Journal of Animal and Veterinary Advances 13 (13): 828-833, 2014
ISSN: 1680-5593

Distribution of Virulence Associated Genes and Type III Secretion System Genes in Motile Aeromonas

[1]Jee Eun Han, [1]Ji Hyung Kim, [1]Jin Woo Jun,
[1]Hyoun Joong Kim, [1]Sae Kil Yun, [2]Ji Young Chai and [1]Se Chang Park
[1]Laboratory of Aquatic Biomedicine, College of Veterinary Medicine,
Research Institute for Veterinary, Science and Seoul National University, 151-742 Seoul, Korea
[2]Department of Rheumatology, Bundang Jesaeng Hospital, 255-2 Seongnam, Korea

Abstract: A total of 28 Aeromonas strains obtained from water and diseased fish in South Korea were examined for virulence gene screening. Strains were tested by multiplex PCR for the presence of 2 *β-hemolysins* genes and 3 Type III Secretion System (*TTSS*) genes. All *A. hydrophila* strains carried both *aerA* genes and *ahh1* genes and 5 out of the 8 *A. sobria* strains carried *asa1* genes for β-hemolysins. Additionally, 11 strains carried *aopB*, *aopD* or *ascV* genes for TTSS (*aopB* gene was detected in 7 *A. hydrophila* strains and 4 *A. sobria* strains, *aopD* gene was detected in 5 *A. hydrophila* strains and 1 *A. sobria* strains and *ascV* gene was detected in 4 *A. hydrophila* strains and 3 *A. sobria* strains). Interestingly, some sequence of *TTSS* genes from tested *A. hydrophila* showed strong genetic variation to those of the reported *A. hydrophila*, the *aopB* genes of strain (SNUFPC-A3) was 94% homologous to *A. salmonicida* (Accession No. AJ616218.2) those of strains (SNUFPC-A10 and CASAH2) were 98% homologous to *A. sobria* (Accession No. EF215451.1) and the *aopD* genes of strain (CASAH2) was 98 homologous to *A. sobria* (Accession No. EF215451.1).

Key words: *Aeromonas hydrophila*, *Aeromonas sobria*, hemolysins, TTSS, virulence gene

INTRODUCTION

Motile Aeromonas is a ubiquitous Gram-negative bacterium of aquatic environments which has been implicated as a causative agent of motile aeromonad septicemia in a variety of aquatic animals (especially freshwater fish species) (Thune *et al*., 1993; Austin and Adams, 1996). They cause gastro and extra-intestinal infections in humans including septicemia, wound infections and gastroenteritis (Janda, 2002).

The pathogenesis of *Aeromonas* sp. is multifactorial in nature and many virulence determinants are involved sequentially for the bacterium to colonize, gain entry to establish, replicate and cause damage in host tissues, evade the host defense systems, spread and eventually kill the host (Smith, 1995).

Recent studies of Aeromonas virulence have identified a Type III Secretion System (TTSS) in *Aeromonas* species (Burr *et al*., 2002; Yu *et al*., 2004). The TTSS plays an essential role in pathogenicity because it facilitates the delivery of toxins directly into the host cells (Hueck, 1998; Winstanley and Hart, 2001; Ramamurthi and Schneewind, 2002).

Here, researchers report the distribution of selected virulence associated genes (β-hemolysins: ahh1, aerA and asa1 and TTSS: aopB, aopD and ascV) in *A. hydrophila* and *A. sobria* strains, isolated from water and diseased fish samples in South Korea. In addition, sequence variations of identified *TTSS* genes from those of the reported strains were described.

MATERIALS AND METHODS

Bacterial strains: A total of 28 motile *Aeromonas* species (20 *A. hydrophila* strains and 8 sobria strains) used in this study was listed in Table 1. These strains were isolated from the diseased fish (n = 23) and environmental water (n = 5) by Laboratory of Aquatic Animal Medicine, College of Veterinary Medicine, Seoul National University and were previously reported in South Korea (Han *et al*., 2012). Strains were cultured for 20 h on tryptic soy agar (TSA, Difco, USA) at 35°C and were kept frozen at -80°C in tryptic soy broth (TSB, Difco, USA) which contained 20% glycerol, until they were used.

DNA extraction: The whole cell DNA was extracted by harvesting the cells with sterile water followed by 10 min of boiling. After a 3 min of centrifugation, at 10,000 g, the supernatants were collected and 1:100 dilutions in sterile water were utilized as PCR templates.

Corresponding Author: Se Chang Park, Laboratory of Aquatic Biomedicine, College of Veterinary Medicine, Research Institute for Veterinary Science, Seoul National University, 151-742 Seoul, Korea

Table 1: Aeromonas strains used in this study

Strains	Species	Origin	*TTSS* genes	*β-hemolysin* genes
SNUFPC-A2	*A. sobria*	Goldfish (*Carassius auratus auratus*)		
SNUFPC-A3	*A. hydrophila*	Koi (*Cyprinus carpio carpio*)	*aopB*	*aerA*, *ahh1*
SNUFPC-A4	*A. sobria*	Yellow swordtail (*Xiphophorus clemenciae*)	*aopB*	
SNUFPC-A5	*A. hydrophila*	Congo tetra (*Phenacogrammus interruptus*)		*aerA*, *ahh1*
SNUFPC-A6	*A. hydrophila*	Sailfin molly (*Poecilia latipinna*)		*aerA*, *ahh1*
SNUFPC-A7	*A. hydrophila*	Koi (*Cyprinus carpio carpio*)		*aerA*, *ahh1*
SNUFPC-A8	*A. hydrophila*	Cherry salmon (*Oncorhynchus masou masou*)	*aopB*, *aopD*, *ascV*	*aerA*, *ahh1*
SNUFPC-A9	*A. hydrophila*	Cherry salmon (*Oncorhynchus masou masou*)		*aerA*, *ahh1*
SNUFPC-A10	*A. hydrophila*	Glowlight tetra (*Hemigrammus erythrozonus*)	*aopB*	*aerA*, *ahh1*
SNUFPC-A11	*A. hydrophila*	Neon tetra (*Paracheirodon innesi*)	*aopB*, *aopD*, *ascV*	*aerA*, *ahh1*
CASAH1	*A. hydrophila*	Albino catfish (*Clarias* sp.)	*aopB*, *aopD*, *ascV*	*aerA*, *ahh1*
CASAH2	*A. hydrophila*	Albino catfish (*Clarias* sp.)	*aopB*, *aopD*	*aerA*, *ahh1*
CASAH3	*A. hydrophila*	Albino catfish (*Clarias* sp.)		*aerA*, *ahh1*
JUNAH	*A. hydrophila*	Loach (*Cobitis biwae*)		*aerA*, *ahh1*
SNUFPC-A16	*A. sobria*	Neon tetra (*Paracheirodon innesi*)	*aopB*, *aopD*, *ascV*	*asa1*
JUNAS	*A. sobria*	Silver arowana (Osteoglossidae *bicirrhosum*)		*asa1*
SNUFPC-AH1	*A. hydrophila*	Koi (*Cyprinus carpio carpio*)	*aopB*, *aopD*, *ascV*	*aerA*, *ahh1*
SNUFPC-AH2	*A. hydrophila*	Koi (*Cyprinus carpio carpio*)		*aerA*, *ahh1*
SNUFPC-AH3	*A. hydrophila*	Koi (*Cyprinus carpio carpio*)		*aerA*, *ahh1*
SNUFPC-AH4	*A. hydrophila*	Koi (*Cyprinus carpio carpio*)		*aerA*, *ahh1*
SNUFPC-AH5	*A. hydrophila*	Koi (*Cyprinus carpio carpio*)		*aerA*, *ahh1*
SNUFPC-AH6	*A. hydrophila*	Koi (*Cyprinus carpio carpio*)		*aerA*, *ahh1*
SNUFPC-A20	*A. hydrophila*	River		*aerA*, *ahh1*
SNUFPC-A21	*A. hydrophila*	Sewage		*aerA*, *ahh1*
SNUFPC-A26	*A. sobria*	Lake		*asa1*
SNUFPC-A27	*A. sobria*	Lake	*aopB*, *ascV*	*asa1*
SNUFPC-A28	*A. sobria*	Lake	*aopB*, *ascV*	*asa1*

Table 2: PCR primers used for β-hemolysins virulence gene detection

Names	Sequences (5'-3')	Target gene	Reference
AHH1F*	GCCGAGCGCCCAGAAGGTGAGTT	*ahh1*	Wang *et al.* (2003)
AHH1R*	GAGCGGCTGGATGCGGTTGT		
AH-aerAF*	CAAGAACAAGTTCAAGTGGCCA	*aerA*	
AH-aerAR*	ACGAAGGTGTGGTTCCAGT		
ASA1F**	TAAAGGGAAATAATGACGGCG	*asa1*	
ASA1R**	GGCTGTAGGTATCGGTTTTCG		

*Primers for *A. hydrophila* strains; **Primers for *A. sobria* strains

Detection of the virulence genes: For the detection of hemolysin and aerolysin genes (ahh1, aerA and asa1), the Multiplex PCR Method and primers were adapted with the paper from Wang *et al.* (2003) (Table 2).

For the detection of *TTSS* genes (aopB, aopD and ascV), 5 primer sets were designed from the type III secretion system gene of *A. hydrophila* and sobria (Accession No. AY394563.2 and EF215451.1, respectively) in this study (Table 3). The multiplex PCR reaction was performed in a final volume of 50 μL containing 100 ng of DNA, 5 μL 10x Taq-Buffer, 4 μL 10 mM dNTP's, 1 U Taq polymerase (Invitrogen, USA) and 2 pmoL of each primer. After initial denaturation at 95°C for 30 sec, amplification was attempted by 30 cycles under the following conditions: 95°C for 30 sec, 1 min at 53°C and 1.5 min at 72°C. Cycling was followed by a final extension for 7 min 72°C.

Sequencing and phylogenetic analysis of *TTSS* genes: Purified PCR products of *TTSS* genes were sequenced by Macrogen Inc. (Korea) and the sequences were analyzed with the AlignX tool in the Vector NTI program (Invitrogen, USA). BLAST searches were conducted using both the blastn and blastx algorithms, provided by the National Center for Biotechnology Information website (http.//www.ncbi.nlm.nih.gov).

Alignment of the nucleotide sequences was generated with BioEdit Software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) using the amplified *TTSS* gene sequences and known *TTSS* genes obtained from GenBank (GU384672.1, AY394563.2, GU 384671.1, AY528667.1 for *aopB* gene of *A. hydrophila* and EF215451.1 for *aopB* gene of *A. sobria*, AJ616218.2 for *aopB* gene of *A. salmonicida*). Additionally, phylogenetic analysis was conducted using Bioedit Software and Molecular Evolutionary Genetics Analysis (MEGA) 5 Software with bootstrap values calculated from 1,000 replicates. The Neighbor-Joining Method was used to construct the phylogenetic tree.

Nucleotide sequence accession numbers: Newly amplified *TTSS* gene sequences are available in GenBank nucleotide sequence databases with the following accession numbers: JQ690874, JQ690875, JQ690876, JQ690877, JQ690878, JQ690879, JQ690880, JQ690881, JQ690882, JQ690872 and JQ690873 for *aopB* gene (from SNUFPC-A3, A4, A8, A10, A11, A16, A27, A28, AH1 CASAH1 and CASAH2, respectively) and JQ690883, JQ690884, JQ690885, JQ690886, JQ690887 and JQ690888 for *aopD* gene (from CASAH1, CASAH2, SNUFPC-A8, A11, A16

Table 3: PCR primers used for TTSS virulence gene detection

Names	Sequences (5'-3')	Target gene	Accession No.	References
AHAOPBF*	GCTACATCCTGCTTGCCTTC	*aopB*	AY394563.2	In this study
AHAOPBR*	GCATAGATCGCCGTGAAGAG			
ASAOPBF*	AACCCGATCAGCAATGAAAG		EF215451.1	In this study
ASAOPBR*	CACTGCCAACGCAATCTCTA			
AHAOPDF**	TCATTGCCGATACCACCTCAG	*aopD*	AY394563.2	In this study
AHAOPDR**	CCAACCATCATGCTATTCCATCC			
ASAOPDF**	CTTCGATACCAAAGGGGTGA		EF215451.1	In this study
ASAOPDR**	CTCGACCTCGTCTTCTTTCG			
ASCVF*' **	CTGCTCGCTTCGCTACTTG	*ascV*	AY394563.2, EF215451.1	In this study
ASCVR*' **	GCCTACAATCCATGCCAACC			

*Primers for *A. hydrophila* strains; **Primers for *A. sobria* strains

and AH1 respectively) and JQ690889, JQ690890, JQ690891, JQ690892, JQ690871, JQ690893 and JQ690894 for *ascV* gene (from CASAH1, SNUFPC-A8, A11, A16, AH1, A27 and A28, respectively).

RESULTS AND DISCUSSION

This study described the distribution of virulence associated genes (*ahh1*, *aerA*, *asa1*, *aopB*, *aopD* and *ascV*) in the isolated Aeromonas strains. Researchers did not fully discuss the virulence of these strains. However, detection and identification of virulence genes in these isolated Aeromonas enhance and elucidate the potential risk for spreading of virulent strains in an aquaculture system because *Aeromonas* sp. are ubiquitous and inhabit a wide range of environments.

Distribution of hemolysin and aerolysin genes: Aeromonas strains are able to secrete two families of β-hemolysins: aerolysin and related β-hemolysins (Howard *et al*., 1987; Husslein *et al*., 1988; Hirono *et al*., 1992; Chopra *et al*., 1993). In this study, the multiplex PCR assay amplified both *hemolysin* and *aerolysin* genes from the *A. hydrophila* and the *A. sobria* strains and these genes were distributed around 90% of the strains (25 out of 28 strains) (Table 1).

Distribution of *TTSS* genes: TTSS are present in a variety of Gram-negative bacteria (Hueck, 1998) and an Aeromonas TTSS was first reported recently in the fish pathogen *A. salmonicida* (Burr *et al*., 2002; Stuber *et al*., 2003). *TTSS* genes were detected from a half of identified strains (7 *A. hydrophila* strains and 4 *A. sobria* strains) and the distribution of these genes in Aeromonas strains are presented in Table 1.

In the previous study concerning the prevalence of TTSS genes in motile Aeromonas by Vilches *et al*. (2004), the TTSS was less prevalent in the environmental strains (26%) than in the clinical strains (56%). However, *TTSS* genes were evenly distributed in the environmental strains (40%) and the clinical strains (39.1%) in the present study.

Sequences of *TTSS* genes: In amplification of *aopB* gene, sequence of *A. hydrophila* strains (SNUFPC-A8, A11, AH1 and CASAH1) were amplified using the primers (AHAOPBF/R) designed from the type III secretion system genes of *A. hydrophila* (AY394563.2) and highly similar (99% similarity) to those of the reported *A. hydrophila* (Accession No. AY394563.2 and GU384672.1).

Interestingly, the *aopB* gene from *A. hydrophila* strains (SNUFPC-A3, A10 and CASAH2) were not detected using the primers (AHAOPBF/R) but amplified using the primers (ASAOPBF/R), designed from the type III secretion system genes of *A. sobria* (EF215451.1). In addition, amplified sequence of *aopB* gene from the strain (SNUFPC-A3) was more related with *A. salmonicida* (AJ616218.2, 94% similarity) and those of the strains (SNUFPC-A10 and CASAH2) were related with *A. sobria* (EF215451.1, 98% similarity). Compared to *A. salmonicida* (Burr *et al*., 2002; Stuber *et al*., 2003) there are few reports regarding TTSS in motile *Aeromonas* species. Because limited *TTSS* gene sequences were available in the Genbank for motile Aeromonas, identified *TTSS* gene sequences from *A. hydrophila* may be matched to those of the other *Aeromonas* species.

For *A. sobria* strains, 4 strains (SNUFPC-A4, A16, A27 and A28) were positive for *aopB* gene detection and the amplified sequences showed 99% similarity to the *aopB* gene of *A. sobria* (Accession No. EF215451.1), 91% sequence similarity to *A. salmonicida* (Accession No. AJ616218.2) and 87% sequence similarity to *A. hydrophila* (Accession No. AY528667.1).

In the amplification of *aopD* gene, 5 *A. hydrophila* strains (SNUFPC-A8, A11, CASAH1, CASAH2, A16 and AH1) were positive and amplified *aopD* gene sequences from 4 *A. hydrophila* strains (SNUFPC-A8, A11, CASAH1, A16 and AH1) were similar to those of *A. hydrophila* (AY394563.2, 99% similarity). However, CASAH2 was amplified with the primer (ASAOPDF/R) which was designed from type III secretion system genes of *A. sobria* (Accession No. EF215451.1) and showed 98% sequence similarity to the reported *TTSS* gene sequence

of *A. sobria* (Accession No. EF215451.1), 91% sequence similarity to *A. salmonicida* (Accession No. AJ616218.2) and 87% sequence similarity to *A. hydrophila* (Accession No. AY528667.1). In addition, only 1 *A. sobria* strains (SNUFPC-A16) was positive for aopD detection and amplified sequence showed 99% sequence similarity to the reported *TTSS* gene sequence of *A. sobria* (EF215451.1) and also showed 91% sequence similarity to *A. salmonicida* (Accession No. AJ616218.2) and 87% sequence similarity to *A. hydrophila* (Accession No. AY528667.1).

In the amplification of *ascV* gene, in *A. hydrophila* strains (SNUFPC-A8, A11, AH1 and CASAH1), ascV sequence were homologous to those of *A. hydrophila* (98%, AY394563.2), *A. sobria* (93%, EF215451.1) and *A. salmonicida* (92%, AJ616218.2) and in *A. sobria* strains (SNUFPC-A16, A27 and A28), ascV sequence were homologous to those of *A. sobria* (99-100%, EF215451.1) and *A. salmonicida* (98%, AJ616218.2) and *A. hydrophila* (93-94%, AY394563.2).

Phylogenetic analysis of *TTSS* genes: Phylogenetic analysis based on the amplified *TTSS* genes (aopB, aopD and ascV) is shown in Fig. 1-3. As shown in the sequence analysis some *TTSS* gene sequences, among the amplified sequences were not closely matched into the known *TTSS* gene groups.

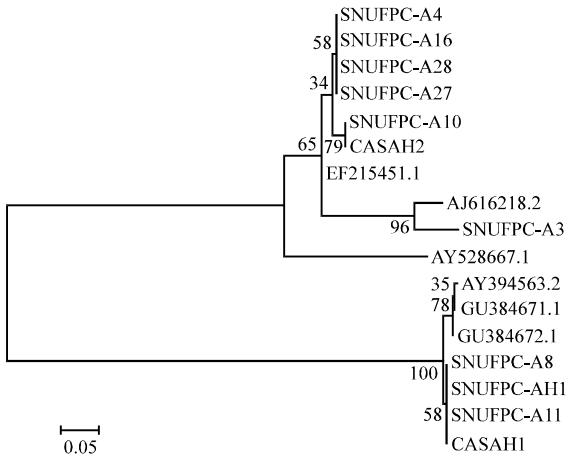

Fig. 1: A phylogenetic tree was conducted on the basis of known aopB genes (GU384672.1, AY394563.2, GU 384671.1, AY528667.1 for *aopB* gene of *A. hydrophila*, EF215451.1 for *aopB* gene of *A. sobria* and AJ616218.2 for *aopB* gene of *A. salmonicida*), 7 amplified *aopB* genes of identified *A. hydrophila* strains (SNUFPC-A3, A8, A10, A11, AH1, CASAH1 and CASAH2) and 4 amplified *aopB* genes of identified *A. sobria* strains (A4, A16, A27 and A28)

Interestingly, in a phylogenetic tree (Fig. 1), aopB sequences of *A. hydrophila* strains (SNUFPC-A3, A10 and CASAH2) were separated from the known *A. hydrophila* group. Amplified aopB sequences of *A. hydrophila* strain (SNUFPC-A3) were clustered into the *aopB* gene of *A. salmonicida* (AJ616218.2) and those of *A. hydrophila* strains (SNUFPC- A10 and CASAH2) were grouped into *A. sobria* (EF215451.1). Additionally, in a phylogenetic tree (Fig. 2), amplified *aopD* gene sequences of *A. hydrophila* strain (CASAH2) was more clustered into a known *aopB* gene sequence of *A. sobira* (EF215451.1) or the *aopB* gene of *A. salmonicida* (AJ616218.2). Other amplified sequences of *TTSS* genes were well matched into the known *TTSS* gene sequences as shown in Fig. 1-3.

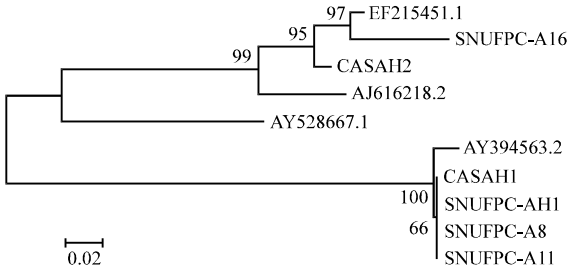

Fig. 2: A phylogenetic tree was conducted on the basis of known *aopD* genes (AY394563.2 and AY528667.1 for *aopD* gene of *A. hydrophila*, EF215451 for *aopD* gene of *A. sobria* and AJ616218.2 for *aopB* gene of *A. salmonicida*), 5 amplified *aopD* genes of identified *A. hydrophila* strains (SNUFPC-A8, A11, CASAH1, CASAH2 and AH1) and 1 amplified *aopD* genes of identified *A. sobria* strain (SNUFPC-A16)

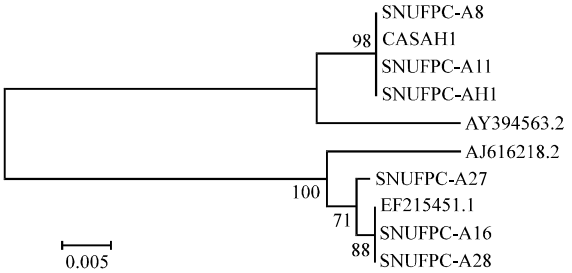

Fig. 3: A phylogenetic tree was conducted on the basis of known *ascV* genes (AY394563.2 for *ascV* gene of *A. hydrophila*, EF215451 for *ascV* gene of *A. sobria* and AJ616218.2 for *ascV* gene of *A. salmonicida*) and 7 amplified *ascV* genes of identified Aeromonas strains (A8, A11, CASAH1, A16, A27, A28 and AH1)

Virulence and pathogenesis are frequently investigated in motile Aeromonas an emergent fish and human pathogen. Especially, TTSS-associated cytotoxicity in motile Aeromonas has been evaluated recently (Vilches *et al*., 2004; Sha *et al*., 2005). However, there has been little data for sequences or polymorphism of *TTSS* gene in these organisms. In the present study, newly identified *aopB* and *aopD* gene of *A. hydrophila* differed from those of *A. hydrophila* but closer to those of *A. sobria* or *A. salmonicida* which means that further investigations were required to get information for *TTSS* genes in *Aeromonas* species. Additionally, distribution and identification of Aeromonas virulence genes in the present study further requires the assessment of virulence phenotypes and complete virulence gene sets.

CONCLUSION

In this study, virulence genes for 2 β-hemolysins such as ahh1, aerA and asa1 and TTSS such as aopB, aopD and ascV were described in motile Aeromonas. Although, researchers only investigated distribution and genetic variation of potential virulence associated genes, identified sequences especially for *aopB* and *aopD* gene showed significant genetic difference between isolates and also with reported strains and these results will provide genetic background for future virulence studies.

ACKNOWLEDGEMENT

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2013R1A1A2006794).

REFERENCES

Austin, B. and C. Adams, 1996. Fish Pathogens. In: The Genus Aeromonas, Auystin, B., M. Altwegg, P.J. Gosling and S. Joseph (Eds.). John Wiley and Sons, Chichester, pp: 197-243.

Burr, S.E., K. Stuber, T. Wahli and J. Frey, 2002. Evidence for a type III secretion system in *Aeromonas salmonicida* subsp. *salmonicida*. J. Bacteriol., 184: 5966-5970.

Chopra, A.K., C.W. Houston, J.W. Peterson and G.F. Jin, 1993. Cloning, expression and sequence analysis of a cytolytic enterotoxin gene from *Aeromonas hydrophila*. Can. J. Microbiol., 39: 513-523.

Han, J.E., J.H. Kim, C.H. Jr. Cheresca, S.P. Shin and J.W. Jun *et al*., 2012. First description of the qnrS-like (*qnrS5*) gene and analysis of quinolone resistance-determining regions in motile *Aeromonas* sp. from diseased fish and water. Res. Microbiol., 163: 73-79.

Hirono, I., T. Aoki, T. Asao and S. Kozaki, 1992. Nucleotide sequences and characterization of haemolysin genes from *Aeromonas hydrophila* and *Aeromonas sobria*. Microb. Pathog., 13: 433-446.

Howard, S.P., W.J. Garland, M.J. Green and J.T. Buckley, 1987. Nucleotide sequence of the gene for the hole-forming toxin aerolysin of *Aeromonas hydrophila*. J. Bacteriol., 169: 2869-2871.

Hueck, C.J., 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol. Mol. Biol. Rev., 62: 379-433.

Husslein, V., B. Huhle, T. Jarchau, R. Lurz, W. Goebel and T. Chakraborty, 1988. Nucleotide sequence and transcriptional analysis of the aerCaerA region of *Aeromonas sobria* encoding aerolysin and its regulatory region. Mol. Microbiol., 2: 507-517.

Janda, J.M., 2002. Aeromonas and Plesiomonas. In: Molecular Medical Microbiology, Volume 2, Part 8, Tang, W.Y., D. Liu, I. Poxton, J. Schwartzman and M. Sussman (Eds.)., Academic Press, San Diego, California, pp: 1237-1270.

Ramamurthi, K.S. and O. Schneewind, 2002. Type III protein secretion in *Yersinia* species. Annu. Rev. Cell. Dev. Biol., 18: 107-133.

Sha, J., L. Pillai, A.A. Fadl, C.L. Galindo, T.E. Erova and A.K. Chopra, 2005. The type III secretion system and cytotoxic enterotoxin alter the virulence of *Aeromonas hydrophila*. Infect. Immun., 73: 6446-6457.

Smith, H., 1995. The State and Future of Studies on Bacterial Pathogenicity. In: Virulence Mechanisms of Bacterial Pathogens, Roth, J.A., C.A. Bolin, K.A. Brogden, F.C. Minion and M.J. Wannemiehler (Eds.). ASM Press, Washington, D.C., pp: 335-357.

Stuber, K., S.E. Burr, M. Braun, T. Wahli and J. Frey, 2003. Type III secretion genes in *Aeromonas salmonicida* subsp. *salmonicida* are located on a large thermolabile virulence plasmid. J. Clin. Microbiol., 41: 3854-3856.

Thune, R.L., L.A. Stanley and R.K. Cooper, 1993. Pathogenesis of gram-negative bacterial infections in warmwater fish. Annu. Rev. Fish. Dis., 3: 37-68.

Vilches, S., C. Urgell, S. Merino, M.R. Chacon and L. Soler *et al*., 2004. Complete type III secretion system of a mesophilic *Aeromonas hydrophila* Strain. Appl. Environ. Microbiol., 70: 6914-6919.

Wang, G., C.G. Clark, C. Liu, C. Pucknell and C.K. Munro *et al*., 2003. Detection and characterization of the hemolysin genes in *Aeromonas hydrophila* and *Aeromonas sobria* by multiplex PCR. J. Clin. Microbiol., 41: 1048-1054.

Winstanley, C. and C.A. Hart, 2001. Type III secretion systems and pathogenicity islands. J. Med. Microbiol., 50: 116-126.

Yu, H.B., P.S. Srinivasa Rao, H.C. Lee, S. Vilches, S. Merino, J.M. Tomas and K.Y. Leung, 2004. Type III secretion system is required for *Aeromonas hydrophila* AH-1 pathogenesis. Infect. Immun., 72: 1248-1256.