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Genotypic Detection of Some Virulence Factors Among *Aeromonas hydrophila* Isolated from Diarrhea Cases (Iraq)

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The present study included the detection of some virulence factors of *Aeromonas hydrophila* under molecular level to clinical isolates were taken from patients suffering from diarrhea during the period from July (2017) to October (2017). Molecular detection of Hemolysin gene (*ahh*) was done for all isolates. The results showed that all isolates (100%) gave positive results for this virulence gene. the positive results were detected by the presence of (130) bp bands when compared with allelic ladder. The genomic DNA of the samples was extracted and bands were observed by performing agarose gel electrophoresis. When PCR was performed, results clearly indicate that all isolated organisms contained serine protease gene and all the amplified products produced a band at the level of (900 bp) when compared with the allelic ladder. Molecular detection of this gene was carried out by using a specific PCR primer were done by comparison with allelic ladder which gave a (309bp) It was found that (Aerolysin) gene present in (12)(75%) of the positive samples. *Lip* gene was also detected in *A. hydrophila* samples and found that all 16 samples (100%) gave positive results to this gene which gave molecular length (382) bp. Molecular study was carried out to show the sequence identity of cytotonic enterotoxins gene in *Aeromonas* spp. to that in *A. hydrophila*. Analysis of the *A. hydrophila* genome revealed a number of a putative virulence factors, including a gene that heat-labile cytotonic enterotoxin (*alt*). Our study showed that all (16) isolates (100%) gave positive results to this gene, which gave molecular length (442)bp. Molecular detection of cytotonic enterotoxins gene (*ast*) was done for (16) *A. hydrophila* isolates and the results showed that all isolates have this gene (100%). The positive results for (*ast*) virulence were detected by the presence of (331) bp band compared with allelic ladder.

Keywords: *Aeromonas hydrophila*, Hemolysin gene (*ahh*) Aerolysin) gene, cytotonic enterotoxins gene (*ast*).

The genus *Aeromonas* is previously placed in the family, *Vibrionaceae* based on its phenotypic expression[1], but they have recently been transferred from *Vibrionaceae* to their new family *Aeromonadaceae* according to Molecular genetics[2]. The genome of *Aeromonas hydrophila* strain ATCC 7966T is completely sequenced[3].The genome is comprised of a single circular (4.744), (448) bp, chromosome with (61.5%) GC content[4]. The complete genome sequence of *Aeromonas hydrophila* reveals mechanisms contributing to virulence and metabolic condition that allow the organism to grow in a variety of environments and explains how *Aeromonas hydrophila* is able to survive in polluted or oxygen- poor environments and to colonize and cause illness in humans and other hosts. Thus, *Aeromonas hydrophila* genome sequence provides valuable insights into its ability to flourish in both aquatic and host environments[3]. 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 aerolysin gene, Hemolysin gene and lipase gene[5]. The PCR amplification of Hemolysin gene from *A. hydrophila* has been reported. The specific, rapid and sensitive PCR technique has been used to

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detect Hemolysin and aerolysin in Indian isolates of *A. hydrophila* (2). An extracellular lipase (EC) is produced by *A. piscicola* AH-3 (formerly *A. hydrophila*); however, the link between virulence and this gene is speculative in *A. hydrophila*[6]. Lipase is also an important extra-cellular virulence factor affecting several immune system functions through free fatty acid generated by lipolytic activity[7], characterize other nine lateral flagellar genes for *A. hydrophila*. The cytotonic enterotoxins do not cause degeneration of crypts and villi of the small intestine-like cytotoxic enterotoxin[8]. Knock out mutations in either the alt or *ast* gene of *A. hydrophila*, and subsequent challenge of mice with these mutant strains, showed significantly reduced accumulation of fluid in the ligated ileal loop of the animal model, compared with that of wild type, indicating a distinct role of these factors in diarrhea[9]. In the isolate *A. hydrophila* B_{32}, a novel serine protease (*ser*) was found that exhibits cytotoxic properties and is thermo stable, both of which hare characteristics that differentiate this protease from known *A. hydrophila* á- hemolysin and ß-hemolysin[10].

The present study is carried out to detection the virulence genes associated with important virulence factors include: hemolysin (*ahh*), aerolysin (*aer*), Lipase (*lip*), Serine protease (*ahp*), thermo labile cytotonic enterotoxin (*alt*), thermo stable cytotonic enterotoxin (*ast*).

MATERIALS AND METHODS

Samples collection and identification of *A. hydrophila*

According to previous study of[11].

DNA extraction form bacterial culture

Genomic DNA was extracted from the *Aeromonas hydrophila* isolates according to instruction provided by manufacturer using Genomic DNA purification kit supplemented by (Geneaid, USA). The viewed using UV-trans illuminator:

1. bacterial cells (in the sediment) was collected from swab samples that diluted in phosphate buffer solution and transferred to a 1.5 ml micro centrifuge tube then centrifuged in high speed centrifuge at 14000-16000 rpm for 1 minute then the supernatant discarded.
2. Add 20 µl of proteinase k (make sure ddH2O was added).incubate at 60ºC for 10 minutes and inverted every 3 minutes through incubation periods
3. GB buffer (200µl) were added to each tube and mixed by shaking vigorously for 5 seconds. Then the tubes were incubated in water bath at 60ºC for 10 minutes and inverted every 3 minutes through incubation periods.Meanwhile, Elution buffer (200µl per sample) was pre-heated at 60°C (to be used in step 9 DNA Elution).
4. Absolute ethanol (200 µl) were added to the clear lysate and immediately mixed by shaking vigorously, and then precipitates broke it up by pipetting.
5. A GD column was placed in a 2 ml collection tube and transferred all of the mixture (including any precipitate) to the GD column. Then centrifuged at 14,000-16,000 rpm for 2 minutes. In addition, the 2 ml collection tube containing the flow-through were discarded and placed the GD column in a new 2 ml collection tube.
6. W1 buffer (400µl) were added to the GD column, then centrifuge at 14,000-16,000 rpm for 30 seconds. The flow-through was discarded and placed the GD column back in the 2 ml collection tube.
7. Wash Buffer (ethanol was added) 600µl were added to the GD column. Then centrifuged at 14,000-16,000 rpm for 30 seconds. The flow-through was discarded and placed the GD column back in the 2 ml collection tube.
8. All the tubes were centrifuged again for 3 minutes at 14,000-16,000 rpm to dry the column matrix.
9. The dried GD column was transferred to a clean 1.5 ml micro centrifuge tube and 150 µl of pre-heated elution buffer were added to the center of the column matrix.
10. The tubes were let stand for at least 3 minutes to ensure the elution buffer was absorbed by the matrix. Then centrifuged at 14,000-16,000 rpm for 30 seconds to elute the purified DNA.
11. The extracted DNA was stored at -16°C. Later on the extracted DNA that used for molecular identification of A. hydrophila and its virulence factors.

Primers Sequences

The primers sequences and PCR conditions that used in study are listed in Table (1).

Contents of Reaction Mixture

Amplification of DNA done in final volume of (25µ) containing the following as in Table (2).

Detection of Amplified Products by Agarose Gel Electrophoresis

Successful PCR amplification was confirmed by agarose gel/ electrophoresis by visualization against UV light (12). Agarose gel was prepared. Then the comb was fixed at one end of the tray for making wells used for loading DNA sample. The agarose was poured gently into the tray, and allowed to solidify at room temperature for 30 min. The comb was then removed gently from the tray. The tray was fixed in an electrophoresis chamber filled with TBE buffer that covered the surface of the gel, 5µl of DNA sample was transferred into each well of agarose gel, and in one well we put the 5µl DNA ladder. The electric current was allowed to pass at 70 volts for 50min. UV trans-illuminator was used 280 nm for the observation of DNA bands, and the gel was photographed using digital camera.

Table 1. The primers, sequences, and PCR conditions

Gene name	Primer sequence (5´- 3´)	Size of Bp	Conditions	References
Aero	F- ATGCTGCAGAAATGA ATAGAATAATTACCGC R- ATGCAAGGCTTGCCCCATAA TCTCCCAGCGAT	301	95/5min/30 cycle 95/2 min 55/1 min 72/1 min 72/7 min	Xai *et at*., 2003
Hly	F- CTATGAAAAAACTAAAAATAACTG R- CAGTATAAG TGGGGAAATGGAAAG	130	95/5min/30 cycle 95/2 min 55/1 min 72/1 min 72/7 min	Xai *et al*, 2003
Ast	F- TCTCCATGCTTCCCTTCCACT R -GTGTAGGGATTGAAGAAGCCG	331	95/5min /30 cycle 95/25Sec 55/30Sec 72/1 min 70/5min	K.Sen *et al*., 2004
Alt	F -TGACCCAGTCCTGGCACGGC R -GGTGATCGATCACCACCAGC	442	95/5min /30 cycle 95/25Sec 55/30Sec 72/1 min 70/5min	K.Sen *et al*., 2004
Lipase (*Lip*)	F- ATCTTCTCCGACTGGTTCGG R- CCGTGCCAGGACTGGGTCTT	382	95/5min /30 cycle 95/25Sec 55/30Sec 72/1 min 70/5min	K.Sen *et al*., 2004
Ahp	F- ATTGGATCCCTGCCTA R- GCTAAGCTTGCATCCG	911	94/5min /30 cycle 94/30Sec 56/30Sec 72/1 min 72/7min	

Table 2. Polymerase Chain Reaction Mixture

No.	Mixture Contents	Volume (µl)
1	Master Mix	12.5
2	Forward Primer	2.5
3	Reverse Primer	2.5
4	Template DNA	5
5	Nuclase -Free Water	2.5
Total		25

RESULTS AND DISCUSSION

Detection of Virulence Factors Genes

A. hydrophila strains have produced a variety of biologically active extracellular products similar to the virulence factors of Enteropathogenic bacteria and these virulence factors associated with health effects in humans[13]. *A. hydrophila* produce virulence factors such as haemolysin, aerolysin, proteases, lipases DNAase and enterotoxins, which are important in the pathogenesis of human and fish disease[14, 15, 16].

Detection of Hemolysin Gene (*ahh*)

Molecular detection of Hemolysin gene (*ahh*) was done for 16 isolates that previously detected as *A. hydrophila*. The results showed that all isolates (100%) gave positive results for this virulence gene. the positive results were detected by the presence of (130) bp bands when compared with allelic ladder as shown Figure (1). The present results agree with[17, 18, 16] confirmed that *A. hydrophila* strains carried genes encoding hemolysin and the â-haemolytic *A. hydrophila* strains in fish and fish products available for human consumption throughout food stores represent a risk to the consumers health. According to[19], these strains could be dangerous and are more likely to cause food poisoning in humans. Hemolysins are exotoxins and the lytic activities on red blood cells are reported to be important for nutrient acquisition or for causing anemia[20]. Hemolytic toxins as hemolysin and aerolysin released by A. *hydrophila*[21] and play significant role in pathogenesis. Hemolysin genes did not appear to be distributed randomly among the different *Aeromonas* species. The hemolytic activity of *A. hydrophila* has been used as an indicator of enterotoxicity and may be responsible for outbreaks

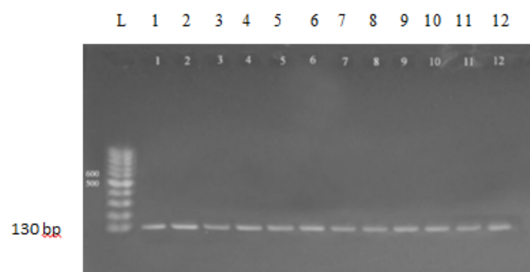

Fig. 1. agarose gel electrophoresis (1.5%) of RCR amplified of *hly* gene (130)bp of *A. hydrophila* for (55)min at (70) volt; L: ladder (DNA marker). (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10) Amplify of 16Sr RNA gene in isolates of *A. hydrophila*

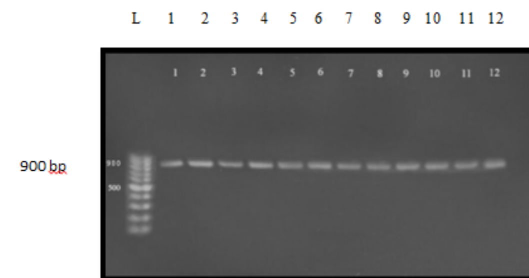

Fig. 2. Agarose gel electrophoresis (1.5%) of RCR amplified of *ahp* gene (900)bp of *A. hydrophila* for (55) min at (70) volt; L: ladder (DNA marker). (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10) Amplify of 16Sr RNA gene in isolates of *A. hydrophila*

Fig. 3. Agarose gel electrophoresis (1.5%) of RCR amplified of *aerA* gene (309)bp of *A. hydrophila* for (55) min at (70) volt; L: ladder (DNA marker). (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10) Amplify of 16Sr RNA gene in isolates of *A. hydrophila*

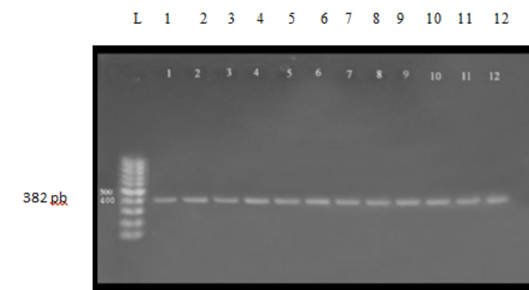

Fig. 4. Agarose gel electrophoresis (1.5%) of RCR amplified of *lip gene* (382)bp of *A. hydrophila* for (55) min at (70) volt; L: ladder (DNA marker). (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10) Amplify of 16Sr RNA gene in isolates of *A. hydrophila*

of diarrhea[22]. Nucleic acid amplification methods targeting virulence genes are used for detection of pathogenic bacteria and to differentiate pathogenic from non-pathogenic strains[23, 24].

Detection of Serine protease Gene (*ahp*)

Molecular studies of serine protease gene were done for all *A. hydrophila* samples by using specific PCR markers. Polymerase chain reaction is a sensitive and specific method for identification of virulence gene. Hence, for the direct detection of pathogenic *Aeromonas* species isolates, virulence determinants are used as a genetic marker. Thus, PCR method is used to detect the virulent gene encoding serine protease using specific primer PCR technique clearly identified the virulent gene as serine protease gene in *Aeromonas hydrophila*. The genomic DNA of the samples was extracted and bands were observed by performing agarose gel electrophoresis. When PCR was performed, results clearly indicate that all isolated organisms contained serine protease gene and all the amplified products produced a band at the level of (900 bp) when compared with the allelic ladder as showed in Figure (2)[25] studied the serine protease and reported the PCR amplified band at (900 bp). The results of the present study also confirm the same[26]; all samples were give positive results 16 positive (100%) for serine protease gene with variable amplicon when it was compared with allelic ladder. Proteases produced by bacteria have been shown to cause tissue damage, aid invasiveness and establishment of infection by overcoming host defenses and provide nutrients for bacterial proliferation. It was reported that *Aeromonas* produced several kinds of protease. Studies of the proteases of *Aeromonas* have mainly been performed with *A. hydrophila*[27]. It has been suggested that proteolytic enzymes of human pathogen, *Aeromonas hydrophila*, play an important role in causing massive tissue damage in the host, which may facilitate establishment of infection[28]. Serine protease or serine endopeptidases

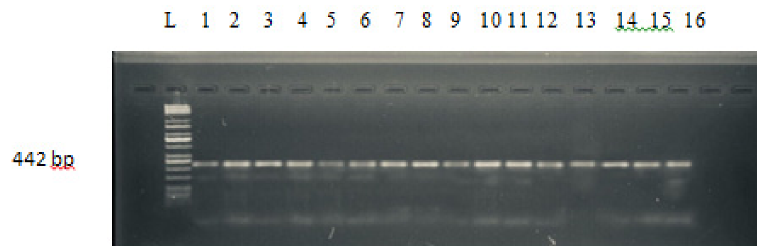

Fig. 5. Agarose gel electrophoresis (1.5%) of RCR amplified of *alt gene* (442)bp of *A. hydrophila* for (55)min at (70) volt; L: ladder (DNA marker). (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10) Amplify of 16Sr RNA gene in isolates of *A. hydrophila*

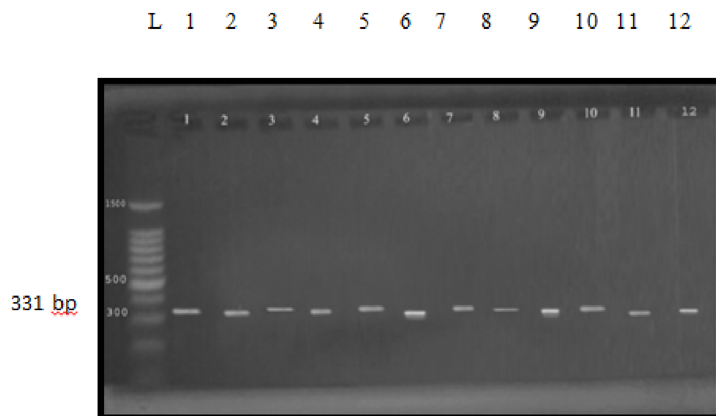

Fig. 6. Agarose gel electrophoresis (1.5%) of RCR amplified of *ast gene* (442)bp of *A. hydrophila* for (55)min at (70) volt; L: ladder (DNA marker). (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10) Amplify of 16Sr RNA gene in isolates of *A. hydrophila*

are proteases (enzymes that cut peptide bonds in proteins) in which one of the amino acids at the active site is serine. Serine protease is known to activate toxins such as aerolysin and Glycerophospholipid Cholestrol acyl transferase (GCAT). This process is called quorum-sensing[29]. Glycerophospholipid cholesterol acyl transferase (GCAT) and Aerolysin both are activated by serine protease. The results also contrary with[23] which stated that aerolysin / hemolysin and serine protease genes were present in all â – hemolytic strains supporting serine protease act as possibly important for the activation of the former gene. PCR detection may prove to be an important tool for the detection, identification, differentiation, and distribution of virulence markers. This method will give microbiologists a substitute way to understand pathogenicity in *Aeromonas* spp. and their distribution in different sources of diarrheal sample. The present report describes PCR method that detects serine protease genes in *A. hydrophila* by using a pair of primers for a serine protease gene the most known virulence gene of *A. hydrophila*.

Detection of aerolysin Gene (*aerA*)

Molecular detection of this gene was carried out by using a specific PCR primer were done by comparison with allelic ladder which gave a (309bp) It was found that (Aerolysin) gene present in (12)(75%) of the positive samples as shown in Figure (3). The results were closely related to those obtained by[30] who mention that *A. hydrophila* have ability to produce aerolysin. Also our results agreement with[31] who reported that the distribution of Aerolysin gene reach to (85.7%). Aerolysin was the factor most commonly described in *A. hydrophila* isolates, and previous studies have shown that two hemolytic toxins: *HlyA* and *aerA* contribute to the virulence of *A. hydrophila*. However, the inactivation of either *aerA* or *hlyA* alone reduced but did not totally cancel the hemolytic and cytotoxic activities of virulent strains of *A. hydrophila* .These activities were removed only when both the *hlyA* and *aerA* genes were inactivated. It is likely that both toxins act by pore-formation and the effects are likely to be synergistic[32]. Current study in agreement with other papers [33, 34]. The presence of aerolysin is a strong indication of virulence in pathogenic isolates of *Aeromonas* spp[35]. The major hemolysin produced by aeromonads is called aerolysin, though it is known by several other names (cytotoxic enterotoxin, Asao toxin, and cholera toxin crossreactive cytolytic enterotoxin). Aerolysin is produced by some strains of *A. hydrophila*[36]. Aerolysin is a pore-forming toxin and is regarded, as the most important virulence factor in *Aeromonas* food poisoning, produced by some strains of *A. hydrophila*, is an extracellular, soluble, hydrophilic protein exhibiting both hemolytic and cytolytic properties[37].

Detection of Lipase Gene (*lip*)

Lipases may be important for bacterial nutrition and they may constitute virulence factors by interacting with human leukocytes or by affecting several immune system functions through free fatty acids generated by lipolytic activity[34]. Lip gene was also detected in *A. hydrophila* samples and found that all 16 samples (100%) gave positive results to this gene, which gave molecular length (382) bp. The amplicon was detected in gel electrophoresis and compared with allelic ladder as shown in Figure (4), our results agree with[38] who mention that *A. hydrophila* have ability to produce lipase. The enzyme have essential role in pathogenicity and enhanced bacterial persist, the direct role of lipase to hydrolysis of phospholipids leading to depletion of structural lipids or generation of lytic reaction products both leading to loss of membrane integrity and cytotoxic. The lipases and hydro lipases were considered as important virulence factors in *Aeromonas* spp. because they alter the structure of the cytoplasmic membrane of the host and thus exacerbate its pathogenicity, especially if the aerolysin gene is present[33]. These genes are important extracellular factors for colonization of host tissues and their necrosis [39,40] demonstrated the potential of the association of elastase and lipase with the damage caused by aerolysin in cell cultures. The capacity of extracellular enzymes to cause lysis to "feed" the bacterial cells in proliferation is very important for *Aeromonas* spp[39]. The lipase gene was also described as an important virulence factor in *Aeromonas* spp. isolates from trout[33,35] proposed that for *A. hydrophila* the presence of two genes (Lipase and aerolysin) might indicate their virulence in animals. These results confirm that this species has a larger matrix of virulence genes when compared with other species of clinical relevance[41].

Detection of cytotonic enterotoxins Gene (*alt*)

Cytotonic enterotoxins may play significant roles in the pathology of *Aeromonas* gastrointestinal disease[41]. Molecular study was carried out to show the sequence identity of cytotonic enterotoxins gene in *Aeromonas* spp. to that in *A. hydrophila*. Analysis of the *A. hydrophila* genome revealed a number of a putative virulence factors, including a gene that heat-labile cytotonic enterotoxin (*alt*). Our study showed that all[16] isolates (100%) gave positive results to this gene, which gave molecular length (442)bp as shown in Figure (5). *Alt* is a Heat-labile cytotonic enterotoxins exhibiting a size of 44 kDa single polypeptide that causes elevation of cyclic AMP and prostaglandin levels in Chinese hamster ovary (CHO) and intestinal epithelial cells. The Cytotonic enterotoxins (*ast, alt*) do not cause degeneration of crypts and villi of the small intestine-like cytotoxic enterotoxin[8]. Mutations in either the alt or ast gene of *A. hydrophila*, and subsequent challenge of mice with these mutant strains, showed significantly reduced accumulation of fluid in the ligated ileal loop of the animal model, compared with that of wild type, indicating a distinct role of these factors in diarrhea[9]. The alt gene however does not manifest any lipase activity[43]. The results from[44] suggest that the product of both the alt and *ast* genes may act synergistically to induce severe diarrhea. Extracellular factor genes and virulence in *A. hydrophila*[32]. In a large survey of enterotoxin genes (act, *alt* and *ast*) in *Aeromonas* spp. Isolates from children with diarrhea, healthy controls and the environment, there was a significant association between the presence of both *alt* and *ast* genes amongst diarrheal children compared with controls[44].

Detection of cytotonic enterotoxins Gene (*ast*)

Molecular detection of cytotonic enterotoxins gene (*ast*) was done for (16) *A. hydrophila* isolates and the results showed that all isolates have this gene (100%). The positive results for (*ast*) virulence were detected by the presence of (331) bp band compared with allelic ladder as shown in Figure (6). The presence of both alt and *ast* genes has been associated with sever watery diarrhea in *A. hydrophila*. However, the prevalence of these genes varies considerably depending on the source, species, geographical location and the number of isolates tested (41). The exotoxins are major virulence factors of *Aeromonas* spp. that include a cytotoxic heat-labile enterotoxin (*act*), a cytotonic heat-labile enterotoxin (*alt*) and a cytotonic heat-stable enterotoxin (*ast*) encoded by act, alt and *ast* gene, respectively (9, 45). In this study, all the isolates among *Aeromonas* spp. carried one or more enterotoxin genes. In the PCR assay, *Aeromonas* strains were found with different virulence gene combinations. The *alt* and *ast* genes showed the highest occurrence in *A. hydrophila* and all the strains carried these enterotoxin genes. The results are closely related to those results obtained by (45) who mention that (88%) of *A. hydrophila* carry the *ast* gene that have a role in diarrhea cases.

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