

Pathogen-specific antigen target for production of antibodies produced by comparative genomics

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Abstract: *Listeria monocytogenes* continues to be a major public health risk and there is a need for improved rapid detection methods. New and highly specific *L. monocytogenes* antibodies are needed to advance current detection and meet the needs of industry. This research compared the *L. monocytogenes* genome with that of *L. innocua* (a nonpathogenic species of *Listeria*) and identified nine surface proteins specific to *L. monocytogenes*. Protein sequences were collected from the database and properties such as hydropathy profile and transmembrane topology were analyzed using TMpred software. Nine peptide sequences were chosen, and synthetic peptides were made and administered to rabbits for antibody production. All nine antibodies were screened against a panel of *L. monocytogenes*, nonpathogenic *Listeria*, and non-*Listeria* bacteria. Two of the nine antibodies, ie, the Lm404 and LmC639 polyclonal antibodies, showed a specific reaction to *L. monocytogenes* internalin B and actin polymerization protein, respectively, and were characterized further by enzyme-linked immunosorbent assay, Western blot, and transmission electron microscopy. In Western blot, both antibodies reacted with the targeted protein and did not cross-react with other *Listeria* spp. The Lm404 polyclonal antibody showed a high reaction with the panel of 41 *L. monocytogenes* strains while the LmC639 polyclonal antibody showed a weak reaction. Both the Lm404 and LmC639 polyclonal antibodies showed potential for use in immunoassays for specific detection of *L. monocytogenes*. This study further indicates that comparative genomics could be used to select pathogen-specific antigen for antibody production.

Keywords: comparative genomics, *Listeria monocytogenes*, polyclonal antibodies, internalin B, actin polymerization protein, proteomics

Introduction

Listeria monocytogenes is an invasive foodborne pathogen that has historically been a major problem in the meat and dairy industries and has now become an issue in the produce industry.^{1,2} In recent years, this organism caused several outbreaks in multiple states in the United States, with numerous fatalities including stillbirths and abortions.^{3–6} The food industry is under constant pressure to detect and eliminate *L. monocytogenes* from finished products and the processing environment. To successfully manage this organism, specific and sensitive detection of *L. monocytogenes* is critical for ensuring food safety. Conventional culture methods, such as those as outlined by the US Department of Agriculture and US Food and Drug Administration, are reliable but take about 5–7 days for detection and confirmation of *L. monocytogenes* from foods.⁷ Antibody-based immunoassays are rapid, but most commercially available antibody-based assay kits are *Listeria* genus-specific because suitable antibodies specific for *L. monocytogenes* are lacking.⁸

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The most common *Listeria* antigens used for antibody production have been flagella, listeriolysin O, phosphatidylcholine-specific phospholipase C (68 kDa), and p60 proteins.^{8–10} Many of these antibodies react to antigens that are either shared by pathogenic and nonpathogenic *Listeria* species or only specific to certain serotypes, or their expression is affected by various environmental factors, such as pH, temperature, and carbon sources.^{11–15} Monoclonal antibodies developed to listeriolysin O and phosphatidylcholine-specific phospholipase C from *L. monocytogenes* showed cross-reaction with *L. ivanovii*.¹⁶ A monoclonal antibody (EM-7G1) specific for *L. monocytogenes* was developed against a 66 kDa surface antigen; however, the monoclonal antibody failed to react with all but three serotypes (3b, 4a, 4c) of *L. monocytogenes*.¹⁷ Sølve et al¹⁸ also developed monoclonal antibodies that reacted with live *L. monocytogenes* and *L. innocua* but showed no reaction with the other *Listeria* species tested. The invasion-associated protein p60 was used to develop polyclonal antibodies and showed reaction with all *Listeria* species,¹⁹ and a monoclonal antibody against the recombinant p60 detected *L. monocytogenes* and other *Listeria* spp.¹⁰ *L. monocytogenes* 4b specific monoclonal antibodies showed cross reaction with serotype 4d and 4e and *L. innocua* strains.²⁰ Paoli et al²¹ developed a single-chain Fv antibody to *L. monocytogenes* that reacted with no other *Listeria* species, but failed to react with several serovars of *L. monocytogenes*. A polyclonal antibody against internalin B was also reported, and this antibody was used in a surface plasmon resonance sensor for detection of *L. monocytogenes*. However, the characteristics of this antibody's reaction with different *L. monocytogenes* strains or other *Listeria* species are unknown.²² A sandwich enzyme-linked immunosorbent assay (ELISA) has been developed using a flagella-specific monoclonal antibody for detection of *Listeria* species.⁹ Recently, a monoclonal antibody against internalin A was found to be highly specific and was used for detection of *L. monocytogenes* and *L. ivanovii* in a fiber optic immunosensor.²³ While many antibodies have been developed for *L. monocytogenes*, there is still a need for more specific and highly reactive antibodies.

The genome sequences of *L. monocytogenes* and *L. innocua* have been published.^{24–26} Genetic analysis indicated that there were 270 *L. monocytogenes*-specific and 149 *L. innocua*-specific genes,²⁵ and genes for major virulence factors, such as internalin A, internalin B, actin polymerization (ActA), listeriolysin O, PlcA, and PlcB in *L. monocytogenes* were conserved.²⁶ Comparative genome sequence analyses further revealed that there

were 22 surface proteins unique to *L. monocytogenes*. Of these 22 proteins, five belonged to the internalin multigene family, one was ActA protein, and the remaining 16 were unknown.²⁷ Our goal was to develop antibodies against these proteins for use in immunoassays for specific detection of *L. monocytogenes*. Furthermore, we also verified if the antibody-specific epitopes would be surface-exposed for interaction with antibody in whole cell ELISA and immunoelectron microscopy. This study shows that comparative genomics could be used as a tool to select pathogen-specific antigens for antibody production.

Materials and methods

Cultures and media

Frozen stock cultures of all *Listeria* species and other non-*Listeria* cultures (Tables 1–3) were subcultured in brain heart infusion broth and maintained on stabs of brain heart infusion agar (Becton Dickinson, Sparks, MD, USA) for 2–3 months at room temperature. For fresh cultures, samples of the stabs were inoculated into brain heart infusion broth and grown at 37°C for 18–20 hours. *L. monocytogenes* DP-L2723 (containing recombinant ActA His-tagged protein, a gift from Daniel Portnoy, University of California, Berkeley, CA, USA) was grown in Luria-Bertani broth with 10 µg/mL of chloramphenicol overnight and shaking.²⁸ Internalin B containing *Escherichia coli* strain BL21 (DE) with pET28b-1 (a gift from Pascale Cossart, Institut Pasteur, Paris, France) was grown overnight with shaking in Luria-Bertani broth with 30 µg/mL of kanamycin.²⁹

Protein sequence analysis and selection of unique peptide epitope as an immunogen

Comparative genome sequencing of *L. monocytogenes* and *L. innocua* indicated that there existed about 22 unique surface proteins of *L. monocytogenes*, the orthologs of which were absent in a nonpathogenic *L. innocua* strain.²⁵ From the 22 unique surface proteins, nine were selected (Table 1). Properties such as hydropathy profile based on the Kyte and Doolittle hydrophobicity scale³⁰ and transmembrane topology were analyzed using the ProtScale and TMPred programs (ExpASy software, Swiss Institute of Bioinformatics, Lausanne, Switzerland). This information was used to select putative surface-exposed 20-mer peptide sequences. A Protein BLAST search (BLASTP) was used to confirm the uniqueness of the chosen peptides in the National Center for Biotechnology Information database.

Table 1 Selected unique target peptide sequences of *Listeria monocytogenes* obtained from *Listeria* genome sequence database as immunogens and respective antibody titers against peptide antigens and whole cells

Target protein	Protein size (amino acids)	Molecular mass (kDa)	Function	Target 20-mer peptide sequence ^a	Antibody designation	Antibody titer ^b	
						Peptide	Whole cells
Internalin A	800	80	Invasion	276 TDLDLANNQISNLAPLSGLT	LmA800	288,000	160
Internalin B	630	63	Invasion	228 GLKNLDVLELFSQECLNKPI	Lm404	163,840	640
Internalin H	548	55	Unknown	181 PLAGLTNLQYLSIGNAQVSD	LmH548	16,000	160
ActA protein	639	90	Cell-to-cell spread	110 QTENAAINEEASGADRP AIQ	LmC639	88,000	160
Lmo0460	689	69	Putative membrane protein	547 LNQDCTNKFDLSPADASIT	Lm405	40,960	160
Lmo1115	862	86	Hypothetical protein	416 HSPNLYLGEYQAVEVSAPAG	Lm406	160	NT
Lmo1290 (internalin K)	598	60	Escape from autophagy	302 SKTDFDNLEKMEYNARIDLS	Lm1290	135,000	160
Lmo2026	626	63	Peptidoglycan binding protein	250 LTPDKTNLGLKSNIPFSQH	Lm407	40,960	640
Lmo2085	562	56	Peptidoglycan binding protein	420 FKQDEANKKGLANAVFDVKS	Lm408	81,920	160

Notes: ^aNumber represents the starting position of the peptide in the protein sequence; ^btiter is the dilution of the antibody that gave 50% of the maximum achieved response on indirect enzyme-linked immunosorbent assay. The peptide concentration was 15 µg per well, and that of bacterial cells was $\sim 10^8$ colony-forming units per well.

Abbreviation: NT, not tested.

Peptide synthesis and antibody production and purification

Peptides for internalin B, Lmo0460, Lmo1115, Lmo2026, and Lmo2085 that consisted of 20 amino acids (Table 1) with an additional cysteine residue (needed for conjugation with keyhole limpet hemocyanin [KLH]) were synthesized in the Laboratory for Macromolecular Structure at Purdue University, purified by high-performance liquid chromatography using a C-18 reverse phase column, and subjected to mass spectrometric analyses. Each purified peptide (2 mg)

from Purdue University was conjugated with 2 mg of KLH using a 77511 kit (Imject[®], maleimide-activated mcKLH, Pierce, Rockford, IL, USA) and injected subcutaneously into two rabbits for antibody production (Animal Science Department, Purdue University). Rabbits were injected with 150 µg of conjugated peptide every 3 weeks, and after the third immunization, the serum was collected every 3 weeks until the rabbits were terminally bled (after four bleeds). The remaining peptides for internalin A, internalin H, ActA, and Lmo1290 (internalin K) were synthesized, conjugated, and antibodies

Table 2 Reaction patterns of eight antibodies to different *Listeria* and non-*Listeria* cultures on indirect enzyme-linked immunosorbent assay (absorbance 490 nm)

Cultures	Lm404	Lm408	Lm407	Lm405	LmA800	LmC639	Lm1290	LmH548
<i>Listeria monocytogenes</i> V7	3.97±0.05	1.01±0.25	0.29±0.08	0.82±0.03	0.87±0.05	0.69±0.31	0.75±0.38	1.56±0.00
<i>L. monocytogenes</i> F4244	1.27±0.21	0.90±0.12	0.38±0.14	0.76±0.02	0.24±0.06	0.34±0.05	0.51±0.08	0.32±0.06
<i>L. monocytogenes</i> Scott A	0.18±0.04	0.40±0.13	0.14±0.04	0.24±0.02	0.13±0.06	0.12±0.06	0.25±0.11	0.14±0.05
<i>L. innocua</i> LA-1	0.27±0.12	0.76±0.05	0.32±0.12	0.74±0.01	0.26±0.03	0.20±0.04	0.51±0.12	0.29±0.06
<i>L. innocua</i> F4248	0.22±0.08	0.74±0.15	0.33±0.13	0.91±0.03	0.24±0.06	0.19±0.03	0.58±0.14	0.38±0.10
<i>L. grayi</i> ATCC19120	0.44±0.10	1.04±0.06	0.33±0.06	0.95±0.03	0.28±0.10	0.42±0.09	0.70±0.08	0.49±0.02
<i>L. ivanovii</i> SE98	0.29±0.13	0.62±0.11	0.25±0.05	0.39±0.02	0.19±0.06	0.15±0.06	0.41±0.05	0.23±0.07
<i>L. seeligeri</i> SE31	0.30±0.06	0.40±0.07	0.18±0.06	0.31±0.01	0.27±0.11	0.25±0.18	0.41±0.18	0.22±0.07
<i>L. welshimeri</i> ATCC35897	0.26±0.05	0.79±0.12	0.30±0.06	0.53±0.10	0.22±0.04	0.34±0.03	0.60±0.16	0.31±0.05
<i>L. marthii</i> BAA-1595	0.34±0.01	0.76±0.03	1.14±0.04	1.59±0.11	0.76±0.03	0.66±0.06	NT	NT
<i>L. rocourtiae</i> CIP109804	0.37±0.01	1.11±0.02	0.69±0.02	1.55±0.15	1.11±0.02	0.71±0.01	NT	NT
<i>Bacillus cereus</i> 4AC	0.71±0.06	0.82±0.04	0.30±0.11	0.80±0.05	0.25±0.04	0.29±0.02	0.60±0.11	0.26±0.04
<i>Citrobacter freundii</i> ATCC3624	0.34±0.16	0.48±0.02	0.45±0.24	1.00±0.05	0.29±0.08	0.48±0.23	0.74±0.35	0.35±0.04
<i>Escherichia coli</i> ATCC51739	1.37±0.28	2.26±0.40	3.07±0.41	2.90±0.20	2.01±0.33	1.77±0.22	2.48±0.33	3.27±0.11
<i>Salmonella enterica</i> serovar Enteritidis ATCC13096	2.24±0.07	2.59±0.32	3.14±0.25	3.43±0.07	2.16±0.34	2.13±0.30	2.84±0.17	3.32±0.10

Notes: Bacterial cell concentration used: 10^8 colony-forming units per well. Antibody dilutions used: Lm404 and Lm407 at 1:1,000; Lm407 and Lm405 at 1:300; and LmA800, LmC639, Lm1290, and LmH48 at 1:100.

Abbreviation: NT, not tested.

Table 3 Analysis of Lm404 and LmC639 polyclonal antibody reaction patterns to different *Listeria monocytogenes* strains grown in brain heart infusion broth on indirect enzyme-linked immunosorbent assay (absorbance 490 nm)

<i>Listeria monocytogenes</i> strain	Serotype	Lm404 ^a	LmC639 ^b
SLCC 2482	7	0.89±0.13	0.18±0.06
V37CE	1/2a	0.73±0.09	0.37±0.03
I03M	1/2a	0.60±0.10	1.01±0.13
I71	1/2a	0.75±0.02	0.33±0.02
F4263	1/2a	0.64±0.20	0.73±0.06
ATCC 35152	1/2a	0.51±0.05	0.23±0.05
ATCC 15313	1/2a	0.68±0.02	0.21±0.08
FSL R2-499	1/2a	0.67±0.17	0.49±0.05
V7	1/2a	3.97±0.05	0.44±0.06
F4233	1/2b	0.75±0.15	0.31±0.06
F4260	1/2b	0.67±0.04	0.37±0.03
ATCC 19112	1/2c	0.62±0.12	0.35±0.10
ATCC 19113	3a	0.69±0.05	0.17±0.02
V47	3a	0.63±0.06	0.32±0.08
V127	3a	0.97±0.21	0.30±0.06
ATCC 19114	4a	0.43±0.11	0.24±0.12
Murray B	4ab	1.95±0.12	0.32±0.05
I01M	4b	1.78±0.24	0.26±0.03
CAP	4b	0.93±0.18	0.25±0.09
F4393	4b	0.65±0.07	0.36±0.01
F4243	4b	2.12±0.12	0.33±0.04
F1057	4b	1.60±0.16	0.28±0.05
F4264	4b	1.14±0.23	0.28±0.11
F4262	4b	0.85±0.08	0.42±0.02
F2379	4b	0.82±0.15	0.28±0.06
ATCC 19115	4b	1.66±0.15	0.25±0.08
FSL NI-227	4b	1.07±0.20	0.33±0.08
FSL NI-225	4b	0.93±0.18	0.28±0.11
F4244	4b	1.27±0.21	0.38±0.08
Scott A	4b	0.56±0.06	0.24±0.08
FSL R2-500	4b	1.00±0.17	0.31±0.14
FSL R2-501	4b	1.40±0.39	0.44±0.17
ATCC 19116	4c	0.82±0.08	0.24±0.08
V53	4c	0.76±0.14	0.33±0.06
ATCC 19117	4d	0.68±0.05	0.34±0.15
ATCC 19118	4e	0.92±0.09	0.44±0.03
C12-S(L)	NK ^a	0.65±0.03	0.30±0.05
V30	NK	0.63±0.11	0.61±0.29
V206	NK	3.23±0.04	0.73±0.42
V207	NK	0.50±0.06	0.36±0.11
ATCC 13932	NK	0.65±0.29	0.45±0.26

Notes: ^aLm404 polyclonal antibody was diluted 1:1,000; ^bLmC639 polyclonal antibody was diluted 1:100.

Abbreviation: NK, not known.

were produced at SynPep Corporation (Dublin, CA, USA). Anti-internalin B (Lm404) and anti-ActA (LmC639) antibodies were also produced in rabbits raised in a specific pathogen-free environment (two rabbits for each antigen) at Lampire Biological Laboratories (Pipersville, PA, USA). Pre-immune serum from each rabbit was found to be negative when tested

against *Listeria* species before the start of the immunization regimen. The antibody titers were determined using synthetic peptides and whole cells as an antigen in ELISA.

Antibodies (immunoglobulin G fractions) from the serum were purified by protein A affinity liquid chromatography. Briefly, 2 mL of serum from each sample was purified on a protein A column using an ÄKTA prime unit (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Undiluted samples were loaded onto the column with 20 mM phosphate-buffered saline (pH 7.4) and eluted with 0.1 M glycine (pH 2.4). The eluent was collected, pH-adjusted to 7.0 with 1 M Tris, and stored at 4°C with 1% bovine serum albumin and 0.01% thimerosal.

ActA and internalin B purification

His-tagged ActA protein was purified from chloramphenicol-resistant *L. monocytogenes* DP-L2723³¹ as described elsewhere²⁸ using a 70239-3 His-Bind[®] purification kit (Novagen, Madison, WI, USA). Fractions were analyzed by Western blot to confirm purification. The final protein concentration was determined using the 23236 Better Bradford assay kit (Pierce). Internalin B was purified from an internalin B-expressing *E. coli* strain BL21 (DE3) containing pET28b-1²⁹ using the His-Bind purification kit as described previously.³² These preparations were used as controls in Western blot assays.

Reaction profile of polyclonal antibodies to synthetic peptides and bacteria by indirect ELISA

Bacterial cells grown in brain heart infusion broth for 20 hours were harvested by centrifugation (9,300× *g* for 10 minutes) and resuspended in equal volumes of 0.1 M carbonate coating buffer (pH 9.6) to yield a final concentration of 1×10⁹ colony-forming units per mL.³³ Peptides were diluted in 0.1 M carbonate coating buffer (pH 9.6) to yield a final concentration of 150 µg/mL. One hundred microliters of the bacteria or the peptides was dispensed per well into a 96-well microtiter plate (Dynex, Chantilly, VA, USA) and stored overnight at 4°C. ELISA was performed as described previously³⁴ using affinity-purified rabbit polyclonal antibodies, a secondary goat anti-rabbit immunoglobulin G-peroxidase conjugate antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), and o-Phenyldiamine substrate (Sigma-Aldrich, St Louis, MO, USA).

SDS-PAGE and Western blot analysis of surface protein extracts from *Listeria*

Fifteen milliliters of *Listeria* cultures were grown in brain heart infusion broth at 37°C for 18 hours. Cell surface

proteins were extracted using Tris-HCL buffer as described before³⁵ and quantified by Bradford assay. Lack of contamination of surface proteins with intracellular proteins were verified by PepC assay.³⁵ Proteins (2.5 µg/well) were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel (7.5% acrylamide) and blotted onto an Immobilon-P membrane (Millipore, Bedford, MA, USA). The membranes were blocked with 5% bovine serum albumin (Sigma-Aldrich) overnight at 4°C and washed four times with phosphate-buffered saline-Tween 20 (PBS-T, 0.5%). The antibodies were diluted in phosphate-buffered saline and incubated with the membrane at 37°C for one hour. The membranes were washed four times with PBS-T followed by incubation for one hour with goat-anti rabbit immunoglobulin G-peroxidase conjugate (1:5,000, Bio-Rad Laboratories Inc., Hercules, CA, USA). After washing three times with phosphate-buffered saline, the substrate containing diaminobenzidine tetrahydrochloride (Sigma-Aldrich) was added and allowed to react for 10 minutes.³⁴

Results

L. monocytogenes proteomic analysis and peptide selection

Based on the comparative genome sequence of *L. monocytogenes* and *L. innocua*, nine of the 22 proteins were selected (Table 1); their amino acid sequences were collected from the database and their hydropathy profile and transmembrane topology were predicted using the ProtScale and TMpred software programs. For the internalin proteins A, B, and H, the predicted N-terminal transmembrane segments were located in amino acids 6–27, 11–31, and 7–26, respectively. Internalin B had no predicted C-terminal transmembrane segment, while amino acids 775–792 of internalin A and 524–541 of internalin B were predicted to be membrane spanning regions. Internalin H had one additional predicted transmembrane segment between amino acids 75 and 97. ActA had both an N-terminal and a C-terminal predicted transmembrane segment at 9–29 and 614–630, respectively. The N-terminal and C-terminal transmembrane segments for the other proteins were as follows: Lmo0460 (8–27, 172–192), Lmo1290 (internalin K; 6–31, 577–594), Lmo1115 (1–18, 835–854), Lmo2026 (5–23, 600–618) and Lmo2085 (4–20, 542–559). The selected peptide segments were predicted to be hydrophilic and possibly located outside the cytoplasmic membrane. The selected peptides showed no homology with proteins from other *Listeria* species or other microorganisms when searched against the National Center for Biotechnology Information BLASTP database.

Reaction of polyclonal antibodies to the synthetic peptides or whole cell antigens

Purified antibodies obtained from antisera collected from third bleeding were tested for antibody reaction titer with prospective peptide antigen and whole cells. All but one antibody (Lm406) had a high titer with respective peptide antigens, indicating successful production of peptide-reactive antibodies (Table 1). No antibody was detected against Lm406, so no further experiments were conducted with this antibody. The ELISA titration data indicated that the peptide antigens were able to induce the immune system to allow successful production of antibodies. In contrast with this, six of eight antibodies showed weak antibody titration (160 titer) against whole live cells of *L. monocytogenes* while two (Lm404 and Lm407) had an antibody titer of 640, indicating that more antibody-reactive epitopes for Lm404 and Lm407 were surface-expressed compared with the other tested antibodies.

Reaction profiles for polyclonal antibodies with different bacterial cultures

All polyclonal antibodies were tested against a panel of eight *Listeria* species and four non-*Listeria* species. The Lm404 polyclonal antibody showed a strong reaction with two of the three *L. monocytogenes* cultures but showed a weaker reaction with other *Listeria* species (Table 2). All other antibodies showed little difference between the reaction with *L. monocytogenes* and the other *Listeria* species tested. A high reaction (Abs₄₉₀ > 1.3) with *E. coli* and *Salmonella enterica* serovar Enteritidis was observed with all nine antibodies. Upon further analysis of protein A-purified preimmune sera, it was determined that background antibodies reacted with a wide spectrum of bacteria including *E. coli* and *Salmonella*.³⁶ Attempts to purify the antibodies with an *E. coli* lysate column, whole cell cross-adsorption, and affinity purification with the target peptide failed to improve the results (data not shown).

Two of the eight antibodies (Lm404 and LmC639) were further tested against 41 *L. monocytogenes* strains in indirect ELISA (Table 3). Antibody reactions were grouped into three categories, ie, high (≥ 1.000), medium (0.500–0.990), and low (< 0.499 , Figure 1). The reaction pattern for Lm404 was 29.3%, 68.3%, and 2.4%, falling into the high, medium, and low categories, respectively. LmC639 had an overall low reaction (90%) with all the tested strains. Only 9.8% were in the combined medium and high categories.

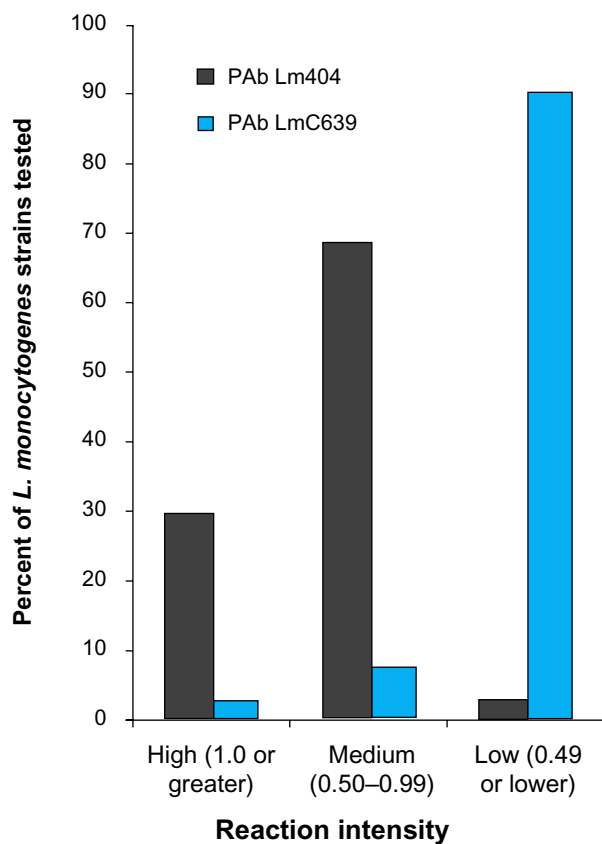


Figure 1 Reaction profiles of Lm404 and LmC639 polyclonal antibody to 41 *Listeria monocytogenes* strains. Enzyme-linked immunosorbent assay values are grouped in high (≥ 1.000), medium (0.500–0.999), and low (≤ 0.499) categories.

Abbreviation: PAb, polyclonal antibody.

Western blot analysis

To confirm if the antibodies react with their respective target antigens, Western blot analysis was done on the surface protein extracts from all eight *Listeria* species. The Lm404 polyclonal antibody showed reaction with the targeted 63 kDa internalin B (Figure 2A) and the LmC639 polyclonal antibody (Figure 2B) showed reaction with its target protein band of 90 kDa (ActA) present only in *L. monocytogenes*. This antibody also showed mild cross-reaction with some other protein bands. As positive controls, these two antibodies showed reaction with affinity purified recombinant internalin B and ActA, confirming the specific nature of these two antibodies (Figure 2C). Western blot analysis with the remainder of the antibodies revealed reaction with multiple protein bands from different *Listeria* species. The Lm407 and Lm408 polyclonal antibodies showed reaction with the target protein bands of 63 kDa (Lmo2026 peptidoglycan binding protein) and 56 kDa (Lmo2085 peptidoglycan binding protein) and also reacted with several additional protein bands from other *Listeria* species (Figure 3A and B). This is not surprising since this is a common protein shared by most *Listeria* species, and

certain amino acids (epitopes) in the peptide sequence used are possibly present in other *Listeria* species. Likewise, the LmH548 polyclonal antibody showed a reaction with the target protein bands of 55 kDa (internalin H); however, it also reacted with several additional protein bands from other *Listeria* species (data not shown). The Lm405 polyclonal antibody (Lmo0460, a 69 kDa putative membrane protein) did not show any reaction with the target protein band (Figure 3C). Likewise, the Lm406 polyclonal antibody (Lmo1115, a 86 kDa hypothetical protein), the LmA800 polyclonal antibody (80 kDa internalin A), and the Lm1290 polyclonal antibody (Lmo1290, 60 kDa internalin K)³⁷ did not also show any reactions with their targeted protein bands (data not shown).

Discussion

Availability of a genome sequence of microbes and comparative genome sequence analysis has made it possible to determine genetic and phenotypic differences between species within a genus.^{25,38} Rapid detection of *L. monocytogenes* from foods or food contact surfaces continues to challenge the food industry. Rapid immunological methods could be improved if a more specific antibody for *L. monocytogenes* existed. Through the use of genomics and proteomics, the present research attempted to develop *L. monocytogenes*-specific antibodies targeted against unique peptide sequences from surface proteins of *L. monocytogenes*. The genomic and proteomic analysis allowed us to select peptide epitopes as potential antigens for production of antibodies.²⁷ Despite detailed analysis, not all of the peptides produced desirable antibody responses. For example, the Lm406 polyclonal antibody developed against the peptide of unknown protein Lmo1115 failed to produce a strong antibody response when tested against the peptide. The low reaction indicated that either peptide synthesis, the immunization process, or peptide selection failed. Although the remaining eight antibodies showed a high titer to their respective peptides, only two (the Lm404 and LmC639 polyclonal antibodies) showed specific reaction to the targeted protein in Western blot. Of the remaining six, some reacted with target antigen along with several more protein bands (see Western blot for the Lm407 and Lm408 polyclonal antibodies, Figure 3A and B), while the Lm405 polyclonal antibody developed against the 69 kDa putative membrane protein did not show any reaction with its target protein band (Figure 3C). In a previous study, the Lm404 and LmC639 polyclonal antibodies were used to monitor differential expression of internalin B and ActA when grown in different *Listeria*-selective enrichment broths.¹⁴ The lack of specific reaction of other

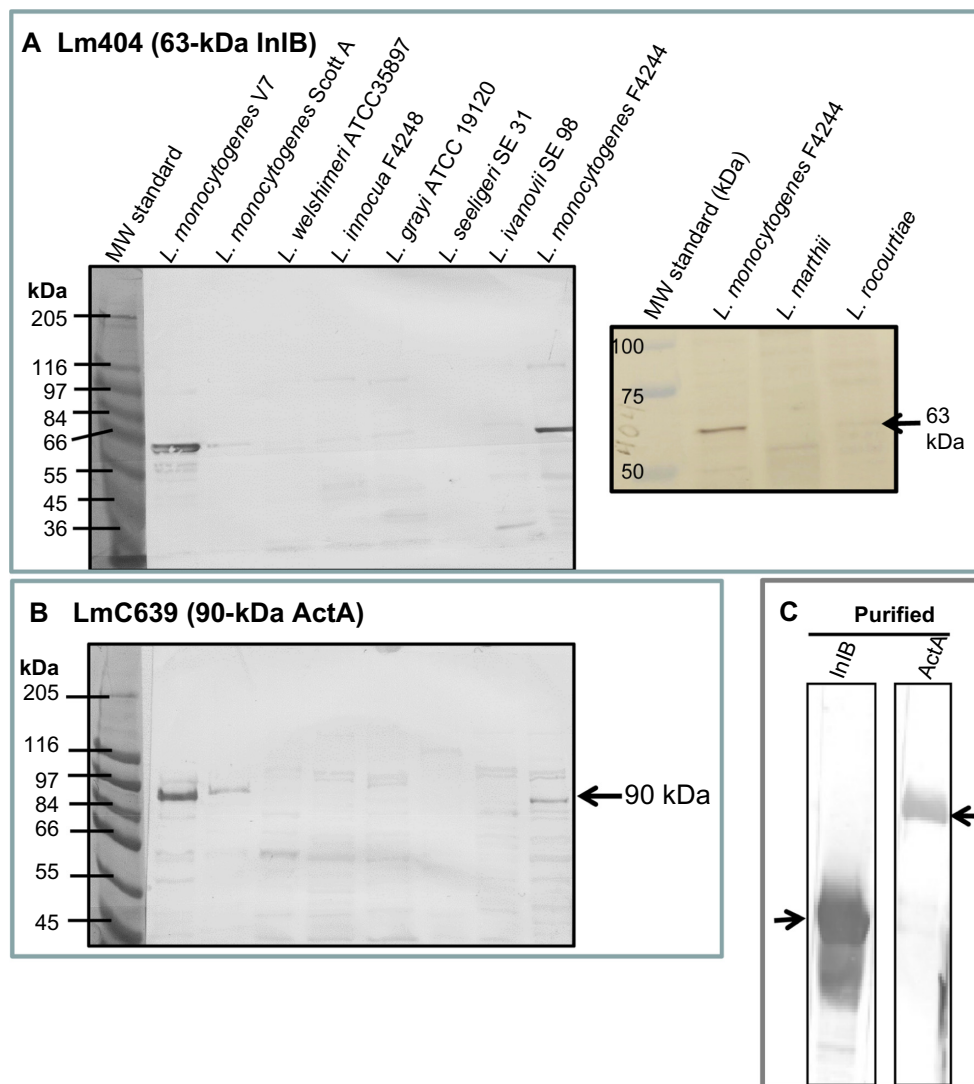


Figure 2 Western blot analysis of *Listeria* surface proteins with (A) Lm404 polyclonal antibody developed against internalin B (63 kDa) and (B) LmC639 polyclonal antibody developed against ActA (90 kDa). (C) Purified internalin B (63 kDa band) and ActA (90 kDa) are included as controls.

Abbreviations: MW, molecular weight; InIB, internalin B; ActA, actin polymerization.

antibodies could be due to denaturation of the target protein. When the Lm404 and LmC639 polyclonal antibodies were tested with a large panel of *L. monocytogenes* strains, the Lm404 polyclonal antibody showed an overall strong reaction while the LmC639 polyclonal antibody was weak. The weak reaction for the LmC639 polyclonal antibody was due to the lower expression of ActA under the current growth conditions (brain heart infusion broth). In an earlier study, we verified that ActA expression was indeed very poor in all 13 serovars of *L. monocytogenes* when grown in brain heart infusion broth and Luria-Bertani broth. However, increased expression was noticed when the same cultures were grown in *Listeria*-selective enrichment broths, ie, buffered *Listeria* enrichment broth, University of Vermont medium, and Fraser broth.¹⁴ Greene and Freitag³⁹ showed that medium with glucose concentrations 5 mM or higher

decreased expression of ActA. Since brain heart infusion has a glucose concentration of 11 mM, changing to a low glucose medium could improve expression of ActA, yielding higher reaction values.

All of the antibodies showed high cross-reaction with *E. coli* and *Salmonella* in ELISA. The protein database was searched to identify all of the peptides and none showed any homology with proteins from other bacteria, so the antibody reaction to these microorganisms was unexpected. After examining the preimmune sera from several rabbits used for antibody production, it was obvious that the cross-reactive antibodies were already present before immunization.³⁶ Use of rabbits raised in a specific pathogen-free environment would be able to reduce cross-reaction problems with nontarget bacteria; however, we observed that sera from several rabbits claimed to be raised

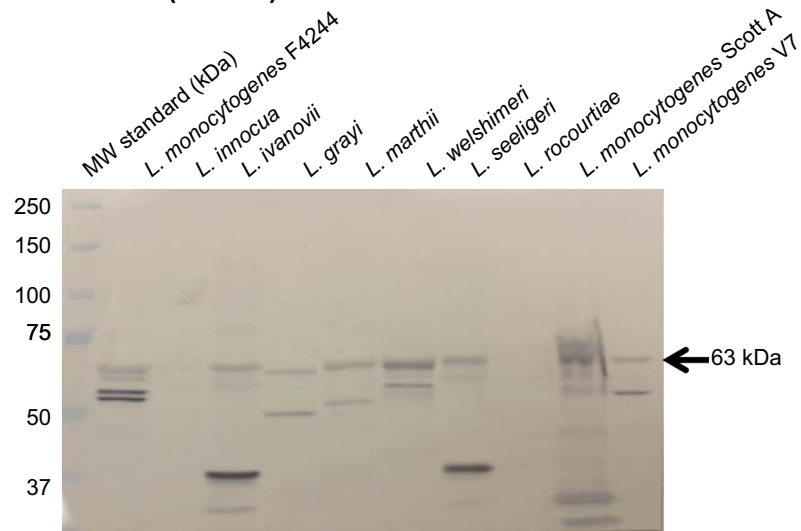
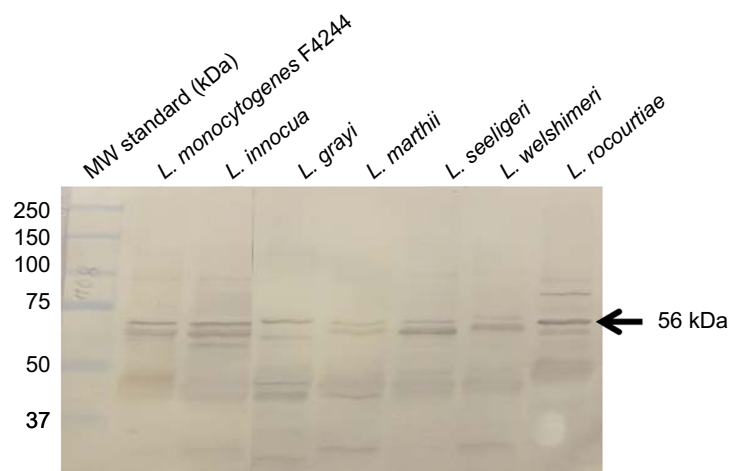
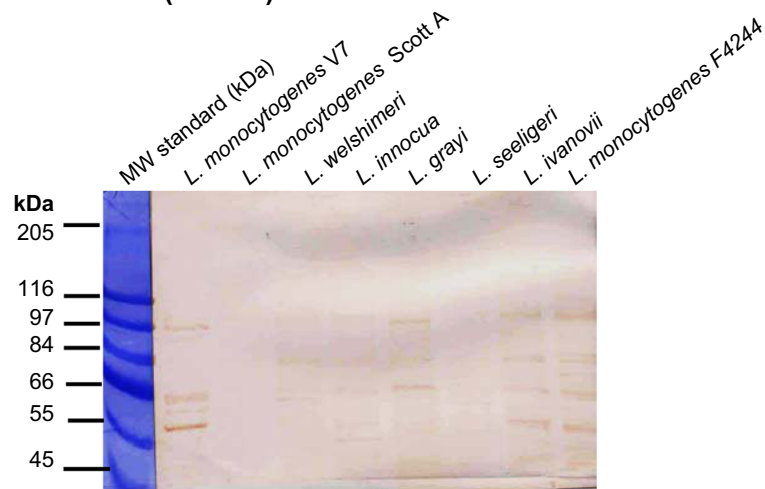
A Lm407 (63 kDa)**B Lm408 (56 kDa)****C Lm405 (69 kDa)**

Figure 3 Western blot analysis of *Listeria* surface proteins with (A) Lm407 polyclonal antibody, (B) Lm408 polyclonal antibody, and (C) Lm405 polyclonal antibody. Lm407 and Lm408 polyclonal antibodies reacted with target proteins and also with other proteins while Lm405 polyclonal antibody showed reaction mostly with nontarget antigens.

Abbreviation: MW, molecular weight.

in a specific pathogen-free environment also showed strong reaction against *Proteus vulgaris*, *Salmonella enterica* serovar Typhimurium, *Bacillus cereus*, *Corynebacterium glutamicum*, *E. coli*, and *Enterococcus faecalis*.³⁶ Attempts to remove cross-reactive antibodies by further purification were unsuccessful. However, cross-reaction to nontarget microorganisms can be overcome by using appropriate selective enrichment broth during preparation of samples before immunoassay.

Conclusion

All polyclonal antibodies except for the Lm406 polyclonal antibody (Lmo1115) showed specific reaction with the peptide antigens, indicating that comparative genomics could be used as a tool to select pathogen-specific antigen for antibody production. Only the Lm404 (internalin B) and LmC639 (ActA) polyclonal antibodies showed specific reactions for the protein from which the peptides were chosen and also showed some minor nonspecific reactions with other protein bands. The remaining polyclonal antibodies reacted with multiple protein bands. Based on Western blot results, the internalin B and ActA peptides appear to be the most promising targets for production of *L. monocytogenes*-specific antibodies. However, LmC639 (ActA) showed weaker reactions in ELISA, indicating epitope access may be obstructed in whole cells under the tested growth conditions. All antibodies, including the Lm404 and LmC639 polyclonal antibodies, showed strong reactions with *E. coli* and *Salmonella* in ELISA. This is because the rabbits had a high background antibody titer against these microbes. Monoclonal antibodies could be produced to avoid background antibodies.

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Disclosure

The authors report no conflicts of interest in this work.

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