Impedance spectroscopy and biochip sensor for detection of *Listeria monocytogenes*

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ABSTRACT

*Listeria monocytogenes* is a deadly foodborne human pathogen. Its ubiquitous nature and its ability to grow at refrigeration temperatures makes this organism a difficult one to control. High-volume processing of food products and poor sanitary conditions of the processing plants often allow this organism to be present in processed, ready-to-eat (RTE) foods. Improved processing along with real-time detection could reduce the incidence of this pathogen. Conventional methods can detect this pathogen accurately, but take several days (2-7d) to complete, which is not practical considering the short shelf-life and cost of storage of RTE foods. Biosensor based approaches were adopted for sensitive detection of *Listeria*. Antibody-coupled fiber optic and microelectrical-mechanical system (MEMS) biochips were designed and examined for direct detection of *L. monocytogenes* from liquid samples. Also, interdigitated microsensor electrode (IME) chip and spectrofluorometer were used to measure *L. monocytogenes* interaction with mammalian cells (cytopathogenic activities) for indirect detection. Preliminary data generated using laboratory cultures of *Listeria* species indicated that *L. monocytogenes* could be detected in 30 min to 1 h 30 min depending on the techniques used.

Keywords: *Listeria monocytogenes*, cytopathogenicity assay, alkaline phosphatase, fiber-optic, interdigitated microsensor electrode-chip, biochip, impedance spectroscopy, spectrofluorometer

1. INTRODUCTION

Consumption of food contaminated with pathogenic or toxigenic microorganisms cause serious illness and even death. On an average, about 6 million people get sick each year from eating contaminated food, of which about 9 thousand die. In addition, in the past several years (between 1986-1993) food products tainted with *Listeria* resulted in 185 recalls involving 970 products and 169 firms. Collectively, economic losses due to human illnesses, product recalls and closure of food processing plants were estimated to be about $8.4-23 billion per year for the US. The estimated cost for *L. monocytogenes* infection alone is about $2 billion. Because of the high morbidity and mortality, the Food and Drug Administration (FDA) has mandated "0" tolerance for *L. monocytogenes* in ready-to-eat (RTE) foods. Immuno-compromised individuals, including infants, elderly, diabetic, cardiovascular patients, patients with organ transplants, cancer patients, HIV infected persons and pregnant women, are highly susceptible to *L. monocytogenes* infection.

*Listeria monocytogenes* is a ubiquitous Gram-positive rod shaped bacterium that can grow at refrigeration temperatures. *Listeria* species have been found in a variety of raw and processed ready-to-eat food products including dairy, vegetable, meat, poultry and seafood. Many of these foods have been implicated in human listeriosis since the 1980s. Numerous outbreaks and product recalls emphasize the urgent need for aggressive research activities for sensitive detection and control of *L. monocytogenes* from RTE foods.

Genus *Listeria* is comprised of six species, of which *L. monocytogenes* and *L. ivanovii* are known to be pathogenic for humans and animals, respectively. *L. monocytogenes*, when present in ready-to-eat foods, is usually in very low numbers (1-1000 colony forming units (CFU)/g or ml), and often associated with other non-pathogenic *Listeria* species, such as *L. innocua*, *L. welshimeri*, *L. seeligeri* or *L. grayi*. Specific and sensitive detection of *L. monocytogenes* is critical for ensuring food safety and preventing economic losses and human lives. Several rapid and automated detection methods have
been developed; however, none of these techniques are capable of real-time detection of a few Listeria cells directly from foods without an enrichment step.

A recent multi-state outbreak emphasized that there is an urgent need to develop technologies for sensitive detection of low numbers of L. monocytogenes cells in food products. Since there is no definitive data available for the infective dose of L. monocytogenes, hence the question arises what should be the acceptable detection limit for this pathogen? It is generally believed that a few microorganisms (10-1000 CFU/g or ml) could potentially cause severe forms of disease in susceptible individuals. Therefore, the obvious challenge is to develop methods, which could sensitively and accurately detect such low numbers of bacteria from foods in a short period (1-2 h) of time. Ideally, the assay could be used on-site during various stages of processing, holding and distribution of food products.

1.1. Conventional Culture Methods

Conventional culture methods as outlined by the USDA or FDA may take about 2-7 days for detection and confirmation of L. monocytogenes from foods. Normally, test samples are homogenized or stomached and added with selective enrichment broth. Enriched samples are then streaked onto selective media such as Oxford (Oxoid Ltd.) or lithium chloride phenylethanol moxalactam (LPM) or PALCAM agar plates. Suspect cultures are further tested for fermentation of various sugars by using API or Micro-ID—Listeria methods or using more sophisticated automated culture identification instruments such as the MicroLog (Biotec Inc), VITEK (bioMerieux Vitek) or MicroScan WalkAway (Dade MicroScan International) systems. The isolates are tested for the production of hemolysin and phospholipases and also characterized serologically for reactions with somatic or flagellar antigens.

1.2. PCR and DNA Based Methods

Rapid detection of Listeria spp. in foods can be achieved by colorimetric DNA hybridization assays. These methods are time consuming involving several steps for DNA isolation and manipulation, and require large numbers of bacterial cells for detection. Therefore, selective enrichment and isolation steps are essential for Listeria detection. With the advent of PCR technology, amplification of specific target DNA, primarily virulence genes or 16S RNA can detect relatively low numbers of L. monocytogenes cells from food. However, contaminating background microflora and food particles may interfere with the assay, therefore isolation and enrichment steps are often necessary to circumvent these problems.

Randomly amplified polymorphic DNA (RAPD), restriction fragment-length polymorphism (RFLP), pulsed-field gel electrophoresis (PFGE) and ribotyping techniques have been used for specific identification of L. monocytogenes. However, these techniques are generally employed with pure isolates from foods. These techniques are not considered as rapid; however, they generate genomic fingerprint data, which can be extremely valuable for tracking foodborne pathogens through the food chain.

1.3. Antibody-Based Detection Methods

Immunoassays such as ELISA, dot or colony-blot are used for rapid detection of Listeria spp. from food samples. The abilities of these assays to detect only pathogenic L. monocytogenes depend on the specificity of monoclonal or polyclonal antibodies. Listeria species in general share a high degree of identity in their surface antigens. The majority of commercially available antibodies react with all Listeria species. Therefore, for specific detection or confirmation of L. monocytogenes, additional tests are required. Monoclonal antibodies developed against a 66-kDa surface antigen were shown to have great promise for successful detection of L. monocytogenes only. Furthermore, antibody-based assays require at least 10^6 bacterial cells for effective detection, which may not always be present in the food samples. Therefore, pre-enrichment or selective enrichment steps are essential to increase bacterial populations.

Use of antibodies coated on the surface of magnetic beads to capture or concentrate Listeria cells from food suspensions appears to be very promising for rapid isolation of low (~10^2 CFU/ml) numbers of L. monocytogenes cells. These captured bacterial cells can be further used for PCR assay, immuno-magnetic PCR, flow cytometry, and selective culturing. The major problems one may encounter with immunomagnetic PCR over other immunomagnetic separation (IMS) methods is that it will not be able to differentiate live from dead bacterial cells. Additionally, antibodies are also used for direct detection of Listeria cells by immunofluorescent techniques.
1.4. Cytopathogenicity-Based Detection

Examination of pathogenic interactions of Listeria isolates with animal or their tissue cells can be used as a means for rapid detection of Listeria species. Among the animal models, the mouse model has been approved by the FDA\textsuperscript{48} and been proven to be efficient in determining the pathogenicity of various food and clinical isolates. However, this procedure takes at least 3 days to complete and requires an animal holding facility thus may not be feasible for routine use. Tissue culture methods such as Caco-2, HT-29, 3T3, HeLa, CHO and J744 cell lines have been proposed as alternatives to the animal model and provide results in 8-24 h.\textsuperscript{14,16,43} Cytotoxicity effects on these cell lines are often determined by differential trypan blue staining or intracellular bacterial counts assay. Considering the complications associated with these procedures, only a limited number of isolates can be tested.

In previous studies, we have used hybridoma B-lymphocyte (Ped-2E9) line to determine the virulence of L. monocytogenes and cytotoxicity results were obtained in 4-6 h. \textsuperscript{7} Using the same cell line, we configured a user-friendly microtiter plate method, where alkaline phosphatase release from Ped-2E9 was used as an indicator for cell cytotoxicity. This colorimetric procedure allowed us to detect L. monocytogenes and some strains of L. ivanovii in 4-5 h with a minimum of experimental setup\textsuperscript{7}. Furthermore, to make this cytotoxicity assay even faster, we showed that the addition of a reducing agent, dithiothreitol (DTT), significantly increased the L. monocytogenes mediated Ped-2E9 cell death (90-95%), which could be detected in just 30 min-1 h. \textsuperscript{58} Recently, we showed that the cytotoxicity effect of L. monocytogenes on Ped-2E9 cells is due to the actions of listeriolysin O or phospholipase C, which cause membrane destabilization (pore formation) and apoptosis in Ped-2E9 cells.\textsuperscript{5}

2. BIOSENSOR-BASED DETECTION

2.1. Antibody-Coupled Fiber Optic Biosensor

In recent years, development of fiber optic biosensors for the real-time detection of biological weapons specially, bacterial cells, toxins or spores is intensely being investigated by the military.\textsuperscript{23,22,52} The basic principles of a fiber optic biosensor are covalent linking of a specific antibody (primary) to the core of a fiber-optic cable, binding of target antigen to the primary antibody, and the detection of antigen-antibody complex by a secondary antibody conjugated to fluorescence molecules.\textsuperscript{41} The formation of a specific antibody-antigen sandwich will emit fluorescence light. When light is transmitted through the core of a fiber-optic cable, it will generate an electromagnetic wave (evanescent wave) in the medium outside the fiber. This wave will stimulate the fluorescence of the antibody-antigen sandwich, which can be measured by a solid state detector such as a spectrometer.\textsuperscript{45,56} Antibody-coupled fiber optic biosensors are being developed for the detection of botulinum toxin A,\textsuperscript{41} staphylococcal enterotoxin B,\textsuperscript{52} Salmonella\textsuperscript{42} and E. coli O157:H7.\textsuperscript{17} A PCR-based fiber optic biosensor has also been reported for the detection of Listeria species.\textsuperscript{49}

We have developed a fiber optic biosensor for Listeria by conjugating polystyrene fiber with a monoclonal antibody (MAB) to Listeria species using carbonate coating buffer. After exposure of fiber to different Listeria species, we added a purified MAB, specific for L. monocytogenes,\textsuperscript{6,8} that was conjugated with cyanine (Cy5) dye (emission $\lambda_{\text{emax}}=668$ nm, excitation $\lambda_{\text{emax}}=635$ nm) (Molecular probes). Specific binding of Listeria cells to the antibody coated fiber optic biosensor and subsequent reaction of Cy5 labeled MAB to L. monocytogenes generated fluorescence that was detected by Analyte 2000 spectrometer (Research International, Woodinville, WA). The Analyte 2000 uses 635 nm laser diode to provide the excitation that is launched into a proximal end of the fiber optic. The evanescent field excites Cy5 molecules located within approximately 100 nm of the fiber and a portion of their emission energy is recoupled into the fiber and quantified by a photodiode in the fluorometer at 668 nm. Using this technology, L. monocytogenes could be detected and differentiated from other Listeria species (Figure 1).

![Figure 1: Detection of Listeria monocytogenes using antibody-coupled fiber optic biosensor](image)

2.2. Antibody-Coupled Biochip Biosensor

In the recent years, there has been a merger of microelectronics and biological sciences to develop micro-scale biosensors or 'biochips'. The term biochip has been used in various contexts but can be defined as 'microelectronic and microfabrication-
inspired devices that are used for processing (delivery, analyses, or detection) of biological molecules and species". These micro-scale biosensors have found increasing use in immunoassay applications due to the advances in the fields of micro-fluidics and microelectrical-mechanical systems (MEMS). Chip-based detection systems are ideal for the possible detection of DNA binding, antigen-antibody interactions, and cell identification and detection when small quantities are to be interrogated. Preliminary research has been performed using avidin, fluorescein labeled avidin, biotin, and fluorescein labeled biotin in our laboratories and has given indication that these proteins can be adsorbed on surfaces of biochips and that their binding can be detected optically. The eventual goal here is to detect the antigen-antibody binding using electrical means in micro-fabricated devices, which could require small sample size and could provide the results rapidly. We have fabricated biochips using a series of deposition, photolithography, and etching techniques in silicon-based wafers. Cavities and channels were formed using anisotropic KOH based etching. RF sputtering of chrome and platinum was performed to form metal electrodes, which were used to measure the impedance of bacteria. Figure 2 shows an optical micrograph of the completed device with micro-fluidic input and output interface ports. The electrode geometry and spacing needs to be optimized to ensure that the bacteria will perturb the electric field above the electrodes significantly and hence will cause a change in the measured impedance. The measured impedance is a function of frequency and the magnitude decreases with increasing frequency. Two possible modes of detection can be used. Firstly, the impedance change can be detected when the bacteria is placed over the electrodes due to the differences in dielectric constant and resistance (hence impedance) of the bacteria itself with respect to the buffer medium. Secondly, the bacteria can cause a change in the ionic concentration and hence the impedance of the medium, which can then be detected. The results shown in figure 3 indicate the possibility of the second mechanism and show that the impedance decreases after the bacteria is added to the buffer. Work continues in our group to optimize the electrode geometry of the interdigitated electrodes to allow for a more sensitive detection of the change in impedance.

**Listeria innocua at -1×10⁹ ml⁻¹**

Well w1-2, pads 74,75. Rectangular well with two finger electrodes.

![Figure 2: Micro-fluidic biochip](image)

![Figure 3: Impedance and phase vs. frequency of Listeria in the biochip.](image)
2.3. Impedance Spectroscopy for Cytopathogenicity

Bacterial interactions with mammalian cell membranes can be measured by electrical methods. Generally, the compartments of a cell are separated from the surrounding medium by a cell membrane, which consists mainly of highly structured electrically insulating phospholipids. The electrical properties of the biological membrane can be modeled as a resistor and capacitor network. Therefore, any (external factor) that affects the integrity of the membrane will cause a change in the conductivity of the cell system and the magnitude of the electrical impedance could be measured. Changes in electrical impedance (complex conductivity) have been used to detect freezing injury of plant cells or infection by viruses.

Similar impedance measurements have been studied to predict the changes in porcine muscle tissue during the rigor process. Using a tetrapolar electrode, accurate measurements of tissue impedance can be made in the muscle. The changes in the impedance over time were correlated to the water holding capacity of the meat at 24 h post mortem. By modeling the cell system as an electrical network and monitoring changes in the electrical impedance at selected frequencies, the degree of cell disruption can be measured.

Interaction of Listeria species with mammalian Ped-2E9 cells was studied by using an interdigitated microsensor electrode (IME) chip in conjunction with an impedance analyzer to measure changes in the cellular (Ped-2E9) membrane potential. The IME is monolithic chip containing gold interdigitated electrodes spaced 15 μm apart (ABTCH Scientific, Inc. Richmond, VA). The current flows in a parallel path between the interdigitated fingers, as well as in an arc spanning over adjacent fingers. Measurements of the resistance (real vector) and capacitance (imaginary vector), as detected by the IME-chip, were used to calculate the magnitude (Z) and the phase (Φ) of impedance of the Ped-2E9 cells using an HP-4194A impedance analyzer, which can provide the source and detection circuits for measurement in the range of 10 Hz to 40 MHz.

Preliminary results indicated that intact Ped-2E9 cells (control) had phase angle values of −83.5° whereas L. monocytogenes infected Ped-2E9 cells had −78.1° after 1 h of exposure. Nonpathogenic L. innocua showed similar values as control Ped-2E9 cells. These values confirm the potential of IME-chip based technique for detection of L. monocytogenes cells.

2.4. Spectrofluorometry for Cytopathogenicity

As indicated before, pathogenic Listeria induces severe damage to Ped-2E9 cells resulting in alkaline phosphatase (AP) release from Ped-2E9 cells. The AP could be assayed with p-nitrophenyl phosphate (pNPP) that would produce colored end product, and is measured by a spectrophotometer. An alternative fluorescence based substrate system was evaluated to increase the sensitivity so that the pathogenic action of few L. monocytogenes cells could be determined. Ped-2E9 cells were exposed to Listeria species and DTT for 1.5 h and the cell supernatants were reacted with 4-methylumbelliferyl phosphate (MUP) as a substrate for AP to produce fluorescence end product. This fluorescence-based substrate gave higher cell cytotoxicity values for L. monocytogenes than the nonpathogenic L. innocua (Figure 4) when compared with the pNPP-based colorimetric data.

Figure 4: Fluorescence-based cytopathogenicity assay for L. monocytogenes and L. innocua with Ped-2E9 cells using 4-MUP as a substrate for alkaline phosphatase after 1.5 h of incubation.
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