MUTATION FREQUENCIES OF MOTILE *AEROMONAS* SPP. FROM CULTURED CARPS AGAINST OXYTETRACYCLINE AND CHLORAMPHENICOL

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Aeromonas spp. are autochthonous in the aquatic environment and known for their aetiological role in motile *Aeromonas* septicemia (MAS) of freshwater fish. In the present study, the susceptibility of five motile *Aeromonas* strains from the aquaculture environment was tested against 12 antibiotics. The mutation frequencies and minimal inhibitory concentrations (MICs) of oxytetracycline, an approved antibiotic and chloramphenicol, a banned antibiotic for aquaculture use were determined against *A. hydrophila* (n=3), *A. caviae* (n=1) and *A. tecta* (n=1). The MICs of oxytetracycline and chloramphenicol were in the range of 0.39 - 6.25 µg/mL and 3.125 - 6.25 µg/mL, respectively. The mutation frequencies to oxytetracycline and chloramphenicol at 25 µg/mL were in the range of 5.94×10^{-7} - 8.25×10^{-7} and 1.20×10^{-7} - 8.33×10^{-7} , respectively. The multiple antibiotic-resistance (MAR) index among the motile *Aeromonas* strains of the aquaculture environment varied between 0.44% and 0.56%, which is of great concern.

Key words: Chloramphenicol, Motile *Aeromonas* spp., Multiple antibiotic resistance, Mutation frequency, Oxytetracycline

West Bengal is one of the highest producers of freshwater table fish and fish seeds in the country (Department of Fisheries, 2019). Aquaculture production of West Bengal has seen an increasing trend with a production of 17.42 lakh tonnes in the year 2017-18, second among the fish producing states of India (Department of Fisheries, 2019). Among the 20 districts of West Bengal, North and South 24 Parganas are the two most aquaculturally important districts, contributing to nearly 70% of the total

aquaculture production of the state (Department of Fisheries, 2019). The Indian major carps (IMCs), viz., *Labeo rohita*, *Catla catla* and *Cirrhinus mrigala* are the mainstay of freshwater aquaculture, which contribute 87% of the total freshwater aquaculture production of the country (Mohanty *et al.*, 2017). Bacterial diseases are one of the main problems of freshwater aquaculture with motile *Aeromonas* septicemia (MAS) causing huge mortalities in IMCs (Stratev and Odeyemi, 2017).

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Aeromonas spp. are Gram-negative and facultative anaerobic bacteria, which are ubiquitous in the aquatic environments (Graf, 2015) and pathogenic to fish as well as humans (Stratev and Odeyemi, 2016). Motile aeromonads, as primary and opportunistic pathogens, have demanded a lot of attention with the use of various antimicrobials in aquaculture (Stratev and Odeyemi, 2017). The use of oxytetracycline (OTC) has been proved to be efficient against MAS (Stratev and Odeyemi, 2017). However, with their extensive use, antimicrobial resistance (AMR) has become a harsh consequence (Romero *et al.*, 2012). Motile *Aeromonas* strains from cultured freshwater fish like *C. catla*, *L. rohita* and *Ctenopharyngodon idella* had been seen to acquire a relatively high resistance to OTC, amoxycillin, ampicillin, novobiocin and polymyxin-B, possibly attributed to the use of antibiotics in aquaculture (Hatha *et al.*, 2005). Most mesophilic motile aeromonads are resistant to ampicillin, cephalothin and trimethoprim and susceptible to ciprofloxacin and ceftriaxone (Bhowmick *et al.*, 2018). In recent years, the prevalence of multiple antibiotic resistance (MAR) among the motile aeromonads has been the major cause for concern (Romero *et al.*, 2012; Graf, 2015). Since West Bengal is one of the major fish producing states of India with high per capita consumption and demand for freshwater fish, the present study attempted to assess the antibiotic sensitivity of motile aeromonads of aquaculture environment, and determine the minimal inhibitory concentration (MIC) and frequencies of mutation against OTC and chloramphenicol.

MATERIALS AND METHODS

Bacterial strains: The chloramphenicol and OTC sensitive (C^s, O^s) motile aeromonad strains, viz., *A. hydrophila* (n=3), *A. caviae* (n=1) and *A. tecta* (n=1) of this study were from the collections of the Department of Aquatic Animal Health, Faculty of Fishery Sciences, Kolkata. The details of these strains, the location and source of the collection are presented in Table 1.

Antibiotic sensitivity: The sensitivity of motile *Aeromonas* spp. to 12 antibiotics (HiMedia, India), viz., amoxyclav (30 µg), azithromycin (15µg), cefalexin (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), co-trimoxazole (25 µg), enrofloxacin (10 µg), gentamycin (10µg), nitrofurantoin (300 µg), oxytetracycline (30µg), sulfafurazole (300µg) 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). Multiple antibiotic resistance (MAR) index of a particular strain was computed as “a/b”, where “a” signified the number of antibiotic groups the strain was resistant to and “b” signified the total number of antibiotic groups against which the antibiotic sensitivity assay was carried out for that particular strain (Magiorakos *et al.*, 2012).

Minimal inhibitory concentration of oxytetracycline and chloramphenicol: Five C^s, O^s strains comprising *A. hydrophila*, *A. caviae* and *A. tecta* were used for determining the MICs of OTC, an approved antibiotic and chloramphenicol, a banned

antibiotic for aquaculture use by agar dilution method (CLSI, 2012). Oxytetracycline dihydrate and chloramphenicol (HiMedia, India) stock solutions (1000 µg/mL) prepared as per CLSI (2011) were used for the preparation of MHA plates with appropriate antibiotics and concentrations ranging from 0 to 100 µg/mL. The MHA plates with OTC and/or chloramphenicol at various concentrations were spot inoculated with 2 µL (~10⁵ cells) of young bacterial culture, incubated for 48 hours at 35±2°C and observed for growth. The lowest concentration of the antibiotic that inhibited the visible bacterial growth was considered as the MIC.

Mutation frequency: The mutation frequencies of *Aeromonas* spp. were determined as per Gustafsson *et al.* (2003) with minor modifications. The MHA plates with desired concentrations of chloramphenicol or OTC at 25 µg/mL each were prepared separately. Freshly prepared bacterial cell suspensions (~10¹⁰ cells) were serially diluted up to 10⁻⁸ level in sterile saline. Aliquots (0.1 mL) of cell suspension from undiluted, 10⁻¹, 10⁻², 10⁻³, 10⁻⁴ and 10⁻⁵ dilutions were then spread plated on to MHA plates containing chloramphenicol or OTC. Simultaneously, 0.1 mL each of 10⁻⁷ and 10⁻⁸ dilutions of the cell suspension was spread plated on to antibiotic-free MHA plates and incubated at 35±2°C for 5-7 days. The number of growing colonies on the plates was counted and mutation frequencies calculated as given below.

Mutation frequency of motile *Aeromonas* = Na/N, where,

Na = Number of colonies on MHA plates containing chloramphenicol or OTC

N = Number of colonies on MHA plates without antibiotic

RESULTS

Antibiotic sensitivity: The antibiotic sensitivity of motile *Aeromonas* spp. are presented in Table 1. *Aeromonas hydrophila* strains (6BM, FBM9 and CR8) were sensitive to chloramphenicol, ciprofloxacin, co-trimoxazole, enrofloxacin, oxytetracycline, sulfafurazole and trimethoprim. *Aeromonas caviae* GHC and *A. tecta* 9NR exhibited a similar resistance pattern as that of *A. hydrophila* 6BM and CR8. Resistance to gentamycin varied among the *A. hydrophila* strains. All the strains were resistant to amoxycylav, azithromycin, cefalexin and nitrofurantoin. The MAR indices for *A. hydrophila*, *A. caviae* and *A. tecta* strains were in the range of 0.44-0.56.

Minimal inhibitory concentrations of oxytetracycline and chloramphenicol: The MICs of OTC ranged between 0.39 µg/mL and 3.125 µg/mL for *A. hydrophila* strains, 6.25 µg/mL for *A. caviae* and 1.56 µg/mL for *A. tecta*. The MICs of chloramphenicol varied between 3.125 µg/mL and 6.25 µg/mL for *A. hydrophila*, *A. caviae* and *A. tecta* strains (Table 1).

Mutation frequencies of motile *Aeromonas* spp.: The mutation frequencies of motile aeromonads to OTC (25 µg/mL) were in the range of 5.94x10⁻⁷ (*A. tecta* 9NR)- 8.25x10⁻⁷ (*A. hydrophila* CR8). The mutation frequencies of motile aeromonads to chloramphenicol (25 µg/mL) ranged from 1.20x10⁻⁷ in *A. tecta* 9NR to 8.33x10⁻⁷ in *A. hydrophila* CR8 (Table 1).

Table 1. Antibiotic sensitivity, minimal inhibitory concentrations of oxytetracycline and chloramphenicol and frequencies of mutation among the motile *Aeromonas* strains of aquaculture environment

Bacterial strain	Location	Source	Antibiotic		MAR index	MIC (µg/mL)		Mutation frequency (x 10 ⁻⁷)	
			Sensitive	Resistant		OTC	C	OTC	C
<i>Aeromonas hydrophila</i> FBM9	Barrackpore farm	<i>Cirrhinus mrigala</i>	C, Ci, Co, E, O, S, T	A, Az, Cn, G, Ni	0.56	0.39	6.25	7.72	3.92
<i>Aeromonas hydrophila</i> CR8	Buderhat bhery	<i>Labeo rohita</i>	C, Ci, Co, E, G, O, S, T	A, Az, Cn, Ni	0.44	3.125	6.25	8.25	8.33
<i>Aeromonas hydrophila</i> 6BM	Buderhat bhery	Water	C, Ci, Co, E, G, O, S, T	A, Az, Cn, Ni	0.44	3.125	6.25	7.85	2.89
<i>Aeromonas caviae</i> GHC	Barrackpore farm	<i>Catla catla</i>	C, Ci, Co, E, G, O, S, T	A, Az, Cn, Ni	0.44	6.25	3.125	7.64	2.86
<i>Aeromonas tecta</i> 9NR	Nalban bhery	<i>Labeo rohita</i>	C, Ci, Co, E, G, O, S, T	A, Az, Cn, Ni	0.44	1.56	6.25	5.94	1.20

Multiple antibiotic resistance (MAR) is defined as resistance to three or more classes of antimicrobials (Magiorakos *et al.*, 2012); A: Amoxycylav (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); OTC: Oxytetracycline; C: Chloramphenicol

DISCUSSION

In the present study, all the C^s and O^s motile aeromonads from the aquaculture environment exhibited a similar antibiotic-resistance pattern, except for gentamicin. All the strains were resistant to amoxycylav, azithromycin, cefalexin and nitrofurantoin. The MAR index observed among the motile aeromonads of aquacultured farms and their environment varied between 0.44 and 0.56, which is of great concern because a sample with MAR index above 0.2 is considered to be exceedingly manifested with antibiotics and having a high-risk factor (Saka *et al.*, 2017). It is worth mentioning here 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. Further, the Nalban bhery used biologically treated sewage, which is considered to be highly contaminated with antibiotic-resistant bacteria [ARB] (Abraham, 2011).

The MICs of OTC were within the range of 0.39-6.25 µg/mL for *A. hydrophila* and *A. tecta* and *A. caviae* strains, which was within the set levels for the susceptible strains (CLSI, 2012). In contrast, Lukkana *et al.* (2011) documented wide variations in the MICs of OTC against *A. hydrophila* in the range of 0.06-64 µg/mL. Baron *et al.* (2017) in their study also showed that MICs of OTC for *Aeromonas* spp. were in the range of 0.062-128 µg/mL. The MICs of chloramphenicol were in the narrow range of 3.125-6.25 µg/mL for motile *Aeromonas* spp. On the other hand, the results of Majeed (2011) documented MICs above 100 µg/mL (128 µg/mL) for chloramphenicol against *A. hydrophila*. The MIC results of the present

study confirmed the susceptibility of motile *Aeromonas* spp. from West Bengal aquaculture environments towards chloramphenicol and OTC.

Mutation frequencies of motile aeromonads of the present study against OTC (5.94×10^{-7} - 8.25×10^{-7}) were comparable with the results of Bharathkumar and Abraham (2013). They demonstrated mutation frequencies in the range of 6.71×10^{-9} and 1.60×10^{-6} against OTC for *A. hydrophila* strains. The frequencies of mutation in motile aeromonads strains against chloramphenicol followed a similar trend and ranged between 1.20×10^{-7} and 8.33×10^{-7} . In nature, bacterial DNA mutations occur at 1 in 10^8 cells (Watford and Warrington, 2017). The results of the present study showed that the C^s and O^s motile aeromonads from the aquaculture environment are mutating at about 1-8 in 10^7 cells, which is comparatively higher than in the natural population (Watford and Warrington, 2017). As opined by Denamur and Matic (2006), the increased mutation rates in motile aeromonads of aquaculture environment may lead to considerably high proportions of the bacterial population with AMR. Theoretical considerations and experimental studies (Giraud *et al.*, 2002) showed that the levels of mutators may be enriched when bacteria are exposed to a changing environment especially while repeated challenges of antibiotics are performed. Also, a positive correlation between MAR and high mutation rates is expected to be frequent in natural populations of bacterial pathogens (Knopp and Andersson, 2018). The bacterial resistance to chloramphenicol and OTC results from genes that are located on

horizontally transferred genetic elements (e.g. plasmids) and a mutator is not expected to increase the rate of plasmid transfer (Gustafsson *et al.*, 2003). The ability to follow the antibacterial susceptibility trend and AMR over a long period of time is crucial to monitor antibiotic-resistance dissemination in the environment like aquaculture systems. In this regard, *Aeromonas* would be a perfect model organism to report on antibiotic-resistance in the freshwater aquaculture environment.

In general, the results of the present study indicated the prevalence of AMR among the motile aeromonads of freshwater aquaculture systems of West Bengal. This study also documented considerably higher

frequencies of mutation among the motile aeromonads. As the usage of antimicrobials is not regulated in India, repeated challenges of antibiotics in freshwater aquaculture may expose the motile aeromonads and other aquatic bacteria to a changing environment, which could be the root source and origin of higher levels of ARB.

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