



Study on Lactose Fermenting Enterobacteriaceae in Captive Star Tortoises (*Geochelone elegans*) from different Captive Facilities in South India, with a Profile of Antimicrobial Drug Resistance in Pathogenic *Escherichia coli*

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ABSTRACT

The present study reported the prevalence of lactose fermenting Enterobacteriaceae in Star Tortoises (*Geochelone elegans*) from three different captive facilities in South India viz., Chennai Snake Park Trust (CSPT), Chennai, Tamil Nadu, Arignar Anna Zoological Park (AAZP), Chennai, Tamil Nadu and Sri Chamarajendra Zoological Gardens (SCZG), Mysuru, Karnataka. A total of forty cloacal swabs and forty fresh faecal samples were collected separately from same captive Star Tortoises of different captive facilities and three water samples each from three different captive facilities. The cloacal swabs were used for cultural isolation of lactose fermenting Enterobacteriaceae and fresh faecal samples were used for direct DNA extraction using QIAamp Fast DNA Stool Mini Kit. The *fimC* (Type I fimbriae) gene was used for identification of Pathogenic *Escherichia coli* through Polymerase Chain Reaction (PCR). *E. coli* (n=11) (27.5%) was the intermittently encountered bacteria, followed by *Klebsiella* spp. (n=8) (20.00%) and *Enterobacter* spp. (n=8) (20.00%) isolated from cloacal swabs through culture method. The *Enterobacter* spp. was more prevalent in water samples, followed by *Klebsiella* spp. while *E. coli* was absent in water samples. A total of 21 samples were positive for *E. coli*, through DNA isolated from fresh faecal samples using QIAamp Fast DNA Stool Mini Kit. This showed that direct DNA isolation was more convenient than traditional culture method of bacterial confirmation. The *E. coli* isolates were tested for antimicrobial sensitivity by using Disc Diffusion Method. Antimicrobial resistance pattern as follows, resistance was recorded against cefotaxime (54.54%), azithromycin (45.45%), gentamicin, tetracycline and amoxicillin-clavulanic acid (36.36%). No isolate was found resistant against enrofloxacin. The *E. coli* isolates also showed multiple drug resistance to different group of drugs.

Keywords: Antibiotic sensitivity test, Enterobacteriaceae, *E. coli*, PCR, Star Tortoise

The Indian freshwater turtles and land tortoises comprises of 16 species of freshwater and semi aquatic batagurines (*Emydidae*), 6 species of softshell turtles (*Trionychidae*) and 4 species of land tortoises (*Testudinidae*) (Choudhury and Bhupathy, 1993). Indian Star Tortoise (*Geochelone elegans*), the land tortoise, is one of the important species

of chelonians (Order- *Testudines*) inhabit a variety of dry vegetation types, including scrublands, grasslands, desert, edges and agricultural landscapes of fields, hedgerows and plantations (de Silva, 2003; Fyfe, 2007) and it was listed as vulnerable by International Union for the Conservation of Nature and Natural resources (IUCN) Red list of



threatened species (IUCN, 2016). The lactose fermenting Enterobacteriaceae include those enteric bacteria that are able to ferment lactose with the production of gas within 24 hours such as *E. coli*, *Klebsiella* spp., *Enterobacter* spp. and *Serratia* spp. *E. coli*, *Klebsiella* spp. and *Enterobacter* spp. are the gram negative, non sporulating facultative anaerobic bacilli. *E. coli* is one of the major bacterial pathogens which affect the gastrointestinal tract of reptiles (Fowler, 1986), usually it lives as a gut commensal of warm and cold blooded animals (Gordon and Cowling, 2003; Tenailon *et al.*, 2010).

Enteritis is one of the primary problems in reptiles. Gram-negative bacteria are the most common agents associated with enteritis, the bacterial isolates include *E. coli*, *Klebsiella* spp., *Salmonella* spp., *Pseudomonas* spp., *Serratia* spp., *Proteus* spp. and *Citrobacter* spp. Some are zoonotic importance especially *E. coli* and *Salmonella* spp. (Mader, 2006).

E. coli was isolated from faecal samples of reptiles especially different chelonians such as fresh water turtles (Mitchell and McAvoy, 1990) and Red-eared sliders (Chiacchio *et al.*, 2014). *E. coli*, *Klebsiella* spp., and *Enterobacter* spp. were isolated from cloaca of free-ranging desert tortoises (Dickinson *et al.*, 2001) and freshwater turtles (*Mauremys rivulata* and *Emys orbicularis*) (Hacioglu *et al.*, 2012). *E. coli* and *Klebsiella pneumoniae* were isolated from cloaca of *Podocnemis expansa* and *P. unifilis* (Morais *et al.*, 2011) and European pond turtle (Nowakiewicz *et al.*, 2015). *E. coli*, *Enterobacter aerogenes* and *E. cloacae* isolated from cloaca of live stranded loggerhead turtles (Foti *et al.*, 2008).

The present study was conducted to know the bacterial population in captive Star Tortoises since there are very few reports from this species. In this study lactose fermenting Enterobacteriaceae such as *E. coli*, *Klebsiella* spp. and *Enterobacter* spp. were isolated from cloacal swabs through culture method and antibiotic sensitivity test was performed for *E. coli* isolates. The DNA was extracted from fresh faecal samples using QIAamp Fast DNA Stool Mini Kit and PCR was carried out to identify pathogenic *E. coli*.

MATERIALS AND METHODS

Samples collection

The cloacal samples and fresh faecal samples were

collected separately from captive Star Tortoises from three different captive facilities in South India viz., 6 samples from CSPT, Guindy, Chennai, Tamil Nadu, 21 samples from AAZP, Vandalur, Chennai, Tamil Nadu and 13 samples from SCZG, Mysuru, Karnataka.

A total of 40 cloacal swabs and 40 fresh faecal samples from captive Star Tortoises were collected separately and one water sample (around 200 ml) collected from each captive facility. The cloacal swabs and water samples were subjected for bacteriological examination using traditional culture method. The fresh faecal samples were subjected for DNA extraction using QIAamp Fast DNA Stool Mini Kit for pathogenic *E. coli* confirmation through PCR.

Bacteriological examination by culture method

The lactose fermenting Enterobacteriaceae from cloacal swabs and water samples were isolated using MacConkey agar and Eosin Methylene Blue (EMB) agar. On MacConkey agar, *Klebsiella* spp. and *Enterobacter* spp. forms large mucoid, pale pink colonies whereas *E. coli* gives bright pink colonies. On EMB agar, *E. coli* forms the unique and characteristic greenish metallic sheen (Quinn *et al.*, 1994). The biochemical tests such as IMViC (Indole, Methyl red, Voges-Proskauer and Simmons citrate) test, lysine decarboxylase, Triple Sugar Iron (TSI) agar were used for bacteriological confirmation. Motility test medium was used to differentiate *Klebsiella* spp. and *Enterobacter* spp.

Polymerase Chain Reaction (PCR) for pathogenic *E. coli*

The QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany) was used to extract DNA from fresh faecal samples. The Type I Fimbriae (*fimC*) gene was used for detection of Pathogenic *E. coli* (Janben *et al.* 2003; Ewers *et al.* 2004) through PCR. The reaction mixture was prepared in 25 µl volumes (12.5 µl of Ampliqon master mix (2X), 1 µl of Forward primer (10 pmole), 1 µl of Reverse primer (10 pmole), 2 µl of sample DNA template (50-100 ng) and 8.5 µl of Nuclease Free Water). The PCR amplification was carried out in Eppendorf Mastercycler (Eppendorf, Germany) with the following thermal programme. Initial denaturation at 94°C for 2 minutes followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 59°C

for 1 minute and primer extension at 72°C for 90 seconds followed by final extension at 72°C for 7 minutes. The amplified PCR products were then electrophoresed (100 V, 20 min) on a 1.5% (W/V) agarose gel and visualized under U.V. light. The images were analysed with PC software (Bio-rad SA, Vitry sur Seine, France) from Bio-rad Gel Doc 1000 imager system.

Antibiotic sensitivity test

The antibiotic sensitivity test for *E. coli* was done using Disc Diffusion Method on Mueller-Hinton agar. The antibiotic discs used were amoxicillin-clavulanic acid (30 µg/disc), cefotaxime (30 µg/disc), azithromycin (15 µg/disc), gentamicin (10 µg/disc), tetracycline (30 µg/disc) and enrofloxacin (10 µg/disc). The *E. coli* isolates were classified as resistance, intermediate resistance and sensitive, according to Clinical and Laboratory Standards Institute (CLSI) standards for most of drugs (CLSI, 2017) and for azithromycin based on work done by Chayani *et al.* (2009).

RESULTS AND DISCUSSION

The *E. coli*, 27.50 per cent (11/40) was most often encountered lactose fermenting Enterobacteriaceae followed by *Klebsiella* spp. 20.00 per cent (8/40) and

Enterobacter spp. 20.00 per cent (8/40) from cloacal swabs (Table 1). The *Enterobacter* spp., 66.66 per cent (2/3) was most frequently isolated lactose fermenting Enterobacteriaceae followed by *Klebsiella* spp., 33.33 per cent (1/3) from water samples of captive Star Tortoises enclosure from different captive facilities (Table 2).

The PCR for *fimC* gene of pathogenic *E. coli* showed positive at 477 bp (Fig. 1). The pathogenic *E. coli* identification by extracting DNA from QIAamp Fast DNA Stool Mini Kit through PCR, showed 21 (52.50%) samples positive, which is higher than the *E. coli* isolated through culture method, the details are given in Table 3.

Antibiotic sensitivity test was performed for 11 *E. coli* isolates from captive Star Tortoises, and drug resistance was observed in six samples. Overall antibiogram pattern of *E. coli* isolates is given in Table 4. Cefotaxime was the least effective antibiotic tested, showed 54.54 per cent antimicrobial resistance followed by azithromycin (45.45%). Amoxicillin-clavulanic acid, gentamicin and tetracycline were responsible for 36.36 per cent of antimicrobial resistance each. Amoxicillin-clavulanic acid showed an intermediate antimicrobial resistance (63.63%) followed by gentamicin (54.54%). Enrofloxacin showed highest antimicrobial sensitivity (54.54%) followed by tetracycline (45.45). Four samples showed multiple drug resistance.

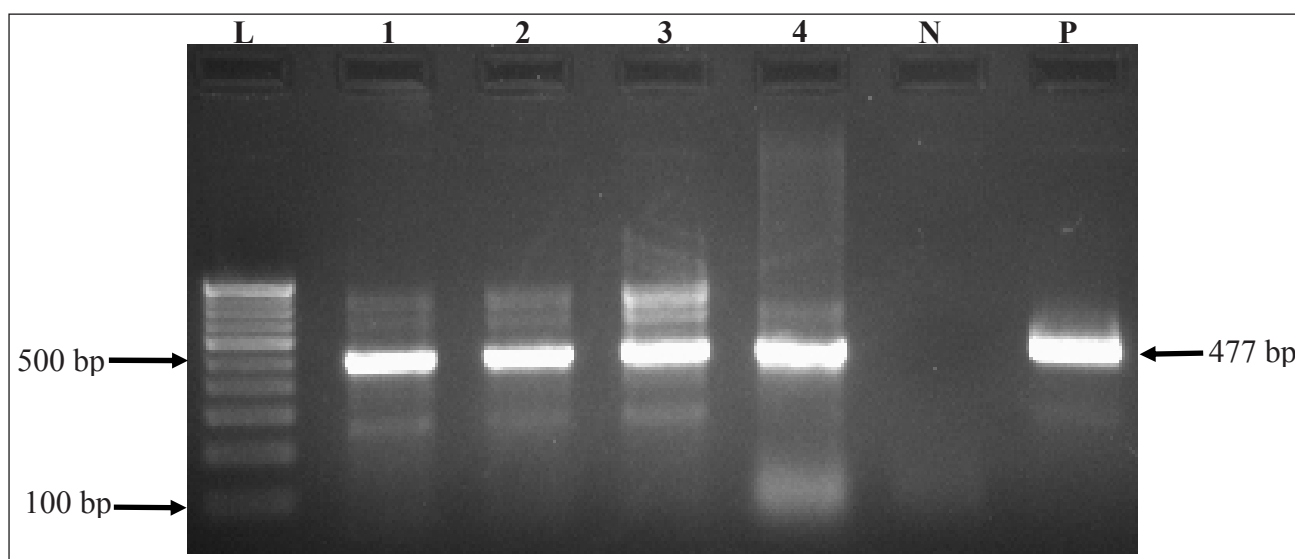


Fig. 1: PCR amplification of *fimC* gene for pathogenic *E. coli*. From left to right: Lane L- 100 bp DNA Ladder (Genei TM, Bangalore); Lane 1-4- Positive Samples from AAZP, Vandalur; Lane N- Negative Control; Lane P- Positive Control.

**Table 1:** Positivity of lactose fermenting Enterobacteriaceae in cloacal swabs from captive Star Tortoises from different captive facilities of South India

Captive Facilities	Total Number of Samples	<i>E. coli</i> (%)	<i>Klebsiella</i> spp. (%)	<i>Enterobacter</i> spp. (%)
Chennai Snake Park Trust, Guindy	06	03 (50.00)	01 (16.66)	02 (33.33)
Arignar Anna Zoological Park, Vandalur	21	04 (19.04)	03 (14.28)	04 (19.04)
Sri Chamarajendra Zoological Gardens, Mysuru	13	04 (30.76)	04 (30.76)	02 (15.38)
Total	40	11 (27.50)	08 (20.00)	08 (20.00)

Table 2: Positivity of lactose fermenting Enterobacteriaceae in water samples from captive Star Tortoises enclosures from different captive facilities of South India

Captive Facilities	Total Number of Samples	<i>Klebsiella</i> spp. (%)	<i>Enterobacter</i> spp. (%)
Chennai Snake Park Trust, Guindy	01	0	01 (100)
Arignar Anna Zoological Park, Vandalur	01	01 (100)	0
Sri Chamarajendra Zoological Gardens, Mysuru	01	00	01 (100)
Total	03	01 (33.33)	02 (66.66)

Table 3: PCR amplification of *fimC* gene of Pathogenic *E. coli*

Captive Facilities	Total Number of Samples	Number of Positives (%)	
		From culture enrichment	From direct faecal samples
Chennai Snake Park Trust, Guindy	06	03 (50.00%)	01 (16.66%)
Arignar Anna Zoological Park, Vandalur	21	04 (19.04%)	12 (57.14%)
Sri Chamarajendra Zoological Gardens, Mysuru	13	04 (30.76%)	08 (61.53%)
Total	40	11 (27.50%)	21 (52.50%)

Table 4: Overall antibiogram pattern of *E. coli*

Results	Antibiotic Discs					
	AMC	CTX	AZM	GEN	TE	EX
Sensitivity (%)	0	0	02 (18.18)	01 (9.09)	05 (45.45)	06 (54.54)
Intermediate Resistance (%)	07 (63.63)	05 (45.45)	04 (36.36)	06 (54.54)	02 (18.18)	05 (45.45)
Resistance (%)	04 (36.36)	06 (54.54)	05 (45.45)	04 (36.36)	04 (36.36)	0

AMC- Amoxicillin-clavulanic acid, CTX- Cefotaxime, AZM- Azithromycin, GEN- Gentamicin, TE- Tetracycline, EX- Enrofloxacin

Foti *et al.* (2008) documented different Enterobacteriaceae species in cloacal swabs from internally hooked live stranded Loggerhead turtles and noted *E. coli* as high prevalent organism and other organisms like *Enterobacter aerogenes*, *E. cloacae* and *E. sakazakii*. The *E. coli* was also reported in fresh water turtles (Mitchell and McAvoy, 1990) and Red eared sliders (Chiacchio *et al.*,

2014). Morais *et al.* (2011) reported *E. coli* as one of the most frequent bacteria found in cloaca and mouth cavity of turtles (*Podocnemis expansa* and *P. unifilis*) and also noted *Klebsiella pneumonia* subsp. *pneumonia* in cloaca and *Enterobacter cloacae* in mouth cavity. Hacıoglu *et al.* (2012) studied on microflora of cloaca and oral cavity of freshwater turtles and noted *E. coli*, *Klebsiella*

pneumonia and *Enterobacter* spp. Nowakiewicz *et al.* (2015) documented *E. coli* as one of the dominant species and reported *Klebsiella pneumoniae* from cloaca of the European pond turtle (*Emys orbicularis*). Similarly in the present study *E. coli* showed the higher prevalence (27.50%) because it is one of the common commensal bacteria present in the gut of animals and also in the soil.

In the present study, DNA extracted using QIAamp Fast DNA Stool Mini Kit from fresh faecal samples showed positive for *fimC* gene (Ewers *et al.*, 2004) of pathogenic *E. coli* (52.50%) through PCR. This showed that direct DNA extraction using QIAamp Fast DNA Stool Mini Kit has higher sensitivity and convenient method compared to the culture method of bacteriological examination.

Gopee *et al.* (2000) conducted a longitudinal study of *E. coli* strains and reported 52 per cent reptiles were positive for *E. coli*, out of which 70 per cent were herbivore chelonians. They also reported antibiogram of *E. coli*, where high resistance to tetracycline (58.2%) and low to gentamicin (9.6%) was observed. In the present study antibiogram pattern showed low resistance to tetracycline (36.36%) and sensitivity of 45.45 per cent and gentamicin showed an intermediate resistance (54.54%). Sylvester *et al.* (2014) reported the occurrence of antibiotic resistant *E. coli* in Green iguanas. Their study on the antibiogram of *E. coli* showed most frequent resistance to amoxicillin-clavulanic acid followed by cefotaxime and tetracycline and multiple drug resistance in 3 isolates. In the present study cefotaxime showed 54.54 per cent of resistance, amoxicillin-clavulanic acid showed an intermediate resistance (63.63%) and tetracycline showed 45.45 per cent of sensitivity and 4 isolates showed multiple drug resistance.

The present study, 27.50 per cent of cloacal swabs were positive for *E. coli* by culture method along with *Klebsiella* spp. (20.00%) and *Enterobacter* spp. (20.00%). On PCR assay, 52.50 per cent of fresh faecal samples were positive for *E. coli* with direct DNA extraction using QIAamp Fast DNA Stool Mini Kit which was more convenient and efficient than traditional culture method. The antibiotic sensitivity test, cefotaxime showed 54.54 per cent of antimicrobial resistance followed by azithromycin (45.45%). This study enlightens the presence of pathogenic organisms in captive Star Tortoises and warrants better hygienic measures in captive areas to prevent infection

and spread of multidrug resistant pathogens to humans and Star Tortoises at captive premises.

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