PHYSICAL PARAMETER AND MICROBIAL SCREENING IN LATA JANGGUT, JELI, KELANTAN

by

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DECLARATION

I declare that this thesis entitled "Physical Parameter and Microbial Screening in Lata Janggut, Jeli, 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 : _____

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Date : _____

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In the name of Allah, the Most Gracious and the Most Merciful

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PARAMETER FIZIKAL DAN MIKROB SARINGAN DI LATA JANGGUT, JELI, KELANTAN

ABSTRAK

Mikrob air boleh didapati secara meluas di kawasan air tawar, laut, tasik atau kawasan takungan air. Hulu Lata Janggut, Jeli, Kelantan dipilih sebagai kawasan kajian untuk membiakkan dan mengasingkan mikroorganisma dari kawasan air tawar. Tujuh mikroorganisma yang dibiakkan telah diperolehi. Berdasarkan ciri-ciri morfologi iaitu bentuk, margin dan ketinggian bakteria dikelaskan. Gram perwarnaan untuk sampel menunjukkan semua bakteria adalah bakteria Gram positif. Bentuk bakteria yang diperolehi adalah kokus, diplobasilus, kokubasilus dan basilus. Tujuh mikroorganisma yang dibiakkan digunakan dalam pengekstrakan DNA. 16S rRNA ialah primer sejagat yang digunakan untuk memperbanyakan salinan jujukan DNA dengan melakukan tindak balas rantai polimerase (PCR). Produk PCR yang dihasilkan dikesan dengan elektroferesis gel agaros. Hasil dari amplifikasi DNA menunjukkan pembentukan pelbagai *band* dalam tindak balas PCR. Pengoptimuman reka bentuk primer boleh mengurangkan pembentukan perbagai *band*. Bilangan kawalan juga perlu dipertimbangkan apabila melakukan proses PCR.

PHYSICAL PARAMETER AND MICROBIAL SCREENING IN LATA JANGGUT

ABSTRACT

Water microbe can be found widely in freshwater, ocean, lake or reservoir. Upstream Lata Janggut, Jeli, Kelantan is chosen as the study area to culture and isolate the microorganism from freshwater. Seven isolates microorganism were obtained. Based on morphology characteristics which are shape margin and elevation, bacteria were characterized. Result from Gram staining for the sample showed all the bacteria were Gram positive bacteria. The shapes of bacteria obtained are coccus, dipplobacilli, cocobacillus and bacillus. Seven isolates microorganism obtains undergo DNA extraction to obtain DNA.16s rRNA universal primer was used to amplify the copy of DNA by performing Polymerase Chain Reaction (PCR). The result from amplifying the copy of DNA showed multiple bands. Optimizations of primer design can reduce multiple bands. Number of control also should be considered when performing PCR.

TABLE OF CONTENT

CHAPTER 3: MATERIAL AND METHOD

LIST OF TABLES

LIST OF FIGURE

LIST OF ABBREVIATION

CTAB	Centyl trimethyl ammonium bromide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
GTE	Glucose Tris EDTA
HCL	Hydrochloric acid
LB	Luria Bertani
MCK	Mac Conkey
NA	Nutrient Agar
NAOH	Sodium hydroxide
O.D	Optocal density
PCR	Polymerase Chain Reaction
pH	potential hydrogen
RNA	Ribonucleic acid
rRNA	ribosomal RNA
TBE	Tris bromide EDTA
TAE	Tris acetate EDTA
TCBS	Thiosulphate-citrate-bile salts sucrose
TE	Tris EDTA
UV	Ultraviolet
WHO	World Health Organization

LIST OF SYMBOL

bp	base pair
-	negative
±	plus-minus
%	percentage
℃	Degree Celsius
µ	micro
µl	microliter
µM	micromolar
g	Gram
M	Molar
mM	milimolar
ml	milliliter
nm	Nanometer
rpm	rotation per minutes
V	volte

CHAPTER 1

INTRODUCTION

1.0 Background of Study

Microorganism can be defined as those organisms that are not readily visible to the stripped eye, requiring a magnifying lens for detailed observation. Almost any freshwater will contain one or many species of microorganism. Although they are mainly microscopic, the present of microorganism plays an equally important in the ecology. Freshwater environment is considered to include all those sites where fresh water occurs. The Antarctic sub-continent is now known to be rich in microorganisms (Vincent, 1988).

Water microorganism can be found widely in freshwater, ocean, lake or reservoir. One of the recreations is selected to determine the diversity of water microbial. Natural areas provide ecosystem services such as recreation, tourism, spiritual experience and sense of place (Lindberg & Fredman, 2015). There are factor that influence the tourist to visit the place such as the element that appears as beautiful, good facilities and organize, maintenance and management of the area (Noralizawati & Noriah, 2012). Tourist willing to pay more if the recreational area is well developed and well managed (Azman & Shahruddin, 2009). Moreover recreational tourism has become a source of income for country. Therefore, study at the area is needed to survey the health condition of the area. The number of tourist will decline if any health issued arises at the area. For example, news report about schistosomiasis cases had decrease the tourist number visiting Lake Malawi in South Africa (WHO, 2003).

Human activity at and nearby recreational area may influence the diversity of water microbial in the area. Upstream Lata Janggut, Jeli, Kelantan is selected as the study area to determine the diversity of water microbial. There are many factors that influence the microbial diversity in freshwater such as temperature, total suspended solid, pH, human activity in the area. Water microorganism population tends to show marked fluctuation in response to environmental factors. Infected water will lead to waterborne disease. The survival of pathogenic microorganism in water is impacted by temperature, light intensity, salinity and water quality (Johnson *et al.*, 1997). Therefore, this study will be focusing on the diversity of water microorganism in upstream Lata Janggut.

1.1 Problem Statement

Upstream Lata Janggut is chosen because the area is one of the recreational areas located in Jeli, Kelantan. Since there is no preliminary data on the diversity of water microorganism had been recorded. So through the study, the data and identifying about the diversity of water microbial will be collected. Therefore from the collected data, the recreation area could be evaluated either the area is contaminated with pathogenic bacteria. Lastly from the study, the data could be used to promote ecotourism activity in the area.

1.2 Objective

1. To culture and isolate the microorganism from water sample in upstream Lata Janggut.
2. To determine the diversity of water microbial in upstream Lata Janggut.

CHAPTER 2

LITERATURE REVIEW

2.0 Water Recreational Area

An activity done by people at their spare times is a sociological need of people. It also can be known as “leisure” or “recreation”. Water recreational area is increasing in popularity nowadays in many countries. Freshwater recreational area could be encased waterways and static, for example lakes for static and running water such as stream. Recreational provide important ecological services. But an individual could be exposed to harmfulness, such as pathogenic micro-organism at contaminated or poorly managed recreational area (Kathy, 2005). Children for few reasons are at some danger catching for recreational waterborne disease at recreational parks. Children tend to be the frequent visitor to recreational water or parks for longer time compared to adults. Therefore higher exposure will increase the possibility to be in contact with contaminated water. An individual can be exposed to pathogen in water through eating, inward breath or penetration of the skin if longer an individual is in the water (Kathy, 2005).

2.0.1 Lata Janggut

Lata Janggut located at Jeli, Kelantan. Jeli is located at the eastern part of Kelantan. Jeli located within central in map of Peninsular Malaysia. Lata Janggut can be considered as a place that has a potential of ecotourism. Lata Janggut is a part of Sungai Long. The rivers is quite shallow, therefore the place is suitable for the water recreational area.

2.1 Water Microbe Organisms

The most abundant organism on Earth is microbe, it lives everywhere in soil, water and animal. Water microbe is concerned with microorganisms that live in water, or can be transported from one habitat to another by water (Sigee, 2005). The major sources of fecal microorganism are from wastewater discharge in fresh waters and costal seawater (Cabral, 2010). Sources of coastal water contamination from point discharge of treated and untreated sewage from shoreline outfalls, and non-point discharge. Run off from naturally vegetated area is the example of non-point sources that discharge pathogens into coastal waters (Pandey *et al.*, 2014). Morover, other sources, including malfuctioning or badly sited septic system likewise introduced significant amount of pathogens (Harvell *et al.*, 2002). At point and non-point sources the concentration of pathogen in freshwater is mainly determined by faecal pollution.

2.1.1 *Vibrio* species

Vibrio species is a microorganism which is motile, Gram-negative rods with a slightly curved and single polar flagellum. Environment samples, including water, sediment, plankton, shellfish and finfish could be found *Vibrio* species (WHO, 2003). This species are both aerobic and some are anaerobic. The only species found in fresh water are *V. cholerae* and *V. mimicus*. Therefore, water quality control measures such as wastewater treatment and disinfection can be controlled the existence of *Vibrio* species in bathing water (WHO, 2003). *Vibrio* species spread disease, *Vibrio* cholera serogroups 01 and 0139, caused cholera. Cholera causes disease in human. Common media growth, such as Mueller-Hinton, blood, and chocolate agar for *Vibrio* species to grow well on. *Vibrio* species also can be growth on a selective media such as thiosulfate-citrate-bile

salts-sucrose (TCBS) medium. According to Kelly *et al*. (1991), not all *Vibrio* species of medical importance grow well on TCBS media. At the moment, there are 12 *Vibrio* species are familiar to induce or to be associated with human infections: *V. alginolyticus, V. carchariae, V. cholerae, V. cincinnatiensis, V. damsela, V. fluvialis, V. furnissii, V. hollisae, V. metschnikovii, V. mimicus, V. parahaemolyticus* and *V. vulnificus* (Kilvington & White, 1994). *V. cholera* mainly cause gastrointestional disease.

2.1.2 Genus Acanthamoeba

According to Page (1988), genus *Acanthamoeba* is a genus of the environmental free-living amoebae can be found in most soil and water habitats. A variety of mammals including humans will produce severe and often fatal consequences when infect with this organism. *A. polyphaga, A. castellanii* and *A. culbertsoni* have been identified as the most commonly cause human disease causing pathogen (Kilvington & White, 1994). There are few factors that affect the presence of *Acanthamoeba* in freshwater which are temperature of the water, presences of a bacterial food sources and an individual activity related with the water (Kilvington & White, 1994).

2.1.3 Leptospira species

General feature are motile and helically coiled bacteria. Conventionally, the genus *Leptospira* appears of two species and they live in the proximal renal tubules of the kidney of transporter animals such as rats, cows or pigs It can be discharge in the urine, which then can pollute surface water such as ponds, lakes, rivers, groundwater soil or mud. Human or animal can become infected either through contact which infected urine or indirectly via contaminated fresh water or soil. Pathogenic leptospires

enter the body through cuts or abrasion of the skin and through mucosal surfaces of the mouth, nose and conjunctiva. Diseases caused by *Leptospira* are termed leptospirosis. Leptospirosis often considered a disease related to animals or contaminated water. They are commonly found in water of below pH 6.8, and at shaded are from direct sunlight. Their endurance in contaminated water and seawater is poor (Alston & Broom, 1958). However, leptospires are still detectable for up to about six months in some condition such as at neutral pH and when favorable temperatures and oxygen supersaturated conditions exist (Alston & Broom, 1958).

2.2 Pathogenic and non-pathogenic bacteria

A pathogen is defined as a specific cause of a disease, while a non-pathogen is considered disarmed. Microbes called pathogen if it capable of causing such infections or microbes that is necessary infectious agents in human or animal. Most bacteria are harmless, some are beneficial but others are pathogenic. Pathogenic bacteria and viruses which are discharged in the excreta of the infected people or carriers may enter a waterway causing a water-borne disease. Human can become infected by ingestion or contact with the water. Pathogen pollution is mostly cause of stream impairments (Pandey *et al.*, 2014).

Some bacteria is non-pathogenic in small number, *Escherichia coli* (*E. coli*) bacteria mostly are harmless. However, some *E. coli* are pathogenic, meaning they can cause illness. *Leptospira* genus is pathogenic species, but some *Leptospira* genus contain a nonpathogenic species (He *et al.*, 2004). Example of genes used for the specific detection of various pathogens is listed in Table 1.0. Still for the classification

of different taxonomy, the 16S and 23S ribosomal RNA (rRNA) genes are commonly convenient (Olsen *et al.*, 1986).

Table 2.1: Virulence factors and gene target to identify waterborne genera (Achbolt, 2003)

Pathogen	Virulence factors	DNA probe	PCR
Diffusely adherent *E. coli* DEAC	DA	Daa	
Enteroaggregative *E. coli* EAggEC	EASTI, AggA	astA, EAggEC	+
Salmonell sp.	Shiga toxin gene stx, aerobatin, Group-specific O antigen	spvABC	+
Shigella sp.			
Vibro cholerae	Cholera toxin	cholera toxin A & B, toxR, toxS	+
Vibro parahaemolyticus	Haemolysin		+
Yesinia enterocoliticia	Heat stable enterotoxin, lipopolysaccharide O side chain		+

2.3 Biological Indicator

Biological indicator is an organism can present as a mark of the quality of the environment, mostly the terms indicator or indicator species is applied. For example, some species are known to have particular requirement of nutrient or level of dissolve oxygen. Moreover the presence of a particular species in a habitat indicates that the parameter is within the tolerance limit of that species. The presence or absence of a species may become the indicator. Biological indicator provides biological status that

integrates chemical and physical stressors over larger spatial and temporal scales. There are three different principal indicator recommended such as benthic macroinvetebrate, fish and algae. Fish assemblages are used to assess the overall health of stream ecosystem. Fish is used as a biological monitoring indicator because fish are long lived and at the top of the food chain in aquatic environment. Moreover, fish are relatively easy to collect and be identified (NJDEP-DWMS, n.d.). Any living organism can be uses as biological monitoring however benthic macroinvetebrate, fish, and algae growths are used frequently, in a certain cases (Engel & Voshell, 2002). The abundance of Diptera; Chironomiidae and Simuliidae in indicated organic pollution in freshwater (Buckup *et al*., 2007). Chironomiidae and Simuliidae were describe as potential macroinvetebrate organism to be used as indicator for water pollution caused by organic pollutants, while Hirudinea and Oligochaeta have the possibility to be used as indicator for other polluted water (Kok Weng & Weng Chee, 2015). Riparian vegetation also serves their interface between the land and body of water. Loss of riparian habitat in freshwater will reduce the biodiversity and productivity of the system and can lead to degradation in water quality and ecosystem health. Others species that may become biological indicator is freshwater mussels. Freshwater mussels are commonly labeled as "good" indicators of biological integrity and water quality by scientists. Freshwater mussel species richness was positively correlated with total dissolved solids and negatively correlated with lead. a significant reduction in mussel species diversity and suggested the change was possibly due to oxygen algal toxins, depletion, application of copper, sulphate, sewage and agricultural spills and runoff, and habitat changes (Jeffrey & Wayne, 2008).

Microorganisms can be uses as direct indicator of pollution and indicates stress by organic chemical, heavy metal. Microorganism is the most diverse biological group and is an appropriate as biodiversity indicator of pollution in river health assessment. The coliforms group of bacteria mostly widely used as an indicator organism. This group of bacteria indicates the existence of faecal contaminated in water. The presence of faecal coliforms bacteria in aquatic environment indicates that the water has been contaminated with the faecal content. Faecal contamination sources may come from human or animal excreta. *Escherichia coli (E. coli*), the thermotolerant and other coliforms bacteria, the faecal streptococci and spores of sulphite-reducing clostridia, are common indicators used for this type. Certain species of algae, example cynobacteria is useful biological indicator of pollution. Cynobacteria constitute a pollutant particularly when present in large number.

There is relationship between microorganism indicator and other biotic indicator. For example, the massive cynobacterial bloom that occurs in the Darling River in 1991, where a numbers factors were postulates as the cause. The factors included high phosphorus concentration, increase light penetration because of reduces turbidity and very low rivers flows providing the right condition for blue-green algal growth.

2.4 Media Agar for Bacteria Growth

A solution containing complex nutrient for survival and continued growth of microorganism is a culture medium (Cappuccino, 2014). Commonly, most culture media are liquid, semisolid, or solid. A liquid medium are less a solidifying agent is called a broth medium while a broth medium supplemented with a solidifying agent called agar result in a solid or semisolid medium (Cappuccino, 2014). When culture the bacteria in media agar, the formation of colour of the colony differs by different type of bacteria (Nandy, Thakur, & Chaudhuri, 2007). *Escherichia coli* produce pink colonies when cultured in chromogenic substrates media agar. *Vibrio* bacteria normally appear as green or yellow colonies when culture on thiosulfate-citrate-bile salts-sucrose (TCBS) medium (Ruangpan, 2004). There are three types of media which are selective media, differential media and enriches media (Cappuccino, 2014). Selective media allows the growth of certain type of microorganism, while inhibiting the growth of other organisms. While differential media used for differentiating related organism or group of organism. Differential media contain certain dyes or chemical, the organism that culture on it will produce certain characteristic changes or growth pattern. Example of differential media is Mac Conkey (MCK) agar. For enriched media, are media that have been provided with highly nutritious materials such as blood or serum for the purpose of cultivating organism (Cappuccino, 2014).

Mac Conkey agar is selective for the growth of Gram-negative species and differential with respect to lactose fermentation (Cappuccino, 2014). Mac Conkey agar contains the dyes eosin and methylene blue. It inhabit Gram positive organism. Mac Conkey agar is used for the detection of coliforms and enteric pathogens based on their

ability to ferment lactose. Lactose-fermenting bacteria appear red to pink while non-lactose fermenting bacteria appear as colorless or transparent colonies. The growth of Gram-positive organisms is inhibited because of the crystal violet and bile salts in the medium (Amritaedu, 2016). The bile salts in the medium precipitate in the immediate neighborhood of the colony, causing the medium surrounding the colony to become hazy appearance. Non-lactose fermenting bacteria such as, *Proteus species, Salmonella*, *Pseudomonas aeruginosa* and *Shigella* cannot utilize lactose in the medium, and will use peptone instead. This results in the formation of ammonia, which raises the pH of the agar, and leads to the formation of white or colorless colonies in the agar plate.

2.5 Bacteria Classification

The most basic technique used for classification of bacteria is based on the bacterium's shape and cell arrangement (Mohammad & Othman, 2012). The most ordinary shapes of bacteria include rod, cocci (round) and spiral forms. Figure 2.1 show the basic shape of bacteria commonly found. For cellular arrangement occur singularly, in series, and in group. For arrangement of cocci it could be oval, elongated or flattened on one side. Cocci may remain attached after cell division. The prokaryotes of the bacteria which include their function and structure such as capsule, peptidoglycan, flagella, pili, and cytoplasmic membranes is others types of bacteria classification. The average diameter of spherical bacteria is 0.5-2.0µ while for rod shaped bacteria, the length is 1-10µ and diameter is 0.25-1.0µ.

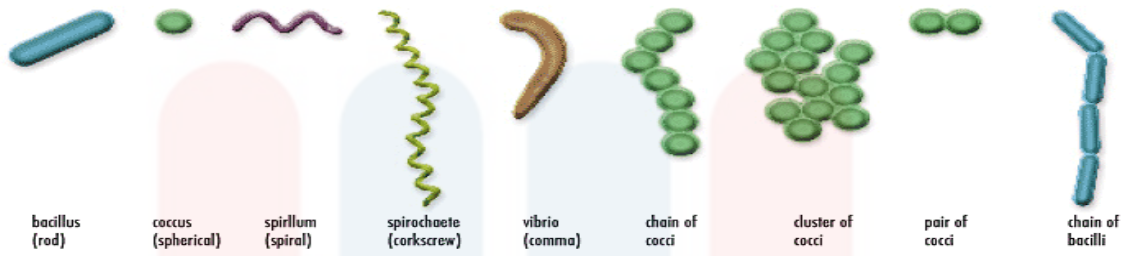


Figure 2.1: The basic shape of bacteria (Retrieved from http://www.microbiologyonline.org.uk/about-microbiology/introducing-microbes/bacteria)

2.6 Gram Staining

Gram stain is a fast and essential method for bacterial differentiation. Staining procedure makes for view bacteria clearly, but it does not distinguish between organisms of similar morphology. In 1884, a Danish Physician named, Christian Gram discover a new technique to differentiate the bacteria of the similar morphology. He used two dyes in, each of a different colour. The organism that retain the colour are called Gram positive and those cannot retain the first dye, but then take on the colour of the second dye are called Gram negative (Ruangpan, 2004). The retain of crystal violet-iodine complex and stains the cells as purple because of the thick peptidoglycan layer of Gram-positive organisms. Gram-negative bacteria have a thinner layer of peptidoglycan but retain the counter stain of Safranin, then are present as reddish or pink. For example *S. aureus* and *Bacillus* spp. was Gram positive while *E. coli* was Gram negative. The characteristics of the bacteria can be influence by some of variables such as culture age, type of media, incubation atmosphere, staining method and the presence of inhibitory substances (Bhattacharyya, 2015).

2.7 Polymerase Chain Reaction (PCR)

Kary Mullis invented PCR in 1978 which granted him Nobel Prize in 1993. PCR is a technique to amplify target genes in *vitro* (Hangbao, 2005). The method is used for identification of new species or strain. PCR amplification has chosen as the method of choice for obtaining rRNA sequence data from microbial communities or pure culture (Stackebrandt, 1992). New phylum from the obtained 16R rDNA clone sequence was recovered from terrestrial soil (Frierish V, 1997). Different primer will be used in PCR. PCR was done using G1/G2 primers for confirmation of pathogenic leptospires (Ridzlan *et al.*, 2010). The PCR product size can be identified by using agarose gel electrophoresis (Hangbao, 2005).

2.8 DNA Extraction

The first step in molecular analytical technique is the extraction of DNA. DNA extraction is still considered difficult and technically challenging for beginner (Martin-Laurent *et al.*, 2001). Many different approach for DNA extraction have been described which include, microwave heating, thermal shocks and sonication (Yang & Hang, 2013). Proper procedure is needed to have a good quality of result. CTAB buffer is used during DNA extraction. CTAB buffer is modified to remove polysaccharide, polyphenols and other secondary metabolites that can inhibit PCR amplification and can lead to error interpretations.

2.9 DNA Quantification

There are a few methodologies use for quantifying the amount of nucleic acid in a preparation of electrophoretic run along with stand DNA, spectrophotometric estimation, flourometric determination and DNA quantification using NanoDrop (Gaikwad, n.d.). Nucleic acids will absorb light at a wavelength of 260 nm. For double stranded DNA, an Optical Density (OD) of one at 260 nm correlates to a DNA concentration of 50 ng/µl, so DNA concentration can be easily calculated from OD measurement (Oxford Gene Technology, 2011). Lower absorbance A260/230 values indicate contamination with salts or some solvent for example phenol (Oxford Gene Technology, 2011)

Agarose gel electrophoresis is a method of quantification based on the ethidium bromide fluorescent staining of DNA. The fluorescent yield of the dye, UV irradiation at 254 nm is absorbed by the DNA and transmitted to the dye. The dye absorbs radiation at 302 nm and 366 nm. The quantify DNA can be estimated by comparing the fluorescent yield of the samples with a series of standard. Others method is DNA quantification by UV spectroscopy. Nucleic acid in the sample will be analysis by UV absorption. The ration of OD_{260}/ OD_{280}, should be determined to assess the purify of the sample. Spectrophotometer is a traditional measurement, a new spectrophotometer called a NanoDrop that requires only about 0.5 µl – 2 µl of sample for quantification. Approximate time needed to quantify DNA using NanoDrop about 10-15 minutes for one sample and add two minutes for each additional sample. NanoDrop spectrophotometer has the capability to measure highly concentrated sample without dilution compare to standard cuvette spectrophotometer.

CHAPTER 3

MATERIAL AND METHOD

3.1 Media preparation for river microbe culturing from upstream of Lata Janggut

3.1.1 Nutrient Agar (NA) for microbes culture

Nutrient agar (NA) brand OXIDS is used for preparation of solid media, 23 grams NA powder were suspended in 1000 ml distilled water. The NA media was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Autoclaved agar which was cooled at room temperature was then poured approximately 30 ml into sterile petri dish. After NA media solidified then the NA was seal and kept into 4℃ chiller. NA was used for cultured water microbes from upstream of Lata Janggut.

3.1.2 Luria Broth (LB) for microbes cultivation

Luria Broth brand MERCK was prepared for the growth of bacteria, 20 grams of Luria broth powder was suspended in 1000 ml conical flask. Distilled water was added until the solution reach 1000 ml. The mixture was heated to dissolve the media completely. The stock was sterilized by autoclaving at 15 lbs pressure (121℃) for 15 minutes. The autoclaved LB was then kept at room temperature.

3.2 River water sampling at upstream of Lata Janggut

The water sample from upstream river at Lata Janggut (Figure 3.1) was taken by using 50 ml sterile falcon tube. The sampling falcon tube bottle was labeled with name of the river water sample, date and sampling sites. The river water samples were taken at five different points which were labeled as Point 1, Point 2, Point 3, Point 4 and Point 5. The water samples were kept in the ice box and sufficient amount of ice packs was

put to keep the samples at cold temperature during transport to the laboratory.

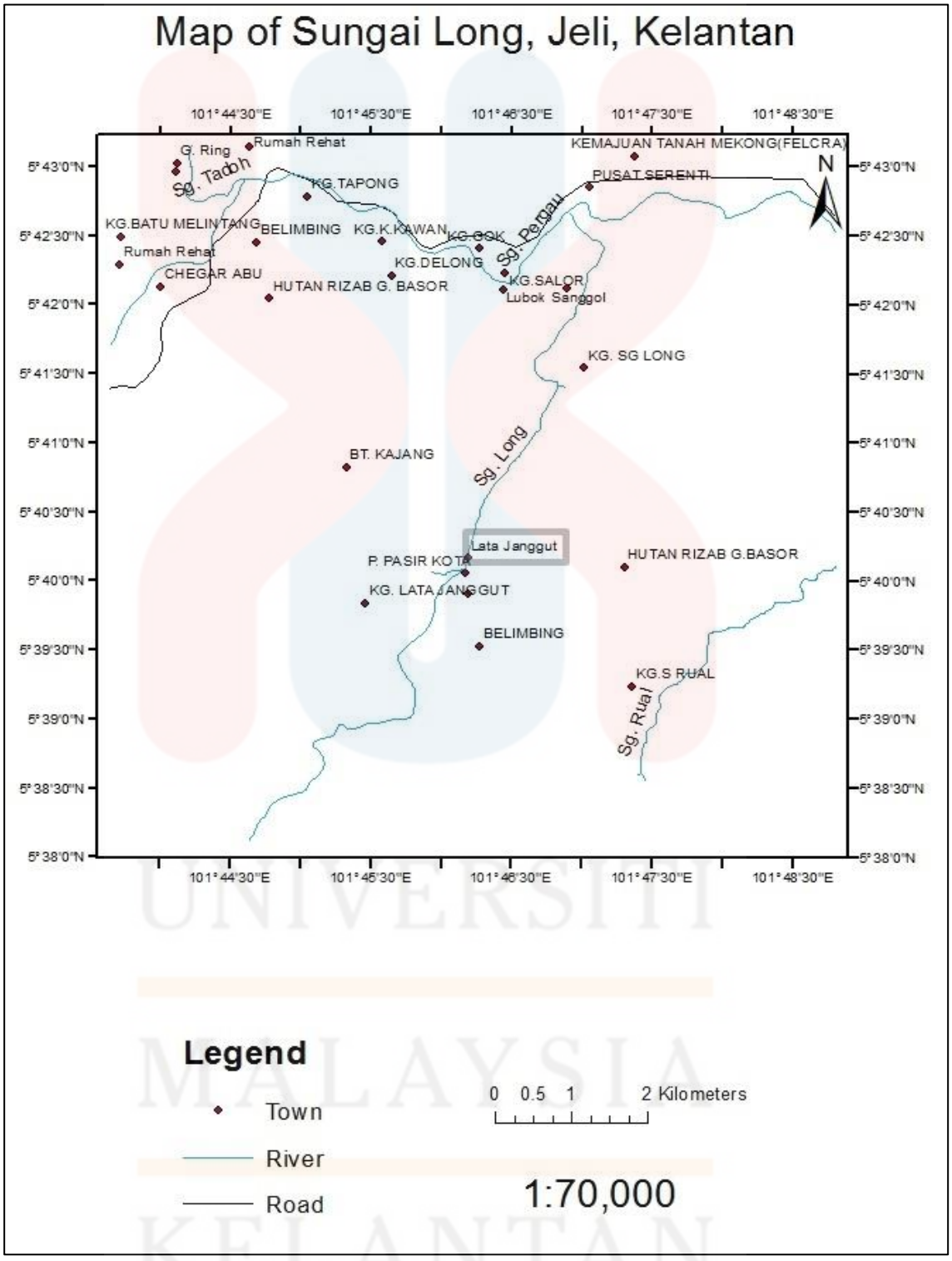


Figure 3.1: Location of Lata Janggut extracted using Arc-GIS 10 (Jabatan Mineral & Geosains Malaysia,2006).

3.2.1 Serial dilution of water microbes from upstream of Lata Janggut

For bacteria isolation, one ml of river water sample was added with nine ml of distilled water in a 15 ml Falcon tube. The mixture were mixed by upside down the falcon tube then were diluted onto 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. Figure 3.2 showed the preparation of apparatus for serial dilution.

Figure 3.2: Set up of apparatus for serial dilution in laminar flow located at Makmal Kimia Bap 1.1, UMK Campus Jeli.

3.2.2 Isolation of water microbes from upstream of Lata Janggut

After serial dilution done as in section 3.1.1, the diluted sample were spread plate onto Nutrient Agar (NA), 200 µL of water sample was pipette from dilution 10^{-5} and spread plate onto nutrient agar. The sample then incubated in room temperature (30°C) for 24-48 hours in the incubator oven. Pure culture colony was obtained as each bacteria colony was isolated from spread plate agar and streaked onto NA plates.

3.3 Morphology identification of microbes isolated from upstream of Lata Janggut

3.3.1 Cell morphology observation from water microbes of upstream Lata Janggut

The morphology identification was done following method as described by Robert (2009). The colour of bacteria, shape and arrangement were observed by using compound microscope. The procedures for Gram staining were done following the protocol from Robert (2009). The bacterial suspensions from pure culture cultured on NA media were smeared on clean dry glass slide. The smear was heated by using Bunsen burner. The slides were smeared with crystal violet and let to stand for one minute. The slide was rinsed with distilled water. The slides were smeared with the Gram's Iodine and let stand for one minute. The slides were rinsed with distilled water. Then 1-2 drops of acetone were dropped and rinsed the slide with distilled water. The slides were smeared with Safranin and kept for one minute and again rinsed with distilled water. The slides were kept air dried and observed under light microscope.

3.3.2 Culture of water microbes in Luria Bertani Broth

Each of single strain isolated as mention in section 3.2.2 was cultured in the 15ml of Luria Bertani broth. The cultures were kept in the incubator shaker located at Makmal Alam Sekitar UMK Campus Jeli at 30℃ for 24 hours at 150 rpm.

3.3.3 Optical Density (OD) of water microbes from upstream Lata Janggut

After culturing water microbes in Luria Bertani broth as in section 3.3.2, Optical Density (OD) of each culture was measured at 600 nm wavelengths by using spectrophotometer. Absorbance at 600 nm was the recommended wavelength for bacterial sample (Quigley, 2008).

3.4 DNA extraction of water microbes from upstream of Lata Janggut

3.4.1 Preparation of buffer for DNA extraction

3.4.1.1 Preparation of 100 M Glucose/Tris/EDTA (GTE) for DNA extraction

The preparation of GTE buffer was modified based on protocol of Vinod (2007). Hundred ml of GTE buffer was prepared by adding 0.9 g of glucose powder with 2.5 mL of 1M Tris buffer with 2 ml of 0.5 M EDTA solution. The buffer solution was stored at 4℃.

3.4.1.2 Preparation of Cetyltrimethylaammonium bromide (CTAB) Buffer for DNA Extraction

The preparation of CTAB buffer was modified based on protocol of Vinod (2007). One liter of CTAB buffer solution was prepared by adding 20 g of CTAB powder with 860 ml distilled water. The solution was added with 81.82 g NaCl, 100 ml of one molar Tris and 40 ml of 0.5 M of Ethylenediaminetetraacetic acid (EDTA). The buffer solution was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. The prepared CTAB buffer was keep in media bottle at room temperature.

3.4.1.3 Preparation of 1 M Tris pH 8.0 for DNA extraction

The preparation of 1 M Tris buffer was modified based on protocol of Vinod (2007). One liter of Tris buffer solution was prepared by adding 186.12 gram Tris

powder with 700 ml distilled water. Tris powder was dissolved using magnetic stir plate. Concentrated HCl was used to adjust the pH to 8.0. The buffer solution was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

3.4.1.4 Preparation of 0.5 M of EDTA for DNA extraction of water microbes

The preparation of 0.5 M of EDTA buffer was modified based on protocol of Vinod (2007). EDTA powder about 186.12 grams was added in media bottle with 750 ml of distilled water. About 20 grams of NaOH pellets was added. NaOH was added until the pH is 8.0. The buffer was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

3.4.2 DNA Extraction of water microbes from upstream of Lata Janggut

3.4.2.1 DNA extraction with standard Cetyltrimethylaammonium bromide (CTAB) method

The DNA extraction method was modified based on protocol of He (2011). One ml of the overnight bacteria culture grows in LB medium was transferred into 1.5 ml Eppendorf tube and centrifuge at10,000 rpm for one minutes to pellet the cell. The supernatant was discarded. The cell pellet was re-suspended in 600 µl lysis buffer and vortexed to completely re-suspended cell pellet. The cell pellet was incubated for one hour at 37℃. The cell pellet was added 600 µl of phenol/chloroform and mixed well by inverting the tube until the phases are totally mixed and centrifuge 14,000 rpm for five minutes.

The upper aqueous phase was transferred into new sterile 1.5 ml Eppendorf tube by using micropipette. The 60 µl cold ethanol was added into the supernatant and was

mixed gently to precipitate the DNA. The supernatant was centrifuge at 14,000 rpm for 15 minutes. The supernatant was discarded and the DNA pellet was rinsed with one ml of 70% ethanol. The mixture was centrifuged at 14,000 rpm for two minutes and the supernatant was carefully discarded. DNA pellet will be left for air dry. The DNA pellets were re-suspended in 50 µl TE buffer.

3.4.2.2 Microbial DNA extraction method with Proteinase K, RNase and Lysozyme

The DNA extraction was modified based on protocol of He (2011). One ml of the overnight bacteria culture that grows in LB medium was transferred into 1.5 ml Eppendorf tube and centrifuged at 13,000 rpm for 15 minute at 4℃ to pellet the cells. The supernatant was discarded. The cell pellet was wash with 1ml of GTE and centrifuge at 10,000 rpm for 5 minutes at 4℃. The supernatant was discarded. GTE was added about 200 µl and kept on ice for 5 minutes. The mixture was incubated for one hour at 37°C after 50 µl RNase and 100 µl lysozyme was added. The mixture was incubated at 50℃ for 30 minutes after 50 µl Proteinase K continued with 50 µl 25% SDS. The mixture was add 200 µl TE buffer and 500 µl Phenol/Chloroform/Isoamy alcohol (25:24:1) and centrifuge at 10,000 rpm for 15 minutes at 4℃. There will be white layer (protein layer) in the aqueous phase. The upper aqueous phase was transferred into new sterile 1.5 ml microcentrifuge tube by using one ml pipette. To precipitate the DNA, 500 µl 80 % ice cold ethanol was added and mixed gently. The mixture was centrifuged at 10,000 rpm for 5 minutes at 4°C. The supernatant was discarded. DNA pellet will be left for air dry. The DNA pellets were re-suspended in 50 µl TE buffer.

3.5 Determination of Microbial DNA Quality and Quantity

3.5.1 NanoDrop Spectrophotometer measurement for DNA quantification of DNA extraction product.

After DNA extraction done as in section 3.4.2, DNA pellet was quantified using NanoDrop spectrophotometer to test purifying of DNA. One µl of DNA extraction was pipetted onto NanoDrop spectrophotometer.

3.5.2 1.0 % Agarose Gel Electrophoresis for DNA quantification and quality of DNA extraction product

After DNA extraction done as in section 3.4.2, DNA extraction product was quantified and qualifies by using Agarose Gel Electrophoresis (AGE). Agarose gel was prepared by mixing 0.8 gram agarose powder with 79 ml TAE (Tris-acetate-EDTA) buffer in 250 ml flask. The mixture was heated in microwave for two second. The solution had completely clear without any visible piece of agarose. The agarose was cooled down at room temperature. The gel tray was prepared with comb fixed completely. The mixture gel was poured into the gel tray when it cold enough to be hold with hand. The gel should be solidified completely in 20-30 minutes. The gel was covered by TAE buffer in electrophoresis bath. The first well were filled with 6 µl of DNA ladder, and the followed with 6 µl of sample. The power source was set at 80 V for 45 minutes. After 45 minutes, the gel was placed on transilluminator and viewed under UV light.

3.5.3 Polymerase Chain Reaction (PCR) with microbial DNA for 16S gene amplification

The PCR was done following method described by Sambrook and Russell (2011). 16s rDNA gene isolated was amplified using two universal primers.

16S forward primer: 5'-CGC TGG CGG CGC GTC TTA AA-3'

16S reverse primer: 5'-TTC ACC GCT ACA CCT GGA A-3

PCR microtubes were prepared and labeled with sample name and date. The entire component listed in Table 3.1 were combined in a sterile 0.5 ml microcentrifuge tube and gently mixed. The preparation of PCR Master Mix was done on ice. The amplification of microbial DNA will be done using the denaturation, annealing and polymerization and temperature listed down in Table 3.2. The withdrawn samples were analyzed by electrophoresis through an agarose gel as in section 3.5.2. The PCR product was stored at -20˚C freezer.

Table 3.1: The volume of component for PCR in 0.5 ml microcentrifuge

Component	25 µl Reaction	Final Concentration
ddH_2O	16.0 µl	-
$MgCl_2$ Solution, 25 mM	2.0 µl	1.0 mM
PCR Nucleotide Mix ($dNTP_S$), 10 mM	1.0µl	0.2 mM each dNTP
5X Green GoTaq$^{@}$ Flexi Buffer	2.5 µl	1.0 X
Forward Primer, 10 µM	0.5 µl	0.2 µM
Reverse Primer, 10 µM	0.5 µl	0.2 µM
GoTaq$^{@}$ DNA Polymerase (5 u/µl)	0.5 µl	1.25 unit
Template DNA	2.0 µl	< 0.5 µg/50 µl

Table 3.2: PCR Condition for amplification of 16S rDNA

Step	Temperature (℃)	Time	Cycles
Pre denaturation	94	2 minute	1
Denaturing	94	1 minute	30
Annealing	55	30 second	30
Extension	72	1 minute	30
Final Extention	72	5 minutes	1

CHAPTER 4

RESULT AND DISCUSSION

4.1 Physical Parameter of Upstream Lata Janggut

Table 4.1: The water sample parameter from upstream of Lata Janggut.

Parameter / Point	Point 1	Point 2	Point 3	Point 4	Point 5	Average
Temperature (℃)	28.97	26.70	26.22	25.21	25.18	26.46 ± 1.38
pH	6.46	6.16	6.43	6.21	5.86	6.22 ± 0.24
Salinity (Sal)	0.03	0.01	0.02	0.01	0.01	0.02 ± 0.0089
Dissolve oxygen (mg/L)	3.23	3.10	3.28	2.63	3.05	3.06 ± 0.26

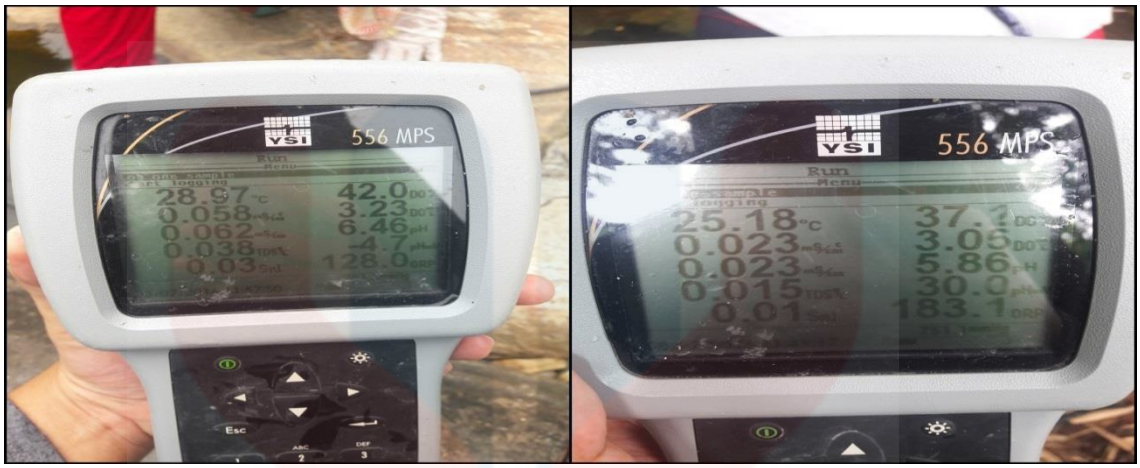
The study aimed to culture and isolate the microorganism from river water sample at upstream Lata Janggut. The temperature of the water was 26.46˚C, ± 1.38 during daytime and the pH value was 6.22 ± 0.24 when sampling was done. The variation in water temperature was mainly due to whether condition related either the areas were shaded area or directly exposed to sunlight. Figure 4.1 showed the location of direct sunlight and shaded are at upstream Lata Janggut.

The lowest dissolved oxygen (DO) value recorded was 2.63 mg/L at Point 4 while the highest was 3.23 mg/L at Point 1.There is no difference in pH for all sampling points, it ranged between 5.86 to 6.46. The physical parameter was taken using YSI Multiparameter. Figure 4.2 showed the reading of YSI Multiparameter at upstream Lata Janggut.

The physical parameter which would influence the microbial growth in the freshwater. Water temperature can affect bacterial mortality rate and growth rate. Increasing in temperature and salinity can lead to fecal coliform survival in the presence of sunlight (Solic & Krstulovic, 1992). The temperature recorded in this study was at 26.46℃ ±1.38 (Table 4.0). Although to indicate fecal contamination temperature 37℃ has been used, this is not generally consider being reliable nowadays (Water - Microbiological Analysis of Water, 2016).

A B

Figure 4.1: The direct sunlight (A) and shaded area (B) at upstream Lata Janggut



A B

Figure 4.2: YSI reading from Point 1 (A) and Point 5 (B) at upstream Lata Janggut

4.2 Isolation and Screening of Water Microbes from upstream Lata Janggut

4.2.1 Morphology Identification of water microbes from upstream Lata Janggut

The isolates were characterized based on morphology characteristics i.e. shape, elevation, and margin of bacterial colony. The result of colony morphology were summarized in Table 4.2

Table 4.2: The morphology characteristics of microbes from Upstream Lata Janggut

ISOLATED	Shape	Elevation	Margin
P.1.A 10^{-5}	Irregular	Convex	Undulate
P.1.B 10^{-5} (1)	Irregular	Flat	Entire
P.1.B 10^{-5} (2)	Irregular	Raised	Lobate
P.3.A 10^{-4}	Irregular	Flat	Entire
P.4.A 10^{-5}	Circular	Raised	Undulate
P.5.A 10^{-4}	Circular	Raised	Undulate
P.5.C 10^{-5}	Irregular	Convex	Undulate

4.2.2 Gram staining of water microbes isolates from upstream Lata Janggut

All the seven sample were stained by method called Gram Staining. All the selected sample appears as purple stain when observed under light microscope (Table 4.3). This indicates that the sample were all Gram positive. Gram positive cells such as cocci and bacilli appear purple due to staining by primary stain. The crystal violet is an insoluble complex that is difficult to remove and results the formation of crystal violet-iodine complex. Thusly, because of higher content in peptidoglycan in cell wall, crystal violet is trapped in cell wall. The Gram positive bacteria do not being recolored pink/red when safranin was being stained because the peptidoglycan layer already have crystal violet-iodine complex. Table 4.3 showed the staining result of water microbe from upstream Lata Janggut. Staining from point P.1.A 10^{-5} showed Gram positive bacteria with dipplobacilli shape when observed under light microscope. Staining from point P.1.B 10^{-5} (1) showed Gram positive bacteria with coccus shape when observe under light microscope. Staining from point P.1.B 10^{-5} (2), P.4.A 10^{-5} and P.5.C 10^{-5} showed Gram positive bacteria with bacillus shape when observed under light microscope. Staining from point P.3.A 10^{-4} and P.5.A 10^{-4} showed Gram positive bacteria with cocobacillus shape when observed under light microscope.

Figure 4.3 indicates Gram positive bacteria from the Point 1 A. The colour observes is purple due to primary staining, crystal violet. The shape of the bacteria is dipplobacilli. Figure 4.4 indicates the Gram positive bacteria from Point 1(B) (1). The colour observes is purple due to primary staining. The shape of the bacteria is coccus. Figure 4.5 indicates the Gram positive bacteria from Point 1 (B) (2). The colour observed is purple due to primary staining, crystal violet. The shape of the bacteria is

bacillus. Figure 4.6 indicates the Gram positive bacteria from Point 3(A). The colour observed is purple due to primary staining, crystal violet. The shape of the bacteria is cocobacillus. Figure 4.7 indicates the Gram positive bacteria from Point 4 (A). The colour observes is purple due to primary staining, crystal violet. The shape of the bacteria is bacillus. Figure 4.8 indicates the Gram staining from Point 5(C). The colour observed is purple due to primary staining, crystal violet. The shape of the bacteria is bacillus. Figure 4.9 indicates Gram staining from Point 5 (A). The colour observes is purple due to primary staining crystal violet and the shape of the bacteria is cocobacillus

Gram positive fecal bacteria were also found in the environment such as lactobacillus, clostridia, bifidobacterium and enterococci (Cabral, 2010). Enterococci are Gram positive and cell occurs singly, in pair or short chain (Cabral, 2010). Presence of enterococci in environment water is due to contamination of fecal into river water (Cabral, 2010). *E. coli* is one of bacteria that were considered as an indicator of fecal pollution in water.

Table 4.3: Staining of water microbe from upstream Lata Janggut

Isolated	Staining
P.1.A 10^{-5}	Gram: Gram Positive Shape: Dipplobacilli
P.1.B 10^{-5} (1)	Gram: Gram Positive Shape: coccus
P.1.B 10^{-5} (2)	Gram: Gram Positive Shape: bacillus
P.3.A 10^{-4}	Gram: Gram Positive Shape: cocobacillus
P.4.A 10^{-5}	Gram: Gram Positive Shape: bacillus
P.5.A 10^{-4}	Gram: Gram Positive Shape: cocobacillus
P.5.C 10^{-5}	Gram: Gram Positive Shape: bacillus

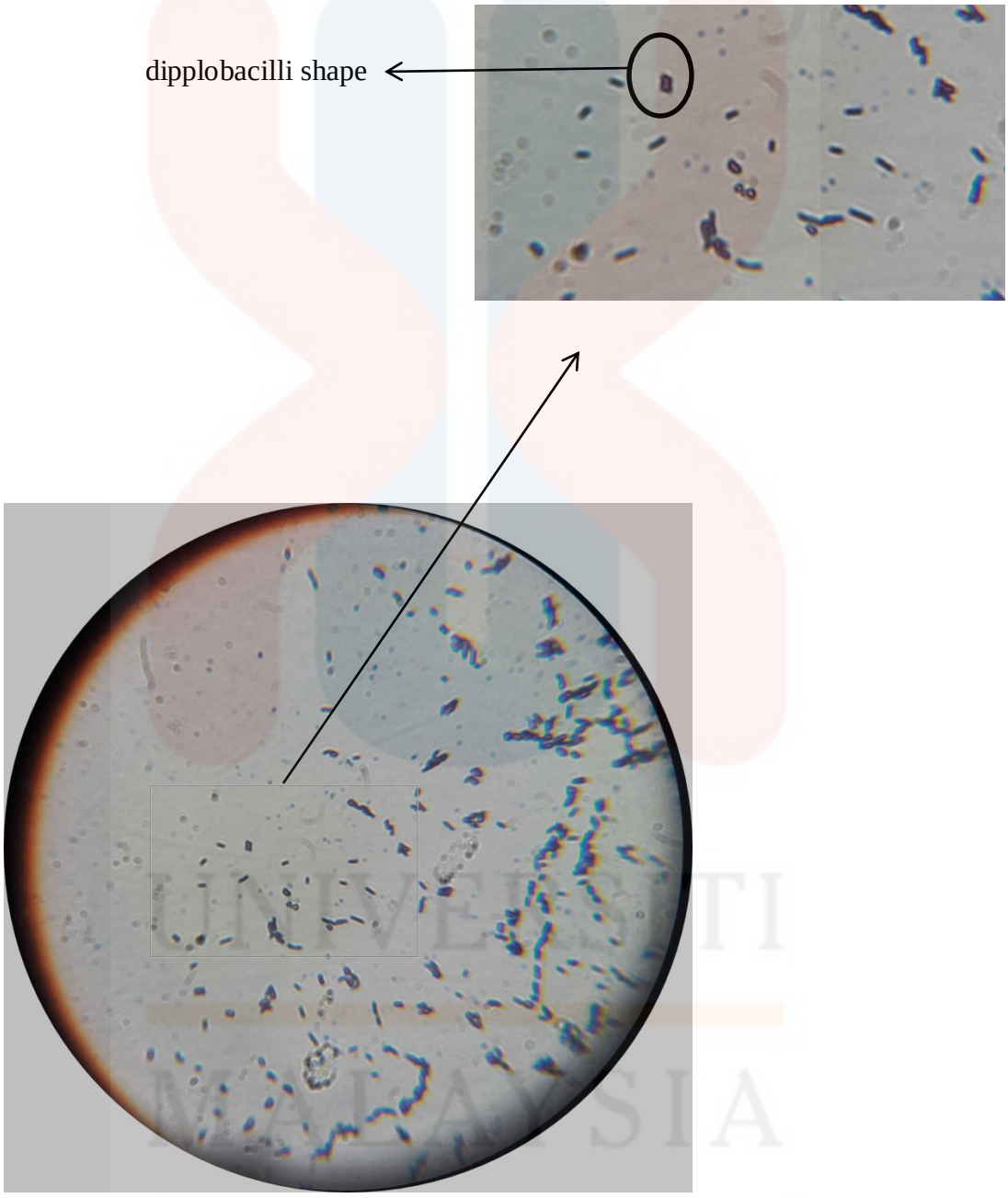


Figure 4.3: Gram positive bacteria from the Point 1 A (100x). The colour observes is purple due to primary staining of Gram stains. The shape of the bacteria is dipplobacilli observed using light microscope.

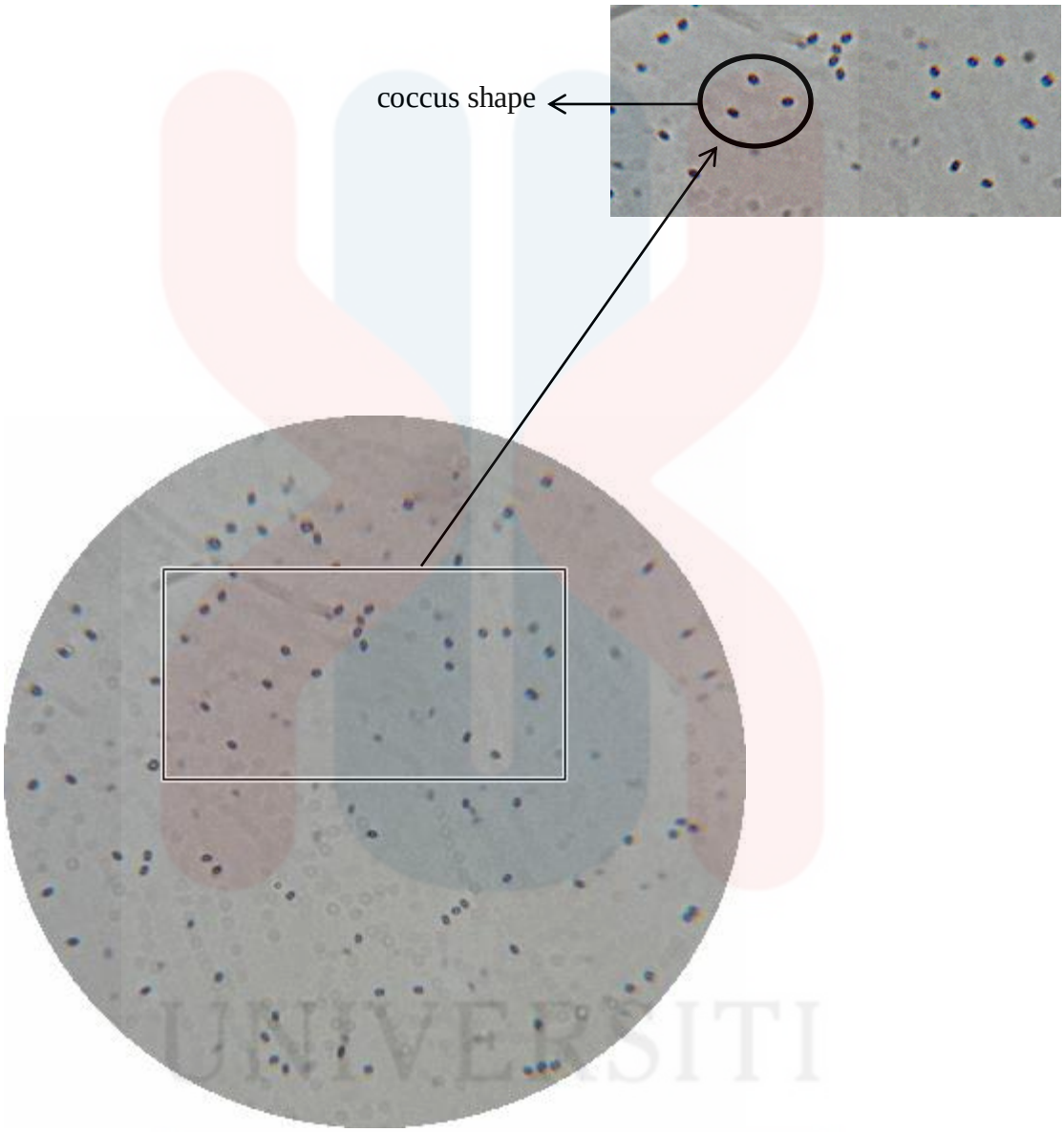


Figure 4.4: The Gram positive bacteria from Point 1(B) (1) under 100x magnification. The colour observes is purple due to primary staining of Gram stain. The shape of the bacteria is coccus observed using light microscope.

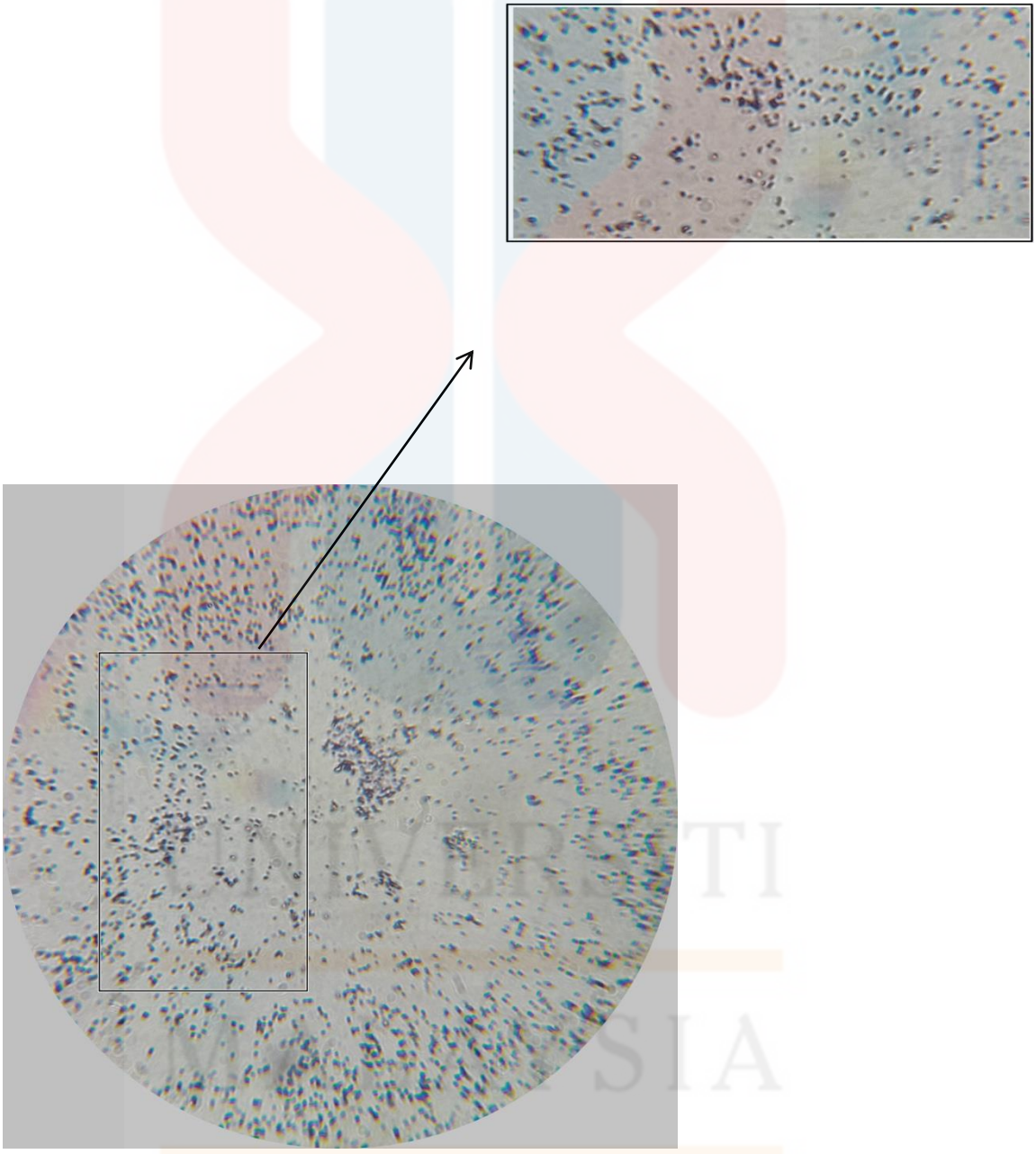


Figure 4.5: The Gram positive bacteria from Point 1 (B) (2) under 100x magnification. The colour observed is purple due to primary staining of Gram stain. The shape of the bacteria is bacillus observed using light microscope

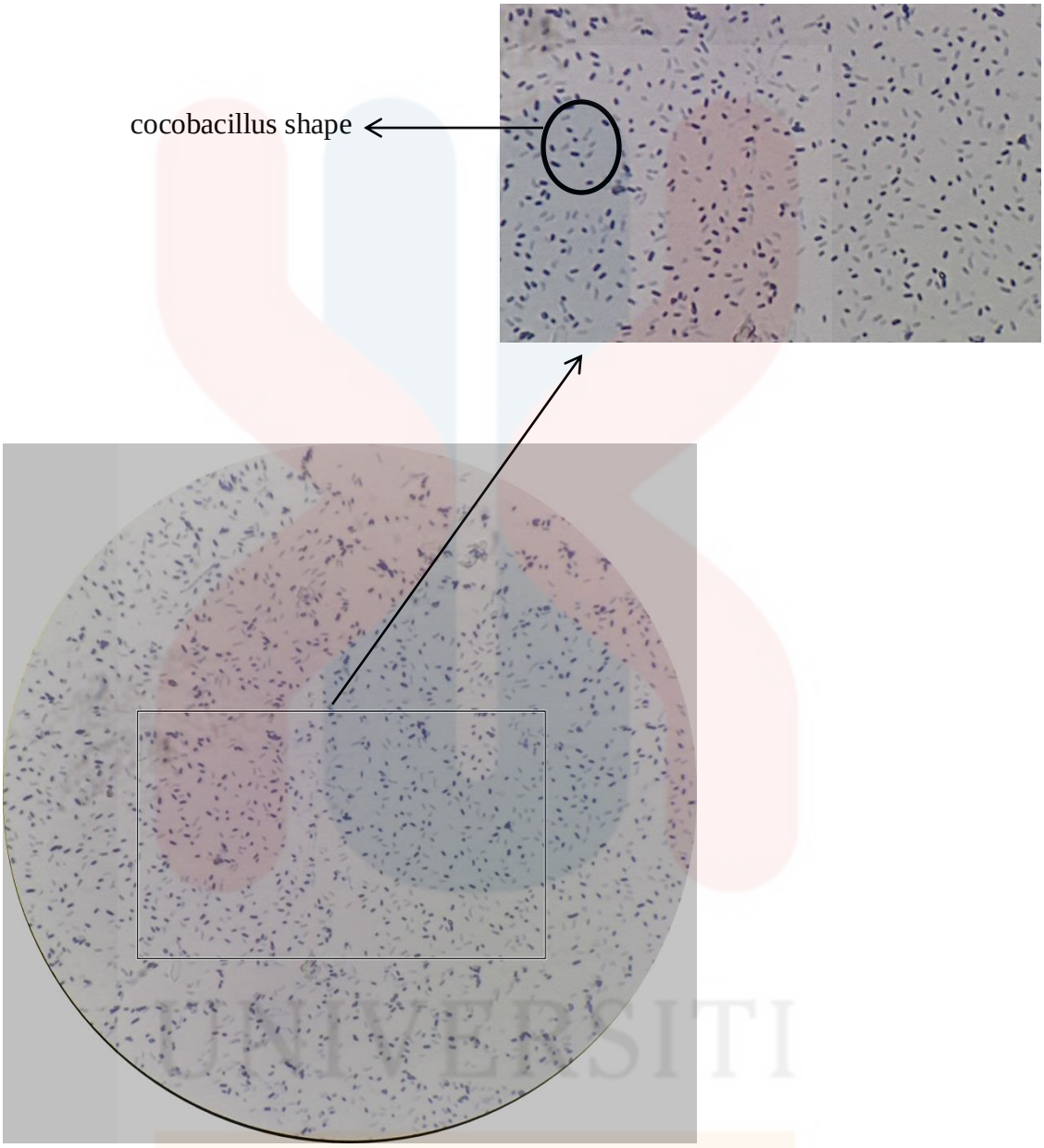


Figure 4.6: The Gram positive bacteria from Point 3(A) under 100x magnification using light microscope. The colour observed is purple due to primary staining of Gram stain. The shape of the bacteria is cocobacillus observed using light microscope.

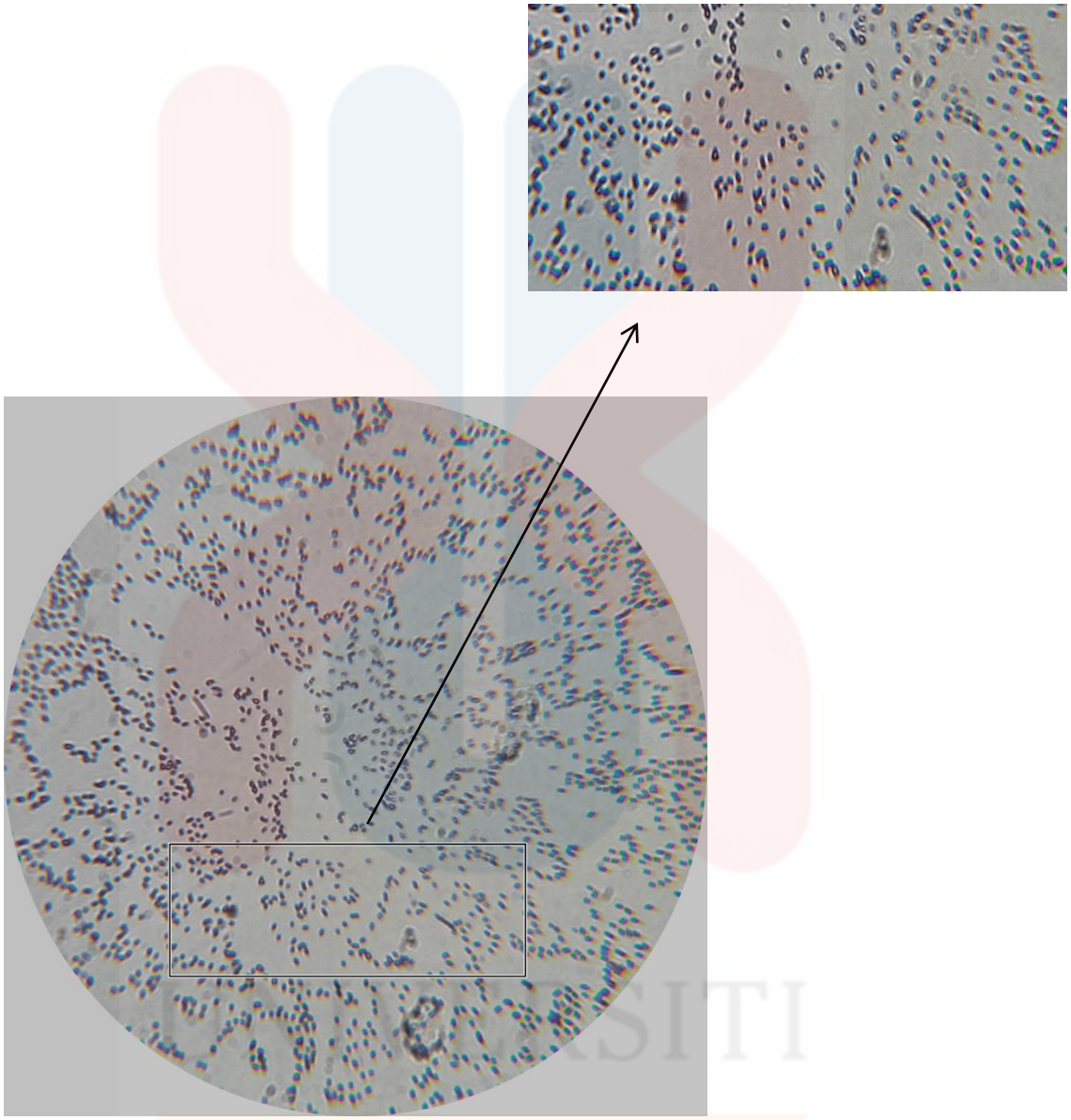


Figure 4.7: The Gram positive bacteria from Point 4 (A) under 100x magnification using light microscope. The colour observed is purple due to primary staining of Gram stain. The shape of the bacteria is bacillus when observed under light microscope.

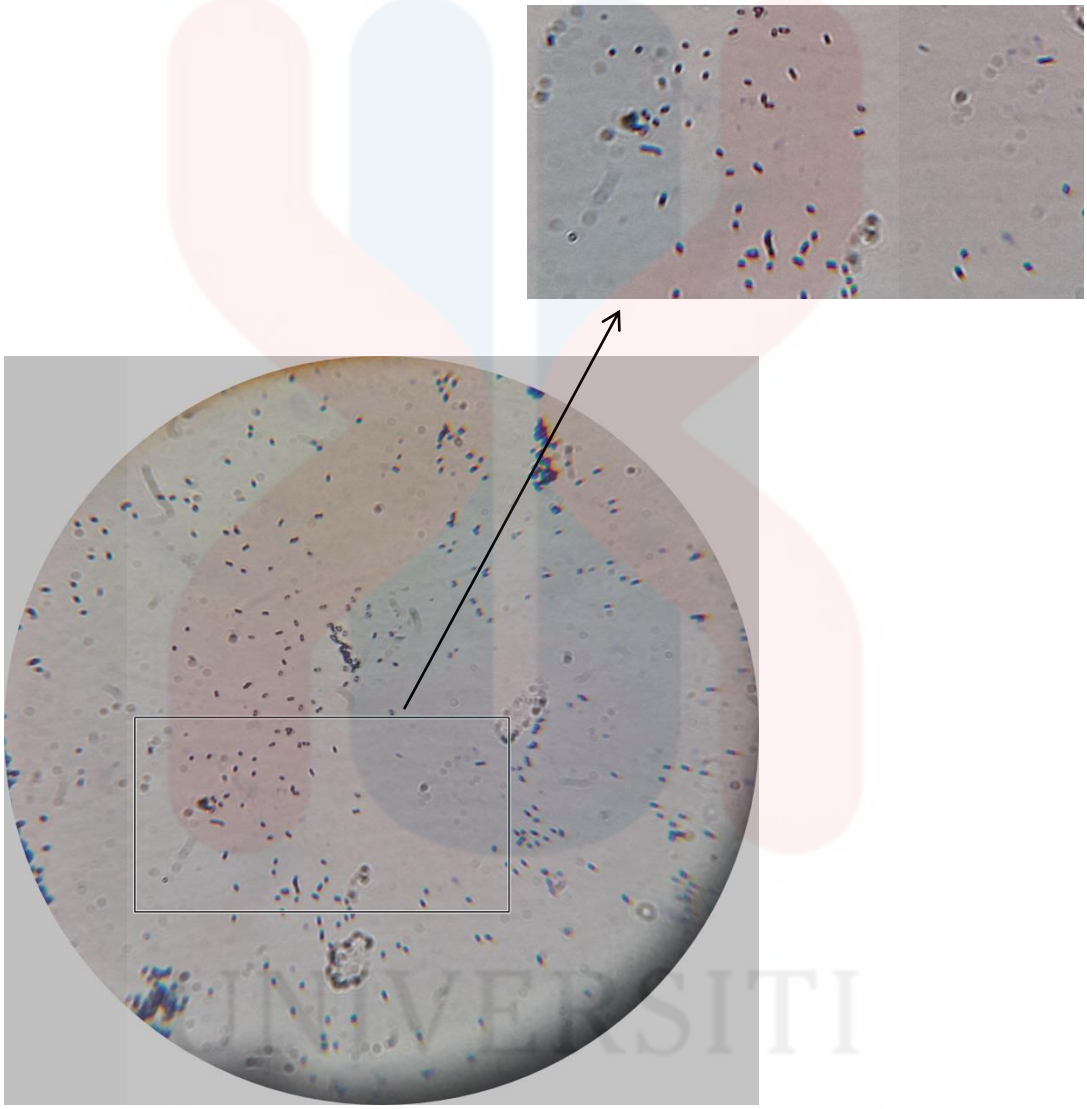


Figure 4.8: Gram staining from Point 5(C) under 100x magnification. The colour observed is purple due to primary staining of Gram stain. The shape of the bacteria is bacillus observed under light microscope.

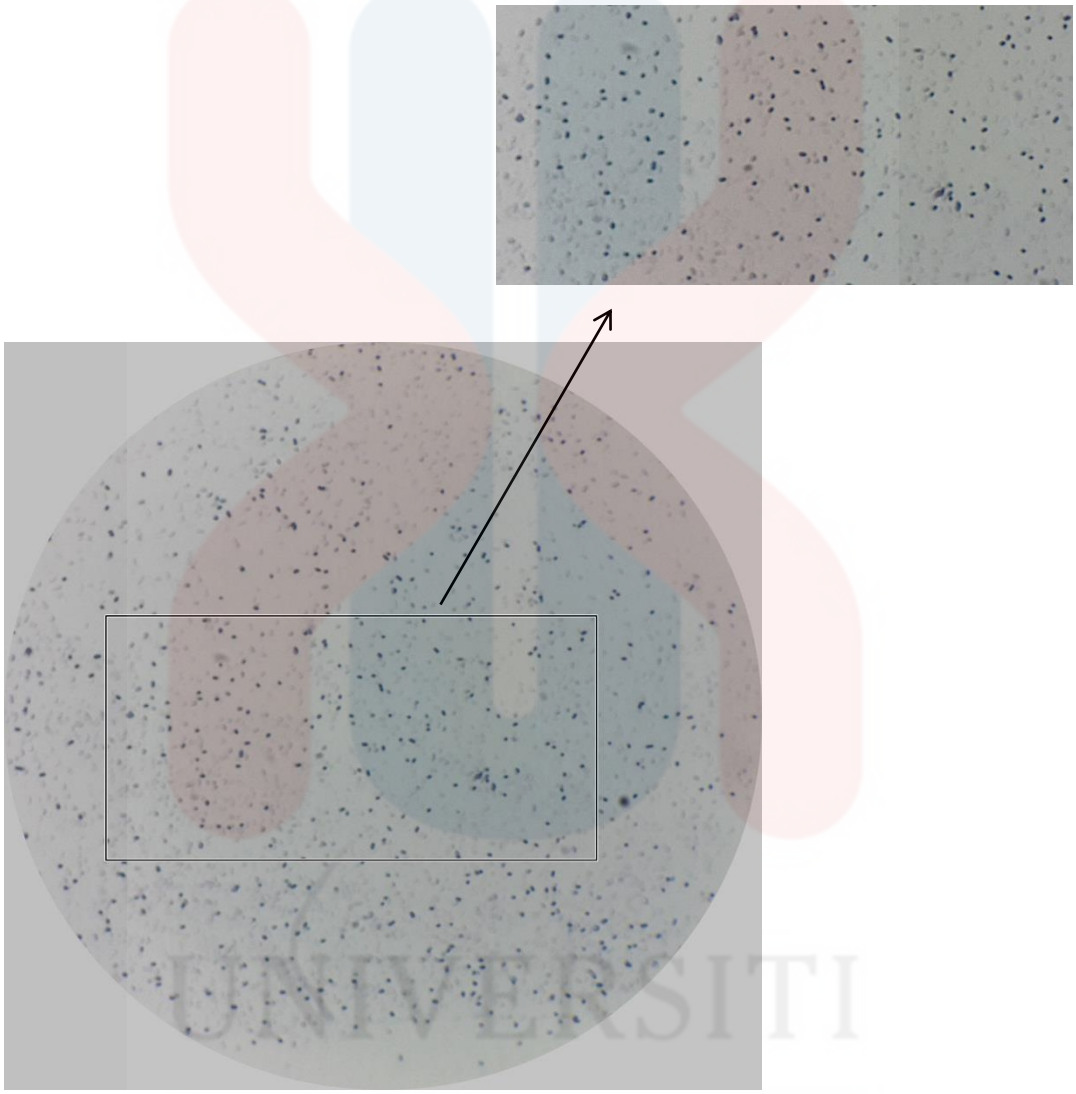


Figure 4.9: Gram staining from Point 5 (A) under 100x magnification. The colour observes is purple due to primary staining, crystal violet. The shape of the bacteria is cocobacillus observed under light microscope.

4.2.3 Optical Density (OD) of water microbes isolated from upstream Lata Janggut

Optical density measure the activity of microorganism at the specific wavelength. The sample bacteria activity was measured at wavelength 600 nm. Absorbance at 600 nm is the recommended wavelength for bacterial sample because at this wavelength, the cells will not be killed as they would under an exorbitant amount of UV light (Quigley, 2008). The resulted are summarized in Table 4.4.

Table 4.4: Optical Density of bacteria

Samples	OD at 600 nm
P.1.A 10^{-5}	0.113 A
P.1.B 10^{-5} (1)	0.330 A
P.1.B 10^{-5} (2)	0.251 A
P.3.A 10^{-4}	0.134 A
P.4.A 10^{-5}	0.414 A
P.5.A 10^{-4}	0.403 A
P.5.C 10^{-5}	0.439 A

4.3 Molecular identification of water microbe isolated from upstream Lata Janggut

4.3.1 DNA Extraction of water microbes isolated from upstream Lata Janggut

Each of 5 µl from seven samples DNA were tested via 1 % agarose gel electrophoresis (Figure 4.4). Bands indicated the DNA was successfully isolated. The 100 bp marker is used to identify the size of the bands.

From figure 4.3, the result obtained is negative. The DNA bands were not present when tested via 1 % agarose gel electrophoresis. This showed that the DNA extraction was unsuccessful. Therefore, the method for the DNA extraction was modified by adding Proteinase K, RNase and Lysozyme. Proteinase K functions to digest the contaminating protein and increase the yield of band (Nadine, 2014).

The DNA extraction method was modified as stated in section 3.4.2. From Figure 4.4 and Figure 4.5, the results obtained illustrated that the bacteria DNA are all of similar size. The DNA bands were clear and the DNA was located at the top which indicated that the DNA are heavy. This proved that the DNA extraction was successful. At lane 4, 5 and 6 (Figure 4.4) DNA bands did not appeared. The method used to extracted DNA used was same with others samples. It is possible that there was contamination during the DNA extraction. Contamination of sample may be cause by air-borne or the laboratory condition. Reagent used for extraction of preparation of sample also potential source extraneous DNA (Newton & Graham, 1997). Pipetting error during extraction method and the step of recombination of enzymes also potential source of contamination (Newton & Graham, 1997). This indicated the reason of the band did not invisible in lane 4, 5 and 6 (Figure 4.4).

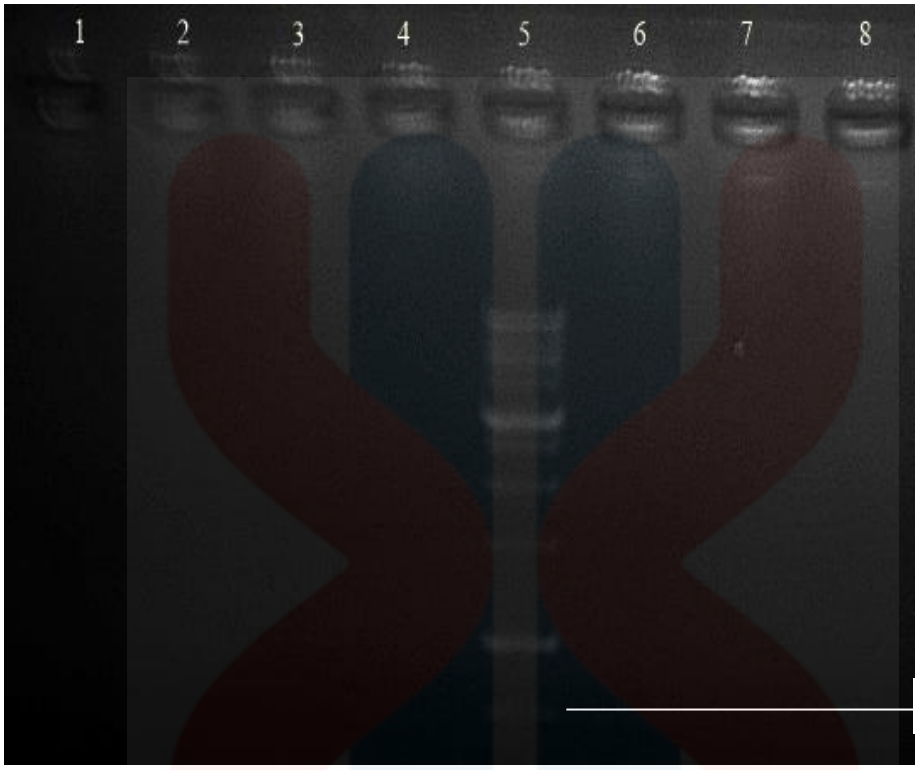


Figure 4.10: DNA extraction bands of seven samples based on DNA extraction protocol of He (2011) after gel electrophoresis on 1.0 % agarose gel at 80 V for 45 minutes

Lane 1. P.1.B 10^{-5} (2)**,Lane 2.** P.1.B 10^{-5} (1) **,Lane 3.** P.5.A 10^{-4} **, Lane 4.** P.4.A 10^{-5}

Lane 5. 1 kb DNA marker, **Lane 6**. P.1.A 10^{-5} , **Lane 7.** P.3.A 10^{-}

Lane 8. P.5.C 10^{-5}

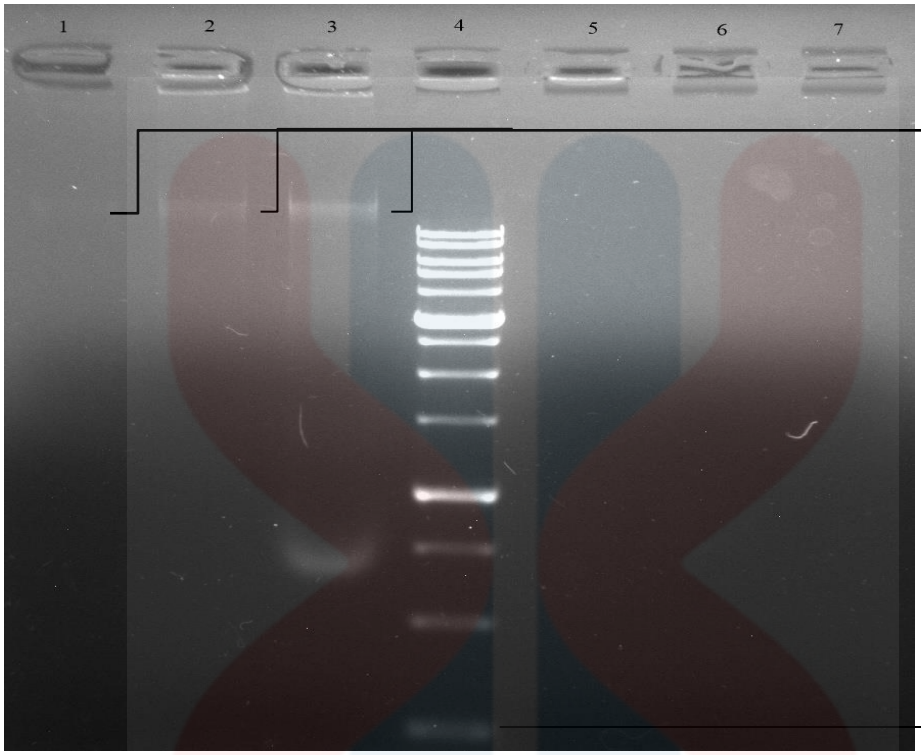


Figure 4.11: DNA extraction bands of six samples based on modified method DNA extraction protocol of He (2011) after gel electrophoresis on 1.0 % agarose gel at 80 V for 45 minutes

Lane 1. P.1.B 10^{-5} (2), Lane 2. P.1.B 10^{-5} (1) , Lane 3. P.5.A 10^{-4} , Lane 4. 1 kb DNA marker , Lane 5. P.4.A 10^{-5}, Lane 6. P.1.A 10^{-5} , Lane 7. P.3.A 10^{-}

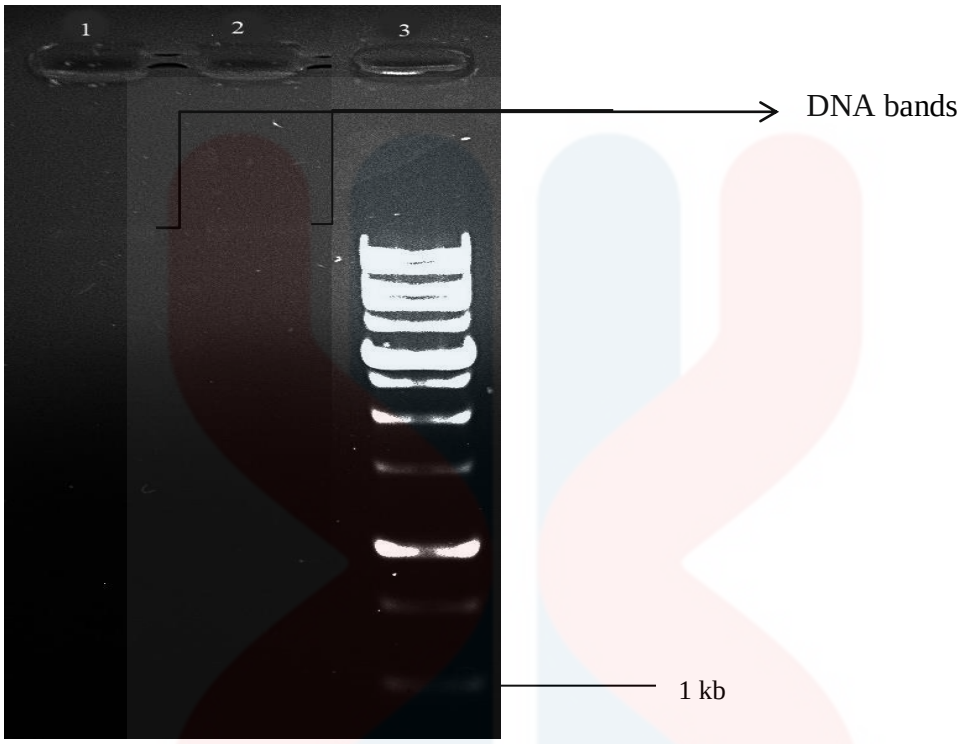


Figure 4.12: DNA extraction bands of two samples based on modified method DNA extraction protocol of He (2011) after gel electrophoresis on 1.0 % agarose gel 80 V for 45 minutes.

Lane 1. P.5.C 10^{-5}, Lane 2. P.5.C 10^{-5}, Lane 3 1 kb DNA marker

4.3.2 Polymerase Chain Reaction (PCR) with microbial DNA for 16S gene amplification

Extracted DNA of one sample is used to perform Polymerase Chain Reaction (PCR) to amplify the copy of DNA product. This is to generate million copies of DNA sequence or contribute to the amplification of DNA sequence. The primer used to perform PCR reactions are universal primers which consist of forward and revere primer. The sequences of the primer are 16S rDNA. Forward: 5'-CGC TGG CGG CGC GTC TTA AA-3' and 16S rDNA Reverse: 5'-TTC ACC GCT ACA CCT GGA A-3 to make nucleotide copies.

The 105 bp PCR marker is used to identify the size of the bands. From Figure 4.4 the result obtained illustrates that multiple band was formed when observed on UV transilluminator. Multiple bands may cause from primer annealing temperature too low, poor primer design, excess primer, contamination with exogenous DNA and premature replication during PCR. According to Newton *et al.* (2004)., multiple bands can be reduced via careful primer design, the amplification of stringent condition and the use of hot-star. Hot start may increase the performance in other application for example multiplex PCR and degenerate multiple bands. Hot starts include the addition of neutralizing monoclonal antibody which specifically binds to *Taq* or N-terminal deletions of *Taq* DNA Polymerase. The antibodies block polymerase action at ambient temperature yet themselves denatured on heating during the first denaturation step of thermal cycling (Newton *et al.*, 2004). Troubleshooting PCR reaction is needed to obtain the desires result (Lorenz, 2012).

Contamination with bacteria DNA during DNA extraction may raise issues of PCR product. Contamination DNA has been accounted for from PCR reagent, kits and water (Newsome *et al.*, 2004), (Salter *et al.*, 2014). Issues of tinting have tormented studies, with example the study of ancient DNA of early human and pathogens (Cooper, 2000). Furthermore, when analyze at different laboratory and facilities, the microbial content of ancient ice core sample has appeared to be inconsistent (Willersiev, Hanse, & Poinar, 2004). With consciousness of regular contamination, cautious gathering off sampling, DNA extraction and PCR reagent it should be conceivable to mitigate the effect of contaminants in microbe studies.

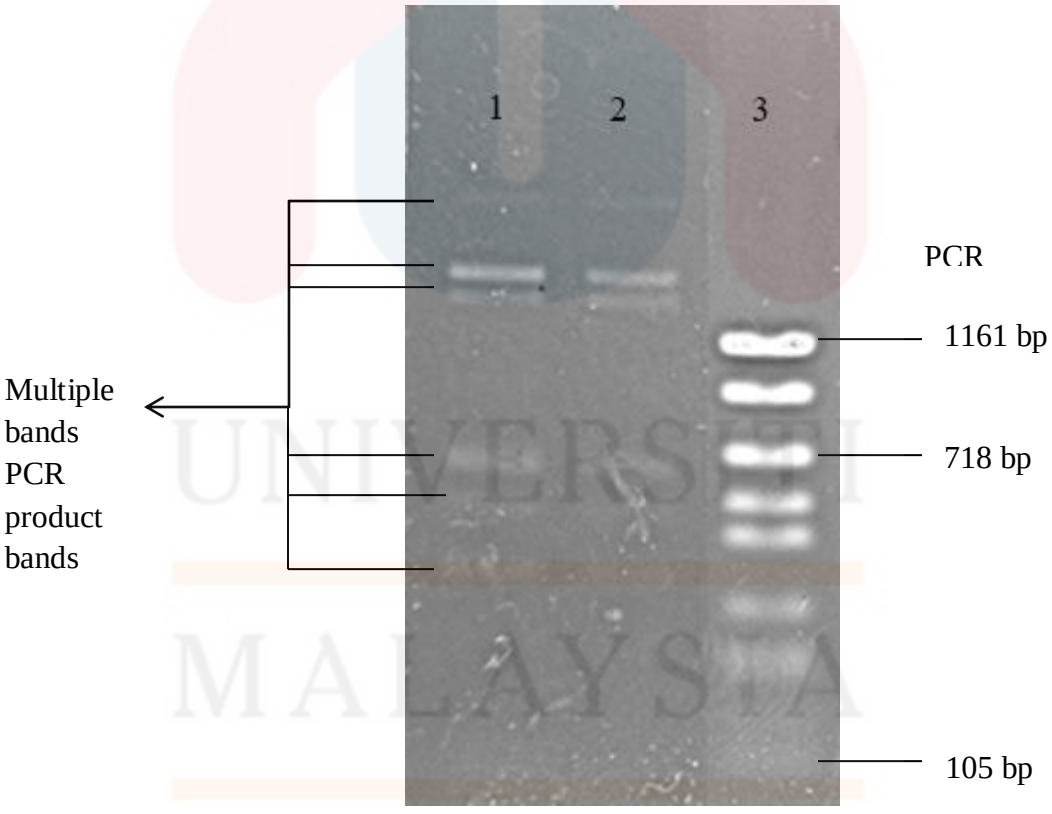


Figure 4.5: PCR product band from sample P.5.C 10^{-5} after gel electrophoresis on 1 % agarose gel at 80 V for 45 minutes

Lane1. P.5.C 10^{-5} ,Lane 2. P.5.C 10^{-5} ,Lane 3. 105 bp PCR Marker

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

From screening of water microbe from upstream Lata Janggut, the staining result shows that the sample were all Gram positive. The shapes of microbes are cocci and bacilli which are dipplobacilli, coccus, bacillus and cocobacillus. A total of seven samples were isolated and identified. A part from that, for molecular analysis the result for PCR 16S amplification, multiple bands was observed on UV transilluminator. Thus, optimization of PCR product is need for further study. The conclusion is, with the physical parameter as mention in section 4.1 of Lata Janggut river water quality obtains is favorable for the growth of Gram positive microbe and optimization of PCR product is needed for further study.

5.2 Recommendation

Culture the water sample that on selective media. Use selective media such as Mac Conkey's agar for Gram negative bacteria or Mannitol salt agar for Gram positive bacteria. Gram negative bacteria can be proves by culture the bacteria again on Mannitol salt agar. The bacteria have been proven to be Gram negative bacteria if the bacteria did not grow. Therefore, for identifying pathogenic bacteria which is Gram negative bacteria can be precise. When performing PCR, number of controls should be consider. The use negative and positive control to check that PCR parameter are suitable and the expected product yell are obtained should be control for set up. Equipment and reagent for use in setting up PCR should be kept as a separate supply from general laboratory equipment.

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