



SHORT COMMUNICATION

Evaluation of Antibiotic Resistance Profile and Multiple Antibiotic Resistance Index in Avian Adapted *Salmonella enterica* serovar Gallinarum Isolates

Rida Haroon Durrani^{1,†}, Ali Ahmad Sheikh^{1,*}, Muhammad Humza^{2,†}, Salman Ashraf¹, Aleena Kokab¹, Tauqeer Mahmood⁴ and Muhammad Umar Zafar Khan^{3,**}

¹Institute of Microbiology, Faculty of Veterinary Sciences, University of Veterinary and Animal Sciences, Lahore 54000, Pakistan; ²Key Laboratory of Agro-products Quality and Safety Control in Storage and Transport Process, Ministry of Agriculture and Rural Affairs/Institute of Food Science and Technology, Chinese Academy of Agricultural Sciences, Beijing 100193, China; ³Institute of Microbiology, Faculty of Veterinary Sciences, University of Agriculture, Faisalabad 38040, Pakistan; ⁴Poultry Research Institute, Rawalpindi 46000, Pakistan

Corresponding author: ali.ahmad@uvas.edu.pk (Ali Ahmad Sheikh); umar.zafarkhan@uaf.edu.pk (M Umar Zafar Khan**)

ARTICLE HISTORY (24-330)

Received: June 15, 2024
Revised: July 29, 2024
Accepted: August 21, 2024
Published online: September 27, 2024

Key words:

Antibiotics
Antibiotic Resistance
Salmonella Gallinarum
Salmonella Pullorum
Amoxicillin, Ampicillin
Sulfamethoxazole
Trimethoprim
Doxycyclin

ABSTRACT

The emergence of antibiotic resistance owing to the imprudent use of antibiotics in food-producing animals has been associated with huge costs and threats, particularly in countries with comparable farming practices like Pakistan. The present study evaluated the efficacy of 10 antibiotics using their standard concentrations against *Salmonella enterica* subsp. *enterica* serovar Gallinarum biovar Gallinarum and Pullorum from three districts of Punjab province in Pakistan. Surprisingly, 66.7% of the isolates were resistant to the β -lactam potentiator, amoxicillin-clavulanate. On the contrary, 23.3% and 33% of the isolates were resistant to the two III-generation aminobenzyl penicillins, amoxicillin and ampicillin, respectively. 56.6% of isolates were found to be resistant to the II-generation aminoglycoside Gentamicin, and 100% resistance was observed against Nalidixic Acid, Ciprofloxacin, or Levofloxacin. A high degree of susceptibility to Sulfamethoxazole-Trimethoprim and Doxycycline was observed. The antibiotics exhibited statistical significance, via correlation and cluster analysis. Conclusively, the study reports the multitude of multidrug resistance in host-adapted *Salmonella*, emphasizing the importance of continued research and monitoring into the use of antibiotics in agro-food animals on a national level.

To Cite This Article: Durrani RH, Sheikh AA, Humza, M, Ashraf S, Kokab A, Mahmood T and Khan MUZ, 2024. Evaluation of antibiotic resistance profile and multiple antibiotic resistance index in avian adapted *Salmonella enterica* serovar Gallinarum isolates. Pak Vet J. <http://dx.doi.org/10.29261/pakvetj/2024.253>

INTRODUCTION

Pakistan's population is growing at a staggering rate of 2.55% annually, reaching 241.49 million (5th most populous country), according to the 7th population and housing census (Pakistan Bureau of Statistics, 2023). The population's expanding nutritional requirements, including protein, are driving an exponential increase in the demand for livestock-derived products, ranking Pakistan 11th among the world's largest poultry producers with an annual growth rate of 7.3% (GOP, 2023–2024). This has led to a massive shift in production systems (i.e., from extensive production to intensive industrialized production), which has coincided with the imprudent and uncontrolled use of antibiotics in food-producing animals (Caneschi *et al.*, 2023).

Despite playing a vital role in the providing a sustainable food supply, avian salmonellosis continues to pose a significant threat associated with productivity (direct loss) and economic losses (i.e., cost of treatment) (Durrani *et al.*, 2022). The widespread use of antibiotics in the poultry sector as a hygiene surrogate and growth promoter has exacerbated the problem and favored the emergence of multi-drug resistant strains in *Salmonella enterica* subsp. *enterica* serovar Gallinarum biovar Gallinarum and Pullorum (Founou *et al.*, 2016; Farhat *et al.*, 2023). The long-term use of single class of antibiotics, exceeding daily dose, sub-therapeutic dose, metaphylaxis, improper withdrawal requirements before slaughter, spread of active drug residues in the food chain, lateral spread of resistance elements, and gut microbiome disruption are well-documented evidence of imprudent

use of antibiotics in food-producing animals (Mohsin *et al.*, 2019; Sun *et al.*, 2019; Ramirez *et al.*, 2020; Caneschi *et al.*, 2023).

Pathogenic microbes and their genes with AMR potential circulate frequently in our environmental surroundings, predominantly evolving from unregulated infectious wastes containing antimicrobial substances released by hospitals, pharmaceutical production units, and livestock farms. The extended-spectrum β -lactamase (ESBL) producing Enterobacteriaceae, *Campylobacter* spp., *Clostridium perfringens*, *Enterococcus* spp., nosocomial and community-associated MRSA and VRSA are notable pathogens (Shokoohi *et al.*, 2018; Khan *et al.*, 2021).

While there is plenty of data available on the antibiotic resistance in human isolates of *Salmonella* the data regarding the animals is insufficient. Therefore, present study aimed to evaluate the efficacy of frequently used antibiotics and provide the preliminary data highlighting the multitude of antibiotic resistance specified as MAR indices in the host-adapted *Salmonella*. The zone of inhibition was used as an interpretative criterion.

MATERIALS AND METHODS

Sampling: A total of 30 ($n=30$) pooled liver and spleen samples were acquired aseptically from necropsied birds presented with a history and clinical signs of Fowl Typhoid/Pullorum Disease (FT/PD). The samples were collected from small and medium-scale privately-owned farms in Lahore, Sheikhpura, and Gujranwala in the Punjab province of Pakistan from 2018 to 2022 as reported. Following collection, the samples were immediately transported to the university diagnostic laboratory (UDL), University of Veterinary and Animal Sciences, Lahore, for further analysis.

Phenotypic Detection of Bacterial Strains: Direct enrichment of isolates was carried out in RV broth (CM0669) at 41.5°C for 48 hours at 120 rpm in a shaking incubator (*SI900R* Robust Technologies), followed by sub-culturing on *Salmonella Shigella* (SS) agar (CM0099) and Xylose Lysine Deoxycholate (XLD) agar (CM0469) as per guidelines mentioned in OIE Terrestrial Animal Health Code 2020. Traditional phenotypic antigenic formula and biotyping were performed as per the White-Kauffmann-Le Minor (WKL) scheme at the university diagnostic laboratory (UDL). Additionally, the isolates were reference typed at the National Reference Laboratory for Poultry Diseases using multiplex polymerase chain reaction and commercially available API (*Biomérieux* Analytical Profile Index API 20E Cat No. 20100) test strips to monitor atypical biochemical variations between biotypes. The confirmed isolates were stored in 50% sterile glycerol at -80°C for further analysis. All the media used were manufactured by Oxoid, UK.

Antibiotic Susceptibility Testing by Kirby Bauer Assay: Antibiotic susceptibility testing (AST) was performed for the positive isolates with selected antibiotics classes that were frequently used in farm

settings, i.e., aminobenzyl penicillins and β -lactam potentiator, aminoglycosides, macrolides, quinolones and fluoroquinolones, sulfonamides and tetracyclines by using pre-infused antibiotic disks of 9mm (Oxoid™ Antimicrobial Susceptibility Disks, Thermo Scientific™ USA). The first group involved three antibiotics including amoxicillin (25 μ g), ampicillin (20 μ g), and amoxicillin-clavulanic acid (30 μ g). Gentamicin (10 μ g) was the only antibiotic from the aminoglycoside group. Clarithromycin (15 μ g) was selected from macrolides class. Nalidixic acid (30 μ g), ciprofloxacin (5 μ g) and levofloxacin (5 μ g) were selected from quinolone and FQ group. Sulfonamide group include sulfamethoxazole-trimethoprim (25 μ g) and the tetracycline group involved doxycycline (5 μ g). The selection of antibiotics was made according to the OIE critically important antibiotics (CIA) list where OIE has defined the β -lactams, aminoglycosides, macrolides, tetracyclines, quinolones and fluoroquinolones as highly important veterinary antimicrobial agents (OIE, 2015).

The susceptibility assay was carried out according to the Kirby-Bauer Method based on the zone of inhibition of the bacterial strains under standard conditions (Clinical Laboratories Standards Institute, 2020b, 2020a). The test isolates were aseptically and uniformly inoculated on Mueller-Hinton agar (CM0337) followed by filter paper discs saturated with a specific antibiotic concentration, which were then placed on the medium. The interpretive criterion is based on the diameter of the zone of inhibition used to indicate susceptibility to a given antibiotic (Table 1).

Table 1: Grading Scale for 10 Antibiotics against *Salmonella entericasubsp. entericaserovarGallinarum* and Pullorum Based on Zone of Inhibition

Antibiotic	Sensitive ^a	Intermediate ^b	Resistant ^c
Amoxicillin	≥ 17	14-16	≤ 13
Ampicillin	≥ 17	14-16	≤ 13
Amoxicillin-Clavulanate	≥ 18	14-17	≤ 13
Gentamicin ^δ	≥ 15	13-14	≤ 12
Clarithromycin	≥ 13	-	≤ 12
Nalidixic Acid	≥ 19	14-18	≤ 13
Ciprofloxacin	≥ 31	21-30	≤ 20
Levofloxacin	≥ 31	21-30	≤ 20
Sulfamethoxazole-Trimethoprim	≥ 16	11-15	≤ 10
Doxycycline	≥ 14	11-13	≤ 10

^a Sensitive isolates are denoted with +++; ^b Intermediate ranges are denoted with a "A" and it also includes a buffer zone for inherent variability in testing methods; ^c Resistant isolates are denoted with a "δ"; Although aminoglycosides may be active *in vitro*, but not therapeutically efficacious and should not be listed as susceptible/sensitive for *Salmonella* and *Shigella*. The zone of inhibition was analyzed using CLSI standards for animals pathogens.

The multiple antibiotic resistance index (MARI) of the test isolates was calculated and interpreted as stated by Krumperman (1983) using the given formula below:

$$MAR\ Index = a/b$$

'a' representing the no. of antibiotics to which an isolate showed resistance, and 'b' represents the total no. of antibiotics examined against the isolate.

Statistical Analysis: Statistical analysis was applied to the data where analysis of variance was done using Statistical Package for Social Sciences (SPSS Version 26 Armonk, USA). The comparisons between means were

performed via Tukey's Honesty Significance Difference (HSD) Test at $P \leq 0.05$. Correlation analysis was performed using dplyr package (Wickham *et al.*, 2023) while cluster analysis was done with the help of heatmaply package (Galili *et al.*, 2018) in R Studio software version 4.0.2 (RStudio Team (2020)).

RESULTS AND DISCUSSION

Among the third generation aminobenzyl-penicillin, the antibiotics AX (76.6%) and AMP (60%) responded with moderate to high degree of susceptibility towards the test isolates, while 66.7% strains showed a high degree of resistance to 3rd generation β -lactamase inhibitor amoxicillin-clavulanate. As illustrated in Figure 1, a positive correlation can be seen between AX and AMP, while a negative correlation can be observed between AX and AMC at their standard concentration. This implies that AX and AMP can be used in combination to have a significant reduction in bacterial numbers. Abdel-Atty *et al.*, 2023 also reported 100% co-amoxiclav resistance in *Salmonella* strains isolated from meat products followed by 66.6% and 33.3% resistance to gentamicin and ciprofloxacin. The results reported by Zige *et al.*, 2023 also confirm the resistance to co-amoxiclav (72.5%) in *Salmonella* spp., isolated from poultry droppings. Hence, establishing the fact that *Salmonella* is a 'priority' pathogen all across the globe due to its expression of β -lactamases.

In case of Gentamicin (2nd generation aminoglycoside), a range of responses were observed where 50% isolates were resistant while the remaining 13.3% and 30% isolates produced zones of inhibition that coincided with an intermediate to moderately susceptible response. The study of Abdel-Atty *et al.*, 2023 also reported 55.1% of their *Salmonella* isolates to be gentamicin resistant. A negative correlation was observed between the amino-benzyl penicillins and aminoglycoside class which suggests that the two classes can produce antagonistic effects if combined (in therapy) with AX and AMC, however, AMP showed positive correlation with CN to produce synergistic effects.

Regarding Clarithromycin, the isolates did not show any kind of susceptibility. (Note: The term "non-susceptible" does not necessarily implies that the isolate(s) possesses resistance mechanism(s). The term "non-susceptible" should not be misinterpreted when the text is describing an organism/isolate in intermediate or resistant interpretative categories, rather the term "not susceptible" must be used). CLR showed a negative correlation with quinolone (NA) and fluoroquinolones (CIP, LEV) while a positive correlation was observed in case of SXT, and DO. The use of macrolides is highly potent against a variety of gram positive and negative bacteria owing to its suppression of protein synthesis. Despite being an attractive alternative in veterinary medicine, clarithromycin has advantages over erythromycin but the data on the rational use of clarithromycin in targeted animal species is not available as reported by Awadallah *et al.* (2016).

A high level of resistance was observed in all the given isolates against the narrow-spectrum synthetic quinolone (technically a naphthyridone – a two nitrogen containing structure as compared to single nitrogen containing quinolone) Nalidixic acid and broad-spectrum second and third generation FQ, ciprofloxacin and levofloxacin, respectively. The correlation analysis confirmed that NA and CIP exhibited a positive correlation with DO while both showed a negative correlation with LEV and SXT. Abdel-Atty *et al.*, 2023 reported 33.3% *Salmonella* isolates to be CIP resistant while Zige *et al.*, 2023 stated that 65.9% of their test isolates were resistant to ofloxacin – a second generation FQ like CIP. Despite showing 100% efficacy against all test strains SXT and DO exhibit negative correlation with each other as can be seen in Figure 1. The findings of Abdel-Atty *et al.*, 2023 coincide with the present study suggesting uniformity in antibiotic resistance patterns all across the globe. Thus, correlation verified that tested antibiotics estimated a significant decline ($p \leq 0.05$) in bacterial progression when using certain combinations to avoid the irrational use.

The current study estimated the multiple antibiotic resistance indices of the current isolates ranging between 0.4 and 0.6 as all 30 isolates (100%) scored more than 0.3. MAR index serves a valuable tool to differentiate high and low risk animal isolates with an index greater than 0.3 indicating the development of antibiotic resistance in the particular area of antibiotic administration. The number of isolates with MAR index with 0.4 (10%), 0.5 (73.3%) and 0.6 (16.7%) highlights the alarmingly high use of antibiotics, creating a selective pressure among bacteria and a high public and animal health hazard. The most frequent resistance patterns observed in the isolates are as follow and Figure 2 illustrates this in the form of heatmap generated via cluster analysis.

- AMC/CN/CLR/NA/CIP/LEV
- CN/CLR/NA/CIP/LEV
- NA/CIP/LEV

The rationale of the present study is to highlight a frequency and diversity of antibiotic resistance patterns in host-adapted *Salmonella* strains suggesting the necessity of continuing research to monitor the inappropriate use of antibiotics and managing then threat of emerging resistance in veterinary pathogens through the alternative and complementary strategies.

Authors contributions: AHS conceived and designed the study. RHD & AK executed the laboratory analysis and finalized the data. TM & SA assisted in animal handling and sampling. MH & MUZK performed statistical analysis. RHD and MH wrote the manuscript. RHD, AAS, MH and MUZK reviewed and edited the manuscript. RHD and MH contributed equally to this work. All the authors read and approved the final draft of the manuscript.

Conflict of Interest: Not Applicable

Data availability statement: The data will be available on request to corresponding author(s).

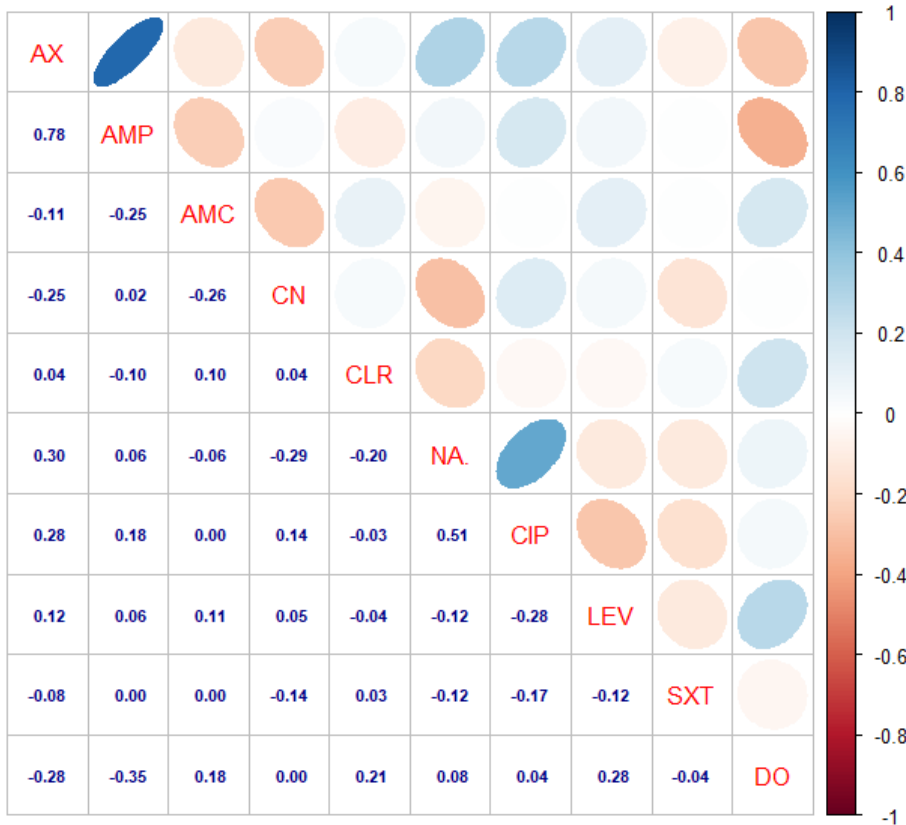


Fig. 1: Correlation analysis among the 10 antibiotics (AX: Amoxicillin, AMP: Ampicillin, AMC: Amoxicillin-Clavulanate, CN: Gentamicin, CLR: Clarithromycin, NA: Nalidixic Acid, CIP: Ciprofloxacin, LEV: Levofloxacin, SXT: Sulfamethoxazole-Trimethoprim and DO: Doxycycline) against *Salmonella enterica* subsp. *enterica* serovar Gallinarum biovar Gallinarum and Pullorum at their standard concentrations. The blue-colored digits and the multi-colored ellipses represent the *r*-value (i.e., correlation value). The positive values suggest synergistic effects between antibiotics as depicted in blue and lighter tones of blue. The negative values propose the antagonistic effect as can be seen in orange and light orange hues as per scale drawn on the right side of the matrix. The value '0' indicates neutral or no association between stated antibiotics.

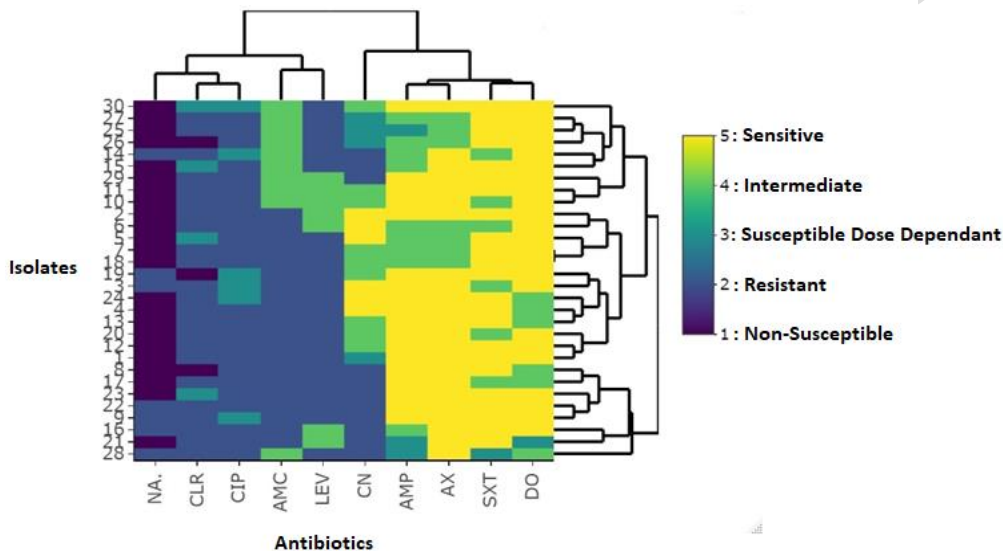


Fig. 2: Cluster analysis of tested 10 antibiotics (AX: Amoxicillin, AMP: Ampicillin, AMC: Amoxicillin-Clavulanate, CN: Gentamicin, CLR: Clarithromycin, NA: Nalidixic Acid, CIP: Ciprofloxacin, LEV: Levofloxacin, SXT: Sulfamethoxazole-Trimethoprim and DO: Doxycycline) against *Salmonella enterica* subsp. *enterica* serovar Gallinarum biovar Gallinarum and Pullorum at their standard concentrations. The heat map form illustrates the cluster analysis of all antibiotics by finding the zone of inhibition. The color scale represents the degree of inhibition caused by antibiotics.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: The authors gratefully acknowledge scientific assistance from Poultry Research Institute, Rawalpindi, Pakistan, Dr. NS (Project Lead, National Reference Laboratory for Poultry Diseases, National Agriculture Research Council, Islamabad, Pakistan) and Dr. AA (Zoonotic Disease Surveillance - National Reference Laboratory for Poultry Diseases, National Agriculture Research Council, Islamabad, Pakistan). The authors further acknowledge the contributions of Assistant Disease Investigation Officers (ADIOs) from the Livestock and Dairy Development Department (L&DD), Punjab for providing data on possible sample locations.

REFERENCES

- Abdel-Atty NS, Abdulmalek EM, Taha RM, *et al.*, 2023. Predominance and antimicrobial resistance profiles of *Salmonella* and *E. coli* from meat and meat products. *J Adv Vet Res* 13(4):647-55.
- Awadallah H, Awidat S and El-Mahmoudy A, 2016. Pharmacokinetics of clarithromycin after single intravenous and intracrop bolus administrations to broiler chickens. *Int J Pharmacol Toxicol* 4:12-8.
- Caneschi A, Bardhi A, Barbarossa A, *et al.*, 2023. The use of antibiotics and antimicrobial resistance in veterinary medicine, a complex phenomenon: A narrative review. *Antibiotics* 12:3, p. 487.
- Agriculture, Economic Survey of Pakistan, Finance Division, Government of Pakistan (2023-24), finance.gov.pk/survey/chapter_24/2_agriculture.pdf. Accessed on 25.06.2024.
- Clinical Laboratories Standards Institute, 2020a. Performance standards for antimicrobial susceptibility testing.
- Clinical Laboratories Standards Institute, 2020b. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard.

- Durrani RH, Sheikh AA, Rabbani M, et al., 2022. Physiological properties of indigenous lytic bacteriophages as monophage suspension and cocktail against poultry-adapted typhoidal Salmonella variants. *Vet Res Forum* 13(4): 481-487.
- Farhat M, Khayi S, Berrada J, et al., 2023. Salmonella entericaserovar Gallinarumbiovars Pullorum and Gallinarum in Poultry: Review of Pathogenesis, Antibiotic Resistance, Diagnosis and Control in the Genomic Era. *Antibiotics* 13(1):23.
- Founou LL, Founou RC and Essack SY, 2016. Antibiotic resistance in the food chain: A developing country-perspective. *Front. Microbiol* 7:1881.
- Galili T, O'Callaghan A, Sidi J, et al., 2018. "Heatmaply: an R package for creating interactive cluster heatmaps for online publishing." *Bioinformatics*.
- Khan MUZ, Humza M, Yang S, et al., 2021. Evaluation and optimization of antibiotics resistance profile against Clostridium perfringens from buffalo and cattle in Pakistan. *Antibiotics* 10:59.
- Krumperman PH, 1983. Multiple antibiotic resistance indexing of Escherichia coli to identify high-risk sources of fecal contamination of foods. *Appl Environ Microbiol* 46(1):165-70.
- Mohsin M, Van Boeckel TP, Saleemi MK, et al., 2019. Excessive use of medically important antimicrobials in food animals in Pakistan: a five-year surveillance survey. *Glob Health Action* 12:1697541.
- Fowl Typhoid and Pullorum Disease, Office International des Epizooties (OIE), 2020. Available at <https://www.oie.int/standard-setting/terrestrial-manual/access-online/>.
- OIE list of antimicrobial agents of veterinary importance, 2015. Office International des Epizooties (World Organization for Animal Health) <https://www.oie.int/app/uploads/2021/03/a-oie-list-antimicrobials-may2018.pdf>.
- RStudio Team (2020). RStudio: Integrated Development for R. RStudio, PBC, Boston, MA.
- Ramirez J, Guarner F, Bustos Fernandez L, et al., 2020f. Antibiotics as major disruptors of gut microbiota. *Front Cell Infect Microbiol* 10:572912.
- Shokoohi R, Samadi MT, Amani M, et al., 2018. Modeling and optimization of removal of cefalexin from aquatic solutions by enzymatic oxidation using experimental design. *Brazilian J ChemEng* 35:943-956.
- Sun L, Zhang X, Zhang Y, et al., 2019. Antibiotic-induced disruption of gut microbiota alters local metabolomes and immune responses. *Front Cell Infect Microbiol* 9:99.
- The Digital Census, 7th Population and Housing Census, Pakistan Bureau of Statistics (March 2023). <https://www.pbs.gov.pk/sites/default/files/population/2023/Press%20Release.pdf>. Accessed on 03.08.2024.
- Wickham H, François R, Henry L, et al., 2023. Dplyr: a grammar of data manipulation. R package version 1.1. 2. Computer software.
- Zige DV and Omeje FI, 2023. Antibiotics profile and public health implication of pathogenic enteric bacteria associated with poultry stool. *Int J Path Res* 12(2):9-15.