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SEROLOGICAL EVIDENCE OF *COXIELLA BURNETII* INFECTION IN THE WHITE RHINOCEROS (*CERATOTHERIUM SIMUM*) IN SOUTH AFRICA

Kyle A. Donnelly, DVM, MPH, DACZM, Michele A. Miller, DVM, PhD, DECZM, Douw Grobler, BVSc, MScDr, Peter Buss, BVSc, MMedVet, PhD, Charles van Niekerk, BVSc, Léanie Kleynhans, PhD, Tanya J. Kerr, PhD, and Scott B. Citino, DVM, DACZM

Abstract: Coxiellosis, or Query (Q) fever, a disease caused by the intracellular bacteria *Coxiella burnetii*, was recently described in a managed breeding herd of white rhinoceros (*Ceratotherium simum*) in the southeastern United States. Clinical disease often results in abortion and could represent a conservation challenge for this species. In addition to the reproductive and herd management consequences, coxiellosis is also a zoonotic disease. Infection or clinical disease in any free-ranging rhinoceros species in a national park setting has not been previously described. In this study, evidence of prior infection was measured by immunofluorescent antibody titers in 89 serum samples collected from white rhinoceros within private reserves and a national park in South Africa. Total seropositivity was 48/89 (53.9% [95% CI, 43.6–63.9%]). Animals on private reserves had a seropositivity of 21/51 (41.1% [95% CI, 27.1–55.2%]), and national park rhinoceros had a higher rate of seropositivity at 71.0% [95% CI, 55.9–86.2%] (27/38; $P = 0.004$). Adults had a higher seropositivity compared with subadults ($P = 0.03$). There was no difference in seropositivity between sexes ($P > 0.05$). Results demonstrate that South African white rhinoceros populations are exposed to *Coxiella,* which could result in underrecognized reproductive consequences. Further studies should investigate potential implications for public health and conservation management of this species.

INTRODUCTION

Coxiella burnetii, the intracellular bacteria responsible for Query (Q) fever, was recently described in a managed population of white rhinoceros (*Ceratotherium simum*) in the southeastern United States.[3] This outbreak was associated with late-term abortions, which is typical for other infected species.[21] In addition to the reproductive and herd management consequences, coxiellosis is also a zoonotic disease, causing fever, flu-like symptoms, pneumonia, abortion and/or stillbirth, and endocarditis in people.[13] This disease was first described in 1937; however, it is considered re-emerging due to recent outbreaks in sheep (*Ovis aries*), goats (*Capra aegagrus hircus*), and camels (*Camelus dromedarius*), with associated human illness worldwide.[21]

Diagnosis of *C. burnetii* infection is challenging due to intermittent bacteremia and shedding in feces, milk, and vaginal fluids.[21,22] Serological tests can be used to assess previous infection; however, seropositive results do not indicate active shedding or current infection, and seronegative animals may also shed in some infection stages.[21] Phase I and phase II antibodies are produced in response to changes from smooth to rough lipopolysaccharides in the bacteria.[24] In humans, phase I and phase II antibodies can help differentiate acute and chronic infections, with phase I more strongly associated with chronic infection, and phase II with acute infection.[8] In infected people studied for 1 y, phase II antibody titers peaked at weeks 12 and 13 after the onset of symptoms, while phase I antibodies were lower compared with phase II but steadily climbed over the course of the year.[8] Tests to detect these phase-specific antibodies are commercially available, however their temporal association with infection status in nonhuman species remains poorly characterized. Both antibody phases were identified in the affected US white rhinoceros herd, with results suggesting a temporal correla-

From White Oak Conservation, 581705 White Oak Road, Yulee, FL 32097, USA (Donnelly, Citino); The Department of Science and Innovation-National Research Foundation Centre of Excellence for Biomedical Tuberculosis Research, South African Medical Research Council Centre for Tuberculosis Research, Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, P.O. Box 241, Cape Town, 8000, South Africa (Miller, Kleynhans, Kerr); Douw Grobler Consulting CC, Wildlife Veterinary Services, Silver Lakes, Pretoria, 0054, South Africa (Grobler); Veterinary Wildlife Services, South African National Parks, Kruger National Park, Private Bag X402, Skukuza, 1350, South Africa (Buss), The Ladysmith Animal Clinic, P.O. Box 75214, Lynnwood Ridge, Pretoria, 0040, South Africa (van Niekerk). Present address (Donnelly): Brevard Zoo, 8225 North Wickham Road, Melbourne, FL 32940, USA. Correspondence should be addressed to Dr. Donnelly, (kyleadonnelly@gmail.com).

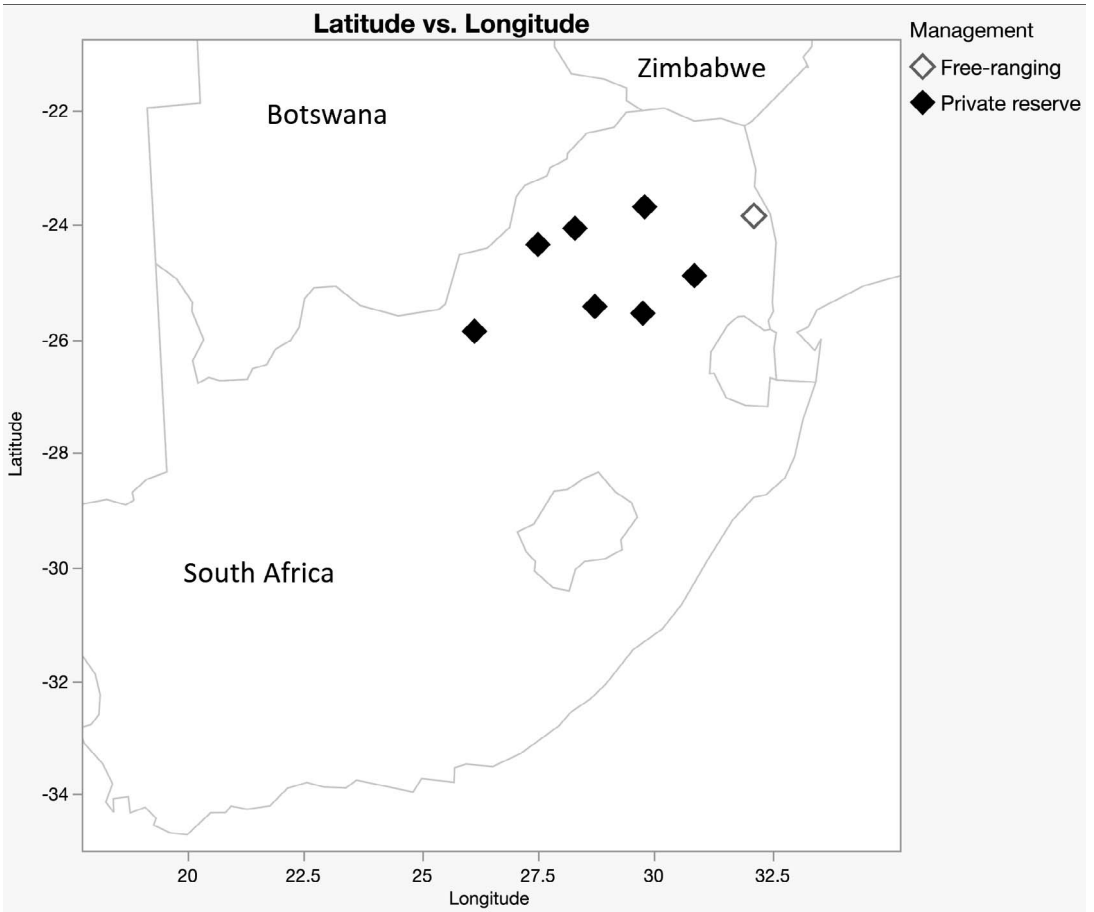

Figure 1. Map of South Africa indicating locations of white rhinoceros (*Ceratotherium simum*) sample collection. Black boxes indicate locations of private reserves ($n = 51$ total white rhinoceros). Grey box indicates location of free-ranging white rhinoceros ($n = 38$) sampled within Kruger National Park.

tion with clinical signs associated with infection.[3] Aside from the outbreak described in the southeastern United States, coxiellosis has not been described in any rhinoceros species in any part of the world, and potential exposure is unknown. Therefore, this work sought to determine if white rhinoceros populations in South Africa have evidence of infection with *C. burnetii*, and to identify potential epidemiologic risk factors including management (private reserve versus national park), age class, and sex.

MATERIALS AND METHODS

Samples ($n = 89$) from a serum bank of white rhinoceros collected during health assessments and/or dehorning procedures were utilized for this retrospective study. Samples were stored at −80°C prior to analysis in a temperature-controlled ultralow freezer. Samples were not defrosted at any time prior to analysis. No hemolysis was present in any sample. All samples were collected between 2017 and 2019 in Kruger National Park (free-ranging rhinoceros) and from private reserves in Cullinan, Lichtenburg, Lydenburg, Middelburg, Polokwane, Thabazimbi, and Vaalwater, South Africa (Fig. 1). Age class was reported as subadults (2–7 y) and adults (≥7 y). Calves were excluded from analyses. Sex was recorded.

Phase I and phase II antibody titers were measured at Stellenbosch University in Cape Town using a commercially available immunofluorescent antibody (IFA) test (Q fever phase I and II) IFA substrate slide, catalog number IF0201, DiaSorin Molecular LLC, Cypress, CA 90630, USA) according to manufacturer's instructions and standard operating procedures from Texas A&M Veterinary Medical Diagnostic Laboratory

Table 1. Overall seropositivity rates among the white rhinoceros (*Ceratotherium simum*) ($n = 89$) across management style (national park vs private reserve), age class (adult vs subadult), and sex (male vs female). Significance between the risk factors is indicated when $P < 0.05$.

Risk factor	Positive/tested (%)	*P*
Overall	48/89 (53.9%)	
Management		
National park	27/38 (71.0%)	**0.0047**
Private reserve	21/51 (41.2%)	
Age class		
Adult	33/52 (63.5%)	**0.032**
Subadult	15/37 (40.5%)	
Sex		
Male	20/42 (47.6%)	0.1029
Female	28/43 (65.1%)	

(College Station, TX 77840, USA). When compared with a standard reference laboratory, this test kit has a 100% sensitivity and 99% specificity. Positive bovine serum controls were sourced from the United States Department of Agriculture National Veterinary Services Laboratories (Ames, IA 50010, USA). Anti-equine fluorescein labeled IgG was used for the conjugate (SeraCare, Milford, MA 01757, USA). The Texas A&M protocol was previously confirmed to detect white rhinoceros antibodies in infected animals.[3] Dilutions of serum samples were prepared in phosphate-buffered saline (PBS). The initial screening dilution was 1 : 64, and samples negative for fluorescence at this dilution were considered negative. Positive samples at the initial screening were prepared at new dilutions of 1 : 64 and two fold serial dilutions up to 1 : 2048 were tested. The final phase I and phase II titers were determined by the last dilution in which specific fluorescence consistent with the positive control was documented. Any positive titer starting at the screening dilution of phase I or phase II antibodies was considered positive. A total of 15 samples were not diluted past the screening dilution due to time and supply constraints. These samples were animals from private reserves, and included 7 subadults, 8 adults, 7 males, and 8 females.

Thirty microliters of diluted serum samples were added to each well. Slides were incubated in a humidified chamber for 30 m at 37°C. The slides were gently rinsed with PBS, and then washed in PBS using a coplin jar for 10–15 m, changing PBS a minimum of two times. Thirty microliters of anti-equine IgG fluorescein isothiocyanate (FITC) diluted 1 : 64 was added to each well, and the incubation and washing process was repeated as previously described. A drop of glycerol, used as a mounting medium, was added to each well and wells were covered with a coverslip. Fluorescence was identified using an Axio Observer 7 inverted microscope (Carl Zeiss, Jena, 07745, Germany). Positive fluorescence was identified based on presence or absence of specific bacterial fluorescence consistent with the positive control.

Statistical software (JMP, Version 13, SAS Institute Inc, Cary, NC 27513, USA) was used to analyze the data. The seropositivity of *C. burnetii* antibodies was calculated from the number of positive samples divided by the total number of samples tested. Ninety-five percent confidence intervals (CI) were calculated. The seropositivity of phase I, phase II, and overall antibody status was compared across the populations by management type (private reserve versus national park), sex, and age (subadult versus adult) using the chi-square test. Titers were log-transformed and normal quantile plots were generated to assess for normality using the Shapiro–Wilk W test. The Wilcoxon rank sum was then used to analyze titers by management type, sex, and age class. Results were considered statistically significant at $P < 0.05$.

RESULTS

Sera from 89 individual white rhinoceros were tested. This cohort included samples from both Kruger National Park ($n = 38$) and private reserves ($n = 51$) and was comprised of both adults ($n = 52$) and subadults ($n = 37$), including 42 males and 43 females. The overall seropositivity was 48/89 (53.9% [95% CI, 43.6–63.9%]), with 47/89 (52.8% [95% CI, 42.5–62.8%]) positive against phase I antibody and 20/89 (22.5% [95% CI, 15.0–32.3%]) positive against phase II antibody (Tables 1 and 2). A total of 19/89 (21.3% [95% CI, 14.1–31.0%]) rhinoceros were positive for both phase I and phase II antibodies. For the entire tested population, phase I titers ranged from 0 to 512 and phase II titers ranged from 0 to 256. Phase I and phase II titers were not normally distributed (Fig. 2). Phase I and II titers in all tested national park rhinoceros were higher than animals from private reserves by Wilcoxon rank sum ($P = 0.004$, $P = 0.03$, respectively). There were no differences in phase I or II titers between sexes or age classes. In seropositive rhinoceros, the median phase I titer was 128 (range 64–512), and the median phase II titer was 64 (range 64–256).

There were 51 rhinoceros tested from private reserves, and 38 from a national park. Seroposi-

Table 2. Seropositivity rates among phase I and phase II antibodies of the white rhinoceros (*Ceratotherium simum*) ($n = 89$) across management style (national park vs private reserve), age class (adult vs subadult), and sex (male vs female). Significance between the risk factors is indicated when $P < 0.05$.

	Phase I antibodies		Phase II antibodies	
Risk factor	Positive/tested (%)	*P*	Positive/tested (%)	*P*
Overall	47/89 (52.8)		20/89 (22.5%)	
Management				
National park	27/38 (71.1%)	**0.0036**	12/38 (31.6%)	**0.0307**
Private reserve	20/51 (39.2%)		8/51 (15.7%)	
Age class				
Adult	33/52 (63.4%)	0.3703	13/52 (25.0%)	0.6121
Subadult	14/37 (37.8%)		7/37 (18.9%)	
Sex				
Male	19/42 (45.2%)	0.0644	9/42 (21.4%)	0.6515
Female	28/43 (65.1%)		11/43 (25.6%)	

tivity in national park white rhinoceros was 71.0% [95% CI, 55.9–86.2%] (27/38), and greater ($P = 0.004$) than seropositivity in rhinoceros from private reserves (41.1% [95% CI 27.1–55.2%] 21/51).

There were 52 serum samples from adult white rhinoceros, and 37 from subadults. Among adults, 63.4% [95% CI, 49.9–77.0%] (33/52) were seropositive, and 40.5% [95% CI, 23.9–57.1%] (15/37) of subadults were seropositive. Adults had a

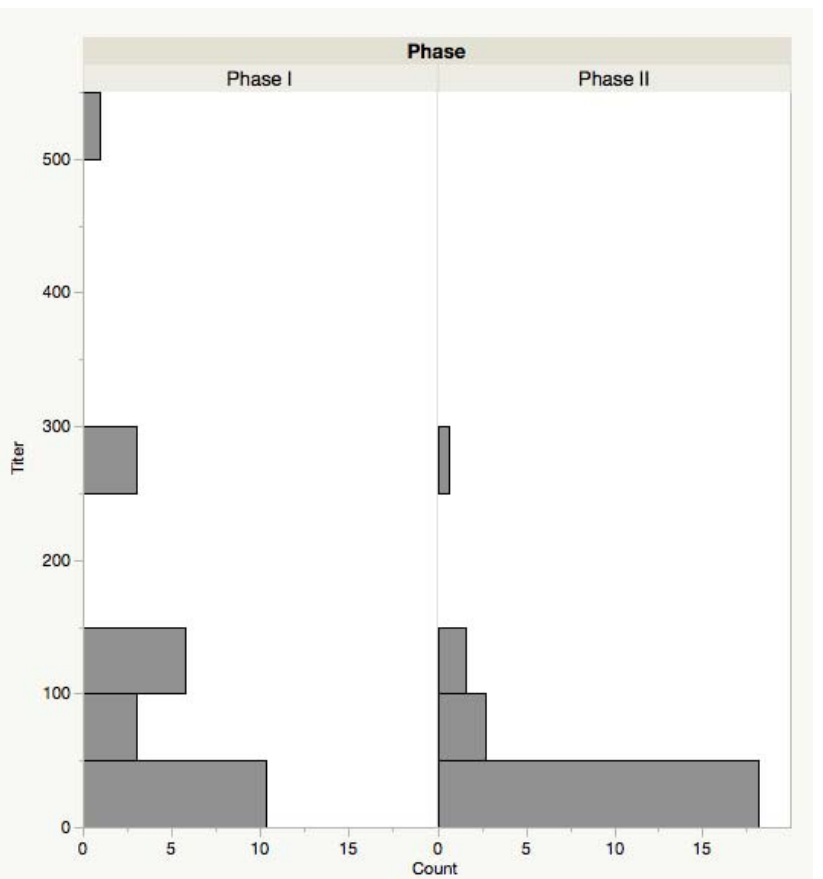

Figure 2. Histogram of phase I and phase II titers of white rhinoceros (*Ceratotherium simum*) ($n = 76$).

higher seropositivity compared with subadults ($P = 0.03$).

Females contributed 43 samples, 42 samples were from males, and 4 samples were from animals without recorded sex. There was no difference in seropositivity in females (65.1% [95% CI, 50.3–80.0%]; 28/43) compared with males (47.6% [95% CI, 31.9–63.4%]; 20/42).

DISCUSSION

Results from this study showed a high seropositivity to *C. burnetii* in white rhinoceros in South Africa. The overall seropositivity was 53.9%, which is higher than reported in other domestic and wildlife species. For comparison, 0.07% of wild saiga antelope (*Saiga tatarica*) were seropositive in Kazakhstan, 13.6% of wild yak (*Bos mutus*) in China, and 14.5% of wild white-tailed deer (*Odocoileus virginianus*) in New York, USA.[14,20,25] In Spain, 15.4% of wild roe deer (*Capreolus capreolus*), 5.6% of wild red deer (*Cervus elaphus*), and 40% of farmed red deer were seropositive.[23] A variety of farmed bovids, caprids, cervids, and ovids in Spain had seropositivity ranging from 1.4% to 23.8%.[6] European wildcats (*Felis silvestris*) inhabiting the same habitat were 33.3% seropositive.[6] Farmed camels, goats, sheep, and cattle in Kenya had seropositivities of 20%, 18%, 13%, and 6%, respectively.[5,15] The comparatively higher seropositivity in white rhinoceros is important because the reproductive implications of coxiellosis could influence the recovery of this threatened species. Given the zoonotic nature of *C. burnetii,* aborted rhinoceros tissue should be handled with caution, and coxiellosis should be considered in the differential diagnosis. The high seropositivity in South African white rhinoceros populations compared with the lower seropositivity in southeastern US population as well as other species studied may be explained by route of transmission and epidemiologic differences in vector and host ecology, as well as overall environmental burden. Additional study on these variables is needed.

Pathological changes associated with coxiellosis in South African white rhinoceros have not been described. There are anecdotal reports of abortion in South African rhinoceros populations on private reserves (Grobler, pers. comm.), however recovery of aborted tissues in South Africa is difficult due to the size of rhinoceros territories and the presence of scavenger species. Late-term abortions were described in a managed population of white rhinoceros in the southeastern United States.[3] Clinical signs and lesions in wild mammals generally paralleled those described in domestic species, including placentitis and reproductive failure.[11]

There was a wide range in titers of phase I and phase II antibodies, reflecting possible variation in acute versus chronic infections. Phase I and phase II titers are used in humans to represent chronic versus acute infection status, respectively.[8] This trend was also noted in a managed white rhinoceros outbreak investigation in the United States.[3] In this population, infected rhinoceros did not maintain phase II antibodies over time, which suggests that animals with both phase I and phase II titers may have been more recently infected.[3] Given that overall seropositivity was significantly higher in adult versus subadult rhinoceros in this study, younger animals may be expected to have higher phase II titers; however, this trend was not identified in this study. Other than higher phase I and II titers in wild rhinoceros compared with those on private reserves, this study did not identify any significant associations with phase I and II titers across age class, sex, or management style. To better determine the phase I and II titer association, cases with known clinical disease would need to be sampled, and younger age classes (i.e. calves) should also be included.

Older animals were significantly more likely to be seropositive, which is a common trend in serosurveys and supports the hypothesis that opportunities for infection increase over the course of an animal's lifetime. There was no significant difference in seropositivity based on sex. Though proximity to livestock appears to be one of the biggest risks for people, the Kruger National Park white rhinoceros population had significantly higher exposure prevalence compared with those in private reserves. Tick-borne disease transmission of coxiellosis is not well-described in people, but *C. burnetii* isolation from ticks has been reported, including ticks sampled in South Africa.[4,18,19] Free-ranging rhinos in the national park typically have a tick burden and there are no interventions to change this. In contrast, depending on the specific management of the private reserve, animals may be treated for ectoparasites during captures or immobilizations. It is hypothesized that these interventions with animals on reserves may result in lower tick burden in the environment. Additionally, there may also be differences in the ecological needs of ticks, e.g. certain vegetation or presence of additional wildlife hosts that may not be as abundant on private reserves. There may be differences in the tick populations in Kruger

National Park versus private reserves that transmit *Coxiella*. Tick sampling from these locations would be needed to better understand their role in disease transmission.

Non–*C. burnetii Coxiella* species are a symbiont in some tick species.[1,9] It is possible that antibodies to these endosymbionts could cross-react in the *C. burnetii* assays, complicating interpretation of true exposure to *C. burnetii;* however, there is limited evidence that these non–*C. burnetii* species are able to infect vertebrate cells.[9,12] There is no evidence that the *Coxiella* tick endosymbiont causes vertebrate pathology.[1,9,12]

Potential routes of infection in rhinoceros are currently unknown. People are typically infected with *C. burnetii* through the inhalation of aerosolized bacteria or ingestion of unpasteurized milk.[2,24] Inhaled bacteria exist in a spore-like form, termed small cell variant, and are responsible for persistence in dust, manure, and the air.[21] Domestic ruminants are considered the most common source of infection for people, though transmission from other domestic animals, including dogs and cats, has also been described.[2,4,16] In contrast to transmission to humans, the sylvatic lifecycle of *C. burnetii* is believed to be primarily tick-borne.[17] One human outbreak described in French Guiana implicated environmental contamination from infected capybara (*Hydrochoerus hydrochaeris*) feces.[7] Natural infection and detection of *C. burnetii* in the semen of a zoo-housed Saharawi dorcas gazelle (*Gazella dorcas neglecta*) suggests horizontal transmission is also possible.[10]

Limitations of this study include a lack of paired clinical data on coxiellosis in South African rhinoceros. Additional data are also needed in black rhinoceros. Future directions include determining the seropositivity of *C. burnetii* in other areas of the United States for a better understanding of the national distribution, and sampling local wildlife and farm animals in the United States and South Africa to investigate levels of local exposure. Sampling ticks for presence of *C. burnetii* may prove a useful determinant for wildlife reservoirs, however the *Coxiella* endosymbiont may complicate interpretation. Genotyping *C. burnetii* isolated from rhinoceros to compare homology between the United States. and South African organisms may be useful, but movement of strains all over the world may limit its utility. Investigation into clinical cases of coxiellosis in free-ranging rhinoceros including testing of aborted fetuses and placenta is warranted. A more comprehensive understanding of the epidemiology of coxiellosis in rhinoceros will inform management practices to reduce transmission, and ultimately conserve the species and protect public health.

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