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CHARACTERIZATION OF *VIBRIO PARAHEMOLYTICUS* RETRIEVED FROM SEABREAM (*SPARUS AURATA* L.) DURING CLINICAL OUTBREAKS AT A SEMI-INTENSIVE FISH FARMS

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ABSTRACT

During 2016, a semi-intensive marine fish farms at Damietta province facing unknown clinical outbreaks. *Vibrio parahemolyticus* was the predominant bacterial isolates recovered from the internal organs of moribund seabream (*Sparus aurata*). Eight *Vibrio parahemolyticus* isolates were identified using phenotypic criteria, biochemical characterization and confirmed by PCR targeting species specific toxR genes. All strains were resistant to ampicillin, oxacillin, tobramycin and kanamycin and were suscetible to levofloxacin, gatifloxacin, doxycycline, oxolinic acid, flumequine and trimethoprim/sulfamethoxazole based on *in-vitro* antibiogram. The LD_{50} of *Vibrio parahemolyticus* isolate (VP3) was 1.0×10^6 CFU/g of fish body weight.

Key words:

Sparus aurata, *Vibrio parahemolyticus*, *Tox*R gene, LD_{50}.

INTRODUCTION

Members of the genus *Vibrio* are defined as Gram negative, motile, straight or slightly curved rods **(Kaysner *et al.,* 2004)**. *Vibrio* is an important stress related opportunistic bacterial pathogen, normally inhabitant the marine environment and incriminated as the potential cause of disease outbreaks among marine fish **(Austin and Austin, 2012)**. *V. parahemolyticus* is compatible with marine/brackish aquatic environment adjusting well to the broad range of salinities. It is commonly found on shellfishes and all varieties of finfish that are traditionally taken from marine and shore areas **(Nithya-Quintoil *et al.,* 2007)**. *Vibrio alginolyticus* and *Vibrio parahemolyticus* are the most prevalent *Vibrios* isolated during mass mortalities of farmed sea bream in some coastal provinces, Egypt **(Abdel-Aziz *et al.,* 2013)**. *Vibrio parahemolyticus* are closely related to *Vibrio alginolyticus* (99.8% of identical nucleotides),

so the differentiation of two isolates based on morpho-chemical criteria are to some extent difficult **(Gomez-gil *et al.,* 2004 and Croci *et al.,* 2007)**. *Vibrio parahemolyticus* can be molecularly identified by the PCR targeting *toxR* gene, a regulatory gene present in all, the strains **(Kim *et al.,* 1999)**. Although regionally there are plenty of literature discussing *Vibrios*, there is scarcity literature in about molecular characterization and pathogenicity of *Vibrio parahemolyticus* isolated from gilthead seabream cultured in semi-intensive marine farms. Hence, the objectives of the present study aimed to molecular characterization and pathogenicity of *Vibrio parahemolyticus* retrieved from tissues of diseased farmed gilthead seabream.

MATERIAL AND METHODS

2.1. Sampling and bacterial isolation:

Moribund seabream (weight 55.6 to 185 g, length 10.8 to 25 cm) were obtained from two different semi-intensive marine farms at Damietta province during 2016. 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, green or blue colonies with green center were picked up then transferred to Brain heart infusion agar (BHIA) (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.

2.2. Phenotypic characterization:

From each plate, single colony was examined by using cytochrome oxidase test, catalase test, and O/129 (150 µg) susceptibility test. The isolate exhibited positive oxidase and catalase tests besides sensitivity to O/129 were characterized using phenotypic and biochemical tests according to **Austin and Austin (2012)**. 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.

2.3. Confirmation of the species level by detection of toxR gene:

Bacterial isolates were routinely grown in tryptic soy broth (TSB) (Lab M, UK) supplemented with 1% NaCl at 37°C for 18 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. The PCR reaction was performed in a 20 µl consisting of 1 µl of purified genomic DNA, 10 µl ready to use PCR reaction mixture, 0.5 µl of each of the primers (50 pmol/ µl) (ToxR-F 5`-GTCTTCTGACGCAATCGTTG-3` and ToxR-R 5`-ATACGAGTGGTTGCTGTCATG-3`) 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 94°C for 10 min (initial activation), followed by 25 cycles of denaturation at 94°C for 60 s, 61°C for 60 s (annealing step), and extension step at 72°C for 60, with final extension step at 72°C for 10 min and the reaction products were stored at 4 °C until further analysis **(Kim *et al.,* 1999)**. The PCR fragments were subjected to electrophoresis on a 1.5% agarose gel in Trisborate-EDTA buffer (0.89 mol/L Tris, 0.89 mol/L boric acid, 0.02 mol/L EDTA, pH 8.0) at 110 V for 40 min, visualized by ethidium bromide staining and a UV transilluminator.

2.4. Antibiogram:

Eight isolates of *V. parahemolyticus* were tested for antimicrobial susceptibility by a standard disk diffusion method on Mueller - Hinton agar 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 the (**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. Infectivity test:

The LD_{50} of *V. parahemolyticus* strain (VP3) isolated from diseased seabream was conducted by intraperitoneal (i.p.) injection as previously described **(Alcaide *et al.* 1999).** Briefly, gilthead seabream (weight 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 all pathological signs and mortalities were observed and recorded daily. All dead fish were examined bacteriologically. Death was considered caused by inoculated bacteria only in case of the inoculated strains 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 Muench 1938).**

RESULTS

1. Biochemical and genetic identification:

Morphological and biochemical characterization of bacterial strains isolated from kidney, spleen, brain, gills and external lesions of diseased cultured sea bream are shown in (Table 1). Eight isolates were recovered and characterized from diseased infected fish. Eight bacterial strains were phenotypically and biochemically identified as *V. parahemolyticus*. Isolating *Vibrio parahemolyticus* were most prevalent from liver, spleen, kidney and external lesions, while the lowest intensities were isolated from gills. Species-specific PCR was carried out to *Vibrio parahemolyticus* isolates targeting toxR gene at 368 bp. All isolates showed the appearance of 368 bp size bands characteristic for *Vibrio parahemolyticus* Fig. (1).

2. Antibiogram:

Regarding the antimicrobial sensitivity of *Vibrio parahemolyticus* strains,all isolates exhibited antibiotic resistance to ampicillin, oxacillin, tobramycin and kanamycin, but low resistance was observed for oxytetracycline, tetracycline, novobiocin and ciprofloxacin (12.5%), and nalidixic acid (25%) (Table 2). None of *Vibrio parahemolyticus* strains were resistant to oxolinic acid, chloramphenicol, gatifloxacin, and levofloxacin, while seven strains were sensitive to doxycycline and trimethoprim/sulfamethoxazole (87.5%) (Table 2). *Vibrio parahemolyticus* revealed a high proportion of intermediate resistance against novobiocin, oxytetracycline and tetracycline (50%), and ciprofloxacin (37.5%) (Table 2).

3. Infectivity test:

Experimentally infected gilthead seabream showed signs and postmortem lesions as naturally infected fish, including skin darkening, reduced appetite, hemorrhages around the head, anal opening and fins, ascetic fluids in the abdominal cavity and distended gallbladder. The onset

of mortalities in *Vibrio parahemolyticus* occurred from third to fifth day post challenge. Neither mortalities nor pathological signs were detected in the control group 7 days' post challenge. The LD_{50} of *V. parahemolyticus* strain (VP3) was 1×10^6 CFU/g fish. All inoculated bacterial strains recovered from dead and/or moribund fish revealed the same morpho-chemical characteristics of the initially injected strain.

DISCUSSION

Vibrio parahemolyticus was the predominant isolates retrieved during clinical outbreaks in semi-intensive earthen pond marine farms at Damietta during 2016 and these may be accredited to feeding habits/type of food used by seabream collected from earthen pond farms which mainly depend on natural feed and live prey (tilapia juveniles, tilapia zilli and shrimp) which are the main reservoir of *Vibrio parahemolyticus* and these findings were in accordance with **Abdel-Aziz *et al.* (2013)** who isolate *Vibrio parahemolyticus* from seabream reared in earthen pond during recurrent episodes of mass mortalities. Eight strains isolated from naturally infected seabream identified as *Vibrio parahemolyticus* 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 parahemolyticus* reported by **Sadok *et al.* (2013)**. Because of the close relation between *Vibrio parahemolyticus* and *Vibrio alginolyticus,* the species-specific primer was used to differentiate between them. A definitive confirmation of *Vibrio parahemolyticus* was made by PCR targeting the toxR gene at 368 bp amplicon specific for this species. **Lin *et al.,* (1993)** stated that, all the strains of *Vibrio parahemolyticus* possess *tox*R gene. **Kim *et al.*, (1999)** also reported that definitive identification of *Vibrio parahemolyticus can be done by the* PCR method targeting *tox*R gene. The interpretation of antimicrobial susceptibility testing in this study revealed that, all *Vibrio parahemolyticus* isolates were resistant to kanamycin, ampicillin, oxacillin and tobramycin. Similarly, high ampicillin resistance was also observed by **Pazhani *et al.* (2014).** Resistance to sulfonamides has been reported in aquaculture and aquatic environments from different geographical regions **(Suzuki *et al.*, 201 3 andYou *et al.*, 2016)**. Doxycycline, levofloxacin, oxolinic acid, oxytetracycline and Trimethoprim/Sulfamethoxazole were still highly effective against *Vibrio parahemolyticus.* 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 parahemolyticus* are susceptible to quinolone and tetracycline **(Han *et al.*, 2007 and 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 infectivity test indicated that *Vibrio parahemolyticus* were virulent for gilthead seabream; the bacterial strains were re-isolated from dead and/or moribund fish. İn addition, moribund and/or dead fish manifested clinical signs as noted in naturally infected fish. Thus, *Vibrio parahemolyticus* isolate fulfilled Koch's postulates. The LD_{50} of *V. parahemolyticus* strain (VP3) was 1×10^6 CFU/g fish. **Khouadja *et al.* (2013)** reported that *Vibrio parahemolyticus* was virulent for seabass with LD_{50} ranging from 3.52×10^4 to 2.29×10^6 CFU/ fish. **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 LD50 values of $>10^8$. In conclusion, the study confirmed the presence of pathogenic *Vibrio parahemolyticus* as major *Vibrio* spp. in cultured gilthead seabream in the semi-intensive farms, Damietta. ToxR gene is needed for confirmatory and rapid identification of *Vibrio parahemolyticus*. Doxycycline, levofloxacin, flumequine and Oxolinic acid are drugs of choice in controlling Vibriosis based on *in-vitro* antibiogram. Unwise use of antimicrobials in fish farms lead to increase multiple antibiotic resistances 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 isolates of *Vibrio parahemolyticus* isolated from diseased cultured gilthead seabream during clinical outbreaks

Morphochemical tests	*Vibrio parahemolyticus* (8 isolates)
TCBS	Green 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	V
Growth at 10% NaCl	–
Growth at 37°C	+
Sucrose fermentation	–
Glucose fermentation	+
Esculin hydrolysis	–
Gelatin hydrolysis	+
Arabinose assimilation	V
Urease	–
Indole production	V
H_2S production	–
Citrate utilisation	+
Voges– Proskauer test	V
Malate assimilation (MLT)	V

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

Table (2): Antimicrobial resistance among *Vibrio parahemolyticus* (8 strains) isolated from naturally infected gilthead seabream during clinical outbreaks.

Antimicrobial	*Vibrio parahemolyticus* No. (%)		
	R	I	S
Chloramphenicol	0	0	8 (100)
Ciprofloxacin	1 (12.5)	3 (37.5)	4 (50)
Doxycycline	0	1 (12.5)	7 (87.5)
Flumequine	0	2 (25)	6 (75)
Gatifloxacin	0	0	8 (100)
Kanamycin	8 (100)	0	0
Levofloxacin	0	0	8 (100)
Nalidixic acid	2 (25)	1(12.5)	5 (62.5)
Novobiocin	1 (12.5)	4 (50)	3 (37.5)
Oxacillin	8 (100)	0	0
Oxolinic acid	0	0	8 (100)
Ampicillin	8 (100)	0	0
Oxytetracycline	1 (12.5)	4 (50)	3 (37.5)
Sulfa-soxazole	6 (75)	2 (25)	0
Tetracycline	1 (12.5)	4 (50)	3 (37.5)
Trimethoprim / Sulfamethazole	0	1 (12.5)	7 (87.5)
Tobramycin	8 (100)	0	0

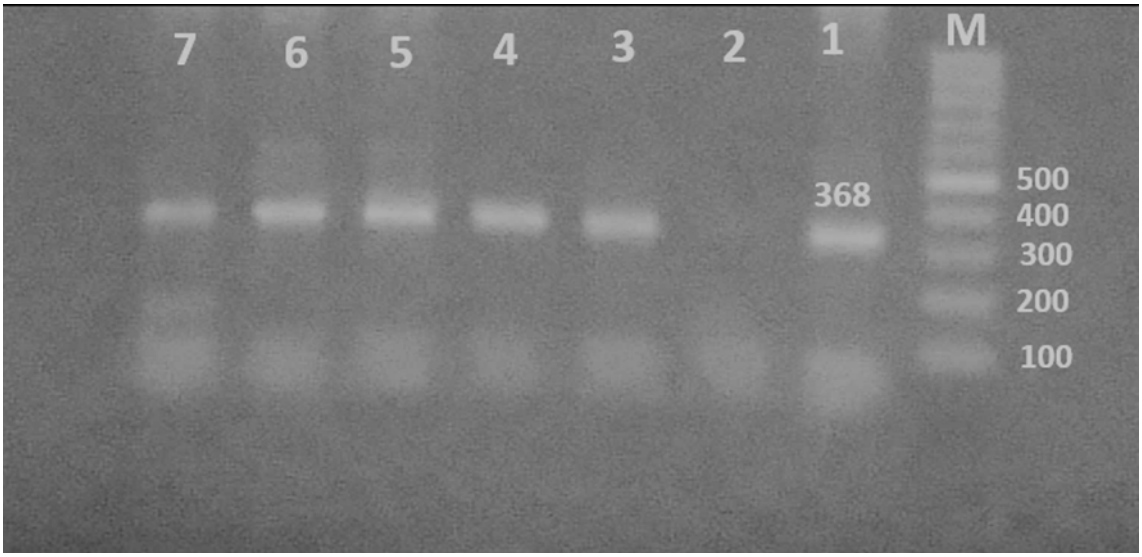

Fig. (1): PCR assay detects the toxR gene of *Vibrio parahemolyticus*. (M) Indicate 100 bp ladder. Lane (1) indicates pathogenic *Vibrio parahemolyticus* isolated from diseased farmed gilthead sea bream with amplicon size of 368 bp. Lane (3) to lane (7) indicate pathogenic *Vibrio parahemolyticus* isolated from diseased farmed gilthead sea bream with amplicon size of 368 bp. Lane (2) indicate *Vibrio alginolyticus* (negative for toxR gene).