Biosight 2025; 07(02): 16-29

Molecular Characterization and Antibiogram of *Salmonella Enterica* and Its Non-Typhoidal Serotypes in Poultry Feed with Seasonal Effects in Karachi, Pakistan.

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Abstract
Salmonella enterica and its non-typhoidal serotypes are major foodborne zoonotic pathogens responsible for salmonellosis, one of the most common gastrointestinal diseases worldwide. This study aimed to molecularly identify S. enterica and its non-typhoidal serotypes (S. Enteritidis and S. Typhimurium), evaluate the seasonal influence on bacterial growth, and determine their antimicrobial susceptibility profiles. A total of 204 poultry feed samples were analyzed using cultural and biochemical methods, followed by confirmation with polymerase chain reaction (PCR). Bacterial load was determined by total viable count (TVC), while antimicrobial susceptibility testing (AST) was performed using the disk diffusion method and minimum inhibitory concentration (MIC). Of the 204 samples, 66 (32.3%) were confirmed as S. enterica. Among these, 31 (46.9%) were identified as S. Typhimurium and 21 (31.8%) as S. Enteritidis. The highest mean bacterial load (7.026×10^8 CFU/g) was recorded during June–August, while the lowest (2.395×10^8 CFU/g) occurred during December–February, with a significant seasonal difference ($p < 0.01$). Antimicrobial susceptibility testing showed that all non-typhoidal S. enterica serotypes were highly resistant to penicillin and its derivatives but remained largely susceptible to fluoroquinolones and aminoglycosides. The high prevalence of Salmonella in poultry feed during hot and humid months highlights an increased risk of foodborne transmission. These findings emphasize the need for improved storage practices, enhanced pre-harvest control measures, and continuous surveillance of microbial contamination and antimicrobial resistance to safeguard public health.

Keywords: Total viable count, Antimicrobial susceptibility test, Polymerase chain reaction, Foodborne pathogens, Seasonal effects.

Received: 29-1-2026
Accepted: 7-7-2026
DOI: *10.67773/bios.v7i2.262*

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Introduction

Foodborne diseases pose a significant public health hazard worldwide, hindering socioeconomic progress [1]. Salmonellosis, the second most prevalent foodborne illness in Europe which is caused by consuming food contaminated with *Salmonella enterica*. It is characterized by bloody diarrhea,

gastroenteritis, fever, vomiting, and nausea. The disease primarily transmits to humans through animal-origin foods, particularly poultry meat and its products [2]

Zoonotically, the most important non-typhoidal *S. enterica* serotypes are *S. Enteritidis* and *S. Typhimurium*, which are responsible for enteric diseases in various animals, including humans [3]. The Centers for Disease Control and Prevention (CDC) estimates that non-typhoidal Salmonellosis leads to roughly 1.35 million illnesses and 420 deaths in the U.S each year [4]. While various vectors can be implicated in the propagation of *S. enterica* to poultry meat, contaminated poultry feed is considered the most common source [5].

Classifying and characterizing foodborne pathogens are crucial for effectively treating and controlling the diseases they cause. Traditionally, conventional culture and biochemical methods have been considered the most reliable ways to trace these microorganisms [6].However, molecular-based techniques like PCR are utilized for organism confirmation due to their specificity, accuracy, and rapid determination [7].

Climatic factors significantly influence the pervasiveness of poultry diseases, as natural or anthropogenic seasonal variations affect pathogen survival [8].For poultry, the period from October to March is generally safer due to a lower incidence of diseases. Conversely, the disease ratio is significantly higher from May to October [9].

The major public health issue in rising countries regarding the production of food animals is antibiotic resistance in non-typhoidal serotypes of *Salmonella* [10].The irrational utilization of antibiotics to meet the demand of poultry products may enhance the spread of resistant strains of *Salmonella* worldwide that consequently affect human health [11,12]

The study was carried out to isolate the causative agent *S.enterica* and its non-typhoidal serotypes (*S.Enteritidis* and *S.Typhimurium*) of foodborne zoonotic disease, salmonellosis, in poultry feed on molecular basis. The effect of seasonal variation was also determined in respect of *S.enterica* contamination in feed. Moreover, the effect of antibiotics was also observed for the control and prevention of the *Salmonella* infection.

Methods

From January 2022 to December 2023, we randomly collected 204 poultry feed samples from 17 farms across different locations including Gadap Poultry State, Bin Qasim, and Surjani. Sterile containers were used for the collection and transport of samples to the Microbiology Department at the University of Karachi. The sample size was determined based on the total number of eligible samples that could be collected during the predefined study period while ensuring adequate representation of different seasons and sampling locations. Since this was an exploratory cross-sectional surveillance study, no formal sample size calculation was performed.

Inclusion Criteria

- Poultry feed samples collected from registered commercial poultry farms and authorized poultry feed mills in Karachi.
- Fresh and unopened feed samples or feed samples collected directly from storage bins under aseptic conditions.
- Feed samples representing different poultry feed types (e.g., starter, grower, finisher, layer feed).
- Samples collected during all four seasons to assess seasonal variation.
- Samples with sufficient quantity for bacteriological, molecular, and antimicrobial susceptibility testing.

Exclusion Criteria

- Spoiled, moldy, water-damaged, or visibly deteriorated feed samples.
- Duplicate samples collected from the same batch at the same sampling time.
- Samples collected in damaged or contaminated containers.
- Samples with incomplete labeling or missing information regarding source or date of collection.
- Samples of insufficient quantity for laboratory analysis.

Isolation &Characterization of *S. enterica*

For *Salmonella,* the isolation standard cultivation method outlined in ISO 6579 was conducted [13].

Pre enrichment Stage

Each five-gram feed sample was mixed with 45ml of Buffered Peptone Water (BPW) and incubated for 24 hours.

Culture Stage

Initially, 0.1ml of the BPW solution was added to 10ml of Rappaport Vassiliadis Soya Peptone (RVS) broth and incubated at 41°C for 24 hours. Subsequently, a loop-full of the RVS broth culture was streaked onto individual agar plates, including SS agar, XLD agar, and brilliant green agar. These plates were then incubated at 37°C for 24 hours.

Biochemical Confirmation

Salmonella enterica suspected colonies were confirmed by microscopic and biochemical characterization.

All suspected *S.enterica* isolates were evaluated microscopically as described by [14]. Biochemical confirmation was also done by performing MR, VP, Indole test, oxidase test ,citrate utilization ,motility test, hydrogen sulfide production test,urease test, carbohydrate (Glucose,Sucrose,Lactose) fermentation test and catalase test [15].

Bacterial count with seasonal effect

Following ISO recommendationsthe total viable count of *Salmonella* was determined [16]. We prepared 10 folds serial dilution ranged from 10^{-1} to 10^{-8} .Then, using the spread plate method, 1ml aliquots from each dilution were spread onto nutrient agar with a bent glass rod and incubated for 24 hours. The total viable count (TVC) data was organized into four quarterly periods across the year.

Molecular Characterization

Positive cultures of *Salmonella enterica* were used for DNA extraction by using a kit (*Invitrogen, Catalog No.K1820*-01) as per manufacturer's instruction. The extracted DNA templates were evaluated by using universal reported primers of genus *Salmonella* (target size 204pb and primers sequences are ATCGCTGACTTATGCAATCG and CGGGTTGCGTTATAGGTCTG). *S. enterica* serovars Typhimurium (target size 401bp and primers sequences are TGTTCACTTTTTACCCCTGAA and CCCTGACAGCCGTTAGATATT) and *S.enterica* serovars Enteritidis (target size 304bp and primers sequences are TGTGTTTTATCTGATGCAAGAGG and TGAACTACGTTCGTTCTTCTGG) were identified by specie-specific PCR [17].After

amplification, the products underwent dideoxy sequencing and their identities were confirmed using NCBI BLAST.

Antimicrobial susceptibility Test

To determine the antibiotic susceptibility of *S. Enteritidis* and *S. Typhimurium* serovars of *Salmonella enterica*, we employed the disc diffusion method in accordance with Clinical and Laboratory Standards Institute guidelines [18] and Minimum Inhibitory Concentration[19] with minor alterations. Commercially prepared discs of oxytetracyclin, moxifloxacin, enrofloxacin, amoxicillin, gentamicin, ciprofloxacin, streptomycin, kanamycin, trimethoprim, neomycin, chloramphenicol, tetracycline, ampicillin and penicillin G were used.

Statistical evaluation

To assess the association between bacterial load and seasonality, a one-way ANOVA was performed using SPSS for Windows (version 22.0). A P-value < 0.05 was considered statistically significant.

Results

Isolation & Identification of *S .enterica*

Out of 204 samples, 66 were positive for *S.enterica* evaluated by cultural method (Fig. 1), microscopic examination (Fig. 2) and biochemical methods as shown in (Table 1).

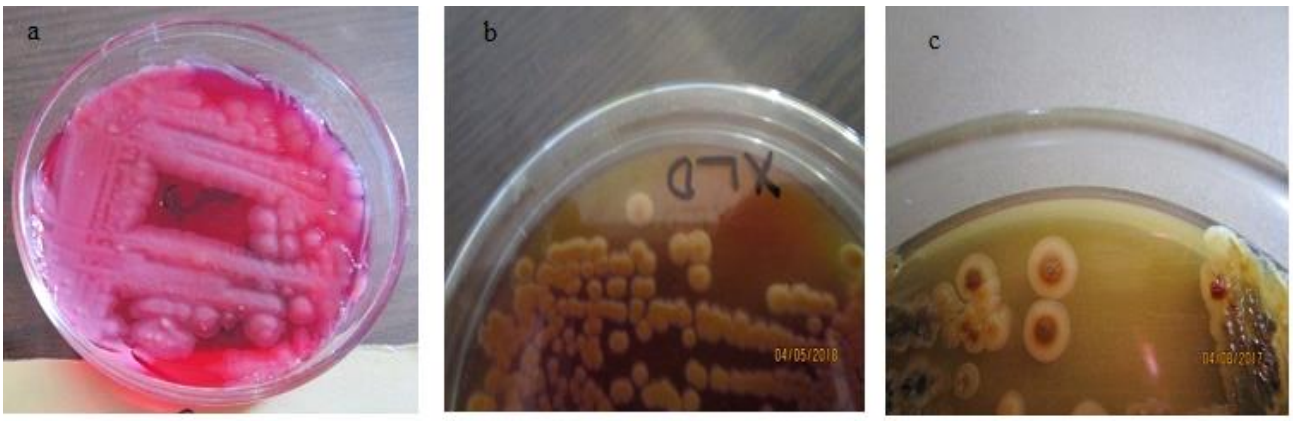

Figure 1: Cultural Evaluation (a): On BGA: Pinkish white Colonies, (b) On XLD: Red colored colonies with dark center (c): On SS agar, *S. enterica* typically formed straw-colored colonies with dark center

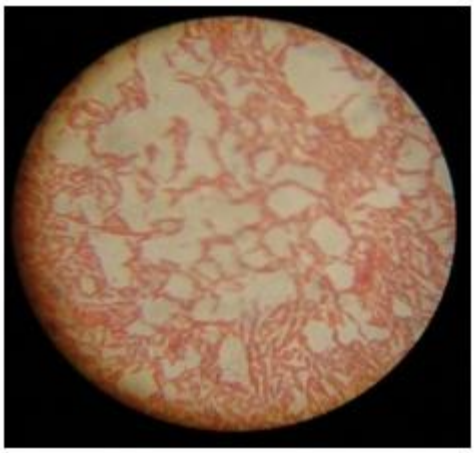

Figure 2: Microscopic Examination of gram-negative, rod shape Bacteria

Table 1: Biochemical features of *S.enterica*

Biochemical Test	Results
Vp Test	-
Indole Test	-
Citrate Utilization Test	+
MR Test	+
H_2S productionTest	+
CatalaseTest	-
Urease Test	-
Oxidase Test	-
Motility Test	+
Glucose Fermentation	+
Lactose Fermentation	-
Sucrose Fermentation	-

Total viable count

A one-way ANOVA revealed a significant difference in the total viable count (TVC) of *Salmonella* isolates across seasons ($p<0.01$, $F=16.186$), as detailed in (Table 2a & b).

Molecular analysis

All culture-positive *S. enterica* samples (32.3%) were also PCR positive when tested with universal primers (Fig. 3). Of these, 46.9% were validated as *S. enterica* serovar Typhimurium and 31.8% as *S. enterica* serovar Enteritidis using species-specific primers (Fig 4 & 5). The remaining 21.2% of samples were non-typable.

Antimicrobial Susceptibility Testing

Antibiogram of all PCR-positive samples of *S. Enteritidis* and *S.* Typhimurium were evaluated to determine the efficacy of antibiotics (Figure 6 and 7).

Table 2 (a):Total viable count of *S. enterica* with seasonal effect

Seasons	Total viable count (Log10 CFUs/g)				95% Confidenc Interval for Mean	
	Positive Samples	Minimum	Maximum	Mean± SEM	Lower Bound	Upper Bound
Dec-Feb	10	1.1	5.7	2.395 ± 0.413	1.4597	3.3303
Mar-May	14	1.33	6.3	3.640 ± 0.554	2.4438	4.8376
Jun-Aug	22	2.39	9.9	7.026 ± 0.384	6.2277	7.8259
Sep-Nov	20	2.09	8.9	5.482 ± 0.512	4.4088	6.5552
Total	66	1.1	9.9	5.138 ± 0.315	4.5087	5.7686

Note: CFU, colony-forming unit; **Log_{10} CFUs/g,** logarithm (base 10) of colony-forming units per gram; **SEM,** standard error of the mean; **Positive samples** represent feed samples with detectable bacterial growth. **Seasons** were classified as December–February, March–May, June–August, and September–November.

Table 2 (b): ANOVA for Total viable count (TVC) of *S.enterica* in different seasons

	df	Mean Square	F	p-value
Between Groups	3	62.494	16.186	0.000
Within Groups	62	3.861		
Total	65			

Note: df: Degrees of freedom. **Mean Square (MS):** Sum of squares divided by the corresponding degrees of freedom**. F:** F-statistic obtained from one-way analysis of variance (ANOVA**). p**-value: Probability associated with the F-test; values < 0.05 indicate statistically significant differences among group means.

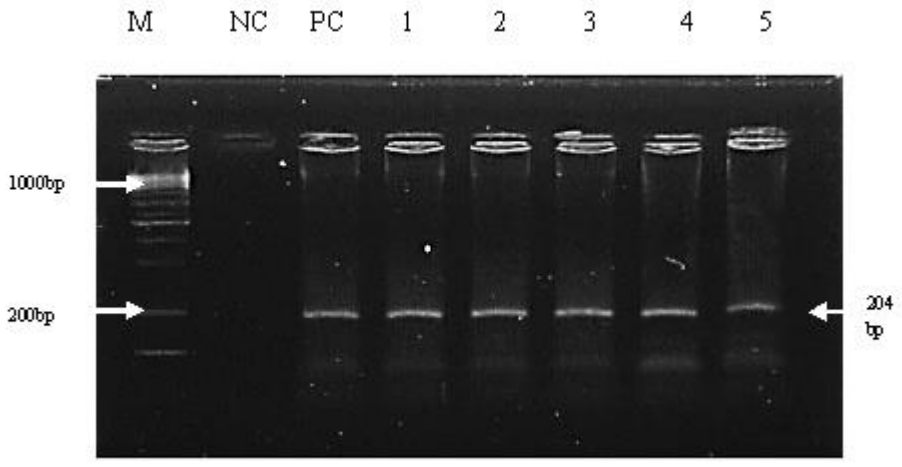

Figure 3: Amplified *S. enterica* products, with a size of 204, are shown. M indicates the marker, NC is the negative control, PC is the positive control, and lanes 1 through 5 are positive samples.

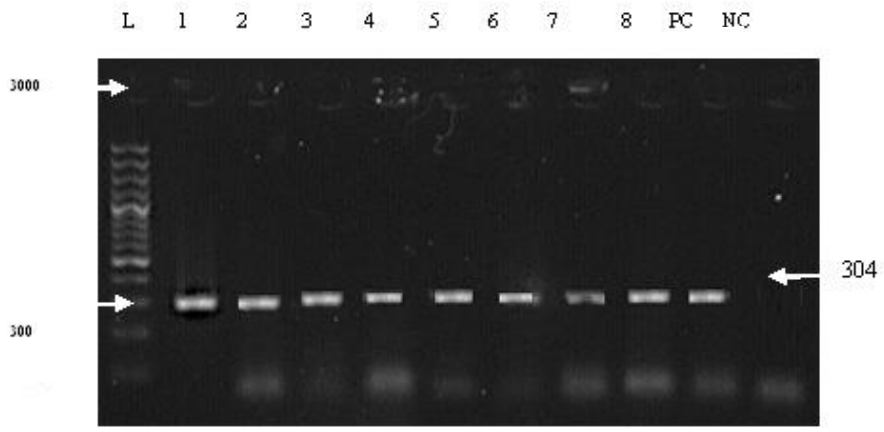

Figure 4: Amplification of S. Enteritidis showing a 304 bp PCR product. L: DNA ladder; lanes 1–8: positive samples; PC: positive control; NC: negative control.

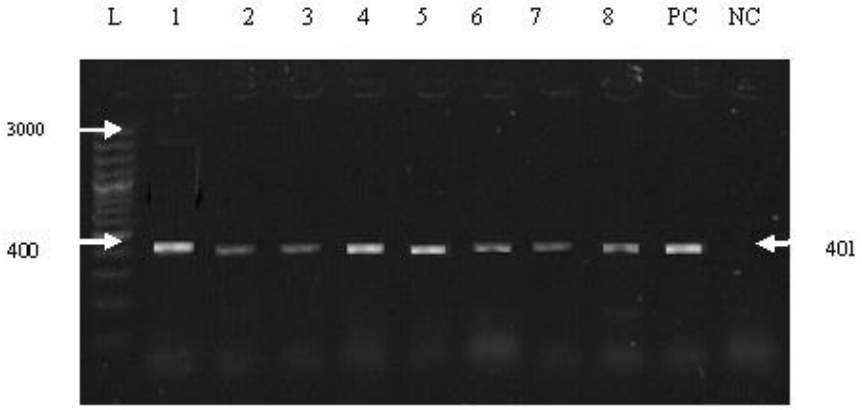

Figure 5: The PCR amplification of S. Typhimurium yielded a 401 bp amplicon. In the gel image, L indicates the DNA ladder, lanes 1–8 represent positive samples, PC is the positive control, and NC is the negative control.

Disc diffusion test

Fourteen antibiotics were used to determine the antibiogram against serotypes of *S.enterica* by disc diffusion test

From (Table 3&4) and (Fig. 6&7),it is evident that *S. enterica* serovars Typhimurium and Enteritidis showed high susceptibility to moxifloxacin, ciprofloxacin, and enrofloxacin. They were also susceptible to chloramphenicol, gentamicin, and streptomycin, though to a lesser extent. Conversely, amoxicillin, ampicillin, and penicillin G were the least effective antibiotics against these isolates.

Table 3: *Salmonella* Typhimurium

Antibiotics	Interpretative one diameter standards by CLS	Sensitive {n (%)}	Intermediat {n (%)}	Resistant {n (%)}
Enrofloxacin (ENR=5µg)	≥23	31(100)	-	-
Gentamicin (CN=30µg)	≥23	29(93.5)	2(6.4)	-
Ciprofloxacin (CIP=30 µg)	≥21	31(100)	-	-
Moxifloxacin (MXF=5µg)	≥20	31(100)	-	-
Chloramphenicol(C=30µg)	≥15	19(61.2)	1(3.2)	11(35.4)
Streptomycin (S=10µg)	≥21	29(93.5)	2(6.4)	-
Oxytetracycline (OT=30µg)	≥22	16(51.6)	-	15(48.3)
Neomycin (N=10µg)	≥17	14(45.1))	-	17(54.8)
Trimethoprim (W=1.25µg)	≥17	14(45.1)	-	17(54.8)
Kanamycin (K=30µg)	≥18	13(41.9)	-	18(58.0)
Tetracycline (TE=30µg)	≥19	15(48.3)	-	16(51.6)
Amoxicillin(AML=10µg)	≥15	2(6.4)	-	29(93.5)
Penicillin G(P_{10}=10µg)	≥22	0	-	31(100)
Ampicillin(AMP=10µg)	≥17	2(6.4)	-	29(93.5)

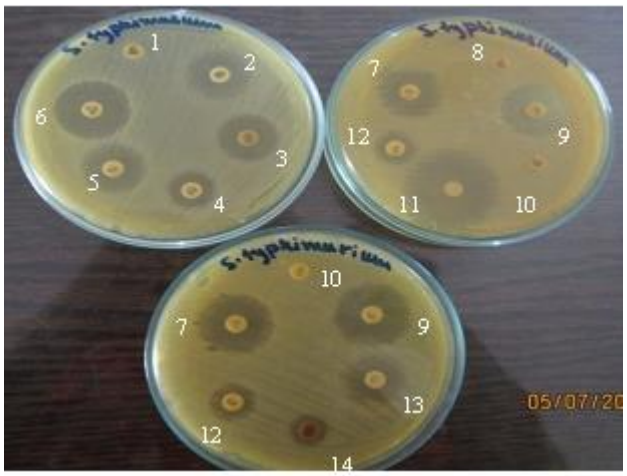

Fig 6: Disc diffusion antibiotic susceptibility test of *S. Typhimurium*

Minimum inhibitory test

MIC was performed to determine the minimum effective dose of the antibiotics. Table 5&6 showed p< 0.005, a significant difference that revealed the fluoroquinolones and aminoglycosides having lower MIC scores indicated high efficacy, whereas, high MIC scores of peniciline derivatives showed lower efficacy for *S.enterica* serovar Typhimurium and *S.enterica* serovar Enteritidis (Table No 5 and 6).

Table4: ***Salmonella Enteritidis***

Antibiotics	**Interpretative zone diameter standards by CLS**	**Sensitive {n (%)}**	**Intermediate {n (%)}**	**Resistant {n (%)}**
Ciprofloxacin CIP=30µg)	≥21	21(100)	-	-
Enrofloxacin (ENR=5µg)	≥20	21(100)	-	-
Moxifloxacin (MXF=5µg)	≥23	21(100)	-	-
Chlorampheniol(C=30µg)	≥21	20(95.2)	1(4.7)	-
Gentamicin (CN=30µg)	≥23	19(90.4)	-	2(9.5)
Streptomycin (S=10µg)	≥15	17(80.9)	4(19.0)	-
Tetracycline (TE=30µg)	≥19	16(76.1)	2(9.5)	3(14.2)
Oxytetracycline (OT=30µg)	≥22	13(61.9)	5(23.8)	3(14.2)
Kanamycin (K=30µg)	≥18	11(52.3)	4(19)	6(28.5)
Neomycin (N=10µg)	≥17	10(47.6)	3(14.2)	8(38)
Trimethoprim (W=1.25µg)	≥17	11(52.3)	4(19)	6(28.5)
Amoxicillin(AML=10µg)	≥15	4(19)	-	17(80)
Ampicillin(AMP=10µg)	≥17	3(14.2)	-	18(85.7)
Penicillin G(P_{10}=10µg)	≥22	1(4.7)	-	20(95.2)

Note; CLSI: Clinical and Laboratory Standards Institute. **n (%):** Number and percentage of *Salmonella enterica* isolates in each susceptibility category. **Sensitive (S):** Isolates susceptible to the tested antimicrobial agent. **Intermediate (I):** Isolates with intermediate susceptibility. **Resistant (R):** Isolates resistant to the tested antimicrobial agent.

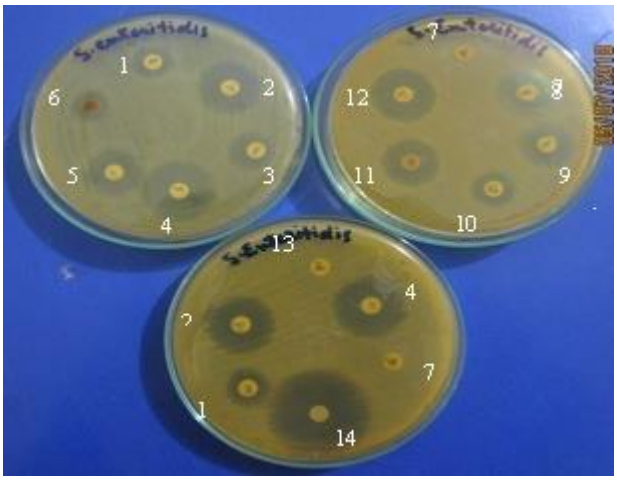

Figure 7: Disc diffusion antibiotic susceptibility test of *S. Enteritidis*. Antibiotic discs are labeled as follows: 1 = N, 2 = MXF, 3 = P10, 4 = C, 5 = S, 6 = TE, 7 = AML, 8 = CN, 9 = K, 10 = W, 11 = OT, 12 = ENR, 13 = AMP, and 14 = CIP.

Table 5: MIC against *S.enterica* serovar Typhimurium

Antibiotics	Mean±SEM	F	P-value
Ciprofloxacin	0.167±0.04		
Enrofloxacin	0.167±0.04		
Moxifloxacin	0.167±0.04		
Amoxicillin	10.66±2.67	9.904	0.000
Chloramphenicol	0.33±0.083		
Ampicillin	13.33±2.66		
Gentamicin	0.208±0.04,		
Streptomycin	3.33±0.67		
Kanamycin	8±0		
Oxytetracycline,	6.67±1.33		
Tetracycline	5.33±1.33		
Penicillin G	16±0		
Neomycin	7.33±4.37,		
Trimethoprim	10.67±2.67		

Note; SEM: Standard error of the mean. **F:** F-statistic obtained from one-way analysis of variance (ANOVA). **P-value:** Probability associated with the ANOVA test.

Table 6: M IC against *S.enterica* serovar Enteritidis

Antibiotics	Mean±SEM	F	p-value
Moxifloxacin	0.167±0.04		
Enrofloxacin	0.167±0.04		
Ciprofloxacin	0.167±0.04		
Chloramphenicol	0.208±0.04	6.04	0.000
Gentamicin	0.208±0.04		
Ampicillin	10.667±2.66		
Streptomycin	9.33±3.52		
Amoxicillin	1.5±0.5		
Oxytetracycline	4.67±1.76		
Neomycin	6.67±1.33		
Kanamycin	6.67±1.33		
Penicillin G	13.33±2.66		
Trimethoprim	8±4		
Tetracycline	3±1		

Note; SEM: Standard error of the mean. **F:** F-statistic obtained from one-way analysis of variance (ANOVA). **P-value:** Probability associated with the ANOVA test.

Discussion

The emergence of foodborne diseases as a significant and rising universal health dilemma has an imperative impact on trade, travel, and economic development worldwide.

The World Health Organization (WHO) reports that roughly 600 million illnesses and 420,000 deaths occur globally each year due to foodborne diseases (FBD). The economic toll of FBD is also

substantial, estimated at $20 billion annually [20] .Among these, Salmonellosis stands out as a critical zoonotic foodborne illness. It is primarily caused by two significant *Salmonella enterica* serotypes, Enteritidis and Typhimurium, which are frequently transmitted from animals to humans [21].

The molecular techniques are considered the most significant diagnostic tool for foodborne pathogens verification [22]. In this study, *S.enterica* and non-typhoidal *Salmonella* isolates (*S. Enteritidis* and *S. Typhimurium*) were detected by specie-specific and Universal PCR due to their reliability and specificity which is in agreement with Resendiz-Nava et al [23]. Results revealed that 31 (46.6%) samples were positive for *S.* Typhimurium and 21(31.8%) were for *S.*Enteritidis. These findings are higher than those recorded in Egypt by Ashraf et al [24] where the prevalence of *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis were 28 (1.8%) and 22 (1.4%) respectively.

Seasonal effects on the incidence of microbes are of prime importance. In the present study, the highest concentration of *S. enterica* (7.026×10^8cfu/g) was recorded during summer season. Likewise, Powell et al., [25] reported high incidence during the months of April to September. However, October to March was considered the low-risk period [26] A study from the UK also demonstrated the pronounced impact of ecological changes on feed contamination, identifying summer as the season associated with the highest risk.

S. Typhimurium and *S.* Enteritidis are highly pathogenic and resistant serotypes, causing salmonellosis, a severe health issue globally [27]. It can be controlled by meticulous sanitation and management measures but it is not always feasible economically because it engages both sectors private and public. Moreover, studies are required for the appropriate communication of scientific knowledge and awareness regarding salmonellosis to all strata of the population [28,29]. On the other hand, antibiotics have proven to be a good remedy but due to multi-drug resistance problem, it may enhance the susceptibility to infection. This alarming condition is due to irrational use of antimicrobial drugs along with insufficient inspection and services to detect MDR [30,31].

Antimicrobial susceptibility for non-typhoidal serotypes of *S.enterica* revealed the high sensitivity to fluoroquinolones. These results are in line with Ammari et al [32] who described very low resistance to fluoroquinolones in *Salmonella enterica* serotypes. The highly resistant antibiotics with *S.Typhimurium* and *S.Enteritidis* were penicillin derivatives. Similar results are recorded by Divek et al [33].

Conclusion

Consequently, there is a need for a more robust food control system and effective strategies for regularly inspecting poultry feed to ensure its quality. Additionally, constant surveillance for the manifestation of multi-drug resistant bacterial strains is crucial to prevent foodborne diseases.

Ethics Approval and Consent to Participate

Not applicable.

Human and Animal Rights

All experimental protocols were performed on the basis of guidelines provided by the National Institute of Care and Use for Animals.

Consent for Publication

Not applicable.

Availability of Data and Materials
Data presented in this study will be available on a personal request to the corresponding author

Funding Source
This research received no grant from any funding agency in the public, commercial, or not–for-profit sector.

Conflict of interest
All authors declare no conflict of interest.

Acknowledgements
None

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