BS. VET. MED. J. JANUARY 2009 VOL. 19, NO.1, P. 44-50

Beni-Suef Veterinary Medical Journal

Studies on Edwardsiella tarda infection in catfish and Tilapia nilotica

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Edwardsiella tarda **was recovered from cultured freshwater catfish** ***(Claries gariepinus)*** **with a prevalence rate of 0.42% in summer season. Experimental infectivity studies of** ***E. tarda*** **in Catfish and** ***Tilapia nilotica*** **were done using the intraperitoneal (I/P) and intramuscular (I/M) routes. Experimentally infected** ***Catfish*** **with a dose of 0.3 ml from** ***E. tarda*** **bacterial suspension of 10^8 CFU/ml manifested pathognomonic clinical signs and postmortem lesions than the experimentally infected** ***Tilapia nilotica*****. Application of PCR technique for rapid detection of** ***E. tarda*** **infection in the experimentally infected fishes gave positive reaction in 4^{th} day post infection in catfish and the 7^{th} day post infection in** ***Tilapia nilotica.***

Outbreaks of infectious disease constitutes a major constrain to aquaculture production and trade with a consequent effect on the economic development (Yunxia *et al*., 2001). Bacterial agents are among the highly encountered causes of diseases in warm water aquaculture. Stressful conditions play an important role in establishing and aggravation of the bacterial diseases in fish farms (Pavanelli *et al.,* 1998; Noga, 2000).

Edwardsiella tarda infection is considered a dangerous septicaemic disease affecting cultured fishes and leading to high economic losses (Meyer and Bullock, 1973). The seriousness of *E. tarda* infection is attributed to its ability to infect different fish species (Mohanty and Sahoo, 2007). It infects catfish and Nile tilapia causing emphysematous putrefactive disease (EPD) (Badran, 1993; Darwish *et al.,* 2000).

Despite the fact that *E. tarda* is considered a bacterial pathogen of fish, reptiles and other cold-blooded animals, it can infect mammals and humans posing a public health threads as it causes gastroenteritis, meningitis, liver and skin abscesses and valvular endocarditis in patient with acquired immune deficiency syndrome (AIDS) (Nettles and Sexton, 1997; Slaven *et al.*, 2001; Mikamo *et al.,* 2003; Mizunoe *et al.,* 2006).

The present study was carried out to spotlight on seasonal prevalence of *Edwardsiella* species in fresh water fishes in Fayoum Governorate, Egypt. Also classical phenotypic and biochemical identification of *E. tarda* isolates with the aid of API20E was done. Evaluation of the infectivity of *E. tarda* isolates by experimental infection in catfish (*Clarias gariepinus)* and *Tilapia nilotica* was carried out. Moreover, PCR technique for rapid detection of *E. tarda* was evaluated.

Material and Methods

Naturally infected fish. A total number of 480 fishes (240 Catfish *[Clarias gariepinus*]) and 240 *Tilapia nilotica)* were randomly collected from different localities in El- Fayoum Governorate. It was collected during the different seasons of the year as shown in (Table 1). The fishes were transferred alive to the Microbiology Department, Faculty of Veterinary Medicine, Cairo University, where the fishes were subjected to clinical, post-mortem and bacteriological examination.

Fish used for experimental studies. The fish used for the experimental infectivity were obtained from Alkhaldiah farm at El. Fayoum Governorate, Egypt. A total number of 80 catfish (*Clarias gariepinus*), with average weight of 120.0g $\pm$ 10.0 gm and 80 *Tilapia nilotica* with average weight of 60.0 $\pm$ 10.0 gm were used in the experiment. Bacteriological examination of random kidney samples proved that the used fishes were free from *E. tarda* and other bacterial pathogens. The used fishes were transferred alive to the wet laboratory of Fish Disease and Management, Faculty of Veterinary Medicine, Cairo University. The fishes were grouped into 16 groups each contain 10 fish of

Table (1): Number and types of collected fish in different seasons.

Season	Water temperature	Fish	
		Catfish (*C. gariepinus*)	*Tilapia nilotica*
Winter	8-12 ˚C	30	30
Spring	12-18 ˚C	70	70
Summer	22-28 ˚C	70	70
Autumn	18-22 ˚C	70	70
Total	-	240	240

the same kind and acclimatized in 16 separate full glass aquaria (30x 40x 80cm) containing Chlorine-free tap water for 14 days before the onset of the experiment according to Innes (1966). Temperature was thermostatically controlled and kept at $24^0C \pm 2^0C$ simulating the water temperature of the fish pond when the bacteria were isolated. Aquaria were supplied with oxygen through an air pump (Sicc- Alis and Pieters, Italy).

Clinical and post mortem examination of the fishes. The naturally and experimentally infected catfish and *Tilapia nilotica* were clinically examined to determine the clinical abnormalities, and the post-mortem changes. The fishes were opened under aseptic condition and three line techniques was adopted according to (Kimberley, 2004). The interior of the body was exposed and examined for abnormal post mortem changes according to (Noga, 2000).

Bacteriological examination. After exposure of the internal organs, sterile samples from skin, liver, spleen, kidney and muscles were collected for bacterial isolation. The preparation of samples was carried out according to (Buller, 2004).

Isolation and biochemical characterization of *Edwardsiella* species recovered from fish. Isolation and identification of pure cultures of *Edwardsiella* species was done using API20E system according to (Soliman *et al.,* 1991).

Preparation of *E .tarda* culture for experimental infection. The isolated strain of *E. tarda* was grown on EIM (Edwardsiella Ictaluri selected Medium) according to (Shotts and Waltman, 1990). Small translucent greenish colonies were picked up and re-grown to ensure purity before being used in this study. The bacterial cells were grown at 32 ˚C for 24 hours. The LD50 value of the *E. tarda* in catfish and *Tilapia nilotica* was determined and calculated following (Reed and Muench,1938). A bacterial culture of *E. tarda* with a density of 10^8 colony forming units (CFU) in nutrient broth was used in the experimental infection in both types of fish.

Experimental infection of fish with the isolated *E. tarda.*

Preparation of bacterial inoculum. Five typical colonies of *E. tarda* were suspended in Mueller Hinton broth, the broth was incubated at 32 ˚C for 24 hours until its turbidity exceeds that of the standard McFarland tube number 1. The turbidity was adjusted to match a McFarland tube number 1 by adding sterile saline and confirmed through plate count according to Sahoo et al., (2000).

Experimental design. The experiment was carried out on 8 groups of catfish (each group of ten fish) with an average weight of 120.0 ±10.0 gm, and 8 groups of *Tilapia nilotica* (each of ten fish) with an average weight of 60.0 $\pm$ 10.0 gram. Two routes of infection were tried, the intraperitoneal and the intramuscular route (Darwish *et al.,* 2000).

As shown in Table (2), the first eight groups of catfish and *Tilapia nilotica* (each consists of 10 fish) were experimentally infected by I/M injection of a dose of 0.3 ml of a bacterial suspension containing 10^8 CFU/ml of the originally isolated strain of *E. tarda*. The second eight groups of catfish and *Tilapia nilotica* species (each consists of 10 fish) were experimentally infected by I/P injection of the same dose of *E. tarda* that has been passaged for one time in fish before used. All fish were kept under observation for three weeks.

Samples were taken from inoculated fish in each group day after day started from first day post inoculation. The clinical, post-mortem examination and re-isolation trial of the inoculated bacteria from the internal organs including liver, kidney, spleen and muscles were recorded among killed and/or dead fishes.

PCR detection of the hemolysin gene fragment in visceral organs of fish challenged by *E. tarda*. PCR was applied for detection of the hemolysin gene fragment of *E. tarda* in visceral organs of experimentally infected fish according to Chen *et al.,* (1996). PCR samples were taken from liver and kidney of catfish and *Tilapia nilotica* that were injected I/P with /0.3ml of 10^8 CFU/ml of *E. tarda* bacterial suspension. The primers used include a forward primer: 5'-CCTTATAAATTACTCGCT, from 744 to 761 bp of ORF II; and reverseprimer:

Fig.1: Catfish experimentally infected with *E. tarda* showing external skin hemorrhages, ulcers, and fin rot.

Fig.2: Catfish experimentally infected with *E. tarda* showing deep ulcers reached the dorsal muscles.

Fig. 3: *Tilapia nilotica* showing signs of septicemia.

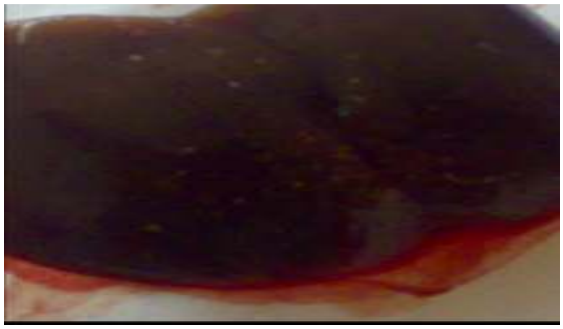

Fig. 4: Liver of Catfish showing multiple tiny white foci and congestion.

5'TTTGTGGAGTAACAGTTT, from 1850 to 1833 bp of ORF III, 400 µM dNTP (Bio-Lab, Beverly, MA, USA), as described by (Chen *and Lai*, 1996).

Results and Discussion

Emphysematous putrefactive disease (EPD) or what is newly termed, Edwardsiella septicemia, is a bacterial disease caused by *E. tarda* that affects mainly water catfish and its incidence increase with the rise in water temperature and environmental stress (Meyer and Bullock, 1973; Roberts, 2001). This disease is considered of highly economic importance as it lower the fish marketability due to the bad appearance and the unacceptable putrid odour produced by the bacteria (Noga, 2000).

E. tarda is a common pathogen, which has been isolated from farmed fish such as eel, *Anguilla Japonica*, Tilapia, flounder, sea bream, striped bass and others (Kubota, 1981, Nakatsugawa, 1983, Herman and Bullock 1986, Chen *et al.* 1996 and Mohanty and Sahoo 2007). In addition, this pathogen has been isolated from mammals such as seals and cetaceans (Regalla, 1982).

Results of clinical and post-mortem examination of fish naturally infected with *E. tarda.* The clinical and post-mortem examination of catfish (*Clarias gariepinus*) and catfish (*Clarias gariepinus*) and *Tilapia nilotica* naturally infected with *E. tarda* revealed the presence of congestion and haemorrhages allover the fish body especially at base of fins, operculum and belly as well as hemorrhagic mouth and vent. Macroscopically the infected fish showed fin and tail rot, skin darkening. The scales were raised or removed, extensive ulceration and excess mucus was also recorded.

The post-mortem examination of naturally infected fish revealed a wide variety of lesions of which, abdominal distention with yellow ascetic fluid was more common. The liver of the examined fish ranged from pale anemic or yellowish in some cases to deep brown in others. The gall bladder was distended with bile and the spleen appeared congested and enlarged in most of examined fishes and the intestinal tract was usually haemorrhagically inflamed. Regarding the skin and muscles of infected catfish; there were ulcers. Similar post mortem findings caused by *E. tarda* were reported by several authors (Miyazaki et al., 1992; Eaves *et al.* 1990; Baya *et al.* 1992 and Eissa and Yassien, 1994).

Results of the studies on the prevalence of *E. tarda* in catfish and Tilapia nilotica. Only one isolate of *E. tarda* was isolated from catfish with a recovery rate of 0.42% in summer season, where water temperature was about

Table (2): Route, dose and bacterial titer in fish experimentally inoculated with *E. tarda*

Type of fish	*Route of inoculation*	*Number of fish (original isolate)*	*Number of fish (re-isolated strain)*	*Dose/fish Using* 10^8CFU/ml
Catfish	**I/P**	**10**	**10**	**0.3ml**
	I/M	**10**	**10**	**0.3ml**
	Control (1)	**10**	**10**	**0.3ml saline**
	Control	**10**	**10**	**-**
	total	**40**	**40**	**-**
Tilapia nilotica	**I/P**	**10**	**10**	**0.3ml**
	I/M	**10**	**10**	**0.3ml**
	Control (1)	**10**	**10**	**0.3ml saline**
	Control	**10**	**10**	**-**
	Total	**40**	**40**	**-**

Table (3): Phenotypic and biochemical characterization of *E. tarda* isolated from catfish

Parameter	Result
Cultural characters: (MacConky's)	Small, circular, transparent, raised and grayish white colonies.
Morphological characters:	Gram– ve, motile, rod shape
Biochemical characters:	
Catalase	+
Citrate	+
Indole	+
H2S	+
Oxidase	-
Urea	-
MR (Methyl red)	+
V . P (Vogus preskauer)	-
Nitrate reduction	+
Glucose	+
Sucrose	-
Mannitol	-
Lactose	-

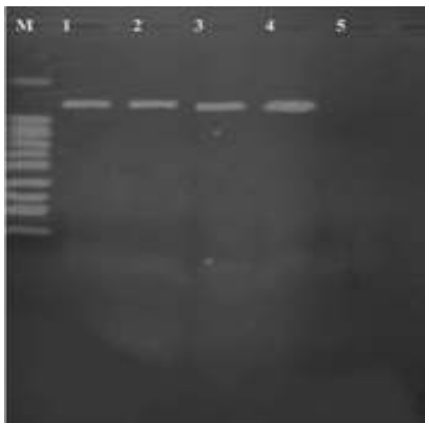

Fig.5: The 1106-Bp PCR product detected from liver, kidney samples taken from infected fish of Cat fish (line 1, 2) and Tilapia nilotica (line 4,5).

24°C. Isolation of *E. tarda,* from *Tilapia nilotica*, however, was unsuccessful. Isolation of *E. tarda* was successful from intestine, kidney and liver, while its isolation from spleen and muscles failed. Marked variation in the recovery rates of this bacterial species among different species of fish (from 2 up to 59%) has been recorded by several authors (Van-Damme and Vandepitte 1980; Eissa and Yassine, 1994; Rashid *et al.,* 1994). The bacterial infection in all fish species appeared to be a reflection of their respective environments (Ogbondeminu and Okaeme, 1986). High temperature and water pollution play a great role for enhancing the infection of fish.

Isolation and identification of the pure culture was done using selective medium according to (Buller, 2004). The morphology and biochemical test results of the isolated bacterium was confirmed to be *E. tarda* on the bases of biochemical characterization using API 20E system assays as shown in Table (3) and as described by (Roberts, 1989; Ling *et al.,* 2001; Quinn *et al.*, 2002).

The isolated strain was grown on EIM (*Edwardsiella Ictaluri* selective medium) according to (Shotts and Waltman, 1990), and the small translucent greenish colonies of *E .tarda* were picked up and re-grown to ensure purity before being used in the experimental infection studies.

Clinical and behavioral abnormalities were recorded in catfish and *Tilapia nilotica* experimentally infected with the originally isolated *E. tarda*, from day one till the end of 3 weeks post infection. However, the clinical signs and gross response were mild. Re-isolation of the injected bacteria was attempted from experimentally infected fish (catfish and *Tilapia nilotica*). The re-isolation was successful only from liver, kidneys, and intestine.

The use of the passaged (re-isolated) *E. tarda* in experimental infection of catfish and *Tilapia nilotica*, induced more strong clinical and behavioral abnormalities from day one till the end of 3 weeks post infection. This agrees with Sahoo and Mukherjee (2002) who re-inject the *E. tarda* isolated from catfish in the same fish species to increase its pathogenicity.

The intraperitoneal injection of *E. tarda* induced high mortality and severe lesions as compared to the intramuscular route. This result was explained by Eissa and Yassien, (1994) and was in accordance with Amandi *et al.* (1982) who recorded that the pathogenicity of *E. tarda* was demonstrated experimentally in salmon by intraperitoneal injection.

In catfish experimentally infected with the passaged *E. tarda* isolate high mortality rate was recorded and reached to 30% of the injected groups starting from the day 7 post infection and the clinical signs and gross lesions in catfish included petechiae and cutaneous ulcers (Fig. 1 and 2). Raised edematous area with liquefaction of underlying tissues; congestion of the fins and petechial hemorrhages all over the body surfaces were observed. Excellent descriptions of the clinical manifestation of *E. tarda* infections in catfish were reported by Meyer and Bullock (1973), Soliman et al., (1991) and Ling et al., (2000). The skin ulcers seen in our experiments were also similar to those reported by Francis-Flody *et al.* (1993) in largemouth bass, *micropterus salmoides*, infected with *E. tarda*.

Experimental infection of *Tilapia nilotica* with *E. tarda* resulted in 0% mortality it induced a range of abnormalities including sluggish movement and loss of escape and defense reflexes. Symptomatically, Scale detachment and pale coloration (Fig. 3), severe edematous swelling at the site of injection, presence of swollen abdomen (Fig. 4) filled with yellowish ascetic fluid; protruded haemorrhagic anus and opaqueness to the eyes were also recorded. These findings are much similar to those reported by (Kubota, 1981).

Internally, in both catfish and *Tilapia nilotica*, severe hemorrhagic enteritis with adhesion between organs was recorded. In catfish; the liver showed a multiple tiny white foci (Fig. 5). These findings were in agreement with (Baya *et al.*, 1992; Noga, 2000)

The pathogenesis of *E. tarda* in fish was investigated by Ullah and Aria (1983a, b)) and was attributed to secretion of haemolysins and

dermatotoxins, (exoenzymes). *E. tarda* produces both an Exotoxin Extracellular Products (ECP) and Endotoxin Intracellular Components (ICC) that are lethal to eels and flounders and play an important role in the pathogenicity of this organism.

Recently, Singh and Tiwari (1996) revealed that all the pathogenic *E. tarda* had either type 1 fimbriae or colonization factor. On iron deficient medium *E. tarda* produced siderophore, which permit the pathogen to scavenge for iron in the blood of the host (Park, 1986; Kokubo *et al.* 1990, and Mathew et al., 2001). Certainly the ability of *E. tarda* to acquire iron or produce toxin is an important part of the infection process. One of the virulence factors of *E. tarda* is hemolysin, which is identified as an endotoxin because it is not secreted outside of the cell (Ullah and Arai 1983a b; Janda *et al.* 1991a, b; Janda and Abbott 1993).

Results of PCR detection of the hemolysin gene fragment in visceral organs of fish challenged by *E. tarda.* The 1106-bp PCR product was detected in liver and kidney samples taken from experimentally infected catfish and *Tilapia nilotica*. The PCR gave positive results 4 days and 7 days post experimental infection of catfish and *Tilapia nilotica*, respectively. This result suggests that live bacteria residing in the visceral organs of infected fish can be easily detected by using the oligomers of the hemolysin gene as primers for PCR assay.

A DNA fragment associated with hemolysin production in the *E. tarda* strain. Direct PCR detection of the hemolysin gene of live bacteria (liver& kidney) is potentially simpler, more accurate, and rapid. Since direct probing for the presence of live *E. tarda* in infected fish by PCR should be very reliable and helpful in anticipating and preventing epidemics, which otherwise occur so frequently in fish farms.

In conclusion*, E. trada* may not be a major clinical disease problem for cultured catfish under proper husbandry and management, but other wise and by increase of other stressors; infection by *E. tarda* may evoke serious ecological and economical problems. The severity of the disease depends on the virulence of the organism. Integrated rearing of catfish and *Tilapia nilotica* may lead to disease outbreaks as the experimental infection cleared that *Tilapia nilotica* can get infected by *E. tarda* isolated from catfish and expressed clear septicemia and mortalities.

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دراسات علي عدوي الإدواردسيللا تاردا في أسماك القرامبط والبلطي

تم عزل الإدواردسيللا تاردا من أسماك العائلة القطية (قرموط السمك) المستزرعة في المياه العذبة بمعدل حدوث (0.42%) في فصل الصيف كما تمت دراسة تأثير العدوي التجريبية لميكروب الإدواردسيللا تاردا في قرموط السمك وكذلك في سمك البلطي بالحقن البريتوني والحقن العضلي و أظهرت قرامبط السمك التي تم عمل عدوي تجريبية لها من الإدواردسيللا تاردا بجرعة (0,3) مليلتر وتركيز (10^8ميكروب/مليليتر) أعراض وصفة تشريحية مميزة بصورة أوضح من أسماك البلطى والتي تم عمل لها أيضاً عدوي تجريبية ، ولقد أعطي تفاعل البلمرة المتسلسل نتائج إيجابية فى اليوم الرابع من إحداث العدوى التجريبية فى القرامبط وفى اليوم السابع فى أسماك البلطى.