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Antimicrobial Resistance and Prevalence of *Salmonella* in Live Bird Markets: A Cross-Sectional Study

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Abstract

The poultry industry of Pakistan holds a substantial economic value, providing bread and butter to 1.5 million individuals and sharing 14% of the national GDP. Poultry diseases, in particular salmonellosis, hinder the advancement and yield of the poultry industry. *Salmonella*, the microbial culprit behind the disease, is a zoonotic food-borne pathogen and is transmitted through eggs, poultry byproducts, hatcheries, and other environmental factors. Multidrug resistance in *Salmonella* serotypes has emerged as a pressing public health problem, attributed to the unchecked use of antibiotics in food animals. *Salmonella* infections can pose notable economic losses to the poultry industry and human health. The immunocompromised individuals, particularly infants and the elderly, encompass the high-risk age groups. Moreover, the shift of the poultry industry towards modernized and intensified farming systems may worsen the issue if not addressed properly. It is, therefore, the need of the hour to implement effective disease control measures, promote responsible antibiotic use, animal husbandry practices, and ensure food safety practices. This study was conducted to evaluate the prevalence of and antimicrobial susceptibility testing of *Salmonella* isolated from live bird markets in Islamabad and Rawalpindi. A total of 180 samples, from the live bird markets in Islamabad and Rawalpindi, were analyzed, with 72 samples (40%) testing positive for *Salmonella*. The prevalence varied across sample types: 28/60 (46.7%) in muscle samples, 26/60 (43.3%) in liver samples, and 18/60 (30%) in cloacal samples. Antimicrobial susceptibility testing revealed resistance to tobramycin, vancomycin, sulfamethoxazole/trimethoprim, piperacillin /tazobactam, and oxytetracycline. The isolates showed intermediate susceptibility against gentamicin and ciprofloxacin, while against florfenicol remained sensitive.

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Introduction

Agriculture is the cornerstone of the economic development of a lower-middle-income country (LMIC) like Pakistan. Crop cultivation and animal husbandry are the key components of this sector. The sector contributes 21% to the country's GDP and has a considerable growth rate of 2.7% annually. It is the breadwinner for about 62% of the agrarian population and employs 44% of the manpower [1]. About 63.1% of the population of Pakistan constitutes the rural population, with agriculture being their only source of earning [2]. The importance of this sector can also be manifested by its contribution of 19% to the country's GNP [3]. These facts and figures have undergone profound changes over seventy years due to multifactorial reasons, with climate change being one of them. Moreover, with the exponential rise in population, food consumption increases, necessitating a surge in agricultural production and shifting the dynamics toward mechanized farming. This shift is crucial in ensuring food security [2]. Livestock is the largest subsector of agriculture, facilitating the daily sustenance of over eight million families, with 35-40% of their income relying on livestock farming. This subsector also contributes to 3.1% of the country's exports and 11.4% to its AGDP [4]. Furthermore, livestock rearing constitutes a substantial 56% of the total agricultural value-added services. With the increase in food consumption and evolving dietary habits over the last decade, increased emphasis is being placed on livestock production and animal husbandry, resulting in economic growth and qualitative improvements. The subsector's growth rate for the year 2014-15 was recorded to be 2.9% [5]. The poultry industry in Pakistan is one of the largest agriculturally based sectors, providing a livelihood for approximately 1.5 million citizens. Pakistan ranks as the 11th largest meat-producing country globally, with an annual production of 1,163 million broilers. Poultry meat accounts for 35% of the total meat production [6]. With a significant investment exceeding Rs. 1056 billion, the poultry industry has experienced substantial growth of nearly 7.3 % per annum over the past decade. The estimated population of commercial poultry reached 1792.46 million (1703 million broilers and 73.28 million layers) by 2022-23 [7]. The livestock sector, which includes poultry, contributes 62% to agricultural output and holds a significant share of 14% of the national GDP. The poultry sector is shifting toward a modernized and intensified farming system [8]. The prevalence of poultry diseases poses a major barrier to the advancement and profitability of Pakistan's poultry sector. The most common bacterial diseases impacting the poultry industry in Pakistan are salmonellosis, colibacillosis, mycoplasmosis, infectious coryza, and fowl cholera [9]. The two most significant diseases of poultry caused by *Salmonella* species are fowl typhoid and pullorum disease. *Salmonella gallinarum* is associated with fowl typhoid, while *Salmonella pullorum* is linked to pullorum disease [10].

Salmonella is a gram-negative, facultative anaerobic bacillus and is a member of the *Enterobacteriaceae* family. Approximately 2600 serotypes of the genus Salmonella have been recognized to date, with most being capable of acclimatizing various animal and human hosts. Salmonella is considered a zoonotic food-borne pathogen known to be isolated from poultry, eggs, and dairy items [11]. The key serovars of avian salmonellosis, *Gallinarum* and *Pullorum*, are transmitted vertically and horizontally through eggs and hatcheries. In addition, environmental factors such as contaminated air, litter, water, and vectors contribute to the contamination of *Salmonella* at poultry farms [12]. Poultry is the primary source of non-typhoidal serotypes of *Salmonella* in food animals, those associated with human salmonellosis [13]. Fowl typhoid and pullorum disease, two avian salmonellosis-related diseases, manifest comparable signs and symptoms. These diseases primarily target two susceptible populations: young and adult birds [14]. Unlike the avian salmonellosis described earlier, zoonotic non-typhoidal salmonellosis causes gastroenteritis, bacteremia, and focal infection, primarily in immunocompromised individuals [15]. The emergence of multidrug resistance in *Salmonella* serotypes is yet another global public health concern and is associated with the widespread use of antibiotics in food animals. Antibiotics are used in food animals for two key purposes: as growth promoters to enhance weight gain, and for the treatment of bacterial diseases [16]. *Salmonella* poses a dual threat, not only causing significant economic losses to the poultry industry but also entailing substantial risk to human health [17]. Salmonella, a food-borne zoonotic bacterium, poses a formidable challenge to both the public health and economic viability of Pakistan's poultry industry. Furthermore, the unchecked and illogical utilization of antibiotics across human and animal healthcare sectors is the dominant force behind the rapid and exponential rise of drug resistance. AMR poses an alarming threat to global health security due to the development, spread,

and resilience of multidrug-resistant strains of pathogens, commonly referred to as 'superbugs,' extended throughout the interconnected triad of animal, human, and environmental ecosystems. Salmonella is a high-priority pathogen listed globally, recognized for intensive monitoring and research, particularly in the quest to discover novel antimicrobials and elucidate the mechanisms driving the development of antimicrobial resistance (AMR). Therefore, estimation of prevalence coupled with antimicrobial screening of *Salmonella* in poultry is significant for unraveling the epidemiology of MDR *Salmonella* isolates and effectively combating AMR at the animal-human interface. This study was conducted to achieve the following objectives: isolation and identification of *Salmonella* from live bird markets in Islamabad and Rawalpindi, and antimicrobial screening of *Salmonella* for antimicrobial resistance against different antibiotics.

Materials and Methods

Study area

The study area comprised the administrative areas of Islamabad and Rawalpindi. A total of 180 samples were collected from commercial broilers. Specifically, four markets (two in Islamabad and two in Rawalpindi) were randomly selected using multi-cluster sampling techniques according to the reference article [18]. Within each market, three bird shops were randomly chosen.

Fig. 1 Criterion of sample collection.

Sample collection

Samples were collected from the cloaca, liver, and muscles of five randomly selected birds. This resulted in 45 samples from each market, taken from 15 birds, ultimately yielding a total sample size of 180 (Fig. 1). All samples were collected and processed separately. Samples were obtained from live bird markets immediately post-slaughter, with strict adherence to sanitary protocols. A lab coat was worn during the retrieval of samples. The samples were then carefully placed in Ziplock bags or sterile containers, labelled, and stored on ice blocks during transport to maintain the cold chain. Upon arrival at the laboratory on the same day, the samples were refrigerated at 4°C for further analysis. Aseptic conditions were maintained to avoid the intrusion of undesirable pathogens or impurities that could hinder lab analysis.

Isolation of *Salmonella*

All samples were cultured onto sterile nutrient agar (HiMedia, Mumbai, Maharashtra, India). This step was followed by inoculation on differential media, *i.e*., MacConkey agar (HiMedia, Mumbai, Maharashtra, India). Presumptive colorless colonies of *Salmonella* isolates were selected and streaked onto selective media, *i.e*., xylose lysine deoxycholate agar (HiMedia, Mumbai, Maharashtra, India). Red colonies with black centers were isolated. Each step of inoculation was followed by 24 hours of incubation at 37°C [19]. These colonies were isolated and subjected to identification.

Identification of *Salmonella*

The bacterial colonies were identified and confirmed based on the colony morphology, Gram staining, biochemical (catalase, citrate utilization test), and sugar fermentation tests [20].

Prevalence of *Salmonella*

The percentage prevalence of *Salmonella* was calculated by dividing the number of positive samples obtained by the total number of samples collected from commercial broilers.

$$\text{Prevalence (\%)} = \frac{Number\ of\ samples\ positive}{Number\ of\ samples\ tested} \times 100$$

Antimicrobial susceptibility test

A bacterial suspension of *Salmonella* isolates was prepared in nutrient broth from an overnight culture**.** The suspension was swabbed evenly onto Müller-Hinton agar (Oxoid, Basingstoke, Hampshire, United Kingdom) using a cotton swab, following the Kirby-Bauer method for an antimicrobial susceptibility test to assess antibiotic resistance. The antibiotic disks were placed onto the agar surface (Table 1). The antibiotic discs (Bioanalyse) were placed on the agar plate, ensuring a minimum distance of 24 mm between each disc. The plates were incubated at 35-

Table 1 Antibiotics used to determine the antimicrobial susceptibility of *Salmonella* species.

Antibiotics	Concentration (µg)
Tobramycin	10
Sulfamethoxazole/trimethoprim	25
Piperacillin/Tazobactam	110
Gentamycin	10
Ciprofloxacin	5
Vancomycin	30
Florfenicol	30
Oxytetracycline	30

37°C for 16-24 hours. The zone of inhibition (clear area around each disk) of antibiotic discs was measured and compared with CLSI standards to determine their resistance patterns [21]. A total of 8 antibiotic discs were used for performing AST, namely: Tobramycin (TOB), Vancomycin (VA), Sulfamethoxazole/trimethoprim (SXT), Gentamycin (CN), Piperacillin /tazobactam (TPZ), Ciprofloxacin (CIP), Florfenicol (FFC), and Oxytetracycline (OT). The experiments were performed in triplicate to calculate the mean zone of inhibition along with the standard error of the mean.

Statistical analysis

Standard deviation, mean, and standard error were used as statistical tools to analyze the results.

Results

Following the culture of samples on nutrient agar, MacConkey agar, and XLD agar, the presumptive *Salmonella* colonies were successfully isolated. On nutrient agar, the presumptive *Salmonella* colonies appeared to be smooth, translucent, off-white to slightly pinkish, and round. On MacConkey agar, the presumptive *Salmonella* colonies exhibited colorless colonies. MacConkey agar, a differential medium, differentiated *Salmonella* from other members of the Enterobacteriaceae family based on lactose fermentation. Colorless colonies on MacConkey agar suggested non-lactose fermenting colonies of *Salmonella,* in contrast to the pink lactose fermenting colonies of the *Enterobacteriaceae* family. On XLD agar, the *Salmonella-positive* colonies showed up as red colonies with a black center (Fig. 2). *Salmonella* appeared as pink or red Gram-negative bacilli on Gram staining under the microscope. The *Salmonella-positive* colonies formed bubbles on the glass slide, in contrast to the catalase-negative bacteria that do not form bubbles. *Salmonella* showed a color change upon the citrate utilization test from green to blue, in contrast to those that do not change color. *Salmonella* positive isolates showed the following reactions on sugar fermentation tests:

Fig. 2 *Salmonella*-positive colonies obtained following the steps of isolation.

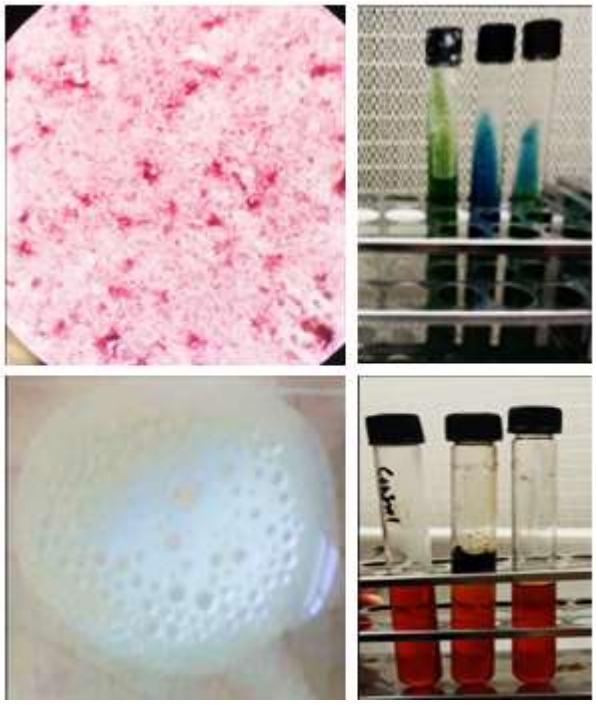

Fig. 3 Gram staining and biochemical tests for the identification of *Salmonella.*

Table 2 Comparison of the prevalence of *Salmonella* in the samples isolated from the twin cities.

Prevalence of *Salmonella* (%)	Islamabad	Rawalpindi
Muscle samples	46.67%	46.67%
Liver samples	23.33%	63.33%
Cloacal Samples	26.67%	33.33%
Overall prevalence	32.22%	53.75%

Table 3 Antibiotic sensitivity test of *Salmonella* isolated from muscle samples.

Antibiotics	Muscle samples			
	Zone of Inhibition (mm)	Antibiotic susceptibility		
		Sensitive	Intermediate	Resistant
Tobramycin	9±0.33	×	×	√
Vancomycin	7±0.33	×	×	√
Sulfamethoxazole/trimethoprim	8±0.58	×	×	√
Gentamycin	13±0.19	×	√	×
Piperacillin /tazobactam	14±0.33	×	×	√
Ciprofloxacin	24±0.09	×	√	×
Florfenicol	31±0.45	√	×	×
Oxytetracycline	14±0.07	×	×	√

Table 4 Antibiotic sensitivity test of *Salmonella* isolated from liver samples

Antibiotics	Liver samples			
	Zone of Inhibition (mm)	Antibiotic susceptibility		
		Sensitive	Intermediate	Resistant
Tobramycin	9±0.09	×	×	√
Vancomycin	7±0.15	×	×	√
Sulfamethoxazole/trimethoprim	7±0.17	×	×	√
Gentamycin	14±0.12	×	√	×
Piperacillin /tazobactam	14±0.17	×	×	√
Ciprofloxacin	24±0.22	×	√	×
Florfenicol	30±0.17	√	×	×
Oxytetracycline	14±0.19	×	×	√

Table 5 Antibiotic sensitivity test of *Salmonella* isolated from cloacal samples

Antibiotics	Cloacal samples			
	Zone of Inhibition (mm)	Antibiotic susceptibility		
		Sensitive	Intermediate	Resistant
Tobramycin	10±0.15	×	×	√
Vancomycin	7±0.1	×	×	√
Sulfamethoxazole/trimethoprim	9±0.15	×	×	√
Gentamycin	14±0.15	×	√	×
Piperacillin /tazobactam	15±0.33	×	×	√
Ciprofloxacin	24±0.17	×	√	×
Florfenicol	32±0.15	√	×	×
Oxytetracycline	14±0.23	×	×	√

alkaline slant (red color of the media indicating no lactose or sucrose fermentation), acidic butt (yellow butt indicating glucose fermentation), and hydrogen sulfide production (indicated by blackening/ darkening of medium). Negative isolates do not show any color change. (Fig. 3). The prevalence of *Salmonella* was calculated by applying the aforementioned formula. Moreover, the comparison of the prevalence calculated from both cities is given in Table 2.

$$Overall\ prevalence\ (\%) = \frac{Number\ of\ samples\ positive}{Number\ of\ samples\ tested} \times 100$$

Following 24 hours of incubation, a clear zone developed around the antibiotic discs. The zone of inhibition of antibiotic discs was calculated (Fig. 4). Statistical tools, namely, mean, standard deviation, and standard error of mean, were applied to analyze the results statistically. *Salmonella* positive isolates, collected from muscle (Table 3), liver (Table 4), and

cloaca (Table 5) showed resistance against tobramycin, vancomycin, sulfamethoxazole/ trimethoprim, piperacillin/ tazobactam, and oxytetracycline. while gentamycin and ciprofloxacin exhibited intermediate susceptibility, only florfenicol was sensitive to the *Salmonella* isolates.

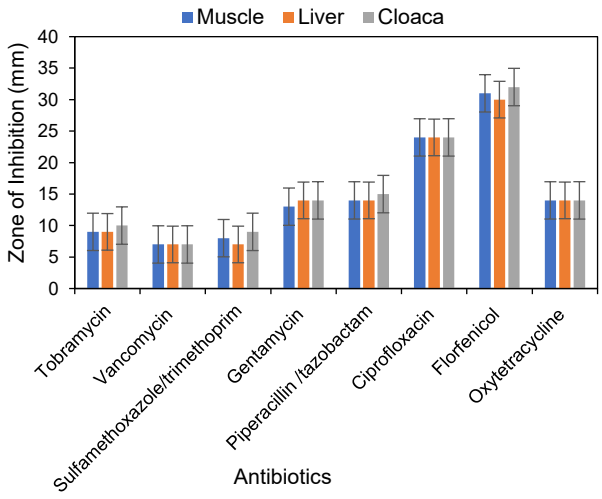

Fig. 4 Graphical depiction of antibiotic sensitivity test against *Salmonella*-positive isolates from commercial broilers.

Discussion

Salmonella species pose a significant health hazard, causing widespread enteropathies and mortalities. Poultry, in particular, is vulnerable to Salmonella species, causing avian salmonellosis that compromises flock health and productivity, ultimately resulting in economic losses. The food chain is a pivotal driver in the transmission of salmonellosis to humans, with poultry and poultry products serving as a chief source of contamination [22]. The emergence of antimicrobial resistance is another alarming threat to public health, as it jeopardizes the effectiveness of treatments and exacerbates the transmission risk. The misuse and overuse of antibiotics in different settings have worsened this budding concern. The connection between antibiotic-resistant *Salmonella* and contaminated animal-based food suggests the call for immediate action. The World Health Organization has identified *Salmonella*, encouraging efforts to develop novel treatments and prevent this burning issue [23].

A total of 180 samples, from live bird markets in Islamabad and Rawalpindi, were analyzed, with 72 samples (40%) testing positive for *Salmonella*. The prevalence varied across sample types: 28/60 (46.7%) in muscle samples, 26/60 (43.3%) in liver samples, and 18/60 (30%) in cloacal samples. Antimicrobial susceptibility testing revealed resistance to tobramycin, vancomycin, sulfamethoxazole/trimethoprim, piperacillin/ tazobactam, and oxytetracycline. The isolates showed intermediate susceptibility against gentamicin and ciprofloxacin, while florfenicol remained sensitive. This study revealed a significantly higher prevalence of *Salmonella* in commercial broilers from Islamabad and Rawalpindi, with an overall prevalence of 40% (72/180 samples). This contrasts with the results from northern Poland, where the total percentage of infected flocks was reported to be 1.57%. Moreover, the Polish study recorded a declining trend in *Salmonella* prevalence from 2.19% in 2014 to 1.22% in 2016. The major difference between our results and the Polish study may be attributed to variations in geographical zone, husbandry practices, biosecurity measures, food safety measures, and regional antibiotic stewardship policies. The higher prevalence in our study suggests the call to action for enhanced surveillance and control measures to diminish avian salmonellosis [24].

This study investigated the prevalence of *Salmonella* to be 40% in commercial broilers compared to the study in Trinidad, which reported a prevalence of 20.5% in chicken carcasses from cottage poultry processors and 8.3% from supermarkets. Additionally, the latter study highlighted the importance of proper handling and storage practices, as non-chilled chickens had a higher frequency of *Salmonella* isolation (22.6%) compared to chilled chickens (8.3%). Whereas our study concentrated on antimicrobial resistance patterns in *Salmonella* isolates, revealing resistance to multiple antibiotics, the Trinidad study relied on estimating prevalence solely [25]. This study found a higher prevalence of *Salmonella* in commercial broilers (40%) in comparison with the study in Ethiopia, documenting a prevalence of 24.3% across various samples, namely cloacal, cecal, feed, and water samples (the environmental factors). While this study relied upon focusing on organ samples from the LBMs. Both studies highlight concerning antibiotic resistance patterns, with our study showing resistance to tobramycin, vancomycin, and oxytetracycline, and the Ethiopian study finding high resistance rates to tetracycline (80.81%), kanamycin (71.72%), and other antibiotics. The Ethiopian study also suggested MDR in *Salmonella* spp. [26]. This study found a higher prevalence of *Salmonella* in commercial

broilers from Islamabad and Rawalpindi, with an overall prevalence of 40% (72/180 samples), in comparison to the study in Shandong, China, which reported the rate of isolation to be 11.2% (67/600 samples). Both studies evaluated antimicrobial susceptibility, revealing alarming resistance patterns. While our study showed resistance to Tobramycin, Vancomycin, and Oxytetracycline, the Chinese study exhibited high resistance rates to Ampicillin (68.7%) and Polymyxin B (100%). The presence of multidrug-resistant Salmonella isolates in the Chinese study (53.7%) and our findings of resistance to multiple antibiotics are indicative of the need for antimicrobial stewardship and enhanced surveillance to alleviate antimicrobial resistance in poultry [27]. This study estimated a higher prevalence of *Salmonella* from commercial broilers (40%) in Rawalpindi compared to the 29% prevalence in pigeons from the live bird market in Chattogram, Bangladesh. The Bangladeshi study researched pigeons as study animals, while this study focused on broilers. In comparison with this study, which collected samples from the cloaca, liver, and muscle, the Bangladeshi study only chose liver samples as the subject for its research. Both studies highlight concerning patterns of antibiotic resistance. The study conducted in Bangladesh documented high resistance rates to ampicillin (93.1%), sulfamethoxazole-trimethoprim (86.2%), and tetracycline (86.2%), whereas our study found resistance to tobramycin, vancomycin, sulfamethoxazole/trimethoprim, piperacillin/ tazobactam, and oxytetracycline. The Bangladeshi study found relatively higher sensitivity to ciprofloxacin (65.5%), whereas our study showed intermediate susceptibility [18]. This study found a higher prevalence of *Salmonella* in commercial broilers (40%) in comparison with the study performed in Timor-Leste, which suggested antimicrobial resistance patterns in both *Escherichia coli* and *Salmonella* species from healthy chickens. In addition, our study highlighted the prevalence among the liver, cloaca, and muscle samples, while the Timor-Leste study was concerned with cloacal and boot swabs. Both studies focus on antibiotic resistance patterns, with this study showing resistance to multiple antibiotics, including Tobramycin, Vancomycin, and Oxytetracycline. Similarly, the Timor-Leste study found high resistance rates to tetracycline and ampicillin. Precisely, the Timor-Leste study reported lower resistance rates to antimicrobials listed in the country's human guidelines, except for ciprofloxacin resistance in *Salmonella* spp. (47.1%). In contrast, our study reported intermediate susceptibility to ciprofloxacin [28].

Conclusion

In a nutshell, the poultry sector is the high-yielding industry of Pakistan, which is challenged by *Salmonella* infections in broilers, which not only compromise flock health, economic stability but also affect human health when they consume the infected poultry and its byproducts. Furthermore, the unchecked practice of using antibiotics in food-producing animals like poultry has caused resistance in *Salmonella* serotypes. These antibiotic-resistant genes can pass down to humans when they consume food products from such treated animals. This study in Islamabad and Rawalpindi found a 40% prevalence of *Salmonella* in commercial broilers, with high resistance to multiple antibiotics. These findings suggest the critical need for improved poultry management, judicious use of the antimicrobials, and active surveillance to alleviate *Salmonella* infection and antimicrobial resistance, safeguarding both one health and the economy. Moreover, immediate action is required to develop alternative therapeutic strategies, other than antibiotics, to combat antimicrobial resistance and address the reduced effectiveness of antimicrobials against the microbial world in the future.

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Conflict of interest

The authors declare no conflict of interest.

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