Request for Report for Projects Awarded in 2013 and 2014 by

Mississippi Center for Food Safety and Post-Harvest Technology

Title: Quantification of high-risk and low-risk *Listeria monocytogenes* serotypes on catfish products

Award year: 2014-2015

PI: Mark L. Lawrence

Co-PI: Attila Karsi, and Frank Austin

Collaborator:

1. Objectives.
   1) **Develop a quantitative multiplex PCR method for identification of high-risk *L. monocytogenes*.**
   2) **Evaluate MALDI-TOF mass spectrometry for identification of serotype-specific *L. monocytogenes* biomarkers.**
2. New Accomplishments toward objectives. Please indicate if all objectives listed were completed.

   In our FY2014 funding from the Food Safety Initiative, we successfully completed our research objective for development of a multiplex PCR method for separation of high-risk serotypes 1/2a and 1/2c from low-risk serotypes 3a and 3c. Primers specific for gene *flaA* were effective in separating serotype 1/2a from serotypes 1/2c and 3c. The *LmoSLCC2372_0308* gene encodes a hypothetical protein and was found to be unique to serotype 1/2c, allowing distinction of 1/2c strains from all serotypes except one 3c strain. Distinction of serotypes 1/2c and 3c is particularly challenging because the genome sequences of serotype 1/2c strain SLCC2372 and serotype 3c strain SLCC2479 have over 96% identity. The *LMLG_0742* gene encodes a hypothetical protein that is present in all of the 3a strains, thus allowing distinction of serotype 1/2a from 3a. The three primer sets were combined and tested in a multiplex format that is effective in distinguishing serotypes 1/2a, 1/2c, 3a, and 3c.

   Our second objective from our 2014 FSI funding was to investigate MALDI-TOF MS as a method to analyze *L. monocytogenes* colonies or/and extracts and establish a library of dominant mass spectral fingerprints from each serotype. MS spectra were obtained for 90 strains of *L. monocytogenes* representing all the serotypes; 89 of these were matched with the reference *L. monocytogenes* spectrum with a sample identification score of over 1.90. A mass signal pattern (MSP) dendrogram was generated using MALDI Biotyper 3.0 with distance levels set to a maximal value of 1,000 according to the manufacturer's recommendation. Two strain clusters were identified based on MSPs. Thus, we were successful in conducting the MALDI-TOF analysis we proposed, and MSP dendogram

analysis demonstrated the potential of MALDI-TOF for intraspecies differentiation of strains. Further analysis of the specific mass spectral patterns for each serotype has potential to identify novel biomarkers that distinguish high-risk *L. monocytogenes* serotypes from low-risk serotypes.

3. Objectives not accomplished and impediments to meeting objectives.

   Despite testing of multiple primer sets, PCR primers for differentiation of serotype 4b from serotypes 4d and 4e were not identified. This prevented our adaptation of the PCR assay into a quantitative PCR format. For this reason, we have changed our focus to the identification of protein biomarkers for differentiation of listerial serotypes. Although we were successful in obtaining MALDI-TOF protein profiles for all the serotypes, the proprietary software with the MALDI Biotyper 3.0 cannot distinguish the serotypes based on protein profiles. However, our manual analysis of the MALDI-TOF data has identified some candidate biomarkers that can differentiate serotypes. We are currently working to identify or develop a software tool to assist in analysis of our MALDI-TOF data to identify potential biomarkers.

4. If continuing project, when will new and/or long term objectives be completed?

   In our continuing 2015-2016 Food Safety Initiative project, we are characterizing a novel hydrolase, Lmof2356_2464, which has potential as a biomarker for differentiating high-risk and low-risk *L. monocytogenes*. We anticipate completion of this research by March 2016 at the conclusion of the current funding period.

5. Students supported

   a. PhDs (% FTE and name)
   b. M.S. (% FTE and name)
   c. Undergraduate (number of students)

   No students were supported in 2014-2015, but the project supported the training of two postdoctoral scientists, Dr. Seongwon Nho (40% FTE), and Dr. Hossam Abdelhamed (25% FTE).

6. Leveraged Funds: External Competitive Funding Applied and Awarded based on findings from this project.
   a. Applied for:
      i. NIH NIAID
      ii. R15
      iii. Lawrence, M. L. and M. Ross. 2014-2016. Regulation of listerial virulence genes by a HD superfamily putative hydrolase. $434,225.
   b. Awarded:
      None

7. Outputs – In addition to the above, please populate the following sections to be included in a report to be compiled in a FSI Research Accomplishment Booklet. The project report will also be posted in a FSI website to be developed.

Please submit reports in Microsoft Word Document (except the published journal articles in pdf format) to Ms. Kaila Peggs by May 15.

Project Summary (Issue/Response)

In the U.S., *Listeria monocytogenes* is responsible for about 2,500 cases of listeriosis each year, with a hospitalization rate of 91% and a case fatality rate of 20%. It is a substantial foodborne pathogen in ready-to-eat food products, causing septicemia, meningitis, encephalitis, abortions, and death, especially in infants, pregnant women, and immunocompromised individuals. Of the thirteen *L. monocytogenes* serotypes, over 98% of isolates from human listeriosis belong to only four serotypes: 1/2a, 1/2b, 1/2c and 4b. A PCR method was developed that allows distinction of four *L. monocytogenes* subgroups: 1/2a-3a, 1/2c-3c, 1/2b-3b-7, and 4b-4d-4e. However, it does not distinguish serotype 1/2a from 3a, 1/2c from 3c, 1/2b from 3b and 7, or 4b from 4d and 4e. Thus, there is a need for development of a rapid PCR method that can distinguish high-risk listerial serotypes 1/2a, 1/2b, 1/2c, and 4b from the low-risk serotypes to enable studies on epidemiology of foodborne disease and the development of prophylactic strategies. In the current project, we focused on developing a multiplex PCR method for differentiating serotypes 1/2a, 1/2c, 3a, and 3c. We compared all of the available fully sequenced *L. monocytogenes* genomes from these four serotypes and identified potential genes unique to different serotypes. We tested multiple primer sets based on these genes and identified three sets of PCR primers that were effective in distinguishing strains from these serotypes. The primers were based on gene sequences from *flaA*, *LmoSLCC2372_0308*, and *LMLG_0742*. The combination of primers from *flaA* and *LMLG_0742* allow differentiation of serotypes 1/2a, 1/2c, and 3a strains. Differentiation of serotypes 1/2c and 3c is the most problematic because of their high genomic identity, but primers from *LMOSLCC2372_0308* allow differentiation of one of the two serotype 3c strains in our panel from the 1/2c strains. We expect that our new method of multiplex PCR based on genes *flaA*, *LmoSLCC2372_0308*, and *LMLG_0742* will be a valuable tool for differentiating listerial serotypes 1/2a, 1/2c, 3a, and 3c and will be useful for epidemiological and diagnostic investigations.

Project Results/Outcomes

To implement effective control and prophylactic measures against *Listeria monocytogenes*, it is necessary to distinguish virulent strains from low virulence strains. *L. monocytogenes* consists of thirteen serotypes, and almost all human listeriosis cases are caused by four of these serotypes. Therefore, serotyping is often used as a first step to discriminate strains associated with human listeriosis. The aim of our current study was to develop a reliable high-resolution subtyping method for differentiating high-risk *L. monocytogenes* serotypes 1/2a and 1/2c from 3a and 3c by multiplex PCR.

To accomplish this, we developed a multiplex PCR method based on variation in three genes to identify high-risk 1/2a and 1/2c serotypes. Gene *flaA* has been used previously in multiplex PCR to distinguish strains from serotypes 1/2a and 3a from serotypes 1/2c and 3c, and our results confirmed its effectiveness. Gene *LMOSLCC2372_0308* is specific for serotype 1/2c. Genetic differentiation of serotype 1/2c from 3c is particularly challenging. In genome comparisons, serotype 1/2c strain SLCC2372 and serotype 3c strain SLCC2479 have a very close genetic relationship based on an alignment of mutually conserved core genes. Based on comparative genome analysis, we selected sixteen potential target gene candidates for potential differentiation of serotypes 1/2c and 3c;

however, only PCR primers based on *LMOSLCC2372_0308* were found to successfully discriminate between serotype 1/2c and other serotypes. The *LMOSLCC2372_0308* gene encodes a hypothetical protein, and *LMOSLCC2372_0308* has no orthologous genes in the other sequenced *L. monocytogenes* genomes (based on reciprocal best-BLAST criteria). Primers based on *LMOSLCC2372_0308* were also successful in distinguishing serotype 1/2c from serotypes 1/2a and 3a with the exception of one serotype 1/2a strain (EGD-e). However, this strain is considered an atypical 1/2a strain. The *LMLG_0742* gene encodes a hypothetical protein that is in a four gene locus unique to one of the sequenced serotype 3a strains (Finland 1998). Together with *flaA*, this gene was effective for distinction of serotype 1/2a and 3a strains.

The three PCR primer sets based on *flaA*, *LMOSLCC2372_0308*, and *LMLG_0742* were combined and tested in a multiplex format and shown to be effective. Thus, with a single PCR reaction based on these three genes, it is possible to distinguish *L. monocytogenes* serotypes 1/2a, 1/2c, 3a, and 3c. This method will be useful as a simplified method for future diagnostic and epidemiologic investigations.

Another objective of this project was to investigate MALDI-TOF MS as a method to differentiate *L. monocytogenes* serotypes. Our goal was to analyze *L. monocytogenes* strains representing each serotype and establish a library of dominant mass spectral fingerprints from each serotype. MS spectra were obtained for 90 strains of *L. monocytogenes* representing all the serotypes; 89 of these were matched with the reference *L. monocytogenes* spectrum with a sample identification score of over 1.90. A mass signal pattern (MSP) dendrogram was generated using MALDI Biotyper 3.0 with distance levels set to a maximal value of 1,000 according to the manufacturer's recommendation. Two strain clusters were identified based on MSPs. Thus, we were successful in analyzing all the listerial serotypes using MALDI-TOF, and MSP dendogram analysis demonstrated the potential of MALDI-TOF for intraspecies differentiation of strains. Further analysis of the specific mass spectral patterns for each serotype has potential to identify novel biomarkers that distinguish high-risk *L. monocytogenes* serotypes from low-risk serotypes.

Project Impacts/Benefits

The Centers for Disease Control and Prevention (CDC) estimates that around 48 million Americans get sick and around 3000 people die of food borne illnesses. *Listeria monocytogenes* has the highest case hospitalization rate and second highest case fatality rate of all foodborne pathogens. It causes about 300 deaths every year in the U.S. *L. monocytogenes* has the ability to adapt to low temperature (2-4°C), low pH, and high sodium concentrations. As a result, *L. monocytogenes* has been isolated from an array of both raw and processed foods, including dairy products, meat products, fresh produce, and fish products. In particular, *L. monocytogenes* can be isolated from channel catfish, but many of the isolates are nonpathogenic.

L. monocytogenes strains are serotyped according to variation in somatic (O) and flagellar (H) antigens. *L. monocytogenes* strains vary in pathogenic potential. In particular, more than 98% of reported human listeriosis cases are caused by strains in four particular listerial serotypes: 1/2a, 1/2c, 1/2b, and 4b. A published PCR method allows distinction of four *L. monocytogenes* subgroups: 1/2a-3a, 1/2c-3c, 1/2b-3b-7, and 4b-4d-4e. However, it does not distinguish serotype 1/2a from 3a, 1/2c from 3c, 1/2b from 3b, or 4b from 4d and 4e. Thus, it does not allow separation of the high-risk serotypes from the low-risk serotypes.

In the current project, we developed a multiplex PCR method for differentiating high-risk *L. monocytogenes* serotypes 1/2a and 1/2c from low-risk serotypes 3a and 3c. We are also working to identify biomarkers for differentiating high-risk and low-risk *L. monocytogenes* serotypes. Development of a molecular method to distinguish high-risk serotypes from low-risk serotypes of *L. monocytogenes* would enable determination of the risk associated with isolation of *L. monocytogenes* from seafood, including channel catfish. It would also facilitate future epidemiologic investigations of *L. monocytogenes* for diagnostic and research purposes.

Project Deliverables

Paul D, Steele C, Donaldson JR, Banes MM, Kumar R, Bridges SM, Arick M, and Lawrence ML. 2014. Genome comparison of *Listeria monocytogenes* serotype 4a strain HCC23

with selected lineage I and lineage II *L. monocytogenes* strains and other *Listeria* strains. Genomics Data 2:219-225.

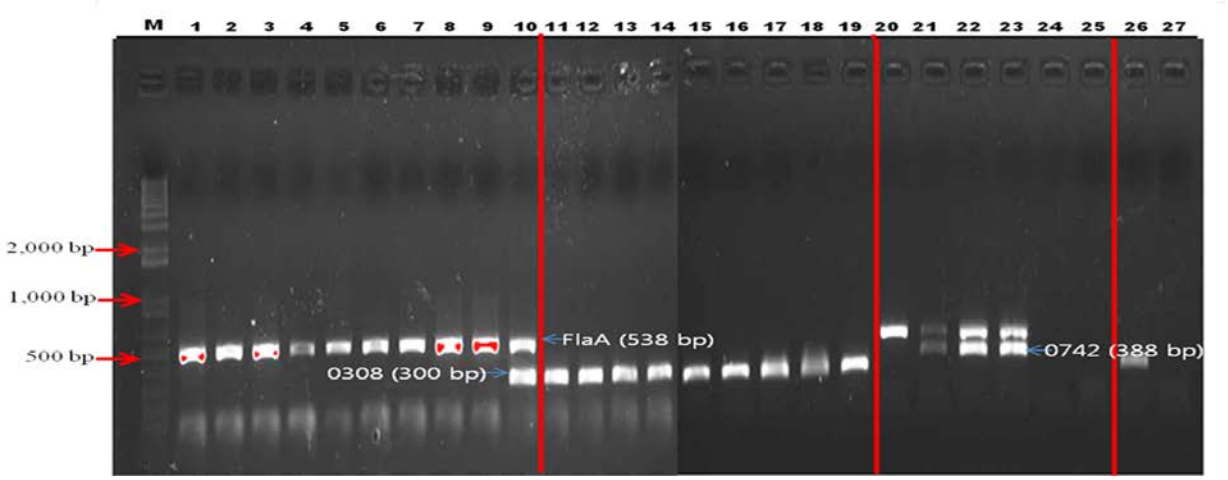
Reddy, S and Lawrence ML. 2014. Virulence Characterization of *Listeria monocytogenes*. In Jordan, K., E. M. Fox, and M. Wagner (eds), *Listeria monocytogenes: Methods and Protocols*, pages 157-166. Humana Press, New York, New York.

Abdelhamed, H, Lawrence ML, and Karsi A. 2014. A novel suicide plasmid for efficient mutation of genes in Gram-positive bacteria. 2014 Annual Meeting of the South Central Branch of the American Society for Microbiology, Fayetteville, Arkansas.

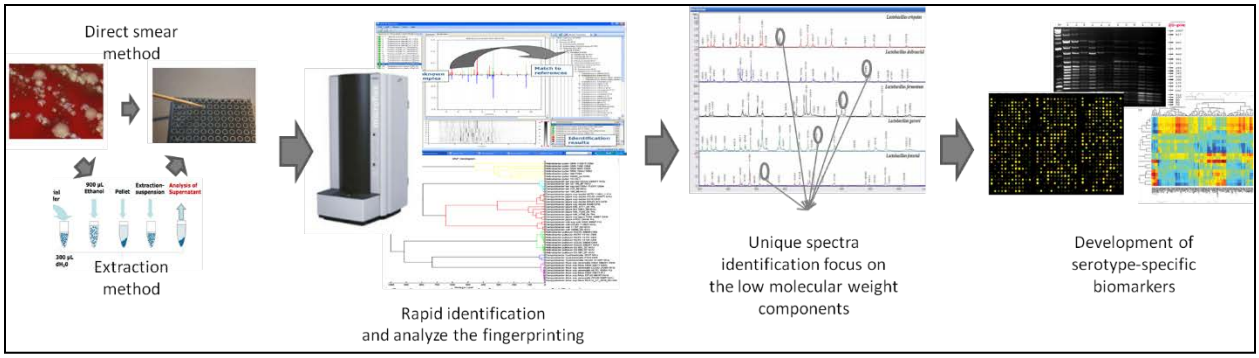
Nho, S and Lawrence ML. 2014. Identification of high-risk *Listeria monocytogenes* serotypes using multiplex PCR. 2014 Annual Meeting of the South Central Branch of the American Society for Microbiology, Fayetteville, Arkansas.

Reddy S, Turaga G, Banes MM, and Lawrence ML. 2013. A HD superfamily putative hydrolase plays a role in *Listeria monocytogenes* virulence. Eighteenth International Symposium on Problems of Listeriosis, Goa, India.

Graphics



Results of multiplex PCR assay to differentiate *Listeria monocytogenes* serotypes 1/2a, 1/2c, 3a, and 3c. Gel electrophoresis of DNA fragments generated by multiplex PCR. Lane M, DNA molecular weight marker (1Kb Plus, Life Technologies, Invitrogen, Carlsbad, CA); Lanes 1 to 25, *L. monocytogenes* strains of serotype 1/2a (35568A, 51772, 12443, FSL-J2-020, FSL-C1-056, FSL-J2-031, FSL-J2-066, FSL-J1-101, F6854, and EGD-e; 10 strains), 1/2c (C622N, G-3321, H9666, H9067, H7973, 57, 9900096, L028, and 33039; 9 strains), 3a (J0095, RM3120, FSL-M1-004, and FSL-C1-115; 4 strains) and 3c (J0096 and FSL-J1-049; 2 strains).



Overview of the MALDI-TOF method for identifying biomarkers for differentiation of *Listeria monocytogenes* serotypes. MALDI-TOF MS allows rapid identification for easier and faster diagnosis of pathogens than conventional phenotypic and molecular identification methods. We analyzed 90 *L. monocytogenes* strains representing all 13 serotypes on a MALDI Biotyper 3.0 (Bruker), and the resulting mass spectra are being analyzed to identify unique biomarkers for the serotypes.

Attached Refereed Journal Publications in Separate Files

Please attached published journal articles (in pdf format if available) for this project. Manuscripts accepted or in review process may be submitted in word files. Thank you very much for your cooperation.