Mass Spectrometry Biotyper System Identifies Enteric Bacterial Pathogens Directly from Colonies Grown on Selective Stool Culture Media[▽]

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We evaluated the performance and cost-effectiveness of a matrix-assisted laser desorption ionization time-of-flight mass spectrometry-based Biotyper system for the routine identification of common enteric bacterial pathogens seen in middle Tennessee from suspicious colonies grown on selective stool culture media. A total of 304 suspicious colonies were selected and further identified from 605 stool specimens. The suspicious colonies were analyzed by the Biotyper system, and the results were compared to those from routine phenotypic methods, which identified 22 *Salmonella* species, 39 *Shigella* species, 3 enterohemorrhagic *Escherichia coli* (EHEC) isolates, 2 *Yersinia enterocolitica* isolates, 2 *Campylobacter* species, and 236 gastrointestinal normal flora isolates. The Biotyper system correctly identified the *Salmonella* species, *Yersinia enterocolitica*, and *Campylobacter* species but failed to distinguish the *Shigella* species and EHEC isolates from *E. coli*. Among the 236 normal flora isolates, 233 (98.7%) and 228 (96.6%) agreed at the genus and species levels, respectively, between the phenotypic and Biotyper methods. Organism identification scores were insignificantly different between colonies directly from selective media and subsequently from pure subculture. The entire Biotyper identification procedure, from smear preparation to final result reporting, can be completed within 30 min. The Biotyper system provides a rapid and simple screening tool for identifying many, but not all, suspicious colonies grown on selective media within 24 h after inoculation, which shortens test turnaround time by 2 to 3 days.

Infectious diarrhea continues to be a worldwide problem and accounts for more than 2 million deaths annually (6, 25, 38). For example, in the United States, it is estimated that there are between 211 and 375 million episodes of acute diarrhea each year, and such episodes are responsible for more than 900,000 hospitalizations and 6,000 deaths annually (18, 21). Stool specimens from patients with acute diarrheal illnesses, particularly with systemic illness, fever, or bloody stools, are routinely cultured for enteric bacterial pathogens. In addition to *Salmonella*, *Shigella*, and enterohemorrhagic *Escherichia coli* (EHEC), the list of potential enteric bacterial pathogens has been expanded to include microorganisms such as *Campylobacter jejuni*, *Aeromonas hydrophila*, *Yersinia enterocolitica*, and *Vibrio* species.

Bacterial stool cultures remain the mainstay laboratory diagnostic method for the evaluation of diarrheal illnesses. Moreover, providing a rapid, accurate, and specific identification of enteric bacterial pathogens is considered essential for directing the antimicrobial therapy of diarrheal illnesses (4, 32, 38). Selective agars are used to facilitate the isolation of suspicious enteric bacterial pathogens. Pure cultures obtained subsequently then allow the microorganisms to be more fully characterized and identified by morphological and biochemical characteristics. These conventional methods, however, are

time-consuming and labor-intensive, and they require experienced clinical microbiologists. A number of different types of selective media have been developed to enhance the recovery of enteric pathogens from stool samples. Unfortunately, these media have been shown to have poor specificity and produce numerous false-positive colonies (11, 16, 33). The identification and confirmation of suspicious colonies is highly nonspecific, and it places a heavy burden on the laboratory in terms of biochemical and serological testing (11, 16, 34). Multiplex PCR-based molecular methods have the potential to provide rapid results, but the detection and identification of a panel of pathogens has been difficult and costly (15, 17, 24, 31).

In recent years, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has emerged as a new technology for species identification. The analyte molecules embedded within the saturated matrix on the target plate are irradiated by a laser of special wavelength and intensity, inducing desorption and ionization; the charged analytes then are accelerated by an electric field in a flight tube to a detector, where they are captured. The separation of various molecules depends on the time of flight, which is reversely proportional to the mass of molecules. After detection signals are processed and interpreted into the mass spectra, the characteristic mass peaks are used to characterize and eventually identify the microorganisms. By measuring the exact sizes of peptides and small proteins, which are assumed to be characteristic for each bacterial species, it is possible to determine the species within a few minutes of when the analysis is started with whole cells, cell lysates, or crude bacterial extracts (8, 14, 22, 26, 27, 36).

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A MALDI-TOF MS-based Biotyper system (Bruker Daltonics, Germany) has been developed for the rapid identification and characterization of microorganisms based on the characterization of biomarker molecules, which are mostly mass signals of ribosomal protein with house-keeping functions in a comparatively more efficient manner (1, 7, 23, 30, 37). In this preliminary study, we explored the application of the Biotyper system for the routine identification of common enteric bacterial pathogens seen in middle Tennessee directly from suspicious colonies grown on selective stool culture media.

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MATERIALS AND METHODS

Clinical specimens. A total of 605 consecutive stool specimens collected from 570 patients with diarrheal diseases from 2 February to 5 November 2009 from the Clinical Microbiology Laboratory at Vanderbilt University Medical Center were included in this preliminary study.

Conventional phenotypic identification. Each stool specimen was inoculated on one Hektoen enteric agar (HEA) plate, two MacConkey (MAC) agar plates, one MACS (MAC sorbitol) agar plate, one *Campylobacter* blood agar (CBA) plate, and with one aliquot of GN (Gram-negative) broth. The HEA, MAC, and MACS plates were aerobically cultivated at 35°C overnight, except for one MAC plate, which was incubated at room temperature for 48 h. The CBA plate was incubated in a microaerophilic environmental chamber (Bio-Bag; Becton Dickinson Microbiology Systems, Cockeysville, MD) at 42°C for 48 h. The GN broth was inoculated onto the HEA and MAC plates after incubation at 35°C for 6 to 24 h. Non-lactose-fermenting colonies on HEA and/or MAC and sorbitol-negative colonies on MACS were selected as suspicious colonies and subcultured on triple sugar iron (TSI), lysine iron agar (LIA), and motility indole ornithine (MIO) slants (4). Suspicious colonies on TSI, LIA, and/or MIO slants were further subcultured on blood agar plates for further identification by using an API 20E system (bioMérieux, Durham, NC) according to the manufacturer's instructions. A bacterial agglutination test was performed on sorbitol-negative colonies on MACS by using the *E. coli* O157 blue latex test reagent kit (Pro-Lab Diagnostics, Round Rock, TX) according to the manufacturer's instructions. Suspicious colonies on the CBA plate were confirmed as *Campylobacter* spp. by spot oxidase, Gram stain, and a *Campylobacter* agglutination test (Oxoid Biochemical Identification System Campy; Oxoid, Hampshire, England). Enteric bacterial pathogens, including *Shigella* spp., *Samonilla* spp., EHEC, *Yersinia enterocolitica*, and *Campylobacter* spp., were specifically identified (4) and further confirmed at Tennessee State Laboratory Services. For each suspicious colony identified, half of each one was used for the phenotypic and Biotyper methods.

MALDI-TOF mass spectrometry method. Suspicious colonies from the original MAC, HEA, MACS, and CBA selective and subculture medium plates were analyzed by the Biotyper system on the same day that phenotypic identification occurred. A single colony of a suspicious isolate from the original or pure culture plates was processed by either a direct smearing treatment or protein extraction, as described previously (23, 29, 30). Identification by MALDI-TOF MS was performed on a Microflex LT instrument (Bruker Daltonics, Germany) according to the manufacturer's instructions. Briefly, spectra were collected using the FlexControl 3.0 software in the linear positive mode in the mass range of 2 to 20 kDa (laser frequency, 20 Hz; ion source 1 voltage, 20 kV; ion source 2 voltage, 18.4 kV; lens voltage, 9.1 kV). Before the specimens were processed, the Bruker bacterial test standard (BTS 255343) was measured for the calibration of the instrument. The automated data analysis of raw spectra was performed with the MALDI Biotyper 2.0 software (Bruker Daltonics) using a library of 3,476 entries, with default settings (23, 29, 30). An identification log (score) value for each specimen was given, ranging from 0 to 3, that indicated the pattern-matching extent according to the specifications of the Biotyper system. Scores of 0 to 1.699 generally indicated no reliable identification; scores of 1.700 to 1.999 indicated probable genus identification; scores of 2.000 to 2.299 indicated secure genus identification, probable species identification; and scores of 2.300 to 3.000 indicated highly probable species identification.

Statistical analysis. The identification results for each isolate obtained from the Biotyper system were compared to those obtained from phenotypic methods. The score values between the original isolates, the pure-culture isolates, and extraction treatment were contrasted using χ^2 (variance analysis) and *t* tests.

TABLE 1. Genus-level identification of pathogenic and nonpathogenic enteric flora

Phenotypic identification	No. of isolates identified by phenotypic methods	No. of isolates identified by Biotyper	Correction rate (%)
Enteric pathogen	68	26	38.2
Normal enteric flora	236	233	98.7
Total	304	259	85.2

Odds ratios (OR), 95% confidence intervals (CI), and *P* values were calculated, and *P* ≤ 0.05 was considered statistically significant.

RESULTS

During the study period, a total of 605 stool specimens were submitted for bacterial culture and 309 suspicious colonies/isolates from 284 patients were selected and further identified by routine phenotypic methods. Among the 309 isolates, five normal enteric flora isolates were not identified by the phenotypic methods and were excluded from further analysis. Of the remaining 304 isolates, 68 (22.4%) were identified as pathogenic enteric isolates, including 22 (7.2%) *Salmonella* species, 39 (12.8%) *Shigella* species, 3 (1.0%) EHEC isolates, 2 (0.7%) *Yersinia enterocolitica* isolates, and 2 (0.7%) *Campylobacter* species. The remaining 236 (77.6%) were identified as nonpathogenic normal enteric flora. Specimen processing and identification by the Biotyper system of the 304 isolates were performed on colonies grown on selective medium and on subculture plates. Compared to the phenotypic methods, the Biotyper system correctly identified 259 isolates to the genus level, giving an overall concordance rate of 85.2%. The genus-level concordance rates were 38.2% in 68 pathogenic enteric isolates and 98.7% in 236 normal flora enteric isolates (OR, 125.5; 95% CI, 33.9 to 549.3; *P* = 0.0000) (Table 1). The identification results were concordant when the test was performed on samples processed by direct smearing and by additional protein extraction. When the direct-smearing treatment was used, a single sample could be identified in approximately 10 min.

Of the 68 pathogenic enteric bacterial isolates, the Biotyper system correctly identified 22 *Salmonella* species, 2 *Y. enterocolitica* isolates, and 2 *Campylobacter* species, but it misidentified 39 *Shigella* species and 3 EHEC isolates as *E. coli*. There were no significant differences between the mean scores generated between isolates on selective medium and subcultured plates by the Biotyper system (*P* > 0.05) (Table 2). The score-oriented dendrogram for randomly selected *Shigella*, EHEC, and *E. coli* isolates indicated that the MALDI-TOF MS-based characteristic spectra of the three enteric pathogens were highly similar and equally distributed (Fig. 1).

Of the 236 nonpathogenic normal enteric flora isolates, 233 (98.7%) and 228 (96.6%) were correctly identified by the Biotyper system into genus and species levels, respectively, compared to results of the routine phenotypic identification methods. *E. coli* was identified in 94 (39.8%) of 236 normal enteric flora isolates. These normal enteric flora isolates, derived from 17 genera, had relatively low identification rates in *Citrobacter*, *Enterobacter*, and *Proteus* at the species level (Table 3). There

TABLE 2. Identification of 68 pathogenic enteric bacteria by the Biotyper on colonies from original selective and subculture plates

Phenotypic identification	No. detected	Detection directly from selective medium		Detection from subculture medium		P
		Biotyper identification	Score (means $\pm$ SD)	Biotyper identification	Score (means $\pm$ SD)	
<i>Campylobacter</i> spp.	2	<i>Campylobacter</i> spp.	2.069 $\pm$ 0.060	<i>Campylobacter</i> spp.	2.230 $\pm$ 0.010	>0.05
<i>Salmonella</i> spp.	22	<i>Salmonella</i> spp.	2.240 $\pm$ 0.304	<i>Salmonella</i> spp.	2.340 $\pm$ 0.127	>0.05
<i>Yersinia enterocolitica</i>	2	<i>Yersinia enterocolitica</i>	2.360 $\pm$ 0.070	<i>Yersinia enterocolitica</i>	2.170 $\pm$ 0.120	>0.05
EHEC	3	<i>E. coli</i> ^a	2.219 $\pm$ 0.028	<i>E. coli</i> ^a	2.327 $\pm$ 0.108	>0.05
<i>Shigella sonnei</i>	32	<i>E. coli</i> ^a	2.176 $\pm$ 0.134	<i>E. coli</i> ^a	2.063 $\pm$ 0.183	>0.05
<i>Shigella</i> spp.	7	<i>E. coli</i> ^a	2.054 $\pm$ 0.141	<i>E. coli</i> ^a	2.063 $\pm$ 0.109	>0.05

^a These pathogens were misidentified as *E. coli* by the Biotyper system.

were no significant differences between the mean scores generated between isolates on selective medium and subcultured plates by the Biotyper system (data not shown). High concordance rates between the Biotyper system and phenotypic methods were revealed in normal enteric flora from isolates directly selected from colonies grown on selective media. Among a total of 304 suspicious colonies identified, 139 (45.7%) non-*E. coli* enteric normal flora isolates were identified by the Biotyper system, which can be ruled out as nonpathogenic normal enteric flora and thus shortens test turnaround time by 2 to 3 days.

DISCUSSION

Stool culture-based enteric bacterial pathogen detection and identification is a time-consuming process with an estimated cost of \$952 to \$1,200 per positive culture (4, 11, 16, 33, 34). Current stool culture procedures for the detection and identification of enteric bacterial pathogens require 3 to 5 days. Providing a rapid, accurate, specific, and economic identification or screening method of enteric bacterial pathogens is essential for the diagnosis and treatment of infectious diarrheal illnesses (38–40).

In this preliminary report, we describe the use of a MALDI-TOF MS-based Biotyper system for the rapid identification of common enteric bacterial pathogens seen in middle Tennessee. It should be noted that common enteric bacterial pathogens for middle Tennessee do not include *Vibrio* species, as these might be seen as stool pathogens in patients from the Gulf Coast states. The Biotyper system has been reported to be able to identify *Vibrio* species (19). Additional studies using laboratories in the Gulf Coast states need to be analyzed for the accuracy of Biotyper results for *Vibrio* species. Other geographic differences in stool pathogens will require further studies as well. Moreover, it should be noted that the identification of different EHEC types by the detection of shiga toxin rather than culture on MACS is not currently done at Vanderbilt. Given the difficulty the Biotyper system had in identifying *Shigella* and EHEC isolates, it must be assumed that different EHEC types would not be readily detected by the Biotyper system. With these limitations, there were no significant accuracy differences in Biotyper results obtained from bacterial colonies directly from selective stool culture media and, subsequently, from pure subculture plates. The Biotyper system correctly identified non-*E. coli* normal enteric flora, which oc-

FIG. 1. Log (score)-derived MSP dendrogram from *Shigella*, EHEC, and *E. coli* using the MALDI-TOF MS-based Biotyper system. The dendrogram was generated with the distance set at Euclidian and linkage set at completion (23).

TABLE 3. Identification of 236 nonpathogenic enteric flora colonies by the Biotyper directly from selective medium

Phenotypic identification	No. collected	No. (%) correctly identified to genus level	No. (%) correctly identified to species level
<i>E. coli</i>	94	94 (100.0)	94 (100.0)
<i>Acinetobacter</i> spp.	1	1 (100.0)	1 (100.0)
<i>Aeromonas</i> spp.	2	2 (100.0)	2 (100.0)
<i>Citrobacter</i> spp.	18	17 (94.4)	15 (83.3)
<i>Enterobacter</i> spp.	14	14 (100.0)	12 (85.7)
<i>Escherichia hermannii</i>	3	3 (100.0)	3 (100.0)
<i>Edwardsiella tarda</i>	1	1 (100.0)	1 (100.0)
<i>Hafnia alvei</i>	8	8 (100.0)	8 (100.0)
<i>Klebsiella pneumoniae</i>	9	9 (100.0)	9 (100.0)
<i>Leclercia adecarboxylata</i>	1	1 (100.0)	1 (100.0)
<i>Morganella morganii</i>	19	19 (100.0)	19 (100.0)
<i>Plesiomonas shigelloides</i>	1	1 (100.0)	1 (100.0)
<i>Proteus mirabilis</i>	24	24 (100.0)	24 (100.0)
<i>Proteus vulgaris</i>	7	7 (100.0)	6 (85.7)
<i>Providencia</i> spp.	9	9 (100.0)	9 (100.0)
<i>Pseudomonas</i> spp.	15	15 (100.0)	15 (100.0)
<i>Serratia marcescens</i>	7	7 (100.0)	7 (100.0)
<i>Vibrio parahaemolyticus</i>	1	1 (100.0)	1 (100.0)
<i>Chryseobacterium meningosepticum</i>	1	0 (0.0)	0 (0.0)
<i>Pantoea</i> spp.	1	0 (0.0)	0 (0.0)
Total	236	233 (98.7)	228 (96.6)

cupied 45.7% of the isolates in the study, directly from colonies grown on selective medium within 24 h after inoculation. The whole Biotyper identification procedure, from smear preparation to final result reporting, can be completed within 30 min. Compared to the current phenotypic identification, the Biotyper system shortened test turnaround time for identification by 2 to 3 days, providing a cost-effective screening tool to rapidly rule out half of the non-*E. coli* normal enteric flora in clinical settings (12, 36).

The coefficient variations of scores from different strains in the same species of bacterial pathogens and normal enteric flora were small, suggesting that the Biotyper system possessed satisfactory reproducibility results in our study. Recent inter-laboratory proficiency testing to identify 480 blind-coded non-fermenting Gram-negative bacterial isolates among eight international laboratories, including ours, achieved 98.8% reproducibility by using the Biotyper system. Only six samples were misidentified due to interchanges, contamination, or insufficient signal intensity (29). It was noticed in our study that excellent reproducible results were produced on colonies directly selected from the selective medium and subsequently selected from the subcultured pure plates. This indicated that the Biotyper system, when used as a screening tool, can be applied directly to suspicious colonies grown on original selective medium plates within 24 h of inoculation.

The Biotyper system identifies bacterial organisms based on the characterization of biomarker molecules, mainly redundant ribosomal protein with mass spectra for a molecular mass range from 2 to 20 kDa (1, 7, 30, 37). This number of peaks has been shown to be sufficient for bacterial identification at the genus and species levels for many enteric bacterial pathogens, such as *Campylobacter*, *Salmonella*, *Yersinia*, *Vibrio*, and *Listeria* (3, 5, 9, 19, 28). The Biotyper system demonstrated polar-

ized capacities in identifying *Salmonella*, *Shigella*, and EHEC. Our study correctly identified all 22 *Salmonella* species, which was consistent with findings reported previously (9). In contrast, all *Shigella* and EHEC isolates included in our study were misidentified as *E. coli* by the Biotyper database. The misidentification of *Shigella* species such as *S. sonnei* as *E. coli* was reported previously due to the absence of *S. sonnei* in the database (12, 36). Our main spectrum projection (MSP) dendrogram covering *Shigella* species, EHEC, and *E. coli* showed that the characteristic mass spectra of these three enteric pathogens were highly similar and cannot be distinguished (Fig. 1). These data suggested that the discriminatory ability based on the redundant ribosomal proteins was limited and cannot be used to differentiate *Shigella* and EHEC from *E. coli* (12). Targeting other pathogen-specific biomarkers has been used for EHEC identification by MALDI-TOF MS (13). Sensitive approaches that directly target functional *Shigella* and EHEC toxins by incorporating an immunoaffinity isolation (10, 35) or an enzymatic digestion (2, 20) are under investigation.

In summary, the Biotyper system provides a rapid and simple screening tool directly from suspicious bacterial colonies grown on selective medium for stool culture. With the Biotyper system implemented, approximately half of the normal enteric flora isolates can be ruled out to speed up the identification process. The other half of the isolates incorrectly identified as *E. coli* could be further identified by other routine or advanced identification techniques incorporated into the Biotyper system. Finally, additional studies are needed to evaluate geographic differences in stool pathogens.

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