Journal of Microbiology (2013) Vol. 51, No. 3, pp. 275–282
DOI 10.1007/s12275-013-2437-x

MINIREVIEW

Development of Diagnostic and Vaccine Markers Through Cloning, Expression, and Regulation of Putative Virulence-Protein-Encoding Genes of *Aeromonas hydrophila*

Vijai Singh[1,2*], Dharmendra Kumar Chaudhary[2], Indra Mani[2,3], Rohan Jain[4], and B.N. Mishra[5]

[1]*Molecular Diagnostics and Biotechnology Laboratory, Division of Crop Protection Central Institute for Subtropical Horticulture, Lucknow - 227107, India*
[2]*National Bureau of Fish Genetic Resources, Canal Ring Road, PO - Dilkusha, Lucknow - 226002, India*
[3]*Department of Biochemistry, Faculty of Science, Banaras Hindu University, Varanasi - 221005, India*
[4]*Department of Environmental Biotechnology, UNESCO-IWE, Netherlands*
[5]*Department of Biotechnology, Institute of Engineering and Technology, Uttar Pradesh Technical University, Lucknow, India*

(Received August 21, 2012 / Accepted November 14, 2012)

Aeromonas hydrophila **is an opportunistic bacterial pathogen that is associated with a number of diseases in fish, amphibians, reptiles, and humans. In fish it causes several disease symptoms including tail and skin rot, and haemorrhagic septicemia; in human it causes soft-tissue wound infection and diarrhoea. The pathogenesis of *A. hydrophila* is multifactorial, but the mechanism is unknown so far. It is considered to be mediated by expression and secretion of extracellular proteins such as aerolysin, lipase, chitinase, amylase, gelatinase, hemolysins, and enterotoxins. A number of the putative virulence-protein-encoding genes that are present in the genome of *A. hydrophila* have been targeted by PCR for molecular diagnosis. These significant genes are also targeted for over-production of proteins by cloning and expression methods. In this review, we emphasize recent progress in the cloning, expression, and regulation of putative virulence-protein-encoding genes of *A. hydrophila* for a better understanding of the pathogenesis and also help to provide effective strategies for control of diseases.**

Keywords: *Aeromonas hydrophila*, PCR, cloning, expression, diagnostic, vaccine

Introduction

Fish and fishery products are an important food component in the human diet all across the globe. These food products are a rich and inexpensive source of protein for poor populations. In Asia, on average, 30% of the total animal protein intake is derived from fish. Among Southeast Asian countries, fish protein provides 45% of the total protein consumed (Prein and Ahmed, 2000). No wonder these products are regarded as "poor man's protein" (Williams, 1996). Apart from being good protein sources, fish and fishery products have a lot of medicinal importance (Din *et al.*, 2004). Over the last two decades, there is an increasing awareness concerning nutritional and health benefits of fish consumption. However, aquaculture is facing many problems such as overcatching feed fish, disease outbreaks, shrinking of natural reserves due to urbanization, dumping of untreated industrial waste water in freshwater reserves – to name a few (Karl *et al.*, 1995; Carr *et al.*, 2004). Among these problems, microbial infections pose a major impediment to successful fish farming and stocking (Singh, 2012). Single infections often affect the whole population converting it to disease carrier, which in turn leads to periodic outbreaks of active disease, high rates of mortality and resultant huge losses to the economy. Therefore, there is a pressing need to prevent or control pathogenic infections in fish populations.

One of the major culprits for infection of aquatic organisms is *Aeromonas hydrophila*, which is a Gram-negative, motile and rod-shaped bacterium belonging to the family *Aeromonadaceae* (Colwell *et al.*, 1986). *A. hydrophila* spreads rapidly because of its ubiquitous presence in the aquatic environment, transmitting infection from one host to another. It is associated with hemorrhagic septicemia in cold-blooded animals, including fishes, reptiles, and amphibians (Janda *et al.*, 1994; Austin and Austin, 1999), which too often results in a high rate of mortality in commercial aquaculture (Chakraborty *et al.*, 1987; Hickman–Brenner *et al.*, 1987; Barghouthi *et al.*, 1989; Janda *et al.*, 1994). *A. hydrophila* secretes a number of extracellular enzymes and proteins such as proteases, DNase, RNase, elastase, lecithinase, amylase, lipase, gelatinase, and chitinase (Merino *et al.*, 1995; Pemberton *et al.*, 1997), cytotoxic/cytolytic enterotoxins (Chopra *et al.*, 1993) and three haemolysins (Howard and Buckley, 1985; Hirono and Aoki, 1991; Hanes and Chandler, 1993; Singh *et al.*, 2009, 2010a, 2011), which are considered to be virulence proteins or factors. Wakabayashi *et al.* (1980) reported that virulence of *A. hydrophila* is related to proteolytic casein and elastin hydrolysis. In their research it was shown that *elastin*-positive strains produced lesions and mortality when injected into channel catfish. Chabot and Thune (1991) suggested that the extra-

*For correspondence. E-mail: vijaisingh15@gmail.com; Tel.: +91-522-2841022; Fax: +91-522-2841025

cellular substances such as hemolysins and proteases might also be involved with the *A. hydrophila* pathogenicity mechanism.

The availability of the complete genomic sequence of *A. hydrophila* has allowed the identification of numbers of new virulence proteins that contribute to infection and pathogenesis. There are different types of hemolysin, including aerolysin, also known as β-hemolysin, [causes the complete lysis of red blood cells (RBCs)], α-hemolysin (causes partial lysis of RBCs), thermostable hemolysin and other unknown hemolysins. These proteins do not show any significant sequence homologies (Seshadri *et al*., 2006; Singh *et al*., 2009, 2010b, 2011). All these virulence proteins produced by *A. hydrophila* play important roles in infection of a host, leading to development of diseases. In this review, we discuss recent progress in PCR-based diagnostic methods for molecular identification of *A. hydrophila.* We describe progress in cloning, expression and regulation of putative virulence-protein-encoding genes of *A. hydrophila* for control of infection and disease outbreaks in fish and other animals.

Molecular detection of putative virulence-protein-encoding genes of *A. hydrophila*

Polymerase chain reaction (PCR) is a rapid and sensitive tool that can be used to amplify a specific fragment of a gene from any organism. The idea is to use primers and probes to amplify virulence-protein-encoding genes present in *A. hydrophila*. This approach has been successfully used to determine and identify various *Aeromonas* species isolated from different sources. The aerolysin-toxin gene (*aerA*) is one of the specific genes in *A. hydrophila* detectable by PCR. Aerolysin was conspicuously absent in the *A. caviae* and *A. veronii* groups whereas it was present in 91% of *A. hydrophila* isolates (Aguilera-Arreola *et al*., 2007). After PCR, a small fragment of the aerolysin gene (209 bp) could be amplified only in hemolytic strains of *A. hydrophila*, while PCR amplification was constantly negative in hemolytic *A. sobria*, *A. veronii*, non-hemolytic *A. hydrophila* and *A. caviae* strains (Pollard *et al*., 1990), providing its high specificity.

Baloda *et al.* (1995) studied 89 strains of *A. hydrophila* and *A. sobria* isolated from drinking water, fish and foods. These strains were found positive for production of virulence factors - hemolysin, protease and cytotoxin. Singh *et al.* (2010a) also identified three virulence genes encoding hemolysin (1,416 bp), aerolysin (309 bp), and lipase (760 bp) from *A. hydrophila* isolated from fish and aquatic environmental samples. A summary of virulence-protein-encoding genes detected by PCR assays from *A. hydrophila* and related *Aeromonas* species is given in Table 1. The table provides a list of groups that have primers designed for amplification of conserved regions of virulence-protein-encoding genes. The table also gives the size of PCR products (amplicons) obtained during amplification, and indicates the sensitivity of each set of primers with genomic DNA of *A. hydrophila.*

Species-specific primers have been reported to amplify a 326 bp conserved region of the aerolysin gene for *A. hydrophila*. A total of 25 isolates of *A. hydrophila* were recovered from fish and pond water and studied for detection of the aerolysin gene. The aerolysin gene was detected in 85% of the isolates during the study. The designed primers were highly specific and showed no cross reactivity with other bacterial species such as *E. coli*, *A. veronii*, *V. cholerae*, *Flavobacterium* spp., *Chyseobacterium* spp., and *S. aureus.* This study indicated the high specificity of aerolysin gene primers and showed a sensitivity limit of 5 pg genomic DNA for amplification (Singh *et al*., 2008). The hemolysin gene was detected in *A. hydrophila* by amplifying a small fragment (196 bp) of the conserved region of the gene using specific primers and showed a 2 ng genomic DNA sensitivity (Singh *et al*., 2007). PCR amplification of thermostable hemolysin has been reported in *A. hydrophila* (Singh *et al*., 2011). A PCR assay was developed to detect hemolysin (*ahh1*) and aerolysin (*aerA*) genes of *A. hydrophila* and *A. veronii* bv. sobria. All of the isolates were divided into five genotypes on the basis of the results of a multiplex PCR. Genotype 1, carrying only the *ahh1* gene was 36% of isolates, genotype 2, carrying the *asa1* gene only, was 8.5% of isolates, genotype 3, carrying both *ahh1* and *asa1* genes, was 4% of isolates, genotype 4, carrying the *ahh1* gene and *A. hydrophila aerA* gene, was 37.5% of isolates and genotype 5, not carrying hemolysin genes, was 14% of isolates (Wang *et al*., 2003). Previously, Heuzenroeder *et al.* (1999) reported the distribution of two hemolytic toxins - aerolysin and hemolysin (*hlyA*) of *Aeromonas* sp. - from clinical and environmental samples.

PCR assay was also used to detect the *aerolysin* toxin (*aer*) gene in *Aeromonas* sp. isolated from faeces of a diarrhea patient. Sixty-eight samples, that constituted 11.3% of total samples, were found to possess the *aer* gene. The different *Aeromonas* species possessing the *aer* gene identified by PCR assays included *A. hydrophila* (55.8%), *A. caviae* (17.6%), *A. veronii* (10.2%), *A. schubertii* (4.4%), *A. jandaei* (2.9%), and 8.8% of *A. trota* (Kannan *et al*., 2001). *A. hydrophila* was also

Table 1. Summary of the major virulence proteins coding genes used in detection of *A. hydrophila* and related *Aeromonas* species

Strains	Virulence protein coding genes	Size of amplicons (bp)	PCR sensitivity	References
A. hydrophila	Aerolysin (*aer*)	326	5 pg	Singh *et al.* (2008)
A. hydrophila	Hemolysin (*hem*)	196	2 ng	Singh *et al.* (2007)
A. hydrophila	Lipase (*lip*)	760	<10 CFU and 0.89 pg	Cascon *et al.* (1996)
A. hydrophila	Aerolysin, hemolysin, lipase	309, 1416, 760	NA	Singh *et al.* (2010a)
A. hydrophila	Aerolysin	209	1 ng	Pollard *et al.* (1990)
A. hydrophila	Aerolysin	209	NA	Baloda *et al.* (1995)
A. hydrophila	Hemolysin	130	NA	Wang *et al.* (2003)
A. hydrophila	Hemolysin	309	NA	Wang *et al.* (2003)
A. trota	Aerolysin	622	NA	Khan *et al.* (1999)
A. salmonicida	Surface array protein gene (*vapA*)	421	10 CFU and 5 fg	Gustafson *et al.* (1992)

NA, Not available; CFU, Colony forming unit; ng, nanogram; pg, pictogram; fg, femtogram

isolated from mineral, drinking and thermal waters. The toxicity was confirmed by cytotoxic assays and detection of the aerolysin gene was performed by PCR. Six strains containing the aerolysin gene, isolated from the mineral waters, were found to be cytotoxic, while out of twelve strains isolated from thermal waters, seven were found cytotoxic, and eleven contained the aerolysin gene (Biscardi *et al*., 2002). Krovacek *et al*. (1998) reported *A. hydrophila* septicemia in grey seal (*Halichoerus grypus*) in the Swedish part of the Baltic Sea. *A. hydrophila* was isolated from both lung and spleen samples. All of the *A. hydrophila* isolates produced hemolysin and also showed the identical patterns of biochemical and antibiotic resistance. The aerolysin gene was detected in all isolates by PCR assay.

The frequency of the *aerolysin* (*aerA*), cytotoxic enterotoxin (*act*) and serine protease (*ahp*) genes in *A. hydrophila* isolates from different sources such as clinical cases (n=40), healthy fish (n=22) and from water (n=21) have been used to assess the prevalence of *aerA*, alt and *ahp* genes by PCR assay. These virulence factors occurred among clinical isolates and among isolates from healthy fish and water, with the majority (97.6%) of the strains examined carrying one or more virulence genes (Li *et al*., 2011). There is a recent report on the distribution of virulence genes in *Aeromonas* isolates from diseased fish, healthy controls and water in China. The frequency of the aerolysin (*aer*), cytotonic enterotoxin (*art*), cytotoxic enterotoxin (*act*), temperature-sensitive protease (*eprCAI*) and serine protease (*ahp*) genes in *Aeromonas* isolates were identified by PCR and their pathogenicity was also determined (Hu *et al*., 2012).

Gonzalez-Serrano *et al*. (2002) tested the virulence-protein-encoding genes in 12 strains of *A. hydrophila* and 3 strains of *A. veronii* biovar *sobria*. Ten strains of *A. hydrophila* were found positive for both *aerA* and *hlyA*, while two strains were positive for *aerA* and negative for *hlyA*. All the *A. veronii* biovar *sobria* strains were negative for both potential virulence genes, *aerA* and *hlyA*. Khan *et al*. (1999) reported the species-specific primers for amplifying the aerolysin gene (*aerA*) of *A. trota*. The size of the amplified aerolysin fragment was 622 bp, confirmed by digestion with *Bam*HI restriction endonuclease, which showed the expected size of digested aerolysin. Gustafson *et al*. (1992) reported on PCR assays for detecting *A. salmonicida* from infected fish tissues, feces and tank water of infected fish. The primers gave amplification of the three regions of the gene (*vapA*) (421 bp) for the surface array protein of *A. salmonicida*. The sensitivity of PCR detection of *A. salmonicida* directly from tissues was less than 10 CFU/mg and 5 fg of genomic DNA (Table 1). A new set of primers has been designed for detection of the thermostable hemolysin gene (645 bp) from *A. hydrophila*, and the sensitivity limit for detection of the *TH* gene was 5 pg (Singh *et al*., 2011).

Shibata *et al*. (1997) reported on the cloning and sequencing of the thermostable lipase (*lipAH*) isolated from clinical strains of *A. hydrophila*. Using PCR, *LipAH* was detected in 80% of the isolates. Cascon *et al*. (1996) also reported on PCR amplification of a thermostable lipase gene (760 bp) from *A. hydrophila*. The sensitivity of the PCR assay was less than 10 CFU and 0.89 pg of genomic DNA (Table 1).

Nam and Joh (2007) isolated and identified species of *Aeromonas* from a water sample of a trout farm and diseased fish. In this study, they used different sets of primers to target six virulence-protein-encoding genes of *Aeromonas* species. The detected virulence genes in *Aeromonas* species include aerolysin (*aer*), GCAT (*gcat*), serine protease (*ser*), nuclease (*nuc*), lipase (*lip*), and lateral flagella (*laf*). In *A. sobria*, the most abundant strain identified in the study, the dominant virulence factors were *aer* and *nuc*. Sen and Rodgers (2004) developed six sets of primers for detection of *Aeromonas* species and determined the presence of six virulence-protein-encoding genes in *Aeromonas* sp. such as elastase (*ahyB*), lipase (*pla/lip/lipH3/alp-1*) flagella A and B (*flaA* and *flaB*), and the enterotoxins *act*, *alt*, and *ast*.

Thus, PCR has been extensively used in various studies and it is the most accepted method for identifying and characterizing *Aeromonas* species from samples of very diverse origins.

Gene cloning, expression, and regulation of the hemolytic-protein-encoding gene

Immunoassays are one of the most widely accepted methods for identifying and characterizing pathogens. This method is based on the unique ability of an antibody to bind with high specificity to one or a very limited group of molecules. It requires either antigen or antibody; in fact, the whole bacterial cell can be used in the immunoassay. However, common antigenic epitopes will then show cross-reactivity. To avoid this problem, the specific purified protein is preferable and is of particular importance when used as a diagnostic for *A. hydrophila*. Several research groups have reported the cloning and expression of the virulence-protein-encoding genes of *A. hydrophila*. These virulence proteins can be used as an antigen in the specific immunoassay for diagnostic purposes. We discuss here, recent progress in using virulence proteins in antigen-based immunoassays and also as useful antigens in vaccination against *A. hydrophila* infections.

Zhu *et al*. (2007) developed a recombinant aerolysin protein, the gene for which was originally derived from of *A. hydrophila* isolated from diseased fish. This recombinant aerolysin (*aerA*) was expressed in *E. coli* under the control of the bacteriophage T7 promoter. The coding region present in the gene of *A. hydrophila* XS91-4-1 was cloned and expressed in *E. coli* BL21 cells, excluding the signal peptide. The protein was expressed in a soluble form and purified using His-Bind resin affinity chromatography. Recombinant aerolysin showed hemolytic activity in blood agar and western blot analysis, demonstrating good antigenicity.

In this context, the complete aerolysin gene of *A. hydrophila* has been cloned and expressed into *E. coli* BL21(DE3)-CodonPlus-RP cells. The protein size was 54 kDa as estimated by SDS-PAGE and it was purified by Ni–NTA affinity chromatography. Anti-His antibodies were used to characterize the expressed aerolysin by western blotting and the aerolysin showed hemolytic activity with fish red blood cells (Singh *et al*., 2010b). The 3-D structure of aerolysin protein has been generated using homology modeling methods for targeting the active amino acids by drugs for prevention of oligomerization on the surface of RBCs. It could be a possible step towards the prevention of hemorrhagic septicemia in hosts (Singh and Somvanshi, 2009). The two extracellular

toxins, both aerolysin and hemolysin from *A. hydrophila*, contribute to pathogenesis. Aerolysin is also known as β-hemolysin, which interacts with eukaryotic RBCs and binds to the glycosylphosphatidylinositol (GPI)-anchored protein receptor, and subsequently causes the complete lysis of the cell (Howard *et al*., 1987; Parker *et al*., 1994). Another hemolysin also plays a vital role in the partial lysis of RBCs. These virulence-protein-encoding genes showed 18% sequence homology with each other, but are located at different positions in the genome of *A. hydrophila* (Wong *et al*., 1998). The 3-D structure, which has been determined using X-ray crystallography, is important for better understanding of the role and mechanism of *A. hydrophila* aerolysin action during infections (Parker *et al*., 1994). Chakraborty *et al.* (1986) reported on the aerolysin of *A. hydrophila* AH2 found by screening a cosmid library for hemolytic and cytotoxic activities. A plasmid containing a 5.8-kilobase *Eco*RI fragment of *A. hydrophila* was required for expression of the hemolytic and cytotoxic phenotypes in *E. coli* K-12. Aerolysin was detected as a 54 kDa protein in *E. coli* maxicells harboring plasmids and also by immunoblotting assays.

Gene regulation and attenuating the function of the *aroA* gene of *A. hydrophila* SO2/2 were carried out using mutagenesis. The *aroA* gene was inactivated by the insertion of a DNA fragment containing a kanamycin resistance determinant and introduced by allelic exchange into the genome of *A. hydrophila* AG2 by means of the suicide vector pSUP202. When inoculated intra-peritoneally into rainbow trout, this highly attenuated mutant of *A. hydrophila* had a 50% lethal dose of $>2\times10^8$ CFU. The strain with the AroA mutation was used as a live vaccine for vaccination of rainbow trout and conferred significant protection against the wild-type strain of *A. hydrophila* (Hernanz-Moral *et al*., 1998).

The role of two unrelated hemolysin toxins of *A. hydrophila* was assessed in a suckling mouse by Wong *et al.* (1998). The first hemolysin gene isolated from an *A. hydrophila* A6 cosmid bank encodes a potential gene product of 621 amino acids with a mass of 69.0 kDa. The inferred amino acid sequence showed 89% identity to the AHH1 hemolysin of *A. hydrophila* ATCC 7966 and 51% identity to the HlyA hemolysin of *Vibrio cholerae* EI Tor strain O17. The second hemolysin gene, aerA that encodes aerolysin - a pore-forming toxin (β-hemolysin), was partially cloned by PCR for the purpose of mutant construction. A suicide vector was used to inactivate both the *hlyA* and *aerA* genes in *A. hydrophila* A6. When these two mutants were assessed in the suckling mouse model, only the *hlyA aerA* double mutant showed a statistically significant reduction in virulence, with a 20-fold change in LD_{50}.

The complete ORF of the hemolysin gene was amplified and cloned by Singh *et al.* (2009). The cloned gene was further sub-cloned in pET28a vector and transformed into *E. coli* BL21(DE3)-CodonPlus-RP cells to express it. The expected size of the expressed protein was 68.0 kDa, as estimated by migration in SDS-PAGE. Anti-His monoclonal antibodies have been used to confirm the identity of the recombinant protein by western blotting. In this report, the 3-D structure was generated using homology modeling for targeting the active pockets with drugs. Hironi and Aoki (1991) have cloned and expressed the extracellular hemolysin (*ahh1*) gene of *A. hydrophila* ATCC7966. The nucleotide sequence of this region contained a single ORF of 1734 bp encoding the 577 amino acids of a 63.6 kDa protein. The protein was analyzed by *in vitro* assay, using [^{35}S] methionine-labelled proteins in *E. coli* CSR603 carrying the ahh1 plasmid coding for a 60 kDa polypeptide.

A study by Erova *et al.* (2007) reported a new *hemolysin* gene in *A. hydrophila* that normally produces a *cytotoxic enterotoxin (Act)*. The new *hemolysin* gene (*hlyA*) was cloned, sequenced and over-expressed in *E. coli.* It was found to encode a 49 kDa protein having 439-amino acid residues, and exhibited 96% amino acids sequences homology with *A. hydrophila* subspecies hydrophila ATCC 7966. The *hlyA* gene did not exhibit any homology with other known hemolysin genes nor with the aerolysin gene detected in *Aeromonas* isolates. Chopra *et al.* (1994) constructed genomic libraries for a diarrheal isolate of *A. hydrophila* using cosmid pHC79 and lambda EMBL3 vectors. The cell lysate of an *E. coli* [pSL24] clone caused enterotoxic activity on Chinese hamster ovary (CHO) cells. The enterotoxin was biologically heat labile and lost all activity at 56°C in 20 min.

Chopra *et al.* (1993) cloned and sequenced cytolytic enterotoxin of *A. hydrophila*. N-terminal amino acid residues of the *Aeromonas* cytolytic enterotoxin were used as a probe to screen a genomic library constructed in lambda EMBL3. All biological activities associated with the cytolytic enterotoxin were neutralized by rabbit homologous polyclonal antibodies. SDS-PAGE and western blot of cell lysates of *E. coli* (lambda CH4) revealed a protein band of 52 kDa, using antisera for cytolytic enterotoxin. The homology of cytolytic enterotoxin either at the DNA or protein sequence level resembled the aerolysin of *Aeromonas* species.

Gene cloning, expression, and regulation of other putative virulence-protein-encoding genes

Chitinase is a digestive enzyme that breaks down glycosidic bonds in chitin, and is considered to be a virulence factor of *A. caviae* strain WS7b - isolated from a soil sample of a black-pepper plantation. The complete nucleotide sequence of the chitinase gene was determined and consists of a 2,748-bp region encoding 864 amino acids. The chitinase sequence showed amino-acid similarity to chiA from *A. caviae* (Malik *et al*., 2003). Lan *et al.* (2006) studied the chitinase (*chiA*) gene isolated from *A. hydrophila* strain SUWA-9. The *chiA* ORF encodes a polypeptide of 865 amino acid residues with a molecular mass of 91.6 kDa. The deduced amino acid sequence showed a high degree of similarity to other chitinases belonging to the glycosyl hydrolase family in bacteria. The chiA was expressed in *E. coli*, the recombinant chitinase (*ChiA*) was purified, and it hydrolyzed the trimer and pentamer forms of N-acetylchitooligomers to produce monomers and dimers. Lan *et al.* (2008) reported a second β-N-acetylglucosaminidase (*nagB*) gene isolated from chitinolytic *A. hydrophila* strain SUWA-9. The nagB ORF encoded 618 amino acid residues with a molecular mass of 68.8 kDa. The *nag*B gene, when expressed in *E. coli*, hydrolyzed the N-acetylchitooligomers into monomers.

Fang *et al.* (2004) reported the gene encoding the 43 kDa major adhesin (*Aha*1) from *A. hydrophila.* Gene sequence analysis of *Aha*1 revealed an ORF encoding a polypeptide of

373 amino acids with molecular weight 40.7 kDa. The amino acid sequences of *Aha1p* showed high degrees of homology with two outer membrane proteins (OMPs) of *A. hydrophila*. The major *Aha*1p was expressed in *E. coli* under control of a bacteriophage T5 promoter. The recombinant Aha1 was confirmed by Western blotting which showed major adhesin conservation among various strains of *Aeromonas* sp. Adhesin was used to immunize Blue gourami, resulting in fish protection, which indicates that it can be used as a potential candidate for control of *A. hydrophila* infection.

Kidd and Pemberton (2002) investigated an amylase enzyme, which breaks starch down into sugar in *A. hydrophila* JMP636. The amylase activity is encoded by multiple genes. A second genetically distinct amylase gene (*amyB*) containing a promoter region, was cloned and expressed in *E. coli*. *AmyB* is a large alpha-amylase consisting of 668 amino acid residues. Significant similarities (80%) were observed between AmyB and α-amylase of *A. hydrophila* strain MCC-1 Gobius and Pemberton (1988) described the amylase gene from *A. hydrophila* JMP636. They further cloned the gene within a partially digested 2.1-kb *Sma*I fragment of genomic DNA. After cloning into *E. coli*, the gene was transcribed from its own promoter and produced copies of a 49 kDa protein. Nucleotide sequencing revealed an ORF of 1,392 bp corresponding to a protein of 464 amino acid residues.

Protease is another significant virulence protein of *A. hydrophila*. It contributes for degradation of cellular proteins, which in turn degrades the repair mechanism of the host. Furthermore, secreted bacterial protease may also act as an exotoxin. An extracellular-protease-producing gene of *A. caviae* Ae6 was cloned and sequenced. Nucleotide sequencing of the 3.5 kb region of pKK3 revealed a single ORF of 1,785 bp encoding 595 amino acids. The N-terminal amino acid sequence of purified recombinant protein (APK) was consistent with the DNA sequence, showing a mature protein of 412 amino acids with a molecular weight of 44 kDa. However, the molecular mass of purified recombinant APK was measured as 34 kDa by SDS-PAGE, indicating that further processing at the C-terminal region took place. The protease showed optimum activity at pH 7.0 and was inactivated by heating at 80°C for 15 min, suggesting that APK belongs to the thermolysin family of metalloendopeptidases, which can be useful for preparation of vaccines (Kawakami *et al*., 2000). Another study demonstrated the existence of extracellular protease in *A. hydrophila* SO2/2 and D13. The gene was cloned into *E. coli* C600-1 using the pBR322 vector. The expressed protein was secreted to the periplasm of *E. coli* C600-1 and purified (Rivero *et al*., 1990).

Maurice *et al*. (1999) reported production of a paracrystalline outer membrane A-layer protein of *A. salmonicida* isolated from ulcerative goldfish. The structural gene of A-protein was cloned into a pET-3d plasmid and expressed in *E. coli* BL21(DE3). The induced protein was isolated from inclusion bodies by a simple solubilization-renaturation procedure and purified by ion exchange chromatography on Q-Sepharose, obtaining more than 95% pure monomeric protein. The immunological similarity of the protein samples was demonstrated by employing polyclonal and monoclonal antibodies in ELISA and Western blot analysis. Thomas and Trust (1995) reported on the paracrystalline surface protein array (S-layer) secreted by *A. hydrophila*. The gene (*ahsA*) encoding the S-protein subunit of *A. hydrophila* TF7 was cloned into lambda EMBL3 and sub-cloned into pUC18. The DNA sequence revealed a 1,406 bp ORF encoding a protein consisting of 448 amino acid residues with a molecular weight of 45 kDa. The Western blot analysis with anti-phosphotyrosine antibody showed that the S-protein of strain TF7 contained phosphotyrosine. Xie *et al*. (2002) studied cloning and expression of outer membrane protein (*ompTS*) of *A. hydrophila*. The size of *ompTS* was 1,024 bp and was amplified without the signal peptide-encoding sequence. The *ompTS* gene was over-expressed using the gene fusion expression vector pRSET system, and the recombinant OMP exhibited a size of 39.9 kDa on SDS-PAGE and Western blot analysis. In ELISA assays, antibody reacted not only to the recombinant OMP, but also to the purified OMPs from *A. hydrophila* and the 36.9 kD OMP in the Western blot, indicating that the recombinant OMP had the same epitope. Khushiramani *et al*. (2007) cloned and expressed the gene for OMP of *A. hydrophila* in *E. coli*. The size of the expressed protein was confirmed by Western blotting and it showed antigenicity when used for immunization of *Labeo rohita*.

Dodd and Pemberton (1996) characterized the DNase (*nucH*) from the genome of *A. hydrophila* strain JMP636. The *nucH* gene was cloned and expressed in *E. coli*. The protein size was observed on SDS-PAGE with the DNA sample as a substrate, revealing a nuclease activity band at approximately 100 kDa. The size was confirmed by *in vitro* transcription and translation, where a single polypeptide of 110 kDa was obtained. Sequencing of a clone predicted an ORF of 3,213 bp encoding a protein of 1070 amino acids with a mass of 114 kDa. Chang *et al*. (1992) cloned and sequenced another extracellular DNase from *A. hydrophila* CHC-1. The 690 bp nucleotide sequence of the DNase gene predicted an ORF encoding 230 amino acids. The expected protein size was 25 kDa based on SDS-PAGE analysis. The culture supernatant of *E. coli* expressing the protein showed DNA hydrolysis activity.

A lipase is a water-soluble enzyme that catalyzes the hydrolysis of ester chemical bonds in water-insoluble lipid substrates. Thus, lipases are a subclass of the esterases. *Aeromonas* species produce lipase to facilitate nutrient absorption from the external medium. The lipase (*apl-1*) gene was identified in a cosmid clone (JM3084) constructed from the genomic DNA of *A. hydrophila*. The *apl-1* gene was sub-cloned from the cosmid JMP3084 with partial Sau3a1 digestion that localized the lipase gene to a 3.4 kb DNA fragment. Southern blot analysis showed that *apl-1*exists in a single copy in *A. hydrophila*. Nucleotide sequence analysis of *apl-1* identified a 2,055 bp ORF predicting a 73 kDa protein. Expression of *apl-1* in the pT7 system identified a single protein of molecular weight 70 kDa. *A. hydrophila apl-1* was inactivated by mutagenesis (vector pJP5603) resulting in the complete removal of phospholipase C activity (Ingham and Pemberton, 1995). An extracellular lipase of *A. hydrophila* H3 was isolated from a female hospitalized patient. The lipase (*lip*) gene was cloned and expressed in *E. coli*. Lipase was purified from both the *A. hydrophila* culture supernatant, and the periplasmic fluids of *E. coli* containing the *lip* gene in the original clone (plasmid pLA2). Sequence analysis showed a

major open reading frame of 2,052 bp, which predicted a polypeptide of 71.8 kDa (Anguita *et al.*, 1993). The lipase gene (*lip*) containing the promoter sequences was isolated from *A. hydrophila* MCC-2, then cloned and expressed in *E. coli*. Nucleotide sequence analysis revealed that the gene was 2,253 bp long, coding for a 79.9 kDa protein. The deduced protein contained a conserved sequence of VHFLGHSLGA showing 67% and 65% overall identity to the amino acid sequences of lipase from *A. hydrophila* strains H3 and JMP636, respectively. The *lip*-encoded lipase was purified from both *A. hydrophila* and recombinant *E. coli*. The purified enzymes showed lipolytic activity with the p-nitrophenyl esters and triacylglycerols (Chuang *et al.*, 1997). The type III secretion system (T3SS) has been shown to play a crucial role in pathogenesis. It is clustered along with one effector (AexT) in *A. hydrophila* strain AH-3. The expression of the T3SS regulon has been investigated by analyzing the activity of the *aopN-aopD* and *aexT* promoters using two different techniques: promoterless GFP fusions and real-time PCR. The expression of the *A. hydrophila* AH-3 T3SS regulon was induced in response to several environmental factors such as calcium depletion, a high magnesium concentration, and a high growth temperature. T3SS was also regulated by lipopolysaccharide, the PhoPQ two-component system, the AhyIR quorum sensing system, and the enzymatic complex pyruvate dehydrogenase (Vilches *et al.*, 2009). The O-antigen and capsule gene clusters of the virulent *A. hydrophila* strain PPD134/91 have been characterized. The O-antigen gene cluster (17,296 bp) comprises 17 genes. Seven pathway genes for the synthesis of rhamnose and mannose, six transferase genes, one O unit flippase gene, and one O-antigen chain length determinant gene have been identified. The purified O-antigen and capsular polysaccharides increased the ability of the avirulent *A. hydrophila* strain PPD35/85 to survive in naive tilapia serum (Zhang *et al.*, 2002; Jimenez *et al.*, 2008).

Lipopolysaccharides (LPS) are a surface component of the outer membrane of *A. hydrophila* and are one of the main virulence factors (Canals *et al.*, 2006; Jimenez *et al.*, 2008; Agarwal *et al.*, 2009). UDP-galactose 4-epimerase (*GalE*) catalyses the last step in the Leloir pathway of galactose metabolism and provides the precursor for the biosynthesis of extracellular LPS and capsule. Cloning, sequence analysis and prediction of 3-D structure of the *galE* of *A. hydrophila* AH17 have been performed and suggest it could be a potential target for drug designing (Agarwal *et al.*, 2009). A single mutation in the gene for UDP N-acetylgalactosamine 4-epimerase (*gne*) confers the O(-) phenotype (LPS without O-antigen molecules) on a strain in serotypes O18 and O34, but not in serotypes O1 and O2. The *gne* gene is present in all the *Aeromonas* strains that have been tested. There are no changes observed for the LPS core in a *gne* mutant from *A. hydrophila* strain AH-3 (serotype O34). Serotype O34 LPS antigen contains N-acetylgalactosamine, whereas no such sugar residue forms part of the LPS core of *A. hydrophila* AH-3. Some of the pathogenic features of *A. hydrophila* AH-3 *gne* mutants are drastically reduced and the *gne* mutants are less virulent for fish and mice as compared to the wild-type strain (Canals *et al.*, 2006).

Elastase has been shown to disrupt tight junctions, causing proteolytic damage to tissue, and breaking down cytokines. Cascon *et al.* (2000) reported the cloning and expression of a gene encoding (*ahyB*) elastolytic activity from *A. hydrophila* AG2. This gene construct was expressed in *E. coli* and in the non-proteolytic species *A. salmonicida* subspecies masoucida. Nucleotide sequence analysis of the *ahy*B gene revealed a 1,764 bp ORF encoding a 588 amino acid, 62.7 kDa protein. The protease activity was shown by hydrolysis of casein and elastin. It had a high degree of sequence homology with metalloproteases of bacteria.

Conclusion

The present review shows that specific and sensitive molecular diagnosis is useful for the control of *A. hydrophila* infection in fish and other animals. Cloning and expression of putative virulence-protein-encoding genes of *A. hydrophila* and other species of *Aeromonas* is useful for production of antigens needed to develop immunoassays for rapid immunodiagnosis and vaccination. In the review, we have also discussed the several virulence proteins that contribute to pathogenesis, and the fine tuning of gene expression for cellular activity. Such knowledge of the virulence proteins would be useful in reducing the virulence effects of these proteins by knocking down part of gene functions. Additional studies would be required for the generation of 3-D structures of virulence proteins of *A. hydrophila* for better understanding their potential function and the mechanism involved during infection of the host. These 3-D structures could used in drug design for neutralizing the function of virulence proteins through specific binding with active amino-acids. Recent progress suggests that *A. hydrophila* and other species of *Aeromonas* are useful in the production of commercial enzymes such as hemolysin, proteases, amylases and lipases. These enzymes have commercial applications that not only include antigens/vaccine production, but also in enhancement of fatty acid based biofuels and production of bioplastics.

Acknowledgements

Authors are grateful to Pawan K. Dhar, Ashok Kumar, A.K. Singh, Satya Prakash and Pritee Singh for providing suggestions, encouragement and fruitful discussion during preparation of the manuscript. Authors would like to thank anonymous reviewers for their valuable comments and suggestions, which improved the quality of the paper.

References

Agarwal, S., Gopal, K., Chhabra, G., and Dixit, A. 2009. Molecular cloning, sequence analysis and homology modeling of galE encoding UDP-galactose 4-epimerase of *Aeromonas hydrophila*. *Bioinformation* **4**, 216–222.

Aguilera-Arreola, M.G., Hernandez-Rodriguez, C., Zuniga, G., Figueras, M.J., Garduno, R.A., and Castro-Escarpulli, G. 2007. Virulence potential and genetic diversity of *Aeromonas caviae*, *Aeromonas veronii*, and *Aeromonas hydrophila* clinical isolates from Mexico and Spain: a comparative study. *Can. J. Microbiol.* **53**, 877–887.

Anguita, J., Aparicio, L.B.R., and Naharro, G. 1993. Purification, gene cloning, amino acid sequence analysis and expression of an extracellular lipase from an *Aeromonas hydrophila* human isolate. *Appl. Environ. Microbiol.* **59**, 2411–2417.

Austin, B. and Austin, D.A. 1999. Bacterial fish pathogens: Diseases in farmed and wild fish. Praxis Publishing, Chichester, UK.

Baloda, S.B., Krovacek, K., Eriksson, L., Linne, T., and Mansson, I. 1995. Detection of aerolysin gene in *Aeromonas* strains isolated from drinking water, fish and foods by the polymerase chain reaction. *Comp. Immunol. Microbiol. Infect. Dis.* **18**, 17–26.

Barghouthi, S., Young, R., Olson, M.O., Arceneaux, J.E., Clem, L.W., and Byers, B.R. 1989. Amonabactin, a noval trypophan- or phenylalanine-containing phenolate siderophore in *Aeromonas hydrophila*. *J. Bacteriol.* **171**, 1811–1816.

Biscardi. D., Castaldo, A., Gualillo, O., and de Fusco, R. 2002. The occurrence of cytotoxic *Aeromonas hydrophila* strains in Italian mineral and thermal waters. *Sci. Total Environ.* **292**, 255–263.

Canals, R., Jiménez, N., Vilches, S., Regué, M., Merino, S., and Tomás, J.M. 2006. The UDP N-acetylgalactosamine 4-epimerase gene is essential for mesophilic *Aeromonas hydrophila* serotype O34 virulence. *Infect. Immun.* **74**, 537–548.

Carr, R.M., Blumenthal, U.J., and Mara, D.D. 2004. Guidelines for the safe use of wastewater in agriculture: revisiting WHO guidelines. *Water Sci. Technol.* **50**, 31–38.

Cascon, A., Anguita, J., Hernanz, C., Sanchez, M., Fernandez, M., and Naharro, G. 1996. Identification of *Aeromonas hydrophila* hybridization group 1 by PCR assays. *Appl. Environ. Microbiol.* **62**, 1167–1170.

Cascon, A., Fregeneda, J., Aller, M., Yugueros, J., Temprano, A., Hernanz, C., Sanchez, M., Rodriguez-Aparicio, L., and Naharro, G. 2000. Cloning, characterization, and inactivation of a major extracellular serine protease gene with elastolytic activity from *Aeromonas hydrophila*. *J. Fish Dis.* **23**, 49–59.

Chabot, D.J. and Thune, R.L. 1991. Proteases of the *Aeromonas hydrophila* complex: Identification, characterization and relation to virulence in channel catfish, *Ictalurus punctatus* (Rafinesque). *J. Fish Dis.* **14**, 171–183.

Chakraborty, T., Huhle, B., Bergbauer, H., and Goebel, W. 1986. Cloning, expression, and mapping of the *Aeromonas hydrophila* aerolysin gene determinant in *Escherichia coli* K-12. *J. Bacteriol.* **167**, 368–374.

Chakraborty, T., Huhle, B., Hof, H., Bergbauer, H., and Goebel, W. 1987. Marker exchange mutagenesis of the aerolysin determinant in *Aeromonas hydrophila* demonstrates the role of aerolysin in *Aeromonas hydrophila* associated systemic infection. *Infect. Immun.* **55**, 2274–2280.

Chang, M.C., Chang, S.Y., Chen, S.L., and Chuang, S.M. 1992. Cloning and expression in *Escherichia coli* of the gene encoding an extracellular deoxyribonuclease (DNase) from *Aeromonas hydrophila*. *Gene* **122**, 175–180.

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

Chopra, A.K., Pham, R., and Houston, C.W. 1994. Cloning and expression of putative cytotonic enterotoxin-encoding genes from *Aeromonas hydrophila*. *Gene* **139**, 87–91.

Chuang, Y.C., Chiou, S.F., Su, J.H., Wu, M.L., and Chang, M.C. 1997. Molecular analysis and expression of the extracellular lipase of *Aeromonas hydrophila* MCC-2. *Microbiology* **143**, 803–812.

Colwell, R.R., MacDonell, M.T., and de Ley, J. 1986. Proposal to recognize the family *Aeromonadaceae* fam. nov. *Int. J. Syst. Bacteriol.* **36**, 473–477.

Din, J.N., Newby, D.E., and Flapan, A.D. 2004. Omega 3 fatty acids and cardiovascular disease-fishing for a natural treatment. *British Med. J.* **328**, 30–35.

Dodd, H.N. and Pemberton, J.M. 1996. Cloning, sequencing and characterization of the *nucH* gene encoding an extracellular nuclease from *Aeromonas hydrophila* JMP636. *J. Bacteriol.* **178**, 3926–3933.

Erova, T.E., Sha, J., Horneman, A.J., Borchardt, M.A., Khajanchi, B.K., Fadl, A.A., and Chopra, A.K. 2007. Identification of a new hemolysin from diarrheal isolate SSU of *Aeromonas hydrophila*. *FEMS Microbiol. Lett.* **275**, 301–311.

Fang, H.M., Ge, R., and Sin, Y.M. 2004. Cloning, characterization and expression of *Aeromonas hydrophila* major adhesin. *Fish Shellfish Immunol.* **16**, 645–658.

Gobius, K.S. and Pemberton, J.M. 1988. Molecular cloning, characterization and nucleotide sequence of an extracellular amylase gene from *Aeromonas hydrophila*. *J. Bacteriol.* **170**, 1325–1332.

Gonzalez-Serrano, C.J., Santos, J.A., Garcia-Lopez, M.L., and Otero, A. 2002. Virulence markers in *Aeromonas hydrophila* and *Aeromonas veronii* biovar sobria isolates from freshwater fish and from a diarrhoea case. *J. Appl. Microbiol.* **93**, 414–419.

Gustafson, C.E., Thomas, C.J., and Trust, T.J. 1992. Detection of *Aeromonas salmonicida* from fish by using polymerase chain reaction amplification of the virulence surface array protein gene. *Appl. Environ. Microbiol.* **58**, 3816–3825.

Hanes, D.E. and Chandler, D.K. 1993. The role of a 40 megadalton plasmid in the adherance and hemolytic properties of *Aeromonas hydrophila*. *Microb. Pathog.* **15**, 313–317.

Hernanz-Moral, C., Flaño del Castillo, E., López Fierro, P., Villena Cortés, A., Anguita Castillo, J., Cascón Soriano, A., Sánchez Salazar, M., Razquín Peralta, B., and Naharro Carrasco, G. 1998. Molecular characterization of the *Aeromonas hydrophila aroA* gene and potential use of an auxotrophic *aroA* mutant as a live attenuated vaccine. *Infect. Immun.* **66**, 1813–1821.

Heuzenroeder, M.W., Wong, C.Y.F., and Flower, R.L.P. 1999. Distribution of two hemolytic toxin genes in clinical and environmental isolates of *Aeromonas* spp.: correlation with virulence in a suckling mouse model. *FEMS Microbiol. Lett.* **174**, 131–136.

Hickman-Brenner, F.W., MacDonald, K.L., Steiferwalt, A.G., Fanning, G.R., Brenner, D.J., and Farmer, J.J. 1987. *Aeromonas veronii*, a new ornithine decarboxylase-positive species that may cause diarrhea. *J. Clin. Microbiol.* **25**, 900–906.

Hirono, I. and Aoki, T. 1991. Nucleotide sequence and expression of an extracellular hemolysin gene of *Aeromonas hydrophila*. *Microb. Pathog.* **11**, 189–197.

Howard, S.P. and Buckley, J.T. 1985. Activation of the hole-forming toxin aerolysin by extracellular processing. *J. Bacteriol.* **163**, 336–340.

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

Hu, M., Wang, N., Pan, Z.H., Lu, C.P., and Liu, Y.J. 2012. Identity and virulence properties of *Aeromonas* isolates from diseased fish, healthy controls and water environment in China. *Lett. Appl. Microbiol.* **55**, 224–233.

Ingham, A.B. and Pemberton, J.M. 1995. A lipase of *Aeromonas hydrophila* showing nonhemolytic phospholipase C activity. *Curr. Microbiol.* **31**, 28–33.

Janda, J.M., Abbott, S.L., Cheung, W.K., and Hanson, D.F. 1994. Biochemical identification of citrobacteria in the clinical laboratory. *J. Clin. Microbiol.* **32**, 1850–1854.

Jimenez, N., Canals, R., Lacasta, A., Kondakova, A.N., Lindner, B., Knirel, Y.A., Merino, S., Regué, M., and Tomás, J.M. 2008. Molecular analysis of three *Aeromonas hydrophila* AH-3 (serotype O34) lipopolysaccharide core biosynthesis gene clusters. *J. Bacteriol.* **190**, 3176–3184.

Kannan, S., Kanna, P.S., Karkuzhali, K., Chattopadhyay, U.K., and Pal, D. 2001. Direct detection of diarrheagenic *Aeromonas* from faeces by polymerase chain reaction (PCR) targeting aerolysin toxin gene. *Eur. Rev. Med. Pharmacol. Sci.* **5**, 91–94.

Karl, T.R., Knight, R.W., and Plummer, N. 1995. Trends in high-frequency climate variability in the twentieth century. *Nature* **377**, 217–220.

Kawakami, K., Toma, C., and Honma, Y. 2000. Cloning, sequencing and expression of the gene encoding the extracellular metalloprotease of *Aeromonas caviae*. *Microbiol. Immunol.* **44**, 341–347.

Khan, A.A., Nawaz, M.S., Khan, S.A., and Cerniglia, C.E. 1999. Identification of *Aeromonas trota* (hybridization group 13) by amplification of the aerolysin gene using polymerase chain reaction. *Mol. Cell Probes* **13**, 93–98.

Khushiramani, R., Girisha, S.K., Karunasagar, I., and Karunasagar, I. 2007. Cloning and expression of an outer membrane protein ompTS of *Aeromonas hydrophila* and study of immunogenicity in fish. *Protein Exp. Purif.* **51**, 303–307.

Kidd, S.P. and Pemberton, J.M. 2002. The cloning and characterization of a second α-amylase of *A. hydrophila* JMP636. *J. Appl. Microbiol*. **92**, 289–296.

Krovacek, K., Huang, K., Sternberg, S., and Svenson, S.B. 1998. *Aeromonas hydrophila* septicaemia in a grey seal (*Halichoerus grypus*) from the Baltic Sea: a case study. *Comp. Immunol. Microbiol. Infect. Dis*. **21**, 43–49.

Lan, X., Zhang, X., Hu, J., and Shimosaka, M. 2006. Cloning, expression and characterization of a chitinase from the chitinolytic bacterium *Aeromonas hydrophila* strain SUWA-9. *Biosci. Biotechnol. Biochem*. **70**, 2437–2442.

Lan, X., Zhang, X., Kodaira, R., Zhou, Z., and Shimosaka, M. 2008. Gene cloning, expression and characterization of a second beta-N-acetylglucosaminidase from the chitinolytic bacterium *Aeromonas hydrophila* strain SUWA-9. *Biosci. Biotechnol. Biochem*. **72**, 492–498.

Li, J., Ni, X.D., Liu, Y.J., and Lu, C.P. 2011. Detection of three virulence genes *alt*, *ahp* and *aerA* in *Aeromonas hydrophila* and their relationship with actual virulence to zebrafish. *J. Appl. Microbiol.* **110**, 823–830.

Malik, A., Wenuganen, S., Suwanto, A., and Tjahjono, B. 2003. Cloning, DNA sequence and expression of *Aeromonas caviae* WS7b chitinase gene. *Mol. Biotechnol*. **23**, 1–10.

Maurice, S., Hadge, D., Dekel, M., Friedman, A., Gertler, A., and Shoseyov, O. 1999. A-protein from achromogenic atypical *Aeromonas salmonicida*: molecular cloning, expression, purification, and characterization. *Protein Expr. Purif*. **16**, 396–404.

Merino, S., Rubires, X., Knochel, S., and Tomas, J.M. 1995. Emerging pathogens: *Aeromonas* spp. *Int. J. Food Microbiol*. **28**, 157–168.

Nam, I.Y. and Joh, K. 2007. Rapid detection of virulence factors of *Aeromonas* isolated from a trout farm by hexaplex-PCR. *J. Microbiol*. **45**, 297–304.

Parker, M.W., Buckley, J.T., Postma, J.P.M., Tucker, A.D., Leonard, K., Pattus, F., and Tsernoglou, D. 1994. Structure of the *Aeromonas* toxin proaerolysin in its water-soluble and membrane-channel states. *Nature* **367**, 292–295.

Pemberton, J.M., Kidd, S.P., and Schmidt, R. 1997. Secreted enzymes of *Aeromonas*. *FEMS Microbiol. Lett*. **152**, 1–10.

Pollard, D.R., Johnson, W.M., Lior, H., Tyler, S.D., and Rozee, R.K. 1990. Detection in aerolysin gene in *Aeromonas hydrophila* by the polymerase chain reaction. *J. Clin. Microbiol*. **28**, 2477–2481.

Prein, M. and Ahmed, M. 2000. Integration of aquaculture into smallholder farming systems for improved food security and household nutrition. *Food Nutr. Bull*. **21**, 466–471.

Rivero, O., Anguita, J., Paniagua, C., and Naharro, G. 1990. Molecular cloning and characterization of an extracellular protease gene from *Aeromonas hydrophila*. *J. Bacteriol*. **172**, 3905–3908.

Sen, K. and Rodgers, M. 2004. Distribution of six virulence factors in *Aeromonas* species isolated from US drinking water utilities: a PCR identification. *J. Appl. Microbiol*. **97**, 1077–1086.

Seshadri, R., Joseph, S.W., Chopra, A.K., Sha, J., Shaw, J., Graf, J., Haft, D., Wu, M., Ren, Q., Rosovitz, M.J., and *et al.* 2006. Genome sequence of *Aeromonas hydrophila* ATCC 7966: Jack of All Trades. *J. Bacteriol*. **188**, 8272–8282.

Shibata, M., Morita, K., Watanabe, N., Okitsu, T., Yamai, S., Itou, K., Shimada, T., and Kanamori, M. 1997. Detection of the thermostable lipase gene (lipAH) in clinical isolates of *Aeromonas hydrophila* belonging to the major O serogroups O11, O16 and O34. *Kansenshogaku Zasshi* **71**, 1172–1174.

Singh, V. 2012. Molecular and structural biology: *Aeromonas hydrophila.* LAP Lambert Academic Publishing GmbH & Co. KG, AV Akademikerverlag, Germany. ISBN: 978-3-659-27704-7.

Singh, V., Chaudhary, D.K., Mani, I., Somvanshi, P., Rathore, G., and Sood, N. 2010a. Molecular identification and codon optimization analysis of major virulence encoding genes of *Aeromonas hydrophila*. *African J. Microbiol. Res*. **4**, 952–957.

Singh, V., Mani, I., Chaudhary, D.K., and Somvanshi, P. 2011. Molecular detection and cloning of thermostable hemolysin gene from *Aeromonas hydrophila*. *Mol. Biol*. **45**, 551–560.

Singh, V., Rathore, G., Kapoor, D., Mishra, B.N., and Lakra, W.S. 2008. Detection of aerolysin gene in *Aeromonas hydrophila* isolated from fish and pond water. *Indian J. Microbiol*. **48**, 453–458.

Singh, V., Rathore, G., Kumar, G., Swaminathan, T.R., Sood, N., Kapoor, D., and Mishra, B.N. 2007. Detection of the hole forming toxin hemolysin gene of *Aeromonas hydrophila* isolates. *Indian Veterinary J*. **84**, 900–902.

Singh, V. and Somvanshi, P. 2009. Inhibition of oligomerization of aerolysin from *Aeromonas hydrophila*: homology modeling and docking approach for exploration of hemorrhagic septicemia. *Lett. Drug Des. Discov*. **6**, 215–223.

Singh, V., Somvanshi, P., Rathore, G., Kapoor, D., and Mishra, B.N. 2009. Gene cloning, expression and homology modeling of hemolysin gene from *Aeromonas hydrophila*. *Protein Expr. Purif*. **65**, 1–7.

Singh, V., Somvanshi, P., Rathore, G., Kapoor, D., and Mishra, B.N. 2010b. Gene cloning, expression and characterization of recombinant aerolysin from *Aeromonas hydrophila*. *Appl. Biochem. Biotechnol*. **160**, 1985–1991.

Thomas, S.R. and Trust, T.J. 1995. Tyrosine phosphorylation of the tetragonal paracrystalline array of *Aeromonas hydrophila*: molecular cloning and high-level expression of the S-layer protein gene. *J. Mol. Biol*. **245**, 568–581.

Vilches, S., Jimenez, N., Tomás, J.M., and Merino, S. 2009. *Aeromonas hydrophila* AH-3 type III secretion system expression and regulatory network. *Appl. Environ. Microbiol*. **75**, 6382–6392.

Wakabayashi, J., Ganai, K., Hsu, T.C., and Egusa, S. 1980. Pathogenic activities of *Aeromonas hydrophila* biovar hydrophila (Chester) Popoff and Veron 1976 to fishes. *Fish Pathol*. **15**, 319–325.

Wang, G., Clark, C.G., Liu, C., Pucknell, C., Munro, C.K., Kruk, T.M., Caldeira, R., Woodward, D.L., and Rodgers, F.G. 2003. Detection and characterization of the hemolysin genes in *Aeromonas hydrophila* and *Aeromonas sobria* by multiplex PCR. *J. Clin. Microbiol.* **41**, 1048–1054.

Williams, M. 1996. The transition in the contribution of living aquatic resources to food security. IFPRI Food, Agriculture and the Environment Discussion Paper, No. 13. p. 41.

Wong, C.Y.F., Heuzenroeder, M.W., and Flower, R.L.P. 1998. Inactivation of two haemolytic toxin genes in *Aeromonas hydrophila* attenuates virulence in a suckling mouse model. *Microbiology* **144**, 291–298.

Xie, J.F., Ye, Q.Z., He, J.G., Dai, W.J., Huang, X., He, M.X., and Chen, C. 2002. The expression of *Aeromonas hydrophila* ompTS gene and the immunogenicity of recombinant OmpTS. *Sheng Wu Gong Cheng Xue Bao* **18**, 300–303.

Zhang, Y.L., Arakawa, E., and Leung, K.Y. 2002. Novel *Aeromonas hydrophila* PPD134/91 genes involved in O-antigen and capsule biosynthesis. *Infect. Immun*. **70**, 2326–2335.

Zhu, D., Li, A., Wang, J., Li, M., and Cai, T. 2007. Cloning, expression and characterization of aerolysin from *Aeromonas hydrophila* in *Escherichia coli*. *Indian J. Biochem. Biophys*. **44**, 204–208.