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Antimicrobial resistance profile of bacterial pathogens isolated from naturally infected fish in some lakes of Ethiopia

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ABSTRACT: Small scale fisheries play important role in providing food and livelihoods for households in Ethiopia. However, prevalence of bacterial fish infection and their antimicrobial resistance are increasing over the world accounting for the major limitations in fish production as well as its consumption. A cross sectional study was carried out to identify bacterial pathogens isolated from naturally infected Nile tilapia (*Oreochromis niloticus*) and 20 were common carp (*Cyprinus carpio*) fish showing clinical signs of disease particularly hemorrhage and skin ulceration and to determine the antibiotic susceptibility of fish associated bacteria in Ethiopia. A total of 42 fish samples from two lakes (Hawassa and Ziway) were aseptically collected and bacteria were isolated from the kidney, liver and intestine. The isolates were identified by their morphological characteristics, biochemical tests and sequencing of 16S rRNA genes. Nine well known fish pathogens were identified from 49 positive samples at the lakes with prevalence rate of *Aeromonas veronii* (21.43%), *Proteus mirabilis* (19.05%), *Edwardsiella tarda* (16.67%), *Shigella flexneri* (11.9%), *Stenotrophomonas maltophilia* (11.9%), *Aeromonas hydrophila* (11.9%), *Pseudomonas putida* (9.5%), *Aeromonas sobria* (7.14%) and *Pseudomonas hunanensis* (7.14%). Of these pathogens, six (6) most prevalent and known potential pathogens (*A. hydrophila*, *A. veronii*, *E. tarda*, *P. mirabilis*, *S. flexneri* and *S. maltophilia*) were evaluated for antibiotic susceptibility by using Kirby Bauer disk diffusion assay. All the isolates tested were resistant to at least three (3) of the eight antibiotics evaluated. High levels of resistance were expressed by the majority (87.5%) of the pathogens against penicillin and vancomycin. The results indicate the presence of potential pathogens and maximum levels of acquired antibiotic resistance in fish bacteria from the study area. Thus, the use of antibiotic in fish farming in Ethiopia should be discouraged. It is also advisable to cook fish properly before consumption, in order to kill bacterial pathogens that may be present.

Keywords/ Phrases: Bacterial pathogens, Common carp, naturally infected fish, Nile tilapia

INTRODUCTION

Fishery sector is a rapidly growing industry that provides massive number of fish for human consumption worldwide (Santos and Ramos, 2018). Fish plays an important role in the human diet with an ever growing need globally (FAO, 2020). Nevertheless, pathogenic microorganisms are a serious threat to fish production all over the world due to the high economic importance of diseases they cause. Environmental dynamics in freshwater ecosystems are fundamental in the development of pathogenic fish bacteria and there has been a steady increase in the numbers of bacterial species associated with fish diseases (Peřkala-Safińska, 2018). Gram-negative bacteria like

Aeromonas spp., *Flavo bacterium* spp., *Pseudomonas* spp., *Edwardsiella* spp., *Vibrio* spp., *Acinetobacter* spp. and *Plesiomonas higeloides* are a great threat to freshwater fish production (Peřkala-Safińska, 2018; Yoseph Kerieet *et al.*, 2019; Ayoub *et al.*, 2021). Most of these bacterial fish pathogens are zoonotic with the potential to infect humans and some are severely serious (Hashish *et al.*, 2018; Haenen *et al.*, 2021).

Different mechanisms are used to produce large stocks of fish, but frequent disease outbreaks occur, and the use of antimicrobials to control these pathogens provides ideal conditions for the emergence of resistant bacterial strains stimulating horizontal gene transfer (Dewi *et al.*, 2022; Ojasanya *et al.*, 2022). There are major concerns in

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use of antimicrobial agents in fishery sectors, since it leads to the development of antibiotic resistant pathogens in fish and the aquatic environment. The passage of antimicrobial resistance genes and resistant bacteria from aquatic to terrestrial animal husbandry and to the human environment can have detrimental effects on both human and animal health in aquatic ecosystems (Santos and Ramos, 2018). Currently, some antimicrobial agents commonly used in fishery are only partially effective against fish pathogens due to the emergence of resistant bacteria (Miller and Harbottle, 2017). Genetic determinants of antimicrobial resistance are commonly found on mobile genetic elements which are recognized as the primary source of antimicrobial resistance for important fish pathogens (Miller and Harbottle, 2017).

The widespread occurrence of naturally resistant bacteria in the aquatic environment could contribute to the passage of antibiotic resistance genes to fish bacteria (Cantas *et al.*, 2013). Besides, high levels of antimicrobial resistances have been reported in bacterial isolates from fish elsewhere (Agoba *et al.*, 2017; Miller and Harbottle, 2017; Gufet *et al.*, 2019; Abedinet *et al.*, 2020; Gazalet *et al.*, 2020; Tsafacket *et al.*, 2021; Dewiet *et al.*, 2022; Ojasanya *et al.*, 2022). Therefore, assessment of the prevalence

of bacterial pathogens in fish and evaluation of their antimicrobial profile were essential to monitor fish health and related potential public health risk of fish-borne bacterial illnesses. Reports on bacterial fish pathogens and their antibiotic susceptibility profile in Ethiopian lakes are scant. Hence, this study was aimed to assess the occurrence, prevalence and identify the bacterial pathogens from naturally infected (clinically sick) Nile tilapia and common carp fish showing clinical signs of disease particularly hemorrhage and skin ulceration, and to determine the antimicrobial resistance profile of selected potential bacterial pathogens in Ethiopia.

MATERIALS AND METHODS

Study area

This cross-sectional study was conducted on two Ethiopian rift valley lakes, Lake Hawassa and Lake Ziway, which are well-known for their common fish catches and tourist destinations. These lakes are some 163 and 275 km to the south of Addis Ababa, respectively (Fig. 1). The information sources were World Lake Database (<https://wldb.ilec.or.jp/>).

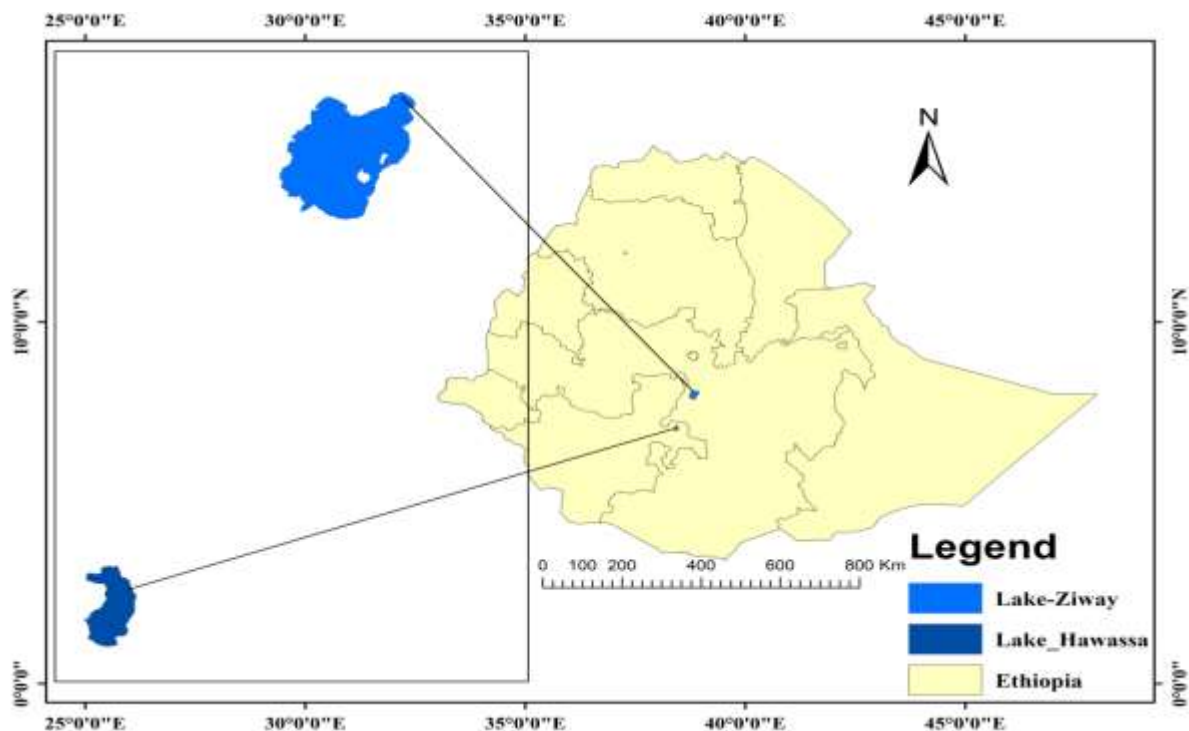


Figure 1. The study area: Lake Hawassa and Lake Ziway.

Sample size

The sample size used to estimate the prevalence of bacterial pathogens in naturally infected fish samples was determined using the formula $n = \frac{Z^2 P(1 - P)}{d^2}$ by (Daniel and Cross, 2013), where 'n' is the sample size, 'Z' the confidence interval (1.96), 'P' the average prevalence estimate of 16.5% calculated from a review report in Ethiopia (Eshetu Yimer, 2000), and 'd' the expected error (0.05).

Study design and sampling strategy

The study design was cross-sectional and its period was from February to June 2022. For sampling, the two lakes were selected purposively that the lakes are known for their common fish catches and from which fish are continuously harvested for subsistence and cash (Daniel and Cross, 2013). Different sampling sites located at different directions of the lakes were selected guided by the available information. The sampling sites are considered major fishing grounds along the shorelines. These sites were located at different directions of the lakes and were assumed to represent each lake. The temperatures of Lake Hawassa and Lake Ziway during sample collection were 25.8°C and 22.5°C respectively. Fish samples were collected by using the fishing boats and gears of local fishermen and finally naturally infected fish were sampled by using stratified sampling techniques. The weight and length of the collected fish were measured. Length is measured from the tip of the nose to the end of the tail in centimeters using a ruler. To measure the weight, the fish were placed on the scale making it balanced and stable, and the weight displayed on the scale were recorded.

Fish samples

The collected fish samples were inspected for any external abnormalities by trained individuals. A total of 42 naturally infected fish were sampled from the study lakes with 22 Nile tilapia (13 from Lake Hawassa and 9 from Lake Ziway) and 20 Common carp (9 from Lake Hawassa and 11 from Lake Ziway). The length and weight of the collected fish were measured by using a ruler and scale respectively. The collected naturally infected fish samples were separately packed in sterile plastic bags and were transported to *Batu Fisheries and other Aquatic Life Research Center located at Batu*

town (formerly Ziway), near Lake Ziway. The fish were euthanized by cervical dislocation in the fish's normal water without using anesthesia. Trained individuals practiced the technique using appropriate equipment. Cervical dislocation is among the methods recommended for fish sacrifice as it is relatively simple and effective for smaller fish (Leal *et al.*, 2009; Robert, 2012).

As our fish are small to medium in size, they were killed by inserting a thumb into the mouth, holding with the opposite hand and displacing it dorsally. Death was recognized by cessation of movement, and confirmed by cessation of respiration (opercular movement) and cessation of heartbeat (palpation); and finally, by destruction of the brain. The fish were dissected under aseptic conditions using sterile dissecting scissors by following well established protocol and standard procedures (SAB, 1957; Holt *et al.*, 1994). Tissue samples of kidney, intestine and liver were taken aseptically using sterile scalpel blade (forceps) and kept in sterile universal bottles of 100ml capacities separately and homogenized in physiological saline solution. Finally, all the tissue samples were packaged and transported on ice to the Health Biotechnology Laboratory of Institute of Biotechnology, Addis Ababa University, and stored at 4°C for bacteriological analysis. Bacterial isolation was started within 48 hours after tissue samples were collected.

Bacteria culture and colony morphology

All bacteriological experiments were performed following the Society of American Bacteriologists Manual of microbiological methods (SAB, 1957), Bergey's manual of determinative bacteriology (Holt *et al.*, 1994) and the respective media manufacturer's instructions. It was under complete aseptic conditions. All incubations were for 24 hours at 37°C.

First, sample suspensions were spread or streaked across nutrient agar medium (Oxoid, England) and incubated under aerobic conditions. Then, specific colonies were picked-up and inoculated on gram-negative selective and differential media Xylose Lysine Deoxycholate (XLD) agar (HIMEDIA, India), and incubated further. Suspected bacterial colonies were picked-up, inoculated into tryptone soy broth (HIMEDIA, India), and incubated. Colony morphology like form, elevation, margin, surface and pigmentation

were examined. Colony color was noted by visual inspection of bacterial agar plate using fresh culture. The cell morphology was screened microscopically using simple and differential (gram) staining techniques. Slides were prepared by staining from cells grown on an agar medium and examined under the microscope (Primo Star, India) using the 100X oil-immersion objective. The morphologically presumptively identified isolates were stored at -20°C in 25% glycerol (Fine Chemical, Ethiopia) using cryovial tubes of 1.8ml capacity (IMEC, China) for biochemical identification.

Biochemical characterization

All the media used in the tests were from HIMEDIA, India. The tests began with inoculating respective media with 24-hours-old pure culture colonies. All incubations were at 37°C for 24 hours and the expected color changes confirmed test positivity. Briefly, indole production was tested by inoculating 10ml of Dev tryptophan broth, incubating and adding two to three drops of indole reagent. Methyl red (MR) test was conducted by inoculating 10ml of MR Voges-Proskauer (MR-VP) medium, incubating, and adding two to three drops of 0.05% MR. Voges-Proskauer (VP) test was done by inoculating 10ml of MR-VP medium, incubating, and adding 2-3 drops of 5% α -naphthol followed by 40% of KOH and shaking and leaving it open for an hour.

For catalase test, a small amount of bacterial colony was transferred to clean glass slide using a sterile loop and a drop of hydrogen peroxide was added, and the formation of bubbles was checked. For citrate utilization test, Simmons citrate agar slant was inoculated and incubated. Hydrogen sulfide or triple sugar iron (TSI) test was done by inoculating TSI by first stabbing through the center of the medium to the bottom of the tube and then streaking the surface of the agar slant, and incubating for 24 hours at 37°C. Similarly, urea production was tested using Christensen's Urea Agar slant. Sugar fermentation test was conducted using sugar broth medium prepared by mixing 1 g peptone, 0.3g meat extract, 0.5g table salt, 0.5g sugar and 0.008g phenol indicator in 100ml distilled water. Three tubes having three different sugars (glucose, sucrose, lactose) in the broth medium were inoculated, and incubated at 37°C for 24 hours.

Molecular Characterization

Nine bacterial isolates were grown overnight in 10ml of TSB broth at 37°C for 24 hours. DNA was extracted using Qiagen DNeasy DNA extraction protocol adapted from Qiagen DNeasy (Hamburg, Germany) handbook, 2006 and according to the manufacturer's instruction. The extracted DNA was checked by gel-electrophoresis and stored at -20 °C till use.

PCR amplification was performed in a DNA thermal cycler (Eppendorf, Hamburg, Germany). PCR reactions were performed in a final volume of 25 μ l containing 20ng of DNA, 0.1-0.3 μ l of each primer (rD1 and fD1), and 1 μ l of Hot Star Taq Master Mix containing MgCl₂, Hot Star Taq DNA polymerase and deoxynucleotide triphosphate mix (dNTPs). The PCR conditions were as follows: initial activation of the Taq-DNA-Polymerase for 9min at 95°C, followed by 30 cycles of 1min denaturation at 95°C, annealing for 1min at 56°C and 35 cycles extension for 10min at 72°C. The PCR products (8 μ l) were analyzed by electrophoresis on 1.5% agarose gels stained with ethidium bromide using 1 μ M Tris-Acetate-EDTA buffer at 100v for 1 hour and were visualized by UV transillumination (Weisburg *et al.*, 1991).

16S rRNA sequencing and phylogenetic tree construction

Sequencing of the amplicon was performed using the universal bacterial primers rD1 (5'-CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC-3') and fD1 (5'-CGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3'). The 16S rRNA was sequenced using Sanger sequencing at BaseClear DNA research laboratory, Leiden, The Netherlands. For sequencing of amplified 16S rRNA directly, four identical 100 μ l amplification reactions were performed on each sample with the resultant material being pooled and purified. A 500ng amount of template (16S rRNA) was combined with 10ng of primer, 2 μ l of Sequence buffer, and water to 10 μ l. This sample was held at 98°C for 7 min and cooled to room temperature for 1 min, and then the labeling reaction was performed at room temperature for 5 min. Chain elongation was terminated with sample loading buffer and sequencing was performed on buffer-gradient gels (Bigginet *al.*, 1983).The 16S rRNA gene sequences obtained were edited using Bioedit and Basic Local

Alignment Search Tool (BLAST) searches carried out in GenBanks especially National Center for Biotech Information (NCBI). Related sequences were obtained and multiple sequence alignments were performed using ClustalW algorithm. The aligned gene sequences are used to constructs phylogenetic tree using the neighbor-joining method.

Antibiotic susceptibility testing of selected bacteria

Antibiotic susceptibility testing was carried out following Kirby-Bauer disc diffusion method on Mueller Hinton agar (HIMEDIA, India), as described by Hudzicki (2009). There are no registered antibiotic formulations for use in fishery sectors in Ethiopia, and therefore, the choice was guided by different classes of drugs reported elsewhere to treat diseases in aquaculture facilities (Austin and Austin, 2016). Moreover, these drugs are commonly used in veterinary and human medicine in Ethiopia. A total of six(6) bacterial pathogens (*A. hydrophila*, *A.veronii*, *E. tarda*, *P. mirabilis*, *S. flexneri* and *S.maltophilia*) were tested due to their high prevalence in the study area and they were considered to represent the isolated gram-negative bacteria, a group to which major bacterial fish pathogens belongs with remarkable resistance to many commonly prescribed antibiotics. Eight (8) commercially available antibiotic disks (Oxoid, UK) were used in the following concentrations: ampicillin (10µg), Chloramphenicol (30µg), erythromycin (15µg), gentamicin (10µg), oxacillin (1µg), penicillin (10µg), tetracycline (30µg) and vancomycin (10µg).

The isolates were plated on Xylose Lysine Desoxycholate (XLD) agar medium (HIMEDIA, India), and incubated at 37°C for 24 hours. Colonies were picked and emulsified in 0.85% NaCl to create a suspension matching 0.5 McFarland standards. The bacterial suspension

was, then, swabbed onto the surface of the Mueller Hinton agar (HI MEDIA, India) to make confluent growth. Antibiotic discs were immediately placed on the surface of the agar plate using forceps and incubated aerobically at 37°C for 18 hours. Inhibition zone diameters (IZD) for various isolates were measured and interpreted as sensitive, intermediate or resistant according to the Clinical Laboratory Standards Institute (CLSI) (CLSI, 2020).

Data Analysis

Bacterial infection status of the different fish tissue samples was determined and the proportion of infected samples was compared between various categories using the Chi-squared test. Bacterial species prevalence in the two fish species, examined tissue samples and lakes was compared using one-way analysis of variance (ANOVA). Statistical analysis was performed using IBM SPSS software version 26 (IBM, Chicago, USA) and $p < 0.05$ was considered the level of statistical significance. The overall antibiotic response of each isolate was calculated as the number of bacteria resistant, intermediate or sensitive to antibiotics over the total number of bacteria isolates tested.

RESULTS

Clinical examination

The body weights and lengths of Nile tilapia and common carp range between 16 cm (139 g) to 23 cm (200 g) and 18 cm (378 g) to 22 cm (462 g), respectively. The clinical examination of the collected naturally infected Nile tilapia (*Oreochromis niloticus*) and Common carp (*Cyprinus carpio*) showed different external abnormalities like skin ulceration and hemorrhages all over the fish body especially at fins and tails (Fig. 2).

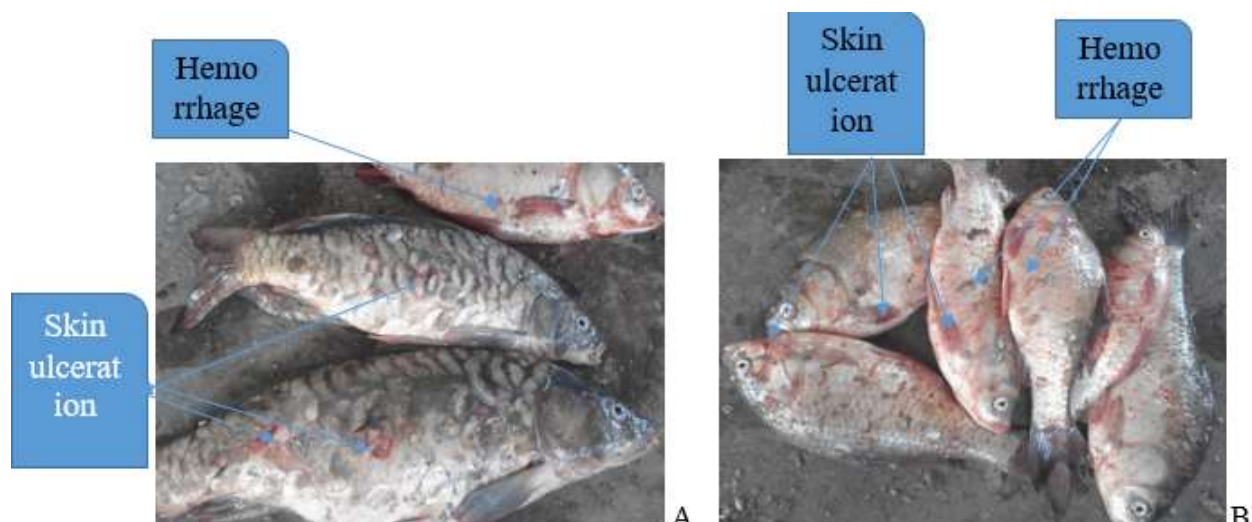


Figure 2. Selected naturally infected (A) *Cyprinus carpio* and (B) *Oreochromis niloticus* showing hemorrhage and skin ulceration.

Isolation of bacterial pathogens and morphology

Totally 126 (3 from each) tissue samples were obtained from the naturally diseased fish of which 38.88% tissue samples were positive for gram-negative bacterial infection. The gram-negative bacteria were further tested and identified into 9 species. All the isolates cell morphology was short rods and non-spore formers. Colonies of the isolates had different form, elevation, margin, surface, colour and optical characteristics. *Aeromonas veronii* was the dominant species with 9 isolates (3 from common carp and 6 from Nile tilapia) followed by 8 *P. mirabilis* (3 from common carp and 5 from Nile tilapia). *Aeromonas hydrophila*, *S. flexneri* and *S. maltophilia* have similar prevalence rate of 11.9%, while *A. sobria* and *P. hunanensis* were the least prevalent isolates (7.14%).

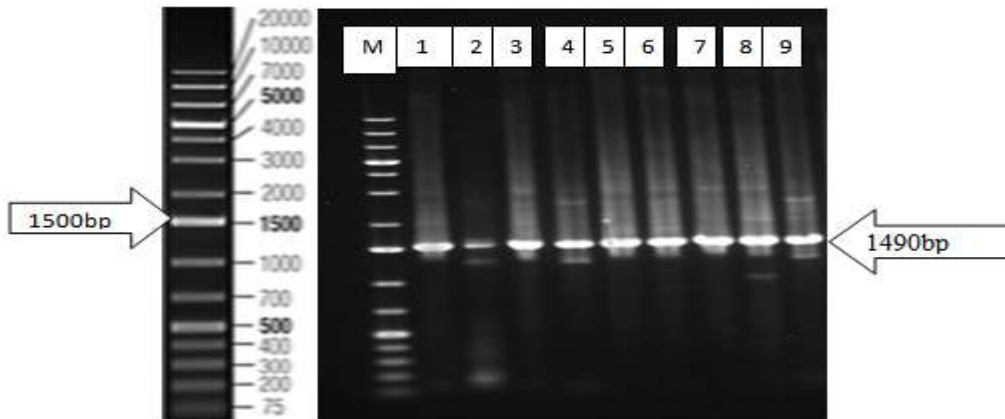
Biochemical tests and molecular identification

The results showed that different groups of isolates that belonged to 9 species were identified (Table 1 and Figure 4). These were *Aeromonas* spp., (*A. hydrophila*, *A. sobria* and *A. veronii*), *E. tarda*, *Pseudomonas* spp., (*P. hunanensis* and *P. putida*), *P. mirabilis*, *S. flexneri* and *S. maltophilia*. Each isolate was confirmed by molecular identification using 16S rRNA gene sequencing and the amplified genomic DNA using the universal primers (rD1 and fD1) of 9 isolates and the control run in 1.5% (W/V) of agarose gel with amplicon size of 1490kb are presented in (Fig 3). Compared with Gen Bank database, the nucleotide sequences of 16S rRNA gene identified the potential bacterial isolates species level, according to levels of homology of nucleotide ranging from 97.65-100%, similarity indices (Fig. 4).

Table 1. Biochemical tests for the selected isolates and their presumptive identity.

Isolate code	Indole	Methyl red	Voges Proskauer	citrate	H2S	Gas	Urease	Sugar fermentation	catalase	Presumptive identity
AhI	+	+	+	+	+	+	-	+	+	<i>A. hydrophila</i>
AsI	+	+	-	+	+	+	-	+	+	<i>A. sobria</i>
AvI	+	+	+	+	+	+	-	+	+	<i>A. veronii</i>
EtI	+	+	-	-	+	+	-	+	+	<i>E. tarda</i>
PhI	-	-	-	+	-	-	+	+	+	<i>P. hunanensis</i>
PpI	+	-	-	-	-	-	-	+	+	<i>P. putida</i>
PmI	-	+	-	+	+	+	+	-	+	<i>P. mirabilis</i>
SfI	v	+	-	-	-	+	-	+	+	<i>S. flexneri</i>
SmI	+	-	-	+	+	+	-	-	+	<i>S. maltophilia</i>

Note: +: positive result, -: negative result.



Note: 1: *A. hydrophila*, 2: *A. sobria*, 3: *A. veronii*, 4: *E. tarda*, 5: *P. humanensis*, 6: *P. putida*, 7: *P. mirabilis*, 8: *S. flexneri* 9: *S. maltophilia*.

Figure 3: Agarose (1.5%) gel electrophoresis analysis of the PCR products from 16S rRNA gene of bacteria with universal primers (rD1 and fD1). Lane 1-9: Bacterial isolates.

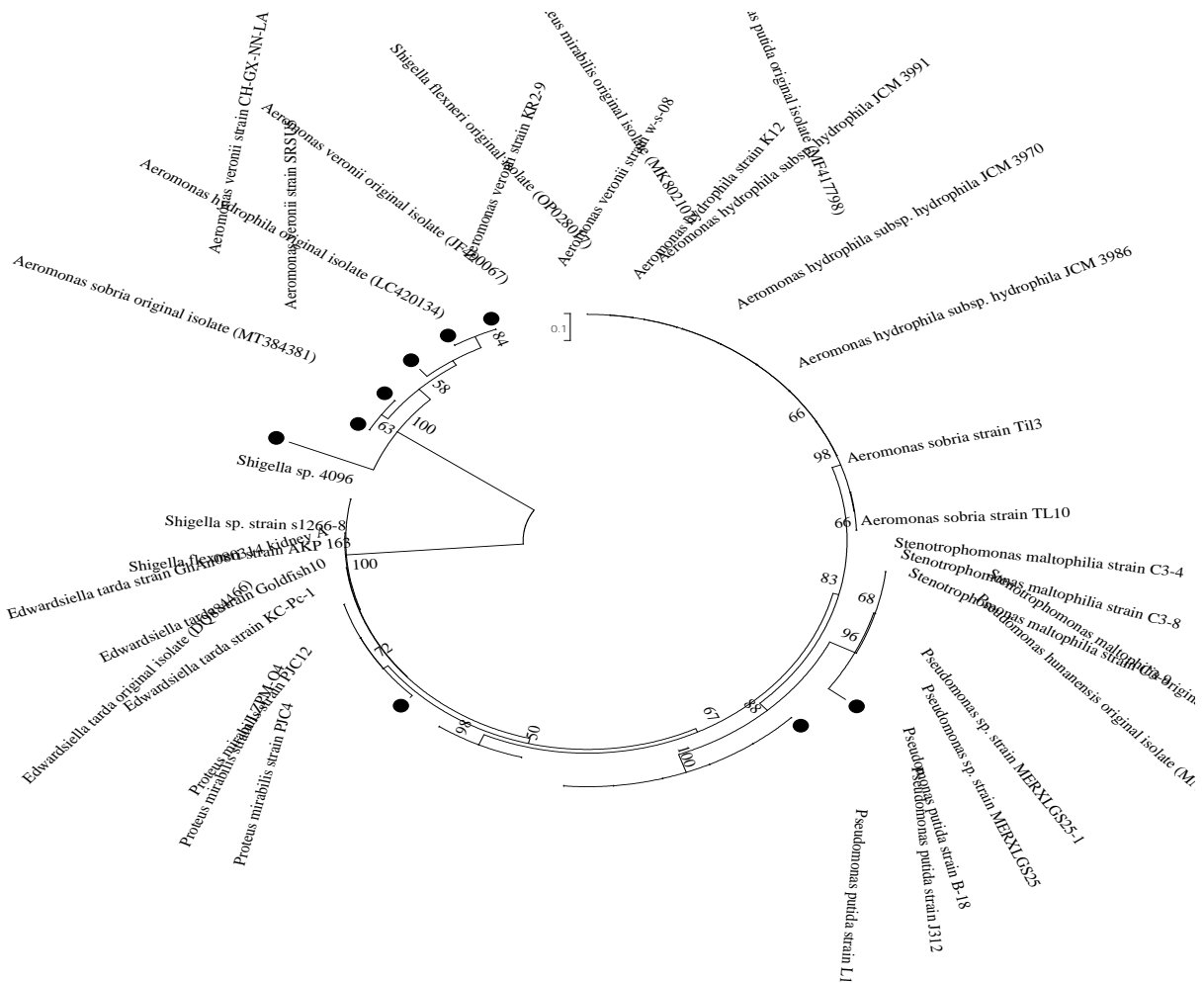


Figure 4. The Unrooted phylogenetic relationships, based on 16S rRNA gene sequences, were constructed by the neighbor-joining method. The bootstrap percentages derived from 500replications. The bacterial isolates from this study with bold dot. Accession numbers of 16S rRNA genes of the bacterial isolates are shown in brackets.

Prevalence of bacterial infections among the examined fish

Except *P. hunanensis* and *S. maltophilia*, all other isolates were recovered from Nile tilapia (*O. niloticus*) and common carps (*C. carpio*). *Edwardsiella tarda* was isolated from 5 Nile tilapia and 2 common carps with overall prevalence of 16.67%. The prevalence of *E. tarda* and *P. hunanensis* were significantly ($p < 0.05$) vary with the occurrence of any other species among naturally infected fish from both Lakes. *Aeromonas veronii* was isolated from 6 tilapia and 3 carps with overall prevalence of 21.43% which was higher than the occurrence of any other species among naturally infected fish from each Lake (Table 1). The highly prevalent bacteria in Nile tilapia (*Oreochromis niloticus*) were *A. veronii* (14.28%) followed by *E.*

tarda (11.9%), *P. mirabilis* (11.9%), *S. maltophilia* (11.9%) and *A. hydrophila* (9.5%). *Aeromonas veronii*, *P. mirabilis* and *S. flexneri* showed similar prevalence rate of 7.14% in common carp among the isolated bacteria.

The highest number of isolates was from Lake Ziway (61.9%) and relatively the smaller number of isolates (54.76%) isolated from Lake Hawassa. The prevalence of bacterial infection in the two Lakes was statistically significant ($p < 0.0001$). Except *P. hunanensis* and *P. putida*, all the others bacterial pathogens (85.7%) were isolated from Lake Hawassa fish samples. Similarly, all of the species were also isolated from Lake Ziway fish samples except *E. tarda* and *A. sobria*. On the other hand, 65.31% of the 9 species were detected in both Lakes fish samples (Table 2).

Table 2. Number (percentage) of bacterial species isolated from two fish species caught from Lakes Hawassa and Ziway (N = 42).

Bacteria spp	Lake Hawassa		Lake Ziway		Total	P-value
	carp	Tilapia	carp	tilapia		
<i>A. hydrophila</i>	-	2(4.76%)	1(2.38%)	2(4.76%)	5(11.9%)	0.578
<i>A. sobria</i>	2(4.76%)	1(2.38%)	-	-	3(7.14%)	0.154
<i>A. veronii</i>	1(2.38%)	2(4.76%)	2(4.76%)	4(9.5%)	9(21.43%)	0.632
<i>E. tarda</i>	2(4.76%)	5(10.2%)	-	-	7(16.67%)	0.042*
<i>P. hunanensis</i>	-	-	-	3(7.14%)	3(7.14%)	0.037*
<i>P. putida</i>	-	-	3(7.14%)	1(2.38%)	4(9.5%)	0.062
<i>P. mirabilis</i>	1(2.38%)	2(4.76%)	2(4.76%)	3(7.14%)	8(19.05%)	0.632
<i>S. flexneri</i>	2(4.76%)	1(2.38%)	1(2.38%)	1(2.38%)	5(11.9%)	0.768
<i>S. maltophilia</i>	-	2(4.76%)	-	3(7.14%)	5(11.9%)	0.078
Total	8(19.05%)	15(35.7%)	9(21.43%)	17(40.47%)	42(100%)	<0.0001

Note: -: Not detected.

Bacteria distribution by fish tissue

The highest number of bacteria occurrences was isolated from the kidney (36.73%) followed by the intestine (32.65%) and liver (30.61%: Table 3). The most frequently isolated bacterium from the examined tissue samples of naturally infected fish was *A. veronii* with similar (8.16%) frequency both

in the intestine and kidney (Table 3). *Edwardsiella tarda* and *P. mirabilis* are the most frequently isolated bacteria from the liver (14.3%). The least frequently isolated bacteria from the examined tissue samples of fish were *A. sobria* and *P. hunanensis* (6.1%: Table 3).

Table 3. Distribution of bacteria in the kidney, liver and intestine of fish caught from Lakes Hawassa and Ziway (N = 49).

Isolated bacteria	Intestine, n (%)	Kidney, n (%)	Liver, n (%)	Total, n (%)	P-value
<i>A. hydrophila</i>	2 (4.1%)	2 (4.1%)	1 (2.0%)	5 (10.2%)	0.714
<i>A. sobria</i>	1 (2.0%)	-	2 (4.1%)	3 (6.1%)	0.064
<i>A. veronii</i>	4 (8.16%)	4 (8.16%)	1 (2.0%)	9 (18.4%)	0.541
<i>E. tarda</i>	3 (6.1%)	1 (2.0%)	3 (6.1%)	7 (14.3%)	0.296
<i>P. hunanensis</i>	-	2 (4.1%)	1 (2.0%)	3 (6.1%)	0.562
<i>P. putida</i>	1 (2.0%)	2 (4.1%)	1 (2.0%)	4 (8.2%)	0.274
<i>P. mirabilis</i>	2 (4.1%)	3 (6.1%)	3 (6.1%)	8 (16.3%)	0.742
<i>S. flexneri</i>	1 (2.0%)	3 (6.1%)	1 (2.0%)	5 (10.2%)	0.536
<i>S. maltophilia</i>	2 (4.1%)	1 (2.0%)	2 (4.1%)	5 (10.2%)	0.391
Total	16 (32.65%)	18 (36.73%)	15 (30.61%)	49 (100%)	0.694

Antibiotic sensitivity testing

Results of sensitivity testing for the eight (8) tested antibiotics against the six (6) bacterial pathogens are indicated in Table 4. The result showed that, except *S. maltophilia* all the others pathogens were sensitive to gentamicin. Majority

(87.5%) of the pathogens were resistant to penicillin and vancomycin. The majority of the pathogens tested were resistant to most of the antibiotics evaluated, suggesting maximum levels of acquired antibiotic resistance in fish bacteria from the study area (Table 4).

Table 4. Antibiotic sensitivity profiles of the bacterial pathogens, based on CLSI's inhibition zones interpretive criterion.

Bacterial strains	Antibiotics and its susceptibility patterns							
	Amp	Chl	Ery	Gen	Oxa	Pen	Tet	Van
<i>A. hydrophila</i>	R	S	S	S	I	R	R	R
<i>A. veronii</i>	R	R	S	S	R	R	R	R
<i>E. tarda</i>	S	S	I	S	R	R	S	R
<i>P. mirabilis</i>	S	S	R	S	S	S	R	R
<i>S. flexneri</i>	R	R	R	S	S	R	R	R
<i>S. maltophilia</i>	R	I	S	R	R	R	S	R

Note: - Amp: Ampicillin, Chl: Chloramphenicol, Ery: Erythromycin, Gen: Gentamicin, Oxa: Oxacillin, Pen: Penicillin, Tet: Tetracycline, Van: Vancomycin, R: Resistance, S: Sensitive, I: intermediate.

DISCUSSION

The prevalence of bacterial fish infection and antimicrobial resistance are increasing all over the world that can be a serious challenge to sustainability of fishery industry. In present study, the two fish species were found to be infected with different groups of bacterial pathogens indicates that these fish species are equally susceptible to these bacterial diseases. Among the bacterial pathogens identified, most of which originated from lake Ziway than Lake Hawassa showing statistically significant variation ($p < 0.0001$).

Overall, the most dominant bacterial pathogens were *A. veronii*, *P. mirabilis* and *E. tarda*. This is in line with the findings of (Zorrilla *et al.*, 2003, El-Barbary and Hal, 2016; Wamala *et al.*, 2018; Tooba *et al.*, 2021; Ayoub *et al.*, 2021) who reported the dominance of fish bacterial pathogens that include *Aeromonas* spp., *E. tarda* and *Pseudomonas* spp., that could pose diseases in different tropical freshwater fishes. Similarly, diverse bacteria are the leading causative agents of diseases in freshwater fishes all over the world (Zorrilla *et al.*, 2003). More severe disease conditions in the fish industry are mostly caused by Gram-negative bacteria (Ojasanya *et al.*, 2022) as noted in the present study.

The present study indicated that, relatively, the major bacterial pathogens were prevalent in the Nile tilapia. This is in line with the finding of (Meron *et al.*, 2020) that showed the sensitivity of Nile tilapia to different potential bacterial

pathogens especially gram negative bacteria *Aeromonas* spp., and *pseudomonas* spp. Bacterial pathogens of fish and zoonotic bacteria such as *Aeromonas* spp (*A. hydrophila*); *Pseudomonas* sp. (*P. fluorescens* and *P. putida*) and *K. oxytoca* were recovered from naturally infected Nile tilapia in El-Serw aquaculture fish farm of Egypt (Austin and Austin, 2016). The author also reported that the higher number of *Aeromonas* isolates commonly infect Nile tilapia than *Pseudomonas* isolates. A study on naturally infected fish in fish farms of district Kasur, Punjab, Pakistan found significantly virulent bacterial pathogens such as *A. hydrophila* from Nile tilapia (Tooba *et al.*, 2021).

In the present study, the highest number of bacterial pathogens (36.73%) was recovered from the kidneys of the two naturally infected fish. Potential bacterial pathogens prevalence was higher in kidneys than in liver and intestine samples. This indicates that different bacterial pathogens have an affinity to specific cells, therefore, for a specific organ. Our findings are in line with (Nilsson and Strom, 2002; Meron *et al.*, 2020) who found that, significantly higher potential pathogenic bacteria were recovered from kidneys than other tissue samples of naturally infected marine fish and variations were found among the fish species, indicating the importance of the environmental conditions on the fish microbiome. The author also reported that *A. veronii* was the most frequently isolated pathogen from the kidney of naturally infected Nile tilapia and Common carp. *Pseudomonas hunanensis* bacterium is not a

well-known fish pathogen. It is unusual isolates and there is no supporting information from the literature as *P. hunanensis* is challenging fish or others animal pathogen. *Pseudomonas hunanensis* represents a novel species of the genus *Pseudomonas*, for which the name *Pseudomonas hunanensis* sp. nov. is proposed. It is commonly isolated from soil samples subjected to long-term manganese pollution (Gao *et al.*, 2014). But in this study it was isolated from naturally infected Nile tilapia. This finding suggests that the naturally infected fish might have been exposed to biological pollutants which have been diluted or neutralized from the environment. Compared to terrestrial animals' epidemiology studies, little research has been done in the aquatic environment and the few existing studies involved with different fish species, different pathogens and different methods. Therefore, comparing results and understanding trends in this field of freshwater fish microbial epidemiology are challenging. This finding indicated that Nile tilapia were relatively more susceptible to the potential bacterial pathogens than carp. This is in line with the finding of Meron *et al.* (2020) who found that Niletilapia was very sensitive to different potential bacterial pathogens especially gram-negative bacteria *Aeromonas* spp., and *pseudomonas* spp.

Among the identified bacteria, the most prevalent potential pathogens (*A. hydrophila*, *A. veronii*, *E. tarda*, *P. hunanensis*, *P. mirabilis*, *S. flexneri* and *S. maltophilia*), were evaluated for their antibiotic susceptibility profiles. The majority of the pathogens evaluated were resistant to most of the antibiotics evaluated. *Aeromonas veronii* and *S. flexneri* were resistant against 75% of the antibiotics evaluated. All the bacterial isolates showed multiple resistances to various drugs tested. Except *S. maltophilia* all the others pathogens were sensitive to gentamicin and this is in agreement with the findings of (Gufe *et al.*, 2019). The majority (87.5%) of the pathogens were resistant to penicillin and vancomycin. The susceptibility levels of each pathogen were relatively low. This result is in line with the finding of (Wamala *et al.*, 2018) who investigated the occurrence and antibiotic susceptibility profiles of fish bacteria infecting *Oreochromis niloticus* (Nile tilapia) and *Clarias gariepinus* (African catfish) in Uganda and found the prevalence of *Aeromonas* spp., (*A. hydrophila* and *A. sobria*) and *E. tarda* in naturally infected Nile tilapia (*Oreochromis niloticus*). Similar

findings have been also reported in Egypt in previous studies (Ayoub *et al.*, 2021).

Antimicrobial resistance is a worldwide public health concern that has drawn attention in the recent time (Roca *et al.*, 2015). Existence of antibiotic resistance amongst these bacterial strains may have public health concern. Fishery sector is a rapidly growing industry providing fish food for human consumption worldwide (Cantas *et al.*, 2013). However, fish farming is confronted with acute problems of disease like bacterial, fungal and viral disease (El-Barbary and Hal, 2016).

The use of antimicrobials has become a customary practice to control those pathogens that provides ideal conditions for the emergence of resistant bacterial strains and stimulates horizontal gene transfer (Santos and Ramos, 2018). The authors further commented that the passage of antimicrobial resistance genes and resistant bacteria from aquatic to terrestrial animal husbandry and to the human environment can have detrimental effects on both human and animal health, and on aquatic ecosystems. Generally, findings from the present study indicate a high frequency of bacterial pathogens from the collected naturally infected fish samples and good evidence of resistance in the antimicrobial susceptibility profiles of evaluated pathogens. But, it has certain notable limitations. It neither quantified the detected bacteria, nor assesses seasonal patterns of fish microbiota. The results would have been more robust if samples from fish skin and gills which are gateway routes of transient or resident microbiota and/or potential pathogenic bacteria have been included. Moreover, fish microbiota variations based on the geographical environment and seasonal variation should be explored in future studies by adding more populations and larger sampling sizes.

CONCLUSION

Bacterial fish pathogens are prevalent in Ethiopia and could partly be responsible for the fish-borne diseases and observed reduction in fish production in the country. The isolated fish bacterial pathogens exhibited high resistance to a number of antibiotics and this would accelerate the emergence and spread of antimicrobial resistance in the aquatic environments from where most of the fish have been harvested for human consumption.

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