ISSN: 2319-8753

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 2, Issue 10, October 2013

Studies on Ulcerative Disease Caused by *Providenciastuartii*Bacteria in Indian Major Carp, *Labeo rohita* (Ham.)

R. Ramkumar[1], M. Anandhi[2], C. Rajthilak[3], T.Natarajan[4], P. Perumal [5]*

Research scholars, Department of Biotechnology, Periyar University, Periyar Palkalai Nagar, Salem – 636 011, Tamil Nadu, India [1, 2, 3, 4]

Professor, Department of Biotechnology, Periyar University, Periyar Palkalai Nagar, Salem – 636 011, Tamil Nadu, India [5*]

Abstract: Rohu (*Labeo rohita*) is a species of fish of the carp family cyprinidae. A survey was carried out from September, 2012 to August, 2013 to study the prevalence of bacterial ulcerative disease in *L. rohita*, cultivated in a freshwater farm located at Moongilthuraipattu Village of Villupuram District, Tamil Nadu, India. To mimic the infection in healthy fish, organs such as liver, gill and ulcerative skin were used to prepare the inoculum in PBS buffer. The inoculum was injected into healthy animals through intramuscular injection, immersion challenge and oral route. The tissue supernatant from infected rohu fish was screened for isolation and screening of the causative organism responsible for ulcerative lesion in *L. rohita*. Based on the biochemical, morphological and molecular features the bacterial culture isolated from infected rohu was tentatively identified as *Providencia* species like bacterium which was further confirmed by the analysis of its 16S rRNA gene using PCR and DNA sequencer and it has been identified as *P. stuartii*. Further, its partial sequence was deposited in GenBank (KF155520.1).

Key words:*Labeo rohita, Providencia stuartii*, 16S rRNA gene, GenBank (KF155520.1)

I. INTRODUCTION

Providencia species are found in multiple animal reservoirs, including flies, birds, cats, dogs, cattle, sheep, guinea pigs, penguins, and are resident oral flora in reptiles such as pythons, vipers, and boas. *Providencia* species are also found commonly in soil, water and sewage. Examples of *Providencia* infections in animals include neonatal diarrhea due to *P. stuartii* infection in dairy cows and enteritis caused by *P. alcalifaciens* infection in dogs. *P. rettgeri* has been isolated in crocodiles with meningitis/septicemia and in chickens with enteritis [1]. *P. heimbachae* has been isolated in penguin feces and from an aborted bovine fetus [2]. The genus *Providencia*, belonging to the family Enterobacteriaceae, consists of 9 species, namely *P. alcalifaciens, P. stuartii, P. rettgeri, P. rustigianii, P. heimbachae, P. vermicola, P. sneebia, P. burhodogranariea*and *P. thailandensis* [1], [3-6]. In human, *Providencia* species have been isolated from urine, stool, blood, sputum, skin and wound cultures. One case study has described *P. stuartii* as the etiology of infective endocarditis,[7].Papadogiannakis [8] studied on the *P. stuartii* infection with severe skin ulceration and cellulitis isolated from a dog.Among *Providencia* species, *P. rettgeri* is the only species isolated from farmed fish (*Hypophthalamichthysmolitrix*) in Israel. Till date, it is the only report implicating this organism as a fish pathogen[9]. In view of acknowledging this, *P. vermicola* was the first species isolated from freshwater fish, *L. rohita* and its partial 16S rRNA sequence was deposited in GenBank by the authors of this study (accession no. KF155518.1.). In the present study, moribund young *L. rohita* with clinical signs of ulcer on the abdomen and pectoral fin surfaces was observed in freshwater fish farms. A work was undertaken to isolate the causative organism responsible for mortality of Indian major carp, rohu. Experimental infection for high mortality of *L. rohita* was carried out to reproduce the infection in healthy fish using the bacterial cultures isolated from the infected fish. Llobrera and Gacutan[3] studied the *Aeromonas hydrophila* associated with ulcerative disease epizootic in Laguna de Bay, Philippines. John Thomas[11] studied the ulcerative disease caused by *A. caviae*-like bacterium in Indian catfish, *Clariasbatrachus*. The

ISSN: 2319-8753

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 2, Issue 10, October 2013

gram negative, rod shaped-bacterium isolated from Indian freshwater carp, *L. rohita* was tentatively identified as *P. stuartii*. In order to acquire more information on the taxonomic position, DNA from the *Providencia* sp. was isolated, polymerase chain reaction was performed and 16S rRNA gene of the sample was amplified and sequenced to confirm the species affecting the freshwater fish, *L.rohita*. The partial gene sequence of 16S rRNA belongs to *P. stuartii* was deposited in GenBank, with accession no. KF155520.1

II. MATERIALS AND METHODS

A. Collection and Maintenance of Naturally Infected L. rohita

The moribund freshwater fish *L. rohita* (56-69g) with the signs of red ulcers on the abdomen and base of pectoral fin were collected from a fish farm located at Moongilthuraipattu Village of Villupuram District, Tamil Nadu and transported within three hours at 30°C in live state to the laboratory in an aerated polythene bag. In laboratory, the infected animals were maintained in 75 L aquarium tanks with tap water at a temperature of 27-30°C. The animals were fed with commercial fish feed.

B. Physico-chemical Parameters of Water

The physico-chemical parameters of the pond water such as temperature, pH, dissolved oxygen (DO), biochemical oxygen demand (BOD) and chemical oxygen demand (COD) of the pond were determined following standard protocols [12].

C. Isolation and Characterization of Bacterial Strain from Infected L. rohita.

The organs liver, gill and ulcerative skin from naturally infected fish were dissected out and homogenized with sterile PBS buffer. The diluted samples were plated on nutrient agar, *Aeromonas* agar and trypticase soy agar by spread plate technique and incubated at room temperature for 24-48 h. Overriding colonies (from trypticase soy agar) were selected and again streaked on trypticase soy agar for locating pure cultures. These pure cultures were maintained on trypticase soy agar for further biochemical and molecular characterization studies. Biochemical characterization was done and the bacterial isolates were identified according to Bergey's manual of Determinative Bacteriology[13].

D. Molecular Identification of Bacterial Strain

1) Bacterial Genomic DNA Isolation:

Genomic DNA was extracted and purified by following the standard techniques [14], [15] with slight alterations. Luria broth was inoculated with a loop full of pure culture and incubated overnight. From this, 1.5 mL of grown culture was centrifuged at 10000 xg for 30 minutes, the resulted pellet was suspended in 600 µL of TE buffer. To this, 45 µL of 10% SDS was added followed by addition of 5 µL lysozyme and mixed well. The mixture was incubated for 1 h at 37° C. After incubation, 500 µL of phenol: chloroform was added and the mixture was mixed well by inverting the tube until the formed two phases completely mix with each other. Then the sample was centrifuged at 10000 xg for 15 min. The aqueous phase was transferred to a new centrifuge tube, to this equal volume of phenol: chloroform was again added and centrifuged at 10000 xg for 15 min. The resulted aqueous phase was mixed with 50 µL of 3M sodium acetate in a new micro centrifuge tube and mixed well. To this, 300 µL of isopropanol was added and mixed gently to precipitate the DNA, then the mixture was centrifuged at 10000 xg for 10min. The resulted pellet was washed with 70% ethanol for 30 sec and centrifuged at 8000 xg for 1-2 min. The obtained pellet was resuspended in 100 µL of TE buffer and stored at 4° C for further analysis. Then the sample (5 µL) was allowed to run in 0.8% agarose gel to confirm the presence of bacterial genomic DNA under UV light.

2) Bacterial PCR and DNA Sequencing:

30-cycle amplification was performed in a DNA thermal cycler (Eppendorf, Germany). For a 30µL reaction: 15µL of *Taq* DNA polymerase PCR master mix, 3µL of amplified DNA, 3 µL of Universal bacterial forward primer

ISSN: 2319-8753

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 2, Issue 10, October 2013

(27f)5'-AGAGTTTGATCMTGGCTCAG,3µL of Universalbacterial reverse primer (1525r) 5'AAGGAGGTGWTCCARCC and 6µL of milli-Q-water was added. DNA sequencing was done by direct sequencing of PCR amplified 16S rDNA gene after purification. DNA sequencing was performed in Sri Ramachandra Medical College, Chennai (India).

E. Collection and Maintenance of Experimental Animals

Disease-free, *Labeo rohita* were collected from the Department of Fisheries, Mettur Dam, Tamil Nadu with no record of ulcerative syndrome. The live fish were transported to laboratory in an aerated bag and maintained in 700 L FRB tank with continuous aeration at room temperature (27- 30°C) with tap water. The animals were fed twice a day with commercial fish feed.

F. Reproduction of Bacterial Infection

Ulcerative skin from the naturally infected fish was cut and homogenized with PBS buffer. The homogenized sample was centrifuged at 1000 xg for 10 min at 4° C. Healthy and active fish were injected intramuscularly with 5U of suspension and maintained for a period of 7days. The control fish were injected intramuscularly with 5U of sterile PBS. Experimental fish were examined often for clinical signs of disease and mortality.

G. Experimental Infection of Bacterial Isolate in Healthy L. rohita

Infectivity of the new bacterial isolate from the naturally infected fish was studied in healthy individuals. Immersion, intramuscular and oral route of administrations were followed to determine the mode of infection and the pathogenicity of bacterial isolate in healthy carp, *Labeo rohita* based on the standard protocols [16], [17].

H. Preparation of Bacterial Inoculum

The bacterial cultures isolated from naturally infected fish was grown on nutrient agar for use in pathogenicity experiments. The pathogenicity of the bacterial isolate was tested by bath immersion, intramuscular injection and oral administration. The bacterial count was determined by standard dilution and plating methods [18].

I. Experimental Infection by Immersion Method

The experiment was carried out by following [11] with some modifications. Healthy fish (12 fish per tank) were reared in aquarium tanks of 75 L capacity containing sterilized freshwater with continuous aeration. Air stones and air tubes were sterilized by immersing them in 5% sodium hypochlorite and by washing them thoroughly with sterilized water before use. The tanks were covered to prevent contamination. Aseptic techniques were used throughout the experiment. Fish were fed with commercial fish feed. For the experimentally induced infection, the fish were exposed to different concentrations of bacterial cells (10^3,10^4, 10^5, 10^6 and 10^7 CFU ml^{-1}). The control consisted of fish exposed to sterilize freshwater alone.

J. Infection by Intramuscular Injection

The experiment was carried out by following [11] with some modifications. Fish (12 per dosage and tank) were maintained in 100 L FRB tank containing sterilized freshwater at room temperature. The bacterial isolate was inoculated into healthy fish through intramuscular injection, near the dorsal fin at doses of 10^3,10^4, 10^5, 10^6 or 10^7 CFU per animal. Control fish were inoculated only with sterile PBS buffer.

K. Oral Infection

Fish were individually secluded in the aquarium tanks and starved for 24 h. Each fish was fed with a piece of fish meat which was injected with 1 ml of bacterial suspension (10^{12} CFU). The fish were fed thrice, with an interval of 8 h. After the last feeding with infected meat, the animals were fed with non-infected meat for 7 days. In the control group, fish were fed only with non-infected meat. In all experiments, animals were examined once in 8 h a day for clinical signs of disease and mortality.

ISSN: 2319-8753

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 2, Issue 10, October 2013

L. Confirmation of pathogenicity

The specific action of the bacterial sample isolated from naturally infected fish as a pathogen was confirmed by re-isolating the bacterium from the liver, gill and ulcerative skin of moribund fish to fulfil Koch's postulates. The samples were inoculated on trypticase soy agar plates by spread plate technique for the isolation of bacterial pathogen. The isolated bacteria were recognized using the earlier described procedure.

III. RESULTSANDDISCUSSION

The physico-chemical features of the fish farm water were determined. The ranges of temperature, pH, DO, BOD and COD were: 26-30 °C, 6.3-8.1, 5.7-7.1 mg/L, 2.36-2.89 mg/L and 183-209 mg/L, respectively. A high temperature of 30 °C was recorded during summer. Death of fish due to bacterial pathogen extended to 99-100% within 2-3 days after the presence of ulcer on the surface of infected fish samples. The clinical signs of the diseased fish contained ulcerative lesions on the abdomen/body surface and bases of the pectoral fin with reddish color on the surface of the infected portions.The bacterial inoculum prepared from ulcerative tissue samples of diseased fish alone produced clinical indication of ulcerative abrasion in the disease free, healthy *L. rohita* fish under experimental condition. Studies were carried out on different organs like ulcerative skin, liver and gill acquired from the infected fish samples. Single prevailing bacterial culture isolated from each of the media was tested for their infectivity in the healthy *L. rohita*, but the bacterial isolate from trypticase soy agar and nutrient agar alone resulted mortality and reproduced the signs of ulcerative lesions in healthy *L. rohita*. This specific bacterial isolate alone was selected and identified based on the colony morphology, biochemical, physiological and molecular identification. Colonies grown on TSA plates were Gram-negative rods, motile, circular, 2.1 – 2.2 mm in diameter, slimy and convex. Colonies are smooth with entire edges and an intense characteristic smell was produced with growth on trypticase soy agar. The biochemical characterization of the bacterial culture resulted in positive for citrate, inositol, glycerol, gelatin, sorbitol, lyxose, D-Mannose, D-Serine, catalase, indole, phenyl alanine, glucose, nitrate reduction, trehalose, and methyl red. Negative results for D-Mannitol, D-Xylose, cellobiose, esculin, sorbitol, pigmentation, oxidase, lactose, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, H_2S, Voges-Proskauer, gas from glucose, L-Arabinose, maltose, raffinose, salicin, D-Xylose, deoxyrinonuclease and lipase.Based on thebiology, biochemical characterization and morphology of the isolated bacteria from infected *L. rohita* was tentatively identified as *P. stuartii* like bacterium. Further confirmation of *P. stuartii*– like bacterium by analyzing its 16S rRNA gene was performed using PCR. For this, bacterial universal primers 27f & 1525r were used for the amplification of 16S rRNA gene and the results wereshown in Fig.1. The sequencing of the strain discovered a homology of 99% with *P. stuartii*. Hence it is a *Providencia* sp. with features of *P. stuartii*.

The exposure of *L. rohita* to *Providencia* like bacterium was tested by immersion, intramuscular injection and oral infection. The maximum concentration of *Providencia* sp. like bacterium (30 x 10^6 CFU ml^{-1}) caused 33.33, 43.33, 56.6, 63.3 and 87.3% of mortalities through immersion method at 18, 30, 48, 84 and 108 h of post exposure, respectively. The LC_{50} value of *Providencia* sp. like bacterium was determined. It was found to be 3.22 x 10^5, 3.69 x 10^5 and 5.85 x 10^6 CFU ml^{-1} at 18, 36 and 54 h of post injectionrespectively.The highest concentration, 54 x 10^5 and 54 x 10^6 possible cells of *Providencia* like bacterium per animal caused 100% death within 96 and 60 h of post infection, respectively. During bath exposure, whereas the lower presentation of 54 x 10^3 and 54 x 10^4 feasible, study bacterial cells, per animal caused 43.33 and 73.3% death within 120 and 108 h of post infection. The LD_{50} value of *Providencia* sp. like bacterium for intramuscular course was defined at different time intervals and was found to be 2.1 x 10^6 and 1.04 x 10^7 per animal after 72 and 96 h of post injection, respectively. There was no mortality in the oral route administered *L. rohita* fish. The infection of *Providencia* sp. like bacterium was established by satisfying Koch's postulate.

Rohu is considered to be the high number of fish cultivated than other major carps in India. It has high market value and are easily available for culture. The risk of diseases in rohu also increased in making the rohu susceptible to diseases in association to the exotic carps [18]. Diseases are the most serious limiting factors in aquaculture, because of increased density of fish in restricted water where the fish pathogens can transmit from one to another [19]. The rohu is

ISSN: 2319-8753

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 2, Issue 10, October 2013

being mainly affected by bacterial pathogens. Among bacterial pathogen, ulcer disease is the major one, followed by columnaris and dropsy.Among bacterial pathogens, *P. rettgeri* was one of them which caused a mass mortality among farmed silver carp, *Hypophthalmichthysmolitrix* in Israel during 1976. Till date, this has been the only report associating this organism as a fish pathogen [9]. This disease tempted severe epidemics of ulcerative diseases in farmed fish. From 2012, an ulcerative disease which is caused by a bacterium has been witnessed in nursery and grow-out ponds located in the Villupuram District of Tamil Nadu, India.The clinical signs of infected rohu fish include large red ulcer lesions on the abdomen and base of pectoral fin. The indications in the infected rohu fish agrees with the reports of [20], [11] who have reported a related clinical indication in other bacterial infections. Isolation and biochemical characterization were performed on organs such as liver, gill and ulcerative skin from infected rohu using nutrient agar (NA), *Aeromonas* agar and trypticase soy agar (TSA). Single pure colony was isolated from TSA and NA plates which exactly revealed the presence of culture colony in the specific medium, which was again studied in healthy rohu fish to confirm that the bacterial sample from infected rohu fish was a pathogen in order to fulfil the Koch's postulates. Many authors have reported that, *Aeromonas* and *Pseudomonas* spp. are causing major infections in *L. rohita* (FAO, 2007) which persuade with severe lesions of ulcerative diseases in fish from Southeast Asia especially in India.In afford with colony morphology, biochemical and molecular identifications the disease caused in *L. rohita* was identified as *Providencia* sp. like bacterium. This is the second report in *L. rohita* for the presence of *Providencia* sp. occurring naturally in cultured freshwater fish. Hence, only one report was available about the infection of *P. rettgeri* isolated and studied in silver carp, *H. molitrix* in 1976 reported by [9]**.** Similar ulcerative lesion induced by *Aeromonas* sp. in many fishes were stated in many reports [21], [22], [11]. Hence in the present study, the bacterial culture *Providencia* sp. like bacterium was confirmed as a pathogen to satisfy Koch's postulates in normal rohu fish. DNA isolation and PCR were executed to detect the bacterial strain at molecular level using universal primers specific to 16S rRNA gene of prokaryotes. The application of 16S rRNA gene sequence is to study the bacterial phylogeny and taxonomy which include important reasons like (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the roles of the 16S rRNA gene over time has not changed, recommending that random sequence changes are a more precise degree of time (evolution); and (iii) the 16S rRNA gene (1500bp) is large enough for informatics purposes [23], [11]. The PCR band (~1500bp) confirmed the presence of *Providencia* sp. Species identification was done using 16S rRNA gene sequencing. Unfortunately, on no account definition for species identification using 16S rRNA gene sequencing exhibited. In not any studies does the definition of a species "match" ever exceed 99% similarity [11]. The species of *Providencia* was identical as *P. stuartii* and it (*Providencia*) is the second report for its infection in *L. rohita*, its 16S rRNA gene partial sequence was deposited in GenBank with accession no. KF155520.1. Whereas, the bacterium *P. vermicola* was first isolated and reported from India by [4] from an infected juvenile nematode, *Steinernemathermophilum*. The authors of this study have submitted the 16S rRNA partial sequence of *P. vermicola* bacteria from *L. rohita*in GenBank with accession no. KF155518.1. The responsibility of rohu fish, *L. rohita* to *P. stuartii* was tested by bath exposure (immersion), intramuscular injection and oral route in *L. rohita*. Finally, the significant gateway of the pathogen is by means of penetration of tissue at the site of wounds or injuries. The expiry by the above experiment showed that the pathogenicity of *P. stuartii* in *L. rohita* depends mainly on the doses and period of exposure.

IV. CONCLUSION

Bacterial diseases are responsible for high mortality in both wild and cultured farm fish. The real role of microorganisms vary from a major pathogen to that of an opportunistic pathogens which makes its host organisms (fish) moribund by commencing infection development. Bacterial flora of fish is directly proportional to its environment. In the present study, clear variations in physico-chemical parameters has been observed which may be considered as conducive condition for the outbreak of disease. Biochemical and molecular characterization of the species confirmed that it is the first report, species *stuartii* of genus *Providencia* causing infection and mortality in the freshwater fish,*L. rohita* infection. From the pathogenicity experiments it is evident that the main portal entry of the pathogen is by means of penetration of tissue at the site of rashes or wounds and the mortality of experimentally infected animals depends on the dosage and time period of exposure.

ISSN: 2319-8753

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 2, Issue 10, October 2013

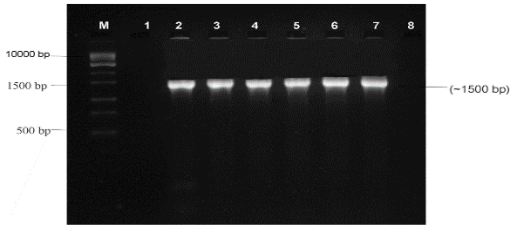

Fig.1Agarose gel showing the amplification of 16S rRNA gene of *P.stuartii*. Lane M-marker; Lane 1- negative control; Lane 2-*P.stuartii* MTCC (4431);

Lane 3to7-*P.stuartii* (isolated from infected fish)

ACKNOWLEDGEMENT

One of the authors[1] thank the Department of Science & Technology, New Delhi, India for providing INSPIRE Fellowship to carry out this study. And the authors of this research work are thankful to the authorities of host Institute for providing necessary facilities throughout the work.

REFERENCES

[1] J.M. Janda and L.S. Abbott, *TheEnterobacteria*, 2nd ed., Washington, D.C: ASM Press, pp. 279-299, 2006.

[2] C.M. O'Hara, A.G. Steigerwalt, D. Green, M. McDowell, B.C. Hill, and D.J. Brenner, "Isolation of *Providencia heimbachae* from human feces," *J. Microbiol.*, 37, 9, 3048-3050, 1999.

[3] H.E. Muller, C.M. O'Hara, G.R. Fanning, F.W. Hickman-Brenner, J.M. Swenson, and D.J. Brenner, "*Providencia heimbachae*, a new species of Enterobacteriaceae isolated from animals," *Int. J. Syst. Bacteriol.*, 36, 252–256, 1986.

[4] V.S. Somvanshi, E. Lang, B. Straäubler, C. Spröer, P. Schuman, S. Ganguly, A.K. Saxena and E. Stackebrandt, "*Providencia vermicola* sp. nov., isolated from infective juveniles of the entomopathogenic nematode *Steinernemathermophilum*," *Int. J. Syst. Evol. Microbiol.*, 56, 629–633, 2006.

[5] P. Juneja and B.P. Lazzaro, "*Providencia sneebia* sp. nov., and *Providencia burhodogranariea* sp. nov., isolated from wild *Drosophila melanogaster*," *Int. J. Syst. Evol. Microbiol.*, 59, 1108–1111, 2009.

[6] S. Khunthongpan, P. Sumpavapol, S. Tanasupawat, S. Benjakul, and A. H-Kittikun, "*Providencia thailandensis* sp. nov., isolated from seafood processing wastewater," *J. Gen. Appl. Microbial*., 59, 185-190, 2013.

[7] P.R. Krake and N. Tandon, "Infective endocarditis due to *Providencia stuartii*," *South Med. J*., 97, 10, 1022-1023, 2004.

[8] E. Papadogiannakis, D. Perimeni, E. Velonakis, V. Kontos, and A. Vatopoulos, "*Providencia stuartii* infection in a dog with severe skin ulceration and cellulitis," *J Small Anim. Pract*., 48, 6, 343-345, 2007.

[9] Y. Bejerano, S. Sarig, M.T. Horne, and R.J. Roberts, "Mass mortalities in silver carp, *Hypophthalmichthysmolitrix* (Valenciennes) associated with bacterial infection following handling," *J. Fish Dis*., 2, 49–56, 1979.

[10] A.T. Llobrera and R.Q. Gacutan, "*Aeromonas hydrophila* associated with ulcerative disease epizootic in Laguna de Bay, Philippines," *Aquaculture*, 67, 3/4, 273–278, 1987.

[11] John Thomas, N. Madan, K.S.N. Nambi, S. Abdul Majeed, A. NazeerBasha, and A.S. Sahul Hameed, "Studies on ulcerative disease caused by *Aeromonas caviae*-like bacterium in Indian catfish, *Clariasbatrachus* (Linn)." *Aquaculture*, 376-379, 146-150, 2013.

[12] APHA, *Standard methods for the examination of water and wastewater*, 16th ed., Washington, DC, USA: 1995.

[13] R.E. Buchanan and N.E. Gibbons, *Bergey's Manual of Determinative Bacteriology*, 8th ed., Williams and Wilkins Co., Baltimore, Maryland: pp.1268.

[14] F. Grimont, and P.A.D Grimont, *Determination of rRNA gene restriction patterns*. In: J. Howard, D.M. Whitcombe editors. *Diagnostic Bacteriology Protocols*. N.J. Totowa: Humana Press, Inc. pp149–164, 1995.

[15] J.E. Clarridge, "Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases," *Clin. Microbiol. Rev*., 17, 4, 840-862, 2004.

[16] E. Egidius, "Vibriosis pathogenicity and pathology-A review." *Aquaculture*, 67, 15–28, 1987.

[17] H.W. Ducklow, H.M. Tarraza Jr., and R. Mitchell, "Experimental pathogenicity of *Vibrio parahaemolyticus* for the schistosome bearing snail *Biomphalariaglabrata*," *Can. J. Microbiol*., 26, 503–506, 1980.

[18] J.H. Lilley, M.J. Philips, and K. Tongutai, *A review of epizootic ulcerative syndrome (EUS) in Asia*. Publ: Aquatic Animal Health Research Institute and Network of Aquaculture Center in Asia-Pacific, Bangkok, Thailand: pp-73, 1992.

ISSN: 2319-8753

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 2, Issue 10, October 2013

[19] Z. Kabata, Z. *Parasites and Diseases of Fish Cultured in the Tropics.* Taylor and Francis Ltd: pp.318, 1985.

[20] T. Lowry and S. Smith, "Aquatic zoonoses associated with food, bait, ornamental and tropical fish," *J Am Vet Med Assoc*, 231, 6, 876–880, 2007.

[21] P.D. Munro, T.H. Birkbeck, and A. Barbour, *Influence of rate of bacterial colonization of the gut of turbot larvae on larval survival*. In: H. Reinertsen, L.A. Dahle, L. Jorgensen, K. Tvinnereim (Eds.), Fish Farming Technology. A.A. Balkema, Rotterdam: pp.85–92, 1993.

[22] H. Sugita, T. Nakamura, K. Tanaka, and Y. Deguchi, "Identification of *Aeromonas* species isolated from freshwater fish with the microplate hybridization method," *Appl. Environ. Microbiol*., 60, 3036–3038,1994.

[23] J.B. Patel, "16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory," *Mol. Diagn*, 6, 313–321, 2001.