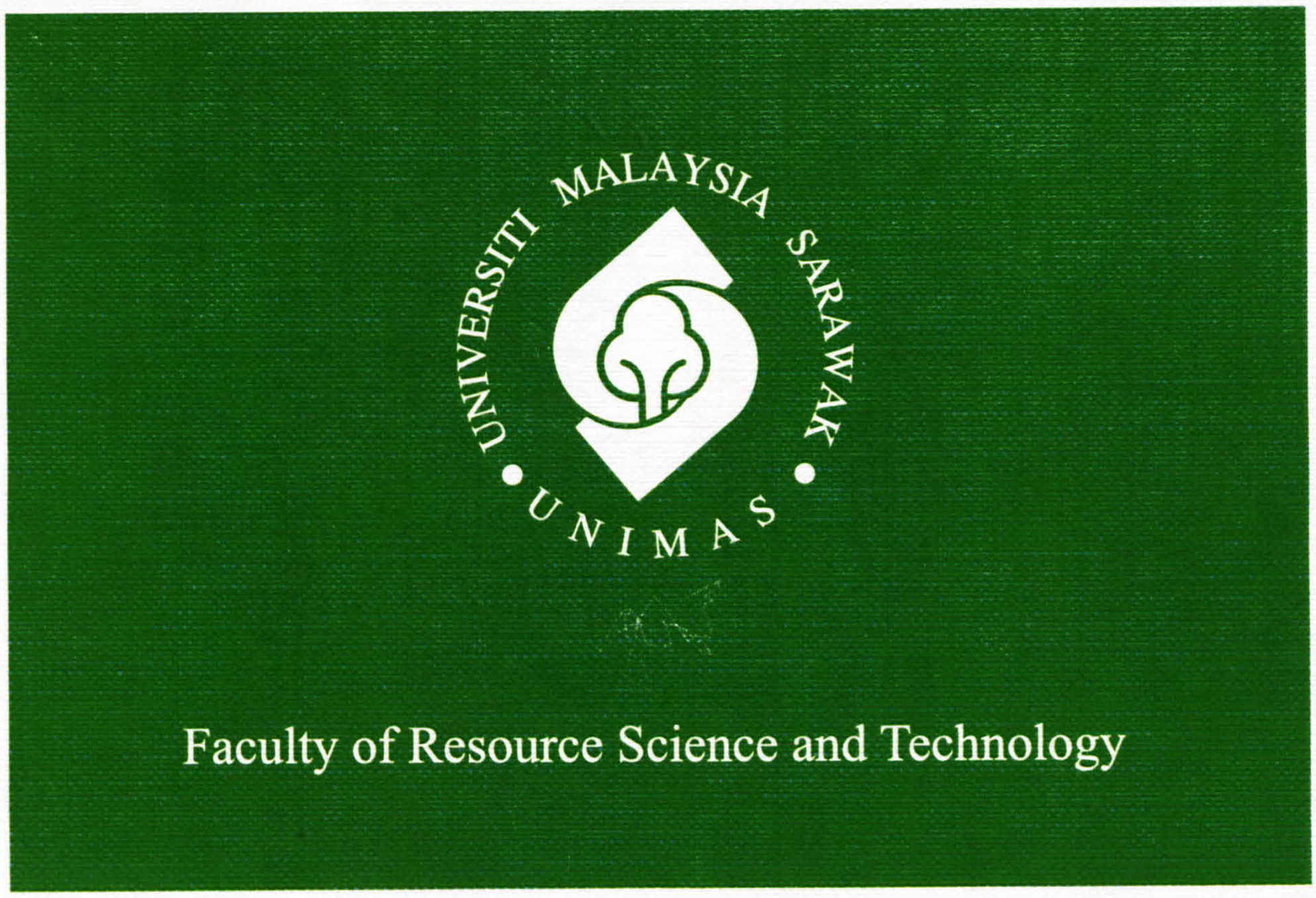

Biofilm Formation and Time Course Study on Growth of Saprophytic *Leptospira*

Alia Diyana Binti Mohamed Hassim

(40486)

Bachelor of Science with Honours
(Resource Biotechnology)
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I would like to thank Allah for the wisdom and strength the He given me in the completion of this project.

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(Name of the supervisor)
Dr Kasing Apun
Professor
Department of Molecular Biology
Faculty of Resource Science & Technology
UNIVERSITI MALAYSIA SARAWAK
94300 Kota Samarahan

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Table of Contents

Acknowledgement I

Declaration II

Table of Contents IV

List of Abbreviations VI

List of Tables and Figures VII

Abstract 1

1.0 Introduction 2

2.0 Literature Review 4

2.1 Discovery of Biofilm 4

2.2 Formation of Biofilm by *Leptospira* 4

2.3 Role of Biofilm in *Leptospira* sp. 5

2.4 *Leptospira* sp. 7

2.4.1 Comparison between Saprophytic and Pathogenic *Leptospira*.7

2.4.2 Features of *Leptospira* 8

2.4.3 Pathogenesis of *Leptospira* 8

2.4.4 Transmission of Leptospirosis 9

3.0 Materials and Method 10

3.1 Bacterial Strains and Growth Condition 10

3.2 Dark field Microscope View 11

3.3 Biofilm formation in 24-well plates 11

3.4 Crystal violet staining 11

3.5 Phase-contrast Microscopy View 12

3.6 Data analysis 12

4.0 Result and Discussions 13

4.1 Dark field Microscopy View 13

4.2 Initial OD reading of the culture 14

4.3 Colour intensity of crystal violet staining..... 14

4.4 Quantification of biofilm 17

4.5 Phase-contrast light microscopy visualization 22

4.6 Data correlation and clinical implications 27

5.0 Conclusion 29

6.0 References 30

List of Abbreviations

nm	-	nanometer
µm	-	micrometer
µL	-	microliter
mL	-	milliliter
CFU/mL	-	Colonies Forming Unit per milliliter
g	-	gram
°C	-	degree Celsius
%	-	percentage
w/v	-	weight per volume
EPS	-	Extracellular Polymer Substances
EMJH	-	Ellison McCullough Johnson Harris
OD	-	Optical Density
PBS	-	Phosphate Buffer Saline

List of Tables

Table		Page
Table 1	- *Leptospira* strains used in formation of biofilm from different sources	10
Table 2	- Optical density reading at OD_{420} for different strains of saprophytic leptospires	14
Table 3	- Observation of attachment at different wells for every isolates of *Leptospira* that show increasing colour intensity	16
Table 4	- Optical density reading at OD_{600} for different strains of saprophytic leptospires	18

List of Figures

Figure		Page
Figure 1	- Image of leptospires from dark field microscope view	13
Figure 2	- Biofilm formation for 11 days by different isolates	18
Figure 3	- Quantification of biofilm formation for different isolates at 600 nm	19
Figure 4	- Visualization of biofilm under phase-contrast light microscope of CFS 12	24
Figure 5	- Visualization of biofilm under phase-contrast light microscope of CFS 34	25
Figure 6	- Visualization of biofilm under phase-contrast light microscope of *Leptospira biflexa* serovar Patoc	26

Biofilm formation and time course study on growth of saprophytic *Leptospira*

Alia Diyana Binti Mohamed Hassim (40486)

Bachelor of Science with Honours
Resource Biotechnology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

Leptospirosis is a zoonotic disease carried by animals especially rodent as the host caused by *Leptospira* that can be transmitted through contaminated environmental surface water. Leptospires exist as saprophytic organisms that are aquatic or as pathogen that are able to survive in water and other sources such as soil, and rat. The main aim for this study is to determine the relationship between contact time and biofilm formation of saprophytic *Leptospira*. A total of six saprophytic *Leptospira* strains were used in this study. Quantification of biofilm was done by crystal violet assay in 24-well plates for every 24 hour based on the value of optical density at 600 nm. Saprophytic leptospires form biofilm from day 3 until day 5 with 1.916 as the highest optical density and correlate with colour intensity at the bottom of the well plates. Visualization of biofilm by phase-contrast microscopy of two representative saprophytic strain show a relationship with the data obtained and colour intensity of stained well plates. The current knowledge from this study helps for better understanding on contact time of biofilm formation by saprophytic *Leptospira*.

Keywords: *Leptospira*, contact time, biofilm formation, crystal violet assay

ABSTRAK

Leptospirosis adalah sejenis penyakit bawaan binatang yang dibawa oleh haiwan terutamanya roden sebagai hos kepada Leptospira yang disebarkan melalui permukaan air yang dicemari oleh bakteria tersebut. Leptospira wujud sebagai organisma saprofitik yang merupakan bakteria akuatik atau sebagai patogen yang mampu hidup di dalam air dan sumber-sumber lain seperti tanah dan tikus. Tujuan utama kajian ini adalah untuk menentukan hubungan antara masa sentuhan dan pembentukan biofilm daripada saprofitik Leptospira. Sebanyak enam jenis Leptospira saprofitik telah digunakan dalam kajian ini. Kuantifikasi biofilm dilakukan melalui protokol ungu kristal untuk setiap 24 jam di dalam 24-plat berlubang berdasarkan nilai ketumpatan optik pada 600 nm. Saprofitik Leptospira mula membentuk biofilm dari hari 3 hingga 5 hari dengan 1.916 sebagai ketumpatan optik tertinggi dan berkaitan dengan keamatan warna di bahagian bawah plat titer. Pengimejan biofilm menggunakan mikroskop fasa-kontrast yang diwakili oleh dua jenis Leptospira saprofitik mempunyai hubungkait dengan data yang diperolehi dan keaamatan warna di dalam lubang plat yang diwarnai. Pengetahuan semasa daripada kajian ini dapat membantu untuk pemahaman yang lebih baik pada masa sentuhan pembentukan biofilm oleh saprofitik Leptospira .

Kata kunci: *Leptospira, masa sentuhan, pembentukan biofilm, protokol ungu kristal*

1.0 Introduction

Leptospirosis is a zoonotic disease spread by infected animals such as mice. It is a re-emerging disease that is widely known caused by pathogenic *Leptospira*. It can be transmitted from infected animal by urine excretion onto the water and soil surfaces (Wójcik-Fatla, 2014). In East Asia, leptospirosis was associated with the people living in regions often flooded after typhoons while common outbreaks of leptospirosis occur in developing countries, when people life with poor urban sanitation, in which they had close contact with the infected rats (Ratet *et al.*, 2014).

The recent outbreak of Leptospirosis in Malaysia was caused by the pathogenic strain of *Leptospira* spp. that can be transmitted through contaminated environmental surface water (Lim *et al.*, 2011). This disease is still spreading when Sarawak Health Department (2015) reported 3 probable cases from Kuching for leptospirosis on 15th to 21st February 2015. Relationship of saprophytic and pathogenic leptospires causes the scientists to characterize the biofilm development by these microorganisms (Ristow *et al.*, 2008).

Formation of biofilm commonly made by the bacteria growing on the surfaces describes the complex adaptation which protects them from the environmental stress (de la Fuente-Núñez *et al.*, 2012). Deb *et al.* (2014) described that the biofilm form when microorganisms attach to a surface and develop as it medium to survive which contain polysaccharide matrix with organic and inorganic substances in it. Saprophytic *Leptospira* exists as aquatic or pathogens that can survive in water and soil that do not cause disease (Benacer *et al.*, 2013).

Formation of biofilm by the spirochaetes can be classified into four stages which are attachment of bacteria, microcolony formation, maturation and dispersion (Crouzet *et*

al., 2014). These stages are essential for course time study to gain knowledge on contact time especially on attachment of the leptospires on a surface. Besides that, duration taken by saprophytic *Leptospira* to form biofilm is also important to fully understand the level of pathogenicity. This study is interesting because saprophytic *Leptospira* has relationship with water, soil and mouse sources. The relationship helps them to survive in the diverse environmental habitats by producing biofilm.

Research problems for this study are:

i. How do the contact time between biofilm and the surfaces affect the level of pathogenicity of *Leptospira* spp.?

ii. What is the duration for biofilm formation by saprophytic *Leptospira* spp.?

The objectives for this study are:

i. To determine the ability of biofilm formation by saprophytic *Leptospira* spp.

ii. To determine the relationship between contact time and biofilm formation of saprophytic *Leptospira* spp.

2.0 Literature Review

2.1 Discovery of biofilm

Biofilm formation can only be made by certain species of microorganisms. Donlan (2002) defined biofilm as "assemblage of microbial cells that is irreversibly associated that cannot be removed by gently rinsing with a surface and enclosed in a matrix of primarily associated". The history began with Antonny van Leeuwenhoek who observed the aggregated microorganism in 'scurf of teeth' and particles from his tongue under microscope followed by Louis Pasteur that saw aggregates of bacteria in wine that leads to pasteurization (Høiby, 2014).

Heukelekian and Heller also discovered that microorganism needs a surface to attach through their observation on the marine microorganism (Heukelekian & Heller, 1940). In the last 40 years, the term 'biofilm' was coined by Costerton in 1985 into medicine and the term 'film' itself refers to adhesion and aggregation of bacteria (Shi & Zhou, 2011).

2.2 Formation of biofilm by *Leptospira*

The ability to form biofilm is important for *Leptospira* spp. as it helps them to survive in their environmental habitats especially inside their host (Ristow *et al.*, 2008). Formation of biofilm can be classified into four stages: attachment of bacteria to surface, the formation of microcolony, maturation of biofilm and dispersal (Bogino *et al.*, 2013). Attachment of gram-negative bacteria such as *Leptospira* must recognize the optimum environmental condition in order for it to attach on a surface. It is different for every organism. *P. flourescens* can attach to the surface under any condition while *E. coli* need amino acid to grow on the surface (O'Toole *et al.*, 2000). After attachment stage, the bacteria will start to grow and divide forming thin layers of biofilm that allow them to

signal each other for cell communication. National Institute of Health (2011) reported that they will also secrete extracellular polymeric substrate (EPS) which consists of DNA, protein, lipid and lipopolysaccharides that will aid the adhesion between cell and the surfaces and developed the microcolonies that act as physical barrier. Beloin *et al.* (2008) also described that the maturation begins when microcolonies form into three-dimension structure as cell replicates and EPS will accumulate. EPS will glue the cell to prevent them from mechanical stress. In the end, some of the cells will detach and disperse from the biofilm and eventually form biofilm in another niche nearby.

Formation of biofilm by other bacteria such as Staphylococci (especially *S. epidermidis*) can lead to device-related infection (Otto, 2008). Implantation of the mechanical heart valve causes circulating platelets, tissue damage and fibrin tend to accumulate where the valve has been attached which have the greater tendency of microorganism colonization (Donlan, 2001). Stoodly-Hall *et al.* (2004) also described that biofilm on the medical device is a strategy of microorganism that helps in the ability to cause infection.

2.3 Role of biofilm formation in *Leptospira* spp.

According to Cahndki *et al.* (2011), biofilm forms on any virtually surfaces that immerse in the natural aqueous environment, involve in the primitive circulatory system, and quorum sensing. An optimum environment like aqueous environment is normal for the microorganism to live that can help in transfer and exchange of products. Characteristics of the aqueous medium such as pH, nutrient levels, ionic strength, and temperature play important function for the bacteria to attach to biofilm (Kokare *et al.*, 2008). The aqueous optimum condition gives *Leptospira* the ability to survive in the environment (Ristow *et al.*, 2008). Kokare *et al.* (2008) also discovered that biofilm attachment varies during the

season due to different temperature and sometimes increase in several cations. The primitive circulatory system helps in facilitating the processing and uptake of nutrients, cross-feeding and also removing the harmful metabolic product by transferring them through water channels between colonies (Chandki *et al.*, 2011).

Cellular communications between microorganisms are regulated by extracellular molecules. This communication involve accumulation of signaling molecules in the medium which enables the bacteria to estimate the total amount of bacteria that exactly define the critical cell density of bacteria, and concentration of autoinductors in the medium to reach the target level for activation of specific genes. This occurrence is known as quorum sensing (Marić & Vraneś, 2007). For pathogenic *Leptospira* spp., quorum sensing helps to minimize immune response from host by postpone the production of virulence factors that can cause damage to the cell until enough mass of bacteria that can overcome host defense mechanism and initiate infection (Deep *et al.*, 2011).

Another crucial role is decreasing of antimicrobial susceptibility in two aspects which are tolerance and resistance (Bjarnsholt, 2013). Biofilms of bacteria is a complex membrane which is dense and semi-impermeable that resists the penetration of antibiotic and tolerates the existence of antibiotic by reducing their growth rate (Hung & Henderson, 2009). The antibiotic resistance will help the *Leptospira* spp. to control their survival and disease transmission (Deb *et al.*, 2014).

There are genes in *Leptospira* that contribute in production of exopolysaccharides that encodes genes such as glycosyltransferases, alginate biosynthesis, and lipopolysaccharide transport systems which helps in colonization in biotic and abiotic surfaces by forming biofilm (Picardeau *et al.*, 2008). O-acetylation of alginate (*AlgI* genes)

of *P. aeruginosa* leads to the formation of biofilm and can be found in *Leptospira* genome (Franklin *et al.*, 2004). Nascimento *et al.* (2004) described that this gene may contribute in finding the genes that responsible in the formation of biofilm in *Leptospira.*

2.4 *Leptospira* sp.

Leptospira sp. is a type of bacteria which classified under spirochaetes phylum (Cinco, 2010). It belongs to Leptospiricae family and which has two genera, *Leptospira* and *Leptonema* (Sthayaparn *et al*, 2013). This type of bacteria is a human pathogen and zoonosis bacteria. *Leptospira* members can be divided into a three types which are saprophytic, intermediate and pathogen (Ristow *et al.*, 2008; Rettinger *et al.*, 2012). An example of saprophytic *Leptospira* is *L. biflexa*, while intermediate *Leptospira* is *L. broomi*, and pathogenic *Leptospira* which is *L. interrogans* (Voronina *et al.*, 2014).

The pathogenic strain of this type of bacteria can cause a re-emerging disease called leptospirosis which is widely known as severe multisystem disease and can cause sudden death if prolonged. Leptospirosis was visualized from a patient who diagnosed from yellow fever through autopsy and did not isolate by the scientist although few years past that occurred in Germany and Japan (Levett & Haake, 2015). Unsure diagnostic of this disease and yellow fever continues and causes scientist such as Stokes and Nuchi dying in order to discover the "causative agent" as discovered by Levett and Haake (2015).

2.4.1 Comparison between saprophytic and pathogenic *Leptospira*

Differences in saprophytic and pathogenic lead to different behavior. *L. interrogans* genome is larger which indicates that more genetic information for survival both within mammalian hosts and aquatic environment, whereas for saprophytic *L. biflexa* is restricted to aquatic and mammalian host environments (Picardeau *et al.*, 2008).

Their ability to survive may help them to form the biofilm on the surface and they are related to waterborne disease. Johnson and Harris (1967) described that saprophytic can grow below the minimal temperature growth of pathogenic *Leptospira*. Thus, different temperature growth causes saprophytic leptospires are more abundant in the environment than pathogenic leptospires because pathogenic species can only live under certain conditions (Ismail *et al.*, 2014).

2.4.2 Features of *Leptospira*

Leptospira is an aerobic helical bacteria that has 0.1 μm diameter and 6.0 to 12.0 μm long (Koneman, 2006). It is visualized by dark field microscopy. It also can be seen as slender, flexuous shape and numerous numbers of primary coils under the microscope (Ren *et al.*, 2003). *Leptospira* has thin characteristic until it can fit through a "membrane filter with a pore size of 0.22 μm" and helicoidal protoplasmic cylinder surrounded by a cell wall that consist of a cytoplasmic-peptidoglycan complex that being enclosed with an outer envelope of at least three layers (Greenwood *et al.*, 1997). Levett and Haake (2015) also discovered that they consisted of two axial flagella lying under the membrane sheath.

2.4.3 Pathogenesis of *Leptospira*

The pathogenic strain of *Leptospira* causes the recent outbreak of leptospirosis. It can be transmitted directly or indirectly from domestic animals to human. Clinical manifestations of this infectious disease had a broad range from mild flu-like illness to hemorrhagic manifestations and multi-organ failure (Disease Control Division MOH, 2011). Levett and Haake (2015) reported that this type of bacteria can enter human body by cuts, mucous membrane, and aerosol inhalation of microscopic droplets and also contaminated lake water as it had been issued at the 1998 Springfield Illinois Trialthon.

Edre *et al.* (2015) reported 29 confirmed cases in 2014 and 22 confirmed cases in 2015 from Kuantan, Pahang that usually exposed to flood because of monsoon season. Universiti Malaysia Sarawak (UNIMAS) and Sarawak Health Department researchers had done some research in Rejang Basin area which lead them to discovery of 30.6 % of samples was positive of leptospirosis associated with farming activities and water activities (Sthayaparn *et al.*, 2013). These cases proved that leptospirosis is a waterborne disease that can easily infect the human.

2.4.4 Transmission of Leptospirosis

There are many factors that contribute in the transmission of leptospirosis. Flooding recorded the highest causes due to water contamination by animal urine. Drainage congestion causes the flood to hit burrows of animal and contaminated the water. Animal and human interface involved when people exposed to occupational related or recreational places to the environment that favour Leptospirosis had the high risk to be infected. Lim *et al.* (2011) reported that in July 2011, 8 peoples were dead after involved in a search and rescue of a drowning victim in a recreational park at eastern Pahang due to leptospirosis infection and other water-borne disease.

Human host risk factor involves a situation where the population did not expose to the bacterial infection or having chronic or open wounds on the skin (Disease Control Division MOH, 2011). As water being a reservoir for pathogenic *Leptospira*, open wound such as cuts on human body can be habitats for the *Leptospira* to form biofilm and incubate inside human body when James and his colleagues succeed to isolate infectious bacteria from open wound (Hung & Henderson, 2009).

3.0 Materials and Methods

3.1 Bacterial strains and growth condition

Saprophytic *Leptospira* strains were obtained from Microbiology Laboratory from Department of Molecular Biology, Faculty Resource Science and Technology, Universiti Malaysia Sarawak. Six isolates of saprophytic *Leptospira* spp. from rat kidney, soil and water were used (Table 1). This method was based on Ristow *et al.* (2008). It was suspended in 10 mL of EMJH (Ellison McCullough Johnson Harris) liquid medium. These cultures were resuscitated by growing in EMJH which contain 0.1 g of 5-flurouracil and enrichment media at room temperature. Final concentration of the culture was 10^6 to 10^8 CFU/mL as mentioned by Ristow *et al.* (2008). All cultures will be incubated at 30°C for 30 days.

Table 1. *Leptospira* strains used in formation of biofilm from different sources

***Leptospira* lab no. designation**	**Isolates strain**	**Source**
CFS 4	*Leptospira meyeri* strain Semeranga_DB49	Soil
CFS 12	*Leptospira meyeri* strain Semeranga_DB49	Water
CFS 16	*Leptospira meyeri* strain 19CAP	Rat kidney
CFS 20	*Leptospira meyeri* strain 19CAP	Water
CFS 21	*Leptospira meyeri* strain 19CAP	Soil
CFS 34	*Leptospira meyeri* strain 19CAP	Rat kidney
Positive control	*Leptospira biflexa* serovar Patoc	

3.2 Dark field microscope view

All of the isolates were pipetted out and transferred on glass slides after 30 days incubation. They were viewed under dark field microscope, Olympus Compound Microscope BX51 (Olympus Corporation, Japan) under 40x magnification at Faculty of Medicine and Health Sciences, UNIMAS.

3.3 Biofilm formation in 24-well plates

This method was adapted from Lambert *et al.* (2012). The optical density was measured at 420 nm before it the biofilm assay was started. This helps to ensure the reading of the isolates was 0.3 to 0.4 that correspond to 10^6 to 10^8 CFU/mL which indicates the mid-exponential phase of the culture (Lourdat *et al.*, 2009). One mL of three different isolates including positive control (*Leptospira biflexa* serovar Patoc) and negative control (PBS buffer) were dispensed into the wells. Every isolates was performed in duplicate for 264 hours (11 days).

3.4 Crystal violet staining

This assay was carried out according to Lee *et al.* (2013). It was carried out for every 24 hours. The cultures were dispensed out of the well and rinsed with 1mL distilled water. The wells were air dried for about 15 minutes in room condition. The wells then were fixed with 1 mL of 2% sodium acetate for five minutes. The sodium acetate was aspirated out from the well and it was set to air dried for 15 minutes again. Then, 0.1% of crystal violet was aspirated into every well and set to stain for 20 minutes. Finally, crystal violet was pipetted out and the wells were rinsed with distilled water for three times. Ethanol/acetone (80/20) was used to solubilize the crystal violet staining at the end of the experiment. The optical density of every isolates was measured by using spectrophotometer (Metertech

Inc.) at 600 nm. To correct the background staining of crystal violet, the mean CV-OD_{600} value obtained for the negative control was subtracted from the mean CV-OD_{600} value obtained for biofilm formation by *Leptospira*.

3.5 Phase-contrast light microscopy view

This procedure was carried out according to Ristow *et al.* (2008). Glass slides were incubated half submerged in a bacterial suspension with 10^6 to 10^8 concentrations. The slides were observed at different interval (1, 6, 24, 48, 64, 72, 96, and 168 hours). It were rinsed three times in distilled water, air-dried, fixed by heating. It was observed by phase-contrast microscopy using a Olympus Inverted IX51 microscope at 60x10 magnification (Olympus Corporation, Japan).

3.6 Data analysis

The graphs were constructed in Microsoft Excel 2010 to demonstrate the quantification biofilm formation. The data used was the mean optical density of the duplicate readings and error bars denote standard error for the mean of duplicate reading.

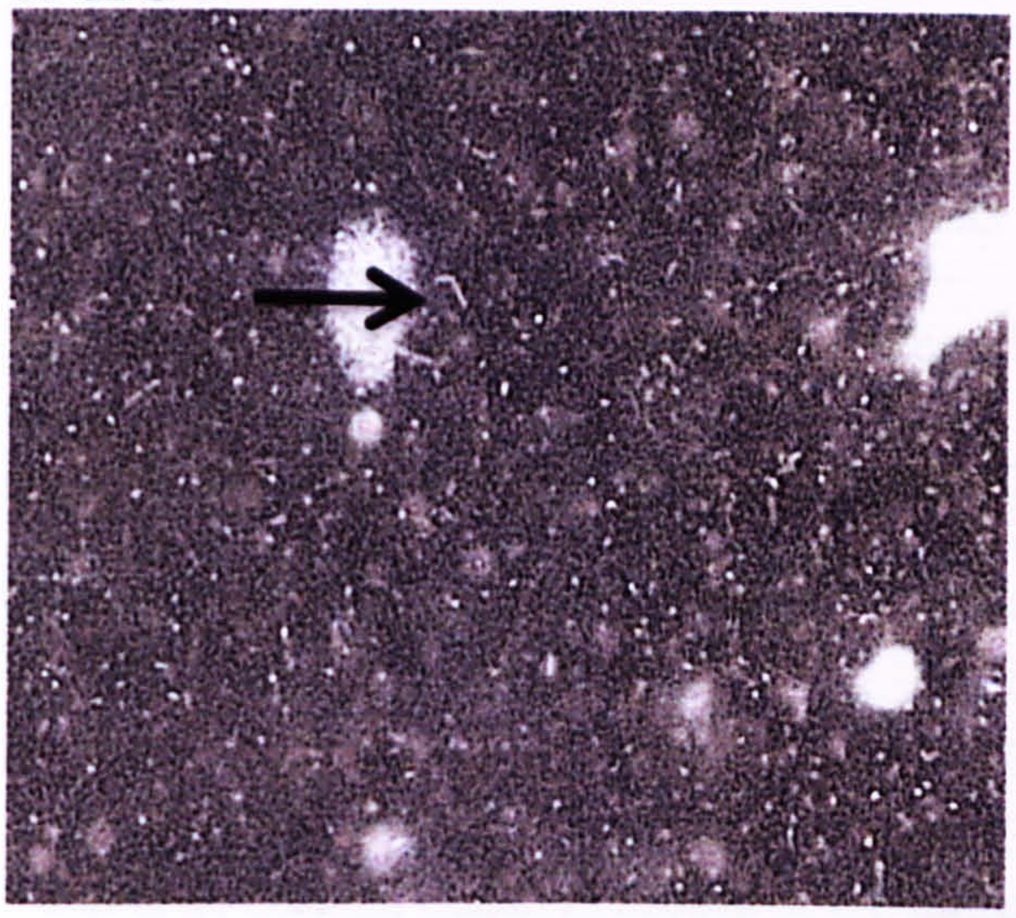
4.0 Result and Discussion

4.1 Dark field microscopy view

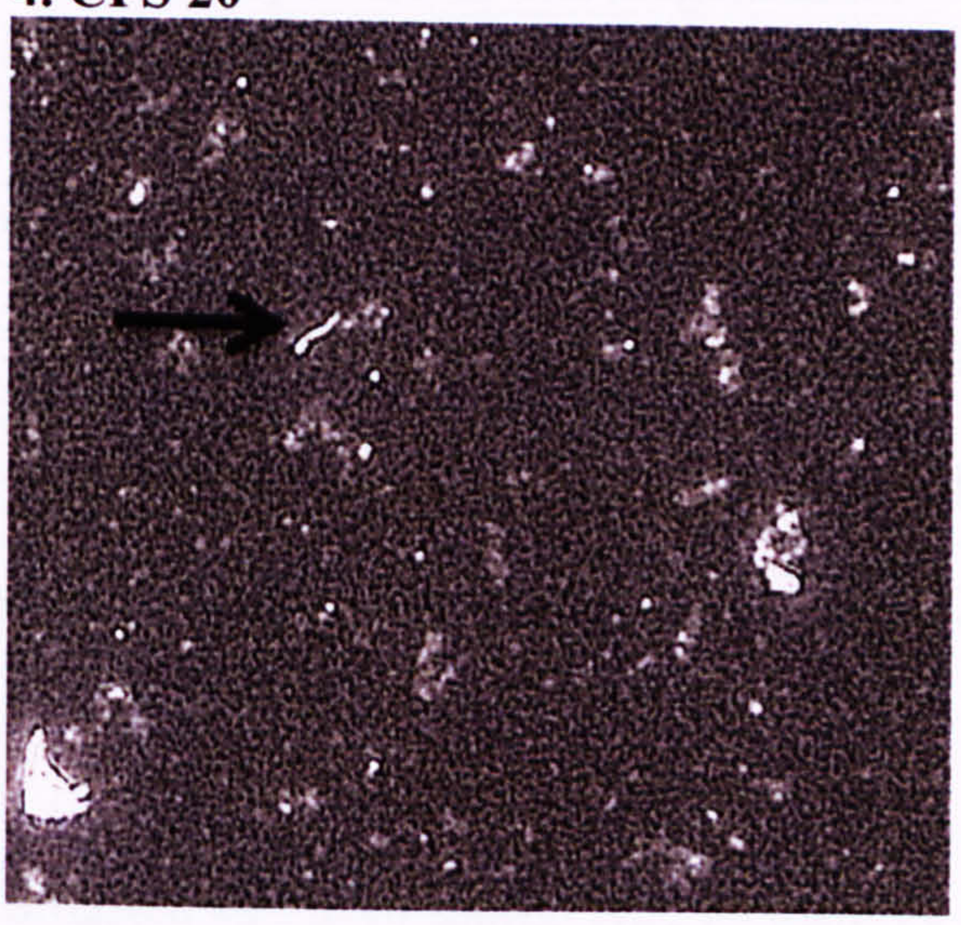
Dark field microscope was done to identify and confirmed the condition of the isolates at concentration of 10^6 to 10^8 CFU/ml. Based on the image shown in Figure 1, hook-like end, thin and motile leptospires were observed under the microscope. Through the observation, it proved that only five strains are positive for viability which was CFS 4, CFS 12, CFS 16, CFS 20 and CFS 21 that was code for *L. meyeri*.

Image of Saprophytic *Leptospira* under Darkfield Microscope

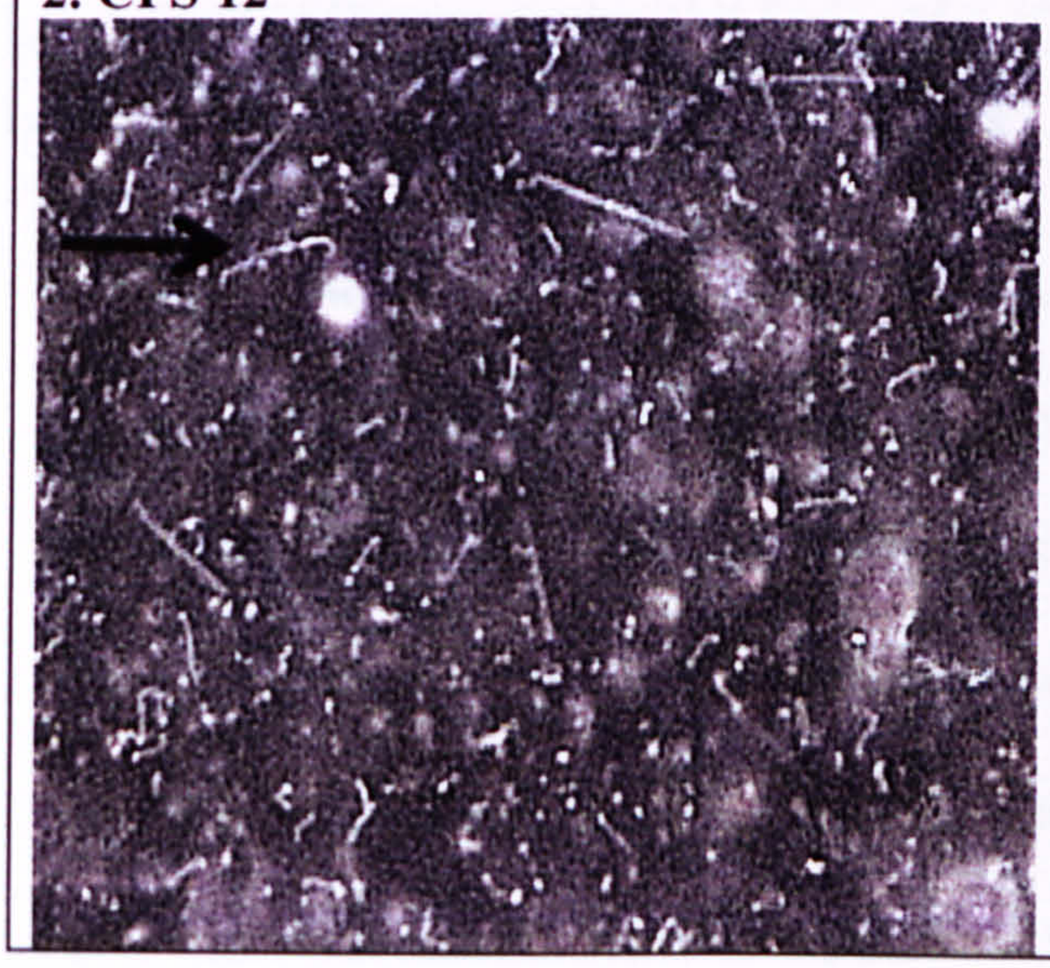
1. CFS 4



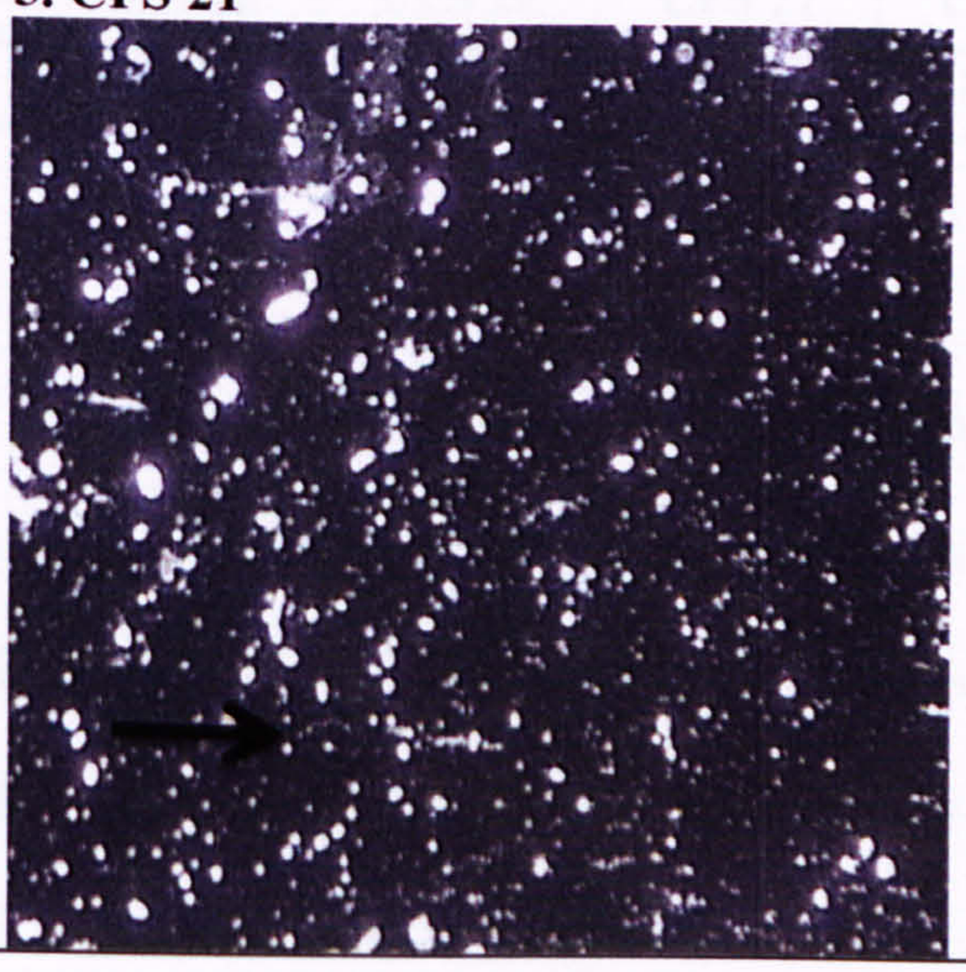
4. CFS 20



2. CFS 12



5. CFS 21



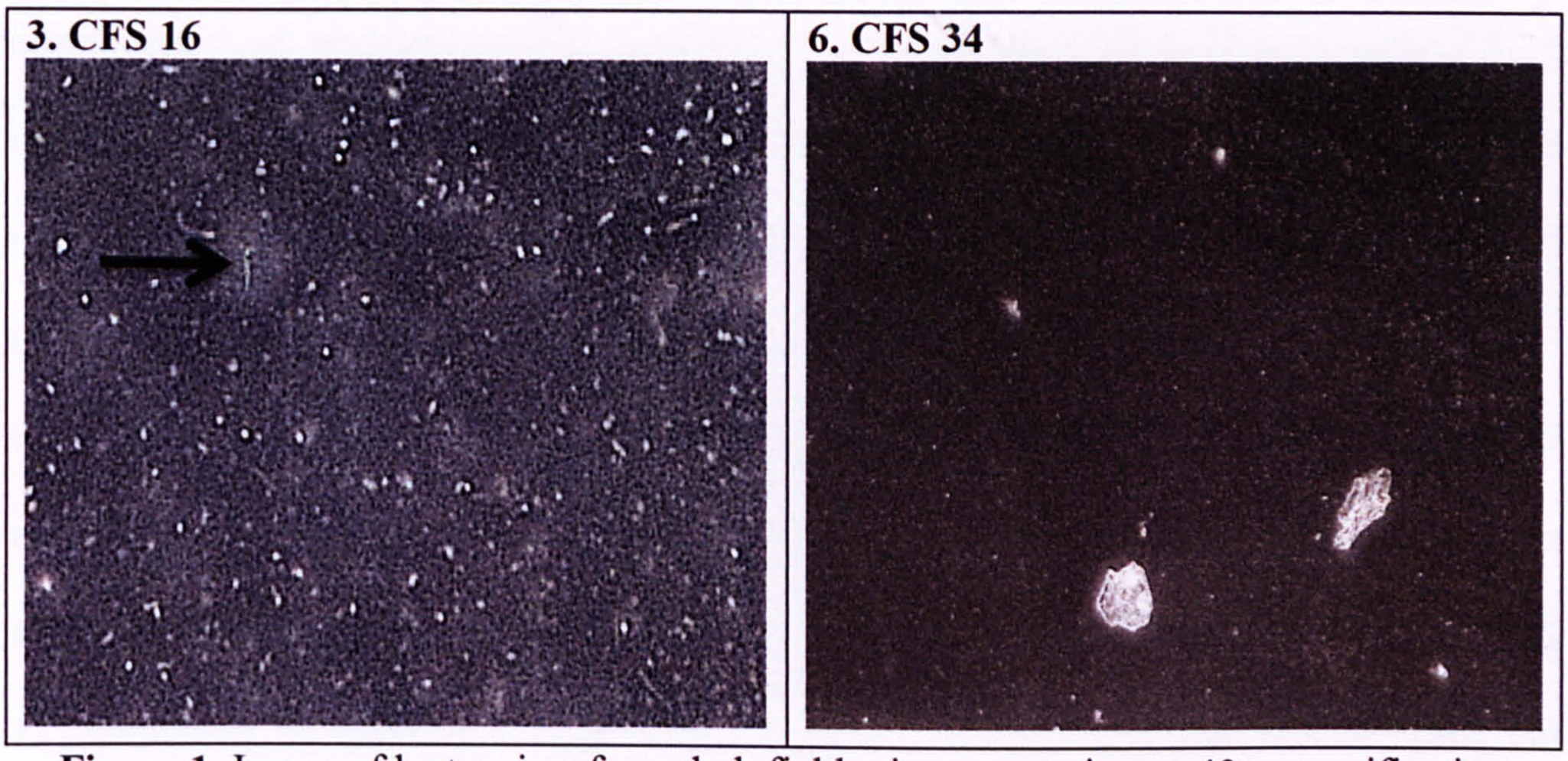


Figure 1. Image of leptospires from dark field microscope view at 40x magnification

4.2 Initial OD reading of culture

Optical density for every culture was measured to ensure the culture has OD range from 0.3 until 0.4 at 420 nm that corresponds to 10^6 until 10^8 concentrations (Lambert *et al.*, 2012). The concentrations indicated the exponential phase of leptospires growth (Bourhy *et al.*, 2011). OD readings of isolates were in exponential state as shown in Table 2.

Table 2. Optical density reading at OD_{420} for different strains of saprophytic leptospires

Isolates	CFS 4	CFS 12	CFS 16	CFS 20	CFS 21	CFS 34
Value OD (OD_{420})	0.493	0.315	0.409	0.384	0.347	0.390

4.3 Colour intensity of crystal violet staining

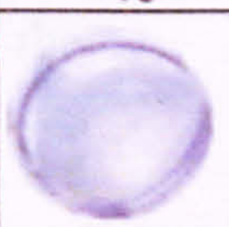
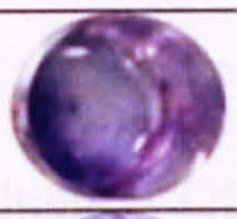
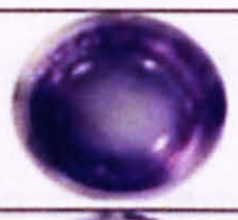
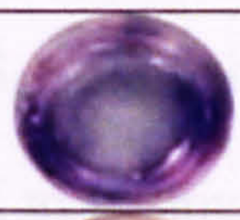
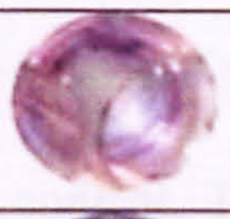
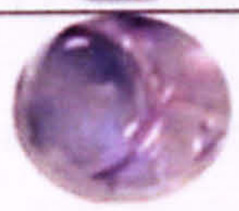
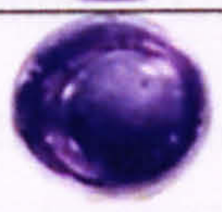
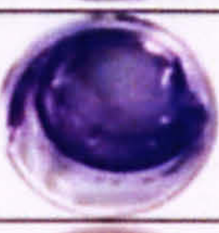
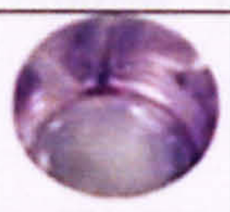
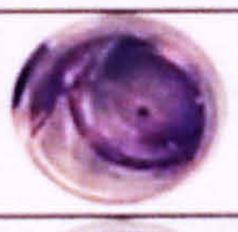
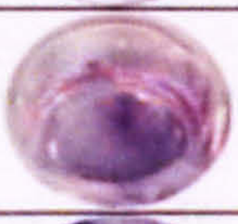
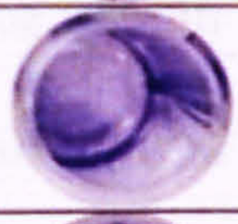
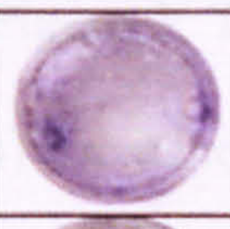
After the crystal violet assay, not all of the isolates have the ability to form biofilm and some of the strains such as CFS 4 only form low attachment during their exponential phase from the observation that had been made earlier. The production of biofilm can be observed from Table 3 which signifies the surface attachment at the bottom wells and air-liquid interface. Colour intensity represents the attachment of biofilm that was stained by

crystal violet. Three isolates which are CFS 12, CFS 16 and CFS 20 show strong attachment (high intensity colour) compared to other isolate. CFS 4, CFS 21, and CFS 34 (low intensity colour) showed less or no attachment. CFS 34 data has relationship between the darkfield microscope as no motile *Leptospira* had been observed. The OD readings of CFS 34 may indicate the growth of the bacterial cells only.

However, CFS 12 had the highest colour intensity starting from day 2 until day 9 especially on day 3 as can be seen in Table 3. It showed high amount of layer that was attached inside the wells. Thus, it signifies higher amount of biofilm production by CFS 12. Formation of attachment layer was not the only observation that can be made but biomass of air-liquid interface also can be observed as purple ring. The biofilms formed by the isolates were resistant to the washes performed in the surfaces at both short and long incubation times (Briheuga *et al.*, 2012). This explained the reason why some of the isolates were able to form attachment at the bottom of the wells although they colour intensity was decreasing until the last day.

In this study, distilled water was used to remove unstained crystal violet in the wells. Distilled water is unreliable solution in washing step after crystal violet staining. This is because inconsistent removal of unstained crystal violet was observed in the wells. Instead of distilled water, phosphate buffer saline (PBS) should have been conducted as it is more reliable and accurate to show consistent removal of unstained crystal violet when performing washing step. According to Das and Dash (2014), the PBS offered low level difference of salinity instead of distilled water by reducing the chances of cell bursting followed by cell death.

Table 3. Observation of attachment at different wells for every isolates of *Leptospira* that showed increasing colour intensity

Days	CFS 4	CFS 12	CFS 16	CFS 20	CFS 21	CFS 34	-ve	+ve
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								

-ve – Negative control (PBS)

+ve – Positive control (*Leptospira biflexa*)