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Genotypic and phenotypic characterization of multidrug-resistant avian pathogenic *Escherichia coli* in poultry

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ABSTRACT

Introduction: Globally prevalent Avian Pathogenic *Escherichia coli* (APEC) causes severe systemic disease, colibacillosis, resulting in multimillion-dollar losses in the poultry. Currently, there is a paucity of information regarding the multidrug resistance of APEC isolates.

Aim of Study: In this study, we examined the occurrence rate, serotype variety, and antibiotic resistance patterns of APEC isolates associated with poultry colibacillosis.

Methods: Post-mortem examinations were performed on 230 colibacillosis-infected chickens from April 1st to September 30, 2025. Chi square tests were used to assess associations, with a p-value < 0.05 considered Significant.

Results: This study expressed 73% prevalence rate of colibacilossis, where 078 strain was considered the most prevalent strain. The Antimicrobial Resistance (AMR) profile showed high resistance to augmentin (100%), colistin (100%), and cefoxitin (93.33%), followed by cefepime (68.89%) and polymyxin B (51.11%). All APEC isolates harboured beta-lactamase genes (blaTEM, blaSHV, and blaCMY) (100%), whereas mphA, catA1, and qnrA were detected in 60%, 44.44%, and 4.44% of the isolates, respectively. A Chi-square test of independence identified a significant disparity in *E. coli* prevalence across various sample sources ($\chi^2 = 34.81$, df = 8, $p < 0.001$), highlighting a strong association between *E. coli* distribution and organ type.

Conclusion: The high prevalence rate of colibacilossis in poultry are the alarming condition for poultry sector in Pakistan that needs proper treatment, management, safety precautions, and long term preventions to control the colibacilossis. This study identifies the potential risk of emerging antibiotic resistance in APEC which is a critical and alarming condition for human health.

KEY WORDS

Colibacilossis; APEC; Antibiotic resistance genes; Broiler; Poultry

ARTICLE HISTORY

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Introduction

E. coli is a gram-negative, facultative anaerobic, rod-shaped bacterium that belongs to the family Enterobacteriaceae. It typically measures 2-3 µm in length and approximately 0.6 µm in width and possesses peritrichous flagella that facilitate motility. Biochemically, E. coli ferments lactose, glucose, mannitol, and galactose with the production of acid and gas. It is indole and methyl red positive, reduces nitrate to nitrite, and is negative for urease production and the Voges–Proskauer test [1,2]. Certain pathogenic strains, collectively known as APEC, are responsible for colibacillosis in poultry, causing significant economic losses to the poultry industry worldwide [3].

The poultry sector is one of the fastest-growing agricultural industries globally and represents a major source of animal protein. In Pakistan, commercial poultry farming has expanded substantially since its establishment in the 1960s, contributing significantly to national food security and the economy [4,5]. However, intensive poultry production systems have also increased the prevalence and transmission of infectious diseases, including avian colibacillosis caused by APEC.

Avian colibacillosis is transmitted through respiratory, fecal–oral, and vertical routes and is associated with poor farm hygiene, contaminated feed and water, rodents, litter, and farm equipment [6]. After entering the host, APEC adheres to epithelial cells using adhesins and colonization factors, enabling bacterial survival and dissemination to internal organs such as the liver, lungs, heart, and air sacs, resulting in systemic infection and tissue damage [7]. The pathogenicity of APEC is mediated by several virulence-associated factors, including adhesins, iron acquisition systems, toxins, protectins, secretion systems, and invasins [8].

AMR has emerged as a major global health concern affecting both human and veterinary medicine. The widespread and often uncontrolled use of antibiotics in poultry production has contributed to the emergence of Multidrug-Resistant (MDR) APEC strains [9]. Resistant APEC isolates and their resistance determinants may disseminate through poultry products and environmental contamination, potentially contributing to the spread of AMR genes among bacterial populations [10,11] . Antibiotics remain the primary

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therapeutic option for controlling colibacillosis; however, increasing resistance to commonly used antimicrobials has reduced treatment effectiveness [12,13].

Several resistance genes have been identified in APEC isolates worldwide, including sul1, sul2, tetA, tetB, floR, mcr-1, CTX-M, and OXA-type β-lactamase genes [14,15]. Although previous studies in Pakistan have reported AMR in poultry-associated E. coli, limited data are available regarding the combined characterization of APEC serotypes, antimicrobial susceptibility patterns, and associated resistance genes in poultry farms of Khyber Pakhtunkhwa. Furthermore, regional surveillance data on MDR and Extensively Drug-Resistant (XDR) APEC strains remain scarce.

Therefore, the present study aimed to isolate and identify APEC from poultry affected by colibacillosis in Khyber Pakhtunkhwa, Pakistan, and to characterize their serotypes, AMR profiles, and associated resistance genes. This study provides updated regional epidemiological data and contributes to the understanding of AMR APEC circulating in poultry production systems.

Methods and Materials

Study design and period

The current research study was conducted from April, 2024 to October, 2024. The laboratory testing and characterization of APEC isolates from poultry samples were performed at the Institute of Biotechnology and Genetic Engineering (IBGE), The University of Agriculture, Peshawar, KP, Pakistan.

Sampling site justification and procedure

A total of 230 clinical suspected cases of colibacilossis infected chickens were collected aseptically from veterinary clinics, control sheds, and poultry farms from the liver, intestine, air sacs, heart, lungs, spleen, egg yolk, and kidney of different ages (2 to 5 weeks) during postmortem in district Mardan, KP Province, Pakistan.

Bacteriological isolation and culture conditions

APEC samples were transported through Peptone Buffered Water (PBW) media and immediately transferred to the Microbiology Laboratory of Biotechnology Institute, The University of Agriculture, Peshawar. The samples were then aseptically incubated for 18-24 h at 37°C on Eosin Methylene Blue (EMB) agar (Biolife), MacConkey agar (OXOID), and Xylene Lactose Dextrose (XLD) agar (OXOID) for identification.

Each sample was cultured on duplicate plates to ensure reproducibility and all isolates were confirmed from at least two independent colonies.

Morphological and biochemical identification of APEC isolates

Initial Identification of APEC isolates performed via microscopy according to [1] and biochemical examination include catalase, oxidase test, Motility Indole Urease (MIU) and Triple Sugar Iron (TSI) [2,3].

Serogrouping of APEC isolates

Serotyping of APEC isolates was conducted using polyvalent and monospecific antisera for routinely encountered poultry serogroups (O1, O2, and O78) according to established protocols and the manufacturer's instructions [4]. A microtiter plate agglutination assay was employed for serotype determination.

Molecular identification (16S rDNA ribotyping)

APEC isolates were identified through ribotyping by using the 16S rRNA universal primer in accordance with the manufacturer's instructions. The extraction kit (Quick-DNA Bacterial Miniprep Kit, Cat No D6005, Zymo Research, Irvine, CA, USA) was used to extract the genomic DNA and identified through the partial 16S rRNA sequencing following PCR. ATCC 32518 strain was used as a positive control in the experiment while nuclease free water used as a negative control in each PCR reaction.

Table 1. Primer used for the detection of APEC.

Primers	Primer name	Nucleotide sequence	Amplicon size
16S rRNA gene	16s-F	5'-AAACTCAAATGAATTGACGG-3'	585 bp
	16s-R	3'-ACGGGCGGTGTGTAC-5'	

Antimicrobial Susceptibility Testing (AST)

A total of 12 potent antibiotics was tested against the APEC isolates to determine their resistivity and sensitivity characteristics, following the guidelines of CLSI, 2024. These are the following, Augmentin (AUG, 30 µg), Ciprofloxacin (CIP, 05 µg), Colistin (CT, 10 µg), Tazobactam (TZP, 10 µg), Meropenem (MEM, 10 µg), Polymyxin-B (PB, 300 µg), Chloramphenicol (C, 10 µg), Cefoxitin (FOX, 30 µg), Tigecycline, (TGC, 15 µg), Imipenem (IMI, 10 µg), Sulbactum-Cepheferezone (SCF, 105 µg), Azithromycin (AZM, 15 µg). This was done following the technique described in [5]. The disc diffusion technique was employed to apply these antibiotics on Mueller Hinton Agar (MHA) media (Oxoid, Basingstoke, Hampshire, UK) [6]. MDR was defined as non-susceptibility to at least one antimicrobial agent in three or more distinct antimicrobial classes, according to recent standardized criteria used in AMR studies [7].

Detection of antibiotic resistant genes of APEC by PCR

Genomic DNA was extracted utilizing the Quick-DNA™ Miniprep Plus Kit from Zymo Research, USA, following the guidelines provided by the manufacturer. The concentration and purity of the extracted DNA were measured with a NanoDrop spectrophotometer, and the samples were kept at -80°C for future use.

For 45 APEC isolates, PCR amplification was conducted to target six genes associated with AMR: blaTEM, blaSHV, blaCMY, qnrA, mphA, and catA1, utilizing gene-specific primers as previously described. Each PCR reaction was set up in a total volume of 25 µL, which included 12.5 µL of 2× PCR Master Mix, 1 µL of forward primer (10 pmol), 1 µL of reverse primer (10 pmol), 2 µL of template DNA, and 8.5 µL of nuclease-free water.

Amplification was carried out using a thermal cycler with the following protocol: an initial denaturation step at 95°C for 5 minutes, succeeded by 35 cycles consisting of denaturation at 95°C for 30 seconds, annealing at temperatures specific to each gene (refer to Table 2) for 30 seconds, and an extension at 72°C for 45 seconds, concluding with a final extension at 72°C for 7 minutes. Table 1 provides the primer sequences and the anticipated sizes of the amplicon for the genes targeted.

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The PCR products that were amplified were separated using a 1.5% agarose gel, which was prepared in a 1× TBE buffer and stained with ethidium bromide. The electrophoresis process was conducted at 100 V for about 45 minutes, and the amplified products were then observed under a UV transilluminator.

Table 2. Primers of antibiotic resistance genes used in the PCR.

S. No		Primer		Primer sequence (5'–3')	Amplified product	References
1	Antibiotic Resistance Genes	blaTEM	F	CATTTCCGTGTCGCCCTTATTC	1150 bp	[8]
			R	CGTTCATCCATAGTTGCCTGAC		
2		blaSHV	F	CACTCAAGGATGTATTGTG	885 bp	[9]
			R	TTAGCGTTGCCAGTGCTCG		
3		blaCMY	F R	TGGCCAGAACTGACAGGCAAAT TTCTCCTGAACGTGGCTGGC	462 BP	[10]
4		qnrA	F	ATTTCTCACGCCAGGATTTG	516 bp	[11]
			R	GATCGGCAAAGGTTAGGTCA		
5		mphA	F	GTGAGGAGGAGCTTCGCGAG	403 bp	[12]
			R	TGCCGCAGGACTCGGAGGTC		
6		CatA1	F	AGTTGCTCAATGTACCTATAACC	547 bp	[13]
			R	TTGTAATTCATTAAGCATTCTGCC		

Table 3. Details of different temperature values used in PCR reactions.

Targeted genes	Denaturation (Initial)	Denaturation (Secondary)	Annealing phase	Extension phase	No of cycles	Final extension
blaTEM	94°C/5 min	94°C/30 sec	55°C/40 sec	72°C/45 sec	35 cycles	72°C/12 min
blaSHV	94°C/5 min	94°C/45 sec	50°C/40 sec	72°C/45 sec	35 cycles	72°C/12 min
blaCMY	94°C/10 min	94°C/30 sec	52°C/30 sec	72°C/30 sec	35 cycles	72°C/10 min
qnrA	94°C/5 min	94°C/30 sec	55°C/40 sec	72°C/45 sec	35 cycles	72°C/10 min
mphA	94°C/5 min	94°C/45 sec	60°C/40 sec	72°C/45 sec	35 cycles	72°C/10 min
catA1	94°C/10 min	94°C/30 sec	55°C/30 sec	72°C/30 sec	35 cycles	72°C/10 min

Statistical analysis

The results were analyzed through one-way ANOVA, followed by Tukey's post hoc test, and graphs were plotted using GraphPad 8.5.0 software. All data are presented as mean Standard Deviation (SD). The chi-squar test was used to determine the correlation between bacterial isolates and sample sources. Statistical value (0.05) was set at $p < 0.05$.

Results

Post-mortem examination and isolation of APEC isolates

Post-mortem examination of colibacilossis suspected chickens having characteristic pathological lesions, including enteritis, perihepatitis, cellulitis and Polyserositis, involving abdominal cavity and heart. Total of 230 poultry samples were collected from various internal tissues, including the intestine (92%), trachea (83%), spleen (83%), liver (76%), feces (72%), lungs (67%), kidney (65%), heart (40%), egg yolk (30%) of chickens suspected of colibacillosis (Figure 1 and Figure 2).

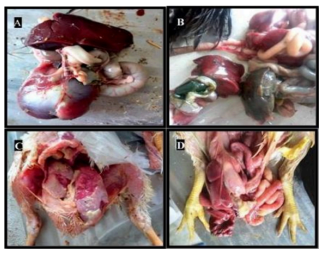

Figure 1. Gross pathological lesions observed in broiler chickens affected with colibacillosis. (A) Enteritis showing intestinal inflammation, (B) Perihepatitis surrounding the liver, (C) Cellulitis involving inflammation of skin and subcutaneous tissues, and (D) Polyserositis affecting the heart and abdominal serosal membranes.

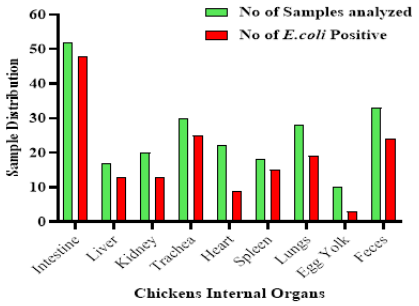

Figure 2. Distribution and prevalence of APEC isolates recovered from different organs and tissue samples of broiler chickens suspected of colibacillosis in Khyber Pakhtunkhwa, Pakistan.

Among the collected samples, 169 isolates were confirmed as APEC, while 61 samples showed no bacterial growth, resulting in an overall prevalence rate of 73% (Figure 3). The highest recovery frequency of APEC was observed in intestinal and tracheal samples.

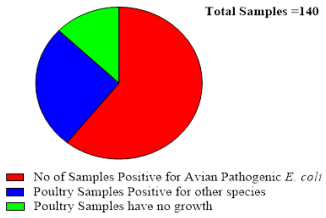

Figure 3. The pie chart illustrates the prevalence of APEC among poultry.

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The isolates produced characteristic metallic green sheen colonies on Eosin Methylene Blue (EMB) agar, pink colonies on MacConkey agar, and yellow colonies on Xylose Lysine Deoxycholate (XLD) agar. Microscopically, the isolates appeared as Gram-negative rods. Biochemical characterization demonstrated that the isolates were catalase-positive, oxidase-negative, lactose-fermenting, gas-producing, urease-negative, indole-positive, and motile in the Motility Indole Urease (MIU) test (Figure 4).

Statistical analysis using the Chi-square test demonstrated a significant association between APEC prevalence and organ type ($\chi^2 = 34.81$, df = 8, $p < 0.001$), indicating that the distribution of APEC varied significantly among sampled tissues (Figure 4).

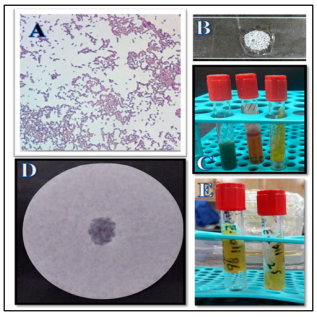

Figure 4. Morphological and biochemical identification of APEC. (A) Gram staining showing gram-negative rods, (B) Catalase test, (C) Motility Indole Urease (MIU) test, (D) oxidase test, and (E) Triple Sugar Iron (TSI) test.

APEC serotyping

A total of 169 confirmed APEC isolates were serotyped using available antisera against O1, O2, and O78 serogroups. The isolates were recovered from broilers, layers, chicks, and breeder flocks. Among the identified serotypes, O78 was the most predominant serotype, detected in 89 of 169 isolates (52.66%), followed by O2 in 47 isolates (27.81%) and O1 in 33 isolates (19.52%) (Figure 5).

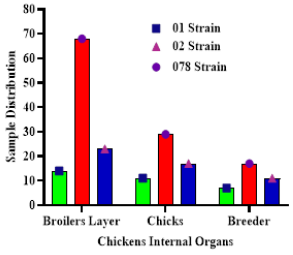

Figure 5: Distribution of APEC serotypes (O1, O2, and O78) isolated from poultry affected with colibacillosis in Khyber Pakhtunkhwa, Pakistan.

The Chi-square test indicated no statistically significant association between APEC serotype distribution and poultry category ($\chi^2 = 4.45$, df = 4, $p = 0.349$).

Antibacterial susceptibility testing

According to the AST results, most isolates were MDR and exhibited resistance to a broad spectrum of antibiotics. Table 4 offers an interpretation of the sensitivity and resistance of the antibiotic profiles, following the CLSI 2021 guidelines. The antibiotics with the highest resistance levels were azithromycin, imipenem, cefoxitin, colistin phosphate, and augmentin, whereas no resistance was found with meropenem, tazobactam, and ciprofloxacin. Additionally, tigecycline (25%), azithromycin (25%), colistin (20%), and polymyxin-B (8.2%) were identified as having the lowest resistance levels (Figure 6).

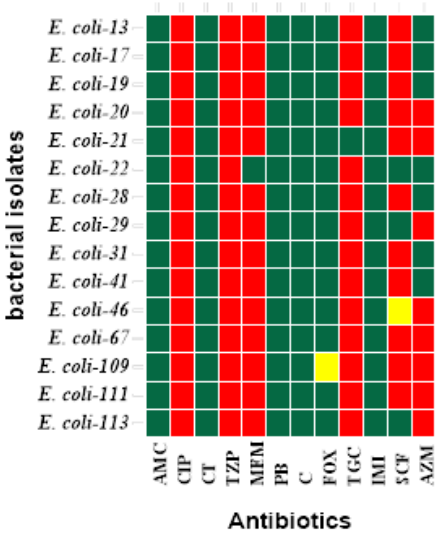

Figure 6. Antibiotic Resistance Pattern of APEC.

According to the disc diffusion method, AMC, CT, FOX, IMI, VA, and FEB showed resistance to APEC isolates, whereas MEM, TZP, CIP, AZM, SCF, and TGC were classified as sensitive. The following are the abbreviations for various antibiotics: Augmentin (AUG), Ciprofloxacin (CIP), Colistin (CT), Tazobactam (TZP), meropenem (MEM), Polymyxin-B (PB), Chloramphenicol (C), Cefoxitin (FOX), Tigecycline (TGC), Imipenem (IMI), Sulbactum-Cepheferezone (SCF), Azithromycin (AZM).

Table 4. Antibiogram pattern of APEC isolates isolated from colibacilossis suspected broiler chickens.

Antibiotic disc	Disc content (ug)	Tested APEC isolates (n=45)					
		Susceptible		Intermediate		Resistant	
		No.	%	No	%	No.	%
Augmentin	30	0	0	0	0	45	100
Ciprofloxacin	15	43	95.56	0	0	2	4.44
Colistin	105	0	0	0	0	45	100
Tazobactam	10	34	75.56	0	0	11	24.44
Meropenem	15	45	100	0	0	0	0
Polymyxin B	30	21	48.89	0	0	23	51.11

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Chloramphenicol	30	34	75.56	0	0	11	24.44
Cefoxitin	30	1	2.22	2	4.44	42	93.33
Tigecycline	10	40	88.89	0	0	5	11.11
Imipenem	110	33	66.67	3	6.67	12	26.67
Sulbactum-Cepheferezone	10	35	77.78	2	4.44	8	17.78
Azithromycin	30	14	31.11	0	0	31	68.89

No: Number of positive cases.

%: was calculated according to the total number of APEC isolates (45).

Ribotyping of 16s rRNA of APEC isolates

Thermocycler PCR was used to amplify 16s rRNA to identify APEC isolates. Gel electrophoresis revealed a 585 bp result, which was verified through sequencing and BLAST analysis with accession number (PV926642). A Gene Ruler 1kb ladder was used in parallel to match the base pairs of the bacterial isolates, as shown in the (Figure 7).

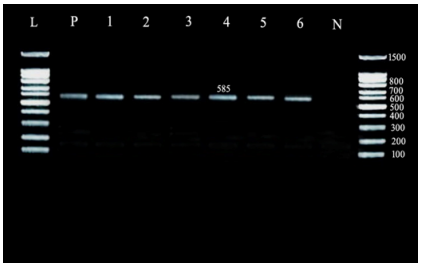

Figure 7. The 16S rRNA gene sequence of APEC was amplified. Lanes 1 through 6 display bands of amplified DNA, each approximately 585 base pairs in length. Lane L contains a DNA Ladder (Gene Ruler 1 kb, Thermo Scientific), while Lane N (Negative Control) and Lane P (Positive Control).

Antimicrobial resistance genes detection by PCR

PCR analysis demonstrated that all tested APEC isolates harbored β-lactamase-associated genes, including blaTEM, blaSHV, and blaCMY (100%), which correlated with the high phenotypic resistance observed against β-lactam antibiotics such as augmentin, cefoxitin, and imipenem. Furthermore, 27 (60%) isolates carried the macrolide resistance gene mphA, consistent with the elevated resistance rate to azithromycin detected during antimicrobial susceptibility testing (Figure 8).

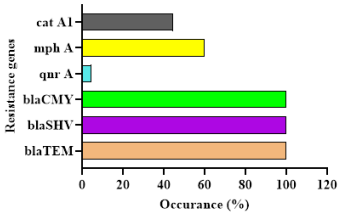

Figure 8. Occurrence of antibiotic resistance genes in the isolated samples from poultry shed.

The quinolone resistance gene qnrA was identified in two isolates (4.44%), which corresponded with the limited phenotypic resistance observed against ciprofloxacin. Similarly, the chloramphenicol resistance gene catA1 was detected in 20 (44.44%) isolates and was associated with phenotypic resistance to chloramphenicol in several isolates. These findings indicate a

The quinolone resistance gene qnrA was identified in two isolates (4.44%), which corresponded with the limited phenotypic resistance observed against ciprofloxacin. Similarly, the chloramphenicol resistance gene catA1 was detected in 20 (44.44%) isolates and was associated with phenotypic resistance to chloramphenicol in several isolates. These findings indicate a substantial correlation between phenotypic AMR patterns and the presence of corresponding resistance determinants identified by PCR analysis (Table 5 and Figure 7).

Statistical analysis further demonstrated a significant association between resistance gene distribution and sample source ($\chi^2 = 95.5$, df = 8, $p < 0.0001$), indicating that the occurrence of AMR genes varied significantly among different organs and sample types.

Table 5. Antibiogram pattern of APEC isolates isolated from colibacilossis suspected broiler chickens.

Isolates name	Resistance phenotype	Resistance genotype				
		Beta-lactamase genes	CIP	AZM	C	TZP
E. coli-13	AMC, CT, PB, C, FOX, AZM, TZP, IMI	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	mphA	catA1	–
E. coli-17	AMC, CT, PB, C, AZM	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	mphA	catA1	–
E. coli-19	AMC, CT, PB, AZM	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	mphA	–	–
E. coli-20	AMC, CT, PB, AZM	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	mphA	–	–
E. coli-21	AMC, CT, PB	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	–	–	–
E. coli-22	AMC, CT, PB, C	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	–	catA1	–
E. coli-28	AMC, CT, PB, AZM	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	mphA	–	–
E. coli-29	AMC, CT, PB, AZM	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	mphA	–	–
E. coli-31	AMC, CT, PB	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	–	–	–
E. coli-41	AMC, CT, PB, C, AZM	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	mphA	catA1	–
E. coli-46	AMC, CT, PB, AZM	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	mphA	–	–

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E. coli-67	AMC, CT, PB	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	–	–	–
E. coli-109	AMC, CT, PB	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	–	–	–
E. coli-111	AMC, CT, PB	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	–	–	–
E. coli-113	AMC, CT, PB, C	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	–	catA1	–
E. coli-122	AMC, CT, PB, C	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	–	catA1	–
E. coli-123	AMC, CT, PB, C, AZM	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	mphA	catA1	–
E. coli-129	AMC, CT, PB, C, AZM	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	mphA	catA1	–
E coli-CHI	AMC, CT, PB, C, AZM	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	mphA	catA1	–
E. coli-133	AMC, CT, PB, C, AZM	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	mphA	catA1	–
E. coli-360	AMC, CT, PB, C, AZM	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	mphA	catA1	–
E. coli-361	AMC, CIP, CT, PB, C, AZM	bla_{TEM}, bla_{SHV}, bla_{CMY}	qnrA	mphA	catA1	–
E. coli-362	AMC, CT, PB, C, AZM	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	mphA	catA1	–
E. coli-363	AMC, CT, PB, C, AZM	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	mphA	catA1	–
E. coli-364	AMC, CT, PB, AZM	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	mphA	–	–
E. coli-365	AMC, CT, PB	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	–	–	–
E. coli-281	AMC, CT, PB, AZM	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	mphA	–	–
E. coli-283	AMC, CT, PB, AZM	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	mphA	–	–
E. coli-288	AMC, CT, PB, C	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	–	catA1	–
E. coli-290	AMC, CT, PB	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	–	–	–
E. coli-294	AMC, CT, PB	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	–	–	–
E. coli-295	AMC, CT, PB, AZM	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	mphA	–	–
E. coli-296	AMC, CT, PB	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	–	–	–
E. coli-297	AMC, CT, PB, AZM	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	mphA	–	–
E. coli-298	AMC, CT, PB, C, AZM	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	mphA	catA1	–
E. coli-299	AMC, CT, PB, C	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	–	catA1	–
E. coli-300	AMC, CT, PB, C	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	–	catA1	–
E. coli-84	AMC, CT, PB, C	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	–	catA1	–
E. coli-F	AMC, CIP, CT, PB, C, AZM	bla_{TEM}, bla_{SHV}, bla_{CMY}	qnrA	mphA	catA1	–
E. coli-HB	AMC, CT, PB, AZM	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	mphA	–	–
E. coli-AN7	AMC, CT, PB, AZM	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	mphA	–	–
E. coli-DY3	AMC, CT, PB, AZM	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	mphA	–	–
E. coli-UJ	AMC, CT, PB, AZM	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	mphA	–	–
E. coli-860	AMC, CT, PB	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	–	–	–
E. coli-DN	AMC, CT, PB	bla_{TEM}, bla_{SHV}, bla_{CMY}	–	–	–	–

+ = Positive detection of the targeted gene by PCR.

- = Negative detection of the targeted gene by PCR.

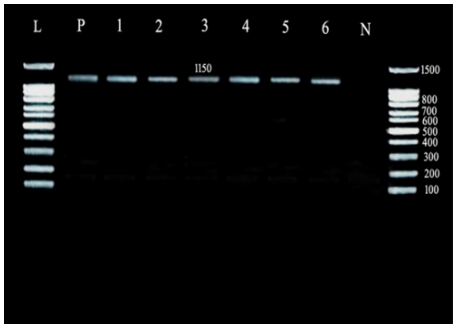

Figure 9: Amplification of 1150 bp primer of blaTEM resistance gene. Lane (N): Negative control. Lane (P): Positive control.

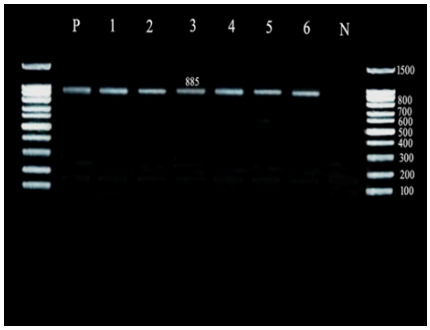

Figure 10: Amplification of 885 bp primer of blaSHV resistance gene. Lane (N): Negative control. Lane (P): Positive control.

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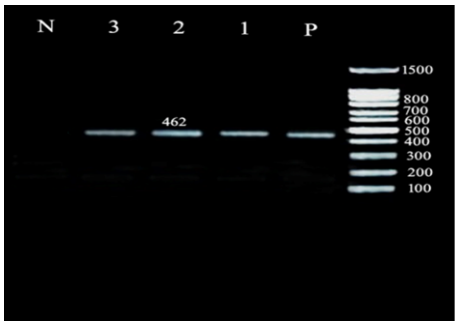

Figure 11: Amplification of 462 bp primer of blaCMY resistance gene. Lane (N): Negative control Lane (P): Positive control.

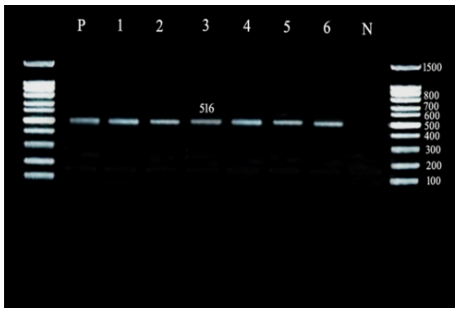

Figure 12: Amplification of 516 bp primer of qnrA resistance gene. Lane (N): Negative control. Lane (P): Positive control.

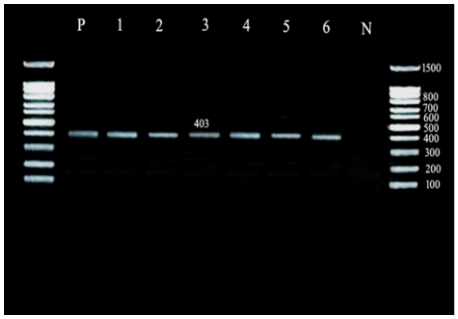

Figure 13. Amplification of 403 bp primer of mphA resistance gene. Lane (N): Negative control. Lane (P): Positive control.

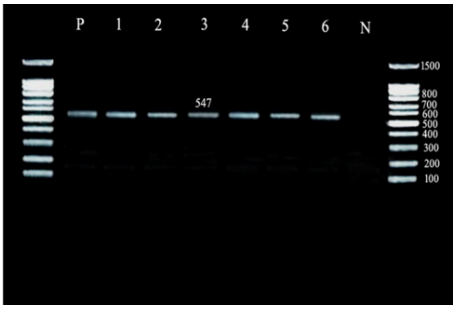

Figure 14. Amplification of 547 bp primer of catA1 resistance gene. Lane (N) Negative control Lane (P): Positive control.

Discussions

APEC remains a major bacterial threat to the global poultry sector, mainly because of its association with colibacillosis, which results in increased mortality rates, reduced productivity, and significant financial losses. This research involved isolating APEC strains from broiler chickens showing colibacillosis symptoms in district Mardan, Khyber Pakhtunkhwa, Pakistan, revealing a high infection rate among the birds studied. Similar infection trends have been noted in various developing countries, where factors like intensive poultry farming, inadequate biosecurity measures, and the indiscriminate use of antimicrobials contribute to the persistent presence and spread of APEC infections [14].

Serotyping analysis indicated that serogroups O78, O2, and O1 were the most common among the isolates, with O78 being the most frequently identified serotype. These results align with earlier studies from Jordan, Egypt, Iran [15,16], and China, where O78 has been recognized as the leading serotype linked to avian colibacillosis [17]. The dominance of these serotypes may be due to their increased virulence, environmental adaptability, and efficient colonization of poultry tissues. The consistent identification of O78 in various geographic areas implies that this serotype plays a significant epidemiological role in *E. coli* infections associated with poultry.

Antimicrobial susceptibility testing revealed that APEC isolates exhibit significant resistance to several widely used antibiotics. The most pronounced resistance was found against cefoxitin, ampicillin, and ciprofloxacin, suggesting that poultry-associated bacteria have been extensively exposed to these drugs [18]. Similar resistance patterns have been documented in research from Jordan, Egypt, Bangladesh, and China, where the overuse and lack of regulation of antibiotics in poultry farming have been associated with the rise of MDR *E. coli* strains [19].

The prevalent MDR found among all isolates in this study is particularly alarming. MDR in APEC greatly restricts treatment options and presents a potential public health threat by facilitating the spread of resistant bacteria or resistance genes through the food supply chain [20]. The significant resistance noted in this research might be linked to the routine use of antibiotics for prevention and growth enhancement in commercial poultry farming. Furthermore, inadequate antimicrobial management and the absence of strict regulations on antibiotic use may further hasten the spread of resistant strains [21].

Molecular analysis identified several key AMR genes, such as blaTEM, blaSHV, blaCMY, qnrA, mphA, and catA1. Notably, blaTEM was the most prevalent, indicating a widespread resistance to beta-lactams among the isolates examined [22-24]. This aligns with previous research from Asian and Middle Eastern regions, where blaTEM is frequently reported as a common beta-lactamase gene in APEC isolates. Conversely, the lower occurrence of blaSHV in this study might suggest regional differences in the distribution of Extended-Spectrum Beta-Lactamase (ESBL) genes [25].

The identification of qnrA signifies the existence of plasmid-mediated resistance to quinolones, potentially leading to decreased effectiveness of fluoroquinolones like ciprofloxacin. Similarly, the detection of mphA and catA1 indicates the spread of resistance factors against macrolides and chloramphenicol-related drugs. The simultaneous presence of various resistance genes in the same isolates underscores the genetic variability and adaptability of APEC strains found in poultry settings [26].

The results of this research highlight the critical necessity for ongoing monitoring of AMR linked to APEC in Pakistan. To reduce the development and dissemination of resistant APEC strains, it is crucial to enforce stringent biosecurity protocols, use antimicrobials judiciously, and implement antimicrobial stewardship initiatives. Additionally, incorporating molecular surveillance of resistance genes into regular poultry disease

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monitoring programs is vital to bolster effective prevention and control measures.

While this study offers valuable epidemiological and molecular insights into APEC in broiler chickens, it is important to recognize certain limitations. The research was confined to a specific geographic area and focused on a limited set of resistance genes. To gain a more thorough understanding of the epidemiology and resistance mechanisms of APEC isolates in Pakistan, future research should include larger sample sizes, multiple regions, virulence gene profiling, and whole-genome sequencing.

Conclusion

This study revealed a high prevalence of MDR APEC in broiler chickens affected with colibacillosis in Khyber Pakhtunkhwa, Pakistan. The detection of ESBL-associated genes, including blaTEM, blaCMY, and blaSHV, highlights the growing threat of AMR in the poultry sector. Serotype O78 was identified as the predominant strain among the isolates. These findings suggest that poultry may act as an important reservoir for the spread of AMR bacteria, emphasizing the need for continuous surveillance, prudent antibiotic use, and strengthened biosecurity measures under a one health framework.

Ethical Approval Statement

No live experimental animals were utilized in this study. Poultry samples were procured from broiler chickens that had either succumbed naturally or were clinically diagnosed with colibacillosis and subsequently submitted by farmers to a poultry clinic for routine diagnostic and post-mortem examination conducted by a licensed veterinary professional. Sample collection for microbiological analysis was conducted post-necropsy as part of standard diagnostic procedures. In accordance with institutional guidelines for diagnostic and post-mortem studies, formal ethical approval was not requisite for this research.

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Disclosure Statement

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Authors' Contribution

Atif Jamal conceived and designed the study. Atif Jamal, Hifsa, Redaina, and Somaira Bibi performed the laboratory experiments and drafted the manuscript, conducted the statistical analysis and data interpretation. Muhsin Jamal supervised the study and critically revised the manuscript. All the authors read and approved the final manuscript.

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