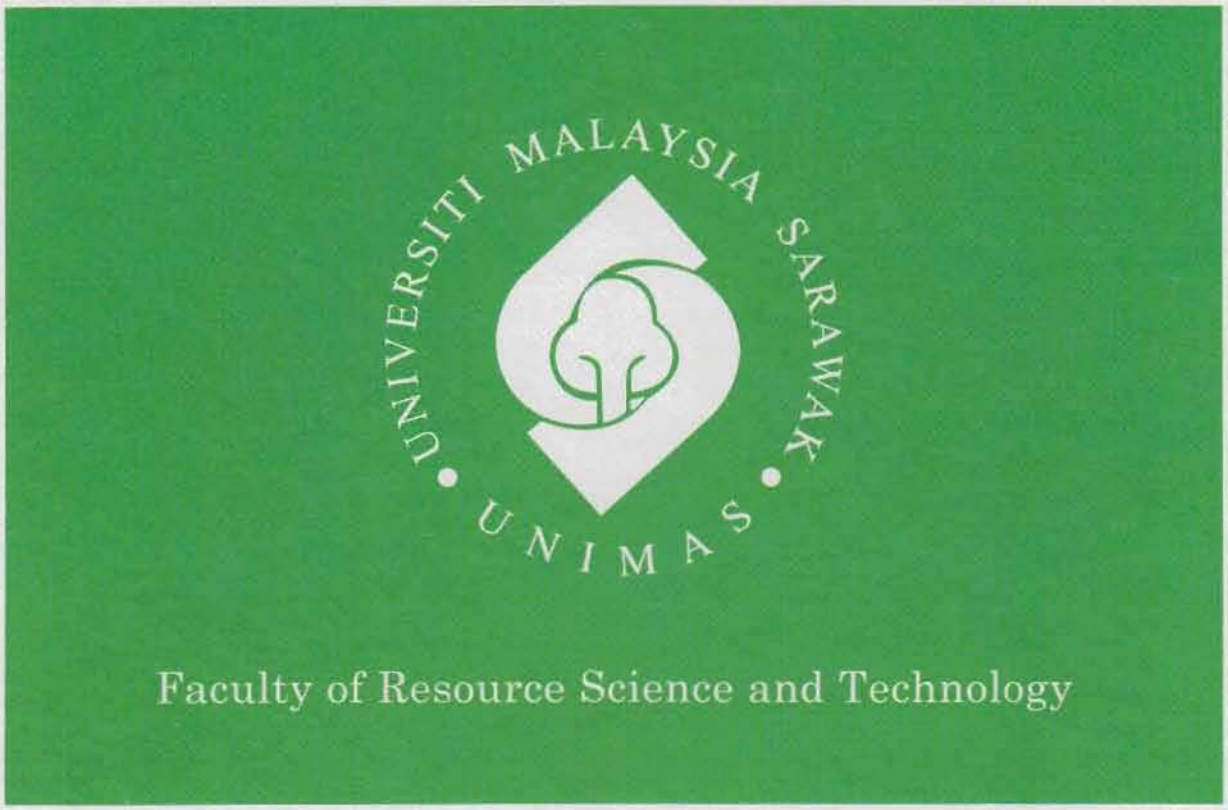

PRESENCE OF *LEPTOSPIRA* SPP IN BATS IN SARAWAK

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(48618)

Bachelor of Science with Honours
(Resource Biotechnology)
2017

Presence of *Leptospira* species in bats in Sarawak

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This project is submitted in partial requirement for degree of Bachelor Science with Honours

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Universiti Malaysia Sarawak

2016/2017

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LIST OF ABBREVIATIONS

°C	-	Degree Celsius
DNA	-	Deoxyribonucleic acid
DNTPs	-	Deoxynucleotide triphosphates
EMJH	-	Ellinghausen-McCullough-Johnson-Haris
EtBr	-	Ethidium Bromide
$MgCl_2$	-	Magnesium chloride
mL	-	Milliliter
µl	-	Microliter
PCR	-	Polymerase chain reaction
MAT	-	Microscopic agglutination test
ELISA	-	Enzyme-Linked Immunosorbent assay
MLEE	-	Multilocus Enzyme Electrophoresis

Presence of *Leptospira* spp. in bats in Sarawak

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ABSTRACT

Leptospira, is a common bacterium found in the environment belongs to the family of *Leptospiraceae* of the *Spirochaetales* order. Animals such as bats, rats, dogs, cattle, pigs, other domestic and wild animals can become a reservoir for the pathogenic *Leptospira* species. Pathogenic *Leptospira* can cause leptospirosis if it infects humans. The increasing scientific study of *Leptospira* has led to the study of the bats as the reservoir of *Leptospira* species. However, not much investigation has been carried out on the presence of *Leptospira* in bats in Sarawak, Malaysia. Thus, the main aim for this study was to detect the presence of pathogenic, saprophytic and intermediate *Leptospira* in bats in Sarawak using Polymerase Chain Reaction (PCR) method using three sets of specifically design primer to target the genes for pathogenic *Leptospira* (*lipL32*), saprophytic *Leptospira* (*rrs*) and intermediate *Leptospira (16S rRNA*). The kidney samples from bats were cultured into modified liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) media with 5-fluorouracil and were incubated at room temperature (28-30 °C) for 3 months. The genomic DNA extraction was conducted using Wizard Genomic DNA purification Kit. A total of 100 bat samples were collected from Gunung Mulu National Park and Gunung Santubong National Park, Sarawak. Intermediate *Leptospira* was detected in 5% (5/100) of bats whereas Saprophytic *Leptospira* was detected in 4% (4/100) of bats. The findings of this study indicate that small mammals such as bats are the reservoirs for *Leptospira* species and are potential vector to emerging disease such as leptospirosis in Sarawak.

Key word: *Leptospira*, leptospirosis, polymerase chain reaction, intermediate, saprophytic

ABSTRAK

Leptospira, bakteria umum yang terdapat dalam alam sekitar tergolong dalam keluarga Leptospiraceae susunan Spirochaetales. Haiwan seperti kelawar, tikus, anjing, lembu, khinzir dan haiwan domestik serta liar boleh menjadi pembawa untuk spesies Leptospira patogenik. Patogenik Leptospira boleh menyebabkan penyakit leptospirosis jika dijangkiti oleh manusia. Peningkatan kajian saintifik terhadap Leptospira telah membawa kepada kajian tentang kelawar sebagai penyimpan Leptospira. Walau bagaimanapun, tidak banyak siasatan yang dijalankan ke atas Leptospira di dalam kelawar di Sarawak, Malaysia. Oleh itu, objektif utama kajian ini adalah untuk mengesan kehadiran patogenik, saprophytic dan perantaraan Leptospira terhadap kelawar di Sarawak mengunakan PCR dan tiga set primer yang mensasarkan gen patogenik Leptospira (lipL32), saprophytic Leptospira (rrs) dan perantaraan Leptospira (16S rRNA). Sampel ginjal dari kelawar telah dikultur dan dimasukkan ke dalam cecair Ellinghausen-McCullough-Johnson-Harris (EMJH) media ditambah dengan 5- Fluoraurasil dan disimpan pada suhu bilik (28-30 °C) selama tiga bulan. Pengekstrakan DNA genomik dilakukan dengan menggunakan Wizard Genomic DNA purification kit. Sebanyak, 100 sampel kelawar telah diambil dari Taman Negara Gunung Mulu dan Gunung Santubong, Sarawak. Perantaraan Leptospira dikesan dalam 5% (5/100) kelawar manakala saprophytic Leptosira dikesan dalam 4% (4/100) kelawar. Hasil kajian ini menunjukan bahawa mamalia kecil seperti kelawar adalah pembawa untuk bakteria Leptospira dan penyebab kepada penyakit seperti leptospirosis di Sarawak.

Kata kunci: Leptospira, Leptospirosis, PCR, patogenik, saprophytic

1.0 INTRODUCTION

Leptospirosis is primarily a zoonotic disease that can be transmitted to human from peridomestic, domestic or wild animals. It is caused by infection with a pathogenic bacteria of the genus *Leptospira* (Levett, 2001). Historically, 100 years ago in Heidelberg, *Leptospira* was first found by Adolf Weil in a renal tubule of a patient thought to have a jaundice fever and in 1886 Adolf Weil had described leptospirosis as a clinical disease (Dutta & Christopher, 2005). Basically, *Leptospira* has a thin, flexible, spiral- shaped, axial flagella, actively motile gram-negative spirochetes with pointed ends like a hook for attachment to the host cell (Safiulah *et al.*, 2009).

According to Mohammed and colleagues (2011), there are over 300 serovars identified and more than 200 of them are pathogenic. There are three categories of *Leptospira* species based on their pathogenicity which are pathogenic, saprophytic and intermediate (Chiriboga *et al.*, 2015). Animals such as rodents, dogs, cats and farm animals are the major contributors to the infection of *Leptospira* species in humans because these animals are the reservoirs for *Leptospira* species. (Mgode *et al.*, 2015). Transmission of *Leptospira* in the environment are through animal urine, soil, water, mucous from infected animals and it will become risk of infection to the human on contact (Levett, 2001).

The first case of leptospirosis that infected human in Malaysia was in 1925 as revealed by Fletcher (El Jalii & Bahaman, 2004). In Malaysia, leptospirosis has been gazetted as a notifiable disease in 2010 under the Prevention and Control of Infectious Diseases Act 1988 (Pui *et al.*, 2015). Sarawak Health Department stated that a total of 616 cases and 24 deaths resulted from leptospirosis had been notified in 2014 whereas 163 cases with six deaths was reported as of 7

March 2015. Statistic from the Health Ministry of Malaysia stated that in 2016 the number of leptospirosis cases were 5,284 and 52 people died from the disease. In Malaysia, leptospirosis has been reported in many natural water spots including waterfalls, ponds and many other contaminated water bodies (Thayaparan *et. al.*, 2011).

Bats have become the hosts for several *Leptospira* species due to the spatial distribution, increase in the bat population and their inter relationship with other animals (Vashi *et al.*, 2009). Other than host specificity, the occurrence of *Leptospira* species inside bats is also due to the food taken by the bats and migration factor (Dietrich *et al.*, 2015). The ability of bats to become reservoir host for many viruses are due to their unique immune system which is influenced by their environment and diet (Calisher *et al.*, 2006). However, the study on the presence of *Leptospira* species in bats is still limited especially in the tropical regions (Dietrich *et al.*, 2015).

Due to increasing deforestation, agricultural activities nowadays had disturbed and changed the habitats of the wildlife. This may cause an increase in contact between wildlife and humans. Several studies show that bats are able to excrete *Leptospira* to the environment through their urine (Dietrich *et al.*, 2015). However, the role played by bats as a potential source of transmission of *Leptospira* to humans is poorly understood. There is not much information about the presence of *Leptospira* species in bats in Sarawak, Malaysia. Thus, this study was conducted to determine the presence of *Leptospira* species in bats.

The objectives of this study are:

1. To detect the presence of *Leptospira* species in bats using modified liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium with 5-fluorouracil.
2. To determine the presence of pathogenic, saprophytic and intermediate *Leptospira* species in bats using Polymerase chain reaction technique targeting *lipL32*, *rrs* and 16S rRNA genes respectively.

2.0 LITERATURE REVIEW

2.1 Classification of *Leptospira*.

Leptospira are spirochaetes which belong to the family of *Leptospiraceae* of the *Spirochaetales* order. *Leptospira* species are categorized according to their serogroups and separated into serovars based on differences of serological features (Mohammed *et al.*, 2011). *Leptospira* are divided into pathogenic, saprophytic and intermediate species. Historically, there are only two species of genus *Leptospira* which are pathogenic *Leptospira interrogans* and non-pathogenic *Leptospira biflexa* (Levett, 2001). Currently, there are 22 species of *Leptospira* have been identify in which ten were pathogenic *Leptospira*, seven were saprophytic and five were intermediate (Dietrich *et al.*, 2015).

Table 1: Genotypic classification of *Leptospira* species (Dietrich *et al.*, 2015).

Pathogenicity	*Leptospira* species
Pathogenic *Leptospira*	*L. interrogans, L. kirschneri, L. borgpetersenii, L. mayottensis, L. santarosai, L. noguchii, L. weilii, L. alexanderi, L. kmetyi, L. astonii*
Saprophytic *Leptospira*	*L. biflexa, L. idonii, L. meyeri, L. terpstrae, L. vanthielii, L. wolbachii L. yanagaweae*
Intermediate *Leptospira*	*L. broomii, L. fainei, L. inadai, L. licerasiae, L. wolffii*

Leptospira species can be identified using several serological methods such as antibodies detection using microscopic agglutination test (MAT), antigen detection using enzyme-linked immunosorbent assay (ELISA) and molecular diagnosis using Polymerase Chain Reaction (PCR) (Ahmad *et al.*, 2005). Recently, an extensive molecular study on genotypic classification of *Leptospira* using DNA hybridization method had revealed 20 species of *Leptospira* (Levett, 2001).

2.2 Morphology of *Leptospira*

Leptospira have a unique shape like a corkscrew with a bent pointed end like a hook. These bacteria have very small dimensions of about 0.1μm in width and 6-20 μm in length. *Leptospira* consists of two internal axial flagella with polar insertion located in the periplasmic space which is responsible for their movement. *Leptospira* can only survive in the presence of oxygen. However, these bacteria do not live in high osmotic pressure and drought environment. The small bodies of *Leptospira* can be observed under dark field microscopy (Mohammed *et al.*, 2011). They consist of a double membrane structure and the lipopolysaccharides (LPS) on the outer surface of *Leptospira* are close together between the cytoplasmic membrane and the peptidoglycan membrane which shows the characteristics of Gram-positive and Gram-negative bacteria (Haake, 2000). *Leptospira* can survive longer and grow well in an optimum temperature of 28-30 °C and are able to produce enzyme catalase and oxidase (Levett, 2001).

2.3 Epidemiology of *Leptospira.*

Leptospirosis is an emerging zoonotic infectious disease that is transmitted from infected animals to humans (Lim *et al.*, 2011). Leptospirosis is recognized as the important emerging worldwide disease in Southeast Asia due to warm and humid condition with high precipitation rate which have met the survival requirements of *Leptospira* (El Jalii & Bahaman, 2004). Leptospirosis

is a common infectious disease which occurs worldwide and is caused by the infection of pathogenic *Leptospira* species (Levett, 2001). *Leptospira* are able to live longer in a warm and humid environment which make it suitable to survive in a tropical country like Malaysia. Leptospirosis can be transmitted through wild and domestic animals such as dogs, rodents, cats, and farm animals (Levett. 2001). It is estimated that the ranges of leptospirosis in Malaysia is from two to five per 100,000 populations. However, the number of people infected by pathogenic *Leptospira* increased significantly in recent years (Lim *et al.*, 2011).

2.4 Transmission of *Leptospira.*

Leptospira can be transmitted through environment such as water, soil, direct or indirect contact of broken skin between infectious organisms to humans (Dutta & Christopher, 2005). *Leptospira* can also enter the human body by drinking contaminated water and through contact with the urine of infected animals or other body fluids from infected animals (Leptospirosis infection, 2015). Exposure of human to the environment or an individual that works at risk such as a farmer, scientist, animal shelter's worker, veterinarian or any other worker who is involved in handling a carrier animal has potential to be infected with leptospirosis (Haake & Levett, 2015).

Previous studies have shown the presence of pathogenic *Leptospira* species from a water sample in the recreational area in Terengganu, Malaysia (Ismail *et al.*, 2014). Another study on the isolation of the pathogenic *Leptospira* species of *Leptospira interrogans* and *Leptospira borgpetersenii* from the urban rat populations of Kuala Lumpur, Malaysia had shown a positive result which has increased the concern of public health risk because rats can transmit leptospirosis (Benacer *et al.*, 2013). Leptospirosis cases may also increase during flooding. For instance, the leptospirosis cases in Kelantan increased from 20 cases to 31 cases during the monsoon season (Azali *et al.*, 2016).

2.5 Clinical manifestation

It is important to understand the effect of Leptospirosis disease in humans. Leptospirosis can cause either an acute febrile illness or a more severe illness if it infects the organs such as liver, kidney, lungs, brain and heart (Cafasso, 2016). There are two different clinical syndromes in which 90% of patients have anicteric leptospirosis which presents influenza-like symptoms and 10% suffered from icteric leptospirosis which is more severe as Weil's disease (Dutta & Christopher, 2005). Anicteric leptospirosis is a mild form of disease in which it may cause an acute febrile illness such as chills, headache, high fever, myalgia, skin rash, nausea, vomiting, conjunctival suffusion and prostration (Dutta & Christopher, 2005).

However, many of these symptoms can be mistaken as other disease and some infected people may not have any symptoms (Leptospirosis infection, 2015). Leptospirosis may occur in two phases which are the septicemic phase and immune phase and it may depend on the patient's immune system (Haake & Levett, 2015). Septicemic phase is the first stage where the patients may experience a febrile illness which lasts for about 5 to 7 days. The second stage is the immune phase where the illness lasts from 4 to 30 days and it is more severe with jaundice, renal impairment, cardiac arrhythmias, pulmonary symptoms, aseptic meningitis, and many more other severe diseases. A patient can get both; that is the septicemic phase and followed by immune phase or the patient may only show symptoms of the second phase of illness (Haake & Levett, 2015).

2.6 Leptospirosis outbreak in Malaysia

In Malaysia, the first leptospirosis case was revealed by Fletcher in 1925 (Lim *et al.*, 2011). In 1984, 16 British cave explorer Gunung Mulu National Park, Sarawak were reported ill due to the leptospirosis (Benancer *et.al.*, 2004). Leptospirosis has been gazetted as a notifiable disease in

2010 under the Prevention and Control of Infectious Diseases Act 1988 (Pui *et al.*, 2015). Sarawak Health Department stated that a total of 616 cases and 24 deaths resulted from leptospirosis had been notified in 2014 whereas 163 cases with six deaths was reported as of 7 March 2015. According to the Health Ministry of Malaysia statistic, the number of leptospirosis cases in 2016 were 5,284 and 52 people died from the disease.

2.7 Relationship between bats and *Leptospira*.

Bats are the only mammals that can fly. They belong to the order *Chiroptera* and can be found abundantly and geographically widespread (Calisher *et al.*, 2006). The emerging scientific study on the bats as reservoir for many pathogenic species has led to the emergence of investigation on the presence of *Leptospira* in wild bats for the last few years (Dietrich *et al.*, 2015). The factor that cause bats to become a host for many viruses and agents for disease are the migration of bats, colony density, diet, roosting behavior, daily movement patterns, ability to echolocate and virus susceptibility (Calisher *et al.*, 2006). A previous study on Serological and molecular detection of *Leptospira* spp. from small wild mammals captured in Sarawak, Malaysia found that the antibodies to different serovars of *Leptospires* was detected in bats (40%; 95% CI 28.5-52.4) (Thayarapan *et. al.*, 2014).

Due to increasing deforestation, agricultural activities nowadays had disturbed and changed the habitats of the wildlife. This may cause an increase in contact between wildlife and humans. Several studies show that bats are able to excrete *Leptospira* species to the environment through their urine (Dietrich *et al.*, 2015). A previous study on the diversity of bat associated with *Leptospira* in the Peruvian Amazon showed that 3.4% of 559 bats kidneys were positive for *Leptospira* (Matthias *et al.*, 2008).

Leptospira usually infected the renal tubules of bats and were excreted through their urine which eventually contaminated the soil and water. *Leptospira* infection has been found in more than 50 bats species belonging to 8 of the 9 species investigated bats families, encompassing various geographical regions in the tropic and subtropics as well as Europe (Dietrich *et al.*, 2015). Previous studies on the molecular epidemiology of pathogenic *Leptospira* species in the straw-colored fruit bats in Zambia showed positive results, where 79 out of 529 kidneys sample of bats were infected with pathogenic *Leptospira* species (Ogawa *et al.,* 2015). Based on the result, it showed that *Leptospira* have evolved and adapted well in the bat population since the investigation had also proved the *rrs* fragments in bats are genetically related to each other without regional variation (Ogawa *et al.*, 2015).

2.8 Polymerase chain reaction (PCR)

Polymerase chain reaction is a scientific technique used to synthesize and amplify the new strand of DNA complementary to the present DNA sequence which can produce million copies of DNA in a short time. Historically, the PCR was first developed by Kary Mullis and colleagues in the early 1980's (Polymerase Chain Reaction, 2011). Nowadays, PCR is an advanced technology in molecular biology because it is cheap, faster and simple (Joshi & J.D, 2010). There are many different techniques which can be used to detect *Leptospira* species. These include dark-field microscopy, serology by Microscopic Agglutination Test (MAT), PCR detection, and bacterial culture (Dietrich *et al.*, 2015). However, PCR detection gives a better diagnostic value in molecular biology as compared to microscopic agglutination test (MAT) especially in detecting the presence of *Leptospira* DNA at the early stage of leptospirosis before the immunoglobulin M (IgM) appeared in the serum (Levett & Haake, 2010).

A study on the comparison between culture and PCR technique proved that PCR gives a fast and accurate identification of the *Leptospira* species but the culture technique is not suitable due to the fact that pathogenic *Leptospira* are a slow grower. This can be proven from the results shown in detection of *Leptospira* using PCR technique, 92 strains of *Leptospira* (89.32 %) were identified whereas only 55 strains of *Leptospira* (53.39 %) were identified using the culture technique (Yasouri & Ghane, 2014).

3.0 MATERIALS AND METHODS

3.1 Study Site

The sampling areas were at Santubong National Park and Gunung Mulu National Park, Sarawak. The sampling date at Santubong National Park was from 24th October 2016 to 30th October 2016 and the sampling date at Gunung Mulu National Park was from 6th February 2017 to 13th February 2017. The Gunung Mulu National Park and Santubong National Park were chosen because they are protected rainforest in Malaysian Borneo which have become tourist attraction spots. The choice of the study sites was based on the leptospirosis outbreak, environmental exposure due to human activities, sustainability of the habitat for bats breeding and its possibility of transmitting the disease. A total 100 bats were collected from Santubong National Park and Gunung Mulu National Park. A total of 25 bats were collected from Gunung Santubong National Park and the kidney tissue samples were collected from both left and right sides of the kidneys. Therefore, the overall number of kidney tissue samples collected from bats at Gunung Santubong National Park were 50 samples. A total of 50 bats were collected from the Gunung Mulu National Park.

3.2 Bats Trapping and Species Identification

All bats were captured using mist nets and harp traps around the roosting area of bats. The bats were caught around the trail of Paku Valley Loop, Botany Loop, Paku Waterfall trail, Deer cave and Kenyalang Trail at the Gunung Mulu National Park. The bats were caught around the track 1 and track 2 in the Gunung Santubong National Park. The mist nets and harp traps were placed along the bats flying path such as across animal walking trails, over the stream, adjacent to fruiting trees and at the mouth of caves.

First of all, the sampling area were observed. The number of bats were counted once the bats leave during the sunset. This is to estimate the roost size. (Epstein *et al.*, 2013). Then the trap was set up and waited until all the bats returned to the roost to collect the caught bats. The bats that were caught in the nets were removed carefully to avoid injury to the bats' wings and then placed into the cloth bags. The sample collected were further identified based on phenotypic characteristic and morphological measurement.

3.2 Bats Samples Collection.

The bats were euthanized humanely by placing them into a zip lock bag containing cotton wool soaked with chloroform. Then the bats were dissected using dissecting kit to collect the kidney samples for culturing and molecular analysis. The kidneys were chopped into small pieces using sterile blade (Thayaparan *et. al.*, 2011). A small piece of tissue was inoculated into liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium modified by Johnson and Haris with 100 µg/ml 5-fluorouracil to reduce contaminants from other bacteria. The EMJH media were incubated aerobically in the dark at room temperature (28- 30 °C) for about 3 months. The culture was examined every month for the presence of *Leptospira* using Polymerase chain reaction (PCR).

3.2.1 Preparation of EMJH medium

One liter liquid EMJH medium was prepared using 2.3 g EMJH base, 100 ml enrichment broth, 0.1 g 5- fluorouracil and 900 ml distilled water. First, the mixture of EMJH base and distilled water was autoclaved and cooled to room temperature. Then 100 ml of enrichment broth was added into the autoclaved liquid medium inside the laminar floor hood. Lastly, a 10 ml each liquid EMJH medium were transferred into the 15 ml falcon tube.

3.3 Genomic DNA extraction for PCR analysis

Genomic DNA from all samples was extracted using Wizard Genomic DNA Purification Kit (Promega, Wisconsin, USA) according to the manufacturer's protocol (Benacer *et. al.*, 2013). Briefly, 1 ml of overnight bacterial culture was added into a 1.5 ml microcentrifuge tube. Then the microcentrifuge tube was centrifuged at 10,700 rpm for 5 minutes to get the pellet and remove the supernatant. After that, 600 µl of Nucleic Lysis solution was added into the tube and vortexed. To lyse the cell, the mixture was incubated at 80 °C for 5 minutes and then cooled to room temperature. Then, 3 µl of RNase solution was added and the tube was inverted 2-5 times to mix the solution. Again, the sample was incubated at 37 °C for 30 minutes. After incubation, the tube was chilled on ice for 5 minutes. Then, 200 µl of Protein Precipitation solution were added and vortexed at 20 seconds. The sample was incubated again on ice for 5 minutes. Then the sample was centrifuged again at 10,700 rpm for 3 minutes. After that, 600 µl supernatant that contains DNA was transferred into a 1.5 ml microcentrifuge tube contained 600 µl of room temperature isopropanol. Then the sample was inverted gently until the thread-like strands of DNA is formed. Again, the sample was centrifuged at 10,700 rpm for 2 minutes and the supernatant was discarded. Then the pellet was washed by adding 600 µl of 70% ethanol. After that, the tube was centrifuged again at 10,700 rpm for 2 minutes. The supernatant was discarded and drained on a clean absorbent paper and the pellet was allowed to air dry for 10-15 minutes. Lastly, 100 µl of DNA rehydration solution was added into the tube. The DNA template was used for molecular analysis by using PCR.