Detection of *Listeria monocytogenes* through real-time PCR and biosensor methods

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ABSTRACT

Listeria monocytogenes is a foodborne pathogen causing listeriosis, especially in sensitive individuals such as children, pregnant women and persons with compromised immune systems. This pathogen has been isolated from different food products, but milk products surely play a major role in the epidemiology of this foodborne disease. Identification traditionally involved culture methods based on selective enrichment and plating followed by the characterization of *Listeria* spp. based on colony morphology, sugar fermentation and haemolytic properties. These methods are the gold standard, but in the last years more rapid tests were developed based on antibodies (ELISA) or molecular techniques (PCR or DNA hybridization). More recently, molecular methods were developed that target RNA rather than DNA, such as RT-PCR, real time PCR or nucleic acid sequence-based amplification (NASBA). In this review, real-time PCR assays, protein chip methods and label-free SPR immunosensors were compared for their application in bacterial detection.

Keywords: *Listeria monocytogenes*; real-time PCR; protein arrays; surface plasmon resonance

Listeria monocytogenes is recognized worldwide as one of the most important food-borne pathogens of concern for the food industries. It is a ubiquitous microorganism and it is commonly isolated from foods of animal origin, mainly meat and milk products (Schuchat et al. 1991), but it can be also found in fresh produce, such as salads (Berrada et al. 2006). However, human listeriosis outbreaks are most often associated with ready-to-eat food products that are consumed without prior cooking (Ryser 1999). Ingestion of foods contaminated with *L. monocytogenes* can result in listeriosis, a severe infectious disease characterized by meningoencephalitis, abortion, septicemia, and a high fatality rate (30%). Listeriosis predominantly affects certain risk groups, including pregnant women, newborns, elderly people and immunocompromised patients (Kathariou 2002, McLauchlin et al. 2004). However, recent reports of a non-invasive form of listeriosis that causes febrile gastroenteritis clearly indicate that persons with no predisposing conditions may be affected (Franciosa et al. 2001). The food safety regulations of most of the countries tolerate no *L. monocytogenes* in ready-to-eat foods (Gallagher et al. 2003), although the minimal infection dose is generally higher than 100 viable cells (Roberts et al. 1996). From January 1, 2006, the new Commission Regulation (EC) No. 2073/2005 entered into force in the European Union that sets the limit of 100 colony forming units (CFU) per g or ml for ready-to-eat products. For foods produced for specific subgroups of the population that are at risk, the absence in 25 g or ml is required.

In addition, bacterial pathogens may contaminate water sources used to irrigate vegetables and sprouts (alfa-alfa, water cress, kaiware daikon) in greenhouses and sprout production sites. In this way, pathogens may arrive in the kitchen and contaminate working surfaces, easily spreading from surface to food. It is important to monitor the presence of such pathogens along the food production lines to avoid their introduction into the kitchens and canteens.

Classical microbiological methods for detection of foodborne bacteria involve the use of pre-enrichment and/or specific enrichment, followed by the isolation of bacteria in solid media and a final confirmation by molecular and/or serologi-

cal tests (Rodríguez-Lázaro et al. 2007). However, these procedures are not always effective and are extremely labour-intensive, requiring long time to yield a result. During the past decades, various alternative methods were developed to overcome these disadvantages, and a number of molecular methods were devised to reveal the presence of undesirable living microorganisms such as NASBA (Malorny et al. 2003, Rodríguez-Lázaro et al. 2007).

The major advantage that molecular techniques offer over conventional methods is that they are based on differences within the genome and do not rely on the expression of certain antigenic factors or enzymes to facilitate identification. They are extremely accurate, reliable and some can be performed in the same time frame as immunoassay methods. There is a wide range of molecular methods available for the identification and characterization of *Listeria*, adaptating conventional PCR methods to the food testing laboratory and these techniques will be applied routinely in the near future (Gasanov et al. 2005).

In this study, the most recent development on available technologies for *L. monocytogenes* identification has been reviewed; real-time PCR assays, protein chip methods and label-free SPR immunosensors have been compared for their application in bacterial detection.

New directions for the detection and identification of *L. monocytogenes*

Real-time PCR

Polymerase chain reaction is a nucleic acid amplification technology. It is based on the isolation, amplification and quantification of a short DNA sequence including the targeted genetic material. Examples of different PCR methods developed for bacterial detection are: real-time PCR (Q-PCR) (Rodríguez-Lázaro et al. 2007), multiplex PCR (Jofré et al. 2005) and reverse transcriptase PCR (RT-PCR) (Deisingh and Thompson 2004a).

Real-time PCR permits to obtain quicker results without too much manipulation. This technique bases its detection in the fluorescent emission with a specific dye as it attaches itself to the targeted amplicon. Given that fluorescence intensity is proportional to the amount of amplified product (Cady et al. 2005); it is possible to follow the amplification in real time, thus eliminating laborious post-amplification processing steps such as gel electrophoresis. Different alternative probes, deriving from this principle, were developed recently (e.g. TaqMan, fluorescence resonance energy transfer or molecular beacon probes) (Yang 2004).

One of the limitations of PCR techniques lies in that the user cannot discriminate between viable

Table 1. Quantitative detection of *L. monocytogenes* in yogurt using the described methods

Approx. *L. monocytogenes* (CFU)[a]	Approx. *L. monocytogenes* genome equivalent/reaction[b]	Signal ratio[c]	Ct value[d]
1×10^5	1×10^4	9/9	23.49 ± 0.22
1×10^4	1×10^3	9/9	26.79 ± 0.23
1×10^3	1×10^2	9/9	30.41 ± 0.26
1×10^2	1×10^1	9/9	33.61 ± 0.18
1×10^1	1×10^0	9/9	36.80 ± 0.19
1×10^0	$< 1 \times 10^0$	0/9	> 45
0[e]	0[e]	0/9	> 45

[a]Results of three independent experiments, with three PCR replicates used in each

[b]Estimated number of *L. monocytogenes* genome equivalents in each PCR run, assuming 100% DNA extraction efficiency (each reaction contained 5 µl of a DNA preparation of 50 µl extracted from 100 ml of yogurt sus pension)

[c]Positive results out of 9 reactions

[d]Cycle number at which fluorescence intensity equals a fixed threshold. Mean value ± standard error of the mean

[e]Non-contaminated sample

and non-viable cells because DNA is always present whether the cell is dead or alive. To circumvent this problem, NASBA and reverse transcription-PCR methods, based on bacterial RNA, are designed to discriminate live cells from dead bacteria. Also a new filtration-based method that can eliminate the DNA from dead and severally damaged *Listeria monocytogenes* cells in food samples was developed. This new method can recover all the viable bacteria in less than 30 min, and is coupled to a subsequent bacterial DNA extraction and PCR. The relative accuracy shown using this method is similar to standard microbiological methods both in pure cultures and using contaminated food samples. After an initial treatment, only viable bacteria can be retained in the filter, and subsequently they can be subject to DNA extraction and PCR (D'Urso et al. 2009).

Artificial contamination of food samples with *L. monocytogenes*. Real-time PCR was used to detect and quantify *L. monocytogenes* in artificially contaminated food samples, using yogurt as a model. Consistent detection of *L. monocytogenes* was possible down to approximately 10 bacterial cells per 10 g of yogurt samples. These detection limits correspond to approximately 1 genome equivalent per reaction (Table 1), similar to the limits obtained when *L. monocytogenes* pure cultures were used as a PCR template (D'Urso et al. 2009).

Yogurts were obtained at the local market and immediately processed. In three replicate experiments, they were spiked with decreasing amounts of *L. monocytogenes*: approximately 1×10^5 colony forming units (CFU), 1×10^4 CFU, 1×10^3 CFU, 1×10^2 CFU, 1×10^1 CFU, and 1 CFU. Spiked yogurt samples (10 g) were diluted ten-fold with 0.1% peptone and 0.85% NaCl, and homogenized for 1 min in stomacher bags. The tubes were centrifuged at 8000 × g for 10 min. The supernatant of each tube was carefully removed and the pellet was resuspended in 100 µl of PBS (150 mM sodium chloride, 10 mM sodium phosphate pH 7.4). Subsequently, DNA was extracted as described above. Five microliters of the resulting DNA extract were used in each RT-PCR assay (D'Urso et al. 2009).

Protein Array

While the use of DNA arrays for bacteria detection and identification is largely documented (Zhou 2003), no studies are available on protein array (PA) based bacteria detection. Microarrays are typically microscope slides with micron-diameter spots separated by micron-sized spacing that contain molecules chemically attached to the surface. They were used extensively in the past years, in particular those containing nucleic acid sequences for gene expression studies (Schena et al. 1995). More recently, microarrays containing proteins were developed and used to study protein – protein interactions (Macbeath and Schreiber 2000). Perhaps the most significant characteristic of microarrays, and the reason for their widespread popularity, is their ability to contain thousands of spots (or features) per slide (or substrate) and, therefore, simultaneously perform thousands of potential analyses with a single sample. Thus, in the past few years, efforts to produce microarray biosensors for diagnostic purposes were undertaken (Rao et al. 2004, Sapsford et al. 2004, Vora et al. 2004).

Protein microarray detection of *L. monocytogenes*. Polyclonal antibodies were produced in rabbits by injection of the lysate of *L. monocytogenes* cells killed by heating. The affinity-purified anti-*L. monocytogenes* antibody was used as a capture antibody, and also in sandwich detection methods.

Antibodies were labeled using commercial kits, as the Cy3-NHS (Amersham). Anti-*L. monocytogenes* antibodies were added in an eppendorf tube with the proportional amount of dye and with 0.1M $NaHCO_3$ pH 9, incubated for one hour at the room temperature covered with aluminum foil to protect from light. After one hour of labelling, gel-filtration was performed in tubes or columns using a G-50 resin, allowing the recovery of pure fluorescent antibodies.

We used epoxy-group surface activation of glass slides for the covalent binding of proteins. After printing the chips with anti-*L. monocytogenes* capture antibodies, a washing step (1 × PBS, 0.1% Tween 20) and a blocking step (1 × PBS, 0.1% Tween 20, 0.5M glycine) were performed to wash away unreacted proteins and to block reactive epoxy species on the slide, respectively. The protein chip was tested with incubation of a solution of *L. monocytogenes* cells. In order to detect the pathogens on the chip, Cy3-labelled anti-*L. monocytogenes* antibodies were used. After washing (1 × PBS), the slides were dried by centrifugation and the signal was revealed using a laser scanner.

Anti-Listeria antibody was spotted in triplicate at serial dilutions, from 1 mg/ml to 0.01 mg/ml. After incubation with *Listeria monocytogenes* extract, a new incubation with Cy3-labelled antibody allowed signal detection (Figure 1). The incubation with the fluorescent antibody provided an effec-

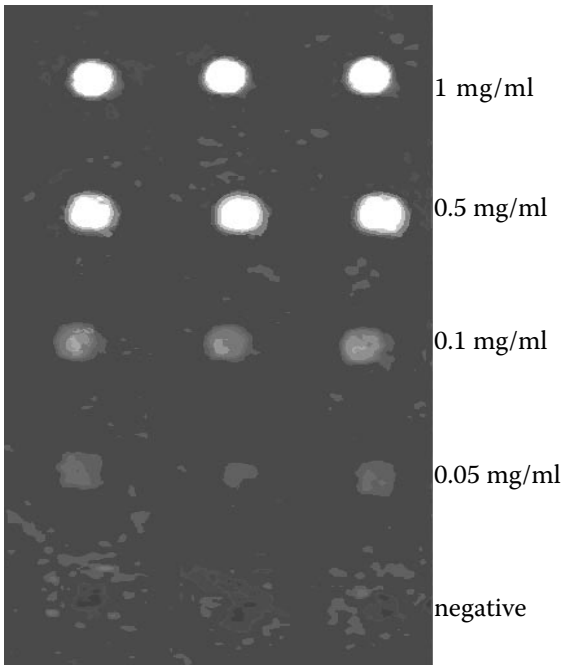

Figure 1. Protein chip using anti-*L. monocytogenes* antibodies spotted at serial dilution. Detection limit of capture antibodies was in the 0.1 mg/ml range. After incubation with a Listeria extract, a further incubation with Cy3 labelled antibody (sandwich method) was performed for signal resolution

tive amplification of the signal due to a high ratio of antibodies bound to each pathogen surface. Further experiments were performed to compare the sandwich detection method (Figure 2, lanes a-c) to the signals from Cy3-labelled antibodies bound to Listeria extracts on the glass.

Duplicate spots of *L. monocytogenes* serial dilutions were observed at decreasing concentration: 10^6, 10^5, 10^4, 10^3 and 10^2 CFU/ml (Figure 2, lanes e-i) and one negative control of *L. innocua* culture at 10^6 CFU/ml (Figure 2, lane l). Serial *L. monocytogenes* dilutions produced signals comparable to those in the control subarrays containing reference bacteria controls at known concentrations (Figure 2, lanes a-e, b-f, c-g). Using the protein

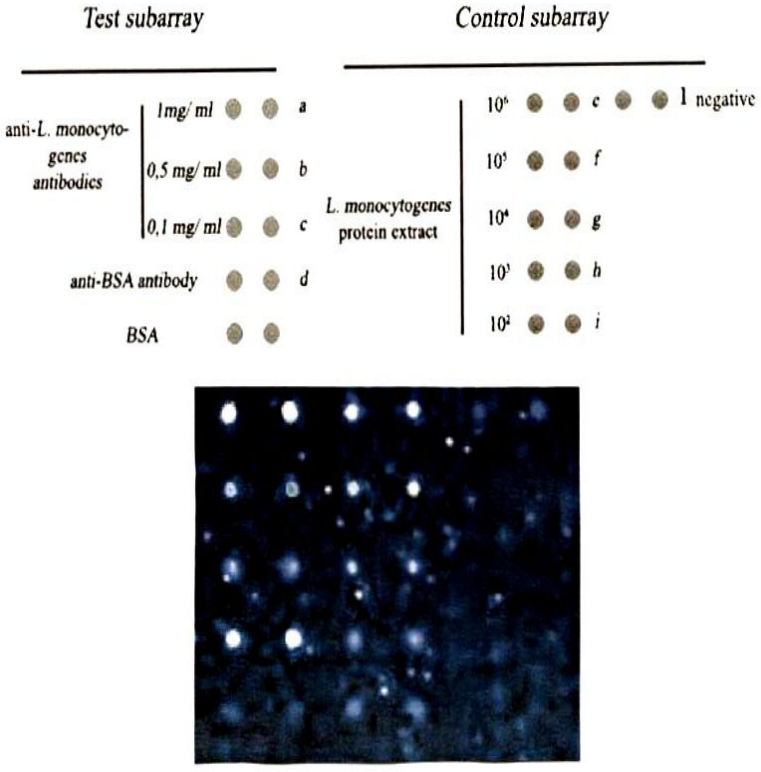

Figure 2. Detection of *L. monocytogenes* using anti-Listeria antibody spotted at decreasing concentration: 1 mg/ml, 0.5 mg/ml, 0.01 mg/ml (lanes a, b, c), and 0.05 mg/ml anti-BSA antibody (lane d), compared to signal resolution using antibody in solution during the recognition of *L. monocytogenes* extracts spotted onto the glass, at serial dilutions from 10^6–10^2 CFU/ml (lanes e, f, g, h, i) and one negative control of *L. innocua* culture at 10^6 CFU/ml (lane l)

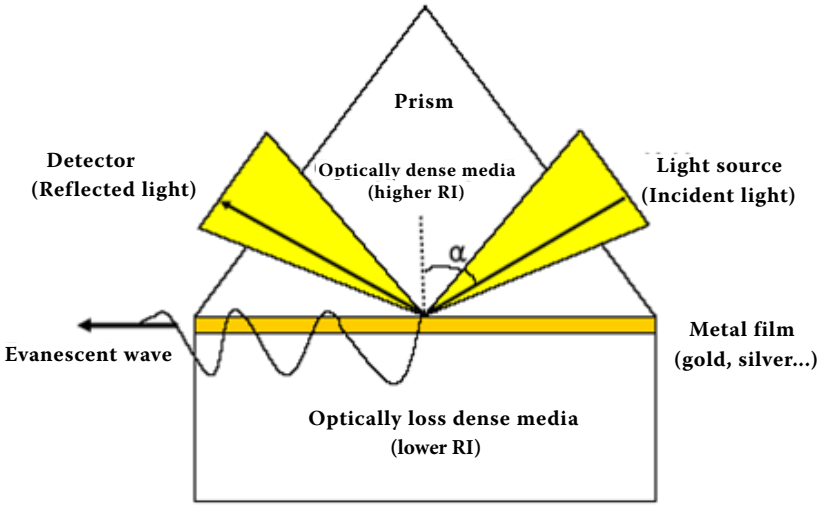

Figure 3. Schematic of the SPR system in the Kretschmann configuration

chips, detection was evident up to the 10^2 CFU/ml solution (Figure 2, lane i).

Surface plasmon resonance (SPR)

A number of methods are used for the rapid detection of biomolecules in solution. These include immunoassays, chromatographic methods, magnetic and biological biosensor methods (using screen-printed biosensors with immobilized enzymes), DNA biosensors and immunosensors based on surface plasmon resonance (SPR). SPR is an optical technique that uses the evanescent wave phenomenon to measure changes in refractive index very close to a sensor surface. The binding of an analyte in solution to its ligand immobilized on the sensor surface results in a change in the refractive index. The interaction is monitored in real time and the amount of bound ligand and rates of association and dissociation can be measured with high precision.

SPR has received a considerable technological impulse for its high sensitivity, especially in biochemical and biomedical fields. A thin metal film is positioned at the interface between two media, and the evanescent wave produced by incident light is able to interact with free electrons (plasmons) in the metal film at a special angle (α) of incident light (SPR angle). The angular position is highly sensitive to any change at the metal-dielectric interface. The

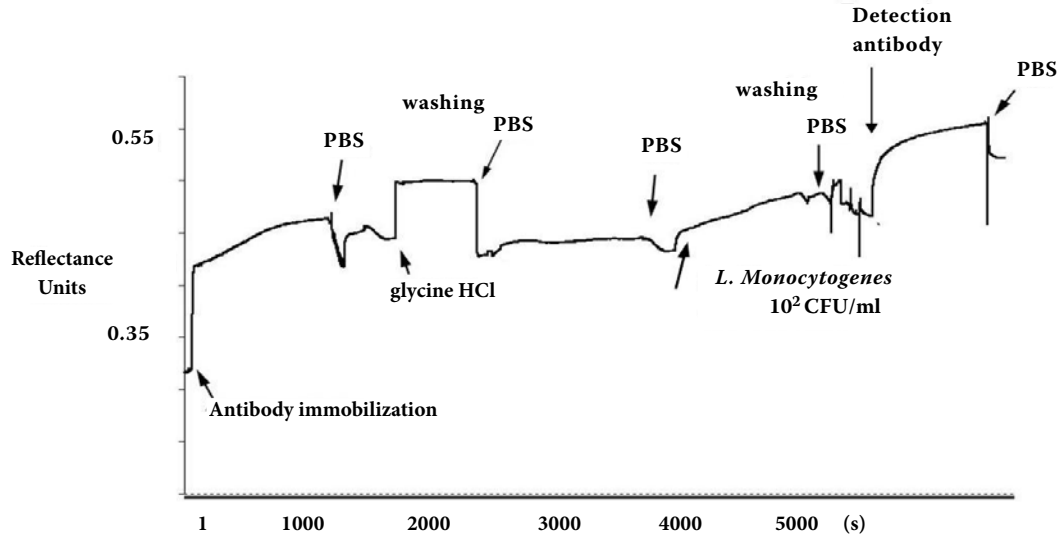

Figure 4. The SPR sensogram for the detection of *Listeria monocytogenes* (10^2 CFU/ml) in PBS buffer, monitored over time. On the y axis detection signal expressed in reflectance units

technique was largely studied in label-free immunosensors for direct detection of bacteria (Deisingh and Thompson 2004b, Mazumdar et al. 2008).

Some works on *L. monocytogenes* bacteria cells were found in literature; they prevalently report investigation by SPR commercial experimental set-up (Perkins 2000, Leonard et al. 2004). Various companies offer a range of instruments, methods and reagents based on SPR, as Bioacore, SurModics, Nomadics, Sensata (ex-Texas Instruments).

Surface plasmon resonance detection of *Listeria monocytogenes*. SPR biosensors were made using a gold electrode with bound anti-*L. monocytogenes* antibodies in the test chamber. The electrode is activated by washing in ethanol, and immersed for 2 h in a 20mM cystamine solution, washed in water, immersed for 1 h in a 2.5% glutaraldehyde solution made in 50mM phosphate solution pH 7.5, and again washed in water (Chou et al. 2002). An antibody solution (100 ppm) was immobilized on the modified electrode. Absorption of receptors on the sensor surface and the antigen binding were observed as a shift in the resonant wavelength. Antigen immobilization and antibody binding can be monitored also by the shift toward higher angles of the minimum of SPR curve, as predicted by SPR theory, when a layer is adsorbed onto a metal film (Figure 3).

The binding of *L. monocytogenes* cells at different dilutions in PBS buffer (from 10^2 to 10^6 cells) was monitored over time. Each analysis was followed by a regenerating cycle with glycine-HCl. SPR could detect the presence of *L. monocytogenes* with detection values ranging from 10^4 to 10^6 CFU/ml. The sensibility was lowered to 10^2 cells with the introduction of a second detection step based on gold-conjugated antibodies, exploiting the signal mass amplification effect (Figure 4). Colloidal gold-antibody conjugates were prepared according to our protocol using a modification of the SPR sandwich method (Dudak and Boyaci 2009). 10 µl of 10 µg/µl unlabelled affinity-purified anti-*L. monocytogenes* antibodies were added to 1 ml of 20 nm colloidal Au suspension (pH 9.6, adjusted by 0.1M K_2CO_3), followed by incubation at room temperature for 10 min. Bovine serum albumin (BSA) was then added to a final concentration of 1%. The conjugate was centrifuged for 1 h at 19 500 × g. The soft sediment was then suspended in 100 µl of PBS containing 1% BSA. SPR could detect directly the presence of *L. monocytogenes* with detection values ranging from 10^4 to 10^6 CFU/ml. SPR sensibility was enhanced using a gold-labelled secondary antibody exploiting the mass amplification effect, allowing to unambiguously detect 10^2 cells (Figure 4).

The protein Microarray and the SPR immunosensor methods showed a good sensibility in bacteria detection, with the threshold of 10^2 CFU/ml. On the other side, the real-time PCR method proved to be useful in the differentiation of viable bacteria distinguishing dead and alive cells. Among the methods used for the rapid detection of biomolecules in solution, the immunosensors based on protein microarrays and the SPR biosensors show to be promising systems in pathogen monitoring and analysis of waters and environments suspect to be a source of pathogen contamination. Through rapid identification using biosensors, it will be possible to test on-line a water supply and its distribution system within a greenhouse or a plant, or, using simple processing protocols, to test vegetables or environmental samples for the detection of pathogen presence.

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