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Prevalence and Molecular Diagnosis of *Escherichia coli* in captive Sloth Bears (*Melursus ursinus*)

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A B S T R A C T

Pathogenic *Escherichia coli* strain from faeces of sloth bears was screened by using culture morphology, Gram's staining, biochemical tests and polymerase chain reaction. Our results showed that out of 60 samples collected, 59 samples (98.33%) were cultured on EMB agar and Gram's stain revealed rod-shaped Gram-negative organism with green metallic sheen - like colonies. The biochemical tests of cultured samples revealed positive to indole production, positive to methyl red test, negative to Voges-Proskauer test, negative to Simmon citrate utilization test, positive to glucose, negative to H_2S production and negative to urea production. All *E. coli* isolates were found sensitive to azithromycin, streptomycin and tetracycline. They were found intermediate to enrofloxacin and gentamicin. The *fimC* gene was amplified by PCR for the pathogenic *Escherichia coli* and were found to be positive of 53.33% among the juveniles, 46.67% among the sub-adults, 53.33% among the adults and 33.33% among the geriatrics were found to be positive. This study may provide information for developing strategies in the future in the control of *Escherichia coli* infections in sloth bears.

Introduction

Ursidae have a complex gut microbiota resulting from a dynamic interplay among diet, host and commensal bacteria, which play an important role in the maintenance of health and disease modulation. The composition of the gut microbiota depends on the physiology of the gut as well as the type of diet and varies among the hosts (Ley *et al.*, 2008). *Ursids*

including Sloth Bear (*Melursus ursinus*) are an enigmatic family. *Escherichia coli* are part of the normal intestinal microbiota and coexist with its host in mutual benefit. Captive animals, especially those acquired from the wild can be asymptomatic carriers of pathogens. Some of these pathogens have also caused morbidity and mortality in animals within zoological gardens. *Escherichia coli* are often a long-term commensal in animals, a part of their normal intestinal microbial community. *Escherichia coli* can cause a variety of diseases including dysentery, haemorrhagic uremia syndrome, bladder and kidney infections, septicemia, pneumonia, and meningitis in humans and animals (Cambre *et al.,* 1980). The intestinal microbiota can also be a reservoir of extra intestinal pathogenic *Escherichia coli* and strains from the intestinal microbiota of the Sloth Bear are scanty. The aim of this study was to investigate and characterize faecal *Escherichia coli* from sloth bear (*Melursus ursinus*).

Materials and Methods

In this study, fecal samples were collected from Bannerghatta Bear Rescue Centre (BBRC), Wildlife SOS, Bangalore, Karnataka. During the sampling period in 2017, the faecal samples from 15 juvenile, 15 sub-adult, 15 adult and 15 geriatric captive sloth bears were collected in nutrient broth using sterile swabs and kept at 4^0C until further processing. The swabs were incubated at 37^0C for 24 hrs. A loop-full of culture was then spread on Nutrient Agar plate and incubated further at 37^0C for 24 hrs. Eosin Methylene Blue (EMB) media was used as a selective and differential culture media. A loop-full of culture was spread on Eosin Methylene Blue (EMB) agar and incubated at 37^0C for 24 hrs to obtain of *Escherichia coli* colonies. The isolated colony was stained with Gram's stain and biochemical tests (HiIMViC^{TM} Biochemical Test Kit, TSI agar and urea agar) and was confirmed by Polymerase Chain Reaction (PCR).

Antimicrobial resistance pattern of pathogen *E. coli* isolates were studied by Modified Kirby-Bauer disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2006) for the following antibiotics: amoxicillin/clavulanic acid (30mcg), azithromycin (15mcg), cefotaxime (30mcg), clindamycin (2mcg), gentamicin (10mcg), enrofloxacin (10mcg), methicillin (5mcg), streptomycin (300mcg) and tetracyline (30mcg).

For the PCR reaction, the DNA was extracted from the isolates by using boiling method (Medici *et al.,* 2003). One ml of the pre-enriched culture was transferred to a 1.5ml micro-centrifuge tube. The cell suspension was centrifuged for 10 min at 10,000rpm. The supernatant was discarded carefully. The pellet was resuspended in 300µl of DNase-RNase- free water by vortexing. The tube was centrifuged at 10,000rpm for 5 min, and the supernatant was discarded carefully. The pellet was resuspended in 200µl of DNase-RNase-free water by vortexing. The microcentrifuge tube was incubated for 15 min at 100°C and immediately chilled on ice. The tube was centrifuged for 5 min at 10,000rpm at 4°C. The supernatant was carefully transferred to a new microcentrifuge tube and incubated again for 10 min at 100°C and chilled immediately on ice. Further, it as stored at -20^0C. An aliquot of 5µl of the supernatant was used as template in the PCR. The molecular weight of the *fimC* gene corresponding to the avian pathogenic *Escherichia coli* was 477bp (Janben *et al.,* 2001). The following primers were used to amplify the *fimC* gene (Forward Primer: 5'–GGGTAGAAAATGCCGATGGTG–3' and Reverse Primer: 5'-CGTCATTTTGGG GGTAAGTGC– 3'). The PCR was performed in a 25µl reaction mixture consisting of 12.5µl

of 2X PCR mastermix, 1µl of each primer, 2µl of extracted DNA and finally volume was adjusted with nuclease free water. Amplification was carried out in thermocycler with initial denaturation 94°C for 2 min followed by 25 cycles each of denaturation at 94°C for 1 min, annealing at 59°C for 1 min, extension at 92°C for 90 sec with a final extension period of 7 min at 72°C. 10µl of each PCR products was electrophoresed on 1.5% agrose gel containing ethidium bromide in the presence of 100bp ladder. The presence specific amplicon of 477bp was viewed under UV transilluminator.

The PCR amplifies the *fim*C gene of the one of the isolate was sequenced using commercial sequencing service.

Results and Discussion

Out of 60 samples collected, 59 samples (98.33%) were cultured on EMB agar and green metallic sheen - like colonies (Figure 1) were picked for Gram's staining and revealed Gram-negative rod-shaped organism.

Usage of biochemical tests in HiIMViC™ Biochemical Test Kit, TSI agar and urea agar revealed that were positive to indole production, positive to methyl red test, negative to Voges-Proskauer test, negative to Simmon citrate utilization test, positive to glucose, negative to H2S production and negative to urea production in cultured samples. All *E. coli* isolates which were studied have been found sensitive to azithromycin, streptomycin and tetracycline.

They were found intermediate to enrofloxacin and gentamicin.

Further, based on the PCR assay, among 53.33% juvenile, 46.67% sub-adult, 53.33% adult and 33.33% geriatric screened for the pathogenic *Escherichia coli* were found positive. In the juvenile sloth bear group, three males and five females harboured the infection (60.00 and 50.00%, respectively), in the sub-adult group, four males and three females harboured the infection (66.67% and 33.33%, respectively), in the adult group one male and seven females harboured the infection (20.00% and 70.00%, respectively) and in the geriatric group three male and two females harboured the infection (27.27% and 50.00%, respectively) (Figure 2 and Table 1).

The *fim*C gene was chosen for amplification since presence of this gene indicates the *E. coli* as a pathogenic gene. All the isolates from the sloth bears were positive for the presence of *fim*C gene indicating the pathogenic nature.

Fig.1 *Escherichia coli* on Eosin Methylene Blue (EMB) Agar

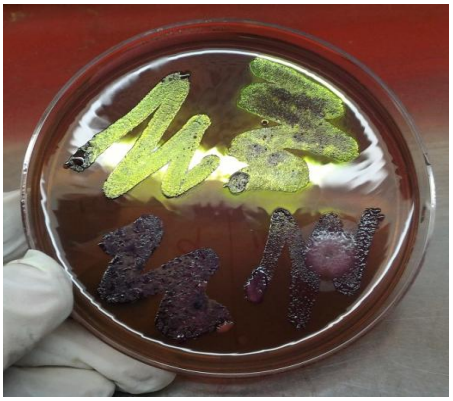
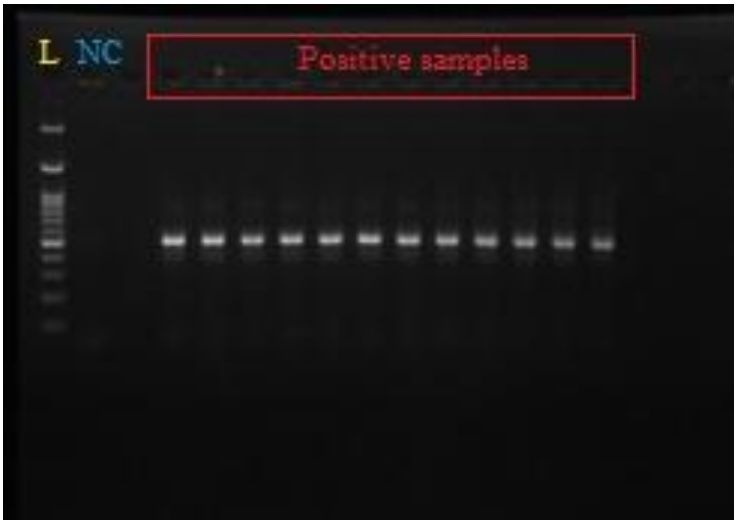


Fig.2 Amplification of *Escherichia coli* by PCR



1.5 % agarsoe showing PCR products of *fimC* gene
Lane L – DNA ladder (500bp)
Lane NC – Negative control
Lane postitive samples – *fimC* gene amplification (477bp)

Supplement: Partial nucleotide sequence of *fim*C gene from *E. coli* isolated from sloth bear (*Melursus ursinus*)

>ConsensusTTTGGGTAGAAAAATGCCGATGGTGTAAAGGATGRKCGKTTTATCGTGA
CGCCTCCTCTGTTTGCGATGAAGGGAAAAAAAGAGAATACCTTACGTATTCTTGATG
CAACAAATAACCAATTGCCACAGGATCGGGAAAGTTTATTCTGGATGAACGTTAAA
GCGATTCCGTCAATGGATAAATCAAAATTGACTGAGAATACGCTACAGCTCGCAATT
ATCAGCCGCATTAAACTGTACTATCGCCCGGCTAAATTAGCGTTGCCACCCGATCAG
GCCGCAGAAAAATTAAGATTTCGTCGTAGCGCGAATTCTCTGACKCTGATTAACCCG
ACACCCTATTACCTGACGGTAACAGAGTTGAATGCCGGAACCCGGGTTCTTGAAAAT
GCATTGGTGCCTCCAATGGGCGAAAGCACGGTTAAATTGCCTTCTGATGCAGGAAGC
AATATTACYTWCCGAACAMTAAATGATTATGGCGCACTTACCCCCAAAATGACGAA

IUPAC nucleotide code	Base
A	Adenine
C	Cytosine
G	Guanine
T (or U)	Thymine (or Uracil)
R	A or G
Y	C or T
W	A or T
K	G or T
M	A or C

Table.1 Prevalence of *Escherichia coli* in Sloth Bears (*Melursus ursinus*) by PCR

Group of animals (n=30)	Sex	Samples collected	Positive for pathogenic *Escherichia coli*	Prevalence (%)
Juvenile (2-7 years) (n=15)	Male	5	3	60.00
	Female	10	5	50.00
	Both	15	8	53.33
Sub-adult (8-13 years) (n=15)	Male	6	4	66.67
	Female	9	3	33.33
	Both	15	7	46.67
Adult (14-18 years) (n=15)	Male	5	1	20.00
	Female	10	7	70.00
	Both	15	8	53.33
Geriatric (above 19 years) (n=15)	Male	11	3	27.27
	Female	4	2	50.00
	Both	15	5	33.33

The sequence results were compared with that of the pathogenic *E. coli* isolate sequences using nBLAST (Nucleotide local alignment service tool) available at https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch. The sequence data is provided as supplement.

The prevalence of 46.67% of pathogenic *Escherichia coli* confirms the presence of infection and ampleness of the bacteria in the Bannerghatta Bear Rescue Centre (BBRC). The isolated pathogenic *Escherichia coli* which could be either enteropathogenic, enterohaemorrhagic or commensal are the possible cause of zoonotic infection in wild animals and in humans. The sources of infection could not be revealed. The possible sources could be food, water, birds, visitors, rodents, lizards and bats (Oludairo *et al.*, 2016). The sloth bear are omnivores animal and has yield a 46.67 % rate of pathogenic *Escherichia coli* infection and which is in agreement with the findings of Gopee *et al.*, (2000) who reported isolation of *Escherichia coli* from omnivores, herbivores, and carnivores at the rate of 87.2%, 70.0%, and 57.3%, respectively, regardless of animal class, were significantly different. Most (99.6%) of the *Escherichia coli* isolates tested for antibiotic sensitivity exhibit resistance to one or more of the antimicrobial agents used. Resistance was generally high to cephalothin (99.2%), ampicillin (62.4%), tetracycline (58.2%), and streptomycin (36.0%) but low to gentamicin (9.6%), chloramphenicol (1.6%), and norfloxacin (0.4%). Captive wildlife in zoo enclosures is potentially exposed to strains of *Escherichia coli* through contact with animal handlers or through the microorganism in their diets.

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