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Concentration of *Listeria monocytogenes* in skim milk and soft cheese through microplate immunocapture

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ABSTRACT

Microplate immunocapture is an inexpensive method for the concentration of foodborne pathogens using an antibody-coated microplate. The objective of this study was to determine the efficacy of microplate immunocapture as an alternative to traditional enrichment for concentrating *Listeria monocytogenes* to levels detectable with selective plating or real-time PCR. *L. monocytogenes* isolates serologically characterized as Type 1 (1/2a) and Type 4 (untypeable) were grown overnight and diluted to 10^0 to 10^6 colony-forming units (CFU)/mL. The isolates were used to optimize microplate immunocapture in tryptic soy broth with 0.6% yeast extract (TSBYE), skim milk, and queso fresco samples. Following microplate immunocapture, the bacteria were streaked onto polymyxin-acriflavine-LiCl-ceftazidime-aesculin-mannitol (PALCAM) agar, followed by incubation at 37 °C for 24 ± 2 h. The bacteria also underwent real-time polymerase chain reaction (PCR). The optimized microplate immunocapture method was tested in triplicate for its ability to capture *L. monocytogenes* in broth and food samples. Overall recovery rates for *L. monocytogenes* in food samples at cell populations of 10^0, 10^2, and 10^4 CFU/25 g using microplate immunocapture with real-time PCR were 88.9%, 94.4%, and 100%, respectively. Recovery in these matrices using microplate immunocapture with selective plating was comparatively lower, at 0%, 44.4%, and 100%, respectively. Conventional culture method showed 100% detection at each inoculation level. Microplate immunocapture combined with real-time PCR shows high potential to reduce the time required for detection, with concentration of *L. monocytogenes* to detectable levels within 1–4 h. The incorporation of a short enrichment step may improve recovery rates at low cell levels.

1. Introduction

Listeria monocytogenes is a facultative anaerobic bacterium that is especially problematic due to its ability to survive and grow at refrigerated conditions (FDA, 2012). This pathogen has the highest hospitalization rate (94.0%) and the third-highest death rate (15.9%) among foodborne pathogens in the United States (Scallan et al., 2011). Common symptoms caused by *L. monocytogenes* are fever, muscle aches, nausea, and vomiting (FDA, 2012). However, in more serious cases it can cause septicemia and meningitis, as well as induce stillbirth or miscarriage in pregnant women. Listeriosis is often linked to raw or ready-to-eat foods, such as fresh produce, unpasteurized milk, smoked fish, and deli meats. There are 13 known serotypes of *L. monocytogenes*, with strains of serotypes 1/2a, 1/2b, and 4b responsible for the majority of foodborne infections. The U.S. Food and Drug Administration (FDA) has a zero-tolerance policy for *L. monocytogenes* in ready-to-eat foods and it is consistently one of the most common pathogens associated with food recalls in the United States (FDA, 2018).

Dairy products, such as milk and cheeses, are a major cause of outbreaks linked to *L. monocytogenes* (CDC, 2017). For example, *L. monocytogenes* was among the top three pathogens linked to 90 foodborne outbreaks associated with cheese in the United States from 1998 to 2011 and it was associated with 5 of the 6 deaths reported (Gould et al., 2014). Mexican-style cheese, including queso fresco, was the main type of cheese associated with illness from *L. monocytogenes* during this time period (Gould et al., 2014). Queso fresco is a soft, unaged cheese that is susceptible to *Listeria* survival and growth due to its relatively high moisture content and low acidity (Moreno-Enriquez et al., 2007).

Cultural methods for the isolation of *L. monocytogenes* involve a series of pre-enrichment and enrichment steps, followed by plating on selective/differential agar (Hitchens et al., 2016). This process is very time-consuming, usually requiring 2–4 days, not including the time required for confirmation of isolated colonies. Bacterial separation and

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concentration methods have the potential to reduce or possibly eliminate the need for pre-enrichment and enrichment steps, thereby significantly shortening the time required for isolation (Stevens and Jaykus, 2004). These techniques are also advantageous because they can be combined with rapid detection methods, such as polymerase chain reaction (PCR) or enzyme-linked immunosorbent assay (ELISA), further reducing the time to detection.

Immunomagnetic separation is a widely used method for bacterial separation and concentration; however, it is relatively expensive due to the need for antibody-coated beads (Amagliani et al., 2006; Chen et al., 2017; Ma et al., 2014). Non-magnetic immunocapture is an inexpensive alternative that relies on the binding of antibodies to a solid plastic support (Arbault et al., 2014a). This technique has been successfully used for the concentration of foodborne pathogens in a limited number of studies (Arbault et al., 2014b; Fakruddin et al., 2017; Molloy et al., 1995). For example, Arbault et al. (2014b) were able to concentrate *Escherichia coli* O157:H7 from ground meat and raw milk cheese samples with an antibody-coated microplate. Using a combination of the microplate and a subculture step (3–5 h), *E. coli* was recovered at levels of 10^5 CFU as compared to 10^3–10^4 CFU with magnetic beads. In another study, microplate immunocapture was evaluated as a potential method for the concentration of *Vibrio cholera, Salmonella enterica* serovar Typhi, and *Shigella flexneri* from a variety of food samples (Fakruddin et al., 2017). Overall, the authors found that microplate immunocapture combined with PCR or selective plating allowed for improved recovery of the target pathogens from foods as compared to traditional culture methods.

PCR is a well-established technique for the rapid identification of foodborne pathogens and it is widely recognized for its specificity and sensitivity (Zhao et al., 2014). Real-time PCR is advantageous over traditional PCR because it enables continuous monitoring of the results as the reaction proceeds and eliminates the need for post-PCR processing steps. There are numerous commercially available kits for the detection of *L. monocytogenes* using real-time PCR (Law et al., 2015) and a real-time PCR assay for detection of *L. monocytogenes* has been published in the FDA's Bacteriological Analytical Method (BAM) (FDA, 2015). Although PCR-based methods are susceptible to inhibition from compounds in the food matrix, concentration methods such as microplate immunocapture can help to overcome this by separating the target organism from the rest of the sample (Fakruddin et al., 2017; Stevens and Jaykus, 2004).

The specific aims of this study were to: (Amagliani et al., 2006) determine the ability of microplate immunocapture combined with selective plating or real-time PCR to detect *L. monocytogenes* in a pure broth solution within 1 workday (8 h), (Arbault et al., 2014a) optimize microplate immunocapture as a means of concentrating *L. monocytogenes* in milk and cheese samples for subsequent detection with selective plating or real-time PCR, and (Arbault et al., 2014b) determine the sensitivity and time to detection for microplate immunocapture combined with selective plating or real-time PCR.

2. Materials and methods

2.1. Media and bacterial strains

All media were obtained from Becton, Dickinson and Company [(BD) (Franklin Lakes, NJ)] unless otherwise stated. Two environmental isolates of *L. monocytogenes* were obtained from the U.S. Food and Drug Administration (FDA) Pacific Regional Laboratory Southwest (Irvine, CA). The isolates were serologically categorized as Type 1 (T1; serotype 1/2a) and Type 4 (T4; untypeable) by a combination of slide agglutination and multiplex PCR (Burall et al., 2011; Doumith et al., 2004) using modifications described in Hellberg et al. (2013). The isolates were streaked to Tryptic Soy Agar (TSA) and incubated overnight at 37 °C, then transferred to tryptic soy broth with 0.6% yeast extract (TSBYE) and incubated overnight at 37 °C to concentrations of 10^8 CFU/mL. Concentration levels were determined by optical density (OD) measurement based on a logarithmic growth curve (not shown) and verified by plate count on TSA. Bacterial cultures from the T1 and T4 isolates were grown separately. The cultures were then serially diluted to concentrations of 10^6 CFU/mL, 10^4 CFU/mL, 10^2 CFU/mL, and 10^0 CFU/mL in TSBYE. For T1 + T4 mixed culture testing, equivalent amounts of the T1 and T4 cultures (10^8 CFU/mL) were combined prior to carrying out serial dilutions.

2.2. Microplate preparation

Polystyrene 96-well microtiter microplates separable into 8-well strips (Fisher Scientific, Waltham, MA) were prepared for the concentration of *L. monocytogenes* according to a protocol from Abcam (**http://www.abcam.com/protocols/sandwich-elisa-protocol-1**). Anti-*Listeria* Polyclonal Antibody, HRP conjugate PA1-73129 (Invitrogen, Carlsbad, CA) was diluted to 1–10 μg/mL in carbonate-bicarbonate buffer. The diluted antibodies were adhered to the inner surface of the microplate by transferring 200 μL of the solution to each of the wells. The plates were then covered with plastic and held overnight (8–16 h) at 4 °C. The following day, the plates were rinsed with phosphate buffered saline solution (PBS), pH 7.4, blocked with a 5% skim milk/PBS solution, held at room temperature for 2 h, and then rinsed a final time with PBS. Following this process, the plates were used in microplate immunocapture, as described below, or stored at −20 °C until needed.

2.3. Optimization of microplate immunocapture

The antibody-coated microplates prepared above were first tested with mixed cultures of *L. monocytogenes* Types 1 and 4 in TSBYE to optimize the method in the absence of a food matrix. The T1 + T4 cultures were prepared as described above to allow for concentrations of 10^6 CFU/mL, 10^4 CFU/mL, 10^2 CFU/mL, and 10^0 CFU/mL (Singh et al., 2012). A blank sample containing TSBYE was run alongside each set of experiments as a negative culture control. Microplate immunocapture was carried out in a biosafety hood and optimized for the number of fill cycles (Amagliani et al., 2006; Arbault et al., 2014a; Arbault et al., 2014b; Burall et al., 2011), hold times (15–60 min), antibody concentration (1–10 μg/mL), and use of a plate shaker (Bio Rad, Hercules, CA) at speeds of 10–120 RPM. For each fill cycle, 1.6 mL of each inoculated broth or control sample were transferred to 8 wells of the antibody-coated plate, resulting in 200 μL of sample per well. The sample was then incubated at room temperature for a specific period of time (i.e., hold time) before being discarded and replaced in the next fill cycle. With each fill cycle, an additional 1.6 mL of the sample (200 μL per well) was added, resulting in a total volume of 6.4 mL per sample (800 μL per well) when 4 fill cycles were carried out.

Following microplate immunocapture, all 8 wells were scraped for each sample using a disposable sterile inoculating loop. The loop was then streaked onto polymyxin-acriflavine-LiCl-ceftazidime-aesculin-mannitol (PALCAM) agar. Next, all 8 wells were scraped again for each sample using a second sterile inoculating loop. The second loop was then mixed with 100 μL sterile water in a sterile Safe-lock microcentrifuge tube (Eppendorf, Hamburg, Germany) to release bacterial cells for DNA extraction, as described below. Positive culture controls were prepared using the 10^8 CFU/mL broth sample, which was streaked directly to PALCAM or transferred to a microcentrifuge tube for DNA extraction using a sterile disposable loop. The PALCAM plates were incubated for 24 ± 2 h at 37 °C. The plates were then examined for typical *L. monocytogenes* growth, consisting of grey-green colonies with accompanied blackening of the agar. Once optimal microplate immunocapture conditions were determined using the PALCAM plates, the T1 + T4 mixed culture as well as individual T1 and T4 cultures were tested in triplicate using the optimized procedure (Table 1).