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Research Article

Extended spectrum and AmpC-Beta-lactamase producing *Escherichia coli* in zoo birds and mammals

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Abstract

Antimicrobial resistance in wildlife, including zoo animals, has received increasing interest recently among researchers as a global public health challenge. In the present study, the prevalence of beta-lactamases in *Escherichia coli* from zoo birds and mammals was investigated. A total of seventy five (n=75) faecal samples were collected from various birds (n=43) and mammalian (n=32) species at Alipore zoo, Kolkata. Sixty *E. coli* were identified from these samples, and they were examined for the presence of bla_{CTX-M} gene and bla_{AmpC} gene by PCR for extended spectrum (ESBL) and AmpC-beta-lactamase production, respectively. Twenty three (38.33 %) isolates harboured ESBL encoding bla_{CTX-M} gene and 35 (58.33 %) possessed bla_{AmpC} gene. Twelve (20.00%) *E. coli* isolates were positive for both bla_{CTX-M} and bla_{AmpC} genes. The current study highlighted *E. coli* from zoo animals as a potential reservoir of beta-lactamase genes.

Key words: bla_{AmpC}, bla_{CTX-M}, *E. coli*, ESBL, Zoo

Highlights

- The presence of bla_{CTX-M} gene for extended spectrum (ESBL) and bla_{AmpC} gene for AmpC-beta-lactamase production was detected by PCR.
- Most of the *E. coli* isolates from zoo birds and mammals harboured genes for ESBL and AmpC beta-lactamases.

INTRODUCTION

Animals kept at the zoo are captured in the wild or acquired from other facilities and raised in captivity. *Escherichia coli* in wildlife harbour pathogenic and antimicrobial-resistant genes in their gut microbiomes. Antimicrobial resistance is a complex global problem involving humans, animals and the environment. However, the role of wildlife in the emergence of antibacterial resistance might be underestimated (Wang *et al*., 2017).

Plasmid-mediated extended-spectrum β-lactamases (ESBLs) and AmpC-type β-lactamases (ACBL) are the most common enzymes that confer resistance to penicillins and broad-spectrum cephalosporins. Since the first report on ESBL-producing *E. coli* isolates from wild animals in Portugal in 2006 (Costa *et al*., 2006), β-lactamase genes in *E.* coli were reported from zoo animals in Japan, China and the Czech Republic (Ahmed *et al*., 2007; Wang *et al*., 2012; Dobiasova *et al*., 2013). At least 80 wildlife species have been found to be carriers of ESBL-producing *Enterobacteriaceae*, most being wild birds (Wang *et al*., 2017). In similarity with isolates from human and domestic animals, the CTX-M family is the most prevalent type of ESBL-producing *Enterobacteriaceae* found in wild animals (Palmeira *et al*., 2021). AmpC beta-lactamases were initially classified as chromosomal beta-lactamases, but since the 1990s, they have been recognized as important plasmid-mediated cephalosporinases associated with *E. coli* and other enteric bacteria (Meini *et al*., 2019).

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There are limited studies on beta-lactamase producing *E. coli* in zoo birds and mammals compared to companion, domestic animals and humans in India (Vinodh Kumar *et al*., 2021a). The ESBL producing *E. coli* isolates were confirmed phenotypically earlier in captive birds and mammals (Amla, 2019). The present study was designed to study further the genotypic prevalence of beta-lactamase producing *E. coli* in zoo animals.

MATERIALS AND METHODS

Sampling: A total of 75 samples were collected from March 2019 to June 2019, comprising the fresh faecal droppings of healthy captive birds (n= 43 of 22 species) and mammals (n= 32 of 22 species) housed in different enclosures of Alipore zoo, Kolkata, India (Table 1, 2). These wildlife species were treated with antibiotics therapeutically when necessary. Antibiotics commonly include preparations with both β-lactam such as amoxicillin/cloxacillin, cefixime, cefpodoxime, ceftriaxone/ tazobactum and non β-lactam groups like tetracycline, sulphonamides, enrofloxacin, ciprofloxacin, amikacin etc. This study did not require ethical approval as per CPCSEA guidelines because faecal samples were collected only after defecation. However, permission was taken from the zoo authority for sampling.

Five to ten grams of faecal sample was collected with peptone water (HiMedia, India) in the morning. The samples were placed in a chilled box, transported to the laboratory, and processed immediately. The samples were cultured on MacConkey's agar and followed by eosin methylene blue (EMB) agar (HiMedia, India), and *E. coli* were identified as per standard procedure (Quinn *et al*., 2011). Beta-lactamase producing *E. coli* strains for positive control were provided by the Department of Veterinary Microbiology, West Bengal University of Animal and Fishery Sciences, Kolkata.

Bacterial DNA extraction: Total DNA was extracted by heating the bacteria in a water bath as described previously (Féria *et al*., 2002). Briefly, isolated bacterial cultures were harvested from 2 mL overnight growth culture, suspended in 200 µL of nuclease-free water and lysed by heating at 100°C for 10 minutes in a water bath followed by immediate chilling on ice. The cell debris was removed by centrifugation at 2000 rpm for 5 minutes. The supernatant was used as template DNA for PCR reaction.

Species-specific PCR: To confirm the isolates at the species level, species-specific 16S rRNA gene forward 5′-GACCTCGGTTTAGTT CACAGA-3′ and reverse 5′-CACACGCTGA CGCTGACCA-3′ primers were used as reported previously (Wang *et al*., 1996). PCR amplifications were performed in a thermocycler (T-100, Bio-Rad). A 25 µL reaction mixture was used containing 2 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate (dNTP), 0.25 mM of each primer, 1 U of GoTaq DNA polymerase (Promega, USA) and 5 µL of extracted *E. coli* DNA samples. The amplification conditions were: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 1 min, elongation at 72°C for 1 min and final extension at 72°C for 10 mins.

PCR Detection of beta-lactamase genes: All *E. coli* isolates were examined by PCR for major beta-lactamases such as $bla_{CTX\text{-}M}$ and bla_{AmpC} genes. Forward primer 5′-CRATGTGCAG YACCAGTAA-3′and reverse primer 5′-CGC RATATCRTTGGTGGTG-3′ for amplification of $bla_{CTX\text{-}M}$ consensus gene as per Weill *et al.* (2004); AmpC forward 5′-CCCCGCTTATAG AGCAACAA-3′ and AmpC reverse primers 5′-TCAATGGTCGACTTCACACC-3′ for bla_{AmpC} gene as per Féria *et al.* (2002) were used along with positive and negative control (PBS). A 25 µL reaction mixture was prepared with 5 µL DNA templates, 50 pmol of each primer, 200 mM deoxynucleoside triphosphate, 1 U GoTaq DNA polymerase (Promega, USA),

Table 1. Extended spectrum and AmpC-beta-lactamase producing *Escherichia coli* from zoo birds

Order	Host Species	Sample No.	No. of positive isolate	Beta-lactamase gene positive	
				bla_{CTX-M}	bla_{AmpC}
Psittaciformes	White ibis (*Threskiornis aethiopica*)	2	2	0	2
	Blue and Yellow macaw (*Ara ararauna*)	4	4	1	3
	Scarlet macaw (*Ara macao*)	2	2	1	2
	Bare-eyed cockatoo (*Cacatua sanguinea*)	2	1	1	1
	Citron-crested cockatoo (*Cacatua sulphurea citrinocristata*)	2	1	0	1
	Goffin's cockatoo (*Cacatua goffiniana*)	2	2	1	1
	Moluccan cockatoo (*Cacatua moluccensis*)	2	1	0	1
	Grey parrot (*Psittacus erithacus*)	2	2	0	2
	Amazon parrot (*Amazona oratrix*)	1	1	0	1
	Rose ringed parakeet (*Psittacula krameri*)	2	2	2	0
Pelecaniformes	Eurasian spoonbill (*Platalea leucorodia*)	3	2	1	1
	Rosy pelican (*Pelecanus onocrotalus*)	1	1	1	1
Columbiformes	Green Imperial pigeon (*Ducula aenea*)	2	1	1	0
	White Dove (*Streptopelia alba*)	2	1	0	0
Galliformes	Grey peacock-pheasant (*Polyplectron bicalcaratum*)	1	1	0	0
	Indian peafowl (*Pavo cristatus*)	1	1	1	0
	Chinese silver pheasant (*Lophura nycthemera*)	2	2	1	0
Piciformes	Keel-billed toucan (*Ramphastos sulfuratus*)	2	2	0	1
Ciconiiformes	Painted stork (*Mycteria leucocephala*)	2	1	1	1
	Lesser adjutant stork (*Leptopilos javanicus*)	2	1	0	1
Anseriformes	Bar-headed goose (*Anser indicus*)	2	1	1	0
Passeriformes	Hill mynah (*Gracula religiosa*)	2	1	0	1
Total		**43**	**33**	**13**	**20**

2 mM $MgCl_2$, and 10% dimethyl sulfoxide. The PCR mixture was subjected to initial denaturation step of 5 min at 94°C; followed by 30 cycles of amplification consisting of 30s of denaturation at 94°C, 30s of annealing at 53°C for bla_{CTX-M} (57°C for bla_{AmpC}), 1 min of

Table 2. Extended spectrum and AmpC-beta-lactamase producing *Escherichia coli* from zoo mammals

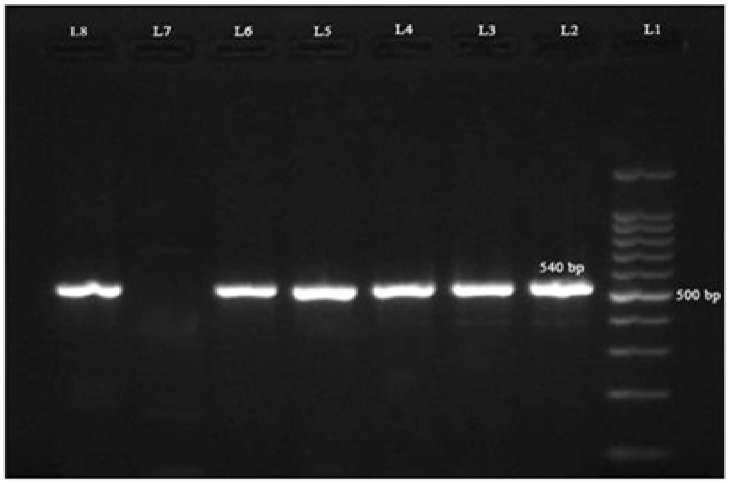
Order	Host Species	Sample No.	No. of positive isolate	Beta-lactamase gene positive	
				bla_{CTX-M}	bla_{AmpC}
Artiodactyla	*Brow-antlered deer* (*Cervus eldii*)	2	2	1	2
	Barking deer (*Muntiacus muntjak*)	2	1	1	0
	Black buck (*Antilope cervicapra*)	1	1	1	1
	Mouse deer (*Moschiola meminna*)	1	1	0	1
	Sambar deer (*Rusa unicolor*)	1	1	1	0
	Swamp deer (*Rucervus duvaucelii*)	3	2	0	1
	Spotted deer (*Axis axis*)	2	1	0	1
	Four horned antelope (*Tetracerus quadricornis*)	1	1	0	0
	Nilga (*Boselaphus tragocamelus*)	1	1	1	0
Perisodactyla	Plains zebra (*Equus quagga*)	1	1	0	1
Carnivora	Bengal tiger (White) (*Panthera tigris tigris*)	1	1	0	1
	Bengal tiger (*Panthera tigris tigris*)	2	2	0	1
	Jaguar (*Panthera onca*)	1	1	1	1
	Striped hyena (*Hyaena hyaena)*	1	1	0	1
	Jungle cat (*Felis chaus*)	1	1	1	0
	Sloth bear (*Melursus ursinus*)	2	1	0	0
	Fishing cat (*Prionailurus viverrinus)*	2	1	0	0
	Golden Jackal (*Canis aureus*)	1	1	1	1
Primates	Chimpanzee (*Pan troglodytes*)	2	2	1	1
	Bonnet macaque (*Macaca radiata*)	1	1	0	1
	Bengal Slow Loris (*Nycticebus bengalensis*)	1	1	0	0
	Eastern Hoolock Gibbon (*Hoolock leuconedys*)	1	1	1	0
	Rhesus Macaque (*Macaca mulatta*)	1	1	0	1
Total		**32**	**27**	**10**	**15**

elongation at 72°C and 10min of final extension at 72°C.

Detection of PCR products: PCR products were electrophoresed in 1.5% agarose gel with 0.5 µL/mL ethidium bromide in 1x TBE buffer at 8V/cm for 1 h. The amplicons were observed under a UV transilluminator (UVP, UK) and were photographed.

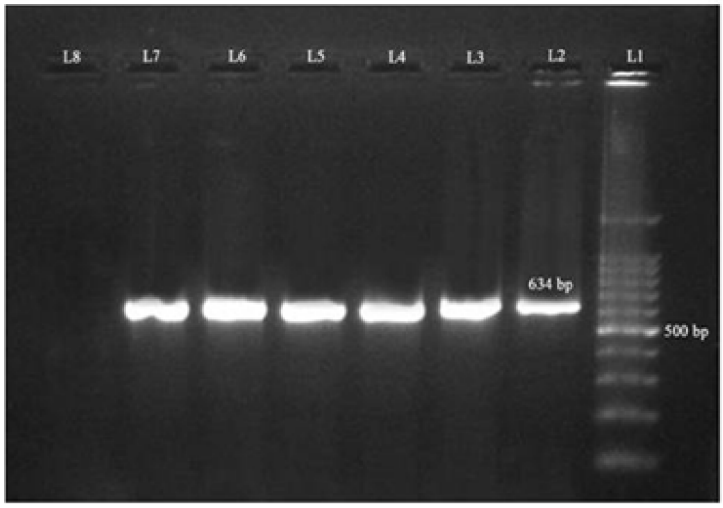
RESULTS

Out of 75 faecal samples, 60 (80.00%) *E. coli* isolates were identified based on Gram staining, colony characteristics, and standard biochemical tests. The isolates were found positive for catalase, indole and methyl red, and negative for oxidase, Voges Proskauer, citrate, urease and H_2S production. They gave positive reactions by16S rRNA gene of *E. coli* specific PCR. Overall, 23



[Lane L1: 100 bp DNA ladder; Lanes L2-L6, L8: bla_{CTX-M} positive samples (540 bp); Lane L7: negative control (PBS)]

Fig. 1. PCR amplification of bla_{CTX-M} ESBL gene of *Escherichia coli*



[Lane L1: 100 bp DNA ladder; Lanes L2-L7: bla_{AmpC} positive samples (634 bp); Lane L8: negative control (PBS)]

Fig. 2. PCR amplification of bla_{AmpC} gene of *Escherichia coli*

(38.33%) ESBL producing *E. coli* isolates harboured bla_{CTX-M} gene (Fig. 1). Among them, 13 (39.39%) were from birds, and 10 (37.04%) were from mammals in the zoo (Table 1, 2). Thirty five (58.33%) *E. coli* isolates were detected positive for bla_{AmpC} gene by PCR (Fig. 2). In more detail, twenty (60.61%) from birds and 15 (55.56%) *E. coli* isolates from mammals were bla_{AmpC} producing *E. coli* (Table 1, 2). Twelve (20.00%) *E. coli* isolates were positive for both bla_{CTX-M} gene and bla_{AmpC} gene. Of the 44 animal species that were sampled, most species carried either of two or both (ESBL/AmpC) beta-lactamases (20 avian and18 mammal species).

DISCUSSION

In the present study, most *E. coli* isolates were found to harbour either bla_{CTX-M} /bla_{AmpC} gene from the zoo birds and mammals. There have been limited studies to compare ESBL and AmpC producing *E. coli* in zoo birds and mammals in India. Like the present study,

bla_{AmpC} gene was previously reported in black buck in India (Vinodh Kumar *et al*., 2021a). In another study, Zaidi *et al*. (2020) observed 95.12% ESBL producing *E. coli* in animals of Bikaner Zoo, Rajasthan, India. Vinodh Kumar *et al.* (2021b) found all ESBL producing *E. coli* (17/45) to harbour bla_{CTX-M} gene in rescued sloth bear (*Meursus ursinus*) in India. In contrast, Shnaiderman-Torban *et al*. (2019) found lower frequencies in the 228 sampled animals; 12% (28/228) carried at least one gene of ESBL/ AmpC in their study of eight petting zoos in Israel. Like the present study, Wang *et al.* (2012) reported 56 (27%, 56/206) bla_{CTX-M} producer *E. coli* isolates from primates in 6 different zoos in China. Predominantly $bla_{CTX-M-1}$ (97.96%, 48/49) gene was detected in ESBL-producing *E. coli* isolates in zoo animals from Czech Republic (Dobiasova *et al*., 2013). Witte *et al.* (2021) also found 64.0% (17/25) CTX-M ESBL-producing *E. coli* in the faecal microbiota of zoo mammals in two zoos in Belgium. Comparison of the frequencies with previous studies is difficult as differences prevailed in number of sampled animals, animal species, geographical area and zoo managemental practises.

The high occurrence of ESBL-producing isolates found in our study could be associated with antibiotic therapy used in the zoo animals for treatment of various infections. In Japan, an investigation reported an association of antimicrobial treatment with the spread of antimicrobial-resistant bacteria among the zoo animals (Ishihara *et al*., 2012). Therefore, the variation in antimicrobial resistance gene profiles in bacteria isolated from zoo animals could be due to differences in antibiotic use in the country and exposure to sources of resistance genes (Wang *et al*., 2012). The extended-spectrum beta-lactamase (ESBL) and AmpC beta-lactamase in wildlife has been reported in 46 countries from all continents (52% in Europe), with descriptions mainly in birds and mammals (Palmeira *et al*., 2021).

The other ways of transmission of ESBL- and AmpC-producing *E. coli* strains may have occurred through multiple pathways of which meat diets may be an important one, also by mechanical ways through zookeepers (Witte *et al*., 2021). Migratory wild birds and rodents that are omnipresent probably in every location were also found to carry ESBL producing *E. coli* (Guenther *et al*., 2010, 2011). However, samples of food (chickens, buffalo, fish or other food sources) and water sources of zoo animals were not analyzed in the present study.

The study of beta-lactamases is important in zoo animals because they may also disseminate resistant bacteria and/or resistance genes to other animals, humans and environment. The zoo animals are sometimes exchanged for breeding programs, and also zoo animals and/or their offspring are reintroduced into the wild.

The prevalence of beta-lactamase producing *E. coli* has been observed in clinically healthy zoo birds and mammals which are potential carriers of antimicrobial-resistant *E. coli*.

Conflict of interest: Authors have no conflict of interest in this study.

Author's contribution: Amla, SD, and DPI: Conceived and designed the study; Amla: Conducted the experiment; SD, KB, SNJ and IS: Performed data analysis. All authors contributed to the draft and approved the final manuscript.

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