Studies on Edwardsiellosis in Some Marine Fishes Using Molecular Diagnosis at Suez bay

Eissa, I.A.M, Maather El-Lamie, M., Mona Ismail M., Abd-Elrehim, A.A.M*

*Dept. of Fish Diseases and Management, Faculty of Vet. Medicine, Suez Canal Univ. *Royal International Inspection Lab. (RIIL), Ain-Elsokhna, Suez governorate*

Abstract

A total of 240 marine fishes *Mugil cephalus, Rastrelliger kanagurta* and *Nemipterus japonicas* were randomly collected from Attaka fishing port, Suez-bay, Suez Governorate (60 in each winter and fall and 120 in spring).The infected fishes showed hemorrhages all over the body surface including gills cover and fins, scales detachment accompanied by slight swelling of abdomen. Isolation was done from gills, liver, kidneys and intestines using Tryptone Soya Agar medium and X.L.D medium. Morphological and biochemical identification of bacterial isolates revealed three different isolates of *Edwardsiella tarda* (A, B and C). Their diagnosis was confirmed using Microbact 24E and 16S RNA gene for PCR. Their virulence was differentiated by H2S, Indole, Catalase test and haemolysis on blood agar (α, β and γ). The total prevalence of *E. tarda* isolates was (9.6%) among all examined marine fishes while seasonal prevalence was (15%) in spring, (3.3%) in winter and (5%) in fall.

Key words: Edwardsiellosis**,** *E. tarda*, *Mugil cephalus, Rastrelliger kanagurta, Nemipterus japonicas,* PCR.

Introduction

Edwardsiellosis is a fish disease responsible for extensive losses in both freshwater and marine aquaculture. *E. tarda* infection is the causative agent of Edwardsiellosis for many commercially important cultured and wild fish **(Thune *et al.*, 1993).** It causes septicemia with extensive skin lesions affecting internal organs such as liver, kidney, spleen and musculature. These bacteria systemically avoid host defense mechanism, thereby, rapidly proliferating within the host and causing death **(Hossain *et al.*, 2011).**

The present study was planned for investigating edwardsiellosis in some naturally infected marine fishes in relation to clinical picture, isolation and identification of the causative organisms using traditional and recent techniques and detection of the total and seasonal prevalence.

Materials and Methods

Fishes:

A total number of 240 fish (80 *Mugil cephalus*, 80 *Rastrelliger kanagurta*, 80 *Nemipterus japonicas*) with an average body weights (228.5 ±10, 124.3 ±10 and90.6 ±10g) respectively. They were randomly collected from Attaka fishing port, Suez-bay, Suez Governorate (60 in winter and in fall and 120 in spring)**.** The collected fish specimens were transported in an ice-box to Royal International Inspection Laboratory (RIIL), Ain El-Sokhna, Egypt to be examined.

Clinical picture:

Fishes were examined clinically by naked eyes according to ***Austin and Austin (1989*****)** for any clinical abnormalities on the external body surface such as small hemorrhages, necrotic lesions, scale detachment, pale coloration, skin ulceration, gas filled blisters, swollen abdomen with yellowish ascetic fluid, protruded hemorrhagic anus with opaque eyes. Internal organs were examined by naked eyes for any gross abnormalities as hemorrhagic enteritis, and foul odor emitted from abdomen.

Bacterial examination:

a. Isolation:

Isolation was done from liver, kidneys, gills, spleen and intestines. According to ***Wei and Musa (2008)***, samples of the examined fishes were taken out aseptically and homogenized separately in sterile MRD (Oxoid). Aliquots of 0.1 ml were inoculated on (XLD) (Oxoid) agar plate with NaCl 4% by spread plate method followed by 48 hr incubation at 28±1^{o}C. Clear colonies with black centre and reddish peripheral ring in diameter 1 to 2 mm on the XLD agar plate were selected and streaked on (TSA) (Oxoid) containing 0.5% NaCl.

b. Identification:

Pure colonies selected for the presumptive tests for morphological and biochemical identification (Gram stain, oxidase, motility, virulence) in addition to biochemical identification was carried-out using Microbact 24E (Oxoid).

PCR technique was applied for detection of *E. tarda* and the primers for 16S rRNA was designed as described according to ***Li et al. (2011).***

Results

Clinical pictures:

The clinical examination of the naturally affected fish showed hemorrhages all over the body surface, gill cover and fins, tail and scales detachment (photo 1) and (photo 2) accompanied by slight swelling of abdomen and opaque eye (photo 3). Other fishes showed anal protrusion and congestion of gills. Internally, liver was pale and swollen with hemorrhagic patches. Intestines were pale with ulceration and degeneration at their end with thick white opaque mucus and kidneys were congested with blood and enlarged (photo 4). In some cases, the internal organs seem to

adhere together with foul odor emitted after opening the fish.

Isolation and Identification:

According to the morphological and biochemical reactions, there were three isolates identified as *E. tarda*. The three isolates were motile and Gram negative, oxidase negative using Microbact 24E for biochemical confirmation revealed that Urease negative while positive results were recorded for Catalase, lysine, ornithine, H2S production, Citrate, and Indole(except isolate C was negative). They were differentiated according to biochemical characters and virulence factors **(Table 1)**.

Total and seasonal prevalence of *E. tarda* in the examined marine fishes:

The total prevalence of *E. tarda* isolates was (9.6%) among all examined marine fishes while seasonal prevalence was (15%) in spring, (5%) in fall and (3.3%) in winter (Table, 2 and Fig 1).

The total prevalence of *E. tarda* isolates for mullet (*M. cephalus*) was (12.5%), threadfin bream (*N. japonicas*) was (12.5%) and for Indian mackerel (*R. kanagurta*) was (3.8%), (Table, 3 and Fig 2).

Molecular diagnosis (PCR technique): PCR technique was done for the confirmation that three isolates (A, B and C) were *E. tarda* by using specific primer, bp 518. (Photo 5).

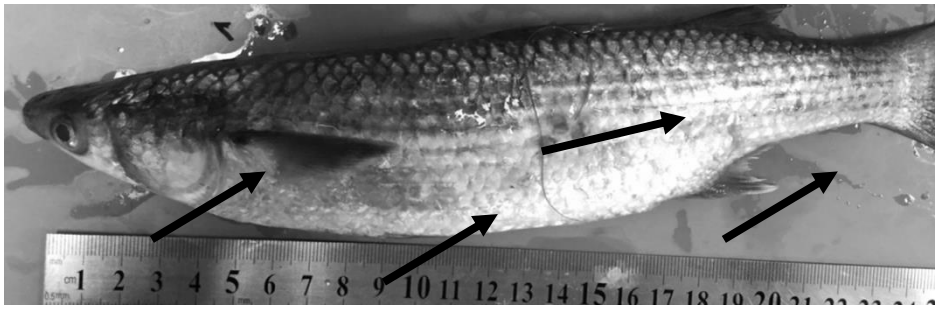

Photo 1: Mullet (*M. cephalus*) showing hemorrhages on different parts of body surface with scale detachment (Arrows).

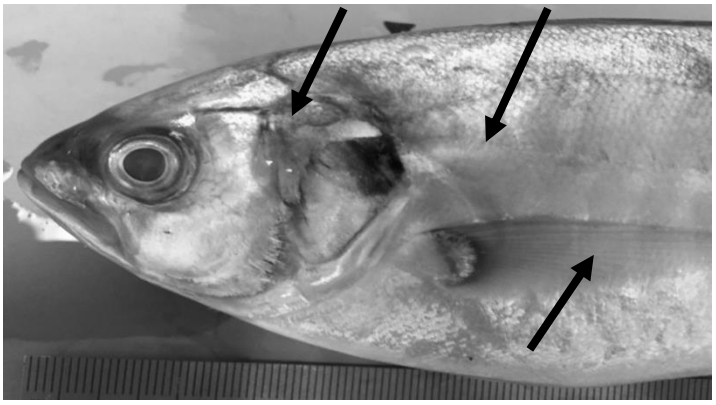

Photo. 2: Indian mackerel (*R. kanagurta*) showing hemorrhages on mouth, near eyes with slight opaqueness, around gill cover and pectoral fin (**Arrows**).

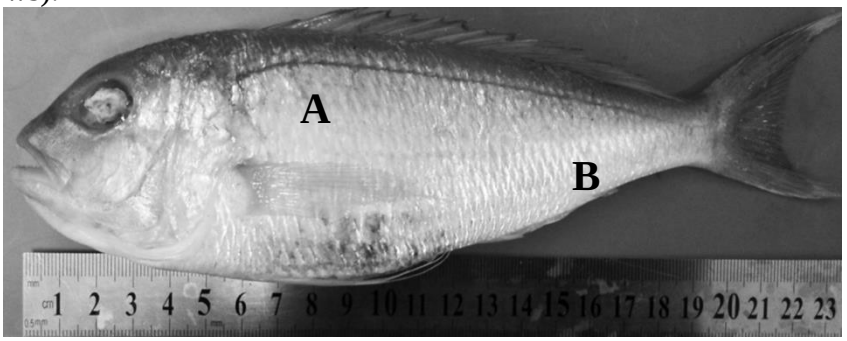

Photo. 3: Threadfin bream (*N. japonicas*) showing eye necrosis (**A)**, scale detachment and abdominal swelling (**B)**.

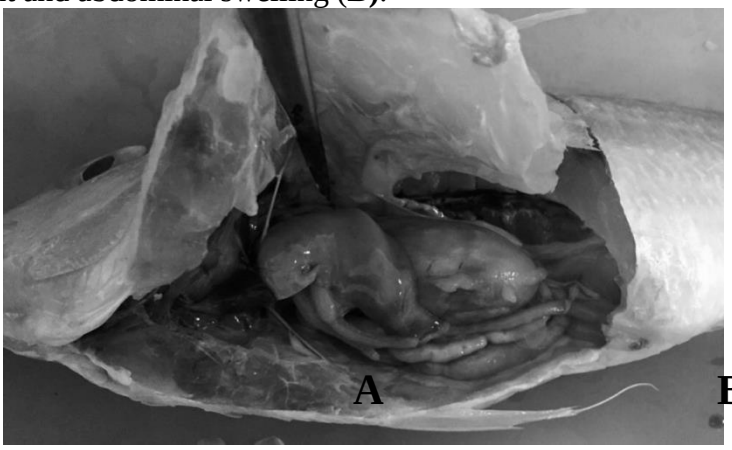

Photo. **(4):** Threadfin bream (*N. japonicas*) showing swollen liver with hemorrhagic spots **(A**), intestine swollen with tiny foci (**B**) and kidneys congested with blood (**C**).

Table (1): *Showing results of virulence factors among the three isolated strains.*

Season	Specimens No.	No. infected fishes	% infected fishes
Spring	**120**	**18**	**15**
Winter	**60**	**2**	**3.3**
Fall	**60**	**3**	**5**
Total	**240**	**23**	**9.6**

(+) positive, (-) negative, (α) narrow zone of haemolysis, (β) wide zone of haemolysis and (γ) no zone of haemolysis.

Table 2: *Showing seasonal and total prevalence of E. tarda among the examined marine fishes.*

Test / Isolate	H_2S	Indole	Haemolysis on Blood Agar	Catalase
Isolate (A)	+	+	**α**	+
Isolate (B)	+	+	**β**	+
Isolate (C)	+	-	**γ**	+

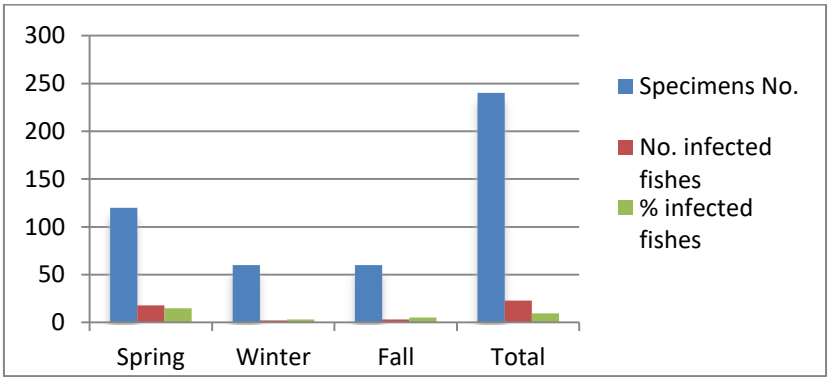

Fig 1: *Showing seasonal and total prevalence of E. tarda among all of the examined marine fishes.*

Table 3: *Showing total prevalence of E. tarda isolates among the examined marine fishes.*

Type of fish	Specimens No.	No. infected fishes	% infected fishes
M. cephalus	80	10	12.5
R. kanagurta	80	3	3.8
N. japonicas	80	10	12.5
Total	240	23	9.6

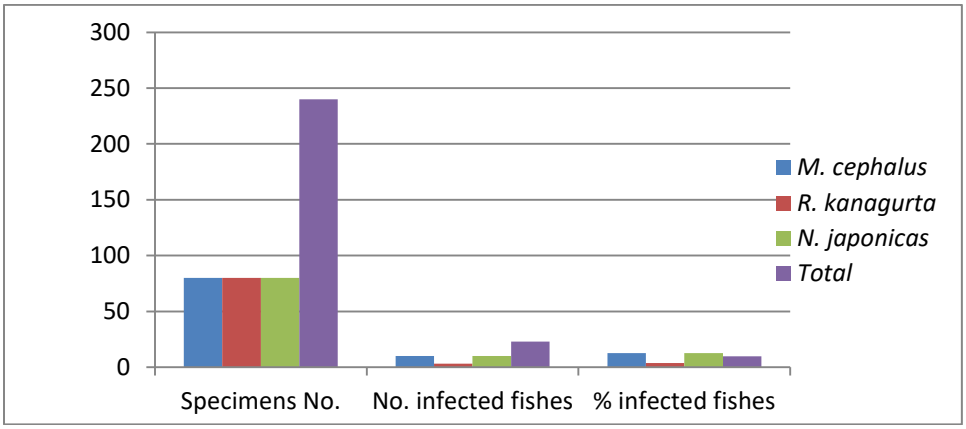

Fig 2: Showing total prevalence of *E. tarda* isolates among the examined marine fishes.

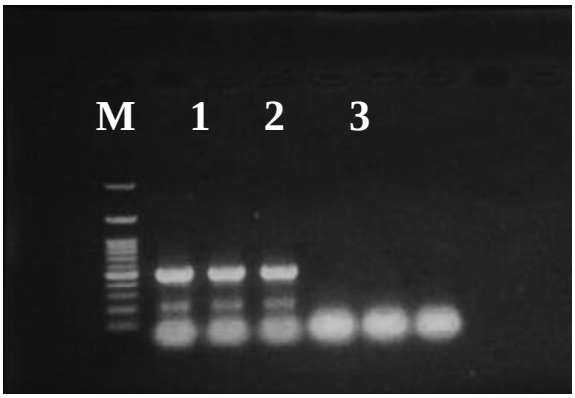

Photo (5): Lane M (Marker); Lane 1 strain A; Lane 2 strain B; Lane 3 strain C, bp 518.

Discussion

In the present study, the external clinical pictures of the naturally infected marine fishes Mullet (*M. cephalus*), Indian Mackerel (*R. kanagurta*), Threadfin bream (*N. japonicas*) were hemorrhages all over the body surface including gill covers, congested gills with hemorrhagic spots; fins and tail; accompanied by slight swelling of abdomen with foul smell; scales detachment; opaqueness of the eye and anal protrusion in some cases. Such obtained results are in agreement with those recorded by ***Alcaide et al. (2006)*** who isolated *E. tarda* from European eels and ***Lan et al. (2008)*** who isolated *E. tarda* from cultured turbot but not agree with those recorded by ***Ibrahem et al. (2011)*** *and* ***Iregui et al. (2012)*** as they recorded the same clinical pictures with presence of large blisters and abscesses filled with liquefied fluid in catfishes and tilapias and this attribution may be due to different fish species , site of study and environmental conditions. The present internal pictures were manifested as pale and swollen liver with hemorrhagic patches; foul smell emitted from abdomen; intestines were pale with ulceration and degeneration and kidneys were congested with blood and enlarged. Such obtained results agree with those recorded by ***Lan et al. (2008); Ibrahem et al. (2011)****;* ***Iregui et al. (2012)*** *and* ***Park et al. (2012)*** while not agree with ***Bullock and Herman (1985)*** who recorded large abscesses that develop in internal organs of Japanese eels which emit a malodorous gas when punctured

Isolation and identification of the causative bacteria revealed three isolates differentiated according to haemolysis on blood agar as (α, β and γ). They were motile and Gram negative, oxidase and Urease negative, positive results were recorded for Catalase, lysine, ornithine, H2S production, Indole(except isolate C), and Citrate. According to citrate utilization these results agree with ***Alcaide et al. (2006)****,* ***Woo and Bruno (2011)****,* ***Hashiem and Abd El-Galil (2012)*** *and* ***Wei et al. (2013)***. However, these results disagree with that of ***Lima et al. (2008), Joh et al. (2011) and Garcia et al. (2012)*** who mentioned that their isolates failed to utilize citrate; while ***Fatma Korni (2012), Das et al. (2014) and El-Seedy et al. (2015)*** found both reactions for citrate utilization (positive and negative) by their isolates in fresh water fishes and this attribution may be due to different fish species, site of study and environmental conditions.

Biochemical analysis and confirmation was done using Microbact 24E, which was used by ***Ling et al. (2000)***. However, ***Ibrahem et al. (2011)****,* ***Hashiem and Abd El-Galil (2012)*** *and* ***El-Seedy et al. (2015)*** used API 20 for biochemical confirmation.

In this study, 16S rRNA gene confirmed the diagnosis of the three isolates of *E. tarda*, this is in agreement with ***Jo et al. (2013)***; ***Pridgeon et al. (2014)*** who used 16S rRNA gene to identify 15 isolate of *E. tarda* and ***Mo et al. (2015)*** who used 16S rRNA gene to identify *E. tarda* during outbreak in cultured giant mottled eel *Anguilla marmorata*. However, ***Ibrahem et al. (2011)*** used only haemolysin gene for diagnosis of *E. tarda* in African catfish and Nile tilapia; ***El-Seedy et al. (2015)*** used major fimbrial subunit gene (etfA) and gyrB gene for identification of *E. tarda* isolates in African catfish and Nile tilapia.

The total prevalence of *E. tarda* isolates was (9.6%) among all examined marine fishes while seasonal prevalence was (15%) in spring, (3.3%) in winter and (5%) in fall. The results of total prevalence is nearly similar to that obtained by ***Alcaide et al. (2006)*** who reported 9% prevalence from wild eels in Spain; ***Woo and Bruno (2011)*** who mentioned that the impact of *E. tarda* in wild fish populations is unknown due to the absence of routine surveillance, monitoring and investigation of the effects of *E. tarda* infection that reveals a wide range of morbidity 5 to 30% ; ***Fatma Korni (2012)*** who also reported that, the prevalence of edwardsiellosis at Beni-Suef Governorate was 13.3% and ***Maysoon Abbas (2014)*** who reported that *E. tarda* infects 12% of total collected samples in Baghdad.

Mainly the higher rate of edwardsiellosis prevalence in warm temperature as in spring (15%) agreed with ***Lan et al. (2008), Lima et al. (2008)*** and ***Ibrahem et al. (2011)*** as they mentioned that the higher mortality and morbidity rates occur during warm seasons as spring and summer. Also, these results come nearly in accordance with ***Hashiem and Abd El-Galil (2012*)** who recorded 16.7% in the clinically diseased fish from May to September. However these results disagree with ***Joh et al. (2011)*** who recorded higher rates of edwardsiellosis in eel which reach 36% on pond level and ***Mo et al. (2015)*** who reported occurrence of edwardsiellosis in over 30% of juvenile eels. Recently, ***El-Seedy et al. (2015)*** reported presence of edwardsiellosis in 5% of the whole freshwater fishes (tilapias and African catfishes). This difference in prevalence may be attributed to location of the study, difference in water temperature, other environmental conditions as well as fish species.

References

Alcaide E., Herraiz S. and Esteve C. (2006): "Occurrence of *Edwardsiella tarda* in wild European eels *Anguilla anguilla* from Mediterranean Spain", Dis. Aquat. Org, 73; 77-81.

Austin B. and Austin D.A (1989): "Methods for the microbiological

examination of fish and shellfish”**;** Ellis Haward limited halsted press, Newyork, Brisban, Toranto, 59-68.

Bullock G. L. and Herman R. L. (1985): *“Edwardsiella* infections of fishes” US Fish & Wildlife Publications, 1-6.

Das B.K., Sahu I., Kumari S., Sadique M. and Nayak K.K. (2014): “Phenotyping and Whole Cell Protein Profiling of *Edwardsiella tarda* strainisolated from infected Freshwater Fishes” Int.J.Curr.Microbiol.App.Sci, 3(1): 235-247**.**

El-Seedy F. R., Radwan I. A., Abd El-Galil M. A. and Sayed H. H. (2015): " Phenotypic and Genotypic characterization of *Edwardsiella tarda* isolated from *Oreochromis niloticus* and *Clarias gariepinus* at Sohag Governorate" Journal of American Science;11(11) 68-75.

Fatma Korni (2012); “Edwardsiellosis in some fresh water fishes” Thesis for PhD to Dept. of Fish Diseases and Management, Beni- Suef University.

Garcia V. N., Iregui – Castro C. A. and **Hirono I.; (2012)** " Edwardsiellosis, common and novel manifestations of the disease: A review" Revista Colombiana de Ciencia Animal, 5 (1) ,82-90.

Hashiem M. and Abd El-Galil M. A. A. (**2012**): "Studies on Edwardsiellosis in *Clarias Gariepinus* Fish at Sohag Governorate", Journal of American Science, 8(4): 438-444.

Hossain M. M. M., Mandal A.S.M.S., Kawai K. and Chawdhury M.B.R.; (2011): “Temperatures effects on virulence of *Edwardsiella tarda* to Japanese eel, *Anguila japonica*” Bangladesh Research publications journal; 5(3) 245-251.

Ibrahem M. D., Shaheed I. B., Abo El-Yazeed, H. and Korani H.; (2011): “Assessment of the susceptibility of polyculture reared African Catfish and Nile tilapia to *Edwardsiella Tarda”* Journal of American Science,;7 (3) 779-786.

Iregui C. A., Guarín M., Tibatá V. M. and Ferguson H. W.; (2012): “Novel brain lesions caused by *Edwardsiella tarda* in a red tilapia (*Oreochromis* spp.)” Journal of Veterinary Diagnostic Investigation, 24(2): 446-449.

Jo G.A., Kwon S.B., Kim N.K., Hossain M. T., Kim Y.R., Kim E.Y. and Kong I.S.(2013): " Species-Specific Duplex PCR for Detecting the Important Fish Pathogens *Vibrio anguillarum* and *Edwardsiella tarda*" Fish Aquat Sci 16(4): 273-277.

Joh S.-J., Kim M.-J., Kwon H.-M., Ahn E.-H., Jang H. and Kwon J.-H. (2011): "Characterization of *Edwardsiella tarda* Isolated from Farm-Cultured Eels, *Anguilla japonica*, in the Republic of Korea", J. Vet. Med. Sci., 73(1): 7–11**.**

Lan J., Zhang X.-H., Wang Y., Chen J. and Han Y. (2008) "Isolation of an unusual strain of *Edwardsiella tarda* from turbot and establish a PCR detection technique

with the *gyrB* gene", Journal of Applied Microbiology 105:644–651.

Li G.Y., Li J., Xiao P., Guo Y.H. and Mo Z.L. (2011): " Detection of type III secretion gene as an indicator for pathogenic *Edwardsiella tarda*", Letters in Applied Microbiology, 52: 213–219.

Ling S.H.M., Wang X.H., Xie L., Lim T.M. and Leung K.Y. (2000): "Use of green fluorescent protein (GFP) to study the invasion pathways of *Edwardsiella tarda* in vivo and in vitro fish models"**,** Microbiology, 146:7-19**.**

Lima L.C., Fernandes A.A., Costa A.A.P, Velasco F.O., Leite R.C., Hackett J.L. (2008): "Isolation and characterizaton of *Edwardsiella tarda* from pacu *Myleus micans"***,** Arq.Bras. Med. Vet. Zootec., 60(1):275-277.

Maysoon S. Abbas (2014):" Isolation of bacteria from fish", International Journal of Advanced Research, 2(3): 274-279.

Mo Z.-Q. , Zhou L. , Zhang X. , Gan L. , Liu L. and Dan X.-M. (2015):" Outbreak of *Edwardsiella tarda* infection in farm-cultured giant mottled eel *Anguilla marmorata* in China**",** Fisheries Science, 81(5):899-905.

Park S. B., Aoki T., and Jung T. S. (2012): "Pathogenesis of and strategies for preventing *Edwardsiella tarda* infection in fish", Veterinary Research, 43(67):1-11**.**

Pridgeon J. W., Klesius P. H., Lewbart G. A., Daniels and Jacob H. V. M.;(2014):*"Edwardsiella tarda* and *Aeromonas hydrophila* isolated from diseased Southern flounder (*Paralichthys lethostigma*) are virulent to channel catfish and Nile tilapia", Journal of Coastal Life Medicine; 2(5): 337-34.

Thune R.L, Stanley L.A and Cooper R.K. (1993): "Pathogenesis of gram negative bacterial infections in warm water fish", Annu Rev. Fish Dis., 3:37-68.

Wei L. S. and Musa N. (2008): "Phenotyping, Genotyping and Whole Cell Protein Profiling of *Edwardsiella tarda* Isolated from Cultured and Natural Habitat Freshwater Fish" American-Eurasian J. Agric. & Environ. Sci., 3 (5): 681-691.

Wei L. S., Wee W., Manan Z. C., Amin M. R., and Hajisamae S. (2013): "A study of *Edwardsiella tarda* colonizing live Asian clam, *Corbiculafluminea*, from Pasir Mas, Kelantan, Malaysia with the emphasis onits antibiogram, heavy metal tolerance and genetic diversity", Aeterinarski Arhiv, 83(3):323-331.

Woo P.T.K. and Bruno D.W. (2011): "Fish diseases and disorders. Volume 3: viral, bacterial and fungal infections. In Edwardsiella septicaemias". 2nd edition Edited by Evans J.J., Klesius P.H., Plumb J.A. and Shoemaker C.A. Wallingford: CABI International: 512–534.