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Short Communication

Free Indian Cormorant at human locality as a reservoir of antimicrobial resistant *E. Coli*

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Abstract

The destruction of wildlife habitats due to human activity is a major environmental issue that threatens biodiversity and ecosystem stability. The Indian Cormorant or Shag (*Phalacocorax fuscicollis*; 'Pankouri' in local language) is primarily found in the Indian subcontinent's inland waters, although it also reaches as far west as the Sind and as far east as Thailand and Cambodia. To the best of our knowledge, little research has been done on antimicrobial resistance (AMR) in wild birds. Public health is compromised with the wild birds' role as reservoir of antimicrobial resistant strains, specially when both of them share common ecosystem. The purpose of this study was to determine the likelihood that the stock of Indian Cormorant birds residing in the trees of Belgachia area would experience AMR of *E. coli*. The present paper showed for the first time that Indian Cormorant harboured *E. coli* in their gut, which had AMR genes, viz. ESBL and ACBL genes that pose potential threat to public health in the area.

Keywords: AMR, Cormorant, *E. coli*, ESBL, PCR

Highlights

- All the *E. coli* samples were sensitive to gentamicin, tetracycline and chloramphenicol, but resistant to higher group of antibiotics.
- A total of 14.28% and 28.85% *E. coli* isolates revealed the presence of bla_{SHV} and bla_{AmpC} genes, respectively.

The Indian Cormorant or Indian Shag (*Phalacocorax fuscicollis*) is a sedentary bird, primarily found in the Indian subcontinent's inland waters. It is found in West as far as Sind and in East as Thailand and Cambodia. It is a sociable species and is easily identified from the tiny Cormorant, which is about the same size, by its blue eyes, small head with a sloping brow, and long, narrow bill with hooked ends. They may act as a vector and reservoir for the infections to spread across the ecosystem because of their mobility and interactions with humans and other animals sharing the habitat. Certain serovars of *Salmonella* and *Escherichia coli* are zoonotic and were detected to be transmitted to zookeepers and caged wildlife (Gopee *et al*., 2000; Zhao *et al*., 2001).

Escherichia coli are Gram-negative rods that are members of the *Enterobacteriaceae* family. They are facultative anaerobes that are oxidase negative, lactose fermenting and do not generate spores. Even though some pathogenic *E. coli* strains have been linked to a wide range of infections in animals and humans, mostly the strains of *E. coli* are opportunistic, found in the gut microflora of humans and animals (Croxen and Finlay, 2010). Numerous eukaryotic cellular processes, including cell signaling, ion secretion, protein synthesis, mitosis, cytoskeletal function, and mitochondrial function, are impacted by the virulence factors (VFs) of *E. coli*. Pathogenicity islands, plasmids, bacteriophages, transposons, and other genetic elements are widely used to encode virulence factors (VFs) of *E. coli*. These elements can be mobilized into different strains to create novel combinations of VFs (Kaper *et al*., 2004).

There is a growing concern among researchers about the pathogenic potential of *E. coli* (Thomson and Moland, 2000; Bhrugubalda *et al*., 2016; Mohammadi-Sardo *et al*., 2017; Paul *et al*., 2023). A number of characteristics, including the ability to form biofilms, slime production and exhibit proteolytic activity (Sheikh *et al*., 2001; Shetty *et al*., 2014), in addition to production of hemagglutination, hemolysin, and hemolytic activities, are linked to the pathogenicity of this bacterium. It is critical to detect the potential virulence traits and pattern of antibiotic resistance among the *E. coli* isolates from the gastrointestinal tracts of various birds and animals, which in turn will aid in understanding the pattern of antibiotic resistance and the frequency of distinct pathogenic features in the normal animal microbiome. The goal of the current investigation was to determine the pattern of antibiotic resistance and presence of ESBL genes in *E. coli* that were isolated from *E. coli* isolates that were collected from Indian Cormorant feces to produce extended

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spectrum β lactamases (ESBL).

The study was carried out with the excreta (faeces/ guano) of Indian Cormorant birds, temporarily taking shelter in the trees of the main campus of West Bengal University of Animal and Fishery Sciences at Belgachia, Kolkata. A sterile plastic sheet was kept under the trees from 4.00 am to 6.00 am in the morning for collection of faeces. The fresh faecal samples (n=4) were collected from plastic sheet by using sterile cotton swab (HiMedia, India). The swabs were transported to the departmental laboratory. The swabs were put in nutrient broth (HiMedia, India) at 37°C overnight. The growth in broth was plated on Eosin methylene blue (EMB) agar and incubated overnight at 37°C. A total of 4 plates were used to process 4 samples. All 4 plates showed growth in selective media and were considered positive for *E. coli*. Out of the 4 positive samples, a total of 7 isolates were generated (Ban-1 to Ban-7) and used for further studies. All the isolates were tentatively identified as *E. coli* by standard biochemical tests (Quinn *et al*., 1994).

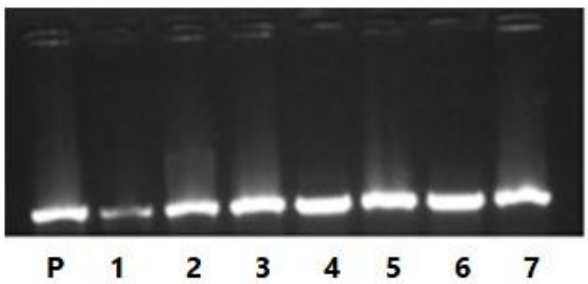
Total DNA was extracted by heating the bacteria in a water bath as described previously (Féria *et al*., 2002). Briefly, isolated bacterial cultures were harvested from 2 mL overnight growth culture, suspended in 200 mL of nuclease-free water, and analyzed by heating at 100°C for 10 minutes in a water bath followed by immediate chilling on ice. The cell debris was removed by centrifugation at 2000 rpm for 5 min. The supernatant was used as template DNA for PCR reaction. To confirm the isolates at the species level, species-specific 16S rRNA PCR was carried out. While doing so, forward (5′ -GACCTCGGTTTAGTTCACAGA-3′) and reverse (5′-CACACGCTGACGCTGACCA-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 MgCl2, 0.2 mM each deoxynucleoside triphosphate (dNTP), 0.25 mM of each primer, 1U 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 30s, annealing at 58°C for 1 min, elongation at 72°C for 1 min and final extension at 72°C for 10 min. The PCR amplification was followed by agarose gel (1.5%) electrophoresis to determine the DNA amplicons.

The isolates were tested for antibiotic sensitivity with commonly used antibiotics like doxycycline (30 mcg), tetracycline (10 mcg), chloramphenicol (30 mcg), gentamicin (10 mcg), amoxycillin (30 mcg), enrofloxacin (10 mcg), and erythromycin (10 mcg), ticarcillin/ clavulanic acid (30/10 mcg), piperacillin/tazobactum (75/10 mcg), cefixime (5 mcg) and co-trimoxazole (25 mcg).

The interpretation of the AST results was performed in accordance with the guidelines provided by Himedia's Antimicrobial Susceptibility test standards (https:// www.himediadownloads.com/EIFU/SD_Products.pdf).

All *E. coli* isolates were examined by PCR for major beta-lactamases such as $bla_{CTX\text{-}M}$, bla_{SHV} and bla_{AmpC} genes. Forward primer 5′ -CRATGTGCAGCACCAGT AA-3′ and reverse primer 5′ -CGCGATATATC GTTGGTGGTTGGTG-3′ for amplification of $bla_{CTX\text{-}M}$ consensus gene as per Weill *et al*. (2004); forward primer 5′ -TTATCTCCCTGTTAGCCACC-3′ and reverse primer 5′ -GATTTGCTGATTTCGCTCGG-3′ for amplification of bla_{SHV} consensus gene as per Cao *et al*. (2002); and forwardprimer 5′ -CCCCGCTTATAGAG CAACAA-3′ and reverse primer 5′ -TCAATGGTCGA CTTCACACC-3′ for bla_{AmpC} gene as per Féria *et al*. (2002) were used along with positive (laboratory obtained *E.coli* isolate) and negative control (PBS). A 25 µL reaction mixture was prepared with 5 µL DNA templates, 50 µM of each primer, 200 µM deoxynucleoside triphosphate, 1UGoTaq DNA polymerase (Promega, USA), 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 30 s of denaturation at 94°C, 30 s of annealing at 53°C for $bla_{CTX\text{-}M}$ (57°C for bla_{AmpC}), 1 min of elongation at 72°C and 10 min of final extension at 72°C. PCR products were electrophoresed in 1.5% agarose gel with 0.5 mL ethidium bromide/mL in 1XTBE buffer at 8V/cm for 1 h. The amplicons were observed under a UV transilluminator (UVP, UK) and were photographed.

All 7 isolates, generated from 4 samples, were found to be positive for 16S rRNA, indicating all the samples were of *E. coli* (Table 1) (Fig. 1). In AST, we found that the bacterial samples were sensitive to aminoglycosides (gentamicin), tetracycline (doxycycline hydrochloride),



[Lane-P: Positive control, Lane-1: Ban-1, Lane-2: Ban-2, Lane-3: Ban-3, Lane-4: Ban-4, Lane-5: Ban-5, Lane-6: Ban-6, Lane-7: Ban-7]

Fig. 1. Gel showing PCR amplicon of 16SrRNA gene of the isolates

Table 1. Results of target gene-specific polymerase reaction (PCR) analysis of the *E. coli* isolates

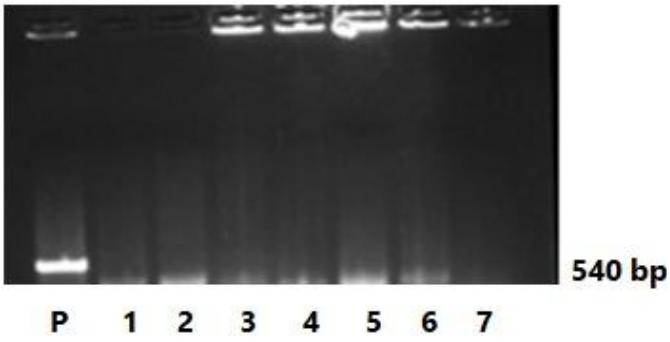
Targeted genes	Isolate no.						
	Ban-1	Ban-2	Ban-3	Ban-4	Ban-5	Ban-6	Ban-7
16S rRNA	+	+	+	+	+	+	+
bla_{CTX-M}	-	-	-	-	-	-	-
bla_{SHV}	+	-	-	-	-	-	-
bla_{AmpC}	+	+	-	-	-	+	-

Table-2. Results of antibiotic sensitivity test (AST) of the *E. coli* samples

Antibiotics	Sample no.			
	S1	S2	S3	S4
Tetracycline (10 mcg)	Sensitive	Resistant	Resistant	Resistant
Doxycycline hydrochloride (30 mcg)	Sensitive	Sensitive	Sensitive	Sensitive
Chloramphenicol (30 mcg)	Sensitive	Sensitive	Sensitive	Sensitive
Gentamicin (10 mcg)	Sensitive	Sensitive	Sensitive	Sensitive
Ticarcillin/Clavulanic acid (30/10 mcg)	Resistant	Resistant	Resistant	Resistant
Piperacillin/Tazobactum (75/10 mcg)	Resistant	Resistant	Resistant	Resistant
Amoxycillin (30 mcg)	Resistant	Resistant	Resistant	Resistant
Enrofloxacin (10 mcg)	Resistant	Resistant	Resistant	Resistant
Erythromycin (10 mcg)	Resistant	Resistant	Resistant	Intermediate
Cefixime (5 mcg)	Resistant	Resistant	Resistant	Resistant
Co-Trimoxazole (25 mcg)	Sensitive	Resistant	Resistant	Resistant

and amphenicol (chloramphenicol) class but resistant to a higher group of antibiotics (Table 2).

While performing PCR to detect ESBL genes in the isolates, no positive sample could be found for bla_{CTX-M} (Fig. 2) (Table 1). However, 1 isolate (14.28%) was positive for bla_{SHV} (Fig. 3), and 3 isolates (42.85%) were positive for bla_{AmpC} (Fig. 4) (Table 1). No literature (data) in Indian Cormorant revealing prevalence of drug resistant *E. coli* was found to compare the present study findings. In an earlier study which was carried out in domestic duck, Banerjee *et al*. (2019) reported the presence of 13.76% bla_{CTX-M}, 5.05% bla_{SHV} and 79.82% bla_{AmpC} genes in *E. coli* isolates. In our laboratory, *E. coli* was isolated from fresh fecal droppings (n=43) of healthy captive birds of 22 species from Alipore Zoological Garden, Kolkata. The *E. coli* was shown to possess 39.39% bla_{CTX-M} gene and 60.61% bla_{AmpC} gene as assessed by PCR (Amla *et al*., 2022). Recently, in our laboratory, while working with duck microbes, we found *E. coli* isolates carried 22.38% bla_{CTX-M}, 37.55% bla_{SHV}, and 90.79% bla_{AmpC} genes (Paul, 2023). Compared to these studies, the prevalence of AMR (antimicrobial resistance) genes was found to be less in the present. Since the habitat of the birds under study was surrounded by human habitat of Belgachia (Kolkata) area and both, human and birds were using the same ecosystem including water bodies, horizontal transfer of resistance genes of *E. coli* is apprehended to the community.



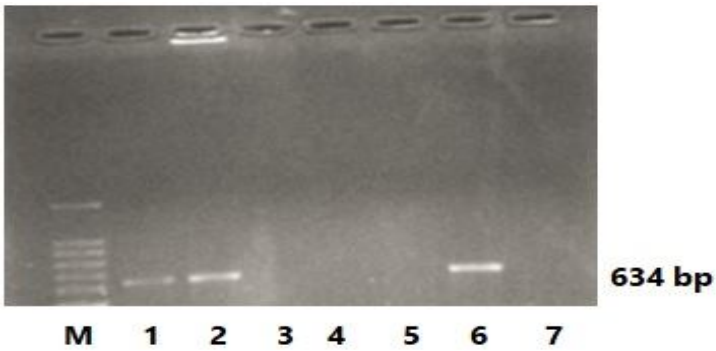
[Lane-P: Positive control, Lane-1: Ban-1, Lane-2: Ban-2, Lane-3: Ban-3, Lane-4: Ban-4, Lane-5: Ban-5, Lane-6: Ban-6, Lane-7: Ban-7]

Fig. 2. Gel showing PCR amplicon of bla_{CTX-M} gene of the isolates

The main drawback of the study is the small sample size, which should be taken care of in

[Lane-P: Positive control, Lane-1: Ban-1, Lane-2: Ban-2, Lane-3: Ban-3, Lane-4: Ban-4, Lane-5: Ban-5, Lane-6: Ban-6, Lane-7: Ban-7]

Fig. 3. Gel showing PCR amplicon of *bla*$_{SHV}$ gene of the isolates



[Lane M: DNA Ladder (100 bp), Lane-1: Ban-1, Lane-2: Ban-2, Lane-3: Ban-3, Lane-4: Ban-4, Lane-5: Ban-5, Lane-6: Ban-6, Lane-7: Ban-7]

Fig. 4. Gel showing PCR amplicon of *bla*$_{AmpC}$ gene of the isolates

such subsequent studies. In short, the present pilot study revealed for the first time that Indian Cormorants harbouring *E. coli* in their gut have AMR genes, viz. ESBL and ACBL genes pose a potential threat to public health and a challenge to One Health.

Conflict of interests: Authors declare that they do not have any conflict of interests.

Authors' contribution: BT, MC: Collected samples and did the experiments; AP, SNJ: Supervised the work; BT, SNJ: Drafted the manuscript; IS: Received the Grant; IS, KB, SD: Corrected the manuscript.

Data availability statement: The corresponding author will provide the raw data upon reasonable request.

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