Immobilization and Detection of *Listeria monocytogenes*

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A procedure was developed for immobilization of *Listeria monocytogenes* cells on metal hydroxides coupled with detection and enumeration using an automated optical system. The results of the immobilization procedure (<1 h) and detection during overnight incubation agreed with calculated plate counts, and this technique is simple and rapid and provides samples that are ready for confirmation of the presence of the pathogen by rapid methods.

Rapid detection and identification of food-borne pathogens are often hampered by the fact that low numbers of the organisms are present. Immobilization on metal hydroxides (MOH) is one of several methods used for microbial separation and concentration (1, 8, 9, 10, 11, 12, 13, 17). Hydroxides of calcium phosphate (hydroxyapatite [HA]), zirconium, hafnium, and titanium are insoluble in aqueous solutions and can act as both affinity agents and solid supports for immobilization of microorganisms (3, 11, 12). Previous studies have suggested that immobilized cells remain viable, that multiple cells are immobilized on each MOH crystal, and that cell immobilization is nonspecific (2, 11).

In previous studies, percentages of immobilization were determined by comparing the optical densities of immobilized cells to the optical densities of control suspensions without MOH (7), directly plating the immobilized cells with the MOH, and comparing the numbers of cells to the initial numbers (4, 12) or, assuming that the immobilized cells were viable, subtracting the numbers of cells left in the supernatants from the total numbers of cells before immobilization, both of which were determined by plate counting (2, 4, 8, 12). The viability of immobilized cells was established by observing oxygen uptake using an oxygen electrode (11), by determining the increase in red coloration of immobilized *Serratia marcescens* cells during enrichment (11), by fluorescent viability staining of immobilized cells (2), or by directly spread plating and incubating samples of immobilized fractions at different times and comparing the numbers of cells to the numbers of cells in controls containing nonimmobilized cells (4). Information on the multiplication patterns of immobilized cells is limited.

In studies with food pathogens in our laboratory we have used the BioSys instrument extensively, whose name was recently changed to Soleris (Centrus International, Ann Arbor, MI). This system enables automated detection and enumeration of microorganisms by registering real-time changes in optical density during cell proliferation (16). We describe here immobilization of *Listeria monocytogenes* Scott A (serotype 4b) on metal hydroxides used in conjunction with this optical system, which is a rapid and simple alternative for obtaining viable cells in liquid media for identification and confirmation by rapid methods.

Hydroxyapatite \( \text{Ca}_5(\text{PO}_4)_3\text{OH} \) (catalog no. C-5267; Sigma-Aldrich) and zirconium(IV) hydroxide \( \text{Zr(OH)}_4 \) (catalog no. 464171; Sigma-Aldrich) were each prepared by suspending 5 g in 50 ml buffered saline. Titanium(III) hydroxide \( \text{Ti(OH)}_3 \) was prepared from titanium(III) chloride (catalog no. 249998; Sigma-Aldrich) (12). All stock solutions of the metal hydroxides were heat sterilized at 121°C for 15 min and stored at room temperature in the dark.

*L. monocytogenes* cells were grown in tryptic soy broth (Difco, Detroit, MI) at 35°C for 18 to 24 h before they were used. Cells were enumerated by spread plating undiluted or diluted samples on plate count agar (PCA) (Difco) or PALCAM agar (Difco). Colonies were counted after incubation at 35°C for 24 to 48 h.

Nonselective nutrient broth (total viable count broth [TVC]; Centrus) and selective *Listeria* broth (SLB) for the genus *Listeria* (5, 14) were used with the optical system. Detection in TVC occurs when the color of the medium (yeast extract, peptone, glucose, bromcresol purple) changes from purple to yellow during incubation (16). *Listeria* cells are detected in SLB by a sharp decrease in light transmittance as the colorless broth changes to black upon hydrolysis of esculin to 6,7-dihydroxycoumarin (esculetin) and the subsequent reaction of the latter compound with ferric ions in the medium (5, 14).

For the uniform immobilization procedure with the three metal hydroxides (Fig. 1), cells available for immobilization \((10^6 \text{ to } 10^8 \text{ CFU of a 24-h culture in 1 ml}) \) were added to sterile centrifuge tubes containing MOH (0.01 g in buffered saline). The numbers of cells available for immobilization were determined by plate counting. The contents of each tube were vortexed at high speed for 10 s and allowed to rest at room temperature for 20 min. Following centrifugation at 830 \( 
\times \) g for 4 min at 4°C in a Super T21 (Sorvall, Asheville, NC), aliquots of the supernatant were removed for cell enumeration and for detection with the optical system, and the remaining supernatant was discarded. The pellet (MOH and immobilized cells) was suspended in sterile broth (TVC or SLB), and the contents were transferred quantitatively to the optical-system vials for overnight incubation at 35°C. Detection times (DT) were determined for cells that were available for immobilization \((\text{DT}_{s})\), were in the supernatant \((\text{DT}_{s})\), and were immobilized \((\text{DT}_{l})\). Tests were carried out in duplicate and repeated at least twice.

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The detection time with the automated system is inversely proportional to the number of cells, and detection occurs when the number of organisms is about $10^6$ CFU. Typical detection curves for available, immobilized, and supernatant cells of *L. monocytogenes* when HA was used (Fig. 2) showed that DT_A and DT_I were within 1 h of each other and that DT_S was delayed. Scattergrams relating the number of available *L. monocytogenes* cells (log CFU) and the corresponding detection times (in hours) were generated for nonselective media (PCA and TVC) (Fig. 3A) and selective media (PALCAM and SLB) (Fig. 3B). In both types of media there was an approximately 3-h change in the detection time for each 10-fold increase or decrease in the number of cells, and bacteria were detected earlier in TVC, the nonselective, inhibitor-free medium.

Since DT_A and DT_I were consistently very similar in each test, scattergrams relating DT_A and DT_I were constructed. Scattergrams for TVC and SLB using data for HA are shown in Fig. 4A and B, respectively. Linear regression analysis resulted in $R$ values of 0.94 and 0.98, respectively. The high correlation between DT_A and DT_I was used to estimate the numbers of immobilized cells and to confirm their viability. Immobilization with all three metal hydroxides was demonstrated by similar scattergrams relating DT_A and DT_I.

The procedure developed for bacterial cell immobilization on metal hydroxides is simple, rapid (<1 h), reproducible, and easy to perform. Combination of this procedure with the automated optical system, which is limited at this time to *L. monocytogenes*, showed that immobilization did not alter the metabolic rate of the organism, judging by the similar growth curves for available and immobilized cells (Fig. 2). Immobilization data from detection times confirmed the data obtained by the plate count method with levels of recovery ranging from 96 to 99%. These recovery values are similar to those reported previously for *L. monocytogenes* and other food-borne bacteria (2, 4, 8, 12). The automated procedure was simple and less time-consuming and gave results sooner (12 to 24 h versus 24 to 48 h). The color change of the indicator in TVC vials originated and intensified at the site of the MOH pellet. A complete change to yellow occurred only after further incubation, demonstrating the strong bacterial cell-MOH interaction and confirming the results of early immobilization studies with *S. marcescens*, in which the red pigment produced by the organism was associated with the insoluble matrix (11). Immobilization of $10^7$ cells of *Streptococcus mutans* was obtained using 1 mg HA (6). In our study, $>10^8$ *L. monocytogenes* cells were immobilized on 10 mg HA. Although not tested with immobilized cells, DNA-based rapid methods (i.e., PCR) could be used directly with positive SLB samples for confirmation of *L. monocytogenes* in less than 24 h, as reported previously (14, 15). A procedure for food samples suspected of contamination with *L. monocytogenes*, consisting of preenrich-

![FIG. 1. Flow chart for immobilization of bacteria on metal hydroxides.](image1)

![FIG. 2. Typical detection curves for *L. monocytogenes* during immobilization on hydroxyapatite. The detection times determined with the automated system were as follows: available (DT_A), 7.4 h; immobilized (DT_I), 8.6 h; and supernatant (DT_S), 15.8 h. The cell numbers as determined by plate counting were as follows: available, $1.3 \times 10^7$ CFU; supernatant, $4.0 \times 10^4$ CFU.](image2)

![FIG. 3. Scattergrams relating *L. monocytogenes* numbers (log CFU) and detection times (in hours) in nonselective media (PCA and TVC; n = 79) (A) and selective media (PALCAM and SLB; n = 38) (B).](image3)

![FIG. 4. Scattergrams relating detection times (in hours) for available and immobilized *L. monocytogenes* cells in TVC (n = 73) (A) and SLB (n = 26) (B). The data represent cell immobilization on hydroxyapatite.](image4)
ment, immobilization, and detection with the automated system followed by PCR confirmation of positive samples, is under investigation.

REFERENCES