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SALMONELLA SURVEILLANCE IN A HERD OF ASYMPTOMATIC CAPTIVE BLACK RHINOCEROS (*DICEROS BICORNIS*) USING FECAL CULTURE AND PCR

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Abstract: Feces were collected from captive black rhinoceros (*Diceros bicornis minor*) housed at Disney's Animal Kingdom to examine the frequency of *Salmonella* spp. shedding in asymptomatic animals using enrichment culture and broth culture- polymerase-chain-reaction (PCR) for detection. Three samples per animal were collected during the first week of each month between February 2001 and December 2003. During the study period, six different individual animals from one herd participated in the study, including two growing calves. A total of 550 cultures, using duplicate samples at two different laboratories, and 464 PCR tests were performed. When culture and PCR results were compared by the same laboratory, similar herd prevalence was found (2.4% positive cultures compared with 2.6% positive PCR tests). However, even though tests were performed on replicate samples, not every sample that was positive by culture was positive by PCR and vice versa. These results suggest that using multiple diagnostic methods and increasing the number of samples submitted may increase the likelihood of finding an asymptomatic *Salmonella* shedder. Although all of the rhinos shared the same environment throughout the study period, only four out of the six animals tested shed *Salmonella* spp. even though a minimum of 37 fecal samples were taken from each of the negative animals. Although this study followed a small number of rhinoceros, it suggests that asymptomatic shedding probably occurs more frequently in captive black rhinoceros than was previously believed. The prevalence appears to be similar to that reported for domestic ungulates.

Key words: Black rhinoceros, *Diceros bicornis*, *Salmonella* polymerase chain reaction, *Salmonella* culture, carrier state, asymptomatic shedding.

INTRODUCTION

Bacteria of the genus *Salmonella* are ubiquitous organisms composed of multiple species and serovars. These Gram-negative bacteria can infect nearly all species of vertebrates and present a zoonotic potential. Human illness has been linked with exposure to contact with animals or animal products including exotic species.[11] Since *Salmonella* organisms can remain viable for extended periods in the environment, there is always the potential for exposure and/or infection.[9,10]

Salmonella infections may be inapparent, enteric, or systemic. Typically, the route of exposure is fecal-oral through contact with feces or contaminated feed or water. Other routes include aerosol or inoculation of wounds. The result of exposure usually depends on the number of organisms, status of the host's immune system, and virulence of the particular serotype. A wide spectrum of clinical diseases has been associated with *Salmonella* infection, including acute or chronic enterocolitis, septicemia, osteomyelitis, abortion, and other organ involvement.[4,9,10] Infected animals may recover and eliminate the organism or become carriers. Since carrier animals may harbor and intermittently shed the organism without apparent clinical signs, these individuals present a risk to other animals. Under stress (such as disease, environmental or social changes, treatment with antibiotics), these animals may also develop clinical disease. Unfortunately, there are no currently accepted methods for identifying carrier animals.

Rhinoceroses have been reported to develop clinical salmonellosis resulting in gastroenteritis and fatal septicemia.[6,14] Due to the severe consequences and difficulty treating clinical infections in these species, prevention and control should be the primary focus.

Surveys of Salmonellosis have been performed in multiple species, including rhinoceroses.[1,4,7,8,13] However, some of these reports reflect the number of animals that have positive cultures associated with clinical signs of disease and may not include asymptomatic animals.

This study was undertaken to provide baseline information on fecal shedding of *Salmonella* in one herd of captive black rhinoceros and evaluate diagnostic methods for detection in feces using serial samples.

MATERIALS AND METHODS

Study animals and sample collection

Four adult captive black rhinoceros (two females, two males) and two male calves (born during the

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study period) were included in the study. Fecal collection from the calves did not start until they were approximately 5–11 months old. All animals were fed a formulated herbivore diet, small amount of produce, freshly cut browse, and a mixture of grass and alfalfa hay. During the day, rhinoceros were housed in exhibits with soil, clay wallow, and other natural vegetation, or holding yards. All animals returned to the barn at night.

The study period was conducted between February 2001 and December 2003. Individual rhinoceros fecal samples were collected from fresh dung piles in the stalls in the morning and placed in replicate sterile bags for submission. Three samples were collected from each individual during the first week of each month of the study period.

Salmonella culture

Fecal samples on ice packs were shipped overnight to laboratories. Fecal samples were inoculated onto Tergitol and XLT agar plates and streaked for isolated colonies. A swab containing approximately 1 g of feces was placed into tetrathionate broth. Plates and broth were incubated overnight at 37°C. On day 2, Tergitol and XLT plates were examined for suspect colonies. The swab from the tetrathionate broth was used to inoculate another XLT plate, and then transferred to Rappaport Vassiliadis R-10 broth. The secondary XLT plate and broth were incubated overnight at 37°C. On day 3, agar plates were examined for suspect colonies. The swab from the R-10 broth was used to inoculate a tertiary XLT plate. The plate was incubated at 37°C overnight and examined the next day for suspect colonies.

Salmonella suspect colonies were confirmed using standard biochemical tests, *Salmonella* polyvalent A-1 and Vi antiserum, and/or the API 20E test kit. *Salmonella* isolates were forwarded to the Molecular Diagnostics Laboratory for polymerase-chain-reaction (PCR) confirmation and to the National Veterinary Services Laboratory (NVSL), Ames, Iowa, for serotyping.

Salmonella PCR

Fecal samples were inoculated into tetrathionate broth and incubated overnight. Genomic DNA extraction was performed using the DNeasy kit (QUIAGEN, Valencia, California 91355, USA). A real-time PCR assay was used to identify *Salmonella* genus DNA using the primers and methods modified from Cohen.[2]

Statistical analyses

Comparison of results between PCR test and culture were made using the Chi-squared test with a cutoff value of $P < 0.05$.

RESULTS

In the first phase of the study (February–August 2001), duplicate samples were submitted to two separate laboratories to compare culture results. Feces were collected from five animals including one calf. Unfortunately, no samples were positive by culture during this phase of the study at either laboratory, and there was only one positive PCR result. The positive PCR result was from an asymptomatic adult male rhinoceros in May 2001. The total number of samples submitted was 173 cultures and 87 PCR tests.

From September 2001 through December 2003, positive PCR results were found in 11 of the 377 samples submitted. During this period, 11 samples were culture positive and 11 were PCR positive, out of 377 samples. Of interest, the youngest rhinoceros (No. 6, born November 2001) had six positive fecal cultures and two positive PCR tests between November 2002 and April 2003. All the cultures yielded *Salmonella miami* serotype. During this period, the juvenile rhinoceros was being introduced to the other calf and dam, and being periodically separated from its dam. This animal and the other rhinoceros in the herd remained clinically normal.

Three of the other five animals had greater than one positive culture and/or PCR result during the study period, with positive results usually separated by weeks or months (Table 1). Clinical signs of diarrhea, colic, or other health concerns were not associated with positive fecal cultures or PCR results. All *Salmonella* cultures were serotyped by the NVSL. The predominant serotype was *Salmonella miami* (7/11), with *S. muenchen*, *S. gaminara*, *S. java*, and a group C2 *Salmonella* species, each isolated once.

Table 2 summarizes the results over the entire study period. Although the overall number of positive PCR and culture results was similar, there were only three occasions on which samples were positive in both tests. On other dates, replicate samples were positive by PCR only on nine occasions or positive by culture only on eight occasions. Calculating the overall number of *Salmonella*-positive fecal samples using either PCR or culture would be 20/464 (4.3%), compared with 2.4% or 2.6% for culture or PCR alone, respectively. Although not statistically significant ($P > 0.05$), the discordant test results would have led to fewer positive fecal samples if only one diagnostic technique had been chosen.

Table 1. Summary of dates and animals for positive test results.

Rhino 1 Culture	Rhino 1 PCR	Rhino 2 Culture	Rhino 2 PCR	Rhino 3 Culture	Rhino 3 PCR	Rhino 4 Culture	Rhino 4 PCR	Rhino 5 Culture	Rhino 5 PCR	Rhino 6 Culture	Rhino 6 PCR
			8 Mar 01								
	5 Sep 01						5 Sep 01				
			6 Nov 01								
			6 Dec 01								
						2 Apr 02[a]					
		3 Jun 02[b]									
1 Jul 02[c]											
8 Aug 02[d]											
										5 Nov 02[e]	
	1 Jan 03									1 Jan 03[e]	
										6 Jan 03[e]	
										9 Jan 03[e]	
										4 Feb 03[e]	
						6 Feb 03[e]	6 Feb 03				
										8 Apr 03[e]	8 Apr 03
											8 May 03
			4 Sep 03								
			9 Oct 03								

[a] *Salmonella* group C2 sp.
[b] *Salmonella java*.
[c] *Salmonella muenchen*.
[d] *Salmonella gaminara*.
[e] *Salmonella miami*.

DISCUSSION

Salmonella is often difficult to diagnose from clinical specimens because of intermittent shedding, selective culture requirements, and potentially low numbers of organisms in samples. This may be especially true when testing asymptomatic animals or environmental samples.

In order to maximize the detection of *Salmonella* by culture, replicate fecal samples from a rhinoceros herd were submitted to two commercial laboratories during the first phase of the study period. In addition, a genus specific primer for *Salmonella* was used in a PCR test to increase the likelihood of finding positive samples. This test has been validated for equine fecal samples and has been previously described.[2,3]

Salmonellosis is a serious and potentially fatal infection in rhinoceros. In a survey of U.S. zoological institutions, it was estimated that rhinoceros had a 10% prevalence of *Salmonella* based on culture.[7] Only six of the sixteen animals were reported to be asymptomatic. It is unknown whether rhinoceros can become asymptomatic shedders or carriers.

Fecal cultures do not appear to be the most sensitive method of detecting asymptomatic shedding of *Salmonella*. An estimate of fecal shedding of *Salmonella* (by culture) in the U.S. equine population was 0.8%, and in dairy cows, 5%.[5,13] Reports of *Salmonella* prevalence in captive wildlife species may include both ill and asymptomatic animals. Surveys of multiple species in zoos and rehabilitation centers have reported 6.5% and 4% prevalence, respectively, similar to this rhinoceros herd.[1,12] Therefore, culture appears to detect a similar, but low, prevalence of *Salmonella* shedding in multiple species.

PCR has been used to increase sensitivity and rapid return of diagnostic tests. Genus-specific primers for *Salmonella* have been used to detect DNA in culture and fecal samples.[2] When culture and PCR were compared using equine fecal samples, the PCR method was positive in 71/110 (64%) hospitalized horses, but only 11 cases (approximately 10%) were culture positive.[3] In contrast, outpatient horses had a prevalence of 17.1% (26/152) by PCR, but none were positive by culture. All culture-positive samples were also PCR positive. These results suggest that culture underestimates *Salmonella* shedding, and that stressed or ill animals may have a higher likelihood of shedding.

Unlike previous reports, detection of *Salmonella* by PCR was not significantly higher than by culture in this study (2.6% vs. 2.4%, respectively; $P > 0.05$). The reason for this observation is unknown but may be due to collection and handling artifacts,

Table 2. Summary of *Salmonella* test results.

Test method	Test results (No. positive results/No. total tests)						
	Total	Rhino 1	Rhino 2	Rhino 3	Rhino 4	Rhino 5	Rhino 6
Lab 1, culture	0/99 = 0%	0/22 = 0%	0/22 = 0%	0/22 = 0%	0/22 = 0%	0/11 = 0%	ND[a]
Lab 2, culture	11/451 = 2.4%	2/92 = 2.2%	1/94 = 1.1%	0/78 = 0%	2/76 = 2.6%	0/74 = 0%	6/37 = 16.2%
Lab 2, PCR[b]	12/464 = 2.6%	4/95 = 4.2%	3/97 = 3.1%	0/81 = 0%	2/79 = 2.5%	0/75 = 0%	3/37 = 8.1%

[a] ND indicates not done.
[b] PCR indicates polymerase-chain-reaction.

the presence of inhibitory substances that interfere with the PCR process, or the unequal distribution of organisms in fecal samples from low shedders. Incongruity in culture and PCR results has been documented for Johne's testing and recognized by the U.S. Department of Agriculture (USDA) during their approval process (Sneed, pers. comm.). By using both PCR and culture to screen asymptomatic rhinoceros for fecal shedding, there was an increased likelihood of detection (4.3%) than by using either method alone. Although the difference was not statistically significant, it is clinically important to detect *Salmonella* to make diagnostic and therapeutic decisions in clinical cases, to minimize the risk of transmission to other animals and people, and to decrease contamination of the environment.

The prevalence of *Salmonella* shedding by individual rhinoceros in this study was similar to prevalence found in domestic ungulates.[3,5] It is difficult to compare these numbers owing to the small sample size used in this study and the number of variables that could influence shedding in other herds. Prevalence for domestic ungulates depends on diagnostic techniques, management systems, clinical status of the herd, and other environmental factors. For example, equine prevalence ranged from 0.1% to 11.9% within one study when different variables were evaluated.[13]

The majority of rhinoceros in this herd (4/6) shed *Salmonella* at some time point in the study, although none developed clinical signs. In comparison, at least 31.4% of dairy farms had at least one *Salmonella*-positive cow when the cattle had one sample submitted.[5] When cattle were sampled five times, 90.9% of farms had at least one positive sample. The fact that multiple rhinoceros shed *Salmonella* at different times probably indicates that there was not a single point source that resulted in pass-through to the feces, although a contaminated environment cannot be completely ruled out. During a concurrent study, environmental samples from feed and soil from another enclosure (elephant yard) were tested using culture and PCR during the same period. Only 2 out of 69 samples submitted for PCR and 0/50 culture samples were reported as positive. Therefore, it appears that environmental levels of *Salmonella* were relatively low. There was a predominant serotype (*S. miami*), which may suggest that intermittent shedding may be associated with a carrier state. Factors affecting shedding were not identified in this study, but should be investigated in the future.

CONCLUSIONS

This preliminary study demonstrates that asymptomatic *Salmonella* shedding in rhinoceros can be detected using culture and PCR methods developed for domestic animals. Although the true prevalence of asymptomatic infection and shedding in rhinoceros will be difficult to determine, it may be higher than originally suspected. Because of the difficulty of detecting *Salmonella* in fecal samples, multiple diagnostic techniques should be employed. These results also emphasize that a positive culture or PCR result should not necessarily be interpreted as the cause of disease but evaluated in conjunction with clinical status of the individual.

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