JOURNAL OF PURE AND APPLIED MICROBIOLOGY, October 2010. Vol. **4**(2), p. 749-753

Virulence Determinants of *Aeromonas hydrophila* Isolated form *Aeromonas septicemia*

A. Lakshmanaswamy[1], M. Karthikeyan[2], A.C. Kiruba Shankari[1], Nancy Sylviya S. Christina[1], N.S. Vasanthi[1] and Arunava Das[1]*

[1]Department of Biotechnology, Bannari Amman Institute of Technology, Sathyamangalam - 638 401, India.
[2]Eurofins Genomics India Pvt Ltd, Whitefield, Bangalore - 560 066, India.

(Received: 30 March 2010; accepted: 08 May 2010)

The study reports the diagnosis of motile *Aeromonas septicemia* caused by *Aeromonas hydrophila* isolated from two cat fish (*Calarius batrachus*) collected from Kodiveri Dam of Erode District, Tamil Nadu. Samples from skin, intestinal contents, kidney, liver, gills, muscle and spleen from the infected fishes were investigated for the isolation of bacteria and their virulence determinants. A total of 14 isolates of *Aeromonas hydrophila* were identified morphologically and biochemically. All the isolates were found to be flagellated and motile. Polymerase chain reaction (PCR) revealed the presence of hemolysin (*ahh*1; 130bp fragment) and aerolysin (*aer*A; 309bp fragment) toxin genes among the isolates. However, out of 14 isolates, 11 (78.5%) isolates were positive for both *ahh*1 and *aer*A genes, and remaining 3 (21.4%) isolates were positive for only *ahh*1 gene. All the isolates were 100% resistance to amphicilin, clindamycin and cephalothin; moderately sensitive to colistin, cholarmphenicol and tetracycline; 100% sensitive to nalidixic acid, nitrofurantoin and cefuroxime. This study confirmed the involvement of both hemolysin and aerolysin toxins producing motile *A. hydrophila* associated with the septicemia in cat fishes. This study suggested that the antibiotics like nalidxic acid, nitrofurantoin and cefroxime can be effective control of *A. hydrophila* infection in cat fishes.

Key words: *Aeromonas hydrophila*, Cat fish, PCR.

In developing countries like India, fishery products contribute a major food item of common man. It is well established that in fisheries, heavy losses are often due to bacterial infections.

Among the bacterial diseases, motile *Aeromonas septicemia* (MAS) in fresh water fishes particularly in cat fishes (*Clarias batrachus*) is very common and is caused by the motile Aeromonads especially *Aeromonas hydrophila* (Yoganath *et al*., 2009).

A. hydrophila spread through water to neighbouring states causing considerable loss to fish farmers. The disease MAS in cat fish is often found in farms where trash fish is used as fish feed or in multiple cultures system where *A. hydrophila* is involved (Areerat *et al*., 1987). Although, cat fish species are banned, however, due to its low price, the consumption rate by the local people is much higher than any other variety of fishes (The Hindu, 2009).

* To whom all correspondence should be addressed.
Tel.: +91-4295-226000/221289 (Extn: 6252/6253/6254)[O],
Fax: +91-04295-226666/ 223775 (O),
E-mail: drarunavadas@rediffmail.com

A. hydrophila is Gram-negative, rod shaped; facultative anaerobe can also sporadically transmit to humans causing wound infections, sepsis and food-borne gastroenteritis (Guadalupe *et al*., 2005). The occurrence of diseases is mainly contributed by the release of two important toxins namely extracellular hemolysin and aerolysin coded by *ahh*1 and *aer*A genes respectively (Chopra *et al*., 1993 & Nordmann & Poirel, 2002). Polymerase chain reaction nowadays has been used a rapid diagnostic techniques to detect the presence of toxin genes and to identify the different strains of *Aeromonas spp* from different variety of fishes (Xia *et al.,* 2003). The present study reports the identification of causative agent associated with the motile Aeromonas septicemia from cat fishes and their virulence determinants.

MATERIAL AND METHODS

History of fish and sample collection

Two fresh water live cat fish (*Clarias batrachus*) showing the symptoms of motile Aeromonas septicemia were collected during the month of December 2009 by casting net from Kodivery Dam in Erode District, Tamil Nadu. Infected fishes were thoroughly examined and any macroscopic and gross lesions observed were recorded. Collected fishes showed swimming abnormalities, pale gills, bloat and skin ulcerations in the body prior to death. The two fishes weighed 343g and 485g respectively. In every case, post-mortem was performed within one to two hours duration after death. Samples were collected aseptically from skin, intestinal contents, kidney, liver, gills, muscle and spleen for microbial investigation.

Isolation and identification of bacteria

All samples were inoculated in sterile Brain heart infusion broth (Himedia, Mumbai) and incubated aerobically at 28^{0}C for 24h. Cultures from BHI broth were inoculated each time in Aeromonas selective agar (Himedia, Mumbai) and Aeromonas dextrin agar plates (Himedia, Mumbai) and incubated at 28^{0}C for 24h. Bacterial colonies were purified based on the size, shape, colour and patterns of haemolysis on blood agar and were subjected to Gram's and flagella staining. All the isolates were identified based on the indole production, esculin hydrolysis, Voges–Proskauer test, cytochrome oxidase, catalase test, acid production from L-arabinose, lactose, sucrose, salicin, m-inositol, D-manitol, triple sugar iron agar slant test for gas and acid production from glucose, sucrose and lactose and hydrogen sulphide production (Altwegg, 1989).

Detection of virulence genes by polymerase chain reaction

To study the virulence of the organism, isolates of *A. hydrophila* were tested to detect the aerolysin gene (*aer*A) and hemolysin gene (*ahh1*) by PCR. The specific forward and reverse primer pairs for *ahh*1 genes of 130bp were 5/-gccgagcgcccagaaggtgagtt-3/ and 5/-gagcggctggatgcggttgt-3/ (Wang *et al*., 2003) and *aer*A genes of 309bp were 5/-caagaacaagttcaagtggcca-3/ and 5/-acgaaggtgtggttccagt-3/ (Wang *et al*., 2003) were commercially synthesized (Bangalore Genei, Bangalore). *A. hydrophila* (MTCC *646) and *E. coli* strain were used as a positive and negative controls respectively.

Freshly grown bacterial colonies from Aeromonas selective agar (Himedia, Mumbai) plates were suspended in 200µl of Milli-Q water in a micro centrifuge tube, gently vortexed and boiled for 15 min in a water bath, after centrifugation at 10000 g for 5 min, supernatant was used as a template DNA. The amplification was carried out in 25µl reaction volume containing 12.5 µl of 2× PCR master mix (Promega, USA) containing 4mM magnesium chloride, 0.4 mM of deoxynucleotide triphosphates (dNTPs), 0.5U of *Taq* DNA polymerase, 150mM trishydrochlroric acid, pH 8.5 (Promega, USA), 2µM concentration of primers (*ahh1*-F and *ahh1*-R), 1.5µM concentration of primers (*aer*A-F, *aer*A-R,) and 2.5 µL of template DNA. The PCR reactions were performed in thermal Cycler (Eppendorf, USA). For both genes, after initial denaturation at 94^{0}C for 5 min, the amplification cycle had denaturation, annealing and extension at 94^{0}C, 56^{0}C and 72^{0}C for 30s, 30s and 30s respectively. Final extension was done at 72^{0}C for 10 min. The PCR amplicons (5µl) were electrophoresed in 1.5% agarose gel in TAE (tris-acetic acid-EDTA, pH 8) buffer, stained with ethidium bromide and observed in gel doc system (Universal Hood, BIORAD, Italy).

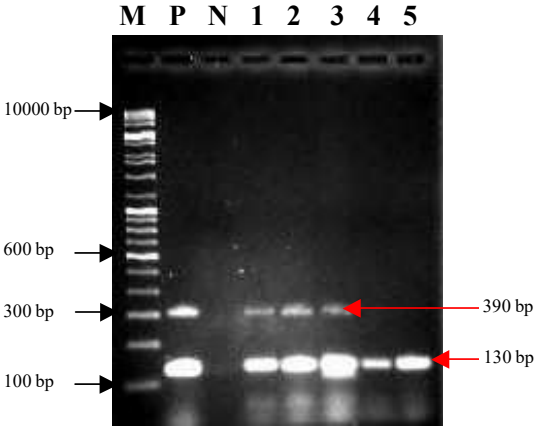
Antimicrobial susceptibility test

Antimicrobial susceptibility test was performed by agar diffusion method (Bauer *et al.,* 1966) using nine commercially available antibiotics. The antimicrobial agents used were Ampicillin (10µg), cefuroximec (30µg), cephalothin (30µg), chloramphenicol (30µg), clindamycin (2µg), colistin (10µg), nalidixic acid (30µg), nitrofurantoin (100µg) and tetracycline (30µg) (Hi media, Mumbai). Data were deleted if poor or no growth occurred in plate or if any contamination was evident.

RESULTS AND DISCUSSION

The symptoms and postmortem findings were suggestive of motile aeromonas septicemia also reported earlier (Wang *et al*., 2003 & Guadalupe *et al*., 2005). Samples on isolation yielded only pure bacterial colonies showing beta hemolysis on sheep blood agar was observed. Isolates were Gram negative, rod shaped positive for indole production, catalase and oxidase test. All the isolates were found to be flagellated and motile. Upon detailed bacteriological investigation, 14 isolates were identified as *A. hydrophila* (Shome *et al*., 2005).

PCR analysis revealed that out of 14 isolates tested, 11 (78.5%) isolates were positive for both *ahh*1 (130bp fragments) and *aer*A (309bp fragments) genes and remaining 3 (21.4%) isolates were positive for only *ahh*1 gene of *A. hydrophila* (Table 1, Fig. 1). Similar detection of *ahh*1 and *aer*A genes from the isolate of *A. hydrophila* associated with motile aeromonas septicemia in various fishes by PCR has been reported (Nordmann & Poirel, 2002) and often single isolates carrying genes for multiple toxins has been also reported (Chopra *et al*., 1993). In this study, out of 14 isolates, *ahh*1 and *aer*A genes were detected in 14 (100%) and 11 (78.5%) respectively. None of the isolates were positive for *aer*A gene alone. These results clearly



Lane P: Positive control (*A. hydrophila* MTCC *646);
Lane N: Negative control (*E. coli*);
Lane 1-3: Field isolates positive for both *ahh*1 and *aer*A genes;
Lane 4-5: Field isolates positive for *ahh*1 gene,
Lane M: High range DNA ruler.

Fig. 1. Detection of *ahh*1 (130bp) and *aer*A (309bp) genes in *A. hydrophila* by PCR

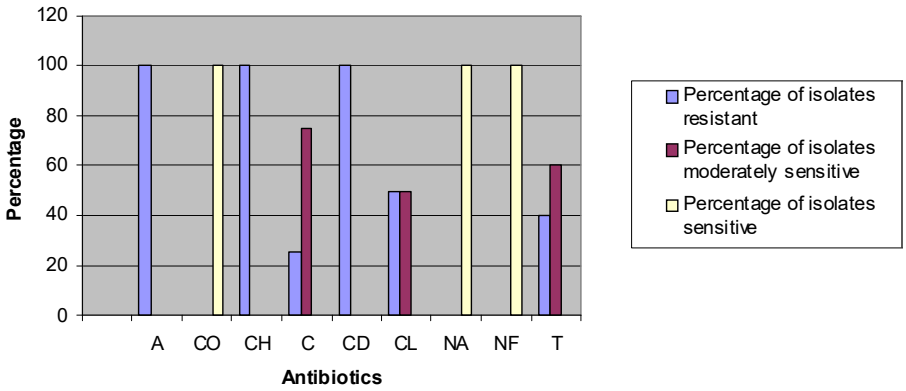


Fig. 2. Antibiotic susceptibility test of 14 *A. hydrophila* isolated from motile Aeromonas septicemia in catfish. A= ampicillin (10µg), CO= cefuroximec (30 µg), CH= cephalothin (30µg), C= chloramphenicol (30µg), CD= clindamycin (2µg), CL= colistin (10µg), NA= nalidixic acid (30µg), NF= nitrofurantoin (100µg) and T= tetracycline (30µg).

Table 1. Sample details and toxin genes of 14 *Aeromonas hydrophila* isolated from Motile Aeromonas Septicemia

S. No.	Sample No	Sample nature	Isolate No	Source with weight (Gram)	Health Condition	Detection of toxin genes by PCR	
						*ahh*1	*aer*A
1	MAS-S1	Skin	AH7e	Cat fish 1 (343)	Motile Aeromonas	+	-
2	MAS-G1	Intestinal contents	AH1d		Septicemia	+	+
3	MAS-IC1	Kidney	AH1h			+	-
4	MAS-L1	Liver	AH4h			+	-
5	MAS-K1	Gills	AH9c			+	-
6	MAS-M1	Muscle	AH8f			+	+
7	MAS-SP1	Spleen	AH8h			+	-
8	MAS-S2	Skin	AH8d	Cat fish 2 (485)	Motile Aeromonas	+	-
9	MAS-IC2	Intestinal contents	AH1a		Septicemia	+	+
10	MAS-K2	Kidney	AH1c			+	-
11	MAS-L2	Liver	AH3a			+	-
12	MAS-G2	Gills	AH1g			+	-
13	MAS-M2	Muscle	AH1b			+	-
14	MAS-SP2	Spleen	AH4e			+	-

+: detected; -: not detected

suggested that *ahh*1 gene is the predominant virulence factor in Aeromonas septicemia in cat fish. This is in complete agreement with a previous observation demonstrating that all the *A. hydrophila* isolates possess *ahh1*, while some carried *aerA*, but none had *aerA* alone (Wang *et al*., 2003 & Shome *et al*., 2005). The presence of both *ahh*1 and *aerA* genes in all virulent *A. hydrophila* strains were earlier reported (Heuzenroeder *et al*., 1999 & Chacon *et al*., 2003).

All the isolates were 100% resistance to amphicilin, clindamycin and cephalothin; moderately sensitive to colistin, cholarmphenicol and tetracycline; 100% sensitive to nalidixic acid, nitrofurantoin and cefuroxime (Fig. 2). This result agrees with the earlier finding reported by Carnahan *et al*., (1991) & Pasquale *et al*., (1994). Based on present investigations, it is suggested that the antibiotics such as nalidixic acid, nitrofurantoin and cefuroxime could be effectively used to control the *A. hydrophila* as 100% of the test strains have been found sensitive to these antibiotics *in vitro*. This study confirmed the involvement of both hemolysin and aerolysin toxins producing motile *A. hydrophila* associated with the septicemia in cat fishes. Further, molecular analysis of the pathogen and the role of both *aer*A and *ahh*1 genes in association with the disease is required to be understood for undertaking the development of control measures, especially for the formulation of cost effecting vaccine.

ACKNOWLEDGEMENTS

This study is a part of the M. Tech project work of the first author. All the authors gratefully acknowledge the Bannari Amman Educational Trust and Principal Bannari Amman Institute of Technology, Sathyamangalam, Erode District, Tamil Nadu, India for providing the facilities to work.

REFERENCES

1. Yoganath, N., Bhakyaraj, R., Chanthru, A., Anbalagan, T. and Mullainila, K. Detection of virulence gene in *Aeromonas hydrophila* isolated from fish samples using PCR techniques. *Global J Biotech and Biochem.,*

2009; **4**(1): 51-53.
2. Areerat, S. Clarias culture in Thailand. *Aquaculture*, 1987; **63**(4): 355-362.
3. The Hindu, http://www.thehindu.com/2009/08/23/stories/2009082354400600.htm.
4. Guadalupe, M.A., Cesar, H.R., Gerardo, Z., Marla, J.F. and Graciela, C.E., *Aeromonas hydrophila* clinical and environmental ecotypes as revealed by genetic diversity and virulence genes. *FEMS Microbiol Lett*, 2005; **242**(2): 231–240.
5. Chopra, A.K., Houston, C.W., Peterson, J.W. and Jin, G.F. Cloning, expression, and sequence analysis of a cytolytic enterotoxin gene from *Aeromonas hydrophila*. *Canadian J. Microbiol.*, 1993; **39**(1): 513–523.
6. Nordmann, P., and Poirel, L. Emerging carbapenemases in Gram-negative aerobes. *Clin. Microbiol. Infect.*, 2002; **8**(6): 321-331.
7. Xia, C, Ma, Z., Habibur, M.R., and Zhi-Guang, W. PCR cloning and identification of the haemolysin gene of *Aeromonas hydrophila* from freshwater fishes in China. *J. Aquaculture*, 2003; **229**(4): 45-53.
8. Altwegg, M. and Geiss, H.K. Aeromnonas as a human pathogen. *Crit. Rev. Microbiol.*, 1989; **16**(4): 253-286.
9. Wang, G.K., Tyler, D., Munro, C.K. and Johnson, W.M. Characterization of cytotoxic, hemolytic *Aeromonas caviae* clinical isolates and their identification by determining presence of a unique hemolysin gene. *J. Clin. Microbiol.*, 2003; **34**(12): 3203-3205.
10. Bauer, A.W., Kirby, W.M., Sherris. J.C. and Turck, M. Antibiotic susceptibility testing by a standardized single disk method. *American J. Clin. Pathol.*, 1966; **45**(4): 493-496.
11. Shome, R., Shome, B.R., Mazumder, Y., Das, A., Kumar, A., Rahman, H., Bujarbaruah, K.M. Abdominal dropsy disease in major carps of Meghalaya: Isolation and characterization of *Aeromonas hydrophila*. *Curr. Sci*., 2005; **88**(12): 1897-1900.
12. Heuzenroeder, M.W., Wong, C.Y. and Flower, R. L. Distribution of two hemolytic toxin genes in clinical and environmental isolates of *Aeromonas* spp. *FEMS Microbiol Lett*, 1999; **174**(1): 131–136.
13. Chacon, M.R., Figueras. M.J., Castro-Escarpulli, G., Soler. and Guarro, J., Distribution of virulence genes in clinical and environmental isolates of *Aeromonas* spp. *Antonie van Leeuwenhoek*, 2003; **84**(4): 269-278.
14. Carnahan, A.M., Fanning, G.R., and Joseph, S.W. *Aeromonas jandaei* (formerly genospecies DNA group 9 *A. sobria*), a new sucrose-negative species isolated from clinical specimens. *J. Clin. Microbiol.*, 1991; **29**(3): 560-564.
15. Pasquale, V., Baloda, S.B., Dumontet, S. and Krovacek, K. An outbreak of *Aeromonas hydrophila* infection in turtles (*Pseudemis scripta*). *App. Environ. Microbiol.,* 1994; **60**(5): 1678-1680.