Faculty of Resource Science and Technology

Assessment of *Leptospira* spp. on the Surface of Packaged Food and Canned Drinks

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Assessment of *Leptospira* spp. on the Surface of Packaged Food and Canned Drinks

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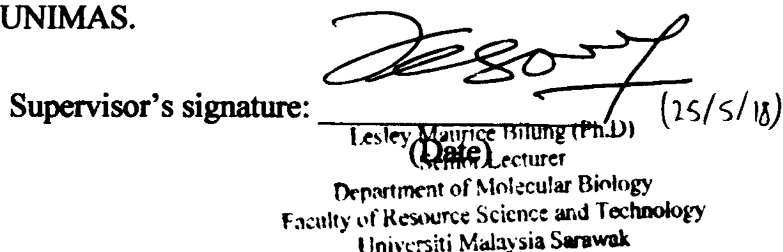

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LIST OF ABBREVIATIONS

PCR	Polymerase Chain Reaction
AGE	Agarose Gel Electrophoresis
LERG	Leptospirosis Burden Epidemiology Reference Group
MAT	Microscopic Agglutination Test
ELISA	Enzyme-Linked Immunosorbent Assay
CAAT	Cross-Agglutinin Absorption Test
NaCl	Sodium Chloride
EMJH	Ellinghausen-McCullough-Johnson-Harris
$MgCl_2$	Magnesium Chloride
dNTP	Deoxyribonucleotide Triphosphate
TBE	Tris-Borate-EDTA Buffer
EtBr	Ethidium Bromide
UV	UltraViolet
BLAST	Basic Local Alignment Search Tool
NCBI	National Center for Biotechnology Information

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Assessment of *Leptospira* spp. on the Surface of Packaged Food and Canned Drinks

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ABSTRACT

Leptospira are shed in the urine of infected animals such as rodents that cause kidney disease, liver disease or reproductive dysfunction in animals. Human can also be infected by *Leptospira* after contact with infected urine on the packaged food and canned drinks or through contact with contaminated water, soil or food. The purposes of this research is to assess the presence of *Leptospira* spp. on the surface of packaged food and canned drinks by using Polymerase Chain Reaction (PCR) method, targeting on *LipL32*, *16S rRNA* and *rrs* genes indicates pathogenic, intermediate and saprophytic *Leptospira*. A total of 30 packaged food and canned drinks samples were purchased randomly from a canteen at a public university, wet market, supermarket, mall, restaurant, Indian Street and Kuching Waterfront. The samples were cultured into Ellinghausen-McCullough-Johnson-Harris (EMJH) broth with 5-fluorouracil and incubated at room temperature (28-30 °C) for 1 month. The DNA isolation was conducted using Wizard Genomic DNA Purification Kit. Saprophytic *Leptospira* was detected on the surface of 27% (8/30) packaged food and canned drinks samples. All the 27% (8/30) isolates were classified as non-pathogenic species which were identified as *Leptospira biflexa*, *Leptospira wolbachii* and *Leptospira* spp. clone NP75. Thus, the findings of this study provide information on the occurrence and distribution of *Leptospira* spp. on the surface of packaged food and canned drinks. The packages and cans must be rinsed and cleaned before eating and drinking to prevent the possible transmission of *Leptospira* spp..

Key words: *Leptospira*, leptospirosis, Polymerase Chain Reaction, saprophytic

ABSTRAK

Leptospira terdapat di dalam air kencing haiwan yang dijangkiti seperti tikus yang menyebabkan penyakit buah pinggang, penyakit hati atau disfungsi reproduktif pada haiwan. Manusia juga boleh dijangkiti oleh Leptospira selepas bersentuhan dengan air kencing yang dijangkiti pada bungkusan makanan dan tin minuman atau melalui kontak dengan air, tanah atau makanan yang tercemar. Tujuan kajian ini adalah untuk mengkaji kehadiran Leptospira spp. pada permukaan bungkusan makanan dan tin minuman dengan menggunakan kaedah Polymerase Chain Reaction (PCR), mensasarkan gen LipL32, 16S rRNA dan rrs yang menunjukkan Leptospira patogenik, perantaraan dan saprophytic. Sejumlah 30 sampel bungkusan makanan dan tin minuman dibeli secara rawak dari kantin di universiti awam, pasar basah, pasar raya, pusat membeli-belah, restoran, Indian Street dan Kuching Waterfront. Sampel tersebut telah dikultur di dalam Ellinghausen-McCullough-Johnson-Harris (EMJH) media dengan 5-fluorourasil dan diinkubasi pada suhu bilik (28-30 °C) selama 1 bulan. Pengekstrakan DNA telah dijalankan menggunakan Wizard Genomic DNA Purification Kit. Leptospira saprophytic dikesan pada permukaan 27% (8/30) sampel bungkusan makanan dan tin minuman. Kesemua 27% (8/30) isolat diklasifikasikan sebagai spesies bukan patogen yang dikenalpasti sebagai Leptospira biflexa, Leptospira wolbachii dan Leptospira spp. klon NP75. Oleh itu, hasil kajian ini memberi maklumat mengenai kehadiran dan pengedaran Leptospira spp. pada permukaan bungkusan makanan dan tin minuman. Plastik bungkusan dan tin harus dibilas dan dibersihkan sebelum makan dan minum untuk mencegah penyebaran Leptospira spp..

Kata kunci: Leptospira, leptospirosis, Polymerase Chain Reaction, saprophytic

1.0 INTRODUCTION

Occurrence and distribution of infected urine shed by rodents on the surface of packaged food and canned drinks as well as in the water, food and soil causes the issues of leptospirosis to increase (Lehmann *et al.*, 2014). The chairman for National Institute for Occupational Safety and Health stated that the number of leptospirosis cases increased between 2011 and 2015 and dropped last year (Othman, 2017). *Leptospira* can survive for long period of time and their survivability are influenced by moisture, pH and temperature of the environment. Nowadays, the common issues in Malaysia are direct transmission of *Leptospira* which is caused by infected urine shed by rodents and contaminated water, food or soil.

In tropical areas, direct contact with contaminated water due to heavy rainfall and flooding can be a major risk of exposure to pathogenic *Leptospira* spp. (Dobigny *et al.*, 2015). In the flood-hit states, 753 suspected leptospirosis infections with 126 confirmed cases in Malaysia were recorded from Jan 1, 2015 reported by the Malaysia Health Minister (Idris, 2015). This infection will increase other diseases too including acute gastroenteritis, diarrhea and upper respiratory tract infections among flood victims. Moreover, it will also increase fatality rates in Malaysia due to slow and less treatment to cure this disease. Mortality rates are totally influenced by the timeliness of treatment interventions.

Recently, several questions have been raised regarding the possibilities of the surface of packages and cans to be susceptible to *Leptospira*. Nevertheless, leptospirosis cases has been controversial due to a lot of issues related to infected urine shed by rodents in the water was exposed on the packaged food and canned drinks, cause death to

thousands of people per year and increased the mortality rate in Malaysia (Lehmann *et al.*, 2014). Case-fatality rates in the world also have been reported to range from <5% to 70%. Even though there have been several issues regarding packages and cans contaminated with *Leptospira* but yet there are still limited data and information available worldwide. As a result, the research was carried out to assess the presence of *Leptospira* spp. on the surface of packaged food and canned drinks under hypothesis that there is a risk of transmission of *Leptospira* from packages and cans to human that cause health threat and mortality.

In addition, packages and cans are usually stored in the warehouse and delivered directly to retail stores without cleaning. The packages and cans also do not have any kind of external protection to prevent them from being infected. So, there is high probability of *Leptospira* infection from the contaminated water or from infected urine shed by rodents itself. Moreover, most of the consumers regularly eat and drink straight from the packages and cans without cleaning. In this case, *Leptospira* or any microorganisms that presence on the surface of packages and cans will directly transmitted into the oral cavity and cause various type of disease.

It is very important to create awareness among Malaysian to understand more about the disadvantages of this disease. Furthermore, people need to be aware and educated about this disease because leptospirosis provide symptoms that may resemble other diseases such as dengue fever, bleeding disorders and jaundice that are caused by different viruses. Molecular detection of 423 bp of *LipL32*, 331 bp of *16S rRNA* and 240 bp of *rrs* genes by using PCR method provide genetic information of pathogenic, intermediate and saprophytic *Leptospira* respectively.

The objective of this study:

1. To isolate pathogenic, intermediate and saprophytic *Leptospira* on the surface of packaged food and canned drinks using PCR method, targeting on *LipL32*, *16S rRNA* and *rrs* genes indicates pathogenic, intermediate and saprophytic *Leptospira* respectively.

2.0 LITERATURE REVIEW

2.1 Morphology of *Leptospira* spp.

Leptospira are known as corkscrew-shaped bacteria and differ from other spirochaetes due to the presence of distinctive hooked ends. They are helix-shaped organisms with a cell diameter of approximately 0.15 µm and 10-20 µm long. According to Plank and Dean (2000), *Leptospira* consists of cytoplasmic membrane, cell wall and surrounded by outer membrane containing porins to allow exchange of solute between periplasmic space and environment. To preserve their integrity, *Leptospira* needs to maintain their shape. The formation of shape and rigid exoskeleton are due to a common essential component, peptidoglycan and a polymer of glycosaminopeptides (Slamti *et al.*, 2011). *Leptospira* has periplasmic space consists of two periplasmic flagella each arising at one end of the bacterium with polar insertions important for the movement. The FlaA and FlaB proteins complement flagella sheath and core separately. Mohammed *et al.* (2011) explained that a FlaB mutant is considered to be deficient in endoflagella and also non-motile under electron microscopy.

2.2 Classification of *Leptospira* spp.

2.2.1 Serological Classification

Serological classification or known as antigenic classification is a system that organizes individual bacterial strains of a species into smaller groups based on their cell surface antigens. In the previous years, *Leptospira* are classified based on serological typing including Microscopic Agglutination Test (MAT) and Cross-Agglutinin Absorption Test (CAAT) (Zhang *et al.*, 2015). There are about 230 pathogenic serovars identified. From previous research, the species are differentiated depending on various growth factors.

Boqvist (2002) explained that pathogenic *Leptospira interrogans* are found in animals and humans while saprophytic *Leptospira biflexa* are found in the favourable environment with 13 °C and 8-azaguanine. On the other hand, *Leptospira biflexa* failed to form cells in spherical shape in 1 M sodium chloride (NaCl).

2.2.2 Genotypic Classification

Genotypic classification of *Leptospira* is based on molecular characterisation which divides *Leptospira* into genome species. Nowadays, this method has been used widely due to the development of facilities and education. Phenotypic tests are not being used anymore because the tests are unable to distinguish and differentiated *Leptospira* species. The new genomic classification system identified an intermediate group of *Leptospira* between pathogenic and saprophytic groups such as *Leptospira inadai* and *Leptospira fainei* (Boqvist, 2002). According to Morey *et al.* (2006) based on the classification, *Leptospira* genus has been classified into 17 species containing both pathogenic and nonpathogenic serovars. The pathogenic genome species such as *Leptospira interrogans*, *Leptospira borgpetersenii*, *Leptospira kirschneri*, *Leptospira noguchii*, *Leptospira alexanderi* and *Leptospira santarosai* has been identified (Letocart *et al.*, 1999).

2.3 Epidemiology of *Leptospira* spp.

Living in urban areas are associated with increased risk of leptospirosis infection rather than in rural areas (Mwachui *et al.*, 2015). Nowadays, around 1.03 million cases of leptospirosis had occurred and caused 2.9 million lost. Lehmann *et al.* (2014) explained that over 500 000 number of human cases of severe leptospirosis per year is the recent estimates by the Leptospirosis Burden Epidemiology Reference Group (LERG) at the World Health Organization. For examples, 0.1–1 cases per 100 000 population per year in

regions with a temperate climate, >10 cases per 100 000 population in humid tropical regions and >100 cases per 100 000 population affected during outbreaks (Hartskeerl *et al.*, 2011). According to Bharti *et al.* (2003), mortality cases remain significant due to lack of infrastructure and adequate clinical suspicion and poor education about the inherent pathogenicity of the *Leptospira* strains.

2.4 Mode of Transmission

Leptospirosis is a life-threatening disease and can be transmitted through dried or wet rat or other animal urine and feces. Mayer-Scholl *et al.* (2014) explained that rodents are the most important reservoirs for both animal and human infection rather than other animals. According to Benacer *et al.* (2013), the main reservoirs for pathogenic *Leptospira* are rodents while for saprophytic *Leptospira* are water and soil. Usually, human will become the incidental hosts that do not act as reservoirs for *Leptospira* infection. Human will become infected after exposure to packaged food and canned drinks that are contaminated with *Leptospira* shed in urine of infected animals (Munoz-Zanzi *et al.*, 2014). It is important to rinse the packages and cans before eating and drinking because they are usually stored in the warehouse and delivered directly to retail stores without cleaning.

2.5 Relationship between Commercialisation and *Leptospira*

Mostly, the infection spreads in working places such as agricultural sectors, waste management sectors and animal farms as well as commercialisation sites such as restaurant, vending machine, mall and supermarket. Different sectors will provide survival of different species of *Leptospira* due to vary of reservoirs, temperature, moisture and pH. Based on previous research conducted by Dantas *et al.* (2006), aluminium cans containing

soda or beer, plastic straws, glass cups and ice collected from polystyrene boxes contained positive saprophytic *Leptospira*, mould and yeasts. This is due to inadequate handling of the packages and cans that lead to contamination. Furthermore, natural disasters such as flooding also cause *Leptospira* infection to increase drastically. Brown *et al.* (2011) explained that *Leptospira* can remain in the water or wet areas for months and this caused the places with insufficient drainage such as commercialisation sites to have higher tendency to the infection. Natural disasters are mostly caused by human activities such as deforestation and countries development.

2.6 Clinical Symptoms

Spirochetes such as *Leptospira interrogans* and *Leptospira biflexa* can cause leptospirosis or known as Weil's syndrome that is infected with dried rat urine. It has been known as a globally important infectious disease because the infection will lead to enlargement of spleen, hepatic and renal failure or massive pulmonary haemorrhage (Kaur & Lal, 2009). *Leptospira* causes a wide variety of signs and symptoms in the infected persons such as fever, myalgias, severe headache, chills, diarrhoea, nausea and vomiting, oliguria or anuria, jaundice, conjunctival suffusion, aseptic meningitis, haemorrhages, joint pain, skin rash, cough, cardiac arrhythmia, psychosis or delirium (Hartskeerl *et al.*, 2011). Levett (2001) explained that leptospirosis is a biphasic infection due to the acute or septicemic phase lasting about a week, followed by the immune phase, characterized by antibody production and excretion of *Leptospires* in the urine. Mostly, the side effects of this infection are begun in the patients at second period of sickness (Levett & Haake, 2009).

2.7 Factors Affecting the Growth of *Leptospira* spp.

2.7.1 Climate

People may maintain *Leptospira* in certain environments such as Peruvian Amazon due to warm and humid climate in that area that favour for *Leptospira* growth. In Latin America and Caribbean region, factor such as climate will contribute to the epidemic outbreaks of *Leptospira* in animal or human populations (Petrakovsky *et al.*, 2014). According to Bertherat *et al.* (2014), in Central Africa especially in mining areas, people always exposed to *Leptospira* infection due to favourable climate as well as very poor living and working conditions. Chomel (2014) explained that *Leptospira* infection has re-emerged due to both climatic changes such as summer and fall and changes in serovars implicated in the animals. Moreover, the mean survival for *Leptospira* is 344 days for $pH < 7$ and 129 days for $pH \geq 7$ (Andre-Fontaine *et al.*, 2015). It will remain virulent and induce lethal disease even though the $pH < 6$, cold and in acidic environment.

2.7.2 Socio-economic

Outbreaks of *Leptospira* are mostly associated with socio-economic and business. According to Browne (1982), businesses such as feed-processing plants, bakeries, butcheries, poultry farms, markets, warehouses and restaurants make ideal habitats for infected rodents and have high risk of leptospirosis. For example, the rodents will consume large amounts of food and contaminate the surface of packaged food and canned drinks through gnawing, urinating and defecating. Furthermore, database showed that leptospirosis was detected in rodents in the USA and Canada in the 1970s, decreased in the 1980s and increased in the 1990s (Chomel, 2014). Almost 65% of food businesses were infested with infected rodents and the owners were unaware of the diseases caused by

rodents (Browne, 1982).

2.8 Ellinghausen-McCullough-Johnson-Harris (EMJH)

EMJH medium is the most commonly used for culturing *Leptospira* because they do not grow on other conventional media and need specific media that favour their growth. Media need to be supplemented with vitamins, ammonium salts and long chain fatty acids which act as carbon source for metabolism process of *Leptospira* with beta oxidation to enhance their growth (Levett, 2001). According to Wuthiekanun *et al.* (2014), EMJH liquid medium must contained 3% rabbit serum while semisolid medium must contained 3% rabbit serum and 0.1% bacteriological agar. To minimize bacterial contamination, EMJH medium must be supplemented with 100-400 mg/mL 5-fluorouracil (Benacer *et al.*, 2013). If the concentration of 5-fluorouracil is too high, the growth of *Leptospira* may be inhibited. Levett & Haake (2009) explained that growth of *Leptospira* takes about several weeks because they grow quite slow in the medium. Turbidity, granular appearance or deposit forms presence in the broth if the growth of *Leptospira* succeed.

2.9 Polymerase Chain Reaction (PCR)

Leptospira is not visible by gram staining but requires dark-field microscopy or special coloration (Lagier *et al.*, 2015). According to Mwachui *et al.* (2015), other methods that can be used to detect *Leptospira* are Microscopic Agglutination Test (MAT), Enzyme-Linked Immunosorbent Assay (ELISA) and Polymerase Chain Reaction (PCR). PCR method performs better than other tests because it can directly detect the genome of various type of *Leptospira* spp. that are present on packaged and canned food and drink in early stage. It also reduces contamination, faster and provides accurate detection of

Leptospira spp. (Pui *et al.*, 2017). For amplifying the target gene, specific primers for specific species are used to detect *Leptospira* spp.. *LipL32* gene in pathogenic *Leptospira* can be detected using *Lip*L32-270F or *Lip*L32-692R primer while *rrs* gene in saprophytic *Leptospira* can be detected using *Sapro1* or *Sapro2* primer (Vein *et al.*, 2012). *rrs*F and *rrs*R primers will be targetted *16S rRNA* gene in intermediate *Leptospira* (Benacer *et al.*, 2013).

3.0 MATERIALS AND METHODS

3.1 Samples Collection

A total of 30 samples of packaged food and canned drinks such as Chipsmore, Zess chocolate, Mamee and Coca Cola canned drinks were purchased randomly from the study sites in Kota Samarahan and Kuching, Sarawak. The study sites include canteen at a public university, wet market, supermarket, mall, restaurant, Indian Street and Kuching Waterfront. The samples were placed and sealed immediately in stomacher bag. The samples were taken to the laboratory immediately for further analysis.

3.2 Media Preparation

First of all, 0.51 g of Difco *Leptospira* medium base EMJH (Becton Dickinson, USA) was mixed with 200 mL distilled water. Next, 0.1 g 5-fluorouracil was added into the mixture to minimize contamination. The liquid EMJH broth was sterilized to ensure all contaminants and bacteria are removed. Hundred mL of Difco *Leptospira* enrichment EMJH (Becton Dickinson, USA) was added into the mixture. The mixture was distributed into 30 test tubes, 10 mL each.

3.3 Collection of *Leptospira* spp.

Swabbing method was used to collect *Leptospira* on the surface of packaged food and canned drinks using the method as employed by Dantas *et al.* (2006). Steriled cotton bud was moistened with liquid EMJH broth with 100 μg/mL 5-fluorouracil. Moistened cotton bud was swabbed on the packages and cans surface. Next, the cotton bud was placed into 10 mL EMJH immediately. Cotton bud was massaged vigorously to release *Leptospira* into EMJH. The cotton bud was discarded and the culture was incubated aerobically at room temperature for 1 month.

3.4 Genomic DNA Extraction

After 1 month of incubation, growth occurs as a turbid or ringed-area below the surface of the medium. DNA isolation was performed using Wizard™ Genomic DNA Purification Kit (Promega Corporation, USA) following manufacturer's instructions for PCR purposes. Briefly, 1.5 mL culture was transferred into 2.0 mL microcentrifuge tube and centrifuged at 10,700 rpm for 5 minutes. Supernatant was removed and 600 μL Nuclei Lysis Solution was added, followed by vortexed for 10 seconds. The tube was then incubated in water bath at 80 °C for 5 minutes and cooled at room temperature. Next, 3 μL RNase A Solution was added and inverted for 2-5 times to mix. The tube was incubated in water bath at 37 °C for 30 minutes and cooled in ice for 5 minutes. Then, 200 μL Protein Precipitation Solution was added, vortexed vigorously for 20 seconds and incubated on ice for 5 minutes. After incubation, the tube was centrifuged at 10,700 rpm for 3 minutes. The supernatant that contained DNA was transferred into 1.5 mL microcentrifuge tube that contained 600 μL isopropanol at room temperature and mixed gently until thread-like strands of DNA was formed. Again, the tube was centrifuged at 10,700 rpm for 2 minutes and the supernatant was discarded. The pellet was drained and washed by adding 600 μL 70% ethanol at room temperature. Next, the tube was inverted gently and centrifuged at 10,700 rpm for 2 minutes. The ethanol was aspirated carefully and the tube was drained on clean absorbent paper. The pellet was allowed to air dry for 10-15 minutes. Lastly, 100 μL DNA Rehydration Solution was added and incubated at 4 °C overnight before proceed to molecular analysis by using PCR.

3.5 Molecular Detection of *Leptospira* spp.

The DNA was amplified using the specific Polymerase Chain Reaction (PCR) method. Pathogenic *Leptospira noguchii* strain LT796, intermediate *Leptospira wolffii* serovar Khorat strain Khorat H-2 and saprophytic *Leptospira biflexa* serovar Patoc strain Patoc 1 was used as positive controls. Three sets of primers were used to detect *LipL32*, *16S rRNA* and *rrs* genes in pathogenic, intermediate and saprophytic *Leptospira* respectively (Pui *et al.*, 2017). The primers sequence used in this study are shown in Table 1.

Table 1: Primers sequence for detection of different genes in *Leptospira* spp.

Genes	Primers sequence	Amplicon size	References
LipL32 gene in pathogenic *Leptospira*	*Lip*L32-270F (5'-CGCTGAAATGGGAGTTCGTATGATT-3') *Lip*L32-692R (5'-CCAACAGATGCAACGAAAGATCCTTT-3')	423 bp	Pui *et al.*, 2017 Pui *et al.*, 2017
16S rRNA gene in intermediate *Leptospira*	*rrs*F (5'-GGCGGCGCGTCTTAAACATG-3') *rrs*R (5'-TTCCCCCCATTGAGCAAGATT-3')	331 bp	Pui *et al.*, 2017 Pui *et al.*, 2017
rrs gene in saprophytic *Leptospira*	*Sapro1* (5'-AGAAATTTGTGCTAATACCGAATGT-3') *Sapro2* (5'-GGCGTCGCTGCTTCAGGCTTTCG-3')	240 bp	Pui *et al.*, 2017 Pui *et al.*, 2017