



Characterization and Antimicrobial Resistance of *Escherichia coli* and Its Serotypes Isolated from Poultry Feed in Relation to Seasons in Karachi, Pakistan

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Abstract

Background: The increasing prevalence of *Escherichia coli* as a foodborne pathogen in poultry poses a high risk to food safety. The resistant strains of *E. coli* may contribute resistant genes to human endogenous flora, causing fatal diseases. Moreover, these pathogens are serious threats to poultry farming.

Objectives: The current study aimed to investigate the effects of seasonal variations on the bacterial load of *E. coli* and to evaluate its antibiogram profile.

Methods: All feed samples were evaluated for the identification of *E. coli* and its serotypes, using conventional culture methods and biochemical characterization. Positive samples were confirmed by polymerase chain reaction (PCR) assay. The bacterial load of *E. coli* was estimated by measuring the total viable count, and the antibiogram data were calculated using two methods, that is, disc diffusion method and minimum inhibitory concentration (MIC) measurement.

Results: Of 204 feed samples investigated, 38 isolates were positive for *E. coli*. All positive samples were also confirmed via universal and species-specific PCR assays, and 8/38 were documented as *E. coli* O157:H7 strains. The bacterial load of *E. coli* was also determined by measuring the total viable count, and the results revealed the highest ratio (6.44×10^8 CFU/g) from June to August and the lowest ratio (2.06×10^8 CFU/g) from December to February. The multidrug resistance of *E. coli* O157:H7 was validated by antimicrobial susceptibility tests since all isolates showed high resistance to chloramphenicol, penicillin derivatives, fluoroquinolones, and oxytetracycline, respectively, and were only susceptible to aminoglycosides.

Conclusions: Considering the high bacterial load of *E. coli* from June to August, the poultry industry needs to establish appropriate and effective hygienic and storage policies, especially during these alarming months. Moreover, surfacing and propagation of resistant strains of these pathogens may obscure future assessments for treatment purposes.

Keywords: Poultry Feed, Antibiogram Profile, Polymerase Chain Reaction, Bacterial Load, Foodborne Pathogen

1. Background

Microbial food safety is an increasing public health concern worldwide. The primary cause of foodborne diseases is the presence of highly potent pathogens, such as *Escherichia coli*, in the poultry (1). Concerning its pathogenicity, *E. coli* is classified into six groups, including enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), verotoxin-producing/Shiga toxin-producing *E. coli* (VTEC/STEC, including its well-known subgroup, enterohemorrhagic *E. coli*), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) (2). Of all these groups, the O157:H7 serotype of EHEC strain is of zoonotic importance (3, 4).

Poultry is the natural reservoir of *E. coli* O157:H7 serotype, causing hemolytic uremic syndrome and hemorrhagic colitis in humans (5). In the United States, 265,000 diseases due to STEC are recorded annually, 36% of which are caused by the O157:H7 strain (6). Moreover, various poultry diseases are attributed to this strain, including avian colibacillosis, yolk sac infection, and meningitis, leading to heavy economic losses (7). Seasonal fluctuations also have tremendous effects on microbial intensification, and rainy seasons are the most alarming period (8).

Another serious problem regarding the treatment of *E. coli* O157:H7 is its multiple drug resistance (MDR) due to the irrational use of antibiotics. In veterinary medicine, antibi-

otics are not only used for treatment and prophylaxis purposes but also as growth promoters (9). Based on the mentioned findings, isolation and determination of the antibiotic resistance of *E. coli* and its Shiga toxin strain (O157:H7) play an important role in the control of their dissemination.

2. Objectives

This study aimed (1) to isolate potentially dangerous bacteria, such as *E. coli*, from the poultry feed in Karachi Province, Pakistan, which has never been done before; (2) to assess the effects of seasonal changes on the growth rate of these pathogens; and (3) to determine MDR by using antibiotics.

3. Methods

3.1. Sample Collection

From 2015 to 2016, a total of 204 feed samples were randomly collected. Seventeen poultry farms were selected randomly in Karachi Province for microbiological evaluation of feed samples. All samples were transferred in sterile labeled bags to the Department of Microbiology, University of Karachi, Pakistan.

3.2. Characterization of Isolates

3.2.1. Conventional Culture Method

Each feed sample was mixed with a nutrient broth at a ratio of 1:9 and incubated overnight. Then, 0.1 mL of this dilution was streaked onto the nutrient agar (Oxoid, UK) and kept in an incubator at 37°C for 24 hours (10).

3.3. Determination of Bacterial Load with Seasonal Variations

The total viable count (TVC) of *E. coli* was determined from 10^{-1} to 10^{-8} . From each dilution, 1 mL of aliquot was inoculated onto eosin methylene blue agar (Oxoid, UK) and MacConkey agar (Oxoid, UK) plates, using the spread plate method and incubated for 24 hours (11). The TVC was recorded seasonally (September-November, December-February, March-May, and June-August).

3.4. Isolation and Identification of *E. coli* by the International Organization for Standardization (ISO) Method

The conventional culture method was carried out based on the recommended ISO 16654 method (12) with slight modifications for *E. coli* serotype identification. This method consists of the following steps.

3.4.1. Pre-Enrichment Stage

Each feed sample (25 g) was placed in 225 mL of modified Tryptone Soya Broth (Oxoid, UK) plus 20 mg/L of Novobiocin (Sigma) and incubated at 41°C for 24 hours.

3.4.2. Concentration Stage

In this stage, 1 mL of each pre-enriched broth was placed in 20 μ L of immunomagnetic beads (Thermo Fisher Scientific, USA), coated with antibodies to *E. coli* O157:H7, and incubated for 24 hours.

3.4.3. Isolation Stage

In this stage, 50 μ L of the prepared suspension was streaked on Cefixime Tellurite Sorbitol MacConkey (CT-SMAC) agar (Oxoid, UK) and incubated for 22 hours at 37°C. Clear and colorless colonies, characteristic of *E. coli* O157:H7, were observed. For further verification, the colonies were streaked again on eosin methylene blue agar (Oxoid, UK) and incubated for the next 24 hours; typical colonies confirmed the presence of *E. coli* O157:H7.

3.4.4. Confirmatory Stage

Biochemical and molecular characterizations were used for further identification.

3.5. Biochemical Characterization

Biochemical tests, including citrate utilization test, Voges-Proskauer test, indole formation, motility test, carbohydrate fermentation test, hydrogen sulfide production, catalase, oxidase, and urease tests, and methyl red tests, were performed on all *E. coli*-positive samples for identification (13).

3.6. Molecular Study of Samples

3.6.1. DNA Mining

A method described in the literature (14) with minor changes was used for DNA extraction of all culturally positive *E. coli* samples. Initially, the bacterial culture (1 mL) was centrifuged at a speed of 13,000 g for 15 minutes, and the supernatant was removed. Then, 80 μ L of proteinase K buffer plus enzyme (Thermo Fisher Scientific, USA), 40 μ L of sodium dodecyl sulfate (SDS; Thermo Fisher Scientific, USA), and 240 μ L of saline water were added and vortexed for two minutes. Next, the mixture was incubated in a water bath for five hours at 55°C. After resting the mixture for 15 minutes, 240 μ L of 6 M sodium chloride (NaCl) was added, and incubation was performed at room temperature. Afterward, the mixture was vortexed for two minutes and centrifuged for five minutes at 3000 g. The supernatant was moved to a second tube and centrifuged for 15 minutes at 13,000 g (in duplicate). After adding 460

μL of isopropanol (Longhong Chemical Co., China) to the mixture, centrifugation was carried out for five minutes, and the supernatant was discarded. In the next step, cold ethanol 70% (1000 μL) was added, and after centrifugation for five minutes, the supernatant was discarded. After the tubes were air-dried for two hours, Tris-EDTA (TE) buffer (25 μL) was added, and the extracted DNA was stored at -80°C .

3.6.2. Polymerase Chain Reaction (PCR) Amplification

Universal primers with a target size of 1500 bp were used for confirmation of *E. coli* at the genus level (15). *E. coli* O157:H7 (target size, 450 bp) was verified by species-specific PCR assay.

3.6.3. Thermal Cycler Conditions for *E. coli* O157:H7 Strain

The thermocycling conditions were as follows: initial denaturation for two minutes at 94°C , denaturation for 30 seconds at 94°C , annealing for 30 seconds at 72°C , extension for 30 seconds at 72°C , and a final extension at 72°C for ten minutes. The dideoxy method was used for the sequencing of amplified products, and the NCBI BLAST tool was used for confirmation. The sequences and product sizes are presented in Table 1.

3.7. Antimicrobial Susceptibility Testing

The disc diffusion test (DDT) was performed as described by the Clinical and Laboratory Standards Institute (CLSI) (16). The minimum inhibitory concentration (MIC) was also measured according to the CLSI guidelines (17) to evaluate the antimicrobial sensitivity of moxifloxacin, ciprofloxacin, gentamicin, enrofloxacin, chloramphenicol, kanamycin, streptomycin, trimethoprim, tetracycline, oxytetracycline, neomycin, penicillin, ampicillin, and amoxicillin by using a commercially prepared disk (Oxoid, UK) against *E. coli* O157:H7 strain.

3.8. Statistical Analysis

A one-way ANOVA test was performed in SPSS version 22.0 to determine the relationship between bacterial load and seasonal variations.

4. Results

4.1. Bacterial load with Seasonal Variations

Positive samples of *E. coli* ($n=38$) were evaluated by TVC in relation to seasonal variations, using one-way ANOVA, as shown in Table 2 and Table 3. As the P value was less than 0.05 ($P = 0.00$) and the F value was above one ($F = 15.56$), the effects of seasonal variations on the bacterial load were significant.

4.2. Isolation of *E. coli* O157:H7 Strain by ISO 16654 Method

For isolation of the pathogenic strain (*E. coli* O157:H7), the method recommended by ISO was used for all *E. coli*-positive culture samples (Figure 1 and Figure 2). Due to colonial variations, some strains of *E. coli* O157 also fermented sorbitol and did not produce colorless colonies; this was also true for the biochemical properties of this strain. Consequently, a low percentage of profile was produced that may be linked to the genetic evolution and the variety of virulence factors. Therefore, all positive and negative samples for *E. coli* O157:H7 were subjected to biochemical characterization and further validated by universal and species-specific PCR to differentiate the pathogenic strains of *E. coli* from non-pathogenic ones.

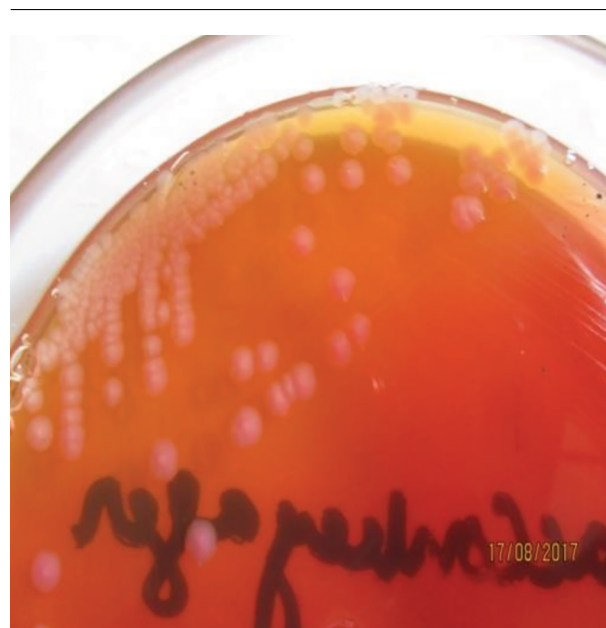


Figure 1. Colorless colonies on CT-SMAC

4.3. Molecular Characterization

All positive culture samples of *E. coli* ($n = 38$) were also positive for the reported universal primers, as shown in Figure 3. However, species-specific primers only confirmed eight positive samples for *E. coli* O157:H7, as shown in Figure 4.

4.4. Antimicrobial Susceptibility Test

The effectiveness of antibiotics against positive samples of *E. coli* O157:H7 was estimated by antimicrobial susceptibility tests.

Table 1. The List of Primers

	Primer sequences	Product size (bp)	Target genes	References
Universal primers	AGAGTTTGATCTGGCTCAG TACGGTTACCTGTACGACTT	1500	16S rRNA	Marchesi et al. (1998), (15)
<i>E. coli</i> O157:H7	AAG CGA CTG AGG TCA CT ACG CTG CTC ACT AGA TGT	450	<i>eae</i> _{O157:H7}	Louie et al. (1994)

Table 2. The total Viable Count (TVC) of *E. coli* in Relation to Seasons

Seasons	Positive samples	TVC (Log ₁₀ CFU/g)			95% Confidence interval	
		Minimum	Maximum	Mean ± SE	Lower bound	Upper bound
December to February	8	1	3.4	2.06 ± 0.29	1.357	2.767
March to May	5	1.21	5.1	3.07 ± 0.70	1.118	5.037
June to August	12	1.98	7.9	6.44 ± 0.45	5.453	7.442
September to November	13	1.77	6.8	4.96 ± 0.47	3.932	6.000
Total	38	1	7.9	4.57 ± 0.36	3.843	5.305

Table 3. ANOVA Results for TVC of *E. coli* (log₁₀ CFU/g) in Different Seasons

	Df	Mean square	F	P
Between groups	3	35.269	15.566	0.000
Within groups	34	2.266		
Total	37			



Figure 2. Small flat colonies with a metallic green sheen on eosin methylene blue agar (EMB)

4.4.1. DDT

By using DDT, the antibiogram profile of *E. coli* O157:H7 was calculated (Table 4). The resistance of *E. coli* O157:H7 to antibiotics was as follows: 100% to enrofloxacin, chloram-

phenicol, trimethoprim, ampicillin, amoxicillin, and penicillin G; 87.5% to tetracycline and oxytetracycline; 50% to streptomycin; 25% to ciprofloxacin; 12.5% to moxifloxacin; and 0% to gentamicin, neomycin, and kanamycin, respectively.

4.4.2. MIC Measurements

The effective minimum concentration of antibiotics against isolates was determined by measuring the MIC (Table 5). The significant difference ($P < 0.003$) between antimicrobial agents indicated the high efficacy of aminoglycosides (gentamicin, kanamycin, neomycin, and streptomycin) with the lowest MICs against *E. coli* O157:H7. On the other hand, fluoroquinolones (ciprofloxacin and moxifloxacin), oxytetracycline, and tetracycline showed low efficacies with high MICs. Also, trimethoprim, enrofloxacin, chloramphenicol, penicillin, ampicillin, and amoxicillin were completely ineffective; therefore, their MICs were not evaluated.

5. Discussion

5.1. High Incidence of *E. coli* O157:H7 in the Animal Feed

This study was conducted to evaluate *E. coli* as a major foodborne pathogen, using the conventional culture method and PCR assay. The results of universal PCR revealed that 18.6% (38/204) of samples were positive for *E.*

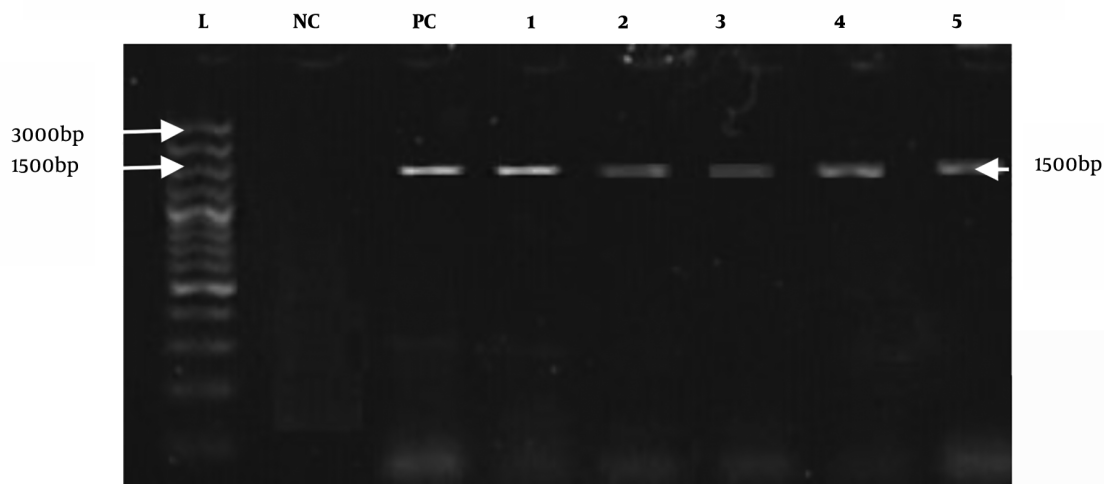


Figure 3. Amplified products of *E. coli* with universal primers (1500 bp). L: DNA ladder; NC: negative control; and PC: positive control (positive samples from 1 to 5).

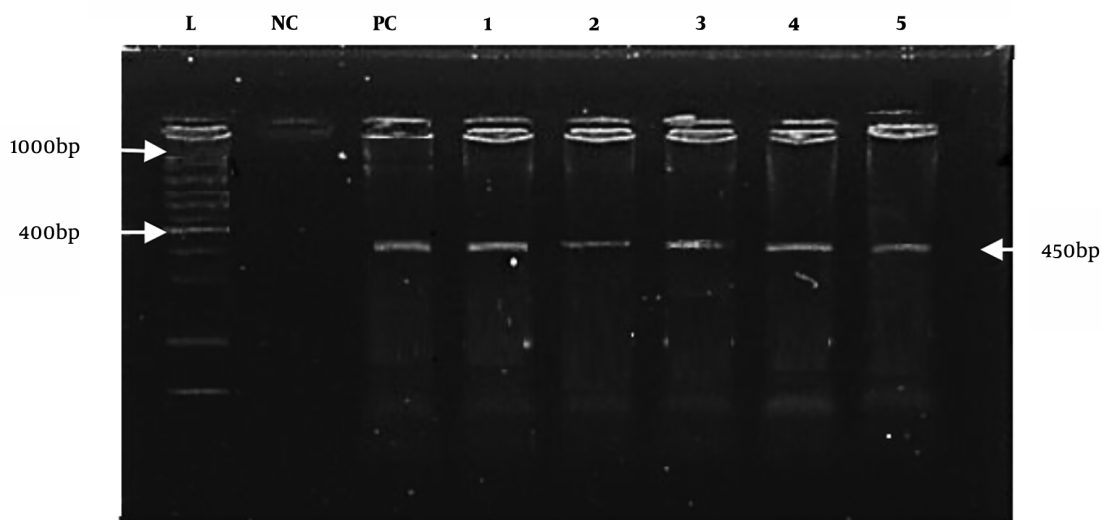


Figure 4. *E. coli* O157:H7 amplified with the reported primer (450 bp). M: DNA marker (positive samples from well 2 to well 5). PC is the positive control, and NC is the negative control.

coli, 21% (8/38) of which were confirmed for *E. coli* O157:H7 through species-specific PCR. This rate is higher than that reported in Argentina, where 5% of chicken product samples were positive for Shiga-toxigenic *E. coli* O157:H7 (18). On the other hand, 4% of meat samples in the UK showed the presence of *E. coli* O157:H7 (19). Overall, the high incidence of foodborne pathogens (*E. coli* O157:H7) in Pakistan, as compared to other countries, is alarming.

5.2. Effects of Seasonal Fluctuations on Bacterial Load

The effects of climatic fluctuations on the propagation of bacterial contamination are important. In the present study, the highest concentration of *E. coli* (7.9×10^8) was recorded in summer, while the lowest concentration (1×10^8) was reported in winter. This finding is consistent with a previous study (20), which demonstrated the effects of seasonal variations on microbial growth during feed storage and transportation.

Table 4. Disc Diffusion Test (DDT) for *E. coli* O157:H7

Antimicrobials	Sensitive, n (%)	Intermediate, n (%)	Resistant, n (%)
Gentamicin (Cn = 30 µg)	8 (100)	-	-
Neomycin (N = 10 µg)	8 (100)	-	-
Kanamycin (K = 30 µg)	8 (100)	-	-
Moxifloxacin (Mxf = 5 µg)	6 (75)	1 (12.5)	1 (12.5)
Ciprofloxacin (Cip = 30 µg)	4 (50)	1 (12.5)	2 (25)
Streptomycin (S = 10 µg)	2 (25)	-	4 (50)
Tetracycline (Te = 30 µg)	1 (12.5)	-	7 (87.5)
Oxytetracycline (Ot = 30 µg)	1 (12.5)	-	7 (87.5)
Enrofloxacin (Enr = 5 µg)	0	-	8 (100)
Chloramphenicol (C = 30 µg)	0	-	8 (100)
Trimethoprim (W = 1.25 µg)	0	-	8 (100)
Ampicillin (Amp = 10 µg)	0	-	8 (100)
Amoxicillin (Aml = 10 µg)	0	-	8 (100)
Penicillin G (P ₁₀ = 10 µg)	0	-	8 (100)

Table 5. The Minimum Inhibitory Concentration (MIC) of Antimicrobials Against *E. coli* O157:H7

Antimicrobials	Mean ± SEM	F	P Value
Gentamicin	0.16 ± 0.04		
Kanamycin	0.29 ± 0.11		
Neomycin	0.25 ± 0.12	5.35	0.003
Moxifloxacin	2.33 ± 0.88		
Ciprofloxacin	3.33 ± 0.66		
Streptomycin	5.33 ± 1.33		
Oxytetracycline	9.33 ± 3.52		
Tetracycline	6.67 ± 1.33		

5.3. MDR of *E. coli* O157:H7

Antimicrobials are used routinely to maintain the health of humans and animals. However, the irrational use of antimicrobials may lead to changes in the intestinal microflora and pathogenic bacteria by creating new resistant microbes that cause public health risks (21, 22). The highest resistance of *E. coli* O157:H7 (100%) to amoxicillin, ampicillin, penicillin, chloramphenicol, trimethoprim, and enrofloxacin has been recorded in recent research. In this regard, Kyabaggu et al. (23) reported the remarkable resistance of *E. coli* O157:H7 to penicillin derivatives and chloramphenicol; susceptibility was only found for aminoglycosides (neomycin, gentamicin, and kanamycin). Also, Altaee et al. (24) and Ari et al. (25) reported similar findings. Overall, microbial analysis of poultry feed is incomplete without appropriate treatment and control measures. Also, the MDR of these microbes has posed treatment challenges.

5.4. Conclusion

The dominance of foodborne pathogens in the poultry feed represents a constant health risk to poultry and humans. Therefore, feed safety and sustained inspection of antimicrobial resistance of pathogens should be major concerns in developing countries, such as Pakistan, to promote poultry farming and human health.

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Footnotes

Authors' Contribution: Study conception and design: Shajeela Iram, Mehmood Ul Hassan, and Tabinda Khawaja; analysis and interpretation of data: Shajeela Iram; drafting of the manuscript: Shajeela Iram and Tabinda Khawaja; critical revision of the manuscript for important intellectual content: Shajeela Iram, Tabinda Khawaja, and Mehmood Ul Hassan; and statistical analysis: Shajeela Iram.

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