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COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

ANTIMICROBIAL RESISTANCE OF *STAPHYLOCOCCUS AUREUS* AND *PSEUDOMONAS AERUGINOSA* IN FISH, AND KNOWLEDGE, ATTITUDE AND HYGIENIC PRACTICES OF FISH HANDLERS IN BISHOFTU, ETHIOPIA

MSc THESIS

BY

KEBADU ENDEG

JUNE, 2024

BISHFOTU, ETHIOPIA

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A THESIS SUBMITTED TO THE DEPARTMENT OF BIOMEDICAL SCIENCE, COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN VETERINARY PHARMACOLOGY

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This is to certify that the thesis prepared by Kebadu Endeg entitled "**Antimicrobial Resistance of *Staphylococcus aureus* and *Pseudomonas aeruginosa* in Fish, and Knowledge, Attitude and Hygienic Practices of Fish Handlers in Bishoftu, Ethiopia**" and submitted in partial fulfillment of the requirements for the degree of Master of Science in Veterinary Pharmacology complies with the regulations of the university and meets the accepted standards with respect to originality and quality.

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STATEMENT OF AUTHOR

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LIST OF ABBREVIATIONS AND ACRONYMS

AMR	Antimicrobial Resistance
AMU	Antimicrobial Use
ATCC	American Type Culture Collection
bp	Base Pair
CLSI	Clinical and Laboratory Standards Institute
DNA	Deoxyribonucleic Acid
KAP	Knowledge, Attitude and Practice
MAR	Multiple Antibiotic Resistance
MARI	Multiple Antibiotic Resistance Index
MDR	Multidrug Resistance
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin Resistant *Staphylococcus aureus*
MR-VP	Methyl Red and Voges-Proskauer
P. aeruginosa	*Pseudomonas aeruginosa*
PCR	Polymerase Chain Reaction
S. aureus	*Staphylococcus aureus*
SXT	Trimethoprim/Sulfamethoxazole

ABSTRACT

Fish, a protein-rich food, can sometimes be contaminated with bacterial pathogens like *S. aureus* and *P. aeruginosa*, known for their resistance to antimicrobials. A cross-sectional study was conducted in Bishoftu, Ethiopia, from November 2023 to May 2024. The purpose of the study was to determine the antimicrobial resistance (AMR) pattern of these pathogens, as well as assess the knowledge, attitude towards antimicrobial use (AMU) and AMR, and hygienic practices of fish handlers. The study used a purposive sampling strategy. *S. aureus* and *P. aeruginosa* were confirmed using polymerase chain reaction and their susceptibility to antibiotics was tested using the Kirby Bauer disk diffusion method. The data was analyzed using STATA version 14, using descriptive statistics, Chi-squared, likelihood ratio, and binary logistic regression. The results of the study found that 8.6% (9) of 105 samples tested positive for *S. aureus* and 7.6% (8) for *P. aeruginosa*. *S. aureus* isolates were 100% resistant to cefuroxime, 44.4% (4/9) to cloxacillin and penicillin G, and 33.3% (3/9) to tetracycline. *P. aeruginosa* isolates were 100% resistant to cefuroxime, doxycycline, erythromycin, florfenicol, kanamycin, nalidixic acid, trimethoprim/sulfamethoxazole and tetracycline. Multidrug resistance was observed in 33.3% (3/9) of *S. aureus* isolates and 100% of *P. aeruginosa* isolates. None of the *S. aureus* isolates were positive for *mecA* gene. The survey revealed that 79% (42/53) and 60% (32/53) of respondents had insufficient knowledge and desirable attitudes about AMU and AMR, respectively and 41% (22/53) had poor hygienic practices. A significant positive correlation was found between respondent's educational level and knowledge and attitude scores. Gender also played a role in attitude and hygienic practices. The study provides insights into *S. aureus* and *P. aeruginosa* resistance in fish production and establishes a baseline understanding of knowledge, attitudes, and hygiene practices among fish handlers. Further research should focus on detecting AMR genes in aquaculture. Additionally, enhancing knowledge about AMU and AMR in aquaculture for fish handlers is of utmost importance.

Key words: *antimicrobial resistance, attitude, fish, fish handlers, hygienic practices, knowledge, P. aeruginosa, S. aureus.*

1. INTRODUCTION

Before antibiotics were discovered in the middle of the 20th century, infectious diseases dominated humankind. The ability to treat infectious disorders has significantly increased since the discovery of numerous antimicrobial drugs (Salam *et al*., 2023). The period of antimicrobial resistance (AMR) began to emerge soon after penicillin was discovered, as evidenced by several treatment failures and the emergence of some bacteria that were no longer susceptible to the antibiotic (Porrero *et al*., 2014).

The threat posed by drug-resistant bacteria to the world community is increasing. They put citizens of poor nations as well as citizens of wealthy societies at risk. The UK review on AMR projected that up to 10 million deaths could be attributable to AMR by 2050 (Trotter *et al*., 2019), whereas the World Bank estimated that AMR could independently result in global GDP falling by 1.1-3.8% by 2050 if current practices continue. AMR is, undeniably, a considerable global health security issue (Medina *et al*., 2020).

Because food animals are the reservoirs for zoonotic and foodborne infections, overuse of veterinary antibiotics causes bacteria to become selectively more resistant, which in turn makes animals the epicenter of AMR (Silbergeld *et al*., 2008; Van Boeckel *et al*., 2015). Animal feed often contains antibiotics in amounts that range from below therapeutic levels to full therapeutic levels, and the antibiotics used come from most of the antimicrobial classes used in humans (Reygaert, 2018).

Fish is a popular choice due to its natural nutritional content, flavor, and ease of digestion. It is a significant source of animal protein in the tropics (Andrew, 2001). However, fish can be contaminated by environmental factors like sewage, water, harvesting areas, and unsanitary handling practices, leading to high bacteria concentrations (Roberts, 2003). Both wild and cultured fish suffer significant mortality rates from bacterial diseases, primarily caused by saprophytes. Over 92 bacterial genera, including *Aeromonas*, *Pseudomonas*, *Mycobacterium*, *Edwardsiella*, *Streptococcus*, *Staphylococcus*, and *Clostridium*, have been linked to infections in freshwater and marine fish (Ali, 2014).

Staphylococcus and *Pseudomonads* species are significant food-borne opportunistic bacteria found in fish samples (Albuquerque *et al*., 2007). *S. aureus* is a major cause of food-borne illnesses, including skin infections, pneumonia, and food poisoning (EFSA and ECDC, 2020). On the other hand, *P. aeruginosa*, commonly found in freshwater, is part of normal fish microbiota. However, under stressful conditions like malnutrition and overcrowding, these bacteria become highly opportunistic and pathogenic, causing serious illnesses (Ardura *et al*., 2013). Methicillin-resistant *S. aureus* (MRSA) is also at present the most commonly identified antibiotic-resistant pathogen globally, with reports of MRSA in fish and fishery products worldwide (Grema *et al*., 2015; Obaidat *et al*., 2015; Murugadas *et al*., 2016; Fri *et al*., 2020).

Globally, the use of antimicrobial agents in aquaculture has led to the transmission of multidrug resistant (MDR) bacterial pathogens between terrestrial and aquatic ecosystems (Cabello, 2006). Ethiopia has a high detection rate (43.5-74.9%) of MDR isolates from bacterium species, with *S. aureus* and *P. aeruginosa* being common MDR pathogens. Like other countries, fish is a major source of protein in Ethiopia and carries additional risks, including the development of antibiotic resistant bacteria in humans and environmental contamination (Muhie, 2019).

There are limited reports on the prevalence of *S. aureus* in fish and aquaculture including 4.80% (Mitiku *et al*., 2023), 65% (Wendwesen *et al*., 2017) and 85% (Admasu *et al*., 2023) from Bahir Dar, Arba Minch and Hawassa, Ethiopia respectively. There is also a single report on *P. aeruginosa* from three Ethiopian rift valley lakes (3.2%, Dissasa *et al*., 2022). The lack of clear regulations regarding antibiotic use in aquaculture farms in Ethiopia poses a risk of inappropriate usage and the long-term consumption of antimicrobial residues in animal food products (Opiyo *et al*., 2018; Brunton *et al*., 2019). The World Organization for Animal Health suggests the continuous monitoring and surveillance of resistant microorganisms in aquatic animals (Smith *et al*., 2013).

However, there is a lack of data on AMR in fish and aquaculture as a result of the limited attention given to this sector in Ethiopia (Biyela *et al*., 2004; Muhie, 2019). Hence, to safeguard consumers from the possible hazards associated with antibiotic-resistant strains

and to ensure the safety of the food supply chain, it is imperative to investigate the prevalence and AMR patterns of highly important bacteria such as *S. aureus* and *P. aeruginosa* in fish and aquatic environments. Information is also needed regarding fish handlers' knowledge and attitudes towards AMU and AMR, and their hygienic practices in fish production. This is important to understand the implications for environmental transmission of AMR and AMR determinants (Madara *et al*., 2022). However, very limited information regarding the knowledge and attitude of fish handlers towards AMU and AMR, and their hygienic practices is available (Bedane *et al*., 2022).

Therefore; the general objective of this study was to detect and determine the AMR pattern of *S. aureus* and *P. aeruginosa* in fish and to assess the knowledge, attitudes and hygienic practices of fish handlers towards AMU and AMR, and hygienic measures.

Specific objectives of this study were:

- To determine the prevalence of *S. aureus* and *P. aeruginosa* in fish, water, utensils and fish handlers.
- To determine the AMR profiles of *S. aureus* and *P. aeruginosa* isolates.
- To determine the MDR patterns and multiple antibiotic resistance indexes (MARI) of *S. aureus* and *P. aeruginosa* isolates.
- To detect *mecA* gene of methicillin-resistant *S. aureus* (MRSA).
- To assess the knowledge, attitude and practices (KAP) of fish handlers towards AMU, AMR, and hygienic measures.

2. LITERATURE REVIEW

2.1. Antimicrobials and Antimicrobial Resistance

2.1.1. Antimicrobial agents

Antimicrobials are drugs that can be natural, synthetic, or semisynthetic and they are specifically toxic to bacteria and other microorganisms. When these antimicrobials are used for preventing or treating bacterial infections, they are referred to as antibacterial or antibiotics. In the agriculture and livestock industries, they are used for various purposes such as prophylaxis, metaphylaxis, infection treatment, and growth promotion (Qiao *et al*., 2018). There are several groups of antibacterial agents, including β-lactams, aminoglycosides, fluoroquinolones, folate pathway antagonists, tetracyclines, phenicol, macrolides, glycopeptides, lincosamides, nitroimidazoles, polymyxins, and quinolones (Cháfer-Pericás *et al*., 2010).

Antibacterial agents are classified into groups based on how they work. They can stop the synthesis of the cell wall, disrupt the cell membrane, prevent protein synthesis, hinder nucleic acid synthesis, or interfere with metabolic pathways in bacteria (Reygaert, 2018). They can also be either bactericidal, causing cell death as by disrupting cell wall construction, or bacteriostatic, only inhibiting bacterial growth by disrupting protein and nucleic acid synthesis. Certain antibiotics, like glycopeptides, are effective against a limited range of bacteria, while others, like β-lactams, target common processes in different bacterial species (Kohanski *et al*., 2010; O'Connell *et al*., 2013). Sulfonamides and fluoroquinolones also have a broad range of action against both gram-positive and gram-negative organisms (Jiang *et al*., 2013).

2.1.2. Antimicrobial resistance

Antimicrobial resistance (AMR) poses a major threat to the progress made in medicine over the past century. The increasing prevalence of AMR in recent decades has raised concerns about effectively treating common infections, leading the World Health

Organization to refer to this as a 'post-antibiotic' era (Reardon, 2014). Recent estimates suggest that in 2019, approximately 3.57 million out of 4.95 million deaths worldwide were attributable to antimicrobial resistance (Murray *et al*., 2022), and there are projections that by 2050, AMR could be responsible for the deaths of 10 million people annually (Trotter *et al*., 2019). While the discovery of new classes of antibiotics in the past has helped alleviate the burden of infectious diseases, pathogenic bacteria have quickly developed resistance to these new compounds (Davies and Davies, 2010).

Antimicrobial resistant microorganisms are those that are no longer inhibited by an antimicrobial to which they were previously sensitive. This resistance is called acquired resistance and is encoded by resistance genes in the microbe's DNA. These genes can arise through spontaneous mutations or natural selection by natural antimicrobials in the environment (Holmes *et al*., 2016). The resistance genes can be transferred between bacteria through transformation, transduction, and conjugation processes (Vikesland *et al*., 2019). If a bacterium is resistant to a certain antimicrobial agent, all daughter cells will be resistant, unless additional mutations occur (Reygaert, 2018). On the other hand, inherent resistance occurs when a species naturally lacks susceptibility to a specific drug. This can be due to the inability of the antibacterial agent to enter the bacterial cell or a lack of affinity between the antibacterial agent and its target (Romero *et al*., 2012).

The ESKAPE pathogens, which include *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* spp., are a group of bacteria that have the ability to develop high levels of resistance. These pathogens are known for causing challenging infections that are difficult to treat, and in some cases, there is currently no available treatment options (Rice, 2008).

2.1.3. Mechanisms of antimicrobial resistance

Bacteria possess an astonishing genetic adaptability that enables them to respond to various environmental threats, such as the presence of antibiotic molecules that could potentially harm their survival. Bacteria employ numerous mechanisms to safeguard

themselves against antibiotics, and comprehending these mechanisms is crucial for resolving the crisis (Yalew, 2020). The mechanisms of drug resistance can be categorized into several broad groups, which include active efflux pumps, drug inactivation or alteration (β-lactams and chloramphenicol), modification of drug binding sites or targets (β-lactams and macrolides), changes in cell permeability leading to reduced intracellular drug accumulation (aminoglycosides), biofilm formation, and mutation (Wilson, 2014; Ali *et al.*, 2018).

Resistance to antimicrobial drugs can occur through various biochemical pathways, with gram-negative and gram-positive bacteria using different mechanisms. For instance, gram-negative bacteria primarily produce β-lactamases to resist β-lactams, while gram-positive bacteria primarily modify their penicillin-binding proteins (PBPs), resulting in resistance to antibiotics (Munita and Arias, 2016). This variation in resistance mechanism varies based on structural and other factors (Reygaert, 2018).

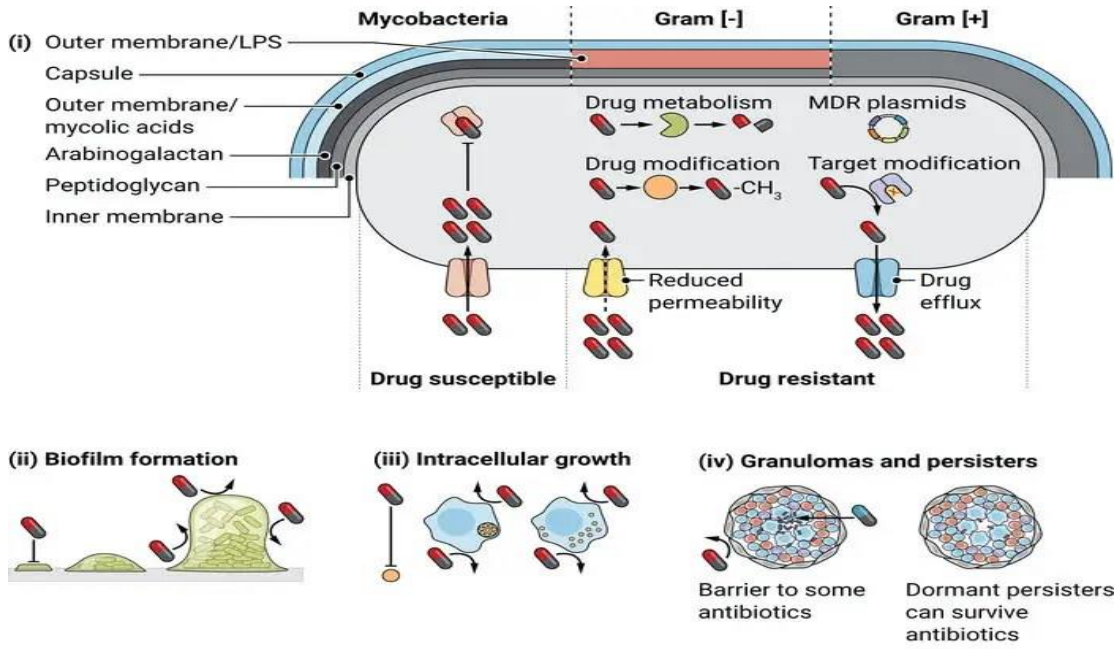

Figure 1: The mechanisms of antibiotic resistance in bacteria [Source: (Khanal, 2024)]

Table 1: Summary of antibiotic resistance mechanisms in *S. aureus* and *P. aeruginosa*

Antibiotic Class	***S. aureus***	***P. aeruginosa***
Penicillin's	Penicillinase, production of PBP2a	AmpC, ESBLs, other b-lactamases
Cephalosporins	PBP2a	AmpC, ESBLs
β-lactamase inhibitors	PBP2a	AmpC
Carbapenems	Development of PBP2a	Class B & D carbapenemases
Tetracyclines	Ribosomal methylation of binding sites, efflux pumps	Efflux pumps
Macrolides and clindamycin	Ribosomal methylation of binding sites, efflux pumps	Efflux pumps
Fluoroquinolones	Mutations in topoisomerase IV and DNA gyrase genes, efflux pumps	Mutations in topoisomerase IV and DNA gyrase genes, efflux pumps
Rifampicin	Mutations in RNA polymerase gene	Mutations in RNA polymerase gene
Trimethoprim-sulfamethoxazole	Mutations in DHPS and DHFR	Efflux pumps
Aminoglycosides	Aminoglycoside degradation enzymes	Aminoglycoside degradation enzymes, efflux pumps
Vancomycin	Altered structure of peptidoglycan precursors from D-Ala-D-Ala to D-Ala-D-Lac	

***Note**: PBP: penicillin binding protein; DNA: deoxyribonucleic acid; RNA: ribonucleic acid; DHPS: dihydropteroate synthase; DHFR: dihydrofolate reductase; ESBL: extended spectrum β-lactamase* [Source: (Kakoullis *et al*., 2021)].

2.1.4. Factors contributing to the emergence of antimicrobial resistance

Antimicrobial resistance (AMR) occurs naturally when microorganisms are exposed to antimicrobial drugs. Under the selective pressure of antibiotics, susceptible bacteria are killed or inhibited, while bacteria that are naturally (or intrinsically) resistant or that have acquired antibiotic-resistant traits have a greater chance to survive and multiply (Prestinaci *et al*., 2015). The misuse of antibiotics in the medical, veterinary and agricultural sectors, which include the inappropriate prescribing of antibiotics, their overuse in the livestock sector, and insufficient hygiene practices in hospital, all contribute to the rise of AMR (Yalew, 2020).

The use of antimicrobials in food-producing animals and aquaculture, both for promoting growth and treating disease, contributes to the issue of resistance (Marshall and Levy, 2011). Administering antibiotics to animals can lead to the emergence of resistant organisms, which can then be transmitted to humans. The resistance patterns seen in animals are a reflection of the types and quantities of antibiotics used (Landers *et al.*, 2012). The irrational use of antibiotics not only results in unwanted residues in animal products but also contributes to the development of AMR. In order to maintain effectiveness and minimize the risk of resistant bacteria, it is crucial to restrict the use of antibiotics (Beyene and Tesega, 2014). Although the use of antibiotics as growth promoters has been completely prohibited in Europe since 2006, it remains a common practice in several other countries (Sanders, 2005).

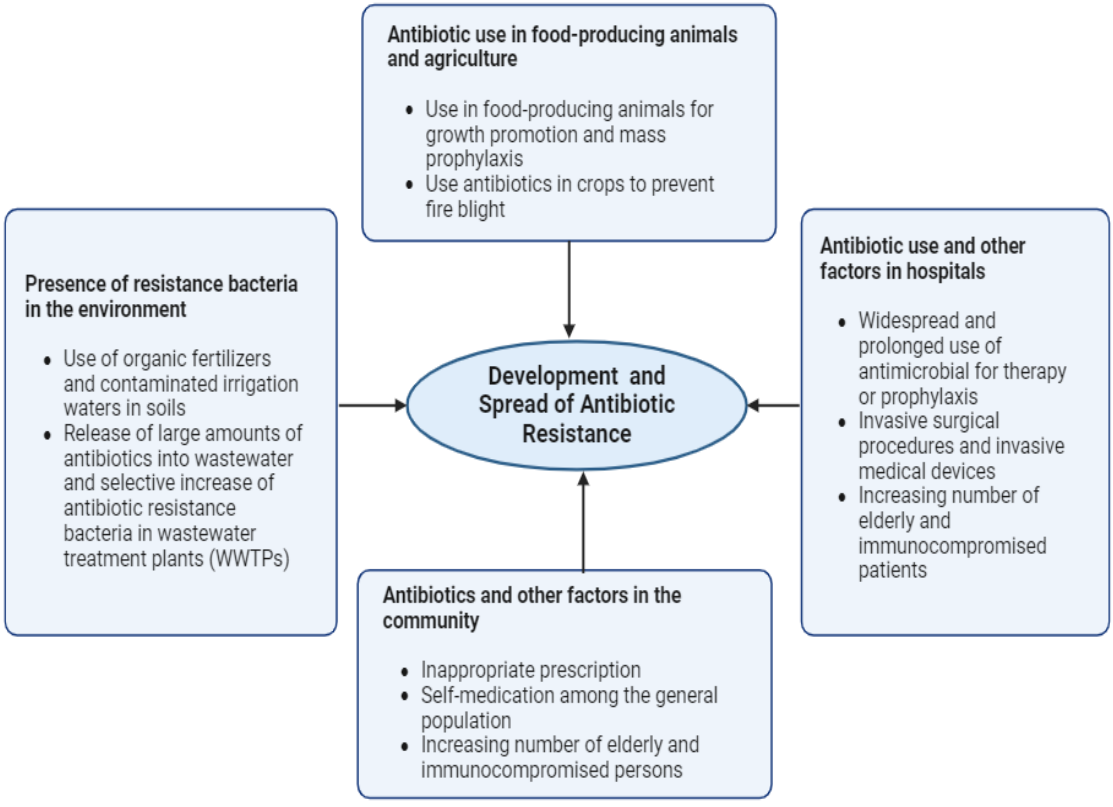

Figure 2: Factors involved in the spread of antibiotic resistance [Source: (Prestinaci *et al.*, 2015)]

2.1.5. Detecting methods of antimicrobial resistance

Agar disk-diffusion method

Agar disk-diffusion, also known as Kirby-Bauer antibiotic testing, is a method used in clinical microbiology laboratories for routine antimicrobial susceptibility testing. It was developed in 1940 (Balouiri *et al*., 2016). The process involves inoculating Mueller-Hinton agar plates with a standardized inoculum (1-2*10^8 CFU/mL) of the test microorganism. Filter paper discs containing the test compound are then placed on the agar surface. The antimicrobial agent diffuses into the agar, inhibiting germination and growth (Hudzicki, 2009). The diameters of the inhibition growth zones are measured with calipers or a ruler and interpreted based on clinical and laboratory standards institute (CLSI) guidelines as susceptible, intermediate, or resistant (Ruangpan and Tendencia, 2004). However, this method cannot determine the minimum inhibitory concentration (MIC) (Balouiri *et al*., 2016).

Dilution method

Agar dilution and broth dilution are commonly used methods to determine the minimal concentration of antimicrobial agents that kill or inhibit the growth of microorganisms (Balouiri *et al*., 2016). Agar dilution involves incorporating varying concentrations of the agent into an agar medium, followed by inoculating a defined microbial inoculum onto the agar plate surface. The MIC endpoint is the lowest concentration that completely inhibits growth under suitable incubation conditions (Jorgensen, 2010).

Broth micro-or macro-dilution is another basic antimicrobial susceptibility testing method. It involves preparing two-fold dilutions of the agent in liquid growth medium, inoculating each tube or well with a microbial inoculum, and incubating under optimum conditions for 16-24 hours (Wiegand *et al*., 2008; Balouiri *et al*., 2016). Antimicrobial effect can be determined using unaided eye or spectro-photometry (Weinstein and Lewis, 2020).

Molecular methods

Molecular analysis is commonly employed to investigate the presence of a gene when phenotypic results are inconclusive or unavailable. Traditional methods such as PCR and hybridization techniques have been utilized for many years, while more recent techniques like Whole-Genome Sequencing (WGS) and Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDITOF MS) are emerging (Anjum *et al*., 2017). PCR is extensively employed for the identification of microorganisms in various environments and the detection of resistance genes (Rohde *et al*., 2017). By utilizing multiplex reactions and real-time PCR (qPCR), PCR techniques can be optimized, enabling the identification and differentiation of multiple microorganisms in a single run, resulting in saving of cost and time (Pishnian *et al*., 2019).

2.2. Antimicrobial Use and Resistance in Fish Production

Antimicrobials are used in aquaculture during both production and processing to prevent and treat bacterial disease contamination (Ladan *et al*., 2021). Authorized antibiotics include oxytetracycline, florfenicol, sulphonamides, erythromycin, and sarafloxacin, while banned ones are chloramphenicol, enrofloxacin, spectinomycin, and rifampin (Serrano, 2005). Tetracyclines are the most used antimicrobial in aquaculture farms (Tuševljak *et al*., 2013), with global consumption of antimicrobial expected to reach 13,600 tonnes by 2030. However, patterns of antimicrobial use are not well documented, limiting antimicrobial stewardship interventions and policies (Schar *et al*., 2020).

Antimicrobial exposure in aquaculture can lead to drug resistance in fish and aquatic pathogens. However, responsible use of antibiotics is often lacking, and control measures have not adequately prevented risks to humans (Mensah *et al*., 2019). Antimicrobials are administered to entire populations through metaphylaxis, leading to bacterial resistance and antibiotic residue accumulation in aquaculture products (Chen *et al*., 2020). Broadcasted medicated feeds are the most common method, causing higher antibiotic doses in aquaculture compared to terrestrial animal farming. The exact levels are difficult to determine due to different distribution and registration systems (Romero *et al*., 2012).

Farm-raised freshwater fishes have been found to carry intestine-associated bacteria that exhibit antibiotic resistance against various drugs including ampicillin, oxytetracycline, amoxicillin and novobiocin (Hatha *et al*., 2005). The efflux pumps in gram-negative bacteria from farmed catfishes are responsible for most of these resistance (Sarter *et al*., 2007). Multiple antibiotic resistance (MAR) has also been observed in fishes with minimal therapeutic applications, including *Salmonella*, *Shigella*, *Pseudomonas*, and *E. coli* (Shah *et al*., 2012b). These MAR pathogens also found in freshwater fishes without antibiotic use (Esther *et al*., 2017), indicating that acquired resistance genes persist in cultured environments (Preena *et al*., 2020).

2.3. Antimicrobial Resistance of *S. aureus* and *P. aeruginosa* in Fish

2.3.1. Antimicrobial resistance of S. aureus in fish

S. aureus is not found in fish's normal microflora and its presence in fish products depends on harvest environment, sanitary conditions, and processing practices (Albuquerque *et al*., 2007; Mohammed *et al*., 2017). Different studies from different country reported the prevalence of *S. aureus* in fish and fishery products including 4.8% (Mitiku *et al*., 2023), 65% (Wendwesen *et al*., 2017) and 85% (Admasu *et al*., 2023) from Ethiopia. Reports from other countries ranges from 15.8% from Japan (Saito *et al*., 2011) to 62.7% from Jordan (Obaidat *et al*., 2015).

Since the 1940s, *S. aureus* has developed resistance mechanisms, becoming resistant to most antibiotics (Kakoullis *et al*., 2021). The widespread use of antibiotics has led to resistance to β-lactam antibiotics and at present, MRSA is a successful modern pathogen, through the acquisition of methicillin-resistant genes (*mecA* and *mecC*) (Wielders *et al*., 2002; Porrero *et al*., 2014). Treatment is difficult due to antibiotic resistance (Cheung *et al*., 2021) and vancomycin was once the gold standard drug, but resistance has limited its clinical utility (Gnanamani *et al*., 2017). There are reports on the resistance of *S. aureus* to different antimicrobials, its MDR profile, and occurrence of MRSA in fish and related samples from different countries (**Table 2**).

Table 2: Resistance profile of *S. aureus* isolated from fish and aquatic samples

Sample type	MDR% (MARI)	MRSA %	Resistance %	Country	Reference
Marine finfish	82 (>0.2)	16.3	Rifampicin and Clindamycin (82) Erythromycin and Ampicillin (67)	Eastern Cape Province, South Africa	Fri *et al.*, 2018; Fri *et al.*, 2020
Fresh fish	71.4 (0.36 to 0.64)		Rifampicin, Ampicillin/Cloxacillin and Meropenem (100) Cefpodoxime (85.7) Vancomycin and Ceftazidime (71.4)	Nairobi, Kenya	Mumbo *et al.*, 2023
Fresh fish from Egypt, India, and Yemen	24.6, 35.5, and 31.4	13.5	Penicillin (86.5) Ampicillin (82.7)	Jordan	Obaidat *et al*., 2015
Fresh fish, fish handlers, utensils and scavenging animals	50	6.6	Cefoxitin (100) Gentamicin (89.5) Ciprofloxacin (94.7) Oxacillin (76.3) Tetracycline (68.4)	Maidugu, Nigeria	Grema *et al.*, 2015
Fish and fish handlers	44		Ampicillin (100)	Brazil	Albuquerque *et al*., 2007
Water and fish	>90		Penicillin, Ampicillin and Flucloxacillin (100) Cefuroxime and Erythromycin (80) Tetracycline (86.1)	Ashanti, Ghana	Esther *et al.*, 2017
Fresh water fish			Ampicillin (91.3) Polymyxin (69.5)	Mosul, Iraq	Ali, 2014
Fishery products			Penicillin G, Chloramphenicol and Ciprofloxacin (100) Tetracycline (82)	Galicia, Spain	Vázquez-Sánchez *et al.*, 2012

***Note**: MDR: multidrug resistance; MARI: multiple antibiotic resistance index; MRSA: methicillin resistant S. aureus*

2.3.2. Antimicrobial resistance of P. aeruginosa in fish

P. aeruginosa is a common gram-negative bacterial pathogen that can survive in a variety of habitats in the environment. It is also a part of normal fish microbiota, but under stressful conditions, the bacteria may become pathogenic, which can lead to economic losses in fish farms (Ardura *et al*., 2013). A single report of 3.2% prevalnce from Lake Hawassa, Langanoo and Ziway, Ethiopia, was reported by Dissasa *et al*. (2022). A range of prevalence including 5% from Iran (Shahrokhi *et al*., 2022) to 100% from Egypt (Abd El-Aziz, 2015), were reported from different parts of the world.

P. aeruginosa is also a known multidrug-resistant gram-negative pathogen (Kakoullis *et al*., 2021). Unlike other bacteria, *P. aeruginosa* possesses unique intrinsic resistance mechanisms that simultaneously confer resistance to multiple antibiotics. Its restricted membrane permeability makes it highly resistant to a wide range of antibiotics, with estimates suggesting its membrane is 12 to 100 times less permeable than that of *E. coli*. Furthermore, *P. aeruginosa* employs efflux pumps and antibiotic-inactivating enzymes to further defend itself against antibiotics that manage to penetrate its cell (Kakoullis *et al*., 2021; Suresh *et al.*, 2023).

Tetracycline and sulphonamides resistance in aquatic-borne bacteria has emerged due to their use in aquaculture for both prophylactic and therapeutic purposes (Aminov, 2013) which is primarily due to extended spectrum beta-lactamases (ESBLs) (Algammal *et al*., 2020). There have been reports on the prevalence of MDR *P. aeruginosa* isolated from fishes and aquatic samples around the world which ranges from 20.0% in Côte d'Ivoire (Benie *et al*., 2017) to 75% in South Africa (Okafor and Nwodo, 2023) (**Table 3**).

Table 3: Resistance profile of *P. aeruginosa* isolated from fish and aquatic samples

Sample type	MDR (%)	MARI	Resistance (%)	Country	Reference
Freshwater fishes	50	0.4	Oxytetracycline (68) Co-trimoxazole (62.8) Ceftazidime (54.2) Cefotaxime (57.1) Ampicillin (48.5)	Andhra Pradesh, India	Suresh *et al.*, 2023
Freshwater fishes	55.5		Amoxicillin (83.3) Cefotaxime (77.7) Tetracycline (75.6) Gentamicin (67.6)	Ismailia Governorate, Egypt	Algammal *et al.*, 2020
Fresh fish and smoked fish	33.1 and 20.0		Kanamycin and Acid Aztreonam (100) Ticarcillin and Ticarcillin-Clavulanic (68.4)	Abidjan, Côte d'Ivoire	Benie *et al.*, 2017
Fresh fish	33.3	0.55 to 0.64	Penicillin, Vancomycin, Ceftazidime, Meropenem and Streptomycin (100)	Nairobi, Kenya	Mumbo *et al.*, 2023
Marine water fish		0.19 to 0.93	Erythromycin (100) Amoxicillin and Oxacillin (88.5) Ampicillin (65.4) Gentamicin (50)	Sharkia Governorate, Egypt	Darwish *et al.*, 2023
Effluent and surface water	55.6	0.38	Aztreonam (86.1) Ceftazidime (63.9) Piperacillin (58.3) Cefepime (55.6)	Mthatha, South Africa	Hosu *et al.*, 2020
Wastewater effluent	75	0.3 to 0.9	Amikacin (69) Ceftazidime (61)	Alice, South Africa	Okafor and Nwodo, 2023

***Note**: MDR: multidrug resistance; MARI: multiple antibiotic resistance index*

2.4. Fish Production and Hygienic Practice in Ethiopia

2.4.1. Fish production in Ethiopia

Fisheries are crucial for developing countries, providing renewable resources and livelihoods for rural communities. They help to reduce poverty and diversify household income (Olale and Henson, 2013). Fish accounts for 19% of total protein consumption,

with sub-Saharan Africa having the lowest consumption globally (Rediet *et al.*, 2022). Egypt and Nigeria are the most productive aquaculture producers in Africa, with Egypt producing nearly 14 million metric tonnes and Nigeria producing 263,000 metric tonnes (FAO, 2000; Kaleem and Sabi, 2021).

Ethiopia's inland waters offer abundant fish stocks, providing a cheap source of animal protein. Major lakes and rivers, including Tana, Ziway, Hawassa, Chamo, Abaya, Koka, Fincha, and various rivers, supply fish dominated by Nile tilapia, African catfish, and *Barbus* species. This fish production supports poor farmer's livelihoods by providing affordable, high-quality protein and diversifying income sources. Fish production in the country is primarily sourced from fishery cooperatives, street traders, fish stores and restaurants (Abdulhakim and Alemayehu, 2020).

Ethiopia's fisheries production is underutilized due to limited access and supply to fish and fishery products, while increasing food demand (Rediet *et al*., 2022). Fish demand is expected to increase from over 67 thousand tonnes in 2003 to nearly 95 thousand tonnes in 2015 and 118 thousand tonnes in 2025 (Janko, 2013). According to Tesfaye and Wolff (2014), the nation's potential for fish production is estimated to be 94,541 tonnes per year for its major bodies of water. Challenges include infectious and non-infectious diseases, post-harvest losses, overfishing, poor infrastructure, marketing constraints, urbanization, agricultural expansion, wetland degradation, climate change, and water hyacinth. Addressing these issues is crucial for overall fish development and production (Abdulhakim and Alemayehu, 2020).

2.4.2 Hygienic practice in fish production

Fish is a highly nutritious food with high protein, vitamins, and minerals, vital for the economy and food security. However, concerns about fish safety arise due to potential bacteria and parasites (Sorsa *et al*., 2019). Contaminated fish can result from aquatic ecosystem pollution and unhygienic handling practices. Consuming raw or undercooked fish increases the risk of fish-borne infections in humans with potential human pathogens (Havelaar *et al*., 2015). In Ethiopia, there have been reports of fish contamination with

foodborne pathogens, but limited information exists on hygienic practices (Bedane *et al*., 2022).

Fishery and aquaculture involve the production and supply chain of fish from water bodies like ponds, lakes, and oceans. The chain includes fishermen, retailers, distributors, transporters, storage facilities, and suppliers (Sengupta *et al*., 2022). Microbial contamination can occur at any stage of the supply chain, including collection, processing, distribution, storage, marketing, and preparation when proper hygienic handling practices are not followed (Bedane *et al*., 2022). Bedane *et al*. (2022) reported a wide range of unsanitary handling practices, such as washing fillets in contaminated lake water, processing multiple fish with a single knife and cutting board without disinfecting, and using unhygienic containers and packaging materials in the fish supply chain which may contribute to contamination.

Proper personal hygiene and knowledge of local food safety and quality laws are crucial for the fishing industry, as they reduce the risk of disease-causing bacteria being transferred from fish handlers to fish (Ward and Beyens, 2012). Increased fish production also leads to increased waste production, including particulate organic and soluble-inorganic excretory waste including fish offal, feed, and chemicals (Pędziwiatr *et al*., 2017; Dauda *et al*., 2019). Governments are enforced to implement waste management programs to address this issue. In Ethiopia, fishery management legislation, proclamation No.315/2003, provides guidelines for resource conservation, food safety, and aquaculture (Janko, 2014).

3. MATERIALS AND METHODS

3.1. Description of Study Area and Study Design

A cross-sectional study was conducted in Bishoftu town, located in the east Shewa zone of Oromia, Ethiopia, from November 2023 to May 2024. Bishoftu is approximately 45km southeast of Addis Ababa, situated in the central highlands. The town has an altitude of 1,900m above sea level and receives an average annual rainfall of 686.9mm. The average temperature in Bishoftu is 18.9°C, with a relative humidity of 60.0% (Ebsa *et al*., 2019). Most of the fish population in Ethiopia is found in the rift-valley lakes, including the crater Lakes of Babogaya, Bishoftu and Hora-Arsedi and shallow Lake Cheleklaka in Bishoftu town. Three fish species, namely *Oreochromis niloticus* (Nile tilapia), *Clarias gariepinus* (African catfish) and *Tilapia zilli* were found and the most dominant species in the study area was *Oreochromis niloticus* (Bedane *et al*., 2022).

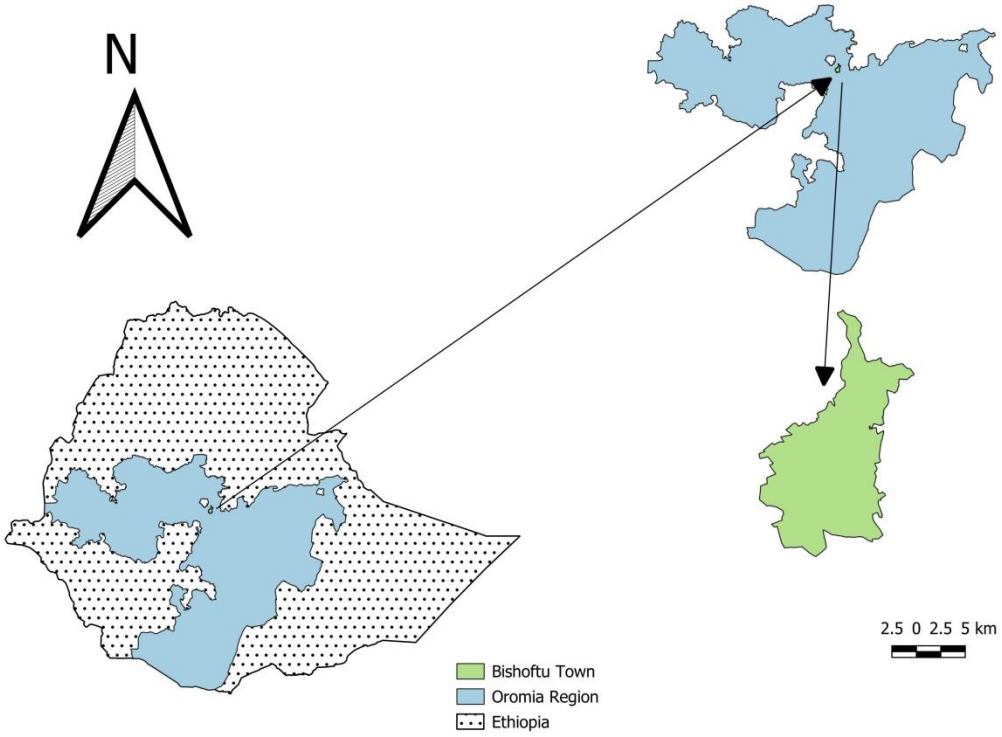

Figure 3: Map of study area; Bishoftu town, Oromia region, Ethiopia.

3.2. Sample Size Determination

The desired sample size for the study was calculated using the formula given by Thrusfield *et al*. (2018) using 3.2% expected prevalence of *P. aeruginosa* from previous study done in three Ethiopian rift valley lakes (Dissasa *et al*., 2022).

$$n = (1.96)^2 \times Pexp\ (1\text{-}Pexp)/\ d^2$$

Where; n = required sample size; Pexp = expected prevalence; 3.2% = 0.032, d = desired absolute precision with 95% confidence interval and 5% absolute precision. Accordingly, the calculated sample size was 48; but 105 samples were included in the study to increase level of precision.

A total of 53 fish handlers, regardless of their educational status and gender, who work on the various lakes and ponds of Bishoftu, were included as information sources for the questionnaire survey.

3.3. Sampling and Sample Type

Samples were collected from fish flesh that had been filleted at the landing sites of various lakes and ponds in Bishoftu, using a purposive sampling technique with sterile swabs. Swab samples were also taken from operator's hand, filleting knife, and filleting table. The swab samples were putted inside a sterile test tube containing 5 mL of peptone water (Himedia, M028). Environmental samples comprised of 25 ml water from different landing sites of the lakes and ponds using sterile universal bottle. The samples were capped, labeled, and stored in an ice-packed cool box. Then, the samples were transported to the National Veterinary Institute (NVI) bacteriology laboratory to be examined for presence of *S. aureus* and *P. aeruginosa*.

3.4. Isolation and Identification of *S. aureus* and *P. aeruginosa*

Cotton swab samples in peptone water (Himedia, M028) were incubated immediately for 24 hours at 37°C. For isolation of *S. aureus* and *P. aeruginosa*, a loopful colony from incubated broth were streaked on mannitol salt agar (Himedia, SMH118D) and cetrimide

agar (Himedia, MH024) respectively, incubated at 37°C for 24 hours. Bacterial colonies with typical *S. aureus* (i.e., colonies with golden yellow pigmentation on mannitol salt agar) and *P. aeruginosa* (i.e., smooth round colonies with a yellowish-green fluorescent pigment on cetrimide agar) characteristics were sub-cultured on tryptic soya agar (Himedia, M1968) for 24 hours at 37°C for further characterization by gram stain and biochemical identification (Garcia, 2016).

All suspected colonies were harvested and purified for phenotypic and biochemical characteristics. Briefly, all isolates were identified morphologically using gram stain and biochemically using various biochemical tests. Bacterial isolates with gram positive cocci grape like morphology, catalase positive, oxidase negative and tube coagulase positive were suspected as *S. aureus* (Ezzeldeen *et al*., 2011). On the other hand isolates which were gram negative short rods, catalase positive, oxidase positive, motile, citrate positive, triple sugar iron negative and MR-VP negative were suspected as *P. aeruginosa* (Algammal *et al*., 2020). The suspected bacterial isolates were stored for subsequent molecular confirmation and antimicrobial susceptibility test using tryptone soya broth (Himedia, LQ508) with 30% glycerol at -20°C.

3.5. Molecular Confirmation of *S. aureus* and *P. aeruginosa*

The DNA extraction was carried out using a Qiagen extraction kit, in accordance with the instructions provided by the manufacturer. To start, a bacterial suspension of 200µl was combined with 20µl of proteinase K and 200µl of AL buffer. The mixture was then vortexed for 15 seconds and incubated at 56˚C for 10 minutes. Next, 200µl of 96% ethanol was added and vortexed. Afterwards, 620µl of the suspension was transferred to a mini spin column and centrifuged at 8,000 rpm for 1 minute. The flow through was discarded, and 500µl of AW1 buffer was added and centrifuged at 8,000 rpm for 1 minute. The flow through was again discarded, and 500µl of AW2 wash buffer was added and centrifuged at 14,000 rpm for 3 minutes. The mini spin column was then placed in a 2 ml collection tube and centrifuged for an additional 1 minute at 14,000 rpm to dry the column matrix. Finally, the mini spin column was placed in labeled eppendorf

tubes and the DNA was eluted by adding 50µl of elution buffer and centrifuging at 8,000 rpm for 1 minute. The eluted DNA was stored at -20˚C until it was ready to be used for polymerase chain reaction (PCR).

For the molecular confirmation of *S. aureus nuc* gene was amplified using a forward primer: *NF1* (5'-CCT GAA GCA AGT GCA TTT ACG A-'3) and a reverse primer: *NR2* (5'-CTT TAG CCA AGC CTT GAC GAA CT-'3). The PCR reaction mixture used was 20µl which contains 10µl IQ super mix, 2µl of each primer, 2µl DNA template and 4µl of nuclease free water. The PCR conditions consisted of initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute. This was followed by final extension at 72°C for 7 minutes.

For the molecular confirmation of *P. aeruginosa oprL* gene was amplified using a forward primer: *oprLF* (5'-ATG GAA ATG CTG AAA TTC GGC-'3) and a reverse primer: *oprLR* (5'-CTT CTT CAG CTC GAC GCG ACG-'3). The PCR reaction mixture used was 20µl which contains 10µl IQ super mix, 2µl of each primer, 2µl DNA template and 4µl of nuclease free water. The PCR conditions consisted of initial denaturation at 95°C for 3 minutes, followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 57°C for 1 minute, and extension at 72°C for 1 minute. This was followed by final extension at 72°C for 5 minutes.

PCR products were resolved using 2% agarose gel. First, 4µl of gel red with loading dye was added to the PCR product then 10µl of it was loaded to the wells of the agarose gel and 10µl of DNA ladder, 50bp was used as a standard. Running of electrophoresis for 1 hour at 120V was done and the result was viewed using UV-light. The primer of *nuc* gene gave a PCR product equal to 166bp and *oprL* 365bp. Confirmed *S. aureus* and *P. aeruginosa* colonies were stored in tryptone soya broth with 30% glycerol at -20°C for antibiotic susceptibility testing and other subsequent tests.

American-type culture collection (ATCC) strains; *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853 were used as a quality control strains.

3.6. Antibiotic Susceptibility Test

The Kirby-Bauer standardized disc diffusion method was used to perform an antibiotic susceptibility test on Muller-Hinton agar (Himedia, M173). The turbidity of the colonies was adjusted to 0.5 McFarland standards using a DEN-1 McFarland densitometer (Biosan). The cultures were then swabbed onto Mueller-Hinton agar, and antimicrobial coated disks were placed on the agar surface using an antimicrobial disc dispenser (Oxoid™, ST6090). The plates were incubated aerobically at 37°C for 24 hours. The diameters of the zones of inhibition were measured in millimeters using a digital caliper (ExGizmo, 12 Inch-300mm) and rounded to whole numbers. The interpretation of the zones of growth inhibition was based on the recommendation of CLSI (CLSI, 2023).

Isolates that show resistance to three or more antimicrobial classes were classified as multiple drug resistance (MDR) bacteria (Magiorakos *et al*., 2012). The MARI (multiple antibiotic resistance index) value for each isolate was calculated using the formula a/b, where 'a' represents the number of antibiotics that the isolate was resistant to and 'b' represents the total number of antibiotics tested. The MARI indexing was also used to evaluate different locations and sample types, using the formula a/(b × c), where 'a' was the cumulative antibiotic resistance score of all isolates from the sample source/type, 'b' was the total number of antibiotics, and 'c' was the number of isolates from the sample source/type (Who *et al*., 2023). Samples with MARI values above 0.2 indicate high antimicrobial overuse (Davis & Brown, 2016).

The antimicrobial agents used for *S. aureus* included cefuroxime, ciprofloxacin, cloxacillin, doxycycline, erythromycin, gentamicin, lincomycin, norfloxacin, penicillin G, tetracycline, trimethoprim/sulfamethoxazole and vancomycin. For *P. aeruginosa*, the antimicrobial agents used were cefuroxime, ciprofloxacin, doxycycline, erythromycin, florfenicol, gentamicin, kanamycin, nalidixic acid, norfloxacin, streptomycin, tetracycline and trimethoprim/sulfamethoxazole (**Appendix II**).

3.7. Molecular Detection of *mecA* gene

To detect the MRSA *mecA* gene, we amplified it using the forward primer *mecAF1* (5'-GTA GAA ATG ACT GAA CGT CCG ATA A-'3) and the reverse primer *mecAR2* (5'-CCA ATT CCA CAT TGT TTC GGT CTA A-'3). The PCR reaction mixture consisted of 20µl, which included 10µl of IQ super mix, 2µl of each primer, 3µl of DNA template, and 3µl of nuclease-free water. The PCR conditions involved an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds, and extension at 72°C for 30 seconds. This was followed by a final extension at 72°C for 7 minutes. We then performed agarose gel (2%) electrophoresis using the PCR products, expecting a size of 533bp for the *mecA* primer.

3.8. Questionnaire Design and Data Collection

The fish handler's knowledge, attitude, and practices regarding AMU, AMR and hygiene were assessed using a structured questionnaire. This questionnaire was developed based on previous literatures and expert opinions (Alhaji *et al*., 2021; Gebeyehu *et al*., 2021; Tufa *et al*., 2023). The questionnaire had four sections and 25 close-ended questions. The first section was about the respondents' socio-demographic characteristics such as age, sex and educational level which contain 5 questions. The second section was focused on the knowledge of respondents towards AMU and AMR which contains 8 questions with yes, no or I don't know answers. The third section of the questionnaire had 6 questions with Likert scale answers ranging from "strongly agree" to "strongly disagree focused on the attitude of respondents towards AMU and AMR. The last section was targeted about the hygienic practice of the fish handlers. This part had 6 questions with yes or no answers (**Appendix IV**).

In addition to the questionnaire, direct observation was conducted to evaluate the hygienic status and practices of fish handlers working in fish processing and preparation across different lakes and ponds. The questionnaire was initially prepared in English and then translated into two commonly spoken local languages (Afan Oromo and Amharic)

for use as needed during data collection. Face-to-face interviews were conducted to administer the questionnaire, and the responses were documented for further analysis.

3.9. Data Management and Statistical Analysis

Isolation frequencies of *S. aureus* and *P. aeruginosa*, the antibiotic resistance pattern of isolates as well as data acquired from the interviews were entered into Microsoft Excel version 2010 for the purpose of cleaning, processing, and conducting further analysis. The frequencies and percentages were calculated by descriptive statistics. The Chi-square test was used to test the association of sample origin and sample type with the frequencies of *S. aureus* and *P. aeruginosa* occurrence.

The responses provided by key individuals in the KAP study were scored and categorized based on a modified Bloom's cut-off score. Scores ranging from 80 to 100% were classified as sufficient, desirable, or good, scores between 50 and 79% were considered moderate, neutral or fair, and scores below 50% were categorized as insufficient, undesirable, or poor ((Bloom *et al.*, 1956; Tufa *et al*., 2023). Each correct answer was awarded one mark, while incorrect or "I do not know" responses received zero marks on the knowledge assessment. The attitude response assessment of the individual was scored using three marks for a "strongly agree/agree" response, two marks for a "neutral" response, and one mark for a "disagree/strongly disagree" response. On the hygienic practice assessment, each "yes" response was given one mark, while "no" responses were given zero marks.

The knowledge score ranged from 0 to 8 points and was categorized as sufficient, moderate or insufficient based on the score. A score of ≥7 points was considered sufficient, 4 to 6 points as moderate, and <4 points as insufficient. The attitude score towards AMU and AMR ranged from 6 to 18 and was graded as desirable, neutral, or undesirable. A score of ≥15 points was desirable, 9 to 14 points was neutral, and <9 points was undesirable. Similarly, the overall hygienic practice score ranged from 0 to 6 and was categorized as good, fair, or poor. A score of ≥5 points was good, 3 or 4 points was fair, and <3 points was poor. The association between the dependent variables

(knowledge, attitude, and practice) and each predictive variable was determined using the likelihood ratio test (LR test) and p-values.

To examine the relationship between demographic variables and KAP scores, we used binary logistic regression. The odds ratio was used to measure the impact of demographic variables. In the analysis, we dichotomized the final KAP scores. Respondents who answered at least 50% of the questions correctly were considered to have sufficient knowledge, desirable attitudes, and good hygienic practices. Conversely, respondents who answered less than 50% of the questions correctly were considered to have insufficient knowledge, undesirable attitudes, and poor hygienic practices (Gebeyehu *et al*., 2021; Tufa *et al*., 2023).

Additionally, we employed Spearman's rank-order correlation coefficient to determine the relationship and direction of the association between the KAP scores of the respondents. Statistical significance was accepted at $p<0.05$. For statistical analysis STATA version 14.2 was used.

3.10. Ethical Approval and Consideration

Ethics approval for the study was obtained from the Institutional Review Board of Addis Ababa University College of Veterinary Medicine and Agriculture (Reference number: VM/ERC/04/21/16/2024) (**Appendix VI**). All participants in the study provided written informed consent, and their names and personal details were kept confidential (**Appendix III**).

4. RESULTS

4.1. Prevalence of *S. aureus* and *P. aeruginosa*

Of the 105 different sample types analyzed, the overall prevalence of *S. aureus* was 8.6% (9/105). Of which, 4 isolates were from Lake Hora-Arsedi, 2 from Lake Cheleklaka and 2 from ponds (**Table 4**). The prevalence was highest in hand swab (20%; 1/5), followed by knife (12.5%; 1/8), and fish (10.8%; 7/65). These differences in prevalence among the sample origin and sample types, however, were not statistically significant ($p>0.05$).

With regard to *P. aeruginosa* the overall prevalence was 7.6% (8/105). Of which, 3 isolates were from Hora-Arsedi, 2 from Lake Bishoftu, 2 from pond and 1 isolate from Lake Cheleklaka. The prevalence was highest in hand swab (20%; 1/5), followed by knife (12.5%; 1/8), and water (8%; 2/25). The same to *S. aureus*, there was no significance difference in the prevalence of *P. aeruginosa* among study samples in relation to sample origin and sample type ($p>0.05$). There were no isolation of both bacteria from Lake Babogaya and samples taken from filleting table (**Table 4**).

Table 4: Prevalence of *S. aureus* and *P. aeruginosa* in the study area

Category		**No. of samples**	***S. aureus***			***P. aeruginosa***		
			Number (%)	X^2	p-value	Number (%)	X^2	p-value
Sample origin	Bishoftu	22	1 (4.5)	3.5527	0.470	2 (9.1)	2.0241	0.731
	Hora-Arsedi	38	4 (10.5)			3 (7.9)		
	Cheleklaka	12	2 (16.7)			1 (8.3)		
	Babogaya	17	0 (0)			0 (0)		
	Pond	16	2 (12.5)			2 (12.5)		
Sample type	Fish	65	7 (10.8)	3.9228	0.417	4 (6.2)	1.7281	0.786
	Water	25	0 (0)			2 (8)		
	Hand	5	1 (20)			1 (20)		
	Knife	8	1 (12.5)			1 (12.5)		
	Table	2	0 (0)			0 (0)		
Total		**105**	**9 (8.6)**			**8 (7.6)**		

***Note**: X^2: Chi-square*

In this study, *S. aureus* and *P. aeruginosa* isolates were further examined to determine the presence of specific gene, *nuc* and *oprL* respectively, using PCR. The nine isolates of *S. aureus* were confirmed to carry the *nuc* gene, with amplification products observed at approximately 166bp (**Figure 4**). On the other hand, the eight isolates of *P. aeruginosa* were confirmed to carry the *oprL* gene, with amplification product of 365bp (**Figure 5**).

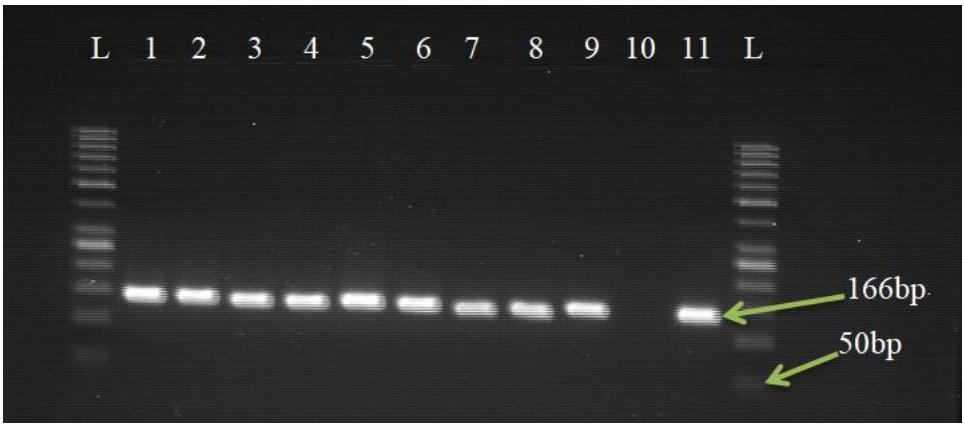

Figure 4: Agarose gel electrophoresis for the detection of the *nuc* gene (166bp) in *S. aureus* isolates. *Lane L: 50bp DNA ladder; Lane 1-9: nuc gene of S. aureus samples at 166bp product size; Lane 10: negative control; Lane 11: positive control for S. aureus.*

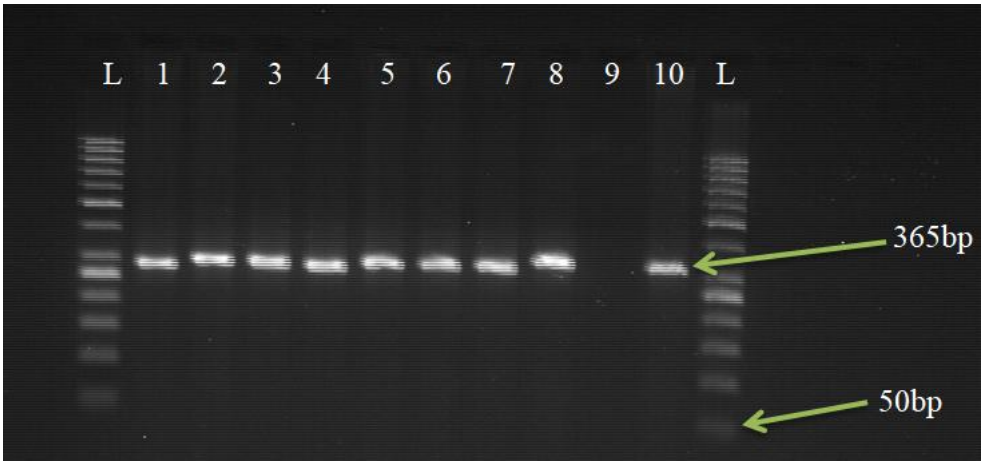

Figure 5: Agarose gel electrophoresis for the detection of the *oprL* gene (365bp) in *P. aeruginosa* isolates. *Lane L: 50bp DNA ladder; Lane 1-8: oprL gene of P. aeruginosa samples at 365bp product size; Lane 9: negative control; Lane 10: positive control for P. aeruginosa*.

4.2. Antimicrobial Resistance Profiles

All nine *S. aureus* isolates were tested for susceptibility against twelve different antimicrobials. The *S. aureus* isolates demonstrated varied levels of phenotypic resistance rates which range from 0% to 100%. The highest resistance was observed against cefuroxime 100%, followed by 44.4% (4/9) resistance to cloxacillin and penicillin G, and 33.3% (3/9) to tetracycline. Intermediate resistance rates were also recorded, ranging from 44.4% (4/9) for erythromycin and tetracycline to 11.1% (1/9) for cloxacillin and trimethoprim/sulfamethoxazole. None of the isolates showed resistance to ciprofloxacin and norfloxacin as seen in Table 5 below.

Table 5: Antimicrobial resistance profiles of *S. aureus* isolates

Antimicrobial agent	Susceptible (%)	Intermediate (%)	Resistant (%)
Ciprofloxacin	9 (100)	0 (0)	0 (0)
Norfloxacin	9 (100)	0 (0)	0 (0)
SXT	8 (88.9)	1 (11.1)	0 (0)
Gentamicin	7 (77.8)	2 (22.2)	0 (0)
Doxycycline	6 (66.7)	2 (22.2)	1 (11.1)
Lincomycin	6 (66.7)	3 (33.3)	0 (0)
Vancomycin	6 (66.7)	2 (22.2)	1 (11.1)
Erythromycin	5 (55.6)	4 (44.4)	0 (0)
Penicillin G	5 (55.6)	0 (0)	4 (44.4)
Cloxacillin	4 (44.4)	1 (11.1)	4 (44.4)
Tetracycline	2 (22.2)	4 (44.4)	3 (33.3)
Cefuroxime	0 (0)	0 (0)	9 (100)

***Note**: SXT: trimethoprim/sulfamethoxazole*

The same to *S. aureus* isolates the eight *P. aeruginosa* isolates were tested for susceptibility to twelve different antimicrobials. All of the eight isolates were 100% resistance for eight numbers of antimicrobials including kanamycin, tetracycline, doxycycline, erythromycin and florfenicol. Intermediate resistance of 12.5% (1/8) was showed for gentamicin and streptomycin. The highest susceptibility rates were recorded for ciprofloxacin and norfloxacin (75%; 6/8), followed by gentamicin (62.5%; 5/8) (**Table 6**).

Table 6: Antimicrobial resistance profiles of *P. aeruginosa* isolates

Antimicrobial agent	Susceptible (%)	Intermediate (%)	Resistance (%)
Ciprofloxacin	6 (75)	0 (0)	2 (25)
Norfloxacin	6 (75)	0 (0)	2 (25)
Gentamicin	5 (62.5)	1 (12.5)	2 (25)
Streptomycin	1 (12.5)	1 (12.5)	6 (75)
Cefuroxime	0 (0)	0 (0)	8 (100)
Doxycycline	0 (0)	0 (0)	8 (100)
Erythromycin	0 (0)	0 (0)	8 (100)
Florfenicol	0 (0)	0 (0)	8 (100)
Kanamycin	0 (0)	0 (0)	8 (100)
Nalidixic acid	0 (0)	0 (0)	8 (100)
SXT	0 (0)	0 (0)	8 (100)
Tetracycline	0 (0)	0 (0)	8 (100)

***Note**: SXT: trimethoprim/sulfamethoxazole*

4.3. Multidrug Resistance Patterns

From the nine *S. aureus* isolates multidrug resistance (MDR) was shown by 3 (33.3%) of the isolates with resistance for three antibiotic classes whereas 6 (66.7%) of the isolates were not MDR. The MDR isolates were resistant to three, four and five number of antibiotics with 0.25, 0.33 and 0.42 MARI value, respectively (**Table 7**).

Table 7: Multidrug resistance patterns of *S. aureus* isolates

No. of AM class	Resistance pattern	No. of isolate	Percent	MARI	Resistance classification
1	CXM (2)	2	22.2	0.08	NMDR
2	CXM, CX (1)	4	44.4	0.17	NMDR
	CXM, P (2)			0.17	
	CXM, TE (1)			0.17	
3	CXM, CX, VA (1)	3	33.3	0.25	MDR
	CXM, CX, P, TE (1)			0.33	
	CXM, CX, DO, P, TE (1)			0.42	
Total		**9**	**100**		

***Note**: AM: antimicrobial; MDR: multidrug resistance; NMDR: not-MDR; MARI: multiple antibiotic resistance index; CXM: cefuroxime; CX: cloxacillin; P: penicillin G; TE: tetracycline and DO: doxycycline.*

With regard to *P. aeruginosa* all of the eight isolates were MDR with resistance to seven (62.5%; 5/8) or eight (37.5%; 3/8) antimicrobial classes. Two of them were resistance to ten numbers of antibiotics with MARI value of 0.83 and two for elven number of antibiotics with MARI value of 0.92 (**Table 8**).

Table 8: Multidrug resistance patterns of *P. aeruginosa* isolates

No. of AM class	Resistance pattern	No. of isolate	Percent	MARI	Resistance classification
7	CXM, DO, E, FFC, K, NA, SXT, TE (2)	5	62.5	0.67	MDR
	CXM, DO, E, FFC, K, NA, S, SXT, TE (2)			0.75	
	CN, CXM, DO, E, FFC, K, NA, S, SXT, TE (1)			0.83	
8	CXM, DO, E, FFC, K, NA, NOR, S, SXT, TE (1)	3	37.5	0.83	MDR
	CIP, CXM, DO, E, FFC, K, NA, NOR, S, SXT, TE (1)			0.92	
	CN, CIP, CXM, DO, E, FFC, K, NA, S, SXT, TE (1)			0.92	
Total		**8**	**100**		

***Note**: AM: antimicrobial; MDR: multidrug resistance; MARI: multiple antibiotic resistance index; CXM: cefuroxime; TE: tetracycline; DO: doxycycline; CN: gentamicin; NOR: norfloxacin; CIP: ciprofloxacin; K: kanamycin; S: streptomycin; E: erythromycin; FFC: florfenicol; NA: naldixic acid and SXT: trimethoprim/sulfamethoxazole*

The three MDR *S. aureus* isolates were obtained from Lake Bishoftu, Hora-Arsedi, and pond. Each location contributed one isolate (33.3%) to the overall *S. aureus* MDR isolates. In terms of sample type, two of the MDR *S. aureus* isolates were derived from fish (28.6% MDR; 2/7), while one isolate was obtained from a knife swab (100% MDR; 1/1). The fish isolates accounted for 66.7% (2/3) of the total MDR isolates, while the knife swab isolate accounted for 33.3% (1/3). It is important to note that the *S. aureus* isolates from Lake Cheleklaka and the hand swabs of fish handlers did not exhibit any MDR characteristics (**Figure 6**).

In the case of *P. aeruginosa*, all of the isolates from various origins and sample types were found to be MDR. Specifically, 37.5% (3/8) of these isolates were from Lake Hora-Arsedi, while both Lake Bishoftu and pond each accounted for 25% (2/8) of the MDR isolates. In terms of sample type, fish and water samples contributed 50% (4/8) and 25% (2/8) respectively to the overall MDR isolates of *P. aeruginosa*. Additionally, isolates from fish handlers hand and knife swabs each contributed 12.5% (1/8) to the total MDR *P. aeruginosa* isolates (**Figure 6**).

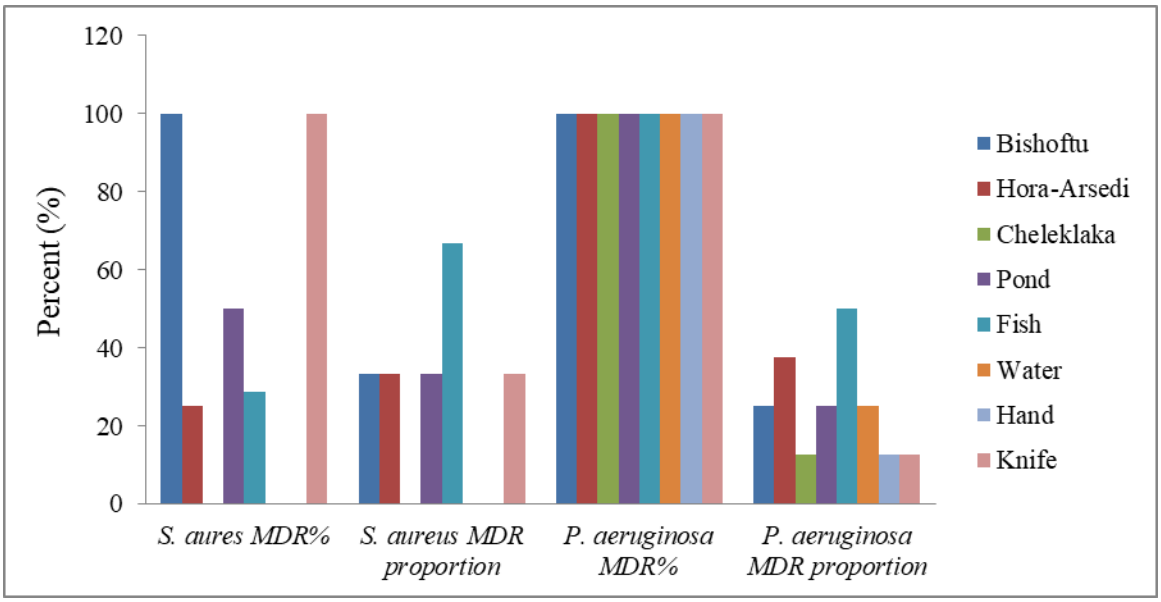

Figure 6: Multidrug resistance of *S. aureus* and *P. aeruginosa* isolates in relation to sample origin and type. *MDR%: multidrug resistance percentage from the total isolates; MDR proportion: percentage of multidrug resistance isolates from the total multidrug isolates.*

MARI value of 0.33 and 0.29 were the highest two values recorded from Lake Bishoftu and ponds, respectively by *S. aureus* isolates. In the case of sample type the highest MARI value for *S. aureus* isolates was 0.25 from knife swab samples. In relation to sample origin the highest MARI for *P. aeruginosa* was from pond (0.87) followed by Lake Cheleklaka (0.83). Hand and knife swab sample types were also having high MARI value of 0.92 and 0.83, respectively (**Figure 7**).

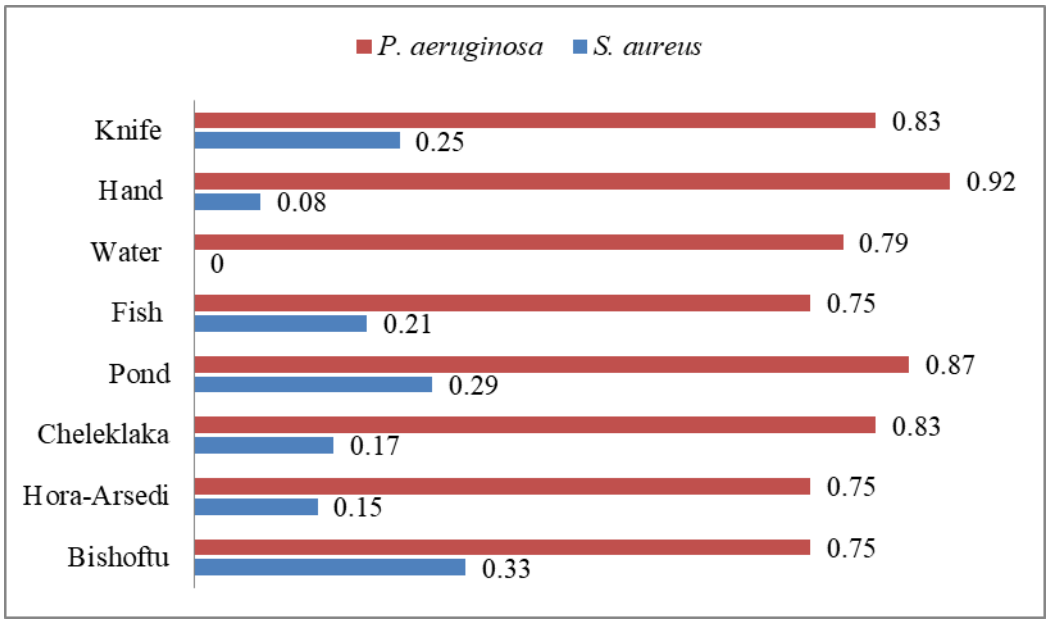

Figure 7: Multiple antibiotic resistance indexes (MARI) of *S. aureus* and *P. aeruginosa* isolates in relation to sample origin and type.

4.4. Molecular Detection of *mecA* gene

In this study, molecular detection of *mecA* gene was done for the *S. aureus* isolates using conventional PCR. However, all of them were negative for it and MRSA were not confirmed.

4.5. Knowledge, Attitude and Hygienic Practices of Fish Handlers

4.5.1. Demographic information of respondents

A total of 53 fish handlers were interviewed. Among them, 21 were from Lake Hora-Arsedi, 11 were from Lake Bishoftu, and 6 were involved in fish production using ponds. Most of the participants were male (44), and 25 of them were aged between 30 and 40 years. Furthermore, 23 of the interviewees had work experience ranging from 3 to 10 years and had completed secondary school education (**Figure 8**).

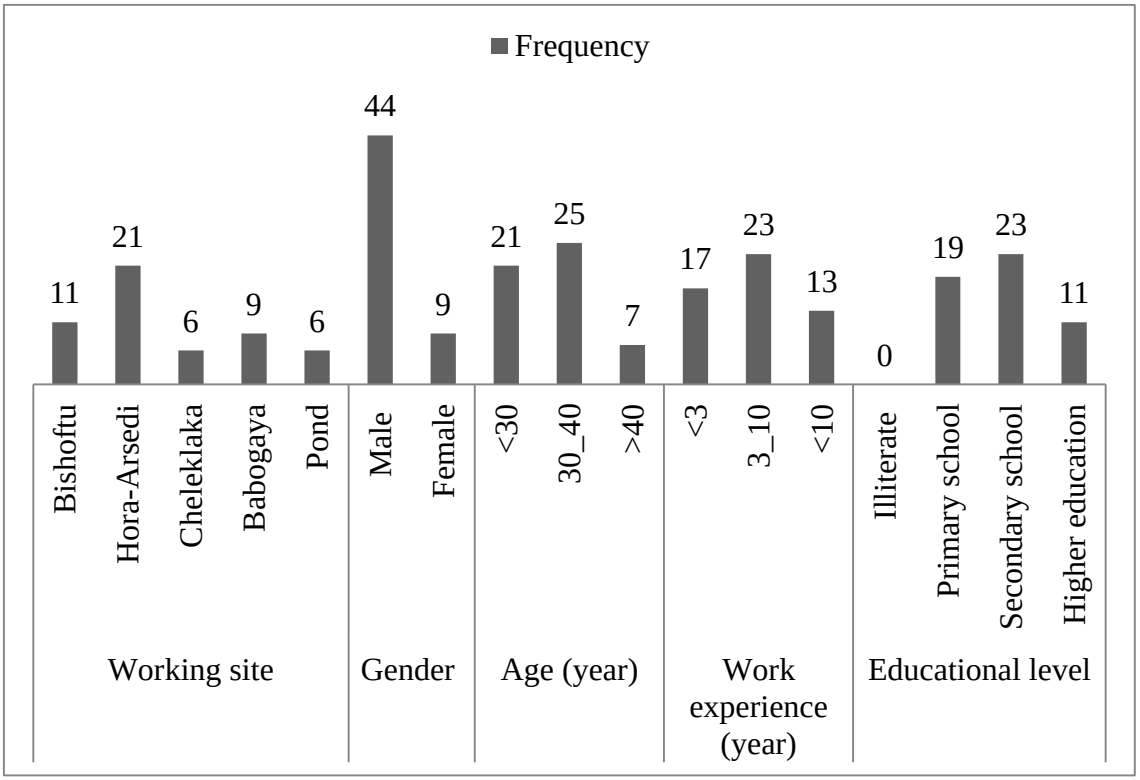

Figure 8: Demographic information of fish handlers in the study area.

4.5.2. Knowledge of fish handlers towards *antimicrobial use and resistance*

In this study, we used eight questions to assess the knowledge of fish handlers regarding AMU and AMR. The results showed that 79% (42/53) of participants had insufficient knowledge, 21% (11/53) had moderate knowledge, and none had sufficient knowledge about AMU and AMR. Approximately 66% (35/53) and 32% (17/53) of respondents had understanding of what antimicrobials/antibiotics and AMR are, respectively. Furthermore, the majority of participants (89%; 47/53) were unaware that the excessive use of antimicrobials in fish can contribute to AMR. Most (70%; 37/53) of participants were aware of the importance of proper storage of antimicrobials, as indicated in Table 9 below.

Table 9: Knowledge of fish handlers towards antimicrobial use and resistance (N=53)

Questions	**Desirable response**	**Percent (frequency)**	**LR-X^2**	**p-value**
Do you know or have heard about antimicrobials/ antibiotics?	Yes	66 (35)	4.5300	0.033
Do you know or have heard about AMR?	Yes	32 (17)	21.9592	0.000
There is a relationship between AMU in fish and development of resistance?	Yes	6 (3)	10.1660	0.001
Can you reduce AMR development by avoiding overuse of antimicrobials in fish production?	Yes	11 (6)	12.8264	0.000
AMR in fish is important for public health.	Yes	26 (14)	5.1407	0.023
Can zoonotic diseases causing agents develop AMR in fish?	Yes	19 (10)	5.5159	0.019
Using of fish origin food products before the end of withdrawal period can promote AMR development in humans?	Yes	6 (3)	3.1746	0.075
Antimicrobial can be stored at any place where easy for use?	No	70 (37)	3.4706	0.062
Overall knowledge score	**Insufficient**	**Moderate**	**Sufficient**	
	79 (42)	21 (11)	0 (0)	

***Note**: N: total respondent; LR: likelihood ratio; X^2: Chi-square value; the higher likelihood ratio test score indicates the higher influence of the respondents response to having insufficient or sufficient knowledge towards AMU and AMR; Scores between 80 and 100% were classified as sufficient; scores between 50 and 79% were classified as moderate and scores less than 50% were classified as insufficient knowledge.*

4.5.3. Attitude of fish handlers towards antimicrobial use and resistance

The majority of respondents (75%; 40/53) agreed that seeking professional advice was crucial for making informed decisions about the use of antimicrobials in fish production. In addition, approximately 59% (29/53) of respondents believed that using alternatives to antimicrobials would help to decrease the development of antimicrobial resistance (AMR). Moreover, 77% (41/53) of respondents were in favor of raising public awareness as an effective measure to reduce the development of AMR. Overall, more than half (60%; 32/53) of the respondents had a desirable attitude that would likely contribute to the reduction of AMR development. However, it is worth noting that 25% (25/13) of respondents had undesirable attitude, which could potentially contribute to the development of AMR (**Table 10**).

Table 10: Attitude of fish handlers towards antimicrobial use and resistance (N=53)

Questions	Desirable and neutral responses	Percent (frequency)	LR-X^2	p-value
Professional advice is crucial for making informed decisions about antimicrobial use in fish production?	Strongly agree/Agree	75 (40)	39.2769	0.000
	Neutral	8 (4)		
Can imprudent AMU result in irreversible loss of drug effectiveness?	Strongly agree/Agree	59 (31)	44.4169	0.000
	Neutral	9 (5)		
Can using antimicrobial alternatives like biosecurity and good hygienic practice reduce AMR development?	Strongly agree/Agree	55 (29)	32.8498	0.000
	Neutral	19 (10)		
Are you concerned about the potential consequences of AMR in fish production?	Strongly agree/Agree	53 (28)	47.3452	0.000
	Neutral	17 (9)		
Can AMU regulations be a solution for the irrational use of antimicrobials in fish production?	Strongly agree/Agree	59 (31)	35.5860	0.000
	Neutral	13 (7)		
Can public awareness creation reduce the development of AMR?	Strongly agree/Agree	77 (41)	49.6494	0.000
	Neutral	15 (8)		
Overall attitude score	**Undesirable**	**Neutral**	**Desirable**	
	25 (13)	15 (8)	60 (32)	

***Note**: N: total respondent; LR: likelihood ratio; X^2: Chi-square value; the higher likelihood ratio test score indicates the higher influence of the respondents response to having undesirable or desirable attitude towards AMU and AMR; Scores between 80 and 100% were classified as desirable; scores between 50 and 79% were classified as neutral and scores less than 50% were classified as undesirable attitudes.*

4.5.4. Hygienic practice of fish handlers

The hygienic practices of the fish handlers were assessed through six questions related to hygiene. The majority of fish handlers (87%; 46/53) had access to water and hand washing facilities. Around 66% (35/53) of them reported washing their hands and cleaning their equipment before and after handling fish. However, only 19% (10/53) of the respondents disposed of their waste products responsibly. Overall, 41% (22/53) had poor hygienic practices, while 38% (20/53) fair practices and 21% (11/53) had good practices as indicated below (**Table 11**).

Table 11: Hygienic practices of fish handlers in the study area (N=53)

Questions	Desirable response	Percent (frequency)	LR-X^2	p-value
Do you have access to clean water and hand washing facilities with soap or sanitizer?	Yes	87 (46)	13.8516	0.001
Do you wash your hand before and after handling fish?	Yes	66 (35)	19.0659	0.000
Do you clean equipment and tools regularly before and after handling fish?	Yes	66 (35)	20.6963	0.000
Do you wear clean and appropriate clothing while handling fish?	Yes	23 (12)	29.3608	0.000
Are waste products disposed responsibly?	Yes	19 (10)	0.9035	0.637
Is the fish handling and production area generally clean and free of debris?	Yes	38 (20)	26.8966	0.000
Overall hygienic practice score	**Poor**	**Fair**	**Good**	
	41 (22)	38 (20)	21 (11)	

***Note**: N: total respondent; n: number, LR: likelihood ratio; X^2: Chi-square value; the higher likelihood ratio test score indicates the higher influence of the respondent's response to having poor or good hygienic practice; Scores between 80 and 100% were classified as good: scores between 50 and 79% were classified as fair and scores less than 50% were classified as poor hygienic practices.*

4.5.5. Association of demographic characteristics and KAP score of respondents

The study found a significant positive correlation between educational level and knowledge and attitude towards AMU and AMR. Individuals with higher education were 21.6 times more knowledgeable than those with primary education ($p<0.05$). Those who completed secondary education also displayed 3.79 times more knowledge, though this finding was not statistically significant. With regards to attitude, secondary school graduates had 6.13 times more desirable attitude compared to those with only a primary education. Graduates with higher education showed a slightly weaker but still positive association with a more desirable attitude (OR=1.56). Gender also showed association with attitude and hygienic practices, with females displaying 0.15 times more desirable attitude and 0.16 times better hygienic practices compared to males (**Table 12**). No statistically significant association was found between the remaining demographic characteristics and the KAP scores fish handlers.

A desirable attitude significantly improves hygienic practices, with individuals with a desirable attitude being 5.79 times more likely to have good hygienic practices (**Table 12**). Correlation analysis showed weak positive linear correlations between knowledge and attitude towards AMU and AMR, and between attitude and hygienic practice scores (0.3>*rho*<0.5). However, minimal positive correlation was found between knowledge and hygienic practice scores (*rho*=0.2285). The correlations between knowledge and attitude, as well as between attitude and hygienic practices, were statistically significant ($p<0.05$) (**Table 13**).

Table 12: The association of demographic characteristics with knowledge, attitude and hygienic practice scores

Demographic variable		**Knowledge**		**Attitude**		**Hygienic practice**	
		OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value
Gender	Male	Ref					
	Female	0.42 (0.05-3.82)	0.445	0.15 (0.03-0.71)	**0.016**	0.16 (0.03-0.88)	**0.035**
Educational level	Primary	Ref					
	Secondary	3.79 (0.38-37.20)	0.253	6.13 (1.09-34.35)	**0.039**	1.44 (0.42-4.89)	0.555
	Higher	21.6 (2.08-223.65)	**0.010**	1.56 (0.31-7.87)	0.593	2.96 (0.59-14.73)	0.184
		Hygienic practice					
Attitude	Undesirable	Ref					
	Desirable	1.41 (0.26-7.61)	0.692			5.79 (1.35-24.85)	**0.018**

***Note**: OR: odds ratio; CI: confidence interval*

Table 13: Correlation between knowledge, attitude and hygienic practice scores

Correlation	**Rho**	**p-value**
Knowledge score-Attitude score	0.3453	0.0113
Knowledge score- Hygienic practice score	0.2285	0.0998
Attitude score-Hygienic practice score	0.3221	0.0187

***Note**: Rho: Spearman rank correlation coefficient*

5. DISCUSSION

Considering the national importance of fishery products and the lack of information on microbial safety in fishery products produced in Bishoftu, Ethiopia, it is crucial to assess the presence of *S. aureus* and *P. aeruginosa* bacteria. These bacteria not only serve as indicators of meat quality, but they can also cause foodborne illnesses (Mitiku *et al*., 2023). While there is limited data on the prevalence of *S. aureus* and *P. aeruginosa* in fishery products in Ethiopia, previous studies (Wendwesen *et al*., 2017; Admasu *et al*., 2023) have reported a high prevalence of *S. aureus* in fish. Additionally, there is a report on the presence of *P. aeruginosa* (Dissasa *et al*., 2022). Unlike previous studies that focused solely on the microbial profile of fish and hygienic practices, this study also determined the resistance pattern of these bacteria.

In this study a total of 105 samples collected were screened for the presence of *S. aureus* and *P. aeruginosa*. The prevalence of *S. aureus* recorded in this study of 8.6% (9/105) is comparable to the findings of 4.8% from Bahir Dar, Ethiopia (Mitiku *et al*., 2023) and 9% from Turkey (Ertas *et al*., 2015). However, our finding is significantly lower than other findings from Ethiopia; 65% from Arbaminch (Wendwesen *et al*., 2017) and 85% from Hawassa (Admasu *et al*., 2023). Higher rates of *S. aureus* prevalence have been also reported globally, with varying percentages in Japan (15.8%, Saito *et al*., 2011), Iraq (17.75%, Ali, 2014), India (24.47%, Bujjamma & Padmavathi, 2015) and Ghana (25%, Esther *et al*., 2017). A prevalence of 62.7, 42.7, and 31.8% in imported fish from Egypt, India, and Yemen, respectively were also reported from Jordan (Obaidat *et al*., 2015).

The present study revealed a 7.6% (8/105) overall prevalence of *P. aeruginosa* with 6.2% (4/65) prevalence in fresh fish which is consistent to 6.8% from Kenya (Mumbo *et al*., 2023) and 5% from Iran (Shahrokhi *et al*., 2022). Our finding is higher than 3.2% prevalence in three rift valley lakes from Ethiopia, reported by Dissasa *et al*. (2022). Higher prevalences have been reported in other parts of the world. This includes a prevalence of 21.5% from Ghana (Esther *et al*., 2017), 37.7% from Côte d'Ivoire (Benie

et al., 2017) and 10% up to 100% from Egypt (Abd El-Aziz, 2015; Salem *et al*., 2018; Algammal *et al*., 2020; Abd-El-Maogoud *et al*., 2021).

Variations in results may be due to differences in isolation methodologies, fishing practices, hygienic handling during processing, water quality and sample types (Shahrokhi *et al*., 2022). Despite not being statistically significant ($p>0.05$), our analysis also revealed that the prevalence of *S. aureus* and *P. aeruginosa* varied depending on the sample types and origins. These findings highlight the need for further research and understanding of *S. aureus* and *P. aeruginosa's* prevalence in fish and aquaculture in the country.

Antimicrobial resistant bacteria pose a significant public health concern (Ertas *et al*., 2015), with studies primarily focusing on cattle, poultry, and swine. However, antibiotic-resistant strains have also been reported in fish and aquaculture systems (Hatha *et al*., 2005). Antimicrobial susceptibility testing aids in drug prediction and therapeutic outcome, guiding clinicians in selecting appropriate treatments and antibiotics for individual patients based on the resistance phenotype of the tested microbial strain (Balouiri *et al*., 2016).

In the current study *S. aureus* and *P. aeruginosa* isolates were tested for susceptibility to 12 antimicrobials each. All *S. aureus* isolates were found to be resistant to at least one antibiotic, consistent with previous reports of 100% from Spain (Vázquez-Sánchez *et al*., 2012) and 88.5% from Jordan (Obaidat *et al*., 2015). The isolates also showed 44.4% (4/9) resistance to cloxacillin and penicillin G, slightly comparable to Kenya's 57.1% resistance (Mumbo *et al*., 2023) and lower than Jordan's 86.5% (Obaidat *et al*., 2015) and Ghana's 100% (Esther *et al*., 2017). Tetracycline resistance was found in 33.3% (3/9) of the isolates, consistent to previous reports of 33.3% from Turkey (Ertas *et al*., 2015) and 30.8% up to 40.6% from Jordan (Obaidat *et al*., 2015). However, Ghana's 84.7% up to 90.8% (Esther *et al*., 2017) resistance report to tetracycline is higher than the study's findings. The variation in results could be attributed to the utilization of varying sets of antibiotics and different breakpoints for determining antibiotic resistance in *S. aureus* isolates (Obaidat *et al*., 2015).

Vancomycin is currently the preferred drug for treating infections caused by *S. aureus.* This study reveals that *S. aureus* strains were resistant to vancomycin (11.1%; 1/9), with 22.2% (2/9) showing intermediate resistance. A study from Kenya found 71.4% resistance and 28.6% intermediate resistance while susceptibility was not observed (Mumbo *et al*., 2023). This raises concerns about multidrug-resistant *S. aureus* infections, especially after consuming raw fish products. Previous studies suggest that thickened cell walls in *S. aureus* can hinder drug penetration, leading to intermediate resistance to vancomycin (Lambert, 2002; Reygaert, 2018).

On the other hand the study found *S. aureus* isolates 100% susceptibility to ciprofloxacin and norfloxacin as well as high susceptibility to trimethoprim/sulfamethoxazole (88.9%; 8/9) and gentamicin (77.8%; 7/9), which is consistent with previous reports of >94% from Jordan (Obaidat *et al*., 2015), and >82% from Iraq (Ghayyib *et al*., 2021). However, a study from Spain reported 100% (Vázquez-sánchez *et al*., 2012) resistance to ciprofloxacin, contradicting the findings of the study. This and other studies have found that fish and aquaculture environments can harbor waterborne resistant microorganisms, which may pose a potential risk to human health, but there was limited information available on the AMR of *S. aureus* found in fish (Vaiyapuri *et al*., 2019).

Regarding to *P. aeruginosa*, the study reveals that all eight *P. aeruginosa* isolates showed resistance to eight of the twelve tested antimicrobials (kanamycin, tetracycline, doxycycline, erythromycin, florfenicol, trimethoprim/sulfamethoxazole, nalidixic acid, and cefuroxime). This resistance is in agreement to previous studies in Côte d'Ivoire (Benie *et al*., 2017) and Egypt (Darwish *et al*., 2023) which reported 100% resistance to kanamycin and erythromycin, respectively. There was also high (75%; 6/8) resistance to streptomycin, lower than a report of 100% from Kenya (Mumbo *et al*., 2023). These findings suggest that *P. aeruginosa* diseases may not respond well to these antibiotics due to mechanisms such as drug efflux, inactivation, uptake limitation, and target modification (Mumbo *et al*., 2023).

On the other hand, the study found low level of (25%; 2/8) resistance by *P. aeruginosa* isolates to gentamicin. A similar finding (29.03%) was reported in Ghana's Ashanti

region (Esther *et al*., 2017), while Egypt's Ismailia and Sharkia Governorates reported 67.6% (Algammal *et al*., 2020) and 50% (Darwish *et al*., 2023) resistance respectively. High susceptibility (75%; 6/8) was also observed to ciprofloxacin and norfloxacin in this study comparable to 66.4% to ciprofloxacin from Côte d'Ivoire (Benie *et al*., 2017) and 88.89% to norfloxacin from Egypt (Algammal *et al*., 2020). A study from Ghana (Esther *et al*., 2017) reported 51.61% resistance to ciprofloxacin which is contrasting to our finding.

The global rise of multidrug-resistant (MDR) bacteria is causing significant concern, as they can render antibiotics ineffective and increase mortality rates (Magiorakos *et al*., 2012; Esther *et al*., 2017). In our study we found MDR isolates of *S. aureus* and *P. aeruginosa* despite no use of antimicrobials in fish production in the study area.

S. aureus has developed resistance to various drugs globally (Pesavento *et al*., 2007), with 33.3% (3/9) of isolates identified as MDR in this study. These isolates exhibit resistance to four classes of antibiotics, with each isolate showing resistance to three classes and up to five specific antimicrobials. Consistent to our findings, Esther *et al*. (2017) from Ghana reported 25.3% resistant to at least five antibiotics and MDR to up to eight antibiotics. Higher MDR reports were found in Brazil (44.4%, Albuquerque *et al*., 2007), Nigeria (50%, Grema *et al*., 2015), South Africa (82%, Fri *et al*., 2020), and Egypt (100%, Seedy *et al*., 2017). In Jordan, 24.6% of *S. aureus* isolates of imported fish from Egypt, 35.5% from India, and 31.4% from Yemen were resistant to three or more classes of antibiotics (Obaidat *et al*., 2015). A study from Galicia, Spain, showed all isolates were resistant to penicillin, chloramphenicol, and ciprofloxacin, with most also being resistant to tetracycline (Vázquez-sánchez *et al*., 2012).

There have been reports of antibiotic resistant bacterial isolates found in fish, ponds, and water sources, even in cases where antibiotics have not been recently used. This resistance may be due to the persistence of previously acquired resistance genes in the aquatic environment (Schmidt *et al*., 2001; Shah *et al*., 2012a) and the source of water or feed, which can transfer resistance to other bacteria (Esther *et al*., 2017).

The MARI values recorded in this study for *S. aureus* isolates were 0.08 and 0.17 for non-MDR isolates, and 0.25, 0.33, and 0.42 for MDR isolates. The majority (66.7%; 2/3) of MDR isolates were from fish while Lake Bishoftu, Hora-Arsedi, and pond contributing 33.3% (1/3) each to MDR isolates. Studies from South Africa (Fri *et al*., 2020), Egypt (Morshdy *et al*., 2022), and Nigeria (Egege *et al*., 2020) have reported MARI value of >0.2, 0.142 to 0.928 and ≥0.2, respectively. These findings align with our own findings.

In our study, we found all *P. aeruginosa* isolates were multidrug-resistant, exhibiting resistance to 7 (62.5%; 5/8) or 8 (37.5%; 3/8) antimicrobial classes and even up to eleven specific antimicrobial agents. This resistance could be attributed to hydrolytic enzyme production and resistance mechanisms of *P. aeruginosa* isolates, and may also be due to improper antibiotic disposal in the surrounding environment, including in animal production areas and aquatic environments (Benie *et al*., 2017). Different reports on the MDR profile of *P. aeruginosa* isolates from fish and aquatic environments exist including 33.3% from Kenya (Mumbo *et al*., 2023), 33.1% and 20.0% from Côte d'Ivoire (Benie *et al*., 2017), 75% (Okafor and Nwodo, 2023) and 55.6% (Hosu *et al*., 2020) from South Africa. Differences in findings may be due to different antimicrobials used, geographical conditions, and improper antibiotic use in aquatic environments.

Samples from Lake Hora-Arsedi, Bishoftu, Cheleklaka, and pond contributed 37.5% (3/8), 25% (2/8), 12.5% (1/8), and 25% (2/8) of *P. aeruginosa* MDR isolates, respectively. The majority of MDR isolates (50%; 4/8) were from fish, 25% (2/8) from water, and 12.5% (1/8) from hand and knife swabs of each. The study found that *P. aeruginosa* isolates had a MARI value of 0.67, 0.75, 0.83 and 0.92, which was assumed as high. Our result is consistent with previous records of 0.19 up to 0.94 (Darwish *et al*., 2023) from Egypt, 0.3 to 0.9 (Okafor and Nwodo, 2023) and 0.08 to 0.69 (Hosu *et al*., 2020) from South Africa. All isolates had a high MARI (>0.2), suggesting they were likely derived from a contaminated source with a high risk of containing multiple antibiotics.

In this work, PCR was used to detect the presence of *mecA* gene for confirmation of MRSA. All nine of the *S. aureus* isolates that we tested for the *mecA* gene molecularly

produced negative MRSA results. As a result, our study did not find any MRSA. MRSA is uncommon in the aquaculture and fish as compared to the clinical sector. According to a study done in Galicia, Spain, fisheries products don't contain any MRSA with no *S. aureus* isolates acquired from the investigation were discovered to possess the *mecA* gene (Vázquez-Sánchez *et al*., 2012), consistent to our result.

Contrasting reports to MRSA occurrence rate in aquatic environment and fish were found from studies in different countries. The findings including 6.6% (Grema *et al*., 2015) and 53.8% (Egege *et al*., 2020) from Nigeria, 3.0% (Sivaraman *et al*., 2022) and 13.4% (Murugadas *et al*., 2016) from India, 5% from Japan (Hammad *et al*., 2012), as well as 22.5% and 30% in processed and raw fish from Brazil (Costa *et al*., 2015). Factors contributing to the occurrence of MRSA include hygiene practices, but it is a complex issue arising from interactions between the host, pathogen, and environment. It is essential to understand the complex nature of MRSA (Mediavilla *et al*., 2012).

Antimicrobial misuse and abuse in veterinary medicine, human medicine, and agriculture has a major impact on the global spread of AMR. Understanding the level of knowledge, attitudes, and behaviors surrounding the use of antibiotics and resistance is essential for fighting global AMR (Dejene *et al*., 2022). In this study, we conducted a questionnaire survey to explore the knowledge, attitudes, and hygienic practices of fish handlers in Bishoftu, Ethiopia, in relation to AMU and AMR.

The study found that overall 79% (42/53) of fish handlers had insufficient knowledge about AMU and AMR, consistent with previous Ethiopian studies including a report of 80.2% (Gebeyehu *et al*., 2021) and 94% (Tufa *et al*., 2023) on animal producers. On the other hand Bulcha *et al*. (2024) reported most respondents had moderate knowledge; Geta & Kibret (2021) reported about half of the respondents had sufficient knowledge while Dejene *et al*. (2022) found 17%, 48%, and 35% of respondents had insufficient, moderate and sufficient knowledge of AMU and AMR, respectively.

In the present study we found that 66% (35/53) of participants were familiar with antimicrobials/antibiotics, which was slightly comparable to previous finding of 72.3%

by Dejene *et al*. (2022). This finding is lower than a report of 95.4% by Moffo *et al*. (2020); 90.1% by Geta & Kibret (2021) and 80.4% by Pham-duc *et al*. (2019) while it is higher than a report of 7% by Tufa *et al*. (2023) and around 20% by Tufa *et al*. (2018). However, in the current study only 32% (17/53) were aware of or had prior knowledge about AMR, which is higher than 9% of Tufa *et al*. (2023) and 14.1% of Tufa *et al*. (2018). This finding is lower than previous findings of 44.1%, 49.9% and 77% by Gebeyehu *et al*. (2021), Moffo *et al*. (2020) and Pham-duc *et al*. (2019), respectively. This difference may be due to fish handlers may have less access to information and training compared to livestock owners, who might be more exposed to veterinary guidance as well as variations in socio-demographic characteristics.

In the current study only 6% (3/53) and 11% (6/53) of participants had sufficient knowledge about the relationship between AMU and AMR and were aware of avoiding overuse of antimicrobial can reduce AMR, respectively. This findings are lower than the findings of 38.0% and 35% by Gebeyehu *et al*. (2021). We also found that 26% (14/53) of them had sufficient understanding about the importance of antimicrobial resistance to public health which is less than the report of 38.9% by Moffo *et al*. (2020) while higher than 19.8% by Geta & Kibret (2021). In this study only 19% (10/53) of participants were aware of that zoonotic disease causing agents can develop resistance in fish which is lower than the report of 38.4%, 43.1% and 45.8% by Geta & Kibret (2021), Gebeyehu *et al*. (2021) and Moffo *et al*. (2020), respectively. This difference may be the consequence of the respondents varying degrees of AMU and AMR awareness in various localities.

Our study revealed that only 6% (3/53) of respondents were aware of the relationship between antimicrobial withdrawal periods and AMR. This is in contrast to previous studies that reported a higher percentage of respondents being aware of recommended withdrawal periods (39.8%, Gebeyehu *et al*., 2021; 56%, Geta & Kibret, 2021; 50.5%, Dejene *et al*. 2022; 17%, Tufa *et al*. 2023). However, 70% (37/53) of respondents were aware of proper storage of antimicrobials. The study suggests that factors such as government focus on aquaculture, educational levels, fish handler exposure to AMU and AMR training, and regulations in the aquatic sector and research areas could explain this disparity.

The study revealed that 75% (40/53) of participants believed professional advice is crucial for making informed decisions about AMU, comparable to 86.9% of Nuangmek *et al*. (2018) and higher than 52.7% of Gebeyehu *et al*. (2021). Nearly 59% (31/53) of respondents believed imprudent AMU resulted in irreversible drug effectiveness loss, higher than 23.5% of Gebeyehu *et al*. (2021) and 42.9% of Geta & Kibret (2021). However, lower than 82.1% and 96.67% of Nuangmek *et al*. (2018) and Bulcha *et al*. (2024), respectively. Additionally, 55% (29/53) of respondents had a desirable attitude towards using antimicrobial alternatives to reduce AMR development, higher than 28.6% (Gebeyehu *et al*., 2021) and 94% (Tufa *et al*., 2023). Almost 53% (28/53) were concerned about the potential consequences of AMR, with 59% (31/53) believing AMU regulations could be a solution for irrational use of antimicrobials. Most (77%; 44/53) respondents also believed public awareness creation could reduce AMR development, consistent with previous studies (Gebeyehu *et al*., 2021; Bulcha *et al*., 2024).

The study found that 60% (32/53) of participants had desirable attitudes towards AMU and AMR, with 15% (8/53) being neutral and 25% (13/53) having undesirable attitudes. Geta & Kibret (2021) and Dejene *et al*. (2022) reported 52.8% and 53.75% of desirable attitude towards AMU, respectively. This aligns with our findings. However, Nuangmek *et al*. (2018), Gebeyehu *et al*. (2021) and Tufa *et al*. (2023) reported undesirable attitudes from 37.1%, 85.29%, and 97% of respondents, respectively. These differences were attributed to differences in sample size, educational attainment, and socio-demographic attributes.

In regard to hygienic practices of fish handlers, the study found that 87% (46/53) of respondents had access to clean water and hand washing facilities, with 66% (35/53) washing their hands before and after handling fish. This finding is in agreement to the findings of 63.33% (Admasu *et al*., 2023) and 69% (Nassor *et al*., 2023) and contrasting to 100% by Wendwesen *et al*. (2017). In the current study 66% (35/53) were found cleaning equipment and tools regularly before and after handling fish, consistent with 63.33% report by Admasu *et al*. (2023). However, only 23% (12/53) of respondents wore clean clothing while handling fish, lower than previous studies of 46.66%, 46.5%

and 85.5% by Admasu *et al*. (2023), Wendwesen *et al*. (2017) and Nassor *et al*. (2023), respectively. Our investigation also found that only 19% (10/53) disposed of waste responsibly, and 38% (20/53) had a generally clean fish handling and production area.

According to the results of the current study, 41% (22/53), 38% (20/53), and 21% (11/53) of respondents in the study area had overall poor, fair, and good hygiene practices, respectively. Bedane *et al*. (2022) found that fish handlers were carrying out the activity without the use of basic processing facilities, such as a clean and impervious processing room or area, clean and potable water, a facility for hand washing and disinfecting equipment, or a facility for handling products. Additionally, 60% of fish workers were not following hygienic procedures, according to Nassor *et al*. (2023), in line with ours.

The current study found a significant association ($p<0.05$) between gender and educational levels and KAP among respondents. Females had a 0.15 times desirable attitude and 0.16 times better hygienic practices compared to males which is in line with Dyar *et al*. (2020) and Gebeyehu *et al*. (2021). It's possible that men's more exposure to meetings, training, and the media made them more knowledgeable about AMU and AMR than females (Gebeyehu *et al*., 2021). Higher education and secondary education resulted in 21.6 and 3.79 times more knowledge and a 1.56 and 6.13 times more desirable attitude, than whom only completed primary education, respectively, consistent with previous studies (Gebeyehu *et al*., 2021; Dejene *et al*., 2022 and Tufa *et al*., 2023).

The study also found weak positive linear correlations between respondents knowledge, and attitude scores (*rho*=0.3453), consistent with previous studies (Dejene *et al*., 2022; Abunna *et al*., 2023 and Tufa *et al*., 2023). A desirable attitude was also found to be 5.79 times more likely to lead to good hygienic practices (OR=5.79) which is consistent with Dejene *et al*. (2022) and contrasting to the finding of Tufa *et al*. (2023).

Overall, the study indicates that despite a desirable attitude towards AMU and AMR, most fish handlers had insufficient knowledge and poor hygienic practices, which could contribute to the emergence and dissemination of AMR (Jin *et al*., 2011). Thus, improving knowledge, awareness, and hygienic practices is crucial to reduce AMR risks.

6. CONCLUSION AND RECOMMENDETIONS

The current study found that *S. aureus* and *P. aeruginosa* were common bacterial pathogens that contaminate lakes and ponds. These could be the source of contamination for fish, fish handlers and utensils in the study area. The study also revealed that MDR strains of *S. aureus* and *P. aeruginosa* had emerged in aquaculture settings in this area. This gives rise to concerns about antibiotics misuse in humans and animals, which may contaminate the environment due to improper disposal system. Several factors may contribute to the occurrence of AMR *S. aureus* and *P. aeruginosa*. Growing resistance may be driven by a lack of knowledge about AMU and AMR. Our study also revealed that a majority of fish handlers had insufficient knowledge and poor hygienic practice. We also found that the respondents' socio-demographic characteristics, such as educational level and gender, influenced their knowledge, attitudes, and hygienic practices. Notably, a desirable attitude was associated with better hygienic practices. Overall, our study provides valuable insights into the occurrence of MDR *S. aureus* and *P. aeruginosa* in freshwater aquaculture settings. Furthermore, it establishes baseline evidence regarding the knowledge, attitudes, and hygienic practices of fish handlers, and offers guidance for designing interventions and policies in the aquaculture sector.

Therefore, based on the aforementioned conclusion, the following recommendations are being presented:

- Detail research should be conducted to detect AMR genes in aquaculture for *S. aureus, P. aeruginosa* and other bacteria with zoonotic importance.
- It is highly recommended to include educational and awareness initiatives to enhance knowledge and promote desirable attitudes towards prudent AMU and to curb AMR.
- Continuous training and awareness-raising efforts should be undertaken to enhance the hygienic practices of fish handlers.
- It is highly recommended to promote a One Health approach as a holistic and effective strategy to combat AMR in fish and aquaculture.

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8. APPENDICES

Appendix I: Type and preparation of bacteriological medias used

1. Peptone water (Himedia)

Ingredients	g/liter
Peptone	10
Sodium chloride	5
Final pH (at 25°C)	7.2±0.2

Preparation: suspend 15 grams in one litter of distilled water. Heat if necessary to dissolve the medium completely. Mix well and dispense into tubes with or without inverted Durham's tubes. Sterilize in autoclave at 121°C for 15 minutes.

2. Mannitol salt agar (Himedia)

Ingredients	g/liter
Peptone	5
Tryptone	5
HM Peptone B	1
Sodium chloride	75
D-Mannitol	10
Phenol red	0.025
Agar	15
pH after sterilization (at 25°C)	7.4±0.2

Preparation: Suspend 111.02 grams in one liter of distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 121°C for 15 minutes. Cool to 45-50°C. Mix well and pour into sterile Petri dishes.

3. Cetrimide Agar (USP, EP) (Dehydrated), Oxoid

Ingredients	g/liter
Gelatin peptone	20
Magnesium Chloride	1.4
Potassium sulphate	10
Cetrimide	0.3
Agar	13.6
pH after sterilization (at 25°C)	7.2 ± 0.2

Preparation: Suspend 45.3g of Pseudomonas Cetrimide Agar in 1 litre of distilled water. Add 10ml of glycerol and boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes. Cool the medium to approximately 50°C and pour into sterile Petri dishes.

4. Tryptone Soya Broth (Himedia)

Ingredients	**g/liter**
Pancreatic digest of casein	17
Papaic digest of soyabean meal	3
Sodium chloride	5
Dextrose	2.5
Dibasic potassium phosphate	2.5
Final pH (at 25°C)	7.3±0.2

Preparation: Suspend 31.50 grams in one liter of distilled water. Heat if necessary to dissolve the medium completely. Dispense 5ml portions into a test tube. Sterilize by autoclaving at 121°C for 15 minutes.

5. Tryptic soya agar (Himedia)

Ingredients	**g/liter**
Tryptone	17
Soya peptone	3
Sodium chloride	5
Dextrose (Glucose)	2.5
Dipotassium hydrogen phosphate	2.5
Agar	15
Final pH (at 25°C)	7.3±0.2

Preparation: Suspend 45 grams in one liter of distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 121°C for 15 minutes. Cool to 45-50°C. Mix well and pour into sterile Petri dishes.

6. Triple Sugar Iron Agar (Himedia)

Ingredients	g/liter
Peptone	10
Tryptone	10
Lactose	10
Sucrose	10
Dextrose	1
Ferrous ammonium sulphate	0.2
Sodium chloride	5
Sodium thiosulphate	0.2
Phenol red	0.025
Agar	13
pH after sterilization (at 25°C)	7.3±0.2

Preparation: Suspend 59.42 grams in 1000 ml purified/ distilled water. Heat to boiling to dissolve the medium completely. Mix well and distribute into test tubes. Sterilize by autoclaving at 121°C for 15 minutes. Allow the medium to set in form of a slope with a butt about 1 inch long.

7. Simmons Citrate Agar (Himedia)

Ingredients	g/liter
Ammonium dihydrogen phosphate	1
Magnesium sulphate	0.2
Dipotassium phosphate	1
Sodium citrate	2
Sodium chloride	5
Bromo thymol blue	0.08
Agar	15
Final pH (at 25°C)	6.8±0.1

Preparation: Suspend 24.28 grams in one liter of distilled water. Heat to boiling to dissolve the medium completely. Mix well and distribute in tubes. Sterilize by autoclaving at 121°C for 15 minutes. Cool the tubes as slants.

8. Motility Test Medium (Himedia)

Ingredients	g/liter
Tryptose	10
Sodium chloride	5
Agar	5
Final pH (at 25°C)	7.2±0.2

Preparation: Suspend 20 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Dispense in tubes and sterilize by autoclaving at 121°C for 15 minutes. Allow tubed medium to cool in an upright position.

9. MR-VP Medium (Himedia)

Ingredients	g/liter
Buffered peptone	7
Dextrose (Glucose)	5
Dipotassium phosphate	5
Final pH (at 25°C)	6.9±0.2

Preparation: Suspend 17.0 grams in one liter of distilled water. Heat if necessary to dissolve the medium completely. Distribute in test tubes in 10 ml amounts or as desired. Sterilize by autoclaving at 121°C for 15 minutes.

10. Mueller Hinton agar (Himedia)

Ingredients	g/liter
Beef infusion from	300
Casein and hydrolysate	17.5
Starch	1.5
Agar	17
Final pH (at 25°C)	7.3±0.2

Preparation: Suspend 38 grams of the medium in one liter of distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 121^{o}C for 15 minutes. Mix well before pouring.

Appendix II: Antimicrobial agents used for susceptibility test

1. Antibiotic discs, disc content and interpretive criteria for *S.aureus*

Antimicrobial agents	**Symbol**	**Disc content/ µg**	**Susceptible**	**Intermediate**	**Resistant**
Cefuroxime	CXM	30	≥18	15-17	≤14
Ciprofloxacin	CIP	5	≥21	16-20	≤15
Cloxacillin	CX	5	≥22	19-21	≤18
Doxycycline	DO	30	≥16	13-15	≤12
Erythromycin	E	15	≥23	14-22	≤13
Gentamicin	CN	10	≥15	13-14	≤12
Lincomycin	L	15	≥21	16-20	≤15
Norfloxacin	NOR	10	≥17	13-16	≤12
Penicillin G	P	10	≥29	-	≤28
Trimethoprim/ sulfamethoxazole	SXT	1.25/23.75	≥16	11-15	≤10
Tetracycline	TE	30	≥19	15-18	≤14
Vancomycin	VA	30	≥17	15-16	≤14

Source: (CLSI, 2023)

2. Antibiotic discs, disc content and interpretive criteria for *P. aeruginosa*

Antimicrobial agents	**Symbol**	**Disc content/ µg**	**Susceptible**	**Intermediate**	**Resistant**
Cefuroxime	CXM	30	≥18	15-17	≤14
Ciprofloxacin	CIP	5	≥25	19-24	≤18
Doxycycline	DO	30	≥14	11-13	≤10
Erythromycin	E	15	≥23	14-22	≤13
Florfenicol	FFC	30	≥19	15-18	≤14
Gentamicin	CN	10	≥15	13-14	≤12
Kanamycin	K	30	≥18	14-17	≤13
Nalidixic acid	NA	30	≥ 19	14-18	≤ 13
Norfloxacin	NOR	10	≥17	13-16	≤12
Streptomycin	S	10	≥15	12-14	≤11
Trimethoprim/ sulfamethoxazole	SXT	1.25/23.75	≥16	11-15	≤10
Tetracycline	TE	30	≥19	15-18	≤14

Source: (CLSI, 2023)

Appendix III: Informed consent for the questioner survey

Informed consent form

Good morning/good afternoon!

I am Kebadu Endeg, a post graduate student at Addis Ababa University College of Veterinary Medicine and Agriculture in the field of MSc in Veterinary Pharmacology. Now I am conducting research on "**Antimicrobial Resistance of *Staphylococcus aureus* and *Pseudomonas aeruginosa* in Fish, and Knowledge, Attitude and Hygienic Practices of Fish Handlers' in Bishoftu, Ethiopia**". I will appreciate your participation in this study. The general objective of this study is to assess antimicrobial use and hygienic practices in fish production and to determine its impact on the antimicrobial resistance pattern of *S. aureus* and *P. aeruginosa* in the study areas. To effectively attain the objective of the research, I am requesting your help. For your participation in the study no payment will be granted or has no any special privilege to you. All information you give will be kept confidential and won't be accessible to any third party; your name won't be registered on the question sheet so that you will not be identified for any reason. You have the right not to participate from the beginning, or you may stop participating at any time after starting the participation. However, your honest answers to these questions will help me in better understanding of antimicrobial usage and hygienic practices in fish production and will eventually help in designing and implementing appropriate interventions to alleviate related problems.

Respondent statement: I have understood the above statements:

□ Yes (Agree to participate) □ No (Not agree to participate)

Informed consent: I have read this form, or it has been read to me in the language I understand all conditions stated above. Therefore, I am willing to participate in this study.

Name of respondent: _____

Signature: _____

Date: _____

Appendix IV: Questioner used for assessing fish handler's knowledge, attitude and practices towards antimicrobial use, antimicrobial resistance and hygiene

Addis Ababa University

College of Veterinary Medicine and Agriculture

Research questioner for assessing fish handler's knowledge, attitude and practice (KAP) towards antimicrobial use, antimicrobial resistance and hygiene.

Questionnaire Serial Number: _____ID of the respondent: _____Date: _____

Section I: **Respondents' socio-demographic information**

1**.** Working site of respondent:

A. Lake Bishoftu B. Lake Hora-Arsedi

C. Lake Chelekleka D. Lake Babogaya E. Pond

2. Gender of respondent: A. Male B. Female

3. Age of respondent: A. <30 B. 30-40 C. >40

4. Educational level of respondent:

A. Illiterate B. Primary school

C. Secondary school D. Higher education

5. Respondents working year of experience: A. <3y B. 3-10yr C. >10yr

Section II: **Knowledge of fish handlers towards AMU and AMR in fish production.**

1. Do you know or have heard about antimicrobials/ antibiotics? A. Yes B. No

2. Do you know or have heard about AMR? A. Yes B. No

3. There is a relationship between antimicrobial use in fish and development of resistance? A. Yes B. No C. I don't know

4. Can you reduce AMR development by avoiding overuse of antimicrobials in fish production? A. Yes B. No C. I don't know

5. Antimicrobial resistance in fish is important for public health.

A. Yes B. No C. I don't know

6. Can zoonotic diseases causing agents develop AMR in fish?

A. Yes B. No C. I don't know

7. Using of fish origin food products before the end of withdrawal period can promote AMR development in humans? A. Yes B. No C. I don't know

8. Antimicrobial can stored at any place where easy for use? A. Yes B. No

Section III: **Attitude of fish handlers towards AMU and AMR in fish production.**

Please tick **(√)** your level of agreement about the questions in the following table. The meaning of the numbers in the first row is defined below.

5= strongly agree 4= agree 3= neutral 2= disagree 1= strongly disagree

No	Questions	1	2	3	4	5
1	Professional advice is crucial for making informed decisions about AMU in fish production?					
2	Can imprudent AMU result in irreversible loss of drug effectiveness?					
3	Can using antimicrobial alternatives like biosecurity, good hygienic practice and vaccination reduce AMR development?					
4	Are you concerned about the potential consequences of AMR in fish production?					
5	Can AMU regulations be a solution for the irrational use of antimicrobials in fish production?					
6	Can public awareness creation reduce the development of AMR?					

Section IV: **Hygienic practices of fish handlers in fish production and processing.**

1. Do you have access to clean water and hand washing facilities with soap or sanitizer?

A. Yes B. No

2. Do you wash your hand before and after handling fish? A. Yes B. No

3. Do you clean equipment and tools regularly before and after handling fish?

A. Yes B. No

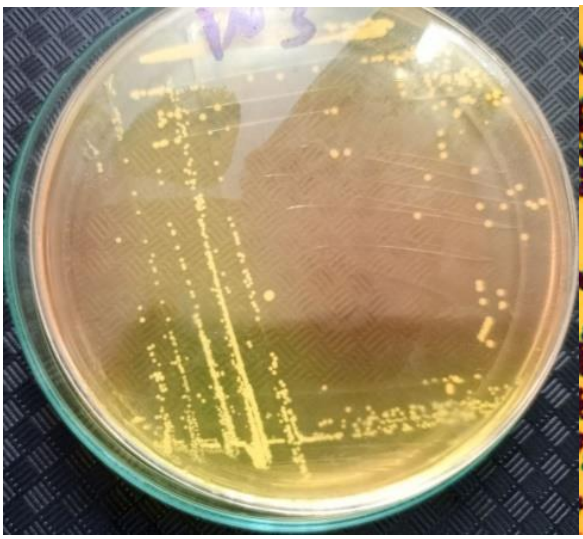
4. Do you wear clean and appropriate clothing while handling fish? A. Yes B. No

5. Are waste products disposed responsibly? A. Yes B. No

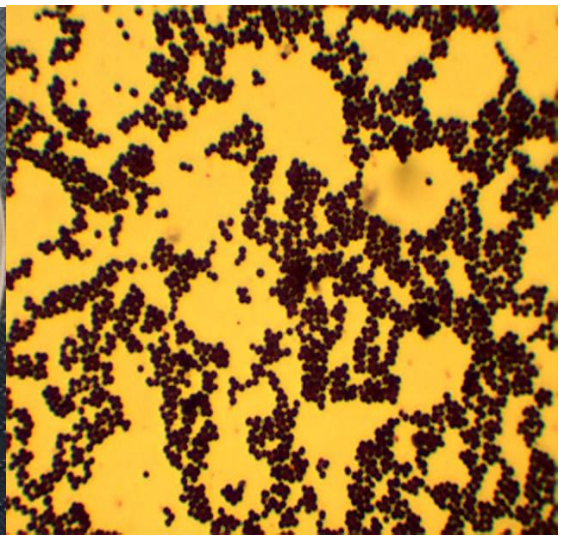
6. Is the fish handling and production area generally clean and free of debris?

A. Yes B. No

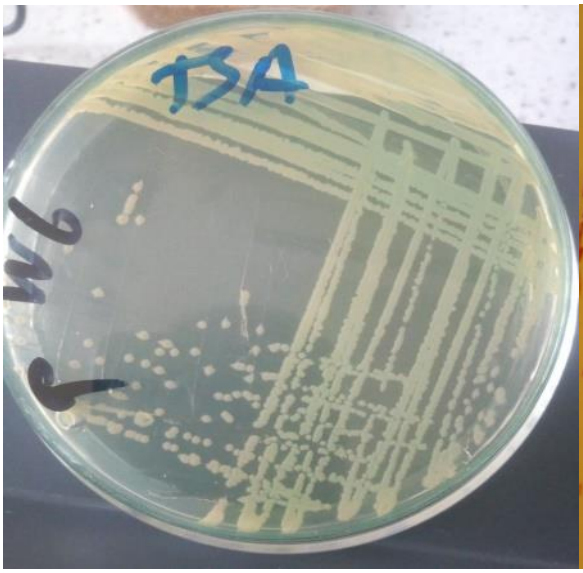
Appendix V: Images taken during the process of this research



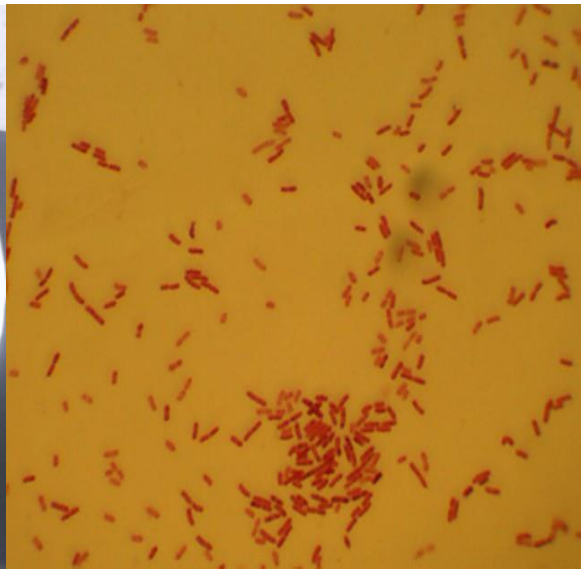
A. *S. aureus* on Mannitol salt agar



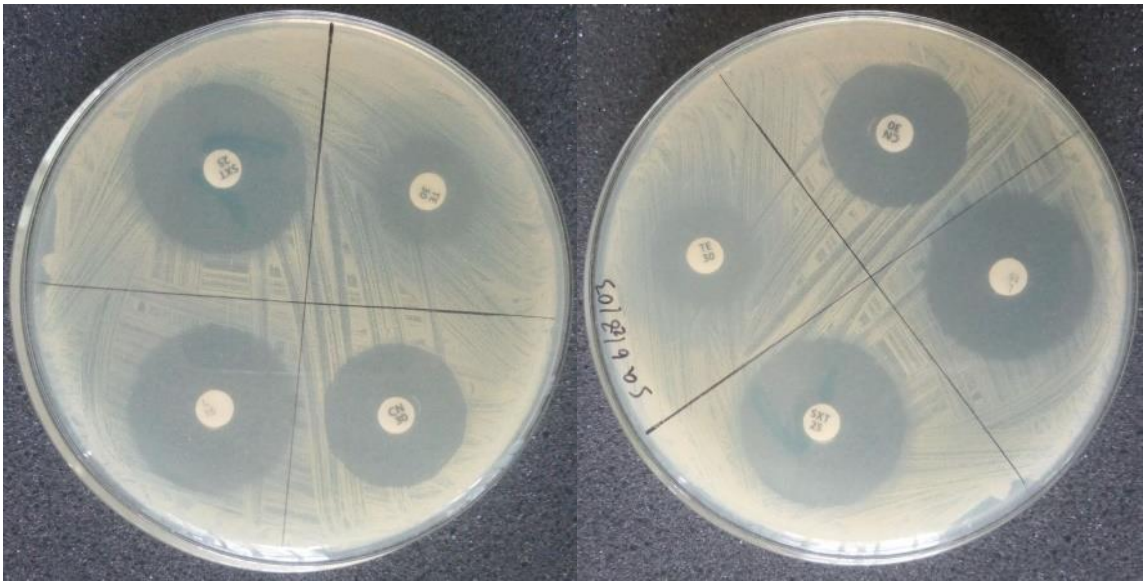
B. *S. aureus* on Gram staining



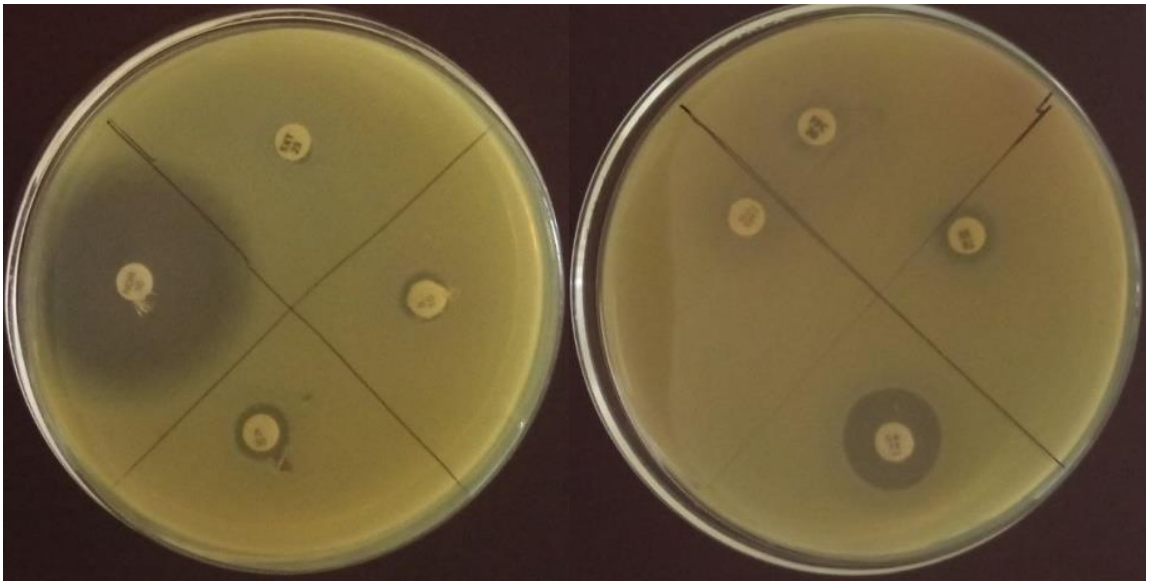
C. *P. aeruginosa* on Tryptic soya agar



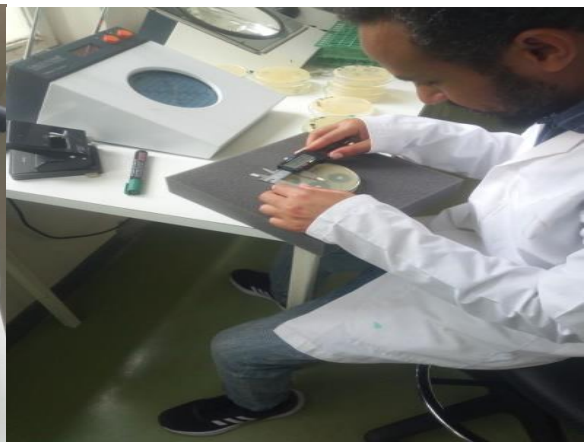
D. *P. aeruginosa* on Gram staining



E. Antimicrobial susceptibility test of *S. aureus*

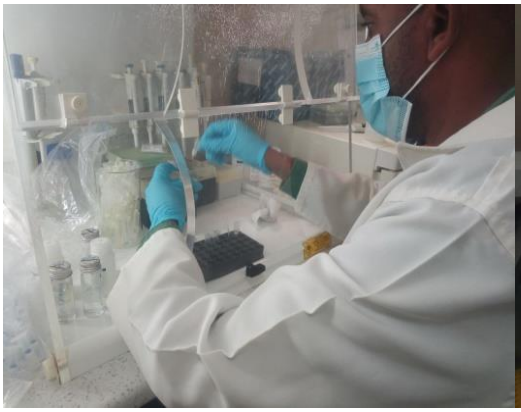


F. Antimicrobial sensitivity test of *P. aeruginosa*



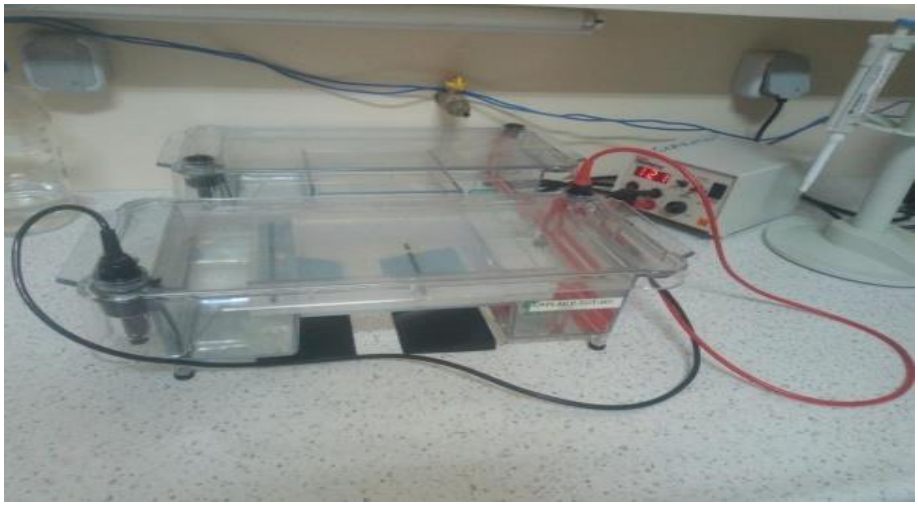
G. 0.5 McFarland bacterial suspension

H. Measuring zone of inhibition



I. DNA extraction

J. PCR running



K. Agaros gel electrophoresis


L. Ponds for fish production


M. Lake Babogaya

N. Lake Hora-Arsedi


O. Unhygienic fish filleting

P. Throwing fish offal's to other animals

Appendix VI: Ethical clearance certificate

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu

Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/04/21/16/2024

Name of Applicant: **Kebadu Endeg (DVM, MSc student)**

Address: Department of Biomedical Sciences, College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: *Antimicrobial resistance of Staphylococcus aureus and Pseudomonas aeroginusa in fish and fish handlers knowledge, attitude and practice in Bishoftu, Ethiopia*

Date of application:	**December, 2023**
Nature of the project:	**Filed investigation and questionnaire survey**
Target animal species:	Fish
Number of animals involved:	**No live fish involved**
Study area:	**Bishoftu, Ethiopia**

Minutes No. and date of review: **VM/ERC/04/16/024, 16/05/2024**

The Institutional Animal Care and Use Committee of the College of Veterinary Medicine and Agriculture of the Addis Ababa University has reviewed the above research project and unanimously approved the application of **Kebadu Endeg.**

Professor Getachew Terefe (DVM, PhD)
Chairman

Signature

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Appendix VII: Plagiarism report

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Antimicrobial Resistance of *Staphylococcus aureus* and *Pseudomonas aeruginosa* in Fish, and Knowledge, Attitude and Hygienic Practices of Fish Handlers in Bishoftu, Ethiopia

MSc THESIS

BY

KEBADU ENDEG

ABSTRACT

Fish, a protein-rich food, can sometimes be contaminated with bacterial pathogens like *S. aureus* and *P. aeruginosa*, known for their resistance to antimicrobials. A cross-sectional study was conducted in Bishoftu, Ethiopia, from November 2023 to May 2024 to determine the antimicrobial resistance (AMR) pattern of these pathogens and assess the knowledge and, attitude towards antimicrobial use (AMU) and AMR, and hygienic practices of fish handlers. *S. aureus* and *P. aeruginosa* were confirmed using polymerase chain reaction and their susceptibility to antibiotics was tested using the Kirby Bauer disk diffusion method. The data was analyzed using STATA version 14, using descriptive statistics, Chi-squared, likelihood ratio, and binary logistic regression. The results of the study found that 8.6% of 105 samples tested positive for *S. aureus* and 7.6% for *P. aeruginosa*. *S. aureus* isolates were 100% resistant to cefuroxime, 44.4% to clinacillin and penicillin, and 33.3% to tetracycline. *P. aeruginosa* isolates were 100% resistant to kanamycin, tetracycline, doxycycline, erythromycin, florfenicol, sulfamethoxazole-trimethoprim, nalidixic acid, and cefuroxime. Multidrug resistance was observed in 33.3% of *S. aureus* isolates and 100% of *P. aeruginosa* isolates. None of the *S. aureus* isolates were positive for *mecA* gene. The survey revealed that 79% and 60% of respondents had insufficient knowledge and desirable attitudes about AMU and AMR, respectively and 41% had poor hygienic practices. A significant positive correlation was found between respondents' educational level and knowledge and attitude scores. Gender also played a role in attitude and hygienic practices. The study provides insights into *S.*

Antimicrobial Resistance of Staphylococcus aureus and Pseudomonas aeruginosa in Fish, and Knowledge, Attitude and Hygienic Practices of Fish Handlers in Bishoftu, Ethiopia

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