

The Open Access Israeli Journal of Aquaculture – Bamidgeh

As from **January 2010** The Israeli Journal of Aquaculture - Bamidgeh (IJA) will be published exclusively as **an on-line Open Access (OA)** quarterly accessible by all AquacultureHub (<http://www.aquaculturehub.org>) members and registered individuals and institutions. Please visit our website (<http://siamb.org.il>) for free registration form, further information and instructions.

This transformation from a subscription printed version to an on-line OA journal, aims at supporting the concept that scientific peer-reviewed publications should be made available to all, including those with limited resources. The OA IJA does not enforce author or subscription fees and will endeavor to obtain alternative sources of income to support this policy for as long as possible.

Editor-in-Chief

Dan Mires

Editorial Board

Rina Chakrabarti	Aqua Research Lab, Dept. of Zoology, University of Delhi, India
Angelo Colorni	National Center for Mariculture, IOLR Eilat, Israel
Daniel Golani	The Hebrew University of Jerusalem Jerusalem, Israel
Hillel Gordin	Kibbutz Yotveta, Arava, Israel
Sheenan Harpaz	Agricultural Research Organization Beit Dagan,
Gideon Hulata	Agricultural Research Organization Beit Dagan,
George Wm. Kissil	National Center for Mariculture, IOLR, Eilat, Israel
Ingrid Lupatsch	Swansea University, Singleton Park, Swansea, UK
Spencer Malecha	Dept. of Human Nutrition, Food & Animal Sciences, CTAHR, University of Hawaii
Constantinos Mylonas	Hellenic Center for Marine Research, Crete, Greece
Amos Tandler	National Center for Mariculture, IOLR Eilat, Israel
Emilio Tibaldi	Udine University Udine, Italy
Jaap van Rijn	Faculty of Agriculture, The Hebrew University of Jerusalem, Israel
Zvi Yaron	Dept. of Zoology, Tel Aviv University, Tel Aviv, Israel

Published under auspices of
**The Society of Israeli Aquaculture and
Marine Biotechnology (SIAMB),
University of Hawai'i at Mānoa Library**

&

**University of Hawai'i at Mānoa
Aquaculture Program**
in association with
AquacultureHub

<http://www.aquaculturehub.org>



UNIVERSITY
of HAWAII
MĀNOA
LIBRARY



AquacultureHub
educate • learn • share • engage

ISSN 0792 - 156X

© Israeli Journal of Aquaculture - BAMIGDEH.

PUBLISHER:

Israeli Journal of Aquaculture - BAMIGDEH -
Kibbutz Ein Hamifratz, Mobile Post 25210,
ISRAEL

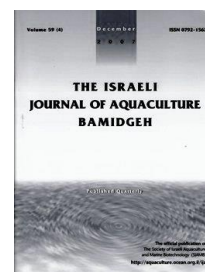
Phone: + 972 52 3965809

<http://siamb.org.il>

Copy Editor **Ellen Rosenberg**



The IJA appears exclusively as a peer-reviewed on-line open-access journal at <http://www.siamb.org.il>. To read papers free of charge, please register online at [registration form](#).



Isolation, Identification, and Pathogenicity of a Virulent *Aeromonas jandaei* Associated with Mortality of Farmed *Pangasianodon hypophthalmus*, in India

Kundan Kumar^{1*}, K. Pani Prasad¹, Gayatri Tripathi¹, R.P. Raman¹, Saurav Kumar¹, Meena Tembhurne², and C.S. Purushothaman¹

¹ Aquatic Environment and Health Management Division, Central Institute of Fisheries Education, Versova, Mumbai, 400061, India

² Fisheries Resources, Harvest and Post Harvest Division, Central Institute of Fisheries Education, Versova, Mumbai, 400061, India

(Received 26.5.2014, Accepted 2.8.2014)

Key words: *Pangasianodon hypophthalmus*, 16rRNA, LD₅₀, *Aeromonas jandaei*, histopathology

Abstract

The present study was conducted to investigate the bacterial pathogens involved in the mortality of cultured *Pangasianodon hypophthalmus*. Diseased fish samples were collected from Maharashtra, India for the isolation of pathogenic bacteria. The pathogenic bacterial isolates were identified using 16S rRNA gene sequencing analysis which revealed that they were 99% identical with *Aeromonas jandaei*. The bacterial isolates were further characterized using biochemical methods. The lowest bacterial dose which caused 50% cumulative mortality (LD₅₀) in *Pangasianodon hypophthalmus* was 8.84 X 10⁵ CFU per fish. This was achieved by injecting the fish intraperitoneally with pure culture of *A.jandaei* isolated from diseased fish. Histopathological studies revealed necrosis hemorrhaging, and other cellular alterations of different tissues of collected organs viz. gill, liver, and kidney of *P. hypophthalmus*, observed with the diseased conditions.

* Corresponding author: email: kundankumar@cife.edu.in

Introduction

The genus *Aeromonas* comprises a collection of gram negative, catalase and oxidase-positive, glucose-fermenting motile bacteria (Abbott et al. 2003). It is a ubiquitous inhabitant of aquatic ecosystems, such as freshwater, coastal water, and sewage, although it has also been described in connection with fish and human diseases (Wu et al. 2007). *Aeromonads* have a broad host range, and have often been isolated from humans with diarrhea, as well as from fish with 'hemorrhagic septicemia' or 'motile *Aeromonas* septicemia' (Rhaman et al. 2001). Within the *aeromonas* group, *Aeromonas hydrophila*, *A. sobria*, *A. veronii* and *A. salmonicida* are considered important fish pathogens (Subagja et al. 1999). In fish, *A. hydrophila* is generally considered an opportunistic pathogen or secondary invader, but in some conditions, such as stress or poor immune function, it may also be a primary pathogen (Thorpe and Roberts, 1972).

P. hypophthalmus is an excellent species for inland aquaculture as it is fast growing and is highly desirable in European markets (Singh and Lakra, 2012). *P. hypophthalmus* is a hardy species and can be cultured at very high stocking density but is susceptible to various microbial and parasitic diseases resulting in poor growth and economic loss to farmers. Disease outbreak is a major constraint in intensive aquaculture systems resulting in mortality and reduced yield. With the increase in the culture of *P. hypophthalmus* in India, the outbreak and spread of bacterial infections is a major concern. Although information from India on bacterial diseases is sparse, studies from other Asian countries suggest that a number of bacterial diseases infect this species. Among these are bacillary necrosis in *Pangasius* (BNP) caused by *E. ictaluri*, and motile *aeromonas* septicemia (MAS) caused by *Aeromonas* spp., mainly *A. hydrophila*, *A. sobria*, and *A. caviae* (Subagja et al. 1999; Ferguson et al. 2001; Crumlish et al. 2001; Yuasa et al. 2003). There has been no systematic study of the diseases of farmed *P. hypophthalmus* in India. Identification of the diseases and their causative agents will help develop strategies to control and prevent the incidence of diseases in farmed *P. hypophthalmus* thus reducing loss to farmers. The aim of this study was to investigate disease outbreaks in cultured *P. hypophthalmus* and identify the etiological agent based on morphological and biochemical characteristics, and 16S rRNA sequence analysis. Pathogenicity was confirmed by challenging healthy *P. hypophthalmus* through intra-peritoneal injection of pure culture of the bacteria, isolated from diseased fish.

Material and Methods

Isolation of bacteria from moribund fish. Diseased samples of *P. hypophthalmus* from Maharashtra, India, were collected for the isolation of pathogenic bacteria. The moribund fish, showing reddish lesions near the pectoral fins and belly region, were used for bacterial isolation. Fish were sacrificed by giving them an overdose (50 µl/l) of anesthesia (clove oil) (Merck, Germany). The fish were then cleaned with alcohol and dissected. Swab samples of liver, kidney, and spleen were taken aseptically and plated directly on BHI Agar (Himedia); then fresh tissues were preserved in neutral buffered formalin (NBF) for histopathology. The plates were incubated for 24 h at 28 °C for colonies to appear. Single colony isolates were selected and re-streaked on fresh BHI plates to obtain a pure culture.

Molecular identification by 16s rRNA sequencing. The genomic DNA was extracted from the bacteria using Qiagen Kit (USA) following manufacturer's instruction. The 16S rRNA gene amplification was performed with primer UFF2: 5' GTTGATCATGGCTCAG 3' and URF2: 5' GGTTCACTTGTTACGACTT 3' (Weisburg et al. 1991). Amplification was performed in thermal cycler (ESCO, USA) using the following programme: 1 cycle of 94 °C for 5 min, 35 cycles of 94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 90 sec, and a final cycle of 72 °C for 10 min. The amplified products were checked through 1% agarose gel. Then, the amplified product was purified using Qiagen kit (USA) and sequenced with Bangalore Genei (India). The 16S rRNA sequences were blast-analyzed with the NCBI database and Ribosomal Database Project.

Bacterial identification and characterization using biochemical methods. The strain that caused the clinical symptoms and mortality was first identified by Gram staining and

then classified according to the methods in Bergey's Manual of Determinative Bacteriology (Krieg and Holt, 1994).

Determination of LD_{50} of isolated bacteria. To determine the cumulative mortality and the lowest bacterial dose which caused 50% mortality (LD_{50}) in *P. hypophthalmus*, fish were divided into seven groups with 20 fish in each group. The experiment was conducted in 150 l plastic tanks. The pathogenic bacteria were sub-cultured in 100 ml BHI broth and the suspension was static-incubated at 28 °C for 16 hours. After incubation, the culture was centrifuged for 15 minutes at 5000 rpm. The supernatant was discarded and pellets were washed twice with NS and finally re-suspended in 10 ml NS. The bacterial count was determined by standard dilution and plating methods. The suspension was serially diluted 10X to obtain the bacterial concentration of 10^4 to 10^9 CFU/ml. The fish in groups 1-6 were injected with 0.2 ml of bacterial suspension intraperitoneally with final concentration of 2×10^3 , 2×10^4 , 2×10^5 , 2×10^6 , 2×10^7 , and 2×10^8 CFU/ml per fish, respectively. The control group (Group 7) was injected with 0.2 ml of NS. The experiment was carried out in duplicate. Fish mortality was recorded every 24 hours for 14 days. The injected bacterial isolate as a pathogen was re-isolated from the kidney of moribund fish to satisfy Koch's postulates. Using the cumulative mortalities, LD_{50} was calculated following Reed and Muench (1938),

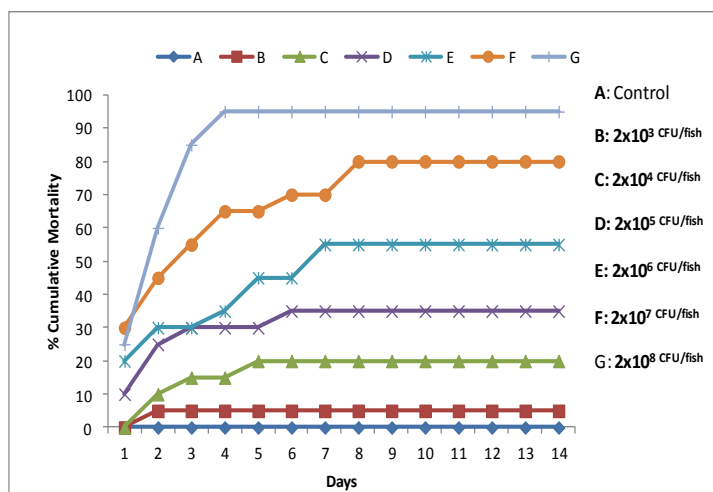
Histopathology. After observing and recording external symptoms, tissues from the infected fish were collected for histological analysis. Internal organs such as liver, kidney, and gill from the fish sampled were collected and fixed in 10% NBF for histopathological studies. The preserved tissues were cut into 1-2 mm pieces and washed overnight under a gentle flow of tap water. The washed tissues were dehydrated with a series of different concentrations of alcohol and cleared in xylene; these were embedded into paraffin following the impregnation technique (Leica EG 1140H, Germany). The paraffin-embedded tissue was sectioned at 5- μ m thickness using a microtome (Leica RM 2125RT, Germany) and stained with hematoxylin and eosin (Luna, 1968). Pathological changes manifested in the tissue sections were noted and microphotographs were taken (Olympus CX-31, Japan), wherever necessary.

Results

Molecular identification and biochemical characteristics of *A. jandaei*. The amplified 16S rRNA gene from isolated bacteria was sequenced and BLAST analyzed and the results demonstrated 99% homology with *Aeromonas jandaei* (GenBank Accession Number: JN644526). The biochemical test showed that *A. jandaei* isolates were Gram negative motile rods. Other tests revealed that they were negative for H_2S production, phenylalanine deamination, ornithine decarboxylase, and methyl red, positive for citrate utilization, lysine decarboxylase, VP, and indole. The isolate also shows hemolytic activity in sheep blood agar plates. The molecular sequence analysis and biochemical analysis confirmed that the isolated bacterial sample is *Aeromonas jandaei*.

Cumulative mortality of *A. jandaei* and determination of LD_{50} . The cumulative mortality rate of *A. jandaei* post infection is shown in fig 1.

Fig 1: Cumulative mortality curves for the determination of LD_{50} values in *P. hypophthalmus* challenged with *A. jandaei* by intra-peritoneal injection at different concentrations



There was no mortality 14 days post injection in the control group injected with NS. Most fish challenged by intra-peritoneal injection developed hemorrhagic subcutaneous ulcers of about 0.6-2.2cm in diameter and reddening at injection sites. Pure culture of injected bacterial isolate were re-isolated from the kidney, spleen, and subcutaneous ulcer, and found to be same as that of the injected strain. The kidneys were shrunk, contracted, brittle, and hemorrhagic. None of the control fish exhibited any clinical symptoms of disease. LD₅₀ value of *A. jandaei* is 8.84×10^5 CFU per fish calculated using the Reed and Muench method.

Histopathology. The clinical symptoms of moribund fish are observed as reddish lesions with hemorrhages on pelvic fin and belly region (Fig. 2).



Fig 2: Diseased fish showing hemorrhages in the belly and pelvic fin of *P. hypophthalmus*

Histopathological examination shows loss of secondary lamellae with progressive hyperplasia at the tip of primary lamellae accompanied with mild congestion of the gill tissue section (Fig. 3A&B). The liver section exhibited necrotized hepatocytes with pyknotic nuclei (Fig. 3C). Necrosis in the tubular epithelium was observed in kidney tissue section (Fig. 3D).

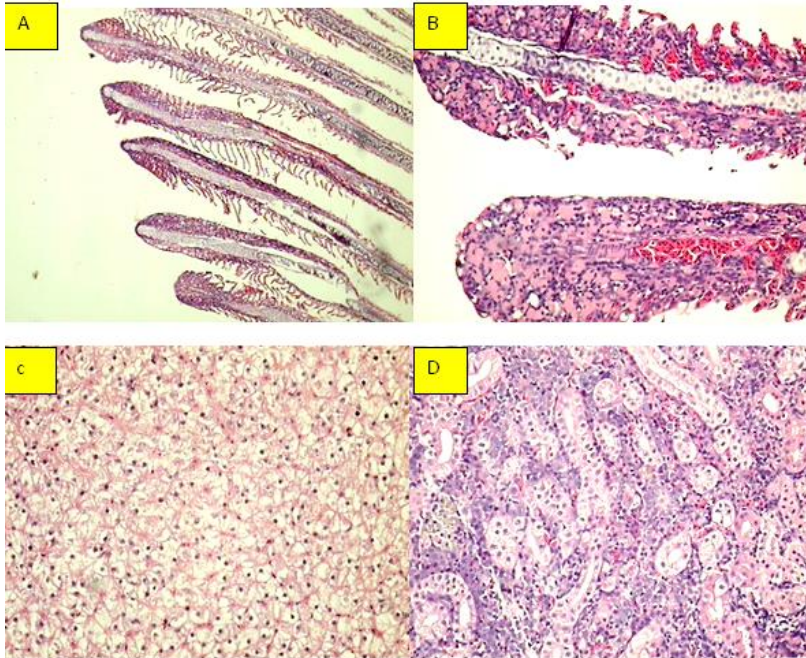


Fig: 3 (A) Gill tissue section showing loss of secondary lamellae with progressive hyperplasia at the tip of primary lamellae, (100X). (B) Marked hyperplasia and fusion of secondary lamellae accompanied with congestion (400X). (C) Liver histological section (natural infected) display necrotized hepatocytes with pyknotic nuclei, (400X) (D) Kidney tissue section (natural infected) exhibiting necrotic tubules (400X). H and E staining.

Discussion

Diseases are major obstacles in the development of sustainable aquaculture practices throughout the world. With the increase in culture of *P. hypophthalmus* in India, the outbreak and spread of bacterial infections is a major concern. In the present study *A. jandaei* bacteria were recovered from both diseased, and apparently healthy, *P. hypophthalmus*. Isolation of these bacteria from ulcerated fish shows its role in the pathogenesis of ulcerative lesions in diseased fish. Our study investigated disease to determine the causative agent involved in the mortality of *Pangasius* stocks in India.

The biochemical test revealed that *Aeromonas jandaei* are gram negative, motile short rods, catalase, and oxidase positive. Other tests indicated that the strains are positive for citrate utilization, VP, methyl red and ONPG, lysine and arginine decarboxylase activity but negative to ornithine decarboxylase enzymes. The differentiating features of *A. jandaei* and *A. hydrophila* were observed in the case of esculine hydrolysis and sucrose fermentation. *A. hydrophila* was positive to both tests whereas *A. jandaei* was negative to both tests. The biochemical results in this study were similar to earlier reports of identification of *Aeromonas* spp. (Carnahan et al. 1991; Abbot et al. 2003; Beaz-Hidalgo et al. 2010).

In the present study gill tissue of diseased *Pangasius* revealed progressive fusion of secondary lamellae, extensive hyperplasia towards the tip of primary lamellae, accompanied by loss of secondary lamellae. Hyperplastic changes in the gill lamellae may be due to cell injury in order to compensate for lost surface area. Infiltration of mononuclear cells along with focal hemorrhages and lamellar fusion on the tips of secondary lamellae were observed in diseased fish. Due to their anatomical location, gills are easily damaged and injury to them causes respiratory dysfunction and osmoregulation. Severe hemorrhagic necrosis of gill epithelium with destruction of gill lamellae and loss of functional integrity of gill tissue with infection of *A. hydrophila* in *Plecoglossus altivelis* has been reported (Huizinaga et al. 1979). Generally, gills are one of the main sites for antigen uptake and retention. Higher infiltration of leukocytes in gill tissue in channel cat fish infected with *A. hydrophila*, have been observed (Grizzle and Kiryu, 1993). This also supports our findings.

The liver tissue sections of infected fish exhibited congested sinusoids as well as pyknotic nuclei in the necrotized hepatocytes, and degenerative changes of pancreatic acinar cells along with vacuolar degeneration. Early acute form of necrosis has been reported to be characterized by diffuse damage of cell membranes and swollen cytoplasm with conspicuous lysis of hepatocytes (Huizinaga et al., 1979). *Clarias batrachus*, *Salmo gairdneri*, and *Ictalurus punctatus*, experimentally infected with *A. hydrophila*, showed signs of necrosis and hemorrhage in the liver and kidney (Angka, 1990; Candan, 1990; Ventura and Grizzle, 1998). *A. hydrophila* toxins have been shown to be cytotoxic (Donta and Haddow, 1978). Release of bacterial toxins cause acute hemorrhage and necrosis of vital organs (primarily liver and kidney), leading to rapid death due to organ failure (Huizinga et al. 1979).

Histological examination of kidney tissues of infected fish reveals necrotic tubules, focal hemorrhages in the interstitium, accompanied with melanomacrophage centers as cellular alteration. Infection caused by aeromonads (members of the genus *Aeromonas*) resulted in diffused necrosis in the kidney (Miyazaki and Kaige, 1985). Diffuse necrosis of the hematopoietic tissue, glomeruli, and renal tubules was observed in channel cat fish (Ventura and Grizzle, 1988). Extensive hemorrhages and diffuse infiltration of mononuclear cell with marked edema in tubules have been reported in post infection of rohu fingerlings infected with *A. hydrophila* (Kumar, 2007). In fish, the kidney is one of the target organs of acute septicemia caused by aeromonads, and this organ is apparently attacked by bacterial toxins and loses its structural integrity (Miyazaki and Kaige, 1985). These findings are in agreement with our results.

In conclusion, our study showed that *A. jandaei* is potentially pathogenic to farmed *P. hypophthalmus* in India therefore more attention should be paid to the effective control of this bacterium through the administration of appropriate antibiotic, or other therapeutic means as well as improving diagnostic measures through the use of advanced molecular techniques. Further research is required to determine the prevalence of this bacterium in culture systems, the virulent factors responsible for infection in fish, and other biotic/abiotic factors that might predispose fish to infection by this opportunistic pathogen.

Acknowledgment

The authors wish to thank Dr. W. S. Lakra, Director and Vice Chancellor, Central Institute of Fisheries Education, Mumbai, India, for providing necessary laboratory and infrastructural facilities required for the present study.

References

- Abbott, S.L., Cheung W.K.W. and Janda J.M.** 2003. The genus *Aeromonas*: biochemical characteristics, atypical reactions and phenotypic identification schemes. *J. Clin. Microbiol.*, 41:2348-2357.
- Angka, S.L.** 1990. The pathology of the walking catfish, *Clarias batrachus* (L) infected intraperitoneally with *Aeromonas hydrophila*. *Asian Fish. Sci.*, 3(3):343-351.
- Beaz-Hidalgo, R., Balboa, S., Romalde, J.L. and Figueras, M.J.,** 2010. Diversity and pathogenecity of *Vibrio* species in cultured bivalve molluscs. *Environmental microbiology reports*, 2(1):34-43.
- Candan, A.A.** 1990. A study on the histopathology of *Aeromonas hydrophila* infections of rainbow trout (*Salmo gairdneri* R.) kept under experimental conditions and the effect of chloramphenicol. *J. Aquat. Prod.*, 4:5-20.
- Carnahan, A.M., Behram S. and Joseph S.W.** 1991. Aerokey II: A flexible key for identifying clinical *Aeromonas* species. *J. Clin. Microbiol.*, 29(12):2843-2849.
- Crumlish, M., Thanh P., Koesling C.J., Tung V.T. and Gravningen K.** 2001. Experimental challenge studies in Vietnamese catfish, *Pangasianodon hypophthalmus* (Sauvage, 1878) exposed to *Edwardsiella ictaluri* and *Aeromonas hydrophila*. *J. Fish Dis.*, 33(9):707-785.
- Donta, S. T. and Haddow A.D.** 1978. Cytotoxic activity of *Aeromonas hydrophila*. *Infection and Immunity*, 21:989-993.
- Ferguson, W.H., Turnbull J.F., Shinn A., Thompson K., Dung T.T. and Crumlish M.** 2001. Bacillary necrosis in farmed *Pangasius hypophthalmus* (Sauvage, 1878) from the Mekong Delta. *Vietnam J. Fish Dis.*, 24 (9):509-513.
- Grizzle, J.M. and Kiryu Y.** 1993. Histopathology of gill, liver and pancreas and serum enzyme levels of channel catfish infected with *Aeromonas hydrophila* complex. *J. Aquat. Anim. Health*, 5:36-50.
- Huizinga, H.W., Esch G.W. and Hazen T.C.** 1979. Histopathology of red-sore disease (*Aeromonas hydrophila*) in naturally and experimentally infected largemouth bass *Micropterus salmoides* (Lacépède, 1802). *J. Fish Dis.*, 2:263-277.
- Krieg, N.R. and Holt J.G.** 1994. Bergy's Manual of Systemic Bacteriology. Volume I, pp. 516-538. Williams & Wilkins, Baltimore
- Kumar, K.** 2007. Clinicopathological and histomorphological studies on induced inflammatory conditions in *Labeo rohita* (Ham.). M.F.Sc. dissertation submitted to Central Institute of Fisheries Education, Mumbai.
- Luna, L. G.** 1968. *Manual of histologic staining methods of the Armed Forces Institute of Pathology*. New York: McGraw-Hill. Pp.
- Miyazaki, T. and Kaige N.** 1985. A Histopathological study on Motile Aeromonad disease of Crucian carp. *Fish Pathol.*, 21:181-185
- Reed, L.J. and Muench H.** 1938. A simple method of estimating fifty per cent endpoints. *American J. Epidemiol.*, 27, 493-497.
- Rhaman, M.H., Suzuki S. and Kawai K.** 2001. The effect of temperature on *Aeromonas hydrophila* infection in goldfish, *Carassius auratus*. *J. Appl. Ichthyol.*, 17:282-285.
- Singh, A.K. and Lakra W.S.** 2012. Culture of *Pangasianodon hypophthalmus* into India: Impacts and present scenario. *Pakistan J. Biol. Sci.*, 15(1):19-26.
- Subagja, J., Slembrouck J., Hung L.T. and Legendre L.** 1999. Larval rearing of an Asian catfish *Pangasius hypophthalmus* (*Siluroidei* *Pangasiidae*): analysis of precocious mortality and proposition of appropriate treatments. *Aquat. Living Resour.*, 12 (I):37-44.
- Thorpe J.E. and Roberts R.J.** 1972. Aeromonad epidemic in brown trout (*Salmo trutta* L.). *J. Fish Biol.*, 4:441-451.
- Ventura, M.T. and Grizzle J.M.** 1988. Lesions associated with natural and experimental infections of *Aeromonas hydrophila* in channel catfish, *Ictalurus punctatus* (Rafinesque). *J. Fish Dis.*, 11:397-407.

- Weisburg, W.G., Barns S. M., Pelletier D. A. and Lane D.J.,** 1991. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol*, 173(2), 697-703.
- Wu, C.J., Wu J.J., Yan J.J., Lee H.C., Lee N.Y., Chang C.M. and Ko W.C.** 2007. Clinical significance and distribution of putative virulence markers of 116 consecutive clinical *Aeromonas* isolates in southern Taiwan. *J. Infect.*, 54(2):151-158.
- Yuasa, K., Edy B K., Panigoro N. and Hatai K.** 2003. First isolation of *Edwardsiella ictaluri* from cultured striped catfish *Pangasius hypophthalmus* in Indonesia. *Fish Pathol.*, 38(4): 181-183.