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Biofilm forming ability of intermediate and saprophytic *Leptospira* on abiotic and biotic surfaces

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ABSTRACT

Aims: *Leptospira* spp. has the ability to develop biofilm communities and this attribute is an essential factor to leptospiral pathogenesis. This study aims to assess and quantify the biofilm forming ability of intermediate and saprophytic *Leptospira* strains.
Methodology and results: The biofilm assay was quantified on microtitre polystyrene plates (abiotic) and wood chips (*Jelutong Paya* hardwood) over a duration of 11 days. Phase contrast light microscope was used to assess the structure of the on the surface. The biofilm production on wood chips surface were approximately one times higher than on polystyrene plate surface indicating *Leptospira* strains were capable of forming higher quantity of biofilm on biotic surface compared to abiotic surface by both intermediate and saprophytic *Leptospira*. A significant difference (p<0.05) exists in biofilms produced by *Leptospira* on wood surface which formed more biofilm than on polystyrene surface. The strongest biofilm producer is intermediate strain G14 with OD_{600} of 2.283±0.180 and OD_{600} of 2.333±0.037, on polystyrene and wood surface, respectively. Visualisation of biofilm by phase-contrast microscopy of two representative strains correlated with the OD values and the colour intensity of stained microtitre plates and wood surfaces. The biofilm formed comprises of a three-step process are adherence (1^{th} to 24^{th} h), maturation ($6t^{h}$ to 7^{th} day) and detachment (9^{th} to 11^{th} day) of biofilms.
Conclusion, significance and impact of study: The contact time of intermediate pathogenic strains was faster compared to saprophytic strain, indicating the biofilm forming ability is related to the level of pathogenicity of *Leptospira* strains.

Keywords: Polystyrene, woods, biofilm formation, *Leptospira*, intermediate, saprophytic, pathogenicity

INTRODUCTION

Leptospirosis is a zoonotic disease spread by infected animals such as mice, fish and birds. It is a re-emerging disease that is caused by pathogenic *Leptospira*. It is transmitted through contact with urine, water, or soil contaminated by urine from animal reservoirs, such as rodents, dogs, and livestock (Guerra, 2009). Direct penetration of *Leptospira* sp. through the conjunctiva or surface epithelium could also cause the transmission to occur (Russ *et al*., 2003). *Leptospira* spp. can be divided into three types which are known as saprophytic, intermediate, and pathogenic (Ristow *et al*., 2008). In Malaysia, the Prevention and Control of Infectious Diseases Act 1988 has officially published leptospirosis as a notifiable disease in 2010.

More than 500,000 cases of severe leptospirosis occur each year, with a mortality rate 5-20% (WHO, 1999). A total of 13,176 leptospirosis cases were reported from the year 2014 until July 2015 in Malaysia, whereby 122 were death cases (Ministry of Health Malaysia, 2015). In Sarawak, a total of 163 Leptospirosis cases were reported caused by leptospirosis according to the Sarawak Weekly Epid News (March 2015). In the recent study conducted by Pui *et al*. (2015), pathogenic and intermediate *Leptospira* strains at two national parks in Sarawak were detected by PCR analysis. However, there are limited epidemiological studies of leptospirosis caused by intermediate strains of *Leptospira.*

Like other spirochaetes, *Leptospira* spp. has the ability to alter their morphology depending on the environmental conditions. The changes include the aggregation and colonization of single planktonic cells into the biofilm mode. In previous studies, bacteria have been proven to be able to exhibit biofilm in both abiotic and biotic surfaces. The potential of *Leptospira* in forming biofilms on these surfaces play an important role, not only for survival strategy but also to ensure it is successful in disease transmission and pathogenesis of these species (Iraola *et al*., 2016). The capability of the bacteria to form biofilm on abiotic and biotic surfaces causes the increase in the persistency of these species.

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To our best knowledge, several studies on biofilm are mainly on saprophytic and pathogenic *Leptospira.* The knowledge about intermediate strains and studies related to its pathogenicity is limited. Hence, this study was conducted to determine the relationship of contact time, biofilm forming ability with the level of pathogenicity of these species on abiotic and biotic surfaces.

MATERIALS AND METHODS

Bacterial strains and growth condition

All intermediate and saprophytic *Leptospira* strains were obtained from Microbiology Laboratory from Department of Molecular Biology, Faculty Resource Science and Technology, Universiti Malaysia Sarawak. The isolates were incubated in EMJH which contain EMJH base, 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 suggested by Ristow *et al*. (2008). All cultures were incubated at 30 °C for 30 days. Tables 1 and 2 show the selected isolates from the culture collection.

Dark field microscopic analysis

Selected cultures were observed at 400× magnification under dark field microscope (Olympus Corporation, Japan) after 30 days. Each strain was placed on sterile glass slides for observation. *Leptospira* were observed as a hook-like end, thin and motile. The images of the motile *Leptospira* were captured and recorded for further analysis. Samples that were positive for *Leptospira* were subcultured into EMJH medium, Difco™ Medium Base EMJH (Becton, Dickinsom and company, USA) for further analysis (Benacer *et al.*, 2013).

Preparation of *Leptospira* isolates culture for biofilm on abiotic surface (microtitre polystyrene surface)

Before performing biofilm assay, the optical density of each isolates was measured at 420 nm with OD values of 0.3 to 0.4 corresponding to 10^6 to 10^8 CFU/mL of cells. Serial dilutions were carried out to ensure the final concentration of cells in each well was standardized to 10^6 CFU/mL which indicating the culture is in mid-exponential phase. The biofilms were formed and measured in 24-well polystyrene plates, Tissue Culture Test Plate 24 (TPP®, Switzerland). Afterwards, the bacterial cultures were filled into the wells and the polystyrene plates were sealed during incubation to avoid from desiccation. Incubation were carried out at different intervals (24, 48, 72, 96, 120, 144, 168, 192, 216, 240, 264 h).

Table 1: Intermediate *Leptospira* strains used in biofilm assay from various sources.

No. designation	Isolates strain	Sources	Locality
G12	*Leptospira wolffii* serovar Khorat strain Khorat-H2	Soil	National Service Training Centre, Miri
G14	*Leptospira wolffii* serovar Khorat strain Khorat-H2	Soil	Kubah National Park, Kuching
G9	*Leptospira* sp. MS341	Water	National Service Training Centre, Miri
G11	*Leptospira wolffii* serovar Khorat strain Khorat-H2	Water	National Service Training Centre, Miri
G29	*Leptospira wolffii* serovar Khorat strain Khorat-H2	Rat liver	Kampung Sungai Mata, Kota Samarahan
G1	*Leptospira wolffii* serovar Khorat strain Khorat-H2	Soil	Tanjung Datu National Park, Kuching

Table 2: Saprophytic *Leptospira* strains used in biofilm assay from various sources.

No. designation	Isolate strain	Sources	Locality
S4	*Leptospira meyeri* strain Semaranga_DB49	Soil	UNIMAS, Kota Samarahan
S11	*Leptospira meyeri* strain Semaranga_DB49	Soil	National Service Training Centre, Miri
S21	*Leptospira meyeri* strain 19CAP	Soil	Medan Niaga Satok, Kuching
S3	*Leptospira meyeri* strain Patoc strain “Patoc	Water	Juara National Service Training Centre, Kuching
S5	*Leptospira meyeri* strain Semaranga_DB49	Water	National Service Training Centre, Miri
S12	*Leptospira meyeri* strain Semaranga_DB49	Water	Kubah National Park, Kuching
Patoc	*Leptospira biflexa* serovar Patoc strain ‘Patoc 1 (positive control)	-	Institute of Medical Research, Malaysia (IMR)

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Biofilm assay on abiotic surface

Microtitre plate assay was conducted in triplicate on 24-well microtitre polystyrene plates. This method was carried out according to Pui *et al*. (2017). Briefly, the cultures were aspired, rinsed and fixed on the polystyrene surface. Crystal violet was used to stain the adherent cells on the surface and the remaining further dissolved with ethanol/acetone solution. By using a spectrophotometer (Metertech Inc. SF-880, Taiwan), the optical density of each isolates was measured at 600 nm.

Preparation of *Leptospira* isolates culture for biofilm on biotic surface (woods chips)

Mid-exponential cultures (10^6-10^8 CFU/mL) were prepared by incubating the cultures in EMJH broth for a week. To ensure the final concentration of cultures is 10^6 CFU/mL, serial dilutions were performed. Study on biotic surface was achieved by using wood surface for attachment and biofilm assay on biotic surfaces. Before biofilm assay, the wood samples known as *Jelutong Paya* Hardwood (*Dyera costulata*) were cut using a fine-toothed saw to provide a precise and accurate size of wood chips (1cm×0.5cm×0.5cm). The hardwood was obtained from the laboratory of department of Plant Science and Environmental Ecology. Subsequently, the chips were rinsed using a detergent and air-dried. Afterwards, the chips were placed into a hot air oven at 75 °C for 30 min. The chips were then filled into the well plates along with 1 mL of bacterial cultures. To prevent from desiccation of the cultures, the plates were sealed with parafilm (Adetunji *et al.*, 2011).

Biofilm assay on biotic surface

Biofilm was assayed in triplicate on the wood chips representing biotic surface. This technique of biofilm assay specifically on wood surface was based on the method of Adetunji *et al.* (2011). At the end of each incubation period at 24 h intervals (24, 48, 72, 96, 120, 144, 168, 192, 216, 240, 264 h), a set of chips were aseptically removed from the bacterial cultures to the new well plates for biofilm quantification using crystal violet binding assay. Each set of wood chips were rinsed once using 1 mL of PBS to remove non-adherent cells on the wood surfaces. Then, 2.5 mL of methanol per chips were used to fix the remaining adhered bacterial cells. Each chip was stained using 0.9 mL of 0.1% crystal violet for 15 minutes and then the excess stain was rinsed three times with 1 mL of PBS. Afterwards, the chips were air-dried for another 15 min before re-solubilized the dye bound to the adherent cells using 2.5 mL of 33% (v/v) glacial acetic acid. Lastly, the re-solubilized liquid was poured into cuvettes for measurement of optical density (OD) using a spectrophotometer (Metertech Inc. SF-880, Taiwan) at wavelength of 420 nm. Glacial acetic acid was used as a blank.

Biofilm formation categories

The optical density of every strain was calculated using optical density cut-off value (ODc) which defined as three standard deviation above the mean optical density of the negative control which adapted from Stepanovic *et al*. (2000). The four classes known as OD ≤ ODc (non-biofilm producer), ODc < OD ≤ 2× ODc (weak biofilm producer), 2× ODc < OD ≤ 4× ODc (moderate biofilm producer) and 4× ODc< OD (strong biofilm producer). The biofilm producers calculated in percentage represent the amount of biofilm formation.

Data analysis

Statistical analysis was performed by using Microsoft Excel (version 14.0, release 1.5, California, US). A significance level of 5% (p<0.05) was set for statistically analyses. Differences between triplicate means for the isolates were evaluated using repeated measures one-way ANOVA, followed by Post-Hoc Bonferoni test to differentiate the biofilm forming ability of *Leptospira*.

RESULTS

Growth of *Leptospira*

The OD_{420} for *Leptospira* species obtained were in the range of 0.3 to 0.4 as shown in Table 3. The OD readings obtained for intermediate isolates at 420 nm were tabulated in Table 3. The acceptable range of OD values for *Leptospira* is between 0.3 to 0.4 which indicates the acceptable number of colonies (10^6-10^8 CFU/mL) to start a biofilm assay (Sutton, 2006). This indicates the microbial population in these samples were in the acceptable range to initiate biofilm experiment.

Table 3: The OD values obtained for *Leptospira* at 420 nm (10^6-10^8 CFU/mL).

Intermediate	OD_{420}	Saprophytic	OD_{420}
G9	0.335	S3	0.367
G11	0.347	S4	0.344
G12	0.382	S5	0.398
G14	0.400	S11	0.379
G29	0.401	S12	0.341
G1	0.371	S21	0.372
-	-	PATOC	0.361

Development of biofilm on abiotic surface (microtitre polystyrene surface)

On microtitres polystyrene surface, this study showed that intermediate strains G1 (positive control), G12 and G14 (Figure 1) formed stronger biofilms compared to G9 and G11. The wells of G14 were stained more with crystal violet than the wells of G9 and G11 which was observed on day 4th. This finding shows that the biofilm produced by G14 is strong to withstand the rinsing process during the assay of G9 recorded as the weakest strain biofilm

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forming on the well plates based on the intensity of crystal violet staining. As for saprophytic strain, S12 showed the highest colour intensity on day 6 with mean optical density values of 2.066 (Figure 2). From the result, 57.14% of isolates developed mature biofilms on day 6 and 42.86% on day 8 of incubation. On the contrary, S3 and S11 had the lowest colour intensity in which the biofilms were only exhibited on day eight with optical density values of 1.450 and 1.565 respectively while rapidly dispersed afterwards. This can be seen in the delayed biofilm formation of S3, S5 and S11 in which these strains required longer time to adapt on the substrata before forming biofilms.

Development of biofilm on biotic surfaces (*Jelutong paya* hardwood)

From Figure 3, strain G14 formed the strongest (2.283±0.180) biofilm on the wood surfaces based on the crystal violet staining. For saprophytic strains, S12 had the highest quantity of biofilm whereas S3 had the lowest amount of biofilm by observing on the mean optical density values of each isolates. The adhesion of bacterial cells on the wood surface started at day 1st till day 6th with the mean optical density values at approximately 2.0. S4 and S12 had the highest starting optical density values (day 1st) with 2.071 and 2.081 respectively. At the highest biofilm cells saturation (day 6th), the mean optical density values of the isolates were relatively at 2.3. From Figure 4, saprophytic *Leptospira*, S12 reported had the highest OD readings (2.310±0.024) while S12 recorded as the lowest optical density value which is 1.980± 0.024 on day 11th.

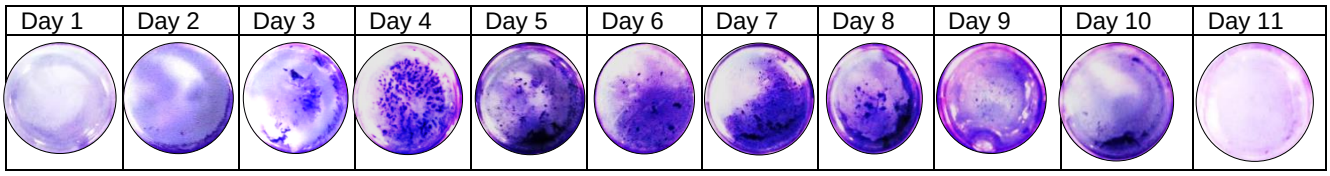

Figure 1: Visualization and quantification of biofilm on microtiter plates of G14 (*Leptospira wolffii* serovar Khorat strain Khorat-H2). Strongest stained biofilm cells were formed on plates and stained with 0.1% of crystal violet solution observed for 11 days.

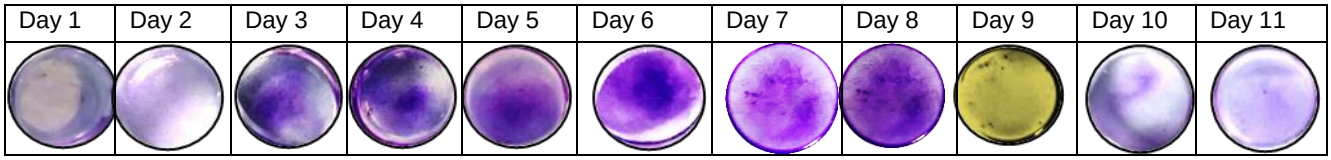

Figure 2: Visualization and quantification of biofilm on microtiter plates of S12 (*L. meyeri* strain Semaranga_DB49) as the strongest biofilm in saprophytic. Stained biofilm cells were formed on plates and stained with 0.1% of crystal violet solution observed for 11 days.

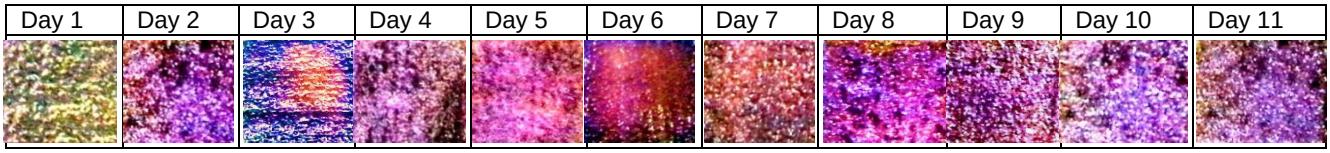

Figure 3: Visualization and quantification of biofilm on *Jelutong paya* hardwood of G14 (*L. wolffii* serovar Khorat strain Khorat-H2) with 2.333±0.037 on day 6th which produced the highest amount of biofilm.

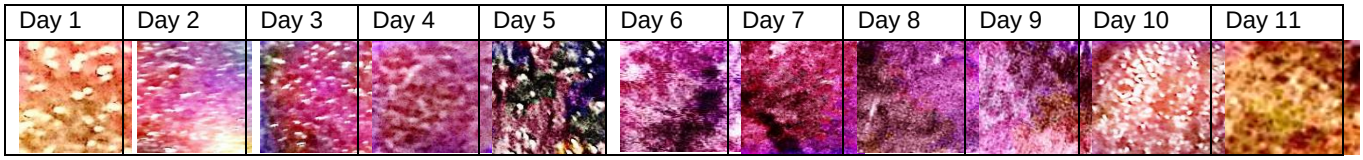

Figure 4: Visualization and quantification of biofilm on *Jelutong paya* hardwood of S12 (*L. meyeri* strain Semaranga_DB49) with 2.283±0.180 on day 8th.

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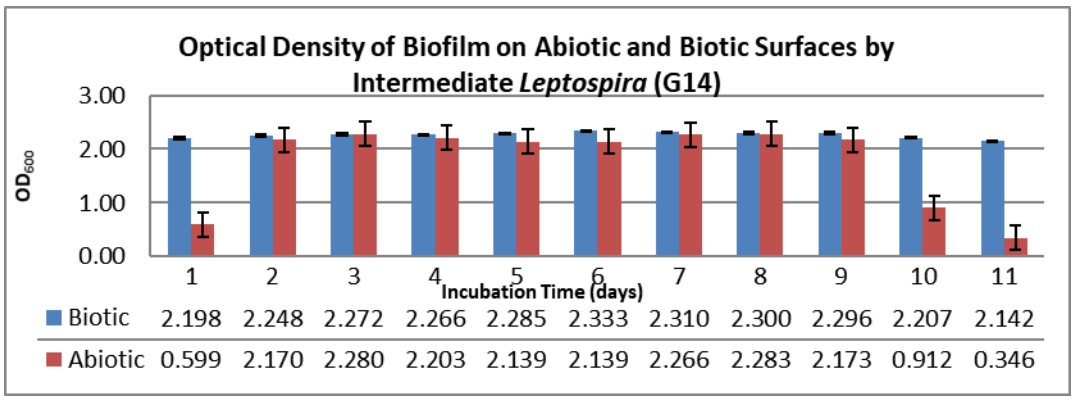

Figure 5: Biofilm formation by strongest intermediate strain, G14 (*L. wolffii* serovar Khorat strain Khorat-H2) environmental isolates incubated at 30 °C for 11 days in EMJH media and measured by 0.1% crystal violet staining with error bars representing ± one standard error.

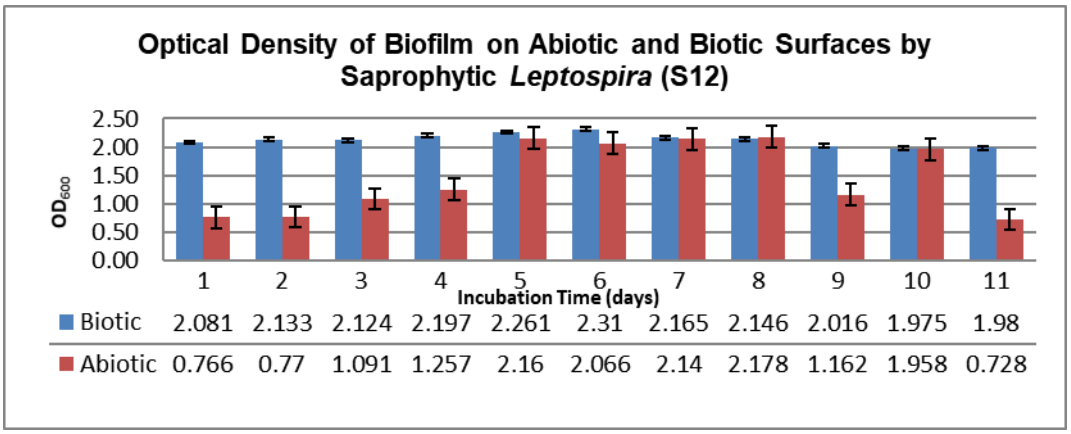

Figure 6: The highest biofilm forming ability by saprophytic strain, S12 (*L. meyeri* strain Semaranga_DB49) environmental isolates incubated at 30 °C for 11 days in EMJH media and measured by 0.1% crystal violet staining with error bars representing ± one standard error.

Optical density of *Leptospira*

The growth pattern from Figures 5 and 6 show the optical density at 600 nm of intermediate and saprophytic *Leptospira* against incubation time for biofilm formation on the abiotic and biotic surfaces, respectively. Both graphs summarized the biotic surface clearly show higher biofilm forming ability in both strains. The biofilms formation trend in intermediate strains was relatively stronger (OD>2.0) from day 2nd while saprophytic attained OD>2.0 at day 5th.

DISCUSSION

In this study, the biofilm formation of intermediate and saprophytic *Leptospira* strains were tested on microtitre plates (abiotic surface) and wood chips (biotic surface). Qualitative analysis by phase contrast microscopy showed that intermediate *Leptospira* produced more adherent bacterial cells than saprophytic *Leptospira.* This finding revealed that biofilm formation by *Leptospira* spp. were able to attach stronger on biotic surfaces and substratum specific than abiotic surface (Figures 3 and 4). Previous studies have reported similar observation where biotic surface produce greater biofilms than abiotic surface (Mueller *et al*., 2007; Adetunji *et al*., 2011). For biotic surface, the strains formed biofilms at the beginning of the experiment and gradually increase in the following days (day 2th to day 7th). Incubation is necessary for the *Leptospira* cells to transform into a complete whole biofilm. Previous study by Chakraborty *et al*. (2011) stated that *Leptospira* strains that were isolated from the environmental samples are considered as a slow growth microorganism. The similar growth pattern was also observed in our study. Statistically, there is significant difference (p<0.05) between the mean of optical density cut-off values and the incubation time in *Leptospira* strains. Therefore, a longer incubation time (11 days) was needed for Leptospires cell to multiply, adapt and completely turns into biofilms. As demonstrated in Figures 5 and 6, the intensity (OD) of the biofilm shown by crystal violet staining increased significantly with the increased of incubation time. It was shown that the attachment of

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intermediate, G14 at the bottom of the wells was strong adhered throughout the duration of biofilm incubation (day 11th). These results agree with Briheuga *et al*. (2012) who reported the biofilms formed by the isolates were resistant to the washes performed in the surfaces at both short and long incubation times.

Biofilm bacteria can form and withstand biofilms at different conditions. The present study found that *Leptospira* spp. developed biofilm on different surfaces, biotic and abiotic with incubation time. Thus, the survival of *Leptospira* in environment for a longer period is due to its ability to colonize into a host and cause disease. The surface characteristics and the attachment efficiency of specific strains may also be the factors that affect the bacterial adhesion on surfaces (Elhariry, 2011). This study showed that the biofilm produced by intermediate *Leptospira* is strong enough to withstand rinsing process, thus indicated the biofilm attachment is irreversible. The strains formed biofilms at the beginning of the experiment and gradually increase in the following days. In this stage, the bacterial cells began to multiply, preconditioning the adhesion surface and adsorb into the surfaces. The surface hydrophobicity increases with the surface roughness where it enhances the bacterial adhesion (Pui *et al*., 2011). Plant surfaces especially wood surface has a rough texture, appearance and irregular surface that favoured bacterial cell attachment which eventually will develop a robust biofilm. Previously, a study reported that the mean values of pathogenic *Leptospira* spp. were six times higher than saprophytes (Pui *et al.*, 2017). This finding agrees with Kumar *et al*. (2015) and implied that saprophytic *Leptospira* is less pathogen in the environment as it lacks the virulence and host invasion genes. This indicated the level of pathogenicity in saprophytes was lesser than pathogenic *Leptospira.*

Interestingly, this study found that intermediate and saprophytic *Leptospira* required 11 days for a complete maturation to detachment of biofilm formation. Previous study showed that pathogenic strains can forms biofilm at approximately OD>21.760 at day 7th (Pui *et al*., 2017). Based on the optical density cut-off value, the biofilm forming ability of pathogenic strains on abiotic surfaces was 10 times higher that intermediate and saprophytic *Leptospira*. In agreement of previous study, biofilm formation of bacteria after an extended period of incubation was correlated with the persistent of host infection and survival strategy (Culler *et al.*, 2014).

CONCLUSION

Leptospira spp., isolated from different sources are capable to form biofilm on abiotic and biotic surfaces. Intermediate strain formed biofilm faster compared to the saprophytic strains. Biofilm formation is an essential factor contributing to bacterial pathogenesis and it can lead to serious implications in medical field, public health, industry and environment. Hence, these results provided better understanding on the potential of biofilm formation by *Leptospira* on abiotic and biotic surfaces.

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