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RESEARCH ARTICLE

SCREENING FOR VIRULENCE GENE MARKERS IN CLINICAL *A. hydrophila* ISOLATES FROM SOUTHERN CHENNAI (INDIA)

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ABSTRACT

The opportunistic and ubiquitous *Aeromonas spp*., is receiving growing importance since its implication in a wide spectrum of human diseases few decades ago. The enteric pathogen *A.hydorphila*, is the most commonly associated pathogen with acute diarrhoea, with a similar prevalence world-wide. While the host susceptibility to the disease depends on various risk factors that includes food and water source contaminations, the myriad of virulence factors- Haemolyin, exotoxins etc., craft the complex pathogenicity of *A.hydrophila* and thus, extensive studies on this bacteria is imperative, especially in developing nations were hygiene measure controls are often neglected. In the present case-control study, PCR was employed to screen two extensively investigated *A.hydrophila* virulence determinants-*aerA* and *hlyA* genes- in clinical bacterial isolates to ascertain the bacterial strain's pathogenicity in acute diarrhoea from the chosen geographical region. 107 (20.89%) out of 512 acute diarrhoeal patients screened harboured the enteric pathogen, *A. hydrophila*, while the asymptomatic control subjects (n=200) showed no faecal carriage of this micro-organism. Consistent with previous reports the clinical *A.hydrophila* isolates screened carried the $aerA^+$, $hlyA^+$, $aerA^+$ $hlyA^+$-virulent genotypes (albeit at varying frequencies) ascertaining the role of the two toxins –Haemolysin (HlyA) and Aerolysin (AerA)-in influencing the pathogenesis of acute diarrhoea in Southern Chennai (India).

INTRODUCTION

The cosmopolitan *Aeromonas spp.* (family *Aeromonadaceae*) surviving and thriving in diverse conditions, have been isolated from different aquatic ecosystems, drinking water (chlorinated and unchlorinated) (Pianetti *et al*., 2005) and, from various food products including fish and shellfish, raw meat, vegetables and raw milk (Pin *et al*., 1996).With increased resistance to antibiotics (Ko *et al*., 1996) and chlorination in water (Burke *et al*., 1984, Fernandez *et al*., 2000), *Aeromonas spp.* is becoming a food- and water-borne pathogen of growing importance, presenting a significant threat to public health.

Among the mesophilic motile *Aeromonas spp., Aeromonas hydrophila* (Chester) is commonly associated with human diseases ranging from gastroenteritis to deep wound infection and septicemia (von Graeventiz *et al*., 1968, and Chopra *et al*., 1999). Worldwide, the incidence of *Aeromonas* associated diarrhoea has been reported to be as high as 10.8% (Khajanchi *et al*., 2010). With a gamut of virulence factors that includes O antigens, capsules, the S layer, flagella, exotoxins (Haemolysin, Aerolysin etc.) and a repertoire of exoenzymes (Janda and Abbott, 1996), the expression of *Aeromonas spp.* virulence is multifactorial and host susceptibility dependent (Kuijper and Peeters, 1991). In India, *Aeromonas spp.* isolated from clinical specimens in Kolkata over a period of 12 months included *A. caviae* (18.7%), *A. veronii* (10.9%), *A. schubertii* (4.6%), *A. jandaei* (3.1%), and *A. trota* (3.1%), with *A. hydrophila* being the most prominent strain occurring at a frequency of 59.3% (Kannan *et al*., 2001). Across Chennai, fecal carriage rates of *Aeromonas spp.* in 6.5% of symptomatic 10 year olds was reported (Komathi *et al*., 1998) and in other prominent Indian cities, Aeromonas associated diarrhoea was reported to range between less than 1% to 13% (Alavandi and Anathan, 2003). The two haemolytic toxins, HlyA (Haemolysin) and AerA (Aerolysin), belonging to a heterogeneous family of cytolytic haemolyisns (Husslein *et al*., 1991) have been described extensively in *A.hydrophila* (Howard *et al*., 1987, Chakraborty *et al*., 1987 and Wong *et al*., 1998 and Wang *et al*., 2003). Although low in homology and believed to be distinct, inactivation of these two haemolytic toxin genes in suckling mouse model attenuates the virulence of the pathogen, suggesting a synergistic mode of action of these two genes and/or gene products (Heuzenroeder *et al*., 1999). Aerolysin (AerA) secreted as a soluble precursor (proaerolysin1) is processed to its mature form upon binding to GPI anchor. Subsequently, the 53.8 kDa mature Aerolysin assembles into a heptameric complex ('membrane-spanning pore') permeabilizing the plasma membrane, triggering a number of

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cellular responses depending on the cell type (Abrami *et al*., 2000 and Fivaz *et al*., 2001). While the *hlyA* toxin gene is a distinct allele of the *ahh1* gene in *Aeromonas*, the 62 kDa gene product- HlyA (Haemolysin) toxin-was observed to be homologous to *A. hydrophila* ATCC 7966 AHH1, *A.salmonicida* 17-2 ASH4 and *V. cholerae* HlyA haemolytic toxins (Wong *et al*., 1998). To appreciate the pathogenicity mechanism of an organism, identification and examination of virulence conferring genes becomes indispensable (Strauss and Falkow, 1997). With routine detection of *Aeromonas spp.* not efficient, molecular tools such as PCR are being employed extensively for detection of species-specific pathogenic strains (Kingombe *et al*., 1999) and virulence marker screening (Chacón *et al*., 2003 and Sen and Rodgers, 2004). The present study reports on the PCR screening of clinical *A.hydrophila* isolates for two prominent virulence gene markers-*hlyA and aerA* to ascertain the pathogenicity of the bacterial strain in acute diarrhoea in the geographical region of study -Southern Chennai, India.

MATERIALS AND METHODS

Bacterial Samples and Isolation

Stool samples from 512 acute diarrhoeal patients and 200 asymptomatic individuals across rural and urban southern Chennai (India) regions were screened for the enteric pathogen, *A.hydrophila.* Clinical samples were collected in sterile containers and were processed within two hours of collection. Detailed clinical history of the patients was duly recorded on a standard form, including duration and frequency of diarrhoea, vomiting, abdominal pain and dehydration status. In this study, the clinical manifestation of the disease was defined as the passage of watery stools three times or more, daily. The reference strain (defined in terms of established toxigenicity)-*Aeromonas hydrophila* MTCC 646-obtained from the Institute of Microbial Technology, Chandigarh was also included in the study. Alkaline peptone water enrichment broth was used for primary isolation of *A. hydrophila*, followed by allowing the growth of enriched bacterial isolates in Starch Ampicillin Agar (SAA) (Palumbo *et al*., 1985) and Rimler-Shott's medium (Shott and Rimler, 1973).

Virulence Gene Screening-Polymerase Chain Reaction (PCR) and Agarose Gel Electrophoresis

Genomic DNA isolation was performed by alkaline lysis method followed by standard phenol:chloroform (24:1) extraction (Sambrook *et al*., 1989). Isolated DNA samples were quantified by determining the optical density (OD) at 260nm (OD_{260}) using SL159 UV VIS Spectrophotometer (ELICO®, India). PCR was used for detection of two virulence determinants-Haemolysin (HlyA) and Aerolysin (AerA) - in the *A.hydrophila* clinical isolates. The HL primer set (Table 1) was designed to amplify a 597-bp fragment of the extracellular gene *hlyA* (Wong *et al*., 1998). The AL primer (Table 1) set amplified a 497-bp fragment of the *A.hydrophila* aerolysin structural gene *aerA* (Howard *et al*., 1987). DNA samples (100ng) were amplified in a 20 µL reaction mixture consisting of 0.2mM dNTP mix (BioTools, Spain); 0.3µM (each) HL and AL primers (Eurofins Genomics, India) (Table 1); 1X ExPrime Taq™ Buffer (GeNetBio™, Korea); and 1U ExPrime Taq™ DNA polymerase (GeNetBio™, Korea).PCR was performed in Eppendorf Master Thermocycler (Hamburg, Germany).Parameters for the amplification(s) included an initial denaturation at 94°C for 5 min, followed by 32 cycles of denaturation at 94°C for 1min, annealing of the primers at 50°C (*aerA* gene)/60°C (*hlyA* gene) for 1min, primer extension at 72°C for 1min 20s and a final extension at 72°C for 7 min was used. 10µL of the reaction mixture(s) was then analyzed by agarose gel electrophoresis in EtBr (30µg/µL) stained 1.5% agarose alongside 0.5µg GeneRuler™ 1Kb DNA ladder (Fermentas, Thermo Fischer Scientific) at 50V and, the amplified products were visualized using LIUVT312 UV transilluminator (Lark Innovative Fine Teknowledge, India) .

RESULTS

Of the 512 acute diarrhoeal patient stool samples screened, the incidence of *A.hydrophila* was observed in 107 samples (20.89%). Prior to virulence gene marker screening, these 107 clinical bacterial isolates were confirmed and established as *A.hydrophila* by conventional biochemical tests and also, the 16S rRNA molecular marker evaluation revealed a 99% similarity of the screened clinical isolates to the reference strain, *Aeromonas hydrophila* MTCC 646, used in this study (Data not shown). Fecal carrier rate of *A.hydrophila* was highest (30.8 %) in 11-15 yr old acute diarrhoeal patients, followed by 29% in 6-10 yr old patients and the prevalence of the enteric pathogen scaled down as the age sampled increased (Fig.1A). Also, disparities in the frequency of *A.hydrophila* associated diarrhoea in urban (64.5%) and rural areas (35.5%) were observed (Fig.1B). However, the susceptibility pattern of the disease was almost equal in males and females across the rural and urban locales (Data not shown). None of the patients had an underlying illness or had been administered antibiotics within 2 weeks prior to the onset of the illness. The sizes of the amplification products obtained by the PCR were identical to those predicated from the design of the primers (Table 1; Fig.2A and 2B). The *Aeromonas spp.* virulence markers *hlyA* and *aerA* occurred at varying frequencies in the screened acute diarrhoeal samples: Of the 107 *A.hydrophila* clinical isolates screened, three virulence genotypes were discerned – $hlyA^+$ (77%), $aerA^+$ (88%) and $aerA^+$ $hlyA^+$ (31.7%) (Table 2).

DISCUSSION

With Aeromonas implicated as primary pathogens in cases of acute diarrhoeal disease in immuno-compromised and healthy hosts of all age groups (Agger *et al*., 1985, Chopra *et al*., 1999, Cumberbatch *et al*., 1993, Janda and Abbott, 1996), extensive studies detecting and characterizing Aeromonas virulence factors employing molecular tools such as PCR (Kingombe *et al*., 1999) has become imperative in recent years. In this case-control study, 107 (20.89%) out of 512 acute diarrhoeal patients screened harboured the enteric pathogen, *A. hydrophila*, while the asymptomatic control subjects (n=200) showed no faecal carriage of this micro-organism. While earlier reports of A.*hydrophila* associated diarrhoea showed less than 1% to 6.5% incidence in Chennai (India) (Komathi *et al*., 1998 and Alavandi *et al*., 1998), the present study demonstrates a much higher incidence (20.89%), reiterating the pathogenic potential of the micro-organism in the geographical area. The role of *Aeromonas spp.* in acute diarrhea has been supported epidemiologically in many case-control studies (Agger *et al*., 1985; Albert *et al*.,

1999) but not in others (Figura *et al*., 1981 and Pitarangsi *et al*., 1982). Such disparities could be attributed to geographical variance, season of sample collection (Burke *et al*., 1984), patient population, food habits and culture methods influencing recovery rates (Dumontet *et al*., 2003). The authors speculate that the higher incidence of the pathogen in 11-15 yrs and 6-10 yrs old acute diarrhoeal patients-30.8% and 29%- respectively (Fig.1A) could be due to the absence of measures promoting hygiene control among younger subjects. The increasing presence of multiple drug-resistant *A. hydrophila* in food and water sources poses a major public health hazard. Our data suggests high-risk sources of origin for the pathogen (MAR index ranged between 0.14 to 0.52,Data not shown) and this is also evident from the urban population appearing to be more susceptible (64.5%) to clinical manifestation of the disease (Fig. 1B) in comparison to the rural subjects (35.5%). While virulence factors observed in *Aeromonas hydrophila* includes cell associated structures (Flagella, Pili etc.) and extracellular products (Haemolysins, Proteases, Lipases etc.) (Janda and Abbott, 1996), the PCR detection of *Aeromonas spp*. virulence determinants by Kingombe *et al*. in 1999 resulted in the classification of these virulence markers in three classes- aerolysins-hemolysins, cytolytic enterotoxins, and cytotonic enterotoxins.

For the virulence gene marker screening PCR assay in this study, the primers used (Table 1) targeted the *hlyA* and *aerA* genes (Fig. 2A and 2B respectively) and clearly detected specific nucleotide sequence in the template DNA extracted from the screened *A.hydrophila* clinical isolates. The amplification specificity of the PCR primers was evident from the comparable amplicon product sizes obtained from the reference strain (*Aeromonas hydrophila* MTCC 646) and the screened clinical *A.hydrophila* isolates. Consistent with previous reports (Pollard *et al*., 1990, Vadivelu *et al*., 1995, Abdullah *et al*., 2003, Zhang *et al*., 2000), the clinical *A.hydrophila* isolates screened in this study carried the $aerA^+$, $hlyA^+$, $aerA^+$ $hlyA^+$ virulent genotypes establishing the definitive role of the two toxins –HlyA and AerA-in the pathogenesis of acute diarrhoea in humans, albeit the contribution of other potential virulence factors cannot be discredited. The data reported in this study indicates varied distribution of virulence genotypes (Table 2) influencing the pathogenicity of *Aeromonas hydrophila* in the geographical area of study. Although earlier reports (Wong *et al*., 1998 and Heuzenroeder *et al*., 1999) suggest a two-haemolytic toxin (HlyA and AerA) model of virulence for *A. hydrophila*, the virulent genotypes -$hlyA^+$ and $aerA^+$- appear to be more prevalent than the $aerA^+$ $hlyA^+$ genotype (Table 2) in the clinical isolates screened in this study. This genotypic heterogeneity could be attributed to the multifactorial nature of virulence of the pathogen and/or the gender-, age-, geographical- bias/variance, progression of the disease at the time of sample collection, seasonal pattern of disease-pathogen association etc.

Recently, suggestive evidence of successful colonization and infection by particular strains of certain *Aeromonas spp.* after transmission from water to humans was reported (Khajanchi *et al*., 2010). Exposure to *Aeromonas spp.* through ingestion of contaminated food and water is continuous, and chronic exposure culminates in gastrointestinal illness and, addressing this issue is of prime concern in developing nations where prevalence of *Aeromonas spp.* is dramatically underestimated. In conclusion, $aerA^+$, $hlyA^+$, $aerA^+$ $hlyA^+$ genotypes are shown to confer pathogenicity (Acute Diarrhoea) in the geographical region of study, although the contribution of other virulence factors cannot be discounted. Assessing and comparing other virulence determinants in this strain could help elucidate the pathogenic potential of clinical isolates plausibly defining the disease spectrum across gender, age and geographical barriers. This study points to the importance of monitoring and evaluating infection-control measures for improved hygiene and could help prevent cross- contaminations in food and water resources. Harnessing the emerging potential of molecular techniques such as PCR would expedite the screening of the pathogen during cases of outbreaks/epidemics and also, in monitoring water and food sources for contamination, in epidemiological typing of various pathogenic strains amongst Aeromonads, thus translating basic scientific research to a public health perspective.

Table 1: Primer pairs used and Amplicon sizes (in bp).

Primer pair	Sequence (5' to 3')	Target gene	Location within gene	Size of PCR amplicon (bp)	Source/ Reference
HL-F HL-R	GGCCGGTGGCCCGAAGATACGGG GGCGGCGCCGGACGAGACGGG	*hlyA*	3' end	597	Wong *et al.*,1998
AL-F AL-R	GCCTGAGCGAGAAGGT CAGTCCCACCCACTTC	*aerA*	3' end	497	Wong *et al.*,1998

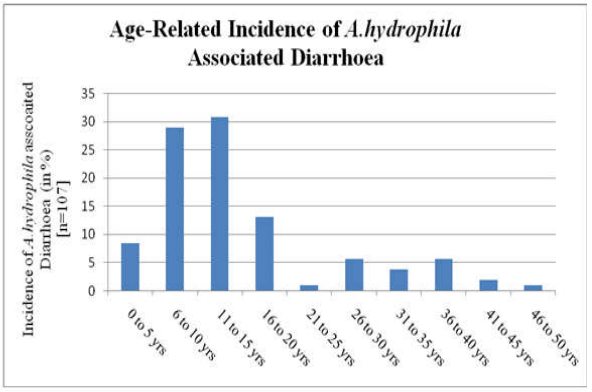
Virulence gene screening in clinical *A.hydrophila* isolates involved identification of two prominent virulence gene markers-*hlyA and aerA* to ascertain the pathogenicity of the bacterial strain in acute diarrhoea in the geographical region of study (Southern Chennai, India)

Table 2: Incidence of *A.hydrophila* virulence genotypes in Southern Chennai (India)

Virulence Genotype	Occurrence (in %) n=107
$aerA^+$	77%
$hlyA^+$	88%
$aerA^+$ $hlyA^+$	31.7%

From the screened clinical *A.hydrophila* isolates, the virulence gene markers-*hlyA and aerA* occurred at varying genotypic frequencies in the geographical region of study (Southern Chennai, India).

A



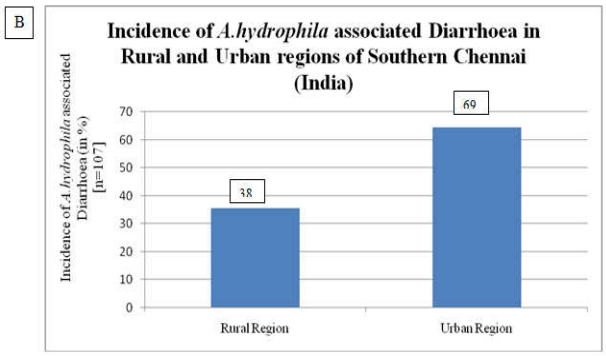


Figure 1: Incidence of *Aeromonas hydrophila* associated Diarrhoea

107 acute diarrhoeal patients were shown to harbour *A.hydrophila* and the occurance of this enteric pathorgen varied acrosse **(A)** different age-groups and **(B)** locality

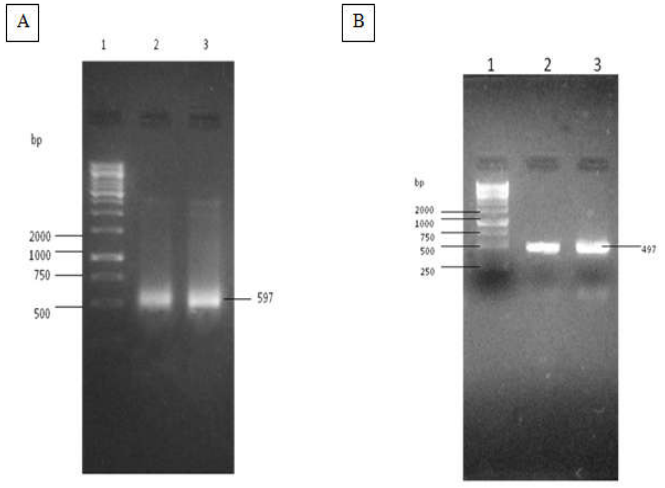


Figure 2: PCR detection of *hlyA* and *aerA* virulence genes in clinical isolates of *A.hydrophila*

1.5% TAE EtBr stained agarose electrophoretogram depicting the amplification of regions of *hlyA* **(A)** and *aerA* **(B)** genes based on the design of the primers (Table 1)

A: Lane 1: GeneRuler™ 1 kb DNA Ladder (Fermentas, Thermo Fischer Scientific) Lanes 2 and 3: *hlyA* gene amplicons (597 bp) from the template DNA obtained from the screened clinical isolates of *A.hydrophila.*

B: Lane 1: GeneRuler™ 1 kb DNA Ladder (Fermentas, Thermo Fischer Scientific) Lanes 2 and 3: *aerA* gene amplicons (497 bp) from the template DNA obtained from the screened clinical isolates of *A.hydrophila.*

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