BACTERIAL EMPIRE

2021, VOL. 4, NO. 4, e310

ANTIBIOTIC-RESISTANT MOTILE AEROMONADS ASSOCIATED WITH CULTURED INDIAN MAJOR CARPS, POND WATER AND POND SEDIMENT

Avishek Bardhan, Thangapalam Jawahar Abraham, Qurratul Ain Qureshi and Ratul Chakraborty*

Address (es):
Department of Aquatic Animal Health, Faculty of Fishery Sciences, West Bengal University of Animal and Fishery Sciences, Chakgaria, Kolkata 700094, West Bengal, India.

*Corresponding author: abrahamtj1@gmail.com

https://doi.org/10.36547/be.310

ABSTRACT

The uproar in antimicrobial resistance (AMR) among the aquacultured environment has led to the isolation of multiple antibiotic-resistant (MAR) *Aeromonas* strains. The current study aimed at the enumeration of antibiotic-resistant *Aeromonas* in carps of aquacultured environment and market samples. Isolation of *Aeromonas* was also done in Rimler-Shotts agar supplemented with novobiocin followed by antibiotic-sensitivity assay against 12 broad-spectrum antibiotics. Five oxytetracycline-resistant strains were examined for the presence of three tetracycline-resistant genes (*tetA*, *tetC* and *tetE*). The presumptive *Aeromonas* counts on starch-ampicillin agar were determined as log 3.00-log 6.45/g in carps, log 3.00l-log 5.06/ml in pond water and log 3.30–log 5.14/g in pond sediment. Higher proportions of motile aeromonads from market carps were resistant to chloramphenicol, cefalexin, gentamycin, oxytetracycline and trimethoprim than the farmed carps. *Aeromonas* strains depicted 57 resistant profiles. About 88.43% of the *Aeromonas* strains were of the MAR group among which 12.15% and 4.67% were resistant to ≥6 and ≥7 antibiotic groups, respectively. Selected oxytetracycline-resistant strains were negative for targeted genes. The current study implied the high prevalence of AMR bacteria in cultured carps in West Bengal, India. Furthermore, the study indicated that motile aeromonads comprise an effective marker for monitoring AMR in freshwater aquatic environments.

Keywords: Aquaculture, *Aeromonas* spp., Antibiogram, Multiple antibiotic resistance, *tet* genes

INTRODUCTION

The genus *Aeromonas* is regarded as an omnipresent aquatic pathogen and also as the anthropogenic etiological agent of food poisoning, sepsis, wound infections and other diseases (**Graf, 2015**). Motile *Aeromonas* strains have been isolated from humans with mild and severe gastrointestinal infections (**Zhou *et al.* 2019; Fernández-Bravo and Figueras, 2020**). The genus can be classified into two groups, viz., the psychrotrophic *Aeromonas*, represented by non-motile *Aeromonas salmonicida* and the mesophilic motile *Aeromonas* spp. (**Graf, 2015**). The mesophilic motile aeromonads are known to cause motile *Aeromonas* septicaemia (MAS) in fish and have been isolated frequently from diseased fish (**Janda and Abbott, 2010**). The members of the genus *Aeromonas* have recently been reorganized with 36 genospecies (**Fernández-Bravo and Figueras, 2020**) most of which belong to the mesophilic and motile groups. *Aeromonas* and carps have had a long history (**Yang *et al.* 2018; Abraham and Bardhan, 2019**); more particularly the motile aeromonads are infamous pathogens under environmental stress and are known to cause mortalities among cultured carps (**Saharia *et al.*, 2019; Nhinh *et al.*, 2021**). They can also lead to worsened quality of products mainly in commercial carp farming (**Ezung and Abraham, 2013**). Today, the genus *Aeromonas* is considered to be synonymous with aquatic environments and isolated from the rivers, lakes, ponds, estuaries, drinking water, groundwater, wastewater and sewage in various phases of treatment (**Figueira *et al.*, 2011**). Concentrations of aeromonads have been reported to vary from lows of <1 cfu/ml in groundwater, drinking water and seawater to highs of 10^8 cfu/ml in crude sewage or domestic sewage sludge (**Pablos *et al.*, 2009**). *Aeromonas* spp. are particularly unsolicited in food because they are active spoilers of fish at ambient temperature and temperatures between 2 and 13ºC (**Hoel *et al.*, 2019**). Competitive growth of *Aeromonas* spp. at 5°C is of considerable importance since refrigeration will not prevent growth and that only minor temperature increases can lead to its rapid propagation (**Janda and Abbott, 2010**).

With the arousal of antimicrobial resistance (AMR) as a global issue (**WHO, 2019**), this ubiquitous microflora is gaining attention as it poses serious threats to the aquaculture environment (**Abraham and Bardhan, 2019**). Antibiotic-resistant motile aeromonads have been isolated from healthy and diseased carps (**Saharia *et al.*, 2019; Nhinh *et al.*, 2021**) and the aquacultured environment (**Zdanowicz *et al.*, 2020**). The rapid increase in the number of resistant and multiresistant *Aeromonas* is due to the ability of these organisms to transfer antibiotic resistance by mobile genetic agents like plasmids, transposons, insertion sequences (IS elements), gene cassettes, class 1 integrons among bacterial populations by the cell to cell contact. The microbial resistance in aeromonads is chromosomally mediated; however, β-lactamases may be coded by plasmids or integrons (**Patil *et al.*, 2016**). *Aeromonas* spp. produce different β-lactamases, which confer resistance to a broad spectrum of β-lactamases antibiotics. Plasmids transfer the resistance genes in the bacterial chromosome or may gather together multiple genes conferring resistance. This results in the emergence of additional bacterial strains resistant to several antimicrobial drugs at a time, i.e., multi-resistance. Further, horizontal gene transfer of these elements can significantly expand their epidemiological capacity leading to the potential transfer of mobile genetic elements across microbiomes of common ecological niches (**Patil *et al.*, 2016**).

Aquatic environments are utilized worldwide for water supply, energy production, irrigation, navigation, aquaculture and primary and secondary contact activities sharing an intricate relationship with the anthropogenic environment (**WHO, 2019**). Freshwater aquaculture in West Bengal, India depends mainly on carp culture practices that have proved sustainable at different levels of production over the years. The Indian major carps (IMCs) are popular staple fish in West Bengal. The IMCs contributed about 80% of the cultured carps followed by exotic carps (**Abraham *et al.*, 2020**). The disease is considered one of the major problems to successful aquaculture in West Bengal and several infectious diseases of cultured carps have been reported (**Abraham *et al.*, 2020**). Different groups of approved and non-approved antibiotics and drugs are used in the treatment of aquatic animal ailments (**USFDA, 2018**) and the indiscriminate use of which leads to upsetting consequences of AMR (**Watts *et al.*, 2017; Nhinh *et al.*, 2021**). **Patil *et al.* (2016)** reported the suitability of the *Aeromonas* genus as an indicator for studying the incidence and development of AMR in fish farms. Though there are reports on the incidence of AMR in known fish pathogens (**Austin and Austin, 2012; Nhinh *et al.*, 2021**), such reports on the ubiquitous bacteria of the freshwater aquaculture system are lacking. This study was, therefore, aimed at quantifying the levels of motile *Aeromonas* as an important component of the microflora in farmed IMCs, pond water and pond sediment, and assess the frequency of AMR in these groups of bacteria.

MATERIAL AND METHODS

Sample collection, isolation and identification of motile aeromonads

The study was carried out for 12 months from April 2018 to March 2019 from 5 different sampling locations encompassing three farms and two retail markets. The sampling locations are Buderhat *bhery* (Lat. 22°28'50" N; Long. 88°24'14" E) and Garia fish market (22°28'57" N; 88°23'06" E) in South 24 Parganas district, and Nalban *bhery* (22°33'12"N; 88°24'42" E), Nilgunj Barrackpore II fish farm (22°46'14"N; 88°22'41" E) and Nonachandanpukur Barrackpore II fish market (22°45'59" N; 88°22'38" E) in North 24 Parganas district. The experimental biota included three species of cultured IMCs (250-350 g) namely *Catla catla*, *Labeo rohita* and *Cirrhinus mrigala*, pond water and pond sediment samples. Besides, the market samples of fresh IMCs (250-350 g) that originated

from the unknown culture systems were also included. The pond water samples at a depth of 30 cm from the surface were collected in sterile 200 ml polypropylene flat bottomed sample containers whereas the sediment samples were collected aseptically with sterile polypropylene containers and transferred to sterile polythene bags. Two fish of each species per farm were collected by cast netting and transferred immediately to sterile plastic zipper bags separately. Healthy IMCs were collected during the monthly harvest time and euthanized with clove oil (0.50 ml/L water), wherever necessary. Likewise, the whole fresh fish samples of the three IMCs were randomly collected from the retail market and transferred immediately into sterile plastic zipper bags separately. The samples were placed in an insulated container with gel ice packs and transported to the laboratory within 2 hours of collection.

The sampling was done monthly. The fish of each species from the farm and/or market were filleted aseptically and collected about 25 g of edible meat with skin. The samples of two fish of each species were pooled and homogenized aseptically in a sterile homogenizer. Ten grams of the homogenized sample of each fish species was then ten-fold serially diluted using a sterile diluent containing 0.85% (w/v) sodium chloride (NaCl). Similarly, the pond water (10 ml) and pond sediment (10 g) samples from each farm were also ten-fold serially diluted in sterile 0.85% NaCl. Loopful of homogenized fish meat, pond water and pond sediment samples from 10^{-1} dilution was streaked onto Rimler-Shotts (RS) agar plates separately and incubated for 24 h at 35±2°C. Yellow, convex, smooth and round colonies were isolated and presumptively considered as *Aeromonas* spp. (**HiMedia, 2009**). These distinct, representative colonies were purified by repeated streaking on nutrient agar (NA) plates and maintained on NA slants. The isolated presumptive *Aeromonas* spp. (n=242) were confirmed based on a series of biochemical tests and identified up to species level (**Collins *et al.*, 2004; Austin and Austin, 2012**). The bacteriological media, chemicals and reagents used were of HiMedia make (HiMedia Mumbai, India).

Enumeration of ampicillin-resistant bacterial counts and presumptive *Aeromonas* counts

A total of 12 samples for each species of fish or water or sediment from the farms and 8 samples for each species of fish from the retail market were used for the enumeration of bacteria during the study period. The enumeration of motile aeromonads was performed in starch ampicillin agar (SAA) plates (**Palumbo *et al.*, 1985**) following the spread plate technique. Aliquots of 10^{-3}, 10^{-4}, 10^{-5} dilutions of fish meat samples and 10^{-2}, 10^{-3}, 10^{-4} dilutions of pond water and pond sediment samples were spread plated on SAA plates, incubated at 35±2°C for 48 h and counted thereafter as total ampicillin-resistant bacterial counts (TABCs). After counting the SAA plates, they were flooded with Lugol iodine solution. Distinct yellow (amylase-positive) to honey-coloured colonies (3-5 mm in diameter) with a clear zone surrounding them were scored as presumptive *Aeromonas* counts (PACs) (**Palumbo *et al.*, 1985**). The counts were expressed as colony-forming units, i.e., cfu/g meat or pond sediment and cfu/ml pond water (**APHA, 1992**).

Antibiotic sensitivity assay and determination of antibiotic-resistance profiles

The sensitivity of 242 motile *Aeromonas* strains from the carps, pond water and pond sediment samples to 12 broad-spectrum antibiotics representing 9 antibiotic groups (HiMedia, India), viz., amoxyclav (30 µg), azithromycin (15 µg), cefalexin (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), co-trimoxazole (25 µg), enrofloxacin (10 µg), gentamycin (10 µg), nitrofurantoin (300 µg), oxytetracycline (30 µg), sulfafurazole (300 µg) and trimethoprim (10 µg) was tested by agar-disc diffusion technique (**CLSI, 2012**) on Mueller Hinton agar (MHA) at 35±2°C. Interpretation of sensitivity was based on the zone size interpretation chart (**CLSI, 2012**).

The resistance profiles and resistance pattern of 242 motile *Aeromonas* strains [180 from carps (farm: 109 and market: 71), 30 from pond water and 32 from pond sediment] were determined from the antibiogram data computed after antibiotic sensitivity assay. Multiple antibiotic resistance (MAR) was determined for those isolates displaying resistance to 3 or more antibiotic groups. The MAR is defined as resistance to three or more classes of antimicrobials (**ECDC/EMEA, 2009**). The MAR index was calculated as 'a'/'b' where 'a' represents the number of antibiotic groups to which the strain was resistant, and 'b' represents the total number of antibiotic groups tested for that strain.

PCR amplification of tetracycline-resistant genes

The bacterial genomic DNA of five OTC-resistant strains was extracted by using a genomic DNA isolation kit (Macherey-Nagel, Germany) as per the manufacturer's protocol and used as PCR templates. These strains were screened for 3 tetracycline-resistant genes (*tet*A, *tet*C and *tet*E), which are the most common among the *tet* group (**Schmidt *et al.*, 2001**). Identification of the target genes was performed using PCR amplification with specific forward and reverse oligonucleotide primers as listed in **Table 1**. The PCR was performed in a Master cycler Pro S system (Eppendorf, Germany) and according to the method described by **Akinbowale *et al.* (2007).** The PCR products were analysed on 1.2% agarose (HiMedia, India) gels containing 0.5 µg/ml ethidium bromide in 1X Tris-acetate- EDTA (TAE) buffer and viewed in Gel Doc system (G-Box Syngene, UK).

Table 1 Polymerase chain reaction primer sets and cycling conditions used for the detection of *tet* genes

Primers	Oligonucleotide Sequence (5'-3')	Cycling conditions	Amplicon size (bp)	References
*tet*A(F)*	GTAATTCTGAGCACTGTCGC	95° – 30s, 62°C – 30s, 72°C – 45s (23 cycles)	956	Deng *et al.*, 2014; Schmidt *et al.*, 2001
*tet*A(R)	CTGCCTGGACAACATTGCTT			
*tet*C(F)	TCTAACAATGCGCTCATCGT	95°C – 30s, 62°C – 30s, 72°C – 30s (30 cycles)	588	
*tet*C(R)	GGTTGAAGGCTCTCAAGGC			
*tet*E(F)	GTGATGATGGCACTGGTCAT	95°C – 30s, 62°C – 30s, 72°C – 45s (23 cycles)	1198	
*tet*E(R)	CTCTGCTGTACATCGCTCTT			

*F: Forward; R: Reverse.

Statistical analyses

The means of TABCs and PACs of carps, pond water and pond sediment samples, after log transformation, as well as the data on AMR among carps (farmed and market samples), pond water and pond sediment samples against 12 broad-spectrum antibiotics. were analyzed by one way ANOVA (Analysis of variance) followed by Tukey's post-hoc tests for pair-wise comparisons using the Statistical Package for Social Sciences (IBM-SPSS) Version: 22.0, considering a probability level of $P<0.05$. Chi-square test was used to compute the significance of difference among MAR of motile aeromonads from carps, pond water and pond sediment samples using an online program (https://www.graphpad.com/quickcalcs/contingency2/).

RESULTS AND DISCUSSION

Enumeration of ampicillin-resistant bacterial counts

The SAA was chosen as the medium for the enumeration of ampicillin-resistant bacteria as well as for presumptive *Aeromonas* because ampicillin-resistance is intrinsic to *Aeromonas* (**Palumbo *et al.*, 1985**). The TABCs and PACs of the

farmed and market IMCs, viz., *L. rohita*, *C. catla* and *C. mrigala* are shown in **Figure 1A**. The mean TABCs of farmed IMCs fluctuated insignificantly between log 5.97±0.64/g in *C. mrigala* and log 6.09±0.53/g in *L. rohita* (*P>0.05*). The mean TABCs of market IMCs also fluctuated insignificantly between log 5.71±1.12/g in *L. rohita* and log 5.83±0.77/g in *C. mrigala* (*P>0.05*). The mean PACs of farmed IMCs fluctuated insignificantly between log 5.76±0.53/g in *C. catla* and log 5.94±0.54/g in *L. rohita* (*P>0.05*). In market samples, it varied insignificantly from log 5.56±0.60/g in *C. catla* to 5.70±0.75/g in *C. mrigala* (p>0.05). The TABCs of pond water ranged from log 3.00/ml to log 5.13/ml with a mean of log 4.36±0.66/ml, while in pond sediment samples, it ranged from log 3.48/g to log 5.22/g, with a mean of log 4.48±0.55/g. The mean PACs of pond water and pond sediment were log 4.24±0.69/ml and log 4.36±0.57/g, respectively (**Figure 1B**). About 97% of the TABCs from the pond water and sediment were presumptive aeromonads. The observed mean TABCs and PACs of farmed and market carps in the peri-urban Kolkata, India were comparable. About 94.89-97.54% and 96.53-97.77% of the TABCs from the farmed and market carps, respectively were presumptive aeromonads. Counts of ampicillin-resistant bacteria including the motile aeromonads were higher than the levels set for total plate counts (TPCs) in fresh, un-iced and frozen fish as per Indian standards (**FSSAI, 2009; EIC, 2012**). **Ezung and Abraham (2013)** reported similar results with more than 70% of the Kolkata retail fish samples showing bacterial counts above Indian standards. *Cirrhinus mrigala*, being a bottom dweller and benthic feeder, recorded the maximum counts of antibiotic-resistant bacterial flora so also in an earlier study (**Haque *et al.*, 2014**). The mean TABCs of market IMCs were about 2.35-5.11% lower than the counts of farmed carps, possibly due to the use of ice to keep the fish cool or the practice of sprinkling water to keep the fish moist. The mean TABCs of pond sediment was slightly higher than the pond water as the benthos harbour more fauna than the water column (**Abraham and Bardhan, 2019**). Presumptive *Aeromonas* counts of the studied samples complied with the works of **Zdanowicz *et al.* (2020)**. The maximum PACs were recorded among the Nalban *bhery* (wastewater aquaculture) samples, signifying the role of effluents in the increased incidence of *Aeromonas* (**Surendraraj *et al.*, 2009**). It has been pointed out that human pathogenic bacteria, mostly intestinal pathogens end up in the sewage water creating an environment where all the bacterial flora share resistant genes and generate multi-resistance (**Sandegren, 2019**). The intrinsic resistance of *Aeromonas* against ampicillin also connoted the 100% prevalence of *Aeromonas* among the carps. **González-Rodríguez *et al.*** (**2002**) depicted similar results on presumptive *Aeromonas* counts, where >60% of the antibiotic-resistant strains were *Aeromonas*. The PACs of pond water samples were lower than those of sediment samples, which corroborate **Igbinosa *et al.*** (**2012**), who reported that groundwater sources enclose significantly lesser *Aeromonas* due to less pollution and contamination. **Huddleston *et al.*** (**2006**) also reported that the apparent absence of strong selection in the environmental water sources is likely to be the key in the absence of ampicillin-resistant *Aeromonas.* However, the proportion of TABCs and PACs of water and sediment samples were analogous (80%) acknowledging *Aeromonas* as the indigenous bacterial flora (**Surendraraj *et al.*, 2009**). The sediments with accumulated organics are regarded as the hot spots of bacterial density and activity **Heß *et al.*** (**2018**), which promote horizontal gene transfer and the spread of antimicrobial-resistant bacterial populations (**Patil *et al.*, 2016**).

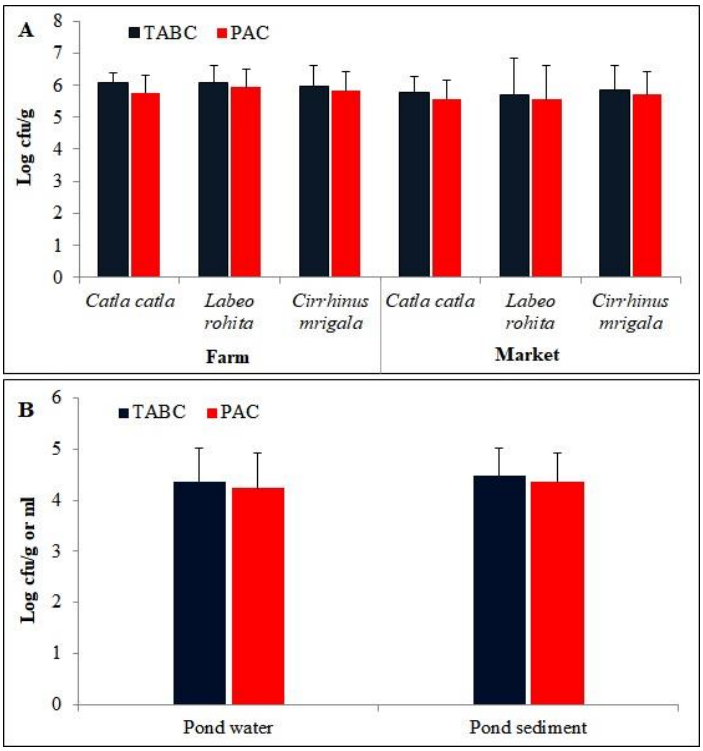

Figure 1 Total ampicillin-resistant bacterial counts (TABCs) and presumptive *Aeromonas* counts (PACs) in the [A] farm and market samples of *Labeo rohita*, *Catla catla and Cirrhinus mrigala* and [B] pond water and pond sediment samples.

Antibiotic sensitivity

Antibiotic resistance among the 242 motile aeromonads of the carps (n=180), pond water (n=30) and sediment (n=32) samples are shown in **Figure 2A** and those of farmed and market carps are shown in **Figure 2B.** In general, motile aeromonads strains exhibited varying degrees of resistance to the 12 antibiotics tested. The motile aeromonads from carps exhibited the maximum resistance to cefalexin (82.25%) and amoxyclav (79.44%) and the least resistance to sulfafurazole (1.67%). Azithromycin resistance (76.67%) was the highest in motile aeromonads of pond water. All strains were sensitive to ciprofloxacin, enrofloxacin and sulfafurazole. The pond sediment motile aeromonads displayed maximum resistance to amoxyclav (75%) and susceptible to ciprofloxacin and sulfafurazole. No marked variations in antibiotic resistance among the motile aeromonads of the farmed and market carps were noted. However, higher proportions of motile aeromonads from market carps were resistant to chloramphenicol, cefalexin, gentamycin, oxytetracycline and trimethoprim than the farmed carps (**Figure 2B**). Most of the antibiotics to which the isolated strains have shown resistance are prescribed popularly for human aeromonad infections (**Igbinosa *et al.*, 2012**). The studied locations are situated in the peri-urban zone of Kolkata and contamination with run-off or urban wastes is likely to be high. Since the farms have no history of antibiotic usage, observance of such high MAR among both farm and market fish samples implies high-risk source contamination along the production or market chain and unhygienic handling (**Ezung and Abraham, 2013**). Resistance to third-generation cephalosporins (cefalexin) is known to be associated with the derepression of chromosomal enzymes (**Zdanowicz *et al.*, 2020**) and anthropogenic *Aeromonas* infections (**Igbinosa *et al.*, 2012**). Oxytetracycline resistance was comparatively less as the change in strains' resistance depends on seasonal variation rather than medication (**Julinta *et al.*, 2019**). The AMR of *Aeromonas* samples exhibited against fluoroquinolones (ciprofloxacin and enrofloxacin) and sulfonamides (co-trimoxazole, sulfafurazole and trimethoprim) were analogous with the studies of **Zdanowicz *et al.* (2020)**.

Resistance against amoxyclav among carps, pond water and pond sediment samples differed significantly (*P<0.05*), so also against gentamycin. The high resistance against amoxyclav observed in this study agrees with the reports of **Sreedharan *et al.* (2012)**, who reported complete resistance to amoxyclav by motile aeromonads from ornamental fish culture system. The accumulation of

antibiotic resistance from fish faeces may also create a more concentrated antibiotic-resistant microflora (**Marti *et al.*, 2013**). Resistance against azithromycin and gentamycin was significantly high in pond water ($P<0.05$) compared to carp and sediment samples. *Aeromonas* spp. of market carps exhibited significantly higher resistance against cefalexin, chloramphenicol, gentamycin, trimethoprim and oxytetracycline than the farmed carps. The studies by **Kozińska and Pękala (2012)** revealed that antibiotic-resistant aeromonads are existent in a huge number of market samples due to some adhesive factors on fish skin. Moreover, the fish vendors' and customers' hand-to-hand contact with fish and cross-contamination with other fish had been an excellent way of transfer of antibiotic-resistance (**WHO, 2019**). Oxytetracycline-resistance among the *Aeromonas* spp. of the market carps was high suggesting that the storage temperatures tend to enhance the growth of microflora (**Janda and Abbott, 2010**). Cefalexin, a third-generation cephalosporin, is widely used in treating clinical infections (**Berglund, 2015**) thus qualifying the role of anthropogenic influence in the spread of antibiotic resistance.

Multiple antibiotic resistance (MAR)

The results on the resistance among the motile aeromonads of farmed and market carps to antibiotic groups are presented in **Table 2.** A total of 214 strains (88.43%) showed resistance to ≥3 antibiotic groups (MAR). Among the MAR group, about 12.15% (n=26) and 4.67% (n=10) of the motile aeromonads were resistant to ≥6 and ≥7 antibiotic groups. It is noteworthy that all the strains of pond water (n=30) were of MAR, exhibiting resistance to 5 antibiotic groups, possibly the pond water shares an adequate linkage with the human microbiome, where the transfer of resistance genes is quite prevalent (**Marti *et al.*, 2013**). There existed a significant difference in MAR among the motile aeromonads of pond water and pond sediment as well as pond water and *L. rohita* ($P<0.05$). The MAR index alternated between 0.33 (resistance to 3 antibiotic groups) and 1.00 (resistance to 9 antibiotic groups) with one strain from *C. mrigala* revealing resistance to 9 antibiotic groups. With MAR as high as 100% and the MAR index in the range of 0.33-1.00, it is plausible that the aquatic environment harbours ARBs with motile *Aeromonas* spp. as the chief flora. The high proportions of antibiotic resistance in *C. mrigala* can be attributed to the sediments, upon which it normally dwells, which accumulate antibiotic-resistant bacteria from fish faeces, water column and unused feed as well as from the surrounding environment (**Marti *et al.*, 2013**). Our results corroborate **Nhinh *et al.* (2021)**, who recorded MAR index in the range of 0.13 - 0.88 and MAR as high as 74.7% in *A. hydrophila* from cultured freshwater fish in Vietnam. The MAR index observed among the motile aeromonads varied between 0.33 and 1.0, which is of great concern because a sample with a MAR index above 0.2 is considered to be exceedingly manifested with antibiotics and highly risky (**Nhinh *et al.*, 2021**). It can also be pointed out that the sampling locations, viz., Buderhat *bhery* and Barrackpore farm were close to highly populated cities, and had hospitals and dumping grounds in their vicinity. Furthermore, Nalban *bhery* used biologically treated sewage, which is considered to be highly contaminated with ARB. Hence, the high MAR indices were observed.

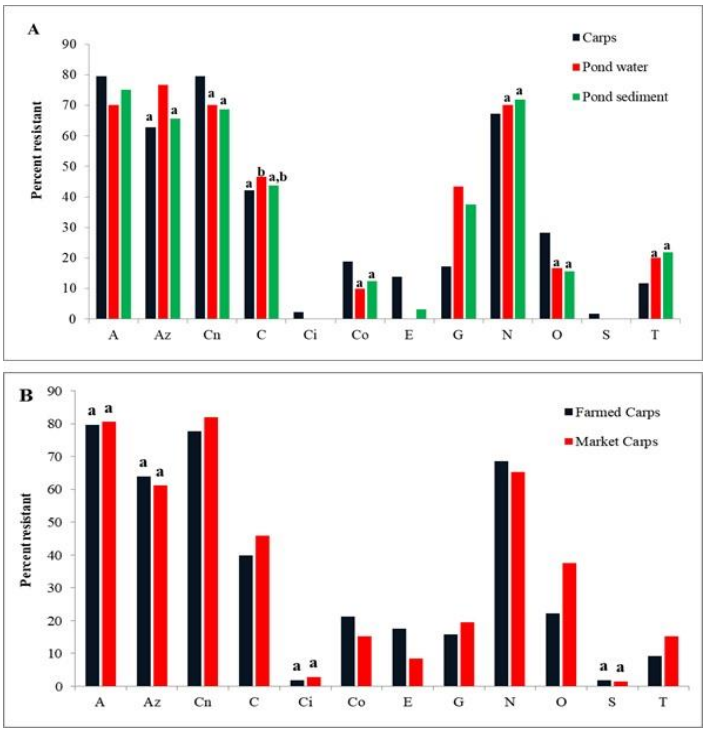

Figure 2 Antibiotic resistance (%) among the motile aeromonads of [A] carp, pond water and pond sediment and [B] farmed and market carp samples. A: Amoxyclav (30 µg), Az: Azithromycin (15 µg), Cn: Cefalexin (30 µg), C: Chloramphenicol (30 µg), Ci: Ciprofloxacin (5 µg), Co: Co-trimoxazole (25 µg), E: Enrofloxacin (10 µg), G: Gentamycin (10 µg), Ni: Nitrofurantoin (300 µg), O: Oxytetracycline (30 µg), S: Sulfafurazole (300 µg), T: Trimethoprim (10 µg). a,b: Bars sharing common alphabets for a particular antibiotic differed insignificantly (p>0.05).

Table 2 Resistance to antibiotic groups among the motile aeromonads of farmed and market carps

Particulars	Number of antibiotic groups to which motile aeromonads demonstrated resistance							Multiple antibiotic resistance (%)	MAR index range
	3	4	5	6	7	8	9		
	Percentage resistance								
Catla catla (n=61)	8.20	47.54	26.23	8.20	0.00	0.00	0.00	90.17	0.33-0.67
Labeo rohita (n=60)	16.67	26.67	26.67	3.33	8.33	1.67	0.00	83.34[a]	0.33-0.78
Cirrhinus mrigala (n=59)	20.34	23.73	25.42	15.25	1.69	1.69	1.69	89.81	0.33-1.00
Pond water (n=30)	13.33	53.34	33.33	0.00	0.00	0.00	0.00	100.00[ab]	0.33-0.56
Pond sediment (n=32)	6.25	43.75	28.13	0.00	3.13	0.00	0.00	81.26[b]	0.33-0.78

Multiple antibiotic resistance (MAR) or multiple drug resistance (MDR) is defined as resistance to three or more classes of antimicrobials (**ECDC/EMEA, 2009**). a,b: MAR values sharing common alphabets differ significantly (p<0.05)

Antibiotic resistance profile

A total of 57 resistant profiles were observed (**Table 3**). The most predominant resistant profile was "A,Az,Cn,N", which represented 26 strains from carps and 11 strains from pond water and sediment samples. This was followed by the profile A,Az,Cn,G,N from 23 motile aeromonad strains. The majority of the strains were resistant to four antibiotic groups. The observed 57 resistant profiles elucidated a wide diversity in antibiotic resistance, which is a cause of major concern. With such a high diversity, the emergence of new combinations of resistant genes is more frequent (**Patil *et al.*, 2016**). Strains from *C. mrigala* had as high as 30 different antibiotic-resistant profiles and a MAR index range of 0.33-1.00 illuminating the association of feeding habit and accumulation of ARBs (**Haque *et al.*, 2014**). Cross-resistance between cefalexin and amoxyclav could have led to the expression of profile "A,Az,Cn,N" resistance profile, which was predominantly observed in carps (n=26), water (n=9) and sediment samples (n=2). Twenty-three strains displayed the resistance profile "A,Az,Cn,G,N" although cross-resistance to third-generation cephalosporins (cefalexin) and aminoglycosides (gentamicin) among aeromonads are not reported. Since the resistance to chloramphenicol and trimethoprim is plasmid-mediated, the aeromonads may probably develop cross-resistance. This explains the resistance profile of "Az,C,G,T", which was present in 15 motile aeromonad strains. Although it is a necessity to determine the antibiotic-resistant profiles of bacteria to provide a brief input about the possible antibiotics that might curb the infection, such high diversity of profiles, as noted from the present study, suggested the decreased effectiveness of the tested antibiotics and the variety of resistance patterns existing within *Aeromonas*. This diversity also advocated that

resistance could be acquired as a cassette containing several determinants or that a single determinant could code for a mechanism that can bid resistance to several different antibiotics simultaneously (**Piddock *et al.*, 2016**).

Table 3 Antibiotic-resistance profiles of motile aeromonads from Indian major carps, pond water and sediment samples

Resistance profile	Number of motile aeromonad strains								
	Catla catla		*Labeo rohita*		*Cirrhinus mrigala*		Pond water	Pond sediment	Pooled sample
	Farm	Market	Farm	Market	Farm	Market			
A,Cn	2	4	1	3	2	2		2	16
A,E			1					1	2
Az,C			4	1					5
C,N						2		3	5
A,Az,C			2						2
A,Cn,G	1					2			3
A,Cn,N	1	2	2	1	2	2	2	2	14
A,Cn,O				1					1
A,E,Co			1						1
Az,C,G			1				2		3
Az,C,N					2				2
Cn,C,O				2	4				6
Cn,N,O	1								1
A,Az,C,G							1		1
A,Az,C,N	1			4	1	1		2	9
A,Az,Cn,N	8	6	7		4	1	9	2	37
A,Az,Cn,G			1		1				2
A,Cn,C,N					1				1
A,Cn,Co,T	2								2
A,Cn,N,Co	1				1	1			3
A,Cn,N,O	1		1		1		2	2	7
A,Cn,N,S	1			1					2
Az,C,E,N	4	2							6
Az,C,G,T						2	3	6	11
Az,C,Cn,O		2		1					3
Cn,C,Co,N	1		1				1	2	5
A,Az,C,E,N	2	1							3
A,Az,C,E,T	3		3	2					8
A,Az,C,G,T						1	4	2	7
A,Az,Cn,C,N	1						2	1	4
A,Az,Cn,Ci,N						1			1
A,Az,Cn,Co,N					1				1
A,Az,Cn,N,O		2			1				3
A,Az,Cn,G,N	2	1	6		3	2	3	6	23
A,Az,C,Cn,O		1			2				3
A,Cn,C,Co,O			1						1
A,Cn,Ci,E,O					2				2
A,Cn,Co,N,O					1				1
Az,Cn,C,N,O				1					1
Cn,C,Co,N,O	2	1	1	1		1	1		7
Cn,Co,G,N,O			1						1
A,Az,Cn,C,Co,N	2	1				2			5
A,Az,Cn,C,G,N					1				1
A,Az,Cn,Co,N,O			1						1
A,Az,Cn,G,O,N	1				1				2
A,Az,Cn,N,O,T				1					1
A,Az,C,Co,N,O					1				1
A,Cn,C,Co,N,O		1			2	1			4
A,Cn,E,N,O,T					1				1
A,Az,Cn,C,Co,N,O				2					2
A,Az,Cn,C,G,N,O				1					1
A,Az,Cn,C,N,O,T				1					1
A,Az,Cn,Co,G,N,O						1			1
A,Az,Cn,E,N,O,T								1	1
A,Cn,Co,E,N,O,S			1						1
A,Az,Cn,C,G,N,O,T				1		1			2
A,Az,Cn,C,Ci,G,N,O,T						1			1

PCR detection of *tet* genes

Though the selected strains were phenotypically resistant, the PCR amplification demonstrated the absence of the target genes, *tet*A, *tet*C and *tet*E (**Figures 3A-C; Table 4**). Similar results were also recorded by **Shivakumaraswamy *et al.* (2019)**. The resistance in such isolates could likely be either due to other mechanisms such as enzymatic inactivation or target modification, which also contribute to tetracycline resistance (**Watts *et al.*, 2017**). The *tet* group of genes are vast in number and the phenotypic oxytetracycline resistance in the selected strains could also be mediated by other genetic determinants of the *tet* group apart from the targeted genes in the current study. **Deng *et al.* (2014)** also reported the absence of *tetC* and *tetE* genes in *Aeromonas* isolates and opined that the presence of *tet* genes was dependent on geographical locations and the history of antimicrobial usage in that area. Resistance to most classes of

antibiotics that are involved in anthropogenic and veterinary use has been found in the aquacultured environment (**Watts *et al.*, 2017; Nhinh *et al.*, 2021**). Our results suggested that motile *Aeromonas* spp. are ubiquitous in aquaculture environments and the enumeration of antibiotic-resistant motile *Aeromonas* spp. can serve as a biological indicator of the quality of the fish as well as the environment.

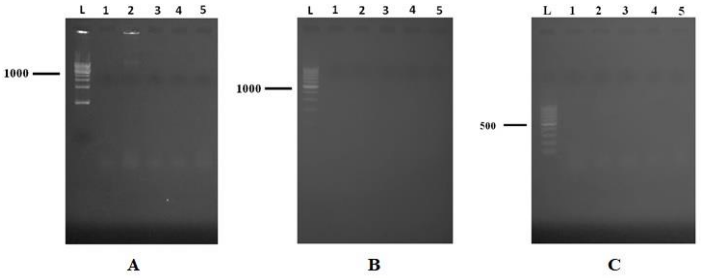

Figure 3 Agarose gel (1.2%) showing electrophoresis of PCR products of five motile aeromonad strains targeting [A] *tetA*, [B] *tetE* and [C] *tetC* genes. Lane L: 1000 bp molecular weight marker for [A] and [B]; Lane L: 100-bp molecular weight marker for [C]. Lanes 1–5: Genomic DNA of motile aeromonad strains.

Table 4 Oxytetracycline-resistant *Aeromonas* strains screened for the distribution of *tet* genes

***Aeromonas* strains**	**Fish species**	**Source**	**Oxytetracycline resistant genes**		
			*tet*A	*tet*C	*tet*E
Aeromonas hydrophila 2CR	*Labeo rohita*	Buderhat *bhery*	-*	-	-
A. caviae 2BMM	*Cirrhinus mrigala*	Barrackpore market	-	-	-
A. hydrophila 3BFC	*Catla catla*	Barrackpore farm	-	-	-
A. tecta 2NS	Pond sediment	Nalban *bhery*	-	-	-
A. diversa 2BFM	*Cirrhinus mrigala*	Barrackpore farm	-	-	-

*-: No bands were obtained

CONCLUSIONS

The current study enlightened the quality of carps and MAR alike in the culture system. The results indicated a high prevalence of AMR among the motile aeromonad strains of farmed and market carps. The high proportion of motile aeromonads and the intriguing proportions of the presence of AMR indicated that motile *Aeromonas* spp. comprise an effective marker for monitoring AMR in freshwater fish like carps and their environment. The high prevalence of MAR *Aeromonas* spp. in the aquacultured environment poses a threat not only to the cultured carps but also to public health.

Author contributions: Authors AB, QAQ and RC performed experiments. AB participated in the manuscript draft. TJA conceived the study, designed the experimental procedures, supervised the experiments, thoroughly checked and revised the manuscript for necessary changes in format, grammar and English. All authors read and approved the final version of the manuscript.

Competing interest: The authors declare that they have no competing interest.

Acknowledgements: The research work was supported by the Indian Council of Agricultural Research, Government of India, New Delhi under the All India Network Project on Fish Health vide Grant F. No. CIBA/AINP-FH/2015-16 dated 02.06.2015. The authors thank the Vice-Chancellor, West Bengal University of Animal and Fishery Sciences, Kolkata for providing the necessary infrastructure facility to carry out the work.

REFERENCES

Abraham, T.J., Sil, S.K., & Nagesh, T.S. (2020). Association of Risk factors and management issues on the occurrence of diseases in carp aquaculture in West Bengal, India. *Proceedings of the Zoological Society*, 73, 243–250. DOI: https://doi.org/ 10.1007/s12595-020-00325-7

Abraham, T.J., & Bardhan, A. (2019). Emergence and spread of antimicrobial resistance in motile aeromonads of the aquaculture environment. *Indian Journal of Animal Health*, 58(2)-Special Issue, 39-52. DOI: DOI: https://doi.org/10.36062/ ijah.58.2SPL.2019.39-52

Akinbowale, O.L., Peng, H., & Barton, M.D. (2007). Diversity of tetracycline resistance genes in bacteria from aquaculture sources in Australia. *Journal of Applied Microbiology*, 103(5), 2016-2025. DOI: https://doi.org/10.1111/j.1365-2672.2007. 03445.x

APHA (1992). *Compendium of Methods for the Microbiological Examination of Foods*. Vanderzant, C., & Splittstoesser, D.F., (Eds), American Public Health Association, Washington, DC. 1219 p.

Austin, B. & Austin, D.A. 2012. *Bacterial Fish Pathogens. Diseases of Farmed and Wild Fish*. Fifth ed. Springer-Praxis in Aquaculture in Fisheries, Praxis Publication Ltd., Chichester, UK, p. 457.

Berglund, B. (2015). Environmental dissemination of antibiotic resistance genes and correlation to anthropogenic contamination with antibiotics. *Infection Ecology and Epidemiology*, 5(1), 28564. DOI: https://doi.org/10.3402/iee.v5.28564

CLSI, (2012). *Performance Standards for Antimicrobial Disk Susceptibility Tests*, Approved Standard, 7th ed., CLSI document M02-A11. Pennsylvania, USA. Clinical and Laboratory Standards Institute, 19087.

Collins, C.H., Lyne, P.M., Grange, J.M., & Falkinham, J.O. III. (2004). *Collins and Lyne's Microbiological Methods*, 8th edition. Arnold, London, UK, p.456.

Deng, Y.T., Wu, Y.L., Tan, A.P., Huang, Y.P., Jiang, L., Xue, H.J., Wang, W.L., Luo, L., & Zhao, F. (2014). Analysis of antimicrobial resistance genes in *Aeromonas* spp. isolated from cultured freshwater animals in China. *Microbial Drug Resistance*, 20(4), 350-356. DOI: https://doi.org/10.1089/mdr.2013.0068

ECDC/EMEA, (2009). *A joint technical report: The bacterial challenge: time to react*. European Centre for Disease Prevention and Control and European Medicines Agency, Stockholm, Sweden & London, United Kingdom.

EIC, (2012). *Compendium of orders of fresh, frozen and processed fish and fishery products*. Export Inspection Council of India, Ministry of Commerce, Government of India, New Delhi, India.

Ezung, R., & Abraham, T.J. (2013). Sensory and microbial quality of retail finfish with emphasis on antibiotic-resistant bacteria. *Journal of Aquatic Food Product Technology*, 22(5), 474-486. DOI: https://doi.org/10.1080/10498850.2012.666711

Fernández-Bravo, A., & Figueras, M.J. (2020). An update on the genus *Aeromonas*: taxonomy, epidemiology, and pathogenicity. *Microorganisms*, 8, 129. DOI: https://doi.org/10.3390/microorganisms8010129

Figueira, V., Vaz-Moreira, I., Silva, M., & Manaia, C. M. (2011). Diversity and antibiotic resistance of *Aeromonas* spp. in drinking and waste water treatment plants. *Water Research*, 45(17), 5599-5611. DOI: https://doi.org/10.1016/j.watres. 2011.08.021

FSSAI, (2009). *Food Safety Standards and Regulations*. Food Safety Standards Authority of India, New Delhi. Available at: http://www.fssai.gov.in//

González-Rodríguez, M.N., Santos, J.A., Otero, A., & García-López, M.L. (2002). PCR detection of potentially pathogenic aeromonads in raw and cold-smoked freshwater fish. *Journal of Applied Microbiology*, 93(4), 675-680. DOI: https://doi.org/10.1046/j.1365-2672.2002.01739.x

Graf, J. (2015). *Aeromonas*. Caister Academic Press, Norfolk, UK.

Haque, S.A., Reza, M.S., Islam, M.A., Roy, V.C., & Alam, M.A. (2014). Effects of antibiotic on bacterial flora in mrigal fish (*Cirhinus cirrhosus*, Bloch, 1795) under laboratory condition. *European Journal of Food Science and Technology*, 2(1), 1-7.

Heß, S., Berendonk, T.U., & Kneis, D. (2018). Antibiotic-resistant bacteria and resistance genes in the bottom sediment of a small stream and the potential impact of remobilization. *FEMS Microbiology Ecology*, 94(9), fiy128. DOI: https://doi.org/10.1093/femsec/fiy128

HiMedia (2009). *The HiMedia Manual: A Manual of Microbiology Laboratory Practice*. HiMedia Laboratories, Mumbai, India.

Hoel, S., Vadstein, O., & Jakobsen, A.N. (2019). The significance of mesophilic *Aeromonas* spp. in minimally processed ready-to-eat seafood. *Microorganisms*, 7, 91; DOI: https://doi.org/10.3390/microorganisms7030091

Huddleston, J. R., Zak, J. C., & Jeter, R. M. (2006). Antimicrobial susceptibilities of *Aeromonas* spp. isolated from environmental sources. *Applied and Environmental Microbiology*, 72(11), 7036-7042. DOI: https://doi.org/10.1128/AEM.00774-06

Igbinosa, I.H., Igumbor, E.U., Aghdasi, F., Tom, M., & Okoh, A.I. (2012). Emerging *Aeromonas* species infections and their significance in public health. *The Scientific World Journal*, 2012, 625023. DOI: https://doi.org/10.1100/2012/625023

Janda, J.M., & Abbott, S.L. (2010). The genus *Aeromonas*: taxonomy, pathogenicity, and infection. *Clinical Microbiology Reviews*, 23(1), 35-73. DOI: https://doi.org/10.1128/ CMR.00039-09

Julinta, R.B., Abraham, T.J., Roy, A., Singha, J., Dash, G., Mali, P., Nagesh, T.S., Sar, T.K., Patil, P.K., & Kumar, K.A. (2019). Effect of oxytetracycline-dosing on the growth, safety and intestinal histology of Nile tilapia, *Oreochromis niloticus* (L.) juveniles. *International Journal of Current Microbiology and Applied Sciences*, 8(8), 2708-2724. DOI: https://doi.org/10.20546/ijcmas.2019.808.313

Kozińska, A., & Pękala, A. (2012). Characteristics of disease spectrum in relation to species, serogroups, and adhesion ability of motile aeromonads in fish. *The Scientific World Journal*, 2012. DOI: https://doi.org/10.1100/2012/949358

Marti, E., Jofre, J., & Balcazar, J.L. (2013). Prevalence of antibiotic resistance genes and bacterial community composition in a river influenced by a wastewater treatment plant. *PloS One*, 8(10), e78906. DOI: https://doi.org/10.1371/journal.pone.0078906

Nhinh, D.T., Le, D.V., Van, K.V., Huong-Giang, N.T., Dang, L.T., & Hoai, T.D. (2021). Prevalence, virulence gene distribution and alarming the multidrug resistance of *Aeromonas hydrophila* associated with disease outbreaks in freshwater aquaculture. *Antibiotics*, 10, 532. https://doi.org/10.3390/antibiotics10050532

Pablos, M., Rodríguez-Calleja, J.M., Santos, J.A., Otero, A., & García-López, M.L. (2009). Occurrence of motile *Aeromonas* in municipal drinking water and distribution of genes encoding virulence factors. *International Journal of Food Microbiology*, 135(2), 158-164. DOI: https://doi.org/10.1016/j.ijfoodmicro.2009.08.020

Palumbo, S.A., Maxino, F., Williams, A.C., Buchanan, R.L., & Thayer, D.W. (1985). Starch-ampicillin agar for the quantitative detection of *Aeromonas hydrophila*. *Applied and Environmental Microbiology*, 50(4), 1027-1030. DOI: https://doi.org/ 10.1128/AEM.50.4.1027-1030.1985

Patil, H.J., Benet-Perelberg, A., Naor, A., Smirnov, M., Ofek, T., Nasser, A., Minz, D., & Cytryn, E. (2016). Evidence of increased antibiotic resistance in phylogenetically diverse *Aeromonas* isolates from semi-intensive fish ponds treated with antibiotics. *Frontiers in Microbiology*, 7, 1875. DOI: https://doi.org/10.3389/fmicb. 2016.01875

Piddock, L., Garneau-Tsodikova, S., & Garner, C. (2016). Ask the experts: how to curb antibiotic resistance and plug the antibiotics gap?. *Future Medicinal Chemistry*, 8(10), 1027-1032. DOI: https://doi.org/10.4155/fmc-2014-0032

Saharia, P.K., Kalita, B., Bhuyan, S., Hussain, I.A., Pokhral, H., Bhagabati, S.K., Dutta, R., Borah, A., Bora, G., & Sharma, R. (2019). Economic impact of bacterial disease caused by motile A*eromonas* (MAS) in carp culture practices of Assam, India: An Analysis. *Journal of Community Mobilization and Sustainable Development*, 14(2), 245-250.

Sandegren, L. (2019). Low sub-minimal inhibitory concentrations of antibiotics generate new types of resistance. *Sustainable Chemistry and Pharmacy*, 11, 46-48. DOI: https://doi.org/10.1016/j.scp.2018.12.006

Schmidt, A.S., Bruun, M.S., Dalsgaard, I., & Larsen, J.L. (2001). Incidence, distribution, and spread of tetracycline resistance determinants and integron-associated antibiotic resistance genes among motile aeromonads from a fish farming environment. *Applied and Environmental Microbiology*, 67(12), 5675-5682. DOI: https://doi.org/10.1128/AEM. 67.12.5675-5682.2001

Shivakumaraswamy, S.K., Deekshit, V.K., Vittal, R., Akhila, D.S., Mundanda, D.M., Raj, J.R.M., Chakraborty, A., & Karunasagar, I. (2019). Phenotypic and genotypic study of antimicrobial profile of bacterial isolates from environmental samples. *Indian Journal of Medical Research*, 149(2), 232. DOI: https://dx.doi.org/10.4103/ijmr.IJMR_2097_17

Sreedharan, K., Philip, R., & Singh, I.S.B. (2012). Virulence potential and antibiotic susceptibility pattern of motile aeromonads associated with freshwater ornamental fish culture systems: a possible threat to public health. *Brazilian Journal of Microbiology*, 43(2), 754-765. DOI: https://doi.org/10.1590/S1517-83822012000200040

Surendraraj, A., Farvin, K.S., Yathavamoorthi, R., & Thampuran, N. (2009). Enteric bacteria associated with farmed freshwater fish and its culture environment in Kerala, India. *Research Journal of Microbiology*, 4(9), 334-344. DOI: https://doi.org/ 10.3923/jm.2009.334.344

USFDA, (2018). *Approved aquaculture drugs*. US Food and Drug Administration. Available at: https://www.fda.gov/animal-veterinary/aquaculture/approved-aquaculture-drugs

Watts, J.E., Schreier, H.J., Lanska, L., & Hale, M.S. (2017). The rising tide of antimicrobial resistance in aquaculture: sources, sinks and solutions. *Marine Drugs*, 15(6), 158. DOI: https://doi.org/10.3390/md15060158

WHO (2019). *In the face of slow progress who offers a new tool and sets a target to accelerate action against antimicrobial resistance*. The World Health Organization. Available at: https://www.who.int/news-room/detail/18-06-2019-in-the-face-of-slow-progress-who-offers-a-new-tool-and-sets-a-target-to-accelerate-action-against-antimicrobial-resistance

Yang, Y., Miao, P., Li, H., Tan, S., Yu, H., & Yu, H. (2018). Antibiotic susceptibility and molecular characterization of *Aeromonas hydrophila* from grass carp. *Journal of Food Safety*, 38(1), e12393. DOI: https://doi.org/10.1111/jfs.12393

Zdanowicz, M., Mudryk, Z.J., & Perliński, P. (2020). Abundance and antibiotic resistance of *Aeromonas* isolated from the water of three carp ponds. *Veterinary Research Communications*, 44(1), 9-18. DOI: https://doi.org/10.1007/s11259-020-09768-x

Zhou, Y., Yu, L., Nan, Z., Zhang, P., Kan, B., Yan, D., & Su, J. (2019). Taxonomy, virulence genes and antimicrobial resistance of *Aeromonas* isolated from extra-intestinal and intestinal infections. *BMC Infectious Diseases*, 19(1), 1-9. DOI: https://doi.org/ 10.1186/s12879-019-3766-0