ISOLATION, SCREENING AND CHARACTERIZATION OF ANTIBIOTIC RESISTANT *Listeria monocytogenes* FROM FOOD SOURCE

By

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ABSTRACT

ISOLATION, SCREENING AND CHARACTERIZATION OF ANTIBIOTIC RESISTANT *Listeria monocytogenes* FROM FOOD SOURCE

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Listeria monocytogenes is a Gram-positive foodborne pathogen that causes listeriosis in human. It affects mainly the pregnant women, newborns, the elderly and immunocompromised individuals with high fatality rate. Since the first outbreak of listeriosis, this disease has become increasingly common nowadays. Besides placing efforts to prevent the outbreak, increase awareness of the emergence of multidrug resistant *L. monocytogenes* strains must be relayed to the public. Treatment for listeriosis has been difficult because there is no effective drug to act against this pathogen, which usually displays multiple antibiotic resistance. In this study, *L. monocytogenes* were isolated from chicken offal in Kampar, Perak using selective enrichment and plating method. Characterization of *Listeria spp*. was done through colony morphology observation and a series of biochemical tests: gram stain, catalase test, oxidase test, Sulfide-Indole-Motility (SIM) test and hemolysis test. Afterwards, ELISA and PCR were carried out for confirmation of *L. monocytogenes* isolates. On the other hand, the antibiotic profiles of presumptive strains were tested against six antibiotics: tetracycline, ampicillin, trimethoprim, chloramphenicol, erythromycin and gentamicin. The isolates demonstrated resistance to most of

the antibiotic tested. PCR of antibiotic resistant genes showed that 83% (5/6) of the isolates carried *bla*SHV gene while none carried *ermA* and *ermC* gene. Thus, erythromycin resistance in the strains were probably due to another category of *erm* gene while ampicillin resistance in the isolates were contributed by the *bla*SHV gene. However, results from ELISA and molecular identification in this study could not confirm *L. monocytogenes* strains due to the arising of various mutant strains of foodborne *L. monocytogenes*. Overall, isolation of *L. monocytogenes* from food source that may come with various foodborne mutant strains can be a lengthy and laborious process. A more advanced technique such as MALDI-TOF mass spectrometry will be more reliable and efficient for the identification of the foodborne *L. monocytogenes* strains.

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DECLARATION

I hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at Universiti Tunku Abdul Rahman or other institutions.

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TANG MEI YIK

APPROVAL SHEET

This dissertation entitled "**ISOLATION, SCREENING AND CHARACTERIZATION OF ANTIBIOTIC RESISTANT *Listeria monocytogenes* FROM FOOD SOURCE"** was prepared by TANG MEI YIK and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman.

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PERMISSION SHEET

It is hereby certified that **TANG MEI YIK** (ID No: **15ADB03851**) has completed this final year project entitled "**ISOLATION, SCREENING AND CHARACTERIZATION OF ANTIBIOTIC RESISTANT *Listeria monocytogenes* FROM FOOD SOURCE**" under the supervision of Dr. Chew Choy Hoong from the Department of Biomedical Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project in pdf format into UTAR Institutional Repository, which may be made accessible to UTAR community and public.

Yours truly,

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(TANG MEI YIK)

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LIST OF ABBREVIATIONS

ALOA	Agar *Listeria* according to Ottaviani and Agosti
AMP	Ampicillin
AnrAB	ABC transporter
bp	Base pair
C	Chloramphenicol
CAMP	Christie, Atkins, and Munch-Peterson
CN	Gentamicin
DNA	Deoxyribonucleic acid
E	Erythromycin
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EN	European
EUCAST	European Committee on Antimicrobial Susceptibility Testing
H_2O	Distilled water
HGT	Horizontal gene transfer
ISO	International Organisation for Standardisation
LB	Luria-Bertani
LLO	Listeriolysin O
MALDI-TOF	Matrix assisted laser desorption ionization-time of flight
MRSA	*Methicillin-resistant Staphylococcus aureus*
NaCl	Sodium chloride

NFP	National Focal Point
NICD	National Institute of Communicable Diseases
PALCAM	Polymyxin Acriflavin Lithium chloride Ceftazidime Aesculin Mannitol
PCR	Polymerase chain reaction
SIM	Sulfide-Indole-Motility
SXT	Sulfamethoxazole-trimethoprim
TBE	Tris/Borate/EDTA
TE	Tetracycline
Tris-HCl	*Tris hydrochloride*
UVM I	Listeria Primary Selective Enrichment Medium
UVM II	Listeria Secondary Selective Enrichment Medium
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Background Information

Listeria monocytogenes is a gram-positive bacteria which belongs to the genus *Listeria spp*. According to a recent study by Orsi and Wiedmann in 2016, there are now a total of 17 Listeria species recognized, including *L. aquatica*, *L. booriae*, *L. cornellensis*, *L. fleischmannii*, *L. floridensis*, *L. grandensis*, *L. grayi*, *L. innocua*, *L. ivanovii*, *L. marthii*, *L. monocytogenes*, *L. newyorkensis*, *L. iparia*, *L. rocourtiae*, *L. seeligeri*, *L. weihenstephanensis* and *L. welshimeri* (Orsi and Wiedmann, 2016). Among these 17 species, only three of them: *L. monocytogenes*, *L. ivanovii* and *L. seeligeri*, have raised the concern of researchers due to their pathogenic properties (Liu, 2008).

Nevertheless, *L. monocytogenes* is the main target of many studies because it is ubiquitous in the environment as a foodborne pathogen. Most of the *L. monocytogenes* strains can endure in environment of wide pH range (4.1-9.6) and high salt concentration up to 14 %. Its ability to survive in such diverse environment renders it chances to contaminate various food sources (Fallah, Saei-Dehkordi, and Mahzounieh, 2013). Food that are highly susceptible to its contamination include soft cheese, raw meat, raw milk and ready-to-eat meat (Swaminathan and Gerner-Smidt, 2007). Secondly, *L. monocytogenes* is the major human pathogen that causes foodborne listeriosis, with 20-30 % high fatality rate and more than 92 % hospitalization (Du, et al., 2017). In contrast,

infection due to *L. ivanovii* and *L. seeligeri* has been described in rare cases (Liu, 2008).

Listeriosis is mostly recognized in immunocompromised individuals, pregnant women, newborns and the elderly (Liu, 2008). As such, emergence of listeriosis has become an important issue as over the decades of 20th century, the aging population has increased due to improvement in medicine and the number of immunocompromised individuals has raised due to cancer and diseases (Lungu, et al., 2011). The principal route of infection is always believed to be through the consumption of contaminated food. However, it is also stated that listeriosis could also be transmitted by direct contact with the environment or the infected animals, or by cross-infection between patients during neonatal period, though these only happened rarely (Liu, 2008). Symptoms of this disease are flu-like and sometimes may include nausea or diarrhea. Headache, stiff neck, confusion, loss of balance or convulsions can occur if infection spread to the nervous system. In fact, infected patients are most likely to get affected at the uterus, the central nervous system and the bloodstream. Therefore, it can lead to severe complications such as spontaneous abortion, listeriosis of newborn, meningitis and septicemia (Gasanov, Hughes and Hansbro, 2005).

1.2 Significance of Study

Nowadays, expansion of bacterial resistance to a wide range of antibiotics has become a threat to global health and development as it had led to the lack of new and effective antibiotics for disease treatment (Mölstad, et al., 2017).

As mentioned, *L. monocytogenes* is ubiquitous in nature. Other than being able to tolerate with wide pH range and high salt concentration, it also has the ability to resist the actions of multidrug. Thus, it has drawn the attentions of researchers nowadays. Based on a review article in 2011, the antibiotic-resistant strain of *L. monocytogenes* was first described in 1988 in France (Lungu, et al., 2011). Since then, many more resistant strains have been found. Over the last decades, this situation has worsened by the increased number of multi-drug resistant *L. monocytogenes strains* isolated from food source (Kuan, et al., 2017). The antimicrobial profiles of multidrug resistant *L. monocytogenes* strains appeared to be very diverse depending on its exposure to the antibiotics in different geographical area and environment factor (Escolar, et al., 2017). Treatment towards these antibiotic-resistant bacteria has become more difficult, particularly if the bacteria are resistant to the current listeriosis treatment.

Hence, it is indeed important to understand the antibiotic profiles of *L. monocytogenes* in different area in order to monitor the environmental spread of antibiotic resistance, revise dosing of antibiotics and decide the suitable treatment for listeriosis (Du, et al., 2017).

1.3 Research Objectives

Therefore, the main objectives for this project are:

a) To isolate *Listeria monocytogenes* from food source in Kampar.

b) To identify and characterize the isolated *Listeria monocytogenes* through biochemical and molecular tests.

c) To detect for the presence of antibiotic resistance profile and its gene determinants in the isolated *Listeria monocytogenes.*

CHAPTER 2

LITERATURE REVIEW

2.1 *Listeria spp.* Characteristics and Listeriosis Pathogenesis

All *Listeria spp.* is rod-shaped Gram-positive, catalase-positive, oxidase-negative (Zhu, et al., 2012) and facultative anaerobes that is able to grow at low temperatures. It can tolerate various environmental stresses. For instances, low pH and high salt concentrations and such features are the reasons that made it a major concern in the food industry (Radoshevich and Cossart, 2018).

L. monocytogenes is a foodborne pathogen (Mehdizadeh, et al., 2015) belongs to the *Listeria spp.* which causes serious listeriosis in human. After ingestion of *L. monocytogenes* contaminated food, the organism can move across the intestinal barrier in the body. After that, it can spread into the bloodstream through lymph nodes. Following the circulation, it will disseminate to body tissues, as an example, the liver (Radoshevich and Cossart, 2018). Surviving bacteria that replicate in liver cells will recruit polymorphonuclear cells which eventually leads to hepatocyte lysis and thereby more bacterial release into the systemic circulation (Hernandez-Milian and Payeras-Cifre, 2014).

Besides, with the use of various host proteins and some internalins as adhesion tool, *L. monocytogenes* is able to invade host cell and survive within it by secreting listeriolysins and phospholipases. These compounds lyse the host vacuolar membrane and allow it to avoid the intracellular killing (Hernandez-

Milian and Payeras-Cifre, 2014). Replication of this organism in the host cell cytosol causes a change in the morphology of host cell organelles and alters their function to promote infection (Radoshevich and Cossart, 2018). Subsequently, cell-to-cell spread also occurs through protrusion of plasma membrane of adjacent cells. This enclosed environment for its movement enables it to escape the human immune system (Hernandez-Milian and Payeras-Cifre, 2014). In immunocompromised individuals, it is able to cross the blood–brain barrier and fetoplacental barrier, causing potentially fatal meningitis, sepsis, premature birth or abortion (Radoshevich and Cossart, 2018).

A review study about *L. monocytogenes* stated that incubation period of this pathogen ranges between 3 and 70 days. This was according to observations made by Linnan et al. from a large listeriosis outbreak associated with Mexican-style cheese in 1988 (Camargo, et al., 2017). In recent years, another study by Goulet et al. demonstrated that incubation periods were significantly different and subjected to the clinical form of the disease. In detail, longer incubation periods are associated with pregnancy cases (median 27.5 days), followed by infections in the central nervous system (median 9 days), sepsis (median 2 days), and febrile gastrointestinal disease (median 24 h) (Goulet, et al., 2013).

2.2 Worldwide Incidence of Listeriosis

Hernandez-Milian and Payeras-Cifre reported in their study that the annual incidence of listeriosis was ranged between 0.1 and 1 case per 100.000 inhabitants. Even though listeriosis is less common compared to other

foodborne diseases, in the USA and France, 19% and 17% of deaths associate with foodborne disease respectively, are caused by *L. monocytogenes* (Hernandez-Milian and Payeras-Cifre, 2014).

In USA, listeriosis was not a prominent disease until 2000. The incidence of listeriosis was decreased by 24% from 1996 through 2001. But since then, there has been an increased incidence. As reported by Silk et al., the incidence rate of listeriosis was 0.27 cases per 100,000 US inhabitants between 2004 and 2009 (Silk, et al., 2012). The incidence rate then increased further to 0.29 cases per 100,000 inhabitants from 2009 to 2011. In adults aged over 65 years, the incidence was 1.3 cases per 100,000 inhabitants (Hernandez-Milian and Payeras-Cifre, 2014).

In Europe countries, despite its low incidence with 0.4 cases per 100 000 population, invasive listeriosis has become a major concern to public health due to its high rate of hospitalization (90%) and fatality (20-30%) (Pontello, et al., 2012). In 2013 alone, 1763 confirmed human cases of listeriosis was reposted by the 27 member states of Europe. Overall, there was total of 191 deaths from the cases (Camargo, et al., 2017).

In Asia country, listeriosis is perhaps not a notifiable disease yet. From 1996 to 2008, Taiwan reported only 48 cases of confirmed listeriosis. Before and after 2005, Huang et al. stated an average of increased incidence from 0.029 to 0.118 cases per 1,000 admissions (Huang, et al., 2011). In China, 479 isolated

of *L. monocytogenes* were reported from 1964 to 2010 and 82 of them were outbreak-related cases (Hernandez-Milian and Payeras-Cifre, 2014).

In 2017, the first case of the current listeriosis outbreak in South Africa was reported in January. Exactly one year later, the South African National Institute of Communicable Diseases (NICD) reported 945 confirmed cases on 27 Feb 2018. Exactly 176 patients had died, resulting in the case fatality rate of 19%. With this, South Africa's listeriosis outbreak has topped the charts as the largest outbreak in history (Ogunbanjo, 2018).

Listeriosis in Malaysia seems to have a very low incidence but concern has been raised in recent due to the outbreak cases in Australia. In March 2018, WHO was notified by the Australian National Focal Point (NFP) on the outbreak of *L. monocytogenes* infection associated with the consumption of rockmelons (World Health Organization, 2018) and the outbreak has cost six lives so far (Megan, 2018). Following the outbreak, a temporary ban on imported rockmelons from Australia has been imposed in Malaysia as a safety measure (Irwan, 2018).

2.3 Foodborne *L monocytogenes* and Its Source in the Food Environment

L. monocytogenes is widely distributed in the natural environment and it can be isolated from many different sources such as soil, water, drainage systems, vegetation, animal feed, farm environments, food processing facilities, various foods, and feces of healthy animals, including humans (Camargo, et al., 2017).

Slaughtered animals are recognized as reservoir for human foodborne pathogen, hence the internal organs of animals are good sources of *L. monocytogenes* (Kuan, et al., 2013). In Malaysia, a research was carried out to determine the prevalence of *L. monocytogenes* in foods in Malaysia in 1994. *L. monocytogenes* contamination was detected in high prevalence (63%) in chicken portions, liver, gizzard and dried oyster (Arumugaswamy, Ali and Hamid, 1994). Another findings in 2013 also showed the presence of *L. monocytogenes* in chicken offal such as chicken liver, gizzard and chicken heart with prevalence of 26.4% (Kuan, et al., 2013).

As it is a foodborne pathogen, cross contamination can readily happen and increases the rate of infection. According to several studies, food contamination by *L. monocytogenes* has high occurrence rate at the food processing level (Camargo, et al., 2017). In Brazil, research has documented different strains of *L. monocytogenes* isolated from different raw meats in samples collected from table, hands of employee, gloves and equipment in the food processing plant (Barbalho, et al., 2005; Barros, et al., 2007; Camargo, et al., 2015). In another study carried out by Zhu in 2012 in China, over 100 isolates also have been found in beef-processing factory, including samples such as carcasses, feces and raw meat (Zhu, et al., 2012).

Presence of *L. monocytogenes* has also been reported in raw milk in different countries such as USA, Canada, England and South Africa. Contaminated milk and cheese product are among the most common cause in outbreak of listeriosis across the world (Pal and Awel, 2016). Study found out that the occurrence of

L. monocytogenes in raw milk is probably through the gastrointestinal tract of animals and the environment, skin of teats, particularly shedding of *Listeria* into milk because of mastitis of animals (O'Donnell, 1995).

Other than meat and raw milk products, there were also research that has successfully isolated *L. monocytogenes* from ready-to-eat vegetables such as cucumber, cabbage, carrot, bean sprouts, lettuce and tomato. Findings by Ajayeoba and colleagues in 2016 in South-Western Nigeria showed the distribution of this foodborne pathogen in cucumber was 23.36%, 28.28% for cabbage, 9.02% for carrot and 19.67% for lettuce and tomatoes (Ajayeoba, et al., 2016). Over the past 25 years in Malaysia, Arumugaswamy isolated *L. monocytogenes* from bean sprouts with frequencies as high as 85% (Arumugaswamy, Ali and Hamid, 1994). Another study by Ponniah and the team in Malaysia also proved the prevalence of *L. monocytogenes* in raw salad vegetables; i.e. 31.3% in yardlong bean, 27.2% in Japanese parsley and 25% in wild parsley (Ponniah, et al., 2010).

By proving the existence of *L. monocytogenes* in various kind of food source, several authors concluded the impossibility to eradicate this pathogen from the food environment since it has a ubiquitous nature and many potential chances for its entry into the facility. However, people can eliminate and exclude it by managing and monitoring through adequate hygienic design of a food-processing plant, effective cleaning and sanitation of food premise, proper personnel practices and transportation of food (Buchanan, et al., 2017).

2.4 The Antimicrobial Resistance Crisis

Antimicrobial resistance is defined as the ability of a microorganism to survive against the growth inhibitory or killing effect of antibiotics (Verraes, et al., 2013). In recent decades, presence of antimicrobial resistance gene in commensal, pathogenic as well as environmental bacteria is causing global health crisis because they form a reservoir of antibiotic resistance genes (ARGs), the resistome (von Wintersdorff, et al., 2016). Basically, bacteria can be intrinsically resistant to a particular antibiotic. Yet, they can also acquire antimicrobial resistance through mutations in chromosomal genes or by horizontal gene transfer (HGT) (Blair, et al., 2015). With resistome, it therefore provides a diverse gene pool for pathogenic bacteria to acquire resistance traits via HGT (Verraes, et al., 2013).

According to findings by the World Economic Forum Global Risks, it is estimated that each year in Europe, 25,000 people die from multidrug-resistant bacterial infections and this can cost the European Union economy up to €1.5 billion annually. In the United States, more than 2 million cases of antibiotic resistant bacterial infection happen annually, with 23,000 deaths reported (Blair, et al., 2015). Although findings in Malaysia was limited in this aspect, antibiotic resistance awareness has been promoted through program such as National World Antibiotic Awareness Week in 2017 (Suresh, 2017). This increased magnitude of antimicrobial resistance in pathogenic bacteria has limited the treatment option for bacterial infections. Consequently, this reduces clinical efficacy with increasing treatment cost and mortality (von Wintersdorff, et al., 2016).

Recently, there is a prominent emergence of antibiotic-resistant bacteria strains in the food industry, including human foodborne pathogens such as *Staphylococcus aureus*, *Salmonella* and *L. monocytogenes* (Lungu, et al., 2011). Indeed, there are several practices in the food industry that can lead to the contamination of food by antimicrobial bacteria or antimicrobial resistance genes. Firstly, the use of antibiotics during agricultural production selectively favored the growth of certain antibiotic resistant bacteria. The second route is during food processing, bacteria that are added intentionally for preservation purpose such as probiotics or bio-conserving microorganisms may possess resistance genes in them. Lastly, cross-contamination of antibiotic resistant bacteria in the food preparation process. In fact, people consuming raw food products that are not prior processed or preserved hold a considerable risk in transferring the antimicrobial resistance of unkilled resistant bacteria. The bacteria can transfer their resistance gene between each other after ingestion (Verraes, et al., 2013).

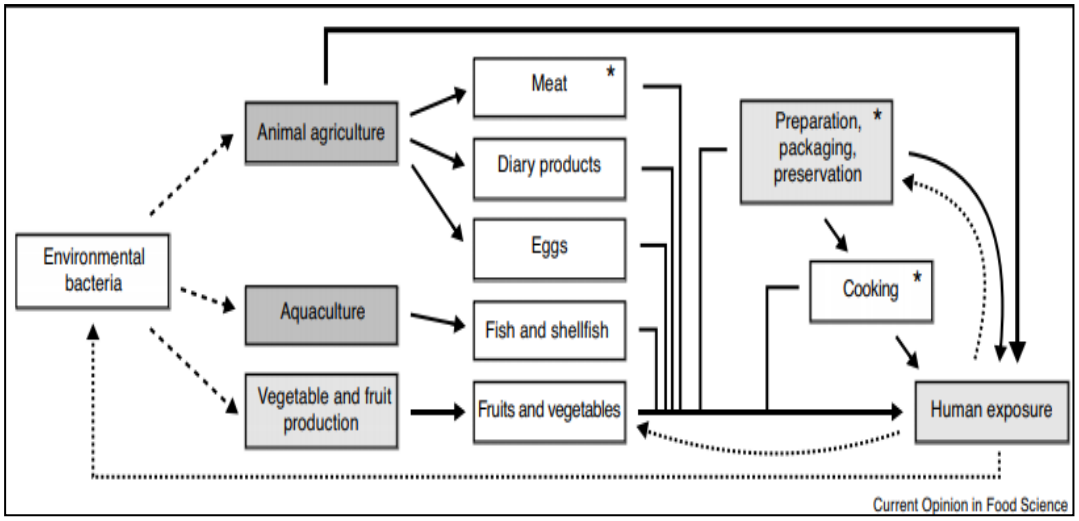


Figure 2.1: A simplified schematic overview of the flow of resistant bacteria and resistance genes in the food supply chain (Bengtsson-Palme, 2017).

2.5 Antimicrobial Resistance Mechanisms of *L. monocytogenes*

According to the study by Charpentier, Gerbaud and Courvalin in 1999, they proved that the antibiotic resistance in *L. monocytogenes* is mainly due to the mobile genetic elements, which include: self-transferable plasmids, mobilizable plasmids, and conjugative transposons (Charpentier, Gerbaud and Courvalin, 1999). Soon afterwards, efflux pumps was found to be responsible for certain antibiotic resistance mechanism in *Listeria spp.* (Godreuil et al., 2003; Lungu, et al., 2011).

There are two important facilitator superfamily efflux pumps in *L. monocytogenes*. MdrL, the first pump, which is responsible in detoxification of the antibiotics, macrolides and cefotaxime. Other than that, it also detoxifies heavy metals and ethidium bromide. (Mata, Baquero and Perez-Diaz, 2000).

Following this, a study by Romanova et al. in 2006 proved that the MdrL pump was partially related to the adaptation of disinfectant, benzalkonium chloride (Romanova, et al., 2006). A second pump, designated Lde, on the other hand, contributes to tolerance with fluoroquinolone and DNA intercalating agents (Godreuil, et al., 2003). In 2010, a third efflux pump, namely AnrAB, was described by Collins et al. This pump belongs to the ABC multidrug resistance transporter family and it confers resistance to nisin, bacitracin, and β-lactam antimicrobials, such as ampicillin, cephalosporins and oxacillin (Collins, et al., 2010).

2.5.1 Antibiotic Resistance Profiles of *L. monocytogenes* in Other Studies

In 2001, Walsh et al. reported 0.6% of *L. monocytogenes* isolates from retail foods were resistant more than one antibiotics, including tetracycline and ampicillin. This proportion of antibiotic-resistant *L. monocytogenes* were still relatively low during that time.

In 2002, Antunes and colleagues observed that isolates of *L. monocytogenes* from poultry carcasses in Portugal also exhibited multi-antimicrobial resistance. With this, it implicated poultry as a potential vehicle for antibiotic-resistant foodborne illness. Another food source that serve as potential vehicle later in 2005 was the dairy farm environment, where Srinivasan et al. isolated *L. monocytogenes* that exhibited multiple resistance to cephalosporin C, streptomycin, and trimethoprim (Srinivasan, et al., 2005).

In recent years, a research carried out to determine the antibiotic susceptibility of *L. monocytogenes* from food and humans in China showed that none of the isolates were resistant to multiple antibiotics. The isolates were all susceptible to most antibiotics, including ampicillin, penicillin, erythromycin, gentamicin, trimethoprim and chloramphenicol. Only 13 isolates were resistant to ceftriaxone and seven to tetracycline (Su, et al., 2016). Some studies of *L. monocytogenes* isolated from India in previous year, however, reported high percentage of resistance to penicillin (100%), tetracycline (90.5%), and erythromycin (85.7%) (Obaidat, et al., 2015). The greatest concern is the finding of vancomycin resistance (26.7%) among the *L. monocytogenes* isolates from dairy-based food product in another research in India because this antibiotic is currently a treatment option for multidrug-resistant Gram-positive bacteria (Harakeh, et al., 2009).

In Australia, *L. monocytogenes* samples encompassing isolates from different food source were recovered from the food production chains between 1988 and 2016. All the isolates were susceptible to penicillin G or tetracycline but a single isolate resistant to erythromycin, which harbored the *ermB* gene. In fact, resistance rate to erythromycin depicted an increased trend in other countries such as Indonesia (6.9%), Iran (14%), Yemen (58.8%), Egypt (62.5%), and India (85.7%) (Wilson, et al., 2018).

From these findings, it can be concluded that there are many variations in the antimicrobial profiles of *L. monocytogenes* depending on different geographical area and environmental factor.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials and Equipment

3.1.1 Chemical Reagents and Manufacturers

Refer to appendix A.

3.1.2 Labware, Equipment and Instrument

Refer to appendix B.

3.2 Food Samples

There were four different kind of food samples selected for this study, the chicken skin, chicken liver, chicken gizzards and bean sprouts. All food samples were collected from stalls in the morning market in Kampar. There were three batches of samples collected in total. Table 3.1 shows the type of food sample, their respective batch number and the assigned code.

Table 3.1: Type of food samples, batch number and the assigned code.

Type of food sample	Batch no.	Assigned code
Chicken skin	First	CS
	Second	SS
	Third	SV
Chicken liver	First	CL
	Second	LL
	Third	LV
Chicken gizzards	First	CG
Bean sprouts	First	BS

3.3 Preparation of Culture Medium and Isolation of Bacteria

3.3.1 Preparation of Culture Medium and Agar

Culture medium used were prepared according to the manufacturers' instructions as shown in Table 3.2.

Table 3.2: Culture medium preparation protocol.

Type of culture medium	Amount of powdered media (g)	Amount of deionized water (ml)
UVM I broth	54.4	1000
UVM II broth	54.4	1000
PALCAM agar	35.4	500
LB agar	35.0	1000
Mueller Hinton agar	38.0	1000
Nutrient broth	8.0	1000
Nutrient agar	20.0	1000
SIM medium	30.0	1000

Powdered media was suspended in deionized water and stirred to mix well until complete dissolution. Prepared media were autoclaved at 120°C, 15 psi before use. Autoclaved agar was allowed to cool to 45-50°C and poured into the sterile petri dishes. Plates were allowed to solidify in a disinfected laminar flow cabinet. Agar plates were then stored in the chiller until further use.

3.3.2 Isolation of Bacteria *L. monocytogenes* from Food Samples

Food samples were cut into smaller pieces using a sterile scalpel. An amount of 25±1g of the food sample was weighed and added into 100 ml of *Listeria* primary enrichment broth, UVM I in a conical flask covered with aluminum foil. Conical flasks containing UVM I and food sample were agitated at 200 rpm at room temperature overnight. One milliliter of primary enrichment culture was pipetted into 9 ml of *Listeria* secondary enrichment broth, UVM II in a universal bottle and then agitated at 200 rpm at room temperature overnight. Secondary enrichment culture was then streaked on *Listeria* selective PALCAM agar and incubated at room temperature for about 48 hours (Fallah, Saei-Dehkordi and Mahzounieh, 2013). Bacteria colonies were purified by continuous streaking of presumptive colonies (grey-green colonies surrounded with black halo) until uniform morphologies of colonies were obtained in a single plate.

3.4 Biochemical Characterization of Bacterial Isolates

From each purified plate, three presumptive *Listeria* colonies were selected for a range of general biochemical tests: Gram staining, catalase test, oxidase test, motility and hydrogen sulfide test, antibiotic susceptibility test, blood agar hemolysis and CAMP test and F48 Microgen^{TM} Listeria Kit test. Fresh cultures of bacterial isolates were prepared by streaking onto new PALCAM agar plate and incubated at room temperature overnight prior to any biochemical test.

3.4.1 Gram Staining

Three presumptive colonies of the bacteria from each purified plate were selected for further analysis. A small bit of selected bacteria colony was thoroughly mixed with one drop of 0.85% (v/v) sterile saline water and smeared on a clean microscopic slide. The slide was allowed to air dry and then heat-fixed by passing the slide rapidly through Bunsen flame three times. After heat fixation, the smear was flooded with crystal violet (purple dye) stain for 1.5 minutes. The stain were rinsed off gently using tap water and covered with Gram's stain iodine (mordant). After 1.5 minutes, the Gram's iodine was rinsed off and excess stain on the glass slide were decolorized with 95 % (v/v) ethanol for 10 seconds. The ethanol was rinsed off and counterstained with safranin for 1.5 minutes. Lastly, safranin stain was washed off. The slide is dried between blotting paper and examined under the oil-immersion objective of light microscope.

3.4.2 Catalase Test

A small amount of presumptive bacteria colony was transferred to a clean, dry slide using sterile wooden stick. A drop of 3% (v/v) hydrogen peroxide solution was added and mixed with the bacteria culture. Rapid evolution of bubbles within 5 to 10 seconds would indicate a positive reaction of the particular bacteria strains.

3.4.3 Oxidase Test

A drop of BactiDrop™ Oxidase reagent was placed onto a piece of filter paper. Using sterile wooden stick, a speck of bacteria culture was scrapped and smeared on the area of filter paper which was soaked with BactiDrop™ Oxidase reagent. Within 10 seconds, any observation of a deep purple-blue color bacteria spot would indicate a positive reaction.

3.4.4 Sulfide-Indole-Motility (SIM) Test

The presumptive colonies were inoculated into SIM medium by stabbing the center of the medium prepared in a polystyrene tube. Diffusion of growth away from the stab in the medium would indicate the presence of motility. Non-motile bacteria, otherwise, would produce a clear stab line. A black precipitation show that there is production of hydrogen sulfide by inoculated bacteria. To check for indole formation, Kovacs reagent was added to the medium and presence of red color at top of medium would indicate a positive reaction.

3.4.5 Blood Agar Hemolysis and CAMP Test-S

Putative *Listeria sp.* colonies were streaked with single line on blood agar and incubated 37°C overnight to determine their β-hemolysis characteristics. β-hemolytic isolates would create clear zone of blood agar in the vicinity of their colonies. CAMP test was carried out by inoculating a single line of *Staphylococcus aureus* ATCC 25923 on the middle of blood agar. Putative bacterial isolates were drawn perpendicular to the *S. aureus* that was streaked.

A distance of one or two lines must be marked out between the inoculated putative bacteria and *S. aureus.* The plates were incubated at 37°C overnight. Positive CAMP test would be indicated by enhanced zone of hemolysis near the *S. aureus.*

3.5 F48 Microgen^{TM} Listeria Latex Kit

A disposable slide containing six wells provided in the kit was placed in sterile laminar flow cabinet. One drop of saline was added to one well on the disposable slide. Putative colonies were emulsified in the drop of saline using sterile mixing stick to produce heavy smooth suspension. Prior to test, *Listeria* Latex Reagent was gently mixed by inverting few times. A drop of latex reagent was then added into same well and mixed with the bacteria suspension. The mixture was spread over the entire area of well and gently rocked. Positive reaction would be seen as agglutination within 2 minutes, indicating the presence of *Listeria* species in the sample.

3.6 Antibiotic Susceptibility Test (Kirby-Bauer Disk Diffusion)

Three colonies from each presumptive pure culture plate were selected and transferred with inoculating loop into 1 ml of 0.85% (v/v) sterile saline water to suspend the inoculum. The saline tube was vortexed to create a smooth suspension. A sterile cotton swab was dipped into the inoculum tube and then rotated against the side of the tube above the suspension to ensure the swab was not dripping wet. The inoculum was then streaked onto Mueller Hinton agar

plate for three times over the entire agar surface. For each time of the streaking, the plate was rotated 60° between each turn for an even distribution of inoculum. Six types of antibiotic discs: tetracycline, ampicillin, trimethoprim, chloramphenicol, erythromycin and gentamicin, were placed onto the surface of agar using sterilized forceps. Inoculated plates were incubated agar side up at 37°C for 16-18 hours. Antibiotic susceptibility profile of bacteria was identified by measurement of zone of inhibition. Inhibition zones were measured in diameters and interpreted according to the guidelines of European Committee on Antimicrobial Susceptibility Testing (EUCAST) 2017.

3.7 Molecular Identification of Isolated Bacteria Strains and Their Antimicrobial Resistance

3.7.1 Total DNA Extraction for Isolated Strains

A volume of 1.2 ml of the overnight bacterial culture was harvested in 1.5 ml microcentrifuge tube and centrifuged at 13000 rpm for 5 minutes. The supernatant was discarded. This procedure was repeated twice in order to concentrate the bacterial cells. Bacterial pellet obtained was resuspended in 180 µl of sterile deionized water. A volume of 20 µl of 50 mg/ml lysozyme chloride was added into the tube and incubated at 37°C for 1.5 hours. After incubation, exactly 600 µl of 6 M guanidine hydrochloride were added. The mixture was incubated at 65°C for 1 hour. Tubes were then centrifuged at 13000 rpm for 5 minutes to pellet the cell debris. Supernatant was collected and 600 µl of 50 % (v/v) isopropanol was added. A maximum of 750 µl of the mixture of supernatant and isopropanol was transferred into silica minicollumn and

centrifuged at 13000 rpm for 1 min. The flow through in the collection tube was discarded. This step was repeated until all the mixture had undergone this process. Next, a total of 750 µl of washing buffer (20 mM Tris-HCl, pH7.4, 1 mM EDTA, 50 mM NaCl, 50% (v/v) ethanol) was pipetted into the minicollumn and centrifuged at 13000 rpm for 1 min. The flow through was also discarded. After the silica minicolumn was reinserted into the collection tubes, the tubes were sent for another round of centrifugation to remove any residual ethanol. About 20 µl of sterile deionized water was pipetted into the silica minicolumn and left to stand at room temperature for 1 minute. Lastly, the silica minicollumn was centrifuged at 13000 rpm for 1 minute. The eluate was used as the template DNA.

3.7.2 Total DNA Extraction for *bla*SHV Control Strain

One colony of bacteria was inoculated into 3 ml of nutrient broth in 15 ml centrifuge tube and incubated in shaking incubator at 37°C, with agitation at 200 rpm for overnight. Exactly 1.5 ml of bacteria suspension was pipetted and transferred into a microcentrifuge tube. The tube was centrifuged at 12000 rpm for 5 minutes. Supernatant was discarded and pellet was resuspended with 300 µl of sterile deionized water. After that, the bacteria suspension was boiled using heat block for 10 minutes and then cooled on ice for 5 minutes. The suspension was then centrifuged at 12000 rpm for 2 minutes in order to precipitate any cell debris. The supernatant containing the DNA was aspirated. Purity and concentration of DNA was measured using a spectrophotometer.

3.7.3 Polymerase Chain Reaction (PCR) Targeting *prs, ermA, ermC* and *bla*SHV

Polymerase Chain Reaction (PCR) allows screening of specific genes. PCR of four types of genes: *prs, erm A, erm C* and *bla*SHV using gene-specific forward and reverse primers were carried out. Positive and negative standard for each primers were included as control in all PCR reaction. PCR amplification for each of the gene of interest was performed in a total of 25 µl reaction volume. The constituents of PCR reaction mixture were listed in Table 3.3. Each primer sequences and expected sizes of amplicons are shown in Table 3.4. PCR cycle conditions for *erm A, erm C, prs* and *bla*SHV are listed in Table 3.5, Table 3.6, Table 3.7 and Table 3.8 respectively.

Table 3.3: The composition of PCR reaction mixture.

PCR reagent	Concentration	Volume (µl)
Taq buffer	10x	2.5
dNTPs	10mM	1.0
Taq polymerase	5 unit/ul	0.2
Forward primer	10 µM	1.0
Reverse primer	10 µM	1.0
DNA template	10 ng/µl	1.0
Sterile distilled water	-	18.3
Total reaction volume		25.0

Table 3.4: Primer sequences of gene of interest and expected size of amplicon.

Gene of interest	Primer sequences (5' → 3')	Expected size (bp)	Reference
prs	F: GCT GAA GAG ATT GCG AAA GAA G	370	(Doumith, et al., 2004)
	R: CAA AGA AAC CTT GGA TTT GCG G		
ermA	F: GTT CAA GAA CAA TCA ATA CAG AG	421	(Lina, et al., 1999)
	R: GGA TCA GGA AAA GGA CAT TTT AC		
ermC	F: GCT AAT ATT GTT TAA ATC GTC AAT TCC	572	(Lina, et al., 1999)
	R: GGA TCA GGA AAA GGA CAT TTT AC		
*bla*SHV	F: TGG TTA TGC GTT ATA TTC GCC	868	(Lee, et al., 2005)
	R: GGT TAG CGT TGC CAG TGC T		

Table 3.5: PCR cycle conditions for *prs* gene.

Primer	PCR stage	Temperature (°C)	Time (s)	No. of cycle	Reference
prs	Initial denaturation	94	180	1	(Doumith, et al., 2004)
	Denaturation	94	24		
	Annealing	53	69	35	
	Elongation	72	69		
	Final extension	72	420	1	

Table 3.6: PCR cycle conditions for *erm A* and *ermC*.

Primer	PCR stage	Temperature (°C)	Time (s)	No. of cycle	Reference
erm A	Initial denaturation	95	60	1	(Lina, et al., 1999)
ermC	Denaturation	95	30		
	Annealing	52	30	30	
	Elongation	72	60		
	Final extension	72	600	1	

Table 3.7: PCR cycle conditions for *bla*SHV.

Primer	PCR stage	Temperature (°C)	Time (s)	No. of cycle	Reference
*bla*SHV	Initial denaturation	96	300	1	(Lee, et al., 2005)
	Denaturation	96	30		
	Annealing	55	30	30	
	Elongation	72	60		
	Final extension	72	300	1	

3.7.4 1.5 % (w/v) Agarose Gel Electrophoresis

After PCR reaction, a small volume of 2.0 µl PCR product was mixed with 1.0 µl of loading dye. The sample was loaded into 1.5 % (w/v) agarose gel well immersed in 1xTBE buffer. The agarose gel was pre-stained with EtBr-out during gel preparation. Electrophoresis was carried out using 80V for about 50 minutes. Upon completion of gel electrophoresis, agarose gel was visualized using Gel Imaging System by Bio-rad, ChemiDoc XRS+ imaging system controlled by Image Lab software.

CHAPTER 4

RESULTS

4.1 Isolation of Bacteria *L. monocytogenes* from Food Samples

Using selective and differential PALCAM agar, only bacteria colonies that were cultured from chicken skin and chicken liver showed positive results. *Listeria spp.* produced colonies surrounded by black halo as shown in Figure 4.1. They were circular form, grey-green colonies with black sunken center. Figure 4.2 shows samples taken from chicken gizzards and bean sprouts. These plates showed negative results. A total of 80 presumptive colonies with *Listeria spp.* morphology were selected from chicken skin and chicken liver culture for Gram staining.

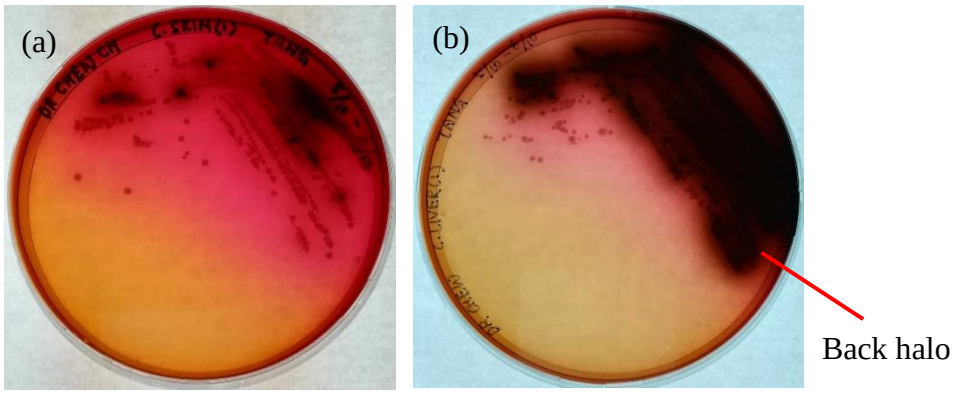


Figure 4.1: Bacterial culture inoculated from (a) chicken skin and (b) chicken liver on *Listeria* selective PALCAM agar.

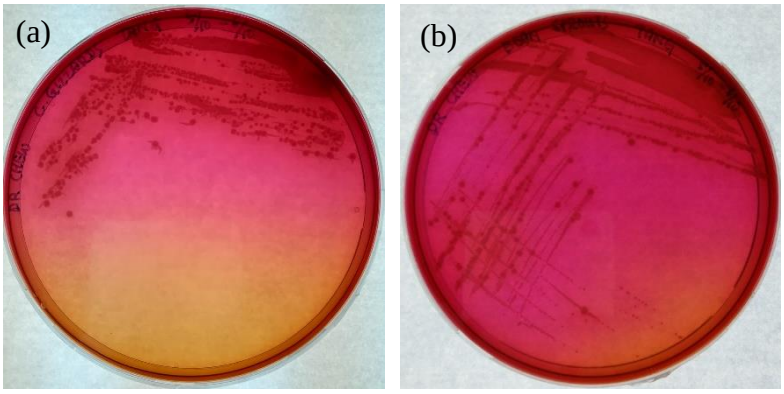


Figure 4.2: Bacterial culture inoculated from (a) chicken gizzards and (b) bean sprouts on *Listeria* selective PALCAM agar.

4.2 Biochemical Characterization of Bacterial Isolates

4.2.1 Gram Staining

Bacteria can be classified into two large groups using the common technique, gram staining. Gram-positive bacteria retain crystal violet-iodine crystal dye after staining, showing violet color under light microscope observation. Gram negative bacteria, on the other hand, is observed with pink colorization. Under observation with 1000X magnification of light microscope, *Listeria spp.* is rod-shaped gram positive bacteria. From the 80 presumptive colonies selected, only 12 of them were observed as violet rod-shaped bacteria. Figures 4.3(a) and 4.3(b) show the results of gram staining. *Bacillus cereus* strain ATCC 11778 (Figure 4.3(c)) and *Pseudomonas aeruginosa* strain ATCC 27853 (Figure 4.3(d)) were used as Gram stain controls.

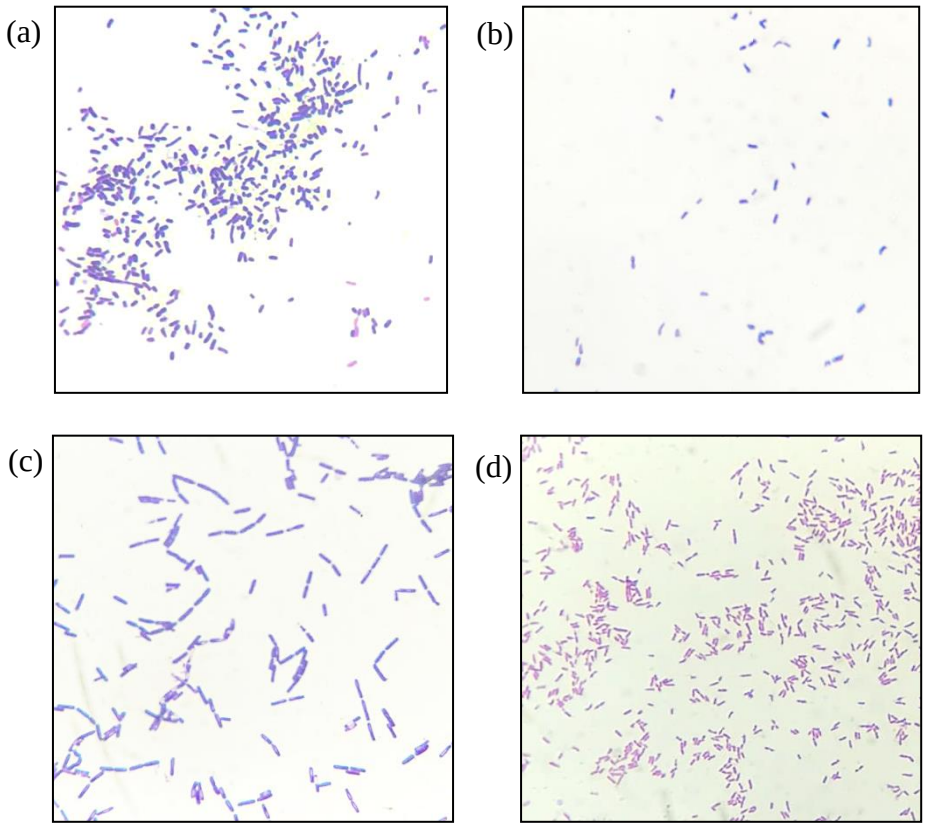


Figure 4.3: Gram staining results under 1000x magnification of light microscope. For (a) and (b), the isolated bacteria; (c) the positive control *Bacillus cereus* strain ATCC 11778; (d) the negative control *Pseudomonas aeruginosa* strain ATCC 27853.

4.2.2 Catalase Test

Upon addition of 3% (v/v) hydrogen peroxide, any catalase positive bacterium would produce rapid formation of bubbles as shown in Figure 4.4. Absence of bubbles indicate a catalase negative bacterium. *Listeria monocytogenes* is catalase positive bacterium. Only six bacteria that were catalase positive were selected and listed in Table 4.1.

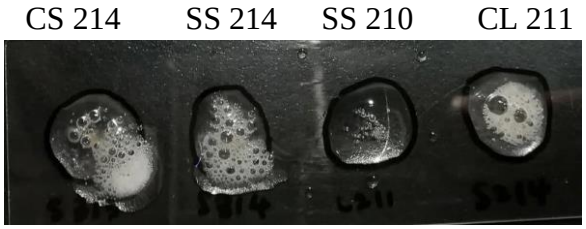


Figure 4.4: Catalase test results. CS 214, SS 214 and CL 211 shows positive results with formation of bubbles. SS 210 shows negative result.

4.2.3 Oxidase Test

Oxidase positive bacteria will cause deep purple-blue coloration on filter paper after adding oxidase reagent while oxidase negative bacteria will not show any color change. Figure 4.5 shows the results of four tested samples. *Listeria monocytogenes* is oxidase negative bacteria. The results for the oxidase tests are listed in Table 4.1.

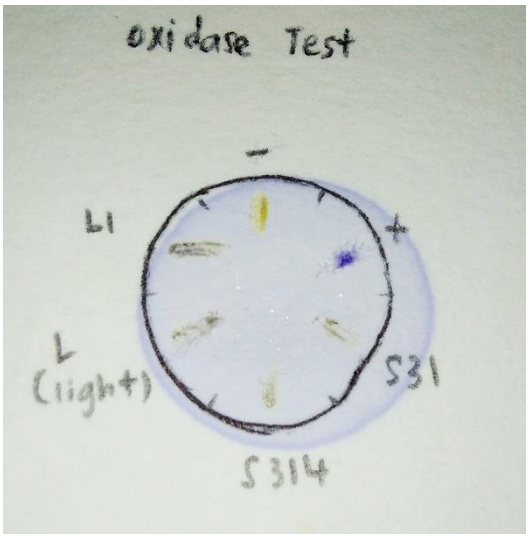


Figure 4.5: Oxidase test results. *P. aeruginosa* was used as the positive control (+) and it gave deep purple-blue coloration. The rest of the samples showed negative results, including the negative control (-), *Escherichia coli*.

4.2.4 Sulfide-Indole-Motility (SIM) Test

SIM test determine production of hydrogen sulfide and indole formation of bacteria. From all the isolates, all bacteria were tested negative for hydrogen sulfide production. There was only one sample, LL 2213, which was tested positively for indole test. Similarly, there was also one positive result for motility test. Figure 4.6 depicts the positive result of SIM test using control strains. Table 4.1 shows the result of SIM test.

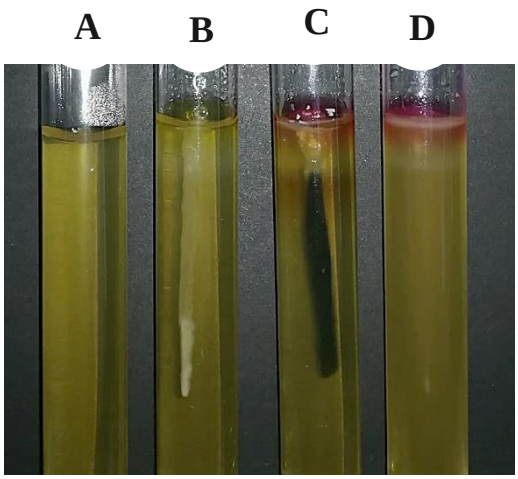


Figure 4.6: Negative and positive control for SIM test.

A: A clear SIM medium was observed in negative control. B: Motile bacteria caused turbidity with diffusion away from the stab (Sample LL2213). C: Black precipitation along the stab line showed the reduction of sulfur by *Salmonella typhimurium*. D: Positive indole test by sample LL2213 with the formation of red color ring at the top of the medium after adding Kovacs reagent.

Table 4.1: Biochemical test results of catalase test, oxidase test and SIM test.

No.	Assigned code	Catalase test	Oxidase test	H_2S test	Indole test	Motility
1	CS 214	+	-	-	-	-
2	SS 214	+	-	-	-	-
3	SS 2111	+	-	-	-	-
4	CL 211	+	-	-	-	-
5	LL 2213	+	-	-	+	+
6	LV 22	+	-	-	-	-

Legend:

(+) = Positive result

(-) = Negative result

4.2.5 Blood Agar Hemolysis and CAMP Test-S

L. monocytogenes is β-hemolytic and some strains cause an enhanced zone of hemolysis in CAMP test. β-hemolytic bacteria produce a clear zone surrounding the growth of colony on blood agar. Bacteria that leave a greenish color is α-hemolytic (Gerhardt, 2007). However, if there was no any changes occur, it is called γ-hemolytic. Five samples were found to be γ-hemolytic. Only one bacteria showed hemolytic characteristics but it was α-hemolytic instead of β-hemolytic. No enhanced zone of hemolysis was observed, and hence indicating negative CAMP test. Figure 4.8 shows the result of blood agar hemolysis and CAMP test.

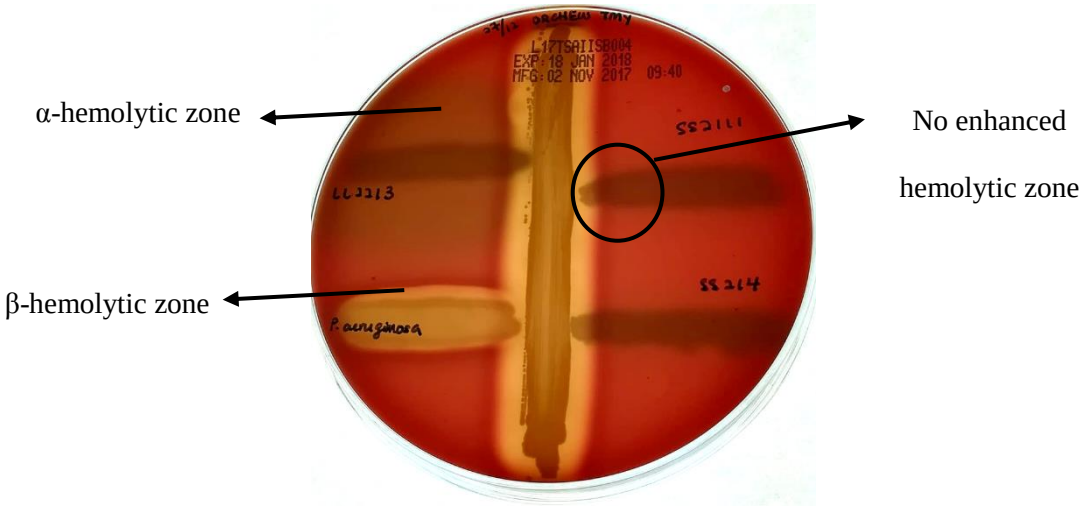
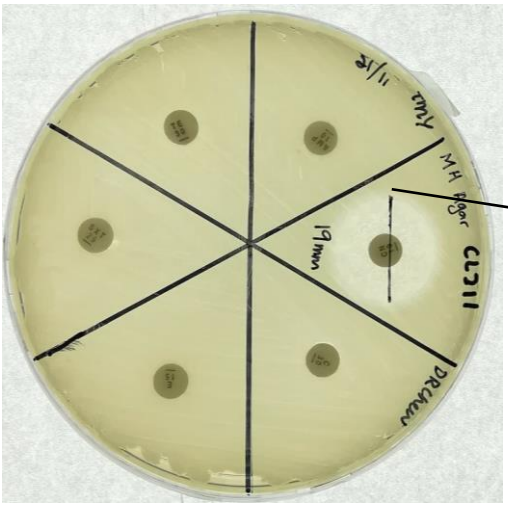


Figure 4.7: Result of blood agar hemolysis and CAMP test. *P. aeruginosa* was used as a control for β-hemolytic strains.

4.3 Antibiotic Susceptibility Test (Kirby-Bauer Disk Diffusion)

The bacteria were tested using Kirby-Bauer disk diffusion for their resistance against six types of antibiotic: tetracycline, ampicillin, sulfamethoxazole-trimethoprim, chloramphenicol, erythromycin and gentamicin. The diameter of inhibition zone was measured to the nearest millimeter and compared with interpretive standards from European Committee on Antimicrobial Susceptibility Testing (EUCAST) 2017 (Figure 4.7). Table 4.2 shows the results of antibiotic susceptibility test. Among the six samples, most of them showed resistance to most of the antibiotic tested. There was only one sample which was susceptible to the antibiotic gentamicin, while the other three samples were intermediate resistant to the antibiotic.



Zone of inhibition diameter

Figure 4.8: Antibiotic susceptibility test using Kirby-Bauer disk diffusion method. Diameter of clear zone surrounding antibiotic disc was measured to determine the zone of inhibition towards the antibiotic.

Table 4.2: Results of antibiotic susceptibility test.

Sample assigned code	Diameter of Inhibition Zone (mm)					
	TE (30 µg)	**AMP (10 µg)**	**SXT (25 µg)**	**C (30 µg)**	**E (15 µg)**	**CN (10 µg)**
CS 214	0 (R)	0 (R)	0 (R)	0 (R)	6 (R)	13 (I)
SS 214	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)
SS 2111	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)
CL 211	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	19 (S)
LL 2213	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	13 (I)
LV 22	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	13 (I)

Legend:

TE = Tetracycline; AMP = Ampicillin; SXT = sulfamethoxazole-trimethoprim;
C = Chloramphenicol; E = Erythromycin; CN = Gentamicin;
R = Resistant; I = Intermediate; S = Susceptible

4.4 F48 Microgen™ *Listeria* Latex Kit

Listeria spp. would cause agglutination in the latex reagent of the kit. Addition of latex reagent to all the bacteria colony samples, however, did not show a positive result. There was no any agglutination found. Figure 4.9 depicts the experiment result using *Listeria* Latex Kit.

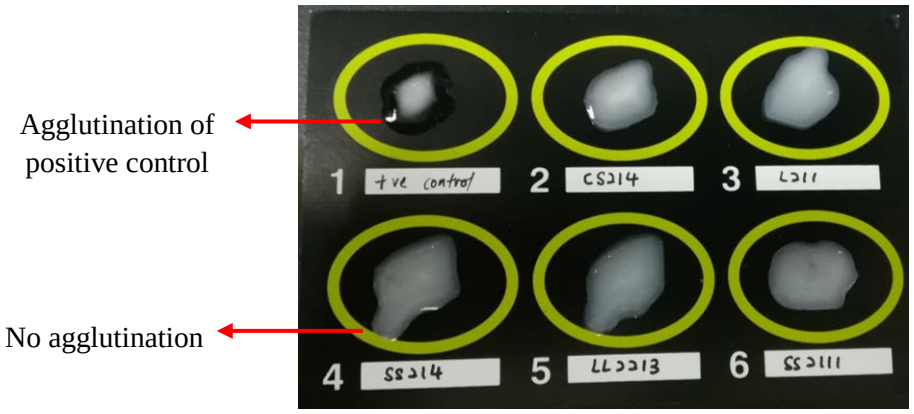


Figure 4.9: Result using ELISA Listeria Latex Kit. Well 1 sample is a positive control provided in the kit. Well 2 to 6 are bacterial isolates from this study.

4.5 Molecular Identification of Isolated Bacteria Strains and Their Antimicrobial Resistance

4.5.1 Purity and Concentration of Extracted DNA

After DNA extraction, the purity and concentration of the isolated DNA were measured using Thermo Scientific Nanodrop Spectrophotometer. Purity of DNA obtained was overall good as their A260/280 ratios fell between the range of 1.8 and 2.2. The concentration of extracted DNA was also sufficient for PCR

amplification. Table 4.3 listed the purity and concentration of extracted DNA from each sample.

Table 4.3: Purity and concentration of the extracted DNA from isolated bacteria strains.

Sample assigned code	DNA concentration (ng/µl)	A260/280 ratio	A260/230 ratio
CS 214	499.2	1.95	1.23
SS 214	409.4	2.09	1.05
SS 2111	398.4	2.05	1.02
CL 211	482.0	2.16	1.42
LL 2213	701.1	2.03	1.36
LV 22	520.8	2.15	1.32

4.5.2 PCR Amplification Targeting *prs, erm A, erm C* and *bla*SHV

The *prs* gene is chosen to confirm the presence of *Listeria spp.* while *ermA* and *ermC* genes were found to associate with macrolide-resistant *Listeria spp.* (Giovanetti, et al., 2002; White, et al., 2002). The *bla*SHV is to screen for the resistance gene which caused by the β-lactamase, in this study, the ampicillin resistance (Overdevest, et al., 2011). The *prs* amplicon with expected size of 370 bp should be observed but there was only non-specific band observed in the gel as shown in Figure 4.10.

Screening of antibiotic resistance *erm* gene was performed targeting *ermA* and *ermC* genes. Amplicon sizes for *ermA* and *ermC* were expected to be 421 bp and 572 bp, respectively. However, none of the isolates showed positive result. Positive control strains for *ermA* (Figure 4.10, lane 2 and lane 3) and *ermC*

(Figure 4.11, lane 10) showed that the amplification was specific and none of the bacterial isolates carried the targeted genes.

On the other hand, the detection for *bla*SHV gene generated positive results for all the six isolates. Bands with the expected amplicon size of 868 bp were observed from lane 3 to lane 7 in Figure 4.17.

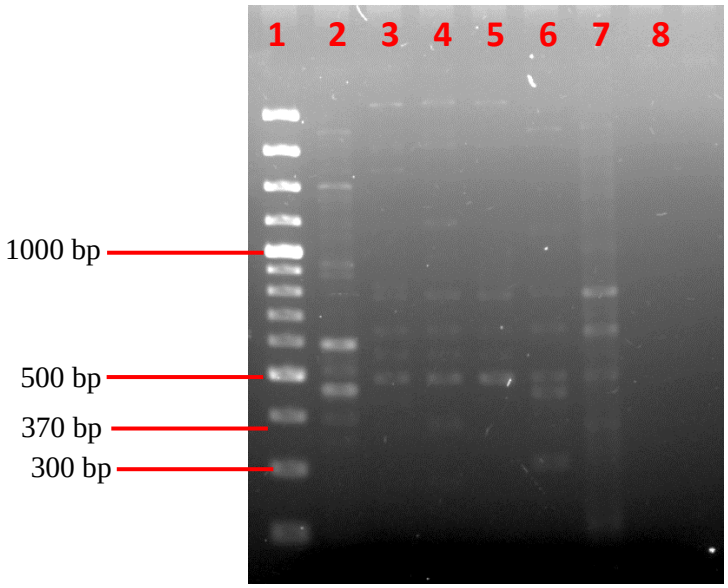


Figure 4.10: Gel image for detection of *prs* gene under visualization of Bio-Rad Gel Imaging System. All observed bands were non-specific bands.

Legend:

1 = 100 kb plus ladder	5 = CL211
2 = LL2213	6 = CS214
3 = SS2111	7 = LV22
4 = SS214	8 = Negative control (H_2O)

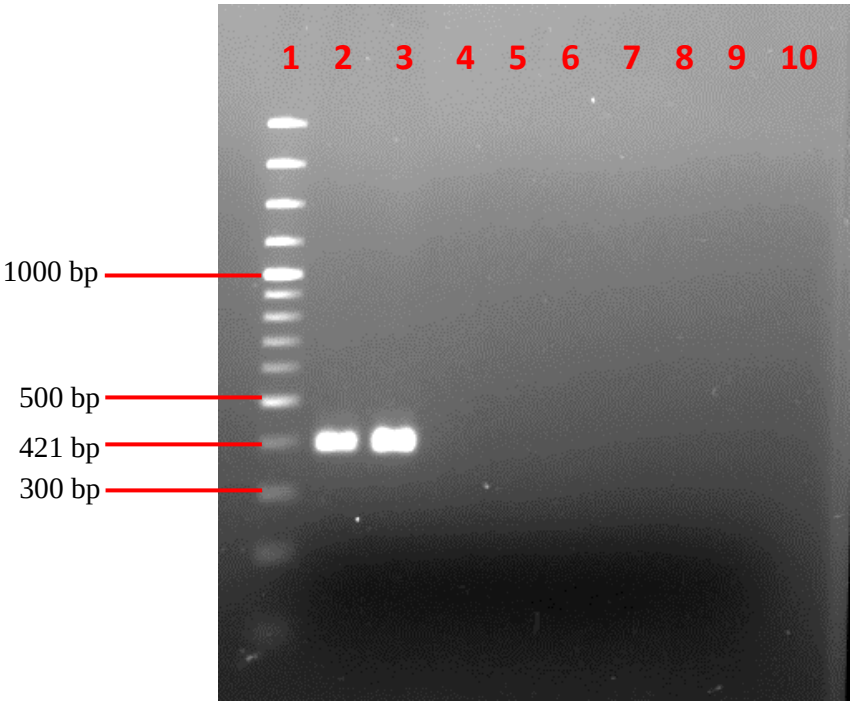


Figure 4.11: Gel image for detection of *ermA* gene under visualization of Bio-Rad Gel Imaging System. No amplicon with expected size (421 bp) was observed.

Legend:

1 = 100 kb plus ladder
2 = Positive control (MRSA 1)
3 = Positive control (MRSA 2)
4 = LV22
5 = CL211
6 = CS214
7 = SS214
8 = SS2111
9 = LL2213
10 = Negative control (H_2O)

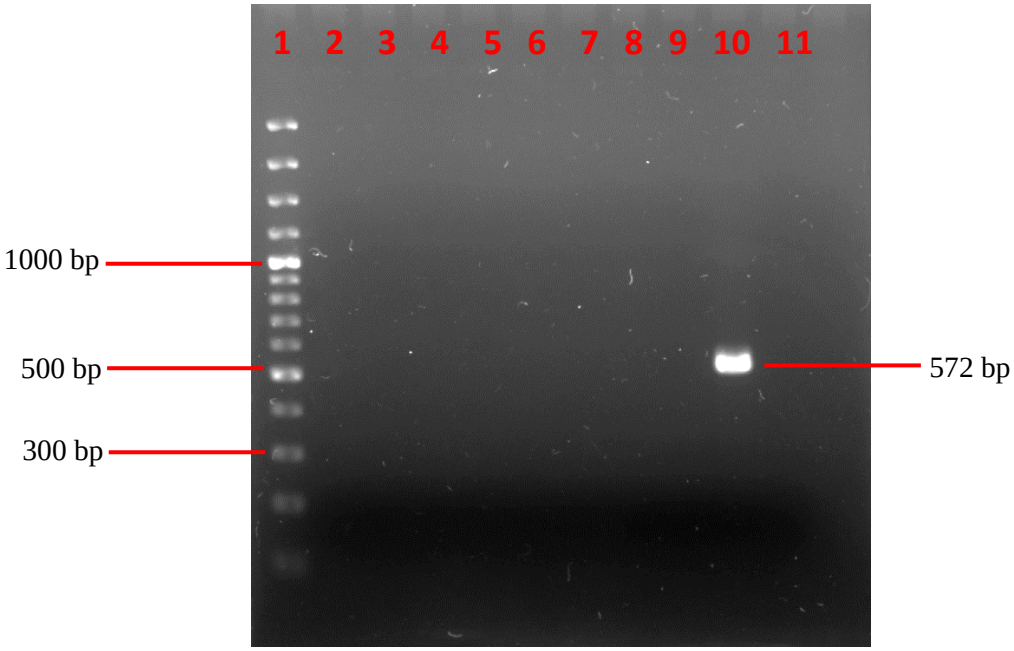


Figure 4.12: Gel image for detection of *ermC* gene under visualization of Bio-Rad Gel Imaging System. No amplicon with expected size (572 bp) was observed.

Legend:

1 = 100 kb plus ladder
2 = LL2213
3 = SS2111
4 = SS214
5 = CL211
6 = CS214
7 = LV22
8 = MRSA 1
9 = MRSA 2
10 = Positive control (MRSA 3)
11 = Negative control (H_2O)

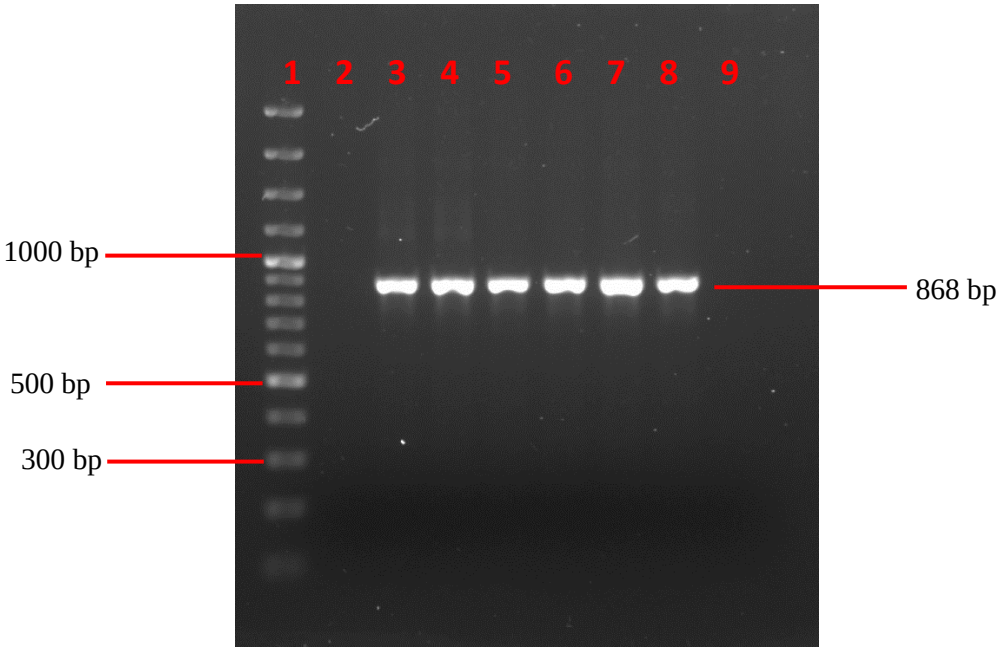


Figure 4.13: Gel image for detection of *bla*SHV gene under visualization of Bio-Rad Gel Imaging System. Amplicons with expected size (868 bp) were observed from lane 3 to lane 7.

Legend:

1 = 100 kb plus ladder
2 = LL2213
3 = SS2111
4 = SS214
5 = CL211
6 = CS214
7 = LV22
8 = Positive control *(Klebsiella pneumoniae)*
9 = Negative control (H_2O)

CHAPTER 5

DISCUSSION

5.1 Aberrant Results of Presumptive *L. monocytogenes* in Biochemical Tests

5.1.1 Indole Test

Presumptive *L. monocytogenes* bacterial colonies that were chosen from *Listeria* selective PALCAM agar and tested with Gram stain, catalase test and oxidase test should not produce hydrogen sulfide (Lamont, et al., 2011) and indole (Kandi, 2017), yet, being motile (Gasanov, Hughes and Hansbro, 2005) in the SIM test. Nevertheless, the strains LL2213 showed positive indole test.

In so many researches and studies about characterization of *L. monocytogenes*, there has not been any result showing positive indole test of this pathogen. Whether it was isolated from dairy-based source, ready-to-eat food or poultry, no supporting evidence has stated a case with mutated indole positive *L. monocytogenes* (Hitchins, Jinneman and Chen, 2016; Islam, et al., 2016; Hossain, et al., 2017). As such, there is a high possibility that sample LL2213 is not the desired bacteria.

5.1.2 Motility

In spite of showing positive indole test, sample LL2213 was the only presumptive bacterial strains that is motile, with the rest of the samples showed negative motility. Presence of motility in *L. monocytogenes*, however, is not a

definite feature that can be used in characterization of this foodborne pathogen (Gasanov, Hughes and Hansbro, 2005). Although flagella seem to play an important role in attachment to surfaces (Nowak, et al., 2017), several studies have come across mutant strains of non-motile *L. monocytogenes.*

In a study by Lemon, Higgins and Kolter, they actually discovered that non-motile *L. monocytogenes* could be divided into two groups of mutants: the flagella-minus mutants and non-functional (paralyzed) flagella mutants. These non-motile strains exhibited impaired biofilm formation at 30°C (Lemon, Higgins and Kolter, 2014).

In 2014, Fieseler et al. studied the motile and non-motile strain of *L. monocytogenes* that grow as a saphrophyte in diverse habitats such as soil and rivers, where they are frequently exposed to the predatory protozoa. In the research, they demonstrated that non-motile *L. monocytogenes* was the less preferred prey of protozoa compared to the motile strains because the lack of motility and ability to utilize amoebal metabolites assisted in avoiding the predator in low nutrient environment (Fieseler, et al., 2014). Another study by Yoshikawa, et al. actually explained that the lack of motility in *L. monocytogenes* was due to the lack of the ActA proteins, which mediate bacterial motility (Yoshikawa, et al., 2009).

5.1.3 Blood Agar Hemolysis and CAMP Test-S

Among the members in *Listeria spp., L. monocytogenes* is the only species that carries the virulence factor, hemolysin (Portnoy, et al., 1992). It has β-hemolytic characteristics (den Bakker, et al., 2010) but its hemolytic activity on blood agar may be demonstrated very slowly or weakly reactive that the hemolysis zone do not extend much beyond the edge of colony. Lysed zone may presents beneath the colony only (Gerhardt, 2007). For the six presumptive *L. monocytogenes* colonies, none of them presented β-hemolysis on blood agar. Sample LL2213, alternatively, produced α-hemolysis zone. For CAMP test, it also produced negative result. These results obtained seem to contradict with the β-hemolytic characteristics of *L. monocytogenes*. Yet, in a research published in 1988, two groups of investigators actually isolated nonhemolytic mutants of *L. monocytogenes.* These mutant strains were proven to be avirulent (Portnoy, et al., 1988).

In another published paper on foodborne *L. monocytogenes*, the strains of *L. monocytogenes* from food showed the absence of hemolysis for 24 hours and presented weak hemolysis after 48 hours of incubation. Such strains of foodborne *L. monocytogenes* are always detected with either gene alteration or deletion of the *hly* gene or its regulatory protein PrfA. Bacteria isolated from processed food or natural sources, in fact, has a higher probability to cause this nonhemolytic phenotype (Pusztahelyi, et al., 2016).

The *hly* gene, previously called *hlyA* and *lis4*, is the gene that codes for hemolysin. The *L. monocytogenes* hemolysin, listeriolysin 0 (LLO), could be

the best-characterized determinant of *L. monocytogenes* pathogenesis. LLO is from the family of sulfhydryl-activated pore-forming cytolysins of which streptolysin 0 is the prototype. According to Potnoy et al., the most likely role for LLO was to mediate lysis of host vacuoles of bacterium-containing. They also demonstrated that LLO-negative mutants were usually found residing in host vacuoles. As consequence, they cannot grow intracellularly (Portnoy, et al., 1992).

The *prfA* gene acts as a positive regulatory factor for *hly* gene when these mutants barely expressed detectable levels of *hly* mRNA. Besides, complementation of the spontaneous deletion mutant with a plasmid carrying *prfA* results in high expression level of not only *hly* but also other genes of *L. monocytogenes*, including *plcA, mpl,* and *plcB.* This somehow proved the function of *prfA* as an activator of other genes intracellularly (Portnoy, et al., 1992)

Therefore, sample LL2213 which showed α-hemolysis may not be the bacteria, *L. monocytogenes* but possibilities of other samples could not be confirmed through blood agar analysis.

5.2 F48 Microgen™ Listeria Latex Kit

Latex slide agglutination test is a rapid identification test for the presumptive *Listeria spp.* The test involves polyvalent antisera, which are the main factors causing agglutination of latex particles. These antisera are prepared against

purified flagellin proteins from *L. monocytogenes* and *L. grayi.* They coat on latex particles and cause agglutination when mix with suspension of *Listeria spp.* (Beumer, 1996).

No agglutination was observed from any sample in this latex agglutination test because this test itself has limitation that culture grown at above 30 °C may not produce flagella, giving a false negative result. Likewise, as mentioned in the motility test, there is possibility that the isolates are non-motile strains which are lack of ActA proteins (Yoshikawa, et al., 2009). Thus, the isolates here may also carry these mutant proteins.

5.3 Absence of *prs* gene for *Listeria spp.*

Since both of the important biochemical tests attempted have limitations in confirming the presence of *L. monocytogenes*, it would be more practical to do molecular detection of the *prs* gene which is only unique towards the strains belong to the genus *Listeria.* The *prs* gene encoded for the putative phosphoribosyl pyrophosphate synthetase in *Listeria spp* (Doumith, et al, 2014).

Many previous studies used *prs* gene in PCR assay as molecular confirmation of *Listeria spp.* and also isolation of *L. monocytogenes*. In 2016, Vongkamjan et al. used *prs* gene as *hly* gene to determine the isolates of *L. monocytogenes* from various ready-to-eat products from retail stores. Overall, 7.5% were positive for *L. monocytogenes* in the study (Vongkamjan, et al., 2016).

In another research, DNA genomic of all the presumptive *L. monocytogenes* were also subjected to PCR amplification to detect the *prs* and *hly gene*. The aim of this study is to isolate and characterize the *Listeria spp.* from raw and processed meat. As a result, a total of 6.42% isolates were found to be *L. monocytogenes* (Doijad, et al., 2016). A recent study published in 2017 also utilized similar genes in determining the occurrence and molecular characterization of *L. monocytogenes* in slaughterhouses in southern Brazil (Iglesias, et al., 2017).

The analyzed gel image as shown in figure 4.10 proved the *prs* gene was absent from the six presumptive *L. monocytogenes* strains. Nevertheless, this could be due to the lack of PCR optimization as the reagents used in the amplification are different from the referred study by Doumith et al. in 2004.

5.4 Specificity of *Listeria* Selective PALCAM Agar

The isolation method used in the present study is following the European and International Standard Method for the detection of *Listeria monocytogenes* as described in EN ISO 11290-1 (International Organisation for Standardisation, Geneva) which was validated by order of the European Commission. The ISO 11290 method involves a two-stage enrichment followed by plating on selective agar (Scotter, et al., 2001).

PALCAM selective agar relies on two indicating system: (i) esculin and ferric salt; (ii) mannitol and phenol red, to diagnose and differentiate *Listeria spp.*

Hydrolization of esculin by *Listeria spp.* to form black halo surround their colonies is a prominent feature (Fernandez, et al., 2013). However, Angelidis, Kalamaki and Georgiadou showed that other species of bacteria could also grow on PALCAM agar and would hydrolyze esculin. Among the list, *Cellulosimicrobium funkei* and *Marinilactibacillus psychrotolerans* presented quite similar morphology with the *Listeria spp.* which may lead to misinterpretation during selection (Angelidis, Kalamaki and Georgiadou, 2015). Culture of these bacteria on PALCAM agar from the study are as shown in Figure 5.1.

C. funkeii is a Gram-positive, catalase-positive and rod-shaped bacteria which belongs to the *Cellulosimicrobium spp.* It was the second identified species and was reported to be motile and capable of hydrolyze esculin (Brown, et al., 2006). So far, *Cellulosimicrobium spp.* have been isolated from soil, human or veterinary clinical specimens, monkey feces, Antarctic snow and also foods (Angelidis, Kalamaki and Georgiadou, 2015).

M. psychrotolerans was firstly isolated from marine organism a decade ago (Angelidis, Kalamaki and Georgiadou, 2015). The *M. psychrotolerans* isolates tested in the experiment by Angelidis, Kalamaki and Georgiadou are Gram-positive rods, catalase-positive, oxidase-negative and non-sporulating. The presence of this bacteria has been reported in food especially cheeses (Angelidis, Kalamaki and Georgiadou, 2015).

Due to their similarities on PALCAM agar, misinterpretation of *L. monocytogens* during selection from PALCAM agar may occur but more confirmation tests are required to rule out the possibility.

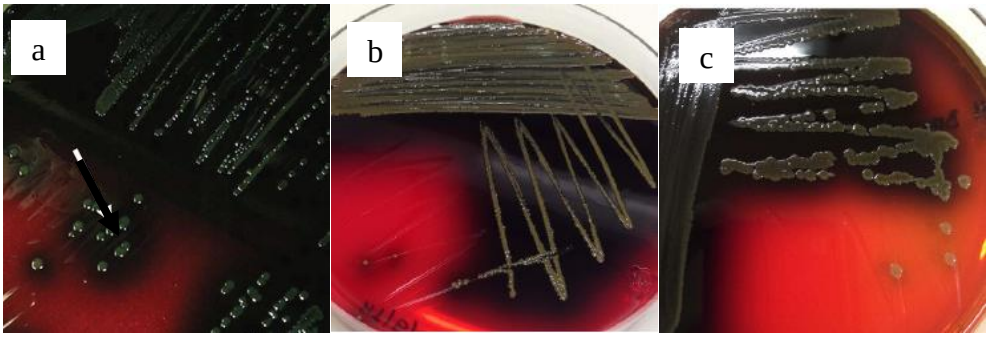


Figure 5.1: Growth of bacteria on *Listeria* selective PALCAM agar (a) *Listeria spp.* (b) *Cellulosimicrobium funkei* and (c) *Marinilactibacillus psychrotolerans.* Adapted from: Angelidis, Kalamaki and Georgiadou, 2015.

5.5 Antibiotic Susceptibility Test

It is important to determine the antimicrobial profile of bacteria from time to time, particularly human pathogen such as *L. monocytogenes* in order to monitor the emergence of bacteria antimicrobial resistance (Kuan, et al., 2017) and to manage infection in patients (Hudzicki, 2016).

Referring to Table 4.1, all tested samples were resistant to five types of antimicrobial agents chosen: tetracycline, ampicillin, trimethoprim, chloramphenicol and erythromycin. Gentamicin, however, showed its effectiveness in inhibiting bacteria sample CL211, and established intermediate

action towards three strains of bacteria samples: CS214, LL2213 and LV22. Sample SS214 and SS2111 were not affected by gentamicin's inhibition action. Resistance of isolated bacteria strains towards almost all of antimicrobial agents chosen indicated that these strains were multi-drug resistance gram positive bacteria (Magiorakos, 2016).

Among the six tested antibiotics in this study, gentamicin is probably more effective than the others. In point of fact, gentamicin shows very high susceptibility towards *L. monocytogenes* in other researches. In three studies on antibiotic resistance *of L. monocytogenes* in Malaysia, Jamali and Thong stated in their study in 2014 that this pathogen is 100% sensitive to gentamicin while in another study by Kuan et al. in 2017, it showed low resistance of 1.7%. In Spain and China, studies have also proven a high susceptibility of *L. monocytogenes* towards gentamicin (Escolar, et al., 2017; Wang, et al., 2015).

Other antibiotics that show high susceptibility are the erythromycin and ampicillin. For erythromycin, two studies in Malaysia showed that it has relatively low resistance (6.3%) (Jamali and Thong, 2014) or intermediate resistant (Kuan, et al., 2017) to erythromycin but in this study, *L. monocytogenes* is completely resistant towards this antibiotic. However, there were study on erythromycin resistance of *L. monocytogenes* where *ermC* gene has been demonstrated to associate with it in 1996 by Robert et al. In 2002, erythromycin-resistant *Listeria spp*. was demonstrated, which were caused by the *ermA* gene transferred from *Streptococcus spp*. (Giovanetti, et al., 2002).

Results of ampicillin resistance in *L. monocytogenes* are varied between studies. In Malaysia, a complete resistance was reported in 2012 by Marian et al. but just recently, 100% susceptible was recorded in another study in Malaysia (Kuan, et al., 2017).

From findings of several studies, sulfamethoxazole-trimethoprim generally showed a high sensitivity but there were also 39.1% and 5.2% of resistant strains found in studies by Kuan et al. and Marian et al respectively (Marian, et al., 2012; Kuan, et al., 2017). Resistance of chloramphenicol in *L. monocytogenes* was found to be 41.2% in study by Wang et al. (2015) in Nanjing, China but a low resistance of 3.1% in research by Jamali and Thong (2014) in Malaysia. According to research from different country, *L. monocytogenes* generally shows some extend of resistance towards tetracycline. In Spain, Escolar et al. determined 48% of resistance in this bacteria (Escolar, et al., 2017) while in China, 26.9% of resistance was demonstrated in one study (Wang, et al., 2015). Overall, the antibiotic resistance profile of the presumptive isolates in my study shows high similarity with those in study by Marian et al. in 2012, in which most of them are resistant towards the particular chosen antibiotics.

5.5.1 Detection of Antibiotic Resistant Genes: *ermA*, *ermC* and *bla*SHV

Antibiotic susceptibility test of the six strains showed resistance towards erythromycin but there were neither positive detection of *ermA n*or *ermC* gene in these bacteria samples after PCR amplification. Possibly, the resistance to erythromycin was due to other categories of *erm* gene, for example, *ermB.*

According to research of Haubert et al., they successfully isolated and detected 10% (five isolates) of multi-resistant *L. monocytogenes* in their study and two of the isolates harbored the antimicrobial resistance genes *tetM and ermB*. These isolates are obtained from food and the processing plant (Haubert, et al., 2016). Another study that found the erythromycin resistance gene *ermB* is by Li et al. in China, in which the multi-drug resistant *L. monocytogenes* isolate was recovered from quick-frozen rice flour product (Li, et al., 2016).

Five strains of bacterial isolates were detected with the presence of *bla*SHV through PCR amplification except sample LL2213, as shown in figure 4.13. The *bla*SHV encodes for the β-lactam antimicrobial resistance (Shaikh, et al., 2015). In this study, the detection of *bla*SHV genes in PCR amplification proved that the ampicillin resistance could be contributed by them.

5.6 Improvement and Future Studies

To aid the initial isolation steps, the Agar *Listeria* according to Ottaviani and Agosti (ALOA) is suggested to co-utilize with PALCAM agar. ALOA is another medium used for the detection and enumeration of *L. monocytogenes* according to the official Internationnal Organization for Standardization (ISO) methods in recent year (Angelidis, Kalamaki, and Georgiadou, 2015). Alternatively, another selective agar such as Oxford agar can also be used together with PALCAM agar as more number of selective media or method used to examine a specific sample is likely to increase the chance to obtain a positive results (Pinto, et al., 2001).

Besides, 16S rRNA sequencing can be utilized in order to identify *L. monocytogenes.* This technique was once utilized in an investigation of listeriosis outbreak case and the application of Resphera Insight for high-resolution analysis of 16S amplicon sequences in the study accurately identified *L. monocytogenes*. The shotgun and 16S rRNA data in the study also supported presence of three slightly variable genome of *L. monocytogens (*Ottesen, et al., 2016).

Lastly, MALDI-TOF mass spectrometry is another advanced technique that allows for rapid identification of L. monocytogenes. This technique could be best suited when using culture from ALOA agar because according to study of Jadhav et al. that was published in 2015, they utilized MARDI-TOF mass spectrometry in identification and source-tracking of *L. monocytogenes* and the study found that successful speciation was highest for isolates (91%) cultured on the chromogenic Agar Listeria Ottaviani Agosti (ALOA) agar (Jadhav, et al., 2015).

CHAPTER 6

CONCLUSION

In this study, 80 presumptive colonies were chosen from the selective PALCAM agar but only six isolates showed higher probability as *L. monocytogenes*. They were examined further using hemolysis test, ELISA and PCR amplification. Sample LL2213 may not be *L. monocytogenes* but the remaining five isolates could not be confirmed as *L. monocytogenes* in this study.

Due to the presence of mutant foodborne *L. monocytogenes* strains have been documented, more advanced and efficient techniques such as 16S rRNA sequencing or MALDI-TOF mass spectrometry are required for the confirmation.

Nevertheless, all six of these presumptive bacteria showed multiple resistance towards the six antibiotic tested (tetracycline, ampicillin, trimethoprim, chloramphenicol, erythromycin and gentamicin) in Kirby Bauer antibiotic susceptibility test. At the molecular level, it is shown that erythromycin resistance in all six bacteria was not conferred by the genes *ermA* or *ermC* and the resistance may be due to the presence of other *erm* determinants. On the other hand, *bla*SHV was detected in five of the isolates and this may contribute to ampicillin resistance in the isolates.

Although the identities of *L. monocytogenes* of the five presumptive strains are yet to be confirmed, the presence of multiple antibiotic resistant bacteria in food source cannot be denied. It is imperative that careful tracking of these foodborne associated antibiotic resistant bacteria must be warranted in future to curb the spread of antibiotic resistance.

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APPENDIX A

LIST OF CHEMICAL REAGENTS AND MANUFACTURES

Chemical Reagents	Manufacturers, Country
100bp Plus ladder	Invitrogen, USA
95% Ethanol	Copens Scientific, Malaysia
Agarose gel powder	Bio-rad, USA
BactiDrop™ Oxidase reagent	Thermo Fisher Scientific, USA
Blood agar	Isolab, Malaysia
Crystal violet	LabChem, Malaysia
dNTPs	Promega, USA
EDTA	Merck, Germany
EtBr-Out gel stain	Yeastern Biotech, Taiwan
Gram stain iodine	R&M Chemicals, UK
Guanidine hydrochloride	Fluka, Switzerland
Hydrogen peroxide	R&M Chemicals, UK
Isopropanol	QRec, Malaysia
Listeria Latex Kit	Microgen, UK
Loading dye	Thermo Fisher Scientific, USA
Luria-Bertani (LB) agar	Conda, Spain
Lysozyme chloride	Nacalai Tesque, Japan
Mueller Hinton (MH) agar	Conda, Spain
Nutrient agar	Merck, Germany
Nutrient broth	Merck, Germany

Oxoid^{TM} antimicrobial susceptibility disc (Ampicillin, Chloramphenicol, Erythromycin, Gentamicin, Tetracycline, Trimethoprim)	Thermo Fisher Scientific, USA
PALCAM agar	Liofilchem, Italy
Primers (*ermA, ermC, prs, bla*SHV)	IDT, Singapore
Safranin	LabChem, Malaysia
SIM medium	Conda, Spain
Sodium Chloride	Merck, Germany
Taq buffer	NanoHelix, South Korea
Taq polymerase	NanoHelix, South Korea
Tris hydrochloride	Fisher Scientific, UK
UVM I	Liofilchem, Italy
UVM II	Liofilchem, Italy

APPENDIX B

LIST OF LABWARE, EQUIPMENT AND INSTRUMENT

Labware/Equipment/Instrument	Manufacturers, Country
1.5 ml microcentrifuge tube	Axvgen® Scientific, USA
15 ml centrifuge tube	Nest biotechnology, China
Biometra PCR machine	Biometra, Germany
Biorad PCR machine	Bio-rad, USA
ChemiDoc XRS+ imaging system	Bio-rad, USA
Conical flask	Pyrex, USA
Freezer (-20°C)	Pensonic, Malaysia
Incubator	Memmert, Germany
Laminar Flow Cabinet Level-1	ESCO, Singapore
Light microcope	Leica microsystems, Germany
Measuring cylinder	FAVORIT®, Malaysia
Micropipette set	NEXTY, Japan
Micropipette tips	Axvgen® Scientific, USA
Nanodrop spectrophotometer	Thermo Scientific, USA
Petri dish	Nest biotechnology, China
Schott bottle	Duran®, Germany
Shaking incubator	Yih Der, Taiwan
Vortex mixer	VELP® Scientific, Europe