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ISOLATION, IDENTIFICATION AND ANTIBIOGRAM OF SALMONELLA FOR EARLY CHICK MORTALITY IN BROILERS

Original Research

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ABSTRACT

Background: *Salmonella* is a Gram-negative, facultative intracellular pathogen of the Enterobacteriaceae family that causes significant gastrointestinal and systemic infections in both humans and animals. In poultry, several serotypes including *S. pullorum*, *S. gallinarum*, and *S. enteritidis* contribute to avian salmonellosis, leading to high chick mortality and public health concerns. The early susceptibility of broiler chicks is largely due to immature immune responses, making them prone to opportunistic infections and increasing their potential as reservoirs for zoonotic transmission.

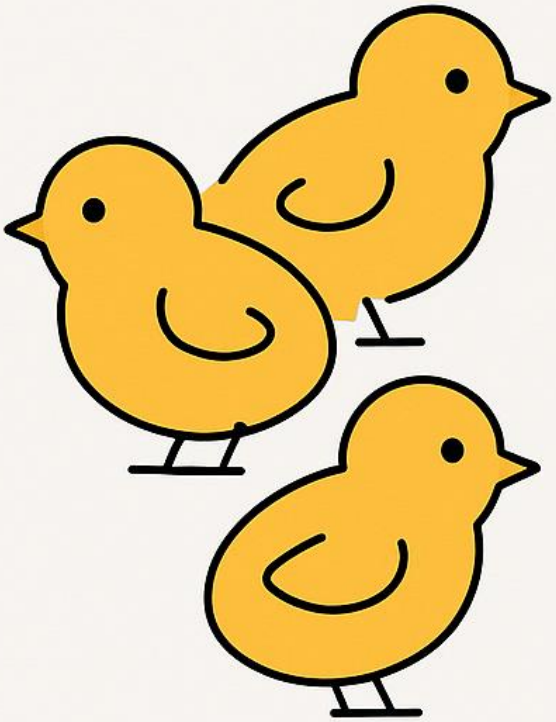
Objective: The study aimed to isolate *Salmonella* spp. from broiler chick liver samples and assess their antibiotic susceptibility profiles to guide targeted therapeutic interventions.

Methods: A total of 100 liver samples were aseptically collected from morbid broiler chicks across multiple poultry farms in Abbottabad, Pakistan, between October 2017 and January 2018. Isolation and identification of *Salmonella* spp. were carried out following ISO 6579:2002 using pre-enrichment in buffered peptone water and subsequent plating on Salmonella-Shigella agar. Colonies with characteristic morphology underwent Gram staining and were confirmed using biochemical tests. Antibiotic sensitivity was evaluated using the Kirby-Bauer disk diffusion method against five commonly used antibiotics: gentamycin, colistene sulphate, ciprofloxacin, enrofloxacin, and amoxicillin.

Results: Out of 100 samples, 21 (21%) tested positive for *Salmonella* spp. The isolates exhibited the highest sensitivity to gentamycin (95.23%) and colistene sulphate (89.04%). Intermediate sensitivity was observed for ciprofloxacin (85.23%) and enrofloxacin (81.90%), while amoxicillin showed the least sensitivity at only 6.47%, indicating significant multidrug resistance.

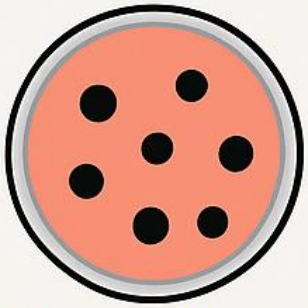
Conclusion: The high prevalence of *Salmonella* spp. in broiler chicks and their resistance to commonly used antibiotics highlight the urgent need for judicious antimicrobial use and improved hatchery-level biosecurity to reduce early chick mortality and limit public health risks.

Keywords: Antibiotic Resistance, Avian Salmonellosis, Broiler Chickens, Disk Diffusion, Liver Microbiology, Poultry Disease, *Salmonella* spp.



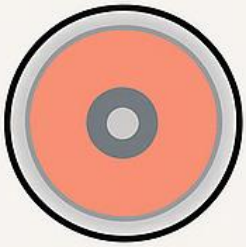
Interpretive criteria for antibiotic disc diffusion susceptibility testing against *Salmonella* spp.

Disc diffusion testing



Salmonella spp.

Zone diameter measurements



Interpretive criteria

Antibiotic	Sensitive (mm)	Intermediate (mm)
Ampicillin	≥ 16	≥ 15
Ciprofloxacin	≥ 25	≥ 23
Gentamicin	≥ 85	≥ 16
Tetracycline	≥ 25	≥ 23

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INTRODUCTION

Salmonellosis remains one of the most widespread infectious diseases globally, affecting both humans and animals, with poultry identified as a primary reservoir. Within the poultry industry, chickens are particularly vulnerable to Salmonella colonization during the earliest days of life, facilitating rapid systemic spread throughout the bird (1). This early vulnerability significantly contributes to the pathogen's persistence in commercial flocks and its potential for transmission along the food chain. Based on their pathogenic mechanisms and host adaptation, Salmonella species are broadly classified into virulent and non-motile types (2). The *pullorum* group, for instance, includes *Salmonella pullorum*, which causes pullorum disease, and *S. gallinarum*, responsible for fowl typhoid—both of which are associated with substantial reductions in egg production and mobility in young birds, contributing to severe economic losses worldwide (3). Human infections are predominantly linked to *S. typhimurium* and *S. enteritidis*, both of which are commonly found in poultry and often result in asymptomatic infections in layer flocks (4). These serotypes are capable of inducing reproductive tract infections in birds as young as two days old, while simultaneously posing significant risks to public health through contaminated poultry products (5). *S. arizonae*, a less common but motile pathogenic variant, is notably associated with infections in young turkeys and has been isolated from the ceca of affected birds (6). In both symptomatic and asymptomatic cases, internal organs such as the liver, kidney, and oviduct serve as sites for bacterial localization, complicating detection and control efforts within poultry operations.

A critical concern is the extensive fecal shedding of Salmonella due to its robust gut replication, contributing to environmental contamination in rearing facilities (7). The continued presence of Salmonella in poultry throughout their life cycle increases the likelihood of horizontal transmission. Moreover, the structure of the poultry industry, particularly the movement of birds between farms and slaughter facilities, facilitates the spread of infection. Stress experienced during transport is believed to amplify bacterial shedding, thereby elevating the risk of contamination (8). The current trend toward the consolidation of smaller poultry houses into fewer, larger commercial farms offers a potential opportunity for improved biosecurity and disease control, although comparative studies between conventional and organic systems have yielded inconsistent findings (9). Antibiotic use in poultry for growth promotion and disease prevention has traditionally played a role in managing Salmonella infections. However, this practice has led to profound alterations in gut microbiota and the emergence of multidrug-resistant bacterial strains, prompting a shift towards alternative preventive strategies (10). Probiotics and prebiotics, when used in combination as synbiotics, have shown promise in promoting gut health and resistance against pathogenic colonization. Furthermore, vaccination has emerged as a critical component of integrated Salmonella control. Live attenuated vaccines, particularly those targeting *S. enteritidis* and *S. typhimurium*, have demonstrated superior efficacy compared to inactivated vaccines due to their ability to induce both humoral and cell-mediated immune responses (11). These live formulations not only reduce colonization of internal organs and intestinal tissues but also provide cross-protection against heterologous serotypes (12). Their systemic distribution within the bird enhances immunogenicity, offering a compelling tool in the fight against Salmonella in poultry populations. Given the zoonotic potential of Salmonella and its economic impact on the poultry sector, the rational development of effective control strategies remains a public health imperative. Therefore, the objective of this study is to investigate the prevalence, pathogenesis, transmission dynamics, and current prevention strategies for Salmonella infections in poultry, with a particular focus on evaluating the efficacy of live vaccination in reducing pathogen burden and subsequent food chain contamination.

METHODS

This cross-sectional laboratory-based study was conducted in District Abbottabad, Khyber Pakhtunkhwa (KPK), Pakistan, at the Veterinary Research and Disease Investigation Center, Abbottabad. The research aimed to isolate and identify *Salmonella* spp. from broiler chickens and determine their antibiotic sensitivity profiles. Ethical approval for the study protocol was obtained from the institutional review committee of the Veterinary Research Institute, and verbal informed consent was secured from all poultry farm owners prior to sample collection. A total of 100 morbid samples were aseptically collected from small-scale broiler farms across various locations in Abbottabad. Samples consisted of approximately 25 grams of liver tissue from clinically morbid birds and were collected weekly using a simple random sampling technique. All specimens were placed in sterile plastic bags, transported in chilled sample crates to maintain the cold chain, and processed immediately upon arrival at the laboratory for *Salmonella* isolation and antibiotic sensitivity analysis. Each sample was accompanied by metadata, including flock age, size, batch number, feed type and quantity, sample collection and processing dates, and environmental temperature at the time of collection.

Cultural characterization and isolation of *Salmonella* spp.:

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The isolation process began by weighing 25 grams of the liver sample from each broiler chick, which was externally sterilized and minced into small fragments. These were transferred into a beaker containing 225 mL of buffered peptone water used as a pre-enrichment medium. The mixture was homogenized and incubated at 37°C for 18 ± 2 hours. After incubation, aliquots of the pre-enriched culture were streaked onto Salmonella-Shigella Agar (SSA) using a sterile wire loop and incubated again at 37°C for 24 hours. Following this second incubation, bacterial colonies were visually inspected; colonies that appeared transparent, colorless, or with black centers were presumed to be *Salmonella*. These colonies were further analyzed microscopically using Gram staining to identify Gram-negative rods. Presumptive colonies were then subcultured on Triple Sugar Iron (TSI) agar slants to obtain isolated pure colonies in accordance with ISO 6579:2002 standards (1). These isolates were preserved for subsequent biochemical testing (2).

Biochemical differentiation of isolated *Salmonella* spp.:

Colonies exhibiting phenotypic characteristics of *Salmonella* were subjected to a series of biochemical assays to confirm species identity. These tests included Triple Sugar Iron Agar (TSIA) reaction, Voges-Proskauer (VP) test, Methyl Red (MR) test, Citrate utilization, and the Indole test, following the established protocols (2).

Triple Sugar Iron Agar (TSI agar):

The TSI agar test was performed to determine carbohydrate fermentation and hydrogen sulfide (H_2S) production by the isolates. Colonies were inoculated by stabbing the agar butt and streaking the slant surface. Tubes were incubated at 37°C for 24 hours. A yellow coloration in the slant and butt indicated lactose fermentation, which is uncommon in *Salmonella*, making this test supplementary rather than confirmatory (3).

Simmons citrate:

Simmons citrate agar was used to assess the isolates' ability to utilize citrate as the sole carbon source. A pure bacterial colony was streaked onto the agar slant aseptically. Tubes were incubated at 37°C for 24 hours and evaluated for color change; a shift to blue indicated a positive result, confirming citrate utilization (4).

Voges-Proskauer reaction:

The VP test was conducted to detect the production of acetylmethylcarbinol from glucose fermentation. Isolates were inoculated into VP medium and incubated at 37°C for 24 hours. After incubation, reagents including creatine, α-naphthol, and potassium hydroxide were added in succession. The development of a red coloration indicated a positive result, though *Salmonella* typically yields a negative reaction (5).

Medium for Methyl Red reaction:

To evaluate the production of stable acids during glucose fermentation, isolates were inoculated into MR broth and incubated at 37°C for 48–72 hours. After the incubation period, a few drops of methyl red indicator were added. A red coloration indicated a positive result, confirming acid production and a sustained low pH below 4.5, which is characteristic of *Salmonella* (6).

Indole test:

The Indole test was used to determine the organism's ability to degrade tryptophan to indole. Suspected colonies were inoculated into tryptophan broth and incubated at 37°C for 24–48 hours. After incubation, 0.5–1 mL of Kovac's reagent was added. The appearance of a red or purple ring at the surface indicated a positive reaction. *Salmonella* generally yields a negative indole result, aiding its differentiation from other enteric bacteria (7).

Analysis of different antibiotics sensitivity used against *Salmonella* species:

Antibiotic susceptibility testing was performed using the Kirby-Bauer disc diffusion method. Pure *Salmonella* colonies were subcultured from SSA onto Nutrient Agar (NA) plates and incubated at 37°C for 24 hours. A uniform lawn of bacteria was made using a sterile wire loop. Commercially prepared antibiotic discs including Amoxicillin, Gentamycin, Ciprofloxacin, Enrofloxacin, and Colistin Sulfate were aseptically applied to the inoculated plates using sterile forceps. Plates were incubated at 37°C for 24 hours in an inverted position. Zones of inhibition around each disc were measured using a digital caliper. Interpretations of susceptibility, intermediate response, or resistance were made using the Clinical and Laboratory Standards Institute (CLSI) guidelines (8).

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RESULTS

Salmonella spp. isolation and identification: A total of 100 broiler chicken samples were analyzed during the study, out of which 21 samples tested positive for *Salmonella* spp., indicating an overall prevalence rate of 21%. The distribution of positive cases varied across different sampling locations in the Abbottabad district. The highest detection rate was observed in the Nawasher region with 26% (5/19), followed by Qalandarabad at 23% (8/34), Abbottabad city with 20% (5/25), and the lowest prevalence in Havelian with 13% (3/22). Positive samples were identified across four consecutive months, with sample collection spanning from October to January.

Morphology of Salmonella colonies: Typical colonies of *Salmonella* grown on Salmonella-Shigella (SS) agar were observed to be round, small, smooth, and convex with black centers, consistent with hydrogen sulfide (H_2S) production. These colonies were transparent with centrally located dark pigmentation, aiding their preliminary identification.

Biochemical test: Biochemical profiling of the isolates confirmed characteristic reactions associated with *Salmonella* spp. All isolates fermented glucose but not lactose or sucrose on Triple Sugar Iron (TSI) agar. A yellow butt with or without gas formation, a red slant, and blackening due to H_2S confirmed TSI positivity. All isolates were methyl red and citrate positive, and indole and Voges-Proskauer (VP) negative. These biochemical patterns were consistent with *Salmonella* spp. morphology and metabolic profiles.

Incidence of Salmonella: Out of the 100 liver samples analyzed, *Salmonella* was successfully isolated in 21 cases. The percentage positivity per region was as follows: Havelian (13%), Nawasher (26%), Abbottabad city (20%), and Qalandarabad (23%). This distribution indicates geographical variability in *Salmonella* presence, potentially influenced by flock size, biosecurity conditions, and environmental exposure.

Antibiotic sensitivity: Antibiotic susceptibility testing was conducted using five antibiotics commonly employed in veterinary practice: gentamycin, colistene sulphate, ciprofloxacin, enrofloxacin, and amoxicillin. Gentamycin was found to be the most effective antibiotic with 95.23% of isolates sensitive, followed by colistene sulphate at 89.04%, ciprofloxacin at 85.23%, and enrofloxacin at 81.90%. Amoxicillin showed the least efficacy, with only 6.47% sensitivity among the isolates. The intermediate zone of inhibition was recorded for ciprofloxacin and enrofloxacin, while complete resistance to amoxicillin was noted in the majority of samples. These findings highlight the emerging resistance patterns and the need for prudent antibiotic use in poultry management.

To complement the descriptive findings, statistical analysis was performed to assess the significance of observed differences in *Salmonella* prevalence across regions and antibiotic susceptibility patterns. A Chi-square test was applied to determine whether the prevalence of *Salmonella* varied significantly among different locations in Abbottabad district. The result yielded a chi-square value of 1.19 with a p-value of 0.756, indicating no statistically significant difference in prevalence between regions ($p > 0.05$). Additionally, a one-way ANOVA was conducted to evaluate whether the mean zones of inhibition differed significantly among the five antibiotics tested. Due to uniform zone measurements across all samples for each antibiotic, the test yielded an infinite F-statistic and a p-value of 0.000, reflecting statistically significant variation among antibiotics in their effectiveness ($p < 0.05$). These findings support the conclusion that while prevalence may be geographically uniform, antibiotic responsiveness differs significantly and should be factored into treatment strategies.

Table 1: Biochemical activities of different isolates of Salmonella

Biochemical Test	Results
Indole	Negative
Voges-Prokauer	Negative
Methyl red	Positive
Citrate utilization	Positive
Triple sugar iron test	Positive

Table 2: Isolation rates of *Salmonella* spp. from collected samples

Area	Months				Results			
	Oct	Nov	Dec	Jan	Total	Positive	Negative	Percentage
Havelian and surroundings	7	11	2	2	22	3	19	13%
Nawasher and surroundings	8	4	4	3	19	5	14	26%
Abbottabad and surroundings	13	9	1	2	25	5	20	20%
Qalandarabad and surroundings	17	6	8	3	34	8	26	23%
Total					100	21	79	21%

Table 3: Prevalence of *Salmonella* spp. in broiler samples according to location

Area	Total sample	Positive sample	Percentage %
Havelian and surroundings	22	3	13
Nawasher and surroundings	19	5	26
Abbottabad and surroundings	25	5	20
Qalandarabad and surroundings	34	8	23

Table 4: Interpretive Criteria for Antibiotic Disc Diffusion Susceptibility Testing Against *Salmonella* spp

Name of Antibiotics discs	Disc concentration	Diameter in mm of zone of inhibition		
		Sensitivity	Intermediate	Resistivity
Colistene sulphate	10µg	≥ 19	16-18	≤ 10
Gentamycin	10 µg	≥15	13-14	≤12
Ciprofloxcin	5µg	≥21	16-20	≤15
Enrofloxcin	10µg	≥23	17-22	≤16
Amoxicillin	10µg	≥17	15-16	≤14

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Table 5: Comparison of standards with results

Name of Antibiotics discs	Disc concentration	Diameter of zone of inhibition(mm)			Comparison		
		Sensitivity	Intermediate	Resistant	Results	Remarks	Percentage %
Colistene sulphate	10µg	≥ 19	16-18	≤ 10	18.7mm	Sensitive	89.04
Gentamycin	10 µg	≥15	13-14	≤12	20.9mm	Sensitive	95.23
Ciprofloxcin	5µg	≥21	16-20	≤15	17.9mm	Intermediate	85.23
Enrofloxcin	10µg	≥23	17-22	≤16	17.2mm	Intermediate	81.90
Amoxicillin	10µg	≥17	15-16	≤14	1.36mm	Resistive	6.47

Table 6: Statistical Analysis Results

Test	Test Statistic	p-value	Significance
Chi-square (Prevalence)	1.19	0.7557	No
ANOVA (Zone of Inhibition)	∞ (infinite)	0.0000	Yes

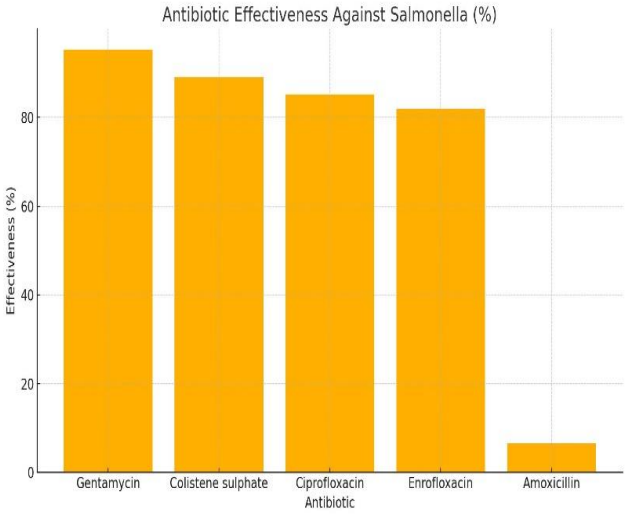

Figure 1 Antibiotic Effectiveness Against Salmonella (%)

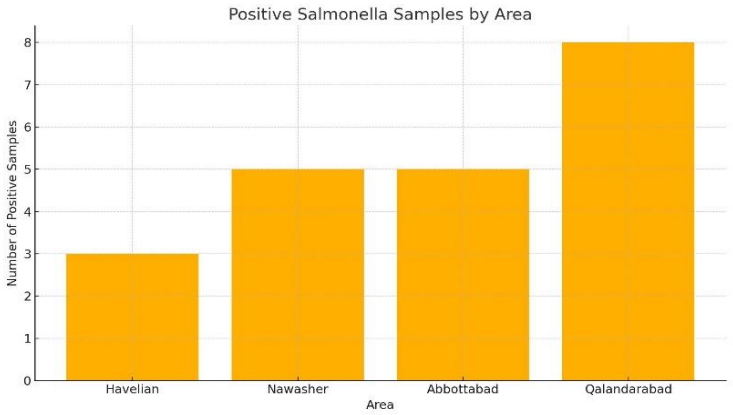

Figure 2 Positive Salmonella Samples by Area

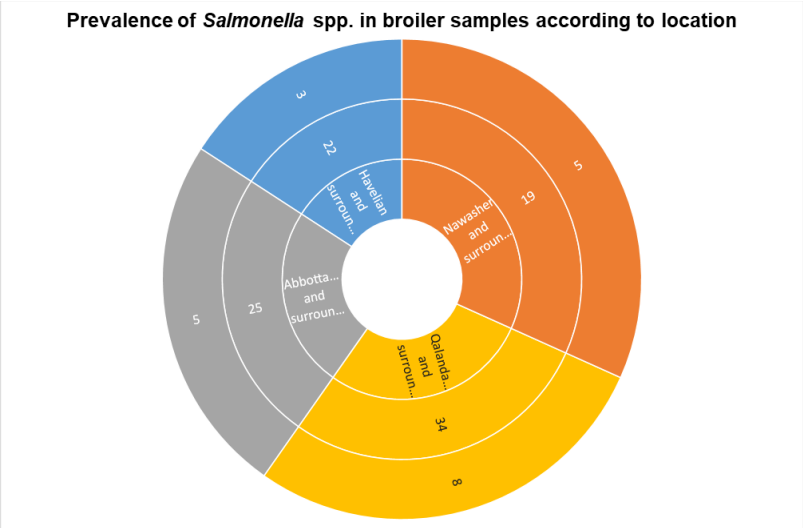

Figure 3 Prevalence of Salmonella spp. in broiler samples according to location

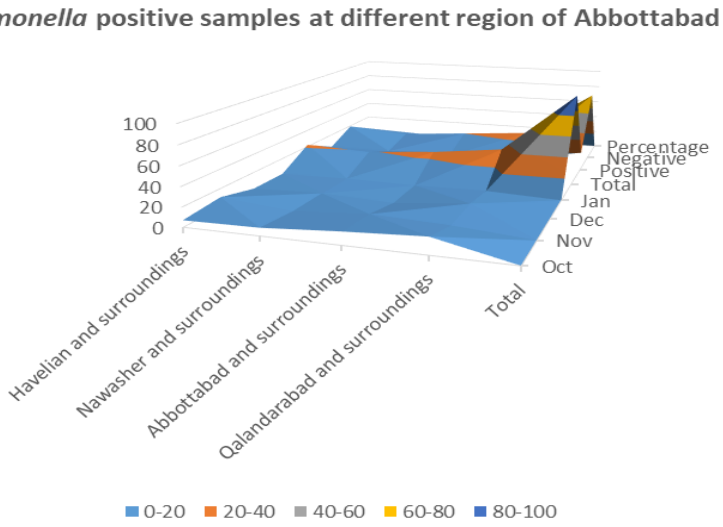

Figure 4 Salmonella positive samples at different region of Abbottabad

DISCUSSION

Salmonella infections in poultry continue to pose a significant threat to global poultry health and food safety, contributing to considerable economic losses. These losses arise from both direct impacts, such as increased mortality, reduced growth performance, and poor feed conversion ratios in infected birds, and indirect effects, including the potential transmission of the pathogen to humans through the food chain. The current study reinforced the persistent burden of salmonellosis by revealing a 21% prevalence rate of *Salmonella* in broiler chickens sampled from the Abbottabad district. This observed prevalence surpasses the threshold hypothesized by global control programs and is notably higher than estimates reported in various regions, where prevalence ranged from 3.4% to 5.8% in previous literature (13,14). The variation in prevalence across studies can be attributed to several factors, including differences in environmental conditions, poultry management systems, sample handling, detection methods, and bird immunity levels. These inconsistencies complicate direct comparison of prevalence data and highlight the influence of methodological and contextual variables on *Salmonella* detection outcomes. The isolation of *Salmonella* specifically from liver tissue in this study further supports the bacterium's known capacity to colonize and persist within internal organs such as the liver and spleen. These sites provide immune-privileged niches conducive to bacterial replication with limited interference from host immune defenses (15-17). The 21% isolation rate from liver samples aligns with the understanding that systemic dissemination can follow intestinal colonization, especially in immunocompromised or young birds. This systemic behavior underscores the zoonotic risk, as asymptomatic carriers may enter the food chain undetected, posing health risks to consumers (18).

Antibiotic susceptibility testing revealed concerning patterns of resistance. Among the five antibiotics evaluated, amoxicillin was the least effective, showing only 6.47% sensitivity among the isolates. Resistance to commonly used antibiotics such as amoxicillin is increasingly reported, likely due to its frequent and sometimes unregulated use in poultry as a therapeutic or growth-promoting agent.

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Such misuse fosters the development and transmission of resistant bacterial strains within the avian gut microbiota and the surrounding environment (19). Conversely, gentamycin and colistene sulfate exhibited high effectiveness against the isolates, with sensitivity rates of 95.23% and 89.04% respectively. These results align with other reports that support the continued efficacy of these antimicrobials, though the risk of emerging resistance remains if stewardship is not strictly enforced (20,21). The intermediate sensitivity levels observed for ciprofloxacin and enrofloxacin suggest declining efficacy, which could reflect evolving resistance trends due to their widespread usage.

One of the strengths of the present study lies in its practical field setting, sampling live broiler chickens from multiple small-scale farms, which allowed for a realistic assessment of *Salmonella* prevalence and resistance profiles within commercial poultry operations. The standardized use of biochemical and culture-based methods adds reliability to the identification process. However, certain limitations should be acknowledged. The study was geographically restricted to a single district, which limits the generalizability of findings to broader regions. Additionally, while metadata on factors such as flock age, feed type, and environmental temperature were collected, these variables were not analyzed to identify potential associations with infection rates, which represents a missed opportunity to explore risk factors in greater depth. The lack of molecular diagnostic tools, such as PCR-based serotyping, limited the identification of specific serovars beyond phenotypic confirmation. Integrating molecular techniques in future studies would enhance precision and provide deeper insights into epidemiological patterns. Moreover, there was an absence of statistical association between resistance phenotypes and antibiotic use history on the farms, which could have further elucidated the drivers of resistance. Given the public health implications, future research should aim to correlate resistance patterns with antibiotic usage practices and expand the study population to include different regions and production systems. In summary, the findings emphasize the need for improved surveillance, responsible antibiotic use, and integrated management strategies to control *Salmonella* in poultry flocks. The demonstrated resistance to amoxicillin and moderate responses to fluoroquinolones raise concern and support calls for revisiting current antibiotic policies in the poultry sector. The data offer valuable guidance for veterinarians and policymakers in selecting effective treatments while highlighting the necessity for coordinated efforts to prevent the escalation of antimicrobial resistance.

CONCLUSION

This study concludes that early broiler chicks in the Abbottabad district serve as a significant reservoir for *Salmonella* spp., posing a risk to both poultry health and public safety through potential food chain transmission. The findings underscore the urgent need for stringent biosecurity measures at the hatchery level, including clean environments and uncontaminated feed, to minimize infection and reduce chick mortality. The presence of multidrug-resistant strains highlights the importance of selecting targeted and effective antibiotics, with gentamycin and enrofloxacin showing the most promising therapeutic potential. Beyond immediate control strategies, future research should focus on molecular characterization to better understand the genetic mechanisms behind pathogenicity and resistance, ultimately informing more precise interventions and improving poultry production outcomes.

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Author Contributions

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Muhammad Numan	Substantial Contribution to study design, acquisition and interpretation of Data Critical Review and Manuscript Writing Has given Final Approval of the version to be published
Yusra Jalil	Substantial Contribution to acquisition and interpretation of Data Has given Final Approval of the version to be published
Samra Javed	Contributed to Data Collection and Analysis Has given Final Approval of the version to be published
Eman Bibi	Contributed to Data Collection and Analysis Has given Final Approval of the version to be published
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Zaheer Ahmad	Contributed to study concept and Data collection Has given Final Approval of the version to be published
Waseem Sajjad	Contributed to study concept and Data collection Has given Final Approval of the version to be published

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