PCR Detection of Putative Hemolysin and Aerolysin Genes in An Aeromonas Hydrophila Isolates from Diarrhea in Babylon Province

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Abstract
Aeromonas spp are considered as opportunistic infection and enterotoxigenic pathogen and can cause severe diarrhea. A total of one hundred and forty two stool samples were collected from patients with diarrhea at Babylon hospital for maternity and pediatrics of a period between December 2012 to June 2013. The 6 (4.22%) strains of Aeromonas hydrophila were isolated from 142 samples of diarrhea stools. The Aeromonas hydrophila identified based on colony and morphological, biochemical tests and confirmed by Api 20E. Then investigation was done on hemolysin. All the isolates of Aeromonas hydrophila have the β-hemolytic activity on the blood agar. The polymerase chain reactions carried out to detect the presence of hemolysin and aerolysin genes in 6 Aeromonas hydrophila isolates. A 100% (6 isolates) of Aeromonas hydrophila isolates were contained hemolysin genes (ahh 1a) and 50% (3 isolates) were contain aerolysin gene (aerA). The band appearance in amplified gene bacteria shows molecular weight of hemolysin (130 p) and the molecular weight of aerolysin (309 bp).

Key word: Aeromonas, aerolysin, PCR

1.Introduction
Aeromonas spp are Gram negative, short rod shape facultative anaerobes resistance to O/129 vibriostatic & non spore forming (Alper et al., 2010). They are ubiquitous microorganisms found in both aquatic and environmental habitats such as estuary sediment, sea water, sea grass, sea food, water used for food and drinking water (Abbott et al., 2003; Matyer et al., 2007; Martinez Mucia et al., 2000). Aeromonads are frequently isolated from different food and drinking water (Ottaviani et al., 2011) Aeromonas spp. Are considered as opportunistic infections and enterotoxigenic pathogen. Aeromonas can cause severe diarrhea, dysentery and bacteremia (Trower et al., 2000; Blair et al., 1999). Virulence of Aeromonas spp. is multifactorial and incompletely understood. Factors contributing to virulence include toxins, proteases, hemolysins, lipases, adhesins, agglutinins, and various hydrolytic enzymes (Janda and Abbott, 1996). Virulence factors are present in two forms, cell-associated structures, and extracellular products. The main route of transmission for Aeromonas gastroenteritis is considered to be fecal oral. Studies on the etiology of travelers’ diarrhea revealed Aeromonas to have a prevalence of about 3% in diarrheic patients returning from Asia and Africa (Shah et al., 2009). Among other clinically relevant aeromonads like Aeromonas caviae, A. trota, and A. veronii biovar sobria, the most frequently isolated pathogen A. hydrophila is mainly associated with diarrheal illness accompanied by abdominal pain and nausea (Adamki et al., 2006). Virulence factors of aeromonas spp. Including toxin , protease S, hemolysin, lipase, adhesins agglutinins, and various hydrolytic enzymes (Janda and Abbott, 1996). A. hydrophila is mainly associated with diarrheal illness is accompanied by abdominal pain and nausea (Adamki et al., 2006).

A. hydrophila is the most commonly involved in human infection such as septicemia and gastroenteritis (Chopra and Houston, 1999). The pathogenicity of A. hydrophila infection by producing virulence factors such as cytotoxin, proteases, S-layer and aerolysin (Rahaman et al., 1997). Some researchers state that the virulence factors are determinant of bacterial pathogenicity (Vadivelu et al., 1995). The virulence of A. hydrophila is closely related to β-haemolysin produced. Screening of hemolysin genes is the most effective way to detecting and characterizing aeromonas virulence factors (Yousre et al., 2007). Two hemolytic toxins have been described the A. hydrophila a hemolysin (hyl A) (Hirono & Aoki, 1991) and aerolysin (aer A) (Howard et al., 1987). Aerolysin produced by some Aeromonas spp. And possesses both hemolytic and enterotoxic activity (hemolysin enterotoxin) (Xu et al., 1994). The present study was therefore carried out to document the presence of pathogenic A. hydrophila in diarrheal stool samples at Babylon province /Iraq. Two hemolytic toxins, hemolysin and aerolysin have been described in A. hydrophila. No report is available from Babylon province, in this study a search was made for the presence of hemolysin and aerolysin genes in the genome of A. hydrophila isolated from diarrhea specimens.
2. Materials and methods.

2.1 Collection of samples

One hundred and forty two (142) stool samples were collected from patients attending to Babylon hospital for maternity and pediatrics during the period from Dec 2012 to April 2013. Samples were collected in screw capped bottles and transported to the laboratory in ice box with ice packs. Information was also obtained from patients regarding age groups. All samples were analyzed within 8 h. for collection.

2.2 Isolation of Aeromonas hydrophila

One gram of each sample was briefly emulsified in 3 ml of sterile 0.85%(w/v) saline and vortexed for 30 sec. Organ debris was allowed to settle down for 5 min. the samples were put in alkaline peptone water (oxoid pH 9) and sub cultured after incubation at 37°C for 6 h. onto to macconkey agar and aeromonas agar at 37°C for 24 h. (Nzeaka et al., 2002; Jatau and Yalubu, 2004). All the isolates were grown on trypticase soy agar (TSA) at 37°C for 18 h. the strains first identified as Aeromonas spp. According to colony morphology on Macconkey and Aeromonas agar and by microscopic Morphology (gram stain) and by chemical tests (Oxidase, Catalase, Motility, H₂S production, Citrate utilization, Indole, Methyl red & Vogas Proskure,) and by String test, Lysine decarboxylase, Arginine dehydrogenase, Ornithine decarboxylation for differentiated from Vibrio cholera and the diagnostic of these strains confirmed by Api 20E (Biomerieux, France).

2.3 Hemolytic activity

The strains were tested for β-hemolytic activity on a blood base agar (oxoid) supplemented with 5% sheep erythrocytes. Five micro liters of each suspension was streaked onto plates and incubated at 22°C and 37°C for 24 h. The presence of clear colorless zone surrounding the colonies indicated β-hemolytic activity (Gerhardt et al., 1981).

2.4 Oligonucleotide primers and PCR conditions

The polymerase chain reaction (PCR) was used to detect the presence of hemolysin in all Aeromonas isolates. The primer used for hemolysine gene and aerolysine gene (table 1). The Ahh1a primer set was designed to amplify a 130-bp fragment of A. hydrophila extracellular hemolysin gene ahh1 (Wang et al., 2003). The Ah-aerA primer set amplified a 309-bp fragment of the A. hydrophila aerolysin gene aerA (Wang et al., 2003).

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Sequence (5to 3)</th>
<th>Target gene</th>
<th>Size of PCR amplicon (bp)</th>
<th>Reference or GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHH1F</td>
<td>GCCGAGCGCCCAGAAGGTGAGTTGAGCGGCTGGATGCGGTTGT</td>
<td>ahh1a</td>
<td>130</td>
<td>Wang et al., 2003</td>
</tr>
<tr>
<td>AHH1R</td>
<td>CAAGAACCAAGTTCAAGTGCCACAGAAGGTTGAGTTGAGCGGCTGGATGCGGTTGT</td>
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<tr>
<td>AH-aerAF</td>
<td>CAAGAACCAAGTTCAAGTGCCACAGAAGGTTGAGTTGAGCGGCTGGATGCGGTTGT</td>
<td>aerA</td>
<td>309</td>
<td>Wang et al., 2003</td>
</tr>
</tbody>
</table>

PCR was carried out on cycler using the following cycle: preheating at 95°C for 5 min followed by 30 cycles at 95°C for 2 min, 55°C for 1 min and 72°C for 1 min followed by 7 min final extension at 72°C. PCR products were examined by electrophoresis in 1.5% agarose gel in TBE buffer. The gel stained with EtBr and saw under U.V. light (Yogananth et al., 2009).

2.5 DNA extraction

DNA extraction from gram negative bacteria was performed according to the genomic DNA purification kit supplemented by the manufacturing company (Gene aid) and it was stored in 2-8°C.

3. Results

3.1 Isolation of Aeromonas hydrophila.

Out of the one hundred and forty two (142) diarrheic stool samples analyzed, 6(4.22%) were found to be positive for Aeromonas hydrophila. The prevalence per age groups as shown in figure (1) showed that the age groups, <6 years having height rate of 2.11%(3 isolates) from total samples analyzed. Age groups 7-12 years having 1.40%(2 isolates) and >13 years having 0.70%(1 isolate).
3.2 Identification of *A. hydrophila*

The colonies of bacteria grown in culture media appeared 1-3 mm in diameter. *A. hydrophila* showed a yellow shine color on TCBS agar and non lactose fermenters on Macconkey agar, and it was smooth, convex, rounded, β-hemolytic colonies and pale white to grey color on blood agar. This bacteria appeared gram negative, rod shaped, singly, in pairs, or even as short chains at the microscopic examination. The biochemical tests used to confirm the initial diagnosis of *A. hydrophila* (Table 2). *A. hydrophila* presented appositive result to each of the oxidase, catalase, indole, methyl red, simmon citrate, motility test, vogas prokauer and gelatin liquefaction this results.

<table>
<thead>
<tr>
<th>Table 2:- biochemical tests of <em>A. hydrophila</em> isolates</th>
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<tbody>
<tr>
<td>No.</td>
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<td>8-</td>
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</tbody>
</table>

To differentiated *A. hydrophila* from *V. cholera* by string test that the all *A. hydrophila* was gave negative results to it and the *A. hydrophila* was gave positive results to argnine dehydrogenase and lysine decarboxylation and negative result to ornithine decarboxylation in compare to *V. cholera* was positive result to string test, and ornithine and lysine decarboxylation and negative result to argnine dehydrogenase (Table3).

<table>
<thead>
<tr>
<th>Table (3):-differentiated between <em>A. hydrophila</em> and <em>V. cholera</em> by string test and amino acid utilization</th>
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<tbody>
<tr>
<td>Test type</td>
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<tr>
<td>-----------</td>
</tr>
<tr>
<td>String test</td>
</tr>
<tr>
<td>Lysine decarboxylation</td>
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<tr>
<td>Argnine dehydrogenase</td>
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<td>Ornithine decarboxylation</td>
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</table>

In this study standard of biochemical tests by API 20Ew were used to confirm identification of *A. hydrophila*. According to the result of API 20E test, the isolates were identical to the reference of Bergey’s Manual of Determinative Bacteriology. Characterization (based on their morphological and biochemical reactions using the API 20E test showed that these isolates were phenotypically identified as *A. hydrophila*. 

3.3 Hemolysin activity

The results of this study revealed that the all *A. hydrophila* positive to the β-hemolysin on blood agar. Nucleic
acid amplification methods targeting virulence genes of hemolysin and aerolysinin A. hydrophila isolates. The specific PCR products corresponding to the 130 bp fragment of the ahh1 gene and the 309 bp fragment of the aerA gene were detected from pure cultures (Figure 2, Figure 3).

4. Discussion

Among bacterial etiologies of diarrhea A. hydrophila is recognized increasingly as a clinically significant enteric pathogen however there are limited data on prevalence and associated severity of diarrheal disease caused by A. hydrophila in many regions (Mansour et al., 2012). Out of the one hundred and forty two (142) diarrheic stool samples analyzed, 6(4.22%) were found to be positive for Aeromonas hydrophila. Our results are agree with the findings of 4.7 % incidence of A. hydrophila in Chennai India (Vila et al., 2003), and higher than the finding of 1.28 % (Vasaiak et al., 2002) and 1.4 % of A. hydrophila from Mumbai, India (Deodhar et al., 1991) and 3.12% in Nigeria (Rogo et al., 2009). Alavandi and Anandhan (2003) reported Aeromonas associated diarrhea in 13 % samples in Chennai, while Kuijper et al. (1987) and Ogunsanya et al. (1994) reported 3.7 % in Netherlands and 1.4 % in Lagos, Nigeria respectively. However, higher prevalence of 17.7 and 28.1 % were recorded during 2000 and 2001 in Kolkata, India (Sinha et al., 2004) the recovery of A. hydrophila from children <6 years represented the highest percent (2.11%) in this study compared with other age groups. It is believed that gastroenteritis caused by A. hydrophila occurred more commonly in children with acute diarrhoea and adults with traveller’s diarrheal (2%), self-limiting watery diarrheal but could be more severe in children (Kuijper et al., 1987). Recovery rates among children with diarrheal varies geographically: 0.62 to 4% in Malaysia (Lee and Puthucheary 2001; Lee and Puthucheary 2002), 0.75% in Nigeria (Kehinde et al., 2001), 2% in Sweden (Svenungsson et al., 2000), 2.3% in Taiwan (Juan et al., 2000), 4.8% in Switzerland (Essers et al., 2000), and 6.8% in Greece (Maltezou et al., 2001) The isolation rates for human fecal specimens vary widely, as geographical areas, patient populations, food habits, level of sanitation, and culture methods influence the recovery rates (Dumontet et al., 2003).

The colonies of bacteria grown in culture media appeared 1-3 mm in diameter and this result agreed with Brenner et al. (2005). A. hydrophila showed a yellow shine color on TCBSagar and non lactose fermenters on macconkey agar, and it was smooth, convex, rounded β-hemolytid colonies and pale white to grey color on blood agar. This is agreement with Janada & Abbott (2010) and Rogo et al. (2009). This bacteria appeared gram negative, rod shaped, singly, in pairs, or even as short chains at the microscopic examination (Brenner et al., 2005).
The biochemical tests used to confirmed the initial diagnosis of *A. hydrophila*. *A. hydrophila* presented appositive result to each of the oxidase, catalase, indole, methyl red, simmon citrate, motility test, vogas prokauer and gelatin liquefaction this results are almost finding in other researchers report (Erdem et al., 2011; Kivanc et al., 2011). To differentiated *A. hydrophila* from *V. cholera* by string test that the all *A. hydrophila* was gave negative results to it (Martin –Carnahan and Joseph, 2005) and the *A. hydrophila* was gave positive results to argnine dehydrogenase and lysine decarboxylation and negative result to ornithine decarboxylation in compare to *V. cholera* was positive result to string test, and ornithine and lysine decarboxylation and negative result to arginine dehydrogenase (Parjia, 2009). In this study standard of biochemical tests by API 20Ewere used to confirm identification of *A. hydrophila*. Indeed Adel et al., (2011) and Orozova et al., (2010) noticed that all suspected colonies were subsequently confirmed to be *A. hydrophila* using API 20E system and analytical profile index give very good identification.

β-hemolysins as an important bacterial virulence factors which promoting channel formation leading to cell death. the results of this study revealed that the all *A. hydrophila* positive to the β-hemolysin on blood agar this results agreements with Janada and Abbott (2010) and EPA (2006). the -hemolytic activity of A. hydrophila has been used as an indicator of enterotoxicity and may be responsible for outbreaks of diarrhea (Rahim et al., 1984) Nucleic acid amplification methods targeting virulence genes are used for detection of pathogenic bacteria and to differentiate pathogenic from non-pathogenic strains (Chacon et al., 2003; Sen and Rodgers, 2004). Two hemolysins toxins have been found, hemolysin and aerolysin. When the genotypes of known virulent strains as defined in Wong et al., (1996) was compared, it was apparent that all the *A. hydrophila* isolates with the a hhaA and aerA genotype were virulent in the suckling mouse model. These isolates also demonstrated <= hemolytic and cytotoxic activities. Due to the fact that the aerA and hemolysin genes were found in the vast majority of the diarrhoeal isolates from this species (Michelle et al., 1999), this results agreement with Howard et al., (1987) The two haemolytic toxins, haemolysin and aerolysin have been described in *A. hydrophila*. When the PCR was performed to detect aerolysin gene (*aerA*), we found that aerA were associated with A hydrophila (52.6%) harbored aerA (Yours et al., 2007). The major hemolysin produced by aeromonads is called aerolysin, though it is known by several other names (cytotoxic enterotoxin, Asao toxin, and cholerla toxin cross-reactive cytolytic enterotoxin). Aerolysin is produced by some strains of *A. hydrophila*. Wang et al., (2003) developed a multiplex PCR method for detection of hemolysin and aerolysin genes in *A. hydrophila* and *A. sobria* The range of virulence of aeromonads is thought to result from the variety of genotypes present in the environment. Both phenotypic and genotypic heterogeneity are common among aeromonads, Xia et al., (2004) cloned the β-hemolysin gene from *A. hydrophila* isolated from freshwater fish in China. The cloned β-hemolysin sequences were used in a PCR assay to survey environmental isolates to detect potential pathogenic *A. hydrophila* strains. (Alperi et al., 2010).

5. Conclusions

*Aeromonas hydrophila* recognized one of the most important factors that cause diarrhea disease especially in children under 6 years old and the *Aeromonas hydrophila* have virulence factors such as hemolysin and aerolysin, that confirm pathogenicity this bacteria.

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