DEVELOPMENT AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES THAT RECOGNIZE A WIDE SPECTRUM OF *LISTERIA MONOCYTOGENES* STRAINS

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ABSTRACT

Listeria monocytogenes is a bacterial pathogen that is typically transmitted to humans through consumption of contaminated foods. Infection with this organism can lead to a severe and life-threatening illness referred to as listeriosis. The goal of this study was to develop monoclonal antibodies (MAbs) with high specificity and affinity to proteins found on the surface of all strains of *L. monocytogenes* while not cross-reacting with non-pathogenic *Listeria* spp. or other major bacterial pathogens commonly found in foods. A literature search was conducted to identify ten candidate surface proteins involved or putatively involved in the virulence of *L. monocytogenes*. Bioinformatics analyses using BLAST on the NCBI website showed that five of the ten candidate proteins were potentially present in *L. monocytogenes* strains but absent from strains of other *Listeria* spp. Genes encoding for these five proteins, ActA, InlA, InlC2, InlJ and LapB, were cloned and expressed in *Escherichia coli*. MAbs were raised against recombinant LapB, InlJ and InlC2 proteins using hybridoma technology. A total of 48 anti-LapB, 33 anti-InlJ and 37 anti-InlC2 MAbs were developed. Based on the comparison of IFM signal of each MAb against *L. monocytogenes* cells, seven anti-LapB MAbs and six anti-InlC2 MAbs were selected for further characterization. All of the anti-InlJ MAbs showed weak IFM signals and negative reactivity in ELISA against *L. monocytogenes* cells. The selected anti-LapB and anti-InlC2 MAbs were further characterized by assessing their ability to bind to cells of 51 strains representing 11 *L. monocytogenes* serotypes using ELISA. Six anti-LapB MAbs (M3484, M3495, M3500, M3509, M3517, M3519) reacted strongly with 44 of 51 strains representing 9 of the 11 *L. monocytogenes* serotypes tested. Five anti-InlC2 MAbs (M3607, M3618, M3630, M3633, M3636) reacted strongly with 47 strains representing 10 of the 11 *L. monocytogenes* serotypes tested. These results indicate that anti-LapB and anti-InlC2 MAbs could potentially be used as diagnostic reagents for isolation and detection of almost all *L. monocytogenes* strains in contaminated foods.

To my husband.

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LIST OF ABBREVIATIONS

A
Ab Antibody
ActA Actin assembly-inducing protein
APS Ammonium persulfate

B
BLAST Basic local alignment sequence tool
BHI Brain heart infusion
BSA Bovine serum albumin

C
CB Coomassie blue
CFA Complete Freunds Adjuvant
CNS Central nervous system

D
DHEAS Dehydroepiandrosterone sulfate
DMEM Dulbecco’s minimal essential medium

E
ELISA Enzyme linked immunosorbent assay
ELFA Enzyme linked fluorescent assay

H
HIS Histidine
HRP Horseradish peroxidase

I
IFA Incomplete Freunds Adjuvant
IFM Immunofluorescence microscopy
Ig Immunoglobulin
InlA Internalin A
InlB Internalin B
InlC2 Internalin C2
InlJ Internalin J
IPTG Isopropyl-β-D-thiogalactopyranoside
IspC Immunogenic surface protein C
IV Intravenous

L
LLO Listeriolysin O
LRR Leucine-rich repeat
LB Luria-Bertani

M
MAb Monoclonal antibody
MW Molecular weight

O
OD Optical density
ORF Open reading frame
OLF Ottawa Laboratory Fallowfield

P
PAb Polyclonal antibody
PBS Phosphate buffer saline
PBS-T PBS containing 0.2% Tween 20
PCR Polymerase chain reaction
PlcA Phospholipase A
PlcB Phospholipase B
PMSF Phenylmethylsulphonyl fluoride
PS Parent strain

R
rInlC2 Recombinant internalin C2
rInlJ Recombinant internalin J
rIspC Recombinant immunogenic surface protein C
rLapB Recombinant *Listeria* adhesion protein B
RTE Ready-to-eat

S
SDS Sodium dodecyl sulfate
SDS-PAGE Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SPR Solid phase receptacle

U
UVM University of Vermont media

W
WB Western blot

FIGURES

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CHAPTER I

INTRODUCTION

1.0 INTRODUCTION

1.1 BACKGROUND

Listeria monocytogenes is a gram-positive intracellular pathogen that is ubiquitous in nature, being commonly found in water, soil and vegetation (1). Consumption of contaminated foods is the most common route of transmission to humans, which can lead to a severe and life-threatening illness referred to as listeriosis (2). The genus *Listeria* is comprised of eight species; *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. grayi*, *L. seeligeri*, *L. welshimeri*, *L. marthi and L. rocourtii. L. monocytogenes* is the major pathogenic species among the *Listeria* genus causing the majority of human cases of listeriosis, whereas *L. ivanovii* mainly causes animal infections. At least 12 serotypes of *L. monocytogenes* have been identified with three serotypes (1/2a, 1/2b and 4b) causing the majority of human listeriosis cases. Although the incidence is low, the high mortality rates (up to 50%) associated with listeriosis make *L. monocytogenes* one of the deadliest human foodborne pathogens. Serotype 4b has been associated with major foodborne outbreaks as well as sporadic cases, suggesting that it may possess unique virulence factors and highlights the importance of studying this strain.Virulence factors of *L. monocytogenes* play an important role in survival of the bacterium during an infection of a host, and thus are expected to be conserved in pathogenic strains of this organism.

1.2 RESEARCH RATIONALE

Conventional and standard procedures for isolation and identification of *L. monocytogenes* are laborious and time consuming, involving primary and secondary enrichment followed by plating on selective agar as well as serological and biochemical tests which can take 5-10 days to complete (3,4). Lack of rapid and effective methods for *L. monocytogenes* isolation from food samples is the major stumbling block to emerging rapid molecular detection technologies designed to reduce the turnaround time from sampling to test results. Antibody-based methods offer a means for faster isolation and detection of *L. monocytogenes* (5-8). A number of monoclonal antibodies (MAbs) that react with *L. monocytogenes* have been produced, however the majority cross-react with other *Listeria* species or do not recognize all 12 serotypes of *L. monocytogenes* (9-14). Low number of *L. monocytogenes* present in food samples can be a challenge for detection of this pathogen. It is rare for *L. monocytogenes* to be the only *Listeria* spp. present in a food sample. Most investigations on food and food-processing environments have found *L. monocytogenes* and *L. innocua* to be the most-prevalent *Listeria* spp. (15). During enrichment of potentially contaminated food, *L. innocua* frequently outgrows *L. monocytogenes* resulting in false negatives (16). Therefore, antibodies that bind uniquely to various strains of all serotypes of *L. monocytogenes,* while not cross-reacting with non-pathogenic *Listeria* spp. are still being sought after for use in the development of rapid and reliable detection and isolation tools for this pathogen in foods. Antibodies with high specificity, affinity and avidity for constitutively expressed *L. monocytogenes* surface proteins are fundamental for the development and optimization of antibody-based isolation and detection methods for this pathogen in a variety of food, environmental and clinical samples.

1.3 HYPOTHESES

This study was undertaken based on the following hypotheses:

1. Certain protein components such as virulence factors are present in all serotype strains of the pathogenic *L. monocytogenes,* but absent from non-pathogenic *Listeria* species.
2. These protein components are expressed on the surface of *L. monocytogenes* and thus form suitable antigen targets against which MAbs can be raised.
3. MAbs generated against these surface proteins bind exclusively to *L. monocytogenes* strains but do not bind to other non-pathogenic *Listeria* species or other bacteria.

1.4 OBJECTIVES

The goal of this study was to identify surface proteins that are present in strains of *L. monocytogenes,* but absent from non-pathogenic species and to produce MAbs that will recognize and bind to these surface proteins on whole cells of *L. monocytogenes*. Antibodies with high specificity and affinity for *L. monocytogenes* will facilitate the isolation, concentration and detection of this pathogen by various antibody-based technologies. The proposed work is expected to identify surface diagnostic markers and develop MAbs to these markers. By targeting only the pathogenic *Listeria* spp., reports will be able to conclude with greater confidence that positive tests indicate a serious health concern.

The four main objectives of this study were:

1. To identify known or putative surface virulence factors (proteins) common and unique to all strains of *L. monocytogenes*.
2. To produce recombinant forms of the identified proteins to be used as the immunizing antigens
3. To assess the expression of identified proteins on the surface of *L. monocytogenes* using polyclonal antibodies
4. To produce and characterize MAbs against three selected surface proteins

CHAPTER II

LITERATURE REVIEW

2.0 LITERATURE REVIEW

2.1 *Listeria* taxonomy

The genus *Listeria* is comprised of eight species; *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. grayi*, *L. seeligeri*, *L. welshimeri*, *L. marthi and L. rocourtii. Listeria* has been categorized into three characteristically hemolytic species (*L. monocytogenes, L. ivanovii and L. seeligeri*) and two non-hemolytic species (*L. innocua* and *L. welshimeri*) (17). *L. rocourtii* and *L. marthii* are two new non-hemolytic *Listeria* species that were identified in 2010 (18, 19). *L. grayi* is another non-hemolytic species; however, due to its apparent divergence from the other *Listeria* spp. it has been suggested that this organism should be put a new genus, *Murraya* (20). Currently, *L. grayi* remains a part of the *Listeria* genus (21). *L. seeligeri* is regarded as non-pathogenic, yet it consists of both hemolytic and non-hemolytic isolates (22). The hemolytic isolates contain a homologue of the *prfA* virulence gene cluster which carries key virulence factors in *L. monocytogenes* and *L. ivanovii*. *L. monocytogenes* is the major pathogenic species for both animals and humans among the *Listeria* genus. *L. ivanovii* has caused disease in domestic animals, particularly in sheep (23) and has caused two cases of human listeriosis in the UK (24). *L. seeligeri* has also caused one case of human listeriosis in Switzerland (22).

L. monocytogenes is classified into 12 serotypes: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, and 7. Molecular subtyping techniques have identified two major phylogenetic lineages and one less common lineage within the species (25-26). Lineage I consists of serotypes 1/2b, 3b, 4b, 4d, 4e and 7, lineage II consists of serotypes 1/2a, 1/2c, 3a and 3c. Lineage III consists of two rare serotypes 4a and 4c. The majority of clinical and food isolates belong to only three serotypes; 1/2a, 1/2b and 4b (25). Although 1/2a strains are isolated more frequently from food, 4b strains cause over 50% of listeriosis cases worldwide, have been associated with a higher number of major foodborne outbreaks and result in more meningoencephalitis cases (27-31). This could suggest that strains of serotype 4b have become more adapted to mammalian host tissues and may possess unique virulence factors that allow the bacteria to spread further in the infected host and cross the blood-brain barrier.

2.2 *L. monocytogenes* and listeriosis

L. monocytogenes is a gram-positive intracellular pathogen that is ubiquitous in nature and is commonly found in water, soil and vegetation (32). Consumption of contaminated foods is the most common route of transmission to humans, which can lead to a severe and life-threatening illness referred to as listeriosis (33). *Listeria* infections are of great concern to public health due to their high hospitalization rates (up to 90%) and mortality rates (up to 50%) amongst foodborne pathogens in human infections (34). *L. monocytogenes* is a unique pathogen having the ability to survive and multiply in a wide range of temperatures (-1.5 to 50°C), pH (4.3 to 9.6), high salt concentrations as well as freezing and drying (32). Due to the growing consumption of ready-to-eat (RTE) foods and the high mortality rate associated with *L. monocytogenes* infections; this bacterium poses a serious threat to human health. A variety of contaminated food products have been the cause of outbreaks of listeriosis including dairy products such as non-pasteurized milk and soft-cheeses, vegetables and RTE foods such as hot-dogs and other processed meats. In the United States there is a zero tolerance policy for *L. monocytogenes* in RTE foods which means that the detectable presence (≥1 colony forming units (CFU) in 25 g of sample) of *L. monocytogenes* requires a recall. In Canada since April 2011, there is a zero tolerance policy for certain RTE foods that have been identified as a high risk product in which *L. monocytogenes* growth can occur throughout its stated shelf life (35). These food products receive the highest priority for industry verification and control with a recall of the product if detection of *L. monocytogenes* in 125 g is confirmed. RTE foods in which have a limited or zero potential for growth of *L. monocytogenes* can have pathogen levels equal to or less than 100 CFU/g. This category of RTE foods are considered as low risk and receive a lower priority with regards to verification and control.

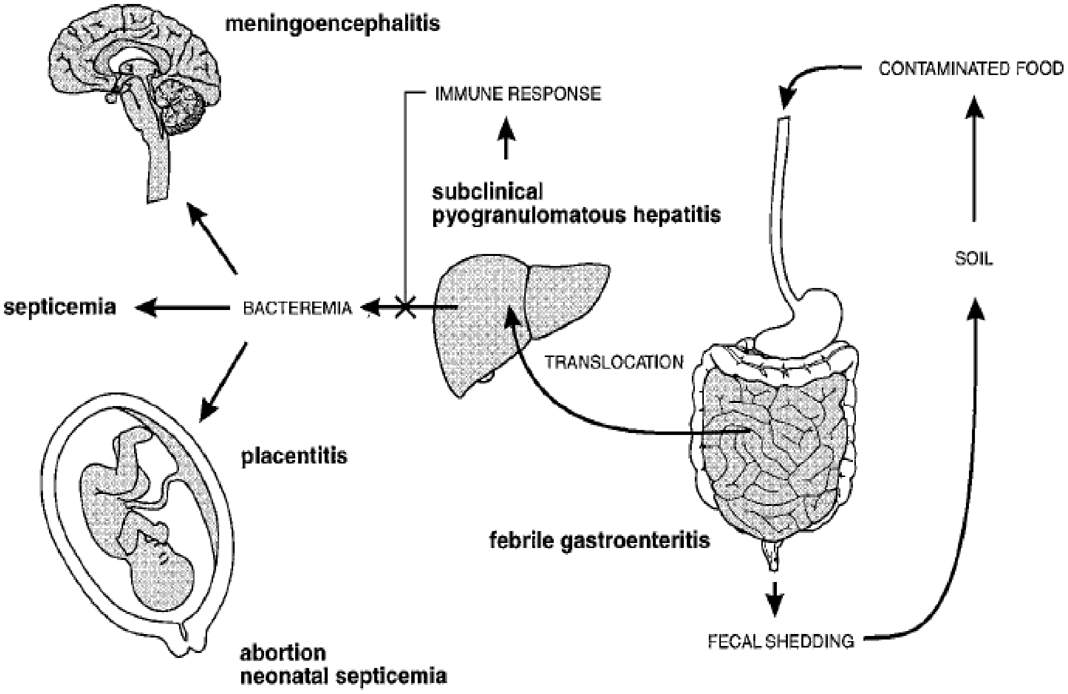
Listeriosis still remains a worldwide disease with several outbreaks being reported annually throughout North America for nearly three decades. In 1985, there was a major outbreak of listeriosis associated with mexican-style cheese in California, which led to a total of 86 cases, 8 deaths and 21 miscarriages (36). From August 1998 to January 1999 the United States experienced their largest outbreak of listeriosis, resulting in 101 cases, 15 deaths and 6 miscarriages across a total of 22 states. The outbreak was caused by the consumption of processed meat contaminated with a rare strain of *L. monocytogenes* serotype 4b (37). In 2002, there was another multistate outbreak resulting in 46 people being infected, seven deaths and three miscarriages (38).

Foodborne outbreaks of disease do not happen frequently in Canada; however, there has been a consistent rise in listeriosis cases in recent years. Between 2005 and 2009 the number of cases of listeriosis reported annually in Canada and Europe had significantly increased. In 2008, Canada experienced a major outbreak of listeriosis originating from deli meats produced by Maple Leaf Foods in Toronto, Ontario causing a total of 57 illnesses and 22 deaths (39). The mortality rate was particularly high because the deli meats were distributed to long-term health care institutions and those infected were mostly elderly people or those with an underlying disease. During this outbreak, the average waiting period to confirm positive contamination with *L. monocytogenes* was seven to ten days (39). It is evident that faster verification of samples positive for *L. monocytogenes* directly influence how quickly contaminated food can be pulled from shelves.

2.3 Infection and pathogenesis

Although the incidence rate of human listeriosis is low, *L. monocytogenes* can cause severe illness and mortality in susceptible individuals. Those who are most at risk are the elderly, pregnant women, neonates and the immunocompromised or individuals with underlying diseases (40). Listeriosis in previously healthy individuals has been reported, but is uncommon and those patients generally survive the infection (41). Contaminated food is the most common source of a Listerial infection. The manifestation of a gastroenteritis infection in an otherwise healthy individual typically begins 20 hours after consumption of highly contaminated foods, whereas the incubation period for an invasive infection in a person with a compromised immune system is usually 20 to 30 days (42). The infectious dose differs depending upon the pathogenicity and virulence of the *L. monocytogenes* strain implicated and the host risk factors. The clinical symptoms are similar in each infected host. Figure 1 illustrates the pathophysiology of an *L. monocytogenes* infection in a human host. The gastrointestinal tract is the primary site of entry for *L. monocytogenes* where the cells encounter a hostile environment including low oxygen pressure, low pH and the presence of bile. *L. monocytogenes* proliferates in the Peyer’s patches of the intestinal cell wall and will translocate to other organs by a passive process. The Listerial cells that cross the intestinal barrier are transported by the lymph or blood to the mesenteric lymph nodes, spleen or liver (43, 44). *L. monocytogenes* cells are quickly removed from the bloodstream by macrophages residing in the spleen and liver (45, 46). These resident macrophages destroy most of the ingested bacteria during the first six hours after infection (47). Antigen-dependent proliferation of T-lymphocytes and the secretion of cytokines are induced, however, not all *Listeria* cells are destroyed and the remaining bacteria start to grow and significantly increase in numbers for 2-5 days in mouse organs. If the infection is not controlled by a sufficient immune response in the liver, which is often the case with immunocompromised individuals, unlimited proliferation of *L. monocytogenes* in the liver may result in the release of bacteria into circulation. *L. monocytogenes* can infect a variety of host tissues; however, the primary clinical forms of listeriosis demonstrate a clear tropism towards the pregnant uterus and the central nervous system (CNS). There are two principal forms of infection: neonatal listeriosis and listeriosis in the adult patient (40). Both types typically display as a disseminated infection or local infection in the CNS. Neonatal listeriosis results from invasion of the fetus via the placenta and results in abortion or the birth of a stillborn. The mother does not always show symptoms but may present as a mild flu-like condition with headache, chills, fatigue and muscular and/or joint pain approximately 2-14 days before miscarriage. Listeriosis in non- pregnant adults most frequently affects the CNS (55 to 70% of infections). Infections typically progresses as a meningoencephalitis accompanied by significant alterations in consciousness, movement disorders and in a few cases paralysis of the cranial nerves. The mortality rate with CNS infections is approximately 20% but can be as high as 40 to 60% if related to an underlying disease (42).

Figure 1. Schematic of *L. monocytogenes* pathogenicity. See text for details.

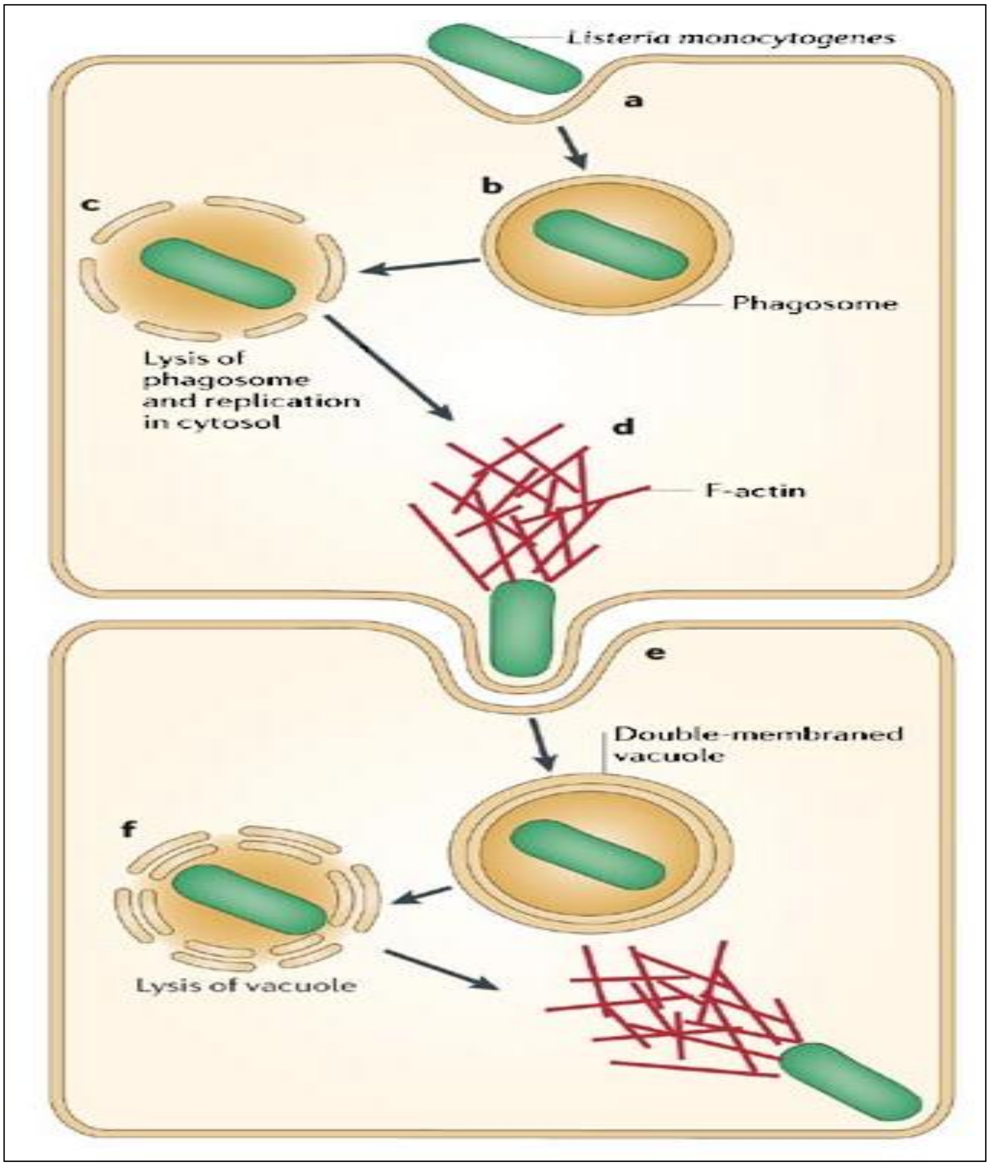

meningoencephalitis
IMMUNE RESPONSE
CONTAMINATED FOOD
subclinical
pyogranulomatous hepatitis
SOIL
septicemia
BACTEREMIA
TRANSLOCATION
placentitis
febrile gastroenteritis
FECAL SHEDDING
abortion
neonatal septicemia

The intracellular life cycle of *L. monocytogenes* is depicted in Figure 2. Entry into phagocytic cells is initiated through binding of the bacterial surface proteins Internalin A (InlA) and InlB to host cell receptors. Shortly after entry the bacteria disrupt the phagosome membrane by means of the toxin listeriolysin O (LLO). Actin assembly-inducing protein (ActA) mediates the formation of polarized actin tails composed of cross-linked actin filaments that propel the bacteria toward the cytoplasmic membrane. This movement is random; thus, in time bacteria reach the cell perimeter and push the membrane causing the formation of finger-like protusions referred to as pseudopods. Once in the cytosol, these pseudopods infiltrate uninfected neighbouring cells and are engulfed by phagocytosis creating a double membrane secondary phagosome. Bacteria escape this secondary membrane again by releasing the LLO toxin, and thus re-starting the cell-to-cell spread of this pathogen (2).

2.4 Virulence factors

The complete genome of *L. monocytogenes* strain EGDe (2,944,528 bp) was sequenced and published (48) which has led to the discovery of hundreds of protein coding genes whose functions have yet to be fully understood. Bacterial surface proteins comprise a large group of molecules that are involved in a diversity of essential functions, such as bacterial growth, detecting and defending against environmental stresses, adhesion and invasion of host cells, signaling, and interaction with the immune syste. The ability of *L. monocytogenes* to survive and multiply in host macrophages is in part due to its complex surface proteome. Proteins that contribute to the pathogenicity of *L. monocytogenes* are commonly referred to as virulence factors. There are a number of important virulence factors which have been indentified and their function elucidated, each playing a key role in the infection process of *L. monocytogenes*.

Figure 2. Schematic representation of the intracellular life cycle of *L. monocytogenes* in an infected host. Entry into the host cell via phagocytosis (a) and development of a surrounding phagocytic vacuole (b). Lysis of phagosome and escape into the cytoplasm where replication can occur (c) and recruitment of host cell actin to the bacterial surface facilitating actin-based motility and formation of a pseudopod (d) followed by phagocytosis of the pseudopods by neighbouring cells and formation of a double-membrane phagosome (e), lysis and escape from the secondary phagosome, thus, starting the cell-to-cell spread of this pathogen over again. Permission to use this schematic was granted by author (2).


Listeria monocytogenes
a
b
c
Phagosome
Lysis of phagosome and replication in cytosol
d
F-actin
e
Double-membraned vacuole
f
Lysis of vacuole

2.4.1 Listeriolysin O

Listeriolysin O (LLO) is a pore-forming cytolysin which facilitates lysis of the phagosomal membrane, allowing bacterial entry into the cytosol (49). LLO is encoded by the hemolysin gene, *hly*, and was the earliest virulence factor to be discovered and sequenced in *Listeria*. Characterization of the *hly* locus resulted in the identification of the 9-kb virulence gene cluster *Listeria* pathogenicity island 1 (LIPI-1) which carries the majority of genes required for the intracellular life cycle of pathogenic *Listeria* spp. Cell culture studies of the effects of *hly* deletion showed that hemolysin is necessary for the survival and proliferation of *L. monocytogenes* within macrophages and non-professional phagocytes (50-52).

2.4.2 PlcA and PlcB

Pathogenic *Listeria* species produce three different enzymes with phospholipase C (Plc) activity that contributes to virulence. Phospholipase A (PlcA) and phospholipase B (PlcB) are two of the secreted proteins in *L. monocytogenes* and *L. ivanovii* necessary to achieve intracellular survival and replication. The third is sphingomyleinase C (SmcL) which is specific to *L. ivanovii*. PlcA and PlcB are encoded by *plcA* and *plcB* genes which reside in the LIPI-1 (53). The *plcA* gene is found in the region upstream of *hly* (54) and its secreted product is a 33-kDa polypeptide. The purified PlcA enzyme is extremely specific for phosphatidylinositol (PI) with a pH optimum between 5.5 and 6.5 suggesting that it would be active in acidified phagocytic vacuoles. PlcB is a zinc-dependent metalloenzyme which is secreted in its inactive form and processed in the extracellular space by proteolytic cleavage to give rise to the faster-migrating active 30kDa form. PlcB has to be secreted in its inactive form to prevent bacterial membrane damage due to degradation of its phospholipids. The major roles of PlcA and PlcB are phagosome disruption. PlcB is required for lysis of the secondary phagosomes formed after bacterial cell-to-cell spread (55). PlcB has also been shown to be required for intracellular spread from macrophages to different cell types, including brain microvascular endothelial cell *in vitro* and in mouse brain tissue *in vivo* (56).

2.4.3 PrfA

PrfA is the major regulator of *L. monocytogenes* virulence genes. This regulatory protein is a member of the cAMP receptor protein (Crp)/fumarate nitrate reductase regulator (Fnr) group of bacterial transcription factors (57). PrfA activates transcription via binding to the palindromic nucleotide sequences (14 bp) referred to as the PrfA box (27). PrfA regulon consists of LIPI-1 encoding the key virulence factors LLO, PlcA, PlcB, ActA, metalloprotease (Mpl), as well as the *inlAB* operon and the *inlC* and hexose phosphase transportase (*hpt)* monocistrons. In addition to these key virulence factors, PrfA regulates the expression of over 145 more *L. monocytogenes* genes during intracellular growth.

2.4.4 ActA

ActA is a surface protein encoded by *actA* which resides in the LIPI-1 and is necessary for establishing an intracellular infection of host cells. Its role is to facilitate bacterial spread to adjacent cells once the bacterium has entered into the cytosol. ActA mediates the formation of polarized actin tails composed of cross-linked actin filaments that propel the bacteria toward the cytoplasmic membrane in order to escape and infect neighboring cells (58) (See Figure 2).

2.4.5 The internalin family

2.3.5.1 InlA, InlB, InlC2 and InlJ

Internalins are the protein products of a family of virulence-associated genes present in pathogenic *Listeria* spp. Of the 25 members of the internalin family, internalin A (InlA) and internalin B (InlB) were the first to be characterized. Their genes are found on same operon (*inlAB*) and together mediate internalization of *L. monocytogenes* into nonphagocytic cells. InlA is a 86.6 kDa surface protein (59) with an N-terminal domain containing a signal peptide and 15 leucine-rich repeats (LRR) units. The LRR domain is believed to be implicated in specific protein-protein interactions. An inter-repeat region separates the LRR region from the C-terminal domain containing three longer B repeats, the LPXTG cell-wall sorting motif and a hydrophobic membrane spanning region (59). InlA exclusively binds to the host cell receptor epithelial cadherin (E-cadherin), a calcium-dependent intercellular adhesion glycoprotein composed of five extracellular domains and a cytoplasmic tail. This transmembrane protein is required for the proper formation of junctions between epithelial cells. InlA binds to the extracellular domain of E-cadherin, although it is the intracellular domain of E-cadherin that is crucial for the cytoskelatal restructuring necessary for bacterial entry. Certain strains of *L. monocytogenes* (L028 was the first to be identified) have a frameshift mutation in the *inlA* gene which results in a nonsense codon and a truncated open reading frame that lacks the LPXTG domain (60). Consequently, InlA cannot be detected on the surface of these bacterial strains.

InlB is a 65 kDa cell-wall-associated protein with seven LRR units in the N-terminal domain (61). The LPXTG motif and the hydrophobic region are absent from the C-terminus of InlB. Alternatively, InlB is comprised of a 232-amino acid region consisting of tandemly ordered repeats around 80 amino acids long, each beginning with the amino acid sequence GW. Infection assays with deletion mutants have revealed a critical role for InlB during invasion of *L. monocytogenes* in endothelial cells (62). The host cell receptor for InlB is Met, a protein tyrosine kinase that is expressed mostly by cells of epithelial variety (63). Met signalling mediated by the binding of GW repeats at the C-terminus of InlB induces cytoskeletal restructuring that is fundamental for the entry of *L. monocytogenes* into non-phagocytic cells (2).

InlC2 and InlJ are two other members of the internalin family that were more recently identified in *L. monocytogenes*. InlC2 is a 58 kDa protein with a N-terminal 35 amino acid signal sequence, seven LRR repeat and two B repeat regions, and a hydrophobic C-terminal end including a modified LPXTG cell wall anchoring motif in which alanine replaces threonine (64). The *inlC2* gene was found clustered on the chromosome with two other internalin genes, *inlD* and *inlE* (64). The specific functions of these internalin proteins remain unclear. The *inlC2* gene was amplified from 37 out of 40 *L. monocytogenes* strains representing serotypes 1/2a (n=21), 1/2b (n=8), 3b (n=1), 4a (n=1) and 4b (n=9) (64). The study found that *inlC2* was present in all isolates tested except three from lineage II (exact strains were not specified), which contained *inlH*, predicted to be a fusion of the 5’ end of *inlC2* with the 3’ end of the adjoining *inlD*. Yu *et al*. showed that InlC2 was expressed as a target for the humoral immune response to *L. monocytogenes* infection in rabbits (65).

InlJ is a 110 kDa protein with an N-terminal LRR domain and a C-terminal LPXTG motif similar to InlA and InlC2 internalin family members. InlJ is a surface protein anchored to the cell wall by sortase A, an enzyme that facilitates the covalent binding of LPXTG surface proteins to the peptidoglycan of gram positive bacteria (66, 67). The *inlJ* gene is conserved in the genome of *L. monocytogenes* strains and is absent from non-pathogenic *Listeria* spp (68). In 2008, Sabet *et al*. revealed that *inlJ* expression stimulates bacterial adherence and InlJ protein is specifically produced and anchored at the bacterial surface during the infection process *in vivo* (69). Quantitative RT-PCR showed that the *inlJ* gene was transcribed in *L. monocytogenes* 1/2a EGDe cells grown in BHI broth; however, InlJ protein was not detected in total protein or cell wall extracts of *L. monocytogenes*. These findings suggest a mechanism of post-transcriptional regulation which could silence the expression of *inlJ* outside the host and induce it specifically during infection to promote bacterial adhesion to host tissues.

2.4.6 LapB

Listeria adhesion protein B (LapB) is a 180 kDa surface protein identified as a new virulence factor involved in adhesion and invasion of *L. monocytogenes* (70). The *lapB* gene encodes a protein of 1711 amino acids with a signal sequence and C-terminal sorting signal including an atypical LPXTG sequence domain, IPALG, which leads to a hydrophobic region and a positively charged N-terminus. LapB is 1 of the 20 *L. monocytogenes* LPXTG proteins that are absent from non-pathogenic *L. innocua* (70). In *L. monocytogenes* strain EGDe, the genes flanking *lapB* (*lmo1666)* are *lmo1665*, a gene encoding a protein of unknown function and *lmo1667*, a gene encoding an L-lactate dehydrogenase. *lapB* is positively regulated by PrfA and up-regulated *in vivo*, as shown by quantitative reverse transcriptase PCR. The levels of *lapB* mRNA were significantly reduced (by 5-fold) in ΔprfA, indicating that PrfA positively regulates *lapB* transcription. The *lmo1666* gene was found to be absent from *L. innocua*, however its flanking genes were conserved. DNA hybridization was performed on 113 strains of *Listeria* spp. strains carrying the *lapB* gene (70). The results showed that *lapB* was exclusive to *L. monocytogenes* lineages I (serotypes 1/2b, 3b, 4b, 4d, 4e and 7) and II (serotypes 1/2a, 1/2c, 3a and 3c), and absent from lineage III (4a and 4c). The *lapB* gene was also absent from *L. innocua*, *L. ivanovii*, *L. grayi* and *L. welshimeri*.

2.5 Detection methods for *L. monocytogenes*

There are a number of detection methods for foodborne *Listeria* spp. but there are advantages and disadvantages for each. The ultimate detection method would be specific for *L. monocytogenes* and sensitive enough to detect 1 CFU in a 25 g sample of food.

2.5.1 Culture based techniques

Culturing is the traditional method used as a standard for comparison. After collection of a suspected food substance or environmental swab, samples are pre-incubated in a selective broth to revive any injured cells (71). Cells are then enriched in selective media for at least 24 hours, typically followed by a secondary enrichment step for another 24 to 48 hours. Cultures are then plated onto selective media and incubated overnight to allow cells to multiply and form colonies. Overall, culture is laborious and can take up to 10 days to confirm a positive contamination and there is a need for development of improved diagnostic tests that promote rapid and reliable test results. Nevertheless, culturing is the standard for comparison to all other tests. The shortcomings of conventional culture based techniques are the changes in phenotype on selective agar under different environmental conditions and interference by other bacteria commonly found in food which can suppress the detection of the target antigens giving false-negative results.

2.5.2 Nucleic acid based techniques

A number of nucleic acid based techniques are available for the detection of *L. monocytogenes*; however, polymerase chain reaction (PCR) is the most extensively used. Sequencing of numerous *Listeria* genomes has expedited the development of specific primers for use with PCR-based detection tests. Additionally, real time or quantitative PCR (qPCR) has shown potential for the detection of *Listeria* as it enables both detection and enumeration in the same tube and reduces the number of steps and the opportunity for contamination (72, 73). Reverse transcriptase PCR (RT-PCR) is another tool used to detect bacterial cells by targeting and amplifying mRNA thus only detecting live cells (74). Although PCR techniques have shown promise as a highly sensitive and specific tool for the amplification and detection of pathogen-specific DNA sequences, such as the *hly* gene in *L. monocytogenes*, there are major limitations to the use of these tests. A serious drawback of nucleic acid techniques is the presence of polymerase inhibitors naturally present in food that can cause false-negative results. Moreover, when testing foodborne pathogens, DNA probes only confirm that a gene is present and not whether a gene is actually expressed. Therefore, DNA-based amplification methods are not suitable for measuring viability as the DNA from dead cells bring about false-positives. Finally, a positive PCR product is not recognized as an acceptable result for confirming contamination of a food sample. Consequently, further biochemical testing such as plating is always necessary to obtain an isolate which can be characterized by various sub-typing on chromogenic agar.

2.5.3 Immunological based techniques

Antigen-antibody interactions are highly specific and typically have a strong affinity and avidity for one another. Immunological based methods can be ideal for detection of bacterial pathogens from food since antibodies can be developed to recognize epitopes on cell surface components. Once antibodies are developed and characterized they can be used in diagnostics for the detection or isolation of bacteria with a variety of immunological based techniques. Some examples are enzyme-linked immunosorbent assay (ELISA), immunomagnetic separation (IMS) and biosensors.

2.5.3.1 ELISA

ELISA is commonly used in food testing as it is straightforward, requires minor treatment of the sample and the results are easily interpreted. ELISA has a minimum limit of detection of 10^5-10^6 cells/ml. Some regarded disadvantages of ELISAs are the relatively high concentration of cells needed for detection, the use of many reagents and the need for enrichment. All samples are enriched to ensure cells in sample with low cell numbers to multiply to detectable levels.

2.5.3.2 IMS

Detection of foodborne pathogens would be greatly improved if the bacteria were separated and concentrated from the complex food matrix before detection. IMS techniques have the potential to achieve this. IMS techniques use paramagnetic polystyrene beads that are covalently linked to antigen-specific antibodies (7). Ideally, the target antigen is on the surface of a pathogen allowing the antibody-bead complex to bind to the pathogen and the pathogen to be easily concentrated following the introduction of a magnetic field. If the antibody-antigen interaction is strong then the cells could be isolated from enrichment culture broths or possibly directly from food samples. Following capture, the beads are plated on selective agar for retrieval of bacteria.

2.5.4 Biosensor based techniques

Biosensor technologies use surface plasmon resonance to examine molecular binding events (6). The main feature of surface plasmon resonance is that it is a ‘label-free’ capturing of ‘real time’ antibody-antigen interaction. It is thought that Biosensor technologies could help accelerate the process of confirming the presence of *L. monocytogenes* in food samples following only a primary enrichment step, circumventing the need for selective plating and subsequent biochemical testing. Antibodies with high specificity and affinity for *L. monocytogenes* strains would facilitate the isolation and concentration of this pathogen for downstream detection by various detection technologies. Biacore is one example of a biosensor technique used for the detection of *L. monocytogenes* from food samples (75). The method was described as being simple, fast and required minimal sample preparation. Biosensors have the potential to shorten the time required to obtain results for diagnostic food testing compared to that for conventional culture based detection methods. However, biosensors are restricted by the binding properties of the bioaffinity ligand used.

2.6 Monoclonal antibodies to *L. monocytogenes*

There have been several MAbs developed for the detection of *L. monocytogenes*. However, the majority of these cross-react with other *Listeria* spp. such as non-pathogenic *L. innocua* which regularly outgrows *L. monocytogenes* during enrichment. A few of the antibodies were able to recognize *L. monocytogenes* serotype 4b, however, these antibodies were not able to recognize other *L. monocytogenes* serotypes, including 1/2a, 1/2b and 1/2c serotypes types which are frequently found in food isolates (76-78). A MAb developed against p60, a protein identified as an essential component for cellular division in *L. monocytogenes*, was able to detect all strains of *L. monocytogenes* tested, but also cross-reacted with non-pathogenic *Listeria* strains tested, with the exception of *L. seeligeri* (73). Furthermore, many of the studies that have produced MAbs against *L. monocytogenes* antigens did not investigate the reactivity of these reagents with all *L. monocytogenes* serotypes or all *Listeria* spp. (74), which is essential for making a conclusive evaluation on the scope of MAb specificity and cross-reactivity. Antibodies that bind uniquely to various strains of *L. monocytogenes* are still being sought for use in the development of rapid and reliable detection tools for this pathogen in foods. The expression of target antigens can be affected in various environmental conditions and differ considerably between enrichment media (75-77). Therefore, antigen targets need to be conserved among *L. monocytogenes* strains and also expressed under different growth conditions, particularly the conditions that bacteria must withstand during food processing (high salt, low pH, freezing and drying etc).

Polyclonal antibodies (PAbs) have commonly been used in diagnostic tests such as ELISA and flow cytometry (79). However, PAbs generally have low specificity and may exhibit cross-reactions with other bacterial species present in samples. Moreover, PAbs are not renewable like MAbs. Thus, developing MAbs with high specificity and affinity for antigens uniquely found on the surface of this bacterium will be useful for *L. monocytogenes* detection methods. A comparison of MAbs and PAbs is reviewed in Table 1.

Table 1. Comparison of monoclonal and polyclonal antibodies in regards to their production, long term reproducibility, specificity, affinity, cross-reactivity and applications.

Category	**Monoclonal**	**Polyclonal**
Production	Produced from one clone of B-lymphocyte.	Produced from different B-lymphocyte lines as a combination of immunoglobulins.
Production time and cost	High cost and can take a year to generate hybridomas and subsequent production of MAbs.	Less expensive and typically generated within 3-4 months.
Long-term reproducibility	Can be generated as a renewable resource and in unlimited quantities.	Antibodies generated to the same antigen using multiple animals will be different. Quantity is restricted by the size of the immunizing animal and its lifespan.
Specificity	Highly specific. Production by numerous B cell clones with each clone generating antibodies to a specific epitope.	Less specific than MAbs. Can bind to many epitopes.
Affinity	Can be measured precisely.	Can only be estimated.
Denatured conditions	Some MAbs may not bind to the single epitope anymore	Binding typically occurs to multiple epitopes (some are likely linear) and thus are not as impacted by conformational changes.
Cross-reactivity	Minimal.	Common.
Useful applications	-ELISA -Biosensor techniques -Flow cytometry	-Immunoprecipitation of proteins -Confirmation of protein expression

2.6 Applications of MAbs for *L. monocytogenes* diagnostics

VIDAS LMO 2, an official test approved by Health Canada is an enzyme-linked fluorescent immunoassay (ELFA) procedure (MFHPB29) used with the VIDAS system for the detection of *L. monocytogenes*. Although VIDAS has been validated for detection of *L. monocytogenes* in a number of foods, it is not validated in RTE meat and poultry, smoked fish, yogurt and fermented dairy drinks. This method is a single dose test composed of a strip containing the pre-made reagents and a Solid Phase Receptacle (SPR) coated with specific antibodies, which takes approximately 70 minutes to complete. The inside of the SPR, which resembles a pipette tip, is pre-coated with anti-*L. monocytogenes* antibodies. The sample is inoculated into the reagent strip and cycled in and out of the SPR for a pre-set time. Target *L. monocytogenes* antigens present in the sample will bind to the MAbs which were coated on the inside of the SPR. Antibodies are conjugated with alkaline phosphatase then cycled in and out of the SPR reacting with the *L. monocytogenes* antigen-antibody complex bound to the SPR wall. The fluorescent substrate (4-methyl-umbelliferyl-phosphate) is subsequently cycled in and out of the SPR being converted to fluorescent 4-methyl-umbelliferone. A relative fluorescence value of ≥ 0.05 signifies the presumptive presence of *L. monocytogenes* in the test sample and all presumptive positive results need to be confirmed with further culturing and plating following MFHPB30. The current sensitivity of the test is 10^5 cells/ml. Antibodies with high affinity for *L. monocytogenes* are required to optimize the capture and the detection of the pathogen.

CHAPTER III

MATERIALS AND METHODS

3.0 MATERIALS AND METHODS

3.1 Bioinformatic analyses

Candidate known or putative virulence surface proteins from *L. monocytogenes* serotype 4b strain F2365 were used as references to perform similarity search using the basic local alignment search tool (BLASTp) on the National Centre for Biotechnology Information (NCBI) website. Sequences were obtained from the NCBI at http://www-ncbi-nlm-nih-gov.proxy.bib.uottawa.ca/. Multiple alignments of the sequences obtained from the BLASTp search were performed with the MegAlign module in the DNAstar Lasergene software.

3.2 Bacterial strains and growth conditions

E. coli DH5α was used as a host strain for molecular cloning. *E. coli* BL21 (DE3)/pLysS was used as a protein expression host. *E. coli* were grown on Luria-Bertani (LB) agar plates or LB broth supplemented with 50μg/mL kanamycin. As required, bacterial agar plates were incubated at 37°C, and liquid cultures were placed in a shaking incubator (LabLine, EnviroShaker) at 225 rpm and 37°C overnight. *L. monocytogenes* serotype 4b (strain L10521) and other strains were maintained on Brain Heart Infusion (BHI) plates at 4°C. A single colony was inoculated into BHI broth and cultured at 37°C overnight. Cell concentrations were determined using OD_{620} of $0.61=1x10^9$ cells/ml for *Listeria*.

3.3 Generation of expression constructs for ActA, InlA, InlC2, InlJ and LapB

Our lab has previously modified the T7-based vector pET30a to create a ligation independent cloning vector pLIC-CHIS for the generation of expression constructs to place the 6X His tag at the C-terminus of recombinant proteins (80). The open reading frames (ORF) of *actA, inlA, inlC2, inlJ* and *lapB* were amplified by polymerase chain reaction (PCR) from the genomic DNA of *L. monocytogenes* serotype 4b strain L10521. PCR was performed with the primers listed in Table 2 for the five genes. Each PCR product was purified with the Wizard PCR Preps DNA purification system (Promega, Madison, Wis., USA) and ligated into pLIC-CHis for *actA, inlA, inlJ* and *lapB* whereas pET30a (Novagen) was used for *inlC2*. Parameters of experiments are detailed in Appendix A. These expression constructs, designated pLIC-ActA, pLIC-InlA, pLIC-LapB, pLIC-InlJ and pET30a-InlC2, respectively, were propagated in *E. coli* DH5α and isolated using a QIAprep Spin Miniprep kit (QIAgen, Mississauga, Ontario, Canada) according to the supplier's instructions. The recombinant plasmids were sequenced with T7 promoter and T7 terminator primers to verify the presence of the correct insert.

3.4 Expression of recombinant proteins in *E. coli*

A single colony of *E. coli* BL21 (DE3)/pLysS containing an expression construct pLIC-InlC2 was cultured in 500 ml of LB broth containing 50 µg/ml kanamycin at 37°C with continuous shaking for 16 hours. A volume of 5 ml from the culture suspension was sub-cultured into 500 ml of fresh LB broth containing 50 µg/ml kanamycin at 37°C with continuous shaking until an OD at 595 nm of 0.5 to 0.8 was obtained. The culture was induced by the addition of 1mM isopropryl-β-D-thiogalactopyranoside (IPTG) to express the recombinant protein for 3 hours. The cells were harvested by centrifugation (Kendro Laboratory Products, Sorvall Evolution RC superspeed centrifuge) at 8,000 x g for 20 min at 4°C and frozen at -80°C until used.

Table 2. List of primers used for the generation of ActA, InlA, InlC2, InlJ and LapB expression constructs.

Plasmid Constructs	**Primers used (5' to 3')**	**PCR product**
pLIC-ActA	Lin905 (F[a]):TTTAAGAAGGAGATATAAGTCATGGCGACAGATAGCGAAGATTC Lin895 (R[b]):AGTGGTGGTGGTGGTGGTGAGTCCCACTATTTTTTCTTAATTGAATG	1682 bp
pLIC-InlA	Lin906 (F):TTTAAGAAGGAGATATAAGTCATGGCTACAATTACACAAGATAC Lin897 (R):AGTGGTGGTGGTGGTGGTGAGTCGTTGTAGGGAGTGAAGCT	2165 bp
pET-InlC2	P402 (F):ACACATATGAAGAAACGGTGGAATTCTGTAT P403 (R):GTGCTCGAGTTTCACTTTGATTGTTTTGCGGAGA	1650 bp
pLIC-InlJ	Lin914 (F):TTTAAGAAGGAGATATAAGTCATGGCAACGAATGATGTGATTGAT Lin915 (R):AGTGGTGGTGGTGGTGGTGAGTCGTATCTGAAGCTTTTTTCAC	2537 bp
pLIC-LapB	Lin912 (F):TTTAAGAAGGAGATATAAGTCATGAAATCGGTGGGAGATATTTC Lin913 (R):AGTGGTGGTGGTGGTGGTGAGTCCCGCTTTCATTTGTAGCGGTA	4928 bp

[a] F, forward primers

[b] R, reverse primers

3.5 Purification of recombinant proteins

3.5.1 Extraction of soluble protein

Frozen cell pellets were resuspended in 10 ml of phosphate buffer saline (PBS) containing 1 mM phenylmethylsulphonyl fluoride (PMSF). The cell suspension was passed through a French Press (SLM Instruments Inc. French Pressurized Cell) twice at 1500 psi. Lysed cells were centrifuged at 27,000 x g for 20 minutes at 4°C and the supernatant (designated the crude protein extract) was collected.

3.5.2 Ni-NTA affinity chromatography

The crude protein extract was mixed with a 1:2 volume of Wash Buffer A (Appendix A) before being applied to a Ni-NTA agarose column (1 cm x 2.5 cm) at 1.0 ml/min which had been pre-equilibrated with 30 ml of Wash Buffer A. The column was washed with 30 ml of Wash Buffer A at 1 ml/min followed by 30 ml of Elution Buffer (Appendix A) containing 250 mM imidazole. Fractions of eluted protein were collected and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fractions containing the recombinant *Listeria* proteins were collected and concentrated by centrifugation at 3000 x g for 30-60 minutes (depending on *Listeria* protein size) using Amicon 30KDa filter centrifugal devices (Millipore).

3.6 Protein quantification

Bradford Assay (81) was used to determine the concentration of purified recombinant proteins using a BioRad protein assay kit and bovine serum albumin (BSA) as a standard.

3.7 SDS-PAGE and Western blotting

SDS-PAGE was carried out as described by Laemmli (82), using a 4% stacking gel and a 10% or 12% separating gel in a BioRad minigel apparatus (Bio-Rad, Mississauga, Ontario). Following electrophoresis, the separated proteins were either stained with Coomassie blue (CMB) or electrotransferred onto a nitrocellulose membrane for Western blotting using a Trans-Blot semidry transfer cell (Bio-Rad) for Western blot (WB) procedure. Prior to WB, successful transfer of proteins was confirmed by staining the membrane with 0.1% (w/v) Ponceau S in 5% (v/v) acetic acid for 2 minutes. The membrane was then blocked with phosphate buffered saline/Triton X-100/Tween 20 (PBS-TT) containing 3% (w/v) BSA for 1 hour at room temperature or overnight at 4°C. The WB was performed using specific primary antibodies followed by peroxidase-conjugated goat anti-mouse IgG (H +L) (Jackson ImmunoResearch Laboratories) and was performed as described previously (12). The substrate solution was prepared from the HRP Conjugate Substrate Kit (BioRad) and used according to the supplier's instructions.

3.8 Extraction of *L. monocytogenes* total cellular proteins

One colony of *L. monocytogenes* from a BHI agar plate was inoculated into 50 ml of fresh BHI broth at 37°C with continuous shaking at 225 rpm for 16 hours. Cells were spun down (at least 15 ml) at 8,000 x g for 10 minutes. The supernatant was decanted and cells resuspended in 500 µl of 0.5 M Tris-HCl/10% SDS/ 1% PMSF. Cell mixture was transferred to a Lysing Matrix B tube (MP Biomedicals) and placed in the Fast Prep machine (Qbiogene) for 40 seconds at speed level 6. The entire mixture was transferred to a new 2.0 ml eppitube and 500 µl of 2X SDS-PAGE sample buffer was added. The eppitube was boiled in a water bath for 10 minutes. The supernatant was then transferred to a new eppitube for further analysis by SDS-PAGE and Western blotting.

3.9 Production of PAbs

Purified recombinant LapB, InlJ and InlC2 (rLapB, rInlJ and rInlC2) antigens (100 µg in 0.5 ml PBS) were emulsified with an equal volume of Incomplete Freunds Adjuvant (IFA) and then inoculated subcutaneously into two white Newzealand female rabbits (Day 0). Booster inoculations were given on days 14 and 28. A test bleed was done on day 42 and rabbits were exsanguinated on day 46. Rabbit serum was spun down at 3000 x g for 30 minutes to separate blood cells from anti-serum. Anti-serum was stored at -20°C until use. Specific antibodies were verified by WB and IFM analysis.

3.10 Production of MAbs

3.10.1 Immunization of mice

Six-week-old CFW and Balb/C mice (Charles River) were immunized with purified recombinant proteins at scheduled intervals over a 2 month period for each protein. Pre-bleeds of mice were performed prior to immunizations with an antigen. On day 0, three CFW and three Balb/C mice were each inoculated subcutaneously with 200 µl of rLapB or InlC2 or InlJ proteins (50 µg/ml) that was emulsified with an equal volume of Complete Freunds Adjuvant (CFA). Booster inoculations using IFA were given intraperitoneally with the same amount of antigen on days 28 and 56. Test bleed of all mice was taken on day 64 to determine specific antibody responses. Mouse serum was spun down at 16,100 x g for 2 minutes to separate blood cells from anti-serum. Anti-serum was stored at -20°C until use. Mice selected for fusion were inoculated intraperitoneally with epinephrine and dehydroepiandrosterone sulfate (DHEAS) and 5 µg of a recombinant protein antigen by intravenous (IV) injection 5 days before scheduled splenectomy.

3.10.2 ELISA for initial screening of anti-*L. monocytogenes* MAb

3.10.2.1 Anti-LapB antibodies

Mouse Sp2/0-Ag14 myeloma cells were fused with spleen cells of two selected CFW and Balb/C mice Tissue culture fluids of parent hybridomas from the two fusions (#573 and #574) were screened by ELISA for reactivity against purified rLapB protein coated on NUNC ELISA plates (Fisher Scientific) at a concentration of 1 µg/ml. Positive hybridomas were selected for and screened by ELISA against whole cell *L. monocytogenes* (4b, 1/2a and 1/2c) coated on NUNC ELISA plates at 5 x 10^8 cells/ml. In addition, IFM analysis (See section 3.10.4) was performed to determine the binding of MAbs to the cell surface of *L. monocytogenes* serotype 4b strain L10521. Based on ELISA and IFM testing, hybridomas were selected for cloning and expansion.

3.10.2.2 Anti-InlJ antibodies

Tissue culture fluids of parent hybridomas from the two fusions (#575 and #576) were screened following the same procedure as LapB antibodies.

3.10.2.3 Anti-InlC2 antibodies

Tissue culture fluids of parent hybridomas from the two fusions (#583 and #584) were screened following a similar procedure as LapB antibodies with some additional changes. Parent hybridomas were screened for reactivity against purified rInlC2 protein coated on NUNC ELISA plates (Fisher Scientific) at a concentration of 1µg/ml or 0.5 µg/ml. Positive hybridomas were selected for screening against one strain of each *L. monocytogenes* serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d and 4e) as well as one strain of *L. innocua* and *L. seeligeri*. Whole cells of formalin killed bacteria washed 3x in PBS and resuspended in fresh PBS and glycerol were coated on NUNC ELISA plates at 1 x 10^8 cells/ml. Based on ELISA testing, hybridomas that were positive against strains of *L. monocytogenes* and negative against *L. innocua* and *L. seeligeri* were selected for cloning and expansion. The final tissue culture fluids were re-tested using IFM and ELISA to determine how strongly each reacted to the InlC2 surface antigen on *L. monocytogenes* cells.

3.10.3 *Listeria* whole cell antigen preparation

Listeria strains were maintained on Brain Heart Infusion (BHI) plates at 4°C or on Tryptic Soy Agar slants at room temperature. A single colony was inoculated into BHI broth and cultured at 37°C overnight. Cell concentrations were determined using OD_{620} of 0.61=1x10^9 cells/ml for *Listeria* (83). Cells were spun down and washed 3x in PBS. Pelleted cells were treated with 0.3% formalin solution for 24 hours. Whole cells of formalin killed bacteria were spun and washed 3x in PBS and resuspended in final solution of PBS and glycerol (1: 1). Formalin killed whole cell antigens were used to coat NUNC ELISA plates at 1 x 10^8 cells/ml.

3.10.4 Fluorescent microscopy analysis

Binding of anti-InlC2, anti-InlJ and anti-LapB antibodies to the cell surface of *L. monocytogenes* was detected by immunofluorescence microscopy (IFM). One colony of *L. monocytogenes* (L10521) from a BHI agar plate was inoculated into 7 ml of fresh BHI broth at 37°C with continuous shaking at 225 rpm for 16 hours. Bacterial cells (3.0 x 10^8 cells/ml) were centrifuged at 15,700 x g for 2 minutes in a microcentrifuge (Eppendorf, Mini Spin Plus) to remove culture supernatant. Cell pellet was resuspended in 500 µl of PBS containing 5% BSA and gently rocked for

45 minutes at room temperature. Cells were spun down to remove blocking supernatant then resuspended in rabbit anti-serum at a 1:500 dilution or mouse anti-serum or MAb tissue culture fluid at 1:50 dilution for 1 hour at room temperature. Cells were washed twice with 1x PBS then incubated with Dylight 488 goat anti-rabbit or anti-mouse (Jackson ImmunoResearch) at a 1:50 dilution for goat anti-rabbit and 1:100 dilution for goat anti-mouse for 1 hour at room temperature. Cells were washed 3 times with 250 µl PBS and resuspended in a final volume of 50µl PBS. The bacterial cell suspension (7µl) was deposited on a microscope slide, sealed with a coverslip and Cryoseal 60 (VWR). The cells were viewed with an epifluorescence microscope (Olympus). A high through-put fluorescent microscopy method was developed by myself and Cathie Elmgren for screening hybridoma tissue culture fluid. Bacterial cells (3.0 x 10^8 cells/ml) were loaded into culture wells of Nunc Maxisorp round bottom ELISA plate and spun down for 10 minutes at 900 x g using a microplate carrier attached to a SX4750 rotor in a Beckman centrifuge machine. The rest of the procedure was followed essentially as described above.

3.10.5 Isotyping of murine immunoglobulins

The subclasses of immunoglobulins (Igs) secreted by the hybridoma cell lines were determined according to the standard operation procedures MC-PR021.02 and MC-PR021.03 (Canadian Food Inspection Agency, Ottawa Laboratory Fallowfield, Ottawa, Ontario, Canada). In summary, 20 µl of hybridoma tissue culture fluid pre-diluted 1:2 with Tris-buffered saline (0.1M Tris/HCl pH 8.0, 0.15M NaCl) containing 0.05% (v/v) Tween and 0.02% (w/v) NaN3, was incubated for 15 minutes at room temperature with 0.7-0.9µm polystyrene beads (IDEXX Laboratories) that were pre-coated with goat anti-mouse Ig (IgM + IgG + IgA, heavy and light (H + L) chain specific) (Southern Biotechnology) in the wells of Fluoricon assay plates (IDEXX Laboratories). Following washing, bound murine Ig was detected with FITC-conjugated goat anti-mouse H + L chain specific antibodies (Southern Biotechnology) using a Baxter fluorescence concentration analyzer (IDEXX laboratories). Positive and negative tissue culture fluid controls were performed simultaneously with the tested hybridoma tissue culture fluids.

3.11 Characterization of MAbs by ELISA

Detection of *L. monocytogenes* by anti-LapB and anti-InlC2 MAbs was assessed by indirect ELISA as described above for screening hybridomas (3.10.2). Fifty one strains of formalin killed whole cell antigens of *L. monocytogenes* (see Tables 7 and 9) were tested. Anti-InlC2 and anti-LapB MAbs were also tested for cross-reactivity with *L. innocua, L. ivanovii, L. grayi, L. seeligeri, L. welschimeri, E. coli* O157:H7, *E. coli* 25922, *S. enterica* DT104, *S. enterica* California and *C. jejuni* ADRI1102 following the same procedure.

3.12 Detection of *L. monocytogenes* cultured in enrichment broths

Primary enrichment of *L. monocytogenes* in broth and secondary enrichment in UVM were described in the MFHPB30 method (the Compendium of Analytical Methods). One colony of *Listeria* was inoculated into 7 ml of Palcam broth (Oxoid) and incubated at 35°C with shaking at 225 rpm overnight. From the overnight culture, 1 ml was transferred into 9 ml of fresh University of Vermont (UVM) broth (BD Diagnostics) and incubated at 30°C with shaking at 225 rpm overnight. Cells were spun down and washed 3x with PBS. ELISA Maxisorp U96 plates (Fisher Scientific) were coated with 2 x 10^8 cells/ml and incubated overnight at 37°C prior to testing. ELISA was performed to determine if the anti-LapB and anti-InlC2 MAbs can detect *L. monocytogenes* after culture enrichment.

CHAPTER IV

RESULTS

PART I: IDENTIFICATION, PRODUCTION AND EXPRESSION CHARACTERIZATION OF CANDIDATE PROTEINS

PART I: IDENTIFICATION, PRODUCTION AND EXPRESSION CHARACTERIZATION OF CANDIDATE PROTEIN ANTIGENS

4.1 Literature review to identify surface proteins common and unique to all serotype strains of *L. monocytogenes*.

Ten *L. monocytogenes* proteins were selected for the proposed study after an extensive review of the current literature (2, 58, 69, 70, 83, 84). The ten surface protein candidates and their functions are listed in Table 3. To be selected, a protein antigen had to meet the following criteria: be a protein localized on the surface of *L. monocytogenes*, be a known or putative virulence factor, be present in the genomes of *L. monocytogenes* and absent from all other *Listeria* species, and be expressed in *in vitro* culture.

4.2 Bioinformatic analyses of candidate proteins.

Ten candidate proteins encoded by the genome of *L. monocytogenes* serotype 4b strain F2365 (85) were used as a reference for similarity search by BLASTp and for amino acid sequence comparison. A total of 28 genomes of *L. monocytogenes* and 10 of other *Listeria* species have been sequenced and are available on the NCBI database. Only the protein candidates that had greater than 80% similarity to homologous proteins found in other *L. monocytogenes* strains and less than 60% similarity to homologous proteins in non-pathogenic *Listeria* species (*L. innocua, L. seeligeri, L. welshimeri, L. grayi and L. ivanovii*) were considered for the purposes of this study. Five (ActA, InlA, LapB, InlJ and InlC2) of the ten candidates were identified to be present in the majority of sequenced *L. monocytogenes* strains but were absent from most non-pathogenic *Listeria* species. Sequence distance charts produced from multiple alignment of ActA, InlA, LapB, InlJ and InlC2 amino acid sequences from *L. monocytogenes* 4b F2365 strain with homologs to these five proteins in other strains of *Listeria* identified in BLASTp search are shown in Figures 3-7. The other protein candidates (OppA, Auto, Ami, and Lmo2026) were not selected based on their BLAST results which revealed four different similarity patterns which all did not satisfy the criteria listed above (data not shown).

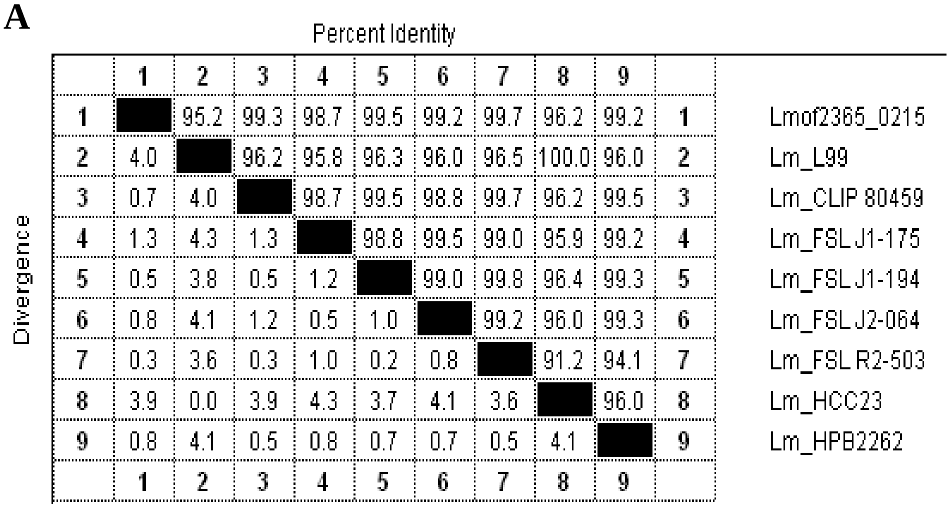
Table 3. List of potential surface protein candidates for the development of MAbs. A comprehensive literature review was performed to identify surface proteins of *L. monocytogenes* that have been recognized as virulence factors or as putative virulence factors for this bacterium.

Protein	**EGD-e gene locus**[a]	**F2365 gene locus**[b]	**Association to bacterial surface**	**Function**	**Reference**
ActA	Lmo0204	Lmof2365_0215	Hydrophobic tail	Intracellular motility	(86)
OppA	Lmo2196	Lmof2365_2229	Lipoprotein	Oligopeptide transport	(87)
InlA	Lmo0433	Lmof2354_0471	LPXTG	Adhesion and invasion	(20)
InlB	Lmo0434	Lmof2365_0472	GW	Invasion and signaling	(2)
InlC2	Lmo0263[c]	Lmof2365_0281	LPXTG	Unknown[d]	(65)
InlJ	Lmo2821	Lmof2365_2812	LPXTG	Invasion	(68)
LapB	Lmo1666	Lmof2365_1690	IPXLG	Adhesion	(70)
Lmo2026	Lmo2026	Lmof2365_0805	LPXTG	Unknown	(67)
Auto	Lmo1076	Lmof2365_2670	GW	Cell wall turnover	(88)
Ami	Lmo2558	Lmof2365_1540	GW	Adhesion	(84)

[a] The sequenced *Listeria monocytogenes* strain EGD-e belonging to serotype 1/2a
[b] The sequenced *Listeria monocytogenes* strain F2365 belonging to serotype 4b
[c] The InlH homolog present in EGD-e
[d] Shown to be expressed as a novel target for the humoral immune response to *L. monocytogenes* infection in rabbits (65)

Figure 3. Sequence distance chart for ActA generated from multiple alignment with similar proteins of *Listeria* strains identified by BLASTp search. A. Of the eight *L. monocytogenes* strains, all had a homolog protein with greater than 80 percent identity to the ActA protein (lmof2365_0215) of the *L. monocytogenes* strain f2365. **B.** Six of the seven non-pathogenic *Listeria* strains had a protein with less than 60 percent identity to ActA in *L. monocytogenes* strain f2365. *L. innocua* strain FSL J1-023 had a protein homolog with 86 percent identity to lmof2365_0215 ActA protein.

A

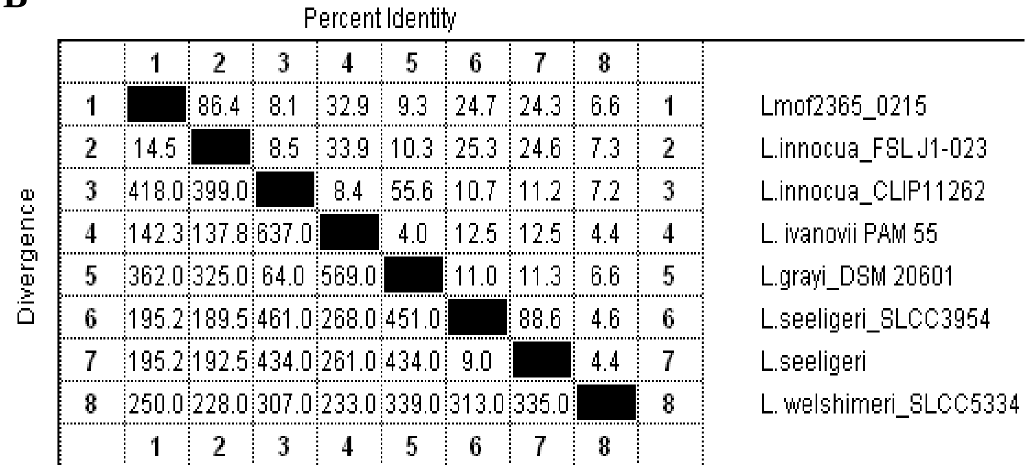


Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9		
1	■	95.2	99.3	98.7	99.5	99.2	99.7	96.2	99.2	1	Lmof2365_0215
2	4.0	■	96.2	95.8	96.3	96.0	96.5	100.0	96.0	2	Lm_L99
3	0.7	4.0	■	98.7	99.5	98.8	99.7	96.2	99.5	3	Lm_CLIP 80459
4	1.3	4.3	1.3	■	98.8	99.5	99.0	95.9	99.2	4	Lm_FSL J1-175
5	0.5	3.8	0.5	1.2	■	99.0	99.8	96.4	99.3	5	Lm_FSL J1-194
6	0.8	4.1	1.2	0.5	1.0	■	99.2	96.0	99.3	6	Lm_FSL J2-064
7	0.3	3.6	0.3	1.0	0.2	0.8	■	91.2	94.1	7	Lm_FSL R2-503
8	3.9	0.0	3.9	4.3	3.7	4.1	3.6	■	96.0	8	Lm_HCC23
9	0.8	4.1	0.5	0.8	0.7	0.7	0.5	4.1	■	9	Lm_HPB2262
	1	2	3	4	5	6	7	8	9		

B



Percent Identity

Divergence

	1	2	3	4	5	6	7	8		
1	■	86.4	8.1	32.9	9.3	24.7	24.3	6.6	1	Lmof2365_0215
2	14.5	■	8.5	33.9	10.3	25.3	24.6	7.3	2	L.innocua_FSL J1-023
3	418.0	399.0	■	8.4	55.6	10.7	11.2	7.2	3	L.innocua_CLIP11262
4	142.3	137.8	637.0	■	4.0	12.5	12.5	4.4	4	L. ivanovii PAM 55
5	362.0	325.0	64.0	569.0	■	11.0	11.3	6.6	5	L.grayi_DSM 20601
6	195.2	189.5	461.0	268.0	451.0	■	88.6	4.6	6	L.seeligeri_SLCC3954
7	195.2	192.5	434.0	261.0	434.0	9.0	■	4.4	7	L.seeligeri
8	250.0	228.0	307.0	233.0	339.0	313.0	335.0	■	8	L. welshimeri_SLCC5334
	1	2	3	4	5	6	7	8		

Figure 4. Sequence distance chart for InlA generated from multiple alignment with similar proteins of *Listeria* strains identified by BLASTp search. A. Of the thirteen *L. monocytogenes* strains identified from BLAST all had a homolog protein with greater than 80 percent identity to the InlA protein (lmof2365_0471) of the *L. monocytogenes* strain f2365. **B.** Five of the six non-pathogenic *Listeria* strains had a protein with less than 60 percent identity to InlA in *L. monocytogenes* strain f2365. *L. innocua* strain FSL J1-023 had a protein with 79.5 percent identity to InlA.

A

Percent identity

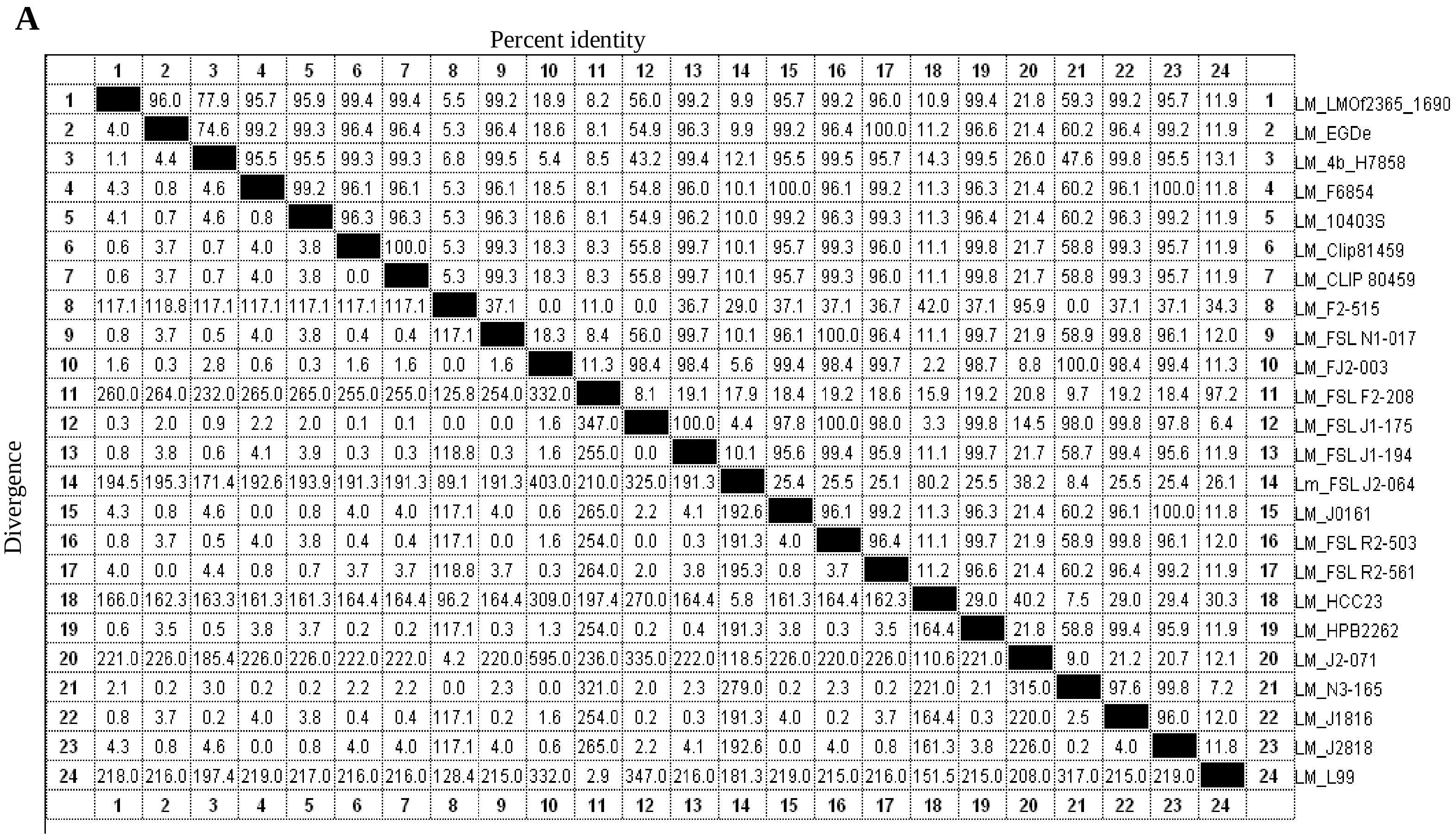
Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
1	■	97.8	100.0	97.8	99.5	99.8	99.8	98.1	99.6	97.9	99.6	97.8	98.9	99.1	**1**	Lmof2365_0471
2	2.3	■	97.8	99.0	97.5	97.8	97.8	96.1	97.6	98.4	97.6	100.0	96.9	97.6	**2**	Lm_EGD-e
3	0.0	2.3	■	97.8	99.5	99.8	99.8	98.1	99.6	97.9	99.6	97.8	98.9	99.1	**3**	Lm_HPB2262
4	2.3	1.0	2.3	■	97.8	98.0	98.0	96.4	97.9	98.1	97.9	99.0	96.9	97.9	**4**	Lm_10403S
5	0.5	2.5	0.5	2.3	■	99.8	99.8	97.9	99.6	97.6	99.6	97.5	98.9	98.9	**5**	Lm_CLIP 80459
6	0.3	2.3	0.3	2.0	0.3	■	100.0	98.1	99.9	97.9	99.9	97.8	98.9	99.1	**6**	Lm_FSL J1-175
7	0.3	2.3	0.3	2.0	0.3	0.0	■	98.1	99.9	97.9	99.9	97.8	98.9	99.1	**7**	Lm_FSL J1-194
8	1.0	3.1	1.0	2.8	1.3	1.0	1.0	■	98.5	97.0	98.5	96.6	97.5	98.2	**8**	Lm_FSL J2-071
9	0.4	2.4	0.4	2.2	0.4	0.1	0.1	1.1	■	97.9	100.0	97.6	98.8	99.0	**9**	Lm_FSL N1-017
10	2.2	1.6	2.2	1.9	2.4	2.2	2.2	2.7	2.2	■	97.9	98.4	97.0	97.5	**10**	Lm_FSL N3-165
11	0.4	2.4	0.4	2.2	0.4	0.1	0.1	1.1	0.0	2.2	■	97.6	98.8	99.0	**11**	Lm_FSL R2-503
12	2.3	0.0	2.3	1.0	2.5	2.3	2.3	3.1	2.4	1.6	2.4	■	96.9	97.6	**12**	Lm_FSL R2-561
13	0.8	2.8	0.8	2.8	0.8	0.8	0.8	1.8	0.9	2.7	0.9	2.8	■	98.4	**13**	Lm_H7858
14	0.9	2.4	0.9	2.2	1.1	0.9	0.9	1.4	1.0	2.5	1.0	2.4	1.6	■	**14**	Lm_HCC23
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**		

B

	1	2	3	4	5	6	7	8		
1	■	79.5	28.1	64.5	19.4	29.1	31.8	32.9	**1**	Lmof2365_0471
2	6.2	■	32.7	67.3	19.8	28.8	33.3	37.7	**2**	L.innocua_FSL J1-023
3	95.1	95.7	■	43.2	25.0	28.9	30.9	39.6	**3**	L.innocua_Clip11262
4	41.5	42.8	99.6	■	18.7	27.8	31.2	30.7	**4**	L.ivanovii FSL F6-596
5	200.0	194.9	192.8	208.0	■	16.0	16.3	18.8	**5**	L.grayi_DSM 20601
6	96.0	91.2	120.5	101.2	238.0	■	42.9	32.8	**6**	L. seeligeri FSL N1-067
7	89.4	73.9	111.9	90.1	241.0	99.5	■	30.8	**7**	L.seeligeri_SLCC3954
8	93.9	89.6	113.0	111.6	222.0	121.0	107.5	■	**8**	L. welshimeri_SLCC5334
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**		

Figure 5. Sequence distance chart for LapB generated from multiple alignment with similar proteins of *Listeria* strains identified by BLASTp search. A. Of the 23 *L. monocytogenes* strains, 13 strains had greater than 80 percent identity to the LapB protein (lmof2365_1690) of *L. monocytogenes* strain f2365: EGDe, F6854, H7858, Clip81459, Clip80459, FSL J1-194, FSL N1-017, FSL R2-503, FSL R2-561, HPB2262, J0161, J1816, and J2818. **B.** A total of 11 non-pathogenic *Listeria* strains were identified from BLAST and of these, one of four *L. seeligeri* strains (FSL S4-171) had higher than 60 percent identity to LapB in *L. monocytogenes* strain f2365.



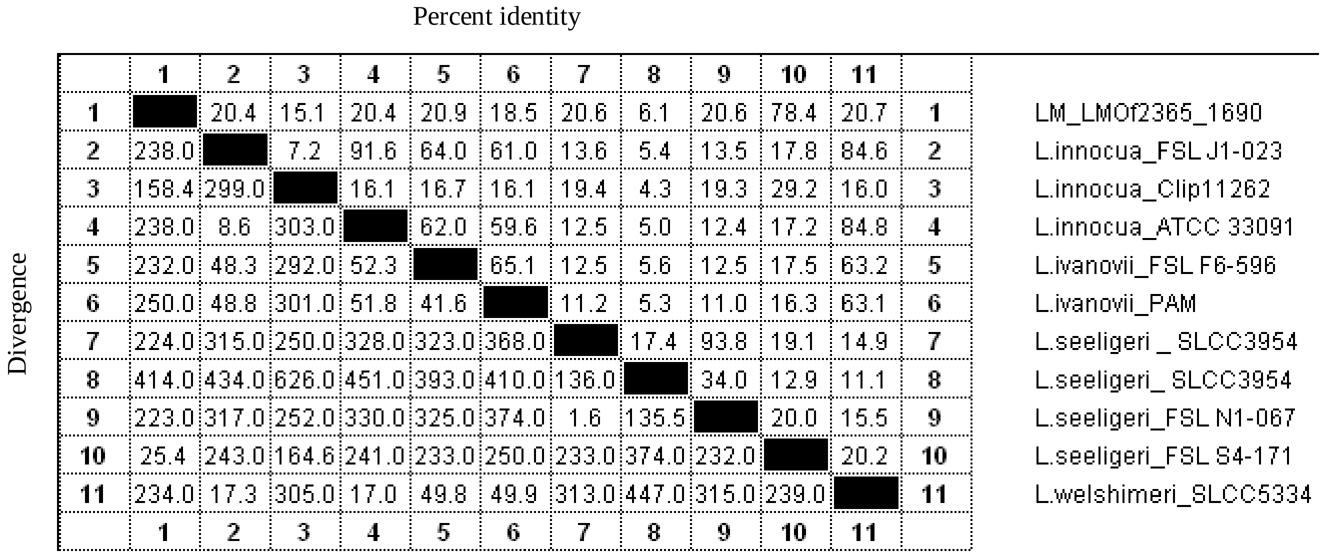
A

Percent identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24		
1	■	96.0	77.9	95.7	95.9	99.4	99.4	5.5	99.2	18.9	8.2	56.0	99.2	9.9	95.7	99.2	96.0	10.9	99.4	21.8	59.3	99.2	95.7	11.9	**1**	LM_LMOf2365_1690
2	4.0	■	74.6	99.2	99.3	96.4	96.4	5.3	96.4	18.6	8.1	54.9	96.3	9.9	99.2	96.4	100.0	11.2	96.6	21.4	60.2	96.4	99.2	11.9	**2**	LM_EGDe
3	1.1	4.4	■	95.5	95.5	99.3	99.3	6.8	99.5	5.4	8.5	43.2	99.4	12.1	95.5	99.5	95.7	14.3	99.5	26.0	47.6	99.8	95.5	13.1	**3**	LM_4b_H7858
4	4.3	0.8	4.6	■	99.2	96.1	96.1	5.3	96.1	18.5	8.1	54.8	96.0	10.1	100.0	96.1	99.2	11.3	96.3	21.4	60.2	96.1	100.0	11.8	**4**	LM_F6854
5	4.1	0.7	4.6	0.8	■	96.3	96.3	5.3	96.3	18.6	8.1	54.9	96.2	10.0	99.2	96.3	99.3	11.3	96.4	21.4	60.2	96.3	99.2	11.9	**5**	LM_10403S
6	0.6	3.7	0.7	4.0	3.8	■	100.0	5.3	99.3	18.3	8.3	55.8	99.7	10.1	95.7	99.3	96.0	11.1	99.8	21.7	58.8	99.3	95.7	11.9	**6**	LM_Clip81459
7	0.6	3.7	0.7	4.0	3.8	0.0	■	5.3	99.3	18.3	8.3	55.8	99.7	10.1	95.7	99.3	96.0	11.1	99.8	21.7	58.8	99.3	95.7	11.9	**7**	LM_CLIP 80459
8	117.1	118.8	117.1	117.1	117.1	117.1	117.1	■	37.1	0.0	11.0	0.0	36.7	29.0	37.1	37.1	36.7	42.0	37.1	95.9	0.0	37.1	37.1	34.3	**8**	LM_F2-515
9	0.8	3.7	0.5	4.0	3.8	0.4	0.4	117.1	■	18.3	8.4	56.0	99.7	10.1	96.1	100.0	96.4	11.1	99.7	21.9	58.9	99.8	96.1	12.0	**9**	LM_FSL N1-017
10	1.6	0.3	2.8	0.6	0.3	1.6	1.6	0.0	1.6	■	11.3	98.4	98.4	5.6	99.4	98.4	99.7	2.2	98.7	8.8	100.0	98.4	99.4	11.3	**10**	LM_FJ2-003
11	260.0	264.0	232.0	265.0	265.0	255.0	255.0	125.8	254.0	332.0	■	8.1	19.1	17.9	18.4	19.2	18.6	15.9	19.2	20.8	9.7	19.2	18.4	97.2	**11**	LM_FSL F2-208
12	0.3	2.0	0.9	2.2	2.0	0.1	0.1	0.0	0.0	1.6	347.0	■	100.0	4.4	97.8	100.0	98.0	3.3	99.8	14.5	98.0	99.8	97.8	6.4	**12**	LM_FSL J1-175
13	0.8	3.8	0.6	4.1	3.9	0.3	0.3	118.8	0.3	1.6	255.0	0.0	■	10.1	95.6	99.4	95.9	11.1	99.7	21.7	58.7	99.4	95.6	11.9	**13**	LM_FSL J1-194
14	194.5	195.3	171.4	192.6	193.9	191.3	191.3	89.1	191.3	403.0	210.0	325.0	191.3	■	25.4	25.5	25.1	80.2	25.5	38.2	8.4	25.5	25.4	26.1	**14**	Lm_FSL J2-064
15	4.3	0.8	4.6	0.0	0.8	4.0	4.0	117.1	4.0	0.6	265.0	2.2	4.1	192.6	■	96.1	99.2	11.3	96.3	21.4	60.2	96.1	100.0	11.8	**15**	LM_J0161
16	0.8	3.7	0.5	4.0	3.8	0.4	0.4	117.1	0.0	1.6	254.0	0.0	0.3	191.3	4.0	■	96.4	11.1	99.7	21.9	58.9	99.8	96.1	12.0	**16**	LM_FSL R2-503
17	4.0	0.0	4.4	0.8	0.7	3.7	3.7	118.8	3.7	0.3	264.0	2.0	3.8	195.3	0.8	3.7	■	11.2	96.6	21.4	60.2	96.4	99.2	11.9	**17**	LM_FSL R2-561
18	166.0	162.3	163.3	161.3	161.3	164.4	164.4	96.2	164.4	309.0	197.4	270.0	164.4	5.8	161.3	164.4	162.3	■	29.0	40.2	7.5	29.0	29.4	30.3	**18**	LM_HCC23
19	0.6	3.5	0.5	3.8	3.7	0.2	0.2	117.1	0.3	1.3	254.0	0.2	0.4	191.3	3.8	0.3	3.5	164.4	■	21.8	58.8	99.4	95.9	11.9	**19**	LM_HPB2262
20	221.0	226.0	185.4	226.0	226.0	222.0	222.0	4.2	220.0	595.0	236.0	335.0	222.0	118.5	226.0	220.0	226.0	110.6	221.0	■	9.0	21.2	20.7	12.1	**20**	LM_J2-071
21	2.1	0.2	3.0	0.2	0.2	2.2	2.2	0.0	2.3	0.0	321.0	2.0	2.3	279.0	0.2	2.3	0.2	221.0	2.1	315.0	■	97.6	99.8	7.2	**21**	LM_N3-165
22	0.8	3.7	0.2	4.0	3.8	0.4	0.4	117.1	0.2	1.6	254.0	0.2	0.3	191.3	4.0	0.2	3.7	164.4	0.3	220.0	2.5	■	96.0	12.0	**22**	LM_J1816
23	4.3	0.8	4.6	0.0	0.8	4.0	4.0	117.1	4.0	0.6	265.0	2.2	4.1	192.6	0.0	4.0	0.8	161.3	3.8	226.0	0.2	4.0	■	11.8	**23**	LM_J2818
24	218.0	216.0	197.4	219.0	217.0	216.0	216.0	128.4	215.0	332.0	2.9	347.0	216.0	181.3	219.0	215.0	216.0	151.5	215.0	208.0	317.0	215.0	219.0	■	**24**	LM_L99
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**		

B



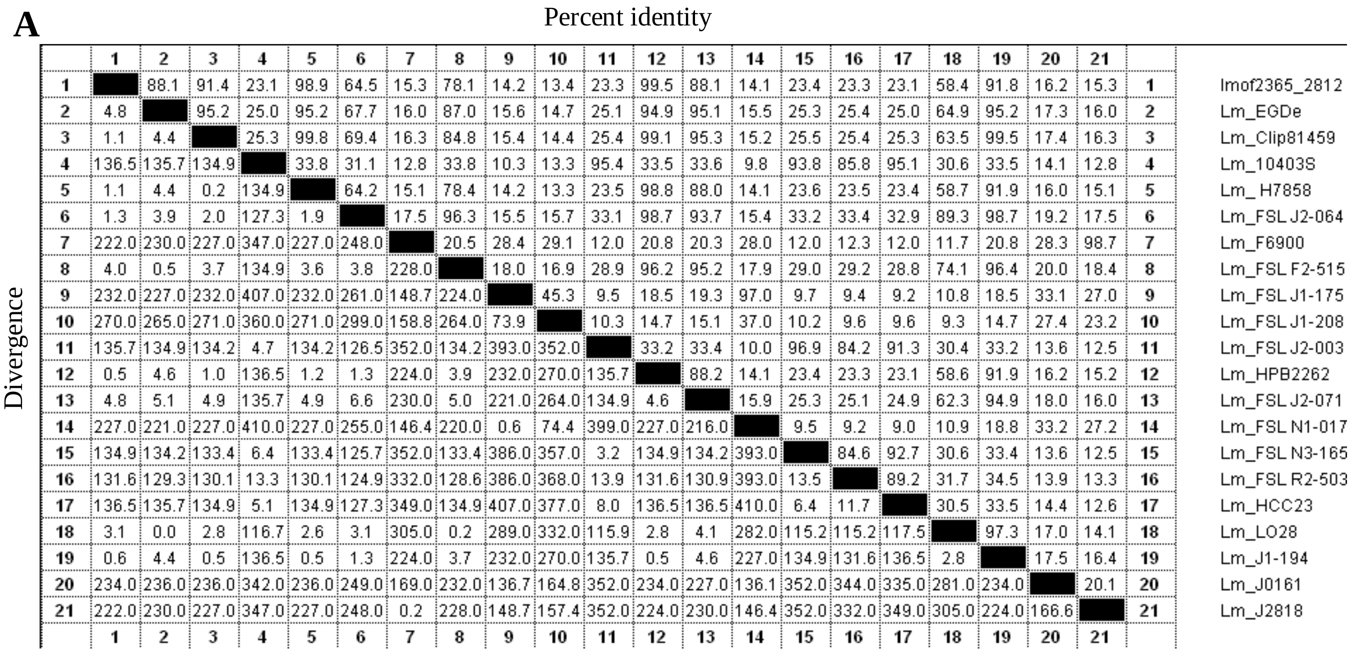
Percent identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11		
1	■	20.4	15.1	20.4	20.9	18.5	20.6	6.1	20.6	78.4	20.7	**1**	LM_LMOf2365_1690
2	238.0	■	7.2	91.6	64.0	61.0	13.6	5.4	13.5	17.8	84.6	**2**	L.innocua_FSL J1-023
3	158.4	299.0	■	16.1	16.7	16.1	19.4	4.3	19.3	29.2	16.0	**3**	L.innocua_Clip11262
4	238.0	8.6	303.0	■	62.0	59.6	12.5	5.0	12.4	17.2	84.8	**4**	L.innocua_ATCC 33091
5	232.0	48.3	292.0	52.3	■	65.1	12.5	5.6	12.5	17.5	63.2	**5**	L.ivanovii_FSL F6-596
6	250.0	48.8	301.0	51.8	41.6	■	11.2	5.3	11.0	16.3	63.1	**6**	L.ivanovii_PAM
7	224.0	315.0	250.0	328.0	323.0	368.0	■	17.4	93.8	19.1	14.9	**7**	L.seeligeri _ SLCC3954
8	414.0	434.0	626.0	451.0	393.0	410.0	136.0	■	34.0	12.9	11.1	**8**	L.seeligeri_ SLCC3954
9	223.0	317.0	252.0	330.0	325.0	374.0	1.6	135.5	■	20.0	15.5	**9**	L.seeligeri_FSL N1-067
10	25.4	243.0	164.6	241.0	233.0	250.0	233.0	374.0	232.0	■	20.2	**10**	L.seeligeri_FSL S4-171
11	234.0	17.3	305.0	17.0	49.8	49.9	313.0	447.0	315.0	239.0	■	**11**	L.welshimeri_SLCC5334
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**		

Figure 6. Sequence distance chart for InlJ generated from multiple alignment with similar proteins of *Listeria* strains identified by BLASTp search. A. Of the 20 *L. monocytogenes* strains, 6 strains had greater than 80 percent identity to the InlJ protein of *L. monocytogenes* 4b strain f2365: EGDe, H7858, Clip81459, HPB2262, FSL-F2-515, J2-071, J1-194. **B.** All nine non-pathogenic *Listeria* strains had less than 60 percent identity to InlJ in *L. monocytogenes* strain f2365.

A

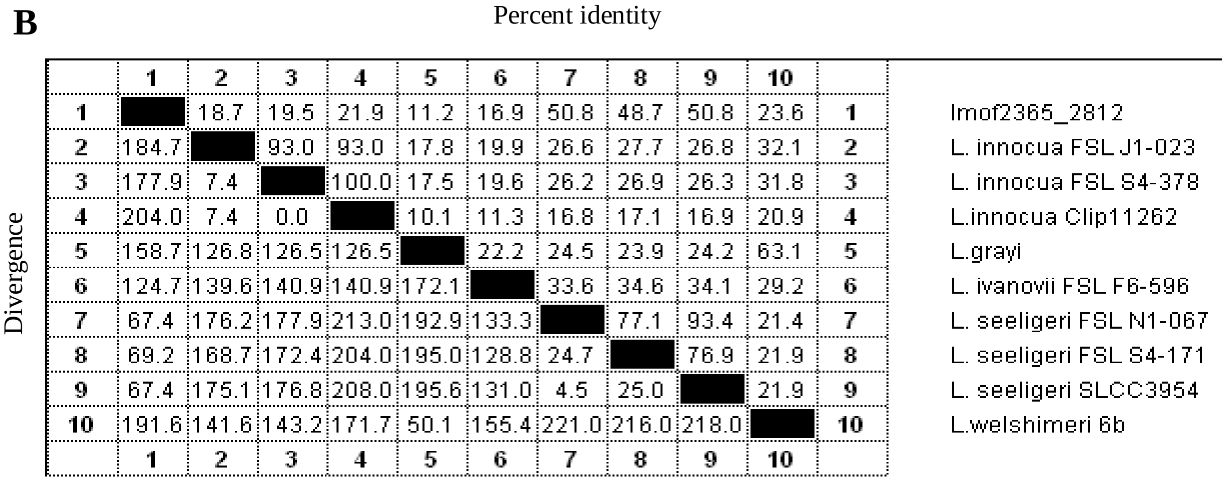


Percent identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21		
1	■	88.1	91.4	23.1	98.9	64.5	15.3	78.1	14.2	13.4	23.3	99.5	88.1	14.1	23.4	23.3	23.1	58.4	91.8	16.2	15.3	1	lmof2365_2812
2	4.8	■	95.2	25.0	95.2	67.7	16.0	87.0	15.6	14.7	25.1	94.9	95.1	15.5	25.3	25.4	25.0	64.9	95.2	17.3	16.0	2	Lm_EGDe
3	1.1	4.4	■	25.3	99.8	69.4	16.3	84.8	15.4	14.4	25.4	99.1	95.3	15.2	25.5	25.4	25.3	63.5	99.5	17.4	16.3	3	Lm_Clip81459
4	136.5	135.7	134.9	■	33.8	31.1	12.8	33.8	10.3	13.3	95.4	33.5	33.6	9.8	93.8	85.8	95.1	30.6	33.5	14.1	12.8	4	Lm_10403S
5	1.1	4.4	0.2	134.9	■	64.2	15.1	78.4	14.2	13.3	23.5	98.8	88.0	14.1	23.6	23.5	23.4	58.7	91.9	16.0	15.1	5	Lm_ H7858
6	1.3	3.9	2.0	127.3	1.9	■	17.5	96.3	15.5	15.7	33.1	98.7	93.7	15.4	33.2	33.4	32.9	89.3	98.7	19.2	17.5	6	Lm_FSL J2-064
7	222.0	230.0	227.0	347.0	227.0	248.0	■	20.5	28.4	29.1	12.0	20.8	20.3	28.0	12.0	12.3	12.0	11.7	20.8	28.3	98.7	7	Lm_F6900
8	4.0	0.5	3.7	134.9	3.6	3.8	228.0	■	18.0	16.9	28.9	96.2	95.2	17.9	29.0	29.2	28.8	74.1	96.4	20.0	18.4	8	Lm_FSL F2-515
9	232.0	227.0	232.0	407.0	232.0	261.0	148.7	224.0	■	45.3	9.5	18.5	19.3	97.0	9.7	9.4	9.2	10.8	18.5	33.1	27.0	9	Lm_FSL J1-175
10	270.0	265.0	271.0	360.0	271.0	299.0	158.8	264.0	73.9	■	10.3	14.7	15.1	37.0	10.2	9.6	9.6	9.3	14.7	27.4	23.2	10	Lm_FSL J1-208
11	135.7	134.9	134.2	4.7	134.2	126.5	352.0	134.2	393.0	352.0	■	33.2	33.4	10.0	96.9	84.2	91.3	30.4	33.2	13.6	12.5	11	Lm_FSL J2-003
12	0.5	4.6	1.0	136.5	1.2	1.3	224.0	3.9	232.0	270.0	135.7	■	88.2	14.1	23.4	23.3	23.1	58.6	91.9	16.2	15.2	12	Lm_HPB2262
13	4.8	5.1	4.9	135.7	4.9	6.6	230.0	5.0	221.0	264.0	134.9	4.6	■	15.9	25.3	25.1	24.9	62.3	94.9	18.0	16.0	13	Lm_FSL J2-071
14	227.0	221.0	227.0	410.0	227.0	255.0	146.4	220.0	0.6	74.4	399.0	227.0	216.0	■	9.5	9.2	9.0	10.9	18.8	33.2	27.2	14	Lm_FSL N1-017
15	134.9	134.2	133.4	6.4	133.4	125.7	352.0	133.4	386.0	357.0	3.2	134.9	134.2	393.0	■	84.6	92.7	30.6	33.4	13.6	12.5	15	Lm_FSL N3-165
16	131.6	129.3	130.1	13.3	130.1	124.9	332.0	128.6	386.0	368.0	13.9	131.6	130.9	393.0	13.5	■	89.2	31.7	34.5	13.9	13.3	16	Lm_FSL R2-503
17	136.5	135.7	134.9	5.1	134.9	127.3	349.0	134.9	407.0	377.0	8.0	136.5	136.5	410.0	6.4	11.7	■	30.5	33.5	14.4	12.6	17	Lm_HCC23
18	3.1	0.0	2.8	116.7	2.6	3.1	305.0	0.2	289.0	332.0	115.9	2.8	4.1	282.0	115.2	115.2	117.5	■	97.3	17.0	14.1	18	Lm_LO28
19	0.6	4.4	0.5	136.5	0.5	1.3	224.0	3.7	232.0	270.0	135.7	0.5	4.6	227.0	134.9	131.6	136.5	2.8	■	17.5	16.4	19	Lm_J1-194
20	234.0	236.0	236.0	342.0	236.0	249.0	169.0	232.0	136.7	164.8	352.0	234.0	227.0	136.1	352.0	344.0	335.0	281.0	234.0	■	20.1	20	Lm_J0161
21	222.0	230.0	227.0	347.0	227.0	248.0	0.2	228.0	148.7	157.4	352.0	224.0	230.0	146.4	352.0	332.0	349.0	305.0	224.0	166.6	■	21	Lm_J2818
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21		

B



Percent identity

Divergence

	1	2	3	4	5	6	7	8	9	10		
1	■	18.7	19.5	21.9	11.2	16.9	50.8	48.7	50.8	23.6	1	lmof2365_2812
2	184.7	■	93.0	93.0	17.8	19.9	26.6	27.7	26.8	32.1	2	L. innocua FSL J1-023
3	177.9	7.4	■	100.0	17.5	19.6	26.2	26.9	26.3	31.8	3	L. innocua FSL S4-378
4	204.0	7.4	0.0	■	10.1	11.3	16.8	17.1	16.9	20.9	4	L.innocua Clip11262
5	158.7	126.8	126.5	126.5	■	22.2	24.5	23.9	24.2	63.1	5	L.grayi
6	124.7	139.6	140.9	140.9	172.1	■	33.6	34.6	34.1	29.2	6	L. ivanovii FSL F6-596
7	67.4	176.2	177.9	213.0	192.9	133.3	■	77.1	93.4	21.4	7	L. seeligeri FSL N1-067
8	69.2	168.7	172.4	204.0	195.0	128.8	24.7	■	76.9	21.9	8	L. seeligeri FSL S4-171
9	67.4	175.1	176.8	208.0	195.6	131.0	4.5	25.0	■	21.9	9	L. seeligeri SLCC3954
10	191.6	141.6	143.2	171.7	50.1	155.4	221.0	216.0	218.0	■	10	L.welshimeri 6b
	1	2	3	4	5	6	7	8	9	10		

Figure 7. Sequence distance chart for InlC2 generated from multiple alignment with similar proteins of *Listeria* strains identified by BLASTp search. A. Of the 20 *L. monocytogenes* strains, 18 strains had a homolog protein with greater than 80 percent identity to the InlC2 protein (lmof2365_0281) of the *L. monocytogenes* strain f2365. **B.** All nine non-pathogenic *Listeria* strains had a protein with less than 60 percent identity to InlC2 in *L. monocytogenes* strain f2365.

A

Percent identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22		
1	■	94.2	60.9	97.8	95.1	95.1	96.7	94.3	100.0	95.1	96.7	95.4	96.7	99.1	96.7	52.7	99.6	99.1	99.8	100.0	96.7	96.7	1	lmof2365_0281
2	6.1	■	61.1	94.0	97.4	97.6	93.6	96.5	94.2	97.4	93.4	93.2	92.0	94.0	93.6	54.7	94.2	94.3	94.0	94.2	93.6	93.6	2	Lm_EGDe
3	49.7	49.3	■	60.6	60.1	60.1	60.6	59.9	60.6	60.1	60.4	60.1	60.6	61.0	60.6	30.3	60.6	60.6	60.6	60.6	60.6	60.6	3	Lm FSL J2-064
4	2.2	6.3	49.7	■	95.3	95.3	98.2	94.5	97.8	95.3	98.5	97.3	95.3	98.0	98.2	52.6	97.8	97.6	97.6	97.8	98.2	98.2	4	Lm4b H7858
5	5.1	2.6	50.8	4.9	■	99.8	94.7	98.5	95.1	100.0	94.5	94.7	92.7	94.9	94.7	53.8	95.1	95.1	94.9	95.1	94.7	94.7	5	Lm 08-5578
6	5.1	2.4	50.8	4.9	0.2	■	94.7	98.5	95.1	99.8	94.5	94.7	92.7	94.9	94.7	53.8	95.1	95.1	94.9	95.1	94.7	94.7	6	Lm 10403S
7	3.4	6.7	49.7	1.8	5.5	5.5	■	94.0	96.7	94.7	98.9	98.5	94.2	96.9	100.0	52.0	96.7	96.5	96.5	96.7	100.0	100.0	7	Lm F6900
8	5.9	3.6	51.1	5.7	1.5	1.5	6.3	■	94.3	98.5	93.8	94.7	92.3	94.2	94.0	53.6	94.3	94.3	94.2	94.3	94.0	94.0	8	Lm Finland 1988
9	0.0	6.1	49.7	2.2	5.1	5.1	3.4	5.9	■	95.1	96.7	95.4	96.7	99.1	96.7	52.7	99.6	99.1	99.8	100.0	96.7	96.7	9	Lm FSL J1-194
10	5.1	2.6	50.8	4.9	0.0	0.2	5.5	1.5	5.1	■	94.5	94.7	92.7	94.9	94.7	53.8	95.1	95.1	94.9	95.1	94.7	94.7	10	Lm_08-5923
11	3.4	6.9	50.1	1.5	5.7	5.7	1.1	6.5	3.4	5.7	■	97.8	94.2	96.9	98.9	52.2	96.7	96.5	96.5	96.7	98.9	98.9	11	Lm FSL J2-071
12	4.7	7.1	50.8	2.8	5.5	5.5	1.5	5.5	4.7	5.5	2.2	■	92.9	95.6	98.5	52.0	95.4	95.3	95.3	95.4	98.5	98.5	12	Lm FSL N3-165
13	0.9	6.0	49.7	2.5	5.2	5.2	3.6	5.6	0.9	5.2	3.6	5.0	■	99.3	96.4	54.2	99.4	100.0	99.1	99.1	96.4	96.4	13	Lm FSL R2-503
14	0.9	6.3	49.0	2.0	5.3	5.3	3.2	6.1	0.9	5.3	3.2	4.5	0.8	■	96.9	52.4	99.5	99.3	98.9	99.1	96.9	96.9	14	Lm HPB2262
15	3.4	6.7	49.7	1.8	5.5	5.5	0.0	6.3	3.4	5.5	1.1	1.5	3.6	3.2	■	52.0	96.7	96.5	96.5	96.7	100.0	100.0	15	Lm J0161
16	3.8	0.0	64.2	4.1	1.7	1.7	5.2	2.0	3.8	1.7	4.8	5.2	3.4	4.5	5.2	■	96.3	96.7	96.3	96.3	95.0	95.0	16	Lm_LO28
17	0.4	6.1	49.7	2.2	5.1	5.1	3.4	5.9	0.4	5.1	3.4	4.7	0.6	0.5	3.4	3.8	■	99.5	99.5	99.6	96.7	96.7	17	Lm_ FSL J1-175
18	0.9	5.9	49.7	2.4	5.1	5.1	3.6	5.9	0.9	5.1	3.6	4.9	0.0	0.7	3.6	3.4	0.5	■	98.9	99.1	96.5	96.5	18	Lm_ FSL N1-017
19	0.0	6.1	49.7	2.2	5.1	5.1	3.4	5.9	0.0	5.1	3.4	4.7	0.9	0.9	3.4	3.8	0.4	0.9	■	100.0	96.7	96.7	19	Lm_ FSLE1124
20	0.0	6.1	49.7	2.2	5.1	5.1	3.4	5.9	0.0	5.1	3.4	4.7	0.9	0.9	3.4	3.8	0.4	0.9	0.0	■	96.7	96.7	20	Lm_CLIP 80459
21	3.4	6.7	49.7	1.8	5.5	5.5	0.0	6.3	3.4	5.5	1.1	1.5	3.6	3.2	0.0	5.2	3.4	3.6	3.4	3.4	■	100.0	21	Lm_F6854
22	3.4	6.7	49.7	1.8	5.5	5.5	0.0	6.3	3.4	5.5	1.1	1.5	3.6	3.2	0.0	5.2	3.4	3.6	3.4	3.4	0.0	■	22	Lm_J2818
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22		

B

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11		
1	■	44.0	48.9	46.5	34.3	47.8	21.7	25.5	35.9	40.1	48.7	1	lmof2365_0281
2	97.3	■	38.6	39.3	30.7	38.4	17.4	20.7	38.4	39.5	49.9	2	L. welshimeri SLCC5334
3	80.3	109.8	■	38.6	28.2	37.5	17.1	20.6	29.4	34.8	39.3	3	L. innocua Clip11262
4	80.6	100.8	92.5	■	32.4	67.3	18.0	21.6	29.1	31.8	38.8	4	L. innocua FSL J1-023
5	83.7	95.5	91.8	70.9	■	48.4	26.4	29.1	32.0	39.1	47.9	5	L. innocua FSL S4-378
6	85.9	113.3	104.3	42.8	84.2	■	15.5	18.8	25.6	26.9	34.0	6	L.ivanovii FSL F6-596
7	118.3	143.1	126.9	115.4	120.9	120.6	■	58.0	22.3	29.0	40.1	7	L.ivanovii InlF
8	172.7	202.0	179.0	155.4	133.6	183.7	60.4	■	14.6	20.7	24.1	8	L.ivanovii InlG
9	80.5	72.1	91.4	78.8	87.9	92.6	124.3	209.0	■	42.6	95.8	9	L.seeligeri FSL N1-067
10	108.7	109.7	110.1	113.0	114.9	130.1	165.4	200.0	100.9	■	37.0	10	L.seeligeri FSL S4-171
11	82.3	76.7	91.7	83.6	85.6	94.5	111.0	164.8	4.3	106.3	■	11	L.seeligeri_ SLCC3954
	1	2	3	4	5	6	7	8	9	10	11		

OppA demonstrated a high homology to a protein in other *L. monocytogenes* strains yet also a high homology to a protein in non-pathogenic *Listeria* strains. Auto demonstrated a low homology to a protein in some *L. monocytogenes* strains as well as low homology to a protein in non-pathogenic *Listeria* strains. Ami showed a mix of high and low homology to proteins in both *L. monocytogenes* and non-pathogenic *Listeria* strains. Lmo2026 had typically a low homology to a protein in other *L. monocytogenes* strains and a high homology to non-pathogenic *Listeria* strains. The InlB protein sequence was not analyzed using BLASTp as it was discovered after the original literature review which identified InlB as a candidate for MAb development, that InlB is buried in the cell wall so it is not accessible to antibodies at the surface (63). One known method of accessing InlB *in vitro* is to digest the cell wall peptidoglycan with muradimase (63). During an infection, InlB needs to be accessible by its host receptor, suggesting that external factors regulate its accessibility.

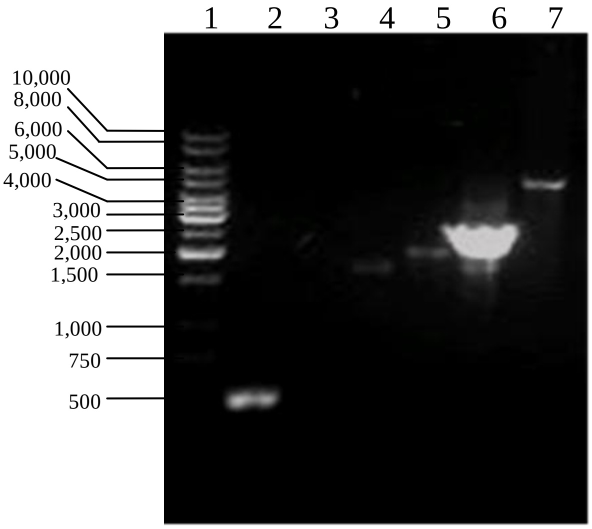
4.3 Cloning and expression of recombinant proteins in *E. coli*

Producing adequate amounts of recombinant proteins (rActA, rInlA, rInlC2, rInlJ and rLapB) was necessary for raising polyclonal antibodies (PAbs) and monoclonal antibodies (MAbs) to these proteins. Four of the five target genes, *actA*, *inlA*, *inlJ* and *lapB* were amplified by PCR (Figure 8A) and cloned into pLIC-CHis vector to create recombinant plasmids pActA, pInlA, pInlJ and pLapB (Figure 8B). All recombinant plasmids were sequenced for confirmation of the correct inserts and used to transform various *E. coli* expression host cells for production of recombinant proteins. Cloning of the *inlC2* gene into the expression vector pET30a was previously performed by our lab.

The expression of recombinant proteins was assessed using an anti-HIS MAb as the probe on Western blotting (WB). Expression levels of InlA, InlJ and LapB were highest with *E. coli* Rosetta(DE3)/pLysS. InlC2 was expressed in *E. coli* BL21(DE3)/pLysS. ActA would only express in *E. coli* BL21(DE3). Figure 9 shows the expression levels of all five target proteins as analyzed by WB. Expression of the rActA protein was not detected in the three *E. coli* hosts that were attempted: BL21(DE3)/pLysS, Rosetta(DE3)/pLysS or C43(DE3)/pLysS (Figure 10). This protein was only expressed in BL21(DE3) (Figure 11). The reason for this still remains unclear.

Figure 8. Cloning of target *L. monocytogenes* genes into pLIC-CHIS vector. **A.** PCR amplification of target genes (*actA*, *inlA*, *inlJ* and *lapB*) from the genomic DNA of *L. monocytogenes* serotype 4b strain L10521 was analyzed on 1.5% agarose gel. Lane 1, 10,000 bp ladder; lane 2, *ispC* as positive control (500 bp); lane 3, no template as negative control; lane 4, *actA* (1682 bp); lane 5, *inlA* (2165 bp); lane 6, *inlJ* (2537 bp); lane 7, *lapB* (4928 bp). All genes were amplified by PCR using 1µl of Pfu polymerase, with the addition of 1µl of Taq polymerase for the amplification of *inlJ*. **B.** Confirmation of the presence of inserts in recombinant plasmids. Lane 1,10,000 bp ladder; lane 2, *actA* (1682 bp); lane 3, *inlA* (2165 bp); lane 4, *inlJ* (2537 bp); lane 5, *lapB* (4928 bp). PCR was performed to confirm the presence of the inserted genes using 1 µl of Pfu polymerase, and 1µl of Taq polymerase with T7 promoter and T7 terminator primers.

A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750
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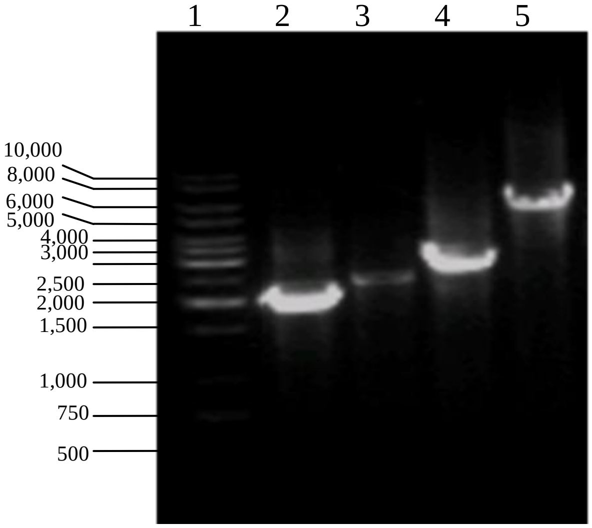
B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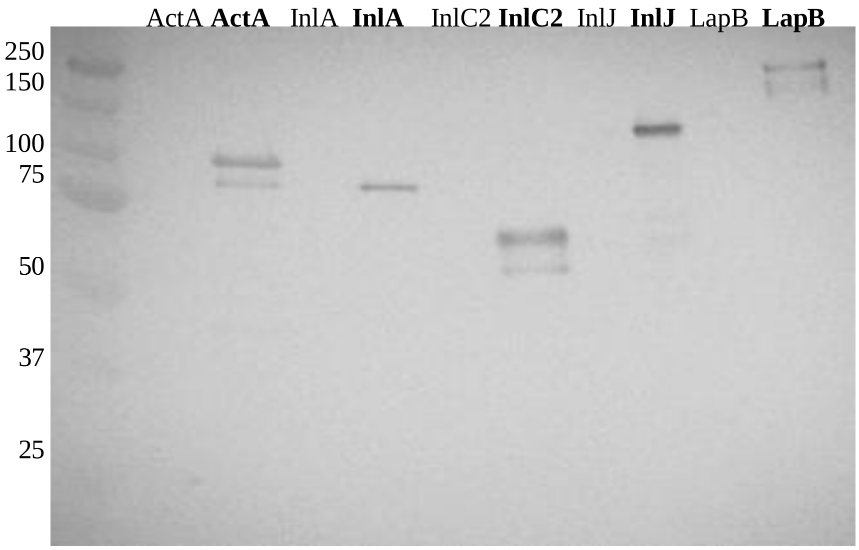
1,000
750
500

Figure 9. Expression of five target proteins in *E. coli*. Proteins in the whole cell lysate were resolved by SDS-PAGE followed by WB analysis using anti-HIS MAb at a 1:1000 dilution. **A**. WB of uninduced and induced (bolded lane heading) samples of ActA expressed in *E. coli* BL21(DE3), InlC2 expressed in *E. coli* BL21(DE3)/pLysS and InlA, InlJ and LapB expressed in *E. coli* Rosetta(DE3)/pLysS. *E. coli* cells containing a recombinant plasmid were grown in LB broth with kanamycin overnight followed by sub-culture to obtain an OD_{600} of ~0.60. Bacterial cells were then induced with 0.1M IPTG for 3 h. The molecular masses of protein standards (in kDa) are shown by the numbers on the left. **B.** Gel stained with CMB showing total protein samples loaded in **A**. Theoretical molecular weights of target proteins are as follows: ActA (65kDa), InlA (87kDa), InlC2 (58kDa), InlJ (110kDa) and LapB (180kDa). Apparent molecular weights are as follows: ActA (92kDa), InlA (87kDa), InlC2 (70kDa), InlJ (140kDa) and LapB (195kDa).

A


ActA
ActA
InlA
InlA
InlC2
InlC2
InlJ
InlJ
LapB
LapB
250
150
100
75
50
37
25

B

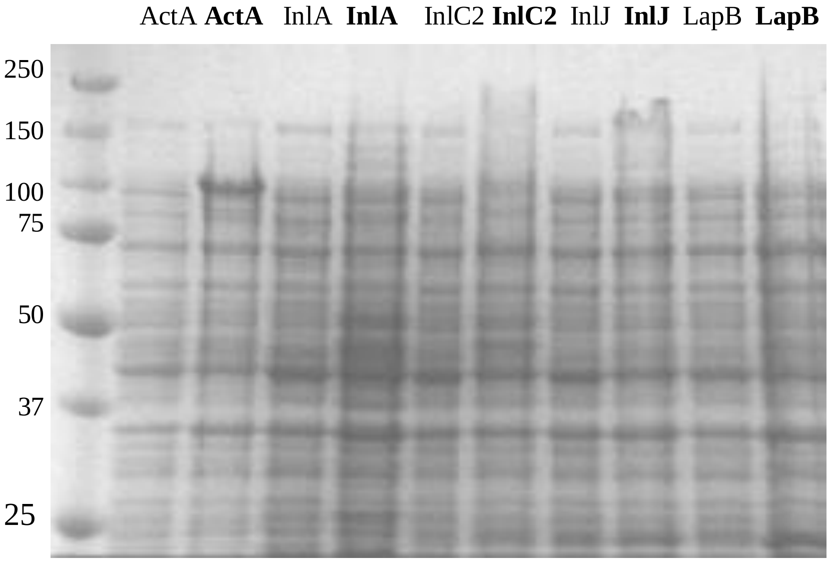
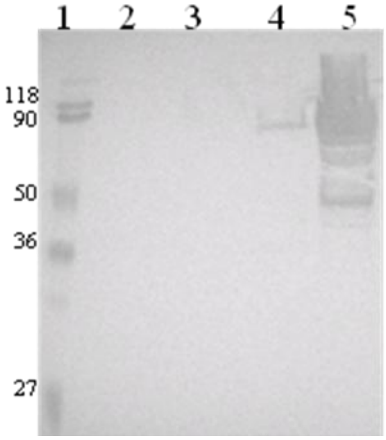
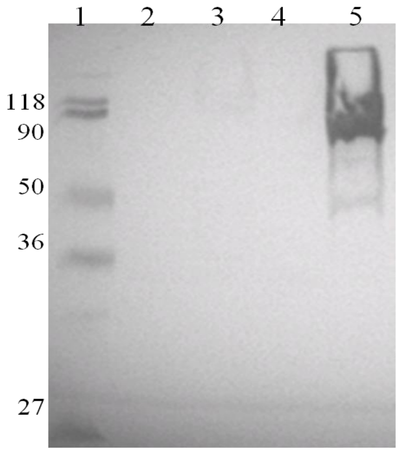

ActA
ActA
InlA
InlA
InlC2
InlC2
InlJ
InlJ
LapB
LapB
250
150
100
75
50
37
25

Figure 10. ActA was not expressed in three different *E. coli* host cells A. Failure to express rActA in BL21(DE3)/pLysS. Whole cell lysates from uninduced and IPTG induced *E. coli* BL21(DE3)/pLysS harboring pActA and pInlA were resolved by SDS-PAGE and analyzed with WB using anti-HIS MAb at a 1:1000 dilution. Lane 1, standard protein marker in kDa; lane 2, uninduced rActA protein; lane 3, IPTG induced rActA protein; lane 4, uninduced rInlA protein; lane 5, IPTG induced rInlA protein. **B. Failure to express rActA in *E. coli* Rosetta(DE3)/pLysS.** Whole cell lysates from uninduced and IPTG induced *E. coli* BL21(DE3)/pLysS harboring pActA, and pInlA were separated with SDS-PAGE and analyzed by WB using anti-HIS MAb at a 1:1000 dilution. Lane 1, standard protein marker in kDa; lane 2, uninduced rActA protein; lane 3, induced rActA protein; lane 4, uninduced rInlA protein; lane 5, induced rInlA protein. **C. Failure to express rActA in *E. coli* C43(DE3)/pLysS.** Whole cell lysates from uninduced and IPTG induced *E. coli* BL21(DE3)/pLysS harboring pActA and pInlA were resolved by SDS-PAGE and analyzed with WB using anti-HIS MAb at a 1:1000 dilution. Lane 1, standard protein marker in kDa; lane 2, uninduced rActA protein; lane 3, induced rActA protein; lane 4, uninduced rInlA protein; lane 5, induced rInlA protein. Theoretical molecular weights of ActA and InlA proteins are 65 kDa and 87 kDa, respectively

A



B



C

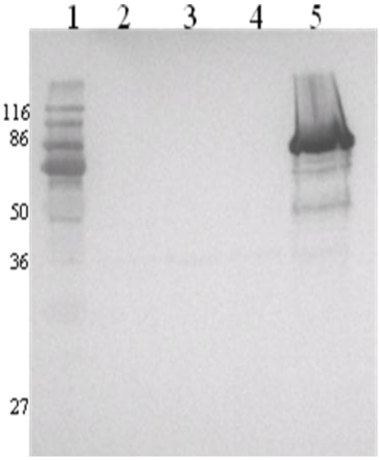
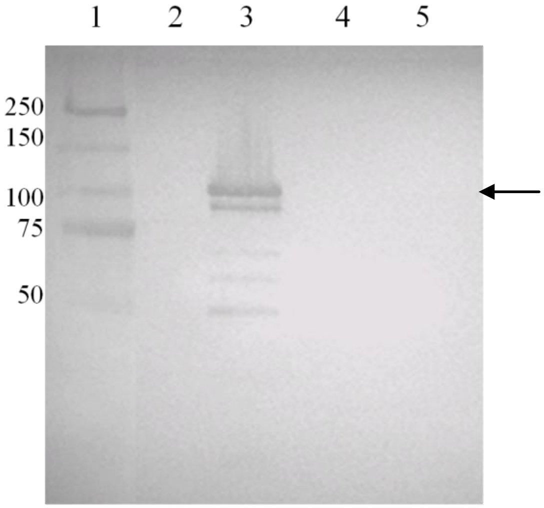


Figure 11. ActA was successfully expressed in *E. coli* BL21(DE3). Whole cell lysates from uninduced and IPTG induced *E. coli* BL21(DE3) and *E. coli* BL21(DE3)/pLysS harboring pActA were resolved by SDS-PAGE and analyzed with WB using anti-HIS MAb at a 1:1000 dilution. Lane 1, standard protein marker in kDa; lane 2, uninduced rActA protein from *E. coli* BL21(DE3); lane 3, induced rActA protein from *E. coli* BL21(DE3); lane 4, uninduced rActA protein from *E. coli* BL21(DE3)/pLysS; lane 5, induced rActA protein from *E. coli* BL21(DE3)/pLysS. Theoretical MW of ActA is 65 kDa whereas apparent MW is approximately 92 kDa. The black arrow indicates the rActA protein.


1
2
3
4
5
250
150
100
75
50

4.4 Purification of recombinant proteins

The rActA protein, expressed from pLIC-actA in *E. coli* BL21(DE3), was purified by Ni-NTA agarose affinity chromatography. The major protein band in the eluted fractions shown by SDS-PAGE (Figure 12A) was smaller than the expected molecular weight (MW) of 92 kDa. WB revealed the degraded rActA protein bands recognized by anti-HIS MAb (Figure 12B). Due to the difficulty in obtaining a high yield of purified rActA protein, this candidate was not selected for the development of MAbs raised against this protein.

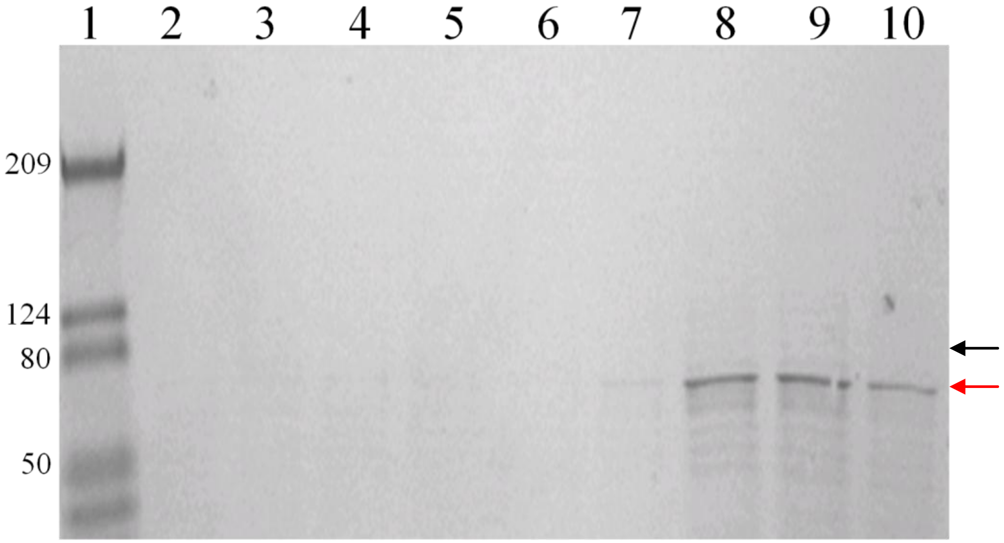
The rInlA protein was expressed at high levels in BL21(DE3)/pLysS, Rosetta(DE3)/pLysS and C43(DE3)/pLysS (Figures 10 and 11). Purification of rInlA from *E. coli* BL21(DE3)/pLysS demonstrated that the rInlA protein was present in the eluted fractions following Ni-NTA agarose affinity chromatography (Figure 13), however, the yield of purified rInlA protein was low and there was a significant amount of contamination with other *E. coli* proteins of as observed with SDS-PAGE (Figure 14). Due to the contamination of the rInlA preparation with *E. coli* proteins, rInlA was not selected for raising MAbs against this protein in this study.

The rLapB protein expressed from *E. coli* Rosetta(DE3)/pLysS was purified by Ni-NTA agarose affinity chromatography. Eluted fractions containing the rLapB protein were collected and analyzed by WB using anti-HIS MAb to confirm the presence of rLapB protein in eluted fractions (Figure 15). Eluted fractions were also analyzed with SDS-PAGE to determine the purity of the rLapB preparation. Figure 15A shows a high amount of rLapB protein in the eluted fractions and Figure 15B confirmed there was only minimal contamination with *E. coli* proteins. Based on the high yield and purity of the protein preparation, rLapB was selected for raising MAbs against this protein.

The rInlJ protein expressed from *E. coli* Rosetta(DE3)/pLysS was purified by Ni-NTA agarose affinity chromatography. Eluted fractions containing the rInlJ protein were collected and analyzed by WB using anti-HIS MAb to confirm the presence of rInlJ protein in eluted fractions (Figure 16). Figure 16A shows a high amount of rInlJ protein in the eluted fractions and Figure 16B confirmed there was only minimal contamination with *E. coli* proteins.

Figure 12. SDS-PAGE and Western blotting analysis of purified rActA protein. Ni-NTA agarose affinity chromatography was performed to purify rActA from the soluble extract of *E. coli* BL21(DE3) expressing the recombinant protein. Fractions containing proteins eluted from Ni-NTA agarose column were resolved by SDS-PAGE (A) and analyzed with WB (B) **A.** Gel stained with Coomassie blue: Lane 1, molecular masses of protein standards (in kDa); lanes 2-10, eluted fractions #2-10 **B.** Western blotting with an anti-HIS MAb: Lane numbers correspond to the same samples as those in (A). Black arrows indicate the expected size of the rActA protein (92 kDa). Red arrows indicate degraded rActA present in eluted fractions.

A



B

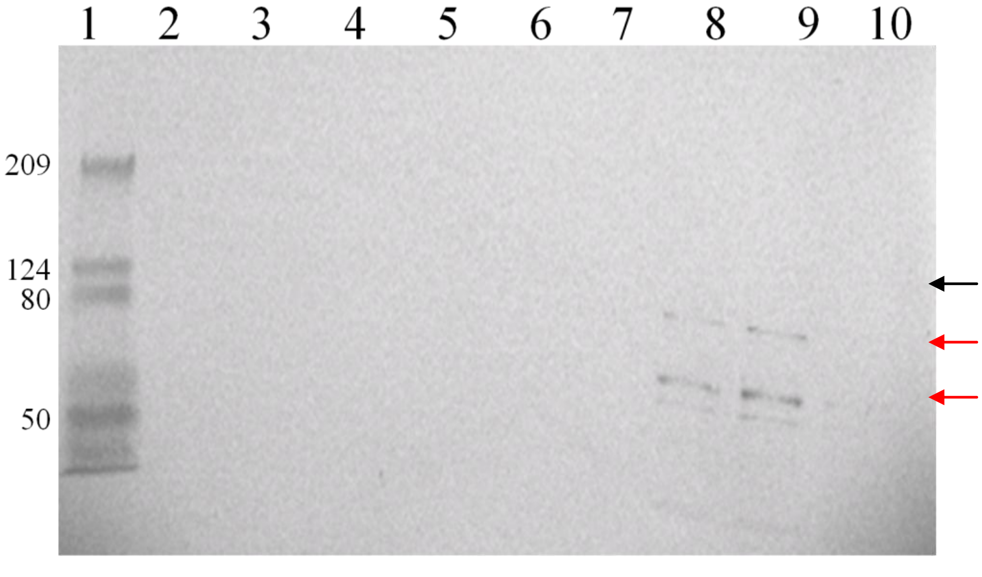


Figure 13. SDS-PAGE and Western blotting analysis of purified rInlA protein. Ni-NTA agarose affinity chromatography was performed to purify rInlA from the soluble extract of *E. coli* BL21(DE3)/pLysS expressing the recombinant protein. Fractions containing proteins eluted from Ni-NTA agarose column were resolved by SDS-PAGE and analyzed with WB.**A.** Gel stained with Coomassie blue: Lane 1, molecular masses of protein standards (in kDa); lanes 2-4, eluted fractions #8-10 **B.** Western blotting with an anti-HIS MAb: Lane numbers correspond to the same samples as those in (A). Black arrows indicate the expected size of the rInlA protein (87 kDa). Red arrows indicate degraded rInlA present in eluted fractions and gray arrows indicate *E. coli* proteins.

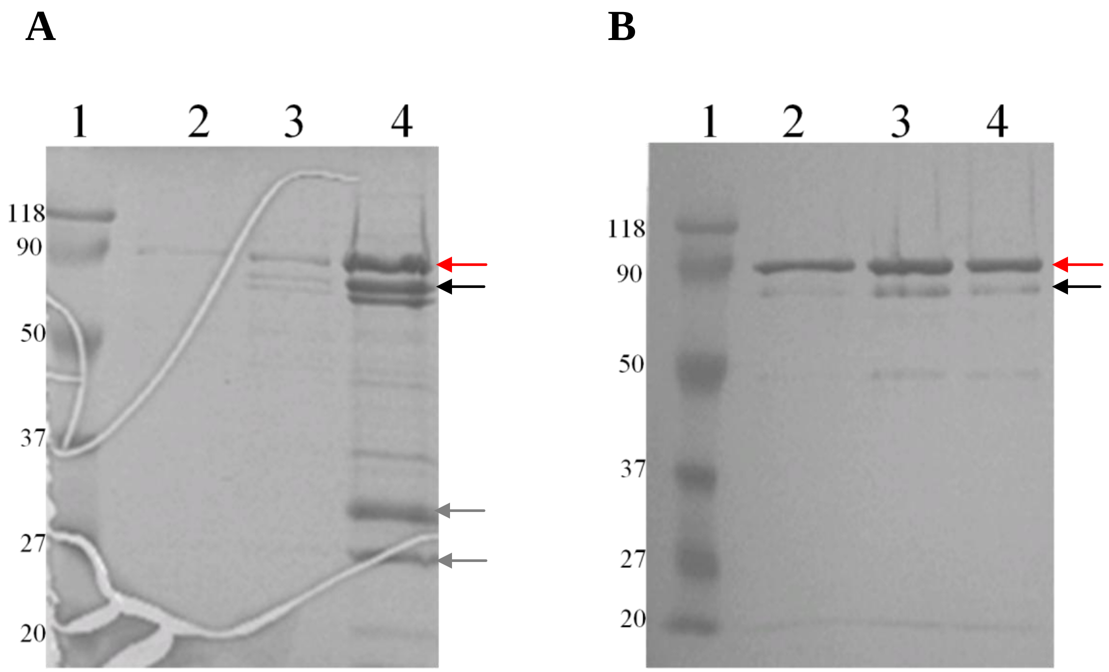
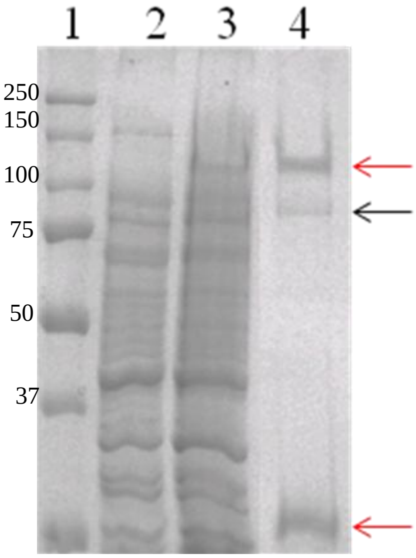

A
1
2
3
4
118
90
50
37
27
20
B
1
2
3
4
118
90
50
37
27
20

Figure 14. Contamination of purified rInlA protein preparation. Ni-NTA agarose affinity chromatography was performed to purify rInlA from the soluble extract of *E. coli* BL21(DE3)/pLysS expressing the recombinant protein. Fractions containing proteins eluted from Ni-NTA agarose column were resolved by SDS-PAGE and analyzed with WB. **A.** Gel stained with Coomassie blue: Lane 1, molecular masses of protein standards (in kDa); lane 2, uninduced rInlA BL21(DE3)/pLysS protein; lane 3, IPTG induced rInlA (BL21(DE3)/pLysS) protein; lane 4, purified and concentrated rInlA (BL21(DE3)/ pLysS). **B.** Western blotting with an anti-HIS MAb: Lane numbers correspond to the same samples as those in (A). Black arrows indicate the expected size of the rInlA protein (87 kDa). Red arrows indicate non-target *E. coli* proteins present in the purified rInlA preparation.


1
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3
4
250
150
100
75
50
37

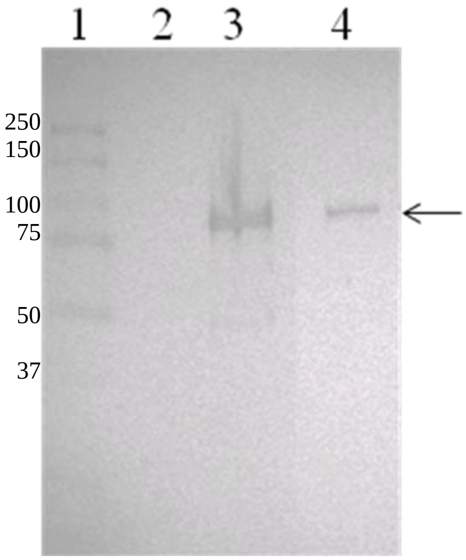

1
2
3
4
250
150
100
75
50
37

Figure 15. **SDS-PAGE and Western blotting analysis of purified rLapB protein.** Ni-NTA agarose affinity chromatography was performed to purify rLapB from the soluble extract of *E. coli* Rosetta(DE3)/pLysS expressing the recombinant protein. Fractions containing proteins eluted from Ni-NTA agarose column were resolved by SDS-PAGE and analyzed with WB **A.** Western blotting with an anti-HIS MAb: rLapB protein fractions (F1-F6) were loaded. **B**. WB analysis of pooled eluted fractions of purified rLapB protein concentrated to 1.04mg/ml. **C.** Gel stained with CMB: concentrated rLapB protein was loaded. Arrows indicate the rLapB protein at ~195 kDa. Theoretical MW of LapB is 180 kDa. Molecular masses of protein standards (in kDa) are shown by the numbers on the left.

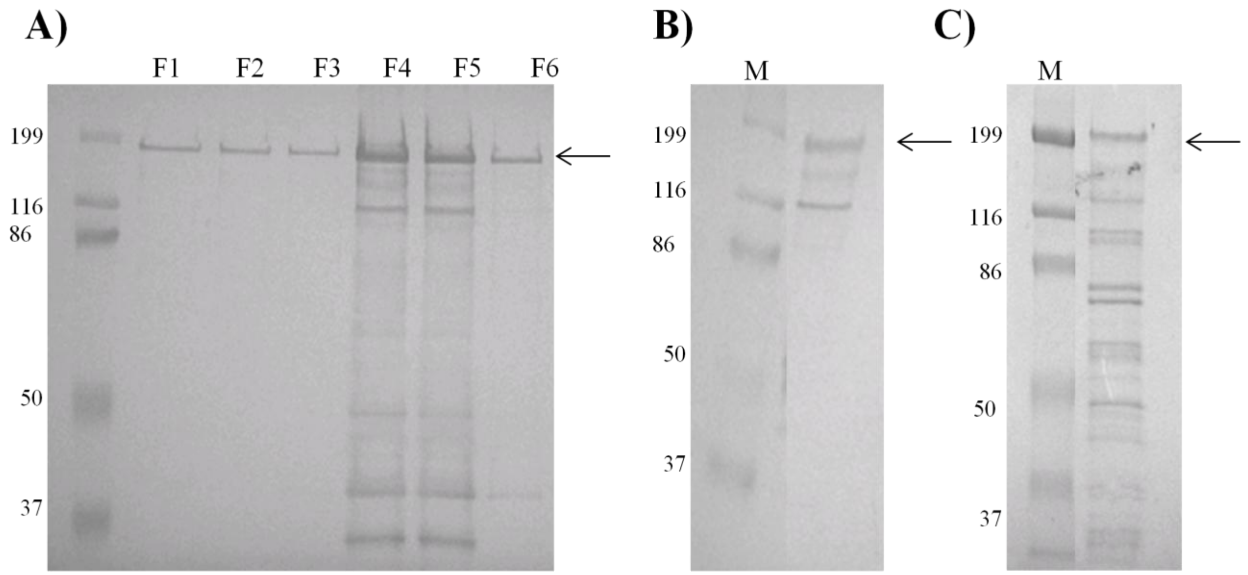
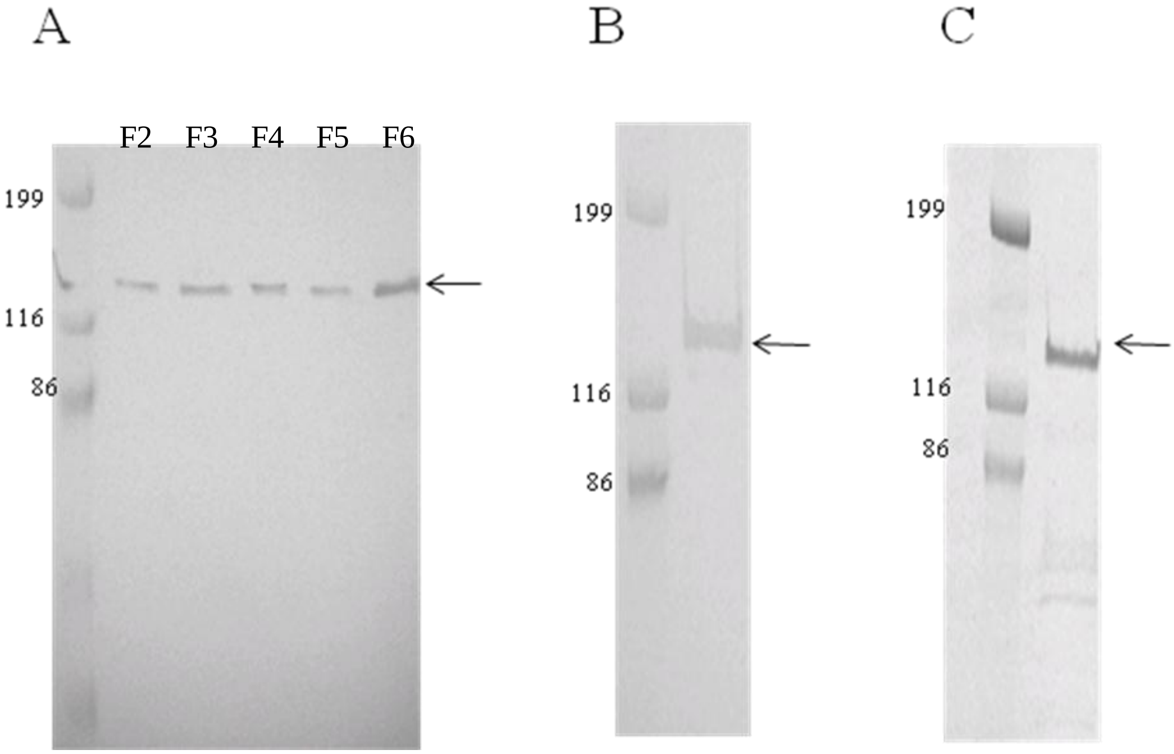

A)
F1
F2
F3
F4
F5
F6
199
116
86
50
37
B)
M
199
116
86
50
37
C)
M
199
116
86
50
37

Figure 16. SDS-PAGE and Western blotting analysis of purified rInlJ protein. Ni-NTA agarose affinity chromatography was performed to purify rInlJ from the soluble extract of *E. coli* BL21(DE3)/pLysS expressing the recombinant protein. Fractions containing proteins eluted from Ni-NTA agarose column were resolved by SDS-PAGE and analyzed with WB. **A.** WB of purified rInlJ eluted fractions #2-6 with an anti-HIS MAb. **B**. WB of pooled eluted fractions of purified rInlJ concentrated to 0.5mL volume and 1.13mg/ml. **C**. SDS-PAGE of concentrated rInlJ sample loaded in (B). Nitrocellulose membrane was immunoblotted with anti-HIS MAb at a 1:1000 dilution. Molecular masses of standard proteins (in kDa) are shown on the left. Black arrows indicate rInlJ target protein bands. Theoretical MW of InlJ protein is 110 kDa and apparent MW is 140 kDa.


A
B
C
F2
F3
F4
F5
F6
199
116
86
199
116
86
199
116
86

Based on the high yield and purity of the protein preparation, rInlJ was selected for raising MAbs against this protein.

The rInlC2 protein expressed from *E. coli* Rosetta(DE3)/pLysS was purified by Ni-NTA agarose affinity chromatography. Eluted fractions containing the rInlC2 protein were collected and analyzed by WB using anti-HIS MAb to confirm the presence of rInlC2 protein in eluted fractions (Figure 17). Figure 17A shows a high amount of rInlC2 protein in the eluted fractions and Figure 17B confirmed there was only minimal contamination with *E. coli* proteins. Based on the high yield and purity of the protein preparation, rInlC2 was selected for raising MAbs against this protein.

4.5 Assessment of LapB, InlJ and InlC2 expression in *L. monocytogenes* cells

Since production and screening of MAbs is a time consuming and expensive process, it was essential to verify that the proteins of interest were suitable surface diagnostic markers for MAb development by examining the expression of LapB, InlJ and InlC2 on the surface of *L. monocytogenes*. PAbs can be easily produced and used to probe the expression and the cross-reactivity of the target proteins among *Listeria* spp. PAbs were raised in rabbits or mice against purified rLapB, rInlJ and rInlC2, respectively, and used to verify the expression of target proteins on the surface of *L. monocytogenes*. WB and immunofluorescence microscopy (IFM) was carried out to evaluate the ability of PAbs to recognize recombinant and native forms of the immunizing proteins.

Localization of LapB on the surface of *L. monocytogenes* cells was demonstrated by IFM. The expression of LapB on the surface of *L. monocytogenes* serotype 4b L10521 was shown to be moderate when compared to the positive control using the MAb M2799 that recognizes the immunogenic surface protein C (IspC) (Figure 18). Pre-immune rabbit serum was negative for detecting the expression of LapB on the cell surface. WB analysis showed that mouse anti-LapB PAb recognized the native protein from total protein extract of *L. monocytogenes* (Figure 19).

Localization of InlJ on the surface of *L. monocytogenes* cells was demonstrated by IFM using rabbit anti-InlJ serum. The expression of InlJ on the surface of *L. monocytogenes* serotype 4b L10521 was shown to be weak to moderate when compared to the expression of IspC (positive control) detected by anti-Isp MAb M2799 (Figure 20).

Figure 17. SDS-PAGE and Western blotting analysis of purified rInlC2 protein. Proteins were resolved by SDS-PAGE and analyzed with CMB and WB using anti-HIS MAb at a 1:1000 dilution **A.** WB analysis: Lane 1, uninduced rInlC2 (BL21) protein; lane 2, IPTG induced rInlC2 (BL21) soluble protein; lane 3, IPTG induced rInlC2 (BL21) insoluble protein and lane 4, purified and concentrated rInlC2 soluble protein following Ni-NTA purification. **B**. CMB of total protein in eluted fractions (F1-F11) from purification by Ni-NTA affinity chromatography. **C**. WB of eluted fractions (F1-F11) following purification. Molecular masses of protein standards (in kDa) are shown by the numbers on the left in (A) and on the right in (B) and (C). Black arrows indicate the rInlC2 protein band (70 kDa).

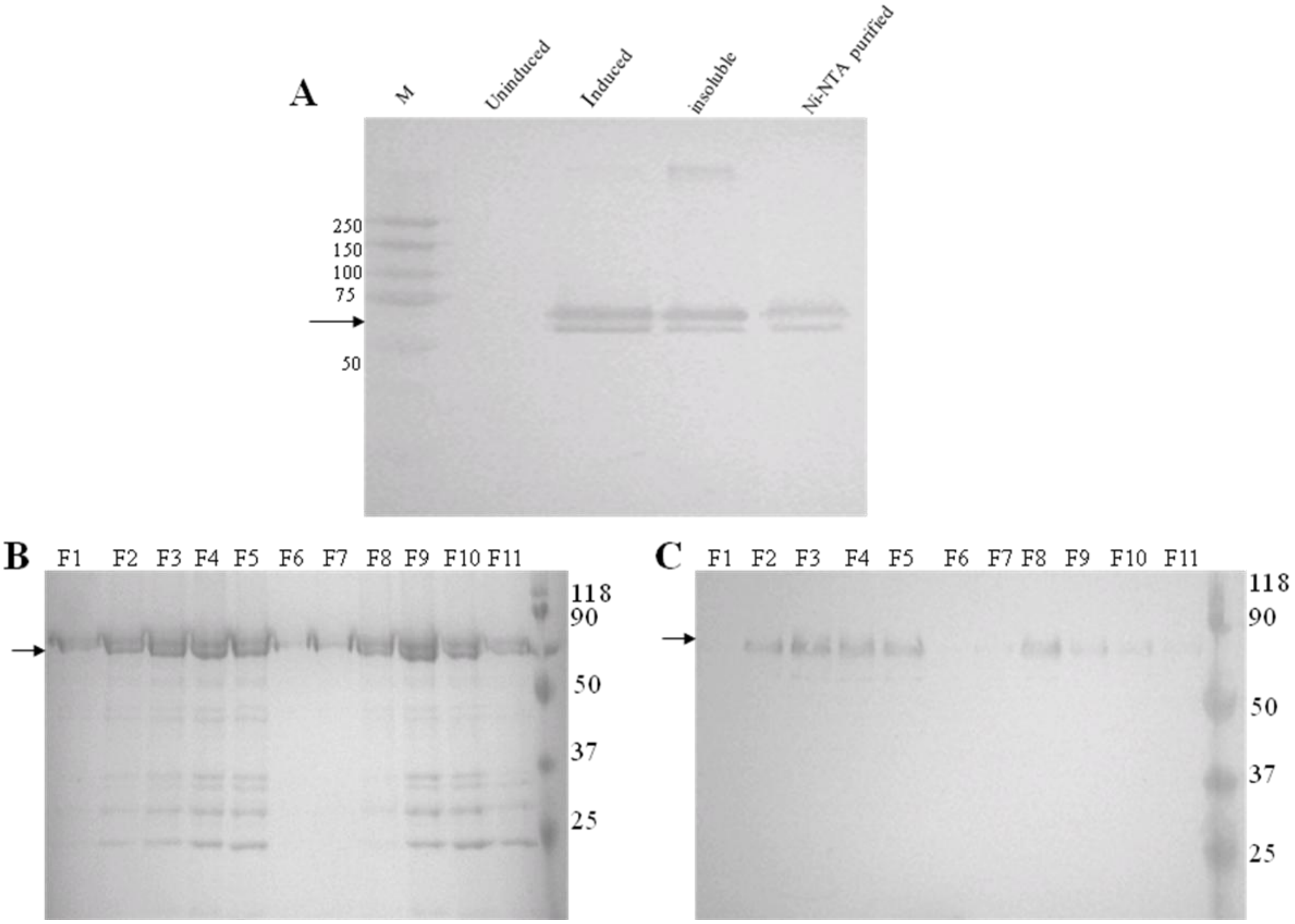

A
M
Uninduced
Induced
insoluble
Ni-NTA purified
250
150
100
75
50
B
F1
F2
F3
F4
F5
F6
F7
F8
F9
F10
F11
118
90
50
37
25
C
F1
F2
F3
F4
F5
F6
F7
F8
F9
F10
F11
118
90
50
37
25

Figure 18. Determining the expression level of LapB in *L. monocytogenes* serotype 4b using rabbit PAb. *L. monocytogenes* serotype 4b incubated with (A) pre-immune serum and (B) anti-serum from day 28 test bleed of the rabbit immunized with rLapB protein. (C) *L. monocytogenes* serotype 4b incubated with purified IspC MAb M2773 which was used as a positive control. A 1:500 dilution of rabbit anti-serum was used followed by a 1:50 dilution of Dylight 488 goat anti-rabbit conjugate. Phase contrast images are shown in the panels on the left and fluorescence images are shown in the panels on the right. Fluorescent images represent cells exposed to UV light for 5 seconds.

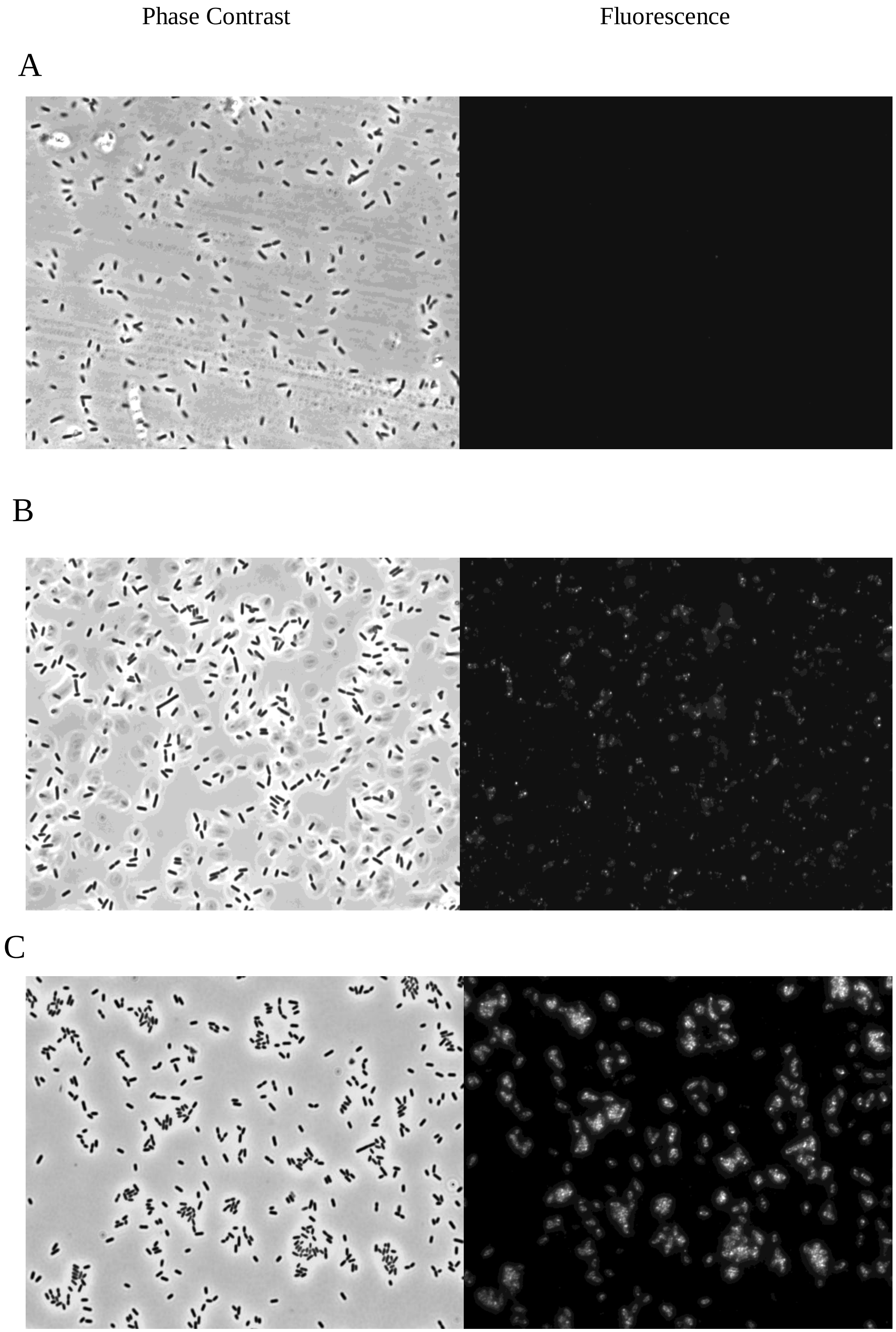

Phase Contrast
Fluorescence
A
B
C

Figure 19. Expression of native LapB in *L. monocytogenes* total cell extract. *L. monocytogenes* serotype 4b total cell extract was resolved by SDS-PAGE (20µl loaded) followed by WB analysis using anti-LapB CFW #1 mouse test bleed (day 64) at a 1:500 dilution. The HRP goat anti-mouse secondary antibody was used at a 1:1000 dilution. Arrow indicates target LapB protein band at ~195kDa. Theoretical MW of LapB is 180 kDa. Molecular masses of protein standards (in kDa) are shown by the numbers on the left.

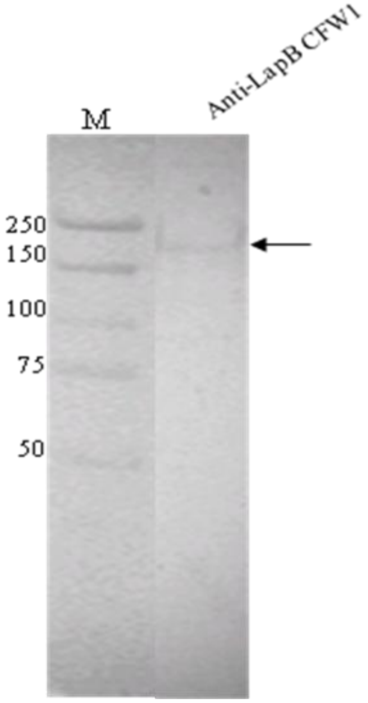
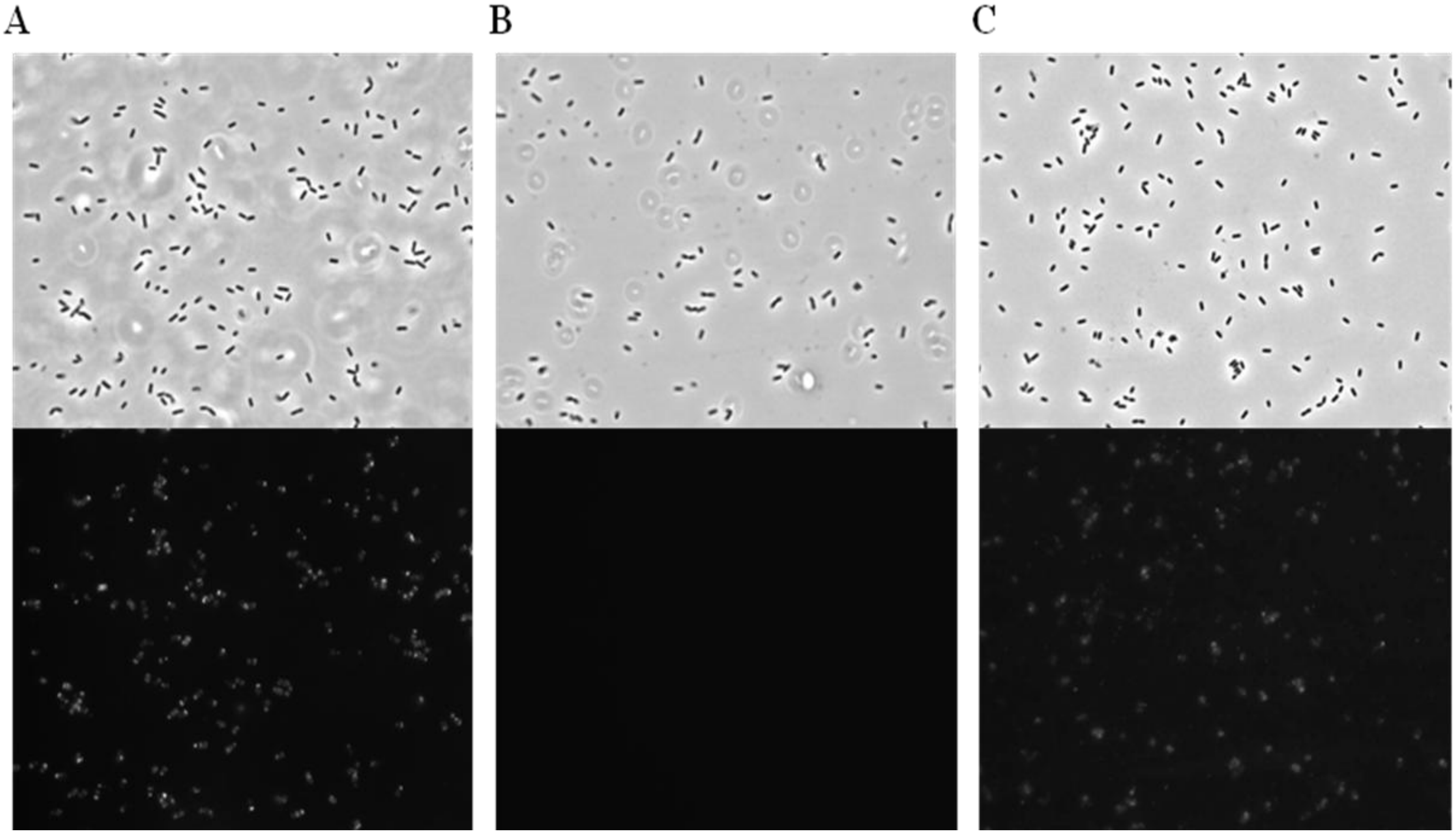

Anti-LapB CFW1
M
250
150
100
75
50

Figure 20. Determining the expression level of InlJ in *L. monocytogenes* serotype 4b using rabbit PAb. *L. monocytogenes* serotype 4b incubated with **(A)** anti-serum from day 28 test bleed of rabbit immunized with rIspC protein **(B)** pre-immune serum and **(C)** anti-serum from day 28 test bleed of rabbit immunized with rInlJ protein. A 1:500 dilution of rabbit anti-serum was used followed by a 1:50 dilution of Dylight 488 goat anti-rabbit conjugate. Phase contrast images are shown in the top panels and fluorescence images are in shown bottom panels. Fluorescent images represent cells exposed to UV light for 5 seconds.


A
B
C

was negative for detecting the expression of InlJ on the cell surface . Furthermore, WB analysis showed that mouse anti-InlJ serum recognized the native InlJ protein from total protein extract of *L. monocytogenes* (Figure 21).

Localization of InlC2 on the surface of *L. monocytogenes* cells was demonstrated by IFM using rabbit anti-InlC2 serum generated in this study. The expression of InlC2 on the surface of *L. monocytogenes* serotype 4b L10521 was shown to be very strong when compared to the expression of IspC (positive control) detected by anti-IspC MAb M2799 (Figure 22). Pre-immune rabbit anti-serum was negative for detecting the expression of InlC2 on the cell surface. Rabbit PAbs were also previously raised by our lab against the rInlC2 protein expressed from *E. coli* BL21(DE3)/pLysS expression host and purified by Ni-NTA agarose affinity chromatography. IFM of *L. monocytogenes* cells using the previously generated anti-InlC2 PAbs was the first work to suggest InlC2 was expressed on the surface of *L. monocytogenes*. However, further testing revealed a cross-reaction of the anti-InlC2 PAb to IspC, another *L. monocytogenes* surface protein (87) that our lab was working on at the time (Figure 23A). InlC2 and IspC are proteins of 70 kDa and 86 kDa, respectively. It was believed that the purified rInlC2 preparation previously used for immunizing rabbits to produce PAbs was contaminated with the rIspC protein due to the use of the same Ni-NTA agarose column during preparation and therefore the animals produced antibodies to IspC as well. For this reason, InlC2 PAbs were generated using the newly purified rInlC2 protein for this study. WB analysis confirmed that the new anti-InlC2 PAbs produced for this study did not cross-react with the rIspC protein (Figure 23B).WB analysis also showed that the new InlC2 PAbs did not detect the native InlC2 protein in total protein extract of *L. monocytogenes* (Figure 24). This could be explained by conformational changes in epitopes on the native InlC2 protein following denatured conditions thereby preventing the PAb from binding.

Figure 21. Expression of native InlJ in *L. monocytogenes* total cell extract. *L. monocytogenes* serotype 4b total cell extract was resolved by SDS-PAGE (20µl loaded) followed by WB analysis using anti-InlJ CFW #3 and Balb/C #1 mouse test bleed (day 64) at a 1:500 dilution. The HRP goat anti-mouse secondary antibody was at a 1:1000 dilution. Theoretical MW of InlJ is 110 kDa. Molecular masses of protein standards (in kDa) are shown by the numbers on the left. Black arrow indicates the native InlJ protein band at 130 kDa.

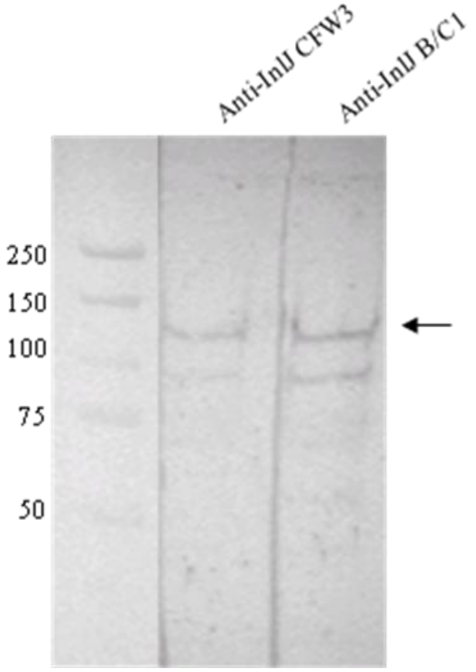

Anti-InlJ CFW3
Anti-InlJ B/C1
250
150
100
75
50

Figure 22. Determining the expression level of InlC2 on the surface of *L. monocytogenes* with IFM. *L. monocytogenes* 4b L10521 grown in BHI broth incubated with pre-immune **(A)** and day 28 test bleed **(B)** of rabbits immunized with rInlC2 protein for the production of PAbs. Phase contrast images are shown in the panels on the left and fluorescence images are in shown in the panels on the right. Fluorescent images represent cells exposed to UV light for 5 seconds.

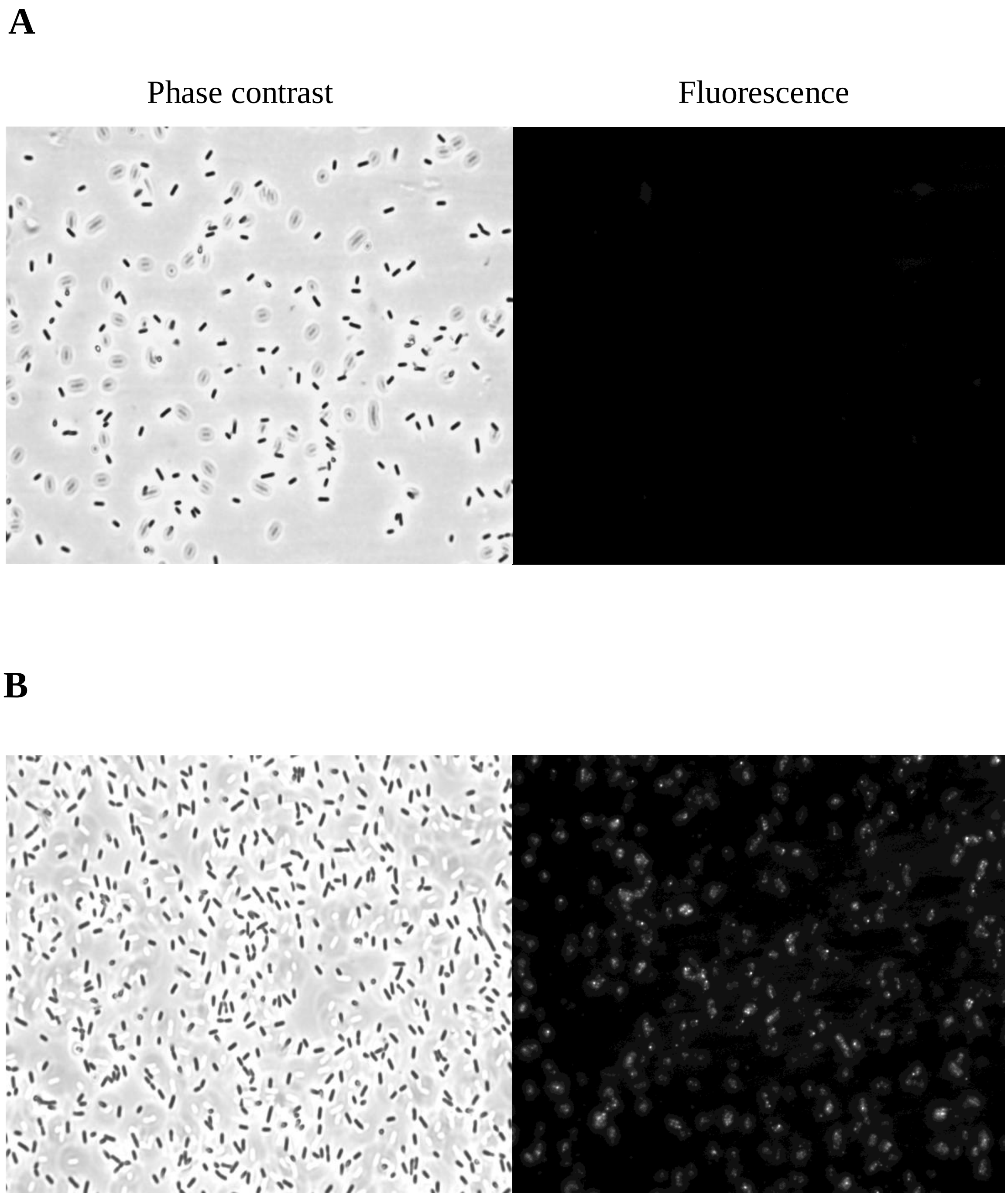

A
Phase contrast
Fluorescence
B

Figure 23. Cross-reactivity of a previously generated anti-InlC2 PAb with another surface protein, IspC. Purified rIspC and rInlC2 proteins, as well as total protein extract of *L. monocytogenes* serotype 4b strain L10521 were resolved by SDS-PAGE followed by WB analysis. **A.** Lane 1, Precision plus protein marker; lane 2, rIspC probed with anti-IspC MAb M2799; lane 3, rIspC probed with the old anti-InlC2 rabbit PAb previously generated in our lab; lane 4, rInlC2 probed with anti-IspC MAb 2799. **B. Verifying no cross-reactivity of newly generated InlC2 PAb with IspC.** Purified rInlC2 and rIspC proteins were resolved by SDS-PAGE (2 µg loaded) followed by WB analysis. rInlC2 and rIspC proteins were both immunoblotted with anti-serum (day 28 test bleed) of rabbit immunized with newly purified rInlC2 protein generated in this study. The rIspC protein was also immunoblotted with anti-IspC MAb M2799. Black arrow indicates rInlC2 protein band (70 kDa) and the red arrow indicates the rIspC protein band (86 kDa).

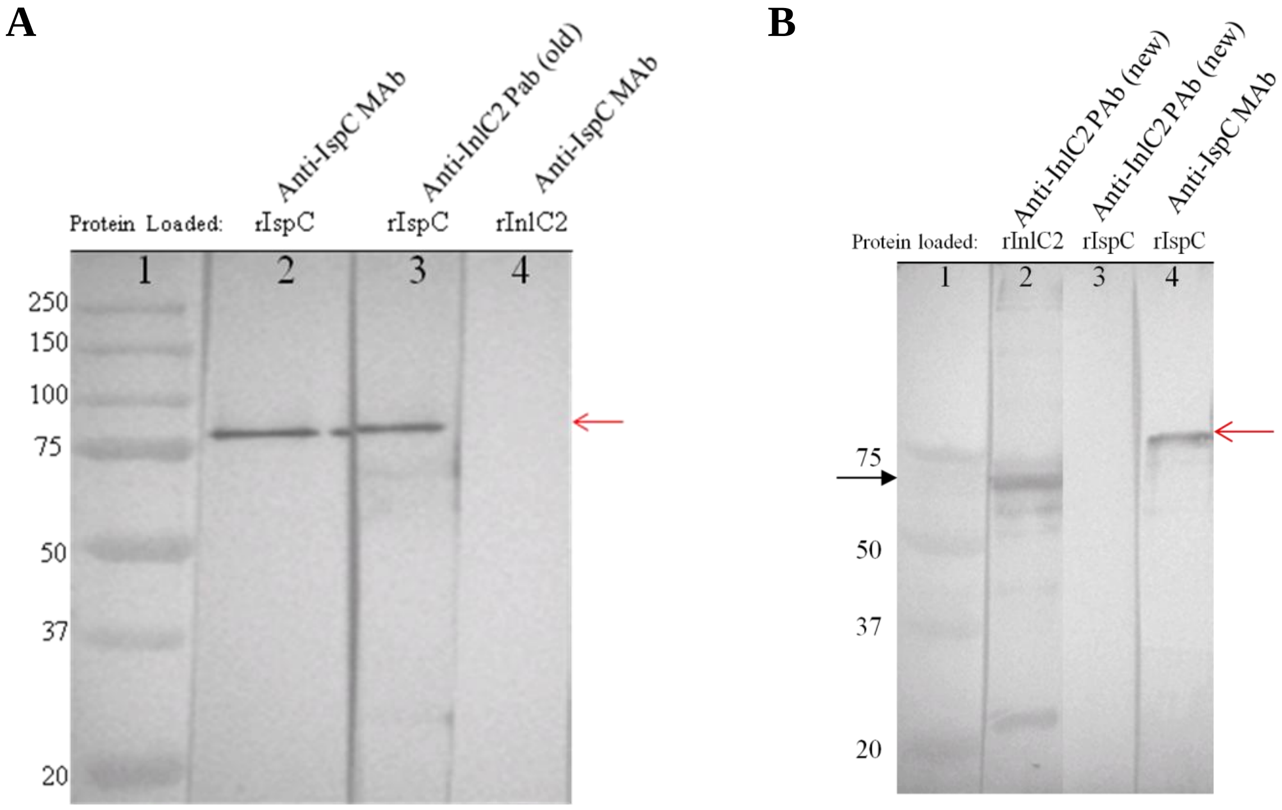
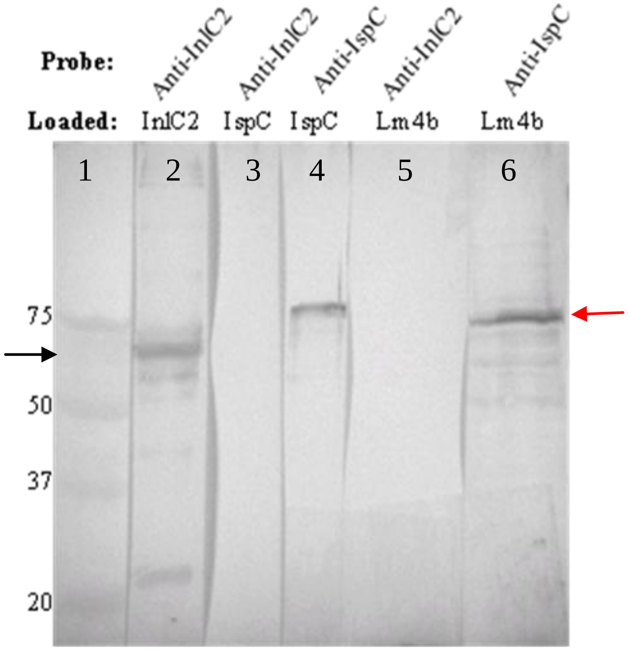

A
Anti-IspC MAb
Anti-InlC2 Pab (old)
Anti-IspC MAb
Protein Loaded:
rIspC
rIspC
rInlC2
1
2
3
4
250
150
100
75
50
37
20
B
Anti-InlC2 PAb (new)
Anti-InlC2 PAb (new)
Anti-IspC MAb
Protein loaded:
rInlC2
rIspC
rIspC
1
2
3
4
75
50
37
20

Figure 24. Native InlC2 protein is not expressed in *L. monocytogenes* total cell extract under denatured conditions. Recombinant proteins and total cell extract of *L. monocytogenes* serotype 4b was resolved by SDS-PAGE followed by WB analysis using anti-InlC2 (new) or anti-IspC antibodies. Lane 1, protein standard marker; lane 2, purified rInlC2 protein (2 µg) immunoblotted with anti-InlC2 rabbit PAb; lane 3, purified rIspC protein (2 µg) immunoblotted with anti-InlC2 rabbit PAb; lane 4, purified rIspC protein (2 µg) immunoblotted with anti-IspC MAb 2773; lane 5, *L. monocytogenes* total cell extract (20 µl) immunoblotted with anti-InlC2 rabbit PAb; lane 6, *L. monocytogenes* total cell extract immunoblotted with anti-IspC MAb 2773. Black arrow indicates the rInlC2 protein (70 kDa). Red arrow indicates rIspC and native IspC protein bands (86 kDa).


Probe:
Anti-InlC2
Anti-InlC2
Anti-IspC
Anti-InlC2
Anti-IspC
Loaded:
InlC2
IspC
IspC
Lm4b
Lm4b
1
2
3
4
5
6
75
50
37
20

CHAPTER IV

RESULTS

PART II:

DEVELOPMENT AND CHARACTERIZATION OF MABS TO LAPB

PART II: DEVELOPMENT AND CHARACTERIZATION OF MABS TO LAPB

4.6 Screening and isotyping of hybridoma clones

Sera from all six mice (day 64 test bleed) receiving rLapB were examined with WB using rLapB protein. All six mice showed comparable antibody response to the antigen. However, CFW mouse #1 and Balb/C mouse #3 showed a slightly stronger antibody reaction to the rLapB on immunoblots and were therefore selected for the fusions. Hybridomas were created using a standard CFIA hybridoma fusion protocol (SOP # MC-PR005.02). Two fusions were carried out using the spleen cells from two selected mice. Hybridoma tissue culture fluid was screened for reactivity with purified recombinant LapB protein and positives were selected for screening against whole cell *L. monocytogenes* (one strain of 4b, 1/2a and 1/2c serotypes) by ELISA. Based on these results, positive hybridomas were selected for cloning and expansion. Of approximately 2000 parent hybridomas, 48 were selected for 2 or 3x cloning and isotyping to ensure they were stable and producing only one subtype of antibody. The following isotypes were accepted: IgG1, IgG2a and IgG2b. IgGMs bind to multiple epitopes and typically are more cross-reactive than IgGs and were therefore selected against. Of the 48 MAbs, 41 belonged to the Ig subclass G1 (IgG1) and 7 were IgG2a (Table 4).

4.6 Reactivity of anti-LapB MAbs with rLapB protein by ELISA

All 48 anti-LapB MAbs were tested for their reactivity with rLapB protein by ELISA. All MAbs reacted strongly to rLapB as ELISA values ranged from 1.0 to 4.0 (Figure 25A).

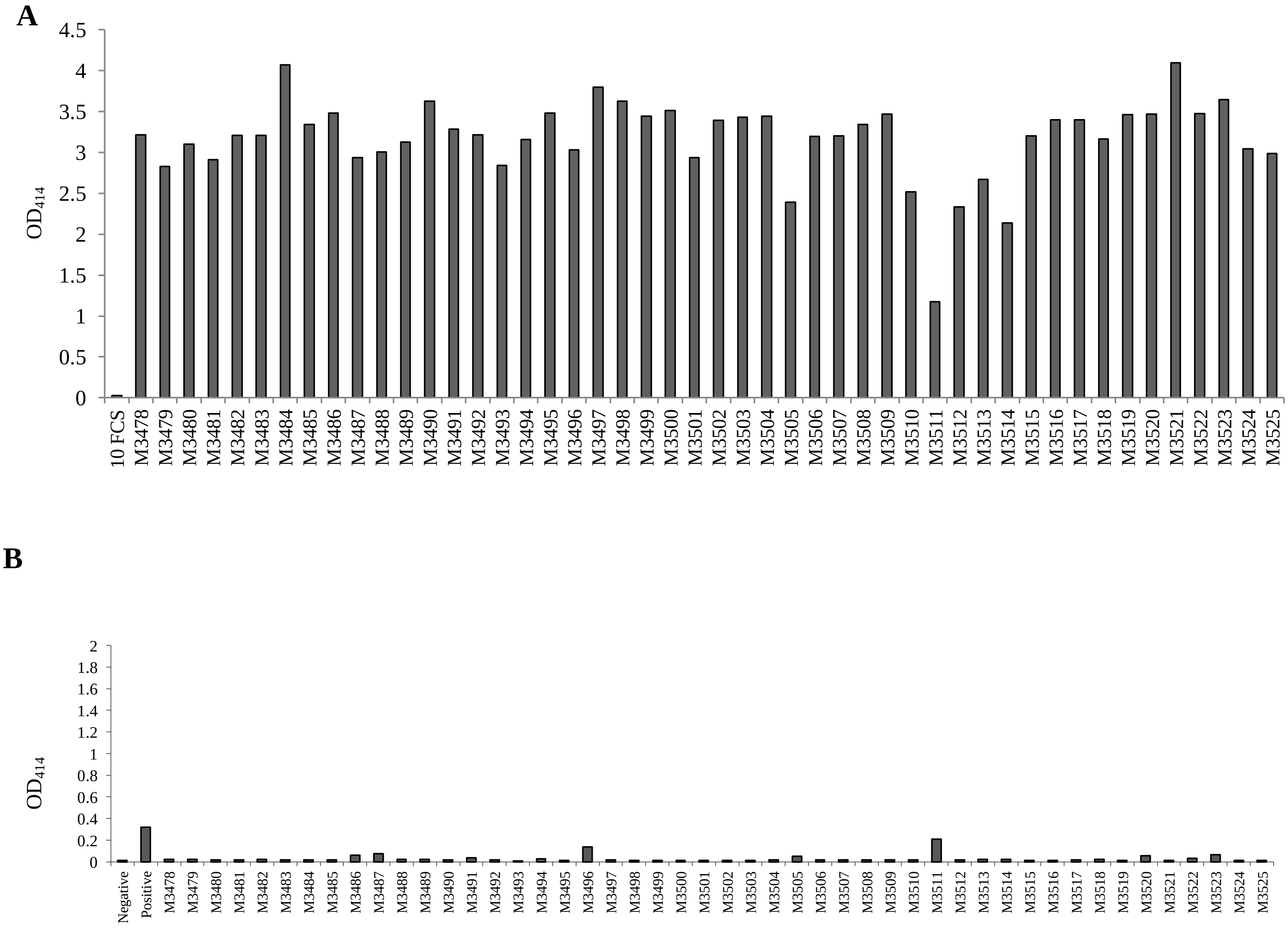
4.7 Testing anti-LapB MAbs for cross-reactivity with *E. coli* proteins

The rLapB protein was expressed in and purified from an *E. coli* host cell. Analysis of the purified protein by SDS-PAGE revealed there was minor contamination with *E. coli* proteins in the rLapB preparation. To ensure that the anti-LapB MAbs did not react with *E. coli* proteins, all 48 anti-LapB MAbs produced were tested for reactivity against soluble proteins of *E. coli* BL21(DE3)/pLysS (with no vector) by ELISA (Figure 25B). No reactivity with *E. coli* proteins was observed with any of the MAbs.

Table 4. List of anti-LapB MAbs with their corresponding hybridoma cell line and final immunoglobulin subclass. Immunoglobulin subclass types were determined by isoptying hybridoma tissue culture fluid.

Anti-LapB MAbs	**Parent**	**Isotype**
M3478	32H3	IgG1
M3479	5C10	IgG1
M3480	31A7	IgG1
M3481	32A5	IgG1
M3482	16F9	IgG1
M3483	11H4	IgG1
M3484	12C11	IgG1
M3485	19A3	IgG1
M3486	25D11	IgG1
M3487	26A12	IgG1
M3488	27C4	IgG1
M3489	28A10	IgG1
M3490	28B5	IgG1
M3491	29B8	IgG1
M3492	30B9	IgG2a
M3493	31A3	IgG1
M3494	31C2	IgG1
M3495	31F2	IgG1
M3496	10F6	IgG2a
M3497	11G9	IgG1
M3498	17G10	IgG1
M3499	20C11	IgG1
M3500	26A1	IgG1
M3501	32B12	IgG1
M3502	31D12	IgG1
M3503	31F10	IgG1
M3504	32D10	IgG1
M3505	32E8	IgG1
M3506	25B8	IgG1
M3507	25E2	IgG1
M3508	26B3	IgG1
M3509	28C2	IgG1
M3510	32A8	IgG1
M3511	32F1	IgG1
M3512	2H2	IgG2a
M3513	16E6	IgG2a
M3514	18F9	IgG1
M3515	6F1	IgG1
M3516	26B7	IgG1
M3517	27C10	IgG1
M3518	29E4	IgG1
M3519	28D4	IgG1
M3520	28F10	IgG2a
M3521	28G4	IgG1
M3522	28G5	IgG2a
M3523	25G6	IgG2a
M3524	25G6	IgG1
M3525	31E12	IgG1

Figure 25. Reactivity of LapB MAbs with rLapB protein and *E. coli* proteins. A. All 48 anti-LapB MAbs (M3578 to M3525) reacted strongly with rLapB protein (1.0 ug/ml) as revealed by ELISA. **B.** All 48 anti-LapB MAbs were tested for cross-reactivity with *E. coli* proteins by ELISA. Soluble proteins of *E. coli* BL21(DE3)/pLysS (with no vector) was extracted following the same procedure as purification of rLapB protein from *E. coli*. Positive and negative controls were CFW mouse anti-LapB serum and 10% fetal calf serum, respectively. ELISA OD_{414} values are an average of duplicate determinations for each MAb.


A
OD414
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
10 FCS
M3478
M3479
M3480
M3481
M3482
M3483
M3484
M3485
M3486
M3487
M3488
M3489
M3490
M3491
M3492
M3493
M3494
M3495
M3496
M3497
M3498
M3499
M3500
M3501
M3502
M3503
M3504
M3505
M3506
M3507
M3508
M3509
M3510
M3511
M3512
M3513
M3514
M3515
M3516
M3517
M3518
M3519
M3520
M3521
M3522
M3523
M3524
M3525
B
OD414
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Negative
Positive
M3478
M3479
M3480
M3481
M3482
M3483
M3484
M3485
M3486
M3487
M3488
M3489
M3490
M3491
M3492
M3493
M3494
M3495
M3496
M3497
M3498
M3499
M3500
M3501
M3502
M3503
M3504
M3505
M3506
M3507
M3508
M3509
M3510
M3511
M3512
M3513
M3514
M3515
M3516
M3517
M3518
M3519
M3520
M3521
M3522
M3523
M3524
M3525

4.9 Binding of anti-LapB MAbs to the surface of *L. monocytogenes* serotype 4b

The 48 anti-LapB MAbs produced were tested again using IFM and ELISA to determine if each reacted with the native LapB protein on *L. monocytogenes* cells. A total of 11 MAbs were positive for ELISA (Figure 26) experiments with *L. monocytogenes* serotype 4b L10521 cells. Of these, four MAbs (M3482, M3493, M3498 and M3499) had negative to weak immunofluorescence signal and seven MAbs (M3484, M3495, M3500, M3509, M3517, M3519 and M3524) had positive immunofluorescence signals indicating good binding to the native LapB on the cell surface (Figure 27). Based on the IFM result alone, M3484, M3500, M3517 and M3519 showed the strongest immunofluorescence signal and M3524 showed the weakest. These seven MAbs were selected for further testing against various strains of *L. monocytogenes*.

4.10 Recognition of the rLapB protein by selected anti-LapB MAbs

The seven LapB MAbs selected for further characterization by IFM as well as the four MAbs that only had weak immunofluorescence signal were tested against the rLapB protein by WB (Figure 28). M3493 and M3498 were the only two MAbs that weakly recognized the rLapB on WB. All seven anti-LapB MAbs (M3484, M3495, M3500, M3509, M3517, M3519 and M3524) selected for further characterization strongly recognized the rLapB protein on WB. The multiple bands detected by anti-LapB MAbs indicate a degradation of the rLapB protein.

4.11 Reactivity of anti-LapB MAbs to various strains of *L. monocytogenes* by ELISA

ELISA results of the seven anti-LapB MAbs were analyzed and given a score (- to +++) based on their ELISA values obtained from the average of three separate experiments (Table 5). Overall, six of the seven anti-LapB MAbs (M3484, M3495, M3500, M3509, M3517, and M3519) recognized 44 of 51 strains which represent 9 of the 11 *L. monocytogenes* serotypes tested. M3524 had a weaker reactivity against the majority of strains and only recognized 19 of 51 strains. This result is not entirely

Figure 26. Reactivity of anti-LapB MAbs with *L. monocytogenes* serotype 4b cells. Reactivity of 48 anti-LapB MAbs (M3478 to M3525) with *L. monocytogenes* serotype 4b L10521 cells grown in BHI broth was tested by ELISA. *L. monocytogenes* cells were coated at a concentration of 5 x 10^8 cells/ml. Positive and negative controls were anti-IspC MAb M2799 and 10% fetal calf serum, respectively. ELISA OD_{414} values are an average of duplicate determinations for each MAb.

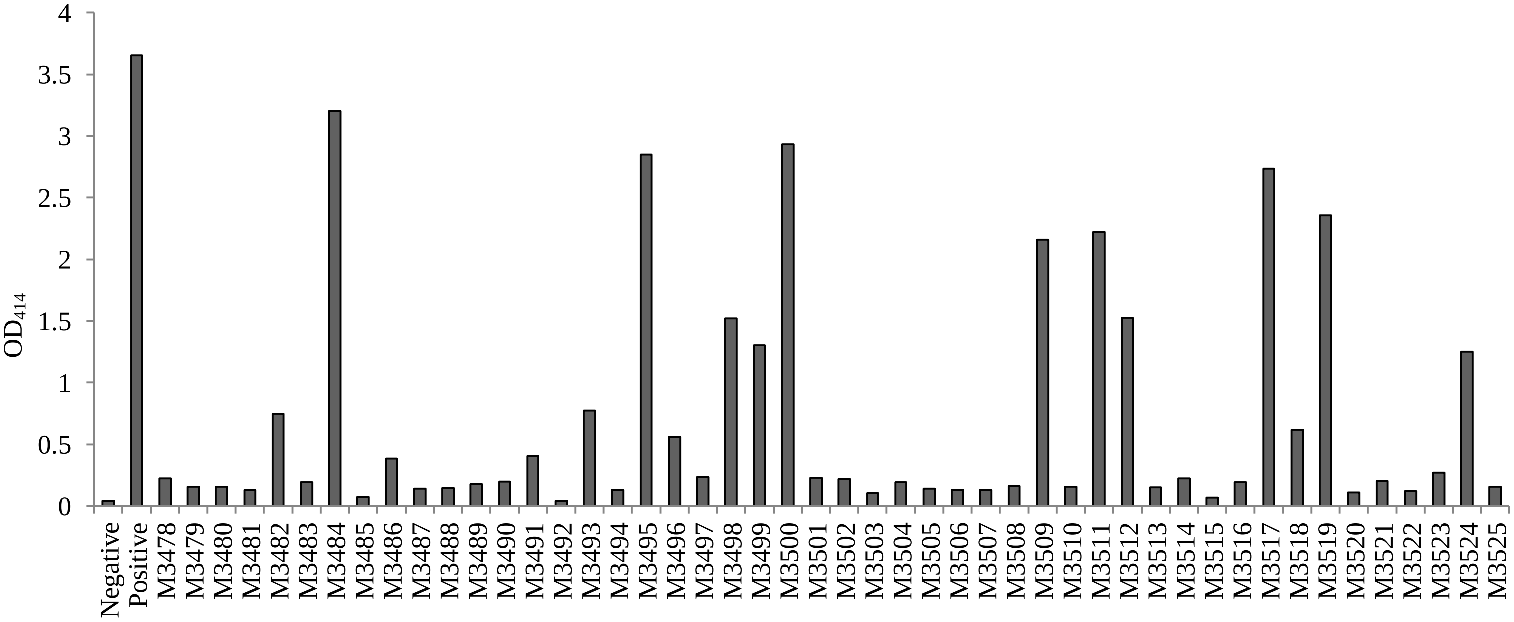
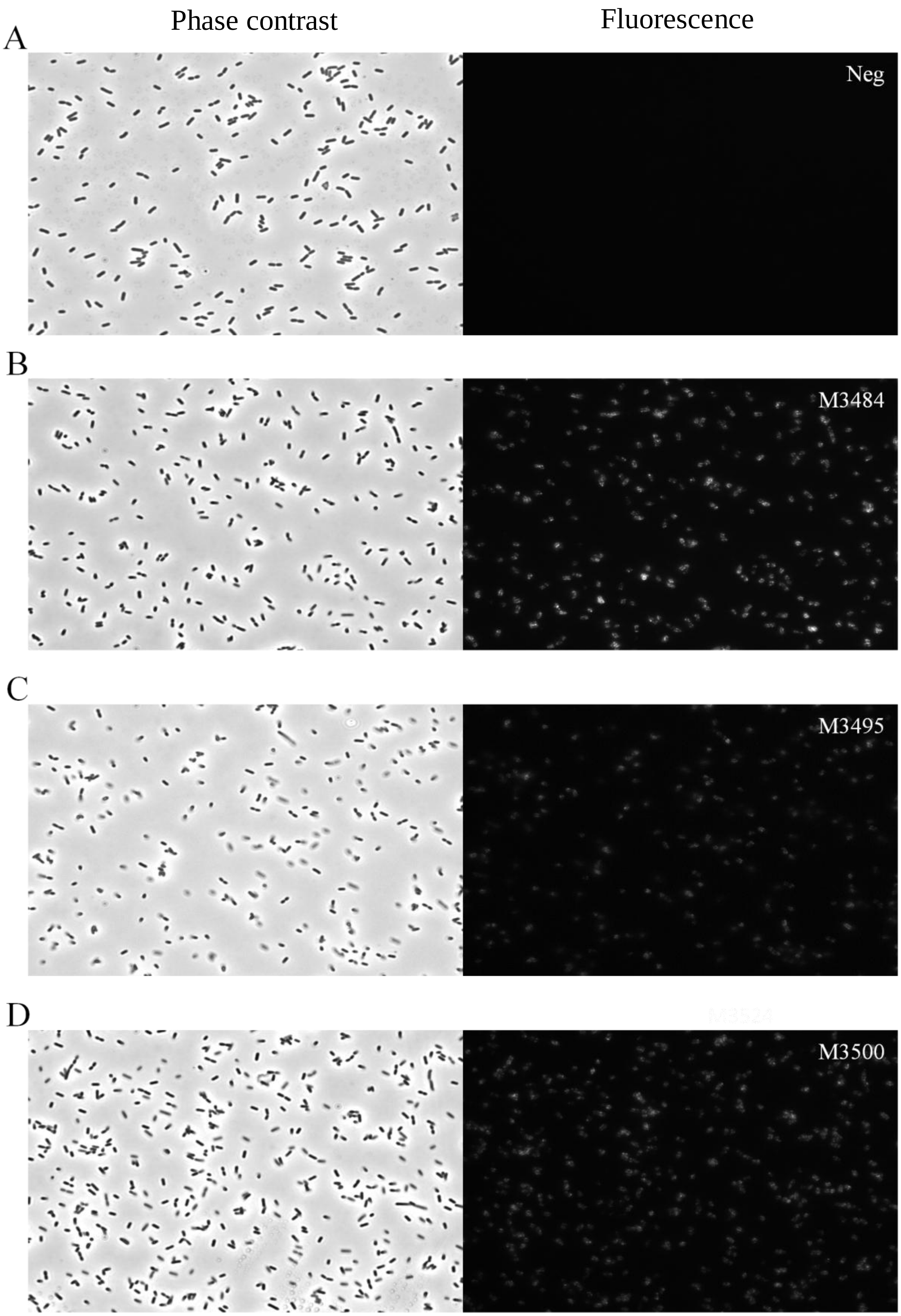
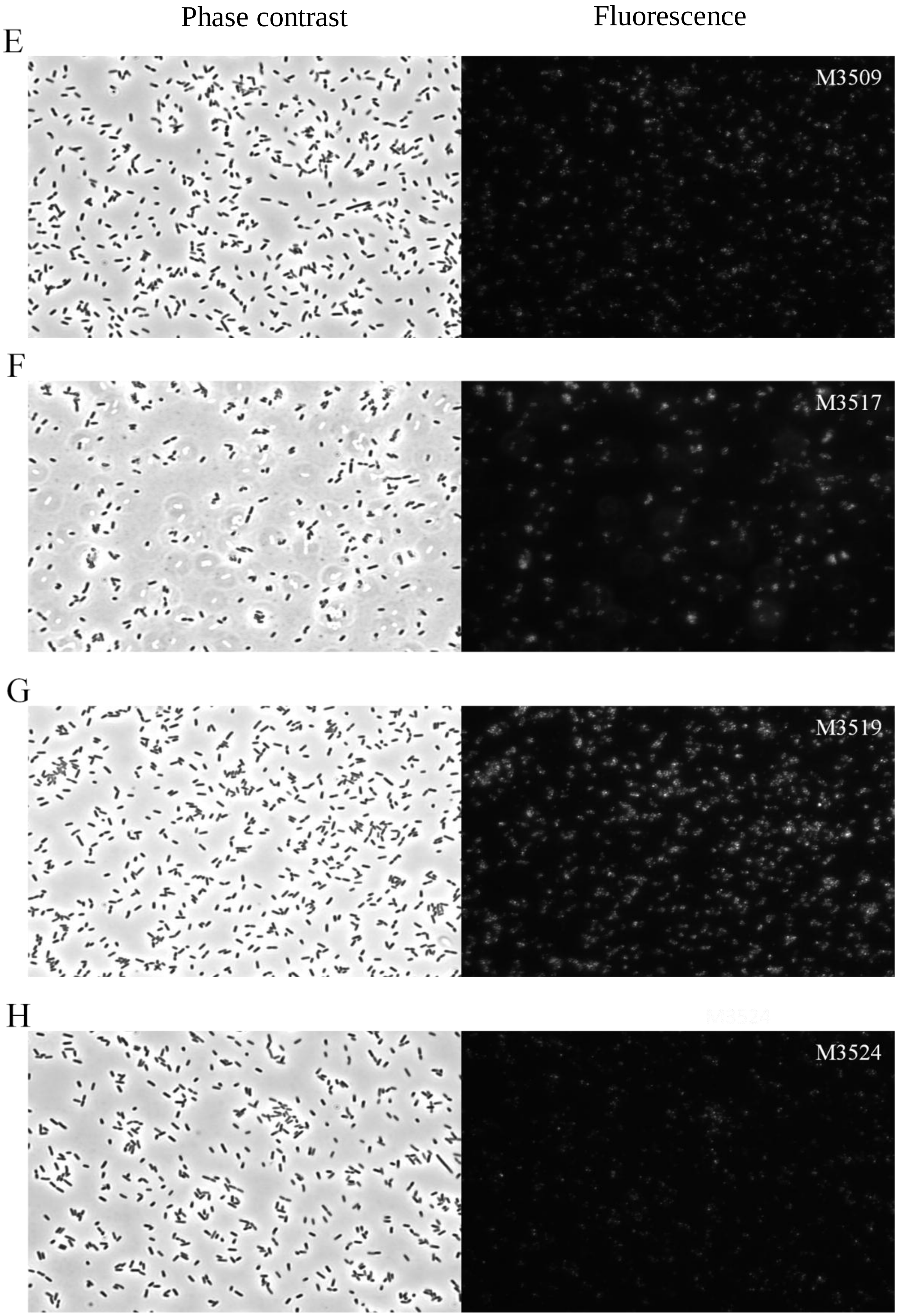

4
3.5
3
2.5
2
1.5
1
0.5
0
OD$_{414}$
Negative
Positive
M3478
M3479
M3480
M3481
M3482
M3483
M3484
M3485
M3486
M3487
M3488
M3489
M3490
M3491
M3492
M3493
M3494
M3495
M3496
M3497
M3498
M3499
M3500
M3501
M3502
M3503
M3504
M3505
M3506
M3507
M3508
M3509
M3510
M3511
M3512
M3513
M3514
M3515
M3516
M3517
M3518
M3519
M3520
M3521
M3522
M3523
M3524
M3525

Figure 27. Binding of anti-LapB MAbs to the surface of *L. monocytogenes* serotype 4b analyzed by IFM. *L. monocytogenes* was probed with pre-immune serum (1:50 dilution) of CFW mouse #1 (A), M3484 (B), M3495 (C), M3500 (D), M3509 (E), M3517 (F), M3519 (G), M3524 (H), M3482 (I), M3493 (J), M3498 (K), M3499 (L). Phase contrast images are shown in the panels on the left and fluorescent images are in shown in the panels on the right. Fluorescent images were taken with an exposure time of 5 seconds under UV light.


Phase contrast
Fluorescence
A
Neg
B
M3484
C
M3495
D
M3500


Phase contrast
Fluorescence
E
M3509
F
M3517
G
M3519
H
M3524

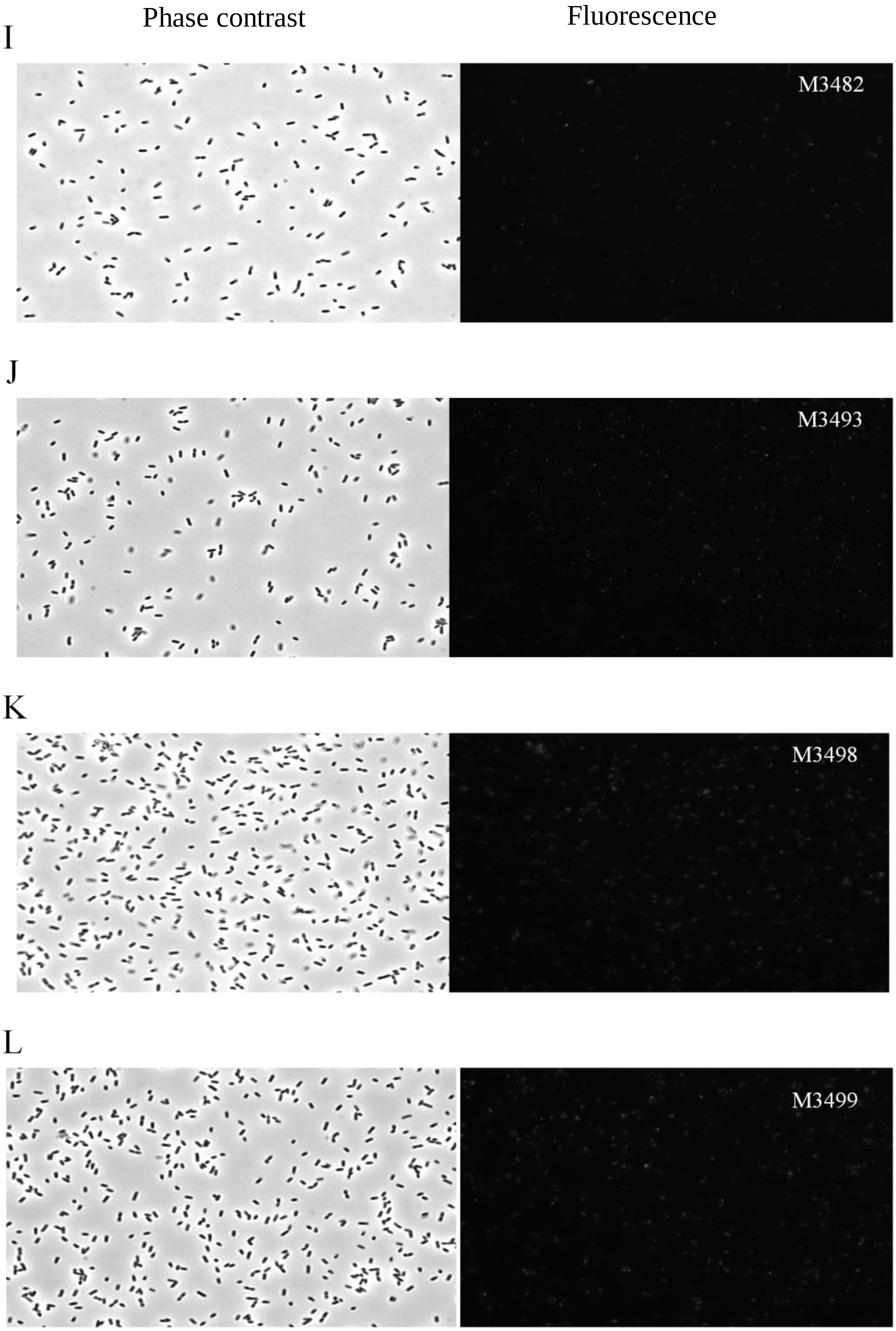

Phase contrast
Fluorescence
I
M3482
J
M3493
K
M3498
L
M3499

Figure 28. Recognition of the rLapB protein by selected anti-LapB MAbs. The rLapB protein was resolved by SDS-PAGE followed by WB analysis using pre-immune serum of CFW mouse #1for negative control, anti-HIS MAb at a 1:1000 dilution for positive control and 11 anti-LapB MAbs tissue culture fluid at a 1:25 dilution. Black arrow indicates the rLapB protein band.

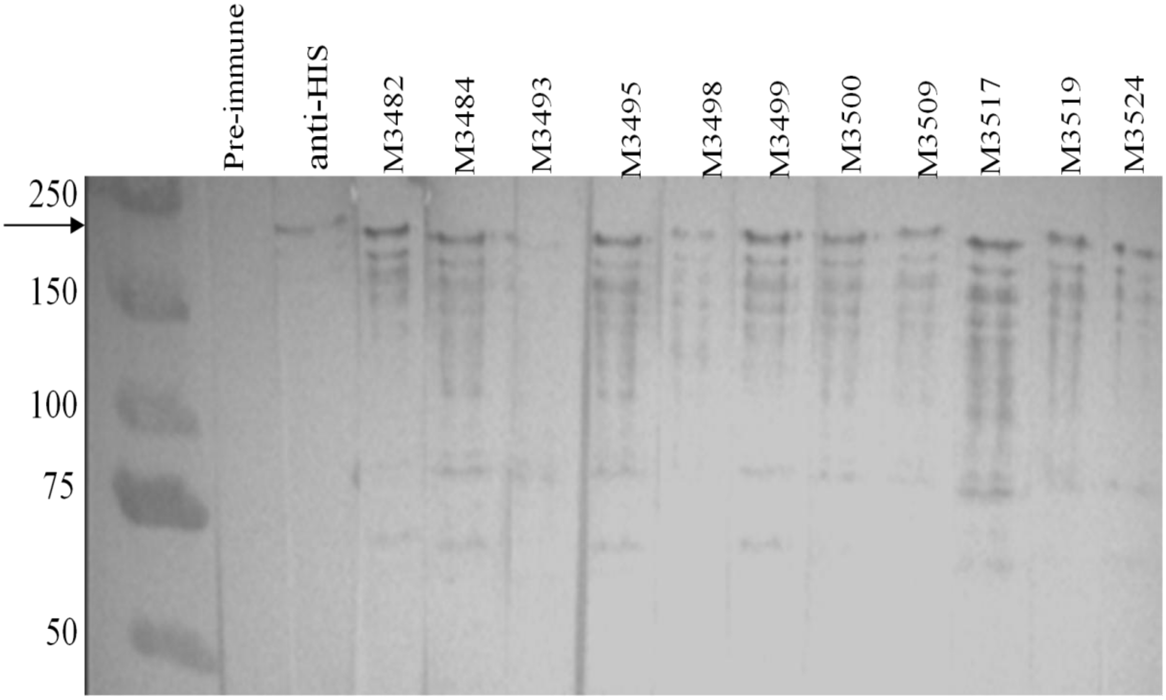

Pre-immune
anti-HIS
M3482
M3484
M3493
M3495
M3498
M3499
M3500
M3509
M3517
M3519
M3524
250
150
100
75
50

Table 5. Reactivity of anti-LapB MAbs to various strains of *Listeria* and other foodborne microorganisms. Reactivity scores (- to +++) were based on ELISA OD_{414} values: -, 0-0.2; -/+, 0.2-0.3; +, 0.3-1.0; ++, 1.0-2.0; +++, 2.0-3.0. All MAbs are IgG1 subclass types. Fetal calf serum (10%) was used as a negative control for each strain and ELISA values were within the range of 0-0.25. All ELISA values represent the average of three separate experiments. Each MAb was tested in duplicate wells in an individual experiment.

Serotype[a] or spp.	**Strain**	**Origin**	**M3484**	**M3495**	**M3500**	**M3509**	**M3517**	**M3519**	**M3524**
1/2a	**4705**		++	++	++	++	++	++	-
1/2a	**5327**		++	++	++	++	++	++	-
1/2a	**6036**	Food	++	++	++	++	++	++	-
1/2a	**6095**	Food	+	+	+	+	+	+	-
1/2a	**L10527**		+	+	+	+	+	+	-
1/2a	**09049**	Environmental	-/+	-	-	-	-	-	-
1/2a	**09015**	Food	++	+	+	+	++	++	-
1/2a	**09033**	Food	++	++	++	++	++	++	+
1/2a	**09011**	Environmental	++	++	++	++	++	++	-
1/2a	**09016**	Environmental	++	++	++	++	++	++	-
1/2b	**4857**		+++	+++	+++	++	+++	+++	+
1/2b	**5328**	Food	++	++	++	+	++	++	+
1/2b	**5913**	Food	++	++	++	++	++	++	+
1/2b	**5330**		++	++	++	++	++	++	+
1/2b	**6027**	Food	++	++	++	++	++	++	+
1/2b	**L10586**		+	+	+	+	+	+	-
1/2b	**09027**	Environmental	+	+	+	+	+	+	-
1/2b	**09040**	Food	+	+	+	+	+	+	-
1/2b	**09060**	Environmental	+	+	+	+	+	+	-
1/2c	**1869**		++	++	++	++	++	++	-
1/2c	**2792**		++	++	+	+	++	+	-
1/2c	**5121**		++	++	++	+	++	++	-
1/2c	**09013**	Environmental	++	++	+	+	++	+	-
1/2c	**09022**	Food	++	++	++	++	++	++	-
3a	**2768**		++	++	++	+	++	++	-
3a	**3058**		+++	+++	+++	++	+++	++	-
3a	**5665**	Food	+	+	+	+	+	+	-
3a	**L10508**		++	++	++	+	++	++	-
3a	**09005**	Food	++	++	++	++	++	++	-
3a	**09039**	Environmental	++	++	++	+	++	++	-
3b	**1031**		++	++	++	++	++	++	+
3b	**4909**		++	++	++	++	++	++	+
3c	**61**		++	++	++	++	++	++	-
4a	**3501**		-	-	-	-	-	-	-
4a	**5041**		-	-	-	-	-	-	-
4a	**5058**	Animal	++	++	++	++	++	++	+
4b	**L10521[b]**	Clinical	++	++	++	++	++	++	+
4b	**3449**		++	++	++	++	++	++	+
4b	**5251**		++	++	++	++	+++	++	+
4b	**5364**	Animal	++	++	++	++	++	++	+
4b	**5816**	Food	++	++	++	++	++	++	+
4b	**5906**	Food	++	++	++	++	++	++	+
4b	**6024**	Food	++	++	++	++	++	++	+
4b	**6092**	Food	++	++	++	++	++	++	+
4c	**4706**	Animal	-	-	-	-	-	-	-
4c	**4497**		-	-	-	-	-	-	-
4c	**5248**	Animal	-	-	-	-	-	-	-
4d	**18**		+	+	+	+	+	+	-/+
4d	**4534**		+++	+++	+++	++	+++	++	+
4e	**1848**		+	+	+	+	++	+	+
4e	**1861**		-	-	-	-	-	-	-
L. innocua	**583**		-	-	-	-	-	-	-
L. ivanovii	**28**		-	-	-	-	-	-	-
L. grayi	**29**		-	-	-	-	-	-	-
L. seeligeri	**24**		+	-/+	-	-/+	+	-	-
L. welshimeri	**92**		-	-	-	-	-	-	-
E. coli	**O157**		-	-	-	-	-	-	-
E. coli	**25922**		-	-	-	-	-	-	-
S. enterica	**DT104**		-	-	-	-	-	-	-
S. enterica	**Californiaa**		-	-	-	-	-	-	-
C. jejuni	**ADRI 1102**		-	-	-	-	-	-	-

[a] Serotypes of *L. monocytogenes*

[b] The *lapB* gene was amplified from the genomic DNA from this strain

unexpected as M3524 showed the weakest IFM reaction to *L. monocytogenes* serotype 4b strain L10521 compared to the other reactive MAbs by IFM. None of the anti-LapB MAbs reacted with the three 4c strains tested. Of the two 4a strains tested, all the seven MAbs reacted with one 4a strain 5058. Of the seven anti-LapB MAbs, M3484 reacted with the highest number of strains (44 of 51) of *L. monocytogenes* tested. ELISA reactivity of M3484 with strains representing 11 *L. monocytogenes* serotypes tested was summarized in Figure 29. M3484 exhibited weak or no binding to the following strains: 09049 (1/2a); 61 (3c); 3501 (4a); 18 (4d); and 1861 (4e) (ELISA OD_{414} values ranged from 0.048 to 0.227).

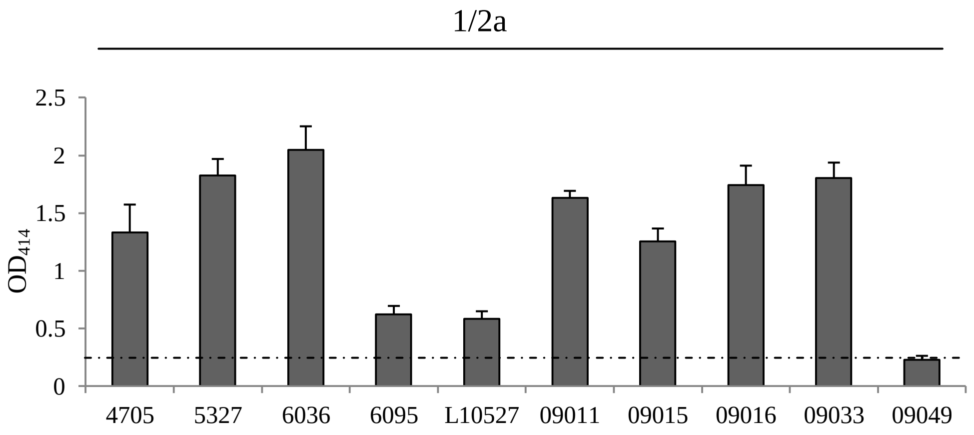
4.12 Cross-reactivity of anti-LapB MAbs to non-pathogenic *Listeria* spp. and other microorganisms

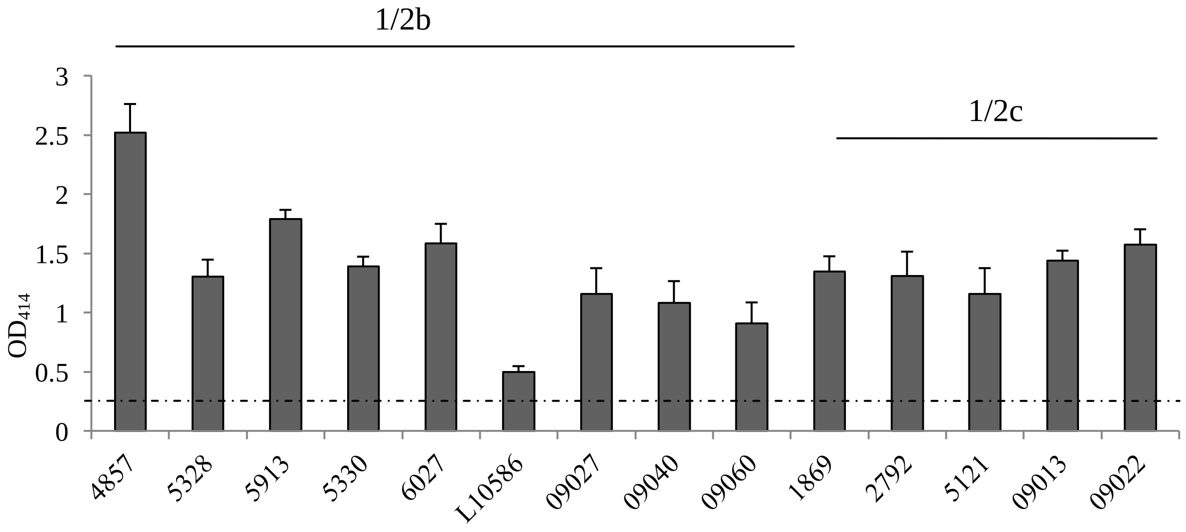
Of the five non-pathogenic *Listeria* strains tested (*L. innocua*, *L. ivanovii*, *L. grayi*, *L. seeligeri* and *L. welshimeri*) four of the seven anti-LapB MAbs (M3484, M3495, M3509 and M3517) had a weak cross-reaction with *L. seeligeri* (Table 5). The ELISA OD_{414} values ranged from 0.227 to 0.320. M3500 and M3519 had negative reactivity with *L. seeligeri* as their ELISA OD_{414} values were 0.177 and 0.197, respectively. None of the seven anti-LapB MAbs reacted with the *Escherichia coli*, *Salmonella enterica* or *Campylobacter jejuni* strains tested (Table 5). Reactivity of M3484 with the non-*L. monocytogenes* strains was tested by ELISA and summarized in Figure 30.

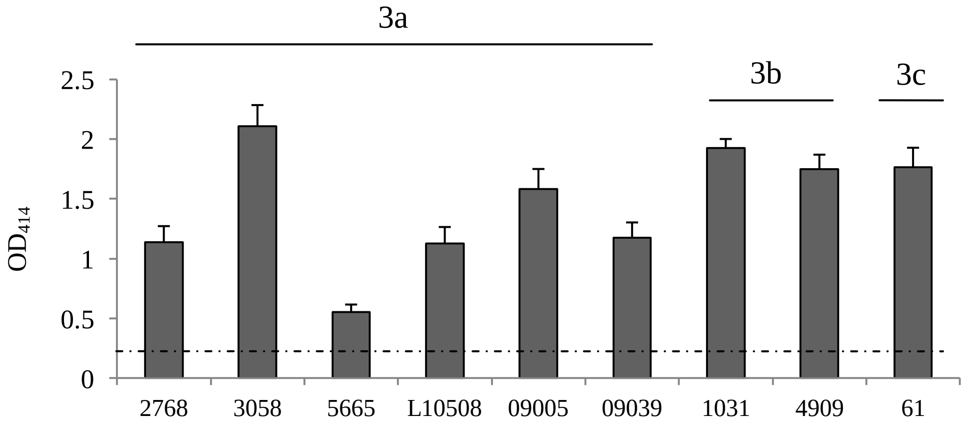
4.13 Conclusion

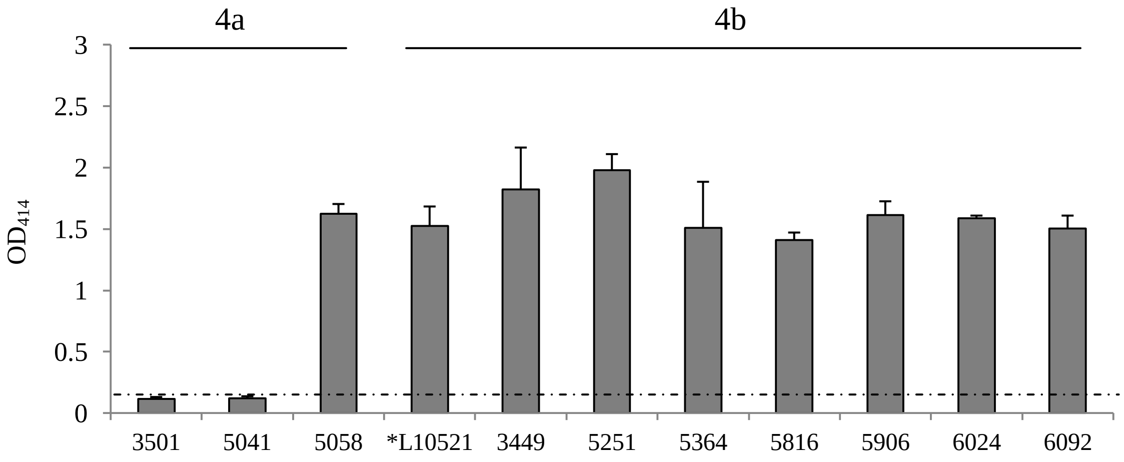
In summary, six anti-LapB MAbs demonstrated strong binding to the majority of *L. monocytogenes* strains tested with the exception of the strains from the two serotypes (4a and 4c) which are predicted to not carry the *lapB* gene. The results indicate that M3484 could be a promising MAb to explore further as a diagnostic reagent for the detection of the majority of *L. monocytogenes* strains (particularly 1/2a, 1/2b, and 4b) without cross-reacting to non-pathogenic *Listeria* strains or other microorganisms commonly found in foods.

Figure 29. Reactivity of anti-LapB MAb M3484 with 51 strains of 11 *L. monocytogenes* serotypes. Strains from 11 of the 12 *L. monocytogenes* serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, and 4e) were tested for reactivity to M3484 (tissue culture fluid) by ELISA. Bacterial cells were prepared as described in materials and methods. ELISA values represent an average of the data from three separate experiments (n=3). The MAb was tested in duplicate wells for each experiment. Dotted line indicates the cut-off for a negative reaction (OD_{414} of 0.200).


1/2a
OD414
2.5
2
1.5
1
0.5
0
4705
5327
6036
6095
L10527
09011
09015
09016
09033
09049


1/2b
1/2c
OD414
3
2.5
2
1.5
1
0.5
0
4857
5328
5913
5330
6027
L10586
09027
09040
09060
1869
2792
5121
09013
09022


3a
3b
3c
OD414
2.5
2
1.5
1
0.5
0
2768
3058
5665
L10508
09005
09039
1031
4909
61


4a
4b
3
2.5
2
1.5
1
0.5
0
OD$_{414}$
3501
5041
5058
*L10521
3449
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5816
5906
6024
6092

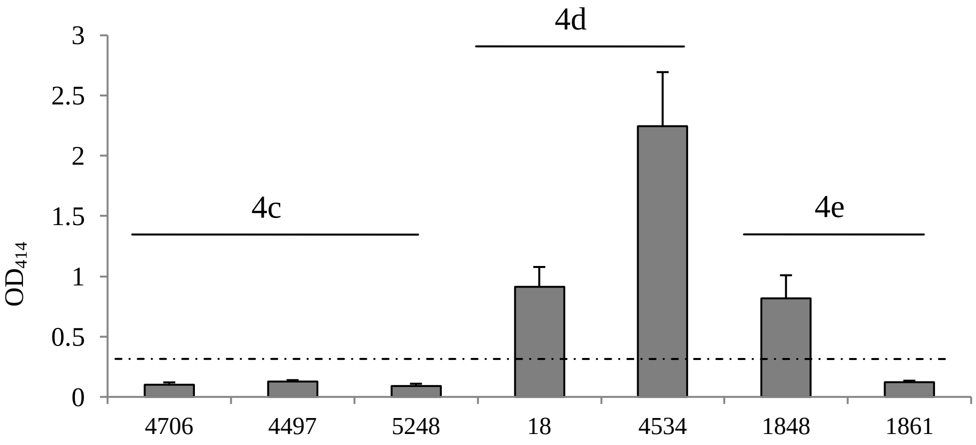
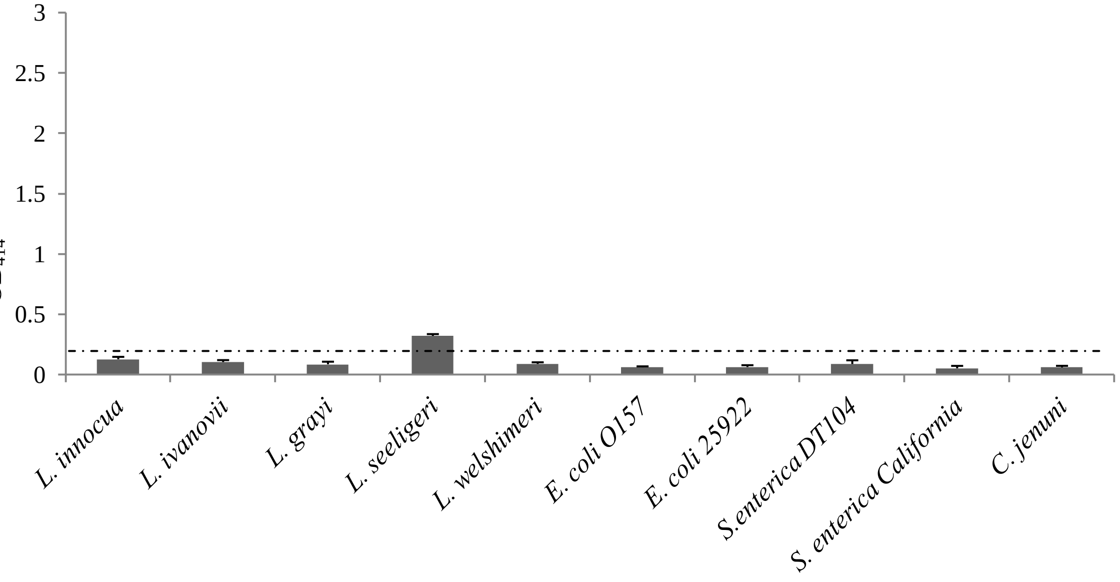

4d
4c
4e
3
2.5
2
1.5
1
0.5
0
OD$_{414}$
4706
4497
5248
18
4534
1848
1861

Figure 30. Analysis of anti-LapB MAb M3484 for cross-reactivity with non-*L. monocytogenes* bacteria. One strain of each non-*L. monocytogenes* spp. (*L. innocua*, *L. ivanovii*, *L. grayi*, *L. seeligeri*, *L. welshimeri*), two *E. coli* strains, two *S. enterica* strains and one *C. jejuni* strain was tested for reactivity to M3484 (tissue culture fluid) by ELISA. Bacterial cells were prepared as described in materials and methods. ELISA values represent an average of the data from three separate experiments (n=3). The MAb was tested in duplicate wells for each experiment. Dotted line indicates the cut-off for a negative reaction (OD_{414} of 0.200).


3
2.5
2
1.5
1
0.5
0
OD$_{414}$
L. innocua
L. ivanovii
L. grayi
L. seeligeri
L. welshimeri
E. coli O157
E. coli 25922
S.enterica DT104
S. enterica California
C. jenuni

CHAPTER IV

RESULTS

PART III: DEVELOPMENT AND CHARACTERIZATION OF MABS TO INLJ

PART III: DEVELOPMENT AND CHARACTERIZATION OF MABS TO INLJ

4.14 Screening and isotyping of hybridoma clones

Sera from all six mice (day 64 test bleed) immunized with the rInlJ showed comparable antibody responses to the recombinant antigen, as revealed by WB. CFW mouse #3 and Balb/C mouse #3 were selected for the fusions as anti-sera from these two animals appeared to react with the rInlJ protein slightly stronger in comparison to other mice. Fusions 575 (Balb/C) and 576 (CFW) were performed using a standard CFIA hybridoma fusion protocol (SOP # MC-PR005.02). Parent hybridomas were screened for reactivity with rInlJ by ELISA following the same procedure as previously described for LapB hybridomas clones. A total of 33 MAbs were produced from the two fusions, with all being of the IgG1 immunoglobulin subclass type except for M3550 which is an IgG2a (Table 6).

4.15 Reactivity of anti-InlJ MAbs with rInlJ protein

All 33 anti-InlJ MAbs (M3526 to M3558) were tested for their reactivity with the rInlJ protein by ELISA. The results showed all MAbs reacted strongly to the recombinant protein as ELISA OD_{414} values ranged from 1.0 to 4.0 (Figure 31A).

4.16 Analysis of anti-InlJ MAbs for cross-reactivity with *E. coli* proteins

The rInlJ protein was expressed in and purified from an *E. coli* host cell. SDS-PAGE revealed there was minor contamination with *E. coli* protein in the rInlJ protein preparation. To ensure that the anti-InlJ MAbs did not react with *E. coli* proteins, all 33 anti-InlJ MAbs were tested for reactivity against soluble proteins of *E. coli* BL21(DE3)/pLysS (with no vector) by ELISA. No reactivity with *E. coli* proteins was observed with any of the MAbs (Figure 31B).

4.17 Binding of anti-InlJ MAbs to the surface of *L. monocytogenes* serotype 4b

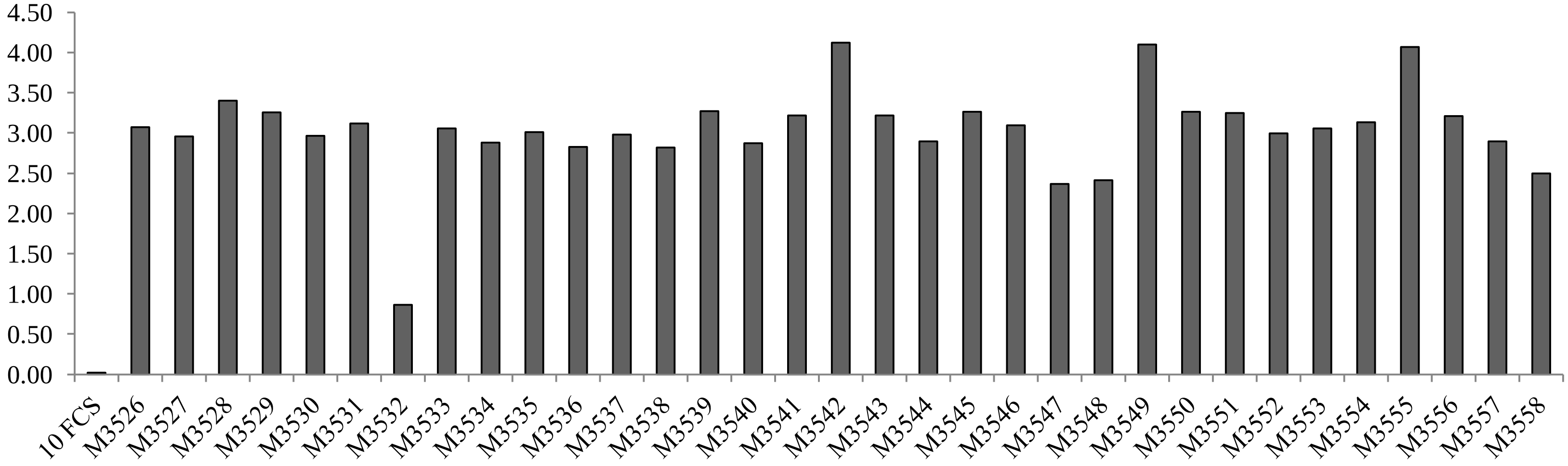
All anti-InlJ MAbs reacted strongly with the rInlJ protein (ELISA values ranged from 0.850 to 4.0) (Figure 31A). However, the MAbs exhibited negative reactivity with the native InlJ on the surface of these *L. monocytogenes* serotype 4b L10521 cells grown in BHI (Figure 32). The highest ELISA OD_{414} value was 0.188 for M3550. The reactivity of anti-InlJ MAbs was also negative with *L. monocytogenes* cells grown in LB and

Table 6. List of anti-InlJ MAbs with their corresponding hybridoma cell line and final immunoglobulin subclass. Immunoglobulin subclass types were determined by isoptying hybridoma tissue culture fluid.

Anti-InlJ MAbs	**Parent**	**Isotype**
M3526	4G6	IgG1
M3527	8C7	IgG1
M3528	9A4	IgG1
M3529	10E3	IgG1
M3530	11D6	IgG1
M3531	11G5	IgG1
M3532	12C5	IgG1
M3533	12C8	IgG1
M3534	12C9	IgG1
M3535	12F8	IgG1
M3536	13G5	IgG1
M3537	14D10	IgG1
M3538	14E5	IgG1
M3539	14G4	IgG1
M3540	14H10	IgG1
M3541	15B12	IgG1
M3542	15C1	IgG1
M3543	15G7	IgG1
M3544	16E5	IgG1
M3545	16F6	IgG1
M3546	16G3	IgG1
M3547	17A11	IgG1
M3548	17C9	IgG1
M3549	18E3	IgG1
M3550	18G2	IgG2a
M3551	19G8	IgG1
M3552	20A1	IgG1
M3553	20A8	IgG1
M3554	20G11	IgG1
M3555	21B10	IgG1
M3556	9F3	IgG1
M3557	21C9	IgG1
M3558	8B2	IgG1

Figure 31. Reactivity of anti-InlJ MAbs with rInlJ protein and *E. coli* proteins. A. All 33 anti-InlJ MAbs (M3526 to M3558) reacted strongly with rInlJ protein (1.0 ug/ml), as revealed by ELISA. **B.** All 33 anti-InlJ MAbs were tested for cross-reactivity with *E. coli* proteins by ELISA. Soluble proteins of *E. coli* BL21(DE3)/pLysS (with no vector) was extracted following the same procedure as purification of the rInlJ protein from *E. coli*. Positive and negative controls were CFW mouse anti-InlJ serum and 10% fetal calf serum, respectively. ELISA OD_{414} values are an average of duplicate determinations for each MAb.

A


4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
10 FCS
M3526
M3527
M3528
M3529
M3530
M3531
M3532
M3533
M3534
M3535
M3536
M3537
M3538
M3539
M3540
M3541
M3542
M3543
M3544
M3545
M3546
M3547
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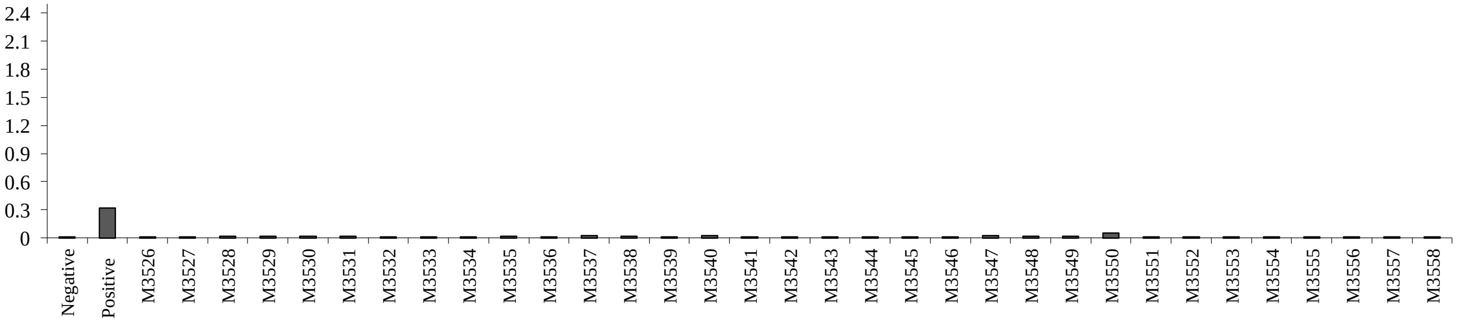
M3554
M3555
M3556
M3557
M3558

B


2.4
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0
Negative
Positive
M3526
M3527
M3528
M3529
M3530
M3531
M3532
M3533
M3534
M3535
M3536
M3537
M3538
M3539
M3540
M3541
M3542
M3543
M3544
M3545
M3546
M3547
M3548
M3549
M3550
M3551
M3552
M3553
M3554
M3555
M3556
M3557
M3558

Palcam/UVM (Figure 32). Failure to detect InlJ on the surface of *L. monocytogenes* cells by anti-InlJ MAbs was also demonstrated by IFM. Of the 33 anti-InlJ MAbs produced, the three MAbs (M3531, M3540 and M3550) with the highest ELISA OD_{414} values, despite those values being negative, were tested with IFM. None of the three anti-InlJ MAbs showed positive immunofluorescence signal with *L. monocytogenes* cells grown in BHI (Figure 33A) or LB (Figure 33B) or Fraser broths (Figure 33C). Due to the neglible binding of these MAbs to the surface of the parent strain *L. monocytogenes* 4b L10521, they were not selected for further characterization with respect to their reactivity with various strains of *L. monocytogenes*.

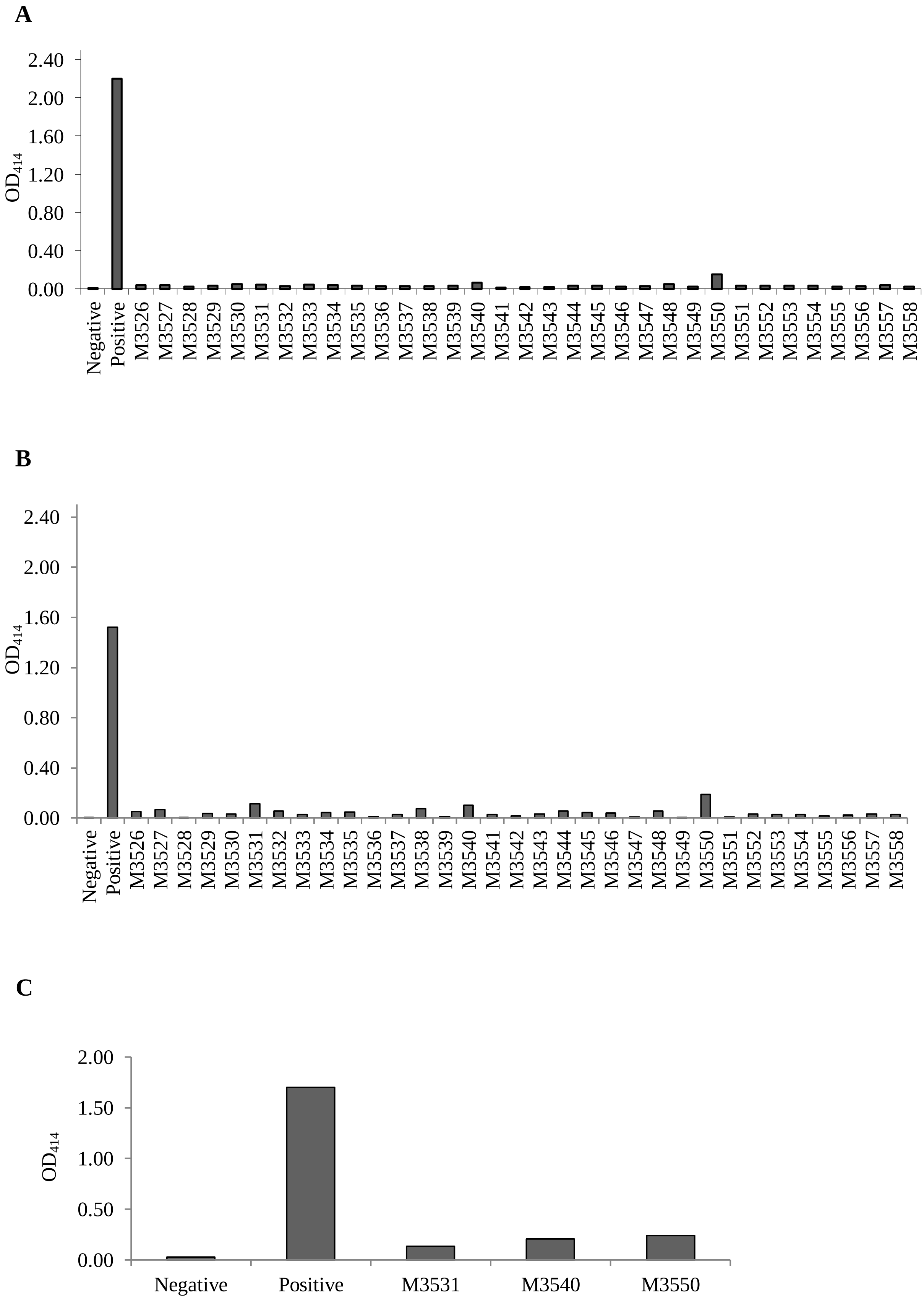
4.18 Recognition of the rInlJ protein by selected anti-InlJ MAbs

Anti-InlJ MAbs M3531, M3540 and M3550 were tested for reactivity with the rInlJ protein by WB (Figure 34). All 3 anti-InlJ MAbs were capable of detecting the rInlJ protein on the WB.

4.19 Conclusion

The expression of InlJ on the surface of *L. monocytogenes* was demonstrated by IFM using anti-InlJ PAbs. Reaction of anti-InlJ MAbs with denatured rInlJ on WB indicated that these MAbs recognized linear epitopes on the protein. One possible explanation for failure to detect InlJ on the surface of *L. monocytogenes* is that the epitopes on the native InlJ are buried within the cell wall and are inaccessible to the anti-InlJ MAbs.

Figure 32. Reactivity of anti-InlJ MAbs with *L. monocytogenes* cells grown in BHI, LB and Palcam/UVM broth. ELISA was performed to evaluate the reactivity of 33 anti-InlJ MAbs with *L. monocytogenes* serotype 4b L10521. **A.** Reactivity of anti-InlJ MAbs (M3526 to M3558) with bacterial cells grown in BHI broth. **B.** Reactivity of anti-InlJ MAbs (M3526 to M3558) with *L. monocytogenes* 4b cells grown in LB broth **C.** Reactivity of three selected anti-InlJ MAbs (M3531, M3540 and M3500) with bacterial cells grown in Palcam/UVM broth. *L. monocytogenes* 4b cells were grown in Palcam and UVM broth essentially following the MFHPB30 method (as described in materials and methods) and were coated at a concentration of 5 x 10^8 cells/ml. The positive and negative controls used were anti-LapB MAb M3484 and 10 % fetal calf serum, respectively.


A
OD414
2.40
2.00
1.60
1.20
0.80
0.40
0.00
Negative
Positive
M3526
M3527
M3528
M3529
M3530
M3531
M3532
M3533
M3534
M3535
M3536
M3537
M3538
M3539
M3540
M3541
M3542
M3543
M3544
M3545
M3546
M3547
M3548
M3549
M3550
M3551
M3552
M3553
M3554
M3555
M3556
M3557
M3558
B
OD414
2.40
2.00
1.60
1.20
0.80
0.40
0.00
Negative
Positive
M3526
M3527
M3528
M3529
M3530
M3531
M3532
M3533
M3534
M3535
M3536
M3537
M3538
M3539
M3540
M3541
M3542
M3543
M3544
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M3546
M3547
M3548
M3549
M3550
M3551
M3552
M3553
M3554
M3555
M3556
M3557
M3558
C
OD414
2.00
1.50
1.00
0.50
0.00
Negative
Positive
M3531
M3540
M3550

Figure 33. Binding of anti-InlJ MAbs to the surface of *L. monocytogenes* cells. A. *L. monocytogenes* serotype 4b L10521 cells grown in BHI broth overnight and probed with anti-InlJ MAbs M3531, M3540 and M3550. **B.** *L. monocytogenes* cells grown in LB broth overnight and probed with anti-InlJ MAb M3550. **C.** *L. monocytogenes* cells grown in Fraser broth overnight and probed with anti-InlJ MAb M3550. **D.** Positive control (Anti-LapB MAb M3495) for each experiment described above. A 1:2 dilution of mouse MAb was used followed by a 1:100 dilution of Dylight 488 goat anti-mouse conjugate. Phase contrast images are shown in the top panels and fluorescent images are shown in bottom panels. Fluorescent images were taken with an exposure time of 5 seconds under UV light.

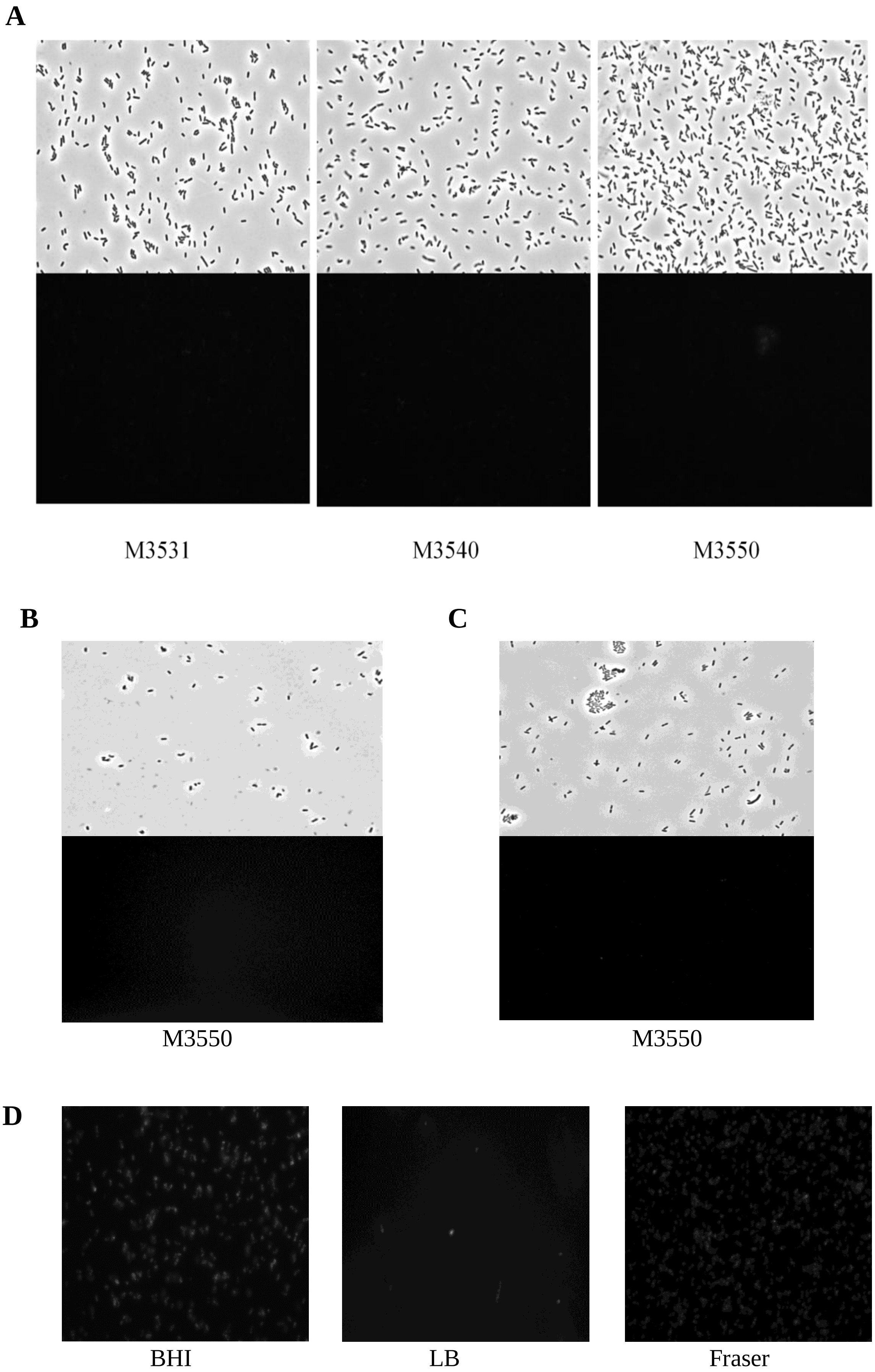
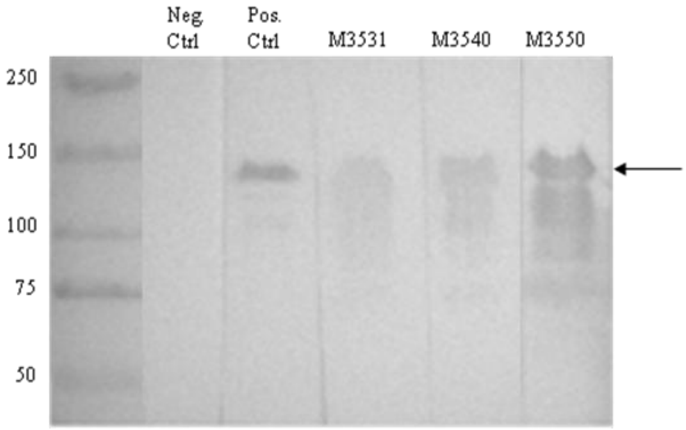

A
M3531
M3540
M3550
B
C
M3550
M3550
D
BHI
LB
Fraser

Figure 34. Recognition of the rInlJ protein by anti-InlJ MAbs. Purified rInlJ protein (2 µg/ml) was resolved by SDS-PAGE and analyzed with WB using the three anti-InlJ MAbs M3531, M3540 and M3550 (tissue culture fluid) at a 1:25 dilution. Anti-HIS MAb at a 1:1000 dilution was used as a positive control. Negative control used was pre-immune serum of CFW mouse #3 immunized with the rInlJ protein. Molecular masses of protein standards (in kDa) are shown by the numbers on the left. Black arrow indicates target rInlJ protein band (130 kDa).


Neg. Ctrl
Pos. Ctrl
M3531
M3540
M3550
250
150
100
75
50

CHAPTER IV

RESULTS

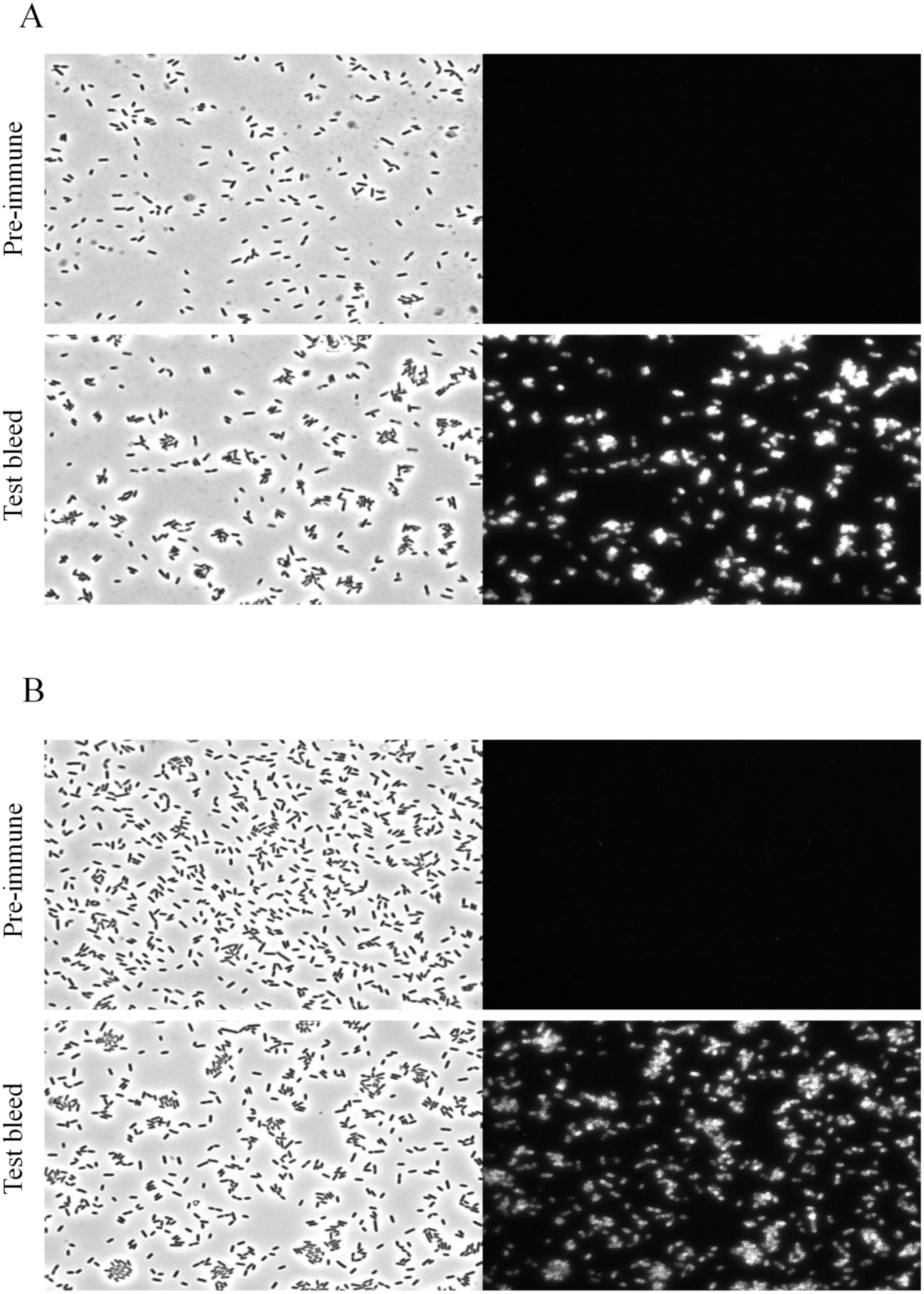
PART IV: DEVELOPMENT AND CHARACTERIZATION OF MABS TO INLC2

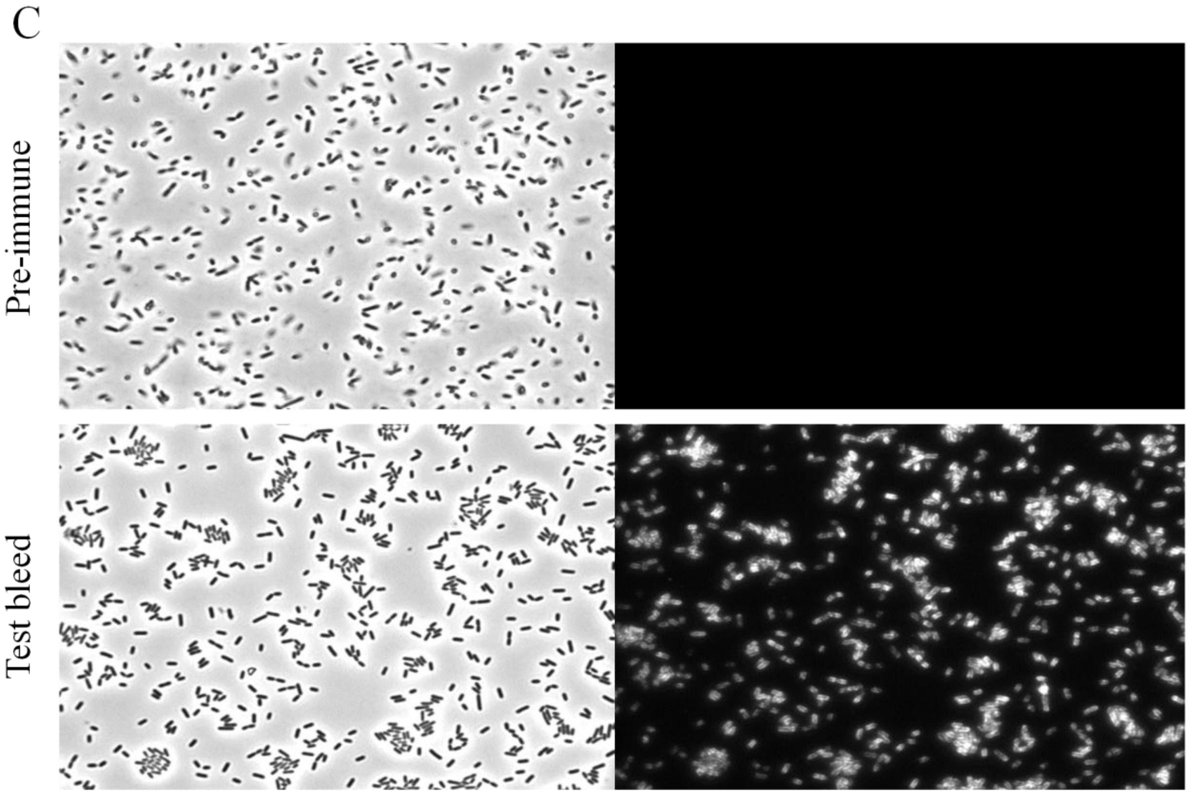
PART IV: DEVELOPMENT AND CHARACTERIZATION OF MABS TO INLC2

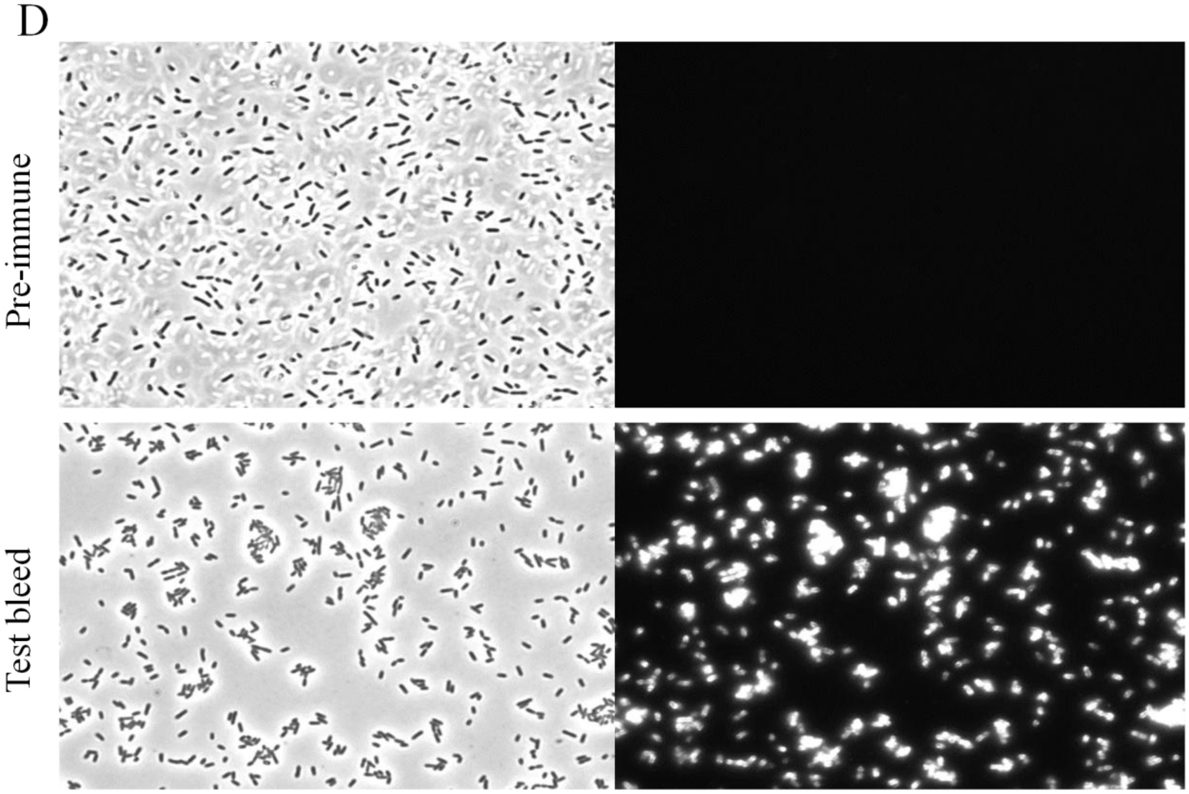
4.20 Screening and isotyping of hybridoma clones

Sera from all six mice (day 64 test bleed) receiving rInlC2 were examined with WB using rInlC2 protein as well as IFM and ELISA using *L. monocytogenes* serotype 4b L10521 cells. All six mice demonstrated an antibody response to the antigen. However, CFW mouse #1 and Balb/C mouse #3 showed the strongest antibody reaction to *L. monocytogenes* cells with IFM (Figure 35) and ELISA (Figure 36) and were therefore selected for the fusions. Fusions 583 (Balb/C) and 584 (CFW) were performed using a standard CFIA hybridoma fusion protocol (SOP # MC-PR005.02). MAbs secreted from hybridoma clones were screened for reactivity to rInlC2 by ELISA. This identified a very high number of positive hybridomas with approximately 90% of the tissue culture fluid samples having an ELISA OD_{414} value range from 1.0 to 3.5. To reduce the number of positive hybridomas down to a workable number they were screened for reactivity to rInlC2 coated at a lower concentration (0.5 µg/ml) as well as *L. monocytogenes* serotype 4b and *L. innocua* cells. This screening approach enabled the selection of hybridomas secreting antibodies that recognize the native in *L. monocytogenes* and select against those that recognize similar epitopes in *L. innocua*. A total of 23 parent hybridomas cross-reacted with *L. innocua* (ELISA OD_{414} values ranged from 0.200 to 0.400). Despite this selection, the number of positives still remained too high. In order to select the best parents cloning one strain from each of 11 *L. monocytogenes* serotypes were use as the ELISA antigen to select for positives that strongly reacted to a wide spectrum of *L. monocytogenes* serotypes. In addition, we tested positive clones for reactivity to *L. seeligeri* to select against any cross-reactors. This approach proved to be very successful and allowed to select some hybridomas that strongly recognized the strains of 1/2a, 1/2b, 1/2c, 3a, 4a, 4b, 4c and 4e serotypes tested and relatively weakly reacted to the strains of 3b, 3c and 4d serotypes tested (Table 7). There were a total of 13 positive hybridomas that cross-reacted with *L. seeligeri* (ELISA OD_{414} values ranged from 0.200 to 1.2) which were selected against. In total, 41 parent hybridomas were cloned in house. Seven days after cloning, the faster growers were screened using cells of *L. monocytogenes* serotype 4b to verify that these clones were stable and capable of recognizing the native InlC2 protein. Four hybridomas were lost due to their failure to continue to secrete the specific antibody.

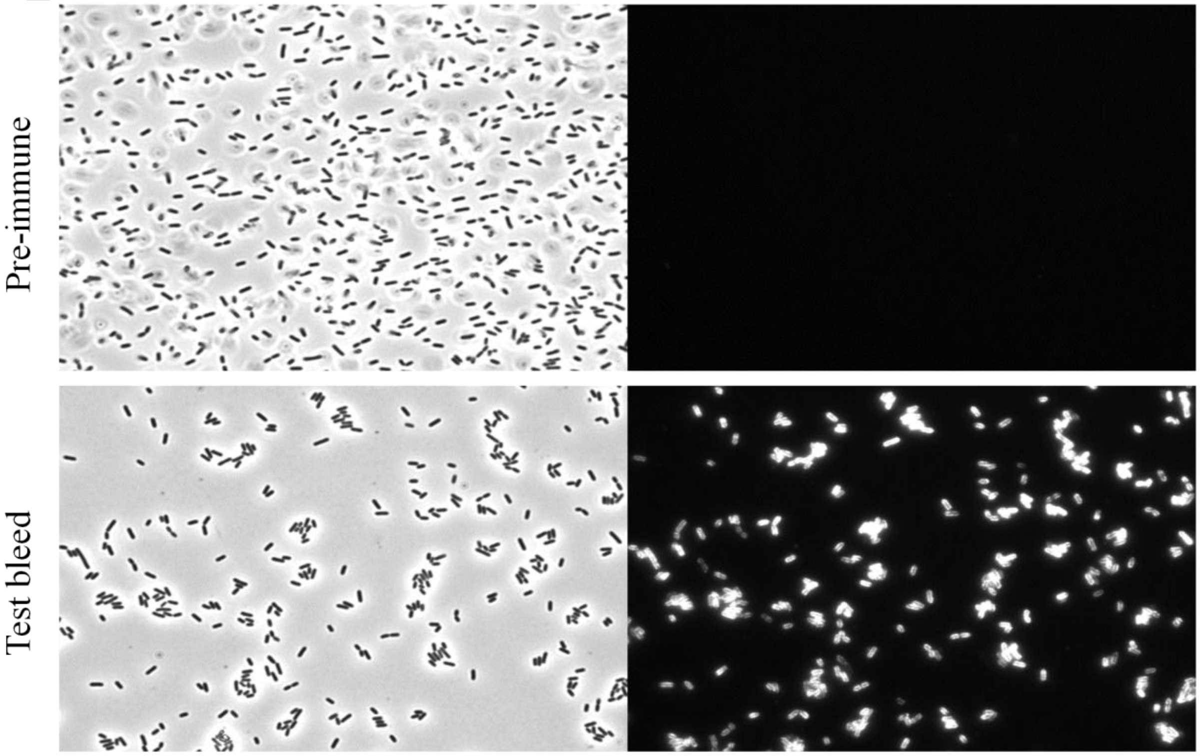
Figure 35. Immunofluorescence staining of *L. monocytogenes* cells using anti-InlC2 mouse sera. *L. monocytogenes* serotype 4b L10521 probed with pre-immune and day 42 test bleed of Balb/C mouse #1 (A), mouse #2 (B), mouse #3 (C), CFW mouse #1 (D), mouse #2 (E), mouse #3 (F). Mouse anti-serum was at a 1:100 dilution in 5% BSA-PBST. Secondary antibody Dylight 488 goat anti-mouse was at a dilution of 1:50 in 5% BSA-PBST. Phase contrast images are shown in the panels on the left and fluorescent images are shown in the panels on the right. Fluorescent images were taken with an exposure time of 5 seconds under UV light.


A
Pre-immune
Test bleed
B
Pre-immune
Test bleed


C
Pre-immune
Test bleed


D
Pre-immune
Test bleed

E



F

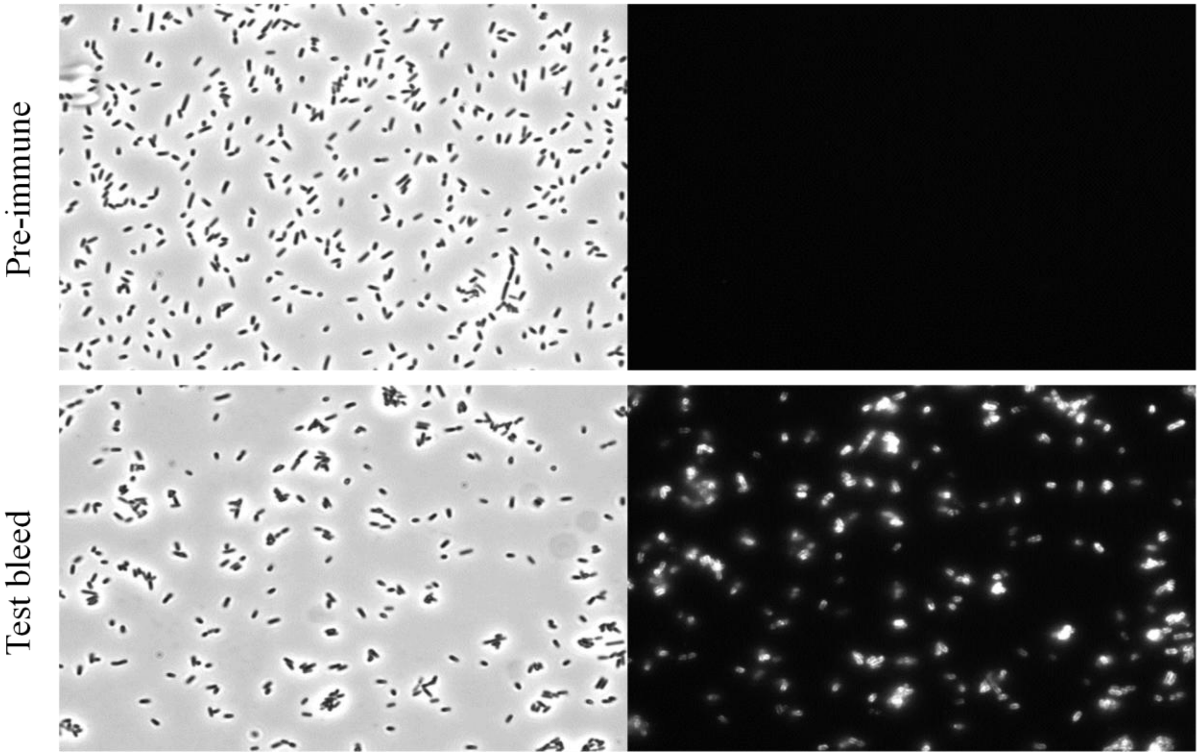
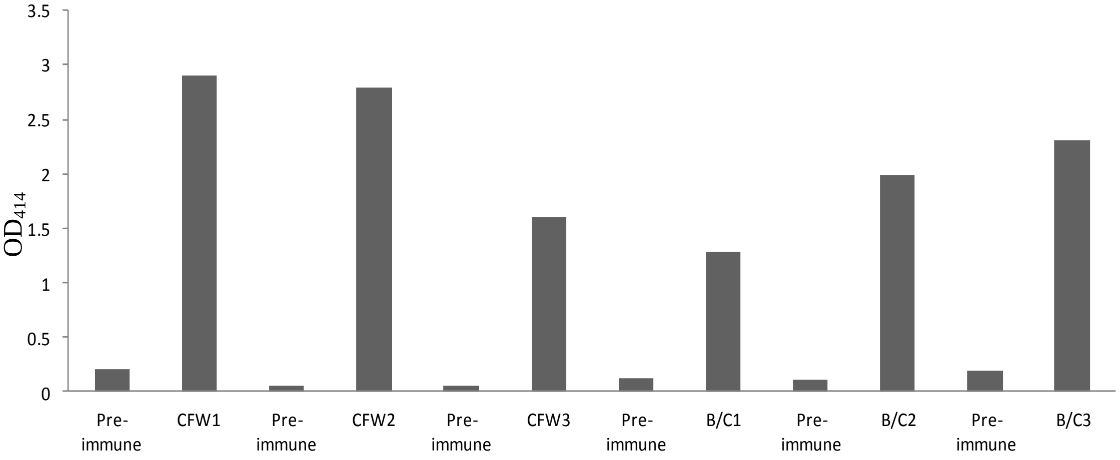


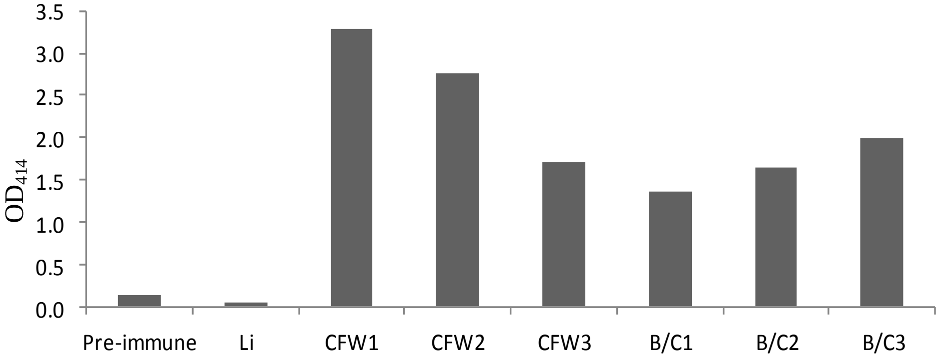
Figure 36. **ELISA of *L. monocytogenes* cells using anti-InlC2 mouse sera**. *L. monocytogenes* serotype 4b L10521 grown in BHI **(A)** and Palcam/UVM **(B)** broths incubated with pre-immune and day 42 test bleed of CFW mouse #1-3 and Balb/C mouse #1-3. Anti-InlC2 mouse sera was also tested for cross-reactivity with *L. innocua* strain 583 cells.

A



OD$_{414}$
3.5
3
2.5
2
1.5
1
0.5
0
Pre-immune
CFW1
Pre-immune
CFW2
Pre-immune
CFW3
Pre-immune
B/C1
Pre-immune
B/C2
Pre-immune
B/C3

B



OD$_{414}$
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Pre-immune
Li
CFW1
CFW2
CFW3
B/C1
B/C2
B/C3

Table 7. Selection of anti-InlC2 MAbs for further characterization based on screening of hybridoma tissue culture fluid. One strain of each of the 11 *L. monocytogenes* serotypes tested were used to screen anti-InlC2 MAbs from hybridoma tissue culture fluid: 1/2a (6036), 1/2b (09060), 1/2c (09022), 3a (3058), 3b (1031), 3c (61), 4a (5058), 4b (L10521), 4c (5248), 4d (18) and 4e (1848). Positive and negative controls were CFW mouse #1 anti-serum from day 64 test bleed and 10% FCS respectively. ELISA OD_{414} values represent an average of duplicate determinations.

		***L. monocytogenes* serotype**										
Hybrid.[a]	**MAb**[b]	**1/2a**	**1/2b**	**1/2c**	**3a**	**3b**	**3c**	**4a**	**4b**	**4c**	**4d**	**4e**
17G6	M3614	0.735	1.182	0.148	1.523	0.264	0.097	1.047	1.770	0.953	0.260	1.457
18F4	M3637	0.638	0.908	0.557	1.112	0.208	0.136	0.853	0.795	0.817	0.182	1.188
18F6	M3618	0.738	1.113	0.715	1.336	0.242	0.145	1.064	1.412	0.938	0.191	1.485
22A7	M3624	0.634	1.167	0.096	1.012	0.213	0.051	1.160	1.772	0.824	0.143	0.687
13D7	M3636	1.634	1.697	1.529	2.509	0.296	0.299	1.731	2.025	1.534	0.215	1.776
11D9	M3634	0.537	1.773	0.551	1.403	0.274	0.131	1.421	1.989	1.262	0.228	2.054
17F3	M3635	0.800	1.401	0.391	1.602	0.296	0.125	1.082	1.150	1.116	0.224	0.513
27F10	M3633	1.523	2.396	1.523	2.518	0.409	0.217	2.243	2.581	2.088	0.285	3.091
10C6	M3630	1.451	1.663	1.457	2.567	0.318	0.233	1.776	1.739	1.610	0.219	1.877
6H3	M3607	1.609	1.959	1.155	2.507	0.425	0.212	0.627	2.847	0.727	0.319	2.298
10FCS[c]	- - -	0.039	0.025	0.040	0.060	0.058	0.074	0.050	0.058	0.042	0.067	0.035

[a] **Hybridoma clone**

[b] **Subsequently assigned MAb number**

[c] **Negative control used was 10% FCS**

Therefore, of approximately 3000 original parent hybridomas, the total number of anti-InlC2 MAbs produced was 37. Of the 37 MAbs, 14 were IgG1, 17 were IgG2a and 6 were IgG2b (Table 8).

4.21 Reactivity of MAbs with rInlC2 protein

All 37 anti-InlC2 MAbs (M3601 to M3637) were tested for their reactivity with the rInlC2 protein by ELISA. All MAbs reacted strongly to the recombinant protein as ELISA OD_{414} values ranged from 0.5 to 2.2 (Figure 37A).

4.22 Testing anti-InlC2 MAbs for cross-reactivity with *E. coli* proteins

The rInlC2 protein was expressed in and purified from an *E. coli* host cell. Analysis of the purified protein by SDS-PAGE (Figure 19) revealed there was minor contamination with *E. coli* proteins in the rInlC2 preparation. To ensure that the anti-InlC2 MAbs did not recognize *E. coli* proteins, all 37 anti-InlC2 MAbs produced were tested for reactivity to soluble proteins of *E. coli* BL21(DE3) pLysS (with no vector) by ELISA . No reactivity with *E. coli* proteins was observed with any of the MAbs (Figure 37B).

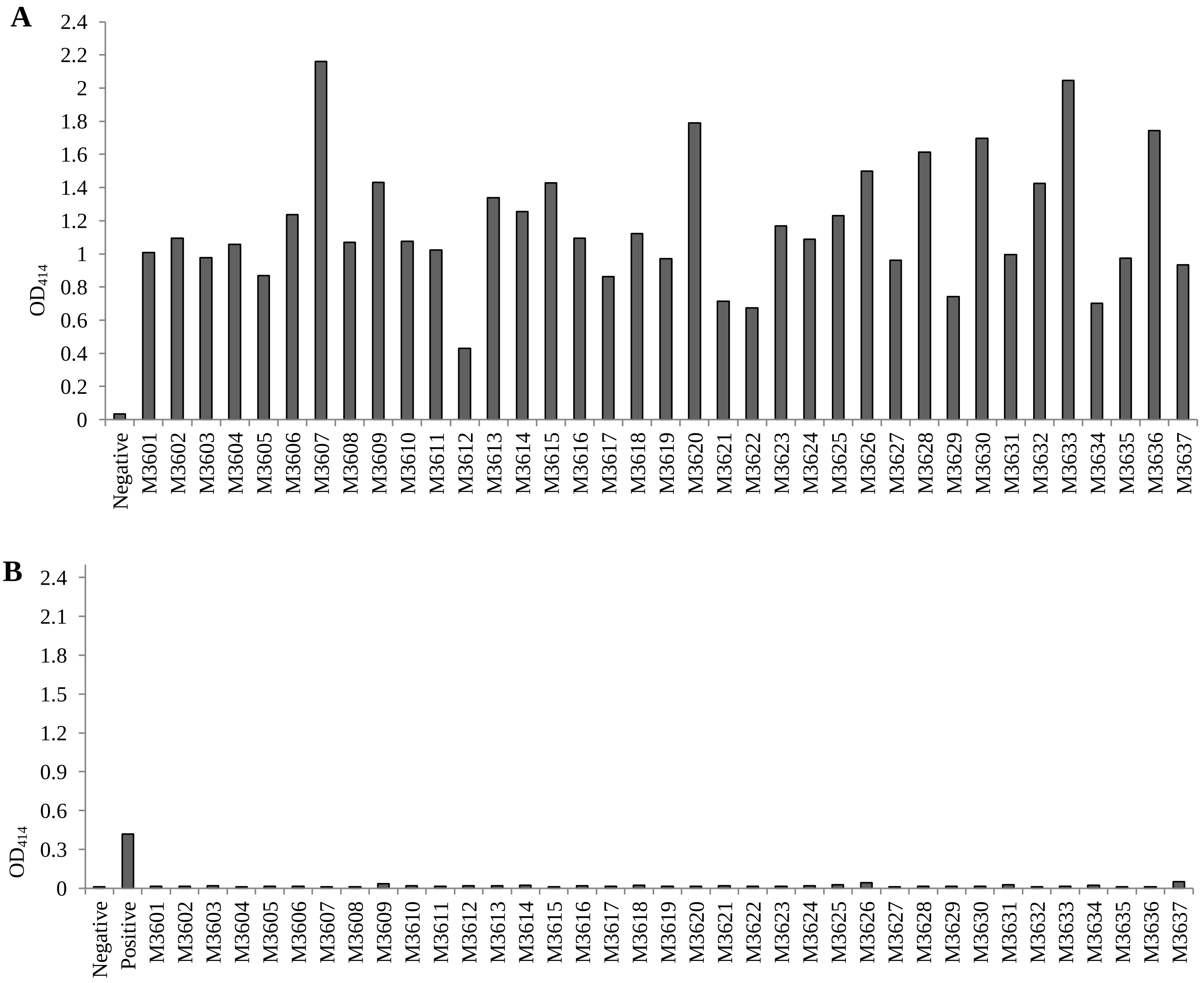
4.23 Binding of anti-InlC2 MAbs to the surface of *L. monocytogenes* serotype 4b

The final 37 MAbs were tested using ELISA and IFM to determine how well each reacted with the InlC2 protein on the surface of *L. monocytogenes* cells. All of the final TCFs were positive as assessed by ELISA (Figure 38) and IFM using cells of *L. monocytogenes* serotype 4b L10521 grown in BHI. However, three MAbs (M3622, M3625 and M3637) did show weak immunofluorescence signal. A total of six MAbs (M3607, M3618, M3630, M3633, M3635 and M3636) exhibited a strong immunofluorescence signal (Figure 38). This, together with the ELISA results from hybridoma screening (Table 7), indicates a good binding of these MAbs to the native InlC2. These six MAbs were selected for further evaluation of their reactivity to against various strains of *Listeria*.

Table 8. List of anti-InlC2 MAbs with their corresponding hybridoma cell line and final immunoglobulin subclass. Immunoglobulin subclass types were determined by isoptying hybridoma tissue culture fluid.

Anti-InlC2 MAbs	Parent	Isotype
M3601	8D9	IgG2a
M3602	9G2	IgG2a
M3603	16E5	IgG1
M3604	4A11	IgG1
M3605	6F1	IgG1
M3606	6H1	IgG1
M3607	6H3	IgG2a
M3608	9A10	IgG1
M3609	9G6	IgG2b
M3610	16F10	IgG1
M3611	16H9	IgG2a
M3612	17C5	IgG2a
M3613	17E12	IgG2a
M3614	17G6	IgG2a
M3615	18B5	IgG1
M3616	18C1	IgG2a
M3617	18F4	IgG1
M3618	18F6	IgG1
M3619	18G11	IgG2a
M3620	19B7	IgG2a
M3621	19D1	IgG1
M3622	19F4	IgG1
M3623	20C10	IgG1
M3624	22A7	IgG1
M3625	25F1	IgG2a
M3626	22E2	IgG2a
M3627	25H8	IgG2a
M3628	27H3	IgG2b
M3629	4A7	IgG2a
M3630	10C6	IgG2a
M3631	19A9	IgG2b
M3632	20G2	IgG1
M3633	27F10	IgG2b
M3634	11D9	IgG2b
M3635	17F3	IgG2a
M3636	13D7	IgG2a
M3637	18F4	IgG2a

Figure 37. Analysis of reactivity of anti-InlC2 MAbs with the rInlC2 protein. A. All 37 anti-InlC2 MAbs (M3601 to M3637) were tested for reactivity with the rInlC2 protein by ELISA. ELISA plates were coated with rInlC2 at 0.5µg/ml. **B.** Testing InlC2 MAbs for cross-reactivity with *E. coli* proteins. Soluble proteins of *E. coli* BL21(DE3)/pLysS (with no vector) was extracted following the same procedure as extraction of recombinant protein from *E. coli*. Positive and negative controls were CFW mouse #3 antiserum and 10% fetal calf serum, respectively. ELISA OD_{414} values are an average of duplicate determinations.


A
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD$_{414}$
Negative
M3601
M3602
M3603
M3604
M3605
M3606
M3607
M3608
M3609
M3610
M3611
M3612
M3613
M3614
M3615
M3616
M3617
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M3632
M3633
M3634
M3635
M3636
M3637
B
2.4
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0
OD$_{414}$
Negative
Positive
M3601
M3602
M3603
M3604
M3605
M3606
M3607
M3608
M3609
M3610
M3611
M3612
M3613
M3614
M3615
M3616
M3617
M3618
M3619
M3620
M3621
M3622
M3623
M3624
M3625
M3626
M3627
M3628
M3629
M3630
M3631
M3632
M3633
M3634
M3635
M3636
M3637

Figure 38. Demonstrating reactivity of anti-InlC2 MAbs with *L. monocytogenes* serotype 4b L10521 by ELISA. ELISA plates were coated with bacteria at a concentration of 1 x 10^8 cells/ml. All 37 anti-InlC2 MAbs (M3601 to M3637) reacted strongly with *L. monocytogenes* cells. Negative control was 10% fetal calf serum. The data represent an average of duplication determinations (i.e. two plate wells).

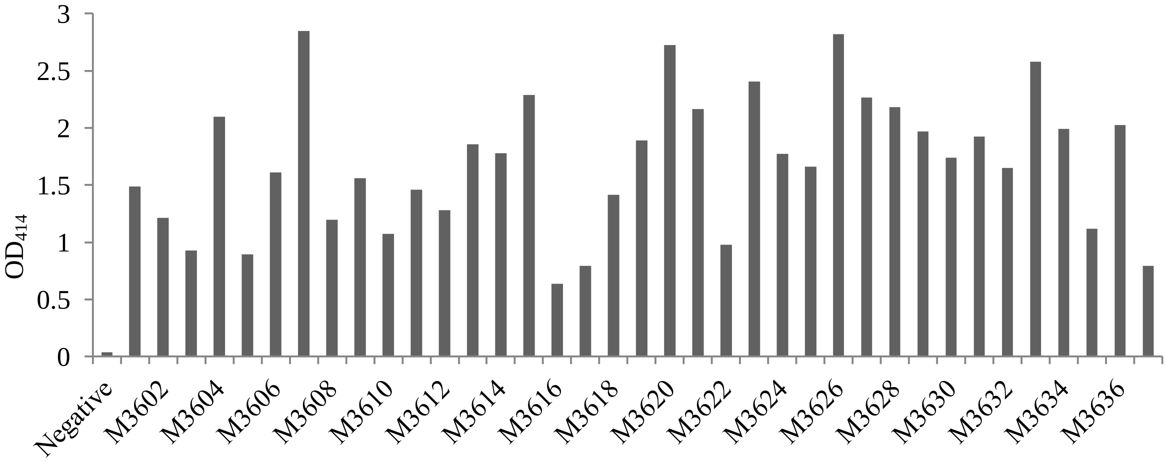
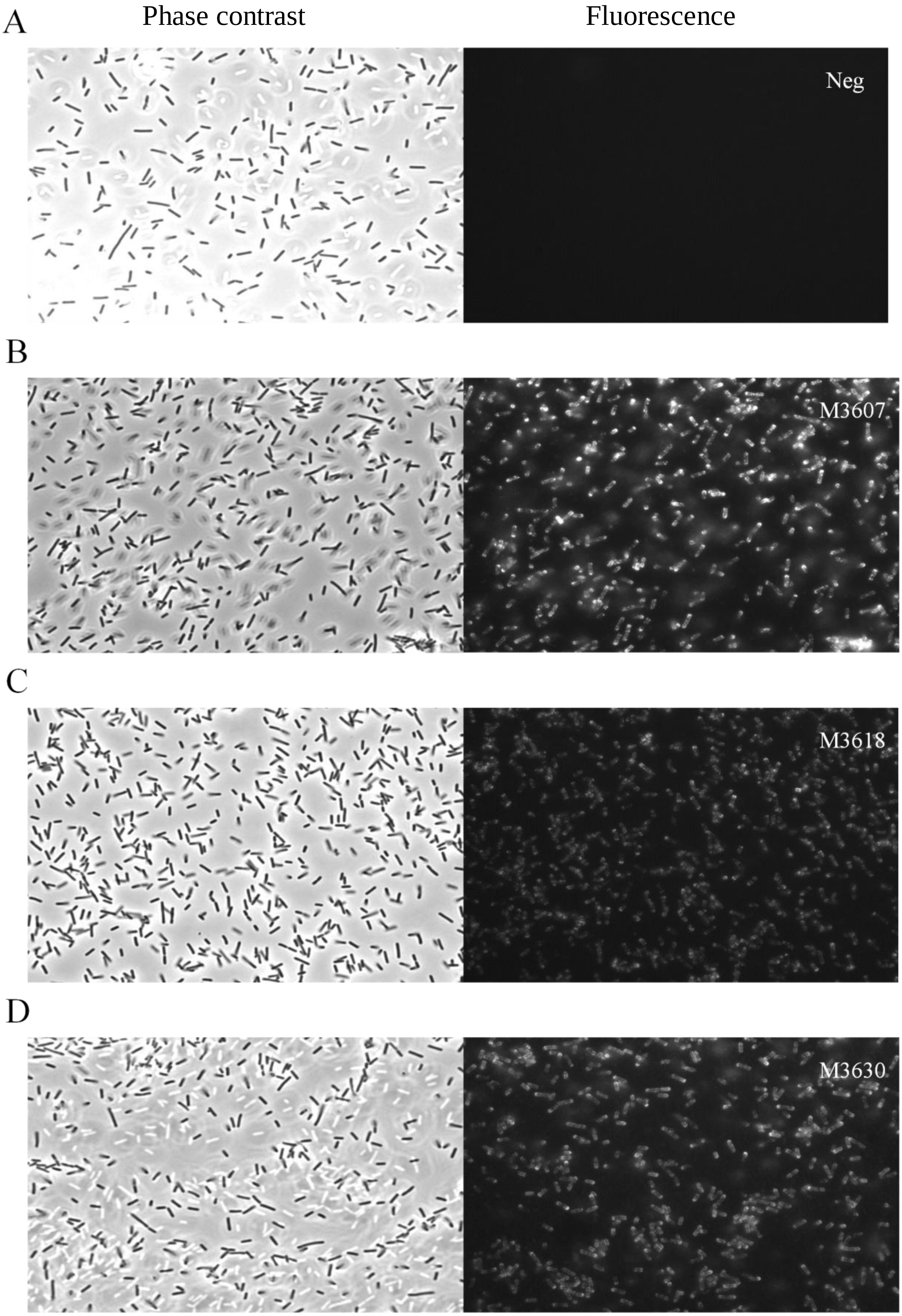
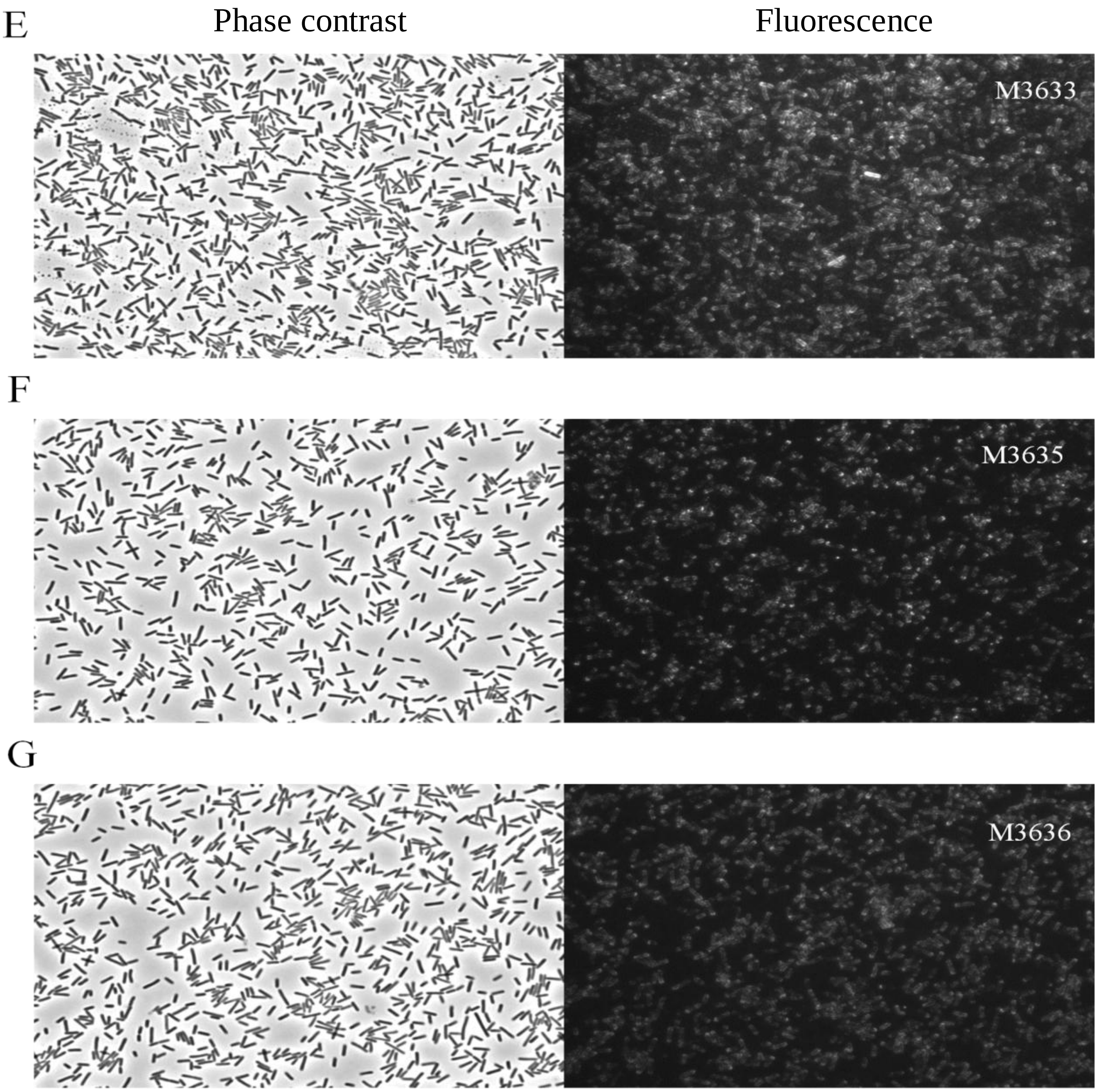

3
2.5
2
1.5
1
0.5
0
OD$_{414}$
Negative
M3602
M3604
M3606
M3608
M3610
M3612
M3614
M3616
M3618
M3620
M3622
M3624
M3626
M3628
M3630
M3632
M3634
M3636

Figure 39. Binding of selected anti-InlC2 MAbs to the surface of *L. monocytogenes*. *L. monocytogenes* serotype 4b L10521 cells were probed with pre-immune serum CFW mouse #3 (A), M3607 (B), M3618 (C), M3630 (D), M3633 (E), M3635 (F), and M3636 (G). Phase contrast images are shown in the panels on the left and fluorescent images are in shown in the panels on the right. Fluorescent images were captured with an exposure time of 5 seconds under UV light.


A
Phase contrast
Fluorescence
Neg
B
M3607
C
M3618
D
M3630


E
Phase contrast
Fluorescence
M3633
F
M3635
G
M3636

4.24 Recognition of the rInlC2 protein by selected anti-InlC2 MAbs

The six anti-InlC2 MAbs selected for further characterization by IFM were tested against the rInlC2 protein by WB (Figure 40). All six anti-InlC2 MAbs strongly detected the rInlC2 protein on WB, indicating they recognize linear epitopes on the protein. The presence of a second immunoreactive protein band of smaller size in the rInlC2 preparation indicates a proteolytic degradation of rInlC2.

4.25 Reactivity of anti-InlC2 MAbs to various strains of *L. monocytogenes*

The six selected anti-InlC2 MAbs were analyzed for their reactivity to various strains of *L. monocytogenes* by ELISA and given a score (- to +++) based on their ELISA OD_{414} values obtained from the average of three separate experiments (Table 9). Overall, five of the six anti-InlC2 MAbs (M3607, M3618, M3630, M3633 and M3636) were capable of recognizing 10 of the 11 *L. monocytogenes* serotypes tested. The only serotype not recognized by any of the anti-InlC2 MAbs was 3c. However, only one strain of serotype 3c was available for testing. Unlike the anti-LapB MAbs, the anti-InlC2 MAbs showed a high degree of variability in reactivity scores. For example, M3607 was the only MAb that did not recognize the 1/2a serotype 6095 strain; M3630 was the only MAb that did not recognize the 4c serotype 4706 strain; and M3635 was the only MAb that did not recognize any of the 1/2c serotypes. Of the six anti-InlC2 MAbs, M3633 with the highest number of *L. monocytogenes* strains tested (47 of 51). ELISA results showing the reactivity of M3633 with strains representing 11 *L. monocytogenes* serotypes were shown as an example in Figure 41. M3633 had weak or no binding to the following four strains: 09049 (1/2a); 61 (3c); 3501 (4a); and 1861 (4e) (OD_{414} readings ranged from 0.078 to 0.217).

4.26 Cross-reactivity of anti-InlC2 MAbs to non-pathogenic *Listeria* spp. and other microorganisms

Five of the six anti-InlC2 MAbs did not react with any non-pathogenic *Listeria* spp. tested (Table 9). M3635 was the only MAb that cross-reacted with *L. ivanovii* (ELISA OD_{414} reading of 0.87). None of the anti-InlC2 MAbs cross-reacted with the *Escherichia coli*, *Salmonella enterica* or *Campylobacter jejuni* strains tested (Table 9). The results showing the ELISA reactivity of M3633 with the non-*L. monocytogenes* strains was shown as an example in Figure 42.

Figure 40. Recognition of the rInlC2 protein by anti-InlC2 MAbs. Purified rInlC2 protein (2µg/ml) was separated by SDS-PAGE and analyzed by WB with the six anti-InlC2 MAbs (M3607, M3618, M3630, M3633, M3635 and M3636) at a 1:25 dilution of MAb producing tissue culture fluid. Positive control used was anti-HIS MAb at a 1:1000 dilution. Negative control used was pre-immune serum of CFW mouse #1 immunized with the rInlC2 protein. Black arrow indicates the rInlC2 protein band.

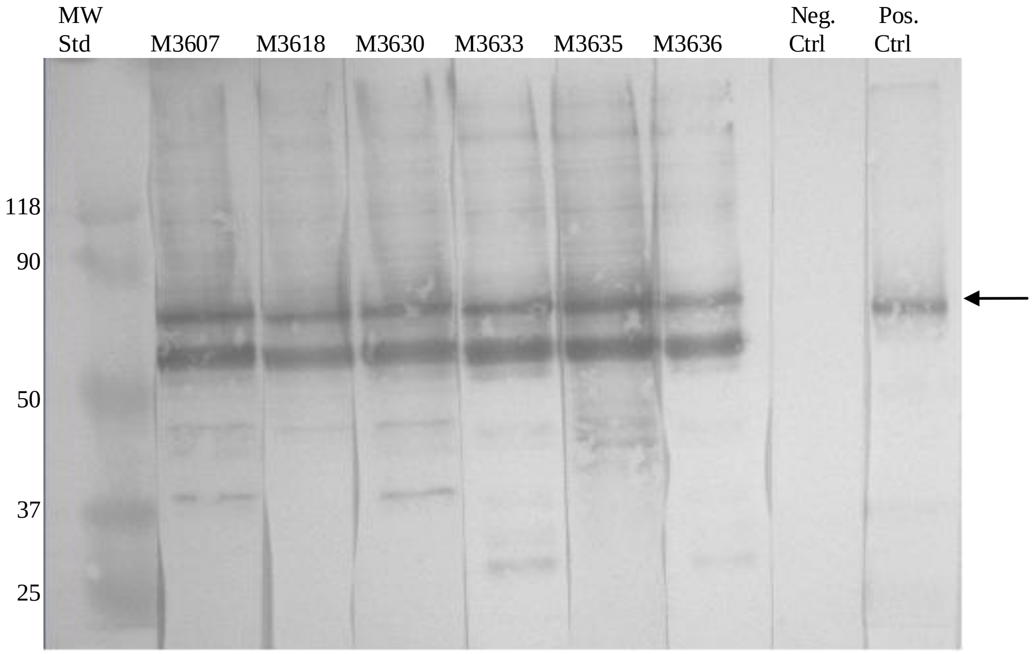

MW Std
M3607
M3618
M3630
M3633
M3635
M3636
Neg. Ctrl
Pos. Ctrl
118
90
50
37
25

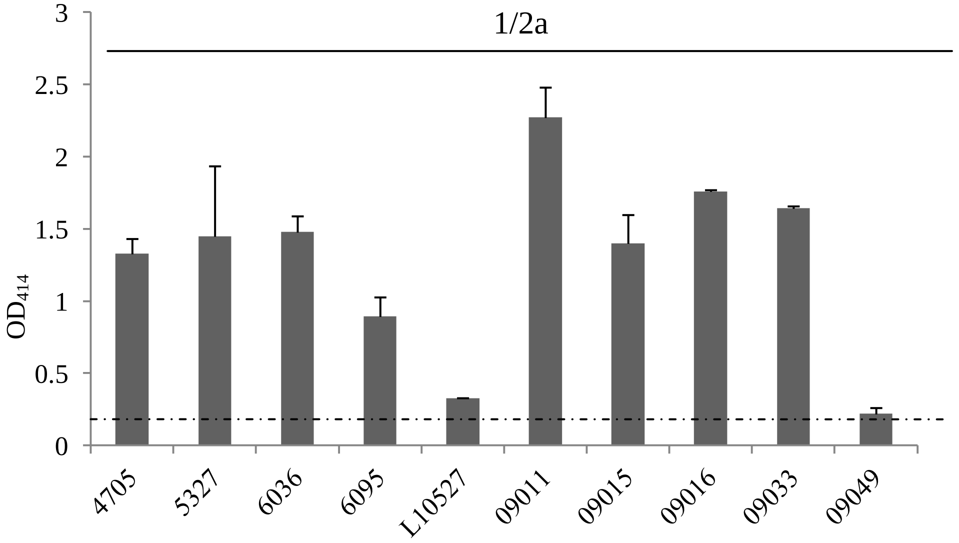
Table 9. Reactivity scores of six anti-InlC2 MAbs with numerous strains of *Listeria* and other foodborne bacterial pathogens. Reactivity scores (- to +++) were based on ELISA OD_{414} values: -, 0-0.2; -/+, 0.2-0.3; +, 0.3-1.0; ++, 1.0-2.0; +++, 2.0-3.0. An irrelevant MAb (anti-*S. enterica* DT104) was used as a negative control for each strain and ELISA OD_{414} values were within the range of 0-0.25. All ELISA data represent the average of OD_{414} readings from three separate experiments. Duplication determinations were obtained for each MAb in each experiment.

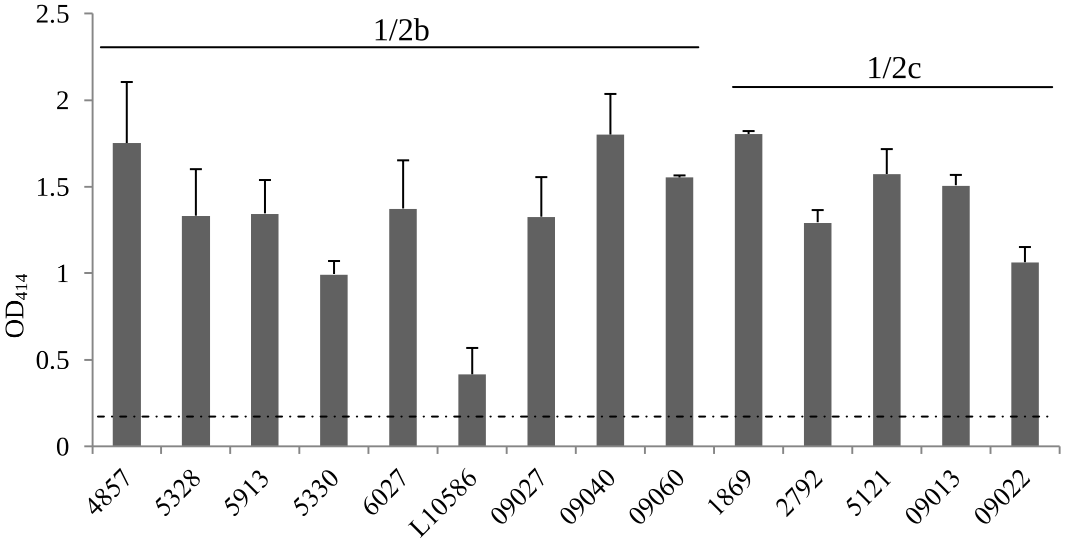
Serotype[a] or spp.	**Strain**	**Origin**	**M3607 (IgG2a)**	**M3618 (IgG1)**	**M3630 (IgG2a)**	**M3633 (IgG2b)**	**M3635 (IgG2a)**	**M363 (IgG2a)**
1/2a	**4705**		++	+	+	++	+	+
1/2a	**5327**		++	++	++	++	+	++
1/2a	**6036**	Food	++	+	++	++	+	++
1/2a	**6095**	Food	-/+	+	+	+	+	+
1/2a	**L10527**		-	-	-/+	+	-	-/+
1/2a	**09049**	Environmental	-/+	-	-	-	-	-
1/2a	**09015**	Food	++	+	+	++	+	++
1/2a	**09033**	Food	++	+	++	++	+	++
1/2a	**09011**	Environmental	++	++	++	+++	++	++
1/2a	**09016**	Environmental	++	+	++	++	+	++
1/2b	**4857**		++	+	++	++	++	++
1/2b	**5328**	Food	++	+	++	++	+	++
1/2b	**5913**	Food	++	+	++	++	++	++
1/2b	**5330**		+	+	+	+	+	+
1/2b	**6027**	Food	++	+	+	++	+	+
1/2b	**L10586**		+	-/+	-	+	+	-/+
1/2b	**09027**	Environmental	++	+	++	++	+	++
1/2b	**09040**	Food	++	+	++	++	+	++
1/2b	**09060**	Environmental	++	+	++	++	++	++
1/2c	**1869**		++	+	++	++	++	++
1/2c	**2792**		+	+	+	++	-	+
1/2c	**5121**		++	+	++	++	-	++
1/2c	**09013**	Environmental	++	+	++	++	-	++
1/2c	**09022**	Food	+	+	+	++	-	+
3a	**2768**		++	+	++	++	+	++
3a	**3058**		++	+	++	++	+	++
3a	**5665**	Food	++	+	++	+++	+	++
3a	**L10508**		+	+	+	+	+	+
3a	**09005**	Food	++	+	+	++	+	++
3a	**09039**	Environmental	++	+	+	++	+	++
3b	**1031**		+	-/+	+	+	-/+	-/+
3b	**4909**		++	+	++	++	+	++
3c	**61**		-/+	-	-/+	-/+	-	-
4a	**3501**		-	-	-	-	-	-
4a	**5041**		+	+	++	++	+	++
4a	**5058**	Animal	+	+	++	++	+	++
4b	**L10521[a]**	Clinical	++	+	++	++	++	++
4b	**3449**		+	+	++	++	++	++
4b	**5251**		+++	+	++	+++	++	++
4b	**5364**	Animal	++	+	++	++	+	++
4b	**5816**	Food	++	+	++	++	++	++
4b	**5906**	Food	++	+	++	++	++	++
4b	**6024**	Food	+	+	++	++	+	++
4b	**6092**	Food	++	+	++	++	++	++
4c	**4706**	Animal	+	+	-	++	+	++
4c	**4497**		+	+	++	++	+	++
4c	**5248**	Animal	+	+	++	++	+	++
4d	**18**		-/+	-/+	-/+	-/+	-/+	-
4d	**4534**		++	+	++	++	+	++
4e	**1848**		++	+	+	++	+	+
4e	**1861**		-	-	-	-	-	-
L. innocua	**583**		-	-	-	-	-	-
L. ivanovii	**28**		-	-	-	-	+	-
L. grayi	**29**		-	-	-	-	-	-
L. seeligeri	**24**		-	-	-	-	-	-
L. welshimeri	**92**		-	-	-	-	-	-
E. coli	**O157**		-	-	-	-	-	-
E. coli	**25922**		-	-	-	-	-	-
S. enterica	**DT104**		-	-	-	-	-	-
S. enterica	**California**		-	-	-	-	-	-
C. jejuni	**ADRI 1102**		-	-	-	-	-	-

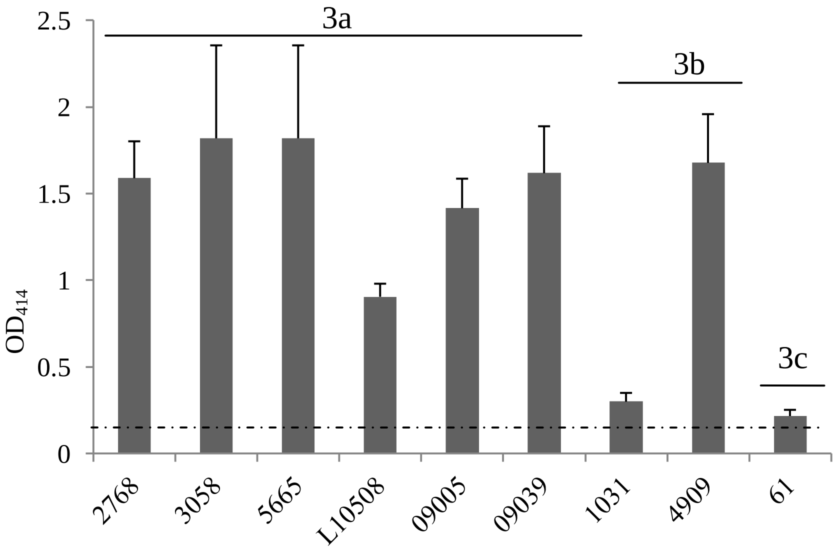
[a] Serotypes of *L. monocytogenes*

[b] The *inlC2* gene was amplified from the genomic DNA of this strain

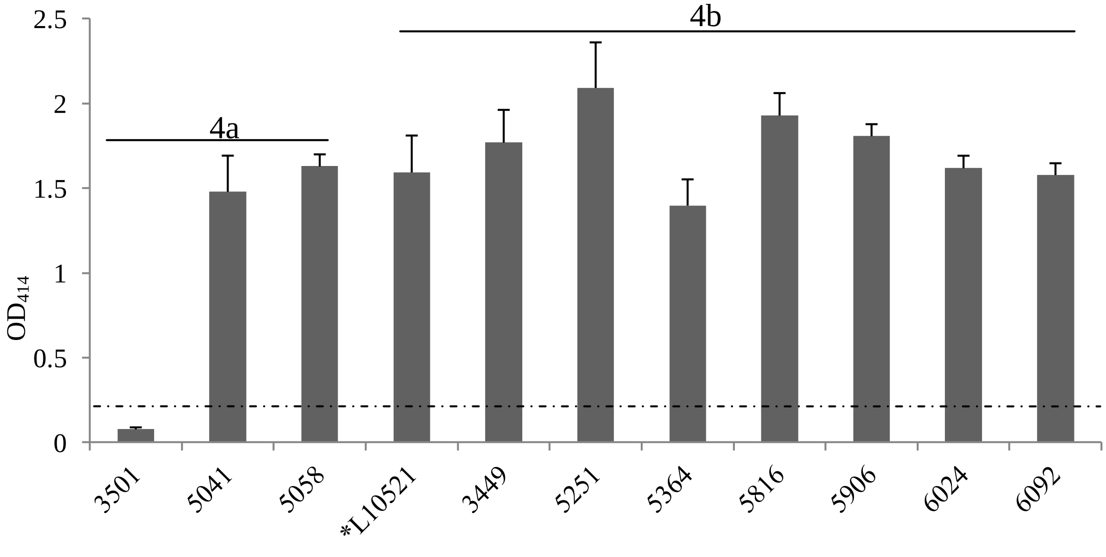
Figure 41. Reactivity of anti-InlC2 MAb M3633 with 51 strains of 11 *L. monocytogenes* serotypes. Strains from 11 of the 12 *L. monocytogenes* serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, and 4e) were tested for reactivity to M3633 (tissue culture fluid) by ELISA. Bacterial cells were prepared as described in materials and methods and coated at a concentration of 1 x 10^8 cells/ml. ELISA values represent an average of the data from three separate experiments +/- SD (n=3). The MAb was tested in duplicate wells for each experiment. Dotted line indicates the cut-off for a negative reaction (OD_{414} of 0.200).


1/2a
OD414
3
2.5
2
1.5
1
0.5
0
4705
5327
6036
6095
L10527
09011
09015
09016
09033
09049

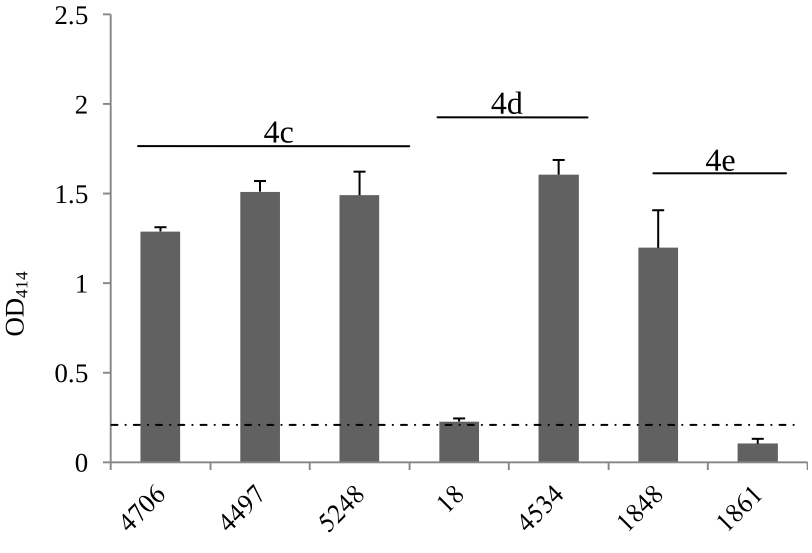

1/2b
1/2c
OD414
2.5
2
1.5
1
0.5
0
4857
5328
5913
5330
6027
L10586
09027
09040
09060
1869
2792
5121
09013
09022



3a
3b
3c
OD414
0
0.5
1
1.5
2
2.5
2768
3058
5665
L10508
09005
09039
1031
4909
61

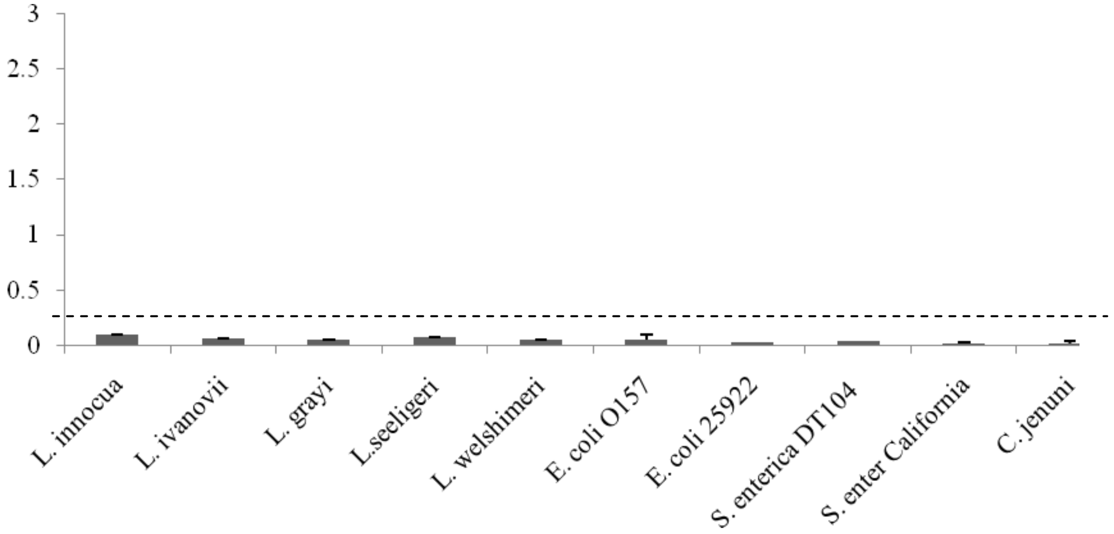


4a
4b
OD414
0
0.5
1
1.5
2
2.5
3501
5041
5058
*L10521
3449
5251
5364
5816
5906
6024
6092



4c
4d
4e
OD414
0
0.5
1
1.5
2
2.5
4706
4497
5248
18
4534
1848
1861

Figure 42. Analysis of anti-InlC2 MAb M3633 for cross-reactivity with non-*L. monocytogenes* bacteria. One strain of each non-*L. monocytogenes* spp. (*L. innocua*, *L. ivanovii*, *L. grayi*, *L. seeligeri*, *L. welshimeri*), two *E. coli* strains, two *S. enterica* strains and one *C. jejuni* strain was tested. Reactivity was determined by ELISA using bacterial cells coated at 1 x 10^8 cells/ml and tissue culture fluid of M3633. ELISA OD_{414} values represent the average of three separate experiments +/- SD (n=3). Each MAb was tested in duplicate for each experiment. Dotted line represents the cut-off for a negative reaction (OD_{414} of 0.200).



3
2.5
2
1.5
1
0.5
0
L. innocua
L. ivanovii
L. grayi
L.seeligeri
L. welshimeri
E. coli O157
E. coli 25922
S. enterica DT104
S. enter California
C. jenuni

4.27 Conclusion

One of the six anti-InlC2 MAbs, M3633, recognizes almost all of the pathogenic *Listeria* strains tested and does not react with any of the 5 non-pathogenic *Listeria* spp. or the other microorganisms tested. Based on these results, it is proposed that M3633 can be used as a diagnostic reagent for detecting and isolating the majority of *L. monocytogenes* serotypes from foods without cross-reacting to other non-pathogenic *Listeria* or other major foodborne pathogenic bacteria.

PART V: EXPRESSION OF LAPB AND INLC2 IN *L. MONOCYTOGENES* CULTURED IN STANDARD DIAGNOSTIC GROWTH CONDITIONS

4.28 Assessment of LapB and InlC2 expression in *L. monocytogenes* cultured in Palcam and UVM broths

Isolation and detection of *L. monocytogenes* in food samples using the procedure MFHPB-30 outlined in the Compendium of Analytical Methods (89) requires primary and secondary enrichment steps where the bacterial cells are grown in Palcam broth at 35°C overnight followed by subculturing the cells (1:10) into UVM broth for growth at 30°C overnight. Cultural enrichment aims to increase the bacterial cell number for the isolation and detection. The expression of LapB and InlC2 in *L. monocytogenes* grown in the diagnostic conditions outlined above must be demonstrated in order to realize the diagnostic potential of anti-Lap and anti-InlC2 MAbs. The ELISA results indicated that LapB and InlC2 were expressed at comparable levels in bacterial cells grown in BHI (Results Part II and Part IV). These two proteins were also expressed when *L. monocytogenes* was grown in Palcam and UVM, as revealed by ELISA (Figure 43 and Figure 44). The top six anti-LapB MAbs were tested for reactivity three strains of *L. monocytogenes* and one strain of *L. innocua* (Figure 43). The results indicated that all six anti-LapB MAbs recognized *L. monocytogenes* serotype 4b strains L10521 and 5906, as well as serotype 1/2a strain 6036. As observed with *L. monocytogenes* grown in BHI (Table 5), the anti-LapB MAbs reacted stronger with strains 5906 and 6036 and did not cross-react with *L. innocua* strain 583. It should be noted that the InlC2 expression in *L. monocytogenes* grown in Palcam and UVM was assessed with ELISA using mouse anti-InlC2 antisera (Figure 44) because anti-InlC2 MAbs were not available at the time of performing these experiments. IFM was not performed to assess the expression of LapB and InlC2 in *L. monocytogenes* cultured in Palcam and UVM broths due to the observation that bacteria grown in Palcam, but not UVM, exhibited a high background of fluorescence signal.

Figure 43. Anti-LapB MAbs react with *L. monocytogenes* grown in Palcam and UVM. ELISA plates were coated with 2x10^8 cells/ml of *L. monocytogenes* cells cultured in Palcam and UVM and probed with six anti-LapB MAbs (M3484, M3495, M3500, M3509, M3517 and M3519) and positive and negative control. The following *Listeria* strains were tested. *L. monocytogenes* serotype 4b strain L10521 (L10521-4b). *L. monocytogenes* serotype 4b strain 4906 (4906-4b), *L. monocytogenes* serotype 1/2a strain 6036 (6036-1/2a), or *L. innocua* strain 583 (583-Li). Fetal calf serum (10%) was used as a negative control and anti-IspC MAb M2799 was used as a positive control for the L10521-4b. All ELISA OD_{414} readings represent the average of duplicate determinations. The ELISA OD_{414} values for *L. innocua* ranged from 0.062 to 0.106.

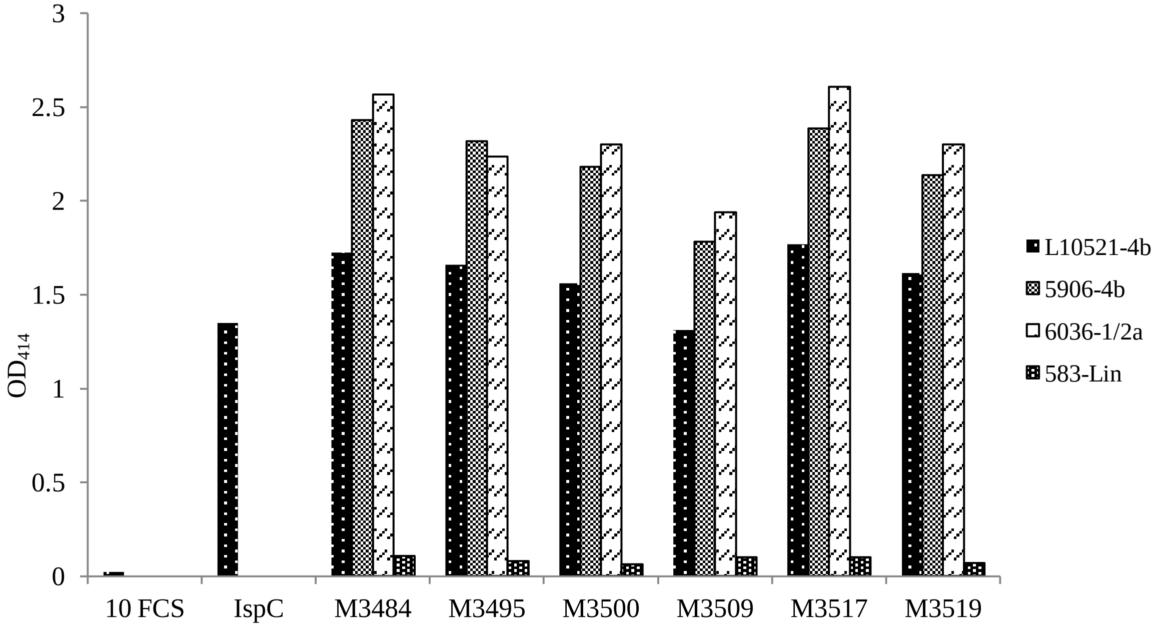
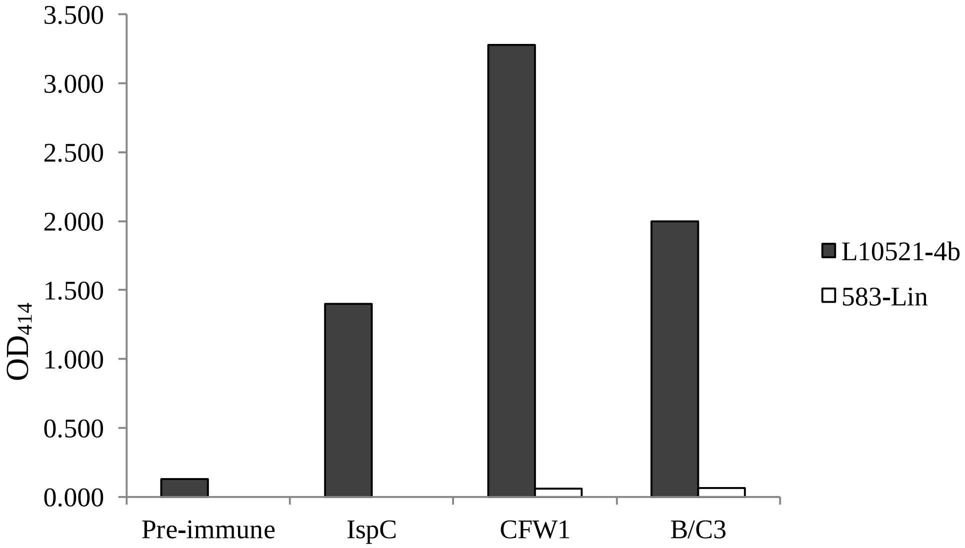

3
2.5
2
1.5
1
0.5
0
OD_{414}
10 FCS
IspC
M3484
M3495
M3500
M3509
M3517
M3519
L10521-4b
5906-4b
6036-1/2a
583-Lin

Figure 44. Reactivity of mouse anti-InlC2 sera with *L. monocytogenes* cells grown in Palcam and UVM broth. *L. monocytogenes* serotype 4b strain L10521 (L10521-4b) and *L. innocua* 583 (583-Lin) cells were grown in Palcam and UVM broth according to the MFHPB30 method and probed with CFW #1 and Balb/C #1 mouse anti-serum to test for reactivity with cells using these growth conditions. Positive control used was an anti-IspC MAb M2799 and negative control was pre-immune serum of CFW #1 mouse.


3.500
3.000
2.500
2.000
1.500
1.000
0.500
0.000
OD414
Pre-immune
IspC
CFW1
B/C3
L10521-4b
583-Lin

CHAPTER V: DISCUSSION

CHAPTER V: DISCUSSION

5.0 General discussion

L. monocytogenes is a common foodborne pathogen that has the capacity to cause severe clinical illness in vulnerable human population groups. The deficit of quick and effective methods for *L. monocytogenes* isolation from food samples is the primary obstacle for emerging molecular detection technologies designed to reduce the turnaround time from sampling to test results. Antibody-based methods offer a means for faster isolation and detection of *L. monocytogenes*. The goal of this study was to develop MAbs with high specificity and affinity to proteins found on the surface of all strains of *L. monocytogenes* while not cross-reacting with non-pathogenic *Listeria* spp. or other microorganisms commonly found in food. Five *L. monocytogenes* surface proteins ActA, InlA, LapB, InlJ and InlC2 were selected from a list of ten known or putative virulence factors of *L. monocytogenes* identified in literature search as potential candidates for MAb development. Surface virulence factors of *L. monocytogenes* play an important role in the interaction of this bacterium with host cells during an infection and therefore are expected to be conserved in all pathogenic strains of *L. monocytogenes* and may serve as useful diagnostic markers. Furthermore, it is not expected that these virulence factors are present in non-pathogenic *Listeria* species.

Constitutive expression of a surface protein is a requirement for the protein to be a useful diagnostic marker for the isolation and detection of *L. monocytogenes* by antibody-based methods. To assess the expression of candidate proteins, recombinant forms of these proteins were produced in sufficient quantity for use in raising PAbs in rabbits. Producing adequate amounts of recombinant forms of the candidate proteins was also necessary for the development of MAbs to these proteins. Despite multiple attempts of purification, sufficient amounts of highly purified rActA and rInlA proteins were not obtained. Based on the BLAST search and sequence comparison results (Figure 3 and 4), ActA and InlA appeared to be strong candidates for the development of MAbs with the ability to recognize all strains of *L. monocytogenes*. However, the inability to express ActA in the three *E. coli* expression host cells (BL21(DE3)/pLysS, Rosetta(DE3/) pLysS and C43(DE3)/pLysS) attempted resulted in an extended period of time before ActA was successfully expressed in *E. coli* BL21(DE3) and subsequently purified (Figure 10). Moreover, there was not a sufficient amount of rActA purified for PAb production. Unlike rActA, rInlA was successfully expressed in the three *E.coli* expression host cells

(BL21(DE3)/pLysS, Rosetta(DE3)/pLysS and C43(DE3)/pLysS) (Figure 10). Yet similar to rActA, not a sufficient amount of purified rInlA was produced (Figure 12). Due to these difficulties, development of antibodies to ActA and InlA was not pursued further. Future work could focus on the optimization of expression conditions for these two recombinant proteins or on the production of truncated recombinant proteins.

In contrast, sufficient amounts of highly purified rLapB, rInlJ and rInlC2 proteins were generated for immunization of animals to raise PAbs and subsequently MAbs. IFM with the rabbit antisera revealed strong expression of LapB, moderate expression of InlJ and very strong expression of InlC2 on the surface of *L. monocytogenes* 4b strain L10521 cells grown in BHI broth. These results indicated that LapB, InlJ and InlC2 are promising surface markers to be targeted for MAb development. Following confirmation of the expression of LapB, InlJ and InlC2 on the surface of *L. monocytogenes* cells, work was successfully conducted to raise MAbs against all three recombinant proteins. A total of 48 anti-LapB MAbs, 33 anti-InlJ and 37 anti-InlC2 MAbs were developed in this work. Further characterization of those MAbs revealed that some of them have great potential for use in the antibody-based methods for *L. monocytogenes.* MAbs capable of recognizing strains of all different *L. monocytogenes* serotypes have not been reported in the literature or commercially available to date. There is a clear need for such antibodies with respect to diagnostic applications. The work described here represented a significant step towards the development of antibody-based methods for isolation and detection of *L. monocytogenes* in food samples.

5.1 Anti-LapB MAbs have the potential to be used for detection and isolation of *L. monocytogenes*

Based on their strong binding to *L. monocytogenes* cells shown by IFM, seven anti-LapB MAbs were selected for further characterization to assess their reactivity with various strains of *L. monocytogenes* by ELISA. The results support the findings by Reis *et al*. (70) that *lapB* is present in all *L. monocytogenes* of lineages I (1/2b, 3b, 4b, 4d, 4e and 7) and II (1/2a, 1/2c, 3a and 3c) but absent from the rare lineage III (4a and 4c) (64). None of the anti-LapB MAbs reacted with the three 4c strains tested. Of the two 4a strains tested all seven MAbs reacted with only strain 5058. The only other *L. monocytogenes* strain that was not detected by any of the anti-LapB MAbs belonged to the 1/2a serotype strain 09049. Interestingly, this strain was also the only 1/2a strain not detected by any of the anti-InlC2 MAbs tested. Further studies are necessary to understand why this strain of serotype 1/2a is not detected by anti-LapB or anti-InlC2 MAbs. It may be possible that LapB and InlC2 genes are absent in this strain or that a premature stop codon is present within the genes leading to the expression of non-functional protein antigens. A premature stop codon has been discovered in the InlA gene (90).

Of the seven anti-LapB MAbs selected for further characterization, M3524 recognized the lowest number of *L. monocytogenes* strains. M3524 only detected 19 of the 51 *L. monocytogenes* strains tested. Of those 19 positives, the ELISA OD_{414} values mostly ranged from 0.31 to 0.6, indicating the binding was not very strong against the majority of *L. monocytogenes* strains tested. For these reasons, M3524 was not recommended for further diagnostic applications. The other five anti-LapB MAbs showed comparable positive results in ELISA, while M3484 reacted with the highest number (44 of 51) of *L. monocytogenes* strains tested. However, M3484 did cross-react with *L. seeligeri* at a low, as did M3517 and M3495. The other two MAbs M3500 and M3519 reacted with a high number of *L. monocytogenes* strains, yet did not cross-react with *L. seeligeri*. The ability of M3484 to bind to the majority of *L. monocytogenes* strains could be explored eventually with a suitable capture technique, such as IMS for the isolation of this pathogen from foods. The minimal cross-reaction of M3484 with *L. seeligeri* is expected to be negligible compared to the strong reactivity that M3484 has with the majority of *L. monocytogenes* strains if this MAb was to be conjugated to magnetic beads for IMS.

5.2 Anti-InlJ MAbs are not suitable for detection and isolation of *L. monocytogenes*

Although the expression of InlJ on the cell surface was detected by IFM with rabbit anti-InlJ PAbs, none of the anti-InlJ MAbs reacted with *L. monocytogenes* serotype 4b L10521 cells grown in BHI broth, LB, Fraser and Palcam/UVM broth. This result, together with the observation that anti-InlJ MAbs was able to detect denatured rInlJ on WB, suggests that immunofluorescence signal by PAbs was due to cross-reactivity on the native protein linear epitopes recognized by these MAbs are not accessible to antibody recognition of *L. monocytogenes*. The findings of Sabbet *et al.* (2008) suggested post-transcriptional regulation may silence the expression of *inlJ* outside the host. In contrast, the data reported demonstrated the expression of InlJ in *in vitro* culture at the transcription (RT-PCR data not shown) and translational levels. Due to the lack of the reactivity of anti-InlJ MAbs with *L. monocytogenes* serotype 4b L10521 they were not further characterized against various other strains of *L. monocytogenes*. The anti-InlJ MAbs reported here were not recommended to be used in the antibody-based methods for *L. monocytogenes*. Nevertheless, previous studies have shown that *inlJ* is present in a number of *L. monocytogenes* strains while being absent from non-pathogenic species (68). Thus, *inlJ* could be a good gene marker for molecular methods such as PCR, which is another commonly used technique in the detection of *L. monocytogenes*.

5.3 Anti-InlC2 MAbs have the potential to be used for detection and isolation of *L. monocytogenes*

Based on the IFM results showing binding to the surface of *L. monocytogenes* serotype 4b L10521 cells, six anti-InlC2 MAbs were further assessed for their reactivity with various strains of *L. monocytogenes*. Overall, five of the six anti-InlC2 MAbs were capable of recognizing 10 of the 11 *L. monocytogenes* serotypes tested. None of the MAbs reacted with the 3c strain tested. Since only one 3c strain was available for analysis it may not be conclusive whether InlC2 is present and expressed in this serotype. Researchers have suggested that 1/2c and 3c serotypes emerge from the ancestral strains of the 1/2a serotype (91, 92). Given that InlC2 was shown to be present in 9 of 10 1/2a strains and 5 of 5 1/2c strains, it could be speculated that InlC2 is present in other strains of serotype 3c.

In contrast to the anti-LapB MAbs, anti-InlC2 MAbs showed a high degree of variability in their reactivity with *L. monocytogenes* strains tested (Table 6). For example, M3607 was the only MAb that did not recognize the 1/2a serotype strain 6095; M3630 was the only MAb that did not recognize the 4c serotype strain 4706; and M3635 was the only MAb that recognized *L. ivanovii* strain 28. It should be noted that *L. ivanovii* is mainly pathogenic to animals (93). In addition, M3635 recognized the lowest number of *L. monocytogenes* strains tested. Interestingly, M3635 did not recognize any of the 1/2c strains tested. This suggested that the epitope recognized by M3635 is different from those recognized by other anti-InlC2 MAbs and that this epitope is not present in serotype 1/2a strains.

The reactivity scores of anti-InlC2 MAbs (Table 7) demonstrated that M3633 reacted with the highest number of *L. monocytogenes* strains, 46 of 51, and had the highest ELISA OD_{414} readings for the majority of those positive strains. Furthermore, M3633 did not cross-react with any of the non-*L. monocytogenes* spp. tested. Although M3635 did not react with most of the 1/2c strains tested, this MAb did react with the majority of 1/2a, 1/2b and 4b strains which account for more than 90% of listeriosis cases. Since *L. ivanovii* is known to be pathogenic to animals, M3635 could be useful for detecting this pathogen from veterinary clinical samples. In addition to infecting animals, *L. ivanovii* has caused rare infections in humans (23) so it could be valuable to have a MAb that recognizes *L. monocytogenes* strains that cause the majority of human *Listerial* disease, and the animal pathogen *L. ivanovii* while not cross-reacting with any of the other non-pathogenic *Listeria* spp. Moreover, M3633 and M3635 could also be used together as a MAb cocktail in the antibody-based methods for better results.

5.4 LapB and InlC2 are expressed in *L. monocytogenes* under standard culture enrichment conditions

Culture enrichment (primary and secondary) steps are necessary to increase the cell number of *L. monocytogenes* in food samples to a level suitable for isolation and detection. For the anti-LapB and anti-InlC2 MAbs to be used in the antibody-based methods, it was important to determine if InlC2 and LapB proteins were expressed in *L. monocytogenes* cells grown in culture enrichment conditions. A standard procedure (MFHPB30), developed by Health Canada and routinely used by CFIA food microbiology laboratories, uses Palcam and UVM selective broths at two different temperatures (35°C and 30°C) for the primary and secondary growth stages. It is well recognized that expression of bacterial proteins is subjected to regulation by environmental conditions. EM-6E11 and C11E9 are examples of two MAbs that do not recognize *L. monocytogenes* cells grown in UVM broth (94, 95). In contrast, the anti-LapB and anti-InlC2 MAbs resulted from this study were capable of binding to *L. monocytogenes* cultured under the conditions of the MFHPB30 method (Figures 50 and 51). Therefore, these anti-LapB and anti-InlC2 MAbs are more suitable for use in the immunological methods for *L. monocytogenes* than other MAbs such as EM-6E11 and C11E9 described by other researchers (94, 95). This indicated that the LapB and InlC2 proteins were expressed in these culture conditions.

5.5 Conclusion

This study has established a strategy for the development of MAbs to a wide spectrum of *L. monocytogenes* strains by identifying the suitable surface targets through bioinformatic analysis and protein expression analysis. It proved to be successful as several MAbs with the diagnostic value were obtained as the result of applying this strategy. The delivery of MAbs that target two surface associated proteins, LapB and InlC2, which appear to be well conserved in various *L. monocytogenes* serotypes is an important achievement in the development of antibody-based detection methods for this foodborne pathogen. Our results indicated that both anti-LapB MAb M3484 and anti-InlC2 MAb M3633 have the potential to be used as diagnostic reagents for isolating and detecting *L. monocytogenes* from potentially contaminated foods. Separately, these two MAbs recognize the major serotypes 1/2a, 1/2b, and 4b which account for about 95% of human listeriosis (26). Together, M3484 and M3633 have the prospective of detecting all serotypes of *L. monocytogenes* without cross-reacting with other major bacterial pathogens and non-pathogenic bacteria commonly found in foods. Table 10 summarizes the number of strains M3484 and M3633 recognized within each of the 11 *L. monocytogenes* serotypes tested (Table 10A) and also the non-*L. monocytogenes* strains tested (Table 10B).

Table 10. Summary of M3484 and M3633 ELISA reactivity with strains of 11 *L. monocytogenes* serotypes (A) and with non-*L. monocytogenes* bacteria (B).

A

MAb	*L.monocytogenes* serotype 1/2a	1/2b	1/2c	3a	3b	3c	4a	4b	4c	4d	4e
M3484	9/10[a]	9/9	5/5	6/6	2/2	1/1	1/3	8/8	0/3	2/2	1/2
M3633	9/10	9/9	5/5	6/6	2/2	0/1	2/3	8/8	3/3	1/2	1/2

[a] number of strains positive/number of strains tested

B

MAb	*L. innocua*	*L. ivanovii*	*L. grayi*	*L. seeligeri*	*L. welshimeri*	*E. coli*	*S. enterica*	*C. jejuni*
M3484	0/1[a]	0/1	0/1	1/1	0/1	0/2	0/2	0/1
M3633	0/1	0/1	0/1	0/1	0/1	0/2	0/2	0/1

[a] number of strains positive/number of strains tested

If successful, a mix of M3484 and M3633 would be the first MAb cocktail ever developed that is capable of detecting all serotypes of *L. monocytogenes* and does not cross-react with non-pathogenic *Listeria* spp. Thus, this cocktail could potentially be used in a rapid screening immunoassay to presumptively detect the presence of *L. monocytogenes* in contaminated food samples.

5.6 Future work

It is necessary to determine if LapB and InlC2 are detectable on the surface of *L. monocytogenes* cells retrieved from food matrices in a future study. In order to use anti-LapB MAb M3484 and anti-InlC2 MAb M3633 for capturing and isolating *L. monocytogenes* from food, it is necessary to achieve the highest purity and concentration of these MAbs. Culturing the hybridomas in serum-free medium is an advantageous method. The Monoclonal Unit at CFIA Ottawa Laboratory Fallowfield (OLF) successfully developed a procedure for this purpose using INTEGRA CELLine CL1000 hybridoma medium. In addition to circumventing the issue of contamination with mouse or bovine immunoglobulins in the MAb preparation, using the serum-free INTEGRA hybridoma culture medium can yield a higher antibody concentration (approximately 10mg/ml of purified MAb) compared to normal complete DMEM culture medium. The future work will focus on the preparation of M3484 and M3633 in the serum-free medium and the methods based on these two MAbs for *L. monocytogenes*. One of the methods to be developed is the use of these MAbs to capture whole cells of *L. monocytogenes* from food samples spiked with this pathogen, as well as other bacteria commonly found in food such as *L. innocua, E. coli and S. enterica*. The proposed capture method is IMS using magnetic beads coated with the highly purified MAbs.

Low affinity or avidity antibodies is one of the major reasons that antibody-based techniques, such as IMS, fail to isolate *L. monocytogenes* cells (7, 96-98). In order to fully characterize the MAbs produced in this study for diagnostic applications, it would be necessary to map the epitopes for the anti-InlC2 and anti-LapB MAbs and also the affinity of the MAbs to their epitopes. These experiments will allow the selection of a MAb with optimal specificity and high affinity for *L. monocytogenes*. The work presented in this study is only the beginning of a complete characterization of these two MAbs. Since the intended application of these MAbs is for diagnostic purposes, this requires a complete characterization with respect to their epitope, specificity, affinity, and avidity. If the affinity or avidity of these MAbs are determined to be stronger than the current MAbs used with standard tests such as VIDAS, then they could potentially improve the sensitivity of the test, or use as reagent MAbs in VIDAS for testing the food types that are not currently validated by this method (i.e. RTE meat and poultry, smoked fish and yogurt).

Furthermore, these MAbs would greatly improve the detection of *L. monocytogenes* if they could separate and concentrate the bacteria from complex food matrices by IMS. If the antibody-antigen interaction is strong then the cells could be isolated from enrichment culture broths or possibly directly from food samples. IMS has not yet been approved in the Compendium of Analytical Methods (Health Canada) for the detection of *L. monocytogenes* from food but further testing with these MAbs could demonstrate their potential to capture a wide spectrum of *L. monocytogenes* strains from contaminated foods.

CHAPTER VI: REFERENCES

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CHAPTER VII
Contributions of collaborators

The Animal Colony Unit at the Canadian Food Inspection Agency (CFIA) Ottawa Laboratory Fallowfield (OLF) assisted in all animal immunizations for this study.

Splenectomies and fusions were carried out by the Monoclonal Antibody Unit at CFIA OLF.

Jennifer Ronholm and I worked together to prepare the formalin killed *L. monocytogenes* cells for ELISA. Jennifer also provided all IspC MAbs used as positive controls for WB, ELISA and IFM experiments.

APPENDIX A

COMMON SOLUTIONS AND BUFFERS USED

LB Agar containing Kanamycin

Per liter:

10 g Tryptone Peptone
5 g Yeast Extract
10 g Sodium Chloride
12 g *Bacto* Agar

Add kanamycin 50 μg/ml

LB Medium

Per liter:

10 g Tryptone
5 g Yeast Extract
10 g Sodium Chloride

SOC Media

Per 100 mL:

Tryptone 2 g
Yeast Extract 0.5 g
NaCl 0.05 g
MQH_2O up to 100 mL

Sterilize this solution by autoclaving. Then add 2 mL of filter sterilized 1 M glucose.

25 x PBS

Per 1 L:

Sodium phosphate dibasic (Na_2HPO_4) 27.5 g
Sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$) 7.88 g
Sodium chloride (NaCl) 212.5 g
MQH_2O up to 1 L

To make 1 x PBS, 40 mL of 25 x PBS was added to 960 mL MQH_2O.

50 x TAE

Per 1 L:

Tris 242.28 g
Acetic Acid (glacial) 57 mL
EDTA 14.61
MQH_2O up to 1 L

To make 1 x TAE, 20 mL of 50 x TAE was added to 980 mL MQH_2O.

1.5% Agarose Gel

Per gel:

Agarose 0.375 g
1 x TAE 25 mL

Bring solution to a boil over heat while providing constant agitation. Re-boil until agarose is dissolved. Cool. Add 0.25 μl SYBr Safe DNA stain (Invitrogen).

10 x TE Buffer

Per 1 L:

Tris 12.1 g
0.5 M EDTA 20 mL
${}_2$O up to 1 L

To make 1 x TE buffer, 10 mL of 10 x TE buffer was added to 90 mL of MQH$_2$O.

4% Stacking Gel

Per 5 mL:

Acrylamide/Bis (30%) 0.65 mL
0.5 M Tris-HCl pH 6.8 1.25 mL
sMQH$_2$O 3.05 mL
10% (w/v) SDS 50 μl
10% (w/v) APS 50 μl
TEMED 5 μl

12% Separating Gel

Per 10 mL:

Acrylamide/bis (30%) 4 mL
1.5 M Tris-HCl pH 8.8 2.5 mL
sMQH$_2$O 3.35 mL
10% (w/v) SDS 100 μl
10% (w/v) APS 50 μl
TEMED 5 μl

2x SDS-PAGE Loading Buffer

Per 10 mL:

0.5 M Tris-HCl, pH6.8 2 mL
Glycerol 4 mL
β-Mercaptoethanol 2 mL
SDS 0.4 g
0.5% (w/v) Bromophenol Blue 400 μl
MQH$_2$O 1.6 mL

Store at -20ºC.

10 x SDS-PAGE Running Buffer

Per 1 L:

Tris 30 g
Glycine 144 g
SDS 10 g
MQH$_2$O up to 1 L

To make 1 x SDS-PAGE Running Buffer, 100 mL of 10 x SDS-PAGE Running Buffer was added to 900 mL sMQH$_2$O.

Protein Transfer Buffer
Per 1 L:

Tris 5.82 g
Glycine 2.93
177
MQH$_2$O up to 1 L

Ponceau S
Per 100 mL:

Ponceau S 1 g
Acetic Acid (glacial) 100 mL

Coomassie Blue Stain
Per 100 mL:

Coomassie Brilliant Blue 0.5 g
Methanol 40 mL
Acetic acid (glacial) 10 mL

Make volume up to 100 mL with MQH$_2$O.

Coomassie Blue Destain soluton
Per 100 mL:

Methanol 40 mL
Acetic acid (glacial) 10 mL

Make volume up to 100 mL with MQH$_2$O.

PBS-T
Per 10 L:

25 x PBS 400 mL
Tween 20 5 mL
MQH$_2$O 9595 mL

PBS-TT
Per 10 L:

25 x PBS 400 mL
Tween 20 5 mL
Triton X-100 20 mL
MQH$_2$O 9575 mL

Wash Buffer A (Immobilized Metal Chelate Affinity Chromatography)
Per 1 L:

Sodium Phosphate monobasic 6.9 g
Sodium Chloride 17.4 g
Imidazole 1.36 g
MQH$_2$O ~500 mL

Adjust the pH to 8.0 using sodium hydroxide. Make volume up to 1 L by adding MQH$_2$O.

Wash Buffer B (Immobilized Metal Chelate Affinity Chromatography)

Per 1 L:

Sodium Phosphate monobasic 6.9 g
MQH_2O ~500 mL

Adjust the pH to 8.0 using sodium hydroxide. Make volume up to 1 L by adding MQH_2O.

Carbonate buffer

Per 1 L:

Sodium Bicarbonate 3.8 g
Sodium Carbonate 1.93 g
MQH_2O ~ 950 mL

Adjust pH to 9.6 and make volume up to 1 L with MQH_2O.

Citrate Buffer

Per 1 L:

Citric Acid 4.6 g
Tri-Sodium Citrate 7.65 g
MQH_2O ~ 950 mL

Adjust pH to 4.5 and make volume up to 1 L with MQH_2O.

ABTS (2, 2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid))

For 10 mL measure the weight of ABTS as close to 0.2195 g as possible and then calculate the volume of water based on V (mL) = W x (10/0.2195). Add required MQH_2O.

ELISA Substrate Solution

Per ~10 mL:

ABTS solution 0.5 mL
3% (v/v) Hydrogen Peroxide 0.1 mL
Citrate Buffer 10 mL

EXPERIMENTAL PROTOCOLS:

Protein G Affinity Chromatography:

1. Set the range of the Econo Recorder (Bio-Rad) to 0.2 or 0.5 and the chart speed to 12 cm/hr.
2. Clean all tubing attached to the Econo Gradient Pump (Bio-Rad) with fresh MQH O and then 1 x PBS.
3. Attach the Protein G Sepharose (GE Healthcare) column to the correct tubing.
4. Equilibrate the column with 20 mL 1 x PBS.
5. Near the end of the equilibration press and hold the Auto Zero the Econo Recorder until it is zeroed.
6. Change the valve to the loading position.
7. Load the TCF containing the antibody at a rate of 0.5 mL/min.
8. Change the valve to the wash position. Wash the column with 20 mL 1 x PBS at a flow rate of 1 mL/min.
9. Place 20 glass tubes in the fraction collector (Bio-Rad). Set the fraction size to 1mL.
10. Elute the antibodies with 20 mL 100 mM Glcyine-HCl pH 2.5 at a rate of 1mL/min.
11. Add 140 µl 1 M Tris-HCl (pH 9.1) to neutralize each fraction and confirm neutralization using pH paper.
12. Pool fractions containing antibodies and place on ice.
13. Estimate the concentration using OD at 278 nM with the absorption coefficient 1.35 A.mL.mg^{-1}.
14. Store at 4ºC.

Immobilized Metal Chelate Affinity Chromatography:

1. Lyse *E. coli* cells with the French Press at 1500 psi.
2. Centrifuge the homogenate at 27 000 x g for 20 min at 4ºC.
3. Store supernatant on ice. Discard pellet.
4. Set the range of the Econo Recorder (Bio-Rad) to 0.5 or 1 and the chart speed to 12 cm/hr.
5. Clean all tubing attached to the Econo Gradient Pump (Bio-Rad) with fresh MQH$_2$O and then Wash Buffer A.
6. Attach the column (Qiagen) to the correct tubing.
7. Equilibrate the column with 25 mL of Wash Buffer A.
8. Near the end of the equilibration press and hold the Auto Zero the Econo Recorder until it is zeroed.
9. Change the valve to the loading position.
10. Load the protein sample at a rate of 0.5 mL/min.
11. Wash the column with 30 mL of Wash Buffer A (or until Econo Reader shows the flow-through is back to base-line absorbance).
12. Place 20 glass tubes in the fraction collector (Bio-Rad). Set the fraction size to 1mL.
13. Elute the recombinant protein with 25 mL Wash Buffer B at 1 mL/min.
14. Store all the collected fractions at 4ºC.
15. Analyze each fraction SDS-PAGE followed by Western Blotting with an anti-HIS antibody.

Bio-Rad Protein Assay Protocol:

1. Warm up the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad) to room temperature.
2. Prepare BSA standards (0 μg/mL, 1 μg/mL, 2.5 μg/mL, 5 μg/mL, 7.5 μg/mL, 10 μg/mL, 12 μg/mL) to a total volume of 800 μl.
3. Dilute protein samples to be measured (dilution factor will depend on protein concentration), to a total volume of 800 μl.
4. Add 200 μl of dye to each standard and sample.
5. Incubate for 10 min at room temperature.
6. Blank the spectrophotometer with 0 μg/mL BSA and measure the absorbance of each sample at 595 nm.
7. Generate a graph of absorbance 595 nm vs concentration μg/mL of the standards.
8. Perform a linear regression to determine x = (y-b)/m and solve for x.
9. Based on the dilution in step 3, use the formula $C_1V_1=C_2V_2$ to calculate the initial sample protein concentration.

Extraction of *L. monocytogenes* genomic DNA Protocol:

1. Innoculate *L. monocytogenes* in 2 mL of BHI broth and grow overnight at 37ºC.
2. Place 1 mL of culture into a 1.5 mL tube and centrifute at 16 100 x g for 2 min.
3. Re-suspend the cells in 100 μl 1x TE buffer.
4. Add 6 μl of 50 mg/mL lysozyme and digest at 37ºC for 30 min.
5. Add 500 μl DNAzol (Invitrogen) and mix.
6. Add 250 μl of ethanol and invert 50 times.
7. Centrifuge at 16 100 x g for 1 min and discard the supernatant.
8. Wash pellet twice with 750 μl of 75% (v/v) ethanol.
9. Briefly air-dry the pellet.
10. Dissolve the pellet in MQH_2O.
11. Store at -20ºC.

QIA MINIPREP Protocol:

1. Grow cells containing plasmid overnight on media with appropriate antibiotics.
2. Spin down 5 mL of cells.
3. Re-suspend the pellet in 250 μl P1 buffer.
4. Add 250 μl of P2 buffer and gently invert the tube 4-6 times.
5. Add 350 μl of P3 buffer and invert the tube 4-6 times.
6. Centrifuge at 16 100 x g for 10 min.
7. Apply the supernatant to the supplied spin column by pipetting.
8. Centrifuge at 16 100 x g for 60 s, discard the flow-through, add remaining supernatant and centrifuge at 16 100 x g. Discard the flow-through.
9. Add 500 μl PB buffer to the spin column, centrifuge at 16 100 x g for 60 s and discard the flow-though.
10. Add 750 μl of PE buffer to the spin column, centrifuge at 16 100 x g for 60 s and discard the flow-through.
11. Centrifuge at 16 100 x g for 60 s.
12. Place the spin column in a clean 1.5 mL tube.
13. Add 50 mL of EB buffer to the centre of the spin column membrane and let stand

for 60 s.
14. Centrifuge at 16 100 x g for 60 s.
15. Store plasmid at -20ºC until use.

Preparation of $CaCl_2$ Competent *E. coli* cells Protocol:
1. Grow *E. coli* in 5 mL of LB broth overnight.
3. Chill the culture on ice for 10 min.
4. Centrifuge cells at 2700 x g and 4ºC for 10 min.
5. Discard the supernatant.
6. Re-suspend the pellet in 24 mL of cold 80 mM $MgCl_2$ – 20 mM $CaCl_2$ and agitate gently on ice for 20 min.
7. Centrifuge at 2700 x at 4ºC for 10 min.
8. Discard the supernatant.
9. Resuspend the pellet in 1.6 mL ice cold 0.1 M $CaCl_2$ in 10% (v/v) glycerol and agitate until re-suspended.
10. Dispense into 50 µl aliquots.
11. Chill aliquots at -20ºC overnight and then store at -80ºC.

Transformation of $CaCl_2$ Competent *E. coli* cells by Heat Shock Protocol:
1. Add 35-50 µl of competent cells to a 1.5 mL tube.
2. Add 5-10 µl of ligation reaction or 2 µl of uncut plasmid DNA to the tube.
3. Mix.
4. Incubate on ice for 30 min.
5. Place tube in 42ºC water bath for 45 s.
6. Incubate on ice for 2 min.
7. Add 250 µl SOC media to the tube.
8. Incubate with agitation at 37ºC for 1 hour
9. Make a 1:100 dilution of 2.5 µl culture in 223.5 µl LB broth and plate on an LB agar plate.
10. Plate the rest of the culture on an LB agar plate containing the correct antibiotics.
11. Incubate the plates overnight at 37ºC.

Preparation of Electroporation Competent *L. monocytogenes* cells Protocol:
1. Inoculate LB broth with *L. monocytogenes* and culture at 37ºC overnight.
2. Subculture at a 1:10 dilution in LB broth containing 0.5 M sucrose, until the OD at 600 nm is ~ 0.2.
3. Add penicillin G to a final concentration of 10 µg/mL.
4. Incubate at 37ºC for 2.5 hours.
5. Calculate the cell concentration using a ratio of an OD at 620 of 0.61 is equal to $1x10^9$ cells/mL.
6. Centrifuge the culture at 8000 x g at 4ºC for 10 min.
7. Wash with sucrose electroporation buffer three times.
8. Re-suspend bacteria in sucrose electroporation buffer to a concentration of $1x10^{11}$ cells/mL.
9. Add sterile glycerol to a final concentration of 15% (v/v) and store cells at -20ºC for future use.

Curriculum vitae

SUMMARY OF QUALIFICATIONS

- Master's of Science degree in Microbiology and Immunology, University of Ottawa
- Over 3 years of practical experience with standard laboratory procedures
- Superior proficiency in Microsoft Word, Excel, PowerPoint and internet research
- Excellent oral and written communication skills

EDUCATION

Masters in Microbiology and Immunology **Sept 2010- present**
Department of Biochemistry, Microbiology and Immunology
Faculty of Medicine, University of Ottawa

Thesis title: *Developing monoclonal antibodies that target surface virulence factors for the rapid detection of Listeria monocytogenes*
The Canadian Food Inspection Agency, Ottawa Laboratory Fallowfield, Ottawa, Ontario
Supervisor: Dr. Min Lin

Baccalaureate, Honours in biology, **Sept 2005- Dec 2009**
Specialization in cellular and molecular biology
Faculty of Science, University of Ottawa, Ontario
Supervisors: Dr. Balu Chakravarthy and Dr. Michael Jonz

Honours project title: *Understanding the role of neurotrophin receptor,* $p75^{NTR}$*, in amyloidogenesis and the development of Alzheimer's disease*
The National Research Council Canada, Ottawa, Ontario

ACADEMIC WORK EXPERIENCE

Teaching assistant- biochemistry laboratory demonstrator **Jan-Mar 2012**
Department of Biochemistry, University of Ottawa
- Demonstrated the enzymes kinetics lab
- Supervised a total of 192 students (16 students in each of the 12 lab sections)
- Was accountable for marking a total of 110 lab reports

Field biologist (university course)
Aug 2008
Koffler Scientific Reserve, Toronto, Ontario
- Collected data and practiced sampling techniques
- Designed and completed an independent field research project on plant reproduction

VOLUNTEER EXPERIENCE

Women's health centre assistant **May-Sept 2008**

The Ottawa Hospital, Riverside Campus, Ottawa, Ontario

- Assisted with clerical paperwork for the five doctors on staff
- Scheduled appointments with patients

Database researcher **Jan-Apr 2008**

Institute of the Environment, Ottawa, Ontario

- Generated a database from a set of 130 wildlife species that are monitored by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC)
- Used the data and sound judgement to answer a number of questions regarding COSEWIC decision making and the content of wildlife status reports

LABORATORY SKILLS

General

- Write and update experimental protocols, lab book data entry and interpretation, ordering consumables and supplies, proper handling and storing of chemicals, using, cleaning and maintaining various laboratory equipment, budgeting project funds

Biochemistry

- Polymerase chain reaction (PCR), reverse transcriptase PCR (RT PCR), chromatography (affinity, ion-exchange)

Molecular biology

- Cloning, tissue culture, cell fractionation, protein and DNA extraction, standard protein assay, gel electrophoresis and western blot

Microbiology

- Sterile techniques, level 1 and 2 biosafety, bacterial culture, extraction and purification of bacterial DNA

Immunology

- Immunofluorescence microscopy, immunoprecipitation of proteins, polyclonal and monoclonal antibody production, enzyme linked immunosorbant assay (ELISA)

Small animal work

- Rabbit and mouse immunizations, bleedings and splenectomy

ASSOCIATIONS

Quality assurance team **July 2011-present**
Canadian Food Inspection Agency

- Enforce lab safety standard operations
- Record all data accurately and according to ISO17025 standards
- Verify and calibrate thermometers, pipettes and incubators

Let's talk science **Dec 2010-present**
University of Ottawa

- Independently organize and perform hands-on science activities with schools across Ottawa (Forensic science, Chemistry magic show and Feast for the senses)
- Mentoring aboriginal students at General Vanier Secondary School in Cornwall, Ontario
- Judge Science Fairs

CERTIFICATES

ISO17025, Radiation Safety Course**,** Canadian Council on Animal Care (CCAC) Institutional Animal User Training Program, Workplace Hazardous Materials Information System (WHMIS)

CONFERENCES

Ontario Municipal Water Association Conference **May 2011**
Blue Mountain Resort, Collingwood, ON

Federal Food Safety Research Conference **Nov 2010**
Chateau Cartier, Gatineau, Quebec

SCHOLARSHIPS

Walkerton Clean Water Scholarship ($10,000)
May 2011
Walkerton Clean Water Center, Walkerton ON

BSc Entrance Scholarship ($16,000) **Sept 2005**
University of Ottawa, Ottawa ON
