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Molecular characterization of *Edwardsiella* species isolated from *Oreochromis niloticus* and *Clarias gariepinus* in Wakiso District, Uganda

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Abstract. *Nantongo M, Mkupasi EM, Byarugaba DK. 2019. Molecular characterization of* Edwardsiella *species isolated from* Oreochromis niloticus *and* Clarias gariepinus *in Wakiso District, Uganda. Bioteknologi 16: 11-20. Edwardsiella tarda* (Ewing & McWhorter, 1965) is well-known as an opportunistic pathogenic bacterium causing Edwardsiellosis in the host body of cultured and wild fish. The disease is among the most important bacterial diseases causing severe economic losses in fish worldwide. Here, we determined the occurrence and performed characterization of *E. tarda* in cultured Nile tilapia and African catfish from selected 17 fish farms in Wakiso District, Uganda. Fish samples were collected in the duration between September 2016 and February 2017. Bacteriological examination of the internal organs (kidney, liver, spleen) was followed by clinical and post-mortem examination of the overall fish body. The bacterium was identified using conventional biochemical tests, API 20E kits, and sequencing of 16S rRNA. Phylogenetic analysis was done by the Neighbor-Joining method in MEGA 7.0.2 against the 16S rRNA gene sequences retrieved from the GenBank. The isolate was further screened for the presence of selected virulence genes by polymerase chain reaction (PCR). One isolate from *Oreochromis niloticus* (Linnaeus, 1758) was confirmed to be *E. tarda* by the 16S rRNA sequencing. The isolate gave 99.9% identity to other members of *E. tarda* compared to known 16S rRNA sequences in the GenBank database. In the phylogenetic analysis, the isolate did not cluster with any of the *E. tarda* isolates, indicating a distant relationship with the isolates whose sequences were included in this study. Six virulence genes that enhance bacterial survival and host pathogenesis were identified in the isolate, including; CitC, muk, gadB, katB, esaV and fima. This study confirmed one positive *E. tarda* isolates. Nevertheless, the isolate possessed several virulence genes indicating their potential to cause disease in fish. Because the bacterium is of public health importance, awareness should be created amongst fish farmers and stakeholders to take precautions to avoid disease outbreaks.

Keywords: *Clarias gariepinus*, *Edwardsiella* species, *Oreochromis niloticus*, Wakiso District

INTRODUCTION

The aquaculture industry is one of the fastest-growing food production sectors globally, with fish production accounting for 44.1% of 2014 (FAO 2016). In Uganda, fish is an important commodity and the source of animal protein for 34.5 million people. Still, the current consumption rate is only 5.7 kg per capita, well below the WHO recommended 12.5 kg per capita (Directorate of Fisheries Resources 2011). Thus, there is a growing need to increase fish production as the population grows rapidly by 3.4% annually (UNBOS 2014). The country needs to be more aggressive in increasing sustainable fish production to reach the goal of the agriculture sector of ensuring sustainable and market-oriented production, food security, and household incomes (NDPII 2015). Uganda's second National Development Plan has identified fish farming as one of the twelve agricultural enterprises on which the focus is to provide food security and contribute to export earnings (NDPII 2015).

Furthermore, the country will have to intensify aquaculture to ensure food security and maximize profits (Öztürk and Altınok 2014). However, fish kept in high densities are more likely to experience stress factors like poor water quality and physical damage, which predispose them to infections. Fish diseases are a major constraint to aquaculture, causing significant economic losses due to mortalities, reduced growth, and increased cost of production through disease management (Faruk et al. 2004). Bacteria are among the most encountered causes of diseases in cultured fish (Mohanty and Sahoo 2007). The *Edwardsiella* species is known to have a major impact on the economy, as it causes mass mortality in a considerable number of commercially important fish populations around the world (Park et al. 2012). The occurrence of *Edwardsiella* in fish has been reported in various countries, including Ethiopia (Kebede and Habtamu 2016); India (Das et al. 2014); Egypt (El-Seedy et al. 2015); Uganda (Walakira et al. 2014); Malaysia (Najiah and Lee 2006) and from the Mediterranean (Katharios et al. 2015).

Edwardsiella tarda (Ewing & McWhorter, 1965) is commonly found in water and healthy fish populations. However, the bacterium is opportunistic, so disease outbreaks usually occur when the environment is imbalanced, such as when the water quality is poor, the population is crowded, or the organic content is high (Park et al. 2012). Wyatt et al. (1979) reported *E. tarda* in African catfish and water bodies, increasing incidence with increasing organic pond content and water temperature. The *E. tarda* is a public health concern as it is known to be zoonotic; it causes disease in fish and other aquatic animals and causes gastroenteritis in humans (Park et al. 2012).

There is little information on the presence and prevalence of *Edwardsiella* species in Uganda, possibly

due to limited studies of fish diseases. Akoll and Mwanja (2012) attribute this to the lack of diagnostic tools and the high cost of identifying and characterizing such pathogens in subsistence aquaculture. Therefore, this study aimed to generate knowledge on *Edwardsiella* infections in farmed African catfish and Nile tilapia in different production systems in selected fish farms, which is useful in planning control measures.

The objectives of this study are: (i) to determine the presence of *Edwardsiella* species in cultured African catfish and Nile cichlids from selected farms in Uganda; (ii) to determine the phylogenetic relatedness of *Edwardsiella* isolates from farmed African catfish and Nile cichlids in Uganda; (iii) Determine the virulence genes present in *Edwardsiella* isolates recovered from Nile cichlids and African catfish in Uganda.

MATERIALS AND METHODS

Study area

This study was conducted in the Wakiso District of Central Uganda, which covers 1,906.7 km^2, as shown in Figure 1. It is located at 004'N North latitude, 32045'E East latitude, and 1,218 m above sea level. One of the main economic activities in the district is fishing on Lake Victoria and fish farming, both commercial and subsistence, in ponds, basins, and cages. The district was selected by FAO (2016) as one of the country's districts suitable for fish farming. The district has 15 sub-counties with a population of over 2 million people and an annual growth rate of 6.6% (UBOS 2014).

Study design and sampling strategy

The study used a cross-sectional research design to characterize *E. tarda* isolates from fish in the Wakiso District. Fish samples were collected and isolated, and bacteria were identified using biochemical assays, API 20E kits, and sequencing. Phylogenetic analysis was performed using MEGA 7.0 software, and isolates were screened for selected virulence genes by PCR.

Fish farms were specifically selected for sampling, with preference given to farms reporting fish diseases and depending on the farmer's willingness to provide samples. Simple random sampling was done to select healthy fish from the farming system, while diseased fish were deliberately selected from the farming system in case of disease.

Sample size

The size of the sample was determined using the formula by Naing et al. (2006);

$$n = \frac{Z^2P\ (1\text{-}P)}{d^2}$$

Where; "n" is the sample size, "Z" is the confidence interval (1.96), "P" is the disease prevalence of 9.67%, as previously reported by Kebede and Habtamu (2016) in Ethiopia and "L" is the expected error (0.05). Hence, sample size (number of fish) = 134. However, one hundred and eleven fish samples of Nile tilapia and African catfish were collected from 17 farms in the Wakiso District, Uganda.

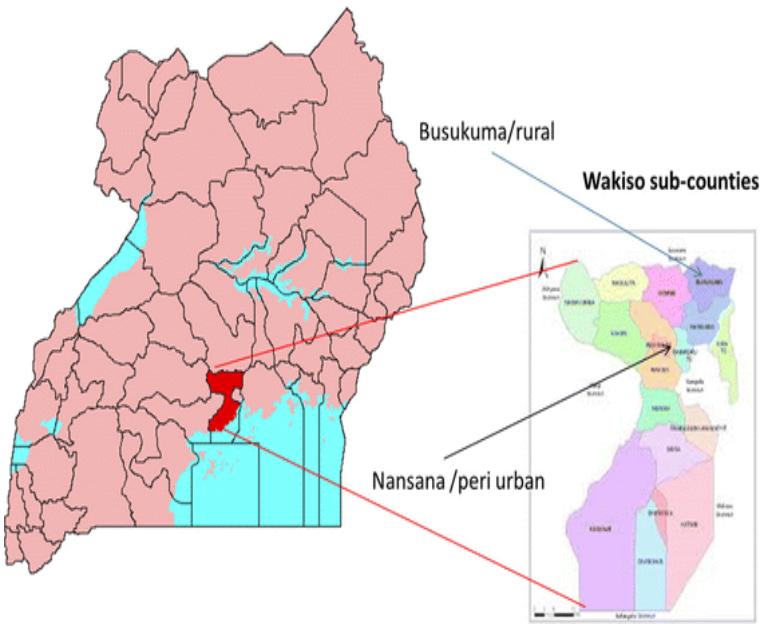

Figure 1. Map of Uganda showing the location of Wakiso District (source: Kalyesubula et al. 2017)

Sample and data collection

Samples were taken from 17 fish farms. A biographical data form was provided to fish farmers whose farms were visited to collect information on management practices and farm history. Five to ten fish were randomly sampled from the culture system (pond, basin, or cage) using a seine net, and if the system was a mixed culture, 10 fish were sampled, 5 of each variety. As many as 111 fish samples were obtained from the 17 farms, including apparently healthy, dying, and recently dead fish. Of the 111 fish samples collected, 81 were Nile tilapia, and 30 were African catfish, as more farms raised Nile tilapia than African catfish. Fish were collected from various culture systems, including in-ground ponds, concrete basins, and cages. Living and dying fish have been transported in plastic pools filled with water and with or without oxygen depending on the distance from the laboratory, while recently, dead fish, animal resources, and biological security at university maker.

Sample examination

There was 7 catfish fry crushed and inoculated directly onto xylose-lysine-deoxycholate (XLD) agar plates. The healthy and dying Nile tilapia was humanly sacrificed by joining together. At the same time, the African catfish was covered over the eyes with a cloth and hit on the head with a hard material. These plus dead fish were weighed, and their length was measured using a digital scale and ruler. The fish then underwent clinical and post-mortem examination to identify external and internal abnormalities. After examination, skin and gill swabs were taken and inoculated directly onto XLD agar and incubated at 370°C for 24 hours. The fish were then dissected using sterilized scissors and forceps by making a transverse incision in front of the anus towards the ventral part of the head. As far as the gill covers, another incision was made at the level of the anus running craniodorsally to the lateral line along the dorsal margin, from the abdominal cavity to the branchial arches, and a third cup connecting the ends of the two cups. The side panel was then removed to reveal the internal organs.

Organ examination was done by looking for any abnormalities, including location, size, color, and other signs of the disease. Next, the digestive tract, gonads, and visceral organs were removed by cutting the esophagus and disconnecting them from the kidneys. Next, samples of organs of interest (kidney, spleen, and liver) were taken using a surgical blade. Finally, organs were cultured separately from gills and skin swabs from the same fish; thus 208 plates were used for 104 fish samples, and the remaining 7 were for crushed African catfish for 215 plates.

Bacterial isolation

The samples were homogenized, seeded on XLD agar plates, and incubated for 24 hours at 37°C, after which the plates were examined for primary cultures. The examination of the plates was carried out by visual inspection for colonial morphology. The *E. tarda* forms small circular colonies 1 mm to 3 mm in diameter with black centers due to lysine decarboxylation (Buller 2004). A single colony well-differentiated from these black colonies was picked and subcultured onto XLD agar plates to produce a pure culture subjected to various biochemical tests.

Phenotypic identification of isolates

Identification of the bacterium was carried out using cultural and morphological characteristics. A series of conventional biochemical tests and the Analytical Profile Index (API 20E) system (BioMerieux, France) were performed according to the manufacturer's instructions. Conventional biochemical tests identifying The *E. tarda* included Gram stain, motility, indole production, cytochrome oxidase, H_2S production, methyl red, Voges-Proskauer, gelatin hydrolysis, urease, citrate, esculin, hydrolysis, and lactose utilization. The suspects from the conventional tests were re-identified using the API 20E kit, and their biochemical profiles were determined. These were preserved in cryovials containing BHI broth and glycerol and then stored in the freezer at -4°C until further testing.

Molecular characterization

Genotypic characterization

Preserved *E. tarda* isolates were recovered and inoculated onto nutrient agar slopes and transported to the Norwegian University of Life Sciences (NMBU) microbiology laboratory. The bacteria were subcultured and partially stored at -80°C as glycerol stock. The remainder was used to extract genomic DNA at Genlab, NMBU, where further molecular identification and characterization were performed.

DNA isolation

According to the manufacturer's instructions, genomic DNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen). Briefly, the bacterium was added to 200 µL of lysis buffer with lysozyme and incubated at 37°C for 30 minutes. Then 20 µL of Proteinase K and 100 µL of buffer AL (lysis buffer) were added and incubated for 30 minutes at 56°C. DNA was precipitated by adding 200 µL of 100% ethanol to the sample and mixed by pulse vortexing for 15 seconds and then centrifuged briefly.

The mixture plus the precipitate were gently applied to the QIAamp Mini spin column in a 2 mL collection tube to bind the DNA to the column. The cap was closed and centrifuged at 6000 x g (8000 rpm) for 1 minute. 500 µL of buffer was added to the QIAamp column and centrifuged at 6000 x g (8000 rpm) for 1 minute. 500 µl of Buffer AW2 was added to the QIAamp column and centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 minutes. The QIAamp column was placed in a new 2 mL collection tube and centrifuged at full speed for 1 minute. The QIAamp column was placed back into a new clean 1.5 mL microcentrifuge tube, and the collection tube containing the filtrate was discarded. 30 µL of Buffer AE was added to the column and incubated for 1 minute at room temperature and then centrifuged at 600 x g (8000 rpm) for 1 minute. The resulting DNA was stored at -200°C.

DNA purification

DNA was purified using a QIAquick® gel extraction kit (cat. 28704 and 28706) according to the manufacturer's instructions as follows; Absolute ethanol was added to the PE buffer and mixed thoroughly. A conventional tabletop microcentrifuge performed all centrifugation steps at 17,900 x g (13,000 rpm). The DNA fragment was excised using a clean, sharp scalpel from the agarose gel. Next, the gel slice was weighed into a colorless tube, and 3 volumes of QG buffer were added to 1 volume of gel (100 mg of gel-100 µL). The sample was incubated at 50°C for 10 minutes after the gel slice had completely dissolved, while the tube was vortexed every 2 minutes to help dissolve the gel. Finally, a volume of isopropanol gel was added to the sample and mixed. Safety glasses were used to protect the eyes from UV rays.

DNA binding was achieved by applying the sample to the QIAquick column and centrifuging for 1 minute until all samples passed through the column. The flow-through was discarded, and the QIAquick column was placed back into the same tube. Next, a 500 µL QG buffer was added to the QIAquick column and centrifuged for 1 minute. The continuous flow was again discarded, and the QIAquick column returned in the same tube. Then 750 µL of Buffer PE was added to the QIAquick column and centrifuged for 1 minute for washing. The continuous flow was again discarded, and the QIAquick column returned in the same tube. The column was left to stand for 5 minutes after adding the PE buffer. The QIAquick column was then centrifuged for 1 minute in the 2 mL collection tube provided to remove residual wash buffer. The QIAquick column was then placed in a clean 1.5 mL microcentrifuge tube. To elute the DNA, 30 µL of Buffer EB (10 mM Tris. Cl, pH 8.5) was added to the center of the QIAquick membrane, and the column was allowed to stand for 1 minute and then centrifuged for 1 minute.

Amplification of the 16S rRNA genes

The 16S rRNA gene was amplified by Polymerase Chain Reaction (PCR) using the 16S universal bacteria primers 27F (5' -AGAGTTTGATCCTGGCTCAG-3'), and 1492R (5'-GGTTACCTTGTTACGACTT-3') with the expected amplicon size of 1465bp (Lane 1991) ordered from Invitrogen, Thermo Fisher Scientific (Waltham, MA USA), PCR machine used in this research was iCycler from Bio-Rad. Each PCR reaction was performed in a final volume of 25 µL containing 2.5 µL of 10x reaction buffer (50 mM KCl, 75 mM Tris-HCl (pH 9.0), 2 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$), 0.5 µL 10 mM deoxyribonucleotide mix, 0.2 µL of *Taq* DNA polymerase, 1 µL 10 mM of each forward and reverse primer, 2 µL of DNA template and 16.8 µL of sterile ultrapure water. PCR conditions included initial denaturation at 94°C for 3 minutes, followed by 30 cycles of amplification as follows; Denaturation at 940°C for 30 seconds, annealing at 560°C for 30 seconds, and elongation at 720°C for 2 minutes. This was followed by a final extension step at 72°C for 5 minutes and left at 40°C until collected for further analysis. The PCR products were analyzed on 1% agarose gel (Ultrapure Agarose from Invitrogen, Thermos Fisher Scientific) using PowerPac 300 (Bio-Rad) at 100 volts for 60 minutes. The amplified DNA on the gel was visualized using Invitrogen's Safe Imager™, and bands of interest were excised using a scalpel blade for gel purification. The ChemiDoc ™ XRS (Bio-Rad) molecular image was used to view and capture gel photos. DNA concentration was measured using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc).

DNA sequencing

The PCR products were sequenced on ABI genetic analyzer with 16S 27F and 16S 1492R universal bacteria primers by Sanger sequencing techniques at GATC Biotech, Germany.

Phylogenetic analysis

The 16S rRNA gene sequence was edited using Bioedit, and Basic Local Alignment Search Tool (BLAST) searches performed in GenBank, specifically the National Center for Biotech Information (NCBI). Related sequences were obtained, and several sequence alignments were performed using the ClustalW algorithm. The gene sequences used in the phylogenetic analysis are shown in Table 3. These sequences were used to construct a phylogenetic tree.

Detection of selected virulence genes in the *Edwardsiella tarda* isolate

To characterize the virulence attribute of the isolate of *E. tarda*, seven virulence genes were screened in this study. These included: *gadB* (glutamate decarboxylase; resists host phagocyte killing activity), *muk* (putative killing factor), *citC*, *esaV*, *fimA* (allows bacteria to attach to host for invasion), *esrB* (helps penetration by encoding a regulatory protein for the type III secretion system (T3SS) and *katB* (provides the bacterium with resistance to the host's phagocytic activity) The genes were amplified by PCR using the primers in Table 1. The PCR reaction was performed in a final volume of 25 µl containing 0.25 µl of 10x reaction buffer (5 mM KCl, 7.5 mM Tris-HCl (pH 9.0), 0.2 mM MgCl2, (NH4)2SO4 2 mM), 0.05 µL of 1 mM deoxyribonucleotide mixture, 0.02 µL of Taq DNA polymerase, 0.1 µL of 1 mM sense and antisense primer, 0.2 µL of template DNA and 1.68 µL of sterile ultrapure water. The PCR program used was as follows: initial denaturation at 94°C for 3 minutes, followed by 32 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for citC, muk, esrB; 580C for catB; 570°C for gadB and 600°C fimA for 1 minute, extension at 720°C for 1 minute and final extension at 720°C for 10 minutes and left at 40°C until collected for analysis further.

PCR products were run on 1% agarose gel by PowerPac 300 (BioRad) at 100Volts for 60 minutes. The amplified products on the gel were observed and captured using ChemiDoc™XRS Molecular imager (Bio-Rad).

Data analysis

Data was saved, summarized, and analyzed using Microsoft Excel and Windows 10. Quantitative data (weight and height) were averaged. The sequences were edited in the MEGA 7.0 software and aligned using the ClustalW algorithm. Finally, a phylogenetic tree was constructed in MEGA 7.0 (Kumar et al. 2016) using the Neighbor-Joining method (Saitou and Nei 1987).

Table 1. Primers used in the amplification of the virulence genes

Target gene	Sequence 5' → 3'	Product size (bp)	Source
gadB (F)	5′- ATTTGGATTCCCGCTTTGGT-3′	583	Wang et al. 2012
gadB (R)	5′- GCACGACGCCGATGGTGTTC-3′		
muk(F)	5′- TTGCTGGCTATCGCTACCCT-3′	357	Wang et al. 2012
muk (R)	5′- TTGCTGGCTATCGCTACCCT-3′		
citC(F)	5′- TTTCCGTTTGTGAATCAGGTC-3′	591	Wang et al. 2012
citC (R)	5′- AATGTTTCGGCATAGCGTTG-3′		
fimA(F)	5′- CTGTGAGTGGTCAGGCAAGC-3′	441	Wang et al. 2012
fimA(R)	5′- TAACCGTGTTGGCGTAAGAGC-3′		
esrB(F)	5′-TCGTTGAAGATCATGCCTTGC-3′	311	Wang et al. 2012
esrB (R)	5′-TGCTGCGGGCTTTGCTT-3′		
katB(F)	5′-CTTAGCCATCAGCCCTTCC-3'	1417	Wang et al. 2012
katB (R)	5′-GCGAGTGCCGTAGTCCTT-3'		
esaV (F)	5'-GGTCAATAGCTGGCTACACAA-3'	955	Li et al. 2010
esaV (R)	5'-GCGCCTCAGCGAGTATGCGAT-3'		

RESULTS AND DISCUSSION

General description and farm history

Seventeen fish farms were visited in the Wakiso District, Uganda, where 111 healthy, dying, and recently dead fish were sampled and examined clinically and post-mortem. The whole Nile tilapia was 81 (72.9%), with an average body weight of 113.1 g and an average length of 17.42 cm. The total number of African catfish was 30 (27%), with an average body weight of 154.34 g and an average length of 25.1 cm. Seven of the 30 African catfish were juveniles, so their weight and length were not measured. Fish were collected from three rearing systems, namely in-ground ponds (10), basins (1), and cages (4). Two farms had both ponds and reservoirs. 82.3% of all farms practiced monoculture, Raising either Nile tilapia or African catfish, while 17.6% had mixed crops where both species were raised in the same system and were in soil ponds systems.

All farms with ponds were located in remote low-lying areas or agricultural areas and their water sources were streams or wells. Cages were located on the lake and river shores, while the farm with basins was only located in a residential area with its water source being tap and rainwater. All farms reported feeding the commercial fish feed twice or once a day, depending on feed availability, time, and the response of the fish. All farms with bottom ponds used organic fertilizers to boost primary production. Most farms used chicken manure (75%) for fertilization, while others used cow manure (25%).

Two farms (11.7%) reported having a history of disease during dry and wet seasons. Others reported observing signs of disease, including loss of appetite, low growth, proptosis, dropsy, abnormal swimming, parasites, and mortality. The farmers also admitted to using antibiotics, formalin, potassium permanganate and salt to treat sick fish. Farms that reported a history of the disease also reported overfeeding and poor water quality

Clinical signs and a post-mortem examination

The infected Nile tilapia showed ascites and abdominal petechial bleeding of the fish examined. In contrast, the African catfish showed abnormalities, including petechial bleeding on the skin and fins, lordosis, pallor of the liver, and ulcers on the abdominal and opercula regions (Figure 2).

Isolation of *Edwardsiella tarda*

Fifty-one plates out of 215 plates had produced clear colonies with black centers surrounded by reddened media due to the decarboxylation of lysine on xylose-lysine deoxycholate (XLD) medium after 36 hours at 370°C (Figure 3), and these have been subjected to conventional treatment biochemical tests submitted.

Phenotypic identification of *Edwardsiella tarda* isolates

Presumptive identification of *E. tarda* by conventional biochemical assays yielded eight (7.2%) *E. tarda* in four (3.6%) tilapia and four (3.6%) African catfish. All isolates were Gram-negative, and microscopic examination of the wet media showed mobile rods 2 µm in length and 1 µm in diameter. All isolates were negative for cytochrome oxidase, citrate, aesculin hydrolysis, gelatin hydrolysis, urease, and lactose fermentation. All isolates produced H2S and tested positive for indole production. There were variations in the MRVP test as only two isolates tested positive for MR and negative for VP (Table 2).

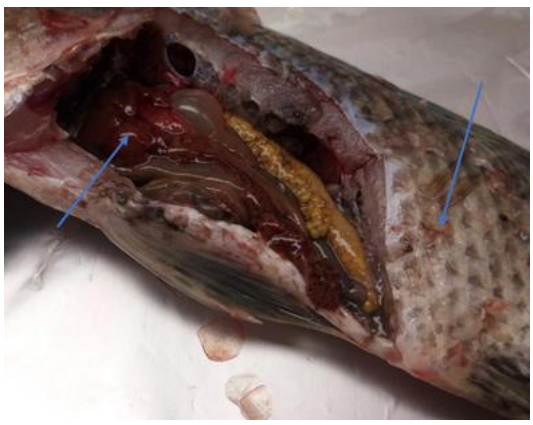

Figure 2. Hemorrhages and accumulation of ascitic fluid in Nile tilapia (arrows)

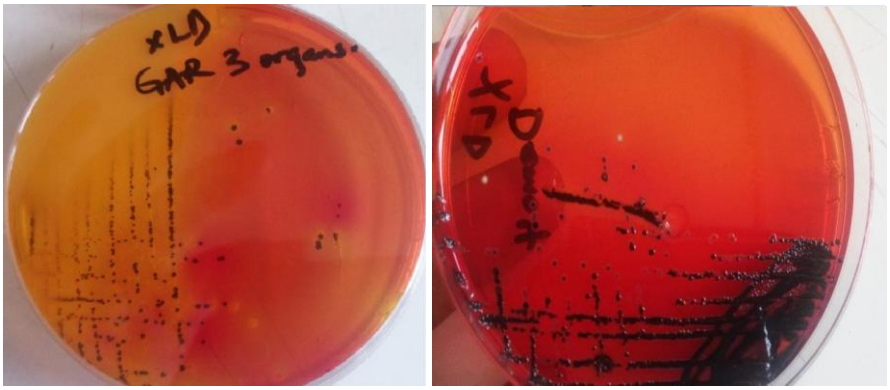

Figure 3. Clear colonies with black centers on XLD agar plates

Table 2. Biochemical properties of the eight *Edwardsiella tarda* isolates

Test	Isolates							
	***O.n*1**	***O.n*2**	***O.n*3**	***O.n* 4**	***C.g*1**	***C.g 2***	***C.g* 3**	***C.g*4**
Gram stain	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-
Indole production	+	+	+	+	+	+	+	+
Citrate	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+	+
Methyl Red	+	-	-	-	+	-	-	-
Voges-Proskauer	-	+	+	+	-	+	+	+
Esculin hydrolysis	-	-	-	-	-	-	-	-
Gelatin hydrolysis	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-
Lactose utilization	-	-	-	-	-	-	-	-
H_2S production	+	+	+	+	+	+	+	+

Note: + means a positive result, - means a negative result.

Identification of the isolates using API 20E kit

One isolate was re-identified as 99.4% *E. tarda* using Analytical Profile Index (API) 20E kit (Code No.4744000) at 37°C. In the biochemical tests, the seven isolates identified as *E. tarda* were identified by the API 20E kit as Plesiomonas shigelloides, a fish pathogen belonging to the family Enterobacteriaceae. Plesiomonashigelloides has a worldwide distribution, and human outbreaks have been reported in Cameroon from cold fish. It has many host plants, including aquatic environments, aquatic animals, mammals, and humans, that cause gastroenteritis (Janda et al. 2016).

Sequence analysis of the 16S rRNA gene for identification of *Edwardsiella tarda*

16S rRNA sequencing has been widely used to understand bacterial evolution and phylogeny and is considered an essential tool in bacterial systematics and identifying new species. Therefore, in this study, the bacterial isolate identified as *E. tarda* using the API 20E kit was identified and confirmed to be *E. tarda* from amplification of the 16S rRNA gene resulting in a PCR product with the expected size of 1465 bp (Figure 4).

Phylogenetic analysis

The *E. tarda* isolates with known 16S rRNA sequences in the GenBank database using the BLAST program showed that the isolate had a 99.9% identity compared to that of other E. late. The phylogenetic relationship of *E. tarda* was probed using the 16S rRNA gene with other strains of *E. tarda* downloaded from NCBI for phylogenetic analysis, including a type *E. tarda* strain ATCC 15947, *Escherichia coli*, *Salmonella enterica* and *Pleisomonas shigelloides* strains. Their taxonomic status, strain collection numbers, and GenBank accession numbers are listed in Table 3. A phylogenetic tree can be seen in Figure 5.

Next to the branches is shown the percentage of replicate trees in which the corresponding taxa are grouped together in the bootstrap test (500 replicates) (Felsenstein 1985).

Detection of virulence genes in the *Edwardsiella tarda* strain

The *E. tarda* strain was screened for 7 virulence genes, including; gadB, muk, citC, fimA, ESRB, katB, and esaV, by Polymerase chain reaction (PCR) technique. Six of the seven virulence genes screened for were present in *E. tarda* isolated in this study, including; CitC, muk, gadB, katB, esaV and fimA.

Table 3. Isolates whose sequences were used in phylogenetic analysis

Name of culture	Accession number
E. tarda strain T1	KX388234.1
E. tarda strain C6	FJ607400.1
E. tarda strain UMT-WD-ON	FJ600537.1
*E. tarda*strainVMCU06	KU860461.1
E. tarda strain C7-5m	HQ663902.1
E. tarda strain ATCC 15947	JX866952.1
E. tarda strain29-907R	KX828266.1
P. shigelloides ATCC 14029	M59159.1
E. tarda strainSY-ED14	KX388234
P. shigelloides ATCC14029T	X74688.1
*E. tarda*strain59-907R	KX828321.1
Salmonella enterica subsp *enterica* ATCC 13076	LSHA01000019.1
E. coli ATCC 11775	NZ_JMST01000035.1

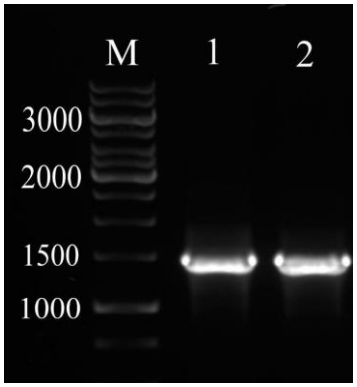

Figure 4. 16S rRNA gene PCR products on a 1% agarose gel. Lane M: Molecular marker, lanes1 and 2: *Edwardsiella tarda*

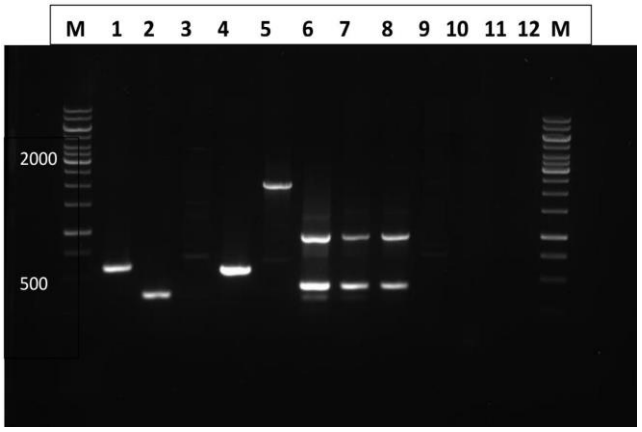

Figure 6. Agarose Gel electrophoresis of PCR of virulence genes for the isolated *Edwardsiella tarda*. Note: lane 1 is citC, lane 2 ismuk, lane 3 isesrB(-ve), lane 4 isgadB, lane 5 is katB,lanes 6, 7 and 8 arefimA and esaV, Lanes 9 to 12 are not considered, and lane M is DNA marker.

Discussion

The present study determined the presence of *Edwardsiella* species at 7.2% by conventional bacteriology and molecular technique. It confirmed *E. tarda* at 0.9% in one of the fish samples from farms in the Wakiso District of Uganda. The *E. tarda* is a significant pathogenic bacterium that is said to cause Edwardsiellosis in more than 20 fresh and marine fish species, both farmed and wild (Bullock and Herman 1985; Mohanty and Sahoo 2007). It reduces the marketability of diseased fish and causes massive mortality in different age groups, leading to severe economic losses in fish farms worldwide (Faruk et al. 2004; Abraham et al. 2015).

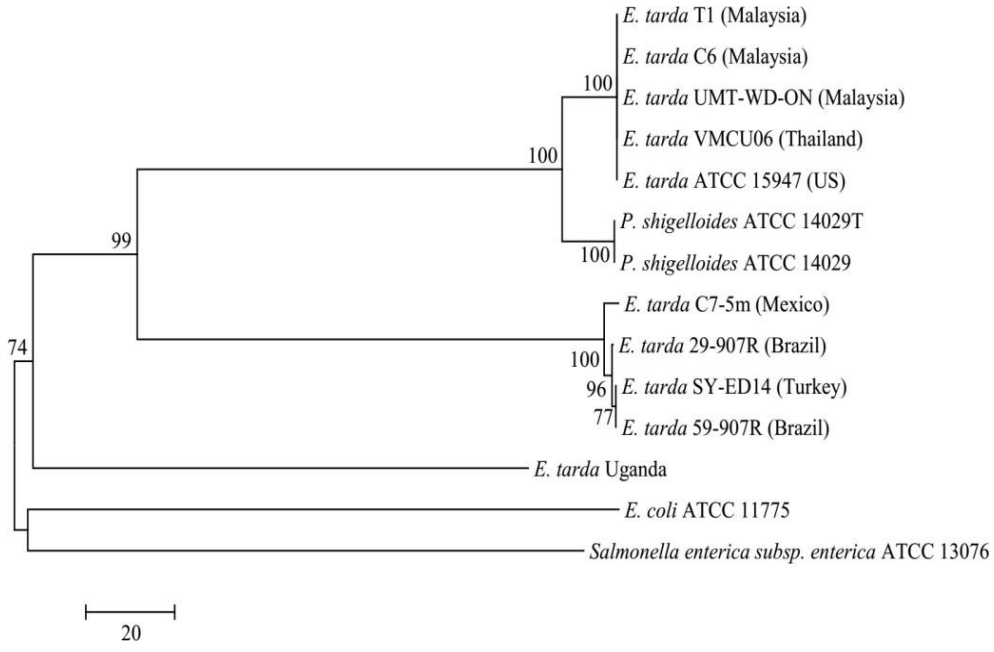

Figure 5. Phylogenetic relationship of *Edwardsiella tarda* from Uganda with other strains

In this study, *Oreochromis niloticus* (Linnaeus, 1758), which produced the *E. tarda* isolates, showed signs of ascites and petechial abdominal hemorrhages. These presentations were similar to those reported for *E. tarda* infection (El-Refaey 2013; El-Seedy et al. 2015). However, Edwardsellosis symptoms and macroscopic lesions are unreliable in arriving at a conclusive diagnosis because several bacterial infections also share similar signs and symptoms (Mohanty and Sahoo 2007). Additionally, diseased fish, with or without clinical signs of disease, are equally important as they can transmit pathogens that pose a risk of disease transmission to other species, including humans. Thereby, detection of these pathogens is crucial for their effective prevention and control (Castro et al. 2014)

The successful isolation and correct identification of a given pathogen depend on the standardized bacteriological culture methods. In the present study, the bacterium was grown on XLD agar and, due to lysine decarboxylation, produced clear colonies with black centers surrounded by reddened backgrounds (Figure 3) (Wyatt et al. 1979; Najiah and Lee 2006; Wei and Musa 2008). All isolates in this study were gram-negative, and microscopic examination of wet slides revealed motile rods 2 µm long and 1 µm in diameter. These results were similar to those of other researchers who isolated *E. tarda* from cultured freshwater cichlids, African catfish, Chinook salmon, and sea bream (Amandi et al. 1982; El-Refaey 2013; Griffin et al. 2013; Abram et al. 2015; Katharios et al. 2015; Emanet al. 2016)

All isolates showed negative results for cytochrome oxidase, citrate, esculin hydrolysis, gelatin hydrolysis, urease, and lactose fermentation. All isolates have been tested positively for H_2S and indole production (Table 2). These results are consistent with those of other studies (Wyatt et al. 1979; Amandi et al. 1982; Joh et al. 2010; El-Refaey 2013; Abraham et al. 2015; Eman et al. 2016). There were variations in MRVP tests as only 2 isolates tested positive for MR and negative for VP. These results did not agree with the literature, which could mean these new strains of E. late. However, variations in phenotypic traits are attributed to the presence and absence of plasmids that control the metabolic traits of the phenotypic traits of the isolates (Acharya et al. 2007).

Biochemical identification using conventional bacteriological techniques failed to distinguish between *E. tarda* and Plesiomonas shigelloides. When the API 20E kit was used on the eight isolates identified as *E. tarda* by biochemical tests, only one isolate was found to be *E. tarda* at 99.4%. Indeed, it tested positive for citrate but was citrate negative in conventional biochemical tests, so it would have been 99.9% *E. tarda*. According to Buller (2004), reaction differences between the API system and conventional biochemical tests are particularly reported for decarboxylase, citrate, urea, indole and Voges-Proskeur tests. El-Seedy et al. (2015) also reported that 22.2% of their *E. tarda* tested positive for citrate utilization and concluded that these isolates were typical strains of *E. tarda*.

More isolates have been confirmed as *E. tarda* by conventional biochemical tests than by molecular techniques, possibly due to the high degree of phenotypic diversity within bacterial species, which limits the accuracy of biochemical identification, especially when only a few isolates are tested, which requires more work for the investigation is needed on the phenotypic diversity of *E. tarda* (Griffin et al. 2013).

Phylogenetic analysis separated strains of *E. tarda* into two groups by excluding *E. tarda* isolated in this study from any group. One group clustered with *Pleisomonasshigelloides* with high bootstrap support (100%). The *P. shigelloides* is deeply rooted in the Enterobacteriaceae family and aligned closer to *E. tarda*. Therefore, this may explain the misidentification in biochemical tests as *E. tarda* in this study (Janda et al. 2016). The second group shares greater similarity with *E. tarda* of this study with 74% bootstrap support than the first group. These results are consistent with other comparative phylogenomic studies comparing two distinct genetic groups of E. spät (Griffin et al. 2013). This finding, therefore, suggests that *E. tarda* isolated from this study is genetically distant from the GenBank included in this study

The ability of a bacterium to cause disease depends on the expression of virulence factors that allow the bacteria to enter the host, produce pathological effects, and evade the host's defenses. The ability of a microorganism to invade the host is the most important aspect of its pathogenicity (Roberts 2012). Identifying the virulence genes needed to establish infection in hosts helps to understand the mechanisms of pathogenesis in bacteria, aiding in developing vaccines and the formulation of therapies to protect fish from infection. The *E. tarda* is believed to be multifactorial, and several authors have reported several potential virulence factors involved in its pathogenesis (Mohanty and Sahoo 2007; Mendez et al. 2012). Several potential pathogenic properties are thought to contribute to the infection process of *E. tarda*, including ability to invade epithelial cells, secretion of degradative enzymes, adhesions, type III and IV secretion systems, production of toxins to spread infection, siderophores, catalase, ability to survive and replicate in phagocytes (Mohanty and Sahoo 2007; Park et al. 2012; Xu and Zhang 2014; Eman et al. 2016). Pathogenic bacteria are thought to have virulence genes absent in non-pathogenic bacteria. While virulence can be present in both pathogenic and non-pathogenic bacteria, they are functional only in pathogenic ones.

In the present study, six of the seven screened virulence genes were present in *E. tarda* isolate, including; citC, muk, gadB, katB, esaV and fimA (Figure 6). Two of these genes (muk and gadB) were also identified by Eman et al. (2016) and are considered specific for *E. tarda*. According to the literature, the genes detected in this study are considered to be mainly virulent genes because they give the bacteria resistance to the host's phagocytic activity (gadB and katB), allow cell adhesion (fimA), which promotes intracellular survival and replication of the bacterium in the host allows and muk is a putative killing factor (Mohanty and Sahoo 2007; Katharios et al. 2015). It has also been suggested that the presence of the fimA gene

indicates the ability of *E. tarda* to bind to specific receptors in fish and thus define the site of entry and colonization (Wang et al. 2009)

It is known that the virulence genes detected are present only in virulent strains and are therefore considered biomarkers in the diagnosis of pathogenic *E. tarda*. Furthermore, these genes determine how pathogenic bacteria interact with the host to cause systemic infections and vaccine development. In addition, they are used to develop new therapies and shared antigens (Mendez et al. 2012). Although the current study did not assess the virulence of the isolate based on in vivo experiments, these virulence genes coupled with clinical signs of disease provide evidence for the presence of Edwardsialosis in fish in which the isolate was found.

The *E. tarda* is a zoonotic bacterium affecting not only fish but also invertebrates, amphibians, reptiles, birds, and mammals, including humans (Bullock and Herman 1985; Park et al. 2012). The *E. tarda* bacteremia (ETB) has been described as a serious food- and water-borne infection that results in high mortality rates, especially in patients with serious underlying conditions (Hirai et al. 2015). In addition, the pathogen was reported by Litton and Rogers (2016) in a fisherman who was pierced by a hook. As no biosecurity measures were adopted in all farms visited in this study, this poses a threat to human health, contributing to the introduction of pathogens from fish to humans and creating potential contamination between humans and animals (Takyi et al. 2012).

In conclusion, this study established the presence of *E. tarda* in *O. niloticus* in the district of Wakiso, Uganda. The isolate has been successfully characterized, which is of the utmost importance in diagnosing the pathogen. Isolation of *E. tarda* from farmed fish intended for human consumption poses a threat to the fishing industry because it causes heavy economic losses and to the public sector because it is zoonotic. The present study results revealed that the *E. tarda* isolate had six virulence genes that play an important role in E. late pathogenicity. This information will facilitate effective control strategies and the development of vaccines to prevent Edwardsialosis in fish.

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