

Full Length Research Paper

Isolation of *Edwardsiella tarda*-like species and its frequency of occurrence in freshwater fish harvested for human consumption from Lake Hawassa and crater lakes around Bishoftu, Ethiopia

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A study was conducted to isolate *Edwardsiella tarda*-like species from apparently healthy freshwater fish (African cat fish, *Clarias gariepinus* and Nile tilapia, *Oreochromis niloticus*) originating from Lake Hawassa and crater lakes around Bishoftu, Ethiopia and thereby determine its distribution. Standard bacteriological examination of tissue specimens (kidney, liver and intestine) aseptically collected from fish harvested for human consumption resulted in a total of 16 isolates (10 from intestine, 4 from liver and 2 from kidney) showing similar colony and biochemical characteristics to *E.tarda* and were presumptively identified as *E.tarda*-like species due to the difficulties in differentiating them from the phenotypically similar new taxa viz. *E.piscicida* and *E.anguillarum*. There was significant variation ($P<0.05$) in the chance of isolating the bacterium among the organs examined with the organism being most frequently isolated from the intestine. Of a total of 210 fish included in the study, the bacterium was isolated from 16 (7.6%) of them with significant difference ($P<0.05$) observed in its occurrence with respect to origin of fish host. The isolation of *E.tarda*-like species from apparently healthy fish in the current study indicates potential sources of disease epidemics in fish as well as safety concerns on local fish products consumed raw or improperly cooked.

Key words: African Catfish, Nile tilapia, *Edwardsiella tarda*, Ethiopia.

INTRODUCTION

Edwardsiella tarda is a zoonotic fish pathogenic bacterium that causes a serious systemic bacterial disease known as Edwardsiella septicemia or Edwardsiellosis. It is a versatile organism causing infection in a variety of fish species of both fresh and marine waters (Austin and Austin, 1999) with significant losses reported in channel catfish (*Ictaluri punctatus*), striped bass (*Morone saxatilis*), eel (*Anguilla anguilla*),

Tilapia (Tilapia species) and flounder (*Paralichthys olivaceus*) (Plumb, 1999). The bacterium also infects reptiles, amphibians, marine mammals and other warm-blooded animals including humans (Plumb, 1999).

Edwardsiella tarda is ubiquitous and can normally be found as part of the normal intestinal micro-biota of fish and other aquatic animals as well as in the aquatic environment (Van Damme and Vandepitte, 1980; Kanai et al., 1988). Isolates identified as *E. tarda* from different host species were found to be genetically heterogeneous with wide genetic divergence between isolates from fish and mainly from humans (Nucci et al.,

2002; Abayneh et al., 2012; Yang et al., 2012).

Recent investigations into the versatile nature of the bacterium revealed that the taxon previously considered as *E. tarda* actually comprise three genetically distinct, yet phenotypically ambiguous taxa, namely; *E. tarda* (Ewing et al., 1965), *E. piscicida* (Abayneh et al., 2013) and *E. anguillarum* (Shao et al., 2015) which are all known to cause Edwardsiellosis in fish.

The disease in fish is characterized by a mild to severe condition, the onset and severity of infection being usually associated with stress factors such as high temperature, poor water quality and high organic fertility. Many epizootics of the disease were reported to occur in aquaculture situation where fish are subjected to environmental stresses and in condition of fluctuating water temperatures (Liu and Tsai, 1980; Amandi et al., 1982) or fish in highly enriched waters (Plumb, 1999).

Apart from being a fish pathogen, *E. tarda* is a health threat to humans when exposed to contaminated water or fish tissues or during ingestion of improperly prepared contaminated fish meal (Clarridge et al., 1980). The clinical picture in humans include usually of diarrhea, gastroenteritis, wound infection but occasionally meningitis (Plumb, 1999), liver abscesses (Zighelboim et al., 1992) and even death (Van Damme and Vandepitte, 1980; Plumb, 1999).

In Ethiopia, the significance of the problem due to the phenotypically similar *E. tarda* like species is not well investigated except for few reports on the isolation of the organism from some freshwater fish species of Lake Zeway (Yimer, 2000, Kebede and Habtamu, 2016) and Tana (Nuru, 2007). Few of the studies so far done indicated the significance of the bacterium as a fish pathogen and public health threat. However, information on the safety of fish products harvested for consumption with regard to contamination with *E. tarda*-like species and its distribution in fish species residing in many of the economically important freshwater rift valley lakes including lakes Hawassa and those around Bishoftu is lacking. Such information would otherwise be useful due to the potential threat of the bacteria for future aquaculture practices as well as public health owing to the practice of consuming raw or partially cooked fish meals in Ethiopia.

This study is, therefore, conducted with the objective of isolating *E. tarda*-like species from apparently healthy African catfish (*Clarias gariepinus*) and Nile tilapia (*Oreochromis niloticus*) harvested for human consumption from Lakes Hawassa and around Bishoftu and thereby determine its distribution.

MATERIALS and METHODS

Study site

Lake Hawassa

Lake Hawassa is located at an altitude of 1680m in the

central part of the Ethiopian Rift Valley (6°33' – 7°33'N and 38°29'E) 275km south of the capital, Addis Ababa. The lake has a surface area of 90km² and catchment area of 1250 km² (Zinabu, 1988) while the mean depth is 11m, the maximum depth being 22m (Zinabu et al., 2002). The lake is primarily fed by a small river named locally "Tikur Weha" that stems from shallow swamp and rivers (streams) on the north and west caldera walls which are ephemeral.

The fish fauna of lake Hawassa consists of about six species which include Nile tilapia (*Oreochromis niloticus*), African catfish (*Clarias gariepinus*), African big barb (*Barbus intermedius*), small barb (*Barbus paludinosus*), cyprinodont (*aplocheilichthyes antinorii*) and the cypinid (*Garra quadrimaculata*) (Desta et al., 2006).

Crater lakes in and around Bishoftu town

Fish fauna of crater lakes around Bishoftu town such as Lakes Hora and Babogaya comprise mainly of Nile tilapia (*Oreochromis niloticus*).

Bishoftu also known as Debre-zeit is located at 47 km South East of the capital at an altitude of 1900 meters above sea level. The mean annual rainfall in the area is 1151.6 mm with an average annual maximum and minimum temperature of 30.7°C and 8.5°C, respectively (National metrological service agency, 2000).

Study animals

The study was conducted between October 2009 and April 2010 at Lake Hawassa and two crater lakes (Hora and Babogaya) around Bishoftu town. A total of 210 fish comprising Nile tilapia, *Oreochromis niloticus* (#197) and African cat fish, *Clarias gariepinus* (#13) were included in the study. All the fish were apparently healthy and were harvested for human consumption.

Tissue sampling

Fish were examined for any external and internal lesions before and after autopsy, respectively and the findings recorded.

Tissue samples were then taken aseptically from liver, kidney and intestine for bacteriological analysis. Ventral approach to kidney was employed during dissection and sampling. Tissue samples were kept in ice box and transported to Faculty of Veterinary Medicine microbiology laboratory for isolation and identification of *Edwardsiella tarda*-like species.

Laboratory Techniques Isolation of *E. tarda*-like species

In the laboratory, tissue samples (kidney and liver) were placed on sterile petri dish and the surface decontaminated with 70% alcohol. Then, an incision

was made into the tissue specimen using sterile scapel blade. Sterile nichrome loop was then inserted into the incision and made to contact adequately to the incised tissue followed by inoculation on Xylose Lysine Deoxycholate (XLD) agar (Quinn et al., 1999). Inoculation from intestinal samples was done by opening the lumen from which swab samples were taken and inoculated on XLD agar plates. Replicate inoculations were made from each of the tissue samples to increase the chance of isolation. Inoculated plates were incubated at 37 °C for 24 hours. The resulting colonies that showed cultural characteristics consistent to *Edwardsiella tarda* were carefully selected and sub-cultured on MacCkonkey agar plates. All lactose non-fermenting colonies (pale colonies) were then selected from MacCkonkey agar plates and sub-cultured on Tryptic Soy Agar (TSA) plates for further identification.

Presumptive Identification of bacteria isolates

Pure overnight cultures of the isolates on TSA plates were subjected to a series of tests to arrive at presumptive identification. Primary identification of isolates was done employing Gram staining, Cytochrome oxidase and Catalase tests as well as test for motility according to the procedures described previously (Quinn et al. 1999). The results of Gram's reaction, oxidase and catalase tests of each isolate were recorded. Motility test was conducted using sulfide-indole-motility (SIM) medium (BBL) by inoculating fresh colony deep into the medium using sterile nichrome microbiological loop followed by incubation at 37°C for 24 hours. Un-inoculated SIM medium was also incubated along with inoculated media as a control. All incubated media were then observed for any turbidity or outgrowths from the line of inoculation and the findings recorded.

In secondary biochemical identification, a range of biochemical tests were carried out using the conventional test tube system.

Demonstration for Indole and hydrogen sulfide (H₂S) production: SIM media (BBL) was used to demonstrate indole and H₂S production characteristic of the isolates by inoculation of the media as described earlier followed by incubation for 24 hours at 37°C. The inoculated culture was observed for blackening of the media and development of red color upon the addition of drops of Kovac's reagent which indicate production of H₂S and indole, respectively.

Triple sugar iron agar (TSI) test: (TSI) test was carried out to demonstrate production of hydrogen sulfide and gas as well as fermentation of lactose, sucrose and glucose by stabbing the butt and streaking the slant TSI agar slant with a colony of the test isolate using microbiological nichrome loop. The results were then recorded after 24 hours of incubation at 37°C.

Citrate test: The ability of the bacterium to utilize citrate as a sole carbon source was determined by stab inoculating an overnight culture onto Simmon's Citrate agar with bromthymol blue as PH indicator. The stab cultures were then incubated at 37°C and monitored for a week for color change to blue which indicates a rise in PH as a result of citrate utilization (Quinn et al., 1999) and the results recorded.

Lysine decarboxylase test: To determine the ability of the bacterium in utilizing the amino acid lysine through the activity of the enzyme lysine decarboxylase was performed by inoculating a pure overnight culture onto lysine decarboxylase broth containing nutrient broth, 0.5% lysine and brom cresol purple indicator as described elsewhere (Quinn et al., 1999). The inoculated medium was then incubated at 37°C for 96 hrs after which the maintenance of the purple color or a color change to yellow is recorded as positive and negative tests, respectively.

Test for carbohydrate fermentations: Phenol red basal broth in test tubes containing sugars rhaminose and, xylose, and alcohols including dulcitol, mannitol, inositol and sorbitol were done according to the standard methods described previously (Quinn et al., 1999). Each test containing the respective substrate (sugar or alcohol) was inoculated with pure overnight cultures of an isolate after which the results are recorded after 24 hour incubation at 37°C. Color changes to yellow indicate positive results while absence of color change as negative.

Isolates showing characteristics that conform to *E. tarda* based on primary identification and secondary biochemical tests were presumptively identified as *E. tarda*-like species due to the fact that it is impossible to differentiate them from phenotypically similar species (*E. piscicida* and *E. anguillarum*) from *E. tarda* based on the currently employed phenotypic characteristics.

RESULTS

Attempts to isolate *E. tarda*-like species from different organs (liver, kidney and intestine) of apparently healthy cat fish, *Clarias gariepinus*, and Nile tilapia, *Oreochromis niloticus*, resulted in 16 isolates with consistent characteristics to *E. tarda* based on primary and secondary identification criteria and thus were presumptively identified as *E. tarda*-like species due to the difficulties to differentiate from the recently described genetically distinct taxa viz. *E. piscicida* and *E. anguillarum* based on the phenotypical tests employed. Morphologically, the colonies on Xylose-Lysine Deoxycholate (XLD) agar had clear appearance with some black center and central depression in some cases surrounded by reddened medium. All the isolates were gram-negative, catalase positive and unable to produce cytochrome oxidase. The biochemical tests showed that they were positive for lysine decarboxylation and produced indole except one isolate which failed to produce indole unlike the typical isolates. On Triple

Sugar iron agar, seven of the isolates produced hydrogen sulfide while the remaining did not (Table 1). All isolates neither utilized simmon's citrate nor fermented mannitol, inositol, dulcitol, xylose, rhaminose and sorbitol. Of the total isolates presumptively identified as *E.tarda*-like species four were non-motile. Table 1 summarizes the results of biochemical characteristics of the isolates.

Of a total of 210 fish (63 from Lake Hawassa and 147 from lakes in and around Bishoftu) examined, *E. tarda*-like species was isolated from 16 (7.6%) of them. The occurrence of *E. tarda*-like species showed significant difference ($P<0.05$) with respect to the two sites with the bacterium being more frequently isolated from fish of lake Hawassa than those from lakes in and around Bishoftu (Table 2). Significant difference ($P<0.05$) was also observed in the distribution of the isolates between African catfish and Nile tilapia (table 3). *E. tarda*-like species was isolated from 1.9% of liver samples ($n=210$), 4.8% of intestinal samples ($n=210$) and 1% of kidney samples ($n=210$) with significant variation ($P<0.05$) in the chance of isolating the bacterium among the organs examined (table 4).

DISCUSSION

E. tarda has long been known to be the cause of Edwardsiellosis in both cultured and wild fish with worldwide distribution as well as an opportunistic pathogen in other animals including humans (Meyer and Bullock, 1973; Ewing et al., 1995; Wakabayashi and Egusa, 1973; Eissa and Yassin, 1994). Recently, however, it was recognized that two more genetically distinct novel taxa namely: *E. piscicida* (Abayneh et al., 2013) and *E. anguillarum* (Shao et al 2015) but are indistinguishable from *E. tarda* based on the common phenotypic identification techniques are known to cause Edwardsiellosis in fish (Austin and Austin, 2016).

Since the common biochemical tests employed in this study cannot distinguish between *E. tarda* and the phenotypically similar novel taxa, the isolates obtained in the current study were considered as *E. tarda*-like species. Most of the isolates found in this study, showed morphological and biochemical characteristics that conform to *E. tarda* except few which did not produce hydrogen sulfide. Such isolates may actually be atypical strains of the bacterium with the ability to produce copious amount of acid from sucrose fermentation which may have prevented precipitate formation (Veron and Gasser, 1963).

The finding of both motile and non-motile *E.tarda*-like strains is consistent with previous reports. Motile *E.tarda* are the most commonly isolated strains in many studies (Austin and Austin,1999) although non-motile strains from red sea bream (*Pagrus major*) (Okuda et al., 2007) and turbot (Lan et al., 2008) were also reported. Both motile and non motile *E. tarda* strains

are known to be virulent, indicating that flagellum is not an essential virulence factor for pathogenicity (Janda et al., 1991). The occurrence of one isolate negative for indole found in this study agrees with previous reports of atypical (non-motile, indole negative and maltose negative) *E. tarda* strains isolated from catfish and their environment (Ewing et al., 1969) warranting further genetic characterization.

The most frequent occurrence of *Edwardsiella tarda* from intestine compared to kidney and liver may be due to the existence of *E.tarda*, in many instances, as part of the normal intestinal micro biota of aquatic animals (Wyatt et al., 1979; Van Damme and Van de pitte, 1980; Kanai et al., 1988). Isolates from intestine may probably be non-pathogenic strains which may later acquire virulence and become a source of infection under certain circumstances such as when the host is stressed. The differences noted in the frequency of *E.tarda* isolates with regard to fish species and site in this study may be attributed to differences in the intestinal micro biota of a specific species owing to the differences in fish digestive systems and the environmental condition, respectively (Cahill, 1990). Differences in the frequency of bacteria occurrence among aquatic environments are associated with variation in bacterial load and/or salinity of the habitats (Cahill, 1990 and Ringo et al., 2003). The characteristics of the micro-environment of the various sections of the alimentary tract of each fish species could also influence the taxonomic composition as well as numerical abundance of the bacteria present (Horsely, 1997). The relative distance and degree of exposure to the nearby source of pollution may also influence the frequency of bacterial isolation (Chill,1990; Ringo et al., 2003). The isolation of *E. tarda*-like bacteria from liver and kidney samples indicates subclinical infection which may eventually develop to disease depending on stress factors that compromise the immune system. Although an infectious disease epidemic is not a common scenario in wild fish due to the sparse population distribution, the finding of infectious pathogens such as *E. tarda*-like species may be a threat to future aquaculture practices. Recently bacteria biochemically identified as *E. tarda* has caused an outbreak with high mortality in tilapia pond culture maintained for research purpose in Ethiopia (personal communication) indicating its potential threat for intensive fish farming.

Due to the difficulty of identifying *Edwardsiella tarda* from the two recently described new species, *E. piscicida* (Abayneh et al., 2013) and *E. anguillarum* (Shao et al., 2015), based on phenotypic characteristics, further work based on molecular identification using species specific PCR is required to know the relative distribution and the potential threats of fish pathogenic *Edwardsiella* species in the Ethiopian freshwater ecosystem particularly of the inland lakes.

In conclusion, the isolation of *E.tarda*-like species from

Table 1. Characteristics of isolates presumptively identified as *Edwardsiella tarda* like species.

Characteristics	Reaction	
	Number of negatives	Number of positives
Oxidase	16	0
Catalase	0	16
Indole	1*	15
Motility	4*	12
Lysine decarboxylase	0	16
H ₂ S production on TSI agar	9*	7
Simmons citrate	16	0
Dulcitol	16	0
D-Rhaminose	16	0
Mannitol	16	0
Sorbitol	16	0
Innositol	16	0
Xylose	16	0

*Isolates which show deviation from characteristics expected of typical *E.tarda* strains.

Table 2. Frequency of occurrence of *Edwardseilla tarda* like isolates with respect to study sites.

Site	Result		Total
	Negative	Positive	
Hawassa lake	51	12	63
Bishoftu lakes	143	4	147
Total	194	16	210

($\chi^2 = 16.701^b$; df =1 & P = 0.00)

Table 3. Frequency of occurrence of *Edwardsiella tarda* like isolates by species.

Species		Result		Total
		Negative	Positive	
Cat fish Tilapia		9	4	13
		189	12	197
Total		194	16	210

($\chi^2 = 10.56$; df =1 & P = 0.01).

Table 4. Distribution of *E. tarda* isolates by organ of isolation.

Organ	Positive (expected values)	Negative (expected values)	Total
Kidney	2 (5.33)	208 (204.66)	210
Liver	4 (5.33)	206 (204.66)	210
Intestine	10 (5.33)	200 (204.66)	210
Total	16	614	630

$X^2 = 6.67$; df=2; P<0.05).

apparently healthy fish indicates their role as potential sources of infection presenting potential risk to future

aquaculture practices in such natural lakes and to humans consuming fresh or improperly cooked fish

products which is a common practice in Ethiopia. The results of the current study suggest further work on fish pathogenic *Edwardsiella* species in different species of fish residing in the different aquatic environments in the country to determine their epidemiology, significance and potential impact on the development of the fishery sector in Ethiopia.

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