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Research Article

Antimicrobial resistance in *Salmonella* spp. from cultured Rohu (*Labeo rohita*), Tilapia (*Oreochromis niloticus*), and Carp (*Cyprinus carpio*): A possible threat to public health

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Abstract

Multidrug-resistant *Salmonella* can infect fish if reared in contaminated water or through manure in ponds that stimulate planktonic biomass. If the fish are not properly cooked, they can become a public health threat. Prior studies have reported the contamination of *Salmonella* in aquaculture products from marketplaces, however, our study determined the contamination of multidrug resistance *Salmonella* spp. through antimicrobial susceptibility in three fish (*Labeo rohita*, *Oreochromis niloticus*, *Cyprinus carpio*) from two retail markets in Lahore, Pakistan. A cross-sectional study was designed from June 2022 to August 2022 in two major fish markets in Lahore. Among 200 samples (*L. rohita* n= 80, *O. niloticus* n= 60, *C. carpio* n= 60), 16% were positive for *Salmonella* spp. with 3.12% of the isolates were resistant to 10 antibiotics, while 100% being resistant to at least two or more antibiotics. We detected high resistance to streptomycin (100%) and oxytetracycline (96.9%). Doxycycline and kanamycin followed a high resistance trend as well (87.5%). Moreover, lincomycin and trimethoprim (84.45%) resistance were also high in the *Salmonella* isolates. However, the lowest resistance was found against azithromycin (50%) and amoxicillin (40.6%). In addition, 100% of the *Salmonella* spp. isolates were multidrug resistant with seven different resistance patterns. Our study highlights the need to continuously monitor pathogens and restrict unnecessary drug use in aquaculture practices.

Introduction

Water bodies are known to be major reservoirs of multidrug-resistant (MDR) pathogens due to the unchecked introduction of sewage water, hospital waste, and untreated industrial effluents (Sivaraman *et al.,* 2020). In fish farming, the bottom of the pond and the dam are made up of soil. The bottom soil is critical for establishing pond equilibrium and for the growth of the fish species because it acts as a biological filter, and stores nutrients for the organisms in the pond ecosystem, especially for the autotrophs and the heterotrophs. Therefore, the characteristics of the soil control the parameters of the overlying water. In Pakistan, water is exchanged regularly in ponds and the fertilizer is frequently added along with lime to improve its quality. After the annual harvest for the next culture, the bottom soil is dried and plowed. There is no practice of determining the chemical and physical changes in the quality of the bottom soil as they vary when the pond ages (Shafi *et al.,* 2021).

In addition as compared to developed countries such as Europe, Japan, and North America (BondadReantaso *et al.,* 2023) where the use of antimicrobials is controlled, in Pakistan they are administered continuously or introduced in the fish feed for longer periods either in lower doses (Wegener, 2003) or high does in case of treatment (Lavanya *et al.,* 2021). Growth promoters are also added (Alexander *et al.,* 2008).

Previous studies have shown that the addition of antibiotics in aquaculture practices is one of the reasons for the spread of antimicrobial resistance in pond microorganisms. It is for this reason that fish in the aquaculture of Pakistan are reported to harbor multidrug-resistant bacteria (Ansari *et al.,* 2022; Singh *et al.,* 2022; Rehman *et al.,* 2023).

Another factor in the contribution of antibiotic resistance is the residues of antibiotics in the environment. Studies have reported that the water samples of the rivers in Punjab, Pakistan contained a high level of antibiotic residues and this was proved by the amplification of antibiotic resistance genes from them (Mohsin *et al.,* 2019). In Pakistan, the use of fish and its products has increased in the last few decades. The major varieties include Carp which are a good source of proteins and a great profitable value (Yaqub *et al.,* 2017). *Labeo rohita* commonly known as Rohu is also one of the major freshwater Carp species reared normally in Pakistan and accounts for about 35% of the total Carp production (Khafagy *et al.,* 2015). It is also the most preferred fish by consumers and therefore preferred by farmers (Bordoloi and Muzaddadi, 2013). Tilapia (*Oreochromis niloticus*) is another fish species that is extensively reared due to the ease of its cultivation (Helmi *et al.,* 2020). Carp locally referred to as *Cyprinus carpio* was first brought to Pakistan from Thailand in 1964 (Achakzai *et al.,* 2022) to overcome food and nutrient shortages (Azhar *et al.,* 2022). Since then it has become very valuable to fish farmers due to its ability to feed on cereals (Masood *et al.,* 2022) along with the ability to resist diseases and possess a rapid growth rate (Azhar *et al.,* 2022).

Earlier studies such as recently done by Shakir *et al.* (2021) and Singh *et al.* (2022) have mainly focused on *Salmonella* spp.

from veterinary sources such as poultry. This is due to the reports of largely unregulated use of antibiotics on poultry farms in Pakistan where veterinarians administered these without prescription. Moreover, the farmers do not comply with the national action plan on AMR set in 2017 (Alhouzani *et al.,* 2021). Considering that information is lacking on the presence of multidrug resistance pathogens in fish meat particularly *Salmonella* in Pakistan the current study was on the isolation and antimicrobial susceptibility of *Salmonella* spp. from the three most demanded fish varieties, Rohu (*L. rohita*), Tilapia (*O. niloticus*) and Carp (*C. carpio*).

Materials and methods

The study area

The current study was conducted in Lahore, the second largest metropolitan area, and capital of Punjab Province of Pakistan Lahore covers an area of around 1772 sq. km and is situated to the northeast of Punjab, and extends between 31°15' N to 31°42' N and between 74°01' E to 74°-39' E. (Rana and Bhatti, 2018; Nasar-u-Minallah *et al.,* 2021). In the current study, fish belonging to three different fish species Rohu, Carp, and Tilapia were collected from two of the fifteen wet fish markets supplying fish to the population of Lahore, Pakistan.

The markets were selected based on their strategic location and customer frequency. The samples were collected from the two main wet fish markets; Urdu Bazar fish market (31.576296 N, 74.309155 E) and Mori Gate fish market (31.577978 N, 74.311894 E). Both wholesale markets are located near Anarkali Bazar (Waheed and Naz, 2023) and are famous for the availability of fish at economical rates.

Sample collection

A cross-sectional study was conducted for four months from June 2022 to September 2022. A total of 200 samples of fish samples were collected from retail shops of Urdu Bazar fish market (n=140) and Mori Gate fish market (n=60) in Lahore. According to different varieties, the total samples included were Rohu (n=80), Tilapia (n=60), and Carp (n=60.). From each retail shop, a 25gm of freshly slaughtered fish meat sample was collected and stored in properly labeled sterile plastic containers and transported to the laboratory in an ice cooler. The samples were processed on the same day.

Microbial analysis

The analysis was carried out for the fish samples according to the method described in our previous study (Sadiq *et al.,* 2022). According to this method, pre-enrichment was carried out using 25 grams of fish meat sample inoculated in 225 ml buffer peptone water and incubated at 37℃ for 24 hours. Enrichment was done by inoculating 1ml of pre-enrichment medium into 9ml of Rappaport Vassilliadis (RV) broth and incubated at 42 ℃ for 24 hours. Following incubation, a loop full was streaked on xylose lysine deoxycholate (XLD) agar and incubated at 37 ℃ for 24 hours Colonies displaying the morphological characteristics of *Salmonella* (Bergey, 1994) were further streaked for purification through quadrant streaking (Cappuccino and Sherman, 2013) and molecular confirmation through the

amplification of *ompC* gene (Puente *et al.*, 1995).

Molecular confirmation of Salmonella genus

For the molecular confirmation of *Salmonella* genus, the *ompC* gene was targeted for amplification (Puente *et al.*, 1995). Briefly, genomic DNA was extracted using the QIAmp DNA Mini Kit following the manufacturer's instructions (Qiagen, USA). The purity and yield of the isolated DNA were determined using an ND-1000 Nanodrop spectrophotometer (Thermo Scientific, USA). For the PCR, amplification was carried out using the primers (FP 5'-ATCGCTGACTTATGCATCG-3' and RP 5'-CGGGTTGCGTTATAGGTCTG-3'.), template DNA, nuclease (DNAase RNAase) free water and Dream Taq green PCR master mix (2X) (Thermo Scientific, USA). The cycling conditions include 30 cycles with initial denaturation at (94°C, 15s), annealing at (57°C, 15s) and final extension at (72°C, 30s.) Negative controls containing all the reagents except template DNA were also run to rule out contamination. The amplified products were visualized by agarose gel electrophoresis using a 1.5% gel. The expected product for *Salmonella* genus was 204bp (Panda *et al.*, 2015; Sadiq *et al.*, 2020).

Antimicrobial susceptibility testing

All isolates confirmed after molecular detection as *Salmonella* were analyzed for antimicrobial susceptibility. Kirby Bauer's disk diffusion method was used to check the susceptibility of 10 commercially available antibiotics as described in our previous study (Sadiq *et al.*, 2021). Briefly, Mueller Hinton agar (MHA) plates were inoculated by gently swabbing by an inoculum of fresh cultures of *Salmonella* strains suspended in normal saline equivalent to 0.5 McFarland standard. The inoculum was allowed to dry and then the antibiotic disks were placed using sterile forceps at equal distance. The antibiotics used were, lincomycin (L) 10µg, amoxicillin (AMC) 30µg, doxycycline (D) 30µg, kanamycin (K) 30µg, streptomycin (S) 10µg, oxytetracycline (OT) 30µg, trimethoprim (TMP) 5µg, ciprofloxacin (CIP) 5µg, Azithromycin (AZM) 15µg, and cefotaxime (CTX) 30µg. The plates were incubated at 37°C for 24 hrs. After incubation, the antibiotics were presented as resistant and sensitive according to the Clinical and Laboratory Standard Institute (Humphries *et al.*, 2021).

Statistical analysis

The prevalence proportion was calculated by Epi-tool software with a 95% confidence interval. The total number of fish samples positive for *Salmonella* was taken as the numerator while the total number of samples collected was taken as the denominator Chi-square test was applied to calculate the significance of association between the prevalence of two markets with *p-value* $\leq$0.05 as statistically significant. Microsoft Excel was used to prepare tables and antimicrobial resistance patterns. QGIS software version 3.36 was used to present the spatial distribution of fish markets (Moyroud and Portet, 2018).

Results

Prevalence of Salmonella in various fish collected from wet markets of Lahore

Out of a total of 200 fish samples, 32 samples were found positive by conventional PCR (Fig. 1), showing a 16% prevalence of *Salmonella* in fish. Overall, species-wise,13.6% samples of Rohu, 16.6% samples of Tilapia, and 18.36% samples of Carp were found positive for *Salmonella* (Table 1). No statistical significance (p=0.1522) was found between the prevalence between the markets of Urdu Bazar 13.57% (CI: 8.86-20.23) and Mori Gate 21.67% (CI: 13.12-33.62). The location of the markets is presented in (Fig. 2).

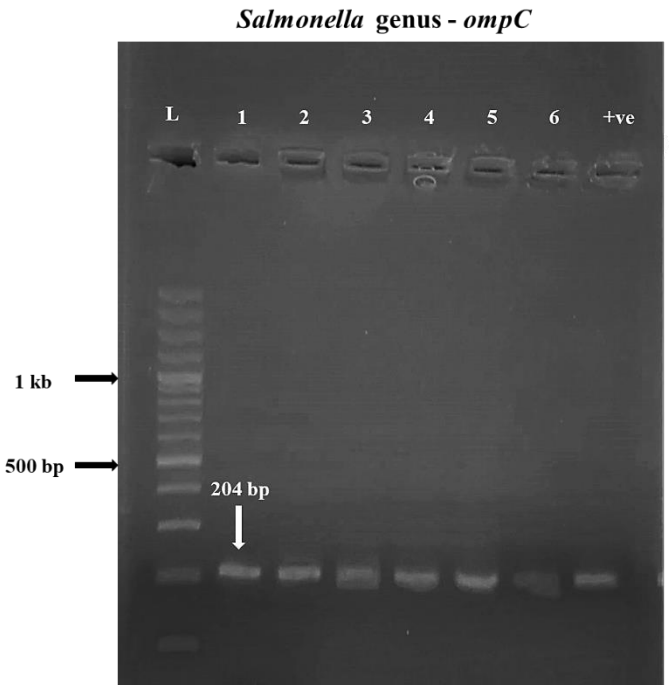

Figure 1: Confirmation of *Salmonella* through conventional PCR. *Salmonella* genus gene *ompC,* was amplified from DNA extracted from samples that tested positive after biochemical testing. An amplicon size of 204bp indicates a positive result. Left to right lane 1: 100 bp DNA Ladder, Lane 2-7: fish isolates positive for *Salmonella,* Lane 8: +ve control for *Salmonella* genus gene *ompC.*

Table 1: Prevalence of *Salmonella* sp. in fish species of two fish markets in Lahore, Pakistan.

Markets	Fish species	Total sample collected	Total positive sample	Species wise prevalence	Prevalence (%)
Urdu Bazar	Rohu	50	6	0.12	12
	Tilapia	50	6	0.12	12
	Carp	40	7	0.17	17
Mori Gate	Rohu	30	5	0.16	16
	Tilapia	10	4	0.40	40
	Carp	20	4	0.20	20
Total		**200**	**32**	**0.16**	**16**

Antimicrobial susceptibility of Salmonella spp.

A total of 3.12% *Salmonella* isolates were found completely resistant to all tested antibiotics while 96.87% were resistant to

at least one of them. Moreover, all strains were resistant to Streptomycin. All strains were checked for resistance against a panel of ten antibiotics selected based on their use either in aquaculture or in the treatment of *Salmonella* infection (Fig. 3). Out of all the strains (32) that were tested against the 10 antibiotics, 100% were resistant to streptomycin, 96.9% of the strains were resistant to oxytetracycline, 87.5% to doxycycline and kanamycin, 71.9% to cefotaxime and ciprofloxacin, 50% to azithromycin. Only 40.6% of the total isolated showed resistance to amoxicillin. (Table 2).

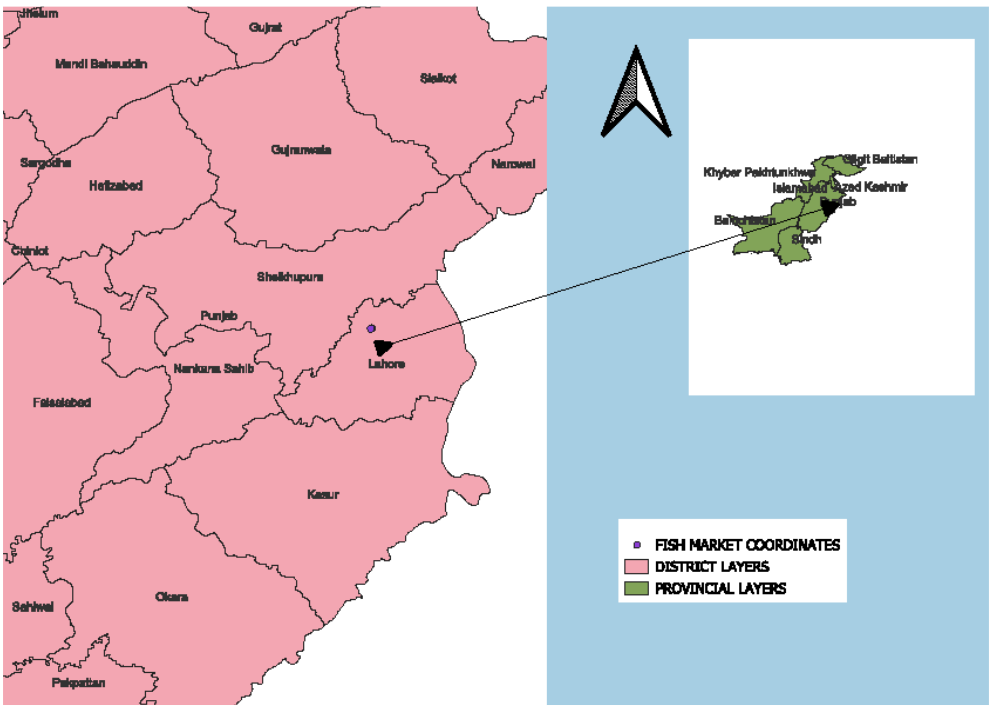

Figure 2: MAP shows the location of two markets in Lahore. Arrow showing the location of Lahore in Punjab.

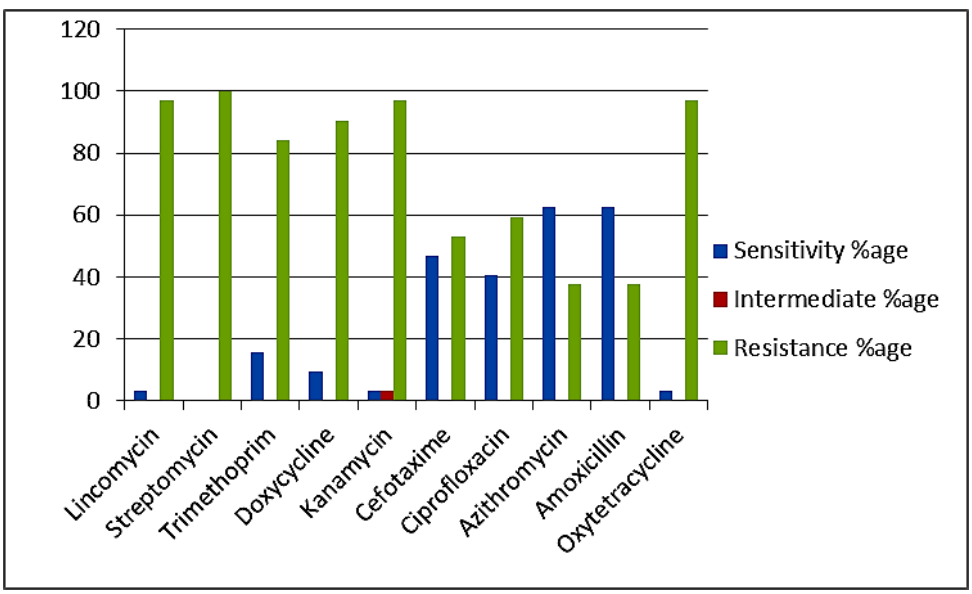

Figure 3: Antimicrobial resistance percentage of *Salmonella* isolates from common fish varieties in Lahore.

Seven resistance patterns were found. The predominant MDR pattern was resistant to streptomycin and oxytetracycline (S+OT), n=31, (96.87%) followed by resistance to lincomycin, trimethoprim, doxycycline, oxytetracycline, kanamycin (L+TMP+D+OT+K), 25 (78.12%). The least resistant pattern was (L+S+TMP+D+K+AMZ+AMC+CTX+OT+CIP), n=1 (3.12%) (Table 3).

Table 2: Antimicrobial resistance percentage of *Salmonella* isolates from common fish varieties in Lahore.

Markets	No. of Positive Samples	Dose of antimicrobial agent along with percentage (%) of resistance *									
		L (10ug)	S (10ug)	TMP (5ug)	D (30ug)	K (30ug)	CTX (30ug)	CIP (5ug)	AZM (15ug)	AMC (30ug)	OT (30ug)
Urdu Bazar	Rohu (06)	16.6 (1)	100 (6)	83.3 (5)	100 (6)	100 (6)	50 (3)	50 (3)	33.3 (2)	50 (3)	83.3 (5)
	Tilapia (06)	100 (6)	100 (6)	85.7 (5)	100 (6)	100 (6)	85.7 (5)	85.7 (5)	66.6 (4)	50(3)	100 (6)
	Carp (07)	100 (7)	100 (7)	85.7 (6)	71.4 (5)	100 (7)	71.4 (5)	71.4 (5)	42.8 (3)	14.2 (1)	100 (7)
	Rohu (05)	100 (5)	100 (5)	80 (4)	100 (5)	20 (1)	40 (2)	40 (2)	40 (2)	40 (2)	100(5)
Mori Gate	Tilapia (04)	100 (4)	100 (4)	100 (4)	100 (4)	100 (4)	100 (4)	100 (4)	100 (4)	50 (2)	100 (4)
	Carp (04)	100 (4)	100 (4)	75 (3)	50 (2)	100 (4)	25 (1)	100 (4)	25 (1)	50 (2)	100 (4)
	Total resistance	**84.4**	**100**	**84.4**	**87.5**	**87.5**	**71.9**	**71.9**	**50**	**40.6**	**96.9**

*L= Lincomycin, S= Streptomycin, TMP= Trimethoprim, D= Doxycycline, K= Kanamycin, CTX= Cefotaxime, CIP= Ciprofloxacin, AZM= Azithromycin, AMC= Amoxicillin, OT= Oxytetracycline

Table 3: Multidrug resistance (MDR) patterns of *Salmonella* isolates from common fish varieties in Lahore.

Sr.no	Pattern of multidrug resistance (MDR)	No. of isolates of fish (n)			Number (%)	Percentage
		Rohu	Tilapia	Carp		
1	S + OT	10	10	11	31	96.87
2	CTX + CIP + AMC	2	5	3	10	31.25
3	TMP + CTX + CIP + AMC	2	2	0	4	12.51
4	S + D + AZM + OT	3	4	3	10	31.25
5	L + TMP + D + OT + K	8	10	7	25	78.12
6	L + S + TMP + D + K	8	9	5	22	68.75
7	L + S + TMP + D + K + AMZ + AMC + CTX + OT + CIP	1	0	0	1	3.12

L= Lincomycin, S= Streptomycin, TMP= Trimethoprim, D= Doxycycline, K= Kanamycin, CTX= Cefotaxime, CIP= Ciprofloxacin, AZM= Azithromycin, AMC= Amoxicillin, OT= Oxytetracycline

Discussion

A study by (Mohsin *et al.*, 2019) indicated that the presence of pathogenic bacteria is a common occurrence in fish in Pakistan due to the high levels of contamination in the rivers of the province of Punjab. This falls in line with the results of our study in which the average rate of *Salmonella* spp. in Lahore was observed to be 16 %. Similar to ours, a study by (Ullah *et al.*, 2023) also indicated that the two fish varieties, Rohu and Carp harbored pathogens of the

Enterobacteriaceae family such as *Escherichia coli* with genes for multiple antibiotics. Another recent study by (Jabeen *et al.,* 2020) reported a 20 % prevalence of *Salmonella* spp. in fish samples of Lahore.

Previous studies have highlighted the fact that the prevalence of *Salmonella* isolates was not limited to the city of Lahore. A study by (Ansari *et al.,* 2022) reported a 10 % prevalence of *Salmonella* spp. from raw fish sold in retail stores in Hyderabad. The situation was not so different from our neighboring country India where 20.7 % prevalence was observed in finfish samples. However, these fin fish were not reared rather they were wild (Prabhakar *et al.,* 2020) whereas the fish in our study were reared.

Studies from the neighboring countries have also reported the incidence of *Salmonella* in fresh fish. A study done in India by (Saharan *et al.,* 2020) described the significant prevalence of *Salmonella* i.e. 45 %. Higher prevalence was also reported in Bangladesh where the fish varieties from the market contained 48.9 % *Salmonella* (Ava *et al.,* 2020) In comparison, our neighboring country Iran reported less contamination of *Salmonella* than only 9 % (Rahimi *et al.,* 2023). It is nevertheless an alarming situation that the incidence of *Salmonella* in fresh fish is now being reported in developing countries with non-typhoidal types being the most frequent (Prabhakar *et al.,* 2020, Mumbo *et al.,* 2023).

It is a known fact that in these countries, the strains of *Salmonella* are of animal origin and while they reside in the animals, they acquire multi-drug resistance. This is due to the common practice of antibiotics use in poultry farms as well as in aquaculture (Zhang *et al.,* 2015) Another common source of *Salmonella* is sewage water as the rivers and coastal waters contain untreated or even partially treated sewage along with highly polluted effluents (Prabhakar *et al.,* 2020). This is apparent in the present study that high antimicrobial resistance is associated with *Salmonella* in popular fish varieties in Punjab. Our data indicated higher resistance levels in the *Salmonella* isolates that were comparable to the resistance levels obtained in the Sindh province of Pakistan by (Rehman *et al.,* 2023). The authors of the study reported that *Salmonella* spp. particularly from Rohu found to be resistant to 4 antibiotics. Similar resistance has been reported from India and the study by (Saharan *et al.,* 2020) is one such example that reported 3 *Salmonella* isolates resistant to all antibiotics tested whereas in our study 1 isolate was resistant to all the ten antibiotics. In our study, 20-25 % of isolates were resistant to 4-5 antibiotics, and is comparable to this study where 21.8% of isolates were resistant to four different antibiotics. Hence, the results of our study highlight the alarming scenario that is developing in fish and related raw products in the region.

We observed a high resistance to streptomycin, oxytetracycline, and doxycycline, which may be due to their routine use in aquaculture feeds and also used as a preventive measure (Zhang *et al.,* 2015). High resistance was also related to lincomycin, which was interesting because most recently in India a study reported by Venkataraman *et al.* (2023), showed 100% resistance to lincomycin in many pathogens

including *Salmonella*. Lincomycin was reported to be the most routinely used antibiotic in poultry feed in Pakistan as well during the years 2013 to 2017 (Mohsin *et al.*, 2019). It has also been used for prophylactic or therapeutic use in poultry farms outside the recommended dosage and unfortunately without a prescription (Alhouzani *et al.*, 2021). This idea is further confirmed by the study by Noreen *et al.* (2022) that found 100 % resistance to lincomycin in poultry-meat-associated members of the Enterobacteraceae family. Another study by Malik *et al.* (2022) reported the resistance to be 96.51%.

Another antibiotic of interest in our study was kanamycin, against which *Salmonella* spp. were observed to be highly resistant. Recent studies carried out on poultry pathogens such as *Clostridium perfringens*, *Campylobacter jejuni*, *Salmonella gallinarum*, *Salmonella enteritidis*, and *Salmonella typhimurium* (Shakir *et al.*, 2021; Achakzai *et al.*, 2022; Suman Kumar *et al.*, 2023) have described the resistance of these strains against kanamycin. It can be assumed that these microbes could be present in the waste products of poultry that are fed to the fish and hence transfer their resistance to other pathogens that are present.

The current study highlights the need for constant surveillance and raising awareness for the use of antibiotics, particularly in poultry and aquaculture. The dissemination of such information will not only help in the development of standards for the appropriate use of antibiotics in aquatic systems but may also result in effective strategies to ensure the safety of our food supplies for public health.

Conflicts of Interest

The authors declare no conflict of interest.

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