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ANTIMICROBIAL RESISTANCE PROFILING AND BIOCHEMICAL ANALYSIS OF SALMONELLA PULLORUM ISOLATES

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ABSTRACT

Pullorum disease (PD), which is caused by Salmonella enterica serovar Gallinarum biovars Pullorum, is regarded as one of the deadliest bacterial illnesses. It undermines food security and results in significant financial losses for the chicken sector. This rod-gram-negative bacterium prefers to dwell in the reproductive system, liver, and spleen. In adult chickens, sulfur-colored (yellow) diarrhea and listlessness are signs of fowl typhoid. In order to treat systemic bacterial infections, the poultry industry has made substantial use of antimicrobial drugs, including lactams, aminoglycosides, and fluoroquinolones. The aim of this study is to isolate *Salmonella Pullorum* from boiler chicken and identify by Biochemical analysis as well as to determine the Antimicrobial Resistance Profiling of *Salmonella Pullorum* isolates. Twenty samples were taken from five different farms' stock of broiler chickens After a whole day at 37 degrees Celsius, the samples were processed and streaked on XLD medium to examine bacterial growth. Gram staining and several biochemical assays, including the lactose fermentation, urease, methyl red, and indole tests, were then used to confirm the presence of bacteria. After that, the bacterial culture was grown for testing for antibiotic susceptibility. In the current investigation, the antibiotics tetracycline, enrofloxicin, azithromycin, and ciprofloxacin were utilized. The isolates' antimicrobial susceptibility tests throughout this investigation showed a variety of resistance patterns. 90% azithromycin, 100% enrofloxicin, and tetracycline. The maximum effectiveness against the investigated isolates was demonstrated by Tetracycline and Enroflaxacin, which showed 0% resistance. Ceftriaxone showed a lower resistance rate of 30%, while ciprofloxacin should have a 58% resistance rate. 10 percent resistant to the Macrolide Azithromycin. According findings, *S. pullorum* is still common in Pakistan and many other countries; thus, effective management and treatment are necessary to completely eradicate this disease.

INTRODUCTION

Salmonella Pullorum a member of the Enterobacteriaceae family, Salmonella is a rod-shaped, facultative anaerobic, gram-negative bacterium that may cause illnesses in both people and animals. *Salmonella pullorum,* which is highly adapted to poultry, produces pullorum disease (PD, white diarrhea), a common and deadly illness (Eriksson *et al.,* 2018). The death rate from pullorum illness in chicks can reach 100%, with the greatest losses occurring during the second week of life. The infection incidence for fowl typhoid ranges from 10% to 90%, and

morbidity is more severe than death (Eng *et al.,* 2015). *S. Pullorum* is eliminated by affected chicks through their feces. This is the main way that it spreads among women. Additionally, direct or indirect contact with respiratory or fecal contaminated bird might spread the infection. Infections spread by hatchery or egg contamination typically cause mortality in the first few days of life and up to two or three weeks (Endris *et al.,* 2013). The symptoms of chicken typhoid and pullorum sickness might include despair, exhaustion, anorexia, huddled bodies, drooping wings, dehydration, difficulty breathing, diarrhea, ruffled feathers, weakness, and fecal adhesion to the vent. In certain situations, the disease may not be visible, and non-specific clinical symptoms such as a decrease in feed intake, a drooping look, or ruffled feathers and pale, shrinking combs may be seen (Terzolo & Chacana, 2016). Pullorum causes severe mortality and characteristic white diarrhea in chicks. Adult carriers who are infected may have internal ovarian lesions (dark, malformed follicles) but may not exhibit clinical symptoms of the illness. The symptoms of fowl typhoid in mature hens include sulfur-colored (yellow) diarrhea and listlessness. The afflicted birds have a widespread infection, resulting in enlarged livers, spleens, and kidneys, along with bleeding in these areas (Berhanu & Fulasa, 2020).

Salmonella evade the stomach's host defenses, enter the intestines, and interact with non-phagocytic cells, including the intestinal mucosa's epithelial cells. Only commercial egg layer hens and meat-producing broilers are permitted to receive antibiotic therapy for chicken typhoid and pullorum disease; breeding lines are not permitted to use this medication, which is required to eradicate sick flocks (Haider *et al.,* 2014). The poultry industry has made extensive use of the preventive use of antimicrobial medications, such as fluoroquinolones, aminoglycosides, and lactams, to treat systemic bacterial infections, including those brought on by *S. gallinarum* and *S. Pullorum* in commercial chickens, or to control intestinal infections (Saha *et al.,* 2012). Gentamicin, Chloramphenicol, Cephradine, Streptomycin, and Kanamycin are the antibiotics used to treat *S. Pullorum* and *S. gallinarum*. Lack of Salmonella antimicrobial resistance evaluations contributes to the global issue of antibiotic resistance. Extended spectrum beta lactamase (ESBL) genes in particular have emerged and been maintained as a result of the widespread and inappropriate use of antimicrobial medications in chicken production (Beyene *et al.,* 2011). This constitutes a major danger to the poultry business as well as public health. Many microorganisms have become resistant to antibiotics as a result of the careless use of these drugs. Continued use of these pathogen-resistant medications may result in the emergence of extremely resistant forms of the

pathogen, which may then spread across the environment and cause massive disease outbreaks in the future (Parvej *et al.,* 2016).

Materials and method

Sample Collecting

From District Mansehra broiler chicken fields, 20 samples were chosen at random. All of the samples including swabs, visceral organs, and droppings were gathered aseptically and brought to Abbottabad University of Science and Technology's microbiology lab.

Identification and Isolation of *Salmonella pullorum*

The samples were cultivated for 24 hours at 37 °C in a non-selective Peptone water broth and for 24 hours at 41.4 °C in 10 ml Tetrathionate broth. After that, the cells were grown on a particular broth (Himedia's Selenite F broth) and incubated for twenty-four hours at 37 °C. MacConkey agar plates were streaked with a loopful of each broth, and they were then incubated for 24 hours at 37 °C. Gram staining and biochemical characterization were used for the further identification of isolated species (Fernandes Queiroga Moraes *et al.,* 2021).

Morphology based Characterization of Isolated *Salmonella Pullorum* Bacterial Strains

Gram Staining

A small amount of distilled water was put to a clear slide in order to carry out the Gram staining. A small quantity of pure culture was applied to the slide using a sterile needle. The needle was used to distribute the culture uniformly on the slide. A drop of crystal violet was applied to the slide smear using sterile water. After achieving a consistent dispersion, the crystal violet was swirled in and allowed to dry for around 30 seconds. After applying crystal violet stain, the slide was gently washed with distilled water that had been sterilized. To get rid of any remaining crystal violet color, a droplet of Lugol's iodine was added to the smear after the slide had been thoroughly cleaned with pure distilled water. Crystal violet and Lugol's iodine work together to hold the stain in place. Acetone was used to clean the slide following the application of Lugol's iodine. Acetone is a decolorizer that helps get rid of extra stains on the slide. A drop of safranin was applied to the slide to hide the smudge. Gram-negative bacteria acquire their characteristic hue from the counter stained safranin stain. The slide was eroded clean after being properly cleaned with water to remove any remaining safranin. The extra liquid was carefully removed from the slide using blotting paper. To preserve the discolored smear, a drop of mounting agent Canada balsam was applied to the slide. A 100X magnification microscope was used to view the slide with the plated smear (Greenwood *et al.,* 2012).

Biochemical Characterization

Biochemical assays, including indole test, methyl red test, urease test, citrate test, carbohydrate fermentation test and motility test were carried out. Briefly described as follow:

Lactose fermentation test

Tests on consumption of lactose demonstrate if bacteria can ferment or oxidize a specific lactose to produce acid. The way an organism utilizes lactose is influenced by its enzyme complement. Following the preparation of a liter of nutrient broth, a little quantity of phenol red indicator which alters the broth color as bacteria proliferate in it was added. Durham tubes were inserted into each test tube after it had been filled with nutritional broth, and the tubes were autoclaved. After autoclaving, the test tubes were filled lactose *S. pullorum* strain cultures that were 48 hours old were placed in test tubes, and the cultures were observed for any change (Reddy *et al.,* 2022).

Urease Test

When amino acids were decarboxylated, urea was produced. Ammonia and CO2 were produced when the urea broke down. Phenol red changed from a pale orange color at a pH of 6.8 to a magenta (pink) tint at a pH of 8.1 when ammonia was added, increasing the alkalinity of the solution. Liquid pH changes were detected using phenol red. After a day, urease-positive bacteria turned the medium pink. Negative bacteria' acid either caused no color shift at all or a yellow hue shift. One or two drops of an overnight brain-heart infusion broth culture can be added to the surface of urea agar medium, or a part of a well-isolated colony can be placed on it. Furthermore, incubate the tube for 48 hours to 7 days at 35 to 37 degrees. Then, for at least seven days, observe for the formation of a pink color (Brink, 2010).

Methyl Red (MR) Test

The Methyl Red (MR) Test was conducted by inoculating a tube with MR broth, which was rich in glucose and peptone, with a culture of the suspected *S. pullorum* strain. Following that, the inoculation tubes were incubated for 24 to 48 hours at 37°C. A colorimetric change was seen in the tubes after the incubation time. The inclusion of methyl red indicator caused the medium's pH to drop and become red if the strain broke down glucose to produce stable, acidic byproducts. However, if the strain was unable to produce enough acidic byproducts, the medium would have remained yellow, indicating that there had not been any major fermentation of glucose and producing a negative MR test result (Tille & Bailey, 2014).

Indole Test

The indole test is used to determine if an organism can convert tryptophan into indole. Indole was identified using the Kovac's reagent, which mixes concentrated hydrochloric acid, isoamyl alcohol, and paradimethylaminobenzaldehyde in an acidic environment. A culture of *S. pullorum* was injected into a tube filled with tryptophan broth and incubated at 37 °C for 24 to 48 hours in order to conduct the Indole test. Add 0.5 ml (5 drops) of Kovac's reagent and mix gently. Look at the topmost layer of the liquid; if there are purple or red rings visible, a favourable result is shown; if yellow rings appear, a negative outcome is shown (KOMAL, 2019).

Antimicrobial Susceptibility Testing

Disk Diffusion Susceptibility Testing

To culture facultative anaerobic and pathogenic aerobic bacteria, Mueller-Hinton agar covered with various antibacterial filter paper disks is employed. By determining the sensitivity or resistance of these microorganisms to different antibiotic medications, the disk diffusion susceptibility test helps physicians choose therapeutic choices for their patients. The growth around the disks suggests indirectly that the medicine has the capacity to suppress that organism (Hudzicki, 2009). The 0.5 McFarland standard was utilized in order to produce bacterial suspensions. Antibiotic disks were placed over Mueller-Hinton agar plates after the suspension was applied. The plates were incubated at 37°C for 16–18 hours in order to determine their antibiotic susceptibility. Next, millimeters were used to measure the inhibitory zones (Hudzicki, 2009).

Results

Sample Collection and Processing: A total of 20 samples were collected from broiler chickens across five farms. Samples included cloacal swabs (5), visceral organs (5), and droppings (10). Each sample was stored in sterile containers and transported under cold chain conditions to maintain bacterial viability. Samples were surface sterilized in a 2% sodium hypochlorite solution for one minute. They were then rinsed three times with sterilized water and immediately transferred to nutritional broth medium (figure 1).

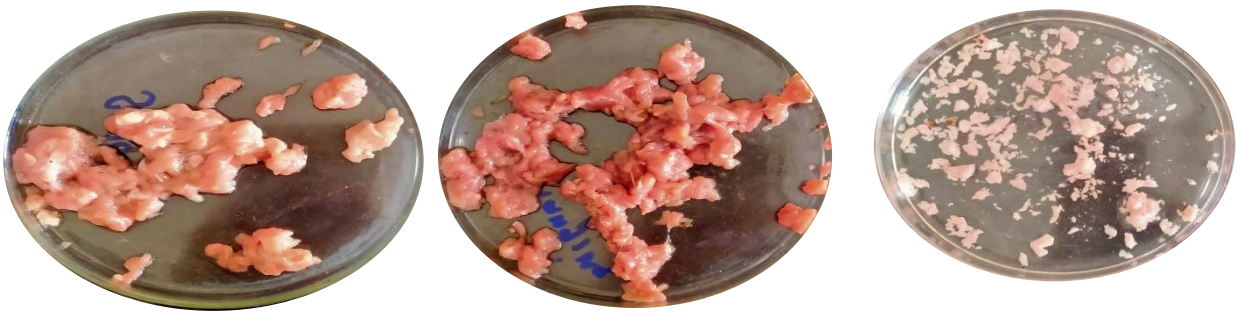
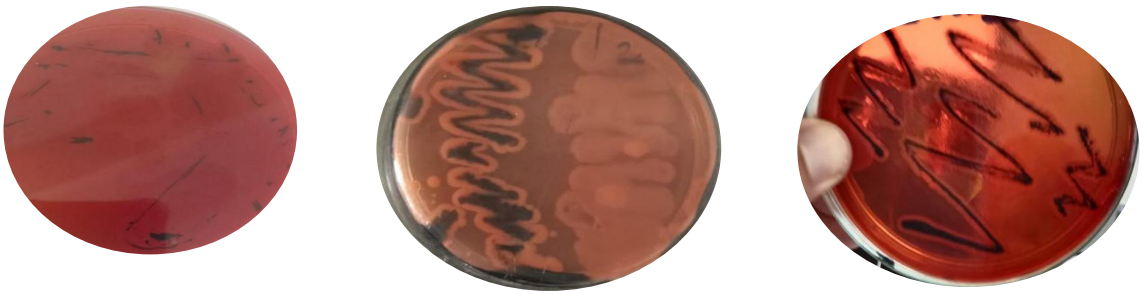


Figure 1: Sample processing

Identification of Bacterial Isolates

Samples were pre-enriched in buffered peptone water and incubated at 37°C for 18–24 hours. Selective enrichment was performed using Rappaport-Vassiliadis (RV) broth and Selenite broth. Culturing was done on Xylose Lysine Deoxycholate (XLD) agar, where black colonies show typical Salmonella morphology. Out of twenty samples three S. pullorum pathogen isolated from chicken ST1, ST2 and ST3 (figure 2).



A **B** **C**

Figure 2: Morphology of Bacterial Isolates ST1 (A), ST2 (B) and ST3 (C)

Gram Staining Results: Gram staining revealed that an isolated species of *S. pullorum* which had been grown for the overnight, was a rod-shaped, Gram-negative bacterium and appear pink color under microscope. The Gram response and cellular arrangement offered the initial evidence that these isolates were *S. pullorum* before doing confirmatory biochemical tests.

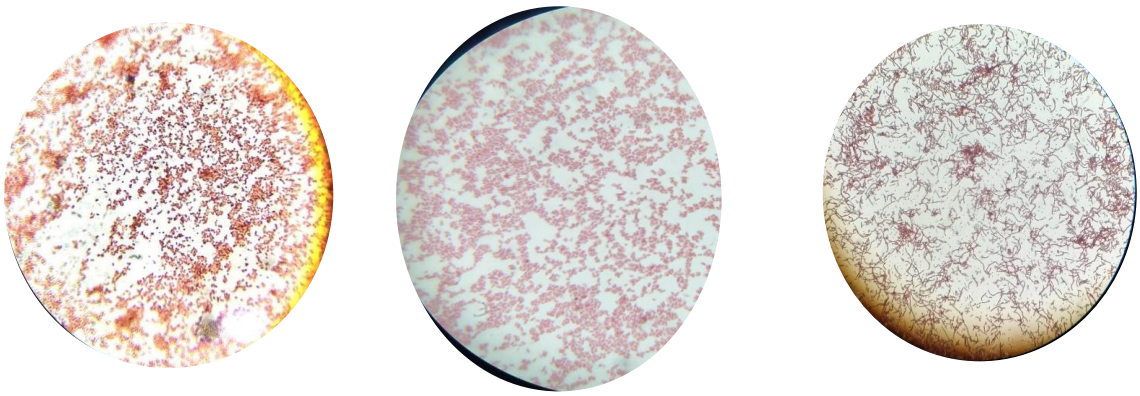


Figure 3*:* Microscopy of bacterial isolates

Biochemical Characterization

Lactose fermentation test

The ability of bacteria to non-ferment or oxidize a particular lactose in order to no create acid is determined by lactose fermentation tests. All strains of *S. pullorum* gave a negative result to lactose utilization test red color show positive result (A) and yellow show negative result. (B) (Figure 4).

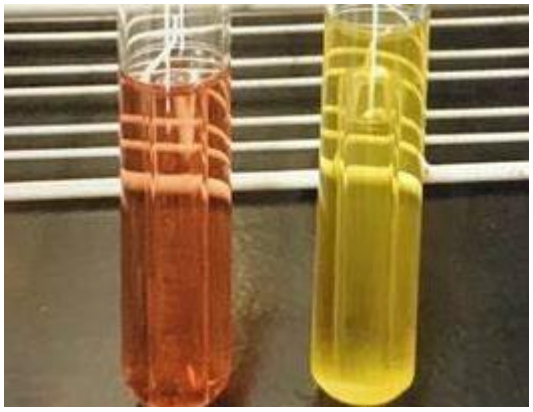


Figure 4: Lactose test result for *S. pullorum*

Urease test

All the bacterial isolates are urease negative, which mean that does not break down urea into ammonia and carbon dioxide (figure 5).

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Figure 5: Urease test for bacterial isolates

Methyl red test

The methyl red test (MR test) test bacteria are cultured in a broth medium that contains glucose. If the bacteria in the broth culture are able to absorb glucose and make a stable acid, the methyl red will cause the culture's yellow to turn into red. The culture provides a satisfactory result for the MR test when the red color of the culture medium results from the fermentation of glucose, which occurs at or below pH 4.4. Yellow in common culture

indicated a negative MR test. The outcome of the Methyl red test for the bacterial isolates of *S. pullorum* is positive show red color (A) and (B) is positive control show yellow color (Figure 6).

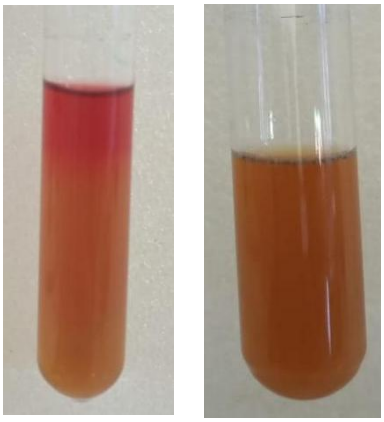

Figure 6: Methyl red test for all bacterial isolates

Indole test

A reddish-colored ring appeared on the glass tube surface as soon as the kovac's reaction was injected, indicating a successful indole test. When indole negativity is present, it is yellow or absent. Upon adding five to six drops of Kovac's reagent, every bacterial isolate had a negative indole test (Figurer 7).

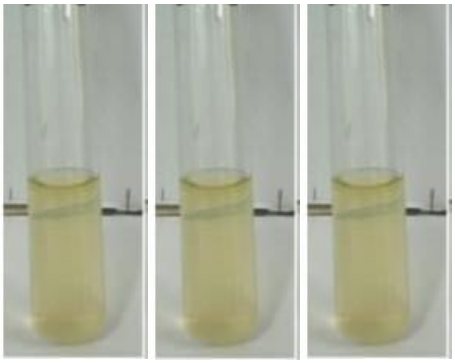

FIGURE 7: Indole Test Results for Bacterial Isolates

Table 1: Biochemical characterization of bacterial isolates

TESTS	ST1	ST2	ST3
Gram staining	-	-	-
Lactose fermentation test	+	+	+

Urease test	+	+	+
Methyl red	-	-	-
Indole	-	-	-

Antimicrobial susceptibility testing

To determine the antibiotic sensitivity pattern of isolated strains, sensitivity testing was performed. The highest antimicrobial Sensitivity rate was substantial, Tetracycline, Enrofloxicin 100%, Azithromycin 90%.

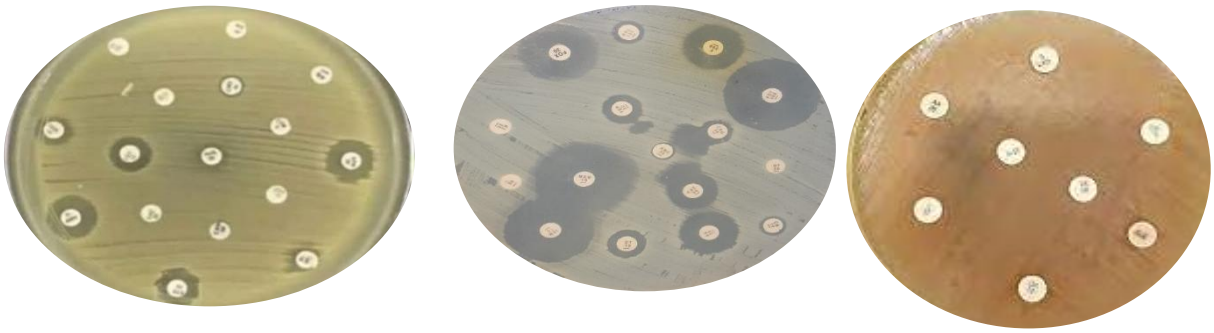

Table 2: Antibiotic resistance pattern of bacterial isolates

Antibiotic	Resistance rate	Sensitivity rate
Tetracycline	0%	100%
Enrofloxicin	0%	100%
Ciprofloxacin	58%	42%
Ceftriaxone	30%	70%
Azithromycin	10%	90%

Discussion

Salmonella pullorum is a significant bacterial infection that causes high death rates in young birds and is linked to decreased egg production, weight loss, and financial losses in adult birds. Pullorum disease (PD, white diarrhea) is a common and deadly sickness caused by *Salmonella pullorum*, which is highly suited to poultry (Zou *et al.,* 2016). Anorexia, sadness, diarrhea, and a chronic cloacal infection are among the signs that infected hens exhibit; PD is

an acute systemic illness that has a high fatality rate (Schat *et al.,* 2021). *S. pullorum* can infect hens of any age, but infection mortality declines with age, and many infected chickens go on to acquire latent and chronic infections. Moreover, *S. pullorum* can survive for several months in the spleen and reproductive system, causing vertical transmission to eggs and offspring, even though infected adult hens may show no symptoms. Although *S. pullorum* can infect hens of any breed and age, infection mortality declines with age, and many infected chickens go on to acquire latent and chronic illnesses (Schat *et al.,* 2021).

The purpose of the study was to isolate *Salmonella Pullorum* from boiler chicken, characterize it using biochemical analysis, and ascertain the isolates' antimicrobial resistance profiles. 20 samples across all were taken from broiler chicks on five different farms. Droppings (10), visceral organs (5), and cloacal swabs (5) were among the samples. In order to maintain bacterial viability, each sample was transported under cold chain conditions and kept in sterile containers. A 2% sodium hypochlorite solution was used to surface sterilize the samples for one minute. Following three rounds of sterile water rinsing, they were immediately placed in nutrient broth medium. AL-Iedani and Khudor (2013) conducted a similar investigation in which they determined 35 poultry samples had an incidence of *S. pullorum* infection. Following sample preparation, samples have been propagated on Xylose Lysine Deoxycholate (XLD) agar, where characteristic Salmonella morphology is apparent by black colonies. Three S. pullorum pathogens have been identified from chickens (ST1, ST2, and ST3) out of twenty samples. On XLD, the surface of every isolate produces black colonies. The culture results are in accordance with Ayesha and Mahmood's (2015) findings, which demonstrate that all Salmonella suspect isolates displayed red and white colonies with black centers on SS agar and XLD media. An isolated species of *S. pullorum* that had been cultured overnight was shown by Gram staining to be a rod-shaped, Gram-negative bacterium that appeared pink under a microscope. Similar study by (Kurts *et al.,* 2013) demonstrates that isolated bacterial pathogens manifest as Gram-negative rod shaped bacterium. Another aspect of this investigation was the biochemical examination of the isolates of *S. pullorum*. The experiment's findings demonstrated that the *S. pullorum* isolates tested negative for urease, positive for metyl red, negative for indole, and negative for lactose consumption. Similar, study was conducted by (Price *et al.,* 2012) which showed that the *S. pullorum* isolates negative for lactose utilization test, negative for urease, positive for metyl red, negative for indole test. The pattern of antibiotic susceptibility in the isolated isolates was determined by sensitivity testing. The greatest rates of antimicrobial sensitivity were 90% for azithromycin, 100% for enrofloxicin, and a significant 90% for tetracycline. The most

effective drugs against the studied isolates were tetracycline and enroflaxacin, which showed zero resistance. Ciprofloxacin should have a 58% resistance rate, whereas ceftriaxone showed a 30% resistance rate. 10% developed resistance to the macrolide azithromycin. Alike study by (Astal & Sharif, 2002) demonstrates that tetracycline, enrofloxicin were the most effective antimicrobial medicines against S. pullorum isolates with high sensitivity rate (81%–100%).

Conclusion

One of the most prevalent illnesses worldwide, pullorum disease (PD) can have disastrous effects. The chicken industry has encountered financial losses. Gallinarum biovar pullorum, a Salmonella enteric subspecies serovar, is the cause. The poultry business is affected by antibiotic resistance, a worldwide health concern, as MDR Salmonella strains have been found to mostly originate from chicken. The development of control measures that lower the danger of antibiotic-resistant infections requires an in-depth understanding of the bacterial resistance in animal and human isolates. Antibiotic resistance must be managed by government organizations, scientists, and chicken farmers via lowering antibiotic usage, actively monitoring MDR strains, and looking for substitutes to stop and stop infectious disease outbreaks.

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