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Isolation and Identification of *Edwardsiella tarda* from Lake Zeway and Langano, Southern Oromia, Ethiopia

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Abstract

A study was carried out from October, 2009 to April, 2010 with the objective of isolating *Edwardsiella tarda* an important fish pathogen from fish harvested for human consumption from Lake Zeway and Langanoo. A total of 372 tissue samples (three from each fish) comprising liver, intestine and kidney were collected from 124 fish (*Clarias gariepinus* and *Oreochromis niloticus* originated from Lake Langanoo and Zeway. Distribution of *E. tarda* infection among the three organs examined indicated that *E. tarda* was isolated most frequently from liver (6.5%) followed by intestine (2.4%) and kidney (0.8%) with significant difference among organs. Statistical significant differences ($P<0.05$) were found in *E. tarda* infection with respect to site although the bacterium was isolated from fish originating from both Lake Zeway and Langanoo with *E. tarda* being more prevalent in fish sampled from lake Zeway. *E. tarda* was isolated more frequently from male fish, the differences in the occurrence of *E. tarda* infection with respect to sex were not significant (P>005) indicating that both sexes are equally susceptible. The isolation of *Edwarsiella* from wild fish population of Lakes Zeway and Langano destined for human consumption in the current study is indicates that *E. tarda* is a potential threat of both the fishery sector/aquaculture and public health. Finally, as is the case for any infectious fish pathogen, there is limited information on *E. tarda* of fish in Ethiopia and hence further study to have comprehensive information on the agent is forwarded.

Keywords: Catfish; *Edwardsiella tarda*; Intestine; Isolation; Kidney; Langanoo; Liver; Tilapia; Zeway

Abbrevations:

µm: Micrometer; BHI: Brain Heart Infusion Agar; CHO: Carbohydrate; EIM: Edwardsiella Isolation Media; *E. tarda: Edwardsiella tarda;* FAO: Food and Agricultural Organization; FISH: Fluorescence *in Situ* Hybridization; g/l: Gram per liter; H_2O_2: Hydrogen Peroxide; H_2S: Hydrogen Sulfide; HPCE: Higher Performance Capillary Electrophoresis; LAMP: Loop-Mediated Isothermal Amplification; LFDP: Lake Fisheries Development Working Paper; ml: Milliliter; PCR: Polymerase Chain Reaction; SIM: Sulfur Indole and Motility Test Media; TSA: Tryptic Soya Agar; TSIA: Triple Sugar Iron Agar; USA: United State of America; V/V: Volume by Volume; WHO: World Health Organization; XLD: Xylose Lysine Deoxycholate

Introduction

Aquaculture is growing rapidly worldwide with fish being the primary sources of animal protein in many countries. The fishery sector plays a significant role in food security through supplementation of food for developing countries. As a whole fish currently make up about 19% of the total protein consumption or just over the 5% of proteins from both plants and animals origin [1].

As in all animal production systems, however, fish are possibly susceptible to microbial diseases which are one of the major problems hampering production, development and expansion of the aquaculture industry. Fish diseases are global problem affecting fresh water, marine water, cultured, sport and even ornamental fish. The problem is extremely important when fish are subjected to intensive culture practices [2].

The control of fish diseases is particularly difficult because fish are often farmed in system where production is dependent on natural environmental conditions. Changes or deterioration in the aquatic environment cause the occurrence of most fish disease and also environmental effects play a great role in influencing the health status of fish. Therefore, the multidisciplinary approaches involving the characteristics of potential pathogenic microorganisms for fish, aspects of the biology of fish as well as a better understanding of the environmental factors affecting such cultures will allow the application of adequate measures to prevent and control the diseases limiting fish production [3,4].

Edwardsiellosis is among the most important bacterial diseases causing severe economic losses in fish farms of many countries. The disease is caused by *E.tarda* which is a gram negative, motile, facultative anaerobic, short rod shaped bacterium (1µm in diameter and 2-3 µm long) pathogenic to a wide range of fish hosts such as Channel cat fish (*Ictaluri punctatus*), Striped bass (*Morone saxatili*), eel (*Anguilla anguilla*), Tilapia (*Oreochromis niloticus*), carp (*Cyprinus cyrpio*) and Flounder (*Paralichthys olivaceus* [5]. The organisms is frequently found in organically polluted water, poor quality water and affect fish stressed by this situations [6,7]. *E.tarda* can be isolated on Edwardsiella Isolation Media (EIM), Brain Heart Infusion (BHI), Tryptic Soya Agar (TSA), Xylose Lysine Deoxycholate(XLD) and MacConkey. It is seen as small, circular, raised, whitish with black center on XLD and pale on MacConkey agar, grow best at a temperature between 25°C-37°C, PH 7-8 and 0.5%NaCl

[7,8] and characteristically, catalase positive, cytochrome oxidase negative, glucose fermentative, indole positive, citrate negative, lysine positive, mannitol, dulcitol, sorbitol, inositol, xylose, rhamnose negative, produce hydrogen sulfide, alkaline slant and acid but on Triple sugar iron Agar [9].

Edwardsiella tarda is a health threat not only to fish and other animals but also to humans [10] with the risk factors being exposure to aquatic environment, pre-existing liver diseases, iron over load and raw sea food ingestion [11].

In humans the bacteria usually cause diarrhea, gastroenteritis, wound infection and even death [5,12]. There are reports of extra intestinal infection with the clinical pictures including a typhoid like illness, peritonitis with sepsis and cellulites with occasionally liver absecess [13] and meningitis [5]. The infection is more severe in immunocompromised individuals.

The practice of consuming partially cooked fish meals, manual handling of fish and unhygienic practice during filleting in Ethiopia indicate that the public is at higher risk of contracting the disease. Therefore, the disease deserves attention due to its impact on the fishery sector as well as its potential threat to future aquaculture industry and public health [14,15]. In Ethiopia, the bacterium has been isolated from apparently healthy fish of Lake Zeway [16] and Tana [17]. However, there is no further work done in covering the different fish species and aquatic environments. Therefore, this study was conducted with the aim of Isolating *E. tarda* from cat fish and Tilapia slauthered at zeway fishery resource center originating from Lake Zeway and Langanoo and elucidates the safety of fish products with respect to *Edwardsiella tarda* contamination.

Materials and Methods

Study site

The study site comprised fish species harvested from lakes Zeway and Langanoo.

Lake zeway: Lake zeway is located on the Eastern side of Zeway town, 163 km South east of Addis Ababa it lies in northern part of the rift valley between 7°51N to 8°7'N and 38'43' E 38°57' E with an open water area of 422 km^2 and shoreline length of 137 km. The lake is fed by two major rivers, i.e. Ketar and Meki River and has one out flow in the South, Bulbula river which flow into Abiyata [18]. Five bigger islands are situated in the lake Viz Tulu Gudo (4.8 km^2), Tsedecha (2.1 km^2), Funduro (0.4 km^2), Debresina (0.3 km^2) and Galila (0.2 km^2). While the latter two have few inhabitants, the three bigger ones are populated with several hundreds of people [19].

The catch from Lake Zeway consists of almost exclusively Tilapia (*Oeochromis niloticus*). Since recent years, however, Cat fish (*Carias gariepinus*) and Crucian carp (*Carcasius gracius*) have appeared in small amounts of the total catch [20]. There are a number of landing points around the lake from where fish is collected either by boat or trucks and brought to the major landing points adjoining Zeway town.

Lake langanoo: Lake langanoo is located 200 km South of Addis Ababa lying between 7°36' N;38°45' E. It is 18 km long and 16 km wide with an open water area of 230 km^2, 7.5 km shoreline and 1600 km^2 catchments area [18]. The main fish species in the lake include *Barbus* species, Clarias species and *Oreochronis niloticus* [14] with the total annual catch of 1000 tones.

Study animals

Study animals used in this study included African cat fish (*Clarias gariepinus*, N=30) and Nile tilapia (*Oreochromis niloticus*; N=94) which were harvested from Lake Zeway and Langanoo for human consumption. The fish were physically examined for any external lesions before necropsy and collecting tissue samples.

Necropsy and Tissue Sampling

In dissecting fish, ventral approach to kidney was employed. The fish sample was cut along the midline of the abdomen starting from the anus up to the mouth using sterile dissecting scissor followed by another dissection from the anus to the lateral line and further along the lateral line up to the gills cover to remove the lateral side of the abdominal wall and expose the internal organs. Internal organs were examined for any gross pathology and the findings recorded. Tissue samples were then taken from intestine (N=124), liver (N=124) and kidney (N=124) aseptically using sterile scalpel blade and forceps kept in sterile universal bottles of 100 ml capacities. All necropsy and tissue sampling procedures were carried out under asceptic condition. The bottles containing the samples were then kept in ice box containing ice packs all the way to School of Veterinary Medicine, Debre Zeit where they are processed for bacterial isolation and identification.

Laboratory Examination

Isolation of *Edwardsiella tarda*

Tissue samples from kidney, liver and intestine of cat fish and tilapia were homogenized in physiological saline. The homogenate was then taken by sterile loop and streaked on xylose lysine deoxycholate agar plate (Titan Biotech) and then incubated at 37°C for 24 hours. Colonies showing or resembling with morphological characteristics of E.tarda were further subcultured on MacConkey agar plates and incubated at 37°C for 24 hours. All lactose non-fermented colonies were further subcultured on tryptic soya agar containing 0.5% NaCl and incubated at 37°C for 24 hours. Presumptive identification of the resulting isolates (colonies) was done employing different tests which included primary bacteria identification techniques and biochemical identification tests.

Primary identification of isolates

Primary Identification of pure culture of the isolates was done based on gram reaction, motility tests, and catalase and oxidase tests. Overnight cultures of pure colony on tryptic soya agar (TSA) plates were used in all of these tests.

Gram reaction: Gram staining was done according to the procedure described by Rowland et al. [21] accordingly, colonies that were gram negative, short rods were considered for further tests.

Catalase test: Catalase test detects whether the bacterium has the enzyme catalase that converts hydrogen peroxide to water and gaseous oxygen. The test was carried out on pure fresh colony on tryptic soya agar plates [22]. Since Edwardsiella tarda is catalase positive, colony showing an elaborated bubble formation was considered positive and taken for further tests.

Oxidase test: This test detects the presence of cytochrome oxidase enzyme in a bacterial cell and characterized by purple colour formation within 10 seconds when the bacterial sample is made in

contact with 1 percent aqueous solution of tetramethyl-p-phenylenediamine dihydrochloride [22]. In this study, oxidase test was conducted employing filter paper method for each bacterial isolates.

Motility test: Motility test was conducted using sulfur, indole and motility test media. The slant of the medium in test tubes was stab-inoculated with fresh colony (isolate) using a straight sterile wire followed by incubation at 37°C for 24 hours [21]. Turbidity of the medium or outgrowths from the line of inoculation was considered indicative of motility and the results recorded.

Biochemical tests

Secondary biochemical identification of bacterial isolates was conducted employing conventional biochemical tests according to the standard procedures described previously [23,24]. These tests are based on the ability of the bacterium to utilize a sugar, an amino acid or an alcohol or any carbon source in the medium where by the byproducts of such reaction if any is detected using appropriate indicators incorporated into the medium. The detailed procedures of the biochemical tests are presented. In all of the tests, care was taken to maintain asceptic procedures to avoid contamination.

Triple sugar iron (TSI) test: Triple sugar iron agar (TSI) test shows hydrogen sulfide production, gas production, fermentation of lactose, sucrose and glucose. *E. tarda* is expected to show red slant and yellow butt with hydrogen sulfide production. In this work, TSI test was carried out by inoculating (by stabbing the butt and streaking the slant) of the test tube of TSI agar slant using straight inoculating wire after which the inoculated tube was loosely capped and the findings recorded after 24 hours of incubation at 37°C [21].

Indole production, motility and H_2S production tests: SIM media (BBL) was used to demonstrate indole production, motility and hydrogen sulfide (H_2S) production. SIM media in test tubes were inoculated with pure overnight grown colonies on TSA plates followed by incubation at 37°C for 24 hours after which the findings were recorded [22]. To demonstrate if there is any indole production, kovac's reagent was added to SIM media after 24 hours of incubation and deep red colour was developed.

Simmon's citrate test: Simmon's citrate slants in test tubes were stab inoculated in same way as SIM media and incubated at 37°C for a week after which the findings were recorded [22,23]. The test detects the ability of the bacterium to utilize citrate as the only carbon source which imparts blue in case of positive cases.

Lysine decarboxylase test: Test tubes with lysine broth were inoculated with pure overnight isolate followed by incubation at 37°C for 4 days. The findings were recorded after 96 hours of incubation. Absence of color change, i.e. the maintenance of purple color indicates the ability of the isolate to utilize the amino acid lysine and produce alkaline PH in the medium [21,23].

Sugar or carbohydrate fermentation tests: Conventional biochemical tests comprising four alcohols (dulcitol, mannitol, inositol and sorbitol) and two sugars (rhaminose and xylose) were used for adequate presumptive identification of *E. tarda*. Phenol red basal broth in Durham tubes containing 1% dulcitol, mannitol, sorbitol, inositol, xylose and rhamnose was prepared and inoculated with the isolates. The inoculates were then incubated at 37°C for 24 hours after which the results were recorded [21]. Bacterial isolates with consistent characteristics of *E. tarda* based on primary and secondary identification criteria were considered presumptively as *Edwardsiella tarda* fish isolates and preserved in 15% glycerol (V/V) at -20°C for further characterization studies.

Data Analysis

Descriptive statistics such as proportions and frequency were employed in summarizing the data. Chi-square test of independence was employed in comparing the prevalence/occurrence of *E. tarda* infection with respect to site, sex, fish species and organ of isolation. A confidence interval of 95% was used to interpret the statistical association and significance was considered when P-value is less than 0.05 [25].

Results

Of the total of 372 tissue samples comprising kidney, liver and intestine collected from 124 fish, *E. tarda* was isolated from 12 tissue samples (8 from liver, 3 from intestine and 1 from kidney). The isolates appeared as small punctuate grayish white colonies on xylose lysine deoxycholate agar after 24 hrs of incubation at 37°C. Except few of the isolates, most showed typical characteristics of *E. tarda* isolated elsewhere, which were gram negative short rods, motile, catalase positive and oxidase negative. In biochemical tests, these typical isolates were positive for indole, H_2S production, and lysine decarboxylase and unable to utilize Simmon's citrate and the different sugars used in this study (Table 1). However, some of the isolates showed variation from the typical characteristics. One isolate was negative for indole and able to utilize Simmon's citrate while the remaining was able to ferment mannitol, rhaminose, xylose and inositol (Table 1). Although these isolates divert in some of the biochemical tests from the typical characteristics expected of *E. tarda* they were considered as variants of *E. tarda* strain due to the fact that the cultural characteristics, growth requirements and the different tests suggested the isolates to be within *E. tarda* species.

Distribution of *E. tarda* infection among the three organs examined indicated that *E. tarda* was isolated most frequently from liver (6.5%) followed by intestine (2.4%) and kidney (0.8%) with statistical significant difference ($P<0.05$) among organs (Table 2).

Statistical significant differences ($P<0.05$) were found in *E. tarda* infection with respect to site although the bacterium was isolated from fish originating from both lake Zeway and Langanoo with *E. tarda* being more prevalent in fish sampled from lake Zeway (Table 3).

Although, *E. tarda* was isolated more frequently from male fish, the differences in the occurrence of *E. tarda* infection with respect to sex were not significant ($P>0.05$) indicating that both sexes are equally susceptible (Table 4).

There was no statistical significant difference ($p>0.05$) in isolation of *Edwardsiella tarda* from Catfish (*Clarias gariepinus*) and Tilapia (*Oreochromis niloticus*) indicating that both fish species are susceptible to the infection.

Discussion

The major species of *Edwardsiella* those infect fish are *E. tarda* and *E.ictaluri*. *E. tarda* infects fish and other animals including human being, while *E. Ictaluri* being a pathogen of fish only [26]. In this study, *E. tarda* were isolated from intestine, kidney and liver of fish indicating that the bacterium is a potential threat to aquaculture as well as to public health. The organism has been isolated from several sources

previously such as from intestine of fish, humans faeces with sporadic cases of diarrhea [12] and from dressed fish samples [27,28].

Parameter	Results	Remark
Cultural characteristics on XLD agar	**Small, circular, grayish white Colonies**	
Morphological characteristics	Gram negative, motile short rods	Two isolates, non-motile
Biochemical characteristics		
Indole production	+	One isolate, indole -ve
H2S production	+	
Oxidase	_	
Catalase	_	
Citrate	_	One isolate, citrate +ve
Lysine	+	
Mannitol	_	Four isolates,mannitol +ve
Dulcitol	_	
Inositol	_	Four isoltes,inositol +ve
Sorbitol	_	
Xylose	_	Four isolates, xylose +ve
Rhaminose	_	Four isolates, rhamnose +ve

Table 1: Phenotypic and biochemical characteristics of *E. tarda* strains.

Organ	Results		Total
	Positive	**Negative**	
Intestine	3	121	124
Liver	8	116	124
Kidney	1	123	124
Total	12	360	372

Table 2: Distribution of *E. tarda* isolates among the organs. (X^2=6.5, df=2, P<0.05).

Parameters		Positive	Negative	Total	X^2 value	P- value
Site	Zeway	7	27	34	6.38	0.012
	Langanoo	5	85	90		
Total		12	112	124		
Sex	Female	3	27	30	0.005	0.945
	Male	9	85	94		
Total		12	112	124		

Table 3: Occurrence of *E. tarda* isolates with respect to site and sex of fish.

Species	Result		Total
	Positive	**Negative**	
Catfish	1	19	20
Tilapia	11	93	104
Total	12	112	124

Table 4: Occurrence and distribution of *E. tarda* with respect to fish species. (X^2=0.59, df=1, P>0.05).

Concerning the morphological and biochemical characteristics of *Edwardsiella tarda* isolates, that showed typical characteristics, the results were consistent with those reported previously [29-31]. The finding of one isolate negative for indole production and positive in Simmons citrate test, however, indicates atypical strain which was also reported in previous studies where variation in Simmons citrate utilization [7,32,33] and indole production [34] was reported among *E. tarda* strains.

The present study showed two isolates were found non motile and this fact is matched with that of Okuda et al. [35]. Although most of the phenotypic characteristics of the isolates were similar as claimed by [36], some isolates showed, however, variation in some of the biochemical tests particularly in the utilization of sugars which included mannitol, rhamnose, xylose, inositol. The finding of such variation contradicts with the study of Baya et al. [37] where no variation was observed with respect to these biochemical tests among fourty four E tarda isolates studied. Variation among *E. tarda* isolates,

however, was reported with respected to utilization of rhaminose [7], mannitol [38].

The occurrence of variation in phenotypic characteristics among *E. tarda* isolates may due to the presence or absence of plasmid that control metabolic activities. Generally, the significance of the incidence of *Edwardsiella tarda* in catfish and tilapia couldn't be substantiated phenotypic characteristics of *E. tarda* [32].

Nowadays, more rapid, relatively accurate and simple molecular based identification techniques have been employed unlike the traditional phenotypic identification methods which rely on bacterial morphology and biochemical characteristics, Nucleic acid probes and the polymerase chain reaction (PCR) have been developed for the identification of pathogens of aquatic animals including *E. tarda*. Molecular methods such as PCR may sometimes yield false positive results as they can be highly subject to laboratory contamination. . Polymerase chain reaction based diagnosis of *Edwardsiella tarda* infection in blood samples of oyster toad fish and more sensitive real time PCR methods were reported as the major way in identifying the genes responsible for virulence of *Edwardsiellosis* [39].

Loop- mediated isothermal amplification (LAMP) is another rapid and sensitive method used for the diagnosis of *Edwardsiellosis* [40].

A fluorescence *in situ* hybridization (FISH) technique using twenty four mer oligonucleotide probe has also been used for detection of bacterial cells belonging to *Enterobacteriaceae* including *E. tarda* without giving false positive reaction [41]. The use of higher performance capillary electrophoresis (HPCE) has also been reported [42] as another technique which identifies, separate and quantifies intact bacteria. They identified *E. tarda* in fish and traced the bacteria in less than ten minutes after injection in to fish fluid using blue light emitting diode induced fluorescence and a cell permeable green nucleic acid strain.

The absence of significant differences in the occurrence of *E. tarda* between males and females indicates that both sexes are equally susceptible to the bacterium. This is an agreement several works where both sexes are equal chance of being infected with *E. tarda* [40,42]. The significant differences in the rate of isolation of *Edwardsiella tarda* between the study lakes may be attributed to differences in the nutritional status of the fish, the environmental condition (Salinity and bacterial load of the water), water quality, changes in temperature, PH and fluctuation in dissolved oxygen which are believed to affect the occurrence of *E. tarda* infection [17,43,44]. Although, *Edwardsiella tarda* affect intestine, liver and kidney of catfish and tilapia, the highest percentage of the pathogen was isolated from liver. This could be due to the metabolic activities of the organs [43].

In conclusion, *Edwardsiella tarda* is one of the most important bacterial diseases among *Oreochromis niloticus* in fish sampled from Lake Zeway and Langanoo. The severity of *Edwardsiella tarda* may have immune suppressive effect which proved by lymphoid depletion induced in spleen.

Conclusion and Recommendations

Edwardsiellosis is the most important bacterial disease causing severe economic loss and hindrance in aqua farming. Apart from veterinary health importance, *Edwardsiella tarda* has also public health significance in people engaged in fishery industry and those depend on fish products for their annual income. The isolation of *Edwarsiella tarda* from wild fish population of Lakes Zeway and Langanoo destined for human consumption is, therefore, indicates that *E. tarda* is a potential threat of both the fishery sector/aquaculture and public health. The finding of certain isolates that divert in their biochemical characteristics warrants further investigation using more advanced methods of bacteria characterization. Generally, assessment of environmental condition, management and other stress factors enhancing the occurrence, distribution and severity of *Edwardsiella tarda* in fish is essential to design an effective disease control and prevention. Since the current state of knowledge of *E. tarda* infection in fish and humans in Ethiopia is almost nil, further study on the epidemiology of *E. tarda* in different hosts and environments as well as comprehensive information on the strains involved should be established for better fish productivity and public health.

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Ethics Approval and Consent to Participate

"Not applicable"

Competing Interests

The authors declare that they have no competing interests.

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