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Residual Agar Determination in Bacterial Spores by Electrospray Ionization Mass Spectrometry

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Presented here is an analytical method to detect residual agar from a bacterial spore sample as an indication of culturing on an agar plate. This method is based on the resolubilization of agar polysaccharide from a bacterial spore sample, enzymatic digestion, followed by electrospray ionization tandem mass spectrometry (ESI-MS^n) analysis for detection of a specific agar fragment ion. A range of *Bacillus* species and strains were selected to demonstrate the effectiveness of this approach. The characteristic agar fragment ion was detected in the spores grown on agar that were washed from 1 to 5 times, irradiated or nonirradiated, and not in the spores grown in broth. A sample containing approximately 10^8 spores is currently needed for confident detection of residual agar from culture on agar plates in the presence of bacterial spores with a limit of detection of approximately 1 ppm agar spiked into a broth-grown spore sample. The results of a proficiency test with 42 blinded samples are presented demonstrating the utility of this method with no false positives and only three false negatives for samples that were below the detection level of the method as documented.

Microbial forensics is an emerging field focused on developing analytical tools that aid in the identification and determination of source origins of materials related to acts of bioterrorism or an inadvertent release of a biological agent.[1] Although considerable capability and expertise has been established in determining the identity of the causal agent in a bioterrorism event or outbreak, additional information and capability is needed to help determine or narrow down the potential source of a sample. In addition to genetic characterization assays for microbial forensics,[2] chemical and physical assays are needed to provide clues that help elucidate the processing, storage, and delivery methods used.[3] There are numerous methods for producing biological materials that range in scale and sophistication. A significant challenge is to identify *how* a given biological material was produced as a means to determine the specific individual or group responsible. In order to narrow down the source of a given sample it is likely that the integration of many sample characteristics will be required. This information may take many forms such as the methods and scale of production used along with any intentional additives or other impurities that may be present.[4] Additionally, there are a number of isotopic, elemental, chemical, and biochemical characteristics of a microbial sample that can provide forensic information. A number of published reports have examined methods for correlating the light isotopic ratio of media components to the final microbial preparation,[5,6] differences in media composition with spore metal ions,[7] small protein profiles,[8,9] and residual media components such as heme.[10] Each microbial characteristic or residual component may provide complementary pieces of information to aid in determining the methods or materials used in production of a microbial sample.

Because the detection of trace agar residue within a suspect microbial sample can indicate that the potential agent was produced using agar-solidified growth media, a method capable of reliably detecting residual agar has been of interest. The presence or absence of agar could imply the scale of production and level of sophistication used to perpetrate a biological attack. Agar is a generic term for a family of polysaccharides produced from marine red-purple algae (class Rhodophyceae) including *Gelidium*, *Pterocladia*, and *Gracilaria*.[11] Agarobiose, the primary constituent of agar, is the low sulfate, neutral gelling fraction consisting of alternating β-D-galactose (Gal) and 3,6-anhydro-α-L-galactose (AGal) monosaccharides with 1−4 linkages

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(1) Budowle, B.; Murch, R.; Chakraborty, R. *Int. J. Legal Med.* **2005**, *119*, 317–330.
(2) Keim, P.; Pearson, T.; Okinaka, R. *Anal. Chem.* **2008**, *80*, 4791–4799.
(3) Budowle, B.; Schutzer, S. E.; Ascher, M. S.; Atlas, R. M.; Burans, J. P.; Chakraborty, R.; Dunn, J. J.; Fraser, C. M.; Franz, D. R.; Leighton, T. J.; Morse, S. A.; Murch, R. S.; Ravel, J.; Rock, D. L.; Slezak, T. R.; Velsko, S. P.; Walsh, A. C.; Walters, R. A. *Appl. Environ. Microbiol.* **2005**, *71*, 2209–2213.
(4) Jarman, K. H.; Kreuzer-Martin, H. W.; Wunschel, D. S.; Valentine, N. B.; Cliff, J. B.; Petersen, C. E.; Colburn, H. A.; Wahl, K. L. *Appl. Environ. Microbiol.* **2008**, *74*, 3573–3582.
(5) Kreuzer-Martin, H. W.; Lott, M. J.; Dorigan, J.; Ehleringer, J. R. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 815–819.
(6) Kreuzer-Martin, H. W.; Jarman, K. H. *Appl. Environ. Microbiol.* **2007**, *73*, 3896–3908.
(7) Cliff, J. B.; Jarman, K. H.; Valentine, N. B.; Golledge, S. L.; Gaspar, D. J.; Wunschel, D. S.; Wahl, K. L. *Appl. Environ. Microbiol.* **2005**, *71*, 6524–6530.
(8) Valentine, N. B.; Wunschel, S. C.; Wunschel, D. S.; Petersen, C. E.; Wahl, K. L. *Appl. Environ. Microbiol.* **2005**, *71*, 58–64.
(9) Wunschel, D. S.; Hill, E. A.; Mclean, J. S.; Jarman, K. H.; Gorby, Y. A.; Valentine, N. B.; Wahl, K. L. *J. Microbiol. Methods* **2005**, *62*, 1327–1330.
(10) Whiteaker, J. R.; Fenselau, C.; Fetterolf, D.; Steele, D.; Wilson, D. *Anal. Chem.* **2004**, *76*, 2836–2841.
(11) Chaoyuan, W. Properties, manufacture and application of seaweed polysaccharides—agar, carrageenan and algin. In *Training Manual on Gracilaria Culture and Seaweed Processing in China*; Chaoyuan, W., Minghuo, J., Renshi, L., Xiaohang, W., Eds.; Chinese Ministry of Agriculture, 1990; Chapter 3.

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between Gal and AGal. AGal is primarily present in the red algae species.[12]

Several methods have been developed to analyze bulk agar[13−19] and agar in the context of microbial forensics.[4,20,21] Previously developed methods required extensive sample preparation and carbohydrate derivatization that may introduce artifacts in the sample. Although still viewed as a valuable complementary technique, the challenges of performing derivatization and gas chromatography/mass spectrometry (GC/MS) for trace agar detection led to the investigation of an alternative method. This alternative method focused on detecting the core dimeric structure of the agar polymer, the repeating Gal−AGal (agarobiose) repeating unit intact. Electrospray ionization mass spectrometry (ESI-MS) was selected due to the capability of this technology to analyze carbohydrates intact and without the need for derivatization for analysis by GC/MS.[22] For more sensitive ESI-MS detection of carbohydrates such as galactose or dimeric sucrose, a metal cation such as sodium can be added to the sample in order to aid in ionization efficiency.[23,24]

Agar gels commonly used in molecular biology for electrophoresis separations are digested via agarase enzyme to cleave the agar carbohydrate backbone.[25−27] The resulting enzymatic cleavage products typically are either agarobiose, agarotetraose, or agarohexaose oligomers depending on the particular agarase and digestion conditions, but all are suitably sized for mass spectrometric detection.

This method was designed to be a qualitative screening to detect the presence of residual agar in *Bacillus* samples from the culturing on agar plates from approximately 1 mg of *Bacillus* spore samples. Characterization of the method was performed against appropriate measures of effectiveness such as specificity and selectivity of the marker of interest over a variety of similar sample types, robustness of the method to changes such as different laboratory personnel, and limit of detection of the signal with standards and in the presence of likely sample background.[28] Characterization of the method presented here includes demonstrating the detectability of agar in bacterial spore samples after successive washings of the bacterial samples, when cultured with different growth media recipes, before and after sterilization by γ irradiation, and for different *Bacillus* species and strains. A final proficiency demonstration with blinded samples was conducted to test the residual agar detection method performance.

EXPERIMENTAL DETAILS

Materials. Agar sample preparation and digestion was conducted in low-retention, sterile 1.5 mL microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA), with the use of Millipore Ultrafree-MC centrifuge filters (Millipore, Bedford, MA), and in 4 mL 13 mm Teflon-lined screw cap glass vials (Grace Davison, Deerfield, IL). Agarase digestion was performed with AgarACE brand agarase enzyme (Promega, Madison, WI) and was aliquoted as delivered. Neoagarotetraose was used for verification of the mass axis calibration (V-Labs, Inc., Covington, LA—distributor for Dextra Laboratories, United Kingdom). Agar standards were prepared with granulated agar (Difco, Franklin Lakes, NJ). Milli-Q-grade water was produced by a Millipore system (Bedford, MA) and NanoPure HPLC-grade water (Sigma-Aldrich, St. Louis, MO) and used for reagent and sample preparation. Acetic acid and sodium chloride were also purchased from Sigma-Aldrich (St. Louis, MO). Disinfection was performed with a 10% bleach solution prepared from stock bleach (Fisher Scientific, Pittsburgh, PA). Alginic acid (sodium salt, 0.001% from Sigma), agarose (LMP from Invitrogen), carrageenan (commercial grade, type 1, Sigma C1013, predominantly κ, lesser amount λ carrageenan), agar (Difco agar granulated), and Telephone brand agar−agar powder (Seng Huad Limited Partnership, Bangkok, Thailand—from a local grocery in Richland, WA) were used. Tryptic soy, brain heart infusion media, and red blood cell agar (SBA) were purchased from Difco.

Bacterial Spore Preparation. *B. anthracis* spores were cultivated and harvested at the Biological Defense Research Directorate (BDRD) at Forest Glen, MD from tryptic soy broth (TSB), tryptic soy agar (TSA), brain heart infusion broth (BHIB), and brain heart infusion agar (BHIA). Four liters of TSB and BHIB were prepared according to manufacturer's instructions with a single source of sterile Milli-Q water and heated until dissolved. Both broths were split into two 2 L aliquots; 30 g of agar was added to one of the 2 L aliquots, and the media was reheated until the agar dissolved. The media was then autoclaved and put into appropriate culture plates or flasks for inoculation.

Starter cultures prepared from stock cultures of *B. anthracis* Sterne and Ames were streaked for isolation on sheep red blood cell agar (SBA) and incubated overnight at 37 °C and then 8 h at room temperature. Three isolated colonies of each isolate were suspended in 500 μL of sterile water (Sigma, product no. W3500, lot no. 113K2331) and heated to 65 °C for 15 min. A 10 μL loop of each heat-shocked suspension was used to inoculate 50 mL of BHIB and TSB. These cultures were incubated overnight at 37 °C in a shaking incubator at 200 rpm.

Five milliliter aliquots of the above starter cultures were used to inoculate three 150 mL broth cultures of both BHIB and TSB. Purity of the starter cultures was confirmed by gram stain and subculture. Aliquots of 50 μL of the BHIB and TSB starter cultures were used to inoculate 20 BHIA and TSA plates for confluent growth. BHIB and TSB cultures were shaken at 37 °C, 100 rpm until day 7, then at 28 °C, 200 rpm. The agar plates were incubated

(12) Jol, C. N.; Neiss, T. G.; Penninkhof, B.; Rudolph, B.; Ruiter, G. A. D. *Anal. Biochem.* **1999**, *268*, 213–222.
(13) Yaphe, W. *Anal. Chem.* **1960**, *32*, 1327–1330.
(14) Yaphe, W.; Arsenault, G. P. *Anal. Biochem.* **1965**, *13*, 143–148.
(15) Lahaye, M.; Yaphe, W.; Viet, M. T. P.; Rochas, C. *Carbohydr. Res.* **1989**, *190*, 249–265.
(16) Kiwitt-Haschemie, K. H.; Heims, H.; Steinhart, H.; Mischnick, P. *Carbohydr. Res.* **1993**, *248*, 267–275.
(17) Quemener, B.; Lahaye, M.; Metro, F. *Carbohydr. Res.* **1995**, *266*, 53–64.
(18) Navarro, D. A.; Stortz, C. A. *Carbohydr. Res.* **2003**, *338*, 2111–2118.
(19) Stevenson, T. T.; Furneaux, R. H. *Carbohydr. Res.* **1991**, *210*, 277–298.
(20) Fenselau, C. In *17th Sanibel Conference on Mass Spectrometry: Forensic Science and Counterterrorism*, Sanibel Island, FL, January 28−February 1, 2005.
(21) Wunschel, D. S.; Colburn, H. A.; Fox, A.; Fox, K. F.; Harley, W. M.; Wahl, J. H.; Wahl, K. L. *J. Microbiol. Methods* **2008**, *74*, 57–63.
(22) Wunschel, D. S.; Fox, K. F.; Fox, A.; Nagpal, M. L.; Kim, K.; Stewart, G. C.; Shahgholi, M. *J. Chromatogr., A* **1997**, *776*, 205–219.
(23) Duffin, K.; Welply, J.; Huang, E.; Henion, J. *Anal. Chem.* **1992**, *64*, 1440–1448.
(24) Sible, E. M.; Brimmer, S. P.; Leary, J. A. *J. Am. Soc. Mass Spectrom.* **1997**, *8*, 32–42.
(25) Swartz, M. N.; Gordon, N. *J. Bacteriol.* **1959**, *77*, 403–409.
(26) Rochas, C.; Potin, P.; Kloareg, B. *Carbohydr. Res.* **1994**, *253*, 69–77.
(27) Ma, C.; Lu, X.; Shi, C.; Li, J.; Gu, Y.; Ma, Y.; Chu, Y.; Han, F.; Gong, Q.; Yu, W. *J. Biol. Chem.* **2007**, *282*, 3747–3754.
(28) Budowle, B. Quality Assurance Guidelines for Laboratories Performing Microbial Forensic Work. *Forensic Sci. Commun.* **2003**, *5*.

Table 1. Cell Counts for *B. anthracis* Sterne Wash Samples

wash no.	brain heart infusion agar (CFU/mL)	brain heart infusion broth (CFU/mL)	tryptic soy agar (CFU/mL)	tryptic soy broth (CFU/mL)
1	8.8×10^9	9.2×10^8	1.176×10^{10}	1.4×10^9
2	9.2×10^9	1.24×10^9	1.6×10^{10}	4.8×10^8
3	9.6×10^9	1.08×10^9	1.12×10^{10}	5.6×10^8
4	9.6×10^9	6.0×10^8	2.6×10^{10}	6.0×10^8
5	1.12×10^{10}	3.2×10^8	1.96×10^{10}	2.8×10^8

for 2 days at 37 °C and then at room temperature until day 7 when harvested (>95% sporulation on the agar plates).

Multiple Washing Samples. The agar plate cultures were harvested by transferring a 5 mL aliquot of sterile Milli-Q water to each plate in a five plate series and resuspending the growth by scraping. The slurries were pooled, and the total end volume was 20 mL for both TSA and BHIA cultures. A 4 mL aliquot of this original suspension was saved and labeled as "wash no. 1". The remaining 16 mL of slurry was centrifuged in a 50 mL sterile polypropylene conical tube at 4000*g* for 15 min. The supernatant was decanted, and 16 mL of sterile water was added, the pellet was resuspended and vortexed, and 4 mL was removed and labeled wash no. 2. This process was repeated three more times to collect aliquots from wash no. 3, no. 4, and no. 5. The washes were stored at −80 °C until the colony counts were conducted.

Harvesting and washing of spores from the broth cultures was performed as follows. The three 150 mL volumes of each broth type were pooled in a sterile polypropylene centrifuge bottle, and a 10 mL raw broth aliquot was saved. The pooled broth cultures were centrifuged at 3000*g* for 15 min. The supernatants were decanted, and the pellets were resuspended in 20 mL of sterile water each. Then aliquots from wash no. 1 through no. 5 were collected as described above.

Colony counts of the *B. anthracis* Sterne and Ames spore washes were performed by plating 10-fold serial dilutions on SRBC agar plates and enumeration of resultant colonies. The resulting counts for each of the wash samples for *B. anthracis* Sterne are shown in Table 1. Similar cell counts for the *B. anthracis* Ames spore samples were obtained.

Instrumentation. Initial method development was performed on the Agilent (Santa Clara, CA) LC/MSD XCT electrospray ionization ion trap mass spectrometer with ChemStation software. Subsequent method development and characterization was performed on the ThermoElectron (Waltham, MA) LTQ electrospray ionization ion trap mass spectrometer with XCalibur Software.

Preparation of Standards and Stock Solutions. Agar stock solutions (1%) were prepared fresh weekly and used to prepare 100, 10, and 1 ppm and 100 ppb solutions upon dilution with Milli-Q water. A 4 mM NaCl solution was prepared in Milli-Q water for addition to each sample after digestion. An 80 μM NaCl solution was prepared as stock solution which was diluted 1:2 daily for use as flow injection eluent.

Agarase Digestion/Sample Preparation for ESI-MS Analysis. Approximately 1 mg of spore material and enough Milli-Q water to suspend to 300 μL was combined into a 1.5 mL Eppendorf microcentrifuge tube. For spore samples in slurry, the cell counts of the different washes were used to estimate the volume of slurry needed to approximate 1 mg of spore material (assuming a spore mass of 1 pg/spore[29]). The sample aliquots were heated at 95−100 °C and 350 rpm for 1 h with an Eppendorf thermomixer to redissolve any residual agar in the sample. After cooling the samples were centrifuged at 13 200 rpm for 2 min to pellet the spores. The supernatant liquid was filtered with a sterile 0.2 μm Millipore Ultrafree-MC centrifuge filter (13 200 rpm, 3 min) and then transferred into a 4 mL glass reaction vial along with 3 μL of agarase and 3 μL of 4 mM NaCl and gently mixed. The sample vials were heated at 45 °C for 2 h in a Pierce (Rockford, IL) Reacti-Therm heat block. The enzymatic digestion was quenched by placing the samples into a 95−100 °C water bath for 2 min, allowed to cool to room temperature, and then analyzed immediately or stored overnight at 4 °C.

A method blank consisting of 3 μL of agarase solution and 3 μL of 4 mM NaCl was put through the digestion procedure daily and analyzed by ESI-MS^n. A process control of 100 ppb or 1 ppm standard agar solution was also prepared and digested with each sample set to ensure the digestion process was working properly.

Analysis by ESI-MS^n. Direct infusion ESI-MS with a 5 μL sample loop and 40 μM NaCl eluent at a flow rate of 5 μL/min was used. Loop cleaning between samples was performed with subsequent flushes of HPLC-grade water, 0.1% acetic acid, and 40 μM NaCl. A separate 10 μL injection of 40 μM NaCl was run as an "analytical blank" between samples. An aliquot of each sample was diluted 1:10 with 40 μM sodium chloride and analyzed prior to the analysis of the undiluted sample in case the sample was too concentrated for ESI-MS analysis. If no detectable agar was present in the diluted sample, then the undiluted sample was analyzed.

Data Collection Criteria and Data Analysis. Neoagarotetraose (10 μg/mL) was used daily as an instrument control standard. The ions at m/z 653.3 from MS mode, m/z 473.1 and 329.1 from MS/MS of 653, and m/z 329.1 from MS/MS/MS of 653 fragmenting to 473 fragmenting to 329 all needed to be within ± m/z 0.3 of the known values before proceeding to the analysis of unknown samples. Following the neoagarotetraose mass calibration check, a method blank and process control digested agar solution were analyzed. At the end of the day the second process control digested agar solution and method blank were run to ensure the instrument was still functioning appropriately with no carryover issues. If either method blank exhibited an ion signal for the MS^3 m/z 329 ion greater than 25 counts then the entire sample set would be reprocessed.

For a positive identification of agar in the sample, the signal intensity for the MS^3 ion at m/z 329 must be greater than 40 counts. This threshold level was determined based on data collected during method development to be above the levels of signal detected for method blanks and to capture as many of the positive controls as possible. The analytical blanks were routinely less than 10 counts of m/z 329 in MS^3 mode.

Blinded Proficiency Samples. A total of 42 blinded samples were prepared by the National Bioforensics Analysis Center (NBFAC, Frederick, MD). All samples were processed and

(29) Fergenson, D. P.; Pitesky, M. E.; Tobias, H. J.; Steele, P. T.; Czerwieniec, G. A.; Russell, S. C.; Lebrilla, C. B.; Horn, J. M.; Coffee, K. R.; Srivastava, A.; Pillai, S. P.; Shih, M.-T. P.; Hall, H. L.; Ramponi, A. J.; Chang, J. T.; Langlois, R. G.; Estacio, P. L.; Hadley, R. T.; Frank, M.; Gard, E. E. *Anal. Chem.* **2004**, *76*, 373–378.

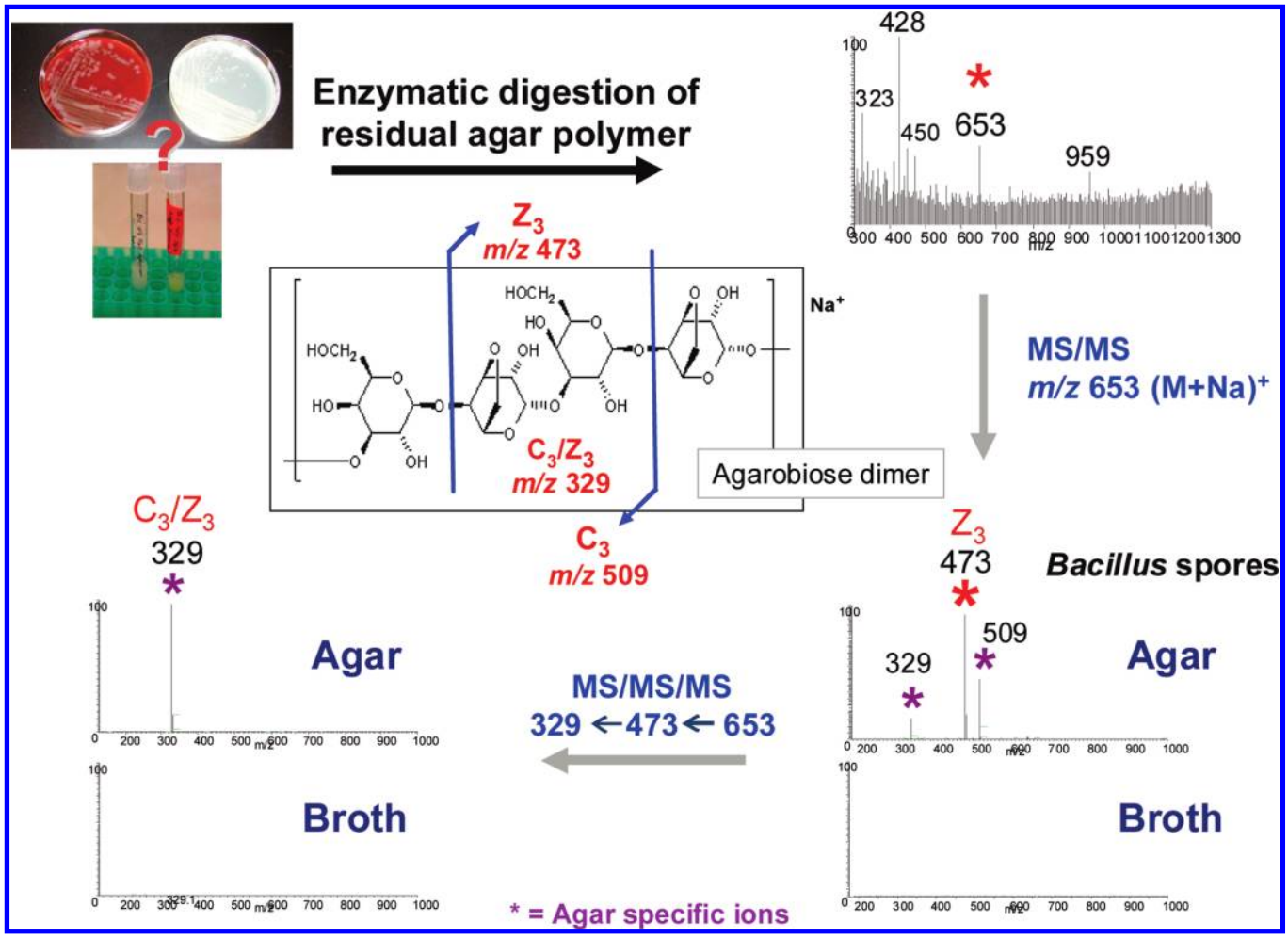

Figure 1. Agarase digestion procedure for ESI-MS analysis.

analyzed in triplicate. The samples were processed in 10 different blocks with only one sample replicate per block and in different orders within the blocks to minimize effects of day-to-day and within day variability. Each sample block contained two water method blanks and agar standards of 100 ppb agar and 1 ppm agar.

Safety Precautions. *B. anthracis* Sterne is the vaccine strain for *B. anthracis* and lacks the plasmid pXO2, rendering it nonpathogenic to healthy individuals. These samples were treated as biosafety level 2 (BSL2) samples. Irradiated, certified, killed *B. anthracis* Ames was also treated as BSL2. All work involving the spores was performed in a certified biological safety cabinet and with sealed rotors in the centrifuge. Appropriate gloves, lab coat, and eye protection were worn at all times when working with the *B. anthracis* and other samples. Access to the laboratory was controlled where samples were stored and where work was performed. All waste associated with *B. anthracis* was autoclaved and then incinerated following laboratory protocols. Samples containing *B. thuringiensis* were autoclaved prior to disposal. The appropriate APHIS permit for receipt and use of *B. anthracis* samples was obtained from the United States Department of Agriculture.

RESULTS

This method was designed to be a qualitative determination of the presence of agar residual on bacterial spores grown on agar plates. An agarase enzyme was used to cleave agar as routinely performed following gel electrophoresis to release separated targets.[30] On the basis of the reported polymeric structure of agar,[11] characterization of the carbohydrate components of agar,[19,20] and previous work we published regarding carbohydrate analysis of agar components[21] we expect the enzymatic digestion to reveal pieces of the agar exhibiting the galactose−anhydrogalactose repeating unit. Under the digestion conditions used with the agarACE brand enzyme, the dominant reproducible digestion products of agar observed as the sodium adducts by ESI-MS were consistent with agarotetraose at m/z 653 (galactose−anhydrogalactose−galactose−anhydrogalactose Gal−AGal−Gal−AGal) and agarohexaose at m/z 959 (Gal−AGal−Gal−AGal−Gal−AGal) (Figure 1). There are other lower m/z ions in this particular MS^1 spectrum, but they are not as consistent or specific to agar presence. The agarotetraose was selected as the primary target for use in the agar identification method due to the stability of this marker over the course of method development and because only three dissociation products were typically observed versus six or more for the agarohexaose dissociation. By adding trace levels of alkali metal to a carbohydrate solution, the affinity of the interactions have been shown to increase ions stability and thus improve ionization efficiency. Consequently, sodium chloride was added to the digestate to aid in ion formation in the electrospray ionization process and

(30) Selman, S.; Knoche, K.; Knuth, M. *Promega Notes Mag.* **1995**, *53*, 6–10.

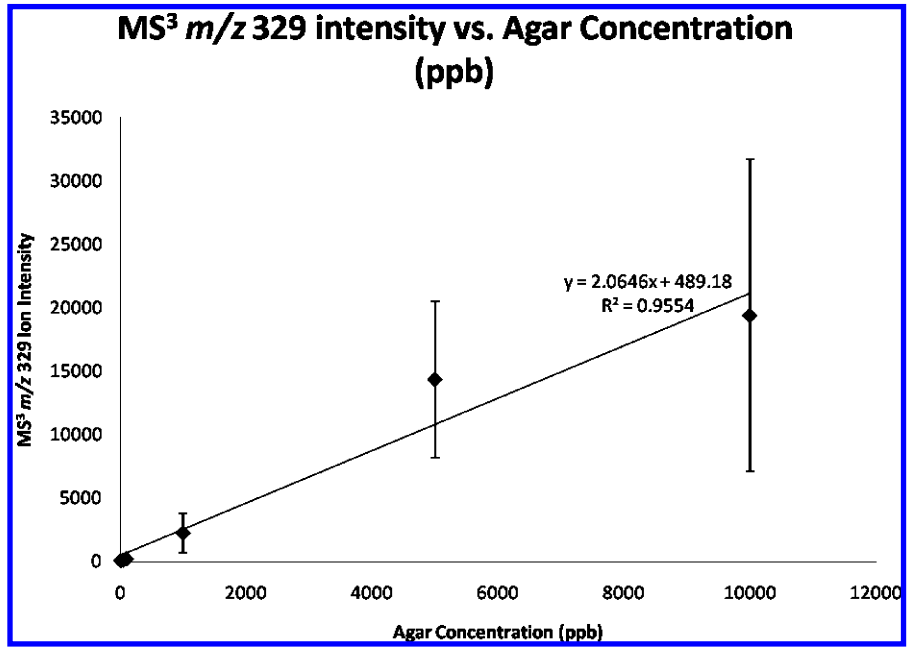

Figure 2. Average ion intensities vs agar concentration.

subsequent fragmentation by tandem mass spectrometry.[31] The resulting sodium adduct ion of agarotetraose (Gal−AGal−Gal−AGal + Na^+) was observed in the mass spectrum at m/z 653.3. The specificity of detecting a single mass in a potentially complex sample background was not considered suitable for selectivity of the assay. Certainly other fragmentation parameters (e.g., collision energy) and instrumentation (e.g., triple quadrupole) may be usable for developing an agar assay, but the key criteria for this application was specificity in an unknown sample versus quantitation. Therefore, multiple stages of tandem mass spectrometry with two consecutive fragmentations were added to increase the specificity of the assay. The m/z 653.3 agarotetraose sodium adduct ion was subjected to gas-phase fragmentation resulting in three major fragment ions observed as products at m/z 509, 473, and 329 from the loss of anhydrogalactose, galactose, and the dimeric agarobiose unit, respectively (Figure 1). The fragmentations are labeled following Domon and Costello nomenclature for carbohydrate fragmentation.[32] The m/z 473 fragment ion was then isolated in the ion trap of the mass spectrometer for further fragmentation. From this secondary fragmentation only one primary fragment ion (C_3/Z_3) was observed at m/z 329 from the additional loss of an anhydrogalactose unit to form a sodium adduct ion of a Gal−AGal fragment. Although the choice of a different instrument such as a triple quadrupole mass spectrometer may provide more abundant MS^2 fragmentation for the m/z 329 ion the specificity may not be as good for unknown complex microbial samples with only one stage of fragmentation.

This method targeted the specific detection of residual agar on bacterial spore preparations with unknown processing or purity. Quantitation of the amount of agar present in the sample would offer minimal additional information during the data interpretation phase. The key information in characterizing the method was the amount of sample or bacterial cell mass needed to provide confidence that agar would be detected if the cells were processed from agar plates. Replicate samples of agar standard solutions at 50 and 100 ppb and 1 ppm and with different lots of agarase were processed and analyzed to determine the detection limit of agar. Numerous lots of the AgarACE enzyme were used throughout the course of this method development with no noticeable difference in performance. A plot of average ion intensity of the diagnostic agar ion at m/z 329 from the tandem ESI-MS^3 analysis of the replicate agar solutions is shown in Figure 2. The lower limit of reproducible detectability with the current instrumental setup was determined to be approximately 50 ppb for standard agar. This ability to detect agar was also tested in the presence of spore background by spiking agar at two known concentrations (100 ppb and 1 ppm) into broth-grown spore samples. Two sets of triplicate samples of *B. anthracis* spore samples (grown in TSB and γ irradiated) for five different wash conditions were spiked with 100 ppb agar and 1 ppm agar. Only 4 out of 15 spore samples spiked at 100 ppb agar were correctly identified as having agar present, whereas all 15 spore samples spiked at 1 ppm agar were positively identified as containing residual agar. Therefore, the detectability of residual agar in the presence of bacterial spores is higher than for agar standards and is conservatively set at 1 ppm agar when spiked into spore samples. Additional separations or sample cleanup prior to the ESI-MS^3 analysis could improve the sensitivity for the spore samples in future method improvements.

A series of experiments were conducted to test the approximate amount of spore sample needed for confident detection of agar if present in agar-grown *Bacillus* spore samples. Three *B. anthracis* Sterne spore samples (one G agar and two aliquots of a Leighton−Doi agar preparation) were used to conduct a series of serial dilutions at 1:2, 1:5, 1:10, 1:100, 1:1000, and 1:10 000 starting with approximately 3×10^9 CFU/mL for the undiluted samples. Agar was correctly identified in the stock and 1:2 diluted *Bacillus* samples. It was also detected in two out of the three spore samples (one Leighton−Doi agar spore aliquot and

(31) Ojima, N.; Masuda, K.; Tanaka, K.; Nishimura, O. *J. Mass Spectrom.* **2005**, *40*, 380–388.
(32) Domon, B.; Costello, C. E. *Glycoconjugate J.* **1988**, *5*, 397–409.

one G agar spore sample) at the 1:5 and 1:10 dilution. Agar was not detected in any of the 1:100 or 1:1000 dilution spore samples. However, it was detected in one of the highest dilution samples at 1:10 000 (Leighton−Doi agar sample) or approximately 10^4 CFU/mL. This may indicate a nonhomogeneous distribution of the residual agar in a bacterial spore preparation.

In a second dilution experiment, a dried powder sample of a *B. anthracis* spore preparation from TSA was weighed out (~58 mg), suspended in distilled water (10 mL), and then aliquoted into three 300 μL portions. Serial dilutions of these three portions were then done at 1:10, 1:100, 1:1000, and 1:10 000 with Milli-Q water. In this case, only the undiluted aliquots containing approximately 1.7 mg of material gave a positive detection for presence of agar in the samples. Agar was not detected with approximately 0.17 mg of material.

In a third experiment designed to obtain similar information, the same dried spore preparation of *B. anthracis* Sterne grown on TSA was weighed out 10 times with end samples ranging from 0.1 to 0.9 mg per sample vial. All 10 aliquots were processed simultaneously and analyzed in the same sample block for direct comparison. Positive agar detection was obtained for seven of the samples weighing between 0.2 and 0.9 mg. At the lowest end, two out of three samples weighing 0.1 mg were false negatives with no indication of residual agar being present.

On the basis of the results of these three experiments, conservatively 0.4 mg of bacterial spore material was required for confident detection of residual agar from culturing. If this sample was a clean, pure spore preparation, and based on the approximation of spore mass from Loferer-Krobacher et al.[33] (0.7 pg per spore) and Fergenson et al.[29] (1 pg per spore) then approximately 5×10^8 spores are needed for confidence in the final results. If increased sensitivity is desired in the future changes to this method can be made such as optimizing the collision conditions, using different mass spectrometers and interfaces that are continually emerging with improved sensitivity, and/or adding a front end separation to minimize sample interferences.

Although the majority of the method characterization and evaluation of the agar method was done with *B. anthracis*, early testing was performed with a variety of *Bacillus* species including *B. cereus*, *B. subtilis*, and *B. thuringiensis*. There is no indication to date that this assay is sensitive to bacterial species or strain (data not shown). *Bacillus* samples cultured on agar gave the characteristic fragment ion at m/z 329 by ESI-MS^3. Both vegetative and sporulated *Bacillus* samples were correctly characterized as having agar present if cultured on agar plates and no agar present if cultured in broth based on the presence or absence of the diagnostic ESI-MS^3 ion at m/z 329.

Several different grades of agars (e.g., food grade, electrophoresis grade) and agar from different manufacturers were evaluated with the agar assay to determine if different types of agar affect the target mass distribution. The various grades of agar and electrophoresis-grade agarose analyzed by the ESI-MS^3 method to date were all readily identified as agar in the assay. In addition, nonagar gelling agents such as alginic acid, xanthan gum, and carageenan were analyzed. The results for the intensity of the diagnostic ESI-MS^3 ion at m/z 329 for agar determination from replicate analyses of a variety of gelling and agar-like materials are summarized in Table 2. The diagnostic agar ions were observed slightly above the acceptance threshold only with carageenan but at approximately 100-fold lower intensity than any of the grades of agar. This is expected as carageenan has the same carbohydrate backbone structure as agar but with significant amounts of sulfation on the carbohydrates. Sulfation could potentially affect the enzymatic digestion efficiency and positive ion formation during the assay, reducing the signal intensity as well as shifting the m/z value of the carbohydrate ions that could be observed by ESI-MS. Other ESI-MS methods targeted at sulfated carageenans exist and could potentially be applied to better distinguish carageenans,[34,35] but was not the focus of this study.

Table 2. Results of ESI-MS/MS/MS Analysis of Other Agars and Gelling Agents

sample (10 ppm)	m/z 329 ion intensity average (SD)
alginic acid	30 (20)
carrageenan	70 (50)
xanthan gum	20 (7)
agarose	7090 (4040)
food-grade agar	18200 (3370)

Another concern in the development of this assay is whether spores from very clean preparations would have enough residual agar in the sample to be detected. For this evaluation, *B. anthracis* spore samples cultured with SBA, BHIA, and TSA that were washed with water 0−5 times after harvesting were collected for analysis. There were over 20 different *B. anthracis* Sterne samples with up to five water washes that were successfully analyzed in triplicate as containing agar. Only one sample, the fourth wash sample of *B. anthracis* Sterne spores from blood agar plates, failed to give a positive agar signal (see Table S-1 in the Supporting Information).

To test the effect of spore viability on the assay, a set of irradiated and nonirradiated *B. anthracis* Sterne spore samples grown on both TSA and BHIA that were washed between 1 and 5 times with water were analyzed. The diagnostic agar ion at m/z 329 was observed at equal or above the threshold level of 40 counts for all of these irradiated and nonirradiated spore samples (Table S-1 in the Supporting Information). The irradiation of the spore samples did not have a deleterious effect on the qualitative results obtained with the agar assay.

During the course of agar method characterization, at least three replicates of *Bacillus* spore samples from different growth media including Leighton−Doi (LD),[36] BHI, G,[37] modified G, TSA, and SBA were prepared and agar was successfully detected by the agar ESI-MS method for the agar-grown samples (data not shown). There was minimal observable difference in the performance of the agar assay with different types of growth media. In

(33) Loferer-Krobacher, M.; Kilma, J.; Psenner, R. *Appl. Environ. Microbiol.* **1998**, *64*, 688–694.

(34) Antonopoulos, A.; Hardouin, J.; Favetta, P.; Helbert, W.; Delmas, A. F.; Lafosse, M. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 2217–2226.

(35) Yu, G.; Zhao, X.; Yang, B.; Ren, S.; Guan, H.; Zhang, Y.; Lawson, A. M.; Chai, W. *Anal. Chem.* **2006**, *78*, 8499–8505.

(36) Doi, R. H. In *Bacillus*; Harwood, C., Ed.; Plenum Press: New York and London, 1989; pp 169−216.

(37) Hashimoto, T.; Black, S. H.; Gerhard, T. *Can. J. Microbiol.* **1960**, *6*, 203–212.

Table 3. Results of ESI-MS^3 Agar Analysis for Blinded Samples Grown on Agar

sample	agar detection	sample identity
2	3/3	mod G agar A
3	2/3	Leighton−Doi agar A
13	3/3	mod G agar A
14	2/3	mod G agar B
23	3/3	mod G agar A
30	2/3	Leighton−Doi agar A
37	3/3	Leighton−Doi agar B
50	3/3	Leighton−Doi agar A
55	3/3	Leighton−Doi agar
	False Negatives	
10	1/3	Leighton−Doi agar A
24	0/3	mod G agar B
34	0/3	mod G agar B

one preliminary experiment with spores produced in LD media, an additional agar adduct ion as the $(M + K)^+$ ion in addition to the typical $(M + Na)^+$ ion was observed presumably due to the high potassium concentration in that LD media recipe. However, this additional ionization did not interfere with the analysis of the designated $(M + Na)^+$ ion per the standard protocol and has not reproducibly been a strong signal for other spore samples cultured from LD agar plates. This observation, however, led to the additional data collection stream for monitoring the $(M + K)^+$ potassium adduct ion in the event that it may be prevalent in an unknown sample.

The ruggedness of the assay in terms of effect of sample physical state, personnel changes, and repeatability over time was put to the test during the course of the method characterization and proficiency testing over a nearly 12 month period. During the course of that time different personnel were used for every aspect of the sample handling, sample preparation, and instrument operation.

A series of blinded samples were prepared at NBFAC to test the agar screening method. Each sample was analyzed in triplicate. For the triplicate sample analysis, two out of three replicates were required to be positive based on the threshold set for the agar fragment ion observed at *m/z* 329. On the basis of these data criteria, agar was found to be present in 9 out of the 12 samples that were cultured on agar plates and was not detected in any of the broth-grown spores or other media samples submitted in the blinded set. Table 3 summarizes the results from ESI-MS^3 agar analysis for the nine samples that tested positive for agar and the three false negatives. No agar was detected in the remaining blinded samples. All proficiency sample results are provided in Table S-2 in the Supporting Information along with the identity of all the blinded samples. One concern was whether a standard microbiology practice of taking agar plate scrapes and using inoculated broth cultures would also result in a positive agar detection. A selection of these samples was included in the blinded sample set. None of the 12 samples that were prepared from plate scrapes of agar plates used to inoculate broth solutions gave any positive agar signal and were correctly identified as no agar detected.

The agar assay failed to identify the presence of agar above the predetermined acceptable thresholds in three of the blinded samples that were produced on agar media. These samples, nos. 10, 24, and 34, are listed in Table 3. However, after completion of the analyses it was revealed that the spore mass in these three samples was below the threshold determined for this agar assay of approximately 0.4 mg of sample, and therefore, we would not have expected a positive result. Samples 24 and 34 were replicates of the same sample and gave consistent false negative results. One analysis of sample 10 met the requirements for a positive result. The other two replicate analyses did show residual agar ion signal but not above the predetermined threshold for positive identification of agar; therefore, sample 10 was reported as no agar detected.

CONCLUSION

The ESI-MS^3-based agar screening method was effective for detection of residual agar on bacterial spore samples. The current method demonstrated a minimum sample requirement of approximately 0.4 mg or 5×10^8 spores. During development of this method two different instruments were used, at least four different personnel conducted the sample processing method, and three different staff operated the mass spectrometer during the different testing phases. Well over 300 samples were analyzed during the final method characterization, evaluation, and blinded proficiency testing. The method is proven to be robust to variables such as irradiation, sample drying, and different culture media and growth conditions and is suitable as a qualitative determination of the presence of residual agar in *Bacillus* spore samples cultured on agar plates. This method was used with both vegetative cells and spores of different *Bacillus* species and strains and should be applicable to other species.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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