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Identification of protein biomarkers for differentiating Listeria monocytogenes genetic lineage III

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Identification of protein biomarkers for differentiating Listeria monocytogenes genetic lineage III

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Identification of protein biomarkers for differentiating Listeria monocytogenes genetic lineage

III

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Listeria monocytogenes causes listeriosis and is one of the most virulent foodborne pathogens. Different subtyping techniques classified *L. monocytogenes* into four genetic lineages (I, II, III and IV) and seventeen serotypes. Most outbreaks of listeriosis are caused by lineage I. Lineage II is mostly associated with sporadic listeriosis, while Lineage III strains are typically lower risk for causing listeriosis. Listeriosis mainly affects elderly, immunosuppressed, children, and pregnant women causing fetal death, miscarriage, meningitis, encephalitis, and septicemia. Therefore, it is important to differentiate high-risk strains from low-risk strains, which will permit early and appropriate interventions. Our main goal in this current work is to detect candidate biomarkers unique to lineage III strains that can be used to differentiate lineage III strains from lineage I and II. To achieve this goal, mass spectrometry, bioinformatics, and comparative proteomics were applied to detect biomarkers unique to lineage III strains.

DEDICATION

To my husband Hossam Abdelhamed and my two handsome boys Ahmed Abdelhamed and Kamal Abdelhamed. To my parents Mahmoud Gomaa and Nesrin Othman and my sister Yasmin Gomaa and my brothers Ahmed and Hussein Gomaa.

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CHAPTER I

LITERATURE REVIEW

Listeria monocytogenes

Listeria monocytogenes was first reported by E.G.D. Murray in 1924 based on six cases of sudden death in young rabbits (Murray et al., 1926). Murray named the bacteria Bacterium monocytogenes. Later, Harvey Pirie changed the genus name to Listeria in 1940.

Listeria was not known as a food-borne pathogen until the end of the 1970s and early 1980s (Schlech et al., 1983). Listeriosis was first reported in Halifax, Nova Scotia, involving 41 cases and 18 deaths, mostly in pregnant women and neonates. It was epidemiologically linked to the consumption of coleslaw that had been produced from cabbage contaminated with *L. monocytogenes* contaminated sheep manure. Since then, several cases of listeriosis have been reported and estimated to reach 3000 case each year from foodborne illness so, *L. monocytogenes* is now widely recognized as an important hazard in the food industry (Ryser and Marth, 1999).

Microbiology of Listeria monocytogenes

Listeria monocytogenes is a small rod-shaped bacterium, facultatively anaerobic, and non-spore forming (Gray and Killinger, 1966). Listeria monocytogenes is catalase-positive and oxidase-negative. Listeria grows on blood agar and expresses incomplete β-hemolysis destroys red blood cells. The genus Listeria is a member of the class Bacilli and the order Bacillales, which also includes Bacillus and Staphylococcus (Sallen et al., 1996).

Optimal growth for Listeria species is between 30°C to 37°C, but, unlike most bacteria, L. monocytogenes can grow at a permissive range of growth temperature from -1°C - 45°C, making it a psychrotroph and a mesophile (Junttila et al., 1988). The bacterium at 20-25°C expresses peritrichous flagella and is motile, whereas at 37°C it is weakly motile or non-motile (Galsworthy et al., 1990).

Listeria monocytogenes and other Listeria species have been reported in at least 37 mammalian species. The most important reservoirs are ruminants (Gray and Killinger, 1966). Listeria is also found in at least 17 species of birds that can serve as a reservoir and can contaminate surrounding environments (Dhama et al., 2013; Kurazono et al., 2003). L. monocytogenes also can be harbored by some species of fish and shellfish (ESTELA et al., 1992).

Listeria monocytogenes is widely distributed in the environment including soil, silage, water, and other environmental sources. Listeria monocytogenes is stable and can resist the deleterious effects of freezing, drying, and heat (Nightingale et al., 2004). It can survive in feces up to 347 days at ambient temperatures and for 27 days in 12% NaCl (Stelma and McCabe, 1992). Since L. monocytogenes is so widespread in the environment and livestock, it is not surprising that it is frequently found in vegetables and grass (Fenlon et al., 1996). However, contaminated agricultural environments and products rarely appear to directly cause human infection (Nightingale et al., 2004),

Listeria monocytogenes can persist in food-processing environments. It can tolerate low pH, high sodium concentrations, and worst of all, survive near freezing temperature (2-4°C) (Gandhi and Chikindas, 2007a; Gandhi and Chikindas, 2007b). It can also grow over a wide range of pH (4.3-9.6), water activity (aw) less than .93 (Farber and Coates, 1992). and salt

concentrations (up to 10%) (Seeliger and Höhne, 1979). This makes Listeria monocytogenes a serious threat to food safety and the food industry (Vazquez-Boland et al., 2001).

The foods that are usually implicated for transmission of L. monocytogenes infection are soft cheeses, dairy products, raw milk, pasteurized fluid milk, cheeses (particularly soft-ripened varieties), ice cream, raw vegetables, fermented raw-meat sausages, raw and cooked poultry, raw meats (of all types), raw and smoked fish, and refrigerated ready-to-eat products that are eaten without reheating (Farber and Peterkin, 1991; Wilesmith and Gitter, 1986). In animals, Listeria monocytogenes infection is transmitted by feeding of spoiled silage, in which the bacteria widely multiply (Vazquez-Boland et al., 1992).

Listeria genus

Initially, genus Listeria was classified in the family of Corynebacteriaceae. In 2004, the genus was moved to the newly created family Listeriaceae based on 16S rRNA sequencing (Somer and Kashi, 2003). For many years the genus Listeria only contained one species, L. monocytogenes. Currently, genus Listeria has seventeen species including L. monocytogenes, L. fleischmannii, L. grayi, L. innocua, L. ivanovii, L. marthii, L. monocytogenes, L. rocourtiae, L. seeligeri, L. weihenstephanensis and L. welshimeri. L. denitrificans (Collins et al., 1991; Rocourt, 1999; Vázquez-Boland et al., 2001). At present only L. monocytogenes is known to be pathogenic to humans and animals, while L. ivanovii is pathogenic only in animals, particularly ruminants (Gouin et al., 1994).

Classification of Listeria monocytogenes

Based on genomic content, ecology, and recombination rates, Listeria monocytogenes is divided into four different phylogenic evolutionary lineages (I, II, III, and IV) (Zhu et al., 2005).

Lineage I includes serotypes 1/2b, 3b, 3c, and 4b and is responsible for outbreaks of human clinical listeriosis (Ward et al., 2010b). Lineage II consists of serotypes 1/2a, 1/2c, and 3a and exhibits a significantly higher prevalence among food isolates, the environment, and animal clinical cases (Gray et al., 2004; Nightingale et al., 2005). Lineage III (serotypes 4a and 4c) formerly consisted of two groups, lineages IIIA/C and IIIB/C, which are now considered lineage III and lineage IV, respectively (Liu et al., 2006). However, limited knowledge exists for lineage IV due to the rarity of strains in this lineage and low strain variability, which contributes to seven unclear serotypes. Lineage III and IV strains account for approximately 1% of human listeriosis cases but are more prominent in animals (Jeffers et al., 2001). Most human listeriosis outbreaks have been linked to serotype 4b (lineage I) strains, while most sporadic human listeriosis cases appear to be caused by serotype 4b (lineage III) or serotype 1/2a (lineage II) strains (Liu, 2006).

Listeriosis and pathogenesis

L. monocytogenes can be found in the gastrointestinal tract of individuals who remain as asymptomatic carriers. Listeriosis may occur in one of two forms: non-invasive and invasive. Non-invasive listeriosis is the mild form of disease and occurs in healthy adults. It is generally limited to gastrointestinal illness, fever, vomiting, and diarrhea. The degree of severity is dependent on characteristics of the host and the strain. Invasive listeriosis is the more severe form of the disease. Most commonly, invasive listeriosis affects high-risk groups, including children, the elderly, pregnant women, and the immunocompromised. With the onset of epidemics such as HIV/AIDS, there has been an increase in the size of the population at risk of morbidity and mortality due to listeriosis (Schlech et al., 1983). Invasive listeriosis is characterized by systemic infection leading to septicemia, meningitis, meningoencephalitis, or febrile gastroenteritis, and it can cause stillbirths and abortions (Vazquez-Boland et al., 2001).

The infective dose is dependent on immunity of the infected individual and the strain. The definite infective dose has not yet been definitively determined, but it may be less than 1000 bacteria in the immunocompromised individual. The incubation period can range from a few days to three weeks and may be preceded by gastrointestinal symptoms that manifest after approximately 12 h incubation (Mead et al., 1999). The rate of normal healthy adult infection from listeriosis is low. There are approximately 0.7 cases per 100,000 persons. However, the infection is more common in children (10 cases per 100,000 persons) and the elderly (1.4 cases per 100,000 persons) (Gellin et al., 1991). Pregnant women are seventeen times more likely than healthy adults to acquire the infection (Jinneman and Hill, 2001).

The Centers for Disease Control and Prevention (CDC) estimates that around 48 million Americans get sick, and around 3000 people die of foodborne illnesses annually (https://www.cdc.gov/foodborneburden/). Listeriosis is estimated by the CDC to be the third leading cause of death from foodborne illness with an approximate 20% case fatality rate; nearly all cases are hospitalized (https://www.cdc.gov/listeria/technical.html).

L. monocytogenes causes about 260 deaths every year in the U.S. (Mead et al., 1999). Although the actual number of infections is low, the mortality rate can be as high as 20-30% regardless of antimicrobial treatment; thus, it has the highest hospitalization rate of the foodborne pathogens.

There have been several sporadic and epidemic outbreaks worldwide that implicate *Listeria monocytogenes* contaminated foods (Murray et al., 1926; Schlech et al., 1983). Foods that are commonly implicated include ready to eat (RTE) foods (deli meats, salads etc.), unpasteurized dairy foods (cheese and milk), cured and raw meats (hot dogs, undercooked chicken), and items such as prepared seafood salads and even raw and unprocessed meats

(Schlech et al., 1983). Treatment of listeriosis depends on the severity of clinical signs. Mild forms of listeriosis require no treatment. Invasive listeriosis can be treated with antimicrobials, but more-serious listeriosis can be untreatable or resistant (Lamont et al., 2011).

Several factors contribute to *L. monocytogenes* pathogenicity and virulence. *Listeria monocytogen*es express Internalin A and Internalin B which helps in internalization and invasion of the host intestinal and endothelial cells (Pentecost et al., 2010). Inside the host cells *L. monocytogen*es express phospholipases and listeriolysin O**.** Both help the bacteria to escape from the phagocytic cells and allowing *Listeria* to replicate inside the cytosol of the host cell. New studies on the factor effect on *L. m* pathogenicity reveled that some surface protein plays critical role in *L. monocytogenes* virulence. ActA is one of *L. m* surface proteins that is best characterized as a virulence factor of *L. m*. It fulfils many essential functions within host cells, allowing *L. m* to escape from autophagy and recruiting an actin polymerization complex that promotes *L. m* motility, cell-to-cell spread and dissemination within host tissues. ActA also acts extracellularly by mediating *L. m* aggregation and biofilm formation in vitro and in vivo (Travier et al., 2014).

Identification of Listeria monocytogenes species

Listeria species possess distinct biochemical properties that can be used for species-specific identification. Therefore, some biochemical tests are useful for discriminating between *Listeria* species. However, biochemical methods for the identification of *Listeria* species are laborious, involving primary isolation with selective and enrichment media, followed by multiple biochemical tests (Rocourt, 1999). Also, biochemical testing of *Listeria* species is time-consuming (up to 6 days to finalize a result) and expensive (Liu, 2006). Also, biochemical tests can be influenced by external factors that affect bacterial growth and metabolic mechanisms.

Although serotyping is not very discriminatory, it is a universally accepted subtyping technique for *L. monocytogenes*. The species is currently classified into 14 serotypes based on variation in the somatic (O) and flagellar (H) antigens (Seeliger and Höhne, 1979). Identification of the strain serotype permits differentiation between important food-borne strains and provides a "gold standard" for comparing isolates (Borucki and Call, 2003; Nho et al., 2015b). *L. monocytogenes* serotypes 1/2a (lineage II), 1/2b, and 4b (lineage I) are responsible for 98% of documented human listeriosis cases, whereas serotypes 4a and 4c are rarely associated with outbreaks of the disease (Wiedmann et al., 1996). Furthermore, while *L. monocytogenes* serotype 4b strains are isolated mostly from epidemic outbreaks of listeriosis, serotypes 1/2a and 1/2b are linked to sporadic *L. monocytogenes* infection (Wiedmann et al., 1996). Because serotyping requires acquiring subtype-specific antisera, serotyping methods are not routinely performed in clinical laboratories. Additionally, serotyping does not correlate with species identities, which limits its widespread clinical use (Liu, 2006).

PCR-based subtyping procedures have provided additional tools for the identification and grouping of *L. monocytogenes* (Doumith et al., 2004; Jinneman and Hill, 2001). PCR-based methods are more rapid, simpler to perform, less costly, and many laboratories with molecular capability already have the necessary equipment, trained personnel, and reagents available. However, because the assay sometimes depends on the selective amplification of a shared gene, size fractionation of the amplicons may be required using standard agarose gel electrophoresis (Versalovic et al., 1991). A multiplex PCR assay (Bubert et al. 1999) facilitates the differentiation of six *Listeria* species based on selective amplification of *iap* gene (Bubert et al., 1999). This assay depends on differentiating small differences in *iap* gene size among various *Listeria* species. Species-specific primers for the six *Listeria* species were developed that

achieved satisfactory specificity in the identification of each of the *Listeria* species (Liu et al., 2003, 2004). Targeting *Listeri*a genes unique to individual species is beneficial because differentiation is based on the presence or absence of an amplicon rather than determining amplicon size (Gilot and Content, 2002). However, co-presence of several *Listeria* species complicates the identification of *L. monocytogenes*.

DNA sequencing methods of one or more particular genes are established and used for genetic subtyping of *L. monocytogenes* (Cai et al., 2002). The accessibility of DNA sequencing data also aids the reconstruction of ancestral and evolutionary relationships among *L. monocytogenes* isolates (Ward et al., 2004, Ward et al., 2010b). For example, a multilocus genotyping (MLGT) method was developed that uses 30 probes to classify *L. monocytogenes* isolates into genetic lineage (I to IV), major serogroup (4b, 1/2b, 1/2a, and 1/2c), and epidemic clone (EC) type (ECI, ECIa, ECII, and ECIII). A panel of 501 *L. monocytogenes* isolates from ready to eat (RTE) meat and poultry products was evaluated using this MLGT and sequencing of the virulence gene *inlA* (encoding internalin A), revealing that the percent of clonal lineages associated with epidemic outbreaks that are contaminants of RTE foods and was less than 7.6% (Ward et al., 2010a). With the cost of DNA sequencing decreasing rapidly, DNA sequencing-based subtyping can play an important role in *L. monocytogenes* subtyping and phylogenetic studies, but it requires DNA sequencing equipment and trained personnel.

Thus, there is an urgent need for the development of a detection method that could be very simple and potentially be used in the field or production plant. Biomarkers have the potential for use in clinical practice for disease diagnoses, monitoring, and patient care. They provide a detached and quantifiable method to diagnose and characterize disease agents.

Identification of bacteria species based on variations in protein sequences (mass spectrometry)

In recent years, proteomics-based approach and mass spectrometry (MS) through mass fingerprinting or peptide sequencing analysis has become an established tool for microbial identification and diagnostics and typing of bacteria (Singhal et al., 2015). As mass spectrometric techniques and proteomic tools become increasingly available and accessible, a broader range of researchers is applying these approaches to fit the specific needs of their projects (Baldwin, 2004).

Both matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) and liquid chromatography-mass spectrometry (LC/MS) have a wide range of applications including rapid bacterial identification in food-borne disease outbreaks, water quality control, antibiotic susceptibility/resistance tests, rapid infectious disease diagnosis, and biomarker discovery that may help accurately identify closely related organisms (Cheng et al., 2016). Moreover, MS has the potential for rapidly distinguishing between pathogenic and nonpathogenic species of bacteria (Barbuddhe et al., 2008; Holland et al., 1996; Karcher et al., 2018; Saenz et al., 1999). Mass spectra obtained from unknown bacterial samples can be used for comparison with reference libraries of known species to facilitate identification (Holland et al., 1996; Barbuddhe et al., 2008).

In general, mass spectrometers employed in proteomic analysis are based on the analysis of peptides generated by proteolytic digestion of whole proteome or proteins of interest (Aebersold and Mann, 2003). The most widely used enzyme is trypsin, which hydrolyzes the protein on the C-terminal side of lysine and arginine (unless the subsequent amino acid in the sequence is a proline) (Peng and Gygi, 2001). After digestion, the peptide mixture is commonly separated by HPLC or by reverse-phase (RP) LC and introduced online into a mass spectrometer

via electrospray ionization (ESI) (Dreger, 2003). Following mass analysis of the fragment ions resulting from peptide dissociation, the experimental data are usually searched *in silico* against theoretical peptides created from predicted protein sequences using database-searching algorithms (Eng et al., 1994). Peptide identifications are then correlated to protein sequences in the database to identify the source proteins.

LC/MS technique provides high molecular specificity and detection sensitivity. LC/MS can be used to analyze complex samples of environmental and biological origin. Therefore, LC-MS may be applied in biotechnology, environment monitoring, food processing, and pharmaceutical industries (Gaspari et al., 2016). Another advantage of using LC/MS is that minor differences among isolates at subspecies level, or different growth conditions for the same isolate, can be observed (Cheng et al., 2013; Kooken et al., 2014; Paul et al., 2007).

Application of MS and proteomics for identifying pathogenic bacteria

Mass spectral-based technologies have potential for the discovery of biomarker peptides or proteins, which must then be validated (Hale et al., 2003). MS has been applied on multiple bacterial species using comprehensive proteomic analysis to identify unique surface molecules, marker proteins, or toxins that allow differentiation between isolates (Fenselau and Demirev, 2001; Krishnamurthy et al., 1999; Wang et al., 2002; Williams et al., 2004). Protein biomarkers from intact bacteria are useful for the identification of bacterial species or intra-species differentiation between different strains of bacteria (Arnold and Reilly, 1998; Demirev et al., 1999; Ho and Hsu, 2002). MALDI-TOF was used to accurately identify 146 strains of different *Listeria* species and correctly classified all *L. monocytogenes* serotypes in agreement with PFGE (Barbuddhe et al., 2008).

In a previous study using mass spectrometry with MALDI-TOF, six protein biomarkers from two strains of *Escherichia coli* O157:H7 and one non-O157:H7 (nonpathogenic) strain of *E. coli* were identified. The six biomarkers were novel for differentiating pathogenic *E. coli* O157:H7 from nonpathogenic strains of *E. coli* using MALDI-TOF, whereas MALDI-TOF failed in differentiating pathogenic *E. coli* O157:H7 from non-O157:H7 in this study. The proteins identified were acid stress chaperone-like proteins; cold shock protein; YbgS (or homeobox) protein; putative stress-response protein YjbJ (or CsbD family protein); and a protein of unknown function (Fagerquist et al., 2010).

In *Campylobacter jejuni*, MS analysis was used to differentiate between 104 previously characterized *C. jejuni* isolates from humans, chicken, turkey, and bovine. All samples were identified as *C. jejuni*, and the method discerned mass peaks to identify subtypes (Zautner et al., 2013). In another study, several protein biomarkers of three *C. jejuni* strains (RM1221, RM1859, and RM3782) were identified by MS and proteomic techniques. The protein biomarkers identified were: DNA-binding protein HU, translation initiation factor IF-1, cytochrome c553, a transthyretin-like periplasmic protein, chaperonin GroES, thioredoxin Trx, and ribosomal proteins (Fagerquist et al., 2006).

Streptococcus agalactiae (170 isolates) was examined by mass spectrometry, and their tryptic peptides were analyzed by LC-MS/MS to identify four biomarkers that were significantly associated with invasive ST17 genogroups (Lanotte et al., 2013). The four biomarkers are: small subunit of exodeoxyribonuclease VII, 50S ribosomal protein L7/L12, CsbD-like protein, and thioredoxin.

To identify a useful protein biomarker to distinguish between a pathogenic *V. parahaemolyticus* O3:K6 strain (VP47) and another distantly related *V.*

parahaemolyticus O4:K55 strain, analysis of the lysates from whole bacteria by LC/MS revealed differences in the masses of several proteins in each strain (Williams et al., 2004).

A number of studies demonstrated that analysis of changes in protein or peptide content in biological fluids such as serum, urine, or milk is useful to discover biomarkers for diagnosis and monitoring of diseases such as renal disorders, cardiovascular disease, mastitis, and diabetes (Good et al., 2010; Metzger et al., 2010). For example, comparison of the peptidome between healthy udders and udders infected with *Staphylococcus aureus* (mastitis milk) identified 154 peptides that have potential for diagnosis of mastitis (Mansor et al., 2013).

Therefore, identification of biomarkers unique to *L. monocytogenes* genetic lineage III will help to distinguish high-risk *Listeria monocytogenes* serotypes from low-risk serotypes. Additionally, it will be useful for studying the epidemiology of foodborne disease and development of preventive strategies that could be very simple and potentially be used in the field or production plant.

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CHAPTER II

DISCOVERY AND UTILIZATION OF PROTEIN BIOMARKERS DIFFERENTIATING *LISTERIA MONOCYTOGENES* GENETIC LINEAGE III

Abstract

Listeria monocytogenes is the causative agent of listeriosis, a severe foodborne illness characterized by septicemia, meningitis, encephalitis, abortions, and occasional death in infants and immunocompromised individuals. *L. monocytogenes* is composed of four genetic lineages (I, II, III, and IV) and at least twelve serotypes. The aim of the current study was to identify proteins that can serve as biomarkers for detection of genetic lineage III strains based on simple antibody-based methods. Liquid chromatography (LC) with electrospray ionization tandem mass spectrometry (ESI MS/MS) followed by bioinformatics and computational analysis were performed on three *L. monocytogenes* strains (Lm33007, Lm33014, and Lm33077), which were used as reference strains for lineage I, II, and III, respectively. Results from ESI MS/MS revealed 42 unique proteins present in Lm33077 and absent in Lm33007 and Lm33014 strains. BLAST analysis of the 42 proteins against a broader panel of >80 sequenced strains from lineages I and II revealed four proteins [membrane protein (ST33077_2770), DUF3916 domain-containing protein (ST33077_1897), DNA methyltransferase (ST33077_1926), and Type IV secretion protein Rhs (ST33077_1129)] that have no homology with any sequenced strains in lineages I and II. The four genes that encode these proteins were cloned into expression vector pET28a, expressed in *E. coli* strain DE3, and purified by His-Bind resin. Polyclonal antibodies

were prepared against purified recombinant proteins. ELISA using the polyclonal antibodies against 12 *L. monocytogenes* lineage I, II, and III isolates indicated that proteins ST33077_2770 and ST33077_1926 detected all lineage III strains with no reaction to lineage I and II strains. In conclusion, proteins ST33077_2770 and ST33077_1926 are potentially useful biomarkers for detection and differentiation of *L. monocytogenes* lineage III strains in clinical, environmental, and food processing facilities. Furthermore, these results validate the approach of using a combination of proteomics and bioinformatics to identify useful protein biomarker.

Introduction

Listeria monocytogenes is a Gram-positive intracellular pathogen of animals and humans (Murray et al., 1926). It has been implicated within the past decade as the causative agent in several outbreaks of foodborne disease, and it currently has the third highest mortality rate (24%) among foodborne pathogens. In the United States *L. monocytogenes* is responsible for about 260 deaths each year. It is ubiquitous in the environment, including water and soil, and it can resist high salt concentration, low and high temperature (-1 - 45 ^{0}C), and low pH (Harrigan, 2001). This capacity for tolerating extreme conditions makes it of particular concern for the food industry, especially because it can be carried asymptomatically by multiple animal species. As a result, it has been isolated from raw and processed foods, including chicken, red meat, seafood, and milk.

Listeria monocytogenes causes listeriosis, a serious foodborne infection with high hospitalization and mortality rates (20 to 30% mortality in high-risk individuals) (Farber and Peterkin, 1991; Schlech et al., 1983). The most common form of the disease occurs mainly in healthy individuals. Illness typically occurs 24 h after ingestion of a large inoculum of bacteria

and usually lasts two days with gastroenteritis-like symptoms. The less common but more severe form of the disease is found mainly among pregnant women and immunocompromised persons, with symptoms of abortion, neonatal death, septicemia, and meningitis (Vazquez-Boland et al., 2001).

Based on classical molecular subtyping methods, such as ribotyping, pulse field gel electrophoresis (PFGE) and multi locus sequence typing (MLST), *L. monocytogenes* strains are grouped into four distinct phylogenetic lineages (I, II, III and IV). Lineage I includes strains of serotypes 1/2b, 3b, 4b, 4d, and 4e; this lineage is more commonly associated with human clinical cases. Most outbreaks of listeriosis are caused by this lineage. Lineage II includes strains of serotypes 1/2a, 1/2c, 3a, and 3c (Nho et al., 2015a), which is mostly isolated from food and environmental samples causing sporadic listeriosis. Lineage III includes strains of serotypes 4a and 4c, as well as certain strains of serotype 4b commonly isolated from animals. Strains in this lineage are typically lower risk for causing listeriosis (Nadon et al., 2001; Rasmussen et al., 1995; Ward et al., 2010a).

L. monocytogenes can be differentiated through culture methods based on selective enrichment and plating, followed by characterization based on colony morphology, sugar fermentation, and hemolytic properties (Jacquet et al., 2002). These methods are the gold standard, but they are lengthy and may not be suitable for testing of foods with short shelf lives. As a result, more rapid tests were developed based on molecular techniques (PCR or DNA hybridization). However, some PCR techniques are complicated and require two or three independent PCR reactions (Mead et al., 2005; Pan et al., 2009). Other PCR techniques are limited and differentiate only two or three serotypes (Borucki and Call, 2003; Tresse et al.,

2007). Thus, there is an urgent need for the development of a rapid method that can distinguish high-risk *L. monocytogenes* serotypes from low-risk serotypes.

Because lineage III strains are low risk for human listeriosis, we sought to identify protein biomarker(s) that could distinguish *L. monocytogenes* genetic lineage III isolates from lineages I and II isolates using simple antibody-based method for distinguishing lineages. This work will enable studies on the epidemiology of listeriosis and the development of preventive strategies.

In the current study, we applied liquid chromatography mass spectrometry (LC/MS) proteomics to identify unique proteins of *L. monocytogenes* lineage III strain ST33077. Mass spectrometry identified a total of 42 distinct proteins in strain ST33077 with no ortholog in representative strains from *L. monocytogenes* genetic lineages I and II. BLAST analysis of lineage III-unique proteins (42) against a panel of 80 *L. monocytogenes* Reference Sequence proteomes (representing lineages I, II, and III) revealed twenty proteins without protein homology with proteins from linages I and II. Four proteins out of twenty were chosen for further studies based on peptide coverage and identity. These proteins have potential to develop antibody-based separation methods for differentiating *L. monocytogenes* genetic lineages III from lineage I and II. Development of simple antibody-based methods for differentiating *L. monocytogenes* genetic lineages, serotypes, and epidemic clones would facilitate identification of listerial subgroups in diagnostic microbiology laboratories and ostensibly in food processing facilities. This increased discriminatory capability would assist in assessment of risk from *L. monocytogenes* isolates and accelerate epidemiological investigations.

Materials and Methods

Bacterial cultures and ethics statement

Polyclonal antibody production in rabbits was performed at Mississippi State University according to a protocol approved by the MSU Institutional Animal Care and Use Committee (IACUC-18-137).

Bacterial strains used are listed in (Table A.1) *L. monocytogenes* genome sequence for strains 33007, 33014 and 33077 were provided by Todd J. Ward. *L. monocytogenes* strain 33077 (genomovar III; serotype 4a) was obtained from USDA-ARS and used for development of a DNA probe-based method for differentiation of *L. monocytogenes* serogroups (Ward et al., 2010b).

Hence, it served as the reference genetic lineage III strain for the current study. *L. monocytogenes* strains were grown on brain heart infusion (BHI) agar and broth (Difco, Sparks, MD, USA) and incubated at 37°C. *Escherichia coli* strain Nova Blue (Novagen, Madison, WI, USA) was used for gene cloning in pET-28a expression vector (Novagen). Three recombinant proteins (WP_012582068.1, WP_003728958.1, and WP_014589151.1) were expressed in BL21(DE3) (EMD Millipore, San Diego, CA), and one protein (WP_077954308.1) was expressed in C41(DE3) (EMD Millipore). All *E. coli* strains were cultured on Luria–Bertani (LB) agar or broth (Difco) at 37°C. Kanamycin (Kan: 30 µg/ml) (Sigma–Aldrich, Saint Louis, MN, USA) was added to culture medium for plasmid selection when needed, and isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma was added for protein induction when needed.

Protein extraction and fractionation

Total proteins were isolated in triplicate from *L. monocytogenes* strains Lm-33007, Lm-33014, and Lm-33077 (lineage I, II and III, respectively) using a phenol-based extraction protocol with some modifications (Hurkman and Tanaka, 1986). Washed *L. monocytogenes* bacteria were resuspended in ice-cold extraction buffer (0.9 M sucrose, 0.5 M Tris-base, 0.05 M Na2-EDTA, 0.1 M KCl, 2% β-mercaptoethanol, 8 mM PMSF, pH 8.7) and sonicated with five 10-s pulses (Fisher Scientific Model 100 Sonic Dismembrator, setting 3) on ice with a minimum of 1 min cooling time between pulses. Lysates were further treated with DNase (85 µg/mL) and RNase (20 µg/mL) at 37ºC for 30 minutes. Proteins were extracted by adding an equal volume of Tris-buffered phenol (pH 8) and homogenizing for 10 minutes at room temperature. Insoluble matter, phenol, and aqueous phases were separated by centrifugation at 5,500 *g* at 4ºC. The phenol phase was collected and extracted with ice-cold extraction buffer two more times using the same procedure. Proteins were then precipitated from the phenol phase with five volumes of 0.1 M ammonium acetate and 2% β-mercaptoethanol in 100% methanol overnight at -20ºC. Precipitated proteins were washed three times with the same solution and three times with 100% acetone. Air-dried protein pellets were stored at -20ºC.

For protein fractionation and digestion, *L. monocytogenes* protein pellets were dissolved in 1x SDS sample buffer (2% SDS, 50 mM Tris-HCl pH 6.8, 10% glycerol; 1% DTT) and quantified using a 2D Quant Kit (GE Healthcare, Chicago, IL). To increase coverage of each proteome, 350 µg of proteins were fractionated into twelve molecular weight (MW) fractions using Sage ELF (Sage Science; Beverly, MA). After fractionation, protein purity was verified using Bioanalyzer (Agilent Technologies, Santa Clara, CA). Fractions were desalted on HiPPR spin columns (Thermo Fisher Scientific, Waltham, MA). In-solution digestion was conducted as follows: each

fraction was reduced with 100 mM DTT (15 min at 65ºC), alkylated with 100 mM iodoacetamide (IAA) 30 min at room temperature in the dark, and digested overnight with Trypsin/Lys-C Mix (Promega, Madison, WI) at 37ºC. Tryptic peptides were acidified with formic acid, concentrated by Speed-Vac, and stored at -80ºC. Immediately be mass spectrometry, they were dissolved in 2% acetonitrile/0.1% formic acid and subjected to LC-MS/MS. Protein fractionation was performed at Institute for Genomics, Biocomputing and Biotechnology (IGBB).

Mass spectrometry and bioinformatics analysis

Trypsin-digested protein fractions from three *L. monocytogenes* strains were subjected to qualitative proteomics analysis using LC-MS. Peptides were separated using C18 column and HPLC directly linked to an LTQ Orbitrap mass spectrometer. Mass spectra were collected in data-dependent analysis (DDA) mode during a three hours-long acetonitrile gradient. Complete open reading frames (ORFs) and translated proteins were predicted using EMBOSS (v 6.6.0). The raw data files were converted to "MGF" format and matched against the ORF database using X!Tandem (v2017.2.1.4). For each strain, search results from the strain specific ORF database were filtered to reduce the false discovery rate to less than 5% using the MSnID R package. Results were filtered further by removing all spectra that matched theoretical ORF databases from ST33007 and ST33014 (genomovars I and II) to yield candidate genomovar III-specific spectra.

Bioinformatics analysis identified 232,470 spectra that were identified in Lm33077 (lineage III) and not in lineage I and II. The unique spectra mapped to 1,522 ORFs with an ORF FDR ≤ 0.05. Of those ORFs, a total of 42 distinct proteins were detected in strain ST33077 that

did not match *L. monocytogenes* strains ST33007 and ST33014 with less than 50% identity. BLAST analysis of the putative Lineage III-specific proteins was conducted against 80 *L. monocytogenes* strains from lineages I and II in the NCBI Reference Sequence proteome database, and 20 proteins were identified that did not match any strains from lineages I and II. The four of these 20 proteins that were most unique to lineage III (based on peptide coverage and identity of all three lineages) are WP_012582068.1, WP_003728958.1, WP_014589151.1, and WP_077954308.1 (ST33077_1897, ST33077_2770, ST33077_1129, and ST33077_1926). These proteins were selected for further analysis to determine their function and suitability as biomarkers for antibody-based detection of *L. monocytogenes* genetic lineage III.
Mass Spectrometry and Bioinformatics analysis were performed at IGBB.

Construction of recombinant plasmids, protein expression and purification.

PCR amplicons for the four genes (ST33077_1897, ST33077_2770, ST33077_1129, and ST33077_1926) were amplified from *L. monocytogenes* ST33077 genomic DNA using the primer pairs shown in (Table A.2). Restriction endonuclease sites were incorporated at the 5' end of each primer. The four amplified products were purified with the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). Cleaned PCR products were digested with two restriction endonucleases, and the size of the digested fragment was analyzed on 1% agarose gel using electrophoresis. Each processed DNA fragment was ligated to pET28a digested using appropriate restriction enzymes. Ligation mixtures were transformed to chemically competent Nova Blue *E. coli* K12 using manufacturer's protocol (Table A.3). Positive clones were selected on LB agar plates with kanamycin. Candidate plasmids with appropriate fragment patterns were

sequenced using T7 promotor and T7 terminator primers to confirm correct orientation of the insert. Three resulting recombinant plasmids pET28a_1897, pET28a_2770, and pET28a_1129 were transformed into *E. coli* BL21(DE3), while (pET28a_1926) was transformed into *E. coli* C41(DE3) because of low level of expression in BL21 (DE3) (Table A.3).

Expression of ST33077_1897, ST33077_2770, and ST33077_1129 proteins in *E. coli* BL21 (DE3) and ST33077_1926 protein in *E. coli* C41 (DE3) were optimized in 250 ml cultures. Expression in the bacteria was induced at an optical density 0.6- 0.8 nm (OD_{600}) by adding IPTG ranging from 1 mM to 100 mM final concentration. Induced cultures were incubated overnight in temperatures ranging from 18-37 ^{o}c. Cell cultures at different time points (2, 4, 6, 8, and 18 h) were prepared and analyzed by electrophoresis in 12% SDS-PAGE. Non-recombinant bacteria and un-induced recombinant clones were used as controls (Abdelhamed et al., 2016).

Purification of the four recombinant proteins ST33077_1897, ST33077_2770, ST33077_1129, and ST33077_1926 containing six histidine tags was done using His-Bind (Novagen) resin column according to the manufacturer's protocols (Abdelhamed et al., 2016). The recombinant clones ST33077_1897 and ST33077_2770 were grown in 250 ml of LB broth and induced by 1 mM final concentration IPTG for 18 h at 18 ^{o}c. Recombinant clones ST33077_1129 and ST33077_1926 were grown in 250 ml of LB broth and induced by 100 mM IPTG. Bacteria were then harvested by centrifugation (6000 g for 20 min at 4 ºC), and the pellet was lysed using 8 M urea, 50 mM Tris–HCl (pH 8.0), 10 mM EDTA, and 10 mg/ml lysozyme, followed by sonication (6 cycles, 30 s) on ice. The sonicated suspension of three proteins (ST33077_1897, ST33077_2770, and ST33077_1129) were subjected to centrifugation and collected for protein purification step. For ST33077_1926 the pellet was washed with 0.2 M sodium phosphate buffer (pH 7.3), 1 mM EDTA, 50 mM NaCl, 5% glycerol, and 1 M urea,

followed by washing with homogenization buffer (50 mM Tris–HCl (pH 8.0), 100 mM NaCl, 0.5 % TritonX-100, 0.1 % sodium-azide). The pellet was solubilized in 6 M guanidinium chloride, 10 mM Tris–HCl (pH 8.0), 500 mM NaCl for 1 h at 4°C followed by centrifugation. The clarified supernatant was loaded onto a His-Bind column prepacked with Ni^{2+}-charged resin that had been pre-equilibrated with 10 ml of binding buffer. Non-specific proteins were removed by applying binding buffer followed by wash buffer (6 M urea, 500 mM NaCl, 20 mM imidazole, and 20 mM Tris-HCl [pH 7.9]). Recombinant proteins were then eluted with 6 M urea, 1 M imidazole, 250 mM NaCl, and 10 mM Tris-HCl. Purity of the proteins was determined by 12% SDS-PAGE analysis. Protein yield was determined on a spectrophotometer at 280 nm.

Polyclonal antibody production and antibody titer analysis

A total of eight 10-week-old specific pathogen free (SPF) New Zealand white rabbits were housed separately in stainless steel cages and allowed free access to complete pelleted rabbit diet and water. Rabbits were acclimated for seven days before use. Two rabbits were used to produce polyclonal antibody against each recombinant protein.

On day 1, 3 ml of blood was collected from the ear vein of each rabbit to determine pre-immunization antibody levels (control). The primary immunization with the purified proteins (ST33077_1926, ST33077_2770, ST33077_1897, and ST33077_1129) was conducted in 1 ml emulsion of sterile antigen and complete Freund's Adjuvant. The ratio of antigen to adjuvant was 4:1 with a final concentration of 250 µg/ml of recombinant protein. Rabbits were administered 0.3 ml of the antigen/adjuvant mixture subcutaneously in four locations on the back and flank regions.

Two booster immunizations were performed at 14- 21-day intervals using emulsion with incomplete Freund's Adjuvant administered subcutaneously in four different locations. The ratio of antigen to adjuvant was 2:1. Post-immunization blood samples were collected 7 and 21 days after the 2nd booster injection to determine antibody titers using an enzyme-linked immunosorbent assay (ELISA).

After a sufficient antibody response was developed, a final blood sample was collected by intracardiac (IC) method under anesthesia with ketamine (15 mg/kg), dexmedetomidine (0.125 mg/kg), butorphanol (0.2 mg/kg), and glycopyrrolate (0.01 mg/kg). Rabbits were euthanized without recovery from anesthesia using dexmedetomidine reversed with atipamezole (as needed).

Analysis of antibody titer in rabbit serum was done using two different methods of the enzyme-linked immunosorbent assay (ELISA). In the first method, a 96-well ELISA plate (Bloomington, MN, U.S.A) was coated with the purified recombinant proteins. The purified protein sample was diluted to 20 mg/ml final concentration using sterile PBS, 100 µl per well. In the second method, a 96-well ELISA plate (Bloomington, MN, U.S.A) was coated with inactivated bacterial suspension. *L. monocytogenes* strains (listed in Table 1) were cultivated to a concentration of 10^8 CFU/ml, heat inactivated for 3 h, washed, and suspended in sterile Hank's balanced salt solution (HBSS).

For both ELISA methods, 100 µl rabbit serum diluted 1:100 was added to each well. After washing, goat anti-rabbit IgG antibody conjugate (Fisher Scientific) was used for detection with p-nitrophenyl phosphate substrate (Sigma 104 phosphatase substrate) dissolved in 10% diethanolamine buffer. Absorbance was measured at 405 nm in an ELISA Microplate Reader

(CA, USA). To standardize, average background absorbance for each plate was subtracted from the measured absorbance for each well.

Automated Western Blot (WES) immunoassays were conducted using a fully automated Simple Western™ capillary instrument (Protein Simple, San Jose, CA) according to the manufacturer's protocol. For ST33077_1926, the assay was set up as follows: final concentration of 5 µg/ml per well of protein diluted in PBS. Antibodies against ST33077_1926 were applied in dilutions of 1:250, 1:500, 1:750, and 1:1000 as one dilution per well. For ST33077_2770, a final concentration of 2. 5 µg/ml of protein diluted in PBS was used per well. Antibodies against ST33077_2770 protein were applied in dilutions of 1:500, 1:1000, 1:2500, 1:5000, 1:7500, 1:10000, and 1:15000 as one dilution per well. Protein samples, antibodies, and reagents were loaded on pre-filled cartridges. Serum (primary antibody) and anti-rabbit HRP-conjugated secondary antibody were used for immunoprobing with chemiluminescent substrate, which took place in the capillaries. Resulting assay data were automatically processed and analyzed by Compass software (Protein Simple).

Automated capillary western blot technique was performed at IGBB.

Statistical analysis

The rabbit serum antibody response against the injected recombinant proteins (measured by ELISA using plates coated with respective recombinant protein) was compared between the preinjected serum and the 21 day post-immunization sera with mixed model logistic regression using PROC GLIMMIX in SAS for Windows 9.4 (SAS Institute, Inc., Cary, NC, USA).

The variance in the mean difference in reactivity between preinjected serum and immune serum for each protein against the mean of all four lineage I strains, four lineage II strains, and

four lineage III strains was compared using PROC GLM Dunnett in SAS for Windows 9.4. Additionally, a multiple variant comparison of the mean difference in reactivity for each genetic lineage III strain was done separately against the mean differences of all four lineage I and all four II strains using PROC GLM with an adjustable P value.

Results

Mass Spectrometry

LC/MS analysis revealed that 232,470 spectra were detected in Lm-33077 (lineage III) and absent in Lm-33007 and Lm-33014 (lineage I and II respectively) with a false discovery rate (FDR) ≤ 0.05. These spectra mapped to 1,522 ORFs, out of which 651 had unique peptides present in Lm-33077 and not in Lm-33014 and Lm-33007. Out of the 651 ORFs with unique peptides, 42 ORFs had no orthologs in Lm-33014 and Lm-33007.

These 42-candidate lineage III-specific proteins were identified based on a comparison of three strains (one from each genetic lineage). To determine which of these 42 have the best potential as biomarkers that would be uniquely present in all lineage III strains (and missing in all lineage I and II strains), these 42 proteins were analyzed by BLAST against the proteomes of 80 sequenced *L. monocytogenes* strains (representing all three genetic lineages) available in NCBI. Results revealed that 20 proteins out of 42 have less than 50% similarity (identity * coverage) to any Lineage I and II proteins (Table A.4). The predicted function of the 20 candidate ORFs was determined and is shown in (Table A.5). Table A.6 shows the number of peptides and the percent coverage of each ORF based on all unique peptides.

Four proteins (ST33077_1897, ST33077_2770, ST33077_1926, and ST33077_1129) out of twenty had the highest peptide coverage in lineage III strains and lowest identity and coverage in lineage I and II strains, and these four were selected for further analysis (See Discussion for biological justification).

Expression and purification of the recombinant proteins.

Four genes from *L. monocytogenes* ST 33077 (*ST33077-1129, ST33077-1926, ST33077-2770,* and *ST33077-1897*) were successfully cloned into pET28a vector. Successful cloning was confirmed by restriction enzyme analysis and DNA sequencing. The result of expression analysis revealed that the induced recombinant bacteria started expression of ST33077-2770 and ST33077-1897 proteins after 6 h of induction with 1 mM final concentration of IPTG and reached maximum expression after 18 h at 18 ^{o}C. On the other hand, induced *E. coli* started expression of ST33077-1129 and ST33077-1926 proteins after 2 h of induction with 100 mM IPTG and reached a maximum level at 6 h at 37 ^{o}C.

Recombinant ST33077-1129, ST33077-1926, ST33077-2770, and ST33077-1897 proteins were estimated to have a molecular weight of 107.73, 33.62, 20.91, and 21.15 kDa, respectively. Each purified recombinant protein yielded a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) which is higher than the expected molecular weights by around 3 KDs for each protein (Figure A.1). This could be because of post translation modification of the protein or because of expression of some vector peptides during the bacterial culture induction with the IPTG. The identities of the four proteins were confirmed using LC-MS analysis. The raw mass spectra data were matched to proteins using SEQUEST algorithm of Proteome Discoverer program and translated ORF database of Lm33077. Sequences of matched proteins were further searched by BLAST against *L. monocytogenes* database at NCBI.

Rabbit serum antibody response

ELISA was used to determine the titers of the polyclonal antibody obtained from each rabbit serum at 21 days after the second booster injection with ST33077-1897, ST33077-2770, ST33077-1129, and ST33077-1926. When ELISA plates were coated with purified proteins, immunized rabbit serum showed a significantly ($p < .0001$) higher antibody titer than the pre-immunized rabbit serum for all four proteins (Figure A.2).

To determine the specificity of polyclonal antibodies for each recombinant protein, reactivity of immune serum for each protein was determined against whole cell lysate from twelve L. monocytogenes strains (four lineage III strains, four lineage I strains, and four lineage II strains). Pre-injected rabbit sera and sera collected 21 days post-injection with the four purified protein were tested against whole cell lysate of each representative strains in lineages III, I, and II using ELISA to determine the reactivity of ST33077_1926, ST33077_2770, ST33077_1897 and ST33077_1129-specific rabbit immune serum against lineage I, II and III strains (Figures A.3, A.4, A.5 and A.6).

To statistically compare the reactivity of immune serum for each recombinant protein to lineage III, lineage I, and lineage II strains, the difference in reactivity between pre-injection serum and immune sera for each L. monocytogenes strain was calculated, and the mean difference in reactivity was determined for each protein against lineage III, lineage I, and lineage II. When the mean difference in antibody reactivity for all four lineage III strains was compared to the mean for all four lineage I and all four lineage II strains, significantly higher reactivity was detected in serum against ST33077_1926 and ST33077_2770 in the plates coated with whole cell lysate of L. monocytogenes genetic lineage III strains compared with the plates coated with whole cell lysate of L. monocytogenes genetic lineages II and I ($p < 0.0001$ and < 0.0018,

respectively) (Figures A.7 and A.8). By contrast, the mean difference in reactivity of serum against Lm33077-1897 and Lm33077-1129 showed no significant difference between the plates coated with whole cell lysate of L. monocytogenes genetic lineage III strains against plates coated with whole cell lysate of L. monocytogenes lineages II and I strains (P = 0.6 and 0.68, respectively) (Figures A.9 and A.10).

When the mean difference in antibody reactivity for each individual lineage III strain was compared to the mean reactivity for all four lineage I and all four lineage II strains using multiple variant comparison, all four lineage III strains (ST33077, RM3030, RM3170, and RM3171) had significantly higher differences in antibody reactivity against ST33077_1926 protein compared to the mean difference of all four lineage I strains and all four lineage II strains. However, ST33077_2770 protein only had significantly higher differences in antibody reactivity in RM3030 and RM3170 compared to the mean of all four lineage I strains and all four lineage II strains. As expected, ST33077_1897 and ST33077_1129 did not show any significant difference in antibody reactivity of any lineage III strain compared by the mean of antibody reactivity for all four lineage I and II strains.

Automated Western Blots

Capillary Western blots were conducted to analyze purity and confirm the size of recombinant ST33077_1926 and ST33077_2770. Compass software generates gel-like images of immunoassays (Figures A.11 and A.12). For ST33077_1926, a single clear band was present at 1:1000 and 1:750 serum dilutions, and the size matched the predicted molecular weight for ST33077_1926 (33.6 kDa). For ST33077_2770, a single clear band was visible at 1:7500 and

1:10000 dilutions of serum. The band was approximately 40 kDa, which is about twice the predicted molecular weight of 20.9 kDa, suggesting the protein may exist as a dimer.

Discussion

Several subtyping techniques using many *L. monocytogenes* strains identified four major genetic lineages (Aarts et al., 1999; Aguilar-Bultet et al., 2018; Bibb et al., 1990; Piffaretti et al., 1989), and specific serotypes are associated with each lineage. Lineage I strains are the most frequently associated with clinical outbreaks of listeriosis. Lineage II strains also commonly cause clinical listeriosis and are routinely found in food and environmental samples. Lineage III strains are not as common. They are often isolated from animals, and they typically pose little risk of causing disease in people (Aarts et al., 1999; Levenson, 2010; Tost, 2009).

Besides serology, differentiation of *L. monocytogenes* lineages currently relies on DNA-based technology. To date, there are no reported biomarkers useful for differentiating *L. monocytogenes* genetic lineages. Because lineage III strains are low risk for human listeriosis, we sought to identify protein biomarker(s) that could distinguish *L. monocytogenes* genetic lineage III isolates from lineages I and II isolates. A protein biomarker could be used for development of a simple antibody-based method for distinguishing lineages.

Another objective of the current study was to determine if mass spectrometry-based shotgun proteomics could be used to identify candidate biomarkers. Our reasoning was that mass spectrometry would be an efficient screening method compared to a purely bioinformatics-based search for unique proteins because we could be assured that any candidate biomarker proteins are actually expressed in detectable quantities prior to conducting labor-intensive cloning and expression of candidate recombinant proteins. To test this method, we used three

strains of *L. monocytogenes*, one representing each of the three genetic lineages. Electrospray ionization tandem mass spectrometry was used to identify peptides from each of the strains, and comparative proteomics analysis between the three strains yielded candidate proteins unique to lineage III.

Proteome coverage was improved by running liquid chromatography-tandem mass spectrometry in triplicate for each strain. Coverage was further improved by running a liquid chromatography gradient based on molecular weight and running mass spectrometry on resulting fractions. Increasing the gradient length enhances the number of protein identifications in complex mixtures by reducing complexity of fractions, which increases sensitivity of mass spectrometry output (Hsieh et al., 2013; Wang et al., 2015). Analysis of brain proteome demonstrated that coupling of prefractionation with long gradient LC-MS/MS yielded higher peptide numbers and increased protein coverage with less than 1% protein false discovery (Wang et al., 2015).

After identification of potential lineage III-specific proteins based on mass spectrometric analysis of three strain, an additional bioinformatic analysis was conducted to help select the best candidate proteins for cloning and expression. BLAST analysis of each candidate lineage III-specific protein against a proteome database of >80 *Listeria monocytogenes* strains (representing all three lineages) was conducted to account for strain sequence variation within each genetic lineage and select candidate proteins consistently present in lineage III strains but with low percent identity and coverage against all lineage I and II strains. The predicted functions of the four candidate proteins include a DNA methyltransferase (ST33077_1926), type IV secretion protein Rhs (ST33077_1129), a membrane protein (ST33077_2770), and a hypothetical DUF3916 domain-containing protein (ST33077_1897).

Membrane proteins are good potential biomarkers due to their surface exposure. Several researchers used membrane proteins as biomarker for differentiating infectious *L. monocytogenes* strains from noninfectious strains and CNS- related strains from non-CNS related strains (Aguilar-Bultet et al. 2018). DNA methyltransferase proteins are potentially useful as universal biomarkers in cancer cell detection (Wallden et al., 2010) and disease-associated changes (Tost 2009).

Type IV secretion system (T4SS) is a large protein complex that plays an important role in *L. monocytogenes* virulence (Schlech et al. 1938). Some T4SS proteins are expressed by *L. monocytogenes* to translocate virulence proteins into the host cell (Green and Mecsas, 2016; Levenson, 2010). Other T4SSs facilitate gene transfer, enabling adaptation to environmental changes and probably in the spread of antibiotic resistance genes (Koskiniemi et al., 2014).

Cloning, expression and purification of the four-candidate lineage III-specific proteins (ST33077_1926, ST33077_2770, ST33077_1897, and ST33077_1129 domain) were very effective using pET28a (5369 bp) in *E. coli* with the T7 promoter. Cloning of the T4SS gene was difficult due to its size (approximately 10 kbp), which is almost twice the plasmid size. Therefore, approximately 2900 bp of the T4SS gene was cloned in pET28a expression vector, which represents the rearrangement hotspot (Rhs) repeat domain. The Rhs domain has Rhs toxin activity (Koskiniemi et al., 2013), which is found in both bacteria and eukaryotes, but the function of this protein is not well studied (Chen et al., 2015). In gram-negative and gram-positive bacteria, Rhs toxin functions as an intercellular competition mediator by inhibiting the growth of neighboring cells (Chen et al., 2015). Rhs also functions as an immunity protein to protect the bacteria from autoinhibition (Chen et al., 2015; Koskiniemi et al., 2013).

ST33077_1897, ST33077_2770, and ST33077_1129 proteins showed a high level of expression in BL21(DE3) *E. coli* in the insoluble fraction. However, ST33077_1926 protein showed no expression after IPTG induction. Additionally, bacterial growth stopped immediately after IPTG induction. Therefore, we used C41(DE3) *E. coli*, which is more resistant to some toxic proteins, to express ST33077-1926, where it was isolated from inclusion bodies.

A lineage III-specific protein biomarker has the potential for development of a simple, field-deployable antibody-based detection method. To begin testing the feasibility of this approach, we developed polyclonal antibodies for each of the recombinant proteins. Some background reactivity was detected against *L. monocytogenes* in all the rabbit sera, but all four proteins stimulated significant antibody titers compared to pre-inoculation serum. The antibody titers in pre-inoculation rabbit's serum may be due to environmental exposure to *L. monocytogenes*, which is common in the environment as a commensal and opportunistic pathogen in rabbit's intestine. Another factor likely contributing to the background antibody reaction is that the ELISA plates were coated with *L. monocytogenes* whole cell lysate, so that some reactivity could occur from other bacterial antigens.

Next, to determine the specificity of the polyclonal antibodies for detection of lineage III *L. monocytogenes*, each was tested for reactivity against whole bacteria from the three original strains (ST33077, ST33014, and ST33007) and nine additional *L. monocytogenes* strains representing the three lineages (Table 1). Importantly, when the mean reactivity of serum against all four lineage III strains was compared to the mean reactivity of serum against all four lineage I and II strains, polyclonal antibodies against two of the proteins, ST33077_1926 and ST33077_2770, showed significantly higher reactivity to *L. monocytogenes* lineage III strains than lineage I and II strains. ST33077_1897 and the Rhs domain of ST33077_1129 did not have

a significant difference in reactivity between lineages III, II, and I. Based on ELISA results using four representative strains from each genetic lineage, ST33077_1926 and ST33077_2770 are promising proteins biomarkers for development of an antibody-based method to differentiate *L. monocytogenes* genetic lineage III from lineage I and II.

Of the two potential biomarkers, ST33077_1926 has higher potential than ST33077_2770 as a biomarker because ST33077_1926-specific serum reacted significantly higher against each of the four individual lineage III strains compared to the mean reactivity against all four lineage I and lineage II strains. By comparison, ST33077_2770-specific serum only reacted significantly higher against two of the four individual lineage III strains compared to the mean reactivity against all four lineage I and lineage II strains.

Western blots based on capillary electrophoresis confirmed that the polyclonal antibodies detected the recombinant proteins ST33077_1926 and ST33077_2770. The size of ST33077_1926 was also confirmed (33.62 kDa). Western blot results suggest that ST33077_2770 (20.9 kDa) exists as a dimer, but little information is published about this protein, so this could not be confirmed. Capillary Western blots provided good sensitivity and protein separation compared to traditional Western blots, and it reduces time and reagent requirements (Beekman et al., 2018).

In conclusion, our results confirmed that ST33077_1926 and ST33077_2770 are promising candidates as proteins biomarkers for differentiating *L. monocytogenes* genetic lineage III from lineage I and II, which will facilitate identification of *L. monocytogenes* subgroups in diagnostic microbiology laboratories and ostensibly in food processing facilities. This increased discriminatory capability would assist in assessment of risk from *L. monocytogenes* isolates and accelerate epidemiological investigations. Biomarkers for differentiating lineage I and II strains

are yet to be identified, but the approach described in the current study shows promise for identifying protein biomarkers for *L. monocytogenes* genetic Lineages I and II.

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APPENDIX A

TABLES AND FIGURES

Table A.1 *Listeria* monocytogenes strains used in this study

Strain No.	Serotype	Genetic Lineage	Source	Reference
Lm33077	4a	III	Unknown	USDA-ARS
Lm33014		II	Unknown	USDA-ARS
Lm33007		I	Unknown	USDA-ARS
RM2707	1/2b	I	Cheese	(Gorski et al. 2003)
RM2995	1/2b	I	Cow brain	(Gorski et al. 2004)
RM3109	1/2b	I	Unknown	(Gorski et al. 2003)
RM3000	1/2c	II	Soil	(Gorski et al. 2004)
RM3020	1/2c	II	Unknown	(Gorski et al. 2004)
RM3102	1/2a	II	Monkey	(Gorski et al. 2004)
RM3030	4c	III	Bull	(Gorski et al. 2004)
RM3170	4c	III	Unknown	(Gorski et al. 2004)
RM3171	4a	III	Unknown	(Gorski et al. 2004)

Table A.2 Primers used for PCR amplification

ORF	Primers	Primer Sequence	Restriction Enzymes	Amplicon size (bp)
ST33077_1897	Forward	AAA**GTCGAC**TTTTCCTATAAACCAAA*	SalI	534
	Reverse	AAA**GGATCC**GGTATGGGGAATAAGGA	BamHI	
ST33077_2770	Forward	AAA**GGATCC**ATTTTCATGTTTAATAA	BamHI	567
	Reverse	AAA**GTCGAC**TTGGGCATCATTCGCTT	SalI	
ST33077_1926	Forward	AAA**GTCGAC**CACCACCCCTGCCTGAT	SalI	920
	Reverse	AAA**GGATCC**GGATCCTTTATTCGTGCA ATTAA	BamHI	
ST33077_1129	Forward	AAA**GTCGAC**GGCACCGTCTTCGTGGT	SalI	2900
	Reverse	AAA**GCTAGC**AAGGACCTGAAGACACA	NheI	

*Bold letters at the 5' end of the primer sequence represent restriction endonuclease site added. AA nucleotides were added to the end of each primer containing a restriction endonuclease site to increase the efficiency of enzyme cut.

Table A.3 *E. coli* strains and plasmids

Strain or Plasmid	Description	Reference or source
E. coli Nova Blue	endA1 hsdR17 (rK12– mK12+) supE44 thi-1 recA1 gyrA96 relA1 lac F′[proA+B+lacIqZΔM15:Tn10] (Tet^R)	Novagen
E. coli BL21(DE3)	F− ompT hsdS gal; expression host F – ompT hsdSB (rB- mB-) gal dcm.	EMD Millipore
E. coli C41 (DE3)	Subgroup from Bl21 (DE3) help plasmid stability	Lucigen
pET-28a	Expression vector; Km^R	Novagen
pET28a_1897	pET28a with cloned ST33077_1897 amplicon	This study
pET28a_2770	pET28a with cloned ST33077_2770 amplicon	This study
pET28a_1129	pET28a with cloned ST33077_1129 amplicon	This study
pET28a_1926	pET28a with cloned ST33077_1926 amplicon	This study

Table A.4 *L.* monocytogenes ST33077 proteins with less than 50% identity and coverage in Lineages I and II strains

ORF	Lineage I (Identity * Coverage) *	Lineage II (Identity * Coverage)
ST33077_0135	NS†	NS
ST33077_0258	WP_003740445.1 (47.77)	WP_012951247.1 (47.32)
ST33077_0314	WP_003725659.1 (10.08)	NS
ST33077_0492	NS	NS
ST33077_0493	WP_023550422.1 (19.28)	NS
ST33077_0937	NS	NS
ST33077_1129	WP_061385917.1 (2.68)	NS
ST33077_1328	WP_003725868.1 (28.53)	WP_014602121.1 (28.15)
ST33077_1569	WP_003728189.1 (16.05)	WP_014601042.1 (10.39)
ST33077_1816	NS	NS
ST33077_1897	NS	NS
ST33077_1926	NS	WP_049962080.1 (26.68)
ST33077_2218	NS	NS
ST33077_2271	WP_003724612.1 (8.91)	WP_003731898.1 (8.91)
ST33077_2323	NS	NS
ST33077_2398	NS	NS
ST33077_2419	NS	NS
ST33077_2617	WP_021496264.1 (8.47)	NS
ST33077_2739	NS	NS
ST33077_2770	WP_003728337.1 (9.27)	NS

*Protein ID of the most similar protein in Lineage I or II and Identity*Coverage
†NS, no significant protein matches found by BLAST.

Table A.5 Predicted functions of candidate Lineage III-specific proteins

ORF	NCBI ID*	Similarity† (%)	Function
ST33077_0135	NS‡	0	
ST33077_0258	WP_012581893.1	97	hypothetical protein
ST33077_0314	WP_039381474.1	10.8	guanylate kinase
ST33077_0492	WP_070220410.1	100	hypothetical protein
ST33077_0493	WP_070220412.1	100	hypothetical protein
ST33077_0937	NS	0	
ST33077_1129	WP_014589151.1	94.2029	type IV secretion protein Rhs
ST33077_1328	WP_003739362.1	28.534	oxidoreductase
ST33077_1569	WP_070221254.1	100	hypothetical protein
ST33077_1816	NS	0	
ST33077_1897	WP_012582068.1	95	hypothetical protein
ST33077_1926	WP_077954308.1	100	DNA methyltransferase
ST33077_2218	NS	0	
ST33077_2271	WP_070779485.1	9.54548	peptide ABC transporter substrate-binding protein
ST33077_2323	WP_070219794.1	100	hypothetical protein
ST33077_2398	WP_070219932.1	100	hypothetical protein
ST33077_2419	WP_070219871.1	100	hypothetical protein
ST33077_2617	WP_070284542.1	13.1763	hypothetical protein
ST33077_2739	NS	0	
ST33077_2770	WP_003728958.1	90	membrane protein

*Best BLAST hits in NCBI database
†Similarity between ST33077 protein and its best BLAST hit.
‡NS, no proteins with significant similarity were identified in the NCBI database.

Table A.6 Number of peptides identified by LC/MS for candidate Lineage III-specific proteins and percent coverage of proteins by identified peptides

Protein	Number of peptides	% Coverage
ST33077_0135	1	19.697
ST33077_0258	1	8.21918
ST33077_0314	1	9.42029
ST33077_0492	7	6.7623
ST33077_0493	1	4.8913
ST33077_0937	3	15.6522
ST33077_1129	1	0.87069
ST33077_1328	12	8.73016
ST33077_1569	7	19.4915
ST33077_1816	1	9.56522
ST33077_1897	3	21.4689
ST33077_1926	31	24.6528
ST33077_2218	1	3.76712
ST33077_2271	1	6.96203
ST33077_2323	16	7.22892
ST33077_2398	1	7.69231
ST33077_2419	4	20.7547
ST33077_2617	1	12.5
ST33077_2739	1	19.1781
ST33077_2770	100	29.8913

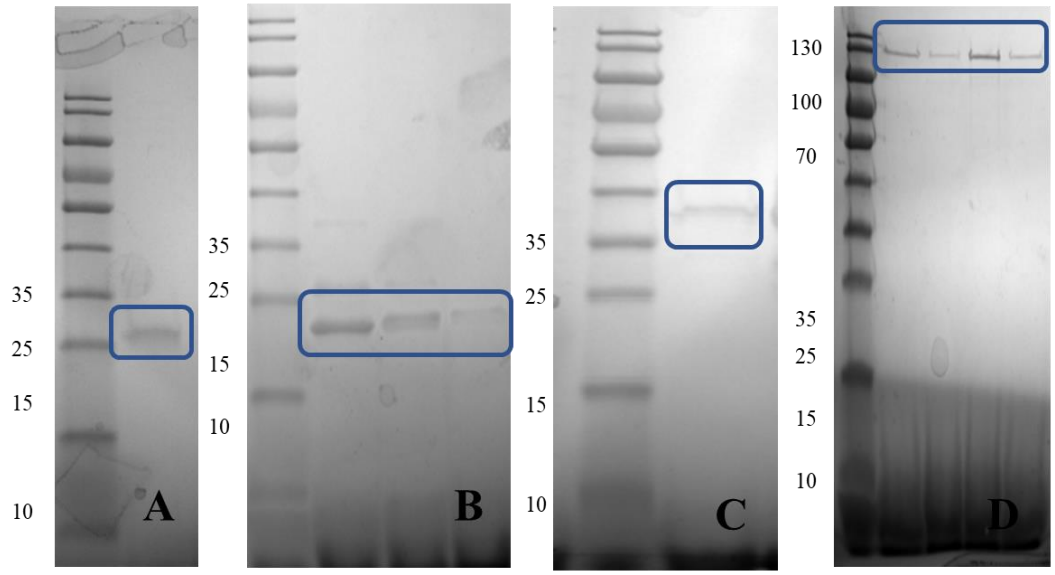

Figure A.1 Confirmation of recombinant protein purity using SDS-PAGE. ST33077_2770 (A), ST33077_1897 (B), ST33077_1926 (C), and ST33077_1129 (D). Molecular weight (KD) of bands in the Page-Ruler Pre-stained Protein Ladder (Thermo Scientific, MAN0011772) are shown to the left of each image.

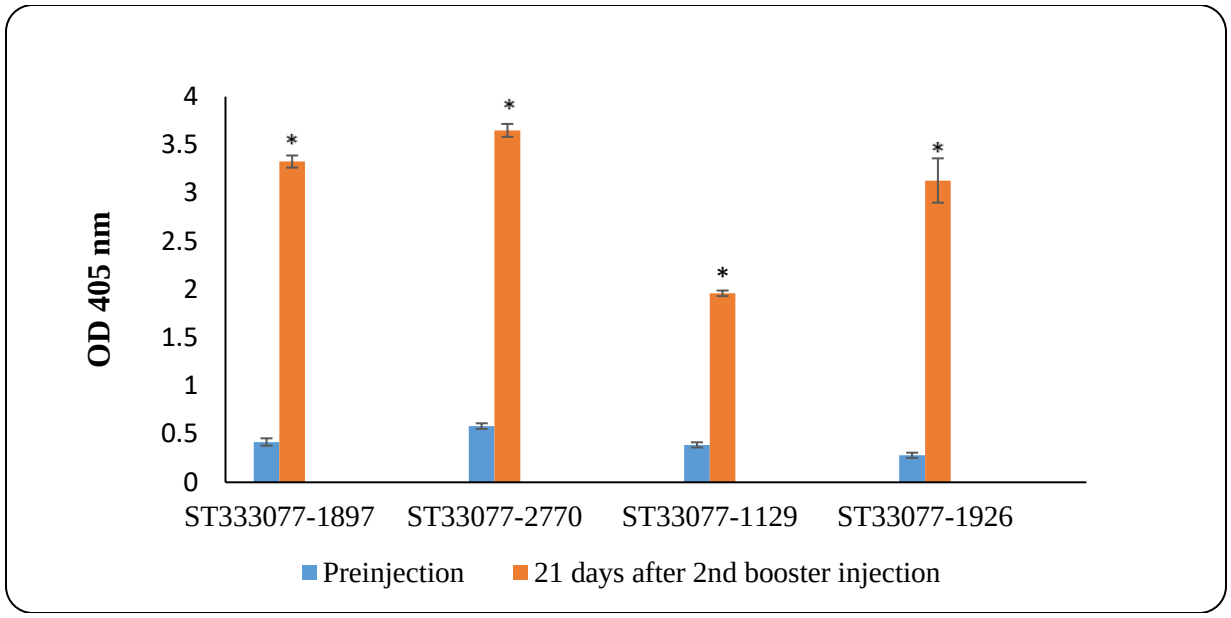

Figure A.2 Serum antibody response against recombinant proteins ST33077_1987, ST33077_2770, ST33077_1129, and ST33077_1926.

Optical densities at 405 nm are means of four replicates of tested serum against the purified protein ($p<0.0001$). Serum was collected 21 days after the second booster injection. Asterisks indicate significantly higher antibody titer compared to pre-injection serum.

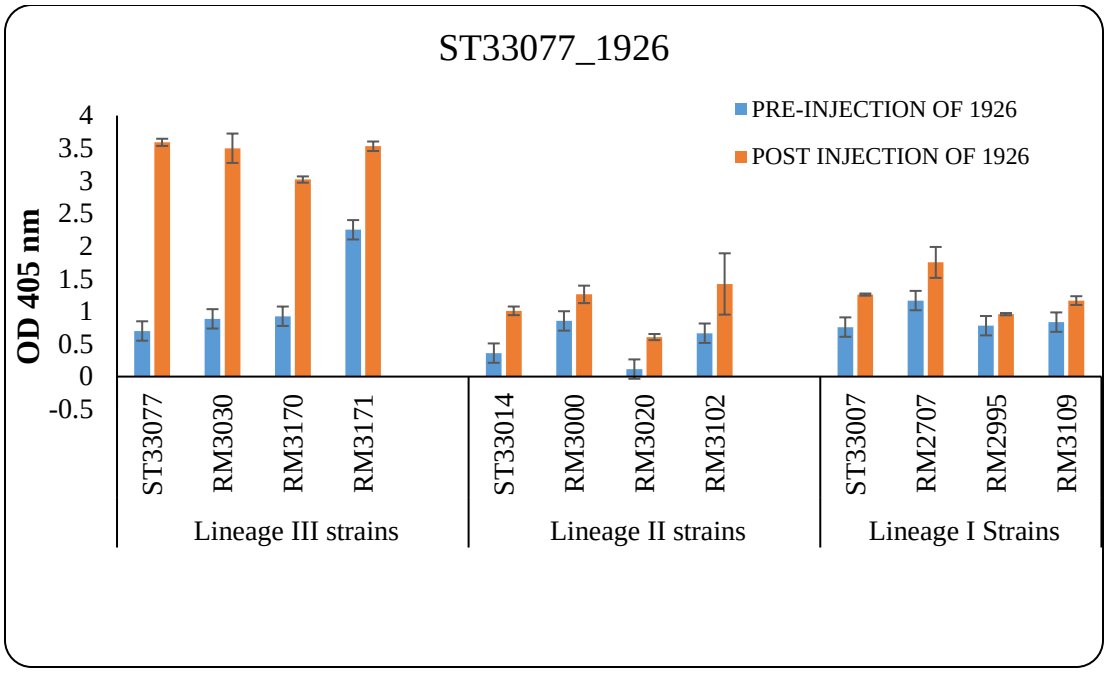

Figure A.3 Reactivity of ST33077_1926-specific rabbit immune serum against representative strains in lineages III, I, and II.

Antibody response was determined by ELISA using plates coated with whole cell lysate of each strain shown using rabbit sera collected 21 days post-injection with ST33077_1926 purified protein. Optical densities at 405 nm are means of two rabbits with four technical replicates each.

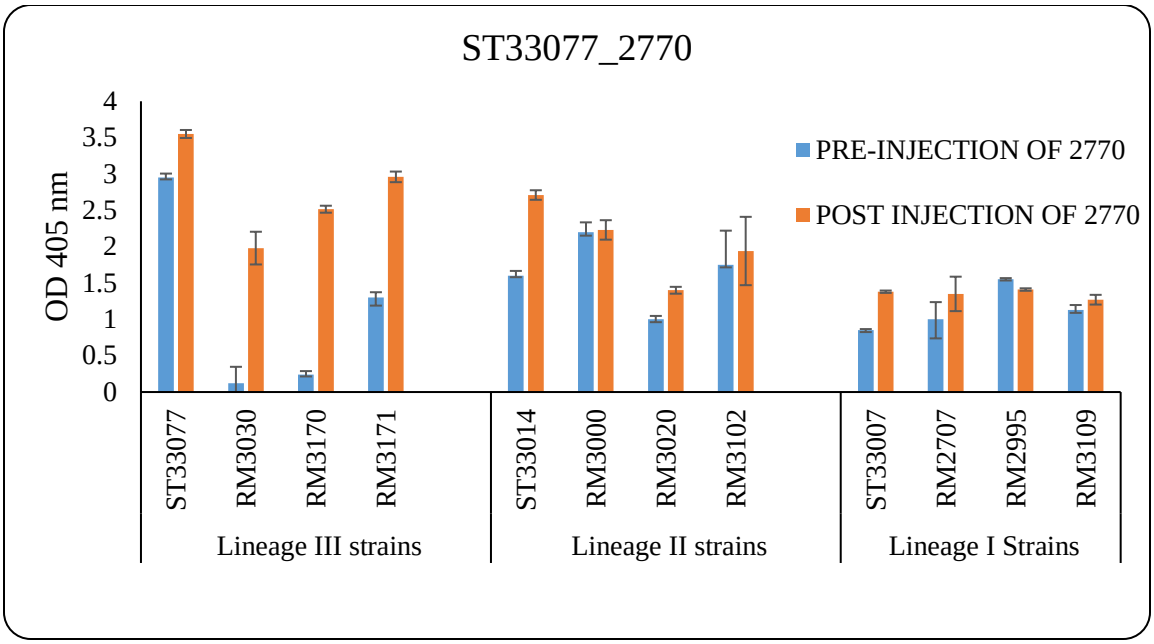

Figure A.4 Reactivity of ST33077_2770-specific rabbit immune serum against representative strains in lineages III, I, and II.

Antibody response was determined by ELISA using plates coated with whole cell lysate of each strain shown using rabbit sera collected 21 days post-injection with ST33077_2770 purified protein. Optical densities at 405 nm are means of two rabbits with four technical replicates each.

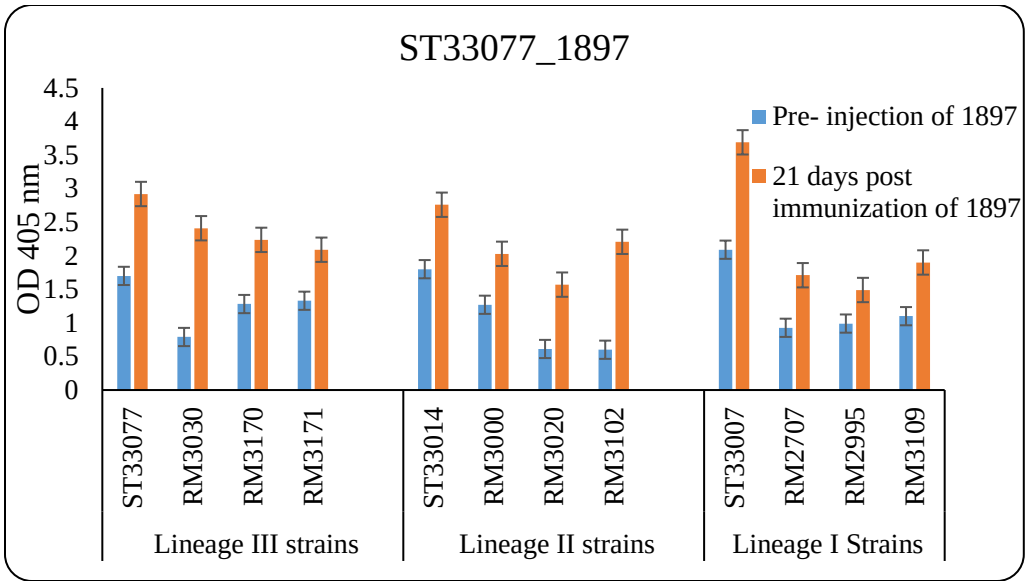

Figure A.5 Reactivity of ST33077_1897-specific rabbit immune serum against representative strains in lineages III, I, and II.

Antibody response was determined by ELISA using plates coated with whole cell lysate of each strain shown using rabbit sera collected 21 days post-injection with ST33077_1897 purified protein. Optical densities at 405 nm are means of two rabbits with four technical replicates each.

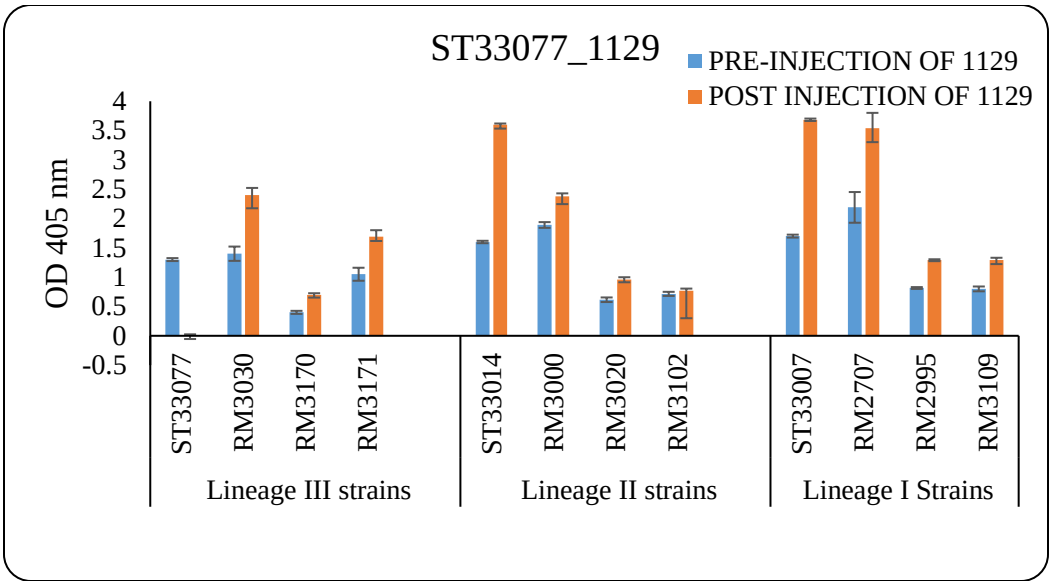

Figure A.6 Reactivity of ST33077_1129-specific rabbit immune serum against representative strains in lineages III, I, and II.

Antibody response was determined by ELISA using plates coated with whole cell lysate of each strain shown using rabbit sera collected 21 days post-injection with ST33077_1129 purified protein. Optical densities at 405 nm are means of two rabbits with four technical replicates each.

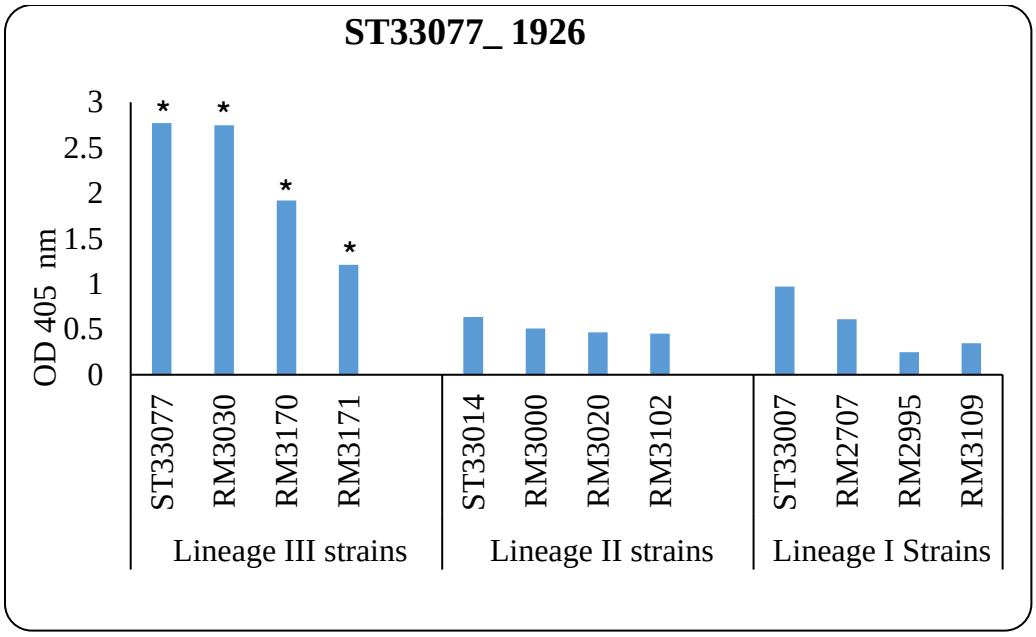

Figure A.7 Difference in mean serum reactivity between pre-injection serum and ST33077_1926-specific serum. Optical densities at 405 nm are the means of two rabbit's post immunization sera titer after subtracting of pre-injection serum titer from post-injection titer. Four technical replicates for each rabbit were used and tested against 12 L. monocytogenes strains. The result shows significantly higher antibody titer in comparing means of lineage III strains to the mean of lineage I and II ($P < 0.0001$). The result also shows significantly higher antibody titer in comparing each strain of lineage III separately to the mean of lineage I and II. Significant differences in reactivity to lineage III strains are indicated by asterisks ($P < 0.05$)

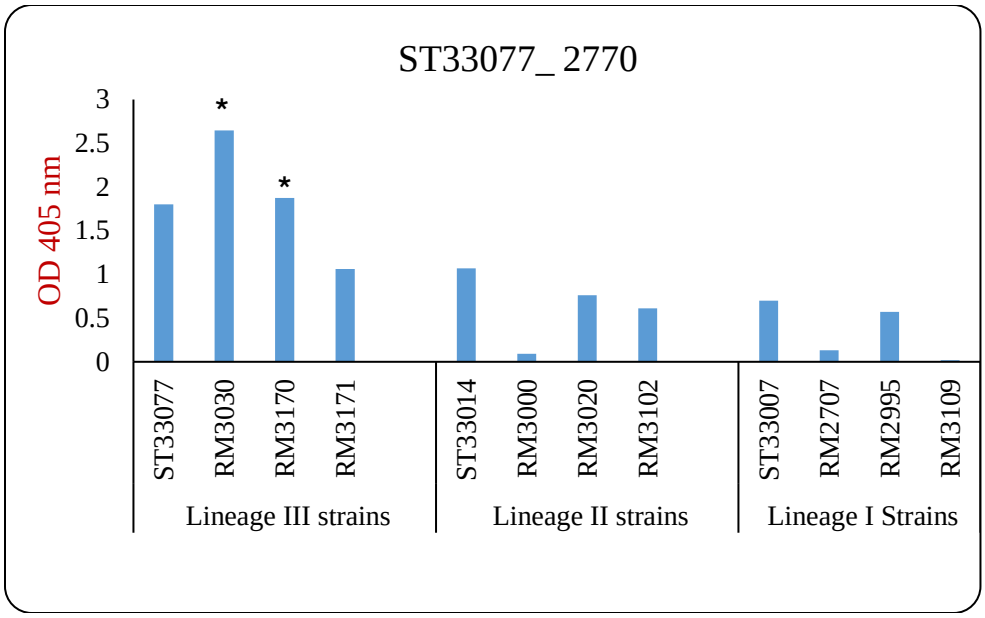

Figure A.8 Difference in mean serum reactivity between pre-injection serum and ST33077_2770-specific serum. Optical densities at 405 nm are the means of two rabbit's post immunization sera titer after subtracting of pre-injection serum titer from post-injection titer. Four technical replicates for each rabbit were used and tested against 12 L. monocytogenes strains. The result shows significantly higher antibody titer in comparing means of lineage III strains to the mean of lineage I and II ($P < 0.0018$). The result also shows significantly higher antibody titer in comparing each strain of lineage III separately to the mean of lineage I and II. Significant differences in reactivity to lineage III strains are indicated by asterisks ($P < 0.05$)

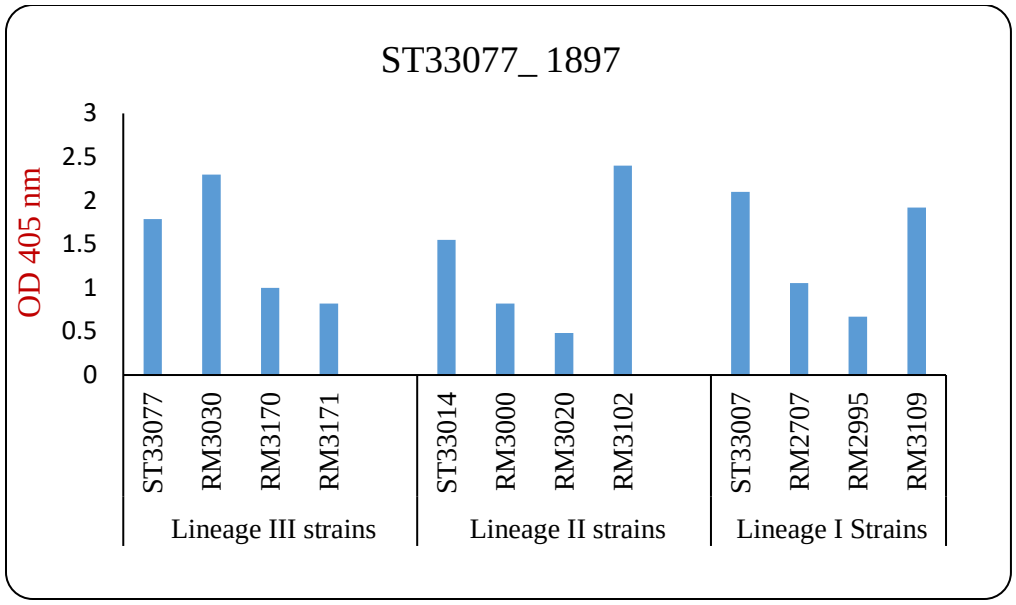

Figure A.9 Difference in mean serum reactivity between pre-injection serum and ST33077_1897-specific serum. Optical densities at 405 nm are the means of two rabbit's post immunization sera titer after subtracting of pre injection serum titer from post injection titer. Four technical replicates for each rabbit were used and tested against 12 L. monocytogenes strains. The result shows no significant difference between the mean of lineage III compared to lineage I and II.

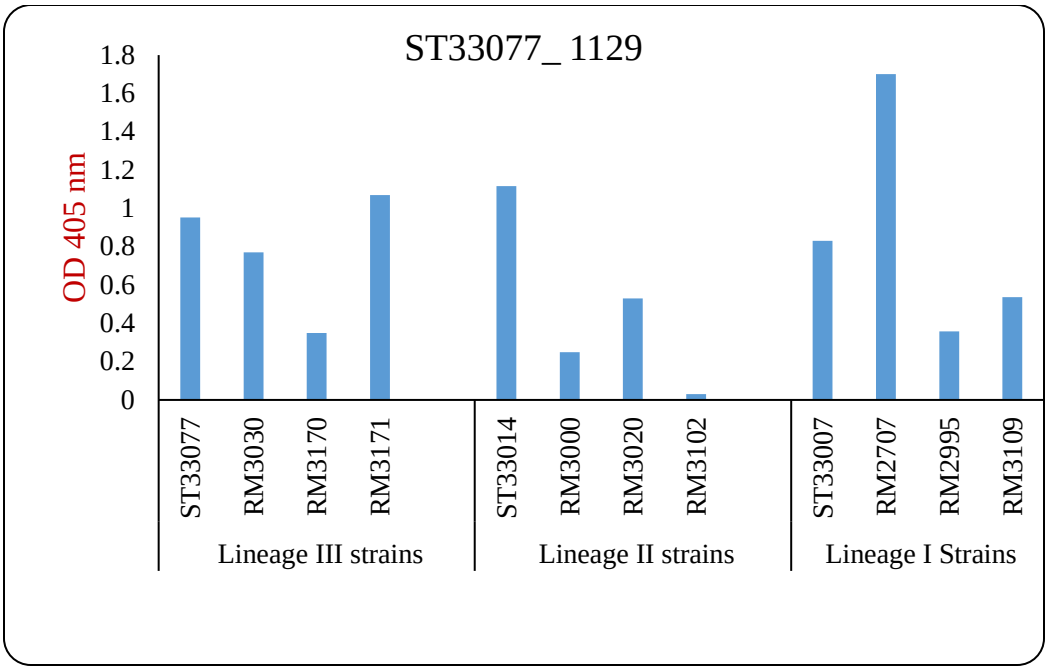

Figure A.10 Difference in mean serum reactivity between pre-injection serum and ST33077_1129-specific serum. Optical densities at 405 nm are the means of two rabbit's post immunization sera titer after subtracting of pre injection serum titer from post injection titer. Four technical replicates for each rabbit were used and tested against 12 L. monocytogenes strains. The result shows no significant difference between the mean of lineage III compared to lineage I and II.

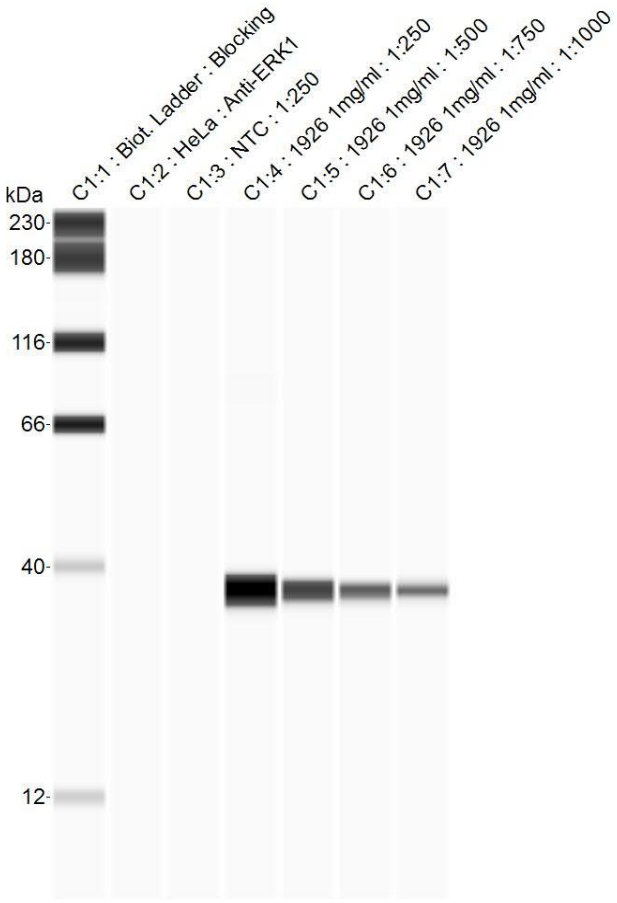

Figure A.11 Automated capillary Western blot gel-like image of ST33077_1926.

The predicted molecular weight of ST33077_1926 is 33.62 kDa. Lane 1: Manfacturer molecular weight standard; lane 2: Manufacturer Hela control; lane 3: Immune serum with no recombinant protein ; lanes 4-7: 5 µg ST33077_1926 with varying dilutions of immune serum.

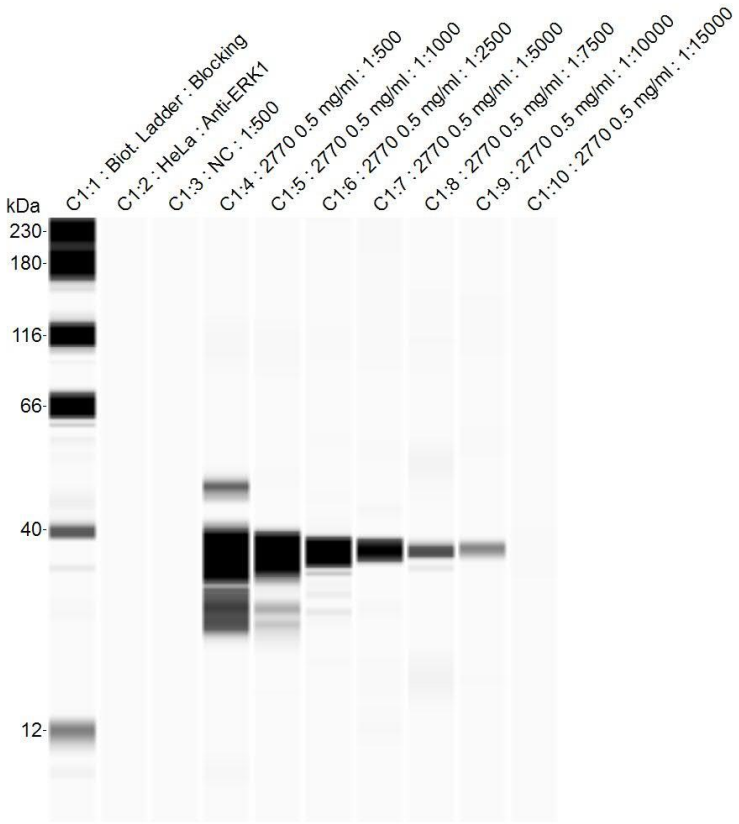

Figure A.12 Automated capillary Western blot gel-like image of ST33077_2770. The predicted molecular weight of ST33077_2770 is 20.9 kDa. Lane 1: Manufacturer molecular weight standard; lane 2: manufacturer Hela control; lane 3: immune serum with no recombinant protein; lanes 4-10: 2.5 µg ST33077_2770 with varying dilutions of immune serum.