Phenotypic and Genotypic characterization of Vibrio species isolated from marine fishes

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A B S T R A C T

Vibriosis is considered the most important threatening disease problem facing aquaculture. The bacteria occur widely in aquatic environment and are part of the normalflora of coastal seawater and are opportunistic pathogens in marine animals.A total number of (311) fish ,*(97)Seabream , (118) Seabass,* (96) *Mugil capito* were collected from Kafr Hamedo and Ezzbet El Borg marine water fish farm (Damietta governorate), El-Manzala and Mansoura Fish Market (EL-Dakahlia governorate)in the period from March 2016 to April 2017 during the four seasons. The clinical examination of diseased fish revealed that, presenceof redness at base of anal fin and erosion of caudal fin, presence of ulcersin skin, darkness of the skin, detached scales, fin erosion, corneal opacity and palegills. Post mortem examination of infected fishes revealed that, liver appearedenlarged, congested or pale with engorged gall bladder, splenomegaly, congestedkidney and hemorrhage in abdominal cavity. Congested gills with excessiveamount of mucous and enlarged liver with hemorrhagic patches on itsedges.The isolated bacteria on thiosulfate citratebile salt sucrose agar (TCBS) gave yellow colonies for *Vibrio alginolyticus* and gave green colonies for *Vibrio parahemolyticus*. The strains were tested according to their susceptibility as resistant, intermediate or sensitive for each antibiotic group. *Vibrio alginolyticus and Vibrio parahemolyticus* revealed sensitivity to ciprofloxacin (Cipro5µg) and novobiocine (NV30µg). Moreover intermediate sensitivity was found to oxytetracycline (OT 30 µg). While resistance was observed to amoxicillin (Aml 10 µg) and cholistine sulphate (CT 10 µg).The isolated bacteria (Vibrio alginolyticus and Vibrio parahemolyticus) were 171/311 (54.98%) from infected fish where the total number of Seabass 72/118 (61%), Seabream 47/97 (48.45%) and Mugil capito 52/96 (54.17%). The highest infection rate was recorded by summer (100% , 82.61% , 80%), winter (73% , 58.33% , 54.54%) , spring (56% , 42.11% , 69.23%) and autumn (36% , 30.23% , 40.38%) for Seabass, Seabream and Mugil capito respectively.PCR yielded a single specific and clear amplificated band ofexpected size 737bp for collagenase gene and 387bp for pR72H gene for *V. alginolyticus* and *V. parahemolyticus* respectively.

Keywords:*V. alginolyticus, V. parahemolyticus,Marine fishes*, specific genes (collagenase and pR72H).

1. Introduction

Egypt's aquaculture production were estimated over 705,490 tones in 2009 as the largest of any African country and 11th in the global production and the importance of this sector as it is providing a cheap source of protein forthe Egyptian people *(Macfadyen et al.,2011; Sadek, 2013).*Fisheries represent an important sector in the Egyptian national income structure. In the fisheries economy, marine fishes represent the major investment choices for the national fishermen. Marine fishes are liable to variable number of environmental stressors, including chemical, natural and biological invaders. Such stressors are the main predisposing factors for the chronic immunosuppression of marine aquatic animals in the affectedmarine habitat. As an ultimate fate for the staggering immuno-

suppression of fishes, bacterial invasion will be the most probable event (*Ellis, 1999).*Further, the bacterial invasion of any marine species could possibly exacerbate under the triggering effect of the fluctuating climatic changes (*Wedemeyer, 1996).*Outbreaks of bacterial diseases are largely responsible for the high mortality of wild and farm-cultured fish.causes severe economic losses to fish farms *(Olsson et al., 1998)*Recently, vibriosis as an important pathogenic cause for outbreaks in Egyptian aquaculture industry was recorded in many studies *(Abd El-Galil and Mohamed, 2012; Abd-Elghanyand Sallam, 2013; Abdel-Aziz et al., 2013; Eissa et al., 2013 ; El-Hady et al.,2015).*Vibriosis is a human illness caused by pathogenic species of the family Vibrionaceae*(CDC 2016).*The genus Vibrio comprises more than 45 species, most of which are widely distributed in marine environment. The Vibrio species affected all typeof fish of either marine or freshwater fish all over the world in different areas ofAsia, America, Australia, Africa and Europe *(Reham, 2009).Vibrio alginolyticus* and *Vibrio parahemolyticus* are responsible for mass mortalities among fish stocks in many marine fish farms throughout the Mediterranean area and severe economic losses in aquaculture worldwide (*Snoussi et al., 2008).*According to *Marhual et al., (2010) and Letchumanan et al., (2015). Vibrio alginolyticus* and *Vibrio parahaemolyticus* are important halophilic Gram negative pathogens, non-spore forming, curved rod shaped bacterium which cause serious episode tomarine fish and shellfish and also naturallylives in marine environments worldwide. Gastroenteritis caused by *V. parahaemolyticus* is mainly characterized by reddish watery bloody diarrhea "Meat Washed", abdominal cramps, nausea, vomiting, headache and low grade fever *(Honda and lida., 1993).* Isolation andidentification of *Vibrio*can be made by using thiosulphate citrate bile salt sucrose agar (TCBS) which is the primary plating medium universally used for the selective isolation of vibrios such as *Vibrio alginolyticus* and *V. parahaemolyticus* from a variety of clinical and nonclinical specimens. The later produces non-sucrose fermenting typical round (2-3 mm in diameter), green or blue center colonies on TCBS Agar *(Elliot et al., 1992).*

Thisstudy aimed to conduct phenotypic and genotypic identification of *Vibrios* isolated from marine fishes and to evaluate the seasonal prevalence of bacterial isolates among the examined fishes.

2. MATERIALS AND METHODS

2.1.Sampling: A total number of (311) fish ,*(97)Seabream , (118) Seabass* ,(96) *Mugilcapito* , with body weight ranging from (70 – 450 g) were freshly collected at random samples from Kafr Hamedo and Ezzbet El Borg marine water fish farm (Damitta governorate) , El Manzala and Mansoura Fish Market (EL-Dakahlia governorate) in the period from March 2016 to April 2017 during the four seasons. Fishes were transferred alive in plastic tank with air blower, freshly dead samples were kept in ice boxes and were transported to Animal Health Research Institute (AHRI) - Al Mansoura branch and subjected to clinical, post mortem and bacteriological examination for isolation of *Vibrio* spp.

2.2-Clinical examination:External clinical examination was performed using the method described by *Schaperclaus et al. (1992)* and internal (Post mortem) examination according

to *Austin and Austin (2007*). 2.3.Bacteriological examination:Sampling and primary isolation of bacteria was carried out under complete aseptic conditions then inoculated into tryptic soya broth with 2% Na Cl and incubated at 25ºC for 24hr. A loopful of incubated broth streaked on thiosulfate citrate bile salt sucrose agar (TCBS) and incubated at 25ºC for 24hr. according to *(Noga, 1996),* Purification of bacterial isolates *(Austin and Austin, 2012),* Identification of bacterial isolates through Gram stain (Shape and arrangement of bacteria) according to method described by *Lucky (1977),* Motility test andOxidase test *(Buller, 2004)*Identification of the isolates by API® 20 E: Analytical profile indexsystem according manufacture guide (BioMerieux, Paris, France), Growth of bacteria indifferent concentration of sodium chloride, other conventional test: Catalasetest, V.P. reaction and Hydrogen sulphide on TSI media.

2.4.Molecular identification of *Vibrio* by polymerase chain reaction (PCR): DNA molecular marker 50 to 2000 bp ladder (Sigma) and procedures according to manufacturer protocol of Omega Co. (USA.LMt.).

Table (1): Target gene, oligonucleotide primer sequence and PCR amplicon (bp) for *Vibrio alginolyticus* and *Vibrio parahemolyticus*.

Gene	Sequence	Molecular weight
Collagenase *(Di pino et al.,2005).*	F: (5-CGAGTACAGTCACTTGAAAGCC-3) R: (5- CACAACAGAACTCGCGTTACC-3)	737 Bp
PR72H *(Lee et al.,1995)*	Vp32:(5-CGAATCCTTGAACATACGCAGC-3) VP33: (5-TGCGAATTCGATAGGGTGTTAACC-3)	387 Bp

Procedures for total genomic of *Vibrio sp.* Samples were done according to protocol of Omega Co. (USA. LMt.). The reaction consists of 40 cycles; each cycle consisted of denaturation at 94 ^{0}C for 30 sec followed by annealing at 30^{0}C for 30 sec and extension at 72^{0}C for 30 sec *(Di pino et al., 2005).* There was an initial delay for 15 min at 95 ^{0}C at the beginning of the first cycle and 10 min delay at 72^{0}C at the end of the last cycle as a post extension step the product was stored at –20 C or 4^{0}C. Gel documentation system was applied for data analysis using Total lab analysis software (Geldoc-it, UVP, England).

2.5. Antibiogram test:

Antibiotic Sensitivity Discs (oxoid) as Oxytetracycline (OT 30ug), Ciprofloxacine(Cipro5ug) , Cholisitine Sulphate (CT 10ug) , Amoxyciline (Aml 10ug) and Novobiocine (NV 30ug) for differentiation between vibrio spp. and other Gram negative bacterial isolates .

The disc diffusion method described by *Koneman et al., (1992) and Quinn et al., (2002).* Commercially available antibacterial disks (Oxoid) was dispensed on the surface of the medium with sterile forceps and incubated for 24 h at 25°C. After incubation of the plates, the degree of sensitivity was determined by measuring the zone of inhibition around each disk which produced by diffusion of antimicrobial agents from the discs into surrounding medium.

3. RESULTS:

3.1. Diagnosis of Vibriosis :

3.1.1 Clinical signs and post mortem changes of infected fishes with *Vibriosis:* Result of clinical examination of diseased fish revealed that, presence of redness at base of anal fin and erosion of caudal fin. Presence of ulcers in skin, darkness of the skin, detached scales, fin erosion, hemorrhages on several parts of the body surface,Hemorrhagic areas around the mouth, corneal opacity and pale gills. The post mortem examination of infected fishes revealed that, liver appeared enlarged, congested or pale with engorged gall bladder, splenomegaly, congested kidney and hemorrhage in abdominal cavity.

3.1.2 Biochemical identification:*Vibrio* spp. is. Oxidase positiveand catalase positive, sensitive to Novobiocine (30 µg) and Vibriostatic disc O/129 (150 µg), grow at wide range of temperature (20-35ºC) and salinity (2- 8 % NaCl). Positive reaction for arginine decarboxylase, indole production, vogus-proskauer tryptophan desaminase, gelatinase, glucose and mannitol fermentation., Negative reaction for H2S production, urea hydrolysis, Ortho-nitro phenylgalactosidase, Lysine and Ornithine decarboxylase, citrate utilization, inositol, sorbitol, mellibiose, and arabinose fermentation. No growth appeared at 0%, Na Cl but positive at 2%, 4% 6%, 8% NaCl as shown in table (2).

Table (2): Growth of Vibrio species on peptone water supplemented with different concentration of sodium chloride:-

Different conc. of salt (NaCl)	Vibrio Parahemolyticus	Vibrio alginolyticus
Peptone water +0% NaCl	-	-
Peptone water +2% NaCl	+	+
Peptone water +4% NaCl	+	+
Peptone water +6% NaCl	+	+
Peptone water +8% NaCl	+	+

- Indicate no turbidity of peptone water (negative result)

+ Indicate turbidity of peptone water (positive result)

3.1.3. Antimicrobial (Antibiogram susceptibility testing):

The strains were tested according to their susceptibility as resistant, intermediate or sensitive for each antibiotic group. *Vibrio alginolyticus and Vibrio parahemolyticus* revealed sensitivity to ciprofloxacin (Cipro5µg), novobiocine (NV30µg). Moreover intermediate sensitivity was found to oxytetracycline (OT

30 µg). While resistance was observed to amoxicillin (Aml 10 µg) and cholistine sulphate (CT 10 µg) as illustrated in table (3).

Table (3): *Showing antimicrobial resistance patterns of Vibrio strains*

Antimicrobial agents	Symbol	Disk potency (µg)	*Vibrio alginolyticus*		*Vibrio parahemolyticus*	
			Inhibition zone (mm)	Interpretation	Inhibition zone (mm)	Interpretation
Oxytetracycline	OT	30 µg	18	Intermediate	13	Intermediate
Ciprofloxacin	Cipro	5 µg	19	Sensitive	17	Sensitive
Amoxicillin	Aml	10µg	-	Resistant	-	Resistant
Colstinsulphate	CT	10µg	-	Resistant	-	Resistant
Novobiocine	NV	30 µg	17	Sensitive	15	Sensitive

3.1.4. Molecular identification**:** The amplified patterns obtained by PCR with tested *V. alginolyticus and V. parahemolyticus* strains. All isolates were positively reacted to the collagenase gene primers. Each strain gave almost a common band with the same molecular weight observed in the different strains. The three isolates of *V. alginolyticus* yielded a single band of amplified product at (737 bp) corresponding to collagenase gene as shown in figure (1).Also The four isolates of *V. parahemolyticus* yielded a single band of amplified product at (387 bp)corresponding to pR72H gene as shown in figure (2).

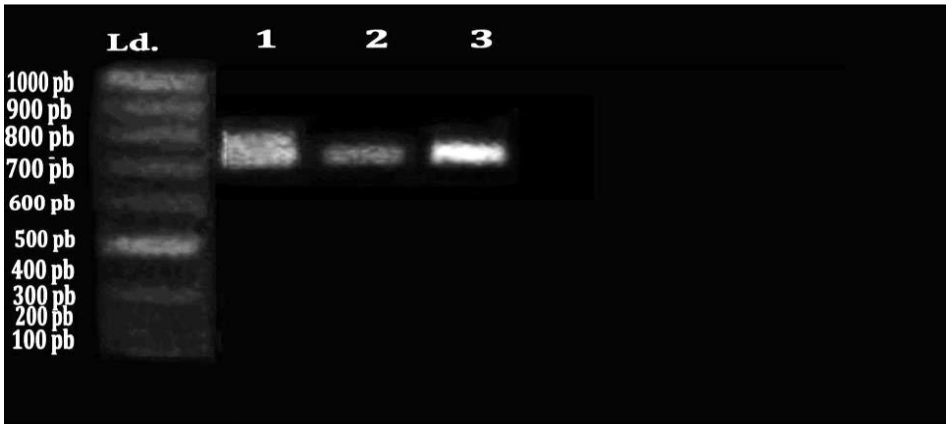

Figure (1): *Agarose gel electrophoresis of products obtained after PCR amplification of collagenase gene of three Vibrio alginolyticus strains extracted from Mugil capito yielded (737 Bp) using forward and reverse primers*.

Ld. Indicate 100bp size ladder.

1 indicate Vibrio alginolyticus strains isolated from spleen.

2 indicate Vibrio alginolyticus strains isolated from liver.

3 indicate Vibrio alginolyticus strains isolated from kidney.

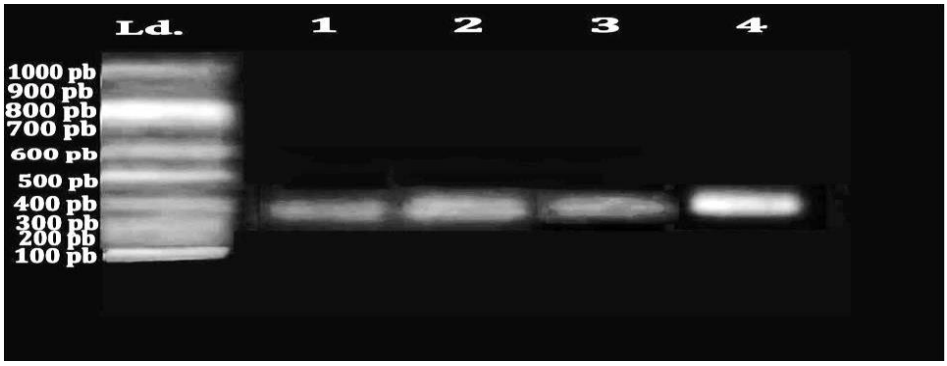

Figure (2): Agarose gel electrophoresis of products obtained after PCR amplification of the pR72H gene of four Vibrio parahemolyticus strains extracted from Mugil capito yielded (387Bp) using forward and reverse primers.

Ld. Indicate 100bp size ladder

1 indicate Vibrio parahemolyticus strains isolated from gill.

2 indicate Vibrio parahemolyticus strains isolated from spleen.

3 indicate Vibrio parahemolyticus strains isolated from liver.

4 indicate Vibrio parahemolyticus strains isolated from kidney.

3.2Prevalence of Vibrio spp. Infection: This study showed some epidemiological characteristics of *Vibriosis* in Sea bass, Sea bream and Mugil capito represented in the etiological agent and its relationship with its prevalence in fish and in between seasons.

As shown in (table 4), which showed seasonal prevalence of *Vibrio spp.* infection in Sea bass, Sea bream and Mugil capito. Total 311 fishes (118 Sea bass, 97 Sea bream, 96 Mugil capito) were examined microbiologically for investigating the seasonal occurrence of *Vibrio spp.* infection. The total number of infected fish was 171 with 54.98 % prevalence rate. For Sea bass, the total number of infected fish was 72 from 118 with 61 % prevalence rate, the highest infection rate was recorded during summer season with prevalence rate 100% followed by winter 73 %, spring 56% and the lowest was autumn with prevalence rate 36%.

For Sea bream, the total number of infected fish was 47 from 97 with 48.45 % prevalence rate, the highest infection rate was recorded during summer season with prevalence rate 82.61 % followed by winter 58.33 %, spring 42.11 % and the lowest was autumn with prevalence rate 30.23 %. For Mugilcapito, the total number of infected fish was 52 from 96 with 54.17 % prevalence rate, the highest infection rate was recorded during summer season with prevalence rate 80 % followed by spring 69.23 %, winter 54.54 % and the lowest was autumn with prevalence rate 40.38 %.Throughout the study two main *Vibrio spp.* were isolated and identified as *Vibrio alginolyticus* and *Vibrio parahemolyticus* infection.As shown in (table 5), the occurrence of *V. alginolyticus* and *V. parahemolyticus* infections in Sea bass in examined organs was , the liverwas the highest infected organwith *V. alginolyticus* 39.13 % followed by spleen 37% and kidney 20 % and gills 4.35 % . Also, the liverwas the highest infected organwith *V. parahemolyticus* 42.3 % followed by spleen 31% and kidney 15.4 % and gills 11.54 %. The occurrence of *V. alginolyticus* and *V. parahemolyticus* infections in Sea bream in examined organs was , the liver was the highest infected organwith *V. alginolyticus* 35.3 % followed by kidney 29.4% and spleen

20.59 % and gills 14.71 % . Also, the liverwas the highest infected organwith *V. parahemolyticus* 38.5 % followed by kidney 30.8% and spleen 23.1 % and gills 7.7 %. The occurrence of *V. alginolyticus* and *V. parahemolyticus* infections in Mugil capito in examined organs was , the liverwas the highest infected organwith *V. alginolyticus* 33.33 % followed by kidney 30.30% and spleen 24.2 % and gills 12.1 % . Also, kidney was the highest infected organwith *V. parahemolyticus* 36.8 % followed by liver 31.6% and spleen 21 % and gills 10.5%.

Table (4): Prevalence of bacteria in different types of fishes in relation to season:-

Fish	Total number of infected fish / Total number of examined fish				Total
	winter	spring	summer	autumn	
Seabass	11 / 15 (73.3%)	13 / 23 (56%)	30 / 30 (100%)	18 / 50 (36%)	72 / 118 (61%)
Seabream	7 / 12 (58.33%)	8 / 19 (42.11%)	19 / 23 (82.61%)	13 / 43 (30.23%)	47 / 97 (48.45%)
Mugilcapito	6 / 11 (54.54%)	9 / 13 (69.23%)	16 / 20 (80%)	21 / 52 (40.38%)	52 / 96 (54.17%)

Table (5): Prevalence of Vibrio species isolated from different organs of naturally infected fishes:-

Isolated bacteria	Fish		Liver		Gills		Kidneys		Spleen	
		Total isolate	No.	%	No.	%	No.	%	No.	%
V.alginolyticus		46	18	39.13%	2	4.35%	9	20%	17	37%
	Seabass									
V.parahemolyticus		26	11	42.3%	3	11.54%	4	15.4%	8	31%
V.alginolyticus		34	12	35.3%	5	14.71%	10	29.4%	7	20.59%
	Seabream									
V.parahemolyticus		13	5	38.5%	1	7.7%	4	30.8%	3	23.1%
V.alginolyticus		33	11	33.33%	4	12.1%	10	30.30%	8	24.2%
	Mugilcapito									
V.parahemolyticus		19	6	31.6%	2	10.5%	7	36.8%	4	21%

4. DISCUSSION:

The results of the clinical signs examination of the infected Seabass ,Seabream and Mugil capito revealed the presence of hemorrhages at base of anal fin , erosion of caudal fin, darkness of the skin, presence of ulcers , detached scales, hemorrhages on several parts of the body surface, Hemorrhagic areas around the mouth, corneal opacity and pale gills, this is in agreement with *Fryer et al.;(1972)and Levin et al. (1972).*While the post mortem examination of the infected fishes revealed that, the liver appeared enlarged, congested, splenomegaly, congestion in the kidney , hemorrhage in abdominal cavity and Congested gills ,this is in agreement with*Umbreit and Tripp.;(1975).*In general all the clinical sings and post mortem finding agreed well with *Khalil and Abd El-Latif ,;(2013)*for Mugil capito. Also These results are in agreement with *Marzouk et al. (2009)* for, Sea bass and Sea bream.

Prevalence of Vibrio spp. infection: The total number of infected fish was 171 from311 with 54.98 % prevalence rate. For Sea bass, the total number of infected fish was 72 from 118 with 61 % prevalence rate, For Sea bream, the total number of infected fish was 47 from 97 with 48.45 % prevalence rate, For Mugilcapito, the total number of infected fish was 52 from 96 with 54.17 % prevalence rate, these results nearly agreed with *El-Gendy(2013*) who recorded 44.1% prevalence of isolated microorganism from Seabream and Seabass.In contrast to the current result was slightly lower than 69.9% prevalence of isolated microorganism from Seabream as recorded by *Zorrilla et al. (2003);Akayli et al., (2008) and Moustafa et al. (2010).* This difference in prevalence might be attributed to different localities and species variation.

For Sea bass, the highest infection rate was recorded during summer season with prevalence rate 100% followed by winter 73%, spring 56% and the lowest was autumn with prevalence rate 36% , For Sea bream, the highest infection rate was recorded during summer season with prevalence rate 82.61 % followed by winter 58.33 %, spring 42.11 % and the lowest was autumn with prevalence rate 30.23 % , For Mugil capito, the highest infection rate was recorded during summer season with prevalence rate 80 % followed by spring 69.23 %, winter 54.54 % and the lowest was autumn with prevalence rate 40.38 % ,the current results were in accordance with that reported by *Sabir et al. (2012)* who recorded 70. 2% prevalencerate also this result was higher than those reported *by Hussain(2002)*; *Zorrilla et al. (2003)* and *El-Adawy(2010) ;*whom mentioned that the total prevalence of Vibrio alginolyticus was 14.61%.

The current results recorded that high prevalence of bacterial infections was correlated with high temperature recorded in summer season and the lowest was recorded in winter season , as reported by *Moustafa et al. (2010)* ; *Nagib (2011)* and *Sabir et al. (2012)* .

This can be explained by higher temperatures reduced immune capability and decreased resistance to infection so fish become susceptible to septicemic diseases *(Lawson 1995).*

The morphological and biochemical properties of *Vibrio alginolyticus and V. parahaemolyticus* from all samples were observed and indicated that the isolated strains are gram-negative, rod-shaped and motile, producing catalase and oxidase and

fermentative bacteria that is in agreement with *Eleonor et al. (1997) ; Buller (2004); Liu et al. (2004) ;Austin (2007); Marudhupandi et al., (2017);Ghenem and Elhadi (2018) and Patel et al .,(2018);*

Vibrio parahemolyticus appeared as green colored colonies on TCBS agar due to hemolytic action of the genus while Vibrio alginolyticus appeared as large yellow convex colonies on TCBS agar as described by *TWEDT et al.; (1969);Ghenem and Elhadi (2018) and Patel et al .,(2018).*

Antimicrobial (Antibiogram susceptibility testing):The current results of *Vibrio alginolyticus* and *Vibrio parahemolyticus* revealed sensitivity to ciprofloxacin (Cipro5µg), novobiocine (NV30µg). Moreover intermediate sensitivity was found to oxytetracycline (OT 30 µg). While resistance was observed to amoxicillin (Aml 10 µg) and cholistine sulphate (CT 10 µg) as reported by *Baumann et al. (1971); Richard and Lhuillier(1977); Ghenem and Elhadi (2018) and Patel et al .,(2018).*

The amplified patterns obtained by PCR with tested *V. alginolyticus* strains. All isolates were positively reacted to the collagenase gene primers. Each strain gave almost a common band with the same molecular weight .The three isolates yielded a single band of amplified product specific and clear band of the suspected size (737bp), internal fragment of the collagenase gene primers these results were agreed with the findings of *Lajnef et al. (2012), Marhual et al. (2010) and Di Pinto et al. (2005);* where the amplified patterns obtained by PCR with tested *V. parahemolyticus* strains. All isolates were positively reacted to the pR72H gene primers. Each strain gave almost a common, specific and clear band of the suspected size (387bp), internal fragment of the pR72H gene primers as agreed with *Lee et al. (1995); Robert-Pillot et al. (2002)*;*Bermúdez-Almada et al.(2014); Li et al. (2016); Chen et al.(2017)*and *Ghenem and Elhadi (2018);*who demonstrated that amplification of the pR72H fragment, for amplicons of 387 bp, is a powerful tool for reliable identification of *V. parahaemolyticus*. which observed in the different strains.

In conclusion, Vibriosis is considered the most important threatening disease problem facing aquaculture specially Seabass, Seabream and Mugil capito.

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