Molecular Detection of Some Virulence Genes in *Aeromonas* Species Isolated from Fishes and Water of Manzala Lake

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Abstract
In a view of increasing the role of *Aeromonas* spp. in fish and human diseases, the present wok was conducted to assay the presence of six virulence genes using PCR in *Aeromonas* species isolated from fishes and water of Manzala Lake. A total of 200 fish samples comprising 100 *Oreochromis niloticus* fish, 100 *Mugil cephalus* fish and 50 water samples were collected from El Gamil region in Lake Manzala. A total number of 258 isolates belonging to *Aeromonas* spp. were recovered from fish and lake water samples and they were biochemically identified into 4 biotypes. PCR assay of 15 representatives, biochemically confirmed *Aeromonas* spp. isolates showed that they were genetically confirmed belonging to genus *Aeromonas* based on specific16S rRNA gene sequence. Virulence properties of the 15 representative strains showed that the majority of the examined strains carried one or more virulence genes. A significant 5 virulence gene have been found in the tested *Aeromonas* isolates. The frequencies distribution of these genes was *aero* (100%), *act* (20%), *ahcytoen* (13.33%), *lip* (13.33%), *alt* (6.67%). Meanwhile, Haemolysin (*hly*) gene could not be detected in any of examined *Aeromonas* isolates.

Key Words: *Aeromonas* spp., Lake Manzala, Virulence genes.

Introduction

Lake Manzala is the largest coastal lake in Egypt, Economically, Manzala Lake is considered as one of the most valuable fish sources in Egypt ***(GAFRD, 2014)***.

Aeromonas spp. is a major bacterial pathogen responsible for hemorrhagic septicemia affecting a wide assortment of freshwater and marine fish causes significant economic losses due to the high mortality in some fish species that limits production (***Paniagua et al., 1990).*** Aeromonads caused a serious problem for the fish farming industry in Egypt as well as in other countries (***Noga, 2010).***

Pathogenicity of *Aeromonas* cannot be ascribed to individual factors there were several virulence factors contribute to the pathogenesis in fish and human diseases caused by *Aeromonas* none of them alone can be responsible for all symptoms of disease stage, ***(Janda and Abbott, 2010).*** These virulence factors are used as survival means, self-defense mechanism and establishment of pathogenicity ***(Odeyemi et al., 2012).*** Identification of several virulence factors of this genus become important because of the complexity in pathogenesis of *Aeromonas* species, as the role of each single factor with respects to pathogenesis varies ***(John and Hatha, 2013).***

PCR has been mostly used for the characterization of isolates either for their virulence properties or for the phylogenetic situations ***(Yanez et al., 2003).*** The 16S rRNA gene is a confirmed method which contributes in signature sequencing for molecular identification of *Aeromonas* species ***(Martinez-Murcia et al., 1992)***. PCR have been broadly used in determining the potential pathogenicity of *Aeromonas* species and virulence-encoded genes ***(Yi et al., 2013).*** Therefore, this study was directed to isolate and identify *Aeromonas* species from fishes and water of Manzala Lake by conventional methods and to study the presence of some virulence genes in the isolates by molecular techniques.

Materials and Methods

2.1. Samples:

A total of 200 fish samples comprising 100 *Oreochromis niloticus* fish, 100 *Mugil cephalus* fish and 50 water samples were collected from El Gamil region, located in the eastern north corner of Manzala Lake. Samples were collected in a sterile container, labeled and transported in insulated ice-boxes with ice to Port Said laboratory for Food Hygiene, Bacteriology Unit for bacteriological examination.

2.2. Isolation of *Aeromonas* species from fishes and water samples:

Isolation of *Aeromonas* spp. from fishes and water samples were carried out according to ***(APHA, 1998)***, first enrichment in alkaline peptone water followed by isolation in *Aeromonas* agar. Typical colonies suspected to be *Aeromonas* species were selected and then purified for further identification.

2.3. Identification of *Aeromonas* isolates:

Identification and biotyping of the isolates to genus and to species level was carried out according to Aerokey II of ***Carnahan et al. (1991a)***.

2.4.1. Molecular characterization of *Aeromonas* species isolates using PCR:

Fifteen representative, biochemically confirmed *Aeromonas* spp. isolates (4 *A. hydrophila*, 4 *A. sobria*, 4 *A. caviae* and *3 A. schubertii*) were identified at the genus level based on their 16S rRNA gene sequences.

2.4.2. Detection of virulence genes by PCR:

The presence of 6 virulence related genes namely; *aer, act, ahcytone, lip, alt* and *hly* were screened by conventional PCR. The Primers sequences, target genes and amplicons sizes showed in **Table (1)**.

Table (1): *Oligonucleotide primers sequences used in PCR:*

Gene	Primer sequence (5'-3')	Length of amplified product	Reference
16S rRNA	F: CTACTTTTGCCGGCGAGCGG	953 bp	Gordon *et al.*, 2007
	R: TGATTCCCGAAGGCACTCCC		
Haemolysin (*hly*)	F: CTATGAAAAAACTAAAAATAACTG	1500 bp	Yousr *et al.*, 2007
	R: CAGTATAAGTGGGGAAATGGAAAG		
Aerolysin (aero)	F: CACAGCCAATATGTCGGTGAAG	326 bp	Singh *et al.*, 2008
	R: GTCACCTTCTCGCTCAGGC		
ahcytoen	F: GAGAAGGTGACCACCAAGAACAA	232 bp	Cagatay and Şen, 2014
	R: AACTGACATCGGCCTTGAACTC		
Lipase (*lip*)	F: ATCTTCTCCGACTGGTTCGG	382 bp	Sen and Rodgers, 2004
	R: CCGTGCCAGGACTGGGTCTT		
act	F: AGAAGGTGACCACCACCAAGAACA	232 bp	Nawaz *et al.*, 2010
	R: AACTGACATCGGCCTTGAACTC		
alt	F: TGACCCAGTCCTGGCACGGC	442 bp	
	R: GGTGATCGATCACCACCAGC		

Results

Table (2): *prevalence of Aeromonas spp. in collected fish and water samples from Manzala Lake:*

Sample	No. of samples	Number and percentage of positive sample for *Aeromonas*	
		No.	%
Oreochromis niloticus	100	62	62
Mugil cephalus	100	46	46
Lake water	50	29	58
Total	250	137	54.8

Table (3): *Identified Aeromonas species recovered from fish and water samples from Manzala lake:*

Identified isolates	No.	%
A. hydrophila	125	48.45
A. sobria	73	28.29
A. caviae	50	19.38
A. schubertii	10	3.88
Total isolates	258	100

Table (4): *The distribution of studied virulence genes among Aeromonas spp. isolates:*

Sample No.	*Aeromonas* spp. Isolates	Origin of isolates	*aero*	*Act*	*ahcytoen*	*lip*	*alt*	*hly*
1	*A. hydrophila*	*O. niloticus*	+	+	-	-	-	-
2	*A. hydrophila*	*M. cephalus*	+	-	-	-	-	-
3	*A. caviae*	*O. niloticus*	+	-	-	-	-	-
4	*A. caviae*	*M. cephalus*	+	-	-	-	-	-
5	*A. caviae*	Lake water	+	-	-	-	-	-
6	*A. sobria*	*O. niloticus*	+	-	-	+	-	-
7	*A. hydrophila*	Lake water	+	-	-	-	-	-
8	*A. schubertii*	*O. niloticus*	+	-	-	-	-	-
9	*A. schubertii*	*M. cephalus*	+	-	-	-	-	-
10	*A. sobria*	Lake water	+	-	-	-	-	-
11	*A. sobria*	*M. cephalus*	+	-	-	-	-	-
12	*A. schubertii*	Lake water	+	+	+	-	-	-
13	*A. caviae*	*O. niloticus*	+	-	-	-	-	-
14	*A. sobria*	*M. cephalus*	+	-	-	-	-	-
15	*A. hydrophila*	*O. niloticus*	+	+	+	+	+	-

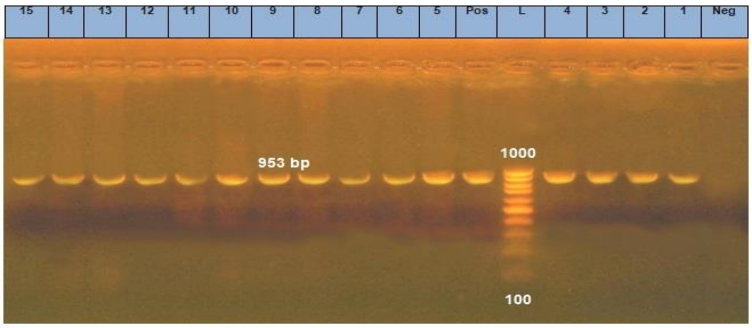

Fig. (1): Agarose gel electrophoresis showing the result of PCR for detection of genus *Aeromonas* (at 953bp).
Lane L: 100-1000 bp. DNA Ladder. Neg.: Negative control. Pos.: Positive control.

In this figure lane 1 to 15 are positive to genus *Aeromonas.*

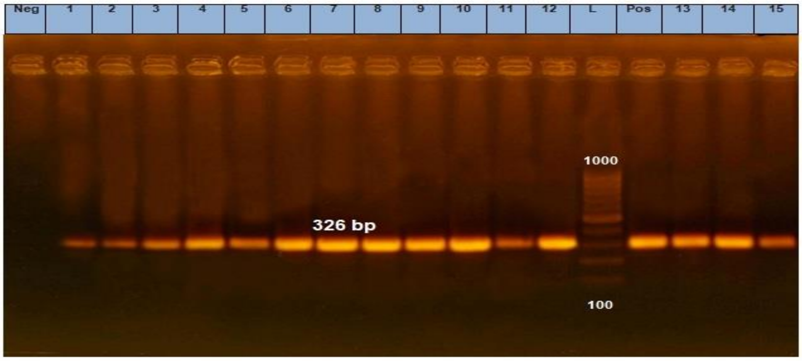

Fig. (2): PCR amplification of Aerolysin (*aero*) gene (at 326 bp).
Lane L: 100-1000 bp. DNA Ladder. Neg.: Negative control. Pos.: Positive control. In this figure lane 1 to 15 are positive to aerolysin gene.

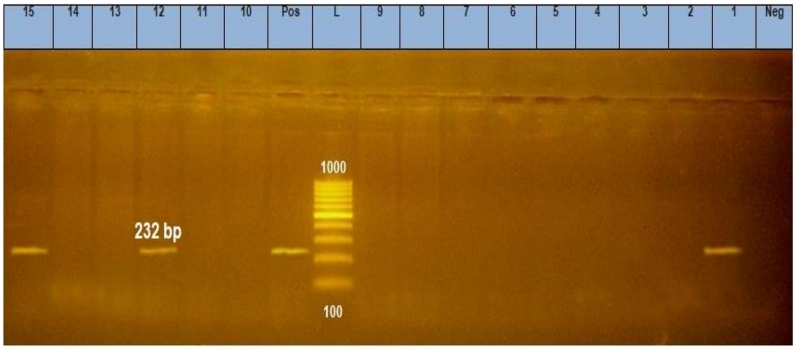

Fig. (3): PCR amplification of Cytotoxic enterotoxin (*act*) gene (at 232 bp.).
Lane L: 100-1000 bp. DNA Ladder. Neg.: Negative control. Pos.: Positive control. In this figure lane 1, 12&15 are (Positive) (*A. hydrophila* from *Oreochromis niloticus*), (*A. schubertii* from Lake water) and *(A. hydrophila* from *Oreochromis niloticus*), while lane 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 13&14 are (Negative) to Cytotoxic enterotoxin (*act*) gene.

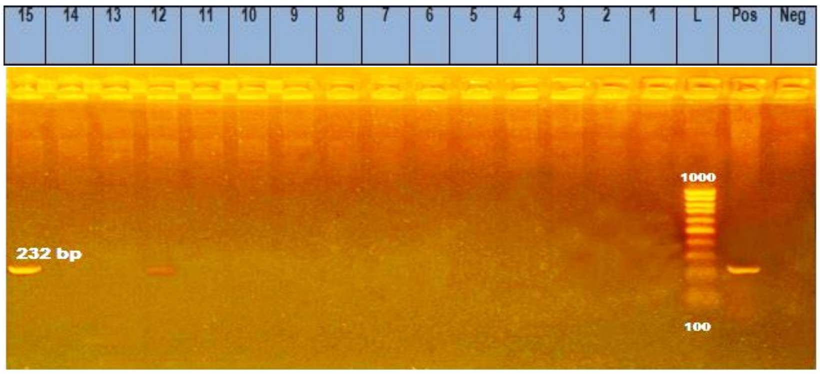

Fig. (4): PCR amplification of *A. hydrophila* cytolytic enterotoxin (*ahcytone*) gene (at 232 bp.).
Lane L: 100-1000 bp. DNA Ladder. Neg.: Negative control Pos.: Positive control. In this figure lane 12&15 are (Positive) (*A. schubertii* from Lake water) and (*A. hydrophila* from *Oreochromis niloticus*), while lane 1, 2, 3,4,5,6, 7, 8,9,10, 11, 13 &14 are (Negative) to A. hydrophila cytolytic enterotoxin (*ahcytone*) gene.

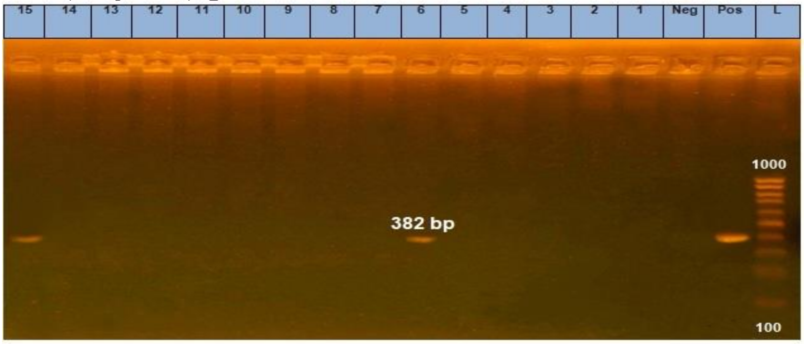

Fig. (5): PCR amplification of extracellular lipase enzyme *(lip)* gene (at 382bp.).
Lane L: 100-1000 bp. DNA Ladder. Neg.: Negative control. Pos.: Positive control In this figure lane 6&15 are (Positive) (*A. sobria* from *Oreochromis niloticus*) and (*A. hydrophila* from *Oreochromis niloticus*),while lane 1, 2, 3,4, 5, 7, 8,9,10 ,11, 12, 13 & 14 are (negative) to extracellular lipase enzyme (*lip)* gene .

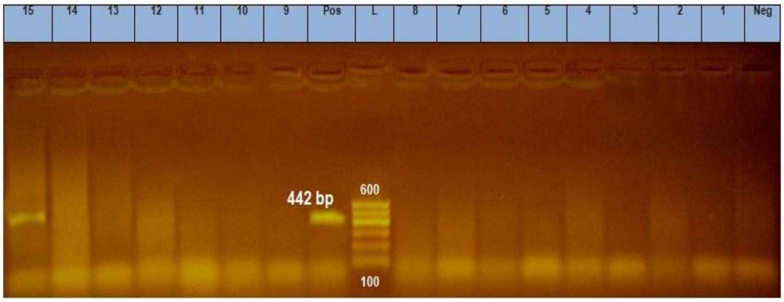

Fig. (6): PCR amplification of cytotonic heat-labile enterotoxin (*alt*) gene at (442 bp.).
Lane L: 100-600 bp. DNA Ladder. Neg.: Negative control. Pos.: Positive control. In this figure lane 15 is (Positive) (*A. hydrophila* from *Oreochromis niloticus*), while, lane1, 2, 3,4, 5,6, 7, 8,9,10 ,11,12,13&14 are (negative) to cytotonic heat-labile enterotoxin (*alt*) gene.

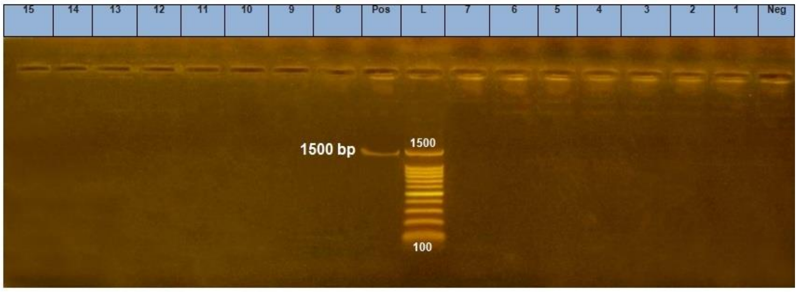

Fig. (7): PCR amplification of Haemolysin (*hly*) gene (at 1500 bp.).
Lane L: 100-1500 bp. DNA Ladder. Neg.: Negative control. Pos.: Positive control. In this figure lane 1 to 15 are negative to Haemolysin (*hly*) gene.

Discussion

The present result in **Table (2)** and **Table (3)** revealed that among the samples investigated, 62%, 46%, and 58% of *Oreochromis niloticus*, *Mugil cephalus* and water samples were found positive by the cultural method. The isolated species from fresh fish are the same types of *Aeromonas* spp. isolated from water and they were biochemically classified into 4 biotypes as *A. hydrophila* which found to be the dominant identified with total prevalence of 48.45% (125/258) followed by *A. sobria* 28.29% (73/258), *A. caviae* 19.38% (50/258) and *A. schubertii* 3.88% (10/258).

Confirmation of *Aeromonas* species may be achieved by sequencing the16S RNA gene ***(Sreedharan et al., 2011)***. **Fig. (1)** revealed that 15 representative, biochemically confirmed *Aeromonas* spp. isolates (4 *A. hydrophila*, 4 *A. sobria*, 4 *A. caviae* and *3 A. schubertii*) were confirmed to belong to the genus *Aeromonas* based on their 16S rRNA gene sequencing and the results were identical to those obtained from conventional identification methods**.** In this concern, ***Diyana and Ina (2016)*** stated that all 22 of *Aeromonas* spp. strains isolated from diseased freshwater fishes were identified using the 16S rRNA.

Since virulence in *Aeromonas* is considered to be multifactorial, the PCR approach to detect virulence genes becomes important. Isolates identified by sequencing of the 16S rRNA were analyzed with respect to the prevalence of some genes responsible for virulence using conventional PCR to confirm their pathogenicity. The presences of the following genes encoding virulence factors were determined in the isolates: Aerolysin (*aer*), cytotoxic enterotoxin gene (*act*), A. hydrophila cytolytic enterotoxin (*ahcytoen*), lipase (*lip*), cytotonic heat-labile enterotoxin (*alt*) and hemolysin (*hly*) as showed in **Table (4)** and **Fig. (2), (3), (4), (5), (6)** and **(7)**.The results revealed that at least 1 of these genes was present in all 15 strains. The frequency distribution of examined genes were *aero* (100%), *act* (20%), *ahcytoen* (13.33%), *lip* (13.33%) and *alt* (6.67%), respectively, while hemolysin (*hly*) gene was not detected (0%).

The distribution of these virulent genes varies among *Aeromonas* strains, but *A. hydrophila* was the main species containing virulence genes. These results agree with ***De silva et al. (2018)*** who indicated that *A. hydrophila* was the species which possessed the highest number of virulence genes. The results showed that at least 1 of these genes was present in all 15 strains. This result supported by ***Sreedharan et al. (2012)*** who reported that *Aeromonas* isolates were found to be associated with at least one virulent gene.

In this study the Aerolysin (*aer*) gene was found to be the most predominant gene among the studied virulence genes as it presents in all examined strains (100%). The present findings were agreed with ***Abdullah et al. (2003*)** who detected aerolysin gene in all isolates and closely similar to ***Hoel et al. (2017)*** who found aerolysin (*aer*A) in (98%) of isolates. These findings supported by ***Nam and Joh (2007)*** who found the dominant virulence factor was *aer* for all seasons. Generally, *aer*A is widely distributed among *Aeromonas* isolates **(*Li et al., 2011).*** Less percent reported by ***Robertson et al. (2014)*** who found the *aer*A in (20%) of strains, respectively. Aerolysin is a pore forming toxin and regarded as the most important virulence factor in *Aeromonas* food poisoning and one of the major virulence factors in gastroenteritis ***(Xu et al., 1998).***

Regarding to the cytotoxic enterotoxin gene (*act*), 20% of our isolates harbored (*act*) gene. This was inconsistent with other studies with higher detection rate of the gene as reported by ***De Silva et al. (2018)*** who detected the gene in 50% of isolates and other with lower rate as reported by ***Robertson et al. (2014) and Ghenghesh et al. (2014)*** who found (*act)* in 13% and 7.7% of isolates, respectively. The cytotoxic enterotoxin gene is one of the primary genes that make *Aeromonas* pathogenic **(*Chopra et al., 2000).***

Concerning to A. hydrophila cytolytic enterotoxin (*ahcytoen*) gene, it was present in (13.33%) of examined strains. This result is closely similar to ***Gupta et al. (2013)*** who revealed that 5 (12.5%) isolates out of 40 were positive for the gene, but lower than result obtained by ***Kingombe et al. (1999)*** who detected *ahcytoen* gene in about 66% of the isolates. *ahcytoen* is commonly detected among pathogenic bacterial isolates and has been previously helpful in differentiating pathogenic strains from non- pathogenic ones ***(Kingombe et al., 1999).*** Regarding to extracellular lipase enzyme (*lip*) gene, it was present in 13.33% of strains. This result is closely similar to ***Khor et al. (2015)*** who found that 16% of isolates were positive for this gene, lower than the result obtained by ***De Silva et al. (2018)*** who found 84% of strains possessed this gene respectively. Lipase plays an important role in invasiveness and establishment of infections ***(Scoaris et al., 2008).*** Concerning to cytotonic heat-labile enterotoxin (*alt*) gene, it was found in 6.67% of examined strains. This result nearly agreed with ***Khor et al. (2015)*** who found *alt* in 8% of *Aeromonas* species isolated from fresh water lakes in Malaysia. This was inconsistent with other studies with higher detection rate of the gene which reported by ***Robertson et al. (2014) and De Silva et al. (2018)*** who detected this gene in (40%) and (77%) of isolates respectively, but ***Ghenghesh et al. (2014)*** could not detect this gene in their study. Cytotoxic heat-labile enterotoxin (*alt*) is one of the enterotoxins which is considered as a major virulence factor of *Aeromonas* spp. ***(Chopra et al., 1993).***

In this study, Haemolysin (*hly*) gene was not detected in any of examined *Aeromonas* isolates. Similar result obtained by ***Khor et al. (2015)*** who could not detect *hly*A genes in *A. hydrophila* and *A. caviae* isolates. In addition ***Sreedharan et al. (2012)*** who recorded that none of the *A. caviae* and *A. veronii* biovar *sobria* isolates possessed haemolytic toxin genes (*hlyA* and *aerA*). Meanwhile, in the other study it was detected in 96% of *A. hydrophila*, 12% of *A. veronii bv. sobria* and 35% of *A. caviae* strains ***(Heuzenroeder et al., 1999).*** The production of haemolytic toxins has been regarded as strong evidence of pathogenic potential Aeromonads ***(Erdem et al., 2010).***

In conclusion the present results revealed that *Aeromonas* isolates found in environmental water and fish of Manzala lake possess a wide variety of virulence-associated genes and this indicate their potential to cause diseases in fishes and humans and this explain the importance of examining as many isolates as possible in order to understand the health risks that these bacteria may cause. The detection of virulence genes is a crucial step for determining the potential pathogenicity of *Aeromonas* isolates and essential to understanding the pathogenesis and epidemiology of *Aeromonas*.

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الملخص العربى

الكشف الجزيئي عن بعض جينات الضراوة لانواع الايروموناس المعزولة من اسماك ومياة بحيرة المنزلة

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في ضوء زيادة دور أنواع الأيروموناس في الأمراض التي تصيب الإنسان والأسماك لما لها من ضراوة تهدف هذه الدراسة الى تحديد بعض جينات الضراوة لأنواع من الأيروموناس المختلفة المعزولة من أسماك البلطي والبوري ومياه بحيرة المنزلة. حيث تم جمع ٢٥٠ عينة من منطقة الجميل في بحيرة المنزلة وتتضمن ١٠٠ سمكة من نوع البلطي و ١٠٠ سمكة من نوع البوري و ٥٠ عينة من المياه التي جمعت عينات الأسماك منها. وتم تحديد أنواع الأيروموناس بناءً على الفحص البكتيرى وكذلك الخواص البيوكيميائية.
تم عزل ٢٥٨ عترة وقد تبين ان العترات المعزولة تنتمى الى أربعة أنواع من جنس الأيروموناس .هذا وقد تم إختيار١٥ معزولة ممثلة من التى تم عزلها وتصنيفها كيميائيا للتاكد من إنها تنتمى لجنس الأيروموناس بالفحص الجزيئى واختبار البلمرة المتسلسل إعتمادا علي تسلسل 16S rRNA gene , وقد تبين أن العترات تنتمى الى جنس الأيروموناس. وبالفحص الجزيئي لجينات الضراوة للمعزولات الممثلة وجد أن غالبية السلالات التي تم فحصها تحمل جينا واحدا أو أكثر من الجينات وتم تحديد ٥ من هذه الجينات وهم *aero* و *act* و *Ahcytoen* و *lip* و*alt* وتتواجد بنسبه ١٠٠٪ و ٢٠٪ و ١٣,٣٣٪ و١٣,٣٣٪ و٦,٦٧٪ علي التوالي. بينما لم يتم العثور علي جين الهيموليسين (*hly*) في أي من عزلات الأيروموناس التي تم فحصها.