Punjab University Journal of Zoology

OPEN ACCESS

40(1): 37-42 (2025)
https://dx.doi.org/10.17582/journal.pujz/2025/40.1.37.42

Research Article

Prevalence of Antibiotic Resistance Non-Typhoidal *Salmonella* in Controlled Layer Poultry Sheds

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Article History
Received: September 21, 2024
Revised: March 10, 2025
Accepted: March 24, 2025
Published: May 13, 2025

Authors' Contributions
MR, AS did the material preparation, lab testing and experiments. MH and RZ analyzed the data. MFA, SFUH wrote the first draft of the manuscript. SA supervised the research. HQA reviewed and edited the manuscript. All authors read and approved the final manuscript.

Keywords
Salmonella, Occurrence, Antibiogram, bla_{TEM} gene, Antimicrobial resistance

Abstract | Salmonella is a major zoonotic pathogen causing salmonellosis in poultry and gastroenteritis in humans. In poultry, *Salmonella* is the leading cause of bacterial infections causing watery diarrhea, inadequate food intake, poor growth, and low egg production. This research sought to identify *Salmonella* in controlled layer poultry sheds from poultry droppings and detect its pattern of antibiotic resistance. A total of 100 samples (25 from each shed) were collected and enriched in selective tetrathionate broth and grown on selective media *salmonella*-shigella agar. *Salmonella* was confirmed by examining the colony morphology, microscopic features, and results of biochemical tests such as the indole test, Voges-Proskauer test, methyl red test, oxidase test, catalase test, triple sugar iron test, and citrate test. Out of 100 samples, 22 samples were confirmed for having *Salmonella*. The Kirby-Bauer disk diffusion method was used to check the susceptibility of different antibiotics and the zone of inhibition was measured and compared to Clinical and Laboratory Standards Institute (CLSI 2023). When susceptibility was measured, all *Salmonella* isolates were resistant to ampicillin, 86.36% of isolates were resistant to Nalidixic acid and 77.27% of the isolated bacteria were resistant to tetracycline. Ceftriaxone and Tazobactam were the most sensitive drugs against the *Salmonella* isolates. PCR results showed that the bla_{TEM} gene was the most detected resistant gene in the *Salmonella* isolates. These results confirm the prevalence of antibiotic resistance *Salmonella* species in the poultry environment. Therefore, to prevent the emergence and spread of drug-resistant *Salmonella* isolates, reasonable utilization and stringent control techniques for antimicrobials in control and treatment are required.

Novelty Statement | This research belongs to importance of zoonosis of *Salmonella* spp., antibiotic resistance which may be caused by the egg consumption from layer sheds used to treat the layer bird. The studies highlight the importance of this aspect and investigated the prevalence in selected area to highlight the potential issue.

To cite this article: Rashid, M., Shahzad, A., Ahmed, H.Q., Hamza, M., Akhtar, M.F., Hassan, S.F. and Zahid, Z., 2025. Prevalence of antibiotic resistance non-typhoidal *Salmonella* in controlled layer poultry sheds. *Punjab Univ. J. Zool.*, **40**(1): 37-42. https://dx.doi.org/10.17582/journal.pujz/2025/40.1.37.42

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Introduction

Increased in Antibiotic resistance is a substantial concern in the poultry industry, specifically about bacteria like *Salmonella*. In Pakistan, the poultry sector is vital for the economy, contributing around 1.3% to the GDP. Commercial production was started in the 1960s, now provides a considerable portion of the daily protein intake for the population (Hussain *et al.*, 2015). However, it faces key challenges, including *Salmonella* contamination and antibiotic resistance. *Salmonella* is a bacterium that can cause foodborne illnesses. Two primary species include: *Salmonella* enterica and *Salmonella* bongori (Hassena *et al.*, 2022). With nearly 2600 identified serovars, some such as *S. typhi* and *S. paratyphi* cause typhoid and paratyphoid fever, while others like *S. typhimurium* and *S. enteritidis* are commonly found in poultry and can infect both birds and humans (Hendriksen *et al.*, 2011; Cui *et al.*, 2021). Typhoid toxin is important in the pathogenicity of these bacteria (Andesfha *et al.*, 2019). *Salmonella* can form biofilms, enabling them to persist for extended periods in animals and humans (Afshari *et al.*, 2018).

Salmonella infections are often occur through consuming contaminated food, eggs, or poultry meat. Approximately 20% of the world's poultry products are contaminated with *Salmonella*. This bacterium not only causes illness in birds and reduces egg and meat production but also potential health risk to humans (Hassena *et al.*, 2022). Young chicks are particularly vulnerable due to their immature immune system and weak intestinal microflora (Pan and Yu, 2013).

Salmonella infections are a common cause of illness in poultry and potential threat to foodborne diseases in humans (Ahmad *et al.*, 2022). Most salmonellosis outbreaks have been linked to chicken products contaminated with S. typhimurium and S. enteritidis (Cui *et al.*, 2021). Poultry diseases, especially those caused by *Salmonella*, result in significant economic losses for the industry (Rosengren *et al.*, 2010).

Antibiotics are normally used to promote growth and treat infections in birds (Beier *et al.*, 2013). However, the use of key antibiotics has been banned in many regions. For instance, antibiotics used as growth promoters were banned in the EU in 2006 and in the US in 2017, though they remain permitted in some countries like China and Brazil (Peruzy *et al.*, 2020).

In Pakistan, while antibiotics for growth promotion are banned, their use for treating sick birds continues. This raises concerns about the emergence of antibiotic-resistant *Salmonella* strains in poultry sheds. However, there is limited data on the prevalence and antibiotic resistance of *Salmonella* in controlled layer sheds in Pakistan. This lack of information hinders the development of effective strategies to control *Salmonella* and protect public health. This study aims to address this gap by examining the prevalence and antibiotic resistance profiles of non-typhoidal *Salmonella* isolated from controlled-layer poultry sheds in Jhang, Punjab, Pakistan.

Materials and Methods

Salmonella isolation and biochemical identification

A total of 100 samples were collected from the feces of egg-laying poultry birds from the four control layer sheds of the Jhang region of Pakistan, following approval from the Institutional Ethical Committee. 1 gram of each sample was measured through weighing balance and added to the 9 ml of buffered peptone water for non-selective bacterial enrichment. Selective pre-enrichment was done for 24 h at 37 °C temperature, followed by streaking a loopful of the enrichment broth on the selective media *salmonella -shigella* agar and incubated for 24-48 h at 37 °C, Figure 1. Suspected *Salmonella* black centered or colorless colonies on SS agar were streaked to xylose-lysine-desoxycholate agar and also observed under a microscope, Figure 2. Colony characteristics showed black dot-centered pinkish colonies on XLD agar, which were further identified by biochemical tests including, the Tripple sugar iron test, IMViC test, and oxidase test (Ashrafudoulla *et al.*, 2021). The entire procedure for isolating and detecting *Salmonella* was carefully followed, as per ISO-6579-2017.

Figure 1: Black-centered or colorless colonies of salmonella on SS agar.

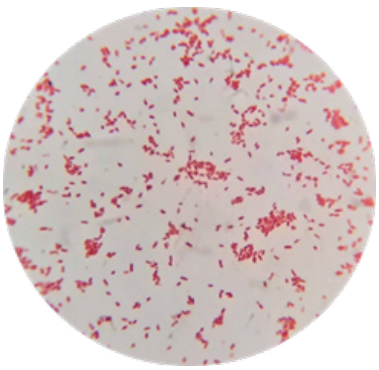

Figure 2: Short pink-colored rods of salmonella under a microscope.

Table 1: Primer sequence used to target *bla*$_{TEM}$ gene.

Gene name	Sequence	AT (°C)	Amplification size (bp)	References
bla$_{TEM}$	F. 5′-ATCAGCAATAAACCAGC-3′ R. 5′-CCCCGAAGAACGTTTTC-3′	54°c	516	(Yukawa *et al.*, 2019)

Antimicrobial susceptibility test

Antimicrobial susceptibility test is used to check the ability of pathogenic microorganisms to develop resistance to different antibiotics. The resistance to antibiotics was checked by the Kirby-Bauer disk diffusion method. Muller Hinton agar was poured into the Petri plates and allowed to cool. A loopful of the bacterial culture was streaked on the agar using sterilized cotton swabs. Antibiotic disks were then placed on the agar using a sterilized tweezer. The plates were then incubated at 37 C for 24 h and inhibition zone was measured (Igbinosa *et al.*, 2022). Susceptibility of various antibiotics was checked: Piperacillin/Tazobactam, Tetracycline, Amikacin, Ciprofloxacin, Nalidixic acid, ceftriaxone, Ampicillin, Chloramphenicol, and Gentamycin.

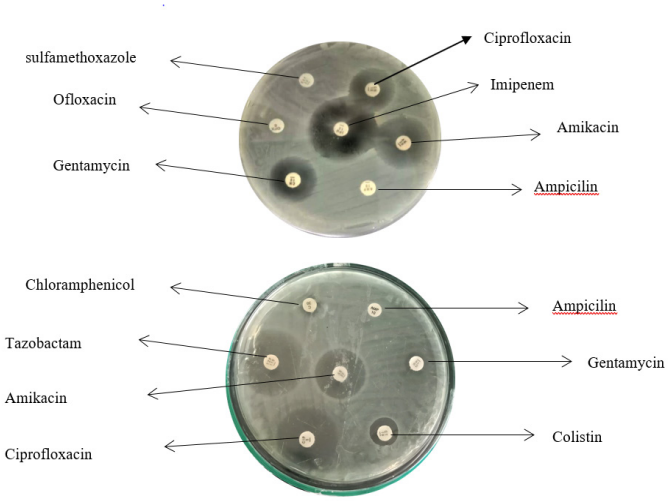

Figure 3: Antibiotic susceptibility test for *Salmonella*.

Molecular detection of antibiotic-resistant gene (bla$_{TEM}$*)*

Polymerase chain reaction was used to detect the *bla*$_{TEM}$ gene in *Salmonella*. The conditions that were used for PCR were: initial denaturation was done at 94°C for 30 sec, annealing was done at 54 °C for 30 sec and extension was carried out at 72°c for 1 min. All isolates of *S*almonella showed the presence of a *bla*$_{TEM}$-resistant gene (Colom *et al.*, 2003).

Results

Antimicrobial resistance profiling

Antimicrobial susceptibility test was performed with commonly used antibiotics as shown in Figure 3. All *Salmonella* isolates were resistant to ampicillin. 86.36% and 77.27% of *Salmonella* isolates showed resistance to Nalidixic acid and tetracycline respectively. Ciprofloxacin, chloramphenicol, gentamycin, and sulphamethoxazole were intermediate sensitive to *salmonella*. Ceftriaxone, tazobactam, and amikacin were the most sensitive drugs to *salmonella* 91%, 82%, and 75%, respectively Figure 4.

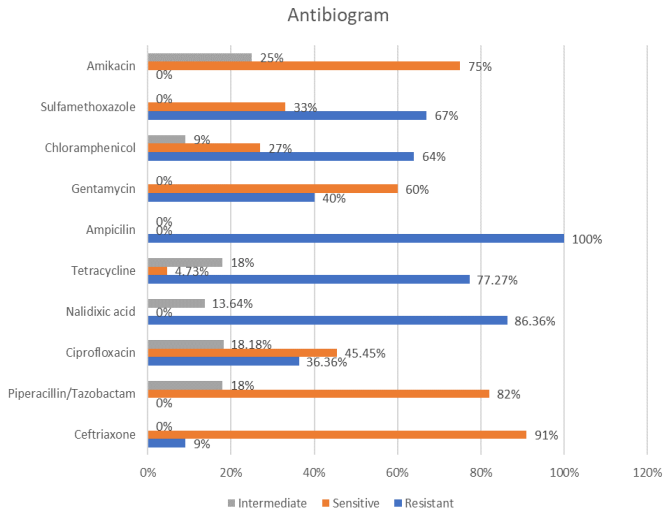

Figure 4: Graphical representation of antibiotic susceptibility pattern.

Molecular detection of antimicrobial-resistant gene

Polymerase chain reaction was used for the detection of *bla*$_{TEM}$ resistant gene in *salmonella* isolates. The primer sequence used to target the gene is given in Table 1. PCR showed most prevalent resistant gene was found to be *bla*$_{TEM}$. Agarose gel electrophoresis results are shown in Figure 5.

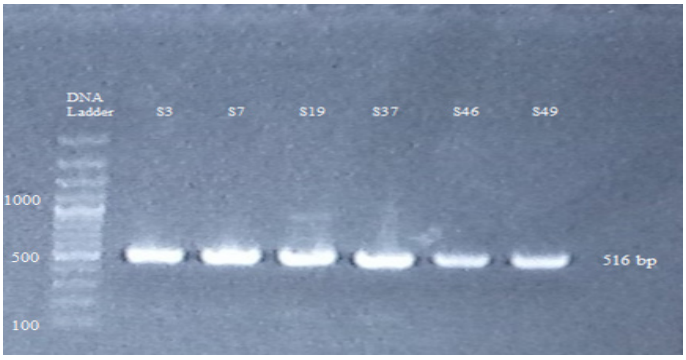

Figure 5: PCR results showing the presence of *bla*$_{TEM}$ in the isolates of *Salmonella*.

Discussion

The poultry sector is facing significant risks due to the prevalence of *Salmonella* in poultry. *Salmonella* is an important zoonotic pathogen that causes various diseases in poultry (Kuźmińska-Bajor *et al.*, 2023). It is transmitted through contaminated food to humans and causes

gastroenteritis and septicemia. According to estimates, *Salmonella* is responsible for 155,000 fatalities and 93 million gastrointestinal infections per year (Yu *et al.*, 2021).

In this study, *Salmonella* in poultry was examined for its prevalence, antibiotic resistance pattern, and the presence of antibiotic-resistant genes for β-lactam antibiotics. The samples were collected from Jhang, Punjab, Pakistan poultry sheds. Out of the 100 fecal samples, 22 found positive for *Salmonella*. Previous research conducted in Pakistan showed a 25.67% prevalence of *Salmonella* (Siddique *et al.*, 2021). This is almost similar to our results. Our results are also comparable to another study on *Salmonella* prevalence in Karachi, Pakistan, which reported a 21.34% rate of *Salmonella* prevalence (Hussain *et al.*, 2020). A previous study showed a higher prevalence of *Salmonella* than our results, finding a 38% prevalence in Hyderabad, Pakistan (Soomro *et al.*, 2010). Another study reported a higher prevalence of *Salmonella* in Punjab, Pakistan, at 44.66% (Yasmin *et al.*, 2020).

The disc diffusion method was used to assess *Salmonella* susceptibility to several antibiotics, revealing the bacterial resistance pattern to drugs. In the present study, all the isolates were found to be resistant to ampicillin, followed by 86.36% being resistant to nalidixic acid, and 72.27% being resistant to tetracycline. This is similar to a previous study in which ampicillin resistance was found in 76.66% of isolates, the highest resistance among other antibiotics (Rafiullah *et al.*, 2018). *Salmonella*'s strong resistance to ampicillin may indicate its overuse in common illnesses. Another study showed that isolated *Salmonella* strains from poultry exhibited 100% resistance to nalidixic acid. In this study, *Salmonella* showed 86.36% resistance to nalidixic acid, which is highly concordant with previous research. In a previous study, *Salmonella* demonstrated 77% resistance to tetracycline (Kayalvizhi and Antony, 2011). The present research showed 72.27% resistance to tetracycline, indicating that resistance to tetracycline has increased to a dangerous level due to the unusual or overuse of antibiotics in poultry.

In our study, Ceftriaxone was the most sensitive drug against *Salmonella* isolates. The previous study also showed that ceftriaxone was the most sensitive drug against *Salmonella* isolates (Veeraraghavan *et al.*, 2021). Another study found that Tazobactam was the most sensitive drug against *Salmonella* (Uddin, 2018). In our study, Tazobactam-Piperacillin was also among the most sensitive drugs against bacteria *Salmonella.*

Furthermore, the presence of the bla_{TEM} gene was detected in all the *Salmonella* isolates. The bacteria displayed resistance to different antibiotics specifically β-lactam antibiotics. The analysis of the genes (in bacteria) causing resistance to β-lactam antibiotics was done by using a polymerase chain reaction. The bla_{TEM} gene was found to be the most prevalent gene in the bacteria causing resistance. Previous studies also demonstrated that the bla_{TEM} gene was the most detected resistant gene in *Salmonella* (Igbinosa *et al.*, 2022). Other studies also confirmed the presence of bla_{TEM} resistant gene is *salmonella* isolates (Siddiky *et al.*, 2021).

This study has some limitations. The sample size was relatively small, with only 100 fecal samples, which might not fully represent the overall prevalence and resistance patterns of *Salmonella* in the region. Additionally, the study was geographically limited to Jhang, Punjab, Pakistan, which may not reflect the situation in other areas. Future research should also investigate the mechanisms behind antibiotic resistance in *Salmonella* and explore alternative strategies to combat this issue, such as the development of new antibiotics or the use of bacteriophage therapy.

Conclusions

This study indicates the high prevalence of antibiotic-resistant *Salmonella* in poultry, which poses a major health threat to the poultry sector. This study indicated that Tazobactam and Imipenem are the most effective drugs against *Salmonella* isolates. *Salmonella* isolates show maximum resistance to ampicillin, tetracycline, and Nalidixic acid. PCR showed positive results for the presence of bla_{TEM} resistant gene. Antibiotics and other antimicrobial agents should only be used when absolutely essential and in the appropriate doses. Overuse or incorrect use of these medications can contribute to the emergence of drug-resistant *Salmonella.* The study emphasizes the concern over the increase and dissemination of *Salmonella* variants that have developed resistance to frequently used antibiotics. Drug-resistant *Salmonella* strains can be difficult to treat because they do not respond to routine antibiotic treatments. There should be procedures put in place to strictly regulate the usage of antimicrobial drugs. This can involve rules for administering antimicrobial agents, controlling their use, and putting in place strategies to limit the spread of antibiotic-resistant microbes.

Declarations

Funding

The study received no external funding.

Data availability

The data supporting the findings of this study is included in the article. Additional datasets generated or analyzed during the study are available from the corresponding author upon reasonable request.

IRB approval and ethical statements

Study was approved by University of Agriculture

Faisalabad Pakistan Institutional Ethical Committee/ research committee with strict observance of animal rights during study.

Declaration of generative AI and AI-assisted technologies in the writing process

No generative AI and AI-assisted technologies were used in the writing process.

Conflict of interest

The authors have declared no conflict of interest.

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