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Full Length Research Paper

Characterization of *Pseudomonas aeruginosa* PB112 (JN996498) isolated from infected *Labeo bata* (Hamilton) by 16S rRNA gene sequence analysis and fatty acid methyl ester (FAME) analysis

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In this study, a total number of fifteen moribund *Labeo bata* were collected from Hooghly, Purba Medinipur, and South 24 Parganas districts of West Bengal, India. The lesions and hemorrhages were recorded at the base of the fins or on the skin due to bacterial infection. Isolation and pure culture of the causative agent were made in the laboratory. The 16S rRNA gene sequence of the bacterial isolate PB112 has been deposited in the NCBI GenBank and was assigned the accession number JN996498. The GC and AT contents of the 16S rRNA gene sequence (JN996498) were 54.22 and 45.78%, respectively. Based on the biochemical properties, 16S rRNA gene sequence analysis and fatty acid methyl ester (FAME) analysis, the causative bacteria were identified as *Pseudomonas aeruginosa*.

Key words: *Labeo bata, Pseudomonas* sp., 16S rRNA gene sequence, fatty acid methyl ester (FAME) analysis.

INTRODUCTION

Bata (*Labeo bata* Ham.) is one of the most favorite and popular fish among minor carps having high market value. It is a benthopelagic and herbivorous column feeder. Although fishery products are of great importance for worldwide human nutrition, these may also act as sources of food-borne pathogens. In commercial farms, unfavorable environmental conditions or poor management practices make them more susceptible to disease outbreaks (Verschuere et al., 2000). Bacterial disease outbreaks impose a significant constraint in fish and shellfish production (Bachere et al., 1995). Clinical signs of the disease may include petechial hemorrhages of the skin (Hossain, 2008), peritoneum and liver (Wiklund and Bylund, 1993).

The reason for the widespread occurrence of *Pseudomonas* spp. in the aquatic environment may be due to its spread through water, which serves as the primary reservoir of infection (Austin and Austin, 1999). Several species of *Pseudomonas* have been reported to cause disease in a number of fish species, including goldfish like *Carassius auratus* (Bullock, 1965), and are associated with septicemia in aquatic animals (Roberts, 1978). These bacteria have been considered as opportunistic pathogens, causing diseases when the host is subjected to stress. A number of aquatic animals like fish, frogs and soft-shelled turtles were recorded to be susceptible to *Pseudomonas* spp. (Somsiri and Soontornvit, 2002). The present study aimed at determining the phenotypic, biochemical and molecular properties of the disease-causing bacterial isolate.

MATERIALS AND METHODS

Sampling of fish

A total of 15 moribund fish (*Labeo bata* Ham.) were collected from fish farms of Hooghly, Purba Medinipur, and South 24 Parganas

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Districts of West Bengal, India. Afterward, the infected fishes were acclimatized in fresh water of glass aquarium in the laboratory conditions approximately at 25°C. The fished were allowed to feed on commercial fish-food daily and were regularly monitored whether any death or ulcerative symptoms occurred.

Bacteria isolation and culture

Bacteria were isolated from affected fish organs by a sterile loop and streaked on the pre-prepared sterilized nutrient agar media (Peptone: 5 g; yeast extract: 3 g; sodium chloride (NaCl): 3 g and agar: 2%; pH: 7.4) following the methods of Poiner and Thomas (1984) and Lacey (1997). The agar plates were incubated at 37°C for 24 h for appropriate colony formation. The predominant bacterial colonies from the media were isolated, purified and characterized following standard methods (Sneath, 1986; Lacey, 1997; Pelczar, 1957).

Preparation of scanning electron micrograph of bacterial smear

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 and 90%, and finally with absolute alcohol for 5 min each. The specimens were gold-coated and observed under a scanning electron microscope.

FAME analysis and 16S rRNA gene sequence analysis

For microbial identification system (MIS, MIDI Sherlock® USA), the fatty acid methyl ester (FAME) analysis is a standard method. Whole cell fatty acids were converted to methyl esters and analyzed by gas chromatography. The fatty acid composition of the bacterial isolate PB112 was compared to a Sherlock Library of known organisms in order to find the closest match. The 16S rDNA technique is an important tool for rapid and accurate detection of bacteria, which can replace conventional, time-consuming biochemical identification method (Uma et al., 2007). In this method, genomic DNA was isolated from the pure culture, and then about 15 kbp rDNA fragment was amplified by polymerase chain reaction (PCR), and finally the product was sequenced. The 16S rRNA gene sequence of *Pseudomonas sp.* has been deposited in the NCBI GenBank and was assigned the accession number JN996498.

Bacterial identification was done by 16S rRNA gene sequence analysis (Jeffry et al., 1992) and FAME analysis (MIS, MIDI Sherlock® USA). Restriction map of 16S rRNA gene sequence of *Pseudomonas sp.* (JN996498) was prepared following the method of Vincze et al. (2003). The sequence data were aligned using the ClustalW submission form (http://www.ebi.ac.uk/clustalw) and analyzed by ClustalW software (Thompson et al., 1994). Evolutionary distances were calculated using the method of Jukes and Cantor (Jukes et al., 1969) and phylogenetic tree was constructed following Tamura et al. (2007).

RESULTS AND DISCUSSION

The lesions and hemorrhages were noted at the base of the fins or on the skin due to bacterial infection. Fluid accumulation in the abdomen is a common feature; the liver and spleen were distended and fluid filled. The isolated bacteria were Gram negative, rod shaped and motile (Table 1 and Figure 1). The organisms were positive for oxidase test, catalase test, pyocyanin production, citrate utilization test, gelatin liquefaction test and negative for indole production, hydrogen sulfide (H_2S) production, Vogues-Proskauer test, methyl red test, ornithine decarboxylase, lysine decarboxylase and arginine utilization test (Table 1). However, the bacteria were unable to ferment glucose. The biochemical properties and the characteristic blue-green appearance of culture due to the mixture of pyocyanin (blue) were indicative of *Pseudomonas aeruginosa.*

For many years, analysis of short chain fatty acids (volatile fatty acids, VFAs) has been routinely used in the identification of anaerobic bacteria. Abel and colleagues suggested that microorganisms could be classified by gas chromatographic analysis (Abel et al., 1963). The Sherlock system-based organism identification solely depends on computer comparison of the unknown organism's fatty acid methyl ester (FAME) profile with the profiles of a predetermined library of known isolates with pattern recognition software. The FAME analysis of the bacterial isolate is shown by Figure 2. According to the FAME analysis, the predominant fatty acids found are C16:0, C18:1 and derivatives. The other predominant fatty acids are the hydroxyl fatty acids; C10:0 3-OH, C12:0 2-OH and C12:0 3-OH. With the presence of these biomarkers, the organism was identified as as *P. aeruginosa.*

The 16S rDNA based identification of bacteria potentially offers a useful alternative when phenotypic characterization methods fail (Drancourt et al., 2000; Lee et al., 2002). It is a scientific and objective method of identification of microorganisms (Tang et al., 1998). The nucleotide base composition of the bacterium is shown in Figure 3; 16S rRNA gene sequence analysis revealed that GC content was 54.22% and AT content was 45.78%. Restriction map of 16S rRNA gene sequence of *Pseudomonas sp.* (JN996498) is shown in Figure 4. A phylogenetic tree based on 16S rRNA gene sequence of *P. aeruginosa* (JN996498) with other 16S rRNA gene sequences has been shown in Figure 5. *P. aeruginosa* (JN996498) branched with *Pseudomonas* sp. TSH1(AB508848), *Pseudomonas* sp. 2A12S4 (HQ246300), *Pseudomonas* sp. HPC326 (AY897410), *Pseudomonas* sp. TSH(AB508847), *Pseudomonas sp*.(EF426443), *Pseudomonas* sp. GD6(GU566307), *Pseudomonas otitidis* strain R6(JQ659846), *Pseudomonas* sp. pt15 (EU887721), *Pseudomonas* sp. AHL 2(AY379974), *P. otitidis* isolate 174 (EU244762), *Pseudomonas* sp. *6.14* (EF426442) and *Pseudomonas* sp. FY6 (JX393019). Thampuran et al. (2005) recorded the prevalence and characterization of typical and atypical *Escherichia coli* from fish sold at retail in Cochin, India. El Hadi et al. (2004) also observed the prevalence of potentially pathogenic *Vibrio* species in the marketed sea food in Malaysia. Members of the genus *Pseudomonas* are a ubiquitous group of Gram-negative, rod-shaped and motile bacteria showing metabolic versatility. They can survive in environments hostile

Table 1. Phenotypic and biochemical properties of the bacterial isolate PB 112.

Characteristics	Observation/Result
Shape	Rod shaped
Gram stain	-
Motility	+
Biochemical properties	
Oxidase test	+
Catalase test	+
Indole production	-
Pyocyanin production	+
H_2S production	-
Urease test	-
Citrate utilization	+
Vogues-Proskauer test	-
Methyl red test	-
Ornithine decarboxylase	-
Lysine decarboxylase	-
Arginine utilization	-
Gelatin liquefaction	+
Lipase (Tween 80 hydrolysis)	+
Growth on NaCl (3% - 10%)	+
Gas from glucose	-
Acid form	
Glucose	-
D-Mannitol	-
L-Arabinose	+
Sucrose	-
Sorbitol	+
Growth on medium	
Aeromonas selective medium	+
Growth on TCBS	-
Growth on *Pseudomonas* Agar Base	+

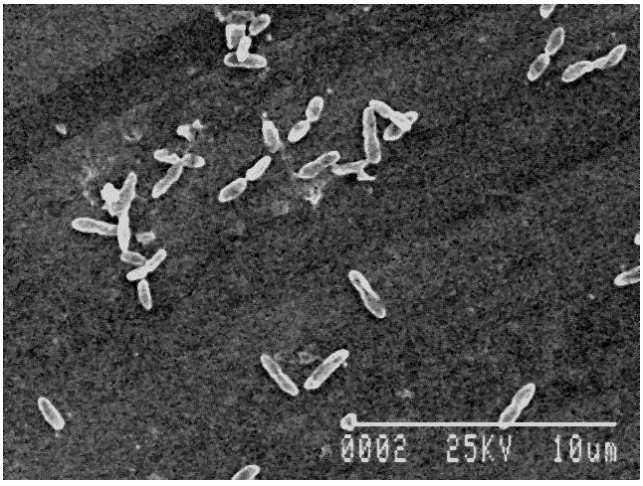

Figure 1. Scanning electron micrograph of bacterial isolate PB112.

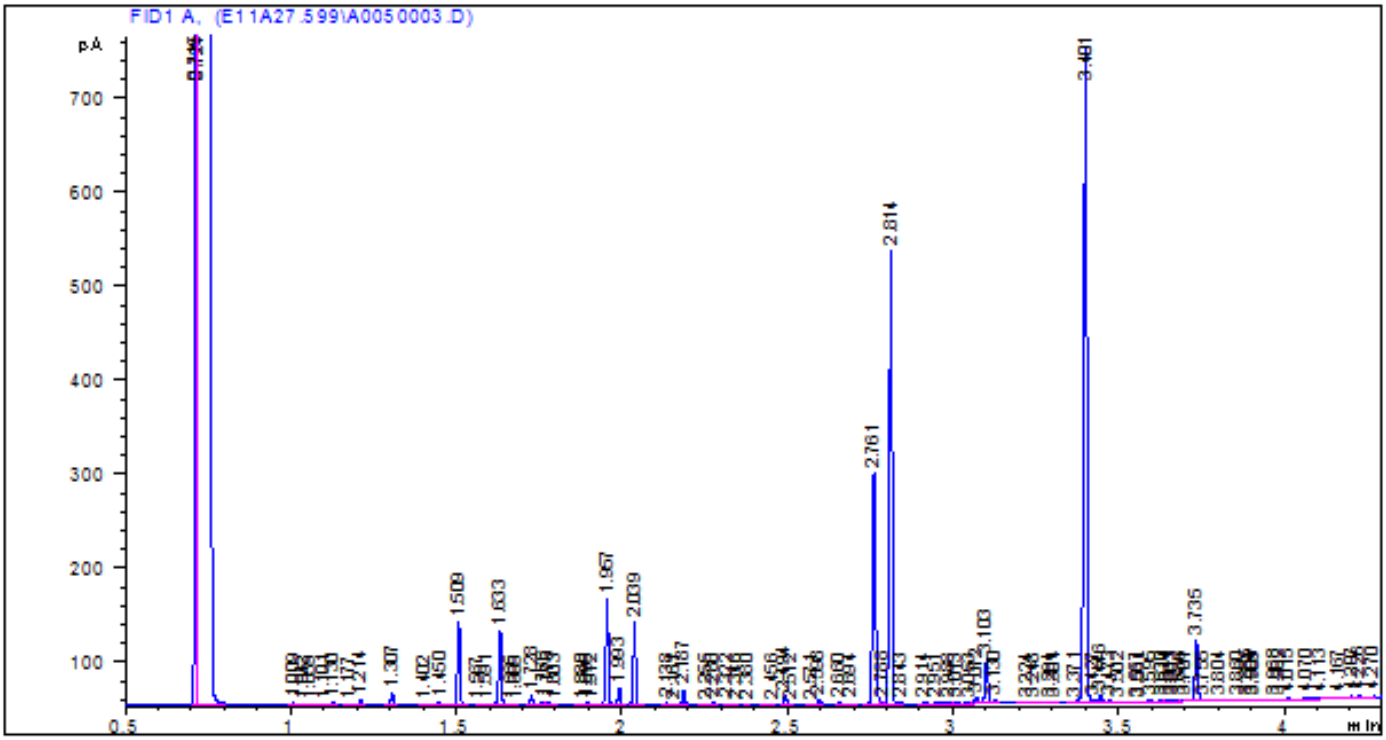

Figure 2. FAME analyses of bacterial isolate PB112.

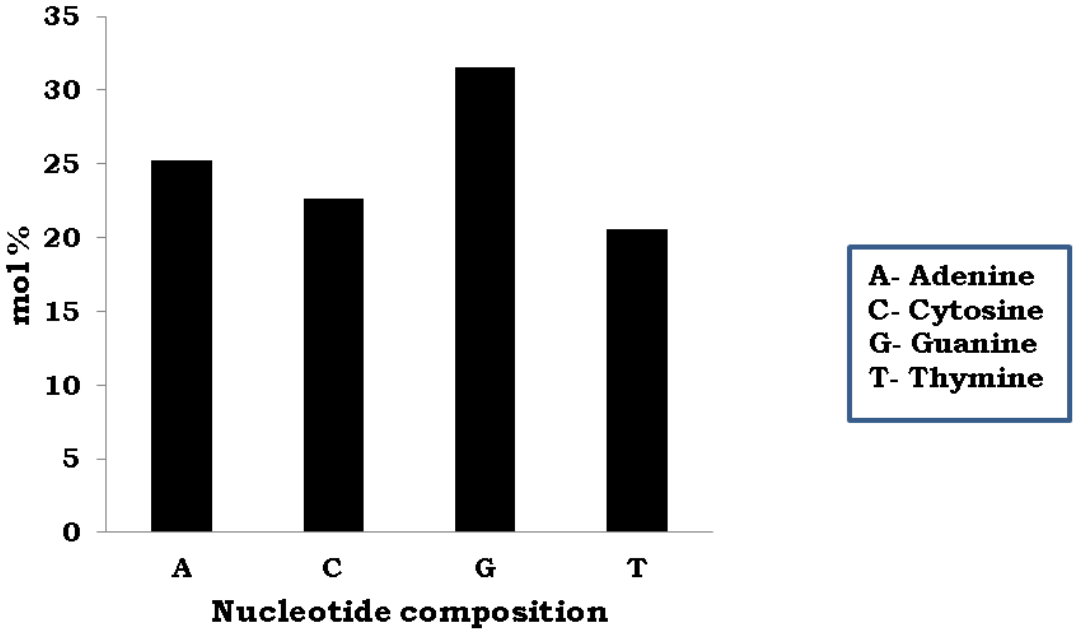

Figure 3. Nucleotide composition of *Pseudomonas aeruginosa* PB 112 (JN996498).

to many other bacteria. This is one of the most diverse bacterial genera, containing over 60 validly described species (Jensins et al., 2004). Some species of *P. aeruginosa* have been reported as opportunistic

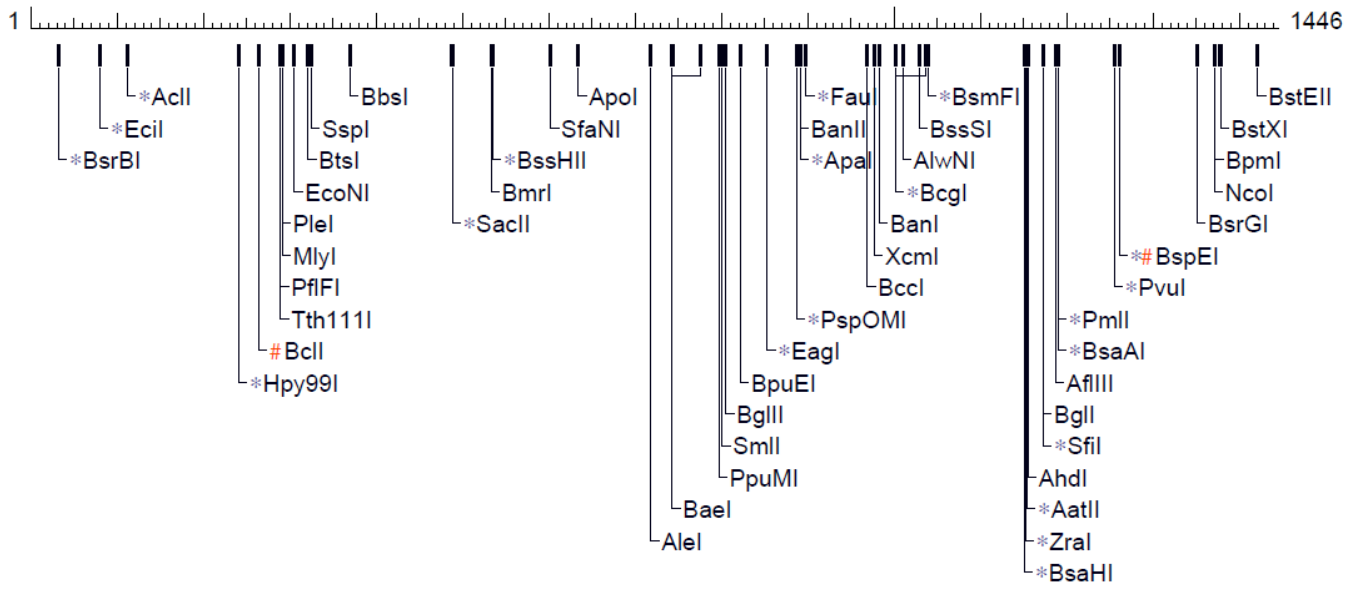

Figure 4. Restriction map of 16S rRNA gene sequence of *Pseudomonas aeruginosa* PB 112 (JN996498).

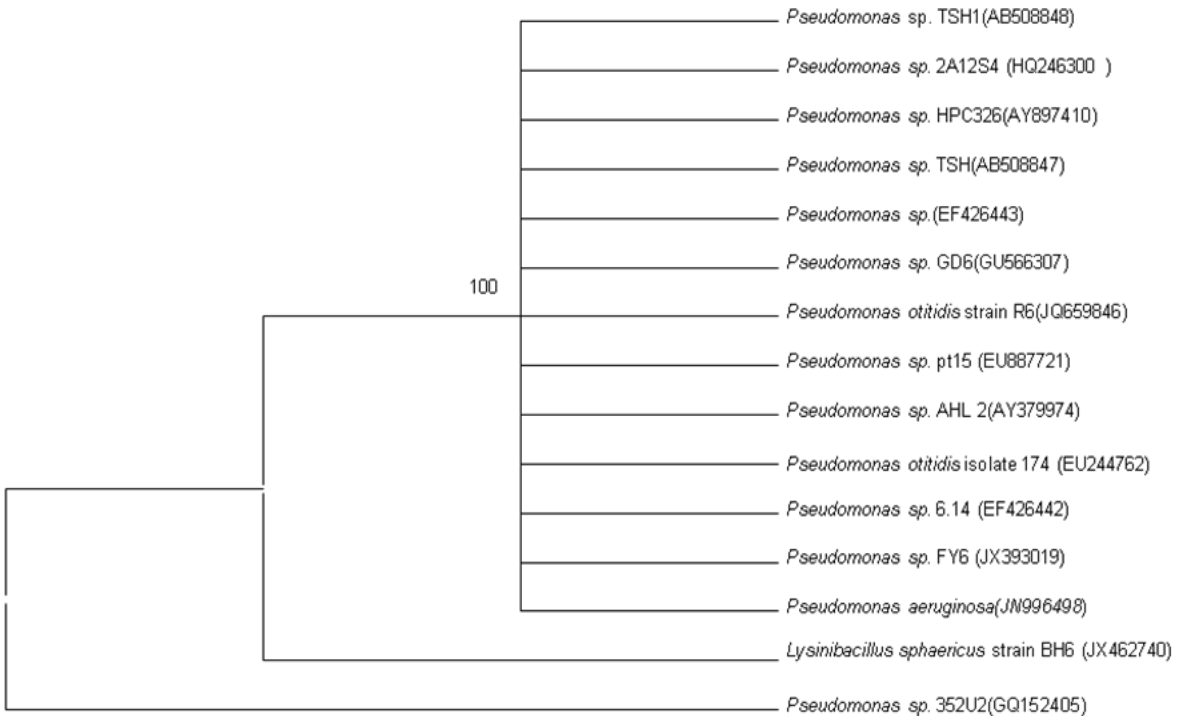

Figure 5. Neighbor-joining tree based on 16S rRNA gene sequence of *Pseudomonas aeruginosa* PB 112 (JN996498) along with other 16S rRNA gene sequences.

pathogens which may create serious and life-threatening infections in patients (Yamagishi et al., 1997; Lang et al., 2004).

In aquaculture, *P. aeruginosa and Pseudomonas fluorescens* especially are the most frequently isolated opportunistic pathogenic species (Shiose et al., 1974; Alderman and Polglase, 1988). Hence, proper control measures should be taken in fish farms to save Bata fish from the infection of *P. aeruginosa.*

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REFERENCES

Abel K, De Schmertzing H, Peterson JI (1963). Classification of microorganisms by analysis of chemical composition. I. Feasibility of utilizing gas chromatography. J. Bacteriol. 85:1039-1044.

Alderman DJ, Polglase JL (1988). Pathogens, parasites and commensals. In: in Freshwater crayfish-Biology, management and exploitation, Holdich DM and Lowry RS, Editors, Timber Press: Portland, Oregon.

Austin B, Austin DA (1999). Bacterial fish pathogens: Disease of farmed and wild fish, 3rd edn. Praxis Publishing Ltd, Chichester, (revised), pp. 29-32.

Bachere E, Mialhe E, Noel D. (1995). Knowledge and research prospects in marine mollusk and crustacean immunology. Aquaculture 132:17-32.

Bullock GL (1965). Characteristics and pathogenicity of a capsulated *Pseudomonas* isolated from goldfish. Appl. Microbiol. 13:89-92.

Drancourtn M, Bollet C, Carlioz A, Martelin R, Gayral JP, Raoult D (2000). 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. J. Clin. Microbiol. 38(10):3623-3630.

El Hadi N, Chen CH, Nishibuchi M (2004). Prevalence of potentially pathogenic *Vibrio* species in the seafood marketed in Malaysia. J. Food Prot. 67(7):1469-1475.

Hossain MA (2008). Isolation of pathogenic bacteria from the skin ulcerous symptomatic gourami (*Colisa lalia*) through 16S rDNA analysis. Univ. J. Zool. Rajshahi Univ. pp. 21-24.

Jeffrey D, Wisotzkey JD, Jurtshuk P, Fox GE, Gabriele D, Poralla K (1992). Comparative Sequence Analyses on the 16S rRNA (rDNA) of *Bacillus acidocaldarius, Bacillus acidoten-estris, and Bacillus cycloheptanicus* and Proposal for Creation of a New Genus, *Alicyclobacillus* nov. Int. J. Syst. Bacteriol. 42:166-170.

Jensins LJ, Skovgaard M, Sicheritz-Ponten T, Hansen NT, Johansson H, et al. (2004) Comparative genomics of four *Pseudomonas* species. in Pseudomonas:Genomics, Lifestyle and Molecular architecture, Ramos J-L, Editor, Juan-Luis Ramos Kluwer Academic Plenum: New York.

Jukes TH, Cantor CR (1969). Evolution of protein molecules. In Mammalian Protein Metabolism. Edited by Munzo. New York, Acad. Press. pp. 21-132.

Lang AB, Horn MP, Imboden MA, Zuercher AW (2004). Prophylaxis and therapy of *Pseudomonas aeruginosa* infection in cystic fibrosis and immunocompromised patients. Vaccine 22:S44-48.

Pelczar MJ, Bard RC, Burnett GW, Conn HJ, Demoss RD, Euans EE, Weiss FA, Jennison MW, Meckee AP, Riker AJ, Warren J, Weeks OB (1957). Manual of microbiological methods. Society of American Bacteriology. McGraw Hill Book Company, Inc., New York. p. 315.

Roberts RJ (1978). Fish Pathology 2nd ed. Bailliere Tindall, London.

Saitou N, Nei M (I987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406-425.

Shiose J, Wakabayashi H, Tominaga M, Egusa S (1974). A report on a disease of cultured carp due to a capdulated *Pseudomonas*. Fish Pathol 9:79-83.

Somsiri T, Soontornvit S (2002). Bacterial diseases of cultured tiger frog (*Rana tigrina*). Diseases in Asian Aquaculture IV, Fish Health Section, Asian Fisheries Society, Manila. pp. 15-17.

Tamura K, Dudley J, Nei M, Kumar S (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24:1596-1599.

Tang YW, Ellis NM, Hoopkins MK, Smith DH, Dodge DE, Persing DH (1998).Comparison of phenotypic and genotypic techniques for identification of unusual aerobic pathogenic Gram-negative Bacilii. J. Clin. Microbiol. 3674-3679.

Thampuran N, Surendraraj A, Surendran PK (2005). Prevalence and characterization of typical *Escherichia coli* from fish sold at retail in Cochin, India. J. Food Prot. 68(10):2208-2211.

Thompson JD, Higgins DG, Gibson TJ (1994). ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673-4680.

Uma A, Reddy YK, Meena S, Saravanabava K, Muralimanohar B (2007). Application of 16S rDNA amplification and sequencing for detection of fish and shrimp bacterial pathogens. Ind. J. Comp. Microbiol. Immunol. Inf. Dis. 28:7-9.

Verschuere L, Rombaut G, Sorgeloos P, Verstraete W (2000). Probiotic bacteria as biological control agents in aquaculture. Microbiol. Mol. Biol. Rev. 64:655–671.

Vincze T, Posfai J, Roberts RJ (2003). NEB cutter: a program to cleave DNA with restriction enzymes Nucleic Acids Res. 31:3688-3691.

Wiklund T, Bylund G (1993). Skin ulcer disease of flounder *Platichithys flesus* in the northern Baltic sea. Dis. Aquat. Org. 17:165-174.

Yamagishi Y, Fujita J, Takigawa K, Negayama K, Nakazawa T (1997) Clinical features of *Pseudomonas cepacia pneumonia* in an epidemic among immunocompromised patients. Chest 103:1706-1709.