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## Isolation and identification of zoonotic bacteria, *Proteus penneri* from a disease outbreak in goldfish, *Carassius auratus* in Kerala

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### Abstract

Moribund goldfish, *Carassius auratus*, from a local ornamental fish farm with 50% cumulative mortality was sampled with the aim of isolating and detecting the causative agent in Ernakulam, Kerala. Five bacterial species viz., *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Bacillus cereus*, *Aeromonas hydrophila* and *Proteus penneri* were isolated, and identified on the basis of biochemical tests and sequencing of 16S rDNA gene using universal bacterial primers. Challenge experiments with these isolates using healthy goldfish suggested that *P. penneri* induced show similar clinical and pathological states within 5 days of injection and confirmed that *P. penneri* was the causative agent. Kanamycin (30µg/disc), Ciprofloxacin (30µg/disc), Cefixime (5µg/disc) and Enrofloxacin (10µg/disc) are sensitive to the pathogen identified by the antibiotic sensitivity test. This study also assessed the toxic activities of extracellular products from *P. penneri* using *in vitro* assays on CCKF cell line. The affected fish recovered from the infection after treatment with the suggested antibiotics.

**Keywords:** *Proteus penneri*, *Carassius auratus*, Challenge study, *in vitro* assays, Kerala

### 1. Introduction

Ornamental fish farming is becoming very popular in many cities in India viz., Kolkata, Mumbai, Chennai, Kochi etc. It is rapidly growing at the rate of 20% per annum in India [1]. Aquaculture production has been hindered by many factors, including diseases [2] and high stocking density of fishes in ornamental fish ponds increase the number of disease outbreaks. Number of pathogenic bacteria, including *Aeromonas* spp., *Bacillus* spp., *Citrobacter* spp., *Edwardsiella* spp., *Flavobacterium* spp., *Klebsiella* spp., *Proteus* spp., *Providencia* spp., *Serratia* spp., and *Vibrio* spp., in ornamental fish were reported worldwide [3]. Many reports on the bacterial diseases of Indian freshwater fishes have been published earlier [4, 5]. Recently, Kumar *et al.* (2015) reported a mass mortality in ornamental Koi carp due to a bacterial pathogen, *Proteus hauseri*, in India [6]. Among ornamental fishes, *C. auratus*, the goldfish, is common and they are the smallest member of the carp subfamily (*Cyprininae*) including Crucian carps (Genus: *Carassius*) and common carp (*Cyprinus carpio*) [7]. Fish are highly susceptible to a wide variety of bacterial pathogens. Most of them can cause disease and are saprophytic in nature. Generally bacterial fish diseases do not develop easily by exposing a host to an infectious agent [8]. In most cases, the disease occurs by the complex interactions between pathogen and fish under environmental stress that affect the host susceptibility to pathogen and cause disease [9].

Human infections are mainly found in the individual having infected edible fish, but it can be also caused from ornamental fish during inappropriate handling, cleaning aquarium with bare hand, injuries from fish such as fish bite etc. [10]. The term zoonosis refers to the infection acquired from animals [11]. Now a day's aquarium is commonly found in hotels, restaurants, conference centers and offices have been associated with the emergence of infection with human and are mostly reported in indoor ornamental fish lovers [12]. Most common organism causing diseases in human from fishes are *Streptococcus iniae*, *Mycobacterium* spp., *Vibrio alginolyticus*, *Photobacterium damsela*, *Erysipelothrix rhusiopathiae*, *Vibrio vulnificus*, *Vibrio parahaemolyticus* and other vibrios, *Vibrio cholera*, *Aeromonas* spp., *Escherichia coli*, *Staphylococcus aureus*, *Plesiomonas shigelloides*, *Clostridium perfringens*, *Edwardsiella*

*tarda*, *Listeria monocytogenes*, *Salmonellosis*, *Clostridium botulinum*, *Campylobacter jejuni*, *Delftia acidovorans*, *Legionella pneumophila*, *Shigella spp.* etc. The present study is based on the disease outbreak in an ornamental fish farm of goldfish during June - July 2017, with a cumulative mortality 70 - 90% over 10 d time along with the clinical signs such as haemorrhages on the tail, fin, dropsy, etc. The aim of this study is to identify the cause of the disease outbreak in goldfish and the virulence of the pathogens by experimental challenge infection trials. The current study also attempts to recover the moribund goldfish in the farm from this outbreak by assessing the antibacterial sensitivity of selected antibiotics.

## 2. Materials and Methods

### 2.1. Sample Collection

Moribund and diseased goldfishes shown hemorrhage symptoms were collected from a disease outbreak in an ornamental fish farm, Ernakulam, Kerala during June- July 2017. The samples were kept in sterile plastic bags and transported to the laboratory on ice within 3-4 h. Infected tissues viz., Fin, gills, spleen, heart, kidney and muscles of the individual fish were excised for isolation of bacteria. Tissues homogenate were inoculated in peptone broth and incubated overnight at 28 °C.

### 2.2. Isolation of Bacteria

Bacteria were isolated using serial dilution method. 1ml of the sample was serially diluted up to  $10^{-7}$  dilutions using 9ml of distilled water. 100µl of sample from  $10^{-5}$ ,  $10^{-6}$  and  $10^{-7}$  dilutions were spread plated into nutrient agar plates and incubated at room temperature for 24 h. From the plates, colonies with different morphology were selected and subculture.

### 2.3 Identification

#### 2.3.1 Morphological identification

Pure cultures of the isolates were streaked on Nutrient agar plates for colony development and examine for size, shape, colour, structure, margin, optical properties, consistency, elevation and pigmentation for the colony morphology. From that single colony, 3% KOH string test and gram's staining performed for the morphological examination. Motility examined by Hanging drop technique.

#### 2.3.2 Biochemical identification

All the isolates were biochemically identified by performing, Indole, Methyl red, Voges proskauer citrate, Triple sugar iron agar, urease, nitrate, catalase, oxidase, mannitol motility, lactose fermentation and sugar fermentation tests.

#### 2.3.3 Molecular identification

For DNA extraction A loop full of culture was inoculated to an Eppendorf tube containing 500µl solution 1 (0.5M EDTA 2%, 1.0M Tris HCl 5%, 10% SDS 20%) and quickly vortexed after the addition of 7µl of proteinase K, then the mix was incubated at 55 °C in water bath (2 h) and chilled on ice (10 min). Then 250µl solution 2 (NaCl 30%) was added and inverted several times to mix, then chilled on ice (5 min) and 500 µl supernatant was transferred to new Eppendorf tube after the centrifugation at 8000 rpm (15 min) at 4°C. Add twice the volume of cold absolute ethanol and kept overnight for incubation at -20 °C. On the next day, it was centrifuged at 11000 rpm (15 min) at 4 °C, then the supernatant was

removed and the DNA pellet was rinsed in 300µl cold 70% ethanol and centrifuged again at 11000 rpm (10 min) at 4°C. Then the supernatant was removed and allowed to dry, then dissolve in 30µl TE buffer.

A small drop of sample examined in Biospectrometer and record the concentration of DNA in 30µl sample. Made each sample concentration to 100µg/ml by using an equation,

$$\text{Initial volume} = \frac{\text{final volume} \times \text{final concentration}}{\text{Initial concentration}}$$

To make the concentration 100µg/ml, the initial volume is made up to 30µl by adding TE buffer.

#### 2.3.3.1 Polymerase chain reaction

PCR was carried out in thermal cycler (Veriti, Applied Biosystems) employing the 16S rRNA bacterial universal primers [13]. In the present work Universal primer 27F and 1492R were used for amplification. The primer sequences are as follows-

**Forward: 5'AGATTTGATCMTGGCTCAG3'**

**Reverse: 5'CGGTTACCTTGTACGACTT3'**

In a sterile 0.5-ml microfuge tube, mix the contents in the following order: Deionized water (18.5µl), 10x buffer (2.5µl), Template DNA (2µl), dNTPs mix (0.5µl (Fermentas)), Forward primer (0.25µl), Reverse primer (0.25µl) and Taq DNA polymerase (0.5µl (Fermentas)). Then place the tubes in the thermal cycler and Amplify the nucleic acids using the denaturation (95 °C - 3 min, 94 °C-1 min and 30 sec), annealing (54 °C- 40 sec), and polymerization (72 °C-1 min and 30 sec, 72 °C-10 min) for 30 cycles. For preservation the tubes were kept in the refrigerator after the amplification. Following the completion of the reaction, 5µl of the reaction solution was subjected to 1.5% Agarose gel electrophoresis to visualize the DNA fragments.

After then the PCR products were sequenced by Sanger's method at an automated sequencing facility (Scigenom Pvt. Ltd, India). For molecular identification, homology comparison of 16S rRNA sequences of five different bacterial strains was performed using NCBI BLAST (Basic Local Alignment Search Tool) <http://www.ncbi.nlm.nih.gov/>.

#### 2.4 Challenge studies of the bacterial isolate

All fish were maintained in 500L capacity glass aquaria containing 400L water. Continued aeration was provided with an exchange of 50% of water daily, and water temperature of the tanks recorded twice daily and ranged from 26°C to 29°C. The fish were acclimatized before challenge studies for 15 days in the laboratory and were injected intra muscularly into healthy goldfish (n=10, body weight, 15.5g ± 1.4g) for fulfilling Koch's postulates.

For the inoculum preparation a loop full of culture inoculated to 9 ml broth and incubated at room temperature, then serially diluted and  $10^{-5}$  dilution centrifuged at 8000 rpm (10 min). 500µl PBS added after discarding the supernatant and mix well. From that 200µl of sample injected to intraperitoneally (100µl each) to each 5 healthy fishes maintained on five tanks.

Clinical symptoms shown by the fish and death time noted, after then by using sterile forceps and scissors aseptically cut out each organ such as the spleen, liver, kidney and gill and they were inoculated to broth separately and incubated at room temperature (20 °C-25 °C). Serially diluted the

incubated broth and from  $10^{-7}$  and  $10^{-5}$ , about 100 $\mu$ l added to the fresh nutrient agar plate to perform spread plate. After the incubation plates were examined for the bacterial colony and counted. The isolates obtained from the sample identified by biochemically and also by using molecular methods of identification. Infected fin and muscle tissue of diseased fish was examined and bacteria re-isolated after the experimental period. All procedures were carried out aseptically.

## 2.5 Preparation of extra cellular protein (ECP) from *Proteus penneri*

For ECP preparation, *P. penneri* was inoculated to 1000 $\mu$ l nutrient broth and incubated at 28 °C overnight in a shaking incubator. Then the supernatant collected followed by the centrifugation at 8000 rpm (15 min) at 4°C and was filtered by using sterile 0.22  $\mu$ m syringe filter and they preserved at -20°C. On the other hand small amount of these filtrated samples inoculated to nutrient broth and incubated for examining the presence of live bacterial cells.

## 2.6 Cytotoxicity Test of Bacterial Extracellular Products

Preparation of CCKF cells [14] on cover slip was made by treating the carp fin fish cell line monolayer with PBS without Ca<sup>2+</sup>/Mg<sup>2+</sup> using a volume equivalent to half the volume of culture medium. Repeat this wash step for 3-5 times and Pipetted trypsin/EDTA onto the washed cell monolayer using 1ml per 25cm<sup>2</sup> of surface area. Rotated flask to cover the monolayer with trypsin. Then decant the excess trypsin and returned flask to the incubator and leave for 2-10 min. Resuspended the cells in a small volume of fresh serum-containing medium to inactivate the trypsin. Removed 100-

200 $\mu$ l and perform a cell count. Place the sterile cover slip on each well of the multiwell plate by using sterile forceps. Transferred the required number of cells to a well plate, about 200 $\mu$ l and 100 $\mu$ l of protein extract of the sample add on the surface of the cover slip. Rinse the content by using a micropipette and 400 $\mu$ l serum added to each of the well. Keep the sample in low temperature incubator and check the plate under an inverted microscope on each day.

## 2.7 Antibiotic susceptibility test

Antibiotic susceptibility test (ABST) of *P. penneri* was tested by the disc diffusion method using a standard procedure from the guidelines of the Clinical and Laboratory Standards Institute [15]. Antibiotic discs used in this study were procured from HiMedia Laboratories, India. A total of 16 antimicrobial agents viz., Cefixime, Chloramphenicol, Bacitracin, Nitrofurantoin, Azithromycin, Erythromycin, Gentamicin, Kanamycin, Cefalexin, Oxytetracycline, Ciprofloxacin, Amoxycillin, Enrofloxacin, Furazolidone, Ampicillin and Cefixime/Clavulanic acid were tested. The zones of inhibition formed around the discs were measured, and antibiotic sensitivity was assayed from the diameter of the zones (in mm). Zone diameters were interpreted as sensitive, intermediate and resistant using CLSI criteria [16] and criteria set by the manufacturer.

## 3. Results

### 3.1. Isolation and identification of isolates

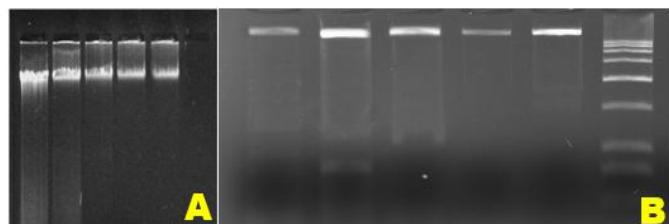
A total of five bacterial isolates (GF1-GF5) were obtained from gold fish by serial dilution and plating method followed by observing their colony characteristics (Table No. 1).

**Table 1:** Colony characteristics of bacterial strains isolated by spread plate method from naturally infected goldfish.

Isolates	Colony characteristics
GF-1	Mucoid, Opaque, White, Small, Round, Entire, Convex colonies.
GF-2	Mucoid, Opaque, Cream coloured, Small, Round, Entire, Raised colonies.
GF-3	Dry, Opaque, Medium sized, Round, Entire, Flat colonies.
GF-4	Moist, Opaque, Creamy, Small, Round, Entire, Flat colonies.
GF-5	Smooth, Opaque, Translucent, Swarming, Flat colonies.

The morphological and biochemical characterization was performed to identify the selected isolate. Biochemical characteristics of the isolates have been summarized in the Table No. 2. As per Bergey's Manual of Determinative Bacteriology (1984) each of the five isolate identified as *Enterobacter*, *Klebsiella*, *Bacillus*, *Aeromonas* and *Proteus*. Among these five isolates *Proteus* constituted 85–90% of the total bacterial flora of samples from individual fish showing clinical signs of disease and remaining isolates only 10-15%. The DNA was isolated from five different bacterial isolates by the mentioned method. The extracted DNA was electrophoresed through 0.8% agarose gel containing ethidium bromide. Approximately 1470 bp of 16S rRNA gene sequences were obtained after assembling the forward and reverse sequences obtained from this goldfish isolate (Fig No. 1A). After then 16S rRNA genes were amplified and checked

in 1.5% agarose gel electrophoresis followed by PCR (Fig No. 1B). The nucleotide sequence of each isolate obtained by Sanger's method were analyzed in BLAST on NCBI for the confirmation and the results are shown in Table No. 3.



**Fig 1:** Molecular identification of five bacteria isolated from diseased goldfish by PCR (A) Genomic DNA isolated from different bacterial isolates; (B) 16S rRNA PCR product in 1.5% Agarose gel.

**Table 2:** Biochemical identification of each bacterial strain isolated from naturally infected fish as per Bergey's Manual of Determinative Bacteriology (1984)

TEST	GF-1	GF-2	GF-3	GF-4	GF-5
KOH mount	+	+	-	+	+
Gram staining	Gram -ve bacilli	Gram -ve bacilli	Gram +ve bacilli	Gram -ve bacilli	Gram -ve bacilli
Motility	Non motile	motile	motile	motile	motile
Indole	-	-	-	+	-
MR	-	-	-	-	+

VP	+	+	-	+	+
Citrate	+	+	+	+	-
Catalase	+	+	+	+	-
Oxidase	-	-	-	+	-
Urease	-	+	-	-	+
Nitrate reduction	+	+	+	+	+
TSI	K/A	A/A	K/A	K/K	A/A with H2S production
Mannitol	-	-	+	+	+
Glucose	AG	AG	+	AG	AG
Lactose	AG	AG	-	+	-
Sucrose	AG	AG	-	+	AG
Maltose	AG	AG	+	-	AG
Identification	<i>Enterobacter</i>	<i>Klebsiella</i>	<i>Bacillus</i>	<i>Aeromonas</i>	<i>Proteus</i>

**Table 3:** Molecular identification of each Bacterial strain (GF-1to GF-5) by using Basic local alignment search tool (BLAST).

Isolates	Organism
GF-1	<i>E. cloacae</i>
GF-2	<i>K. pneumonia</i>
GF-3	<i>B. cereus</i>
GF-4	<i>A. hydrophila</i>
GF-5	<i>P. penneri</i>

### 3.2. Experimental challenged study/ Pathogenicity test

The pathogenicity studies (Table No. 4) using live bacterial cells indicated that the mortality of fish is dose dependent pattern.  $10^5$ cells/ml was observed as lethal dose for *P. penneri*. All the dead fish injected with *P. penneri* displayed several symptoms showed in Fig No. 2.

**Table 4:** Details of the experimental challenge infection of the bacteria isolated from disease goldfish

Bacterial strain	Experimentally infected fish	Mortality time (hours)
<i>P. penneri</i>	Fish 1	15 h
	Fish 2	19 h
	Fish 3	14 h
	Fish 4	13 h
	Fish 5	10 h



A.



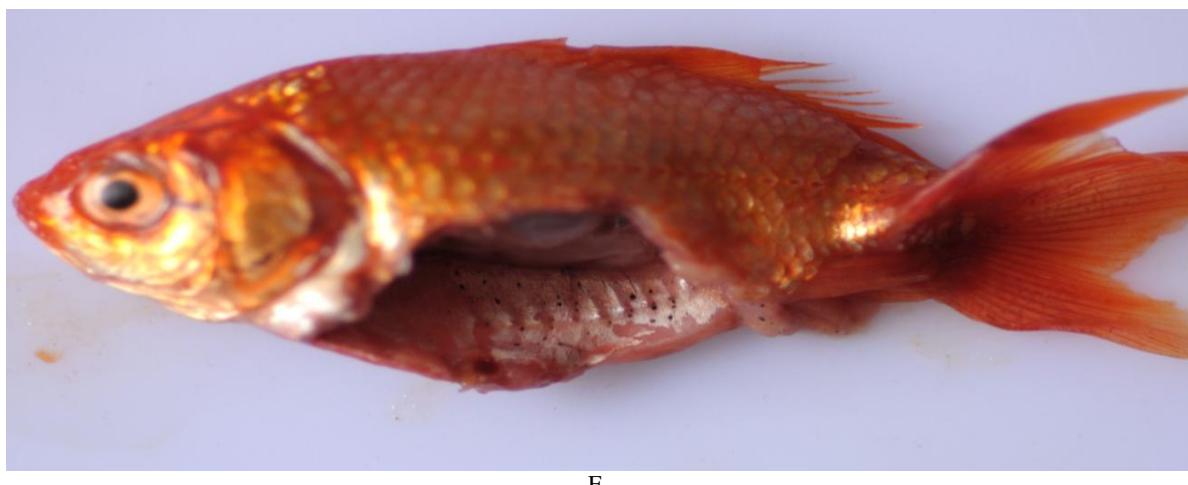
B.



C.



D.



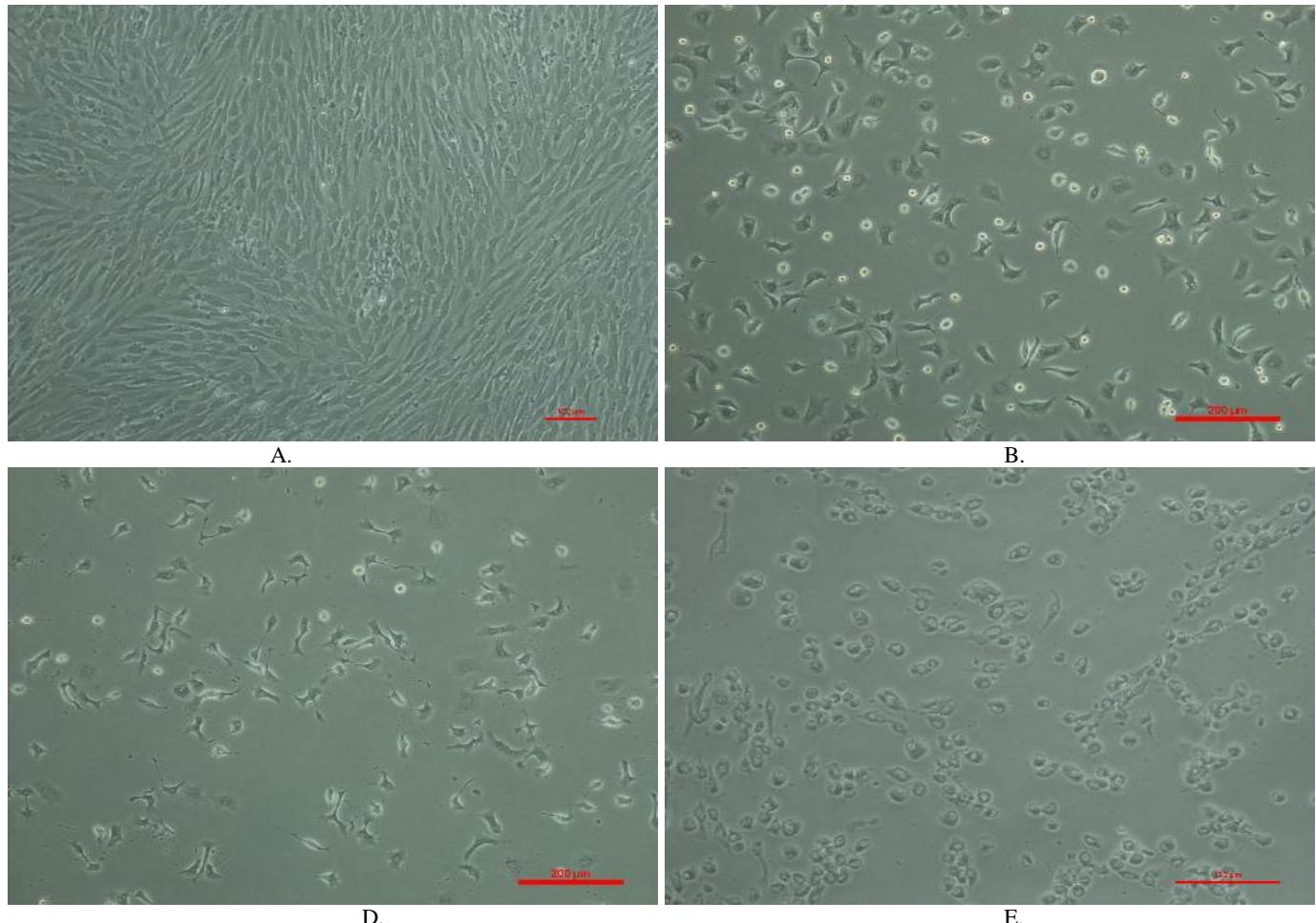
E.

**Fig 2:** Symptoms shown by experimentally infected Gold fish with *P. penneri* A) Pale coloured gills; B) Internal haemorrhage at the caudal fin; C) Congested internal organs; D) Haemorrhagic patches at the base of the pelvic fin; E) Black coloured spotted marks on the abdominal wall.

### 3.3. Cytotoxicity Test

The ECP from *P. penneri* were cytotoxic for the gold fish cell line. Cytotoxic effects were started after 24 h of inoculation of ECPs, then the gold fish cells became rounded, having

serrated edges, degenerative changes were manifested by pyknotic nuclei, shrinking, dendritic elongations and detached from the surface leading to the destruction of the monolayer by 72 h. (Fig No. 3).



**Fig 3:** Cytotoxicity assays of the ECP of *P. penneri* on CCKF cell line. A) Uninoculated CCKF cell line; B) CCKF cells at 1 day post inoculation to ECP of *P. penneri*; C) 2<sup>nd</sup> day; D) 3<sup>rd</sup> day.

### 3.4. Antibiotic-susceptibility test

The results of the agar disc diffusion tests of *P. penneri* to different antimicrobial compounds are presented in Table No. 5 In antibiotic susceptibility testing it was found that the *P.*

*penneri* was sensitive to Kanamycin (30 $\mu$ g/disc), Ciprofloxacin (30 $\mu$ g/disc), Cefixime (5 $\mu$ g/disc) and Enrofloxacin (10 $\mu$ g/disc).

**Table 5:** The zone of inhibition (in mm) by the respective antibiotics against fish pathogen *P. penneri*

Antibiotics with symbols	Zone of inhibition (mm)		
	Resistant	Sensitive	<i>P. penneri</i>
Oxytetracycline O30	≤18	≥25	-
Cephalexin CN30	≤17	≥22	-
Enrofloxacin EX10	≤15	≥21	26mm
Cefixime CFM5	≤15	≥19	16mm
Amoxycillin AMX25	≤13	≥18	-
Ciprofloxacin CIP30	≤15	≥21	31mm
Nitrofurantoin NIT100	≤11	≥15	11 mm
Kanamycin K30	≤13	≥18	17mm
Ampicillin AMP25	≤22	≥30	-
Gentamycin GEN30	≤21	≥25	20mm
Furazolidone FR100	≤20	≥25	-
Erythromycin E15	≤13	≥23	-
Chloramphenicol C25	≤21	≥25	19mm
Furazolidone FR50	≤20	≥25	-
Bacitracin B10	≤12	≥22	-
Azithromycin AZM30	≤24	≥30	11mm

#### 4. Discussion

Sample of diseased fan tail goldfish is collected from a local ornamental fish farm in Ernakulam, Kerala, shows hemorrhage by macroscopic examination. In this study, from each fish, liver, kidney, gills and spleen were separate and inoculated to nutrient broth. By spread plate technique after serial dilution five different isolates were obtained. By biochemical test and genome sequencing, the isolates were identified as *E. cloacae* (GF1), *K. pneumoniae* (GF2), *Bacillus cereus* (GF3), *A. hydrophila* (GF4) and *P. penneri* (GF5). Each of these isolates is already reported as normal flora of fish, but due environmental stresses it can be pathogenic to fish [17]. Among these isolate four of them belong to *Enterobacteriaceae* family, such as, *E. cloacae*, *K. pneumonia*, *A. hydrophila* and *P. penneri* and those considered as an indicator of sewage pollution [18]. Now antibiotic resistant varieties are also reported [19]. *E. cloacae* is also reported as common water-borne bacterium and is also present in the tissue of normal fish [20]. *K. pneumonia* is ubiquitous in nature and it exists as normal flora in the gastrointestinal tract of humans and animals. Mainly gram negative bacteria are reported as pathogenic. *B. cereus* is a gram positive, have inhibitory effect against *Vibrio carchariae* and *A. hydrophila* is reported, and is used as probiotics in fishes [21]. Naturally, it found in the intestinal tract and does not induce any infection. By spread plate technique *P. penneri* found in large number, studies show that *P. penneri* as a causative agent of red body disease in *Penaeus vannamei* (white shrimps), cultivated in China. It is isolated in large number from the each organ by spread plate technique [22]. It's most distinguishable property is the formation of swarming growth [23]. But this result cannot be taken for confirmation of pathogen, as a result challenge study performed.

By challenge study, only *P. penneri* injected fishes only died along with several symptoms. The lethal dose value of the experimentally infected fish is about  $220 \times 10^5$  cfu/ml once and the median lethal time of mortality is estimated to be 12 h. One of the major symptoms is the internal hemorrhage in caudal fin and at the base of the pelvic fin. Additionally gills of the fishes are the primary target of contaminants [24], here it shows a pale colour. The experimental infection assays proved that *P. penneri* (GF-5) was virulent to gold fish and produced disease.

In this study *P. penneri* is identified as pathogen in fishes. But due to direct contact with this diseased fish can develop infection in humans. It is considered as opportunistic pathogenic bacteria in humans, cause diarrheal disease, wound infection, urinary tract infection and they were mainly found in soil, water, and sewage and the majority of them reported in males [25]. The bacterium, which is the causative organism of blotch disease, red spot and spottiness of skin in fish, has been isolated from skin lesions of the Indian major carp [26].

Bacterial ECPs are reported to be produced by pathogenic and non-pathogenic organisms and are lethal when they produced by pathogenic organism. These enzymes while release in the body results in invasion, establishment of infection by providing nutrients for cell proliferation [27] and cytolytic exotoxins considered as an important virulence factors [28]. Here from *P. penneri*, ECP was extracted and are tested on CCKF cell line show cytopathic effect within 5 h, as a result of the production of virulent toxin.

In order to prevent this disease outbreak, treatment should be done as fast possible. Kanamycin, Ciprofloxacin, Cefixime and Enrofloxacin are the effective drug has been identified by antibiotic sensitivity test among the sixteen antibiotics tested. Antibacterial agents are mainly administered as supplementary feed for the treatment of diseased fish. However, due to loss of appetite in the severely affected fish, the antibiotics may not be consumed by the fish. So in this study, we suggested Ciprofloxacin injection, intra muscularly and the fish recovered after 5 days of treatment.

A bacterial pathogen in fish could develop serious health issues in humans by the direct exposure, such as, when cleaning an aquarium with bare hands, exposure to fish tank water, injuries from fish by bite, from spines etc. So there is a possibility for human beings to acquire bacterial infection from the infected ornamental fish while handling them. Regular screening of aquaculture facilities can serve as an effective means of preventing the spread of such pathogens to human being.

#### 5. Conclusion

In the present study, it has been found that *P. penneri* (GF-5) is pathogenic to goldfish and cause typical haemorrhage, leading to death of fish. Injury when cleaning an aquarium with bare hands, exposure to fish tank water, by contact with tropical fish, injuries from fish, e.g. by spines and fish bite, etc are the number of possible routes through which man gets infected by bacterial pathogen from fish. So fish farm workers may acquire bacterial infection from the infected ornamental fish while handling them. Regular screening of aquaculture facilities can serve as an effective means of preventing the spread of such pathogens to human being.

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