2020-06

Characterization of aeromonads and development of vaccine candidate from aeromonas hydrophila isolated from tilapia fish farms in Tanzania

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CHARACTERIZATION OF AEROMONADS AND DEVELOPMENT OF VACCINE CANDIDATE FROM *AEROMONAS HYDROPHILA* ISOLATED FROM TILAPIA FISH FARMS IN TANZANIA

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Life Sciences of Nelson Mandela African Institution of Science and Technology

Arusha, Tanzania

June, 2020

ABSTRACT

Tanzania produces less than half of the country annual demand in fish. Therefore, there is an urgent need to produce more fish, particularly through fish farming. However, aeromonads infections cause major lose in aquaculture worldwide and especially in developing countries, including Tanzania, lacking advanced capacity for fish disease control and prevention. Poor fish farming management practices, lack of data on prevalence, emergence of resistances to commonly used drugs, drug residues and limited capacity to control aeromonads bacterial infections emerged as major health problems in fish farming in Tanzania. This study aimed to characterise the aeromonads species circulating in fish farms and then develop a monovalent vaccine candidate from selected prevalent aeromonads specie for supporting tilapia fish farming improvement in Tanzania. A cross sectional study was conducted in Ruvuma, Mbeya, Iringa and Kilimanjaro regions between February 2017 and October 2018. A questionnaire was administered to 32 selected fish farmers to explore their knowledge on pond, fish health and diseases management practices. The results showed that the selected farmers had limited knowledge on pond, fish health and disease management practices. On-farm training on the same to these farmers would improve their knowledge. A total of 816 whole fish samples were aseptically collected from these 32 fish farms to detect and identify aeromonads using molecular methods in order to establish the prevalence and characterise their virulence properties. The overall prevalence of 24.6% was recorded. Seventy five percent of the isolates had virulence genes of varying combinations and the in-vivo study showed high mortality (98.3%) to isolates with more virulence genes indicating their capacity to establish disease in a favourable environment. The *Aeromonas hydrophila* strain TZR7-2018 was selected and attenuated using a novel thermo-continuous sub-culturing method to develop a vaccine candidate. The experimental study was carried out to assess its protective efficacy. The results showed that the vaccine candidate had acceptable protective efficacy of 82.3% and 71.4% when given through intraperitoneal injection (IP) and immersion (IM); respectively. To the best of my knowledge this study reports the development of thermo-attenuated and stabilized *A. hydrophila* vaccine candidate for the first time in Tanzania or elsewhere.

DECLARATION

I, **Alexanda Mzula** do hereby declare to the Senate of the Nelson Mandela African Institution of Science and Technology that this thesis is my own original work and that it has neither been submitted nor being concurrently submitted for degree award in any other institution.

Alexanda Mzula

Name of the candidate	**Signature**	**Date**

The above declaration is confirmed

Gabriel M. Shirima

Supervisor 1	**Signature**	**Date**

Philemon N. Wambura

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Robinson H. Mdegela

Supervisor 3	**Signature**	**Date**

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CERTIFICATION

This is to certify that this thesis titled "Characterization of aeromonads and development of vaccines from *A. hydrophila* isolate from tilapia fish farms in Tanzania" is written by Alexanda Mzula under the supervision of Prof. Philemon N. Wambura & Prof. Robinson H. Mdegela (from SUA) and Dr. Gabriel M. Shirima from NM-AIST. I approve the thesis for submission to the NM-AIST Senate for award of the PhD degree in Life Science (Health and Biomedical Sciences).

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ACKNOWLEDGEMENTS

I'm grateful to the African Development Bank (AfDB) for the sponsorship they provided to me that made this research successful and the Sokoine University of Agriculture (SUA) for permitting me to pursue a PhD.

I would like to extend a personal and special thanks to my supervisors, Professor Philemon N. Wambura & Professor Robinson H. Mdegela of the College of Veterinary Medicine and Biomedical Sciences at (SUA) and Dr. Gabriel M. Shirima of School of Life Science and Bioengineering at NM-AIST; The supervisors have played a great role in guiding, encouraging and supporting me during the entire period of my research.

Grateful appreciation is extended to members of staff in the School of Business, Studies and Humanities (BUSH) at NM-AIST for contributing to stimulating lectures in very important courses that have grounded me in research, innovation and entrepreneurship. I would like to mention Dr. Liliane Pasape, Dr. Eliaman Laltaika, Dr. Ahmad Kipacha, Mr. Emmanuel S. Mollel, Mr. Gabriel C. Malima and Mr. Baraka Shuma for the same.

Special mention goes to Mr. Beatus Lyimo who is the laboratory manager at NM-AIST and Mr. Jeremiah Mugusi, a Laboratory Technologist at Microbiology section in the Department of Veterinary Microbiology, Parasitology and Biotechnology, SUA. Their support was crucial to my success because they were very happy to see me practice and conduct laboratory activities on my own and they didn't hesitate to help me when I was in need of their support; I say thank you all.

I'm deeply grateful to my fellow researchers and students for their cooperation shown during the period of my study; Ms. Elizabeth Gachanja and Dr. David D. Maleko. The former showed great studentship cooperation when we were in a molecular biology laboratory and the later contributed a lot on knowledge about the analytical software and spent his time in coaching of the same.

My gratitude and special love should go to my wife Sophia P. Kumburu and daughters; Agness A. Mzula, Anna A. Mzula, Abriana A. Mzula and Ancy A. Mzula for their prayers, support, sacrifice and patience throughout the study period. My beloved parents, relatives and neighbours are gratefully thanked for their sacrifice, encouragement, prayers, and enthusiastic support.

Above all, I’m thankful to my Rock and Redeemer, the Almighty God in whom we live and prosper.

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AAT	Aquaculture Association of Tanzania
BLAST	Basic Local Alignment Tool
DFAT	Direct Fluorescent Antibody Test
ELISA	Enzyme Linked Immune-Sorbent Assay
EMA	European Medicinal Agency
ICT	Information and Communication Technology
IFAT	Indirect Fluorescent Antibody Test
IHNV	Infectious Haemopoietic Necrosis Virus
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry
MAR	Multiple Antimicrobial Resistances
MAS	Motile Aeromonads Septicemia
MHC	Major Histocompatibility Complex
MLF	Ministry of Livestock and Fisheries
MLFD	Ministry of Livestock and Fisheries Development
MLPA	Multiple Locus Phylogenetic Analysis
LPS	Lipopolyssacharids
NFTI	National Fisheries Training Institute
NM-AIST	Nelson Mandela African Institution of Science and Technology
OIE	World Organization for Animal Health
OMPs	Outer Membrane Proteins
RPS	Relative Percent Survival
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SUA	Sokoine University of Agriculture
TAFIRI	Tanzania Fisheries Research Institute
TCRA	Tanzania Telecommunication Regulatory Authority

UDOM	University of Dodoma
UDSM	University of Dar es Salaam
EU	European Union
URT	United Republic of Tanzania

CHAPTER ONE

INTRODUCTION

1.1 Background information

Fish contributes and serves as a quick source of animal protein worldwide (Food and Agriculture Organization of the United Nations [FAO], 2016a). In Tanzania fish contributes more than 27% of the animal proteins in take (Béné & Heck, 2005). In several African and Asian countries, the impact and contribution of fish to improved food security may be greater than it was before (United States Agency for International Development [USAD], 2016). This is because fish culture is practiced in an eclectic scale, from small farming with the low initial cost, semi-intensive to intensive farming. Tanzania in particular practices fish farming in a form of subsistence aquaculture, hence contributing directly to household food security (Ministry of Livestock and Fisheries Development [MLFD], 2013). Fish farming practices not only contribute to providing animal protein but also these farms stand as a way of erosion control, water supply for livestock, fire control, irrigation in vegetable gardens, swimming, picnicking and wildlife enhancement. It is noteworthy that while farmed fish serve to provide animal protein and income to households; in Tanzania, almost 80% of the fish supplied for food and cash are obtained from the seas and lakes available in the country. Only limited number of fish farms contributes to national income in the fisheries industry.

In Tanzania, there are about 20 000 public and private ponds that produce approximately 10 000 tones of fish per year which is not enough to meet the consumers' demand (FAO, 2009; MLFD, 2013). Fish farming is increasingly growing in the country though at a low pace and it is faced with several challenges including limited or poor fish research, fish diseases-diagnosis, treatment and control (MLFD, 2013). Bacterial fish infection and diseases are the major problems to fish health and farming industry in the world (Pridgeon & Klesius, 2012). There are several genera and species of bacteria which cause economic loss to the fish farming industry; some of the bacteria are adapted to warm freshwater or cold freshwater while others are found in marine water. In freshwater fish, *A. hydrophila* is one of the most important pathogen (Pridgeon & Klesius, 2012). Other *Aeromonas* species include: *Aeromonas caviae, Aeromonas veronii, Aeromonas sobria,* and *Aeromonas dhankesis*. *Aeromonas* infection in fish causes a hemorrhagic septicemia and it has been reported worldwide to cause mortality of up to 100% in cultured fish (Paniagua *et al*., 1990).

Aeromonads are ubiquitous bacteria of the aquatic environment and therefore, serve as opportunistic and even primary pathogens. Antibiotic resistance in aeromonads has been reported by several researchers. Shayo *et al*. (2012) conducted phenotypic virulence and antimicrobial susceptibility study on *Aeromonas* spp isolated at the Mtera hydropower dam in Tanzania and found that the bacteria were susceptible to the antibiotics tested. Another study conducted by Shah *et al*. (2012) showed that *Aeromonas* spp isolated from farmed fish in Tanzania have developed resistant against several antibiotics where as 90% demonstrated resistance between one and eight of the nine tested antibiotics.

Biosecurity measures, good pond management practices, disease treatment and vaccination are of paramount importance towards sustainable aquaculture. In Tanzania the first two are moderately implemented by fishpond farmers. Whereas the last two practices are commonly practiced in developed countries, but in developing countries, Tanzania they are minimal or not done at all. A study by Chenyambuga *et al*. (2014) at Mbarali, Mbeya, revealed that fish farmers had little knowledge of pond management practices. Following the copiousness nature of aeromonads, and current concern on antimicrobial resistance due to; constantly use of antibiotics in treatment, prophylaxis and integrated aquaculture and antimicrobial residue in fish products, extra and alternative approach in control of aeromonads diseases in fish farms is needed for the sustainability of the industry. Vaccination is the novel approach that need to be combined with the proper biosecurity measures and good fish farming management practices for control of aeromonads disease to improve fish health and production (Feng *et al*., 2017; Gong *et al*., 2015; Marsden *et al*., 1998). Several types of vaccines have been used in controlling bacterial fish diseases. These includes: killed vaccines, live attenuated vaccines, recombinant live vaccines, recombinant protein vaccines and DNA vaccines. While some of the recombinant vaccines and DNA vaccines against *A. hydrophila* in particular, have been licensed, some are under trials (Ma *et al*., 2019). Despite some added advantages these vaccines have over the conventional killed and live attenuated vaccines, their availability and accessibility in developing countries, like Tanzania, are costly. Besides most of them have been developed to serve the purpose of high value fish species such as common carp and salmons and also have been developed from antigens appropriate to the regions of origin. Locally developed vaccines based on local antigens would be the most appropriate because will provide appropriate protection. In addition, the vaccine is cheap in terms of cost to farmers and easily accessed.

1.2 Statement of the problem

Tanzania produces less than half of the country annual demand in fish. Therefore, there is an urgent need to produce more fish, particularly through fish farming. However, aeromonads infections cause major lose in aquaculture worldwide and especially in developing countries, including Tanzania, lacking advanced capacity for fish disease control and prevention. Therefore, poor fish farming management practices, lack of data on prevalence, emergence of resistances to commonly used drugs, drug residues concern as well as limited capacity to control aeromonads bacterial infections emerged major health problems in fish farming in Tanzania.

1.3 Rationale of the study

This study was carried out in a sense that development of local vaccine could help to resolve some of the challenges above as currently no live commercial aeromonads' vaccines are available for wide coverage worldwide partly due to vaccine strain specificity. Development of local vaccine against aeromonads disease is very important for supporting fish farming improvement in Tanzania in a cost-effective and environmentally friendly manner while ensuring production of antibiotic residue free fish and fish products by smallholder farmers.

In view of the above, this research was focused on assessment of management practices and isolating, characterizing and attenuating the selected isolate of aeromonads to have vaccine candidate against aeromonads infection in Nile tilapia fish farms in Tanzania.

1.4 Objectives

1.4.1 General objective

To characterise the aeromonads species circulating in fish farms and then develop a monovalent vaccine candidate from a selected prevalent aeromonads specie for supporting tilapia fish farming improvement in Tanzania.

1.4.2 Specific objectives

(i) To explore on the knowledge and awareness of tilapia fish farmers on pond, fish health and disease management practices.

(ii) To establish the prevalence of aeromonads infection in tilapia farms in Tanzania.

(iii) To carry out phenotypic and molecular characterization of putative virulence of aeromonads isolates obtained in Tanzania.

(iv) To perform attenuation, immunogenicity and efficacy studies from selected *A. hydrophila* isolate.

1.5 Research questions

(i) What is the level of understanding of fish farmers in the selected areas of study on pond, fish health and disease management practices?

(ii) What is the magnitude of aeromonads infection in tilapia farms in the selected regions of Tanzania?

(iii) Do these circulating and more prevalent Aeromonas have virulence attributes potential to establish diseases when environmental situation allows?

(iv) Can the isolates be successfully attenuated and serve as a vaccine manage MAS?

1.6 Significance of the study

This study was designed to contribute to the body of knowledge on significant understanding of the prevalent circulating aeromonads species and their virulence characteristics in tilapia fish farms in Tanzania. The study was also done to contribute solutions for preventing and controlling aeromonads diseases in tilapia fish farms, which was highlighted as one of a major constrains in aquaculture (National fisheries policy of 2015). This was done through development of a vaccine which is going to be useful for fish farming industry. The outcomes of this study are envisaged to be beneficial to fish farmers through improved fish production and hence improved household food security, nutrition and finally national economy.

1.7 Delineation of the study

The study is delimited to the following:

(i) The work focused on characterizing the circulating aeromonads in tilapia fish farms in four regions namely; Ruvuma, Iringa, Mbeya and Kilimanjaro and development of a vaccine candidate from a selected isolate of *A. hydrophila* for control of MAS in Tanzania

(ii) The laboratory experimental study on attenuation and testing for vaccine immunogenicity and efficacy generated useful results. The possibility to conduct shelf life, reversion to virulence study and assessment of the induced mutation at genomic level would generate useful information that could enhance field trial.

(iii) Two species of *Aeromonas* were prevalent, *A. hydrophila* and *A. veronii*. Possibility to develop vaccine candidate from selected *A. veronii* could facilitate formulation of a bivalent vaccine against the two etiological agents.

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview of fish farming in Tanzania Mainland

Aquaculture in Tanzania mainland started in the late 1920s, after the introduction of trout from Scotland to the streams in the Kilimanjaro and Mbeya regions (Balarin, 1985). In the 1950s, fish farming started using experimental ponds at Korogwe (in Tanga Region) and Malya (in Mwanza Region) (FAO, 2012; Nilsson &Wetengere, 1993). During those times, tilapia fingerlings were supplied from wild stocks in Lake Victoria and the Congo and Pangani Rivers (Rothuis *et al*., 2014). Later, Nile tilapia (*Oreochromis niloticus*) fingerlings were supplied by the Hombolo Center to all over the Tanzania Mainland (Coche *et al*., 1994). These fingerlings were distributed by the government to fish farms (both public and private) as well as to public water reservoirs (Madalla, 2008). Tanzania Mainland is dominated by the tilapia species of the genus *Oreochromis*and *O. niloticus* has become a predominant cultured species because of its superior growth characteristics (Chenyambuga *et al*., 2014; Mdegela *et al*., 2011) (Fig. 1). Other species include; trout, and catfish in freshwater, and milkfish and prawns in mariculture (United Republic of Tanzania [URT], 2016).

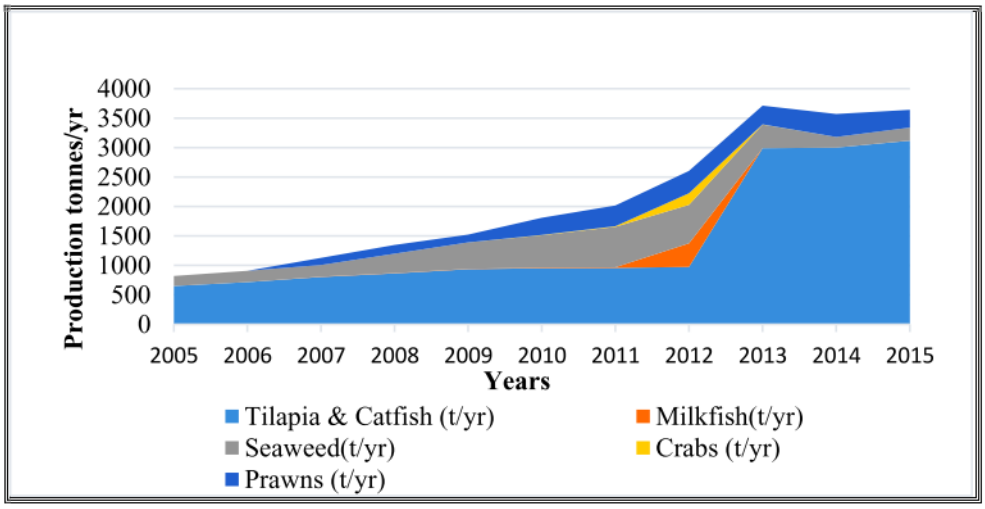


Figure 1: Trend in aquaculture production with regard to fish species (FAO, 2016b)

Aquaculture development in Tanzania Mainland has been moving with changes in organizational structure, administration, and regulatory instruments. Up to the 1990s, the industry was handled under the Ministry of Tourism, Natural Resources and Environment in the Fisheries Division (Coche *et al*., 1994). Later it passed under several regulatory

authorities (Ministries) following political changes and decisions. These regulatory ministries were; Ministry of Agriculture, Ministry of Agriculture, Livestock and Fisheries, Ministry of Livestock and Fisheries Development and now the Ministry of Livestock and Fisheries. In the last two ministries, aquaculture operates administratively under the established Directorate of Aquaculture Division (Shoko *et al*., 2011).

The National Fisheries Policy of 2015 is a review of the Fisheries policy of 1997. The former was published by the Government to boost the development of fisheries and aquaculture sectors. The policy objective is to develop the sectors to significantly contribute to improving food security and nutrition and promote the national economy. The policy is executed by key documents; the Fisheries Sector Development Programme, Fisheries Management Plans for the prawn, octopus, tuna and small-scale artisanal pelagic fisheries and the National Aquaculture Development Strategy (URT, 2015). Legal and regulatory frameworks related to aquaculture are implemented through the enacted Fisheries Act no. 22 of 2003, which is an amendment of the Fisheries Act no. 6 of 1970. In addition, other related acts and regulations have been put in place to complement the Fisheries Act, including the Tanzania Fisheries Research Institute (TAFIRI) Act of 2016. The move towards the establishment of independent Aquaculture Development Act of 2019 is in the final stages to be published in the Government Gazette. In the proposed Act, matters related to diseases such as notification and biosecurity measures have been put in place under the section of Health and Welfare of Aquaculture Organisms.

Aquaculture in Tanzania Mainland is still at its infant stage but it has enormous potential for expansion (Mdegela *et al*., 2011) as the demand is high and production is increasing (Fig. 2). However, fish farming in the country was traditionally practiced by smallholder farmers who owned small fish farms of up to an average size of 10 m x 15 m (150 m^2). Recently, large-scale fish farms are being opened to attract industrial investment in the country and this is demonstrated by Chenyambuga *et al*. (2014) in their study at Mvomero and Mbarali districts with an increase in average pond size of about 345 m^2 and 631 m^2 ; respectively. Such pond sizes are bigger than the size of 150 m^2 reported by (FAO, 2012) and 300 m^2 reported by Kaliba *et al*. (2006) from Southern and Northern Highlands. Tanzania is currently estimated to have a total of more than 20 000 freshwater fish ponds (Fig. 3) distributed across the mainland (Rukanda, 2018).

In Tanzania Mainland, fish ponds have been distributed and concentrated in certain geographical regions because of factors such as water availability especially from rivers, suitable land for fish farming, awareness and motivation within the community on the economic benefits of fish farming. The industry is subjugated by integrated freshwater fish farming and most of the farmers own an average of one small fish pond. It is still subsistence and a part time operation characterized by household ownership. Fish farming is largely practiced in five regions in Tanzania each having more than 1000 fish ponds. The regions are: Ruvuma (4942), Iringa (3137), Mbeya (1176) and Kilimanjaro (1660) (Fig. 4). Production has been low due to small pond size coupled with poor management but it kept increasing. In 2013; 3600 ton of fish were produced and currently, the production is estimated to be over 4000 ton per year (Rothuis *et al*., 2014; Rukanda, 2018; Ubwani, 2018). This increase explained by the fact that fish farming is now practiced widely in Tanzania, from small-scale ponds to large ones and the farming systems are moving from extensive normal operation (low input demand) to intensive farming (high input demand). However, the industry is largely still operating at subsistence level. Tanzania Mainland produces 336 821 ton of fish annually, less than the demand of 731 000 ton, a deficit of approximately 480 886 ton (Mirondo, 2017; Nachilongo, 2019).

Fish farming practices not only contribute to providing animal protein but also stand as a way of erosion control by conserving sloping land surrounding the pond against rainfall erosion, livestock watering, fire control, irrigation, picnicking, swimming and wildlife enhancement (Wetengere, 2010). In addition, Wetengere (2010) in his study revealed that fish farmers in the study area even acquired political positions because of their involvement in fish farming.

It is evident that the expansion and growth of the aquaculture industry will occur consequent to the efforts made by the public and private sectors to improve fish farming in the country. This will demand improved fish research, fish diseases-diagnosis, treatment and control (Akoll & Mwanja, 2012; MLFD, 2013) as these may become major challenges to sustainable aquaculture development in the country in the future (URT, 2015).

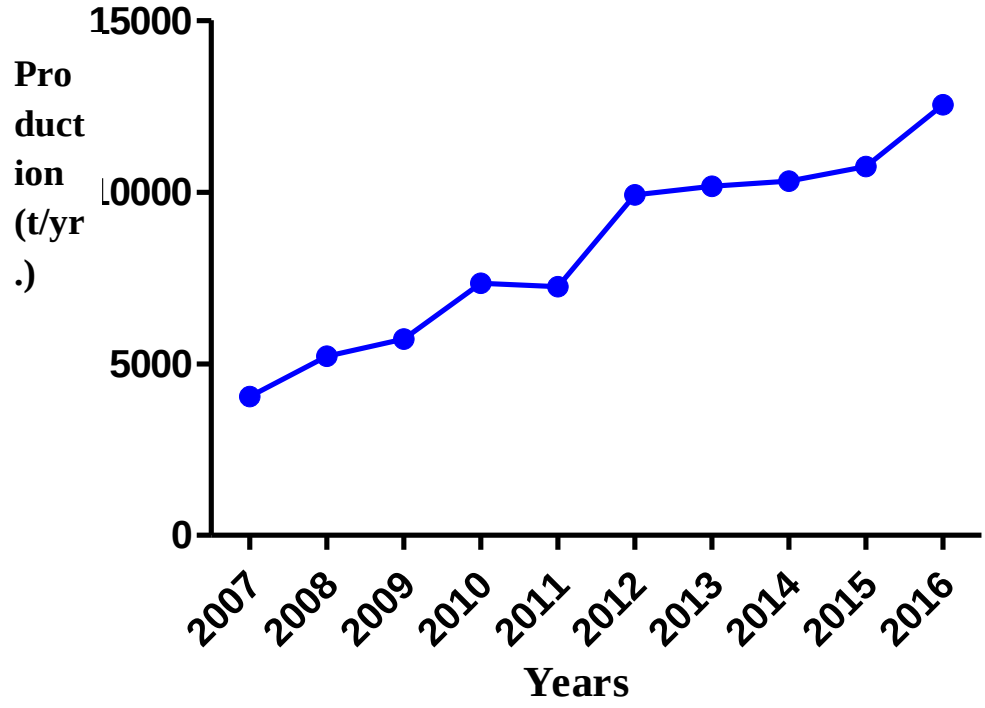


Figure 2: The trend in overall aquaculture production per year in Tanzania (FAO, 2018a)

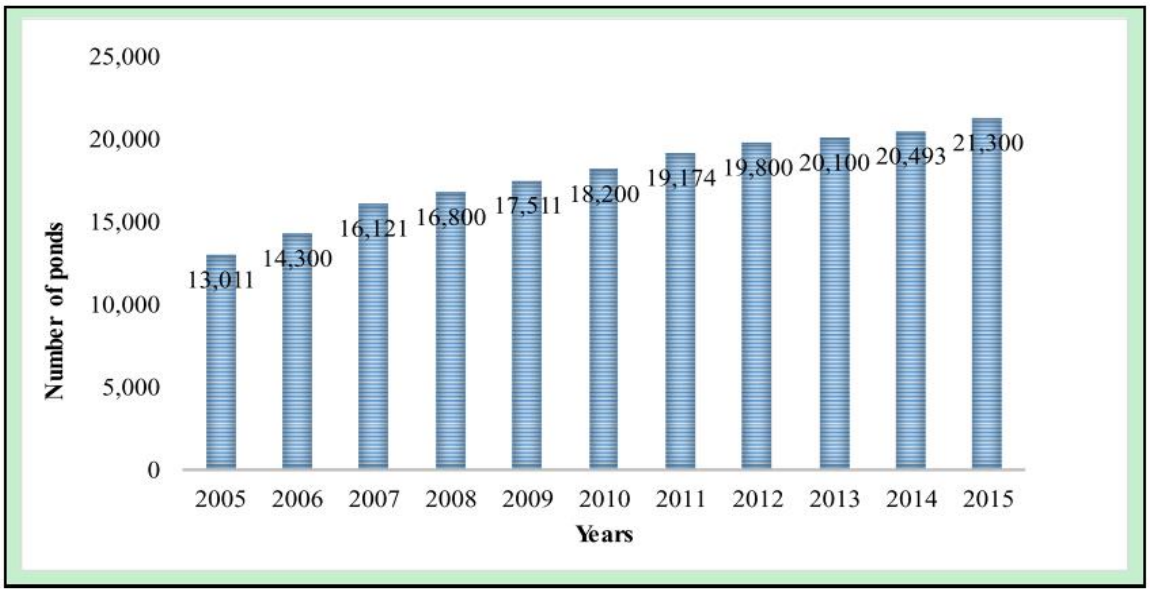


Figure 3: Synopsis of number of ponds since 2005 to 2015 (Rukanda, 2018)

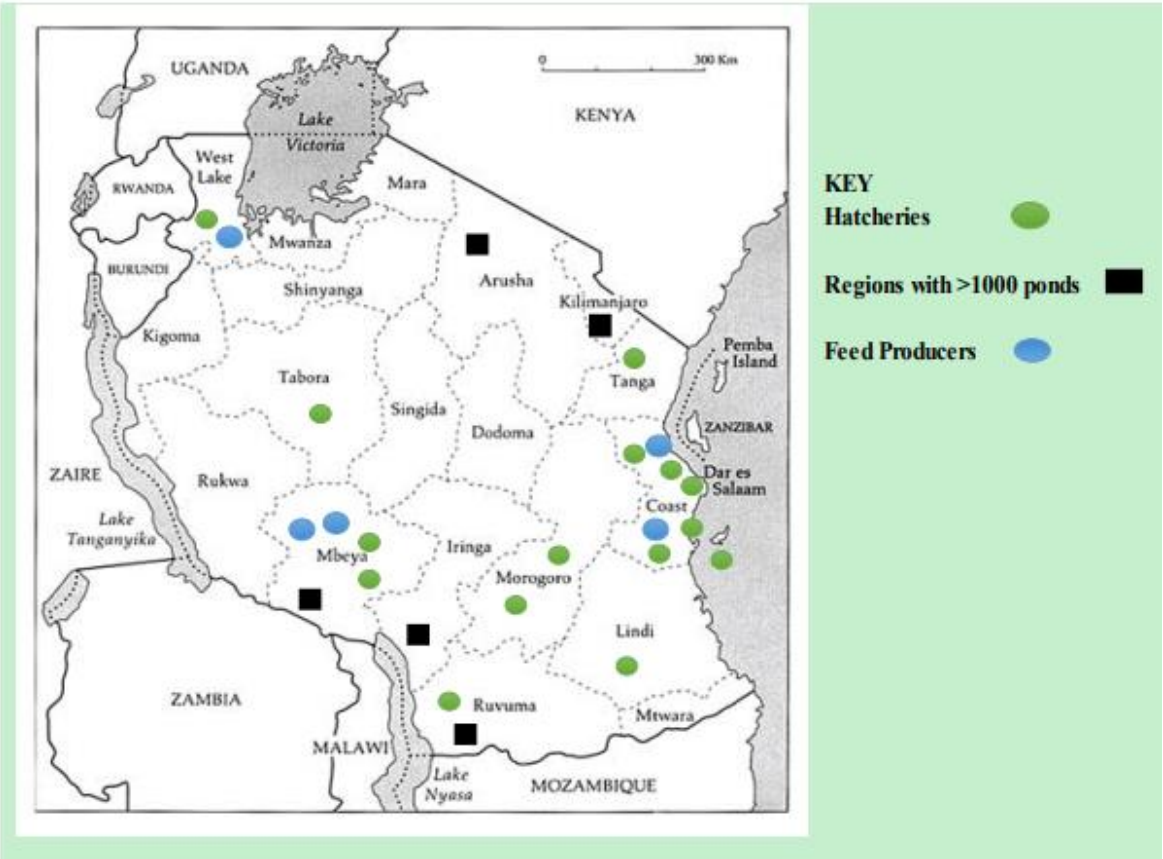


Figure 4: The map of Tanzania mainland showing major regions practicing aquaculture production (Rukanda, 2018)

2.2 Challenges and prospects to sustainable aquaculture development in Tanzania

2.2.1 Farmers' knowledge on fish pond management practices and biosecurity measures

Biosecurity measures, good pond management practices topped with other fish disease control methods such as vaccination are important especially during this era of antimicrobial resistance. While these are greatly implemented in developed countries, in developing countries like Tanzania efforts must be made to provide knowledge to fish farmers on biosecurity measures and pond management practices and create awareness on potential risks of bacterial diseases if the same are not employed. A study conducted by Chenyambuga *et al*. (2014) revealed that fish farmers had little knowledge of biosecurity measures and pond management practices. From their finding, 25% of fish farmers from the study site sourced fingerlings and fries from neighbours and from little known Non-Governmental Organisation (NGOs). However, Rukanda (2018) pointed out that the reason for the collection of the fries and fingerlings from these untrusted sources is championed by the low availability of well

managed hatcheries across the country augmented by lower production than the demand. For sustainability of the industry, it is therefore accentuated that, efforts should be made by appropriate authorities to strengthen extension services, such as increasing the number of on-farm training and workshops to fish farmers in Tanzania, to effectively use biosecurity measures and proper pond management practices such as collection of fingerlings from trusted sources and well-managed hatcheries; monitoring and assessing the quality of pond water, disinfecting equipment used in handling fish, improving pond workers hygiene, reducing stress level in fish and restriction of fish movement from one body of water to another something which is advocated by legislation but it lacks follow-up policies. All these are possible if extension and advisory services are adequate.

2.2.2 Extension and Advisory Services in Tanzania

Extension and advisory services are crucial for sustainable aquaculture industry, however, due to inadequate number of extension staff in this field, extension services do not reach the majority of fish farmers (URT 2011; Mlozi *et al*., 2012). Ragasa *et al*. (2016) reported that the country is having 8000 extension agents; however, the demand is projected to be greater than 20 000 outreach agents. In addition, even those extension staff available and expected to deliver the skills and knowledge to fish farmers, are often hindered by a long distance to and transport problems. The use of Information and Communication Technologies (ICTs) has made a substantial impact towards improved extension and advisory services and hence revolutionized agriculture in India, Ghana and South Africa (Tarimo & Sanga, 2017). It is, therefore, believed that if strategies are made by the extension centres and fish farmers are motivated to use ICTs in seeking for help on good pond management practices and fish health management, extension services will be improved. The use of ICTs in Tanzania for outreach services to fish farmers is also advocated by Tarimo and Sanga (2017) as over 40 million Tanzanians possess mobile phones (Tanzania Communications Regulatory Authority [TCRA], 2018). Nevertheless, social platforms such as WhatsApp and Skype will facilitate interactive communication between the fish farmers and extension officers, therefore, farmers should be encouraged to form and join those networking platforms for quick access to information.

2.2.3 Bacterial diseases of fresh water farmed fish

Infectious diseases are a major concern in fish farming practice and can broadly be categorized as parasitic, bacterial, viral and fungal. Diseases are usually linked to high morbidity and mortality, resulting into negative impacts for farmers, consumers and the environment (Hasan *et al*., 2013; Toranzo *et al*., 2005). The microorganisms which cause these diseases range from primary pathogens to opportunistic microorganisms (Richards & Roberts, 1978). Bacterial infection in fish farms is accelerated by a number of factors including variation in physical-chemical parameters of pond water, such as increased turbidity, temperature, salinity, pH, water conductivity and low dissolved oxygen (FAO, 2018; Jacobs & Chenia, 2007; Najiah, 2014). These environmental factors induce stress to fish and therefore fish can easily succumb to infections. Due to the current nature of aquaculture in Tanzania, the industry has to deal with the growing problem of bacterial diseases (Romero *et al*., 2012) by putting proper strategies on how to provide knowledge and skills on proper pond management practices and how to address fish diseases once outbreaks occur.

Globally, more than 13 bacterial genera have been reported to cause bacterial diseases in the aquaculture industry. Of these, five genera have been known to cause infection in freshwater farmed fish in Tanzania. In this chapter, bacterial diseases affecting freshwater farmed fish have been discussed with the goal of assessing available knowledge and to contribute towards diagnosis and control strategies for these infections. These important fish pathogens involved include: *Aeromonas*, *Pseudomonas*, *Edwardsiella*, *Flavobacterium* and *Streptococcus*.

(i) *Edwardsiellosis*

Edwardsiellosis is one of the important bacterial septicemic diseases in farmed fish. It is caused by a gram-negative bacterium, *Edwardsiella* spp, (Nadirah*et al*., 2012). The disease is becoming a serious problem in catfish in Tanzania as it emerges as secondary infection following lesions developed by the lack of Vitamin C. Catfish are particularly vulnerable when farmed in ponds cast out of concrete. Supplementing vitamin C in feed would minimize the problem. *Edwrdsiella tarda* was isolated during a 2016 outbreak in catfish in Dakawa, Morogoro (E. D. Mwega, personal communication, April 8, 2016). Although no reports on the occurrence of Edwardsiellosis in tilapia in Tanzania have been published so far, further investigation is required in this host.

(ii) *Flavobacteriosis*

Flavobacterium species are another cause of devastating bacterial disease in Tilapia farms and the disease is said to be highly contagious, especially to fingerlings resulting in high mortality (Intervet, 2007). Flavobacteria are gram-negative, rod-shaped bacteria that save as both opportunistic and primary pathogen of fish in freshwater. *Flavobacterium columnare, F. johnsoniae*, *F. Branchiophilum* and *F. psychrophilum* are the most known pathogenic *Flavobacterium* spp (Austin & Austin, 2016; Nematollahi *et al*., 2003; Pridgeon *et al*., 2013; Starliper, 2011). It is noteworthy that, *F. indicum, F. hydatis, F. aquatile, F. succinicans,* and others are also reported to be opportunistic pathogens of fish (Bernardet & Grimont', 1989). *Flavobacterium* spp infections have been reported to occur in tilapia ponds in the Lake Victoria, Mwanza and at Morogoro; respectively (Mwega *et al*., 2019). Despite the fact that no outbreak of Flavobacteriosis has been reported in Tanzania, a further survey to cover a large area is vital.

(iii) *Streptococcosis*

This disease is caused by *Streptococcus* spp in several freshwater cultured fish species such as tilapia. The most pathogenic species affecting fish is *Streptococcus iniae*. *Streptococcus* spp are gram-positive bacteria, cocci in shape arranged in chains. *Streptococcus agalactiae* is another species which is reported to affect tilapia and is linked to the intensive culturing of broodstock (Hernández *et al*., 2009). Streptococcosis can cause mortality of up to 50-70% in tilapia farms (LuMaiXin, 2010) leading to dramatic economic loss results from outbreaks (Fawzy *et al*., 2014).

Streptococcosis has been reported to occur in fish farms in Africa including Egypt (Fawzy *et al*., 2014), but in Tanzania *Streptococcus* spp has been recovered in apparently healthy tilapia fish in few farms (unpublished data), however the disease outbreaks have never been reported. E extensive surveillance is recommended.

(iv) Red skin disease

Pseudomonas spp is the aetiological agent of red skin disease and affects a wide range of freshwater fish species, including tilapia. *P. anguilliseptica* is believed to be one of the most significant pathogens for cultured fish (Mastan, 2013). Other important Pseudomonas species found in fish cultures are *P. aeruginosa* and *Pseudomonas fluorescens* which are ubiquitous

in freshwater ecosystems. Shayo *et al*. (2012) reported *Pseudononas* spp to cause ulcerative diseases and haemorrhagic septicaemia in tilapia in Mtera hydropower Dam in the Iringa region, Tanzania. Due to the presence of Pseudomonas pathogens in the environment, further surveillance over a larger part of the country is needed.

(v) Motile Aeromonas Septicemia (MAS)

Aeromonads disease outbreaks are now becoming a common phenomenon in farmed fish worldwide (Bebak*et al*., 2015; Harikrishnan & Balasundaram, 2005). Aeromonads are gram-negative, rod-shaped facultative bacteria which cause various diseases in fish also known as haemorrhagic septicemia, dropsy, epizootic ulcerative syndrome, haemorrhagic enteritis, and red body disease of fish (Abdelhamed *et al*., 2017; Igbinosa *et al*., 2012). These bacterial species are ubiquitous of the aquatic environment but now have become a challenging pathogen of cultured fish (Chaix *et al*., 2017; De Jagoda *et al*., 2014; Janda & Abbott, 2010; Joseph *et al*., 2013). Nile tilapia (*Oreochromis niloticus*) is one among a wide range of fish species infected by aeromonads (Baumgartne *et al*., 2017). Five important Aeromonas species are well known to cause disease in freshwater farmed fish. These are *A. hydrophila*, *A. caviae*, *A. veronii*, *A. sobria* and *A. dhakensis* (Cipriano *et al*., 2001; Skwor *et al*., 2014). *Aeromonas. hydrophila* is the main cause of disease outbreaks in farmed fish, contributing to food insecurity and economic losses worldwide (Aboyadak *et al*., 2015; Baumgartner *et al*., 2017). It has been well noted that semi-intensive and intensive fish farming coupled with poor fish pond management can result in aeromonads disease outbreak in a farm (Najiah, 2014). Since the 2000s there were severe mortalities and morbidities of cultured freshwater fish in several African countries including Egypt (Beaz-Hidalgo *et al*., 2010). These cases were most seen in cultured Nile tilapia (*O. niloticus*) and *A. hydrophila* had a prevalence of up to 70% of fish examined. In Tanzania, the outbreak of disease characterized by haemorrhagic septicemia symptoms like those caused by *A. hydrophila* occurred in 2009 at Mtera hydroelectric power dam and caused substantial loss of *O. niloticus* in the dam (Shayo *et al*., 2012). After repeated outbreaks took place, the aetiological agent was then confirmed in 2012 at the same site (Shayo *et al*., 2012).

Despite the occurrence of few sporadic cases of unknown origin in the Southern Highlands of Tanzania, in which fish had clinical signs similar to haemorrhagic septicaemia (B. Tarimo, Personal communication, January 16, 2017), the prevalence of aeromonads infections in farmed fish is yet to be explored. To avoid losses that tilapia fish farmers might encounter,

information on the magnitude of infection and characteristics of the aetiological agent is vital. Further surveillance using a combination of diagnostic methods is required in farmed fish in different regions of Tanzania, especially in areas where sporadic cases have been reported to occur with similar symptoms to those displayed in the Mtera catchment area.

2.2.4 Surveillance systems and monitoring of fish bacterial diseases in Tanzania

In Tanzania, surveillance and monitoring of animal diseases is the mandate of the Ministry of Livestock and Fisheries (MLF). The focus and priority are several livestock diseases that affect a range of animals from cattle to poultry.

In comparison to the well-developed program of monitoring livestock diseases, little has been done on surveillance of bacterial and other diseases found in cultured fish (Akoll & Mwanja, 2012). The reason was that the aquaculture industry was not well established and bacterial diseases that meet the World Organization for Animal Health (OIE) notifiable criteria were rare. But recently, however, clustered cases and outbreaks have been occurring on a seasonal basis (Mwega & Tarimo, personal communication, 2017). Therefore, the Ministry of Livestock and Fisheries and the District Veterinary Officers should set up a national guideline and procedures for existing and emerging fish diseases surveillance. This should also involve research-based institutions such as Universities, Livestock Training Agencies and Research Institutes, the Tanzania Fisheries Research Institute (TAFIRI) and the National Fisheries Training Institutes (NFTI). The guidelines should cover sample size and sampling, tests and test procedures and measures to be taken when positive diagnosis occurs.

In addition, the guidelines should adhere to the Guideline for Aquatic Animal Health Surveillance established by the OIE. This is important because it will assist in disease documentation, monitoring and to control the disease at a level where it can be tolerated economically (Hastein *et al.*, 2001). It has been observed that several countries have developed their own surveillance systems to monitor prevalent fish diseases in addition to those listed by the Aquatic Animal Health Code (OIE, 2018a). Tanzania therefore, has a responsibility to establish which bacterial fish diseases are of particular economic and food security concerns in aquaculture farming systems.

Tanzania has several reasons contributing to the poor surveillance of fish diseases. A few are: (a) Absence of aquaculture Act before the establishment of the industry, (b) Inadequacy of funds to carry out fish disease research and implement a surveillance system, (c) Little

expertise in fish disease diagnosis, treatment and management options such as restriction of unregulated live fish movement from one water body to another (URT, 2015). These challenges need to be looked at in-depth as the aquaculture industry keeps growing and the risk of farmed fish bacterial diseases is becoming higher with time.

2.2.5 Fish disease diagnostic facilities and diagnostic methods

Specialized fish diagnostic laboratories recognized by OIE are lacking in Tanzania. Currently, fish disease outbreak investigation and diagnosis are largely performed by universities and public research organisations. However, these institutions approach the problem in an academic and a research oriented way (Akoll & Mwanja, 2012). Necessary efforts are needed to establish fish disease diagnostic facilities in Tanzania for sustainable regional aquaculture.

The methods which have been used to carry out the diagnosis of most fish diseases are those which are categorized as levels I and II diagnostic tools which include observation of fish and environment, clinical examination and gross pathology for level I. Farmers should be well trained on these simple methods to primarily identify diseases once they occur in their farms before further diagnosis to take place. However, under intensive aquaculture conditions, it is preferable to detect a bacterial pathogen in carrier fish to fasten the management option. Thus, sensitive, and specific system that are cost effective are required to detect pathogen carrier fish for surveillance and monitoring of fish populations. The advantages and weaknesses of diagnostic methods used in identifying fish bacterial etiological agents during surveillance and monitoring of fish diseases are hereby briefly reviewed.

(i) Clinical signs and symptoms

The clinical signs of an infection can be observed and applied as part of the surveillance of fish bacterial diseases. This is especially true in situations where the diagnostic test for a specific pathogen is not available or in situations of new pathogen emergence. These simple methods can be effective and serve as a diagnostic test if performed in a standardized manner (OIE, 2018b). However, one of the disadvantages of this method is that most of fish bacterial diseases pose similar clinical signs, hence complicating differential diagnoses.

(ii) Microscopy

Direct fluorescent antibody test (DFAT) has been used to detect antigens from fish specimens using labeled monoclonal antibodies (Lipton *et al*., 1998). However, this method is only efficient when substantial quantities of etiological agents are available in a clinical sample. While indirect fluorescent antibody test (IFAT) utilizes secondary labeled antibodies in the detection system, the disease is difficult to detect in early stages.

(iii) Histopathological examinations

Histopathological lesions can be used as a diagnostic tool for specific bacterial pathogens (Bernardet *et al*., 1990), however, this method does not directly target the pathogen itself but rather identifies the specific effects caused by the pathogen in the tissue or organ. Furthermore, this diagnostic technique can fail to provide the correct diagnosis of diseases with similar histopathological characteristics.

(iv) Serology

Rapid agglutination tests and Enzyme-linked immunosorbent assay (ELISA) are diagnostic techniques which apply the antigen-antibody binding principle and have been widely used in fish disease diagnosis (Kumar *et al*., 2014). While the first utilizes the particulate bacterial antigen, ELISA can detect either an antigen or antibody directly or indirectly while also quantifying it. These immuno-serological techniques can solve the problem of the diagnostic test mentioned above; it is also sensitive and specific to pathogen detection.

(v) Isolation of the cultured organism

Isolation of an etiological agent is the gold standard screening assay that can also be used in surveillance of bacterial fish diseases (Kumar *et al*., 2014; OIE, 2018b). This method can also be time consuming and some strains could be difficult to isolate. Furthermore, as it has been with other tests, some bacteria share phenotypic characteristics, making it difficult to distinguish between similar species.

(vi) DNA based diagnostic tests

Recently following advances made in genomics of fish pathogens, molecular biology has been a useful routine tool in diagnosis and epidemiology of bacterial fish diseases.

Molecular techniques such as conventional Polymerase chain reaction (PCR), Multiplex PCR, Real time PCR (RT-PCR), restriction enzymes, probe hybridization, western blotting, microarray and sequencing are increasingly being used as routine diagnostic and confirmation techniques in the primary stages of fish bacterial disease and during disease monitoring. These methods are more efficient when coupled with other diagnostic test such as isolation of the etiological agents. Additionally, these techniques are more sensitive and specific as they can discriminate fish pathogens down to species level and identify individual strains. Detection and diagnosis should occur as early as possible and should be conducted in a standardized manner to avoid contamination and false positives. The only challenge of these techniques is detection of etiological agents which are not viable in a host cell. Scientific efforts are being made to solve this hurdle. Soejima *et al*. (2008) managed to develop Ethidium monoazide (EMA) based PCR that discriminate live and dead cells.

Because most of the bacterial etiological agents have strain diversity, molecular detection and characterization of etiological agent is an important method and should be combined with other conventional methods when conducting surveillance in aquaculture (OIE, 2018b).

2.2.6 Disease treatment implementation and the need of a novel control strategy of bacterial diseases in fish culture

It is well established that treatment of bacterial fish diseases should be done using selected antibiotics recommended in aquaculture by the authorized government. In the USA for example, the Food and Drug Administration (FDA) has recommended three antibiotic preparations for aquaculture. These antibiotics are oxytetracycline, florfenicol and Sulfadimethoxine/ormetoprim (Romero *et al*., 2012). Fish farmers in Tanzania seem not to use antibiotics in aquaculture (Shah *et al*., 2012); because the majority of fish farmers have no prior knowledge of effective bacterial fish disease treatment. However, relatively high multiple antimicrobial resistant (MAR) index values were observed by Shah *et al*. (2012) in Tanzanian isolates from fish farms, indicating antibiotic contamination of the aquaculture facilities (Mdegela *et al*., 2011). Treatment guidelines should be put in place for management officers and aquaculturists tasked to assist fish farmers. Combined antibiotic treatment and vaccination using killed and or live vaccines after cost-benefit analysis would be the best approach.

2.2.7 Success and prospects towards enhanced fresh water farmed fish in Tanzania

Tanzania Mainland has a population of about 50 million people who depend on fish as the source of protein. But due to population expansion, wild fish from freshwater and marine capture fisheries are not enough to meet the growing demand for improving food security and household income. The effort which has been made by the government of Tanzania through the Ministry of Livestock and Fisheries has now started to revolutionize aquaculture. These efforts include the establishment of the National Fisheries policy in 2015, the Directorate of Aquaculture Division and the proposed aquaculture development Act. Universities have been able to build capacity in terms of human resources by establishing bachelor degrees in Aquaculture at graduate level at the University of Dar es Salaam (UDSM), Sokoine University of Agriculture (SUA) and the University of Dodoma (UDOM) and at the postgraduate level the MSc. Health of Aquatic Animal Resources at SUA as well (URT, 2016). Expansion in aquaculture from small scale to intensive fish farming could lead to increased occurrences of fish diseases especially bacterial diseases.

Therefore, reliable measures towards sustainable aquaculture industry in Tanzania should be taken. These include: (a) strengthening collaborative bacterial fish disease researches to identify emerging and re-emerging bacterial diseases in fish farms (b) develop or strengthen fish disease surveillance for monitoring of bacterial diseases in fish farms and (c) provision of extension services to farmers on basic control strategies such as biosecurity measures and proper management practices. Furthermore, the initiative to strive for innovative technologies towards control of these fish diseases should be taken.

2.3 Aeromonads; their diseases, host diversity, characterization and control strategies

2.3.1 Aeromonas species classification and nomenclature

Up until 1970s, aeromonads have been classified into two major groups based on physiological characteristics and host ranges. The optimum growth temperature groups aeromonads into two groups; motile aeromonads which grow at the optimum temperature of 35–37^{o}C, *A. hydrophila* being one of them and non-motile aeromonads which grows at 22–28^{o}C, of which *A. salmonicida* is an example to mention (Igbinosa *et al*., 2012). Further differentiation can be done based on motility, indol-production, and melanin like pigment on the tyrosine medium (Igbinosa *et al*., 2012). Thereafter, several new species of the genus

Aeromonas have been added in the course of reclassification of pre-existing taxa. In the previous classification, *Aeromonas* spp were placed alongside with other species which belonged to the genus; *Vibrio* and *Plesiomonas* in the family *Vibrionaceae* , however, following advances in genetic and molecular biology, aeromonads were rightly placed in their perspective group and assigned a family called *Aeromonadaceae* (Colwel *et al*, 1986; Igbinosa *et al*., 2012). While all genera in the family *Aeromonadaceae* are gram-negative, small rod-shaped, motile bacteria and they share certain growth and biochemical characteristics, their classification and scientific names are constantly under review (Camus *et al*., 1998).

The family *Aeromonadaceae* includes the genus *Aeromonas, Tolumonas* (*incertaesedis*), *Oceanimonas*, and *Oceanisphaera*.These genera were grouped when the classification was based on DNA-DNA hybridization and 16S ribosomal DNA relatedness (Huys, 2014). However, the comprehensive phylogenetic classification of this group of bacteria is critical because of their complex and challenging taxonomy due to the occurrence of micro-heterogeneities in the 16S rRNA gene (Alperi *et al*., 2008). This hurdle can now be circumvented by targeting the bacterial housekeeping gene called RNA polymerase sigma factor 70 domain (rpoD) gene, which is considerably more accurate for the phylogenetic classification of aeromonads (Alperi *et al*., 2008).

Aeromonas hydrophila, *A. veronii*, *A. sobria* and *A. caviae* are said to be the main secondary pathogens, however, recent studies have reported certain strains of *A. hydrophila* to be primary pathogens of human and farmed fish causing high mortalities (Bravo *et al*., 2003; Esteve *et al*., 1993; Jing Li *et al*., 2011; Pridgeon and Klesius, 2011). *Aeromonas hydrophila* ST251 clonal group marked to highly virulent which has caused an outbreak in channel catfish farms in the USA (Pang *et al*., 2015). Some of these members of the family *Aeromonadaceae* such as *A. hydrophila* are also known to be emerging zoonotic pathogens of humans causing a wide range of diseases such as gastroenteritis, wound infections, septicaemia, meningitis, peritonitis, endocarditis and osteomyelitis (Al-Fatlawy & Al-Ammar, 2013). Despite the susceptibility observed in both scaled and unscaled fish, frogs and other vertebrates are also infected by aeromonads (Camus *et al*., 1998).

With the exception of *A. salmonicida* which is a non-motile aeromonad, most of the bacterial infections which are common in fish raised in ponds are caused by motile members of the genus *Aeromonas* (Deen *et al*., 2014; Azad *et al*., 2001). These bacteria are widely distributed

and are ubiquitous in the aquatic environment. Therefore, any stress posed to fish in intensive culture predisposes them to infections that may sometimes lead to mortalities of up to a 100 percent. Fish infections caused by motile aeromonads bacteria have existed for many years and have been given different names such as motile aeromonads septicemia (MAS), motile aeromonads infection (MAI), hemorrhagic septicemia, red pest, and red sore.

2.3.2 Phenotypical and molecular characterization of aeromonads

Recent taxonomy has established more than 30 genospecies of the genus *Aeromonas* (Erdem *et al*., 2011). It has been always difficult to identify the species phenotypically due to the existing complexity in growth and biochemical characteristics especially to very closely related species (Beaz-Hidalgo *et al*., 2010; Chandran *et al*., 2002; Puthucheary *et al*., 2012). Previously, with the use of a profile of sugars, matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF) and API systems, the only *Aeromonas* spp recognized were *A. hydrophila, A. veronii, A. sobria* and *A. caviae*. However, their subphenospecies could not be differentiated using conventional biotyping (Khor *et al*., 2015). No consensus has been reached yet in assigning the *Aeromonas* genus to the recognized species through conventional biotyping, hence, the use of kits and phenotypic schemes as the sole option is recommended for precise classification and identification (Abbott *et al*., 1992; Carnahan *et al*., 1991; Erdem *et al*., 2011; Joseph & Carnahan, 2000).

In recent times, the use of molecular approaches provided advanced identification of *Aeromonas* species, supplementing to conventional approaches, and indeed have presented some improvement. The use of DNA/DNA homology data and sequencing data was common (Figueras *et al*., 2000; Figueras *et al*., 2000; Martinez-Murcia, 1999; Martinez-Murcia *et al*., 2011; Soler *et al*., 2004; Yáñez *et al*., 2003), however, inconsistencies in grouping aeromonads using DNA hybridization probes and 16S rRNA Restriction Fragment Length Polymorphisms has arisen. The use of housekeeping genes in the identification of *Aeromonas* species has recently gained attention to most scientists. These housekeeping genes are believed to have high discriminatory and resolving power and upon precise identification of aeromonads at the genus level, a phylogenetic analysis of either one of them could be used to reveal the genospecies. However, Zhou *et al*. (2019) suggested the use of five or more housekeeping genes in the multilocus phylogenetic analysis (MLPA) to ascertain or identify *Aeromonas* spp. Some of these housekeeping genes employed in inferring the taxonomy of

the genus *Aeromonas* include but not limited to *gyrB, rpoD, recA, dnaJ, gyrA, dnaX* and *atpD* (Zhou *et al*., 2019)

Although the isolation and identification of aeromonads based on growth and biochemical characteristics have been extensively done worldwide, only two studies have been done to identify aeromonads using phenotypic and molecular characteristics in Tanzania. Shayo *et al*. (2012) reported the occurrence of ulcerative infections at Mtera hydroelectric power dam caused by aeromonads identified to species level using 16S rRNA sequence data. However, in the same year Shah *et al*. (2012) conducted a study on the prevalence of antimicrobial-resistance genes to bacterial flora of integrated fish environment in Pakistan and Tanzania and managed to isolate and test the antimicrobial profile of aeromonads. However, attempt to identify the species of the organism was not successful. This indicates that little has been done with regard to molecular characterization of aeromonads in Tanzania using appropriate molecular tags that are currently and widely employed elsewhere (Furmanek-blaszk, 2014; Puthucheary *et al*., 2012).

It is important to note that, if development of vaccines is the priority control strategy then knowledge is required to facilitate its development including characterizing the pathogen in order to understand its strains and serotypes, infectivity, virulence, antigenicity, and the nature of essential immunogens (Committee on Issues and Priorities for New Vaccine Development, 1986).

2.3.3 Clinical signs of diseases caused by aeromonads

The clinical signs of diseases caused by *Aeromonas* spp are not typical and may be easily misdiagnosed with other diseases. Symptoms and signs for the disease can be revealed either in the skin only or as septicemia and occasionally in combined form (Janda & Abbott, 2010). The disease may be presented in a chronic form and if that happens, it normally affects only small numbers of fish. However, in acute form, it is normally accompanied by mass mortality. In scaled fish such as *O. niloticus*, hemorrhages appear in the skin lesions particularly in scale pockets, which can extend to larger areas and form ulcers. Sometimes external signs do emerge which include: abdominal swelling, exophthalmia (popeye), and pale gills (Janda & Abbott, 2010). Afterward the scaled fish accumulate fluid (oedema) in the body and create a roughened or bristled appearance (lepidorthosis)

2.3.4 Factors causing motile aeromonads disease outbreaks in fish farms

Motile aeromonads, *A. hydrophila,* in particular, are ubiquitous bacteria of freshwater aquatic surroundings which are rich in organic matters, such as ponds, but their abundance gets reduced as the salinity increases above 15 parts per thousand. ″A pond is referred to as a man-made or natural water body obtaining its water from either a river or from spring or from rain" (Bhavimani & Puttaiah, 2014). Aeromonads can survive for a long time without the host in the aquatic environment in a pond simply because it can utilize nutrients present in water. These bacteria can also be isolated from healthy fish and for that case are regarded as opportunistic pathogens of fish, making elimination of this group of bacteria difficult. The survival of farmed fish especially in intensive culture is largely governed by physical-chemical characteristics of water and their stability in the pond.

Aeromonads disease development is enhanced by a number of environmental factors that contribute to induce stress in fish. They include those associated with poor water quality conditions such as high ammonia and nitrite levels, low dissolved oxygen levels, high water temperature, and pH variations (Camus *et al*., 1998). These factors do not only lead to fish immunosuppression but save as the intrinsic factors in virulence genes expression (Abreu *et al*., 2018; Shakya & Labh, 2014).

Small fingerlings and fry are the most affected, however, the infection can occur at all ages (Camus *et al*., 1998). In tropical countries, A. *hydrophila* outbreak, for example, can occur in any month of the year depending on the predisposing factors; however, the outbreaks are usually seasonal, with a peak in the hot season. An outbreak can also occur in the winter season following extensive handling and transport of young fish.

2.3.5 Virulence factors and disease pathogenicity of aeromonads

The pathogenicity of aeromonads in fish and humans is contributed by a number of virulence factors working in a multifactorial manner making the phenomenon complex (Galindo *et al*., 2006; Li *et al*., 2011; Sha *et al*., 2009). Detection of virulence factors through their phenotypic activity and/ or presence of their genes in clinically sick fish or apparently healthy fish have become a crucial and common measure of putative virulence and pathogenicity of several species of the genus *Aeromonas* (Hoel *et al*., 2017; Khajanchi *et al*., 2010; Oliveira *et al*., 2012; Silva *et al*., 2017). Li *et al*. (2011) showed that the phenotypic characteristics of virulence factors and the presence of their genes in different combinations correlate with in-

vivo animal disease pathogenicity, hence their potential use as virulence markers. These virulence factors include but not limited to outer membrane proteins (OMPs), lipopolysaccharides (LPS), adhesive structures and extracellular factors such as siderophore, enterotoxin, aerolysins, haemolysins proteases and lactamases (Al-Fatlawy *et al*., 2013; Janda & Abbott, 2010). The virulence genes have been broadly saved as a determinant of pathogenicity of *Aeromonas* species (Kingombe *et al*., 1999; Li *et al*., 2011). On the other hand, the majority of the *A. hydrophila*, *A. veronii* and other species are virulent and proved to be pathogenic, while some strains or genotypes are avirulent and posed little or no detrimental effects to the host (Li *et al*., 2011). There is a great variation of virulence gene occurrence, possession and distribution of aeromonads within and between genus and species. The differences may also be linked to differences in geographical location (Ghenghesh *et al*., 2014). Therefore, assessment of occurrence, possession, and distribution of virulence genes and their phenotypic characteristics based on geographical location is important for improved control and prevention strategies of disease occurrence.

2.3.6 Antibiotics and chemotherapy use and its implication in aeromonads

The use of antimicrobials in the treatment of infectious bacterial diseases have made a tremendous revolution in the field of medicine in several ways, however, in past few decades, their massive application has led to rapid emergence and increase of resistant strains, which have now become a global health threat. (Baron *et al*., 2017; Zhou *et al*., 2019)

In developed countries, where regulations with regard to antimicrobial use are strictly followed, no one is allowed to license more than two or three antimicrobial agents for use in aquaculture (Deng *et al*., 2014; Smith, 2008). However, developing countries, such as Egypt have a problem of implementing antimicrobial regulations. As a result, resistance of pathogens to these antimicrobials in aquaculture in those countries has been well documented (Deng *et al*., 2014).

Despite successful management of diseases in aquaculture for more than 20 years, prophylaxis and chemotherapy have greatly contributed to the emergence of multiple-drug resistant strains of pathogens and residue in the aquatic environment (Mitchell & Plumb, 1980). In addition, the resistance in those selected pathogens is always being transferred to other related or unrelated bacteria through R-plasmid (Kim *et al*., 1993).

Several findings have reported a high prevalence of drug-resistant *Aeromonas* spp from fish, environment, foods and human clinical samples (Alcaide *et al*., 2010; Aravena-Román *et al*., 2012; Čížek *et al*., 2010; Deng *et al*., 2014) in different parts of the world, showing their resistance to a number of antibiotics such as ampicillin and penicillin. However, they are also susceptible to other antibiotics such as tetracycline, aminoglycosides, quinolones, trimethoprim-sulfamethoxazole and chloramphenicol, and second and third-generation cephalosporins (Igbinosa *et al*., 2012; Vivekanandhan *et al*., 2002). In addition, it has been observed that resistance to one antibiotic can induce the same to several antibiotics. Nygaard *et al*. (1992) for example, reported that exposure to oxolinic acid or oxytetracycline introduced a cross-resistance to flumequine and oxytetracycline.

Even though antimicrobial resistance in aeromonads is chromosomally mediated, the existence of mobile genetic elements such as plasmids, integrons and transposons in *Aeromonas* isolates facilitates the quick horizontal transfer of resistance to non-resistant isolates. These mobile elements are passed to subsequent bacteria through transformation, transduction or conjugation (Romero *et al*., 2012; Stratev & Odeyemi, 2016). The methods of resistance acquisition and mechanism of antibiotic-resistant have been illustrated in Fig. 5 and 6. It is, therefore, important to monitor antibiotic usage in aquaculture and advocate the use of alternatives and novel control strategies such as vaccination and biological control such as the use of phages.

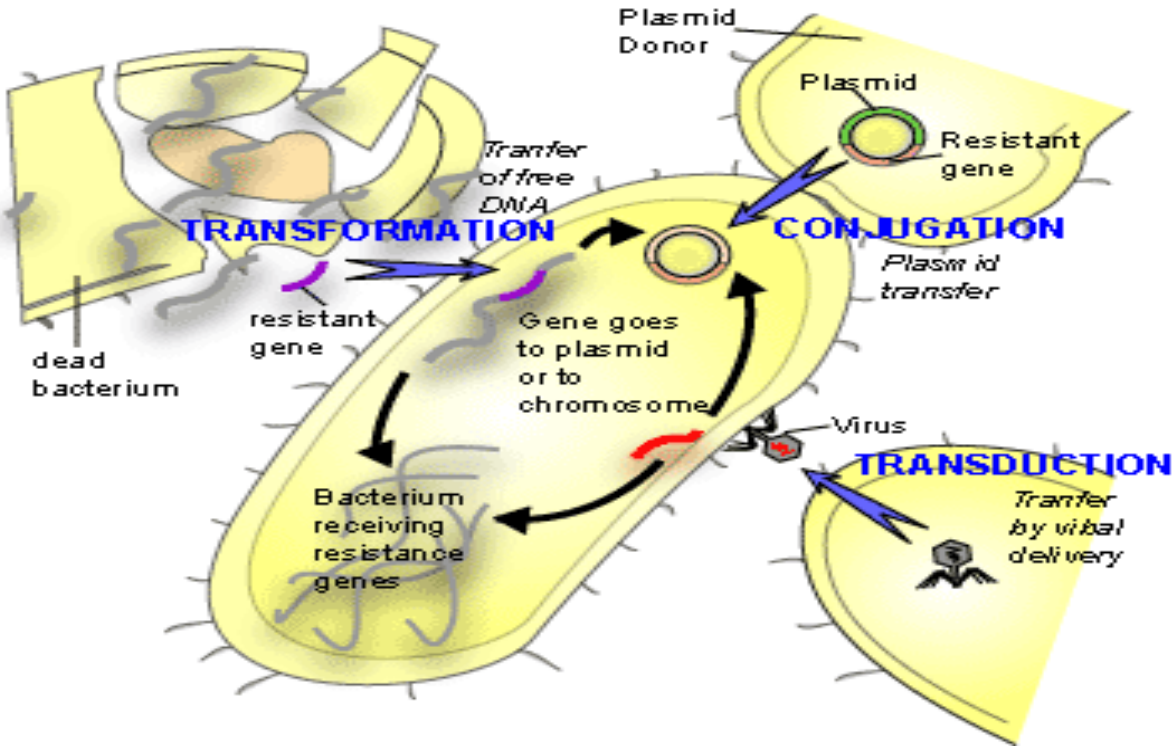


Figure 5: Horizontal antibiotic resistance gene acquisition and gene transfer methods (Yim, 2006)

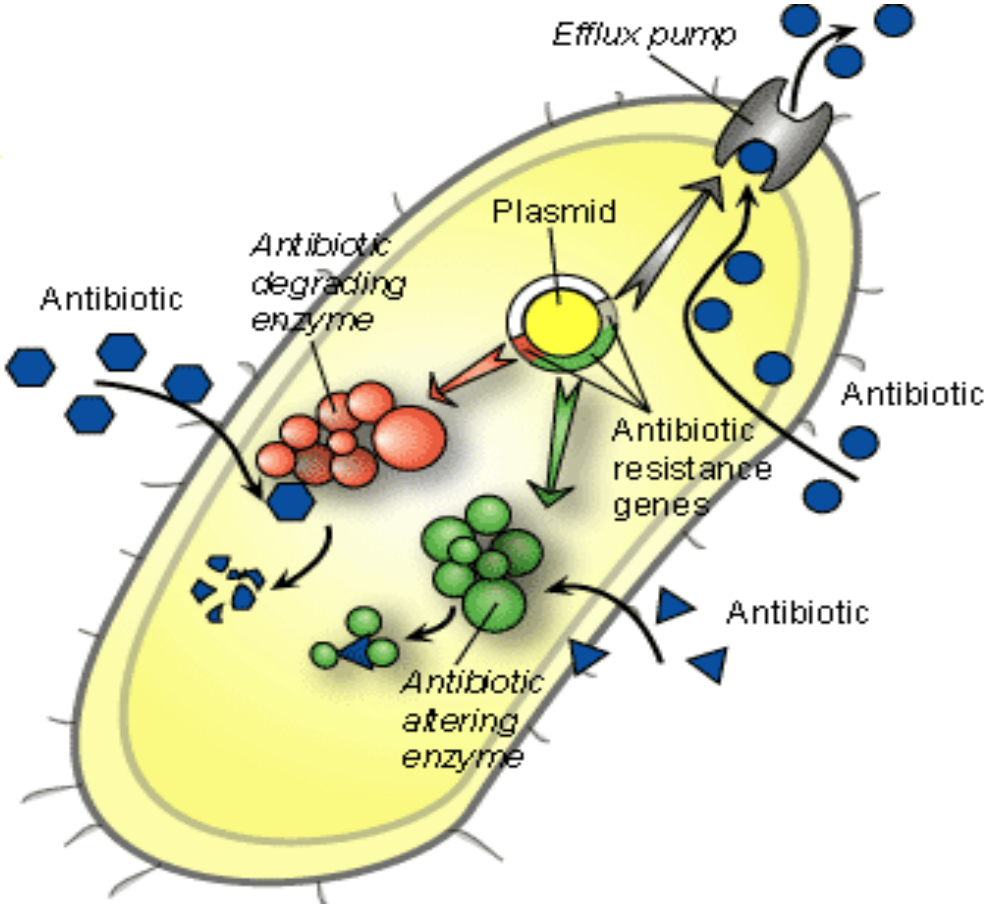


Figure 6: Mechanisms of antibiotic resistance by different bacterial species (Yim, 2006)

2.3.7 Vaccines development and vaccination against *A. hydrophila*

(i) Vaccine application

Following increase in fish farming practices during mid to late 1970s, scientists thought of developing vaccines for controlling or preventing fish diseases. This is because use of antimicrobials and chemotherapy not only raised public health concerns and antibiotics resistance threat in fish but also are not cost-effective and are environmentally unfriendly (Goni-Urriza, 2002). Since then, vaccination is regarded as the most effective tool in the prevention of diseases (Chandran *et al*., 2002; John *et al*., 2002) and has become an integral tool in fish health management strategies. Live attenuated vaccines are efficacious to stimulate protective immunity with induced or natural avirulence and have long been successfully used to prevent many animal and human diseases. It is believed that the most promising preventative strategy to combat the infectious diseases of fish is by using live attenuated vaccines.

Despite the fact that vaccination represents the most effective strategy to prevent diseases in the aquaculture industry (Chandran *et al.* 2002), commercial vaccines for *A. hydrophila* in fish have remained a challenge (Dash *et al.* 2014). One of the problems that limit the development of commercial *A. hydrophila* vaccines is strain diversity (Moral *et al.* 1998) and failure of the vaccine to confer protection to heterologous strains (Ni *et al*. 2010). However, efforts were made to develop vaccines in different regions worldwide and initially focusing on inactivated products and live attenuated organisms. Following advancement made in Molecular biology, biotechnology, vaccine immunology and reverse vaccinology, new high-tech vaccines are being developed and experimentally tested against *A. hydrophila* in different fish species.

(ii) Steps in vaccine development

Vaccine development can follow a number of steps that can be summarized in different ways by researchers/scientists. Mitchell (2003) outlines the steps required in developing a vaccine. These are: (a) Isolation and characterization of the aetiological agent (b) Experimental infection of a suitable or susceptible animal in the laboratory to confirm reoccurrence of disease signs (c) Challenge trial in a model animal (d) Preliminary "bench-top" fermentation experiments (e) processing of small-volume downstream culture (f) Wet laboratory vaccination, safety trials (g) Reviewing, modifying and refining the above techniques (h)

Scaling-up (i) Implementing clinical field trials (j) Developing regulatory submission documents and serial batches (k) Marketing, gathering feedback and refining the formulation. These steps are crucial and failure to fulfill one of it stops the whole process.

(iii) *Aeromonas hydrophila* vaccine types

Whole organism vaccines (killed and attenuated vaccines) has advantages over other types of vaccines and hence great potential in aquaculture. Live attenuated vaccines provide a simulation model of infection and the vaccine strain could spread to a non-vaccinated fish population over a prolonged period of time. Live attenuated pathogens carrying epitopes of the pathogen promote a potent immune response as it mimics natural infections and has intrinsic adjuvant properties than non-replicating products (Marsden *et al*. 1998). Furthermore, live vaccines have the advantage that they stimulate humoral and cellular immunity significantly in fish. However, not all these vaccines completely prevent disease and in addition, they raise safety concerns, and are time-consuming process, which delays the timely development of vaccines against emerging and re-emerging pathogens of fish (Marsden *et al*. 1998). Therefore, novel approaches through advances made in genetics, biotechnology, immunology and molecular biology were needed for the development of newer types of effective vaccines in the aquaculture field (Delany *et al*., 2014; Finco & Rappuoli, 2014; Effio & Hubbuch, 2015).

Advances in molecular biology, biotechnology, and reverse vaccinology have enabled the development of different types of *A. hydrophila* vaccines which have recently been experimentally tested in fish. They include; subunit vaccines, plasmid DNA vaccines, the recombinant live vector vaccines, and recombinant protein vaccines.

DNA vaccines against a wide range of pathogens have been investigated in various fish species especially against viral diseases but limited in bacterial diseases. In spite of having several advantages such as conferring immediate, safe and a durable protection against several viral diseases such as infectious hematopoietic necrosis virus (IHNV) (Ballesteros *et al.,* 2015; Assefa & Abunna 2018) in farmed fish, this type of vaccine seemed to be less adopted in bacterial diseases and especially in controlling diseases caused by *A. hydrophila* in farmed fish. Among others, one reason given by researchers was bacteria having genes involved in the production of carbohydrates and highly glycosylated proteins of which transcription and production of plasmid DNA encoding these genes is not feasible but only

possible for non-glycosylated proteins (Tonheim *et al*., 2008). Thus DNA vaccines could not be a good substitute for the more traditional polysaccharide containing vaccines in triggering immune responses against microbes that have an outer membrane made of, for example, lipopolysaccharides (Jorgensen *et al*. 2001). The reported possibilities of developing myositis upon intramuscular injection of plasmid DNA (pDNA) vaccine, is another challenge limiting its use against bacterial infection in fish.

Limited studies focused on recombinant live vectored vaccines against *A. hydrophila* in fish. One of the studies utilised non-pathogenic recombinant *Lactococcus lactis* to carry Aerolysin gene from *A. hydrophila*. However as it has been explained by Vaughan *et al*. (1993) immunization with such vaccines unavoidably infers the release of recombinant organisms into the surrounding environment, thus based on European Union (EU) and other guidelines, such organisms are classified as genetically modified organisms (GMO), limiting their potential utilisation in aquaculture.

Recombinant protein vaccines seem to take a wide coverage in controlling most of the bacterial diseases in fish. This is depicted by a number of studies on recombinant protein vaccines against *A. hydrophila* diseases in fish. These vaccines are prepared by inserting the immunogenic regions of a pathogen in an expression host t to obtain the protein in large scale and the protein purified as a vaccine (Nascimento & Leite, 2012). Initially, the development of this type of vaccine was challenging in the characterization of the immunogenic component of the pathogen, however, following advancement in reverse vaccinology; vaccine development can take not more than two years. Vaccine safety is guaranteed by appropriate vaccine delivery systems and adjuvants in different fish species.

(iv) *Aeromonas hydrophila* vaccine delivery methods

Vaccine administration in fish is done through different routes such as oral administration, intramuscularly, intraperitoneal injection and through immersion (Fig. 7). While efforts are made by researchers, to improve vaccine carriers in a way that can accommodate mass vaccination of fish, vaccine delivery for most of the bacterial fish vaccines through intraperitoneal injection. For DNA vaccines delivery is best achieved through intramuscular injection.

Intraperitoneal injection gives a higher protection compared to other delivery systems; however, this delivery method poses stress to fish, it is labour intensive, costly and suitable for only large size fish (Plant & LaPatra, 2011).

Contrary to the injection method, dip and bath immersion is applied to vaccinate fish of all sizes using a different concentration of vaccines. However, this method gives a relatively low protection due to poor vaccine -antigen uptake through skin and gills. Nakanishi *et al*. (2002) reported high protection of a vaccine against *Streptococcus iniae* in rainbow trout (*Oncorhynchus mykiss*) using a skin puncture followed by immersion delivery system. However, skin puncture has been disputed for causing stress to fish.

Oral administration is another useful method for mass vaccination of fish through feeds. Findings have revealed that naked antigens are prone to degradation in the foregut of the fish before reaching to the hindgut where adherence and immune responses are elicited (Embregts & Forlenza, 2016). This is particularly the case with inactivated and un-encapsulated vaccines. Also, oral vaccine administration does not give reliable protection because of inconsistent in vaccine uptake by the fish. Therefore, emphasis is placed on targeted delivery strategies for *A. hydrophila*, similar to those used for humans and other animal species.

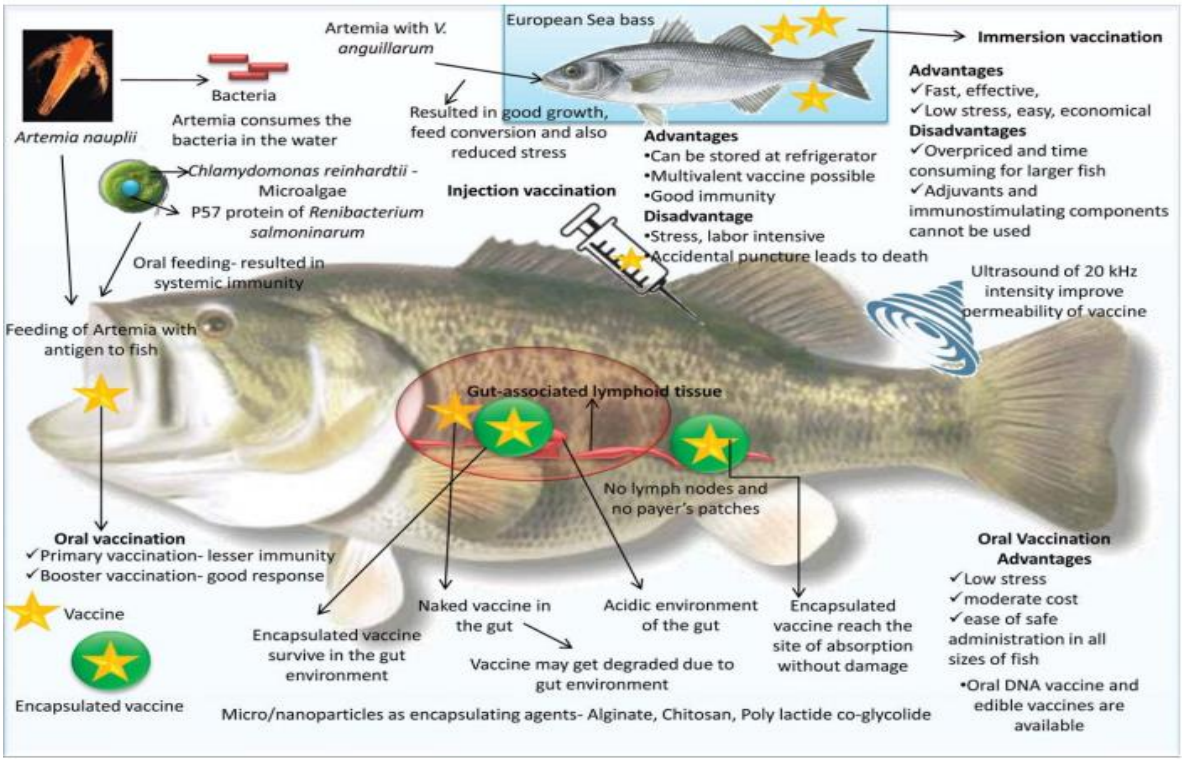


Figure 7: Different vaccine administration routes, their advantages and disadvantages (Dadar *et al*., 2017)

(v) Adjuvant/vaccine carrier system in aeromonads vaccines

An immunologic adjuvant is applied to accelerate, prolong, or enhance antigen-specific immune response when combined with specific antigens (Tafalla *et al*., 2013). Search for safer and potent vaccine adjuvants and carrier system has resulted in the formulation of antigens into different carrier systems from those of historical solution form to modern adjuvants and carrier system in particulate form. These adjuvants and carrier systems range chemically-based to biological ones (Sudheesh & Cain, 2017). Despite the reported efficiency, conventional chemical adjuvants and vaccine carriers also produce adverse effects to the host, such as chronic peritonitis, adhesions, and granulomas in extreme conditions (Midtlying*et al*., 1996; Poppe & Breck, 1997; Dash *et al*. 2014).

Due to that, the search for better carrier systems that provide improved vaccine efficacy especially in new generation vaccines such as subunit, DNA and recombinant protein vaccines was instigated. The use of biological adjuvant such as molecular adjuvants i.e. Plasmid-encoded cytokine adjuvants in DNA vaccines (Hølvold *et al*. 2014) and herbal based adjuvants such as that of *Asparagus racemosus* extracts (Thangaviji*et al*., 2012), nanotubes and nanoparticles has gained special attention in human and animal vaccines (Dubey *et al*. 2016) but not to a large extent in *A. hydrophila* vaccines of fish.

Micro-encapsulation of vaccines in polymers such as chitosan, MicroMatrix™, alginates, liposome and Poly D, L-lactic-co-glycolic acid (PLGA) are the current novel approaches towards improving oral vaccines incorporated in the feed (Embregts & Forlenza, 2016). The application of Biodegradable PLGA nanoparticles, for example, has attracted interest as an antigen carrier system for oral vaccines because of their ability to enhance antigen uptake and ability to allow the slow release of antigens in vivo (Dubey *et al.* 2016) and, therefore, research on nanomaterial carrier systems for oral vaccines against *A. hydrophila* in fish continues alongside injectable vaccines in mass vaccination of fish which is more complicated.

Even though commercial vaccines for aquaculture work really well in terms of protecting the fish against certain diseases, it is also agreed that all of these vaccine development strategies have merits and demerits, and their use will depend on nature of the mechanisms of infection of the particular pathogen and respective immune response required for protection (Dalmo,

2018). Therefore, vaccination should be part of the general fish health management program in combination with other preventative practices (Vinitnantharat *et al*., 1999).

(vi) Fish immunology

The physiology of the immune system of fish is comparable to that of higher vertebrates, however, although significant variations exist (Tort *et al*., 2003) (Table 1). The body compartments and cell organization play a great role in the existing differences. For example, the generative and secondary lymphoid organs are common in both mammals and fish, with the exception of the lymphatic nodules and the bone marrow, which exists in mammals but not in fish (Biller-Takahashi & Urbinati, 2014).

Table 1: Immune response differences between jawed fish and mammals

	Jawed Fishes	Mammals
Biotic constrictions		
Temperature range	-2 to 35℃	36.5 to 37.5℃
Primary environment	Water	Air
Metabolism	Poikilothermia Endothermia (eg. bluefin tuna and some pelagic fishes)	Homeothermia
External interfaces	Mucous skin, gills	Respiratory tree
Humoral diversity		
Ig isotypes	IgM, IgD? *(Teleostei)* IgM, IgX/IgR, IgW, NAR(C) *(Chondrichthyes)* IgM redox forms	IgM, IgA, IgD, IgE, IgG
Ig gene rearrangement	Multicluster *(Chondrichthyes* and some *Teleostei)*	Translocon
Non-specific diversity	Several C3 isoforms *(Teleostei)*	No C3 isoforms
Overall performance		
Antibody affinity	Low	High
Antibody response	Slow	Fast
Memory response	Weak	Strong
Affinity maturation	Low or absent	High
Low temperatures	High dependence, immunosuppressive response (only in poikilothermic fish)	Low dependence
Lymphoid organs		
Haematopoietic tissue	Head kidney *(Teleostei)* Epigonal and Leydig organs, meningeal tissue, orbital and subcranial hematopoietic tissue *(Chondrichthyes)*	Bone marrow
Thymus	Involution species-dependent, influenced by seasonal changes and hormonal cycles	Involution with age
Lymphoid nodes	Absent	Present
Gut-associated lymphoid tissues	Not organized, lymphoid aggregates Leydig organ and spiral valve *(Chondrichthyes)*	Organized, Peyer patches
Germinal centres	Absent (melanomacrophage centres?), dendritic cells probably present	Present

Tort *et al*. (2003)

Ontogenically, the fish immune system, especially that of teleost is somewhat primitive compared to other vertebrates such as mammals. This is because the divergence took place about 400–500 million years back (Secombes & Wang, 2012; Tort *et al*., 2003). Some fish immune organs such as the anterior kidney and thymus of teleosts have been reported to be

completely developed even before hatching happens. The kidney is the first organ to be formed followed by spleen and thymus, the latter being the first organ to become lymphoid (Razquin *et al*., 1990; Zapata *et al*., 2006); however, the variation exists between species (Magnadottir *et al*., 2005).

Fish depend largely on the innate immune system for survival than the acquired or adaptive immunity (Ellis, 1990; Rombout *et al*., 1986; Zaki *et al*., 2011). Non-specific immunity is essential and plays a key role in the acquired immune response in fish. This is backed by the poikilothermic nature of the host, limited repertoire of antibodies, the confinement nature of the adaptive immune system, and the slow proliferation, maturation, and memory of their lymphocytes (Whyte, 2007). The roles of nonspecific immune responses have been placed to work in three categories as physical barriers, in cellular and humoral immune responses.

The physical barriers such as the gills, skin, and alimentary can have a substantial role in preventing infection in fish (Magnadottir, 2010). In addition to these structures, t there are contains soluble antimicrobial molecules which assist in inhibiting the penetration of pathogens (Alexander & Ingram, 1992; Aranishi & Nakane, 1997; Boshra *et al*., 2006; Rombout *et al*., 1993; Saurabh & Sahoo, 2008). These include complement proteins, antibacterial peptides, lectins, lysozymes, pentraxins and immunoglobulin M (IgM).

Phagocytosis is implemented by neutrophils and macrophages and the process (Secombes & Fletcher, 1992) rarely influenced by temperature (Blazer, 1991; Lange & Magnadóttir, 2003; Magnadottir *et al*., 2005). Natural antibodies do exist in fish prior to stimulation by antigen and they are said to provide wide protection against bacterial and viral pathogens during a nonspecific immune response (Boes, 2000).

Populations of different lymphocytes do interact to execute specific or adaptive immunity in fish. These lymphocytes are somewhat analogous to B-cells, cytotoxic cells, T cells, and antigen representing cells (macrophages and dendritic cells). The fish body responds specifically and at high affinity after interacting with the pathogen following the complex networking of the immune cells, proteins, genes, and biochemical signals.

Antibodies are glycoproteins, also called immunoglobulin (Ig), presented in the membrane of the B lymphocyte. The IgM is the predominant class in teleosts blood plasma there is no diversity has been demonstrated in fish due to the restricted profile of isotypes (Tort *et al*., 2003). The simplicity and lack of flexibility of the Ig profile are justified by the evolutionary

data that fish was the first group to show antibody activity before the complex and increased number of Ig isotypes observed in other vertebrates such as amphibians reptiles birds and mammals (Magnadottir, 2010; Tort *et al*., 2003). However, in recent years researchers have reported the existence of other immunoglobulin isotypes.

These isotypes have been identified in some different species of fish (Fig. 8), and they include IgD (Wilson *et al*., 1997), IgZ (Danilova *et al*., 2005) and the IgT (Hansen *et al*., 2005). The concept of the diversity of the isotypes is based on the fact that only one gene can generate more structural isoforms following the structural organisation of the immunoglobulin rather than genetic variability (Tort *et al*., 2003).

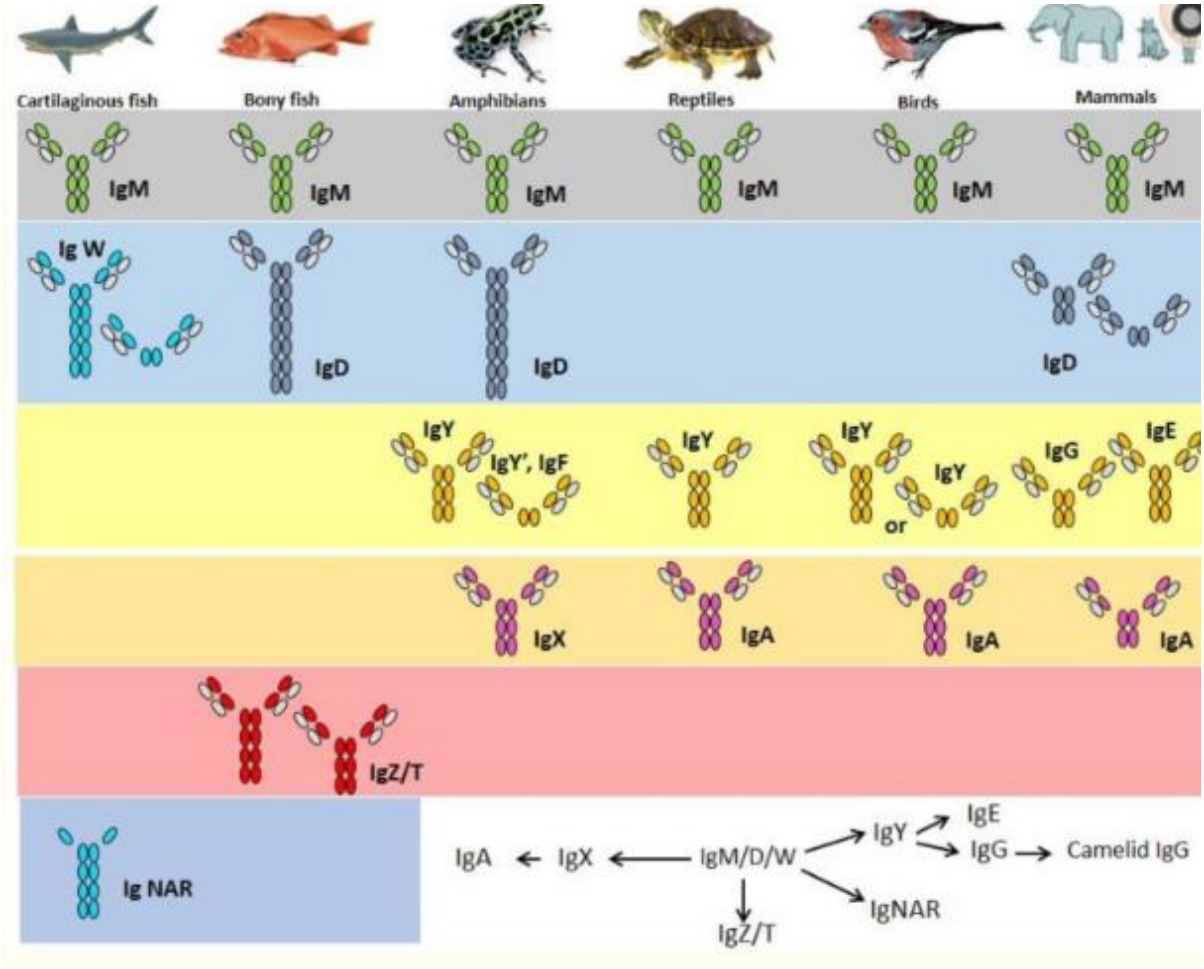


Figure 8: Different Ig isomers of fish, amphibians and mammals (Mashoof & Criscitiello, 2016)

The predominant tetrameric IgM in teleost exhibits structural heterogeneity due to disulfide bonds linkages variability of monomers and or halfmer subunits referred as to redox forms (Kaattari *et al*., 1998). The number of hydrogen disulfide bonds is determined by the affinity

of B-cells receptors (BCRs) upon interaction with the specific pathogen (Mashoof & Criscitiello, 2016).

Moreover, following inconsistency in structure of the tetrameric IgM which results in existence of redox forms, the measurement of antibody titre in immune responses by using agglutination, precipitation or ELISA techniques may not save as a good indicator of antibody response efficiency, especially if a particular redox form is required to react with a pathogen and the form is serologically indistinguishable from other forms (Kaattari *et al*., 1998; Tort *et al*., 2003).

Similar to mammals, cell-mediated immune response in fish is facilitated by different types of immune cells, including T-lymphocytes, which encompass cytotoxic T-lymphocytes (CTLs) and T helper cells (Th) (Kato *et al*., 2013).These cells possess different cell surface markers, notably is the T cell receptor (TCR), which serves as a borderline to distinguish them from other lymphocytes (Ashfaq *et al*., 2019; Forlenza *et al*., 2008).

Teleosts possess the major histocompatibility complex (MHC) which works together with T cell receptor as MHC/TCR system. On the other hand, T cells designated as T helper (Th1 and Th2) cells have a trans-membrane glycoprotein expressed on the surface known as CD4. Despite the structural and functional differences, both classes of the MHC receptor, the MHC I and MHC II are involved in initiating the specific immune response through presentation of the antigenic determinants to the T cells (Nakanishi *et al*., 1999). Class I MHC molecules in association with the Th1 cells present peptides derived from intracellular pathogens to CD8+ cytotoxic T cells in cell-mediated immunity (Nakanishi *et al*., 1999).

The working mechanism of cell-mediated immunity in fish is believed to be analogous to that of mammals. In short, antigen-specific T cells are activated and react with the pathogen that is presented to them by antigen-presenting cells via their MHC molecules. The cytotoxic T cells can kill the host cell infected with a viral or bacterial agent. The T cell produces cytokines to activate the innate defenses which destroy the intracellular microbes (Laing & Hansen, 2011). A number of cytokines are known to be involved in cell-mediated immunity in fish (Litman *et al*., 2010). Some of these include type I and type II IFN which drives the Th1 cell differentiation, IFN-g a potential effector of Th1 responses. Others are IL-12 , IL-18, and IL-2 (Secombes & Wang, 2012).

One of the important characteristics of the adaptive immune system is immunological memory (Secombes & Wang, 2012). Fish develop a memory response after the first encounter for the next exposure of an antigen (Arkoosh & Kaattari, 1991; Whittington *et al*., 1994). In some circumstances, some species of fish require two exposures for them to respond vigorously and rapidly to T-dependent antigens than to the T-independent antigen where it needs only one exposure (Uribe *et al*., 2011).

The immune system of fishes is habituated by the particular environment, but also by their poikilothermic condition. Natural and artificial environmental stress factors, can affect the immune response together with other physiological functions in fish (Bly *et al*., 1997). The natural environmental stressors include; seasons, temperature, pH and salinity while the artificial one includes the man-made such as acid rain, heavy metals and organic compounds (Bly *et al*., 1997). All forms of the environmental factors are believed to affect the innate (non-specific) as well as adaptive. Dominguez *et al*. (2005) reported that environmental factors such as temperature, pH and salinity affect the lysosome activity non-specific immunity in Nile tilapia (*Oreochromis niloticus*). Some inorganic minerals such as copper which accumulate in water through anthropogenic activities can also affect the production of antibodies from the B-cells during the humoral immune response (Anderson, 1996). However, several food additives and immunostimulants can enhance the efficiency of innate immunity and hence adaptive immunity (Magnadottir, 2010).

(vii) *Aeromonas hydrophila* vaccine working mechanisms and protection

Vaccines work by inducing either humoral immunity or both humoral and cellular immunity. Few studies have assessed the humoral and adaptive cellular immune response of vaccines against *A. hydrophila* as compared to innate and antibody-mediated immunity (Munang'andu, 2018; Munang'andu & Evensen, 2018).

Although it is well known that the immune response in fish resembles that of mammals with some specific differences between them (Newman, 1993), assessment of the immune responses in fish is not straight forward. The measurement of humoral immunity can be easier carried out than cell-mediated immunity (Abdelhamed *et al*., 2017).

In line with that, the challenges in designing vaccines using different strategies that will elicit the appropriate cellular immunity (Munang'andu, 2018; Nascimento & Leite, 2012) and the extracellular nature of the bacterium could be other reasons of assessing the humoral

immunity rather than cellular immunity. Correlate of protection (CoP) based on antibody titres has been established for some of licensed human and animal bacterial vaccines (Dalmo, 2008). However, the same is yet to be established in most if not all fish vaccines.

A study conducted by Abdelhamed *et al*. (2017) on recombinant *A. hydrophila* vaccine in fish revealed that antibody response did not correlate with the protection level while the relative percent survival (RPS) showed fish to be protected following challenge. They, therefore, explained the scenario by acknowledging that antibodies do not account for all of the protection and the predominance of cellular immunity over the antibody response cannot be undervalued.

It has been observed in most studies that have experimented on *A. hydrophila* vaccines, that vaccine efficacy was assessed in terms of relative percent survival (RPS) without assessing vaccine immunogenicity. Nonetheless understanding the immunological mechanism of the vaccine under study, especially on how the vaccinated fish prevent bacteria colonization on mucosal surfaces, blocking bacteria entrance into the systemic environment and averting tissue damage in target organs is important (Munang'andu *et al*., 2015).

(viii) Fish vaccination and experimental design

Fish vaccination protocol and procedure requires the vaccinologist to choose the type of vaccine to be used, the vaccination method to be employed, the time of vaccination with regard to the production cycle, water temperature and the size of fish species in question (Lillehaug, 1997). During the vaccination process, fish need to be in their immune-competent state. The time required for the immune response to take place depends on several factors.

The best time for vaccination in the production cycle is the time before potential exposure to actual pathogens, which is just after hatching, beginning to feed or first growth period (Lillehaug, 2014). It is therefore, recommended that vaccination should not be done during the grow-out phase in ponds and net pans.

Temperature plays a great role in the immune response following vaccination (Bowden, 2008). There is a contradiction on the optimum temperature to which immune responses may effectively be evoked following vaccination. However, it has been revealed that the overall protection does not seem to rely on the ambient temperature. The range of temperature between 21^{o}C – 29^{o}C is recommended for warm freshwater fish.

Limited data do exist on the exact age when fish can fully mount the immune response following vaccination. Few species of commercial value such as carps and salmonids have its time for full immune response established (Nakanishi *et al*., 1999). In sub-tropical freshwater species, channel catfish develop full immune response seven days post-hatch.

A vaccine by injection is the most successive delivery method especially to adult fish of commercial value such as carp and salmonids. This method requires specialized setup and fish need to be netted and anesthetised one to two minutes in an oxygenated facility. Oral administration is observed to be the best approach in fish vaccination as it can induce both local and systemic protection. However, this method requires large amounts of the antigens and has low efficacy due to variation of vaccine uptake and gastric degradation (Mutoloki *et al*., 2015). In the immersion vaccine delivery method, the surface of fish is exposed to a diluted vaccine and antigen internalization is via the skin, the gills, and the lateral line (Nakanishi *et al*., 2002; Nakanishi & Ototake, 1997). The method is useful for fries, fingerling, and sub-adult fish and is performed by dip or bath techniques with the later utilizing a much-diluted solution of a vaccine for an extended time interval.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study sites

Fish samples were collected from randomly selected fish ponds and farms in four regions of Tanzania; namely Kilimanjaro, Iringa, Mbeya and Ruvuma (Fig. 9). Selection of regions was purposive because these are the big four regions where fish farming is mostly practiced having more than 1000 fish ponds each and it is where clustered and sporadic outbreaks characteristic to hemorrhagic septicemia have been reported to occur (Tarimo, personal communication).

Ruvuma is one of the five regions of the southern highlands of Tanzania together with Iringa, Mbeya, Njombe and Rukwa. Ruvuma region is situated between latitudes 9° 35' to 11° 45' South of Equator and longitudes 34° 35' to 38°10' Meridian (URT, 1997). Ruvuma is bordered to the east by the Mtwara Region, to the north by the Morogoro Region, to the northeast by the Lindi region and to the northwest by the Njombe region. It covers a total of 63 669 km^2 with a population size of approximately 1.377 million (Tanzania Census, 2012). This region has a number of rivers including:Ruvuma River in southern coast basin is having of five major river systems. The major river systems include: Ruvuma, Mavuji, Lukuledi, Mbwemkuru and Matandu. Of these perennial rivers includes Ruvuma, Mavuji and Lukuledi while Matandu and Mbwemkuru are Seasonal which empties water into Lake Nyasa. Other perennial rivers in the region are Ruhuhu, Chiwindi, Mnywamaji, Yola, Lukali, Lwika, Liweta, Ngano, Lumumba, Ndumbi, Yungu, Mbuchi, Mbawa, Luhekei and Nkalachi.

Iringa region in the southern highlands of Tanzania, is located between latitudes 6o 55’ and 9° 00' and longitude 33° 45' and 36° 55’ (URT), 2013). The region shares borders with Morogoro region to the east, Singida and Dodoma regions to the North, Njombe region to the south and Mbeya region to the West. This region is largely drained by the Little Ruaha and the Great Ruaha rivers and their tributaries.

Mbeya Region is situated between latitudes 7° and 9° 31’ and longitudes 32° and 35° to the east of Greenwich (URT, 1997). This region shares borders with countries of Malawi and Zambia to the south, Rukwa region to the West; Singida and Tabora regions to the North and Iringa region lies to its east. Mbeya region has a substantial number of rivers and an

upstanding number of fish ponds. The Southern plateau in the Southern Highlands of Tanzania provides a watershed of the most important rivers supplying water in this region. Chimala, Igurusi and Kimani serve to be the main rivers that supply its water to the Great Ruaha. Furthermore, river Songwe and river Zira channels its water into lake Rukwa, while Mmbaka, Lufilyo and Kiwira supplies to the south and ends in lake Nyasa (URT, 1997). These rivers and their tributaries form the main source of water for aquaculture in Mbeya region.

Kilimanjaro region is located in the north eastern part of Tanzania Mainland and it shares borders with Tanga region to the southeast, Arusha region to the southwest, and Kenya to the north. The region is l located between 36º 25' 30''and 38º10' 45'' east of Greenwich and between latitudes 2º 25' and 4º 15' south of the equator (URT, 1998).

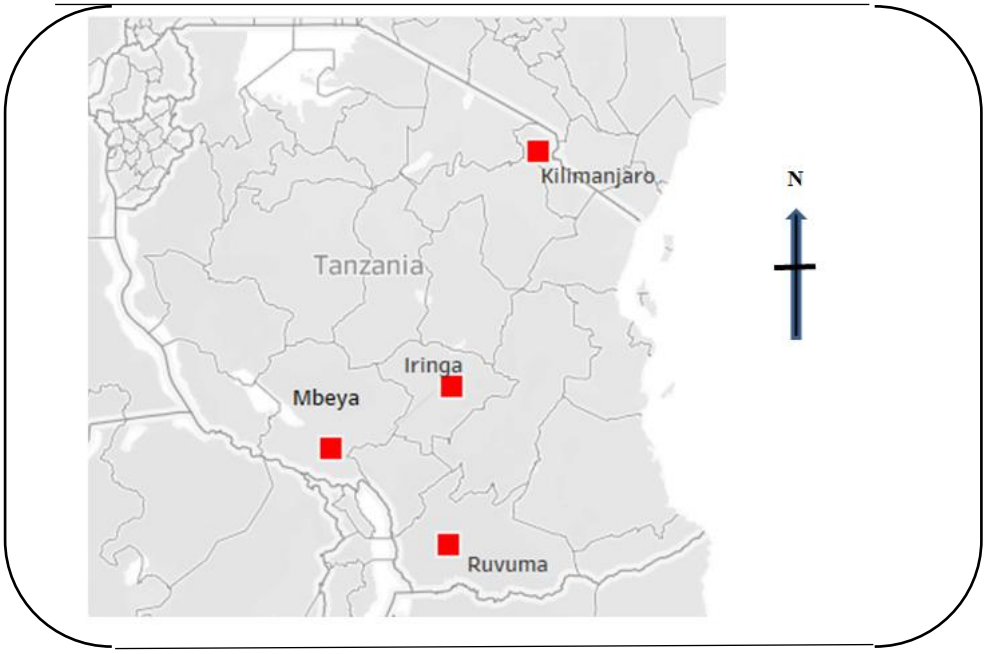


Figure 9: Map of Tanzania showing regions where farmed fish sampling was conducted

Note: The map was developed using Tableau software

3.2 Sample size and sampling

A total of 816 whole fish samples were collected from 32 randomly selected fish farms in Mbeya, Iringa, Ruvuma and Kilimanjaro regions (eight farms from each region). The sample size was determined according to the method developed by Ossiander (1973) which recommends that, for an estimated fish disease incidence of 10% in a fish pond with a population of 2500 (adopted from Egypt), a minimum of 27 fish are sampled.

From the pond (Fig. 10A), fish were sampled by scooping using small sized fish net (Fig. 10B). Morphometric measurements (weight and length) of the fish were done using a portable digital balance and a millimeter ruler. Fish were then dissected on the spot (Fig. 10C) and internal organ; Liver, Kidney, Spleen and Gills were removed and placed in bijou bottles containing Cary Blair transport medium (Fig. 10D). The samples were placed in a cool box and transported to the microbiology laboratory at the College of Veterinary Medicine and Biomedical Sciences - SUA for bacterial isolation and later to the School of Life Science and Bioengineering at Nelson Mandela African Institution of Science and Technology (NM-AIST) for molecular analysis of the isolates. The Cary Blair transport medium was chosen because of its ability to maintain the gram negative bacteria for a considerable period (Koehler & Ashdown, 1993).

During sampling, physical and chemical parameters of the water were recorded in each pond using a portable multiparameter meter (HI98229, HANNA Instruments, Woonsocket, USA). The assessed parameters were; pH, dissolved oxygen, turbidity, water conductivity, water salinity and temperature.

Figure 10: Sample collection (A&B), fish dissection and storage in transport medium (C&D) at the field before transportation to the microbiology laboratory

3.3 Awareness on fish health and fish pond management

Along with fish sample collections, the 32 fish farmers were interviewed with the aid of a semi-structured questionnaire on general pond management practices, fish bacterial diseases and fish health management. The questionnaire was pre-tested by administering to 10 fish farmers in Morogoro region. The questionnaire aimed at collecting demographic data of the owners, knowledge on pond management practices such as fish farming systems, stocking rate and densities, pond fertilization, pond cleaning and water exchange. In addition, knowledge on bacterial fish diseases and fish health management practices was also assessed.

They included clinical signs, disease prevalence, farmer's ability to diagnose disease, season of disease occurrence, fish disease prevention and treatment.

3.4 Laboratory activities

3.4.1 Culture, isolation and identification

For isolation and identification of bacteria, internal organs (liver, spleen and kidneys and gills) obtained after dissecting the fish were cultured on MacConkey agar, Tryptic soy agar supplemented with 5% sheep blood, Tryptic soy agar and *Aeromonas* isolation agar medium (M884) for between 24 and 48 hours at 28 ^{o}C. Classical identification of bacterial colonies and biotyping was done according to the method described by Abbott *et al*. (2003) and Deen *et al*. (2014) with slight modifications. Briefly, the isolates were conventionally studied for their macro-micromorphological characteristics and then by biochemical assays that consisted of 21 phenotypic characteristics tests. The assays included; lactose, raffinose, trehalose, dulcitol, maltose, mannose, D-mannitol, melibiose, sucrose, citrate, urea, indole, catalase, motility, ampicillin resistance, m-inositol, oxidase, nitrate, cellobiose and xylose. All isolates suggestive of aeromonads were stored in cryovials containing 20% glycerol Tryptic soy broth for further molecular typing.

3.4.2 Molecular genotyping and identification

The genomic DNA extraction was performed by the boiling method according to Carriero *et al*., (2016). The integrity of the extracted genomic DNA was assessed in one percent agarose gel while the concentration of DNA and the purity were spectrophotometrically measured using Nano drop (Thermo Scientific, Waltham, U.S.A) and stored at -20°C until used.

Polymerase Chain Reaction amplification of DNA targeting a high resolving power RNA polymerase sigma factor gene (*rpoD*) was performed in a T1000^{TM} thermocycler (BIORAD). The amplification process followed a protocol used by Carriero *et al*. (2016) with some adjustments as follows; PCR amplification for the *rpoD* gene (820 bp) was carried out in a concoction that included 3.0 µL of 10–50 ng of genomic DNA, 12.5 µL of 2X OneTaq Quick Load Standard Buffer (New England BioLab), 0.5 µL of each primer (0.2 µM) and 8.5 µL Nuclease free water to give a final volume of 25 µL. The reaction mixture was subjected to a PCR regimen of 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 s and extension at 72°C for one minute preceded by an initial denaturation step at 95°C for

three min and followed by terminal extension at 72°C for three min. The amplified product was gel electrophoresed on 1.5% agarose TBE gel stained with EZ-vision In-Gel dye for band size determination through gel documentation system.

The amplicons were submitted to Mbeya Referral Hospital where the nucleotide sequences of PCR products were determined using Sanger method in ABI 3500 Genetic analyzer (Applied biosystem^{TM}, Foster City, California, U.S.A) according to manufacturer's instructions and established protocol). The sets of primers that were involved in the PCR and sequencing are given in Table 2.

Table 2: Primers for detection of *A. hydrophila*

Gene	Primers	Sequence 5'-3'	Position	References
rpoD	rpoD70F	ACGACTGACCCGGTACGCATGTA	280–302	Yamamoto *et al*. (2000)
	rpoD11R	ATGCTCATGCGRCGGTTGAT	1100–1081	Martinez-Murcia *et al*. (2011)

3.4.3 Molecular virulence factor characterization

The presence of virulence factors was determined by assessing their respective genes in the isolates by PCR (Senderovich *et al*., 2012): Aerolysin (*aer*), cytotoxic enterotoxin (*act*), elastase (*ahy*), Hemolysin (*hly*), serine (*ser*) and polar flagella (*fla*). Specific primers for the virulent genes have been given in Table 3.

Table 3: Primers for virulence factors

Gene	Primer	Sequence (5'-3')	Size(bp)	References
Haemolysin	AHH1F	GCCGAGCGCCCAGAAGGTGAGTT	130	Wang *et al*. (2003)
	AHH1R	GAGCGGCTGGATGCGGTTGT		
Elastase	*ahyB*-F	ACACGGTCAAGGAGATCAAC	540	Sen (2005)
	ahyB-R	ATCTTCTCCGACTGGTTCGG		
Flagella	*fla*-F	TCCAACCGTYTGACCTC	608	Sen and Rodgers (2004)
	fla-R	GMYTGGTTGCGRATGGT		
Aerolysin	*aer*-F	CCTATGGCCTGAGCGAGAAG	431	Howard *et al*. (1987)
	aer-R	CCAGTTCCAGTCCCACCACT		
Enterotoxin	AHCF1	GAGAAGGTGACCACCAAGAACA	232	Kingombe *et al*. (1999)
	AHCF2	AACTGACATCGGCCTTGAACTC		
Serine	Ser F	ACGGAGTGCGTTCTTCCTACTCCAG	211	Nam and Joh (2007)
	Ser R	CCGTTCATCACACCGTTGTAGTCG		

For elastase and aerolysin, the PCRs employed the same amplification conditions for the first single denaturation step at 94°C for two min and then a 35-cycling regimen that consisted of denaturation at 94°C for 30s and an extension step at 72°C for 30s. The difference consisted of the annealing temperature which was 60.6°C for elastase and 55.5°C for aerolysin. After the end of the cycles, one final extension step at 72°C for 10 min was added.

Parameters for the amplification of hemolysin gene used an initial denaturation at 95°C for five min, followed by 50 cycles of denaturation at 95°C for 30s, annealing of the primers at 59°C for 30s, and extension at 72°C for 30s (Wang *et al*., 2003). A final extension at 72°C for seven min was used. The PCR amplification for cytotoxic enterotoxin gene was done following the temperature regimen: One cycle of denaturation for 10 minutes at 95°C; 35 cycles of melting at 95°C for 15s, annealing at 66°C for 30s, and elongation at 72°C for 30s; and a final extension round at 72°C for 10 minutes (Kingombe *et al*., 1999).

The amplification conditions for Flagella (flaA/flaB) consisted of an initial single cycle at 95^{o}C for five min, followed by 35 cycles of melting for 25s at 95^{o}C, annealing for 30s at 55^{o}C, elongation for one minute at 72^{o}C and a final single cycle at 72^{o}C for five min (Sen & Rodgers, 2004). The cycling requirement used for serine protease gene was adopted from the work conducted by Nam and Joh (2007). All the implications used the same reaction mixture procedure and setup as in the identification PCR above. The amplified products ware gel electrophoresed on 1.5% agarose TBE gel stained with EZ-vision In-Gel dye for band size determination through gel documentation system.

3.4.4 Phenotypic biotyping of virulence factors

Six virulence factors characteristics were assayed phenotypically as described by Al-Fatlawy *et al*. (2013), Aljanaby and Alfaham (2017) and Osman *et al*. (2018). Briefly, isolates were tested for haemolytic activity by streaking on 7% horse blood agar medium. Lipase activity was done on Tween 20 agar and a colour change on the colonies on the media was characterised using $CuSO_4.5H_2O$ solution. Protease hydrolysis was assayed by streaking on a 2% agar-agar containing 10% (w/v) skimmed milk. Gelatinase was assessed by inoculating the colonies in tubes with medium containing 1.2 g of gelatin in 100 mL of nutrient broth. Motility test was done in sulphide, indole motility (SIM) medium by stabbing a sterile needle containing a well-isolated colony one centimetre to the bottom of the tube. Incubations were done at 37°C for 24 hours. Capsule possession was demonstrated through staining the slide with India ink and counterstained with crystal violet.

3.4.5 In-vivo virulence study with selected virulence factors frequencies of *A. hydrophila* in Nile tilapia fingerlings

The virulence study involved 120 Nile tilapia fingerlings, sourced from SUA, weighing 5 to 10g. The fingerlings were randomly distributed in four treatment groups with two replication tanks, each tank with 15 fingerlings. After five days of acclimatization, the fingerlings were inoculated by the intraperitoneal route with *A. hydrophila* in a combination having aerolysin and haemolysin (B), aerolysin, haemolysin, elastase and enterotoxin (C) and aerolysin, haemolysin, enterotoxin, elastase, flagella and serine (D) virulence genes. All combination contained the aerolysin and haemolysin genes. The inoculum contained bacterial concentration of 10^8 CFU/mL as proposed by Oliveira *et al*. (2012) and the injection dose was 0.2 mL/fish. The same dose of normal saline was given to a control group (A).

The tanks were aerated and physical chemical parameters; pH, temperature and dissolved oxygen were monitored. All fingerlings were fed three times in a day. Water samples were collected from the tanks before inoculation took place for sterility checking and mortality was and culture of dead fish was conducted to recover the bacterium. One-way ANOVA was used to assess variation of the treatments.

3.4.6 Attenuation of selected virulent *A. hydrophila* isolate

Based on the virulence gene possession, phenotypic virulence characteristics, and in-vivo virulence study, the *A. hydrophila* strain TZR7-2018 was selected for vaccine development. This strain has all the six assessed virulent genes; it is encapsulated and causes high mortality in the in-vivo virulence experiment. Attenuation of *A. hydrophila* strain TZR7-2018 here referred to as parent strain TZR7-2018^+, was performed by inoculating the isolate in the tryptic soy broth (TSB) and incubated at 28ºC for 24 h. The culture was then distributed in 1.5 mL eppendorf tubes containing sterile normal saline in 1:1 ratio and preheated in a water bath at a relatively higher than the normal incubation temperature of 28ºC before inoculation on a tryptic soy agar (TSA). Subsequent subculture in TSA was performed proceeded by preheating the passage in the water bath at increasing temperature. A one-fold raise in temperature was used after every two passages. This thermal continuous sub-culturing was done and reaching a total of 40 passages and a maximum temperature of 45ºC. During subsequent sub-culturing the bacterium was evaluated for loss of capsule, motility,

haemolytic activity, cell morphological change and bacterial growth rate as compared to the parent strain.

3.4.7 Preparation of bacterin of *A. hydrophila* strainTZR7-2018^{+}

Bacterial isolate of the parent strain TZR7-2018^{+} was inoculated into the TSB and incubated at 28ºC for 24 h and then inactivated by addition of 40% (W/V) formalin to the broth culture at a final concentration of 0.5 % (V/V) and left at room temperature for 48 h. The suspension was centrifuged at 4000 x g for 10 min to collect the inactivated cells pellet which was then washed twice in a PBS solution and resuspended at a concentration of McFarland standard tube No3 (approx.10^{8} cells/mL). The preparation was checked for sterility by inoculating in TSA at 28ºC for 48 h according to Kamelia *et al*. (2009).

3.4.8 Vaccination of Nile tilapia fingerlings with *A. hydrophila* TZR7-2018^{-}

The experimental setup (number of fish, weight, and source) was similar to the *in vivo* virulence study, above, with slight modifications. Briefly, the fish were randomly grouped into four groups of which three were experimental groups and one control group constituting 30 fish in two replication tanks (each15 fish). Group four (G4) remained unvaccinated and served as a control group. Group one (G1) got the attenuated *A. hydrophila* TZR7-2018^{-} through the intraperitoneal (IP) route at the dose of 1.6x 10^{8} CFU /mL) at the injection volume of 0.1 mL. Group two (G2) fish were immersed in a attenuated *A. hydrophila* TZR7-2018^{-} diluted vaccine in a separate vaccine tank at a ratio of 1 volume of vaccine to 10 volumes of tank water at the same dose of 1.6x 10^{8}CFU/mL for 30 min) (Kamelia *et al*. 2009). Group three (G3) were given *A. hydrophila* TZR7-2018^{+} bacterin mixed with Freund's complete adjuvant at the same dose of 1.6x 10^{8} CFU / mL at the total volume of 0.1mL via IP route. A booster dose of bacterin was given to G3 in day 14 of the observation period which took 28 days before the challenge trial.

3.4.9 Immunogenicity and efficacy of *A. hydrophila* TZR7-2018

Guideline on the design of the studies to evaluate the immunogenicity, efficacy and safety of fish vaccines (EMA/CVMP/IWP/314550/2010) were adhered to. Briefly, in determining the humoral response, antibody titres against *A. hydrophila* TZR7-2018^{-} were measured at intervals of 7, 14, 21 and 28 days after vaccination respectively while day zero served as the baseline. A maximum of 1 mL blood sample from the fish was drawn using a syringe through

the caudal vein into eppendorf tubes and stored at 4°C. Sera were separated by centrifuging the clotted blood at 6000 rpm for 10 min. Each serum sample was heat-inactivated on a water bath at 55°C for 30 min. A two-fold serial dilution of the serum (25µL) was titrated against equal volumes of the heat-inactivated TZR7-2018^{-}bacterial suspension (10^9 CFU/mL). The titre was recorded as the highest dilution indicating a clear agglutination and then it was expressed as $\log_2$ values (Kalita *et al*., 2006).

Fish were challenged with a parent virulent *A. hydrophila* TZR7-2018^{+} at day 28 post vaccination at a dose of 10^9 CFU/mL (established LD_{50}) by IP injection and immersion. The challenge process was conducted through intraperitoneal injection (IP). Mortalities were recorded for 15 days after challenge and internal organs were collected from dead fish and cultured to check for the presence or absence of *A. hydrophila*.

The results of the protective efficacy was presented as relative per cent of survival (RPS) that was calculated according to the formula described previously by Jeong *et al*. (2016) and Zhang *et al*. (2014).

$$RPS = 1 - \left(\frac{\% \text{ Mortality in vaccinated}}{\% \text{ Mortality in control}} \right) * 100$$

3.5 Data handling and analysis

The statistical package for social sciences (SPSS) program was used in descriptive statistical analysis and in a chi-square of independent variables to determine the association between fish size groups developed based on fish weight and infection status. Graph pad Prism 5 software was used for assessing the variation between treatment groups in the vaccination trial, using one-way ANOVA, and differences in antibodies titers between treatment and control groups using Newman-Keuls Multiple comparison test at a level of $p< 0.05$. Data were presented in Tables, graphs and figures using the same software.

Molecular data were analysed by performing alignment of the *rpoD* gene sequences generated from this study and those obtained from the National Center for Biotechnology Information (NCBI) by blasting it on a Basic Local Alignment Search Tool (BLAST) to identify sequence similarity. Sequence editing and assembly was done using Bioedit version 7.2 program and phylogenic tree was constructed using MEGA X program.

(i) Ethical statement

All fish farmers consented to be involved in a semi-structured questionnaire interview before interviewing them. Sampling of fish, dissections and all *in-vivo* experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986 and associated guidelines, the European and the National Institutes of Health – Office of Laboratory Animal Welfare Policies and Laws and the Tanzania Animal Welfare Act of 2008 was complied with. This study also complied with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

4.1.1 The objective one

In this objective, fish farmers were interviewed through semi-structured questionnaire in order to explore selected fish farmers knowledge on pond, fish health and disease management practices. The results on respondent characteristics, pond management characteristics, pond management practices, awareness and knowledge about pond management practices and fish health and water quality parameters of their pond are hereby described.

(i) Characteristic of respondents

Thirty-two (32) fish farmers were interviewed in the all four regions (eight in each region), 87.5% (28/32) were males and the rest were females. Their age ranged from 27 years to 65 years with an average of 39.7±1.5 years. The education levels of the farmers were: Primary (43.8%, 14/32), secondary (31.3%, 10/32) and college (15%, 5/32). Only 3.1% (1/32) possessed vocational training. The majority of them were peasants (62.5%, 20/32) and 25% (8/32) were Government employees, while 12.5% (4/32) were businessmen.

These fish farmers had experience in fish farming ranging from 1 to 11 years with an average of 4.6 ±0.4 years of experience. They own earthen ponds ranging from 90 m^2 to 864 m^2 in size with an average pond size of 454 m^2 and a stocking density ranging from 150 to 10 000 fish per pond. Monoculture fish farming system is the most commonly practiced by fish farmers (68.8%, 22/32) followed by those practicing both monoculture and polyculture (21.9%, 7/32) and polyculture (9.4%, 3/32).

(ii) Pond management practices at the study areas

The majority of the farmers (81.2%, 26/32) reported to fertilize their ponds regularly. Out of them 69.2% (18/26) reported to use cow dung while 3.9% (1/26) mentioned to have used urea and DAP which is inorganic fertilizer (Table 4). These farmers apply the dung either directly from the source (50%) or dry them first before use (50%). Out of those who fertilize their

ponds, 50% spread the fertilizing material on the surface of the pond water while the rest reported to reduce water and dip in the pond. Sixty eight percent have reported to change water and clean their ponds in different circumstances such as after a long stay, discharge of bad smell, water becoming too greenish and when they notice oxygen deficiency in the pond. It was observed that most of farmers stoked their ponds above the recommended stocking rate (Table 4).

Table 4: Pond management practices performed by fish farmers in the study areas

Practice	Category	Frequency	%
Stocking rate	Above recommended (2fish/m^2)	24 (n=32)	75
	Recommended (≤2fish/m^2)	8 (n=32)	25
Pond fertilization	Yes	26 (n=32)	81.2
	No	6 (n=32)	18.8
	Cow dung	18 (n=26)	69.2
	Urea and DAP	1 (n=26)	3.9
	Poultry manure	3 (n=26)	11.5
	cow dung and poultry manure	4 (n=26)	15.4
Fertilizer application	Reduce pond water and apply	13 (n=26)	50.0
	Spread over the surface	13 (n=26)	50.0
	Direct from the source	13 (n= 26)	50.0
	Dry	13 (n= 26)	50.0
Change water and cleaning ponds	Yes	22 (n=32)	68.8
	No	10 (n=32)	31.2
Circumstances of changing and cleaning	Long stay	7 (n= 26)	26.9
	Smelling	9 (n= 26)	34.6
	Too greenish (dark green)	9 (n= 26)	34.6
	Experience oxygen deficiency	8 (n= 26)	30.8

(iii) Awareness and knowledge about pond management practices and fish health

Few farmers (28.1%, 9/32) mentioned to have previously encountered diseases outbreaks in their farms. Of these, 66.7% experienced disease outbreak between May and August, 22.2%

between September and December whereas 11. 1% reported to occur between January and April. Out of 32 farmers, 18 (56.3%) experienced fish death in their farms prior to commencement of this study (Fig. 11). Haemorrhages, slow swimming, pope-eye and reddening were the major clinical signs mentioned and identified by farmers in all study areas (Fig. 12). According to the respondents, 47% could state the reasons for mortality whereas, 18.8% mentioned low oxygen concentration, 12.5% bird injury, 6% bad transportation, 6.3% sudden death and 9.4% mentioned inadequate water and feed supply.

The majority (84.4%, 27/32) of the respondents confessed were ill-informed about control methods. However, a small proportion used other methods, including antibiotics (9.4 %), herbs (6.3%) and separation of infected fish (6.3%).

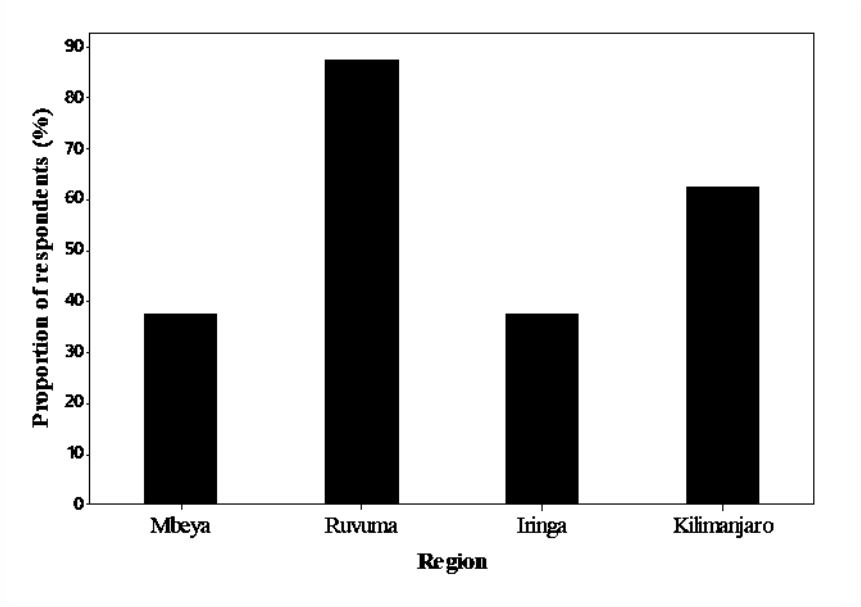


Figure 11: Proportion of respondents who experienced mortality in their fish farms in the four regions

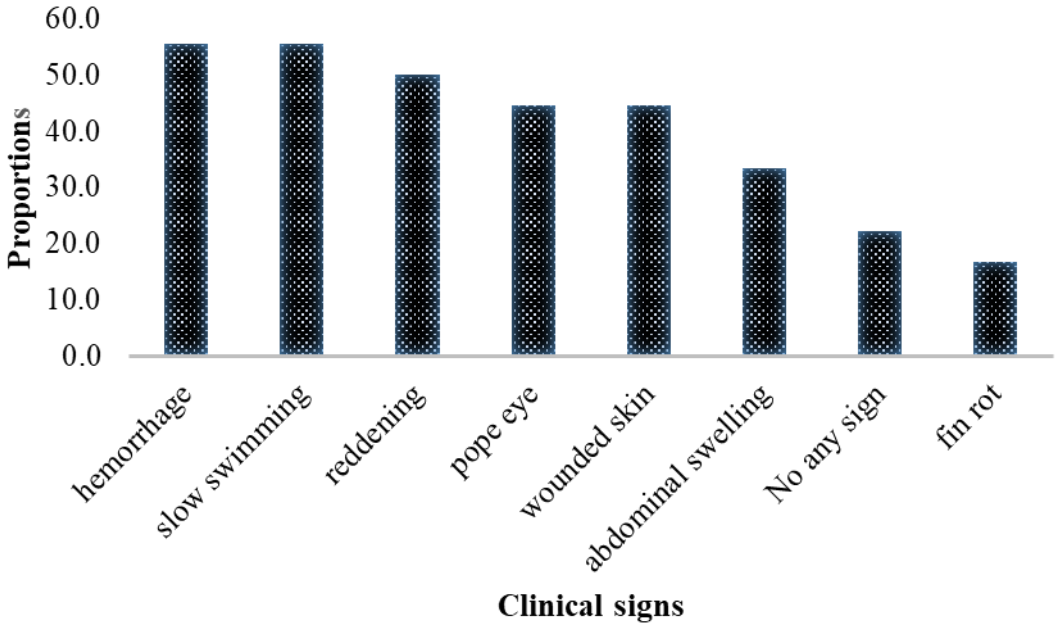


Figure 12: Proportions of fish farmers who reported to have seen clinical signs of fish disease in their farms

(iv) Pond water quality parameters

Generally, the average temperature ranged from 24.9± 0.5^{o}C (Ruvuma) to 26.2±0.4^{o}C (Mbeya). The highest average level of dissolved oxygen in all the four regions was recorded in Mbeya (7.7 ± 0.5 mg/L) and the lowest was in Ruvuma (6.5 ± 0.5 mg/L). Conductivity levels varied between fish ponds within the region and between regions. The average conductivity in fish ponds in all regions ranged between 143.4±32.7 µS/cm and 182.3±49.8 µS/cm. Mbeya region had the highest average fish ponds pH (7.0±0.3) while Kilimanjaro had the lowest (6.6±0.1). The findings for water turbidity can be accessed in Table 5. There was a significant regional variation in temperature and turbidity water parameters ($p<0.05$).

Table 5: Mean physical - chemical parameters in fish ponds by region

Variable	Region	Mean	SE Mean	ANOVA	Preferred range	Stressful range
Temperature (ºC)	Iringa	25.11[b]	0.11			
	Kilimanjaro	25.71[b]	0.22			
	Mbeya	26.24[a]	0.42	*P=0.035	20 to 30	<12, >35
	Ruvuma	24.86 [c]	0.48			
DO (mg/L)	Iringa	7.36	0.61			
	Kilimanjaro	6.81	0.46			
	Mbeya	7.73	0.55	P=0.405	5 to 8	<5, >8
	Ruvuma	6.53	0.52			
pH	Iringa	6.74	0.30			
	Kilimanjaro	6.58	0.12			
	Mbeya	7.03	0.33	P=0.580	6 to 9	<4, >11
	Ruvuma	6.88	0.09			
Turbidity (NTU)	Iringa	33.02[a]	4.26			
	Kilimanjaro	16.05 [b]	1.03			
	Mbeya	18.74 [b]	2.26	*P=0.000	30 to 80	30 to 80
	Ruvuma	10.73 [b]	1.14			
Conductivity (µS/cm)	Iringa	143.4	26.7			
	Kilimanjaro	174.6	39.3	P=0.809	150 to 500	-
	Mbeya	139.6	32.7			
	Ruvuma	182.3	49.8			

Note: The same letter in superscript within the column indicate no significant difference and * indicates a P value < 0.05. The abbreviation DO=Dissolved oxygen

4.1.2 Objective two

In this objective 816 fish samples and their internal organs; liver, spleen, kidney and gills were recovered to isolate, detect and identify aeromonads to specie level through conventional and molecular methods to establish the prevalence. The findings of their morphometric parameters, isolation outcomes, molecular analysis results and the prevalence are described:

(i) Morphometric parameters of sampled fish

Weight and length of fish sampled displayed variability due to random sampling employed at the final stage. The overall fish weight ranged between 10-250 g while that of length ranged from 2 to 15 cm. When fish were grouped based on weight scale in accordance with FAO (FAO, tilapia nutrition requirements) in categories of “fingerlings”, “sub adults” and “adults”,

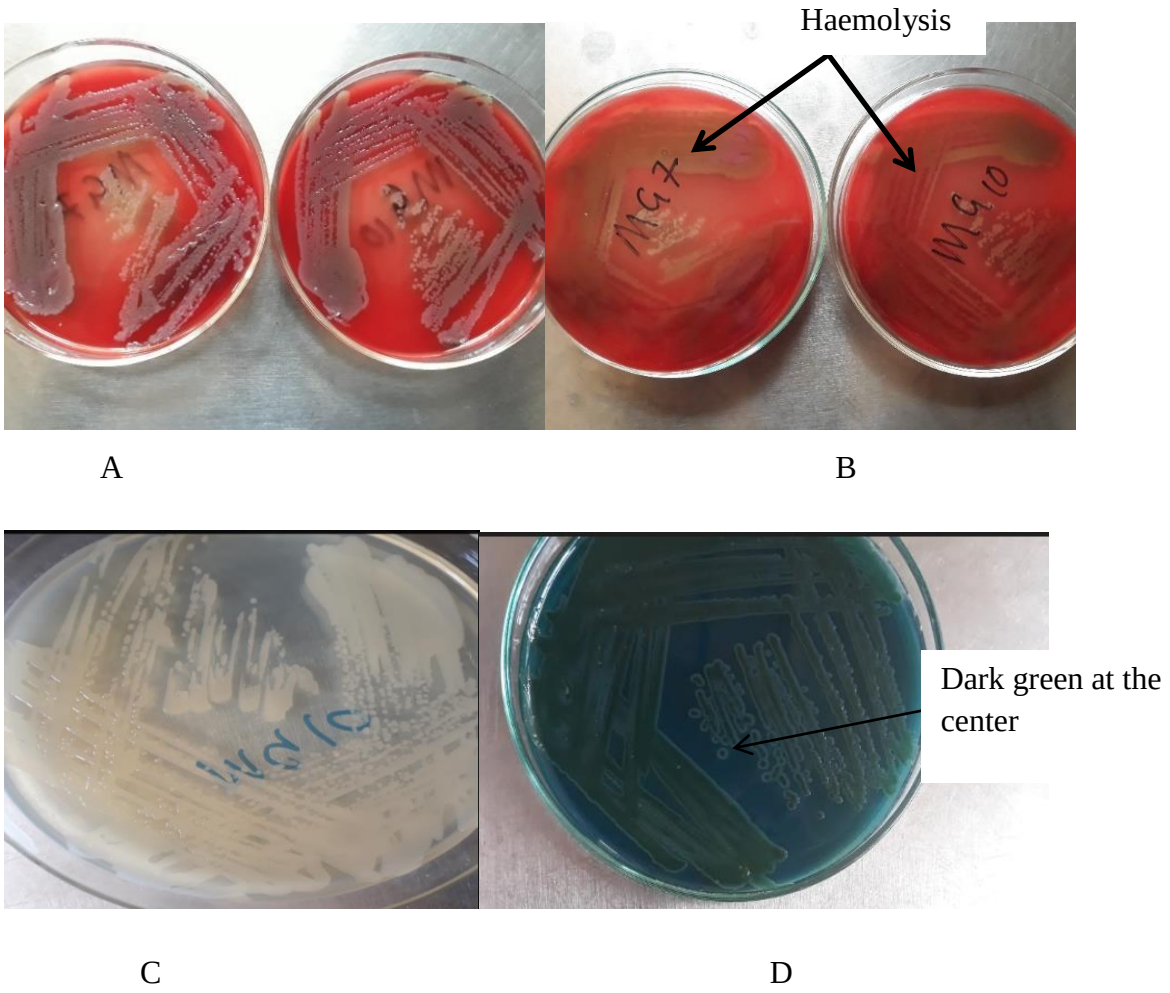
it was revealed that the high percentage (46.5%) were fingerlings (Table 6) as most farmers had mixed sex stocks.

Table 6: Sampled fish grouped based on weight and length

Weight (g)	Category (size)	No of fish	Percentage (%)
1-10	Fingerlings	379	46.5
10-25	Sub adults	231	28.3
>25	Adults	206	25.2
Total		**816**	**100**

(ii) Macro-morphological and microscopic findings

The bacterial colonies assumed to be of aeromonads had medium size (1-3 mm diameter), grayish in color with total hemolysis in blood agar; relatively small and pale colonies (non-lactose fermenter) on MacConkey agar; smooth, shining, creamy colonies on TSA and dark green, opaque with dark centre colonies on Aeromonas isolation medium (M884) (Fig. 13). Upon staining, bacteria were gram negative, rod shaped, in singles and few in pairs.



A B

C D

Figure 13: Colony morphologies of aeromonads in different media. A and B are horse blood agar with B showing total haemolysis characteristics, C is the TSA and D is Aeromonas Isolation Agar (M884)

(iii) Biochemical identification

All suspected aeromonad colonies when subjected to different biochemical tests gave reactions which are characteristic to the genus. The bacteria produced positive catalase, oxidase, D-glucose, citrate, arabinose and mannose reactions (Table 7).

Table 7: Biochemical sugar profile of *Aeromonas* spp

Biochemical test/ Bacteria	*Outcome*
Catalase	+
Oxidase	+
m-Inositol	-
Raffinose	-
Lactose	-
Xylose	-
Cellobiose	-
Maltose	+
Mannose	+
D-Mannitol	+
Melibiose	-
Sucrose	+
Citrate	+
Urea	-
Indole	+
Motility	motile
Ampicillin^{R}	+
Nitrate,	+
D-sorbitol	-
Trehalose	+
Dulcitol	-
Salicin	+

(iv) Prevalence of aeromonads infection in fresh water farmed tilapia

Bacteriological testing of 816 apparently healthy tilapia fish was done from 32 fresh water ponds in Songea Municipality (Ruvuma region), Mbarali District (Mbeya Region), Mafinga Township (Iringa Region) and Rombo District (Kilimanjaro Region). Out of the 816 fish samples, 250 (30.6%) were identified to have been naturally infected with Aeromonas species.

A conventional PCR for identification of Aeromonads was done by amplifying the RNA polymerase gene sigma 70 domain (*rpoD gene*). A total of 201 (80.4%) out of 250 isolates that were conventionally identified using biochemical tests confirmed to be Aeromonads by amplification of 820 bp rpoD gene (Fig. 14), making the overall molecular prevalence of 24.6% (201, n=816), higher in Iringa and Mbeya and least in Ruvuma (Fig. 15A). *Aeromonas* spp were isolated from in gills (40%, 135/339) in Kidneys (17%, 57/339) (Fig. 15B).

When the relationship between fish groups (fingerlings, sub adults and adults) and infection of *Aeromonas* spp was tested using χ^2 test of independent, a statistical association was observed between infection and size groups with fingerlings being more significantly infected with aeromonads than other size groups [χ^2 (1, N=816) = 23.3, P < 0.00001] (Fig. 15C).

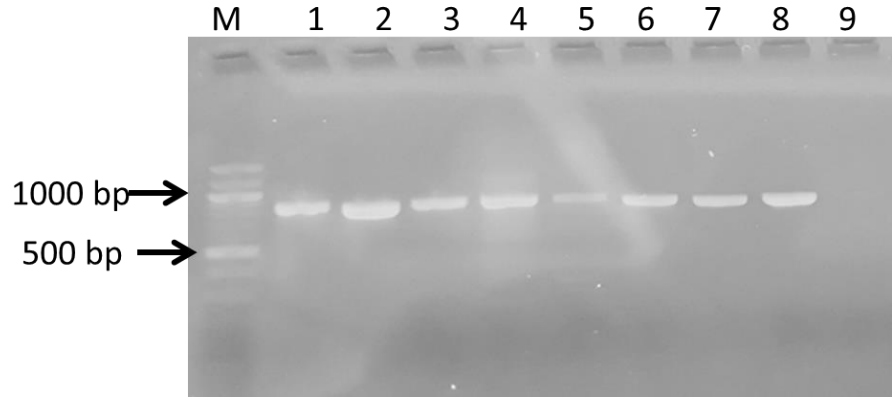


Figure 14: PCR amplification of rpoD gene (820 bp) from aeromonads isolates

Note: Lane 1-7 are representative bacterial isolates, lane 8 is the +ve control, lane 9 is the -ve control and lane M is the DNA size marker (100 bp) DNA ladder (sourced from Inquba Biotec)

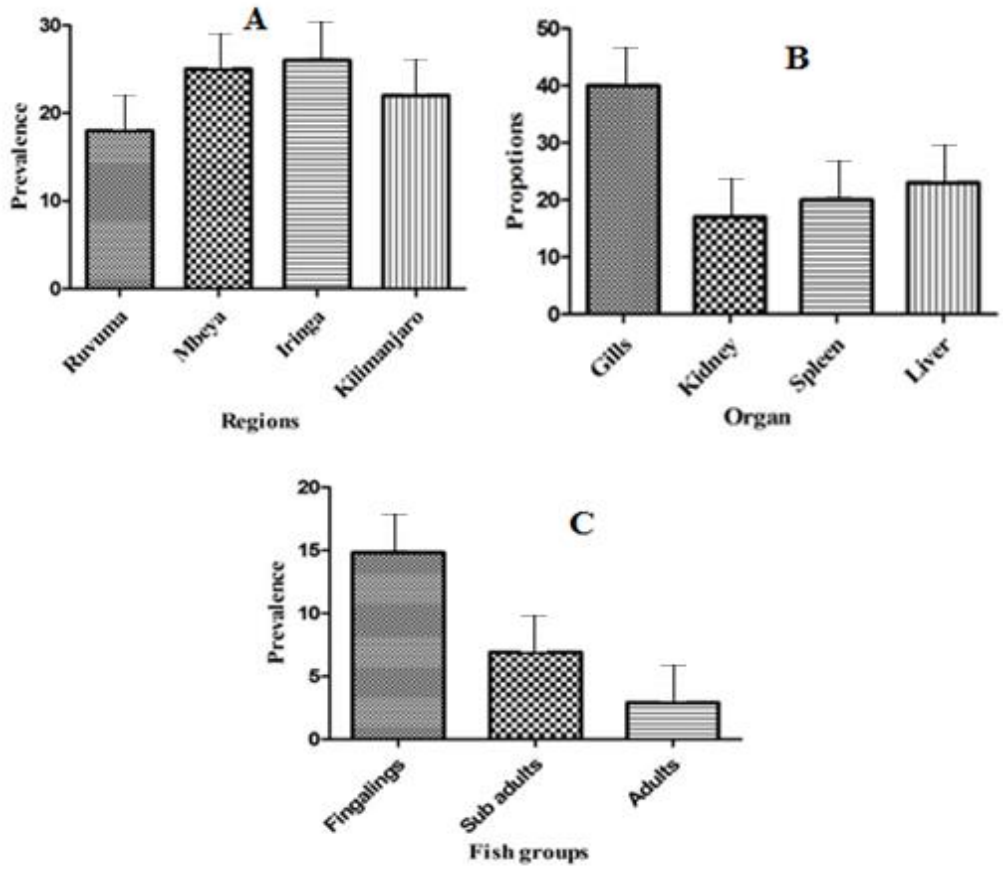


Figure 15: Prevalence of *Aeromonas* spp based on geographical regions (A), fish internal organs (B) and fish groups by size (C)

The phylogenetic analysis of the *rpoD* gene from the isolates displayed sequence homology of 97–99 % with several *rpoD* sequences of *Aeromonas* spp from the GenBank. However, the 201 sequences from this study displayed very minimum variation within species in the two species when phylogenically analysed. The phylogeny grouped the isolates from this study into the clusters of *A. hydrophila* (19.5%) and *A. veronii* (5.1%) in relation to reference sequences from the GenBank (Fig.16).

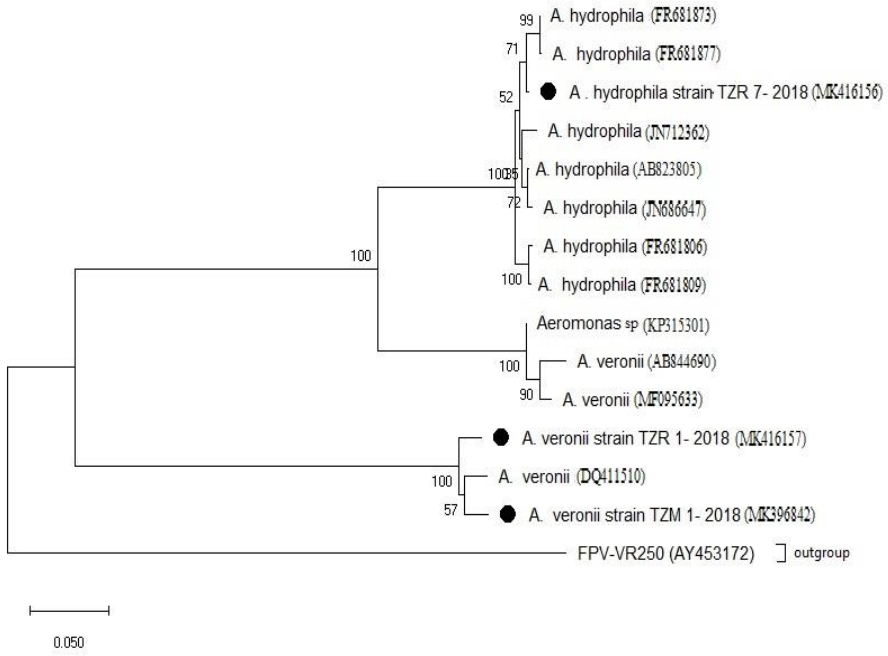


Figure 16: Phylogenetic tree of representative aeromonads isolates from this study (black circle) and closely related taxa from the GenBank

Note: The tree was generated using Neighbor-Joining method (p-distance model), bootstrap values expressed as percentages of 1000 replication. *Fowlpox virus* (FPV-VR250) served as an out-group

4.1.3 Objective three

This objective aimed at assessing the virulence characteristics of the isolated aeromonads phenotypically, molecularly and through in-vivo study in order to determine their inherent attribute in establishing disease and select appropriate isolate for attenuation to serve as a vaccine candidate. The results are hereby described;

(i) Phenotypic characterization

Different phenotypic approaches were used to investigate virulence factors; hemolysis, lipase activity, protease hydrolysis, gelatin liquefaction, capsule possession and motility. Highest proportion of isolates (75.1%, 151/201) displayed protease hydrolysis with least proportion being those possessing capsule (37.8%, 76/201) (Table 8).

Table 8: Outcome (%) of phenotypic biotyping of selected virulence factors of *Aeromonas* genospecies

Virulence| factor	Observation	Outcome (n = 201)
Hemolysin	Presence of colourless zone surrounding the colonies (total haemolysis)	147 (73.1)
Lipase	Turbid zone around colonies with a blue colour change	148 (73.6)
Protease	Presence of transparent zone around the colonies	151 (75.1)
Gelatinase	Absence of liquefaction upon refrigeration	149 (74.1)
Motility	Red turbid area extending away from the line of inoculation	131 (65.2)
Capsule	Unstained clear holo surrounding individual bacilli	76 (37.8)

(ii) Virulence gene detection

Out of 201 isolates confirmed by PCR to be aeromonads, 50 isolates (24.9%) did not possess any of the assessed virulent genes. Of the six assessed virulence genes, haemolysin (*hly*), flagella and aerolysin (*aer*) were observed to occur in most of the isolates of aeromonads with the occurrences being 97%, 87% and 83%, respectively (Table 9). Haemolysin being one of the virulent factors, few (4/151) isolate did not possess it. However, it was observed that 151 (75.1%) of the aeromonad isolates had at least one virulent gene where 120 isolates were *A. hydrophila* and 31 isolates were *A. veronii*. The number of isolates of the two genospecies in a given virulence factors is shown in Table 10. Of the 151 isolates 25.2% had a combination of two genes while 37.7% had a combination of three genes and more. The distribution or possession of virulence genes in aeromonads isolates are shown in Table 11. Detection of these virulent genes resulted to amplification of their respective fragment sizes. Aerolysin gene had a 431 bp, flagella gene 608 bp, enterotoxin 232 bp, haemolysin gene 130 bp, elastase gene 540 bp and serine gene 211 bp (Fig. 17).

Table 9: Occurrence of virulence factors of aeromonads genospecies in the study areas as determined by PCR method

	Ruvuma (n =17)		Mbeya (n = 47)		Iringa (n = 50)		Kilimanjaro (n = 37)		Total % (n =151)
V/genes	# isolates	%	# isolates	%	#isolates	%	# isolates	%	
Hemolysin	16	94	47	100	48	96	36	97	97
Aerolysin	14	82	38	81	43	86	30	81	83
Enterotoxin	7	41	25	53	38	76	11	30	54
Elastase	8	47	27	57	24	48	23	62	55
Serine	10	59	31	65	28	56	12	32	54
Flagella	11	65	39	83	44	88	37	100	87

Table 10: A summary of occurrence of virulence factors between genospecies

Virulent factors	Genospecies [No. (%) positive]		
	A. hydrophila	*A. veronii*	**Total**
	(n=120)	(n=31)	(n=151)
Hemolysin *(hly)*	120 (100)	27 (87.1)	147 (97.4)
Aerolysin *(aer)*	99 (82.5)	26 (83.8)	125 (82.9)
Enterotoxin *(act)*	68 (56.7)	13 (41.9)	81 (53.6)
Elastase *(ahy)*	55 (45.8)	27 (87.1)	82 (54.3)
Serine *(ser)*	52 (43.3)	29 (93.5)	81 (53.6)
Flagella *(fla)*	118 (98.3)	13 (41.9)	131(86.8)

Table 11: Genospecies virulence possession by isolates in the study areas

V/ genes possession	Ruvuma (n = 28)		Mbeya (n=56)		Iringa (n=68)		Kilimanjaro (n=49)	
	Frequency	%	Frequency	%	Frequency	%	Frequency	%
0	11	39.29	9	16.07	18	26.47	12	24.49
1	9	32.14	6	10.71	17	25.00	24	48.98
2	2	7.14	11	19.64	17	25.00	8	16.33
3	2	7.14	25	44.64	11	16.18	4	8.16
4	2	7.14	1	1.79	3	4.41	1	2.04
>4	2	7.14	4	7.14	2	2.94	0	0.00
Total	**28**	**100.00**	**56**	**100.00**	**68**	**100.00**	**49**	**100.00**

Key: V=virulence

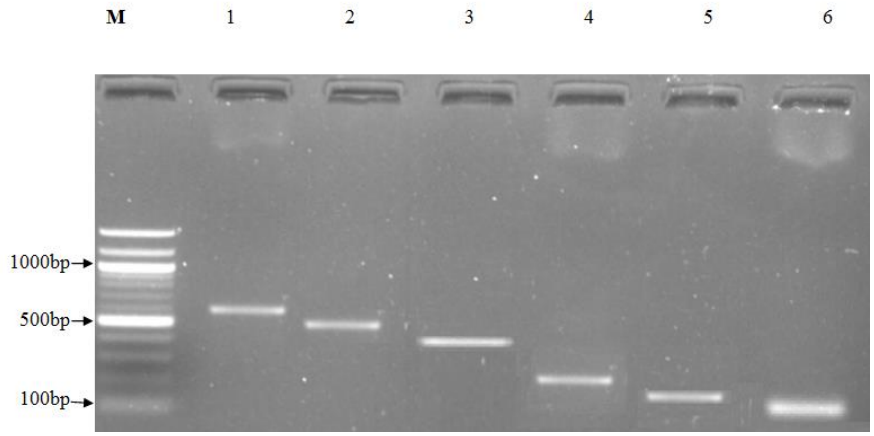


Figure 17: PCR amplification products of the six assessed virulence genes: Flagella (608bp), Elastase (540 bp), Aerolysin (431 bp), Enterotoxin (232 bp), Serine (211 bp) and Hemolysin (130 bp); respectively. Lane M is DNA size marker (100 bp DNA ladder)

(iii) Combination patterns of virulent genes of isolated aeromonads

Generally, there was a varied combination of virulence genes in most of the isolates obtained from samples collected from the four geographical regions of Tanzania namely; Ruvuma, Mbeya, Iringa and Kilimanjaro. Sixty-three percent of the isolates had at least two virulent genes while two isolates (1.3%) had the six virulent genes assessed. Thirteen different combinations were revealed with the virulence gene pattern of *aer /hly/fla* and *aer/ser/hly* being the most prominent with the prevalence of 12.6% and 10.6%, respectively (Table 12).

Table 12: Generalised combination pattern of virulence factors of two *Aeromonas* genospecies

Name of the gene	No of isolates detected n=151	Percentage (%)
hly	18	11.9
act	3	2.0
fla	8	5.3
aer	10	6.6
Ser	9	6.0
ahy	8	5.3
hly / act	5	3.3
hly / fla	11	7.3
hly / aer	12	7.9
act/ fla	6	4.1
act/ aer	4	2.6
aer /hly/fla	19	12.6
hly/act/ fla	7	4.6
aer/ser/hly	16	10.6
hly/act/fla/aer	4	2.6
hly/ser/aer/act	2	1.3
ahy/aer/act/fla	1	0.7
ahy/aer/fla/act/hly	6	4.0
ser/aer/fla/hly/act/ahy	2	1.3
Total	**151**	**100**

KEY: *hly* = Hemolysin gene; *act* = Cytotoxic enterotoxin gene; *fla* = Flagella gene; *ahy* = elastase gene; *ser* = Serine gene and *aer* = Aerolysin gene

(iv) In-vivo virulence study of selected *A. hydrophila* in Nile tilapia fingerlings

A high mortality (98.3%) of fish was observed in the three experimental groups against only 6.7% and 3.3% in the control group at day one and day two respectively. Generally, a higher mortality was recorded in day two. The mortality increased based on the number of virulence genes the *A. hydrophila* isolate possessed. However, no significant difference in mortality was observed between the treatment groups administered with the isolate possessing four

virulence genes and six virulence genes combinations (Fig. 18). No *A. hydrophila* was isolated in the water prior to the commencement of this *in-vivo* study. The bacterium was recovered from internal organs of the dead fish in all treatment groups and none in the control group.

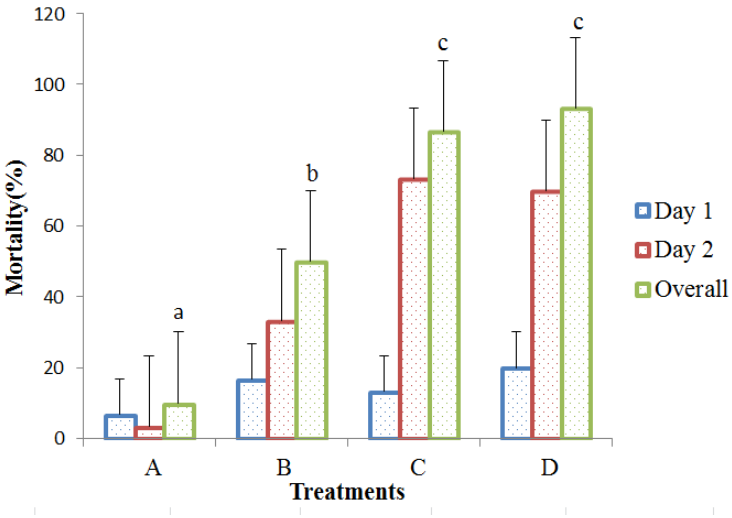


Figure 18: Daily and overall mortality of Nile tilapia fingerlings injected with *A. hydrophila* isolates

Note: Treatment B (two virulence genes), treatment C (four virulence genes), treatment D (six virulence genes) and group A (control, no any *A. hydrophila* injected)

4.1.4 Objective four

The purpose of this objective was to attenuate or reduce the virulence of the selected *A. hydrophila* and test for its immunogenicity and efficacy in order to evaluate its quality of being the vaccine candidate. The attenuation was performed through a novel thermal continuous sub-culturing technique and antibody response assessed using quantitative serological agglutination test (qSAT) while protective efficacy was evaluated through in-vivo challenge with a parent virulent strain. The findings of these assays are summerised below;

(i) Attenuation of *A. hydrophila* strain TZR7-2018

The attenuated *A. hydrophila* TZR7-2018^{-} was assessed for motility, haemolysis, cell size, colony appearance and capsule possession. The isolate was shown to lose the capsule at the 30th passage and no motility was observed. No haemolysis was seen at the 25th passage and colonies appeared smaller in size as compared to the parent strain TZR7-2018^{+} (Table 13). No difference in cell morphology was observed, however, the cells of TZR7-2018^{-} appeared

smaller than the *A. hydrophila* parent strainTZR7-2018^{+} (Fig.19). Bacterial load increased with time of incubation and was higher in parent *A. hydrophila* TZR7-2018^{+} than TZR7-2018^{-} (Fig. 20).

Table 13: Number of passages and changes observed in *A. hydrophila* TZR7-2018- following attenuation in comparison with the parent strain TZR7-2018^{+}

	Passages/days							
Factor	5	10	15	20	25	30	35	40
Haemolysis								
TZR7-2018+	+	+	+	+	+	+	+	+
TZR7-2018-	+	+	+	+	−	−	−	−
Colony appearance								
TZR7-2018+	Large	Large	Large	Large	Large	Large	Large	Large
TZR7-2018-	Large	Large	Large	Small	Small	Small	Small	Small
Motility								
TZR7-2018+	+	+	+	+	+	+	+	+
TZR7-2018-	+	+	+	+	+	−	−	−
Capsule								
TZR7-2018+	+	+	+	+	+	+	+	+
TZR7-2018-	+	+	+	+	+	−	−	−
Cell size								
TZR7-2018+	Large	Large	Large	Large	Large	Large	Large	Large
TZR7-2018-	Large	Large	Large	Large	Large	Small	Small	Small

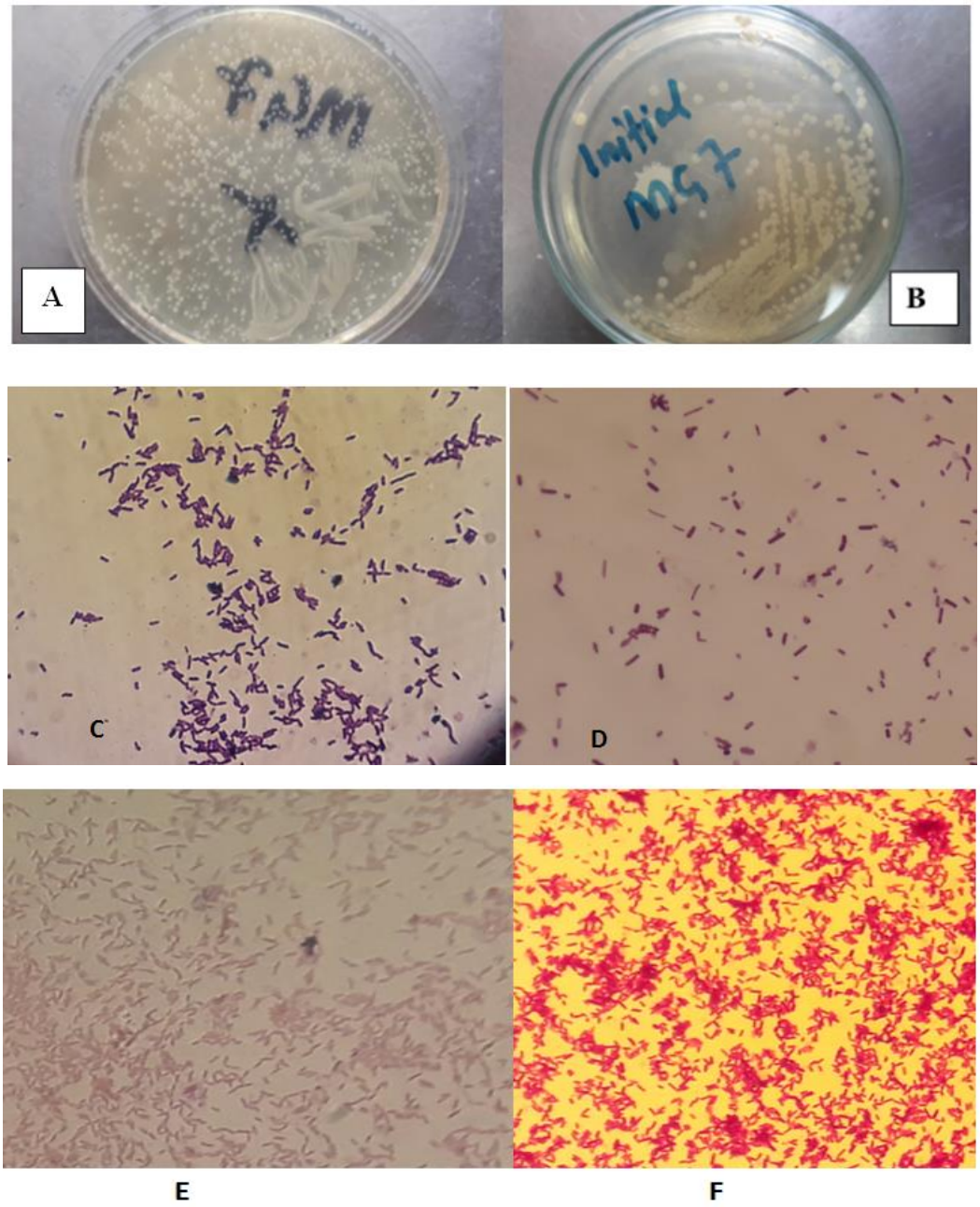


Figure 19: Changes of morphological characteristics in a passaged *A. hydrophila* TZR7-2018$^-$ in comparison to parent strain TZR72018$^+$

Note: Fig.19A and Fig. 19B show colony size in TSA, being smaller in TZR7-2018$^-$ (A). Fig.19C and Fig.19D is Indian ink staining showing presence of capsule in parent *A. hydrophila* TZR7-2018$^+$ (C) and absent in *A. hydrophila* TZR7-2018$^-$ (D). Fig.19E and Fig.19F indicate smaller cell size in TZR7-2018$^-$ (F) compared to TZR7-2018$^+$ (E)

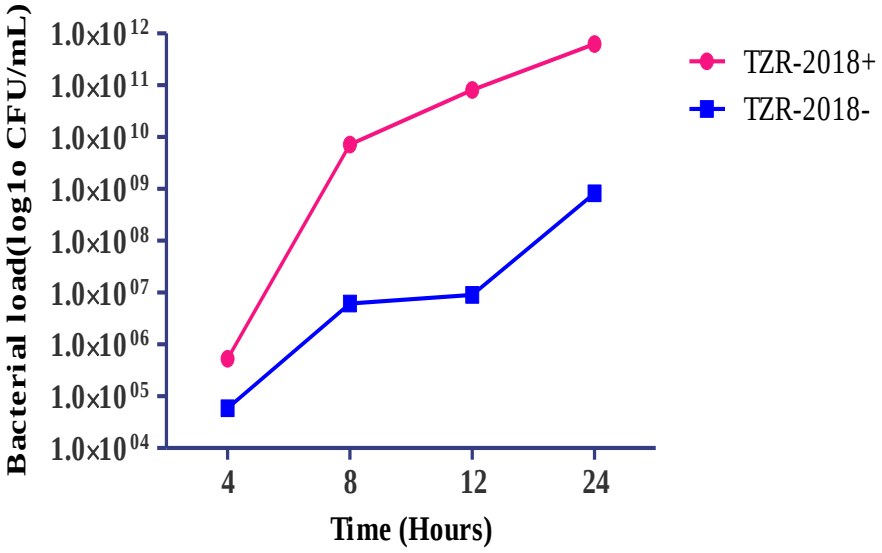


Figure 20: Bacterial load at different incubation time between parent *A. hydrophila* TZR7-2018^{+} and TZR7-2018^{-}

(ii) Immunogenicity and efficacy of the *A. hydrophila* TZR7-2018-

The sera were collected from fish blood and analysed to determine the antibody levels using qSAT. The geometric mean titre (GMT) increased with time during the observation period in all the treatment groups and the maximum titre (GMT log2 6.4) was observed in group one administered with attenuated *A. hydrophila* TZR7-2018^{-} through IP route at the 28th day post vaccination. Lower (GMT log2 4.4) antibody titres were observed in fingerlings in the experimental group vaccinated with attenuated *A. hydrophila* TZR7-2018^{-} via immersion throughout the period of observation (Fig. 21A) as compared to those vaccinated via IP route. The overall results showed no significant difference in antibody levels between the treatment groups ($p > 0.05$), however, marked differences were recorded between all the treatment groups and the control group ($p < 0.05$, Fig. 21B). No mortality or clinical signs characteristic to *A. hydrophila* were observed during the entire study period.

The protective efficacy assay was conducted by challenging all treatment and control groups with a virulent parent *A. hydrophila* strain. In the efficacy trial, the mortality and relative percent survival (RPS) indicated high cumulative mortality in the control i.e. unvaccinated group during the 15 days of observation after challenge. The bacterin showed high protective efficacy having RPS of 85.1% (Fig. 22) while the attenuated *A. hydrophila* TZR7-2018^{-} given by immersion showed a lower relative percent survival (71.4%). However, no significant difference in protection (RPS) was observed between the three treatment groups ($p > 0.05$).

Aeromonas hydrophila were recovered and confirmed by PCR in the fish that died after challenge. Most fish of the control group that died showed scattered skin haemorrhages and exophthalmia.

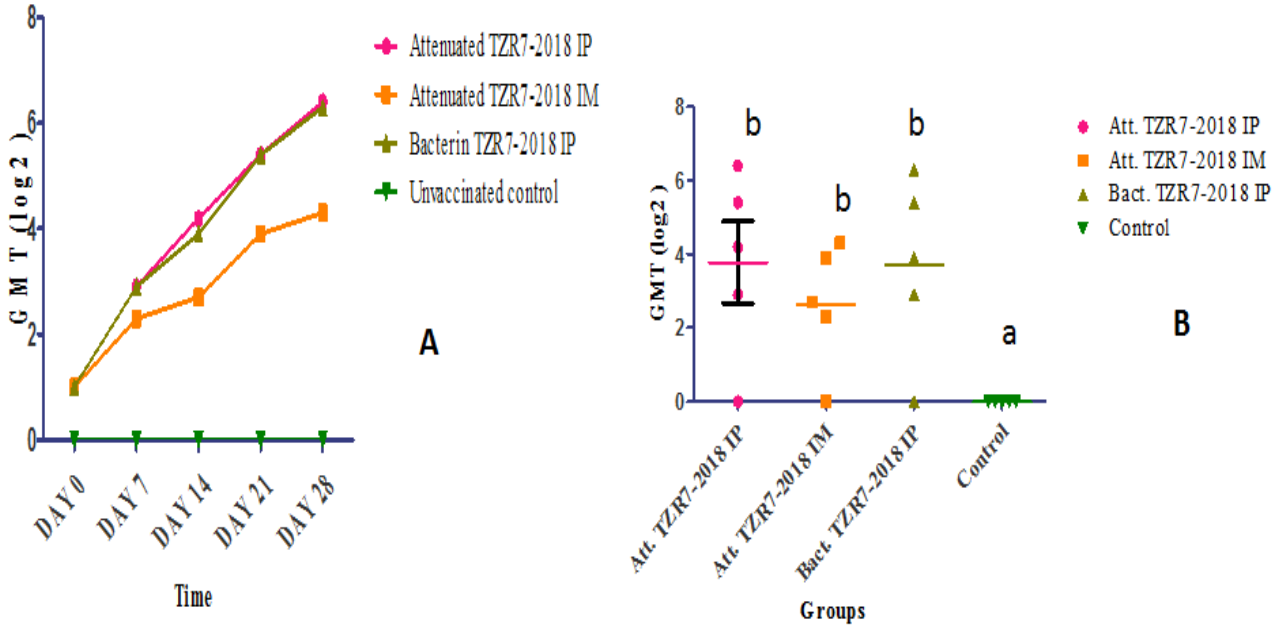


Figure 21: Level of Abs GMT according to route of administration.

Note: The Fig. 21A and 21B indicate antibody increase during observation period and the overall performance of each treatment respectively

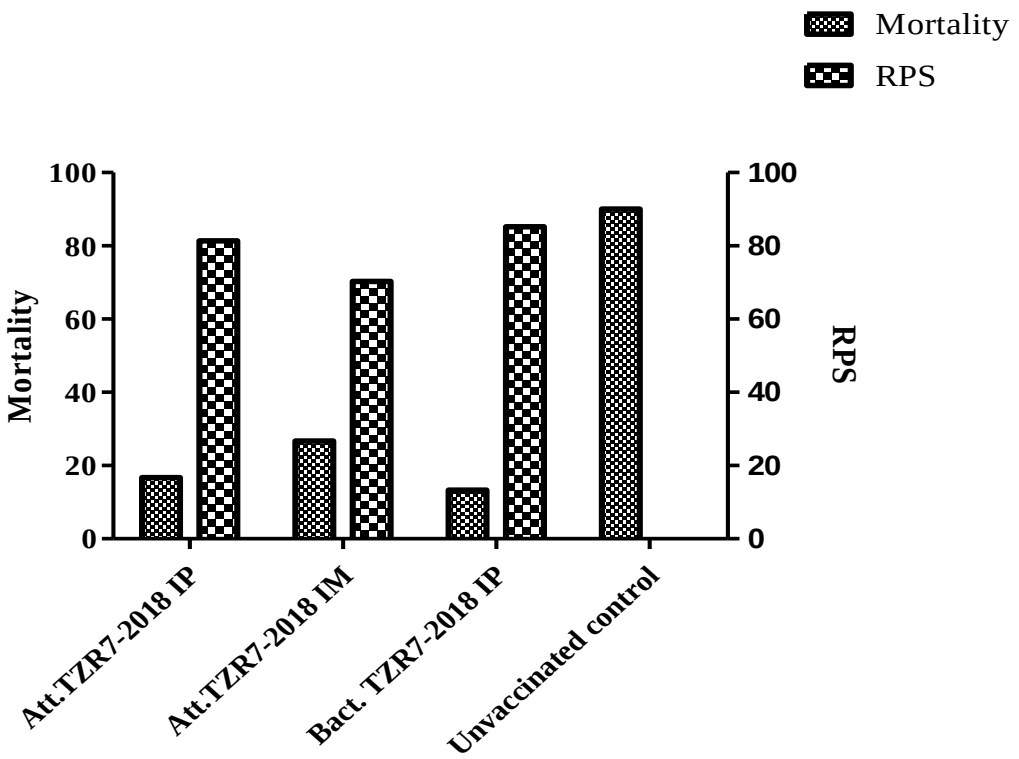


Figure 22: Mortality and RPS of the fish vaccinated with attenuated and bacterin of *A. hydrophila* TZRR7-2018- by IP and IM routes

4.2 Discussion

This study was carried out to characterise, identify and establish the prevalence of aeromonads, the group of negative bacteria which cause haemorrhagic septicemia or motile aeromonad septicemia in farmed fish leading to economic loss to fish farmers. Assessment of knowledge of selected fish farmers on pond, fish health and disease management practices were also conducted. The findings from these studies were necessary to support the development of a vaccine candidate for controlling aeromonads outbreaks and improve tilapia production in Tanzania. The development of vaccine candidate was achieved through attenuation *of A. hydrophila* strain TZR7-2018 through thermo-continuous sub-culturing technique and its immunogenicity and efficacy was successfully tested.

Knowledge on how to play, control and balance between environmental conditions and human interaction is vital. Farmers interviewed on knowledge of pond management practices and fish health management revealed that they have inadequate knowledge and are not aware of some pond management practices (Chenyambuga *et al*., 2014). High stocking rate and

poor ways of fertilizing pond are some of them. Assessment of knowledge and awareness on fish health management showed that the majority of these selected farmers lack knowledge on disease diagnosis based on clinical signs. The exception were farmers from Ruvuma region who were familiar with the most common clinical signs based on previous experience of fish mortalities in their farms. One of the most common methods for managing diseases on fish farms is the application of antibiotics (Chitmanat *et al*., 2016), however, the majority of fish farmers in the study areas were ill-informed of any method of managing, and controlling fish diseases. Biosecurity measures, good pond management practices coupled with other fish disease control methods such as vaccination are of paramount importance towards climate smart aquaculture. Tanzania requires policy guidance and sector empowerment in fish farming. Therefore, efforts must be made to train farmers on biosecurity measures, pond management practices, and on potential risks of bacterial diseases.

Aeromonads disease outbreaks are one of the important limiting factors to sustainable fish farming worldwide (Ibrahem *et al*., 2008). This study reports the occurrence and identification of aeromonads for the first time in farmed tilapia in Southern Highlands and Northern Tanzania regions at an overall prevalence of 24.6% without clinical disease being reported in the farms. The prevalence is close to that reported by Deen *et al*. (2014) in Egypt.

As it was explained by Lio-Po *et al*. (2001) disease occurrence in fish farms is a function of the pathogen, host and the environment. Favourable environment could explain the absence of the disease the time of this study. The two *Aeromona*s species identified from farmed tilapia in this study (*A. hydrophila* and *A. veronii*) are known etiological agents of disease outbreaks in freshwater tilapia farms. However, detection in kidneys, the liver and spleen of apparently healthy fish are not startling because they are ubiquitous in the aquatic environment. The high proportion of infection in gills in comparison to other organs is due to constant exposure of the organ to microbiota (Mwega *et al*., 2019).

Identification of members of the family *Aeromonadaceae* in apparently healthy fish corroborates with a previous report by Omeje and Chukwu (2014), who found these bacteria in both apparently healthy as well as diseased fish. Despite being detected in apparently healthy fish, these species remain a potential risk to disease outbreaks where pond management practices are poor. It is well-known that aeromonads affect all ages and sizes of fish (Camus *et al*., 1998); however, the current findings reveal that fingerlings are relatively more affected (16.9%) compared to other age groups (sub-adults = 9.3% and adults = 4.4%).

This is in agreement with the report by Camus *et al*. (1998). The outbreaks of aeromonad diseases are seasonal being experienced more in summer (Ibrahem *et al*., 2008). In this study, fish farmers reported previous outbreaks occurred between May and August, which is a warm and dry season in Tanzania.

Detection of virulence factors phenotypically and by the presence of virulence genes in fish with clinical disease or in apparently healthy fish. have become common measures of putative virulence and pathogenicity of several species of the genus *Aeromonas* (Hoel *et al*., 2017; Khajanchi *et al*., 2010; Oliveira *et al*., 2012; Silva *et al*., 2017). Li *et al*. (2011) showed that the phenotypic characteristics of virulence factors and presence of their genes in different combinations correlates very well with in-vivo pathogenicity study, stressing on their potential use as virulence markers.

In this study we put in evidence six virulence factors in 201 *Aeromonas* isolates of which 151/201 (75.1%) had at least one virulence gene. A total of 120 isolates were *A. hydrophila* and 31 isolates were *A. veronii.* However, 63% of these aeromonad isolates had at least two virulence genes. These figures closely fall to those reported by Oliveira *et al*. (2012), indicating potential these isolates in establishing diseases in farmed fish if suitable environmental conditions are favourable (Hoel *et al*., 2017; Silva *et al*., 2017).

In addition, infections by potentially pathogenic *Aeromonas* may not necessarily lead to disease in situations where the host responses are strong and the bacterial challenge (infectious dose) is low. Nonetheless, the absence of the six virulence genes in 24.9% of the isolates does not exclusively eliminate them from being potential pathogens of fish. This is because different species and isolates may possess other different pathogenicity instruments (Silva *et al*., 2017).

Haemolysin, aerolysin and flagella genes were the most prevalent virulence genes regardless of the geographical region studied, demonstrating that the circulating aeromonads in the four study regions are closely related in terms of putative virulence. Possession of capsule is one of the important virulence factors of bacterial pathogenesis. This virulence factor was observed mostly in clinical isolates from humans (Al-Fatlawy *et al*., 2013). However, in this study few isolates (37.8%) from apparently healthy farmed tilapia were found to possess the capsule indicating the bacterial e potential to escape the host immune cells and resist antimicrobial agents.

While cytotoxic enterotoxins, extracellular haemolysins and aerolysins are known to be the major contributor to pathogenicity of *Aeromonas* spp, multifactorial interaction of these virulence factors and other virulence factors cannot be undervalued. Observations of the 151 isolates from this study revealed 13 combinations having two (25.2%) and more (37.7%) of the virulence genes, with aeroA being core virulence factor in these combinations. While some studies proposed a combination of two genes as an indicator of virulence to their host animals, others have reported the likelihood of causing diseases in the host to be positively correlated with the increasing numbers of virulence gene they possess in a pathogen (Sha *et al*., 2009). Similar observations were reported by Li *et al*. (2011) and Oliveira *et al*. (2012) in their studies who found more mortalities in experimental fish injected with aeromonads isolate with more virulence factors and so does in this study, making this to be the best explanation.

Attenuation of the selected *A. hydrophila* strain TZR7-2018 to serve as a local vaccine candidate was effective through thermo-continuous-sub-culturing technique. The process led to the loss of some virulence factors such as motility, haemolysis and capsule. Reduced multiplication rate, reduced colon size and changes in cell size were also observed in the attenuated strain TZR7-2018^{-}. These effects were also demonstrated by Pridgeon (2012) using a novobiocin selection. Although Jiang *et al*. (2016) and Pridgeon (2012) reported successful attenuation with antibiotic selection after 20 passages, this study has achieved successful attenuation after more than 20 passages and at different passage in point.

In assessing the performance of the attenuated vaccine candidate in Nile tilapia fingerlings, antibody levels were shown to increase in titre sup to day 28 of the observation period. However, there was gradual elevation in antibody titres as from day 7 to day 28 in the three treatment groups, indicating maintenance of potential immunogenicity of the passaged TZR7-2018^{-} strain.

Despite the statistically marked difference in humoral response between treatment groups and the unvaccinated control group, no significant variation was observed among the three-treatment group themselves. However, lower immune response was observed when the attenuated vaccine through IM compared to the IP route. This could be because most antibodies are localised to mucosal part (IgT/Z) and cannot be detected. The poor penetration of the vaccine agent can lead to low antibody level to the circulation system. As time of

exposure and vaccine concentration can be also factors, further study is needed to optimise its performance.

The *in-vitro* attenuation outcome and humoral response results of the two-vaccine formulation (attenuated and bacterin) of strain TZR7-2018 given through IP and IM routes were confirmed through protective efficacy in the in-*vivo* study. Bacterin provided through injection showed a higher protection level (85.1%) followed by attenuated vaccine given through IP (82.3%), however, the difference was not statistically significant. Contrary to the findings of this study where immersion recorded a lower RPS of 71.4% compared to IP, Kamelia *et al*. (2009) reported high protective efficacy to the vaccine given through immersion than by oral route and injection. Other researchers have explained the variability of vaccine efficacy when administered through immersion (Nakanishi & Ototake 1997). This route largely depends on the fish species, exposure time and vaccine concentration. In addition, as it mimics natural infection through skin, gills and oral cavity, the maximum dose that induces optimal immune protection may sometime not be attained. Nonetheless, the use of immersion if successful is a stress free, user friendly and an economically viable method in terms of cost and labour (Munang'andu *et al*. 2015). According to Varvarigos (1999), the immersion vaccine used in this study, giving a protective efficacy (71.4%) showed successful outcome that is economically acceptable.

Furthermore, the application use of antibiotic resistance selection as the method of attenuation has been a common procedure in the development of *A. hydrophila* vaccine candidate. Rifampicin and novobiocin have been used to attenuate *Flavobacterium columnare, Edwardsiella ictaluri* and *Streptococcus iniae* (Jiang *et al*. 2016; Pridgeon, 2012). However, the application of thermo-continuous-sub-culturing technique which has shown to be effective in this study is of interest and would be helpful as this will reduce the risk of spill-over of resistant strains of bacteria in the aquatic environment. As it was stated by Jiang *et al*. (2016) and Pridgeon (2012), the mechanism of attenuation of *A. hydrophila* with antibiotic selection is not well understood; and so are, the mechanisms of attenuation using thermo-continuous-sub-culturing technique. This is because passaging of bacterial isolates with the two approaches does not necessarily end up in partial or complete attenuation.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusion

The infection rate of aeromonads in apparently healthy tilapia fish coupled with limited knowledge and awareness on proper pond management practices and fish health management by the selected fish farmers in the study area poses the risk of disease outbreaks in their farms. Therefore, the selected farmers in the study regions needs to be trained on basic pond and fish health management practices and control strategies while striving for full development, registration and licensures of the local vaccine.

The prevalence of 24.6% of aeromonads (*A. hydrophila and A. veronii*) infections in tilapia farms in the four studied regions of Tanzania has been established.

Among the isolated aeromonads, 75.1% have been identified to possess virulence factors with haemolysin, aerolysin and flagella genes being at high prevalence. This suggests a close relatedness in terms of putative virulence, while *in-vivo* pathogenicity study shows the potential of these specie to cause disease under favourable conditions.

The selected *A. hydrophila* strain TZR7-2018 has been successfully attenuated through thermo-continuous subculture technique. It proved to be efficacious when the bacterin was given through IP than by immersion. To the best of my knowledge, this is the first time the thermo-continuous sub-culturing technique has been used in Africa or elsewhere to develop a vaccine candidate for controlling aeromonads diseases of fish.

5.2 Recommendations

The assessment of the changes that occurred to the attenuated TZR-2018^{-} strain at genomic level in comparison to the parent TZR-2018^{+}strain is also required to add up to knowledge of this inducible attenuation. Optimization of the immersion route of administration with both homologous and heterologous virulent strain of *A. hydrophila* is also recommended. In addition, further work is required to carry out, safety, shelf life and a possible reversion to virulence for this vaccine candidate, under field conditions.

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APPENDICES

Appendix 1: Questionnaires

Instrument Title: A questionnaire on investigation of fish bacterial diseases and health management in Southern High lands and Northern regions of Tanzania

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SECTION A: DEMOGRAPHIC DATA

We would like you to respond to the following questions. The questionnaire is meant to be anonymous although your responses to the demographic questions could possibly identify you. The questionnaire will not be linked to your name. You do not have to answer every question.

1. What is your age? _____(years)
2. What is your gender? Male ☐ Female ☐
3. What is your education level?
   (a) Primary level ☐
   (b) Secondary level ☐
   (c) High school level ☐
   (d) College level ☐
   (e) Other vocational training ☐
4. What is your occupation?
   a. A government employee ☐
   b. A farmer ☐
   c. A NGO employee ☐
   d. A businessman ☐
5. How many years have you been in this field? _____ years
6. What is the size of your pond area _____ M^2

7. What type of culture system are you practicing?
   a. Polyculture
   b. Monoculture

SECTION B: FISH DISEASES

8. Did you experience any fish disease problem in your farm?
    a. Yes ☐
    b. No ☐
9. In what season (s) of the year did you experience disease problem in your farm
    a. January to April ☐
    b. May to August ☐
    c. September to December ☐
10. Can you estimate the stocking density of your farm?
11. Can you tell the number of fish that died after introduction of fingerlings in your farm?
12. What do you think can be the cause of mortality other than infectious disease……
    ……
    ……
    ……..
13. Tick the clinical signs that commonly appear in your farm when experiencing a disease problem
    i. Pop eye ☐
    ii. Ventral reddening ☐
    iii. Tail and fin rot ☐
    iv. Hemorrhages ☐
    v. Wounded skin ☐
    vi. Gill rot ☐
    vii. Slow swimming ☐
    viii. Abdominal swelling ☐
    ix. No any signs ☐

SECTION C: HEALTH MANAGEMENT

14. What methods do you know in managing diseases on fish farms
    i. Apply antibiotics ☐
    ii. Treat with KMnO4 ☐
    iii. Apply herbs ☐
    iv. Treat with formalin ☐
    v. Separate infected fish ☐

vi. Apply vaccine ☐

vii. I don't know any method ☐

15. If you are using antibiotics, which one are you commonly using

……

……

……

……..

16. Where do you get assistance when there is a problem in your farm?

i. From the government organization (GO) ☐

ii. From non-governmental organization (NGO) ☐

iii. Neighbours ☐

iv. Fisheries officers ☐

v. Radio ☐

vi. No assistance ☐

17. Which problems do you face in controlling fish diseases?

i. Lack of assistance ☐

ii. Lack of proper knowledge ☐

iii. Unavailability of medicine/vaccine ☐

iv. Lack of training ☐

v. Lack of money ☐

18. Do you fertilize your pond?

i. Yes ☐

ii. No ☐

19. By using what?

……

…….

20. How do you apply your material for pond fertilization……

……

……..

21. Do you clean and change water in your pond(s)?

Yes ☐

No ☐

22. Under what circustances ……………………………….…………………………….

………………………………………………………………………………………..