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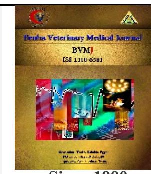

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*Original Paper*

# 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%), difloxacine (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 *qnr*A, *bla*TEM, *aad*A1 and *ere*A 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 (Guijarro 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 Kafrelsheikh (Abdel–latif, 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

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*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 Kafr-Elsheikh governate 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 28ºC. Next, a loopful was extracted from each tube and cultured on ribose ornithine desoxycholate 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 Tobback *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), gentamycin (CN, 10 mg), flumequine (UB, 30 mg), enrofloxacin (ENR, 5 mg), sulphamethoxazole-trimethoprim (SXT, 23.7 + 1.25 mg), difloxacine (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 QIA amp 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. RR310A 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 $^0$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

| Gene | | Primer Sequence 5'-3' | Amplified product (bp) | Reference |
|---|---|---|---|---|
| blaTEM | F: | ATCAGCAATAAACCAGC | 516 | Colom et al., 2003 |
| | R: | CCCCGAAGAACGTTTTC | | |
| qnrA | F: | ATTTCTCACGCCAGGATTTG | 516 | Robicsek et al., 2006 |
| | R: | GATCGGCAAAGGTTAGGTCA | | |
| aadA1 | F: | TATCAGAGGTAGTTGGCGTCAT | 484 | Randall et al., 2004 |
| | R: | GTTCCATAGCGTTAAGGTTTCATT | | |
| ereA | F: | GCCGGTGCTCATGAACTTGAG | 420 | Nguyen et al., 2009 |
| | R: | CGACTCTATTCGATCAGAGGC | | |

Table (2) Cycling conditions of PCR amplification

| Gene | Primary denaturation | 2nd denaturation | Annealing | Extension | Number of cycles | Final extension |
|---|---|---|---|---|---|---|
| *bla*TEM | 95˚C 5 minutes | 94˚C 30 seconds | 54˚C 40 seconds | 72˚C 45 seconds | 35 | 72˚C 10 minutes |
| *qnr*A | 95˚C 5 minutes | 94˚C 30 seconds | 55˚C 40 seconds | 72˚C 45 seconds | 35 | 72˚C 10 minutes |
| *aad*A1 | 95˚C 5 minutes | 94˚C 30 seconds | 54˚C 40 seconds | 72˚C 45 seconds | 35 | 72˚C 10 minutes |
| *ere*A | 95˚C 5 minutes | 94˚C 30 seconds | 60˚C 40 seconds | 72˚C 45 seconds | 35 | 72˚C 10 minutes |

## 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, erythematic 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 28ºC.

### *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 towards ciprofloxacin (63.5%), difloxacine (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

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

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

### *3.4. Antibiotic resistance genes*

Four antibiotic resistance genes *qnr*A, *bla*TEM, *aad*A1 and *ere*A were identified by multiplex PCR at 516, 516, 484 and 420 bp respectively in all 5 tested *Yersinia ruckeri* isolates (Fig 1 and 2).

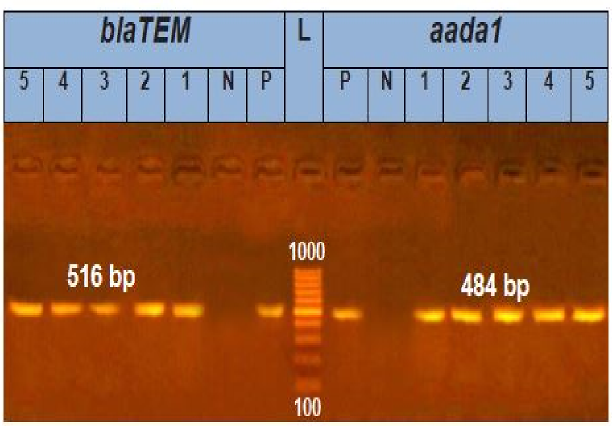


Fig (1). Displays the results of *bla*TEM and *aad*A1 resistance genes PCR amplification on 1.5% agarose gel. Lane L: 100-1000 base pairs of DNA Ladder. Lane N was designated as the Negative Control Resistant *Yersinia ruckeri* strain. Lane P served as a Positive Control coming from a CLQP poultry production lab in Egypt. Lane 1-5 positive samples showed bands of *bla*TEM resistance gene at 516bp and aadA1 resistance gene at 484bp.

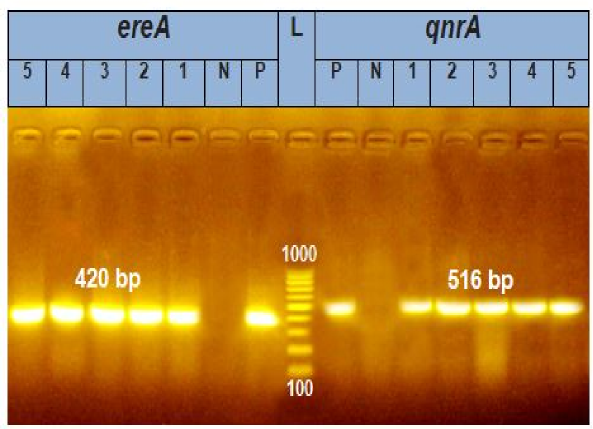


Fig (2). Displays the results of *ere*A and *qnr*A resistance genes PCR amplification on 1.5% agarose gel from samples in Lanes L-5. Lane L contained a 100-1000 bp DNA Ladder. Lane N was designated as the Negative Control Resistant *Yersinia ruckeri* strain. Lane P served as a Positive Control coming from a CLQP poultry production lab in Egypt. Lane 1-5 positive samples showed bands of *ere*A resistance gene at 420 bp and *qnr*A 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 (Gophen 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 exophthalmia, 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%), difloxacine (70.8%) and sulphamethoxazole-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 sulphamethoxazole-trimethoprim mixture, moderately sensitive to enrofloxacin, erythromycin and doxycycline and less sensitive to Gentamycin, Flumequine, 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, Sulphamethoxazole/Trimethoprim, Ampicillin, Chloramphenicol and Erythromycin) except for Difloxacine. In addition, Khafagy et al. (2023) recorded that, multi-drug resistance genes (*bla*TEM*, qnr*S*, tet*Agene) in all *Y. ruckeri* isolates. While, Altun et al. (2013) declared that the *Y. ruckeri* isolated from rainbow trout, *Oncorhynchus mykiss* were resistant to florfenicol, erythromycin, oxytetracycline and trimethoprim-sulphamethoxazole, 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 sulphamethoxazole-trimethroprim, oxytetracycline, ampicillin and enrofloxacin. In contrast, according to Duman *et al.* (2017), over 80% of *Y. ruckeri* strains were sensitive to florfenicol (FFC), sulfamethoxazole-trimethoprim (SXT), and tetracycline. Also, Abdel-Latif et al. (2014) recorded that ciprofloxacin or a combination of sulphamethoxazole-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 Önalan 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, florfenicol: these include amoxicillin; oxolonic acid; oxytetracycline; and sulphadiazine.

Antibiotic *bla*TEM 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 *bla*TEM gene did not express among *Yersinia ruckeri* strains isolated from rainbow trout. The antibiotic *qnr*A, *aad*A1 and *ere*A resistance genes were observed in all 5 examined *Y. ruckeri* isolates. This result differed from Shah et al. (2012) who stated that expression of *qnr*A gene did not exhibit among *Yersinia ruckeri* strains isolated from Atlantic salmon (Salmo salar L.).

## 5. CONCLUSIONS

This study concluded that Quinolone resistance gene (*qnr*A), Beta lactamases resistance gene (*bla*TEM), Streptomycin resistance gene (*aad*A1), in addition, macrolides resistance gene (*ere*A), 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.

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