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Original article

Migratory birds as the vehicle of transmission of multi drug resistant extended spectrum β lactamase producing *Escherichia fergusonii*, an emerging zoonotic pathogen

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ABSTRACT

The acquisition of multi-drug resistance (MDR) genes by pathogenic bacterial bugs and their dispersal to different food webs has become a silent pandemic. The multiplied use of different antibacterial therapeutics during COVID-19 pandemic has accelerated the process among emerging pathogens. Wild migratory birds play an important role in the spread of MDR pathogens and MDR gene flow due to the consumption of contaminated food and water. *Escherichia fergusonii* is an emerging pathogen of family Enterobacteriaceae and commonly causes disease in human and animals. The present study focused on the isolation of *E. fergusonii* from blood, saliva, and intestine of selected migratory birds of the Hazara Division. The sensitivity of isolated strains was assessed against ten different antibiotics. The isolation frequency of *E. fergusonii* was 69%. In blood samples, a high rate of resistance was observed against ceftriaxone (80%) followed by ampicillin (76%) whereas, in oral and intestinal samples, ceftriaxone resistant strains were 56% and 57% while ampicillin resistance was 49% and 52% respectively. The overall ceftriaxone and ampicillin-resistant cases in all three sample sources were 71% and 65% respectively. In comparison to oral and intestinal samples, high numbers of ceftriaxone-resistant strains were isolated from the blood of mallard while ampicillin-resistant strains were observed in blood samples of cattle egrets. 16S rRNA-based confirmed strains of *E. fergusonii* were processed for detection of *CTX-M* and *TEM-1* gene through Polymerase chain reaction (PCR) after DNA extraction. Hundred percent ceftriaxone resistant isolates possessed CTX-M and all ampicillin-resistant strains harbored TEM-1 genes. Amplified products were sequenced by using the Sanger sequencing method and the resulted sequences were checked for similarity in the nucleotide Database through the BLAST program. *TEM-1* gene showed 99% and the *CTX-M* gene showed 98% similar sequences in the Database. The 16S rRNA sequence and nucleotide sequences for TEM-1 and CTX-M genes were submitted to Gene Bank with accession numbers LC521304, LC521306, LC521307 respectively. We posit to combat MDR gene flow among the bacterial

Abbreviations: CIP, ciprofloxacin; CRO, ceftriaxone; AMP, ampicillin; E, erythromycin; S, streptomycin; VA, vancomycin; CN, gentamicin; IPM, imipenem; MEM, meropenem; ATM, aztreonam.

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pathogens across different geographical locations, regular surveillance of new zoonotic pathogens must be conducted.

1. Introduction

Pakistan is located over the transitory Indus, a flyway route for birds to migrate from one area to another and every winter season Pakistan welcomes millions of different migratory birds, including Waterfowls, Cranes, Gulls, and Mallards. Birds migrate due to the harsh winter season of Siberia and Central Asia nest in various wetland territories of different areas in Pakistan (Umar et al., 2018). Wildlife associated directly or indirectly with humans or animals may increase the chances of transmission of microorganisms within the population as a result of migration from diseased regions (Elmberg et al., 2017).

The spread of resistant genes in human microflora is an important risk factor for public health (Mahoney et al., 2021). Domestic or wild birds are susceptible to several bacterial pathogens and can get the infection through resistant microorganisms common to humans and livestock (Rahman et al., 2020). The process of reverse zoonosis or zooanthroponosis is seldom investigated but its evidence has been reported (Adesokan et al., 2019). Human intervention and fragmentation of the natural habitat of wild fauna have accelerated the evolution of resistance genes in microflora. Human and animals are connected through environment considering the Global One Health perspective (Lebov et al., 2017).

Escherichia fergusonii was recognized by Farmer et al. (1985) and the genomic sequence of nonpathogenic strain was accessed in the survey of *E. coli* genomic evolution (Cantón et al., 2012; Bonnedahl and Jarhult, 2014). After the formal classification of *E. coli,* separate species status was assigned to *E. fergusonii* that commonly cause disease in animal and human. Since 1985, several studies were reported on its pathogenicity and describe that it causes Ferguson's disease in human and also show resistance to the extended-spectrum of antibiotics (Farmer et al., 1985; Parin et al., 2018). *E. fergusonii* has been isolated from the wound, urinary tract infection, bacteremia patients, diarrhea, endophthalmitis, and pleuritis in humans (Lindsey et al., 2017; Baek et al., 2019). It has also been isolated from fecal droppings of domestic animal, fresh produce and fish (Gaafar et al., 2015). *E. fergusonii* has been a cause of spleen and liver infection in calf and chicken (Beli and Duraku, 2017), non-human primates (Glover et al., 2017) and cattle pneumonia (Rimoldi and Moeller, 2013). Several investigations demonstrate that wild birds uptake the pathogenic bacteria from wastes and shed them into new locality which results in broad-range outbreaks as well as endemic spreads of multidrug-resistant strains (Sharif et al., 2020; Yuan et al., 2021). The plasmid contains resistant genes that may transfer horizontally to completely different microorganisms and bring structural changes (Dolejska and Papagiannitsis, 2018). The beta-lactamase-producing genes have been acquired by many Gram-negative enteropathogens associated with avian environment (Ngaiganam et al., 2019). It is investigated that wild birds act as a reservoir for pathogens such as *Campylobacter*, *Salmonella*, and toxin-producing *Escherichia species* (Sanches et al., 2017). Interaction between migratory birds and humans may cause zoonotic infection. Seasonal migration of birds has an important role in the epidemiology of human infections and the spread of resistant bacteria (Liakopoulous et al., 2016).

Currently, there is little information regarding the molecular basis of antibiotic resistance in *E. fergusonii* strains isolated from migratory birds from Pakistan, and therefore this study was conducted to assess the prevalence, isolation, and molecular characterization of ESBL producing *Escherichia fergusonii* from wild migratory birds in different wetland habitats of Hazara Division.

2. Materials and methods

2.1. Sample collection

Blood, oral and intestinal samples of migratory birds were collected from different sites of Tarbella Dam (including Bheer, Ghazi, Khalabat) and Siran Valley Mansehra, Pakistan.

2.2. Sample processing

In the period of six months (September 2018 to March 2019), three different types of samples were collected from migratory birds captured in different watery sites of the Hazara division. A total of one hundred and eighty-three samples were collected from different sites of Tarbella Dam. Three samples (oral, intestinal, and fecal) were collected from each bird. Blood samples were taken through sterile syringes and oral and intestinal samples were obtained through sterile swabs. Samples were taken from a watery site and all the samples were collected in dawn time to avoid cross-contamination of birds samples with that of environment. All samples were kept in separate containers to avoid cross-contamination. Samples were kept in a refrigerator at 4 °C until further processing. Biochemical and molecular characterization through PCR was performed in the Microbiology lab of The University of Haripur, Khyber Pakhtunkhwa, Pakistan.

2.3. Enrichment

Samples were enriched in MacConky broth and incubated at 37 °C for 24 h. After incubation, the enriched samples were inoculated on Eosin methylene blue agar and incubated at 37 °C for 24 h. Grown colonies were separately streaked on the EMB Agar medium to obtain a pure culture.

2.4. Identification

Purified bacterial colonies were identified through colony morphology, microscopic examination, and various manual biochemical tests including catalase, oxidase, and Indole. After initial scrutiny of isolates, further confirmation was done through the Biomerieux API20 E kit.

2.5. Antibiotic sensitivity testing

Antibiotic Sensitivity was checked on Muller Hinton agar by using the Kirby-Bauer disc diffusion method. A fresh bacterial colony was spread over the entire Petri plate containing Muller Hinton agar and ten different antibiotics including ciprofloxacin (5 μg), ceftriaxone (30 μg), ampicillin (10 μg), erythromycin (15 μg), streptomycin (10 μg), vancomycin (10 μg), gentamicin (10 μg), imipenem (10 μg), meropenem (10 μg) and aztreonam (30 μg) were placed on the surface of the agar. Plates were incubated for 24 h at a temperature of 37 °C and the zone of inhibition

was measured after incubation. The resistance and susceptibility profile of each isolate was determined as described by European Committee on Antimicrobial Susceptibility Testing (EUCAST).

2.6. DNA extraction

DNA of ceftriaxone and ampicillin-resistant strains were extracted by following these steps by the Phenol-Chloroform Method as described by Ausubel et al., (1994). An overnight 1 mL bacterial suspension was centrifuged for 2 min at 8000 g and supernatant was discarded. Pellet was resuspended after adding 400 μL STE buffer and cells were centrifuged at 8000 g for 2 min. The supernatant was discarded again and 200 μL TE buffer was added to the pellets. 10 0 μL Tris-saturated phenol was added and tubes were vortexed for 1 min. Samples were centrifuged at 13,000 g for 5 min at 4 °C so that the aqueous phase is separated from the organic phase. Then 40 μL TE buffer was added to 160 μL upper aqueous phase and mixed with 100 μL chloroform and centrifuged at 13,000g for 5 min at 4 °C. Again 40 μL TE buffer was added to 160 μL upper aqueous phase and mixed with 100 μL chloroform and centrifuged for 5 min at 13,000 g at 4 °C. 150 μL upper aqueous phase was taken into a clean Eppendorf tube and that contained the required purified DNA.

2.7. PCR amplification of resistant genes

Detection of *CTX-M* and *TEM-1* was done through Polymerase Chain Reaction (PCR) in a thermocycler (Multigene Optimax, USA). Different primers were used for the amplification of each gene (Table 1). Conditions for TEM-1 genes amplification were 94 °C (5 min), final denaturation at 94 °C (45 sec) for 35 cycles, annealing at 52 °C (45 sec), initial extension at 72 °C (1 min), and final extension 72 °C for 10 min.

Conditions of *CTX-M* gene: Initial Denaturation 94 °C (5 min), Final Denaturation 94 °C (45 sec) for 35 cycles, Annealing 54 °C (45 sec), Initial Extensions 72 °C (1 min), Final Extension 72 °C (10 min) and a 4 min hold.

2.7.1. PCR mixture

25 μL PCR mixture contained 3 μL DNA Sample, 1 μL each forward primer and reverse primer, 10 μL master mix and 10 μL distilled water.

2.7.2. Gel electrophoresis of extracted DNA and PCR samples

The mixture of 2 μL loading dye and 3 μL extracted DNA/ PCR product was loaded into the wells of 1% agarose gel stained with 2 μg/mL ethidium bromide with the help of micropipette and was run after setting the voltage at 90 V and 30 A current for 45 min. The gel was placed in a gel documentation system (CSLUVTL312) to visualize the bands of targeted amplified DNA sequences under UV light.

Table 1
Primers used for detection of antibiotic resistant genes.

Primers	Primer sequence	Product size	Reference
TEM-1 gene Forward	5́ TGGGTGCACGAGTGGGTTA 3́	508 bp	Gangoué-Piéboji et al. (2005)
TEM-1 gene Reverse	5́ AATTGTTGCCGGGAAGCTA 3́		
CTX-M gene Forward	5́ACCGCCGATAATTCGCAGAT 3́	588 bp	Kaftandzieva et al., (2011)
CTX-M gene Reverse	5́GATATCGTTGGTGGTGCCATAA 3́		

2.8. 16S RNA sequencing

16sr RNA sequencing was performed for the confirmation of *Escherichia fergusonii*. The presumed Sorbitol non-fermenters and citrate utilizing *E. fergusonii* were selected for 16S RNA sequencing and the rest of the samples were confirmed through biochemical analysis.

2.8.1. Sequencing and analysis

Amplified products were sequenced through Sanger Sequencing Technique and the resulted sequences were analyzed by the BLAST (Basic Local Alignment Search Tool) program to check similarity in the sequence database. The existence of similarity to desired resistant genes confirmed the presence of *CTX-M* and *TEM-1* genes in *Escherichia fergusonii*.

The 16Sr RNA and CTX-M and TEM-1 genes sequences were submitted to the NCBI Gene Bank database for Accession No. LC521304, LC521306, LC521307.

3. Results

A total of one hundred and eighty-three samples of different migratory birds were collected. These birds migrate from Siberia to India, Afghanistan, and Pakistan across Karakoram ranges during the winter season. The route of migratory birds and sample collection sites is shown in the following map (Fig. 1). The seasonal avian species observed during the study duration include Common teal, Northern pintail, Mallard, Eurasian collard dove, Water rail, White wagtail, Common moorhen, Cattle egret, Common pochard, Oriental turtle dove, and Northern shoveler (Table 2).

Out of 183 samples, only 126 (69%) samples were positive for *Escherichia fergusonii* while 57 (31%) samples were positive for *Escherichia coli* isolates. The frequency of positive samples showed that most of the isolates (58%) were obtained from blood samples of avian species (Table 3).

3.1. Morphology and biochemical characterization

In-vitro culture of *Escherichia fergusonii* shows green metallic sheen on Eosin methylene blue agar (Fig. 2a). Isolated strains were examined under a microscope after Gram staining and *E. fergusonii* appeared pink rods. The colony and cell morphology resembled that of *E. coli* (Fig. 2b). Biochemical confirmation shows that *E. fergusonii* is Gram-negative bacilli, catalase-positive (Fig. 2c), and negative for oxidase (Fig. 2d), sorbitol (Fig. 2e), and Indole tests.

3.2. Molecular characterization

Sequencing revealed that most of the isolated strains were *Escherichia fergusonii*. The sequence was submitted to NCBI, Gene Bank with accession No. LC521304 (Fig. 3).

DNA of ceftriaxone and ampicillin-resistant strains was visualized as clear distinct bands on 1% gel (Fig. 6). Ceftriaxone (71%) and ampicillin-resistant (65%) genes were detected in antibiotics resistant strains of ESBL producing *E. fergusonii*. *CTX-M* and *TEM-1* gene amplified products were observed on 1% agarose gel to visualize bands (Fig. 7a & b).

BLAST results show 99% nucleotide sequence similarity for and 98% similarity for the *CTX-M* gene (Fig. 8a) and *TEM-1* gene (Fig. 8b) of *Escherichia coli*. The sequence for *TEM-1*gene was submitted to NCBI, Gene Bank with accession No. LC521307. The alignment was done through Clustal Omega that recognized partial variations on some bases. *E. fergusonii* may be classified

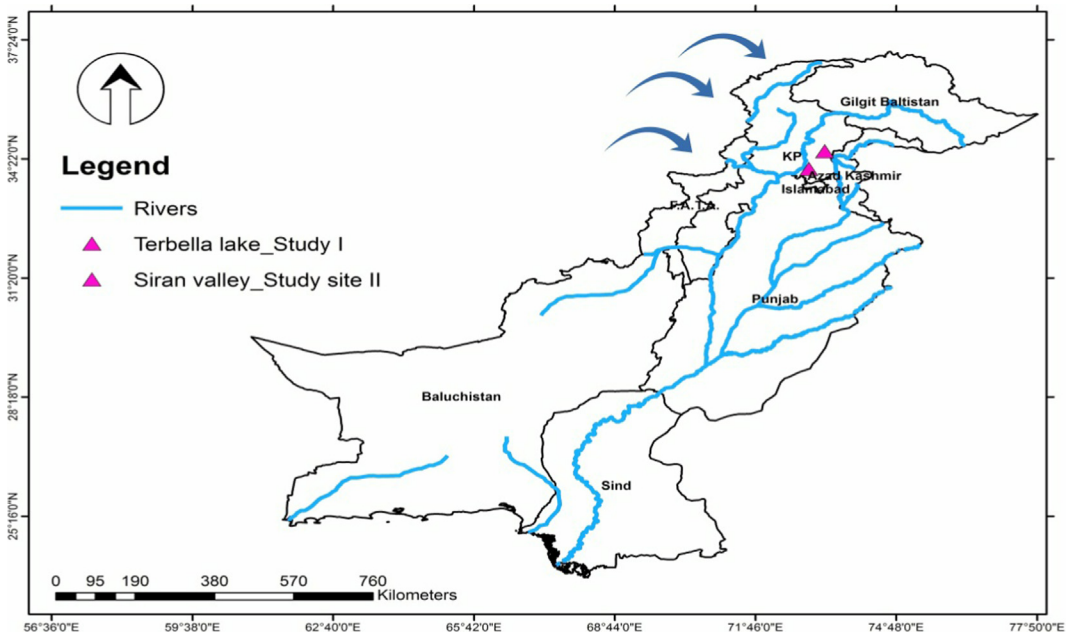

Fig. 1. Study area for sampling of migratory birds to isolate *Escherichia fergusonii.*

Table 2
List of migratory birds observed during field visit.

S.No.	Common Name	Scientific Name	Family Name	Resident status [13]
1.	Common teal	*Anas crecca*	Anatidae	Wintering
2.	Northern pintail	*Anas acuta*	Anatidae	Wintering
4.	Mallard	*Anas platyrhynchos*	Anatidae	Wintering
5.	Eurasian collard dove	*Streptopelia decaocto*	Columbidae	Summer breeder
6.	Water rail	*Rallus aquaticus*	Rillidae	Wintering/passage migrant
7.	White wagtail	*Motacilla alba*	Motacillidae	Wintering
8.	Common moorhen	*Gallinula chloropus*	Rallidae	Year-round resident
9.	Cattle egret	*Bubulcus ibis*	Ardeidae	Year-round resident
10.	Common pochard	*Aythya ferina*	Anatidae	Wintering
11.	Oriental turtle dove	*Streptopelia orientalis*	Columbidae	Wintering/Year-round resident
12.	Northern shoveler	*Anas clypeata*	Anatidae	Wintering

Table 3
Isolation percentage of *Escherichia fergusoni* from migratory birds.

Sample type	Total no. of positive samples	Isolation frequency %
Blood	73	58%
Oral Cavity	30	32%
Intestine/feacal	23	18%

within the genus *Escherichia* based on phenotypic characterization, DNA hybridization, and molecular phylogenetic data. The sequence was submitted to NCBI, Gene Bank with accession No. LC521306.

3.3. Antibiotics sensitivity pattern

Antibiotic sensitivity pattern showed an overall high resistance against ceftriaxone (71%) followed by ampicillin (61%) (Fig. 4). In *E. fergusonii* blood isolates exhibited a high rate of resistance against ceftriaxone (80%) and ampicillin (76%) whereas oral and intestinal isolates showed 56% and 57% resistance against ceftriaxone respectively. Ampicillin resistance was 49% (oral isolates) and 52% (intestinal isolates). The overall data depicts 71% and 65% resistance for ceftriaxone and ampicillin in all three types of sample sources respectively (Fig. 5). High numbers of ceftriaxone-resistant strains were isolated from mallard blood while ampicillin-resistant strains were mostly isolated from the blood of cattle egrets. It was also noted that these isolates were also co resistant to other antibiotics tested (Fig. 5). MEM (meropenem) seemed to be most effective against *E. fergusonii* (Table 4).

Chi-Square test (x^2) was implied through Vassar Stat.net to estimate the significance level between sources of *E. fergusonii* isolates. *Chi-Square* test (x^2) shows significant resistance for all the antibiotics against *E. fergusonii* obtained from blood, oral and intestine sources except vancomycin at $p \leq 0.05$ (Table 4).

4. Discussion

Different migratory birds were observed during the reporting period. Their abundance has been reduced with passage of time due to multiple ecological changes and this finding is parallel with the studies of Umar et al., (2018). One hundred and twenty-six samples from all studied free living birds harbored *E. fergusonii* (69%). This result varies from the findings of previous investigation

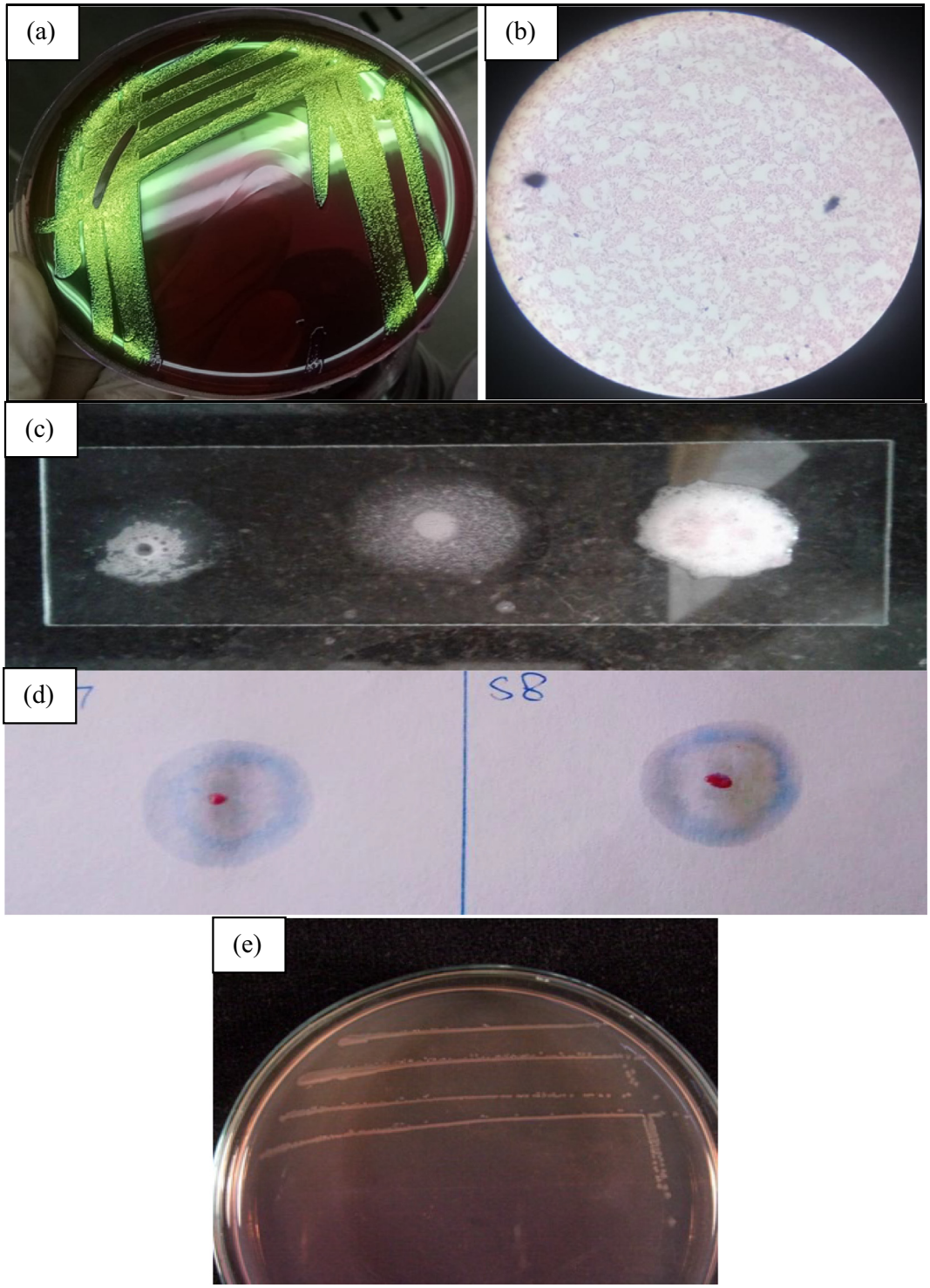

Fig. 2. (a) Growth of *Escherichia fergusonii* on Eosine Methylene Blue Agar; (b) microscopic view of *Escherichia fergusonii*; (c) catalase test for *Escherichia fergusonii*; (d) oxidase test for *Escherichia fergusonii* and (e) growth of *Escherichia fergusonii* on Sorbitol McConkey agar.

in Pakistan related to MDR bacterial pathogens. Antimicrobial resistant *E. coli, Klebsiella pneumoniae* and *Salmonella enterica* serovar Typhi have been detected in few reports (Mohsin et al., 2017; Raza et al., 2017; Sharif et al., 2020). *E. fergusonii* has been proposed as a new species in the *Enterobacteriaceae* family and *Escherichia* genus. *E. fergusonii* is closely allied to *E. coli* based on DNA hybridization. The present study revealed some phenotypic differential features that demarcate it from other species of the Escherichia genus. The current study indicates that *E. fergusonii* is distinct from *E. coli* as it does not ferment lactose, sucrose, raffinose, or sorbitol. Since the discovery of *E. coli* its various phenotypic and genotypic diversity has been observed (Beli and Duraku, 2017). Present results suggest indole negative result of *E. fergusonii* that might have evolved this character as its ancestor bacterium. Moreover,

Sequencing Primer Name Primer Sequences	PCR Primer Name Primer Sequences
785F 5' (GGA TTA GAT ACC CTG GTA) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct.(%)
CU928158.2	Escherichia fergusonii	4588711	481856	483337	0	2697	0.0	1475/1482	99

Kingdom	Family	Genus	Species
Bacteria	Enterobacteriaceae	Escherichia	Escherichia fergusonii

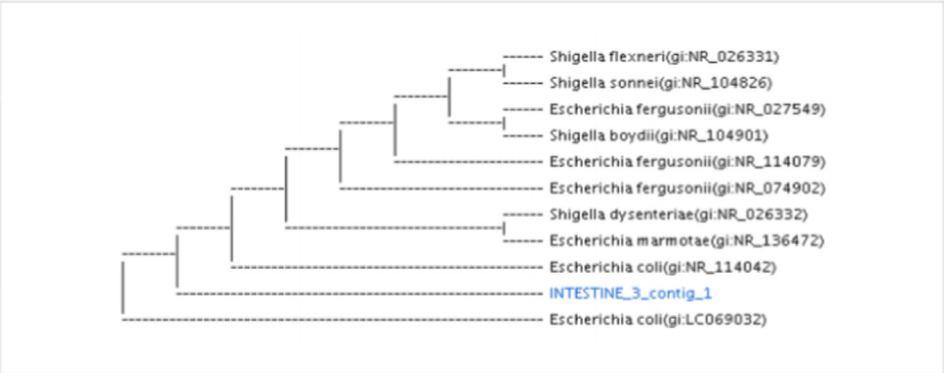

Fig. 3. 16S rRNA sequencing of *Escherichia fergusonii* through BLAST.

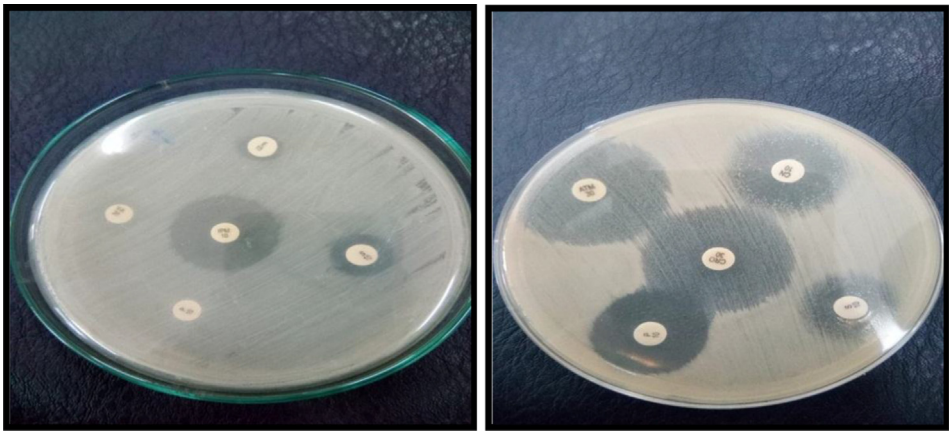

Fig. 4. Antibiotic sensitivity pattern of *Escherichia fergusonii.*

it is an imperative fact that microbial enzyme production depends upon environmental factors, and being an emerging pathogen *E. fergusonii* is yet to be well characterized. Although *E. fergusonii* is detected in specimens such as blood, urine, and feces, its virulence of clinical significance has not been investigated enough (Parin et al., 2018). The food chain and environment may be infested by the colonization of this potential pathogen (Rimoldi, and Moeller, 2013).

E. fergusonii are opportunistic microorganisms in wildlife (Glover et al., 2017), also linked with causing different diseases in chickens (Beli and Duraku, 2017). The antimicrobial extensive use has spilled the MDR genes in natural environment. Humans explore natural resources and shed garbage and human waste in open space. Birds do not take antibiotics directly but human-domestic animals–wild fauna interface contributes to MDRs dissemination. More blood isolates were resistant to different antibiotics as compared to oral and intestinal isolates in present study. This may be attributed to fact that the sampled birds were having some sort of prior infection. CTX and TEM genes were confirmed by polymerase chain reaction (PCR) in our study. Both CTX and TEM genes were verified through BLAST. The sequence data of resistance genes indicated that these genes have a similar sequence to that of *E. coli*. *E. fergusonii* might have acquired these genes from *E. coli.* This outcome is comparable to the findings of Lindsey et al., (2017) and Mohsin et al., (2017). Mohsin et al. (2017) studied wild birds in Southern Punjab and identified

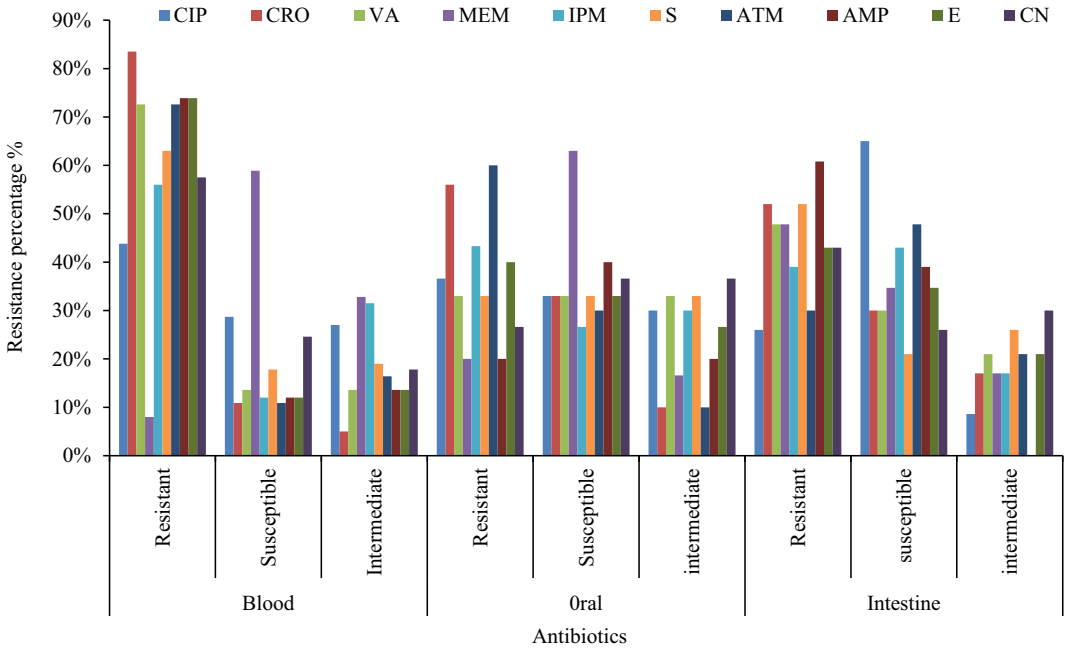

Fig. 5. Antibiotics resistance (%) pattern of *Escherichia fergusonii* obtained from blood, oral cavity and intestine of migratory birds.

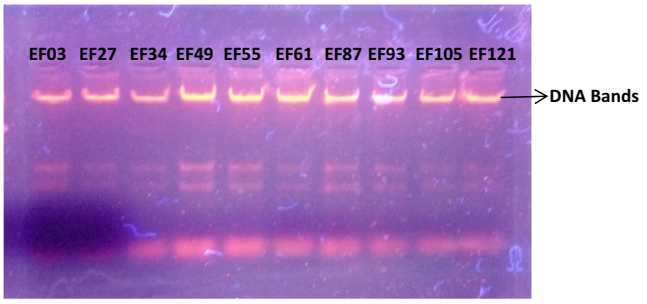

Fig. 6. DNA of *Escherichia fergusonii* isolates obtained from seasonal avian species.

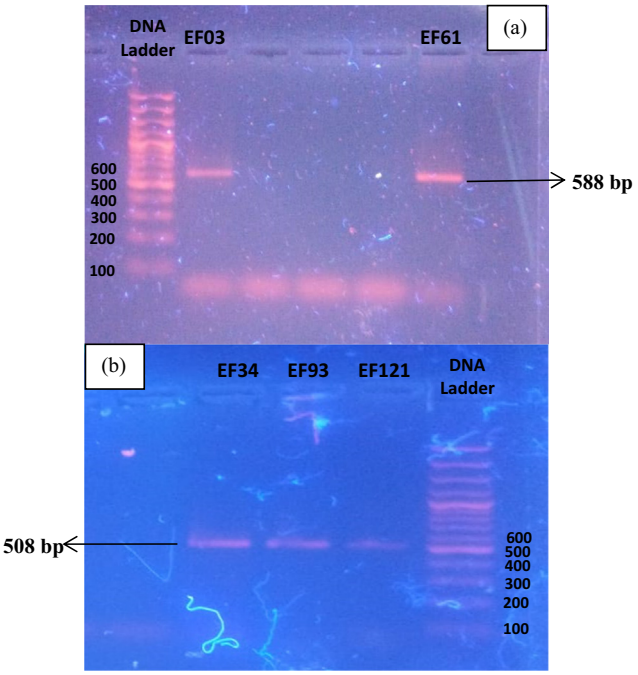

Fig. 7. (a) Amplicons of CTX-M gene observed in isolates EF03 (mallard) and EF61 (Cattle egret) and (b) Amplicons of TEM-1 gene in EF34 (Common teal), EF93 (White rail) and EF 121(White wagtail).

multidrug-resistant *E. coli* in wild birds in Pakistan. High rates of resistance were observed against ampicillin followed by tetracycline. Shobrak and Abo-amer (2014) also identified the resistance mechanism of *E. vulneris* in migratory and non-migratory birds in different provinces of Saudi Arabia. They reported that all isolates of non-migratory birds were resistant to oxacillin, whereas migratory birds isolate showed resistance to oxacillin, chloramphenicol, oxytetracycline and lincomycin (MDR). Our study has parallel findings to that of Shobrak and Abo-amer (2014) and Mohsin et al. (2017). *E. fergusonii* isolates were mostly sensitive to meropenem (MEM) indicating cell wall permeability to MEM (Simmons et al., 2016). Sharif et al., (2020) detected TEM-1 and CTX-M genes in avian isolates of *Salmonella enterica* of seasonal birds. Although wild birds are not using any antibiotics yet a higher rate of antibiotic resistance has been observed in migratory birds. Hernandez et al. (2013) demonstrated the incidence of ESBL-producing *E. coli* among seagulls and humans sharing specific types of gene sequences that indicate transmission. Loncaric et al. (2013) reported that among ESBL-producing isolates blaCTX-M was the most prevalent genes in wild Rooks bacterial isolates in Austria.

The co-existence of both blaCTX-M and blaTEM was detected in the present research. Similarly, Guenther et al. (2012) examined different wild avian species and detected CTX-M together with blaTEM among the *E. coli* isolates. Various prevalence rates of ESBL producing *E. coli* in different birds might be explained by different ecological niches, human influence, and antibiotic exposure. Parallel to our results, Malekian et al. (2021) have confirmed wild birds as reservoir for enteric zoonotic pathogens. The worldwide distribution of CTX-M is explained by the localization of these genes on a plasmid, which facilitates their dissemination within species and among genera.

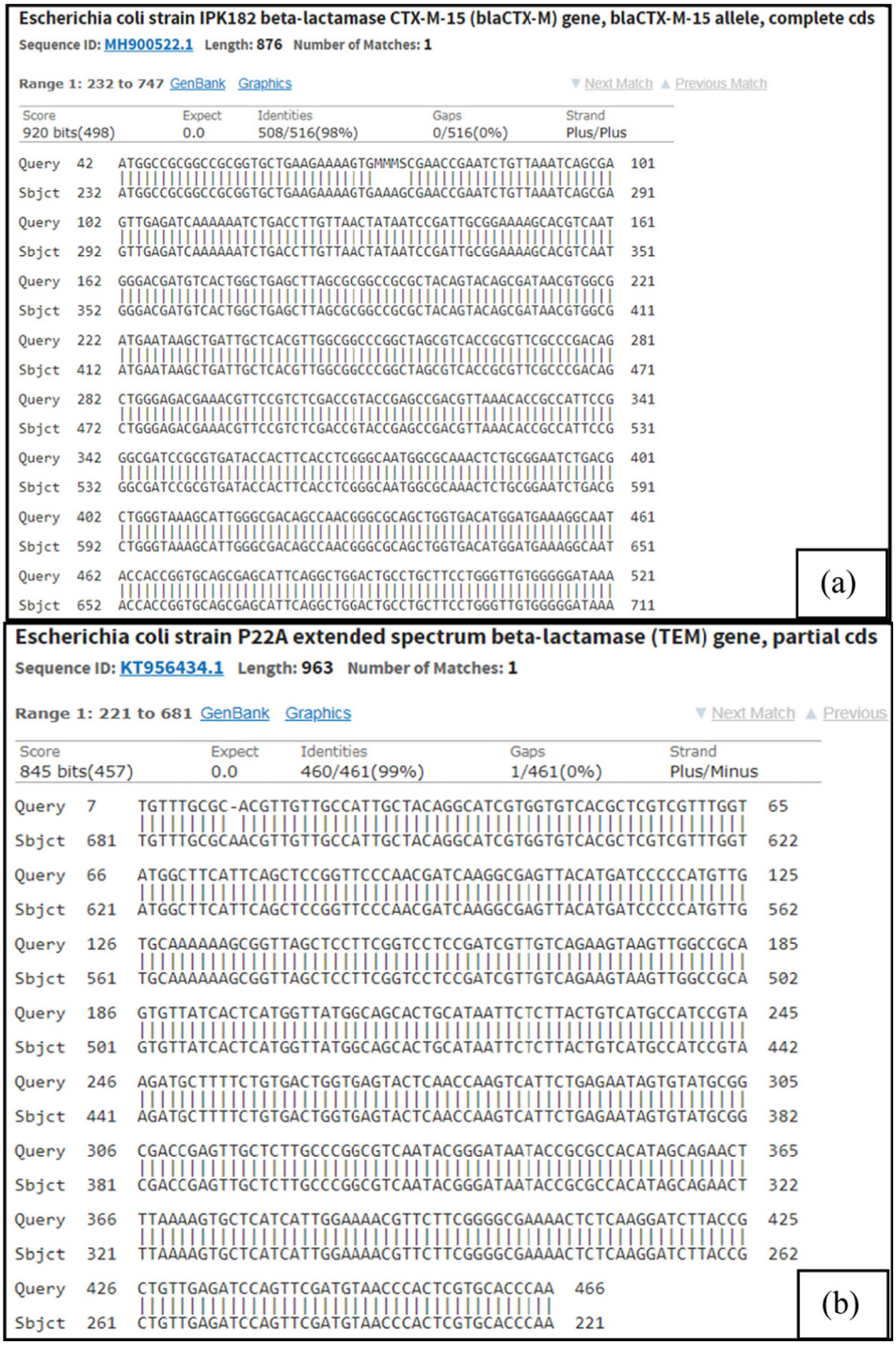

Fig. 8. (a) Confirmation of *CTX-M* gene sequence through BLAST and (b) Confirmation of *TEM-1* gene sequence through BLAST.

According to present knowledge the *E. fergusonii* has been detected from seasonal wild fauna for the first time from the reported region in Pakistan. Ben Yahia et al. (2020) reported the first evidence of ESBL-producing *E. coli* with the blaCTX-M-15 genes in the wildlife of Tunisia and Africa. Yuan et al. (2021) also demonstrated role of migratory birds in disseminating MDR genes. Our study indicates that birds containing pathogenic and antibiotic-resistant genes may disperse them to the new locality and cause infection in other birds, farm animals, and humans. Monitoring studies on birds could be very useful for the early detection of these genes in the environment and are of significant public health concern. Further research regarding whole genome sequencing of this new emerging zoonotic pathogen must be conducted to unveil the virulent traits of *Escherichia fergusonii.*

5. Conclusion

Our study highlights the isolation of Multidrug-resistant *Escherichia fergusonii*, an emerging pathogenic bacterium from different migratory birds of different origins in the Hazara region of Pakistan. CTX-M and TEM-1 genes were harbored by many isolates indicating the genetic pollution of these isolates. This paradigm may lead to the ultimate therapeutic failure of lifesaving drugs.

Table 4
Chi Square test (x^2) for *E. fergusonii* resistance to different antibiotics.

S.No.	Antibiotics	Blood	Oral	Intestine	Chi-square (x^2)	P – Value
1	CIP	31/73 (42%)	11/30 (37%)	6/23 (26%)	9.56	0.0485
2	CRO	58/73 (80%)	17/30 (56%)	13/23 (57%)	23.11	0.0001
3	VA	47/73 (65%)	11/30 (37%)	9/23 (40%)	9.46	0.0506
4	MEM	5/73 (8%)	4/30 (13%)	10/23 (45%)	41.3	0.0001
5	IPM	41/73 (56%)	13/30 (43%)	8/23 (35%)	12.07	0.0168
6	S	44/73 (60%)	8/30 (26%)	9/23 (40%)	9.63	0.0471
7	ATM	45/73 (62%)	14/30 (46%)	7/23 (30%)	17.98	0.0012
8	AMP	55/73 (76%)	15/30 (49%)	12/23 (52%)	21.64	0.0002
9	E	41/73 (56%)	6/30 (20%)	10/23 (43%)	35.15	0.0001
10	CN	41/73 (56%)	6/30 (20%)	10/23 (43%)	11.61	0.0205

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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