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Full Length Research Paper

Detection of *Salmonella*, *Clostridium perfringens* and *Escherichia coli* from fecal samples of captive animals at the National Zoological Gardens of South Africa

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***Escherichia coli*, *Clostridium perfringens* and *Salmonella* spp. are enterotoxic, ubiquitously distributed bacteria. Diseases caused by these pathogens are well recognized in humans, livestock, companion and zoo animals and can result in morbidity, mortality, and significant economic losses. The aim of the study was to identify and simultaneously amplify enterotoxigenic *E. coli*; *Salmonella* spp. and *C. perfringens* using multiplex polymerase chain reaction (PCR). Bacterial DNA was isolated from faeces of captive animals, birds (n=17), primates (n=6) and antelopes (n=26) using the Zymo Research Faecal DNA kit. The overall frequency of bacterial isolation was 8%. This low frequency could have resulted from the highly standardized management carried out at the zoo as well as other factors such as lack of contact with other animals and spatial buffers.**

Key words: *Clostridium perfringens, Escherichia coli, Salmonella*, multiplex PCR.

INTRODUCTION

Animals kept at the zoo are usually bred in captivity, acquired from other facilities or captured in the wild and have been reported to be associated with bacterial infections, which are major health hazard, as their excretion result in contamination of the environment leading to morbidity and mortality of other animals as well as significant economic losses for the zoo (Gopee et al., 2000; Thachil et al., 2010; Adesiyun et al., 1998). Bacterial pathogens such as *Salmonella* spp., *E. coli* and *C. perfringens* are zoonotic and can therefore be interchanged between zoo keepers and captive wildlife (Gopee et al., 2000; Zhao et al., 2001). These pathogens are commonly associated with outbreaks of diarrhoea, septicaemia, enteritis, fever, dysentery, abortion and numerous other infections individually or in association (Gyles and Henton, 2004). The current study was aimed at identification and simultaneous amplification of enterotoxigenic *E. coli*; *Salmonella*; and *C. perfringens* using multiplex PCR from faeces of animals at the National Zoological Gardens of South Africa, Pretoria (NZG). The zoo has been in existence for 112 years and still there is a dearth of information on the prevalence of enteropathogens in the animals kept at the facility. Probable sources of infection for zoo animals are fruits and foods indiscrimately provided by zoo keepers, visitors, native rodents and wild small birds which gain access to the enclosures (Goope et al., 2000).

Salmonella spp. and *E. coli* are closely related Gram-negative bacteria of the proteobacterial family Enterobacteriaceae (Coetzer and Tustin, 2004; Vanderhust and Hunter, 1992) whereas *C. perfringens* is a Gram-positive, histiotoxic bacterium belonging to the family Clostridiaceae (Bagge et al., 2009; Coetzer and

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Tustin, 2004; Daly and Rotert, 2007). The genus *Salmonella* has two species that is, *S. bongori* and *S. enterica* with more than 2,600 serovars identified in this genus to date (Edwin et al., 1992) and they have become increasingly significant due to their ubiquitous distribution, wide host range, complex pathogenesis and their complicated epizootiology involving humans, animals and the environment (Salehi et al., 2010). Non-pathogenic *E. coli* is present in the intestinal tracts of vertebrates and forms a normal component of the gut microflora (Ozaki et al., 2011), however there are several subtypes that can become pathogenic. The most commonly reported diarrhoeagenic *E. coli* strains are the enterotoxigenic *E. coli* (ETEC), defined as strains that produce one or more enterotoxins that are either heat-labile (LT) enterotoxin or heat-stable (ST) enterotoxin (Ahmadi et al., 2000). *C. perfringens* can be found in the intestinal tracts of humans, domestic and wild animals as well as of insects and as a normal component of decaying vegetation, marine sediment and in the soil, the infecting organisms may be from an exogenous source, but are often endogenous (Kalender et al., 2005). In small amounts, these bacteria are generally harmless in the intestine, but under the right conditions they may grow and proliferate, resulting in enterotoxemia (Daly and Rotert, 2007).

Such bacterial pathogens have the ability to acquire multiple resistance genes (Ford et al., 2003) and cause major diseases as well as a number of minor diseases, the prevention of which depends largely on the efforts of medical, veterinary and agricultural bacteriologists (Singleton, 2004).

MATERIALS AND METHODS

Study area and sampling method

The study was conducted throughout September 2010 at the National Zoological Gardens of South Africa (NZG) situated in Pretoria (25°44.349' S, 28°11.329' E) [http://www.nzg.ac.za/map/index.php]. The present study was conducted to document information on the existence of *Salmonella* spp., *E. coli*'s heat labile (LT) and heat stable (STa and STb) strains as well as *C. perfringens* using a multiplex PCR system. A total of forty nine fresh fecal samples representing birds (n=17); antelopes (n=26); and primates (n=6) were collected from the animal enclosures at the zoo. The sampled animals are listed in Tables 1-3. The experimental design was in such a way that samples should be collected from members of species that had five or more representatives in an enclosure. The freshly voided fecal samples were collected using sterile wooden applicators and placed into sterile transport media for processing in the laboratory.

DNA extraction and PCR amplification

DNA was isolated using the Zymo Research Faecal DNA Kit (Zymo Research Corporation, South Africa) and the bacterial DNA detected with the Porcine Diarr-B PCR Detection Kit according to the manufacturer's instructions (Seeplex, USA). The multiplex PCR kit simultaneously amplifies five bacterial pathogens that is, *Salmonella* spp., *C. perfringens* and *E. coli*'s LT, STa and STb strains based on primers designed from the 16S rRNA gene coding region of each bacterial pathogen (Oh et al., 2009). Amplification was performed in a thermal cycler and the cycling conditions were as follows: initial denaturation at 94°C for 15 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 90 s, elongation at 72°C for 90 s, and final extension 72°C for 10 min according to the GeneAmp PCR system 9700 of Applied Biosystems. Amplified products were subjected to electrophoresis at 100V on a 2% agarose gel stained with gel red and visualized under UV light.

RESULTS

PCR positive results were all obtained from the bird samples (n=17). Two samples (from a vulture and a bald ibis) out of seventeen were positive by PCR for the presence of *C. perfringens* DNA (534 bp). A sample from a fulvous duck was positive by PCR for the presence of *E. coli* (STb) DNA (198 bp). *Salmonella* spp. DNA (372 bp) was detected from the faeces of the same vulture that was infected with *C. perfringens* above. Ninety four percent of the sampled animals that is, birds, primates and the antelopes were all negative for *C. perfringens, Salmonella* spp., *E. coli's* STa, STb and LT strains by PCR (Tables 1, 2 and 3).

DISCUSSION

C. perfringens isolation from the vulture and bald ibis could be attributed to their raw meat diet as bacterial spores can survive and germinate in contaminated food under circumstances of poor temperature control, particularly a lack of cooling and insufficient reheating (Daly and Rotert, 2007; Eriksen et al., 1992). If food that contains high values (>10^5 cfu/g) of *C. perfringens* vegetative cells is consumed, the bacterial cells can sporulate and produce illness, poor personal hygiene in catering staff is also a risk factor for this foodborne pathogen (Eriksen et al., 2010). Artificial diets given to animals in captivity might be positively correlated (r^2=0.90) with the increase in intestinal *C. perfringens* as well as they are reported to contain high calories and proteins which create optimal conditions for bacteria to thrive and contain less fibre which is said to proportionally decrease *C. perfringens* in the intestinal microflora (Fujita and Kageyama, 2006). As a normal component of the gut microflora of many warm-blooded animals (Jores et al., 2008) the low prevalence of infection in this case is noteworthy.

Most classes of vertebrates are known to be carriers of *E. coli* and it forms a normal component of the gut flora. Before infections with *E. coli* can occur, transfer of LT and ST toxins from plasmids or occasionally transposons, among Gram-negative enterobacteria has to take place (Adesiyun et al., 1998; Ozaki et al., 2011). The virulence of enterotoxigenic *E. coli* (ETEC) is believed to be associated with the production of fimbrial adhesins and enterotoxins. Fimbrial adhesions mediate the attachment

Table 1. Detection of *Clostridium perfringens* from captive animals at Pretoria Zoo.

Common name	Scientific name	No. of animals tested	No. (%) positive
			***C. perfringens* (N=49)**
Springbok	*Antidorcas marsupialis*	8	0
Lechwe	*Kobus leche*	5	0
Nyala	*Tragelaphus angasii*	5	0
Greater Kudu	*Tragelaphus strepsiceros*	8	0
Fulvous duck	*Dendrocygna bicolor*	7	0
Bald ibis	*Geronticus calvus*	5(20)[a]	1(2.0)
Vulture	*Gyps* spp.	5(20)[a]	1(2.0)
Black-eared marmoset	*Callithrix penicillata*	6	0

[a]percentage of positive samples per group of sampled animals. *C. perfringens* was isolated from two samples: One, a fecal sample that was pooled amongst a mixture of Cape and White-backed vultures that were enclosed in one cage and the other, from a bald ibis, each forming 20% of the total sampled vultures and bald ibises respectively, with an overall 4% *C. perfringens* detection from the 49 samples.

Table 2. Detection of *E. coli* from captive animals at Pretoria Zoo.

Common name	Scientific name	No. of animals tested	No. (%) positive		
			***E. coli* (N=49)**		
			STa	**STb**	**LT**
Springbok	*Antidorcas marsupialis*	8	0	0	0
Lechwe	*Kobus leche*	5	0	0	0
Nyala	*Tragelaphus angasii*	5	0	0	
Greater Kudu	*Tragelaphus strepsiceros*	8	0	0	0
Fulvous duck	*Dendrocygna bicolor*	7(14.3)[b]	0	1(2.0)	0
Bald ibis	*Geronticus calvus*	5	0	0	0
Vulture	*Gyps* spp.	5	0	0	0
Black-eared marmoset	*Callithrix penicillata*	6	0	0	0

[b]percentage of positive samples per group of sampled animals. *E. coli* (STb) DNA (an overall 2%) was detected in a sample collected from a fulvous duck (14.3% of sampled fulvous ducks), all other samples were negative for *E. coli* STa and LT strains.

Table 3. Detection of *Salmonella* species from captive animals at Pretoria Zoo.

Common name	Scientific name	No. of animals tested	No. (%) positive
			***Salmonella* species (N=49)**
Springbok	*Antidorcas marsupialis*	8	0
Lechwe	*Kobus leche*	5	0
Nyala	*Tragelaphus angasii*	5	0
Greater Kudu	*Tragelaphus strepsiceros*	8	0
Fulvous duck	*Dendrocygna bicolor*	7	0
Bald ibis	*Geronticus calvus*	5	0
Vulture	*Gyps* spp.	5(20)[c]	1(2.0)
Black-eared marmoset	*Callithrix penicillata*	6	0

[c]percentage of positive samples per group of sampled animals. *Salmonella* spp. DNA was detected by PCR from the feces of the same vulture that was infected with *C. perfringens* above indicating co-infection. Giving an overall 2% of *Salmonella* positive samples of the 49 sampled animals.

of bacteria to the surface of host epithelium cells and allow bacterial colonization. Fimbriae produced by different ETEC strains are quite diverse and they apparently bind to glycoconjugates in the enterocyte brush borders, and the absence of the respective glycoconjugate renders the animal resistant to bacterial

colonization and consequent diarrheal diseases (Zhang et al., 2006), this could explain the absence or low frequency of isolation (6% positive) of *E. coli* from the 17 birds sampled.

The low frequency of isolation of *Salmonella* spp. from avian species (6% positive) was not unexpected as similar results have been reported by other investigators, e.g. in a study of captive avian wildlife in individual households in Trinidad, none of the birds were carriers of *Salmonella* spp. but a 5% infection rate detected in racing pigeons was attributed to observed poor sanitary practices at the two lofts which yielded *Salmonella* spp. positive racing pigeons (Gopee et al., 2000). In another study waterbirds among others had the highest frequency (6%) of detection of *Salmonella* spp. which were isolated from digestive tracts of flamingos and a waterbird, and mortality due to the microorganism was reported (Wobeser, 1997). The intermittent shedding of *Salmonella* makes it difficult to identify carriers but the most commonly known are reptiles (snakes, turtles and lizards such as chameleons and iguanas) and rodents and they remain a potential source of *Salmonella* infections for other animals, the microorganism has been isolated from wild mammals and birds (Salehi et al., 2010). Although salmonellae may survive for long periods in the environment, it is the carrier state that provides the major source of infection for animals and humans and various carrier states are recognized (Dvorak et al., 2008; Keen et al., 2006). In this case the spread of the infection was restricted or limited as different groups of animals at the zoo have different keepers.

A retrospective study of necropsy cases done in 2004-2008 by NZG revealed that the incidences of infections with *Salmonella* spp. and *E. coli* at the zoo are more common in birds, this is in line with the findings in the current study. *C. perfringens* was not implicated in any deaths during that period, this is unexpected since the bacterium (*C. perfringens*) seemed to be slightly more prevalent than the other two in this study. Death in primates has not been reported by any of these particular pathogens but has been reported to be caused by others such as *Yersinia enterocolitica.* In 2008 none of the animals sampled in this study were reported dead as a result of these bacterial infections. The absence of *Salmonella* and *C. perfringens* in primates is in agreement with previous studies, it is considered rare for free-living wild primates to be infected at the time of capture but they frequently become infected in captivity (Gopee et al., 2000). Although *Salmonella*, *E. coli* and *C. perfringens* are not part of routine screening for new or resident animals, quarantine policies may protect against introduction and transmission of these non-targeted zoonotic agents as well. Animals at the zoo may be relatively free from these enteric zoonotic bacteria only by chance, because of this possibility and because these infections are usually clinically silent, zoological parks should consider implementing a specific preventive zoonotic microbial screening programme on a routine basis (Keen et al., 2006). Other bacteria such as *Lactobacilli plantarum* are known to play a major role in decreasing the number of and preventing colonization by *E. coli* and *C. perfringens* and other pathogens, and the prevalence of alpha toxin gene of *C. perfringens* in faeces (Takahashi et al., 2008), therefore even if the animals are infected the pathogens may be below detectable limit because of this factor.

The overall low frequency of infections at the NZG could be because of the fact that these bacterial have been proven to show variable seasonal prevalence e.g. *Salmonella* spp. have been reported to cause infection in colder months (Murray and Miller, 2008) while *C. perfringens* and *E. coli* are known to be more prevalent in warmer months (Zhao et al., 2001). The peaking at different seasons remains undetermined and it is said that results of bacteriologic cultures of faeces obtained from individual animals may at first be positive (that is, growth of the organism detected), then negative, and then positive again when samples are collected at intervals for analysis (LeJeunne and Davis, 2004), contrary to this Suresh et al. (2004) conducted a statistical significance test that showed that there is no significant variation in the incidence levels during different seasons. Therefore it is safe to say that the low incidence of infection with *C. perfringens*, *Salmonella* spp. and *E. coli* at the zoo could result from the standardized management and facility conditions, generally high hygiene levels, low animal stress due to exhibit permanency (e.g., lack of transport stress), and low rate of new animal introductions and animal mixing compared to temporary or reoccurring types of animal exhibits or production livestock settings. The institution is in an urban location, distant from farms with endemically infected livestock. This spatial buffer may insulate them from rural infection pressures (Keen et al., 2006). This low incidence (8%) of bacterial infections in the animals sampled is comparable with that of Emperor Valley Zoo and that of confined wildlife farms across Trinidad 6.5 and 7% respectively (Adesiyun et al., 1998). Typing of *Salmonella* strains and sequencing of the other bacteria may aid in determining the zoonotic significance as some serotypes are human adapted (Woodward et al., 1997), in that way the transmission from humans to captive animals can be controlled. A comparison of the incidences of infections at the zoo with other zoos in the country could be helpful in making a statistically significant conclusion about the prevalence of infections at the zoo and whether or not the animals are at risk or if there is a possibility of an outbreak.

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