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Identifying bacteria associated with diseased *Oreochromis niloticus* in Lake Kariba, Zambia

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Abstract. *Sakala T, Mdegela RH, Hangombe BM. 2022. Identifying bacteria associated with diseased* Oreochromis niloticus *in Lake Kariba, Zambia. Bioteknologi 19: 62-70.* The recent intensified aquaculture projects in Lake Kariba, Zambia, have brought about disease outbreaks in cultured *Oreochromis niloticus* (Linnaeus, 1758). This study aimed to identify bacteria associated with diseased *O. niloticus* and establish their antibacterial resistance patterns. Caged fish were identified as diseased based on behavioral and physical abnormalities, including swimming in circles, swimming in lateral or dorsal recumbency, ocular opacity, hyperpigmentation, fin erosions, and ulcerations. A total of 25 sick and 4 healthy fish were sampled. Samples from the liver, spleen, brain, abdomen, kidney, blood, and ulcers were inoculated on 10% sheep-blood agar and nutrient agar. Then the isolates were classified by genera using biochemical tests and standard culture. Furthermore, the bacterial isolates were tested for resistance to commonly used antibacterial compounds in aquaculture using the disc technique; the spleen, eyes, and liver had the highest number of pathologies in descending order. A total of 15 bacteria genera were identified, where *Lactococcus/Streptococcus* genera had the highest prevalence with 46.2%, then *Aeromonas* at 11.5%. All isolates have been observed to have multiple drug resistance, with two isolates, each of *Lactococcus Streptococcus* and *Aeromonas* exhibiting complete resistance to most antibiotics tested. The results suggest that increased biomass in diseased cages may be the main risk factor for the disease, with the immune and regulatory organs being first to succumb. The lake environment is a mixing vessel of various microorganisms that show multiple antibacterial resistance. Therefore, risk factors surrounding the existence of these bacteria genera must be accessed, and a more comprehensive, comparative study in antibiotic resistance on farmed, in contrast to wild species, in Lake Kariba, Zambia.

Keywords: *Aeromonas*, bacteria, Kariba, *Oreochromis niloticus*, *Streptococcus*

INTRODUCTION

Fish are a crucial food source worldwide, providing energy, protein, and a range of essential nutrients as they are eaten by over 2.9 billion people (FAO 2014). As people are consuming more fish, the dependence on the fisheries and aquaculture sector has increased (FAO 2016). Aquaculture has intensified in many parts of the world, including Chile, Brazil, China, India, Norway, Bangladesh, Morocco, Nigeria, Uganda, Ghana, and Egypt (FAO 2014; FAO 2016). This intensive production has brought with it the development of disease outbreaks. The host (fish), pathogen, and environment triad are generally balanced-relationship, regulated primarily by the immune system. However, some aquaculture-related practices and consequences, such as high densities, can cause stress leading to lowered immunity and, ultimately, disruption of the balanced triad relationship (Huicab-Pech et al. 2016). That can result in bacterial disease outbreaks in which microflora found within the natural aquatic environment may be implicated (Austin 2006; Helmy and Atallah 2015). Antibacterial agents have been used in small sub-therapeutic doses in feed as growth promoters or to prevent disease in fish. For example, they have been used practically to treat an entire population to protect healthy individuals until the sick fish die, and the infection subsides. This form of oral treatment leads to sub-therapeutic doses, enabling selection for resistance in bacteria (Thorsen 2014).

Currently, the most common species farmed in Zambian aquaculture are from the Cichlid family, namely, *Oreochromis andersonii* (Castelnau, 1861) (64%), *Tilapia rendalli* (Boulenger, 1897) (20%), *O. niloticus* (Linnaeus, 1758) (5.2%), and *O. macrochir* (Boulenger, 1912) (5%). Commercial *O. niloticus*, due to its hardy nature and economical production (Popma and Masser 1999), has been a profitable source of income on Lake Kariba, Zambia. However, with reports of resistant bacteria recently have been investigated and confirmed in *O. niloticus* and catfish in aquaculture within Africa (Ekundayo et al. 2014; Tiamiyu et al. 2015), this study aims to lay a foundation for resistance to antibacterials and the prevalence of bacteria associated with sick fish in cages in Lake Kariba.

The growing cage culture of Nile tilapia (*O. niloticus*) on Lake Kariba, Zambia, has risen because of the many advantageous qualities of the species. However, there has been an outbreak of the bacterial disease in cultured *O. niloticus*, with reported cases from 2015 to the present. On-going anthropogenic activities on Lake Kariba and recently established aquaculture projects may have affected the aquatic environment. These may have changed the environmental setup, leading to a situation where bacteria

populations increase as the host, *O. niloticus*, is concentrated in one location. Therefore, there is a need to investigate the disease in fish cultured commercially, the sensitivity to antibacterial agents of the cultured fish, and the establishment of baseline information. Furthermore, there is no information on public health issues surrounding fish found in this lake.

This study aimed to investigate the most prevalent bacterial isolates present on Lake Kariba and their sensitivity towards commonly used antimicrobials in aquaculture or present within the lake environment.

MATERIALS AND METHODS

Study area

The study area for this research was a commercial cage farm within the Siavonga town in the Southern Province of Zambia (Figure 1). The site is located at latitude 16°S 28.318", longitude 28°E 38.52", on Lake Kariba. Lake Kariba is one of the world's largest artificial lakes and reservoirs by volume, covering an area of 5,580 square kilometers, which lies 1.300 kilometers upstream from the Indian Ocean, along the border between Zimbabwe and Zambia.

Lake Kariba is home to a variety of freshwater fish species, including Tigerfish (*Hydrocynus vittatus* Castelnau, 1861), Catfish (*Clarias gariepinus* Burchell, 1822), Barbel (*Barbus barbus* Linnaeus, 1758), Labeo (*Labeo rohita* Hamilton, 1822), Jack (*Caranx lugubris* Poey, 1860), Vundu (*Heterobranchus longifilis* Valenciennes, 1840) and Bream/Tilapia (*O. niloticus*). These were introduced and reared under cage aquaculture facilities (Mudenda 1994).

Sample size

Sampling was such an outbreak investigation. In line with this, to enable detection at a 95% confidence level, infected animals must have a case of clinical infection which requires sampling 10 diseased fish; combined to form pools of a maximum of 5 fish each. These include detecting asymptomatic carriers that involve samples combined in pools of no more than 5 fish per pool (OIE 2003). According to Midlyng et al. (2000), the recommended minimum number of adult fish (>150g) to be sampled for outbreak investigation is a sample size of 5.

Sampling technique

A total of 29 fish were captured during feeding time using a disinfected scoop net from four different cages on Lake Kariba. The subtotal of 25 'sick' fish and four 'healthy' fish were selected purposively from four of the nine cages experiencing disease and increasing mortalities. Fish were initially thoroughly examined for external lesions, measured (total length and circumference) using a standard measuring tape, and weighed using an electronic balance.

Swabs were collected from the abdomen, spleen, liver, brain, eye, gonads, blood, and skin lesions on each fish. The swabs were then inoculated onto freshly prepared, appropriately labeled Nutrient agar and 10% sheep blood agar (HiMedia Laboratory Pvt, Mumbai, India) plates by streaking using sterile disposable loops on-site. Individual organ samples were then collected and stored in 10% buffered formalin, and blood smears were prepared. Culture plates and samples were stored at room temperature for 24 hrs and then transported to the laboratory under icepacks at -4°C. The organisms were initially grown on Nutrient and Blood Agar media (HiMedia Laboratory Pvt, Mumbai, India) for primary isolation. Next, culturing was done on-site by streaking with a sterile inoculating loop.

Isolation and classification of bacteria

Culture of bacteria

Samples were processed at the School of Veterinary Medicine, Department of Paraclinical Studies, University of Zambia (Woodland 2004). Isolation involved three stages; firstly, the different primary bacteria isolates were individually sub-cultured on Nutrient and Blood agar (HiMedia Laboratory Pvt, Mumbai, India). Then, to ensure that possible contaminants were absent, they were incubated at room temperature (25°C) for 24 hrs. Finally, based on colony morphological appearance, all different bacteria colonies from all organs of each sampled fish were each noted and labeled clearly.

Gram stain and morphology characteristics

The pure colonies produced were then Gram-Stained to determine Gram-Positive or Gram-Negative nature and microscopic morphological appearance. Each pure colony was first emulsified in sterile normal saline on a well-labeled, clean, dry glass slide. That was then air-dried and fixed under a Bunsen burner. After that, the slides were stained with Crystal Violet solution for 45 seconds and washed under gently running water. The slides were then flooded with iodine solution for 45 seconds and washed under gently running water again. The slides were then decolorized with 70% alcohol solution, followed by gentle washing under running water. Finally, the slides were counter-stained with safranin solution for 45 seconds, followed by gentle washing under running water. They were then air-dried and viewed at X100 magnification under oil immersion. The microscopic morphology characteristics and Gram stain were then viewed and noted (Buller 2014). Finally, the following biochemical tests were performed to confirm the suspected isolates: Simmons Citrate, SIM (Sulphur, Indole, Motility) test, Triple Sugar Iron test, Urease, and carbohydrate 'sugar' utilization tests (Esculin, Galactose, Raffinose, Salicin, Maltose monohydrate, Xylose, Mannitol, Trehalose, Insulin, Sorbitol, Lactose monohydrate, and Glucose).

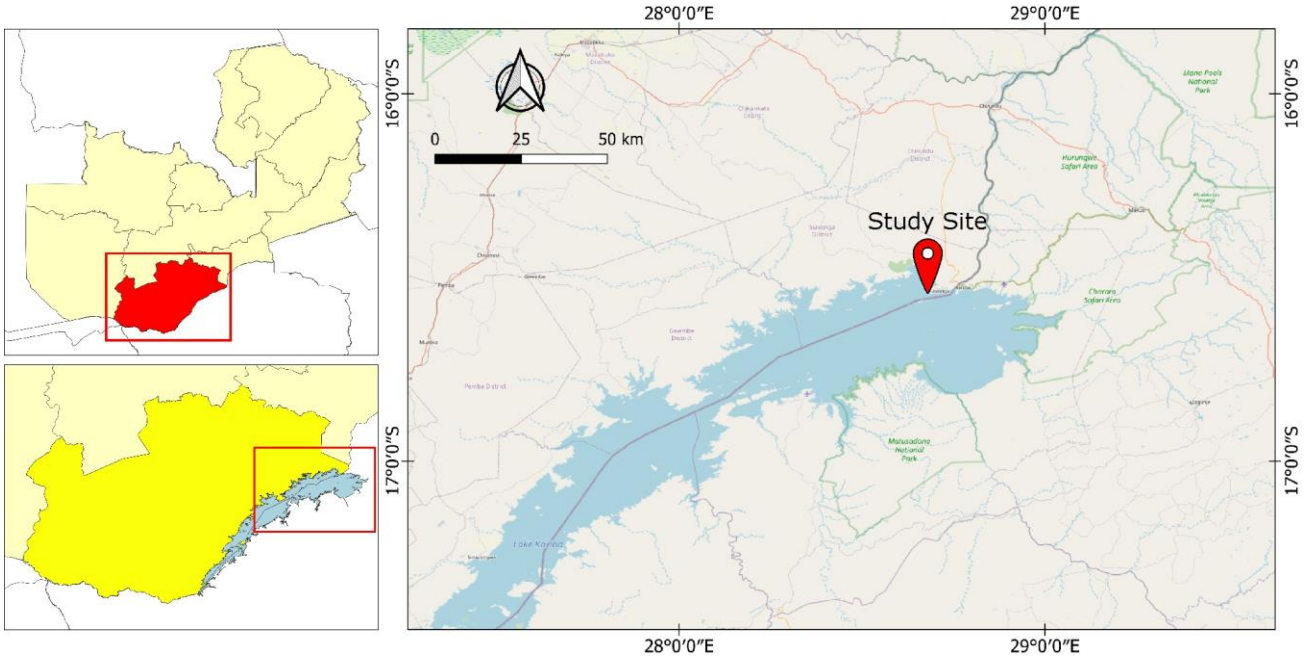


Figure 1. Study area- commercial cage farm on Lake Kariba, Zambia

Phenotypic identification of bacterial isolates

Sulphur indole motility test. The SIM media was prepared according to the manufacturer's protocol (HiMedia Laboratories, India). It was distributed in short tubes and autoclaved at 121°C for 15 minutes. The tubes were allowed to solidify for 24 hrs, producing a clear, light-yellow colored media. The pure isolates grown on nutrient agar were then collected using a sterile straight-wire loop and inoculated into the media by stabbing once under a Bunsen burner to maintain sterility. That was followed by incubation for 24 hrs at 37°C. After 24 hrs, the media was viewed for motility (cloudy appearance) and production of Sulphur gas (blackening). That was followed by adding two to three drops of Kovac's reagent to the suspension using a dropper and waiting briefly for 5-10 seconds for the reaction. The formation of a pink-colored ring that rises to the surface indicated a positive Indole result, whereas the presence of no pink-colored ring meant a negative Indole result.

Simmons citrate test. The Simmons citrate agar was prepared according to the manufacturer's protocol (HiMedia Laboratories, India). The agar was prepared according to the manufacturer's protocol. It was distributed in long tubes and autoclaved at 121°C for 15 minutes. The tubes were then allowed to solidify for 24 hrs, producing a clear, crystal-green colored media. Pure isolates of the organisms were then collected from nutrient agar and inoculated into the agar using a sterile straight-wire loop by gently streaking the media slant under the Bunsen burner to maintain sterility. The media was then incubated for 24 hrs at 37°C. The Citrate agar was green in color before inoculation. However, a positive result was obtained when the color changed to blue, meaning that the citrate was utilized, whereas in a negative result, there was no color change, and the media remained green.

Triple sugar iron test. The TSI agar was prepared according to the manufacturer's protocol (HiMedia Laboratories, India). First, it was distributed in long tubes and autoclaved at 121°C for 15 minutes. Next, the tubes were allowed to solidify for 24 hrs, producing a light-orange colored media. Next, the pure isolated colony was picked with a sterile, straight-wire loop, followed by the first stabbing of the agar and then gently streaking the slant under the Bunsen burner to maintain the surface of the slant sterility. It was then incubated at 37°C for 24 hrs. The results were read and interpreted according to Table 2.

Urease test. The urease media was prepared according to the manufacturer's protocol (HiMedia Laboratories, India). First, it was distributed in short tubes and autoclaved at 121°C for 15 minutes. Next, the tubes were allowed to solidify for 24 hrs, producing a yellowish-orange clear media. The pure isolates grown on nutrient agar were then collected using a sterile, straight-wire loop and inoculated into the media by stabbing once under a Bunsen burner to maintain sterility. That was followed by incubation for 24 hrs at 37°C. After 24 hrs, the media was observed for color change; a brick-red color meant a positive result, while a yellowish-orange color meant a negative result, meaning that the bacteria did not utilize the media.

Identification using sugars

The different strains were tested for the biochemical reaction using 12 sugars and alcohol; disaccharides (maltose monohydrate, trehalose), hexoses (glucose, mannose, and galactose), pentose (xylose), polyhydric alcohols (mannitol, sorbitol, inulin, and salicin), trisaccharides (raffinose) and Esculin (HiMedia Laboratories, Mumbai, India), were prepared according to manufacturer's protocol. That was performed in a tube of phenol red broth containing either one percent sugar or

alcohol, followed by inoculation with a single bacterial isolate using a sterile straight wire. The broth tubes were incubated at 30±0.5°C for 48 hrs, and the results were recorded as positive if the production of acid condition induced a change in the red phenol indicator from pink to yellow.

Antibacterial susceptibility test of bacterial isolates

The Kirby-Bauer disc diffusion antibacterial sensitivity test method was used to test the antibacterial resistance of the bacterial isolates. A sum of 9 antibacterial agents belonging to 5 antibacterial classes (Beta-lactam Penicillins, macrolides, tetracyclines, aminoglycosides, and sulphonamides) were used to determine the antibiograms of the isolates. Ten antimicrobial drugs were used, among them Penicillin-G (P 10 µg), Amoxiclav (AMC 30 µg), and Amoxicillin (AMX 10 µg) (HiMedia Laboratory Pvt, Mumbai, India), selected for being readily available and efface against Gram-Positive bacteria. In addition, cefotaxime (CTX 30 µg), Ciprofloxacin (CIP 5 µg), and Norfloxacin (NX 10 µg) (HiMedia Laboratory Pvt, Mumbai, India) have known efficacy against Gram-Negative bacteria. In contrast, Tetracycline (TE 30 µg), Erythromycin (E 5 µg), and Co-trimoxazole (COT 25 µg) (HiMedia Laboratory Pvt, Mumbai, India) are broad in their effect (Thompson MICROMEDEX 2003).

Mueller-Hinton agar was prepared according to the manufacturer's protocol (HiMedia Laboratory Pvt, Mumbai, India). First, the organisms were purified on nutrient agar. Next, a loop full of bacterial colonies was collected using a sterile, round-wire inoculating loop, which was then streaked onto the Muller Hinton agar surface plate until their surface was thoroughly covered under Bunsen flame to ensure a sterile environment. Next, using a pair of sterile forceps, the antibiotic discs were removed from the dispensers and then gently placed on the agar while ensuring each disc was fixed on the agar surface. Next, the discs were placed equidistant from each other, with only five antibiotic discs placed per plate to ensure clarity of results. The plates were then placed upside down and incubated for 24 hrs at room temperature (NCCLS 2000). The sensitivity of each isolate was then read by measuring the clear, circular diameter around each disc. These results were recorded in millimeters and later classified as susceptible, intermediate, and resistant.

Data analysis

Microsoft Excel 2010 was used for data storage and computation of prevalence. Chi-square tested the relationship between weight and disease severity at a 95% confidence interval.

RESULTS AND DISCUSSION

Field observations of sampled fish

Based on appearance and behavior, observations indicating the presence of disease included abnormal swimming, positioned in dorsal or lateral recumbency, and swimming in circles. The skin was abnormal, with a highly pigmented or black external appearance. The ulcers were present around the mouth and body with eroded fins. The most affected fins were the dorsal and tail fins. The eyes were blind (appearing white) and exophthalmos or protruding eyes.

Bacterial isolates

Primary isolation was performed on-site. Out of the initial 462 plates inoculated, 98 plates did not grow, and 364 plates had growth; 179 isolates were on Blood Agar, and 185 isolates were on Nutrient Agar. These were sub-cultured in the laboratory on Nutrient and Blood agar giving pure colonies (Figure 2). Gross morphology on both media was then recorded (Table 3).

Chi-square test

χ^2calculated value = 0.529

χ^2 expected value = 3.841

P (α) = 0.05; χ^2 Calculated value < 3.841 reflects no statistical significance H_O has been accepted

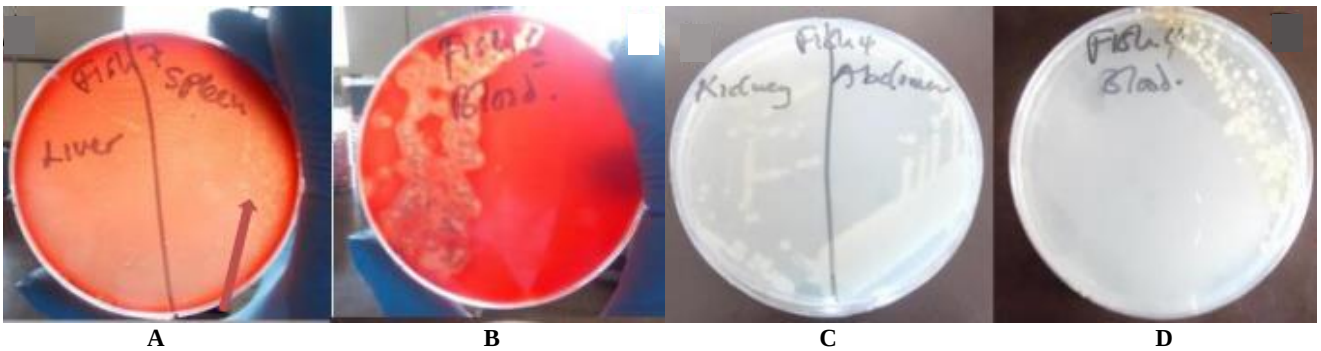
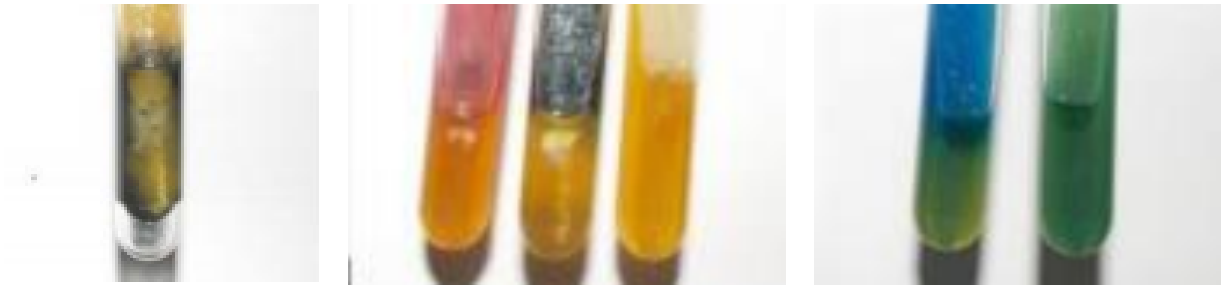


Figure 2. Labeled bacterial isolates: A. Liver and spleen isolates of fish 7 showing hemolysis on blood agar, B. Blood isolates of fish 6 showing hemolysis on blood agar, C. Kidney and abdominal isolates of Fish 4 on nutrient agar, D. Blood isolate of fish 4 on nutrient agar



A B C

Figure 3. Biochemical test results of labeled bacterial isolates: A. TSI illustration of acid throughout medium, hydrogen sulfide production (blackening) and gas production, B. TSI result, from left; acid butt and alkaline slant, middle; acid throughout medium and hydrogen sulfide production (blackening), right; acid throughout medium (Table 1), C. Simmons citrate result, left; blue slant positive test result, right; no color change negative test result

Gram-staining and morphology

The sub-cultured (purified) colonies were then Gram-Stained and observed under oil emersion at X100 magnification. Bacteria were classified based on gram-staining and morphology characteristics. Based on gross colony description and Gram-Stain, 78 bacterial isolates were selected and segregated as representative of all different isolates present from each fish. Of these, 56 were Gram-Positive, and 22 were Gram-Negative.

Biochemical tests

A sum of 16 biochemical tests was performed on each fish's representative bacterial colonies (Figure 3). The biochemical test results were compared to those stated in the literature to identify the bacterial colonies (Buller 2004).

Table 1. Gross pathologies observed in the organs of sick fish during post-mortem

Sites sampled	Frequency of observed pathologies (n=31)	Percentage (%)
Brain	3	9.7
Eye	9	29.0
Abdominal cavity	4	12.9
Spleen	7	22.6
Liver	6	19.4
Gonads	0	0
Kidney	2	6.5
Blood	0	0

Note: n: Total number

Table 2. Interpretation of triple sugar iron agar reaction slants

Appearance	Reactions
Acid butt: yellow, alkaline; slant: red	Glucose fermented
Acid throughout medium: butt and slant yellow	Glucose, sucrose and/or lactose fermented
Gas bubbles in butt and medium frequently split	Gas production
Butt shows blackening	Hydrogen sulfide produced
Unchanged or alkaline butt and slant: medium red throughout	None of the three sugars fermented

Source: Carter (1984)

Antibiograms

Table 6 summarizes the resistance profiles of bacteria isolates to the selected antibacterials.

Table 3. Prevalence of bacterial isolates at genus level based on morphological characteristics, Gram-Staining, and biochemical test screening

Identified bacterial isolates (genus)	No. of isolates (n=78)	Prevalence %
Aeromonas spp.	9	11.5
Aequorivita spp.	1	1.3
Enterococcus spp.	2	2.6
Serratia spp.	1	1.3
Lactococcus/Streptococcus spp.	36	46.2
Citrobacter spp.	1	1.3
Corynebacterium spp.	6	7.7
Edwardsiella spp.	2	2.6
Acinetobacter spp.	1	1.3
Bacillus spp.	2	2.6
Klebsiella spp.	1	1.3
Staphylococcus spp.	3	3.9
Norcardia spp.	1	1.3
Carnobacterium spp.	1	8.0
Rhodococcus spp.	1	1.3
Unidentified Bacteria	5	6.4

Note: n: Total number, %: percentage

Table 4. Frequency of bacteria identified at genus level in fish sampled

Bacteria identified (genus)	No. of fish associated with the bacteria
Aeromonas spp.	8
Aequorivita spp.	1
Enterococcus spp.	2
Serratia spp.	1
Lactococcus/Streptococcus spp.	25
Citrobacter spp.	1
Corynebacterium spp.	3
Edwardsiella spp.	1
Acinetobacter spp.	1
Bacillus spp.	2
Klebsiella spp.	1
Staphylococcus spp.	3
Norcardia spp.	1
Carnobacterium spp.	6
Rhodococcus	1

Table 5. Frequency of bacteria isolated from the internal organs of diseased *Oreochromis niloticus* at the genus level

Identified bacterial isolates	Brain	Eye	Abdominal cavity	Spleen	Liver	Gonads	Kidney	Blood
Aeromonas	-	3	1	1	1	-	2	1
Aequorivita	-	-	-	-	-	1	-	-
Enterococcus	-	1	-	1	-	-	-	-
Serratia	1	-	-	-	-	-	-	-
Lactococcus/Streptococcus	4	4	3	6	4	3	4	4
Citrobacter	-	-	1	-	-	-	-	-
Corynebacterium	2	-	-	-	-	-	3	1
Edwardsiella	-	-	1	-	1	-	-	-
Acinetobacter	-	-	-	1	-	-	-	-
Bacillus	1	-	-	-	-	-	-	1
Klebsiella	-	-	-	-	-	-	-	1
Staphylococcus	-	1	1	-	-	1	-	-
Norcardia	-	-	-	-	-	1	-	-
Carnobacterium	-	-	2	1	-	1	-	2
Rhodococcus	-	1	-	-	-	-	-	-
Total	8	10	9	10	6	7	9	10

Table 6. Antibacterial resistance profiles of bacteria isolates

	Bacteria% resistance														
A/B	**Aer**	**Aeq**	**Et**	**Ser**	**Lt/Stp**	**Cit**	**Cor**	**Ed**	**Acn**	**Bac**	**Kle**	**Stph**	**Nr**	**Carn**	**Rhd**
P 10µg	88.9*	-	100	100*	61.1	-	83.3	100*	-	100	100*	100	-	66.7	100
AMC 30 µg	100*	100*	100	100*	36.1	-	50.0	50*	-	100	50*	33.3	-	33.3	100
AMX 10 µg	100*	-	100	100*	72.2	-	83.3	50*	100*	100	50*	100.0	-	66.7	100
CTX 30 µg	44.4	-	100*	-	61.1*	-	50.0*	50	-	50*	100	66.7*	-	50.0*	-
TE 30 µg	55.6	-	50.0	-	13.9	-	33.3	50	-	100	50	-	-	33.3	-
E 5 µg	100	-	50.0	-	30.6	-	66.7	100	-	100	50	100.0	-	50.0	-
CIP 5 µg	55.6	-	100*	-	41.7*	-	33.3*	-	-	50*	50	33.3*	-	50.0*	-
COT 25 µg	33.3	-	100	-	75.0	100	50.0	50	-	50	100	33.3	-	66.7	100
NX 10 µg	44.4	-	100	-	69.4	100	16.7	-	-	50	100	33.3	-	66.7	-

Note: P: Penicillin, AMC: Amoxiclav, AMX: Amoxicillin, CTX: Cefotaxime, TE: Tetracycline, E: Erythromycin, CIP: Ciprofloxacin, COT: Co-trimoxazole, NX: Norfloxacin, A/B: Antibiotic, Aer: *Aeromonas*, Aeq: *Aequorivita*, Et: *Enterococcus*, Ser: *Serratia*, Lt/Stp or *Lacto/Strep.*: *Lactococcus/Streptococcus*, Cit: *Citrobacter*, Cor: *Corynebacteria*, Ed: *Edwardsiella*, Acn: *Acinetobacter*, Bac: *Bacillus*, Kle: *Klebsiella*, Stph: *Staphylococcus*, Nr: *Norcardia*, Carn: *Carnobacteria*, Rhd: *Rhodococcus*, *Probable Natural resistance

Discussion

The importance and significance of this study have been driven mainly by reports of disease outbreaks in commercially cultured *O. niloticus* on Lake Kariba, Zambia. These outbreaks have been recorded from as far back as 2014, thus the need to study bacterial organisms that may be associated with these outbreaks.

The fish was first observed for abnormal features and behavior in their natural habitat. Upon careful observation, 'sick' fish were singled-out. That was based on common clinical signs, including abnormal swimming, positioned in dorsal or lateral recumbency, swimming in circles, skin that was highly pigmented, giving an almost black external appearance, eroded fins, dorsal and tail fins visible, ulceration around the mouth and body, blind eyes (appearing white) and exophthalmos or protruding eyes (Austin and Austin 2007; Noga 2010; Parker 2012).

The clinical signs that were observed in the cultured *O. niloticus* have been associated with infection by bacteria, namely; *Aeromonas* (Belém-Costa et al. 2006), *Pseudomonas* (Amutha and Kokila 2016), *Edwardsiella* (Amal and Zamri-Saad 2011; Dong et al. 2015), *Flavobacterium* (Al-harbi et al. 2005; Huicab-Pech et al. 2016) and *Streptococcus* (Iregui et al. 2004; Musa et al. 2009; Anshary et al. 2014; Pretto-Giordano et al. 2015) species.

It has been said that waters with a high organic load, which favors the multiplication of bacteria, rapidly changing temperatures, overcrowding, trauma, and transportation are the most encountered environmental stress factors which predispose to clinical disease in fish. Intensive fish culture systems, such as Lake Kariba, are particularly likely to give rise to these factors (Roberts 2012; Huicab-Pech et al. 2016); as fish grow within the cage, their body size increases. The farm management reported that sick fish originating from cages delayed harvest by 2-3 months. A market size of 700 g is attained in about 4 months, according to Anonymous (2016), though market traders, restaurants, and the public generally require varying weights regularly. In Zambia, the ideal harvest size of fish is 400-500 g. Therefore, it could be concluded that the sick fish's delay in harvest resulted in increased biomass beyond that which the caged environment could support. These resulted in a stressful condition that opened the opportunity for infection by opportunistic organisms.

A Chi-square test was performed to determine any correlation between the sick fish's weights and infection severity. Mild infection was characterized by petechial hemorrhage and fin erosion. Severe infection was characterized by clinical signs, including blindness, open wounds, ulcers, and abscesses, among those presented with mild infection. Results revealed no significant relationship between the parameters; the weight of the sick fish was not related to the severity of outward clinical signs observed.

The organs with the most abnormalities included the eye, spleen, and liver, with 29.0%, 22.6%, and 19.4%, respectively (Table 5). The eye is one of the most sensitive organs, with the retina having among the highest oxygen demands of any tissue in the body (Helfman et al. 2009). With any homeostatic imbalances, the eye is often one of the first to show signs of underlying disease. That was clearly observed during the examination, with signs of blindness, opacity, and protrusion (exophthalmos) in most sick fish. The spleen is one of the organs primarily responsible for immunity, with the liver involved in blood chemistry maintenance. Perhaps for these reasons, they were severely affected by the disease.

Based on bacterial culture, morphology, Gram-Staining characteristics, and a series of biochemical evaluations and classification, an overwhelming majority, at 46.2%of these isolates, were identified as *Lactococcus* or *Streptococcus* species. Furthermore, 11.5% were identified as being *Aeromonas* species. Among the other 15 bacterial genera identified, *Corynebacterium* was 7.7%, and *Staphylococcus* was 3.9%. The rest was belonged to other bacteria. Therefore, based on the study, the pathogens most likely present in diseased *O. niloticus* on Lake Kariba include *Streptococcus*/*Lactococcus*, *Aeromonas*, *Corynebacterium*, *Carnobacterium*, and *Staphylococcus* species. *Streptococcus*/*Lactococcus* and *Aeromonas* species have been some of the most widely implicated bacteria in disease outbreaks, mainly due to their opportunistic nature (Belém-Costa et al. 2006; Austin and Austin 2007; Musa et al. 2009; Amal and Zamri- Saad 2011; Abdelsalam et al. 2013; Ahmed and El-Refaey 2013; Haenen et al. 2013; Anshary et al. 2014; Pretto-Giordano et al. 2015; Amutha and Kokila 2016; Noga 2010; Roberts 2012; Huicab-Pech et al. 2016). They can survive in the natural environment in a dormant state and invade host tissues once there is destabilization in the environment and/or host. *Streptococcus*/*Lactococcus* was the most isolated across all eight organ sampling sites. The fish observed as being 'sick' had manifested full-blown disease due to stressful cage conditions, which was proven by the clinical signs and post-mortem lesions documented in sampled fishes 1, 8, and 14. These lesions included fin, nasal and buccal erosion and ulceration, ascites, and under-belly and petechial body hemorrhages. These are lesions characteristic of Aeromonas infection (Austin and Austin 2007; Ibrahim et al. 2008; Woo 2011; Roberts 2012).

Bacteria from the family Streptococcae are found within the natural aquatic environment and are known to be naturally opportunistic. Disease caused by these bacteria has been associated with poor husbandry and excessive stocking densities (Roberts 2012). Specific causative agents of disease outbreaks worldwide include *Lactococcus garvieae* (Woo 2011; Roberts 2012; Helmy and Atallah 2015), *Streptococcus iniae* (Mcnulty et al. 2003; Baiano and Barnes 2009; Pretto-Giordano et al. 2015) and *Streptococcus agalactiae* (Iregui et al. 2004; Jafar et al. 2008). Disease outbreaks usually occur when fish have been exposed to stress, including increased water temperature, suboptimal water oxygen levels, or overcrowding for a long time. *Streptococcosis*, theoretically, affects all fish sizes; however, bigger fish (from 100 g to market size) are usually most susceptible to the disease (MSD Animal Health 2006). *Lactococcus*/ *Streptococcus* was isolated in sick fish, with an average weight of 547.8 g. These fish exhibited clinical signs of abnormal swimming, positioned in dorsal or lateral recumbency, spiral swimming, and lesions, including numerous hemorrhages all over the body, wounds, ulcers, and hyperpigmentation. That is documented in the literature (Al-harbi et al. 2005; Musa et al. 2009; Noga 2010; Roberts 2012; Ahmed and El-Refaey 2013; Huicab-Pech et al. 2016) as being characteristic of *Lactococcosis*/ *Streptococcosis*.

The eye is also a major point of bacterial infiltration, being one of the most sensitive organs of the fish (Helfman et al. 2009). In particular, *Streptococcus agalactiae* is known to cause unilateral and bilateral ocular lesions and has a tropism for the central nervous system (CNS) (Iregui et al. 2004; MSD Animal Health 2006; Jafar et al. 2008; Roberts 2012). The study revealed that nine (9) out of the twenty-five (25) fish from which *Lactococcus*/*Streptococcus* was isolated had either ocular lesions or both ocular and brain lesions. These findings show to the possible causative agent is *Streptococcus agalactiae*. *Lactococcosis*/*Streptococcosis* generally leads to inflammation and necrosis of the liver, spleen, kidney, eye, and brain, and septicemia as a hematogenous infection (MSD Animal Health 2006; Roberts 2012). That has been evidenced by the organs in which bacteria were isolated from the various fish.

The sick fish were overdue for harvest by 2 months with maintained regular feeding. Caged fish have a relatively small surface area to volume ratio compared to ponds and raceways. That was coupled with a more restricted food supply (dispersing feed over a comparatively small area of the cage) that resulted in greater competition and more pronounced disparity in food acquisition among individuals (Beveridge 2004). In this case, stocking the 900 m3 cages was on the upper limit, thus more contact between individuals. Competitive and defensive feeding behavior such as high speed, jaw protrusion, and spreading of the fins (Helfman et al. 2009), coupled with biomass beyond that which the cage is meant to support, would result in a high incidence of injury, leading to open wounds. Diseases such as Streptococcosis are transmitted horizontally from fish to fish (via cannibalism and skin injuries) and from the aquatic environment to fish (MSD Animal Health 2006).

Among the bacteria isolated in the fish included *Bacillus* (Table 4), which has been shown to have probiotic properties as lactic acid-producing bacteria. The same as

Carnobacterium and *Rhodococcus*, which have also been discovered to have the same probiotic properties (Takyi et al. 2012). Bacteria, including *Norcardia* and *Citrobacter*, are known to be commensals in the aquatic environment and surrounding soil (Roberts 2012; Takyi et al. 2012). *Staphylococcus*, *Serratia*, and *Klebsiella* are opportunistic bacteria and may manifest in heavily stressed fish, evidenced by the study. These bacteria may pose public health risks associated with human pollution, as with some Enterococci species (Marcel and Sabri 2013). *Aequorivita* is a genus under the Flavobacteria family, mostly associated with living and dead phytoplankton in the natural environment (Bowman and Nichols 2017). *Edwardsiella septicemias* and ulcerative conditions have been documented in various fish species worldwide (Austin and Austin 2007; Roberts 2012; Huicab-Pech et al. 2016). Bacterial species under the *Corynebacteria* genera have been implicated in widespread disease conditions affecting the kidney in fish species other than *Oreochromis* (Woo 2011; Buller 2014). *Acinetobacter* species have been labeled as emerging fish pathogens in other fish species. These strains have been commonly known as microorganisms transmitting antibiotic resistance genes, which may greatly impact the resistance transfer in aquaculture (Kozińska et al. 2014).

With disease outbreaks in Zambia in *O. niloticus* cage aquaculture, the active surveillance of disease pathogens has been a priority. Disease-causing pathogens isolated and confirmed at the molecular level include *Streptococcus iniae*, *Lactococcus garvicae*, and *Aeromonas hydrophila* (Hangombe and Ndashe 2015). The bacteria isolated were tested for sensitivity against some commonly used antibacterial agents in aquaculture. Some documented antibacterials include ROMET 30® or ROMET TC® (sulphadimethoxine and Ormetoprim), Aquaflor® (Florfenicol), and Terramycin® in feed formulations (Sekkin and Kum 2011; Kelly 2013). Intensification and the advent of disease outbreaks have occasion for developing resistant bacterial strains. The study looked at nine (9) commonly used antibacterial compounds, which were meant to give an overview of the profiles of antibacterial classes on the market as treatment options in fish health. According to the research findings, the thirty-six (36) *Lactococcus/Streptococcus* expressed varying levels of antibacterial resistance, with one isolate having total resistance to all the antibacterials tested. The results also showed a set of two having the same resistance patterns and another three having the same pattern, which could indicate them being the same strain. The most effective antibacterial compound was Tetracycline, with the bacterial isolates showing the lowest resistance of 13.9%. Co-trimoxazole was the most effective against the *Aeromonas* spp. (33.3%). Two isolates from different fish had the same resistance profile, indicating that these could be one in the same strain. All six (6) isolates of the Corynebacteria expressed different levels of resistance, with Norfloxacin being the most effective against it (16.7% resistance), Carnobacteria being the most sensitive to Amoxicillin (33.3% resistance), and *Staphylococcus* was most sensitive to Co-trimoxazole, Amoxicillin, and Norfloxacin (all 33.3% resistant).

Multiple resistances have been expressed towards antibacterials commonly used worldwide in aquaculture practices. These results otherwise suggest the possibility of undocumented and/or unregulated use of antibiotics by aquaculture communities on Lake Kariba. There currently have been no records of antibacterial use by fish farmers on Lake Kariba (Pers. comm. 2015). On the contrary, the antibiogram profiling of the isolates revealed multiple resistance to most of the antibacterial agents. Potential sources of this resistance include antibiotics flushed into the lake from surrounding human settlements and animal husbandry practices. In addition, bacterial populations within the water and the fish may have gained resistance through mutation upon exposure to these antibacterials. Alternatively, the bacteria within the ecosystem may have an innate resistance to the selected antibacterials. These phenomena may only be answered by further in-depth antibacterial screening and testing of the lake and surrounding environment.

This study isolated fifteen (15) bacterial genera from diseased *O. niloticus* on commercial cage fish farms on Lake Kariba. It reflected the vast number of ubiquitous, opportunistic bacterial organisms in the aquatic environment. This profile is relevant to the future of aquaculture establishments on Lake Kariba as intensification practices advance. Furthermore, the bacterial isolates all expressed varying levels of resistance to commonly used and available antibacterials, which ought to be revered in the different lakes' practices and could also be a potential concern to public health.

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