Prevalence and Genotypic Characterization of *Vibrio Alginolyticus*in Somemarine Fishes

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Abstract

This study aimed to determine the prevalence of ***V. alginolyticus*** isolated from mullet, seabream, and seabass marine fishes in addition to study the phenotypic and genotypic identification of the isolated strains. A total of 180 samples were examined bacteriologically for detection of ***V. alginolyticus***. Six isolates were identified biochemically as ***V. alginolyticus*** (3. 33%).The highest prevalence of ***V. alginolyticus***was in seabreamfollowed bymullet and seabass in a percentage of 5.00% 3.33% and 1.60 % respectively. The highest prevalence of***V. alginolyticus***was isolated from liver and spleen with percentages of 50% in mullet fish. While in seabream fish the highest prevalence of***V. alginolyticus***was isolated fromliver andkidneywith percentages 66.67% and 33.33% respectively. On other hands the highest prevalence of***V. alginolyticus***was isolated fromliver of seabass fish samples in a percentage of 100%. PCR was used for confirmation of ***Vibrio*** spp. by detection of *16S rRNA* and***V. alginolyticus***by detection of collagenasegene.All 6 isolates were positive for *the16S rRNA* andcollagenasegene which specific for ***V. alginolyticus***.All ***V. alginolyticus***isolates were tested for the detection of*tdh* and *trh*which is responsible for its virulence. The results showed that all ***V. alginolyticus***isolates were negative for*tdh* and *trh*genes.

Introduction

In Egypt, fish remains a growing, vibrant and have an additionalimportance as being the main source ofanimal protein where it is available onlarge scale and in suitable prices ***(Edriset al., 2013)***. However, a major setback in aquaculture is the sudden outbreak of diseases, especially those caused by ***Vibrio*** spp., which are considered significant economic and public health problems ***(Abd Ell-Razeq and Khaliel, 2014)***.

The genus ***Vibrio*** is a gram-negative, a curved-rod shape

bacteria that occur naturally in estuarine or marine environments, that inhabit estuarine ecosystems ***(Schärer et al., 2011)***.

Vibrio is widespread in the estuarine and coastal marine environments and show seasonal dynamics in their population ***(Thompson et al., 2004)***. In these environments, ***Vibrio*** plays a significant role in the degradation of organic matter ***(Damiret al., 2013)*** hence, regulates the dissolved organic carbon to higher trophic levels of the marine food web ***(Grossartet al.,2005)***. However, some members of the genus ***Vibrio*** are also opportunistic pathogens that have been associated with infections in humans and marine animals ***(Austin, 2010)***.

Vibriosis, an economically important disease especially in the mariculture industry, affects large number of fish and shellfish species, both cultured and feral.The genus ***Vibrio*** is a ubiquitous bacteria present in almost of aquatic and marine habitats cause infections in human ***(Baker-Austin et al., 2018)***.

Numerous studies have been conducted to determine the relationship between ***Vibrio*** spp. abundance and environmental factors such as temperature, salinity, nutrients and dissolved oxygen. As a result, these water quality characteristics can be used in a predictive manner to determine when these pathogens may be present ***(Khalil et al., 2014)***. The outbreaks of vibriosis were a common problem among cultured marine fish particularly at summer season, as a result of the deterioration of basic water parameters as temperature, pH, dissolved oxygen, and salinity ***(Albert and Ransangan, 2013)***.

Thus with the rapid extension of the intensive mariculture and the consequent deterioration of culture condition, vibriosis is considered one of the most prominent pathogens frequently affecting a wide range of fish spp. ***(Alcaide, 2003)***.

V. alginolyticusis considered one of the most dangerous pathogens in marine aquaculture causing severe losses among a large numbers of fish and shellfish species ***(Austin and Austin, 2012)***. ***V. alginolyticus*** has been suggested to be a pathogen of humans ***(Bauer and Young, 2000)***.

In recent years, PCR have overcomeproblems associated with culture-based techniques, enabling the detection of bacteria directly in clinical samples without the need for previous culturing ***(Gonzalez et al., 2004).***

V. alginolyticus was isolated during recurrent episodes of mass mortalitiesamong different stages of gilthead seabream (*Sparusaurata*) and Europeanseabass (*Dicentrarchuslabrax*) ***(Abdel-Aziz et al., 2013)***. So this study aimed to make phenotypic and genotypic identification of ***V. alginolyticus***whichisolated from some marine fishes at different farms in Port-Said Governorates.

Materials and Methods

Samples

Totally 180 marine fish samples include 60 of each seabass*(Dicentrarchuslabrax),* seabream(*Sparusaurata*) and mullet (*Mugilcephalus*) were collected randomly from different fish farms at Port-Said governorates. Samples were taken under aseptic condition from the lesions, in the external body surface, gills, liver, kidneys, muscle, and spleen. The samples put in sterile polythene bags and transferred to the laboratory, as soon as possible, in an ice-box to be examined bacteriologically for detection of ***V. alginolyticus***.

Bacteriological identification of *V. alginolyticus*

Primary isolation was done according to ***(Kaysner and DePaola 2004).*** Loopful of culture from pellicle (surface growth) of each flask was then streaked onto TCBS agar plates and incubated at 35°C for 18-24 h.5-10 yellow colonies from TCBS media suspected to be ***V. alginolyticus***were selected randomly for characterization. Cultures examined quickly after removal from the incubator as the yellow coloration of the colonies may revert to a green color when left at room temperature. Morphological and biochemical identification of the genus ***Vibrio***were done according to ***Elliot et al., (2001)***.

Molecular detection of *V. alginolyticus*

Extraction of DNA was done according to QIAamp DNA Mini kit (Qiagen, Germany, GmbH) instructions. Preparation of PCR Master Mix was done according to Emerald Amp Max PCR Master Mix (Takara, Japan), Code No. RR310Akit. Temperature and time conditions of the primers during PCR are shown in **Table (1).**

Oligonucleotide primers sequences are shown in **Table (2).** The products of PCR were separated by electrophoresis. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table (1): *PCR conditions for detection ofVibrio spp. andV. alginolyticus and some virulence genes.*

Target gene	Primary denaturation	Secondary denaturation	Annealing	Extension	Final extension	Reference
16S rRNA	94˚C 5 min.	94˚C 30 sec.	56˚C 45 sec.	72˚C 45sec.	72˚C 10 min.	**Tarr*et al.*, (2007)**
***V. alginolyticus* Collagenase**	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 45sec.	72˚C 10 min.	**Abu-Elala*et al.*, (2016)**
trh	94˚C 5 min.	94˚C 30 sec.	54˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	**Mustapha *et al.*, (2013)**
tdh	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	

Table (2): *primers used for identification of V.Vibrio spp. andalginolyticus and some virulence genes.*

Target gene	Primers sequences 5'-3'	Amplified segment (bp)	Reference
Vibrio 16S rRNA	CGGTGAAATGCGTAGAGAT TTACTAGCGATTCCGAGTTC	**663bp**	**Tarr*et al.*, (2007)**
***V. alginolyticus* Collagenase**	CGAGTACAGTCACTTGAAAGCC CACAACAGAACTCGCGTTACC	**737 bp**	**Abu-Elala*et al.*, (2016)**
trh	GGCTCAAAATGGTTAAGCG CATTTCCGCTCTCATATGC	**250 bp**	**Mustapha *et al.*, (2013)**
tdh	CCATCTGTCCCTTTTCCTGC CCAAATACATTTTACTTGG	**373 bp**	

Results

Table (3): *Prevalence of V. alginolyticusin different marine fish samples*

Types of fish	Examined samples	Positive ***V. alginolyticus***	
	No.	No.	%
Mullet	60	2	3.33
Seabream	60	3	5.00
Seabass	60	1	1.60
Total	180	6	3.33

Table (4): *Distribution of V. alginolyticus at different organs of marine fish's samples.*

Fish samples	No. of strains	Type of samples											
		Surface		Internal organs						Muscle (Flesh)		Gills	
				Liver		Kidney		Spleen					
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Mullet	2	0	0	1	50	0	0	1	50	0	0	0	0
Seabream fish	3	0	0	2	66.67	1	33.33	0	0	0	0	0	0
Seabass	1	0	0	1	100	0	0	0	0	0	0	0	0

Table (5): *Molecular identification of Vibrio spp. and V. alginolyticusin mullet, seabream andseabass.*

Genes	1	13	22	24	26	37
16S rRNA Genral for ***Vibrio*** spp.	+	+	+	+	+	+
Collagenase gene for ***V. alginolyticus***	+	+	+	+	+	+
tdh	-	-	-	-	-	-
trh	-	-	-	-	-	-

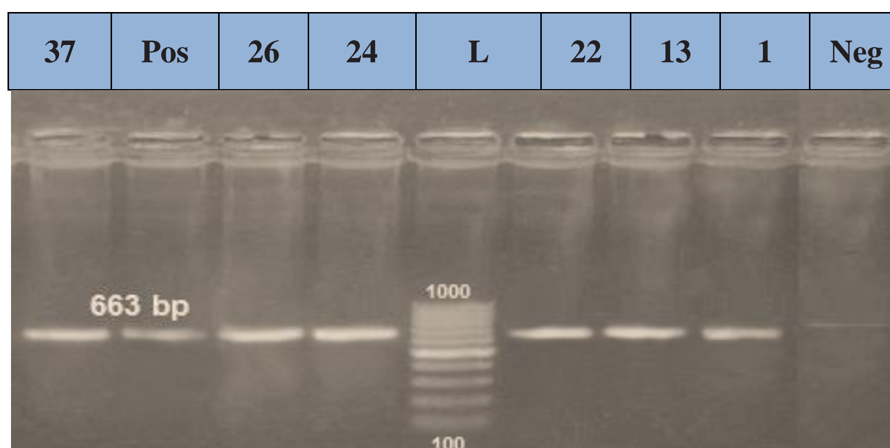

Figure (1): Agarose gel electrophoresis showed that isolates (1 and 13) from mullet fish, (22, 24 and 26) from seabream fish and (37) from seabass were positive for *16S rRNA*gene for *Vibrio* spp. at 663 bp. L = DNA ladder (100 – 1000 bp), Pos = positive control, Neg = Negative control.

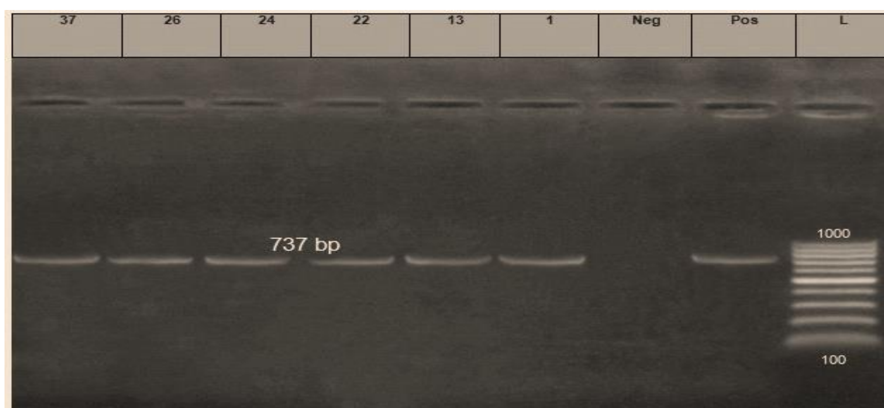

Figure (2): Agarose gel electrophoresis showed that 6 tested isolates from different marine fish samples were positive for *collagenase* gene *V. alginolyticus* at (737 bp). L = DNA ladder (100 – 1000 bp), Pos = positive control, Neg = Negative control.

Discussion:

V. alginolyticusis highly abundant in marine environments, including estuaries, marine coastal waters and sediments, and aquaculture settings ***(Vandenbergheet al., 2003)***. From the public health significance ***V. alginolyticus*** considered the most important species affecting human being fed on fish and crustacean meals ***(mustaphaet al., 2013).*** In Egypt, mariculture represents an important investment for fishermen, but diseases and high feeding cost are the main obstacles facing sustainability. The marine environment, which includes both native and externally introduced microbial contaminants and plays an important role in ecological and epidemiological studies as it acts as a reservoir not only for the persistence, dissemination, and evolution but also the transmission of pathogenic microbes to humans ***(Khalil and Abd El-Latif, 2013)***.

The results in **Table (3)**shownedthat the bacteriological examination of 180 samples of marine fishes for presence of ***V. alginolyticus***. Six isolates were identified biochemically as ***V. alginolyticus*** (3.33%).The highest prevalence of ***V. alginolyticus***was in seabream followed bymullet and seabass in a percentage of 5.00 % 3.33% and 1.60% respectively.Nearly similar results were obtained by**Saad*et al.,* 2015**who isolated ***V. alginolyticus***in a percentage of 4%. The current results were less than **Jaksic*et al.,* 2002**whoisolated ***V. alginolyticus***in a percentage of 14% and **Edris*et al.,* 2013**whoisolated ***V. alginolyticus***in a percentage of25.7%.This difference in prevalence percentages may be related to difference in area, fish

species, changein the fish immune system and time and methods of sampling and water quality characters.

Table (4) showed that most of ***V. alginolyticus*** isolates were recovered from internal organs of examined fish samples. The highest prevalence of ***V. alginolyticus*** was isolated from liver and spleen with percentages of 50% in mullet fish. While in seabream fish the highest prevalence of ***V. alginolyticus*** was isolated fromliver andkidneywith percentages 66.67% and 33.33% respectively. On other hands the highest prevalence of ***V. alginolyticus*** was isolated from seabass fish liver in a percentage of 100%. These results were agreed with ***(Mahmoudet al., 2017)*** who isolated the ***V. alginolyticus*** from internal organs of marine fish.

The results of agarose gel electrophoresis using *16S rRNA* gene in **Table (5)** and **Figure (1)**revealed that the all tested isolates were ***Vibrio*** strain with molecular weight 663bp. These results are approximately similar that recorded by ***Mohamed et al. (2017)*** who used *16S rRNA*gene sequence as accurate identification and confirmation of all tested strains and ***Montieriet al. (2010)*** who used 16S rRNA genefor confirmation of biochemically identified ***V. alginolyticus*** However this gene has low discriminatory power to differentiate closely related ***vibrio*** species that has nearly identical sequences**.**

PCR assays were developed with specific primers for the detection collagenase which specific for ***V. alginolyticus*** was found in 6 isolates**Table (5)** and **Figure (2)** revealed that the all tested isolates were ***V. alginolyticus*** with molecular weight737bp.The incidence of ***V. alginolyticus*** isolated from mullet, seabream andseabass were 2. 3 and 1 isolates respectively.All 6 isolates of ***V. alginolyticus*** did not show any virulence, as all of it showed negative detection for *tdh*and *trh* genes.Our results agree with results recorded by ***Mohamed et al. (2017)*** who identified ***Vibrio*** spp. by molecular identification using species specific primers for collagenase categorized 10 isolates belong to ***V. alginolyticus*** specific detection of ***V. alginolyticus*** was confirmed via collagenase gene ***(Miyoshi, 2013)*** that produce a specific and clear band at 737bp.The results of molecular detection and determination of *tdh*and*trh* virulence genes of ***V. alginolyticus*** strains were not detected in 6strains in the examined fish samples. Our results agree with the results recorded by ***Serracca et al., 2011***.

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الملخص العربي

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استهدفت هذه الدراسه تحديد مدى تواجد ميكروب الفيبريو الجينوليتيكس في بعض أسماك المياه المالحه بوري دنيس ,قار وصب الاضافه الدراسة التعريف الظاهري والجيني للعزلات ولهذا تم تجميع 180 عينه لفحصهم بكتريولوجيا. تم تعريف 6 عزلات بيوكيميائياً بنسبة 3.33% على أنهم فيبريو الجينوليتيكس وكانت اعلى نسبة تواجد للميكروب في سمك الدنيس ثم البوري والقاروص بنسب 5% , 3.33% و 1.6% على التوالي. وكانت أعلى نسبه للعزل من الكبد والطحال بنسبة 50% لكل منهما في سمك البوري ومن الكبد والكلى بنسبة 66.67% و 33.33% على التوالي في سمك الدنيس بينما كانت نسبة العزل 100% من الكبد في سمك القاروص. وباستخدام تفاعل انزيم البلمره المتسلسل للتأكيد على عزلات الفيبريو باستخدام جين 16s rRNAوللتأكيد على عزلات الفيبريوالجينوليتيكس باستخدام جين collagenase كانت نتيجة ال 6 عزلاتموجبهلكلمنهما , كما تم اختبار عزلات الفيبريوالجينوليتيكس لجينات الضراوه tdh , trh وكانت النتيجه سلبيه لكل منهما.