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IDENTIFICATION OF *EDWARDSIELLA TARDA* ISOLATED FROM TILAPIA NILOTICA (OREOCHROMIS NILOTICUS) BY POLYMERASE CHAIN REACTION (PCR). THE EFFECT OF GARLIC OIL AND DIFFERENT ANTIMICROBIALS ON THE IDENTIFIED ORGANISM IN VITRO.

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ABSTRACT

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Edwardsiella tarda is considered as a serious pathogen of fish and it is also important due to its zoonotic aspects as infected fish processed for human consumption is a source of gastroenteritis and meningitis. One hundred and thirty samples of cultured apparently healthy Nile tilapia were collected from private farms in Alexandria governorate and examined bacteriologically to isolate *Edwardsiella tarda*. *E. tarda* could be isolated from the kidney, liver, spleen, muscles and intestinal contents of examined fish with in a percentage of 3.9 %. The five isolates of *E. tarda* were identified morphologically and biochemically then subjected to confirmation by conventional Polymerase Chain Reaction (PCR) with the amplification product of 415 bp using gyrB gene as a taxonomic marker. Pathogenicity test of the isolate and histopathological examination of the experimentally infected fish were carried out. In vitro, sensitivity test of the isolate against antimicrobials and garlic oil showed that it was resistant to garlic oil, Neomycin, Nitrofurantoin, Streptomycin, Tetracycline, Amoxicillin, Ampicillin/ sulbactam and Colistin; and sensitive to Chlormphenicol, Enerofoxacin, Flumequine Gentamycin; while antimicrobials combined with garlic oil revealed that garlic oil decreased the potencies of antimicrobials. proper aquarium hygiene, appropriate regulations of physical conditions in the aquarium are recommended and fish must be cooked sufficiently before eating to protect human health from such hazards.

Key words: *E. tarda- tilapia- PCR- antimicrobials- garlic oil.*

INTRODUCTION

Bacterial agents are among the highly encountered causes of diseases in stressed warm water aquaculture (Pavanelli *et al.,* 1998; Noga, 2000). *Edwardsiella tarda,* a member of the family *Enterobacteriaceae,* is the causative agent of septicaemia in a variety of fish species. Although it is commonly classified as opportunistic, it is considered as a serious pathogen of fish because of its expanding fish host range (Alcaide *et al.,* 2006; Mohanty and Sahoo, 2007) and it is also important due to its zoonotic aspects as infected fish processed for human consumption is a source of gastroenteritis, meningitis, liver and skin abscesses and valvular endocarditis in patient with Acquired Immune Deficiency syndrome (AIDS) (Inglis *et al.,* 2001; Mikamo *et al.,* 2003; Mizunoe *et al.,* 2006).

It is the causative agent of Edwardsiellosis in many commercially important freshwater and marine fishes (Lan *et al.,* 2008) and it is a common pathogen which has been isolated from tilapia (Kubota *et al.,* 1981).

Stress factors such as overcrowding, sudden change of temperature, pH and dissolved oxygen fluctuations might contribute to the development of the disease (Choresca *et al.,* 2011).

Sporadic cases of human diarrheal disease associated with *E. tarda* were reported from Zaire by Makulu *et al.* (1973) and by Vandepitte *et al.* (1974). With respect to the most recent diagnostic methods for Edwardsiellosis, polymerase chain reaction (PCR) represents a widely-used alternative to traditional identification methods (Ibrahem *et al.,* 2011).

Histopathological examination of Nile tilapia infected with *E. tarda* revealed hydropic degeneration in most of hepatocytes, the kidneys revealed necrobiotic changes in the convoluted tubules and there were increase in melano-macrophages centers and depletions in lymphoid follicles of spleen and congestion of blood vessels (Ibrahem *et al.,* 2011).

The pathogenic *E. tarda* isolates were often found to be naturally resistant against multiple antimicrobial compounds which increases the difficulty of antibiotic-based treatment (Stock and Wiedemann, 2001; Yoo *et al.,* 2003 and Alcaide *et al.,* 2006). In addition to, using of antibiotics in fish farms may introduce potential hazards to public health and to the environment by the emergence of drug-resistant microorganisms and antibiotic residues. Furthermore, the normal microbial flora in the digestive tract, which is beneficial to fish are also killed or inhibited by oral chemotherapy (Sugita *et al.,* 1990) that leading to increasing of the interest in the request for natural alternatives, so researchers are looking at plants that have been used as alternative therapies for generations (Srinivasan *et al.,* 2009).

Allium vegetables, particularly garlic (Allium sativum L.) exhibit a broad antibiotic activity against both Gram-positive and Gram-negative bacteria (Whitemore and Naidu, 2000). The raw juice of garlic was effective against many common pathogenic bacteria (Kumar and Sharma, 1982), against the strains that have become resistant to antibiotics (Jezowa *et al.,* 1966) and even toxin production by some pathogenic strains was prevented by garlic (Dewitt *et al.,* 1979). Therapeutic effect of garlic is possible because of its oil- and water- soluble organosulfur compounds (Thiosulfinates), which are responsible for its typical odour and flavour; and play an important role in the antibiotic activity of garlic (Srinivasan *et al.,* 2009).

The objectives of the present study are to isolate and identify *E. tarda* by PCR, re isolate the organism from experimentally infected fish, and to evaluate the sensitivity of the isolated organism against some antibiotics or garlic oil or both in vitro infected fish, and to in vitro sensitivity test of the isolate against different antimicrobials, garlic oil and antimicrobials combined with garlic oil.

MATERIALS and METHODS

Collection of samples:
One hundred and thirty cultured tilapia nilotica weighted 50 ± 3 gm were collected from private farms in Alexandria governorate and transported alive in large plastic bags filled with water and brought to the laboratory.

Clinical and post mortem examination:
Fish were examined for any external abnormalities and opened under aseptic conditions; interior of the body was exposed and examined for changes according to Noga, (2000) and Kimberley (2004).

Bacteriological examination:
A loopful was taken from the internal organs (kidney, liver, spleen), muscles and intestine inoculated into tryptic soya broth (TSB) (Difco) with 3% NaCl and incubated at 25ºc for 24 hours., then streaked on Salmonella Shigella agar (SS) and incubated at 25ºc for 72 hrs. One single suspected colony that showing small, black centre to predominant black colony was purified on brain heart infusion (BHI) agar and stored on nutrient agar slope for further identification.

Bacterial identification:
The purified colonies were identified by colony morphology and cultural behaviour on BHI, Trypticase soya agar (TSA), MacConkey agar, SS agar and biochemical tests.

PCR detection:
The methods of Choresca *et al.* (2011) for bacterial DNA extraction, primers and PCR amplification were used. A colony of overnight culture of bacteriologically positive isolates was added into 100 µl of distilled water; the mixture was boiled for 10 min. and centrifuged at 1000 xg for 10 min. to sediment the cell debris. The DNA supernatants were transferred to fresh Eppendrof tubes and subjected to PCR technique using two pairs of primers specific for *Edwardsiella tarda* gene*:*

The forward primer, gyrBF1 5'-GCATGGAGACCTTCAGCAAT-3'
The reverse primer, gyrBR1 5'-GCGGAGATTTTGCTCTTCTT-3'. The expected length in polymerase chain reaction (PCR) is 415 bp.

The PCR amplification was performed in a thermocycler (Boeco- Germany) in a final volume of 50 µl using 10 µl of extracted DNA as template, 25 µl Dream Taq Green Master Mix (Fermentas), 1 µl of forward primer, 1 µl of reverse primer and 13 µl of DNase/ RNase-Free Distilled water. Thermal cycling involved an initial denaturation at 94 º C for 5 min. then 30 cycles at 94ºC for 1 min., 51.5ºC for 30 s and 72ºC for 30 s, and then an extra extension step of 72ºC for 10 min.

A volume of 5 µl from each PCR product resulted from the amplification were loaded on 2% agarose in Tris-EDTA buffer (TEB) containing 0.5 µg of ethidium bromide per millilitre. After electrophoresis, the gels were photographed under ultraviolet light. The 100 bp DNA ladder was used as a molecular weight marker (Fermentas).

To avoid contamination, sample preparation, DNA extraction, and PCR amplification steps were performed in separate areas. Aerosol filter pipette tips were used for handling all liquids. All applicances, containers, and the work areas were cleaned and irradiated with UV light for at least 60 minutes. Nuclease- free water control was included and results were negative in all cases.
DNA extraction, PCR amplification and detection of amplification products were done in the department of microbiology, High institute of public Health.

Experimental infection:
The virulence characteristics of *E. tarda* were assessed by challenging Nile tilapia (*Oreochromis niloticus).* Ten fish weighted 60 ±2 gm proved to be

free from *E. tarda* were injected intraperitoneally (I/P) with 0.2 ml of 10^4 bacteria/ ml and another ten fish were injected I/P with 0.1 ml PBS (phosphate buffered saline and served as controls, the temperature of the aquaria adjusted at 25 ± 1º C and fish were fed twice daily on commercial diet (Ibrahem *et al.*, 2011). Samples of muscles, liver, and spleen were aseptically removed and streaked on SS agar to confirm the infection of *E. tarda.*

Histopathological examination:

Fresh tissue specimens from the liver, spleen and muscle were collected in the day 3 PI (post infection) from morbid experimentally infected fish. Specimens were fixed in 10 % neutral buffer formalin, processed by conventional method , embedded in paraffin, sectioned and stained with Haematoxylin and Eosin stain.

In vitro sensitivity test of the isolate to antimicrobials:

Agar disc diffusion method was carried out according to Bauer *et al.* (1966):

amoxicillin (10µg), ampicillin/ sulbactam (20µg), chlormphenicol (30µg), colistin (10µg), enerofloxacin (5µg), flumequine (30µg), gentamycin (10µg), Neomycin (30µg), nitrofurantoin (300µg), streptomycin (10µg), tetracycline (30µg) and Trimethoprim/ sulfamethoxazole(25µg) were distributed over the surface of Muller-Hinton agar plate swabbed with the inoculum of the isolate and incubated at 37ºc for 18-24 hours then the diameter of inhibition zone was measured and interpreted according to CLSI (Clinical and Laboratory Standards Institute) criteria for animal isolates (CLSI, 2006).

In vitro sensitivity test of the isolate to garlic oil:

The garlic oil used in the study was obtained from El-Captain Company (Cairo- Egypt). Disc diffusion assay was followed according to Hood *et al.* (2003):

1. Ten ml Tween 80 in sterile nutrient agar (final concentration of Tween 80 of 0.1% and 1%) were poured on to a 10 ml prepared nutrient agar plate.
2. An over night culture of bacteria (0.1 ml) was spread over the surface of the agar plate using a sterile glass rod and incubated at 37ºC for 30 min.
3. Tween 80 (final concentration of 0.5%, 1%, 2.4% or 5%) was added to the oil prior to application to the susceptibility disc.
4. Ten µl of oil treated with Tween 80 was added on susceptibility discs that forming from sterile blotting paper of 6 mm diameter.
5. The oil impregnated discs were placed on the surface of the agar plate.
6. The agar plate was incubated over night at 37ºC and the zones of bacterial inhibition were recorded.

In vitro sensitivity test of the isolate to antimicrobials combined with garlic oil:

Disc diffusion method was followed. Each previously mentioned antimicrobial disc was impregnated with 10 µl of garlic oil treated with tween 80 of 0.5% concentration then distributed over nutrient agar plate previously treated with tween 80 of 0.1% concentration and coated with over night culture of the isolate. The agar plate was incubated over night at 37º C and the zones of bacterial inhibition were measured.

RESULTS

Table 1: Percentage of *E. tarda* isolated from examined Nile tilapia.

Isolate	Number of examined fish	Positive	%
E. tarda	130	5	3.9

Table 2: Biochemical profile of the isolated *E. tarda.*

Test	Reaction
Gram stain	-
Catalase	+
Oxidase	-
Indole	+
Methyl red	+
Vogus Proskaur	-
Simmon's citrate	+
TSI	K/AG
H2S production	+
Urease	-
Glucose	+
Sucrose	-
Motility	+
Growth in BHI with 3% NaCl	+

K= alkaline A= acid production G= gas production

Table 3: In vitro sensitivity test of *E. tarda* for antimicrobials and garlic oil

Antimicrobial	Inhibitory zone diameter(mm)	Interpretation
Amoxicillin (10µg)	0	R
Ampicillin/ sulbactam (20µg)	0	R
Chlormphenicol (30µg)	25	S
Colistin (10µg)	0	R
Enerofloxacin (5µg)	31	S
Flumequine (30µg)	27	S
Gentamycin (10µg)	19	S
Neomycin (30µg)	15	R
Nitrofurantoin (300µg)	15	R
Streptomycin (10µg)	17	R
Tetracycline (30µg)	7	R
Trimethoprim/ sulfamethoxazole (25µg)	17	S
Garlic oil	0	R

S= susceptible R= resistant

Table 4: In vitro sensitivity test of *E. tarda* against antimicrobials combined with garlic oil

Antimicrobial	Inhibitory zone diameter (mm)
Amoxicillin (10µg)	0
Ampicillin/ sulbactam (20µg)	0
Chlormphenicol (30µg)	19
Colistin (10µg)	0
Enerofloxacin (5µg)	19
Flumequine (30µg)	25
Neomycin (30µg)	15
Nitrofurantoin (300µg)	15
Streptomycin (10µg)	7
Tetracycline (30µg)	0
Trimethoprim/ sulfamethoxazole (25µg)	15

Post mortem examination:
Some fish had necrotic foci on liver and intestine.

Experimental infectivity of Nile tilapia:
Clinical abnormalities were clear in experimentally infected Nile tilapia with *E. tarda*, from day one tell the end of 3 days post infection as sluggish movement and loss of escape and defence reflexes, Scale detachment, skin discolouration, severe oedematous swelling at the site of injection, protruded hemorrhagic vent, fin rot and corneal opacity. Internally, Nile tilapia showed severe hemorrhagic enteritis, congested liver, kidney and spleen.

Histopathological examination:
Histopathological changes in experimentally inoculated *O. niloticus* with *E. tarda* at day 3 PI were summarized as hepatic degeneration, fatty changes and necrosis of liver, proliferation of mononuclear cell infilteration, haemosiderosis, dilatation and congestion of centeral vein. Spleen showed congestion and dilatation of spleenic blood vessels, thrombi formation inside the blood vessels, haemolysis of RBCs and haemosiderin deposition. Changes in muscles were in the form of hyalinization, sarcoplasmolysis, oedema and lymphocytic infiltration.

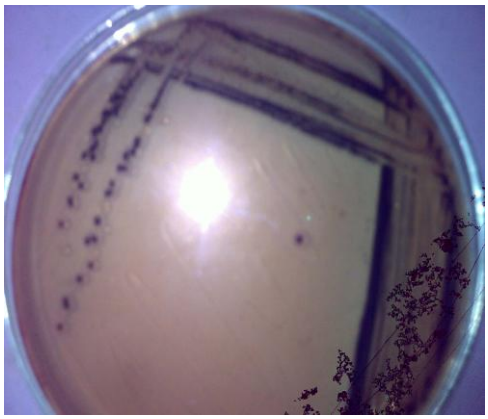


Fig. (1)

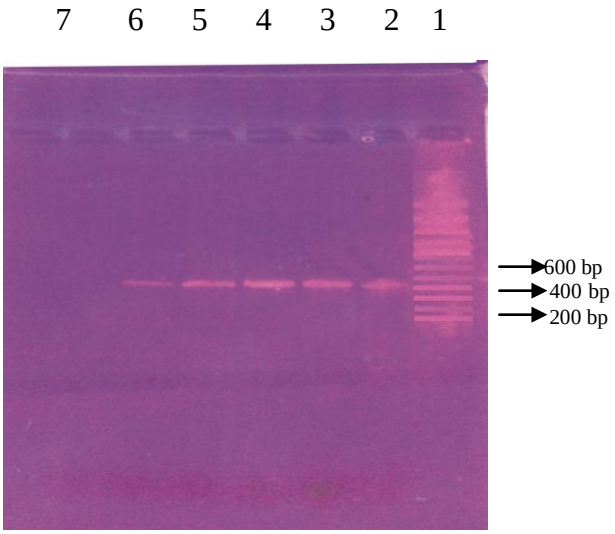
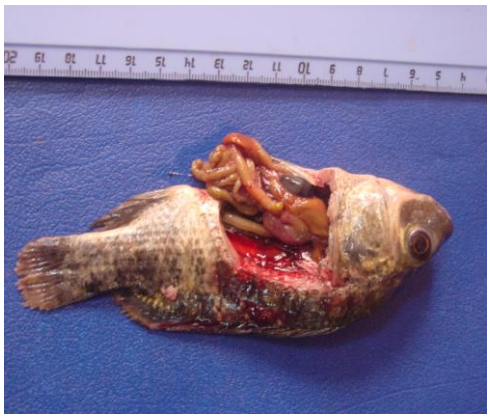


Fig. (2)

Fig. (3)



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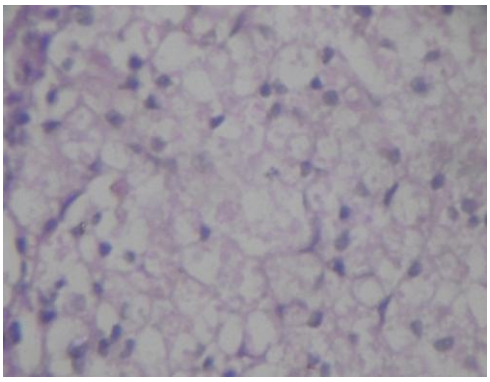

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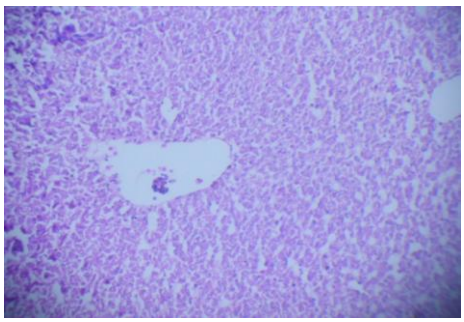

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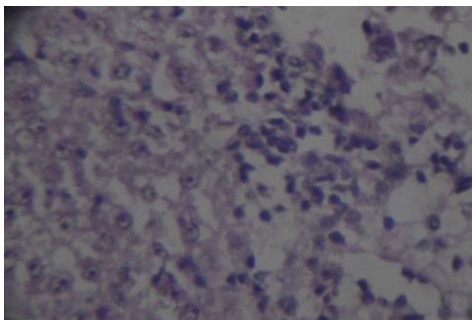

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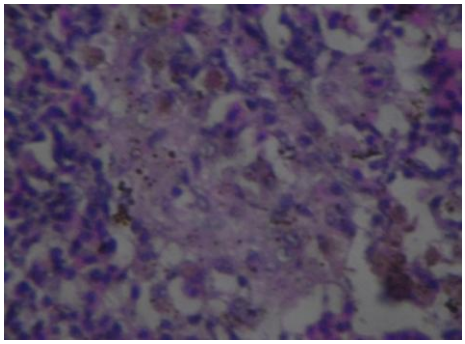

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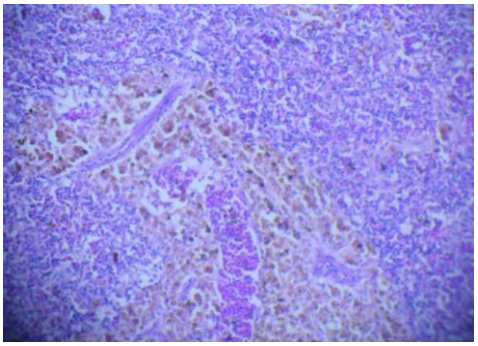

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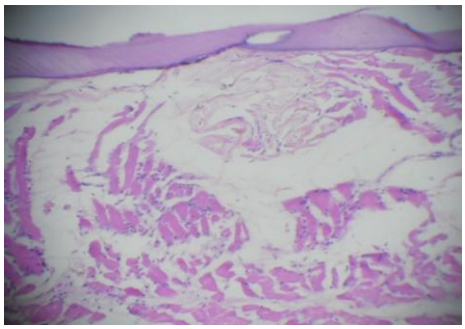

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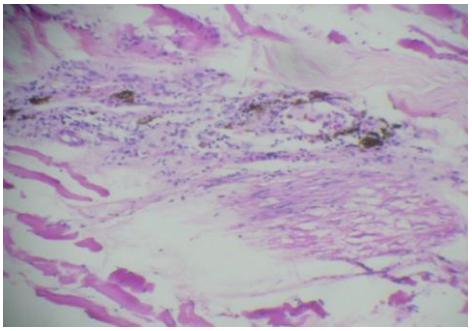

Fig. (11)

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Fig. (1): Colonies of *E. tarda* with black centres on Salmonell Shigella agar plate.

Fig. (2): PCR amplification products profile of *E. tarda* gyrB gene (415 bp) isolated from different organs of Nile tilapia.
Lane 1: 100 bp DNA ladder (Fermantas)
Lanes 2, 3, 4, 5. 6: positive PCR samples.
Lane 7: negative control.

Fig. (3): Nile tilapia experamintaley infected by *E. tarda* showing signs of septicaemia expressed by loss of skin colouration.

Fig. (4): Nile tilapia expermintely infected by *E. tarda* showing haemorrhagic enteritis.

Fig. (5): Liver of Nile tilapia experimentally infected I/P by *E. tarda* showing fatty changes in the most of hepatocytes.

Fig. (6): Liver of Nile tilapia experimentally infected I/P by *E. tarda* showing congestion and dilatation of centeral vein, hydropic degeneration, haemosiderosis.

Fig. (7): Liver of Nile tilapia experimentally infected I/P by *E. tarda* showing proliferation of mononuclear cells, hydropic degeneration.

Fig. (8): Spleen of Nile tilapia experimentally infected I/P by *E. tarda* showing area of necrosis, and haemosiderosis.

Fig. (9): Spleen of Nile tilapia experimentally infected I/P by *E. tarda* showing haemorrhage, congestion and dilatation of spleenic blood vessels, thrombi formation inside the blood vessels and haemolysis of RBCs.

Fig. (10): Muscle of Nile tilapia experimentally infected I/P by *E. tarda* showing infiltration of mononuclear cells, necrosis of muscle bundles, hyalinization and loss of muscle striation.

Fig. (11): Muscle of Nile tilapia experimentally infected I/P by *E. tarda* showing degenerative changes, loss of normal muscle striation, oedema of muscle bundles and area of hyalinization.

DISCUSSION

E. tarda is a common pathogen which has been isolated from tilapia (Kubota *et al.,* 1981), cat fish (Hashiem and Abd- El- Galil, 2012) and human (Vandepitte *et al.,* 1983). It is one of the main causative agents of enteric septicaemia or putrefactive diseases (EPD) with extensive skin lesions affecting internal organs such as liver, spleen and muscles, many cases reported all over the world such as North America, Japan, Taiwan, Thailand, and, Africa (Baya *et al.,* 1997). Edwardisella septicaemia is a mild to severe systemic bacterial disease primarily of warm water fishes which is characterized by the presence of gas–filled malodorous lesions in the muscles of fish. (Hawke *et al.,* 1981). Edwardsiella septicaemia caused by *E. tarda* is the currently accepted name for the disease caused by this pathogen. Other synonyms were bound as fish gangrene, Emphysematous Putrefactive Disease of Catfish (Meyer and Bullock, 1973) and red disease of eels (Egusa, 1976).

It was reported that a case of diarrhoea in human infant has been traced to *E. tarda* in the home aquarium (Vandepitte *et al.,* 1983).

Edwradsiella tarda could be isolated from kidney, liver, spleen, muscles and intestinal contents of 130 apparently healthy tilapia with in a percentage of 3.9 % (Table, 1), the isolate was identified morphologically as a Gram negative cocco bacilli showing small, black centre to predominant black colony (Fig. 1) and biochemically as it was motile, positive catalase, indole, methyl red, Simmon'citrate, H2S production, motility glucose fermentation and growth on BHI with 3 % Na cl while negative urease, oxidase, Vogus proskauer and sucrose fermentation (Table, 2), these results were nearly similar to that recorded by Nagla *et al.* (2005); Wei and Musa (2008).

The results of PCR detection revealed that all five morphologically and biochemically identified isolates of *E. tarda* were positive for *E. tarda* gyrB gene marker (Fig. 2) which is responsible for formation of the enzyme essential for DNA replication (Choresca *et al.,* 2011).

The experimental infection of Nile tilapia resulted in sluggish movement and loss of escape and defence reflexes, Scale detachment, discolouration of the skin (Fig. 3), severe oedematous swelling at the site of injection, protruded hemorrhagic vent, fin rot and corneal opacity. Internally, Nile tilapia showed severe hemorrhagic enteritis (Fig. 4), congested liver, kidney and spleen and these results were nearly similar to that reported by Kubota *et al.* (1981); Baya *et al.* (1997); Noga (2000).

The histological examination of experimentally infected Nile tilapia showed hepatic degeneration, fatty changes and necrosis of liver, proliferation of mononuclear cell infilteration, haemosiderosis, dilatation and congestion of centeral vein (Fig. 5, 6, 7); Spleen showed congestion and dilatation of spleenic blood vessels, thrombi formation inside the blood vessels, haemolysis of RBCs and haemosiderin deposition (Fig. 8, 9); and muscles were in the form of hyalinization, sarcoplasmolysis, oedema and lymphocytic infiltration (Fig. 10, 11), these findings nearly similar to those reported by Areechan and Plump (1983); Soliman *et al.* (1991); Nagla *et al.* (2005); Ibrahem *et al.* (2011).

The gross and histopathological lesions may be due to septicaemia induced by two exotoxins (haemolysins and dermatotoxins) that cause diseases and the most important one of them is haemolysin (Ullah and Arai, 1983; Hirono *et al.,* 1997; Mathew *et al.,* 2001).

Stress factors such as overcrowding, sudden change of temperature, pH and dissolved oxygen fluctuations might contribute to the development of the disease (Choresca *et al.,* 2011).

Table (3) illustrated that *E. tarda* in the present study was susceptible to enerofloxacin, flumequine, gentamycin, chloramphenicol and trimethoprim/sulfamethoxazole with diameter zones of 31, 27, 19, 25 and 17 mm respectively while resistant to amoxicillin, ampicillin, streptomycin, neomycin, tertracycllin, colistin and nitrofurantoin. The obtained results were nearly similar to that recorded by Choresca *et al.* (2011).

Table (4) revealed that the diameter of inhibition zones of Amoxicillin, Ampicillin/ sulbactam, Chlormphenicol, Colistin, Enerofloxacin, Flumequine, Neomycin Nitrofurantoin, Streptomycin, Tetracycline and Trimethoprim/ sulfamethoxazole combined with garlic oil were 0, 0, 19, 0, 19, 25, 15, 15, 7, 0 and 15 mm respectively against isolated *E. tarda* and when compared these results with the previously obtained in Table (3), it can be concluded that garlic oil decreased the potencies of antimicrobials used in this study.

Control of fish disease is currently based almost entirely on chemotherapy and it will entirely retain a role in the management of fish culture systems (Roberts, 1995). Anti-bacterial chemotherapy has been applied in aquaculture for over 50 years (Inglis, 1996). Antibiotics are also used prophylactically in carp culture at times of year when haemorrhagic septicaemia is most likely to occur (Inglis *et al.,* 2001). But habitual use of anti-bacterials can lead to problems with bacterial resistance and unacceptable residues in aquaculture products and environment. The resistant bacterial strains could have a negative impact on the therapy of fish diseases or human diseases and environment of fish farms (Smith *et al.,* 1994). This situation actually brings human to new medical dilemma (Muniruzzaman and Chowdhury, 2004). Medicinal plants possess therapeutic properties; exert beneficial pharmacological effects on the animal body, widely available in nature and

eco-friendly. Garlic could be used as an alternative therapeutic measure against bacterial infection of fish (Rahman, 2005).

An experiment was conducted by Rahman *et al.* (2009) to compare the efficacies of some selected antibiotics and medicinal plants against common bacterial fish pathogens: *Aeromonas hydrophila, Pseudomonas fluorescens and Edwardsiella tarda.* Four different antibiotics: CFCIN (ciprofloxacin), Renamycin (oxytetracycline), DT-10 (doxycicline) and sulfatrim (sulphadiazine + trimethoprim) were exposed in different doses (100, 75, 50 and 25 ppm) to the culture of freshly isolated bacteria under the in vitro condition for sensitivity test and they recorded that garlic offered the best result with 90.00 ± 2.89% recoveries of challenged fish in aquarium trial. *E. tarda* was resistant to garlic oil and many antimicrobials used in the present study.

From the previously mentioned results, it can be concluded that proper aquarium hygiene and appropriate regulations of physical conditions in the aquarium are required to avoid the stress and occurrence of diseases. Also, more researches are needed to find another natural antibacterial compound that can overcome the resistance of *E. tarda* to garlic oil and antimicrobials, studying other forms of garlic and trials of garlic administration in vivo and finally fish must be cooked sufficiently before eating to protect human health from such hazards.

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التعرف على الادورديسيلا تاردا المعزولة من اسماك البلطى بتفاعل البلمرة المتسلسل تاثير زيت الثومو المضادات الحيوية المختلفة على الميكروب المصنف خارجيا

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تم تجميع 130 عينة من سمك البلطى النيلى المستزرع السليم ظاهريا واجرى الفحص البكتيريولوجى للعينات لعزل ميكروب الادورديسيلا تاردا وتصنيفه باختبار تفاعل البلمرة المتسلسل وقد كانت نسبة الادورديسيلا تاردا التى تم عزلها من الاعضاء الداخلية للسمك (الكبد- الطحال- الامعاء- الكلى- العضلات) 3.9%. وكذا تم اجراء اختبار الضراوة والهيستوباثولوجى للميكروب المعزول وايضا اختبار الحساسية باستخدام المضادات الحيوية المختلفة وزيت الثوم كلا على حدى وايضا المضادات الحيوية وزيت الثوم معا وقد اوضحت النتائج ان ميكروب الادورديسيلا تاردا الذي تم عزله مقاوم لزيت الثوم ومعظم المضادات الحيوية. كما ان زيت الثوم قلل من تاثير المضادات الحيوية المستخدمة فى هذه الدراسة هذا وقد تمت مناقشة النتائج تفصيليا.