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Paper

Antibiotic resistance, virulence genes, and phylogenetic groups of bacteria isolated from wild passerine birds in Iran

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Abstract

Wild passerine birds may serve as environmental reservoirs and as vectors for the long-distance dispersal of microorganisms and resistance determinants. However, there is no much knowledge on pathogenic bacteria in wild birds in Iran. The present study aimed to analyze antibiotic resistance in wild passerine birds collected from the northeast region of Iran as the rich breeding bird fauna with a special focus on *Escherichia coli* virulence, integron, and phylogenetic groups. A total of 326 isolates were collected and identified from the cloaca of wild birds using a swab. The results showed a high percentage of resistance to tetracycline (45.8%) and ampicillin (26.7%). The resistance genes, *tet*(A), *tet*(B), *tet*(M), and *tet*(L) were detected in tetracycline-resistant isolates, while the bla_{TEM} gene was the most prevalent in ampicillin-resistant isolates (38.6%). Out of the 129 *E. coli* isolates examined, 99 isolates were found to have virulence gene, with the highest prevalence of the fimbriae (*fim*H) gene (22.4%). Additionally, the *E. coli* strains were most often classified into phylogenetic groups B1 (48.8%) followed by B2 (19.3%). Also, the highest average frequency of class 1 integron was detected among our isolates. Results indicated that wild birds are reservoirs of multidrug resistance and virulence genes that may have the potential to be transferred to other organisms, including humans.

Keywords

Antibiotic resistance, Bacteria, Virulence genes, Wild passerine birds, Phylogenetic group, Integron

Introduction

Wild birds can serve as reservoirs of pathogens that may have the potential to affect domestic birds, animals, and humans (Franklin et al. 2020, Gargiulo et al. 2018, Jones&James Reynolds 2008). Due to their ability to move across various distances, from local movements to long-distance migrations, across national and intercontinental borders, they can act as dispersers of pathogenic microorganisms on both spatial and temporal scales (Ahlstrom et al. 2021, Altizer et al. 2011, Benskin et al. 2009, Wang et al. 2017). Furthermore, many birds including several common passerine species such as house sparrow, *Passer domesticus*, and barn swallow, *Hirundorustica*, have adapted to farm environments where their proximity to domestic animals may also play a role in human disease (Atterby et al. 2016, Capua&Alexander 2002, Carter et al. 2018, Marzluff 2001, Tsiodras et al. 2008).

Among the Enterobacteriaceae family, *Escherichia coli, Yersinia* spp., *Klebsiella* spp., *Salmonella* spp., and some species of *Enterobacter* genus such as *E. aerogenes* and *E. cloacae* are examples of established and putative pathogenic bacteria (Botti et al. 2013, Davin-Regli 2015, Droual et al. 1997, Fu et al. 2021, Islam et al. 2021, Pasquali

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et al. 2014). These bacteria have been used for antibiotic analysis in previous studies (Fu et al. 2021, Janecko et al. 2018, Kruse et al. 2004, Liao et al. 2019, Sáenz et al. 2004).

Antimicrobial resistance is a significant public health concern and the Enterobacteriaceae family has experienced a notable increase in resistance in recent years greatly due to the extensive use of antibiotics in the treatment of humans, animals, and agriculture. This has, lead to the choice and universal spread of resistant profiles (Ojer-Usoz et al. 2017, Organization 2014, Roca et al. 2015, Shaikh et al. 2015).

Antimicrobial resistance genes may transfer among various organisms through various pathways. Bacteria with antibiotic resistance may disseminate into the environment through contact between wild birds and farm animals, eventually reaching animals that have not previously been exposed to antibiotic-resistant bacteria (Marinho et al. 2013, Poirel et al. 2012). Therefore, a comprehensive study is required to find out the emergence and dissemination of antibiotic resistance genes across different sectors.

Wild birds as biological indicators of environmental pollution have an important role in widespread dissemination and changing the number of antibiotic resistance genes in humans and livestock due to their migratory lifestyle (Allen et al. 2010, Lin et al. 2020, Wang et al. 2017).

Many studies have investigated the patterns of bacterial transfer from birds to other organisms and the environment (Hird et al. 2015, Zhao et al. 2017). Among the various bacteria, *Escherichia coli* is an important common bacteria found in both birds and humans as part of the intestinal microflora in birds (Rahman et al. 2020). Avian pathogenic *E. coli* (APEC) can cause disease in birds and the presence of the disease is associated with several virulence genes, including *fim* C, *fim* H, and *pap* C (Ievy et al. 2020, Johnson et al. 2008). These virulence factors are found on bacterial chromosome and plasmids and transferred horizontally or vertically between bacteria (Piatti et al. 2008). Horizontal gene transfer is a major route for the transmission of antibiotic resistance genes by bacteria, which is transferred by mobile genetic elements (MGEs) such as integrons, transposons, and plasmids. Integron, as a type of MGE, can integrate multiple drug resistance gene cassettes to confer multiple drug resistance. Integrons play an important role in the spread of drug resistance in bacteria, in particular among gram-negative bacteria (Rowe-Magnus et al. 2002).

Based on phylogenetic groups, *Escherichia coli* is divided into eight phylogenetic groups: A, B1, B2, C, D, E, F, and clade 1 . The majority of ExPEC strains are related to the B2 and D groups whereas most intestinal infection strains belong usually to other groups (Krawczyk et al. 2015, Yair&Gophna 2018).

Most studies of putative zoonotic bacteria in birds have focused on Europe and North America, with fewer studies from elsewhere, including important biodiversity areas in the Middle East and Asia. Iran, with its rich breeding bird fauna and hosting migratory and wintering bird populations emanating from areas of northern Eurasia, serves as a bridge between the Palearctic, Oriental, and Afrotropical regions, making it an important area for avifauna exchanging (Aliabadian et al. 2005).

In light of this, the objective of this study was to investigate the presence of bacteria from the Enterobacteriaceae family, antimicrobial-resistant bacteria, and corresponding resistance genes in isolates obtained from wild birds in Iran, with a particular focus on *E. coli* virulence and phylogenetic groups.

Materials and Methods

The isolation of bacteria

A total of 184 cloacal samples were collected from wild birds from June to September in both 2018 and 2019. All sampled birds were captured using two mist nets from nine farms, six residential areas, and 21 agricultural field locations in northeast Iran. Swabs were put in Amies transport medium (Difco Co., Italy) and kept cool in an icebox until they were transferred to the laboratory. All sampled birds were released back into the wild after sampling.

For bacterial isolation, the swabs were placed in nutrient broth and subsequently incubated at 37°C for 24h. The cultures were then streaked onto MacConkey agar (Difco Co., Italy) and Eosin Methylene Blue (EMB) agar and incubated at 37°C for 18–24h. Target gram-negative colonies were plated on Hekton enteric agar to identify *Salmonella* spp., and *Shigella* spp., colonies. Furthermore, Gram staining and cell morphology were used to confirm pure colonies . Microgen™ kit was performed to identify bacteria using 122 Microgen Identification System (MID-60) software. The bacteria isolates were stored with glycerol at -80°C.

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Antimicrobial susceptibility testing and detection of resistance genes

The antibiotic susceptibility of the isolates was determined using the disk diffusion method following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (Clinical&Institute 2017). The following 15 antibiotics commonly used in human and veterinary medicine, were tested: ampicillin (10 mg), amoxicillin (20 mg), streptomycin (10 mg), cefoxitin (30 mg), cefotaxime (30 mg), ceftazidime (30 mg), gentamicin (10 mg), amikacin (30 mg), nalidixic acid (30 mg), ciprofloxacin (5 mg), trimethoprim/sulfamethoxazole (SXT) (1.25 mg+23.75 mg), imipenem (10 mg), kanamycin, tetracycline (30 mg) and chloramphenicol (30 mg) (Ferraro 2001).

Isolates resistant to one or more antibiotics were chosen for further analysis. All resistant isolates were analyzed by polymerase chain reaction (PCR) to identify the following resistance genes: *tet*(A), *tet*(B), *tet*(M), *tet*(L), *tet*(C), *tet*(D), and *tet*(E) for tetracycline-resistant isolates, and bla_{TEM}, $bla_{SHV}$and bla_{CTX} genes for ampicillin-resistant isolates, as previously recommended (Santos et al. 2013, Sarker et al. 2019).

Virulence genes, phylogenetic groups and integrons

In *E. coli* isolates, we further identified some known virulence genes. DNA extraction was carried out using the boiling method and then, virulence genes, haemolysin (*hly*A), fimbriae (*fim*H), afimbrial adhesion(*afa*), cytotoxicnecrotizing factor type 1 (*cnf*-1), aerolysin (*aer*) and pyelonephritis-associated pili C (*pap* C) were detected by PCR (Allami et al. 2022). The PCR products were assayed using 1% agarose electrophoresis gel.

The *E. coli* strains were tested to determine phylogenetic groups based on the Clermont method. The multiplex PCR method was performed as described before (Clermont et al. 2013, Lin et al. 2020). The PCR products were classified into one of the eight major *E. coli* phylogenetic lineages: A, B1, B2, C, D, E, F, and clade 1 (Nagachinta&Chen 2009). The primers used are shown in the table I. Also, the *E. coli* strains were screened to determine the prevalence of class I, II, and III integron genes (Cocchi et al. 2007, Rehman et al. 2017).

Primer name	Primer sequence	size	PCR programs	m- PCR 25	Reference
Fim h	F:TGCAGAACGAT AAGCCGTGG R:GCAGTCACCTGC CCTCCGGTA	508	1 cycle: 95 °C: 5 min 30 cycle: 94°C: 1 min 62°C: 30s 72°C: 1 min 1 cycle: 72°C: 10 min	9 µl 2x PCR Master MIX Red 1 µl of each primers F& R 2 µl DNA template	(lee et al, 2016)
afa	F:GCTGGGCAGCAA ACTGATAACTCTC R:CATCAAGCTGTT TGTTCGTCCGCCG	750			Sawma_Aouad et al., 2009)
*hly*A	F: AACAAGGATAAG CACTGTTCTGGCT R:ACCATATAAGCG GTCATTCCCGTCA	1177			Sawma_Aouad et al., 2009)
Pap C	F:GTGGCAGTATGA GTAATGACCGTTA	200	1 cycle: 95 °C: 5 min 30 cycle: 94°C: 1 min 60°C: 30s 72°C: 45 min 1 cycl: 72°C: 5 min	9 µl 2x PCR Master MIX Red 1 µl of each primers F& R 2 µl DNA template	(lee et al, 2016)
Cnf 1	F:AAGATGGAGTTT CCTATGCAGGAG R:CATTCAGAGTCC TGCCCTCATTATT	498			Sawma_Aouad et al., 2009)
aer	F:TACCGGATTGTC ATATGCAGACCGT R: AATATCTTCCTC CAGTCCGGAGAAG	602			Sawma_Aouad et al., 2009)
Chu A	F:GACGAACCAACG GTCAGGAT R:TGCCGCCAGTAC CAAAGACA	279	1 cycle: 94 °C: 4 min 30 cycle: 94°C: 30s 55°C: 30s 72°C: 30s 1 cycl: 72°C: 7 min	10 µl 2x PCR Master MIX Red 1 µl of each ChuA and YjaA primers F& R 1.5 µl of each TspE4. C2 primers F& R2 µl DNA template	Miranda-Estrada et al 2017
*yja*A	F: TGAAGTGTCAGG AGACGCTG R: ATGGAGAATGCG TTCCTCAAC	211			Miranda-Estrada et al 2017
TspE4.C2	F: GAGTAATGTCGG GGCATTCA R: CGCGCCAACAAA GTATTACG	152			Miranda-Estrada et al 2017

Table I. Primer pairs used.

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Results

Bacteria isolation

Bacteria were isolated from 171 out of the 184 sampled passerine birds tested (92.9%). *E. coli* was the most abundant isolated bacterium, isolated from 129 birds, followed by the *Enterobacter* genus, which was found in 99 sampled birds. The prevalence of other isolated bacterial species was as follows: 52 (16.3%)*Serratia*spp., 16 (8.6%) *Hafnia* spp., 15 (5.9%) *Klebsiella* spp., 6 (3.2%) *Salmonella* spp., and 6 (3.2%) *Proteus* spp., (Table II).

Bacteria isolated	Isolates from each bird family												
	Passeridae	Fringillidae	Hirundinidae	Motacillidae	Muscicapidae	Corvidae	Alaudidae	Emberizidae	Paridae	Sturnidae	Sylviidae	Turdidae	Total
	n=63 (%)	n=12 (%)	n=2 (%)	n=13 (%)	n=7 (%)	n=3 (%)	n=3 (%)	n=2 (%)	n=13 (%)	n=7 (%)	n=53 (%)	n=6 (%)	n=184 (%)
	47 (74.6)	5 (41.6)	0 (0)	9 (69.2)	3 (42.8)	3 (100)	1 (33.3)	0 (0)	5 (38.4)	4 (57.1)	48 (90.5)	5 (83.3)	129 (70.1)
Escherichia coli	43 (68.2)	3 (8.3)	2 (66.6)	3 (0)	3 (28.5)	1 (50)	1 (33.3)	2 (0)	5 (7.6)	7 (14.2)	25 (20.7)	4(33.3)	99 (21.7)
Enterobacter spp.	5 (7.9)	3 (25.0)	1 (33.3)	1 (7.6)	0 (0)	2 (100)	1 (33.3)	0 (0)	0 (0)	0 (0)	2 (3.7)	1 (16.6)	16 (8.6)
Hafnia alvei	18 (17.4)	7 (25.0)	2 (0)	6 (46.1)	1 (0)	0 (0)	1 (0)	1 (0)	3 (23.0)	3 (28.5)	10 (9.4)	0 (0)	52 (16.3)
Serratia spp	3 (4.7)	1 (8.3)	0 (0)	0 (0)	0 (0)	1 (50)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (16.6)	6 (3.2)
Salmonella typhimurium	6 (6.3)	0 (0)	0 (0)	2 (7.6)	1 (14.2)	1 (50)	0 (0)	1 (50)	0 (0)	1 (14.2)	3 (3.7)	0 (0)	15 (5.9)
Klebsiella spp	1 (1.5)	1 (8.3)	0 (0)	2 (15.3)	0 (0)	1 (50)	0 (0)	0 (0)	0 (0)	1 (14.2)	0 (0)	0 (0)	6 (3.2)
Proteus spp	63 (100)	10 (83.3)	2 (66.6)	13 (100)	5 (71.4)	3 (100)	2 (66.6)	2 (100)	11 (84.6)	7 (100)	48 (90.5)	6 (100)	171 (92.9)
Enterobacteriaceae													

Table II. Prevalence of Enterobacteriaceae in wild passerine birds in the north-east of Iran.

Antimicrobial resistance

The results of the observed resistance phenotype are summarized in Table III. Generally, resistance to tetracycline was the most prevalent (45.8%) among all bacterial genera, followed by resistance to ampicillin (26.7%). All isolates were susceptible to cefotaxime, imipenem, and cefixime (Table III). Overall, multidrug resistance (MDR) to three or more antibiotics was observed in *E. coli, Enterobacter* spp., *Serratia* spp., and *Klebsiella* spp., isolates with 34%, 20%, 8%, and 2%, respectively. None of the *Hafnia* spp., S*almonella* spp., and *Proteus* spp., isolates were multiple drug resistant. The presence of specific antibiotic-resistance genes was assayed by PCR. The rate of antibiotic resistance genes in all isolates is shown in Table IV. Among tetracycline-resistant isolates, the presence of the *tet*(B) was detected in 56 isolates, *tet*(A) gene in 37 isolates, *tet*(M) gene in 14 isolates, and *tet*(L) gene in two isolates. Combinations of two tetracycline genes were detected in 20 isolates. The highest percentage of tetracycline resistance genes was detected in *E. coli* isolates (97.7 %) (Table IV).

Antimicrobial agent	Number and percentage of antimicrobial resistance distributed by isolates							
	E, coli n=129 (%)	*Enterobacter* n=99 (%)	*Hafnia* n=16 (%)	*Serratia* n=52 (%)	*Salmonella* n=6 (%)	*Klebsiella* n=15 (%)	*Proteus* n=6 (%)	Total n=329 (%)
Amikacin	0 (0)	0 (0)	1 (6.2)	5 (9.6)	0 (0)	1 (6.6)	0 (0)	7 (2.12)
Amoxicillin	9 (6.9)	2 (2.0)	1 (6.2)	10 (19.2)	0 (0)	3(20.0)	3 (50)	28 (8.5)
Chloramphenicol	2 (1.5)	0 (0)	0 (0)	3 (5.7)	0 (0)	0 (0)	0 (0)	5 (1.5)
Cefotaxime	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Streptomycin	5 (3.8)	0 (0)	0 (0)	4 (7.6)	0 (0)	0 (0)	0 (0)	9 (2.7)
Imipenem	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Cefixime	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Trimethoprim	2 (1.5)	3 (3.0)	1 (6.2)	5 (9.6)	0 (0)	2 (13.3)	0 (0)	13 (3.9)
Nalidixic acid	12 (9.3)	5 (5.0)	3 (18.7)	12 (23.0)	0 (0)	3 (20.0)	1 (16.6)	36 (10.9)
Ampicillin	41 (31.7)	21 (21.2)	3 (18.7)	15 (28.8)	2 (33.0)	4(26.6)	2 (33.3)	88 (26.7)
Ceftazidime	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (6.6)	0 (0)	1(0.3)
Tetracycline	87 (67.4)	30 (30.0)	6 (37.5)	21 (40.3)	1(16.6)	3 (20.0)	3 (50%)	151 (45.8)
Gentamicine	5 (3.8)	0 (0)	1 (6.2)	0 (0)	0 (0)	3 (20.0)	0 (0)	9 (2.7)
Ciprofloxacin	13 (10.07)	10 (10.0)	2 (12.5)	3 (5.7)	0 (0)	0 (0)	0 (0)	28 (8.5)
Kanamycin	2 (1.5)	0 (0)	1 (6.2)	0 (0)	0 (0)	0 (0)	0 (0)	3 (0.9)
Susceptible to all antibiotics	178	71	18	78	3	20	9	377

Table III. Antibiotic resistance in Enterobacteriaceae strains isolated from wild birds from northeast of Iran.

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Antibiotic	Phenotype of resistance	Number of isolates (%)	Resistance genes	Number of isolates (%)
E. coli	Tetracycline	87 (67.4)	*tet*(A)	20 (22.9)
			tet(B)	38 (43.6)
			tet(A) + *tet*(B)	8 (9.1)
			tet(M)	10 (11.4)
			tet(L)	2 (2.2)
			tet(M) + *tet*(L)	7 (8)
	Ampicillin	41 (31.7)	bla_{TEM}	30 (73.1)
			bla_{SHV}	5 (12.1)
Enterobacter	Tetracycline	30 (30.3)	*tet*(A)	9 (30)
			tet(B)	14 (46.6)
			tet(M)	3 (10)
			tet(M) + *tet*(L)	2 (6.6)
	Ampicillin	21 (21.2)	bla_{SHV}	4 (13.3)
			Bla_{TEM}	1 (3.3)
Hafnia	Tetracycline	6 (37.5)	*tet*(B)	3 (50)
	Ampicillin	3 (18.7)	bla_{TEM}	1 (33.3)
Serratia	Tetracycline	21 (40.3)	*tet*(A)	6 (28.5)
	Ampicillin	15 (28.8)	*tet*(A) + *tet*(B)	3 (100)
			tet(M)	1 (6.6)
Salmonella	Tetracycline	1 (16.6)	*tet*(A)	1 (100)
	Ampicillin	2 (33.3)	bla_{TEM}	1 (50)
Klesiella	Tetracycline	3 (20)	*Tet*(B)	1 (33.3)
	Ampicillin	4 (26.6)	bla_{SHV}	1 (25)
Proteus	Tetracycline	3 (50)	*tet*(A)	1 (33.3)
	Ampicillin	2 (13.3)		

Table IV. Resistance genes detected in antibiotic resistant isolates obtained from wild birds in Iran.

The percentage of beta-lactamase genes of 88 ampicillin-resistance strains isolated in the present study were as follows: bla_{TEM}(18.23 %) was observed in 33 isolates, bla_{SHV} (5.52%) gene was detected in 10 isolates, and the bla_{CTX} (0%) gene was not detected in any of the ampicillin-resistant isolates (Table IV).

Virulence factors, phylogenetic groups and integrons genes among E. coli isolates

Among the 129 isolated *E. coli* isolates, 11 different virulence factor profiles were observed, in which, 65.1% carried at least one virulence gene, two isolates carried three different virulence genes, and none of them carried the *aer* gene (Table V).

The virulence factor, *fimH* showed the highest frequency with 22.4% (n=29) among *E. coli* isolates followed by *hylA* gene (6.9%), *afa* (5.4%), *pap C* (6.2%), and *cnf-1* (2.3%). The *aer* virulence factor was not detected in any of 129 the isolates (Table V). Our results showed an association between the presence of the *fimH* gene and strains' resistance to ampicillin and tetracycline. Furthermore, all isolates with combinations *of hylA/fimH* and of *hylA/fimH/afa* genes were resistant to ciprofloxacin and ampicillin, respectively (Table V).

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Pattern codes	Virulence Patterns	Antibiotic resistance										Number of Isolates (%)
		C	AMX	S	SXT	NA	AMP	TE	GM	CP	K	
E1	*fimH*	1	2	1	1	5	21	20	5	9	0	29 (22.4)
E2	*Aer/papeC*	0	1	0	1	0	2	1	0	1	1	2 (1.5)
E3	*HylA/fimH*	0	2	0	2	0	2	3	0	4	0	4 (3.1)
E4	*HylA /fimH /afa*	0	0	0	0	0	2	0	0	0	0	2 (1.5)
E5	*Cnf-1*	0	0	0	0	0	3	1	0	0	1	3 (2.3)
E6	*papC*	0	1	0	0	0	3	7	2	1	0	8 (6.2)
E7	*Cnf-1/ papC*	0	2	0	1	0	6	7	2	0	2	13 (10)
E8	*hylA*	0	0	0	0	2	4	1	3	3	1	9 (6.9)
E9	*afa*	1	0	0	0	0	1	1	2	2	1	7 (5.4)
E10	*Afa/fimH*	0	0	2	1	0	3	4	1	1	0	7 (5.4)
E11	*Aer/cnf-1*	0	0	0	0	2	2	1	1	0	0	4 (3.1)

Table V. Prevalence of virulence patterns among 129 E. coli isolates.

Phylogenetic analysis of the 129 *E. coli* isolates revealed that group B1 was the most prevalent phylogroup (48.8%) followed by group B2 (19.3%), group A (10.8%), group D (5.4%), group E (6.2%), group C (5.4%) and clade I (1.5%). 2.3% of the isolates did not belong to any of the studied phylogenetic groups. Moreover, we found phylogenetic groups in the *E. coli* strains resistant to most of the studied antibiotics, except for chloramphenicol. Our results showed that isolates belonging to group B1 were resistant to tetracycline (Table VI). Regarding integron genes, the prevalence in this study was as follows: class 1, 33% of strains; class 3, 18% of strains; class 2, 7% of strains, and 13% of strains lacked the integron genes. The resistance profile of integron-positive isolates is shown in Table VII. The integron genes were absent in gentamicin, kanamycin, imipenem, and cefixime resistance isolates.

Antibiotic	Phylogenetic group							
	A (n=14)	B1 (n=63)	B2 (n=25)	C (n=7)	D (n=7)	E (n=8)	Clade1 (n=2)	Unknown (n=3)
Chloramphenicol	0	0	0	0	0	0	0	2
Amoxicillin	0	3	2	2	0	1	0	1
Streptomycin	0	0	0	0	0	3	0	2
Trimethoprim	0	1	1	0	0	0	0	0
Nalidixic acid	1	5	0	2	0	2	0	2
Ampicillin	8	21	11	2	3	3	2	0
Tetracycline	2	36	19	0	2	0	1	0
Gentamicin	1	2	0	1	0	1	0	0
Ciprofloxacin	2	4	4	1	1	1	0	0
Kanamycin	0	1	0	0	1	0	0	0

Table VI. Distribution of antibiotic resistance among phylogenetic groups

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Antibiotic	Integron genes		
	INT1	INT2	INT3
Streptomycin	2	1	2
Chloramphenicol	0	0	1
Nalidixicacid	4	0	5
Ciprofloxain(CP)	3	1	3
Amoxicillin	5	1	2
Gentamicine	0	0	0
Kanamycin	0	0	0
Ampicillin	16	7	8
Imepenem(IPM)	0	0	0
Cefixime(CFM)	0	0	0
Tetracycline	23	6	11
Trimethoprim	0	1	0
Total	52	16	32

Table VII. Integron genes detected in antibiotic resistant isolates obtained from wild birds in Iran.

Discussion

In the present study, we isolated putative zoonotic bacteria such as *E. coli, Yersinia* spp., *Klebsiella* spp., and *Salmonella* spp., from apparently healthy wild passerine birds. It indicates that wild passerine bird species may act as serious environmental reservoirs or bridge hosts of potentially pathogenic microorganisms subclinical (Di Francesco et al. 2014, Stenkat et al. 2014).

We also detected a dominance of *E. coli* isolates (71.0%) among the cloacal samples of wild birds. Our results on the high prevalence of *E. coli* isolates align with performed studies in Brazil (63.3%) and Germany (88.5%) (Guenther et al. 2010b, Matias et al. 2016), however, our findings are inconsistent with studies conducted in Italy, which reported lower prevalence rates (0% and 33.9%) (Dotto et al. 2016). These discrepancies in prevalence rates may be due to variations in sampling methods, seasonal distribution, and geographical conditions.

In our study, the low prevalence of *Salmonella* spp., as one of the main causes of gastrointestinal disease is in agreement with an earlier study on wild passerine birds (Botti et al. 2013, Matias et al. 2016). This result may be related to the collection of samples from apparently healthy birds. Nevertheless, some studies have reported high rates of *Salmonella* (20.8%-27.5%) in gulls, which could be linked to feeding habits, such as foraging at dumps and sewage treatment plants (Moré et al. 2017).

Several studies have shown that antibiotic resistance rates in wild birds may affect the antibiotic resistance profile in wild animals (Bonnedahl & Järhult 2014, Dolejska 2020, Gargiulo et al. 2018). Moreover, a correlation has been observed between the level of antimicrobial resistance in wild birds, farm animals, and humans (Skurnik et al. 2006). However, data on the susceptibility of pathogenic bacteria to antimicrobial agents in healthy wild animals is still rather limited (Guenther et al. 2010a, Silva et al. 2010).

In the present study, we detected several antimicrobial resistances in wild birds for the first time in Iran. Our results showed the highest antibiotic resistance rate for tetracycline with 45.8% in *E.coli* isolates, which is in agreement with other studies reporting a high presence of tetracycline resistance in 50.0% - 70.0% of *E. coli* isolates (Nowaczek et al. 2021, Radhouani et al. 2012), however, our results are inconsistent with other similar studies that reported a lower rate

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of antibiotic resistance for tetracycline (Guenther et al. 2010b, Santos et al. 2013, Sigirci et al. 2019). These discrepancies may be due to differences in bird types or geographical distribution.

In the present study, out of 329 isolates, 151 isolates were identified as tetracycline resistant, and 109 isolates carried the studied tetracycline resistance genes. Among the tetracycline resistance genes, *tet*(A) and *tet*(B) are the most commonly reported genes in *E. coli* isolates obtained from birds (Santos et al. 2013, Sigirci et al. 2019, Srinivasan et al. 2008, Vuthy et al. 2017) in addition, the *tet(B*) gene is frequently reported in Enterobactericeae isolates (Sigirci et al. 2019, Srinivasan et al. 2008) that is in line with our findings.

TEM b-lactamase production has been frequently known as the mechanism of ampicillin resistance (Machado et al. 2007, Mendonça et al. 2016, Ngaiganam et al. 2019, Silva et al. 2010, Yassin et al. 2017). In our study, we found that bla_{TEM} at the predominant rate (73.1%) followed by bla_{SHV} (11%) in *E. coli* strains is in agreement with previous studies (Islam *et al.* 2022, Ngaiganam *et al.* 2019).

Our data on the phylogenetic analysis showed the dominance of group B1 among *E. coli* isolates which is in agreement with previous studies analyzing *E. coli* isolates from wild birds (Escobar-Páramo et al. 2006, Kuczkowski et al. 2016, Nowaczek et al. 2021, Staji et al. 2017). Fifteen percent of isolates were assigned to groups C, E, clade 1 and group f was not observed among our isolates that low percentage of these groups were reported previously (Abbasi et al. 2022, Gioia-Di Chiacchio et al. 2016, Staji et al. 2017). Also, other studies have reported that extraintestinal or enteropathogenic *E. coli* isolates belong to groups B2 and D while commensal *E. coli* isolates without any pathogenic features most often belong to other groups (Köhler&Dobrindt 2011, Tivendale et al. 2010).

Virulence genes are essential for the identification of pathogenic microorganisms (Chui et al. 2010). In this study, 68.2% of all *E. coli* isolates were positive for at least one virulence gene. However, the combination of *hylA /fimH /afa* virulence genes was only found in two isolates. Previous studies conducted in Italy and Slovakia have indicated the presence of virulence genes in migratory birds (Bertelloni et al. 2019, Kmet et al. 2013). Considerably, the presence of virulence genes in wild birds in our study has also been reported in wild mammals and humans (Frömmel et al. 2013).

All of the *E. coli* strains resistant to the studied antibiotic were screened for the presence of integrons. Fifty-eight percent of the isolates were positive for integrons, and among them, integrons class 1 was more prevalent, which is in agreement with previous studies (Nebbia et al. 2008, Sacristán et al. 2014) considering that wild passerine birds do not naturally in contact with antibiotics, 58% rate of occurrence integrons among resistant strains is a very high number. Research emphasizing the occurrence of integrons, which have a role in the transfer of resistance genes, potentially provide beneficial data for a deeper understanding of the resistance mechanisms and also for the development of strategies related to antimicrobials.

Although wild birds are not normally exposed to antibiotic drugs, our results showed the presence of antibiotic resistance in wild birds. This can be due to their interaction with farm and urban areas and close contact with domestic animals and humans, so birds may acquire antibiotic resistance from a variety of sources and potentially disseminate them to domestic animals and human, and vice versa (Atterby et al. 2016). This poses a significance problem in countries such as Iran, which is biogeographically located among four avifauna regions, the western-eastern Palearctic, Oriental, and Afrotropical regions, making it an important area for avifauna exchange and hosting a rich breeding bird fauna.

Given the globally increase in antibiotic resistance, especially in underdeveloped countries, the presence of antibiotic resistance in the environment can affect human health. Therefore, accurately predicting the source of emerging resistant bacteria is of utmost important.

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