Molecular Characterization and Multidrug Resistance of Yersinia Species in Fish

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Abstract:

A total of, (100) samples of *O. Niloticus* and (100) samples of *C. Gariepinus* were gathered from multiple areas at Dakahlia governorate from April 2019 to April 20. Fish samples were subjected to clinical and microbiological examination from kidney, liver and spleen. Isolates were characterized by cultural characters, some biochemical tests, Api 20 system and PCR. 24 diseased fish were characterized as infected with *Y. ruckeri* [10 from 100] *O. niloticus* (10%) and 14 from *C. Gariepinus* (14%)] with 44 *Yersinia ruckeri* isolates with percentage of 22%. 16srRNA gene (specific common gene) was demonstrated in all *Y.ruckeri* isolates by PCR in addition to detection of virulence genes (*yrp*,*yrIlm*,*yhlA*,*yhlB*,*yrInv*) in them by PCR assay and multi-drug resistance genes (*blaTEM*,*qnrS*, *tetA*gene). The study results showed that PCR is rapid and reliable method for *Y. ruckeri* isolates identification which will be useful in prevention and control of Yersiniosis.

Keywords: *Yersinia ruckeri, Oreochromis niloticus, Clarias gariepinus*, MDR and PCR.

Introduction

Infectious diseases of fish continue to cause major losses on aquaculture production ***(Stentiford G. et al., 2017).***

Yersiniosis is an infectious disease which leads to gross mortalities and large economic losses in freshwater fish farms. Heavy mortalities were found in *Oreochromis niloticus*, *Clarias gariepinus* at Dakahlia governorate. A total number of 200 freshly dead fish were sampled and transferred as soon as possible to the lab for clinical examination and bacteriological identification. The examined fish showed the septicemic signs of yersiniosis

which appeared as generalized erythema, petechial hemorrhages on the skin and characteristic feature of red mouth and lips. Postmortem examination revealed hemorrhagic gills, engorged gall bladder and GIT.

After bacterial isolation from spleen, kidney, liver and heart on specific culture media, sure diagnosis was achieved by conventional biochemical tests, serotyping and PCR. Antimicrobial tests were conducted on bacterial isolates in order to find the most suitable antibiotic for controlling this bacterial infection. From our study, it was found that the recommended antibiotic for controlling this bacterial infection is ciprofloxacin or sulphamethoxazole-trimethoprim combination.

In this study *Yersinia ruckeri* is a very common pathogen affecting fish; it causes one of the worst septicemic diseases responsible for gross mortality in freshwater fish leading to gross economic losses. This study was performed to asess *Y.R*. prevalence among *O. Niloticus* and *C. Gariepinus* at dakahlia governorate and to assess phenotypic and genotypic characterization of the isolates.

Yersinia ruckeri belongs to Yersiniaceae family. It is a Gram negative, facultatively anaerobic, non-spore-forming, rod-shaped bacterium ***(Adeolu M. et al., 2016).***

Yersinia ruckeri can be easily cultivated from internal tissues of diseased fish on media such as Nutrient Agar, Tryptic Soy Agar, McConkey Agar, and Brain Heart Infusion Agar **(*Wortberg F. et al., 2012 and Wrobel A. et al., 2019).***

Yersiniosis (ERM) is one of the most common diseases which affect salmonids and causes huge economic losses. It was isolated primarily from rainbow trout (*Oncorhynchus mykiss*) in Idaho, USA and is currently found throughout North and South America, the Middle East and China, Australia, Europe and South Africa. ***(Kumar G. et al., 2015).***

Aim of the work

This study was conducted to Study molecular characterization and multidrug resisting genes of Yersinia isolated from Fish.

Material and Methods:

Fish samples:

Atotal of (200) fresh water fish (100) Oreochromis niloticus and (100) *Clarias gariepinus* were collected from different fish farms in Dakahillia government , apparentely healthy;(20) *O.niloticus* and (30) *C. gariepinus*, diseased;(30) O.niloticus and (20) *C. gariepinus* and freshly dead;(50) *O.niloticus* and (50) *C. gariepinus* through the different seasons of the year. Fish were examined to determine the clinical abnormalities indicating bacterial fish diseases.

Clinical examination and Postmortem examination

Examination of internal organs was done as described by ***Schaperclaus et al., (1992).***

Isolation of *Yersinia ruckeri*:

A loopful from tryptic soy broth was streaked on to Tryptic soy agar, nutrient agar and MacConkey's agar then was incubated at 25-30°C for 24 h. After 1-2 days of incubation, the bacteria were identified.

Bacteriological identification:

According to ***(Bergey's Manual of Systematic Bacteriology, 2005)***

Biochemical characteristics:

The following methods of biochemical tests used for identification of microorganism were carried out according to the schemes described by ***(Austin&Austin., 2007).***

Identification by using the analytical profile index of API20E system *(Buller, 2004)*

Antimicrobial sensitivity test of *Yersinia ruckeri* : according to *(Finegold and martin.1982).*

Methods of PCR

1.Extraction of DNA

2.Preparation of Master Mix (table 1) Agarose gel electrophoreses ***(Sambrooket al., 1989)***

Table (1) *showing preparation of Master Mix*

Component	Volume/reaction
Emerald Amp GT PCR mastermix (2x premix)	12.5 μl
PCR grade water	5.5 μl
Forward primer(20 pmol)	1 μl
Reverse primer (20 pmol)	1 μl
Template DNA	5 μl
Total	25 μl

Results

1. Bacteriological Examination of Yersinia ruckeri

Yersinia ruckeri is a Gram-negative, rod-shaped, non-capsulated, non-spore-forming and most often flagellated bacterium. It can be isolated from the internal organs of infected fish, and can be cultured on various bacteriological media such as Nutrient Agar, Tryptic Soy Agar, Columbia Blood Agar, Brain Heart Infusion Agar, and McConkey Agar. After 1-2 days of incubation, the bacterium formed circular, smooth and shiny colonies. The cells grew rapidly in a wide temperature range from 22 ◦C to 37 ◦C.

2. Biochemical Characters of Y.ruckeri :

All *Y. ruckeri* isolates were oxidase negative and biochemically homogenous. *Y.ruckeri* were positive for Voges–Proskauer, Methyl Red, Catalase ,citrate ,maltose fermentation , glucose fermentation, gelatinase, ornithine decarboxylase, arginine dehydrolase and reduce nitrate to nitrite but negative for lactose fermentation ,sucrose , urease, indol, H2s production ,inositol fermentation,sorbitol fermentation,lysine decarboxylase and tryptophan deaminase.

3. Confirmatory biochemical identification of the isolates by using API 20E:

The isolates were identified by using the numerical profile supplied in the API20 E system.

4. Prevalence of fish infected with *Y. ruckeri* in both *O.niloticus* and *C.gariepinus*:

The total prevalence of Yersinia ruckeri isolated from *O.niloticus* was 10% while its prevalence in *C.gariepinus* was 14%, the total prevalence of Yersinia ruckeri in both *O.niloticus* and *C.gariepinus* were 12% as shown in table 2 and figure 2.

5. Seasonal Variation of fish infected with Y. ruckeri.

Y. ruckeri were isolated from fish during spring, summer, autumn and in winter. The summer season showed the highest prevalence with 45.8% then the spring season with 25%, autumn 16.7% finally winter 12.5% as shown in table 3 and figure 3.

6. Distribution of *Y. ruckeri* in internal organs of examined *O.niloticus* tilapia fish:

The highest distribution of *Y.ruckeri* was in liver (55.6%) then kidney (38.9%) then spleen (5.5%) as shown in table 4 and figure 4.

7. Distribution of *Y. ruckeri* in internal organs of examined *C.gariepinus*:

The highest distribution of *Y.ruckeri* was in liver (53.8%) then in kidney (34.6) and spleen (11.6%) as shown in table 5 and figure 5.

8. Distribution of *Y. ruckeri* in apparent healthy, diseased and freshly dead fish.

Number of isolated Y.R. from *O.niloticus* and *C.gariepinus* varied according to healthy status of the examined fish as shown in table 6.

9. Antimicrobial sensitivity results:

Y. ruckeri isolates vary in their antimicrobial sensitivity pattern to sixteen (16) antimicrobial discs used in present study showed that most of isolates were sensitive to ciprofloxacin, florfenicol, gentamycin, colistin sulphate, neomycin, trimethoprim-sulphamethaxazol, nalidixic acid, conversely most of isolates were resistant to erythromycin, cefotaxime, tetracyclin, amoxicillin, ampicillin, streptomycin, lincomycin, enrofloxacin and norfloxacin as shown in table 7 and figure 6.

11. Molecular characterization of *Y. ruckeri* isolates

Agarose gel electrophoresis was made for the products of PCR and then the amplified DNA was observed. Isolates were positive as *Y.R.* contains virulance genes and multidrug resistance genes.

11.1. Detection of (16SrRNA) gene in *Y.ruckeri* isolates:

All *Y.ruckeri* isolates showed positive amplification of 575 bp fragment specific for16S rRNA gene (common gene) with a total percentage of 100% as shown in **Figure (7)**.

11.2. Results of the PCR for amplification of virulence genes in *Y.ruckeri* isolates.

The *yrp* gene as shown in table(8), Figure (8),was not identified by PCR in 2 isolates (0%).the *yrInv* gene, was identified in 2 out of 2 isolates (100%) as shown in table(8), Figure (8).

11.2.1. Detection of (*yrp*) gene and (*yrInv*) gene in *Y.ruckeri* isolates:

The results observed in Figure (8) revealed that the *yrInv* gene was detected in Y.ruckeri isolates with percentage of 100% and the *yrp* gene wasn't detected in Y. isolates.

11.2.2. Detection of (*yhlB*) gene, (*yhlA*) gene and (*yrIlm*) gene in *Y.ruckeri* isolates:

The results observed in Figure (9) revealed that the *yhlB* gene was detected in Y.R. isolates with percentage of 100%, the *yhlA* gene was detected with percentage of 100% and the *yrIlm* gene was detected with percentage of 100%.

11.3. Results of the PCR for amplification of (Multidrug resistance gene) in *Y.ruckeri* isolates.

The *blaTEM* gene was identified in 2 out of 2 isolates (100%) by PCR, *tetA* gene was identified in 2 out of 2 isolates (100%). *qnrS* gene was identified in 2 out of 2 isolates (100%) as shown on table(9).

11.3.1. Detection of (*bla TEM*) gene in *Y.ruckeri* isolates:

The results observed in Figure (10) revealed that the *blaTEM* gene was detected in *Y.ruckeri* isolates with a percentage of 100%.

11.3.2. Detection of *tetA (A)* gene and (*qnrS*) gene in *Y.ruckeri* isolates:

The results observed in Figure (11) revealed that the *tetA(A)* gene was detected in *Y.ruckeri* isolates with a percentage of 100%, and the *qnrS* gene was detected in *Y.ruckeri* isolates with a percentage of 100%.

Table 2: *Prevalence of Yersinia from O.niloticus & african cat fish.*

Species of examined fish	No. of examined fish	No.of Infected fish	% of infected fish
O.niloticus	100	10	10
C.gariepinus	100	14	14
Total	200	24	12

Table3: *Seasonal Variation of fish infected with Y. ruckeri.*

NO of isolates	Summer		Spring		Autumn		Winter	
	NO	%	NO	%	NO	%	NO	%
24	11	45.8%	6	25%	4	16.7%	3	12.5

Table4: *Frequency distribution of Y. ruckeri recovered from internal organs of O.niloticus*

Y.ruckeri* in *O.niloticus	**Total no. of isolates**	**Number and distribution of isolates in internal organs**					
	No	**Liver**		**Kidney**		**Spleen**	
		No	%	No	%	No	%
	18	10	55.6	7	38.9	1	5.5

Table 5. *Frequency distribution of Y. ruckeri recovered from internal organs of c.gariepinus:*

***Y.ruckeri* in *C.gariepinus*:**	**Total no. of isolates**	**Number and distribution of isolates in internal organs**					
	No	Liver		Kidney		Spleen	
		No	%	No	%	No	%
	26	14	53.85	9	34.61	3	11.54

Table 6: *Isolation rate of Y. ruckeri in accordance to fish status*

	Apparently				**Diseased**				**moribund**				**Total**	
Species of	***O.niloticus***		***C.gariepinus***		***O.niloticus***		***C.gariepinus***		***O.niloticus***		***C.gariepinus***			
examined	**N**	**%**	**No**	**%**	**N**	**%**	**No**	**%**	**N**	**%**	**N**	**%**	**N**	**%**
	20	**10**	**30**	**15**	**30**	**15**	**20**	**10**	**50**	**40**	**5**	**40**	**2**	**1**
isolated	**N**	**%**	**No**	**%**	**N**	**%**	**No**	**%**	**N**	**%**	**N**	**%**	**N**	**%**
	2	**1**	**8**	**4**	**8**	**4**	**10**	**5**	**8**	**4**	**8**	**4**	**4**	**2**

Table 7: *Antimicrobial sensitivity of recovered Y. ruckeri strains*

Antibiotic classes	Specific tested antibiotic	Interpretation					
		Sensitiv		Intermediat		Resistanc	
		N	%	N	%	N	%
Penicillins	Amoxicillin Ampicillin	0 0	0 0	4 0	9.1	4 44	93.3 100
Cephalosporin	Cefotaxime	2	4.5	4	9.1	38	86.4
Macrolides	Erythromycin	4	9.1	2		38	86.4
Aminoglycosides	Gentamycin	4 0 25	93. 3 6.8	2 6 8	4.5 13.6 18.1	2 35 11	4.5 79.6 25
Fluoroquinolones	Ciprofloxacin	24 8 18	54. 5 18	3 4 2	6.8 9.1 45	17 32 24	38.6 72.7 54.2
Polymyxins	Colistin sulfate	40	90. 9	2	4.54	2	4.55
Tetracycline	Tetracycline	0	0	4	9.1	40	90.9
Sulfonamides	Trimethoprim-Sulfamethoxazo	0	0	4	9.1	40	90.9
Lincosamides	Lincomycin	4	9.1	11	25	29	65.9
Phenicols	Florfenicol	32	63. _	8	18.1	8	18.1

Table (8): *Incidence of virulence genes in Y.ruckeri isolates.*

Virulence gene	No. of positive isolates	Incidence%
yrp	0	0
yrInv	2	100
yrIlm	2	100
yhlA	2	100
yhlB	2	100

Table 9: *incidence of MDR in Y.ruckeri isolates.*

MDR gene	No. of positive isolates	Incidence%
blaTEM	2	100
tetA	2	100
qnrS	2	100

Figure (1): Biochemical identification of the isolates by using API 20E.

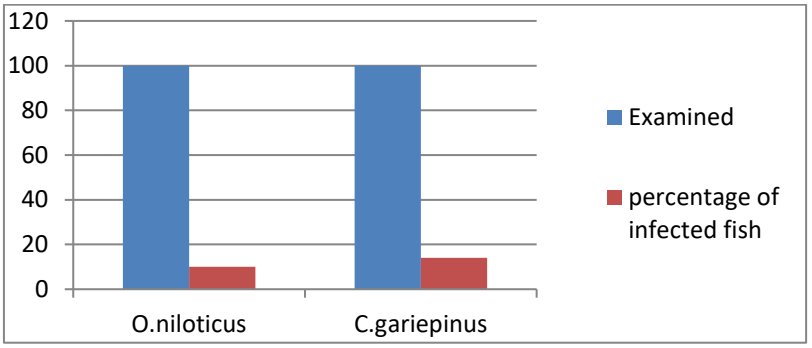

Figure 2. Prevalence of infection from *O.niloticus* and *C.gariepinus*.

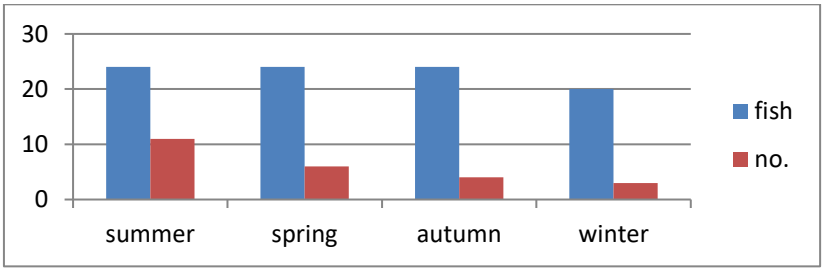

Figure 3. Seasonal Variation of infected fish with. *Y.ruckeri*

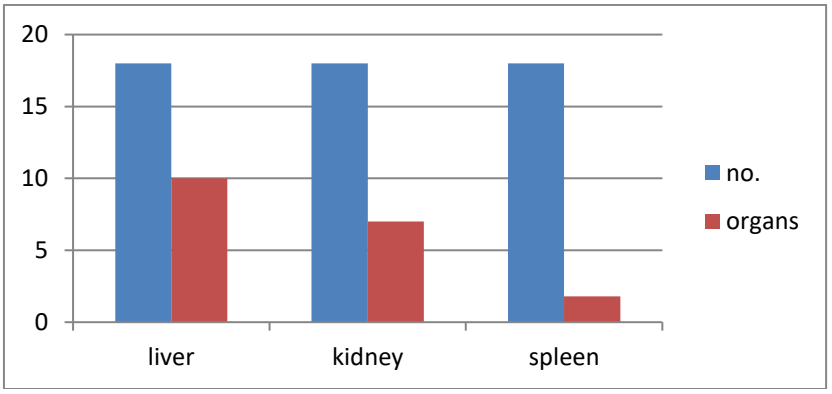

Figure 4. Frequency distribution of *Y.ruckeri* recovered from internal organs of *O.niloticus*

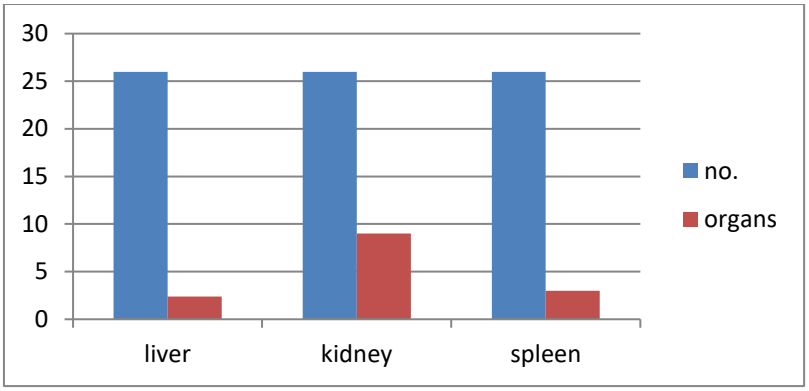

Figure 5. Frequency distribution of *Y.ruckeri* recovered from internal organs of *C.gariepinus*

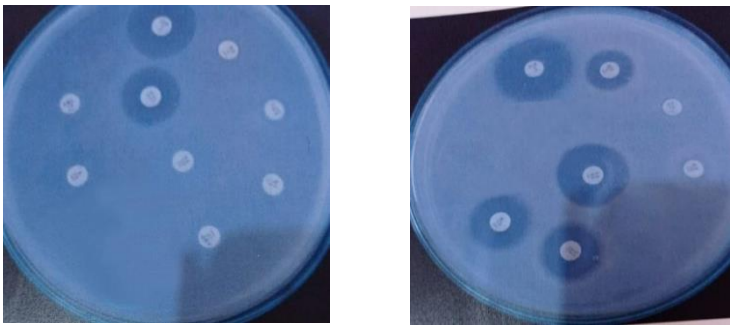

Figure 6. Antimicrobial discs were gently placed on the surface of the Muller Hinton agar.

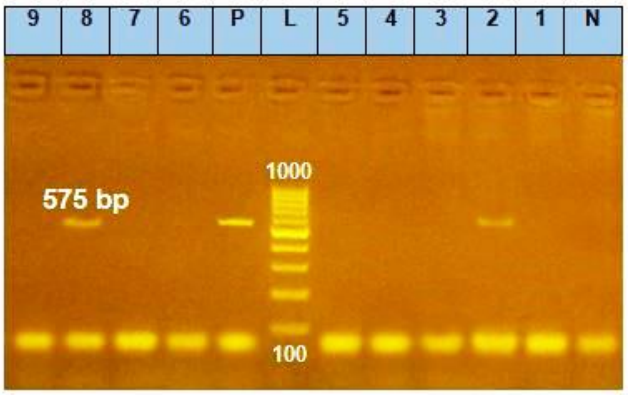

Figure 7. Agarose gel electrophoresis showing specific PCR of *Y.ruckeri* isolates using primer set for *16SrRNA* (575bp).

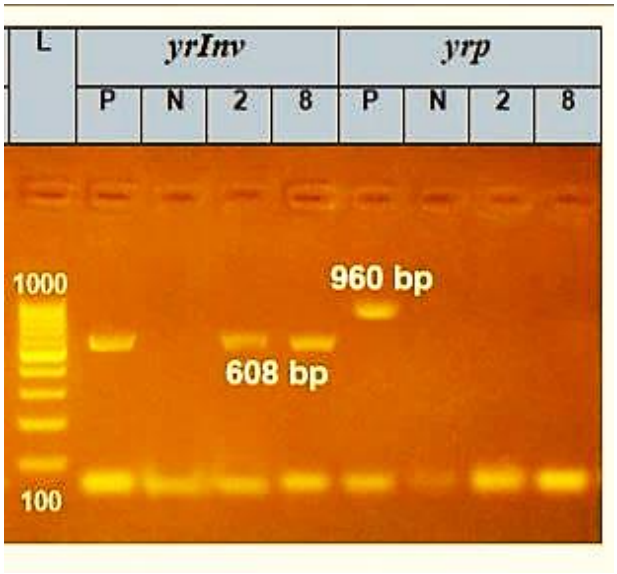

Figure 8: Agarose gel electrophoresis non showing virulence gene of *Y.ruckeri* isolates using primer set for *yrp* gene (960bp) and *yrInv* gene was shown at (608bp)

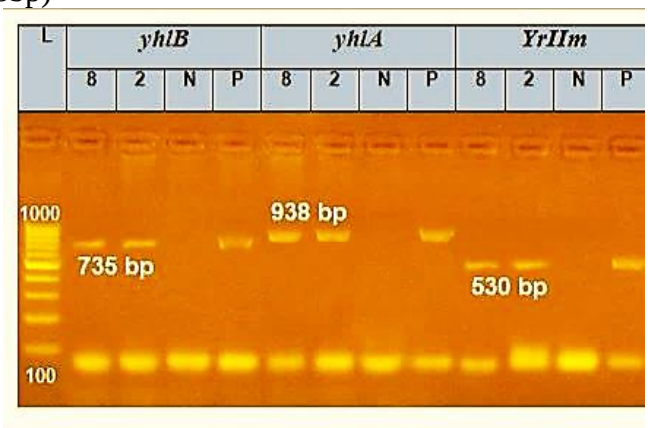

Figure 9 Agarose gel electrophoresis showing virulence gene of *Y.ruckeri* isolates using primer set for *yhlB* gene (735bp), *yhlA* gene was shown at (938bp) and *yrIlm* gene was shown at (530bp).

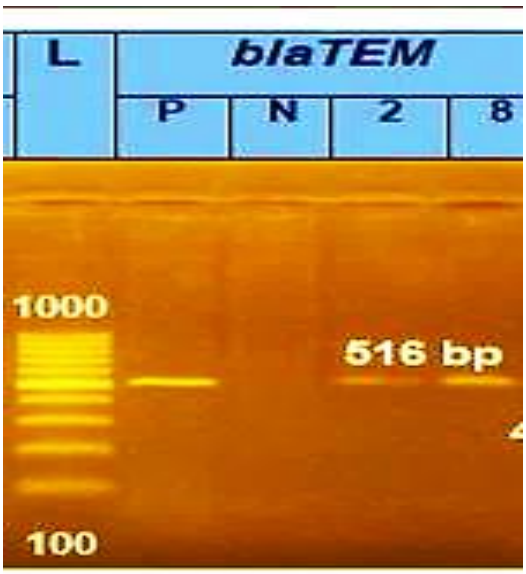

Figure 10. Agarose gel electrophoresis non showing MDR gene of *Y.ruckeri* isolates using primer set for *ermB* gene (425) and *blaTEM* gene was shown at (516bp).

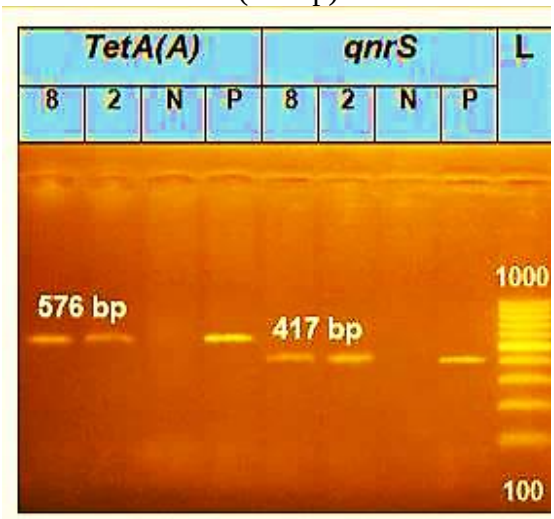

Figure 11 Agarose gel electrophoresis showing MDR gene of *Y.ruckeri* isolates using primer set for *tetA (A)* gene (576bp) and *qnrS* gene was shown at (417bp).

Discussion

Examination of *O. niloticus* infected with *Y. ruckeri* showed presence of extensive bleeding on the skin, congestion of fins, snout region, lips, oral cavity and erosions in oral tissues. Internally, there was severe hemorrhagic gastroenteritis. These clinical findings were similar to those reported by ***Abd El-Latief et***

al. (2001), El Gamal et al. (2005) and Eissa et al. (2008).

The characteristic red mouth feature of ERM wasn't seen in all the affected *C. gariepinus* and this finding was similar to that reported by ***Horne and Barnes (1999).***

In this study, *Y. ruckeri* colonies on TSA have small round, white and creamy features and this result was in line with the results of ***Ross et al. (1966), Seker et al. (2011) and Seker et al. (2012)***. Microscopically, the isolates were G-ve short motile bacilli and coccibacilli. These findings were in line with the results reported by ***Abd El-Latif et al. (2001)*** and to some extent not in line with the results obtained by ***Bastardo et al. (2011)*** who have isolated non-motile *Y.R.* strains in Peru.

This study showed that; identification of *Y.ruckeri* by PCR using specific primers target 575 - bp fragment of *16SrRNA* gene is a rapid and a reliable method in diagnosis of Yersiniosis and this is useful in its control and prevention and subsequently lessening the huge economic losses in aquaculture.

In the present study, *yrInv,yrIIm,yhlA,yhlB* genes characteristic to *Y.R.* were detected in all the isolates. Detection of *yrInv,yrIIm,yhlA,yhlB* genes by PCR can be used as a specific and a rapid method for identifying pathogenic *Y.ruckeri* isolates.

In our study, *qnrS* genes were identified in all 2 isolates of *Y.ruckeri* (100%). Also the *tet A* (tetracycline resistance gene) and The *bla TEM* gene (β-lactamases resistance gene) were reported with a percentage of 100%.

In the antimicrobial susceptibility tests, all of the isolates showed marked sensitivity to ciprofloxacin and Sulphamethoxazole-Trimethoprim combination, and these results were in line with the results obtained by ***Joon Joh et al. (2010).***

Conclusion and recommendations

From the present study, it could be concluded that:

- *Y.ruckeri* isolates vary in pathogenicity according to their virulence.
- *Y.ruckeri* sensitive to sulphamethaxazol with trimethoprim, ciprofloxacin, gentamycin, erythromycin, amoxicillin,flumequine, enrofloxacin, doxycycline and oxytetracycline that could be used to decresed mortalities.
- PCR method can be used as an important technique in the diagnosis of Virulence genes *(yrp,yrInv,YrIIm,yhlA,yhlB)* of *Y.ruckeri* isolates and MDR *genes*(*tetA(A), blaTEM, qnrS*) .In addition to the importance of *(16SrRNA)* that can be used as marker for rapid and accurate detection of *Y.ruckeri* isolates.

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التوصيف الجزيئي لأنواع اليرسينيا ومقاومتها للعديد من الأدوية فى الأسماك

الملخص العربى

في هذه الدراسة ، تعتبر" اليرسينيا" أحد مسببات الأمراض الشائعة للأسماك ، وهو يتسبب في أحد أهم أمراض التسمم الدموى المسؤولة عن الوفيات الجماعية في أسماك المياه العذبة وبالتالي خسائر اقتصادية عالية. أجريت هذه الدراسة لمعرفة مدى انتشار مرض اليرسينيا فى السمك البلطى النيلى وسمك القراميط فى محافظة الدقهلية؛ لمعرفة الخصائص الظاهرية والجينى للمعزولات، وكما تبينا عن طريق اجراء اختبار تفاعل البلمرة المتسلسل، معرفة جينات الضراوة وهم *yrp,yrIlm,yhlA,yhlB,yrInv* والجينات المقاومة للأدوية المتعددة وهم blaTEM,qnrS, tetAgene.وعليه قد تم جمع) 100 (عينة من سمك البلطى النيلى و) 100 (عينة من سمك القراميط التي تم جمعها من مزرعة اسماك مياة عذبة بمحافظة الدقهلية خلال الفترة من أبريل 2019 إلى أبريل 2020 .و تم إخضاع عينات الأسماك للفحص الظاهرى والفحص ما بعد الذبح ثم الفحص البكتريولوجي لعينات من الكبد والكلى والطحال. وتميزت العزلات المشتبه بها بالخصائص المورفولوجية وبعد الاختبارات البيوكيميائية التقليدية ونظام API 20E ثم اجراء اختبار تفاعل البلمرة المتسلسل. وجد 24 اصابه من السمك) 10 من من سمك البلطى النيلى بنسبة 10 ٪ و 14من من سمك القراميط بنسبة 14 % والمعزولات بأنة اليرسينيا 22 %(و أظهر التوصيف البكتريولوجي للمعزولات أنها متجانسة. علاوة على ذلك ، تم توضيح جين16SrRNA) جين شائع محدد (في جميع عزلات اليرسينيا بواسطة اجراء اختبار تفاعل البلمرة المتسلسل)