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Isolation, identification and antibiotic resistance of *Vibrio alginolyticus* isolated from *Mugil seheli* - Suez Governorate, Egypt

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ABSTRACT

Vibrio alginolyticus was isolated from *Mugile seheli* collected from private farm in Suez Governorate. Clinically examined fish exhibited dark colouration, erosions and haemorrhages of body parts. The target organs for isolation were kidney (52%), liver (48%) and spleen (15%). Isolates were biochemically and molecularly identified using species specific primers targeting collagenase gene and tdh gene. Investigation revealed that, 17 isolates were identified as *V.alginolyticus* which detected at 737 bp fragment, and only one positive amplicon for tdh gene that was detected at 373bp fragment. The sequence results of *V.alginolyticus* isolate showed 99% identity with the reference strain *V.alginolyticus* reported on Gene Bank with accession number (**MG271844**). The antibiotic sensitivity test of virulent strain of *V. alginolyticus* showed that, the bacterium was highly resistant to lincomycin 2µg, streptomycin 10µg, ampicillin10µg, trimethoprim 5µg and ampicillin/ sulbactam combination, and intermediate sensitivity to Naldixic acid 30µg, polymyxin B 300u, erythromycin 15µg, Tobramycin 10µg, ciprofloxacin 5µg, kanamycin 30µg and chloramphenicol 30µg. It was also found that, the bacterium was highly sensitive to imipenem 10µg, Oxolonic acid 2µg, oxytetracyclin 30µg, gentamycin 10µg, gatifloxacin 5µg and piperacillin/ tazobactam combination.

Key words: *Vibrio alginolyticus, Suez Governorate, Virulent gene, Antibiotic resistance*

Introduction

Mugil Seheli belongs to family Mugilidae that consists around the world of more than 72 species from 17 fish genera. It is one of the commercially important fish species in Suez Bay and Egypt, although it has a lower growth rate, fetches a higher market price compared to the other mullet in Egypt because of its highly appreciated taste **(Nelson, 2006).** Farmed fish are more susceptible to disease agents than wild fish due to their intensive rearing conditions **(Salinas *et al*., 2006).** And bacterial diseases are considered one of the most important problems facing fish industry, that are responsible for heavy mortality not only in cultured fish but also in wild too and affect badly the economics since the beginning of marine fish culture **(Khalil and Abd El-Latif 2013).** The majority of bacterial diseases in aquaculture production system are caused by some causative agents include bacteria from short, Gram-negative rods belonging to the families *Enterobacteriaceae*, *Pseudomonadaceae (Pseudomonas*) and *Vibrionaceae* (*Vibrios*) **(Aly, 2009 and Barbosa *et al*., 2011)**. And *Vibrionacea*e is a large and complex group of marine bacteria that can significantly affect the health of aquatic animals **(Carson *et al*., 2009)** and some *Vibrio* species are pathogenic for fish, eels and frogs as well as other vertebrates and invertebrates **(Todar, 2005).** Antibiotic susceptibilities differ between species but almost all strains are sensitive to chloramphenicol, tetracycline, and quinolones. However, some strains of *V. alginolyticus* are sometimes multiply resistant to these antibiotics including chloramphenicol, tetracycline and cefotaxime.(**Musa and Wei 2008; Costinar *et al*., 2010; Ransangan, *et al*., 2013 and Younes *et al*., 2016)**. But the use of antibiotics and disinfectants not only for disease control but also for the improvement of growth and efficiency of feed conversion have been found to have a negative impacts in aquaculture such as the development of bacterial resistant, the change of microbial composition in ecosystem and residual antibiotics or disinfectants in aquaculture products and thus the appearance of multiple antibiotic resistance (MAR) **(Levy, 2001; Cabello, 2006 and Esposito *et al*., 2007)**.So, this study was planned for isolation of *V. alginolyticus* from *Mugile Seheli* collected from private farm in Suez Governorate with phenotypic and molecular characterization of the isolated species. Furthermore, to investigate the

antibiotic susceptibilities of isolates against the most common antibiotics used for controlling of *Vibrio* infection

Materials and methods

Fish samples:

A total of twenty five (25) of naturally infected *Mugil Seheli* collected from private farm in Suez Governorate. The collected fish samples were submitted directly to the laboratory for clinical and bacteriological examination **(Buller, 2004; Austin and Austin, 2012).**

Clinical and postmortem examination:

External and internal examination of fish samples were performed to record clinical abnormalities according to **(Austin and Austin, 2012 and Noga and Dvm 2010).**

Bacteriological examination:

An inoculum from kidney, spleen and liver samples were taken under complete aseptic conditions and inoculated into TSB with 2% NaCl then a loopful of bacterial suspension was re-streaked over TCBS (Thiosulphate Citrate Bile Salt Agar, OXOID) plates. All plates were incubated at 28 ºC for 18–24 hr. then a single colony from each suspected isolate was picked up and re-streaked on a new plate of TCBS culture media and re-incubated at the same conditions for purification. Ordinary biochemical identification of bacterial isolates was performed using Gram's stain, oxidase, catalase and IMVC tests followed by Analytical Profile index 20NE (Biomerieux) **(Buller, 2004)**. The purified strains were stored in BHI with 15% (vol/ vol) glycerol at -20 ^{o}C[4].

Molecular identification and Partial sequences of 16SrRNA gene:

The retrieved *Vibrio* species were cultured on tryptic soya agar with 2% NaCl for genomic DNA extraction according to QIA amp DNA mini kit instructions. The reaction for PCR amplification of each sample was performed in a total volume of 25 µl containing: 12.5 µl 2X Dream Taq Green buffer, 5.5 µl PCR grade water, 1 µl of each primer (20 pmol) and 5 µl Template DNA. Internal fragment of the 16SrRNA gene was amplified using primer sets designed by (**Tarr *et al.*, 2007**). Collagenase gene was used for species specific detection of *V. alginolyticus*, (**Mustapha *et al.*, 2013)**. tdh gene was used for confirming the pathogenicity of *V. alginolyticus* isolates **(Abu-Elala *et al.*, 2016)**. The primer sets and the cycling conditions used in this study

are described in **(Tables 1 and 2)**. The amplified fragment of 16SrRNA gene of *Vibrio* species were sequenced using **MEGA5** program for checking purity and blasted at the NCBI BLAST home page (https://blast.ncbi.nlm.nih.gov/Blast/).

Antimicrobial susceptibility test of the suspected isolate:

Screening of antimicrobial susceptibility of the bacterial isolates was tested using the standard disk diffusion method **(Kirby Bauer test).** The test performed on Mueller-Hinton agar (Oxoid) according to the instruction of Clinical Laboratory Standards Institute **(CLSI, 2012).**

Results

Clinical signs and Necropsy findings of naturally infected fish:

Clinically infected fish showed dark coloration of skin with detached scales, Hemorrhages at the base of the fin and some fishes showed eroded fins. Postmortem findings revealed hemorrhagic, friable liver and congested kidney **(Fig. 1)**

Bacteriological Examination:

Large, greenish yellow colonies of *V. alginolyticus* were observed on TCBS agar plates. The bacterial isolates were Gram-negative; short comma shaped curved rods, motile, oxidase and catalase positive. Biochemical identification of *V. alginolyticus* isolates by API (20NE) revealed about 99.1% probability with a code of **(7447444).**

Table (1): Identification of *V.alginolyticus* by biochemical test:

Test	Reaction
Oxidase test	Positive
Catalase test	Positive
Indole test	Positive
Methyl red test	Positive
Vogus Proskauer	Negative
Citrate test	Positive

Table (2): showing the API profile of *V.alginolyticus*

Biochemical test	*V.alginolyticus*
NO3	+
TRP	+
GLU	+
ADH	-
URE	-
ESC	+
GEL	-
PNPG	-
(GLU)	+
ARA	+
MNE	+
MAN	+
NAG	-
MAL	-
GNT	+
CAP	-
ADI	-
MLT	+
CIT	-
PAC	+

Molecular identification and partial sequences of 16SrRNA gene:

The PCR produce specific and identical amplicons to the size of the target gene sequence. In particular, electrophoresis of the partial gene sequence of 16SrRNA gene produce amplicons at 663 bp. PCR amplifications of partial gene sequence of collagenase gene of *V. alginolyticus*, were observed at 737 bp, **(Fig. 2)**. The obtained sequences of 16SrRNA gene were blasted with the highly similar sequences in the Gene Bank database of National Center for Biotechnology Information Center (NCBI). The sequencing results revealed that sequences of *V. alginolyticus* isolates showed a close relationship with almost 99% identity with the reference strains of *V. alginolyticus* reported on Gene Bank .The obtained nucleotide sequences were submitted with accession number (**MG271844**) in the Gene Bank.

Table (3): The primer sets used in this study

Gene	primer	Sequence	Amplified product	Reference
16SrRNA	V.16S-700F	CGGTGAAATGCGTAGAGAT	663 bp	**Tarr *et al*., 2007**
	V.16S-1325R	TTACTAGCGATTCCGAGTTC		
Tdh	F .tdh	CCATCTGTCCCTTTTCCTGC	373 bp	**Mustapha *et al*., 2013**
	R.tdh	CCAAATACATTTTACTTGG		
***V. alginolyticus* Collagenase**	Collagenase F	CGAGTACAGTCACTTGAAAGCC	737 bp	**Abu-Elala *et al*., 2016**
	Collagenase R	CACAACAGAACTCGCGTTACC		

Table (4): cycling conditions of used primer

Gene	Initial denaturation	Amplification				Final extension
		Secondary denaturation	Annealing	Extension	No. of cycles	
16SrRNA	94˚C 5 min.	94˚C 30 sec.	56˚C 45 sec.	72˚C 45 sec.	35	72˚C 10 min.
tdh gene	94˚C 5 min.	94˚C 1 min.	54˚C 1 min.	72˚C 1 min.	35	72˚C 10 min.
***V. alginolyticus* Collagenase**	94˚C 5 min.	94˚C 1 min.	50˚C 1 min.	72˚C 1 min.	35	72˚C 10 min.

Fig. 1: Necropsy Findings in naturally infected *Mugil Seheli* showing congested kidney and lacerated and congested liver.

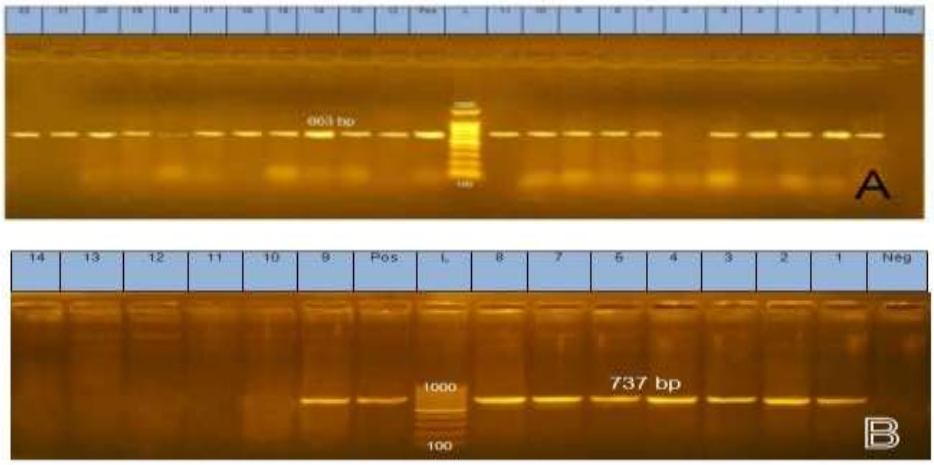

Fig 2: Ethidium bromide stained agarose gel of PCR products representing (A)amplification of 700 bp amplicons of universal 16SrRNA housekeeping gene of *Vibrio* species, (B) amplification of 737 bp amplicons of collagenase gene of *V. alginolyticus***.**

Antimicrobial sensitivity test:

The result of antimicrobial susceptibility test of virulent strain of *V. alginolyticus* showed that the bacterium was highly resistant to lincomycin 2µg, streptomycin 10µg , ampicillin10µg and ampicillin/ sulbactam combination while it show intermediate sensitivity to naldixic acid 30µg , polymyxin B 300u, erythromycin 15µg, tobramycin 10µg, ciprofloxacin 5µg, kanamycin 30µg and

Table (5): result and interpretation of antibiotic sensitivity test:

Antimicrobial agents	Symbol	Disc conc.	Diameter of inhibition zone			Result
			Resistant (R) mm or less	Intermediate (M) mm range	Sensitive (s) mm or more	
Ampicillin	(AM)	10 µg	14	15-16	17	4.5
Ampicillin/ sulbactam	(SAM)	10 µg / 10 µg	11	12-14	15	6.5
Piperacillin/ tazobactam	(TPZ)	100µg / 10 µg	17	18-20	21	24.5
chloramphenicol	(C)	30 µg	12	13-17	18	16.5
Gatifloxacin	(GAT)	5 µg	14	15-17	18	27.5
Ciprofloxacin	(CIP)	5 µg	15	16-20	21	17
lincomycin	(L)	2 µg	11	12-16	17	No zone
Gentamycin	(CN)	10 µg	12	13-14	15	20
Kanamycin	(K)	30 µg	13	14-17	18	17.5
Tobramycin	(TOB)	10 µg	12	13-14	15	13
Erythromycin	(E)	15 µg	No zone	<15	>15	14.5
streptomycin	(S)	10 µg	<11	12-14	>15	No zone
Oxytetracycline	(TE)	30 µg	14	15-18	19	20
Trimethoprim	(TMP)	5 µg	10	11-15	16	No zone
Trimethoprim / sulphamethoxazole	(SXT)	25 µg	10	11-15	16	11.5
Polymyxin B	(PB)	300 U	11	12-13	14	11.5
Oxolinic acid	(OA)	2 µg	No zone	<15	>15	23.5
Naldixic acid	(NA)	30 µg	13	14-18	19	17
Imipenem	(IPM)	10 µg	19	20-22	23	34

chloramphenicol 30µg. it was also found that the bacterium was highly sensitive to imipenem 10µg, oxolonic acid 2µg, oxytetracyclin 30µg, gentamycin 10µg, gatifloxacin 5µg and piperacillin/ tazobactam combination. The inhibition zone is shown in **(Fig 3).**

Fig (3): Inhibition zones of antibiotic susceptibility test.

Discussion

Bacterial diseases are the major constraint affecting production and sustainability of aqua-culture operation **(Khalil and Abd El-Latif 2013).** ***Vibriosis*** is the most common bacterial disease affecting Mari-culture fishes worldwide causing severe economic losses. The obtained results of naturally infected fishes indicating disease problem and the clinical signs varied from dark coloration of skin with detached scales, and hemorrhage at the base of the fins with some erosion Which support the findings of **El-Bouhy *et al*. (2016) and Al-Taee *et al*. (2017)** in *Mugil* and *Tilapia*. Post mortem examination the fish revealed clear internal typical lesions. Internally there was congested friable enlarged liver and congested kidney **(Fig1).** These findings were agreed with those reported by **Younes, *et al*. (2016); Abdel-Aziz *et al*., (2017) and Bluford *et al*. (2017).** Bacteriological examination of isolated *Vibrio* species depends mainly on using TCBS agar as a selective media to differentiate between sucrose and non-sucrose fermenter colonies. The result of identification revealed that *V.alginolyticus* is sucrose fermenter showing yellow color. These finding were in lines with results obtained by **Zulkifli *et al*. (2009); Shionda (2011) and Abdellrazeq and khaliel (2014).** The result of biochemical identification showed that *V.alginolyticus* was oxidase, catalase, indole, methyl red and citrate test positive while they were Vogus Proskauer test negative **(Tab 1)** and these finding go in parallel with **Snoussi *et al*., (2008) and Abu-Elala *et al*., (2016).** The result of identification of

V.alginolyticus by API (20NE) revealed about 99.1% probability with a code of 7447444 **(Tab 2).** These finding were agreed with result found by **Martins *et al*., (2008); El-Bouhy, (2016) and Abu-Elala *et al*., (2016).** The results of molecular identification of *Vibrio* isolates by using 16SrRNA results in PCR product with positive amplicons at 663bp as shown in **Fig (2).** And these results are agreed with **Younes *et al*., (2016); You *et al*., (2016) and Abdelaziz *et al*., (2017).** 16SrRNA gene was used for confirmation of biochemically identified *vibrio* species; however the result showed that this gene has low discriminatory power to differentiate closely related *vibrio* species that were nearly identical as *V.parahaemolyticus* and *V.alginolyticus* **(Montieri *et al*. 2010, Younes *et al*. 2016).** The specific identification of *V. alginolyticus* by using specific primer targeting collagenase gene resulted in PCR product with production of positive amplicons that were detected at 737 bp as shown in **Fig (2).** Similar result was obtained by **Khamesipour *et al.* (2014); Moustafa *et al*. (2015) and El-Hady *et al*. (2015).** The result showed that only one strain of *V.alginolyticus* produces positive tdh gene with positive amplicon at 373bp and this result is with agreement with **Natividad-Bonifacio *et al*., (2013), Mustapha *et al*., (2013), and Hernández - Robles *et al*., (2016).** Multiple sequence alignment of 16S rRNA partial gene sequence of *V.alginolyticus* showed 99% identity with other *Vibrio* species recorded on gene bank with accession number **(MG271844)**. The result for in-vitro sensitivity test of virulent strain of *V.alginolyticus* showed that the bacterium was highly resistant to lincomycin 2µg, streptomycin 10µg, and ampicillin 10µg, ampicillin/ sulbactam combination. On the other hands, it show intermediate sensitivity to naldixic acid 30µg , polymyxin B 300u, erythromycin 15µg, tobramycin 10µg, ciprofloxacin 5µg and chloramphenicol 30µg. Also it was also found that the bacterium was highly sensitive to oxolonic acid 2µg, oxytetracyclin 30µg, gentamycin 10µg, gatifloxacin 5µg and piperacillin/ tazobactam combination. This result was agreed with those of**, Abdel-Aziz *et al*., (2013); Khaliel *et al*., (2014); Younes *et al*., (2016) and Hernández - Robles *et al*., (2016).**

It could be concluded that *V.alginolyticus* is significant threat to *Mugil Seheli* aquaculture in Egypt. PCR is sensitive and accurate method for diagnosis of *Vibriosis.*

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عزل وتصنيف ومقاومة المضادات الحيوية لفبريوالجينوليتكس المعزولة من سمكة السهلية بمحافظة السويس – مصر

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٣-معمل الميكروبيولوجي , شعبة البيئة البحرية, المعهد القومي لعلوم البحاروالمصايد (الاسكندرية).

الملخص العربى

اجريت هذه الدراسة علي عدد ٢٥ سمكة مريضة من اسماك السهليه والتي تم تجمعها من مزرعة خاصة في محافظة السويس حيث تم فحص هذه الاسماك اكلينيكيا وتشمل التغيرات المرضية المصاحبة للاصابة ببكتريا الفبريوالجينوليتيكس. وقد اظهر الفحص الاكلينيكي للاسماك وجود دكانة في لون الجلد,انزفه وتأكل في الزعانف. وقد تم اخذ عينات من الكلى والكبد والطحال. تم استخدم تفاعل البلمرة المتسلسل والتسلسل الجيني كوسيله تأكيدية لعزلات الفبريو و ذلك بأستخدام بادئات لجين كولاجينز وكان حجم المنتج٧٣٧ bp وتحديد الانواع التي تحتوي علي جين الضراوة باستخدام بادئات لجين tdh وكان حجم المنتج ٣٧٣bp. وأظهرت نتائج تسلسل عزل الفبريوالجينوليتيكس تطابق بنسبة٩٩ ٪ مع سلالة الفبريوالجينوليتيكس المسجلة على بنك الجينات (accession number MG271844). أظهر اختبار الحساسية أن البكتيريا كانت عالية المقاومة لللينكوميسين ٢مجم ، الستربتوميسين ١٠مجم ، أمبيسيلين ١٠مجم ، تريميثوبريم ٥مجم ومجموعة الأمبيسلين / سولباكتام ، والحساسية الوسيطة لحمض نالدكسك ٣٠ مجم، بولي ميكسين ب ٣٠٠ وحدة دولية ، الإريثروميسين ١٥مجم ، توبراميسين ١٠مجم ، سيبروفلوكساسين٥مجم ، كاناميسين ٣٠مجم و كلورامفينيكول ٣٠ مجم. وجد أيضا أن البكتيريا كانت حساسة للغاية للاميينيم ١٠ مجم ، حمض الاوكزولينك ٢ مجم ،الاوكسي تتراسيكلين ٣٠ مجم ، جينتاميسين ١٠ مجم ،جاتي فلوكساسين ٥ مجم ومجموعة بيبراسيللين/ تازوبكتام .