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Short Communication

Occurrence of Shiga toxin producing *E. coli* in zoo animals of Rawalpindi and Islamabad zoos

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Abstract

Shiga toxin-producing *E. coli* (STEC) are considered pathogens of zoonotic importance. Zoo animals have been reported as reservoirs of STEC and many STEC human outbreaks have been linked with zoo animals. Available information about the occurrence of STEC in zoo animals in Pakistan is limited. Therefore, the current study was executed to estimate the occurrence of STEC in zoo animals of two zoos of Rawalpindi and Islamabad cities in Pakistan. Total of 110 faecal samples were collected from 24 species of zoo animals. The samples were analysed for determination of *eae*, *stx1*, *stx2*, and *ehxA* genes using multiplex PCR. The positive samples for any of these genes were further analysed for isolation using sorbitol MacConkey agar. Out of 110 fecal samples, 15 samples (13.6%) contained targeted virulence genes (*stx1*, *stx2*, *eae*, *ehxA*). Six different combinations of virulence genes were observed in positive samples. Only two *E. coli* isolates with targeted virulence genes could be isolated from PCR positive samples. The study indicated that the wild animals maintained in zoos of Rawalpindi and Islamabad are carriers of STEC and may be the source of infection for humans.

Keywords: STEC, Wild animals, Zoo, Virulence genes, Pakistan

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Introduction

Wild animals keeping in zoos are getting popularity in Pakistan for recreation, entertainment, knowledge, research, and conservation purposes. This increasing trend of zoo animal keeping is posing an increased chance of spreading zoonotic diseases from wild animal to susceptible human (Friedman et al., 1998; Bender and Shulman, 2004). Shiga toxin producing *Escherichia coli* (STEC) are highly zoonotic in nature and considered important foodborne pathogens. In humans, they cause various clinical manifestations including diarrhea, hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), and death (Paton and Paton, 1998b; Buelli et al., 2019; Rafique et al., 2022) The major STEC virulence factors linked with the emergence of disease in humans are Shiga toxins (encoded by *stx* gene) intimin (encoded by *eae* gene) and entero-hemolysin (encoded by *ehxA* gene) (Schmidt et al., 1995; Hofinger et al., 1998; Kaper and Strains, 1998; Gyles, 2007).

STEC transmission pathway is very complex. Various species of animals act as reservoir, spill-over host and dead-end host. Ruminants (sheep, goats, and cattle) and poultry birds are considered important reservoir of STEC and play an important role in spreading the disease to humans (García et al., 2010; Siddique et al., 2021). Wild animals kept in zoos have also been reported to be involved in the transmission of STEC to humans (Committee, 2013). Several outbreaks of STEC O157 in 17 different states of the USA have been reported due to zoo animals, pet exhibition and festivals (Crump et al., 2003; Durso et al., 2005; Lee et al., 2016; Varma et al., 2003).

STECs have been recovered from different wild animals kept in various zoological parks of the world (Milton et al., 2018). There is no data available in Pakistan related to occurrence of STEC in zoo animals. Therefore, a study was carried out to estimate the occurrence of STEC in zoo animals kept in zoos of Islamabad and Rawalpindi. Zoos in Rawalpindi and Islamabad maintain different species of animals such as chinkara deer, white fallow deer, hog deer, spotted deer, red deer, blackbuck, Australian sheep, Mouflon sheep, Punjab urial, and blue bulls while non-ruminants include zebras, tigers, lions, monkeys, bears, emu, ostrich, and pony horses. The information obtained from this study would help in identifying the significance of zoo animals in dissemination of STEC to humans and environment.

Material and Methods

Freshly voided fecal samples (n=110) from the ground were collected from twenty-four species of the wild animals with the help of zookeepers. These animals were maintained at two different zoos of Rawalpindi and Islamabad. On average 2000 persons/day visit these zoos and this number increases to 50,000 to 60,000 persons/day in public holidays.

These collected samples were placed into zip lock bags and transferred to Animal Health Program, Animal Sciences Institute, Islamabad within 2 hours of collection in cold conditions. The samples were enriched with Buffer Peptone Water (BPW) for 24 hours at 37 °C.

Simple boiling method was used to extract the DNA from one ml aliquot of broth culture from each sample (Reischl et al., 2002; Irshad et al., 2012; Momtaz et al., 2013; Tahmasby et al., 2014). The extracted DNA was analyzed for the detection of *eae*, *ehxA*, *stx1*, and *stx2* genes using multiplex PCR (Paton and Paton, 1998a; Sharma and Dean-Nystrom, 2003). The details of primers' sequences used for the detection of these genes are given in Table 1. Briefly, PCR reaction was carried out for initial denaturation at 95°C for 7 mins, 40 cycles of denaturation at 95°C for 45s, annealing at 60°C for 45s and extension at 72°C for 45s, followed by final extension at 72°C for 8 mins. After completion of reaction PCR, amplicons were examined by gel electrophoresis using 2% agarose gel. The PCR amplicons were visualized using ethidium bromide used as intercalating dye under ultra-violet light. The positive samples for any of virulence gene were cultured on sorbitol MacConkey agar (SMAC) to get colonies of STEC. Inoculated SMAC plates were placed in incubator for 24 hours at 37°C. Two different colors colonies (Grey and pink) were obtained on plates. Five sorbitol fermenting (pink) and five non-sorbitol fermenting colonies (grey) colonies from each culture plate were tested for the detection of *stx1*, *stx2*, *eae*, and *ehxA* genes using multiplex PCR. The positive isolates for virulence genes were further sub-cultured on the SMAC plates to obtain pure culture and preserved at -80°C in glycerol broth [nutrient broth having 15% (v/v) glycerol]. The 95% confidence intervals around the prevalence were calculated using Microsoft Excel.

Table-1. Primer sequences of virulence genes used for PCR during the study.

Primer	Sequence (5′-3′)	PCR product (bp)	Reference
stx1 (Forward)	GAC TGC AAA GAC GTA TGT AGA TTC G	*stx1* (150)	(Sharma and Dean-Nystrom, 2003)
stx1 (Reverse)	ATC TAT CCC TCT GAC ATC AAC TGC		
stx2 (Forward)	ATT AAC CAC ACC CCA CCG	*stx2* (200)	
stx2 (Reverse)	GTC ATG GAA ACC GTT GTC AC		
eae (Forward)	GTA AGT TAC ACT ATA AAA GCA CCG TCG	*eae* (100)	
eae (Reverse)	TCT GTG TGG ATG GTA ATA AAT TTT TG		
ehxA (Forward)	GCA TCA TCA AGC GTA CGT TCC	*ehxA* (534)	(Paton and Paton, 1998)
ehxA (Reverse)	AAT GAG CCA AGC TGG TTA AGCT		

Results and Discussion

Out of 110 fecal samples, targeted virulence genes (*eae*, *stx1*, *stx2*, *ehxA*) were identified in 15 (13.6%) samples. The presence of virulence genes in fecal samples obtained from zoo animals indicated their potential in spreading pathogenic *E. coli* including STEC to humans. Previously many STEC outbreaks in humans have been reported to be linked with contact with wild animals through handling, petting and feeding of zoo animals (Conrad et al., 2017; Schlager et al., 2018). The zoos in Islamabad and Rawalpindi are popular among visitors especially children as these zoos provide opportunity to come close to the animals for feeding and petting. Therefore, these zoo animals could be a source of transmission of STEC to visitors, especially children. A study carried out in India reported high occurrence of STEC in wild ruminants (7.14%, 9/126) compared to wild non-ruminants (3.48%, 3/86) (Milton et al., 2018). In our study the occurrence of samples positive for STEC virulence genes was higher in wild ruminants 12/79 (15.1%: 95% CI=9-21) compared to wild non-ruminants 4/31 (12.9%: 95% CI=1.5-22) (Table 2). However, this difference was non-significant. More number of positive samples were observed in Zoo A (16.9%; 95% CI=4-36.3) compared to Zoo B (7.6%: 95% CI=1-13), but the difference was non-significant. In total six different virulence gene combinations were identified in positive samples (Table 3). The virulence gene profiles observed in positive samples were *stx1* (8 samples), *stx1, ehxA* (2 samples), *stx1, stx2, ehxA* (2 samples), *stx1, stx2* (1 sample), *stx2, ehxA* (1 sample), *stx2* (1 sample) and *ehxA* (1 sample). Three of the four virulence genes were observed in the samples collected from wild zoo animals. Most of the samples were positive only for *stx1* (53.3%), while others carried combination of virulence genes. The detection of virulence genes of clinical significance in the samples indicate that the wild zoo animals may be source of infection for humans especially zookeepers and children visiting the zoos. Out of 15 positive samples, two isolates with virulence genes could be recovered. These isolates were recovered from hog deer and spotted deer. The isolates obtained from hog deer contained *ehxA* gene while the isolates obtained from spotted deer contained *stx2 and ehxA* genes. The recovery of isolates with virulence genes was very low (2/15; 13.3%). PCR has been considered as a sensitive method compared to culturing (Stefan et al., 2007) and can detect genetic material of the dead organism. Therefore, STEC with virulence genes of interest may be present in the samples but cannot be isolated.

Table-2. Fecal samples (n=110) obtained from zoo animals of two different zoos of Rawalpindi and Islamabad were examined using multiplex PCR for detection of *stx1*, *stx2*, *eae* and *ehxA* genes of Shiga toxin-producing *E. coli*

Species	Scientific Name	Samples collected from zoos				Total samples	
		A (Rawalpindi)		B (Islamabad)			
		Samples collected	Samples positive	Samples collected	Samples positive	Collected	Positive
Australian Sheep	*Ovis aries*	6	0	0	0	6	0
Blue Bull	*Boselaphus tragocamelus*	5	0	0	0	5	0
Black Buck	*Antilope cervicapra*	6	2	7	0	13	2
Chinkara Deer	*Gazella bennettii*	3	1	0	0	3	1
Fallow Deer	*Cervus dama*	0	0	4	0	4	0
Hog Deer	*Axis porcinus*	5	2	8	2	13	4
Mouflon Sheep	*Ovis orientalis*	1	1	0	0	1	1
Red Deer	*Cervus elapus*	0	0	3	0	3	0
Spotted Deer	*Axis axis*	8	3	2	1	10	4
Urial	*Ovis vignei*	8	0	8	0	16	0
White Fallow Deer	*Dama dama*	5	0	0	0	5	0
African Lioness	*Panthera leo*	2	0	0	0	2	0
Baboon Monkey	*Papio ursinus*	2	1	0	0	2	1
Bengal Tiger	*Panthera tigris tigris*	3	0	0	0	3	0
Bear	*Ursidae*	2	0	1	0	3	0
Emu	*Dromaius novaehollandiae*	0	0	3	0	3	0
Miniature Horses	*Equus caballus*	5	0	0	0	5	0
Ostrich	*Struthio camelus*	3	1	0	0	3	1
Pony Horses	*Equus ferus caballus*	0	0	2	0	2	0
Rhesus Monkey	*Macaca mulatta*	2	0	0	0	2	0
Vervet Monkey	*Chlorocebus pygerythrus*	2	0	0	0	2	0
White Lioness	*Panthera leo krugeri*	1	0	0	0	1	0
White Tiger	*Panthera tigris*	1	1	0	0	1	1
Zebra	*Equus quagga*	1	0	1	0	2	0
Total		71	12 (16.9%; 95% CI=4-36.3)	39	3 (7.6%: 95% CI=1-13)	110	15 (13.6%: 95% CI 7-19)

Table-3. Distribution of targeted genes (*stx1*, *stx2*, *eae* and *ehxA*) in positive samples recovered from wild animals of two different zoos of Islamabad and Rawalpindi

Specie	Zoo	Animal type	Virulence genes
Black Buck	A	Wild ruminant	*stx1, ehxA*
Black Buck	A	Wild ruminant	*stx1*
Chinkara Deer	A	Wild ruminant	*stx1*
Spotted Deer	A	Wild ruminant	*stx1, stx2, ehxA*
Spotted Deer	A	Wild ruminant	*stx2, ehxA*
Spotted Deer	A	Wild ruminant	*stx1*
Spotted Deer	B	Wild ruminant	*stx1*
Hog Deer	A	Wild ruminant	*stx1*
Hog Deer	A	Wild ruminant	*stx1, stx2*
Hog Deer	B	Wild ruminant	*stx1, ehxA*
Hog Deer	B	Wild ruminant	*stx1*
Mouflon Sheep	A	Wild ruminant	*ehxA*
Baboon Monkey	A	Wild non-ruminant	*stx2*
Ostrich	A	Wild non-ruminant	*stx1*
White Tiger	A	Wild non-ruminant	*stx1, stx2, ehxA*

Conclusions

To our knowledge this is the first-ever report of occurrence of STEC in captive zoo animals in Pakistan. It was a small-scale study but provided evidence of occurrence of STEC of human health importance from captive wild ruminants and wild non-ruminants of zoos of Islamabad and Rawalpindi. The

study showed that these zoo animals may be the source of infection for zookeepers and visitors visiting zoos especially children. These animals may also act as a source of dissemination of STEC to environment as their feces is disposed in the environment. It is suggested that measures should be adopted to minimize the exposure of zookeepers and visitors especially children with these animals to avoid STEC related illness.

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Contribution of Authors

Rasheed MB: Data collection and analysis and laboratory analyses.
Ahsan A & Irshad H: Project administration, analysis of data and samples and article write up.
Shahzad MA & Usman M: Data collection and analysis of samples in laboratory
Riaz A, Chaudhry TH, Amir A, Zubair M & Khan A: Data collection, data analysis and article write up.
Yousaf A: Conceived idea and supervised the study and approved final draft of article.