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OCCURRENCE, ANTIMICROBIAL SUSCEPTIBILITY AND PUBLIC HEALTH IMPORTANCE OF SHIGELLA FROM FISH VALUE CHAIN IN BAHIR DAR CITY, NORTH WEST ETHIOPIA

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COLLEGE OF AGRICUTLURE AND ENVIROMENTAL SCIENCE

SCHOOL OF ANIMAL SCIENCE AND VETERINARY MEDICINE

DEPARTMENT OF VETERINARY SCIENCE

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MSc Thesis

By

Mekidm Tamer Wubayehu (DVM)

July, 2020

Bahir Dar, Ethiopia

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SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN VETERINARY PUBLIC HEALTH

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THESIS APPROVAL SHEET

As member of the Board of Examiners of the Master of Sciences (M.Sc.) thesis open defense examination, we have read and evaluated the thesis prepared by Mekidm Tamer Wubayehu entitled **Occurrence, Antimicrobial Susceptibility and Public health importance of *Shigella* from fish value chain in Bahir Dar Town, North West Ethiopia.**

We hereby certify that, the thesis is accepted for fulfilling the requirements for the award of the Degree of Master of Sciences (M.Sc.) in Veterinary Public Health.

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DECLARATION

This is to certify that this thesis entitled "**Occurrence, Antimicrobial Susceptibility and Public health importance of *Shigella* from fish value chain in Bahir Dar city, North West Ethiopia.**" submitted in partial fulfillment of the requirements for the award of the degree of Master of Science in "**Veterinary Public Health**" to the Graduate Program of College of Agriculture and Environmental Sciences, Bahir Dar University by (Mekidm Tamer Wubayehu) (ID. No. BDU1100534) is an authentic work carried out by him under our guidance. The matter embodied in this project work has not been submitted earlier for award of any degree or diploma to the best of our knowledge and belief.

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STATEMENT OF AUTHOR

First, I declare that this thesis is my *bonafide* work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for MSc degree at Bahir Dar University; College of Agriculture and Environmental Science; School of Animal science and Veterinary Medicine and which is deposited at the University/College library to be made available to borrowers under rules of the Library. Brief quotations from this thesis are allowable without special permission provided that accurate acknowledgement of source is made.

Name of student

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Date of Submission: _____

DEDICATIONS

This thesis is dedicated to my father, Mr Tamir Wubayehu and mother Miss Abezu Mekonnen for their encouragements and patience, and to my friend Dr. Taye and Dr. Estibel who have been a great source of motivation and inspiration. This thesis is also dedicated to all those who believe in the richness of learning.

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LIST OF ABBREVIATIONS

APHA	American Public Health Association
BFEA	Bahir Dar Fisher men Enterprise Association
CDC	Center for Disease Control and Prevention
CFS	Centre for Food Safety
CLSI	Clinical and Laboratory Standards Institute
CSA	Central Statistical Agency
FAO	Food and Agricultural Organization of United States
FBD	Foodborne diseases
GFI	Global Foodborne Infections
GFN	Global Foodborne Infections Network
HEA	Hektoen Enteric agar
HPA	Health Protection Agency
ICMSF	International Commission on Microbiological Specifications for Foods
MAC	MacConkey agar
MoH	Ministry of Health
RTE	Ready to Eat
SSA	*Salmonella-Shigella* agar
TSI	Triple Sugar Iron
UNIDO	United Nations Industrial Development Organization
XLD	Xylose Lysine Desoxycholate Agar

OCCURRENCE, ANTIMICROBIAL SUSCEPTIBILITY AND PUBLIC HEALTH IMPORTANCE OF *SHIGELLA* FROM FISH VALUE CHAIN IN BAHIR DAR CITY, NORTH WEST ETHIOPIA

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ABSTRACT

The microbiological hazard of *Shigella* contamination of fish and fish products during improper handling or cooking of fish can lead to human food-borne illness. Hence, this cross-sectional study was conducted from December 2019 to May 2020 to investigate the occurrence and antimicrobial susceptibility profile of *Shigella* in fresh and ready to eat fish products at Bahir Dar City and Lake Tana selected fish landing sites. A total of 170 samples of fish products represented by fresh fish (40), fileted fish (40) and ready to eat fish (90) like *asa lebileb*, *asa dulet* and *asa firfir* samples were collected using systematic random sampling technique. The data was analyzed according to the recommended standard procedure to isolate and identify *Shigella*. In addition, questionnaire survey was conducted to assess the current status of knowledge and handling practices in the fish value chain. Out of the total 170 samples examined, 36 (21.2%) were positive for *Shigella.* The proportion of *Shigella* isolated was 15 (37.5%) from fileted fish, followed by fresh fish 11(27.5%) and ready to eat fish products 10 (11.1%). From this result statistically significant difference was observed along fish supply chain (X^2 = 12.806, P = 0.002). The study also revealed that, a varying level of resistance of *Shigella* against the seven commonly used antimicrobials. All the isolates of *Shigella* were susceptible to Gentamicin Ciprofloxacin and Chloramphenicol but resistant to Streptomycin (100%) and Tetracycline (91.6%). All the fishermen and retailor responded (100%) that source of water for washing of fish were Lake Tana, Observational practical data has shown occurrence of fly on fresh fish, fileted and retailer area were (100%), (100%) and (27.5%) respectively. All main actors in fish value chain had sufficient knowledge (70.58%) concerning fish handling and food safety rules, but those main actors had insufficient practice (57.7%). In general, the study showed the occurrence of *Shigella* along fish value chain with relatively high isolations in fileted fish. Eventually, good hygienic practices along the fish value chain

and training for retailer to safeguard the public from the associated risks of food borne *Shigella*. Further study to identify the sources of the bacteria establishes the clear link between fish chains and stockholders and also investigating the serotype of the isolates were recommended.

Key Words Antibiotic susceptibility, Bahir Dar, Ethiopia, Fish, *Shigella*

Shigella, fish Lake Tana,

CHAPTER 1: INTRODUCTION

1.1. Background and Justification

Fish is an important part of a healthy diet due to its high quality protein, other essential nutrients and omega 3-fatty acids, and its low fat content as compared to other meats (Rhea, 2009; Pal, 2010). It also provides high biological value in term of high protein retention within the body, low cholesterol level and presence of essential amino acids (Emikpe *et al*., 2011; Petronillah *et al*., 2013). It is man's most important source of high quality protein, providing approximately 17% of the animal protein consumed by the world's population (FAO, 2014). In addition sixty percent of the developing countries derive 30% of their annual protein from fish (Abisoye *et al*., 2011; Emikpe *et al*., 2011). Fishes are generally considered safe, nutritious and beneficial nevertheless artisanal fishery and fish products have sometimes been related to certain food issues of safety (WHO, 2007).

Even though having those advantages fish and its products are more liable to contamination with pathogenic bacteria from human reservoir which may contaminate from the water depending on the fishing and also may be further contaminated during handling, processing and packaging (Noha *et al*., 2019). Personal hygiene practices and hygienic production processes are equally important to ensure that fish products are safe to eat. Personal hygiene rules for people handling the catch along the supply chain, from capture or harvest to consumption, including personal hygiene and dress codes (UNIDO, 2017). The quality of fish degrades due to a complex system in which physical, chemical and microbiological types of deterioration are implicated. Fish of good first-class have bacterial count much less than 105 per gram and the greatest risk to human health occurs due to the consumption of raw, inadequately cooked or insufficiently processed fish, and fish product (Pal *et al*., 2016).

Bacterial infection of fish and fish products influence human health by inducing infection and intoxication (Han *et al*., 2001). The microbiological hazard of *Shigella* contamination of fish and fish products were associated with fishes derived from the consumption of raw or insufficiently heat treated fishes, which may be contaminated with bacteria from water environment or terrestrial sources. (e.g. of pathogens those contaminate fish origin foods are

Vibrio spp., C. botulinum C. perfringens, *Salmonella spp*., *Shigella spp*., *Staphylococcus spp*) (Novothy *et al*., 2004; (Pal, 2012; Pal and Mahendra, 2015).

Among the above pathogens; *Shigella* is one of the human pathogen that contaminates frequently fish origin foods (Gosa Girma, 2015). *Shigella* cause severe health problem in human with compromised immune systems. The infection in human cause watery and bloody diarrhea, fever, abdomen cramps and nausea. Often, patients experience vomiting, seizures (young children), or post infectious manifestations, together with reactive inflammatory disease, glomerulonephritis, and enteric perforation. Hemolytic azotemia syndrome will occur when infection with Shiga toxin-producing strains, particularly *Shigella dysentery*1 (CDC, 2019).

In Ethiopia, fish is eaten as substitute for meat especially at time of fasting in those areas that have high fish production like northwestern Ethiopia (Shimels Tesfaye *et al*., 2018).
There are scarce data regarding public health impact and burden of *Shigella* as fish food borne infection. But there were some studies of *Shigella* as human origin ranging from 2.7% (Gizaw Tadesse *et al*., 2019) to 16.9% (Huruy *et al*., 2008) and *Shigella* as meat borne reported 10.5% by (Legesse Garedew *et al*. 2016). These studies indicate the existence of *Shigella* infection in the country associated with human diarrhea patients and food handler at different palace. But there is no previous recorded document for contribution of fish origin foods as source of *Shigella* infection in Ethiopia.

Extensive and uncontrolled prescription of antibiotics has led to the emergence of multi drug resistant to *Shigella* strains. These in turn has created it troublesome within the choice of applicable antibiotics and effective treatment of bacillary dysentery (DeRoeck *et al*., 2005). Empirical prescription of antibiotics by treating physicians is common in Ethiopia because of the shortage of microbiological laboratory facilities to check antimicrobial condition. As a result multi drug resistant strains of *Shigella* have been reported from different regions of the country (Debas Getachew *et al*., 2011; Gebrekidan Atsebaha *et al*., 2015; Dadi Marami *et al*., 2018). Evidently studies in Ethiopia at different hospital shows all isolates of *Shigella* species had characteristics of antibiotic resistance from human origin. These characterized the basis of antibiotic resistance in clinical isolates of *Shigella* from human origin. However, to date no

such characterization has been reported for *Shigella* species of food origin in Bahir Dar Ethiopia.

1.2. Statement of the Problem

The fish value chain actors in many countries including Ethiopia often rely on simple low cost equipment and live and work in remote areas where basic services and facilities are not available. Particularly in Bahir Dar most of the fish catches from the Lake Tana and reach the market by non-motorized boats or traditional wood boat without any preservation facilities. Even fishermen hook tight at fish together with a string and carry them by hand to the market for immediate cash income and others put the fish in a basket, cover them with fresh leaves and carry them by hand (Lemma Abera, 2017).

Shigella spp are extremely sourced in fish contamination throughout harvest to home on faced risky than others foods as a result of vulnerable to surface or tissue contamination originating from the water surroundings, sanitation throughout harvested, fileting, guttering and the way they are ready and served. Even little information on key actors within the fish value chain to be aware of and consistently implement proper handling and storage of fish prior to processing and distribution to consumers.

Thus microbiological hazard of *Shigella* contamination of fish and fish products during improper handling or cooking of fish can lead to human food-borne illness. It is therefore vital to investigate the association between the food safety knowledge and practices of fish actors, and isolation of *Shigella* from fish value chain in the study area. Basically this research claimed that there had been no report on the *Shigella* species and their antimicrobial resistance patterns on fresh and ready to eat food of fish products. Therefore this research aims to investigate on occurrence, antimicrobial status and public health implication of *Shigella* throughout fish value chain in Bahir Dar City, North Western Ethiopia.

1.3. Objectives of the Study

1.3.1. General objective

Occurrence, bacterial load and antimicrobial resistance patterns of *Shigella*, from Fish value chain in Bahir Dar city; North West Ethiopia.

1.3.2. Specific objectives

- To isolate and identify *Shigella* from fish value chain at selected fish landing ports in Lake Tana and in Bahir Dar city
- To determine the *Shigella* load in fish value chain and assess its public health significance in RTE fish
- To determine antimicrobial resistance patterns of *Shigella*
- To assess the hygienic knowledge and practice of actors involved in fish value chain

1.4. Research Questions

- ✓ Is there any *Shigella* that was contaminated and identified from fresh and RTE fish?
- ✓ Which food item was more exposed to *Shigella* of fish foods like *asa lebileb, asa dulet, asa wet, asa tibs*?
- ✓ What was the antimicrobial resistance pattern of *Shigella?*
- ✓ Was fish value chain in the study area has public health risk and used as source of Shigellosis?

CHAPTER 2: LITERATURE REVIEW

2.1. Overview of Food Hygiene and Food Safety

Foodborne diseases remain a true and formidable problem in both developed and developing countries, causing great human suffering and significant economic losses. Up to one third of the population of developed countries is also laid with foodborne diseases annually; even though the problem is elevated to be more widespread in developing countries, where food and water-borne diarrheal diseases kill an estimated 2.2 million people annually, most of them are Children (FAO/WHO, 2006). It is severe in developing countries due to difficulties in securing optimal hygienic food handling practices. In developing countries, up to an estimated 70% of cases of diarrheal disease are related to the consumption of contaminated food (Knife Zeru and Abera Kumie, 2007).

2.1.1. Food hygiene and food safety practices in Ethiopia

Foodborne diseases are common in developing countries including Ethiopia due to prevailing poor food handling and sanitation practices, inadequate food safety laws, weak regulatory systems, lack of financial resources to invest in safer equipment and lack of education for food handlers (WHO, 2004). National hygiene and sanitation strategy program (MoH, 2005) reported that in Ethiopia more than 250,000 children die every year from sanitation and hygiene related diseases and about 60% of the disease burden was associated with poor hygiene and sanitation in Ethiopia.

Unsafe sources, contaminated raw food items, improper food storage, poor personal hygiene during food preparation, inadequate cooling and reheating of food items and a prolonged time lapse between preparing and consuming food items were mentioned as contributing factors for outbreak of foodborne diseases (Linda du and Irma, 2005). Studies conducted in different parts of Ethiopia showed poor sanitary conditions of catering establishments and presence of pathogenic organisms like *campylobacter, Salmonella, Staphylococcus aureus, Shigella and Escherichia coli,* (Tefera Woldemariam *et al*., 2009 and Legesse Garedew *et al*., 2016).

Of the foods intended for humans, those of animal origin tend to be most hazardous unless the principles of food hygiene are employed. Animal products such as meats, fish and their products are generally regarded as high-risk commodity in respect of pathogen contents, natural toxins and other possible contaminants and adulterants (Knife Zeru and Abera Kumie, 2007). Bacterial contamination of fish products is an unavoidable consequence in fish value chain. Microbial contamination on environmental surfaces may be transferred to the food products directly through surface contact or by vectors such as personnel, pests, air movements or cleaning regimes during harvesting to final serves to customer. Bacteria may also infect the fish from outside during careless handling of landed fish, its stowing and cutting. Among major external sources of bacterial contamination are ice and salt, crushed ice is known to carry heavy bacterial loads (Pal, 2010).

2.2. History and discovery of *Shigella*

The numerous types of *Shigella* spp bacteria have been named after the lead workers who discovered each one (DuPont, 2000; CDC, 2009; Kelmani and Chidre, 2018). For the first time isolation of this bacterium from faces of individuals with acute dysentery was described by Chantemesse and Widel in 1888 (reviewed by Singh, 2006). A detailed description of the organism that caused the dysentery called *Shigella dysentriae*, was named after Kiyoshi Shiga, a Japanese scientist who discovered it in 1896 as investigating a large epidemic of dysentery in Japan. The bacterium was also referred to a genus of generally as the dysentery bacillus of rod shaped bacteria of which *Shigella* is a member) (Trofa *et al*., 2009).

2.2.1. Taxonomic Classification of *Shigella*

Taxonomy

Domain	Bacteria
Phylum	Proteobacteria
Class	Schizomycetes
Order	Eubacterials
Family	Enterobacteriaceae
Genus	*Shigella*

The evolution of taxonomy classification of *Enterobacteriaceae* has changed with the accumulation of knowledge about the organism. The family *Enterobacteriaceae* resides within the phylum Gamma proteobacteria is the largest most heterogeneous collection of medically important gram negative rods. The classification of *Enterobacteriaceae* has been controversial and there have been successive change in there grouping and nomenclature. The three widely used system of classification of *Enterobacteriaceae* are Bergey's manual, Kauffmann, Edwards-Ewing.

The oldest method of classification was based on the action of organism on the lactose. In 1972, Edwards and Ewing describe 11 genera and 26 species belong to the family *Enterobacteriaceae*. Later nomenclature and classification of genera, species subspecies, bio-groups and serotypes of *Enterobacteriaceae* were defined by morphology, biochemical characteristics and antigen analysis. In 1985 Farmer and Associate described 22 genera 69 species and 29 enteric groups (Joklik *et al*., 2002; Patrik *et al*., 2007). Today with the application of new technologies such as nucleic acid hybridization and nucleic acid sequencing to the study the taxonomy of these organisms has led to rapid increase in the number of genera and species of bacteria that fit the general criteria of *Enterobacteriaceae* and has led to basic understanding of phylogenetic relationship of genera and species. At present there are more than 44 genera and 176 species in the family *Enterobacteriaceae* (Patrik *et al*., 2007).

2.2.2. Genus of *Shigella*

Shigella is a Gram-negative, non-motile, non-spore forming, non-lactose-fermenting, facultative anaerobic bacillus belonging to the family *Enterobacteriaceae* (Yang *et al*., 2005). It causes bacillary dysentery or shigellosis as do Enteroinvasive *Escherichia coli* strains (EIEC). The disease naturally occurs only in humans and primates. It is characterized, in its classical form, by acute colonic and rectal mucosal inflammation that leads to the triad of the dysenteric syndrome: fever, intestinal cramps and passage of blood and mucus in diarrheal stools (Levinson, 2006).

Shigella dysenteriae 1 was the first of the four *Shigella* species to be described in 1898 by the Japanese microbiologist K. Shiga, thus the name of Shiga bacillus (DuPont, 2000; Trofa *et al*., 2009). This disease is a well-known agent of bacillary dysentery, infantile diarrhoea all over the world and more serious than the common "stomach flu." *Shigella* species was the second most widespread bacterial agents causing diarrhea next to *Escherichia coli* (Esmaeili *et al*., 2014). The agent has four species and they share several major characteristics such as lack of motility, optimal growth at 37°C, oxidase negative character and lack of growth in synthetic media containing salts and a simple carbon source, unless glucose and nicotinic acid are added. Some isolates may require the addition of certain amino acids, purines or vitamins (Germani and Sansonetti, 2006).

The *Shigella* genus is very closely related to the species *Escherichia coli*; because the degree of homology of their chromosomal DNAs is close to 100%, considering *Shigella* as a member of the genus *Escherichia* is regarded as justifiable (Yang *et al.,* 2005). However, whereas *Escherichia coli* isolates are usually prototrophic, motile, and able to ferment many sugars, *Shigella* isolates are auxotrophic, non-motile, ferment few sugars and, with a few exceptions no H_2S gas production in the presence of glucose. These distinct species can be distinguished based on the variations in their O- polysaccharide portion of their LPS, the species were further classified into several serotypes, as *S. dysentriae* known to have 15 serotypes, *S. flexneri* have 14 serotypes and sub serotypes, *S. boydii* 20 serotypes and *S. sonnei* with a single serotype (Niyogi, 2005; CDC, 2007).

2.3. Pathogenesis of *Shigella*

Upon ingestion, the bacteria survive the gastric environment of the stomach and make its way to the large intestine; where it attaches and penetrates the epithelial cells of the intestinal mucosa. Post invasion, *Shigella* multiplies intracellular and spreads to neighboring epithelial cells resulting in tissue destruction and representative pathology of shigellosis. Generally, *Shigella* adheres to the membrane of the cell and is internalized by an endosome which it subsequently lyses to gain access to the cytoplasm where multiplication occurs (Presterl *et al*., 2003; Schroeder and Hilbi, 2008; Carneiro *et al*., 2009).

Pathogenesis is initiated by the invasion through the basal face of the intestinal epithelium by *Shigella*. The bacteria use the type III secretion system to invade the epithelium cells and involve approximately 20 proteins which are encoded by the 210 kb virulent plasmid (Germani and Sansonetti, 2006). Among the proteins secreted are VirA, OspB to OspG and invasion plasmid antigens (Ipa) proteins like IpaB, IpaC, IpaD and IpaH (Singh, 2006).Thus, upon reaching the large intestine, *Shigella* is taken up in vacuoles by microfold cell (M cells) which are specialized structure of the follicle associated epithelium which covers the mucosal lymphoid follicles, the stimulating site of the mucosal immune system (Levinson, 2006). Extracellular *Shigella* is not motile but intracellular it is able to move occupying the entire cytoplasm of the infected cell and between cells. It uses a mechanism for its motility by which its *IcsA* and *IcsB* proteins trigger actin polymerization in the host cell in a rocket propulsion fashion for cell-to-cell spread (Niyogi, 2005; Levinson, 2006). Specifically, movement between adjacent cells is facilitated by the *IcsA* protein (Kelmani and Chidre, 2018).

After successful epithelial cell invasion and penetration of the colonic mucosa by the bacteria, there is degeneration of the epithelium and inflammation of the lamina propria culminating in desquamation and ulceration of the mucosa and subsequent leakage of blood, inflammatory elements and mucus into the intestinal lumen (Yang, 2005). Thus the characteristic passage of frequent and scanty dysenteric stool mixed with blood and mucus. Absorption of water by the colon is inhibited under these conditions (Kelmani and Chidre, 2018).

Some strains of *Shigella* produce enterotoxins and shiga-toxins similar to the verotoxin of *E. coli* O157:H7. The toxin has a molecular weight of 68kDa and is a multi-subunit protein consisting of one molecule of an A subunit (32,000 MW) and five molecules of the B subunit (7,700 MW). Both shiga-toxins and verotoxins are associated with hemolytic uremic syndrome (HUS), Hemolytic colitis and dysentery (Presterl *et al*., 2003; Levinson, 2006). The names of these conditions are dependent on the causative organism and symptoms range from severe diarrhea, abdominal pain, vomiting and bloody urine. No antidote exists for these toxins.

Thus supportive care requires maintenance of fluid and electrolyte levels and monitoring and support for kidney function. Inactivation of the toxin is achieved by steam treatment,

oxidizing agents such as bleach and chemical sterilizing agents such as glutaraldehyde. The toxin acts on the lining of the blood vessels, the vascular endothelium. The B subunits of the toxin bind to a cell membrane component, Gb3, and the complex enters the cell (Germani and Sansonetti, 2006; Amenyedor, 2013). Once inside, the A subunit interacts with the ribosomes to inactivate them. The A subunit of the shiga-toxin is an N-glycosidase that modifies the RNA component of the ribosome to inactivate it and thereby bring a halt to protein synthesis of the cell leading to cell death (Germani and Sansonetti, 2006).

The vascular endothelium has to continually renew itself. Hence, cell death leads to breakdown of the lining leading to hemorrhage. The primary response is characteristically bloody diarrhea. For unexplained reasons, the toxin is seemingly effective against small blood vessels such as found in the digestive tract, kidneys and lungs but not against vessels such as the arteries or major veins. A specific target for the toxin appears to be the vascular endothelium of the glomerulus destroying the structures and concluding in kidney failure and the development of the often deadly and frequent debilitating hemolytic uremic syndrome. Food poisoning with shiga-toxin often has an effect on the lungs and the nervous system (Schroeder and Hilbi, 2008; Amenyedor, 2013). The pathogenic mechanism of shigellosis is complex and has been studied extensively by Sansonetti and colleagues (Sansonetti *et al*., 2000; Jennison and Verma, 2004; Germani and Sansonetti, 2006).

2.4. Epidemiology of *Shigella*

Worldwide, *Shigella* is calculable to cause 80-165 million cases of sickness and 600,000 deaths annually; of those 20-119 million sicknesses and 6,900-30,000 deaths are because of foodborne transmission. *Shigella* bacteria spp. is endemic in temperate and tropical climates. Transmission of enteric bacteria spp. is presumably once hygiene and sanitation are inadequate. Bacillary dysentery is caused preponderantly by *S. sonnei* in industrial countries, whereas *S. flexneri* prevails within the developing world. Infections caused by *S. boydii* and *S. dysenteriae* are less common globally however will form up a considerable proportion of *shigella spp*. isolated in Black Africa and South Asia (Shane *et al*., 2017).

Shigella dysenteriae typically presents in epidemic and happening varieties of the illness. *S. flexneri* and *S. sonnei* are principally accountable for disease in developing and developed nations, severally, while *S. boydii* is restricted to Asian nation and neighboring countries. *S. flexner*i was found to be the foremost normally isolated species (68%) in an exceedingly multi-centric study from six Asian countries wherever *S. sonnei* was the most common (84%), while *S. dysenteriae* that is most frequently seen in Southern Asia and sub-Saharan Africa, recognized solely four percent of the isolates (Neelam and Abhishek, 2016). For never-ending transmission in humans, the bacteria should be passed from one person to a different because it doesn't survive in other organism rather than humans and primates which are the first reservoir of *Shigella*, it has been isolated from varied sources viz. aquatic bodies (rivers, surface waters furthermore as coastal waters), life style amoebae, insects, fish part for long outside the body (Niyogi, 2005).

2.5. Occurrence of *Shigella* on Foods

Food process is a vital trade worldwide. One in every of the main issues threatening food trade is that the contamination with foodborne microbes of human origin ensuing from improper handling and process. Microbes contamination reduces period of time and food quality resulting in food infection and poisoning outbreaks, a number of that are life threatening. Continuous observance of food process is important to avoid potential health issues (Al- Bahry *et al*., 2014). *Shigella* is one of the most important foods borne pathogen causing diarrheal disease in both developing and developed countries. Epidemiology reports show that about 140 million people suffer from shigellosis with estimated 600,000 deaths per year worldwide (WHO, 2001).

In United States, an estimated 450,000 people are infected each year, majority in children of 1-5 years age group (Taneja, 2007). *Shigella* are the foremost common reason for shigellosis, this term has become substitutable with all clinical displays of bacillary dysentery though these displays vary from symptomless carriage to gentle, watery diarrhea to raw infectious disease, additionally together with associate degree acute page inflammation related to nausea, fever, anorexia, dehydration, body fluid and bloody diarrhea tenesmus (Ergonul *et al*., 2004). Even though water is the common vehicle for this infection in humans; it has been

isolated from big variety of foods like beef, chicken, egg, milk, fish, vegetables and fruits (Goud *et al*., 2018).

The growth and survival of *Shigella spp*. in foods is influenced by variety of things like temperature, pH, salt content and therefore the presence of preservatives. As an example, survival of *S. flexneri* has been shown to extend with: decreasing temperature, increasing hydrogen ion concentration, and decreasing NaCl concentration (Zaika and Phillips 2005). The temperature range for growth of *Shigella* spp. is 6-8 to 45-47°C; rapid inactivation occurs at temperatures around 65°C. In contrast, under frozen (-20°C) or refrigerated (4°C) conditions *Shigella* spp. can survive for extended periods of time (Warren *et al*. 2006).

There is extremely very little revealed policy investigation and knowledge on the presence of *Shigella* in food. Some international surveys were performed within which *Shigella* spp. are found directly on foods. For example, Ghosh *et al*. (2007) isolated *Shigella* spp. from 15% of coconut slices (n=150), 9% of ready-to-eat salads (n=150) and 7% of samples of coriander sauces (n=150) from Indian street vendors. *Shigella* spp. have also been detected in 11% of raw meat samples (n=250) from retail outlets in Pakistan (Hassan *et al*. 2010). In Mexico, *Shigella* spp. have been isolated from 6% of freshly squeezed orange juice samples (n=100) and from the surface of 17% of oranges sampled (n=75). All four *Shigella* spp. were isolated from the surface of the oranges, whereas only *S. sonnei* and *S. dysenteriae* were isolated from the orange juice samples (Castillo *et al.* 2006).

The sources related to the health problem were drinking, laundry utensils and consumption contaminated food with enterobacteria, and therefore the happening due to subsided following repair of the pipeline. Food-borne outbreaks of *S. sonnei* have additionally been according. More than 300 people suffered in Kerala in south India in 2009 where local food made of rice, lentils, milk, and fish was implicated as the source (Neelam Taneja and Abhishek Mewara, 2016).

Nygren *et al.* (2012) analyzed 120 reported foodborne shigellosis outbreaks in the United States between 1998-2008. The contributory factors known in these outbreaks enclosed infected food handlers (58%), bare-handed contact of the food handler with prepared to-eat

food (38%), inadequate cold-holding temperatures (15%), and inadequate cleanup of food preparation instrumentation (15%). It ought to be noted that quite one issue is concerned in a deadly disease. Even though contaminated water is major vehicle for transmission of *Shigella* spp. it's been conjointly isolated from big variety of foods like beef, chicken, egg, milk, fish, vegetables and fruits (Goud *et al*., 2018). This can occur because of inadequately treated contaminated water and unsanitary preparation getting used for drinking and food preparation, ooze of waste matter through the planet or dirty contamination of recreational water (Warren *et al*., 2006).

2.6. The role of Fish Value Chain in Bahir Dar Ethiopia

The term value chain describes the full range of activities which are required to bring a product or service from conception, through the different phases of production (involving a combination of physical transformation and the input of various producer services), delivery to final consumers and final disposal after use. Basic definitions of value chains add incremental value to the product in the nodes of a chain either by value addition or value creation (Hempel, 2010).

For example, within fisheries and aquaculture, the term value-chain is used to characterize adding value in products through some type of processing method essentially converting raw fish to a resulting finished or semi-finished product that has more value in the market place. Basically fisheries especially small-scale fisheries and aquaculture are a globally significant source of employment and livelihoods. According to the most recent estimate, 58.3 million people were engaged in the primary sector of capture fisheries and aquaculture in 2012 (FAO, 2014a). The other stages of the value chain, fish processing and trading are estimated to employ more than twice as many people and many of them are women (World aBank/FAO/World Fish, 2012).

In Ethiopia, fisheries are acknowledged as an important strategy in the drive for poverty reduction; they also promote greater economic development. In 2010, Ethiopia realized about USD 14 million from its capture fishery while a total of 40000 livelihoods were positively impacted upon by the fishery sector in the same year (FAO, 2016). Lake Tana is one of the

largest fishing sites in the Amhara region and the country which is almost dominated by artisanal fishermen. It has a surface area of 32,000km^2 with a maximum and mean depth of 14m and 8m. The Lake provides commercially important three delicious fish species namely, African Cat fish (*Clarius gariepinus* also locally called "Ambaza"), Nile tilapia (*Oreochromis niloticus*, locally called "Kereso") and *Labeobarbus* spp. (locally called "Nech Asa") (Tewabe Derege, 2015).

The numbers of actors of fish value chains in Lake Tana vary from season to season. According to the reports of the 9 district there are almost well over 4556 fisher men and women without counting the number of people employed in the retailer and RTE processer as fishery value chain (Amare Dagninet *et al*., 2018). Value creation is used to characterize fish and fishery products that have incremental value in the marketplace by differentiating them from similar products based on product attributes such as: geographical location; environmental stewardship, fair trade, organic products; and food safety system, free from antibiotics and heavy metals (De Silva, 2011; FAO, 2014).

For the microbiologists and food scientists, a value chain analysis affords an opportunity to study the growth and activity of spoilage and pathogenic microorganisms at each stage of the value chain; this would enhance better traceability and allow design and implementation of better quality control and fish safety methods (Popovic *et al*., 2010). Each country has different value chains based on the number of actors involved in the process. The smooth functioning of value chains requires not only factors of production and technology but also efficient transport, market information systems and management (De Silva, 2011). In case of our Country Ethiopia there is cross sector challenges affecting fisheries value chain like; insufficient institutional and management capacity, limited resource allocation and investment, poor policy and regulatory framework.(FAO, 2015).

Basically in Bahir Dar city and around Lake Tana fish consumers have preference for whole fresh fish; as a result, the bulk of the fish harvest from the lakes is sold as fresh. However, frozen filets are increasingly being marketed in lakeside towns as well as in the capital city, Addis Ababa by Bahir Dar fishery enterprise 1 (Amare Dagninet *et al*., 2018).

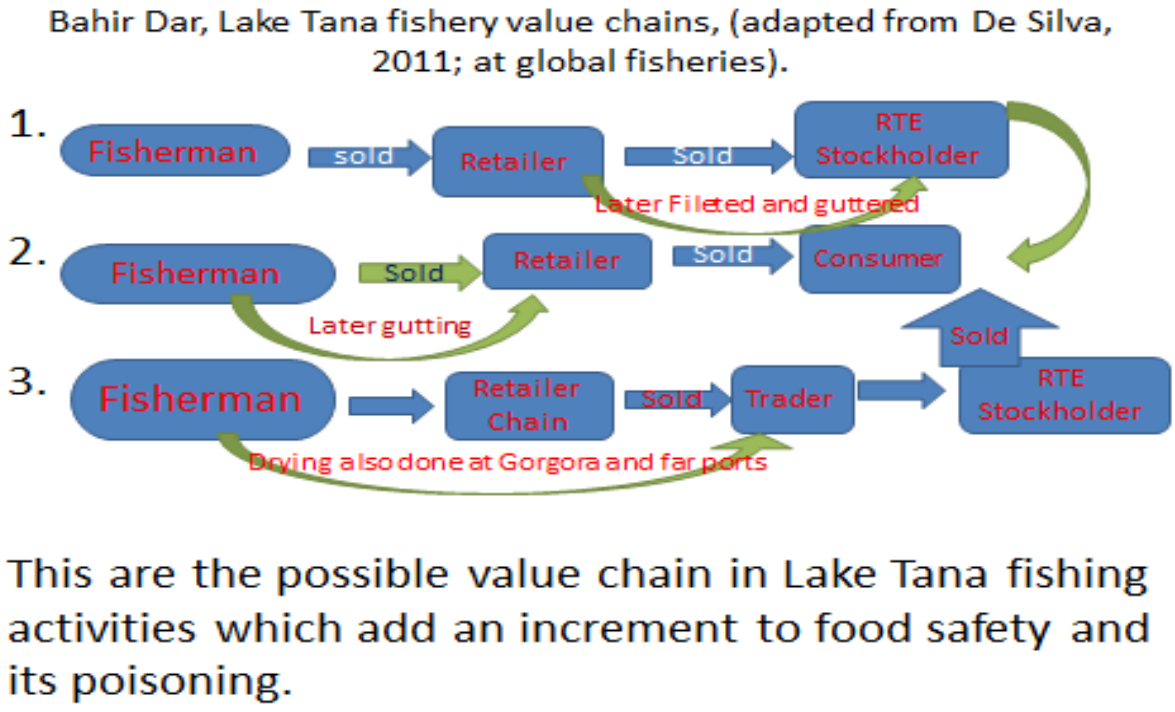

Figure:1: The fishery value chains in Bahir Dar, Lake Tana.

2.7. *Shigella* Load in fish Value Chain and its Public Health Importance

Literature extended over many years pointed out that fish and its products are liable to contamination with various kinds of microorganisms from different sources. Such contamination may render fish unsafe to the consumers or impair its utility, especially in undeveloped countries, where the hygienic measures are still underway. Many efforts needed to keep the fish free from pathogens of public health hazard (Ghanem *et al*., 2019*)*.

Shigella should be emphasized because of its low dose infecting ability and the severity of the associated disease, accounting for 140 million cases globally per year and 60,000 deaths annually of which 60% occur in children below 5 years of age. The geographical distribution, frequency of occurrence and the pathogenicity of the four *Shigella* spp. are different by country and also different among populations within a country. A few studies have demonstrated the prevalence of *Shigella* in different parts of the world as fish borne. According to Group *et al*. (2018) study the incidence of *Shigella* as fish borne in Telangana

India was 12.5% (Goud *et al*., 2018); and higher incidence (39.7%) findings were recorded from Winam Gulf of Lake Victoria, Kenya by Onyango *et al.* (2009) whereas low incidence (2.2%) reported by Yagoub (2009) from Khartoum state.

As Legesse Garedew *et al*. (2016) study in Gonder Northwestern Ethiopia *Shigella* prevalent in meat and butcher shops 10.5 percent. This has been show that more investigation needed for interrupting *Shigella* spp and its epidemiological pattern. According to the study of (Hendawy *et al*., 2016) 23.6% of the study fish sample was contaminated by *Shigella* and Hendawy *et al* (2016) suggest that isolation of *Shigella* species may be attributed to sewage pollution of human origin or unsatisfactory hygienic conditions during catching, handling and marketing of the fish. In all circumstance of fish value-chain; products contamination has great public health significance due to its liable nature to pathogenic bacteria from human reservoirs during handling, processing and packaging of fish (Ghanem *et al*. 2019).

2.8. Antimicrobial Resistance in *Shigella*

Antimicrobial resistance in pathogenic bacteria is developing and increasing risk to human health. Physicians are progressively attentive that antimicrobial resistance is increasing in bacterial pathogens; as a result, patients who are recommended antibiotics are at increased risk for promoting antimicrobial-resistant various infections. Certainly 'increased frequency of treatment failures for acute illness and increased relentless of infection may be signified by continued duration of illness, increased frequency of bloodstream infections, increased hospitalization or expanded fatality (Angulo *et al*., 2004b). Although most diarrheal diseases are self-resolving and should not need treated with antimicrobial agents, invasive or protracted infections require chemotherapy and are typically managed empirically (Okeke *et al*., 2007).

Over the past decades, *Shigella* species show a pattern of steadily increasing and became even more immune to most widely-used antimicrobials (Berhanu Andualem *et al.,* 2006). This resistance pattern of *Shigella* species differ consistent with geographic area and within the same place over time, resulting in therapeutic problems. Periodic regional monitoring of disease with serotype breakdown and regular periodic antibiotic-susceptibility testing mode of

isolates to conduct local empirical therapy are important factors for the adequate control of shigellosis (Njunda *et al*., 2012). The clinical consequences of antibiotic resistance differ among the pathogenic bacterial diarrhea agents. For Shigellosis, antibiotics are the primary treatment. Patients treated with an ineffective antibiotic may have more complications than condition that, they had not been treated, since the antibiotic is likely to affect the normal intestinal flora, thus actually supporting the growth of the resistant *Shigella* (Germani and Sansonetti, 2006; Kelmani and Chidre, 2018).

In recent years, Fluoroquinolones, especially Ciprofloxacin have been very successful in combating Shigellosis but unfortunately, resistant strains have emerged. The emergence of high-level Ciprofloxacin resistance in *Shigella* spp has also been reported in India (Capoor and Nair, 2010). One of the reasons for emergence of multi-drug resistant in *Shigella* spp is the unique capability of the pathogen to acquire resistance factors (transmissible genes) from the environment or from other bacteria (Kelmani and Chidre, 2018).

2.9. Prevalence of *Shigella* infection in Ethiopia

Most *Shigella* infections were result of sporadically, but huge *Shigella* epidemics have been traced to contaminated food and water. The CDC estimates that 450,000 total of Shigellosis appear in the developing countries every year (CDC, 2009; Haley *et al*., 2010). Evidently its prevalence is highest in tropical and subtropical parts of the world where living standards are very low and access to safe and adequate drinking water and proper excreta disposal systems are often limited (Girma Gosa, 2015).

As developing country, Shigellosis is an important public health problem in Ethiopia, where there is substandard hygiene and unsafe water supplies (Debas Getachew *et al*., 2011). As worldwide among the four species of *Shigella*, *Shigella dysentriae* and *Shigella flexnerii* were more predominant one (Haley *et al*., 2010). This is too in Ethiopia, where *Shigella dysenteriae* and *Shigella flexineri* have been identified as the species that account for about 80% of *Shigella* isolates and diarrheal are the major causes of morbidity and mortality in the country especially in children younger than five years old (Kahsay Huruy *et al*., 2008).

Recently, many studies have demonstrated the prevalence of *Shigella* in different parts of Ethiopia. For instance, study conducted in Gonder Town showed that *Shigella* species were isolated from stool samples of four food-handlers (3.1%) out of 127 food handlers (Gashaw, Andargie *et al*., 2008). A study conducted by Reda Ayalu *et al*. (2011) has reported (6.7%) *Shigella was* isolated in Harer. Besides this prevalence rate, *Shigella* were also studied in food of meat items conducted in Gonder Ethiopia 10.5% by Legesse Garedew *et al.* (2016), Shigellosis also characterized by seasonality with the largest percentage of accounted cases occurring between July and October and the smallest percentage occurring in January, February and March (Gupta *et al*., 2004).

2.10. Control Measure of *Shigella* as food borne

Because the only enormous source of *Shigella* infection is transmission of most often in oro-fecal way and sanitary measures are integral for disease prevention. These measures should consist of hand washing with soap, chlorination of water, perfect disposal of feces, and protection of meals from contamination, in particular by flies. Patients, specifically those preparing foods, must be isolated ideally until their stool cultures have turn out to be negative (Kelmani and Chidre, 2018).

The greatest hazard to human health occurs due to fish were the consumption of it as raw, inadequately cooked or insufficiently processed fish and cross contamination of fish products. Sensory techniques are the most first-rate for assessing the spoilage and freshness of fish and fishery products (Pal, 2016). A most important intention for the food processing enterprise is to provide safe, wholesome and suitable meals to the consumer. Control of microorganisms exerted via high degree of hygiene, efficient cleaning and disinfection practices throughout the processing and preservation methods are fundamental to meet this goal (Pal and Mahendra, 2015).

Proper anti-biotherapy, which shortens the length of *Shigella* excretion, also may additionally help minimize the spread. However, control also cannot be easy in the most impoverished areas in which the disease is endemic or in emergency conditions where implementation of proper personal and widely wide-spread hygiene standards is difficult. This may be

complicated by the high incidence of unapparent infections in such situations and the low inoculum of *Shigella*, which is required to elicit the disease. These considerations, added to the multiplied resistance to antibiotics, have led to consideration of vaccination in opposition to shigellosis as an efficient and cost effective approach for prevention (Germani and Sansonetti, 2006).

CHAPTER 3: MATERIAL AND METHODS

3.1. Study Area Description

The study was conducted in Bahir Dar city and Lake Tana. Bahir Dar city is located in Northwestern Ethiopia; it far 580 km Northwest of Addis Ababa. Lake Tana is one of the largest fishing sites in the region and the country which is almost dominated by artisanal fishermen. This is, found in Amhara Region and has a surface area of 32,000km2 with a maximum and mean depth of 14m and 8m. The Lake occupied with African Cat fish (*Clarius gariepinus* also locally called "Ambaza"), Nile tilapia (*Oreochromis niloticus*, locally called "Kereso") and *Labeobarbus* spp. (locally called "Nech Asa") fishes were harvested. The total population of Bahir Dar city is 750,991. Geographically Bahir Dar is located at latitude of 11.35°-11.59° North and 37.23°-37.39°East and its average elevation is estimated to be 1810 meter above sea level (CSA, 2018).

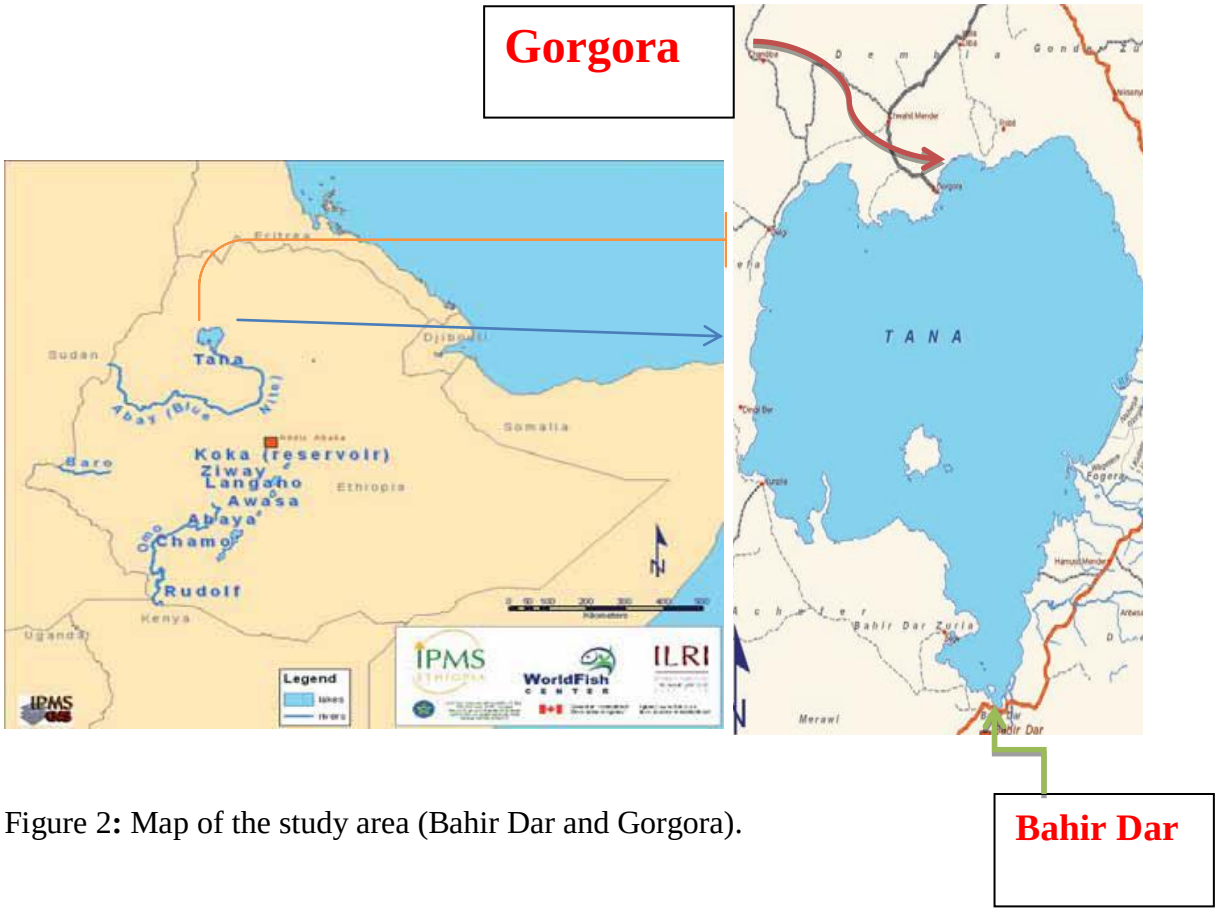

Figure 2**:** Map of the study area (Bahir Dar and Gorgora).

3.2. Study Design and its Study population

A cross-sectional study was conducted from November 2019 to May 2020 to generate the desired data, along the fish value chain in the study area. The study populations were fishes harvested in Lake Tana and landed on Bahir Dar and Gorgora fish landing ports and also fileted fish marketed at different informal fish markets area Bahir Dar fish enterprise 1 and *Gorgora* street road. RTE fishes presented to hotel and restaurant at Bahir Dar town originated from Bahir Dar landing ports were included in the study. In addition, fish value chain actors (fishermen, retailer and RTE producer) were also included.

3.3. Sample size Determination

Fresh fish bacteriological prevalence was found on *S. sonni* (0.8%) from Southern gulf of Lake Tana by Anwar Nuru *et al*. (2012). Therefore; the expected prevalence were taken as 0.8%. According to Thrusfield (2007) fresh fish sample size were become: 11 from one port lands. Since the researches were conducted in two ports of fish landing area and two fish market, the calculated sample size become 44. But to increase the chance of accuracy sample size were increase to be 80. The distribution of sampling to study area can be proportion to its number of production. For isolation and identification of *Shigella*, from cooked fish the sample size were determined by using 95% confidence interval and 5% desired level of precision. Because there was no previous studies conducted in cooked fish products in the city. So the expected prevalence of *Shigella* was taken as 50% and the sample size determined by the formula for infinite population given below (Thrusfield, 2007).

$$n = \frac{1.92^2 \exp(1-\exp)}{d2}$$

Where

n = required sample size,

Pexp = expected prevalence,

d = desired absolute precision.

Based on the above given formula the total sample size of cooked fish become 384. But, in relatively small populations, the required sample size was adjusted (n_{adj}) by the following formula for the same degree of precision (Thrusfield, 2007).

$$n_{adj} = \frac{N \times n}{N + n}$$

Where:- n = the sample size based on an infinite population (384)

N = the size of the study population (70 = Number of hotel and restaurants prepared fish for RTE). Based on the above calculation, the sample size from cooked fish was 59; but to increase the chance of accuracy sample size was increase to be 90. And the total sample size in the population were (90 cooked fish plus 80 fresh harvested and fileted fish = 170).

3.4. Sampling Technique

Sampled fish and fish products from all value chain were selected by systematic random sampling technique. The study examined the microbiological quality of three locally consumed fishes at different stages of their respective value chains. The sample taken for bacteriological analysis was fresh fish muscles from fisher men, fresh-fileted fish from retailor and items like *asa lebileb*, *asa dulet. asa wet*, *asa tibis* from hotel and restaurant. Fresh fish samples were taken from Lake Tana shore ports; *Gorgora* and Bahir Dar. These samples were selected based on their availability and proportionality from different fish species at value chain in study area.

Each sample measured greater than (100g) was kept in a separate homogenizing plastic bag and at a moment insert in to an insulating refrigerated ice box container under complete aseptic condition to avoid any changes in the quality of the sample. Samples were transported to Veterinary Microbiology laboratory; School of Animal Science and Veterinary Medicine, Bahir Dar University. The isolation of *Shigella*, were started immediately after arrival to the laboratory. The study participant's on questionnaires part for hygienic knowledge, awareness and practices assessment were selected purposively.

3.5. Sample preparation

Twenty five grams of each sample was aseptically weighed into sterile stomacher bags with the addition of 225 ml buffered peptone water (HCM008, Guangdong Haunki Microbial Sci. and Tech. Co. Ltd). Because of their larger sizes muscle from the thickest part of fish muscle were taken with sterile scissors and aseptically weighed to obtain the final mass. The weighed samples were then homogenized for up to 2 minutes in a stomacher blender.

3.6. Isolation of *Shigella*

Detection and isolation of *Shigella* were conducted by incubation of the above homogenized sample for 24 hours at 37ºC. After overnight incubation, a loop full of the enriched culture was directly streaked onto Hektoen Eneteric Agar (HEA) (HCM016, Guangdong Haunki Microbial Sci. and Tech. Co. Ltd) using the streak plate technique, and then incubated aerobically at 37°C for 24 h. The culture plates were examined for the presence of *Shigella*. (Small greenish colonies) (Trofa *et al*., 2009; FDA, 2017).

Colonies suspected to be *Shigella* (small greenish colonies) were sub cultured for pure colony isolation. Isolation of *Shigella* was done according to microbiology of food and animal feeding stuffs horizontal method for the detection of *Shigella* spp for little modification (FDA, 2017; FAO, 2018). The little modification made for isolation of *Shigella* in this study was that *Shigella* broth and novobiocin acted for better *Shigella* spp growth were not used due to unavailability. Cultured positives were characterized by standard biochemical tests; including (TSI) (HCM014, Guangdong Haunki Microbial Sci. and Tech. Co. Ltd), Urea Agar Base (Oxoid Ltd., Basingstoke, Hampshire, England) and SIM test (Himedia Laboratories Pvt. Ltd. India) (H_2S production, motility). The result of each biochemical test culture was read after incubation for 24-48 h at 37°C (WHO, 2015).

3.7. Standard plate counts

Enumeration of *Shigella* were conducted by weighing 1 gram of the fish sample which was dissected out, blended and mixed properly in a mortar. It was aseptically transferred to a sample bottle containing 9mls of 0.1% sterile buffered peptone water (HCM008, Guangdong

Haunki Microbial Sci. and Tech. Co. Ltd). The bottle was closed and shaken thoroughly for 10 minutes and allowed to stand for 20 minutes, after which a 10 fold serial dilution was carried out. This result was recorded as dilution of 10^{-1}. Samples (1ml) were mixed and transferred to dilutions tubes (9ml) using a fresh sterile pipette with each transfer prepares dilution of 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. Shake all dilutions until uniformly homogenized. Samples were plated in duplicate to agar plates in order to determine CFU/ml. using new pipette, apply 0.1mL of this dilution to a previously marked Petri dish. Flame spreader and gently spread liquid around plate. Allow plates to dry and then invert and incubate at 35°C for 24 hours (ICMSF, 1996). The results were recorded and the numbers of bacteria from plate counts were calculated as to the CFU/ml or CFU/gram.

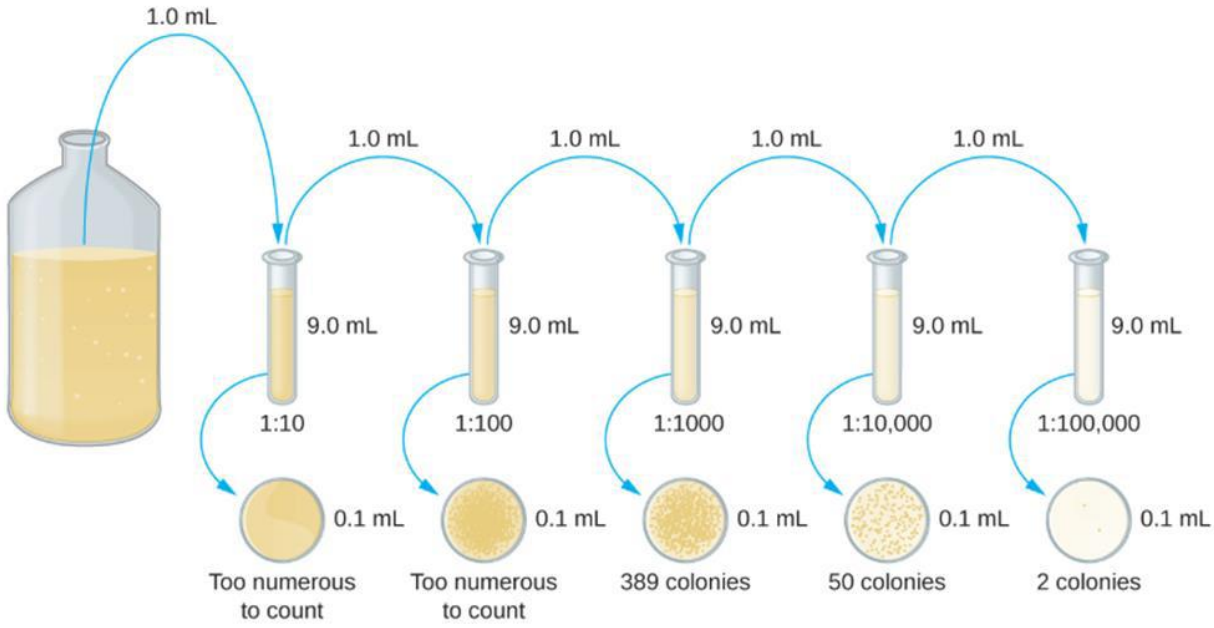

Figure 3: Sample of Serial dilution (OpenStax CNX, 2018)

3.8. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility test of *Shigella* was performed using Mueller-Hinton agar disc diffusion technique (HiMedia Laboratories Pvt. Ltd. India) using seven antimicrobial disks: Tetracycline (10µg/disk), Gentamicin (10µg/disk), Ciprofloxacin (5µg/disk), Chloramphenicol (30µg/disk), Trimethoprim-Sulfamethoxazole (25µg/disk), Erythromycin (15µg/disk) and Streptomycin (10µg/disk) based on Clinical and Laboratory Standards

Institute (CLSI) guidelines (CLSI, 2018). The inoculum was prepared by making a direct sterile saline water suspension of isolated colonies selected from a 24-hour agar plate (nutrient agar). The suspension was adjusted to match with 0.5 McFarland turbidity standard using sterile saline water and a vortex mixer. Sterile swab were dipped into the suspension and roll the swab firmly against the side of the tube several times to remove excess swab.

This was then swabbed on the entire surface of Mueller-Hinton agar. Then antimicrobial disks with known concentration of antimicrobials were placed on the cultured Muller Hinton agar plate of appropriate distance of each disc with flamed forceps, inverted and incubated with agar side up for 18-20 hours at 37ºC. After incubation, the zones of inhibition were measured with caliper in millimeter (mm). The plates were held just above a non-reflecting surface. Clear zones produced by antimicrobial inhibition of bacterial growth were measured to the nearest millimeter using a straight-line ruler. Zone sizes were recorded and growths up to the edge of the disc were reported as a zone of zero mm. The zones of inhibition were uniformly circular. The results were interpreted according to standard of CLSI as susceptible, intermediate or resistant (CLSI, 2018). The thresholds for the antibiotics are shown in Appendix V.

3.9. Questionnaire and observational survey

To trace the individual fish value chains, the fishermen were the first point of contact. They were asked which groups of people they handed their catch to; the trail was then followed to identify the next group of main actors until the fish reached the final RTE. The knowledge, attitudes and practices of hygienic fish handling along fish value chain from fishermen to RTE producer were assessed using a semi-structured questionnaire. Questionnaires were interviewed to 36 fishermen, 32 retailers and 40 RTE processors. The questionnaire was divided into two sections; questioner and observational points. All questionnaires were interview in Amharic language by translated face to face from English language and responses given were entered by the researcher. The information collected included; age, level of education; information on fish hygiene, fish handling knowledge, attitude and practice to the food safety (clean and cross contamination).

Each correct answer within the closed ended responses carried a score of 2 while correct responses in the open ended questionnaires carried a score of 3 and each observational point score 5. 'Wrong' or 'don't know' answers were given a score of zero. For each respondent, the score of questions were summed up and converted into percentages (0 to 100). A representative score of 70% and above was considered "sufficient knowledge/practice" while a score of <70% was considered "insufficient knowledge/practice". The scoring system applied here was adapted from similar studies by Osaili *et al.* (2013); Zanin *et al*. (2015) and Aboagye (2016). The detailed questionnaire and observation point format is described in the appendix I.

3.10. Data Quality Management

The quality of data were assured by immediate and follow up recorded and also the reliability of test results were assured by following standard procedures for each microbiological test. The sterilization of prepared media was checked by incubating some randomly selected plates for 24-48 hours at 37^{o}C.

3.11. Statistical Analysis

All data collected during the study period were checked, coded and entered in to a computer Excel spread sheet. Descriptive statistics such as frequencies and percentage were used to summarize the result. Chi-square test with 95% confidence interval was used to assess the association of the sample with positivity of *Shigella.* P-value less than 0.05 were considered statistically significant. All statistical analyses were done using SPSS version 23.

3.12. Ethical Review

The study protocol was reviewed and approved by Institutional Review Board of Bahir Dar University, College of Agriculture and Environmental science, School of Animal science and Veterinary Medicine. A letter of support was obtained from Bahir Dar University and official permission was requested from the involved higher officers Bahir Dar Town Administration Health Department and Culture and Tourism Enterprise department. The sampled owner permission and interviewee's willingness were asked to participate in the research. After

thoroughly explaining the objectives and relevance of the study, the procedure, benefit and their right, informed consent were obtained from the participants.

3.13. Dissemination of the Result

The result of this study will be disseminated to Bahir Dar University College of Agriculture and Environmental Science, School of Animal science and Veterinary Medicine. In addition to this, the study result will be delivered to Health Bureau, and Culture and Tourism office of Bahir Dar city administration based on their interest. Moreover, it could disseminate to those of stockholders (Health Bureau, Fishery and Aquaculture) and final users broadly through publishing in international scholar journals.

CHAPTER 4: RESULTS AND DISCUSSION

4.1. Occurrences of *Shigella* isolates among the value chain of fish origin foods.

Out of a total of 170 fish sample; *Shigella* was isolated from 36 (21.2%) samples. Of all those fileted fishes 15 (37.5) were the highest isolated followed by fresh harvested fish 11 (27.5%) and the rest 10 were RTE (11.2%). Based on landing site proportion *Shigella* were isolated 13(52%) and 19(34%) at *Gorgora* and Bahir Dar Michael landing site respectively.

Table 1: The Occurrence and isolation of *Shigella* in fish value chain at Bahir Dar city 2019/2020.

Samples Type	No of samples examined	Positive (%)	X^2	P value
Fresh Fish	40	11 (27.5)		
Fileted	40	15 (37.5)		
RTE	**90**	**10 (11.2)**		
Dulet	17	3 (17.6)	12.806	0.002
Firfir	13	4 (30.8)		
Lebileb	24	3 (12.5)		
Asa wet	12	0		
Tibs	14	0		
Total	170	36 (21.2)		

In the present study high percentage of *Shigella* isolation were recorded from fileted and fresh fish samples; this is might due to Lake Tana washing water added chance to more contamination. In fact high bacterial loads found in the raw fish at the source point are most likely to have a multiplier effect as the caught fish are poorly handled and stored until they are consumed and cooking (frying or roasting) were apparently efficient in killing these pathogens (Sichewo *et al.,* 2014).

Since RTE fish has direct exposure to consumer and cause infection even in small number of *Shigella*; among the RTE fish *asa firfir, asa lebileb* and *asa dulet* were revealed to cause an

incremental carrier of *Shigella* (30.8%), (12.5%) and (17.6%) respectively. The reason that fresh fileted had more bacterial in this study might be suggested that they were more liable for contamination during fileting and washing with questionable water for its bacteriological quality from Lake Tana. RTE has smaller percentage compared to fresh and fileted fish samples; this is due to in RTE preparation use washing frequently with water and cooking temperature may in addition onion and spice have great effect for decrease it proportion. Similar study to our finding were done by Onyango *et al*. (2009) on isolation of *Shigella* from fish harvested from the Winam Gulf of Lake Victoria, Kenya and who's reveled that Nile tilapia infected by *Shigella spp* were isolated 39.7 percent.

Another study which has similar result with this study was also done by Hendawy *et al*. (2016) at Egypt out of the total fish samples (23.6%) were contaminated by *Shigella*. He suggests that isolation of *Shigella* species may be attributed to sewage pollution of human origin or unsatisfactory hygienic conditions during catching, handling and marketing of the fish. In all circumstance of fish value-chain; products contamination has great public health significance due to its liable nature to pathogenic bacteria from human reservoirs during handling, processing and packaging of fish (Ghanem *et al*. 2019). In general the results obtained from this study gives evidence to the unsatisfactory microbiological quality and safety of fish from the local artisanal fish value chain. Of particular concern was the occurrence of key pathogenic bacteria such as *Shigella* as fish and its products contain hazards microorganism.

4.2. *Shigella* Concentration and Its Public Health Risk

In this study, the total colony count (CFU) for all the fish samples were ranged between $1.2x10^1$ to $7.3x10^4$cfu/g as shown in (Table 2). Of which all positive samples were employed for CFU, the fileted had the highest number of bacteria with $7.3x10^4$cfu per gram. *Dulet* food items had the lowest isolation with $1.2\ x10^1$cfu/g from hotel and restaurant sites.

Out of the total fish muscle traced 36 (21.2 %) were contaminated with *Shigella*, of which all positive samples were employed for plate count enumeration of *Shigella*. As compared to many studies on *Shigella* detection in fish products, the isolation of *Shigella* in fish muscle in

this study was of high levels and this was might hygienic keeping status and fly control strategies in this study area is very low as demonstrated in questionnaire part. The current load of *Shigella* from the examined samples of fish was higher than those obtained by Sichewo *et al.* (2014) at Zimbabwe who was isolated a load of $1.4\text{-}3.8\times10^3$ from all examined samples.

Table 2: RTE fish, fileted and fresh fish products contaminated by *Shigella* and its average colony count percentage study result in 2020.

Types of sample	No. Positive for *Shigella*	Average load *Shigella* cfu/g (Percentage of samples)		
		$1.2\times10^1\text{-}1.7\times10^2$	**$2\times10^2\text{-}3\times10^3$**	**$3\times10^3\text{-}7.3\times10^4$**
Fileted	15	0	33.3	66.7
Fresh	11	0	54.5	45.5
Dulet,	3	100	0	0
Firfir	4	75	25	0
Lebileb	3	66.6	33.3	0

The *Shigella* organisms are highly contagious, causing bacillary dysentery after ingestion of as few as 10-100 organisms (Kosek *et al*., 2010). Outbreaks of Shigellosis are associated with poor sanitation and contaminated food (Bardhan *et al*. 2010; Kosek *et al*. 2010. According to the standard of center food safety (CFS, 2014) and American Public Health Association (APHA, 2004) the current result of *Shigella* was not compatible and which shows great public health risk.

4.3. Antimicrobial Resistant patterns of *Shigella* isolates

The antibiotic resistance patterns of *Shigella* isolates was shown in Table 3. In this study the resistant patterns of seven different commonly applied antibiotics were used: *Shigella* spp. was 100% sensitive to ciprofloxacin, gentamycin and chloramphenicol and 86.1% to

trimethoprim sulfamethoxazole; while 100% resistant to Streptomycin and (94.5%), (91.6%) were resistance to Erythromycin and Tetracycline respectively.

Table 3: Antimicrobial Resistance patterns of *Shigella* for commonly used antibiotics in the study of *Shigella* from fresh and RTE fish product at Bahir Dar City 2019/2020.

Antimicrobials	Number of *Shigella* isolates N = 36		
	Resistance strains in (%)	Intermediate R Strain in (%)	Susceptible strains in (%)
Streptomycin 10µg	100	---	---
Tetracycline 30µg	91.6	8.4	---
Ciprofloxacin 5µg	---	---	100
Trimethoprim Sulfamethoxazole 1.25µg (23.75µg)	---	13.9	86.1
Chloramphenicol 30µg	---	---	100
Gentamycin 10µg	---	---	100
Erythromycin 15µg	94.5	5.5	---

The emergence and dissemination of antimicrobial resistance is an important issue in public health, animal health, and food safety. Thus, antimicrobial susceptibility test was performed to all of the bacterial isolates. Of 36 *Shigella* spp. strains, 33 (91.7%) were resistant to one or more antibiotics. In this study, most of the bacterial isolates were susceptible to gentamycin, ciprofloxacin and chloramphenicol which were compatible to many studies Legesse Garedew *et al*. (2016) and Huruy *et al*. (2008) at Gonder and Atsebaha Gebrekidan *et al*. (2015) at Mekelle. However, relatively high resistant *Shigella* strain isolates for gentamicin (100%) was found by Rahimi *et al*. (2017) Iran; (18.8%) and (53.1%) resistant strain for gentamicin and chloramphenicol respectively were recorded by Debas Getachew *et al*. (2011) at Felege hiwot hospital Bahir Dar.

Similar to the present study many studies have recorded *Shigella* strains were 100% sensitive to ciprofloxacin like Debas Getachew *et al*. (2011) at Felege hiwot hospital Bahir Dar.
, Mulatu Getnet *et al*. (2014) at Hawassa and Huruy *et al*. (2008) Gonder from Ethiopia and Brazil Nunes *et al.* (2012) (0.0%), Gahna Opintan and Newman (2007) (0.0 %) of *Shigella* strains were sensitive to ciprofloxacin. But some *Shigella* strains were resistance to ciprofloxacin from other countries: India by Bhattacharya *et al*. (2012) (82 %) and at Iran by Rahimi *et al*. (2017) (38.46%).

In the present study the resistance profile of Streptomycin (100%) and Tetracycline (91.6%) were similar with Debas Getachew *et al*. (2011) at Bahir Dar felege hiwot hospital who reported (96.9%) and (93.8%) respectively. High levels of resistance to streptomycin, tetracycline and Erythromycin observed. This alarming rise in multiple antimicrobial resistances in the study area may be related to the indiscriminate use of antimicrobial agents
The current study revealed a multidrug resistance pattern of (42.9%) in the isolates, which is less than report of Addis Aklilu *et al.* (2015) (100%) at Addis Ababa and Getenet Beyene and Haimanot Tassew (2014) (100%) from Jimma Ethiopia. The multidrug resistance of the isolates in the present study was alarming in that all *Shigella* isolates were developing multidrug resistance to different antibiotics which could be the consequence of indiscriminate use of antibacterial in clinical practice. The multidrug resistance pattern recorded in the current research might be due to the emergence of antibiotic resistant strains of *Shigella* due to mutation or antibiotic pressure from the unrestricted use of antibiotics by the community and animal health professionals.

4.4. Questionnaires Survey Results

4.4.1. Demographic Character of fish value chain main actors in Bahir Dar city.

Every stage in the fish value chain was dominated by women with the exception being the fishermen who were all male. Wholesalers or retailers of fresh and fileted fish were predominantly female (81.2%). RTE Processors were also almost all female, recording 92.5% in all areas surveyed in this study. The demographic characteristics of the stakeholders are depicted in (Table 4). As many as (52.5%) of fishermen had never received formal education.

Similarly, (34.3%) of retailers as well as (37.5%) of RTE processors also had no formal education.

Table 4: Demographic characteristics of the fish value chain actors in our study area 2019/2020.

Questionnaires Variables	**Variable Category**	**Frequency of Respondents** N (%)		
		Fisher men	Retailer	RTE
Sex	Male	36(100)	6 (18.7)	3 (7.5)
	Female	0	26 (81.2)	37 (92.5)
Age	18-24 yrs	9 (25)	6 (18.7)	36(90)
	30-39	14 (38.9)	18 (56.3)	4(10)
	≥40	13 (36.1)	8 (25)	0
Education standard	None	21 (52.5)	11 (34.3)	15 (37.5)
	Primary/2nd	15 (37.5)	15 (46.8)	20 (50)
	Degree	0	6 (18.75)	5 (12.5)
Experience in fish related activity	1-5yrs	6 (16.7)	17 (53.2)	40 (100)
	6-10yrs	13 (36.2)	15 (46.8)	0
	Above 10 yrs.	17 (47.2)	0	0

Table 5. Responses to questions on fish hygiene and safety

Questionnaires	**Variables**	**Frequency of Respondents** N (%)		
		Fishermen	Retailor	RTE
Have you know about food born disease?	Yes	31 (86)	19 (59.4)	27 (67.5)
	No	5 (14)	13(40.6)	13 (32.5)
Washing hands before and after handling of fish can minimize?	Yes	20 (55.6)	10(31.25)	29 (72.5)
	No	16 (44.4)	22(68.75)	11 (27.5)
Have you heard about *Shigella* as a food-born pathogen?	Yes	19 (52.8)	20 (62.5)	31 (77.5)
	No	17 (47.2)	12 (37.5)	9 (22.5)
What do you do to minimize	Washing	24 (66.7)	10 (31.3)	33 (82.5)

contamination?	Nothing	12 (33.3)	22 (68.7)	7 (17.5)
Do you experience fish spoilage or	Yes	36 (100)	29 (90.6)	4 (10)
contamination	No	0	3 (9.4)	36 (90)
Do you have fridge for fish storage?	Yes	0	5 (15.6)	30 (75)
	No	36 (100)	27 (84.4)	10 (25)

Table 6. Observational check points on fish hygiene and safety

Practical Observation	**Variables**	**Frequency of Respondents** N (%)		
		Fishermen	Retailor	RTE
The hygiene of fileted, fresh landed and the time retail fish?	Yes	7 (19.4)	2 (6.25)	____
	No	29 (80.6)	30 (93.75)	_____
Occurrence of fly seen on fish	Fly	36 (100)	32 (100)	11 (27.5)
	No fly	0	0	29 (72.5)
Cleanness of fish carrying basket during harvesting and transportation?	Clean	0	0	____
	Not clean	36 (100)	32 (100)	____
During cooking fresh fish and cooked fish has different room?	Yes	_____	_____	13 (32.5)
	No	_____	_____	27 (67.5)
Food handlers wear appropriate personal protective equipment?	Yes	0	0	28 (70)
	No	36 (100)	32 (100)	12 (30)
Source of water used for washing of fish	Lake Tana	36 (100)	30 (93.75)	0
	Pipeline	0	2 (6.25)	40 (100)

Out of thirty six fishermen (83.4%) had experience more than ten years in fishery process were included in this study. All of them experience fish spoilage and 66.7% of fishermen responded prevent fish from direct sunlight activities were taken to minimize fish spoilage. Eighty six percent of responded were correctly answered about foodborne disease the rest does not have clue about food borne disease. 72.5% of RTE worker respondent that washing

their hands before and after handling of fish could decrease fish contamination. But no one in retailor respondent believed that hand washing before and after fish handling can prevent fish contamination. All the fishermen and retailor responded that source of water for washing of fish were Lake Tana, occurrence of fly on fish and dirty retailer area could not cause fish contamination. Direct observations revealed the absence of fridge, pipeline water and clean fileting area in the retailor.

4.5. Food Safety Knowledge, Attitude and Practices in Stakeholders

The mean hygienic and fish handling knowledge score for all stakeholders found from this survey were (70.58%) with 8.7±10.34 while the mean practice score were (**57.7**%) 10.34 ±11.48 (Table 7).

Generally sufficient levels of food safety knowledge but not practice which suggested insufficient levels of food safety practices. Fishermen, Retailer and RTE processors had mean practice scores of (53.75%), (55.78%) and (63.25%) respectively.

Table 7: Fish hygiene and safety knowledge and practice of stakeholders in the value chains.

Dependent Variables	Variables	No.	Mean score in %	Std. Deviation	95% CI	
					L. bound	U. bound
Correctness	Fisher men	36	72.25	7.481	69.109	72.280
	Retailer	32	70.58	15.180		
	RTE prod	40	69.08	7.426		
	Total	108	70.58	10.342		
Practice	Fisher men	36	53.75	9.741	56.678	59.139
	Retailer	32	55.78	6.153		
	RTE prod	40	63.25	10.335		
	Total	108	57.87	9.938		

It can be observed from Table 7 that, 72.25% fishermen, 70.58% of retailers and 69.08% RTE of the value chain had sufficiently knowledge and have food safety rules concerning fish handling and food borne disease awareness but only 53.75%, 55.78% and 63.25% of these

fishermen, retailer and RTE worker respectively had sufficient practice on food safety. Studies assessing food safety knowledge and practices had reported a similar trend, with this study where good knowledge did not always translate to good practices. This is more exemplified by the findings of Omemu and Aderoju (2008), whose study on the food safety knowledge and practices of street food vendors in Nigeria, revealed that a good knowledge in the importance of hand washing did not translate into improved handling practices.

Another contrary study on current study was by (Aboagye, 2016) whose study on microbial quality of fish along the tilapia and artisanal value chains in Ghana, revealed that even though almost 90% of retailers in the fish value chain sufficiently practiced food safety rules concerning fish handling, only 30% of these individuals had sufficient knowledge about food safety and Aboagye, (2016) had argue that many of the stakeholders practiced good handling practices without necessarily understanding the basis or importance of their actions.

4.5. Limitation of the study

- The study cannot address *Shigella* at spices and strain level due to budget limit. So antimicrobial susceptibility done at genus level and need further study for future.
- Due to COVID-19; un-able to get full permission from major fish value chain actors to take cooking temperature of fish products.

CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

The present study revealed that *Shigella* is a contaminant of fish in the study area and its occurrence in fish could represent a risk to the consumers. *Shigella* isolates were identified in one fifth of the fish samples taken from raw and cooked fish samples. In general the results obtained from this study gives evidence to unsatisfactory microbiological quality and safety of fish from the local artisanal fish value chain.

Load of *Shigella* in fish muscle in this study was of high levels and this was might hygienic keeping status and fly control strategies in this study area is very low as demonstrated in questionnaire part. The load of *Shigella* was above the recommended level according to center for food safety standards. In this study, all isolates of *Shigella* were susceptible to drug ciprofloxacin, chloramphenicol and gentamycin. However, more than half of isolates were resistance to tetracycline and erythromycin. But all isolates were resistance to streptomycin also was observed.

This study found inadequate level of food safety and poor handling practices at every stage of the fish value chain. The poor handling practices of major actors particularly retailer and fishermen were lack of fridge for storage and use of unjustifiable water for washing after fileting of fish from Lake Tana could be associated with the high estimated for occurrence of *Shigella* reported in this study.

Therefore, based on the above conclusion, the following recommendations were forwarded:

- Proper attention should be paid for construction of quality water pipe for washing of fresh and fileted fish.
- Fish handlers and sellers should be controlled by regulatory body and follow up for best practices on handling of fish throughout value chain since they have sufficient knowledge about fish handling
- Governmental organs should be constructing fly trap semi slaughter or fileting area for those fishermen and retailers.
- NGO and governmental bodies could be addressed cold chain materials like deep fridge.

- The treatment of choice for *Shigella* suspected cases should be ciprofloxacin chloramphenicol and gentamycin.
- Further investigations should be conducted to investigate pathogenic spices and strains of *Shigella*

CHAPTER 6: REFERENCES

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APPENDIXES

Appendix I: Sample Collection sheet for laboratory analysis

SN	Type of sample	Date of collection	Collection site	Sample code	Species of sample
1					
2					
3					
4					
.					
.					
170					

Appendix II: Record sheet format for *Shigella* isolation and identification during laboratory class

Sample code	Date of sample collected	Colony characteristics on	Biochemical tests						Sample +/-
		HEA	urase	TSI				SIM	
				But	Slant	Gas	H_2S	Motility	
1									
2									
3									
.									
.									
170									

Appendix III: Questionnaire and Background Information

Bahir Dar University School of Animal Science and Veterinary Medicine; VPH Program: Occurrence, Antimicrobial Susceptibility and Public Health Importance of *Shigella* from Fish Value Chain in Bahir Dar city, North West Ethiopia:

Dear respondent, this questionnaire is aimed at collecting information on the hygienic status, fish contamination level and actions done to prevent from spoilage starting at the first chain of harvesting to its final process as RTE It is part of MVPH Thesis on the topic above. Your identity protected to the best of my ability. Your name will not appear on any record including the questionnaires and reports. Only I and my academic advisors involved in execution, review and examination of this project may access the research records. Thank you.

Date: _____

Location: _____

Respondent's Code: _____

1. Sex A. Male B. Female

2. Age …….

A Case Study Questions

3. Educational Status A. Illiterate B. Literate,

If the answer is "B", state the level A. Primary B. Secondary C. Tertiary D. Degree

4. How long have you been in this business? A. 1-2 years B. 3-5 years C. 6-10 years D. above 10 years

5. Do you remove your personal jeweler such as rings, necklaces, and watch while harvesting, fileting and cooking of fish? A. Yes B. No

6. Do you believe people with, gastroenteritis and dysentery diseases can allowed in handling of fish harvesting, fileting and cooking of fish? A. Yes B. No

7. Who are your key customers? A. Retailer B. Restaurant and hotels C. Consumers

8. Do you experience fish spoilage/contamination? A. Yes B. No

9. If yes what indications do you look out for spoilage? _____

10. What do you do to minimize contamination and spoilage of fish? _____ A,

11. Do you wash your hands before and after handling of fish? A. Yes B. No

12. Do you think the ways fishes are handled could contribute to their spoilage? A. Yes B. No

13. What illnesses are feel by eating **contaminated** fish? A. dysentery B, Typhoid C. none ?

14. Have you heard about food born disease? A. Yes B. No

15. If yes, what will be the cause of the disease? _____

16. Have you heard about **dysentery** as a food-born pathogen? A. Yes B. No

A: Observational and exceptional points for fishermen

1. Cleanness of fish carrying boat during harvesting and transportation? A. Clean B. Not Clean

2. Cleanness of fish collecting equipment/ Basket? A. Clean B. Not Clean

3. Risk for contamination of fish during landing area? A. high risk B. low risk

4. Risk for contamination and spoilage of fish during more than one hour transportation? A. high risk B. low risk

5. Is there observable physical contamination on boat during fish transportation? A. Yes B. No

6. Do you think un-ice transportation of fish can be a source of fish spoilage and contamination? A. Yes B. No C. No idea

7. How long does it take you to transport caught fish from point of fishing to landing?

A. Less than 2 hours C. 6 - 12 hours B. 2 hours to 6 hours D. More than 12 hours

8. How do you transport the fish to next chain to wholesaler, retailer or direct to the market?

A. On ice C. In a refrigerated van

B. Without ice D. other specifies.

B: Observational and exceptional points for only Retailer man

1. Source of water used for washing of fish A Pipe water B. Lake Tana water

2. The hygienic of fileted fish during the retail period? A. Hygiene B. Unhygienic

3. Occurrence of fly on fileted fish from fish retail place

4. Personnel hygiene keeping during fileting and selling

5. Are you always able to process the quantities you procure in a batch? A.Yes B. No

6. If no how you handle the quantities you are not able to sell a day?

C: Observational points for only Hotel and Restaurants

1. Workers/food handlers wear appropriate personal protective equipment's (PPE)

2. Cleanliness of food handlers PPE. A. Clean B. Not clean

3. Presence of any hand washing facilities or disinfectant. A. Yes B. No

4. During cooking fresh fish and cooked fish has different room? A. yes B. no

5. General sanitation? Cooking room, tables, wall and roof A. yes B. no

6. Occurrence of fly on fileted fish from fish storing

7. Can you list methods are helpful to prevent fish contamination at RTE?

A. Washing hands with soap and water before and after touching raw fish during cooking

B. Use higher heat for *asa lebileb, asa dulet…*etc. during cooking C. Use of spice for *asa lebleb, asa dulet…* etc. for preparation foods D. don't know

Appendix IV: Media and Biochemical test procedures

A. Result of *Shigella* isolates and its Biochemical test

Isolation of *Shigella* was confirmed by series of biochemical test TSI, Urease and SIM (H_2S production and by its negative motility and also by aiding excellent selective media Hektoen Enteric Agar which seems green (colorless) due to inability utilizing of lactose (Figure 4). Differentiation for TSI Agar slants is based on carbohydrate fermentation patterns. Organisms capable of fermenting only dextrose (e.g *Shigella* spp) will result in an alkaline (red) slant and an acid (yellow) butt. Organisms capable of fermenting dextrose and lactose and/or sucrose will result in an acid (yellow) slant and acid (yellow) butt (Figure 5). Another system of differentiation is based on H_2S production. Positive H_2S reaction appears as a black precipitate (ferrous sulfide) in the medium or a black ring near the top of the tube.

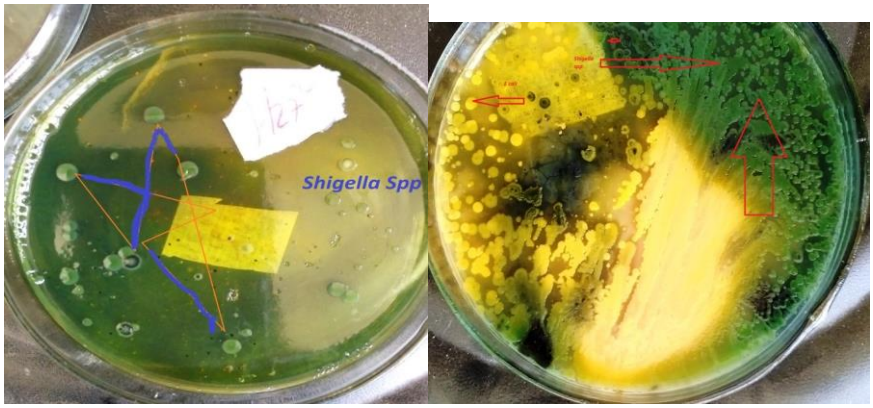


Figure 4: Culture of *Shigella* spp produce green colony on Hektoen Enteric Agar media.

SIM Medium enables determination of three characteristics by which enteric bacteria can be differentiated. Peptonized iron and sodium thiosulphate are the indicators of H_2S production. This H_2S reacts with peptonized iron to form black precipitate of ferrous sulphide. Motile organisms intensify the H_2S reaction. Motile organisms grow away from line of inoculation showing diffused growth while non-motile organisms grow along the stab line. Motility detection is possible due to the semisolid nature of the medium. Growth radiating out from the central stab line indicates that the test organism is motile (HiMedia Technical data, 2018). But *Shigella* is an exceptional bacterium that it does not produce H_2S gas and also it does not show any motility during inoculum so this is good way for isolation of *Shigella* (Figure 5, C). The urease test identifies those organisms that are capable of hydrolyzing urea to produce ammonia and carbon dioxide. It is primarily used to distinguish urease-positive bacteria like *Proteeae* from other *Enterobacteriaceae*. The culture medium will remain a yellowish color if the organism is urease negative like *Shigella* (Figure. 5, B).

Figure.5: Biochemical test of TSI (A), Urease (B) and SIM (C) for isolation of *Shigella*.

Hektoen Enteric Agar Medium HCM016

Hektoen Enteric Agar Medium is recommended for differential and selective isolation of *Salmonella* and *Shigella* species from enteric pathological specimens in accordance to United States Pharmacopoeia.

Composition and Ingredients Gms / Litre

Protease peptone	12.000
Yeast extract	3.000
Lactose	12.000
Sucrose	2.000
Salicin	9.000
Bile Salts mixture (Equivalent to Bile Salt No. 3)	9.000
Sodium chloride	5.000
Sodium thiosulfate	5.000
Ferric ammonium citrate	1.500
Acid fuchsin	0.100
Bromothymol blue	0.065
Agar	14.000
Final pH	7.5±0.2

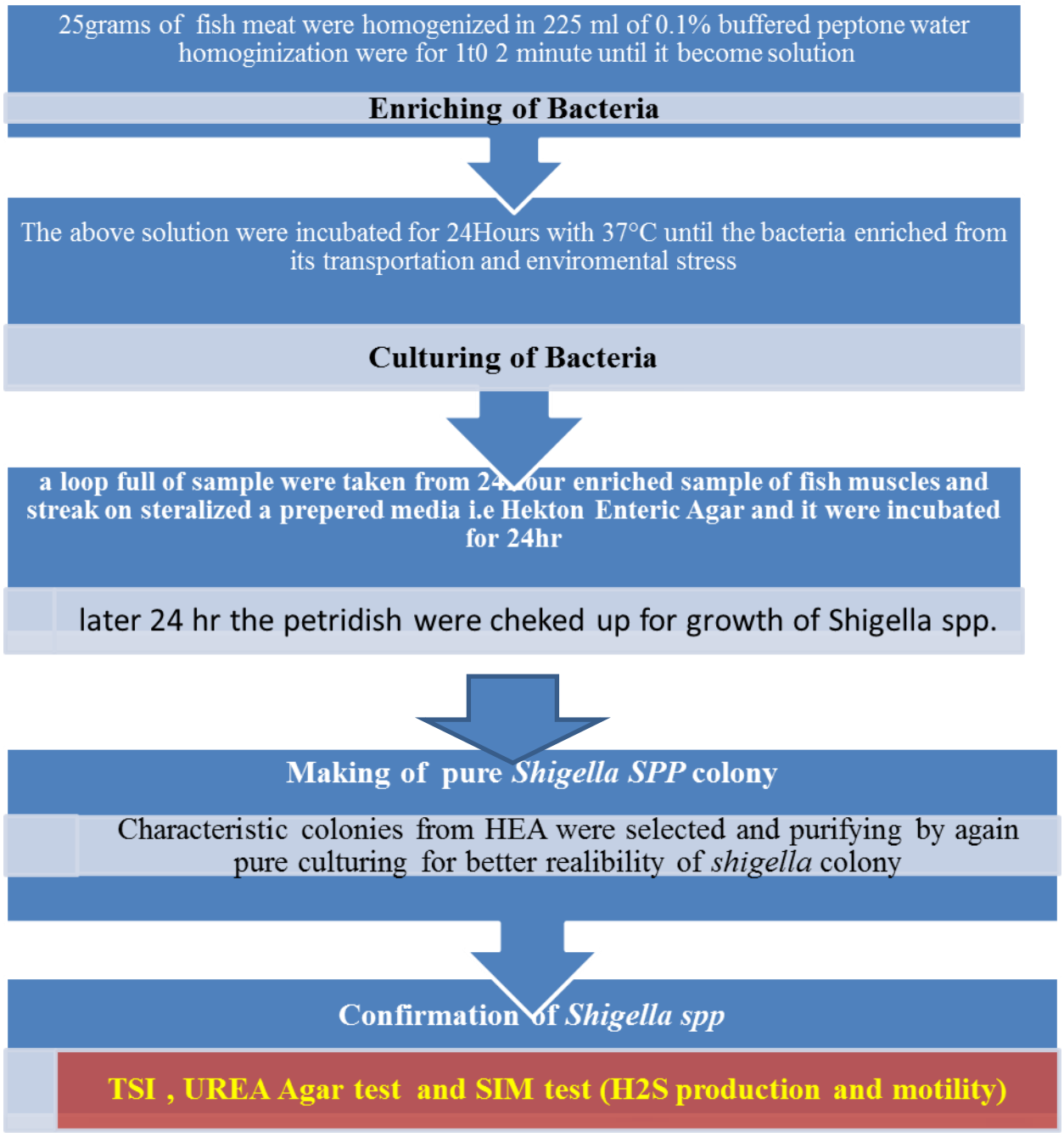
Principle and Interpretation

Hektoen Enteric Agar, a selective and differential medium designed to isolate and differentiate members of the species *Salmonella* and *Shigella* from other *Enterobacteriaceae* and was developed by King and Metzger (King and Metzger, 1968; USPC, 2009). When compared with other selective medium, this medium inhibits the growth of *Salmonella* and *Shigella* very slightly; thus giving high yields of these microorganisms, but at the same time inhibits accompanying gram positive and other microorganisms.

Hektoen Enteric Agar can also detect the production of hydrogen sulfide gas, which turns parts of the medium black. Ferric ammonium citrate serves as iron source, which cause production of hydrogen sulfide from sodium thiosulphate and also aids in the visualization of hydrogen sulfide production by reacting with hydrogen sulfide gas to form a black precipitate. Enterobacters that are capable of fermenting one or more of the carbohydrates produces

yellow or salmon-orange colored colonies like *Klebsiella pneumoniae*, which ferments lactose. Non-fermenters will produce blue-green colonies. Organisms that reduce sulfur to hydrogen sulfide will produce black colonies or blue-green colonies with a black center. *Salmonella* reduce sulfur to hydrogen sulfide, producing a black precipitate.

Appendix V: Flow chart for isolation of *Shigella* from fresh and RTE fish samples.



Appendix VI: Detailed procedures for enumeration of *Shigella* count

By using standard plate count method (Harley and Prescott; 2002; Collins *et al*., 2004)

- To count bacteria from solid fish sample, weigh 10 gram of the sample and place in a Stomacher.
- Add 90 ml of peptone diluent and homogenize
- Allow to settle. Until the bacteria were evenly distributed between the solid and liquid. These dilutions represent of; 10, i.e. 1 ml contains or represents 0.1 g. shake very well to distribute the bacteria and break up any clumps of bacteria that may be present and remove 1 ml to from original tube and aseptically transfer it tube 1 to make the dilution 10^{-2}. Further dilutions were prepared in similar manner and the dilutions were like here.

Tube No.	Original	1	2	3	4	5	6	7	8	9
Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}

- Label the bottom of petridishes with the following the corresponding dilutions and transfer aseptically into petridishes. The inoculums were spread on the surface of agar using a thin, bent in an L shape iron rod by flame sterilized. I was plated four inoculum plates per dilution.
- Retain plates in upright position until inoculum is absorbed by agar medium (about 10 min on properly dried plates).
- The plates were incubated for 24-48 hours at 37 °C.
- At the end of the incubation period, select all of the petri plates containing 10-250 colony were calculated as (CFU) per gram of sample.

Appendix VII: Procedures for anti-microbial susceptibility testing of *Shigella*

(CLSI, 2018)

- ❖ Three to five well-isolated colonies of the same morphological type were selected from the nutrient agar medium (Oxoid, England) (non-selective medium), from 18 to 24 hours agar plate, was touched with the loop, and transferred into a tube containing 4 to 5 ml of sterile saline solution.

- Just the top of the colonies are touched and the growth transferred to a tube containing saline suspension.
- The turbidity of suspension is adjusted by comparison with a 0.5 McFarland turbidity standard.
- The standard and the test suspension were placed in similar 4-6 ml thin, glass tube or vials.
- The turbidity of the test suspension were adjusted with saline and compared with the turbidity standard, against a white background with contrasting black lines, until the turbidity of the test suspension equates to that of the turbidity standard.
- A sterile, non-toxic swab on an applicator stick is dipped into the standardized suspension of bacteria and excess fluid is expressed by pressing and rotating the swab firmly against the inside of the tube above the fluid level.
- The swab were streaked in three directions and continuously brushed over the Mueller Hinton agar
- The inoculated plates were allowed to stand for 3-5 minutes, but no longer than 5 minutes and the discs are placed onto the agar surface using sterile forceps. Each disc was gently pressed with the point of a sterile forceps to ensure complete contact with the agar surface.
- The discs should were placed no closer together than 24mm (center to center).
- After incubation, the diameters of the zones of inhibition were measured to the nearest mm using a ruler or calipers.
- The diameters were read from the back of the plate and the zones were read across the center of the discs.

An interpretation of the size of the zones of inhibition were made with reference according to (CLSI, 2018) and reported as susceptible, intermediate or resistant to each antimicrobial agent used in the test which were indicated in the following table.

Table 8: Antimicrobial drugs its disk content and inhibition zone of diameter interpretive standards for *Shigella*.

Antimicrobials Drugs/ Disk	Zone Diameter Interpretive Criteria (mm)		
	Susceptible MID	Intermediate MID (mm)	Resistance
Gentamycin 10µg	≥15	13-14	≤12
Streptomycin 10µg	≥15	12-14	≤11
Tetracycline 30µg	≥15	12-14	≤11
Ciprofloxacin 5µg	≥21	16-20	≤15
Trimethoprim Sulfamethoxazole 1.25µg (23.75µg)	≥16	11-16	≤10
Chloramphenicol 30µg	≥18	13-17	≤12
Erythromycin 15µg	≥23	14-22	≤13
Penicillin 10µg	≥29	-------	≤28

Source: - (CLSI, 2018)

Appendix VIII: Galleries, which are collected at time of laboratory work and field work sample collection 2020.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TO PORT HOTEL