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Molecular Characterization and Antibiotic Resistance Patterns of *Vibrio Cholerae* isolated from ornamental Gold Fish (*Carassius Auratus*)

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Abstract

The main objective of this study was to determine the presence and density as well as the antibiotic profile of *Vibrio cholerae* bacterium isolated from ornamental gold fish (*Carassius auratus*). A total of 175 ornamental gold fish (*Carassius auratus*) were analyzed for the presence of V. cholera. Infected and moribund gold fishes were collected during the period between January 2020 and October 2020 at a local breeding farm in Chennai, Tamilnadu. The obtained samples were confirmed by polymerase chain reaction-plating on TCBS agar methods, *V. cholerae* was detected in 55 samples and *V. cholerae* O139 was detected in 14 samples, with a density ranging between <3.5 to 85.0 MPN/g and <3.5 to 11.3 MPN/g respectively. The isolated *V. cholerae* was further subjected to antibiotic susceptibility test in Mueller-Hinton agar using disc diffusion method of 10 different antibiotics and the results interpreted as per the Clinical and Laboratory Standards Institute guidelines. All the *V. cholerae* isolates evinces highly resistant to Penicillin, Amoxyclav, Kanamycin and Cefotaxime when compared with other antibiotics used in this study, The MAR index values of 0.2 to 1.0 indicates that the isolates were exposed to high risk sources in the environment. This study recommends aquarium fish may disseminate the bacteria in the aquatic environment and may transfer it to water birds that consume them. Therefore, aquarium fishes are the reservoirs of *V. cholerae* and may play a major role in its global dissemination.

Keywords: *Vibrio cholera*; Ornamental gold fish; Molecular characterization; Antibiotic resistance; Public health.

Introduction

Vibrio cholerae, the causative agent of cholera, is a natural inhabitant of aquatic environments. Only two serogroups of *V. cholerae* O1 and O139 are known to cause epidemics and pandemics [1]. Vibrio infection is the most prevalent bacterial disease in ornamental fishes that leads to extensive economic loss. The main clinical signs of infected fishes evinces dropsy, exophthalmia, scale detachment and haemorrhages on the body surfaces [2]. Besides several reports of pathogenic *Vibrio spp.* in seafoods, to date few studies have characterize the occurrence, molecular assay and antibiotic resistance patterns in ornamental fishes [3,4].

In India, the demand for ornamental fish has increased manifold because of the increasing popularity of aquariums. Ornamental fish sector generates huge potential for female employment in the ancillary industry of aquarium decoration, and providing other accessories, as well as medicines. Ornamental goldfishes (*Carassius auratus*) are the most cheerful among other fishes and also the highest preference among hobbyists, hence its breeding dominates among the Indian Ornamental Fish Sector [5].

Zago et al. reported, the potentially pathogenic and zoonotic risks caused by V. cholera of both non O1/non O139 isolated from ornamental fishes mainly originated from Southeastern region of Italy, Asia between 2000 and 2015. V. cholera bacteria were recovered from 104 septicemic goldfish in Australia [6,7]. Other countries such as Japan and Iran, [8,9] isolated *V. cholerae* of non-O1/O139 from diseased ornamental fishes internal organs of ayu (*Plecoglossus altivelis*) and guppy fish (*Poecilia reticulate*). Similarly *Vibrio cholera* O1 was detected by using conventional molecular approaches of aquarium water containing ornamental goldfish aquarium shops in Rhode Island [10]. Ashok Kumar et al. [11] demonstrated, the *V. cholerae* non-O1 and non-O139 in fresh water sediments by using the most probable number (MPN) method. Epidemiological studies evinces, fish carry the cholerae bacteria from one place to another, eventually, water birds feed on the fish, V. cholera may transfer in some water bird species digestive tracts and that leads to global spread [12].

Worldwide, V. cholera considered as virulent pathogen and possibly transmitted among aquarists in countries which are outside cholerae endemic areas [13]. Antibiotics plays crucial role in decreasing illness and death associated with bacterial infections in humans and animals. Recently, usage of antibiotics in aquaculture and companion animals is restricted in many countries [14]. In India, ornamental fishes are frequently subjected to various treatments without a veterinary prescription, treatments includes the use of number of antibiotics applied inappropriately [15,16]. Bacterial infection in fishes is occasionally chronic, it may need months of antibiotic treatment, if effective at all, and this would easily induce resistant genes.

According to [17] research reports, antibiotics are easily available via the five gram regulation, that would be recommended, provided all antibiotics be solely on prescription via a veterinarian, to avoid selection of resistance. Moreover, the presence of antibiotic resistance of enteric

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bacteria in ornamental fish needs careful interpretation. Nevertheless, Aquarium water, with or without antimicrobials in it are drained into surface water or sewage plants, which are ideal sites for exchange of antimicrobial resistance genes, because there is a constant influx of enteric bacteria of large numbers are present and in close contact with each other. Therefore, considering these factors, the present study is designed to continuous monitor the characteristics of frequently occur pathogenic *V. cholerae* strains in ornamental fishes with multiple antibiotic resistance patterns that become significant to protect and promote the global public health.

Materials and Methods

Sample collection

A total of 175 infected and moribund ornamental gold fish (*Carassius auratus*) were collected in sterile polythene bag brought to the laboratory in an ice box within 2 hours. Aseptic procedures were strictly followed during collection, transportation and analysis of the samples.

Most probable number (MPN) procedure

Most probable number (MPN) procedure was followed the US FDA Bacteriological Analytical Manual (BAM) was employed with some modifications [18]. Briefly, 10 gms portion of flesh along with the gut sample were homogenized with 225 ml of Alkaline Peptone Water (APW) with 3% NaCl in a sterile polythene bag (Hi-Media, Mumbai) for one minute, then pre-enriched mixture was diluted tenfold for three successive times and maintained at room temperature for 18 to 24 hours prior to MPN analysis. For the MPN analysis, about one ml of each dilution tube was transferred into three tubes set containing 9 ml of APW and further incubated at 37°C for 18 to 24 hours. After the incubation period, the turbid portion was streaked onto Thiosulphate Citrate Bile salt Sucrose (TCBS) agar (Hi-Media, Mumbai). After the incubation in APW broth, a loopful of enrichment broth was aseptically streaked onto sterile surface of dried Thiosulphate Citrate Bile salt Sucrose (TCBS) agar. The presumptive flat yellow colonies (Figure 1) appeared on TCBS agar plates were picked and further confirmed by Polymerase Chain Reaction (PCR).

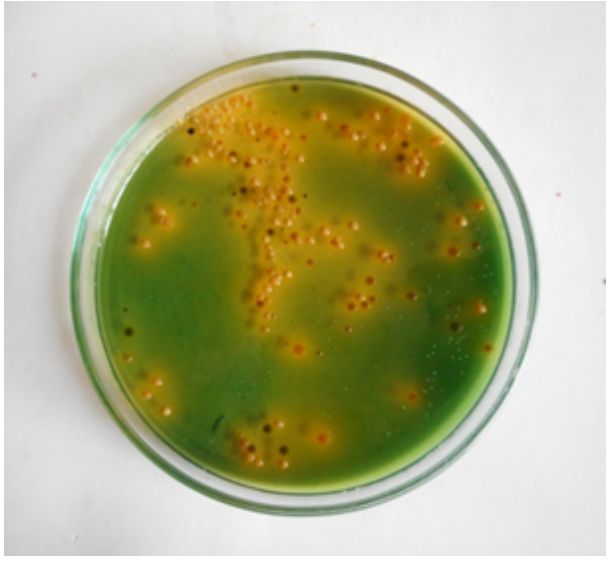

Figure 1: *Vibrio cholerae* in TCBS

Genomic DNA extraction and PCR assay

The isolated single colony was transferred to 2 ml of Tryptic Soy Broth (TSB) with 1% sodium chloride (NaCl) and incubated overnight in an incubator shaker. A one ml portion of the cultures was centrifuged at 15,000 x g for 1 min. Further the pellet was re-suspended in 500 µl sterile distilled water and vortexed vigorously. The obtained cell suspension was boiled for 10 min and chilled immediately on ice for 10 min and centrifuged again at 15,000 x g for 1 min. Subsequently, the obtained supernatant crude DNA was transferred into a new 1.5 ml tube and 5 µl was used as DNA template in PCR, using the specific primer pairs and the PCR conditions as described by [19,20]. The PCR product was checked for purity by electrophoresis on 2% agarose gel, stained with 0.3 µg/ml ethidium bromides for visualization of the amplicons under UV light gel documentation system.

Antibiotic Susceptibility Test (AST)

The susceptibilities of 50 *V. cholerae* isolates to antibiotics were determined via disc diffusion method [21] as per the CLSI guidelines. Briefly, the colony was directly suspended into 1 ml of normal saline inoculum of 0.85% NaCl, and adjusted to 0.5 McFarland turbidity standards. The inoculum was swabbed evenly on Mueller– Hinton (MH) agar plate (Hi-Media, Mumbai) using sterile cotton swab and allowed to dry for 3–5 min at 37°C. Antibiotic discs were arranged accordingly onto the plate, and incubated at 37°C overnight. The inhibition zone was measured, and the results were interpreted based on the CLSI recommendation M45-2A. Ten selective antibiotic discs from (Hi-Media, Mumbai) were used for the susceptibility test viz., Amoxyclav (Amc) 30 µg, Penicillin (P) 10 µg, Cefotaxime (Cef) 30 µg, Chloramphenicol (C) 30 µg, Erythromycin (E) 15 µg, Gentamicin (G) 10 µg, Kanamycin (K) 30 µg, Norfloxacin (N) 10 µg, Streptomycin (S) 10 µg and Doxycycline (Do) 30 µg.

Determination of MAR index

The multiple antibiotic resistances (MAR) index for single isolates followed the procedure as described by [22] in which a total number of antibiotics to which isolates are resistant to (a) is divided by the total number of the antibiotics used in the study (b). The calculating formula is MAR Index=a/b.

Results

In this study, a total of 175 goldfish (infected and moribund) were investigated for the presence of V. cholera, fifty five samples was positive for *V. cholerae* and 14 samples were positive for *V. cholerae* O139 strains. The obtained positive samples of *V. cholerae* and V. cholera O139 strains evinces the density ranged between <3.5 to 85.0 MPN/g and <3.5 to 11.3 MPN/g, respectively. Fifty five *V. cholerae* strains were randomly picked from 37 positive samples were tested for their susceptibility to all 10 selected antibiotics. However, the 14 positive samples was detected using PCR assay of the MPN turbid tubes, but unable to pick any of the *V. cholerae* O139 isolates on the TCBS agar. Fifty five isolates of *V. cholerae* were found to be resistant to Penicillin, Amoxyclav, Kanamycin and Cefotaxime, with 13 isolates from different samples showing resistance to Penicillin and Cefotaxime antibiotics tested and 38 antibiotic resistance patterns (Table 1). Otherwise the isolates were sensitive to Doxycycline.

Antibiotic resistant patterns	Multiple antibiotic resistance index (MAR)	No. Isolates
PAmc	0.2	2
KCef	0.2	1
PAmcK	0.2	2
PAmcK	0.2	1
PAmcK	0.2	1
PAmcK	0.2	2
PAmc	0.2	1
PAmc	0.2	1
PAmc	0.2	1
PAmc	0.2	2
KCef	0.2	1
KCef	0.2	1
KCef	0.3	1
KCef	0.3	2
KCef	0.3	1
PAmc	0.3	2
PAmc	0.3	1
PAmc	0.4	1
PAmc	0.4	2
PAmc	0.4	1
PAmc	0.4	1
PAmc	0.4	1
PAmcK	0.5	1
PAmcK	0.5	1
PAmcK	0.5	1
PAmcK	0.5	1
PAmcK	0.5	1
PAmcK	0.6	1
PAmcK	0.6	1
PKCefAmc	0.6	1
PKCefAmc	0.6	1
PKCefAmc	0.6	2
PKCefAmc	0.6	1
PKCefAmc	0.7	1
PKCefAmc	0.7	1
PKCefAmc	0.7	1
PKCefAmc	0.7	1
PKCefAmc	0.2	1
PKCefAmc	0.2	
Gentamicin (Gn) 10µg; Norfloxacin (Nor) 10 µg; Erythromycin (E) 15 µg; Chloramphenicol (C) 30 µg; Streptomycin (S) 10 µg; Amoxyclav (Amc) 30 µg; Penicillin (P) 10 µg; Cefotaxime (Cef) 30 µg; Kanamycin (K) 30µg .		

Table 1: Antibiotic susceptibility patterns and multipleantibiotic resistance index of V.cholerae isolated from goldfish

Discussion

Next to the viral infection, bacterial infections are the most important causes of disease problems in Indian aquaculture [23]. The Gram negative bacteria such as Vibrio infections are very common in ornamental fish settings. *Vibrio spp.* inhabitants in healthy ornamental fish systems which in turn opportunistic or secondary pathogenic invaders that induce mortalities ranging from trivial to 100% [24,25]. However, the etiologic agent of cholerae is *V. cholerae*, an autochthonous to various aquatic environments, in India, studies on the investigation of *V. cholerae* in ornamental fishes are extremely scanty. Similarly, various research conducted by transmission pathway of this vibrio pathogen in marine fish and shellfish, investigation on aquarium fishes contamination was unclear. In our study, the data indicates that the presence of V. cholera and V. cholera O139 isolates in goldfish maintains similarity with the seasonal regularity of cholera epidemics in developing countries. Norshafawati et al. (2017b) reported aquatic environments are more supportive to disease causing bacteria independently of their host than the terrestrial environment, which leads to the pathogen reach high densities among fish population, eventually ingest them or contaminating the fishes through harvesting. Smith et al. reported strains which produce the cholera toxin belonging to the O1 serogroups, but non-O1/O139 serogroups also produce toxins and disease [25]. In this study the selected fish species evinces the presence of targeted bacteria and also the aquarium water contamination, the prevalence of *V. cholerae* bacterium is high. It is beyond this scope of this paper to discuss about the aquatic environment through sewage and other sources of water contamination. Thirty-three different countries had exported seafood/fish on which an alert was reported, the highest alerts were received on fish sent from India. Contamination through sewage is a very common practice around the world and especially in India. Overboard sewage discharge into seafood harvest areas, illegal harvesting from sewage-contaminated waters and sewage runoff from land after heavy rains or flooding are the many ways, (unpublished data) of India First report, Dec-2019. Our study evinces *V. cholerae* isolates with MAR value more than 0.2 indicated samples originating from a high risk source of contamination with potentially hazardous to human health, this is in agreement with recent studies of [26] prevalence, multidrug resistance patterns MAR of Vibrio parahaemolyticus isolated from different types of seafood in Selangor, Malaysia. PCR-based detection targets the specific region of DNA, for identification of bacterial strains and also less labor intensive and much faster than conventional methods. This study in agreement with [27] molecular identification and of *V. cholerae* from marine fishes from local fish market Thanjavur, Tamil Nadu, The antibiogram profile revealed that isolates, all isolates showed multi-drug resistant to Penicillin, Amoxyclav, Kanamycin and Cefotaxime, and they are susceptible to the doxycycline used in this study which is commonly used to treat many different bacterial infections, such as acne, urinary tract infections, intestinal infections, respiratory infections, eye infections, gonorrhoea, chlamydia, syphilis, periodontitis (gum disease). Sandrine Baron et al. reported, antimicrobial susceptibility of 50 environmental isolates of *Vibrio cholerae* non-O1/non-O139 collected in surface waters in Haiti in July 2012, nearly all isolates were sensitive to amoxicillin-clavulanic acid, cefotaxime, ciprofloxacin, and gentamicin,

only doxycycline antibiotic evinces smaller inhibition zone (15 mm) [28]. Roychowdhury et al. also reported tetracycline resistant in *V. cholerae* strains in Kolkata during the year 2005 [29]. There is an agreement between the results that evinces high individual and multiple antibiotics resistance MAR among all examined Vibrio strains [30]. Despite, antibiotics provide the main basis for the therapy of bacterial infections, high genetic variability of microorganisms enables them to rapidly evade the action of antibiotics by developing antibiotic resistance. On the other hand, compared with toxigenic *V. cholerae* O1 and O139 strains isolated over the same period from 1993 to 2009 evinces few toxigenic O1 strains were resistance to ampicillin, chloramphenicol and azithromycin, whereas resistance to these antibiotics was common for toxigenic O139 strains [31]. Julian Davies and Dorothy Davies, [32] reported, man's overuse of antibiotics to exploit every source of resistance genes and every means of horizontal gene transmission to develop multiple mechanisms of resistance. In this study we found the unabsorbed antimicrobials and secreted antimicrobial metabolites in aquatic water and sediments around fish farms. This study supported by [33] stated that even low concentrations of antibiotic from fish excreta leads to major alterations in the biodiversity of the sediments. Monteiro SH et al. have also reported there is a sparse knowledge about the great amount of fish excreta in aquaculture, that containing none digested antibiotics, is able to stimulate the genetic variability and horizontal gene transfer in the sediment around aquaculture. However, there is an urgent need for the discovery of new and novel antimicrobial drugs to effectively eradicate the diseases producing microorganisms; the emergence of AMR requires awareness raising, improving farm management (practices and monitoring), stricter regulation and controls [34].

Conclusion

Our work demonstrates the occurrence; molecular characterization and antibiotic susceptibility pattern of *V. cholerae* isolated from ornamental gold fish species. Molecular assay of the MPN method could be rapid and concise methods for the detection of foodborne bacteria. This study also revealed the development of multidrug resistance except doxycycline (tetracycline-class antibiotics). However, we are unable to pick the *Vibrio cholerae* O139 strain on TCBS agar; their presence indicates a need for quality microbiological surveillance in the ornamental fish industry. Finally, the results of this study indicate over-use of antibiotics and emergence of AMR requires awareness raising, improving farm management (practices and monitoring), stricter regulation and controls. The findings of this study can also serve as baseline information for the antibiotic resistance of *Vibrio cholerae* isolated from ornamental goldfish samples to monitor trends in the future.

Conflicts of Interest

All authors declare to have no conflict of interest.

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