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Original article

Incidence and molecular characterization of extended-spectrum beta-lactamase (ESBL)-producing *Salmonella enterica* and *Escherichia coli* of avifauna origin in Pakistan

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Abstract

Members of Enterobacteriaceae are known to produce extended-spectrum beta-lactamases (ESBL) which hydrolyze the beta-lactam group of antibiotics. The existence of ESBL-producing *Salmonella enterica (S. enterica)* and *Escherichia coli (E. coli)* harbored by urban avifauna was investigated in this study. Dropping samples (n= 180) were collected from six different bird species in the district Jhang, Punjab province, Pakistan. Isolation and identification of ESBL isolates were made by using cefotaxime- (4 mg/L) supplemented MacConkey agar and double disc synergy test (DDST). Polymerase chain reaction (PCR) was performed for the detection of four different ESBL genes including bla_{CTX-M}, bla_{TEM}, bla_{SHV} and bla_{OXA}. A total of 42.69% isolates were confirmed as ESBL via DDST including 30.64% *S. enterica* and 49.54% *E. coli*. The incidence of ESBL *S. enterica* and ESBL *E. coli* was found highest in egret (*Ardea alba*) and pigeon (*Columba livia*) as 64.28% and 78.95%, respectively. The bla_{CTX-M} gene was detected in 57.89% and 64.81% of isolates of *S. enterica* and *E. coli,* respectively. Among other genes in *S. enterica* and *E. coli*, bla_{TEM} (21.05%, 20.4%); bla_{SHV} (15.78%, 9.26%), and bla_{OXA} (5.26%, 5.56%) were detected, respectively. All of the tested isolates were found resistant to at least one of the thirteen antimicrobial agents except meropenem. To the best of our knowledge, this is the first study reporting the incidence and genetic diversity of ESBL bacteria associated with urban avifauna in Pakistan. The urban avifauna can serve as a potential subject of bio-surveillance to monitor the emergence of antimicrobial-resistant bacteria.

Keywords: ESBL, genotypic characterization, *Salmonella enterica*, *Escherichia coli*, avifauna, Pakistan

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Introduction

Antimicrobial resistance (AMR) is a complicated and emerging public health threat that claimed 4.95 million lives in 2019 worldwide. The death toll is expected to rise to 10 million by the year 2050 (Murray et al. 2022). The unjudicial use of antibiotics in human and veterinary practice has facilitated resistant microbes to flourish in environmental niches and across multiple host species (Ivey et al. 2020). Free-flying birds, especially migratory birds, cover long distances across the globe, seasonally to find shelter and/ or breeding sites resulting in interaction with other avian as well as non-avian species (Islam et al. 2022). The extensive movement of these birds facilitates the spread of AMR microbes evolved from one geographical location to another, across species and in the environment (Zurfluh et al. 2019). The fecal samples of apparently healthy wild birds have been confirmed to carry potential pathogenic microbes including *Escherichia coli* (*E. coli*), *Shigella,* and *Salmonella* spp. (Bin et al. 2018, Tardone et al. 2020). The urban avifauna lives in close association with domestic animals, pets, poultry, and human beings. Although these free-flying urban birds are not fed antimicrobials directly yet their feeding habits often expose them to sewage water, landfills, biowaste (originated from hospitals, laboratories, pharmaceuticals, etc.), insect vectors, agriculture and industrial waste containing antimicrobials or their residues (Poirel et al. 2012).

Extended-spectrum beta-lactamase (ESBL) and carbapenemase-producing bacteria of the *Enterobacteriaceae* family have been recognized as an emerging public health threat (Castanheira et al. 2021). The ESBL bacteria are known to be widely disseminated across clinical settings, environments, animals and birds including domestic and wild species (De Lucia et al. 2018, Athanasakopoulou et al. 2022). The amide bond present in the chemical structure of the β-lactam ring of the β-lactam antibiotics is cleaved by a wide variety of β-lactamases comprising twelve families and mainly found in gram-negative bacteria (Castanheira et al. 2021). Integrons, transposons, and plasmids are thought to be the mobile genetic components in bacterial populations that promote the horizontal transmission of the acquired resistome (Bevan et al. 2017). The β-lactamase (*bla*) genes encoding ESBL enzymes originate at plasmids as well as on chromosomes. The most frequent and broader categories of ESBL gene groups include cefotaxime-hydrolysing β-lactamase isolated in Munich (bla_{CTX-M}), β-lactamase temoneira (bla_{TEM}), β-lactamase sulfhydryl reagent variable (bla_{SHV}) and β-lactamase oxacillinase (bla_{OXA}) while other relatively less frequent groups including bla_{IRT}, bla_{CMT}, bla_{GES}, bla_{PER}, bla_{VEB}, bla_{BEL}, bla_{TLA}, bla_{SFO} and bla_{OXY} have also been reported (Castanheira et al. 2021). Studies conducted in various parts of the globe have reported the existence of beta-lactamase-producing bacteria in various species of urban birds (Báez et al. 2015). In a study conducted in France, the $bla_{CTX-M-15}$, $bla_{CTX-M-1}$, bla_{TEM-1} ESBL genes were detected in yellow-legged gulls conferring resistance to various antimicrobials (Ngaiganam et al. 2019). Another study conducted in Spain reported *Salmonella* spp. in 12.3% of samples from urban birds and 86.7% strains of bacteria were multi-drug resistant (Martín-Maldonado et al. 2020). The prevalence of ESBL *E. coli* isolates was recorded as 38.18% in migratory birds in Bangladesh with the detection of multiple ESBL genes including bla_{CTX-M}, bla_{SHV}, bla_{TEM} and bla_{CMY} (Islam et al. 2022).

In Pakistan, CTX-M-15 type beta-lactamases were reported in migratory birds (Mohsin et al. 2017). However, the data is scarce regarding urban bird species carrying ESBL microbes. The present study aimed to determine the incidence of both ESBL and non-ESBL *Salmonella enterica* (*S. enterica*) and *E. coli* in multiple species of urban avifauna in Pakistan. The isolates found in urban avifauna were examined for ESBL genes (bla_{CTX-M}, bla_{SHV}, bla_{TEM} and bla_{OXA}) and their antibiotic susceptibility profiles were also determined.

Materials and Methods

Sample collection

The study was conducted on urban avifauna residing in district Jhang, Punjab province, Pakistan. In total, 180 samples of fresh droppings were collected from six different bird species including crow (*Corvus splendens*), common myna (*Acridotheres tristis*), pigeon (*Columba livia*), Japanese quail (*Coturnix japonica*), egret (*Ardea alba*) and house sparrow (*Passer domesticus*) residing as urban avifauna. From each representative bird species, freshly passed dropping samples (n=20) were collected via sterile swab sticks. Each swab was homogenized in 500 µL of sterile phosphate buffered saline (PBS, ThermoFisher, USA) and stored at 4°C for further processing.

Isolation and phenotypic identification of ESBL *S. enterica* and *E. coli*

Each homogenized sample was enriched in 9 ml of Luria Bertani (LB) broth (Invitrogen 12795027, USA) and Rappaport Vassiliadis (RV) broth (Solus RVS001, UK), both broths supplemented with cefotaxime (Caisson C032-100G, USA) as 4 mg/L of the broth and incubated at 37°C for 18 hours. One loopful

from LB and RV broth was streaked directly on MacConkey agar (Oxoid CM0007, UK) supplemented with cefotaxime as 4 mg/L (Mac-Cef) (Eshrati et al. 2020) as well as Xylose Lysine Deoxycholate (XLD; Oxoid CM0469, UK) agar supplemented with cefotaxime (4 mg/L) (XLD-Cef), respectively. The colonies of *S. enterica* and *E. coli* were identified by their typical colony characteristics and confirmed by biochemical tests including Gram staining, Catalase test, Oxidase test, Triple Sugar Iron Agar test (TSIAT), Indole test, Methyl red test, Voges Proskauer test and Citrate utilization test (Effendi et al. 2020).

Following identification as *S. enterica* and *E. coli*, the isolates were phenotypically confirmed as ESBL producers via double disc synergy test (DDST) as described in the performance standards for antimicrobial susceptibility testing (M100) protocol (CLSI 2018). Briefly, overnight broth cultures of isolated bacteria were standardized as equivalent to 0.5 McFarland standard and swabbed on Mueller-Hinton (MH) agar plates. Antibiotic discs cefotaxime-30 µg (CTX-30, Oxoid, UK) and amoxicillin/clavulanic acid as 20/10 µg (AMC-30, Oxoid, UK) were placed at 20 mm apart and plates were incubated at 37°C for 24 hours. DDST-positive samples were recorded on the basis of the expansion of the zone of inhibition of CTX-30 toward the AMC-30 disc.

ESBL genes detection via PCR

Phenotypically confirmed isolates were subjected to genotypic characterization by monoplex polymerase chain reaction (PCR). Pure enriched broth culture (1 ml) was used for the isolation of DNA as previously described (Dashti et al. 2009). Briefly, enriched broth culture (1 ml) was centrifuged at 14,000 × g for 6 min. The supernatant was discarded and the pellet was resuspended in 200 µL of nuclease-free water and incubated for 2 minutes before pelleting again via centrifugation (washing step). The washed pellet was resuspended in 200 µL of nuclease-free water, boiled at 95°C for 10 minutes and recentrifuged at 14,000 × g for 6 min. The supernatant (50 µL) was collected and used as template DNA in PCR. PCR was performed for the ESBL genes (bla_{CTX-M}, bla_{TEM}, bla_{SHV} and bla_{OXA}) by using primers as described in Table 2. Briefly, PCR reaction mixture (50 µL) was prepared using 25 µL master mix (Dream Taq Green 2x, K 1081, ThermoFisher, USA), 10 pmol of each primer (2 µL), template DNA (4 µL) and nuclease-free water (17 µL). Amplification of targeted genes was carried in a thermal cycler (Biorad, T100, USA) with reaction conditions including initial denaturation at 95°C for 15 min followed by 30 cycles of denaturation at 94°C for 30 s; annealing (temperature as mentioned in Table 2) for 30 s; extension at 72°C for 2 min and a final extension at 72°C for 10 min. The PCR products were further processed with agarose gel electrophoresis at 100 V for 35 minutes by using agarose gel (1.2 %) stained with ethidium bromide at 0.5 µg/ml of the gel. Stained gels were examined with a gel documentation system (Syngene Ingenius 3, USA).

Antimicrobial susceptibility testing

Kirby-Bauer disk diffusion test was performed to determine antimicrobial resistance among isolates of *S. enterica* and *E. coli*. Selected clinically used antimicrobial drug discs (Oxoid, UK) including cephalosporins (ampicillin, AMP 10 µg; amoxicillin/clavulanic acid, AMC 30 µg and cefotaxime, CTX 30 µg), tetracyclines (doxycycline, DO 30 µg), aminoglycosides (gentamicin, CN 10 µg; streptomycin, S 10 µg and neomycin, N 30 µg), macrolides (erythromycin, E 15 µg), fluoroquinolones (ciprofloxacin, CIP 5 µg and enrofloxacin, ENR 5 µg), carbapenem (imipenem, IPM 10 µg; meropenem, MEM 10 µg) and polypeptides (colistin, CT 10 µg) were used for susceptibility testing. For each isolate, the inoculum was prepared from an overnight growth of pure culture suspension standardized to 0.5 McFarland units. Standardized suspensions were swabbed homogenously onto the surface of Mueller-Hinton agar plates and let to dry for 15 minutes. Antimicrobial discs were placed aseptically and plates were incubated at 37°C for 24 hrs. The diameter of the zone of inhibition was measured in millimeters and interpreted as sensitive, intermediate or resistant as per CLSI M100s manual (Bayer et al. 1966, CLSI 2018).

Results

Prevalence of *S. enterica*, *E. coli* and ESBL phenotypes

In this study, a total of 62 and 109 isolates of *S. enterica* and *E. coli* were identified, respectively. On the basis of DDST, the overall prevalence of ESBL isolates was found to be 42.69%. The prevalence of ESBL character was recorded higher in *E. coli* (49.54%) as compared to *S. enterica* (30.64%). The prevalence of ESBL isolates was recorded highest in pigeon (*Columba livia*) followed by egret (*Ardea alba*), common myna (*Acridotheres tristis*), crow (*Corvus splendens*), house sparrow (*Passer domesticus*) and Japanese quail (*Coturnix japonica*) as summarized in Table 1.

Table 1. Frequency of non-extended-spectrum beta-lactamase (ESBL) and ESBL isolates in studied bird species.

Bird Species	Total dropping samples	No. of *Salmonella enterica* isolates	No. of *Escherichia coli* isolates	DDST Positive		Total ESBL positive isolates
				Salmonella enterica	*Escherichia coli*	
Crow (*Corvus splendens*)	30	18	22	4 (22.22%)	12 (54.54%)	16 (40%)
Common myna (*Acridotheres tristis*)	30	05	13	1 (20%)	8 (61.54%)	9 (50%)
Pigeon (*Columba livia*)	30	11	19	4 (36.36%)	15 (78.95%)	19 (63.33%)
Japanese quail (*Coturnix japonica*)	30	09	14	0	4 (28.57%)	4 (17.39%)
Egret (*Ardea alba*)	30	14	24	9 (64.28%)	11 (45.83%)	20 (52.63%)
House sparrow (*Passer domesticus*)	30	05	17	1 (20%)	4 (23.53%)	5 (22.72%)
Total	180	62	109	19 (30.64%)	54 (49.54%)	73 (42.69%)

Table 2. Primers used for genotypic characterization of ESBL genes found in *Salmonella enterica* and *Escherichia coli*.

Target Gene	Primers	Primer Sequences (5′- 3′ strands)	Annealing temperature (°C)	Amplicon Size	Reference
bla_{CTX-M}	CTX-M-U1	ATGTGCAGYACCAGTAARGTKATGGC	58	593 bp	(Monstein et al. 2007)
	CTX-M-U2	TGGGTRAARTARGTSACCAGAAYCAGCGG			
bla_{TEM}	TEM-F	TTTCGTGTCGCCCTTATTCC	57	403 bp	(Ahmed et al. 2014)
	TEM-R	ATCGTTGTCAGAAGTAAGTTGG			
bla_{SHV}	SHV-F	CGCCGGGTTATTCTTATTTGTCGC	68	1016 bp	(Coculescu et al. 2016)
	SHV-R	TCTTTCCGATGCCGCCGCCAGTCA			
bla_{OXA}	OXA-F	ATTATCTACAGCAGCGCCAGTG	56	296 bp	(Uysal et al. 2018)
	OXA-R	TGCATCCACGTCTTTGGTG			

ESBL genotypic characterization

ESBL-confirmed isolates of *S. enterica* (19) and *E. coli* (54) were tested for the presence of four different genes responsible for beta-lactamase production. ESBL genes were detected via PCR through amplification of specific size amplicon by using primer sets as described in Table 2. Based on the PCR detection, bla_{CTX-M} gene predominated among *S. enterica* (57.89%) and *E. coli* (64.81%) as shown in Fig. 1. Among other genes in *S. enterica* and *E. coli*, bla_{TEM} (21.05%, 20.4%); bla_{SHV} (15.78%, 9.26%) and bla_{OXA} (5.26%, 5.56%) were detected, respectively. The prevalence of the bla_{CTX-M} gene varied (25-100%) among different bird species. The bla_{TEM} gene was detected in *E. coli* isolates of only two bird species, pigeon (*Columba livia*) and egret (*Ardea alba*). Only the isolates originated from crow (*Corvus splendens*), common myna (*Acridotheres tristis*) and pigeon (*Columba livia*) contained the bla_{SHV} gene while bla_{OXA} was only detected in isolates originated from pigeon (*Columba livia*) and egret (*Ardea alba*) (Table 3).

Antimicrobial susceptibility testing

Antibiotic sensitivity test was conducted on all 171 isolates obtained in this study (Table 4). All isolates were found resistant to at least one type of antibiotic except meropenem. The mean value of resistant phenotypes of *S. enterica* (29.85% ± 23.66) and *E. coli* (37.53% ± 27.29) differ significantly ($p = 0.02$) against tested antibiotics. *S. enterica* isolates (≥ 50%) were resistant to three antibiotics, gentamicin, neomycin and erythromycin while *E. coli* (≥ 50%) were resistant to six out of thirteen tested antibiotics.

Discussion

The present study was conducted in an effort to investigate the potential of urban free-flying bird species (avifauna) to carry and disseminate antimicrobial-resistant bacteria. The study was conducted in the central district of Jhang, in the most populous province

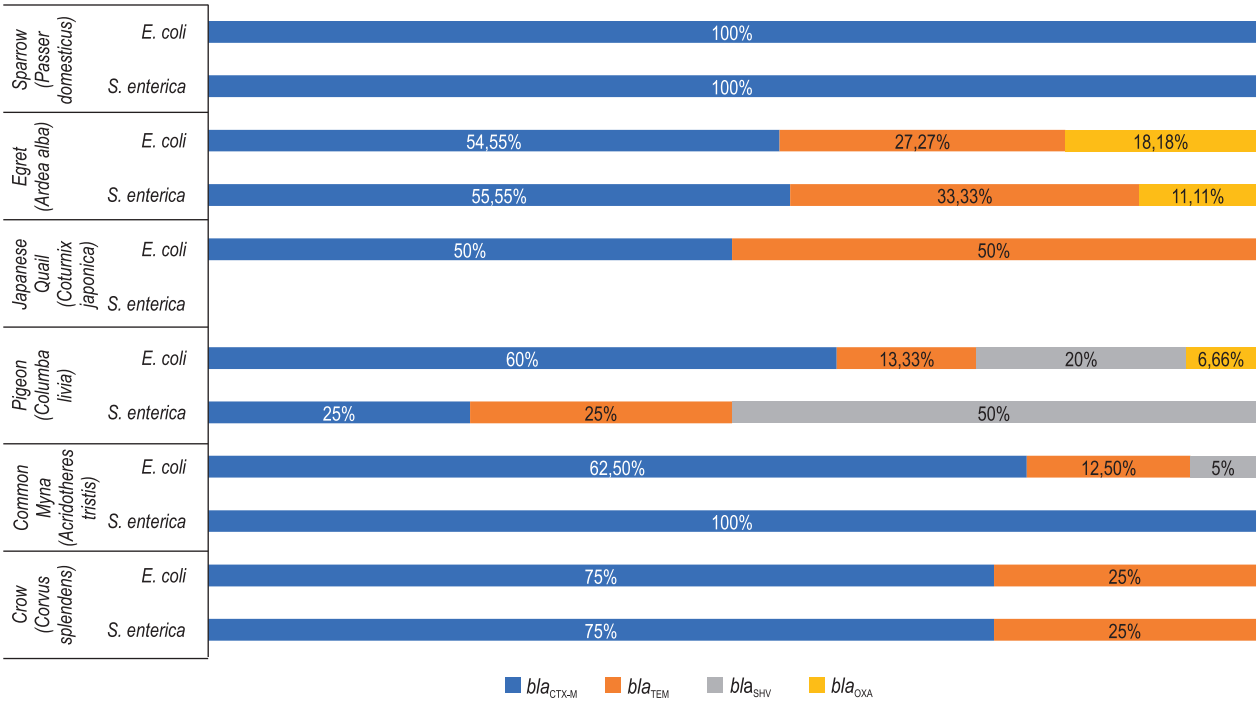

Fig. 1. ESBL genes (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{OXA}$) prevalence among different bird species. ESBL genetic diversity of *S. enterica* and *E. coli* has been shown in bars with color codes as described above for each bird species.

Table 3. Distribution of ESBL genes among *Salmonella enterica* and *Escherichia coli*.

Avian Species	Bacteria Species	Genes identified				
		No. of isolates	*bla*$_{CTX-M}$	*bla*$_{TEM}$	*bla*$_{SHV}$	*bla*$_{OXA}$
Crow (*Corvus splendens*)	*S. enterica*	4	3 (75%)	0	1 (25%)	0
	E. coli	12	9 (75%)	3 (25%)	0	0
Common myna (*Acridotheres tristis*)	*S. enterica*	1	1 (100%)	0	0	0
	E. coli	8	5 (62.5%)	1 (12.5%)	2 (25%)	0
Pigeon (*Columba livia*)	*S. enterica*	4	1 (25%)	1 (25%)	2 (50%)	0
	E. coli	15	9 (60%)	2 (13.33%)	3 (20%)	1 (6.66%)
Japanese quail (*Coturnix japonica*)	*S. enterica*	0	0	0	0	0
	E. coli	4	2 (50%)	2 (50%)	0	0
Egret (*Ardea alba*)	*S. enterica*	9	5 (55.55%)	3 (33.33%)	0	1 (11.11%)
	E. coli	11	6 (54.545%)	3 (27.27%)	0	2(18.181%)
House sparrow (*Passer domesticus*)	*S. enterica*	1	1 (100%)	0	0	0
	E. coli	4	4(100%)	0	0	0
Total Isolates	*S. enterica*	19	11 (57.894%)	4 (21.052%)	3 (15.789%)	1 (5.263%)
	E. coli	54	35 (64.815%)	11 (20.4%)	5 (9.26%)	3 (5.56%)

(Punjab) of Pakistan. This study included six different free-flying bird species found commonly in the urban area of the district Jhang. Fecal dropping samples were tested for the presence of ESBL and non-ESBL-producing enterobacteria *S. enterica* and *E. coli* known to cause food-borne illness and severe gastroenteritis in animals as well as in humans.

In this study, 34.44% (62/180) and 60.55% (109/180) isolates were identified as *S. enterica* and *E. coli*, respectively. Earlier studies meant for the isolation of *S. enterica* reported variable prevalence among different geographical regions and bird types. Findings of the current study showed a high prevalence of *S. enterica* (34.44%) when compared to previous results from aquatic or wild birds as the prevalence of *S. enterica* was reported to be 2.31% (12/519) in Chile (Tardone et al. 2020), 0.99% (15/1510) in Singapore (Aung et al. 2019), 2.75% (3/109) in Brazil

Table 4. Antibiotic resistance/sensitivity profiles of *Salmonella enterica* and *Escherichia coli* isolates as tested via Kirby-Bauer disk diffusion test.

Antibiotic	*Salmonella enterica* isolates (n=62)			*Escherichia coli* isolates (n=109)		
	S	I	R	S	I	R
AMP-10	35(56.4%)	6(9.67%)	21(33.8%)	28(25.68%)	4(3.66%)	77(70.64%)
AMC-30	40(64.5%)	5(8.06%)	17(27%)	18(16.5%)	34(31.19%)	57(52.29%)
CTX-30	24(38.71%)	12(19.35%)	26(41.93%)	26(23.85%)	16(14.68%)	67(61.46%)
DO-30	29(46.77%)	20(32.25%)	13(20.96%)	49(44.95%)	38(34.86%)	22(20.18%)
CN-10	8(12.9%)	5(8.06%)	49(79.03%)	6(5.5%)	16(14.67%)	87(79.81%)
S-10	56(90.3%)	2(3.22%)	4(6.45%)	87(79.8%)	13(11.92%)	9(8.25%)
N-30	14(22.5%)	12(19.35%)	36(58.06%)	18(16.5%)	36(33.02%)	55(50.45%)
E-15	11(17.74%)	17(27.41%)	34(54.8%)	16(14.67%)	24(22.01%)	69(63.30%)
CIP-5	51(82.2%)	0(0%)	11(17.7%)	44(40.36%)	33(30.27%)	32(29.35%)
ENR-5	33(53.22%)	8(12.90%)	21(33.87%)	42(41.28%)	28(25.68%)	39(35.77%)
IPM-10	58(93.5%)	1(1.61%)	3 (4.83%)	103(94.49%)	4(3.66%)	2(1.83%)
MEM-10	62 (100%)	0 (0%)	0(0%)	107(98.16%)	2(1.83%)	0(0%)
CT-10	54 (87.09%)	2(3.22%)	6(9.67%)	89(81.6%)	4(3.66%)	16(14.67%)

S – Sensitive, I – Intermediate, R – Resistant

(Matias et al. 2016) and 6.4% (64/1000) in Poland (Krawiec et al. 2015), but comparable to the studies conducted on urban birds in various parts of the world as De Lucia et al. (2018) reported that *Salmonella* spp. isolation percentage varied as 7.4-44.3% in free birds in the UK in the vicinity of livestock farms. In Spain, 12.3% of samples from urban birds were positive for *Salmonella* (Martín-Maldonado et al. 2020) and in the USA 27% of white ibis (*Eudocimus albus*) were found positive for *Salmonella* (Murray et al. 2021). In general, antimicrobial-resistant *S. enterica* prevalence was found low in wild birds as compared to free-flying avifauna of urban regions (Ngaiganam et al. 2019). In addition, the present study further explores the prevalence of *S. enterica* in various species of birds and revealed the highest prevalence of *Salmonella* in crow (*Corvus splendens*) (10%) while the lowest in common myna (*Acridotheres tristis*) and house sparrow (*Passer domesticus*) (2.77% each). The findings of this study revealed a high prevalence of *E. coli* (60.55%) as compared to *S. enterica* (34.44%). Similar findings were reported from Canada where authors reported a higher isolation rate of *E. coli* among urban robins (44.8%) and crows (92%) and concluded a significantly higher recovery rate of MDR *E. coli* isolated from urban locations as compared to countryside free-flying birds (Parker et al. 2016).

Each wild bird species provides a unique microenvironment and habitat which encourages the colonization by a wide variety of microorganisms in their guts (Grond et al. 2016). More recently, a comparative study of the fecal microbiota of six different bird species revealed *E. coli* to be a principally dominating bacterial species as compared to the other enterobacteria, including *Salmonella*. This variation may depend upon the living environment, type of feed and eating habits of individual bird species (Gao et al. 2021). In the present study highest prevalence of *E. coli* was recorded in egret (*Ardea alba*) (13.33%) and the lowest in common myna (*Acridotheres tristis*) (7.22%) which suggests the variation dependency on host bird species.

Wild and domestic free-flying birds have been documented as a reservoir and potential carriers for the dissemination of evolving antimicrobial-resistant (AMR) microbes. Due to their wide variety of feeding sources, movement and frequent interaction with other species spanning over large environmental landscapes, wild birds are considered a potential source for the environmental and cross-species spread of AMR (Wang et al. 2017). Unlike wild and feral birds residing in distant areas with minimal human interaction, the bird species belonging to urban avifauna are in close contact with human settlements, sewage channels, domestic animals and birds, this situation facilitates the spread of acquired genetic elements of resistance in their microflora (Martín-Maldonado et al. 2020). This study investigated the existence of ESBL-producing enterobacteria which is supported by the evidence provided by other regional studies conducted in Asia. Beta-lactam resistant *E. coli* (43.7%) were isolated

from wild migratory birds in China with the detection of bla_{CTX-M} and bla_{TEM-1} genes (Yuan et al. 2022). The prevalence of bla_{CTX-M} (7.8%), bla_{TEM} (5.6%) and bla_{SHV} (4.4%) genes were reported from *E. coli* associated with migratory wild birds of Al-Asfar lake in Saudi Arabia (Elsohaby et al. 2021). The occurrence of ESBL *E. coli* was found to be 15.5% in wild birds from Mongolia (Schierack et al. 2020). Migratory birds in Bangladesh were reported to carry bla_{SHV} (42.86%), bla_{TEM} (95.24%) and bla_{CTX-M} (85.71%) genes with an overall prevalence of ESBL *E. coli* as 38.18% (Islam et al. 2022). In this study, the overall prevalence of ESBL isolates was found to be 42.69% (73/171), comprising of *S. enterica* (30.64%) and *E. coli (*49.54%). The present study's findings revealed the presence of all tested ESBL genes in *S. enterica* isolates obtained from different bird species. The prevalence of bla_{OXA} (5.263%) was the lowest, while bla_{SHV} (15.789%) and bla_{TEM} (21.052%) were moderately high. bla_{CTX-M} (57.894%) gene predominated in *S. enterica* isolates. Previously, a study conducted on migratory birds in Pakistan reported 17.3% ESBL *E. coli* with reports of bla_{CTX-M} gene and bla_{TEM} gene in 92.3% and 73.07% samples, respectively (Mohsin et al. 2017). The rising burden of ESBL *E.* coli-associated birds as indicated by our study can be attributed to the factors like different host bird species under study, urban habitat, environmental exposure and feeding habits. In this study, multiple ESBL genes have been detected however bla_{CTX-M} (64.81%) has been found to be the most frequent gene, others include bla_{TEM} (20.4%), bla_{SHV} (9.26%) and bla_{OXA} (5.56%) genes. This finding is consistent with the global molecular epidemiological analysis of ESBL clinical isolates, which reports the existence of multiple different genetic determinants and dominance of bla_{CTX-M} encoded beta-lactamases (Bevan et al. 2017, Castanheira et al. 2021). These findings are in agreement with the findings of Fuentes-Castillo et al. (2019) who reported that CTX-M-8 and CTX-M-65 types of genes were more frequent in the wild owls. Global epidemiological data of ESBL isolates from the last decade suggests the SHV and TEM types of ESBL which predominated since the 1980s are gradually replaced by the CTX-M types of ESBL (Doi et al. 2017). However, beta-lactamases are evolving at a rapid pace into new types as the molecular assays have identified multiple variants of SHV (228), TEM (243), CTX-M (172) and OXA (27) (Paterson and Bonomo 2005, Koirala et al. 2021). On contrary, predominating ESBL genes in *S. enterica* isolates originating from commercial poultry were lacking bla_{CTX-M}, while bla_{SHV} was detected in 100% of the tested samples along with bla_{TEM} gene found in 87.5% of tested samples (Ibrahim et al. 2022). These findings reflect the high degree of variation in the existence of ESBL genes among *S. enterica* as originated from different host bird species. The transmission of ESBL genetic determinants between bacteria found in the environment and of clinical origin is enormous. Recently, in a study conducted on samples of surface and wastewater in the urban territory of Islamabad (capital of Pakistan), bla_{CTX-M} (33.33%) and bla_{TEM} (40%) ESBL genes were identified in isolates of *E. coli* (Ahsan et al. 2022). These findings advocate the spread of AMR microbes in urban avifauna through the consumption of contaminated water.

The antibiotic sensitivity profile of ESBL isolates obtained in our study showed almost absolute susceptibility towards carbapenem antibiotics (meropenem and imipenem). These findings are in agreement with a previous study aimed at genomic characterization of AMR genes in chicken origin *E. coli* (Rafique et al. 2020). However, the resistance trend found previously regarding ciprofloxacin (72%) and gentamicin (11%) as reported by Azam et al. (2019) was found contrary as isolates resistant to ciprofloxacin and gentamicin were found as 29.35% and 79.81%, respectively, in our study. This shift might be due to the limited use of ciprofloxacin in animal practice and the progressive increase in the use of gentamicin and colistin in Pakistan, thus expressing resistance in microflora.

The present study was conducted for comparative analysis of four different ESBL-coding genes in six different species of urban avifauna in Pakistan for both *S. enterica* and *E. coli*. Previous studies conducted in Pakistan undertook commercial chicken, human beings and livestock species. To the best of the authors' knowledge findings of this study provided the first evidence for exploring emerging antimicrobial resistance in free-flying urban birds.

Conclusions

This study confirms the existence of extended-spectrum β-lactamase (ESBL) producing *Salmonella enterica* and *Escherichia coli* harbored by multiple free-flying avian species in the urban area of Pakistan. The ESBL isolates have been confirmed to carry bla_{CTX-M}, bla_{TEM}, bla_{SHV} and bla_{OXA} genes which encode beta-lactamases. The bacteria have been found resistant to most of the clinically used antibiotics in human and veterinary medicine except the carbapenem class. The urban avifauna can act as a potential reservoir and spreader of antimicrobial-resistant bacteria. There is a dire need to enhance active surveillance and strengthen the ties of the one-health concept in Pakistan. This is essential to undertake effective preventive measures to address the possible future outbreaks of resistant bacteria.

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