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Full Length Research Paper

Molecular identification and codon optimization analysis of major virulence encoding genes of *Aeromonas hydrophila*

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Aeromonas hydrophila **is an opportunistic pathogen of aquatic and terrestrial animals and human beings. The aim of this study is to investigate 25 isolates of *A. hydrophila* from 40 fish muscles and 12 water samples. All isolates are screened for the presence of virulence factors such as aerolysin, hemolysin and lipase both phenotypically as well as genotypically. All the isolates produced lipase, whereas only 60% of isolates produced β-hemolysis with RBCs. The presence of 3 virulence genes aerolysin, hemolysin and lipase were confirmed by PCR assay, which gave positive PCR amplification for conserved fragment. These were also codon optimized for over expression in *Escherichia coli* and obtained the high codon adaptation index in comparison to wild type of DNA sequences. These finding may help to better expression of gene for scale up production of vaccine candidates.**

Keywords: *Aeromonas hydrophila*, virulence factor, PCR, codon optimization, vaccine.

INTRODUCTION

Aeromonas hydrophila is an opportunistic bacterial pathogen. It is Gram negative, motile and rod-shaped bacterium which belongs to Aeromonadaceae family (Colwell et al., 1986). It has been associated with soft tissue infections and several diseases in fish that is, hemorrhagic septicemia, fin and tail rot (Austin and Austin, 1999). Fishes are the major source of food as it has essential nutritional and medicinal values. Nowadays, aquaculture is facing lots of problems such as disease outbreaks, high mortality resulting in loss of economy. Virulence factors are common in bacteria, which contribute to their pathogenicity and ability to attach the host cells in the development of diseases. These factors can be used for detection and characterization of pathogens. *A. hydrophila* secretes many extracellular proteins such as β-hemolysin (aerolysin), hemolysin, proteases, enterotoxin and acetylcholinesterase (Chakraborty et al., 1987; Rivero et al., 1981; Singh et al., 2009). Aerolysin is cytolytic extra cellular water-soluble protein of *A. hydrophila*, secreted during active growth phase. During invasion, the aerolysin is inserted into lipid bilayer of host red blood cell and destroys its membrane permeability barrier, thereby causing hemolysis. It can be detected by the presence of zone of hemolysis on blood agar (Howard and Buckley, 1985).

Identification and confirmation of *A. hydrophila* by conventional methods is laborious and time consuming (Rathore et al., 2005), whereas detection of *A. hydrophila* by amplification of virulence genes through PCR is rapid, sensitive and less time taking.

Detection of *A. hydrophila* has been carried out by amplification of lipase gene (Cascon et al., 1996) and aerolysin gene (Baloda et al., 1995; Xia et al., 2004; Singh et al., 2008; Singh and Somvanshi, 2009). Lipase is also an important extra-cellular virulence factor affecting several immune system functions through free fatty acid generated by lipolytic activity. The amplification

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of small fragment of *lip* H3 gene was used to confirm *A. hydrophila* isolated from aquatic environment (Swaminathan et al., 2004). The PCR amplification of hemolysin gene from *A. hydrophila* has been reported (Singh et al., 2007). The specific, rapid and sensitive PCR technique has been used to detect aerolysin and lipase gene in Indian isolates of *A. hydrophila*. The species specific primers have been selected for this study was based on published nucleotide sequence of aerolysin gene (Wang et al., 2003) and lipase gene (Cascon et al., 1996).

In most synonymous case, codons are not used in the same frequencies; it is known as codon usage bias. Codon bias is generally control by a balance between the mutation, natural selection and genetic drift. If gene contains the codon which is rarely used by host, its expression level will be maximized in heterologous system (Gustafsson et al., 2004; Basak et al., 2008; Fuglsang, 2003). The heterologous protein expression in *Escherichia coli* can be diminished by biased codon usage and normally used to minimize this problem including targeted mutagenesis to remove rare codons or the addition of rare codon tRNAs in specific cell lines.

Expression patterns in *E. coli* of 30 human short-chain dehydrogenase/reductase genes (SDRs) were analyzed in three independent experiments, comparing the native and synthetic (codon-optimized) versions of each gene. Expression of the native and synthetic gene constructs was compared in two isogenic bacterial strains, one of which contained a plasmid (pRARE2) that carries seven tRNAs recognizing rare codons. Although we found some degree of variability between experiments, in normal *E. coli* synthetic genes could be expressed and purified more readily than the native (Burgess-Brown et al., 2009). To improve the expression of *Chlamydia* protein in mammalian cells, the DNA sequence encoding the major outer-membrane protein (MOMP) of MoPn was modified to substitute the human-preferred codons for rarely used codons. The human-optimized MOMP gene was synthesized and cloned into the pcDNA3 vector, as was the wild-type MOMP gene. The protein expression levels of the human-optimized MOMP and wild-type MOMP genes were compared. The experiments showed that the human-optimized MOMP gene produced significantly higher levels of MOMP protein than the wild-type MOMP (Zheng et al., 2007).

In this work, the comparison of the expression of three constructs of a multistage candidate vaccine (FALVAC-1) against *Plasmodium falciparum* in an *E. coli* system: a synthetic gene with *P. falciparum* codons, a synthetic gene with optimized *E. coli* codons and a synthetic gene with *P. falciparum* codons co-transformed with a RIG plasmid, which encodes three tRNAs (AG(A/G), ATA, GGA) that recognize rare *E. coli* codons. The expression of the protein increased at least threefold with codon optimization. The codon optimization increases the yield of *P. falciparum* candidate vaccines in the *E. coli* expression host (Zhou et al., 2004). Therefore, on the basis codon which is used the more is abundant in host.

It is selected for optimization of codon of our target genes. The aim of this study was to examine the virulence factors aerolysin, hemolysin and lipase of motile *A. hydrophila* from different types of fish and water isolates. Optimization of codon is important for the over expression of *A. hydrophila* genes in *E. coli* for use in vaccine rationale.

MATERIALS AND METHODS

Sampling

Finfish and water samples were collected from fish markets, fish farms, ponds and canal located 50 km in and around Lucknow, India. *Labeo rohita, Catla catla*, *Cirrhinus mrigala* (IMC) and *Channa punctatus* were collected in a sterile polythene bags and transported on ice while 50 ml water samples were collected in a sterile tubes.

Isolation and identification of *A. hydrophila*

Ten grams of fish muscle tissue was cut and homogenized in 90 ml alkaline peptone water. Homogenates were serially diluted up to 10^{-6} in sterile normal saline solution and 100 µl aliquots of each dilution were plated on starch ampicillin agar (Palumbo et al., 1985) using spread plate technique in duplicate. The plates were incubated at 37°C for 12 - 16 h. Yellow and amylolytic colonies (Kidd and Pemberton, 2002) 2 - 5 mm in diameter were subjected to biochemical tests as per Barrow and Feltham (1992). Aero-key suggested by Joseph and Carnahan (1994), was used for confirmative identification of *A. hydrophila*. The cultures were stored at -80°C in 15% (v/v) glycerol for further characterization.

Determination of hemolytic activity

The strains were tested for β hemolytic activity on nutrient agar base (Hi media) supplemented with 5% rabbit erythrocytes with incubation at 37°C for 24 h. For quantification of hemolysin, methods have been described (Burke et al., 1983). Briefly, bacteria were cultured in tryptone Soya broth at 37°C with shaking 210 rpm for 18 h, cells were pelleted by centrifugation at 10,000 X g for 10 min at 4°C, and 1 ml of the supernatant (filtered through 0.22 um pore diameter filter) was added to 1 ml of a 1% suspension of washed rabbit erythrocytes in Phosphate Buffered saline. This mixture was incubated at 37°C for 1 h and additional 1 h at 4°C and absorbance at 540 nm was measured to determine erythrocyte lysis. A positive hemolysin test was defined as production of an O. D. reading of samples more than O. D. reading of 20% hemoglobin standard in a standard curves plot between the hemoglobin percentage and erythrocytes optical density (Figure 1).

Lipolytic activity

All the strains of *A. hydrophila* were analyzed for the presence of lipolytic activity in 0.1% Tween 80 agar medium. Test cultures were inoculated on agar surface of medium and incubated at 37°C for 24 – 48 h. The opaque haloes around the colonies were indicated lipolytic activity of bacteria.

Genomic DNA isolation

Genomic DNA of *A. hydrophila* was isolated by lysis of bacterial

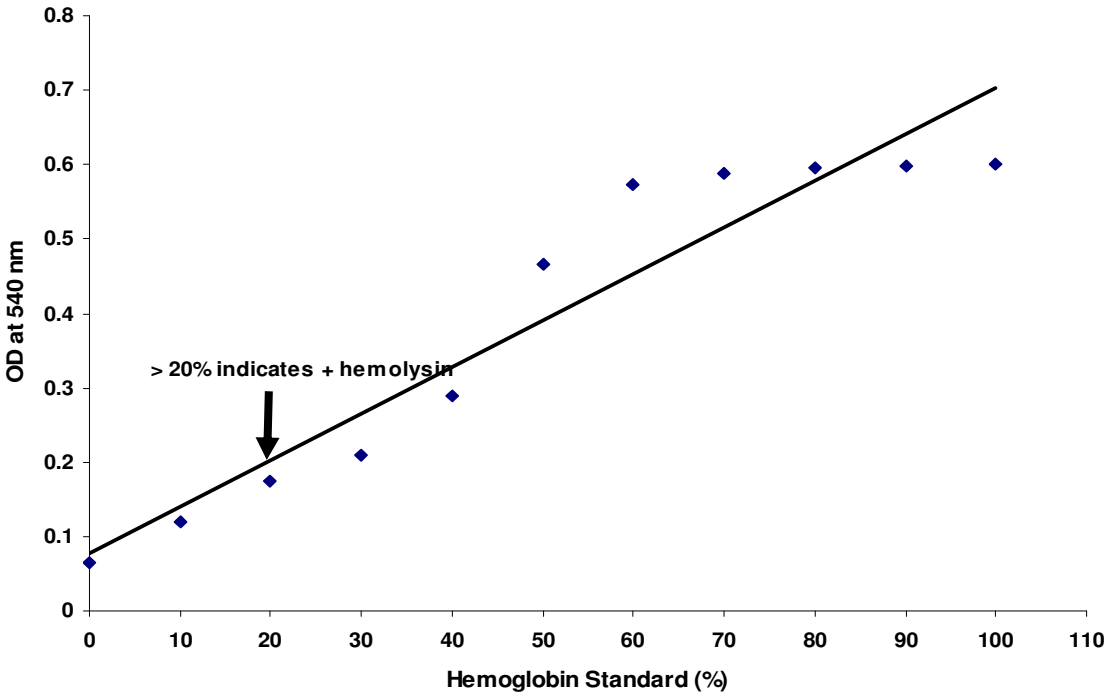

Figure 1. Standard curve showing % hemoglobin standard and O.D. value of rabbit RBCs for the estimation of hemolysin production in *A. hydrophila* isolates.

cells in lysis buffer containing 50 mM Tris. Cl, 10 mM EDTA, 1.5% NaCl, 0.25% sucrose, 1% SDS and 10 mg/ml proteinase-K. Twice extraction of lyste with phenol; chloroform; isoamylalcohol (25:24:1); then lysate was precipitated with absolute alcohol and finally dissolved in T.E. pH 8.0 (10 mM Tris-HCl and 1 mM EDTA). The genomic DNA content was quantified by determining optical density at 260 nm and stored at -20°C for further study (Hiney et al., 1992).

PCR amplification and conditions

PCR amplification of aerolysin and lipase gene was carried out following Wang et al. (2003) and Cascon et al. (1996), respectively. Hemolysin gene of *A. hydrophila* was amplified by PCR. The reaction mixture (50 µl) consisted of 10 ng of bacterial genomic DNA, 1.5 units of Taq DNA polymerase, 5 µl of 10X PCR amplification buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl. pH 8.3), 200 µM deoxynucleotide triphosphate (dNTP) and 5 pmoles of each primer (AHF8/ AHR8) (Singh et al., 2009). Amplification included initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing of primers at 50°C for 30 s and extension at 72°C for 1 min. A final extension at 72°C for 10 min was used. Ten µl of the reaction mixture was then analyzed by submarine gel electrophoresis in 1.0% agarose with ethidium bromide run at 8 V/cm. The PCR products were visualized under gel documentation system (UVP).

Codon optimization and analysis

All these three gene of *A. hydrophila* were retrieved from NCBI. The gene designer (https://www.dna20.com/index.php?pageID=220), optimizer (Puigbo et al., 2007), CAIcal, MrGene were used for optimization of DNA sequences at maximum threshold. Codon optimization was on the basis of 10 - 15% threshold level of host which was used as template. Codon adaptation index (CAI) was also calculated for each gene. It is widely acceptable as an effective measure of potential level of gene expression (Sharp and Li, 1987).

Nucleotide sequence accession number

The nucleotide sequence of the codon-optimized aerolysin, hemolysin and lipase genes have been submitted in the DDBJ/GenBank/EMBL databases under accession number GQ406050, GQ335447 and GQ406051.

RESULTS AND DISCUSSION

In present study, the sampled fish did not show any gross lesions on the body surface but still tested positive for *A. hydrophila.* A total of 25 *A. hydrophila* isolates were isolated from 40 fish muscles and 12 water samples. Out of 25 *A. hydrophila* isolates, a total of 13, 8 and 4 no. of isolates were recovered from *Channa striatus,* IMC and water samples, respectively. All isolates of *A. hydrophila* were Gram negative, motile, rod shaped and produced oxidase, catalase, acid and gas from glucose and indole and hydrolyzed arginine, lysine and aesculin.

All the twenty-five isolates of *A. hydrophila* were biochemically tested for virulence factors like aerolysin (β-hemolysin) and lipase. Sixty percent of isolates were positive for β-hemolysis on rabbit blood agar, whereas 40% isolates produced the α-hemolysis. Hemolysin factor of *A. hydrophila* was quantified by microtiter method and

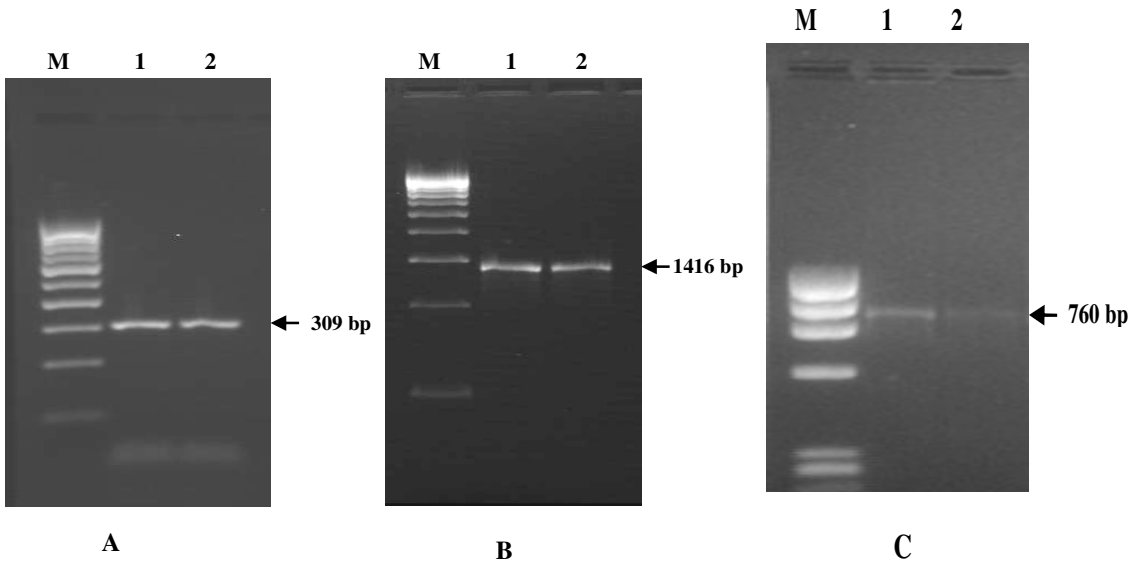

Figure 2. Molecular identification of *A. hydrophila* by PCR. A, Lane M: 100 bp DNA ladder, Lane 1 and 2 amplification of aerolysin gene (309 bp); B, Lane M: 500 bp DNA ladder, lane 1 and 2 amplification of hemolysin gene (1416 bp); C, Lane M: Phi X 174 DNA *Hae* III digest marker, Lane 1 and 2 amplification of lipase gene (760 bp).

approximately 60% isolates gave the higher titer (>20% hemoglobin standard) with red blood cells of rabbit, while others gave the very low titer (<20% hemoglobin standard) (Figure 1).

Potential virulence factors of mesophlic motile *A. hydrophila*, which contribute to their pathogenicity, include the production of endotoxins, extra cellular enterotoxins, hemolysin, cytotoxins and protease, the ability to adhere the cells, and the possession of certain surface proteins (Howard and Buckley, 1985). In the present study, all the twenty-five isolates of *A. hydrophila* were biochemically tested for virulence factors like hemolysin (β-hemolysin) and lipase. There are two types of hemolysin that us, α-hemolysin and β-hemolysin. β-hemolysin is known as aerolysin (Ljungh et al., 1987). Sixty percent of isolates were positive for β-hemolysis on rabbit blood agar, whereas 40% isolates produced the α-hemolysis. The production of hemolytic toxins has been regarded as strong evidence of pathogenic potential in *A. hydrophila*. We have used rabbit erythrocytes for the determination of the hemolytic activity both on agar plates and in quantification assays. Brenden and Janda (1987) described that the routine use of sheep blood agar appears to be inappropriate, considering that sheep erythrocytes are less sensitive than erythrocytes from other mammals. Santos et al. (1999) observed stronger hemolytic activity of *Aeromonas* sp against rabbit blood, either on agar plates or in quantification assays.

The PCR amplification of 309 bp fragment of *aerolysin* gene was amplified from all isolates of *A. hydrophila* but only 60% isolates showed β-hemolytic activity either on blood agar or quantification assays (Figure 2A). The PCR amplification of 1416 bp fragment of hemolysin gene was obtained in all isolates during the course of our investigation (Figure 2B). All isolates showed positive lipolytic activity on agar media and 760 bp fragment of *lipH3* gene was amplified from all the test strains (Figure 2C). Previous studies have shown *A. hydrophila* isolates produced more than one hemolytic toxin: *ahh1*, *aerA* or *asa1* (Wang et al., 2003).

It is possible that the nonhemolytic isolates carried hemolysin genes either that could not be expressed or that had mutations affecting domains responsible for the hemolytic phenotype. All isolates showed positive lipolytic activity on agar media and 760 bp fragment of *lipH3* gene was amplified from all the test strains. Cascon et al. (1996) described strains of *Aeromonas* spp. that were negative in the PCR amplification of *lip* gene and positive for lipolytic activities. This was due to the fact that more than one lipolytic factor was being secreted into the medium which resulted in demonstration of lipolytic activity in all strains of *Aeromonads*. It was concluded that *A. hydrophila* produced two lipases, belonging to the lipase super-family that is, lipase H3 and lipase non-H3 genes. Only lipase H3 producers were positive in the PCR assay because of amplification of H3 lip gene. However, strains of *Aeromonas* spp. that was negative in the PCR amplification assay and positive for lipolytic activity lack production of lipase H3, which might be masked by other lipolytic activities. In this study, we have described the presence of *lipaseH3* (760 bp) gene by PCR amplification which resulted in lipolytic activity in all isolates of *A. hydrophila.* There was no cross-reactivity of *lip* gene in *Edwardsiella tarda, Escherichia coli* and *A. sobria*.

Table 1. Comparison of different genes of *A. hydrophila* expression level in *E. coli* of wild-type and codon-optimized sequences.

Gene designation	Wild type DNA			Optimized DNA		
	CAI	GC %	AT %	CAI	GC %	AT %
Aerolysin	0.478	56.3	43.7	0.779	54.8	45.2
Hemolysin	0.514	63.3	36.7	0.812	58.6	41.4
Lipase	0.654	65.1	34.9	0.795	57.6	42.4

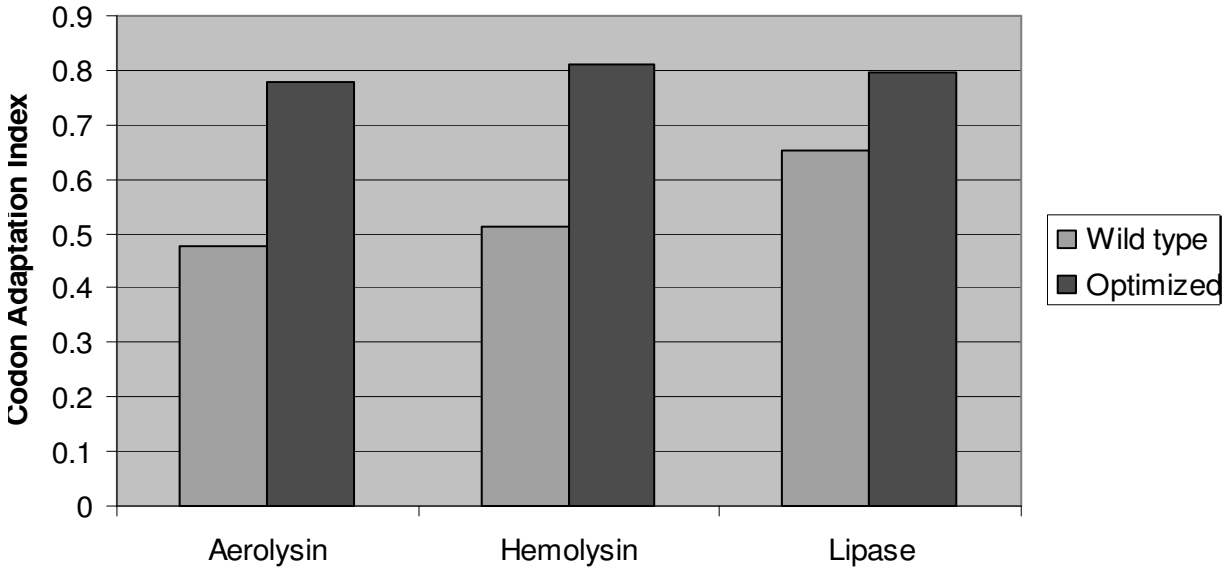

Figure 3. The graphical comparison of the wild and optimized types of DNA sequences of different genes in *A. hydrophila*.

In this study, we used the complete genes sequences *A. hydrophila* for optimization experiments. Therefore, it can be completely expressed in *E. coli* for adequate amount of vaccine. The codon adaptation index of aerolysin, hemolysin and lipase genes of *A. hydrophila* were obtained viz 0.478, 0.514 and 0.654. These sequences were codon optimized and obtained the high values of CAI viz 0.779, 0.812 and 0.795 in comparison to wild type. Whereas the C+C were also changed after the optimization of DNA sequences (Table 1). The graphical representation of wild and optimized DNA of these genes was also shown (Figure 3). The wild type of gene sequences show low CAI compare to optimized. The G+C content of all three genes of *A. hydrophila* was high and after optimization, it was less according to the *E. coli* system.

Codon optimization has been considered an effective strategy for improving the expression levels of heterologous genes that contain codons rarely used in the host organism. The expression of functional proteins in heterologous hosts is a cornerstone of modern biotechnology (Gustafsson et al., 2004). *In silico* study has been done on the low and high level of expression of gene of *V. cholerae*. The value of CAI has been more considered as the high level of expression of gene in host (Basak et al., 2008). The wild type of gene may not over express in other heterologous host. Therefore, it needs to optimize the codon according to host machinery for better expression.

In conclusion, the presence of an aerolysin, hemolysin and lipase genes were demonstrated by PCR and phenotypic methods in *A. hydrophila*. Thus a combination of phenotypic methods and gene amplification may be used to screen virulence factors of *A. hydrophila*. Since all the *A. hydrophila* were isolated from apparently healthy fishes, normal fish may pose as carriers for source of infection. Sometimes, the gene contains the codons that are rarely used in the desired host, come from organisms that use non-canonical code or contain expression-limiting regulatory elements within their coding sequence. Improvements in the speed and cost of gene synthesis have facilitated the complete redesign of entire gene sequences to maximize the likelihood of high protein expression.

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