WATER MICROBE SCREENING FROM RIVER BANK ALONG LATA JANGGUT, KELANTAN

by

NOR SHAKIRAH BT RAMLI

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DECLARATION

I declare that this thesis entitled “Water Microbe Screening from River Bank along Lata Janggut, Kelantan”is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

Signature : _____

Name : _____

Date : _____

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LIST OF ABBREVIATIONS

CO_2	Carbon Dioxide
CTAB	Cetyltrimethylammonium Bromide
dNTPs	Deoxynucleotide Triphosphate
E’	East
EDTA	ethylene-diamine-tetraacetic acid
Et-Br	Ethidium Bromide
LB	Luria Broth
N’	North
NaCI	Sodium Chloride
NaOH	Sodium Hydroxide
OD	Optical density
pH	Potential hydrogen
sp.	Species
SDS	Sodium Dodecyl Sulfate
SPM	Semi Permaeble Membrane
TE	Tris-EDTA
TBE	Tris-Borate
Tris	Trisaminomethane

LIST OF SYMBOLS

mmol/L	Millimoles per litre
V	Volt
mm	Milimeter
nm	Nanometer
mL	Milliliter
M	Mol/Molar
µL	Microliter
%	Percentage
~	Approximately
°	Degree
℃	Degree Celcius

PEMERIKSAAN MIKROBIOLOGI AIR DARI TEBING SUNGAI LATA JANGGUT, KELANTAN

ABSTRAK

Mikrobiologi air adalah tentang mikroorganisma yang hidup di dalam air. Antara kepentingan kajian ini adalah untuk menetukan kepelbagaian mikrob di dalam air. Kajian ini adalah untuk memeriksa dan mengenal pasti mikrob air dari Lata Janggut, Kelantan. Kaedah pemencilan mikrob air menggunakan media agar nutrient, kultur yang menggandungi Luria Bertani (LB) larutan dan sampel air dari pencairan bersiri. Keputusan dari pewarnaan gram, enam daripada lapan isolat dikenal pasti sebagai Gram Positif manakala 2 lagi adalah Gram Negatif. Morfologi bagi koloni isolat yang terbentuk adalah cocci dan bacili. Sampel tersebut diuji menggunakan UV Spectrophotometer dan Nanodrop Spectrophotometer untuk menilai ketumpatan sampel-sampel tersebut. Kajian diteruskan dengan 1% gel agarose elektroporesis di mana keratan-keratan DNA dapat dilihat menggunakan cahaya UV. Dalam Reaksi Rantai Polimer, pelbagai keratin DNA dihasilkan. Oleh itu, pengoptimuman dalam Reaksi Rantai Polimer diperlukan untuk meningkatkan penghasilan produk Reaksi Rantai Polimer.

WATER MICROBE SCREENING FROM RIVER BANK ALONG LATA JANGGUT, KELANTAN

ABSTRACT

Water microbiology is about the microorganisms that live in the water. The significance of this research is to determine the diversity of microbes in water. This study aim to screen and identify water microbes from Lata Janggut, Kelantan. The research started from taking the water samples from Lata Janggut, isolation of bacteria, bacteria morphology identification, DNA extraction, gel electrophoresis and polymerase chain reaction (PCR). The isolation of water microbes is using enrichment culture that contains Nutrient Agar (NA) media, Luria Bertani (LB) broth, and water sample from serial dilution. The result from Gram staining showed, 6 out of 8 isolates were Gram positive while the other 2 were Gram negative after staining. The isolated colonies morphology are both coccii and bacilli. The samples were measured with UV spectrophotometer and Nanodrop Spectrophotometer to obtain their optical density. The research was continued with 1% agarose gel electrophoresis (AGE) which the DNA bands can be visualized under UV light. In Polymerase Chain Reaction (PCR), multiple bands were formed. Hence, future application for screening and identification of water microbes will be investigated further.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Microorganisms build up a large zone of the planet's living material which plays a crucial role in maintaining the ecosystem of the Earth. Zion National Park (2014), has defined microorganisms as a living thing which is too small and need to be viewed by microscope. The specific domain of microorganism consist of Archae, Eukarya and Bacteria which known as eukaryote (Pepper & Gentry, 2015). Microorganisms include viruses, bacteria, fungi, algae and protozoa which vary in size and shape (Mara & Horan, 2003).

According to Boundless (2016), microorganisms are divided into seven types of groups which archae, protozoa, algae, fungi, viruses and multicellular animal parasites known as helminth. Microorganism can be found almost on the planet including hot spring, deep ocean, deserts or even in our body.

Microorganisms can be classified based on a few factors such as their shape, nutrient requirements, cell wall staining, cell appendages and a few more factors. There are various type of bacteria including *Cocci* which spherical or oval cells shape, Bacilli which is look like rod shape cells, meanwhile *Vibrios* are comma shaped and curved rods which is based on their vibratory motility, *Spirilla* is a cell with spirals forms, Spirochetes which means coil and chaite. Besides, *Actinomycetes* are being named as the resemblance to the radiating rays of the sun when seen in tissue lesions.

Microorganisms can lead to several of diseases. One of them is waterborne disease. According to National Institute of Environmental Health Sciences (2013), waterborne diseases are caused by various types of microorganism especially pathogenic, biotoxins or toxic contaminants. Waterborne disease resulted from direct and indirect exposure to water. It may be from the application or from skin exposure during bathing or recreational water use (Smith, 2016). The disease can lead to the illness such as cholera, schistosomiasis or other gastrointestinal problems.

According to Smith (2016), the agents of water-related disease are microbiological pathogens or toxic substances. Water-related diseases or water-associated diseases is a widespread disease which able to give a bad effect on human health such as death, disability, illness or disorder. The disease may be caused by direct or indirect condition or the changes in quantity or quality of the water. Trachoma is the example of a disease which is spread due to poor hygiene.

Water-related disease is caused by the consumption or exposure to contaminated water. Water exposure can be divided into a few types, including drinking water which is divided into treated drinking water and untreated drinking water, recreational water use and aerosols.

In this study, Lata Janggut which is a recreational area that is located in Jeli, Kelantan was chosen as the study area. As a recreational area, this place is highly potential to be used as research area for determination of the diversity of environmental microbes.

1.2 Problem Statement

Lata Janggut, Jeli, Kelantan has been chosen as the study area since there is no previous study or research on water microbiology has been done there. Lata Janggut has been known as recreational area among local people especially Jeli residents. So, it is important to do the research this area since it may help to give awareness about the water status to people who visit that area.

1.3 Objective

The objective of this study is to determine the diversity of microbes in water of Lata Janggut.

CHAPTER 2

LITERATURE REVIEW

2.1 Lata Janggut's background

Lata Janggut is situated in Jeli district. Jeli is located at the eastern part of Kelantan. Jeli is located within central belt in map of Peninsular Malaysia. Jeli also bordered by the state of Perak to the west, Thailand to the north, Tanah Merah district to the north east and Kuala Krai district to the south east.

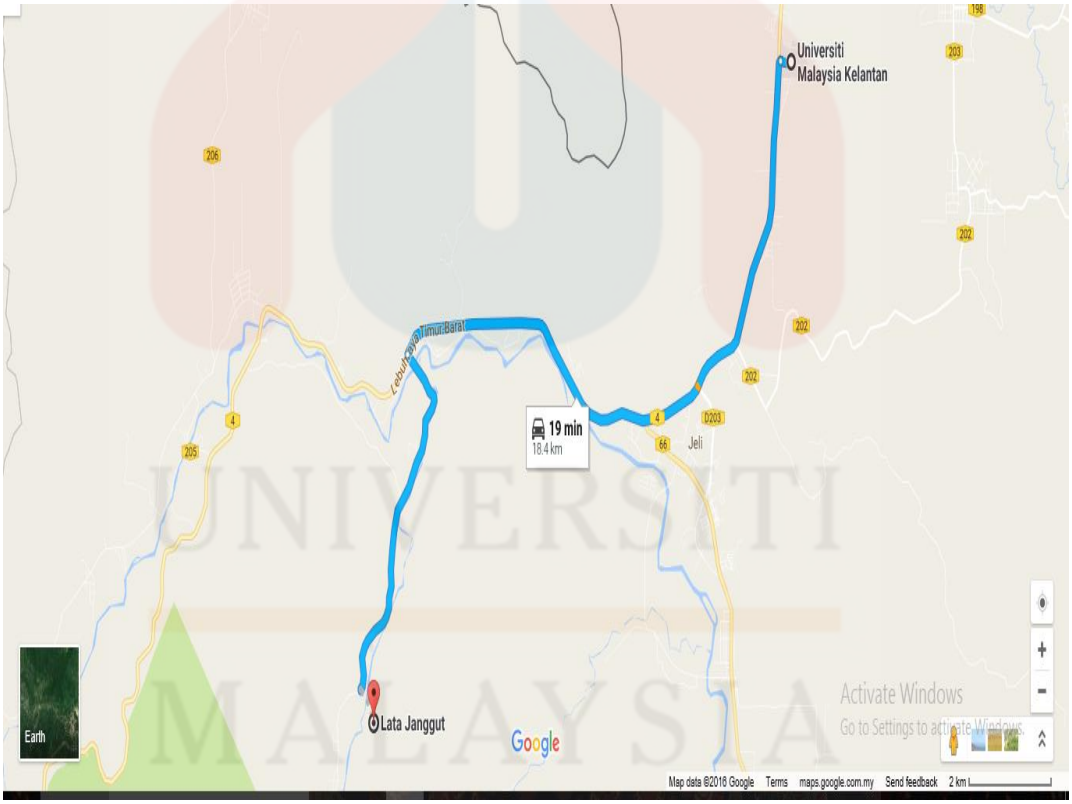

Figure 2.1: The figure shows the location of Lata Janggut from Universiti Malaysia Kelantan, Kampus Jeli which takes 19 minutes by using car. 1 cm indicates 2 km. (Sources: Google Maps, 2016)

2.2 Freshwater ecosystems

Freshwater ecosystem is one of the part of Earth's aquatic ecosystems which including lakes, ponds, rivers, stream, springs and wetlands. Freshwater ecosystems can be divided into standing water and flowing waters. Standing water includes lentic systems which ponds, lakes, marshes and other enclosed water bodies. Meanwhile, flowing water includes lotic systems such as river, estuaries and canals. Freshwater ecosystems are support by physical habitat, energy sources, water quality biotic interactions, hydrology and connectivity (Hitt *et al.*, 2015). Besides that, freshwater habitats also can be classified by different factors, including temperature, light penetration, and vegetation.

Alexopuolos, Plessas and Bezirtzoglou (2011) argue that, there are three domain kingdom of bacteria that are usually form within freshwater environment includes Bacteria, Archae and Eukarya. Bacteria present almost in all freshwater environment which contain single kingdom, Eubacteria which also includes bacteria, actinomycetes and blue-green algae. Besides, domain Archae will be limited presence in extreme freshwater ecosystem.

Freshwater microorganisms are divided base on their feeding habits, autotrophs and heterotrophs. Autotrophs synthesize carbon compounds from external CO_2. They obtain their nutrients supplies as nitrogen and phosphorus from inorganic compounds. Meanwhile, heterotrophs use organic compounds as their source of carbon. Basically, bacteria, protozoa and fungi are heterotrophic among freshwater microorganisms.

2.3 Morphology of bacteria in identification

Tshikhudo, Nnzeru, Ntushelo and Mudau (2013), they have been argued that bacteria are grouped according to their shape, presence or absence of flagella, and arrangement of flagella. Besides, microorganisms are also classified based on their other morphological characteristics including their color, cell size, colonial morphology and other characteristics as it is the easiest method to identify the microorganisms. The size and shape of bacteria are determined by using microscopic observation method (Shaha *et al.*, 2016).

Basically, the microorganisms were identified in order to the pathogenic potential because some of them have the ability to spread and distribute diseases. They may be cocci, bacilli or spirals. Cocci may be divided into streptococci, tetrads, sarcinae and staphylococci. Cocci may be oval, elongated or flattened which is attached after cell division.

Based on figure 2.2, the figure shows the bacterial morphology. Cocci after cell division may be diplococci, streptococci, staphylococci, streptococci, sarcinae or tetrad. Besides, Bacili are consist of genus of bacteria namely as *Bacillus* sp., a bacteria with a rod shape. Single Bacillus may be appeared as a single rod. Based on Figure 2.2, Bacilli also divided into few other shapes after cell division such as diplobacilli, streptobacili and palisades.

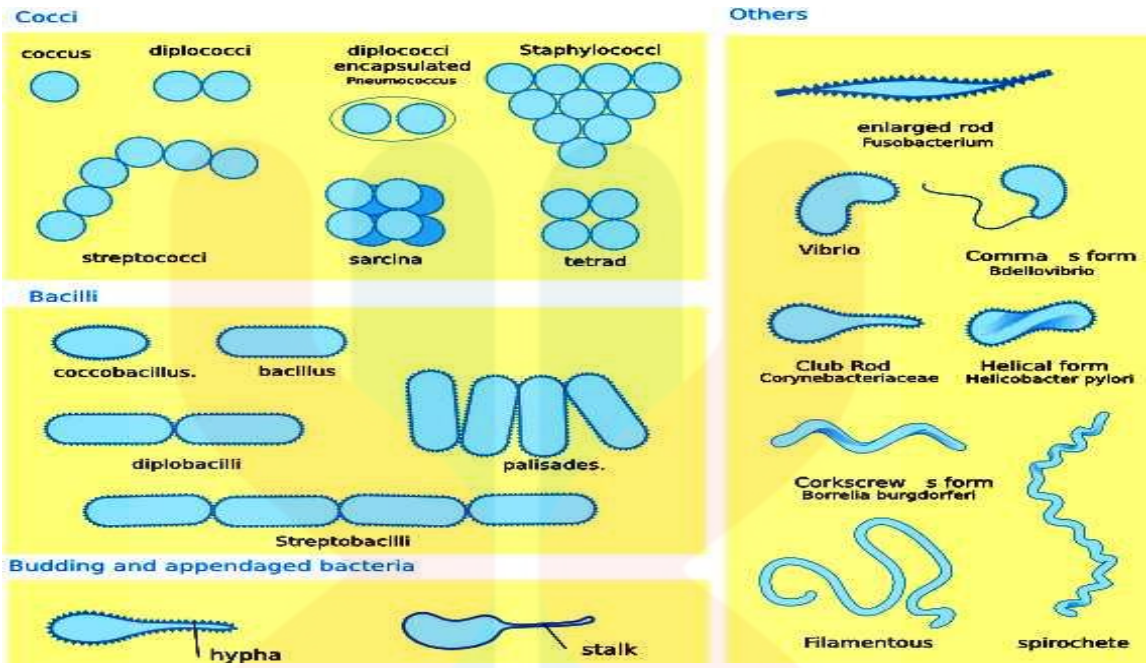

Figure 2.2: The figure shows the bacteria morphology (Sources: Medical Bacteriology, 2016)

2.4 Physical factor that affect growth of microorganism

The growth of the microorganism was affected by various factors. It is including physical factors involving temperature, pH, osmotic pressure, hydrostatic pressure and radiation. Meanwhile, chemical factors include oxygen, carbon, nitrogen, phosphorus, sulfur and others also can affect the growth of the bacteria.

2.4.1 Temperature

Temperature is the important factors that determine the rate of growth of, multiplication, survival and death of all living organisms. High temperatures will damage the microbes by denaturing enzymes, transport carriers and other proteins. Meanwhile, at low temperature, the membrane will be solidified and the enzymes will

not function properly. There are three types of temperature, minimum, optimum and maximum growth temperature. Minimum growth temperature is the lowest temperature. Optimal growth temperature is the temperature which multiplication occurs rapidly.

2.4.1.1 Classification of Bacteria according to growth temperature

i. Psychrophiles

Extremophilic organism that is able to grow and reproduce at cold temperatures. The temperature ranges between – 20℃ to + 10℃. *Oscillatoria*, *Chlamydomonas nivalis*, *Methanogenium* are the examples of Psychrophiles.

ii. Mesophiles

Bacteria in this group are best grows in moderate temperature which range between 20℃ to 45℃. The example of these bacteria is *Escherichia coli* and *Streptococcus pneumoniae.*

iii. Thermophiles

Bacteria in this group are able to grow at high temperature. Their optimum temperatures are usually around 45℃. Literally, their optimum temperature is between 50℃ and 80℃. *Thermus aquaticus* and *Geogemma barossi* are the example of the bacteria of this group.

iv. Hyperthermophiles

Hyperthermophiles are the bacteria that are able to live in extremely hot environments. The range of temperature is between 80℃ to 113℃. These bacteria contain high levels of saturated fatty acid in order to retain its shape at high temperature. *Sulfolobus*, *Methanococcus jannaschii* and *Thermotoga* are the example of the bacteria of Hyperthermophiles.

2.4.2 pH

Microbial growth also can be effected by the pH as there were drastic variation in cytoplasmic pH will disturb the plasma membrane or inhibit the activity of enzymes and membrane transport proteins. Basically, bacteria prefer media of pH near neutrality as it cannot tolerate with the pH values below 4 to 5. Acidophiles will be growed between pH 0 to 5.5. *Ferroplasma*, *Thiobacillus thioxidans*, *Sulfolobus acidocaldarius* are the example of acidophiles. Meanwhile, alkalophiles is the bacteria which grow between pH 7.5 to 14. The example of these bacteria is *Thermococcus alcaliphilus*. Neutrophils grow at pH 5.5 to 8.0.

2.4.3 Osmotic pressure

Osmotic pressure is the minimum pressure that needs to be applied to a solution to prevent the inward flow of water across a SPM. There are three types of solution, hypotonic, isotonic and hypertonic osmotic pressure. Besides that, the bacteria also will be classified based in osmotic pressure. Osmotolerant referred to microorganisms that can grow at high salt concentration. For example *Aeromonas sp*. and *Staphylococcus sp*.

Meanwhile, halophiles are the bacteria which can grow in the presence of 0.2 to 0.6 of salt concentration. Halobacterium halobium is the example of halophiles.

2.4.4 Hydrostatic pressure

The bacteria will be classified based on hydrostatic pressure. Barotolerant bacteria will not affected by increased pressure. Meanwhile, barophiles or piezophilesis the bacteria that is able to grow at high hydrostatic pressure. *Halomonas salaria* and *xenophyophores* are the example of barophiles.

2.5 Pathogenic and Non-pathogenic bacteria

Pathogenic bacteria refer to bacteria which can lead to infection. Pathogen is a microorganism which able to cause disease in almost everything including, animal, plant, human or insect. Virulence genes of pathogenic bacteria are coding for toxins, adhesins, invasins or other virulence factor (Hacker, 1997). Virulence is the term which refers to the level of pathogenicity of the microbe. The determinants of virulence of pathogens of its genetic or biochemical or structural features will enable it to produce disease in a host.

There are two type of pathogenic bacteria which able to cause disease, invasiveness and toxigenesis. Invasiveness means the ability to invade tissues which including the mechanism for colonization, production of extracellular substances which facilitate invasion (invasins) and ability to bypass or overcome host defense mechanisms. Meanwhile, toxigenesis are defined as the capability to produce toxis. A few pathogenic bacteria which commonly can lead to food poisoning such as Salmonella, *Staphylococcus aureus*, *Clostridium perfringens*, *Clostridium botulinum* and

Bacillus cereus. These bacteria may be from pests and domestics pests, dirt and soil and also from food waste.

Meanwhile, non-pathogenic bacteria are being defined as a bacteria which incapable of causing disease. For example, nonpathogenic *E. Coli sp.* is the bacteria that do not cause disease, but instead live naturally in the large intestine.

According to Wilson *et al.* (2007), they have been discussed about the mechanism of pathogenic bacteria. Once pathogen entered a human host, bacterial pathogen may illegal to a few or several host responses and uses a variety of mechanisms to avoid the host defenses. There are a few steps involved in the pathogenesis of the bacteria including transmission, colonization, adhesion, invasion, survival in the host and tissue injury.

2.6 Gram staining of water microbes

Gram staining method has been developed by Danish bacteriologist J.M.C Gram (1853-1980), by using a dye called crystal violet which help in distinguishing different types of bacteria. Gram staining is a common method that is used to differentiate Gram positive and Gram negative bacteria (Dash & Payyappilli, 2016). Through gram staining, the bacteria are separated into two group based on their cell wall composition. As gram positive bacteria has thick layer of peptidoglycan, it will stain purple of crystal violet. Meanwhile gram negative bacteria will stain red/pink of counter stain safranin as it has thin layer of peptidoglycan. Different cell wall composition of Gram negative and gram positive showed the differences in gram staining. Gram staining can be viewed by

the aid of light microscope since staining is able to visualize the bacteria more clearly. Therefore, gram staining is the easiest and faster method to identify the bacteria.

2.7 Polymerase Chain Reaction (PCR) of DNA

The Polymerase Chain Reaction (PCR) is a powerful and sensitive technique for DNA amplification (Saiki, 1985). PCR methods are designed to allow rapid and accurate detection of microbial pathogens. It will produce multiple copies of target DNA by using thermostable polymerase enzyme such as *Taq* enzyme. *Taq* DNA polymerase is an enzyme widely used in PCR (Powell *et al.*, 1987). The detection of target DNA will be achieved by using short section of synthetic, single stranded known as oligonucleotide. Besides, PCR has the ability to produce extremely large number of specific nuclei acid. There are three major steps involved in PCR involving denaturation, annealing and extension. According to Antler (2003), PCR is an important tool especially for medical diagnosis as it can detect and identify bacteria and viruses that cause infections. Hence, in PCR genes from various related organism are amplified, sequenced and analyzed for similarities or differences.

2.8 Luria Broth (LB) in microbe cultivation

Luria broth (LB) is a nutritionally rich medium which is also known as Lennox Broth,or Lysogeny Broth. Luria-Bertani (LB) broth is the most widely used medium for the growth of bacteria (Bertani *et al.*, 1951). This broth is usually used in growth and maintenance of bacteria within molecular microbiology procedures. LB medium is a rich medium that is commonly used to culture bacteria. Besides, it is also suitable for the growth of bacteria since it has been designed for the growth of pure cultures of

recombinant strains. LB consists of peptides and casein peptones, vitamins which also including vitamins B, trace elements such as nitrogen, sulfur and magnesium and minerals which the ingredients used also act in promoting the growth of bacteria.

2.9 Optical Density (OD)

According to Matlock *et al.* (2016), they have been defined optical density (OD) as the measurement of bacterial cultures which is a common technique used in microbiology. Commonly, they used spectrophotometers to make the measurements which the measurement are relied on the amount of light scattered by the culture compared to the amounts of light absorbed. However, the bacterial cells may have different light scattering angle according to their size that will affect the OD reading (Maia, 2016). The spectrophotometer tests will includes reverse-optical systems utilizing array detection, traditional monochromator-based systems and monochromator-based system with an integrating sphere accessory (ISA). The growth curves will be measured on each of the instruments by determining the optical density at 600 nm (OD600).

2.10 Gel Electrophoresis of DNA

According to Aurpita Shaha (2016), gel electrophoresis is a technique used for the analysis of nuclei acid and proteins. Electrophoresis is a technique used in laboratory to separate charged molecules. DNA is a negatively charged and able to be move through agarose matrix by electric current. Shorter molecules will be migrated easily and faster than longer molecules through the pores of the gel for the sieving process. The gel is used to see the DNA in order to quantify or isolate particular band. The DNA will be visualized in the gel as ethidium bromide was added. Ethidium bromide will be act as

intercalating agent which is will intercalates between nuclei acid bases and allows the detection of DNA fragments in gel. As it were exposed to UV light, it will fluorescence with orange color. After running the DNA through an EtBr-treated gel, any band which containing more than ~20 ng DNA will be able to see under UV light. As precaution step, the voltage should be limited to avoid the gel from melting.

CHAPTER 3

MATERIAL AND METHOD

3.1 Study area of water microbial sampling

The study was carried out at Lata Janggut, Jeli Kelantan. Lata Janggut is located at 12 kilometers south-west of Jeli. The coordinate of Lata Janggut is between N 5°40’0’’ to N 5°42’30’’ and E 101°44’30’’ E 101°47’00’’.

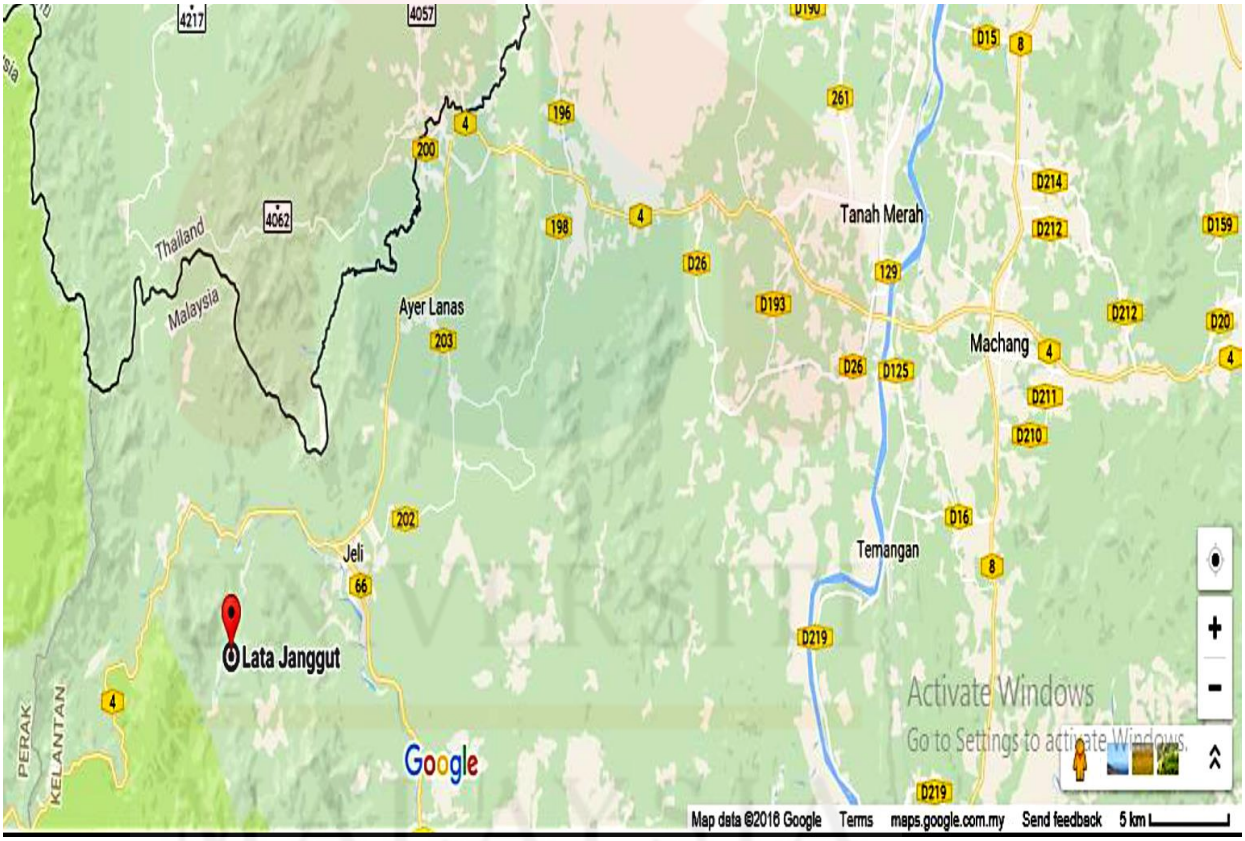

Figure 3.1: The figure shows the location of Lata Janggut, Jeli, Kelantan map. 1cm indicates 5 km (Sources: Google Map)

3.2 Water sampling at Lata Janggut, Jeli, Kelantan

River water samples were taken at the surface of the water by using 50ml falcon tube. The water sampling was taken randomly at five chosen points at the riverbank of Lata Janggut. The falcon tube was labeled with name, date and location. As precaution steps, rubber gloves were used in sampling process in order to reduce contamination. The water samples were stored in a cooler box with sufficient amount of ice and were brought back to laboratory within 24 hours for storage. The temperature of the site were measured and recorded.

3.3 Media preparation for bacteria culture

3.3.1 Preparation of Nutrient Agar (NA)

Nutrient Agar (NA) is used for the cultivation of microbes. For preparation of NA, 28 gram of nutrient agar powder was added with 1000 ml of distilled water into 1 liter of Scott bottle. The nutrient agar solution was sterilized by autoclaving at 121℃ for one hour. The autoclaved NA was left at room temperature for cooling. After that, the solutions were poured into petri dish inside laminar flow cupboard that has been rinsed with 70% ethanol to avoid contamination. Once the NA in plate has solidified it was stored in refrigerator.

3.3.2 Preparation of Luria Broth (LB)

Luria broth was prepared by adding 20 g of LB powder into 1000 ml of distilled water in Scott bottle. The solution was mixed well in order to dissolve all components. The Scott bottle containing LB solution was labeled and was sterilized by autoclaving at 121℃ (15 lbs) for one hour. After sterilized, the Scott bottle containing LB was closed by using aluminium foil and stored in room temperature.

3.3.3 Serial dilution of river water samples

Serial dilution is a series of sequential dilution of water samples which is used to reduce a dense culture of cells to usable concentration. Each dilution will reduce the concentration of bacteria by specific amounts. Serial dilution for river water collected from riverbank of Lata Janggut has done by adding 1ml of water samples into 9 ml of sterile distilled water. After that, the initially diluted water sample was further diluted to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} of distilled water. From the serial dilution, 0.2 ml of 10^{-5} prepared dilutions was pipetted into NA plate and spread equally by using spread plate technique. The plate was incubated at range of 30℃ until 37℃ for 24 hours.

3.4 Isolation of bacteria colonies from spread plate

An amount of 0.2 ml of water sample were pipetted from each of the dilution and spread into NA plates in order to grow the single colony of the bacteria. Then the plates were incubated at 30℃ for 24 hours. After 24 hours, the pure culture colony obtained was isolated and streak into new plate of NA. The grown bacteria were examined under light microscope in order to determine their morphology characteristics.

3.5 Morphology identification of bacteria

According to Christopher and Bruno (2003), identification of bacteria is based on many factors including cell and colony morphology, chemical composition of cell walls, biochemical activities and nutritional requirements.

In gram staining, firstly, one piece of slide was taken. After that, the loop was heated for a while. A drop of water was taken by using loop and smeared on the slide. The loop was heated again and let it cooled for a few seconds. The sample was taken by using loop and smeared on the slide which contains water. The slide was dried by using heat fix method which is passing the slide on heat for a few times. After the sample was dried, it was stained with crystal violet dye for one minute and rinsed with slowly flowing water. After that, iodine solution was added on the sample for one minute and rinse with water. Acetone solution was added on the slide for five second and the slide was rinsed quickly with slow flowing water. Lastly, safranin solution was added on the slide for one minutes and the slide was dried. The prepared slide of microbes was then viewed under light microscope to determine the either the bacteria was gram positive or gram negative bacteria.

3.6 Cultivation of bacteria in Luria broth (LB)

A single colony from each pure culture plate was taken by using a loop. The selected colony was inoculated into previously prepared LB solution in bijuour bottle. The bacterial culture was incubated at 30℃ for 12 to 18 hours in shaking incubator at 150 rpm.

3.7 DNA extraction of Lata Janggut microbes

3.7.1 DNA extraction with standard Cetyltrimethylammonium Bromide (CTAB)

Method

The pure cultured of bacteria isolated from Lata Janggut water samples which grown in Luria Brown was measured by using 600nm (OD_{600}) spectrophotometer to determine their active phase (He, 2011). As they achieved their active phase, one ml of the bacteria culture will be transferred into two micro centrifuge tubes (1ml). The cultured microbes were centrifuged until a pellet was formed. According to Amani et al. (2011), the pellet was dried and dissolved in 100 µl of TE buffer. After pellet form, discard the supernatant. By using 567µl TE the pallet was re-suspend by repeating the pipette. 30 µl of 10% of SDS and 3 µl of 20 mg/ml proteinase K were added in order to form final concentration of proteinase K. The solution was incubated for 1 hour at 37℃. In order to remove cell wall debris, denatured protein, and polysaccharides complex to CTAB while retaining the nucleic acids in solution, 100 µl of 5 M NaCI were added and mixed thoroughly. After that, 80 µl of CTAB solution was added into the solution and incubated for 10 minutes at 65℃. 0.8 ml of chloroform or isoamyl alcohol was added and mixed thoroughly in order to removes the protein. The tubes were centrifuged for 5 minutes. After that, phenol or chloroform was added into the tubes with samples and centrifuged for 5 minutes to separate aqueous and viscous supernatant. Upper layer of supernatant was transferred into a fresh tube as 0.6 vol of isopropanol were added to precipitate nucleic acid. The tube was inverted back and forth until a stringy white DNA become clearly visible. At this point, transfer the pellet into a fresh tube which containing 70% of ethanol. After that, the DNA was washed by using 70% ethanol to

remove residual of CTAB and centrifuged it for 5 minutes to form pellet. The pellet was dried in laminar flow cupboard. Lastly, the pellet will be redissolve in 100 µl TE buffer.

3.7.2 DNA extraction with Glucose-Tris-EDTA (GTE) and Proteinase K

Using modification of previous method, one ml of cultured colony was transferred into 1.5 ml microcentrifuge tube and it was centrifuged at 2000 rpm for 15 minutes. After centrifugation, the supernatant were removed. The microbes pellet were washed twice by using 1ml Glucose-Tris-EDTA (GTE) buffer and centrifuged at 10,000 rpm for 5 minutes. After that, the pellet was mixed with 200 µl GTE. The mixture was kept on ice for 15 minutes. After that, 50 µl of RNAse and 100 µl of lysozyme were added and the mixture was incubated in water bath at 37˚C for 2 hours. After incubation, 50 µl of Proteinase K and 50 µl of Sodium Lauryl Sulphate (SLS) were added and incubated in water bath at 50˚C for 30 minutes. After incubation, 200 µl of Tris-Hcl-EDTA (TE) buffer and 500 µl of Phenol chloroform Isoamyl alcohol (25:24:1) were added and the tube containing the mixture inverted for several times. Then, the mixture was centrifuged for 15 minutes at 10,000 rpm. After centrifugation, two layer mixtures were formed and the upper layer was transferred into a new 1 ml micro centrifuge tube. Next, 400 µl of sodium acetate and 800 µl of isopropanol were added and the mixture was incubated at room temperature for 10 minutes before centrifuged at 10,000 rpm for 15 minutes. The DNA pellet then washed with 500 µl of 80 % cold ethanol by centrifuged at 10,000 rpm for five minutes. After centrifugation, the pellet was air dried and dissolved in 50 µl TE buffer and kept at -20˚C.

3.7.3 Optimization DNA extraction with Glucose-Tris-EDTA (GTE) and Proteinase K

The DNA extraction method was optimised with one ml of cultured colony was transferred into two ml micro centrifuge tube and it was centrifuged at 13,000 rpm for 15 minutes. After centrifugation, the pellet was formed. The supernatant was removed. The pellet was washed with 1ml Glucose-Tris-EDTA (GTE) buffer and centrifuged at 10,000 rpm for five minutes. The mixture was added with 200 µl GTE and the mixture was kept on ice for 5 minutes. After that, 50 µl of RNAse and 100 µl of lysozyme were added and the mixture was incubated in water bath at 37˚C for 1 hours. After incubation, 50 µl of Proteinase K and 50 µl of 25% Sodium Lauryl Sulphate (SLS) were added and incubated in water bath at 50˚C for 30 minutes. After incubation, 200 µl of Tris-Hcl-EDTA (TE) buffer and 500 µl of Phenol chloroform Isoamyl alcohol (25:24:1) were added and inverted for several times. Then, the mixture was centrifuged for 15 minutes at 10,000 rpm. After centrifugation, two layer mixtures are formed and about 150 µl of upper layer solution was transferred into a new tube. Next, 400 µl of sodium acetate and 800 µl of isopropanol were added and the mixture was immediately inverted for several times before incubated at room temperature for 10 minutes before it undergo centrifugation process at 10,000 rpm for 10 minutes. The DNA pellet then washed with 500 µl of 80 % cold ethanol by centrifuged at 10,000 rpm for five minutes. After centrifugation, the pellet was air dried and dissolved in 50 µl TE buffer and kept at -20˚C. Modification was made to optimize to minimize time during incubation step.

3.8 DNA Quantification and Quantities Determination

3.8.1 Nanodrop Spectrophotometer Measurements of Cultured Bacteria

The concentration of DNA was determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer. The accurate absorbance readings fall at 260 nm at 0.15 and 1.0. Pure DNA has an A_{260}/A_{280} ratio of 1.8–2.0 in 10 mM Tris·Cl, pH 8.5. Strong absorbance at A_{280} for A_{260}/A_{280} indicates the presence of contaminants such as proteins. Meanwhile, strong absorbance at 270 nm and 275 nm results in the presence of contaminating phenol from extraction step.

3.8.2 Preparation of TAE buffer and 1% Agarose Gel Electrophoresis

TAE electrophoresis buffer (10×) was prepared by adding 48.4g Tris Base, 11.4ml glacial acetic acid, 3.7g EDTA and 988.6ml of distilled water. 1×TAE buffer was prepared by taking 10 ml of TAE and diluted it with 990ml of distilled water. Agarose gel was prepared when 0.8 g of agarose powder and 79.2 ml of 1×TAE were added into 250 ml conical flask. The solution was heated in microwave for 1 minute to dissolve the agarose. The solution was allowed to cool for 5 minutes. 3 µL of ethidium bromide (10 mg/ml) was added and the solution was swirled to mix the solution. The gel was poured slowly in the tank. Agarose gel set was left for at least 30 minutes to 1 hour with the lid on it. The comb was removed carefully and placed in the tray into electrophoresis chamber. After that, 1× TAE was poured into the gel tank to submerge the gel to 2 to 5 mm depth as the running buffer. The gel was run at 80 to 150 V until the dye line is about 75 to 80% of the way down the gel. The electrode was disconnected

from the power source and the gel was removed from the gel box. By using UV gel doc system, the DNA fragments were able to be visualized.

3.8.3 Polymerase Chain Reaction of DNA Isolated from Microbes in Water Samples

By using modification method by Appalasamy (2012), the amplification of DNA preparation contained DNA, 1X buffer, MgCl2 solution, dNTPs in equimolar ratio, 16 sRNA and Flexi GoTaq (Promega). The amplification was conducted by using BioRad Thermal Cycler. By using 1% of agarose gel, the amplified products were electrophoresed with 100V for 50 minutes. The gel was stained by using 1% ethidium bromide and distained in sterile distilled water and the gel was viewed and documented by using Gel Doc XR System (Bio-Rad). Referring to Table 3.1, it is showed the component, concentration and volume of PCR.

Table 3.1: The table shows the component for preparation of PCR

Component	25µl Reaction	Final Concentration
5× Green or colorless GoTaq® Flexi Buffer[1]	2.5µl	1.0× 10µl
MgCl2 solution, 25 mM[1]	2.0µl	1.0mM
PCR Nucleotide Mix, 10mM each	1.0µl	0.2mM each dNTP
Primer (Forward)	0.5µl	0.2µM
Primer (Reverse)	0.5µl	0.2µM
GoTaq® DNA Polymerase (5u/µl)	0.5µl	1.5unit
Template DNA	2.0µl	<0.5µg/50 µl
ddH2O	16.0µl	-

Table 3.2: The table shows the Recommended Thermal Cycling Condition for GoTaq®DNA Polymerase-Mediated PCR Amplification

	Temperature (℃)	Time (Min)	Cycles
Pre-Denaturation	95	2	1
Denaturation	95	1	
Annealing	42-65	1	
	25-35		
Extension	72	1	
Final Extension	72	5	1
Soak	4	-	1

CHAPTER 4

RESULT AND DISCUSSION

4.1 Physical Parameters for Water Quality at Lata Janggut

Table 4.1 shows the data of water quality in Lata Janggut. The data were collected by using YSI Fields Meter. The water samples were taken at five different points at riverbank of Lata Janggut.

The average temperature of Lata Janggut waterfall was 25.88 ℃. The main factors that affect the temperature of freshwater ecosystems are distribution of organisms, behavior and reproduction of organisms. Water temperature is one of the important abiotic drivers of aquatic ecosystems which effect the organism's existence including feeding, metabolic and growth rates, fecundity, emergence, behavior and ultimately survival (Dallas & Gillespie, 2015).

Meanwhile, the pH reading showed the average of 6.35. The pH reading is between 0-6 which mean is acidic. The pH reading can be influenced by both man-made and natural. Carbon dioxide can cause the acidity in water. Besides that, as the area was a recreational area, the usage of toiletries such as shampoo and soap can increase the pH of the surface water.

Referring to the table 4.1, the dissolved oxygen (DO) for Lata Janggut showed the minimum value recorded was 4.93 and the highest was 7.08. DO is the oxygen gas molecule (O2) presence in water. DO was influenced by water temperature, time of day,

depth or flow of the water. Besides, it is also can be caused by human factor such as the addition of chemical or increasing the water temperature.

Table 4.1: The table shows the average data of water quality at Lata Janggut

Location	Point 1	Point 2	Point 3	Point 4	Point 4	Average
Temperature(℃)	24.74	25.89	25.90	26.41	26.50	25.88 ±0.70091
DO (mg/l)	6.80	7.08	4.93	5.98	6.95	6.35 ±0.9012
pH	6.27	6.52	6.22	6.20	6.20	6.28 ±0.13609

4.2 Isolation of water samples from river water

The water samples were isolated by using serial dilution method. The samples of 10^{-5} and 10^{-4} dilution were used to spread into nutrient agar media. The plate was incubated in room temperature between 25℃ to 30℃ to let the bacteria grow for 12 hours. The growth of bacteria in the plate was streaked into other plates. However, not all of the water samples took 12 hours to grow on the media. Some of them take less and more than 12 hours. Single colony of selected bacteria with different morphology were selected and cultured in the Luria Broth (LB) in orbital shaker with 150rpm at 30℃ for 12 hours. As the LB color was changed, the OD readings were noted. After the LB solution turns cloudy, the OD reading was taken using UV spectrophotometer. Based on table 4.2.1, it shows the reading of OD reading of water microbes in Lata Janggut. The

table shows that reading of the OD was different because as the reading of OD of the microbes depends on the phase of the bacterial growth. The greater light scatter indicates that more bacteria present (Bae *et al*., 2016). Basically, the OD reading was taken to determine particular wavelength that correlates with different phase of bacterial growth which is the bacteria were preferred in mid-log phase.

Table 4.2: The table shows the Optical Density (OD) reading of water microbes in Lata Janggut

Point	Reading
Point 2 10^{-4}	0.524A
Point 3 10^{-4} (A)	0.446A
Point 310 $^{-4}$ (B)	0.403A
Point 510 $^{-4}$ (A)	0.453A
Point 510 $^{-4}$ (B)	0.330A
Point 510 $^{-4}$ (C)	0.437A
Point 4 10^{-4}	0.186A

4.3 Gram Staining of Water Microbes

The water microbes were determined through gram staining method, as it was able to differentiate the water microbes into two groups, gram positive and gram negative. Gram staining methods accounts either Gram positive or Gram negative bacteria stain the crystal violet (Budin *et al*., 2012). Gram positive was takes up the violet stain and appear as purple color when seen through microscope as it composed of thick layer of peptidoglycan with numerous teichoic acid cross linking which resists the decolorization. Meanwhile, gram negative cannot retain the violet stain but it was takes up safranin and appeared as pink under microscope as it composed of thin layer of peptidoglycan. From the selected samples, 6 out of 8 samples were gram positive while

two others were gram negative. Mostly, gram positive bacteria of water microbes in Lata Janggut were cocci which can be diplococcus, streptococcus or staphlococci and gram negative bacteria were bacillus. The samples were tested using one biochemical method called Gram Staining. Some of the selected samples appear as purple stains while others appears as red or pink stains when observed under microscope. Figure 4.1 and Figure 4.2 shows the results of the bacteria from gram staining under 100 × magnifications using light microscope.

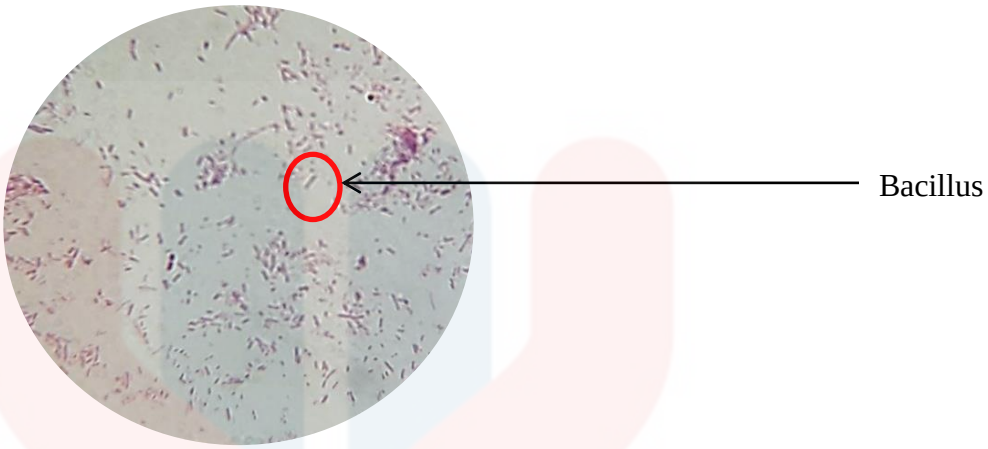

Figure 4.1: The figure indicate the morphology of Gram Negative Bacillus (magnification 100×) using light microscope

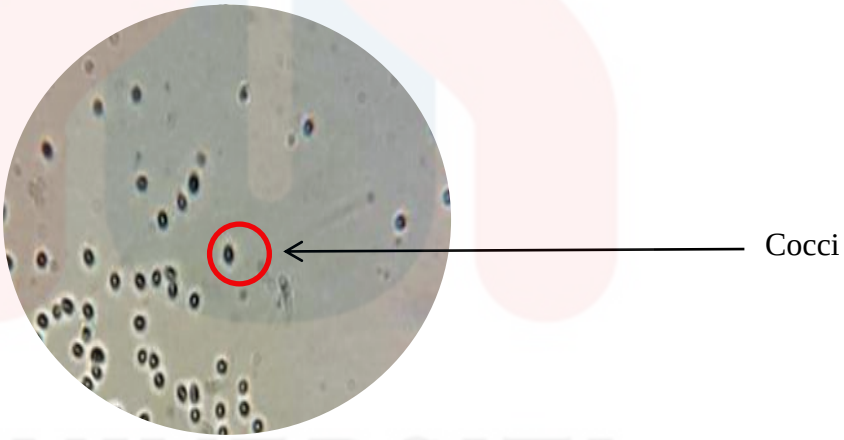

Figure 4.2: The picture indicate the morphology of Gram Positive Cocci (magnification 100×) using light microscope

4.4 DNA Extraction and Agarose Gel Electrophoresis (AGE) of Water Microbes

5µl of isolated DNA was pipetted into the well of the agarose electrophoresis gel. The isolated DNA were tested by using 1% Agarose gel electrophoresis, as it is an effective ways to separate DNA fragment of varying sizes ranging from 100 bp to 25 kb (Py *et. al*, 2012). 100 bp is used in order to identify the size of the DNA bands. The band was visualized under UV light with the aid of Ethidium bromide (EtBr).

Figure 4.3 shows the result for DNA extraction with standard Cetyltrimethylammonium Bromide (CTAB) method which does not showed any band of the DNA. The band of DNA indicates either the DNA extraction success or not. This showed that the DNA extraction using CTAB method was unsuccessful.

Hence, the DNA extraction was modified by applying DNA extraction with Glucose-Tris-EDTA (GTE) and Proteinase K. This method includes the usage of lysozyme, RNase and Proteinase K which are able to break the chemical bonds in the outer cell wall of the bacteria, meanwhile Proteinase K were able to digest protein contamination.

Based on Figure 4.4, the figure shows that the bands appeared in lane 1. This proved that, the DNA extraction was successful. Meanwhile at lane 2, the bands were not clearly visualized and at lane 3 there were no DNA bands. This may be caused by several reasons such as contamination or may be caused other reasons such as pipetting error.

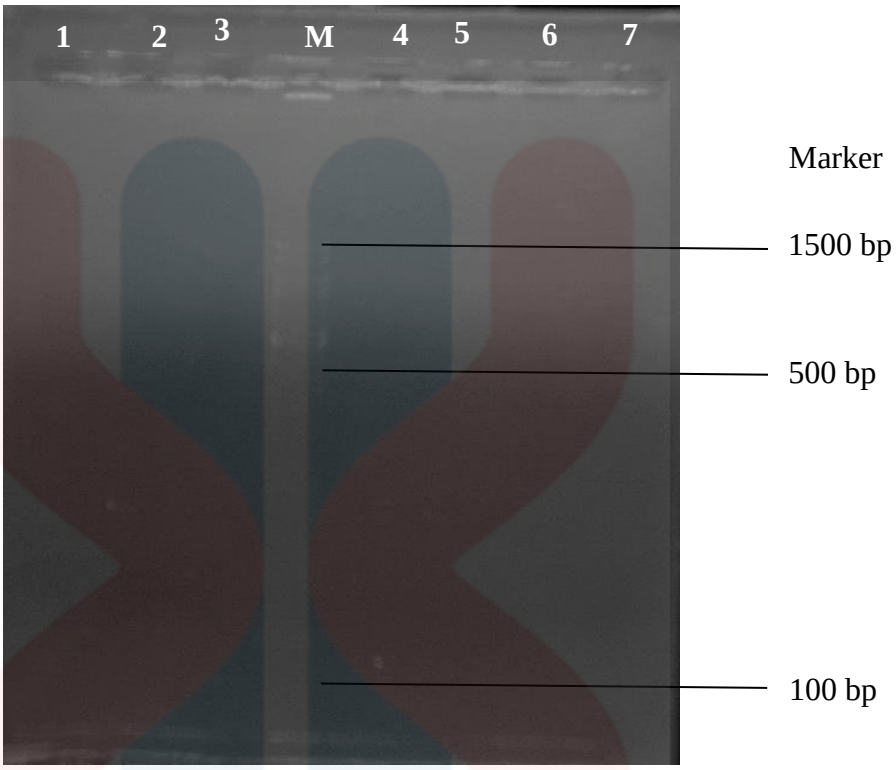

Figure 4.3: Gel Electrophoresis of DNA extraction from seven river water sample. The figure shows there was no DNA bands present after being visualized under UV transilluminator of 1% agarose gel at 80V for 45 minutes. Lanes 1, 2, 3, 4, 5, 6 and 7 represents as DNA sample and M represents as marker.

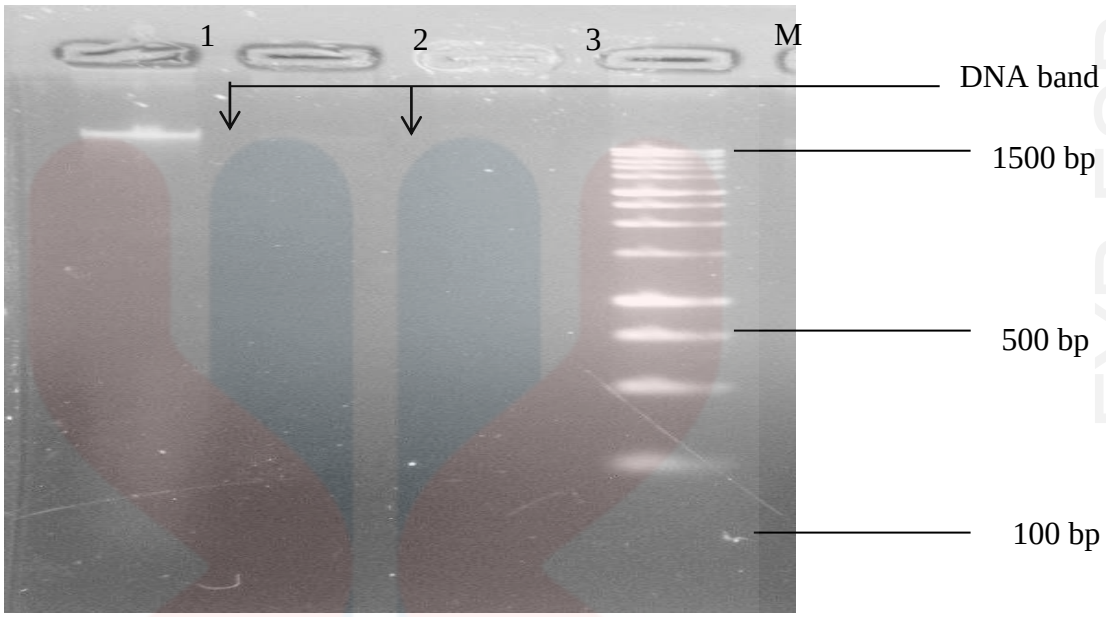

Figure 4.4: Electrophoresis of DNA extraction on 1% agarose gel in TAE 1× buffer at 80V for 45 minutes. M represents as marker. Meanwhile 1, 2 and 3 represents as DNA lane

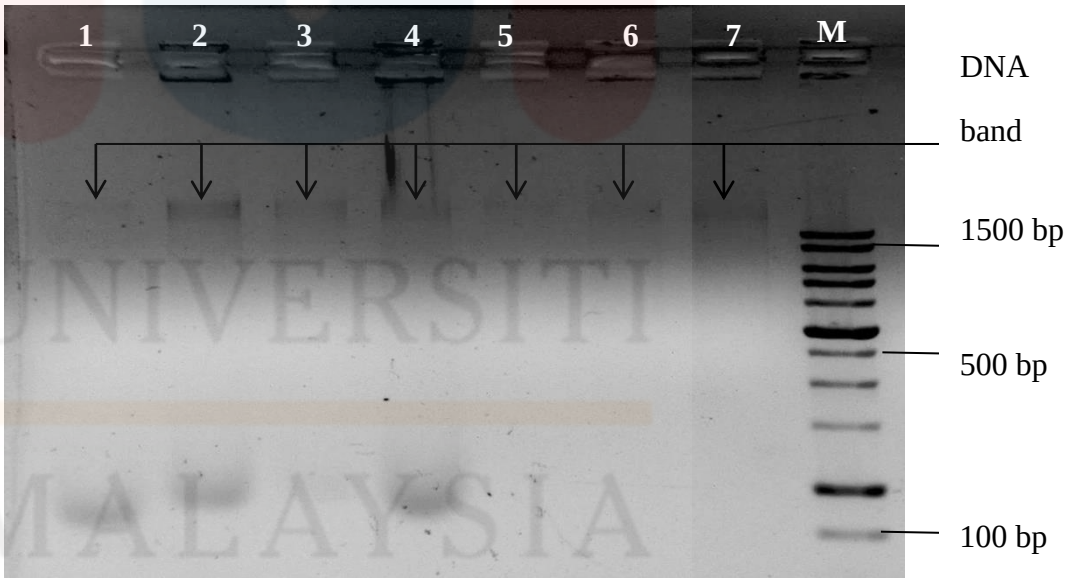

Figure 4.5: The figure shows the electrophoresis of DNA extraction on 1% agarose gel in TAE 1× buffer of seven samples. Ethidium Bromide (EtBr) has been added before electrophoresis, followed by separation by 8 V for 45 minutes. 1, 2, 3, 4, 5, 6 and 7 represents as the lane of DNA samples. Meanwhile, M represented for marker.

4.5 Polymerase Chain Reaction (PCR) of Water Microbes

The quality of the isolated DNA was further confirmed by PCR analysis. Extracted DNA of the samples was used for PCR for the amplification of DNA. The multiple copies of DNA were used in DNA sequencing. 16S rDNA were used as a sequence of the DNA. Meanwhile, the primer of PCR consists of reverse and forward primer. For forward primer 5'-CGC TGG CGG CGC GTC TTA AA-3' meanwhile for 16S rDNA reverse 5'-TTC ACC GCT ACA CCT GGA A-3' to make nucleotide copies.

In PCR, 105 bp of PCR marker were used in order to identify the size of DNA. Besides that, 1 kb of DNA ladder was served as a molecular standard for the agarose and gel electrophoresis. Based on figure 4.6, it was show that, there were multiple band were form. Multiple bands may be caused by the temperature of annealing primer which is too low or can be caused by the contamination from DNA extraction. Multiple bands can be reduced by use less primer, DNA template or *Taq*, increase annealing time, increase annealing temperature, increase extension time or increase extension temperature.

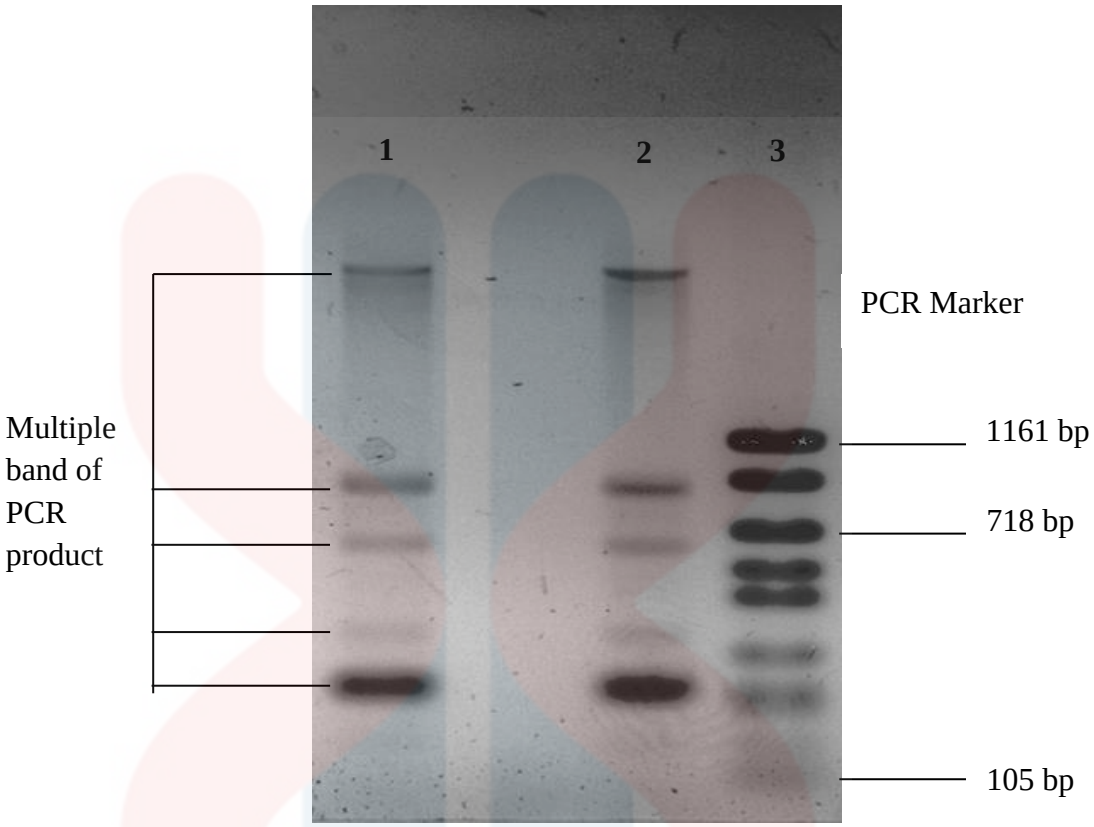

Figure 4.6: Electrophoresis of PCR products using GoTaq DNA polymerase on 1% agarose gel showing the PCR amplification of representative DNA samples. W1 and W2 showed the well of the samples. Meanwhile M represents as marker.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

As a conclusion, based on the result, there are water microbes in Lata Janggut. Through gram staining method, it was able to recognize Gram-positive and Gram-negative bacteria. Gram positive bacteria, the shape were including cocci, diplococci and streptococci. Meanwhile, for Gram negative bacteria, bacillus shape was identified. Eight samples has been isolated and identified. A part from that, by using modification of DNA extraction with Glucose-Tris-EDTA (GTE) and Proteinase K, the DNA bands were able to be visualized under UV transilluminator remarks the success of DNA extraction. As for 16s rDNA identification, multiple band were formed. Hence, the optimization of PCR was required for the future study.

5.2 Recommendation

Based on the results, in order to identify more specific bacteria or microorganisms, the samples need to be isolated or cultured in more specific media or selective media, as nutrient agar (NA) is the simplest media which not all of the bacteria prefers to grow on it. Besides that, for the growth of the bacteria, some of them grow within 12 hours and some of them take more than 12 hours. Through this research, the bacteria were let to grow out of incubator. The bacteria were let to grow in room temperature to avoid from overgrowth. For the future study, the usage of rich media, Luria Broth (LB) can be change with other broth as their nutrient content do not fulfill the growth requirements for certain bacteria. The optimizations for Polymerase Chain Reaction (PCR) also need to be done to avoid inaccurate and ultimately meaningless results. With the reference to the findings of this study, long-term monitoring and screening programs for the detection of water microbes in Lata Janggut are required as it was reported Leptospirosis's case has been reported at that area. The research is needed as precaution to people especially the villagers about the water disease that may be have at the area.

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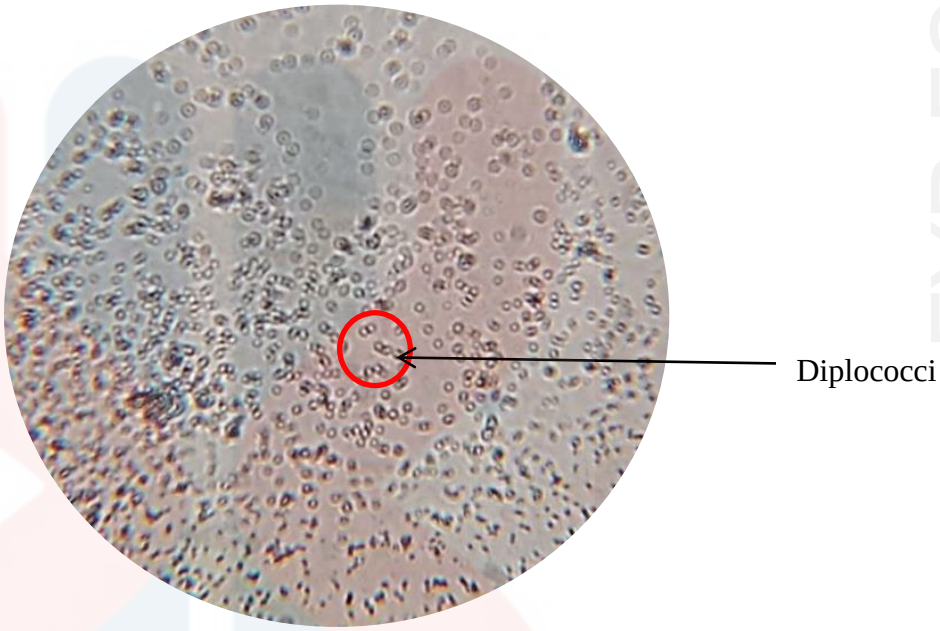
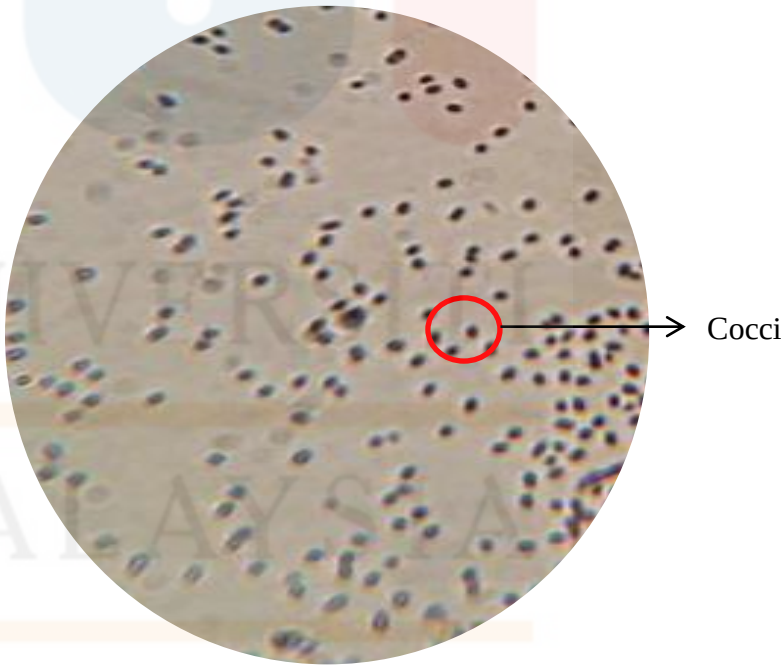
APPENDIX

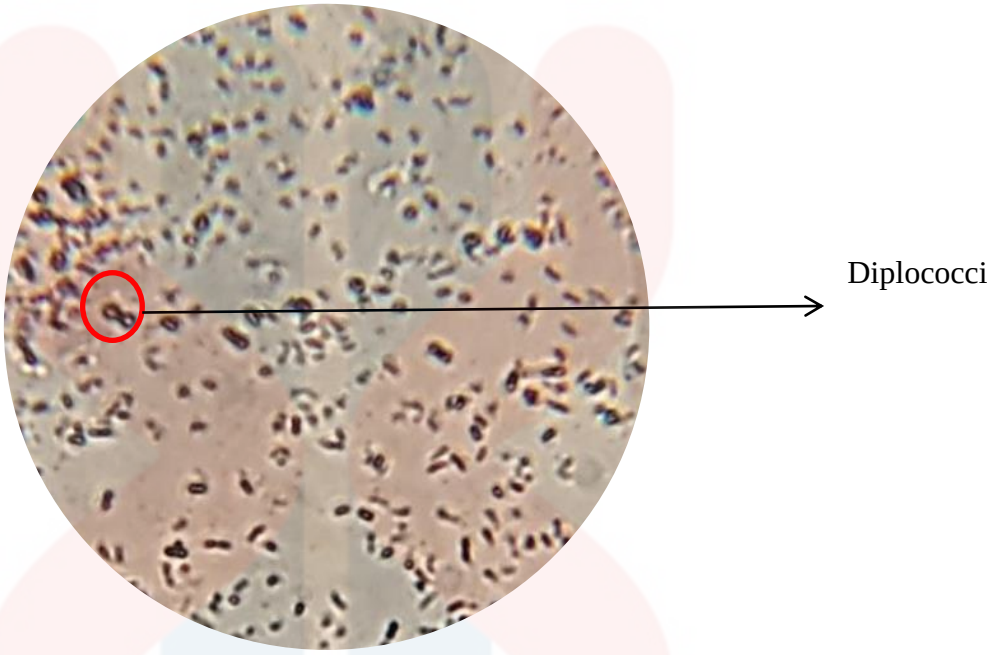
The figure show a few locations where the water samples were taken in Lata Janggut



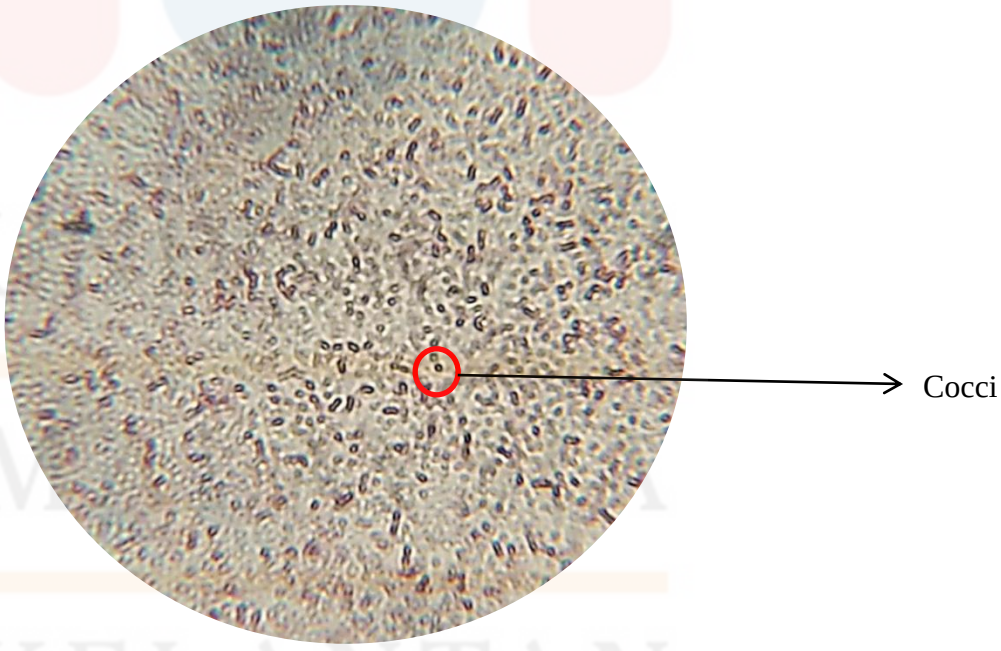
The figure indicates morphology of a Gram Positive (100×) Diploocci



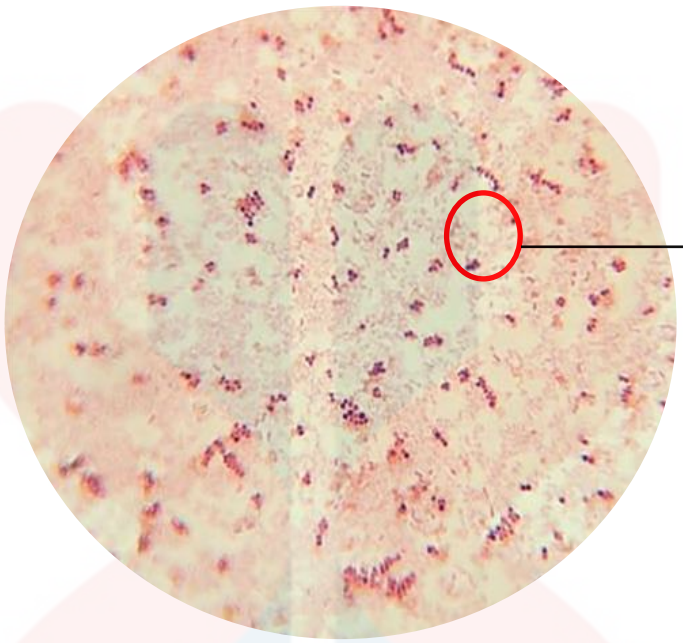
The figure indicates morphology of Gram positive (100×) Cocci



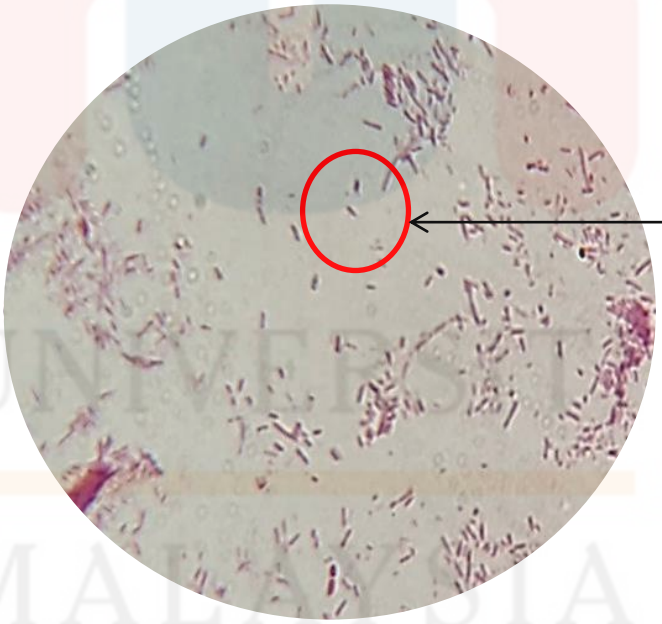
The figure indicates morphology of Gram Positive (100×) Diplococcus



The figure indicates morphology of a Gram Positive (100×) Cocci



The figure indicates morphology of a Gram positive (Oil) Streptococi



The figure indicates morphology of Gram Negative (Oil) Bacillus