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Namita Shukla
College of Veterinary and Animal Sciences, G.B.P.U.A.T., Pantnagar, Uttarakhand, India

Rajesh Kumar
Department of Veterinary Microbiology, College of Veterinary and Animal Sciences G. B. Pant University of Agriculture & Technology, Pantnagar, Uttarakhand, India

AK Upadhyay
College of Veterinary and Animal Sciences, G.B.P.U.A.T., Pantnagar, Uttarakhand, India

Aakanksha Tiwari
College of Veterinary and Animal Sciences, G.B.P.U.A.T., Pantnagar, Uttarakhand, India

NK Singh
College of Veterinary and Animal Sciences, G.B.P.U.A.T., Pantnagar, Uttarakhand, India

Anupama Mishra
College of Community Sciences, CAU (Imphal), Tura, Meghalaya, India

Prakash Bhatt
College of Veterinary and Animal Sciences, G.B.P.U.A.T., Pantnagar, Uttarakhand, India

Corresponding Author
Rajesh Kumar
Department of Veterinary Microbiology, College of Veterinary and Animal Sciences G. B. Pant University of Agriculture & Technology, Pantnagar, Uttarakhand, India

Antimicrobial resistance pattern of shigatoxigenic *E. coli* (STEC) and enteropathogenic *E. coli* (EPEC) isolated from wild Felidae in India

Namita Shukla, Rajesh Kumar, AK Upadhyay, Aakanksha Tiwari, NK Singh, Anupama Mishra and Prakash Bhatt

Abstract
The purpose of this Study was to assess the antibiotic resistance profile of Shigatoxigenic and Enteropathogenic *Escherichia coli* isolated from captive wild Felidae i.e., Tiger, Lion, Leopard and Panther. Faecal samples (n=60) collected from various zoological gardens and enclosures in India were processed for the isolation of *E. coli*, followed by pathotyping by multiplex PCR targeting Shigatoxigenic (*stx*1 and *stx2*), intimin (*eaeA*), and enterohaemolysin (*ehyA*) genes. All STEC and EPEC isolates were subjected to *in vitro* antibiotic sensitivity test by disc diffusion method against twelve commonly used antibiotics. *viz*., chloramphenicol, Cefuroxime, Azithromycin, Cephalothin, Ciprofloxacin, Ceftriaxone, Norfloxacin, Ticarcillin+ clavulanic acid, Co-Trimoxazole, Tetracycline, Gentamicin, Piperacillin + Tazobactam. Total 28 *E. coli* isolates were recovered from fecal samples, which were found to be resistant to tetracycline, Norfloxacin, cefuroxime, Gentamicin, Chloramphenicol, Co-Trimoxazole and Azithromycin. No resistance was observed for cephalothin, ciprofloxacin, ceftriaxone, Ticarcillin+ clavulanic acid and Piperacillin + tazobactam. In the present study 21.42% isolates from captive wild felines were found to be resistant to more than two antibiotics.

Keywords: Antimicrobial resistance, STEC, EPEC, wild felines

Introduction
Escherichia coli is a commensal bacteria found in the gastrointestinal tract of human and animals, but some strains are pathogenic. Enteropathogenic (EPEC) and Shigatoxigenic (STEC) represent two important classes of enteric pathogens that can cause enteritis and enterotoxaemia in human and animals. Production of A/E lesion is foremost virulence property of EPEC, that causes inflammatory reaction and diarrhea (Moon *et al*., 1983) [15]. EPEC Strains are important cause of infantile diarrhoea in developed and developing countries and are responsible for thousands of deaths world-wide (Chen and Frankel 2005; Ochoa *et al.*, 2008) [4, 17]. The main virulence feature of STEC strains is the production of shigatoxin (stx) causing clinical syndrome mainly in piglets and humans. However, they are also being isolated from various domestic and wild animals, which may be asymptomatic healthy carriers (Wieler and Bauerfeind, 2003) [21]

Animals are undeniably the prominent carriers of STEC as these strains are continuously being isolated from various domestic and human-associated animal species (Persad and Lejeune 2014; Espinosa *et al*., 2018) [19, 6]. Wildlife surveillance has gained importance in recent decades, as most of emerging zoonotic pathogens are of wildlife origin (Jones *et al*., 2013). [11] and increasing number of wild animals have been shown to be potential STEC reservoirs (Espinosa *et al.*, 2018) [5].

High antimicrobial resistance in STEC and EPEC strains is another important factor which complicates the outcome of the infection. The prevalence of antimicrobial- resistance (AMR) bacteria is one of the most important concerns for human as well as animal health worldwide. As widespread and irrational use of antimicrobials in human and veterinary medicine is an impediment in combat against AMR therefore, reducing the use of antimicrobials is an important approach for preventing the spread of AMR (Hiki *et al*., 2015; Kurita *et al*., 2019) [10, 13]. Environments contaminated with human and animal wastes have become breeding ground to drug resistant bacteria and wild animal also play a definite role in the spread of AMR (Alexender 2020; Guyomard -Rabenirina *et al.*, 2020; Heuer *et al.*,2007) [1, 7, 9].

Therefore, surveillance of antimicrobial resistant bacteria in wild animals is important for one health approach (McEven *et al*., 2018) [14]. This study attempts to find out antibiotic resistance profile of STEC& EPEC isolates from captive wild felines.

Materials and Methods

Sampling and Isolation of *E. coli*: The study was carried out in seven zoological gardens and wildlife enclosures, in India *viz*., Kannan penderi Zoo, Bilaspur, Chhattisgarh; Nandan Kannan Zoo Raipur, Chhattisgarh; Jungle Safari Raipur Chhattisgarh; Kanpur Zoo, Kanpur, Uttar Pradesh; Lucknow zoo, Lucknow Uttar Pradesh; Nainital Zoo, Nainital, Uttarakhand; Almora Zoo, Almora, Uttarakhand. Total 60 fecal sample were collected from 17 tigers, 12 Lions, 20 leopards and 11 panthers during January 2021 to Feburary 2022 from three different geographical locations in India. After collection, samples, were immediately transported to the laboratory within 48 hrs. under optimum condition.

The collected samples were inoculated in buffered peptone water and incubated at 37 °C for 24 h**.** The growth in the nutrient broth was transferred to MacConkey's agar followed by incubation at 37 °C overnight. The next day 2-3 pink colonies were randomly picked and transferred to EMB agar (Hi-Media) followed by overnight incubation at 37 °C. The colonies were observed for metallic sheen The well separated single colonies were picked up and transferred on nutrient agar slant to obtain pure culture and subjected to standard morphological and biochemical tests.

Multiplex PCR for detection of STEC and EPEC isolates: All the isolates exhibiting expected morphological and biochemical characteristics for *E. coli* were subjected to multiplex PCR for the detection of Shigatoxigenic (*stx*1 and *stx2*), intimin (*eaeA*), and enterohaemolysin (*ehyA*) genes (Paton and Paton, 1998).

PCR assay was carried out in a 25 µl volume reaction containing 3 µl of DNA template prepared from each isolate, 3 µl of 10XPCR buffer with Mgcl2, 1 µl each primer with in the four-primer set (Table-1) at a concentration of 250nM, 3 µl of10mM each of dNTPs. 0.5 µl of 5 U of Taq DNA polymerase and the rest NFW (8.5 µl) to make up to the volume. The PCR reaction was performed in thermal cycler (Eppendorf, USA) with following conditions: initial denaturation at 95 °C for 5 min followed by 29 cycles of denaturation at 94 °C for 1 min, primer annealing at 59 °C for 1min and extension at 72 °C for 1min followed by final extension at 72 °C for 6 min. The amplified PCR products were analyzed by agarose gel (2%) electrophoresis at 80 V/cm for 45 min and stained with ethidium bromide (0.5 µg/ml). The product was visualized under ultraviolet transilluminator and documented by gel documentation system. (Syngene, G- Box). A known molecular weight marker (100bp DNA ladder) was used in each run to determine the amplicon size. The PCR was performed thrice to ensure the repeatability of the technique and to make sure that *E. coli* isolate was correctly assigned to respective pattern.

Antibiotic sensitivity test: All STEC and EPEC isolates were subjected to *in vitro* antibiotic sensitivity test (CLSI 2008) by disc diffusion method using twelve commonly used antibiotic *viz* Chloramphenicol (30µg). Cefuroxime (30µg), Azithromycin (15µg), Cephalothin (30µg), Ciprofloxacin (30µg), Ceftriaxone (30µg), Norfloxacin (10µg), Ticarcillin+ clavulanic acid (75/10 µg), Co-Trimoxazole (23.75/1.25µg), Tetracycline (10µg), Gentamicin (30µg), Piperacillin + Tazobactam (100/10µg) (Himedia, India). STEC and EPEC isolates were inoculated in nutrient broth and incubated at 37 °C for 5 hours. The broth was then diluted in normal saline solution to a density of 0.5 McFarland turbidity standard. Cotton swabs were used for spreading the diluted broth onto Mueller-Hinton agar plates. After air drying, antibiotic discs were placed 30mm apart and 10mm away from the edge of the plate. Plates were incubated at 37 °C for 16 - 18 hours. The measurements were compared with zone size interpretative chart furnished by the manufacturer and the zones were graded as sensitive, intermediate and resistant.

Table 1: Primers sequences used in multiplex PCR reaction to identify pathotype

Primers	Sequence 5–3	Target gene	PCR product	Reference
stx1F	ATAAATCGCCATTCGTTGACTAC			
stx1R	AGAACGCCCACTGAGATCATC	*stx1*	180 bp	Paton & Paton (1998) [18]
stx2F	GGCACTGTCTGAAACTGCTCC	*stx2*	255bp	Paton & Paton (1998) [18]
stx2R	TCGCCAGTTATCTGACATTCTG	*eaeA*	384bp	Paton & Paton (1998) [18]
eaeAF	GACCCGGCACAAGCATAAGC	EHEC	534bp	Paton & Paton (1998) [18]
eaeAR	CCACCTGCAGCAACAAGAGG	*hlyA*		
hlyAF	GCATCATCAAGCGTACGTTCC			
hlyAR	AATGAGCCAAGCTGGTTAAGCT			

Result and Discussion

Total of 105 *E. coli* isolates were recovered from 60 fecal samples collected from tiger, lion, leopard and panther. These isolates were pathotyped by multiplex PCR targeting 4 genes and 28 isolates were successfully pathotyped. Among 28 isolates AE-STEC was found to be the predominant pathotype with an isolation rate of 13.33% followed by STEC (8.57%) and EPEC (4.76%).

All the pathotyped isolates were tested for antibiotic resistance and all were found to be resistant to Tetracycline (100%) followed by Norfloxacin (25%), Cefuroxime (21.42%), Gentamicin (17.85%), Chloramphenicol (10.71%) Co-Trimoxazole (7.14%) and Azithromycin (3.57%). No resistance was observed for cephalothin, ciprofloxacin, ceftriaxone, Ticarcillin+ clavulanic acid and Piperacillin along with tazobactam.

Antimicrobial resistance has been recognized as a worldwide challenge in human and veterinary medicine worldwide (Bager and Helmuth, 2001; Hammerum and Heuer, 2009) [2, 8]. The aim of this was to study antimicrobial drug resistance profile of STEC and EPEC isolated from wild felines. We found that all the 28 isolates were resistant to tetracycline. This is consistent with fact that tetracycline is among the most widely used antibiotic in farm animals, from which resistant bacteria can emerge and spread to the environment. A study, carried out in non-O157 STEC isolate from farm and abattoir sources, reported 87% of isolates resistant to antimicrobials used in veterinary and agriculture practice. (Kennedy *et al.*,

2017) [12]. Another study conducted in Mexico showed that 25.7% STEC strain from healthy farm cattle were not sensitive to Tetracycline, trimethoprim and penicillin (Navarro *et al*., 2018) [16]. STEC/EPEC isolates in present study were shown to be resistant against Norfloxacin, Cefuroxime, Gentamicin, Chloramphenicol, Co-Trimoxazole and Azithromycin and 21.42% of the isolates were found to be resistant to more than two antibiotics. Another study suggests that wild birds could act as carriers of multidrug-resistant EPEC and STEC (Borges *et al*., 2017). [2] The fact that wild animals are normally not in close contact with antibiotics explains low level of resistance (Thaller *et al*., 2010). [20] Therefore, resistance against some of antibiotics in our findings be explained by condition of captivity, persistence of antibiotic in the environment and exposure to antimicrobial chemical in their food intake.

Table 2: Antibiotic resistance profile of isolates of STEC and EPEC strains

S. No	Strain ID	Antibiotic resistance profile	Virulence gene profile				Source of isolation
			Stx1	*Stx2*	*eaeA*	*hiyA*	
1	STECT1	TE, NX, GEN	+	-	+	-	Tiger
2	STECT2	TE, GEN, CXM	+	-	+	-	Tiger
3	STECL1	TE, NX	+	-	+	-	Lion
4	STCL2	TE, NX	+	-	+	-	Lion
5	STECL3	TE	+	-	+	-	Lion
6	STECL4	TE, COT, GEN	+	-	+	+	Lion
7	STECL5	TE	+	-	+	+	Lion
8	STECLp1	TE, GEN, C	+	-	+	+	Leopard
9	STECLp2	TE	+	+	+	-	Leopard
10	STECLp3	TE, CXM	+	+	+	-	Leopard
11	STECP1	TE, COT	+	-	-	+	Panther
12	STECP2	TE, AZM NX	+	-	-	-	Panther
13	STECP3	TE	+	-	-	-	Panther
14	STECBT1	TE, CXM, NX	+	-	-	-	Tiger
15	STECBT2	TE	+	-	-	-	Tiger
16	STECBL1	TE	+	-	-	-	Lion
17	STECBL2	TE, NX	+	-	-	-	Lion
18	STECBP1	TE	+	-	-	-	Panther
19	STECBP2	TE, CXM	+	-	-	-	Panther
20	STECBT3	TE	-	+	-	-	Tiger
21	STECBL3	TE, CXM	-	+	-	-	Lion
22	STECBP3	TE	-	+	-	-	Panther
23	EPECT1	TE	-	-	+	+	Tiger
24	EPECT2	TE, C,	-	-	+	-	Tiger
25	EPECLp1	TE	-	-	+	-	Leopard
26	EPECLp2	TE, C, NX	-	-	+	-	Leopard
27	EPECLp3	TE, CXM, GEN	-	-	+	-	Leopard
28	STEChT1	TE	-	-	-	+	Tiger

TE: Tetracycline, CXM: Cefuroxime, C: Choramphenicol, AZM: Azithromycin, GEN: Gentamicin, NX: Norfloxacin, COT: Co-Trimoxazole

Conclusion

The results of our study indicate that captive wild Feline in India can carry STEC and EPEC strains that are potentially pathogenic for humans and contribute to environmental contamination by this strain. Further the low prevalence of antimicrobial resistant STEC and EPEC in captive wild Feline suggest that wild Felines are not currently likely to be source for the emergence and transmission of antimicrobial resistance in natural environment. However, more studies involving wildlife are required for assessing their AMR status.

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