Antibiotic Resistance and Antibiotic Resistance Genes Among Edwardsiella Tarda Isolated from Fish

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Abstract

Edwardsiella tarda is a common fish pathogen, causing significant septicemic diseases in fish. This study was carried out to investigate prevalence of E. *tarda* among *Tilapia zilli at* EL manzla lake, Dakahlia governorate and characterize the isolates phenotypically and genotypically in addition to detection of multi-drug resistance genes (B-lactames genes and plasmid-mediated quinolone resistant genes by PCR assay. Therefore, (100) samples of *Tilapia zilli* collected from different localities. Fish samples were subjected to clinical and post-mortem examination then bacteriological examination from liver, kidney and spleen. The suspected isolates were characterized by cultural and morphological characters, some conventional biochemical tests and API 20E system then by PCR assay. Eighteen % isolates were characterized as *E. tarda*. Furthermore, detection of antimicrobial resistance genes by PCR, the recovered isolates harbored *blaSHV* and *blaOXA-1* with a prevalence of 50% and 25%, respectively with no detection of *aac (6')-Ib-cr and qepA* genes in examined isolates. The results of antibiotics sensitivity showed resistance to Nalidixic acid, Lincomycin, Amoxicillin and Norfloxacin and most of isolate were found to be sensitive to Florfenicol and Neomycin.55.6% of recovered isolates showed resistance to three antibiotic classes and 27.7% of recovered isolates showed resistance to four antibiotic classes and considered as MDR. The emergence of MDR-strains represents a threat-alarm and PCR is very rapid method for identification of *E. tarda* isolates which may be helpful in control of Edwardsiellosis.

Keywords: *Edwardsiell tarda*, *Tilapia zilli*, PCR and Antibiotic resistance genes

Introduction

Edwardsiella is enteric pathogen includes strains of three species (*E. tarda*, *E. ictaluri* and *E. hoshinae*). *E. tarda* is primarily a pathogen of fish associated with fish gangrene, emphysematous putrefactive disease of catfish, red disease of eels ***(Evans et al., 2011).*** *E. tarda* is mainly cause of a dangerous systemic disease, Edwardsiellosis which is one of the most important bacterial diseases in fish and it causes mass mortality in the various populations and age groups of fish in freshwater and marine fishes of both farmed and wild population all over the world ***(Enany et al., 2018).*** *E. tarda* is a Gram-negative, short, rod-shaped bacterium of about 1cm in diameter and 2-3 cm in length ***(Evans et al., 2011).*** *E. tarda* strains isolated from various geographical sources exhibit little variation in the phenotypic characters ***(Austin and Austin, 2007)*** and several studies have demonstrated a wide degree of intraspecific diversity in the isolates from the different geographic regions and host species ***(Nucci et al.,2002 and Wang et al., 2011).***

E.tarda has been recognized as a normal guest of a wide range of animals, including fish, reptiles, crustaceans, chickens and warm-blooded animals as human and contains essential virulence factors which increase bacterial survival and disease in hosts ***(Castro et al., 2016 and Park et al., 2012).*** *E. tarda* strain possess several virulence and toxin secretion associated genes which illustrate to some extent its ability to survive within phagocytic cells and to infect a wide range of hosts due to a highly virulent and multidrug resistant strain ***(Verjan et al., 2013).*** Pathogenesis of *E. tarda* is multifactorial and several potential virulence factors have been recognized to contribute to the infection process have been reported ***(Mohanty and Sahoo, 2007).*** Also, it is well known that diagnosis of a particular infection depends on detection and identification of its causative agent ***(Das et al., 2014).***

Nowadays, one of the main threats to public health is the antibiotic resistance (WHO, 2002). Multi-drug resistant (MDR) was defined as resistance to only one agent in three or more antimicrobial classes. ***(Basak, et al.,.2016).*** Several previous investigations revealed the emergence of multidrug-resistant bacterial pathogens from different origins especially fish that increases the need for new natural immunostimulants

and antimicrobial alternatives to the commonly used old antimicrobial agents ***(EL-Sayed et al., 2019; Abouelmaatti et al., 2013; Algammal et al., 2020a; Algammal et al., 2020b; Algammal et al., 2020c; Algammal et al., 2021).*** This work was carried out to isolate and characterize *E. tarda* from brackishwater fish *Tilapia zilli* in Dakahlia governorate, Egypt. In the current study we detect the presence of *E. tarda*, determine antibiotic sensitivity and screened the presence of antimicrobial resistance genes of *E. tarda* isolated from diseased, dead and healthy fishes using molecular technique of conventional PCR.

Materials and methods

Fish sampling:

A total number of 100 of brackishwater fish of *Tilapia zilli* (healthy, diseased and morbid)) were collected randomly from EL-Manzla in Dakhlia governorate, Egypt during the period from April 2019 to April 2020. Samples were transported directly in plastic bags to the microbiological department of animal health research institute in EL-Mansoura branch to be full clinical, postmortem and bacteriological examination.

Clinical and postmortem examination

Fish were examined clinically for the presence of external and internal lesions according to ***(Schaperclaus et al.,1992)*** The examined fish were placed on right side. Sterilization of the skin of fish by70% ethyl alcohol. The first cut was made in front of the anus through the abdominal wall with blunt sterile Scissors. The second cut was made perpendicular to the first directly behind the branchial cavity and the third cut was from the anus to the head parallel to middle line where the abdominal wall was removed and the internal organs become visible and examined for detection of any abnormalities as change in color, size, hemorrhages ascetic fluid and other abnormalities. fish were examined in a sterile manner using a three-line.

Bacteriological examination

Samples from kidney, liver and spleen were aseptically inoculated on tryptic soya broth (Oxoid, UK) and incubated at 25-30°C for 24 hrs. then inoculated on Salmonella-Shigella agar (SS agar; Oxoid CM0099) incubated at 35°C for 24- 28 hrs. according to **(Bergey's *et al.*, 2005)**. For purification and further identification, recovered isolates streaked on TSA slope and incubated at 37°C for 24 hrs. All recovered isolates morphologically were detected with Gram's stain and biochemically according to. **(Austin&Austin.,2007)** and by

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

Antibiotic susceptibility test for *E. tarda*:

The sensitivity test was done on examined isolates of *E. tarda* using disc diffusion method according to **(Finegold and martin.1982)** using various antimicrobial agents: Amoxicillin (10µg), Nalidixic acid (30µg), Florfenicol (30µg), Neomycin (30µg) Lincomycin (2µg) and Norfloxacin (10µg). The inhibition zone diameter was measured and interpreted according to (NCCLS, 2007). Isolates showed resistance to more than two different antimicrobial classes were multiple drug resistant (MDR).

Detection of antimicrobial resistance genes by polymerase chain reaction

The DNA was extracted using QIAamp DNA mini-Kit Primers (Catalogue no. 239035),USA) and used for the detection of *Edwardsiella tarda* antibiotic resistance related genes to β-lactams (bla_{SHV} and $bla_{OXA}1$), Fluroquinolones(*aac(6')-Ib-cr and qepA* (Metabion, Germany) as shown in (Table 1). Separation of amplified products by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) and photographing gels by gel documentation system (Alpha Innotech, Biometra).

Statistical analysis

The obtained findings were analysed using the Chi-square test with SAS software, version 9.4, SAS Institute, Cary, NC, USA) (Significance-level; $P < 0.05$).

Results

Clinical and postmortem examination of fish

Samples represented *Tilapia zilli* were randomly collected and analyzed. based on the type of fish, the most clinical signs observed in the examined *Tilapia zilli* were the appearance of abdominal distension, hemorrhage on the surface of the body, congestion of the fins, skin ulcers and congested vent. Accumulation of the ascitic fluid and congestion of the internal organs, including the liver, gallbladder, spleen and kidney, occurred internally. As shown in the (Fig. 1a,b).

Prevalence of *E.tarda* in the fish and Their distributions in Different Internal Organs

All examined strains were reported as *Edwardsiella tarda*. Under microscope, the bacteria appeared as Gram-negative, short, rod-shaped bacterium. The bacteria grew well Salmonella shigella agar appear as small transparent with black center on TSA. All recovered isolates were oxidase negative and biochemically homogenous. *E.tarda* were positive for Indole,

Methyl Red, Catalase, H2s production , glucose fermentation and reduction nitrate to nitrite but negative for Lactose fermentation, sucrose, Urease, Voges – Proskauer . Also, the presence of *E. tarda*, identified using API20 E as shown in Fig.(2). The results exhibited that 18 isolates recovered from (zero from healthy fish ,10 from diseased fish and 8 from mourbid fish) as shown in table (2) and highest distribution of *E. tarda* was in liver (60%) then kidney (40%) then spleen (0%). Statistically, there is no significant difference in distribution of *E. tarda* among different organs.

Seasonal Variation of *E. tarda*.

Recovered strains of *E. tarda* were detected during spring, summer and autumn, while in winter not detected in any samples. The summer season showed the highest prevalence with 58.3% then the spring season with 25%, finally autumn 16.7%. Statistically, there is no difference in seasonal variation of *E. tarda*.

Antibiotic Sensitivity of recovered *E. tarda* strains:

E. tarda isolates vary in their antimicrobial sensitivity pattern to different used antimicrobial. In present study, all recovered isolates showed resistance to Nalidixic acid, Lincomycin, Amoxicillin and Norfloxacin and most of isolate were found to be sensitive to, Florfenicol and Neomycin. Statistically, there is a significant difference ($P < 0.05$) in the resistance and susceptibility of the recovered *E. tarda* strains to various antimicrobial agents as shown in table (3). in the present study, 55.6% of the recovered isolates showed resistance to three antibiotic classes and 27.7% of recovered isolates showed resistance to four antibiotic classes were multidrug resistant (MDR)

Molecular characterization of *E. tarda* isolates

Two isolates of representative four isolates (2/4) showed positive amplification of 392 bp fragment specific for bla_{SHV} with a total percentage of 50%, one (1/4) isolate showed positive amplification of 619 bp fragment specific for bla_{OXA1} with a total percentage of 25%, no recovered isolates detect *aac(6)-Ib-cr* and *qepA* amplified at 113 and 403 bp fragment respectively, as shown in Fig (3,4,5,6)

Table (1): *Oligonucleotide primers sequences of genes among recovered isolates*

Gene	Sequence	Actual cycle 35 cycles	Amplified product	Reference
aac(6')-Ib-cr	CCCGCTTTCTCGTAGCA	Denaturation:94˚C/30sec Annealing:52˚C/30sec Extension:72˚C/30sec	113 bp	**Lunn *et al.*, 2010**
	TTAGGCATCACTGCGTCTTC			
blaSHV	AGGATTGACTGCCTTTTTG	Denaturation:94˚C/30sec Annealing:54˚C/40sec Extension:72˚C/40sec	392 bp	**Colom *et al.*, 2003**
	ATTTGCTGATTTCGCTCG			
blaOXA-1	ATATCTCTACTGTTGCATCTCC	Denaturation:94˚C/30sec Annealing:54˚C/40sec Extension:72˚C/45sec	619 bp	
	AAACCCTTCAAACCATCC			
qepA	CGTGTTGCTGGAGTTCTTC	Denaturation:94˚C/30sec Annealing:54˚C/40sec Extension:72˚C/45sec	403 bp	**Cattoir *et al.*, 2008**
	CTGCAGGTACTGCGTCATG			

Table 2. *prevelance of Edwardsiella tarda in accordance to fish status:*

Fish	Apparently healthy	Diseased	morubid	Total
No of examined sample	**20**	**30**	**50**	**100**
No of isolated *E.tarda*	zero	10	8	**18**

Table 3. *Antibiotic sensitivity of recovered E. tarda strains*

Specific tested antibiotic	Interpretation					
	Sensitive		Intermediate		Resistance	
	N	%	*N*	%	*N*	%
Amoxicillin	0	0	2	11.2	16	88.8
Neomycin	11	61.1	2	11.2	5	٢7.7
Norofloxacin	4	22.2	4	22.2	10	55.6
Nalidixic acid	3	16.6	8	44.4	7	38.9
Lincomycin	4	22.2	2	11.2	12	66.6
Florfenicol	11	61.6	3	١6.6	4	22.2

Figure 1a. *Naturally infected Tilapia zilli with E. tarda showing, external hemorrhages and congested fins and gills.*

Figure 1 b. *Naturally infected Tilapia zilli with E. tarda showing hemmorhage with congested liver*

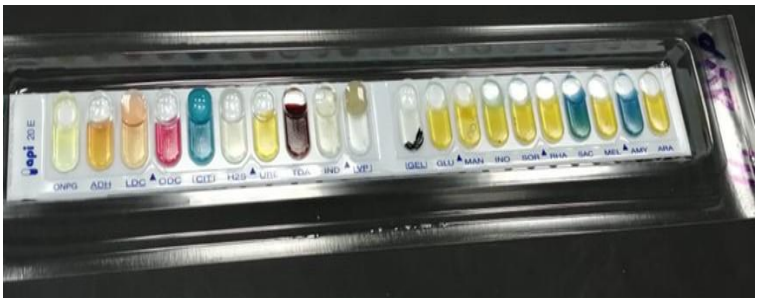

Figure 2. Biochemical identification of the isolates by using API 20E.

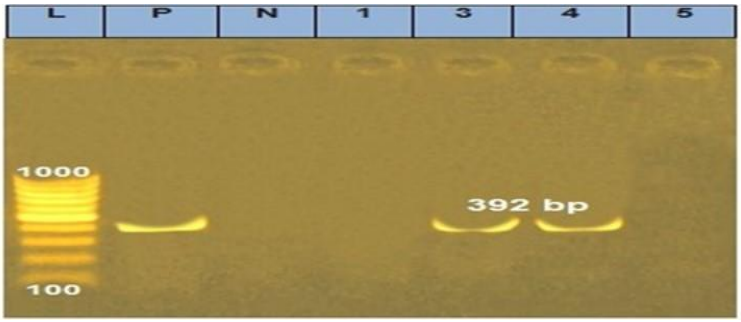

Figure 3. Agarose gel electrophoresis showing MDR gene of *E. tarda* isolates using primer set for *bla*SHV gene (392bp).
LaneL: 100-1000bp ladder. P: control positive. N: control negative.
Lanes3, 4: *E. tarda isolates Positive the blaSHV gene.*
Lanes1, 5: *E. tarda isolates Negative the blaSHV gene*

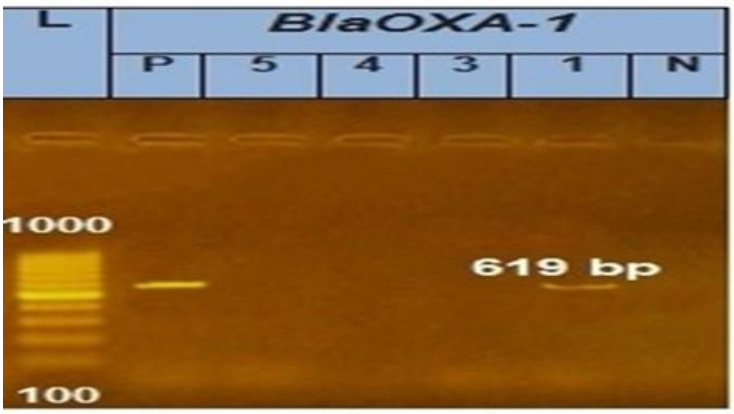

Figure 4. *Agarose gel electrophoresis showing MDR gene of E. tarda isolates using primer set for blaOXA-1 gene (619bp).*
LaneL: *100-1000bp ladder.* ***P:*** *control positive.* ***N:*** *control negative.*
Lanes 1: *E. tarda isolates Positive for the blaOXA-1 gene*
Lanes 3, 4,5: *E. tarda isolates Negative for the blaOXA-1 gene.*

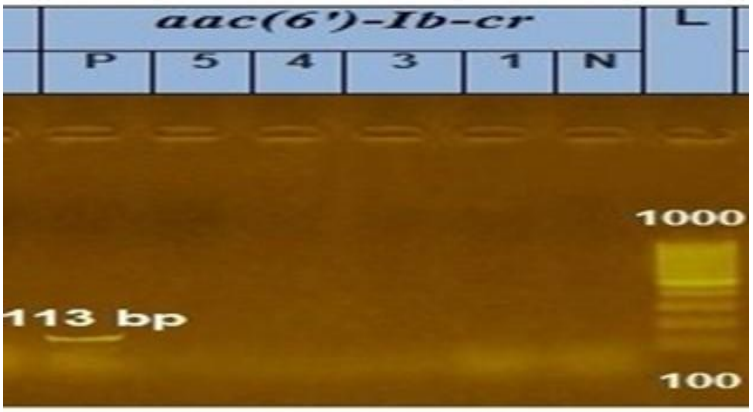

Figure 5. *Agarose gel electrophoresis of MDR gene of E. tarda isolates using primer set for aac(6-)-Ib-cr gene (113bp).*
LaneL: *100-1000bp ladder.* ***P:*** *control positive.* ***N:*** *control negative.*
Lanes1, 3, 4, 5: *E. tarda isolates Negative for the aac(6-)-Ib-cr gene.*

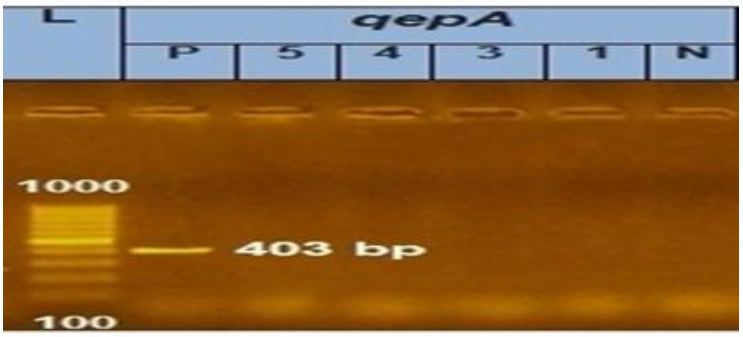

Figure 6. *Agarose gel electrophoresis of MDR gene of E. tarda isolates using primer set for qepA gene (403bp).*
LaneL: *100-1000bp ladder.* ***P:*** *control positive.* ***N:*** *control negative.*
Lanes1, 3, 4, 5: *E. tarda isolates Negative for the qepA gene.*

Discussion

Edwardsiellosis is one of the most important bacterial diseases in fishes causing massive mortalities in the various populations and age groups of fish consequently high economic losses ***(Plumb, 1993 and Jun and Yin, 2006).*** In the current study, the prevalence of *E. tarda* infection among the examined *Tilapia zilli* was 18% agree with results of ***(Korni et al., 2012)*** who recorded that prevalence of Edwardsiellosis among the cultured *Nile Tilapia* in spring season at Beni-Suef governorate was 13.33 % and higher than ***(Ali etal., 2008)*** who reported that incidence of *E.*

tarda among the diseased *Tilapia zilli* at Beni-Suef Governorate was 3.7%. Our results also were disagreed with ***(Abd El-Mageed et al, 2002)*** who recorded that incidence of *E. tarda* in *Tilapia zilli* collected from different localities in Egypt was 0% The difference in prevalence of *E. tarda* may be attributed to the difference in water temperature, stocking density, water quality and/or location of the study.

Examination of *Tilapia zilli* infected with *E. tarda* revealed number of the clinical signs as abdominal distension, hemorrhages on the body surface, congestion of the fins, presence of skin ulcers and congested vent. Internally, there were accumulation for the ascitic fluid and congestion of internal organs including liver, spleen and kidney. These clinical signs and post-mortem lesions were similar to those reported by ***(Kubota et al., 1981; Eissa and Yassien 1994; Galal et al, 2002; Saad El-Deen et al., 2005; El-Deeb et al., 2006; Ramadan et al., 2009; Yu et al., 2009 and Hashiem and Abd El-Galil ,2012).***

Multidrug resistance could be partly attributed to the inadequate dose, extensive use and sub-active concentration of the drug used in fish farms. Furthermore, widespread use of antibiotics in medical, veterinary, agricultural and aquacultural settings as prophylactic measures and growth promoters have resulted in proliferation of antibiotic resistant genes in horizontal gene pool ***(Meervenne et al., 2012).***Our results detected that the recovered isolates showed resistance to nalidixic acid, Lincomycin, amoxicillin and Norfloxacin and this agree with those reported by ***(Noor ELDeen et al., 2017 ;Nagy et al., 2018)*** and most of isolate were found to be sensitive to, florfenicol and neomycin**.** ***(Ahamad et al., 2013; Anyanwu et al.,2014; Thangapalam Jawahar Abraham et al.,2015; Pankaj Kumar et al., 2016).*** These resistance results may be attributed to mutations in the gyrase or to poisomerase antibiotic genes. resistance genes or by horizontal gene transfer of antibiotic resistance determinants ***(Poole,2004) and (Sorum,2006).***

Plasmid mediated quinolone resistance (PMQR) has been shown to play an important role in resistance not only to quinolones, but also B-lactamase and aminoglycosides. In fact, quinolones resistant genes represent one of the most important PMQR mechanisms and frequently carried along with B-lactamase on the same plasmids also *aac (6-)-Ib-cr* genes. *qepA* was identified

PMQR gene encoding efflux pump ***(Yamane et al.,2007)*** was detected in *Edwardsiella* isolates resistant to quinolones. DNA sequencing of *qepA* revealed that the gene includes three alleles of *qipao (qepA1, qepA2, qepA3),* ***(Cattoir et al.,2008 and wang et al., 2015).*** In the present work *qepA* gene could not be identified in any isolates of *edwardsiella* and disagree with ***(Liu et al., 2011)*** who found *qepA* gene in isolates of *E. tarda*. Quinolone resistance genes are widely distributed among bacteria ***(Flach et al.,2013).*** In this study *aac(6-)-Ib-cr* genes were investigated using specific primers and PCR, could not be detected in any one of the isolates and this results are in disagreement with these results recorded by***(Sudu et al.,2018)*** who isolate 11 out of 30 isolates of *E.tarda* from fish in japan, ***(Huang et al., 2012 and Yu et al.,2012)*** who identified this gene in one isolate and mentioned that this gene was located on large plasmid. While ***(Liu et al., 2011)*** mentioned that quinolones resistant genes were acquired from chromosomal genes in bacteria and are usually associated with mobilizing or transposable elements on plasmids.

The high levels of resistance to the β-lactam antibiotics in several Gram-negative bacteria has been attributed to their intrinsic resistance, often chromosomal mediated and transferable to new generations ***(Kümmerer, 2009).*** on the other hand, bla_{OXA-1} was present in one isolate only with a percentage of (25%) and our results detect bla_{SHV} with a percentage of (50%) disagree with ***(Kees et al., 2008).*** Who recorded 0% and 25%, respectively in *E. tarda* while disagree with ***(Goudarzi et al., 2013)*** who could not detect bla_{SHV} genes in isolates and ***(Shahcheraghi et al., 2010)*** found bla_{SHV} genes among 6% of 200 isolates.

Conclusion

Edwardsiellosis infection leads to high morbidity and mortality rate resulting great economic losses. The total prevalence of *E. tarda* in brackishwater fishes is high may be due to pollution and stress factors so that overcrowded, bad environmental condition, bad water quality and high organic matter in fish farms. *E. tarda* has also public health significance in people engaged in fishery industry and those depend on fish products for their annual income. Overuse or miss use of antibiotics increase *Edwardsiella* resistance to most antibiotics. PCR method can use as an important technique in the diagnosis of antibiotics resistance genes of MDR *E.tarda* isolates *aac(6-)-Ib-cr, qnrA,* bla_{SHV}, bla_{OXA-1},*qepA* -

based techniques are used increasingly in food-microbiology research as they are well developed and when applied as culture confirmation tests, they are reliable, fast and sensitive which measure epidemics occurrence and subsequently decreasing the economic losses.

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المقاومة للمضادات الحيوية والجينات المقاومة للمضادات الحيوية الادواردسيلا تاردا المعزوله من السمك

يعتبر ميكروب الادواردسيلا من الميكروبات التى تسبب مشاكل مرضية خطيرة وتؤثر على الثروة السمكية . ولذلك تهدف هذة الدراسة الى تحديد الاصابة بهذا المرض الضار وكيفية عزل الميكروب المسبب لة وتحديد الجينات المقاومة للمضادات الحيوية من المعزولات ولذلك قد تم عزل ميكروب الادواردسيلا من عدد ١٠٠ سمكة بلطي والتى جمعت من بحيره المنزله بمحافظة الدقهلية فى الفترة من ابرايل ٢٠١٩ الى ابرايل ٢٠٢٠ واظهرت الدراسة عزل ميكروب الادواردسيلا ١٨% وقد تم اجراء الفحوصات الظاهرية والتشريحية والبكتيريولوجية و تم اخذ العينات من الطحال والكبد والكلى للفحص البيكتريولوجى. فقد ظهر على الاسماك المصابة نزيف على سطح الجسم يشمل ووجود تقرحات على بعض الاسماك،كما تبين وجود بقع نزيفية فى العضلات الخارجية بالاضافة الى ظهور حالات من الاستسقاء وانتفاخ فى البطن حيث ظهر التهاب و احتقان الكلى والطحال بالدماء وتورم فى الكبد ووجود علامات نزيفية وتم اجراء اختبار الحساسية للمضادات الحيوية المختلفة لتلك العترات وكانت النتايج ان معظم العترات حساسة فلوروفينيكول والنيوميسين بينما اتضح ان معظم العترات مقاومة الاموكسيسلين والنورفلوكساسين واللينكوميسين ونيلديكسيك و٥٥,٦% بكتريا مضاده لعده مضادات الحيوية. وتم اجراء اختبار البلمرة الجزيئية على ٤ من المعزولات التى تم عزلها باستخدام البادئ المتخصص لكل جين من الجينات المقاومة للمضاد الحيوية بمعزولات الادواردسيلا(qepA aac(6-)-Ib-cr الخاصة بالكينولونات - الخاص بالبيتا لاكتام (blaOXA, blaSHV وحيث اظهرت النتايج تواجدهم فى العينات المعزولة بالنسب الاتية: ٥٠%(blaSHV)٢٥%blaOXA ولم يظهر فيqepA aac(6-)-Ib-cr