Molecular Characterization of Vibrio spp. Isolated from Naturally Infected Larvae of the Delicate Round Herring Spratelloides delicatulus (Pisces: Clupeidae) in the Red Sea

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Abstract. This study aimed to investigate the incidence of bacterial infection in the early stages of the delicate round herring, Spratelloides delicatulus in the Red Sea, Egypt. Fish larvae were collected seasonally by light trap off the National Institute of Oceanography and Fisheries (NIOF) on the Egyptian Red Sea coast from January to December 2018. The clinical and bacteriological examinations were done to analyse the incidence of infection on the larval fish collected. The retrieved bacterial isolates were phenotypically and biochemically identified as V. alginolyticus (20%), V. vulnificus (18.6%), V. parahaemolyticus (21.4%), Vibrio harveyi (22.9%), V. cholera (11.4%) and unidentified vibrio species (5.7%). Further molecular characterization of Vibrio species using different species-specific primers for virulence genes of the former biochemically identified bacteria where the molecular identification was identical with the biochemical identification. It is concluded that Vibrio species could be a cause of infections and mortalities in the delicate round herring fish larvae, inhabiting the Red Sea.

Keywords: Fish pathogen, Vibrio, Fish larvae, Spratelloides delicatulus, Red Sea.

1. Introduction

The delicate round herring, Spratelloides delicatulus is an economic pelagic fish species of the Egyptian Red Sea coasts. Mortalities of larvae have been attributed to environmental factors, feeding conditions, predation and bacterial infection (Garrido et al., 2015). The outer surface of eggs is an excellent habitat for bacterial colonization and multiplication, and after egg hatching, the yolk sac larva become exposed directly to the bacteria present on the eggs surface. In addition, larvae are also exposed to bacteria from the surrounding water, which may cause disease and mortalities (Oppenheimer, 1955; Olafsen 2001). Gram negative bacteria that are considered as the most common pathogens of fish include Aeromonas spp., Pseudomonas sp. and Vibrio spp. Other Gram-positive bacterial genera such as Streptococcus, Lactococci, and Vagococci also cause serious infection. Vibrio is not only one of the bacterial pathogens of marine fishes, but it is also one of the most important causes of economic losses (Sudheesh et al., 2012). Vibrio spp. are more common in the marine habitats and predominant in organic matter at
water temperatures in the mesophilic range (Ramkumar et al., 2011). They have been isolated from many diseased marine animals, including shrimps (Vandenberghhe et al., 1998), Asian seabass (Tendencia 2002), abalone (Nicolas et al., 2002), marine teleost’s (Thompson et al., 2002), red drum (Liu et al., 2003) and sea horse (Tendencia 2004). However, the initiation of the disease may be related to inadequate environmental conditions such as low dissolved oxygen and high ammonia and pH values because of increased human activities and thus increasing the microbiological pollution (Haenen et al., 2014). Studies related to bacteria that infect the early stages of fishes in the Red Sea are almost lacking, this may be greatly attributed to the difficulties in the sampling and identification (Abu El-Regal et al., 2014).

One of the major causes for the low and unpredictable survival in the early stages of marine fish are outbreaks of bacterial diseases. Vibriosis, a bacterial disease caused by genus Vibrio, is one of the most challenging bacterial diseases that attack marine fish eggs and larvae.

The purpose of this study was to detect the bacteria that infect the early stages of the delicate round herring and demonstrate the effect of pathogenic bacteria on the fish stock in the Red Sea during different seasons.

2. Materials and Methods

2.1 Study Area

The area where the fish larvae were collected is located at 27° 17´ 6´´ N and 33° 46´ 22´´E in front of the National Institute of Oceanography and Fisheries about 5 km north of Hurghada city (Fig.1).

The shore of the area is bordered by a narrow sandy strip and is mostly composed of an unsorted mixture of stones, gravel, sand and mud admixed with coral fragments and shell remains. The intertidal zone has a rocky fossil reef covered with a thin layer of soft deposits. This site contains fringing reefs extending from the shore for about 450 m with many lagoons. It extends to about 150 m seaward and ends with a lagoon of about 5 m depth whose bottom is covered with seagrass beds. The area is characterized by the presence of coral reefs and different species of reef fish.

2.2 Hydrographic Parameters

Temperature (°C), salinity (psu), the concentration of hydrogen ion (pH) and dissolved oxygen (DO; mg/L) were measured simultaneously by using the Multi-probe device (Aquaread AP 5000).

Water samples for ammonium determination were collected in dark brown bottles. The reagents of 1 ml citrate solution (480g.L⁻¹), 1 ml phenol reagent, (38g.L⁻¹) and 1 ml of hypochlorite reagent (5%) were added immediately to 35 ml of the sample in the field. The mixture was allowed to stand overnight (10-12 h) and the blue color of indophenols formed was measured using a spectrophotometer at 630 nm.

2.3 Sampling of Fish Larvae

Plankton samples were collected seasonally by light trap during the period from January to December 2018. The trap was deployed in the water for two hours just after the sunset. All the trapped fish larvae settled at the bottom of the collecting bucket and were taken immediately to the laboratory.

Fish larvae were sorted, identified and counted, then larvae of the delicate round herring were separated, counted and subjected to clinical examination and bacteriological studies (Fig. 2a). Larvae were identified to species based the morphological features (Maaty, 2015, Abu El-Regal, 2017, Abu El-Regal et al., 2019).

2.4 Bacterial Isolation
Inoculum for bacterial isolation was taken from the skin and external skin ulcers of the fish larvae and were inoculated on 1.5% NaCl Trypton Soy Agar (TSA) and brain heart infusion agar (BHI) plates (Oxoid). The inoculated plates were incubated at 25.5 °C for 2-5 days (Farmer and Brenner, 1992).

2.5 Morphological and Biochemical Characterization and Identification of the Isolated Bacteria

The pure bacterial colonies were sub cultured on Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar plates (Oxoid, UK), and incubated at 25 °C for 18-24 hr. The presumptive bacterial isolates were characterized using morphological and cultural characteristics as well as biochemical tests as described by Alsina and Blanch (1994) and Nicky (2004). Commercial API 20E strips (BioMerieux, France) were further used for the identification of the organisms according to the manufacturer’s instructions. The purified bacterial strains were then stored in semisolid nutrient agar slant and kept in the refrigerator at -20 °C for further molecular diagnosis.

2.6 Molecular Identification of Vibrio spp. via PCR

DNA extraction from bacterial cells is an important step in a PCR mediated molecular approach in characterization. Genomic DNA was extracted using commercial kits (QIAamp DNA Mini Kit), (Qiagen, Hilden, Germany) according to the manufacturer’s instruction from the overnight broth culture of each of the organisms. The extracted DNA was purified and stored at -20 °C.

2.7 DNA Amplification

The purified DNA of the isolates was amplified using species specific primers for virulence genes for each of the organisms. The process was done using a gradient thermal cycler. Oligonucleotide primers used to amplify virulence gene were demonstrated in Table 1. The PCR reaction was performed in a total 50 µl reaction system consisting of 100ng of DNA template, 30 nM each primer and 25µl of COSMO PCR RED M.Mix, (Willowfort, Birmingham, England). The condition for PCR amplification includes a 5 minutes denaturing step at 95°C then 35 cycles of 20 sec at 95°C, 45 sec at 60°C and 30 sec at 72°C followed by a final extension stage for 5 minutes at 72°C.

Fig. 1. Map showing the sampling site in Hurghada.
Table 1. Primers used for the PCR amplification.

<table>
<thead>
<tr>
<th>Targeted genus or spp.</th>
<th>Primer sequences (5’-3’)</th>
<th>Amplicon Size (bp)</th>
<th>References and targeting gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vibrio vulnificus</td>
<td>F-5’ CAGCC GGAGC TCGTCCATT T TG 3’ R-5’ ATGAG TAAGC GTCCGAGCCG T</td>
<td>484 bp</td>
<td>Kim et al. (2015) vvhA gene</td>
</tr>
<tr>
<td>V. parahaemolyticus</td>
<td>F-5’ AGCTT ATTG CGGTTTCTGT CGG–3’ R-5’ CKCAA GACCA AGAAAAGCCG TC 3’</td>
<td>297 bp</td>
<td>Kim et al. (2015) Tdh gene</td>
</tr>
<tr>
<td>Vibrio alginolyticus</td>
<td>F-5’ ACGGC ATTGG AAATTGCGAC TG 3’ R-5’ TACCC GTCTC ACGAGCCCA A GC 3’</td>
<td>199 bp</td>
<td>Kim et al. (2015) ToxR gene</td>
</tr>
<tr>
<td>Vibrio cholera</td>
<td>F-5’ CAAGC TCCGC ATGTCCAGAA GC 3’ R-5’ GGGGC GTGAC GCGAATGATT 3’</td>
<td>154 bp</td>
<td>Kim et al. (2015) ctx gene</td>
</tr>
<tr>
<td>V. harveyi</td>
<td>F-5’ CTTCACGCTTTGATGGCTACGT CG 3’ R-5’ GTCACCCAATGCTACGACCT 3’</td>
<td>235bp</td>
<td>Raissy et al. (2015) Vvh gene</td>
</tr>
</tbody>
</table>

2.8 Detection of the Amplified product

The final amplified products were analyzed using electrophoresis on a 1.5% agarose NA gel (Pharmacia, Uppsala, Sweden) in Tris-borate-EDTA (TBE) buffer (its composition is; 10mM Tris-HCl, 10mM NaCl, 1mM EDTA, pH 7.8), stained using ethidium bromide and then visualized using a UV light transilluminator. A 100–1000 bp DNA Ladder which is ideal for determination of a double stranded DNA was used throughout. The 500bp fragment is present at increased intensity to allow easy identification.

3. Results

3.1 Hydrographic Parameters

Concentration of hydrogen ion (pH) varied from 8.03 in summer to 8.82 in winter. The temperature varied from the lowest of 20.4 in winter to the highest of 31 in summer. Salinity had the highest value in summer (40.6) and the lowest value in winter (39.4). The values of dissolved oxygen reached its maximum in winter (8.32) and its minimum in autumn (7.01). Ammonia had the highest concentration in summer and the lowest concentration in winter (Table 2).

3.2 Prevalence of Infection of Fish Larvae with Different Vibrio spp. During Different Seasons

A total of 120 larvae, 30 larvae in each season, were examined for the prevalence of bacterial infection of which 70 larvae constituting 58.3% of all larvae were infected with certain bacterial species of *Vibrio*. The highest number of infected larvae was recorded in summer where 28 larvae forming 93.3% of all larvae collected in this season were infected. On the other hand, the lowest number of infected larvae was recorded in winter with only 4 (13.3%) infected larvae. The examined *Vibrio* species were found in all seasons but *V. vulnificus* was absent in winter specimens. The most frequently encountered *Vibrio* sp. was *V. harveyi* which was isolated from 16 fish larvae followed by *V. parahaemolyticus* which was isolated from 15 fish larvae. (Table 3).

3.3 Clinical Picture

The larvae showed variation in movement from rapid to sluggish movement with petechial hemorrhage in skin small ulcers in tail region and internal gut necrosis delicate round herring larvae showing petechial hemorrhage on the skin (Fig. 2b).

3.3.1 Bacterial isolation

A total of 70 bacterial isolates were isolated from skin and external skin ulcers of delicate round herring. The colonies on Trypton...
Soy Agar appeared as smooth, rounded, buff white-to-cream-colored and 2-5 mm in diameter, whereas on TCBS, the colonies were yellow-greenish, smooth, circular, and convex (Fig. 3).

3.3.2 Identification of the isolated bacteria

- Morphological characterization and Biochemical identification of the isolated bacteria

Most of the isolates showed initial phenotypic properties typical of the genus *Vibrio*; being Gram-ve curved rods, oxidase positive, have requirements for sodium chlorides and motile.

The biochemical and physiological characteristics (Table 2) of all isolates were almost different and allowed the presumed identification of the bacteria as *V. alginolyticus* (14 isolates, 20%), *V. vulnificus* (13 isolates, 18.6%), *V. parahaemolyticus* (15 isolates, 21.4%), *V. harveyi* (16 isolates, 22.9%), *V. cholera* (8 isolates, 11.4%) and unidentified *Vibrio* species (4 isolates, 5.7%).

- Molecular characterization of the isolated *Vibrio* spp.

The primers selected for PCR amplification of the previously biochemically characterized *Vibrio* spp. showed maximum identity for their respective targeted gene sequence with 100% homology with their reference strains; *ToxR* genes of *V. alginolyticus*, *Tdh* gene of *V. parahaemolyticus*, *Vh* gene of *Vibrio vulnificus*, *ctx* gene of *V. Choleraand*, *vhh* gene of *V. harveyi*, that were detected at 199 bp, 297 bp, 484bp, 154bp and 235bp, respectively (Fig. 4, 5, 6, 7 and 8).

Table 2. Seasonal values of physicochemical characteristic of Sea water samples.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Winter</th>
<th>Spring</th>
<th>Summer</th>
<th>Autumn</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>8.82</td>
<td>8.4</td>
<td>8.03</td>
<td>8.32</td>
</tr>
<tr>
<td>Temp.(°C)</td>
<td>20.42</td>
<td>27.86</td>
<td>31</td>
<td>25.78</td>
</tr>
<tr>
<td>Salinity (PSU)</td>
<td>39.4</td>
<td>40</td>
<td>40.6</td>
<td>40.5</td>
</tr>
<tr>
<td>DO (mg/L)</td>
<td>8.32</td>
<td>8.06</td>
<td>7.7</td>
<td>7.01</td>
</tr>
<tr>
<td>NH₄(mg/L)</td>
<td>0.006</td>
<td>0.019</td>
<td>0.116</td>
<td>0.058</td>
</tr>
</tbody>
</table>

Table 3. Prevalence of infection of the early stages of delicate round herring with different *Vibrio* spp. during deferent seasons.

<table>
<thead>
<tr>
<th>Season</th>
<th>No. of larvae</th>
<th>No. infected larvae</th>
<th>% of infected larvae</th>
<th><em>V. vulnificus</em></th>
<th><em>V. parahaemolyticus</em></th>
<th><em>V. alginolyticus</em></th>
<th><em>V. harveyi</em></th>
<th><em>V. cholera</em></th>
<th>Unidentifid. vibrio sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. %</td>
<td>No. %</td>
<td>No. %</td>
<td>Infected larvae</td>
<td>Infected larvae</td>
<td>Infected larvae</td>
<td>Infected larvae</td>
<td>Infected larvae</td>
<td>Infected larvae</td>
</tr>
<tr>
<td>Winter</td>
<td>30</td>
<td>4</td>
<td>13.3</td>
<td>0.0</td>
<td>0.0</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>0.0</td>
</tr>
<tr>
<td>Spring</td>
<td>30</td>
<td>13</td>
<td>33.3</td>
<td>2.67</td>
<td>3.0</td>
<td>2.3</td>
<td>3.0</td>
<td>3.0</td>
<td>2.3</td>
</tr>
<tr>
<td>Summer</td>
<td>30</td>
<td>28</td>
<td>93.3</td>
<td>7.23</td>
<td>6.2</td>
<td>7.2</td>
<td>7.2</td>
<td>9.3</td>
<td>2.7</td>
</tr>
<tr>
<td>Autumn</td>
<td>30</td>
<td>25</td>
<td>83.3</td>
<td>4.13</td>
<td>5.16</td>
<td>4.13</td>
<td>6.2</td>
<td>5.20</td>
<td>5.16</td>
</tr>
<tr>
<td>Total</td>
<td>120</td>
<td>70</td>
<td>58.3</td>
<td>13.18</td>
<td>21.4</td>
<td>14.20</td>
<td>16.22</td>
<td>8.11</td>
<td>4.5</td>
</tr>
</tbody>
</table>
Fig. 2: a) 6mm total length of *S. delicatulus* from the Red Sea, b) 14 mm larvae of *S. delicatulus* from the Red Sea.

Fig. 3. *Vibrio* growth (colonies) on TCBS media 24 hr 25.5°C.

Fig. 4. The amplified PCR products from chromosomal DNA recovered from pure culture colonies determined by the agarose gel electrophoresis as following: 100-1000 bp DNA ladder, lane 2,3 are 199 bp of *V. alginolyticus* and lane 1 is a negative control.
Fig. 5. The amplified PCR products from chromosomal DNA recovered from pure culture colonies determined by the agarose gel electrophoresis as following: 100-1000 bp DNA ladder, lane 1 is 297 bp of *V. parahaemolyticus* and lane 2 is negative control.

Fig. 6. The amplified PCR products from chromosomal DNA recovered from pure culture colonies determined by the agarose gel electrophoresis as following: 100-1000 bp DNA ladder, lane 1 is 484 bp of *V. vulnificus*, lane 2 is negative and lane 3 is negative control.
4. Discussion

Since the studies concerning with the ecology of coral reef fish larvae are scarce, very little is known about the diseases affecting the early stage of coral reef fish in general and in the the Red Sea in particular. Studies on the infectious bacterial diseases affecting the Red Sea coral reef fishes, and their ecological and economic importance in Egypt, are also few (Abd El-Galil and Mahmoud 2012). Therefore, this work was designed mainly to detect the bacterial fish pathogens incriminated in mortalities of the early stages of coral reef fishes especially those of delicate round herring fish larvae (Family: Clupeidae). In this
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Investigation, *V. alginolyticus*, *V. vulnificus*, *V. harveyi* and *V. parahaemolyticus* and *V. cholerae* were accused of diseases and mortalities of delicate round herring fish larvae (Pisces: Clupeidae). The clinical examination of those larvae showed sluggish swimming with small ulcers in tail region and Petechial hemorrhage in skin and internal organ necrosis. This is in agreement with Olafsen (2001), Mahmoud et al. (2017) and Rodrigo et al. (2016) where it was reported that *V. tubiashii* accompanied with an outbreak of massive larval mortalities in Scallop *Argopecten purpuratus* associated with necrosis of digestive tissue of scallop larvae.

The analysis of water quality parameters during the period of study showed a decrease in dissolved oxygen and an increase in ammonia concentration in summer and autumn. These variations in *O*₂ and ammonia were accompanied with high prevalence rate of infection with different *Vibrio* spp. This finding is in an accordance with Haenen et al., (2014) who attributed the increased rate of infection to the decrease of dissolved oxygen and elevation of ammonia and *pH* values due to the increased human activities and thus increasing the microbiological pollution. The high rate of infection incidence may be also related to the elevated temperature in summer as indicated by other studies (Moustafa et al. 2004, and Mahmoud et al., 2017). The increase in the ammonia level in summer and autumn, the physical contact and the sharp decrease in the dissolved oxygen are the most possible predisposing factors for initiation, colonization and spread of infection in addition to suppress fish immune system Suomalainen et al. (2005). Moreover, this is more or less in agreement with Beatrise (2003) and Pujalte et al. (1999) who reported that although a seasonal variation was not confirmed in *Vibrio* spp., a great variety was recognized in spring. This may be attributed to the slight elevation in the water temperature during warm seasons, which stimulates the multiplication of a larger group of mesophilic *Vibrio* species such as *V. mediterranei*. This could also show why psychrotrophic bacteria, such as *V. splendidus* was commonly detected in winter while a large number of *Vibrio* spp. associated with Mediterranean oysters are detected during warmer seasons. The 70 pure bacterial isolates from naturally infected early stages of delicate round herring fish were identified as *V. alginolyticus*, *V. vulnificus*, *V. parahaemolyticus*, *V. harveyi*, *V. cholera* and unidentified species of *Vibrio* by the colony, morphological characters, and biochemical reactions including the API20E tests.

These findings were in agreement with those of Nicky (2004) and Martins et al. (2010). *Vibrio harveyi*, and *V. parahemolyticum* were the most prevalent isolates (22.9% and 21.4%, respectively) followed by *V. alginolyticus* and *V. vulnificus* (20% and 18.6%, respectively). This was in agreement with Tendencia (2002) and Hansen and Olafesn (1999) who recorded that *Vibrio* spp. including *V. fluvialis*, *V. alginolyticus*, and *V. parahaemolyticus*, *V. scophthalmi*, *V. splendidus*, constitute the main bacterial groups isolated from the intestinal micro flora of turbot larvae (*Scophthalmus maximus*). Yet, *Vibrio* spp. are one of the main predominant genera on sardine eggs, the establishment of *Vibrio* in association with eggs in a natural marine ecosystem is logical, taking in consideration that *Vibrio* spp. are normal inhabitants of the marine aquatic environment and estuarine habitats. In addition, *Vibrio* spp. have excellent colonization efficiency on the mucous surfaces and hence easily colonize on the eggs surface (Beatrise 2003, Bergh et al., 1992, Strom and Olafsen 1990).

Regarding the molecular characterization that were concerned with identification of the former biochemically identified *Vibrio* using conventional single PCR, as pieces-specific
genes were used for the amplification. A species specific vhh gene (V. harveyi) (Raissy et al., 2015), vvhA gene (V. vulnificus), Tdh gene (V. parahaemolyticus) ToxR gene (Vibrio alginolyticus) and ctx gene (Vibrio cholera) (Kim et al., 2015) were used. In conclusion the result of our investigation indicates that the early stages of delicate round herring fish larvae (Clupeidae) are susceptible to infection by *Vibrio* sp. Also, the infection is present all over the year irrespective of the season. *Vibrio* is considered as a potential pathogen for delicate round herring fish larvae.

References


التشخيص. تهدف هذه الدراسة إلى بحث معدل الإصابة البكتريا في المراحل الأولى لسمكة السردين (سيراتللويدس ديليكاتيليس) بالبحر الأحمر. حيث تم تجميع بركات الأسماك موسميًا باستخدام الشبكة الضنوئية من شواطئ البحر الأحمر أمام المعهد القومي لعلوم البحار والمايا، ثم بالقربة بمصر من يناير حتى ديسمبر 2018. وقد تم إجراء الفحص الإكلينيكي والكيميائي لتحديد معدل إصابة اليرقات التي تم تجميعها، وقد تم تعرف البكتريا المعزولة باستخدام الخصائص المورفولوجية والبيوكيميائية على أنها فيريبيوس الجونليكس (20%) وفيريبيوس فلفكس (18,7%) وفيريبيوس بارهيلولينيكس (21,4%) وفيريبيوس هارفاي (22%”) وفيريبيوس كورنا (21,7%) وفيريبيوس غير معرفة النوع (5%)، إضافة إلى ذلك تم تعرف هذه اليرقات بالبكتريا باستخدام البيولوجيا الجزيئية باستخدام برمجات مختلفة ونوعية لجينات الدرو أو أجسام الفيروي المعروفة سابقاً باستخدام الطرق البهميائية، وقد كانت نتائج التعرف متطابقة. وقد خلصت الدراسة أن أجسام الفيروي مسؤولة عن أغلب حالات العدوى والنجاح في بركات أسماك السردين (سيراتللويدس ديليكاتيليس) بالبحر الأحمر.