

**MOLECULAR CHARACTERIZATION OF *EDWARDSIELLA* SPECIES
ISOLATED FROM AFRICAN CATFISH (*Clarias gariepinus*) AND NILE
TILAPIA (*Oreochromis niloticus*) IN WAKISODISTRICT, UGANDA**

MARY NANTONGO

**A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE
REQUIREMENTS FOR THE AWARD OF A DEGREE OF MASTER OF
SCIENCE IN HEALTH OF AQUATIC ANIMAL RESOURCES OF
SOKOINE UNIVERSITY OF AGRICULTURE. MOROGORO, TANZANIA.**

ABSTRACT

Edwardsiella tarda is an opportunistic pathogenic bacterium that causes Edwardsiellosis in cultured and wild fish. It is among the most important bacterial diseases causing severe economic losses in fish worldwide. This study was carried out to determine the occurrence and characterize *E. tarda* in cultured Nile tilapia and African catfish from selected fish farms in Wakiso district, Uganda. A total of 111 fish samples were collected from 17 fish farms between September 2016 and February 2017. Clinical and post mortem examination of sampled fish was done followed by bacteriological examination of the collected internal organs (spleen, kidney and liver), gills and skin swabs. Identification of the bacterium was done using conventional biochemical tests, API 20E kits and sequencing of *16S rRNA*. Phylogenetic analysis was done by Neighbor-Joining method in MEGA 7.0.2 against the *16SrRNA* gene sequences retrieved from the GenBank. The isolate was screened for presence of selected virulence genes by polymerase chain reaction (PCR). From this study, one isolate from *O. niloticus* was identified and confirmed to be *E. tarda* by the *16S rRNA* sequencing. The isolate gave an identity rate of 99.9% to other members of *E. tarda* on comparison with known *16S rRNA* sequences in the GenBank database. In phylogenetic analysis, the isolate did not cluster with any of the *E. tarda* isolates suggesting a distant relationship with the isolates whose sequences were included in this study. Six virulence genes were detected in the isolate that enhance bacterial survival and pathogenesis in the host including; *CitC*, *muk*, *gadB*, *katB*, *esaV* and *fimA*. Although the study confirmed only one *E. tarda* isolate, the isolate tested positive for several virulence genes indicating their potential to cause disease in fish and since the bacterium is of public

health importance, awareness should be created amongst fish farmers and stakeholders to take precaution to avoid disease outbreak.

DECLARATION

I, MARY NANTONGO, do hereby declare to the senate of Sokoine University of Agriculture that this dissertation is my own original work and that it has neither been submitted nor concurrently being submitted in any other institution.

Mary Nantongo

(MSc. Candidate)

Date

The declaration above is confirmed by;

Dr. Ernatus M. Mkupasi

(Supervisor)

Date

Prof. Denis K. Byarugaba

(Supervisor)

Date

COPYRIGHT

No part of this dissertation may be reproduced, stored in any retrieval system, or transmitted in any form or by any means without prior written permission of the author or Sokoine University of Agriculture in that behalf.

ACKNOWLEDGEMENTS

Firstly, I give special thanks to the Lord Almighty who granted me good health, wisdom, strength and endurance to accomplish this research. I am indebted to my supervisors; Dr. Ernatus M. Mkupasi, Prof. Denis K. Byarugaba and Dr. Samuel Wamala for their tireless supervision, guidance and sincere commitment throughout my work. My sincere gratitude also goes to Prof. Robinson H. Mdegela for his sincere dedication, guidance and advice throughout this work. May the good Lord bless you abundantly. I am grateful to the Norwegian Agency for Development Cooperation (NORAD) through the Training and Research in Aquatic and Environmental Health in Eastern and Southern Africa (TRAHESA; TAN_13/0027) project for funding this study. I am truly thankful to the staff of Bacteriology laboratory of the College of Veterinary Medicine, Animal Resources and Biosecurity of Makerere University where I carried out the laboratory work. My sincere thanks to the laboratory manager Mr. Nathan Lubowa, Mr. Deus Kanya and Mr. Arthur Kalyebi Watelo for their guidance, assistance and commitment throughout sample analysis. I am also grateful to Dr. John Walakira of National Fisheries Resources Research Institute, Mr. Sekayi (Wakiso Fisheries Officer), all the extension workers and fish farmers that assisted in field work. Lastly, I thank my family and friends for the patience, moral support and encouragement they rendered to me during the course of this study. May God bless you all.

DEDICATION

I dedicate this work to my beloved parents Rev. Daniel and Mrs. Florence Victor Kiggundu for their endless support and love. And also to my greatest inspiration, my daughter, Alexis Priscilla Nakitto.

TABLE OF CONTENTS

COPYRIGHT	v
ACKNOWLEDGEMENTS	vi
DEDICATION.....	vii
TABLE OF CONTENTS	viii
LIST OF TABLES	xi
LIST OF FIGURES	xii
LIST OF APPENDICES	xiii
LIST OF ABBREVIATIONS AND ACRONYMS	xiv
CHAPTER ONE.....	1
1.0 INTRODUCTION	1
1.1 Background	1
1.2 Problem statement and justification of the study	3
1.3 Objectives of the study	4
1.3.1 Main objective	4
1.3.2 Specific objectives	4
CHAPTER TWO	5
2.0 LITERATURE REVIEW	5
2.1 Aquaculture industry in Uganda	5
2.2 Diseases in aquaculture.....	5
2.3 <i>Edwardsiella</i> infections	7

2.3.1 <i>Edwardsiella tarda</i>	7
2.3.1.2 <i>Edwardsiella tarda</i> virulence	11
2.3.2 <i>Edwardsiella ictaluri</i>	12
2.4 Diagnosis of <i>Edwardsiella</i> infections.....	12
2.5 Economic and health impacts of <i>Edwardsiella</i> infections.....	13
2.6 Proposed control measures	13
CHAPTER THREE	15
3.0 MATERIALS AND METHODS.....	15
3.1 Study area.....	15
3.2 Study design and sampling strategy	16
3.2.1 Sample size.....	17
3.2.2 Sample and data collection.....	17
3.2.3 Sample examination.....	18
3.3 Bacterial isolation.....	19
3.3.1 Phenotypic identification of isolates.....	20
3.3.2 Molecular characterization.....	20
3.3.2.1 Genotypic characterization.....	20
3.3.2.2 DNA isolation	20
3.3.2.3 DNA Purification	21
3.3.2.4 Amplification of the <i>16S rRNA</i> genes	22
3.3.2.5 DNA Sequencing	23
3.4 Phylogenetic analysis	24
3.5 Detection of selected virulence genes in the <i>Edwardsiella tarda</i> isolate	24

3.6 Data analysis	26
CHAPTER FOUR.....	27
4.0 RESULTS	27
4.1 General description and farm history	27
4.2 Clinical signs and post-mortem examination.....	28
4.3 Isolation of <i>Edwardsiella tarda</i>	29
4.4 Phenotypic identification of <i>Edwardsiella tarda</i> isolates.....	30
4.5 Identification of the isolates using API 20E kit	31
4.6 Sequence analysis of the <i>16S rRNA</i> gene for identification of <i>E. tarda</i>	31
4.7 Phylogenetic analysis	32
4.8 Detection of virulence genes in the <i>Edwardsiella tarda</i> strain.....	34
CHAPTER FIVE.....	35
5.0 DISCUSSION	35
CHAPTER SIX	41
6.0 CONCLUSION AND RECOMMENDATIONS	41
6.1 Conclusion	41
6.2 Recommendations	41
REFERENCES.....	43
APPENDICES	56

LIST OF TABLES

Table 1:	Primers used in the amplification of the virulence genes.....	25
Table 2:	Biochemical properties of the eight <i>Edwardsiella</i> isolates	30
Table 3:	Isolates whose sequences were used in phylogenetic analysis	33

LIST OF FIGURES

Figure 1: Conceptual diagram of equilibrium among hosts, pathogens and the environment in aquatic systems.....	7
Figure 2: Map of Uganda showing the location of Wakiso district (source: Kalyesubula <i>et al.</i> , 2017).....	16
Figure 3: Hemorrhages and accumulation of ascitic fluid in Nile tilapia (arrows)	28
Figure 4: Clear colonies with black centers on XLD agar plates	29
Figure 5: <i>16S rRNA</i> gene PCR products on a 1% agarose gel	32
Figure 6: Phylogenetic relationship of <i>E. tarda</i> from Uganda with other strains	33
Figure 7: Agarose Gel electrophoresis of PCR of virulence genes for the isolated <i>E. tarda</i>	34

LIST OF APPENDICES

Appendix 1: Biodata Form 56

Appendix 2: Identification of *E. tarda* by API 20E kit, the isolate tested
positive for citrate utilization (arrow) 58

Appendix 3: Isolates earlier identified as *E. tarda* turned out to be
Plesiomonas shigelloides by API 20E kit 59

LIST OF ABBREVIATIONS AND ACRONYMS

µl	microliter
API 20E	Analytical Profile Index System
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CDL	Central Diagnostic Laboratory
DFR	Directorate of Fisheries Resources
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ESC	Enteric Septicemia of Catfish
FAO	Food and Agriculture Organization
FISH	Fluorescent <i>in Situ</i> Hybridization
H ₂ S	Hydrogen Sulphide
IFA	Indirect Fluorescent Antibody
MRVP	Methyl Red Voges-Proskauer test
NCBI	National Center for Biotechnology Information
NDPII	The Second National Development Plan
PCR	Polymerase Chain Reaction
RRNA	Ribosomal Ribonucleic acid
T3SS	Type III Secretion System
UNBOS	Uganda National Bureau of Statistics
WHO	World Health Organisation
XLD	Xylose Lysine Deoxycholate

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Aquaculture is one of the fastest growing food production sectors in the world with fish production amounting to 44.1% of the total fish production in 2014 (FAO, 2016). In Uganda, fish is an important source of animal protein diet for about 34.5 million Ugandans although the current consumption rate is 5.7kg per capita being below the WHO recommended rate of 12.5kg per capita (Directorate of Fisheries Resources, 2011). Therefore, the need to increase fish production is becoming increasingly urgent as the country's population is growing by 3.4% annually (UNBOS, 2014). This calls for more aggressive efforts to efficiently increase sustainable fish production so as to reach the goal of the agriculture sector of ensuring sustainable and market-oriented production, food security and household incomes in the country (NDP II, 2015). The Uganda's second National Development Plan selected fish farming as one of the twelve agricultural enterprises on which focus is to be placed for high potential for food security and high contribution to export earnings (NDP II, 2015).

In order to meet the planned goals of ensuring food security and profit maximization in the country, intensification in aquaculture is inevitable (Öztürk and Altınok, 2014). However, fish stocked at high densities are subjected to stress factors like poor water quality and physical damage predisposing them to a wide range of infections. Fish diseases are one of the major constraints of aquaculture which often cause 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). Among the most annihilating bacteria are the *Edwardsiella* species that cause major economic losses due to mass mortality in a considerable number of commercially important fish populations worldwide (Park *et al.*, 2012). Occurrence of *Edwardsiella* in fish has been reported in various countries including Ethiopia (Kebede and Habtamu, 2016); Egypt (El-Seedy *et al.*, 2015); India (Das *et al.*, 2014); Malaysia (Najiah and Lee, 2006); Spain (Alcaide *et al.*, 2006); Uganda (Walakira *et al.*, 2014) and from the Mediterranean (Katharios *et al.*, 2015).

Edwardsiella tarda is commonly present in water and can be isolated from healthy fish as normal fauna. However, the bacterium being opportunistic in nature, disease outbreaks usually occur under imbalanced environmental conditions like poor water quality, overcrowding, high organic content and high temperatures (Park *et al.*, 2012). A study by Wyatt *et al.* (1979) reported cases of *E. tarda* in African catfish and in water with incidences increasing with increase in the organic content of the pond and water temperature. *Edwardsiella tarda* has a public health importance as it is known to be zoonotic; producing disease not only in fish and other aquatic animals but also causes gastroenteritis in humans (Park *et al.*, 2012).

There is scanty information about the occurrence and prevalence of *Edwardsiella* species in Uganda, possibly due to limited studies on fish diseases in the country. Akoll and Mwanja (2012) attributed this to lack of diagnostic tools and high cost of identifying and characterizing such pathogens in subsistence

aquaculture. Therefore, this study looked to generate knowledge about *Edwardsiella* infections in cultured African catfish and Nile tilapia in different production systems in selected fish farms which is useful in planning control measures.

1.2 Problem statement and justification of the study

Despite some reports of disease outbreaks in farmed fish and *Edwardsiella* infections being one of the most common cause of diseases, information about the epidemiology of this infection in Uganda was scanty. This was possibly due to lack of studies conducted in the country despite of increased fish farming. Studies conducted elsewhere have shown that different strains and genotypes of *Edwardsiella* organisms vary in their ability to cause disease in aquaculture. Hence, accurate identification and characterization of pathogens is important for epidemiological investigations, diagnosis and control of bacterial diseases (Abowei and Briyai, 2011). The virulence factors in *Edwardsiella* species that are responsible for disease development were not known. Understanding these factors will facilitate development of disease protective measures. Therefore, this study has generated more knowledge about the occurrence, phylogenetic relationships and virulence properties of *E. tarda* and the results are to be used by the relevant stakeholders in aquaculture industry as a decision-making tool to improve the health of the fish, increase fish production and ultimately ensure food security in the country.

1.3 Objectives of the study

1.3.1 Main objective

Isolation and characterization of *Edwardsiella* species in farmed African catfish and Nile tilapia in Wakiso district, Uganda.

1.3.2 Specific objectives

The specific objectives of this study are:

- i. To determine the occurrence of *Edwardsiella* species in farmed African catfish and Nile tilapia from selected farms in Uganda;
- ii. To determine the phylogenetic relationship of *Edwardsiella* isolates from farmed African catfish and Nile tilapia in Uganda;
- iii. To determine the virulence genes present in the *Edwardsiella* isolates recovered from Nile tilapia and African catfish in Uganda.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Aquaculture industry in Uganda

Aquaculture is one of the fastest growing source of animal protein to fulfill the high demand due to the declining wild fish stocks worldwide (Takyi *et al.*, 2012). Uganda has a high potential for aquaculture development due to the availability of water and favorable climatic conditions (Directorate of Fisheries Resources, 2011). The industry has been growing substantially probably due to increasing interest in fish farming and realizing its higher profitability. There are some degrees of intensification with current fish production from aquaculture estimated to be at 111,023 metric tonnes/year (Kasozi *et al.*, 2017). However, high stocking density, poor farming management practices and poor water quality cause chronic stress and immunosuppression in farmed fish which predispose fish to infectious diseases and epizootic outbreaks caused by pathogens that would not have high expression in natural environments (Sebastião *et al.*, 2015).

2.2 Diseases in aquaculture

Fish are most susceptible to diseases compared to other domesticated animals and this is one of the major problems hindering production, development and expansion of the aquaculture industry. Aquaculture sector is plagued by infectious diseases with a global annual economic loss estimated to be US\$3 million annually (Faruk *et al.*, 2004). The major pathogens affecting aquaculture industry are bacteria, fungi, viruses and parasites. The sources and modes of infection in fish are variable but factors that influence occurrence of disease in aquaculture include the characteristics

of the pathogen, host and the environment which is determined by stocking density of fish in culture system and the physico-chemical parameters of water (Austin and Austin, 2007; Parker, 2012). The host variable includes the immune system, genetic make-up and nutrition which determine the degree of individual resistance or susceptibility to diseases as shown in Figure 1. (Huicab-Pech *et al.*, 2016).

Pathogenic bacteria are of most importance in fish health because they lead to mass mortality of both wild and cultured fish (Abowei and Briyai, 2011; Takyi *et al.*, 2012). Most pathogenic bacteria are short Gram-negative rods belonging to families Enterobacteriaceae, Pseudomonadaceae and Vibrionaceae. Outbreak of bacterial diseases in fish remains one of the most significant limiting factors affecting fish culture worldwide (Mohanty and Sahoo, 2007; Marcel *et al.*, 2013). Opportunistic bacteria become pathogenic mostly when fishes are physiologically unbalanced, nutritionally deficient, or there are other stressors like poor water quality and overstocking (El-Refaey, 2013).

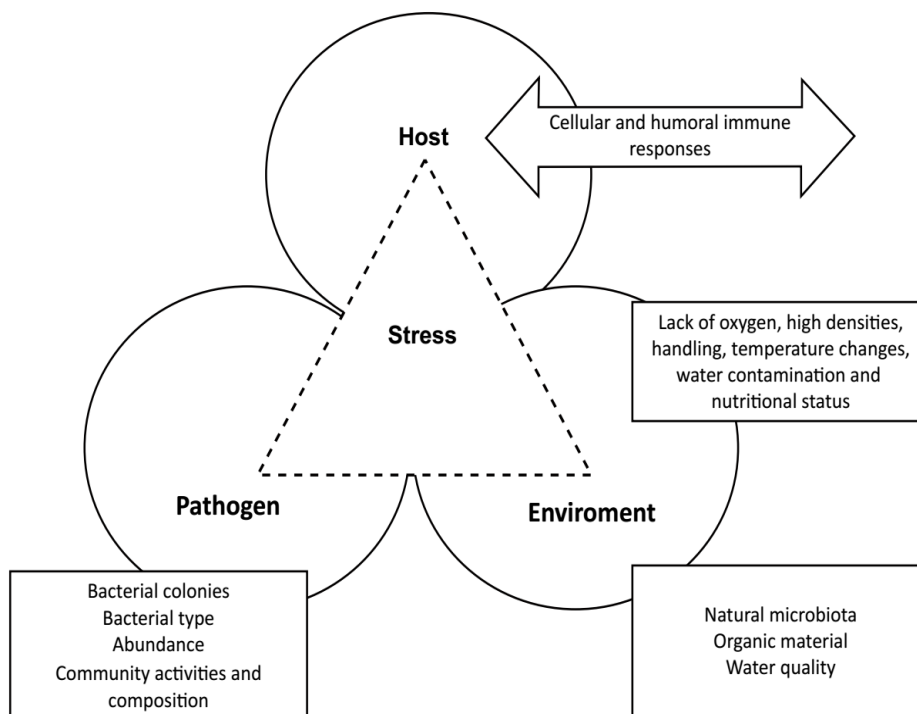


Figure 1: Conceptual diagram of equilibrium among hosts, pathogens and the environment in aquatic systems

Source: Huicab-Pech *et al.*, 2016

2.3 *Edwardsiella* infections

The genus *Edwardsiella* is composed of four species; *E. tarda*, *E. ictaluri*, *E. hoshinae* and *E. piscicida* which contains strains previously identified as *E. tarda*. Fish are usually infected with *E. tarda*, *E. piscicida* or *E. ictaluri*, whereas *E. hoshinae* infection is usually reported in reptiles and birds (Park *et al.*, 2012).

2.3.1 *Edwardsiella tarda*

Edwardsiella tarda is a Gram-negative short rod, measuring 1 µm in diameter and 2-3 µm in length. It is a facultative anaerobe and is motile at 25 and 35°C. It can survive between 0-4% sodium chloride, pH of 4-10, and a temperature range of 14 to

45⁰C. The bacterium is cytochrome oxidase negative, indole positive, Methyl Red positive, Voges-Proskauer negative, citrate (Simmons) negative, phenylalanine deaminase negative, lysine decarboxylase positive, ornithine decarboxylase positive, urease negative, catalase positive, produces Hydrogen Sulphide and hydrolyses gelatin. It produces acid from glucose, rhamnose and trehalose and not from lactose, mannitol, salicin, inositol, arabinose, erythritol, mannose, maltose, sucrose, dulcitol, adonitol, sorbitol, raffinose, xylose, cellobiose and esculin (Mohanty and Sahoo, 2007; Garcia *et al.*, 2012; Park *et al.*, 2012).

2.3.1.1 Epidemiology of Edwardsiellosis

Edwardsiella tarda is the causative agent of Edwardsiellosis in fish (Ewing *et al.*, 1965). It is synonymous to emphysematous putrefactive disease of Catfish, Red disease of Eels and *Edwardsiella* septicemia of Salmon, Tilapia and Stripped bass (Xu and Zhang, 2014). *Edwardsiella tarda* has been characterized as one of the leading bacterial pathogens affecting both freshwater and marine fish worldwide (Austin and Austin, 1987). Source of *E. tarda* is the intestinal contents of carrier animals although it can be a common inhabitant of the aquatic environment. The bacterium is usually transmitted through water from carrier animal feces, water or mud to susceptible host (Wyatt *et al.*, 1979).

The bacterium is zoonotic and causes enterohemorrhagic septicemia in amphibians, reptiles, marine mammals and in human (Park *et al.*, 2012). Makulu *et al.* (1973) found the incidence of *E. tarda* in humans in Zaire to be 1 in 400 cases (0.25%). *Edwardsiella tarda* is transmitted to humans through contamination with

infected water and the infections normally manifests as gastrointestinal infection. The bacterium has been isolated from fecal specimens in both symptomatic and asymptomatic people (Clarridge *et al.*, 1980).

Edwardsiella tarda is normally more prevalent in environments with high temperature, poor water quality and high organic content which allow its adherence to and replication in cell lines (Park *et al.*, 2012). Fish infected with *E. tarda* show several clinical signs. Diseased fish normally manifests as systemic hemorrhagic septicemia, internal abscesses in organs like liver, spleen and kidney and skin lesions (Katharios *et al.*, 2015). The infected fish also display spiral movement, exophthalmia, excessive mucus secretion and small skin lesions that progress into abscesses within muscles and produce a foul odor when opened (Mohanty and Sahoo, 2007; Garcia *et al.*, 2012). In a study by Abraham *et al.* (2015), diseased *Clarias gariepinus* fingerlings exhibit a loss of pigment, swelling of the abdominal surface, petechial hemorrhages in the fins while internally the fish showed bloody ascites and inflamed liver, spleen and kidney.

Edwardsiella tarda is phenotypically more polymorphic and capable of adapting to survival within a broad range of hosts hence it is said to be host and habitat specific (Park *et al.*, 2012). It infects several fish species and its most predominant in eels and catfish (Meyer and Bullock, 1973). Its occurrence in both Nile tilapia and African catfish has been reported in Uganda (Walakira *et al.*, 2014). Although the disease prevalence in ponds is said to seldom exceed 5% and 50% in tanks (Mohanty and Sahoo, 2007), some studies have reported its prevalence to be as high

as 70%. Abel-Latif and Sedeek (2017) reported its occurrence in Egypt at a prevalence of 10.42% in diseased Nile tilapia whereas another study again in Egypt on experimental infection of *E. tarda* in Nile tilapia and African catfish resulted in mortalities of up to 60% and 70%, respectively. Another study by Aly (2013) in Egypt reported incidences of *E. tarda* in Nile tilapia and African catfish at 34% and 50%, respectively, in 2006 which was higher than that reported by Abel-Latif and Sedeek (2017).

In Nigeria, the bacterium was isolated from *C. gariepinus* at a prevalence of 3.5% (Efuntoye *et al.*, 2012). The bacterium is also known to occur in other continents as reported by Kumar *et al.* (2016) in finfish and shellfish in India where the overall incidence was 14.14% with incidences being higher in wild fish (15.30%) than in cultured fish (13.26%). A study by Joh *et al.* (2010) in Korea reported the occurrence of Edwardsiellosis in cultured eels to be at 72% which is much higher than those reported elsewhere. *Edwardsiella tarda* was also reported to cause disease in cage-cultured sharpnose sea bream (*Diplodus puntazzo*) in the Mediterranean (Greece) with a cumulative mortality rate reaching 5.3% (Katharios *et al.*, 2015).

Edwardsiella tarda is an intracellular pathogen and its pathogenicity mechanisms in terms of adherence, penetration, survival and replication in host cells are not clearly understood (Xu and Zhang, 2014). Therefore, to understand its pathogenesis, identification of virulence-related genes is essential. *Edwardsiella tarda* pathogenesis is believed to be multifactorial and there are a number of potential pathogenic properties that contribute to its infection including adhesins, Type III secretion system (T3SS), Type VI secretion system (T6SS), ability to survive and

replicate in phagocytes, dermatotoxins, antiphagocyte killing, hemolysins, serum resistance, quorum sensing, ferric uptake regulator and ability to invade epithelial cells (Park *et al.*, 2012; Xu and Zhang, 2014). Wang *et al.* (2009) suggested that the most important properties for the initiation of *E. tarda* infection process are surface structures mediating motility, adherence and pathogen-host recognition. The virulence properties of pathogens depend on the presence of plasmids. Plasmid profiles of *E. tarda* are related to geographical locations and year of isolation (He *et al.*, 2011). Several comparative phylogenomic studies of *E. tarda* identified two distinct genetic groups of the bacterium much as they are phenotypically classified as *E. tarda* (Yang *et al.*, 2012).

2.3.1.2 *Edwardsiella tarda* virulence

Pathogenic bacteria are known to have virulence genes that are absent in non-pathogenic bacteria. Some virulence genes are specific to pathogenic *E. tarda* and the more the number of virulence genes the more the pathogenicity of the bacterium (Emanet *et al.*, 2016). Some studies indicate variations in virulence of *E. tarda* strains from different origins whereby strains from marine fish are more virulent than those from freshwater fish (Xu and Zhang, 2014).

Edwardsiella tarda is an opportunistic bacterium causing infection when the fish are faced with stressful conditions (poor water quality, high organic content and overcrowding) and high temperatures affect the production of some substances that contribute to its pathogenicity (Hossain *et al.*, 2010). Temperature also affects the expression of the two protein secretion systems that are involved in *E. tarda* virulence (Guijarro *et al.*, 2015).

2.3.2 *Edwardsiella ictaluri*

Edwardsiella ictaluri has a limited host distribution compared to *E. tarda*. It causes enteric septicemia of catfish (ESC). The bacterium is a short, Gram-negative rod with a dimension of 0.8 by 3µm. *Edwardsiella ictaluri* is oxidase negative and it attains its best growth between temperatures of 25 to 30⁰C. The disease outbreaks occur in temperature range of 18-28⁰C, whereas a low-level mortality and carrier status have been observed at temperatures outside this range (Zhang, 2007).

Fish infected with *E. ictaluri* normally show clinical signs including petechial hemorrhages on skin, small cutaneous lesions, abnormal posture where infected fish hang with a head-up-tail-down and spin while swimming. Internally, the peritoneal cavity may contain bloody ascetic fluid and the intestines have petechial hemorrhages, multifocal necrosis of liver and swollen trunk and kidney. The brain can swell and become ulcerated if the infection is chronic developing an open lesion through the skull hence a “hole in the head” disease (Zhang, 2007).

2.4 Diagnosis of *Edwardsiella* infections

Diagnosis involves identification of the bacterium using microbial culture techniques followed by biochemical characterization of the isolates. The bacteria can be isolated from infected tissues using general culture media like Brain Heart Infusion agar (BHI), trypticase soy agar (TSA), MacConkey, Blood Agar, Xylose Lysine Deoxycholate (XLD) agar or a selective medium (*E. ictaluri* medium or EIM) for the recovery of *E. ictaluri* (Zhang, 2007). *Edwardsiella tarda* is normally seen as small, circular, raised, clear colonies with black centers on XLD and pale on

MacConkey agar. Alternative techniques to direct detection of the bacteria can also be used like serological methods which include using Enzyme immunoassay (ELISA), loop-mediated isothermal amplification (LAMP), indirect fluorescent antibody (IFA) test, fluorescent *in situ* hybridization (FISH) and use of molecular techniques like Polymerase Chain Reaction (PCR) (Mohanty and Sahoo, 2007).

2.5 Economic and health impacts of *Edwardsiella* infections

Occurrence of diseases in fish farms lowers production in aquaculture. Cost of fish diseases is approximately 15% to income of fish farmers in Bangladesh from fish (Faruk *et al.*, 2004). Effects of disease occurrence in terms of economic loss is felt more in small-scale farms compared to medium and large-scale farms. This is partly due to small-scale farmers having limited knowledge in aquaculture and are generally reluctant to use new technologies for disease control and prevention like antibiotics (Faruk *et al.*, 2004). Intensification of aquaculture especially in large-scale farms has led to over stocking fish in the culture systems which increases stress in the fish. This negatively affects the health of the fish as they are predisposed to opportunistic bacterial infections like *E. tarda* which manifests during poor environmental conditions (Mohanty and Sahoo, 2007).

2.6 Proposed control measures

The control of fish diseases in aquaculture is predominantly difficult as fish are cultured in systems where production depends on natural environmental conditions. Occurrence of fish diseases greatly depends on changes in the aquatic environment.

Since the bacteria are prevalent in fish farms, Mohanty and Sahoo (2007) suggested the best way to prevent diseases is to maintain proper environmental conditions in the culture systems. This includes maintaining physico-chemical parameters at optimum, proper hygiene and sanitation and avoid overcrowding. Antibiotics like Ormetoprim-sulfadimethoxine, Aquaflor and oxytetracycline are widely used in treatment of *Edwardsiella* infections much as there are studies that reported the development of drug-resistant strains.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study area

This study was carried out in Wakiso District in central Uganda which covers a total area of 1,906.7km² as shown in Figure 2. It is located at 0°4'N North latitude, 32°45'E East longitude and 1218m elevation above sea level. Among the main economic activities taking place in the district is fishing mainly on Lake Victoria and fish farming both commercial and subsistence in ponds, tanks and cages. The district was selected by FAO (2005) as one of the districts in the country 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).

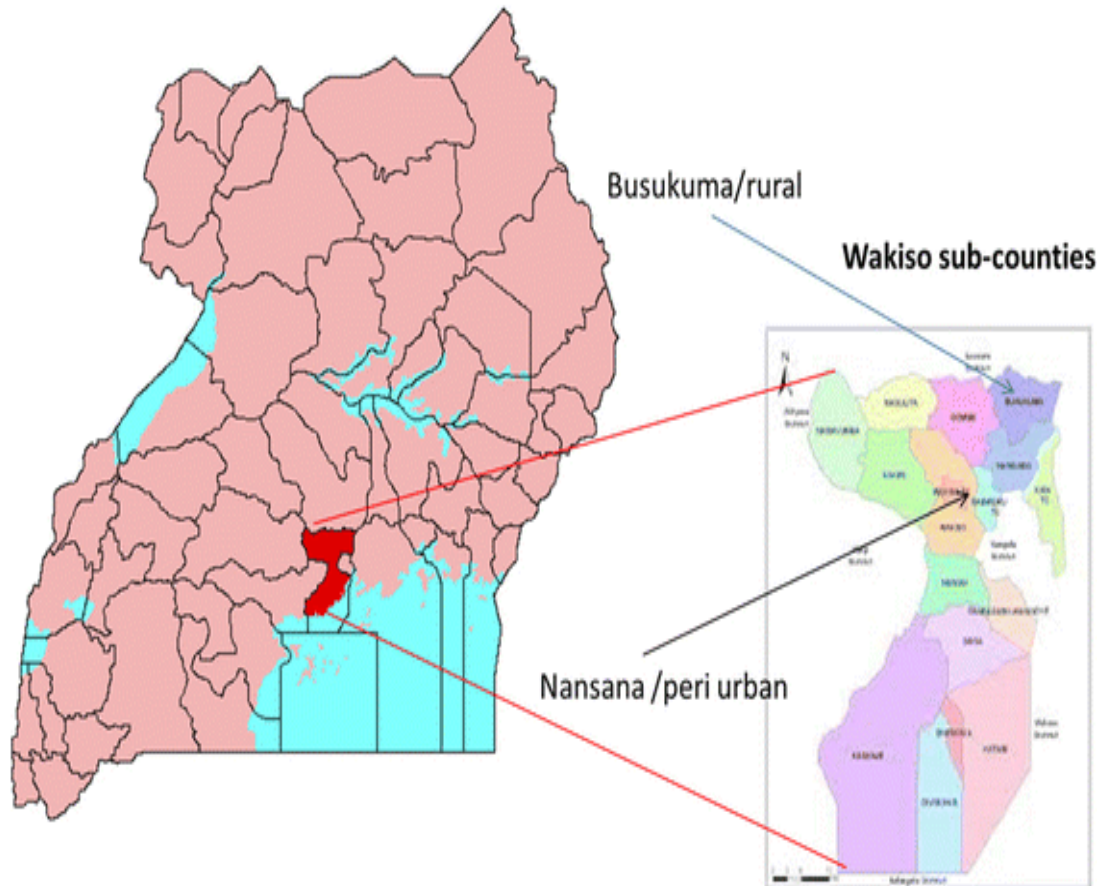


Figure 2: Map of Uganda showing the location of Wakiso district (source: Kalyesubula *et al.*, 2017)

3.2 Study design and sampling strategy

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

For sampling, fish farms were selected purposively with the farms that reported fish disease being given priority and also depending on the willingness of the farmer to provide samples. Simple random sampling was done to select healthy fish from the culture system whereas in case of disease occurrence, diseased fish were purposively selected from the culture system.

3.2.1 Sample size

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

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

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 both Nile tilapia and African catfish were collected from 17 farms in Wakiso district.

3.2.2 Sample and data collection

Samples were collected from 17 fish farms. A biodata form (Appendix 1) was administered to fish farmers whose farms were visited to gather information about the management practices and farm history. A total of 5 to 10 fish were randomly picked from the culture system (pond, tank or cage) using a seine net, and in case the system was mixed culture, 10 fish were picked; 5 of each species. A total of 111 fish samples were obtained from the 17 farms and these included apparently healthy,

moribund and recently dead fish. Out of the 111 fish samples collected, 81 were Nile tilapia and 30 were African catfish because there were more farms rearing Nile tilapia than African catfish. The fish were collected from different culture systems including earthen ponds, concrete tanks and cages. The live and moribund fish were transported in plastic tanks filled with water and with or without oxygen depending on the distance to the laboratory while recently dead fish were transported in insulated ice boxes containing ice to the Central Diagnostic Laboratory (CDL) at the College of Veterinary Medicine, Animal Resources and Biosecurity at Makerere University.

3.2.3 Sample examination

There were 7 catfish fry that were crushed and inoculated directly on Xylose Lysine Desoxycholate (XLD) agar plates. Apparently healthy and moribund Nile tilapia were humanely sacrificed by pithing while African catfish were covered on the eyes by a cloth and hit by a hard material on the head. These plus the dead fish were weighed and their length measured using a digital weighing scale and a measuring ruler, respectively. The fish were then subjected to clinical and post mortem examination to determine any abnormalities both externally and internally.

After examination, skin and gill swabs were taken and also directly inoculated on XLD agar and incubated at 37⁰C for 24 hours. The fish were then dissected using sterilized scissors and forceps by making a transverse incision anterior to the anus towards the ventral part of the head up to the gill covers, another incision was made at the anus cutting craniodorsally toward the lateral line following the dorsal margin

of the peritoneal cavity up to the gill arches and finally a third incision connecting the ends of the two cuts. The sidewall was then removed to expose the internal organs.

Examination of organs was done by observing for any abnormalities including their position, size, color and other signs of the disease. The digestive tract, gonads and visceral organs were removed by cutting the esophagus thus, disconnecting them from kidneys. Samples of organs of interest (spleen, kidney and liver) were collected using a surgical blade. Organs were cultured separately from gills and skin swabs of the same fish; hence 208 plates were used for 104 fish samples and the remaining 7 were for the crushed African catfish fry, hence a total of 215 plates.

3.3 Bacterial isolation

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

3.3.1 Phenotypic identification of isolates

Identification of the bacterium was done using cultural and morphological characteristics, a number of conventional biochemical tests and Analytical Profile Index (API 20E) system (BioMerieux, France) was done according to manufacturer's instructions. Conventional biochemical tests that were done in the identification of *E. tarda* included Gram stain, motility, indole production, cytochrome oxidase, H₂S production, Methyl Red, Voges-Proskauer, gelatin hydrolysis, urease, citrate, esculin hydrolysis, and utilization of lactose. The suspects from the conventional tests were again identified using the API 20E kits and their biochemical profiles were determined. These were preserved in cryovials containing BHI broth and glycerol and then stored in the freezer at -4⁰C until further testing.

3.3.2 Molecular characterization

3.3.2.1 Genotypic characterization

The preserved *E. tarda* isolates were retrieved and inoculated out on nutrient agar slants and transported to the Microbiology laboratory at the Norwegian University of Life Sciences (NMBU). Here, the bacteria were subcultured and part preserved at -80⁰C as glycerol stocks while the rest was used to extract genomic DNA at the Genlab, NMBU where further molecular identification and characterization was carried out.

3.3.2.2 DNA isolation

Genomic DNA extraction was done using QIAamp DNA mini kit (Qiagen) following manufacturer's instructions. Briefly, the bacterium was added to 200µl of lysis buffer with lysozyme and incubated at 37⁰C for at least 30 minutes. Then 20µl of

Proteinase K and 100µl of Buffer AL (lysis buffer) were added and incubated for 30 minutes at 56⁰C. The DNA was precipitated by adding 200µl of 100% ethanol to the sample and mixed by pulse-vortexing for 15 seconds and then briefly centrifuged.

The mixture plus the precipitate were carefully applied to the QIAamp mini spin column in a 2ml collecting tube for binding the DNA to the column. The cap was closed and centrifuged at 6000xg (8000rpm) for 1 minute. 500µl buffer was added to the QIAamp column and centrifuged at 6000xg (8000rpm) for 1 minute. 500µl of Buffer AW2 was added to the QIAamp column and centrifuged at full speed (20,000xg; 14,000rpm) for 3 minutes. The QIAamp column was placed in a new 2ml collection tube and centrifuged at full speed for 1 minute. The QIAamp column was again placed in a new clean 1.5ml microcentrifuge tube and the collecting tube containing the filtrate was discarded. 30µl of Buffer AE was added to the column and incubated at room temperature for 1 minute then centrifuged at 600xg (8000rpm) for 1 minute. The resultant DNA was stored at -20⁰C.

3.3.2.3 DNA Purification

DNA was purified using a QIAquick® gel extraction kit (cat. nos. 28704 and 28706) following the manufacturer's instructions as follows; Absolute ethanol was added to Buffer PE and thoroughly mixed. All centrifugation steps were carried out at 17,900xg (13,000 rpm) in a conventional table top microcentrifuge.

The DNA fragment was excised from the agarose gel using a clean sharp scalpel. The gel slice was weighed in a colorless tube and 3 volumes of Buffer QG was

added to 1 volume gel (100mg gel, 100µl). This was incubated at 50°C for 10 minutes when the gel slice had completely dissolved while vortexing the tube every 2 minutes to help dissolve the gel. One gel volume of isopropanol was added to the sample and mixed. Safety goggles were used to protect eyes from the UV light.

DNA binding was done by applying the sample to the QIAquick column and centrifuged for 1 minute until all the samples have passed through the column. The flow-through was discarded and placed the QIAquick column back in the same tube. A 500µl Buffer QG was added to the QIAquick column and centrifuged for 1 minute. The flow-through was again discarded and placed the QIAquick column back in the same tube. Then 750µl Buffer PE was added to QIAquick column and centrifuged for 1 minute for washing. The flow-through was again discarded and placed the QIAquick column back in the same tube. The column was left to stand for 5 minutes after addition of Buffer PE. The QIAquick column was then centrifuged in the provided 2ml collection tube for 1 minute to remove the residual wash buffer. The QIAquick column was then placed into a clean 1.5ml microcentrifuge tube.

To elute DNA, 30µl Buffer EB (10 mM Tris.Cl, pH 8.5) was added to the center of the QIAquick membrane and the column was let to stand for 1 minute and then centrifuged for 1 minute.

3.3.2.4 Amplification of the *16SrRNA* 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 25µl containing 2.5µl of 10x reaction buffer (50mM KCl, 75mM Tris-HCl (pH 9.0), 2mM MgCl₂, 20mM (NH₄)₂SO₄), 0.5µl 10mM deoxyribonucleotide mix, 0.2µl of *Taq* DNA polymerase, 1µl 10 mM of each forward and reverse primer, 2µl of DNA template and 16.8µl of sterile ultrapure water. PCR conditions included initial denaturation at 94⁰C for 3 minutes, followed by 30 cycles of amplification as follows; denaturation at 94⁰C for 30 seconds, annealing at 56⁰C for 30 seconds and extension at 72⁰C for 2 minutes. This was followed by a final extension step at 72⁰C for 5 minutes and left to stand at 4⁰C until collected for further analysis.

The PCR products were analyzed on 1% agarose gel (Ultrapure agarose from Invitrogen, Thermo Fisher Scientific) using PowerPac 300 (Bio-Rad) at 100Volts for 60 minutes. The amplified DNA on the gel was visualized using Safe Imager™ from Invitrogen and bands of interest excised using a scalpel blade for gel purification. ChemiDoc™ XRS Molecular image (Bio Rad) was used for viewing and capturing gel pictures. The concentration of the DNA was measured using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc).

3.3.2.5 DNA Sequencing

The PCR products were sequenced with 16S 27F and 16S1492R universal bacteria primers by Sanger sequencing techniques at GATC Biotech, Germany on ABI genetic analyzer.

3.4 Phylogenetic analysis

The *16S rRNA* gene sequence obtained was edited using Bioedit and Basic Local Alignment Search Tool (BLAST) searches carried out in GenBanks especially National Center for Biotech Information (NCBI). Related sequences were obtained and multiple sequence alignment was performed using ClustalW algorithm. The gene sequences used in phylogenetic analysis are shown in table 3. These sequences were used to construct a phylogenetic tree.

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

To characterize the virulence attribute of *E. tarda* isolate, seven virulence genes were screened for in this study. These included: *gadB* (glutamate decarboxylase; resists host's phagocyte killing activity), *muk* (putative killing factor), *citC*, *esaV*, *fimA* (enables adherence of the bacterium to the host for invasion), *esrB* (assists in penetration by encoding for a regulator protein for type III secretion system (T3SS), and *katB* (provides resistance to the bacterium against host phagocyte killing activity). The genes were amplified by PCR using primers in Table 1 below.

PCR reaction was performed in a final volume of 25 μ l containing 0.25 μ l of 10x reaction buffer (5mM KCl, 7.5mM Tris-HCl (pH 9.0), 0.2 mM MgCl₂, 2 mM (NH₄)₂SO₄), 0.05 μ l 1mM deoxyribonucleotide mix, 0.02 μ l of *Taq* DNA polymerase, 0.1 μ l 1mM of each forward and reverse primer, 0.2 μ l of DNA template and 1.68 μ l of sterile ultrapure water.

The PCR program used was as follows: initial denaturation at 94⁰C for 3 minutes, followed by 32 cycles of denaturation at 94⁰C for 1 minute, annealing at 55⁰C for *citC*, *muk*, *esrB*; 58⁰C for *katB*; 57⁰C for *gadB* and 60⁰C *fimA* for 1 minute, extension at 72⁰C for 1 minute, and final extension at 72⁰C for 10 minutes and left to stand at 4⁰C until collected for further analysis.

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

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

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

3.6 Data analysis

Data was stored, summarized and analyzed using Microsoft Excel, Windows 10. Quantitative data (weight and length) data were reported in mean. Sequences were edited in MEGA 7.0 software and aligned using ClustalW algorithm. A phylogenetic tree was constructed in MEGA 7.0 (Kumar *et al.*, 2016) using the Neighbor-Joining method (Saitou and Nei, 1987).

CHAPTER FOUR

4.0 RESULTS

4.1 General description and farm history

Seventeen fish farms were visited in Wakiso District where a total of 111 apparently healthy, moribund and recently dead fish were sampled and subjected to clinical and post mortem examination. The total number of Nile tilapia was 81 (72.9%) with average body weight of 113.1g and mean length 17.42cm. The total number of African catfish was 30 (27%) with average body weight of 154.34g and mean length of 25.1cm. Seven of the 30 African catfish were fingerlings hence their weight and length were not measured. The fish were collected from three culture systems, namely, earthen ponds (10), tanks (1) and cages (4). Two farms had both ponds and tanks. 82.3% of the total number of farms practiced monoculture; rearing either Nile tilapia or African Catfish, while 17.6% of the farms had mixed cultures rearing both species in the same system and these were in earthen ponds. All culture systems, except one hatchery from which the African catfish fingerlings were collected were production (growout) systems.

All farms that had ponds were located in either low lying isolated areas or agricultural fields and their sources of water were either streams or springs. Cages were located on the lake and river shores while the farm that had tanks only was located in a residential area with its water source being tap and rainwater. All farms reported feeding fish with commercial feeds twice or once a day depending on the availability of the feed, weather and response of the fish. All farms that had earthen ponds used organic fertilizers to stimulate primary production. Most farms used chicken droppings (75%) for fertilization where as others used cow dung (25%).

Two (11.7%) farms reported having a history of disease in both dry and wet seasons, while others reported observing some signs of disease including loss of appetite, dropsy, low growth rate, abnormal swimming, exophthalmia, parasites and mortality. The farmers also admitted to use antibiotics, formalin, potassium permanganate and salt to treat the sick fish. The farms that reported a history of disease also reported overfeeding and poor water quality.

4.2 Clinical signs and post-mortem examination

Of the examined fish, the infected Nile tilapia had ascites and petechial abdominal hemorrhages, whereas African catfish showed signs of abnormalities including petechial hemorrhages on the skin and fins, lordosis, a pale liver, ulcers on the belly and opercula region (Figure 3).

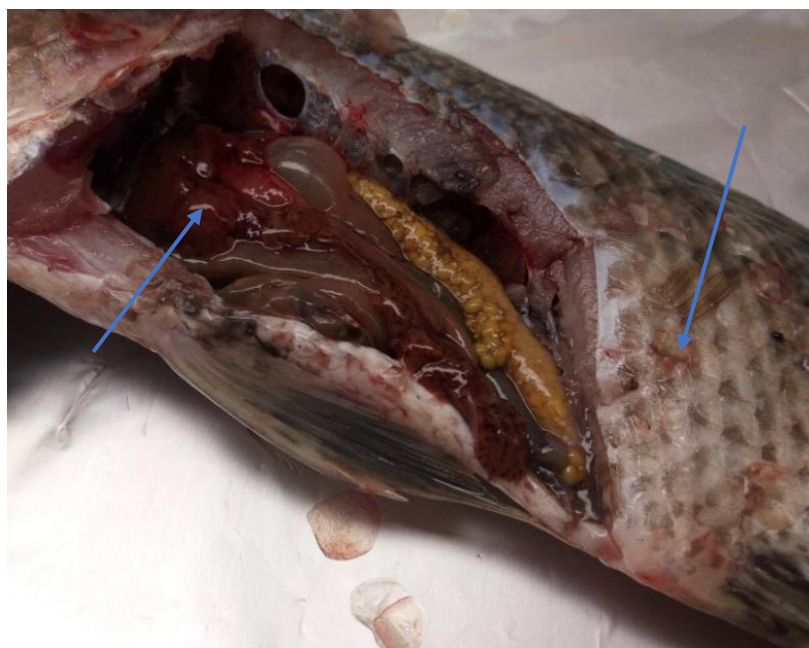


Figure 3: Hemorrhages and accumulation of ascitic fluid in Nile tilapia (arrows)

4.3 Isolation of *Edwardsiella tarda*

Fifty-one plates out of 215 plates had produced clear colonies with black centers surrounded with reddened media due to decarboxylation of lysine on Xylose Lysine Deoxycholate (XLD) media after 36 hours at 37°C (Figure 4) and these were subjected to conventional biochemical tests.

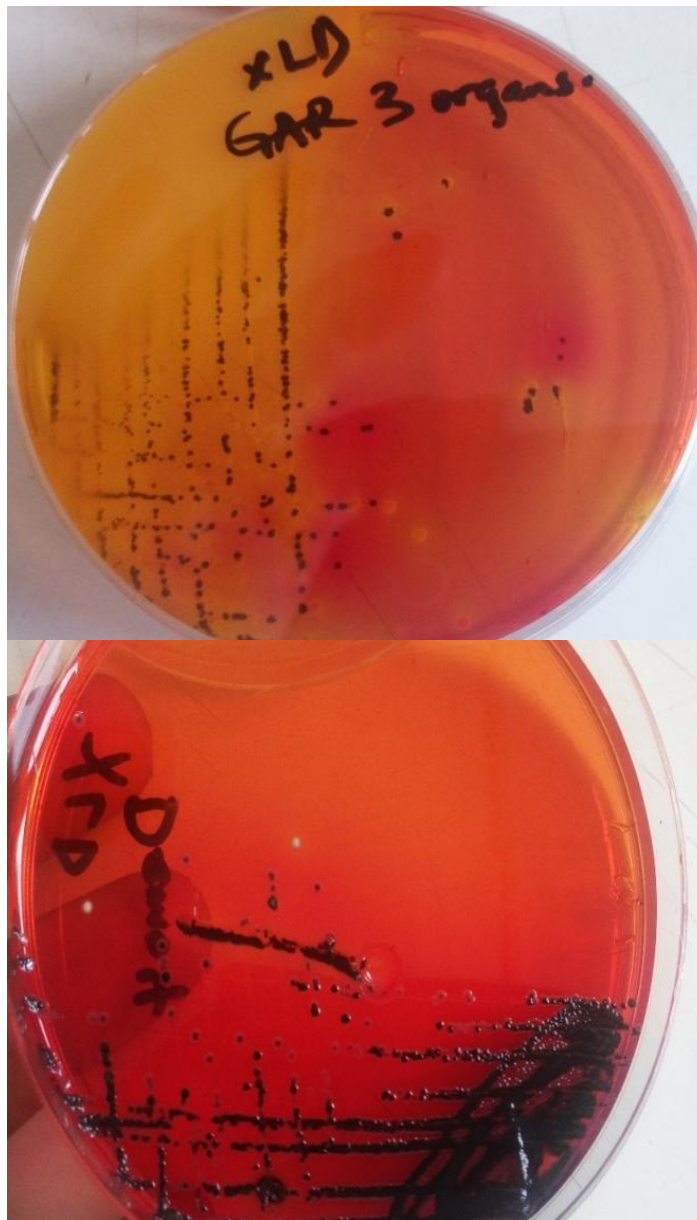


Figure 4: Clear colonies with black centers on XLD agar plates

4.4 Phenotypic identification of *Edwardsiella tarda* isolates

Presumptive identification of *E. tarda* using conventional biochemical tests gave eight (7.2%) *E. tarda* from four (3.6%) tilapia and four (3.6%) African catfish. All the isolates were Gram negative and microscopic examination of the wet mounts showed motile rods that measured 2µm in length and 1µm in diameter. All isolates tested negative for cytochrome oxidase, citrate, esculin hydrolysis, gelatin hydrolysis, urease and lactose fermentation. All isolates produced H₂S and tested positive for indole production. There were variations in the MRVP tests as only two isolates tested positive for MR and negative for VP (Table 2).

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

Test	Isolates							
	<i>O.n1</i>	<i>O.n2</i>	<i>O.n3</i>	<i>O.n 4</i>	<i>C.g1</i>	<i>C.g 2</i>	<i>C.g 3</i>	<i>C.g4</i>
Gram stain	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-
Indole production	+	+	+	+	+	+	+	+
Citrate	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+	+
Methyl Red	+	-	-	-	+	-	-	-
Voges-Proskauer	-	+	+	+	-	+	+	+
Esculin hydrolysis	-	-	-	-	-	-	-	-
Gelatin hydrolysis	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-
Lactose utilization	-	-	-	-	-	-	-	-
H ₂ S production	+	+	+	+	+	+	+	+

Key: + means a positive result, - means a negative result

4.5 Identification of the isolates using API 20E kit

One isolate was again presumptively identified to be *E. tarda* at 99.4% using the Analytical Profile Index (API) 20E kit with code number 4744000 at 37⁰C (Appendix 2). The seven isolates identified as *E. tarda* in the biochemical tests were identified by the API 20E kit as *Plesiomonas shigelloides* which is a fish pathogen belonging to family Enterobacteriaceae (Appendix 3). *Plesiomonas shigelloides* has a worldwide distribution and outbreaks in humans have been reported in Cameroon from cold fish. It has a wide host range including, aquatic environments, aquatic animals, mammals and humans causing gastroenteritis (Janda *et al.*, 2016).

4.6 Sequence analysis of the 16S rRNA gene for identification of *E. tarda*

Sequencing the 16S rRNA has been extensively used in the understanding of bacterial evolution and phylogeny and it is regarded as an essential tool in bacterial systematics and identification of new species. Hence in this study, the bacterial isolate identified to be *E. tarda* by API 20E kit was further identified and confirmed as *E. tarda* by the amplification of the 16S rRNA gene which resulted in PCR product at the expected size of 1465bp.

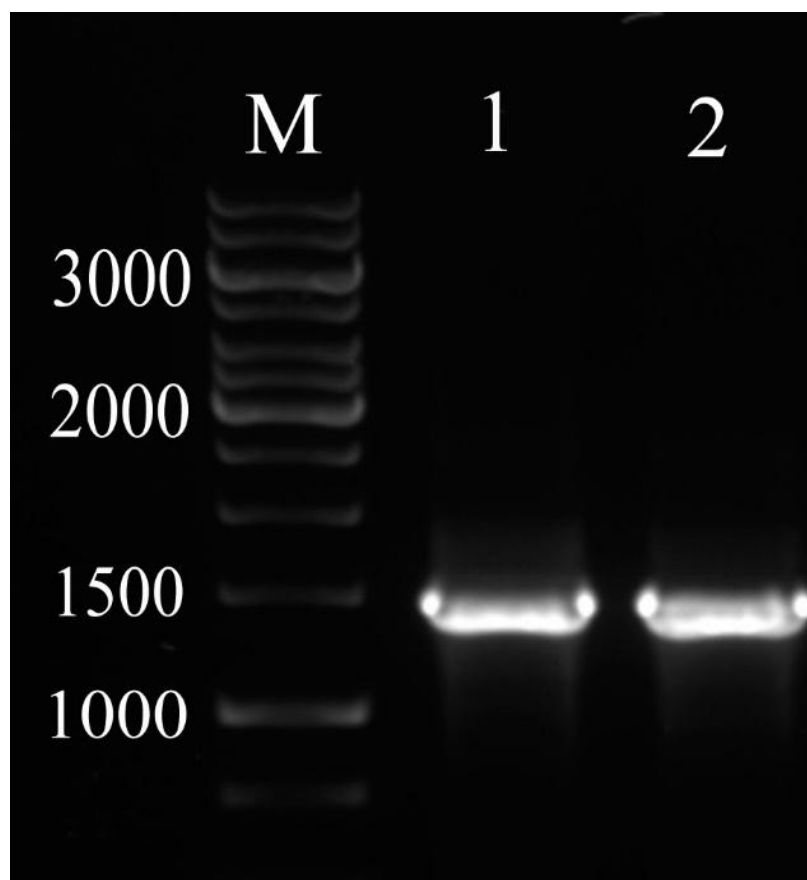


Figure 5: *16S rRNA* gene PCR products on a 1% agarose gel

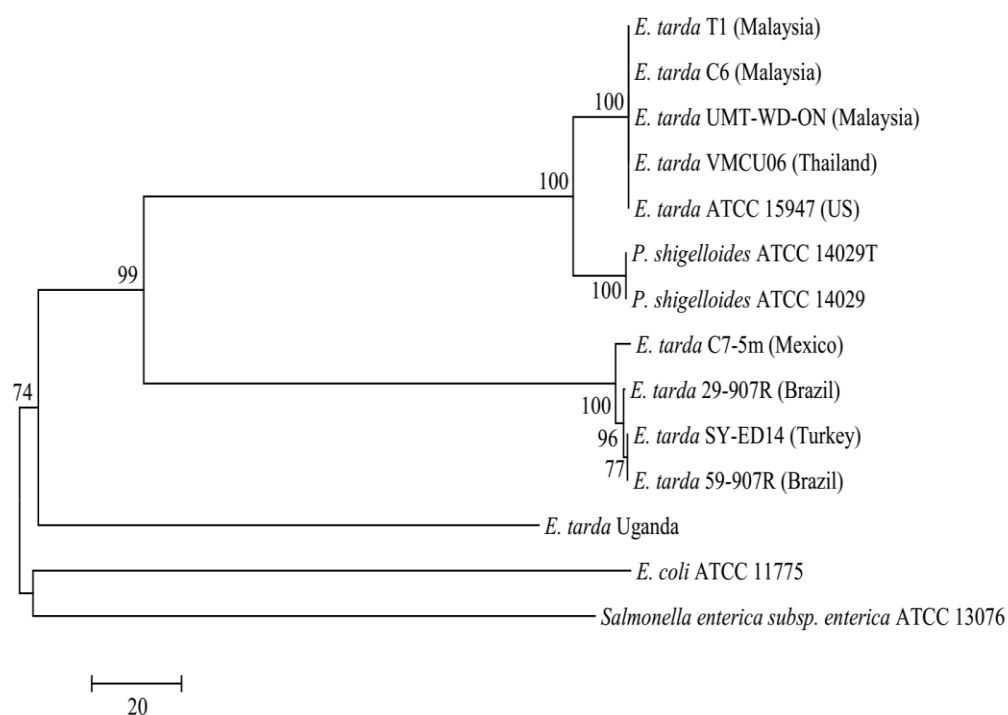
Lane M: Molecular marker, lanes 1 and 2: *Edwarsiella tarda*.

4.7 Phylogenetic analysis

Comparison of the *E. tarda* isolate with known *16S rRNA* sequences in the GenBank database using the BLAST program showed that the isolate had an identity rate of 99.9% to those of other members of *E. tarda*. The phylogenetic relationship of the *E. tarda* isolate was studied using the *16S rRNA* gene with other *E. tarda* strains that were 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 shown in Table 3.

Table 3: Isolates whose sequences were used in phylogenetic analysis

	Name of culture	Accession number
1	<i>E. tarda</i> strain T1	KX388234.1
2	<i>E. tarda</i> strain C6	FJ607400.1
3	<i>E. tarda</i> strain UMT-WD-ON	FJ600537.1
4	<i>E. tarda</i> strain VMCU06	KU860461.1
5	<i>E. tarda</i> strain C7-5m	HQ663902.1
6	<i>E. tarda</i> strain ATCC 15947	JX866952.1
7	<i>E. tarda</i> strain 29-907R	KX828266.1
8	<i>P. shigelloides</i> ATCC 14029	M59159.1
9	<i>E. tarda</i> strain SY-ED14	KX388234
10	<i>P. shigelloides</i> ATCC14029T	X74688.1
11	<i>E. tarda</i> strain 59-907R	KX828321.1
12	<i>Salmonella enterica subsp enterica</i> ATCC 13076	LSHA01000019.1
13	<i>E. coli</i> ATCC 11775	NZ_JMST01000035.1

**Figure 6: Phylogenetic relationship of *E. tarda* from Uganda with other strains**

The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985).

4.8 Detection of virulence genes in the *Edwardsiella tarda* strain

The *E. tarda* strain was screened for the presence of 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* that was isolated in this study including; *CitC*, *muk*, *gadB*, *katB*, *esaV* and *fimA*.

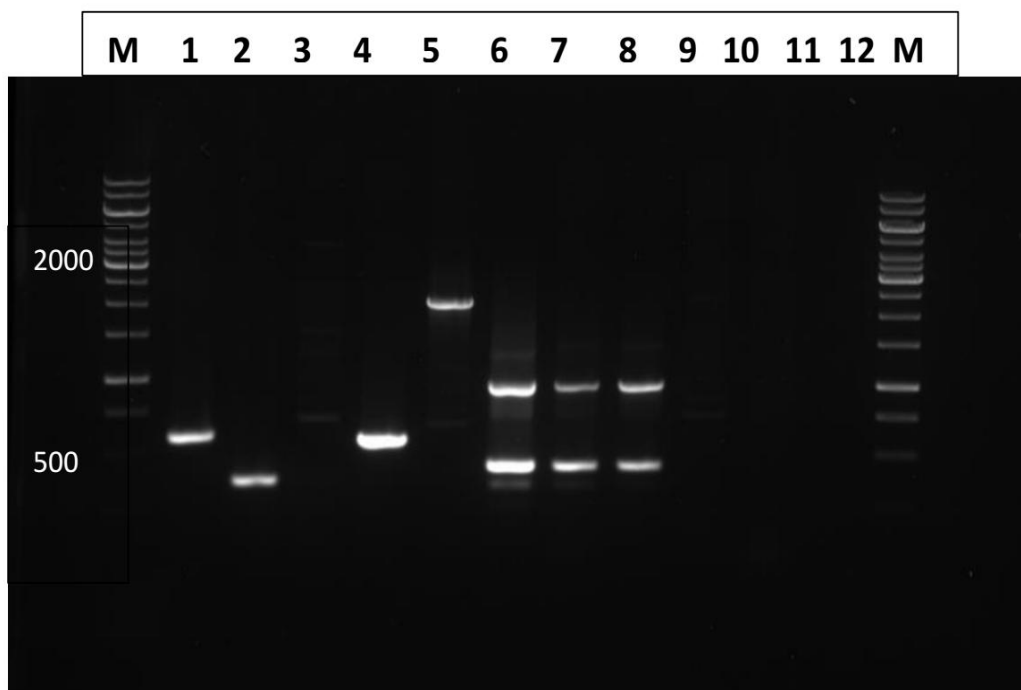


Figure 7: Agarose Gel electrophoresis of PCR of virulence genes for the isolated *E. tarda*

Key: Lane M is DNA marker, lane 1 is *citC*, lane 2 is *muk*, lane 3 is *esrB*(-ve), lane 4 is *gadB*, lane 5 is *katB*, lanes 6, 7 and 8 are *fimA* and *esaV*, Lanes 9 to 12 are not considered and lane M is DNA marker.

CHAPTER FIVE

5.0 DISCUSSION

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

In this study, *O. niloticus* that produced the confirmed *E. tarda* isolate showed signs of ascites and petechial abdominal hemorrhages. These presentations were similar to what others reported for *E. tarda* infection (El-Refaey, 2013; El-Seedy *et al.*, 2015). However, Edwardsiellosis symptoms and gross lesions are not reliable in arriving to a conclusive diagnosis as several bacterial infections also have similar signs and symptoms (Mohanty and Sahoo, 2007). Furthermore, diseased fish with or without any clinical signs of disease are equally important as they may carry pathogens causing a risk of spreading disease to other species including humans. Therefore, detection of these pathogens is crucial for their effective prevention and control (Castro *et al.*, 2014).

Successful isolation and correct identification of a particular pathogen greatly relies on the standardized bacteriological culture methods used. In the current study, the bacterium was grown on XLD agar and produced clear colonies with black centers surrounded with reddened media (Figure 4) due to decarboxylation of lysine (Wyatt *et al.*, 1979; Najiah and Lee, 2006; Wei and Musa, 2008). All the isolates in this study were Gram negative and microscopic examination of the wet mounts showed motile rods that measured 2 μm in length and 1 μm in diameter. These findings were similar to those of other researchers who isolated *E. tarda* from cultured freshwater tilapia, African catfish, chinook salmon and sharpnose seabream, (Amandi *et al.*, 1982; El-Refaey, 2013; Griffin *et al.*, 2015; Katharios *et al.*, 2015; Abraham *et al.*, 2015; Emanet *et al.*, 2016).

All isolates tested negative for cytochrome oxidase, citrate, esculin hydrolysis, gelatin hydrolysis, urease and lactose fermentation. All isolates tested positive for H₂S and indole production (Table 2). All these findings concur with those from other studies (Wyatt, 1979; Amandi *et al.*, 1982; Joh *et al.*, 2010; El-Refaey, 2013; El-Seedy *et al.*, 2015; Griffin *et al.*, 2013; Abraham *et al.*, 2015; Emanet *et al.*, 2016). There were variations in the MRVP tests as only 2 isolates tested positive for MR and negative for VP. These results were not in agreement with any literature and this could possibly mean that these maybe new strains of *E. tarda*. However, variations in phenotypic characteristics are attributed to the presence and absence of plasmids that control the metabolic traits of the phenotypic characteristics of the isolates (Acharya *et al.*, 2007; Das *et al.*, 2014).

Biochemical identification using conventional bacteriology techniques was inconclusive in discriminating between *E. tarda* and *Plesiomonas shigelloides*. When the API 20E kit was used on all the eight isolates identified as *E. tarda* by the biochemical tests, only one isolate turned out to be *E. tarda* at 99.4% (Appendix 2). This is because it tested positive for citrate yet in the conventional biochemical tests it was citrate negative, hence it would have been 99.9% *E. tarda*. According to Buller (2004), differences in reactions occur between the API system and conventional biochemical tests and this is especially reported in decarboxylases, citrate, urea, indole and Voges-Proskauer tests. El-Seedy *et al.* (2015) also reported that 22.2% of their *E. tarda* isolates tested positive for citrate utilization and concluded that those isolates were typical strains of *E. tarda*.

There were more isolates confirmed to be *E. tarda* by conventional biochemical tests than in molecular techniques possibly due to the high degree of phenotypic diversity within bacterial species which limits the accuracy of biochemical identification especially when few isolates are tested hence more work is required to investigate the phenotypic diversity of *E. tarda* (Griffin *et al.*, 2013).

Phylogenetic analysis separated the *E. tarda* strains in two groups without placing *E. tarda* isolated in this study in any group. One group clustered with *Plesiomonas shigelloides* with high bootstrap support (100%). *Plesiomonas shigelloides* is deep rooted in the family Enterobacteriaceae and is aligned closer to *E. tarda*. Hence, this could explain its misidentification in the biochemical tests as *E. tarda* in this study (Janda *et al.*, 2016). The second group shares greater similarity to the *E. tarda* isolate from this study with bootstrap support of 74% than the first

group. These findings concur with those of other comparative phylogenomic studies which identified two distinct genetic groups of *E. tarda* (Panangala *et al.*, 2006; Griffin *et al.*, 2013). This finding therefore, suggests that the *E. tarda* isolate from this study is genetically distant from the GenBank that are included in this study.

The ability of a bacterium to cause disease depends on the expression of virulence factors which enable the bacteria to invade the host, produce pathological effects and evade host defenses. A microorganism's ability to invade the host is the most important aspect of its pathogenicity (Roberts, 2012). Identification of virulence genes required for establishment of infection in hosts aids in understanding the mechanisms of pathogenesis in bacteria which aids in the development of vaccines and formulating therapeutics to protect fish against the infection. *Edwardsiella tarda*'s virulence is believed to be multifactorial and several authors have reported a number of potential virulence factors that play a role in its pathogenesis (Mohanty and Sahoo, 2007; Mendez *et al.*, 2012; Park *et al.*, 2012). Several potential pathogenic properties are believed 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 for dissemination of the infection, siderophores, catalase, ability to survive and replicate in phagocytes (Mohanty and Sahoo, 2007; Park *et al.*, 2012; Xu and Zhang, 2014; Emanet *et al.*, 2016). It is believed that pathogenic bacteria may have virulence genes that are absent in non-pathogenic bacteria and although virulence may be present in both pathogenic and non-pathogenic bacteria, they are only functional in pathogenic ones.

In the current study, six of the seven virulence genes screened for were present in the isolated *E. tarda* including; *citC*, *muk*, *gadB*, *katB*, *esaV* and *fimA*(Figure 7). Two of these genes(*muk* and *gadB*) were also detected by Emanet *al.* (2016) and are believed to be specific to pathogenic *E. tarda*. According to literature, detected genes in this study are regarded as main virulent genes as they provide resistance to the bacterium against host phagocyte killing activity (*gadB* and *katB*), allow cell adhesion (*fimA*)which enables intracellular survival and replication of the bacterium in the host and *muk* is a putative killing factor(Mohanty and Sahoo, 2007; Katharios *et al.*, 2015).It was also suggested that the presence of *fimA* gene indicates the ability of *E. tarda* to bind to specific receptors in fish hence defining the site of entry and colonization (Wang *et al.*,2009).

The detected virulence genes are known to be present only in virulent strains and therefore they are considered as biomarkers in the diagnosis of pathogenic *E. tarda*. These genes are used in determining how pathogenic bacteria interact with host to cause systemic infections and vaccine development as they are used in designing novel therapeutics and common antigens (Mendez *et al.*, 2012).Although the current study did not assess the virulence of the isolate based on *in vivo* experiments, the presence of these virulence genes coupled with the clinical signs of the disease proves the presence of Edwardsiellosis in the fish in which the isolate was found.

Edwardsiella tarda is a zoonotic bacterium affecting not only fish but also invertebrates, amphibians, reptiles, birds and mammals including human (Bullock and Herman, 1985; Park *et al.*, 2012). The *E. tarda* bacteremia (ETB) was reported

as a severe food and water-borne infection resulting in high mortalities especially in patients with severe underlying illnesses(Hirai *et al.*, 2015). The pathogen was reported by Litton and Rogers (2015) in a fisherman who was punctured by a fish hook. As there was no any biosecurity measure taken in all farms visited in this study, this poses a threat to human health which could contribute to introduction of pathogens from fish to human hence causing potential contaminations between humans and fish (Takyi *et al.*, 2012).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This study established the occurrence of *E. tarda* in *O. niloticus* in Wakiso district, Uganda. The isolate was successfully characterized which is of paramount importance in the diagnosis of the pathogen. Isolation of *E. tarda* from farmed fish destined for human consumption demonstrates a threat to the fishery sector as it causes heavy economic losses and also to the public sector as it is zoonotic. The results of the present study revealed that the *E. tarda* isolate had six virulence genes which play an important role in *E. tarda* pathogenicity. This information facilitates formulation of effective strategies for control and development of vaccine for the prevention of Edwardsiellosis in fish.

6.2 Recommendations

- Further studies are required to characterize the *E. tarda* strain found in this study and also more pathogenicity studies should be carried out to establish its virulence using in vivo experiments.
- The results from this study do not indicate the prevalence of *E. tarda* in the study area. This is because, due to limited resources, the fish samples collected in this study were less than the required sample size of n=134. Hence, a study should be carried out to determine the prevalence of *E. tarda* in the study area.

- *Edwardsiella tarda* is an opportunistic bacterial pathogen which is known to occur under stressful conditions. The present study did not determine the quality of the water from which the fish were sampled. Aspects of water quality in terms of temperature and organic matter content need to be checked as they may lead to the occurrence of the infection and development of the disease. In this study, however, this was not done due to lack of the kit necessary to measure water quality parameters. Therefore, more research is required to investigate the role of water quality in the occurrence of *E. tarda* in the study area which will form baseline information for future studies.
- The occurrence of disease is an interaction of the host, the environment and the pathogen and therefore, fish farmers are encouraged to maintain good aquaculture practices to ensure that fish is kept healthy and safe for human consumption.
- The study confirmed the occurrence of the pathogen in the study area and since the bacterium is of public health importance, awareness should be created amongst fish farmers and stakeholders to take precaution to avoid disease outbreak.

REFERENCES

- Abdel-Latif, H. M. R. and Sedeek, E. K. (2017). Diversity of Enterobacteriaceae retrieved from diseased cultured *Oreochromis niloticus*. *International Journal of Fisheries and Aquatic Studies* 5(1): 29-34.
- Abowei, J. F. N. and Briyai, O. F. (2011). A Review of Some Bacteria Diseases in Africa Culture Fisheries. *Asian Journal of Medical Sciences* 3(5): 206-217.
- Abraham, T. J., Mallick, P. K., Adikesavalu, H. and Banerjee, S. (2015). Pathology of *Edwardsiella tarda* Infection in African Catfish, *Clarias gariepinus* (Burchell 1822), Fingerlings. *Archives of Polish Fisheries* 23: 141-148.
- Acharya, M., Maiti, N. K., Mohanty, S., Mishra, P. and Samanta, M. (2007). Genotyping of *Edwardsiella tarda* isolated from freshwater fish culture system. *Comparative Immunology, Microbiology and Infectious Diseases* 30: 33-40.
- Akoll, P. and Mwanja, W. W. (2012). Fish Health Status, Research and Management in East Africa: Past and Present. *African Journal of Aquatic Science* 3(2): 117-129.

- Aly, S. M. (2013). A Review of Fish Diseases in the Egyptian Aquaculture Sector. Working Report. [www.livestockfish.cgiar.org] site visited on 20th June 2017.
- Amandi, A., Hiu, S. F., Rohovec, J. S. and Fryer, J. L. (1982). Isolation and Characterization of *Edwardsiella tarda* from Fall Chinook Salmon (*Oncorhynchus tshawytscha*). *Applied and Environmental Microbiology* 43(6): 1380-1384.
- Austin, B. and Austin, D. A. (1987). Enterobacteriaceae Representatives. Bacterial Fish Pathogens: Disease in Farmed and Wild Fish. Ellis Horwood Ltd., Chichester England. pp 196-224.
- Austin, B. and Austin, D. A. (2007). Bacterial Fish Pathogens: Diseases of Farmed and Wild Fish. Chichester: Praxis Publishing Ltd. 593 pp.
- Buller, N. B. (2004). Bacteria and Fungi from Fish and Other Aquatic Animals: A Practical Identification Manual. 2nd edition. 920 pp.
- Bullock, G. L. and Herman, R. L. (1985). *Edwardsiella* Infections of Fishes. *US Fish and Wildlife Publications*. [<http://digitalcommons.unl.edu/usfwpubs/132>] site visited on 14th March 2017.

- Castro, N., Toranzo, A. E. and Magariños, B. (2014). A Multiplex PCR for the Simultaneous Detection of *Tenacibaculum maritimum* and *Edwardsiella tarda* in Aquaculture. *Journal of International Microbiology* 17: 111-117.
- Clarridge, J. E., Musher, D. M., Fainstein, V. and Wallace, R. J. (1980). Extraintestinal Human Infection Caused by *Edwardsiella tarda*. *Journal Of Clinical Microbiology* 11(5): 511-514.
- Das, B. K., Sahu, I., Kumari, S., Sadique, M. and Nayak, K. K. (2014). Phenotyping and Whole Cell Protein Profiling of *Edwardsiella tarda* Strains Isolated from Infected Freshwater Fishes. *International Journal of Current Microbiology and Applied Science* 3(1): 235-247.
- Directorate of Fisheries Resources. (2011). Directorate of Fisheries Resources Annual report 2010/2011. Entebbe, Uganda. 47pp. [<http://aquaticcommons.org/20470/1/DFR%20ANNUAL%20REPORT%202012.pdf>] site visited on 4th January 2017.
- Efuntoye, M. O., Olurin, K. B. and Jegede, G. C. (2012). Bacterial Flora from Healthy *Clarias gariepinus* and their Antimicrobial Resistance Pattern. *Advance Journal of Food Science and Technology* 4(3): 121-125.

- El-Refaey, A. M. E. (2013). Studies on Major Bacterial Diseases Affecting Fish; Tilapia *Oreochromis niloticus*, Catfish *Clarias gariepinus* and Mulletts in Port Said, Egypt with Special References to its Pathological Alterations. *Researcher* 5(2): 5-14.
- 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.
- Eman, M. M., Omar, A. A. E. and Abdo, W. S. (2016). Insight into the Virulence-Related Genes of *Edwardsiella tarda* Isolated from Cultured Freshwater Fish in Egypt. *World Veterinary Journal* 6 (3): 101-109.
- Ewing, W. H., McWhorter, A. C., Escobar, M. R. and Lubin, A. H. (1965). *Edwardsiella*, a New Genus of Enterobacteriaceae Based on a New Species, *E. tarda*. *International Bulletin of Bacterial Nomenclature and Taxonomy* 15: 33-38.
- FAO. (2016). State of World Fisheries and Aquaculture 2016. Contributing to food security and nutrition for all. Rome. 200 pp.
- Faruk, M. A. R., Sarker, M. M. R., Alam, J. and Kabir, M. B. (2004). Economic

Loss from Fish Diseases on Rural Freshwater Aquaculture of Bangladesh. *Pakistan Journal of Biological Sciences* 7 (12): 2086-2091.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39: 783-791.

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

Griffin, M. J., Quiniou, S. M., Cody, T., Tabuchi, M., Ware, C., Cipriano, R. C., Mael, M. J. and Soto, E. (2013). Comparative Analysis of *Edwardsiella* Isolates from Fish in the Eastern United States Identifies Two Distinct Genetic Taxa Amongst Organisms Phenotypically Classified as *E. tarda*. *Veterinary Microbiology* 165: 358-372.

Guijarro, J. A., Cascales, D., Garcia-Torrico, A. I., Garcia-Dominguez, M. and Mendez, J. (2015). Temperature-dependent Expression of Virulence Genes in Fish Pathogenic Bacteria. *Frontiers in Microbiology* 6: 700-711.

He, Y., Xu, T., Han, Y., Shi, X. and Zhang, X. H. (2011). Phenotypic diversity of *Edwardsiella tarda* isolated from different origins. *Letters in Applied Microbiology* 53: 294-299.

- Hirai, Y., Asahata-Tago, S., Ainoda, Y., Fujita, T. and Kikuchi, K. (2015). *Edwardsiella tarda* Bacteremia. A Rare but Fatal Water and Foodborne Infection: Review of the Literature and Clinical Cases from a Single Centre. *Canadian Journal of Infectious Diseases and Medical Microbiology* 26(6): 313-318.
- Hossain, M. M., Mondal, S. A. S. M. and Kawai, K. (2010). Temperature Effects on Pathogenicity of Selected *Edwardsiella tarda* Strain to Japanese Eel, *Anguilla japonica*. *Bangladesh Journal of Fisheries Research* 14(1-2): 65-73.
- Huicab-Pech, Z. G., Landeros-Sánchez, C., Castañeda-Chávez, M. R., Lango-Reynoso, F., López-Collado, C. J. and Platas-Rosado, D. E. (2016). Current State of Bacteria Pathogenicity and their Relationship with Host and Environment in Tilapia *Oreochromis niloticus*. *Journal of Aquaculture Research and Development* 7: 428-438.
- Janda, J. M., Abbott, S. L. and McIver, C. J. (2016). *Plesiomonas shigelloides* revisited. *Clinical Microbiology Reviews* 29: 349-374.
- Joh, S., Kim, M., Kwon, H., Ahn, E., Jang, H. and Kwon, J. (2010). Characterization of *Edwardsiella tarda* Isolated from Farm-Cultured Eels, *Anguilla japonica*, in the Republic of Korea. *Journal of Veterinary Medical*

Science 73(1): 7-11.

Kalyesubula, R., Nankabirwa, J. I., Ssinabulya, I., Siddharthan, T., Kayima, J., Nakibuuka, J., Salata, J. A., Mondo, C., Kamyra, M. R. and Hricik, D. (2017). Kidney disease in Uganda: a community based study. *BMC Nephrology* 18: 116-125.

Kasozi, N., Rutaisire, J., Nandi, S. and Sundaray, J. K. (2017). A Review of Uganda and India's Freshwater Aquaculture: Key Practices and Experience from Each Country. *Journal of Ecology and the Natural Environment* 9(2): 15-29.

Katharios, P., Kokkari, C., Dourala, N. and Smyrli, M. (2015). First Report of Edwardsiellosis in Cage-Cultured Sharpsnout Sea Bream, *Diplodus puntazzo* from the Mediterranean. *BMC Veterinary Research* 11: 155-161.

Kebede, B. and Habtamu, T. (2016). Screening for the Presence and Prevalence of *Edwardsiella tarda* Infection in Fish Harvested from Lakes Zeway and Langano, Southern Oromia, Ethiopia. *Fisheries and Aquaculture Journal* 7: 184-190.

Kumar, S., Stecher, G. and Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for Bigger Datasets. *Molecular Biology and Evolution* 33: 1870-1874.

- Lane, D. J. (1991). 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, editors. *Nucleic acid techniques in bacterial systematics* John Wiley and Sons. Chichester, pp 115-175.
- Li, G.Y., Li, J., Xiao, P., Guo, Y.H. and Mo, Z. L. (2010). Detection of type III secretion gene as an indicator for pathogenic *Edwardsiella tarda*. *Letters in Applied Microbiology* 52: 213-219.
- Litton, K. M. and Rogers, B. A. (2016). *Edwardsiella tarda* Endocarditis Confirmed by Indium-111 White Blood Cell Scan: An Unusual Pathogen and Diagnostic Modality. *Case Reports in Infectious Diseases* 2016. [<http://dx.doi.org/10.1155/2016/108216>] site visited on 28th May 2017.
- Makulu, A., Gatti, F. and Vandepitte, J. (1973). *Edwardsiella tarda* infections in Zaire. *Annales De La Societe Belge De Medecine Tropicale* 53(3): 165-172.
- Marcel, G., Sabril, M. Y., Siti-Zahrah, A. and Emikpe, B. O. (2013). Water Condition and Identification of Potential Pathogenic Bacteria from Red Tilapia Reared in Cage-cultured System in Two Different Water Bodies in Malaysia. *African Journal of Microbiology Research* 7(47): 5330-5337.

- Méndez, J., Reimundo, P., Pérez-Pascual, D., Navais, R., Gómez, E., Cascales, D. and Guijarro, J. A. (2012). An Overview of Virulence-Associated Factors of Gram-Negative Fish Pathogenic Bacteria. *Health and Environment in Aquaculture* 5: 133-156.
- Meyer, F. P. and Bullock, G. L. (1973). *Edwardsiella tarda*, a New Pathogen of Channel Catfish (*Ictalurus punctatus*). *Journal of Applied Microbiology* 25: 135-156.
- Mohanty, B. R. and Sahoo, P. K. (2007). Edwardsiellosis in fish: a brief review. *Journal of Biosciences* 32: 1331-1344.
- Naing, L., Winn, T. and Rusli, B.N. (2006). Practical Issues in Calculating the Sample Size for Prevalence Studies. *Archives of Orofacial Sciences* 1: 9-14.
- Najiah, M. and Lee, S. W. (2006). Phenotypic Characterization and Numerical Analysis of *Edwardsiella tarda* in Wild Asian Swamp Eel, *Monopterus albus* in Tereengganu. *Journal of Sustainability Science and Management* 1(1): 85-91.
- NDPII. (2015). Second National Development Plan (2015/16 – 2019/20) Uganda. 344 pp. [<http://npa.ug/wp-content/uploads/NDPII-Final.pdf>] site visited

on 6th January 2017.

Nei, M. and Kumar, S. (2000). *Molecular Evolution and Phylogenetics*. Oxford University Press, New York. 333 pp.

Öztürk, R. Ç. and Altınok, İ. (2014). Bacterial and Viral Fish Diseases in Turkey. *Turkish Journal of Fisheries and Aquatic Sciences* 14:275-297.

Panangala, V. S., van Santen, V. L., Shoemaker, C. A. and Klesius, P. H. (2005). Analysis of 16S-23S intergenic spacer regions (ISRs) of the rRNA operons in *Edwardsiella ictaluri* and *Edwardsiella tarda* isolates from fish. *Journal of Applied Microbiology* 99: 657-669.

Park, S. B., Aoki, T. and Jung, T. S. (2012). Pathogenesis of and Strategies for Preventing *Edwardsiella tarda* Infection in Fish. *Veterinary Research* 43: 67-78.

Parker, R. (2012). *Aquaculture Science*. 3rd Edition. Delmar, United States. 668 pp.

Roberts, R. J. (2012). *Fish Pathology*. 4th Edition. John Wiley and Sons, Limited, United Kingdom. 587 pp.

Saitou, N. and Nei, M. (1987). The Neighbor-joining Method: A New Method for Reconstructing Phylogenetic Trees. *Molecular Biology and Evolution* 4: 406-425.

- Sebastião, F. A., Furlan, L. R., Hashimoto, D. T. and Pilarski, F. (2015). Identification of Bacterial Fish Pathogens in Brazil by Direct Colony PCR and 16S rRNA Gene Sequencing. *Advances in Microbiology* 5: 409-424.
- Takyi, R. F., Nunoo, K. E., Ziddah, P., and Oddoye, J. (2012). Occurrence of Bacterial Infection in Two Commonly Cultured Fish Species on Two Fish Farms in Southern Ghana. *World Journal of Biological Research* 5(2): 81-92.
- UNBOS. (2014). Uganda National Bureau of Statistics Statistical Abstract. 305 pp. [http://www.ubos.org/onlinefiles/uploads/ubos/statistical_abstracts/Statistical_Abstract_2014.pdf] site visited on 20th February 2016.
- Walakira, J., Akoll, P., Engole, M., Sserwadda, M., Nkambo, M., Namulawa, V., Kityo, G., Musimbi, F., Abaho, I., Kasigwa, H., Mbabazi, D., Kahwa, D., Naigaga, I., Birungi, D., Rutaisire, J. and Majalija, S. (2014). Common fish diseases and parasites affecting wild and farmed Tilapia and Catfish in Central and Western Uganda. *Uganda Journal of Agricultural Sciences* 15(2): 113-125.
- Wang, K., Liu, E., Song, S., Wang, X., Zhu, Y., Ye, J. and Zhang, H. (2012). Characterization of *Edwardsiella tarda* rpoN: Roles in Family Regulation, Growth, Stress Adaptation and Virulence Toward Fish.

Archives of Microbiology 194(6): 493-504.

Wang, Q., Yang, M., Xiao, J., Wu, H., Wang, X., Lv, Y., Xu, L., Zheng, H., Wang, S., Zhao, G., Qin, Q. and Zhang, Y. (2009). Genome Sequence of the Versatile Fish Pathogen *Edwardsiella tarda* Provides Insights into its Adaptation to Broad Host Ranges and Intracellular Niches. *PLoS ONE* 4(10): e7646.
[<http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0007646>]
6] sited visited on 21st March 2017.

Wang, X., Yan, M., Wang, Q., Ding, L. and Li, F. (2012). Identification of *Edwardsiellatarda* isolated from duck and virulence genes detection. *African Journal of Microbiology Research* 6: 4970-4975.

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 Journal of Agricultural and Environmental Science* 3(5): 681-691.

Wyatt, L. E., Nickelson, R. and Vanderzant, C. (1979). *Edwardsiella tarda* in Freshwater Catfish and Their Environment. *Applied And Environmental Microbiology* 38(4): 710-714.

Xu, T. and Zhang, X. (2014). *Edwardsiella tarda*: an intriguing problem in aquaculture. *Aquaculture* 431:129-135.

Yang, M., Lv, Y., Xiao, J., Wu, H., Zheng, H., Wu, H., Zheng, H., Liu, Q., Zhang, Y. and Wang, Q. (2012). *Edwardsiella* Comparative Phylogenomics Reveal the New Intra/Inter-Species Taxonomic Relationships, Virulence Evolution and Niche Adaptation Mechanisms. *PLoS ONE* 7(5): e36987. [<http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0036987>] site visited on 9th April 2017.

Zhang, Y. (2007). Molecular characterization of *Edwardsiella* species and *Flavobacterium columnare*. PhD Thesis. Auburn University. 144 pp.

APPENDICES

Appendix 1: Biodata Form

a) Farm History

Date.....

District.....

Subcounty.....Parish.....

Village.....

Name of Farmer.....

Contact.....

Number of ponds.....

Water source.....

Culture system: Ponds Tanks Cages

Purpose: Subsistence Commercial Both

Species.....

If both Tilapia and catfish; Mixed Separate ponds Both

Type of feed.....

Farm location.....

History of disease outbreak: Yes No

Frequency of disease outbreak: Low High Moderate

Common in which season: Dry Rain

b) Fish samples

S/N	Species	Sex	Weight	Length	Clinical signs	Site	Description
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							

Appendix 3: Isolates earlier identified as *E. tarda* turned out to be *Plesiomonas shigelloides* by API 20E kit

