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CHARACTERIZATION AND PATHOGENICITY OF *VIBRIO ALGINOLYTICUS* ISOLATED FROM DISEASED CULTURED GILTHEAD SEABREAM

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ABSTRACT

Fifty farmed gilthead seabream and two water samples were collected from two semi-intensive marine farms at Damietta province, Egypt during 2016. Fourteen *Vibrio alginolyticus* isolates were recovered and presumptively identified using morpho - chemical characterization then definitely confirmed by PCR targeting species specific collagenase gene. The total prevalence of the examined fish was 28% (14/50). All strains were resistant to ampicillin, oxacillin, tobramycin and kanamycin. On the other hand, all strains were sensitive to levofloxacin, gatifloxacin, doxycycline, oxolinic acid, flumequine and trimethoprim/sulfamethoxazole. The LD_{50} of *Vibrio alginolyticus* isolate (VAS7) were 3×10^5 $CFUg^{-1}$ of fish body weight.

Key words:

Seabream, *Vibrio alginolyticus, collagenase* gene, Antibiogram, LD_{50}.

INTRODUCTION

Gilthead seabream (*Sparus aurata* L.) is the predominant marine fish species cultured in on a large-scale in the Mediterranean region especially Greece, Turkey, Spain, Egypt and Italy **(FAO, 2014)**. Vibriosis has been incriminated as the most detrimental diseases affecting cultured seabream **(Zorrilla *et al.*, 2003 and Roberts 2012)**. *Vibrio alginolyticus* is the most prevalent *Vibrios* isolated during mass mortalities of farmed seabream in some coastal provinces, Egypt **(Abdel-Aziz *et al.*, 2013)**.Characterization of *Vibrios* based on conventional methods are less specific **(Tang *et al.*, 1998 and Thompson *et al.*, 2004)**. In contrast, the application of PCR may provide an easy, rapid, more sensitive and specific tools for detection of a low number of bacteria and/or non-culturable organisms **(Coleman and Olive, 1996)**. A definitive confirmation of *Vibrio alginolyticus* can be obtained by molecular detection of collagenase gene, 737 bp amplicon **(Di Pinto *et al.*, 2005)**. Although regionally there are

plenty of literature discussing *Vibrio alginolyticus*, there is scarcity literature about molecular characterization and pathogenicity of *Vibrio alginolyticus* isolated from semi-intensive cultured gilthead seabream. Hence, the objectives of the current study are morpho-chemical, molecular characterization and pathogenicity of *Vibrio alginolyticus* isolates retrieved from tissues of diseased farmed gilthead seabream.

MATERIAL AND METHODS

2.1. Fish and water sampling:

Fifty moribund and freshly dead cultured gilthead seabream (*Sparus aurata*) were collected from two different semi-intensive marine farms located at dibah triangular zone (DTZ), Damietta province, Egypt during the mortality episode occurring at 2016. The weight and length of randomly collected fish were ranged from 55.6 to 185 g and 10.8 to 25 cm, respectively. Clinical and postmortem examination was performed on naturally infected gilthead seabream **(Noga, 2010)**. Two water samples were collected from the investigated farms in clean and dry bottles **(APHA, 1998)**. Dissolved oxygen, ammonia, nitrite, pH and iron concentration were measured **(WHO, 1993)**.

2.2. Bacterial isolation and purification:

Samples were taken from external lesions, kidney, spleen, brain and gills of examined moribund sacrificed fish and/or freshly dead marine fish, and then streaked onto Thiosulphate citrate bile salt sucrose agar (TCBS, Lab M). The inoculated plates were incubated at 28 °C for up to 24 h. From each plate, yellow colonies were picked up then transferred to Tryptic soy agar (TSA) (Lab M, UK) supplemented with 2% (w/v) NaCl for purification of bacterial isolates. These plates were incubated at 28 °C for 24 to 48 h.

2.3. Phenotypic and biochemical characterization:

From each plate, single colony was examined by using cytochrome oxidase test, catalase test, and O/129 (150µg) susceptibility test. The isolate exhibited positive oxidase and catalase tests besides sensitivity to O/129 were characterized using phenotypic and biochemical tests according to **Buller (2004)**. Miniaturized API 20 NE and API 20 E system (Biomerieux, France) were also used according to manufacturer's instructions. Also, Bacterial colonies were inoculated into peptone water supplemented with 0%, 3%, 5%, 7 and 10% NaCl as another presumptive criterion for identification of the bacterial isolates.

2.4. PCR assay of *Vibrio* strains:

2.4.1. Isolation of genomic DNA:

Bacterial isolates were routinely grown in tryptic soy broth (TSB) (Lab M, UK) supplemented with 2% NaCl at 28°C for 24 h. One milliliter of the culture was centrifuged at 10,000 rpm (Centrifuge, Eppendorf, Germany) at room temperature to obtain the bacterial pellets then extraction of genomic DNA was performed using bacterial DNA extraction kit (Vivantis) manufacturer's instructions. The DNA samples were stored at -20 °C until use.

2.4.2. Detection of 16SrRNA gene by PCR:

Two sets of primers were used for identification of *Vibrio* spp. The primer pairs were 63f (5'- CAGGCCTAACACATGCAAGTC-3') and 763r

(5'- GCATCTGAGTGTCAGTATCTGTCC-3')basedon 16SrRNA gene producing700-bp fragment **(Marchesi *et al*., 1998 and Montieri *et al*., 2010)**. The PCR reaction was performed in a 20 µl; 1 µl of purified genomic DNA, 0.5 µl of each of the primers (50 pmol/ µl), 10 µl ready to use PCR reaction mixture, and the final volume was adjusted to 20 µl with DNase-free water then the mixture was processed in a gradient thermal cycler with one cycle of initial denaturation at 95°C for 10 min, followed by 30 cycles of denaturation at 95°C for 60 s, annealing at 55°C for 60 s, and extension at 72°C for 90 s and a final extension at 72°C for 5 min and the reaction products were stored at 4 °C until further analysis **(Marchesi *et al*., 1998 and Montieri *et al*., 2010)**.

2.4.3. Detection of Collagenase gene by PCR:

Two sets of primers were used for identification of *V. alginolyticus*. The primer pairs were VA-F (5`-CGAGTACAGTCACTTGAAAGCC-3`) and VA-R

(5`-CACAACAGAACTCGCGTTACC-3`) based on collagenase gene producing 737 - bp amplicon **(Di Pinto *et al.,* 2005)**.The PCR reaction was performed in a 20 µl as aforementioned then the mixture was processed in a gradient thermal cycler with initial activation step at 95°C for 10 min, followed by 35 cycles at 94°C for 30 s (denaturation step), 57°C for 30 s (annealing step), and 72°C for 60 s (extension step), and a final extension at 72°C for 5 min and the reaction products were stored at 4 °C until further analysis **(Di Pinto *et al.,* 2005).**

2.4.4. Detection of amplicons:

The amplicons were analyzed by electrophoresis on a 1.5% (w/v) agarose gel in 1X TAE buffer at 110 V for 40 min and visualized by ethidium bromide staining (0.5 µl/ml) and a UV

transilluminator. A NEX Gen 100-bp DNA Ladder consisting of DNA fragments ranging in size from 100 bp to 3000 bp was used as a molecular weight marker.

2.5. Antimicrobial susceptibility testing:

All isolates of *V. alginolyticus* were tested for antimicrobial susceptibility by a standard disk diffusion method on Mueller - Hinton agar (Oxoid, UK) with 2% (w/v) NaCl according to the method described by **Jorgensen and Turnidge (2007)**. Each antimicrobial was classified as susceptible (S), resistant (R), or intermediately resistant (I), according to guidelines developed by **(CLSI, 2016)**.

The following antimicrobial discs (Oxoid) were used: ciprofloxacin 5 µg (CIP 5), chloramphenicol 30 µg (C 30), nalidixic acid 30 µg (NA 30), novobiocin 30 µg (NV 30), oxolinic acid 10 µg (OA 10), Oxytetracycline 30 µg (TE 30), tetracycline 30 µg (T 30), doxycycline 30 µg (DO 30), trimethoprim/sulfamethoxazole 1.25 + 23.75 µg (SXT 25), flumequine 30 µg (UB 30), kanamycin 30 µg (K 30), ampicillin 10 µg (AM 10), oxacillin 1 µg (OX 1), levofloxacin 5 µg (LEV 5), gatifloxacin 5 µg (GAT 5), tobramycin 10 µg (TOB 10) and sulfa-soxazole 300 µg (ST 30).

2.6. Pathogenicity assay (LD50):

The 50 % lethal dose (LD_{50}) of *Vibrio alginolyticus* strain (VAS7) isolated from diseased cultured gilthead seabream was determined following the methodology described by **Nieto *et al.* (1984) and Zorrilla *et al.* (2003)**. Briefly, thirty-five apparently healthy gilthead seabream (30 ± 5 g) were acclimated for two weeks in the aquaria prior to the initiation of the experiment. Five random samples were taken for microbiological examination to check any bacterial contamination. Groups of five fish were inoculated intraperitoneally with the bacterial isolates using 0.2 ml of serial bacterial dilutions containing 10^4-10^8 CFU/ml. A control group was provided using the same number of fish inoculated with 0.2 ml sterile phosphate buffer saline (PBS) (pH 7). The fishes were kept in aquaria (32% salinity) at 24 °C for 7 days and observed daily for pathological signs and all mortalities were recorded. Bacteriological analyses of dead fish were carried out in all the cases, considering only the bacterial origin when the bacterial strain was re-isolated in pure culture from internal organs of dead fish to prove the Koch's postulates **(Walker *et al.*, 2006)**. The LD_{50} was calculated by simple method for estimating 50% endpoints **(Reed and Müench 1938)**.

RESULTS

1. Clinical and postmortem examination:

The clinical and postmortem examination of diseased fish revealed detached scales, extensive external hemorrhages around the head and at the base of fins, external excessive mucous secretion, skin darkening, congested spleen and kidney, pale liver, empty stomach with enlarged gallbladder and accumulation of serous fluids in the abdominal cavity.

2. Water samples:

The examined two water samples revealed decreased level of dissolved oxygen (2.9-3.5 mg/L), with elevated levels of nitrite (0.4-0.6 mg/L), ammonia (0.7-0.9 mg/L) and iron (0.9 - 1.3 mg/L), while the pH level was within normal range (7.5 - 8).

3. Morpho-chemical characterization:

Morphological and biochemical characterization of bacterial strains isolated from kidney, spleen, brain, gills and external lesions of diseased cultured seabream are shown in (Table 1). Fourteen bacterial isolates were recovered and characterized from eighteen infected fish with a total prevalence of 28 %. Most of the frequently isolated bacterial strains were phenotypically and biochemically identified as *Vibrio alginolyticus*. Isolating of *V. alginolyticus* was most prevalent from kidney, spleen, brain and external lesions, while the lowest intensities were isolated from gills.

4. Molecular characterization:

Vibrio species were confirmed by PCR targeting the genus-specific 16SrRNA gene at 700 bp. All isolates were confirmed to be *Vibrio* species by 16SrRNA using PCR Fig. (1). *Vibrio alginolyticus* was confirmed by species-specific PCR using collagenase gene. All fourteen tested isolates showed the characteristic *Vibrio alginolyticus* amplicon size of 737 bp Fig. (2).

5. Antibiogram:

Antibiogram of *Vibrio alginolyticus* isolates showed that,all strains demonstrated susceptibility to levofloxacin, gatifloxacin, and flumequine. There were no strains sensitive to kanamycin, tobramycin, ampicillin and oxacillin (Table 2). Almost all *Vibrio alginolyticus* isolates showed sensitivity to oxolinic acid (92.9 %), chloramphenicol and doxycycline (85.8 %), and trimethoprim/sulfamethoxazole (71.4%).High percentages of intermediate susceptibility were showed in novobiocin (71.4%), sulfa-soxazole (57.1%) and nalidixic acid (35.7 %) (Table 2).

6. Pathogenicity:

Experimentally infected fish showed external signs resembling those observed in outbreaks. During the determination of the LD_{50}, Pure culture of *V. alginolyticus* was isolated from kidney, liver and skin of moribund fish, and Neither mortalities nor pathological signs were detected in the control group 7 days' post challenge. Signs of infection began to appear on the second day. The onset of mortalities in *Vibrio alginolyticus* challenged seabream occurred from second to the fourth day. The LD_{50} of *Vibrio alginolyticus* strain (VAS7) was 3×10^5 CFU g^{-1} fish. All inoculated bacterial strains recovered from dead and/or moribund fish revealed the same morpho - chemical characteristics of the initially injected strain.

DISCUSSION

The clinical symptoms and necropsy recorded in this study ranged from darkening of the skin up to hemorrhagic head and liver with ascitic fluid in the abdominal cavity were similar to those reported by **Zorrilla *et al.* (2003); Toranzo *et al.* (2005) and Abdel-Aziz *et al*., (2013).** Pathological alterations and mortalities were induced by the different extracellular products (collagenase, chondroitin sulfatase, different hemolysins, cytotoxins, enterotoxins, lytic enzymes and protease), these ECP were very toxic and responsible for invasive and proliferative processes of *Vibrios* in fish. Furthermore, destruction of critical components of both circulatory and immune systems **(Jun *et al*., 2003)**. Most fish diseases are stress related initiated by deterioration in water quality as increase un-ionized ammonia, nitrite and iron associated with a sharp decrease of dissolved oxygen. This water quality results lead to suppression of fish immune system giving the chance to opportunistic pathogens to invade the fish resulting in the emergence of outbreaks of fish diseases **(Roberts, 2012)**. These results were in accordance with those reported by **Chen *et al.* (2011)** who stated that sharp decrease in the dissolved oxygen with high levels of ammonia and iron are the most possible triggering factors for the emergence of infection as well as jeopardize fish immune system.Fourteen strains isolated from naturally infected seabream identified as *Vibrio alginolyticus* by colony morphology on TCBS, motility, sensitivity to O/129 (150µg), salt tolerance and biochemical characters by both API20E and API20NE tests.These findings were completely in accordance with the standard criteria of *Vibrio alginolyticus* reported by **Sadok *et al.* (2013)**. All tested strains were confirmed to be belonging to genus *Vibrio* by using PCR detection of 16 SrRNA amplifying 700 bp amplicon simulating that of **Marchesi *et al.* (1998) and Montier *et al.***

(2010). *Vibrio alginolyticus* strains were molecularly confirmed using collagenase gene where the entire tested isolates produce an amplicon of 737 bp that were consistent with **Di Pinto *et al.* (2005)**. The overall prevalence of bacterial infections (28%) was in accordance with **El-Gendy (2013)** who reported that, the prevalence of bacterial infections from naturally infected seabream was 37 %. These results were lower than those reported by **Abdel - Aziz *et al.* (2013)** who stated that the total prevalence of bacterial infections in seabream was 86.33%. Lower prevalence could be attributed to the immune status of fish and disease resistance and season of sampling. **Zorrilla *et al.* (2003)** reported that *Vibrio alginolyticus* was the most frequently isolated *Vibrio* species (21.35%). **Ben-Kahla-Nakbi *et al.* (2006)** reported that *Vibrio alginolyticus* have been implicated in several outbreaks observed along the Mediterranean seacoasts.The interpretation of antimicrobial susceptibility testing in this study revealed that, all *Vibrio alginolyticus* isolates were resistant to kanamycin, ampicillin, oxacillin and tobramycin. Resistance to sulfonamides has been reported in aquaculture and aquatic environments from different geographical regions **(Suzuki *et al.*, 2013 and You *et al.*, 2016)**. A few *V. alginolyticus* isolates showed resistance to nalidixic acid, oxolinic acid, tetracycline, oxytetracycline, novobiocin, and ciprofloxacin while none of the isolates showed resistance to levofloxacin, flumequine, Chloramphenicol, gatifloxacin. Several studies stated that *Vibrio* spp. is susceptible with the great extent to tetracycline group **(Vignesh and Muthukumar, 2012)**. Also, these results were concordant with the literature stating that *Vibrio alginolyticus* are susceptible to quinolone and tetracycline **(Sudha *et al.*, 2014)**. The emergence of antimicrobial resistance may be attributed to the unwise application of these antibiotics in the treatment of diseased farmed fish or could be due to contamination of the aquatic environment with antimicrobials from agricultural, animal and human sources. The results of experimental infection indicated that *Vibrio alginolyticus* strain (VAS7) was virulent for gilthead seabream. The bacterial strain was re-isolated from dead and/or moribund fish. İn addition, moribund and/or dead fish manifested clinical signs such as skin darkening, reduced appetite, hemorrhages around the head, anal opening and fins, congestion of liver and ascetic fluids in the abdominal cavity as noted in naturally infected fish. Thus, *Vibrio alginolyticus* isolates fulfilled Koch's postulates. **Balebona *et al.* (1998)** stated that LD_{50} of *Vibrio alginolyticus* ranging from 5.4×10^4 to 1.0×10^6 CFU/g of gilthead seabream. **Santos *et al.* (1988)** reported that isolates with LD_{50} between 10^4 and 10^6 CFU/g of fish have

been considered as highly virulent and a nonvirulent showing LD_{50} values of $>10^8$. In conclusion, the study confirmed the presence of pathogenic *Vibrio alginolyticus* as major *Vibrio* spp. in cultured gilthead seabream in the semi-intensive farm, Damietta. Doxycycline, levofloxacin, flumequine and Oxolinic acid are antimicrobials of choice in controlling Vibriosis based on *in-vitro* antibiogram. Unwise use of antimicrobials in fish farms may lead to increase antibiotic resistance among the most commonly used antibiotics. Therefore, a vaccination programs against *Vibrio* spp. should be applied.

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Table (1): Morpho-chemical characterization of *Vibrio alginolyticus* strains recovered from diseased cultured gilthead seabream.

Morphochemical tests	*Vibrio alginolyticus* (14 isolates)
TCBS	Yellow colonies
Oxidase	+
Catalase	+
Motiltity	+
Senstivity to O/129 (150 µg)	+
Swarming on TSA	+
Growth at 0% NaCl	–
Growth at 3% NaCl	+
Growth at 5% NaCl	+
Growth at 7% NaCl	+
Growth at 10% NaCl	V
Growth at 37°C	+
Sucrose fermentation	+
Glucose fermentation	+
Esculin hydrolysis	+
Gelatin hydrolysis	V
Arabinose assimilation	–
Urease	–
Indole production	+
H_2S production	–
Citrate utilisation	–
Voges - Proskauer test	–
Malate assimilation (MLT)	+

(+): test positive; (–): test negative; (V): variable reaction.

Table (2) : Antimicrobial susceptibility among *Vibrio alginolyticus* (14 strains) isolated from diseased cultured gilthead seabream.

Antimicrobial	*Vibrio alginolyticus* No. (%)		
	R	I	S
Chloramphenicol	0	2 (14.2)	12 (85.8)
Ciprofloxacin	1 (7.1)	4 (28.6)	9 (64.3)
Doxycycline	1 (7.1)	1 (7.1)	12 (85.8)
Flumequine	0	0	14 (100)
Gatifloxacin	0	0	14 (100)
Kanamycin	14 (100)	0	0
Levofloxacin	0	0	14 (100)
Nalidixic acid	3 (21.4)	5 (35.7)	6 (42.9)
Novobiocin	0	10 (71.4)	4 (28.6)
Oxacillin	14 (100)	0	0
Oxolinic acid	1 (7.1)	0	13 (92.9)
Ampicillin	14 (100)	0	0
Oxytetracycline	2 (14.3)	4 (28.6)	8 (57.1)
Sulfa-soxazole	4 (28.6)	8 (57.1)	2 (14.3)
Tetracycline	2 (14.3)	4 (28.6)	8 (57.1)
Trimethoprim / Sulfamethazole	0	4 (28.6)	10 (71.4)
Tobramycin	14 (100)	0	0

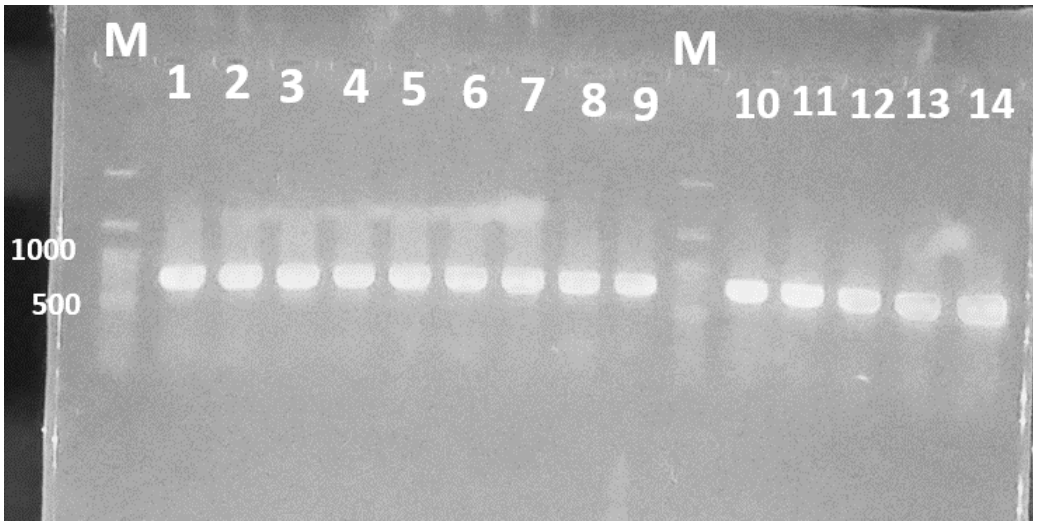

Fig. (1): PCR assay detects the 16SrRNA gene of *Vibrio* species. (M) Indicate 100 bp ladders. Lane (1) to lane (14) indicates *Vibrio* species isolated from diseased farmed gilthead seabream with amplicon size of 700 bp.

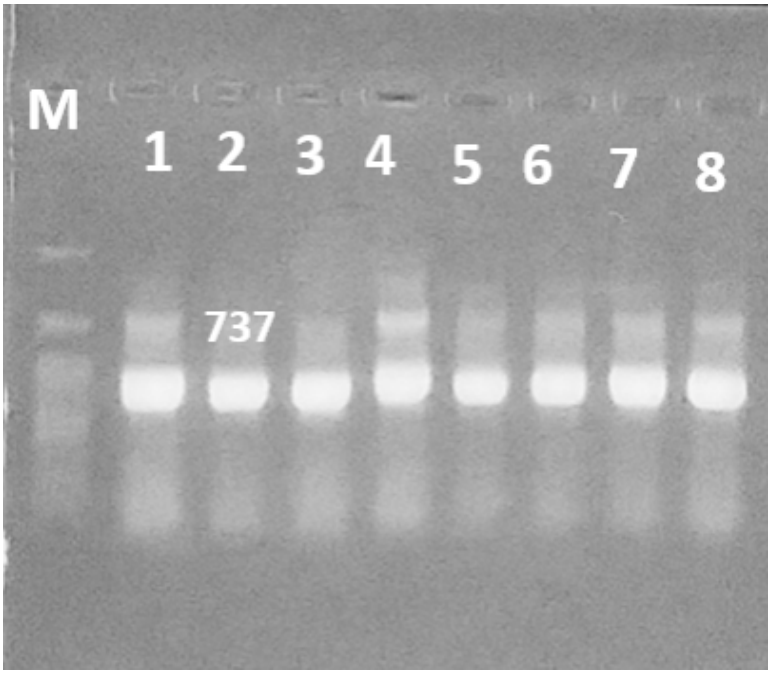

Fig. (2): PCR assay detects the collagenase gene of *Vibrio alginolyticus*. (M) Indicate 100 bp ladders. Lane (1) to lane (8) indicates pathogenic *Vibrio alginolyticus* isolated from diseased farmed gilthead seabream with amplicon size of 737 bp.