Studies on yersiniosis in cultured Mugil seheli for the first time in Suez Governorate
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Abstract
A total of 360 cultured Mugil seheli had clinical signs were collected from private farms in Suez Governorate, Egypt. M. seheli was subjected to clinical, post-mortem and bacteriological examinations for detection of Yersinia ruckeri. Yersinia ruckeri was identified by biochemical reactions and polymerase chain reaction (PCR). One isolate of Y. ruckeri. The results revealed that the presence of Yersinia ruckeri infection was 68.1%. The highest prevalence of Y. ruckeri infection in cultured M. seheli was during autumn season (80%) followed by spring (72.2%), summer (70%) and then winter (50%). PCR amplification of DNA from Y. ruckeri isolates using 16s rRNA (YER8/10) specific primers for Y. ruckeri resulted in PCR products size of 575 bp. All Y. ruckeri isolates were found to be sensitive to Ciprofloxacin, Tobramycin and Trimethoprim. While resisted Erythromycin, Amoxycillin and Novobiocine.

Introduction
Fish constitute a major source of protein, fatty acids, vitamins, minerals and essential micronutrients for an expanding segment of the world population. Consequently, aquaculture is the fastest growing food production sector and accounts for approximately 50% of the fish consumed worldwide (Mathiesen, 2015). Mugil seheli (M. seheli) is recognized as economically-important marine and brackish water fish and abundantly cultured in Suez Governorate, Egypt, for domestic consumption. M. seheli may be an excellent candidate for aquaculture especially in North Egypt regions because of its nutritional value.

Disease outbreaks have become a major constraint to the expansion of aquaculture and have a significant impact on the economic development of many countries. Bacterial pathogens are among the most important serious fish diseases in aquaculture causing 80% of fish mortalities (Woo and Bruno, 1999). Most of the bacteria, associated with these diseases, are saprophytic and widely distributed in the aquatic environment (El-Ashram and Abd El-Rahman 2006; Austin and Austin 2007 and
Studies on yersiniosis in cultured Mugil seheli for the first time in Suez Governorate

Plumb and Hanson 2011). Enteric redmouth disease (ERM, yersiniosis) is one of the most important diseases of salmonids and leads to significant economic losses (Horne and Barnes 1999). The disease is caused by Yersinia ruckeri, It's a Gram-negative rod-shaped enterobacterium, which was first isolated from rainbow trout (Oncorhynchus mykiss) in USA (Ross et al., 1966) and is currently found throughout North and South America, Europe, Australia, South Africa, the Middle East and China (Tobback et al., 2007 and Shaowu et al., 2013).

16s rRNA gene (YER8/10) primer was used to identify Y. ruckeri and gave a 575 bp band when tested by means of PCR using these primers (Eissa et al., 2008). The aim of the current investigation was to throw light on detection of Y. ruckeri in cultured M. seheli in Suez Governorate, Egypt with special reference to detection of 16s rRNA gene as diagnostic tool.

MATERIALS AND METHODS

Naturally infected 360 M. seheli were collected from private fish farms in Suez Governorate using gill nets method described by Portt (2006). The average body weight and length were 39.11gm and 15.17cm respectively. Fish samples were subjected to clinical, post-mortem and bacteriological examinations (Noga, 2010, Meyers, 2006 and Austin and Austin, 2007). The prevalence of Y. ruckeri seasonally infected fish and organs also were recorded.

Clinical and Postmortem Examinations:

Clinical and post-mortem examinations were carried out according to Noga, (2010) and Meyers, (2006).

Isolation and identification of Y. ruckeri

Under complete aseptic condition, samples of gills and internal organs (liver, kidney and spleen) were collected from diseased M. seheli and cultivated on tryptic soy agar (Micromaster) ® supplemented with 1% NaCl and incubated at 25°C for 24-48 hours (Buller, 2014). Colonies from general media were streaked on Yersinia selective agar base (Micromaster) ® supplemented with Yersinia selective supplement. The plates were incubated at 25°C for 24 hrs. Separated colonies were described and pure culture was identified using phenotypical and biochemical characters according to The biochemical tests were used for identification of bacterial isolates.

DNA extraction and PCR assay
Genomic DNA was extracted from bacterial isolates as described by Devi et al., (2009). The broth culture of bacterial isolates incubated at 37 °C for 16–18 hrs. Then centrifuged (10,000 rpm, 1 min) to obtain a pellet, which was then washed with normal saline (0.85 w/v) and suspend pellet of cells in sterile distilled water (0.5 ml). Suspended pellets were placed in boiling water bath at 98±2 °C for 15–20 min in a water bath to lyse the cells. The lysate was centrifuged to remove the cell debris (10,000 rpm, 5 min). The supernatant was obtained and stored (−20 °C) until further use. Primer specific for 16s rRNA of Yersinia ruckeri was used for PCR protocol under specific condition which was done twice. The first one was done exactly according to Gibello et al., (1999) and the second trial was achieved as previous but with some modification of the annealing temperature. The modification was 25 cycles of denaturation for 1 min at 92°C, annealing was raised to 62°C for 1 min, and extension for 1 min at 72°C, followed by a final extension step of 72°C for 5 min. PCR amplified product was subjected to Gel electrophoresis in 1.5% (w/v) agarose gel, using TAE (tris-acetate-EDTA) as running buffer and the power was supply adjusted at 100 volt for 20-30 minutes (Lee et al., 2012).

Challenge test

A total of 20 apparently healthy acclimated M. seheli were divided into two equal groups in glass aquaria filled with pond water supplemented with continuous aeration using electrical pump for a week before injection. First group injected intraperitoneally with 0.1 ml of Y. ruckeri 5 × 10^5 CFU (Berc et al., 1999). The second group injected with sterile saline by using one ml insulin syringe. The experimentally infected fish were daily observed up to 14 days for any abnormal clinical signs and mortalities. Mortality rate was calculated for each group and re-isolation of injected bacteria was done.

Sensitivity test to antibiotic

A total of randomly selected five Y. ruckeri isolates recovered from naturally infected M. seheli were used in antimicrobial susceptibility test. Susceptibility was determined by the disc-diffusion technique on Mueller-Hinton agar plates (CLSI, 2013). Y. ruckeri pure cultures were grown in brain heart infusion (BHI) broth for 24 hours at 37°C (Samal et al., 2014). The suspension was swabbed on Mueller-Hinton medium plates. Discs were placed on inoculated plates and incubated for 24 hours at 37°C (Thakur et al., 2003 and Aravena-Román et al., 2012). The antibiotic discs were used Amoxicillin, Ciprofloxacin, Erythromycin, Naldixic Acid, Novobiocin, Tobramycin and Trimethoprim. Isolates were
judged as sensitive (S), intermediately resistant (I) or resistant (R) on the basis of the size of the zone of bacteria growth inhibition according to the guidelines of the CLSI (2013).

**Results**

**Clinical findings**

The most common findings were hemorrhages on skin at different parts of the body, hemorrhages at the base of fins, on operculum and anal opening, swelling of abdomen (Plate 1, A). Some fish exhibited sluggishness, darkening of the fish coloration, swimming near the water surface with air gasping and bulging eyes. Affected fish showed loss of equilibrium and exhibited abnormal swimming behavior.

**Post-mortem findings**

The post-mortem finding of naturally infected *M. seheli* showed petechial hemorrhages of the most internal organs and erythematous intestinal mucosa (Plate 1, B). In other cases, the intestines voided of feed and contained yellowish mucus. Congested and enlarged spleen also was observed (Plate 1, C). Moreover, enlarged and congested kidney and liver were noticed.

**Plate (1):** Naturally infected *M. seheli* showing hemorrhages on external body surface (A), petechial hemorrhages on intestinal external surface (B) and enlarged and congested spleen (arrow) and pale liver with hemorrhages (C).
**Bacteriological examination**

The phenotypic and biochemical characters of isolated bacteria from naturally infected *M. seheli* were illustrated in (Table 1).

**Morphological and biochemical identification (Table 1)**

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram staining</td>
<td>-</td>
</tr>
<tr>
<td>Colonies character on Yersinia selective agar base</td>
<td>Pink colonies with red center</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>-</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
</tr>
<tr>
<td>Ornithine decarboxylase</td>
<td>+</td>
</tr>
<tr>
<td>Indol</td>
<td>-</td>
</tr>
<tr>
<td>H$_2$S</td>
<td>-</td>
</tr>
<tr>
<td>Glucose fermentation</td>
<td>+</td>
</tr>
<tr>
<td>Citrate utilization</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin liquification</td>
<td>-</td>
</tr>
<tr>
<td>Methyle red</td>
<td>+</td>
</tr>
</tbody>
</table>

**Prevalence of *Y. ruckeri* isolates in naturally infected *Mugil seheli*:**

Total prevalence of *Y. ruckeri* infection in naturally infected *M. seheli*:

The total prevalence of *Yersinia ruckeri* infection was 68.1% among the examined fish.

**Seasonal prevalence of *Y. ruckeri* infection in naturally infected *M. seheli*:**

The prevalences of *Y. ruckeri* infection in cultured *Mugil seheli* were 80% in autumn followed by 72.2% in spring, 70% in summer and 50% in winter.
Studies on yersiniosis in cultured *Mugil seheli* for the first time in Suez Governorate

**Prevalence of *Y. ruckeri* infection in organs of naturally infected *M. seheli***:

The majority of *Yersinia ruckeri* isolates were recovered from gills 41.7% followed by liver 29.55% and kidney 25.1%. While the lowest percentage was recovered from spleen 3.64%.

**Experimental infection of *M. seheli* with *Y. ruckeri***

The mortality rate of experimentally infected *M. seheli* with *Y. ruckeri* isolate was showed in (Table 2). The most cases of experimentally infected fish suffered from abnormal swimming, darkening of the skin and loss of equilibrium within 24hrs post-injection. Also, severe diffused external hemorrhages on the body of experimental infected fish, redness of all fins in some cases; severe congestion and redness of the mouth were observed (Photo 1).

![Photo 1](image-url) *Mugil seheli* artificially infected with *Yersinia ruckeri* showed sever hemorrhage of the mouth area.

**Table (2): Results of pathogenicity of *Y. ruckeri* used in challenge test**

<table>
<thead>
<tr>
<th>Injected bacteria</th>
<th>No. of infected fish/control</th>
<th>Average weight of fish (g)</th>
<th>Dose (CFU/Fish)</th>
<th>Method of infection</th>
<th>No. of dead fish</th>
<th>Mortality (%)</th>
<th>Post-infection days of mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Y. ruckeri</em></td>
<td>10</td>
<td>30.5</td>
<td>$5 \times 10^5$</td>
<td>Intraperitoneal</td>
<td>6</td>
<td>60</td>
<td>3-7</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td></td>
<td>0.1 ml saline</td>
<td></td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>
Molecular identification by PCR

Tested seven bacterial isolates gave the expected bands of 575 bp size in lane one to seven when the \textit{Y. ruckeri} specific 16s rRNA primers were used for molecular identification (Photo 2) by modified protocol (Table 3,4).

Table (3) Specific primers of \textit{Y. ruckeri}, Annealing temperature and number of cycles used in DNA amplification of each isolate:

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Name of primers</th>
<th>Gene product</th>
<th>Forward/ Reverse</th>
<th>AT</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Yersinia ruckeri}</td>
<td>YER 8, YER 10</td>
<td>16s rRNA</td>
<td>F:GCGAGAGGAAGGGTTAAGTG</td>
<td>62°C</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: GAAGGCACCAAGGCATCTCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Gibello \textit{et al.}, 1999 and Altinok 2001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

AT= Annealing temperature C= Number of cycles

Table (4) Standard MyTaq™ Mix Protocol

<table>
<thead>
<tr>
<th>Template</th>
<th>200ng</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primers (20pmol)</td>
<td>1μl</td>
</tr>
<tr>
<td>Reverse primers (20pmol)</td>
<td>1μl</td>
</tr>
<tr>
<td>MyTaq Mix, 2x</td>
<td>25μl</td>
</tr>
<tr>
<td>Water</td>
<td>Up to 50μl</td>
</tr>
</tbody>
</table>

Photo (2) PCR amplification of DNA from \textit{Yersinia ruckeri} isolates using specific primers for \textit{Y. ruckeri} resulted in PCR products size of 575 bp.
Antibiogram sensitivity of *Y. ruckeri*

All *Yersinia ruckeri* isolates were sensitive to Ciprofloxacin, Tobramycin and Trimethoprim. While resisted Erythromycin, Amoxycillin and Novobiocine (Table 5).

**Table (5) Antibiogram profile of *Y. ruckeri***

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Break point (mm)</th>
<th>Average inhibitory zone of isolates (mm)</th>
<th>Sensitivity of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aml (10μg)</td>
<td>&gt;18 14-17 &lt;13</td>
<td>10</td>
<td>R</td>
</tr>
<tr>
<td>Cipro (5μg)</td>
<td>&gt;31 21-30 &lt;20</td>
<td>45</td>
<td>S</td>
</tr>
<tr>
<td>E (15μg)</td>
<td>&gt;23 14-22 &lt;13</td>
<td>10</td>
<td>R</td>
</tr>
<tr>
<td>NA (30μg)</td>
<td>&gt;19 14-18 &lt;13</td>
<td>10</td>
<td>R</td>
</tr>
<tr>
<td>NV (30μg)</td>
<td>&gt;22 18-21 &lt;17</td>
<td>10</td>
<td>R</td>
</tr>
<tr>
<td>TMP (5μg)</td>
<td>&gt;16 11-15 &lt;10</td>
<td>35</td>
<td>S</td>
</tr>
<tr>
<td>TOB (10μg)</td>
<td>&gt;15 13-14 &lt;12</td>
<td>16</td>
<td>S</td>
</tr>
</tbody>
</table>

Aml = Amoxycillin, cipro = ciprofloxacin, E = erythromycin, NA = naldixic acid, NV = novobiocin, TMP = trimethoprim, TOB = tobramycin, S = sensitive, I = intermediate resistant, R = resist.

**Discussion**

The most common clinical signs of naturally infected fish were swimming near the surface of water, congested or pale gills, hemorrhages at the base of fins, abdominal part and gill cover, hemorrhages around the vent and mouth and some fish displayed abdominal distension. Similar picture was noticed by Oren (1981), El-Ashram and Abd El-Rahman (2006) and Enany *et al.*, (2011).

The results of postmortem examination of naturally infected fish revealed pale liver in some cases and in other cases liver were hemorrhagic. Kidneys and spleen were enlarged and congested. These results went hand in hand with that observed by El-Ashram and Abd El-Rahman (2006) and Enany *et al.*, (2011).

Regarding to the bacteriological examination, *Yersinia ruckeri* was Gram negative short rod bacilli, grew on *Yersinia* selective agar base that not agreed with Buller, (2014) who reported that *Yersinia ruckeri* didn't grow on *Yersinia* selective agar base. *Yersinia ruckeri* in present study was oxidase negative and catalase positive. It was negative for hydrogen
sulphide production, indol production and gelatin liquefaction tests while positive for citrate utilization, ornithine decarboxylase and methyle red test. This finding was supported by Altun et al., (2013).

The mortality rate of Yersinia ruckeri in experimentally infected M. seheli fish was 60% in the present study. This result was nearly in line with that obtained by Ohtani et al., (2016) who reported that the mortality of Y. ruckeri IP experimentally rainbow trout (Oncorhynchus mykiss) was 64%.

Yersinia ruckeri PCR assay using YER 8, YER 10 primers according to Gibello et al., (1999) showed that the use of annealing temperature 60°C as indicated by them led to the appearance of non-specific bands. Therefore the temperature of annealing step was raised to 62°C in the present study (higher than that used by Gibello et al., 1999) to avoid the appearance of non-specific bands, bacterial isolates gave the expected bands of 575 bp.

Yersinia ruckeri was isolated from cultured Mugil seheli which reared in brakish water in Suez governorate, Toranz, (2004) cited that Yersinia ruckeri was documented in cultured fresh and seawater fish. The total prevalence of Yersinia ruckeri in cultured Mugil seheli was 68.1%. These results were nearly in line with that obtained by Eissa et al., (2008) who reported the prevalence of Y. ruckeri infection in cultured Nile tilapia, Oreochromis niloticus in Egypt was 66.6%. While Aly (2013) mentioned that incidence of Y. ruckeri in grey mullet was 20%.

All Y. ruckeri isolates were found to be sensitive to Ciprofloxacin, Tobramycin and Trimethoprim. While resisted Erythromycin, Amoxycillin and Novobiocine, these resulted supported by Akhlaghi and Sharifi Yazdi (2008) and Lazar et al., (2010).

It could be concluded that yersiniosis is one of the most important bacterial disease of cultured M. seheli and leads to significant economic losses. Ciprofloxacin, Tobramycin and Trimethoprim are the drugs of choice to control the disease. PCR is most sensitive and rapid method for identification Yersinia ruckeri pathogen.

REFERENCES

Studies on yersiniosis in cultured *Mugil seheli* for the first time in Suez Governorate


Clinical and Laboratory Standards Institute (CLSI) (2013): Informational Supplement; CLSI document M100-S21, Wayne, PA, USA. Vol. 31 No. 1 Pages 13, 43 to 46.


Studies on yersiniosis in cultured *Mugil seheli* for the first time in Suez Governorate


دراسات عن مرض الفم الأحمر المعوى لأول مرة في أسماك السهلية المستزرعة

بمحافظة السويس

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الملخص العربي

أجريت هذه الدراسة على (360) سمكة من أسماك السهلية متوسط أوزانها (39,11 جرام) و متوسط أطوالها (15,17 سم) و المجمعة من المزارع الخاصة بمحافظة السويس في الفترة ما بين نوفمبر 2014 إلى أكتوبر 2015، خضعت العينات للفحص من حيث الفحص الإكلينيكي و الصرف التشريحي المصاغية والإصابات و الفحوص البكتيرية لعزل بكتيريا (يرسينا روكري) وإستخدام تقنية البلمرة المتسلسل في التشخيص.

أظهرت العلامات المرضية للأسماك المصابة وجود أنزفت مختلفة الأحجام و منتشرة على السطح الخارجي للسمكة ووجود أنزفة حول الفم و الانتفاخ السرجي و إنتفاخ البطن و تأثر القشر مع ظهور تآكل بدن الأسماك. تأثر كل لون الجسم، العوم، البطن، السباحة بالقرب من سطح الماء، أظهرت أن الزعانف و جروح العين، فقدان التوازن، والجسم بصورة عصبية.

تمثلت الصفة التشريحية للأسماك المصابة في وجود سائل استسقائي، نزف مع معظم الأحشاء، وانزفت الزعانف و حروف اللون و انزفات الخياشيم و الكبد، بعض الأسماك أظهرت فقدان الشهية، لون الجسم، العوم، السباحة بالقرب من سطح الماء، تآكل الزعانف و جروح العين، فقدان التوازن، و انزفات الزعانف و حروف اللون.

تمت تصنيف البكتيريا من خلال إستخدام صبغة جرام و زرع البكتيريا على البيئة البكتيرية الخاصة باليرسينيا، وكذلك تم دراسة الخصائص البيوكيميائية للعزلات. و قد سجلت الدراسة أن معدل إصابة أسماك السهلية ببكتيريا اليرسينيا روكري 68/٪، و تبين أن أعلى نسبة من اليرسينيا روكري تم عزلها من الخياشيم 41,7٪ يتبعها الكبد 29,55 ٪ ثم الكلي 25,1 ٪ و اخيرا الطحال 3,64 ٪. و تم تأكيد نتائج العزل و التصنيف للبكتيريا بإستخدام اختبار البلمرة المتسلسل لعزلات اليرسينيا روكري باستخدام برايمر خاص لليرسينيا روكري.

أوضحت نتائج العدوى المعملية لأسماك السهلية المصابة ببكتيريا اليرسينيا روكري انها تسبب التفوق في الأسماك خلال 7 يوم، و تتضمن العلامات المرضية في ظهور حركات دائرية، نزف في مرحلة خاصة، تأثر كل لون الجسم، الزعانف و جروح العين، فقدان التوازن، و انزفت الزعانف و حروف اللون و انزفت الزعانف و حروف اللون و انزفت الزعانف و حروف اللون و انزفت الزعانف و حروف اللون و انزفت الزعانف و حروف اللون.

تم إجراء اختبار الحساسية لعزلات اليرسينيا روكري للمضادات الحيوية، و أوضحت النتائج أنها حساسة للسيبروفلوكساسين و التراميتربريم و التوبراميسين و مقاومة للنوفوبيسين، أيرثروميسين، حامض النالدكسيك وأموكسيسلين.