Research Article **Microbiology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF BUBBLE EYE GOLD FISH PATHOGENIC AMPICILLIN RESISTANT *BACILLUS* SP. (GF1)

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ABSTRACT

In this study a total number of 15 moribund gold fish (*Carassius auratus*) were collected from the fish farms of Hooghly district, West Bengal, India. The bacterial infection in the eyes of the affected fishes was recorded. Isolation and biochemical characterization of the bacterial isolate was done in laboratory. The bacterial isolate (GF1) was found to be positive for catalase, methyl red, nitrate reduction test, citrate, starch hydrolysis test and negative for urease, voges proskauer, indole, oxidase, gelatin hydrolysis, casein hydrolysis and lipase test. GF1 utilized glucose and sucrose as carbon source. The bacteria could tolerate upto 10% NaCl in the nutrient broth medium. The bacteria were found to be sensitive to the recommended doses of ciprofloxacin, gentamycin, levofloxacin, streptomycin, tetracycline and nalidixic acid. Poly acrylamide gel electrophoresis (PAGE) analysis revealed that the isolate contained 11 discrete bands ranging from 24.853 to 73.941kDa proteins. Phylogenetic analysis of the 16S rRNA gene sequence was also done. Based on the morphological, biochemical and phylogenetic analysis the GF1 isolate was identified as *Bacillus* sp.

KEY WORDS: ***Carassius auratus auratus,*** **infection, eyes, biochemical characterization, bacterial isolate (GF1), poly acrylamide gel electrophoresis (PAGE), phylogenetic analysis, *Bacillus* sp.**

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INTRODUCTION

Bubble eye gold fish (*Carassius auratus*) is one of the most favourite and popular fish among aquarium fishes. It is a dorsal fin less fish, having a clean back and eye bubbles well matched for colour and size. Although aquarium fishes have a good economic importance, they may act as vectors of human pathogens also. Some facultatively pathogenic bacteria have been reported for both fish and man[1]. According to Takyi *et al.*[2], there was no significant difference in both pathogenic and non-pathogenic bacteria occurred in the gill, stomach and liver of fish which indicated that all the organs are susceptible to bacterial infections. The infection source may be fish-food or as a hobby[3]. The prevalence of potentially pathogenic *Vibrio* species in the Malaysian sea food was also observed by Hadi *et al.*[4]. Contamination of hands and surfaces during cleaning and evisceration of fish is a common route for pathogenic infection in humans[5]. Bacterial disease outbreaks impose a significant constraint in fish and shellfish production[6]. There has been a steady increase in the numbers of bacterial species associated with fish diseases[7]. Cloudy eye is a common bacteria-borne disease that may lead to blindness in case of fresh water fishes. Most bacterial pathogens of fish are rod shaped or flagellated[8] .*Aeromonas spp. Bacillus* spp. *Pseudomonas spp.* and *Aerobacter* spp. are the common fish pathogens causing severe diseases. Some human pathogens such as *Aeromonas, Escherichia, Klebsiella, Pseudomonas, Salmonella* and *Vibrio* have been found to survive and multiply in the gut, mucus and tissues of fish and thus render fish a potential vector of human disease over long periods[9]. *Vibrio cholerae* infection has been associated with consumption of raw fish[10] in several countries[11-14]. According to Wedemeyer *et al.*[15], bacterial diseases Outbreaks are influenced by the susceptibility of the host, virulence of the pathogens, and quality of the environment. According to Ahmed *et al.*[16], seasonal variations in pH, temperature and dissolved oxygen play important roles in the multiplication of pathogens thus leading to diseases in fish. The present study was aimed to phenotypic and biochemical characterization of the bacteria isolated from the infected eyes of bubble eye gold fish.

MATERIALS AND METHODS

A total of 15 moribund bubble eye gold fish were collected from some fish-farms of Hooghly district of West Bengal, India. Then the infected fish specimens (Fig1) were acclimatized in fresh water -glass aquarium in the laboratory conditions at 25±1°C and were allowed to feed on commercial fish-food daily and were monitored regularly. Bacteria were isolated from the eyes of the affected fish by sterile loop and plated on nutrient agar (peptone: beef extract: NaCl : agar at 5:3:3:1 g/l) plates and incubated at 30 ± 1^0 C in a BOD incubator for 24 hours to obtain the isolated colonies. Pure culture was maintained on agar slants for further characterization and identification. The shape, size, colour, margin and opacity of the isolated colonies were recorded. Phenotypic and biochemical characterization of the isolate (GF1) was done following the standard microbiological methods[17-19]. To study the bio-chemical properties, catalase, citrate utilization, nitrate reduction, indole production, methyl-red, voges-Proskauer, urease, oxidase, NaCl tolerance and carbohydrate metabolism (acid-gas production) tests were done. For qualitative determination of enzymes, starch hydrolysis, lipase, protein hydrolysis, gelatin hydrolysis, casein hydrolysis tests were done. Sensitivity of the isolate to recommend doses of antibiotics were also studied Brown *et al.*[20]. Smear preparation of bacterial suspension was done on a cover slip and heat-fixed over a flame for 1-2 s, followed by 2.5% glutaraldehyde (aqueous) for 45 min. The cover slips were then dehydrated passing through 50%, 70%, 90% and absolute alcohol for 5 min each. After that the cover slips were passing through the iso-amyl acetate and absolute alcohol solution (1:1) and finally through the iso-amyl acetate. The specimens were gold-coated and observed under a scanning electron microscope. Total protein samples were extracted from the bacterial isolate, described by Bushuk *et al.*[21]. Total protein analysis was carried out by using

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) following the method of Laemli[22].Genomic DNA was isolated from the pure culture pellet using a genomic DNA isolation kit (DNA-Xpress TM Kit, Himedia-MB501). The~1.5 kb rDNA fragment was amplified by Thermal Cycler (ABI2720) using high-fidelity PCR polymerase. The PCR product was sequenced by Genetic Analyzer (ABI 3130 Genetic Analyzer) bidirectionally using the forward and reverse primer (*Bacillus*-specific universal primer). The isolate was identified both phenotypically and on the basis of 16S rRNA gene sequence analysis. Fingerprinting of the nucleotide was done following Lou and Golding[23]**.** The sequence data were aligned using the "ClustalW SubmissionForm"(http://www.ebi.ac.uk/clustalw/) and analyzed by ClustalW[24]**.** Evolutionary distances were calculated using the method of Jukes and Cantor[25] and the phylogenetic tree was prepared following the"neighbor-joining"method[26].

RESULTS

The colony of the bacterial isolate (GF1) was off-white, round with an average diameter of 5.75 mm, opaque and flat (Table 1). The scanning electron micrograph showed that the bacteria were spore-forming, rod shaped (Fig2) The organisms were gram-positive. The bacterial isolate (GF1) showed positive result for catalase, methyl-red, nitrate reduction, citrate utilization, starch hydrolysis, gelatin hydrolysis and negative for urease, voges proskauer, indole, oxidase and lipase test (Table 2). The bacteria were found to be sensitive to the recommended doses of ciprofloxacin, gentamycin, levofloxacin, streptomycin, tetracycline, nalidixic acid, doxycyclin but resistant to ampicilin, chloramphenicol, rifampicin, and vancomycin (Table 2). The bacterial isolate (GF1) could not ferment mannitol and lactose but utilized glucose and sucrose. (Table 2). The bacteria could tolerate upto 10% NaCl present in the nutrient broth medium (Table 2). Based on the morphological, biochemical and phylogenetic analysis, the GF1 isolate was identified as *Bacillus* sp. PAGE analysis revealed that the isolate contained 12 discrete protein bands having the molecular weight of 24.853, 31.909, 35.398, 41.849, 48.336, 52.222, 56.922, 59.598, 61.867, 66.441, 70.853 and 73.941 kDa (Fig3). The restriction map of the 16 S rRNA gene sequence of GF1 was shown in Fig4. The fingerprinting of the 16S rRNA gene sequence has been shown in The fingerprinting of the 16S rRNA gene sequence has been shown in Fig5. The Mol% of A,T, G and C of the 16S rRNA gene was shown in Fig6. The GC content was 53.33% and AT content was found to be 46.67%. The phylogenetic analysis of the 16S rRNA gene sequence revealed that the GF1 isolate branched with all other *Bacillus* spp. in the tree (Fig7). The cluster containing the *Bacillus* sp. GF1 and other *Bacillus* spp. branched with *Bacillus aryabhattai* B8W22 (EF114313.2) and *Bacillus macroides* (AJ628749.1) with 0.02% bootstrap value.

DISCUSSION

Some species of *Bacillus* have been established as bacterial pathogens of fish. *Bacillus cereus*, found to be present on the necrotic gills of common carp *Cyprinus* carpio[27] produces toxins that cause disseminated intravascular coagulation[28,29]**.** *Bacillus mycoides*, established as a bacterial pathogen of channel catfish in Alabama, can cause muscle lesions in fish without interaction with other pathogens[30] **.** Piscine Tuberculosis (caused by *Mycobacterium* sp.), Dropsy (*Aeromonas* sp. and *Pseudomonas* sp.) are the most common bacterial diseases occurred in gold fish[31]. Some strains of *P. aeruginosa* have also been reported as opportunistic pathogens[32,33]**.** Streptococcal diseases were first reported from *Oreochromis* spp. and *Sarotherodon* spp., in Japan[34]**.** Gold fish *C. auratus* is highly susceptible to aeromonads[35]**.** *Aeromonas hydrophila* is responsible for red fin disease, haemorrhagic septicaemia, motile aeromonad septicaemia and other infections in *C. auratus*[36]**.** The production of endotoxins, extracellular enterotoxins, haemolysin, cytotoxins and protease, the ability to adhere the cells, and the possession of certain surface proteins are the virulence factors of *Aeromonas hydrophila* which contribute to their pathogenicity to the fish[37].The bacterial isolate GF1 has the protease enzymes and it may produce some exoproteins which may take part

in the pathogenicity to the gold fish. Amin *et al.*[38] reported the myxobacterial skin lesions and gill-rot in tilapia (*Oreochromis niloticus*) reared in Egypt. The causative organism of bacterial kidney disease was a small Gram-positive bacteria named *Renibacterium salm*oninarum[39] which would be transmitted from fish to fish[40]**.** In the Czech Republic, one of the most commonly diagnosed bacterial diseases of aquarium fish was Mycobacteriosis[41]**.** The prevalence of *Escherichia coli* from fish sold in Cochin, India, was recorded by Thampuran *et al.*[42]. For prevention and cure of different bacterial infections, antibiotic treatment has been proved as an important method. Almost all the Corynebacterium strains were resistant to penicillin, oxolinic acid, nalidixic acid, furazolidone, and nitrofurantoin[43]. Oxytetracycline and erythromycin proved to be useful antibiotics for the control of fish infections by Gram positive bacteria such as *Renibacterium salmoninarum*[44-46]

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Figure1
Bacteria infected gold fish showing blindness due to deformities in eye.

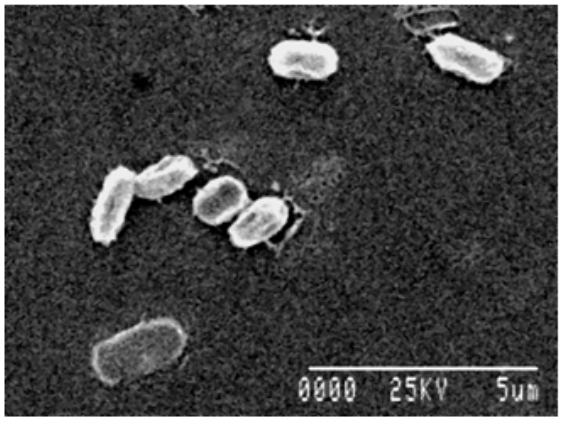

Figure2
scanning electron micrograph of the bacterial isolate GF1.

Table 1
Colony characteristics of GF1

Bacterial isolate	Colour	Shape	Size	Transparency	Elevation
GF1	Off white	Round	5.75 mm	Opaque	Flat

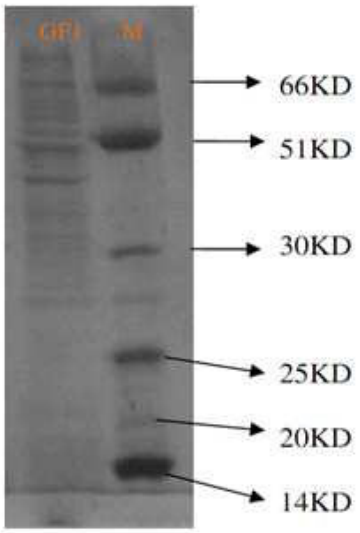

Figure3
SDS-PAGE protein profiling of the bacterial isolate GF1.

Table 2
Biochemical properties of bacterial isolate GF1

Biochemical tests -	**Result**
Catalase	**+**
Indole	**-**
MR	**+**
VP	**-**
Nitrate	**+**
Urease	**-**
Citrate	**+**
Oxidase	**-**
Enzymatic activity test -	
Starch hydrolysis	**+**
Gelatin hydrolysis	**+**
Lipid hydrolysis	**-**
Growth on different media-	
Nutrient agar	**+**
EMB agar	**-**
Mac' Conkey agar	**-**
Carbon source utilisation-	**Gas/Acid**
Glucose	**+ / +**
Lactose	**- / -**
Mannitol	**- / -**
Sucrose	**- / +**
NaCl tolerance-	Upto 10%
Antibiotic sensitivity test-	
Ampicillin (10 mcg)	R
Tetracycline (30 mcg)	S
Streptomycin(10 mcg)	S
Ciprofloxacin (5 mcg)	S
Chloramphenicol (30 mcg)	R
Gentamycin (10 mcg)	S
Levofloxacin (5 mcg)	S
Rifampicin (5 mcg)	R
Nalidixic acid (30 mcg)	S
Doxycyclin (30 mcg)	S
Vancomycin (30 mcg)	R
Ofloxacin (5 mcg)	S
Gatilofloxacin (5 mcg)	S

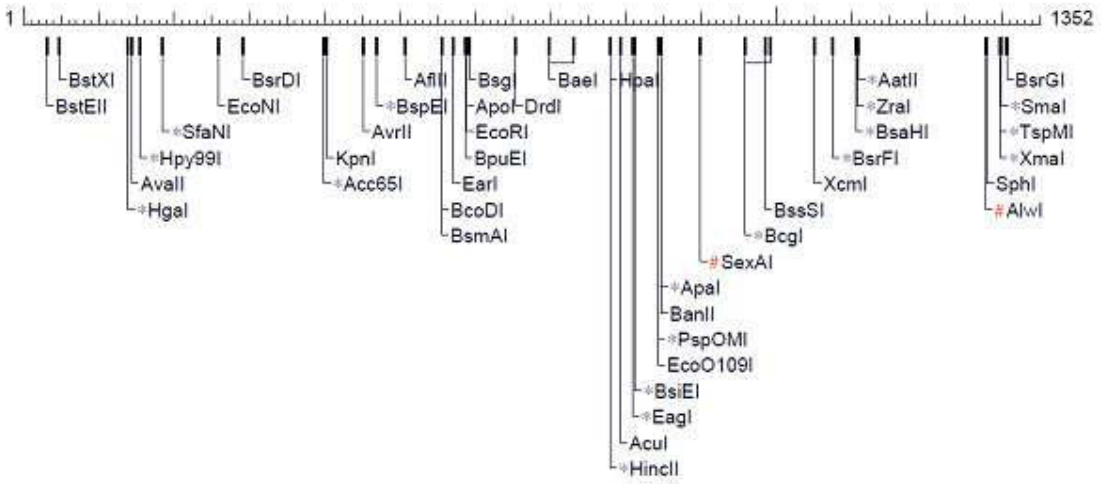

Figure4
Restriction Map of 16S rRNA gene sequence of the bacterial isolate GF1.

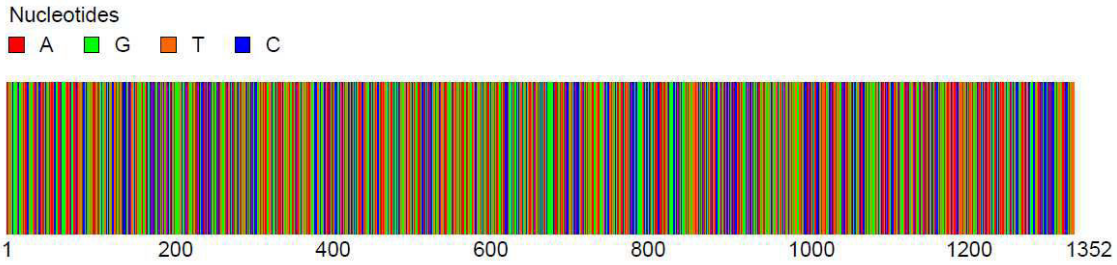

Figure5
Fingerprint of nucleotide composition of 16S rRNA gene of the bacterial isolate GF1.

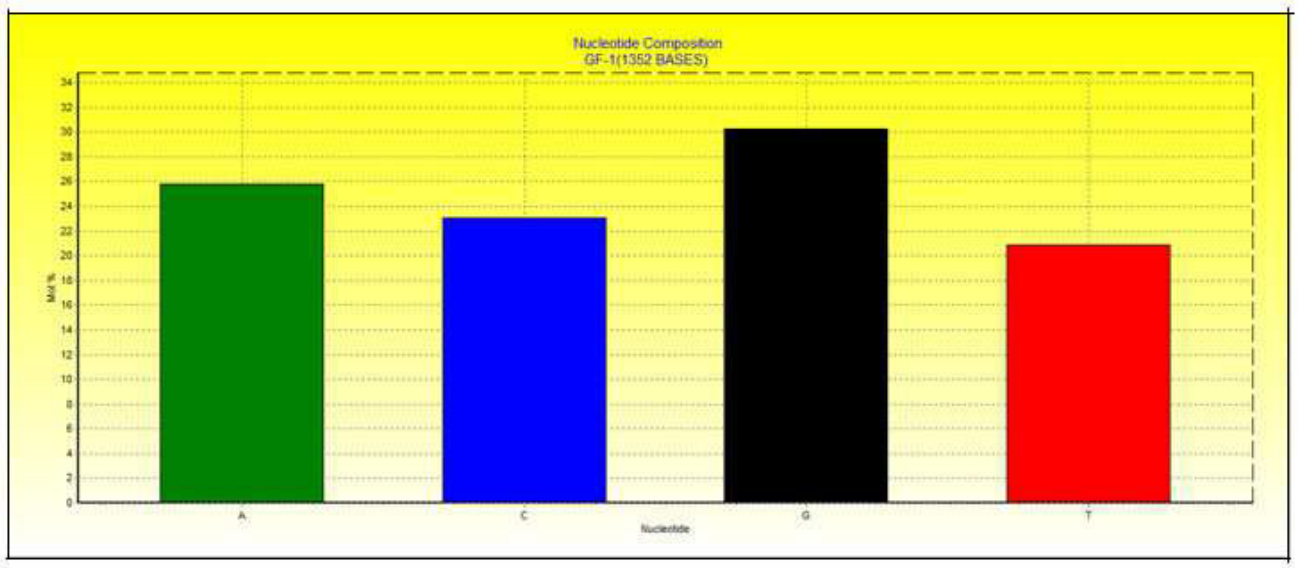

Figure6
A, C, G and T content (Mol%) of the 16S rRNA gene of GF1.

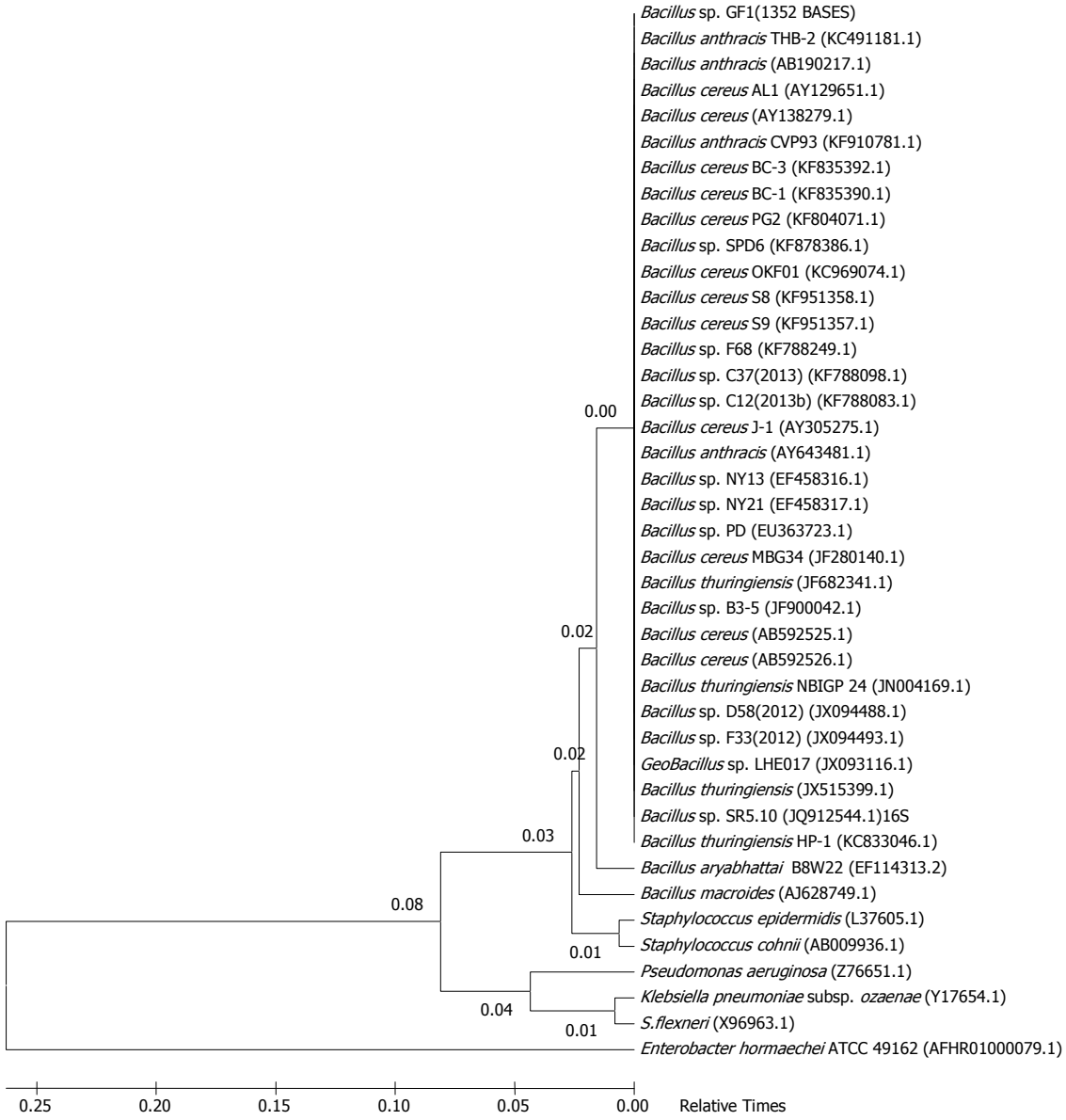

Figure7
Phylogenetic tree of the bacterial isolate GF1 based on 16S rRNA gene analysis.

CONCLUSION

As the bacterial isolate (GF1) causes pathogenicity to gold fishes causing great loss in aquarium business, proper control measure should be taken either by using antibiotics or plant extracts to which the isolate would be sensitive and or by proper management programme to prevent the infectious bacterial disease.

CONFLICT OF INTEREST: Declared none

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