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Isolation and characterization of bacterial pathogens, *Pseudomonas aeruginosa* and *Enterobacter cloacae* from the moribund fish, *Etroplus maculatus*

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Abstract

The fishes can be affected by different pathogens such as bacteria that affect mainly different internal organs with detriment in fish health that affect the production for ornamental or human food production. The ornamental fish *Etroplus maculatus* "Orange Indian cichlid" is widely distributed in India and Sri-Lanka, and it is widely cultured for ornamental purposes. The aim of the present study was study the presence of bacterial species *Pseudomonas aeruginosa* and *Enterobacter cloacae* from the moribund fish, *E. maculatus*. The results revealed the existence of both bacteria, specifically strains PSA1 and EMS1 respectively in internal organs of studied fishes, the reported strains were not resistant to antibiotic. The phylogenetic studies based denoted marked differences in strains of both bacterial species. The results would be similar with reports for other fishes species with interest for fisheries and aquaculture.

1. Introduction

A disease occurs when a susceptible fish is exposed to a virulent pathogen under unfavorable environmental circumstances [1]. The incidence of a disease is thus the result of a complex interaction between the fish, the disease agent, and the aquatic environment, a communicable disease can occur only when a susceptible virulent pathogen meets an environmental condition conducive for disease induction [2].

Fishes are exposed to a complex array of infectious agents like viruses, bacteria, fungi and parasites that inhabit and exploit them as abundant source of nutrients. Of all the infectious agents, bacteria are known to cause heavy mortalities in wild as well as in culture conditions [3]. Bacterial infection of fish constitutes a huge menace for aquaculture, leading to disastrous economic loss [4]. The normal bacterial flora of fish reflects the bacterial flora of the water in which they are reared. Some common bacterial populations found on the surface of the freshwater fishes include representatives of *Aeromonas* spp.

Pseudomonas anguilliseptica, *P. aeruginosa*, *Micrococcus* spp., or *Vibrio fluvialis* [5, 6]. Usually, a disease manifests when the fish is subjected to a shift in its physiological condition like infection of a pathogen in the spawning time or due to an external stressor.

A stressed fish population becomes vulnerable to a potential pathogen either from the environment or a carrier fish and ultimately succumbs to the infection [5]. Before an infection can be established, pathogens must penetrate the primary barrier. Hence stressors are the main predisposing factors for the chronic immune suppression of aquatic animals in the affected habitat. As an ultimate fate for the staggering immuno-suppression of fishes, bacterial invasion will be the most probable event [7]. The three major routes of infection are through skin, gills [8] and gastrointestinal (GI) tract [9].

Clinical signs (external and internal) caused by each pathogen are dependent on the host species, fish age and stage of the disease (acute, chronic, sub clinic carrier) [5]. In addition, in some cases, there is no correlation between external and internal signs [6]. In fact, systemic diseases with high mortality rates cause internal signs in the affected fish but they often present a healthy external appearance [7]. On the contrary, other diseases with relatively lower mortality rates cause significant external lesions, including ulcers, necrosis, exophthalmia which make fish unmarketable [8]. Therefore, studies on the clinical signs (external and internal) of the disease become one of the prerequisites in pathological investigation in addition to isolation and characterization of pathogenic strains [9]. Since the DNA sequencing and utilization of PCR tools, comparison of the gene sequences of bacterial species showed that the 16S rRNA gene is highly conserved within a species and among species of the same genus and hence can be used as the new "gold standard" for determination of the species of bacteria [8, 9]. By use of this new standard, phylogenetic trees, based on base differences between species, are constructed. Bacteria are classified and reclassified into new genera [10].

The ornamental fish *Etroplus maculatus* is very important for ornamental aquaculture in Asia, mainly in Indian subcontinent, that is a very important as economic activity [11, 12]. The literarure described the presence of viral and bacterial diseases [13], that would affect fish production [11, 12]. The fishes affected by bacterial infestations, are characterized by reddish patches and skin eruptions (Fig. 1). In the present chapter pathogens isolated from moribund *Etroplus maculatus* fishes were characterized based on the cultural, morphological and biochemical methods. The potent disease-causing strains were further characterized up to the species level by using the 16S r RNA sequencing.

Fig. 1. *Etroplus maculatus* showing red lesions on the skin, from which bacteria were isolated

2. Material and Methods

Isolation of bacterial pathogen: Live and moribund symptomatic fishes were carefully removed and used for isolating the pathogens. [14]. They were examined for external symptoms. The fishes

showing such ulcerative symptoms were surface sterilized with ethanol and the exudates from the ulcer was taken in a sterile inoculation needle and inoculated into a conical flask containing 25 ml of sterile nutrient broth (Hi Media) and incubated in shaker at 50 rpm at room temperature 27°C for 1 h. After taking bacterial samples, the fishes were cut open and examined. Most of the fishes were found in anorexic condition. The broth containing the bacterial population was taken for streaking on nutrient agar plates (Hi Media) for isolation of the causative bacteria using the standard streak plate method. The plates were incubated overnight and examined for size and shape of the colonies [14, 15]. Two colonies were dominant, and those were re- isolated in nutrient agar plates, incubated and the pure cultures were obtained by streaking on nutrient agar slants and stored in refrigerator. Samples of both colonies of cultures were streaked in agar slant tubes once in a month, and these were inoculated through the same species of fish for retaining the virulence. These both kind of isolated cultures were designated as PSA1 and EMS1.

The bacteria were cultured separately in nutrient broth (Hi Media) medium (beef extract 0.3 g, peptone, 0.5 g sodium chloride, 5 g and distilled water, 100 ml, pH 7.0). A small amount of both the cultures from the agar slants were taken and transferred separately into the sterile culture media [14, 15]. After this, the freshly inoculated culture media were kept in shaker at 50 rpm at 27±2 º C for 24 h. These cultures were used for characterization studies and for studying the pathogenicity of the isolates PSA1 and EMS1.

2.1. Characterization of the bacterial isolates - Physical and cultural characteristics: Observations on morphological status like shape, form, surface elevation, edge and pigmentation in nutrient agar were recorded. The suspected colonies on Nutrient agar plates were Gram stained and observed under oil immersion objective of a light microscope (Magnus) [14]. Gram's reaction, shape and arrangement of bacilli were recorded. Motility using hanging drop method was recorded [15].

2.2. Biochemical characteristics: The colonies which resembled Pseudomonas aeruginosa and Enterobacter cloacae on the basis of their cultural and morphological characters were subjected to different biochemical tests including catalase, oxidase and the sugar fermentation reactions and tentatively identified up to the genus level [14]:

a. Catalase test: A drop of 3% hydrogen peroxide was placed on the colony to be tested. Formation of bubbles indicated a positive catalase reaction [15].
b. Carbohydrate fermentation test: The nutrient broth suspended in different test tubes with inverted Durham's tube was added with 1% (w/v) of different sugars and phenol red. The overnight grown cultures to be identified was inoculated (1%; v/v) into the respective test tubes and incubated at 37°C for 24 h. Acid and gas production was noted at the end of incubation. Sugars used for identification were: glucose, sucrose, maltose, mannitol, mannose, sorbitol, fructose, lactose, arabinose and inositol [15].
c. Oxidase test: Oxidase disc was placed in the centre of a clean glass slide. Using a glass rod, culture was picked by touching the colony and immediately transferred by pressing the glass rod on the surface of the oxidase disc. After 20 seconds the disc was examined [15].
d. Amino acid utilization: The decarboxylase media was prepared by mixing the following ingredients: Peptone-5g. Yeast extract-3g, Glucose-1g and 10 ml of 20% bromocresol purple. The pH was adjusted to 6.7 and sterilized at 115ºC for 20 minutes. The media was divided into 4 groups of equal

volume and three were mixed with the three amino acids (L- arginine, L-lysine and L-ornithine) at a concentration of 0.5%, while the fourth served as control.Tubes were inoculated with a straight wire and incubated in room temperature and the results were recorded after 4 days [14, 15].

e. Gelatinase activity: Gelatin agar plates were prepared by dissolving 4 g of gelatin (Hi Media) in 50 ml of distilled water, to which 1000 ml of nutrient agar (Hi Media) was added. The media was sterilized at 115ºC for 10 minutes. The organisms were inoculated on the plate in a pinhead size. After incubation at 27±2ºC overnight, the plates were flooded with acid mercuric chloride solution. Acid mercuric chloride was prepared by mixing 12 g of $HgCl_2$ with 80 ml of distilled water, to which 16 ml of conc. HCl was added. The zone of liquefaction was recorded [16].
f. Casein hydrolysis: Skim milk agar plates were prepared by mixing 50 ml of 10 % skim milk powder solution, which was sterilized at 15 lbs for 5 minutes and added with 100 ml of sterile nutrient agar (Hi Media). This media mixture was poured into sterile plates. The PSA1 and EMS1 strains were inoculated as two single pinpoints and incubated at room temperature overnight. Acid mercuric chloride solution was poured on the plates to make the zone clear and the zone was measured and the area calculated [17].
g. Indole Test: The test organism was cultured in a medium, which contains tryptophan. With the presence of the enzyme tryptophanase, organism can ferment tryptophan and produce indole. Kovac's reagent that reacts with the indole to produce a red coloured compound indicates the indole production. Absence of red colour indicates the negative result. Using sterile straight inoculation loop, a smooth colony was inoculated of the test organism in to the tryptophan broth and incubated at 35 to 37ºC over night. After the incubation period, 5 ml of Kovac's reagent was added in to the broth to see the colour reaction [18].
h. Methyl Red Test: The methyl red test was employed to detect the ability of the test organism to produce the mixed acid fermentation. The test was performed by inoculating a colony of the isolates in 0.5 ml of sterile glucose phosphate broth. After overnight incubation at 35-37ºC, few drops of methyl red solution was added. The positive result was recorded by the appearance of bright red colour indicating acidity. Appearance of yellow or orange colour indicated negative result [18].
i. Voges Proskauer (V-P) Test: The V-P test was done to detect the production of acetone by the test organism. Sterile glucose phosphate peptone water (2 ml) was inoculated with the test organisms and incubated at 35-37º C for 24 h. After incubation, 1 ml of 40% potassium hydroxide (plus creatine) and 3 ml of the 5% solution of alpha-naphthol in absolute ethanol were added. A positive reaction was indicated by the development of a pink colour. Absence of pink red colour indicated negative result [19].
j. Citrate Utilization Test: The bacterial isolates were detected for the ability to utilize sodium citrate as the sole source of carbon. The isolates were inoculated in a Simmon's Citrate agar medium and incubated at 37ºC for 24 h. The formation of blue colour indicates the positive result [20].
k. Urease Test: The isolates were cultured in a medium containing urea and the indicator phenol red. If the strain is produced, the enzyme will break down the urea and the indicator phenol red. The change of medium to alkaline as indicated by changes in colour of the indicator to red-pink gave positive result, absence of colour change indicated negative result [21].
l. Triple Sugar Iron (TSI) Test: TSI test was used to differentiate the test organism according to their ability to ferment sucrose or lactose or glucose or none. Production of gas in fermentation of carbohydrate was accompanied by acid formation [20].

2.3. Antibiogram of PSA1 and EMS1 isolates: The isolates PSA1 and EMS1 were inoculated into MRS (Hi Media) broth individually and incubated for 24 h at 30°C. The culture from the broth was swabbed on the solidified MRS agar medium in the petri dish. Antibiotic discs (approximately 30µg concentration) either individually or as OCTA discs (8 antibiotics in a single ring; Hi Media) were placed upside down, pressed on the top of the agar plates and kept at 40°C for 1 h [15]. The plates were incubated at 30°C overnight. Resistance was defined as the absence of a growth inhibition zone around the discs [14].

2.4. Characterization by 16S rRNA gene sequencing: Molecular approach was employed for the confirmation of the strains Pseudomonas aeruginosa and Enterobacter cloacae [14]. The 16S rDNA gene of isolates were sequenced (ABI 3100 sequencer and genotyper; Genei make) after the DNA isolation and amplification. PCR was used to amplify the 16S rRNA gene of strains using eubacterial primer. The 16S rDNA sequence was determined by direct sequencing [14]. Total DNA was isolated by using genomic DNA purification kit (Qiagen, Germany). Primers used for PCR and DNA sequencing are given in Table 1. The PCR amplification was performed with the primer targeted against regions of 16S rDNA [21]. Amplification of DNA was performed in an Eppendorf Master cycler Gradient thermal cycler (Applied Biosystems – 2720). PCR conditions included an initial denaturation at 96°C (5 min.), denaturation at 96°C (1 min), 25 cycles consisting of annealing at 50°C (1 min), polymerisation at 72°C (2 min.), denaturation at 96°C (1 min) and a final extension at 72°C (10 min.). PCR products were resolved by electrophoresis in 1% (w/v) agarose gel and visualized on UV transilluminator after staining with ethidium bromide (1 µl/10 ml). 16S rDNA PCR amplicons were purified following the Qiagen kit protocol and sequenced. The automated sequencing of both strands of the PCR products was done on an ABI 3100 automated gene sequencer (Genei). The sequences were then subjected to Distance matrix based on nucleotide sequence homology using the Kimura2 parameter [22]. The last step for identification is the Phylogenetic Tree based on Nearest Neighbor joining method (MEGA 5.03), which identified the homology of the organism. Phylogenetic analysis was realised by an alignment of sequence consensus of the 16S rDNA genes collected in an international database (GenBank) [22]. The results were then expressed in percentage of homology between the submitted sequence and the sequences resulting from the database. The sequences were deposited in GenBank.

Table 1. Primers used for amplification of the genome

	PCR Primers
Fwd Primer	5'-AGAGTRTGATCMTYGCTWAC-3'
Rev primer	5'-CGYTAMCTTWTTACGRCT-3'

BLAST: Sequenced results were compared to those databases that were already deposited in the GenBank using Basic Local Alignment Search Tool (BLAST) algorithm to identify the sequences with maximum similarity. Ten sequences having the maximum sequence homology towards the submitted sequence were selected and aligned [14].

Phylogenetic analysis: Phylogenetic Tree Builder uses sequences aligned with System Software aligner. A distance matrix was generated using the Jukes-Cantor corrected distance model. When generating the distance matrix, only alignment model positions were used, alignment inserts were ignored and the

minimum comparable position is 200 (Prabha et al., 2021. The tree was created using Neighbor with alphabet size 4 and length size 1000. Sequences were aligned at each branch point, starting from the least distant pair of sequences [22].

3. Results.

Occurrence of disease: Diseased *E. maculatus* showed septicemia hemorrhage in the skin of the mouth region, opercula and both sides of the body and detachment of scales and skin ulceration, abdominal distention and bulged intestine (Fig. 2). Internally these fishes showed abdominal dropsy with reddish ascetic exudates, liver paleness and enlargement in some fishes and congestion with necrotic patches in other fishes, spleen was congested with enlarged and hemorrhagic enteritis in some fishes. The infected fishes exhibited sluggish movement, swimming disorders and weakened escaping reflex.

Isolation and characterization of pathogenic bacteria: The results of various physical, cultural and biochemical tests carried out to determine the identity of both the pathogenic isolate PSA1 and EMS1 are depicted in the Table 2. The isolate PSA1 was recorded as Gram-negative, straight or slightly curved rods, motile and arranged singly, non-sporing, non-capsulated and facultative anaerobes. Culturally, it produced large wrinkled, β haemolytic, low convex with irregular margin colonies. It produced K/N and did not produce H_2S in TSI medium. The organism utilized glucose, fructose and mannitol, but not xylose, maltose and inositol sugars. Production of blue-green pigment was indicative of *P. aeruginosa*. The isolate was also exhibited oxidase negative and nitrate reductase positive activity.

Fig. 2. Diseased *Etroplus maculatus* showing enlarged intestine

The other bacterium isolated from moribund fish was a short, Gram- negative, motile rod. This bacterium grew well at pH 6 to 8 (optimum pH, 7.0) and at 20 to 40°C (optimum, 30°C). This bacterial strain was designated as EMS1. It was determined that the strain EMS1 belonged to the family Enterobacteriaceae as it was oxidase negative and nitrate reductase positive. On the basis of the morphological, biochemical properties and characterization by 16S r RNA sequencing this bacterium was identified as E. cloacae at a confidence level of 99%. The size of this bacteria ranges from 0.3-0.6 x 0.8-2.0 µm. Enterobacteriaceae that can utilize citrate will extract nitrogen from ammonium phosphate releasing ammonia. Citrate utilization was recorded a key biochemical property. Antibiogram of the selected isolates PSA1 and EMS1: The sensitivity of the isolates PSA1 and EMS1 against various antibiotics were detected by antibiotic disc diffusion method (Figs. 3 to 5).

The results indicated that the strain PSA1 was resistant to several antibiotics when compared to that of EMS1 (Tables 3 and 4).

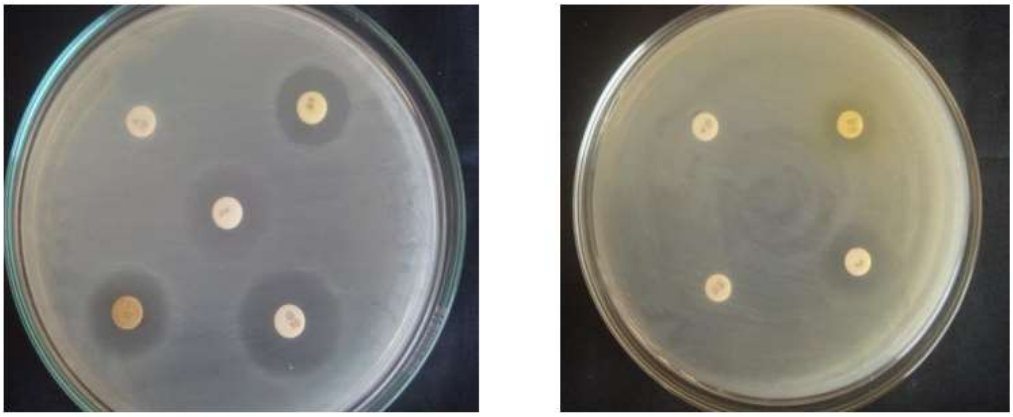

Fig. 3. Sensitivity of *E. cloacae* strain (EMSI)

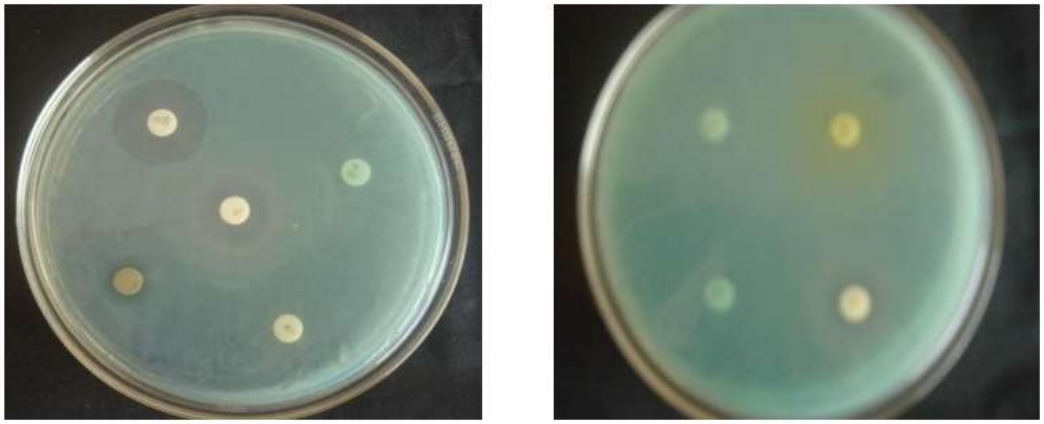

Fig 4. Sensitivity of *P. aeruginosa* strain (PSA1).

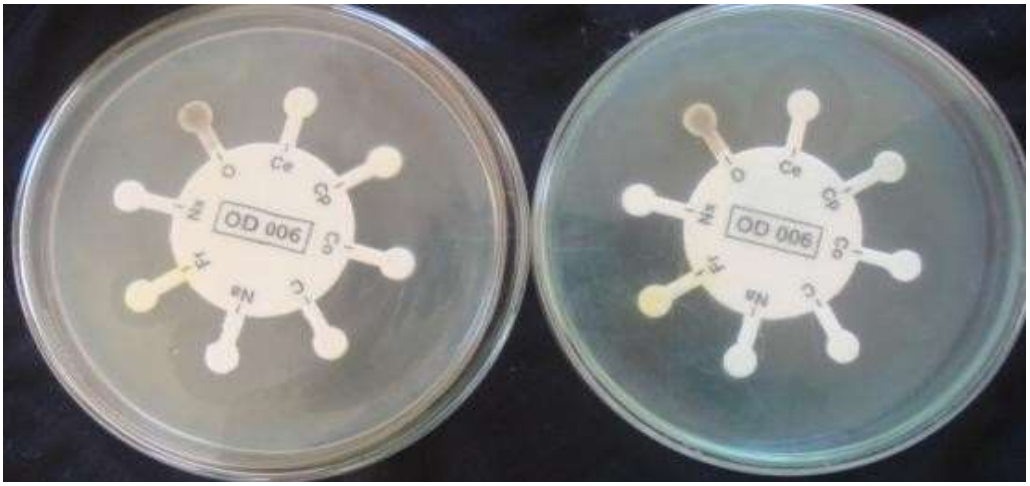

Fig. 5. Octa disc representing sensitivity of *E. cloacae* and *P. aeruginosa* strains

Identification of pathogenic strains using 16S r RNA gene sequencing: The pathogenic strains PSA1 and EMS1 isolated from the moribund fish *E. maculatus* were characterized as *P. aeruginosa* and *E. cloacae* after the DNA sequencing technique using the universal 16Sr DNA primer and subsequent alignment of the sequences.

Table 2. Morphological and Biochemical characteristics of PSA1 and EMS1

Test	PSA1*(Pseudomonas aeruginosa)*	EMS1*(Enterobacter cloacae)*
Gram staining	Gram negative	Gram negative
Oxidase	+	-
Indole	_	-
Methyl Red	_	+
Voges Proskauer	_	+
Simmons Citrate	+	+
Triple Sugar Ion	AK/AK	A/A
Urease	_	+
Mannitol motility	+	+
Nitrate reduction	+	+
Arginine Dihydrolase	_	+
Ornithine Decarboxylase	_	+
Catalase	_	+
Protease	_	_
Gelatinase	_	_
Amylase	_	+
Sugar Fermentation		
Lactose	_	+
Adonitol	_	+ (weak)
Dextrose	_	+
Trehalose	_	+
Melibiose	_	+
Raffinose	_	+
Arabinose	_	+
Sucrose	_	+
Cellobiose	_	+
Maltose	_	+
Manitol	_	+
Xylose	_	+
Sorbitol	_	+

Table 3. Sensitivity of *P. aeruginosa* strain to various antibiotics

Antibiotics	Disc Content (µg)	Inhibition Zone (mm)	Inference
Nitrofurantoin (NF)	100	No Zone	R
Ampicillin (A)	10	No Zone	R
Erythromycin (E)	15	13	R
Furazolidone (FR)	50	No Zone	R
Gentamycin (G)	10	24	S
Tetracycline (T)	30	10	R
Vancomycin (VA)	10	No Zone	R
Cefotaxime (CE)	30	21	I
Cephalexin (CP)	30	No Zone	R
Co-trimoxazole (CO)	25	16	S
Chloramphenicol (C)	30	20	S
Nalidixic acid (NA)	30	13	R
Furazolidine (FR)	50	No zone	R
Norfloxacin (Nx)	10	25	S
Oxytetracycline	30	11	R

Table 4 Sensitivity of *E. cloacae* strain to various antibiotics

Antibiotics	Disc Content (µg)	Inhibition Zone (mm)	Inference
Nitrofurantoin (NF)	100	13	R
Ampicillin (A)	10	No Zone	R
Erythromycin (E)	15	15	R
Furazolidone (FR)	50	16	R
Gentamycin (G)	10	23	S
Tetracycline (T)	30	21	S
Vancomycin (VA)	10	No Zone	R
Cefotaxime (CE)	30	30	S
Cephalexin (CP)	30	No Zone	R
Co-trimoxazole (CO)	25	26	S
Chloramphenicol (C)	30	18	S
Nalidixic acid (NA)	30	25	R
Furazolidine (FR)	50	12	R
Norfloxacin (Nx)	10	32	S
Oxytetracycline	30	12	R

R-Resistant M – M.S–Sensitive

The 16S ribosomal DNA (rDNA) sequence of the strain PSA1 (Fig. 6) isolated was found to be most similar to another strain of *Pseudomonas* species 9 GUW; (EU449119). Hence, the isolated organism was characterized and designated as *Pseudomonas aeruginosa* PSA1 and are Gram-negative, straight or slightly curved rods, motile and arranged singly, non- sporing, non-capsulated and facultative anaerobes. The Enterobacter strain EMS1 isolated from the moribund fish *E. maculatus* was characterized on the basis of biochemical tests and the 16S rRNA sequencing as *E. cloacae*. The phylogenetic analysis of this strain revealed similarity of the strain EMS1 with *Enterobacter cloacae* subsp. *cloacae*; MSA1 (HM131220) through the Neighbor Joining Method (Fig. 7).

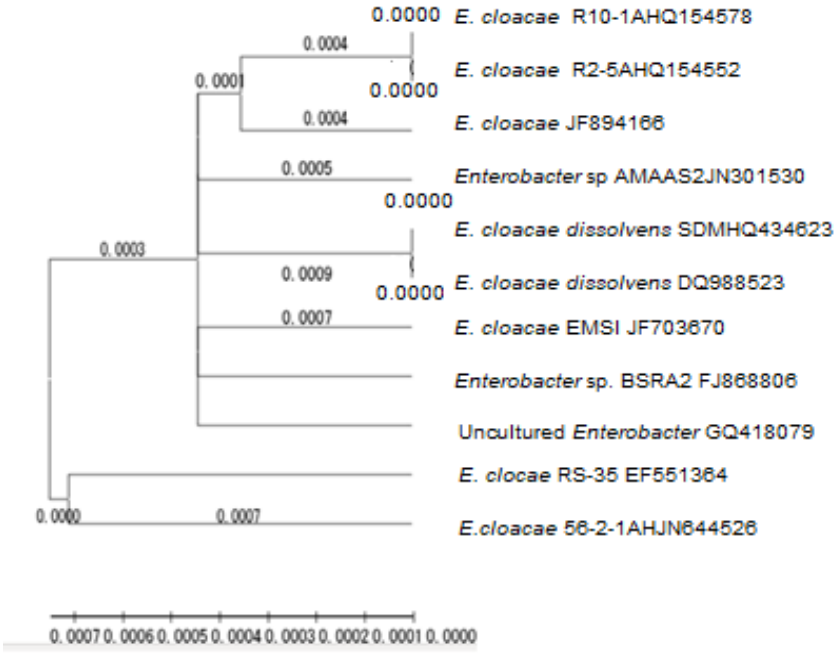

Fig. 6. Phylogenetic tree in MEGA 5.03 software using Neighbour Joining Method- *Enterobacter cloacae* PSA1

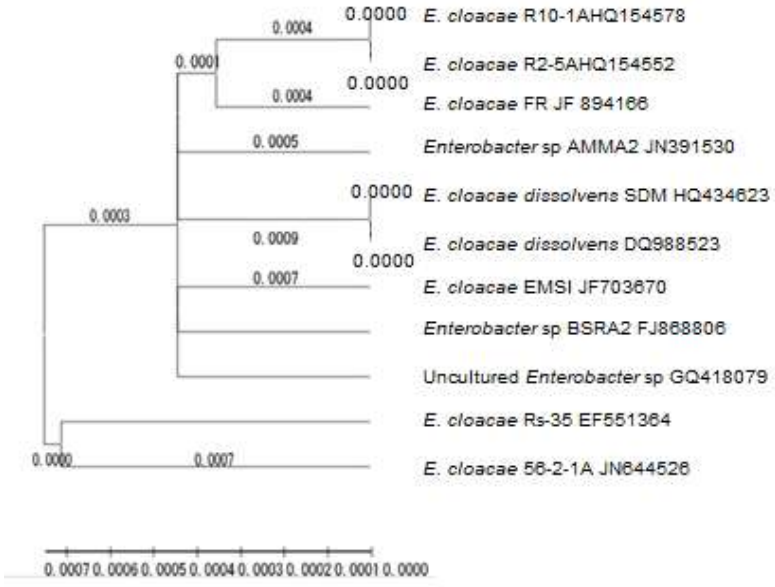

Fig. 7. Phylogenetic tree in MEGA 5.03 software using Neighbour Joining Method- *Enterobacter cloacae* EMS1

4. Discussion

In ornamental fish industry, disease outbreaks have been a perennial threat to their growth and productivity, diseases also bring heavy economic loss for the culturists and investors due to mass mortalities and shutting down the hatchery/culture operations [11, 12]. The complex nature of these disease outbreaks has necessitated the need for an understanding of the causative pathogens and their metabolism and the progress of their infection in vivo in order to apply control measures and managerial strategies. The present study revealed the fact that outbreaks of the disease are usually caused by stress and changes in environmental conditions which could have favored the pathogen. Soltani and Burke [23] stated that, environmental factors are of crucial importance in the expression of the pathogenic potentials of the disease producing microorganisms and thereby linked to the occurrence of bacterial diseases. Bacterial diseases were considered the main cause of high mortalities and economic losses among fish and fish farms [5].

Based on the morphological and biochemical tests, the pathogens were characterized as *P. aeruginosa* and *E. cloacae*. The scarce literature about bacterial pathogens in gut of *E. maculatus* described the presence of *Bacillus, Lactobacillus* and *Vibrio* [24], also it was reported the presence in the gut of *Micrococcus, Moraxella, Corynobacterium, Bacillus* and members of Enterobacteriaceae [25]. Among the test species, E. maculatus was found to be a susceptible host to bacterial infections depending on the seasonal variations and that the physico-chemical parameters of the environment could influence the bacterial load [24, 25]. In this scenario, the presence of P. aeruginosa and E. cloacae in gut of E. maculatus reported in the present study is a first description of it. The detailed external symptoms of the ulcerative disease including the reflex pattern of the infected fish as given in the results section corroborated with those of some of the bacterial ulcerative diseases reported from India and abroad. The experimental fishes showed signs of lassitude, swimming disorders and weakened escaping reflex, which are due to outbreak of bacterial infection as stated by [26]. Similar observations were made by [27, 28]. Also, in according to the literature [29] observed relatively similar symptoms except those of oozing pus in Indian Major Carps affected with ulcer disease. Fish pathogenic bacteria have developed different strategies to infect the host fish species. Adhesion to the epithelial tissue of host, resistance

against body surface mucus and serums of host are the important mechanisms during initial stages of infection [30].

Changes in the skin are the most readily observed clinical features of fish. The result of the current study demonstrated that clinical picture of septicemia characterized by irregular hemorrhages all over the body surface, especially at the ventral part of the abdomen as well as detachment of the scales as stated by [31]. Internally these fishes showed abdominal dropsy with reddish ascetic exudates, liver paleness and enlargement in some fishes and congestion with necrotic patches, spleen was congested with enlarged and hemorrhagic enteritis. The ulcerative condition is different from that of Epizootic Ulcerative Syndrome (EUS). In the case of EUS, initially pinhead size red spots develop on the body surface with no noticeable hemorrhages or ulcers, which further advances to necrotic open ulcers ([27, 28]. Annie [32] reported that vibriosis infected blue damsel, *Pomacentrus caeruleus* exhibited skin discoloration, presence of red necrotic lesions in the abdomen and erythema at the base of the fins and around the vent, sloughing off scales and reduced activity.

A perusal of literature has revealed that the freshwater forms have been infected with the bacterial species such as *Aeromonas* sp, *P. aeruginosa, Edwardsiella* sp and *Enterobacter cloacae* [33]. Also, Hettiarachchi and Cheong [34] described *A. hydrophila* as the major pathogenic bacteria causing disease among freshwater ornamental fish.

Jongjareanjai *et al.* [34] showed that the majority of the bacteria isolated was *A. hydrophila* (27/30) and one strain of *Enterococcus durans, Flavobacterium* sp. and *Serratia marcescens* from sick ornamental fish. Mosharrof Hossain [36] isolated six strains of Aeromonas spp bacteria from the gourami (*Colisa lalia*) by 16S r DNA sequencing analyses that are pathogenic to freshwater fish. Among them, three were grouped under A. veronii species, two were Aeromonas sp ATCC and one was A. hydrophila. According to Noga [37, 38], motile aeromonad infection (MAI) was likely to be the most common bacterial disease of freshwater fish. *A. hydrophila* causes skin ulcers at any site on the fish and often they are surrounded by a bright red rim of tissue [39]. However, ulcer caused by P. putida can be observed almost exclusively on the dorsal surface of the fish. Eels and several other species, including goldfish, have been shown to be experimentally susceptible to *P. anguillid septica* [40, 41, 42]. Thampi Raj [43] isolated *V. harveyi* from infected seahorse (*Hippocampus kuda*). Pramila [44] reported LD50 of marine ornamental fish, pathogen, *Serratia marcescens* injected to clownfish was 1 × 105 cells/g of fish and the median lethal time (LT50) taken for 50 % mortality was recorded as 45.0 h.

Though *Pseudomonas aeruginosa* and *Enterobacter cloaca* were isolated as causative agents, it doesn't mean that the infection was only caused by *Pseudomonas aeruginosa* and *Enterobacter cloacae*. Cheng and Cheng [45] reported that the crustacean Macrobrachium rosenbergii was infected with *Enteroccocus bacterium*. Wakabayashi and Egusa [46] reported that *Pseudomonas* is a common bacterial pathogen of fishes. Premalatha [47] isolated *P. fluorescens* and *A. hydrophila* from *Carasius auratus*. In Bangladesh, the major carp species have been found to be suffering from ulcer type diseases of different expressions, including epizootic ulcerative syndrome (EUS), bacterial hemorrhagic septicemia, tail and fin rot, bacterial gill rot, dropsy, columnaris disease, fungal disease and parasitic disease [48]. The pathogenic bacterial isolates of fish such as *Pseudomonas aeruginosa* and *A. hydrophila* were tested for their pathogenecity [49]. The author reported that the fish isolate of *P. aeruginosa* had a lethal dose of 1.5 $\times 10^5$ cells/fish for *Cyprinus carpio* and 4.2 $\times 10^5$ cells for *O. mossambicus*. The fish pathogen *A. hydrophila* had lethal doses of 2.1 $\times 10^6$, 6.8 $\times 10^5$ and 3.2 $\times 10^6$ cells/ fish, respectively, for *C. carpio*, *L. rohita* and *O. mossambicus*.

Mixed bacterial infections with *Aeromonas* and *Pseudomonas* sp was also recorded by Ahmed and Shoreit [49]. A. hydrophila alone was isolated from gills of the naturally infected male monosex *O. niloticus* suffering from Motile A. septicemia (MAS) in floating cages [50]. It was associated fin rot to overcrowding, dirty and muddy water and other parameters engendered to poor water quality. There has been a steady increase in the numbers of bacterial species associated with fish diseases, with new pathogens regularly recognized in the scientific literature [5]

Selvin et al. [51] suggested that bacterial disease outbreaks particularly vibriosis and black shell disease impose a significant constraint on the sustainable production of shrimp, *P. monodon, Vibrio alginolyticus* and *V. harveyi* posed a serious disease problem in cultured black tiger shrimp in India [52]. Larval mortalities occurring in molluscan hatcheries have often been associated with bacterial contamination and more specifically with vibrios [51, 52]. The hatchery production of *P. fucata* was seriously affected by massive larval mortalities caused by *Vibrio* sp [53]. Subhash et al. [54] also revealed that the total bacterial load in the culture tank water was found to increase in the hatcheries of *P. fucata* during disease out breaks resulting in heavy larval mortality.

The three major routes of infection are through skin, gills [8] and gastrointestinal (GI) tract [9]. In order to prevent microbial entry, fish might have evolved various protective mechanisms. These include production of mucus by goblet cells, the apical acidic micro-environment of the intestinal epithelium, cell turnover, peristalsis, gastric acidity, lysozyme and antibacterial activity of epidermal mucus. Although not all of these protective mechanisms have been reported in fish, lysozyme has been reported in most of the fishes but not in Atlantic cod (*Gadus morhua* L.) and spotted wolfish (*Anarhichas minor* Olafsen) [55]. At the same time, pathogenic microorganisms have evolved mechanisms to target skin, gills or gastrointestinal tract as points of entry.

Cultural and biochemical characterization of the isolated *P. aureginosa* were parallel to previous studies which identified the same organisms from other fish species [5, 56]. Pseudomonads are opportunistic Gram negative pathogens, naturally occurring in aquatic environment and as a part of normal gut flora of healthy fish, it cause outbreak when the normal environmental conditions changed [57]. *P. fluorescens, P. angulliseptica, P. aeruginosa* and *P. putida* were identified in various species of fish as causative agents of pseudomonas septicemia. The disease is characterized by petechial hemorrhage, darkness of the skin, detached scales, abdominal ascitis and exophthalmia [5]. El-Moghazy [58] reported that the prevalence of *Pseudomonas septicemia* was 41.66% among the diseased tilapia. Tripathy et al. [59] *P. aeruginosa* was isolated from pond sediments as well as from intestine of freshwater fishes. El Hady et al. [60] isolated *Pseudomonas fluorescence* from 24% of diseased *Oreochromis niloticus*.

Several researchers [46, 61] studied the pathogenicity of bacteria to fish. The biochemical tests conducted inferred that the strain *P. aeruginosa* PSA1 was Gram-negative, straight or slightly curved rods, motile and arranged singly, non-sporing, non-capsulated and facultative anaerobes. Culturally, it produced large wrinkled, β haemolytic, low convex with irregular margin colonies. It produced K/N and did not produce H2S in TSI medium.

Members of the genus *Pseudomonas* are important phytopathogens and agents of human infections, while other strains and species exhibit bioremediation and biocontrol activities. Species-specific detection of *Pseudomonas* species in the environment may help to gain a more complete understanding of the ecological significance of these microorganisms. Comparative analysis of biochemically and PCR-ribotyping and PAGE revealed that there was extensive heterogeneity at the genetic and protein levels. Tripathy *et al.* [59] reported that SDS-PAGE clearly demonstrated the differences between fish and sediment isolates as evident from the higher range of protein profiling.

Enterobacter cloacae (EMS1) is a rod-shaped, Gram-negative bacterium from the Enterobacteriaceae family. As per the observations, the size of *E. cloacae* (EMS1) ranged from 0.3-0.6x0.8-2.0 µm. E. cloacae lives in the mesophilic environment with its optimal temperature at 37 °C and uses its peritrichous flagella for movement. This organism is oxidase negative but catalase positive and is facultative anaerobic. In other words, this organism can make ATP by aerobic respiration when oxygen is present but can switch to fermentation in the absence of oxygen. E. cloacae, an enteric bacterium that belongs to the family Enterobacteriaceae, has been reported as an opportunistic pathogen in humans [62] and other organisms such as fish [63] and insects [64]. Kasing *et al.* [65] isolated *Enterobacter aerogenes* from intestine of four freshwater fish species belonging to the family Cyprinidae reared in experimental ponds. Hansen et al. [63] have isolated *E. agglomerans* from the kidney of infected dolphin fish, *Coryphaena hippurus* L showing that this enteric bacterium is pathogenic to fish. Enteric bacteria are not the normal flora in the intestinal tract of fish and hence their presence in the infected fish may be the result of the association of fish with the polluted waters [66]. Troast [67] reported that Escherichia coli and E. cloacae have been shown to have possible involvement in the infection of fish.

The genomes of several strains of *E. cloacae* have been partially sequenced with the 16S ribosomal RNA gene. Unfortunately, there are no genomes of E. cloacae that have been fully sequenced. The present study revealed that *Enterobacter cloacae* is positive for beta-galactosidase, arginine dihydrolase, ornithine decarboxylase, citrate utilization, nitrate reduction, and Voges-Proskauer reaction. Although acid was produced from many carbon sources, this bacterium did not produce lysine decarboxylase, hydrogen sulfide, urease, tryptophan deaminase, and indole.

In the present study *P. aeruginosa* isolate exhibited sensitivity towards gentamycin, chloramphenicol and norfloxacin. However, it was resistant to nitrofuratonin, ampicillin, erythromycin and furazolidine. E. cloacae was more sensitive to norfloxacin and cephotaxime. Ghosh *et al.* [68] found that the antibiotic sensitivity profile of Pseudomonas fluorescence was resistant to amoxycillin, cloxacillin, penicillin-G and ampicillin, on this view point these antibiotics cannot be used as therapeutic agents. El-Bouhy and Khalil [69] mentioned that Gentamycin was one of the effective choices for treatment of *Staphylococcus* infection in some freshwater fish. According to El-Hady and El-Katib [69] *Pseudomonas fluorescens* isolates are highly susceptible to oxytetracycline. Wolska *et al.* [70] also suggested that 99% of *Pseudomonas aeruginosa* strains were susceptible to ciprofloxacin. Sarker et al. [31] performed drug sensitivity test and found that 50% of the *Aeromonas sobria* isolates were highly sensitive to oxytetracycline, oxolinic acid and chloramphonical and resistant to erythromycin and sulphamethoxazole. Liao et al. [71] used oxytetracycline in aquaculture as bactericide. Lio-Po and Sanvictores [72] found positive effect of oxytetracycline in controlling *Pseudomonas* sp. in Tilapia fry. According to Shariff *et al.* [73] oxytetracycline (about 20 ppm) in a dip or bath solution is used against bacterial disease in Malaysia and Singapore.

Since biochemical properties do not accurately reflect the genomic complexity of a given species and the diagnostic results may be influenced by physical parameters, such as pH, temperature, and growth substrate concentrations, unambiguous identification of the different members of the genus by biochemical reactions is impossible. Hence molecular method, restriction digestion of the 16S rRNA, could be considered as characterizing the isolates PSA1 and EMS1. Both the isolates were subjected to characterization up to the species level using molecular method. The identification of the isolates was accomplished after the DNA sequencing using the universal 16S rDNA primer and subsequent alignment of the sequences. It could be noted from the phylogenetic tree that the strain *P. aeruginosa* PSA1 was most similar to another strain *Pseudomonas* sp. 9 GUW (EU449119) and the pathogen *E. cloacae* EMS1

was closely similar to another strain *E. cloacae* subsp. *cloacae*; MSA1 (HM131220). The present study revealed that mortality occurred in moribund *E. maculatus* were mainly due to disease caused by the pathogenic bacteria viz., *P. aeruginosa* and *E. cloacae.* As conclusion, the mortality occurred in moribund *E. maculatus* were mainly due to disease caused by the pathogenic kind of bacteria viz., *P. aeruginosa* and *E. cloacae*, that affect also other fish's species under natural environment as well as under aquaculture or laboratory conditions.

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