IMMUNOPROTECTION OF NILE TILAPIA (*Oreochromis niloticus*) AGAINST *EDWARDSIELLA TARDA* INFECTION IN UGANDA

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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

2018

ABSTRACT

Edwardsiella tarda is a gram negative bacterium belonging to the family Enterobacteriaceae, it is the causative agent of edwardsiellosis a disease which is among the major constraints in aquaculture worldwide. It causes mass mortalities of fish that results into high economic losses in both aquaculture and fisheries. This study aimed at determining the immunoprotection of Nile tilapia against *E. tarda*. A total of 50 fish in five groups were used to determine the LD_{50}, fish were exposed to *E. tarda* intraperitoneally by injection with dilutions $1.5x10^{6}$ to $1.5x10^{9}$. In the second phase, two groups of 20 fish each were vaccinated with formalin-killed *E. tarda* and phosphate buffered saline for control; a booster dose was given two weeks after the first dose. Blood was collected weekly from six fish in each group for serum to determine antibody titer by agglutination in microtiter plates. Two weeks after the booster dose, all fish were challenged with 100µl of 10^{8} CFU/ml *E .tarda* (LD_{50}). Fish were monitored for 4 weeks; dead fish were recorded, examined for clinical signs and pathological changes. Bacteriology was done to confirm the presence of the pathogen in freshly dead or moribund fish. Bacterial load in the liver kidney and spleen was determined by drop plate counting from 10-fold serial dilutions of homogenized tissues. LD_{50} of $1.6x10^{8.1}$ was determined in this study. Infected Fish showed signs of skin and fin hemorrhages, ulcers, depigmentation, exophthalmia, erosion and distended abdomen externally. Grayish nodules in the spleen, kidney, congested internal organs, fluid filled intestines, black spots in the liver, mottled liver were observed internally. The lesions were more severe in the non-vaccinated groups. There was high bacterial load in the kidneys than in the spleens and livers. All the sampled dead fish were *E. tarda* positive which was confirmed using API 20E kits. Significantly high antibody titers were found in vaccinated fish and the Relative Percentage Survival was 32.4% indicating relative protection. No significant difference in percentage mortalities was

found between groups ($p>0.05$), there was high bacterial load in the kidney than in the liver and spleen and the bacterial load in non-vaccinated fish was highly significant than in vaccinated fish ($p<0.05$). The antibody titers in the vaccinated fish were highly significant than in non-vaccinated ($p<0.05$). Results indicate that formalin-killed cells enhance production of specific antibodies, induce specific immunity and can confer protection to the fish. These results can be used as a baseline for vaccine development after a series of studies on different age groups of fish and doses of different formulations of vaccines under optimized conditions.

DECLARATION

I, EZRA BYAKORA, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution.

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ACKNOLEDGEMENTS

I express my heartfelt gratitude to the almighty God for the gift of life, good health and the spirit of endurance and perseverance he granted me throughout my research work. My sincere appreciation to my supervision team Prof. Byarugaba, D. K. of Makerere University, Prof. Philemon N. Wambura of Sokoine University of Agriculture and Prof. Evensen, Ø of Norwegian University of Life Sciences for their guidance and Dr. Mugimba, K. K. and Dr. Wamala, S.P. for their remarkable guidance during my work. I would also like to thank Prof. Mdegela, R. H. and Dr. Mkupasi, E. M. for their commitment in championing Aquatic Animal Health. I thank the team of the Microbiology Laboratory of the College of Veterinary Medicine Animal Resources and Biosecurity of Makerere University, Mr. Musisi, N. L., Mr. Kamya, D., Mr. Alafi, S., Mr. Kasirye, J and Mr. Ngolobe, B. for their kind and tireless assistance in laboratory analysis methods. My sincere appreciation also goes to Mr. Ddungu, R. for his guidance and assistance in data analysis. I am thankful to Mr. Kityo, G the farm manager of Aquaculture Research and Development Center – Kajjansi for his assistance and cooperation during fish sample collection. I extend my appreciation to Mr. Mulyazaawo, F. for his assistance with laboratory animals (rabbit and guinea pigs) and techniques. I am really grateful to my family, relatives and friends for their encouragement and spiritual support they gave me as i was doing this work. I also want to appreciate Bushenyi District Local Government for releasing me to proceed for further studies. Lastly, am extremely grateful to the Norwegian Agency for Development Cooperation (NORAD) for funding this study through the Training and Research in Aquatic and Environmental Health in Eastern and Southern Africa (TRAHESA) project.

DEDICATION

I dedicate this work to my family; the family of Mr. and Mrs. Yosamu Ssemakula for their great encouragement. And to my late mum Mary Mbabazi (R.I.P).

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LIST OF ABBREVIATIONS AND ACRONYMS

µg	microgram
µl	microliter
µm	micrometer
AIDH	Autotransport adhesin
API 20E	Analytical Profile Index
ARDC	Aquaculture Research and Development Center
AU-IBAR	African Union – Inter African Bureau for Animal Resources
BHIA	Brain Heart Infusion Agar
BHIB	Brain Heart Infusion Broth
CFU	Colony Forming Units
CP	Crude Protein
CRD	Completely Randomized Design
DFR	Directorate of Fisheries Resources
DNA	Deoxyribonucleic acid
ECP	Excretion of cytosolic proteins
ELISA	Enzyme-Linked immunisorbent Assay
FAO	Food and Agricultural Organization
FKC	Formalin-Killed Cells
HA	Hemagglutination
HAU	Hemagglutinating Unit
HI	Hemagglutination Inhibition
ip	Intraperitoneal
LD_{50}	Median Lethal Dose
LPS	Lipopolysaccharides

MAAIF	Ministry of Agriculture Animal Industry and Fisheries
mm	millimeters
MS222	Tricane methanosulfate
NaFIRRI	National Fisheries Resources Research Institute
OIE	Office International des Epizooties/World Organization for Animal Health
OMP	Outer Membrane Proteins
PBS	Phosphate Buffered Saline
pH	Hydrogen potential
RBCs	Red Blood Cells
RPM	Revolutions per Minute
RPS	Relative Percentage Survival
SPSS	Statistical Package for Social Sciences
T3SS	Type III secretion System
T6SS	Type VI Secretion System
TSA	Tryptic Soy Agar
TSI	Triple Sugar Iron
USA	United States of America
WHO	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Global fish production has increased over the last several years with fish supply increasing at an average annual rate of 3.2 percent, outpacing world population growth at 1.6 percent (FAO, 2014). In 2016, production was estimated at about 171 million tonnes. World per capita apparent fish consumption increased from an average of 9.9 kg in the 1960s to 20.2 kg in 2016 (FAO, 2018). China increased an average annual rate of 6.0 percent in the period between 1990 and 2010 to about 35.1 percent in 2010 (FAO, 2016).

Fish is a valuable source of animal protein, minerals and vitamins that are essential requirements in human diets. Fish occupies a unique position in the agriculture sub-sector in the world economy. It contributes immensely to the food and nutritional security, employment and national revenues (AU-IBAR, 2016). A global population of 3.2 billion people consumed fish that accounted for 17 percent of the total animal protein that was consumed globally in 2016 (FAO, 2018). The continuing impressive growth in fish supply for human consumption has been attributed to aquaculture as the capture fisheries production has either declined or remained static since 1980s. As wild stocks continue to decline while demand for fish is increasing, aquaculture fish production is important to compensate for the gap for the required fisheries resources (FAO, 2014). Aquaculture has the potential to make a significant contribution to food, nutritional security and income generation.

In Africa, aquaculture started at beginning of the 20th century in Egypt, Kenya and Malawi in 1940, Rwanda, Uganda Zambia, Zimbabwe and Tanzania in 1960. The main

aquaculture systems in practice are; monoculture, polyculture of Nile tilapia and African catfish as the main species in ponds and the most recent cage farming of Nile tilapia especially on Lake Victoria. The contribution of aquaculture to global fish production increased continuously from 25.7 percent in 2000 to 46.8 percent in 2016, and about 17 to 18 percent of the total fish production in Africa was from aquaculture (FAO, 2018).

The Eastern African Region has high potential for aquaculture production due to the presence of lakes, rivers, wetlands and Indian ocean coastline and availability of suitable native species such as Nile tilapia and the African catfish. The availability of inputs for feed production, suitable temperatures for fish growth, access to local, regional and international market and trade can facilitate the development of aquaculture industry (AU-IBAR, 2016). To meet the growing demand, there has been an effort to increase fish production through intensive farming in ponds and cages especially on Lake Victoria in Uganda.

Due to increased intensification and rapid development of aquaculture in East Africa, the risk of fish diseases and disease outbreaks has also increased. Aquatic animal disease outbreaks impact on aquaculture investments at farm and national levels in terms of costs associated with disease management and reduce levels of production and returns (FAO, 2000). Bacteria are the most common pathogens of cultured warm water fish, and cause major losses to the freshwater aquaculture industry in the world. They are also the most prevalent cause of morbidity and mortality among wild fish populations. Out of the most annihilating bacteria, motile Aeromonads and *Edwardsiella sp*. are the most significant. *Edwardsiella tarda* is one of the the commonly found pathogens causing diseases that lead to mass mortalities in various populations and age groups of fish (Mohanty and Sahoo, 2007).

1.2 Problem Statement and Study Justification

Cases of aquatic diseases incidences leading to mortality rates of 60% have been reported in hatcheries and grow-out systems in Uganda (Akoll and Mwanja, 2012). Infectious parasites and bacteria are reported to affect private and public fish farms with profound effects. *Edwardsiella tarda* has been isolated in many fish farms in East Africa in both symptomatic and asymptomatic fish (Walakira *et al.*, 2014; Wamala *et al.*, 2018). Evidence from a number of studies shows that disease incidences in the aquaculture and fisheries industry have the capacity of causing huge economic loss as occurred in Asian countries where massive expansion in aquaculture industry preceded fish health capabilities which cost the industry heavily (AU-IBAR, 2016). Intensification of fish farming in Uganda and East Africa as a whole is on the rise and is expected to shoot up especially in Low Volume High Density facilities like cages with the increased demand for fish. Due to this, diseases incidences especially bacterial are increasing as well.

In fish diseases management, antibiotics are currently being used in aquaculture for treatment of bacterial diseases especially by farmers practicing intensive fish farming. With the increasing importance of aquaculture industry, there might be increased antibiotics use resulting into microbial resistance, and increasing consumption of products from aquaculture could lead to increased human exposure to resistant bacteria and resistance genes (World Health Organisation, 2006). Research indicates that 70-80% of the drug used in aquaculture ends up in the environment (Hernandez, 2005). Preventive measures such as use of vaccines and improved biosecurity are appropriate in disease management. Disease prevention will decrease the amount of antibiotics that are needed in aquaculture and will also decrease the pressure that induces and amplifies antibiotic resistant bacteria (WHO, 2006). Protection of Nile tilapia against *Edwardsiella tarda* infections using inactivated bacterins was evaluated against this background. Results from

this research give a baseline for possibility of vaccine development through applied, advanced and intensive research.

1.3 Objectives of the study

1.3.1 Overall objective

Evaluation of immunoprotection of Nile tilapia against *Edwardsiella tarda* following immunological challenge with inactivated bacterins.

1.3.2 Specific objectives

i. To assess the immunogenicity of inactivated *Edwardsiella tarda* in Nile tilapia,
ii. To assess the protection against homologous challenge of vaccinated Nile tilapia.

1.3.4 Research questions

i. To what extent can inactivated *Edwardsiella tarda* protect Nile tilapia against infection and mortality?
ii. What are the pathological changes in vaccinated Nile tilapia following homologous challenge with *Edwardsiella tarda*?

1.3.5 Hypotheses

i. Formalin-killed *Edwardsiella tarda* bacterins induce protective immunity against homologous challenge in Nile tilapia

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Nile Tilapia Farming

Global production from aquaculture was 110.2 million tonnes with an estimated value of 243.5 billion dollars (FAO, 2018). Nile tilapia is one of the most cultured fish species in the world since the 1990s and the production capacity of 2.5 million tons was estimated in 2007. In 2008, it ranked fifth and in 2014 it reached above 3.5 million tons after carp and salmon (Munang'andu *et al.*, 2016). In FAO 2018 report, Nile tilapia is the 4th most cultured species globally at 8 percent (4.2mt) after Common carp, Silver carp and Grass carp at 8 percent (4.5mt), 10 percent (5.3mt) and 11 percent (6.0mt), respectively. In Africa, Egypt, Nigeria and Uganda are the leading fish producers from aquaculture with Nigeria producing more of African catfish and Egypt and Uganda producing more of Nile tilapia (FAO, 2016). Total annual aquaculture production in Uganda is estimated to 111,023 mt per year (Kasozi *et al.*, 2017). In Uganda, Nile tilapia is still the most cultured and valuable fish species followed by African catfish and its production and general aquaculture production have increased sustainably with increased intensification and development of cage farming especially in the central region (Directorate of Fisheries Resources, 2011).

This rapid expansion has resulted into an increase in the number of diseases infecting tilapia due to intensified farming systems, high stocking densities that are aimed at increasing productivity and production. Using high stocking densities induces stress-related immunosuppression, rendering fish highly susceptible to disease infections, and increased transmission of infectious pathogens (Munang'andu *et al.*, 2016). The outbreak of pathogenic bacterial diseases is one the biggest challenges in Aquaculture in Uganda

and worldwide (AU-IBAR, 2016). *Edwardsiellosis* is one the bacterial diseases that impact on Nile tilapia production causing mass mortalities and it has been reported in several studies in Uganda (Akoll and Mwanja, 2012; Nantongo, 2017; Wamala *et al*., 2018). These challenges of bacterial diseases in Nile tilapia production have resulted into increased use of antibiotics and other drugs, raising concerns on antimicrobial resistance environmental issues related to drugs.

2.2 *Edwardsiella tarda*

Edwardsiella tarda is a Gram negative, motile, short, rod-shaped bacterium (1 μm in diameter and 2–3 μm long) which belongs to the family Enterobacteriaceae (Ewing, 1965). *Edwardsiella tarda* is the etiological agent of Edwardsiellosis that leads to extensive losses in a number of commercially important fresh and marine water fish worldwide. Since the first outbreak of *E. tarda*, reported outbreaks have increased in most geographical areas in the world over the years (Baxa *et al.*, 1990).

Edwardsiella tarda has been isolated in many cases and many isolates have shown high virulence potential (Nantongo, 2017), the pathogenic mechanisms have also been described. In countries where there is increased use of antibiotics, drugs resistance *E. tarda* has been reported (Castro *et al.*, 2008). Because of this, there is an urgent need to find other approaches for controlling the infection (Castro *et al.*, 2008). Fish species commonly affected by Edwardsiellosis include carp, tilapia, eel, catfish, mullet, salmon, trout and flounder. *Edwardsiella tarda* has also been isolated in reptiles and amphibians which might be a source of infection to cultured fish (Xu and Zhang, 2014). Edwardsiellosis is one of the most serious systemic bacterial diseases in fish, resulting in substantial losses in the fish farming industry all over the world (FAO, 2006). Its infections in tilapia have been reported in many studies, causing chronic mortalities in

Nile tilapia in some farms. It has a zoonotic potential especially in immunocompromised individuals, it causes liver abscess, mild diarrhea and wound infection (Park *et al.*, 2012).

2.2.1 *Edwardisiela tarda* virulence

Numerous features and strategies of bacteria determine virulence within a host. A critical aspect in the success of a bacterial pathogen is the ability to attach to and penetrate host surfaces. Bacteria have evolved several mechanisms to aid in the crucial initial step of motility, adherence, colonization and expression of genes (Wu *et al.*, 2018). Proteinaceous, rod-like structures such as pili and fimbriae are produced by bacteria, especially Gram-negative species, to maintain contact with host cell surfaces. Other surface proteins, known as afimbrial adhesins, may not arrange structurally in the manner of pili or fimbriae, but still function in the adherence of the bacterium to a host cell (Salyers and Whitt, 2002). Outer surface polysaccharide molecules forming the glycocalyx also contribute to adherence of bacterial cells to the host and other bacteria. *Edwardsiella tarda* may derive much of their virulence from the ability to invade epithelial cells (Janda *et al.*, 1991; Salyers and Whitt, 2002). In addition, some bacterial pathogens have developed mechanisms to allow survival and multiplication intracellularly within phagocytic cells through the prevention of the phagosome-lysosome complex, escape from the phagosome by disruption of the membrane, or direct resistance. A potential host possesses a multifaceted system of innate and acquired immune defenses the potential bacterial pathogen must overcome in order to cause disease. Numerous bacterial pathogens may rely on the production of extracellular proteins, especially enzymes, to evade immune responses and inflict damage to host cells (Mohanty and Sahoo, 2007). Several enzymes implicated in the virulence of bacterial pathogens include catalase, hemolysins, and hydrolytic enzymes (Xie *et al*., 2014).

The infection of *E. tarda* as in other Enterobactereaceae family members is considered to be due to a number of factors. Virulence factors responsible for its pathogenicity include stable enterotoxin and hymolysins (Du *et al.*, 2007), dermatonecrotic toxin, chondroitinase activity, complement-mediated resistance, hemagglutination mediated by non-fimbrial adhensins and siderophore production (Kokubo *et al*., 1990; Janda and Abbott, 1993; Michael and Abbott, 1993), invasive ability and cytotoxicity to HEp-2 cell lines (Park *et al*., 2012). *Edwardsiella tarda* survives in the host by utilizing several important substances and abilities that serve as virulence factors in the host. Type III Secretion System (T3SS) and Type VI Secretion System (T6SS) play an important role in adherence, penetration, survival and replication of *E. tarda* in the epithelial cells and phagocytes (Eman *et al*., 2016). T6SS of *E. tarda* comprises of 16 genes and 13 of the encoded proteins are involved in the secretion of *E. tarda* virulence proteins. T3SS is a multiprotein complex that is essential for the host and pathogen interactions (Mendez *et al*., 2012).

Several reports indicate that motility related proteins such as flagellin and autotransport adhensin AIDA, a fimbrial adhensin-like protein are important for the attachment and penetration into the epithelial cells of the host (Park *et al*., 2012). *Edwardsiella tarda* is able to survive and adapt to various host environmental conditions including host hormonal changes, temperature, pH, salinity and variations in several important nutritional elements such as iron, phosphate and magnesium ions (Mg^{2+}). As the complete elimination of pathogens from the environment is not possible, efforts have been made to develop methods of treatment and prevention. However, the pathogenic count that amounts to LD_{50} in Nile tilapia is somewhat unclear as it varies in different studies. This study therefore estimated the LD_{50} as the virulence evaluation for dose that was used for challenge after vaccination.

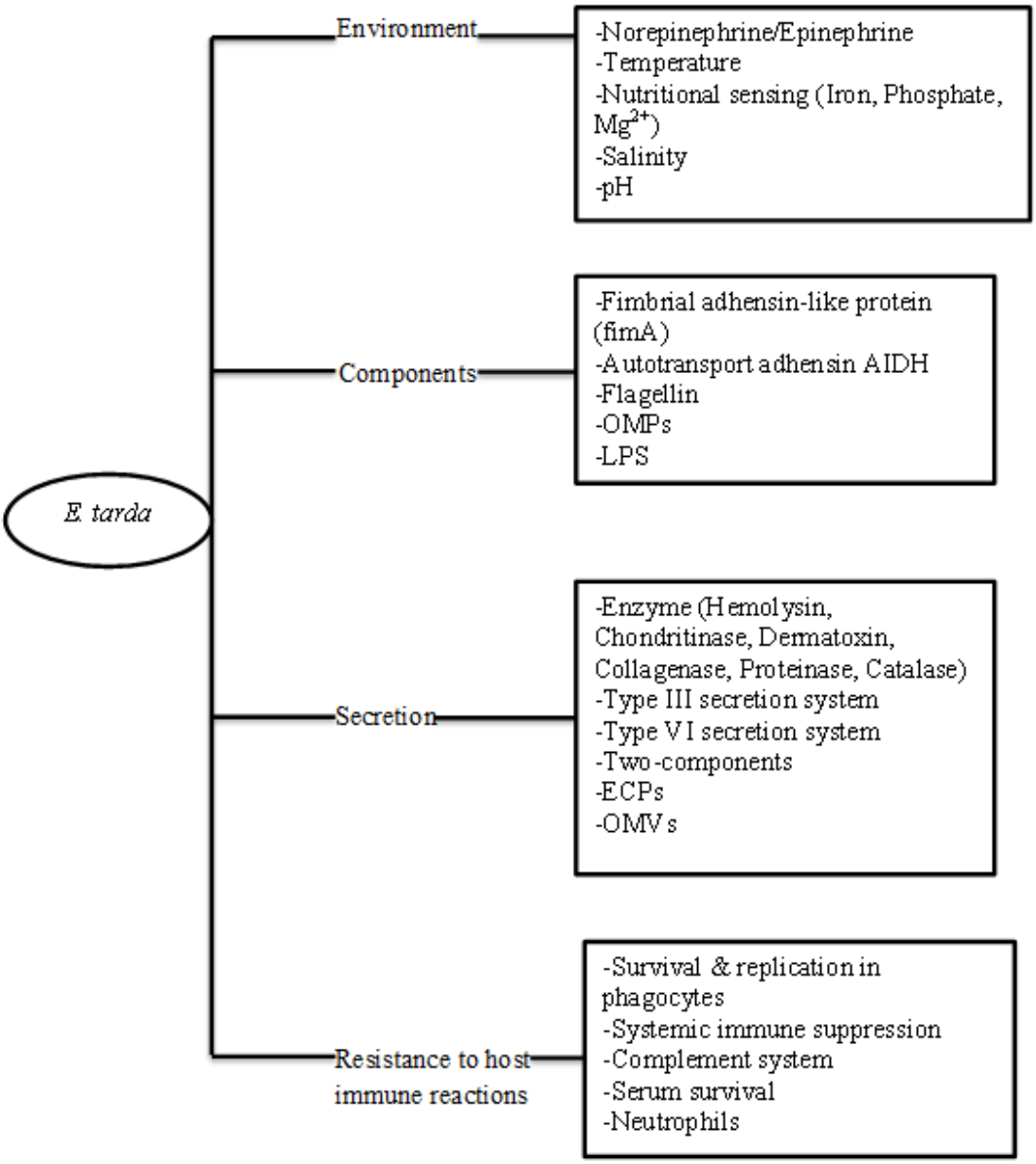

Figure 1: Factors contributing to *Edwardsiella tarda* survival and infection (modified from Park *et al.*, 2012)

2.2.2 Epidemiology of *Edwardsiella tarda*

Edwardsiella tarda presumably resides in the intestinal contents of fish and other carrier aquatic animals such as snakes, some amphibians and reptiles and in the mud at the bottom of many water bodies. In USA, *E. tarda* has been isolated from mud, water, frogs, turtles and crayfish from catfish ponds (Plumb and Evans, 2006; Michael and Abbott,

1993). It is transmitted through water, mud and faeces, where it probably infects susceptible hosts through trauma of the epithelium or through the intestines (Nagy *et al*., 2018). The infection is usually triggered by a number of predisposing/stress factors including high temperature, poor water quality, overcrowding, low dissolved oxygen, high levels of ammonia and high organic matter content (Meyer and Bullock, 1973). All these factors contribute to the onset and severity of the disease. *Edwardsiella tarda* infects a wide variety of fish, the most predominant hosts are eels, catfishes and tilapia, but many other fish species are also susceptible. At least 21fish species are known to have been infected but all species of fish may be susceptible under certain conditions. *Edwardsiella tarda* infections are not limited to fish, but often exist as part of the normal intestinal micro flora in especially fish eating birds, reptiles, cattle and swine (Wyatt *et al*., 1979; Sharma *et al*., 1974). The infection may cause lesions in the dermis, musculature and visceral organs of the infected fish. A foul odor is realized on piercing the skin lesions which appear as gas-filled hollow areas or nodules (Meyer and Bullock, 1973).

Edwardsiella tarda may spread from fish to fish through water by organisms being shed in feces, cannibalism of infected fish and feeding on dead or infected fish (Wyatt *et al*., 1979). Birds are involved in spreading the pathogen by predating on infected or dead fish from one place to another where they end up dropping the infected fish carcass in the facility that contains healthy fish. Other possible channels of transmission include; contaminated nets and equipment moved from facility to facility without being disinfected. *Edwardsiella tarda* is said to be an ubiquitous pathogen and is found in fish cultured worldwide but common in Venezuela, Japan, Taiwan, Korea, India, Thailand, Egypt, Israel, South and Central America and many other developing countries (Plumb and Evans, 2006). The organism has also been successfully isolated from some wild fish

species in USA, Australia and Canada and Africa, including Ethiopia (Habtamu and Kebede, 2017) and Uganda (Nantongo, 2017; Wamala *et al.*, 2018).

2.3 Immunoprophylaxsis

With the ever increasing intensity of fish farming, immunoprophylaxsis, as a method of stimulating non-specific and specific immunity, has become the most important method of bacterial disease prevention (Gudding, 1999; Håstein *et al*., 2005). Vaccines can be administered to fish by injection, typically intraperitoneally, by immersion in a vaccine solution, or orally, with the selection of immunization route based on factors such as feasibility, level of protection conferred, potential side effects, and cost. Critical to the design of an efficacious vaccine is the initiation and optimization of host immune responses and recognition of possible virulence factors of the target pathogen. Vaccine formulations consisting of inactivated cell bacterins, live, attenuated cells, and DNA recombinant products have been developed for use in many fish species (Gudding *et al*., 1999).

At the present time, licensed vaccines against approximately 15 bacterial pathogens are employed in aquaculture worldwide, while numerous others are currently being researched (Håstein *et al.*, 2005). Although many potential vaccines have been evaluated, one effective in the prevention of Edwardsiellosis has not yet been developed in Africa. Currently, a number of antigens, including Lipopolysaccharides (LPS), Excretion of Cytosolic Proteins (ECP), and Outer Membrane Proteins (OMP), are thought to be involved in inducing protection against *E. tarda* in fish, however; an effective, commercial vaccine is not presently available, a few studies have been conducted using formalin-killed bacterins only in Egypt in Africa and none has been conducted in East Africa.

2.4 Vaccination Against *Edwardsiella tarda* Infection

Over the years, vaccination has been used for prevention of some infectious diseases in farmed fish (Yan *et al.*, 2018), including Nile tilapia with much focus on bacterial and viral diseases. For Edwardsiellosis, several vaccination strategies and attempts have been made to induce protection against *Edwardsiella tarda* with vaccines composed of whole cells, disrupted cells, cell extracts and attenuated strains as immunogens but results on protection efficacy have been varying among studies in different places and fish species. Kwon *et al.* (2006) immunized tilapia with *Edwardsiella tarda* ghosts (ETG) and formalin killed *Edwardsiella tarda* (FKC) and results showed significantly higher survival rate than in control fish. Fish vaccinated with ETG showed significantly higher survival rate than fish vaccinated with FKC.

El-Jakee *et al.* (2008) reported that infection of *O. niloticus* with *E. tarda* (0.5 ml / fish, 10^6 CFU /ml) was effective in producing mortality rate that reached to 41.64%. The most common clinical disease manifestations were hemorrhages all over the fish body, skin darkening, pale skin areas with detached scales and hemorrhagic protruded vent. As the disease progressed, there was bilateral exophthalmia as well as abdominal dropsy. The intraperitoneal administration of outer membrane vaccine caused a gradual increase in mean serum antibody titers over the sampling periods. However, there was a significant rise in mean antibody titers from days 21 to 28 sampling for outer membrane protein vaccine. Vaccination with *E. tarda* formalin killed cells (FKCs) delayed mortality following experimental *E. tarda* infections (Song *et al.*, 1982). Vaccination with FKC was found to enhance phagocytic activity of Japanese eel leucocytes *in vitro*. Gutierrez and Miyazaki (1994) reported that vaccination with FKCs of *E. tarda* resulted in a survival of 40% when challenge doses of *E. tarda* did not exceed 10^5CFU/fish.

In Africa, studies on immunoprotection using inactivated bacteria or immunostimulants have only been done in Egypt and lately in Nigeria, giving a research gap in other major fish producing countries under intensive culture such as Uganda. The study therefore focused on determining the antigenicity of inactivated *E. tarda* against infections of virulent strain of *E. tarda* to assess the effectiveness of this prophylaxis and give recommendations for further vaccine development approaches.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

This study was conducted in Uganda at Makerere University, College of Veterinary Medicine, Animal Resources and Biosecurity. Makerere University is one of the leading research institutions in the region and Africa. It is the corresponding institution in the implementation of capacity building for training and research in aquatic and environmental health in Eastern and Southern Africa in Uganda with the required facilities and expertize appropriate for this research. Fish samples were collected from a government research center in Wakiso district in the central Uganda the biggest fish producing region.

3.2 Study Design

The study was entirely experimental and Completely Randomized experimental Design (CRD) was used (random selection and random assignment to treatment and control groups)**.** However, fish samples for the experiment were collected purposively as the target was on the source/farm with no history or signs and symptoms of parasites and diseases especially *Edwardsiella tarda.*

3.3 Sample size

The Resource Equation Method based on law of diminishing return (Charan and Biswas, 2013) was used to determine the sample size. This method was used as an option to the power analysis method which requires standard deviation and effect size that must be got from similar previous studies or pilot studies of the same nature.

Sample size = Number of individual animals targeted in each group - Number of groups intended

$$= (21 \text{ X } 10) – 10 = 200.$$

The risk of attrition from collecting blood samples from vaccinated and challenged fish, 5 fish from 8 groups which makes it 40 (40/200) = 20% fish was calculated.

Considering the Attrition factor of 20% therefore,

$$\text{Real Sample Size} = 200/(1 – 0.2) = 200/0.8$$

$$= 250 \text{ fish.}$$

However, 50 fish in 5 groups of 10 each were used for the LD_{50} experiment and 80 fish in 4 groups of 20 fish each for the vaccination/challenge experiment basing on the resources that were available.

3.4 Median Lethal Dose (LD_{50}) determination

To determine the LD_{50} of *E. tarda* for use in challenge studies, 50 apparently healthy fish were used. Live fish of 75g were collected from Aquaculture Development and Research Center of National Fisheries Resources Research Institute (NaFIRRI-Kajjansi) and transported in an open tank with aeration to Makerere University College of Veterinary Medicine, Animal Resources and Biosecurity for experimentation. Samples were divided into 5 equal groups of 10 fish and each group was randomly assigned to treatments of 10^6, 10^7,10^8 and 10^9 cfu/ml dilutions with one overall control. Before treatment, fish were acclimatized for a period of two weeks in dechlorinated municipal water in 60L plastic tanks and a quarter of the water was changed twice daily. *Edwardsiella tarda* KOCT4’ was cultured in Tryptic Soy Broth and incubated at room temperature (25-27 °C) for 24 hours, the

concentration of the stock culture $1.5x10^9$ cfu/ml was determined by drop plate counting. The stock culture was subjected to serial 10-fold dilution to obtain 10^8, 10^7, 10^6 dilutions which were used immediately. Fish were intraperitoneally exposed by injecting them (25guage needles on 1ml syringes) with 0.1ml bacterial suspensions in their respective dilution groups and 0.1ml PBS for the control group after anesthesia with MS222 at a concentration of 100mg/l. Monitoring was done for a period of 4 weeks for mortalities. Freshly dead fish were removed from tanks and recorded on a daily basis in case of any. Four freshly dead/moribund fish from each group were dissected and their liver, spleen, gut, gills and head kidney inoculated on Tryptic Soy Agar by stamp plating to confirm the presence of *E. tarda*. All the fish were fed on 30% CP feed (2mm pellets) at a rate of 3% body weight except two days prior to infection. Dissolved oxygen was maintained by continuous aeration and pH and temperature were monitored on a daily basis using a pH/Temperature meter throughout the experimental period. The LD_{50} was calculated following Reed and Muench (1938) method; LD_{50} = [(mortality at dilution next above 50%) - 50%] / [(mortality next above 50%) - (mortality next below 50%)].

3.5 Vaccine Preparation

Laboratory stock *Edwardsiella tarda* was resuscitated and cultured in TSI, incubated at 37 °C for 24hrs at 200rpm. After 24hrs, the cultures were centrifuged at 2500rpm for 10minutes. The pellet was suspended in 10ml of 1X PBS. CFU count was done on TSA. Bacteria were killed using 3% formaldehyde with viability checks every 12 hrs until no

growth was observed on TSA. The inactivated bacteria were dialyzed in 1XPBS that was changed every 12hrs for a total of 72hrs. The dialyzed inactivated whole bacteria was then mixed with an oil based adjuvant (ISA 763 VG -SEPPIC) and thoroughly mixed. CFU was maintained at 10^8 and the vaccine was stored at 4°C until used.

3.6 Vaccination

Eighty (80) apparently healthy fish of average body weight of 75g were used for experimentation. All the fish were acclimatized for a period of two weeks in dechrolinated water in 170L glass tanks prior to treatment. The fish were divided into four equal groups of 20 each and each group was randomly assigned vaccination or control in duplicates. Two groups were intraperitoneally vaccinated (after anesthesia with MS222-100mg/l) with formalin-killed oil adjuvanted (0.1ml containing 10^8cells/ml) *E. tarda* and the other two groups received Phosphate Buffered Saline (PBS) for control. After two weeks, fish were given a booster dose ip. Fish were monitored for a period of 4 weeks prior to challenge, fed with 30% CP pelleted feed at a rate of 3% body weight with continuous aeration for oxygen supply. About quarter of the water volume was changed twice every day and pH and temperature recorded every morning and late afternoon.

3.7 Blood Sample Collection and Serum Preparation

Random samples of 6 fish from each group were bled from the caudal vein weekly and 0.3ml of blood collected using 25guage needles and1ml syringes into vacutainers without anticoagulant. For separation of

serum, blood samples were centrifuged at 5000RPM for 5minutes and serum was harvested and kept in eppendorf tubes at -20°C until required for use.

3.8 Challenge of Vaccinated and Non-vaccinated Fish

Edwardsiella tarda was sub-cultured on Tryptic Soy Agar and incubated at room temperature (25-27° C) for 24 hours. Colonies were homogenized in sterile Phosphate Buffered Saline (PBS) and the turbidity adjusted to correspond to 0.5 McFarland's turbidity standard equivalent to $1.5x10^8$ CFU/ml, the LD_{50} determined from the previous experiment. Fish were then intraperitoneally exposed by injection (after anesthesia with MS222 100mg/l) with *Edwardsiella tarda* (0.1 ml suspension) for both vaccinated and control groups after two days of starvation. The challenge was done two weeks post vaccination with the booster dose. After challenge, half the vaccinated fish were stocked with half of the non-vaccinated (non-vaccinated fish were marked by a small cut on the caudal fin) to eliminate tank effects. Fish were then monitored on a daily basis for a period of 4 weeks for clinical signs, mortalities and other abnormalities. In case of mortalities, dead fish were removed from the tanks and recorded. Percentages of mortalities in both vaccinated and control groups were worked out to calculate the relative percentage survival (RPS) that was used to evaluate the antigenicity of inactivated bacteria with the following formula:

RPS% = 1 – (Mortalities in vaccinated group/Mortalities in control group) X 100

3.8.1 *Edwardsiella tarda* re-isolation

Freshly dead or moribund fish were collected for re-isolation following procedures described by Austin and Austin (2016). Fish were dissected and the liver, head kidney, spleen, gills and the gut aseptically removed. These were cultured by stamp platting on TSA plates and incubated at room temperature for 18-24hours. Single colonies from TSA were sub cultured on BHIA, incubated under the same conditions and confirmed by biochemical tests using API 20E kits (Appendix 1).

3.8.2 Pathological examination

Gross pathological changes were determined by observation of clinical and postmortem signs of the disease. For histopathological changes, the liver, kidney, spleen tissue sections were prepared. Tissue sample were collected from freshly dead or moribund fish, preserved in 10% formalin until required for use. After 2 weeks of complete fixation in buffered formalin, tissues were trimmed and loaded in cassettes. Tissues were then taken through a series of ethanol; 70% (1hr), 80% (1hr), 90% (1hr), 95% (1hr), Absolute 1 (1hr), Absolute 2 (1hr) and Absolute 3 (1hr) then into Xylene 1 (1hr) and finally Xylene 2 (1.5hrs) in a Histokinette. Tissues were then embedded in paraffin wax, allowed to cool and sectioned using a microtome. Before staining, tissues were dewaxed in xylene for 2 minutes, rehydrated in 100% ethanol first then in 95% for 3 minutes. Staining was done using haematoxylin and eosin. The pathological changes were compared between vaccinated and non-

vaccinated groups under the microscope at different magnifications (X4 to X100).

3.8.3 Sampling for bacterial load

Freshly dead or moribund fish were collected from all groups, examined for gross lesions on all external body parts and dissected for examination of the internal organs and determination of the bacterial load in CFU/g. Liver, gills, kidney, spleen and the brain were aseptically removed, weighed and homogenized in 1ml of sterile normal saline. Ten-fold dilutions of the solutions were made in sterile normal saline up to the 15^{th} dilution. 10µl of each dilution was drop plated on divided plates of Tryptic Soy Agar and plates were then incubated for 18-24 hours at room temperature (25-27°C) after which colonies were counted and the bacterial load calculated as CFU/g of the tissue.

3.9 Generation of Positive Control Serum

A female rabbit was inoculated intramuscularly with 0.6ml of $1.5x10^9$ CFU/ml live *E. tarda* on day zero and boosted with the same dose after 2 and 4 weeks. After 3 and five weeks, the rabbit was bled from the ear and the collected blood was centrifuged at 5000RPM for 5minutes. Serum was harvested and preserved in eppendorf tubes at -20°C until required for assay.

3.10 Purification of the Positive Control Serum

Before use in the hemagglutination inhibition assay, the serum was purified by decomplementation and adsorption with red blood cells. The serum was thawed and inactivated by heating in a water bath at 56°C for 30 minutes. After inactivation, the serum was tested for red blood cells agglutination with guinea pig RBCs and there was minimal

agglutination. The serum was then further purified by mixing it with a pellet of guinea pig RBCs and gently mixing every after 15 minutes for 2hrs at 4° C. Serum was recovered by centrifuging at 1200 RPM for 5minutes. This procedure was repeated 4 times and non-specific inhibitors were removed successfully.

3.11 Determination of Serum Antibody Titers

Hemagglutination was first performed to determine the hemagglutinating unit of the antigen. Two-fold dilutions of 25µl of live *E. tarda* were made in PBS in a 96-well V-shaped microtiter plate, 25µl of 1.5% guinea pig RBCs were then added to each well and the plate was incubated at room temperature for 30 minutes. The HA titer was determined as the highest dilution showing complete hemagglutination of the antigen with the RBCs in the last well with no tear-shaped streaming of RBCs. The hemagglutinating unit (HAU) was calculated by dividing the HA titer by 4 to get the 4HAU/25µl of the antigen. The concentration of the antigen was previously determined by plate counting.

Serum agglutinating antibody titers against *E. tarda* were determined by the microtiter method according to the procedure described by Hirst and Ellis (1994). Briefly, two-fold dilution series of 25µl of each serum sample were made in PBS in wells of a 96 well microtiter plate. Then 25µl of live *E. tarda* suspensions containing $2.8x10^7$ bacterial cells/ml were added to each well. Each plate had a row of positive and negative control sera and the RBCs control. Plates were then incubated at room temperature for 30 minutes after which 25µl of guinea pig RBCs were added to each well. Plates were again incubated at room temperature for 30 minutes. The HI titer was scored as the highest dilution of serum causing complete inhibition of hemagglutination. All the titers were expressed in reciprocal and averages in each treatment were worked out.

3.12 Data Analysis

Statistical analysis was performed in SPSS® 20 with 95% level of confidence. At first the data on mortalities, bacterial load and antibody titers were checked for normality and homogeneity of variance by Shapiro-Wilk test and Levene's test, respectively. Data that obeyed the rules of normality and equal variances were analyzed by one way analysis of variance to detect the effect of the treatment. Data that were not normal were transformed by their natural log and analyzed by Independent-Samples T-test to detect the effect of the treatment for bacterial load. Kruskal-Wallis test analysis was conducted to detect the effect of the treatment among different sampling period for titer values after log transformation. Data on LD50 mortalities was analyzed using Kruskal-Wallis test in Epiinfo 7.

CHAPTER FOUR

4.0 RESULTS

4.1 Median Lethal Dose

The first death in the Nile tilapia LD_{50} experiment was recorded in 10^7 dilutions four days post infection and the first manifested clinical signs were hemorrhages on the skin and caudal fin and pathological features included tiny black spots in the liver and the kidney. The clinical signs observed were generally much more severe in fish infected with dilutions 10^8 and 10^9 regardless of days post infection than in other dilutions.

On the fifth day, the second mortality was recorded in 10^6 dilution followed by one mortality in 10^9 and last dilution to have first mortality was 10^8 at day seven post infection. The highest mortality (3 fish) in a single day was recorded in 10^9 at day eleven post infection. No mortality was recorded in the control group that was injected with phosphate buffered saline throughout the experimental period. At day nine and day thirteen, no mortality was recorded in any of the dilutions. The LD_{50} value was determined at the 14^{th} day and the calculated value was $1.6x10^{8.1}$. Results are summarized in Table 1 and Figure 2.

The experiment was further monitored up to day 28 post infection and after day fourteen, no mortality was recorded for four consecutive days in all dilutions until day nineteen in 10^8. At twenty days post infection, all the fish in 10^9 dilution had died and in 10^8 the last fish died on day twenty two. By the end of the twenty eight days of the experiment, four fish and seven fish survived in dilutions 10^7 and 10^6, respectively. All the fish in the control survived. Dead fish were subjected to bacteriology and *E. tarda* was recovered from dead fish after stamp plating the liver, kidney, gills, spleen and the intestines on

nutrient agar plates and sub culturing on TSA plates. Small round raised whitish-cream colonies developed after 18-24hrs of incubation at room temperature (25-27°C). The mean water temperature and pH throughout the experiment was 22±1.6°C and 7±0.6, respectively. The mortalities data was analyzed by non-parametric Independent-Samples Kruskal-Wallis test with 95% level of confidence to detect the effect of the treatment. There was no significant difference in % mortality of treatment among the different concentrations, p =0.406.

Table 1: Daily and total cumulative mortalities and their percentages 14 days post infection

Dilution	Total Number. of fish	Number of mortalities per day														Total mortality	Percentage mortality
		1	2	3	4	5	6	7	8	9	10	11	12	13	14		
$1.5x10^6$	10	0	0	0	0	1	0	1	0	0	0	0	0	0	0	2	20
$1.5x10^7$	10	0	0	0	1	0	0	0	1	0	0	2	0	0	0	4	40
$1.5x10^8$	10	0	0	0	0	0	0	1	2	0	0	2	0	0	0	5	50
$1.5x10^9$	10	0	0	0	0	0	1	0	0	0	2	3	1	0	1	8	80
PBS	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

4.2 Clinical Signs Manifestations

The experimental infection of vaccinated and non-vaccinated Nile tilapia with *E. tarda* LD_{50} resulted into a number of clinical signs (Figure 3 to 8). The clinical signs ranged from mild to severe as the number of days post infection increased in some cases. The first mortality was recorded at three days post infection and on gross examination, it did not show any signs of the disease on the external body parts. The internal organs however showed some changes that included pale liver, fluid filled intestines and a few whitish to greyish nodules in the spleen. In general, the most affected fish exhibited sluggish swimming, isolation in the corners of the tank and slow movements of the operculum, fish also stopped feeding at least two days before death and would not easily swim away on touching them or the outer surface of the tank at their position. Moribund fish also showed

slight vertical hanging from the water surface especially on the sides of the tank with very slow movements of the operculum.

Clinical signs that were observed from the external body surfaces of examined fish included; pin point or slightly larger hemorrhages on the skin and fins, skin ulcers from about 0.4 to 2cm in length with loss of scales, caudal fin erosion, exophthalmia, mouth erosion, depigmentation of the skin, swollen abdomen, erosion at the base of the dorsal fin, congestion of the fin rays and cataracts that was observed in only two fish. There was also liquefaction of the muscle underlying the skin ulcers areas which was only observed in one fish from the non-vaccinated group.

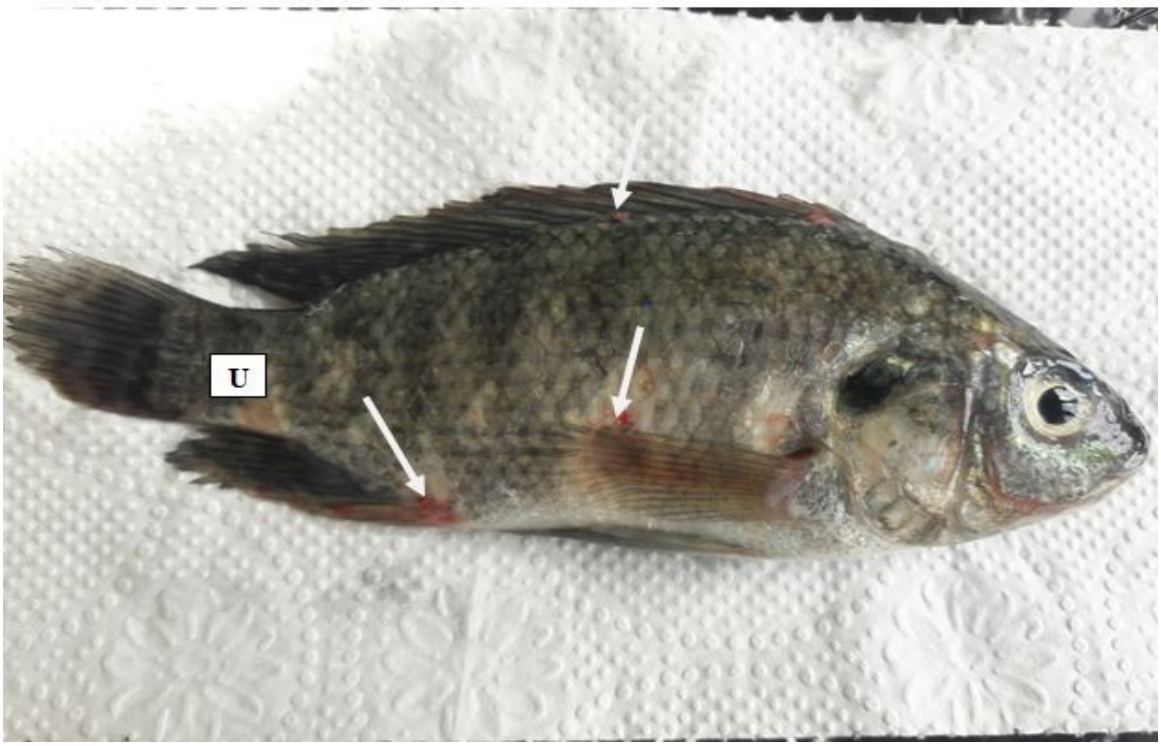

Figure 2: Skin ulcers (U) and hemorrhages on the skin an fins (arrows) and caudal fin erosion in an *E. tarda* infected Nile tilapia

The manifestation of the infection/disease was more pronounced in the internal organs. The observations from these were; mottled liver, pale liver, numerous black spots in the liver, greyish to whitish nodules in the spleen and kidney, pale clamped gills, congestion of the visceral organs, fluid/gas filled intestines, hemorrhages in the stomach and intestines and fluid-filled abdomen.

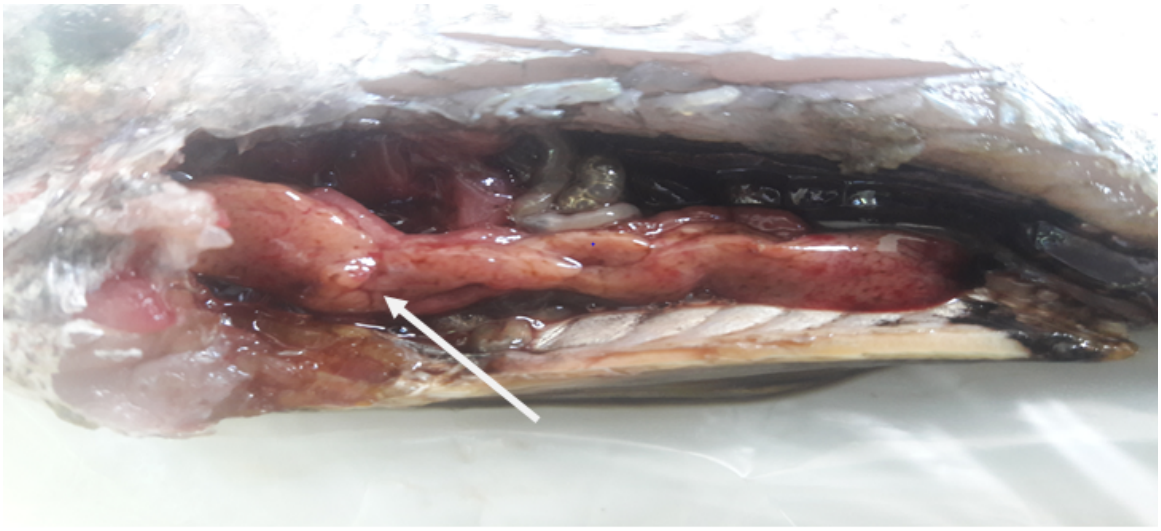

Figure 3: Slightly pale mottled liver observed in an infected Nile tilapia (Arrow)

Figure 4: Fluid-filled stomach and intestines and congestion of organs in the abdominal cavity of *E. tarda* infected Nile tilapia (Arrows)

In addition, the spleen and the kidney of some fish appeared swollen and severely damaged. The greyish nodules were much more numerous in the kidney than in the spleen except in only two fish.

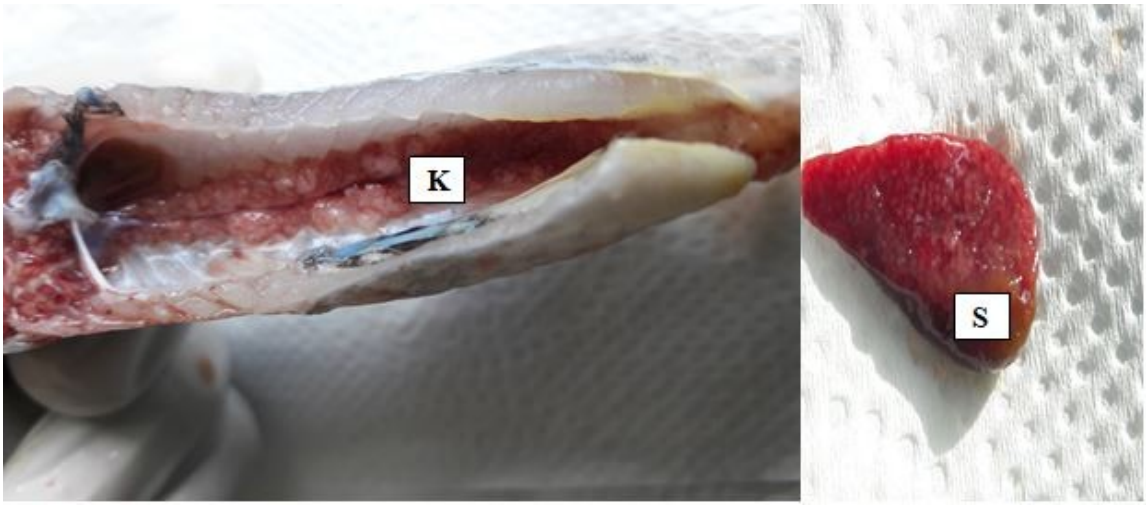

Figure 5: Swollen, nodular and severely damaged kidney (K) and spleen (S) of *E. tarda* infected Nile tilapia

Figure 6: Pale liver with black spots (white arrow) and greyish nodules in the spleen (blue arrows) of Nile tilapia infected with *E. tarda*

In some cases, the kidney was severely damaged to the extent of becoming greyish entirely from the head kidney to the proximal kidney and a smelly pus-like fluid would be released on squeezing especially in fish that died between ten to fifteen days post

challenge in non-vaccinated groups. In a few cases, hemorrhages were observed in the swim bladder and very small black spots in the muscles surrounding the proximal kidney area. Clinical signs such as exophthalmia, erosion at the base of the dorsal, cataracts and hemorrhages in the gut and the swim bladder were only observed in fish from the non-vaccinated groups.

Figure 7: Exophthalmia (bulging eye) and erosion at the base of the dorsal fin (arrows) of a Nile tilapia infected *with E. tarda*

On the other hand, some fish showed the clinical signs of the disease precisely exophthalmia, pin point hemorrhages on the skin but did not die. This was observed in only two fish in the non-vaccinated groups where the signs appeared at about three weeks post challenge.

4.3 Histopathological Changes

Tissues from both vaccinated and non-vaccinated fish were collected and processed to determine changes and compare them between the groups to ascertain whether there is a difference in the damage caused by the bacterium. Major changes were observed in the kidneys, livers and spleens. Most spleens of non-vaccinated fish showed follicular necrosis, lymphoid hyperplasia, increased hemosiderin (Figure 9), increased histeocytes in pale appearing areas/white pulp.

Increased glycogen storage in areas that appeared pale, infiltration of heterophils around the hepatic area, vascular damage indicating necrosis and diffuse irregular cytoplasmic vacuolization were observed in the livers from some non-vaccinated fish (Figure 10). The livers also showed diffuse, severe fatty change and hemorrhages indicated by red cells mixed with heterophils. The kidneys of non-vaccinated fish showed damaged tubules indicating necrosis and those of some of the vaccinated ones showed acidophilic granules in the cytoplasm of the tubule cells.

The Histopathological changes were more severe in the tissues of non-vaccinated fish except in the spleen of one vaccinated fish that died three weeks post challenge. The spleens of most non-vaccinated fish showed severe extensive multifocal active necrosis with a ring of pus cells around the necrotic debris and some resolving necrosis. The necrotic areas observed in the spleen of vaccinated fish were resolving with no puss cells but with macrophages and the necrotic areas were few compared to those in non-vaccinated fish (Figure 11). High diffuse fatty changes were observed in the liver of the non-vaccinated fish that died fifteen days and above post challenge. The livers also showed increased vacuolization and damage/necrosis of hepatocytes (Figure 12). More inflammatory cells and macrophages were observed in tissues of vaccinated fish than in

the non-vaccinated fish especially those that died at three weeks post challenge and above. Multifocal necrosis damaging the tubules were observed only in non-vaccinated fish (Figure 14), mild resolving necrotic areas were noticed in the vaccinated fish.

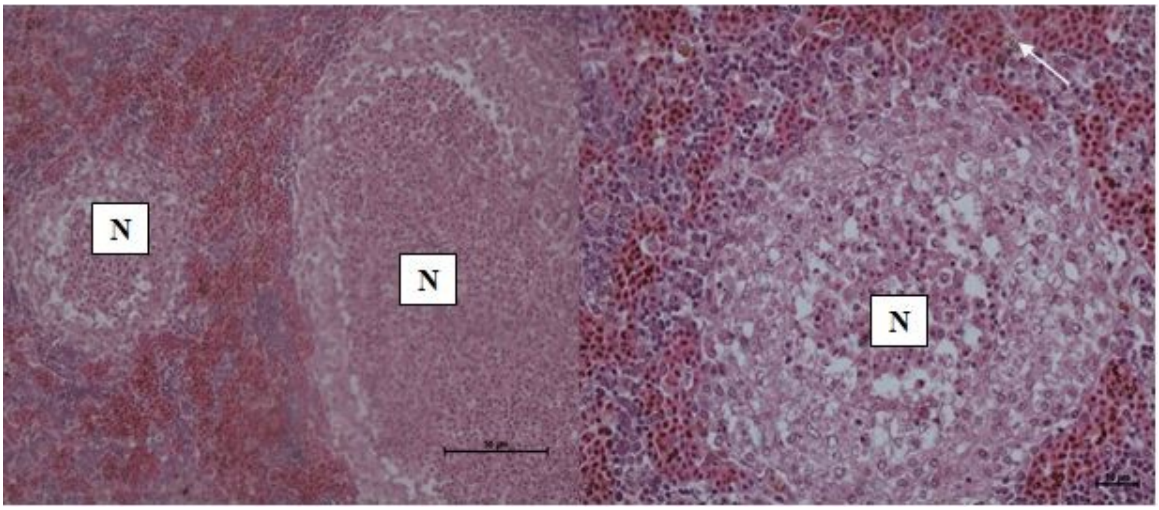

Figure 8: Spleen of non-vaccinated fish showing multifocal extensive necrosis (N) and increased hemosiderin (arrow). X20

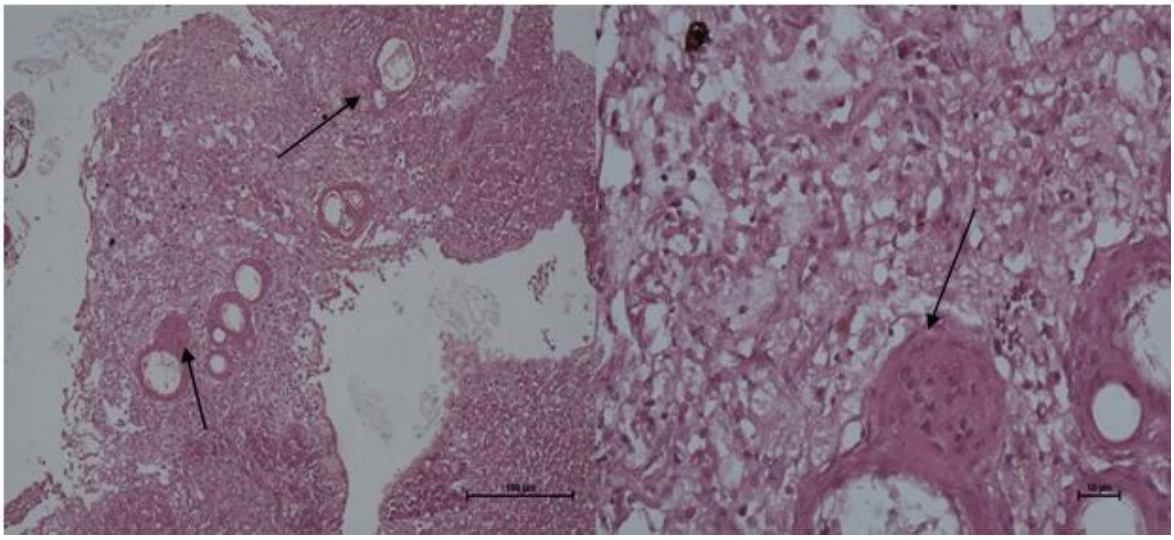

Figure 9: Liver of non-vaccinated fish with vacuolization indicating necrosis (arrows). X10, X40

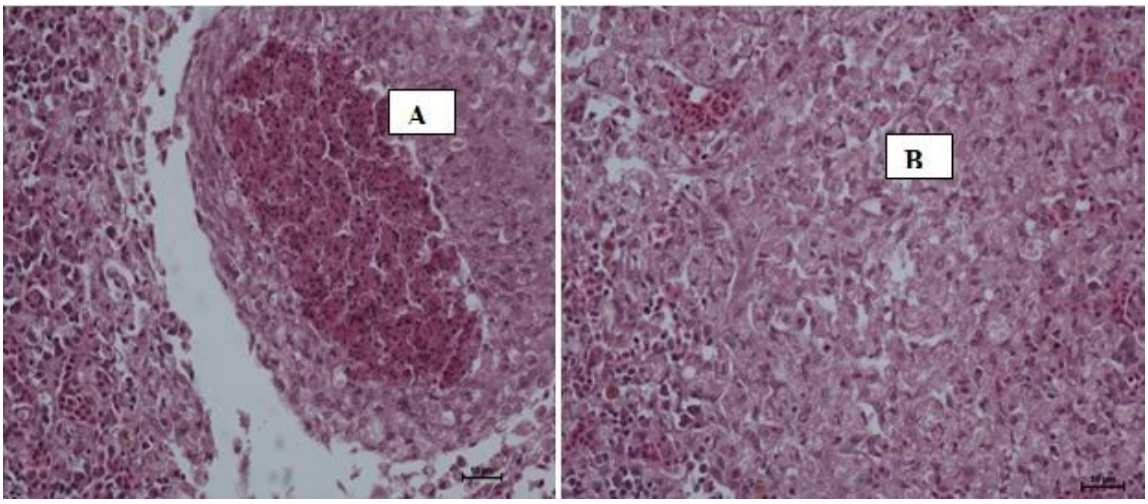

Figure 10: Focally extensive active necrosis, A (control) and resolving necrosis, B (vaccinated) in the spleen. X40

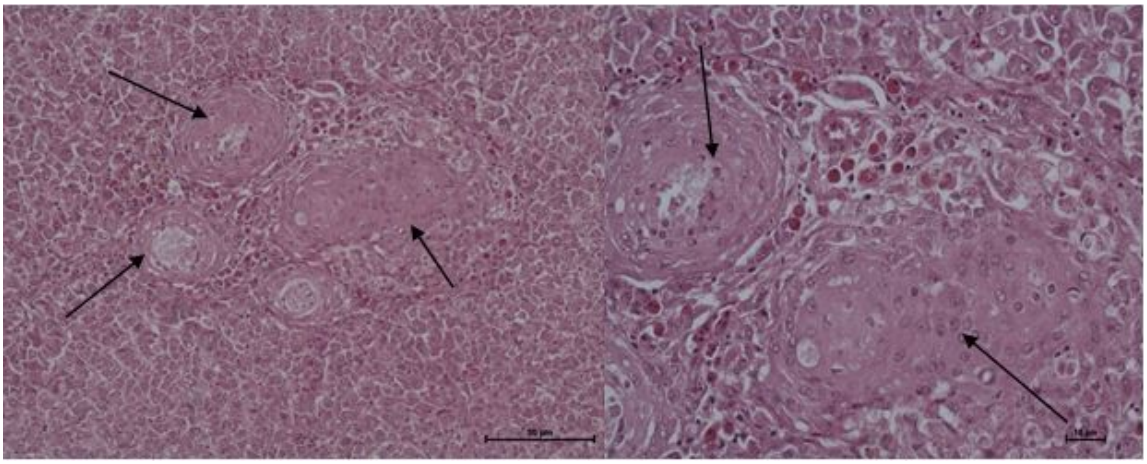

Figure 11: Liver of non-vaccinated fish with multifocal necrosis and surrounding heterophils (arrows). X20, X40

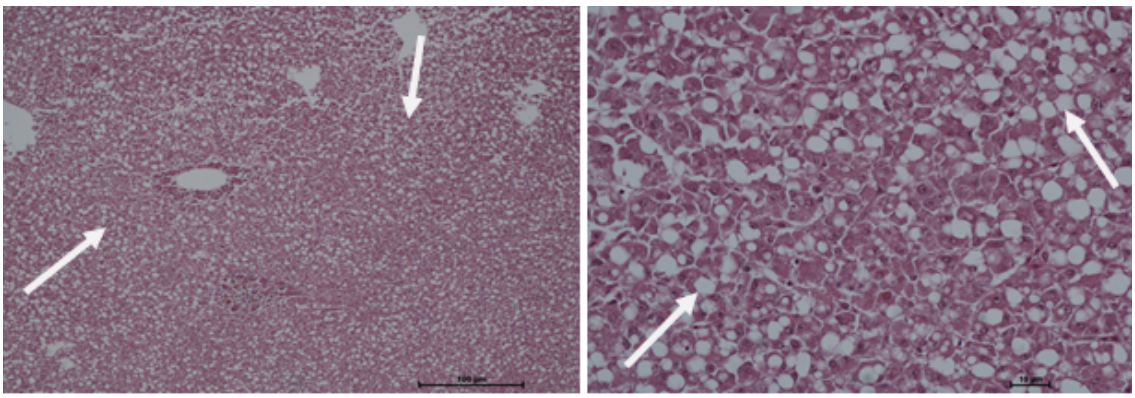

Figure 12: Liver of non-vaccinated fish indicating severe, diffuse fatty changes. X10, X40

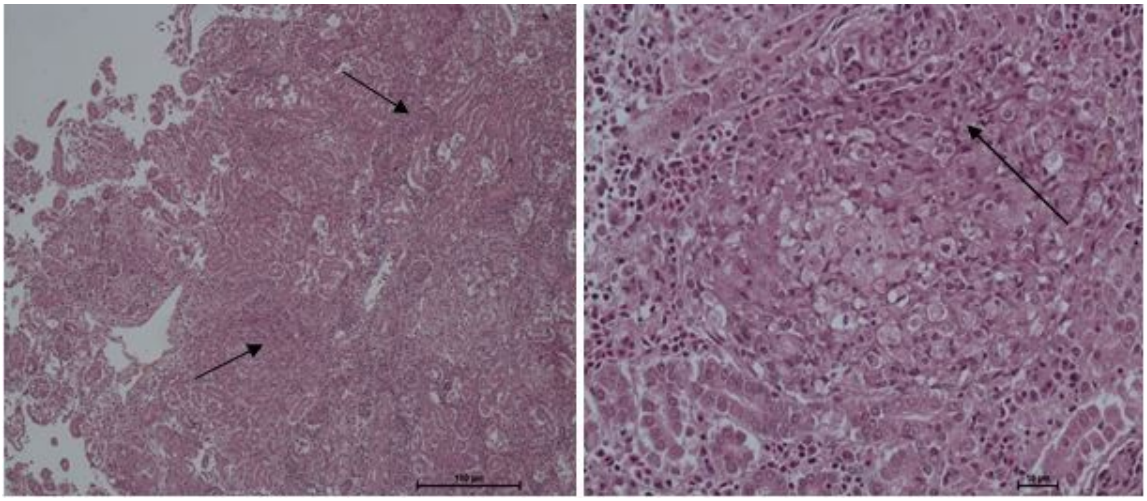

Figure 13: Kidney showing multifocal areas of necrosis damaging the kidney tubules (arrows). X10, X40

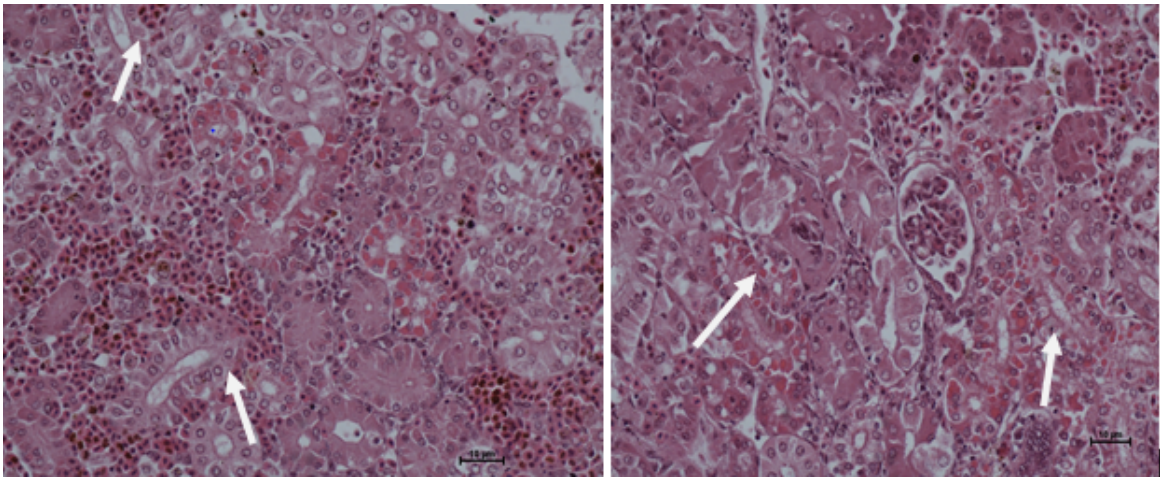

Figure 14: Acidophilic granules in the renal tubule but with no necrosis in the kidney of vaccinated fish. X40

4.5 Fish Mortalities Post Challenge

All vaccinated and non-vaccinated fish in all groups were challenged with the same concentration of *E. tarda* fourteen days after the booster dose for the vaccinated groups. The challenge resulted into development of the disease that resulted into a number of clinical signs and eventually death in some cases in both vaccinated and the non-vaccinated groups. The first and the second mortalities were recorded in one non-vaccinated and vaccinated group at third and fifth day post challenge, respectively. Fish in

the non-vaccinated groups continued dying at six days post challenge for seven consecutive days without registering any mortality in the vaccinated groups. The rate at which the fish in non-vaccinated groups were dying was much higher than the rate in the vaccinated groups. More than one dead fish were recorded in the non-vaccinated groups in several consecutive days which were not observed in the vaccinated groups.

Also, most of the fish in the non-vaccinated groups usually died between one to three days after the appearance of clinical signs but the fish from vaccinated groups died at least three or more days after showing clinical signs. Fish in the control groups showed most of the gross pathological signs than fish in the vaccinated groups and the severity was much more depicted in the moribund and dead fish from the non-vaccinated fish than in the vaccinated groups especially in the liver, kidney and the spleen.

Table 2: Fish mortalities, survival and relative percentage survival

Treatment	Total No. of fish	Mortality	Survival	%age Survival	Total %age survival	Total mortality	RPS
V1	20	12	8	40	42.5	23	32.4
V2	20	11	9	45			
C1	20	16	4	20	15	34	
C2	20	18	2	10			

Key: RPS = Relative percentage survival, V= Vaccinated group, C= Control group.

Table 3: Descriptive for mean percentage mortalities in vaccinated and non-vaccinated groups

			Observations	Total	Mean	Variance	Std Dev
1			2.0000	115.0000	57.5000	12.5000	3.5355
2			2.0000	170.0000	85.0000	50.0000	7.0711
	Minimum	**25%**	**Median**	**75%**	**Maximum**		**Mode**
1	55.0000	55.0000	57.5000	60.0000	60.0000		55.0000
2	80.0000	80.0000	85.0000	90.0000	90.0000		80.0000

Key: 1 = Vaccinated, 2 = Non-vaccinated, % = Percentage, Std Dev = Standard deviation.

The data was analyzed by non-parametric Kruskal-Wallis test for two groups test with 95% level of confidence to detect the effect of the treatment. The average percentage mortality of treatment C (2) (85±7.0) was not significantly higher than the average percentage mortality of treatment V (1) (57±3.5) ($p = 0.1213$), ($p > 0.05$).

4.6 Bacterial Load

Results on the bacterial load in different tissues are shown in Table 4. The bacterial load was determined from liver, kidney and spleen of six fish samples from the vaccinated and non-vaccinated groups by getting the average value of the calculated colony forming units from two different dilutions from each tissue sample.

In general, the highest bacterial load was obtained from the kidney followed by the liver and the spleen in both vaccinated and non-vaccinated groups. It is only in one vaccinated fish where the bacterial load was higher in the liver than in the kidney and the spleen and in another vaccinated fish where the load in the spleen was slightly higher than that in the kidney and the liver. The bacterial load in the non-vaccinated group was much higher than the one in the vaccinated groups in the kidney, liver and the spleen regardless of the days at which the fish died post challenge.

Table 4: Bacterial load count from liver, kidney and spleen of challenged fish

Treatment	No. of colonies (CFU/g)		
	Liver	Kidney	Spleen
V1	$5.9X10^{6}$	$2.7X10^{7}$	$3.1X10^{6}$
V1	$2.64X10^{7}$	$1.63X10^{7}$	$1.63X10^{7}$
V1	$5.4X10^{7}$	$1.44X10^{9}$	$4.15X10^{8}$
V2	$5.45X10^{8}$	$7.55X10^{7}$	$5.2X10^{7}$
V2	$5.45X10^{6}$	$3.55X10^{7}$	$6.9X10^{9}$
V2	$4.6X10^{8}$	$2.2X10^{8}$	$4.3X10^{8}$
C1	$7.6X10^{13}$	$1.99X10^{18}$	$7.25X10^{8}$
C1	$6.8X10^{9}$	$3.25X10^{9}$	$4.55X10^{7}$
C1	$7.45X10^{10}$	$2.99X10^{19}$	$7X10^{9}$
C2	$1.25X10^{8}$	$1.08X10^{9}$	$5.2X10^{7}$
C2	$4.9X10^{13}$	$6.8X10^{11}$	$5.9X10^{10}$
C2	$2.7X10^{14}$	$2.54X10^{16}$	$6.85X10^{8}$

Key: V=Vaccinated, C=Control/Non-Vaccinated, 1=group 1, 2=group 2, No. = Number.

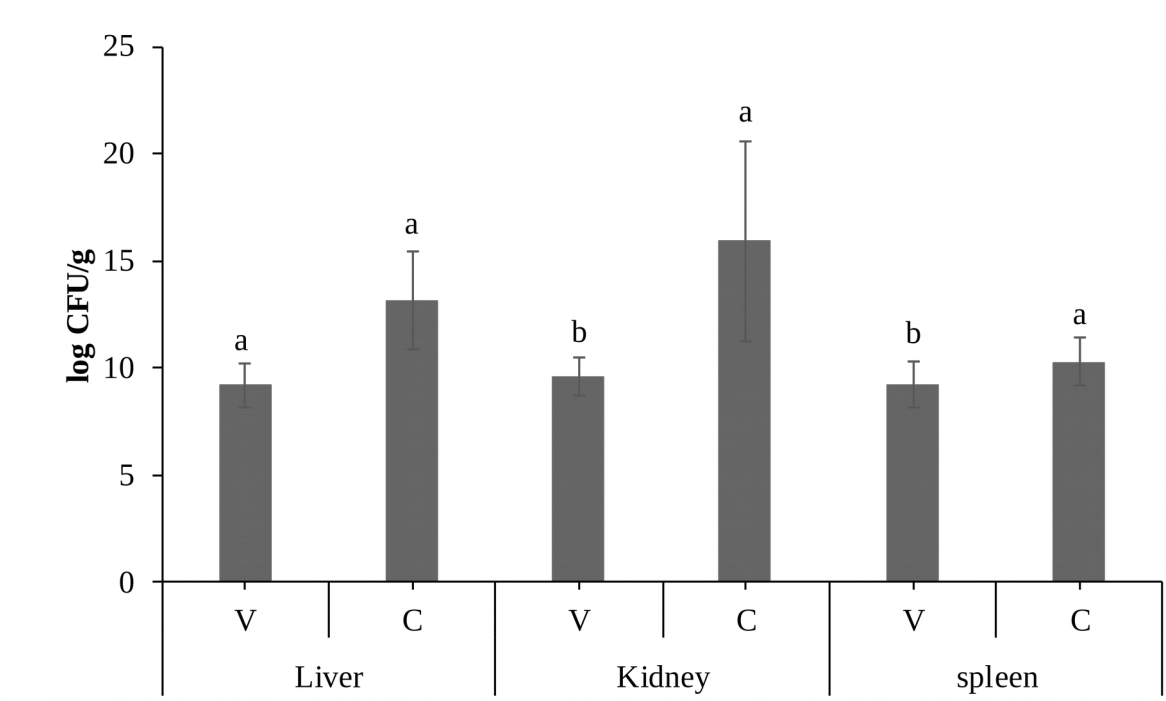

Figure 15: Mean Log CFU/g in different organs among treatments

Key: a,b = different alphabet letters indicating significant difference ($P<0.05$), V=Vaccinated, C=Non-vaccinated, CFU/g = Colony forming units per gram.

4.7 Serum Antibody Titers

There was increased serum antibody titers at weeks one, two, three and four post vaccination in vaccinated groups. The highest increase in antibody titers was observed at

week three post vaccination. The results showed highly significant ($p<0.05$) serum antibody titers in vaccinated groups than in the non-vaccinated groups. Also, there was a highly significant difference ($p<0.05$) in mean serum antibody titers at week two and three than at other weeks. Results are summarized in Table 5.

Table 5: Mean antibody titter values in different treatments among different sampling periods

	Treatments	
Week	**V**	**C**
1	21.3 ± 7.9^{d}	0.2 ± 0.6^{a}
2	56.0 ± 14.5^{cd}	0.7 ± 1.3^{a}
3	229.3 ± 63.8^{ab}	0.8 ± 1.3^{a}
4	341.3 ± 126.0^{a}	0.8 ± 1.3^{a}

Key: V=Vaccinated group and C = Control/non-vaccinated, a,b,c,d = different alphabet letters indicating significant difference (P<0.05).

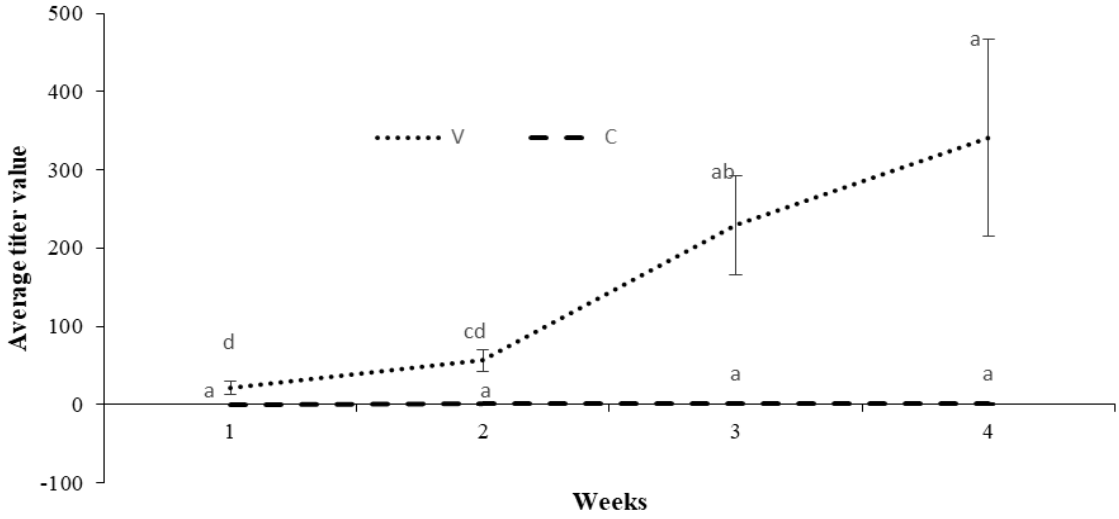

Figure 16: Mean antibody titter values in different treatments among different sampling periods

Key: a,b,c,d = different alphabet letters indicating significant difference (P<0.05). V= vaccinated, C=non-vaccinated.

CHAPTER FIVE

5.0 DISCUSSION

Edwardsiella tarda is the causative agent of Edwardsiellosis, a disease which is among the major constraints in aquaculture worldwide. This study was conducted to determine the pathogenicity of virulent *E. tarda* and the immunogenicity of formalin-killed *E. tarda* in Nile tilapia by intraperitoneal experimental infection. The median lethal dose of this bacterium in Nile tilapia was found to be $1.6x10^{8.1}$ cfu/ml at day 14 post infection. These LD_{50} results reveal that the *E. tarda* strain KOCT4' was moderately virulent according to the degree of virulence described by Pu *et al*. (2007).

The median lethal dose of bacterium is the assessment of the virulence which is determined by a number of mechanisms. These results are more or less similar to those reported by Baxa *et al*. (1990) who determined the LD_{50} at $7.9x10^8$ cfu/ml and the fish exhibited similar disease clinical signs and symptoms. Mostafa *et al*. (2008) reported LD_{50} of *E. tarda* to be $2.8x10^8$ cfu/ml in Nile tilapia at 5-7 days and 7-15 days post infection. However, results in this study differ from LD_{50} that has been determined in a number of other studies. Abraham *et al*. (2015) calculated the value of LD_{50} to be $1.68x10^7$ cfu/ml in a twenty two days experiment. Also, Pridgeon *et al.* (2014) reported an LD_{50} value of Southern flounder *E. tarda* isolates in Nile tilapia at $1.1x10^7$ cfu/ml CFU when Nile tilapia was exposed by intraperitoneal injection. The variations in the values of the median lethal dose could probably be due to differences in the involved strains. Variation in strains of Nile tilapia used in these experiments could have been different bringing about differences in response to the infection and development of immunity against the pathogen overtime. Furthermore, environmental conditions under which the experiments were conducted may influence the virulence and pathogenicity of *E. tarda* (Leung *et al.*, 2012).

Infected fish clinically presented with hemorrhages, ulcers, swollen abdomen, vertical hanging, and loss of pigment. These observations are similar to what was reported earlier although varied in LD_{50} values. Variations in the days at which the fish died and the number of fish that survived as the days of the experiment increased suggests the differences in the development of immunity in individual fish's body against the pathogen, which could include response to stress by other fish in the same tank among others. The fish that survived in pathogen concentrations of 10^6 and 10^7 until day 20 of the experiment could have fully developed immunity against *E. tarda*. This is supported by the fact that there were neither clinical signs nor pathological features of the disease in fish after 20 days of the experiment. These findings suggest that the pathogen can as well be found in fish that appear to be healthy.

The ability of a pathogen to cause a disease depends on a number of factors and processes that interact and result into a disease (Park *et al.*, 2012; Roberts, 2012). Different clinical signs of the disease are attributed to different virulence factors of a particular pathogen. A number of virulence factors are known to facilitate the pathogenicity of *E. tarda*, the major factors are reported to be T3SS and T6SS which are essential for the survival, replication and virulence of the bacteria in the host's body (Nakamura *et al.*, 2013; Xie *et al.*, 2014). They produce different effectors that exploit the host's environment. T3SS secretes needle complex proteins that make the pathogen able to avoid killing by phagocytes. *Edwardsiella tarda* also has a range of regulatory systems that sense environmental changes such as pH, temperature, osmolarity, presence of antimicrobial peptides and nutritional value changes or shortages that contribute to its survival in the host (Leung *et al.*, 2012; Mendez *et al.*, 2012; Park *et al.*, 2012). The initiation and expression of the pathogen's virulence factors depend on these environmental factors (Leung *et al.*, 2012).

Ullah and Arai (1983) demonstrated that *E. tarda* has exo-enzymes with haemolysins and dermatoxins activities which confer pathogenicity on the pathogen and they have been associated with clinical signs like hemorrhages, ulcers and depigmentation in infected fish. These clinical signs were among those that were observed in this study, this together with the confirmed re-isolation of *E. tarda* from the kidney, liver, spleen and intestines of dead fish confirms that the development of the disease clinical signs and mortalities were due to inoculated *E. tarda*.

The development and manifestation of Edwardsiellosis in the experimental infection in this study resulted in different clinical signs and pathological lesions that have been associated with the disease. Exophthalmia, cataracts, skin ulcers, hemorrhages on the skin and fins, depigmentation, loss of scales, swollen abdomen were observed. These results concur with Kubota *et al.* (1981) who reported scale detachment, swollen abdomen and opaqueness in the eyes associated with *E. tarda* exposure. Other reported clinical signs were sluggish movement, loss of escape and defense which were also observed in this study. The results are also in agreement with those of Ling *et al*. (2000) and Griffin *et al*. (2017) who found out that the experimental infection of fish resulted into hemorrhages all over the body, detached scales, pale skin which is reported as depigmentation and abdominal dropsy which were referred to as swollen abdomen in the current study. El-Seedy (2015) also reported similar findings in Nile tilapia infected with *E. tarda*. On the other hand, the clinical signs observed in this experimental study are in line with those of Meyer and Bullock (1973) and Bullock and Herman (1985) on Edwardsiella septicemia diagnosis in channel catfish and in largemouth bass reported by Francis-Flody *et al.* (1993). Similar findings have also been reported in many studies on the natural fish infections with *E. tarda.*

However, this study reports mouth erosion and erosion at the base of the dorsal fin that have not been reported before on experimental infection of Nile tilapia and other species with virulent *E. tarda*. The only erosion that was reported in this study and has been reported in Nile tilapia and catfishes is the caudal fin erosion. These erosions can probably be compared to deep cutaneous ulcers that have been reported in other studies. Production of excessive mucus reported in previous experimental infections was also not observed in fish in the current study.

Internal pathological changes in infected Nile tilapia in this study showed hemorrhages in the intestines, congestion of the internal organs, pale liver, grayish nodules in the kidney and spleen and abdominal fluid which were also similarly reported in other studies (Kubota *et al*., 1981; El-Refaely, 2013; El-Seedy, 2015; Nagy *et al.*, 2018). Fish in this study showed significant damage in the liver, kidney and spleen which results are similar to those reported by Darwish *et al.* (2000), Darwish *et al.* (2001) and Xue and Xu (2018). From these findings, it can be suggested that the pathogen most likely targets the hematopoietic and immune tissues of the host to easily weaken and flourish in the host's body. Miwa and Mano (2000) and Mathew *et al.* (2001) reported congestion in the liver and kidney of the examined fish which they associated with nephric and hepatic virulence factors of *E. tarda.*

Ideally, the ability of this bacterium to progress and replicate could also be due to its ability to acquire iron or producing toxins as part of its infection process which factors contribute to clinical and postmortem lesions. The explanations by Braude (1964), Nowotny (1979) and Park *et al.* (2012) in their findings support this statement. Similarities in the nature of pathogenesis caused by all Gram-negative bacteria have been reported in a number of studies and said to be a result of endotoxins and exotoxins they produce.

However, Ullah and Arai (1983) stated that endotoxins were not produced by *E. tarda* like other Gram-negative bacteria but it produced two exotoxins which are responsible for a number of lesions.

In some moribund that died few days post challenge, had no lesions on the external body surfaces and a few on the internal organs. This could probably be due to rapid progression of the disease/infection which could not allow enough time for formation of pathological lesions. Similar findings have been reported by Meyer and Bullock (1973) and Pressley *et al*. (2005) in channel catfish and Zebra fish following intraperitoneal injection with *E. tarda*.

The survival of fish up to the end of experiments in the non-vaccinated groups and in the LD_{50} experiment can be attributed to the development of immunity against the disease with time post infection. As the pathogen may possess several factors that enhance its infection ability, survival and replication in the host's body, the host also has different mechanisms in the body that facilitate protection against the pathogen. In this case, when the host's mechanisms suppress the progression of the infection by the pathogen, the host fish can recover and may not be killed or severely affected by the pathogen under optimum environmental conditions in the fish facility. However, the pathogen may be recovered from fish that do not show any signs and symptoms of the disease. Defoirdt *et al*. (2011) stressed that in some cases, fish can develop immunity, combat and clear the infection of *E. tarda* when they are reared under good environmental conditions. Reducing stress inducing factors and injuring fish and using proper fish husbandry and management practices such as biosecurity are important in preventing infections and their spread.

The severe necrosis and tissue damage, infiltration of macrophages, heterophils, vacuolization of hepatocytes in the liver, damaged kidney tubules with necrosis observed

in histopathology tissues are similar to observations in other studies (Darwish *et al*., 2000; Guo *et al.,* 2014; Aznan *et al.,* 2018). The histopathological changes correlate with the gross pathological changes and the high bacterial load counts. However, the bacterial load was much higher in the kidney and the liver than in spleen but the histopathological changes were more severe in the spleen. This suggests that the spleen could be an organ that plays an important role in fish immunity. The spleen of the non-vaccinated fish showed severe multifocal splenic necrosis, this suggests that the immune role played by the spleen could have been suppressed by heavy *E. tarda* infection as many spleens of non-vaccinated fish had numerous greyish nodules that contained the bacterium. The tubular necrosis and damage observed in the kidney indicates less protective effect of the kidney against *E. tarda* infection and reduced functioning of the kidney that could have resulted into death. Aznan *et al.* (2018) suggested less protective effect of the kidney against *E. tarda* in channel catfish after observing disarrangement of the tubules architecture, glomerular expansion, degeneration of the tubules and renal corpuscles. On the other hand, the increased number of macrophages and inflammatory cells observed in the liver, kidney and spleen of the vaccinated fish could be a result of functional immunity or activation of immune system (Abde-Baki *et al.*, 2015; David and Kartheek, 2015). The increase in diffuse fatty changes observed in the control fish that died 15 to 28 days post challenge suggest utilization of fat in the liver as a result of starvation, this could have resulted from anorexia from diseases-related stress and onset of illness. This is supported by the fact that some fish stopped feeding two to several days before death. The resolving necrosis observed in the spleen of the vaccinated fish than the active necrosis in the non-vaccinated fish indicates some degree of recovery which could be the effect of the vaccine. The increased vacuolization of hepatocytes in the liver indicating degeneration could have resulted into metabolic malfunction leading to death. However, Al-Salahy and Mahmoud (2003) suggested that vacuolization can be a defense response against injury and damage.

Prevention of fish diseases by vaccination is increasingly becoming an important aspect in aquaculture and it is considered less costly compared to other approaches. Evelyn (2002) stated that, combined with proper health management, prophylactic immunization is an indispensable tool in controlling diseases in the aquaculture industry. Different monovalent and multivalent vaccines against viral and bacterial diseases have been developed and commercialized (Bostock, 2002; Evelyn, 2002). Studies on the protective efficacy in different fish species with inactivated *E. tarda* cells against *E. tarda* infections have been reported (Swain *et al.*, 2007).

In this study, Nile tilapia was vaccinated with 0.1µl containing10^8 cells of formalin-killed oil-adjuvanted *E. tarda* intraperitoneally, given a booster dose after fourteen days and challenged fourteen days after the booster dose. Injectable vaccines are said to be superior to other vaccines as they can be quickly taken up for adaptive immune responses induction compared to for example mucosal vaccines that have to cross the mucosal barriers to enter into the systemic environment of fish's body (Munang'andu *et al.*, 2016). Furthermore, the vaccine was oil-adjuvanted, oil adjuvants in vaccines generate a depot effect which facilitates slow release of the antigen into the blood or tissues that results into humoral immunity enhancement and prolonging (Anderson, 1997). In some cases, this is good enough that the booster dose or revaccination may not be required given good environmental conditions. A higher degree of success has been achieved against a number of fish diseases in some studies (Newman, 1993; Rahman *et al.*, 2000).

In the current study, there was significantly higher serum antibody titers in fish that were vaccinated than in the control fish ($p<0.05$). This suggests that the used formalin-killed cells induced specific humoral immunity in Nile tilapia. In addition, the antibody titers increased as weeks past vaccination also increased, this was more significant at one week

post the booster dose and three weeks after the initial dose. The increase in the titers can be attributed to the deposit effect of the oil adjuvant and the boosting at two weeks after the initial dose. El-Jakee *et al.* (2008) reported an increase in the number of serum antibody titers against *E. tarda* using agglutination and ELISA in Nile tilapia following immunization with formalin-killed *E. tarda* cells. Inactivated bacteria have been reported to be antigenically valid and to induce specific antibodies and/or systemic humoral immune responses in a number of fish species (Romalde *et al.*, 2004).

A relative percentage survival of 32.4% was determined from this study after Nile tilapia was exposed to *E. tarda* intraperitoneally two weeks after the vaccine booster dose. This protection level is relatively low compared to some previous studies which have reported RPS of 50-100% using the same inactivated cells. These differences can be attributed to use of different challenge doses as some studies did not ascertain the median lethal doses experimentally to determine the challenge dose. Gutierrez and Miyazaki (1994) reported that vaccination with formalin-killed *E. tarda* cells resulted into survival of 40% when the challenge dose of *E. tarda* did not exceed 10^5 CFU/fish. On the other hand, Shoemaker and Klesius (1997) reported high mortalities in vaccinated fish regardless of the antibody titers. Igarashi and Iida (2002) reported the same results in Nile tilapia four weeks after challenging Nile tilapia with $1.8x10^7$ and $2.9x10^8$ CFU of two different strains of *E. tarda*. They concluded that formalin-killed cells enhanced the production of antibodies against *E. tarda* but did not give good protection. More so, Mekuchi *et al.* (1995) reported similar results on vaccination of Japanese flounder with formalin inactivated *E. tarda* where there was increased antibody titers but low protection. These results suggest that production of antibodies or increased antibody titers as a result of vaccination may not mean high protection levels. However, a number of studies have associated increased survival

rates/RPS with high antibody titers in vaccination experiments (Carrias *et al.*, 2008; Castro *et al.*, 2008).

Certainly, the increased survival (though relatively low) of the vaccinated fish than the non- vaccinated fish in this study can be attributed to antibody production following vaccination with two doses. In addition, vaccination in this study delayed mortality in the vaccinated groups following infection. There was also reduced severity of the clinical signs and pathological changes in vaccinated fish and some clinical signs such as exophthalmia, dorsal fin base erosion and cataracts were not observed in vaccinated fish but were present in non-vaccinated groups. This is also evidenced by the fact that there was a significantly high bacterial load counts in tissues of the non-vaccinated fish than in their vaccinated counterparts.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

(i) From the results, it can be concluded that; The *E. tarda* strain used in this study was moderately virulent according the determined LD_{50}. The isolate used in this study was formerly isolated from Nile tilapia and literature says that *E. tarda* isolated from tilapia are less virulent compared to those isolated from catfish.

(ii) Intraperitoneal route of injection used to expose fish to infection in this study was effective as the effects of the infection including clinical signs and pathological changes were observed few days post exposure/infection. This study identified, characterized and described disease clinical signs caused by *E. tarda* but did not score the clinical signs or quantify them per individual fish to ascertain their prevalence and statistical differences in different fish treatment groups.

(iii) Results from the study showed that there was high bacterial load in the internal tissues of liver, kidney and spleen with kidney being the most affected. It can therefore be concluded that the pathogen targets these tissues and it can be successfully isolated from these tissues if at all the fish has the pathogen. Some fish in both median lethal dose and vaccination/challenge experiment survived throughout the experimental period and beyond and some did not show clinical signs and pathological changes. This indicated that fish can develop immunity against Edwardsiellosis and recover fully. Also, the pathogen can be found in both symptomatic and asymptomatic fish.

(iv) It can also be concluded that vaccination of fish with formalin killed *E. tarda* can induce immunity and offer protection to the fish against the infection as it enhanced development/production of specific antibodies. In this experiment, the

reported results of protective efficacy were determined for four weeks post challenge.

6.2 Recommendations

(i) Further studies need to be done focusing on different age groups of fish and using one and several booster doses of the vaccine for a long period of time to ascertain which age group is more protected by a certain concentration and doses for a certain period of time.

(ii) Formalin-killed *E. tarda* cells were used as a vaccine in this study to assess the protective efficacy. I recommend that further studies of the same nature should be done to compare the protective efficacy of different vaccines such as outer membrane proteins, lipopolysaccharides and others to further evaluate the best vaccine that can be used in advanced and applied vaccine development.

(iii) *E. tarda* strain KOCT4' that was used in this was formerly isolated from Nile tilapia, the literature indicates that *E. tarda* strains isolated from catfishes are more virulent than those isolated from other fish species. This calls for studies that target on comparing the virulence of *E. tarda* strains isolated from catfishes with those isolated from other species. Also, this study used Nile tilapia being the most cultured and valued species in Uganda and East Africa, catfish should be considered for the same study as it is the second cultured species.

(iv) The pathogen can be isolated from both symptomatic and asymptomatic fish. Studies on screening and prevalence should not only consider symptomatic fish as they may not be conclusive enough.

(v) Awareness on proper fish husbandry, management practices and biosecurity should be created among farmers and handlers to avoid outbreaks and spread from facility to facility or farm to farm since the pathogenicity is enhanced by variations from

the normal environmental factors/water quality parameters. This can also prevent the spread of the pathogen from fish to humans since it is zoonotic.

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APPENDICES

Appendix 1: Analytical Profile Index 20 E results

ONPG	Negative
ADH	Negative
LDC	Positive
ODC	Positive
CIT	Positive
H2S	Positive
URE	Negative
TDA	Negative
IND	Positive
VP	Negative
GEL	Negative
GLU	Positive
MAN	Negative
INO	Negative
SOR	Negative
RHA	Negative
SAC	Negative
MEL	Negative
AMY	Negative
ARA	Negative

Appendix 2: Hemagglutination (HA) and Hemagglutination Inhibition (HI) plates