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Prevalence, Antimicrobial Resistance and Resistance Gene Cassettes Detection in Bacterial Pathogens Isolated from Freshwater Ornamental Fishes

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ABSTRACT

Background: Antimicrobial resistance (AMR) is a rising concern in the global aquaculture sector due to the rampant prophylactic use of antibiotics. This study is aimed to determine the AMR pattern in freshwater ornamental fishes.

Methods: Fish pathogens were isolated and identified from infected Guppy and Molly collected from ornamental fish farms. Antibiotic susceptibility testing (ABST) using disc diffusion with 36 antibiotics was performed following the disc diffusion method. The resistance gene cassettes such as Class 1 and Class 2 integron were also detected from resolved isolates.

Result: Fish pathogens were isolated and identified as *Aeromonas hydrophila*, *A. dhakensis, A. veronii*, *A. sobria*, *Bacillus subtilis*, *Comamonas testosteroni*, *Edwardsiella tarda*, *Enterobacter cloacae*, *Enterococcus faecalis, Kurthia gibsonii* and *Klebsiella aerogenes*. Shannon Weiner diversity index of resolved isolates was found to be 1.366 and 2.101 for Guppy and Molly, respectively. ABST results showed an elevated resistance pattern for *A. veronii, E. faecalis* (Guppy), *K. aerogenes, B. subtilis, E. faecalis, C. testosteroni* (Molly) with higher multiple antibiotic resistance indexes (>0.33). Meanwhile, all the recovered isolates were susceptible to sulphafurazole, enrofloxacin, norfloxacin and ciprofloxacin. Detection of class 1 integron in genomic and plasmid DNA reminds the rapid spread of the AMR gene through horizontal gene transfer.

Key words: Antimicrobial resistance, Fish pathogens, Integrons, Multiple antimicrobial resistance, Ornamental fish.

INTRODUCTION

Ornamental fish production is a major source of income and employment globally. More than one billion ornamental fish are traded internationally and have become the most important global trade sector (Hatha and Nifty, 2012). Guppy and Molly are the most popular and commonly available pet fishes in tropical countries because of their beautiful coloration and easy maintenance. Like other ornamental fishes, these fishes are also prone to bacterial, fungal, viral and parasitic infections. Most bacterial infections in aquaculture are especially caused by Gram-negative species (Lewbart, 2001). Poor water quality, improper handling and stress are the common factors that cause diseases in fishes cultured in captive conditions. This enforces the farmers to use antibiotics in the aquaculture system therapeutically and prophylactically to control bacterial infections. Erythromycin, penicillin, ampicillin, tetracycline, oxytetracycline, gentamicin, neomycin, kanamycin, amikacin, nalidixic acid, oxolinic acid, nitrofurantoin, nitrofurazone, furanace, furazolidone, ormetoprim and sulfadimethoxine are commonly used in the ornamental sector for treating bacterial diseases (Yanong, 2011). This unregulated use of antimicrobials in the system leads to the emergence of antimicrobial resistance (AMR) (Hemamalini *et al*., 2021). The emergence of AMR pathogens has been increasingly reported, which decreases the ability to treat the infections. The resistant determinants from fish pathogens can be transmitted to clinically important pathogens *via* horizontal gene transfer (HGT). AMR gene dissemination is mainly mediated by integron gene cassettes such as class 1, 2 and 3 (Soufi *et al*., 2011). Of the 2152 studies published by Indian institutions on AMR, only 11 were on fishes (Taneja and Sharma, 2019). To control the AMR, considerable baseline data are essential. In Tamil Nadu, not many studies were carried out for detectingAMR in the

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aquaculture sector. Therefore, the present study was carried out to determine the bacterial diversity, antimicrobial susceptibility pattern, and AMR gene cassette detection from infected Guppy and Molly.

MATERIALS AND METHODS

Bacterial isolation and dendrogram construction

Infected Guppy (*Poecilia reticulata*) and Molly (*P. sphenops*) were collected (n=30 each) from ornamental fish farms in Chennai, India. Clinical signs were observed and the infected tissue samples were pooled and inoculated into the nutrient broth for bacterial isolation. Bacterial isolation was carried out by the conventional spread plate method. Bacterial colonies were randomly selected based on colony morphology and sub-cultured. Biochemical tests such as gram staining, motility, catalase, oxidase, sugar fermentation, indole, Voges-Proskauer and citrate utilization were performed. The results of the biochemical tests were recorded as numerical values for dendrogram construction using NTedit, version 1.2 and NTSYSpc, version 2.10e (Exeter Software, Setauket, NY, USA). Shannon Weiner diversity index was calculated using Primer-E software (Clarke and Gorley, 2015).

Molecular characterization and phylogenetic tree construction

Genomic DNA from representative isolates of each cluster was extracted following the salting-out method (Miller *et al*., 1988). Polymerase chain reaction (PCR) was performed with 16SrRNA gene universal primers fD1 (5′CCG AAT TCG TCG ACA ACA GAG TTT GAT CCT GGC TCA G 3′) and rD1 (5′CCC GGG ATC CAA GCT TAA GGA GGT GAT CCA GCC 3′) (Weisburg *et al*., 1991) for bacterial identification. The PCR products were sequenced by Sanger dideoxy chain termination method at Eurofins Genomics India Pvt. Ltd., Karnataka, India. The raw sequence data were trimmed and aligned using BioEdit Alignment Editor, version 7.2.5 (Hall, 1999). The sequences were identified by comparing the sequences in the NCBI database using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the final sequences were deposited to GenBank. MEGA7.0 software was used for determining the distance matrices of 16SrRNA sequences and phylogenetic trees were constructed using UPGMA statistical method with Kimura 2- parameter substitution model (Kumar *et al*., 2016).

Antibiotic susceptibility test (ABST) and detection of AMR gene cassettes

Thirty-six antibiotics were used to detect the antimicrobial susceptibility pattern of resolved isolates. ABST was performed following the disc diffusion method on Muller Hinton agar (MHA) plates. 0.5 McFarland standards (10^6 CFU/ml) were prepared for all the isolates and the bacterial lawn was prepared using sterile cotton swabs (Himedia, India). *Escherichia coli* ATCC 25922, CLSI reference strain, was used as a positive control. The zone of inhibition (ZOI) was measured and interpreted by CLSI (2018) and manufacturer guidelines. The multiple antibiotic resistance (MAR) index was calculated as the ratio of the number of antibiotics to which the bacterium was resistant to the total number of antibiotics used in the study (Krumperman, 1983).

The PCR was performed for the detection of class 1 and class 2 integron gene cassettes from genomic and plasmid DNA using specific primers [class 1 integron primers 5′CSF (5′GGCATCCAAGCAGCAAG3′) and 3′CSR (5′AAGCAGACTTGACCTGA3′) and class 2 integron primers Hep74F (5′CGGGATCCCCGGCATGCACGATTTGTA3′) and Hep51R (5′GATGCCATCGCAAGTACGAG3′)]. Plasmid DNA from bacterial isolates was extracted using the HiPurA plasmid DNA miniprep purification kit (Himedia, India). Class 1 and class 2 integron gene cassettes were detected following the PCR conditions suggested by Lévesque *et al*. (1995) and White *et al*. (2001). The PCR products class 1 and class 2 integron gene cassettes were detected in 1% agarose gel.

RESULTS AND DISCUSSION

Bacterial diversity

Clinical signs from infected fishes were observed, including skin ulcers, hemorrhagic patches, fin rot, lesions on internal organs (liver, spleen and kidney) and ascetic fluid accumulation in the abdomen on infected Guppy and Molly. The conventional spread plate technique provided more than 100 colonies for all the fish samples at 10-6 dilution. Dendrogram construction using biochemical test results generated 4 and 9 clusters for Guppy and Molly, respectively. Shannon Weiner diversity index was calculated based on species richness, dominance and evenness (Kim *et al*., 2017). The Shannon Weiner diversity was calculated as 1.366 and 2.101 for Guppy and Molly, respectively. Knowing the bacterial diversity in infected fishes will help to improve disease treatment methods.

Molecular identification of bacterial isolates

16SrRNA sequences of recovered isolates were compared with NCBI databases using BLAST and the sequences were identified as *Enterobacter cloacae*, *Aeromonas veronii*, *A. hydrophila* and *Enterococcus faecalis* in Guppy; *A. dhakensis*, *B. subtilis*, *E. faecalis*, *Kurthia gibsonii*, *Comamonas testosteroni*, *A. sobria*, *Edwardsiella tarda*, *E. cloacae* and *Klebsiella aerogenes* in Molly. The diversity of bacterial isolates derived from infected fish samples are depicted in Fig 1. The 16SrRNA sequences were submitted to GenBank and the accession numbers were given in Table 1. Most of the isolates derived from Guppy and Molly are gram-negative. It was reported that most of the infections caused in aquaculture are mainly by gram-negative bacteria, while some diseases were caused by gram-positive microbes such as *Streptococcus* sp. and *Staphylococcus* sp. (Lewbart, 2001). In the present study, enteric bacteria such as *E. cloacae, K. aerogenes* and *E. tarda* were reported in Guppy and Molly. Human enteric pathogens such as *E. cloacae* could lead to severe mortality in cultured fishes.

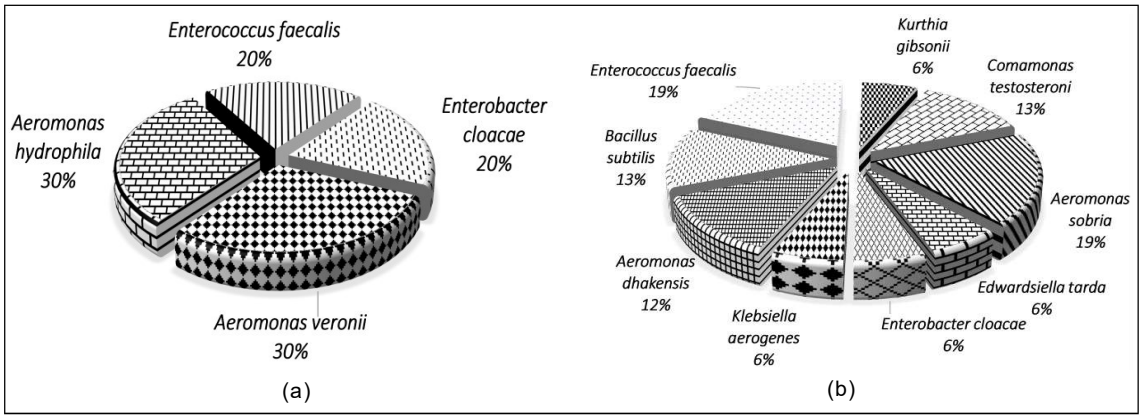


Fig 1: Diversity of bacterial isolates derived from infected fishes. A) Guppy; B) Molly.

Table 1: MAR index and resistant antibiotics of bacterial strains isolated from infected fishes.

Fish sample	Bacterial species	Accession number	Recovered isolates	Resistant antibiotics	MAR index
Guppy	*Enterobacter cloacae*	OL454678	2	Ampicillin, Cefazolin, Aztreonam, Gentamycin, Erythromycin, Azithromycin, Vancomycin, Polymyxin-B and Rifampicin	0.25
	Aeromonas veronii	OL454679	3	Amoxicillin, Ampicillin, Piperacillin, Amoxyclav, Cefalexin, Ceftazidime, Trimethoprim, Sulphadiazine, Nalidixic acid, Furazolidone, Nitrofurantoin, Vancomycin, Bacitracin and Rifampicin	0.39
	Aeromonas hydrophila	OL454680	3	Ampicillin, Piperacillin, Furazolidone, Vancomycin and Bacitracin	0.14
	Enterococcus faecalis	OL454682	2	Cefalexin, Cefoxitin, Cefixime/clavulanic acid, Ceftazidime, Cefoperazone, Cefepime, Aztreonam, Kanamycin, Streptomycin, Nalidixic acid, Polymyxin-B and Rifampicin	0.34
Molly	*Aeromonas dhakensis*	OL454683	2	Amoxicillin, Ampicillin, Cefazolin, Cephalothin, Furazolidone and Bacitracin	0.17
	Bacillus subtilis	OL454685	2	Amoxicillin, Ampicillin, Penicillin-G, Amoxyclav, Cephalothin, Cefixime/clavulanic acid, Cefotaxime, Ceftriaxone, Cefepime, Aztreonam, Trimethoprim, Sulphadiazine and Bacitracin	0.37
	Enterococcus faecalis	OL454686	3	Cefalexin, Cefoxitin, Cefixime/clavulanic acid, Ceftazidime, Ceftriaxone, Cefoperazone, Cefepime, Aztreonam, Kanamycin, Streptomycin, Nalidixic acid, Polymyxin-B and Rifampicin	0.37
	Kurthia gibsonii	OL454687	1	Trimethoprim, Sulphadiazine and Furazolidone	0.09
	Comamonas testosteroni	OL454688	2	Ampicillin, Cephalothin, Cefoperazone, Aztreonam, Streptomycin, Trimethoprim, Sulphadiazine, Azithromycin, Furazolidone Nitrofurantoin, Vancomycin, Bacitracin and Rifampicin	0.37
	Edwardsiella tarda	OL454689	1	Ceftazidime, Azithromycin, Vancomycin, Bacitracin and Rifampicin	0.14
	Enterobacter cloacae	OL454690	1	Cephalothin, Cefoxitin, Cefoperazone, Streptomycin, Gentamycin, Pefloxacin, Vancomycin and Rifampicin	0.23
	Klebsiella aerogenes	OL454691	1	Amoxicillin, Ampicillin, Piperacillin, Amoxyclav, Cefazolin, Cephalothin, Cefalexin, Cefoperazone, Cefepime, Trimethoprim, Sulphadiazine, Erythromycin, Azithromycin, Nalidixic acid, Vancomycin and Rifampicin	0.45
	Aeromonas sobria	OL454692	3	Amoxicillin, Ampicillin, Chloramphenicol, Trimethoprim, Sulphadiazine, Furazolidone and Bacitracin	0.2

The source of these pathogens may be fecal contamination, which poses a high health risk to humans and cultured animals (Gufe *et al*. 2019). The presence of enteric bacteria in the current study might be due to the fecal matter of birds and animals.

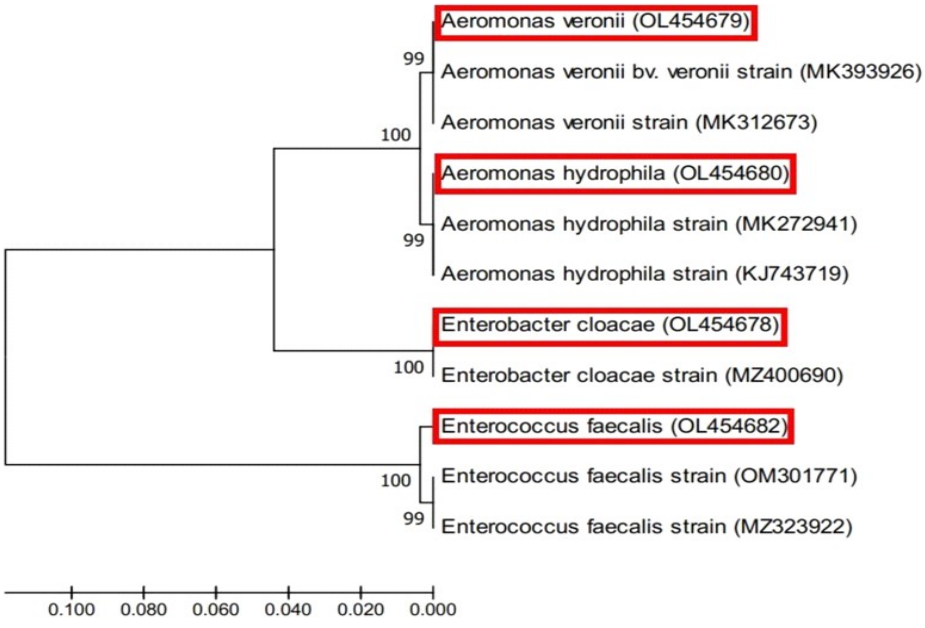
A wide range of diseases in aquaculture is caused by *Aeromonas* sp., including *A. hydrophila*, *A. veronii* and *A. caviae* (Lewbart, 2001). Aeromonas infection in ornamental and food fishes significantly threatens human health. The first report of *A. dhakensis* pathogenicity in Nile tilapia was reported by Soto-Rodriguez *et al*. (2013). Gram-positive bacteria, *B. subtilis* was isolated in this study. *K. gibsonii* was isolated from infected Molly; although this bacterium is not the main pathogenic bacteria, it can enter fish via skin ulceration and may aggravate the host's condition. No information was available to us about the pathogenicity of *C. testosteroni* in fish. However, it can cause human diseases (Tsui *et al*., 2011).

Phylogenetic trees of resolved isolates were constructed using MEGA 7.0 software. It was revealed that the cladogram consisted of 4 and 9 operational taxonomic units (OTUs) with the corresponding bacterial strains from GenBank for the bacterial isolates derived from Guppy and Molly, respectively. The distance coefficient of phylogenetic trees ranged from 0.10 to 0.0 for Guppy and 0.12 to 0.0 for Molly. The phylogenetic tree generated for all the bacterial isolates from Guppy and Molly mainly consists of two branches. In Guppy, two branches were separated at the distance coefficient of 0.12r (Fig 2). In Molly, branch 1, containing 6 clusters, was separated from branch 2, having 3 clusters at the distance coefficient of 0.140r (Fig 3). It was found that the mean distance between the 16SrRNA gene sequences of Guppy and Molly isolates was calculated as 0.02.

Antimicrobial susceptibility testing

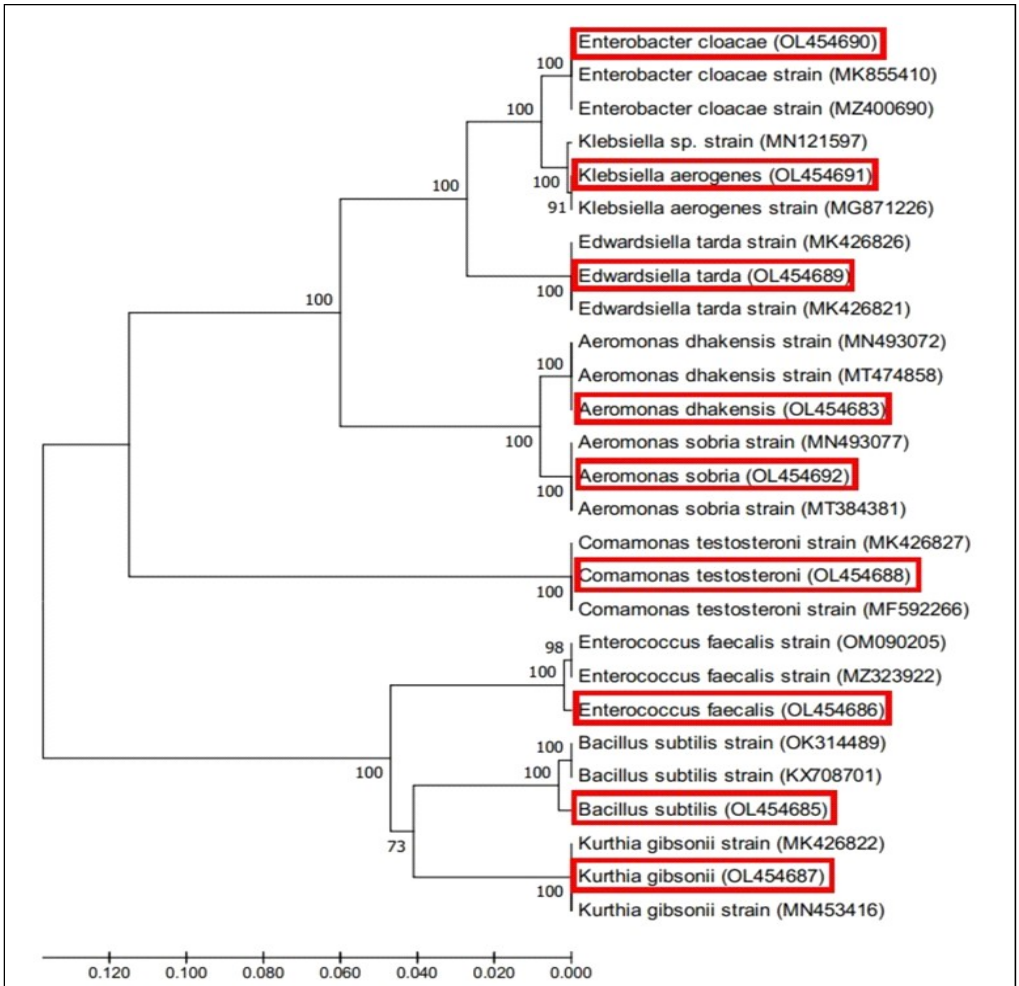
Antibiogram profiling of bacterial isolates was performed using 36 antibiotics. Bacterial isolates from Guppy were resistant to minimum of 5 and maximum of 14 antibiotics, isolates from Molly were resistant to minimum of 3 and maximum of 16 antibiotics tested. *A. veronii* (0.39) isolated from infected Guppy and *K. aerogenes* (0.45) from Molly has the highest MAR index. *K. aerogenes* isolated from moribund goldfish collected from ornamental fish farms in Kerala and Tamil Nadu have a higher MAR index (0.67) (Preena *et al*. 2020). MAR index, resistant antibiotic and respective antibiotic classes are listed in Table 1. The MAR index of >0.2 indicates a higher risk of AMR and antibiotic contamination in aquaculture systems (Krumperman, 1983). In the present study, all the isolates possessed a MAR index >0.2 except *A. hydrophila, A. dhakensis, K. gibsonii* and *E. tarda*. This highlights the heavy usage of antibiotics and the occurrence of AMR pathogens in the ornamental fish culture system. Similar to the present study, the highest MAR profiles have been detected by Preena *et al*. (2020) in goldfish.

All the isolates from Guppy were resistant to at least one antibiotic from cephalosporin 1st generation and polypeptides class. More than 50% of isolates from Guppy were resistant to at least one antibiotic of penicillin, cephalosporin 3rd generation, monobactam, aminoglycoside,



The UPGMA tree constructed were computed using Kimura 2- parameter substitution model with 1000 bootstrap replications.

Fig 2: Phylogenetic analysis of the 16SrRNA gene sequences from Guppy isolates (highlighted) using MEGA 7.0 software.



The UPGMA tree constructed were computed using Kimura 2- parameter substitution model with 1000 bootstrap replications.

Fig 3: Phylogenetic analysis of the 16SrRNA gene sequences from Molly isolates (highlighted) using MEGA 7.0 software.

sulphonamides, quinolone, nitrofuran, glycopeptides and rifamycin class. In contrast, all the isolates were susceptible to phenicol, macrolides and fluoroquinolones class antibiotics. In Molly, the highest resistance (78%) was recorded in the polypeptide class and more than 50% of isolates were resistant to minimum of one antibiotic from penicillin, cephalosporin 1st and 3rd generation, sulphonamide and rifamycin class. The percentage of antimicrobial resistance exhibited by bacterial isolates toward different antibiotic classes is depicted in Fig 4.

All the isolates were susceptible to sulphafurazole, enrofloxacin, norfloxacin and ciprofloxacin. Nalidixic acid from the quinolone class was ineffective against >20% of isolates from Guppy and Molly. Contrary results were obtained by Preena *et al*. (2020), where nalidixic acid was ineffective against almost 62% of the tested isolates. The percentage of antibiotic resistant isolates against 36 antibiotics tested are listed in Table 2. *E. faecalis* from Guppy, *B. subtilis, E. faecalis* and *K. aerogenes* from Molly have exhibited resistance against 4th generation cephalosporin antibiotic cefepime. Similar to our results, *K. aerogenes* isolated from infected goldfish also exhibited resistance to fourth-generation cephalosporin, cefepime (Preena *et al*., 2020). The production of extended spectrum beta-lactamases in fish pathogens results in the resistance toward new generation cephalosporins, making it difficult to control the diseases (Verner-Jeffreys *et al*., 2009). Thus, the ineffectiveness of new generation antibiotics towards the fish pathogens could raise major challenges in the aquaculture sector. The emergence of AMR towards new generation antibiotics such as cephalosporins increases the risk and forces the development of better alternatives to antibiotics.

In Guppy, *E. faecalis* was susceptible to all the penicillin antibiotics. *A. hydrophila* was susceptible to all the antibiotics tested in cephalosporin 1st, 2nd, 3rd, 4th generation, monobactam, aminoglycoside, phenicol, sulphonamide, macrolide, quinolone, fluoroquinolone and rifamycin group. *A. hydrophila* is the most common opportunistic pathogen in aquaculture and is associated with water quality-related

Table 2: Percentage of antibiotic resistant isolates against 36 antibiotics tested.

Antimicrobials	Disc content (µg)	% of resistant isolates	
		Guppy (no. of isolates tested-10)	Molly (no. of isolates tested-16)
		Penicillin	
Amoxicillin	25 µg	25	45
Ampicillin	10 µg	75	56
Penicillin-G	10 units	0	12
Piperacillin	100 µg	50	12
Amoxyclav	30 µg	25	23
		Cephalosporin	
		Cephalosporin 1st generation	
Cefazolin	30 µg	25	23
Cephalothin	30 µg	0	56
Cefalexin	30 µg	50	23
		Cephalosporin 2nd generation	
Cefoxitin	30 µg	25	23
		Cephalosporin 3rd generation	
Cefixime/clavulanic acid	5/30 µg	25	23
Ceftazidime	30 µg	50	23
Cefotaxime	5 µg	0	12
Ceftriaxone	30 µg	0	23
Cefoperazone	75 µg	0	45
		Cephalosporin 4th generation	
Cefepime	30 µg	25	34
		Monobactam	
Aztreonam	30 µg	50	34
		Aminoglycosides	
Kanamycin	30 µg	25	12
Streptomycin	10 µg	25	34
Gentamycin	10 µg	25	12
		Phenicols	
Chloramphenicol	30 µg	0	12
		Sulphonamides	
Trimethoprim	5 µg	25	56
Sulphadiazine	100 µg	25	56
Sulphafurazole	5 µg	0	0
		Macrolides	
Erythromycin	15 µg	25	12
Azithromycin	30 µg	25	34
		Quinolones	
Nalidixic acid	30 µg	25	23
		Fluoroquinolones	
Enrofloxacin	10 µg	0	0
Pefloxacin	5 µg	0	12
Norfloxacin	10 µg	0	0
Ciprofloxacin	30 µg	0	0
		Nitrofurans	
Furazolidone	100 µg	50	45
Nitrofurantoin	100 µg	25	12
		Glycopeptides	
Vancomycin	30 µg	75	45
		Polypeptides	
Polymyxin-B	300 units	50	12
Bacitracin	10 µg	50	56
		Rifamycins	
Rifampicin	5 µg	75	56

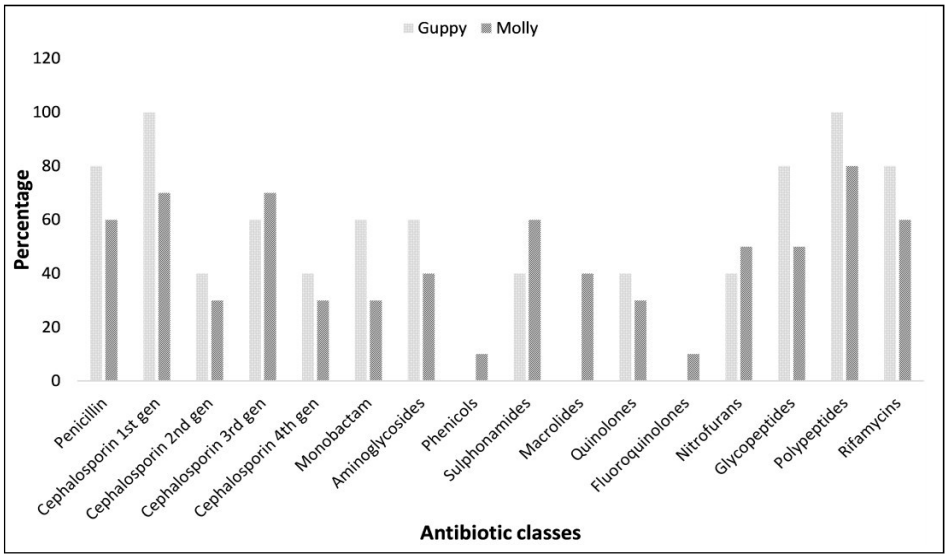
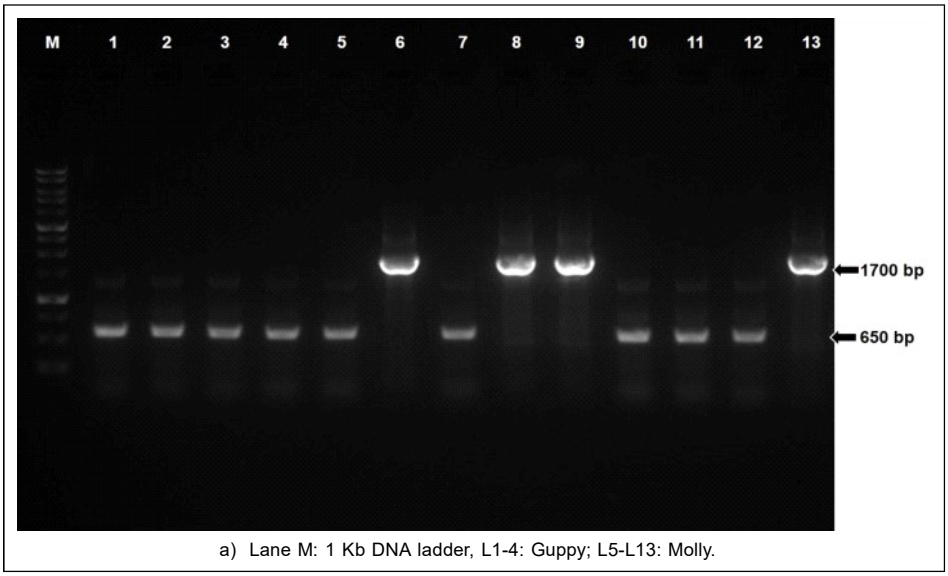


Fig 4: Percentage of antimicrobial resistance towards different antibiotic classes.



a) Lane M: 1 Kb DNA ladder, L1-4: Guppy; L5-L13: Molly.

Fig 5: Agarose gel electrophoresis of Class 1 integron.

diseases in cultured fish. *E. cloacae* was sensitive against the antibiotics belonging to cephalosporin 2nd, 3rd and 4th generation, phenicol, sulphonamide, quinolone, fluoroquinolone and nitrofuran class, whereas antibiotics tested from monobactam, macrolides, glycopeptides and rifamycin exhibited resistance. All the bacterial isolates derived from Guppy were sensitive to all the antibiotics of the fluoroquinolone group. In Molly, *E. faecalis, K. gibsonii, E. tarda, E. cloacae* exhibited susceptibility towards all the antibiotics in the penicillin group. *K. aerogenes* exhibited resistance and *K. gibsonii*, *A. sobria* and *E. tarda* exhibited susceptibility towards all the antibiotics belonging to the cephalosporin 1st generation class. The MAR index of E. *tarda* was minimal (Aoki and Kitao, 1981).

A. dhakensis, *K. gibsonii* and *A. sobria* were susceptible to all the antibiotics belonging to the cephalosporin 3rd and 4th generation class. *A. dhakensis, B. subtilis, K. gibsonii, A. sobria, E. tarda* and *K. aerogenes* were susceptible to all the antibiotics belonging to the aminoglycoside class. *A. dhakensis, E. faecalis, E. tarda* and *E. cloacae* were susceptible to all the antibiotics in the sulphonamide group. *A. dhakensis, B. subtilis, E. faecalis, K. gibsonii, A. sobria* and *E. cloacae* were susceptible and *K. aerogenes* exhibited resistance to antibiotics of macrolides. Except for *E. cloacae,* all the other isolates were sensitive to fluoroquinolone antibiotics. *B. subtilis, E. faecalis, E. tarda*, *E. cloacae* and *K. aerogenes* were susceptible and *C. testosteroni* were resistant to all nitrofuran antibiotics. *K. gibsonii, E. cloacae* and *K. aerogenes* were susceptible to all the antibiotics from the polypeptide group. Thus, this study provides information about AMR in the aquaculture system, which helps to formulate alternative measures to control the disease in the aquaculture system.

Screening of Integron gene cassettes

In this study, class 1 integron was detected from all the bacterial isolates derived from Guppy and Molly. Two different product sizes of class 1 integron (650 bp and 1700 bp) were detected (Fig 5). Similarly, class 1 integrons were detected in respective bacterial plasmid DNA. Meanwhile, class 2 integron was not detected in the genomic and plasmid DNA of all the isolates from Guppy and Molly. Similar results were reported in fish pathogens isolated from infected Guppy collected from an ornamental fish farm, Cochin (Preena *et al*., 2019a). It was reported that the prevalence of class 1 integron in aquaculture systems is higher than class 2 and class 3 integron (Stalder *et al*., 2012). Both plasmid and genomic DNA were found to have class 1 integron indicating the AMR genes are plasmid-borne. The presence of AMR genes in mobile genetic elements indicated the risk of gaining new resistant determinants from other species and enabled potential gene transfer to other clinically important pathogens via horizontal gene transfer (Jacobs and Chenia, 2007). The AMR genes integrated into gene cassettes may be detected by further molecular characterization. However, integrons with empty gene cassettes were also detected in *Y. ruckeri* (Balta *et al*., 2010) isolated from rainbow trout. Hence, further molecular characterization of integron is necessary for confirmation.

CONCLUSION

The present study was conducted to isolate AMR fish pathogens from infected Guppy and Molly. ABST of resolved isolates revealed that most fish pathogens exhibited a MAR index of >0.2, indicating the heavy usage of antibiotics in aquaculture systems of Tamil Nadu and increases the risk of AMR in fish and human health. Some of the isolates from all the three fishes exhibited resistance to new generation antibiotic cefepime, which reminds the need for surveillance and continuous monitoring programs. Meanwhile, all the recovered isolates were susceptible to sulphafurazole, enrofloxacin, norfloxacin and ciprofloxacin. Based on the results, these antibiotics can be used in the ornamental fish culture system. The presence of integron gene cassettes in both genomic and plasmid DNA increases the risk of AMR gene dissemination to clinically important bacteria *via* gene transfer. Our study provides baseline data on the AMR level in fish pathogens from ornamental fish, which will be essential to mitigate the potential risks to human and fish health.

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Conflict of interest

No conflict of interest was declared by the authors.

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