Molecular identification of antibiotic resistance genes in *Yersinia ruckeri* isolated from diseases Nile tilapia, *Oreochromis niloticus*

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**ABSTRACT**

*Yersinia ruckeri* is the causative pathogen of a systemic disease called enteric red mouth disease. It can infect different fish species causing considerable economic losses in fish farms. This research was conducted to evaluate antimicrobial susceptibility of isolated *Yersinia ruckeri* (Y. ruckeri) from diseased Nile tilapia as well as molecular detection of some antibiotic resistance genes was carried out. In this study, a total of ninety-six Y. ruckeri isolates were recovered from 150 examined fish samples. Results of antibiotic sensitivity test revealed that isolates were sensitive to ciprofloxacin (63.5%), difloxacin (70.8%) and sulphamethoxazole-trimethoprim (55.2%), but isolates were moderately sensitive to enrofloxacin (52%), erythromycin (82.2%) and doxycycline (61.4%). Meanwhile, high resistance to oxytetracycline, gentamycin, amoxicillin and flumequine was observed. Antibiotic resistance genes were screened in 5 isolates by multiplex PCR and recorded resistance genes qnrA, blaTEM, aadA1 and ereA at 516, 516, 484, 420 bp respectively in all isolates. This observation revealed that Y. ruckeri isolates have multiple resistance to antibiotics and hence difficult control and treatment of disease. Consequently, there is a necessity to develop an innovative strategy for controlling *Yersiniosis* outbreaks in diseased farms.

**1. INTRODUCTION**

Fish are an immense source of protein, vitamins, minerals, fatty acids and other important micronutrients for a large percentage of the global population. Therefore, natural resources and aquaculture have reached incredibly high levels and this sector is predicted to provide more food in the future and will prove to be increasingly essential for sustaining a nourished populace (FAO, 2022). *Yersinia ruckeri* is the main cause of enteric red mouth infection (ERM), which causes an immense bacterial septicemia in salmonids. It is a Gram-negative, straight, facultative intracellular enterobacterium. All growth stages of the fish are vulnerable to disease, resulting in high mortality rates (Gujarro et al., 2018). Swollen spleen, inflammation of intestine, exophthalmia and hemorrhaging on the skin and mucous membranes in addition to darkening of skin demonstrate the symptomatic clinical signs (Kumar et al., 2015). In Egypt, Y. ruckeri was firstly isolated in Giza Province from the Nile River coming from seemingly whole and dropping-in-quality Nile tilapia (Hussein et al., 1997). Subsequently it has been detected from the Nile Delta region of healthy and disease common carp cyprinus carpio and African catfish Clarias gariepinus, at a prevalence rate (12.5%, 8% and 5%) respectively (Abd-El Latief, 2001). It was also identified from semi-intensive earthen ponds rearing tilapia at Sharkiya Province (Eissa et al., 2008), as well as from Nile tilapia private fish farms at Kafreshelikhe (Abdel-latief, et al., 2014). Also, Aly et al. (2021) recoded Y. ruckeri in Nile tilapia numerous farms in Alexandria. Y. ruckeri serves as a serious bacterial disease to aquaculture producers all over the world, as these infections can bring significant financial losses. Until now, disease control is based on medicinal treatments using antibiotics. A popular fish vaccine for ERM was prepared from inactivated formaldehyde whole cells of Y. ruckeri and was certified back in 1976 in USA as recorded by Gudding and Van Muiswinkel (2013). However, reports of outbreaks are being increasingly reported around the globe (Kumar et al., 2015; Wrobel et al., 2019). Furthermore, all researches exhibit how fish infections increase due to aquaculture, offering assistance in creating organizational practices to enhance security protocols and ultimately limit financial losses (Ormsby and Davies, 2021; Yang et al., 2021). Önalan and Çevik (2020) reported that phytochemicals indicate promising results for treatment rather than continuing to rely solely on antibiotics, as the threat of antibiotic resistance bacteria. The economic influence of Y. ruckeri on the fish farming sector in Egypt is extensive, so the current research aimed to isolate *Yersinia ruckeri* from diseased cultivated Nile tilapia, determining the antimicrobial susceptibility of gained isolates, then molecular analysis for antimicrobial resistance genes.

**2. MATERIAL AND METHODS**

2.1. Approval Ethics

All experimental methods were approved by the Benha University Animal Ethical Committee with ethical approval number (BUFVTM03-06-23) of the Faculty of Veterinary Medicine, Benha University.
2.2. Fish sampling
A total of 150 Nile tilapia weighing 100-250 g was collected from six farms (25 fish per farm) suffered from high mortalities at Kaf\-El-Shich governorate during wintertime in 2019. The diseased fish were transported in a sterile bag which placed inside an insulated container for immediate transfer to the lab for further examination following Abd El Tawab et al., (2022) procedures.

2.3. Clinical signs and postmortem examination
All fish were subjected for examination to record abnormal clinical signs and postmortem lesions according to El-Bably (2015).

2.4. Bacterial isolation and identification
Under a completely sterile environment in a laminar airflow chamber, sterile cotton swabs were separately used to collect samples from the liver, kidney, spleen, and heart. Each swab was added to a tube containing tryptic soya broth from Difco (USA), then incubated for 24 hours at a temperature of 28°C. Next, a loopful was extracted from each tube and cultured on ribose ornithine deoxycholate agar and xylose lysine deoxycholate (Oxoid). Then, the colonies that exhibited typical characteristics of Y. ruckeri were further cultured on sheep blood agar employing the protocols described by Tobbback et al. (2007) and Carson et al. (2019).

3.5. Antibiotic sensitivity test of Yersinia ruckeri isolates:
The antimicrobial activity of the examined bacterial isolates were evaluated with an agar disc diffusion method as described by Ali et al. (2018). Herein, the overnight broth cultures were adjusted to 0.5 McFarland measure and then spread onto the Mueller–Hinton agar plate. After 10 min, antibiotic discs were cautiously put on it followed by 24h incubation at 35 °C.

The susceptibility of Y. ruckeri was tested by utilizing antibiotic discs (Oxoid, England) for ciprofloxacin (CIP, 5 mg), doxycycline (DO, 30 mg), gentamicin (CN, 10 mg), fluomune (UB, 30 mg), enrofloxacin (ENR, 5 mg), sulphamethoxazole-trimethoprim (SXT, 23.7 + 1.25 mg), difloxacin (DIF, 10 milligram), oxytetracycline (OT, 30 milligram), amoxicillin (AMK, 10 milligram) in addition to erythromycin (E, 15 milligram). For assessing the diameter of the zones of inhibition with precision to 0.5mm, a graduated rule was used and it was measured twice at perpendicular angles.

3.6. Extraction of DNA
The genomic DNA was extracted using QIAamp DNA Mini Kit (Catalogue no.51304), following manufacture instructions. PCR assay was carried out at a 25 μl reaction volume consisting of 3μl of the extracted DNA, 12.5 μl of 2xMaster Mix (Intron, Korea), 1.25 μl of each forward and reverse primer, as well as 7 μl of nuclease-free water were combined for total PCR reactions.

The primers used in this molecular study are presented in Table (1). Furthermore, Table (2) represents the PCR reactions which were conducted taking into account Emerald Amp GT PCR Main Mix (Takara) kit Code No. RK310A as reported by Hassan et al. (2020)

PCR products were analyzed by electrophoresis on agarose gel (1.5 %). Briefly, a sterilized flask containing 1.5 g of electrophoresis grade agarose and 100 ml TBE buffer was placed in a microwave and heated to melt the agarose completely, then cooled down to 70 °C. Afterwards, 0.5 µg/ml ethidium bromide was added and mixed thoroughly. Then, the liquid agarose was put into a gel casting device with an appropriate comb at room temperature for it to solidify.

After that, the comb was carefully removed and, the electrophoresis apparatus was filled with TBE buffer. Fifteen microliters of each individual PCR product sample, both a negative and a positive control, were loaded into it. An electrical source of 1 to 5 volts per centimeter of the vessel size maintained the current. After around thirty minutes, it had stabilized. Using a gel imaging set up the image was captured and then put to analysis by computer software.

Table 1 Oligonucleotide primers sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence 5’-3’</th>
<th>Amplified product (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>blaTE</td>
<td>F: ATCACGCAATAAACCCAGC</td>
<td>516</td>
<td>Colom et al.,2003</td>
</tr>
<tr>
<td>M</td>
<td>R: CCCCCGAGAACGTITCTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>qnrA</td>
<td>F: ATTTCTCACGGCAGATTGG</td>
<td>516</td>
<td>Robiesek et al.,2006</td>
</tr>
<tr>
<td>R: GATCGGCAAAGGTAGGTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aadA1</td>
<td>F: TATCGAGGJGTTTGGCTCAT</td>
<td>484</td>
<td>Randall et al.,2004</td>
</tr>
<tr>
<td>R: GTCCATAGAGTITTTTGAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ereA</td>
<td>F: GCCGTTGCTCATAGACTTG</td>
<td>420</td>
<td>Nguyen et al.,2009</td>
</tr>
<tr>
<td>R: CGACTTATTGCGATCAGG</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2 (2) Cycling conditions of PCR amplification

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primary denaturation</th>
<th>2nd denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>Number of cycles</th>
<th>Final extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>blaTEM</td>
<td>95°C, 5 minutes</td>
<td>94°C, 30 seconds</td>
<td>54°C</td>
<td>72°C</td>
<td>35</td>
<td>72°C</td>
</tr>
<tr>
<td>qnrA</td>
<td>95°C, 5 minutes</td>
<td>94°C, 30 seconds</td>
<td>40 seconds</td>
<td>45 seconds</td>
<td>35</td>
<td>10 minutes</td>
</tr>
<tr>
<td>aadA1</td>
<td>95°C, 5 minutes</td>
<td>94°C, 30 seconds</td>
<td>40 seconds</td>
<td>45 seconds</td>
<td>35</td>
<td>10 minutes</td>
</tr>
<tr>
<td>ereA</td>
<td>95°C, 5 minutes</td>
<td>94°C, 30 seconds</td>
<td>60°C</td>
<td>72°C</td>
<td>35</td>
<td>10 minutes</td>
</tr>
</tbody>
</table>
3. RESULTS

3.1. Clinical and postmortem investigation
The examined diseased fish showed darkening of skin color and corneal opacity, obvious congestion to widespread hemorrhages alongside the dorsal musculature, whole fins, erythematous appearance of oral cavity with bilateral exophthalmic eye. Internally these fishes showed petechial hemorrhages on liver, spleen, heart, kidneys, intestine, and subcutaneous and underlying musculature.

3.2. Bacterial isolation
Ninety-six Y. ruckeri specimens cultured on CIN agar exhibited moderate-sized colonies with deep pink to red centers and light pink edges, following 48 hours of incubation at a temperature of 28°C.

3.3. Antibiotic sensitivity test
The results of Y. ruckeri sensitivity to different antibiotics are given in Table (3). Y. ruckeri isolates were significantly sensitive to ciprofloxacin (63.5%), diflucoxacine (70.8%) and combination of sulphamethoxazole-trimethoprim (55.2%). Meanwhile, moderately responsive to enrofloxacin (52%), erythromycin (82.2%) and doxycycline (61.4%) was recorded. Resistance to other antibiotics including gentamycin, flumequine, oxytetracycline and amoxicillin were observed.

Table (3) In-vitro antimicrobial sensitivity for Yersinia ruckeri isolates

<table>
<thead>
<tr>
<th>Antimicrobial disc</th>
<th>Disc concentrations</th>
<th>Sensitive No. %</th>
<th>Intermediate No. %</th>
<th>Resistant No. %</th>
<th>Inhibition zone</th>
</tr>
</thead>
<tbody>
<tr>
<td>ciprofloxacin</td>
<td>5 µg</td>
<td>61 63.5</td>
<td>35 36.4</td>
<td>0 0.0</td>
<td>2.4 S</td>
</tr>
<tr>
<td>doxycycline</td>
<td>30 µg</td>
<td>13 13.5</td>
<td>59 61.4</td>
<td>24 25</td>
<td>1.4 I</td>
</tr>
<tr>
<td>enrofloxacin</td>
<td>5 µg</td>
<td>38 39.5</td>
<td>50 52</td>
<td>8 8.3</td>
<td>1.4 I</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>30 µg</td>
<td>0 0.0</td>
<td>17 17.7</td>
<td>79 82.2</td>
<td>- R</td>
</tr>
<tr>
<td>sulphamethoxazole-trimethoprim</td>
<td>25µg</td>
<td>53 55.2</td>
<td>43 44.79</td>
<td>0 0.0</td>
<td>2.4 S</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>10 µg</td>
<td>0 0.0</td>
<td>15 15.625</td>
<td>81 84.3</td>
<td>- R</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>15 µg</td>
<td>11 11.4</td>
<td>79 82.2</td>
<td>6 6.25</td>
<td>1.5 I</td>
</tr>
<tr>
<td>Diflucoxacine</td>
<td>10µg</td>
<td>68 70.8</td>
<td>28 29.1</td>
<td>0 0.0</td>
<td>2.4 S</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>10µg</td>
<td>0 0.0</td>
<td>24 25</td>
<td>72 75</td>
<td>- R</td>
</tr>
<tr>
<td>Flumequine</td>
<td>30 µg</td>
<td>0 0.0</td>
<td>32 33.3</td>
<td>64 66.6</td>
<td>- R</td>
</tr>
</tbody>
</table>

AA: Antibogram activity  No.: Number of specimens  %: proportion compared to the whole number of specimens (96)

3.4. Antibiotic resistance genes
Four antibiotic resistance genes qnrA, blaTEM, aadA1 and ereA were identified by multiplex PCR at 516, 516, 484 and 420 bp respectively in all 5 tested Yersinia ruckeri isolates (Fig 1 and 2).

**Figure 1:** Displays the results of blaTEM and aadA1 resistance genes PCR amplification on 1.5% agarose gel. Lane L: 100-1000 base pairs of DNA Ladder. Lane N was designated as the Negative Control Resistant Yersinia ruckeri strain. Lane P served as a Positive Control coming from a CLQP poultry production lab in Egypt. Lane 1-5 positive samples showed bands of blaTEM resistance gene at 516bp and aadA1 resistance gene at 484bp.

**Figure 2:** Displays the results of ereA and qnrA resistance genes PCR amplification on 1.5% agarose gel from samples in Lanes 1-5. Lane L contained a 100-1000 bp DNA Ladder. Lane N was designated as the Negative Control Resistant Yersinia ruckeri strain. Lane P served as a Positive Control coming from a CLQP poultry production lab in Egypt. Lane 1-5 positive samples showed bands of ereA resistance gene at 420 bp and qnrA resistance gene at 516 bp.
4-DISCUSSION
Massive aquaculture production has been linked to increased levels of infectious disease outbreaks, such as Yersinia ruckeri bacterial infections, which lead to extensive economic losses, as highlighted by (Gopheren 2017; Ormsby and Davies 2021). A study was conducted to analyze how prevalent Yersinia ruckeri infections are in Trachinotus marginatus (pompano), with the purpose to observe and note any symptoms that would be linked to bacteria, including exophthalma, darkening of skin and bleeding under the epidermis around the jawline and snout (Roman et al., 2012). In the present work, the Y. ruckeri isolates demonstrated markedly sensitivity to ciprofloxacin (63.5%), diflucloxacin (70.8%) and sulphonamethoxazole-trimethoprim combination (55.2%). However, moderately sensitive to enrofloxacin (52%), erythromycin (82.2%) and doxycycline (61.4%) was observed. Less sensitive to oxytetracycline, gentamycin and amoxicillin were recorded. These results agree with El-Bably (2015) who recorded that Y. ruckeri was markedly sensitive to ciprofloxacin in addition to sulphonamethoxazole-trimethoprim mixture, moderately sensitive to enrofloxacin, erythromycin and doxycycline and less sensitive to Gentamycin, Fluomoxine, Oxytetracycline and Amoxicillin.

On the other hand, Aly et al. (2021) explained that the antimicrobial susceptibility of Y. ruckeri strains showed a higher resistance pattern for all antibiotics (Oxytetracycline, Sulphonamethoxazole/Trimethoprim, Ampicillin, Chloramphenicol and Erythromycin) except for Difloxacine. In addition, Khafagy et al. (2023) recorded that, multi-drug resistance genes (blaTEM, qnrS, tetAgene) in all Y. ruckeri isolates. While, Altun et al. (2013) declared that the Y. ruckeri isolated from rainbow trout, Oncorhynchus mykiss were resistant to fleroxinicol, erythromycin, oxytetracycline and trimethoprim-sulphonamethoxazole, whereas Bastardo et al. (2011) discovered in the drug susceptibility tests that all 11 strains of Yersinia ruckeri isolated from Atlantic salmon Salmo salar L. farmed in Chile had strong susceptibility to sulphonamethoxazole-trimethoprim, oxytetracycline, ampicillin and enrofloxacin. In contrast, according to Duman et al. (2017), over 80% of Y. ruckeri strains were sensitive to fleroxinicol (FFC), sulphonamethoxazole-trimethoprim (SXT), and tetracycline. Also, Abdel-Latif et al. (2014) recorded that ciprofloxacin or a combination of sulphonamethoxazole-trimethoprim may be the most effective antibiotics for controlling Yersinia ruckeri in Nile tilapia. It is quite probable that variations in the geographical distribution, fish type or protocols used can account for the disparities. The performed antibiotic susceptibility test on the Y. ruckeri isolates revealed that they were mostly not vulnerable to amoxicillin and oxytetracycline, which suggests the presence of beta-lactamase and tetracycline antibiotic resistance. This concurs with earlier studies conducted by Grandis and Stevenson (1985) and Onalan and Çevik (2020). In contrast, Alderman and Hastings. (1998) and Michel et al. (2003) have revealed that just a limited range of compounds are generally employed in combination with trimethoprim and lately, fleroxinicol: these include amoxicillin; oxolonic acid; oxytetracycline; and sulphadiazine.

Antibiotic blaTEM resistance gene in Y. ruckeri strains in the current study revealed that it was successfully amplified in all 5 tested isolates, similarly to prior research by Feng et al. (2022), who declared that every one of their 16 isolates could ascertain the gene with 81.25 % accuracy. On contrary, Balta et al. (2010), recorded that blaTEM gene did not express among Yersinia ruckeri strains isolated from rainbow trout. The antibiotic qnrA, adaA1 and ereA resistance genes were observed in all 5 examined Y. ruckeri isolates. This result differed from Shah et al. (2012) who stated that expression of qnrA gene did not exhibit among Yersinia ruckeri strains isolated from Atlantic salmon (Salmo salar L.).

5. CONCLUSIONS
This study concluded that Quinolone resistance gene (qnrA), Beta lactamases resistance gene (blaTEM), Streptomyacin resistance gene (aadA1), in addition, macrolides resistance gene (ereA), were detected in all of the screened Y. ruckeri isolates. The sensitivity examination showed that the studied Y. ruckeri samplings were resistant to multiple antibiotics. To impede the evolution of antibiotic-resistant bacterial infections, it is essential to limit the improper use of antibiotics in veterinary medicine.

6. REFERENCES


34. Wrobel, A., Leo, J.C., and Linke, D., 2019, Overcoming fish defences; the virulence factors of *Yersinia ruckeri*. *Genes (Basel)*, 11(9), 700.