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From Reservoirs to Potential Vectors of Superbugs: Wild Birds Co-Harboring Extended- Spectrum Beta-Lactamases and Plasmid-Mediated Quinolone Resistance determinants in Enterobacteriaceae isolates in Pakistan

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Title: From Reservoirs to Potential Vectors of Superbugs: Wild Birds Co- Harboring Extended-Spectrum Beta-Lactamases and Plasmid-Mediated Quinolone Resistance determinants in *Enterobacteriaceae* isolates in Pakistan.

Running title: ESBL and PMQR co-carriage in *Enterobacteriaceae* from wild birds.

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Abstract

Antimicrobial resistance (AMR) is a significant health issue of the 21st century. Wild birds are increasingly recognized as reservoirs of multidrug-resistant (MDR) bacteria. They are ecologically important because they have travelling ability across globe, and with no direct antimicrobial interaction making them an important part of One Health interface to predict environmental contamination of MDRs and its dissemination. This study aimed to assess the prevalence and co-occurrence of extended-spectrum beta-lactamase (ESBL) and plasmid-mediated quinolone resistance (PMQR) determinants in *Enterobacteriaceae* isolates from the gut of wild birds in Islamabad region. Using standard bacterial characterization techniques in total, 342 Gram-negative *Enterobacteriaceae* isolates were obtained from fresh fecal droppings collected seasonally at eight locations with various types of facilities in Islamabad Pakistan including hospitals. zoo, parks and other public areas. Disk Diffusion test was performed to confer quinolones resistance and double-disk synergy test (DDST) was used to confirm ESBL production. Multiplex PCR was used to detect PMQR genes (*QnrA, QnrB, and QnrS*), whereas *aac (6')-Ib-cr* was detected using conventional PCR. Statistical analysis was used to assess inter-variable associations. A total of 342 isolates were obtained, of which 118 (34.5%) were ESBL producers. Among ESBL-positive isolates that were phenotypically co-resistant to ciprofloxacin and/or nalidixic acid (Quinolones), 73 (61.8%) carried at least one gene of the PMQR family. The most common PMQR determinants were *QnrS* (76.7%) and *aac (6')-Ib-cr* (47.9) %. The co-harboring isolates were dominated by *Escherichia coli* (82.2%). *K. pneumoniae* showed a high carriage of *aac (6')-Ib-cr* (90%) and *QnrB* (60%) genes. The highest levels were observed in autumn and winter. Multi-gene co-carriage patterns, where ESBL and PMQR genes occur on a single plasmid, were inferred and were similar to those found in other major plasmid families, IncF, IncI, and IncHI. Wild birds are important reservoirs and active spreaders of MDR *Enterobacteriaceae* carrying ESBL and PMQR determinants on transferable plasmids in the urban environment of Islamabad. Based on these results, the need to involve wildlife surveillance in One Health-based AMR surveillance programs and increase antibiotic stewardship in Pakistan is highlighted.

Keywords: Antimicrobial resistance, *Enterobacteriaceae*, Extended-spectrum beta-lactamases (ESBL), Multi-drug resistance (MDR), plasmid-mediated quinolone resistance (PMQR), One Health, and wild birds.

1. Introduction

Antimicrobial resistance (AMR) has become one of the greatest public health challenges and threatens to compromise the effectiveness of antibiotics, which have been the basis of modern medicine for more than 70 years [1]. The World Health Organization (WHO) has classified AMR as a critical global priority, projecting that without immediate and coordinated action, drug-resistant infections could cause up to 10 million deaths annually by 2050 [2]. At the molecular level, resistance is perpetuated, amplified, and spread quickly horizontally by conjugative plasmids, transposons, and integrons, which are mobile genetic elements that carry resistance genes from one phylogenetically distant bacterial species to another and from ecological niche to niche [3]. Extended-spectrum beta-lactamases (ESBLs) are enzymes produced by gram-negative bacteria (mainly in the family Enterobacteriaceae) that deactivate penicillins, third- and fourth-generation cephalosporins, and monobactams. CTX-M is the most widely disseminated and prevalent ESBL type in clinical and environmental isolates, including CTX-M-15, which is the most widely disseminated and resistant ESBL type [4]. ESBL-producing bacteria have been isolated from various non-human reservoirs such as livestock, companion animals and wildlife and

prevalence rates among wild birds in Europe vary from 5% in areas with low human-wildlife contact, to over 40% in areas with high human-wildlife contact [5]. The co-location of ESBL-encoding genes with other resistance determinants in transferable plasmids makes it difficult to treat and manage multi-drug resistance. The ESBL problem is complicated by the co-occurrence of Plasmid-Mediated Quinolone Resistance (PMQR). Fluoroquinolones have been considered key drugs for treating severe infections caused by gram-negative pathogens; however, this option has been gradually compromised with the emergence of PMQR genes, including those encoding the Qnr protein family (*QnrA*, *QnrB*, *QnrS*, QnrC, and QnrD) and the aminoglycoside acetyltransferase variant aac(6')-Ib-cr [6]. PMQR genes contribute to low-level resistance; however, when combined with chromosomal target-site mutations, they can increase the minimal inhibitory concentrations (MICs) to clinically relevant levels. Moreover, PMQR genes are often found on the same plasmids that contain ESBL-encoding genes, leading to the co-transfer of both classes of resistance and the formation of MDR strains that cannot be managed therapeutically [7].

Within the One Health paradigm, which recognizes the interrelation of human, animal, and environmental health systems, the importance of environmental reservoirs in the dissemination of AMR has been increasingly recognized. Wild birds are given special attention within this framework. They are not restricted to any particular ecological niches. Bird species found in urban and peri-urban environments that regularly visit hospitals, sewage outlets, and open waste dumps are particularly at risk of acquiring MDR bacteria from contaminated food or the environment. After acquisition, these birds release resistant organisms in several locations, polluting food chains, soil, and water on a large geographical scale [8]. Resistance genes can be geographically dispersed in days by long-distance migratory species, making geographically limited interventions inadequate. The carriage of ESBL-producing Enterobacteriaceae in urban birds, such as gulls, pigeons, crows, storks, and others, has been confirmed in Sweden, Portugal, Spain, Japan, and sub-Saharan Africa, and this research has shown that these birds are capable of being vectors of AMR at both local and global levels [9].

The prevalence and spread of AMR in wild birds are also affected by environmental and seasonal factors. Changes in temperature, rainfall, and seasonal changes in bird congregation and foraging habits affect the survival of resistant bacteria in the environment and the number of bird-environment contact events. The monsoon (June-July-August) and post-monsoon (September-October) seasons are linked to high water contamination and increased bird foraging near human settlements in the Islamabad region, which may result in peaks in MDR throughout the season. As a low- and middle-income country (LMIC), Pakistan's compounding AMR risk factors include the availability of unrestricted over the counter (OTC) antibiotics, widespread usage of antibiotics for human and veterinary/agricultural purposes with significant misuse, poor hospital effluent management, and lack of AMR surveillance capacity. Therefore, this study was conducted to characterize the prevalence of co-resistance among *Enterobacteriaceae* in wild bird fecal droppings collected from eight ecologically different sites in Islamabad, Pakistan, examine the genetic distribution of PMQR genes (*QnrA*, *QnrB*, *QnrS*, and aac (6')-Ib-cr), deduce the basis of co-resistance based on co-occurrence and literature evidence, and provide an evidence base to support One Health-based AMR surveillance frameworks in Low- and Middle-Income Countries.

2. MATERIALS AND METHODS

2.1. Study sites and Sample collection.

Eight geographically and ecologically different areas across Islamabad, Pakistan, were selected to cover a spectrum of human-wildlife interfaces, including two tertiary care hospitals (Polyclinic Hospital and Pakistan Institute of Medical Sciences PIMS), three public recreational parks (Lakeview Park, Chattar Park, Fatima Jinnah Park and Shakarparian Park), zoo (Margallah National Park and Zoo) and Quaid-i-Azam university campus.

Sampling was done during four seasons of the year, namely, Autumn (October-November 2016), Winter (December 2016-February 2017), Spring (March-April 2017), and Summer (May-August 2017). A total of 360 fecal samples were collected with 10-12 samples per season at each sampling site. Fecal samples were collected aseptically using sterile spatulas into sterile capped test tubes containing phosphate-buffered saline (PBS). The samples were immediately carried under cool chain (2-8°C) condition to Medical Microbiology Research Laboratory of Quaid-i-Azam University for immediate processing.

2.2 Isolation and Identification of Enterobacteriaceae

The samples were cultured on MacConkey Agar (Oxoid, UK) by the spread plate method and were incubated at 37°C for 18-24 hours. The streak plate method was used for further purification of the morphologically distinct colonies. Isolates of *E. coli* were sub-cultured onto Eosin Methylene Blue (EMB) agar with green metallic sheen being indicative of *E. coli* identity at the preliminary level. All isolates were tested using a full panel of conventional biochemical tests (IMVIC, SIM, TSI agar, Urease, Oxidase) which were interpreted as per Bergey's Manual standards [10].

2.3 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was done using the disk diffusion method on Mueller Hinton Agar (MHA, Oxoid, UK), according to the Clinical and Laboratory Standards Institute (CLSI) 2017 guidelines. The antibiotic disks used were ceftazidime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), ciprofloxacin (5 µg) and nalidixic acid (30 µg). All the plates were incubated at 37°C for 18-24 hours and the zone of inhibition was measured by a calibrated ruler. *Escherichia coli* ATCC 25922 was used as a quality control strain.

2.4 Phenotypic confirmation of ESBL production

The ESBL production was confirmed by Double Disk Synergy Test (DDST). The isolates with reduced susceptibility (inhibition zone ≤22 mm) to any of the three third generation cephalosporins in primary screening were tested for DDST on MHA turbidity of 0.5 McFarland. Ceftazidime 30-µg, cefotaxime 30-µg, and ceftriaxone 30-µg disks were positioned 20-25 mm (center to center) from an amoxicillin-clavulanate 20/10-µg disk placed in the center. ESBL production was confirmed by the enhancement of the inhibition zone towards the amoxicillin-clavulanate disk (≥5 mm; the 'keyhole' effect). As a quality control strain, *E. coli* ATCC 25922 was used.

2.5 Molecular detection of PMQR genes.

ESBL-positive isolates showing phenotypic co-resistance to ciprofloxacin and/or nalidixic acid were subjected to genomic DNA extraction by boiling lysis (boiling for 10 minutes, after colony suspension in 1mL sterile distilled water, centrifuged at 13,000 rpm for 10 minutes, the supernatant was stored at -20°C until use). The multiplex PCR was performed for the simultaneous detection of *QnrA*, *QnrB* and *QnrS* genes with validated primers

developed by Cattoir et al. (2007) [11]. The detection of aac(6')-Ib-cr was performed by conventional singleplex PCR, with primers described in Park et al. (2006) [12] as well as amplicon sizes are provided in Table 1.

Table 1: Primers sequences for Plasmid-mediated Quinolone's resistance genes.

Primers	Sequences (5'-3')	Product size (bp)	Reference
QnrA-F *QnrA-R*	AGAGGATTTCTCACGCCAGG TGCCAGGCACAGATCTTGAC	580	[11]
QnrS-F *QnrS-R*	GCAAGTTCATTGAACAGGGT TCTAAACCGTCGAGTTCGGCG	428	[11]
QnrB-F *QnrB-R*	GGMATHGAAATTCGCCACTGa TTTGCYGYYCGCCAGTCGAAa	264	[11]
aac (6') Ib-cr-F *aac (6') Ib-cr-R*	TTGCGATGCTCTATGAGTGGCTA CTCGAATGCCTGGCGTGTTT	482	[13]

Multiplex PCR thermocycling conditions: initial denaturation, 95°C for 10 min; 35 cycles, 94°C for 45 s, 55°C for 1 min, 72°C for 1 min; final extension, 72°C for 10 min. Initial denaturation was at 95°C/10 min; 35 cycles of 94°C/45 s, 56°C/1 min, 72°C/1 min; final extension was at 72°C/10 min. PCR products were resolved on 2% agarose gels stained with ethidium bromide and visualized under UV illumination. The 100 bp DNA ladder (New England BioLabs) was used as the molecular weight marker. All PCR runs had positive and negative controls.

2.6 Statistical Analysis

All prevalence parameters were calculated as proportions with 95% confidence intervals (95% CI) using the Wilson score method. Associations between categorical variables such as species-specific distribution of PMQR genes, and prevalence of co-resistance of ESBL/PMQR genes were evaluated with chi-square (χ^2) tests, with Fisher's exact test used when the expected cell frequencies were <5. A p-value of $p<0.05$ was deemed to be statistically significant. The association between ESBL positivity and co-harboring of PMQR was quantified in terms of odds ratios (OR) with 95% CI. A SPSS v25.0 (IBM Corp.) and Microsoft Excel 2019 software were used for all analyses.

3. Results

3.1 Isolation and identification of *Enterobacteriaceae.*

Fecal droppings from wild birds were successfully isolated at 342 Gram-negative Enterobacteriaceae from eight locations spread over four seasons. Colonial morphology and also the conventional biochemical characterization identified four species, *Escherichia coli* (72.8%), *Klebsiella pneumoniae* (18.1%), *Enterobacter spp.* (6.1%), and *Klebsiella oxytoca* (2.9%).

3.2 Antimicrobial Susceptibility Profile

All 342 isolates were subjected to antimicrobial susceptibility testing against five antibiotics. Resistance rates for each antibiotic, overall and by species, are presented in figure 1. Nalidixic acid recorded the highest overall resistance rate (44.4%), followed by ceftazidime (42.4%), cefotaxime (39.2%), ceftriaxone (38.6%), and ciprofloxacin (35.1%). *K. pneumoniae* consistently exhibited the highest resistance rates across all five antibiotics tested, while *K. oxytoca* showed the lowest resistance prevalence. Out of all the 342 isolates, (21.3%) demonstrated combined resistance to both ciprofloxacin and nalidixic acid, and it was this co-resistant phenotype among ESBL-positive isolates that was selected for molecular characterization of PMQR genes.

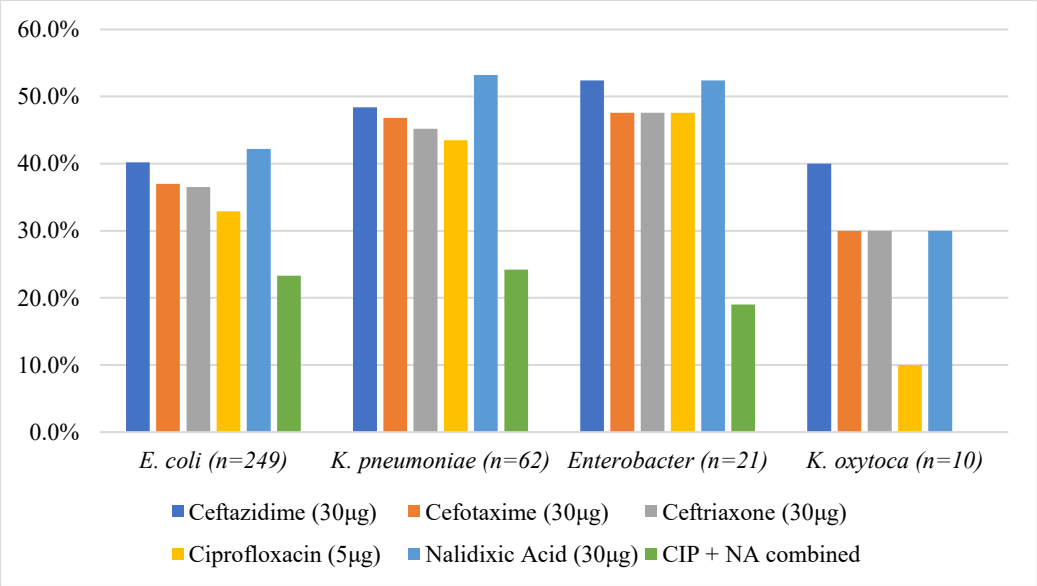


Figure 1. Antimicrobial resistance among *Enterobacteriaceae* isolated from wild bird faecal droppings in Islamabad, Pakistan (n = 342). Bars show the percentage of isolates resistant to each of the five antibiotics tested (ceftazidime, cefotaxime, ceftriaxone, ciprofloxacin, and nalidixic acid), presented for the overall isolate collection and separately for each of the four species recovered (*Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, and *Enterobacter spp.*). Resistance was determined by the disk diffusion method on Mueller-Hinton agar, interpreted by CLSI.

3.3 Prevalence of ESBL Production

The total number of 342 *Enterobacteriaceae* screened by DDST, (34.5%; 95% CI: 29.5-39.8%) 118 isolates were confirmed ESBL producers. Among ESBL-positive isolates, *E. coli* accounted for (70.3%), *K. pneumoniae* (19.5%), *Enterobacter spp*. (8.5%), and *K. oxytoca* (1.7%). CLSI standards were applied.

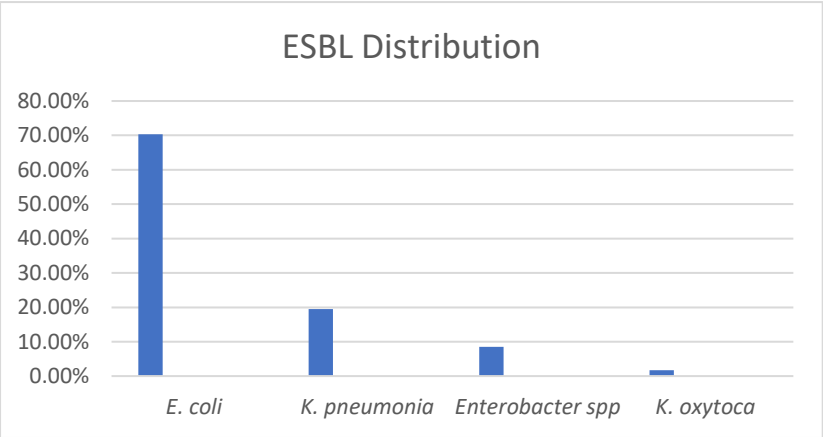


Figure 2: The bar chart shows the species-wise *ESBL* production among *Enterobacteriaceae* isolates (n = 118).

Table 2. Corresponding prevalence values and 95% confidence intervals are given in Table 2

Bacterial Species	95% CI
Escherichia coli	27.5-39.5
Klebsiella pneumoniae	25.2-50.3
Enterobacter spp.	26.2-69.7
Klebsiella oxytoca	3.6-56.1
Total	29.5-39.8

3.4 Co-harboring of ESBLs with PMQR Genes

ESBL-producing isolates that were phenotypically co-resistant with ciprofloxacin and/or nalidixic acid, (61.8%, 95% CI: 52.3-70.6%) were found to harbor one or more PMQR genes by PCR. The species composition of these co-harboring isolates was as follows: *E. coli* (82.19%), *K. pneumoniae* (13.69%) and *Enterobacter spp.* (4.10%), and *K. oxytoca* (1.36%). The odds of ESBL-positive isolates being co-harboring PMQR genes were 3.4 times greater than chance (OR = 3.41; 95% CI: 1.89-6.15; $p < 0.001$).

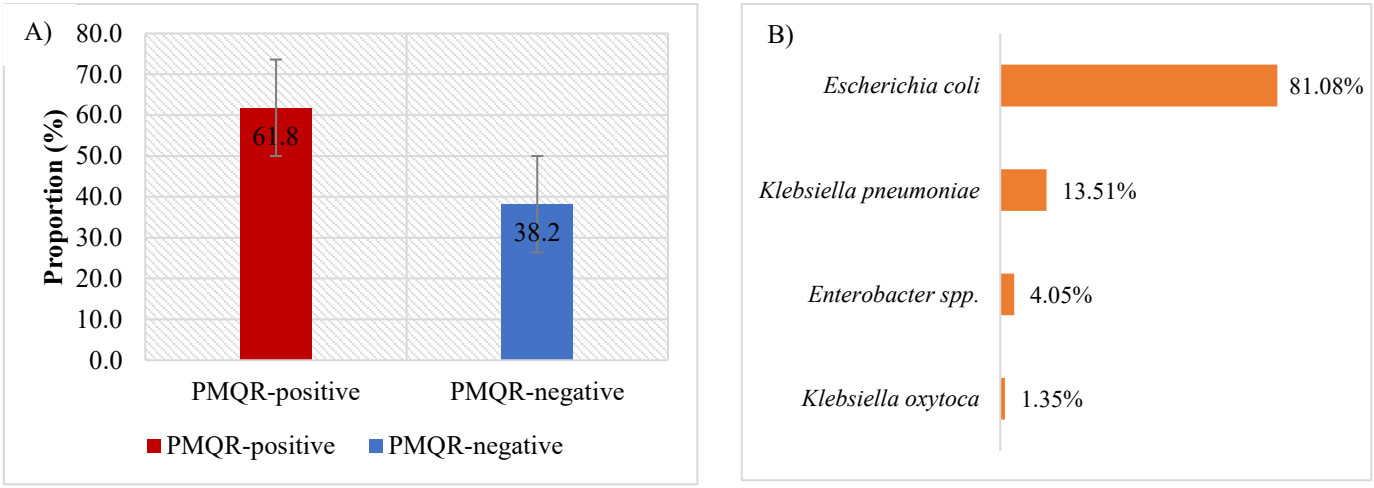


Figure 3: Species distribution and gene carriage among ESBL/PMQR co-harboring isolates (n = 73).

(A) In total of 118 ESBL positive isolates, graph shows proportion of ESBL co-harboring PMQR determinants (carry at least one *QnrA*, *QnrB*, *QnrS*, or *aac(6')-Ib-cr*) versus those in which no PMQR gene was detected. **(B)** Proportional species composition of isolates that were phenotypically ESBL-positive and co-resistant to ciprofloxacin and/or nalidixic acid (*Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter spp.*, and *Klebsiella oxytoca*).

3.5 Prevalence of Individual PMQR Genes

The presence of individual PMQR genes was examined. Among the 73 ESBL/PMQR co-harboring isolates, *QnrS* was the most prevalent determinant (56/73; 76.7%; 95% CI: 65.4-85.2%), followed by aac(6')-Ib-cr (35/73; 47.9%; 95% CI: 36.4–59.6%), *QnrB* (21/73; 28.8%; 95% CI: 19.0-40.2%), and *QnrA* (5/73; 6.8%; 95% CI: 2.5-15.5%). Data are presented in Table 3. Co-carriage of PMQR genes was common; the detailed multi-gene combinations are presented in Section 3.8 and Table 5.

Table 3. Prevalence of individual PMQR genes among ESBL positive isolates (n = 73).

PMQR Gene	Number (total=73)	Prevalence (%)	95% CI
QnrS	56	76.7%	65.4-85.2
aac(6')-Ib-cr	35	47.9%	36.4-59.6
QnrB	21	28.8%	19.0-40.2
QnrA	5	6.8%	2.5-15.5

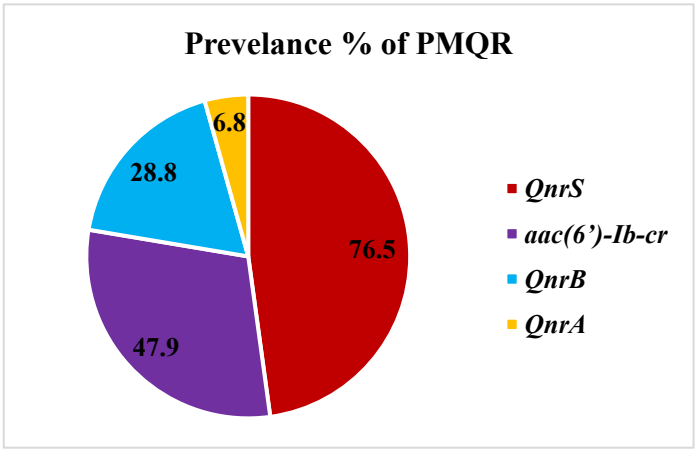


Figure 4. Prevalence of individual plasmid-mediated quinolone resistance (PMQR) determinants among ESBL positive isolates.

3.6 Seasonal Distribution of Individual PMQR Genes

Seasonal analysis of individual PMQR gene detection revealed divergent patterns. The gene aac (6')-Ib-cr was the most frequently detected gene in Autumn. *QnrS* achieved its highest absolute prevalence in Winter, consistent with the overall dominance of *E. coli* in that season and the strong association between *QnrS* and *E. coli* in this dataset. *QnrB* detections were distributed across Autumn and Spring seasons, coinciding with the higher *K. pneumoniae* counts in those seasons. *QnrA* detections, though low overall, were recorded principally in Autumn and Winter.

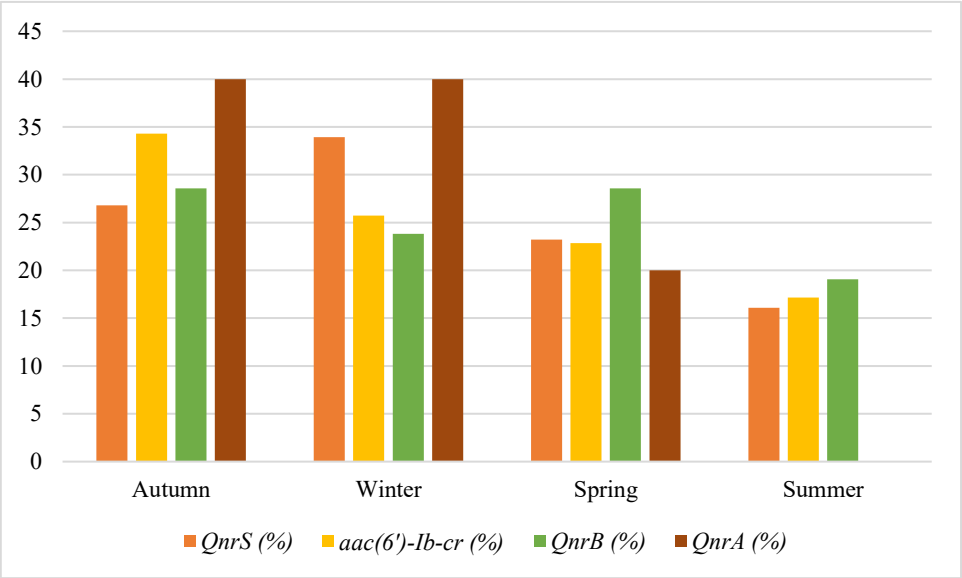


Figure 5. Seasonal distribution of individual PMQR genes (*QnrA*, *QnrB*, *QnrS*, and *aac(6')-Ib-cr*) among the 73 ESBL/PMQR co-harboring *Enterobacteriaceae* isolates, grouped by the four sampling seasons (Autumn, Winter, Spring, and Summer; October 2016-August 2017). Bars represent the number of isolates in which each gene was detected per season.

3.7 Species-Specific PMQR Gene Distribution

The distribution of species-specific PMQR genes is presented in the following section. There were also differences in resistance level between species ($\chi^2 = 14.36$; df = 6; p = 0.026) identified by species-level analysis. *E. coli* (n = 60) predominantly carried *QnrS* (80%), with lower prevalence of aac (6')-Ib-cr (43.3%), *QnrB* (25%), and *QnrA* (6.8%). In contrast, *K. pneumoniae* (n = 10) showed the highest rate of aac (6')-Ib-cr (90%) and *QnrB* (60%), with *QnrS* in 60% and *QnrA* in 10%. *Both K. oxytoca* (n = 1) and *Enterobacter spp.* Only 2 of these (n = 2) harboured *QnrS* alone. The patterns show variation in plasmid content among host species.

Table 4. Species-specific distribution of PMQR genes among ESBL/PMQR co-harboring isolates (n = 73).

Species (n)	*QnrS* (%)	aac (6')-Ib-cr (%)	*QnrB* (%)	*QnrA* (%)

E. coli (60)	80.0	43.3	25.0	6.8
K. pneumoniae (10)	60.0	90.0	60.0	10.0
K. oxytoca (1)	100.0	0.0	0.0	0.0
Enterobacter spp. (2)	100.0	0.0	0.0	0.0

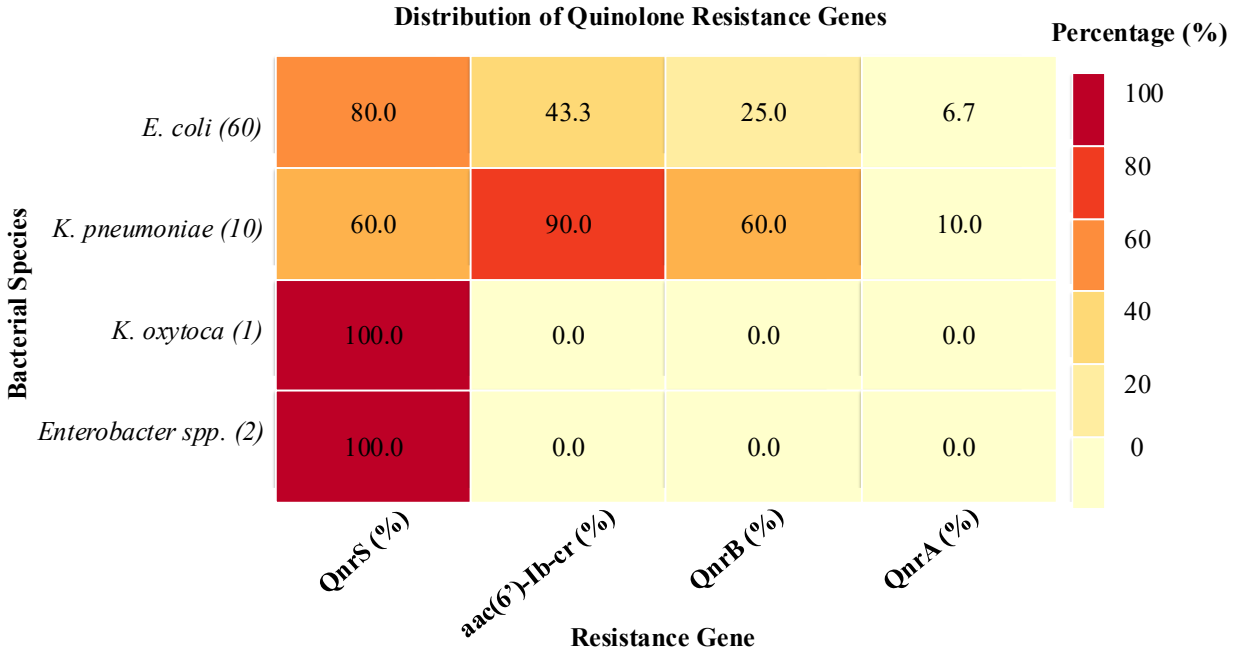


Figure 6. Heatmap of species-specific PMQR gene prevalence among ESBL/PMQR co-harboring isolates (n = 73). Rows correspond to bacterial species (*Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, and *Enterobacter spp.*) and columns to the four PMQR determinants screened (*QnrA*, *QnrB*, *QnrS*, and *aac(6')-Ib-cr*). Cell shading intensity is proportional to the within-species percentage prevalence of each gene (0-100%), with darker shading indicating higher prevalence.

3.8 Multi-Gene Co-Carriage Patterns

Multiple PMQR gene co-carriage was a frequent finding within the 73 co-harboring isolates. The co-occurrence combinations are detailed in Table 5. Two *E. coli* isolates simultaneously harbored all four PMQR determinants (*QnrA* + *QnrB* + *QnrS* + aac(6')-Ib-cr), representing the highest complexity of multi-gene carriage (2.7% of co-harboring isolates). The most common combination was aac(6')-Ib-cr co-occurring with *QnrS* (27/73; 37.0%), followed by *QnrB* + *QnrS* co-carriage (12/73; 16.4%). Four of the five *QnrA*-positive isolates also carried aac(6')-Ib-cr, either as part of the four-gene combination (2/5) or alongside *QnrB* or *QnrS* individually (1/5 each); the gene profile of the remaining *QnrA*-positive isolate should be confirmed against raw PCR records, but the pattern overall suggests co-location on a common plasmid replicon. One isolates each harbored *QnrA* + *QnrB* and *QnrA* + *QnrS* combinations without additional PMQR genes. The remaining 30 isolates (41.1%) carried a single PMQR determinant only.

Table 5. Multi-gene PMQR co-carriage combinations among ESBL positive isolates (n = 73).

Gene Combination	No. of Isolates (n)	Percentage % of Co-harboring Isolates
QnrA + QnrB + QnrS + aac(6')-Ib-cr	2	2.7
QnrB + QnrS	12	16.4
QnrS + aac(6')-Ib-cr	27	37.0
QnrA + QnrB	1	1.4
QnrA + QnrS	1	1.4
Single gene only (*QnrS, aac, or QnrB)*	30	41.1
Total	**73**	

3.9 Co-Location of ESBL and PMQR Genes within Plasmids

Plasmid incompatibility typing and whole genome sequencing were not performed for the present study, but a number of conserved features suggest that ESBL and PMQR genes are co-harboring within the same plasmid(s) in the co-harboring isolates. Firstly, the high frequency of multi-gene co-carriage (including the three isolates that carry all four PMQR genes in addition to ESBL genes) is consistent with co-location on large conjugative plasmids, as described in IncF, IncI and IncN-type plasmids worldwide. Second, all five *QnrA*-positive isolates contained *aac (6')-Ib-cr* in addition to *QnrA*, which encodes a protein that is genetically linked to the blaCTX-M-15 gene, as previously reported for IncF-type plasmids carrying blaCTX-M-15 and aac (6')-Ib-cr in clinical and environmental isolates. Third, *K. pneumoniae* isolates with the largest PMQR gene repertoire (*QnrS* + *QnrB* + aac (6')-Ib-cr; 60% each) are consistent with large multi-resistance IncF or IncHI plasmids reported in clinical *K. pneumoniae* sequence types (STs) ST258 and ST11 that typically carry *blaCTX-M*, *blaSHV* and several PMQR genes on a single replicon. Fourth, literature consistently reports that the PMQR genes (particularly *QnrS*, *QnrB* and *aac(6')-Ib-cr)* are co-located with the CTX-M-type ESBL genes on conjugative plasmids of the Enterobacteriaceae family; this is important because it allows co-transfer and thus a wide dissemination in clinical and environmental settings [14]. The co-harboring rate detected in this study, coupled with the observations above, suggests that genes for ESBL and PMQR are transferred concurrently using the same or co-existing transferable plasmids.

4. Discussion

The present study gives in-depth surveillance information on prevalence and co-infection of ESBL production and PMQR genes in the Enterobacteriaceae collected from wild bird fecal droppings at eight ecologically different locations of Islamabad, Pakistan. The results largely align with and, in some aspects, surpass the global results of similar wildlife surveillance studies, highlighting the specific AMR burden on this urban Low and middle-income countries context.

The overall ESBL prevalence found in this study was comparatively high (34.5%) compared to other wild bird studies conducted in Western European and North American urban areas which have typically reported rates of 5-15%, but is comparable to the prevalence reported from more environmentally contaminated urban areas in Asia and Africa [15]. A study conducted in the Czech Republic in 2013 found ESBL prevalence of around 30-40%

among zoo animals [16] and a survey of urban gulls in Spain found ESBL prevalence of around 40-51.5% [17]. This is compared to the 3.9% rate observed with studies of waterbirds in Poland, and as low as 0.5% in rural European populations in which contact with wildlife is rare [18]. There is a high proportion in our Islamabad group and it is possible that this is because the foraging environment is highly polluted, with resistant bacteria being common in effluents, waste streams and on surfaces in and around tertiary care hospitals. The high prevalence of *E. coli* ESBL reported in this study (33.3%) is in accordance to the high prevalence of ESBL reported in community acquired (1.53%) and HA (61.8%) isolates in clinical setting [19]. *QnrS* was found in 83.9% of the ESBL co-resisters in wild birds from South Korea [20, 21], while in our study it was 76.7%, and in the Netherlands [22], of all wild birds, 10.8% of ESBL producing *E. coli* were found to be PMQR positive.

This 61.8% rate is among the highest levels recorded in the world for wild birds, and is similar to the high background AMR in Pakistan. This is a clinically concerning high co-resistance rate as organisms with both ESBL and PMQR determinants on transferable plasmids are resistant to both the two most important antibiotic classes for empirical treatment of serious infections with Gram-negative bacteria [23]. The majority of PMQR determinants (76.7%) are *QnrS*, which is in harmony with data from environment and wildlife worldwide. A high prevalence of co-resistance with *QnrS* was observed in South Korea [20] with 83.9% of wild bird isolates being co-resistant with *QnrS*, while in Europe, co-resistance with *QnrS* was detected in up to 14.5% of ESBL-positive isolates from rook (Corvus frugilegus) isolates from various countries [24]. The background resistance pressures of European wild bird populations are low, and the *QnrS* rates are very low in most cases. The prevalence of *QnrS* in our study is similar to other studies of clinical and environmental isolates of Enterobacteriaceae, which indicated high prevalence in *E. coli* and *Klebsiella* species, respectively, from South and Southeast Asian countries, carrying IncI and IncF plasmid containing *QnrS*, respectively [25]. The alarming high rate is likely due to the high selection pressure of ciprofloxacin in the clinical and agricultural settings of Pakistan because ciprofloxacin is one of the most commonly and indiscriminately used antimicrobials in Pakistan [26]. The prevalence of *QnrA* (6.8%) in our study agrees with global trends that put *QnrA* as the least prevalent determinant found in environmental isolates. The first reported cases of *QnrA* were in U.S. clinical isolates of *K. pneumoniae* in 1998 [27], and *QnrA* has not been identified in wildlife surveillance studies to date. No *QnrA* was detected in waterbirds in Poland [28] and little or no positivity for *QnrA* was found in most countries in a multi-country European study of the wildlife, with 14.3% of PCR positive isolates being positive for *QnrA* for American crows [29]. This rate of 6.8% was only seen in *E. coli* and might be due to spill over from clinical strains commonly found in hospital settings in Islamabad. Interestingly, the five isolates in our study that were carrying *QnrA* were also aac(6')-Ib-cr positive, and this combination was identical to that of the IncF-type plasmids that were originally found in clinical isolates of Enterobacteriaceae from Southeast Asia [30].

Further epidemiological information is provided in the species-level analysis. In the co-harboring dataset, *E. coli* is the most abundant bacterium (82.19%), much like it is in the world at large, commensal and a reservoir of resistance genes, while the *K. pneumoniae* co-resistance profile is of significant concern due to fewer occurrences (13.69%). WHO has designated *K. pneumoniae* as a critical priority pathogen for the urgent need to develop new antibiotics, and the wide variety of PMQR genes found in the sampling of *K. pneumoniae* isolates from environmental wildlife. The limited number of *Enterobacter spp.* and *K. oxytoca* that were resistant only to *QnrS* may indicate a limited but potentially widespread resistance problem in these species. This should be investigated

336 further as there are seasonal variations, especially in terms of the most co-resistance in the autumn and winter.
337 The relatively low number of summer counts is surprising, particularly in light of the generally higher rates of
338 bacterial survival in warmer temperature, and transmission of conjugative plasmid transfer. The potential
339 explanation is that samples were collected in Summer when monsoon rains are likely to be high and hence dilute
340 environmental contamination, or that monsoon rains induced bird foraging changes that summer. In contrast, the
341 maximums in the Autumn and Winter periods might be due to post-monsoon flocks of birds focusing on water
342 sources and food that remain in the vicinity of the hospital, leading to increased exposure to surroundings of the
343 hospital. These results have important public health implications. Due to higher MDR rates in hospital birds
344 compared to non-hospitals urban birds, in Islamabad wild birds are likely to develop resistance to these antibiotics
345 from the effluents or waste stream from hospitals.

346 This study has several limitations, including the fact that only phenotypic (DDST) typing was performed for
347 complete strain typing, meaning the ESBL gene types (CTX-M, TEM, SHV), plasmid incompatibility groups and
348 clonal lineages were not determined. Plasmid mediated co-transfer did not get directly confirmed by an absence
349 of conjugative transfer experiments. The study should be replicated in the future with plasmid profiling
350 (incompatibility typing, pMLST), WGS of representative co-resistant strains and comparison of the resistance
351 gene flow between the present study and the contemporaneous clinical isolates from hospitals in Islamabad.

352 **5. Conclusion**

353 A high prevalence of ESBL-producing Enterobacteriaceae and exceptionally high co-resistance of ESBL-PMQR
354 of wild bird fecal droppings in urban Islamabad, Pakistan is reported in this study. The major PMQR determinants
355 were *QnrS* and aac (6')-Ib-cr, while the most common species and the most extensive PMQR repertoire was found
356 in *E. coli*. The patterns of multi-gene co-carriage observed, and the species-specific resistance profiles confirmed
357 the co-location of the ESBL gene and the PMQR gene products on conjugative transferable replicons which allows
358 for the horizontal spread of dual-class resistance. Seasonal analysis showed that there were more birds with co-
359 resistance in the autumn/winter season, perhaps because in this season, birds gather to feed at contaminated sites
360 after the monsoon. The findings are in line with other studies which also show the existence of MDR bacteria in
361 urban wild birds in Pakistan and their active participation at the One Health interface. Adding wildlife surveillance
362 to national AMR monitoring systems, enhancing existing hospital effluent monitoring and antibiotic stewardship
363 strategies based on evidence are complementary and imperative for containment of AMR in Pakistan and other
364 LMICs.

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Ethical approval: The study involved the collection of fresh fecal droppings from wild birds and did not involve the capture, handling, or invasive sampling of birds. This study did not involve human and animal participants or clinical specimens; fresh fecal droppings were collected non-invasively from the environment.

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Author contributions:

Sumair Khan: Conceptualization, Methodology, Investigation, Supervision, Sample collection, Laboratory analysis, Molecular characterization, Data curation, Formal analysis, Visualization, writing original draft, and Writing review & editing.

Rida Riaz: Sample collection, Laboratory analysis, and Molecular characterization.

Salmana Ahmed: Statistical analysis and data visualization. All authors reviewed and approved the final manuscript.