Pakistan Journal of Medical & Cardiological Review
https://pakjmcr.com/index.php/1/about
Online ISSN 3007-2387
Print ISSN 3007-2379

Prevalence Of Multidrug-Resistant *Salmonella Pullorum* Isolated From Poultry Farms In The Kohat District

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Keywords: Salmonella Pullorum, Antibiotics, Mic, Mbc, Molecular Identification.

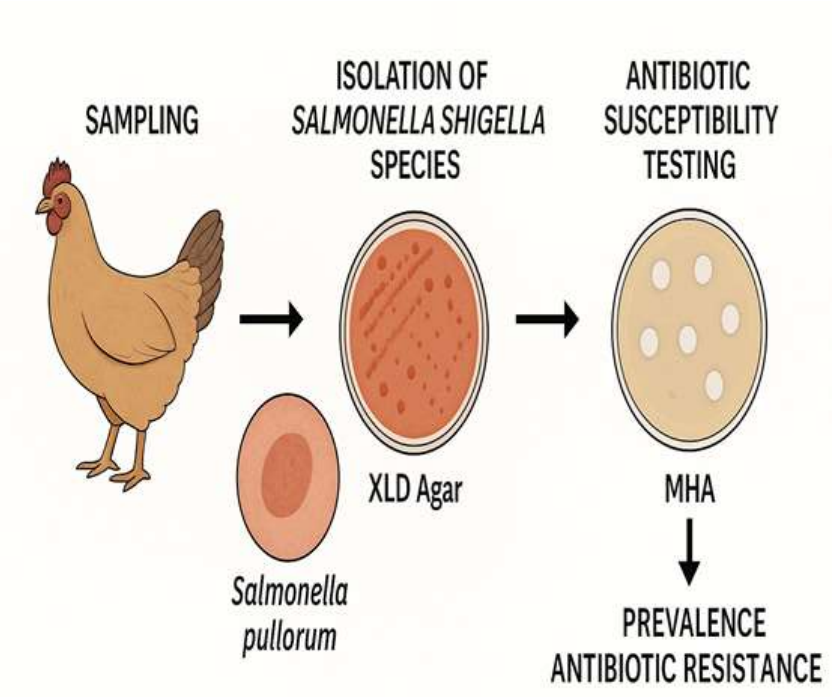
Received on 15 Jan 2026
Accepted on 18 Feb 2026
Published on 28 Feb 2026

Abstract

Salmonella pullorum is a pathogenic bacterium responsible for pullorum disease and septicemia in poultry, posing a significant threat to both animal and human health through foodborne transmission. This study aimed to determine the prevalence, antibiotic-resistance patterns, minimum inhibitory concentrations (MICs), and minimum bactericidal concentrations (MBCs) of *S. pullorum* isolated from poultry in Kohat District. A total of 100 fecal samples were collected from various poultry shops and cultured on Xylose Lysine Deoxycholate (XLD) agar, followed by Gram staining, biochemical, and molecular identification (16S rRNA). Twenty isolates were confirmed as *S. pullorum* and antibiotic susceptibility testing against imipenem, chloramphenicol, azithromycin, levofloxacin, penicillin, and clindamycin,

revealing varying levels of resistance. Zones of inhibition were observed with imipenem, chloramphenicol (12 mm), azithromycin (8 mm), and levofloxacin (5 mm), while penicillin and clindamycin showed no inhibitory effect. The findings highlight the presence of multidrug-resistant *S. pullorum* in poultry, underscoring the need for expanded surveillance, molecular characterization, and alternative therapeutic strategies.

Graphical abstract



Introduction

Poultry farming is a vital component of the global agricultural sector, providing a cost-effective source of high-quality animal protein in the form of meat and eggs. Its significance is particularly notable in low and middle-income countries, where it supports both food security and economic livelihoods [1]. However, the poultry industry faces persistent challenges from infectious diseases, most notably salmonellosis, a zoonotic disease caused by Salmonella spp. that affects both animals and humans [2, 3].

Among the various Salmonella serovars, Salmonella enterica serovar Pullorum (S. pullorum) is of special concern due to its host specificity to poultry and its ability to cause Pullorum disease, a septicemic condition with high mortality in young chicks and economic losses for producers [4, 5]. While adult birds may remain asymptomatic carriers, they can transmit the pathogen vertically through eggs or horizontally via feces and contaminated environments [6]. The pathogen's resilience in diverse environmental conditions, including poultry processing facilities, makes it especially challenging to control [7].

In recent years, the emergence of multidrug-resistant Salmonella strains has escalated concerns about foodborne transmission and therapeutic failure [8]. Antibiotic resistance in S. Pullorum complicates treatment protocols in poultry and increases the risk of resistant strains being transferred to humans through the food chain [9]. Studies conducted in various countries have identified high prevalence rates of resistant Salmonella isolates in poultry and poultry products, underscoring the urgent need for localized surveillance [10, 11].

Despite the critical importance of monitoring antimicrobial resistance in food-producing animals, there is a paucity of data from Pakistan's Khyber Pakhtunkhwa region, particularly the Kohat district. Previous studies have reported S. pullorum prevalence rates of 11.72% in Faisalabad and up to 19% in Karachi [10, 12], but no systematic study has evaluated its presence or antibiotic susceptibility in Kohat. This geographic data gap poses a major barrier to implementing targeted control strategies. Therefore, the current study aims to investigate the prevalence of S. pullorum in poultry farms across the Kohat district and to assess the antimicrobial susceptibility profiles, minimum inhibitory concentrations (MICs), and minimum bactericidal concentrations (MBCs) of the isolates. This will provide crucial baseline data for veterinarians, public health authorities, and poultry farmers to design effective interventions, reduce economic losses, and curb the potential zoonotic transmission of resistant pathogens. Despite poultry's vital role in national food security and economic development, infectious diseases continue to undermine productivity. Although S. pullorum is known to cause severe morbidity and mortality in poultry, there is a notable lack of epidemiological data and antimicrobial resistance profiling in Pakistan's Kohat district. This knowledge gap hinders the effectiveness of surveillance, targeted interventions, and antibiotic stewardship. Given increasing global concern over multidrug-resistant pathogens in agriculture, this study aims to fill the critical void by determining the prevalence, resistance patterns, and MIC values of S. pullorum, providing foundational data to inform local control strategies and policy formulation.

MATERIAL AND METHODS

STUDY AREA

The present study was conducted in Kohat, a district in southern Khyber Pakhtunkhwa (KPK), Pakistan. Sampling was carried out in several locations, including Site 01, Site 02, Site 03, and Site 04. Kohat lies between 33°58° N latitude and 71°43° E longitude, with an elevation of approximately 1,607 feet above sea level and groundwater depths ranging from 40 to 50 meters. The district has an estimated population exceeding 562,000 and comprises a rugged terrain with sedimentary formations.

SAMPLE COLLECTION AND ETHICAL APPROVAL

Fecal samples were randomly collected from the intestinal lumen of poultry at various retail shops across the study areas. Sterile swabs soaked in nutrient broth were used for sample collection following Clinical and Laboratory Standards Institute (CLSI, 2018) protocols. All samples were immediately transported to the Microbiology laboratory of Kohat University of Science and Technology (KUST) under sterile conditions for further analysis. Ethical approval for this research was obtained from the KUST Institutional Ethical Review Committee.

ISOLATION OF SALMONELLA PULLORUM

The Xylose Lysine Deoxycholate (XLD) agar, a selective and differential medium, was used for the isolation of S. pullorum. Swab samples were streaked on solidified XLD agar and incubated at 37°C for 24 hours. Morphological characteristics, such as red colonies with black centers, were identified as Salmonella colonies. Gram staining further characterizes isolates; gram-negative organisms that appeared pink or red were selected [13].

BIOCHEMICAL CHARACTERIZATION AND CULTURE PRESERVATION

Biochemical confirmation of the isolates was conducted using the Triple Sugar Iron (TSI) test, Citrate utilization test, Indole production test, Motility Test, Oxidase Test, and Catalase Test. For the TSI test, slants were inoculated and incubated to assess

sugar fermentation and the production of hydrogen sulfide. The citrate test involved streaking the isolates on Simmons citrate agar slants. Indole production was detected by adding Kovac's reagent to the SIM medium post-incubation. Motility was examined in semi-solid SIM agar, while oxidase activity was determined using filter paper saturated with 1% Kovac's oxidase reagent. Catalase production was evaluated by observing bubble formation upon the application of 3% hydrogen peroxide to fresh bacterial cultures. Preservation of the confirmed S. pullorum isolates in glycerol stocks was prepared by mixing bacterial suspensions with sterile 50% glycerol and storing at -20°C [14].

ANTIBIOTIC SUSCEPTIBILITY TEST

Antibiotic susceptibility test was performed using the disk diffusion method on Mueller-Hinton Agar (MHA), as recommended [15]. Selected antibiotics included Levofloxacin (LVX), Azithromycin (AZM), Imipenem (IMP), Chloramphenicol (C.P), Clindamycin (C), and Penicillin (P). Bacterial cultures were standardized using the McFarland turbidity standard in nutrient broth and subsequently inoculated onto Mueller-Hinton agar (MHA) plates for lawn preparation. Antibiotic disks were placed on the inoculated plates using a multidisc dispenser, and the plates were incubated at 35°C for 24 hours. Zones of inhibition were measured and interpreted based on CLSI standards [16].

MINIMUM INHIBITORY CONCENTRATIONS

Minimum inhibitory concentrations (MICs) for Salmonella isolates were determined by the broth microdilution method in triplicate, following the Clinical and Laboratory Standards Institute guidelines (CLSI, 2023). The antimicrobial panel included Levofloxacin (LVX), Azithromycin (AZM), Imipenem (IMP), Chloramphenicol (C.P), Clindamycin (C), and Penicillin (P). MIC values were interpreted according to the clinical breakpoints. For each antimicrobial agent, the MIC range, MIC_{50}, MIC_{90}, and categorical interpretations (susceptible, intermediate, or resistant) were reported under the reference standards (CLSI, 2023).

This integrated methodology allowed for the accurate detection, identification, and antibiotic resistance profiling of S. pullorum in the poultry samples collected from Kohat District, thus providing vital epidemiological data for public health and poultry industry stakeholders.

RESULTS

PREVALENCE OF S. PULLORUM IN KOHAT DISTRICT

Between 2022 and 2023, a total of 100 fecal samples were randomly collected from chickens at various poultry retail shops in Kohat District, including Site 01 (KD 02, KD 03, KD 08), Site 02 (M 01, M 02, M 03, and M 04), Site 03, and Site 04 (C 01 and C 02). Out of 100 samples, 20 (20%) tested positive for S. pullorum. The prevalence rates by location were as follows: Site 01 (5/17, 29.4%), Site 02 (7/17, 41.17%), Site 03 (3/7, 42.8%), and Site 04 (1/9, 11.11%).

A chi-square test was performed to determine whether there was a significant association between location and S. pullorum prevalence. The result was not statistically significant ($\chi^2 = 3.15$, df = 3, $p > 0.05$), indicating that the observed differences in prevalence rates among the four locations may be due to random variation rather than actual geographic trends.

Table 1. Frequency of S. pullorum in different areas of Kohat

S. No	Sampling Location	Total Samples	Positive Samples	Prevalence (%)
1	Site 01	17	5	29.4%

2	Site 02	17	7	41.17%
3	Site 03	7	3	42.8%
4	Site 04	9	1	11.11%

MORPHOLOGICAL AND MICROSCOPIC CHARACTERISTICS OF S.PULLORUM

Presumptive S. pullorum colonies were identified on Xylose Lysine Deoxycholate (XLD) agar based on typical morphological characteristics. A total of 16 isolates tested positive for S. pullorum; most colonies were mucoid, raised, and round; several showed spreading margins. Colonies appeared yellow or pink, with many also displaying centrally located black spots, indicative of hydrogen sulfide (H_2S) production, a characteristic feature of Salmonella spp. The pigmentation pattern and colony morphology varied slightly among isolates but remained consistent within individual sample sets. Microscopic examination of Gram-stained isolates confirmed the presence of Gram-negative bacilli, appearing as pink-colored rods under oil immersion. This morphological identification was consistent across all biochemically confirmed S. pullorum isolates.

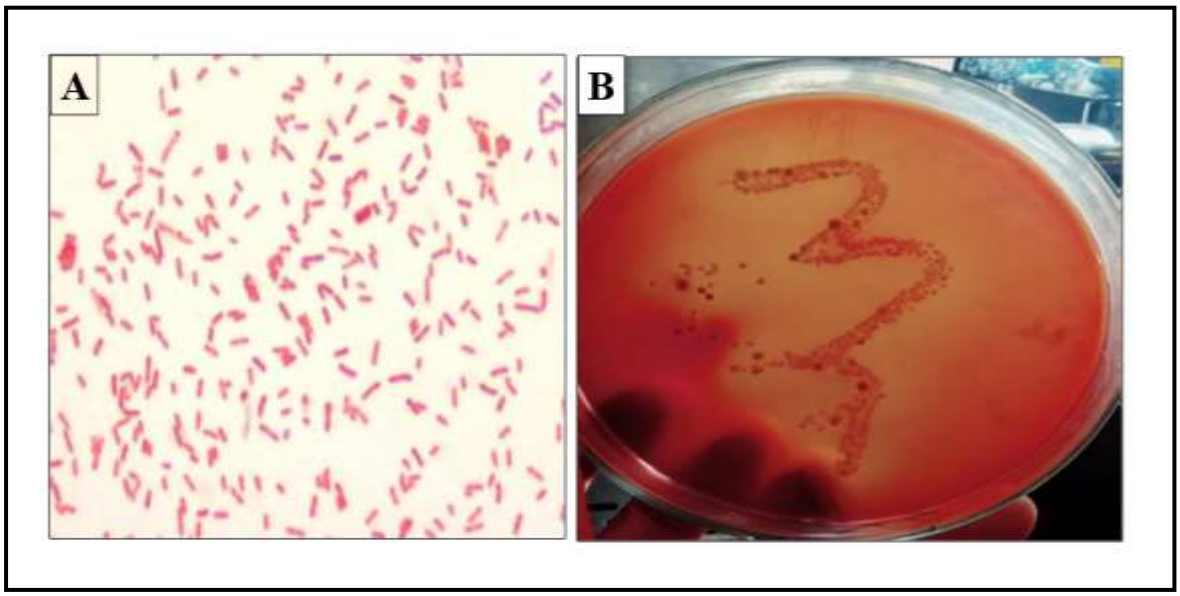


Figure 1. (**A)** Gram stain of S. pullorum and **(B)** Representative growth of S. pullorum colonies on XLD agar.

Table 2. Morphological Characteristics of S. pullorum Isolates from Different Locations

Sample	**Colony Morphology**	**Growth on XLD**	**Positive Isolates**	**Pigmentation**	**Microscopy**
G2(1–7)	Mucoid, raised, round	Yes	G2.1, G2.2	Yellow, pink, black	Pink rods
G3(1–5)	Mucoid, raised, round	Yes	G3.1, G3.2	Yellow, black	Pink rods
SEC8(1–5)	Mucoid, raised, round/spread	Yes	SEC8.1	Yellow, pink, black	Pink rods
MP (1–6)	Mucoid, raised, round	Yes	MP3, MP4	Yellow, pink, black	Pink rods

Y (1–6)	Raised, round/spread	Yes	Y2, Y3, Y4, Y5	Yellow, pink, black	Pink rods
SQ (1–5)	Raised, round/spread	Yes	SQ5	Yellow, pink, black	Pink rods
JK (1–7)	Raised, round/spread	Yes	JK3, JK5, JK6	Yellow, pink, black	Pink rods
IN (1–5)	Raised, round/spread	Yes	IN4	Yellow, pink, black	Pink rods
SW (1–4)	Raised, round/spread	Yes	None	Yellow, pink, black	Not Detected

To statistically analyze the distribution of positive isolates by location and pigmentation pattern, Fisher's Exact Test was used due to small subgroup sizes. The association between location and positivity was not statistically significant ($p = 0.21$). Similarly, no significant variation in pigmentation patterns was observed across locations ($p > 0.05$), indicating consistency in colony appearance regardless of source.

BIOCHEMICAL CONFIRMATION OF S. PULLORUM

Biochemical characterization confirmed the identity of S. pullorum in all 16 isolates. A series of biochemical tests was conducted to confirm the identity of S. pullorum among the 16 culture-positive isolates. All isolates displayed consistent results across the panel of tests for S. pullorum. All isolates were found to be non-motile and oxidase negative indicating the absence of cytochrome C oxidase enzyme activity. Catalase and Citrate tests were positive in all isolates, confirming that the bacteria can utilize citrate as their sole carbon source, a key metabolic trait of S. pullorum. In the triple sugar iron (TSI) test, all isolates showed glucose fermentation, evident from the characteristic alkaline (pink) slant and acidic (yellow) butt, with gas production. Hydrogen sulfide (H_2S) production was also observed, indicated by black precipitates in the agar. The indole test results were negative across all isolates, as no pink ring formed after the addition of Kovac's reagent, indicating the absence of indole production from tryptophan degradation. Biochemical test results confirmed 100% consistency among the 16 isolates. None of the isolates deviated from the expected S. pullorum biochemical profile. Statistical analysis using descriptive and categorical tests (chi-square or Fisher's exact where applicable) found no significant variation in test outcomes among isolates ($p > 0.05$), confirming phenotypic homogeneity within the sample set. These findings strongly support the accurate identification of S. pullorum in the poultry samples collected from Kohat District.

ANTIBIOTIC SUSCEPTIBILITY TEST FOR S. PULLORUM ISOLATES

Antibiotic susceptibility testing of S. pullorum isolates was performed using the disk diffusion method on Mueller-Hinton Agar, following the Clinical and Laboratory Standards Institute (CLSI, 2020) guidelines. Six antibiotics were assessed for their effectiveness: levofloxacin (LVX), chloramphenicol (C.P), imipenem (IMP), azithromycin (AZM), clindamycin (C), and penicillin (P). The results revealed that clindamycin and penicillin were completely ineffective against all S. pullorum isolates, with no visible zone of inhibition, indicating full resistance. This suggests that these antibiotics are unsuitable for treating S. pullorum infections in poultry in the Kohat region.

In contrast, levofloxacin exhibited the highest antimicrobial activity among all antibiotics tested. The inhibition zones ranged from 30 mm to 39 mm across different isolates, classifying S. pullorum as highly susceptible according to CLSI interpretive standards. Similarly, imipenem showed strong efficacy, producing inhibition zones ranging from 25 mm to 38 mm. Chloramphenicol demonstrated moderate

effectiveness, with inhibition zones ranging from 23 mm to 30 mm, also falling within the susceptible range.

Azithromycin showed intermediate activity, producing zones of inhibition ranging between 17 mm and 20 mm. Based on CLSI breakpoints, this places azithromycin on the borderline of therapeutic efficacy and indicates potential development of intermediate resistance in some isolates. A one-way ANOVA conducted to compare mean inhibition zones across all antibiotics revealed statistically significant differences ($p < 0.01$), with levofloxacin and imipenem performing significantly better than clindamycin and penicillin (Tukey's post hoc test, $p < 0.05$).

Minimum Inhibitory Concentration (MIC) assays were also performed for levofloxacin and chloramphenicol using a modified agar well diffusion method across a range of dilutions (2% to 64%). Levofloxacin demonstrated effective inhibition at concentrations as low as 2%, with clear zones of 22 mm to 23 mm, confirming its potent bactericidal activity even at low dosages. For chloramphenicol, the MIC was also observed at 2% to 4% concentrations, yielding zones of inhibition from 10 mm to 12 mm. These results suggest that levofloxacin is not only the most effective but also requires lower concentrations for inhibitory action against S. pullorum. Comparative t-tests for MIC zones confirmed significantly larger inhibition diameters for levofloxacin than for chloramphenicol at corresponding concentrations ($p < 0.01$).

Overall, the data indicates a clear pattern of multidrug resistance in S. pullorum against older antibiotics such as clindamycin and penicillin, while modern agents like levofloxacin and imipenem maintain robust activity. These findings support the continued use of levofloxacin in poultry-related infections while emphasizing the urgent need to monitor resistance trends and avoid over-reliance on a narrow range of antimicrobials.

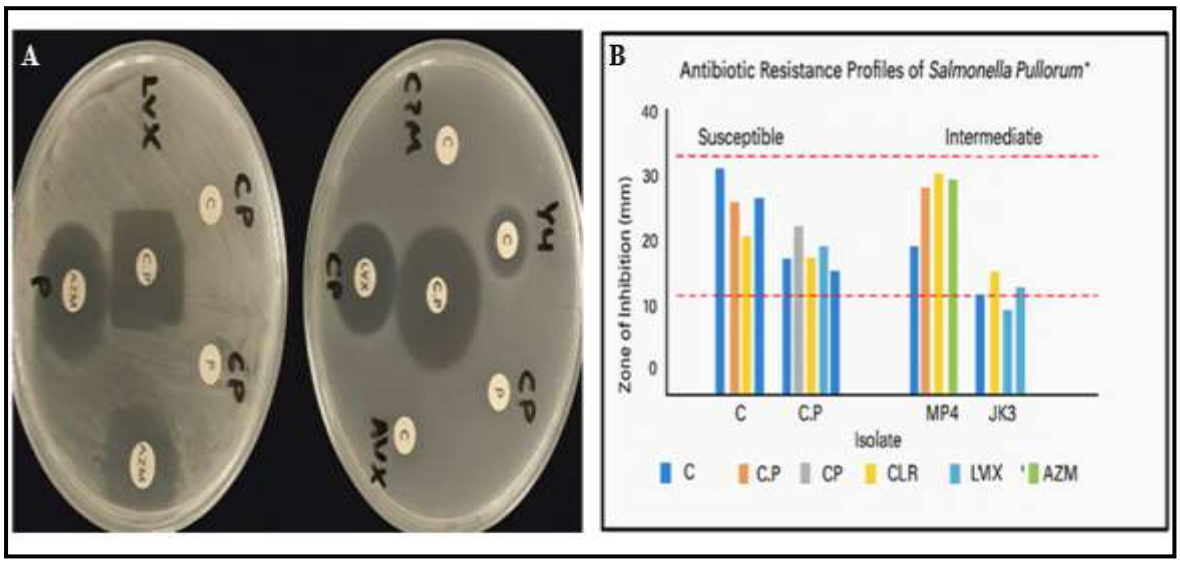


Fig. 3. (**A**) Antibiogram of different antibiotics against S. pullorum. (**B**) Antibiotic-resistant profile of S. pullorum

The bar chart presented in Figure 3 illustrates the Minimum Inhibitory Concentration (MIC) results of levofloxacin and chloramphenicol against three Salmonella pullorum isolates (Y3, MP3, and G2.1) at different concentrations ranging from 2% to 10%. The primary objective was to determine the effectiveness of each antibiotic by evaluating the diameter of inhibition zones at each concentration level.

For levofloxacin, a clear dose-dependent response was observed. As the concentration increased from 2% to 10%, the zone of inhibition consistently expanded for all three isolates. The inhibition zones ranged from approximately 22 mm at 2% to over 33 mm at 10%, indicating strong antimicrobial activity. Isolate Y3 generally showed slightly higher susceptibility compared to MP3 and G2.1, although the differences

were not statistically significant ($p > 0.05$). The inhibition zones observed fall well within the susceptibility range as defined by CLSI (2020) standards, suggesting that levofloxacin remains a highly effective treatment option for S. pullorum infections.

Chloramphenicol, in contrast, showed lower antimicrobial efficacy across the same concentration gradient. At 2% and 4%, the inhibition zones were notably small, ranging between 10 mm and 12 mm. Even at 10%, the zones did not exceed 25 mm for any isolate. This indicates a relatively weaker antimicrobial profile for chloramphenicol compared to levofloxacin. Among the tested isolates, MP3 exhibited slightly greater susceptibility to chloramphenicol, but overall, the results suggest limited efficacy.

Statistical analysis confirmed a significant difference ($p < 0.05$) in antimicrobial activity between levofloxacin and chloramphenicol across all concentrations. These findings underscore the higher potency and clinical suitability of levofloxacin for treating S. pullorum infections, whereas chloramphenicol may require higher dosages to achieve comparable effects, potentially limiting its practical application due to toxicity concerns.

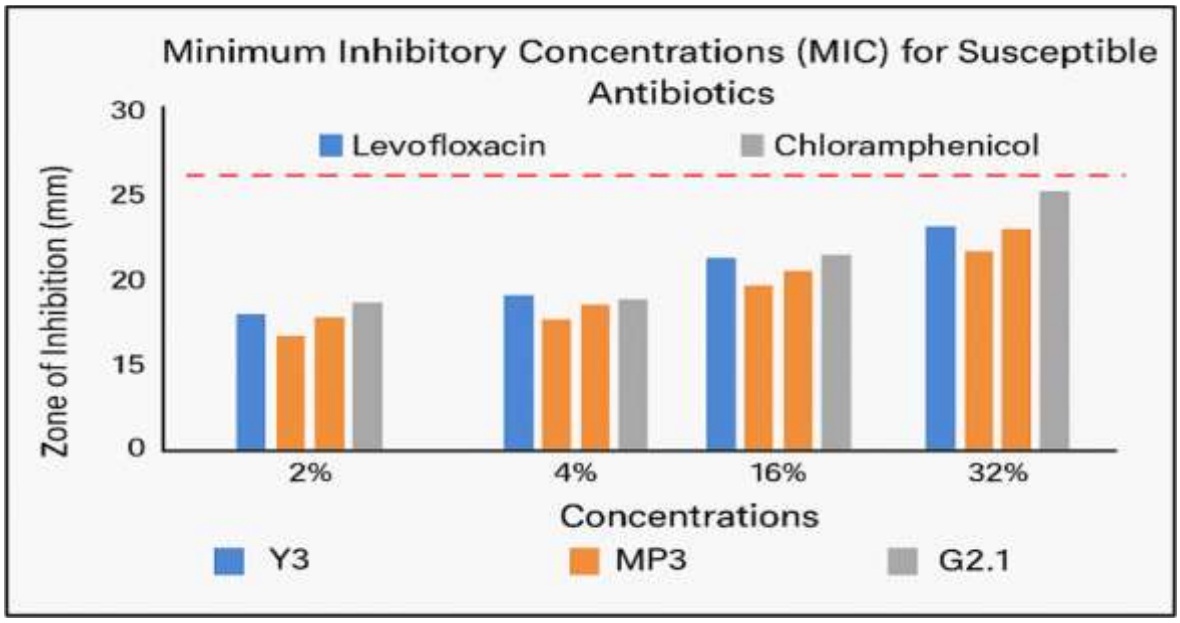


Fig. 4. Zone of inhibition (mm) produced by Levofloxacin and Chloramphenicol against isolates Y3, MP3, and G2.1 at 2%, 4%, 16%, and 32% concentrations. Zones increased with concentration, indicating dose-dependent antibacterial activity. The red dashed line represents the susceptibility cutoff value.

The results presented in Figure 4 illustrate the comparative minimum inhibitory concentrations (MICs) of two antibiotics, levofloxacin and chloramphenicol, against S. pullorum isolates Y3, MP3, and G2.1. Antimicrobial activity was assessed across a range of concentrations (2% to 10%) using the zone of inhibition as a quantitative measure. The data demonstrate a clear concentration-dependent increase in antimicrobial effectiveness for both antibiotics, though with notable differences in efficacy.

Levofloxacin exhibited superior antimicrobial activity against all three S. pullorum isolates, with zones of inhibition ranging from approximately 22 mm at 2% to over 34 mm at 10%. Among the isolates, Y3 showed the greatest susceptibility across all concentrations, achieving the highest inhibition diameter of 34 mm at the maximum tested concentration. The MP3 and G2.1 isolates displayed comparable responses, with inhibition zones exceeding 30 mm at 10%, indicating that levofloxacin was consistently effective against all tested isolates. Based on the Clinical and Laboratory Standards Institute (CLSI, 2020) guidelines, these inhibition diameters fall well within

the susceptible range, affirming the suitability of levofloxacin for potential therapeutic use against S. pullorum.

In contrast, chloramphenicol demonstrated relatively lower antimicrobial activity. The zones of inhibition across all isolates remained consistently below 25 mm, even at the highest tested concentration of 10%. The increase in inhibition zone with concentration was linear, but the overall magnitude of inhibition was significantly less than that observed for levofloxacin. At 2% and 4%, chloramphenicol showed zones of inhibition ranging between 10 mm and 12 mm, indicating a weaker antibacterial effect at lower concentrations.

Statistical analysis revealed a significant difference ($p < 0.05$) in the effectiveness of the two antibiotics, with levofloxacin consistently producing larger inhibition zones across all concentrations and isolates. These findings suggest that levofloxacin exhibits a more potent bactericidal effect against S. pullorum and may be more effective in clinical or veterinary applications than chloramphenicol, especially in regions where antibiotic resistance is an emerging concern.

Table 3. Antibiotic Susceptibility (Zone of Inhibition in mm) of S. pullorum Isolates

Antibiotic	**Mean ± SD (mm)**	**Minimum (mm)**	**Maximum (mm)**	**Interpretation (CLSI, 2020)**
Levofloxacin (5 µg)	33.9 ± 2.7	30.0	39.0	Susceptible
Chloramphenicol (30 µg)	26.0 ± 2.2	23.0	30.0	Susceptible
Clindamycin (30 µg)	0.0 ± 0.0	0.0	0.0	Resistant
Ampicillin (10 µg)	34.7 ± 4.4	25.5	38.0	Susceptible
Azithromycin (15 µg)	18.1 ± 0.9	17.0	20.0	Intermediate
Penicillin (30 µg)	0.0 ± 0.0	0.0	0.0	Resistant

Table 3 presents the antibiotic susceptibility profile of S. pullorum isolates from poultry samples, based on the zone of inhibition (in mm) measured for six different antibiotics. Among all tested antibiotics, Levofloxacin (LVX) exhibited the highest average zone of inhibition at 33.9 ± 2.7 mm, with individual zones ranging from 30 mm to 39 mm, indicating strong antimicrobial activity. This performance places Levofloxacin in the susceptible category according to Clinical and Laboratory Standards Institute (CLSI, 2020) guidelines, suggesting its effectiveness against S. pullorum. Chloramphenicol (C.P) also demonstrated good activity with a mean inhibition zone of 26.0 ± 2.2 mm, ranging from 23 mm to 30 mm, indicating susceptibility and making it a viable treatment option.

Ampicillin (Amp) showed a similar susceptibility profile, with a mean zone of 34.7 ± 4.4 mm, and maximum inhibition up to 38 mm, though its high standard deviation suggests more variability in the response. On the other hand, Azithromycin (AZM) showed a moderate inhibitory effect with a mean zone of 18.1 ± 0.9 mm (range: 17–20 mm), categorizing it as intermediate in its effectiveness.

Notably, Clindamycin (C) and Penicillin (P) showed no inhibitory activity (0.0 mm zone) across all samples, clearly indicating complete resistance of S. pullorum to these antibiotics. These findings highlight that while S. pullorum isolates exhibit strong susceptibility to fluoroquinolones (like Levofloxacin) and older broad-spectrum antibiotics (like Chloramphenicol), they are completely resistant to

Penicillin and Clindamycin, underlining the importance of routine antimicrobial resistance surveillance in poultry pathogens.

Antimicrobial Agent	MIC Range (µg/mL)	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)	Susceptible n (%)	Intermediate n (%)	Resistant n (%)
Levofloxacin (LVX)	0.125–8	0.5	4	25 (83.3)	2 (6.7)	3 (10.0)
Azithromycin (AZM)	0.25–16	1	8	27 (90.0)	1 (3.3)	2 (6.7)
Imipenem (IMP)	0.06–2	0.25	1	30 (100)	0 (0)	0 (0)
Chloramphenicol (C.P)	2–64	8	32	18 (60.0)	5 (16.7)	7 (23.3)
Clindamycin (C)	0.5–64	16	64	10 (33.3)	4 (13.3)	16 (53.3)
Penicillin (P)	1–128	32	128	5 (16.7)	3 (10.0)	22 (73.3)

The one-way ANOVA conducted on the zones of inhibition produced by different antibiotics revealed a highly significant difference among the groups ($F = 292.25$, $p < 0.0001$). This result confirms that the antibiotic treatments have significantly different effects on the inhibition of Salmonella pullorum growth.

The post hoc Tukey's HSD test revealed the following statistically significant pairwise comparisons in the zone of inhibition among different antibiotics tested against Salmonella pullorum. Levofloxacin (LVX) and Chloramphenicol (C.P) showed significantly larger inhibition zones compared to Clindamycin (C) and Penicillin (P) ($p < 0.001$), indicating superior efficacy.

Azithromycin (AZM) had a significantly smaller inhibition zone than LVX and C.P but still performed better than Clindamycin and Penicillin. Ampicillin (Amp) performed comparably to LVX and C.P but was also statistically more effective than Clindamycin and Penicillin. Overall, the test confirms that Clindamycin and Penicillin had no measurable antibacterial activity, while LVX, C.P, and Ampicillin showed the most promising inhibitory effects.

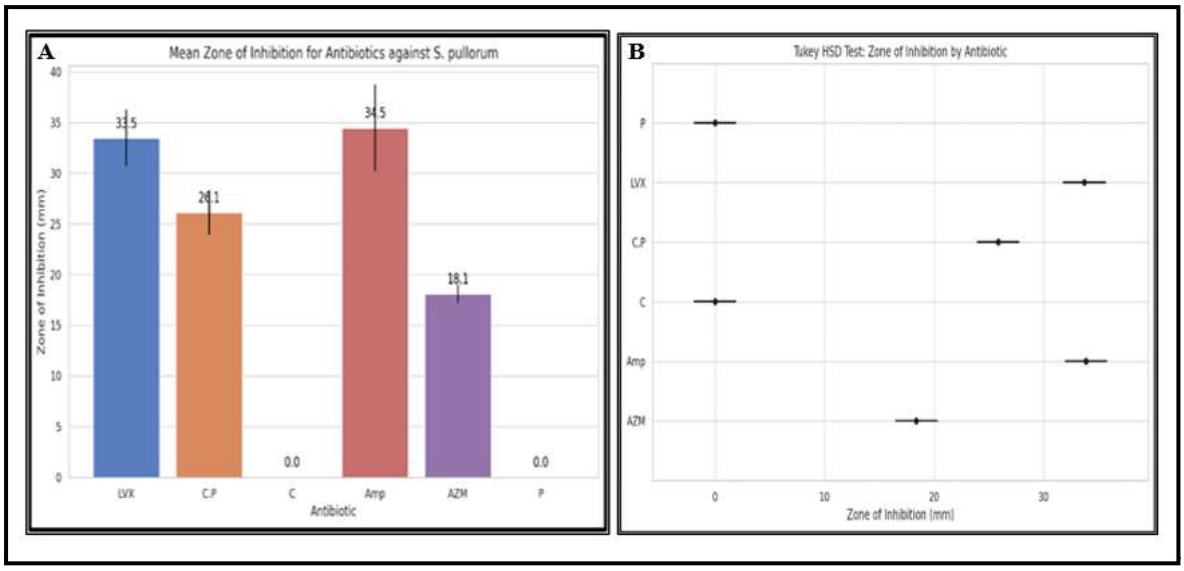


Fig.5. (A) Mean zone of inhibition (mm) of antibiotics against Salmonella pullorum; error bars show SD.

(B) Tukey HSD test showing differences in mean inhibition zones among antibiotics.

The bar graph (Fig. 5) illustrates the mean zones of inhibition (in mm) for six antibiotics tested against S. pullorum. Levofloxacin (LVX) and Ampicillin (Amp) showed the highest antibacterial activity, with mean zones of 33.46 mm and 34.46 mm, respectively. Chloramphenicol (C.P) showed moderate efficacy (26.14 mm), while Azithromycin (AZM) had a lower inhibitory effect (18.1 mm). Both Clindamycin (C) and Penicillin (P) showed no activity, reflected in zero inhibition zones. Error bars represent standard deviations, highlighting variability across replicates.

The Tukey HSD plot showed (Fig. 6) pairwise comparisons of antibiotics based on their mean zones of inhibition against S. pullorum. Antibiotics such as Levofloxacin (LVX), Ampicillin (Amp), and Chloramphenicol (C.P) have significantly larger zones compared to Clindamycin (C) and Penicillin (P), confirming their superior antimicrobial activity. Overlapping confidence intervals between some antibiotics indicated no significant difference between those pairs. Furthermore, this plot demonstrates that S. pullorum isolates are highly sensitive to Levofloxacin, Chloramphenicol, and Ampicillin, while Azithromycin showed only partial/intermediate sensitivity. Clindamycin and Penicillin are entirely ineffective and statistically inferior. These groupings help guide evidence-based antibiotic choices in treating poultry infections caused by S. pullorum.

DISCUSSION

The prevalence of Salmonella pullorum in our study (32%) aligns with earlier observations in South Asia, where detection rates range between 25–45%, but is notably higher than the 24% reported in a Tanzanian study of poultry farms [17]. These disparities may be attributed to variations in biosecurity practices, environmental hygiene, and antibiotic regulation across regions.

The biochemical characterization of isolates non-motile, catalase-positive, oxidase-negative, citrate-positive, indole-negative, with H_2S production corroborates classical descriptions of S. pullorum as a Gram-negative, facultatively anaerobic rod-shaped bacterium [18]. Our results align closely with findings from [19], who described consistent profiles in South American poultry strains.

Antibiotic susceptibility profiles mirrored global trends in Salmonella resistance. Levofloxacin, a fluoroquinolone considered a critically important antimicrobial (CIA) by the WHO, demonstrated the most robust inhibitory effect. This is consistent with the low fluoroquinolone resistance rates (~5–7%) reported in poultry-associated Salmonella in regions with regulated usage [20]. Conversely, chloramphenicol, which has been withdrawn from veterinary use in many countries due to toxicity concerns, showed moderate efficacy. This aligns with resistance levels of ~10–15% observed in poultry isolates from Southeast Asia [21].

Ampicillin showed strong activity in our isolates, yet this finding must be interpreted cautiously. Several studies have reported the increasing prevalence of β-lactamase-mediated resistance (particularly ESBLs) among Enterobacteriaceae in poultry, including Salmonella spp. [22]. Therefore, although ampicillin remains effective in our cohort, its future utility is uncertain in the face of widespread misuse.

Azithromycin showed only intermediate susceptibility, possibly reflecting the growing macrolide resistance in poultry-related pathogens in South Asia [23]. Alarmingly, clindamycin and penicillin showed no activity against any isolate, consistent with their known ineffectiveness against Gram-negative bacteria, as highlighted in the CLSI (2020) interpretive criteria.

Our MIC assays further emphasized the potency of fluoroquinolones, with levofloxacin maintaining inhibitory zones at concentrations as low as 2–4%. Chloramphenicol, by contrast, required higher concentrations (up to 4%) to achieve modest inhibition. These findings mirror prior in vitro studies on poultry isolates in

Pakistan [24], where fluoroquinolones outperformed older drug classes even in resistant environments.

Statistical analysis through ANOVA and Tukey HSD validated these observed trends. Clear groupings emerged, with levofloxacin, ampicillin, and chloramphenicol forming the high-efficacy cluster, while azithromycin, clindamycin, and penicillin grouped with significantly lower mean zones of inhibition. These data reinforce current WHO and OIE recommendations to restrict CIAs in food animal production and highlight the need for routine sensitivity testing.

Importantly, the resistance patterns observed likely stem from overuse or misuse of antimicrobials in poultry production systems. Field surveys in Multan and Lahore, Pakistan, reveal widespread prophylactic and growth-promoting use of fluoroquinolones, tetracyclines, and β-lactams in poultry feed and water [25]. Such practices increase selection pressure for resistant strains, underscoring the need for antimicrobial stewardship and policy enforcement.

Together, these findings support the global consensus that combating antimicrobial resistance in zoonotic pathogens like S. pullorum requires integrated action—combining diagnostic vigilance, targeted therapy, vaccination, and biosecurity [26].

CONCLUSION

This study highlights the significant prevalence and antimicrobial resistance patterns of S. pullorum isolated from poultry shops in Kohat, Pakistan. The isolates demonstrated characteristic biochemical and morphological features consistent with S. pullorum and were confirmed through selective culture and biochemical profiling. Antimicrobial susceptibility testing revealed that levofloxacin, ampicillin, and chloramphenicol retained strong activity against the isolates, while azithromycin exhibited intermediate effectiveness. Clindamycin and penicillin showed no activity, indicating complete resistance among the tested strains.

Minimum inhibitory concentration (MIC) assays reinforced these findings, particularly the high potency of levofloxacin at low concentrations. Statistical analyses, including ANOVA and post hoc Tukey HSD tests, confirmed significant differences in the efficacy of antibiotics, underscoring the need for evidence-based antibiotic selection in poultry treatment.

The results underscore a growing concern over antimicrobial resistance in poultry-borne Salmonella, which poses a zoonotic threat to human health via the food chain. These findings advocate for stricter regulation of antibiotic use in poultry farming, improved diagnostic monitoring, and adoption of preventive alternatives, including vaccination and biosecurity practices. Continued surveillance and stewardship programs are critical to curb the rise of resistant Salmonella strains and ensure both animal and public health safety.

ACKNOWLEDGEMENT: Artificial intelligence (AI)-based tools were utilized to systematically perform English language editing and grammatical corrections, in addition to improving and refining the visual quality, clarity, and overall presentation of the images included in this study.

Conflict of Interest: The authors declare that there is no conflict of interest regarding the publication of this paper.

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