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Original Research Article

Comparative Assessment of Selective Antibiotics for Managing Salmonellosis in Rabbits (*Oryctolagus cuniculus*)

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Abstract

Salmonella, major food-borne illness among human and animals where poultry being primary source of infection. Current strategies, vaccination, antibiotics, feed additives, help to reduce the infection in poultry but insufficient for long-term protection. This study's aim to compare the efficacy of three antibiotics used for salmonellosis treatment in poultry. Experimentally, rabbits (n=12) 8 weeks old, were divided into four (4) groups (A, B, C and D), three animals in each group. Animals initially kept for five days in their respective wooden cages, fed on commercial diet. The blood samples from typhoid infected human patients (n=5) were collected from Jinnah hospital Lahore to isolate bacteria by culturing on blood agar media. Culture filtrate (5 mL) of *salmonella typhi* was injected to experimental rabbits except control group. After 48 hours collected blood samples of three antibiotics treated groups were subjected for genomic DNA isolation, PCR amplification of *flipC* gene. For experiment trail, the experimental groups were subjected on three antibiotics treatments with oral dose (50mg/kg) for ten days. Group-A (control) untreated, group-B (ciprofloxacin), group-C (azithromycin) and group-D (cefotaxime). During drug delivery, feces of rabbits were collected on 1st, 4th, and 7th day for comparative analysis of drug efficacy by calculating CFU/mL grown on blood agar medium. Body weight analysis showed an increase in weight of untreated group while gradual decrease for experimental groups, which indicated the effect of infection and poor absorption of nutrients. S*almonella* infection was confirmed through PCR gene mapping test which was observed in all infected animals. Lesser CFU/mL (68.33) with grey-white colonies were observed in animal of group-B, 107 CFU/mL with opaque colored colonies (group-C) and 89 CFU/mL with moist, circular, smooth convex surface colonies (group-D). Thus ciprofloxacin (group-B) revealed as most effective antibiotic against *Salmonella* infection with more efficacy. These findings would be helpful for the farmers to use this antibiotic at poultry flocks against salmonellosis.

Keywords: *Salmonella,* infection; *flipC* gene; antimicrobial resistance, CFU, salmonellosis.

INTRODUCTION

Salmonellosis, a pathogenic bacterial infection, in poultry industry is a significant public health concern due to its impact on food safety and the potential for zoonotic transmission to humans. *Salmonella* is a gram negative, facultative anearobic bacterium, belong to family Enterobacteriaceae. Globally more than 2500 *Salmonella* serotypes have been reported, where some are the common cause of this disease among human and poultry like two *Salmonella* strains: *Salmonella enterica* ser. Enteritidis and *Salmonella enterica* ser. Typhimurium. Poultry products, particularly meat, milk, sea foods and eggs, are major sources of *Salmonella* infections, which can lead to severe gastrointestinal illness in humans. Worldwide, salmonellosis is highly prevalent in poultry across various regions. In Africa, the pooled prevalence estimate of poultry salmonellosis is 14.4%, with *S.* Enteritidis and *S.* Typhimurium being the most common serotypes from more than 170 serotypes (Kabeta, Tolosa, Duchateau, Van Immerseel, & Antonissen, 2024). In China, the prevalence of *Salmonella* in raw poultry meat varies, with chicken showing a prevalence of 26.4% (Sun *et al.,* 2021). In Asia, its prevalence is particularly high in developing countries, with high rates of anti-microbial resistance, poor sanitation and hygiene conditions (Iyer, Kumosani, Yousef, Barbour, & Harakeh, 2018). In United States, the second main cause of bacterial food borne illness is Salmonellosis, where *S.* Enteritidis and *S.* Heidelberg are commonly associated serotypes with poultry-related human infections (Foley, Lynne, & Nayak, 2008).

Poultry are the most common carrier of *Salmonella* infection while the consumption of this

contaminated product is the primary cause of human illness (Antunes, Mourão, Campos, & Peixe, 2016). Large number of poultry flocks have been associated with numerous *Salmonella* outbreaks due to close contact with infected poultry. Golbalization of poultry food products poses additional challenges for controlling salmonellosis (Antunes, *et al.,* 2016). Antibiotics are the most conventional approach to treat salmonellosis however, it is necessary to identify a suitable antibiotic based on the pathogen's susceptibility. Many first- and second-line typhoid antimicrobials therapies could be administered for the treatment of infected poultry, helping to alleviate its clinical symptoms and limiting infection spread.

However, the emergence of multidrug-resistant (MDR) *Salmonella* strains against these first-, second- and third-line drugs therapies is a growing concern in poultry health and production. The misuse of antibiotics in treating salmonellosis has led to the spread of MDR strains, complicating its treatment efforts. The use of lytic phages, natural medicinal compounds, like probiotics and phyto-compounds, against MDR *Salmonella* are the new emerging sustainable approaches for poultry which showed alternative approaches in the infection management (Khan and Rahman, 2022; Galán-Relaño *et al.,* 2023). Rabbit farming is widespread in many tropical countries which supports rural development through agriculture. *S.* Typhi and *S.* Enteritidis are reported as the common strains found in rabbits, ducks, chickens and pigeons in these farms. In Pakistan, first outbreak of multiple drug-resistant typhoid fever was reported from Sindh followed by Punjab, this highlighted the urgent need to investigate this incidence in Pakistan, considering the relationship between the development of anti-microbial resistance (AMR) and other factors (Shaikh, *et al.,* 2023). The current study was designed to investigate the efficacy of three different third generation antibiotics (ciprofloxacin, azithromycin and cefotaxime) in induced *Salmonella* infected rabbits to analyze the impact of these antibiotics in disease control.

MATERIAL AND METHODS

For this study**,** 12 healthy male rabbits (pre-vaccinated) aged 8 weeks were purchased from University of Veterinary and Animal Sciences **(**UVAS) Lahore. Pre-weighed rabbits were kept in wooden boxes with adequate ventilation and hygiene, fed similar diet for 10 days to get them acclimatized with the controlled environment conditions. Food was served twice in feeding bowls (6 am to 5 pm) placed in front of them with access to fresh water round the clock. For experimental purpose rabbits were divided into four groups (A, B, C and D) with 3 animals in each group. Group-A (control) where no dose of *Salmonella typhi* was given to the animals while three treatment groups (B, C & D) were given same dose (5mL per animal).

Collection of *Salmonella typhi* samples

To induce *S. typhi* infection, the blood samples (5 mL) of infected human patients were collected from Pathology department, Jinnah hospital Lahore in sterilized blood cultured bottles. All the blood samples were mixed, homogenized and a composite sample of this mixture was sent to laboratory for culture on blood agar plates and further confirmatory tests (genomic DNA isolation, gram staining and microscopic morphological observation) were performed as mentioned in Berger's manual ("The Protebacteria," 2006). The glycerol stocks were prepared from isolated *S. typhi* culture and kept at -20 ^{0}C till further use.

Induction of pathogens in rabbits

The experimental groups were get infected from isolated *S. typhi* culture via sterile injection syringes. For this purpose, the bacterial culture was thoroughly mixed in distilled water and filtered (0.4 µm membrane filter). The obtained filtrate (5 mL) was injected to experimental groups rabbits (B, C, & D) to develop infection within 24 hours. The infection symptoms including diarrhea, lethargy, loss of appetite, weight loss, fever, abdominal discomfort and pain, started to appear. Infected rabbits were subjected for treatment with respective antibiotics.

Detection of salmonellosis in rabbits

To detect salmonellosis, each animal blood samples were collected for the isolation of genomic DNA followed by selected bacterial gene amplification through PCR. For DNA isolation, the bacterial culture (100 mL) was grown overnight in LB broth at 37°C with shaking. Cells were harvested by centrifuging the culture at 8000 rpm for 5 minutes. Discarded the supernatant and resuspended the bacterial pellet in sterile PBS (180 µL). Further added 20 µL of lysozyme (10 mg/mL) to the suspension and mixed by vortexing. Incubated the mixture at 37°C for 30 minutes. For digestion of cellular proteins, added lysis buffer (200 µL) and Proteinase K (20 µL) solution to the sample and vortexed. Incubated the mixture at 56°C for 30 minutes to ensure complete lysis. For DNA precipitation 200 µl of chilled ethanol (96-100%) was added to the above lysed sample and mixed by vortexing, incubated the reaction mixture and centrifuged (12,000 rpm for 5 minutes) to get DNA pellet. Washed the pellet with 70% ethanol, briefly centrifuged the tube to remove any ethanol droplets from the lid. For DNA purification carefully purified the gel bound DNA to a Qiagen mini spin column with binding solution, placed in a 2 mL collection tube and spin at 8,000 rpm for 5 minutes to bind DNA on the column. Washed the column with wash buffer to remove salts and finally eluted the DNA in deionized water (100 µL). Stored the eluted DNA at -20°C or 4°C for short-term use.

In *Salmonella enterica,* serovar typhi, the flagellin protein is the major component of bacterial

flagella and essential for its mortality and virulence. This protein is encoded by *fliC* gene. To confirm *salmonella* infection 1521 bp *flicC* gene (GenBank ID AE014613.1) was amplified using gene specific 20 bp forward primer (5′ACAGGCGGTGAGCTTAAAGA3′) having 50% GC content and 60 ^{0}C T_m and 20 bp reverse primer (5′ATTGCCCAGGTTGGTGATAG3′) with 50% GC content and 59.8 ^{0}C T_m by Primer3Plus online software. PCR product get separated eletrophoretically and visualized through ethidium bromide-stained agarose gel (1%) under UV trans-illuminator and quantified spectrohotometrically at 260 nm.

Selection of antibiotics

Following three antibiotics, fluoroquinolone-based ciprofloxacin 500 mg (Organopharma), azithromycin dihydrate azithromycin 500 mg (Azilife), and -amino-4-thiazolyl based cefotaxime 500 mg (Mankind Pharma). All were third generation antibiotics for the gram positive and negative bacterial infections, and purchased from local marked to treat the infected rabbits (Figure 1). Antibiotic doses (50 mg/L) of each selected antibiotics were prepared in distilled water. Antibiotics dose optimization was done by using drug to body weight ratio standards. Oral drug delivery, once per day, was ensured for 7 days, for each antibiotic.

Figure 1: Antibiotics (available locally) used in the study for treatment of Salmonellosis in rabbits

Microbial analyses of animal's feces

During the drug delivery week, feces of all rabbits were collected in clean plastic bags and brought to laboratory on 1st, 4th and 7th day for comparative analysis of drug efficacy by calculating colony forming unit (CFU/ml) and colonial morphology was observed on selective media. After that gel electrophoresis was also performed for the detection of *S. typhi* in collected samples.

RESULTS

Isolation of S. *typhi* from patients' blood samples

Blood samples from typhoid suffering patients (n= 5) were collected and streaked on blood agar medium. The growth of bacterial (bluish black colonies) were detected on petri dishes after culturing them for 24 hours (Figure 2a) and counted them as per the procedure explained by Kaur, Kapil, Elangovan, Jha and Kalyanasundaram (2018). Following two tests were performed for strain confirmation; gram staining and microscopic and colonial morphology of *S. typhi*. In gram staining, *S. typhi* colonies appeared pink (Figure 2b) which confirmed the gram-negative cell wall composition of bacteria. This bacterium was found rod shaped with peritricus flagella under the microscope. Their colonies (Figure 2c) were in circular form with convex patches and smooth translucent margins.

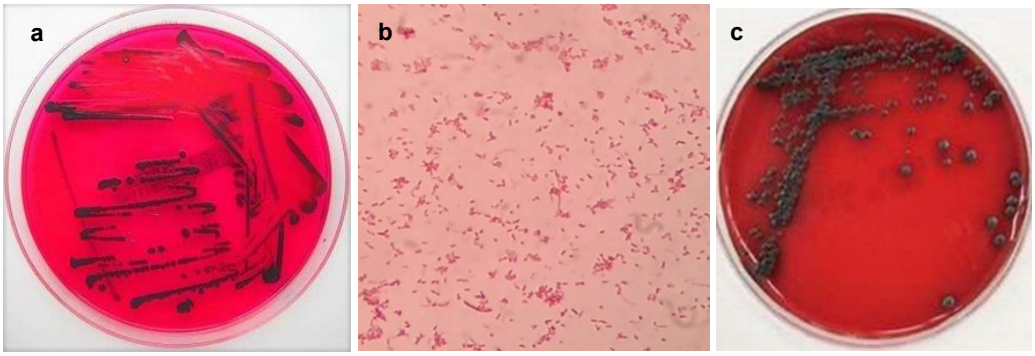

Figure 2a: Isolation of *S. typhi* from typhoid blood samples. b: Gram staining result of isolated bacterial cells (40X). c: Colonial morphology of *S. typhi* on blood agar

Impact of selective antibiotics on body weights of rabbits

The influence of antibiotics intake on body weights of rabbits were observed on following days: 0 (initial), 3rd, 6th, and 9th day of drug intake, the measurements were noted and obtained results showed in control group (A) with no inoculation of *salmonella* strain, gradual increased in body weights of rabbits was observed whereas the body weight of rabbits within treatment groups B (treated with ciprofloxacin), C (treated with azithromycin) and D (treated with cefotaxime), was decreased which confirmed the *salmonella* infection in experimental rabbits. Body weight observations are shown in Table 1.

Table 1: The impact of selective antibiotics on body weight (gram) of rabbits.

Groups	Initial weight	Day 3 (g)	Day 6 (g)	Day 9 (g)	Net weight (g)
Control group (A)	992.33	1076.66	1162	1252	259.33 (increased)
Ciprofloxacin (B)	996.33	993	985	980	16 (decreased)
Azithromycin (C)	996.33	992.33	985	982	14 (decreased)
Cefotaxime (D)	997.33	994	989	983	14 (decreased)

The results in experimental groups showed a decline in body weights of the animals due to the side effect of antibiotic intake and induced salmonellosis which most probably interfered the normal body metabolism. The reported data suggested that oral intake of antibiotics destroyed the intestinal microbiota and indirectly hindered in proper absorption of nutrients which led to a malnourished state of animals and resulted in gradual body weight reduction.

Detection of *S. typhi* in rabbits by PCR

To ensure that rabbits were properly infected with salmonellosis, blood sample (0.5 ml) was withdrawn and centrifuged (3,000 x g, 15 minutes). The harvested bacterial cells were lysed and genomic DNA was isolated and quantified (as mentioned in methodology). The selected *S. typhi* gene sequence, *flagellin C* (*fliC)* (code for flagellin protein, responsible for bacterial motility), gene sequence was retrieved from NCBI (Figure 3). *fliC* was amplified using gene specific primers in 30 cycles of PCR. Amplified gene fragment 336 bp was confirmed by agarose gel electrophoresis (Figure 4).

>AE014613.1:1013788-1015308 *Salmonella* Enterica subsp. enterica serovar Typhi
ATGGCACAAGTCATTAATACAAACAGCCTGTCGCTGTTGACCCAGAATAACCTGAACAAATCCCAGTCCGC
ACTGGGCACTGCTATCGAGCGTTTGTCTTCCGGTCTGCGTATCAACAGCGCGAAAGACGATGCGGCAGGAC
AGGCGATTGCTAACCGTTTTACCGCGAACATCAAAGGTCTGACTCAGGCTTCCCGTAACGCTAACGACGGT
ATCTCCATTGCGCAGACCACTGAAGGCGCGCTGAACGAAATCAACAACAACCTGCAGCGTGTGCGTGAAC
TGGCGGTTCAGTCTGCGAATGGTACTAACTCCCAGTCTGACCTCGACTCCATCCAGGCTGAAATCACCCAG
CGCCTGAACGAAATCGACCGTGTATCCGGCCAGACTCAGTTCAACGGCGTGAAAGTCCTGGCGCAGGACA
ACACCCTGACCATCCAGGTTGGTGCCAACGACGGTGAAACTATCGATATTGATTTAAAAGAAATCAGCTCT
AAAACACTGGGACTTGATAAGCTTAATGTCCAAGATGCCTACACCCCGAAAGAAACTGCTGTAACCGTTGA
TAAAACTACCTATAAAAATGGTACAGATCCTATTACAGCCCAGAGCAATACTGATATCCAAACTGCAATTG
GCGGTGGTGCAACGGGGGTTACTGGGGCTGATATCAAATTTAAAGATGGTCAATACTATTTAGATGTTAAA
GGCGGTGCTTCTGCTGGTGTTTATAAAGCCACTTATGATGAAACTACAAAGAAAGTTAATATTGATACGAC
TGATAAAACTCCGTTGGCAACTGCGGAAGCTACAGCTATTCGGGGAACGGCCACTATAACCCACAACCAA
ATTGCTGAAGTAACAAAAGAGGGTGTTGATACGACCACAGTTGCGGCTCAACTTGCTGCAGCAGGGGTTAC
TGGCGCCGATAAGGACAATACTAGCCTTGTAAAACTATCGTTTGAGGATAAAAACGGTAAGGTTATTGATG
GTGGCTATGCAGTGAAAATGGGCGACGATTTCTATGCCGCTACATATGATGAGAAAACAGGTGCAATTACT
GCTAAAACCACTACTTATACAGATGGTACTGGCGTTGCTCAAACTGGAGCTGTGAAATTTGGTGGCGCAAA
TGGTAAATCTGAAGTTGTTACTGCTACCGATGGTAAGACTTACTTAGCAAGCGACCTTGACAAACATAACT
TCAGAACAGGCGGTGAGCTTAAAGAGGTTAATACAGATAAGACTGAAAACCCACTGCAGAAAATTGATGC
TGCCTTGGCACAGGTTGATACACTTCGTTCTGACCTGGGTGCGGTTCAGAACCGTTTCAACTCCGCTATCAC
CAACCTGGGCAATACCGTAAATAACCTGTCTTCTGCCCGTAGCCGTATCGAAGATTCCGACTACGCAACCG
AAGTCTCCAACATGTCTCGCGCGCAGATTCTGCAGCAGGCCGGTACCTCCGTTCTGGCGCAGGCGAACCAG
GTTCCGCAAAACGTCCTCTCTTTACTGCGTTAA

Figure 3: FASTA gene sequence of *flicC* gene. The forward and reverse primers are highlighted in yellow color. Only 336 bp DNA fragment was selected to be amplified by PCR

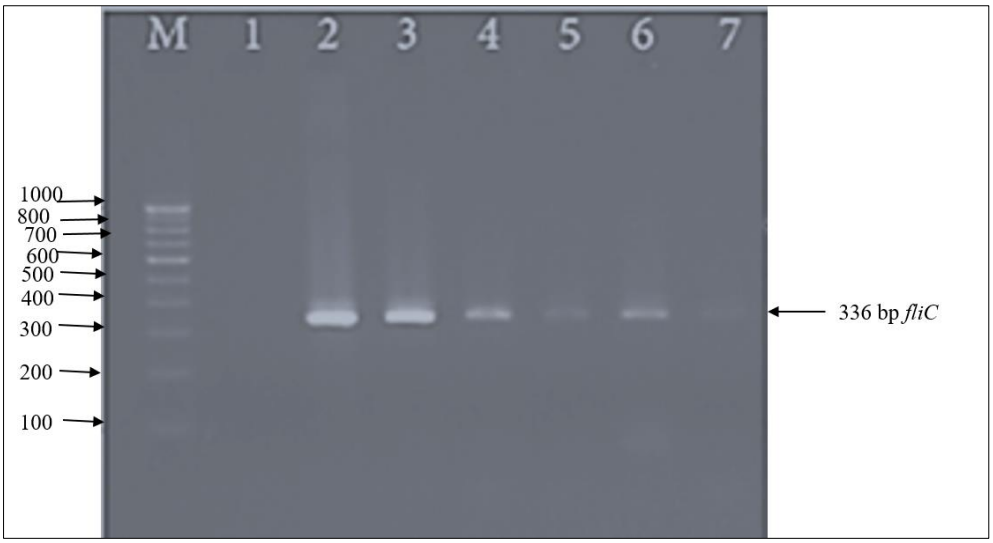

Figure 4: Agarose gel showing PCR amplification of the targeted *flagellin* (*fliC*) gene of *S. typhi*. Lane M, 100 bp DNA ladder (Cat No.15628019, Fisher Scientific); lane 1, negative control; lane 2, positive (*S. Typhi* MTCC 3216); lanes 3-7, positive PCR amplicons)

Colony Forming Units (CFU/ml) in infected rabbits

To measure the effect of antibiotics on infected rabbits, their feces were collected during medicinal doses delivery on 1st, 4th and 7th day of infection. Colony forming units (CFU, measures the number of viable bacterial cells) of *S. typhi* were calculated from fecal samples on petri dishes of infected rabbits to indirectly estimate the comparative efficacy of selected antibiotics against salmonellosis. Though statistically results were not found significant but minimum number of surviving bacterial colonies was noted in group B (Table 2). Moreover, less efficient antibiotic effect was observed for group C.

Table 2: CFU/ml of study groups and their colony morphologies.

Experimental groups	CFU/ml	Morphology
B (Ciprofloxacin)	68.33	Grey-white colonies
C (Azithromycin)	107	Opaque
D (Cefotaxime)	89	Moist, circular with smooth convex surface

DISCUSSION

Salmonella is an acute and pathogenic infection in poultry birds with high mortality rate and cause of latent infection transfer in human through infected meat and egg products consumption. Antimicrobial therapeutics are the most conventional treatment for salmonellosis in poultry including enrofloxacin, ciprofloxacin, azithromycin, ceftriaxone, chloramphenicol, neomycin, polymxin, nitroflurazolidone, amoxicillin, tetracycline etc. The use of broad-spectrum antibiotics is the common choice, however, antibiotic sensitivity of these strains, using disc diffusion assay, showed the highest resistance against first-line antibiotics (amoxicillin-clavulanic acid, ampicillin) while lowest resistance was observed with second generation antimicrobials including gentamicin, ciprofloxacin, and norfloxacin. Furthermore, genetic analysis showed the presence of *sopB* virulence gene in these strains (Hamed *et al.,* 2023). Third-generation antibiotics such as cephalosporins, azithromycin, are the recommended treatment options for typhoid fever in developing countries (like Pakistan, Bangladesh, Nepal, Liberia) but they are gradually removing from the arsenal due to acquired resistance of typhoid infection. High content imaging and machine learning approach also predicted the Ciprofloxacin susceptibility in *S.* Typhimurium isolates without prior exposure to this drug. This antibiotic is used to treat *Salmonella* infections in spite of widespread resistance, especially in low-resource settings where the medication is administered haphazardly (Jabeen *et al.,* 2023; Tran, *et al.,* 2024).

In current study the body weight analysis of infected rabbit's vs control group revealed the gradual decreased in body weight of all *Salmonella* infected groups (14-16 g) which indicated the effect of *Salmonella* infection and its confirmation through *flicC* gene amplification. The body weight loss in all infected groups have occurred due to poor absorption of nutrients. Antibiotics treatment efficacy was further evaluated through CFU/ml counts in contaminated feces of all treated groups. This analysis showed the lowest CFU/ml in animal of group-B (68.33 with grey white colonies), 107 CFU/ml for group-C with opaque colored colonies and 89 CFU/ml with moist, circular with smooth convex surface colonies for group D. This indicated that group-

B (Ciprofloxacin treated) showed the most effective antibiotic therapy against S*almonella* infection than other two antibiotics.

Ciprofloxacin, a fluoroquinolone based broad spectrum antibiotic, effectively discontinue DNA replication by hindering the A subunit of DNA gyrase (crucial for DNA replication) and having an additional influence on bacterial cell walls. Although the resistance has been increased in several pathogens but its new derivatives compounds along with other antibacterial agents may enhance its effectiveness against bacterial resistance and biofilm formation (Shariati *et al.,* 2022). The study on the combination of deep learning and high-resolution imaging, as a potential tool to predict Ciprofloxacin susceptibility in *S.* Typhimurium, revealed the less changes in growth and morphology in *S.* Typhimurium isolates with ciprofloxacin use (Tran *et al.,* 2024). Another study showed that in acute typhoid fever, ciprofloxacin induced a significant cytokine alteration including IL-6 and IL-10 with some TNF-beta cells (Kadhim, Saeed, Al-Ganahi, 2022). In addition, microorganisms that exposed to sub-minimal inhibitory concentration of ciprofloxacin therapy led to a complete reduction in the expression of sifA and sifB or other *Salmonella* lethal genes, which are important for intestinal *Salmonella* lineage system and lead to lower mortality rate of bacteria. Ciprofloxacin minimal inhibitory dose helped to reduce sifA and sifB genes expressions which are required for bacterial filaments formation so provide additional mechanism for controlling *Salmonella* infection (Askoura and Hegazy, 2020). Our study also showed the decrease in colony forming units in presence of ciprofloxacin.

CONCLUSION

The current study supported the strong efficacy of ciprofloxacin antibiotic against *S. typhi* infection. These findings would be helpful for the farmers to use this antibiotic at their poultry farms against salmonellosis. The findings also contribute for the management and treatment of salmonellosis in the context of rabbit farming and will be very helpful for those farmers who wish to improve the operational and health aspects of their farm's animals.

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