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Occurrence of Antimicrobial Resistant Bacteria Isolated from Farmed Nile Tilapia (*Oreochromis niloticus*) in Zanzibar, Tanzania

Forekomst av antibiotikaresistente bakterier isolert fra oppdrettet Nile tilapia (*Oreochromis niloticus*) i Zanzibar, Tanzania

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Preface

This study is our final year project in the veterinary medicine program at the Norwegian University of Life Sciences (NMBU). We were very lucky to get the opportunity to travel to Zanzibar for the study of antimicrobial resistance (AMR). AMR poses a significant threat to public health, yet the study of this in Zanzibar is scarce. It was exciting to get the opportunity to contribute to the available literature on this topic. We were accompanied and assisted by the helpful professors and scientists at the Institute of Marine Sciences (IMS) and NMBU, without whom this project would not have been possible.

Summary

Title: Prevalence of Antimicrobial Resistant Bacteria Isolated from Farmed Nile Tilapia (*Oreochromis niloticus*) in Zanzibar, Tanzania

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Antibiotic resistance is an increasingly significant problem and a serious threat to both public and animal health. Overuse and misuse are key driving factors behind the development of resistance. Antibiotic resistance can occur in fish farming due to the antibiotic treatment of fish, or contamination from the environment or humans. This study investigated the occurrence of antibiotic resistant bacteria in Nile tilapia farming in Zanzibar. Samples were collected from three different sites. A total of 30 fish were examined, and samples were taken from skin/mucus, the gut, and the head kidney. Antibiotic susceptibility testing (AST) was conducted using a disk diffusion method on Muller-Hinton agar. Bacteria were identified

through 16S sequencing. Overall, the occurrence of antibiotic resistance was low to moderate. The bacteria samples from one of the three sites showed no resistance to the antibiotics tested for. Within the Enterobacteriaceae family, the highest level of resistance was observed against tetracyclines and sulfonamides. Examining the prevalence of resistance for each antibiotic in relation to the overall resistance, the highest levels of AMR were observed against tetracycline, amoxicillin, and sulfonamides.

Definitions and abbreviations

Abbreviation	Definition
AMR	Antimicrobial resistance
AMU	Antimicrobial use
AST	Antimicrobial susceptibility testing
BHI	Brain heart infusion
bp	Base pairs
BTB agar	Bromothymol blue lactose agar
CLSI	Clinical and Laboratory Standards Institute
cm	Centimeters
EUCAST	European Committee for Antimicrobial Susceptibility Testing
g	Grams
IMS	Institute of Marine Sciences
kg	Kilograms
LB	Lysogeny broth
mA	Milliampere
MDR	Multi-drug resistant
MIC	Minimum inhibitory concentration
NCBI	National Center for Biotechnology Information
NFW	Nuclease-free water
NMBU	Norwegian University of Life Sciences
rpm	Rotations per minute
TSA	Tryptone Soya Agar
V	Volts

WHO	World Health Organization
µL	Microliters

Introduction

Species information

Tilapia fish are within the Family Cichlidae, a diverse group of freshwater fishes. The Family Cichlidae are naturally distributed across Africa, the Middle East, the Neotropics and the Indian subcontinent (Genner et al., 2008). There are representatives of three "Tilapia tribes" in Tanzania, with the most species rich tribe being Orechromini, consisting of at least 20 species. This tribe includes species in the genera *Alcolapia* and *Oreochromis*, among others *Oreochromis niloticus* (Nile tilapia) (Genner et al., 2008). Nile tilapia is a large deep-bodied tilapia, with a relatively small head. Males are bluish pink (contrasting colors) while females are usually brownish. In the wild they are found in lakes and slow flowing stretches of streams and rivers (Genner et al., 2008). Nile tilapia are described as being particularly resistant to various diseases, harsh environments, poor water qualities, handling and stress. They have a short generation interval and rapid growth (El-Sayed, 2020 p. 1).

Tilapia farming

Nile tilapia has been introduced to Tanzania through fish farming and are now found in most catchments in the country (Genner et al., 2008). The fish farming industry in Africa grew slowly from the 1950s to the 1980s, due to poor farming methods and technology. The industry has had rapid growth the last few years, with Egypt being in the forefront. In recent years, the fish farming industry in Tanzania has also gained popularity, with the most cultured fish being Nile tilapia. Despite increased popularity, the majority of Sub-Saharan countries, such as Tanzania, report relatively low fish farming production (Mmanda et al., 2020).

Nile tilapia aquaculture can be done intensively or semi-intensively. The goal of intensive production is to maximize production with minimal use of water. It is highly dependent on artificial feeding and water reuse and/or exchange (El-Sayed, 2020 p. 103). Semi-intensive culture is a simpler, cheaper, and more natural production method. Such systems are widely used for fishes low on the food chain, such as Nile tilapia (El-Sayed, 2020 p. 69).

Production practices of tilapia may vary, but they are usually cultivated in artificially built ponds. The ponds can be large or small depending on production scale. Some productions also have cages in the water, and others use recirculating culture systems (El-Sayed & Fitzsimmons, 2023).

Harvest methods depend on the scale of production, pond size, culture system and levels of technology applied. Many small-scale farms adopt partial harvesting techniques using locally available gear, while large-scale tilapia producers use more advanced harvesting tools. Partial harvesting is designed to remove large fish to provide smaller fish more space for growth (El-Sayed, 2020 p. 245).

Reproduction

Nile tilapia are “mouthbrooders”, meaning fertilized eggs are incubated in the female’s buccal cavities. The eggs can be fertilized inside or outside the female's mouth (El-Sayed, 2020 p. 174). Eggs and fry are harvested, either partially or completely, at intervals ranging from 6 to 60 days.

Growth of tilapia fry depend on factors such as stocking density, food type, feeding regimes, photoperiods, water flow and replacement. Mixed-sex culture of tilapia has been a common

practice for decades, but there's been a shift to monosex cultures during the past two decades (El-Sayed, 2020 p. 189).

Age of sexual maturation depends on size and environmental conditions, food supply and water body area. In natural conditions, Nile tilapia mature at 20–30 cm (150–250g), but in fish farms, females can mature at 30-50g (El-Sayed, 2020 p. 175). Females usually grow faster and larger than males (El-Sayed, 2020 p. 189).

An attempt has been made to find information about what age the tilapia is considered large enough for consumption, without success. Through personal conversations with fish farmers in Zanzibar there seems to be a consensus that the fish reach market size around 4-5 months.

Impact and importance of production

Nile tilapia is one of the most farmed fish globally. It has several health-promoting benefits, containing essential bioavailable macro- and micronutrients, high-quality protein, omega-3 fatty acids and minerals (Munguti et al., 2022).

Fish in general provide roughly 16% of the animal protein consumed by the world's population, and approximately 60% of developing countries derive 30% of their annual protein from fish. Fish is also one of the cheapest sources of protein in Africa (Marijani, 2022). Paradoxically, Africans are highly dependent on fish for animal protein, but rank relatively low in per capita fish consumption (Chan et al., 2019). In Tanzania fish consumption is 8.0 kg per capita, and make up 19.7% of the country's animal protein intake (FAO, 2016). In Zanzibar, fish consumption is higher, as much as 20 kg per capita. Nile tilapia, and fish in general, has a significant potential to enhance and contribute to improving

local livelihoods of marginalized communities and promoting socioeconomic growth (Munguti et al., 2022).

Rising antimicrobial resistance

Antimicrobial resistance (AMR) is one of the world's largest threats to global health, food safety and development (Centers for Disease Control and Prevention, 2022). An increasing number of bacterial infections are more difficult to combat as the antibiotics previously used to treat them have become less effective. AMR occurs naturally in bacteria, but misuse, overuse and abuse of antibiotics for animals and humans accelerate the process of resistance development (World Health Organization, 2020). Antimicrobial use (AMU) is one of the major drivers of emerging AMR. Surveillance of AMU helps devise strategies to mitigate AMR (Sangeda et al., 2021).

AMU in animals and its potential effects on human health has been a topic discussed for decades. Although the discussion is somewhat restricted to terrestrial animals, aquaculture is also important (Laxminarayan et al., 2013).

There are several possible pathways for transmission of antimicrobial resistant bacteria between animals and humans. Resistant genes can be transferred between commensal bacterial species, and from commensal to pathogenic bacteria. Exposure through food is an important transmission route. Exposure through environmental routes is less explored, but still an important transmission route. Contamination of surface water can occur through run-off from fertilized land or directly from sewage. Further spread to humans and animals is possible through contact with soil, irrigation of crops, contact with water or wildlife (Laxminarayan et al., 2013).

Several antibiotics used in aquaculture are critical in human medicine according to the World Health Organization (WHO). Resistance to all microbiological classes has been observed in a wide range of bacteria, including those pathogenic to humans (Marijani, 2022).

This thesis has not successfully been able to obtain exact information about AMU in Zanzibar or Tanzania, nor about regulation and control. These are subjects which would be beneficial and interesting to gain further information about.

There exists some information about the prevalence of antibiotic resistant bacteria from tilapia farming, but the knowledge is still fragmented. Farmers in Tanzania can buy antibiotics over-the-counter, leading to misuse, overuse and wrong dosages, which further promotes the development of AMR (Mdegela et al., 2021). As a result, it is hard to keep track of how much and where the antibiotics are being used. Studying the prevalence of AMR bacteria can indicate how prevalent antibiotic use is and identify potentially unacceptable levels and motivate more restrictive administration.

Objective

The objective of this study was to gain more knowledge about antibiotic resistance in Zanzibar. The specific objective was to assess the prevalence of antibiotic resistant bacteria in farmed Nile tilapia in Zanzibar.

Material and method

Sampling

Sampling was done in May 2023. Ten Nile tilapia were sampled from three different sites (A - C) in Zanzibar for a total of 30 fish (10 fish from each site). The sites were located in the south-western part of the island. The fish were selected randomly, they were healthy and without significant exterior lesions. None of the sites reported any known history of disease or antimicrobial treatment.

Site A had farmed Nile tilapia for about three years, and they also farmed catfish. Three tanks were in operation on the day of sampling. They fed a commercial diet. The fish sampled for this study were from the same tank and were about 2 months old.

The farmer in site B did not know how long they had farmed Nile tilapia, or how old the fish in the tank were. The site had one tank. They fed a homemade diet.

Sampled fish from site C were about 1 month old. They farmed Nile tilapia in a pond. The fish were fed bread.

The fish from site A were euthanized and sampled in the field. The samples taken in the field from site A were transported back to the Institute of Marine Sciences (IMS) in plastic bags in a cooling box with cooling elements. The fish from sites B and C were transported in water-filled buckets to the laboratory at IMS where they were euthanized and sampled.

All fish were euthanized prior to sampling. The fish from site A were sedated by blunt force trauma to the dorsal skull, and then euthanized by severing the cervical spine with a scalpel. The fish from sites B and C were sedated by submersion in a water tank with dissolved clove powder, and then euthanized by severing the cervical spine with a scalpel. The fish' weight and length were registered prior to sampling. The weight ranged from 21g to 136g. The length ranged from 11cm to 20.5cm. Both sexes were accepted.

Samples were taken from three anatomical locations from each fish. Sample 1 was taken by stroking a sterile cotton swab over the dorsal fin. Sample 2 was obtained using a sterile inoculation loop that was pushed into the vent after expanding the vent-opening with a sterile scalpel, and sample 3 was taken with a sterile inoculation loop from the head kidney after sterile dissection of the fish.

Laboratory analysis in Zanzibar

Obtained samples were streaked on blood agar (0.5% NaCl) and bromothymol blue lactose agar (BTB agar) using sterile swabs and inoculation loops for a total of six streaks per fish. The sample swabs were streaked on blood agar before the BTB agar to prevent components from the selective media transferring to the blood agar. The petri dishes were incubated inverted at room temperature (circa 25 °C) overnight.

Secondary smears were transferred onto nutrient agar or blood agar to obtain pure cultures using sterile inoculation loops. The number of colonies selected for secondary smears varied depending on growth from the different samples and different agar plates, and all fish yielded samples from secondary smears. The morphological features of the bacterial colonies selected

were as followed: color, size, shape, hemolysis, and whether they were opaque or clear. The petri dishes were incubated inverted at room temperature (circa 25 °C) overnight.

Kirby-Bauer Test (disk diffusion method)

Colonies were tested for AMR. The colonies were diluted with sterile water to a McFarland standard 0.5. The bacterial solutions were spread evenly on Mueller-Hinton agar plates by using a sterile cotton swab for AMR analysis using the disk diffusion method. Standardized antimicrobial discs (10 mm) were applied using a disc dispenser. The available antimicrobials were neomycin 120µg, amoxicillin 30µg, trimethoprim 5µg, tetracycline 30µg, florfenicol 30µg and sulfonamides 240µg. Because of limited availability of sulfonamide and florfenicol discs, these were only available for n=50 and n=150 of the samples respectively. The Mueller-Hinton agars were incubated inverted overnight at room temperature (circa 25 °C). The analysis followed the Kirby-Bauer method, a disc diffusion method measuring inhibition zones with a ruler (Hudzicki, 2009). The Clinical and Laboratory Standards Institute (CLSI), the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Rosco Diagnostica A/S (NEO-SENSITABS) have guidelines for determining antibiotic sensitivity based on zone diameters (CLSI, 2016; European Committee on Antimicrobial Susceptibility Testing, 2023; Rosco Diagnostica A/S, 2011).

The colonies tested for AMR were transported back to the Norwegian University of Life Sciences (NMBU) for genus or species identification by sequencing of the 16S-gene. The colonies were transported by cutting out a piece of the nutrient agar using a sterile inoculation loop and putting it in a sterile Eppendorf tube before sealing the tube with parafilm. Upon arrival at the laboratory at NMBU in May 2023, the samples were preserved by adding 1mL brain heart infusion (BHI) broth to each Eppendorf tube. The tubes were incubated overnight

at 25-37 °C, and then 0.5mL broth was transferred to new Eppendorf tubes and stored at –20 °C. This was done to await further analysis in September 2023.

The cultures were regrown by using sterile inoculation loops to streak the bacteria solutions on Trypton Soya Agars (TSA). The agar plates were incubated inverted at 37 °C for 24 hours. The plates were then stored at 4 °C awaiting further analysis.

16S Sequencing

DNA extraction

Single colonies were transferred to 15mL centrifuge tubes containing 4-5mL lysogeny broth (LB) using a sterile inoculation loop. The tubes were incubated at 30 °C overnight.

DNA was extracted using a Qiagen DNeasy® Blood & Tissue Kit. The following steps were done for all 72 samples:

1. 1mL of the LB-bacteria solution was added to a 1.5mL Eppendorf tube and centrifuged at 2500 rotations per minute (rpm) for 5 minutes.
2. The supernatant was discarded.
3. The precipitated material was resuspended in 200µL PBS followed by adding 20µL proteinase K and then 200µL Buffer AL.
4. The tube was vortexed before being incubated at 56 °C for 10 minutes.
5. 200µL ethanol (96-100%) was added and the sample vortexed.
6. The mixture was transferred to a DNeasy Mini spin column placed in a 2mL collection tube. This was centrifuged at 8000rpm and 4 °C for 1 minute. The flow-through and collection tube was discarded.

7. The spin column was placed in a new 2ml collection tube. 500µL Buffer AW1 was added. This was centrifuged at 8000rpm and 4 °C for 1 minute. The flow-through and collection tube was discarded.
8. The spin column was placed in a new 2mL collection tube. 500µL Buffer AW2 was added. This was centrifuged at 14000rpm and 4 °C for 3 minutes. The flow-through and collection tube was discarded.
9. The spin column was placed in a new 2mL collection tube and centrifuged at 8000rpm and 4 °C for 1 minute. The flow-through and collection tube was discarded.
10. The spin column was put in a 1.5mL Eppendorf tube and 35µL Nuclease-free water (NFW) was added to the center of the column membrane. The sample was incubated for 2 minutes at room temperature before being centrifuged for 1 minute at 8000rpm and 4 °C.
11. The spin column was discarded and the Eppendorf tube with the DNA-material was stored at –80 °C awaiting further analysis.

The DNA concentration (ng/µL) and purity (260/280nm) of the samples were measured by absorbance spectrophotometry using a VWR mySPEC micro-volume spectrophotometer, see appendix D. The machine was calibrated using NFW before testing the samples. 2µL was applied from each sample.

Polymerase chain reaction

The hypervariable region (V1-V9) with a product size of about 1460 bp of the 16S gene was amplified by polymerase chain reaction (PCR). The 16S gene was amplified by using a specific primer set consisting of forward (27F) and reverse primer (1492R) manufactured by Eurofins Genomics, Germany. The details of the primer sequence was as followed:

- 27 F- 5'-AAGAGTTTGATCCTGGCTCAG-3'
- 1492 R- 5'-GGTTACCTTACGACTT-3'

The PCR mix contained the following components:

- 10µL Master Mix (Thermo Fisher, Thermo Scientific Mix with HF)
- 1µL primer (specific primer set consisting of forward (27F) and reverse primer (1492R) manufactured by Eurofins Genomics, Germany).
- 7µL NFW
- 2µL DNA sample

The master mix, primer and NFW was combined and mixed by centrifugation before being distributed in PCR tubes (18µL in each tube). The DNA sample was added to the PCR tubes with Master Mix, primer and NFW. The total volume was 20µL. The entire PCR mix was mixed by centrifugation.

PCR was run using a Veriti 96 Well Thermal Cycler by Thermo Fisher Scientific. The PCR program was set in the following way:

- Step 1: one cycle at 98 °C for 10 seconds.
- Step 2:
    - 98 °C for 30 seconds
    - 55 °C for 30 seconds
    - 72 °C for two minutes
    - Step 2 was repeated 34 times.
- Step 3: one cycle at 72 °C for five minutes.
- When all three stages were completed, the machine was automatically set to 4 °C until the samples were removed.

The samples were stored at 4 °C awaiting further analysis.

Gel agarose electrophoresis

The samples were run through gel agarose electrophoresis to verify the presence and purity of the amplicon. 1% agarose gel was created by mixing 1g agarose in 100mL 1xTAE-buffer and heated to a boil in a microwave before adding 10µL Thermo Fisher SYBR Safe DNA gel stain.

2-3µL Thermo Scientific 6X Orange DNA Loading Dye was added to each PCR tube with DNA sample and mixed with a pipette before transferring 20µL dyed sample to the agarose gel wells. 5µL of the marker Thermo Scientific GeneRuler 1kb Plus DNA Ladder was added to the last well. The electrophoresis was run at 90 volts (V) and 400 milliampere (mA) for 45 minutes.

After the electrophoresis, the gel was transferred to a gel imaging system by Azure Biosystems to detect the different bands of DNA representing different sizes of DNA-fragments. The gel was cut at the area of the 16S amplicon using a sterile scalpel and transferred to Eppendorf tubes.

Gel extraction

The DNA fragment was extracted from the gel samples using a QIAGEN QIAquick Gel Extraction Kit. The following steps were performed for each sample:

- 300µL Buffer QG was added to the Eppendorf tube with a cut out gel sample.
- The tube was incubated at 50 °C for 10 minutes.
- 100µL isopropanol was added to the tube.

- The sample was transferred to QIAquick spin columns in 2ml collection tubes and centrifuged for 1 minute at 13000rpm and 4 °C. The flow-through was discarded and the spin column was placed back in the collection tube.
- 500µL Buffer QG was added to the QIAquick spin column and centrifuged for 1 minute at 13000rpm and 4 °C. The flow-through was discarded and the spin column was placed back in the collection tube.
- 750µL Buffer PE (with added ethanol (96-100%)) was added to the QIAquick column and incubated for 2 minutes at room temperature before centrifuging for 1 minute at 13000rpm and 4 °C. The flow-through was discarded and the spin column was placed back in the collection tube.
- The sample was centrifuged again for 1 minute at 13000rpm and 4 °C. The flow-through was discarded and the QIAquick spin columns were transferred to sterile 1.5mL Eppendorf tubes.
- 30µL NFW was added to the center of the QIAquick membrane and incubated for 2 minutes at room temperature before centrifuging for 1 minute at 13000rpm and 4 °C. The QIAquick column was discarded.

Sequencing

Samples were sent for sequencing at Eurofins Genomics, Germany. The results were analyzed using the National Center for Biotechnology Information (NCBI) nucleotide BLAST Database.

Data management

Data was entered in Microsoft excel. All graphs were made in Excel.

Results

In the laboratory at IMS a total of 191 colonies were selected for secondary smears: 92 from the vent, 74 from the dorsal fin and 25 from the head kidney. Of the 191 colonies selected, 20 did not result in pure cultures and were therefore excluded from further sampling. These samples could have been redone from the primary smears, but this was not possible because of contamination of the primary plates by ants in the laboratory.

Following the Kirby-Bauer test, 12 samples were removed from further analysis either because of insufficient growth on the Mueller-Hinton plates or because of suspected contamination (Hudzicki, 2009).

A total of 159 samples were regrown at the laboratory at NMBU. Six samples were excluded from further analysis because they were not pure cultures. The morphology of these 153 colonies was recorded, see appendix C.

Of the 153 samples, 72 were selected for 16S PCR and sequencing. A total of 25 samples were selected from site A, of which 16 were from the gut, eight from the skin and one from the head kidney. A total of 25 samples were selected from site B, of which nine were from the gut, nine from the skin and seven from the head kidney. A total of 22 samples were selected from site C, of which eight were from the gut, 10 from the skin and four from the head kidney. Samples were included from all 30 fish.

Following PCR and gel electrophoresis, the PCR products deviating from expected size for the 16S-gene were discarded. This was the case for two samples.

The sequencing results were analyzed in the NCBI´s nucleotide BLAST Database. The bacteria species or genus with the highest sequence homology >97% was accepted. Ten samples had no conclusive matches. This left a total of 60 samples identified at genus or species level. Table 1 and figures 1 - 3 show an overview of the identified bacteria from the different sites.

Table 1: Overview and count of the identified bacteria from site A, B and C.

	Site			
Bacteria	A	B	C	Total
Acinetobacter johnsonii		5	1	6
Acinetobacter junii		4	3	7
Acinetobacter sp.		1	3	4
Aeromonas caviae	3	1		4
Aeromonas enteropelogenes			1	1
Aeromonas hydrophila		1	3	4
Aeromonas jandaei		1		1
Aeromonas sobria			1	1
Aeromonas sp.		1	2	3
Aeromonas taiwanensis			1	1
Aeromonas veronii	2			2
Bacillus albus			1	1
Bacillus cereus			1	1
Citrobacter freundii		1		1
Citrobacter murliniae	1			1
Citrobacter sp.	1			1
Escherichia coli	7	1		8
Escherichia fergusonii	1			1
Plesiomonas shigelloides	3	3		6
Pseudomonas hunanensis			1	1
Pseudomonas plecoglossicida			1	1
Pseudomonas sp.		1	1	2
Raoultella ornithinolytica	1			1
Vibrio mimicus			1	1
Total	**19**	**20**	**21**	**60**

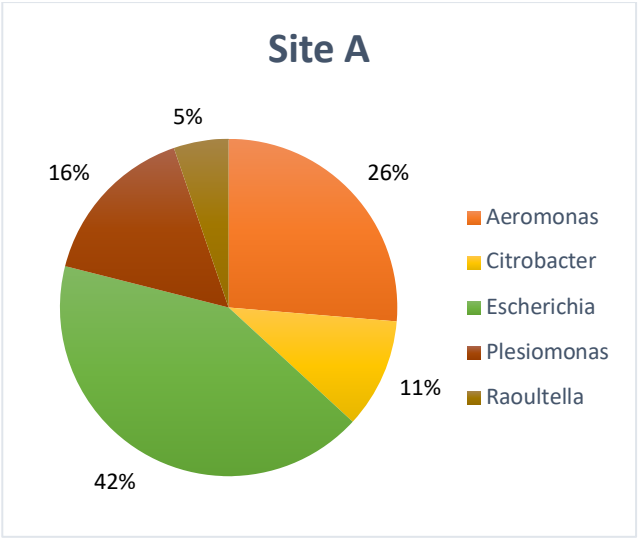

Figure 1: Distribution of bacteria genera at site A.

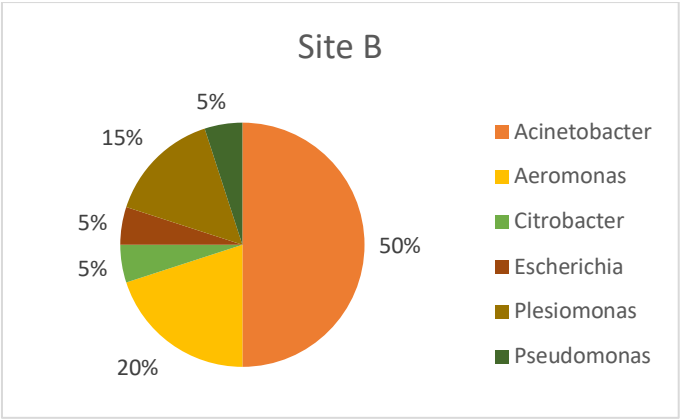

Figure 2: Distribution of bacteria genera at site B.

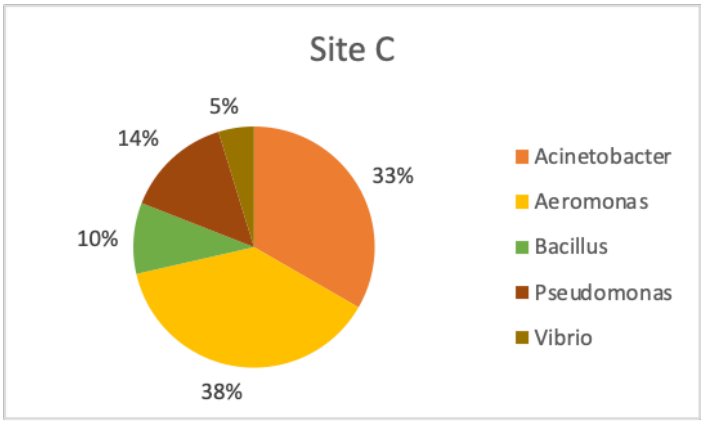

Figure 3: Distribution of bacteria genera at site C.

Of the 60 samples, 19 were from site A, 20 from site B and 21 from site C. Twenty-eight were from the gut, 21 from the skin and 11 from the kidney. From site A, 13 samples were from the gut, five from the skin and one from the kidney. From site B, seven samples were from the gut, seven from the skin and six from the kidney. From site C, eight samples were from the gut, nine from the skin and four from the kidney. Table 2 and figure 4 show the distribution between organs and sites. There were more samples from the gut than the other organs (46.7%), with the majority being from site A.

Table 2: Distribution of samples from different sites and organs.

	Organ			
Site	Gut	Kidney	Skin	Total
Site A				
Count of samples	13	1	5	19
%	21.7%	1.7%	8.3%	31.7%
Site B				
Count of samples	7	6	7	20
%	11.7%	10.0%	11.7%	33.3%
Site C				
Count of samples	8	4	9	21
%	13.3%	6.7%	15.0%	35.0%
Total count of samples	**28**	**11**	**21**	**60**
Total %	**46.7%**	**18.3%**	**35.0%**	**100.0%**

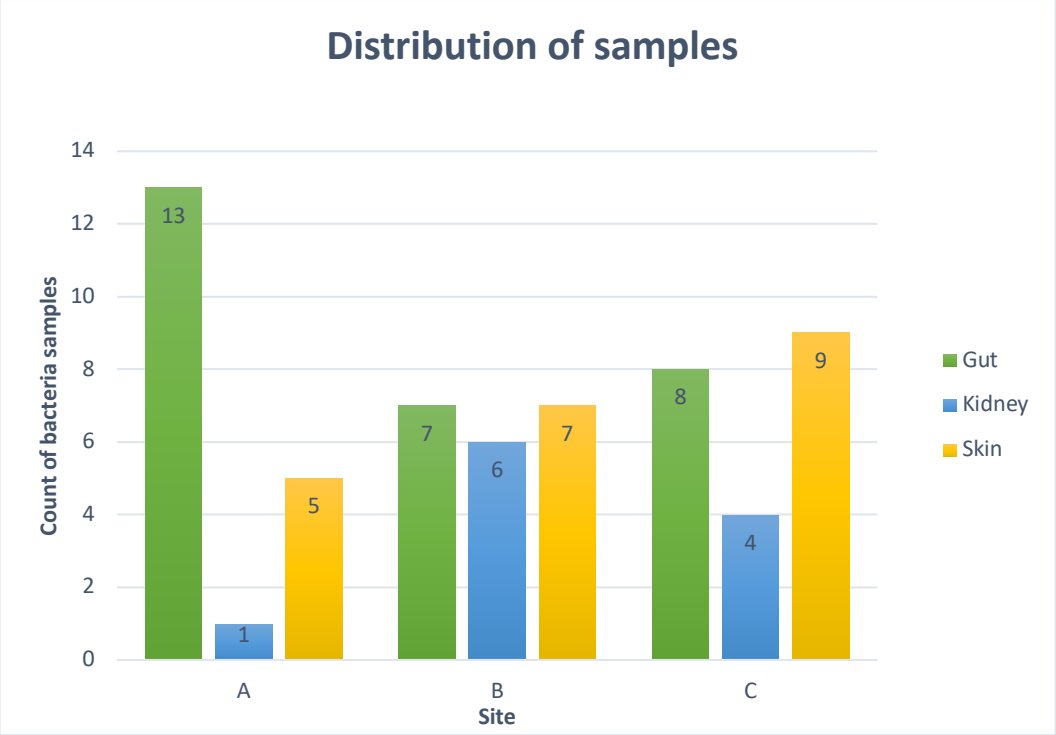

Figure 4: Distribution of samples from site A, B and C.

Aeromonas sp.

Seventeen samples were identified as *Aeromonas* at genus or species level, of which four were identified as *Aeromonas caviae*, two as *Aeromonas veronii*, and four as *Aeromonas hydrophila*. Seven samples were either identified as other *Aeromonas* species (*A. jandaei, A. enteropelogenes, A. sobria, A. taiwanensis*) or only at genus level. Table 3 gives an overview of *Aeromonas* sp. from different sites and organs. Most of the samples were from the gut, and from site C.

Table 3: Distribution of *Aeromonas* sp. samples between sites and organs.

Site and bacteria	Organ: Gut	Kidney	Skin	Total
Site A	3		2	5
Aeromonas caviae	2		1	3
Aeromonas veronii	1		1	2
Site B	2	1	1	4
Aeromonas caviae			1	1
Aeromonas hydrophila	1			1
Aeromonas jandaei	1			1
Aeromonas sp.		1		1
Site C	4	3	1	8
Aeromonas enteropelogenes	1			1
Aeromonas hydrophila	1	2		3
Aeromonas sobria			1	1
Aeromonas sp.	1	1		2
Aeromonas taiwanensis	1			1
Total	**9**	**4**	**4**	**17**

CLSI has guidelines for determining antibiotic sensitivity based on zone diameters for *A. hydrohila, A. caviae* and *A. veronii* (CLSI, 2016). Of the antibiotics CLSI have studied, only tetracycline is applicable for this study. The zone diameters [mm] for tetracycline are defined as:

- Sensitive: ≥ 15
- Intermediate: 12-14
- Resistant: ≤ 11

Applying these criteria, seven samples are classified as sensitive and three as resistant, *i.e.*, 70% sensitive and 30% resistant. There are no CLSI or EUCAST guidelines available for the remaining seven samples (identified as other species or only at genus level). *Aeromonas* spp. are uniformly resistant to amoxicillin as used in this study (CLSI, 2016).

Acinetobacter sp.

Seventeen samples were identified as *Acinetobacter* at genus or species level. Seven samples were identified as *Acinetobacter junii*, six samples as *Acinetobacter johnsonii,* and four samples as *Acinetobacter* sp. Table 4 shows an overview of distribution of *Acinetobacter* sp. between organs and sites. Most of the samples were from the skin. There are no available sensitivity guidelines from CLSI or EUCAST based on the antimicrobials used in this project. EUCAST notes that *Acinetobacter* spp. in most cases are resistant to penicillins and that sensitivity testing to penicillins are unreliable (European Committee on Antimicrobial Susceptibility Testing, 2023).

Table 4: Distribution of *Acinetobacter* sp. samples between sites and organs.

	Organ			
Site and bacteria	Gut	Kidney	Skin	Total
Site B	3	2	5	10
Acinetobacter johnsonii	1	2	2	5
Acinetobacter junii	2		2	4
Acinetobacter sp.			1	1
Site C	1	1	5	7
Acinetobacter johnsonii			1	1
Acinetobacter junii			3	3
Acinetobacter sp.	1	1	1	3
Total	**4**	**3**	**10**	**17**

Table 5 gives an overview of the measured inhibition zone diameters for *Acinetobacter* sp. Two of the samples (*Acinetobacter johnsonii* (skin, site C) and *Acinetobacter junii* (gut, site B)) measured 10mm towards trimethoprim (*i.e.,* no inhibition zone around the antibiotic disc). Because of no available sensitivity guidelines, it is not possible to tell if these samples are resistant to trimethoprim or not.

Table 5: Zone diameters of *Acinetobacter* sp.[1]

Site	Bacteria	Organ	Neomycin [mm]	Amoxicillin [mm]	Trimethoprim [mm]	Tetra-cycline [mm]	Florfenicol [mm]	Sulfonamides [mm]
C	*Acinetobacter junii*	Skin	25	28	14	14	13	N.a.
C	*Acinetobacter sp.*	Kidney	24	23	17	20	13	N.a.
B	*Acinetobacter johnsonii*	Gut	26	34	14	26	20	N.a.
B	*Acinetobacter johnsonii*	Kidney	21	30	12	25	18	N.a.
B	*Acinetobacter johnsonii*	Kidney	25	32	12	26	20	N.a.
B	*Acinetobacter johnsonii*	Skin	27	32	13	27	20	N.a.
B	*Acinetobacter johnsonii*	Skin	25	32	14	27	27	N.a.
C	*Acinetobacter johnsonii*	Skin	24	28	10	22	20	N.a.
B	*Acinetobacter junii*	Gut	24	26	10	22	17	N.a.
B	*Acinetobacter junii*	Gut	27	30	13	23	15	N.a.
B	*Acinetobacter junii*	Skin	27	30	13	24	18	N.a.
B	*Acinetobacter junii*	Skin	28	30	12	23	16	N.a.
C	*Acinetobacter junii*	Skin	26	30	12	22	N.a.	N.a.
C	*Acinetobacter junii*	Skin	25	27	13	19	14	N.a.
C	*Acinetobacter sp.*	Gut	26	14	11	22	N.a.	N.a.
B	*Acinetobacter sp.*	Skin	28	27	18	23	22	N.a.
C	*Acinetobacter sp.*	Skin	23	25	12	20	N.a.	N.a.

Bacillus sp.

Two samples were identified as *Bacillus* sp. Both samples were from site C and the gut. One sample was identified as *Bacillus cereus*, and the other as *Bacillus albus*. There are no available antimicrobial sensitivity guidelines from CLSI or EUCAST for *Bacillus* sp. using zone diameters for the antibiotics used in this study. CLSI note that *Bacillius cereus* are usually resistant to penicillins, which correspond with the *B. cereus* sample in this study having a zone diameter of 11mm to amoxicillin, see table 6. There were no inhibition zone around the trimethoprim disc for both samples.

[1] N.a. = Not applicable due to a lack of available florfenicol and sulfonamide discs.

Table 6: Zone diameters of *Bacillus* sp.[2]

Site	Bacteria	Organ	Neomycin [mm]	Amoxicillin [mm]	Trimethoprim [mm]	Tetra-cycline [mm]	Florfenicol [mm]	Sulfonamides [mm]
C	*Bacillus albus*	Gut	25	17	10	25	N.a.	N.a.
C	*Bacillus cereus*	Gut	23	11	10	24	28	N.a.

Enterobacteriaceae family

There were 19 samples identified as belonging to the Enterobacteriaceae family, distributed between the genera *Citrobacter* sp., *Escherichia* sp., *Plesiomonas* sp. and *Raoltella ornothinolytica*, see table 7. There were no samples from the Enterobacteriaceae family from site C.

Table 7: Distribution of samples from the Enterobacteriaceae family between sites and organs.

	Organ			
Site and bacteria	Gut	Kidney	Skin	Total
Site A	10	1	3	14
Citrobacter murliniae	1			1
Citrobacter sp.	1			1
Escherichia coli	5		2	7
Escherichia fergusonii			1	1
Plesiomonas shigelloides	2	1		3
Raoultella ornithinolytica	1			1
Site B	2	3		5
Citrobacter freundii		1		1
Escherichia coli		1		1
Plesiomonas shigelloides	2	1		3
Total	**12**	**4**	**3**	**19**

[2] N.a. = Not applicable due to a lack of available florfenicol and sulfonamide discs.

There are antimicrobial sensitivity guidelines from CLSI and EUCAST for the Enterobacteriaceae family (CLSI, 2016; European Committee on Antimicrobial Susceptibility Testing, 2023). There are also sensitivity guidelines from NEO-SENSITABS (Rosco Diagnostica A/S, 2011). The available guidelines provide sensitivity thresholds at family level (Enterobacteriaceae), not individual genera or species.

Table 8 list the zone diameters as determined by CLSI, EUCAST or NEO-SENSITABS. The breakpoints listed are used to estimate levels of sensitive (S), intermediate (I), and resistant (R) bacteria in the following paragraphs describing each Enterobacteriaceae genus/species separately.

Table 8: Antibiotic sensitivity, zone diameters in the Eneterobacteriaceae family

Antibiotic sensitivity, zone diameters, Enterobacteriaceae			
Antibiotic	**Zone diameters breakpoints [mm]**		
	S	**I**	**R**
Amoxicillin	≥14	-	<14
Trimethoprim	≥15	-	<15
Neomycin	≥25	24 - 21	≤20
Tetracycline	≥22	21 - 19	≤18
Sulfonamides	≥17	16 - 13	<13

Table 9 shows an overview of AMR bacteria in the Enterobacteriaceae family across sites, organs, and different types of antibiotics. Figure 5 shows the resistant pattern across the Enterobacteriaceae family.

Table 9: Overview of antimicrobial resistance in the Enterobacteriaceae family.

Site, organ and bacteria	Neomycin	Amoxicillin	Trimethoprim	Tetracycline	Florfenicol	Sulfonamides
Site A	1	5	3	7	0	5
Gut	1	5	2	6	0	5
Citrobacter murliniae	0	1	0	0	0	0
Citrobacter sp.	0	1	0	0	0	0
Escherichia coli	0	1	2	4	0	3
Plesiomonas shigelloides	1	1	0	2	0	1
Raoultella ornithinolytica	0	1	0	0	0	1
Kidney	0	0	0	1	0	0
Plesiomonas shigelloides	0	0	0	1	0	0
Skin	0	0	1	0	0	0
Escherichia coli	0	0	1	0	0	0
Escherichia fergusonii	0	0	0	0	0	0
Site B	0	1	1	1	0	0
Gut	0	0	0	1	0	0
Plesiomonas shigelloides	0	0	0	1	0	0
Kidney	0	1	1	0	0	0
Citrobacter freundii	0	1	1	0	0	0
Escherichia coli	0	0	0	0	0	0
Plesiomonas shigelloides	0	0	0	0	0	0
Total	**1**	**6**	**4**	**8**	**0**	**5**

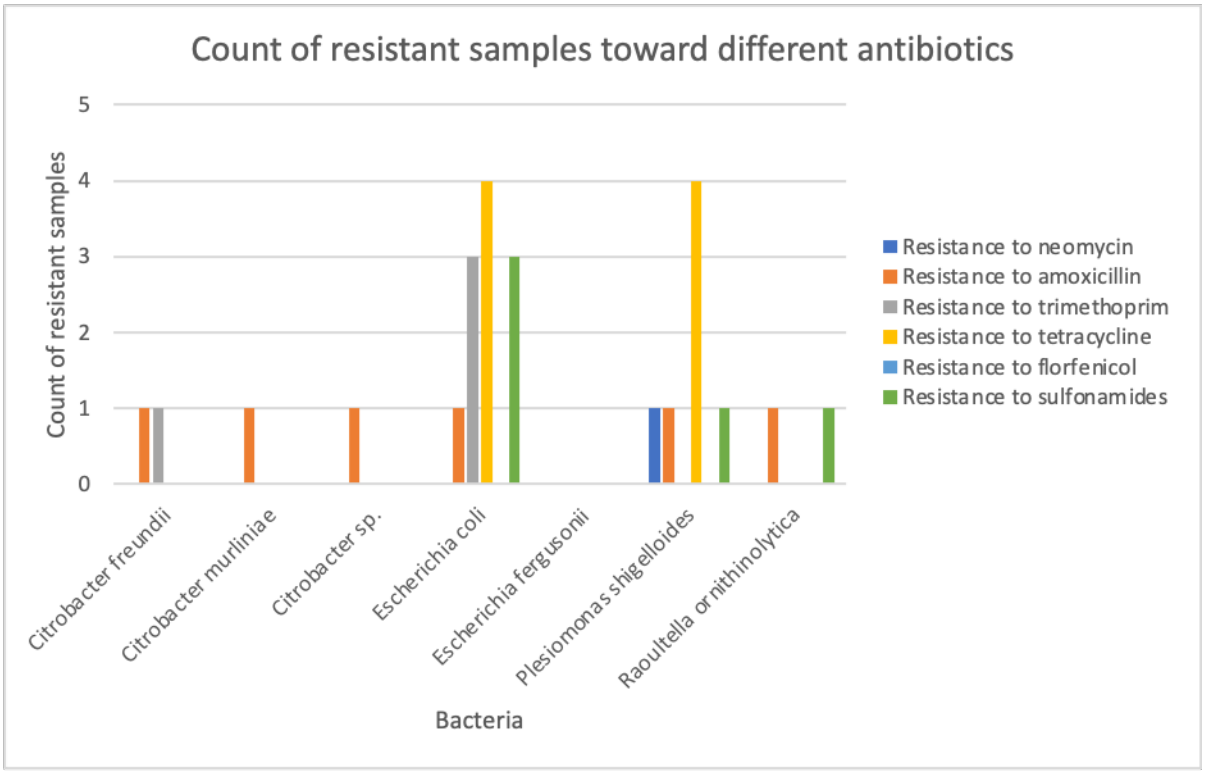

Figure 5: The resistance pattern across the Enterobacteriaceae family. One bacterium sample can be counted in several columns if they are resistant to more than one antibiotic.

Escherichia sp.

Nine samples were identified as *Escherichia* sp., eight from site A and one from site B, as seen in table 7. Of the *Escherichia* sp. from site A, three were from the skin and five from the gut. The sample from site B was from the kidney. Eight of the nine *Escherichia* sp. samples were *E. coli*, except for one being *E. fergusonii*.

Eight of the nine samples from *Escherichia* sp. were sensitive, and only one resistant to amoxicillin, *i.e.*, 88.9% sensitive and 11.1% resistant. Six of the nine samples from *Escherichia* sp. were sensitive, and three were resistant to trimethoprim, *i.e.*, 66.7% sensitive and 33.3% resistant. Seven of the nine samples from *Escherichia* sp. were sensitive, and two had intermediate sensitivity to neomycin, *i.e.*, 77.8% sensitive and 22.2% intermediate. All

Escherichia sp. were sensitive to neomycin. Three of the nine samples from *Escherichia* sp. were sensitive, two intermediate and four resistant to tetracyclines, *i.e*., 33.3%, 22.2% and 44.4% respectively. Because of limited availability of sulfonamide discs, only eight of the nine *Escherichia* sp. samples were tested against sulfonamides. Five of the eight samples from *Escherichia* sp. were sensitive and three resistant, *i.e*., 62.5% sensitive and 37.5% resistant.

Plesiomonas shigelloides

Six samples were identified as *Plesiomonas shigelloides*, three from site A and three from site B, as seen in table 7. Of the *Plesiomonas* sp. from site A, two were from the gut and one from the kidney. The distribution was the same from site B.

One of six samples identified as *Plesiomonas shigelloides* was sensitive, and the other samples were resistant to amoxicillin, *i.e*., 16.7% sensitive and 83.3% resistant. All six samples were sensitive to trimethoprim. One of six *Plesiomonas shigelloides* samples was sensitive, four intermediate and one resistant to neomycin, *i.e*., 16.7%, 66.6% and 16.7% respectively. Two of the six samples were sensitive and four samples were resistant to tetracyclines, *i.e.,* 33.3% sensitive and 66.7% resistant. Because of limited availability of sulfonamide discs, only two of the six *Plesiomonas shigelloides* samples were tested against sulfonamides. One was sensitive and the other resistant.

***Citrobacter* sp.**

Three samples were identified as *Citrobacter* sp., two of which were from site A and the gut, and one from site B and the kidney, as seen in table 7. One sample was only identified at

genus level as *Citrobacter* sp., one was identified as *Citrobacter murliniae* and one as *Citrobacter freundii*. The latter was isolated from the kidney.

The sample only identified at genus level was sensitive to trimethoprim, tetracycline, and sulfonamides; intermediate sensitivity to neomycin; and resistant to amoxicillin. The sample identified as *Citrobacter murliniae* was sensitive to neomycin, trimethoprim and tetracycline; and resistant to amoxicillin. It was not tested against sulfonamides. The sample identified as *Citrobacter freundii* was sensitive to neomycin; intermediate sensitivity to tetracycline; and resistant to amoxicillin and trimethoprim.

Raoultella ornithinolytica

Only one sample was identified as *Raoultella ornithinolytica*. It was sensitive to trimethoprim and tetracycline; intermediate sensitivity to neomycin; and resistant to amoxicillin and sulfonamides.

Antimicrobial sensitivity pattern across the Enterobacteriaceae family

Figure 6 shows the distribution and number of sensitive, intermediate, and resistant samples from the Enterobacteriaceae family.

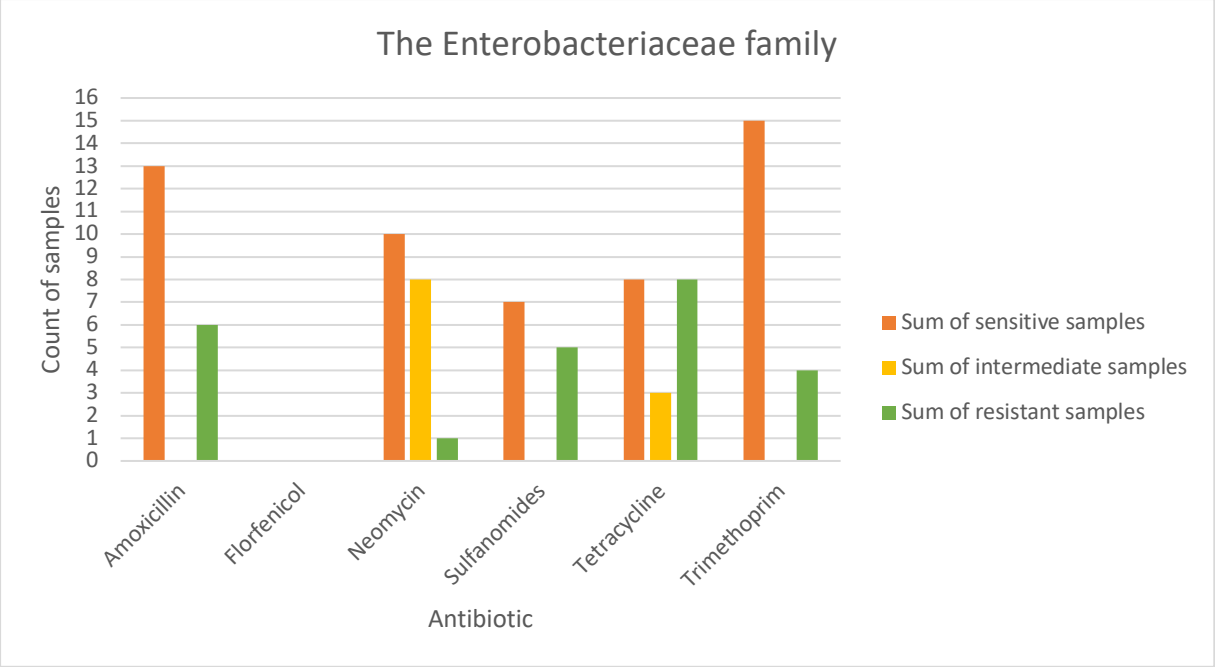

Figure 6: Number of sensitive, intermediate, and resistant samples from the Enterobacteriaceae family. In this figure, individual samples can be subjected to multiple counts as they were tested against different antimicrobial discs (appendix A) – consequently they can be sensitive, intermediate, and resistant to different antibiotics.

Thirteen samples from the Enterobacteriaceae family were sensitive and six were resistant to amoxicillin, *i.e.*, 68.4% sensitive and 31.6% resistant. Fifteen samples from the Enterobacteriaceae family were sensitive and four were resistant to trimethoprim, *i.e.*, 78.9% sensitive and 21% resistant. Ten samples from the Enterobacteriaceae family were sensitive, eight were intermediate and one was resistant to neomycin, *i.e.*, 52.6%, 42.1% and 5.2% respectively. Eight samples from the Enterobacteriaceae family were sensitive, three were intermediate and eight were resistant to tetracyclines, *i.e.*, 42.1%, 15.8% and 42.1% respectively. Because of limited availability of NEO-SENSITABS sulfonamide discs, only 12 of the 19 Enterobacteriaceae samples were tested against sulfonamides. Seven samples were sensitive and five were resistant, *i.e.*, 58.3% sensitive and 41.6% resistant.

Pseudomonas sp.

Four samples were identified as *Pseudomonas* at genus or species level. One sample being *Pseudomonas hunanensis* and one being *Pseudomonas plecoglossicida,* both from the skin from site C. Two samples were only identified at genus level as *Pseudomonas* sp., both from the skin from site B and C. There were none available sensitivity guidelines from CLSI or EUCAST based on the antimicrobials used in this project. Table 11 shows the measured inhibition zones for *Pseudomonas* sp.

Table 10: zone diameters of *Pseudomonas* sp.[3]

Site	Bacteria	Organ	Neomycin [mm]	Amoxicillin [mm]	Trimethoprim [mm]	Tetra-cycline [mm]	Florfenicol [mm]	Sulfonamides [mm]
C	*Pseudomonas hunanensis*	Skin	23	10	10	17	10	N.a.
C	*Pseudomonas plecoglossicida*	Skin	24	13	10	15	N.a.	N.a.
C	*Pseudomonas* sp.	Skin	22	10	10	15	N.a.	N.a.
B	*Pseudomonas* sp.	Skin	24	11	10	18	10	N.a.

Vibrio sp.

One sample from the gut (site C) was identified as *Vibrio mimicus.* Sensitivity guidelines for tetracycline 30µg and sulfonamides 250µg or 300µg exists for the *Vibrio* spp (CLSI, 2016).

Tetracycline 30µg, zone diameters [mm]:

- Sensitive: ≥ 15
- Intermediate: 12-14
- Resistant: ≤ 11

Sulfonamides 250µg or 300µg, zone diameters [mm]:

[3] N.a. = Not applicable due to a lack of available florfenicol and sulfonamide discs.

- Sensitive: ≥ 17
- Intermediate: 13-16
- Resistant: ≤ 12

The *Vibrio mimicus* sample was sensitive to tetracycline. Because of limited availability of sulfonamide discs, this study was unable to test the *V. mimicus* sample against sulfonamides.

Results across the entire sample set

Figure 7 shows the overall number of sensitive, intermediate, and resistant samples across the entire sample set. This includes the bacteria with available sensitivity guidelines; *Aeromonas* sp., the Enterobacteriaceae family and *Vibrio* sp.

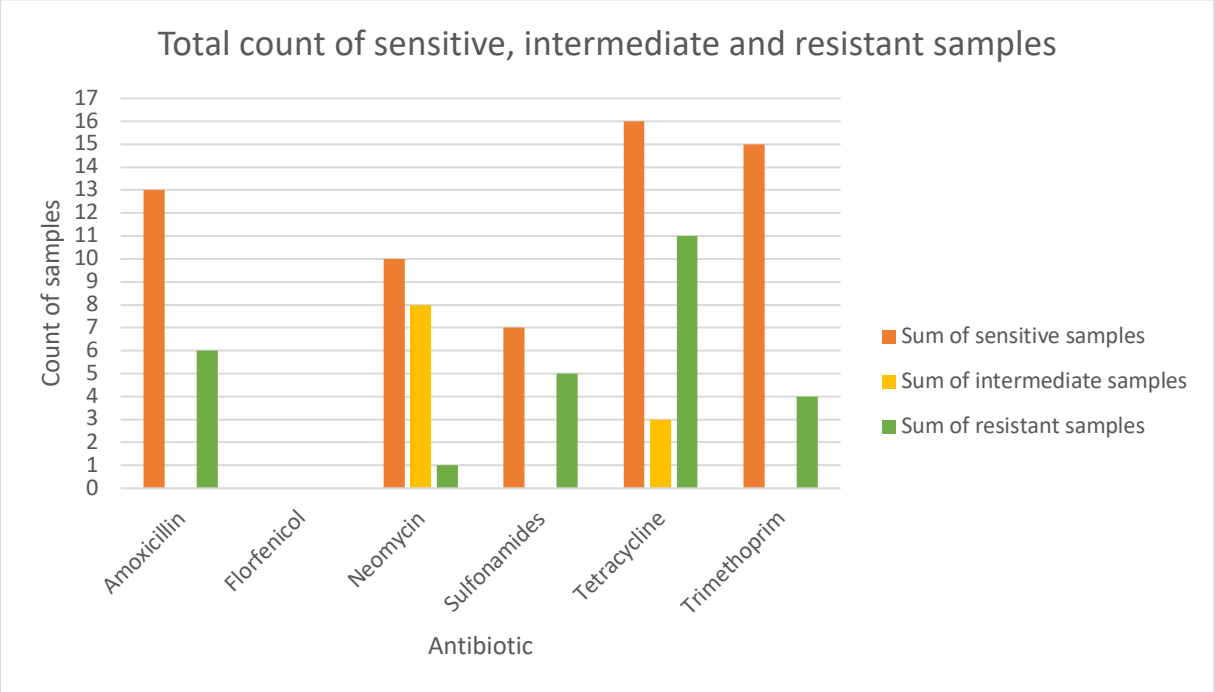

Figure 7: Total count of sensitive, intermediate, and resistant samples. In this figure, individual samples can be subjected to multiple counts as they were tested against different antimicrobial discs (appendix A) – consequently they can be sensitive, intermediate, and resistant to different antibiotics.

Discussion

The results were dominated by *Aeromonas* sp. (28.3%), *Acinetobacter* sp. (28.3%) and bacteria from the Enterobacteriaceae family (31.6%). The remaining samples were identified as *Bacillus* sp. (3.3%), *Pseudomonas* sp. (6.6%) and *Vibrio* sp. (1.6%).

Fish are exposed to a variety of bacteria that can be classified as indigenous and nonindigenous. Nonindigenous bacteria contaminate the fish or the aquatic environment, for example *Escherichia coli, Clostridium botulinum, Salmonella* or *Staphylococcus aureus.* Indigenous bacteria live naturally in the aquatic environment and include *Vibrio* and *Aeromonas* species. Therefore, the type of microorganisms associated with a particular fish depends on its habitat (Haenen et al., 2013; Marijani, 2022).

Aeromonas sp. (gram negative, facultative anaerobe, rod-shaped) is commonly found in nature, and is normal to isolate from water (both fresh and saltwater) (Folkehelseinstituttet, 2023). Some species can cause disease in fish, and some can be transferred to humans and cause illness, most commonly gastroenteritis. The latter is the case for *A. hydrophila*, *A. veronii*, *A. caviae* and *A. dhakensis* (Folkehelseinstituttet, 2023).The three former mentioned species were all identified in this study. In the sampled *Aeromonas* sp., 30% of the bacteria were resistant to tetracycline. The resistant species were either *A. caviae* or *A. hydrophila*, bacteria that can be transferred to humans. This is interesting from a public health standpoint.

Acinetobacter sp. (gram negative, aerobic) is widely distributed in nature, and commonly found in soil and water. It can also be part of the human skin flora (Abo-Zed et al., 2020). The samples identified at species level in this study, were identified as *Acinetobacter junii* and *Acinetobacter johnsonii*. Both species can cause disease in humans. *Acinetobacter* sp.

also has the ability to accumulate different mechanisms of antibiotic resistance (Kanafani & Souha, 2022). The occurrence of *Acinetobacter* sp. in this study can either be the result of contamination from the environment (water or soil) or from humans through handling. Further analysis would need to be done to determine its specific origin. Unfortunately, there are no antimicrobial susceptibility thresholds available for *Acinetobacter* sp. It would have been interesting to assess the level of sensitive and resistant *Acinetobacter* sp., especially considering it can cause disease in humans and is commonly known to develop antibiotic resistance.

The Enterobacteriaceae family is a family of gram negative, rod shaped and facultative anaerobe bacteria. It compromises different organisms, some of which are commensals in the intestinal microbiota, others opportunistic pathogens and some principal pathogens (Donnenberg, 2015). From the Enterobacteriaceae family, nine of the 19 samples were identified as *Escherichia* sp. (47.3%), six as *Plesiomonas shigelloides* (31.5%), three as *Citrobacter* sp. (15.8%) and one as *Raoultella ornithinolytica* (5.2%). The majority of the bacteria belonging to the Enterobacteriaceae family in this study were identified as *E. coli* (42.1%).

Five of the nine samples (55.5%) of *Escherichia* sp. were resistant to one or more antibiotics; two samples (22.2%) had intermediate sensitivity to one or more antibiotics; and two *Escherichia* sp. samples (22.2%) were sensitive to all antibiotics tested. It was interesting to note that eight of the nine *Escherichia* sp. samples were from site A, only one from site B and none from site C. *E. coli* is commonly found as a part of the normal flora in the intestinal tract in warm-blooded organisms (Bøvre, 2021). The primary smears from site A were conducted on-site, and the high frequency of *E. coli* might be a result of human or

environmental contamination at the time of sampling. There was no available information about the water supply or -treatment of site A, and this might also be a reservoir for *E. coli*. Further study of the *E. coli* would need to be done to determine its origin. The *E. coli* sample from site B was from the kidney, which could indicate a systemic infection with the bacteria. This sample was sensitive to all antibiotics tested. It would be interesting to study this *E. coli* further, especially focusing on virulence factors to evaluate potential differences from the other *E. coli* making systemic infection possible.

Four of the six *Plesiomonas shigelloides* samples (66.6%) were resistant to one or more antibiotics tested, with the remaining two (33.3%) being sensitive to all antibiotics tested except for having intermediate sensitivity to neomycin. *Plesiomonas shigelloides* is commonly found in aquatic environments and can cause disease in fish and humans. It is not considered part of the normal human gastrointestinal flora (Morris & Horneman, 2021; Murdoch & Lang, S.a.). Two of the *Plesiomonas shigelloides* samples were isolated from the kidney, which is interesting and can suggest a systemic infection with the bacteria. The remaining samples were isolated from the gut, and none from the skin, which makes environmental contamination less likely.

Citrobacter sp. are normally found in water, soil, food and the gastrointestinal tract of animals and humans (Wang & Shan-Chwen, s.a.). They can cause disease in humans as well as fish, and can be transferred to humans eating contaminated food (Cortés-Sánchez et al., 2023). All three *Citrobacter* sp. samples in this study were resistant to one or more antibiotics. All were resistant to amoxicillin. Two of the *Citrobacter* sp. in this study stemmed from the gut, and one from the kidney. The bacteria from the gut could be part of the normal bacterial flora of the fish, while the bacteria from the kidney could be an

indication of systemic infection and disease. The *Citrobacter* sp. isolated from the kidney was identified as *Citrobacter freundii*, which can cause severe disease in humans and is increasingly becoming multi-drug resistant (MDR) (Balasubramanian, 2021). The *C. freundii* sample in this study was sensitive to neomycin, had intermediate sensitivity to tetracycline, and was resistant to amoxicillin and trimethoprim.

Only one sample was identified as *Raoultella ornithinolytica*, which is commonly found in aquatic environments, soil, and fish. The sample in this study stemmed from the gut and is most likely a part of the fish´ normal intestinal flora. Although rare, it can cause human infections (Hajjar et al., 2020). The bacterium in this study was sensitive to trimethoprim and tetracycline, had intermediate sensitivity to neomycin and was resistant to amoxicillin and sulfonamides. It is hard to extrapolate the findings for the one bacterium in this study for *Raoultella ornithinolytica* as a whole.

Unfortunately, there are no available antimicrobial sensitivity thresholds for several of the bacteria identified and the antibiotics tested against in this study. This goes for *Pseudomonas* sp., *Acinetobacter* sp. and *Bacillus* sp. *Pseudomonas aeruginosa* is resistant to a wide range of antimicrobials due to its outer membrane with low permeability (Pachori et al., 2019). Developing antimicrobial sensitivity thresholds for *Pseudomonas* sp. is therefore less relevant.

It was interesting to note that none of the sequenced bacteria from site C showed resistance to any of the antimicrobials tested where there are available sensitivity thresholds. This was despite the number of sequenced bacteria from site C being almost identical to the number sequenced from site A and B. The reason for this is unknown. A high number of the resistant

bacteria from site A and B belong to the Enterobacteriaceae family, of which there were no samples from site C, which could be a contributing factor.

When looking at the resistance for each antibiotic compared to the total occurrence of resistance, most AMR was seen toward tetracycline (41%), amoxicillin (22%) and sulfonamides (18%), followed by trimethoprim (15%) and neomycin (4%). This distribution is shown in figure 8. There was no registered resistance against florfenicol due to there being no available sensitivity thresholds for florfenicol and the bacteria sequenced.

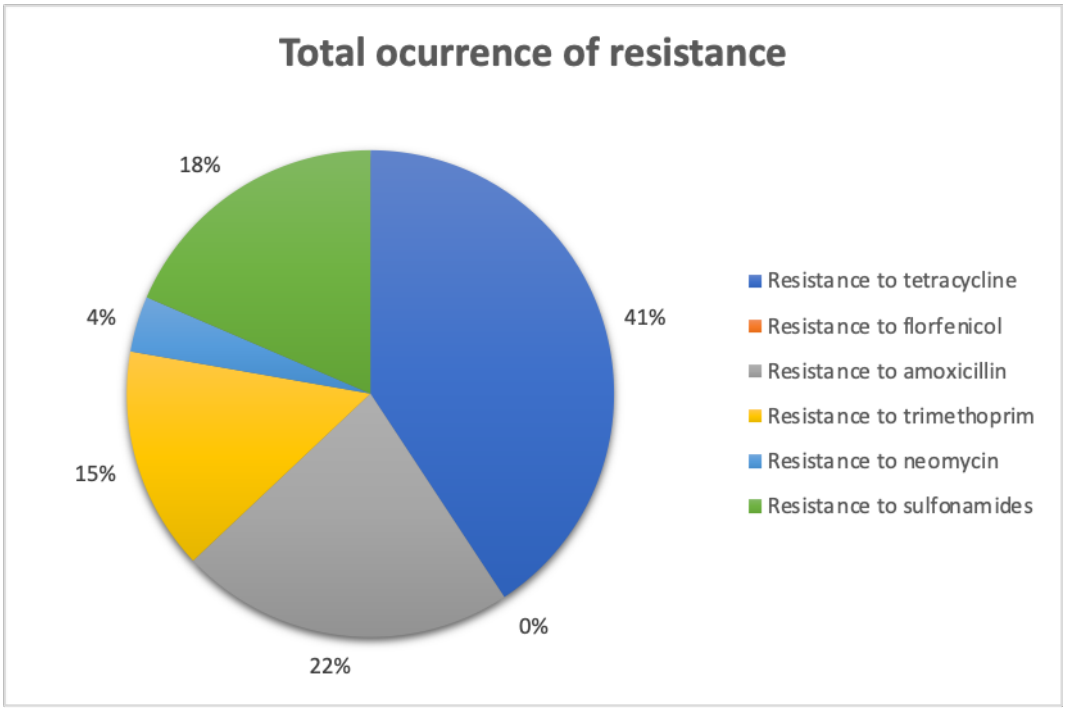

Figure 8: AMR to each antibiotic compared to total resistance.

None of the sites sampled reported any illness or having used antibiotics to treat their fish. The resistance found in the sampled bacteria can therefore be the result of natural resistance in the bacteria; contamination of antibiotics from water, manure or feed to the fish ponds and

subsequent resistance development; or contamination of bacteria from humans that are already resistant. Further study would need to be done to pinpoint the exact resistance mechanisms present, and to be able to say something about their origin.

Limitations

Possible contamination

The day after reading the primary smears at the laboratory at IMS, ants were discovered on the agar plates. This means the ants could have contaminated the plates, and even cross contaminated between bacteria colonies, although there were no obvious signs of this at the time of transferring them to the secondary smears.

At times it was challenging to work aseptically in the field in Zanzibar and at the laboratory at IMS. This could have led to human or environmental contamination of the samples. It was also challenging to maintain sterile dissection of the smallest fishes, which could have led to contamination of the kidney samples.

Incubation temperature

The incubators were not working properly during the laboratory work at IMS. The samples were therefore incubated at room temperature, with the air condition at 25 ^{o}C. Because the samples were incubated overnight with no supervision, there is no guarantee the temperature was stable the entire incubation time. This was most likely not a significant issue as there was growth on the primary and secondary as well as the Mueller-Hinton agars after one day of incubation.

McFarland standard

When performing the AMR testing with the Kirby-Bauer method, the bacteria dilution was compared with a McFarland standard of 0.5 based on macroscopic appearance only. This led to a strictly subjective evaluation of turbidity and bacteria concentration, which furthermore could cause false negative and/or positive results if the concentrations were grossly inaccurate compared to McFarland standard 0.5. It would have been more precise to measure the turbidity using spectrophotometry, but that was not possible at the time.

Sample size

171 samples were brought back to NMBU for further testing. Only 72 samples were selected for sequencing. This decision was made due to time restrictions. The selection was based on morphological description of the bacteria and focused on including a representative selection from the three sites and sample locations on the fish. An attempt was made to exclude potentially overlapping bacteria based on morphological description. However, this is a limitation of this study, as it could have caused a possible misrepresented selection of the samples. It would have been preferable to include and sequence all acquired samples.

In addition, the sample size of 30 fish is arguably too small and makes it challenging to extrapolate the findings to antibiotic resistant bacteria in tilapia farming in Zanzibar as a whole. It would have been preferable to sample at least 60 fish.

Suboptimal timeline

The study had a somewhat suboptimal timeline, where the antimicrobial susceptibility testing (AST) was performed before the bacteria was sequenced and identified. This was due to logistical, practical and time restrictions. It would have been better to perform the AMR testing after sequencing, where it would have been possible to test relevant antibiotics according to the bacteria genus and/or species identified.

Kirby-Bauer method

The Kirby-Bauer method was used in this study due to logistical, practical and time restrictions. The method is quick, easy, and affordable. Limitations to the method are that not all slow or fastidious bacteria will grow, and there is also a subjective execution of the method with a lack of automation (Reller et al., 2009). Furthermore, the method does not give information about the minimum inhibitory concentration (MIC) of the tested antimicrobial to the given bacteria. Other methods that could have been used are the broth dilution method or the antimicrobial gradient method (Etest) (Reller et al., 2009). These methods will both provide a MIC value. They were not chosen due to time restrictions and resource availability.

Antibiotic sensitivity breakpoints

Some of the results were challenging to interpret because there were no established antibiotic sensitivity breakpoints for all bacteria identified and the antimicrobials sampled. Some of the bacteria identified in this study do have breakpoints from CLSI or EUCAST, but not for any of the antimicrobials used in this study. Ideally, greater consideration should have been given to the selection of antibiotics, based on the available literature and guidelines, prior to sampling and testing in Zanzibar. The choice of antibiotics in this study was based on

availability at NMBU and their representativeness in field application. The choice of antibiotics to use can vary depending on the specific goals and desired outcomes of the thesis. In this thesis, where the primary goal was to investigate AMR in general in farmed Nile tilapia, it would have been more beneficial to first know the identity of the bacteria present and then determine which antibiotics to use.

As mentioned in the earlier sections of this thesis, acquiring information about AMR, and monitoring its occurrence is of great significance. Consequently, the development of breakpoints and methodologies to measure AST hold an important role. The two most widely used systems worldwide, CLSI and EUCAST, lack harmonization, which makes it more complicated to validate and interpret the results in this study (Cusack et al., 2019).

Application and validity

The findings of this study give a good indication of the prevalence of AMR bacteria in farmed Nile tilapia in Zanzibar. However, it is important to note that AST was applied to a relatively low number of bacteria, which may not be sufficient to extrapolate to all fish farms in Zanzibar, let alone Tanzania as a whole. Furthermore, is it essential to note that the fish sampled were only collected from three different sites that were pre-decided. The sites might not be representative for the tilapia operations in Zanzibar overall. Little information is known about the sites, and it is not certain that the fishes sampled were a representative selection. This study cannot provide specific insights into the origins of the bacteria identified. To gain a better understanding in this regard, further research involving sequencing and exploring how the bacteria are related is necessary.

Despite the small sample size and small number of sample sites, the selection of fish was completely random, and therefore holds validity to the bigger picture of AMR bacteria in farmed Nile tilapia in Zanzibar. Although not all-encompassing, this study is a good starting point in the important research of AMR bacteria in the fish farming industry in Zanzibar. Further investigation is needed to make further assessment of AMR in fish farms in Zanzibar. Together with other studies this thesis gives important insight and contribute to the knowledge in this field.

Conclusion

The objective of this study was to assess the prevalence of antibiotic resistant bacteria in farmed Nile tilapia in Zanzibar. Estimates of AMR could only be made for *Aeromonas* sp. and the Enterobacteriaceae family, because the other identified bacteria genera and species do not have available antimicrobial sensitivity thresholds for the antibiotics tested, or because the number of identified genera or species were too few to estimate a prevalence of resistance. Samples identified as *Aeromonas* sp. showed a prevalence of resistance of 30% against tetracyclines. Samples identified as Enterobacteriaceae family had a prevalence of resistance of 31.6% against amoxicillin; 21% against trimethoprim; 5.2% against neomycin; 42.1% against tetracyclines; and 41.6% against sulfonamides. Considering the resistance for each antibiotic compared to the total occurrence of resistance, most AMR was seen toward tetracycline (41%), amoxicillin (22%) and sulfonamide (18%), followed by trimethoprim (15%) and neomycin (4%).

Further research with a higher number of samples is recommended to strengthen the findings of this study, and to be able to extrapolate the findings to farmed tilapia in Zanzibar as a whole. It would be interesting to study the exact resistance mechanisms to evaluate whether they are natural or acquired. It would also be beneficial to analyze the bacteria identified further to determine their origin, which can give indications to the degree of human or environmental contamination.

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Oppsummering

Tittel: Forekomst av antibiotikaresistente bakterier isolert fra oppdrettet Nile Tilapia (*Oreochromis niloticus*) i Zanzibar, Tanzania

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Veiledere: Øystein Evensen og Stephen Mutoloki, institutt for parakliniske fag

Antibiotikaresistens er et stadig økende problem, og en alvorlig trussel mot både folke- og dyrehelsen. Overforbruk og feilbruk er viktige drivkrefter bak resistensutvikling. Antibiotikaresistens kan forekomme i fiskeoppdrett på grunn av antibiotikabehandling av fisken eller på grunn av kontaminasjon fra miljøet eller mennesker. Denne studien undersøkte forekomsten av antibiotikaresistente bakterier i oppdrettet Nile Tilapia i Zanzibar. Prøver ble tatt fra tre ulike anlegg. Det ble totalt undersøkt 30 fisk, og det ble tatt prøver fra slim/hud, gatt og hodenyre. Testing av antibiotikafølsomhet ble gjort ved en disk-diffusjonsmetode på Muller-Hinton agar. Bakteriene ble identifisert ved hjelp av 16S-sekvensering. Generelt var forekomsten av antibiotikaresistens lav til moderat. Bakterieprøvene fra det ene av de tre anleggene viste ingen resistens mot antibiotikaene testet for. Innen Enterobacteriaceae familien var det sett høyest grad resistens mot tetrasykliner og sulfonamider. Forekomsten av resistens for hvert antibiotikum i forhold til total resistens viste høyeste nivåer AMR mot tetrasyklin, amoxicillin og sulfonamider.

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Appendices

Appendix A: Excel worksheet

Appendix B: Metadata

Appendix C: Morphology

Appendix D: Absorbance spectrophotometry

Appendix E: Overview of S, I, R and inhibition zones

Appendix A

Excel work sheet with overview of sites, weight, length, colony number, inhibition zones, results from PCR, match for sequence homology from BLAST.

Fish	Weight	Length	Organ	Agar	Colony number	Neo-mycin	Amoxi-cillin	Trime-thoprim	Tetra-cycline	Flor-fenicol	Sulfo-namides	Result PCR 16S	% match
A-1	82	16,5	g	Blood agar	3	20	13	28	0	20	0	*Plesiomonas shigelloides*	99.48
					4	24	0	31	0	32	0		
				Blue agar	5	25	16	30	0	21	28		
			s	Blood agar	1	24	0	0	26	30	20	*No match*	
					2								
				Blue agar									
			k	Blood agar									
				Blue agar									
A-2	112	20	g	Blood agar	6	22	16		30		30		
					7								
				Blue agar	9	24	0	25	30	36	26	*Aeromonas caviae*	97.67
			s	Blood agar	8	33	40				34		
				Blue agar	10	28	16	16	20	0	36	*Escherichia fergusonii*	99.69
			k	Blood agar									
				Blue agar									
A-3	117	18,5	g	Blood agar	11	27	0	0	12	33	20		
					12	30	32	21	28	32	34	*Escherichia coli*	99.50
					13	24	15		30		28		
				Blue agar	16	22	0	25	22	17	22		
			s	Blood agar	14								
					15								
				Blue agar	17	23	11	21	24	20	24		
			k	Blood agar									
				Blue agar									
A-4	110	18,5	g	Blood agar	18	22	0	27	25	25	0	*Raoultella ornithinolytica*	99.57
				Blue agar	20	23	17	30	0	23	23	*Escherichia coli*	99.07
			s	Blood agar	19	34	16	0	14	36	28		
				Blue agar	21	28	17	17	24	0	36	*No match*	
			k	Blood agar									
				Blue agar									
A-5	98	18	g	Blood agar	22	28	0		34	38	30		
				Blue agar									
			s	Blood agar	23								

Fish	Weight	Length	Organ	Agar	Colony number	Neo-mycin	Amoxi-cillin	Trime-thoprim	Tetra-cycline	Flor-fenicol	Sulfo-namides	Result PCR 16S	% match
				Blue agar	24	30	17	14	26	19	30	*Escherichia coli*	99.14
			k	Blood agar									
				Blue agar									
A-6	119	19	g	Blood agar	25	24	0	12	0	30	0	*Aeromonas caviae*	97.42
					26	25	18		20	42	28		
				Blue agar	29	25	18	0	0	23	0	*Escherichia coli*	97.76
					30	22,5	0	20	12	29	15		
			s	Blood agar	27								
					28	27	0	30	34	36	28		
				Blue agar	31	23	0	24	28	30	24	*Aeromonas caviae*	99.66
			k	Blood agar									
				Blue agar									
A-7	89	17,5	g	Blood agar	32	26	0	0	0	32	0	*No match*	
					33	23	16	36	32	40	30		
				Blue agar	37								
					38	23	0	26	30	34	22		
			s	Blood agar	34	25	0	0	27	32	24		
					35	23	11	26	30	36	22		
				Blue agar	39	25	0	0	0	25	0		
					40	26	23	23	21	16	33	*Escherichia coli*	99.22
			k	Blood agar	36	25	17	36	13	40	24	*Plesiomonas shigelloides*	99.58
				Blue agar									
A-8	93	18,5	g	Blood agar	41	23	16	34	16	40	24	*No match*	
				Blue agar	43	26	0	0	0	30	0	*Escherichia coli*	99.40
					44	24	0	30	25	23	36	*Citrobacter sp.*	97.71
			s	Blood agar	42	32	38				32		
				Blue agar	45	19	0	18	25	24	0		
					46	23	14	22	23	19	30		
			k	Blood agar									
				Blue agar									
A-9	59	15	g	Blood agar	47	22	17	32	14	40	34		
				Blue agar	50	23	27	32	0	26	0	*Escherichia coli*	99.51
					51	24	0	30	30	36	30	*Aeromonas veronii*	97.87
			s	Blood agar	48	22	0	30	31	34	24		
					49	28	19	16	22	0	30	*No match*	
				Blue agar	52	25	0	20	28	32			
					53								
			k	Blood agar									
				Blue agar									
A-10	87	17,5	g	Blood agar	54	24	15	36	15	40			

Fish	Weight	Length	Organ	Agar	Colony number	Neo-mycin	Amoxi-cillin	Trime-thoprim	Tetra-cycline	Flor-fenicol	Sulfo-namides	Result PCR 16S	% match
					55	24	17	34	12	40		*Plesiomonas shigelloides*	97.49
				Blue agar	58	25	0	30	26	24		*Citrobacter murliniae*	99.63
					59	26	0	32	11	34			
			s	Blood agar	56	24	42	40					
					57								
				Blue agar	60	24	0	24	16	34		*Aeromonas veronii*	99.67
			k	Blood agar									
				Blue agar									
B-1	55	15	g	Blood agar	61	23	14	0	26	30			
					62	25	0	0	12	0			
				Blue agar	66	24	25	0	24	18			
			s	Blood agar	63	25	27	11	24	18		*No match*	
				Blue agar	67								
			k	Blood agar	64	30	52	28	30	30			
					65	24	0	16	28	24		*Aeromonas sp.*	99.73
				Blue agar	68	28	25	17	24	16		*Escherichia coli*	99.65
B-2	91	18	g	Blood agar	69	25	18	22	30	38			
					70	24	26	0	22	17		*Acinetobacter junii*	99.82
				Blue agar	72	24	17	42	12	42			
			s	Blood agar	71	27	31	16	22	16			
				Blue agar	73	24	11	0	18	0		*Pseudomonas sp.*	99.24
			k	Blood agar									
				Blue agar									
B-3	128	20	g	Blood agar	74	23	0	21	20	24		*No match*	
				Blue agar	76								
			s	Blood agar	75	25	28	12	24	21			
				Blue agar	77	20	20	0	0	13		*Aeromonas caviae*	99.49
			k	Blood agar									
				Blue agar									
B-4	136	20,5	g	Blood agar	78	27	22	0	30	38			
					79	24	26	11	21	18			
				Blue agar	81	16	0	0	0	0			
					82	27	30	13	23	15		*Acinetobacter junii*	99.03
			s	Blood agar	80	28	27	18	23	22		*Acinetobacter sp.*	99.49
				Blue agar	83								
			k	Blood agar									

Fish	Weight	Length	Organ	Agar	Colony number	Neo-mycin	Amoxi-cillin	Trime-thoprim	Tetra-cycline	Flor-fenicol	Sulfo-namides	Result PCR 16S	% match
				Blue agar	84	22	28	15	21	16		*No match*	
B-5	118	20	g	Blood agar	85	27	17	0	31	35			
					86	23	16	42	12	30		*Plesiomonas shigelloides*	99.65
				Blue agar	88								
					89								
			s	Blood agar	87	23	21	0	20	16		*No match*	
				Blue agar	90	23	24	0	22	17			
					91	26	11	0	17	0			
			k	Blood agar									
				Blue agar									
B-6	57	14,5	g	Blood agar	92	24	0	15	0	20			
				Blue agar	94								
			s	Blood agar	93	27	32	13	27	20		*Acinetobacter johnsonii*	99.56
				Blue agar	95	28	21	20	24	20			
			k	Blood agar									
				Blue agar									
B-7	28	12	g	Blood agar	96								
				Blue agar	99	25	0	20	0	27		*Aeromonas jandaei*	98.59
					100	22	0	29	30	32			
			s	Blood agar	97	30	32	14	24	15			
				Blue agar	101	26	24	0	0	15			
			k	Blood agar	98	21	30	12	25	18		*Acinetobacter johnsonii*	99.15
				Blue agar	102								
					103	23	20	32	34	40		*Plesiominas shigelloides*	99.74
B-8	28	12,5	g	Blood agar	104	26	34	14	26	20		*Acinetobacter johnsonii*	99.9
				Blue agar	107	27	11	0	19	0			
					108	23	0	26	0	34			
			s	Blood agar	105	27	34	15	28	22			
				Blue agar	109	25	32	14	27	27		*Acinetobacter johnsonii*	99.56
			k	Blood agar	106	27	15	13	24	0			
				Blue agar	110	28	0	0	20	0		*Citrobacter freundii*	99.58
					111	28	34	18	19	30			
B-9	21	11	g	Blood agar	112	24	15	30	30	36		*Plesiomonas shigelloides*	99.32
				Blue agar	115								
			s	Blood agar	113	27	30	13	24	18		*Acinetobacter junii*	98.97
				Blue agar	116	24	30	12	24	20			
			k	Blood agar	114	25	32	12	26	20		*Acinetobacter johnsonii*	98.56

Fish	Weight	Length	Organ	Agar	Colony number	Neo-mycin	Amoxi-cillin	Trime-thoprim	Tetra-cycline	Flor-fenicol	Sulfo-namides	Result PCR 16S	% match
				Blue agar	117	21	20	12	25	19			
B-10	48	14	g	Blood agar	118	22	0	22	30	34			
				Blue agar	120	20	0	31	30	30			
					121	24	0	29	0	34		*Aeromonas hydrophila*	98.91
			s	Blood agar	119	28	32	15	25	17			
				Blue agar	122	28	30	12	23	16		*Acinetobacter junii*	99.41
			k	Blood agar									
				Blue agar									
C-1	91	18	g	Blood agar	123								
					124	25	0	30	33	34		*Aeromonas enteropelogenes*	99.66
					125								
				Blue agar	128	20	0	16	24	30			
					129	21	0	20	27	28			
			s	Blood agar	126	24	28	11	22	19			
				Blue agar	130	25	28	14	14	13		*Acinetobacter junii*	98.79
			k	Blood agar	127	28	44	38	36	36			
				Blue agar									
C-2	58	15	g	Blood agar	131	23	11	0	24	28		*Bacillus cereus*	99.30
					132	22	0	21	28	32			
				Blue agar	136	20	0	22	21				
			s	Blood agar	133	23	28	0	23	20			
				Blue agar	137	24	13	0	15			*Pseudomonas plecoglossicida*	99.73
			k	Blood agar	134	26	0	23	30	36		*Aeromonas hydrophila*	99.41
					135	30	44	54	30	40			
				Blue agar	138	24	26	12	21				
C-3	50	15	g	Blood agar	139	26	14	11	22			*Acinetobacter sp.*	99.33
					140	30	32	38	27				
				Blue agar	142	24	24	14	20				
			s	Blood agar	141	23	26	11	21				
				Blue agar	143	23	0	12	0			*Aeromonas sobria*	99.41
			k	Blood agar									
				Blue agar									
C-4	104	19	g	Blood agar	144	25	24	0	30				
					145	20	0	25	30				
					146								
				Blue agar	151	23	0	21	27			*Aeromonas hydrophila*	99.38
			s	Blood agar	147	24	30	11	21				
				Blue agar	152	26	30	12	22			*Acinetobacter junii*	99.24

Fish	Weight	Length	Organ	Agar	Colony number	Neo-mycin	Amoxi-cillin	Trime-thoprim	Tetra-cycline	Flor-fenicol	Sulfo-namides	Result PCR 16S	% match
			k	Blood agar	148	32	36	32	30				
					149								
					150	18	42	0	22				
				Blue agar	153	25	0	21	30			*Aeromonas sp.*	99.25
C-5	56	15	g	Blood agar	154	25	24	28	28				
					155								
				Blue agar	157	24	24	19	24	25			
			s	Blood agar	156	23	22	0	19	13			
				Blue agar	158								
					159	25	27	13	19	14		*Acinetobacter junii*	99.00
			k	Blood agar									
				Blue agar									
C-6	54	14,5	g	Blood agar	160	24	16	0	30	34			
					161	24	0	17	26	30		*Aeromonas sp.*	99.32
				Blue agar	163	23	18	11	22	18			
			s	Blood agar	162	25	24	12	21	18			
				Blue agar	164	25	30	12	26	34			
					165	23	0	0	17	0		*Pseudomonas hunanensis*	99.49
			k	Blood agar									
				Blue agar									
C-7	73	16	g	Blood agar	166								
					167								
				Blue agar	170	22	0	24	28	30		*Aeromonas taiwanensis*	99.83
			s	Blood agar	168								
					169	25	23	13	19	14			
				Blue agar	171	24	28	0	22	20		*Acinetobacter johnsonii*	99.10
			k	Blood agar									
				Blue agar	172	24	23	17	20	13		*Acinetobacter sp.*	99.47
C-8	38	13,5	g	Blood agar	173	22	18	28	22			*Vibrio mimicus*	100.0
				Blue agar	176	20	15	20	24				
			s	Blood agar	174	22	36	22	22				
					175	24	21	12	18	13			
				Blue agar	177	22	0	0	15			*Pseudomonas sp.*	99.91
			k	Blood agar									
				Blue agar									
C-9	37	13	g	Blood agar	178								
					179								
				Blue agar	182	22	26	0	22				

Fish	Weight	Length	Organ	Agar	Colony number	Neo-mycin	Amoxi-cillin	Trime-thoprim	Tetra-cycline	Flor-fenicol	Sulfo-namides	Result PCR 16S	% match
			s	Blood agar	180	23	25	12	20			*Acinetobacter sp.*	99.65
				Blue agar	183	24	28	13	24				
			k	Blood agar	181	26	26	28	30			*Aeromonas hydrophila*	99.49
				Blue agar	184	24	22	28	27				
C-10	23	11	g	Blood agar	185	25	17	0	25			*Bacillus albus*	99.56
					186								
				Blue agar	190	24	25	0	21				
			s	Blood agar	187	26	20	0	30				
					188	25	26	13	18				
				Blue agar	191	22	15	15	18			*No match*	
			k	Blood agar	189								
				Blue agar									

Appendix B

Metadata.

g	Gut
k	Head kidney
s	Skin
Weight	Gram
Length	Cm
SIR	Sensitive, intermediate, resistant
Colony nr	1-250

Appendix C

Morphology of the colonies recultured at NMBU.

Fish	Organ	Colony number	Morphology 21/09/2023	Comments	Result PCR 16S
A-1	g	3	Medium size, mucoid		*Plesiomonas shigelloides*
		4	Medium size, mucoid		
		5	Medium size, mucoid		
	s	1	Large, dry		*No match*
		2		Not pure culture on secondary smear	
	k				
A-2	g	6	Medium size, mucoid	Trimethroprim og florfenicol not readable (large zone)	
		7		Not pure culture on secondary smear	
		9	Medium size, mucoid		*Aeromonas caviae*
	s	8	Large, dry	Trimepthoprim, tetracycline and florfenicol not readable (large zone)	
		10	Medium size, mucoid		*Escherichia fergusonii*
	k				
A-3	g	11	Large, dry		
		12	Large, dry, yellow shear		*Escherichia coli*
		13	Small, mucoid	Trimethroprim og florfenicol not readable (large zone)	
		16	Small, mucoid	Lost during gel electrophoresis	
	s	14		Not pure culture on secondary smear	
		15		Not pure culture on secondary smear	
		17	Mucoid, medium size, white-ish	Possible mixed colony 21/09	
	k				
A-4	g	18	Large, dry		*Raoultella ornithinolytica*
		20	Medium size, mucoid		*Escherichia coli*
	s	19	Medium size, mucoid		
		21	Medium size, mucoid		*No match*
	k				
A-5	g	22	Medium size, mucoid, yellow-ish	Trimethroprim not readable (large zone). Possible contmination	
	s	23		Not pure culture on secondary smear	
		24	Medium size, mucoid		*Escherichia coli*
	k				
A-6	g	25	Large size, dry, irregular borders		*Aeromonas caviae*
		26	Medium size, mucoid, white-ish-grey	Trimethroprim not readable (large zone). Contaminated colony 28	
		29	Medium size, mucoid, white/grey		*Escherichia coli*
		30	Medium size, mucoid, white/grey		
	s	27		Not pure culture on secondary smear	
		28	Medium size, mucoid, white/grey	Contaminated colony 26	
		31	Medium size, mucoid, white/yellow		*Aeromonas caviae*
	k				
A-7	g	32	Large size, dry, irregular borders		*No match*
		33	Small/medium, mucoid		
		37		Not pure culture on secondary smear	
		38	Large size, dry		
	s	34	Large size, dry, irregular borders		
		35	Medium size, mucoid		
		39	Medium size, mucoid		
		40	Pinpoint colonies, convex, mucoid		*Escherichia coli*
	k	36	Large size, dry		*Plesiomonas shigelloides*
A-8	g	41	Pinpoint colonies, mucoid, yellow tinge		*No match*

Fish	Organ	Colony number	Morphology 21/09/2023	Comments	Result PCR 16S
		43	Medium size, mucoid, irregular borders		*Escherichia coli*
		44	Small/medium size, mucoid, white		*Citrobacter sp.*
	s	42	Medium/large size, mucoid, green-ish	Trimethroprim, tetracycline and florfenicol not readable (large zone)	
		45	Medium size, mucoid, yellow-ish		
		46	Small/medium, mucoid, white		
	k				
A-9	g	47	Small/medium, mucoid, white		
		50	Small size, mucoid, white/grey		*Escherichia coli*
		51	Medium size, mucoid, yellow-ish		*Aeromonas veronii*
	s	48	Medium size, mucoid, white/grey		
		49	Dry, yellow-ish		*No match*
		52	Small size, mucoid, yellow-ish		
		53		Suspected not pure culture.	
	k				
A-10	g	54	Small size, mucoid, yellow-ish		
		55	Medium size, dry		*Plesiomonas shigelloides*
		58	Small size, dry		*Citrobacter murliniae*
		59	Small size, dry		
	s	56	Medium size, dry	Tetracycline, florfenicol and sulfonamide not readable (large zone)	
		57		Suspected not pure culture.	
		60	Small size, mucoid		*Aeromonas veronii*
	k				
B-1	g	61	Small size, dry		
		62	Small size, dry, yellow		
		66	Small size, dry, irregular borders		
	s	63	Medium size, dry, white		*No match*
		67		Not pure culture on secondary smear	
	k	64		Not pure culture 21/09	
		65	Medium size, mucoid, yellow-ish		*Aeromonas sp.*
		68	Medium size, mucoid, white/grey		*Escherichia coli*
B-2	g	69	Small size, dry, yellow-ish		
		70	Small size, dry, yellow-ish		*Acinetobacter junii*
		72	Small/medium, mucoid, yellow-ish		
	s	71	Small size, dry, irregular borders		
		73	Medium size, mucoid, white/grey		*Pseudomonas sp.*
	k				
B-3	g	74	Small/medium, mucoid, yellow-ish		*No match*
		76		Not pure culture on secondary smear	
	s	75	Small/medium size, dry, yellow-ish		
		77	Medium size, mucoid, yellow-ish		*Aeromonas caviae*
	k				
B-4	g	78	Small/medium, dry, yellow-ish		
		79	Small size, yellow-ish		
		81	Small size, mucoid, yellow-ish		
		82	Pinpoint/small colonies, mucoid		*Acinetobacter junii*
	s	80	Dry, irregular borders		*Acinetobacter sp.*
		83		Not pure culture on secondary smear	
	k				
		84	Dry, irregular borders		*No match*
B-5	g	85	Small size, dry, white/grey, irregular borders		
		86	Small size, mucoid, yellow-ish		*Plesiomonas shigelloides*
		88		Not pure culture on secondary smear	
		89		Not pure culture on secondary smear	
	s	87	Small size, white/grey		*No match*
		90	Medium size, mucoid, white/grey		
		91	Small/medium		
	k				
B-6	g	92	Small/medium	Lost during gel electrophoresis	

Fish	Organ	Colony number	Morphology 21/09/2023	Comments	Result PCR 16S
		94		Not pure culture on secondary smear	
	s	93	Small size, white/grey		*Acinetobacter johnsonii*
		95	Medium size, mucoid, white/yellow		
	k				
B-7	g	96		Not pure culture on secondary smear	
		99	Dry, grey/white, irregular borders		*Aeromonas jandaei*
		100	Medium size, mucoid, yellow/white		
	s	97	Dry, grey/white, irregular borders		
		101	Small/medium size, mucoid, yellow/white		
	k	98	Dry, grey/white, irregular borders		*Acinetobacter johnsonii*
		102		Not enough material on Muller-Hinton agar.	
		103	Small/medium size, mucoid, yellow/white		*Plesiominas shigelloides*
B-8	g	104	Small size, dry, yellow-ish		*Acinetobacter johnsonii*
		107	Small/medium size, dry, yellow-ish, irregular borders		
		108	Small/medium size, dry, yellow-ish, irregular borders		
	s	105	Small size, dry, yellow-ish		
		109	Small/medium size, dry, yellow-ish, irregular borders		*Acinetobacter johnsonii*
	k	106	Small size, mucoid, white/grey		
		110	Small/medium size, dry, yellow-ish, irregular borders		*Citrobacter freundii*
		111	Small/medium size, dry, yellow-ish, irregular borders		
B-9	g	112	Small/medium size, dry, yellow-ish		*Plesiomonas shigelloides*
		115		Not pure culture on secondary smear	
	s	113	Small/medium size, dry, white/grey, irregular borders		*Acinetobacter junii*
		116	Small size, yellow-ish		
	k	114	Medium size, dry, white		*Acinetobacter johnsonii*
		117		Not pure culture 21/09	
B-10	g	118	Small size, yellow-ish		
		120	Medium size, mucoid, white/yellow, almost translucent		
		121	Medium size, mucoid, white/yellow, almost translucent		*Aeromonas hydrophila*
	s	119	Small size, orange, round, convex		
		122	Pinpoint colonies, mucoid, white/yellow		*Acinetobacter junii*
	k				
C-1	g	123		Not enough material on Muller-Hinton agar.	
		124	Medium size, mucoid, yellow-ish, round		*Aeromonas enteropelogenes*
		125		Not enough material on Muller-Hinton agar.	
		128	Medium size, mucoid, yellow-ish, round		
		129	Small/medium, mucoid, yellow		
	s	126	Small size, dry, white/yellow, round		
		130	Dry, white/yellow		*Acinetobacter junii*
	k	127		Not pure culture 21/09	
C-2	g	131	Dry, white/yellow		*Bacillus cereus*
		132	Small/medium, mucoid, yellow		
		136	Mucoid, white/grey		
	s	133	Small size, dry, white/grey		
		137	Pinpoint colonies, dry, orange		*Pseudomonas plecoglossicida*
	k	134	Medium size, mucoid, yellow		*Aeromonas hydrophila*
		135		Not pure culture 21/09	
		138	Small size, white/grey		
C-3	g	139	Mucoid, white/yellow, round, convex		*Acinetobacter sp.*
		140	Medium/large, mucoid		
		142	Small/medium size, mucoid, orange, round		
	s	141	Medium size, mucoid, orange, convex		
		143	Small/medium, mucoid, orange, round		*Aeromonas sobria*
	k				
C-4	g	144	Dry, white/grey, irregular borders	Amoxicillin: some colonies in the inhibition zone	

Fish	Organ	Colony number	Morphology 21/09/2023	Comments	Result PCR 16S
		145	Dry, white/grey		
		146		Not enough material on Muller-Hinton agar.	
		151	Mucoid, white/grey		*Aeromonas hydrophila*
	s	147	Medium size, mucoid, yellow, round, convex		
		152	Mucoid, yellow, round		*Acinetobacter junii*
	k	148		Not enough material on Muller-Hinton agar.	
		149		Not pure culture on secondary smear	
		150		No growth 21/09	
		153	Medium size, mucoid, yellow, round, convex		*Aeromonas sp.*
C-5	g	154	Medium size, mucoid, yellow, convex		
		155		Not enough material on Muller-Hinton agar.	
		157	Small size, dry, white/grey		
	s	156	Small/medium, mucoid, white/grey		
		158		Not pure culture on secondary smear	
		159	Medium/large, mucoid, yellow		*Acinetobacter junii*
	k				
C-6	g	160	Large, dry, irregular borders		
		161	Medium/large size, mucoid, yellow		*Aeromonas sp.*
		163		Contaminated by colony number 169 21/09	
	s	162	Medium/large size, mucoid, yellow		
		164	Pinpoint colonies, dry		
		165	Pinpoint colonies, dry		*Pseudomonas hunanensis*
	k				
C-7	g	166		Forgot to apply antibiotic tablets	
		167		Not enough material on Muller-Hinton agar.	
		170	Medium size, mucoid, white/grey, round		*Aeromonas taiwanensis*
	s	168		Not pure culture on secondary smear	
		169		Contaminated by colony number 163 21/09	
		171	Medium size, mucoid, white/grey, round		*Acinetobacter johnsonii*
	k				
		172	Medium size, mucoid, white/grey, round		*Acinetobacter sp.*
C-8	g	173	Medium size, mucoid, yellow, round		*Vibrio mimicus*
		176	Medium size, mucoid, orange, round		
	s	174	Medium size, mucoid, orange, round, convex		
		175	Medium size, mucoid, orange, round		
		177	Medium size, mucoid, white		*Pseudomonas sp.*
	k				
C-9	g	178		Not pure culture on secondary smear	
		179		No growth	
		182	Small size, almost translucent		
	s	180	Small size, grey, almost translucent		*Acinetobacter sp.*
		183	Small size, grey, almost translucent		
	k	181	Large size, mucoid, orange		*Aeromonas hydrophila*
		184	Medium size, mucoid, orange		
C-10	g	185	Dry, grey/white		*Bacillus albus*
		186		Not pure culture on secondary smear	
		190	Pinpoint/small colonies, dry, white/grey		
	s	187	Dry, grey/white		
		188	Pinpoint colonies, mucoid, orange, round		
		191	Small size, mucoid, white, round		*No match*
	k	189		Not pure culture on secondary smear	

Appendix D

Absorbance spectrophotometry.

Colony number	Concentration of DNA (ng/µL)	Purity of DNA (260/280 ratio)
1	81.68	1.90
3	94.33	1.86
9	152.54	1.85
10	83.78	1.87
12	142.39	1.91
16	68.70	1.70
18	165.78	1.87
20	159.62	1.86
21	127.85	1.94
24	123.20	1.90
25	130.12	1.84
29	145.65	1.87
31	153.93	1.83
32	79.90	1.89
36	134.13	1.84
40	136.73	1.89
41	139.63	1.90
43	169.40	1.89
44	186.87	1.81
49	91.51	1.89
50	74.80	1.78
51	193.68	1.88
55	228.56	1.89
58	95.04	1.85
103	75.62	1.85
86	108.83	1.86
104	32.96	1.85
110	55.57	1.72
73	99.13	1.87
93	48.97	1.86
109	46.24	1.65
98	44.04	1.94
60	136,00	1.83
63	174,00	1.85
114	34,00	1.92
77	89.33	1.89
87	112.41	1.81
113	23.87	1.64
92	132.85	1.87
112	82.25	1.9

Colony number	Concentration of DNA (ng/µL)	Purity of DNA (260/280 ratio)
65	40.25	1.84
84	49.25	1.88
68	35.81	1.78
70	59.79	1.85
80	116.62	1.92
82	46.26	1.78
99	116.87	1.83
74	124.53	1.86
172	49.68	1.92
131	40.45	2.14
177	259.00	1.86
143	132.00	1.84
130	91.62	1.88
173	173.83	1.85
137	101.85	1.86
185	40.78	1.85
134	88.74	1.85
180	114.09	1.87
122	56.17	1.84
191	127.09	1.88
121	181.57	1.86
161	82.29	1.85
170	301.97	1.85
139	59.65	1.85
124	117.12	1.83
151	96.49	1.83
171	24.93	1.86
181	116.19	1.85
159	109.79	1.83
153	126.78	1.85
165	120.90	1.86
152	108.61	1.84

Appendix E

Identified bacteria, S, I, R, and measured inhibition zones.

Site	Fish	Organ	Colony number	Neomycin	SIR	Amoxicillin	SIR	Trimethoprim	SIR	Tetracycline	SIR	Florfenicol	SIR	Sulfonamides	SIR	Result PCR 16S
A	A-1	g	3	20	R	13	R	28	S	10	R	20		10	R	Plesiomonas shigelloides
A	A-2	g	9	24		10		25		30	S	36		26		Aeromonas caviae
A	A-2	s	10	28	S	16	S	16	S	20	I	10		36	S	Escherichia fergusonii
A	A-3	g	12	30	S	32	S	21	S	28	S	32		34	S	Escherichia coli
A	A-4	g	18	22	I	10	R	27	S	25	S	25		10	R	Raoultella ornithinolytica
A	A-4	g	20	23	I	17	S	30	S	10	R	23		23	S	Escherichia coli
A	A-5	s	24	30	S	17	S	14	R	26	S	19		30	S	Escherichia coli
A	A-6	g	25	24		10		12		10	R	30		10		Aeromonas caviae
A	A-6	g	29	25	S	18	S	10	R	10	R	23		10	R	Escherichia coli
A	A-6	s	31	23		10		24		28	S	30		24		Aeromonas caviae
A	A-7	s	40	26	S	23	S	23	S	21	I	16		33	S	Escherichia coli
A	A-7	k	36	25	S	17	S	36	S	13	R	40		24	S	Plesiomonas shigelloides
A	A-8	g	43	26	S	10	R	10	R	10	R	30		10	R	Escherichia coli
A	A-8	g	44	24	I	10	R	30	S	25	S	23		36	S	Citrobacter sp.
A	A-9	g	50	23	I	27	S	32	S	10	R	26		10	R	Escherichia coli
A	A-9	g	51	24		10		30		30	S	36		30		Aeromonas veronii
A	A-10	g	55	24	I	17	S	34	S	12	R	40				Plesiomonas shigelloides
A	A-10	g	58	25	S	10	R	30	S	26	S	24				Citrobacter murliniae
A	A-10	k	60	24		10		24		16	S	34				Aeromonas veronii
B	B-1	k	65	24		10		16		28		24				Aeromonas sp.
B	B-1	k	68	28	S	25	S	17	S	24	S	16				Escherichia coli
B	B-2	g	70	24		26		10		22		17				Acinetobacter junii
B	B-2	s	73	24		11		10		18		10				Pseudomonas sp.
B	B-3	s	77	20		20		10		10	R	13				Aeromonas caviae
B	B-4	g	82	27		30		13		23		15				Acinetobacter junii
B	B-4	s	80	28		27		18		23		22				Acinetobacter sp.
B	B-5	g	86	23	I	16	S	42	S	12	R	30				Plesiomonas shigelloides
B	B-6	s	93	27		32		13		27		20				Acinetobacter johnsonii
B	B-7	g	99	25		10		20		10		27				Aeromonas jandaei
B	B-7	k	98	21		30		12		25		18				Acinetobacter johnsonii
B	B-7	k	103	23	I	20	S	32	S	34	S	40				Plesiomonas shigelloides
B	B-8	g	104	26		34		14		26		20				Acinetobacter johnsonii
B	B-8	s	109	25		32		14		27		27				Acinetobacter johnsonii
B	B-8	k	110	28	S	10	R	10	R	20	I	10				Citrobacter freundii
B	B-9	g	112	24	I	15	S	30	S	30	S	36				Plesiomonas shigelloides
B	B-9	s	113	27		30		13		24		18				Acinetobacter junii
B	B-9	k	114	25		32		12		26		20				Acinetobacter johnsonii
B	B-10	g	121	24		10		29		10	R	34				Aeromonas hydrophila
B	B-10	s	122	28		30		12		23		16				Acinetobacter junii
C	C-1	g	124	25		10		30		33		34				Aeromonas enteropelogenes
C	C-1	s	130	25		28		14		14		13				Acinetobacter junii
C	C-2	g	131	23		11		10		24		28				Bacillus cereus
C	C-2	s	137	24		13		10		15						Pseudomonas plecoglossicida
C	C-2	k	134	26		10		23		30	S	36				Aeromonas hydrophila
C	C-3	g	139	26		14		11		22						Acinetobacter sp.
C	C-3	s	143	23		10		12		10						Aeromonas sobria
C	C-4	g	151	23		10		21		27	S					Aeromonas hydrophila
C	C-4	s	152	26		30		12		22						Acinetobacter junii
C	C-4	k	153	25		10		21		30						Aeromonas sp.
C	C-5	s	159	25		27		13		19		14				Acinetobacter junii
C	C-6	g	161	24		10		17		26		30				Aeromonas sp.
C	C-6	s	165	23		10		10		17		10				Pseudomonas hunanensis
C	C-7	g	170	22		10		24		28		30				Aeromonas taiwanensis
C	C-7	s	171	24		28		10		22		20				Acinetobacter johnsonii
C	C-7	k	172	24		23		17		20		13				Acinetobacter sp.
C	C-8	g	173	22		18		28		22	S					Vibrio mimicus
C	C-8	s	177	22		10		10		15						Pseudomonas sp.
C	C-9	s	180	23		25		12		20						Acinetobacter sp.
C	C-9	k	181	26		26		28		30	S					Aeromonas hydrophila
C	C-10	g	185	25		17		10		25						Bacillus albus

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