Characterization of Coliforms from Water of Ranchan River, Serian Sarawak

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A thesis submitted in partial fulfilment of the Requirement of the Degree Bachelor of Science with Honours

(Resource Biotechnology)

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Characterization of Coliforms from Water of Ranchan River, Serian Sarawak.

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ABSTRACT

Coliform bacteria are Gram-negative belongs to the genera such as Citrobacter, Enterobacter, Klebsiella and Escherichia. Coliform bacteria are capable to cause pneumonia, respiratory diseases and urinary tract infections. As water provides as a potential transmission channel for pathogenic coliform bacteria, the rising cases of waterborne disease have raised concerns over the years. This research was carried out to isolate, identify, and characterize coliforms bacteria from water samples of Ranchan river, Sarawak. A total of 15 isolates were cultured, identified by 16S rRNA sequencing and characterized against 8 antibiotics. From the results, most bacteria (81.82%) present are resistant to Ampicillin (AMP,10). Result of the identification of bacteria by 16S rRNA sequencing revealed bacteria from the *Enterobacteriaceae* family, *Pseudomonas aeruginosa*, and *Aeromonas.* The antibiotic susceptibility test showed 100% of isolates were susceptible to Norfloxacin (NOR,10).

Key words: Coliform, Enterobacteriaceae, identification, Characterization

ABSTRAK

Bakteria koliform adalah Gram-negatif tergolong dalam genera seperti Citrobacter, Enterobacter, Klebsiella dan Escherichia. Bakteria koliform mampu menyebabkan radang paru-paru, penyakit pernafasan dan jangkitan saluran kencing. Memandangkan air membekalkan sebagai saluran penghantaran yang berpotensi untuk bakteria koliform patogen, peningkatan kes penyakit bawaan air telah menimbulkan kebimbangan selama ini. Penyelidikan ini dijalankan untuk mengasing, mengenal pasti, dan mencirikan bakteria koliform daripada sampel air sungai Ranchan, Sarawak. Sebanyak 15 isolat telah dikultur, dikenal pasti oleh penjujukan rRNA 16S dan dicirikan terhadap 8 antibiotik. Daripada keputusan, kebanyakan bakteria (81.82%) yang hadir adalah tahan terhadap Ampicillin (AMP,10). Hasil pengenalpastian bakteria melalui penjujukan rRNA 16S mendedahkan bakteria daripada keluarga Enterobacteriaceae, Pseudomonas aeruginosa, dan Aeromonas. Ujian kerentanan antibiotik menunjukkan 100% pencilan terdedah kepada Norfloxacin (NOR,10).

Kata kunci: Coliform, Enterobacteriaceae, pengenalan, Pencirian

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LIST OF ABBREVIATIONS

MHA	Mueller Hinton Agar
CLSI	Clinical Laboratory Standards Institute
PCR	Polymerase Chain Reaction
rpm	revolutions per minute
PBS	Phosphate Buffer Saline
DNA	Deoxyribonucleic acid
AGE	Agarose Gel Electrophoresis
C. *freundii*	*Citrobacter freundii*
S. *fonticola*	*Serratia fonticola*
E. coli	*Escherichia coli*
P. fulva	*Pseudomonas fulva*
A. *hydrophila*	*Aeromonas hydrophila*

CHAPTER 1

1.0 Introduction

Water covers an estimated 71% of the Earth's surface, and the oceans contain approximately 96.5 percent of all water on the earth ("How Much Water Is There on Earth? | U.S. Geological Survey," 2018). Water cycle is known as hydrologic cycle which flows consistently in Earth such as evaporation process, transpiration process, condensation process, precipitation process and runoff. Precipitation over land is impacted by evaporation and transpiration (Britannica, 2019).

Coliform bacteria are gram-negative bacteria that belong to the genera of *Enterobacteriaceae* which are *Citrobacter freundi, Enterobacter cloacae, Enterobacter aerogenes* and *E. coli* (Halkman, 2014). The Ranchan River is a densely populated area near Serian Sarawak, where people always have picnics and spend more time at the river waterfalls. As a result, coliforms can cause diseases such as pneumonia, respiratory illness, and urinary tract infections (Todar, 2007). Most *E. coli* are harmless to humans, but some can cause mild illness and some can cause serious water-borne infections (Bryan, 2016).

Coliform bacteria are found in the intestine of all warm-blooded animals and humans ("Coliform Bacteria in Drinking Water | Washington State Department of Health," 2019). *E. coli* bacteria are unlikely to be harmful. The vast majority of microbes that can contaminate water supplies are found in feces. *E. coli* bacteria come in three varieties. Whole coliform bacteria occur naturally and are generally harmless, such as in the ground or in vegetation. Faecal coliforms are among the coliforms. They are found in human and animal feces and in the intestines. *E. coli,* for example, belongs to the group of coliform bacteria. Most of *E. coli* bacteria are harmless and live in the intestines of humans and warm-blooded animals. However, Ranchan

river is potential channel for the transmission of coliform bacteria as it may be contaminated with faeces of human or animals that have been infected with coliform bacteria.

Pathogenic bacteria are dangerous bacteria that cause disease. Bacterial diseases occur when pathogenic bacteria enter and infect the body. Medical professionals may prescribe antibiotics for bacterial diseases (Festa & McBratney, 2022). Antimicrobial drugs prevent bacteria from multiplying and spreading. Antibiotics work against different types of bacteria. Thus, antimicrobial resistance is currently one of the most serious threats to global health, food security and development.

Therefore, contaminated water is a potential to transmission pathway of coliform bacteria. The presence of faecal coliform bacteria in a water sample frequently indicates recent faecal contamination, implying that pathogens seem to be more likely to be present than that if only total coliform bacteria are detected. Hence, this makes the water becomes potentially contaminated with coliform bacteria.

The objectives of this study are;

1. To isolate and identify the coliform bacteria from water samples collected at Ranchan river
2. To determine the antibiotic susceptibility pattern of the coliform isolates using disk diffusion method.

CHAPTER 2

2.0. Literature Review

2.1 Ranchan River, Serian Sarawak

Water sample were obtained from Ranchan river and used for the testing of antimicrobial activity in Molecular Laboratory in Institute of Biodiversity and Environmental Conservation and Virology Laboratory, Faculty of Resource Science and Technology, University Malaysia Sarawak. Figure 1 show the location of how far Ranchan river from Kuching town.

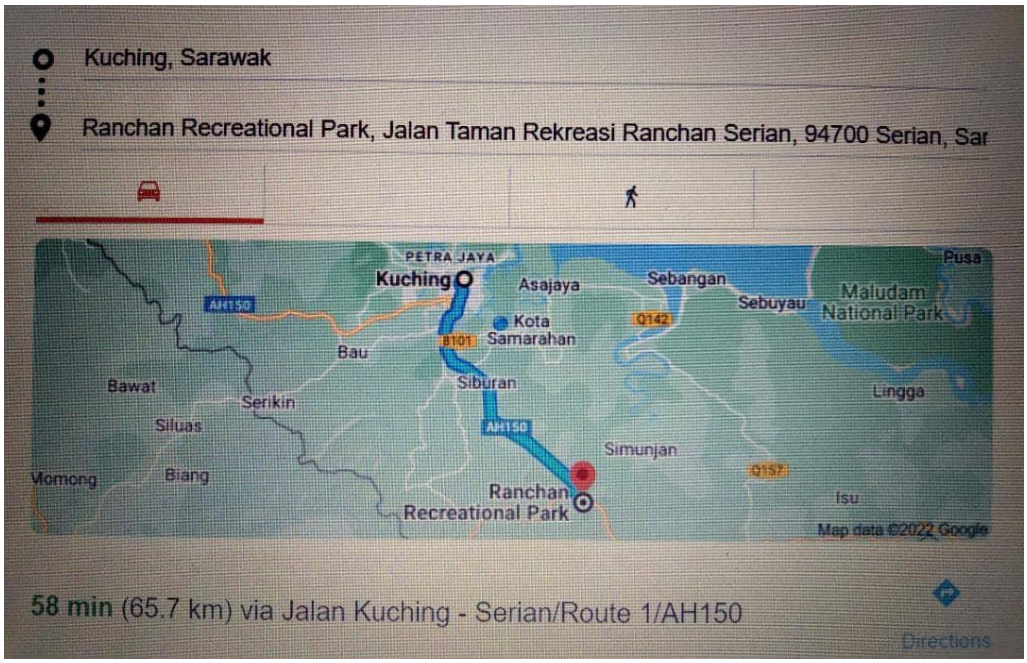

Figure 1: View of how far Ranchan river from Kuching town

2.2 Waterborne Disease

Waterborne illness is caused by pathogens or disease-causing microbes in recreational water. A variety of symptoms can be caused by waterborne illnesses. For example diarrhoea and vomiting are the most commonly reported symptoms of waterborne illness, other symptoms can include skin, ear, respiratory, or eye problems. Besides, usually water that contaminated with human or animal faeces poses the greatest microbial risk (Cabral, 2010).

2.3 Bacterial Diseases

Bacterial infections result from the spread of harmful strains of microorganisms in or on the body. Microorganisms have the ability to contaminate all proteins in the body. Pneumonia, meningitis, and food poisoning are just a few of the diseases caused by virulent bacteria. Bacterial pneumonia was most likely the leading cause of death in the elderly until the mid-20th century ("Bacterial Disease | Definition, Types, & Mechanisms | Britannica," 2022). Bacterial diseases are contagious and can be transferred from one person to the next. Bacterial diseases are infectious diseases spread by water, air, food, and bodily fluids. Even with advances in medical research, bacterial diseases continue to be one of the leading causes of death in humans.

2.4 Resistance Bacterial Strains

Antimicrobial resistance is a major public health problem. Antibacterial resistance occurs when bacteria develop the ability to kill them or resist antibiotics designed to stop their growth altogether (Nikaido, 2009). Some pathogens develop resistance to common antibiotics. Antibiotics are drugs used to treat bacterial infections by inhibiting or preventing bacterial growth. Antibiotic resistance occurs when bacteria develop their ability to kill antibiotics or resist antibiotics designed to block their growth.

2.2 Coliform

According to the Washington State Department of Health (n.d.), coliforms are organisms, microbes, and feces found in all warm-blooded animals and humans. Coliform bacteria are abnormally harmful to the human body. On the other hand, their presence in water indicates the presence of pathogens in the water system. Total coliforms, coliforms, and *E. coli* are indicators of water quality. The entire coliform group contains a variety of bacteria. Fecal coliforms are total coliforms found primarily in stool. Coliforms are coliforms. *E. coli* is a type of coliform bacteria.

2.3 Antimicrobial Activity

Antibacterial activity refers to the process of killing or inhibiting pathogenic bacteria. This fact is accomplished using a variety of antibacterial drugs. An anti-toxin is a synthetic substance that has the ability to be restorative against the development of microscopic organisms while also being safe for the host (Antimicrobial, n.d.). In 2013, 9.2 million passings were accounted for because of microbial contaminations (Khameneh et al., 2019).

2.4 Enterobacteriaceae

Enterobacteriaceae is a huge family of bacteria that known as pathogens such as *Salmonella, Shigella, and Escherichia coli*. For example, *Bacillus* (rod-shaped) facultative anaerobes produced by digesting lactic acid and other end products belong to the family Enterobacteriaceae. Usually, they are normally 1-5 μm long and have gram-negative stains (Guentzel, n.d.).

2.4.1 *Citrobacter freundi*

Citrobacter freundii (Citrobacter genus, Enterobacteriaceae) is a known opportunistic pathogen. Urinary tract infections, diarrhea, pneumonia, and rarely meningitis and intracranial abscesses are caused by *C. freundii* (Liu et al., 2017).

2.4.2 *Enterobacter cloacae*

Enterobacter cloacae is a common pathogen that can cause pneumonia, or urinary tract infections. With the advent of multidrug resistance (MDR), including resistance to the last-choice carbapenem, meropenem, imipenem, and ertapenem, interest in these microorganisms has increased significantly (Annavajhala et al., 2019).

2.4.3 *Enterobacter aerogenes*

Aerobacter aerogenes was renamed *Enterobacter aerogenes* in 1960 after being classified into the genus Enterobacter. The phenotype of *E. aerogenes* may be due to horizontal gene transfer from other Enterobacteriaceae species. It is also known as an in-hospital pathogen that causes bacterialemia, endocarditis, septic arthritis, and infections of the lower respiratory tract, urinary tract, and abdominal cavity (Davin and Pages, 2015).

2.4.4 *E. coli*

Escherichia coli is the most common species of fecal coliforms (*E. coli*). Only *E. coli*, one of the five common groups of bacteria that make up the total number of coliforms, is usually not observed to grow and grow in the environment. As a result, *E. coli* is widely recognized as the best indicator of fecal contamination and the presence of pathogens. *E. coli* and other fecal coliforms are pathogens. The presence of *E. coli* colonies is confirmed by the addition of the urea substrate. This bacterium is a favorite indicator of freshwater recovery as it indicates fecal contamination by

warm-blooded animals. *E. coli* is rarely dangerous, but it can cause meningitis, sepsis, urinary tract infections, and bowel infections.(Water science school, 2018).

2.5 Molecular Identification and Characterization of Enterobacteriaceae

2.5.1 16S rRNA Sequencing

The 16S rRNA gene sequencing method is widely used in complex biological mixtures such as environmental samples to identify, characterise, and quantify microorganisms. The 16S rRNA gene is a highly conserved component of the transcription factors of all DNA-based organisms, making it an ideal target gene for sequencing the DNA of samples containing thousands of different species (Cox et al., 2013). Universal PCR primers can target the conserved region of the 16S gene, enabling the gene to be amplified by a wide range of microorganisms from a single sample.

CHAPTER 3

3.0 Material and methods

3.1 Study Area

This research focused on the characterization coliform bacteria from water in Ranchan river, Serian Sarawak. Fifteen samples of water from Ranchan river. Figure 1 show the location of water sample collection which was at Ranchan river (1.143439,110.584451).

Figure 2: View of First sampling location, Ranchan river as pointed by the coordinates.

Each of the samples were then labeled according to where they were collected. For example, RRL which indicates that the samples was collected from Ranchan river location while STS stands for station sample. The list of samples is shown in Table 1.

Table 1: RRL stands for Ranchan river location, ST stands for Station sample

Sample code	Source
RRL STS1	Water
RRL STS2	Water
RRL STS3	Water
RRL STS4	Water
RRL STS5	Water

3.2 Media preparation

3.2.1 HiCrome™ Agar

Approximately 27 gram of Hichrome agar powder was dissolved in 1L distilled water and mixed by string. The mixture was heated to properly dissolve the agar and autoclaved at 121℃ for 15 minutes. Once autoclaved, sterilized agar then poured into the petri dishes and let to solidified. The agar plates were put in the fridge before use.

3.2.2 Nutrient Agar Preparation

Approximately 28 gram of nutrient agar powder was dissolved in 1L distilled water and mixed by stirring. The mixture was heated to properly dissolve the agar and autoclaved at 121 degrees Celsius for 15 minutes. Once autoclaved, sterilized agar then poured into the petri dishes and let to solidify. The agar plates were put in the fridge before use.

3.2.3 Mueller-Hinton Agar Preparation (MH agar)

Approximately 38 gram of MH agar powder was dissolved in 1L distilled water and mixed by string. The mixture was heated using microwave to properly dissolve the agar and autoclaved at 121℃ for 15 minutes. Once autoclaved, sterilized agar the poured into petri dishes and let to solidified. The agar plates were put in the fridge before use.

3.2.4 Mueller-Hinton Broth Preparation (MHB)

Approximately 21 gram of MHB powder was dissolved in 1L distilled water and mixed by string. The mixture was heated using microwave to properly dissolve the agar and autoclaved at 121℃ for 15 minutes. Once autoclaved, sterilized broth the poured half-full into 4ml aluminium screw top glass bottles.

3.3 Water Sample Preparation

First, HiCrome™ agar was prepared. Then, 2 µL of water sample was added using a 10 µL micropipette into each microcentrifuge tube through white grid Whatmann sterile membrane filters paper (0.45 µm) and water was distributed evenly on top of the filters with vacuum open. Next, the filters was removed by sterile forceps and was placed in the middle of agar. Then, the step was repeated for 5 plates agar and labeled accordingly. Place 5 completed plates agar and were incubated for 24 hours in 37℃.

3.4 Bacteria Colony Purification

Nutrient agar was prepared for 15 petri plates. Then, One bacterial colony from 15 sample plates was taken using inoculating loop. Streaking was done on the surface of new Nutrient agar plates. The step was repeated for 15 plates agar with 15 different colonies of coliform bacteria and labeled accordingly. The loop was heated using Bunsen burner before taking the colony and each time before doing the streaking. Then, Place 15 completed plates agar side up and were incubated for 24 hours in 37℃.

3.5 Stock Culture Preparation

Nutrient agar was prepared for 1L. Once done autoclaved, sterilized agar then poured half-full into 15 bijou bottles. Then, The bijou bottles half filled with agar were kept at slanted angle as the agar solidified. One pure bacteria colony was taken from sample agar plates using inoculating loop and streaked on the slanted agar surface. Then, the slanted agar then accordingly labeled and incubated for 24 hours at 30℃ to create stock culture. After incubation, stock cultures were kept in the fridge before use.

3.6 DNA Extraction

15 2mL microcentrifuge tubes and 15 100 µL tips were autoclaved at 121℃ for about 45 minutes by placing the tubes in a clean beaker and covering it with aluminium foil. Then, 40 µL of sterilized water was added using a 10-100 µL micropipette into each microcentrifuge tube after the bottles were autoclaved near the flame of the Bunsen burner. One colony of bacteria was taken using a sterilized loop from one Nutrient agar petri plate and put into microcentrifuge tube. The step was repeated for 15 microcentrifuge tubes with 15 different colonies of coliform bacteria and labeled accordingly. Then, the microcentrifuge tubes were heated using a pcr machine for 15 minutes at 95℃ and spun at maximum speed at 1350rpm for a minute.

3.7 Thermocyler PCR Machine

PCR master mix for 16S rRNA sequencing was prepared using the reagents below for one prep with 4 µL buffer, 4 µL MgCI2, 0.3 µL dNTP, 2 µL primer forward, 2 µL primer riverse, 25.4 µL ddH2O and 0.3 µL taq. Table 2 shows reagents of master mix for 16S rRNA sequencing.

Table 2: Reagents of master mix for 16S rRNA sequencing.

Reagents	Volume (µL)
Buffer	4
$MgCl_2$	4
dNTP	0.3
Primer –F (27F)	2
Primer-R (519R)	2
ddH_2O	25.4
DNA template	2
Taq Polymerase	0.3
Total	40

Next, 38 µL of the pcr master mix and 2µL of the supernatant from DNA extraction step was added into another 15 sterilized microcentrifuge tubes respectively and labeled accordingly. Then, the microcentrifuge tubes were put in the thermocycler PCR machine for about 1.5hours.

3.8 Agarose Gel Electrophoresis

10X TAE buffer was diluted by adding 50mL of the 10X TAE buffer and 450mL of ddH2O into a sterilized 1L Scott's bottle. 50mL of the diluted 10X TAE buffer was measured and poured into a sterilized conical flask. 0.5g of agarose gel powder was weighed and kept in the sterilized conical flask containing the diluted 10X TAE buffer. Then, the conical flask was heated for a minute in the microwave. After 1 minute in the microwave, the solution was poured into the casting tray with the well combs attached. The agarose gel was solidified after 20 minutes. A volume of 3 µL of 100bp DNA ladder was added into the first well of agarose gel and 5 µL of the 15 PCR products into each well, respectively, using sterilized micropipette tips. Then, the electrodes were fixed accordingly as red color for positive electrode and black color for negative electrode. The agarose gel electrophoresis was turned on for 40 minutes at 200℃ and 90V. After 40 minutes, the agarose gel was carefully submerged into EtBr for staining for 20-30 minutes and was then replaced with water to destain for about 10-20 minutes. DNA fragments were visualized under UV light as bands.

3.9 Antibiotic Susceptibility Tests

Disk diffusion method was used. Antibiotic disks used were Ticarcillin-clavulanic acid (85µg), Streptomycin (10 µg), Amikacin (30 µg), chloramphenicol (30 µg), Piperacillin (100µg), Norfloxacin (10 µg), nalidixic Acid (30 µg), ampicillin (10 mca).

The disc diffusion method was used. Then, Mueller Hinton broth was prepared by suspending 21g of the Mueller Hinton Powder in 1L distilled water. In order for the medium to completely dissolve, it was heated to boil. Once the medium was mixed well, it was sterilized by autoclaving at 121℃ for 12 minutes. After the autoclaving, 5mL of the broth was poured into each of the 15 bijou bottles that have been sterilized earlier. Next, one colony of bacteria from 15 agar was inoculated into the MH broth. *E. coli* ATCC 25922 which was prepared on a LB agar was used as a positive control. Bacterial culture broth was shaken overnight to allow it to grow. Bacterial growth is indicated by cloudy broth. The cotton bud was then dipped into the broth and swabbed uniformly on the MH agar surface. For 5 minutes, the plates were allowed to dry. The agar plates were then divided into sections for each antibiotic disc and labelled with a marker pen. Using forceps, antibiotic discs were placed on each of the agar plates. The plates were then incubated at 37°C for 24 hours. Following incubation, the diameter of the inhibition zone on MH agar was measured, recorded, and classified as susceptible (S), intermediate (I), or resistant (R) using Clinical and Laboratory Standards Institute (CLSI, 2012) antimicrobial susceptibility testing standards M02-A11.