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Prevalence of *Escherichia coli* and *Salmonella* spp. in Captive Niligiri Langur (*Trachypithecus johnii*) in South India

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A B S T R A C T

The Niligiri Langur (*Trachypithecus johnii*) is a threatened black faced colobine which is endemic to Western Ghats in south India. The present study is designed to study the bacterial flora of gut from this non-human primate. The captive non-human primates usually suffer from gastrointestinal disturbances which often go unnoticed but they harbor variety bacterial flora in the gut. The Enterobactericeace such as *E. coli*, *Shigella* spp. and *Salmonella* spp. are some of the important bacterial species of gut flora. A total of 21 animals studied, of which 56 feacal swabs were collected from 18 animals from Arignar Anna Zoological Park (AAZP), Vandalur, Chennai and 8 feacal swabs from 3 animals from Sri Chamarajendra Zoological Park (SCZP), Mysore. The 64 fresh feacal samples were screened for prevalence of *E. coli* and *Salmonella* spp. by conventional cultural method and molecular techniques. The prevalence of *E. coli* was found to be 100 percent (n-64) by cultural and PCR assay whereas *Salmonella* was isolated from 67.18% (43/64) *viz*., 37/56 (66%) in AAZP and 6/8 (75%) in SCZP by cultural method and PCR. The isolates were further subjected to antibiotic sensitivity test and found that isolates are sensitive to gentamicin, azithromycin and ciprofloxacin but few isolates found resistant to amoxicillin and cefotaxime. The present study concludes that *E. coli* is highly prevalent compared to *Salmonella* in gut of Niligiri Langur.

Introduction

Nilgiri Langur (*Trachypithecus johnii*) is a threatened black faced colobine that is endemic to Western Ghats in South India. Its range includes Kodagu in Karnataka, Kodayar Hills in Tamil Nadu and many other hilly areas in Kerala and Tamil Nadu. Nilgiri Langur has been classified as vulnerable under International Union for Conservation of Nature (IUCN) Redlist (ICUN, 2008). This endangered colobines would benefited from captive breeding programs, maintaining the healthy captive population of colobines can be difficult since they commonly suffer from morbidity and mortality due to gastrointestinal(GI) distress of unknown cause (Shelmidine *et al.,* 2013). The gut microbiota is important in mammalian nutrition (Mackie, 2002). This gut microbiota help the animals to convert indigestible plant structural components such as cellulose to fatty acid chains which aids in animal energy meets (Flint *et al.,* 2012). The colobines have sacculated foregut, which help in pregastric fermentation of food by gut microbiota (Davies and Oates, 1994).The normal inhabitant of non-human primate carry some of pathogenic organisms which includes, *E. coli, Salmonella spp.*, *Shigella* spp., *Klebsiella* spp., *Campylobacter* spp. and more (Nizeyi *et al.,* 2001). The presence of pathogenic bacteria in non-human primate has public health significance due to their close interaction with humans (Marshall, 1991). The primates carrying this organism may affect the health and the fatal infection from its excretion in faeces can carry out diseases to humans or as silent shredders (Mohan *et al.,* 1973).

Escherichia coli are a Gram-negative bacteria and known gut commensal of animals, including non-human primates (NHPs). This diverse organism not only plays a role in the maintenance of gut health by helping to prevent the establishment of pathogenic bacteria in the gastrointestinal (GI) tract, but can also exist in a number of pathogenic forms (Jonathan *et al.,* 2014). *Salmonella* spp. are gram negative bacteria that occur worldwide, inhabiting the intestinal tracts of many species including humans, nonhuman primates, birds, horses, pigs, dogs, cats, rats, mice, hamsters, guinea pigs and other species. There are more than 2000 recognized serotypes of *Salmonella*. Among these, *S. typhimurium* and *S. enteritidis* have been most commonly encountered in the gastrointestinal infections. The present study was taken to understand gut microbiota of Nilgiri Langur (*Trachypithecus johnii*) on an account of *E. coli* and *Salmonella* spp. from different captive facility in South India.

Materials and Methods

Sample collection

The samples were collected from the Arignar Anna Zoological Park, Vandalur, Chennai for isolation and identification of enteric bacterial micro flora in apparently healthy, captive Nilgiri Langur (*Trachypithecus johnii*) between June 2017 to January 2018. The study population includes 18 Nilgiri Langur held in captivity. The samples were fresh excreta from the Niligiri Langur in duplicates and collected by deeply inserting and rotating the sterile swab (Himedia, India) 15 cm long into freshly passed faeces. The samples were transported to the laboratory under refrigeration temperature within four hours. The samples were kept in refrigeration until further processing.

Bacterial Isolation and Identification

The bacterial isolation and identification of *E.coli* and *Salmonella* spp. bacteria by cultural method, the feacal samples were enriched aerobically by inoculating the feacal swab into

nutrient broth for *E. coli* and Rappaport vassiliadis (RV) medium for *Salmonella*, incubated at 37°C overnight. The selective plating was done on Eosin Methylene Blue (EMB) Agar (Himedia) for *E. coli* and on Xylose Lysine Deoxycholate (XLD) Agar for *Salmonella*, incubated at 37°C, 24-36 hours. The colony characteristic of *E.coli* was green metallic sheen on EMB and *Salmonella* spp. were slightly transparent halo with a black centre surrounded by pink-red zone on XLD. The standard biochemical tests performed for confirmation as per the Bergeys manual of determinative bacteriology (Cowan *et al.,* 1993).

DNA extraction

The presumptive colonies of *E.coli* and *Salmonella* spp. grown on selective agar were used for DNA extraction. In brief, single colony from the selective media was suspended in 200 uL of nuclease free water and washed twice. The DNA extraction was done using DNeasy Blood & Tissue Kit (QIAGEN^{TM}) as directed by the manufacturer.

Polymerase Chain Reaction

The *E.coli* was confirmed by PCR targeting *uspA* gene (Chenand Griffiths,1998) and the genus *Salmonella* spp. was confirmed by PCR targeting *invA* gene (Shanmugasamy *et al.,* 2011). The primer sequence and cycling condition are given in (Table: 1).The PCR amplification was optimized using Eppendorf Master cycler personal as thermal cycler.

The 25µL PCR reaction mix was prepared using12.5µL of Taq DNA polymerase 2x Master Mix RED (Ampilqon, India) which provides Tris-HCl (pH 8.5), ammonium sulphate, 2 mM $MgCl_2$, 0.2% Tween 20, 0.4 mM (each) deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), 2U of TaqDNA polymerase and 1µl of each oligonucleotide primer along with 3µl of template DNA. The PCR products were electrophoresed on 1.5% agarose gel pre-stained with ethidium bromide (0.5ug/mL) and viewed under UV light using a UV Trans illuminator with the DNA bands sized by extrapolation based on mobility of 100 bp DNA markers co-electrophoresed.

Antibiotic sensitivity test

Antimicrobial susceptibility test was performed by the disc diffusion method on Mueller-Hinton Agar (Himedia, Mumbai, India) using antimicrobial discs (Himedia, Mumbai, India) according to the instructions of Clinical and Laboratory Standards Institute (CLSI, 2015). Isolates were grown for 6 hours on nutrient broth (Himedia, Mumbai, India) and adjusted to 0.5 McFarland using sterile physiological saline, swabbed onto the Mueller-Hinton medium (Himedia, Mumbai, India), and incubated with antimicrobial discs at 37^0C for 18-24 hours. A total of 5 antibiotic discs (Himedia, Mumbai, India) i.e Amoxicillin (AMX-10 mg), Gentamicin (GEN-10 mg), Azithromycin (AZM-30), Cefotaxime (CTX-5mg) and Ciprofloxacin (CIP-5 mg) were used. After incubation, the zone of inhibition was measured and compared with zone diameter interpretative chart to determine the sensitivity of the isolates to the respective antibiotics standards.

Results and Discussion

Prevalence of *E. coli* and *Salmonella* spp. by cultural method

Out of 64 samples collected from the captive Nilgiri Langur showed the prevalence of *E. coli* was 100 percent (64/64) from cultural and PCR assay in both the study areas whereas *Salmonella* isolated was 67.18 percent (43/64) by cultural method *viz*., 37/56 (66%) in AAZP and 6/8 (75%) in SCZP.

E. coli was isolated and identified from the samples after cultivation on EMB agar. The prevalence of *E. coli* in the study was 100% (table: 2) the colony characteristic were yellow green with metallic sheen on agar plates and the staining characteristic were Gram negative, pink color, small rod shaped appearance arranged in single or paired short.

The biochemical test characters identified were Indole positive (+ve), Methyl red positive (+ve), VogesProskauer test negative (-ve), TSI test yellow butt and slant yellow(y/y), H2S negative (-ve), gas production positive (+), citrate utilization test positive (+ve).

Salmonella spp. was isolated and identified from the samples culturing on XLD agar. The prevalence of *Salmonella* spp. in the study was 67.18percent (43/64) (table 2). The colony characteristic was red with black centre on agar plates and the staining characteristic were Gram negative, pink color, small rod shaped arranged singly or paired short. The biochemical test characters identified were Indole negative (-ve), Methyl red positive (+ve), VogesProskauer test negative (-ve), TSI test yellow butt and slant red(y/r), H2S positive (+ve), gas production positive (+), citrate utilization test positive (+ve).

Prevalence of *E. coli* and *Salmonella spp.* by PCR

E. coli was confirmed by pcr targeting *uspA* gene (universal stress protien) as described by (Chen and Griffiths, 1999). Similarly, *Salmonella* spp. was confirmed by *invA* gene (Shanmugasamy *et al., 2011*). The positive culture samples were screened by PCR to amplify the *uspA* for *E. coli* and *invA* gene for *Salmonella* spp., the amplified product found to be 884bp (Figure 1) for *uspA* and 284 bp (figure 2) for *invA* gene fragment. The results shown in table 2.

Antibiotic sensitivity test

The bacterial isolates of 43 *Salmonella* and 64 *E. coli* were subjected disk diffusion assay for further detection of antibiotic sensitivity test against antibiotics such as gentamicin, amoxicillin, azithromycin, cefotoxime and ciprofloxacin. The resistance pattern of *E. coli* and *Salmonella* spp. against different antibiotics given in table: 3.

The isolation of *E.coli and Salmonella* spp. from apparently healthy Niligiri Langurs is first attempt to study microbiota from these captive Langurs. The prevalence of this organism has no doubt they are normal inhabitants of the gut microflora and some of them may be enteric pathogens. The presences of E. coli are indicators of potential hazardous infections of surrounding human communities (Bailey and Mansfield, 2010). Toxin producing *Escherichia coli* such as shiga toxigenic *Escherechia coli* (STEC) has potential to infect from mild diarrhoea to severe disease, in animals (Mansfield and Kemnitz, 2008). Colobine numbers in the wild are declining at a rapid rate with no signs of reprieve (IUCN, 2015), and while captivity offers colobines protection from external threats such as hunting and habitat destruction (Mittermeier *et al.,* 2009), it is also associated with a distinct gut microbiota that may influence susceptibility to GI illness.

In this study, we aimed to determine the prevalence of *E.coli* and *Salmonella* in a captive population of Nilgiri Langur. The present study revealed the higher prevalence of *E.coli* (100%) than *Salmonella* spp. (67.18%), respectively.

Similar results were recorded in some studies, like the prevalence of *E. coli* is 100 percent (n-33) and *Salmonella paratyphi* A is 87.9 percent (29/33) in non-human primates (NHPs) (okwari *et al.,* 2014).

Table.1 The primers sequences and cyclic conditions used in the present study

Organism	GENE	SEQUENCE					REFERRENCE
E. coli	***uspA***	Forward 5-3- CCGATACGCTGCCAATCAGT Reverse 5-3-ACGCAGACCGTAGGCCAGAT					Chen and Griffiths, 1999
		Cycling condition					
		94°C for 5 min	94°C for 1 min	57°C for 1 min	72°C for 2 min	72°C for 5 min	Product size 884bp
			30 cycles				
***Salmonella* spp.**	***invA***	Forward 5-3- GTGAAATTATCGCCACGTTCGGGCAA Reverse 5-3-TCATCGCACCGTCAAAGGAACC.					Shanmugasamy *et al.*, 2011
		Cycling condition					
		94°C for 1 min	94°C for 1 min	64°C for 30 sec	72°C for 30 sec	72°C for 7 min.	Product size 284bp
			35 cycles				

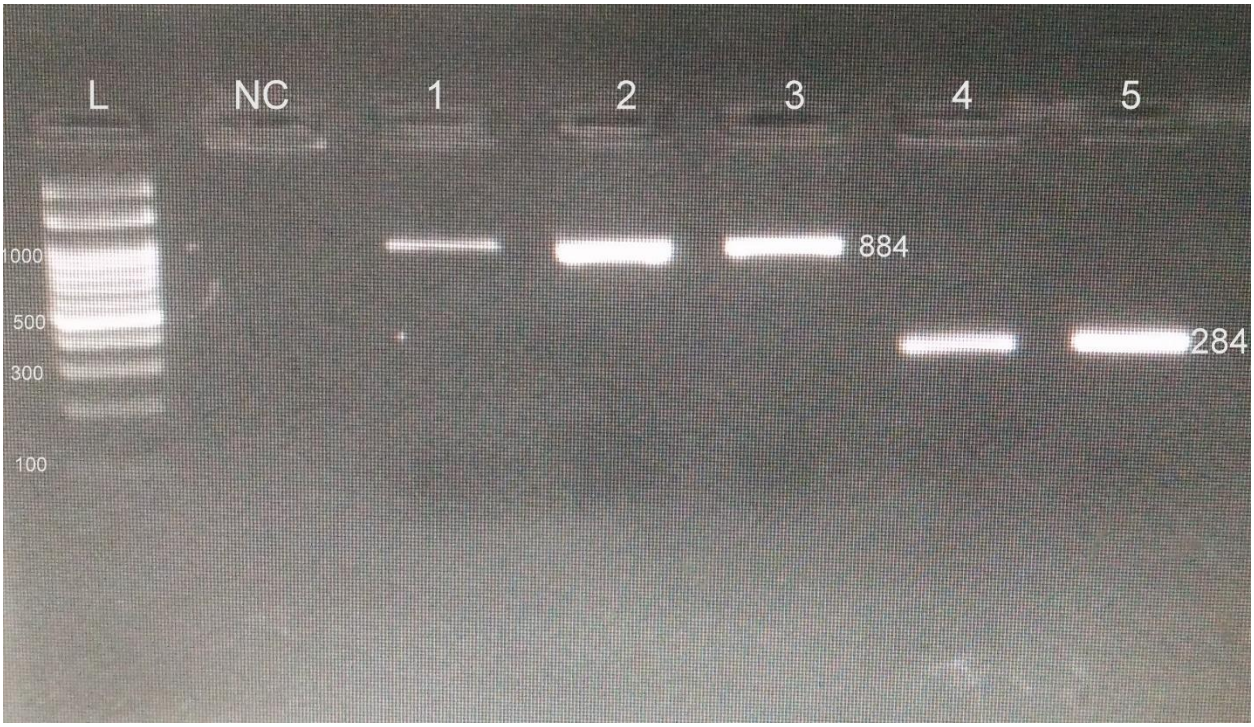
Table.2 Prevalence of *E. coli* and *Salmonella* spp. of captive Niligiri Langurat different facility

Sl No	Sampling site	*E.. Coli* spp.		*Salmonella spp.*	
		+Ve	%	+Ve	%
1	Arignar Anna Zoological Park (Chennai) (n=56)	56	100	37	66
2	Sri Chamarajendra Zoological Gardens (Mysore) (n=8)	8	100	6	75
Total		64	100	43	67.18

Table.3 Antibiogram for *E. coli* and *Salmonella* spp. isolated from captive Niligiri Langur

Organism	Gentamicin (10mg) MIC: <12->15 mm			Amoxicillin (10mg) MIC: <19->25 mm			Cefotoxime (30mg) MIC: <29->35 mm			Azithromycin (30mg) MIC: <24->30 mm			Ciprofloxacin (5mg) MIC: <16->20 mm		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
E. coli	55	9	-	-	34	30	-	51	13	60	3	-	64	-	-
Salmonella spp.	40	3	-	-	9	34	-	40	3	34	9	-	40	3	-

Fig.1 PCR gel image of *E. coli* and *Salmonella* spp. isolated from captive Niligiri langur



(L: Ladder, NC: Negative control, 1-3: E. coli, 4-5: Salmonella)

Enteropathogenic *Escherichia coli* (EPEC) infection was isolated in adult monkeys (rhesus macaques) (Sestak K *et al.,* 2003). Isolation of *Salmonella* species been previously reported from Non-Human Primates (NHPs) (Robert *et al.,* 1969). The mode of transmission is contaminated water, flies, infected dust; fruits and vegetables and human waste, they aid in transmission and may also contribute through animals diet. The prevalence in NHPs may thus indicate an anthropozoonotic transmission of these organisms. The prevalence of *Salmonella* paratyphi, Campylobacter and Yersinia, organisms are similar to findings of (Mikov, 1994).

The *E. coli* has high resistant against amoxicillin and cefotaxime whereas *Salmonella* spp. has resistant against amoxicillin and also showed intermediate resistance against cefotaxime. The isolates are sensitive against gentamicin, azithromycin and ciprofloxacin. The prevalence of antibiotic resistant *E. coli*in wild gorillas reported that ampicillin resistance followed by streptomycin resistance and tetracycline resistance in national park (Benavides *et al.,* 2012).This tendency suggests the transmission of human-borne resistant bacteria to wildlife. In emperor tamarins showed highest prevalence of ampicillin resistance (32.3%) and in white faced sakis (29.6%) for E. coli (Jonathan *et al.,* 2014).

This study shows the prevalence of *E. coli* and *Salmonella* spp. in healthy captive Niligiri Langur and it's reported for the first time. The prevalence of *E. coli* was 100 percent whereas *Salmonella* spp is 67.18 percent respectively. This current study may serve as guideline for future studies in the same species and other important gut microbiota. This study aided to know the gut microbiota in captive Nilgiri Langur supporting Asian colobine conservation

efforts. The present data helps to formulate and treat animals with antimicrobials in captivity with GI disturbance.

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