Research Note

Application of a Novel Hydrophilic Infrared-Transparent Membrane to the Differentiation between Microcolonies of Enterobacter sakazakii and Klebsiella pneumoniae

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ABSTRACT

A proof-of-concept study is reported for the differentiation between microcolonies of Enterobacter sakazakii and Klebsiella pneumoniae by means of a novel sample preparation for infrared (IR) analysis. A disposable, IR-transparent, microporous (0.2-μm pores), hydrophobic, polyethylene (PE) membrane (51 μm thick) was plasma treated under an oxygen atmosphere and used to (i) filter or print microarrays of dilute aqueous foodborne bacterial suspensions and (ii) subsequently grow bacterial microcolonies when the treated, hydrophilic PE membrane was placed over brain heart infusion agar medium and incubated. Because this unique membrane is transparent to IR light, isolated microcolonies (200 μm) of bacterial cells grown on this PE substrate for the first time could be directly fingerprinted by IR microspectroscopy in the transmission mode. Hence, time-consuming bacterial cell transfer from culture plates to an IR sample holder for subsequent measurement by IR spectroscopy was eliminated. Multivariate analysis of the observed IR spectra for microcolonies allowed the rapid differentiation between E. sakazakii and K. pneumoniae.

Infrared (IR) spectroscopy has been used to identify microorganisms for many years (2, 8, 14, 16). However, improved IR protocols for the isolation, sample preparation, and identification of bacteria are needed.

Identification of bacteria by IR spectroscopy is a multidisciplinary research field that entails (i) growing each isolated bacterium in pure culture, (ii) transferring bacterial cells from a culture plate to an IR spectrometer (“sample preparation”), (iii) measuring bacterial cells by IR spectroscopy, and (iv) identifying bacteria by multivariate statistical analysis. The scope of the present study was limited to the reporting of a novel sample preparation protocol that allows the direct measurement of individual bacterial “colonies,” thus eliminating time-consuming and poorly reproducible sample preparation procedures for cell transfer from a culture medium to an IR instrument.

The conventional IR bacterial sample preparation protocol (14) requires the application of aqueous bacterial cell suspensions on optical crystals and the slow (1-h) drying of these suspensions prior to IR measurement. With this procedure, bacterial deposits did not always adhere to IR crystals and were likely to crack or even peel off upon drying. A fundamental requirement for reliable bacterial identification by IR spectroscopy and multivariate analysis is the availability of reproducible IR databases, which, in turn, require reproducible sample preparation protocols. To optimize the IR sample preparation step, several procedures were developed (10, 12) in which a disposable, hydrophobic, microporous, polyethylene (PE) substrate that was transparent to most wavelengths of IR radiation (see “Materials and Methods”) was used instead of expensive optics (e.g., a zinc selenide slide). The use of the PE substrate led to the formation of bacterial deposits with uniform thickness that irreversibly adhered to the membrane, thus forming an ideal deposit (10, 12). However, the drying of deposits on the PE substrate was still slow (1 h). To accelerate this step, aqueous bacterial suspensions were mixed with 1:1 (vol/vol) ethanol for 3 s to reduce surface tension (10). This allowed the suspensions to be filtered through the hydrophobic PE membrane. However, in the present study, the use of alcohol was avoided, because it has the ability to disrupt lipid membranes and denature proteins and may interfere with the identification of intact bacteria by IR. More effective refinements in the sample preparation procedure were developed in the present proof-of-concept study. These modifications eliminated the need to either prepare bacterial suspensions (14) or stamp bacterial colonies onto an IR crystal (8). Instead, they allowed the direct IR measurement of bacterial microcolonies.

In the present proof-of-concept study, a novel IR bacterial sample preparation procedure was proposed. It entailed the use of the same PE material but after low-pressure surface ionization plasma treatment under an oxygen at-
Inoculation and preenrichment. The IFM products were locally purchased. Prior to inoculation, a single isolated colony was removed from the incubated BHI plate and placed in a tube containing 1 ml of sterile distilled water. The resulting suspension was serially diluted, and each dilution was streaked on BHI agar medium and incubated at 37°C overnight. Next, the number of colonies was counted. *E. sakazakii* was then inoculated from the dilutions estimated to give a bacterial level of 1 CFU/10 g into an IFM sample that consisted of a 10-g portion dissolved in 90 ml of sterile distilled water prewarmed to 45°C. This inoculated IFM sample in water was preenriched by incubation overnight at 36°C. The same procedure was repeated for *K. pneumoniae*. The IFM was used in this study not as a representative food matrix, but only as a substitute for a growth medium that is low in the number of bacterial cells. Literature surveys indicate that the incidence of *E. sakazakii* in IFM commercial products is low, namely 20 of 141, 8 of 120 (15), and 2 of 82 IFM (4), and its isolation levels range from 0.36 to 66.0 cells per 100 g (13).

Filtration and incubation of bacterial cells and growth of microcolonies on plasma-treated PE membrane. The preenriched IFM test samples of *E. sakazakii* and *K. pneumoniae* were each serially diluted to 1:10⁶. Optimal dilution ranges had to be determined. Each of the 100-µl portions of the 1:10⁷ and 1:10⁸ serially diluted samples was slowly dispensed over an area 0.5 by 0.5 in. (ca. 1 by 1 cm²) of a plasma-treated, hydrophilic PE membrane (3M, St. Paul, Minn.), which is approximately 0.002 in. or 51 µm thick. Each side of the membrane surface (area, ca. 9 by 11 in. [ca. 22 by 27 cm²]) was plasma treated at room temperature with low-pressure (below ~1 torr [~1 mm of mercury]) oxygen at a flow rate of 60 ml/min for 5 min.

FIGURE 1. Flow chart of the various steps of the assay used.

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<th>Step</th>
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<td>2.</td>
<td>Removal of a single colony and preparation of a suspension in 1 ml water followed by serial dilution</td>
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**MATERIALS AND METHODS**

**Organisms and growth conditions.** Isolates from *E. sakazakii* and *K. pneumoniae* were obtained from the U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition culture collection. *E. sakazakii* is a gram-negative, rod-shaped bacterium that has been associated with neonate deaths and outbreaks of a rare form of infant meningitis and other diseases (7), and the presence of *E. sakazakii* in powdered infant formula milk (IFM) has been of particular concern (3). For this feasibility study, a single strain of each organism was used. This method requires the testing of multiple dilutions of each sample (as detailed below), especially when unknown samples are used. The stock cultures were streaked onto brain heart infusion (BHI) agar plates and incubated at 37°C for approximately 24 h; see the flow chart in Figure 1.

Imaging and photography of the microcolonies. The microcolonies were examined with an Olympus BX51 fluorescence microscope (Olympus Optical Co., Ltd., Tokyo, Japan) equipped with the following components: a ×40 objective; a mercury HBO 100-arc lamp within a reflected light illuminator; and an epicondenser with a filter cube having excitation, dichroic, and barrier filters with wavelengths, respectively, of 450 to 480, 500, and 515 nm. Images of the microcolonies (Fig. 2a) were recorded and stored with a digital camera (Spot model 2.3.1, Spot Diagnostic Instruments, Inc., Sterling Heights, Mich.) interfaced with a Dell.
FIGURE 2. (a) Observed fluorescence image of microcolonies of *E. sakazakii* grown on a disposable, microporous, plasma-treated, hydrophilic, IR-transparent polyethylene membrane; microcolonies were produced by the filtration and incubation-over-agar procedure (see text). (b) First IR chemical images of an intact bacterial microcolony of *E. sakazakii* indicating a diameter of approximately 250 μm (left, two-dimensional image) and a hemispherical topology (right, three-dimensional image). IR images were recorded just before submission of this article for publication on a newly acquired Varian (Randolph, Mass.) FTS 7000e IR spectrometer operating under Resolution Pro software and interfaced to a Varian UMA 600 IR microscope equipped with a Schwarzschild ×15 Cassegrain objective and a 32 × 32-pixel focal plane array mercury-calcium-telluride detector. Images were generated by plotting the intensity at 1,223 cm⁻¹ for a microcolony obtained from an archived test sample. The background was recorded from an adjacent colony-free region of the treated PE substrate. For each image acquisition, a total of 1,024 FTIR spectra were collected simultaneously by coadding 16 scans at a resolution of 8 cm⁻¹ between 4,000 and 900 cm⁻¹.

Optiplex GX 260 computer operating with Spot RT version 3.1 software.

**Microarray printing on plasma-treated PE membrane.** Optimal dilution ranges had to be determined. Aliquots of the preenriched IFM test samples of *E. sakazakii* or *K. pneumoniae* were each diluted to 1:10² and 1:10³. Portions (30 μl) from the various diluted suspensions of each sample were transferred to a microtiter plate, which was placed in a PixSys 5000 contact microspotting robotic system (Cartesian Technologies, Inc., Irvine, Calif.). With a CMP10B microspotting pin (ArrayIt-TeleChem International, Inc., Sunnyvale, Calif.), the suspensions were printed on a section measuring 1 by 3 in. (2.5 by 7.5 cm²) of the plasma-treated, hydrophilic PE membrane after placing it over a zinc selenide slide of similar dimensions that served only as a solid support during printing. For each test sample, a 600-nl uptake volume of bacterial cell suspension was taken by the microspotting pin from one of the 384-microtiter plate wells, and five 5.2-nl replicate droplets were sequentially printed on the surface of the treated PE membrane. These uptake and printing volumes had been calibrated for DNA solutions by the pin manufacturer and may represent approximate, yet consistent volumes for dilute cell suspensions. After printing five replicates of each test sample, the pin was cleaned automatically with water and vacuumed three times. The PE membrane, on which a microarray of dilute bacterial suspensions was printed, was subsequently placed on BHI agar media and incubated at 36°C for 3 to 4 h until the formation of a microarray of microcolonies was observed.

**FTIR microspectroscopy with single-point mercury-cadmium-telluride detection.** A Magna 550 Fourier transform (FTIR) spectrometer equipped with a Continuum IR microscope (Thermo, Madison, Wis.) and a 0.25-mm mercury-cadmium-telluride detector were used to record the FTIR spectra of air-dried microcolonies observed on the plasma-treated PE substrate. FTIR spectra were collected over a wave number range of 4,000 to 600 cm⁻¹ at a resolution of 8 cm⁻¹. The PE spectrum consisted of an intense feature due to hydrocarbon stretching vibrations from 2,940 to 2,840 cm⁻¹ and two weak deformation bands near 1,465 and 725 cm⁻¹. These bands fell outside the frequency range of 1,172 to 995 cm⁻¹ that was used to discriminate between the two bacteria in the present study (see Fig. 3). To enhance the signal-to-noise ratio, 64 scans were coadded (ca. 1 min), and the signal was averaged for each microspot with a 100- by 100-μm² aperture. The estimated signal-to-noise ratio was 5,000:1. A reference background spectrum was measured from a clear portion of the PE substrate with no bacterial colonies.

**Data analysis and presumptive identification of microcolonies.** The acquired FTIR spectra were imported as absorbance (*.SPC) files into the multivariate statistics program PIROUETTE 3.11 (InfoMetrix, Inc., Bothell, Wash.). Spectral data were trans-
FIGURE 3. Each of the displayed 28 representative FTIR spectra was obtained by measuring a microcolony of E. sakazakii or K. pneumoniae from IFM test samples inoculated with one microorganism (see text). The wave number region of 1,172 to 995 cm$^{-1}$ exhibited unique spectral features and was used to discriminate between the two bacteria. A Magna 550 FTIR spectrometer equipped with a Continuum infrared microscope (Thermo) and a 0.25-mm single-point mercury-cadmium-telluride (MCT) detector was used.

FIGURE 4. Filtration experiments on treated PE membranes resulted in a dendrogram that illustrated the successful differentiation between microcolonies of E. sakazakii and K. pneumoniae measured by IR microspectroscopy.

The IR spectra of microcolonies (Fig. 3) of the foodborne pathogens E. sakazakii and K. pneumoniae were sequentially recorded for the various test samples, namely those that had been inoculated with each bacterium. Isolates were cultured five times and analyzed for 3 months to assess the reproducibility of this procedure. It is noted that inspections of all the observed IR spectra for microcolonies indicated that no residual agar in the membranes after removal from the plates was ever found. The spectral region spanning the observed spectral range was considered; however, the optimal region for differentiation between the two microorganisms was 1,172 to 995 cm$^{-1}$ (Fig. 3) and was used to discriminate between the two bacteria (Fig. 4). An HCA dendrogram that illustrates the successful differentiation of E. sakazakii and K. pneumoniae measured by IR microspectroscopy is shown in Figure 3. HCA, which is based on calculations of the pairwise distances (e.g., Euclidian) of the observations in multidimensional data space, allows a determination of (i) the number of clusters and (ii) which object belongs to which cluster. Hence, the HCA algorithm groups data into clusters with similar attributes, and the dendrogram is a visual presentation of the clustering. Distances between pairs of samples are calculated and compared. Similar samples are separated by relatively small distances (left side of the dendrogram), while dissimilar samples are separated by relatively large distances (right side of the dendrogram). Euclidian distances and the centroid linkage method were used to generate the dendrograms presented in this study. An index at the top of each dendrogram is an arbitrary scale used to compare similarities at different distances.

An inspection of Figure 4 indicates that there is a complete separation of the spectral data into two main clusters for E. sakazakii and K. pneumoniae. The colony-to-colony variability (from all five batches originating from a single culture that were cultured and analyzed for 3 months) was much smaller than the between-microorganism variability,
thus meeting the fundamental requirement for reliable presumptive bacteria identification by IR microspectroscopy.

The recent application of high-throughput microarray printing to automatically deposit intact bacterial cells on IR substrates was developed in our laboratory (1, 5, 11). As a substitute for the manual filtration step, the microarray printing of aliquots of diluted, preenriched IFM test samples on treated PE membranes was also explored. In this preliminary study, the printing of microarrays of diluted IFM test samples preenriched with either *E. sakazakii* or *K. pneumoniae* was successful and could be routinely carried out. The observed sizes of the microarrays of microcolonies of *E. sakazakii* or *K. pneumoniae* varied; microcolonies that originated from printing suspensions that had been diluted to 1:10^3 were approximately 200 μm in diameter, while those that had been diluted to 1:10^2 were larger (ca. 500 μm in diameter). Multivariate analysis of IR spectra measured for microarrays of microcolonies (from five batches cultured from a single culture for 3 months) resulted in a dendrogram similar to that shown in Figure 4. The potential application of printing microarrays of bacterial cells for different microorganisms on the plasma-treated PE membrane for subsequent incubation and the high-throughput IR screening of microarrays of microcolonies of food pathogens will be explored further.

The treated PE membrane used in this study is an IR substrate that is ideally suitable for the cultivation of cells of foodborne pathogens and their subsequent measurement in IR transmission mode. It supports cell growth because of its hydrophilic property, which allows nutrients from the agar medium to reach the cells collected on its top surface. On the other hand, for IR reflection-absorption measurements, an aluminum-coated glass microscope slide has been commercially available and was recently used to grow cells and assess the response of lung cancer cells to a chemotherapeutic agent (17). However, it was labor-intensive and reportedly required placing a cell suspension on a slide, seeding, attaching cells to the slide, supplementing the attached cells with more medium, and using its aluminum-coated surface as an incubation vessel. The culture medium was then washed off, and the cells were air dried and measured by IR in the reflectance mode.

A novel bacterial sample preparation procedure for subsequent IR measurement was developed. A plasma-treated hydrophilic PE material that is IR transparent was successfully fabricated. This PE substrate was used to filter (or print microarrays of) dilute suspensions of foodborne bacteria and grow microcolonies that could be directly measured by IR transmission microspectroscopy. In this preliminary study, *E. sakazakii* and *K. pneumoniae* were differentiated by applying HCA analysis to the observed IR spectral data. More research is planned to demonstrate discrimination among species and strains of each organism. The growth time and conditions and the resulting size of the microcolonies obtained on the treated PE membrane will be further evaluated and optimized for measurement by IR microspectroscopy.

**REFERENCES**


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