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Bio-Plex suspension array immuno-detection of *Listeria monocytogenes* from cantaloupe and packaged salad using virulence protein inducing activated charcoal enrichment media

J.B. Day*, T.S. Hammack

U.S. Food and Drug Administration Center for Food Safety and Applied Nutrition 5001 Campus Dr., College Park, MD, 20740, USA

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ABSTRACT

Listeria monocytogenes, the causative agent of listeriosis in humans, is a Gram-positive bacterium that is contracted via the ingestion of contaminated foods. Two of the largest outbreaks of listeriosis occurred following consumption of tainted cantaloupe and packaged salads. Molecular methods and immuno-based techniques for detection of *L. monocytogenes* in these food matrices can be difficult due to the presence of assay inhibiting elements. In this study, we utilized a novel enrichment media containing activated charcoal as the key ingredient that induces hyperactive expression and secretion of *L. monocytogenes* virulence proteins. The Bio-Plex suspension array system, based on Luminex xMAP technology, was subsequently employed to specifically detect accumulated *L. monocytogenes* secreted and membrane bound proteins via paramagnetic microsphere-antibody complexes. Cantaloupe and packaged salad samples were treated with a dilution series of *L. monocytogenes* and incubated in activated charcoal media following a short pre-enrichment step in Buffered Listeria Enrichment Broth. Secreted L. *monocytogenes* lysteriolysin O was captured using magnetic microsphere-antibody conjugates and measured using the Bio-Ple × 200 analyzer. As few as 10^0 CFU/g of *L. monocytogenes* was detected from both spiked cantaloupe and packaged salad samples. In addition, antibody conjugated microspheres targeting a membrane protein present on both pathogenic and non-pathogenic *Listeria* species was used to identify as few as 10^0 CFU/g of both pathogenic and nonpathogenic species in cantaloupe and packaged salad. This method presumptively identifies *L. monocytogenes* from cantaloupe and packaged salad in less than 24 h and non-pathogenic *Listeria* species within 22 h.

1. Introduction

A reported 3423 foodborne outbreaks occurred in the United States from 2013 to 2016 leading to 56,067 illnesses and 69 deaths (CDC, 2017). The leading cause of death in the United States from foodborne illness during this time was from infection with the Gram-positive bacterium *Listeria monocytogenes* (CDC, 2017). The elderly, infants and immunocompromised individuals are most susceptible and the mortality rate is estimated at 20–30% (Fonnesbech Vogel et al., 2001; Roberts and Wiedmann, 2003; Radoshevich and Cossart, 2017). Severe symptoms occur during hematogenous spread of the organism in these individuals to the heart and brain leading to endocarditis and meningitis/encephalitis, respectively (Alonzo et al., 2011; Camejo et al., 2011; Doganay, 2003). Neonates are particularly predisposed to severe *L. monocytogenes* infection with a high incidence of septicemia, pneumonia and/or meningitis (Lamont et al., 2011). Pregnant women are also vulnerable with a high risk of miscarriage, stillbirth or abortion of the fetus when *trans*-placental migration of the bacteria occurs *in utero*.

The genus *Listeria* is composed of 17 known species although *L. monocytogenes* is responsible for the vast majority of listeriosis outbreaks in humans (Renato and Wiedmann, 2016). There are 13 serotypes of *L. monocytogenes* of which 1/2a, 1/2b and 4b are responsible for over 90% of human outbreaks (Ward et al., 2004). *L. monocytogenes* is able to initiate infection, in part, due to its ability to invade and replicate within a broad range of host cells including epithelial cells, hepatocytes, fibroblasts, macrophages and endothelial cells (Braun et al., 1998; Vázquez-Boland et al., 2001). Lysteriolysin O (LLO) is one of the central virulence factors that is essential for infection in human hosts. Secretion of LLO is stimulated during infection in a host organism and functions as a pore forming toxin that allows *L. monocytogenes* escape from the macrophage phagosome into the cytoplasmic environment where it can proliferate unchecked by the host immunity. LLO is encoded from the *hlyA* gene which is expressed during certain external stimuli including an environmental temperature of 37 °C (Ermolaeva et al., 2004).

The ubiquitous nature of *L. monocytogenes* in the environment and the potential to form sanitizing agent resistant biofilms adds to the challenge of eliminating the organism from food matrices and food processing facilities despite implementation of improved current good manufacturing practices (CGMPs) and monitoring by government agencies (Nyenje et al., 2012; Pan et al., 2006). Indeed, *Listeria* biofilms have the potential to develop in various

* Corresponding author.
E-mail address: james.day@fda.hhs.gov (J.B. Day).

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locations within food processing factories such as conveyor belts, storage tanks and drains (Chmielewski and Frank, 2003). In addition, *Listeria* is exceedingly resilient and can survive and proliferate in extreme environments such as at refrigeration temperatures and in high salt as (Fonnesbech Vogel et al., 2001; Nakamura et al., 2013).

Various foods have been shown to harbor *L. monocytogenes* and human outbreaks have occurred through ingestion of contaminated dairy products (raw milk, cheeses, ice cream), vegetables (sprouts, packaged salads, green peas, sweet corn, celery), meats (deli meat, frankfurters, pâté) and fruits (cantaloupe, caramel apples, stone fruits). One of the largest outbreaks of listeriosis occurred in 2011 from consumption of tainted cantaloupe and involved 147 illnesses in 28 states with 33 deaths (McCollum et al., 2013). Cantaloupe contamination can be particularly hazardous due to robust *L. monocytogenes* attachment and proliferation on cantaloupe surfaces and the formation of biofilm complexes in as little as 2 h (Fu et al., 2017; Martinez et al., 2016; Michelle et al., 2014; Nyarko et al., 2016; Salazar et al., 2017). Internalization of L. *monocytogenes* from the cantaloupe rind into the pulp region has been shown to occur by both direct entry through the stem area and by indirect transfer via cutting during fruit preparation (Macarisin et al., 2017; Ukuku and Fett, 2002). In addition, *L. monocytogenes* exhibited more rapid growth in cantaloupe flesh compared to growth in other cut produce tested (Salazar et al., 2017). Leafy green vegetables have also been shown to be a potential source of *L. monocytogenes* and *L. ivanovii* contamination (Mercanoglu Taban and Halkman, 2011; Soriano et al., 2001; Vongkamjan et al., 2016). Indeed, evaluations of packaged salads from supermarkets revealed high frequencies of *Listeria* species including *L. monocytogenes* (Francis and O'Beirne, 2006). Moreover, packaged salads were shown to support growth of *L. monocytogenes* which increases the risk of cell numbers reaching the infectious dose to trigger listeriosis in susceptible populations (Zeng et al., 2014). A recent outbreak of listeriosis was linked to consumption of packaged salads resulting in 19 cases with one death reported (Self et al., 2015).

Due to the difficulties in eliminating *L. monocytogenes* form food matrices, rapid and specific detection of this organism is essential to prevent adulterated food from reaching consumers. Unfortunately, efforts to detect *L. monocytogenes* in food matrices has been problematic due, in part, to the physical nature of foods which render sample preparation and subsequent analysis extremely difficult. Traditional culture methods continue to be the gold standard for identification of *L. monocytogenes* (Magalhães et al., 2014). However, these techniques are laborious and time-consuming that can require several days for final results. DNA and immunological-based techniques are being developed to enhance the time to detection without loss of sensitivity or specificity (Jadhav et al., 2012). Real-time PCR has been widely tested as a detection method and shown to achieve low limit of detection as well has high specificity (O'Grady et al., 2008; Petrauskene et al., 2012; Rodriguez-Lazaroa et al., 2014). However, real-time PCR reactions are readily impeded by circulating food components leading to false-negative results (Bhagwat, 2003; Perelle et al., 2004; Powell et al., 1994; Rodriquez-Lazaro et al., 2005; Rossen et al., 1992). In addition, most PCR detection methods require a lengthy enrichment step with extensive DNA template processing to clean the target sufficiently to allow DNA amplification. Immunological methods, based on recognition of *Listeria* markers using specific antibodies, have also gained popularity. However, these methods are also susceptible to interference by food elements (Kim et al., 2010). Immunomagnetic separation (IMS) techniques, which utilize antibody-magnetic bead conjugates to separate the target bacteria from interfering food components and surrounding microflora, have been partially successful (Brandão et al., 2015). One drawback of immunological techniques is that current *Listeria* specific antibodies used do not distinguish *L. monocytogenes* from other *Listeria* species due to the high homology of the target proteins (Bhunia et al., 1991; Geng et al., 2006; Lathrop et al., 2003). In addition, due to the low quantities of *Listeria* present in foods, a protracted enrichment step is required to increase bacterial numbers above the minimum threshold of detection.

In this study, we incorporated an enrichment media with an immunocapture system to rapidly and specifically identify *L. monocytogenes*. The enrichment media contains activated-charcoal, which induces hyperactive expression and secretion of *L. monocytogenes* virulence proteins, and results in an amplified pool of antibody targets available for immunoreactions (Ermolaeva et al., 1999). Magnetic bead-based immunecapture and detection technology using the Bio-Rad Bio-Plex format can then allow for the identification of low quantities of bacteria due to the high virulence protein target output stimulated by the enrichment media. The Bio-Plex suspension array system is based on Luminex xMap technology that utilizes microspheres that contain a mixture of two fluorescent dyes which can display 100 distinct colors depending on the concentration of each dye. Using classification lasers, the Bio-Plex 200 unit can differentiate among the 100 distinct microsphere sets as well as measure and quantitate fluorescence derived from a reporter fluorophore bound to a microsphere set via a target analyte. We utilized this method for specific identification of *L. monocytogenes,* as well as *L. ivanovii* and non-pathogenic *Listeria* species in cantaloupe and packaged salad.

2. Materials and methods

2.1. Bacterial strains and growth cultures

Working cultures of *L. monocytogenes* were grown overnight on Brain Heart Infusion (BHI) agar plates (Becton Dickinson, Sparks, MD) at 37 °C. *Listeria* from BHI plates were inoculated into 50 mL of BHI broth and grown shaking (100 rpm) overnight at 37 °C. Cultures were harvested by centrifugation at 10,200 × *g* for 5 min and washed once with Dulbecco's phosphate buffered solution (D-PBS, pH 7.4) (ATCC, Manassas, VA). *L. monocytogenes* isolates were preserved in BHI broth with 25% glycerol at −80 °C. *L. ivanovii,* non-pathogenic *Listeria* species and non-*Listeria* control bacteria used for inclusivity and exclusivity testing were grown and processed as described above. Activated Charcoal Media (ACM) was prepared using 10 mM glucose-6-phosphate, 5 mM MOPS, and 0.2% (w/v) activated charcoal in Luria-Bertani broth (Yeung et al., 2005).

2.2. Food products

Whole cantaloupe and ready-to-eat packaged salad (shredded iceberg lettuce) were purchased from local supermarkets and kept at 4 °C until use.

2.3. Secretion of lysteriolysin O from L. monocytogenes grown in diverse enrichment media

L. monocytogenes was prepared as described above and inoculated into 10 mL of activated-charcoal media (ACM), Luria-Burtani broth (LB), Brain-Heart Infusion broth (BHI), Buffered Listeria Enrichment Broth (BLEB) or Fraser broth at 10^2 CFU/mL and grown for 14 h at 37 °C. Cell pellets and culture supernatants were separated by centrifugation at 10,200 × *g* for 10 min. Culture supernatant proteins were precipitated with 10% (v/v) trichloroacetic acid (TCA) (for 1 h, on ice) and collected by centrifugation at 10,200 × *g* for 10 min at 4 °C. Protein pellets were dried for 5 min and resuspended in 100 μL of 100 mM Tris-HCL-1 mM EDTA, pH 8.0. Resuspended supernatant proteins were mixed 1:1 (v/v) with 2 × electrophoresis sample buffer (20 mM Tris-HCL, 1 mM EDTA, 4% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) β-mercaptoethanol) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis using LLO specific capture (α-LLO-1) antibodies. Densitometric analysis of chemiluminescent images were quantified using Image Lab Touch software (Bio-Rad).

2.4. Listeriolysin O capture and detection antibody specificity

A total population of 10^3 CFU/mL of pathogenic *Listeria* species, non-pathogenic *Listeria* species and a selection of non-*Listeria* foodborne bacterial pathogens (Fig. 2) were inoculated into 30 mL of ACM and grown at 37 °C for 14 h. Culture supernatants were separated from whole cell pellets by centrifugation and were prepared as described above. Secreted proteins were analyzed by SDS-PAGE and Western immunoblot using LLO-1 (capture) and LLO-2 (detection) specific antibodies.

2.5. SDS-PAGE and immunoblot procedures

Secreted proteins prepared as described above were loaded onto 4–15% Mini-PROTEAN TGX precast protein gels (Bio-Rad, Hercules, CA) and electrophoresed for 30 min at 200 V. Proteins were then transferred onto PVDF membranes (Millipore Sigma, Billerica, MA) and blocked with 5% non-fat milk (NFM) in TBS (20 mM Tris, 150 mM NaCl, pH 7.4) for 30 min at room temperature. Anti-LLO-1 and anti-LLO-2 antibodies were used as the primary antibodies and were diluted 1:5000 in TBS containing 1% bovine serum albumin (BSA) and 0.05% Tween 20. Blots were incubated at room temperature for 1 h and washed 3 × with TBS containing 0.05% tween 20 for 5 min. Secondary antibodies (anti-rabbit IgG or anti mouse IgG horseradish peroxidase conjugated) were diluted 1:8000 in TBS containing 0.05% tween 20 and 1% NFM and incubated with blots for 1 h at room temperature. Blots were then washed 3 × as described above, developed with Clarity Western blotting ECL (Bio-Rad, CA) and visualized with the ChemiDoc Touch MP imaging system (Bio-Rad).

2.6. Preparation of mag-plex microsphere capture antibody conjugate and biotinylated detection antibody

Listeria monoclonal LLO specific capture antibody, LLO-1 (catalog #ab81141), was purchased from Abcam (Cambridge, MA). Monoclonal antibody targeting a *Listeria* membrane protein (LMP-1) (catalog #20-511-241651) was purchased from Genway Biotech, Inc. (San Diego, CA). Both antibodies were filtered using Bio-Rad (Hercules, CA) Micro Bio-Spin 6 Tris chromatography columns according to the manufacturer's instructions. Bio-Plex Pro Magnetic COOH bead regions #37 and #55 were purchased from Bio-Rad and covalently coated to LLO-1 and LMP-1 antibodies, respectively using the Bio-Rad amine coupling kit according to the manufacturers' instructions. In brief, a 0.1 mL preparation of magnetic bead regions #37 and #55 from the 10 × stock solution (approximately 1.25×10^6 microspheres) were washed and resuspended in 80 μL of activation buffer. A 500 μg aliquot of N-hydroxysulfosuccinimide (S-NHS) and 1-(3-Dimethylaminopropyl) carbodiimide (EDAC) were suspended separately in activation buffer and sequentially added to the MagPlex beads and incubated at room temperature for 20 min. The beads were then washed three times in PBS, pH 7.4 and resuspended in 100 μL of PBS. A 10 μg preparation of LLO-1 and LMP-1 antibodies were added to bead regions #37 and #55, respectively, in a final volume of 500 μL PBS and incubated at room temperature for 2 h with mild rotation. The beads were then washed once with PBS and resuspended in 250 μL of blocking buffer for 30 min. The beads were then washed once in storage buffer and resuspended in a final volume of 150 μL of the same buffer. The quantity of recovered antibody coupled microspheres were then determined using the TC20 Automated Cell Counter (Bio-Rad, Hercules, CA). The microsphere-antibody coupling efficiency was evaluated using anti-mouse secondary antibody-phycoerythrin conjugates and measured on the Bio-Plex 200 analyzer.

The detection antibody LLO-2 was raised in rabbits by Pacific Immunology Corporation (Ramona, CA) using a unique *L. monocytogenes* Listeriolysin O N-terminal amino acid sequence as the immunogenic peptide. The resultant custom detection antibody was affinity column purified and biotinylated by Pacific Immunology Corporation. The detection antibody for LMP, LMP-2 (catalog #20-511-241694), was purchased and biotinylated by Genway Biotech Inc.

2.7. Sample preparation

For the preparation of artificially contaminated cantaloupe and packaged salad, *L. monocytogenes, L. innocua* and *S.* Typhimurium were grown as described above and 10-fold serially diluted in Dulbecco's Phosphate Buffered Saline (D-PBS). Dilutions of L. *monocytogenes, L. innocua* and *S.* Typhimurium from D-PBS samples were spread plated onto BHI agar plates and grown for at least 1 day at 37 °C to determine approximate numbers of the initial inocula. A series of Whirl-Pak filter bags (Seward, London, UK) were packed with 25 g of cut cantaloupe or packaged salad and inoculated with the corresponding bacterial dilution of *L. monocytogenes, L. innocua* or *S.* Typhimurium to obtain a range of samples containing from 10^3 to 10^0 CFU/g. Uninoculated Whirl-Pak filter bags containing 25 g of cantaloupe or packaged salad were also prepared as negative controls. After 10 min at room temperature, 225 mL of Buffered Listeria Enrichment Broth (BLEB) was added to each filter bag and stomached for 20 s in a stomacher 400 (Seward, London, UK). Filtered homogenates were transferred to 500 mL flasks and incubated at 30 °C for 1 h. A 0.4% (v/v) portion of Modified Listeria Selective Enrichment Supplement (Oxoid, Hampshire, England) was added to each sample and incubation continued for an additional 5 h at 30 °C. A 35 mL aliquot of each sample was transferred to 50 mL conical tubes and centrifuged at 10,200 × *g* for 5 min. Supernatant fractions were decanted and the remaining pellets were resuspended in 35 mL of ACM and incubated at 37 °C overnight for 14 h. Cell pellets and culture supernatants were separated by centrifugation at 10,200 × *g* for 5 min. Culture supernatants were precipitated on ice with 10% (vol/vol) TCA for 1 h. The resultant TCA protein pellets were washed once with ice-cold acetone and resuspended in 1 mL of PBS. Whole cell fractions were resuspended in 2 mL of PBS and boiled for 5 min. Samples were cooled 5 min and centrifuged at 10,200 × *g* for 5 min. The supernatant fractions of the boiled samples were used for analysis of LMP.

2.8. Bio-Plex suspension array procedure

The Bio-Rad pro reagent kit was used for the Bio-Plex suspension array technique. LLO and LMP specific capture antibody coated microspheres were added to secreted protein and cell fraction samples, respectively at 5000 microspheres per bead region along with internal control microspheres and incubated by gentle rotation at room temperature in the dark for 30 min. Microspheres were then collected using a magnetic rack and transferred to a 96-well flat bottom plate. The microspheres were then washed 3 × using the Bio-Rad Pro II Wash Station. LLO and LMP specific biotin conjugated detection antibodies were added to secreted protein and cell fraction samples, respectively at a concentration of 4 μg/mL in a total volume of 30 μL of antibody diluent solution. After a 30 min incubation period, the beads were washed 3 × followed by the addition of 50 μL of a 1 × stock solution of streptavidin-phycoerythrin conjugate in assay buffer for 10 min with vigorous shaking. Microspheres were washed 3 × and resuspended in 125 μL of assay buffer. Microspheres were then examined using the Bio-Plex 200 apparatus. Fluorescent output from each test sample is calculated as a mean fluorescent intensity (MFI), with a positive result recorded when the MFI value is greater than twice the background level (Day and Basavanna, 2015). MFI values were expressed as means of two replicate experiments performed in duplicate.

2.9. Internal controls

AssayChex process control panel internal controls (catalog #PCP-M-01) were purchased form Radix Biosolutions (Georgetown, TX) and used in all experiments in conjunction with *Listeria* specific antibody-microsphere complexes. Internal controls consist of 4 magnetic bead regions (bead sets # 75,76,77 and 78) which monitor instrument function, fluorescent reporter addition, biotin-conjugated detector antibody addition and non-specific interactions (false positive control), respectively. The manufacturer's recommended threshold metrics acceptance ranges for each microsphere region were applied. The validity of the assays were confirmed when MFI values from microsphere regions 75, 76, and 77 exceeded the manufacturer's recommended MFI threshold value of 300 whereas threshold MFI values below 75 from microsphere region 78 indicated an acceptable range.

2.10. Specificity of Bio-Plex immuno-detection of listeria species and non-listeria bacterial pathogens

Pathogenic and non-pathogenic *Listeria* species as well as a selection of non-*Listeria* foodborne bacterial pathogens (Table 1) were used to inoculate 10 mL of ACM and incubated at 37 °C for 14 h. Cultures were harvested by centrifugation at 10,200 × *g* for 10 min at room temperature. Supernatant were transferred to 15 mL conical tubes and proteins were precipitated with 10% (v/v) TCA for 1 h on ice. Samples were centrifuged for 10 min and washed with ice cold acetone. Protein pellets were resuspended in 1 mL of D-PBS and analyzed by the Bio-plex suspension array procedure using LLO capture and detection antibodies as described above. Whole cell fractions were resuspended in 1 mL of PBS and boiled for 5 min. Samples were centrifuged at 10,200 × *g* for 5 min and supernatants were analyzed by the Bio-plex suspension array procedure using LMP capture and detection antibodies as described above.

3. Results

3.1. ACM induced hypersecretion of L. monocytogenes listeriolysin O (LLO)

Activated charcoal has previously been shown to induce overexpression and hypersecretion of *L. monocytogenes* virulence proteins (Yeung et al., 2005; Ermolaeva et al., 1999; Ripio et al., 1996). To determine the level of LLO secretion stimulated by growth in activated charcoal media (ACM), we compared LLO secretion from *L. monocytogenes* that was grown in ACM with LLO secretion from *L. monocytogenes* grown in other common growth media (LB, BHI, BLEB and Fraser broth). LLO secretion from *L. monocytogenes* grown in ACM was over 31 × greater than when grown in all other media tested (Fig. 1). No discernible LLO secretion was observed when *L. monocytogenes* was grown in Fraser broth.

3.2. Specificity of LLO-1 (capture) and LLO-2 (detection) antibodies

The capture and detection antibody specificities against secreted proteins from several *Listeria* species and a selection of common foodborne bacterial pathogens were determined using SDS-PAGE and immunoblot analysis. The capture antibody identified secreted LLO from *L. monocytogenes* and cross reacted with secreted *L. ivanovii* Ivanolysin O (ILO), the *L. monocytogenes* LLO homolog. However, LLO capture antibody failed to detect the presence of LLO in supernatants from other *Listeria* species or in

supernatants from the control bacterial pathogens tested (Fig. 2). The detection antibody identified secreted LLO from *L. monocytogenes* but failed to detect LLO from other *Listeria* species. In addition, the detection antibody failed to detect LLO from the other foodborne bacterial pathogens tested and did not cross react with *L. ivanovii* ILO (Fig. 2).

3.3. L. monocytogenes LLO and LMP detection specificity using the Bio-Plex suspension array system

Capture (LMP-1) and detection (LMP-2) antibodies were utilized to identify a *Listeria* membrane protein (LMP) present in both pathogenic and non-pathogenic *Listeria* membranes while capture (LLO-1) and detection (LLO-2) antibodies were used to identify secreted LLO using the Bio-Plex system. To determine the detection specificity of the Bio-Plex suspension array, supernatants and whole cell fractions from several *Listeria* species and a selection of non-*Listeria* foodborne bacterial pathogens grown in ACM were analyzed using LLO and LMP capture/detection antibody sets. The LLO specific capture/detection antibody set identified secreted LLO from all 85 strains of *L. monocytogenes* tested but failed to recognize any secreted proteins from *L. ivanovii* strains or non-pathogenic *Listeria* species (Table 1). In addition, MFI values above the positive threshold were not observed in any of the non-*Listeria* bacterial pathogens tested (Table 1). The LMP specific capture/detection antibody combination identified LMP from all strains of *L. monocytogenes, L. ivanovii* and non-pathogenic *Listeria* species except *L. grayi* (Table 1). MFI values above the positive threshold were not observed in any of the non-*Listeria* bacterial pathogens tested (Table 1). Internal controls from each sample were all within acceptable ranges (data not shown).

3.4. Bio-Plex detection of L. monocytogenes and L. Innocua in cantaloupe

To evaluate the effect of food matrices on detection of *Listeria* using the Bio-Plex system and to determine the minimum bacterial load required to elicit a positive result, cantaloupe slices were inoculated with a dilution series of *L. monocytogenes* or *L. innocua* ranging from 10^3 CFU/g to 10^0 CFU/g. A dilution series of *Salmonella enterica* serovar Typhimurium inoculated in cantaloupe slices was also tested as a negative control. Bio-Plex analysis of the LLO and LMP conjugated MagPlex microspheres collected from supernatants and cell fractions, respectively, obtained from *L. monocytogenes* growth in ACM revealed a detection limit of 10^0 CFU/g from both supernatant and cell fractions (Table 2). Analysis of the LLO and LMP conjugated MagPlex microspheres collected from supernatants and cell pellets, respectively, obtained from *L. innocua* growth in ACM revealed a detection limit of 10^0 CFU/g in cell fraction samples but no detection of LLO was observed in TCA precipitated supernatant samples (Table 2). MagPlex microspheres collected from supernatants and cell fractions of *S. enterica* ser. Typhimurium grown in ACM and uninoculated samples (negative controls) failed to elicit a MFI signal above the positive threshold level (Table 2). Internal controls from each sample were all within acceptable ranges (data not shown). These data demonstrate the high detection sensitivity of *L. monocytogenes* and *L. innocua* in cantaloupe using the Bio-Plex suspension array technique following enrichment in ACM.

Table 1
L. monocytogenes and non-*L. monocytogenes* strains used to determine the specificity of the Bio-Plex suspension array assay against secreted Lysteriolysin O (LLO) and cellular *Listeria* membrane protein (LMP).

Organism	Number of strains tested	Bio-Plex target (% positive)	
		Secreted LLO	LMP
Listeria monocytogenes	85	100	100
Listeria ivanovii	17	0	100
Listeria innocua	7	0	100
Listeria welshimeri	12	0	100
Listeria seeligeri	15	0	100
Listeria grayi	5	0	0
Bacillus cereus	1	0	0
Bacillus subtillus	1	0	0
Salmonella Enteritidis	1	0	0
Salmonella Typhimurium	1	0	0
Yersinia pestis	1	0	0
Yersinia enterocolitica	1	0	0
Yersinia pseudotuberculosis	1	0	0
Francisella tularensis subsp. holarctica	1	0	0
Francisella tularensis subsp. novicida	1	0	0
Vibrio parahymolytica	2	0	0
Vibrio cholerae	2	0	0
Enterobacter cloacea	1	0	0
Enterococcus faecalis	2	0	0
Shigella dysenteriae	1	0	0
Shigella sonnei	1	0	0
Shigella boydii	1	0	0
E. coli 0157:H7	2	0	0
Campylobacter jejuni	1	0	0
Campylobacter coli	1	0	0
Klebsiella pneumoniae	1	0	0

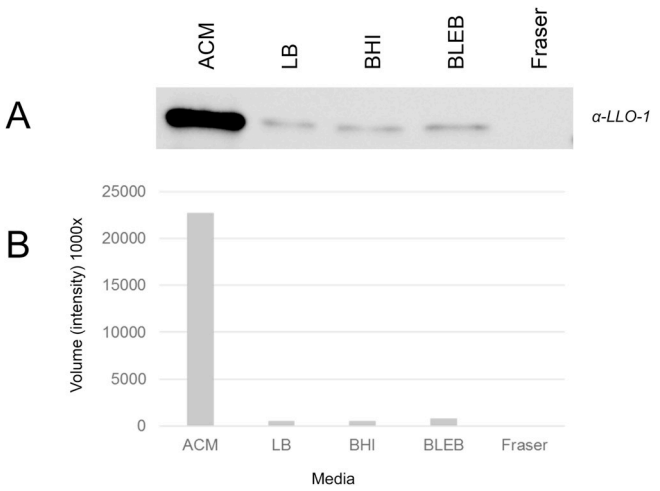

Fig. 1. Immunoblot analysis of culture supernatants from *L. monocytogenes* grown at 37 °C in ACM, LB, BHI, BLEB or Fraser broth using lysteriolysin O specific capture antibody (α-LLO-1). (A) TCA precipitated secreted proteins from *L. monocytogenes* grown in diverse media were analyzed by SDS-PAGE and immunoblot using LLO specific capture antibody. (B) Volume intensity values from densitometric analysis of Western blot bands of *L. monocytogenes* grown in diverse media probing with LLO specific capture antibody.

3.5. Bio-Plex detection of L. monocytogenes and L. Innocua in packaged salad

To evaluate the effect of food matrices containing a high level of natural resident microbiota on detection of *Listeria* using the Bio-Plex system and to determine the minimum bacterial load required to elicit a positive Bio-Plex result, packaged salad was inoculated with a dilution series of *L. monocytogenes* and *L. innocua* ranging from 10^3 CFU/g to 10^0 CFU/g. A dilution series of *S. enterica* ser. Typhimurium inoculated in packaged salad was also tested as a negative control. Bio-Plex analysis of the LLO and LMP conjugated

Fig. 2. Immunoblot analysis of culture supernatants from *Listeria* species and select non-*Listeria* bacterial pathogens grown at 37 °C in ACM probing with capture (α-LLO-1) and detection (α-LLO-2) antibodies. Molecular weight values are indicated on the left.

Table 2
MFI values of *L. monocytogenes*, *L. innocua* and *S.* Typhimurium detection from artificially contaminated cantaloupe by ACM enrichment and Bio-Plex analysis targeting cellular LMP and secreted LLO.

Initial inoculum (CFU/g)	Mean MFI[a]					
	L. monocytogenes		*L. innocua*		*S. Typhimurium*	
	LLO target	LMP target	LLO target	LMP target	LLO target	LMP target
10^3	8799 ± 2362	3541 ± 498	16 ± 05	2292 ± 80	21 ± 4.2	19 ± 0.8
10^2	6236 ± 3265	3222 ± 369	16 ± 0.5	839 ± 287	23 ± 5.2	19 ± 0.0
10^1	829 ± 148	2446 ± 495	16 ± 0.8	382 ± 116	25 ± 2.8	20 ± 1.0
10^0	120 ± 120	296 ± 125	16 ± 0.9	241 ± 46	23 ± 4.8	19 ± 1.0
0	18 ± 0.5	20 ± 0.6	15 ± 0.5	18 ± 1.5	26 ± 7.2	18.0 ± 0.6

[a] MFI values are expressed as means of two replicate experiments performed in duplicate with ± SD.

MagPlex microspheres collected from supernatants and cell fractions, respectively, obtained from *L. monocytogenes* growth in ACM revealed a detection limit of 10^0 CFU/g from both supernatant and cell fractions (Table 3). Analysis of the LLO and LMP conjugated MagPlex microspheres collected from supernatants and cell pellets, respectively, obtained from *L. innocua* growth in ACM revealed a detection limit of 10^0 CFU/g in cell fraction samples but no detection of LLO was observed in TCA precipitated supernatant samples (Table 3). Microspheres collected from supernatants and cell fractions of *S. enterica* ser. Typhimurium grown in ACM and uninoculated samples (negative controls) failed to elicit a MFI signal above the positive threshold level (Table 3). Internal controls from each sample were all within acceptable ranges. These data demonstrate that high sensitivity of *L. monocytogenes* detection in a food matrix known to comprise immunoassay inhibiting components as well as high quantities of competing microbiota using the Bio-Plex suspension array technique following enrichment in ACM.

4. Discussion

The physical nature and composition of food matrices is a significant impediment to the effective performance of molecular and immunological detection methodologies. Therefore, inhibitory food particles must be at least partially removed prior to measuring the presence of a specific target analyte. In this study, we utilized specific antibody-magnetic microsphere complexes to segregate and identify secreted lysteriolysin O from *L. monocytogenes* grown in activated charcoal enrichment media. The detection limit attained was 10^0 CFU/g in all foods tested and the time to detection was achieved in less than 24 h. The utilization of antibody conjugated magnetic microspheres that bind target microorganisms have previously been moderately successful in separating various bacterial pathogens such as *Listeria*, *Campylobacter, Salmonella, Escherichia coli*, and *Shigella* from the original food sample using magnets (Kim et al., 2010). Nevertheless, homogenized food matrices release elements such as proteins, carbohydrates and other macromolecules that have been shown to weaken assay detection sensitivity by obstructing the interactions between the antibody conjugated microspheres and target organism (Kim et al., 2010; Ogunjimi and Choudary, 1999). In fact, *L. monocytogenes* detection sensitivity was shown to be significantly diminished when using magnetic microspheres in various foods including lettuce, spinach and milk compared to detection in PBS alone (Kim et al., 2010). Indeed, many fruits and vegetables possess elevated levels of polyphenols which impede binding between antibody and target cells (Ogunjimi and Choudary, 1999). The high detection sensitivity achieved in this study is likely facilitated not only by the prolific secretion of LLO induced by activated charcoal but by the removal of inhibiting food components prior to addition of antibody/MagPlex bead complexes as well. After L. *monocytogenes* incubation in ACM, centrifugation of growth samples effectively separate secreted proteins from residual food particles and competing food microbiota which allows for a cleaner sample for Magplex/antibody capture of LLO. Although only one secreted analyte was tested in this study, the Bio-Plex suspension array technique has the potential to evaluate 100 analytes simultaneously in a common sample in a high throughput and real time testing format which reduces reagent costs and labor. The four bead internal controls are also added directly to the sample tests to ensure the integrity of the assays.

The presence of low numbers of *L. monocytogenes* in food matrices presents a major obstacle for detection of these bacteria during an outbreak of listeriosis. Use of traditional culture reference methods, such as those described in FDA's *Bacteriological Analytical Manual* and in the International Organization for Standardization's (ISO) 11290:2017 (Hitchins et al., 2017; ISO, 2017) can overcome this complication by allowing the organism to multiply in pre-enrichment media followed by selective enrichment media over a prolonged period. Although these methods reach high sensitivity levels, results are not obtained until several days after initial food sample processing and the procedure can be labor intensive. Immunological detection techniques have more recently been developed to reduce the time for detection while maintaining high specificity and sensitivity levels. The Vitek Immuno Diagnostic Assay System (VIDAS) is an automated ELISA based technique that can identify *L. monocytogenes* with detection limits of 0.2–2 CFU/25 g in various food matrices (Johnson et al., 2013). However, an enrichment period of 26–30 h is required which delays acquisition of results. Use of fiber-optic immuno-sensors is an alternative immunological-based method that exhibits a reduction in the overall detection time to under 24 h but is limited due to susceptibility to false positive results (Geng et al., 2004). Immuno-detection of *L. monocytogenes* using the Bio-Plex suspension array system has previously been utilized in various food matrices following enrichment in a macrophage based system (Day and Basavanna, 2015). Although the time to detection was just 24 h, the detection limit was restricted to 10 CFU/mL or g and cross-reactivity was observed with *L. ivanovii* (Day and Basavanna, 2015). The detection limit using these methods is dependent

Table 3
MFI values of *L. monocytogenes*, *L. innocua* and *S.* Typhimurium detection from artificially contaminated packaged salad by ACM enrichment and Bio-Plex analysis targeting cellular LMP and secreted LLO

Initial inoculum (CFU/g)	Mean MFI[a]					
	L. monocytogenes		*L. innocua*		*S. Typhimurium*	
	LLO target	LMP target	LLO target	LMP target	LLO target	LMP target
10^3	5063 ± 1505	781 ± 111	21 ± 6.8	377 ± 154	19 ± 0.5	19 ± 4.8
10^2	4234 ± 1045	536 ± 15	22 ± 6.1	216 ± 121	20 ± 1.4	18 ± 3.6
10^1	1948 ± 397	138 ± 28	25 ± 5.7	136 ± 67	25 ± 4.1	19 ± 3.4
10^0	156 ± 27	69 ± 17	24 ± 5.8	77 ± 30	27 ± 6.3	18 ± 3.1
0	14 ± 0.0	18 ± 2.6	25 ± 7.2	16 ± 1.7	27 ± 4.1	19 ± 5.4

[a] MFI values are expressed as means of two replicate experiments performed in duplicate with ± SD.

on the amount of bacteria present after enrichment and the expression and accessibility of bacterial surface antigens to specific antibodies. In this study, we utilized antibodies that react to LLO, a virulence protein that is abundantly secreted under temperatures that activate the *L. monocytogenes* virulence regulon. In addition, activated charcoal media is utilized in the enrichment broth, which has previously been shown to stimulate hyperactive expression and secretion of *L. monocytogenes* toxins. Indeed, we show that growth of *L. monocytogenes* in ACM induced over 31 × the amount of LLO secretion than in other common enrichment broth tested. The secreted L. *monocytogenes* virulence factors Phosphatidyl-inositol phospholipase C (PI-PLC) and Internalin C (InlC) also showed much greater secretion in ACM than in other media tested (data not shown). Detection of secreted proteins has the added benefit of confirming the viability of *L. monocytogenes* present in a food sample as nonviable cells fail to express and secrete virulence proteins.

There is a high degree of homology between the genomes of *L. monocytogenes* and other *Listeria* species, particularly *L. ivanovii*, which increases the level of difficulty in distinguishing between them using molecular and immuno-detection techniques. Although antibodies directed against *L. monocytogenes* targets such as p60, PI-PLC, surface autolysin IspC, and *Listeria* adhesion Protein B (LapB) have previously been used as diagnostic tools for *L. monocytogenes*, the utility of these antibodies has been limited due to restricted specificity (Boivin et al., 2016; Suryawanshi et al., 2017; Ronholm et al., 2013; Yu et al., 2004). On the other hand, LLO has been a popular target for identifying *L. monocytogenes* as it is expressed in all clinical isolates and is unique to *L. monocytogenes* serotypes (Suryawanshi et al., 2017; Erdenlig et al., 1999; Law et al., 2015). However, although non-pathogenic *Listeria* species do not express LLO, *L. ivanovii* encodes a protein referred to as Ivanolysin O (ILO) with high sequence similarity to *L. monocytogenes* LLO and specific LLO antibodies have been shown to cross-reacts with ILO (Day and Basavanna, 2015; Erdenlig et al., 1999). Indeed, protein alignments of LLO with ILO show 80% identity and 92% similarity. Nevertheless, the LLO specific detection antibody did not cross react with *L. ivanovii* ILO. In contrast, the commercially available LLO specific antibody, which we utilized as the capture antibody, did show cross reactivity to ILO of *L. ivanovii*. Bio-Plex suspension array detection experiments of 17 strains of *L. ivanovii* using the capture and detection antibody combination failed to show MFI values greater than the positive threshold indicating that the LLO detection antibody is sufficient to impart specificity to *L. monocytogenes* detection. Similarly, 39 non-pathogenic *Listeria* strains tested were also negative. Interestingly, it has been shown that *L. monocytogenes* serotypes 4a and 4c often escape detection using immuno-assays with antibodies directed against p60 and LapB (Boivin et al., 2016). In our case, Bio-Plex detection analysis of all *L. monocytogenes* serotypes tested were positive, including serotypes 4a and 4c. Testing of 25 non-*Listeria* bacterial pathogens also failed to produce MFI values above the positive threshold which highlights the highly specific quality of the assay.

L. ivanovii and non-pathogenic *Listeria* species are often found in various food matrices and are capable, although very rarely, of initiating human infection (Cummins et al., 1994; Guillet et al., 2010; Liu, 2013; Snapir et al., 2006; Rocourt et al., 1986). However, *L. ivanovii* is more commonly recognized as a potential source of infection in ruminants which can result in substantial economic loss in the livestock industry (Alexander et al., 1992; Chand and Sadana, 1999; Gill et al., 1997; Sergeant et al., 1991; Ramage et al., 1999). Therefore, we included a second set of commercially available capture and detection antibodies that are directed against an unidentified *Listeria* membrane protein (LMP) present on both pathogenic and non-pathogenic *Listeria*. The Bio-Plex assay conducted using the LMP specific antibodies produced positive results when tested against both pathogenic and non-pathogenic *Listeria* except L. *grayi* (Table 1). Interestingly, previous studies have revealed that antibodies directed against *Listeria* species do not recognize *L. grayi* antigens (59). Indeed, evolutionary history suggest that *L grayi* is a much more distant relative of other *Listeria* species based on whole genome sequences (Hain et al., 2007; Sauders et al., 2012). The use of two sets of capture and detection antibodies facilitates the distinction between *L. monocytogenes* and other *Listeria* species. Specifically, utilization of the two capture/detection antibody sets allows us to determine the presence of *L. monocytogenes* serotypes (LLO+, LMP+), *L. ivanovii* and some non-pathogenic *Listeria* species (LLO-, LMP+) or the absence of *Listeria* species (LLO-, LMP-). The method, however, does not rule out the possibility of the presence of *L. grayi* or contamination with multiple *Listeria* species. Use of the LMP specific antibody set not only increase the degree of specificity for *Listeria* species detection but also reduces the time to detection by 2 h as TCA precipitation is not utilized for extraction of LMP during whole cell processing. In the future, other non-pathogenic *Listeria* species that have recently been described will require testing to further determine the specificity of antibodies.

In conclusion, this study demonstrates the utilization of a novel enrichment media that induces increased production of an *L. monocytogenes* specific toxin that can be used as a marker to identify the bacterium present in specific food matrices. The method has the advantage of inducing very high concentrations of target protein from low levels of *L. monocytogenes* as well as isolating the target protein from the environment to produce a clean sample for subsequent immuno-detection. The method also enables the differentiation between *L. monocytogenes* and *L. ivanovii* as well as from nonpathogenic *Listeria* species. This technique could be implemented as a rapid preliminary screen of foods for the presence of viable *L. monocytogenes* in case of an outbreak and would lead to enhanced responding efforts.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.05.009.

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