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Isolation of *Escherichia coli* from faecal matter of wild captive animal species of Rajasthan

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Abstract

Background: *Transmission of Zoonotic diseases of antimicrobial-resistant bacteria from wildlife to humans is a matter of serious global concern. Among these, Escherichia coli (E. coli) serves as an effective indicator of faecal contamination and antimicrobial resistance in all the major species including both human and animal populations.*

Objectives: *This study aimed to isolate and characterize E. coli from the faecal samples of wild captive animals in two zoological parks in Rajasthan, India–Bikaner Zoo and Machia Biological Park, Jodhpur–and assess their antimicrobial resistance profiles.*

Methods: *A total of 66 faecal samples were collected aseptically from various captive wild species residing in two biological parks of Rajasthan. Isolation was performed as per protocol using standard culture techniques on MacConkey and EMB agar. Identification was done by Gram staining and Confirmation using biochemical profiling through the VITEK 2 system and genotypic confirmation via 16S rRNA gene PCR amplification. Antimicrobial susceptibility was examined using the disc diffusion method against 15 commonly used antibiotics, and resistance was interpreted following CLSI guidelines.*

Results: *Out of 66 samples, 59 isolates were confirmed as Escherichia coli, yielding an overall recovery rate of 89.39%. Bikaner Zoo showed a higher prevalence (92.38%) compared to Machia Biological Park (84%). Antibiogram results indicated high sensitivity to ciprofloxacin and gentamicin, while significant resistance was observed against polymyxin B, cefaclor, and cefepime. VITEK 2 accurately identified 100% of the isolates, and 16S rRNA PCR confirmed their genetic identity.*

Keywords: *Escherichia coli, Captive wild animals, Zoonotic pathogens, Antimicrobial resistance, VITEK 2 identification, 16S rRNA PCR, Disc diffusion assay*

INTRODUCTION

Wildlife is the term used for animals which are either free roaming like in forests or maintained in captivity like in zoos (mammals, birds, fish, reptiles, and amphibians), and the term zoonosis is applied for infectious disease transmittable between animals and humans. The total number of zoonoses is still unknown, but according to Taylor et al., who in 2001 catalogued 1,415 known human pathogens, 62% were of zoonotic origin. The list of human pathogens which are found to be of animal origin is continuously increasing with time. Wild animals are likely to be involved in the epidemiology of most zoonoses (Kruse et al.,2004). The most probable reason for this can be that they shed a variety of pathogens without showing any clinical symptoms. Therefore, animals who are even well maintained, or appear to be healthy and physiologically normal, may act as a reservoir of pathogenic organisms, and thus can act as a carrier of infectious disease.

Although all kind of animals are at risk for shedding infectious agents, but our study was mainly focussed on wild animals maintained in captivity like in zoos and biological parks. As the humans are in direct contact with such animals and so there is a high risk of transmission of these infectious agents between them.

Recently, zoos have aimed to focus on animal welfare and species conservation (Ward et al., 2018); however, some still use strategies such as petting for zoo management and increasing public interest as a result it has become possible to become infected with zoonotic pathogens through the ingestion of animal waste through the mouth, direct contact with animals or contaminated surfaces (Conrad et al.,2017).

On the other hand, Humans specially children can easily approach and feed captive wildlife and domestic animals in petting zoos so such wildlife species can result in several zoonotic outbreaks, including infections caused by *Escherichia coli* O157:H7, Salmonellae and Coxiella burnetii (Bender et al., 2004). Contact with human handlers specially caretakers of the zoo and humans continue to contact wildlife through hunting or trade in wild animals so there is always a increased risk of pathogen spill over into human populations (Daszak et al., 2000). Exposure to captive wild animals at circuses or zoos can also be a source of zoonotic infection.

Another concern is animal-to-human transmission of antimicrobial-resistant bacteria (Collignon et al.,2016). A strong positive correlation may exist between antibiotic use and antibiotic resistance in *E. coli* (Chantziaras et al., 2014) and several studies have reported the horizontal transmission of zoonotic diseases from zoos or petting farms (McMillion et al., 2007).

Although captive wild animals can shed variety of pathogens. Our study was mainly focussed on *E. coli*. This notorious bacterium is mainly found in the intestinal and extra intestinal region of the gut and is one of the potent infectious agents resulting in dysentery and other complications so it can easily spread through faecal matter contaminated with them. Research and data available in the past has shown that it is responsible for causing numerous outbreaks of diseases at petting zoos, or similar public animal contact places (Bender et al.,2004). So suitable protocol or procedures are much needed to isolate and identify *E. coli* from faecal matter of wild captive animal species in order to facilitate its further study.

MATERIALS AND METHODS

Sample Collection - A total of 66 freshly voided faecal samples were collected aseptically from wild captive animals housed in Bikaner Zoo (July 2018) and Machia Biological Park, Jodhpur (August 2018), with formal permission from the Department of Forest and Environment, Government of Rajasthan. The animals sampled included Chinkara (Gazella gazella), Black buck (Antelope cervicapra), Blue bull (Boselaphus tragocamelus), Jackal (Canis aureus), Lion (Panthera leo), Panther (Panthera pardus), and Jungle cat (Felis chaus). Approximately 100 g of each faecal sample was collected in sterile vials and transported to the laboratory in insulated ice boxes for immediate processing.

Samples were enriched by inoculation into nutrient broth and incubated overnight at 37°C in a shaking incubator. Species-wise distribution of collected samples is summarized in Tables 1 and 2.

Table 1: Faecal samples collected from Bikaner Zoo

S. No.	Species	No. of Samples
1	Chinkara (Gazella gazella)	18
2	Black buck (Antelope cervicapra)	18
3	Blue bull (Boselaphus tragocamelus)	5
	Total	**41**

Table 2: Faecal samples collected from Machia Biological Park, Jodhpur

S. No.	Species	No. of Samples
1	Chinkara (Gazella gazella)	8
2	Black buck (Antelope cervicapra)	10
3	Jackal (Canis aureus)	1
4	Lion (female) (Panthera leo)	1
5	Lion (cub) (Panthera leo)	3
6	Panther (Panthera pardus)	1
7	Jungle Cat (Felis chaus)	1
	Total	**25**

Isolation and Identification of *E. coli*:

Isolation and identification of *Escherichia coli* were performed following the protocols of Cowan and Steel (1975). Enriched broth cultures were streaked onto MacConkey agar and incubated at 37°C for 18–24 hours. Pink, lactose-fermenting colonies were selected and subcultured onto Eosin Methylene Blue (EMB) agar plates. Colonies exhibiting a characteristic green metallic sheen on EMB were presumptively identified as *E. coli*.

Well-isolated colonies were then subjected to Gram staining and biochemical identification. Gram staining revealed pink-colored, rod-shaped bacilli arranged singly or in pairs, consistent with *E. coli*. Biochemical confirmation was performed using the VITEK 2 Compact automated identification system (bioMérieux India Pvt. Ltd.), which provided definitive identification of *E. coli* isolates. Confirmed isolates were maintained on nutrient agar slants at 4°C. To preserve viability, repeated subculturing was carried out as required for further molecular analysis.

Chromosomal DNA extraction from *E. coli* isolates:

Chromosomal DNA from confirmed *Escherichia coli* isolates was extracted using the method described by Chen and Kuo (1993), with minor modifications. After extraction of the DNA, the DNA was stored at -20 degree.

Molecular identification by PCR

All 59 *Escherichia coli* isolates were genotypically confirmed by amplification of the 16S rRNA gene using primers described by Khaled et al. (2010). The primer sequences used for ribotyping were:

- **Forward Primer (Primer 1):** 5'-GCTTGACACTGAACATTGAG-3'
- **Reverse Primer (Primer 2):** 5'-GCACTTATCTCTTCCGATT-3'

PCR Reaction Mixture

PCR was performed in a 25 µL reaction volume using sterile, nuclease-free PCR tubes (ABDOS). The reaction components are listed in Table 3.

Table 3: Components and Quantity of PCR Reaction Mixture for Genotypic Confirmation of *E. coli*

Component	Quantity (µL)
Master Mix (2X)	12.5

Forward Primer	1.0
Reverse Primer	1.0
Nuclease-Free Water	7.5
Template DNA	3.0
Total Volume	**25.0**

PCR Amplification Conditions

PCR was carried out in a **Veriti™ 96-well Thermal Cycler** (Applied Biosystems) with a heated lid. The thermocycling profile is detailed in Table 4.

Table 4: PCR Cycling Conditions for 16S rRNA Amplification

Step	Action	Temperature (°C)	Time	No. of Cycles
1.	Initial Denaturation	95	3 minutes	1
2.	Denaturation	95	1 minute	30
3.	Annealing	50.8	1 minute	30
4.	Extension	72	1 minute	30
5.	Final Extension	72	7 minutes	1

The amplification reaction targeted a fragment of approximately **662 base pairs**. PCR products were analyzed using agarose gel electrophoresis to verify the expected amplicon size.

Storage of *E. coli* isolates:

For purpose of short preservation, bacteria were stored under refrigeration temperature.

For long term preservation, cultures were freezed at -80°C in Glycerol.

Antimicrobial susceptibility test

Antibiogram pattern of the *E. coli* isolates was studied by disc diffusion technique as described by Bauer et al. (1966) against a panel of 15 antibiotics (Table 5). Antimicrobial testing results were recorded as sensitive, intermediate and resistant according to zone diameter interpretative standards provided by (CLSI 2011).

Table 5: Concentration (ug) and class of the Antibiotics used in the experiments

S. No.	Antibiotics	Class	Concentration (µg)
1.	Ampicillin (AMP)	Penicillin	10
2.	Cefaclor (CF)	Cephalosporin (IInd generation)	30
3.	Cefixime (CFM)	Cephalosporin (IIIrd generation)	5
4.	Cefepime (CPM)	Cephalosporin (IVrd generation)	30
5.	Cephalothin (CEP)	Cephalosporin (Ist generation)	30
6.	Ciprofloxacin (CIP)	Fluoroquinolone	5
7.	Chloramphenicol (C)	Lincosamide	30
8.	Co-trimoxazole (CoT)	Sulfonamide	1.25/23.75
9.	Colistin (CL)	Polypeptides	10
10.	Gentamicin (GEN)	Aminoglycosides	10
11.	Imipenem (IPM)	Carbapenem	10
12.	Nalidixic acid (NA)	Fluoroquinolone	10
13.	Piperacillin (PIT)	Penicillin	100
14.	Polymyxin B (PB)	Polypeptides	300
15.	Tetracycline (TE)	Tetracycline	30

RESULT AND DISCUSSION

Isolation and identification of *Escherichia coli* from faecal samples of wild captive animals A. Isolation of *E. coli* from faecal matter of wild captive animals of Bikaner zoo - From Bikaner region, out of 41 different samples from captive animals, only 1 sample of black buck was found negative on MacConkey and EMB agar, while 2 samples of black Buck and one sample of blue bull were negative after genotypic confirmation using 16S rRNA sequence based identification using PCR which gave an overall recovery rate of 92.38%. The results are summarized in the table 6.

Table 6: Details of samples, sources of samples and recovery of *E. coli* isolates from faecal matter of wild captive animals at Bikaner zoo.

S.no.	Source of Samples	Total no. of samples	No. of samples positive on MacConkey agar	No. of samples positive on EMB agar	No. of genotypically confirmed isolates	Percentage of confirmed isolates
1.	Chinkara (Gazella gazelle)	18	18	18	18	100
2.	Black buck (Antelope cervicapra)	18	17	17	16	88.88
3.	Blue bull (Boselaphus tragocamelus)	5	5	5	4	80
	Total	41	40	40	38	92.38%

B. Isolation of *E. coli* from faecal matter of wild captive animals at Machia biological park, Jodhpur - A total of 25 freshly voided faecal samples of chinkara, black buck, lion (cub and female), panther and jungle cat were collected under the supervision of biological park authorities. Out of 25 samples, 21 were genotypically confirmed as *E. coli* which gave an overall recovery rate of 84%. One sample of chinkara and black buck were found negative on genotypic confirmation. No colonies were observed in faecal samples of panther and jungle cat. The results are summarized in table 7.

A total of 59 *E. coli* isolates were recovered from different wild captive animal species of Bikaner and Jodhpur regions with an overall recovery percentage of 89.39.

Table 7: Details of samples, sources of samples, and recovery of *E. coli* isolates from faecal matter of wild captive animals of Machia biological park, Jodhpur

S.no.	Source of samples	Total no. of samples	No. of samples positive on MacConkey agar	No. of samples positive on EMB agar	No. of Genotypically confirmed isolates	Percentage of confirmed isolates
1.	Chinkara (Gazella gazelle)	8	8	8	7	87.5
2.	Black buck (Antelope cervicapra)	10	10	10	9	90
3.	Lion (cub) (Panthera leo)	3	3	3	3	100
4.	Lion (female) (Panthera leo)	1	1	1	1	100
5.	Panther (Panthera pardous)	1	0	0	0	0
6.	Jackal (Canis aureus)	1	1	1	1	100
7.	Jungle Cat (Felis chaos)	1	0	0	0	0
Total	25	23	23	21	Recovery = 84%	

In our results, although high prevalence of *E. coli* isolates was observed in both the captive areas of Rajasthan, but Bikaner Zoo showed greater no. of isolates (92.38%) as compared to biological park of Jodhpur region. There could be several reasons which explain the higher prevalence of *E. coli* isolates in

Bikaner Zoo. The most probable reason of which could be high shedding of *E. coli* in diarrhoeal cases. Location of Bikaner Zoo being situated in the centre of Bikaner city, surrounded by the urban locality could be another probable reason for such observation as it could result in transmission of more *E. coli* contamination from humans to animals.

Wild captive animals at biological park of Jodhpur also presented a high satisfactorily maintained, there is a limited access by visitors (visitors can only see animals from a distance). Moreover, Machia biological Park is situated far away from the urban areas of Jodhpur, which could be the main reason for restricted (although high) transmission of *E. coli* in captive animals of Jodhpur as compared to Bikaner area. Katakweba et al. (2015) isolated *E. coli* in cattle and wildlife faeces in Tanzania and examined higher counts in wildlife faeces than the cattle faeces. Similarly, Chandran and Mazumdar (2013) also isolated a total of 593 E. coli strains from faecal samples of wild animals during a 3 year period (2004 to 2006) from the Okanagan lake of Canada. The animals included bison (11), black bear (48), buffalo (5), cow (49), coyote (121), deer (89) dog (48), horse (19), donkey (25), big-horned sheep (16), llama (5), pony (19), rabbit (5), mountain goat (20), cougar (20), elk (29), grizzly bear (20), and groundhog (19). Oludairo et al. (2016) collected 160 samples from different wild animals, of which 58 (36.3%) were confirmed as *E. coli.*

Reasons for high prevalence of *E. coli* in wild animals could be attributed to contaminated food and water, visitors, lizards or bats. Unhygienic practices by caretakers could also serve as a possible source of transmission of *E. coli* in such captive areas.

Biochemical identification *of E. coli* by Vitek 2 system

All the 59 *E. coli* isolates were correctly identified by the VITEK 2 GN method, representing 100 % correct identification *of E. coli.*

Our results were also in consistence with the results obtained by Crowley et al. (2012) who observed 100% correct identification of 60 *E. coli* isolates. Other Gram-negative and Gram-Positive bacteria were also correctly identified by Vitek 2 system. Crowley et al. (2012) concluded that Vitek 2 automated system is an accepted, convenient and rapid system for correct identification of bacteria. The overall reliable performance was produced by Vitek 2 system.

The method is much faster than conventional identification biochemical tests, which are usually reported in many days, and the process was much lesser labour intensive

Genotypic confirmation of *E. coli* isolates

Properly diluted primers specific for amplification of 16S rRNA gene fragment of *E. coli* were used and PCR was performed in standard condition as per protocol suggested by Khalid et al. (2010). Molecular ladder of 100 bp was used. All the 59 *E. coli* isolates produced amplicon of 662 bp on gel electrophoresis at 80V (Fig.). Clear bands of 662 bp visualized in gel doc confirmed the presence of *E. coli* in the isolates. Islam et al. (2016) also identified the *E. coli* phenotypically as well as DNA extracted from *E. coli* isolates was used in the PCR assay. PCR primers targeting 16S rRNA gene of E. coli amplified primers binding sequences on DNA confirmed the identity of *E. coli*. In another similar study on spotted dear at Bikaner zoo was done by (axis axis) Rathore et al. (2016), it was observed that out of total 18 samples, 16 isolates were genotypically confirmed as *E. coli* with a recovery of (80%). Our results were also similar to those of Shahrani et al. (2014) who obtained 630 *E. coli* isolates from 824 samples (76.45%) collected from diarrhoeic calves in Iran.

Result of antimicrobial susceptibility testing

A total of 15 antibiotics from various classes were used on all the isolates to develop antibiograms using disc diffusion method. The antibiograms of 38 isolates from Bikaner Zoo revealed that the most effective antibiotics was ciprofloxacin against which 90.2% of the isolates were sensitive, followed by nalidixic acid (75.6%), chloramphenicol (68.4%), gentamicin (68.3%) and Imipenem (53.6%) and very less sensitivity was observed for other antibiotics. Resistance towards polymyxin B was shown by 80.5% of the isolates while 78.1% and 78% of the isolates were resistant to cephalothin and cefepime, respectively. The antibiogram of *E. coli* isolates from wild captive animals of Machia biological park, Jodhpur revealed that the most effective antibiotics was ciprofloxacin against which 100% of the isolates were sensitive followed by Co-trimoxazole (98%), polymyxin B (98%), chloramphenicol (94%) and gentamicin (89%). Ninety percent of the *E. coli* isolates were resistant to cefaclor and cefepime both.

The resistance observed in captive wildlife of Rajasthan may be due to regular exposure of animals to the human strains of bacteria possibly through contact with care takers, visitors, contaminated food or water consumed by animals. Captive wild animals residing in areas adjacent to urban locality are mainly at the high risk of infection of *E. coli* and thus transfer of antibiotic resistance genes. Inappropriate chemotherapeutic measures overuse or misuse of antibiotic drugs, visitors, zookeepers, unhygienic conditions which may involve contaminated food and water supply are some of the reasons responsible for transfer of drug resistant *E. coli*. Some animals could also be already resistant to antibiotic drug at the time of their introduction to the zoo, so the antibiotic resistance pattern of animals should be carefully monitored throughout their life span in order to prevent its further spread into vicinity.

CONCLUSION

In conclusion, high prevalence of *E. coli* in wild captive animals of Bikaner zoo and Machia biological park, Jodhpur indicated that these animals are important reservoir of E. coli. Prevalence of *E. coli* in both the areas observed in present study were almost similar, so these animals may be equally important source for human infection particularly those living in close proximity of these animals. It is also possible that due to lack of awareness, there is high transmission of *E. coli* from human and contaminated feed or water to the animals living in captivity. The organisms were 100% typable using 16S rRNA ribotyping and this method identified all the isolates with specificity. All the 59 *E. coli* isolates were correctly identified by the VITEK 2 GN method, representing 100% correct identification of *E. coli*, thus an overall reliable performance was produced by Vitek 2 GN method. The isolates of both the places showed highest sensitivity against ciprofloxacin while polymyxin B, cefaclor and cefepime were the most resistant antibiotics observed in isolates of Bikaner and Jodhpur respectively. Public Health awareness including safe and hygienic practices, are of prime importance in decreasing the occurrence of *E. coli* infection and its transmission especially between the individuals closely associated with management of captive areas.

Conflict of Interest The authors declare no conflict of interest.

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