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PHENOTYPIC, MOLECULAR DETECTION AND ANTIBIOGRAM ANALYSIS OF *AEROMONAS HYDROPHILA* FROM *OREOCHROMIS NILOTICUS* (NILE TILAPIA) AND READY-TO- EAT FISH PRODUCTS IN SELECTED RIFT VALLEY LAKES OF ETHIOPIA

MSC Thesis

By:

NEBIYU KASSA

JUNE, 2021

BISHOFTU, ETHIOPIA

PHENOTYPIC, MOLECULAR DETECTION AND ANTIBIOGRAM ANALYSIS OF *AEROMONAS HYDROPHILA* FROM *OREOCHROMIS NILOTICUS* (NILE TILAPIA) AND READY-TO- EAT FISH PRODUCTS IN SELECTED RIFT VALLEY LAKES OF ETHIOPIA

A thesis submitted to the College of Veterinary Medicine and Agriculture, Addis Ababa University in partial fulfillment of the requirements for the Degree of Master of Science in veterinary science (MVSc) in Veterinary Epidemiology

By

Nebiyu Kassa Kerigano

June, 2021

Bishoftu, Ethiopia

ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

DEPARTMENT OF CLINICAL STUDIES

As MVSc research advisor I hereby certify that I have read and evaluated this Thesis prepared under my guidance by **Nebiyu Kassa** entitled: **Phenotypic, Molecular Detection and Antibiogram Analysis of *Aeromonas Hydrophila* from *Oreochromis Niloticus* (Nile Tilapia) And Ready-To- Eat Fish Products in Selected Rift Valley Lakes of Ethiopia,** I approved for submission to dissertation assessment committee.

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As members of Examiners Board of the final MVSc open defense, we certify that we have read and evaluated this Thesis prepared by **Nebiyu Kassa** entitled: **Phenotypic, Molecular Detection and Antibiogram Analysis of *Aeromonas Hydrophila* from *Oreochromis niloticus* (Nile Tilapia) And Ready-To- Eat Fish Products in Selected Rift Valley Lakes of Ethiopia,** and recommend that it is accepted as fulfilling the thesis requirement for the degree of Master of Veterinary Science in Veterinary Epidemiology.

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STATEMENT OF THE AUTHOR

First, I affirm that this thesis is my solely work and that all sources of material used for this MVSc thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for Masters of Veterinary Science (MVSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic award.

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LIST OF ABBREVIATIONS

BPW: Buffered peptone water

CDC: Centers for Disease Control and Prevention

DNA: Deoxyribonucleic acid

ELISA: The enzyme-linked immunosorbent assay

HGs: Hybridization groups

LFCDP: livestock and Fishery Sector Development Project

MALDI-TOF: The matrix-assisted laser desorption/ionization time-of- flight mass spectrometry

MDP: Muramyl dipeptide

MIC: Minimum Inhibitory Concentrations

MLST: Multilocus Sequence Typing

NaCl: Sodium chloride

NAHDIC: National Animal Health Diagnostic and Investigation Center

OMPs: Outer membrane proteins

PMQR: Plasmid-Mediated Quinolone Resistance

RFLP: Restriction Fragment Length Polymorphism

RNA: Ribonucleic acid

TCBS: Thiosulphate Citrate Bile salts

TSA: Tryptic soy agar

TSI: Triple sugar iron

ABSTRACTS

Aeromonas hydrophila is a zoonotic bacterial pathogen that frequently causes disease and mass mortalities among cultured and feral fishes worldwide. In Ethiopia, *A. hydrophila* outbreak was reported in Sebeta fish ponds and in lake Tana fishery. However, there is no to little information on the molecular, and phenotypical characteristics of *A. hydrophila* in Ethiopian fisheries. Therefore, a cross-sectional study was conducted from November 2020 to May 2021 in selected Ethiopian Rift valley lakes namely Koka, Ziway, Langano and Hawassa Lakes with aim of isolation and determination of phenotypic and genotypic features of *A. hydrophila* infecting tilapia and fish products in respective towns. A total of 140 samples were collected aseptically from fish (Muscle, Gill, Intestine, Spleen and Kidney) from fish landing sites, market and restaurants from respective towns with purposive sampling methods. Aeromonas selective media (AMB), morphological and biochemical tests were used to isolate and identify *A. hydrophila*. Accordingly, the pathogen was isolated from 81 (60.45%) of samples. Among the isolates 92.59% expressed virulence trait through β hemolysis on blood agar media with 5% sheep blood. Moreover, 54 strains (66.67%) were further confirmed with Real-Time PCR (qPCR) using *ahaI* gene specific primers and optimized protocol. The highest (68.51%) were detected from live fish, (24.07%) were from market fish and the lowest (7.4%%) were from RTE. Antibiogram analysis was conducted on ten representative isolates. Accordingly, *A. hydrophila* isolates were susceptible to ciprofloxacin (100%), chloramphenicol (100%) and ceftriaxone (100%). However, all ten isolates were resistant to Amoxicillin and Penicillin. The present study revealed virulent, Amoxicillin and Penicillin resistant *A. hydrophila* stains in fish and fish products. Interestingly adhesive (*ahaI)* gene was detected in majority of the isolates. Moreover, we optimized the first Real time PCR protocol that would be useful for molecular epidemiological studies of *A. hydrophila*. In conclusion, *A. hydrophila* strains carrying virulence *ahaI* gene that were ß-hemolytic and resistant to antibiotics commonly used in human and veterinary medicine are circulating in the fishery. The detection of the pathogen in 140 of the sampled fish population is alarming for potential outbreaks and zoonosis. Therefore, further molecular epidemiology of the disease should be studied to establish potential inter host transmission and antibiotic resistance traits. Therefore, raising the public awareness on risk associated with consuming undercooked or raw fish meat is pertinent. Designing prevention and control strategies against *A. hydrophila* to safeguard the fishery sector is highly recommended.

Key words: *A. hydrophila, Ethiopia, Nile tilapia, qPCR, Rift valley lake*

1 INTRODUCTION

Nile tilapia (*Oreochromis niloticus*) is one of the commercially important fast-growing and well adapted freshwater fish that is produced extensively and intensively all over the world (FAO, 2014). Tilapia are increasingly used in aquaculture and is currently the second most important freshwater fish farmed worldwide with an annual global production of 6.4 MT (Nicholson et al., 2020). It is characterized by their reasonable resistance to diseases and its suitability for intensive farming which subsequently leads to increased production and makes it as a cheap protein source for all people (Abdel-latif, 2020). Nowadays this high protein source is threatened by bacterial diseases especially those caused by drug resistance and highly virulent bacteria such as *A. hydrophil*a (Mansour et al., 2019). Significantly impeding both economic and socioeconomic developments in regions dependent on aquaculture and fisheries and zoonotic implications as well (Fowoyo and Achimugu, 2019).

A. hydrophila is facultative anaerobic, Gram-negative bacteria that belong to the family Aeromonadaceae which is cosmopolitan in distribution and have a broad host spectrum with both cold and warm blooded animals including humans (Dong et al., 2020). *A. hydrophila* is a well-known bacterial pathogen that frequently causes disease and mass mortalities among cultured and feral fishes worldwide (Y. Zhou et al., 2019). *A. hydrophila* has gained increased attention due to pathogenicity to humans and emerged as a foodborne pathogen of extreme importance (Zhong, 2019). *A. hydrophila* resulting serious health condition and death associated with consumption of frozen fish in market-sold sushi products containing raw fish (Wu and Ko, 2019). High antibiotic resistance is seen in *A. hydrophila* infections (Aa and Najiah, 2013) and regarded universally exhibit resistance to the penicillin for quite a long time (Stratev and Odeyemi, 2016) nowadays, becoming a serious public health concern. In Ethiopia however, less attention has been given to pathogens of fish including those which have zoonotic importance except few isolated cases (Nuru et al., 2012). For instance, a survey of bacterial and parasitic fish pathogens was conducted in Lake Ziway but *A. hydrophila* was not included (Yimer, 2000). *A. hydrophila* was reported as the most frequent isolate from Lake Tana and also the pathogen was associated with outbreak and mortality in Sebeta fish ponds (Almaw et al., 2014).

In Ethiopia, intensive and semi-intensive aquaculture is becoming an emerging business in the country. The number of private investors interested in fish farming in the country is evolving and

some of them have even already started the process. The Great Renaissance Dam and several other dams and reservoirs are being constructed in the country for hydropower generation, irrigation and other purposes apart from providing water for their primary uses, these water bodies could also be stocked with different fish species which could provide a source of livelihood to many rural young Ethiopians engaged in fishing (Eshetu et al., 2019 unpublished). Despite the potential contribution of fisheries in the country emerging zoonotic bacterial pathogen like *A. hydrophila* could constrain the productivity and safety of the fish industry in the country. This calls for proactive investigation into important pathogens in water bodies with high fish sources in Rift Valley lakes of Ethiopia.

According to FAO (FAO,2014), majority of fish catch in Ethiopia originate from Rift valley lakes. Therefore, knowing the infection status and characteristics of *A. hydrophila* in fish and ready-to-eat fish products is paramount to the understanding of the epidemiology and associated risks to public health. To this end the present study was intended to isolate and determine phenotypic and genotypic features of *A. hydrophila* infecting tilapia in selected Rift Valley Lakes and fish products in respective towns.

The specific objectives of the study were:

- To isolate *A. hydrophila* from fish and ready-to-eat fish products
- To determine the susceptibility of *A. hydrophila* isolates to major antimicrobials of veterinary and human importance
- To reveal phenotypic and genotypic traits of *A. hydrophila* isolates

2 LITERATURE REVIEW

2.1 Historic Perspective: Past and Present

The first isolates thought to date back to 1891 were reported by Sanarelli who named the bacteria as *Bacillus hydrophilus fuscus* (now *A. hydrophila*) (Ottaviani et al., 2006). However, the International Committee of Systematic Bacteriology established the authorship of the genus to Stanier in 1943. Then genus Aeromonas was included in the family Vibrionaceae in 1965 with the genera Vibrio and Plesiomonas. In 1986 Colwell carried out sequence analysis of the 16S rRNA and 5S rRNA genes and DNA–DNA hybridization studies and demonstrated that Aeromonas formed a different phylogenetic branch thus, creating the Aeromonadaceae family (Igbinosa et al., 2012).

In the mid-1970s aeromonads could be broken down roughly into two major groupings based upon growth characteristics and other biochemical features (Ma et al., 2020). The mesophilic group typified by *A. hydrophila* consisted of motile isolates that grew well at 35 to 37°C and were associated with a variety of Fish and human infections. The second group referred to as psychrophilic strains caused diseases in fish were non-motile and had optimal growth temperatures of 22 to 25°C. This group contained isolates that currently reside within the species *A. salmonicida.* Beginning in the mid-1970s and continuing for almost 10 years thereafter several groups including the Institute Pasteur in Paris, the Centers for Disease Control and Prevention (CDC) in Atlanta GA, and the Walter Reed Institute of Research in Washington DC, spearheaded an effort to re-define the mesophilic group based upon DNA relatedness studies. Over that period DNA hybridization investigations revealed that multiple hybridization groups (HGs) existed within each of the recognized mesophilic species (*A. hydrophila*, *A. sobria*, and *A. caviae)* (Janda and Abbott, 2010). These unnamed HGs were represented by reference strains, since in each case they could not be separated unambiguously from each other by simple biochemical means. The term "phenospecies" was coined to refer to a single heterogeneous species (such as *A. sobria*) containing multiple HGs within it. Hybridization groups were given numbers for either defined species (*A. hydrophila* // HG1) or reference strains representing unnamed species. In general, there was consensus agreement on the first 12 HGs between the Institute Pasteur and CDC. At a later time when phenotypic markers were recognized that clearly separated these groups from one another (Rangel et al., 2019).

The genus Aeromonas currently comprises 36 species that have been described since 1943 species (Zhou et al., 2019). *A. hydrophila* was historically considered an opportunistic pathogen associated with secondary bacterial infections after a 2009 outbreak of motile Aeromonas septicemia (MAS) in farmed catfish in West Alabama and East Mississippi a clonal population of virulent *A. hydrophila* (vAh) was attributed to the emergence of the MAS cases. Tens of millions of pounds of market-sized catfish have been lost due to vAh and it has emerged as a primary pathogen associated with catfish and carp aquaculture in the United States (Zhang et al., 2016).

2.2 Aeromonas hydrophila as a pathogen in human

A. hydrophila is an important cause of zoonotic diseases (Rodrigues et al., 2019). In recent studies *A. hydrophilia* has been associated with four types of human illnesses, a general infection in which the organisms spread throughout the body (septicemia), extra-intestinal, wound, and gastrointestinal infections. Extra intestinal infections such as meningitis, peritonitis, otitis, or of sites such as eye or urinary tract. Wound infection or cellulitis caused by *A. hydrophila* are linked circumstantially with injuries incurred during recreation, handling fishes with bare hands or other activities in the aquatic environment. *A. hydrophia* has also been implicated as food-associated gastroenteritis which is the most common clinical manifestation in immunocompromised and immunocompetent human hosts and manifested by diarrhea (Roges et al., 2020).

Infections in humans with bacteremia (Climent et al., 2017), respiratory tract infections (Igbinosa et al., 2012), gastroenteritis (Song et al., 2019), septicemia (Fu et al., 2020), urinary tract infection (Hasan et al., 2018), traveler's diarrhea (Farzadnia and Naeemipour, 2019), Panophthalmitis (Thanapaisal, 2019), Osteomyelitis (Doganis et al., 2016) and Post-op wound infection (Spp et al., 2017; Ugarte-torres et al., 2018) have been associated with *A. hydrophila.*

The knowledge on the mechanism of pathogenesis of this microbe is poor but it is known that several different factors influence the development of the disease caused by it (Katz et al., 2015). Recent studies indicated that *A. hydrophila* causes a range of pathologies they are emerging as an enteric pathogen of public health concern (Roges et al., 2020). Nowadays results from diarrheal outbreak studies further showed that the infective dose of Aeromonas is very low (Thomas et al., 2020).

2.3 Aeromonas hydrophila as a pathogen in fish

A. hydrophila is believed to primarily causing motile aeromonad septicemia (MAS) in aquatic animals and affects a wide variety of freshwater fish species including carp, tilapia, perch, catfish, and salmon and occasionally from marine fish e.g. ulcer disease of cod (Wang et al., 2020). Epidemic disease in fish caused by *A. hydrophila* resulting in millions of dollars of losses reported to the aquaculture industry worldwide (Ali et al., 2020).

A. hydrophila is a predominant bacterium and more common in warm water and temperate species than in cold-water fish which causing high mortalities even without symptoms in the per-acute phase (Abdel-latif, 2020). Infections can occur in any age fish but losses are usually most severe in fry and small fingerlings. The symptoms of *A. hydrophila* infections in the acute form of the disease is fatal septicemia which may occur so rapidly that fish die before they have time to develop anything but a few gross signs of the disease include swelling of tissues, dropsy, red sores, necrosis, ulceration, and hemorrhagic septicemia (Rodrigues et al., 2019). When clinical signs of infection are present affected fish may show exophthalmia, reddening of the skin (muscle), and an accumulation of fluid in the scale pockets (V.O and C.C., 2012). There may also be severe bronchitis, as indicated by leukocytic infiltration and dilation of the central venous sinus (Woo, 2011). The severity of the disease is influenced by several interrelated factors including bacterial virulence, the kind and degree of stress exerted on a population of fish, the physiologic condition of the host, intensification of fish and the degree of genetic resistance inherent within specific populations of fishes (Amsaveni et al., 2016).

2.4 Epidemiology

A. hydrophila is widely distributed across numerous ecosystems although, it is more commonly found in various aquatic environments (Ali et al., 2016). These microorganisms have also been isolated in terrestrial animals, several environmental and clinical samples including human beings (Vega-sánchez et al., 2014).

2.4.1 Aeromonas hydrophila in Aquatic Environments

A. hydrophila is indigenous to aquatic environments it has been isolated from freshwater environments and distributed throughout the world and it is not restricted to freshwater systems

but also present in saline waters. A more recent study having salinity values of up to 35 %. Since aeromonads are found in water systems they are also present in both chlorinated and unchlorinated potable water (Miyagi et al., 2016).

In a study of rivers and lakes in Finland 116 strains of Aeromonas spp. were recovered among *A. hydrophila* the dominant percentage which was thought to be linked to cyanobacterial blooms (Ottaviani et al., 2006) and these strains were re-identified by (Beaz-hidalgo et al., 2015). The incidence of *A. hydrophila* in wastewater is high (Igbinosa and Okoh, 2012). Conventional treatments to reclaim water at wastewater treatment plants (WWTPs) such as a primary and secondary or biological treatment do not greatly reduce the concentration of *A. hydrophila* (Samantaray, 2008). Depending on the microbiological quality of the reclaimed water it can be used for irrigation (Rather et al., 2019). Irrigated waters containing the bacteria can contaminate fruits and vegetables (Stratev and Odeyemi, 2016) and thus cause infection in humans. There is epidemiological evidence that water acts as a vehicle for the dissemination of this bacterium because it has been possible to identify the same *A. hydrophila* strains in drinking water samples and in the feces of individuals with diarrhea (Qamar et al., 2016). In another study the same strains isolated from lettuce and tomatoes were recovered from the irrigation water (Rather et al., 2019). There are numerous studies in which the presence of *A. hydrophila* was detected in drinking water (Latif-eugenín et al., 2016). A common source of *A. hydrophila* in outbreaks of gastroenteritis is from water supplies such as natural mineral springs, marine water environments, chlorinated and unchlorinated domestic supplies and watersheds polluted by sewage effluents. *A. hydrophila* is not considered a normal inhabitant of the human gastrointestinal tract. The fecal carriage rate of *A. hydrophila* from asymptomatic hospitalized patients is from 0-8%. These results suggest that the bacteria are transient inhabitants of the human intestinal tract and humans are not the major contributor of *A. hydrophila* in the environment although, the bacteria that are shed can multiply in sewage lines to significant numbers before discharge into the receiving waters (Nishikawa, 2019).

2.4.2 Aeromonas hydrophila in Foods of Animal Origin

Sources other than water from which this organism can be readily isolated include seafood, shellfish, fishes, foods of terrestrial animal origin such as meats, dairy products and poultry, and

vegetables and fruits (Sharma, 2014). The presence of *A. hydrophila* in the food chain should not be ignored because it is a potential cause of food poisoning (Buchanan, 2020).

The temperature, salinity or pH are the factors that determine the number of *A. hydrophila* in the foods (Austin, 2012). In fact, in the case of temperature the organism is of great concern because several authors reported that the bacteria survive and capability of growing at refrigerated temperatures in low temperatures (2–10 ◦C). Additionally, salt or sodium chloride (NaCl) is a common preservative for meat products and raw fish however, *A. hydrophila* can grow in NaCl concentrations of up to 4%. Finally, pH is another parameter that controls bacterial growth in food it was demonstrated that the bacteria can survive at pH = 5-9 (Austin and Austin, 2016).

Many studies have investigated the incidence of *A. hydrophila* in meat products (Roges et al., 2020). In the case of fish intended for human consumption tilapia and salmonids harbor the bacteria (Attia et al., 2018). However, as several authors reported *A. hydrophila* infections are often under-diagnosed or even misdiagnosed changing the values based on the diagnostic methods (Ali et al., 2020).

2.4.3 Aeromonas hydrophila in Human

Asymptomatic and immuno-compromised human carriers involved with the handling of foods are another potential source (Nishikawa, 2019). The incidence of *A. hydrophila* in human infections worldwide is unknown but a study in California in 1988 showed that the annual incidence of *A. hydrophila* infections was 10.5 cases per million people (Elsayed and Mohamed, 2018). Janda and Abbott (2010) also reported that in 2004 in England the estimated incidence of *A. hydrophila* was 1.5 cases per million people. In France in 2006 it was estimated that 0.66 cases per million (Elsayed and Mohamed, 2018). Another study conducted in Taiwan between 2008 and 2010 showed that the incidence of bacteremia by *A. hydrophila* was 76 cases per one million individuals (Wu et al., 2019). Those studies therefore, shows that the incidence of diseases caused by *A. hydrophila* vary by geographical location and can be related to bad hygiene habits in undeveloped regions (Bhattacharjee and Bhowmick, 2018). However, based on the molecular identification of 817 strains obtained from different studies around the world 75% of the strains associated with clinical cases from Aeromonas spp. correspond to *A. hydrophila* (Climent et al., 2017).

2.4.4 Worldwide distribution and Risk factors

A. hydrophila is universally distributed and widely isolated from clinical cases (Wu et al., 2019), water treatment Plants (Magdalena et al., 2017), freshwater environments (Talagrand-reboul, 2020) and food samples where they may grow even at low temperatures (Hoel et al., 2017). Some minor seafood-borne outbreaks have also been observed in *A. hydrophila* (Ali et al., 2020).

Contaminated pond water, diseased fish and diseased frogs, as well as convalescent frogs and fish, maybe reservoirs of infection (Austin, 2007). Certain algae and other protozoa that are grazed upon by fish can also harbor motile aeromonads. In the latter study, Tetrahymena pyriformis was shown experimentally to graze on populations of *A. hydrophila* can be a risk factor for the epidemiology of the bacteria (Woo, 2011).

2.4.5 Transmission of Aeromonas hydrophila

The bacteria are universally distributed in freshwater and sediments as well as the intestinal tract of fish. The pathogen is readily transmitted horizontally through the water and by direct contact (Mummah et al., 2020; Smith, 2019). The mode of transmission of pathogenic *A. hydrophila* from the environment to humans is still not clearly understood (Miyagi et al., 2016). The primary route for transmission to a clinician or persons handling fish in bare hands in contact with mucus and tissues from infected or carrier fish, cuts and abrasions that are already present on the hands of the handler as well as wounds caused by handling fish directly are possible routes of infection. Another way of transmission by consumption of *A. hydrophila* infected raw fish or undercooked fish meat and products (Lowry and Smith, 2007).

2.4.6 Host range

Wild and cultured freshwater fish worldwide are susceptible to *A. hydrophila* infection with cultured fish being particularly vulnerable. Also, *A. hydrophila* has caused disease in amphibians, reptiles, cattle and humans throughout the world (Smith, 2019).

2.5 Virulence Factors

A. hydrophila virulence is complex and multifactorial since several factors contribute significantly to the development of an infectious process (Zhou et al., 2019), as the efficacy of the host immune system decreases (Sharma, 2014), structural components, toxins and extracellular products (Ma et

al., 2020), acting jointly or individually (Liu et al., 2020), to overcome the host immune response and enable these microorganisms to colonize and infect hosts (Fernandez-Bravo and Figueras, 2020).

2.6 Antimicrobial Resistance

Resistance to antimicrobial agents is a genetic–evolutionary response mediated by the presence of genes some of which are found in plasmids, integrons or the genome of the bacteria. Outer membrane proteins (OMPs) play essential roles in antibiotic resistance particularly in Gram negative bacteria (Sun et al., 2019). High antibiotic resistance is seen in bacterial infections caused by *A. hydrophila* (Aa and Najiah, 2013). *A. hydrophila* have been regarded universally to exhibit resistance to the penicillin's (penicillin, ampicillin, carbenecillin, and ticarcillin) for quite a long time (Stratev and Odeyemi, 2015). Antibiotic resistance in *A. hydrophila* in aquatic environments is associated especially with plasmids of the IncU family (Nwaiwu and Aduba, 2020). Most IncU plasmids described up to date share a highly conserved backbone represented by genes for plasmid replication, stability, and conjugative transfer and one variable region for resistance genes to antibiotics, such as tetracycline, sulfonamides, trimethoprim, streptomycin, and chloramphenicol (Mulani et al., 2019). Quinolones are broad-spectrum antimicrobial agents widely used in human and veterinary medicine, and they are among the most widely used antibiotics in aquacultures. *A.hydrophila* isolates harboring quinolone resistance (PMQR) genes on IncU plasmids have been recently reported in river water, lake water, and fish (Stratev and Odeyemi, 2016). Thus, highlighting the role of this plasmid family in the dissemination of clinically relevant resistance mechanisms in a wide range of aquatic environments (Dobiasova et al., 2016).

Antibiotic resistance of *A.hydrophila* to multiple antibiotics is becoming a serious public health concern (Lim et al., 2019). Tetracycline resistance has been reported for *A. hydrophila* isolated from a river that receives wastewater discharge (Kluga et al., 2019). Similarly, *A. hydrophila,* isolated from human diarrheic stool in Mexico (Baron et al., 2017) showed variable resistance to tetracycline. Furthermore, variable resistance of *A. hydrophila* isolates to other antibiotics was observed in several studies which includes Erythromycin, Nalidixic acid and Gentamicin are among others (Igbinosa and Okoh, 2012).

As per Ali et al., (2016) *A. hydrophila* isolates were sensitive to Chloramphenicol 30 µg (100%), Streptomycin 10 µg (90%), Tetracycline 30 µg (66.7%), Gentamycin 10 µg (60%), Norfloxacin

10 µg (55%) and Erythromycin 10 µg (33.3%), while, those isolates were resistant to Amoxicillin 10 µg (100%), Penicillin 10 µg (100%) and Novobiocin 30 µg (100%). Wimalasena et al., (2017) shown that *A. hydrophila* isolates were susceptible to Cefotaxime (30 µg), Ciprofloxacin (5 µg), Streptomycin (10 µg) and resistant to Amoxicillin, Ampicillin, Cephalothin, Chloramphenicol and Tetracycline.

Ndi and Barton, (2011) reported that there were an increment seen in resistance to beta-lactam antimicrobials (Penicillin and derivatives, Cephalosporin, Carbapenems, and Monobactams) by the presence of genes that code for the production of beta-lactamases. Three β-lactamases were mainly described in *A. hydrophila* among class B Metallo-β-lactamase, class C cephalosporin, and class D penicillinase. Esbl, (2003) characterized the β-lactamases associated with *A. hydrophila* complex strains expressing class B, C, and D β-lactamases.

Recently, the studies in antimicrobial profiles of *A. hydrophila* has increased due to the necessity of responsible use of antibiotics (Dahdouh et al., 2016). Previous studies comparing clinical isolates demonstrated that the resistance genes were species and isolation site dependent. The resistant strains were mostly detected from peritoneal fluids besides, Zhou et al., (2018) demonstrated that clinical strains isolated from extra-intestinal infections were resistant to several antibiotics however, third-generation cephalosporin, fluoroquinolones, and aminoglycosides could be an option to treat these infections (Baron et al., 2017). Data from different studies agree that resistance could be related to mobile genetic elements such as plasmids, insertion elements, pathogenicity islands or cassettes associated with integrons (Wimalasena et al., 2017).

Mcauliffe et al., (2015) revealed the incidence of class 1-integron and β-lactamases genes in *A. hydrophila* isolates from rainbow trout. Zdanowicz et al., (2020) studied the variety of beta-lactamases genes present in *A. hydrophila* isolated from wastewater finding. A high number of genes encoded by plasmids suggest that *A. hydrophila* antibiotic resistance strains could disseminate from wastewater to other environments. Recently Ramadan et al., (2018) observed a higher incidence of different antimicrobial resistance genes and class 1-integron gene cassettes in resistant *A. hydrophila* isolates. Several studies have been conducted to prevent the consequences of inappropriate antimicrobial use (Fernandez-Bravo and Figueras, 2020). Since 2019 epidemiological cut-off values for Aeromonads are available at CLSI, before the only

interpretation criteria to interpret minimum inhibitory concentrations (MIC) available used were clinical breakpoints for Enterobacteriaceae.

2.7 Diagnosis

Detection of fish pathogenic bacteria like *A. hydrophila* can be achieved by traditional culturing techniques, immunological methods like ELISA, and genetic tools like polymerase chain reaction (Hong et al., 2017). An identification method that is both rapid and reliable is crucial to the surveillance, prevention, and control of *A. hydrophila* infection (Elsheshtawy et al., 2019). Diagnosis and treatment may be difficult especially because of emerging antibiotic resistance in fish pathogens. Several approaches have been applied to investigate the bacterial pathogens in the aquaculture and fishery world (Gotesman et al., 2018).

2.7.1 Conventional methods

2.7.1.1 Biochemical identification of A. hydrophila

For the isolation and identification of *A. hydrophila* samples inoculated on Nutrient agar media or tryptic soy agar (TSA) media in Petri dishes and incubated at 37°C for 24 hours (Goni et al., 2020). Culture methods subjected to phenotypic differential analysis by conventional procedures by their morphological, physiological and biochemical plate and tube tests (Khor et al., 2015). All isolates observed for colony morphology, hemolytic ability, bacterial cell shape, Gram staining and growth capacity on differential medias and use a basal medium for all biochemical tests. Carbohydrate metabolism determination using MAO slant phenol red as indicator and carbohydrate (Glucose, Sucrose, Lactose, Mannitol, Arabinose, Maltose, and Trehalose), oxidase, catalase, resistance to Vibriostatic agent O/129 (150 µg) Voges-Proskauer reaction, and resistance to Cephalothin (30 µg). Additional tests such as Nitrate, Citrate, Urease and decarboxylase tests were also performed to detect the ability of *A. hydrophila* to produce nitrate reductase, Citrates', Urease and decarboxylase enzymes respectively and growth in broth without and with 6%NaCl (Shivakumaraswamy et al., 2019).

Biochemical characters

Colonies suspected of *A. hydrophila* appeared round, convex, shiny and creamy on TSA media. Gram staining of these colonies gives gram-negative coccobacilli to rod-shaped and motile

(Mohammed et al., 2018). Aeromonads gave a positive reaction for oxidase, catalase, and indole test also ferment glucose with the production of acid and gas. They gave negative results toward H_2S production, urea hydrolysis, and non-lactose fermentation and produced variable results with V.P, Indole production, Gelatin hydrolysis, Nitrate reduction and Sugar fermentation (Mannitol, Inositol, Sorbitol, and Arabinose).

Cultural characteristics of *A. hydrophila* (Goni et al., 2020)**.**

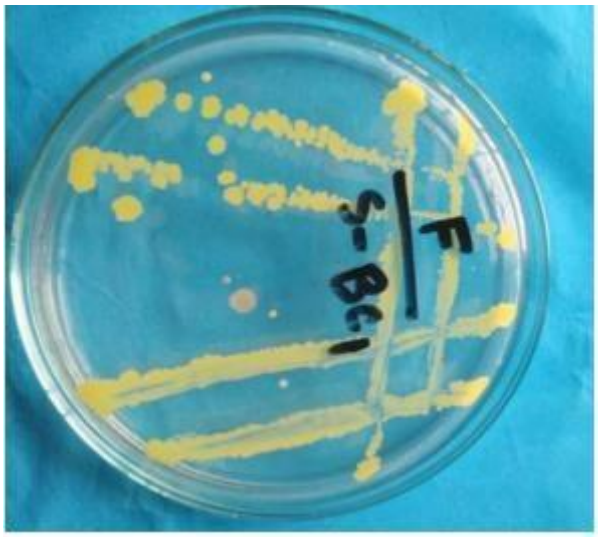


Figure 1: *A. hydrophila* on Nutrient agar.

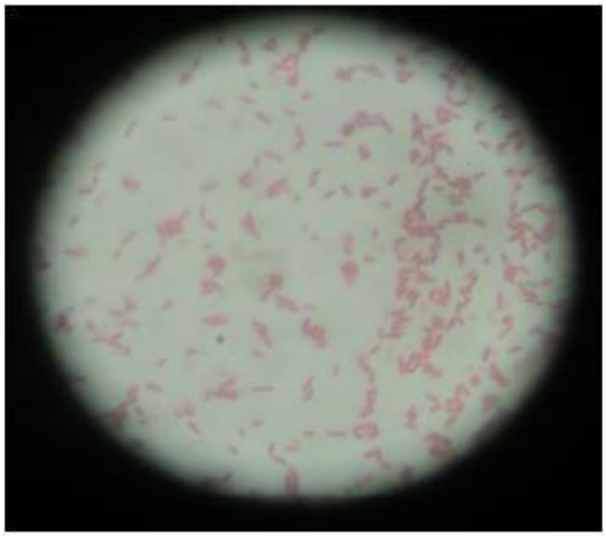


Figure 2: *A. hydrophila* found gram negative.

Figure 3: *A. hydrophila* found motile on a microscope.

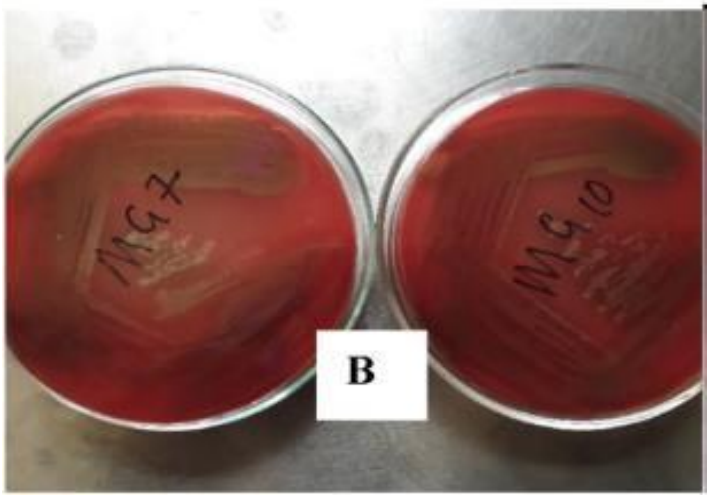


Figure 4: *A. hydrophila*, Blood Agar showing β-hemolytic characteristics

Biochemical tests of *A. hydrophila* (Goni et al., 2020)**.**

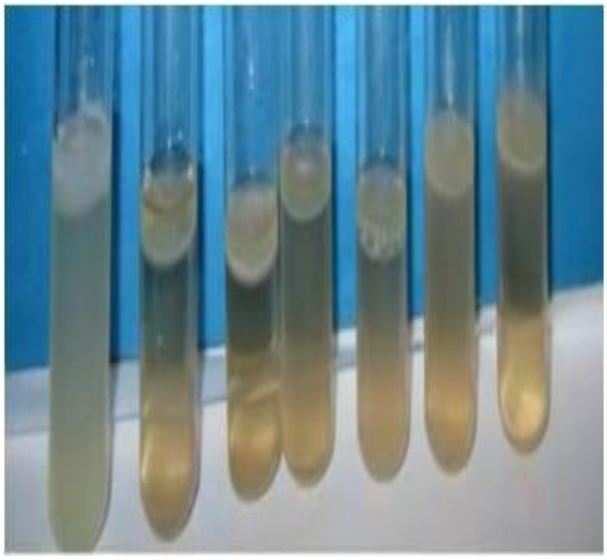


Figure 5: *A. hydrophila* found positive on sugar fermentation test. Figure 6: *A. hydrophila* showing negative on MR test

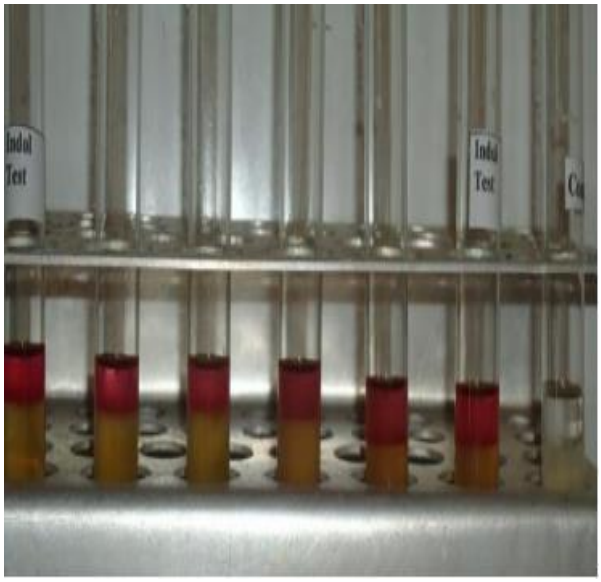


Figure 7: *A. hydrophila* showing positive on the VP test. Figure 8: *A. hydrophila* showing positive on the Indole test.

2.7.1.2 VITEK system

Automated bacterial identification system was introduced now days. This system is ahighly automated and rapid identification system that uses several types of small plastic reagent cards each capable of identifying a limited set of bacteria. The system requires growth before interpretation and cards are read spectrophotometrica-lly every 20 minutes and are compared to a database until an identification can be made (Park et al., 2003).

2.7.1.3 API 20strep

The API 20NE test kit (BioMerieux) were used and the manufacturer's instructions were followed by incubating bacteria at 30°C. The API 20E test strip consists of 20 microtubules containing dehydrated substrates. These tests are inoculated with a bacterial suspension that reconstitutes the media. During incubation metabolism produces color changes that are either spontaneous or revealed by the addition of reagents. The examination of the strips conducted after 24 and 48hr. The reactions read according to the interpretation table and the identification is obtained by referring to the identification profile. The identification of strains carried out according to the API 20NE identification manual (Tokajian and Hashwa, 2004).

2.7.1.4 Biolog Microlog System

Pure bacterial isolates from agar plates taken and one or two days before inoculating in to Biolog GN plates (Biolog, Inc.), the isolates streaked on BUG agar plates. The wells of the Biolog GN plates inoculated with a specific amount (µl) of the bacterial suspension adjusted to the appropriate density and incubated at 30°C for 24 and 48 hr. The development of color was automatically recorded using a microplate reader with a 590-nm wavelength filter. Identification from (Biolog Microlog 3.70 database) and file output of test results applying the automatic threshold option performed using Biolog software (Fouz et al., 2006).

2.7.1.5 *Histopathological*

Formalin-fixed tissues were processed using standard histological methods stained with hematoxylin and eosin (H&E) and examined at 40x and 200x magnification on a light microscope (Mehdi et al., 2016).

2.7.2 Molecular Techniques

During the last two decades' substantial progress has been achieved in molecular disease diagnosis for the aquaculture field and this includes the application of polymerase chain reaction (PCR). Among these techniques the most common technique involves the amplification of 16S rRNA by PCR (Smith, 2019).

2.7.2.1 Amplification of 16S rDNA by PCR DNA

The most common technique involves the 16S rRNA gene the main component of the 30s ribosomal subunit (Zhou et al., 2018). Its sequencing is universally known about the establishment of phylogenetic relationships among bacteria, however, the accuracy of this method is limited when analyzing strains whose sequences are very similar. The amplification of 16S rRNA for sequencing is generally done by conventional polymerase chain reaction (PCR) using extracted bacterial DNA. However, it has been reported that sequencing of the 16S rRNA gene amplified by direct PCR colony, a method that excludes DNA extraction and purification of the PCR product is not only cheaper but also more efficient in the taxonomic classification of Aeromonas species (whose sequences are not similar) than the amplification of the 16S rRNA gene using bacterial DNA previously extracted and purified (Sebastião et al., 2015).

2.7.2.2 *Restriction Fragment Length Polymorphism (RFLP)*

All the phenotypically identified *A. hydrophila* was also identified based on restriction fragment length polymorphism patterns obtained from the amplified 16S rDNA gene. RFLP analysis allows the study of small variant regions or polymorphisms in DNA using restriction enzymes (Algammal et al., 2020). The application of this technique in 16S rRNA analyses has been reported as a fast and effective way to identify some *A. hydrophila* (Hoel et al., 2017).

2.7.2.3 *The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry*

The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) method is considered a powerful tool in the identification of *A. hydrophila* (Ndi and Barton, 2011). This technique consists of the ionization of samples and generation of gas-phase ions which after particle acceleration in an electric field are detected according to their speed (depending on mass and ionic charge) upon arriving at the detector (Hartman, 2018). MALDI-TOF MS allows

for the analysis of the protein content of a microorganism constituted largely of ribosomal proteins but also cytosolic proteins such as heat-shock proteins. Particular differences in the protein composition among microorganisms are what effectively guarantee the success of the technique in the identification of the bacteria (Mzula et al., 2019a). The application of MALDI-TOF in the phylogenetic study and protein characterization in *A. hydrophila* has already been reported (Cerd et al., 2018).

2.7.2.4 Multilocus Sequence Typing

Multilocus Sequence Typing (MLST) capable of accurately and universally providing bacterial characterization through the analysis of the nucleotide sequence of multiple chromosome sites. This technique detects changes in DNA quickly and reproducibly without the need for reagent exposure and does not require living bacterial suspensions or even high-quality genomic material sparing (Bastos et al., 2019).

2.8 Prevention and control

Fish diseases can be controlled to a large extent by various ways such as the wise use of drugs and antibiotics either prophylactically or therapeutically, enlightened husbandry strategies, genetic selection of stress and disease-tolerant strains and prevention by employing vaccines. Of all these strategies for prevention of disease is much more desirable than intervention to stop and reverse disease processes once they have begun (Monir et al., 2020).

2.8.1 Antibiotics and Chemotherapy

In fish culture the ensuing 30 to 40 years is rightly termed as the "era of chemotherapy" because a large number of antibiotics, Sulpha drugs, and even mercury-based antimicrobial agents were routinely used. Of the many antimicrobial agents used to combat the diseases Terramycin, potassium permanganate, and malachite green are the most frequently applied drugs although, complete control has not been achieved. Resistance in *A. hydrophila* has been recorded to a wide range of new antimicrobial compounds. Bacteriological studies showed the presence of *A. hydrophila* in the internal organs of infected tilapia fingerlings dip treatment in 50 ppm formalin solution significantly controlled the mortality (Treves-Brown, 2000).

Antibiotic and chemical resistance is a persistent problem in *A. hydrophila* management in aquaculture. Despite the emergence of multiple drug-resistant strains of pathogens the antibiotics and chemicals used in aquaculture have residual effects and undergo bio-magnification in the aquatic environment when used over an extended period. Resistance may be transferred to other related or unrelated bacteria through R-plasmid. The use of antibiotics for controlling *A. hydrophila* infection in farmed fish pose threats to humans and increased the incidence of antibiotic-resistant bacteria from the environment can be observed (Oliveira et al., 2019).

In therapeutics in addition to direct toxicological risks concern has been raised about the potential for antibiotic residues in water since they are typically found in the aquatic environment at sub therapeutic concentrations promoting the emergence of resistant bacteria and subsequent development of more resistant and virulent pathogens. These bacterial resistances through horizontal gene transfer may end up in human pathogens raising questions on human health and the stability of the ecosystem. This emergence of bacterial resistance presents one of the major emerging threats to human health and is by far the highest risk for humans of having medicinal product residues in the environment. Furthermore, historical evidence appears to indicate that in the aquatic environment resistance might be acquired faster than in the terrestrial environment (Pereira et al., 2020).

2.8.2 Vaccines

Disease prevention through vaccination and immune-stimulation is extremely effective and will continue to play a major role in fish disease management since it circumvents pollution associated with chemotherapy and the emergence of multiple drug-resistant strains. Disease prevention by vaccination is economic, environmental and ethical grounds and the most appropriate method for pathogen control currently available to the aquaculture industry (Bøgwald and Dalmo, 2019). It was only in the mid to late 1970s with an increased interest in fish farming particularly marine fish farming attention was once again turned to the possibility of developing a vaccination as a means of preventing/controlling fish diseases and to the development of commercially available vaccines. The reasons for this turn of events were varied because the high cost of using chemotherapy, the short-term nature of the protection obtained with antibiotics, the increasing appearance of antibiotic-resistant fish pathogens, and to some extent concerns about the environmental impacts of antibiotic use (Ma et al., 2019).

Fish vaccines are licensed and closely regulated in the same manner as all other veterinary vaccines to ensure safety, potency, and efficacy. Even though commercial vaccines for aquaculture works well in terms of protecting the fish against certain diseases they should be only used as part of the overall fish health management program because fish vaccines are not a cure-all. Fish husbandry is still the key to success in aquaculture (Mutoloki et al., 2015).

Some attention has been devoted to developing vaccines with commercial products becoming available. Simple preparations of inactivated whole cells, ECPs or OMPs (outer membrane proteins) which may be administered by immersion, injection, or via the oral route appear to work quite well (Maiti et al., 2020). Using formalized whole cells applied by I.P. injection recorded complete protection in Nile tilapia within only 2 weeks and protection of 53– 61% occurred only 1 week after vaccination (Mzula et al., 2019b). Vaccination dose is critical in fish and its importance was highlighted by (Austin and Austin, 2016).

Methods of vaccine preparation have varying results on the pathogen. For instance, vaccination even with crude lipopolysaccharide (LPS) induced better protection against *A. hydrophila* infection in the common carp, *C. carpio* than the formalin- killed vaccine (Hwang et al., 2020). The Indian major carps *Catla catla*, *Labeo rohita*, and *Cirrhinus mrigala* were immunized against the potent *A. hydrophila* intra-peritoneally in field conditions. Two different polyvalent antigen preparations namely whole-cell and extracellular products (ECP) were used. Upon challenge with virulent strains relative percent survival was as high as 80–90% (Mohd-aris et al., 2019). An enteric red mouth bacterium (ERM) is one of the first commercially produced fish vaccines and then, the formalin-killed and whole-cell vaccine continues to be highly effective whether administered by immersion, spray, injection or oral routes (Maiti et al., 2020). The nonspecific immune response due to vaccination seems to be more important than the specific immune response. This is because specific immunity requires a longer time for antibody build-up whereas, with the nonspecific immune response phagocytosis and the production of oxidative radicals are quickly activated by the immuno-stimulants to protect the fish against pathogens. Several immune-potentiators have been described and some of these include killed bacteria, bacterial products, levamisole, muramyl dipeptide (MDP), lipopolysaccharide (LPS), chitin, and chicken egg products (Austin, 2007; Nya and Austin, 2009).

Oral vaccines administered along with feed supplements are desirable but oral vaccines yield low protection against diseases and this is reflected by the non-availability of any commercial oral vaccine for fish (Mohd-aris et al., 2019). The use of immune-stimulants (adjuvant) concomitant with a vaccine is an elegant means of eliciting a superior protective capability in fish. It not only heightens antibody response but also elevates nonspecific components of the immune system (Alexandra, 2016). The aquaculture industry needs to enhance its global production and efficiency to meet the increasing consumer needs for fish and shellfish products. Unfortunately, infectious diseases have been a major impediment to the development and profitability of fish farms. While vaccines offer the most efficient way to control infectious pathogens current products have only been successful against some diseases. These are mostly bacterial however, there are still several important diseases mainly of viral and parasitic origin for which no prophylactic treatment exists (Smith, 2019).

DNA vaccines compared to traditional antigen vaccines have several practical and immunological advantages that make them very attractive for the aquaculture industry. Alternative methods of administration also have to be developed for small fish and low-valued species for which IM injection is not practical and/or cost-effective (Jeney, 2017). Vaccination of fish in aquaculture has been particularly successful against several bacterial diseases. Using attenuated as well as live vaccines have been particularly successful against specific bacterial diseases. Mono and multivalent vaccines have been developed against several bacterial diseases in fish. Salmonid fish are usually immunized with multivalent vaccines by intraperitoneal injection. During the last 10 to 20years vaccination has become established as an important method for the prevention of infectious diseases in farmed fish mainly salmonid species. So far, most commercial vaccines have been inactivated vaccines administered by injection or immersion. Bacterial infections caused by gram-negative bacteria such as Vibrio sp., Aeromonas sp., and Yersinia sp. have been effectively controlled by vaccination (Austin, 2012).

The overall positive effect of vaccination in farmed fish is reduced mortality. A potential side effect associated with injectable vaccines is local reactions in the peritoneal cavity. However, one of the major constraints in vaccination is as new diseases and pathogens emerge from time to time, it is impossible to develop proactive strategies using vaccines and there are strains of pathogens that do not convey for vaccine development (Le et al., 2018).

An ideal fish vaccine is one that is safe for the fish and environment, economical for large-scale production, easy to administer, capable of inducing strong immunity throughout periods of greatest susceptibility, and demonstrates minimal side effects. New and alternative fish vaccines are adapting advanced technologies often developed based on needs in animal or human medicine but have shown great promise for aquaculture. Those that meet the criteria for an effective aquaculture vaccine will provide the most benefit and have the greatest potential for commercialization. New fish vaccines using alternative technologies (beyond just killed cellular preparations) can be expensive to develop but given the limited success of traditional approaches for new disease problems so, it is essential to further explore such approaches. As aquaculture continues to grow globally there will be a need for new vaccines long into the future and the application of all available biotechnology towards solving emerging disease issues will be critical (Ma et al., 2019).

2.8.3 Immuno-stimulants/ Dietary Supplements

Vitamins in diets are of value for controlling the infection. Evidence has been presented which pointed to the role of vitamin C in stimulating the humoral and cell-mediated immune response in fish vaccinated with *A. hydrophila* vaccines (Austin and Austin, 2016).

2.8.4 Probiotic-feeding

The definition by Fuller of a probiotic being "A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" is commonly used (Abomughaid, 2020). Probiotic-feeding continues to be a promising strategy to control bacterial pathogens in aquaculture (Gong et al., 2017).

2.8.5 ß-glucans

ß-glucans enhance the non-specific resistance to *A. hydrophila* infection (Kumari and Sahoo, 2006).

2.8.6 Ginger

Success occurred with ginger (*Zingiber officinale*) which led to immunomodulation and protection against *A. hydrophila* (Nya and Austin, 2009).

2.8.7 American ginseng

American ginseng (Panax quinquefolium) was effective as a dietary supplement when dosed at 1.0, 2.0 [optimal dose], or 5.0 g/kg diet for 8-weeks to Nile tilapia with the data revealing improved growth and survival from challenge of *A. hydrophila* (Saleh et al., 2017).

2.8.8 Honey Bee Pollen

Honey bee pollen which was fed at 2.5% for 20 or 30 days improved growth, stimulated the immune response (phagocytic and serum bactericidal activity hematocrit, leucocyte, the numbers of lymphocytes, monocytes and neutrophils, serum total protein, albumin, and globulin ratios), and protected Nile tilapia (survival = 93 %) against experimental challenge with *A. hydrophila* (Austin and Austin, 2016).

2.8.9 Bacteriophages

Bacteriophages are naturally-occurring bacterial viruses that can kill specific bacteria with no chemical residues and do not affect other flora are one of the potential alternatives in *A. hydrophila* infection (Liu et al., 2020).

2.9 Epidemiology in Ethiopia

Few diseases have been described from fish of Ethiopian waters (Yimer, 2000). In Ethiopian water bodies including lakes the only group of relatively well-studied pathogens of fish is helminths however, less attention has been given to bacterial pathogens of fish including those which have zoonotic importance (Nuru et al., 2012). Anwar Nuru and his colleagues in 2012 isolated *A. hyrophila* from Lake Tana and *A. hyrophila* were the most frequent isolates from examined fishes and the bacteria were also isolated from water samples collected at different fish habitats. In 2011 Outbreak of disease was observed in a pond of African catfish (Clarias gariepinus) fingerlings at Sebeta the outbreak was investigated by using a combination of methods that included clinical observations, gross and histopathology examination and bacterial isolation. *A. hydrophila* was found to be the cause of the outbreak (Almaw et al., 2014).

3 MATERIALS AND METHODS

3.1 Study area

The current study was conducted in selected Rift Valley Lakes of Ethiopia, Koka, Ziway, Langano, and Hawassa from November 2020 to June 2021 from lake fishes, market fish and Restaurants.

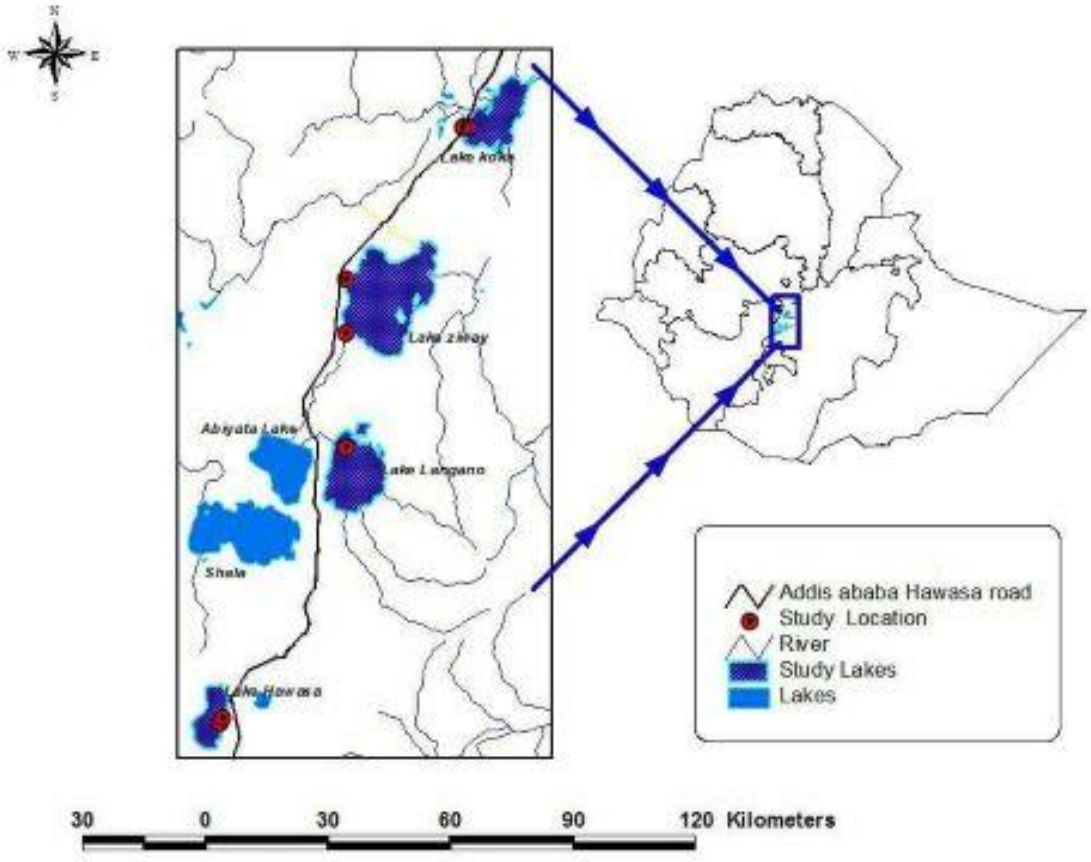


Figure 9: Map of the study Area

Lake Koka

The tropical Lake Koka (also called Lake Galilea in the past) is located in the Ethiopian Rift Valley (08°23'22" N - 39°05'15" E) at an altitude of 1590 m.a.s.l., about 90 km Southeast of Addis Ababa. It has a surface area of about 255 km^2 with a maximum and mean depth of 14m and 9m respectively (Dadebo et al., 2015). Water in-and outflow is mainly provided by the Awash River in addition,

the Mojo River may also generate inflow during the rainy season. The climate is characterized by a four-months dry season (November-February) and an eight-months rainy season (March-October), the total annual rainfall varies from 600-800 mm in the Rift Valley area and the mean annual temperature ranges from 20-26^{o}C (an average of about 23 °C) (Tesfaye and Wolff, 2015). The lake provides multiple services to the local communities and the country at large in fishing, drinking water supply, sanitation, ecotourism, irrigation, hydropower generation, and industrial purposes. The mean annual landings for the past 11 years (2002 – 2012) was about 600 metric tons per year which is about 7% of the total annual landings in Ethiopia (Kebede et al., 2014). The target species of the artisanal fishery include the native Tilapia *(O. niloticus),* Catfish (*Clarias gariepinus*), Barbus (*Labeobarbus intermedius),* and the introduced common Carp (*C. carpio*), the lake ecosystem also supports other non-exploited species including small fishes (Garra denbecha and Garra makiensis) and aquatic birds (Tesfaye et al., 2015).

Lake Ziway

Lake Ziway is found in Adami Tulu Jido Kombolcha district which is a part of Great East African Rift Valley Lakes of Ethiopia, that lies 165 km south of Addis Ababa at an altitude of 1636 m.a.s.l. It has an open water area of 434 km^2 and a shoreline length of 137 km, a minimum and maximum depth of 2.5m and 9m respectively (Bayissa et al., 2021). The Ziway watershed falls in between 7°15'N to 8°30'N latitude and 38°E to 39°30'E longitude covering a total area of about 7300 km^2. It is fed by two inflowing rivers the Meki River from the north-west and the Katar River from the east, and drains towards the Lake Abijata through the Bulbula River (Desta and Lemma, 2017). The area has an average annual rainfall of 700mm which is bimodal with a short rainy season from March to May and a long rainy season from June to September followed by the dry season from October to February. The area has maximum and minimum temperatures of 27.2ºC and 12.7ºC respectively and relative humidity of 60% (Desta, 2021). Five bigger islands are situated in Lake Ziway: Tulu Gudo (4.8 km^2), Tsedecha (2.1 km^2), Funduro (0.4 km^2), Debre Sina (0.3 km^2) and Galila (0.2 km^2) (Dadebo et al., 2013). The fish that inhabit Ziway Lake are Nile tilapia *(Oreochromis niloticus)*, Barbus species, Cat fish *(Clarias gariepinus),* Carp species, Redbelly tilapia *(Tilapia zillii),* African big barb (*Barbus intermedius),* African sharptooth catfish *(Clarias gariepinus),* and Carassius spp. (*Carassius carassius and Carassius auratus)* (Nakayama and Ishizuka, 2014). The landings of Lake Ziway used to be dominated by *O. niloticus,* but species of

C. gariepinus, *T. zillii*, and Carassius spp. *(C. carassius and C. auratus)* are increasingly becoming a part of the catch. The potential yield of all the species of the lake is estimated to range between 2500 and 6680t/yr (Desta et al., 2017).

Lake Langano

Lake Langano is located 200 km South of Addis Ababa lying between (7°40′N, 38°50′E; 1582 m. a.s.l.) occupies a 20km long and 16km wide asymmetric half-graben in a central basin (48m maximum water depth) and western littoral plateau (34m maximum water depth) which is drained by the Hora Kallo river empties into the adjacent Lake Abijatta (Gibert, 2021). Lake langano with an open water area of 230 km^2, 7.5 km shoreline and 1,600 km2 catchments area. The main fish species in the lake include *Oreochromis niloticus,* Barbus species, Clarias species and with the total annual catch of 1,000 tones (Garoma et al., 2013).

Lake Hawassa

Lake Hawassa is located in the south-eastern part of Ethiopia at 6°33′–7°33′ N; 30°22′–38°29′ E; 1,680m above sea level with surface area of 90 km^2, mean depth of 11m and a maximum depth of 22m (Worako, 2015). It is one of the many Ethiopian wet land resources situated in the middle of a series of Rift Valley lakes. The area receives a mean annual rainfall of 950mm and a mean annual air temperature of 19.8°C (Degife et al., 2019). The area is characterized by three main seasons which are long rainy season (locally called Kiremt) in the summer from June-September (mean annual total rainfall accounts from 50-70%), dry period (locally called Bega) extends between October and February and small rain season (locally called Belg) during March and May when about 20−30% of the annual rainfall falls. Mean monthly rainfall is above 100mm from April to September with August showing the highest 124mm and the lowest rainfall occur in November, December and January (Worako and Dilla, 2015). Lake Hawassa Without outlet, it is the smallest lake in the Ethiopian Rift Valley with the only perennial stream, the wetland provides water to the lake through Tikur Wuha River at its northeastern shore (Kebede and Belay, 2015). The lake lies to the west of Hawassa town and the existence and protection of this lake is very critical to the city and the population residing within the entire catchment. It supports commercial fishing activities and the dominant fish in the lake is *Oreochromis niloticus* (Tilapia nilotica) for which a sizeable commercial fishery exists (Sorsa et al., 2016).

3.2 Study population

The present study was conducted on the Nile tilapia (*Oreochromis niloticus*) of fish having various size and weight collected from lake and different fish markets and restaurants at study area. Nile Tilapia were selected because of the fish population density and the trends of consumption preferences in the area.

3.3 Study design

A cross-sectional study was conducted from November 2020 to May 2021 at Koka, Ziway, Langano and Hawassa Lakes. The lakes were selected because of the bulk of the fish catch that contributes to 79% of the total fish catch in the country (FAO, 2014). Restaurants in respective areas based on the accessibility to public transport transit areas and presence of recreational activities around the lake.

3.4 Sampling procedure

Purposive sampling strategy was followed in selecting fishes i.e. fish with suggestive lesions (hemorrhages on the external surface, the base of pectoral and tail fin, ulcer on the skin, abdominal distention, unilateral or bilateral exophthalmia, prolapsed anus, and fin rot) of *A. hydrophila* infection were picked for sampling. Tissue samples (muscle, gill, intestine, spleen and kidney) were collected from those fish having suggestive lesions.

All the fishes were caught using gillnets with mesh size ranging from (10 cm to 14 cm) that were used for the exploratory fishing work at the lakes. Samples were carried in Autoclavable sterile plastic bag containing water from the lake where they were caught and transported alive for the case of Koka, Langano and Ziway lakes samples after collection immediately transported to Batu fishery and other aquatic life research center laboratory for post mortem examination and in the case of Hawassa lake, to Hawassa University Biology Department laboratory for post mortem examination and were analyzed immediately.

3.5 Field Examination (Clinical and P.M examination)

Sampled fish were subjected to the clinical examination of the gross external signs as described by Austin and Austin (2016). Fish was killed by transecting the spinal cord behind the skull. Autopsy and examination of the internal organs were carried out according to the method described by Wu et al., (2019). The organs sampled was muscle, gill, intestine, kidney, and spleen for bacterial

culture and molecular analysis. First, the external body surface of the fish was examined for the presence of lesions, the gills, tail, and fins was observed for visible signs of infection and samples from muscle and gill were taken aseptically. After opening the body, the internal organs were exposed with care not to puncture any part of the intestinal tract by using ventral approach. In the absence of any visible lesions samples of a kidney, spleen and Intestine was taken after searing the surface of the organs with a hot scalpel blade. 2gm of each specimen were aseptically taken into the falcon tube (50ml) containing 20ml of Alkaline peptone water PH 8.5 (Oxoid, England) which were kept cool at 4^{o}c.

All raw fish was purchased and collected from traditional markets and supermarkets from respective towns by purposive selection based on the amount of fish stock kept on specific seller, market availability and customer's choice store based on informal collection of data in the area. Each market fish sample was individually packed in a clear sterile polyethylene bag immediate after sampling while, RTE fish samples collected as take away order and bagged in sterile plastic bags. 2gm of each specimen from market fish and RTE added to falcon tube containing 20ml of peptone water and preserved in an icebox. All the specimens from fish, market and RTE were labeled and transferred to the laboratory under aseptic condition with a minimum of delay in Batu and Hawassa University laboratories and finally brought to National Animal Health Diagnostic and Investigation Center (NAHDIC) with Electrical cooler jugs (icebox) for further studies.

3.6 Bacteriological Examination (Phenotypic Identification)

A standard operating protocol was used for isolation and identification of *A. hydrophila* from fish and water samples (Dahdouh et al., 2016; Janda and Abbott, 2010). Aseptically taken 2gm of each fish sample (muscle, gill, intestine, kidney, and spleen) was thoroughly mixed (vortexed) from 20ml of samples in alkaline peptone water which is used as enrichment and transport media as per the method described by (Buller, 2014). The homogenates were incubated for 24 hrs. at 35°C. A loopful from each enriched homogenate was streaked on to Aeromonas Medium Base (Oxoid, England) and incubated for 24 hrs. at 35°C, a single colony from each suspected isolate was picked up and re-streaked on a new plate of its perused selective culture media and re-incubated at the same conditions. presumptive colony from Aeromonas medium base inoculated in to Brain heart infusion broth (Oxoid, England) and incubated for 18-24hrs at 35^{o}C then loopful from the broth

cultured on Nutrient agar media and incubated for 24hrs at 35ºC, each pure colony from the nutrient agar medium used as a stock culture for further biochemical identification.

A. hydrophila were identified biochemically to species level based on colonial characteristics (colony morphology and arrangement) and by using 14 chosen biochemical test including gram staining of the microorganisms, cytochrome oxidase, catalase, motility, sugar utilization, indole, methyl red test, hemolysis production, Voges- Proskauer test, DNase test, gas production from Glucose, acid production from Sucrose, Mannitol and Xylose. Then the phenotypic and biochemical characteristics of the isolates were characterized according to the guideline indicated in Bergey's manual on fish and other aquatic animal practical identification manual (Buller, 2004).

3.7 Phenotypic characterization of A. hydrophila virulence determinants

The collected isolates were examined for their hemolytic activity on 5% whole sheep blood agar medium and results was recorded after 24 hours of incubation at 35°C and checked for the type (α or ß) of hemolytic activity.

3.8 Molecular Detection of A. hydrophila

3.8.1 DNA extraction

Genomic DNA was extracted using the DNA extraction kit (DNeasy kit, Qiagen, Germany) following the manufacturer's instructions. Qiagen DNeasy DNA extraction protocol for bacterial cultures adapted from Qiagen DNeasy handbook, 2020. Briefly, 200µl of the sample suspension was incubated at 70°C for 10 min after the addition of 20µl of proteinase K and 200µl (AL) Buffer or lysis buffer by vortexing. Then, 200µl of 100% ethanol was added to the lysate and mixed thoroughly by vortexing. Washing and centrifugation of the sample was performed following the manufacturer's recommendations. Then, nucleic acid was eluted with 200µl of elution buffer provided in the kit.

3.8.2 Real-time qPCR amplification

Real-time qPCR was performed using a thermocycler for real-time PCR (Applied Biosystems - Model Real time - 7500) and the marker used was Eva green Super mix (Bio-Rad, USA). The Amplification reactions were performed in a reaction mixture of 20 µl volumes consisting of 1µl of each ahaI primer (F and R), 10µl of 10x master mix including buffer, MgCl2, dNTPs, Evagreen and DNA polymerase, 6µL of

RNase-free distilled water and 2µl of genomic DNA template. The PCR program consisted of an initial step at 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15s and annealing at 60°C for 1 minute. At the end of each cycle, a DNA melting curve of the amplified products was performed between 65°C and 95°C, 95ºC for 15sec, 65^{O}c for 1min and 95ºC for 15sec with an increase of 0.5 °C in a stepwise manner to evaluate the melting temperature (Tm) and to check the random amplification of untargeted regions.

Primer design

Two specific primers used here were previously described by (Gertrudes et al., 2018) based on the sequence of the ahaI gene from the strain *A. hydrophila subsp. hydrophila* ATCC 7966. Sequences are shown in Table 1**.**

Table 1: Primer sequences (5' to 3') used to amplify the gene ahaI in A. hydrophila, yielding a 200bp amplicon.

Primer	Primers sequences (5'-3')	T_m (°C)	Reference
ahaI Forward	5- GAGAAGGTGACCACCAAGAACA-3	57.8	(Gertrudes et al., 2018)
ahaI Reverse	5- GAGATGTCAGCCTTGTAGAGCT-3	54.2	

3.9 Antibiogram analysis

A. hydrophila strains was subjected to antibiotic sensitivity test using the Kirby-Bauer disc diffusion method according to the National Committee for Clinical Laboratory Standards (NCCLS) recommendations for Aeromonas species (CLSIM45, 2020). *A. hydrophila* isolates was inoculated in TSB and incubated at 35ºC for 16-20 h, the turbid broth was inoculated in Muller Hinton broth (Oxoid, CM0405), the turbidity was adjusted according to McFarland obesity tube No. 0.5. Isolates was streaked on Muller Hinton agar (Oxoid, CM0337) and disks were placed, incubation was done at 37ºC overnight. The used antibiotics were Amoxicillin-clavulanate (AMC, 30 µg), penicillin (P,10µg), Ampicillin (AMP, 10µg), Ceftriaxone (CRO, 30 µg), Gentamicin (CN, 10µg), Streptomycin (S, 10µg), Tetracycline (TE, 30µg), Ciprofloxacin (CIP, 5µg), Trimethoprim-Sulfamethoxazole (SXT, 25µg) and Chloramphenicol (C, 30µg). Antimicrobials are selected based on the importance and common use in preventing and treating diseases in both veterinary and human medicines. After a period of 24hr. incubation, the zones of inhibition were compared and

measured according to the manufacturer's instruction (CLSIM45, 2020). The result was interpreted as sensitive, intermediate and resistant according to the reference values.

The formula below is used to calculate the Multiple Antibiotic Resistances (MAR index) of the present isolates against tested antibiotics.

MAR index = X/(Y×Z)

Where; X–Total of antibiotic resistance case

Y–Total of antibiotic used in the study

Z–Total of isolates. When the use of antibiotics is seldom or of low dose use for animal of treatment, the MAR value is usually equal to or less than 0.2. In contrast, the elevated rate of use or the high risk of exposure of antibiotics for animal treatment will yield an MAR index value which is more than 0.2.

3.10 Ethical clearance

Ethical clearance was obtained from the animal research ethical review committee of Addis Ababa University College of Veterinary Medicine and agriculture for collecting samples from fishes during this study under the animal welfare guidelines.

3.11 Data management and analysis

The collected data were entered into Excel spreadsheet (Microsoft® office excel 2016) spread sheets and descriptive statistics was used.

4 RESULT

4.1 Clinical and post-mortem Findings

Fishes suspected of infection with *A. hydrophila* showed hemorrhages all over the body especially at the base of fins and tail. Clinical presentations observed include fins rot, cloudiness of both eyes, detachment of scales and skin ulceration and abdominal distention. Internally these fishes showed abdominal dropsy with reddish ascetic exudates, liver paleness and enlargement in some fishes and congested with necrotic patches in other fishes, spleen was congested, enlarged and hemorrhagic enteritis in some fishes as shown in (Figure 10).

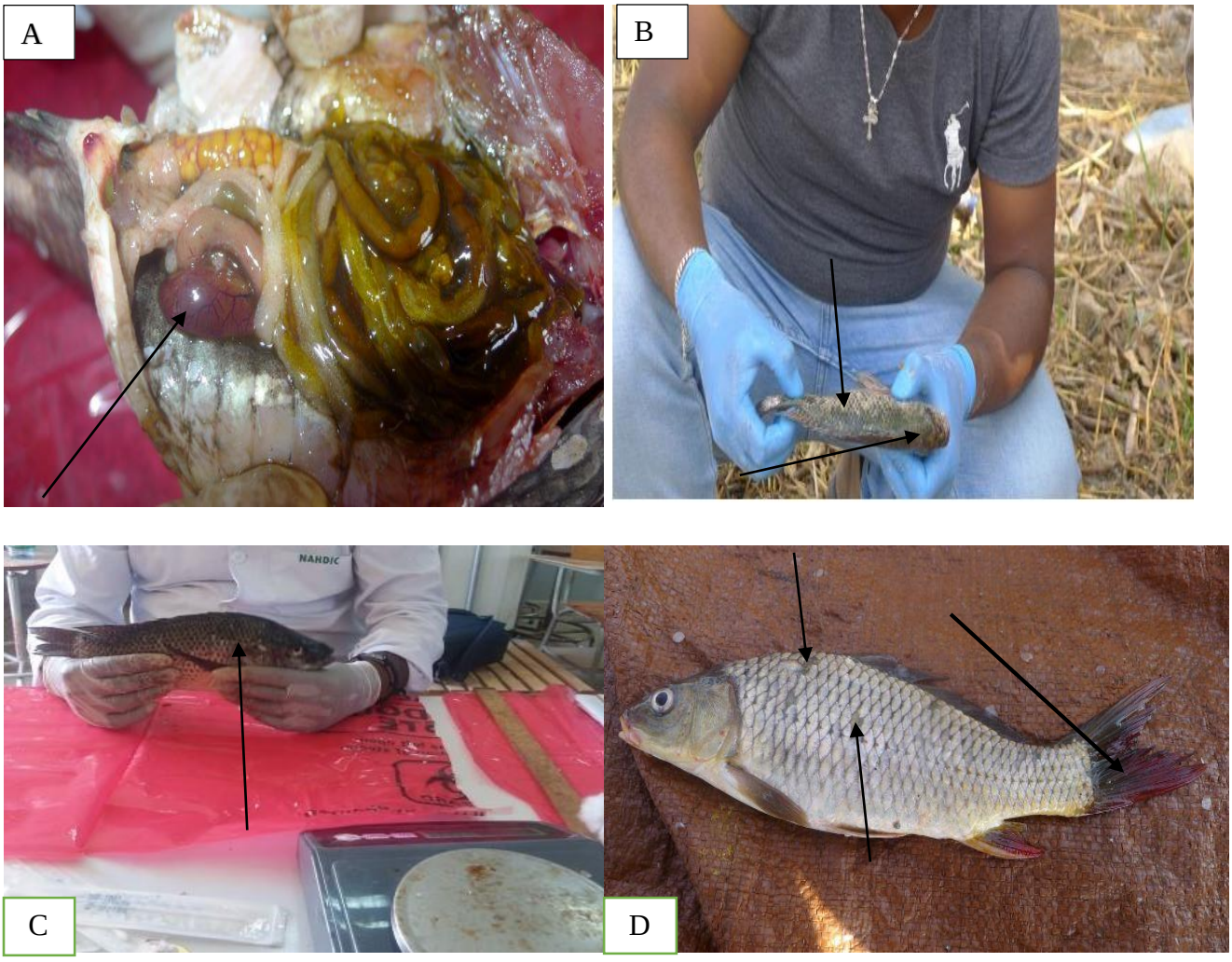
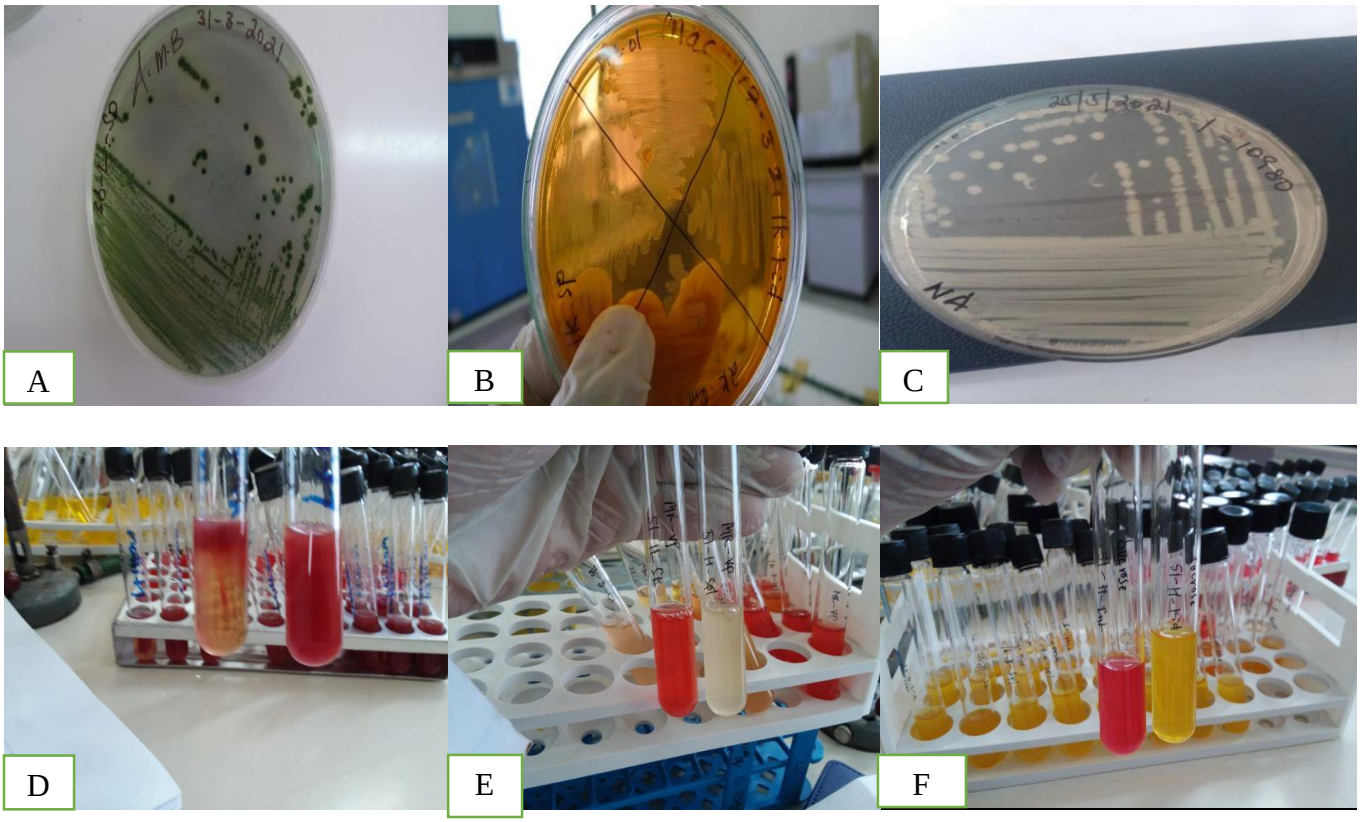


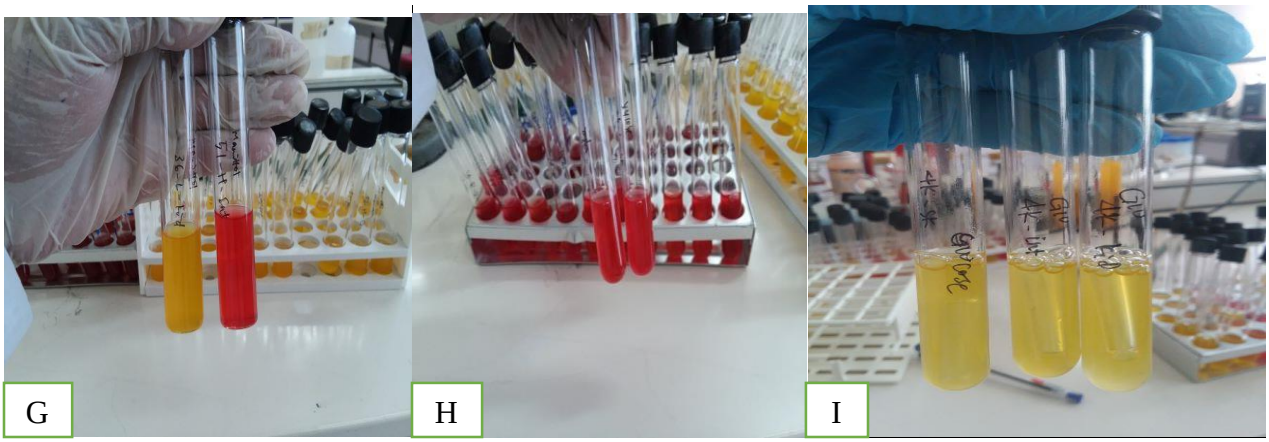
Figure 10: Clinical picture and post mortem findings

An arrow in A) shows abdominal dropsy with reddish ascetic exudates B) shows skin hemorrhage at the base of pectoral fin with hemorrhagic skin ulcer under the dorsal and tail fin C) dark discoloration in the skin D) shows skin ulcer.

4.2 Bacteriological identification and biochemical characterization of A. hydrophila

The presumptive identification of the bacteria in the current study was carried out from the colony morphology over Aeromonas Medium Base, a selective medium for *A. hydrophila*. Accordingly, based on 14 morphological and biochemical tests, a total number of 81(60.45%) isolates were presumptively identified as *A. hydrophila.* They appeared rounded smooth colonies 2-3mm in diameter and dark green with a darker center in Aeromonas medium base and creamy white on Nutrient agar. Colonies were gram-negative short rods, they gave a positive reaction for oxidase, catalase, DNase, Indole production, also ferment glucose with production of acid and gas, sugar utilization K/A, Acid production from (Sucrose and Mannitol) and Motile. They gave negative results toward xylose, urea hydrolysis, and non-lactose fermentation and produced variable results with MRVP as presented in (Figure 11).





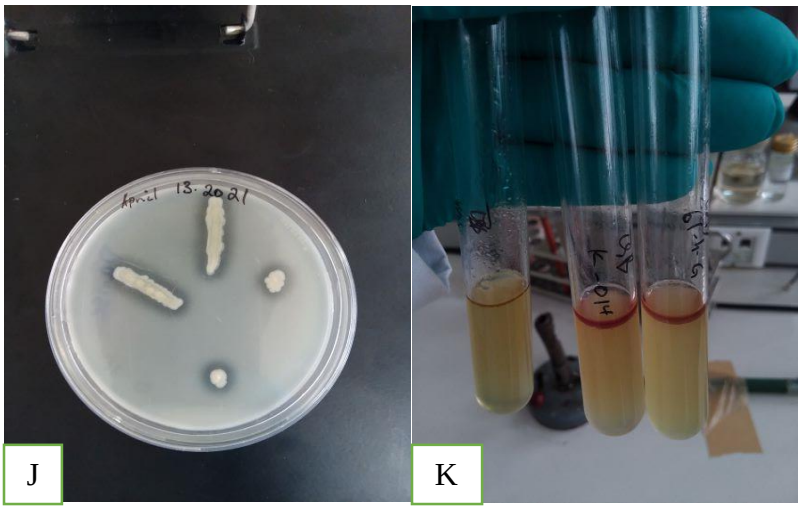


Figure 11: Biochemical characteristics of *A. hydrophila* isolate

A) Colony morphology in Aeromonas medium base B) growth on mac-Concey agar C) growth on Nutrient Agar. D) Motility E) MR positive F) sucrose positive G) mannitol positive H) Xylose negative I) acid fermentation with gas production J) DNase positive K) Indole positive

4.3 Hemolysis assay

Hemolytic activity of the isolates was determined for its importance as a virulent factor. *A. hydrophila* produced hemolysis on blood agar base with 5% sheep blood. Accordingly, from the current study found that 93.33% (n=56/60), 94.11% (n=16/17) and 75% (n=3/4) isolates from the life fish group, market fish and RTE fish show β hemolysis respectively and 6.66% (n=4/60), 5.88% (n=1/17) and 25% (n=1/4) show α hemolysis. The hemolysis pattern results in the media displaying clear halos around bacterial colonies as

shown in (Fig 12). Table 2. Represent hemolytic activities of *Aeromonas hydrophila* from the current study found over all isolates 92.59% (n=75/81) show β hemolysis and only 7.4% (n=6/81) show α hemolysis.

Table 2: Hemolytic characteristics of the isolates

Hemolytic activity of *A. hydrophila* isolated from fish samples Source			
Source	**Total**	**β**	**α**
Live fish	60	56 (93.33)	4 (6.66)
Market fish	17	16 (94.11)	1(5.88%)
RTE	4	3 (75%)	1 (25%)
Total	81	75 (92.59%)	6 (7.4%)

β: beta, α: alpha

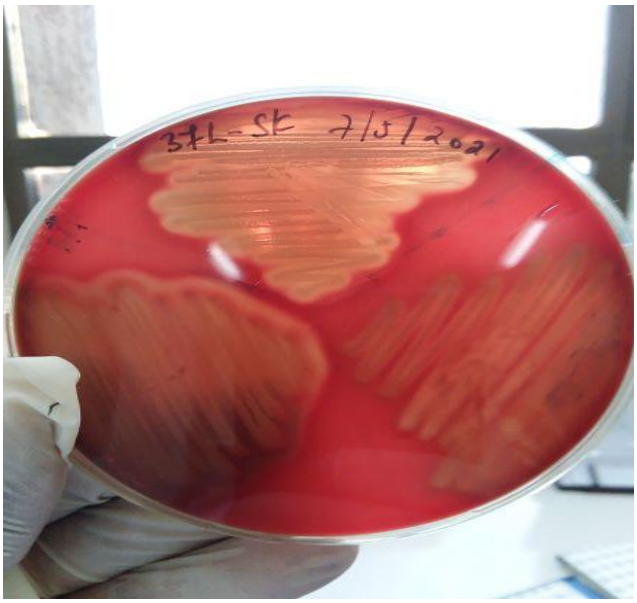


Figure 12: ß-hemolysis activity by *A. hydrophila* on blood agar base with 5% sheep blood

4.4 Molecular Detection

4.4.1 Quantitative Real-time PCR detection of A. hydrophila and virulence gene

Molecular detection with Real-Time PCR (qPCR) using specific primers based on the sequence of the ahaI gene coding for adhesive surface protein mainly present in virulent *A. hydrophila* strain. From the total of 81 *A. hydrophila* isolates, 54 were confirmed by real-time PCR for presence of the ahaI gene. The threshold cut off value for classifications of the samples as positive or negative by the real-time PCR was set to a cycle threshold (Ct) value of 34. Samples giving a Ct value of $\leq$ 34 with a sigmoid shape of the analysis curve were classified as positive (Figure 13). Samples with a Ct value > 34 were classified as negative. The Ct value of real time PCR positive samples ranges between 19-34. A no-template control and positive control were included in every reaction. The melting curve analysis of the PCR products showed typical melting profiles at 85^{o}C (Figure 14), while the negative samples did not show any melting curve.

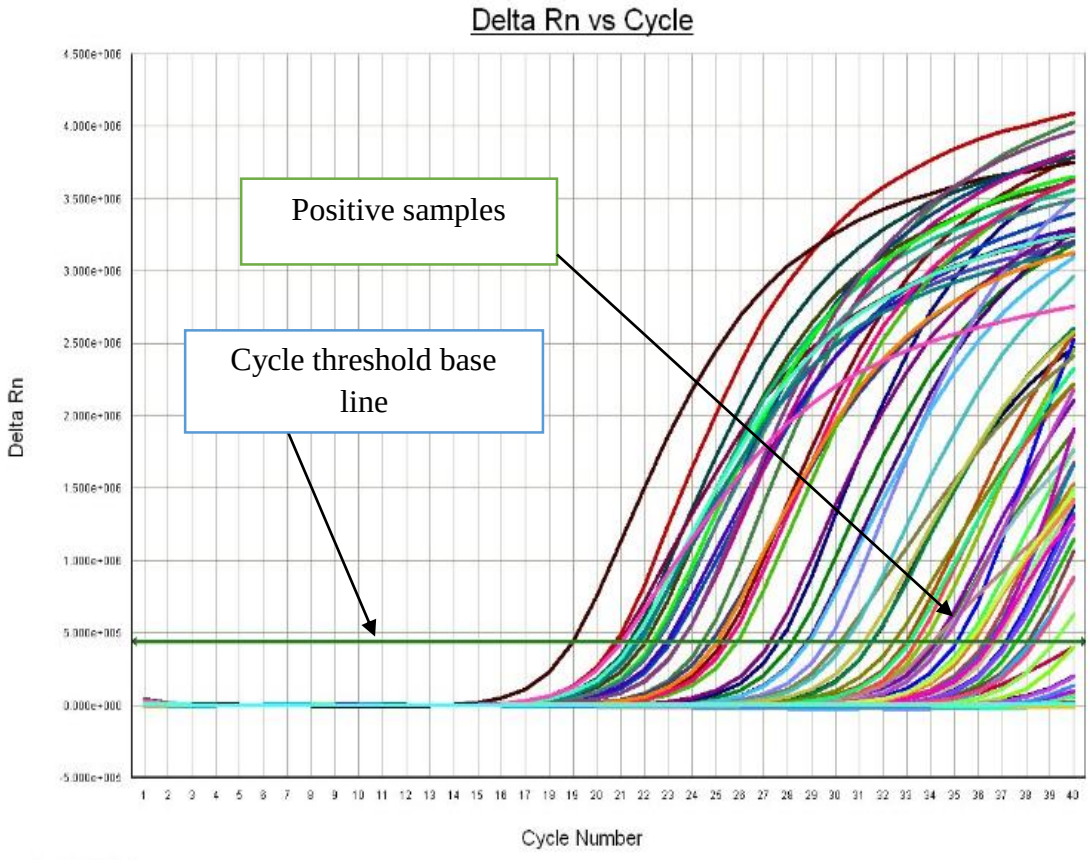


Figure 13: Real time PCR positive samples of *A. hydrophila*

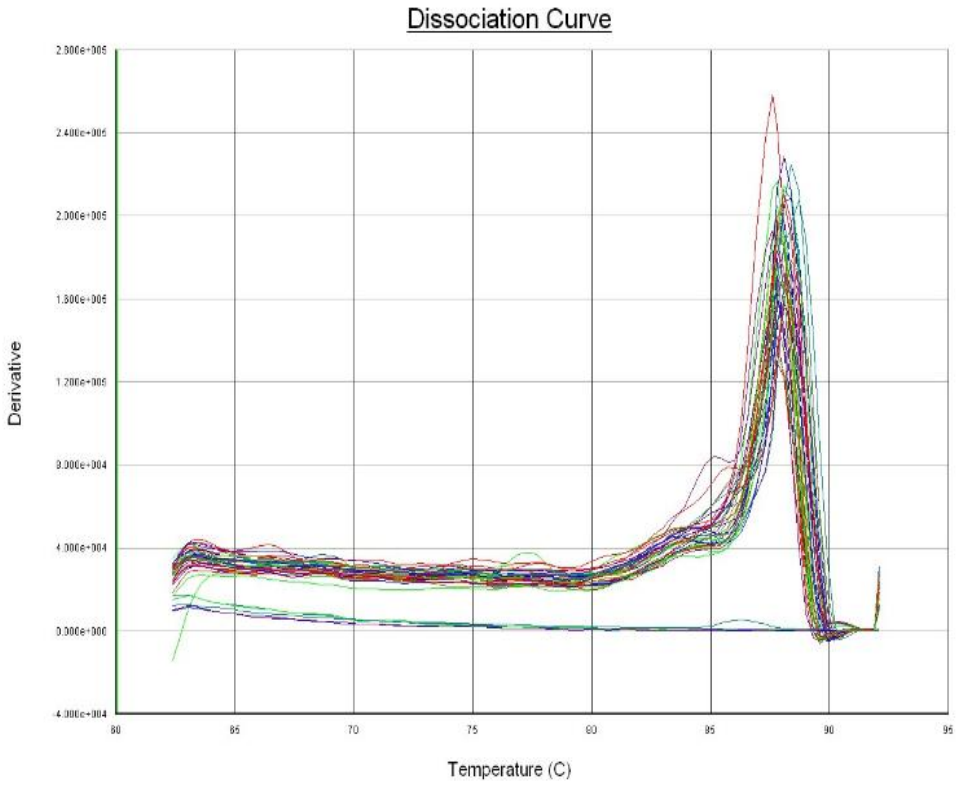


Figure 14: Melting curve analysis

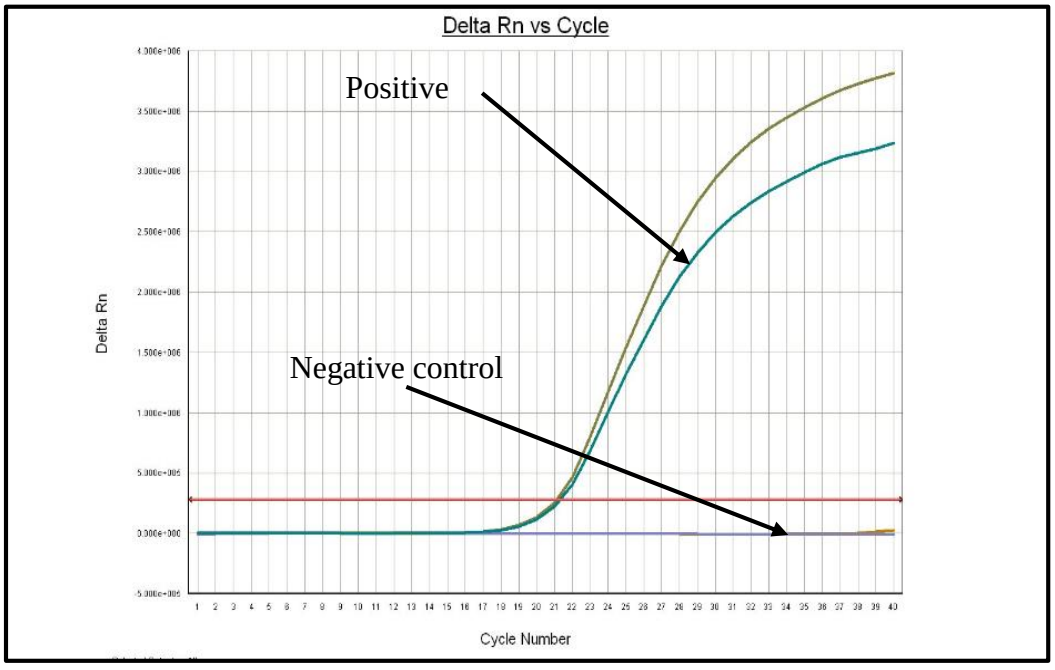


Figure 15: Positive and Negative Controls

4.4.2 Detection of A. hydrophila from different source

A total of 140 samples were collected from different sources and subjected to culture on *A. hydrophila* selective media (AMB). From these, 81 (57.86%) isolates were presumptively identified as *A. hydrophila* by morphological and biochemical examination. These isolates were further confirmed as *A. hydrophila* by qPCR 54 (66.67%) positive as shown in (Figure 13) based on specific primers on the sequence of the ahaI gene from the strain *A. hydrophila subsp. hydrophila* ATCC 7966.

Table 3: Detection of *A. hydrophila* based on source

Factors	**No of sample cultured**	**CP**	**qPCR**
Live Fish	100	60	37 (61.67%)
Market	20	17 (85%)	13 (76.47%)
RTE	20	4 (20%)	4 (100%)
Total	140	81 (60.45%)	54 (66.67%)

CP: culture positive, qPCR: quantitative polymerase chain reaction

4.4.3 Detection of Aeromonas hydrophila isolated from examined fishes based on the Organs

In the current study, *A. hydrophila* was detected on the basis of their organ's location. Accordingly, the highest detection (40.54%) was assessed in both Muscle and gill, and the lowest (2.7%) was observed in Spleen (Table 4)

Table 4: detection of *A. hydrophila* in respect to the organs

Organ	**No of culture positive**	**Total qPCR positive**
Muscle	17	15/37 (40.54%)
Gill	18	15/37 (40.54%)
Intestine	5	4/ (10.81%)
Spleen	8	1/37 (2.7%)
Kidney	12	2/37 (5.4%)
Total	60	37/60 (61.67%)

4.5 Antibiogram Analysis

In the present study, antibiogram assay for the examined *A. hydrophila* isolates concerning 10 antibiotics revealed that all the tested isolates were completely sensitive to ciprofloxacin (100%), chloramphenicol (100%) and ceftriaxone (100%). In addition, amoxicillin and penicillin did not exhibit any bactericidal activity (100% resistant) as shown in (Figure 17).

Figure 16: McFarland Densitometer

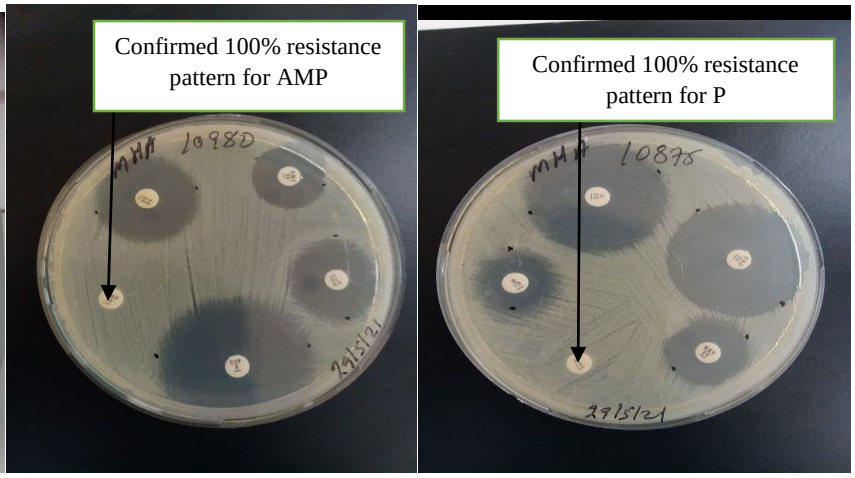


Figure 17: Confirmed complete drug resistance pattern for P and AMP.

Table 5: Antibiotic susceptibility of *A. hydrophila*

Source	Isolate ID	Antimicrobial Agents Concentration µg									
		AMP,10 µg	CN 10µg,	AMC 30µg	TE 30µg	CIP 5µg	S 10µg	C 30µg	SXT 25µg	CRO 30µg	P 10µg
Life fish	10881	R	S	S	S	S	I	S	I	S	R
	10875	R	S	R	S	S	S	S	S	S	R
	10886	R	S	I	S	S	I	S	S	S	R
	10970	R	S	S	S	S	I	S	S	S	R
	11060	R	S	R	S	S	I	S	I	S	R
Market fish	10880	R	S	R	R	S	S	S	S	S	R
	10976	R	S	R	S	S	I	S	I	S	R

RTE	10980	R	S	R	R	S	I	S	I	S	R
	10895	R	S	R	I	S	I	S	S	S	R
Water	11071	R	S	S	S	S	S	S	S	S	R

Table 6: Frequency distribution of multidrug resistant *A. hydrophila* isolates

Resistance pattern	***A. hydrophila* isolates (no = 10)**		
	No. of *A. hydrophila* isolates	**Percentage of *A. hydrophila* isolates**	**MAR index**
Resistance to 2	10	100	0.28
Resistance to 3	6	60	0.2
Resistance to 4	2	20	0.08
Average MAR = 0.18			

MAR: Multi – Antibiotic Resistance

5 DISCUSSION

Bacterial diseases are considered to be the most serious disease problem among freshwater fishes (Fernandez-Bravo and Figueras, 2020). *A. hydrophila* has gained increased attention due to pathogenicity to humans and the ubiquity of the organism in the environment, food and water (Skwor et al., 2020). Isolation of *A. hydrophila* from four freshwater lake fishes along its value chain during the current study adds more evidence for the wide geographical distribution of the bacteria.

Concerning the clinical picture and postmortem findings observed in the current study of Nile tilapia showed hemorrhages on the external surface, the base of pectoral and tail fin, ulcer on the skin, abdominal distention, prolapsed anus, and fin rot. Postmortem examination revealed that the accumulation of yellowish watery fluid in the abdominal cavity, pale anemic, and friable liver with some hemorrhagic patches with the distended gall bladder. The observed clinical and postmortem findings were nearly similar to those described by (Abd-el-malek *et al*., 2019; Austin and Austin, 2016; Ramadan et al., 2018).

The phenotypic and biochemical characteristics of *A. hydrophila* isolates recorded were in line to those reported in Bergey's manual of determinative bacteriology (Garrity and Holt, 2001). Similar phenotypic and biochemical findings with current study were also reported by (Abdel-latif, 2020; Austin and Austin, 2016; Buller, 2014; Mohammed et al., 2018; Skwor et al., 2020).

Hemolytic activity of the isolates was determined for its importance as a virulent factor. Accordingly, from the current study found that 93.33%, 94.11% and 75% isolates from the live fish group, market fish and RTE fish showed β hemolysis respectively and 6.66%, 5.88% and 25% showed α hemolysis. From over all isolates 92.59% show β hemolysis and only 7.4% show α hemolysis. These toxins are responsible for lethality, hemolysis and entero-toxigenicity. Their production by organisms found in food signals public health concern. The secretion of these extracellular proteins hemolysin associated with bacterial virulence (hemolytic toxins) contribute to the virulence of *A. hydrophila* in fish and human host. The bacterium could be entero-toxigenic and may be responsible for outbreaks of diarrhea if the fish are consumed without proper cooking in humans.

Molecular characterization of isolate using real-time PCR for the first time provided evidence for presence of ahal gene in *A. hydrophila* infecting fish of Ethiopia. The optimized qPCR protocol which uses ahaI gene. Accordingly, qPCR revealed presence of adhesin gene in 66.67% of the A. *hydrophila* isolated from samples. The adhesin gene is a virulence gene that code for bacterium surface protein useful to surface

binding, colonization and infection of the host tissue. Targeting this adhesin gene (ahaI) constitutes an interesting and valuable study, not only to identify the specie, but also, enables future projects regarding recombinant adhesin as potential vaccine against Aeromonadaceae (Sebastião et al 2018). From the total of 54 (66.67%) of qPCR positive samples, 37 (68.51%), 4 (7.4%), and 13 (24.07%) were from Fish source, RTE, and market fish respectively with no disease outbreak reported in all lakes at the point in time. As it was explained by Gilda (2001), that disease occurrence in fish is a function of the pathogen, host and the environment. These results were at par with those reported by Aun and Alzainy (2011) in Iraq who found that over all detection rate of 65% *A. hydrophila*, Ullmann et al. (2005) in Berlin, Germany who found 63.% cytotoxin producing *A. hydrophila*. However, lower prevalence were detected by Nagarajan and Ramaiyan (2008) in Tamilnadu, India who found 40% of detection rate; Abd-el-malek (2017) who found 40% of *A. hydrophila* from wild fish in Assiut, Egypt, Mohammed et al. (2018) in Moshtohor Egypt, who detected the total prevalence of bacterial infection (55.3%), Dahdouh et al. (2016) who found the prevalence of *A. hydrophila* 47% in Alexandria, Egypt, and Rodrigues et al. (2019) in Brazil who found the total prevalence of 46.66% *A. hydrophila*. However, a higher prevalence of *A. hydrophila* (95.06%) was reported by Gobat and Jemmi (1993) in LiebefeM-Bern, Switzerland and Aboyadak et al. (2015) in Kafrelsheikh governorate, Egypt who found a total prevalence of 75%. Variations in the incidence level of *A. hydrophila* in the fish worldwide can be attributed to sampling time and geographical range (Vivekanandhan et al., 2005). Difference in the current study may be attributed to the number of examined fish, the size of fish and environmental conditions, geographical range, seasons of the study, sensitivity, and specificity of the techniques used to identify the bacteria.

Overall *A. hydrophila* (24.07%) contaminations in the market fish and RTE (7.4%) was observed in the current study. These results are in accordance with Yucel et al. (2004), who identified *A. hydrophila* (22.6%) from market fish in Ankara (Turkey). Sousa and Silva-souza (2001) who detected 22.9% *A. hydrophila* from market fish samples in Brazil. However, lower prevalence was detected by Santos et al. (2002) who isolated 13 % *A. hydrophila* from market fish samples in Brazil. Different studies have reported inconsistent detection rates of *A. hydrophila* for instance, Minana et al. (2002) identified 2% of market fish in Spain, While, in India, 15.6% detection rate of *A. hydrophila* was reported in marketed fish samples by (Seethalakshmi et al., 2008). However, a higher prevalence recorded by Alhazmi (2015) in Riyadh, Saudi Arabia who found 34% from fish market samples, Abd-el-malek (2017) who found (40%) of *A. hydrophila* from market fish in Assiut, Egypt and Attia et al. (2018) who reported overall higher *A. hydrophila* (51.4%) contaminations in the market fish in Sharkia Governorate, Egypt. This may be due to post-harvest

contamination during selling through fishermen improper handling and transportation from the catching area. Fish in retail in the current study area are considered potential source for infection of human consumers. Although, the source of the organism may be ambient environment, secondary contamination during catching, handling and transportation may also contribute for its distribution.

Fish products ("leb-leb", fish salads, "gulash", smoked fish, etc.) are some of the most popular RTE choices in Ethiopia. Concerning the detection of *A. hydrophila* in RTE fish, the current study revealed 7.4%. This results are in accordance with Attia et al. (2018), who detected the prevalence of *A. hydrophila* in RTE grilled fish 8.6%. Mohamed (2012) in Assiut Egypt, reported that *A. hydrophila* 20 and 10%, detection rate in grilled and fried fish samples respectively. A lower percentage (2.3%) of *A. hydrophila* was reported in RTE fish product in India by Gupta (2013). Whereas, a higher percentage (77.3%) in RTE fried fish in India was also reported by Manna et. al (2013). The contamination rate in RTE fish may suggesting contamination after cooking caused by lack of hygiene, contaminated water or contaminants from uncooked produce. The presence of *A. hydrophila* in RTE products again may be attributed to rapid grilling which could be insufficient to kill *A. hydrophila* that may be present in raw fish before preparation.

Regarding the frequency of detecting *A. hydrophila* from the different parts of the fish, out of 37 (68.51%) fish tissue samples, it was noticed that the highest (40.54%) gene detection was recorded from both gill and muscle respectively, (10.81%) from intestine, 5.4% from kidney and the lowest (2.7%) gene detection was recorded from spleen. The high proportion of infection in gills and muscle in comparison to other organs is due to the exposed nature of the organ to microbiota. The current findings are supported by the observations of (Gobat and Jemmi, 1993; John and Hatha, 2012; Nahar et al., 2016; Rahman et al., 2002) who reported that *A. hydrophila* has detected from wild fish, pond cultured edible and ornamental fish from different parts of the fish. These attributed to the ubiquitous nature of the microorganism in the aquatic environment. The predominance of *A. hydrophila* in the gill and muscle of fishes may be attributed to the presence of *A. hydrophila* in contaminated water in which the fish lives (Fowoyo and Achimugu, 2019).

With the steady expansion of the fishery industry, the vast use of antibiotics will be unavoidable. The continuous and extensive use of antibiotics in humans also led to the emergence of antimicrobial-resistant strains worldwide. Ten antibiotics namely; Ampicillin, Penicillin, Tetracycline, Ciprofloxacin, Chloramphenicol, Streptomycin, Gentamicin, Ceftriaxone, Amoxicillin-clavulanate, and Trimethoprim-Sulphamethoxazole were used in the current study mainly due to their routine usage in veterinary and human medicine. Fish treatments are not practiced almost in all fishery and aquaculture sectors of Ethiopia

but, Tetracycline is commonly applied for the treatment of bacteremia in fishery research centers of Ethiopia (observation).

In the present study, antibiogram assay for the examined *A. hydrophila* isolates concerning 10 antibiotics revealed that all the tested isolates were completely sensitive to ciprofloxacin (100%), chloramphenicol (100%) and ceftriaxone (100%). In addition, amoxicillin and penicillin did not exhibit any bactericidal activity (100% resistant) against the tested isolates. These results are nearly agreed with those obtained by (Aa and Najiah, 2013; Baron et al., 2017; Dahdouh et al., 2016; Fernandez-Bravo and Figueras, 2020; Igbinosa and Okoh, 2012; Lim et al., 2019; Ramadan et al., 2018; Stratev and Odeyemi, 2016; Zdanowicz et al., 2020). Freshwater streams are usually receptors of many industrial, domestic and agricultural wastes, which could contain antimicrobial agents and antimicrobial-resistant bacteria (Mahsa Ansari, 2011; Von Graevenitz, 2007). Due to diverse microbial population in such ecosystems freshwater environment provides favorable conditions for the spread of antimicrobial resistance. The resistance to penicillin in *A. hydrophila* mainly attributed to β -lactamase production that encoded in their chromosomes. The antibiotic resistance has a public health concern it mainly results from the improper intensive use of antibiotics (Aa and Najiah, 2013). The aeromonads have been regarded as being universally resistant to penicillin (Aa and Najiah, 2013), in the current study penicillin and ampicillin resistance were confirmed. In the present study the multi-drug resistant (MAR) of the *A. hydrophila* were 0.18 and this finding are in accordance to the previous study of (Aa and Najiah, 2013; Stratev and Odeyemi, 2016).

6 CONCLUSION AND RECOMMENDATIONS

The present study provided first evidence infections of fish and fish products with virulent *A. hydrophila* strains. The pathogen was isolated and identified in 81 samples. On phenotypical assessments 92.59% (n=75) of the isolate expressed virulence trait of ß – hemolysis. Molecular characterization using real-time PCR revealed presence of the adhesin gene (ahaI) in 54 (66.67%) of the isolates. Meanwhile, antimicrobial susceptibility test on selected *A. hydrophila* strains revealed the presence of resistance to amoxicillin and penicillin. The phenotypic and genotypic analysis provided epidemiological evidences for dissemination of a virulent *A. hydrophilia* strain among the fish population in rift valley lakes. The detection of the pathogen in hemopoetic organ of the sampled fish population is alarming for potential outbreaks. The identified *A. hydrophilia* isolates carry virulence trait that aids in colonization, infection and pathogenicity with ability to resist antibiotics commonly used in human and veterinary medicine. *A hydrophila* is a zoonotic emerging pathogen and fish in lakes and fish products from Lake Koka, Zeway, langano and Hawassa are a potential sources of infection for humans in the area.

Based on the above conclusive remarks, the following recommendations are forwarded:

- Public awareness concerning risks of handling fish, improperly grilling or frying fish, or eating raw fish must be enhanced to ensure fish food safety and public health.
- Further molecular epidemiology study to establish potential inter host transmission and antibiotic resistance traits is important.
- Effective control strategies of the disease should be implemented across fishery value chains.

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8 ANNEXES

Annex 1: Protocol: Purification of Total DNA from Bacterial Culture

Important point before staring

- Before using the DNeasy Tissue kit for the first time, read “important notes” on page 10-17
- All centrifugation steps are carried out at room temperature (15-25c)
- Vortexing should be performed by pulse vortexing for 5-10s
- Optionally, RNase A may be used to digest RNA during the procedure. RNase A is not provided in the RNeasy tissue kit (see “co-purification of RNA”, page 14).

Things to do before starting

- Buffer AL* may form a precipitate upon storage. If precipitate has formed, incubate the buffer 55C until the precipitate has fully dissolved.
- Buffer AW1* and AW2* are supplied as concentrates. Before using for the first time, add the appropriate amounts of ethanol (96-100%) to buffer AW1 and AW2 as indicated on the bottles.
- Prepare a 70C water bath or heating block for use in step 2.
- Prepare PBS, PH7.2 (50Mm potassium phosphate; 150mM NaCl), for use in step 1.

Procedure

1. Centrifuge the appropriate number of cells (maximum 5x10^6) for 5min at 300xg.resuspend pellet in 200µl PBS (not supplied).

   When using a frozen cell pellet, before adding PBS allow cells to thaw until the pellet can be dislodged by gently flicking the tube.

   Ensure that an appropriate number of cells is used in the procedure. For cell lines with a high degree of ploidy (e.g Hela cells) it is recommended to use less than the maximum number of cells listed in Table 1(page 10)

   Optional: if RNA free genomic DNA is required, add 4µl RNase A (100mg/ml) and incubate for 2 min at room temperature. If residual RNA is not a concern, continue with step 2.
2. Add 20 µl proteinase K and 200 µl buffer AL to the sample, mix thoroughly by vortexing and incubate at 70C for 10min.

It is essential that the sample and buffer AL are mixed immediately and thoroughly by vortexing and pipetting to yield a homogeneous solution.

Note: do not add proteinase K directly to buffer AL.

3. Add 200µl ethanol (96-1005) to the sample and mix thoroughly by vortexing

   It is important that the sample and ethanol are mixed thoroughly to yield a homogenous solution

   A white precipitate may form an addition of ethanol .it is essential to apply all of the precipitate to the DNase mini spin column. This precipitate does not interfere with the DNeasy procedure.
4. Pipet the mixture from step 3 (include any precipitate) into DNeasy mini spin column placed in a 2ml collection tube (provided). centrifuge at>=6000xg(8000rpm) for 1min. discard flow through and collection tube.
5. Place the DNeasy mini spin column in a new 2ml collection tube (provided), add 500µl buffer AW1, and centrifuge for 1min at >=6000xg(8000rpm). Discard flow-through and collection tube.
6. Place the DNeasy mini spin column in a new 2ml collection tube(provided) add 500µl buffer AW1, and centrifuge for 3min at 20,000x g (14,000rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.

   It is important to dry the membrane of the DNeasy mini spin column, since residual ethanol may interfere with subsequent reaction. This centrifugation step endures that no residual ethanol will be carried over during the following elution.

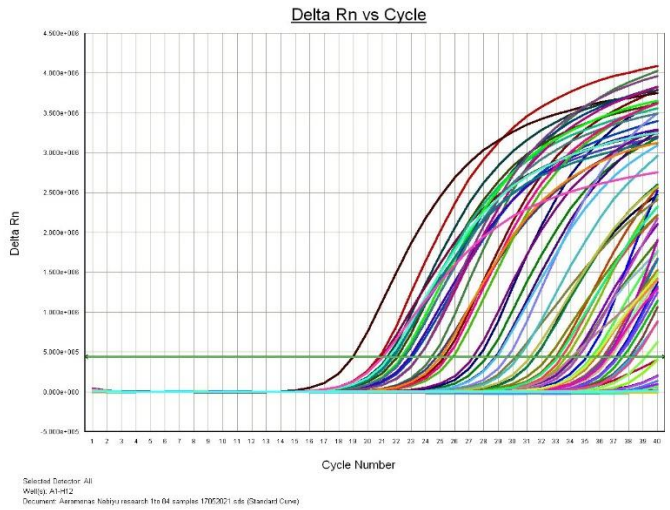
   Following the centrifugation step, remove the DNeasy mini spin column carefully so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol. If carryover of ethanol occurs, empty the collection tube, then reuse it in another centrifugation for 1 min at 20,000xg (14000rpm)
7. Place the DNeasy mini spin column in a clean 1.5 ml or 2ml micro-centrifuge tube (not provided) and pipet 200µl buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1min, and then centrifuge for 1min at >=6000xg (8000rpm) to elute.

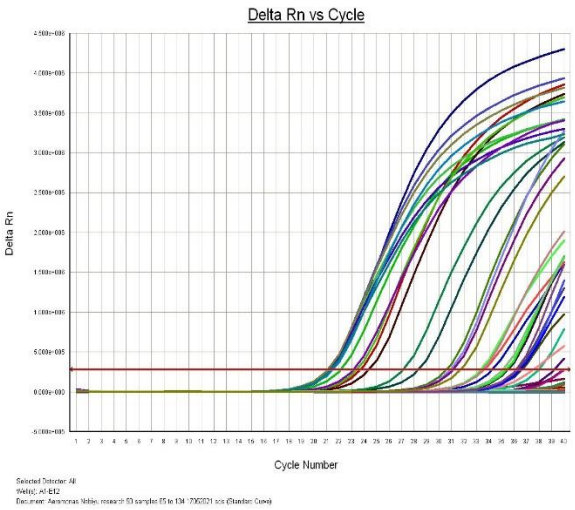
   Elution with 100µl (instead of 200µl) increase the final DNA concentration in the eluate, but also decrease the overall DNA yield (see the figure 2, page 14)
8. Repeat elution once as described in step 7.

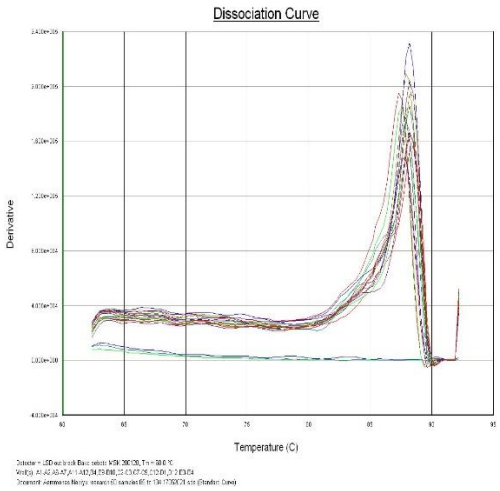
   A new micro-centrifuge tube can be used for the second elution elution step to prevent dilution of the first eluate. alternatively, to combine the eluates, the micro-centrifuge tube from step 7 can be reused for the second elution step.

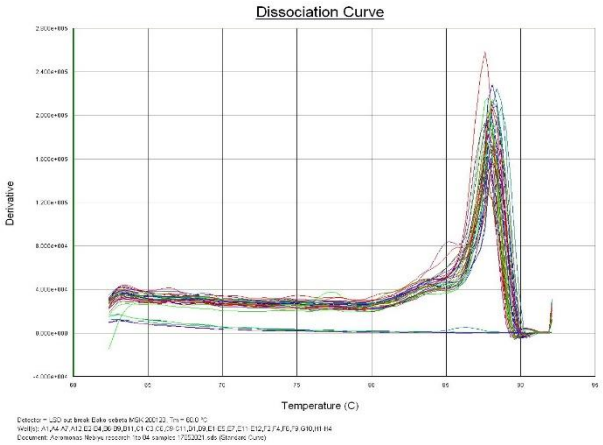
Note: more than 200µl should not be eluted into a 1.5 ml micro-centrifuge tube because the DNeasy mini spin column will come into contact with the eluate.

Annex 2: Real Time results.



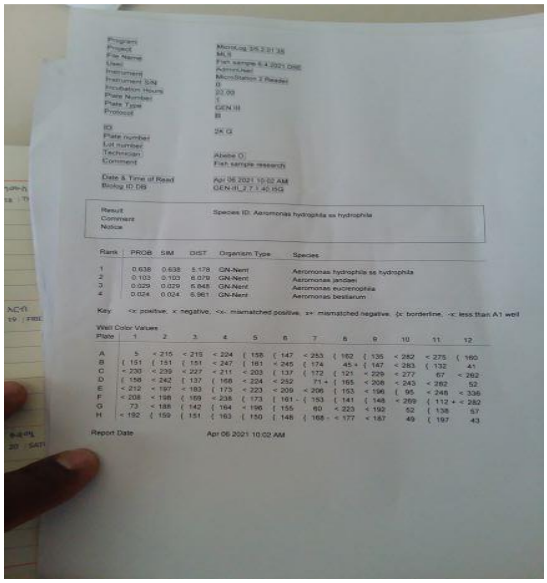






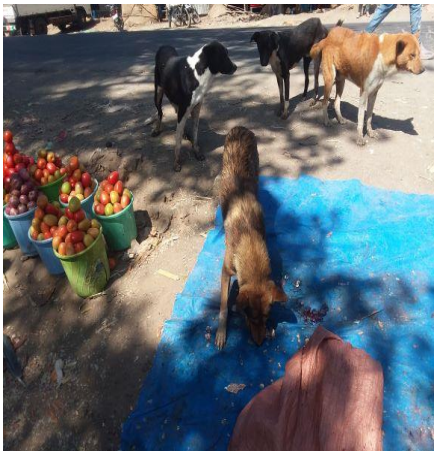
Annex 3: Photo Gallery

Samples confirmed by Biolog Micro log system



Fish marketing in the study areas area



Raw fish eating practice in shore line


Collecting information (Discussing) about fish disease in the study areas


Fish transportation with out keeping cold chain

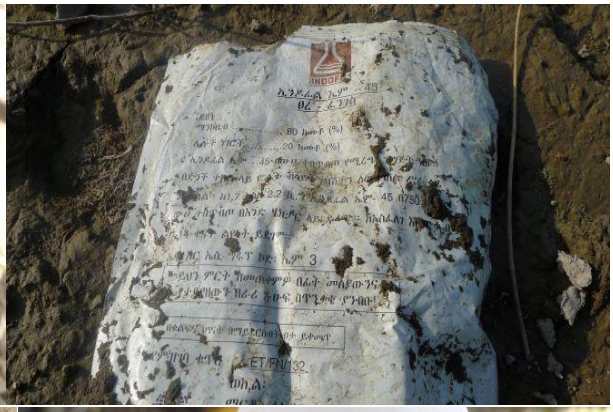

Effluents from factory and sewarage from towns, chemicals from farm activity dirrectly discharged in to the lakes





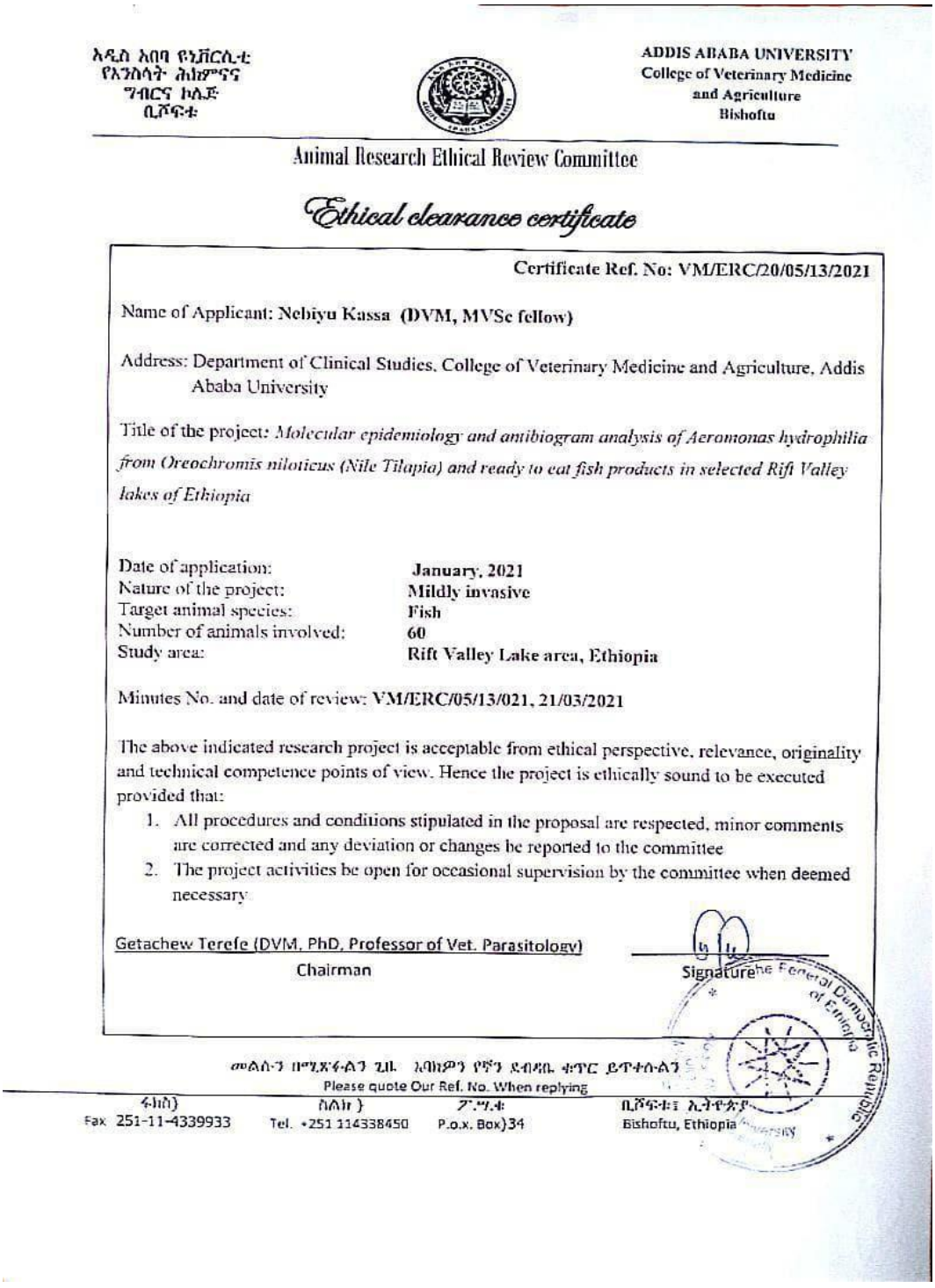



Some of chemicals used in shore line farming in the study areas



9 APPENDICES

Appendix 1: Ethical clearance certificate for sample collection from animals



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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu

Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/20/05/13/2021

Name of Applicant: **Nebiyu Kassa (DVM, MVSc fellow)**

Address: Department of Clinical Studies, College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: *Molecular epidemiology and antibiogram analysis of Aeromonas hydrophilia from Oreochromis niloticus (Nile Tilapia) and ready to eat fish products in selected Rift Valley lakes of Ethiopia*

Date of application:	**January, 2021**
Nature of the project:	**Mildly invasive**
Target animal species:	**Fish**
Number of animals involved:	**60**
Study area:	**Rift Valley Lake area, Ethiopia**

Minutes No. and date of review: **VM/ERC/05/13/021, 21/03/2021**

The above indicated research project is acceptable from ethical perspective, relevance, originality and technical competence points of view. Hence the project is ethically sound to be executed provided that:

1. All procedures and conditions stipulated in the proposal are respected, minor comments are corrected and any deviation or changes be reported to the committee
2. The project activities be open for occasional supervision by the committee when deemed necessary

Getachew Terefe (DVM, PhD, Professor of Vet. Parasitology)
Chairman

Signature

The Federal Democratic Republic of Ethiopia

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Please quote Our Ref. No. When replying

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