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Original article

Extended spectrum beta lactamases-producing Escherichia coli in retail chicken meat from Khyber Pakhtunkhwa, Pakistan

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ABSTRACT

In human diet, poultry meat is an important component due to the presence of vitamins, proteins and minerals. But poultry meat can be contaminated by pathogenic bacteria which are responsible for food borne infections. The current study was therefore aimed at identification of *Escherichia coli*, a common pathogen causing food borne infections, in chicken samples (n = 400) collected from three districts of KhyberPukhtunkhwa; Peshawar, Kohat and Nowshera. The isolates were identified by Gram staining, API strips and through PCR (Universal Stress Protein). A total of 174 samples were positive for *E. coli* among the collected chicken samples. The isolates were resistant to TE, NOR and NA while were sensitive to MEM, TZP and FOS. The results were statistically significant having value $P \leq 0.05$ in ANOVA. The isolates showed different antibiotic resistance genes; OXA-1, CTX-M15, blaTEM, QnrS, TetA, AAC, AAD, sul1 and sul2 which is the molecular explanations of their antibiotic resistance pattern. The PCR products were sequenced by Next Generation Sequencing (NGS) and the results revealed mutations in AAC gene (M120T and R197T) and CTX-M15 (A85V, N122D, A148S and G247D).

To prevent and treat pathogenic diseases, the use of antimicrobial agents in animal husbandry are of utmost concern. The over-use and misuse of antimicrobial agents has made pathogenic *E. coli* multi drugs resistant making it a causative agent for many diseases in human beings. The results of the current study may be helpful for the physicians the better management of the diseases caused by *E. coli*.

1. Introduction

Worldwide poultry meat is the consumers' first choice due to its high reproductive ability, nutritional value, relatively low sales prices, excellent space utilization and its feeding treatment (Kalakuntla et al., 2017). Currently poultry meat production and consumption is rapidly growing in almost every developing and developed countries of the world (Cooreman-Algoed et al., 2022). The food products made from chicken meat is globally popular among people and is considered better choice for consumers because it can be quickly prepared and can be mixed with a variety of foodstuffs. The modern days consumers in developed and developing countries are dependent on chicken meat products as their usual choice of meal due to their lifestyle. The main advantage of chicken meat over red meat is the presence of low caloric value and little amount of saturated fats. Individuals that suffers from coronary/cardiac diseases can also consume chicken due to its nutritional profile. Chicken meat also contain low level of collagen making it easy for digestion (Marangoni et al., 2015). The chicken meat is also a rich source of different vitamins; niacin (vitamin B3), vitamin B6 and A in comparison to other types of meat and is also cost effective, around the globe (Garg et al., 2017).

Many pathogenic microorganisms like fungi and bacteria are still a major threat and the main competitors to human beings. The diseases caused by pathogenic bacteria like *E. coli* are usually treated by antibiotics and can decreased morbidity and mortality rates. *E. coli* is a common cause of human urinary tract infections and septicemia. *E. coli* is a major pathogen with a wide range of importance in commercially raised poultry, causing significant

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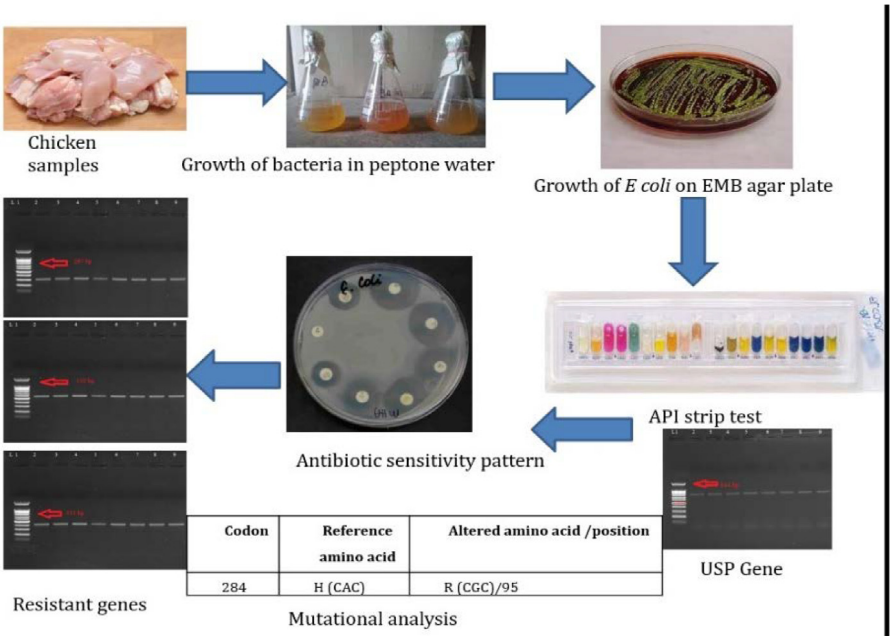
economic losses. *E. coli*, on the other hand, is a highly versatile bacterium that has been used as a model microorganism for detecting antimicrobial resistance (Benameur et al., 2019).

Unfortunately, due to self-medication, misuse or over-use of antibiotics, these pathogenic bacteria are becoming resistant, making antibiotics ineffective (Nakayama et al., 2022). The production of different enzymes, most importantly β-lactamases, which degrade the structure of β-lactams antibiotic (an important class of antibiotics), are one of the major tools conferring antibiotic resistance.

In developed countries many regulations have been established to minimize the risk of antimicrobial resistance in poultry (Cogliani et al., 2011) however, in developing countries the problem is drastically increasing (Dahshan et al., 2015) resulting in major health problems. The current study was therefore aimed to determine the prevalence of *E. coli* in chicken meat and their antibiotic resistance pattern hence giving clues to the physicians for better management and treatment of food borne diseases caused by *E. coli*.



Flowchart of the methods used in the study

2. Materials and methods

2.1. Sample collection and transportation

A total of 400 chicken samples (spleen, liver and meat) were aseptically collected from different poultry shops and farms of district Peshawar, Kohat and Nowshera in sterilized zipper bags for bacteriological analysis. The samples collected in zipper bags were tightly sealed, labelled and were transported to laboratory for bacteriological analysis. No ethical approval was deemed necessary for this study. Verbal permission was obtained from the shopkeeper as well as the slaughterhouse/farms manager before sampling.

2.2. Detection of Escherichia coli

On sterile Eosin Methylene Blue (EMB) agar plates the samples were streaked and incubated at 37 °C for 24 h. Metallic sheen color colony on EMB agar showed positive results for *E. coli* (Anderson et al., 2007).

2.3. Gram staining and biochemical identification of bacterial isolates

The *E. coli* isolates were identified as Gram Negative Rods (GNR) by Gram staining. Analytical Profile Index (API 20E) kit was used to identify the isolates. Pure bacterial culture suspension was inoculated in the wells of the strips followed by incubation at 37 °C for 24 h followed by identification using the codes provided with the API strips and API reading scale (Atieh et al., 2015).

2.4. Extraction of DNA and molecular level identification

For molecular level identification of *E. coli* using specific primer for Universal Stress Protein (USP), the DNA was extracted by Vivantis Genome extraction kit. The extracted DNA was also used for the detection of antibiotic resistance genes in *E. coli* isolates (Elsharawy et al.2022). On 1.5% agarose gel, the amplified PCR product stained with ethidium bromide were run and was visualized with the help of gel documentation system (Cattoir and Nordmann, 2009).

2.5. Antibiotic susceptibility pattern of bacterial isolates

Kirby-Bauer disc diffusion method was used to check the antibiotic susceptibility pattern of the identified *E. coli* isolates using specific antibiotic discs (Table 1). As per Clinical and Laboratory Standard Institute (CLSI) 2019 standards, the results were interpreted as sensitive, resistant and intermediate (Clinical Laboratory Standard Institute, 2019).

2.6. Determination of Minimum inhibitory concentrations

MICs of the selected antibiotics (Table 2) were determined by using the MICs test strips. On sterile MHA agar plate exponential

Table 1
List of antibiotics used in the present study.

S. No	Antibacterial Agent (abbreviations)	Sensitive (mm)	Intermediate (mm)	Resistant (mm)
1	Cefotaxime(CTX)	≥=26	23–25	≤=22
2	Colistin(CT)	≥=2	–	≤=2
3	Amikacin(AK)	≥=17	15–16	≤=14
4	Cefepime(FEP)	≥=25	19–24	≤=18
5	Meropenem(MEM)	≥=23	20–22	≤=19
6	Amoxicillin + Clavulanic acid (AMC)	≥=18	14–17	≤=13
7	Trimethoprim-Sulfamethoxazole(SXT)	≥=16	11–15	≤=10
8	Fosfomycin(FOS)	≥=16	13–15	≤=12
9	Norfloxacin(NOR)	≥=17	13–16	≤=12
10	Gentamicin (CN)	≥=15	13–14	≤=12
11	Cefoperazone/Sulbactam(SCF)	≤ 10	11–15	≥ 16
12	Ceftriaxone(CRO)	≥=23	20–22	≤=19
13	Ciprofloxacin (CIP)	≥=21	16–20	≤=15
14	Nalidixic Acid(NA)	≥=19	14–18	≤=13
15	Piperacillin/Tazobactam(TZP)	≥=21	18–20	≤=17
16	Ceftazidime(CAZ)	≥=21	18–20	≤=17
17	Tetracycline(TE)	≥=15	12–14	≤=11

Table 2
Determination of MICs using different E-test strips.

Antibiotics	Symbols	MIC Strips	Breakpoints		
			S	I	R
Cefotaxime	CTX	E – CT	≤ 1	2	≥ 4
Co-Trimoxazole	SXT	E – TS	≤ 2/38	–	≥ 4/76
Meropenem	MEM	E – MP	≤ 1	2	≥ 4
Ciprofloxacin	CIP	E – CL	≤0.25	0.5	≥ 1
Amikacin	AK	E – AK	≤ 16	–	64
Gentamicin	CN	E – GM	≤ 4	8	≥ 16
Ceftazidime	CAZ	E – TZ	≤ 4	8	≥ 16

Key: S = Sensitive, I = Intermediate, R = Resistant.

Table 3
Sequences of primers, along with optimized conditions, used in the current study (Wen-juanLiang et al., 2018).

Gene	Specific Primers	Product Size (bp)	Annealing Temperature (°C)	Cycles
BLA-TEM 1	**F:** TGCGGTATTATCCCGTGTTG **R:** TCGTCGTTTGGTATGGCTTC	297	55 for 30 sec	35
Qnr-S	**F:** ACGACATTCGTCAACTGCAA **R:** TAAATTGGCACCCTGTAGGC	550	55 for 30 sec	35
TET-A	**F:** GGTTCACTCGAACGACGTCA **R:** CTGTCCGACAAGTTGCATGA	577	58 for 30 sec	35
CTXM 15	**F:** CGATGTGCAGTACCAGTAA **R:** TTAGTGACCAGAATCAGCGG	586	52 for 30 sec	35
OXA 1	**F:** ACACAATACATATCAACTTCGC **R:** AGTGTGTTTAGAATGGTGATC	814	57 for 30 sec	35
AAC	**F:** TTGCGATGCTCTATGAGTGGCTA **R:** CTCGAATGCCTGGCGTGTTT	482	58 for 30 sec	35
SUL 1	**F:** TTCGGCATTCTGAATCTCAC **R:** ATGATCTAACCCTCGGTCTC	822	58 for 30 sec	35
SUL 2	**F:** CCTGTTCGTCCGACACAGA **R:** GAAGCGCAGCCGCAATTCAT	435	58 for 30 sec	35
AAD	**F:** GCAGCGCAATGACATTCTTG **R:** ATCCTCGGCGCGATTTTG	282	58 for 30 sec	35

gradient of antimicrobial agents test strips were placed and incubated at 37 °C for 24hrs and MIC was measured (Kuo et al., 2009).

2.7. Phenotypic analysis of resistant pattern

For phenotypic determination of ESBL producing *E. coli* isolates, synergy test was performed using discs of CRO, AUG and TZP as per reported procedure while phenotypic determination of carbapenemase production was determined by Modified Hodge test (Saito et al., 2015).

2.8. Detection of antibiotic resistant genes

After phenotypic detection, the presence of antibiotic resistant genes (blaOXA-1, blaTEM-1, blaCTX-M-15, AAD, AAC, SUL-1, SUL-2, Qnr-S and TET-A) in *E. coli* isolates was detected with the help of PCR (Applied biosystem thermocycler (A24811) using specific primers (Table 3) under optimized conditions (Sheikheldinet al., 2018) followed by running on 1.5% agarose gel along with 100 bp DNA ladder and visualization by gel documentation system (Bio Rad (Universal Hood II) (Pei et al., 2012).

2.9. DNA sequencing and mutational analysis

The amplified PCR products of antibiotic resistant genes, after purification through Purification Kit (Thermo Scientific™ GeneJET PCR Purification Kit), were sequenced at Rehman Medical Institute (RMI), Peshawar, Pakistan. After sequencing the FASTA sequences of the selected genes were recovered from GenBank–National Center for Biotechnology Information (NCBI) database. Through Basic Local Alignment Search Tool (BLAST) and BioEdit Software the sequence of PCR products were compared with FASTA sequences of the selected genes to confirm its presence in *E. coli* isolates and its mutational analysis (Sacramento et al., 2018). By using I-mutant software the pathogenic effects of the identified mutations were predicted (https://folding.biofold.org/cgi-bin/i-mutant2.0.cgi).

2.10. Statistical analysis

A chi-square analysis was conducted using SPSS version 20 to find the association between expected value of *E. coli* with the observed $p \leq 0.05$. For that, number of sample was (n) set at 150 and the degree of freedom was taken at n-1. For comparative analysis, one way analysis of variance (ANOVA) among the continuous values of antibiotics with *E. coli* was performed respectively and $P \leq 0.05$ values were considered statistically significant.

3. Results

3.1. Isolation of bacterial isolates in chicken samples

Different isolates from the collected chicken samples (spleen, meat and liver), in district Peshawar, Kohat and Nowshera obtained are mentioned in Figs. 1, 2 and 3.

3.2. Identification of E. coli isolates

As *E. coli* was the most common of all isolates, further analysis was focused on it. After identification by Gram staining (pink coloured rods in microscope) and API strips (as per API codes and reading scale), the Universal Stress Protein (USP), amplified by PCR, confirmed the *E. coli* isolates on molecular level (Fig. 4).

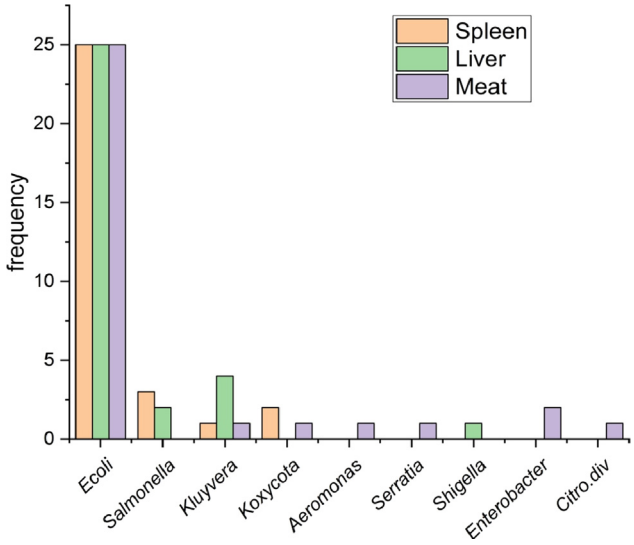


Fig. 1. Different bacterial isolates from district Peshawar.

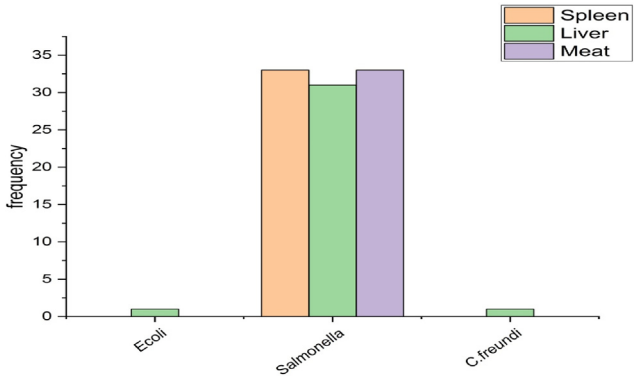


Fig. 2. Different bacterial isolates from district Kohat.

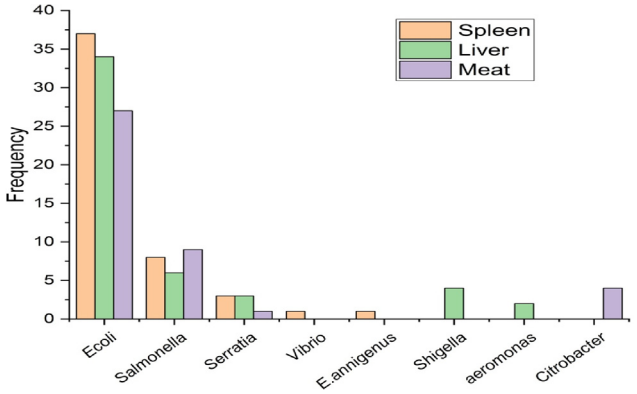


Fig. 3. Different bacterial isolates from district Nowshera.

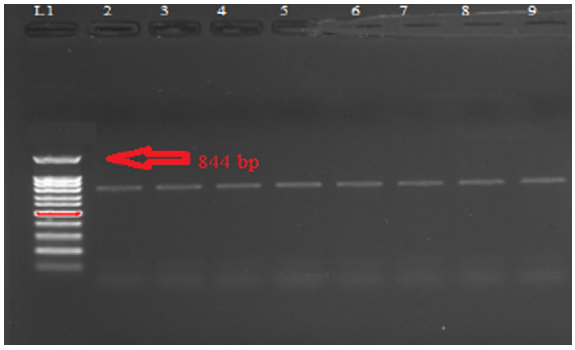


Fig. 4. Gel image of USP (884 bp) for identification of *E. coli*.

3.3. Antibiotic sensitivity pattern of E. coli isolates

The results of antibiotic sensitivity pattern of *E. coli* isolates from different districts are mentioned in Table 4.

3.4. Calculations of Minimum Inhibitory Concentration (MIC)

Antibiotics potency depends on Minimum Inhibitory Concentration (MIC) values the lower the MICs value; the drug will be more powerful while higher the MICs value, the drug will be less potent. The MICs values of β-lactam drugs were high against ESBLs producing *E. coli* isolates showing their resistance but all the isolates were sensitive to MEM as indicted by low MIC value (Table 5).

Table 4
Percentage of antibiotic sensitivity pattern of *E. coli* isolates.

S. No	Abbreviations	Sensitive (%)			Resistant (%)			Intermediate (%)		
District Peshawar										
		S	L	M	S	L	M	S	L	M
1	MEM	46.6	31.2	27.2	10	12.5	18.1	43.3	46.8	45.4
2	CAZ	26.6	18.7	12.1	26.6	31.2	48.4	46.6	46.8	30.3
3	CTX	20	18.7	18.1	53.3	71.8	66.6	26.6	12.5	18.1
4	AK	23.3	9.3	21.2	50	71.8	54.4	23.3	21.8	21.2
5	SCF	33.3	18.7	15.1	33.3	34.3	36.3	33.3	46.8	54.4
6	NA	0	0	0	100	100	100	0	0	0
7	CT	0	0	0	100	100	100	0	0	0
8	TE	0	0	0	100	100	100	0	0	0
9	AMC	0	0	3	93.3	100	93.3	6.6	0	3.3
10	SXT	10	9.3	12.1	86.6	78.1	90.9	0	3.1	0
11	CIP	0	0	3.3	76.6	81.2	75.7	13.3	15.6	15.1
12	CRO	16.6	18.7	6	40	40.6	51.5	16.6	43.7	45.4
13	NOR	3.3	0	0	96.6	100	100	0	0	0
14	TZP	33.3	21.8	18.1	30	31.2	39.3	36.6	46.8	42.4
15	FOS	73.3	53.1	72.7	20	34.3	21.2	6.6	12.5	6
16	FEP	86.6	15.6	9	13.3	34.3	48.4	0	50	51.5
17	CN	6.6	6.2	12.1	93.3	90.6	84.8	0	0	3.1
District Nowshera										
1	MEM	20.5	36.3	33.3	71.7	51.5	57.5	7.6	12.1	9
2	CAZ	12	21.2	12.1	87.8	78.7	87.8	0	0	0
3	CTX	12	15	18	89.7	71.7	81.8	0	0	0
4	AK	0	6	0	100	93.9	100	0	0	0
5	SCF	7.6	3	9	92.3	90.9	90.9	0	0	0
6	NA	0	0	0	100	100	100	0	0	0
7	CT	0	0	0	100	100	100	0	0	0
8	TE	0	0	0	100	100	100	0	0	0
9	AMC	0	0	0	100	100	100	0	0	0
10	SXT	0	0	0	100	100	100	0	0	0
11	CIP	0	0	0	100	100	100	0	0	0
12	CRO	0	12.1	21.2	100	87.8	78.7	0	0	0
13	NOR	0	0	0	100	100	100	0	0	0
14	TZP	30.7	12.1	6	69.2	69.2	93.9	0	6	0
15	FOS	12.1	3	15.1	89.7	96.9	78.7	0	0	6
16	FEP	17.9	12.1	27.2	79.4	81.8	72.7	2.5	6	0
17	CN	0	0	0	100	100	100	0	0	0

Table 5
Minimum Inhibitory Concentration of different antibiotics against ESBL producing *E. coli*.

Antibiotics	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MIC Range (μg/ml)
CTX	64	256	4–256
CAZ	128	256	16–256
MEM	0.19	0.75	0.25–1
CN	4	16	0.38–140
AK	32	256	0.25–256
CIP	16	256	0.38–256
SXT	24	256	1.0–256

3.5. Phenotypic analysis of resistant pattern

In synergy test, the zone of inhibition of corner antibiotics (AUG and TZP) diffused into the center antibiotic (CRO) showing positive result for ESBL production (20–25 mm from corner to center). For the carbapenemase production, the two antibiotics disc (MEM and IPM), after incubation presented leaf like flattening at the center showing positive results for carbapenemase production.

3.6. Detection of antibiotic resistant genes by polymerase chain reaction

The representative images of different antibiotic genes along with their band size are depicted in Fig. 5 and Table 6 is showing the number of antibiotic resistant genes in *E. coli* isolates.

3.7. Sequencing and mutational analysis of antibiotic resistant genes

After sequencing of the antibiotic resistant genes, the data was further analyzed for non-synonymous and synonymous mutations and the predictions of the I-mutant (Tables 7 and 8).

3.8. Statistical analysis

The Chi square test showed a significance level of association between type of bacteria in different districts and hence proved our null hypothesis where $p \leq 0.05$. One way ANOVA test presented a significant association of dependent to independent value.

4. Discussion

The present study showed that the collected chicken samples (meat, spleen and liver) were contaminated as 174 (43.5%) samples showed the growth of *E. coli* out of 400 collected samples. In the present study, *E. coli* showed 100% resistance to NA, TE and NOR while varied resistance was recorded against CRO, AK, CTX, SCF, FEP, CAZ, TZP, FOS and MEM. In a reported study, QNRs gene were mostly found in *E. coli* isolates from chicken making it in line with our results (Lenart Boron et al., 2016). According to National Central Plan of Drug in Portugal, in veterinary the appearance of antibiotic resistance is because of the usage of tetracycline, quinolones and Sulfonamide. In poultry high resistance of the *E. coli* isolates was recorded against ampicillin (69.4%), trimethoprim (66.7%), TE (88.9%) and Sulfonamide (75.0%) (Wang et al.,2012). The *E. coli* iso-

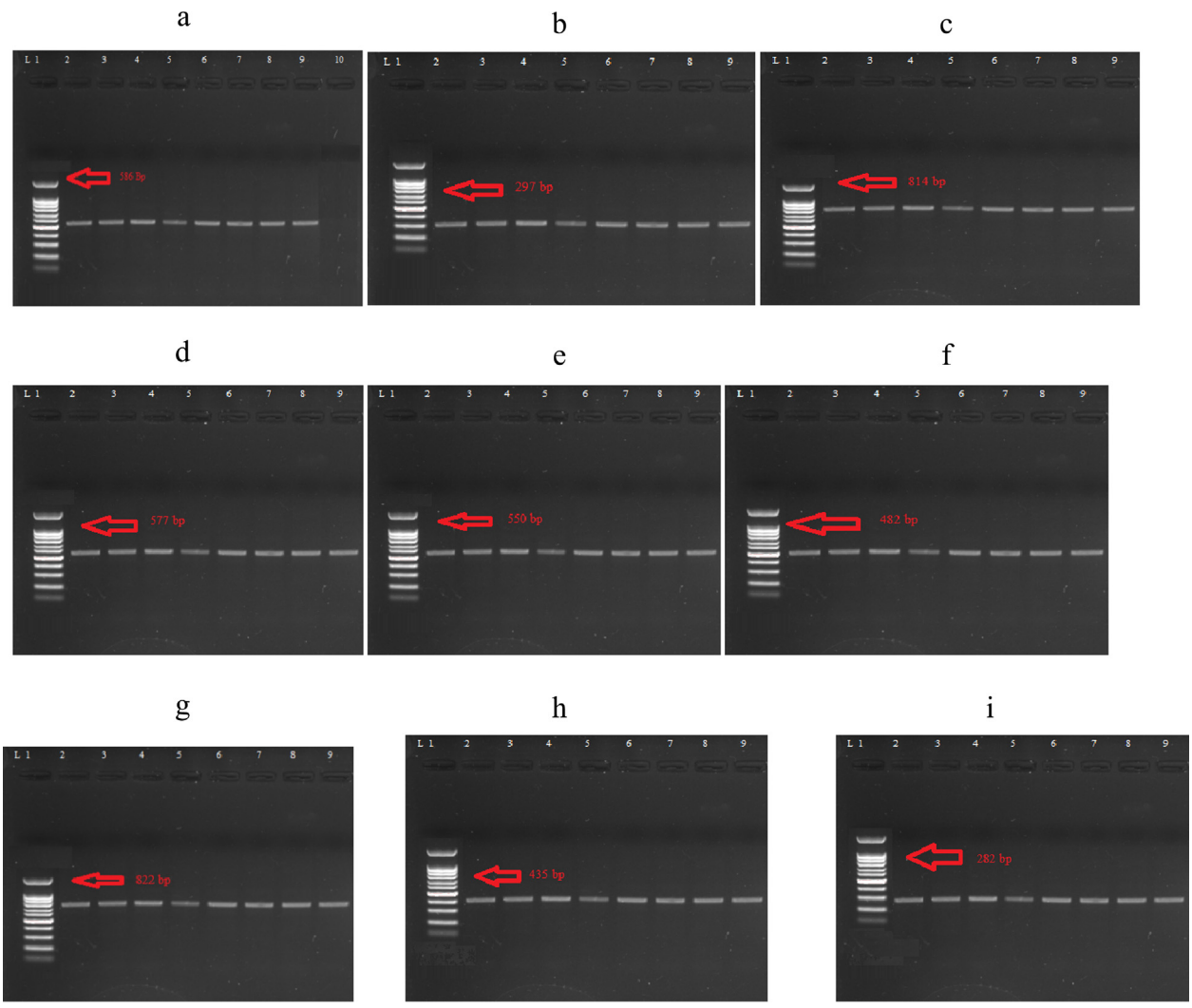


Fig. 5. CTXM 15: 1; DNA Ladder, 2; positive control, 3–9; positive for CTXM-15 (bp = 586), b. TEM 1: 1; DNA Ladder, 2–9; positive for TEM 1 (bp = 297), c. OXA 1**:** 1; DNA Ladder, 2; positive control, 3–9 positive for OXA 1 (bp = 814), d.TET A: 1; DNA Ladder, 2; positive control, 3–9; positive isolates for TET A (bp = 577), e. QNRS: 1; DNA Ladder, 2–9; positive isolate for QNR S (bp = 550), f. AAC(6)-Ib-cr: 1; DNA Ladder, 2–9 positive isolate for AAC(6)-Ib-cr (bp = 482), g. SUL 1: 1; DNA Ladder, 2–9; positive isolate for SUL 1 (bp = 822), h. SUL 2: 1; DNA Ladder, 2–9 positive isolate for SUL 2 (bp = 435), i. aad A1: 1; DNA Ladder, 2–9 positive isolate for aad A1(bp = 282).

Table 6
Number of resistant genes in *E. coli* isolates of selected districts.

Sample No	CTXM 15	TEM 1	OXA 1	TET A	QNRs	SUL 1	SUL 2	AAC	AAD
Peshawar									
S1	+	–	+	+	+	–	–	+	–
L11	+	–	+	+	+	–	+	–	+
M14	+	+	–	+	+	+	+	+	–
S17	+	–	–	+	+	+	+	–	+
L20	+	–	+	+	+	+	+	–	+
M20	+	–	+	+	+	+	+	+	+
S23	+	+	+	+	+	+	+	+	–
L25	–	–	+	+	+	–	–	–	+
M28	–	–	–	+	+	–	–	–	+
S34	–	–	–	+	+	+	–	–	+
L38	+	–	+	+	+	+	+	+	+
M35	+	+	+	+	+	–	–	+	+
S40	+	–	–	+	+	+	+	–	–
L42	+	–	–	–	–	+	–	–	+
M43	+	–	–	+	–	–	+	+	–
Kohat									
21L	+	–	+	+	+	+	+	+	+

Table 6 (*continued*)

Sample No	CTXM 15	TEM 1	OXA 1	TET A	QNRs	SUL 1	SUL 2	AAC	AAD
Nowshera									
3L	+	+	+	+	+	–	+	+	+
6S	+	+	+	+	+	–	+	–	+
7M	+	–	+	+	+	+	–	–	+
14S	–	+	–	+	+	–	–	+	+
15M	–	+	–	+	+	–	+	+	–
20S	–	–	–	+	+	+	–	+	–
24L	+	+	–	+	+	+	+	+	–
28M	+	–	–	+	+	–	–	+	–
30L	+	+	+	+	+	+	+	–	+
33M	+	+	+	+	+	+	+	–	+
37L	–	+	+	+	+	–	+	–	+
38S	–	–	–	+	+	+	–	+	–
42L	+	+	–	+	+	+	–	+	–
44S	+	+	–	+	+	+	+	+	–
50M	+	–	+	+	+	–	+	+	–

lates from chicken in China, showed resistance against ampicillin (98.9%) and TE (97.6%) (Grave et al., 2010). In many countries like Brazil, India, Canada and China the resistance of *E. coli* to various antibiotics in the poultry meat are increasing gradually (Overdevest et al., 2011) and all these are in line with the present study. In Pakistan, 200 samples from poultry chicken were obtained and analyzed for ESBL production. Results showed that 87% bacterial isolates were *E. coli* having blaOXA-48, blaNDM, blaCTXM and blaSHV2 as the most reported genes (Ali et al., 2021) making it in line with our results.

Table 7
Synonymous and non-synonymous mutations.

Synonymous mutation of *AAC* gene			
Nucleotide position	Reference - altered amino acid	Amino acid	Amino acid position
400	CCT – CCA	P	134
405	GTC – GTT	V	135
Non synonymous mutation of *AAC* gene			
Codon position	Reference amino acid (Sequence)	Altered amino acid (Sequence)	Altered Amino acid Position
359	M (ATG)	T (ACG)	120
590	R (AGA)	T (ATA)	197
Non synonymous mutation of *CTX-M15*			
Codon position	Reference amino acid	Altered amino acid	Altered Amino acid Position
254	A (GCG)	V(GTG)	85
364	N (AAT)	D (GAT)	122
442	A (GCT)	S (TCT)	148
740	G (GGC)	D (GAC)	247
Synonymous mutation of *CTX-M15*			
Nucleotide position	Reference - altered amino acid	Amino acid	Amino acid position
328	CTT – TTG	L	110
597	CGG – CGT	R	199
624	GGC – GGT	G	208

5. Conclusion

The appearance of MDR *E. coli* in poultry is due to the over and misuse of antimicrobials and has severely affected the public health in the form of various diseases. The presence of antibiotic resistant genes, especially to the first line antibiotic, is a serious concern for the health authorities and for the provision of data regarding antimicrobial use, in poultry production a longitudinal monitoring program should be implemented. In poultry farms, good clean practices and poultry litter treatment may decrease MDR prevalence and can optimize poultry production and human health conservation. The present study was therefore part of the effort to identify the most common disease causing bacteria, in chicken meat and their antibiotic resistance pattern hence giving clues to the physicians for better management and treatment of food borne diseases caused by *E. coli*.

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.

Table 8
I-Mutant software prediction result.

Wild type	New type	I-Mutant prediction effect	Reliability Index (RI)	pH	Temperature (°C)
		AAC gene			
Cysteine	Threonine	Increase	4	7	25
Arginine	Threonine	Increase	1	7	25
		CTX M 15 gene			
Cysteine	Valine	Increase	1	7	25
Alanine	Aspartic acid	Decrease	3	7	25
Glycine	Serine	Decrease	2	7	25
Glycine	Aspartic acid	Decrease	3	7	25

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