temperature).

Another important knowledge gap was whether *M. bovis*-infected rhinoceros were infectious (i.e., capable of transmitting *M. bovis*). This was investigated by collecting nasal swab samples from rhinoceros with known IGRA results, which were processed for mycobacterial culture and species determination by PCR. In addition, swab samples were tested directly using the Ultra qPCR assay, to determine whether this could be a potential method to detect mycobacteria

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in respiratory secretions. *Mycobacterium tuberculosis* complex DNA was identified in 14/64 IGRA positive individuals and no IGRA negative individuals. These preliminary findings provide evidence that shedding of *M. bovis* by infected rhinoceros may be possible, although further investigation is required to fully evaluate this hypothesis.

The significance of this research is that it includes the first estimate of *M. bovis* infection prevalence in a free-ranging rhinoceros population in a *M. bovis* endemic environment, provides evidence that presence of bTB maintenance hosts (such as buffaloes) may increase risk of infection in rhinoceros, and provides preliminary data suggesting that infected rhinoceros may be able to shed *M. bovis* (i.e., be potentially "infectious"). In addition, the requirement for same-day processing of blood samples for the QFT-IGRA was confirmed. This body of work will be discussed below.

MTBC Prevalence

Tuberculosis has been identified in captive rhinoceros as early as the late 1800s (Dwyer et al., 2020; Miller et al., 2017, Rookmaaker et al., 1998). However, historical cases of TB in zoo rhinoceros were often only identified at necropsy or late in the course of disease, once clinical signs developed. In addition, identification of the specific MTBC member causing infection was often absent. This highlights the limited knowledge of TB in rhinoceros, especially in free-ranging populations. With the lack of sensitive diagnostic tools, and in the absence of dedicated surveillance programs, it is likely that subclinical cases of MTBC infection were missed, resulting in an under representation of the disease in the global population.

Following the discovery of *M. bovis* infection in KNP rhinoceros (Miller et al., 2017a, 2018), a population-based study of TB epidemiology was undertaken in 437 African rhinoceros (*Diceros bicornis, Ceratotherium simum*) to provide the first prevalence estimate in a free-ranging rhinoceros population. The *M. bovis* prevalence of 15.4% in KNP rhinoceros, based primarily on QFT-IGRA results (Chileshe et al., 2019b; Dwyer et al., 2022a), suggested that infected rhinoceros may have been present in KNP for an unknown period of time prior to its discovery. This is not unexpected since KNP has been considered endemic for TB for at least two decades (Michel et al., 2006). The initial prevalence estimate provides a valuable reference point to monitor changes and impact of *M. bovis* infection on KNP rhinoceros in the future. In addition, this approach could be used to assess *M. bovis* infection in other rhinoceros populations in TB endemic areas.

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While prevalence is a measure of the overall infection burden in a specific population, incidence is a more appropriate measure to investigate trends in infection. Incidence provides a measure of new cases in a population over a specified span of time and can be used to estimate the probability of acquiring infection (Rothman et al., 2008). Although it has not yet been possible to estimate infection incidence in the KNP rhinoceros population (based on repeated sampling of individuals over time), the current dehorning program (which requires repeated immobilizations every 1.5-2 years) may provide the opportunity to further investigate incidence and changes in *M. bovis* infection status. Additionally, application of advanced statistical modelling methods may facilitate calculation of incidence estimates based on population prevalence and age structure over time (Yao et al., 2023). Therefore, continued surveillance of the KNP rhinoceros population may provide insights into changes in infection rates in the population and its impact on population numbers in the future. This is especially important as environmental changes related to climate, such as drought, may alter susceptibility of rhinoceros to *M. bovis* infection and disease (Dwyer et al., 2022a).

Demographic Risk Factors

Review of TB literature in captive rhinoceros had prompted the hypotheses that, independently, the characteristics of being male, an adult rhinoceros, or black rhinoceros increased the risk of TB compared to being female, younger or a white rhinoceros, respectively (Dwyer et al., 2020; Miller et al., 2017b). However, we found no evidence for differences in infection status across species, sex, or age in KNP rhinoceros (Dwyer et al., 2022a). This is not surprising since the hypotheses were based primarily on literature reporting TB resulting from *M. tuberculosis* infection in clinically-affected, captive rhinoceros. Consequently, any perceived patterns of heightened risk in certain demographic groups may be impacted by the under reporting of subclinical cases (in part due to the limited sensitivity of diagnostic tests used), confounding factors of captive management, biases inherent in captive population demographics, or a difference in susceptibility or exposure to *M. tuberculosis* versus *M. bovis* infection.

Most historical cases of TB in captive African rhinoceros were only identified following the development of clinical signs of disease, resulting in the death of the rhinoceros due to euthanasia or disease complications (Espie et al., 2009; Hofmeyr, 1956; Keep and Basson, 1973; Rookmaaker, 1998). This was also the case for the initial discovery of TB cases in KNP rhinoceros (Miller et al., 2017a, 2018). Subclinical MTBC infections have been reported in rhinoceros (Michel et al., 2017). However, subclinical cases in captive rhinoceros were only identified following diagnostic investigation of individuals that had been exposed to known

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TB cases and were considered to be at risk of infection (Duncan et al., 2009; Mann et al., 1981; Stetter et al., 1995). It is plausible that more historical cases of subclinical TB in captive rhinoceros occurred than were reported; such cases may have remained undetected, either due to the limited availability of resources and sensitive diagnostic tests, or the lack of diagnostic investigation of rhinoceros without clinical signs. This might bias the perceived risk factors in captive rhinoceros toward those that influence TB disease but may not be indicators of infection risk in free-ranging populations.

Importantly, management of rhinoceros may have an impact on their immune function and affect susceptibility to diseases (such as tuberculosis). For example, white rhinoceros that were maladapted to confinement after capture demonstrated significant clinical, hematologic, and serum biochemical changes, suggestive of physiological compromise (Miller et al., 2016, 2022). This has been demonstrated in other wildlife species; for example, captive dolphins (Fair et al., 2017), hyenas (Flies et al., 2015) and zebras (Seeber et al., 2020) exhibited diminished immune function compared to their free-ranging counterparts. It is conceivable that rhinoceros managed in captivity are more susceptible to TB disease than free-ranging rhinoceros, and drivers of infection and disease would vary between these groups. For example, rhinoceros in captivity may have prolonged close contact with infected animals or humans; examples of TB outbreaks in captive wildlife populations support inter-species transmission (Chandran, 2023; Mozter, 2022; Oh et al., 2002; Sternberg Lewerin et al., 2005; Stetter et al., 1995; van Zijll Langhout et al., 2009; Zlot et al., 2016). In these cases, *M. tuberculosis* was the predominant cause of infection in rhinoceros and other species (Barbiers, 1994; Duncan et al., 2009; Miller et al., 2015b, 2017a, 2017b; Oh et al., 2002; Valandikar and Raju, 1996; Witte pers. comm., 2020), with only a few caused by *M. bovis* (Dalovisio et al., 1992; Espie et al., 2009; Mann et al., 1981; Stetter et al., 1995). Notably, all four recorded historical cases of *M. bovis* infection in captive rhinoceros affected individuals that originated from the wild in southern or eastern Africa (Dwyer et al., 2020; Rookmaaker, 1998). Therefore, it appears that extrapolating risk profiles from captive animals to free-ranging rhinoceros is limited due to the multitude of differing risk factors.

Since no sex, species, or age-related predilections for TB infection in KNP rhinoceros were detected in this study (Dwyer et al., 2022a), results suggest that the entire rhinoceros population is susceptible to *M. bovis* infection. It is possible that true associations of demographic variables with *M. bovis* infection existed but may have been masked by sampling biases or confounding factors, such as disrupted social organisation or population demography due to

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poaching (Ferreira et al., 2015) or prolonged drought (Ferreira et al., 2019). In other free-ranging wildlife, age and sex have been associated with risk of *M. bovis* infection, disease, and excretion (shedding) (O'Brien et al., 2002; Vicente et al., 2007). In these reports, associations appeared to result from variations in social organisation and movement dynamics rather than biological differences in susceptibility (Vicente et al., 2007). For example, in a study of *M. bovis* epidemiology in free-ranging white-tailed deer in Michigan (O'Brien et al., 2002), older male deer were more likely to be infected. The authors postulated that TB may be maintained at low prevalence in matriarchal groups, with disease dissemination primarily occurring as a result of dispersal and movement of adult male deer (O'Brien et al., 2002). African rhinoceros, especially white rhinoceros, have a similar herd structure, with females being more social than males and adult bulls dispersing (Mukinya, 1973; Pienaar, 1994). Therefore, the role of rhinoceros social organization in TB epidemiology should be explored, since this has been demonstrated to impact TB in other wildlife species (Patterson et al., 2017; Vincente et al., 2007).

Despite the lack of statistical evidence of any association between demographic variables and *M. bovis* infection risk in KNP rhinoceros, significant effect modification was identified across the sampling years by species. Species-specific models identified risk factors that differed for the two species in KNP, with proximity to buffalo herds a risk for white rhinoceros and year of sampling for black rhinoceros. This was expected since the two species exhibit different ecological behaviour (Dwyer et al., 2020; Joubert and Eloff, 1971; Owen-Smith, 1973, 1975; Roche, 2000, 2001), and their KNP populations are distributed differently over the landscape (Ferreira et al., 2015, 2019). These findings emphasise the importance of separately investigating TB risk factors for these two species in specific environments.

Spatiotemporal Risk Factors for *M. bovis* Infection

The population-wide study of KNP rhinoceros revealed that *M. bovis* prevalence in rhinoceros did not have a uniform spatial distribution across the landscape, but differed significantly according to ecozones (Dwyer et al., 2022a; Gertenbach, 1983). This is likely due to different levels of *M. bovis* exposure of rhinoceros living in different ecozones. Potential factors affecting infection pressure would include the infection status and level of *M. bovis* excretion by susceptible host species found in that ecozone, the extent to which the environment is contaminated, and the distribution of rhinoceros (Dwyer et al., 2020). The role of environmental contamination in indirect transmission has been reported in other multi-host systems. For example, *M. bovis*-infected badgers in the UK have been shown to sporadically

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shed viable *M. bovis* in their faeces, which results in contamination of pastures, and have been implicated in transmission of *M. bovis* to cattle sharing the same land (Hutchings and Harris, 1997, 1999; Murphy et al., 2020). Similarly, in Portugal, presence of *M. bovis*-infected red deer and wild boar has been linked to contamination and spread to cattle in communal environments (Barasona et al., 2017; Santos et al., 2009, 2015a, 2015b).

In KNP, the *M. bovis* prevalence in rhinoceros was highest in the Pretoriuskop Sourveld ecozone, where the only significant case cluster occurred (Dwyer et al., 2022a). This area contains abundant vegetation types favoured by both African buffaloes and white rhinoceros (de Vos et al., 2001; Dwyer et al., 2020; Ferreira et al., 2015). It is also one of the areas in which high bTB herd prevalence (> 60%) has been identified in buffaloes (de Vos et al., 2001). This suggests that rhinoceros living in ecozones preferred by buffaloes may be at higher risk of exposure to *M. bovis* shed by infected buffaloes. This hypothesis is supported by the lower prevalence in rhinoceros living in thicket/woodland-type ecozones, which have lower utilization by buffaloes (Funston et al., 1994).

The first direct evidence for a link between presence of *M. bovis* maintenance hosts and infection in rhinoceros was shown by the increased risk of TB infection in white rhinoceros associated with greater numbers of buffalo herds near rhinoceros sampling locations (Dwyer et al., 2022a). This finding is based on an approximation of buffalo spatial distribution from census data. The crude nature of these data may have led to some misclassification error of buffalo distribution, which would be expected to bias associations towards the null hypothesis (Rothman et al., 2008). Therefore, it is possible that the magnitude of the true associations with TB infection could be even larger than was estimated (OR=1.77 for each additional buffalo herd near the rhinoceros sampling location). This can be interpreted as an estimated 77% increase in the odds of a rhinoceros being *M. bovis* infected with each additional buffalo herd in the vicinity of the sampling location. Since this study did not include direct measurements of buffalo-rhinoceros interactions, further studies are needed to understand and better quantify this association.

Studies in other systems have investigated the role of the environment in persistence of *M. bovis* infection. These commonly include monitoring movement and interactions of susceptible animal hosts using camera traps and other methods, to determine patterns that may explain TB infection in those populations (Payne et al., 2016, 2017); this may be a useful approach to further investigate the role of buffalo in *M. bovis* infection of KNP rhinoceros in the future. In

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addition, determination of bTB status of buffalo herds in rhinoceros home ranges would provide additional insights on the role of maintenance hosts in the context of rhinoceros TB.

Although an association between buffalo herd numbers and *M. bovis* infection in white rhinoceros was demonstrated, this was not a significant risk factor for black rhinoceros (Dwyer et al., 2022a). Since black rhinoceros are browsers, this could be due to the difference in preferred habitat and vegetation types/ecozones between the species (Ferreira et al., 2019). Therefore, the presence of *M. bovis*-infected buffalo may be less of a concern for black rhinoceros in KNP. However, differences in management of species, for example, on game farms or small reserves, may change the risk level.

Regarding temporal risk factors, there was a significantly higher odds of *M. bovis* infection in black rhinoceros in the early years of the study (2016-2017) compared to the latter three years (Dwyer et al., 2022a). Plausible explanations include changes in infection incidence or clearance rates associated with environmental factors (including vegetation distribution), co-morbidities such as nutritional stress (during drought), changes in contact patterns or density of infected host species, sampling bias associated with poaching occurrence, or other unrecognised variables (Dwyer et al., 2022a).

Environmental factors, especially those influencing nutritional status, have been shown to influence TB susceptibility in humans, livestock, and wildlife (Abrantes et al., 2021; Biratu et al., 2014; Downs et al., 2008; Field et al., 2002; Schaible and Kaufmann, 2007; van Crevel et al., 2002; Wint et al., 2002). In a study of a TB-endemic Mediterranean population of wild boars, increasing drought severity was significantly associated with increased occurrence of TB-like lesions (Abrantes et al., 2021). Another study reported a positive association between climatic factors and risk of bTB in cattle in Great Britain using remotely sensed satellite data (Wint et al., 2002). These examples highlight the importance of environmental changes in animal TB epidemiology.

A severe drought began in KNP in the winter (rainy season) of 2014/2015 and continued through 2015/2016 (Ferreira et al., 2019; Miller et al., 2018). This resulted in limited availability of water and nutritional resources for the animal population, including rhinoceros. Anecdotally, increased aggregation of animals was observed in areas in which water or vegetation was present (Buss, pers. comm.). This may have led to increased exposure of rhinoceros to *M. bovis*-infected hosts, either directly or indirectly, thereby increasing risk of infection. In addition, many animals, including rhinoceros, had loss of body condition, due to

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nutritional stress, which could contribute to immunocompromise (Field et al., 2002; Schaible and Kaufmann, 2007) and increased susceptibility to TB (Biratu et al., 2014; Downs et al., 2008; van Crevel et al., 2002).

It is unclear why the temporal risk factor was only significant in black rhinoceros. One possibility is that the significance of this variable in white rhinoceros was obscured by the more important predictor variable in this species, specifically, the association with buffalo herds. It is also possible that black rhinoceros experienced a more acute decline in health and immunocompetence in response to the drought as browsers, leading to heightened susceptibility to infection, and therefore a significant increase in infection odds. Interestingly, this is in contrast with the predicted species-specific effects of drought on African rhinoceros in KNP, in which drought appeared to have a negligible impact on browsers (black rhinoceros) compared to grazers (buffaloes and white rhinoceros) (Abraham et al., 2019). This was based on the expected rapid, intense short-term impact of drought on available grasses and herbaceous vegetation, with a comparably less significant short-term impact on trees and browse condition (Ferreira et al., 2019; Higgins et al., 2000; Sankaran et al., 2004; Vetter, 2009). Therefore, further studies are needed to understand risk factors for TB in KNP black rhinoceros.

IGRA: A Diagnostic Tool for Detecting *M. bovis* Infection in African Rhinoceros

Studies on TB epidemiology require accurate available tools, especially ante-mortem diagnostic tests. The QFT-IGRA, developed for detection of *M. bovis* infection in African rhinoceros, has been invaluable for surveillance in the KNP rhinoceros population. This blood-based assay allows for high sample-throughput and has a relatively fast turnaround time (24 hours for QFT processing, and <12 hours for completion of the ELISA, once samples arrive at the laboratory) (Chileshe et al., 2019b). The IGRA is a standard method for diagnosis of active and latent *Mycobacterium tuberculosis* infection in humans (Lewinsohn et al., 2017; Walzl et al., 2018), and for *M. bovis* infection in animals (Palmer et al., 2020; Smith et al., 2021), including rhinoceros (Chileshe et al., 2019a, 2019b; Michel et al., 2017; Parsons et al., 2017), and is suitable for population-level screening. However, the test has limitations, and sole reliance on this diagnostic method may introduce the potential for misclassification of infection status. Similar to other IGRAs, the rhinoceros QFT-IGRA may not detect cases of recently acquired infection (Parsons et al., 2017). A study using *M. bovis* experimentally infected rhinoceros showed that an immune response (detected by IGRA) was measurable within 1 to 2 months post-infection, with waning responses between 5 and 12 months, and reversion to

negative results between 12 and 16 months (Parsons et al., 2017). Therefore, naturally infected animals with recent infection or advanced disease, leading to anergy, may have false negative IGRA results.

The preliminary validation of the QFT-IGRA, including determination of the diagnostic cutoff value, was based on a small number of culture-confirmed, *M. bovis*-infected and uninfected rhinoceros (Chileshe et al., 2019b). This resulted in a range of antigen-specific IFN-γ cutoff values, which introduced uncertainty in determination of infection status (Chileshe et al., 2019b). In cases in which the test result falls in this "grey zone", there is a need for follow-up tests to confirm a positive or negative result, especially when findings are used for surveillance, diagnostic screening, and management decisions (Chileshe et al., 2019b). Therefore, as results become available from additional rhinoceros with culture confirmed infection status, a more accurate diagnostic cutoff value will be generated to improve confidence in the assay.

Since the IGRA has imperfect sensitivity and specificity, additional biomarker-based assays should be developed and used in conjunction with the IGRA. Immune signatures and algorithms using other cytokines, such as IFN-γ-induced protein 10, have been tailored to enhance sensitivity or specificity in African buffaloes, humans, and cattle (Clarke et al., 2022a; Garlant et al., 2022; Palmer et al., 2020). Another approach would be to incorporate other biomarkers of inflammation or humoral responses. In humans, the measurement of serum C-reactive protein (CRP) has been used as a screening tool for tuberculosis (Saripalli and Ramapuram, 2022). Rapid field-friendly diagnostic tests, such as lateral flow chromatographic assays, like the STAT-PAK and Dual Path Platform VetTB which detect antibodies to *M. bovis*-specific antigens (MPB83 and ESAT6/CFP-10), have been used to identify *M. bovis*-infected badgers, elephants, cervids, and rhinoceros (Ashford et al., 2020; Lyashchenko et al., 2018; Miller et al., 2017a). A pilot study investigating host CMI biomarkers in African rhinoceros with an equine PCR array identified antigen-specific *CXCL10* expression as a promising TB biomarker (Chileshe et al., 2021). Therefore, developing additional blood-based TB biomarkers in rhinoceros would expand diagnostic tools for these species.

Blood-based cytokine assays provide the currently available and potentially future methods for *M. bovis* surveillance in KNP rhinoceros. Therefore, it is important to optimise field applicability to overcome any logistical challenges related to testing, while still maintaining standardised implementation. The IGRA is performed in two steps, with a requirement for same-day processing of heparinised rhinoceros whole blood samples using the QFT stimulation platform. Rhinoceros in national parks and game reserves (like KNP) are often located in

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remote areas, without easy access to sample transport systems and laboratory facilities. Therefore, it was considered important to investigate the impact of extended refrigerated storage of whole blood, to mimic transport conditions, on the ability of stored immune cells to produce IFN-γ and affect IGRA results (Dwyer et al., 2022b).

The impact of time delays and sample storage conditions on IGRA results appears to vary according to host species. Some studies have shown that extended storage of blood, from humans and cattle prior to stimulation, has a negative effect on cytokine responses (Doherty et al., 2005; Gormley et al., 2006; Smith et al., 2009). For KNP rhinoceros, it was found that the relative concentrations of IFN-γ decreased significantly with increased time that blood was stored at 4°C prior to mitogen stimulation (Dwyer et al., 2022b). Should the relative concentrations of IFN-γ similarly decrease with increased storage time, then this may result in a failure to detect animals that are truly infected. Although the impact on antigen-specific IFN-γ responses was not directly tested, the findings using mitogen stimulated samples support the need for same-day processing of rhinoceros blood for the QFT-IGRA. This will ensure optimal performance and reproducibility of the test.

The QFT platform provides a convenient commercially available method for stimulation of whole blood, as well as incorporating standardised MTBC specific antigens (Hoffmann et al., 2016). Since this stimulation platform is used for human TB diagnosis globally, one of the reasons that this was selected for use in rhinoceros was that it could be available across rhinoceros range countries. The use of PPD antigens for stimulation, like IGRAs developed for cattle (Gormley et al., 2006), has been investigated along with the QFT system in rhinoceros, with the latter appearing to provide more consistent results (Parsons et al., 2017). The other advantage to QFT stimulation is that it can be performed by non-laboratory personnel since whole blood can be directly collected or aliquoted into tubes, incubated, then centrifuged and frozen or transported to facilities to perform the IFN-ɣ ELISA. This provides a potential solution to testing rhinoceros in remote locations by performing in-field same-day whole blood stimulation.

Mycobacterial Shedding in Rhinoceros

A key aspect of animal TB epidemiology is identification of hosts that can transmit MTBC. Individuals which are not infectious (i.e., not shedding) are considered dead-end hosts, which suggests that their presence does not present a risk to susceptible contacts (Turner et al., 2017). However, based on cases reported in the literature, it appears that intra-species MTBC

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transmission in rhinoceros is plausible (Dwyer et al., 2020). Therefore, this was investigated in the population-level study by evaluating the distance to the nearest *M. bovis*-infected rhinoceros as a risk factor in adjusted multivariable models, although no association of this variable with TB infection was found (Dwyer et al., 2022a). This initial exploratory analysis included only crude evaluations of landscape-level effects and did not include the potential for infection in more than one nearby rhinoceros (Dwyer et al., 2022a). Additionally, no information was available for infection status of all (unsampled) individuals within rhinoceros social groups, which may further have led to misclassification of exposure that could have masked associations (Dohoo et al., 2009). More refined, individual-level measures that include longitudinal social network effects may improve understanding of the potential for intra-species transmission; this work is ongoing but is beyond the scope of this thesis. Nevertheless, the question of whether transmission of *M. bovis* from infected rhinoceros occurs, prompted an initial study to investigate potential shedding of *M. bovis* in respiratory secretions of presumed infected rhinoceros (based on positive IGRA results).

In this study, evidence of MTBC was detected in 14 of the 64 (22%; 95% CI: 13 – 34%) nasal swabs from IGRA positive, free-ranging KNP rhinoceros that were tested by at least one of three selected direct detection methods (Chapter 5). This result refutes the previous prevailing hypothesis, supported by limited studies, which suggested that subclinically infected rhinoceros are unlikely to shed *M. bovis* (Dwyer et al., 2020; Michel et al., 2017; Parsons et al., 2017). The application of enhanced techniques for direct detection of *M. bovis* utilised in this study may have enabled more sensitive detection of the pathogen in rhinoceros respiratory samples than was previously possible.

The presence of *M. bovis* in nasal secretions of rhinoceros could be indicative of shedding by infected individuals. However, *M. bovis* detected in these samples could also have resulted from environmental contamination, or possibly pathogen exposure/colonization of the animal without true infection. Nonetheless, the detection of *M. bovis* in nasal secretions from a proportion of study individuals with immunological evidence of infection (IGRA positive), and not from any IGRA negative individuals, supports the hypothesis that at least some of the IGRA positive individuals are truly infected, and may be shedding mycobacteria. This should raise concern that *M. bovis*-infected rhinoceros could be a source of exposure for other susceptible hosts that share their environment, as well as potential introduction to naïve populations after translocation of an infected animal (Barasona et al., 2017; Meiring et al., 2021; Santos et al., 2015a).

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The possibility that infected rhinoceros may sporadically excrete viable *M. bovis* into the environment provides another potential explanation for the significant case cluster observed in white rhinoceros along the southwest border of KNP (Dwyer et al., 2022a). Although the presence of buffalo herds was shown to increase risk of *M. bovis* infection in white rhinoceros, the finding of possible shedding of *M. bovis* by infected rhinoceros supports the possibility that intra-species transmission could contribute towards the rhinoceros infection burden as well. While not as gregarious as African buffaloes, white rhinoceros in KNP have been observed in social groups of up to 10 individuals (Buss pers. comm., 2020). Therefore, it is plausible that shedding individuals may pose a transmission risk to other white rhinoceros nearby, either directly via aerosols, or indirectly through their shared environment.

Interestingly, the proportion of white rhinoceros with MTBC in nasal secretions (12/60; 25%) was lower than black rhinoceros (2/4; 50%), although the black rhinoceros sample population was very small. However, this observation warrants further investigation in larger study populations in the future. The source(s) of *M. bovis* infection in black rhinoceros remain unknown; however, other browsers, such as *M. bovis*-infected greater kudu, have been speculated to play a role in environmental contamination of browse, leading to exposure in other animals that forage on similar foods in the same areas (Bengis et al, 2001). Therefore, if black rhinoceros shed *M. bovis*, it is feasible that indirect transmission could occur within their shared environment, which may play an important role in the epidemiology of TB in this species.

The preliminary data presented in Chapter 5 warrant additional investigations of potential *M. bovis* excretion, and potentially transmission, from rhinoceros. Advanced techniques, including whole genome and targeted deep sequencing of *M. bovis* isolates from rhinoceros and other species, may provide greater insight into epidemiology of TB in the KNP. These results can provide resolution for molecular epidemiologic studies of TB and outbreak investigations in multi-host systems, such as the KNP (Biek et al., 2012; Price-Carter et al., 2018).

Study Limitations

The findings in this dissertation are limited to the KNP white and black rhinoceros population. Differences in management, environment, prevalence of *M. bovis*, presence of maintenance hosts, and surveillance techniques would limit extrapolation of findings, especially prevalence and risk factors, to other rhinoceros populations, such as in HiP. However, the approach can provide a model for investigating TB in multi-host systems containing rhinoceros. It is

important to highlight that the samples collected in this study were obtained opportunistically when KNP rhinoceros were immobilised for management (e.g., dehorning) or veterinary interventions, including necropsy. Therefore, there is likely to be some sampling bias introduced. For example, ante-mortem sampling was skewed towards adults, which comprised the majority of samples for the epidemiologic analyses (Buss et al., 2017; Dwyer et al., 2022a). Some animals were treated or euthanised due to injuries or illness, and sampling would be influenced by the probability of having these rhinoceros reported to and found by the VWS staff in an expansive park. Locating dead rhinoceros for necropsy is limited due to carcass decay and consumption by scavengers. Additionally, while the use of GIS spatial data was valuable for this study, it can only provide crude measures of variables, which can affect the identification of risk factors and the magnitude of their estimates (Rothman et al., 2008). While these kinds of sampling bias may be unavoidable, it is important to be aware of their existence when designing research studies and interpreting findings, especially in free-ranging wildlife populations.

One of the major limitations was the availability and reliance on results from a single ante-mortem diagnostic tool, the QFT-IGRA, which has only been partially validated for African rhinoceros (Chileshe et al., 2019a, 2019b). The use of a range of diagnostic cutoff values meant that results from animals that fell in the grey zone had to be excluded from the epidemiologic study, thus decreasing the sample size and losing information on 38 individuals. Additionally, although initial same-day processing of whole blood for QFT stimulation was achieved, plasma samples required freezing for long distance transport from Skukuza to Stellenbosch University, Tygerberg campus in Cape Town. This makes quality assurance (e.g., maintaining cold chain) a logistical challenge, and samples may have been subjected to variable freeze-thaw cycles, which could have compromised sample integrity and impacted IGRA results. However, it is recognised that this reflects the real-world implementation of this test for rhinoceros, which has been conditionally approved by the South African Department of Agriculture, Land Reform, and Rural Development to screen KNP rhinoceros for translocation (Buss et al., 2017).

Due to logistics of field sampling, the primary ante-mortem respiratory samples collected from rhinoceros were nasal swabs. Other studies from our research team have used a variety of techniques, including bronchoalveolar lavage, oropharyngeal or nasal swabs for direct detection of *M. bovis* infection to evaluate potential mycobacterial shedding (Bernitz et al., 2021; Clarke et al., 2021, 2022b; Goosen et al., 2020; Meiring et al., 2020; Miller et al., 2015a). Although the nasal swab sample is cheaper and less labour-intensive to obtain in the field, it is

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more prone to environmental contamination; therefore, other ancillary methods of assessing infection status are required to avoid misclassification of *M. bovis* infection and shedding status (Clarke et al., 2022b; Meiring et al., 2021). Bronchoalveolar lavage samples are less prone to environmental contamination, and consequently, may more reliably reflect the infection and respiratory shedding status of the test individual, and have been performed in rhinoceros (Goosen et al., 2020; Michel et al., 2017). However, it was not feasible to obtain these samples from the large, screened cohort of rhinoceros in the field. Techniques to obtain respiratory samples from deeper in the airways should be explored for use in rhinoceros to improve confidence in findings.

The recognised suboptimal performance of mycobacterial culture as the gold-standard diagnostic test for direct detection of MTBC was also considered a limitation, especially when using heavily contaminated and paucibacillary nasal swab samples. Therefore, direct detection of MTBC DNA was also performed using the Ultra qPCR assay, which has been shown to have greater sensitivity with respiratory samples (Goosen et al., 2020; Kolia-Diafouka et al., 2019). Surprisingly, in this study, the Ultra qPCR assay detected fewer positives than the TiKa-enhanced culture method, which suggests that he Ultra qPCR may be a less sensitive assay. The presence of PCR inhibitors in these samples is a plausible explanation for the culture-positive, but Ultra-negative results. Future studies that focus on the detection of *M. bovis* in antemortem samples (like swabs or BALFs) should consider utilising both (if available) direct molecular detection tools (e.g. Ultra qPCR) and culture methods. The combined use of these methods may enhance the overall sensitivity of detection.

Discussion Summary

Although African rhinoceros are known to be susceptible to MTBC infection, there is much that remains unknown about TB epidemiology and pathogenesis in these species. Although historical TB cases in captive rhinoceros have been associated with clinical disease (Dwyer et al., 2020), the majority of rhinoceros sampled for this study appeared to be “healthy”, or potentially subclinically infected (i.e., no clinical signs but evidence of MTBC specific immune responses or presence of *M. bovis*), as previously observed (Michel et al., 2017). This should not be unexpected – in humans, approximately one quarter of the global population is estimated to have latent TB (Houben and Dodd, 2016); however, most infected individuals do not develop disease, and some will clear the infection with time (Behr et al., 2019; Drain et al., 2018; Emery et al., 2021). While the possibility of latent TB and potential clearance of infection in animals remains an open question (Domingo et al., 2014; Ncube et al., 2022; Sabio y García et al.,

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2020), there is evidence that supports the possibility of latent animal TB (Flynn et al., 2015; Gormley and Corner, 2018; Lin et al., 2009; Ncube et al., 2022; Pollock and Neill, 2002; Sabio y García et al., 2020). Therefore, it is plausible that apparently healthy rhinoceros, like humans, may contain and clear the infection without progressing to clinical disease.

Disease associated with MTBC infection has been reported in black and white rhinoceros with comorbidities, such as nutritional stress, capture/transport/management–related stress, and advanced age (Dwyer et al., 2020; Espie et al., 2009; Miller et al., 2015a; Miller et al., 2018). Although this study did not find an association between *M. bovis* infection and morbidity or mortality in KNP rhinoceros, other factors, such as inability to continuously observe free-ranging rhinoceros or high mortality rate due to poaching (Ferreira et al., 2015) could have confounded the results. Further investigation of TB progression is needed in free-ranging rhinoceros to begin to unravel the potential long-term impact on the population.

Overall, the outcome of *M. bovis* infection in African rhinoceros seems to be more complex than simply a progression to disease; rather, it is more likely to consist of a spectrum of different immunological, clinical, and microbiological manifestations, similar to what is observed in humans (Drain et al., 2018; Walzl et al., 2011). The findings in this dissertation highlight the requirement for targeted research focused on characterising the temporal changes in immune responses and pathology in *M. bovis*-infected rhinoceros.

This study has significantly contributed to an understanding of the epidemiology of TB in rhinoceros and elucidated techniques that can be used for future surveillance. The first prevalence study provides a foundation for future monitoring of the population and evaluation of impact of changing conditions on transmission and disease. It also provides support for the current SANParks Rhinoceros Tuberculosis Management Plan (Buss et al., 2017), which relies on the QFT-IGRA results to minimise risk of spread through translocation of *M. bovis*-infected rhinoceros. The identification of risk factors will allow veterinarians and wildlife managers to develop strategies for preventing and managing TB in rhinoceros populations. Evidence that suggests presence of *M. bovis* DNA in respiratory (nasal) secretions also emphasises the need to screen rhinoceros for infection prior to translocation and in multi-host systems to minimise the risk of introduction and intra- and inter-species transmission. Therefore, this dissertation contributes important information that supports improved conservation of African rhinoceros.

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Chapter 7

Conclusion and Future Directions

This dissertation describes *M. bovis* infection in African rhinoceros, providing new insights on the epidemiology of the pathogen, screening tools, risk factors for infection, and potential mycobacterial excretion by infected individuals. For the first time, the prevalence of *M. bovis* infection in black and white rhinoceros in KNP was determined. The confirmation of a substantial, widespread level of *M. bovis* infection in KNP rhinoceros supports the continued implementation of quarantine and testing prior to translocation of animals from this population. Furthermore, this research provided SANParks Veterinary Wildlife Services, DALRRD, and other partners with evidence to institute proactive measures to minimise the risk of spreading *M. bovis* from infected rhinoceros, while still supporting their translocation for conservation and tourism purposes.

The association of *M. bovis* infection status in white rhinoceros with number of buffalo herds provided the first evidence for potential indirect transmission, through a contaminated environment. This under recognised route could also be a source of infection for other susceptible hosts in the ecosystem, particularly herbivores. Studies to investigate the role of maintenance hosts, such as buffalo, should include determining frequency of *M. bovis* excretion from infected animals, persistence of viable bacilli in different environmental conditions, and confirmation of infected rhinoceros and buffalo herds contemporaneously. Importantly, research focused on inter-species transmission, using advanced molecular tools, can highlight interactions that influence risk of spread.

This research also supports the hypothesis that infected rhinoceros can excrete *M. bovis* in respiratory secretions. Although preliminary, the findings suggest that rhinoceros may contribute to environmental contamination or even intra-species transmission. Factors that can influence excretion, such as stress associated with translocation or advanced age, need further exploration to provide a foundation for mitigation strategies.

Tuberculosis has not been considered a major direct threat to the survival of African rhinoceros populations, but this may change with future adverse ecological events such as drought, comorbidities, and climate change. In addition, changes in bTB prevalence in African buffaloes and other maintenance hosts may impact levels of exposure of KNP rhinoceros, and subsequently infection. Identification of factors that contribute to *M. bovis* infection and

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continued surveillance of rhinoceros populations will assist stakeholders and decision-makers to anticipate changes that could threaten these species.

Future research should focus on expanding the epidemiological characterisation of rhinoceros TB. Serial sampling of KNP rhinoceros, during the dehorning or other programmes provide an opportunity to further investigate incidence and changes in IGRA status. Additionally, advanced modelling methods could be applied to estimate incidence and detect impact of changes under different scenarios. Efforts to improve *M. bovis* diagnosis and surveillance would benefit from investigation to identify and validate additional biomarkers that could be used in conjunction with the IGRA and direct detection methods. These assays could be included in diagnostic panels or algorithms to enhance overall sensitivity and specificity, according to contextual requirements. Finally, preliminary data generated by this study that suggest potential excretion of *M. bovis* by infected rhinoceros warrant additional investigations to unravel transmission risks. These studies could incorporate *M. bovis* culture isolates from rhinoceros in molecular epidemiologic studies, using WGS, to elucidate spread and evolution of the pathogen through ecosystems.

Overall, continued research on tuberculosis in KNP rhinoceros could provide important insights on its impact on this population and other populations exposed to *M. bovis* and other MTBC. For example, the TB burden of rhinoceros populations in *M. bovis*-endemic HiP is, to our knowledge, yet unknown. A similar approach to that used in the epidemiological study in Chapter 3 could be applied to determine the *M. bovis* burden and risk factors for rhinoceros living in this different ecosystem. Additionally, similar epidemiological studies may be conducted for other infectious diseases that affect rhinoceros populations. These studies may enable stakeholders to improve the design and early implementation of rhinoceros management strategies, as well as infectious disease control at wildlife-livestock-human interfaces. In summary, this dissertation provides a foundation to improve disease surveillance and contribute to evidence-based management strategies and conservation of African rhinoceros.

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Addendum A

Supplementary material: Chapter 2

Table S2.1: Globally recorded historical cases of tuberculosis in black rhinoceros (*Diceros bicornis*) and white rhinoceros (*Ceratotherium simum*) (1. Hofmeyr, 1956; 2. Keep and Basson, 1973; 3. Mann et al., 1981; 4. Dalovisio et al., 1992; 5. Barbiers, 1994; 6. Stetter et al., 1995; 7. Valandikar and Raju, 1996; 8. Rookmaaker, 1998; 9. Oh et al., 2002; 10. Duncan et al., 2009; 11. Espie et al., 2009; 12. Morar et al., 2013; 13. Miller et al., 2015; 14. Miller et al., 2017, 15. Miller et al., 2018; 16. Witte, 2020)

Year of TB diagnosis	Location	Living conditions (captive/ semi-captive/free -ranging)	Additional notes on rhinoceros movements	Species	Age (years) at time of death	Sex	MTBC Species	Key Finding	Place of origin *	Ref (s)
1892	Wroclaw, Poland	Captive (zoo)	... → Travelled with Hagenbeck's Nubian African show for 10 years → Wroclaw, (1888).	Black rhinoceros	≥14	Unknown	Unknown	Died of TB	From wild	8
1944	Dresden, Germany	Captive (zoo)	... → Dresden (1928)	Black rhinoceros	≥16	Male	Unknown	Died of TB	From wild East Africa	8
1956	Pretoria, South Africa	Captive (zoo)	Mkhuze → NZG (1944)	Black rhinoceros	12-13	Male	Unknown	Died of TB	From wild – Mkhuze, South Africa	1, 8

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1957	Cologne, Germany	Captive (zoo)	... → Cologne (1953)	Black rhinoceros	≥4	Male	Unknown	Died of TB	From wild	8
1961	Osaka Japan	Captive (zoo)	... → Osaka (1955)	Black rhinoceros	≥6	Male	Unknown	Died of TB	From wild	8
1969	Prague, Czech Republic	Captive (zoo)	... →Imported by Behrend (dealer?) → Prague (1954)	Black rhinoceros	≥15	Male	Unknown	Died of TB	From wild	8
1970	Hluhluwe-iMfolozi, South Africa	Free-ranging	No record of any translocation	Black rhinoceros	Old; age not specified	Female	Unknown	Condition deteriorated up to death, general poor health. Acid fast *Mycobacterium* sp. isolated from lung granulomas at necropsy.	From wild (HiP)	2
1978	USA Washington DC	Captive (zoo)	... → purchased from “Dealer A” Washington D.C. (1961)	Black rhinoceros	20	Female	*M. bovis*	Ante-mortem diagnosis- died of TB after 3 day-illness during a time period of extreme heat & humidity.	From wild East Africa	3, 8
1979	USA	Captive (zoo)	... → purchased from “Dealer A”	Black rhinoceros	Unknown	Male	*M. bovis*	Ante-mortem diagnosis –	From wild	

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	Washington DC		→ Washington D.C. (1960)					*M. bovis* isolated from lung biopsy. Euthanised.	East Africa	3, 8
1984	San Diego USA	Captive (zoo)	Hannover, Germany (1968) → Budapest (1969) → San Diego Wild Animal Park (1983)	Black rhinoceros	16	Female	*M. tuberculosis*	Died of TB	Captive born - Hannover, Germany	8, 14, 16
1990	Hluhluwe iMfolozi, South Africa	Free-ranging	-	Black rhinoceros	Unknown	Unknown	Unknown	Necropsy reveals lesions consistent with TB; no culture confirmation	From wild (HiP)	12
1990	USA	Captive (zoo)	-	Black rhinoceros	Unknown	Unknown	*M. tuberculosis*	-	-	13
1991	Audubon, New Orleans, USA	Captive (zoo)	Umfolozi, South Africa → NY Bronx (1962) → New Orleans (1974)	White rhinoceros	29	Male	*M. bovis*	Died of TB and pneumonia	From wild (HiP)	4, 6, 8
1992	Mysore, India	Captive (zoo)	No translocation. Parents were purchased by the zoo from the	Black rhinoceros	13	Female	*M. tuberculosis*	Died of TB	Captive born - Mysore, India	7, 8

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			same dealer, were captured as adults from wild.							
1993	Chicago, USA	Captive (zoo)		Black rhinoceros	31	Female	*M. tuberculosis*	Positive *M. tuberculosis* cultures from sputum and gastric lavage. Treated – RIF[1], INH[2], PZA[3]. Died 1994: *M. tuberculosis* cultured from thoracic lymph node, tracheal exudates, and gastric lavage.	Origin unknown	5, 10
1994	Mysore, India	Captive (zoo)	No translocation. Born to same parents as the rhinoceros in which the previous reported TB case at this	Black rhinoceros	22	Male	*M. tuberculosis*	Died of TB	Captive born Mysore, India	7, 8

[1] Rifampicin

[2] Isoniazid

[3] Pyrazinamide

			zoo (1992) occurred.							
1998	Los Angeles, USA	Captive (zoo)	Housed in same facility as previous known TB cases	Black rhinoceros	24	Female	*M. tuberculosis*	Nasal wash in 1998 was positive for *M. tuberculosis*, treated with RIF[1], INH[2], PZA[3]. Observed weight loss and nasal discharge in 2000, no improvement so it was euthanised in 2001.	Origin unknown	9, 10
2007	Limpopo, South Africa	Captive (zoo)	Mkhuze (free-ranging)→HiP→ Limpopo NZG's Mokopane Biodiversity Conservation Centre (displaced in Mkhuze by another bull) (2002)	Black rhinoceros	Old (Estimated 35-40)	Male	*M. bovis*	Animal euthanised due to poor condition, old age, dental attrition. Two lung granulomas culture positive for *M. bovis*.	From wild → Mkhuze	11

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2013	Texas, USA	Captive (zoo)		Black rhinoceros	33	Male	*M. tuberculosis*	Died after non-specific illness. *M. tuberculosis* isolated from lungs at necropsy, spoligotype uncommon in animals but present in humans worldwide.		1, 13
2014	Private Reserve, South Africa	Free-ranging	-	Black rhinoceros	Adult	Male	*M. bovis*	Dies of pneumonia *M. bovis* culture positive.	From wild-private reserve, South Africa	15
2016	Kruger National Park, South Africa	Free-ranging	-	Black rhinoceros	Adult	Female	*M. bovis*	Animal euthanised due to poor prognosis. Lung tissue was culture- and PCR-positive/ confirmed *M. bovis*.		15
					Subadult	Male	*M. bovis*			15

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				White rhinoceros	Adult	Female		Poor condition of animals attributed drought or poaching wounds – animals were euthanised. bTB was not considered a cause of poor condition, and no generalised disease or infection in these animals. *M. bovis* culture positive from one or more of lymph nodes, and/or the lungs. Strain type: SB02121, the most common		
					Adult	Male				
					Subadult	Female				
2017	Kruger National Park, South Africa	Free-ranging	-	White rhinoceros	Subadult	Female				
					Adult	Male				

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								strain in the KNP.		

*Where a rhinoceros originated "from wild" and no additional information is provided, the individual originated from an unrecorded location on the African continent.

This data was located in the Rhinoceros Resource Centre (RRC) literature database, or through extensive web searches with (Miller et al., 2017) as a guide

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Addendum B

Supplementary material: Chapter 3

S3.1.

Table S3.1.1. Demographic characteristics of the sampled African rhinoceros population in Kruger National Park, South Africa (2016 – 2020), according to *M. bovis* infection status, species, sex and age, n=475

		Black rhinoceros (*Diceros bicornis*) (n = 128)			Total	White rhinoceros (*Ceratotherium simum)* (n = 347)			Total
		Calf	Subadult	Adult		Calf	Subadult	Adult	
M. bovis positive	Male	1	2	5	8	4	5	17	26
	Female	0	1	10	11	4	13	20	37
	Total	1	3	15	**19**	8	18	37	**63**
M. bovis negative	Male	7	12	32	51	17	38	46	101
	Female	10	9	31	50	22	48	83	153
	Total	17	21	63	**101**	39	86	129	**254**
Unknown *M. bovis* status	Male	0	0	2	2	2	4	7	13
	Female	0	3	3	6	5	4	8	17
	Total	0	3	5	**8**	7	8	15	**30**

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S3.2. Different iterations of continuous home range variables describing relative exposure of rhinoceros to buffaloes (Table S3.2.1) and kudus (Table S3.2.2)

Table S3.2.1.

Modelled variable	Home Range buffer radius (km)	*M. bovis* positive individuals (n = 82) Median (IQR)	*M. bovis* negative individuals (n = 351) Median (IQR)	Odds ratio (95% CI)	p	AIC
Number of buffalo herds[i]	23	129 (102 – 161)	119 (86 – 159)	1.78 (1.05 – 3.04)	0.026	419.34
	17.25[ii]	86 (46 – 111)	64 (41 – 102)	1.79 (1.16 – 2.77)	0.006	416.71
	11.5	35 (21 – 58)	24 (16 – 48)	1.54 (1.11 – 2.14)[iii]	0.007	417.12
	5.75	9 (5 – 17)	7 (2 – 12)	1.44 (1.09 – 1.91)[iii]	0.010	417.56
Number of buffaloes[i]	23	5494 (4397 – 6063)	5232 (3882 – 5901)	1.66 (0.90 – 3.06)	0.083	421.27
	17.25	3465 (2211 – 4617)	3106 (2123 – 3951)	1.57 (0.98 – 2.51)	0.040	420.08
	11.5[ii]	1372 (961 – 2157)	1388 (744 – 1977)	1.30 (1.01 – 1.67)[iii]	0.022	419.05
	5.75	425 (92 – 648)	334 (34 – 605)	1.08 (0.96 – 1.23)[iii]	0.199	422.64
Buffalo density (number of buffaloes per km²)[i,iv]	23	1.5 (1.0 – 3.9)	1.7 (1.0 – 4.1)	0.96 (0.71 – 1.32)	0.818	424.23
	17.25	1.4 (0.9 – 4.3)	1.3 (0.8 – 4.8)	1.03 (0.79 – 1.35)	0.802	424.22
	11.5	1.4 (0.9 – 2.7)	1.3 (0.8 – 4.0)	0.91 (0.63 – 1.31)[iii]	0.594	424.00
	5.75[ii]	1.2 (0.6 – 2.3)	1.1 (0.7 – 2.6)	0.85 (0.58 – 1.25)[iii]	0.407	423.60

[i]*model fit parameters and odds ratios based on log transformation of the associated variable;* [ii]*selected variable for further evaluation;* [iii]*added 1 before log transformation of the measured variable for the study population if it is equal to 0 for any of the study individuals),*[iv]*Derived according to estimates from the Poisson logistic regression model in (1).*

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Table S3.2.2.

Home range radius (km)	Number of kudu herds in home range (according to quartiles)	Number of *M. bovis* positive rhinoceros N = 82 (% of total in category)	Number of *M. bovis* negative rhinoceros N = 351 (% of total in category)	Odds ratio (95% CI)	p	AIC
23	197 - 264	15 (18)	89 (25)	0.6 (0.3 – 1.2)	0.42	425.49
	166 – 196	23 (28)	84 (24)	1.0 (0.5 – 1.8)		
	121 – 165	20 (24)	94 (27)	0.7 (0.4 – 1.4)		
	0 – 120	24 (29)	84 (24)	Reference		
17.25	51 – 66	23 (28)	91 (26)	1.4 (0.7 – 2.7)	0.76	427.10
	43 – 50	21 (26)	92 (26)	1.2 (0.6 – 2.5)		
	32 - 42	22 (27)	82 (23)	1.4 (0.7 – 2.9)		
	0 – 31	16 (20)	86 (25)	Reference		
11.5	24 - 37	24 (29)	113 (32)	1.2 (0.6 – 2.4)	0.45	425.65
	21 – 23	20 (24)	69 (20)	1.6 (0.8 – 3.4)		
	17 – 20	22 (27)	78 (22)	1.6 (0.8 – 3.3)		
	0 – 16	16 (20)	91 (26)	Reference		
5.75[i]	8 – 14	24 (29)	95 (27)	1.1 (0.5 – 2.3)	0.30	424.65
	5 – 7	23 (28)	132 (37)	0.7 (0.4 – 1.6)		
	3 – 4	21 (26)	64 (18)	1.4 (0.7 – 3.0)		
	0 – 2	14 (17)	60 (17)	Reference		

[i]selected variable for further evaluation

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S3.3. Final, combined-species multivariable model and evaluation of effect modification.

Since environmental risk factors for black and white rhinoceros were hypothesised to be similar due to sharing of resources, a combined-species model was initially fit to the data. This approach also allowed us to evaluate whether effects differed by species of rhinoceros. Model-fitting methods are described in detail in the Materials and Methods section.

The combined-species multivariable model highlighted two variables that were significantly associated with *M. bovis* infection: the year of sampling, and the number of buffalo herds within 17.25 km of the rhinoceros (Table S3.3.1). While controlling for other factors, the odds of *M. bovis* infection in rhinoceros increased as the number of (log transformed) buffalo herds within a rhinoceros' home range (17.25 km radius) increased. For each additional log-transformed buffalo herd in the home range, the odds of infection increased by 80% (OR = 1.8; 95% CI: 1.1 – 2.8). This corresponds to a probability of *M. bovis* infection of 0.053 when the number of buffalo herds in the home range is at the minimum (n buffalo herds = 6) and all other factors in the model are held at their mean. This probability increases to 0.178 when the number of buffalo herds is at the median (n nearby buffalo herds = 67) and all other factors are held at their mean.

The year that the individual rhinoceros was sampled was also significantly associated with odds of *M. bovis* infection in the final adjusted model. Animals sampled in years 2016 (OR = 4.3; 95% CI: 1.5 – 12.0), 2017 (OR = 3.5; 95% CI: 1.4 – 8.5) and 2019 (OR = 2.2; 95% CI: 1.0 – 4.7) had higher odds of infection compared to the reference year 2020 (p = 0.01). The estimated OR for animals sampled in 2018 (OR = 1.9; 95% CI: 0.8 – 4.5) was similar in magnitude and direction to those sampled in 2019; however, the association was not significantly different from the reference year of 2020. Species of rhinoceros, age, and sex were included in the final, combined-species model to adjust for species and demographic factors (age and sex) but were not statistically significant.

Each of the significant variables was explored for effect modification by species, age and sex by inserting a corresponding interaction term into the model. Table S3.3.2 shows a similar combined-species model (to that shown in Table S3.3.1), with a combined reference category for years 2019 and 2020. The sampling years 2019 and 2020 were combined for purposes of evaluating statistical interaction between species and year due to small number of black rhinoceros in these categories and the potential for missing covariate patterns in that group. Table S3.3.3. shows the same model as presented in Table S3.3.2, but includes a species*year

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interaction term. Significant interaction between species and year of sampling with *M. bovis* infection was identified ((p =0.038; Table S3.3.3). We report associations for all species combined, as well as for black rhinoceros and white rhinoceros, separately (see Main Text, Figure 2, and Supplementary information S3.4, Table S3.4.1). Additional effect modification by species or other factors was not identified, and other variable combinations did not improve the fit of the model or indicate additional sources of confounding.

Table S3.3.1. Final combined-species multivariable model of factors associated with *M. bovis* infection in black and white rhinoceros in Kruger National Park, South Africa (2016 – 2020) (n = 433)

Variable	Beta coefficient	SE	Odds ratio (95% CI)	p
Species				
White rhinoceros (*Ceratotherium simum*)	0.391	0.299	1.48 (0.82 – 2.66)	0.18
Black rhinoceros (*Diceros bicornis*)			Reference	
Sex				
Female	-0.134	0.271	0.87 (0.51 – 1.49)	0.62
Male			Reference	
Age				
Adult	0.567	0.402	1.76 (0.80 – 3.88)	0.16
Subadult	0.098	0.445	1.10 (0.46 – 2.64)	
Calf			Reference	
Sampling year				
2016	1.482	0.522	4.40 (1.58 – 12.26)	0.02
2017	1.208	0.451	3.35 (1.38 – 8.11)	
2018	0.547	0.457	1.73 (0.71 – 4.23)	
2019	0.766	0.387	2.15 (1.01 – 4.59)	
2020			Reference	
Number of buffaloes within home range (radius = 17.25 km)[i]	0.557	0.233	1.75 (1.11 – 2.75)	0.01

[i]*Model fit parameters and odds ratios based on log transformation of the associated variable; null model AIC = 424.35; fitted model AIC = 416.99*

Table S3.3.2. Combined-species multivariable model of factors associated with *M. bovis* infection in black and white rhinoceros in Kruger National Park, South Africa (2016 – 2020) (n = 433), with combined reference category for year

Variable	Beta coefficient	SE	Odds ratio (95% CI)	p
Species				0.218
White rhinoceros (*Ceratotherium simum*)	0.361	0.298	1.43 (0.80 – 2.57)	
Black rhinoceros (*Diceros bicornis*)			Reference	
Sex				0.876
Female	-0.042	0.265	0.96 (0.57 – 1.61)	
Male			Reference	
Age				0.226
Adult	0.561	0.400	1.75 (0.80 – 3.83)	
Subadult	0.194	0.442	1.21 (0.51 – 2.89)	
Calf			Reference	
Sampling year				0.066
2016	0.943	0.434	2.57 (1.10 – 6.01)	
2017	0.690	0.352	1.99 (1.00 – 3.97)	
2018	0.050	0.366	1.05 (0.51 – 2.15)	
2019 and 2020			Reference	
Number of buffaloes within home range (radius = 17.25 km)[i]	0.581	0.225	1.79 (1.15 – 2.78)	0.007

[i]*Model fit parameters and odds ratios based on log transformation of the associated variable; null model AIC = 423.22; fitted model AIC = 416.81*

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Table S3.3.3. Combined-species multivariable model of factors associated with *M. bovis* infection in black and white rhinoceros in Kruger National Park, South Africa (2016 – 2020) (n = 433), with combined reference category for year and a species*year interaction term

Variable	Beta coefficient	SE	Odds ratio (95% CI)	p
Species				0.218
White rhinoceros (*Ceratotherium simum*)	1.203	0.507	3.33 (1.23 – 8.99)	
Black rhinoceros (*Diceros bicornis*)			Reference	
Sex				0.476
Female	-0.196	0.274	0.82 (0.48 – 1.41)	
Male			Reference	
Age				0.271
Adult	0.568	0.406	1.77 (0.80 – 3.91)	
Subadult	0.257	0.446	1.29 (0.54 – 3.10)	
Calf			Reference	
Sampling year				0.066
2016	3.056	1.045	21.25 (2.74 – 164.62)	
2017	1.463	0.713	4.32 (1.07 – 17.48)	
2018	1.348	0.676	3.85 (1.02 – 14.48)	
2019 and 2020			Reference	
Species * sampling year				0.038
white rhinoceros*2016	-2.572	1.159	0.08 (0.01 – 0.74)	
white rhinoceros*2017	-0.951	0.833	0.39 (0.08 – 1.98)	
white rhinoceros*2018	-1.855	0.821	0.16 (0.03 – 0.78)	
white rhinoceros*2019 & 2020			Reference	
Number of buffaloes within home range (radius = 17.25 km)[i]	-1.855	0.821	1.76 (1.12 – 2.74)	0.010

[i]*Model fit parameters and odds ratios based on log transformation of the associated variable; null model AIC = 423.22; fitted model AIC = 416.8*

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S3.4. Species-specific multivariable models of factors associated with *M. bovis* infection African rhinoceros in Kruger National Park, South Africa (2016 – 2020).

Table S3.4.1.

Variable	Beta coefficient	SE[iii]	Odds ratio (95% CI)	P
White rhinoceros (*Ceratotherium simum*) (n = 314)				
Sex				0.31
Female	-0.326	0.322	0.72 (0.38 – 1.36)	
Male			Reference	
Age				0.38
Adult	0.465	0.446	1.59 (0.66 – 3.82)	
Subadult	0.080	0.485	1.08 (0.42 – 2.80)	
Calf			Reference	
Sampling year				0.15
2016	0.928	0.578	2.53 (0.81 – 7.86)	
2017	0.926	0.504	2.52 (0.94 – 6.78)	
2018	-0.125	0.540	0.88 (0.31 – 2.55)	
2019	0.656	0.416	1.93 (0.85 – 4.35)	
2020			Reference	
Number of nearby buffalo herds[i,ii]	0.569	0.257	1.77 (1.07 – 2.92)	0.02
Black rhinoceros (*Diceros bicornis*) (n = 119)				
Sex				0.82
Female	-0.142	0.613	0.87 (0.26 – 2.88)	
Male			Reference	
Age				0.42
Adult	1.264	1.117	3.54 (0.40 – 31.59)	
Subadult	0.893	1.247	2.44 (0.21 – 28.11)	
Calf			Reference	
Sampling year				0.01
2016	2.896	1.103	18.11 (2.09 – 157.15)	
2017	1.472	0.725	4.36 (0.98 – 19.41)	
2018	1.527	0.725	4.60 (1.11 – 19.05)	
2019 and 2020 (combined)			Reference	
Number of nearby buffalo herds[i,ii]	0.239	0.582	1.27 (0.41 – 3.97)	0.68

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Addendum C

Supplementary material: Chapter 4

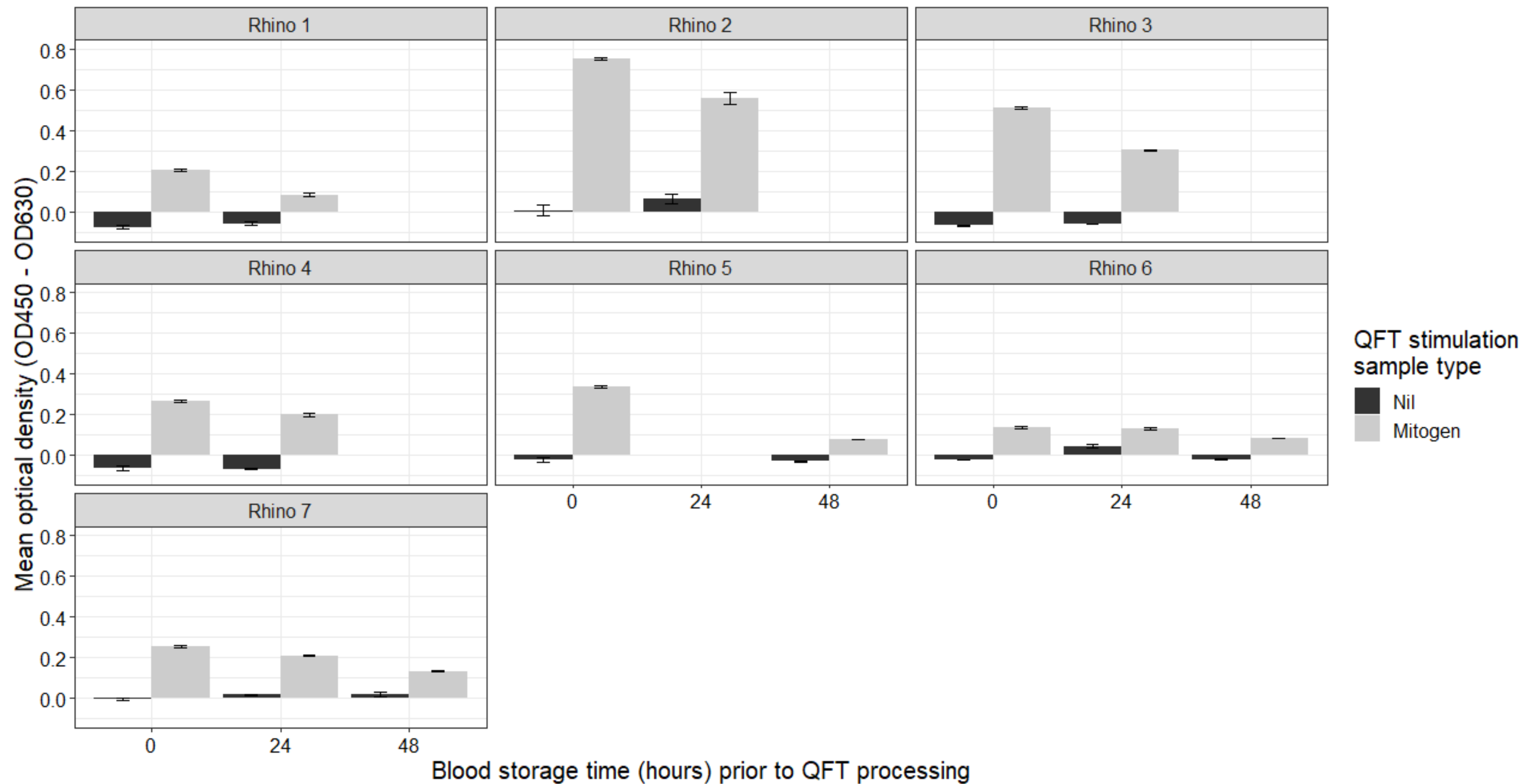

Figure S4.1. Mean optical density (OD) values of Nil and mitogen plasma samples corresponding to different rhinoceros whole blood storage time points prior to processing for the QuantiFERON Gold In-Tube (QFT) equine interferon-gamma release assay (Chileshe et al., 2019b). All Nil samples were diluted 1:2 prior to measurement. Mitogen plasma samples from Rhinos 2 and 3 were diluted 1:100, and mitogen plasma samples for all other rhinos (Rhinos 1, and 4 – 6) were diluted 1:1000 to achieve OD values within the linear range of the assay. Error bars indicate standard deviation of duplicate wells for each sample.

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Addendum D

Published manuscripts (Chapters 2-4)

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frontiers in Veterinary Science

REVIEW
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Epidemiology of Tuberculosis in Multi-Host Wildlife Systems: Implications for Black (*Diceros bicornis*) and White (*Ceratotherium simum*) Rhinoceros

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Cases of tuberculosis (TB) resulting from infection with *Mycobacterium tuberculosis* complex (MTBC) have been recorded in captive white (*Ceratotherium simum*) and black (*Diceros bicornis*) rhinoceros. More recently, cases have been documented in free-ranging populations of both species in bovine tuberculosis (bTB) endemic areas of South Africa. There is limited information on risk factors and transmission patterns for MTBC infections in African rhinoceros, however, extrapolation from literature on MTBC infections in other species and multi-host systems provides a foundation for understanding TB epidemiology in rhinoceros species. Current diagnostic tests include blood-based immunoassays but distinguishing between subclinical and active infections remains challenging due to the lack of diagnostic techniques. In other species, demographic risk factors for MTBC infection include sex and age, where males and adults are generally at higher risk than females and younger individuals. Limited available historical information reflects similar age- and sex-associated patterns for TB in captive black and white rhinoceros, with more reports of MTBC-associated disease in black rhinoceros than in white rhinoceros. The degree of MTBC exposure in susceptible wildlife depends on their level of interaction, either directly with other infected individuals or indirectly through MTBC contaminated environments, which is dependent on the presence and abundance of infected reservoir hosts and the amount of MTBC shed in their excreta. Captive African rhinoceros have shown evidence of MTBC shedding, and although infection levels are low in free-ranging rhinoceros, there is a risk for intraspecies transmission. Free-ranging rhinoceros in bTB endemic areas may be exposed to MTBC from other infected host species, such as the African buffalo (*Syncerus caffer*) and greater kudu (*Tragelaphus strepsiceros*), through shared environmental niches, and resource co-utilization. This review describes current knowledge and information gaps regarding the epidemiology of TB in African rhinoceros.

Keywords: epidemiology, *Mycobacterium bovis*, rhinoceros, TB risk, TB transmission, tuberculosis

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INTRODUCTION

Tuberculosis (TB) is a chronic infectious disease that affects a broad range of host species (1). It is caused by members of a group of closely related pathogenic mycobacteria known as the *Mycobacterium tuberculosis* complex (MTBC) (1). Bovine tuberculosis (bTB) is caused by *Mycobacterium bovis* (*M. bovis*), which is known to infect livestock as well as captive and free-ranging wildlife species (2, 3). For most wildlife, however, little is known about susceptibility to MTBC infection, pathogenesis, and its impact on affected populations.

Rhinoceros are iconic species which are under threat due to habitat destruction and heavy poaching pressure. Cases of TB resulting from infection with *M. tuberculosis* and *M. bovis* have been recorded in captive, semi-captive (maintained in private reserves and more intensively managed), and free-ranging rhinoceros worldwide (4–19); TB was implicated as the cause of death in some of these cases.

Kruger National Park (KNP) and Hluhluwe–iMfolozi Park (HiP) are home to large populations of free-ranging black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceros in South Africa. As of 2016, HiP housed ∼1,500 white rhinoceros and 360 black rhinoceros (20). In 2017, the Kruger National Park contained ∼5,150 white rhinoceros and 500 black rhinoceros (21), at which time the global populations totaled 20,300 white rhinoceros and 5,200 black rhinoceros (22). The HiP and KNP populations have been and continue to be central to the "Integrated Strategic Management of Rhinoceros" plan, introduced by the South African Department of Environmental Affairs (23, 24). Part of this strategy relies on the translocation of rhinoceros from the feeder populations in these parks to newly developing rhinoceros safeguarding strongholds around the country. However, KNP and HiP are endemic for bTB, and these rhinoceros populations share habitat ranges and various resources with *M. bovis*–infected wildlife (over 20 species in KNP), including important bTB maintenance hosts such as African buffaloes (*Syncerus caffer*) (2), and greater kudu (*Tragelaphus strepsiceros*) (25–27). The identification of disease in free-ranging wildlife is often challenging due to limited resources and access to these populations for diagnostic testing, and it was only with the increase in poaching and associated veterinary interventions that evidence of MTBC infection in white and black rhinoceros in KNP was discovered (9, 19).

Although *M. bovis* and *M. tuberculosis* have not been considered an immediate threat to the world's African rhinoceros populations, the potential impact of these pathogens on their health and conservation is largely unknown. Because bTB is a World Organization for Animal Health (OIE) and nationally notifiable disease (1), animals with *M. bovis* infection are subject to regulatory requirements which limit their movements between populations; this hampers conservation efforts that are reliant on translocation of rhinoceros from bTB-endemic to bTB-free areas.

Since the discovery of *M. bovis* infection in the free-ranging rhinoceros populations in KNP (13, 23), knowledge gaps regarding the risk of MTBC infection, intra- and inter-species transmission, and disease progression in these species have become apparent. This review describes the current knowledge regarding TB in African rhinoceros and provides information on epidemiological aspects of this disease in other relevant species, especially free-ranging populations, to improve understanding of the disease and inform management strategies.

ASSESSMENT OF INFECTION RISK IN AFRICAN RHINOCEROS

With any infectious disease, the risk of becoming infected is based on an individual's susceptibility when exposed to an infectious dose of the etiological agent, as well as the likelihood of exposure to the pathogen (28). These factors provide a foundation for investigating infection risks and will be discussed as they pertain to *M. tuberculosis* and *M. bovis* infection in black and white rhinoceros.

Outcomes and Detection of MTBC Infections: Epidemiological Implications

MTBC infections are typically chronic and once disease occurs, usually progressive. In various human and animal hosts, infections have been observed to move between various stages in a dynamic host-pathogen interaction network, where clinical manifestations of the infection vary between stages (29–32). Following infection, the host's innate immune system may eliminate the mycobacteria. If the mycobacteria are not eliminated, T helper type-1 cell-mediated immune (CMI) responses develop and are followed by the T helper type-2 humoral immune response through B-lymphocyte activation and an increase in circulating antibodies (33–35). Immunological responses of the host play a key role in determining the outcome of infection, and in humans, these include latent, incipient and subclinical stages (30). These stages have not been clearly defined in animal hosts, but subclinical MTBC infections have been reported in a variety of species (36–39). Although controversial, there is speculation that latent infection of animals with *M. bovis* may also occur (35, 40). These complex and dynamic interactions between host and pathogen can lead to elimination of infection, or an asymptomatic stage in which the mycobacteria are either dormant or result in localized disease, or progression to active disease, which has been observed in a multitude of species (29, 30, 41, 42).

Although there is a paucity of information on outcomes of MTBC infection in rhinoceros, a hypothesized scenario is shown in **Figure 1**; however, further investigation is required to verify these stages. Most cases of TB in zoos have only been detected once disease is sufficiently advanced to detect clinical signs, resulting in the death of the rhinoceros either due to euthanasia or disease complications (**Supplementary Table 1**) (4–6, 17). However, a study involving three experimentally *M. bovis*-infected white rhinoceros, monitored serially over 2 years, suggests that although immunological responses could be detected, the animals appeared to contain, and potentially eliminate, the infection (43, 44). Similarly, in the limited cases of natural *M. bovis* infection in white rhinoceros, pathological lesions were localized in lymph nodes or other tissues (19). The outcome of infection appears to be more complex than simply

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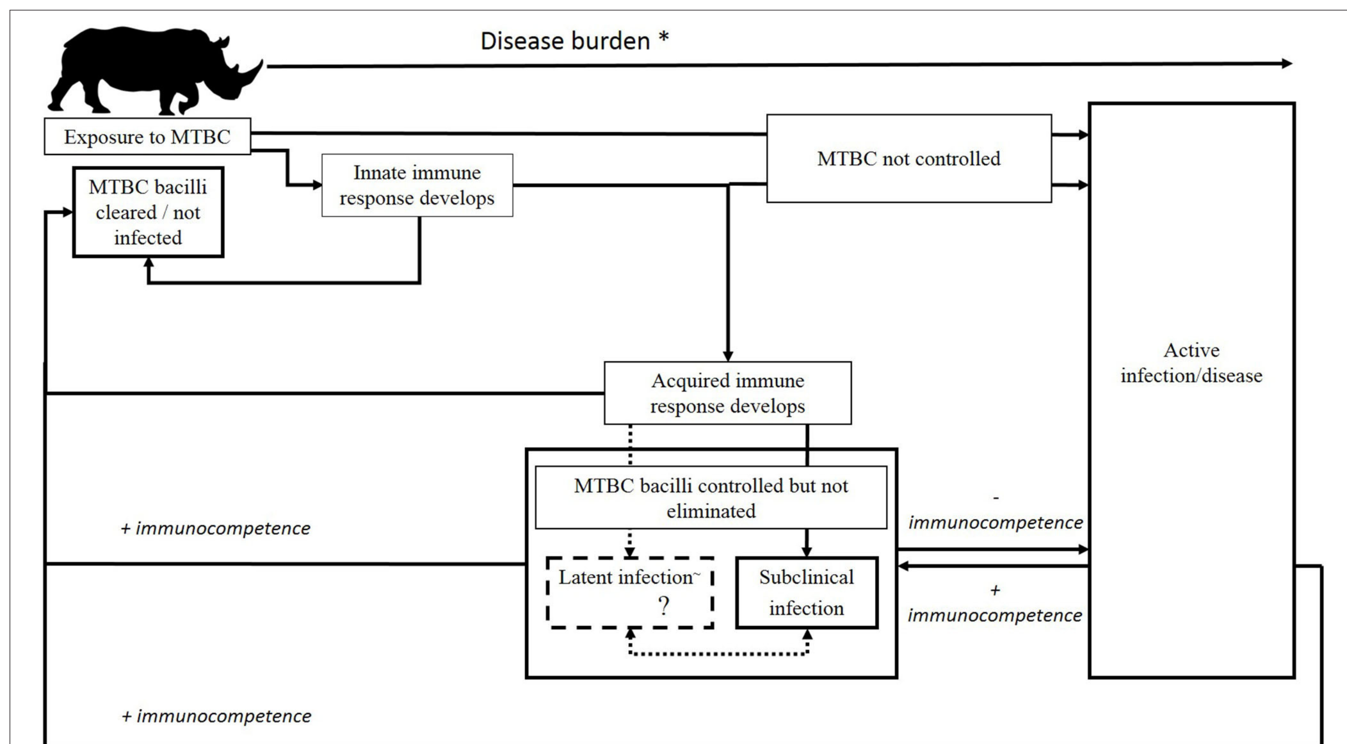

FIGURE 1 | Possible outcomes of *M. bovis* infection in African rhinoceros. After initial exposure, MTBC may be eliminated by the host's immune response, persist as a subclinical or latent~ infection, or progress to active infection/disease. Following the establishment of subclinical or latent infection, the host's immune response may clear the MTBC, or infection may persist in this form, either naturally progressing in a slow or rapid fashion to active tuberculosis, or cycling through subclinical and latent states, before development into symptomatic disease or eventual eradication of the infection by the host's adaptive immune response. *Rising disease burden implies an increase in abundance of TB and/or MTBC biomarkers, immunological changes characteristic of stage of infection, and increasing pathology, with a declining recovery prognosis. ~Although controversial, there is speculation that latent infection of animals with *M. bovis* may occur. More research is required.

a progression to disease, based on these observations as well as reports of immunological responses (without evidence of disease) in rhinoceros that have been exposed to other known TB cases (7, 13, 15). In addition, decreases in immunocompetence as a result of comorbidities, drought, capture/transport–induced stress, increased age, or other factors may be associated with greater susceptibility to disease as sequelae of acute infection or activation of subclinical infection in rhinoceros, although tools to identify these stages need to be developed.

In order to characterize the epidemiology of TB in a population, it is important to have accurate diagnostic methods that can distinguish between various stages of infection and disease, since animals in different stages of infection may present with altered levels of transmission risk (45). Risk factors associated with acquiring an infection may be different from those that increase the likelihood of disease progression, or the maintenance of a subclinical or latent infection (28, 30). This important distinction may have implications for understanding the epidemiology of TB and could impact subsequent management decisions.

In vivo and *in vitro* indirect detection methods for early MTBC infection primarily rely on the detection of TB-specific adaptive immune responses of the host, including the tuberculin skin test (TST) and MTBC antigen stimulated cytokine assays (46, 47). In addition, serological assays for the detection of host-specific antibodies to MTBC antigens have been useful for diagnosis of TB in certain animal species (48–50), although they are considered unreliable for TB diagnosis in humans (51). In rhinoceros, the TST is unreliable due to cross-reactivity with environmental mycobacteria (7, 13, 52); therefore, a white rhinoceros whole blood MTBC antigen-specific interferon-gamma release assay (IGRA) for *M. bovis* infection has recently been developed (44, 53). Serological assays for the detection of antigen-specific antibodies have also been shown to be useful for the diagnosis of MTBC infection in rhinoceros (15, 18). However, the use of these indirect immunological diagnostic assays alone may not distinguish between recent infection, latent/incipient/subclinical infection, and active disease.

One way to overcome the challenges posed by these indirect tests is to directly detect the pathogen by mycobacterial culture and nucleic acid amplification tests (NAATs) (54, 55). Mycobacterial culture and speciation are useful as both pre- and post-mortem diagnostic tests for MTBC infection. Ante-mortem samples obtained for culture include bronchoalveolar, tracheal and gastric lavages, as well as nasal and fecal swabs, although culture of tissue obtained during necropsy may be more sensitive for detection of bacilli. Although this method is highly specific, culture of ante-mortem samples has low sensitivity, which may

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be related to the site of infection and whether the individual is shedding at the time of sampling (56). For example, in a study evaluating shedding in three experimentally *M. bovis*-infected rhinoceros, only one of 36 tracheal lavage samples collected monthly over a 2-year period was *M. bovis* culture positive (43). In humans and recently in wildlife, mycobacterial culture has been supplemented with NAATs including the automated GeneXpert MTB/RIF Ultra qPCR assay (Ultra) (57, 58). This rapid ancillary test may enable the direct detection of MTBC DNA in some tissues (59), as well as animal respiratory samples (57). Regardless, the direct detection of MTBC organisms alone also does not provide information on the host's stage of infection or disease. The presence and classification of lesions detected by macroscopic and microscopic examination provides important information for staging disease, although this is primarily used post-mortem (43, 60).

Molecular methods such as spoligotyping, mycobacterial interspersed repetitive unit-variable number of tandem repeat (MIRU-VNTR) genotyping, or whole genome sequencing of *M. bovis* isolates may be useful not just for diagnosis, but also for tracing the origin of MTBC infection in multi-host systems (26, 61–64). The application of these techniques in rhinoceros and other free-ranging wildlife, though useful, is challenging due to limited samples. Nonetheless, these techniques have been employed to investigate the distribution and transmission of MTBC strains in some wildlife multi-host systems (63), including between brushtail-possums (*Trichosurus volpecula*) (65), badgers (65, 66), deer (67) or African buffaloes (68) and cattle at the livestock/wildlife interface (65–68). Therefore, use of both direct and indirect detection methods should be included in investigations of transmission in rhinoceros.

Investigating Susceptibility of Black and White Rhinoceros to Infection With *M. tuberculosis* and *M. bovis*

As a result of the popularity of rhinoceros for zoological exhibition, they have historically been globally distributed through importation. Reports of TB in captive rhinoceros in zoological gardens worldwide date back to the late 1800's. Historical cases of bTB and TB in black and white rhinoceros are summarized in **Figure 2**. While TB is still considered a rare occurrence in domestic perissodactyls (69), these cases provide evidence for susceptibility of black and white rhinoceros.

According to limited information, most TB cases have been recorded in black rhinoceros, with an apparent paucity of cases identified in white rhinoceros (4–19). This observation may be related to differences in numbers, demographics, housing or management of these species in zoological collections, exposure to other infected animals, employees or visitors, differences in species-specific susceptibility to the different pathogens, or the impact of individual or species-specific co-morbidities on immunocompetence and TB susceptibility. Black rhinoceros in captivity are known to suffer from a variety of syndromes (70); afflicted individuals may have compromised immunity that increases their susceptibility to TB, which could explain the apparent higher prevalence in this species compared to white rhinoceros.

Most reports of TB in captive rhinoceros have been caused by infection with *M. tuberculosis*, with only a few caused by *M. bovis*. This is hypothesized to be a result of a high level of exposure of captive animals to *M. tuberculosis* through direct or indirect interactions with humans (especially in high TB burden countries), while in captivity or during exportation (4) (**Supplementary Table 1**). Another explanation might be differences in virulence of *M. tuberculosis* and *M. bovis* in rhinoceros, although limited studies suggest that *M. bovis* has greater virulence in other species, such as mice and goats, compared to *M. tuberculosis* (71, 72).

The susceptibility of white rhinoceros to *M. bovis* was studied in experimentally infected animals (43, 44). The results confirmed susceptibility to infection and potential to shed bacilli, albeit based on the detection of viable *M. bovis* in only one of 36 tracheal lavage samples collected over the course of the study. None of the individuals developed clinical signs or evidence of disease based on gross and histological examination, although *M. bovis* DNA was detected by PCR in lung tissues of two animals at necropsy (43). The immune response kinetics and pathological findings suggested that the rhinoceros were able to contain and possibly clear *M. bovis* infection (44). This observation is consistent with the historical lack of TB cases in white rhinoceros, which may be due to the ability to contain and clear the infection before the onset of disease. However, it should be noted that the response of rhinoceros to experimental inoculation in this study may not reflect naturally occurring infection, which could occur through one or more exposure events over time, each with variable numbers of MTBC bacilli. Additionally, the individuals in this study were not subject to stressful conditions and were young (4–7 years old); all individuals had adapted to living in managed care by the time of initial infection, and were not exposed to the seasonal variations in food availability that they might have been if free-ranging. It is unknown whether the lack of disease development in these white rhinoceros was a consequence of low susceptibility to disease resulting from *M. bovis* infection, or the conditions associated with the experimental infection. In contrast, a naturally *M. bovis* infected 29-year-old white rhinoceros in a zoo developed weight loss, cough and nasal discharge and succumbed to the infection (13), demonstrating that this species can develop disease.

In contrast to white rhinoceros, evidence of TB disease in black rhinoceros has been reported more frequently. An elderly (estimated 35–40 years old) black rhinoceros, euthanized due to loss of condition, had small non-encapsulated pulmonary granulomas associated with *M. bovis* infection (17). Similarly, the free-ranging black rhinoceros in KNP, infected with *M. bovis*, had evidence of significant pulmonary pathological changes (9). Interestingly, the first case was in an elderly animal and the second rhinoceros case was discovered after a prolonged period of drought. However, cases of *M. bovis*-associated disease in zoo black rhinoceros have been reported in animals that were ∼20–25 years old (7). In addition, post-mortem pulmonary changes consistent with *M. tuberculosis* disease were observed in multiple zoo black rhinoceros aged 13–33 years old (14, 15, 18). The

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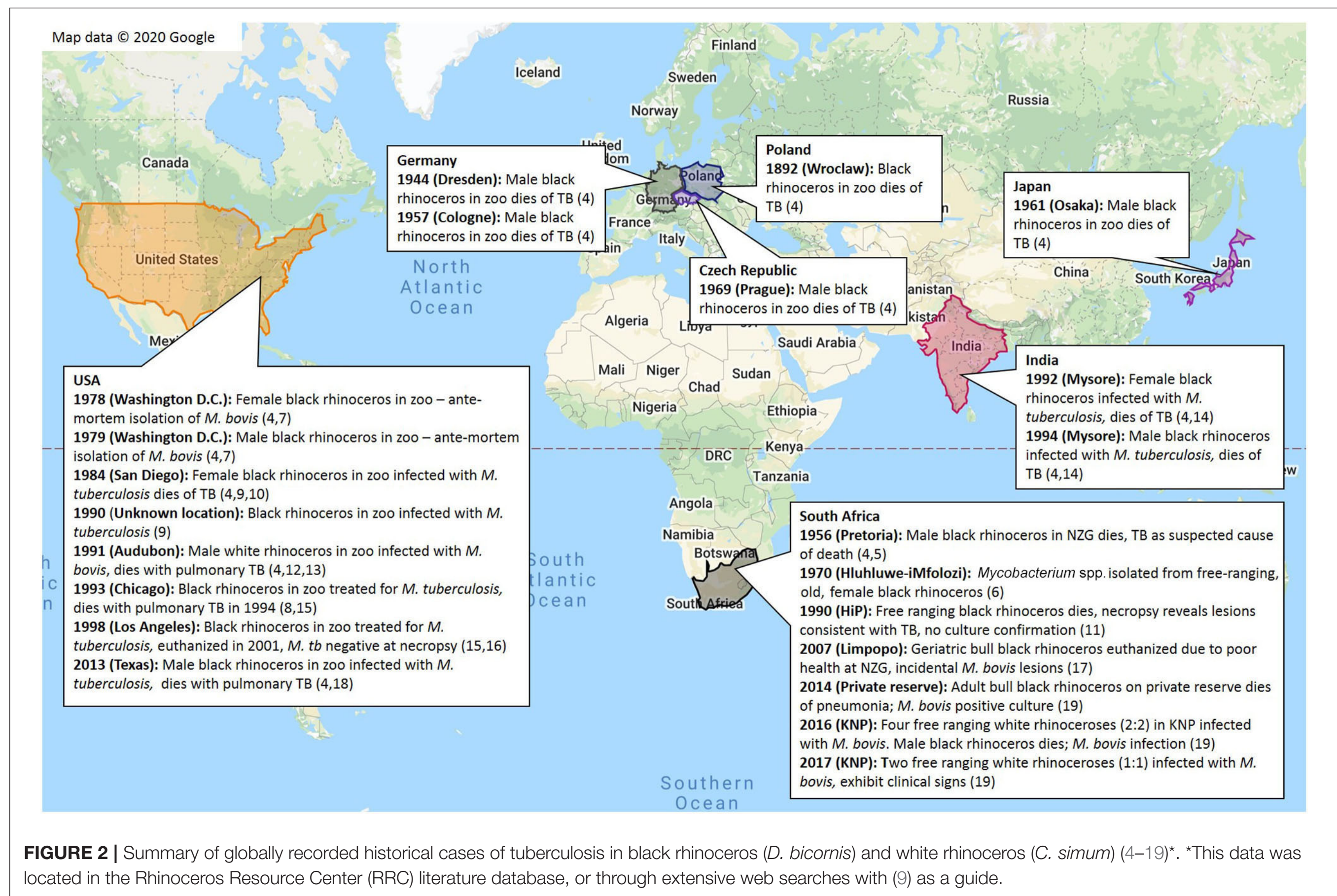

FIGURE 2 | Summary of globally recorded historical cases of tuberculosis in black rhinoceros (*D. bicornis*) and white rhinoceros (*C. simum*) (4–19)*. *This data was located in the Rhinoceros Resource Center (RRC) literature database, or through extensive web searches with (9) as a guide.

reports of clinical signs and presence of changes post-mortem in black rhinoceros infected with *M. bovis* or *M. tuberculosis* suggests this species may be more prone to TB disease.

Assessing Risk Factors for Infection and Transmission Patterns of MTBC in African Rhinoceros Populations

Demographic Risk Factors

There is limited literature that characterizes risk factors of MTBC exposure and transmission patterns in free-ranging black and white rhinoceros populations, likely a result of the logistical and technical difficulties associated with disease surveillance and sporadic cases in these animals. Therefore, extrapolation from literature on TB in other species and multi-host systems may aid in understanding the epidemiology of MTBC in African rhinoceros populations. Demographic risk factors for MTBC infection, such as sex and age, have been described in humans, mice, cattle, and limited species of wildlife (48, 49, 73–82). Results from these studies may inform hypotheses regarding demographic patterns of MTBC infection in rhinoceros.

In many TB-susceptible species, sex is considered a risk factor for infection (48, 49, 73–78). In humans, the global male to female ratio for individuals that develop TB is 2:1 (73). While this has partially been due to socioeconomic and cultural barriers in access to healthcare (74), inherent biological characteristics are also implicated. Sex-based differences in susceptibility are usually observed in adults, but not in children or adolescents (83). This suggests that the relative male: female difference in susceptibility is related to the effect of steroid sex hormones and their regulatory activities on immune cells (84).

Both testosterone and progesterone are immunosuppressive. These hormones impair macrophage activation and may increase TB susceptibility (85, 86). In contrast, estrogen is a pro-inflammatory mediator that stimulates the production of tumor necrosis factor alpha (TNF-α) (87), and interacts with the IFN-γ promoter (88). In mice, increased susceptibility to TB has been observed in post-adolescent males relative to post-adolescent females, with this difference partially mitigated by castration (84).

In various wildlife species, studies have shown a higher frequency of MTBC infection in males, which could be linked to hormonal differences, but behavioral differences may also play a role. In a cohort of free-ranging African elephants (*Loxodonta africana*) tested in KNP, overall TB seroprevalence was higher in males than in females (48). Another study reported a higher risk for both bTB infection and disease in male badgers (*Meles meles*) than in females (75). An epidemiological study of white-tailed deer (*Odocoileus virginianus*) in Michigan also reported a higher odds of being bTB test-positive in males compared to females (76). This study also reported a dramatic effect of sex

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on the association of increasing age with positive TB test status. In fawns and yearlings, no significant difference in TB incidence between males and females was found; however, in age groups of 2 years and above, males were increasingly more likely to test positive for TB than females of the same age class. This is a similar trend to what is observed in humans, and may be due either to sex-based hormonal differences, or to the contrasting social/reproductive behavior between mature males and females (76, 89). Therefore, it may be difficult to separate risk factors associated with hormonal and behavioral differences in adults.

Most wildlife epidemiological studies report a higher risk of bTB in males than in females; however, this association does not strictly hold true for all species. Studies of different populations of wild boar, for example, have yielded conflicting results with respect to the association between sex and bTB risk. One study on wild boars in Portugal (82) reported a significantly higher bTB incidence in female than males in all age groups. In a different wild boar population in Spain (81), studies showed a significantly higher bTB prevalence in males. Studies of wild boar populations in France (77) and Italy (78) reported no significant association between sex and TB risk. Variable findings from different populations of the same species illustrate the complexity of determining sex-associated bTB risk. Hormone-derived TB susceptibility of a species may be largely conserved between different multi-host systems; however, sex may also be a mediator of pathogen exposure due to sex-related differences in social, reproductive, and territorial behavior as well as movement patterns. The degree to which such sex-related factors alter rhinoceros' exposure to MTBC may depend on the unique characteristics of dispersal and transmission in that particular host system (89).

Most historical reports of TB in captive rhinoceros have occurred in black rhinoceros males (**Figure 2** and **Supplementary Table 1**). It is unknown whether this observation represents a true species and sex predilection for TB, or whether reports are biased for other reasons, such as the skewed natal sex ratios in captive black rhinoceros (90), or a disproportionate number of black rhinoceros and/ or males kept in zoos. However, available records show a preference for import and exhibition of female white rhinoceros over males, and no substantial preferential import of male vs. female black rhinoceros for exhibition during the twentieth century (4). Records for USA, Mexico, and Australia show a near-equivalent sex ratio of black rhinoceros currently kept in captivity (91, 92), and the sex ratio of white rhinoceros in captivity in Canada, the USA, Mexico, Chile, and Singapore is substantially skewed toward females (93). These records also indicate that a higher number of white rhinoceros are kept in captivity compared to black rhinoceros; there are currently 278 white rhinoceros in captivity in Canada, the USA, Mexico, Chile, and Singapore (93), compared to 96 black rhinoceros in the USA, Mexico, and Australia (91, 92). These data do not support an apparent bias toward males or black rhinoceros in captivity, which suggests that reports (**Figure 2** and **Supplementary Table 1**) may reflect a true increased risk for TB in these groups. While the absolute historical numbers of rhinoceros housed in captivity globally are unknown, and therefore cannot be used to draw conclusions on TB risk in rhinoceros, these observations provide avenues for further investigation into species and sex-specific susceptibility.

In humans, TB occurs in individuals of all ages, although the highest burden is in men past adolescence ($\geq$15 years old) (73). Human susceptibility to TB shows an increase with age, which may be due to age-related effects on the immune system or possibly the outcome of multiple exposures over time (79). A similar age-related bTB trend has been observed in cattle, with a peak in incidence after 12 months of age (80). Adult warthogs (*Phacochoerus africanus*) and African elephants (>25 years old) in bTB endemic regions also showed a higher seroprevalence than their younger counterparts (48, 49). Increasing age had the greatest effect on TB disease risk in a meerkat population in South Africa (94).

While most studies in wildlife show increasing bTB prevalence with age, studies conducted in the Iberian Peninsula report higher prevalence in juvenile wild boar than adults in high-prevalence multi-host systems (81, 95). This could be due to higher susceptibility in juveniles compared to fully-grown adults in this species, possibly related to immunological maturity, or age-related changes in behavior that result in increased exposure to the pathogen. Interestingly, historically reported cases of TB in captive African rhinoceros appear to have occurred exclusively in adults (**Figure 2** and **Supplementary Table 1**). These observations suggest that infection can take years to manifest in these species (18), or that there is an increase in susceptibility and/or repeat exposures with age. Based on other species, it is likely that both sex and age are risk factors for MTBC exposure and infection in black and white rhinoceros.

Transmission of *M. bovis* in Multi-Host Systems

Investigation of TB transmission has been limited in free-ranging rhinoceros until recently because of a lack of diagnostic assays and paucity of samples. Therefore, characterization of transmission depends largely on extrapolation using patterns observed in other multi-host systems. Some of the predictors for persistence and transmission of a pathogen within a multi-host system appear to be related to patterns of movement, migration, and different modes of interactions between host species (28, 96, 97). Importantly, the presence of an infected reservoir species in the system has been shown to increase the risk of spill-over to other susceptible hosts. Wildlife reservoir hosts for bTB are present globally, including African buffaloes in South Africa (27) [and possibly other areas in Africa where the species occurs (98, 99)], greater kudu in South Africa (27), brush-tailed possums (*T. volpecula*) in New Zealand (100), European wild boar (*Sus scrofa*), red deer and fallow deer (*Dama dama*) in Spain (95), white-tailed deer (*O. virginianus*) in the USA (101), elk (*Cervus canadensis*) (102) and American bison (*Bison bison*) (103) in Canada, and European badgers (*M. meles*) in the United Kingdom (97). In wildlife populations with *M. bovis*, there are numerous examples of intra- and inter-species transmission (27, 104–107). Direct intra-species *M. bovis* transmission can occur through respiratory droplets in social species like African buffaloes (107) or through antagonistic or territorial behaviors like those that occur between white-tailed deer (76).

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The mechanism of inter-species *M. bovis* transmission to herbivores is largely unknown but has been attributed to indirect interactions through contamination of pastures, feed, or browse with MTBC shed by infected hosts. Various studies have demonstrated that infected hosts shed *M. bovis* into the environment (106, 108). In one study, intranasal administration of *M. bovis* to calves resulted in intermittent shedding for up to 38 weeks (108). A European study of infected wild boar and red deer demonstrated shedding by oronasal, bronchial-alveolar, fecal and urinary routes (106). In that study, 83% of wild ungulates with bTB had mycobacteria isolated in at least one type of excretion, which suggests a high level of shedding into the environment. In a study in Spain, interactions between four different species (cattle, domestic pigs, red deer, and wild boar) in a bTB endemic system found that although there was a low percentage of direct interactions between these species, there was a high percentage of indirect interactions over the 3-day time frame investigated, suggesting a high risk of indirect transmission (109). A similar study in France detected a high frequency of indirect interactions between badgers, wild boar, and red deer at waterholes and baited locations (110). Therefore, environmental contamination may present risks for transmission to susceptible hosts sharing the same resources as infected individuals. However, in addition to the presence of an infected host that is shedding, the pathogen must remain viable in the environment for enough time to encounter the susceptible host.

Routes of Transmission of *M. bovis* to Rhinoceros in Sub-Saharan Africa

Predicting routes of transmission of MTBC requires an understanding of patterns of shedding, movement patterns, social behavior, and resource utilization of susceptible hosts in relation to infected hosts, and the persistence of the pathogen in a contaminated environment.

Wildlife Maintenance Hosts as a Source of M. bovis Infection in African Rhinoceros in Sub-Saharan Africa

Domestic livestock (such as cattle) are implicated as bTB maintenance hosts where spillover into wildlife occurs (25, 111). However, since KNP and HiP have perimeter fencing in place to prevent disease interactions between wildlife and cattle, transmission from livestock is unlikely to be a major mode of *M. bovis* infection acquisition in rhinoceros in these areas. In South Africa, the African buffalo is a recognized bTB reservoir host that is implicated in the spill-over of *M. bovis* to other susceptible hosts, both directly, and indirectly through shedding into the environment (27, 68, 105, 112). There is evidence that greater kudu can also be maintenance hosts in the Kruger National Park and possibly in other bTB endemic areas where the species occurs (27). Since these large herbivorous hosts are often found in similar ranges and utilize the same resources as white and black rhinoceros, interactions between these species are likely to occur. These interactions may be a potential route for transmission of *M. bovis* to African rhinoceros.

According to recent biodiversity statistics, KNP African rhinoceros share the park with an estimated 37,130 African buffaloes (21). Similarly, HiP has a buffalo population of ∼3,500 (113). African buffaloes are socially organized into herds, which can be as large as 1,000 individuals (112, 114). A study that investigated seasonal movements and habitat use by these animals revealed home ranges varying between 73 and 601 km^2 (115). Due to the size of their home ranges, interactions with other species (including rhinoceros), particularly at aggregation points such as water sources or shared feeding areas, are likely to occur at a relatively regular frequency. Dispersal events, though less frequent in adult females, occur in adults of both sexes of buffalo (116). Natal dispersal events occur at least once in most adult male buffaloes, and can be driven in both sexes by seasonal (water and nutrient) or social resource limitations (117). Additionally, bTB disease may influence individual health and body condition in buffaloes, which could indirectly impact dispersal events (118). The resulting frequency of dispersal events may influence the probability of pathogen exposure opportunities resulting in spillover from buffaloes to other susceptible species, including white and black rhinoceros in bTB endemic areas.

Investigation of preferred vegetation and habitat of buffaloes showed the strongest association with open to closed herbaceous vegetation on temporarily flooded land, closed shrubs, open shrubs or with 40–65% crown cover (115). The white rhinoceros, like the African buffalo, is a grazing species (119, 120). Their vegetation preference closely mirrors that of buffaloes. In wet months, white rhinoceros may concentrate their grazing in the short grass-dominated grasslands, while in the dry seasons, they move to tall grass grasslands, with a general preference for shaded grasses. Thickets are generally rejected in favor of open grassland vegetation. The black rhinoceros is a browsing species, and their vegetation preference has less in common with that of African buffaloes. They tend to associate closely with thickets (closed shrubland or low forest areas) for access to food (120). For this reason, pathogen exposure interactions with buffaloes due to aggregation at shared feeding areas may be more likely to occur in white rhinoceros than in black rhinoceros.

Available statistics indicate that there are between 11,200 and 17,300 greater kudu in KNP (21). For HiP, a recent estimate of the greater kudu population was not found. The greater kudu is a browsing species of antelope that is socially organized into small bachelor, cow, or mixed herds, typically of fewer than ten individuals (121–123). The home ranges of these herds are typically small and stable, and male home ranges often overlap; the greater kudu social system appears to be based on absolute social dominance (according to age) and territoriality is not evident in this species. Due to their small and stable home ranges, interactions with other species (even indirect) are likely to be less frequent than those observed in buffaloes, who range more widely. However, black rhinoceros share their vegetation preference of thickets or more woody, covered vegetation with this species (124); as a result, indirect interactions with infected kudu (e.g., via shedding of *M. bovis* through fistulated lymph nodes in kudu leading to contamination of vegetation during browsing) may be an important mode of bTB transmission to black rhinoceros. Buffaloes, greater kudus, black and white rhinoceros share water pans, which may also

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increase the frequency of interactions within and between these species (125–128).

Overall, the wide ranges traversed by buffaloes on a seasonal basis and the potential for contamination of browse by infected kudu, coupled with evidence supporting their integral roles as maintenance hosts for bTB, support the potential for *M. bovis* transmission to white and black rhinoceros (115, 129). While overlapping vegetation preference, mud wallow usage, and ranges may be important infection predictors, indirect transmission of *M. bovis* from maintenance hosts may not be the only risk factor for infection of rhinoceros. Risk factors for infection should be considered as part of a multi-dimensional network, with the potential for transmission from other infected species, or possibly intra-species transmission.

Intra-Species Transmission of MTBC in Rhinoceros

Initial *M. bovis* infection in free-ranging rhinoceros in KNP was likely a result of spread from African buffaloes or other infected wildlife species, since the strains of *M. bovis* isolated from rhinoceros cases were the same as those identified in other KNP wildlife, based on comparison of spoligotypes in different studies (19, 64). However, it is unclear whether these infections were the immediate result of inter- or intra-species transmission in rhinoceros. Although rhinoceros have been translocated extensively, it is interesting that the only reported cases of bTB in rhinoceros in South Africa are in animals that originated from or spent time in parks with *M. bovis*-infected reservoir hosts (9, 17, 19). Therefore, further investigation is needed to determine if there is a risk of intra-species spread in rhinoceros.

As with inter-species transmission, the risk of intra-species transmission is dependent on whether the infected host is shedding MTBC, and the frequency of interactions between shedding and susceptible individuals, either directly or indirectly through utilization of shared resources. Evidence suggests that rhinoceros can shed MTBC into their environment in respiratory secretions, or at least that mycobacteria are present in the respiratory system of infected individuals (43, 130). Necropsies of two black rhinoceros in an Indian zoo revealed the presence of acid-fast organisms and large volumes of purulent material in the lungs (14). One of these rhinoceroses was sneezing and had a yellow muco-purulent nasal discharge in the days before its death. *M. tuberculosis* has also been isolated from nasal secretions from an infected black rhinoceros in a zoo which had diagnosed TB in several different species of animals (16). In addition, *M. tuberculosis* was isolated from a gastric lavage sample of a captive black rhinoceros with pulmonary disease, which suggests that like in humans, infected material may be coughed up and swallowed, leading to potential shedding of mycobacteria in feces (8, 15). These observations suggest the possibility that infected rhinoceros may transmit MTBC in secretions, presenting a risk for spread to other animals, and possibly humans, that are in close prolonged contact, such as in a zoo setting.

In free-ranging African rhinoceros, characteristics of social organization may inform the frequency of interactions between shedding and susceptible individuals. Adult female rhinoceros tend to occupy home ranges of up to 70 km^2, whereas adult bulls are often territorial, and occupy ranges up to 40 km^2 with little to no overlap, although young bulls may share their territory (131, 132). Young adult females generally range more widely than males, then settle into a similar, smaller home range to have their first calf (131). Home ranges are typically based on permanent water sources and food availability. African rhinoceros may move beyond their usual home ranges during dry periods and peak mating months (124, 133). Although there are some cases in which social groups have been observed in black rhinoceros (134), they tend to be more solitary. In general, cohesive social groups of white rhinoceros are mostly pairs—these can be adolescent-adolescent, cow-adolescent, cow-cow, and cow-calf pairs (132). However, social groups of up to ten individuals have been observed in white rhinoceros in the KNP (134). As density of white rhinoceros in a home range increases, the range occupied by individual cows or territorial bulls decreases. Groups of up to four adult cows with their offspring generally have smaller home ranges than solitary cows (132).

Behavioral characteristics of rhinoceros may also influence the risk of intra-species transmission of pathogens. Territorial behavior is prominent in adult bulls, with frequent olfactory territorial marking, or urine "spraying." Additionally, both defecation and urination are ritualized in territorial bulls, using specific locations, called "middens," scattered around the territory (131, 133, 135). Therefore, if *M. bovis* is excreted in feces, like in wild boar and red deer (106), middens might serve as a contaminated site where MTBC bacteria persist. In both black and white rhinoceros, mud wallowing is a behavior practiced more frequently during summer, and in the heat of the day, but can occur at any time (133). Therefore, shared use of wallows may be a potential source of intra-species transmission of *M. bovis* excreted in respiratory secretions or feces.

In summary, both direct and indirect interactions occur between individual rhinoceros, with direct contacts likely occurring at a higher frequency in white rhinoceros. Social exchanges, as well as overlap of ranges, and shared utilization of water sources, middens, and mud wallows are likely to result in indirect interfaces that carry potential for pathogen transmission. However, the apparent low prevalence and lack of disseminated disease in affected free-ranging populations of rhinoceros suggest that the risk of intra-species transmission of MTBC is lower than in captive rhinoceros (19), due to differences in duration and frequency of contact.

Environmental Contamination as a Route for Indirect Transmission of M. bovis

For indirect transmission of bTB to occur, an area, shared by the recipient host, must be contaminated with MTBC by an infected individual (109). Several studies have successfully isolated pathogenic mycobacterial DNA from various environmental substrates, including water, soil, sediments, and grass; this finding supports the possibility of a *M. bovis*-contaminated environment as a source of exposure for rhinoceros (136). Recipient host exposure risk likely increases with an increase in the mycobacterial load shed by infected hosts into the environment.

Factors affecting persistence of *M. bovis* in the environment have been investigated but are still poorly understood. Because

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of the low sensitivity associated with culture of MTBC from the environment, qPCR specific for MTBC DNA has been adapted as a supplementary quantification method (136, 137), and 16S rRNA has also been used as a proxy for viable MTBC (138). In addition, the type of samples collected within a system also appears to influence detection of *M. bovis*. For example, using PCR, MTBC DNA has been more frequently recovered in sediments around waterholes than in water in environments with *M. bovis*-infected hosts (139).

Seasonal changes in environmental conditions appear to influence persistence of *M. bovis* in the environment. Both air and soil temperatures affect detection of MTBC DNA in environmental samples (136, 137, 139). Soil concentrations of *M. bovis* DNA were higher in spring compared to all other seasons in the Iberian Peninsula (139). Regardless of soil type, *M. bovis* DNA concentrations were higher when air and soil temperatures were moderate (averaging ∼15°C and 17°C, respectively), and with greater soil moisture content (∼50%) in spring, compared to higher air and soil temperatures (maximum averages 32.6 and 26.4°C, respectively) and lower soil moisture content (∼2%) in summer in this region (139). A study in Michigan (USA) showed that the persistence of *M. bovis* (measured by PCR and culture) in contaminated environmental substrates, which varied between 4 weeks and 6 months, was shortened by exposure to high ambient temperatures, increased intensity of solar radiation, and higher loss of substrate moisture through evapotranspiration (136). In addition, when soil was spiked with *M. bovis*, persistence was longer at 4°C than at 22°C (137). This was in agreement with field studies that showed that *M. bovis* persisted longer in soil in autumn/winter than in summer in Michigan and New Zealand (140, 141), though in these cases it is not clear whether this was only correlated to temperature, or a combination of variables associated with certain seasons. One previous study done under controlled conditions presented contradictory findings related to the correlation of *M. bovis* persistence with temperature; *M. bovis* persisted longer in spiked soils at 37°C than at 4°C (138), highlighting some of the continued knowledge gaps related to persistence of *M. bovis* in the environment. Physico-chemical properties in soil, such as the proportional contribution of clay, silt, sand and organic matter to overall composition, as well as pH, and mineral content (137), may also affect *M. bovis* persistence in the environment. Presence of shade has also been associated with the persistence of environmental *M. bovis,* likely due to maintenance of higher water content and moderate temperatures of the soil and vegetation (142). Additionally, reduced ultraviolet radiation in shade results in less cell stress and fewer genetic mutations, improving bacterial survival (142). These areas may play a role in exposure to *M. bovis* because resting rhinoceros and other species often occupy shady areas during the hottest times of the day, which may promote concentration of bacteria shed in secretions.

In addition to abiotic factors, there are other biological reservoirs that are ubiquitous in the environment and may play a vital role in environmental persistence and subsequent transmission of MTBC. MTBC bacilli have been isolated from free-living amoeba, found frequently in soil (143). It has also been discovered that earthworms (*Lumbricus terrestris*) can disseminate *M. bovis* from contaminated animal feces to the surrounding soil through casting egestion (144). These worms can shed bacteria for up to 4 days after initial ingestion of contaminated feces. The presence of these organisms in areas where grazing by *M. bovis*-infected and susceptible hosts occurs may promote exposure through indirect interactions.

It is hypothesized that, due to the influence of environmental variables on the ability of *M. bovis* to persist in the environment, there may be a seasonal variation in bTB transmission risk. Environmental persistence of *M. bovis* under cold and wet conditions, as well as seasonal changes in the presence of shade and vegetation, may influence *M. bovis* exposure risk in rhinoceros; however further studies are needed to determine pathogen persistence in the natural habitats of these species.

DISCUSSION

The Importance of MTBC Infections in African Rhinoceros

Today, the largest free-ranging populations of African rhinoceros in South Africa are located in bTB endemic areas. While poaching of African rhinoceros for their horns is currently the most substantial threat to their conservation (23, 24), the presence of bTB in rhinoceros presents a considerable barrier to conservation due to the inability to translocate animals from bTB endemic areas to safeguarding areas that are bTB free. Without the tools to screen rhinoceros in endemic areas for *M. bovis* infection, regulations prevent translocation to bTB-free areas. Individuals that cannot be moved for safeguarding purposes are then exposed to risk of mortality resulting from poaching incidents. Although bTB is not currently recognized as a major cause of morbidity or mortality or a threat to rhinoceros population health, understanding the epidemiology and pathogenesis of this disease in rhinoceros will provide a foundation for studying the impact of bTB on these species. African rhinoceros populations in zoos around the world may also be at risk of MTBC infection, as individuals in these populations have exhibited morbidity and mortality (4–19). Based on limited case reports, both *M. tuberculosis* and *M. bovis* infect black and white rhinoceros, although the epidemiology and sources of these infections may differ. In addition to the impact on individual rhinoceros health, these infections may also result in spread to other animals, as well as humans, in the zoo environment (12, 13, 130). Therefore, it is essential to investigate the epidemiology of MTBC infections in rhinoceros in various settings to inform the most appropriate disease management and control strategies.

Hypothesized TB Risk Factors

As in other species, it is expected that the risk of MTBC infection in rhinoceros will be based on factors influencing susceptibility of the individual and exposure to the MTBC (28).

M. tuberculosis vs. *M. bovis*

Most reported cases of TB in captive rhinoceros resulted from infection with *M. tuberculosis*, with only a few cases caused by *M. bovis*. This is likely due to differing levels of exposure to each pathogen according to its prevalence, as well as the likelihood of

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interaction with an infected host, including both other animals and humans. TB affects human populations worldwide, and most human cases are caused by infection with *M. tuberculosis* (145). Captive rhinoceros may therefore have a higher likelihood of exposure to *M. tuberculosis* than *M. bovis* through their prolonged close contact with infected human caregivers. In these cases, transmission may occur through aerosols or through a contaminated environment, and possibly both.

While *M. bovis* can cause TB in humans, the pathogen is less efficient than *M. tuberculosis* at propagating through human hosts (146). This animal-adapted MTBC species predominantly occurs in livestock and wildlife populations, and is maintained in certain endemic areas by susceptible host populations (3). Free-ranging rhinoceros in bTB endemic areas may be exposed to *M. bovis* through infected hosts or a contaminated environment; the latter is likely to occur more frequently, especially for inter-species transmission, as free-ranging animals are less likely to have close prolonged contact with other infected host species than they are to share aggregation points in their environments (e.g., water sources) with these species. Exposure to *M. tuberculosis* is less likely in free-ranging populations than in captive rhinoceros, as they have little to no interaction with humans, which are the most affected by this pathogen.

Species-Specific Susceptibility

The more frequently reported occurrence of TB disease in captive black rhinoceros than in white rhinoceros, from the case series outlined in **Figure 2** and **Supplementary Table 1**, lends credence to the hypothesis that black rhinoceros are more prone to TB disease than white rhinoceros. However, this observation may be due to other confounding factors, including inapparent differences in numbers of black and white rhinoceros in captivity, and differences in *M. tuberculosis* or *M. bovis* exposure. Disease surveillance and reporting biases also exist because not all institutions that house rhinoceros conduct post-mortem TB surveillance or have equal diagnostic capabilities, not all cases of TB in rhinoceros are recorded in the literature, and non-cases are often not reported (147). Nevertheless, the data presented provides foundational hypotheses for further evaluation of TB risk in black and white rhinoceros.

Sex as a Risk Factor for MTBC Infection and TB

Limited available data from scientific reports on rhinoceros show more cases of TB in males compared to females (**Figure 2** and **Supplementary Table 1**). It is unknown whether this observation represents a true sex predilection for TB, or whether reports are biased toward males for other reasons. That said, a proposed hypothesis is that African rhinoceros males have a higher susceptibility to TB than females, and this is further supported by males in other species tending to have higher rates of TB compared to females (refer to section Demographic Risk Factors).

In free-ranging African rhinoceros populations in South Africa, it is hypothesized that females have a higher exposure to *M. bovis* due to their wider home range, and more consistent association in cohesive groups than males, and it is unknown how this may contribute to the overall TB risk of males and females in these populations. More controlled investigation in free-ranging populations is required to test these hypotheses.

Age as a Risk Factor for MTBC Infection and TB

All recorded TB and bTB cases (**Figure 2** and **Supplementary Table 1**) have occurred in adults. One explanation for this observation is the chronic and recurring nature of this disease. It is possible that young individuals are just as susceptible to MTBC infection as older individuals, but that the disease takes extended time to clinically manifest. An alternative explanation is that diminishing immunocompetence with age (as occurs with age-based hormonal changes in humans as well as the immune effects of old age) could render adults more susceptible to infection and onset of disease than young rhinoceros. It is possible that this observation is attributed not only to age-based changes in susceptibility, but is also due to age-related changes in exposure to either *M. tuberculosis* and *M. bovis* due to translocation of captive rhinoceros for management and breeding (**Figure 2** and **Supplementary Table 1**) (4). Based on this limited information, and age-related TB risk trends observed in most other species, it is hypothesized that susceptibility to MTBC infection and disease progression with exposure increases with age in black and white rhinoceros species.

In free-ranging populations of African rhinoceros in SA, incidence of *M. bovis* is expected to increase with age, due to more intense, and consistent exposure of adults in their smaller, more "settled" home ranges compared to that of the more nomadic young, and the accumulation of repeat exposures over their lifetime. The prevalence of *M. bovis* may also be higher in older animals, reflecting the chronic nature of disease in these long-lived species. The impact of increasing age on the overall TB risk of these populations is still unknown, and requires more investigation.

Environmental and Seasonal Factors Affecting Exposure to and Transmission of *M. bovis* in Free-Ranging Rhinoceros in South Africa

The characteristic closer association of black rhinoceros with closed, shady environments than white rhinoceros may increase exposure to *M. bovis* in this species; this is expected to occur as a result of their sharing of this habitat with the suspected bTB maintenance host, the greater kudu, as well as the longer persistence of *M. bovis* in shady (vs. irradiated) conditions. Conversely, the more frequent association of white rhinoceros with soil-associated bTB reservoirs and African buffaloes (a prominent bTB maintenance host) with whom they share their environment and food source, is likely to increase their *M. bovis* exposure. Additionally, because the white rhinoceros is considered a more social species than the black rhinoceros, it is hypothesized that *M. bovis* risk resulting from intra-species interactions will be comparatively higher in white rhinoceros. Overall, because of the higher population of buffaloes (the maintenance host with which white rhinoceros is expected to more frequently associate) than greater kudus (with which the black rhinoceros is expected to more frequently associate), as well as the expected occurrence of more intra-species interactions in white rhinoceros, it is hypothesized that there will be a higher risk

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M. bovis exposure in white rhinoceros populations than in black rhinoceros populations in KNP and HiP.

In free-ranging African rhinoceros populations in South Africa, it is hypothesized that there is an association between seasonal fluctuations in environmental conditions, rhinoceros spatial patterns, and *M. bovis* infection risk. During hot or dry periods, the *M. bovis* exposure of rhinoceros (and other species) may increase due to increased aggregation of *M. bovis*-infected and susceptible hosts at water sources. During hotter periods, specifically, the aggregation of infected and susceptible hosts in shady areas and/or mud wallows may be associated with increased *M. bovis* exposure. Seasonal fluctuations in soil-associated bTB reservoirs such as free -living *M. bovis* in the soil, as well as earthworms and amoeba, may also be associated with seasonal changes in incidence *M. bovis* infection in African rhinoceros and other animals.

Future Research

The most pressing concern related to TB in African rhinoceros is the acquisition of knowledge and the development of tools to inform surveillance and control strategies for the disease, as well as conservation plans.

The development of sophisticated diagnostic tools may allow for early detection of infection; this would enable earlier interventions that could improve the prognosis of infected individuals and mitigate the spread of the infection in captive and free-ranging systems. Of particular consideration is logistical feasibility and fitness-for-purpose of a test. Capture and immobilization of rhinoceros, especially in free-ranging populations, is extremely costly and is a source of stress for the animal. Therefore, the use of a test like the TST, in addition to being unreliable in this species, would be ill-advised, as it involves immobilization for both administration and interpretation of the test on separate occasions, increasing the cost and the stress for the rhinoceros undergoing testing. Development of blood-based cytokine release assays for bTB in African rhinoceros is currently ongoing; these tests require a single capture and immobilization, and once validated, may be reliable, cost effective diagnostic methods for bTB in rhinoceros.

Coordinated studies in captive populations may help to clarify demographic factors (e.g., age, sex, species) as risks for *M. bovis* infection and disease progression in African rhinoceros. This would involve ante-mortem monitoring for MTBC infection, as well as thorough post-mortem exams that include histopathology and ancillary diagnostic tests. Because zoological facilities tend to keep curated medical records, retrospective, longitudinal data may already be available to address these knowledge gaps. Careful, standardized data curation across institutions could inform and enumerate a study population and be used to identify cases and non-cases. In any such study, attention to confounding factors such as differences in exposure to MTBC based on animal origin, movement history, and TB prevalence in human populations should be considered.

In South Africa, population-based epidemiological studies of bTB in free-ranging African rhinoceros populations are currently ongoing. Findings from such studies could help identify major drivers of bTB infection in free-ranging populations as well as identify low risk individuals, which would have immediate benefit to current conservation and translocation efforts. This knowledge could be applied to inform management decisions for these populations, e.g., to minimize the probability of moving a false-negative infected animal out of bTB endemic areas and inadvertently spreading bTB to other areas. Such studies may also aid in identifying important bTB risk mitigation opportunities aimed to decrease continued spread of bTB in black and white rhinoceros living in these fragile ecosystems.

CONCLUSION

This review has focused on available literature that could help to characterize the risk posed by MTBC (including *M. bovis* and *M. tuberculosis*) to African rhinoceros species. It has also drawn attention to major knowledge gaps pertaining to TB in rhinoceros. By identifying and systematically addressing each of these knowledge gaps, advances will inform management decisions for conservation of African rhinoceros, and South African biodiversity.

AUTHOR CONTRIBUTIONS

RD, MM, WG, and CW reviewed available literature and wrote and edited the manuscript. PB edited the manuscript. RD and MM generated **Figures 1, 2**. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.580476/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer BB declared a past co-authorship with one of the authors PB to the handling editor.

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PNAS RESEARCH ARTICLE | POPULATION BIOLOGY

Epidemiology of *Mycobacterium bovis* infection in free-ranging rhinoceros in Kruger National Park, South Africa

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***Mycobacterium bovis* infection, which is a prominent cause of bovine tuberculosis, has been confirmed by mycobacterial culture in African rhinoceros species in Kruger National Park (KNP), South Africa. In this population-based study of the epidemiology of *M. bovis* in 437 African rhinoceros (*Diceros bicornis*, *Ceratotherium simum*), we report an estimated prevalence of 15.4% (95% CI: 10.4 to 21.0%), based on results from mycobacterial culture and an antigen-specific interferon gamma release assay from animals sampled between 2016 and 2020. A significant spatial cluster of cases was detected near the southwestern park border, although infection was widely distributed. Multivariable logistic regression models, including demographic and spatiotemporal variables, showed a significant, increasing probability of *M. bovis* infection in white rhinoceros based on increased numbers of African buffalo (*Syncerus caffer*) herds in the vicinity of the rhinoceros sampling location. Since African buffaloes are important maintenance hosts for *M. bovis* in KNP, spillover of infection from these hosts to white rhinoceros sharing the environment is suspected. There was also a significantly higher proportion of *M. bovis* infection in black rhinoceros in the early years of the study (2016–2018) than in 2019 and 2020, which coincided with periods of intense drought, although other temporal factors could be implicated. Species of rhinoceros, age, and sex were not identified as risk factors for *M. bovis* infection. These study findings provide a foundation for further epidemiological investigation of *M. bovis*, a multihost pathogen, in a complex ecosystem that includes susceptible species that are threatened and endangered.**

bovine tuberculosis | rhinoceros | risk | epidemiology | prevalence

Significance

African rhinoceros survival is threatened by poaching, habitat loss, and climate effects. The presence of *Mycobacterium bovis* in wild populations creates an additional potential threat to health and conservation programs. This study reports a large survey of *M. bovis* infection in free-ranging rhinoceros. Our findings confirm a widespread, high infection burden in the rhinoceros population of Kruger National Park, South Africa and identify risk factors for infection. These findings provide a foundation for understanding the spread of bovine tuberculosis in complex ecosystems. This study reflects the complexity of investigating a multihost pathogen in a previously naïve system. It provides an opportunity to increase awareness of the global impact that tuberculosis can have on animal populations, food security, and conservation.

African rhinoceros (*Diceros bicornis*, *Ceratotherium simum*) are currently under threat due to poaching activity and habitat destruction, as well as the underrecognized threat of infectious diseases (1, 2). *Mycobacterium bovis* infection has been confirmed in the African rhinoceros population in Kruger National Park (KNP), South Africa (3–6). The discovery of *M. bovis* infection in this population has led to a quarantine of rhinoceros intended for translocation from the park to other protected areas, which has significant conservation consequences (1). The paucity of knowledge regarding the epidemiology and risk of transmission from infected rhinoceros has become evident when assessing impact on the KNP population and potential for spread to other populations (1).

Because *M. bovis* infection is chronic and may not cause clinical signs of disease for months to years, its presence in an ecosystem with multiple susceptible host species may not be recognized for decades, as has been documented in several bovine tuberculosis (bTB) afflicted wildlife populations worldwide (7–9). KNP is considered endemic for bTB, with African buffaloes (*Syncerus caffer*) being the key maintenance hosts (8–10). Historically, *M. bovis* is believed to have originated from infected cattle adjacent to the park boundaries in the 1960s and 1980s, but was not detected until the 1990s in infected buffalo herds (11). Since then, 15 additional wildlife species in KNP have been documented with infection (12), including rhinoceros, in which infection was confirmed using mycobacterial culture and *M. bovis* species confirmation (3–6).

The epidemiology of bTB in a complex system that contains multiple hosts with varying susceptibility results in an array of opportunities for infection spread. Black and white rhinoceros in KNP share environmental resources (including browse/grazing, and water sources) with potentially *M. bovis*–infected African buffaloes (8–10), greater kudu (*Tragelaphus strepsiceros*) (13, 14), warthogs (*Phacochoerus africanus*) (15), and other species (12). A recent review of potential scenarios for interspecies transmission has suggested that rhinoceros may become infected with *M. bovis* in ecosystems containing other infected hosts (1). Interspecies spread has been demonstrated in other systems, including badgers and cattle in the United Kingdom (16), wild boars, deer, and

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cattle in Spain (17), and deer and cattle in the United States (18). Mechanisms of transmission between herbivores are poorly understood but have been attributed to indirect interaction through shared resources such as pastures, feed, or water holes that are contaminated by *M. bovis*–shedding hosts (17, 19, 20). The potential for intraspecies transmission between rhinoceros is also plausible based on antemortem detection of mycobacteria in respiratory secretions (1, 21).

For species (like white and black rhinoceros) that are considered threatened or endangered, the presence of a controlled infectious disease can significantly hamper conservation efforts and potentially impact population health and survival (22–24). Regulations imposed by the Department of Agriculture, Land Reform and Rural Development in KNP, due to the diagnosis of *M. bovis* in rhinoceros and other species, are an additional barrier to the movement of rhinoceros from the park to other national or private reserves. This can have a significant impact on conservation of the species, as KNP has historically been an important source population of rhinoceros for other conservation strongholds in South Africa and other African countries. In order for a captured rhinoceros to be moved out of KNP, it must first be placed in a quarantine facility for 3 mo, and must test negative for *M. bovis* infection during repeated testing events.

It is therefore critical to be able to assess infection status in these populations. Recent advances in the development of diagnostic tests (12, 25) for *M. bovis* infection in wildlife beyond conventional mycobacterial culture and *M. bovis* species confirmation has allowed for antemortem testing. The QuantiFERON TB Gold In-Tube Plus (QFT) (Qiagen)–interferon gamma release assay (QFT–IGRA) was recently validated for use in white rhinoceros (4), and has been used for testing KNP rhinoceros for *M. bovis* infection. These results provide the opportunity to generate an understanding of epidemiological determinants and risk factors for infection and disease transmission within the rhinoceros population.

Here, we report a population-based study on the epidemiology of *M. bovis* infection in free-ranging African rhinoceros. We investigated the distribution of *M. bovis* infection in rhinoceros over the KNP landscape and identified the demographic, spatial, and temporal factors that may drive infection in this population (1). Our findings begin to uncover the complex epidemiology of bTB for rhinoceros in a multihost system where bTB is endemic. Results from this study emphasize the importance of disease surveillance in managed wildlife systems and support current quarantine and testing requirements for rhinoceros in KNP. These findings are important for preventing the spread of *M. bovis* infection to other rhinoceros populations (2, 3). In a broader sense, this study reflects the complexity of investigating a multihost pathogen that has been introduced into a previously naïve system. It provides an opportunity to increase awareness of the global impact that TB and other zoonotic pathogens can have on domestic and wild animal populations, food security, and conservation of species and ecosystems.

Results

Prevalence of *M. bovis* Infection and Spatial Clustering. The study population consisted of 475 free-ranging African rhinoceros that were opportunistically sampled in KNP from 2016 to 2020, as described in *Materials and Methods*. The *M. bovis* infection status could be determined for 437 (92%) of 475 rhinoceros, largely based on antemortem test results from IGRA (n = 428) (4, 5), with a few individuals' case statuses confirmed using (postmortem) mycobacterial culture of tissues with *M. bovis* species confirmation using a rapid diagnostic multiplex PCR (RD-PCR) (n = 9) (3, 26) (Table 1). Fifty-eight of the 437 study population individuals with a known infection status were tested after recent translocation out of the park to quarantine areas. Of these, only five were *M. bovis* positive according to the IGRA result. All five of these individuals were sampled for testing within hours of their translocation out of the park; therefore, their positive status reflects infection acquired in KNP.

The apparent *M. bovis* prevalence was estimated and adjusted according to the sensitivity and specificity of the IGRA assay, as described in *Materials and Methods* (27). The overall adjusted prevalence (based on the IGRA) during the study period was 15.4% (82/437: 95% CI: 10.4 to 21.0%). The majority of *M. bovis*–positive cases were considered clinically normal (83%, 68/82), based on veterinary clinical assessment at the time of immobilization. Species-specific prevalence was 17.0% (63/317; 95% CI: 11.0 to 23.9%) for white rhinoceros and 11.2% (19/120; 95% CI: 3.1 to 22.2%) for black rhinoceros, and were not statistically different (Fisher's exact $P = 0.41$). The 38 rhinoceros that were classified as having an "unknown" infection status were excluded from prevalence calculations and further analyses. Further description of demographic characteristics of the study population, according to *M. bovis* status, species, sex, and age, is shown in *SI Appendix*, Table S1.

Prevalence of *M. bovis* infection (adjusted based on IGRA sensitivity and specificity) in rhinoceros according to ranger area and ecozone is shown in Fig. 1. In total, 420 individuals were included in prevalence calculations across the different areas. For ranger area, the highest *M. bovis* prevalence was in Pretoriuskop (28.1%), and the lowest was in Tshokwane (9.7%; Fig. 1*A*); however, no significant differences in prevalence across ranger areas were identified (Fisher's exact $P > 0.05$ for all comparisons). Similarly, the highest prevalence by ecozone was in the Pretoriuskop Sourveld (36.4%), and the lowest prevalence was in the Lebombo Mountain Bushveld (13.3%; Fig. 1*B*). The Pretoriuskop Sourveld ecozone had a significantly higher prevalence of *M. bovis* infection than the Sabie/Crocodile Thorn Thickets ($P < 0.001$), the Mixed Bushwillow Woodlands ($P = 0.02$), and the Lebombo Mountain Bushveld ($P = 0.01$) ecozones. No other significant differences were detected (Fisher's exact $P > 0.05$ for all other area comparisons). Ecozone and ranger area were not evaluated in the univariate and multivariable analyses, due to the numerous categories and the potential for missing covariate patterns.

Further exploration of differences in geographical distribution of *M. bovis* infection using Kulldorff's spatial scan statistic (28) showed significant spatial clustering of infection in white rhinoceros. A single, statistically significant cluster of radius 6.5 km was detected toward the northern border of the Pretoriuskop ranger area (Fig. 1*A*). Twelve cases of *M. bovis* were identified in this cluster, whereas the model predicted only four (relative risk = 3.5, $P = 0.036$). No other significant spatial clustering was detected.

Univariate Analyses. Since environmental risk factors were hypothesized to be similar for black and white rhinoceros due to sharing of resources, the initial univariate analyses were performed with data from the two rhinoceros species (black rhinoceros, or *D. bicornis*, and white rhinoceros, or *C. simum*) combined. In total, 13 variables were evaluated in the univariate analysis using logistic regression (as outlined in Tables 2 and 3). This included three spatial variables: number of buffalo herds, number of kudu herds, and buffalo density. Because the spatial scale for these variables was unknown, we used our data

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Table 1. Mycobacterial QFT-IGRA test and culture results for 475 African rhinoceros in KNP, South Africa (2016–2020)

M. bovis status	Test result for IGRA and culture*	White rhinoceros	Black rhinoceros	Total
M. bovis positive (n = 82)	IGRA+/no culture performed	55	19	74
	IGRA+/culture+	3	0	3
	IGRA−/culture+	5	0	5
M. bovis negative (n = 355)	IGRA−/no culture performed	253	101	354
	IGRA−/culture-	1	0	1
Unknown (n = 38)	IGRA status undetermined/no culture performed	30	8	38
	Total	347	128	475

IGRAs were completed on plasma from QuantiFERON-stimulated rhinoceros whole blood (4, 5) to detect immune sensitization to mycobacteria. Rhinoceros were classified as IGRA positive, negative, or unknown for *M. bovis* infection based on criteria outlined in *Determination of* M. bovis *infection status*.

**M. bovis* was isolated in tissues obtained at necropsy from eight of nine white rhinoceros through conventional mycobacterial culture using BACTECMGIT platform, with *M. bovis* species confirmation using a rapid diagnostic multiplex PCR (RD-PCR) (26). Culture positive animals included three adult males (one necropsied in 2016, two in 2018), one subadult male (necropsied in 2016), two adult females (necropsied in 2016 and 2018), and two subadult females (necropsied in 2016 and 2018).

from repeated captures to estimate a plausible spatial scale over which rhinoceros in the study population could potentially move. We then evaluated the three spatial variables across different potential circular home ranges, with radii of 5.75, 11.5, 17.25, and 23 km. For each variable, a single home range size was ultimately chosen for further statistical evaluation based on the best-fitting single-variable logistic regression model. Chosen home ranges included 17.25 km for number of buffalo herds and 5.75 km for both number of kudu herds and buffalo density (Table 3). *SI Appendix*, Tables S2 and S3 show all results from evaluations using home ranges with various distances; methodology is described in detail in *Materials and Methods*.

Four of the 13 variables met screening criteria ($P < 0.2$; Tables 2 and 3). Two of these factors were statistically significant ($P < 0.05$) in the univariate analyses, including sampling year and number of buffalo herds within the rhinoceros home range (circular buffer; radius = 17.25 km). Rhinoceros species, age group, sex, and nearest permanent water source type were also considered important demographic covariates or plausible effect modifiers and were evaluated in the final multivariable model(s).

Even though 82 of the 437 study individuals tested positive for *M. bovis* infection, no significant association between *M. bovis* infection and (apparent) clinical health status was detected. The majority of the test-positive individuals appeared

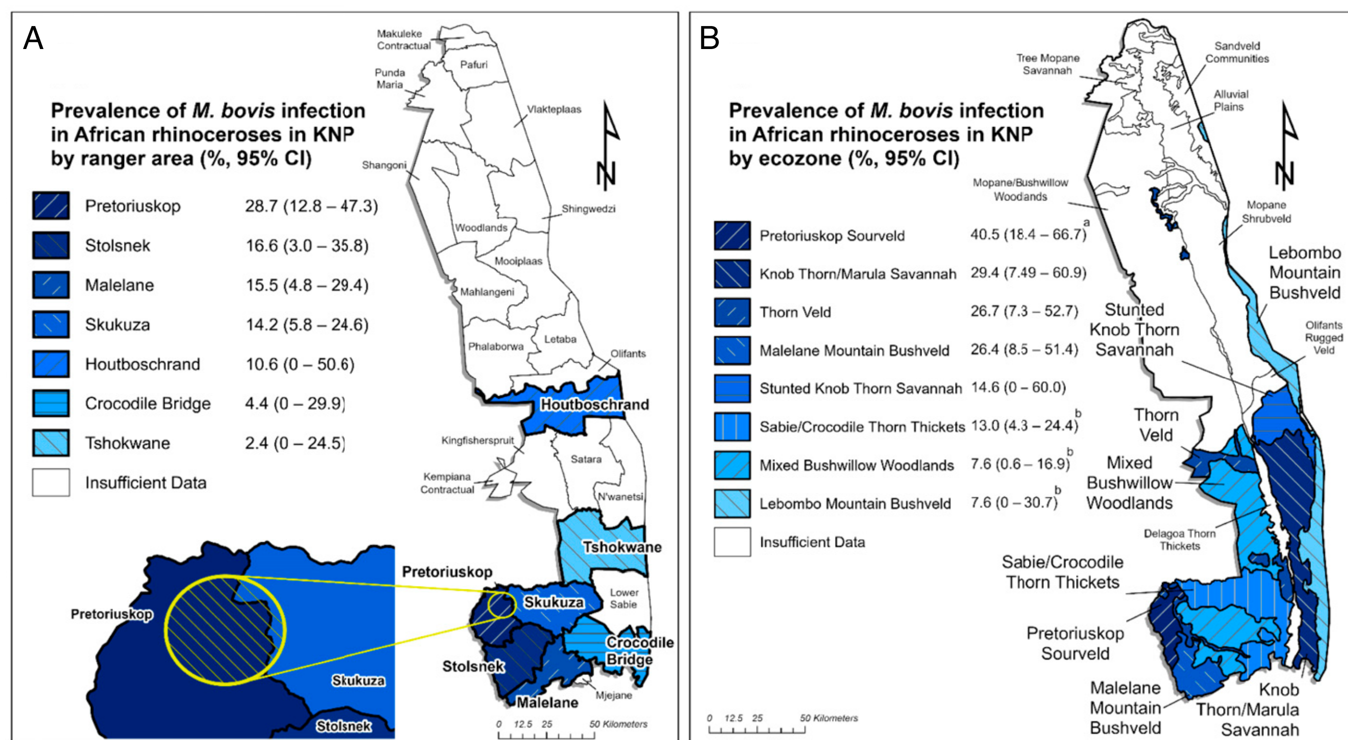

Fig. 1. Prevalence (percent) of *M. bovis* infection in rhinoceros in KNP, South Africa, 2016–2020 (n = 420). Prevalence estimates are reported in the key in descending order. Areas with insufficient data ($n \leq 10$ sampled animals) are shown in white. (*A*) The prevalence of *M. bovis* in the study population according to ranger management area. No significant differences in *M. bovis* prevalence according to ranger area were identified (Fisher's exact $P > 0.05$ for all comparisons). A single, statistically significant cluster of radius 6.5 km is depicted by a hatched yellow circle based on Kulldorff's spatial scan statistic (28); 12 cases of *M. bovis* were identified in this cluster, whereas the model predicted only 4 (relative risk = 3.5, $P = 0.036$). (*B*) The prevalence of *M. bovis* according to ecozone (76). Significant differences in *M. bovis* infection prevalence were detected between the ecozones with the same superscript letter. Prevalence in the Pretoriuskop Sourveld ecozone was significantly different from in Sabie/Crocodile Thorn Thickets ($P < 0.001$), Mixed Bushwillow Woodlands ($P = 0.02$), and Lebombo Mountain Bushveld ($P = 0.01$). Fisher's exact $P > 0.05$ for all other area comparisons.

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Table 2. Frequency distributions and univariate analyses of potential risk factors (measured as categorical variables) for *M. bovis* infection in African rhinoceroses in KNP, South Africa, 2016–2020 (*n* = 437)

Risk factor	Number of *M. bovis*–positive rhinoceros *n* = 82 (percent of total in category)	Number of *M. bovis*–negative rhinoceros *n* = 355 (percent of total in category)	OR (95% CI)	*P*
Species				0.33*
White rhinoceros (*C. simum)*	63 (77)	254 (72)	1.3 (0.8–2.3)	
Black rhinoceros (*D. bicornis*)	19 (23)	101 (28)	Reference	
Sex				0.82*
Female	48 (59)	203 (57)	1.1 (0.6–1.7)	
Male	34 (41)	152 (43)	Reference	
Age				0.27*
Adult	52 (63)	192 (54)	1.7 (0.8–3.6)	
Subadult	21 (26)	107 (30)	1.2 (0.5–2.8)	
Calf	9 (11)	56 (16)	Reference	
Orphan status (calves only, *n* = 65)				0.61
Orphaned	2 (22)	17 (30)	0.7 (0.1–3.5)	
With mother	7 (78)	39 (70)	Reference	
Health status				0.38
Injured/abnormal health	14 (17)	47 (13)	1.3 (0.7–2.6)	
Appear healthy	68 (83)	307 (87)		
Sampling year				0.02*,†
2016	10 (12)	19 (5)	4.5 (1.7–12.1)	
2017	15 (18)	42 (12)	3.1 (1.3–7.2)	
2018	13 (16)	59 (16)	1.9 (0.8–4.5)	
2019	33 (40)	141 (40)	2.0 (1.0–4.2)	
2020	11 (13)	94 (26)	Reference	
Season				0.61
Dry	43 (52)	197 (55)	0.9 (0.5–1.4)	
Wet	39 (48)	158 (45)	Reference	
Nearest permanent water source type‡				0.93*
Waterhole	36 (44)	156 (44)	1.0 (0.6–1.6)	
River	46 (56)	195 (56)	Reference	
Number of kudu herds nearby‡,§,¶				0.30
8–14	24 (29)	95 (27)	1.1 (0.5–2.3)	
5–7	23 (28)	132 (37)	0.7 (0.4–1.6)	
3–4	21 (26)	64 (18)	1.4 (0.7–3.0)	
0–2	14 (17)	60 (17)	Reference	

*Selected for inclusion in multivariable model.
†Met screening criteria.
‡Here *n* = 433, and excludes individuals with unknown capture locations.
§Within home range of 5.75 km radius.
¶Categories created according to quartile distribution of measure over the study population.

clinically normal (83% of infected animals were clinically normal, 68/82), and all but 2/14 *M. bovis*–positive individuals with recorded clinical abnormalities had afflictions that were associated with poaching or fighting injuries, rather than evidence of infection.

Multivariable Analyses. The final, combined-species model with all rhinoceros included 433 individuals, and consisted of five variables, namely, species, sex, age, sampling year, and number of buffalo herds within a 17.25-km radius of the capture location (*SI Appendix*, Tables S4–S6).

This model indicated that the year of sampling and the number of buffalo herds within a 17.25-km radius of the capture location were significantly associated with *M. bovis* infection in rhinoceros. Specifically, rhinoceros sampled in years 2016 (odds ratio [OR] = 4.4; 95% CI: 1.6 to 12.3), 2017 (OR = 3.4; 95% CI: 1.4 to 8.1), and 2019 (OR = 2.2; 95% CI: 1.0 to 4.6) had higher odds of infection compared to the reference year 2020 (*P* = 0.01). Additionally, for each additional log-transformed buffalo herd in the rhinoceros home range, the odds of *M. bovis* infection increased by 75% (OR = 1.75; 95% CI: 1.1 to 2.8). However, there was significant effect modification identified across the sampling year by species (*SI Appendix*, Table S6). Therefore, we also constructed species-specific models with the same variables. The final species-specific models are reported in Fig. 2, and in greater detail

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(including model fit parameters) in *SI Appendix*, Table S7. Additional effect modification by species or other factors were not identified, and other variable combinations did not improve the fit of the model or indicate additional sources of confounding.

Species-Specific Models. Variables found to be significantly associated with *M. bovis* infection differed between white and black rhinoceros. For the white rhinoceros, each additional log-transformed buffalo herd in the home range resulted in an increase in odds of infection by 77% (OR = 1.77; 95% CI: 1.07 to 2.92, $P = 0.02$; Fig. 2 and *SI Appendix*, Table S7). This corresponds to a probability of *M. bovis* infection of 0.057 when the number of buffalo herds in the white rhinoceros home range is at the minimum (number of buffalo herds = 6) and all other factors in the model are held at their mean. This probability increases to 0.192 when the number of buffalo herds is at the median (number of buffalo herds = 66) and all other factors are held at their mean.

Importantly, the numbers of buffalo herds should be considered a relative, rather than an absolute, measure of exposure to buffaloes, since we do not have a precise measure of exposure to buffalo herds for each rhinoceros; however, our data support the hypothesis that white rhinoceros in areas with more buffalo herds are at an increased risk of *M. bovis* infection compared to those in areas with fewer buffalo herds, while controlling for other factors. Sampling year was not significantly associated ($P = 0.15$) with *M. bovis* infection in white rhinoceros.

Conversely, for the black rhinoceros, the year of sampling was significantly associated with *M. bovis* infection ($P = 0.01$; Fig. 2 and *SI Appendix*, Table S7), while controlling for other factors in the model; individuals sampled in years 2016 (OR = 18.11; 95% CI: 2.09 to 157.15), 2017 (OR = 4.36; 95% CI: 0.98 to 19.41), and 2018 (OR = 4.60; 95% CI: 1.11 to 19.05), compared to years 2019 and 2020 (note that the years 2019 and 2020 were combined for black rhinoceros, due to small numbers of animals in those categories). However, the number of buffalo herds nearby (within 17.25 km) was not significantly associated ($P = 0.68$) with *M. bovis* infection in the black rhinoceros (Fig. 2 and *SI Appendix*, Table S7).

ORs from final models reporting adjusted associations for *M. bovis* infection among white rhinoceros and black rhinoceros, separately, are reported graphically in Fig. 2. Species-specific model estimates, including coefficients and SEs, are included in *SI Appendix*, Table S7.

Discussion

A considerable and widespread *M. bovis* infection burden was reported for the KNP rhinoceros population (15.4%), with similar rates of infection found in males and females of all age groups and in both black and white rhinoceros. Although demographic factors were not associated with risk, an increasing number of buffalo herds in the white rhinoceros home range, and year of sampling in black rhinoceros, increased the risk of *M. bovis* infection in this population. The KNP rhinoceros are central to the "Integrated Strategic Management of Rhinoceros" plan introduced by the South African Department of Environmental Affairs (29, 30). This strategy relies, in part, on translocation of individuals from the KNP population to newly developed safeguarding strongholds around the country. Therefore, the findings in this study support the decision to impose quarantine (31) on all rhinoceros (regardless of demographics) prior to translocation, in order to mitigate the risk for inadvertent *M. bovis* spread to other ecosystems outside KNP.

The distribution of *M. bovis* infection in KNP rhinoceros is similar to that reported for other species in the park. For example, a 1991–1992 survey of bTB in 1,122 African buffaloes in KNP showed widespread bTB in the central and southern regions of the park (including Houtboschrand and regions to the south of it), with individual herd bTB prevalence up to 67% (10). A later study showed spread of *M. bovis* infection to African lions ($n = 70$) sampled in 2012/2013 in the same areas of KNP, with an overall infection prevalence of 44% (32). Such extensive infection is increasingly observed in additional species in KNP, including warthogs (33), African wild dogs (*Lycaon pictus*) (23), and African elephants (34), with cases identified in more than 15 species in the park, to date (12). Taken together, these findings suggest that spillover of bTB is not a new occurrence and support the need for ongoing bTB surveillance across species to continuously assess disease risk and conservation impact, and to better understand transmission within and from the multihost system in KNP.

The detection of a single statistically significant *M. bovis* infection cluster, with a 6.5-km radius, in white rhinoceros toward the northern border of the Pretoriuskop ranger area (Fig. 1*A*) is in concordance with the higher infection prevalence in the Pretoriuskop Sourveld ecozone, compared to other areas in the east and the north (Fig. 1*B*). The identified cluster is in close proximity to the KNP border with the surrounding Mpumalanga province. Importantly, the region outside of the southern KNP borders is primarily farmland and home to livestock herds, specifically, cattle. Livestock in areas around the southern border of the park have historically been implicated in spillover of *M. bovis* to wildlife in KNP, including African buffaloes (10, 35). Now recognized as maintenance hosts for *M. bovis* in KNP (10, 35–37), African buffaloes share similar vegetation preferences to that of white rhinoceros. Interestingly, favored vegetation is abundant in the Pretoriuskop Sourveld ecozone where the highest *M. bovis* prevalence in rhinoceros, as well as the only significant case cluster, occurred.

Results from this study suggest a role for buffalo in *M. bovis* infection, specifically, in white rhinoceros. Adjusted associations showed an increasing risk of *M. bovis* infection in white rhinoceros with increasing numbers of nearby buffalo herds. This suggests that the African buffalo in KNP may serve as a potential source for spillover of *M. bovis* infection into white rhinoceros, as observed in other species (1, 10, 14, 38, 39). The importance of buffalo as a predictor of infection in white rhinoceros as compared to black rhinoceros was expected, as the landscape and preferred vegetation of white rhinoceros closely mirror that of buffaloes (40, 41); both are grazing species, and, therefore, white rhinoceros are more likely to share habitat with buffaloes than are black rhinoceros.

Interestingly, the association was only significant when examining buffalo herds—the number of individual buffaloes nearby and buffalo density were not significantly associated with *M. bovis* infection. Importantly, the crude nature of the available data makes it difficult to define the exact relationship of the buffalo variable with *M. bovis* infection. It is possible that the identification of a buffalo "herd" is a more precise measure compared to estimating total number of buffalo in a herd from aerial surveys. Alternatively, this finding could simply be due to variation in individual versus herd-level prevalence in KNP buffaloes (37). Our findings could also suggest that effective contact rates between infected buffalo and susceptible rhinoceros that lead to transmission do not depend on buffalo herd size.

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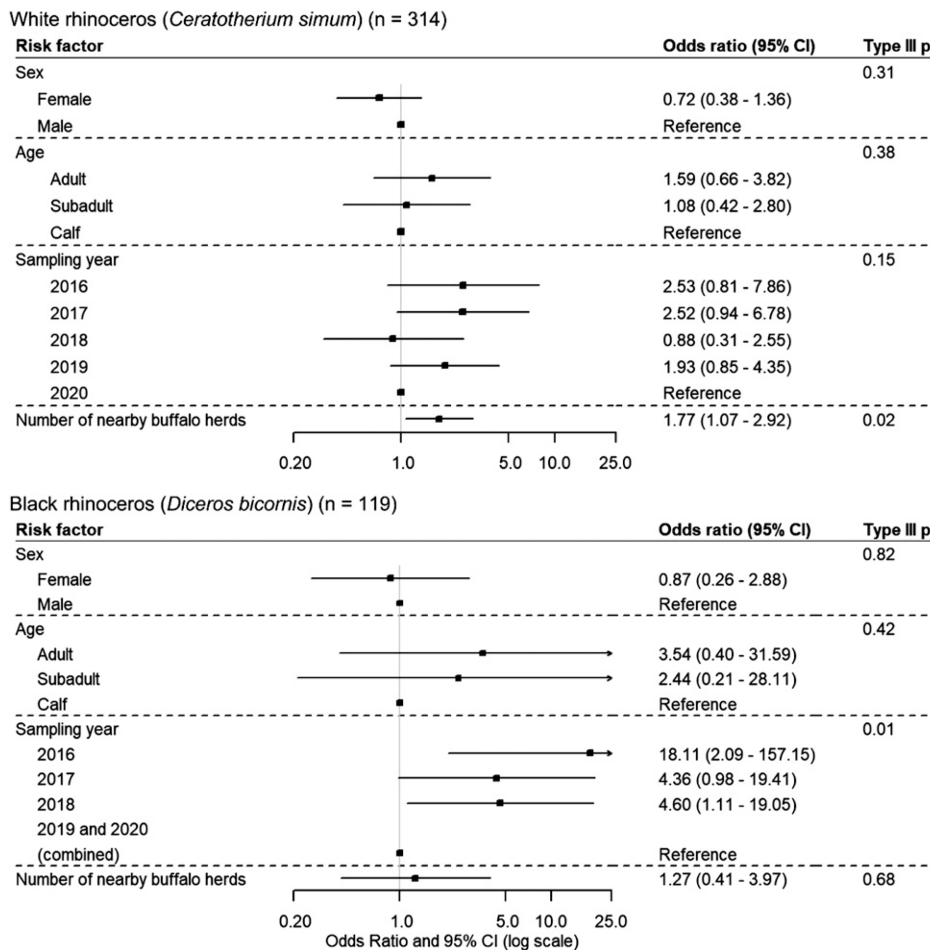

Fig. 2. Forest plots depicting species-specific multivariable models of factors associated with *M. bovis* infection African rhinoceros in KNP, South Africa (2016–2020). Parameters for both models, including coefficients, SEs, and fit statistics, are reported in detail in *SI Appendix*, Table S7.

Presence of infected buffalo herds was likely to increase environmental contamination with *M. bovis* due to shedding, consequently increasing exposure of rhinoceros to the pathogen. This phenomenon is observed in other bTB multihost systems, which involve wild boar, red deer, and cattle populations, across continental Europe (17, 19, 20). Mechanisms of transmission between herbivores are unclear but have been attributed to indirect interaction through shared resources such as pastures, feed, or water holes that are contaminated by *M. bovis*–shedding hosts (19, 20). In the current study, we did not find any associations with distance to nearby water source or water type. Prospective evaluations with refined measures of frequency of individuals at particular water sources may further elucidate exposure to contaminated environments.

Potential transmission of TB between rhinoceros was also plausible (1, 21, 42), but we did not find an association in adjusted models when evaluating distance to a nearby infected rhinoceros. For our initial exploratory analyses, our models included only crude evaluations of landscape-level effects and did not include the potential for infection in more than one nearby rhinoceros. We also did not have information on infection status of all (unsampled) individuals within a rhinoceros group, which may further lead to misclassification of exposure that could mask associations (43). More refined, individual-level measures that include longitudinal social network effects may improve understanding of the potential for intraspecies transmission.

Our findings also show a strong temporal association with risk of *M. bovis* infection, especially among black rhinoceros, albeit data were sparse for this group. Infection prevalence and odds of *M. bovis* infection were significantly higher in the earlier years of the study compared to 2019 and 2020. The magnitude of the adjusted OR was substantial in 2016 (OR = 18.1) and high in 2017 and 2018 (OR = 4.4 and OR = 4.6, respectively), compared to the most recent years of 2019 and 2020 (combined). It is unclear whether the observed decreasing prevalence over the study period was a result of changes in infection incidence or infection clearance rates, changes in contact patterns, an artifact of sampling bias, or another unobserved cause. All of these scenarios could be considered plausible.

Importantly, severe drought occurred in the year preceding and the first year of this study (2015–2016) (44). Associations between drought and *M. bovis* infection or disease have been detected in other multihost systems. In a large study of Mediterranean wild boars (n = 3,923), which are known bTB reservoirs, the occurrence of drought and increasing drought severity were significantly associated with increased occurrence of TB-like lesions (45). Another study investigating risk factors for bTB in cattle in Great Britain showed a positive association between atmospheric dryness and areas of high risk for *M. bovis* infection (46). Drought may impact rhinoceros body condition, with adverse consequences for immune responses (47, 48), and susceptibility to TB (49–51). Alternatively, reduced availability of water sources during periods of drought could lead to greater congregation of animals at the limited available drinking or wallowing sites. This could increase the risk of transmission through indirect interaction via *M. bovis*–contaminated water sources or surrounding resources, as is posited

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to occur in other multihost systems (19, 20). Multiyear, longitudinal investigations may elucidate potential interactions between disease, climate change, and other factors that vary over time, on rhinoceros' susceptibility and exposure to *M. bovis* infection.

Of the 18.8% of rhinoceros that tested positive for *M. bovis* infection, most appeared clinically normal (83% of infected individuals were clinically normal, 68/82, and only 2/14 *M. bovis*–positive individuals with clinical abnormalities showed possible evidence of infection); this is consistent with reports that *M. bovis* infection does not always lead to clinical disease in rhinoceros. Findings from a study of experimentally infected white rhinoceros suggest that individuals that are healthy may be able to contain and clear the infection before developing active or overt disease (21, 52). However, it was difficult to confirm that there was no association between *M. bovis* infection and clinical disease or death in KNP rhinoceros; if present, this association could be obscured by the currently high mortality rate due to poaching (53).

Whether or not *M. bovis* infection is likely to progress to disease in KNP rhinoceros, the substantial infection burden in this population should raise concern that changes in (unknown) factors impacting disease progression could lead to increased TB-related morbidity and mortality in this population. This could have further negative consequences for survival of this population, which is already experiencing pressures associated with habitat loss, climate change, and poaching (44, 53, 54). Overall, more research that utilizes sensitive indicators of rhinoceros clinical health is required to improve understanding of *M. bovis* infection and pathogenesis.

In recent years, there have been numerous important diagnostic advances for the detection and characterization of *M. bovis* infection and disease in wildlife species, including free-ranging populations (12). These tools have been used in bTB surveillance efforts in wildlife species and, importantly, provide platforms to investigate bTB at the population level. Mycobacterial culture of tissue or secretions, followed by *M. bovis* confirmation using PCR, is a highly specific, gold standard diagnostic method, which can confirm true infection. However, this technique is most accurate when applied postmortem, and tissues from mortalities in wildlife populations are often unavailable. For this reason, most of our study population was defined according to IGRA (5) results, which were used to classify rhinoceros infection status antemortem. Importantly, there is a potential for misclassification of *M. bovis* infection status, due to the sole reliance on this single available diagnostic platform. The IGRA is a standard method for diagnosis of active and latent *Mycobacterium tuberculosis* infection in humans (55, 56), and for active *M. tuberculosis* complex (MTBC) infection in animals (25, 57), including rhinoceros (4, 5, 21, 52). A limitation of this test is that it may not always detect cases of recently acquired infection (52). A study using *M. bovis* experimentally infected rhinoceros showed that an immune response (detected by IGRA) was measurable within 1 mo to 2 mo after exposure by airway inoculation, decreasing gradually between 5 and 12 mo postinfection and reverting to negative results between 12 and 16 mo postinfection, indicating clearance of active infection (52). In general, the chronic nature of *M. bovis* infection makes it difficult to reliably estimate infection incidence, although the presence of a positive IGRA result signifies current infection and, therefore, can be used to estimate prevalence. To minimize misclassification of infection status, 38 individuals that could not be defined as positive or negative were removed (refer to *Determination of* M. bovis *infection status*). Efforts focused on improving *M. bovis* diagnosis in rhinoceros are currently ongoing and may lead to more accurate and reliable case classification in future studies.

The spatial analyses in this study relied on capture location data for the study population as proxy for the location or area where each rhinoceros may have been exposed during the study period, since information on the individual ranges was not available. Our rationale for using our own location data for individuals captured multiple times over the study was to provide contemporaneous and contextual estimates of the spatial scale in KNP over which study individuals could move (and therefore be exposed to spatial factors). The chosen sizes for potential home ranges around each individual's capture location were approximated from our data based on the distances that rhinoceros traveled, as indicated by repeated captures, with 95% of point-to-point distances traveled falling within 23 km. These were consistent with limited reports of rhinoceros movement within home ranges, typically reported to have sizes between 5 and 65 km^2, in the literature (41, 58–68). However, the single-time-point capture locations do not reflect the movement patterns of rhinoceros and provide only a crude representation of the area that the rhinoceros regularly inhabited. Similarly, movements of other rhinoceros, and potential maintenance hosts (buffalo and kudu), were based on single-time-point location data for a cross-sectional sample of individuals, and the true rhinoceros home ranges are likely to vary over time according to the landscape and the resource availability, and by individual (40, 41, 59–68). Additionally, information on the infection status of these potential maintenance hosts was also not available; therefore, the applied model assumed a uniform risk over space and time, which is unlikely to be the case. Lack of precision in these measures would be expected to misclassify exposure variables, often biasing associations toward the null (43). The fact that we detected associations between numbers of nearby buffalo herds and risk of *M. bovis* infection in rhinoceros may suggest that the true magnitude of the association is higher than our estimate. Future studies could circumvent these challenges by tracking each rhinoceros over the course of the study, using satellite trackers to record their movement over time and space.

Conclusion

This study examines the epidemiology of *M. bovis* in a free-ranging population of rhinoceros and includes a large sample population from what is historically the world's largest population of free-ranging rhinoceros in KNP. We detected evidence of widespread *M. bovis* infection in African rhinoceros in KNP, with a substantial infection burden (*M. bovis* prevalence was 17.0% [63/317; 95% CI: 11.0 to 23.9%] for white rhinoceros and 11.2% [19/120; 95% CI: 3.1 to 22.2%] for black rhinoceros), the extent of which was previously unknown. This emphasizes the importance of cross-species surveillance in bTB-afflicted multihost systems. Since bTB can affect wildlife, domestic animals, and humans, its spread to different areas could have serious consequences for human and animal health and, consequently, the agriculture and tourism industries in southern Africa. For rhinoceros specifically, translocation to other populations is an integral part of conservation strategies but may be accompanied by the risk of introducing novel pathogens, including *M. bovis*, into other ecosystems. Due to the presence and widespread impact of *M. bovis* in KNP rhinoceros, imposed quarantine and testing requirements prior to translocation are warranted across both rhinoceros species and all age groups.

Results from this study also highlight the potential role of different factors in infection risk for each species. In black rhinoceros, we found temporal associations, with a higher risk of infection in individuals sampled in the early years of the study compared to the final years (2019-2020). This may suggest the

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Table 3. Frequency distributions and univariate analyses of potential risk factors (measured as continuous variables) for *M. bovis* infection in African rhinoceroses in KNP, South Africa, 2016–2020 ($n = 437$)

Continuous variables	Median for *M. bovis*–positive rhinoceros $n = 82$ (IQR)	Median for *M. bovis*–negative rhinoceros $n = 355$ (IQR)	OR (95% CI)	*P*
Distance to nearest water source (km)*	2.34 (1.33–3.62)	2.41 (1.32–4.08)	0.9 (0.8–1.0)	0.15[†,‡]
Distance to nearest *M. bovis* infected rhinoceros (km)*[,§,¶]	2.76 (1.00–4.81)	2.54 (1.35–4.89)	0.8 (0.6–1.1)	0.11[†,‡]
Number of buffalo herds nearby*[,§,]#	86 (46–111)	64 (41–102)	1.8 (1.2–2.8)	0.006[†,‡]
Surrounding buffalo density (estimated buffalo per square kilometer)*[,§,¶,‖]	1.21 (0.55–2.30)	1.13 (0.67–2.60)	0.9 (0.6–1.3)	0.41

IQR, interquartile range.
*Here $n = 433$, and excludes individuals with unknown capture locations.
[†]Selected for inclusion in multivariable model.
[‡]Met screening criteria.
[§]Odds ratio and CI calculated with a log transformation of the associated variable.
[¶]Added one before log transformation of the measured variable for the study population as variable is equal to zero for at least one of the included individuals.
#Within home range of 17.25 km radius.
[‖]Within home range of 5.75 km radius.

involvement of drought and changing climatic conditions in infection risk in black rhinoceros. In white rhinoceros, we found an association with distribution of buffalo in the individual's surrounding vicinity. This highlights the potential role of buffalo, a recognized bTB maintenance host, in infection of white rhinoceros. Further study of *M. bovis* risk in these populations is warranted.

Future related work should focus on the development of diagnostic tools that may improve surveillance in these species. These techniques would also enhance the ability to classify cases and improve resolution to understand the epidemiology of bTB in complex systems. In particular, a targeted cohort study that tracks individual rhinoceros longitudinally and measures their resource usage in relation to their infection status could aid in further developing the understanding of infection and transmission risk. Characterizing threats to the survival of these species in the KNP ecosystem is vitally important for conservation and protecting other vulnerable populations.

Materials and Methods

Source Population and Data Collection. Black and white rhinoceros populations in the KNP were sampled opportunistically during postmortem examinations ($n = 9$) or immobilizations performed as part of management and veterinary activities conducted in 2016–2020. In total, 528 rhinoceros (130 black rhinoceros and 398 white rhinoceros) were sampled and considered for inclusion in this study. Data collected for individual rhinoceros included date of sample collection, GPS coordinates for capture locations, demographic characteristics (sex, species, and age class), and general health status of the animals prior to immobilization.

During immobilization, whole blood was collected from the auricular or radial vein of the rhinoceros in lithium heparinized vacutainer tubes (BD Biosciences), as previously described (3). Postmortem tissue samples were collected from nine white rhinoceros during necropsy. These samples included submandibular, retropharyngeal, cervical, prescapular, axillary, inguinal, mediastinal, tracheobronchial, and mesenteric lymph nodes and lung, which were frozen at $-20\,°C$ for transport to Stellenbosch University for further laboratory testing under biosafety level 3 conditions, as previously described (3).

All living animals ($n = 519$) were immobilized by wildlife veterinarians for management, or by other approved procedures according to KNP's Wildlife Veterinary Services' standard operating procedures for the capture, transportation, and maintenance in holding facilities of wildlife (South African National Parks). Ethical approval for this project was granted by the Stellenbosch University Animal Care and Use Committee (ACU-2020-19019), and a section 20 research permit was issued by the Department of Agriculture, Land Reform and Rural Development (DALRRD; 12/11/1/7/2).

Study Design and Study Population. A cross-sectional retrospective study design was used to identify factors associated with *M. bovis* infection in rhinoceros from KNP. An individual rhinoceros from the sampled source population (described above) was included in this study if 1) a blood sample was obtained from the individual during immobilization, or tissues were sampled from the individual at necropsy, and 2) it was sampled while free ranging in KNP or within 2 mo [this is the expected length of time after infection with *M. bovis* within which an immune response is detectable using QFT-IGRA (52)] after translocation out of the park to a quarantine location. A total of 475 out of the 528 rhinoceros (90%) from the source population met these inclusion criteria.

Determination of M. bovis infection status. The *M. bovis* infection status of individual rhinoceros was determined using one of the previously described methods: 1) QFT-IGRA (4, 5) (sensitivity = 78%; 95% CI: 52.3 to 93.5%: specificity = 92%; 95% CI: 63.9 to 99.8%) or 2) mycobacterial culture for *M. bovis* isolation from a (postmortem) tissue (3, 69) with RD-PCR for *M. bovis* species confirmation (26). QFT-IGRA is a standard method for diagnosis of active or latent *M. tuberculosis* infection in humans (56, 70), and for MTBC infection in animals, including rhinoceros (4, 5, 21, 52). A rhinoceros was classified as *M. bovis* infected if it had a positive IGRA result (antigen-specific TB response ≥ 21 pg/mL) or positive BACTEC mycobacterial growth indicator tube (MGIT) culture result with subsequent PCR identification of *M. bovis.* An individual was assigned a negative infection status if it had a negative IGRA result (antigen-specific TB response ≤ 21 pg/mL, mitogen response ≥ 21 pg/mL, nil response ≤ 21 pg/mL) and, if conducted, any MGIT culture result with subsequent PCR that did not identify the presence of *M. bovis*. Individuals for whom the *M. bovis* status could not be defined according to these criteria were classified as "unknown" infection status.

A small number of rhinoceros ($n = 38$) had multiple immobilizations and QFT-IGRA results during the study period. For these individuals, a negative infection status was assigned if all test results (QFT-IGRA and mycobacterial culture, if applicable) were negative. A positive infection status was assigned if any of the tests (QFT-IGRA, and/or mycobacterial culture with RD-PCR confirmation of *M. bovis*) were positive. Rhinoceros were considered positive for *M. bovis* infection on the date of the first positive test result. Importantly, data assigned to each of the individuals in this subset were associated with a single GPS point location at which the animal was sampled–either the point of capture at which an *M. bovis*–infected individual first tested positive for *M. bovis*, or, for individuals that consistently tested negative for *M. bovis*, a randomly selected point from their multiple capture locations.

Evaluated Risk Factors. Risk factors hypothesized to be associated with *M. bovis* infection in black and white rhinoceros populations from KNP (1) were evaluated in this study based on availability of data at each individual's sampling event. For *M. bovis*–positive rhinoceros that were sampled multiple times, the

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immobilization date (and corresponding data) associated with the first positive sample was included. For *M. bovis*–negative rhinoceros that were sampled multiple times, a single date (and corresponding data) was randomly selected from among all of that individual's capture dates. Evaluated factors are further described below and include the following 13 variables: species, sex, age class, orphan status, health status at time of sampling, sampling year, sampling season, nearest permanent water source type, distance to nearest permanent water source, distance to the nearest *M. bovis*–infected rhinoceros, number of nearby kudu herds, number of nearby buffalo herds, and buffalo density.

Demographic, health, and temporal risk factors. Demographic factors selected and evaluated in this study included species (white or black rhinoceros), sex (male, female), and age. Age was estimated by field veterinary staff and categorized as follows: adult (>7 y), subadult (>2 y to 7 y), and calf (0 y to 2 y). Calves were further classified as orphaned or with their mother at the time of sample collection. Health status at the time of sampling was assessed by veterinary staff as normal or abnormal. Examples of conditions associated with abnormal health status included poor body condition, visible injuries, or any treatment undergone for illness or injury at the time of sampling. The health status variable was used to determine whether there was an association between *M. bovis* infection and health status. Temporal factors like year of sampling (2016–2020) together with rainfall season, that is, dry (March–August) or wet (September–February), were evaluated.

Spatial risk factors. Spatial risk factors were based on a single GPS point for each rhinoceros' immobilization location plotted onto a map using geographic information system software (GIS; ArcGIS Pro, version 2.8; Environmental Systems Research Institute). Spatial data were further processed and evaluated in the GIS software. Rhinoceros that did not have a GPS point recorded ($n = 4$) were omitted from the spatial analyses.

Spatial data describing the distance between the rhinoceros capture point and the nearest water source, or other *M. bovis*–infected rhinoceros were summarized. Risk of *M. bovis* infection was then evaluated as a function of distance (continuous predictor).

A circular polygon buffer was placed around each rhinoceros capture location to approximate a crude home range, in which exposure to African buffalo and greater kudu, which are known bTB maintenance hosts (14), could occur. The radius of the home range was derived from a subset of rhinoceros ($n = 38$) with GPS coordinates from multiple immobilization events occurring within a maximum of 3 y of each other. The distribution of distances between pairs of capture points for the same individual ($n = 70$ total pairs of capture points for the 38 rhinoceros individuals; 4 individuals had four immobilization events, 7 individuals had three immobilization events, and 27 individuals had two immobilization events) was examined, and 95% of pairwise distance observations occurred within 23 km of each other. This distance served as an approximation of the upper limit of the distance that a rhinoceros would travel; however, it is assumed that most of the movement probably occurs within a smaller core area (3, 41, 68) of unknown size. Therefore, the size of the circular home ranges was varied to include radii at 75%, 50%, and 25% of the maximum, corresponding to 17.25, 11.5, and 5.75 km, respectively. Variables characterizing relative exposure to African buffalo and greater kudu (further described below) were summarized for each of the four circular home range sizes.

African buffaloes in home range. Two different spatial data layers were available to estimate the presence and density of African buffaloes in each rhinoceros' assigned home range. The first dataset was a zero-inflated Poisson model generated by Hughes et al. (71) for prediction of buffalo density per square kilometer across KNP. The predicted buffalo density map was overlaid with rhinoceros home ranges to derive an estimated buffalo density (per square kilometer) value for each rhinoceros as a proxy for *M. bovis* exposure due to the presence of these maintenance hosts (9, 10, 14, 72).

The second dataset was obtained with permission from SANParks GIS Scientific Services, and contained buffalo census data (describing distribution of herds and individuals) that were collected across KNP in 2015 and 2017 using aerial line transect sampling and distance analysis methods, as previously described (73). The census data for the 2 y were combined into a single mapped data layer, which was applied as a crude estimate of buffalo distribution in KNP. The combined census data were then overlaid with the rhinoceros' home ranges to estimate the number of buffalo individuals and herds within the rhinoceros home range.

Greater kudu in home range. Overlap of rhinoceros distribution with kudu was evaluated because greater kudu are considered *M. bovis* maintenance hosts (14). Census data on greater kudu were collected in three separate years (2014, 2016, and 2017), using aerial line transect sampling and distance analysis methods as previously described (73), and made available by SANParks GIS Scientific Services. The three datasets were combined into a single mapped data layer, providing a crude measure of kudu distribution in KNP. The combined kudu census data were then overlaid with each rhinoceros' home range to estimate the number of kudu individuals and herds that each rhinoceros may have been exposed to.

Proximity to water source(s). Mapped datasets of the rivers and water holes in KNP were provided by SANParks GIS Scientific Services. The river dataset described main and secondary rivers and was compiled in 2018 using older data sources in combination with National Geo-spatial Information aerial imagery (74). The water hole dataset described the location of available water holes in KNP, updated through June 2016.

The distance (kilometers) between each rhinoceros' capture location and the nearest water source (river or water hole) was determined in the GIS; this value was used to represent the proximity of each rhinoceros to water. This variable was tested based on the hypothesis that aggregation of infected hosts at available water sources may result in increased infection exposure of susceptible hosts living in close proximity to these water sources, either through direct interactions with infected hosts or due to mycobacterial loads shed into the environment. The nearest water source type (river or water hole) was also evaluated as an independent risk factor.

***M. bovis* status of nearby rhinoceros.** A continuous variable was created to evaluate whether the risk of *M. bovis* infection was a function of the infection status of other nearby rhinoceros. All rhinoceros capture location points were plotted in ArcGIS, and the infection status of each animal was determined. The distance (kilometers) from each study subject to the nearest *M. bovis*–positive rhinoceros was then determined with ArcGIS and ascribed to the study subject.

Data Analyses. Apparent *M. bovis* infection prevalence was estimated for the full KNP study population (number of test-positive rhinoceros/total number of study rhinoceros), as well as within different ranger sections (75) and different ecozones (number of test-positive rhinoceros in specific area/total number sampled in specific area) (76). Within ranger sections and ecozones, prevalence calculations were limited to the areas where >10 animals were sampled. These apparent prevalence values were then adjusted to account for the sensitivity and specificity of the IGRA (sensitivity = 78%; 95% CI: 52.3 to 93.5%: specificity = 92%; 95% CI: 63.9 to 99.8%), thereby estimating true prevalence, using the following equation: Estimated true prevalence = (apparent prevalence + IGRA specificity – 1)/(IGRA sensitivity + IGRA specificity – 1) (based on formulas implemented in ref. 27). Prevalence values were compared across ranger areas and ecozones using Fisher's exact tests.

Differences in geographical distribution of *M. bovis* infection were further explored using Kulldorff's spatial scan statistic (28). The statistic was applied using a Bernoulli based model and SatScan software (version 10.0). SatScan is a trademark of Martin Kulldorff and developed under the joint auspices of Martin Kulldorff, the National Cancer Institute, and Farzad Mostashari of the New York City Department of Health and Mental Hygiene (28). The methods identify significant case clustering by moving a circular window over the geographic area; the maximum spatial cluster size was set to half of the population. For this statistic, the null hypothesis assumed that the relative risk of *M. bovis* infection is the same inside the geographic area compared to outside. Significance was determined by comparing likelihood ratio tests from 999 iterations of a Monte Carlo simulation. We performed this evaluation among all rhinoceros, and then among black and white rhinoceros separately.

Univariate logistic regression was used to screen for associations between each factor and *M. bovis* infection (Tables 2 and 3). Crude ORs, 95% CIs, and type III Wald's P values were estimated. Evaluation of the association between *M. bovis* infection status and orphan status was completed only within the subset of calves. Functional forms of continuous variables were determined by fitting a logit-transformed Loess curve for single-variable models. Natural log transformations were used for covariates that were not normally distributed. If there was evidence of nonlinearity in the logit, then the variable was categorized into quartiles. Important demographic covariates, potential effect modifiers, and associations with $P \leq 0.2$ were further examined in multivariable analyses.

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The three factors derived from rhinoceros areas of exposure (i.e., number of kudu herds, number of buffalo herds, buffalo density) were further evaluated to determine the optimal spatial scale for each variable separately. For each variable, a single radius distance for area of rhinoceros exposure was selected for further evaluation in the multivariable model. The chosen distance was based on the strongest association and the best-fitting single-variable logistic regression using the Akaike information criterion (AIC) statistic (77).

Multivariable logistic regression analyses evaluated associations between multiple factors and *M. bovis* infection. A backward stepwise approach was used to fit models that included species and demographic factors (age and sex) as well as those that met inclusion criteria ($P \leq 0.2$). Effect modification was evaluated by including an interaction term between plausible effect modifiers (species, age, and sex) and the other factors in the model. We also examined whether water type was an effect modifier of the association between distance to the nearest water source and *M. bovis* infection. Competing models with similar predictors were chosen based on the AIC. The final model included covariates (species, age, and sex) as well as other significant risk factors. Since significant interaction between species and year of sampling with *M. bovis* infection was identified in the final multivariable model with all rhinoceros, the same model was further explored for black and white rhinoceros separately, to describe potential differences in risk factors between species.

Spatial data processing and analyses were performed in ArcGIS (version 2.8), except for tests of spatial clustering performed with SatScan, as described above. Statistical analyses were performed in R (version 4.0; R Core Team); true prevalence was calculated with the package EpiR with the function epi.prev (27), and univariate and multivariable models were fit with the glm function (78). Associations with $P < 0.05$ were considered statistically significant.

Data Availability. Summarized data are included in the manuscript and supplementary information. Rhinoceros data are highly sensitive due to the ongoing crisis of poaching for rhinoceros horn and data restrictions apply.

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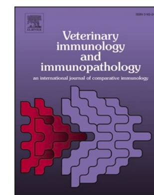

Short communication

Reduced capability of refrigerated white rhinoceros whole blood to produce interferon-gamma upon mitogen stimulation

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ABSTRACT

Ante-mortem surveillance for *Mycobacterium bovis* (*M. bovis*) infection in the Kruger National Park (KNP) rhinoceros population currently relies on results from the QuantiFERON-TB Gold (In-Tube) Plus (QFT)–interferon gamma (IFN-γ) release assay (IGRA). However, same-day processing of rhinoceros blood samples for this test is a logistical challenge. Therefore, a pilot study was performed to compare mitogen-stimulated and unstimulated IFN-γ concentrations in plasma from rhinoceros whole blood processed within 6 h of collection or stored at 4°C for 24 and 48 h prior to incubation in QFT tubes. Replicate samples of heparinized whole blood from seven subadult male white rhinoceros were used. Results showed no change in IFN-γ levels in unstimulated samples, however the relative concentrations of IFN-γ (based on optical density values) in mitogen plasma decreased significantly with increased time blood was stored post-collection and prior to QFT stimulation. These findings support a need for same-day processing of rhinoceros blood samples for QFT-IGRA testing as per the current practice. Further investigation using TB-antigen stimulated samples is warranted to properly assess the impact of blood storage on TB test results in rhinoceros.

1. Introduction

The survival of white rhinoceros (*Ceratotherium simum*) populations is threatened by poaching, habitat loss, drought, and potentially infectious diseases, such as bovine tuberculosis (Miller et al., 2018; Dwyer et al., 2020). *Mycobacterium bovis* infection, which is the primary cause of bovine tuberculosis (TB), is present in African rhinoceros in Kruger National Park (KNP), South Africa (Miller et al., 2018; Goosen et al., 2022). Ante-mortem surveillance of *M. bovis* infection in this population currently relies on an in vitro cytokine release assay called the QuantiFERON-TB Gold (In-Tube) Plus (QFT)–interferon gamma release assay (IGRA), which has been recently validated for white rhinoceros (Chileshe et al., 2019a, 2019b). This process requires that heparinized whole blood samples collected from immobilized rhinoceros are transported to laboratories and processed the same day to perform whole blood incubation in QFT tubes.

This requirement is a logistical challenge for using the QFT-IGRA for TB detection. Rhinoceros in national parks and game reserves are often located in remote areas, without easy access to laboratory facilities or sample transport options, or with inconsistent availability of personnel to process these samples timeously upon their arrival at laboratories. Therefore, it is important to investigate the impact of extended whole blood storage (at 4 °C to mimic transport conditions/extended delays) on the ability of immune cells to produce interferon-γ (IFN-γ) when stimulated in vitro. The goal of this pilot study was to measure and compare mitogen-stimulated IFN-γ production in rhinoceros whole blood processed within 6 h of collection, to that in replicate samples stored at 4°C for 24 and 48 h prior to stimulation. These results will inform whether transport/processing delays could impact QFT-IGRA results in rhinoceros.

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2. Materials and methods

2.1. Animals

Whole blood samples were opportunistically collected from the metacarpal vein of seven clinically normal subadult male white rhinoceros that were chemically immobilised by experienced wildlife veterinarians in Kruger National Park (KNP), South Africa, for dehorning procedures. Immobilisations were performed according to the Standard Operating Procedures for the Capture, Transportation and Maintenance in Holding Facilities of Wildlife (South African National Parks). Ethical approval for this project was granted by the Stellenbosch Animal Care and Use Committee (SU-2020–19019), and a section 20 research permit was issued by the Department of Agriculture, Land Reform and Rural Development (DALRRD;12/11/1/7/2).

2.2. Whole blood stimulation

Rhinoceros whole blood was collected in 9 mL lithium heparin vacutainers (BD Biosciences, Franklin Lakes, New Jersey, USA), as described by Chileshe et al. (2019a, 2019b). Samples were transported at ambient temperature (approximately 18–23 °C) in a Styrofoam container to the Veterinary Wildlife Services laboratory within 6 h of collection. A 2 mL aliquot of blood was removed (t = 0 h) after arrival at the laboratory and 1 mL added to each of the QFT (Qiagen, Venlo, Limburg, The Netherlands) nil (containing saline), and mitogen (containing phytohaemagglutinin) tubes. To ensure sufficient stimulation, additional pokeweed mitogen (Sigma Aldrich, St. Louis, Missouri, USA) was added to the QFT mitogen tube (10 μL) at a final concentration of 10 μg/mL. Tubes were thoroughly inverted 10 times then transferred to a 37 °C incubator for 24 h. The remaining heparinized whole blood was refrigerated for 24 h at 4 °C, then allowed to warm to room temperature, prior to adding aliquots to a set of QFT tubes, as described above. For the three rhinoceros with sufficient remaining volume of heparinized whole blood, an additional aliquot was stored at 4 °C for 48 h prior to adding the sample to a set of QFT tubes. All QFT tube sets were incubated at 37 °C for 24 h. After incubation, plasma was harvested following centrifugation at 800 x *g* for 10 min, transferred to a 2 mL microcentrifuge tube, and frozen immediately at − 80 °C until testing (completed within 1 month).

2.3. Interferon-gamma (IFN-γ) ELISA

Interferon gamma detection in the nil and mitogen plasma samples was performed using the anti-equine IFN-γ ELISA^{PRO} kit (Mabtech Ab, Nacka Strand, Sweden; custom precoated plate using reagent product 3117 – 1 H – 6). The procedure, as previously described (Chileshe et al., 2019a, 2019b), was conducted with slight modifications; all mitogen plasma samples were serially diluted 1:10, 1:100, 1:1000 and 1:10,000, and nil plasma samples were diluted 1:2, in ELISA sample diluent prior to adding each sample to duplicate wells. The remainder of the assay steps were followed as previously reported (Chileshe et al., 2019b).

2.4. Data analysis

The mean optical density (OD) values for each sample were calculated after subtracting OD_{630} value from OD_{450} value for each well. The mean OD values of the serial dilutions for each rhinoceros was then compared to the working range of OD values of the standard curve in order to select the appropriate dilution for comparison of IFN-γ concentrations in the replicate samples at each time point. This step was performed to use empirical OD values for comparison rather than extrapolating IFN-γ concentrations in the mitogen samples, which were expected to be outside the linear range of the assay. A linear mixed effects model (fit with R statistical software (R Core Team, 2020) using packages lme4 (Bates et al., 2015) and lmertest (Kuznetsova et al., 2017) was then used to compare mean OD values in mitogen plasma from whole blood stored at 4 °C for 0, 24, and 48 h. The overall model outcome was the mean OD value at the selected dilution, and the main fixed effect was storage time. Random effects for intercept were included for each animal to account for repeated measures on the same individual. Likelihood ratio tests compared models with and without the fixed effect of storage time to generate a p-value. A p-value < 0.05 was considered statistically significant.

3. Results and discussion

The mean OD results for unstimulated (nil) rhinoceros samples, diluted 1:2, were all near zero (OD < 0.01), which was below the assay's limit of quantification based on IFN-γ concentration (LOQ = 7.8 pg/mL; Chileshe et al., 2019b). These results were similar at all three storage time points for all individual rhinoceros. Since these results indicated that there was no production of IFN-γ in the unstimulated rhinoceros samples, no further analyses were undertaken. Therefore, refrigerated storage of rhinoceros whole blood for up to 48 h did not appear to affect background levels of IFN-γ.

The mitogen sample mean OD values were all significantly higher than mean OD values for the corresponding nil samples (Supplementary Figure 1), which suggested stimulation of IFN-γ production in blood processed after all storage time points. To compare empirical measurements of IFN-γ production in mitogen samples across time points, the dilution factor resulting in an OD value in the range of 0.07–1.00 was selected for each set of samples from an individual rhinoceros. The dilution factor chosen was 1:100 plasma dilution for two rhinoceros and 1:1000 plasma dilution for five rhinoceros. Since the dilution factor was kept constant for a given individual rhinoceros, selecting different dilution factors for different rhinoceros did not affect the comparisons. Mean OD values for each set of mitogen samples from an individual rhinoceros decreased as whole blood storage time increased (Fig. 1). The results from a linear mixed model, that included storage time as the fixed effect and individual rhinoceros as a random effect, are shown in Table 1. These findings confirmed that there was a significant decrease in mean mitogen sample OD values when whole blood was stored at 4 °C for 24 and 48 h prior to stimulation (−2 log likelihood of the reduced model containing the intercept only compared to the full model with time as a fixed effect = 13.63; $\chi^2 = 12.75$, $p = 0.002$). Therefore, these pilot data suggest that storage of rhinoceros whole blood at 4 ºC for 24–48 h may impact the ability of immune cells to produce IFN-γ when stimulated in vitro.

A diagnostic cut-off value for mitogen induced interferon gamma responses in rhinoceros has been previously determined (Chileshe et al., 2019a, 2019b) to be > 84 pg/mL, although IFN-γ concentrations are typically greater than 400 pg/mL. All specimens in this study met this criterion at each time point, indicating that processed blood retained a sufficient, albeit reduced, ability to produce IFN-γ upon mitogen stimulation. Antigen-specific IFN-γ concentrations in *M. bovis* sensitised rhinoceros have ranged between 22 and 700 pg/mL (Chileshe et al., 2019b; Dwyer et al., 2022), and were usually lower than Mit-stimulated IFN-γ concentrations. One of the limitations of this study was the inability to select known *M. bovis* sensitized rhinoceros at the time of sample collection since the animals were free ranging. Therefore, data are unavailable to determine whether delays in processing decreases IFN-γ production enough to change the test positive to negative classification; this would require further investigation to confirm.

The rationale for this pilot study was based on the limited resources available to veterinary staff collecting and transporting samples in the context of Kruger National Park and other areas in South Africa, as well as incorporating the manufacturer's guidelines for use of the QFT system with human blood (Qiagen, 2019) for IGRA. These state that human blood samples should be kept at 17–25 °C, for a maximum of 12 h, or transferred to storage at 2–8 °C within 3 h of collection for a maximum of 48 h prior to processing. However, limited studies conducted in

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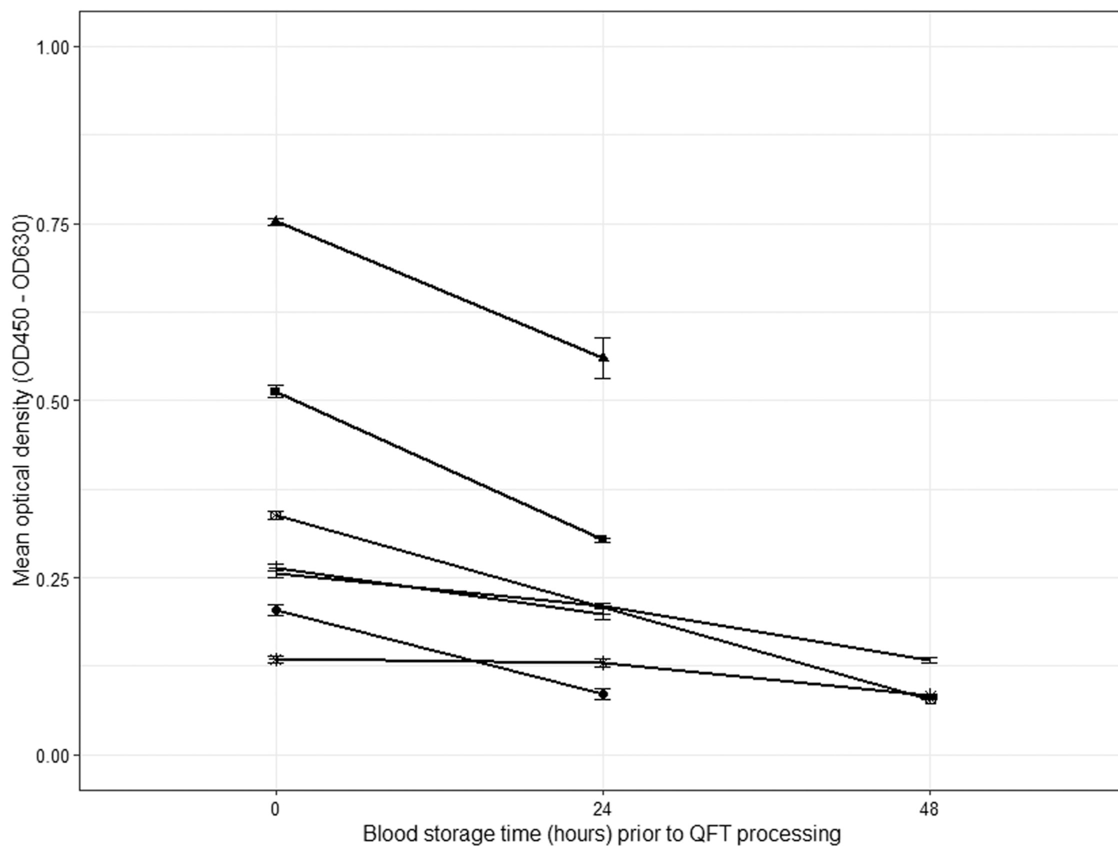

Fig. 1. Mean optical density (OD) results for plasma harvested from QuantiFERON-TB Gold (In-Tube) Plus (QFT) mitogen tubes and measured in the equine interferon-gamma (IFN-γ) ELISA are shown. A fixed plasma dilution factor for each of seven white rhinoceros (represented by different symbols) was selected and OD values shown for each whole blood storage time point (0, 24, 48 h at 4 °C prior to stimulation). Error bars indicate standard deviation across two replicates for each mean OD result. Note: for one individual, there was insufficient plasma volume to complete the IGRA on the Nil and Mit samples from the 24 h test point; hence, only the 0 and 48 h timepoint samples could be tested for this animal.

Table 1
Linear mixed model of the relationship between mean optical density (OD) values of plasma from QuantiFERON-TB Gold (In-Tube) Plus (QFT) mitogen-stimulated rhinoceros whole blood screened with an equine interferon-gamma (IFN-γ) ELISA, and the time blood was stored at 4 °C prior to stimulation (n = 17 observations). Random effects for intercept were included for individual animals.

	Coefficient	Standard error	df[$]	p[%]	-2 log likelihood[*]
Intercept	0.35	0.065	8		7.251
24 h storage vs. 0 h	-0.11	0.029	9	0.004	
48 h of storage vs. 0 h	-0.17	0.039	9	0.002	

[$] df – degrees of freedom;
[%] p – p-value for fixed effect;
[*] Storage time was a significant indicator of mean OD value in the uniformly diluted mitogen plasma samples, as determined by comparing the − 2 log likelihood statistic from this model to that of a reduced model containing the intercept and random effects only (−2 log likelihood of the reduced model = 13.63; $\chi^2 = 12.75$, p = 0.002).

humans and other animal species have shown that delays in processing of blood samples (kept at ambient temperature) for antigen and mitogen stimulation can result in compromised viability of cells, and therefore, a reduced capacity for cytokine production in the stimulated sample (Doherty et al., 2005; Gormley et al., 2006; Smith et al., 2009). For example, a study conducted on human blood found that the number of spot-forming cells (T-cells) detected using the ELIspot assay was significantly reduced in samples where processing delays of 4 h (at ambient temperature) occurred, compared to immediately processed aliquots (Smith et al., 2009). A similar study showed that 2-hour delays in processing human blood samples (held at ambient temperature) had a negative impact on the production of IL-4 and IFN-γ following immune stimulation (Doherty et al., 2005).

Various other studies in humans and animals have reported similar negative impacts on the sensitivity of cytokine release assays with delayed sample processing (Gormley et al., 2006). One study showed that delays in sample processing resulted in a significant increase in the expression of several cytokines (e.g., IL-1, IL-6, and IL-8) measured in unstimulated blood (Duvigneau et al., 2003). In this case, both the delay in processing (h) and the temperature at which samples were stored prior to processing (4 °C or ambient temperature) had an impact on the measured cytokine expression. Interestingly, the effects differed according to the cytokine measured.

Conversely, other studies have shown no impact of delays in sample processing on sensitivity of cytokine release assays (Gormley et al., 2006). Since the effect of storage of blood at different temperatures on cytokine concentration appears to differ according to the host context, a pilot study using rhinoceros whole blood was necessary to determine how sample processing delays would affect IFN-γ production using conditions relevant to the rhinoceros QFT-IGRA. Due to the variability of ambient temperatures under field collections and transport, a single practical temperature condition was used, after receiving samples at the laboratory. In the current study, whole blood samples were refrigerated at 4 °C for 0–48 h prior to processing in accordance with the QFT manufacturer guidelines for human blood. Specifically, this study was designed to mimic conditions in which blood is collected in the field,

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transported to the clinic at ambient temperature within 6 h, and then placed on ice packs for shipment to a laboratory for further processing. In South Africa, many wildlife veterinarians would not have access to equipment required for initial sample processing and therefore, would need to rely on a courier or other service to get the samples to a laboratory.

The comparison of IFN-γ production in mitogen-stimulated rhinoceros whole blood in the present study used mean OD values rather than IFN-γ concentrations extrapolated from the standard curve. This approach was selected because the mitogen samples contained high levels of IFN-γ that were beyond the linear range of the assay, which would lead to the extrapolated concentrations having greater variability and potential inaccuracy. Such variability could bias the results towards not showing a change in assay performance with blood storage time when there truly was one.

To minimize this effect and use empirical values, the mitogen samples were serially diluted. The dilution factor for each rhinoceros, for comparison of replicate samples over storage time points, was selected based on a mean OD value in the working range of the assay. Therefore, empirical changes in OD values (rather than extrapolated concentrations) were compared between time points within an individual rhinoceros sample set, which created a more sensitive approach for detecting change in relative IFN-γ concentrations.

A limitation of this study was that samples were used from rhinoceros with unknown *M. bovis* infection status, and therefore, the impact of blood storage on antigen-specific IFN-γ results could not be evaluated. Since the concentrations of antigen-specific IFN-γ are typically lower than that of the mitogen-stimulated samples (Chileshe et al., 2019b), the decrease associated with blood storage may lead to a false negative result in *M. bovis* sensitised rhinoceros, and reduced assay sensitivity. Therefore, future studies should investigate QFT-IGRA results using stored blood from a cohort of known *M. bovis* sensitized rhinoceros. Based on the preliminary findings of this study, it is recommended that blood samples from white rhinoceros are processed the same day as collection and kept at room temperature prior to stimulations in the QFT platform, to maintain sensitivity of the QFT-IGRA. Delayed processing should be avoided as this could have negative consequences for test interpretation and confidence in reporting results.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetimm.2022.110485.

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