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RESEARCH ARTICLE

Detection of Hemolysins(*hlyA and aerA*) Genes in *Aeromonas hydrophila* Isolated from Raw and Drinking water in Baghdad City

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Abstract

A simplex and duplex PCR assay was used to amplify the *Aeromonas hydrophila* hemolysin and aerolysin genes. The assay was evaluated by using 63 *A.hydrophila* .A simplex PCR assay was performed to determine whether a597 bp *hlyA* gene and 416bp *aerA* gene fragments could be detected in sixty three Aeromonas isolates. Four genotypes among *A. hydrophila* isolates were found in this study ,the *hlyA*+ *aerA*+ genotype was the most common genotype.A survey was performed on the sixty three *A. hydrophila* isolates by duplex PCR to detect hemolysin genes presence. Four genotypes among *A. hydrophila* isolates also were found.When the presence of both toxin genes was investigated the $hlyA^+$ $aerA^+$ genotype was the most common

Introduction:

Species of Aeromonas are Gram-negative, non-spore-forming, rod-shaped, facultatively anaerobic bacteria that occur ubiquitously and autochthonously in aquatic environments widely distributed in aquatic environments such as surface waters (Ashbolt et al., 1995, Janda and Abbott ,2010). as well as food (Pinto et al., 2011**)** Thus, food and water are probable sources of human infection (Khajanchi et al., 2010).

These organisms have long been known to cause disease in fish, reptiles and amphibians . In recent years, however, their role as opportunistic pathogens implicated in human illnesses such as gastroenteritis, wound infections, septicaemia ,pneumonia and soft tissue conditions, has gained increasing interest (Mukhopadhyay et al., 2003, Janda and Abbott, 2010).

Aeromonas species can be found in various numbers in drinking water. The chronic exposure of immunocompromised persons to Aeromonas via contaminated waters could potentially lead to invasive disease,such as septicemia (Leclerc, **,** and Dei-Cas 2002).

Several studies revealed that these organisms can produce b-hemolysins , (Sen and Rodgers, 2004; Singh et al.,2009).ß- hemolysin, which typically belongs to a larger group of poreforming bacterial cytolysins, causes leakage of the cytoplasmic contents from target cells via disruption of the normal integrity of the cell membrane(Abrami et al., 1998) .

Most Aeromonas hemolysins described are related to *hylA*,and *aerA* toxin. Beta-hemolytic isolates of Aeromonas were found to cause significantly more fluid accumulation in the ileal loops of experimentally infected rabbits. Mutagenesis of *A. hydrophila* isolates that carry two hemolysin genes (*hlyA* and *aerA*) revealed that the hemolytic activity of the isolate on horse blood agar was eliminated only following double mutations in the *hlyA* and *aerA* genes (Wong et al., 1998). A subsequent study suggested that all virulent *A. hydrophila* isolates carried both the *hlyA* and the *aerA* genes (Heuzenroeder et al., 1999). The aim of this study was detection of Aeromonas hemolysins(*hlyA* and *aerA*) by PCR technique.

Material and methods

Bacterial identification

One hundred and thirty one raw water samples (from Tigris river)and four hundred and twenty from five water treatment plants(WTPs) in different area in Baghdad) were collected during the of period April 2011 till February 2012. Drinking water were filtered by filtration apparatus then the membranes were then transfered carefully to a Petri dish of Ampicillin Dextrin Agar(ADA),incubate the Petri dishes at 37 °C for 24 hours.Aloop full of river water samples were cultured directly on Ampicillin Dextrine Agar(ADA) and incubate at 37 °C for 24 h.
Bacterial isolates were identified by the procedures described in Bergey's Manual of Systematic Bacteriology (Martin-Carnahan**,** 2005).Typical colonies (yellow on ampicillin dextrin agar) submitted to biochemical screening :Oxidase and Catalase test; H2S and gas production; fermentation of (glucose, sucrose, arabinose, maltose), indole production ,lysine,argenin and ornithine decarboxylation; motility , triple sugar iron agar test, string test, esculin hydrolysis test, DNase test and detection of haemolysin .For further identification of the *Aeromonas hydrophila* isolates, two API strips: API 20 E and , ID32 E, were used .

Isolation of Genomic DNA

A. hydrophila isolates were cultured on ampicilin dextrin agar then DNA was extracted from all isolates cell using a commercial purification system(Genomic DNA Mini Kit,geneaid ,Thailand) as described by the manufacture instructions . The nucleic acid content was quantified by determining theoptical density (OD) at 260 nm (OD260) **(**Maniatis *et al.*,1982). To determine the purity of the DNA and the degree of contamination with protein, spectrophotometer was also used to estimate the purity ratio of DNA; an additional measurement was made at 280 nm and the purity ratio estimate according to the following formula,(Purity between 1.7 and 2.0 is usually accepted): DNA purity = O.D260 / O.D280 . Gel electrophoresis was used for DNA visualization(agarose 1%) with ethidium bromide and UV transilluminator (Sambrook *et al.*,1989).

Simplex PCR for detection of hemolysin genes.

Simplex PCR have been used to detect the presence of hemolysin genes (*ahhl, AerA*))among all *A.hydrophila* isolates . According to the Heuzenroeder et al.,1999;Aslani, and Hamzeh 2004 and Seethalakshmi ,*et al.*,2008 the forward and reverse primers , 597(*ahhl)*F 5GGCCGGTGGCCCGAAGATACGGG-3, R5-GGCGGCGCCGGACGAGACGGG-3and416bp fragments of the gene (*AerA)*F5-GCCTGAGCGAGAAGGT-3,R5-CAGTCCCACCCACTTC-3 were used for detection the gene ,Lyophilized forward and reverse primers were suspended withD.D.W as recommended by Bionear company protocol.
Optimization of polymerase chain reaction of accomplished, PCR reaction was conducted in 25 µl of a reaction mixture contataining 5 µl PCR premix (Accupower, Bionear), 2µL of each primer(10 picomole/ µl), 3µL of extracted DNA(50 ng/ µl), and 8µl of of distilled water.PCR was performed under the following conditions: 1 cycle at 95C° for5min , ,35cycles of 30 sec at 94 C°,annealing for 30 sec at 62 C°*(ahhl*)or 52 C°(*AerA*),and 2min extention at 72 C°,with a 1min final extention at 72 C°.
A 10µl of PCR product was separated on 1.5% agarose gel electrophoresis, the size of amplified products were compared with the 100pb DNA ladder to determine the exact size of these products

Duplex PCR for detection of hemolysin genes.

Duplex PCR including two genes have been used to detect the presence of hemolysin genes (*ahhl, AerA*))among all *A.hydrophila* isolates . According to the method described by Wang *et al.(*,2003), and Choresca *et al.(*,2010) , the forward and reverse primers were used for detection,*ahhl* 130 bpF5-GCCGAGCGCCCAGAAGGTGAGTT-3, R5 -GAGCGGCTGGATGCGGTTGT-3 and *AerA* 309 bp F5 -CAAGAACAAGTTCAAGTGGCCA-3,
R 5- ACGAAGGTGTGGTTCCAGT-3 were used . Lyophilized forward and reverse primers were suspended with D.D.W as recommended by Alpha- DNA company protocol. Optimization of polymerase chain reaction was accomplished after several trials with some modifications in annealing temperature(62 instead of 59) and number of cycles(35 instead of 50) table 1. PCR reaction was conducted in 25 µl of a reaction mixture containing 5 µl PCR premix (Accupower, Bionear), 2µL of each primer(10 picomole/ µl) , 3µL of extracted DNA(50 ng/ µl), and the rest volume was completed with sterileD. D.W. Negative control contained all material except DNA template. PCR reaction tubes were placed into thermocycler PCR instrument.DNA was amplified as indicating in table1. 10µl of PCR product was separated on 1.5% agarose gel electrophoresis, the size of amplified products were compared with the 100pb DNA ladder for determination the exact size of these products.

Results

Isolation and identification of *A. hydrophila*

Forty five *A. hydrophila* isolates from raw water (Tigris river)
and eighteen isolates from drinking water were obtained .

DNA Extraction

DNA from all isolates of *A. hydrophila* (63) isolates was successfully extracted by using genomic DNA mini kit (Geneaid, Thailand).

Simplex PCR for detection of hemolysin genes.

A PCR assay was performed ,DNA bands were confirmed and analyzed by gel electrophoresis , (figures 1,2) . The results of aerolysin and hemolysin genes percentage in *A.hydrophila* isolates was shown in table 2 . Four genotypes among *A. hydrophila* isolates were found in this study (*hlyA*$^+$ *aerA*$^+$, *hlyA*$^-$ *aerA*$^+$, *hlyA*$^-$ *aerA*$^-$ and *hlyA*$^+$ *aerA*$^-$ with percentages 52.38%, 15.87% ,4.76%, 26.98% respectively. The *hlyA*$^+$ *aerA*$^+$ genotype was the most common genotype. These results is in accordance with Heuzenroeder, *et al.,* (1999) ; and (Aslani and Hamzeh, 2004).Other investigations are carried out to detect *hlyA* and *aerA* genes in *A. hydrophila* strains isolated from drinking water samples (Sen, and Rodgers, 2004).Castro-Escarpulli *et al*. (2003) reported that 96% of strains were positive for aerolysin/hemolysin genes. The results of hemolysin genes presence in the this study was confirmed by several researchers (Heuzenroeder,*et al.,* 1999 ;Aslani, and Hamzeh 2004 and Seethalakshmi ,*et al.,* (2008).

Table: 1 Program used in thermocyler PCR(duplex PCR).

Stage	Temperature (time)	
Initial denaturation	95˚C (5min) 1 cycle	
Denaturation	94˚C (30 sec)	35 cycles
Annealing	62˚C (30 sec)	
Extension	72˚C (30 sec)	
Final extension	72˚C (7 min)	

Table 2 : Percentage (No.)of hemolysin genes of *A.hydrohpila*

PCR assay	*hlyA*$^+$ *aerA*$^+$	*hlyA*$^-$ *aerA*$^-$	*hlyA*$^+$ *aerA*$^-$	*hlyA*$^-$ *aerA*$^+$	*hlyA*$^+$	*aerA*$^+$	Hemolytic strains on blood agar	Positive hemolysin genes strains by PCR	Positive hemolysin genes strains but non hemolytic on blood agar
Simplex	**52.38% (33)**	**4.76% (3)**	**26.98% (17)**	**15.87% (10)**	**79.36% (50)**	**68.25% (43)**	**88.88% (56)**	**95.23% (60)**	**3 strains carried *hlyA*$^+$ *aerA*$^+$, one strain carried *hlyA*$^+$ *aerA*$^-$ genotype**
Duplex	**50.79% (32)**	**6.34% (4)**	**26.98% (17)**	**15.87% (10)**	**77.77% (49)**	**66.66% (42)**	**88.88% (56)**	**93.65% (59)**	**Two strains carried *hlyA*$^+$ *aerA*$^+$, one strain carried *hlyA*$^+$ *aerA*$^-$ genotype**

Figure 1 : Agarose gel electrophoresis (1.5%) of amplified aerA gene (416 bp)of A. hydrophila for 1.5 hour at 5 V/cm, stained with ethidum bromide and visualized on a UV transilluminator.Lanes 1,8: molecular weight marker using 100 bp ladder ,Lane 6 :negative control (had all PCR mixture including water instead of DNAtemplate), Lane 2,3,4,5,7,9,10,11 PCR amplification products of *A. hydrophila* aerolysin gene *aerA* 416 bp.

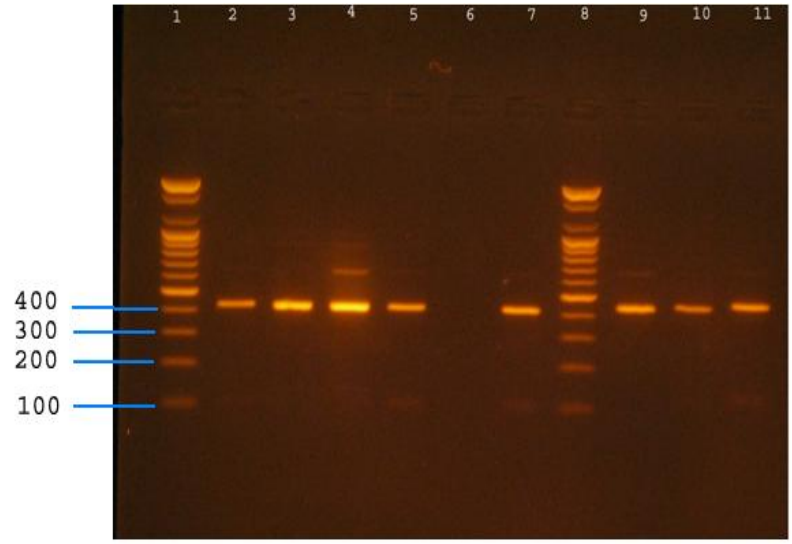
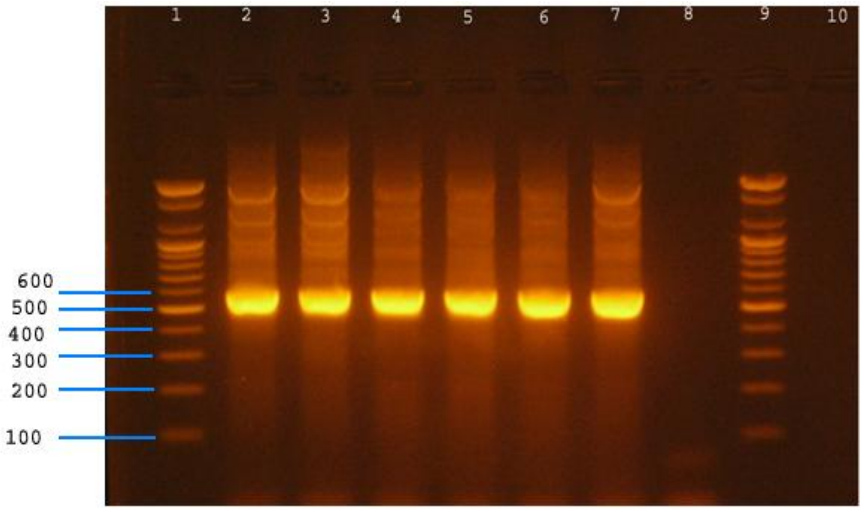


Figure 2 :Agaros gel electrophoresis (1.5%) of amplified *hlyA* gene (597bp) and of *A. hydrophila* for 1.5 hour at 5 V/cm, stained with ethidum bromide and visualized on a UV transilluminator.Lanes 1,9: molecular weight marker using 100 bp ladder.



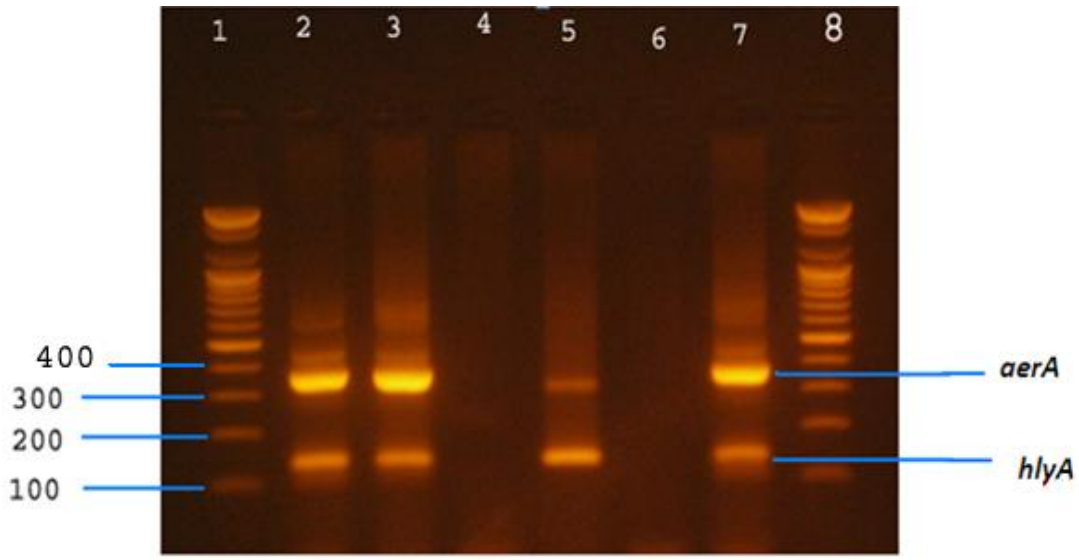
Lane 8 negative control (had all PCR mixture including water instead of DNA template).,Lane 2-3,4,5,6,7 PCR amplification products of *A. hydrophila* hemolysin gene *hlyA A(*597 bp).

Duplex PCR for detection of hemolysin genes.

A survey was performed on the sixty three *A. hydrophila* isolates by duplex PCR using primers *hlyA* (130 bp) and *aerA* (309bp) to detect hemolysin genes presence ,the result summarized in table 2 ,DNA bands were confirmed and analyzed by gel electrophoresis ,(figure 3). Four genotypes among *A. hydrophila* isolates were found in this study (*hlyA*$^+$ *aerA*$^+$, *hlyA*$^-$ *aerA*$^+$, *hlyA*$^-$ *aerA*$^-$ and *hlyA*$^+$ *aerA*$^-$with percentages 50.79%, 15.87%, 6.34%, 26.98%

respectively. When the presence of both toxin genes was investigated the *hlyA*$^+$ *aerA*$^+$ genotype was most common among *A. hydrophila* 50.79 %(32 isolates). The presence and frequency of *hlyA* and *aerA* genes in *A. hydrophila* isolates in this study was in agreement with previous studies (Wang *et al.*, 2003 ; Choresca *et al.*, 2010**).** Some researchers suggested that the occurrence of hemolytic factors in *A. hydrophila* was widespread (Heuzenroeder, *et al.*, 1999).

Figure: 3 Agaros gel electrophoresis (1.5%) of amplified hlyA gene (130 bp) and *aerA* (309 bp) of *A. hydrophila* for 1.5 hour at 5 V/cm, stained with ethidum bromide and visualized by a UV transilluminator.Lanes 1,8:. molecular weight marker using 100 bp ladder ,Lane 4: negative *A. hydrophila* isolate of *hlyA* and *aerA* genes , Lane 6: negative control (had all PCR mixture except DNA template ,Lane: 2,3, 5, 7, PCR amplification products of *hlyA* (130 bp) and *aerA*(309 bp).



Discussion

The high incidence of *Aeromonas spp* in drinking water samples, highlights the importance of detection for hemolysin genes to determine potential pathogenic isolates , which represent a risk for human health (Seethalakshmi ,*et al.*, 2008).In this study showed that 88.88% (56) of *A. hydrophila* strains are β-hemolytic on human blood agar, but 95.23% (60) PCR(simplex) positive for hemolysin genes(*hlyA* and *aerA*) , in other word , PCR is more sensitive, rapid and reliable analytical tools to detection hemolytic activity . Notable that four nonhemolytic isolates on blood agar but carried hemolysin genes were found (three belonged to genotype *hlyA*$^+$ *aerA*$^+$ and one belonged to genotype *hlyA*$^-$ *aerA*$^+$).It is possible that the nonhemolytic isolates carried hemolysin genes either could not expressed because of other factors affect gene expression such as mutations **(**wang *et al.*, 2003).On other hand in duplex PCR , three nonhemolytic isolates on blood agar but carried hemolysin genes were detected (two belonged to genotype *hlyA*$^+$ *aerA*$^+$ and one belonged to genotype *hlyA*$^-$ *aerA*$^+$), this observation supported by Wang *et al.*, 2003**),** this may due to such factors affect gene expression or that had mutations. Although there was no drastic differences observed between frequency results of the *hlyA* and *aerA* genes carried by simplex and duplex PCR , simplex PCR appeared more reliable than duplex PCR in amplification but in other hand duplex PCR more easy in technical side, the same observation reported by Jones *et al.*, 2012.

Conclusion :From these data revealed that using of PCR assay was interesting approach for the direct detection of toxin-producing *A. hydrophila* isolates which isolated from drinking and raw water by use virulence determinants as genetic markers such as hemolysin genes**.**

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