

Rapid Identification and Differentiation of Non-O157 Shiga Toxin–Producing *Escherichia coli* Using Polymerase Chain Reaction Coupled to Electrospray Ionization Mass Spectrometry

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Abstract

A polymerase chain reaction (PCR)–mass spectroscopy assay was developed to identify non-O157 Shiga toxin–producing *Escherichia coli* (STEC) with Plex-ID biosensor system, a platform identifying short PCR amplicons by specific base compositions. This assay simultaneously amplifies five fragments of two housekeeping genes, two subunits of *stx2* gene, and four other virulence genes of STEC. A total of 164 well-characterized STEC isolates were examined with the assay to build a DNA base composition database. Another panel of 108 diverse STEC isolates was tested with the established database to evaluate the assay's identification capability. Among the 108 isolates, the assay specificity was 100% for three (*stx1*, *eae*, and *aggA*) out of five tested virulence genes, but 99% for *stx2* and 96% for *hlyA*, respectively. Main *stx1/stx2* subtypes and multiple alleles of *stx1/stx2* could be differentiated. The assay successfully identified several clinically significant serotypes, including O91:H14, O103:H25, O145:H28/NM, O113:H21, and O104:H4. Meanwhile, it was able to group isolates with different levels of pathogenic potential. The results suggest that this high-throughput method may be useful in clinical and regulatory laboratories for STEC identification, particularly strains with increased pathogenic potential.

Introduction

NON-O157 SHIGA TOXIN–PRODUCING *Escherichia coli* (STEC) are emerging pathogens associated with foodborne illnesses and outbreaks (Hughes *et al.*, 2006). The “top six” O serogroups (O26, O111, O103, O145, O121, and O45) accounted for about 71% of all human non-O157 isolates in the United States from 1983 to 2002 (Brooks *et al.*, 2005). All STEC strains produce one or more Shiga toxins (Stx1 and Stx2), which are encoded by *stx1* and *stx2*, respectively. The *stx1* gene has three subtypes (*stx1a*, *stx1c*, and *stx1d*), whereas *stx2* are more heterogeneous and include several subtypes: *stx2a*, *stx2b*, *stx2c*, *stx2dact*, *stx2e*, *stx2f*, and *stx2g* (Karmali *et al.*, 2010). Other virulence factors include intimin (*eae*) and enterohemolysin (*hlyA*). Aggregative adherence fimbriae I (*aggA*), an attribute of enteroaggregative *E. coli*, was harbored by the German outbreak *E. coli* O104:H4 strain (Frank *et al.*, 2011).

Identification and characterization of STEC isolates during foodborne outbreak investigations can help establish the link between clinical isolates and those from food or environmental samples, therefore outlining the possible source. Polymerase chain reaction (PCR) coupled to electrospray ionization mass spectrometry (PCR-MS) is a novel technique for microbial detection and identification (Ecker *et al.*, 2008). This technology uses mass spectrometry–derived base composition obtained from PCR amplicons of targeted genome fragments to identify microorganisms. The method is demonstrated to be fast and effective, with high-throughput detection capability (Ecker *et al.*, 2008). To date, it has been successfully applied to clinical microbial characterization (Lamson *et al.*, 2006; Sharma *et al.*, 2011). It can potentially be very useful in other fields, including food safety.

Previously, we developed a 96-well Plex-ID foodborne pathogen assay (FBP) to identify *Salmonella*, *Shigella*, *E. coli*, and *Listeria* using eight pairs of primers, of which six pairs targeted

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mutS and *mdh* genes in Gram-negative enteric bacteria, and the other two were specific for *L. monocytogenes* (Pierce *et al.*, 2012). The second version of FBP (Foodborne Bacteria v2) was developed in the present study by replacing primers irrelevant to *E. coli* with those that target major STEC virulence factors, including *stx1*, *stx2*, *eae*, *hlyA*, and *aggA*. The aim of this study was to (1) build an extensive database for STEC using a panel of well-characterized isolates with diverse genetic background, and (2) assess the identification abilities of the new assay.

Materials and Methods

Bacterial isolates

A collection of 272 well-characterized STEC isolates including 99 from humans, 103 from animals, and 70 from food were used, of which 10 were outbreak strains. In addition to our collection (Xia *et al.*, 2010; Ju *et al.*, 2012), these isolates were kindly provided by the Center for Veterinary Medicine of the U.S. Food and Drug Administration (FDA), U.S. Center for Disease Control and Prevention, and the STEC center at Michigan State University. Serotypes were initially determined by conventional serotyping and confirmed by molecular methods (Machado *et al.*, 2000; Ju *et al.*, 2012), whereas virulence genes were identified using molecular methods described previously (Beutin *et al.*, 2007; Zheng *et al.*, 2008; Xia *et al.*, 2010). Since *Shigella* produce Shiga toxin, and share high homology with *E. coli*, 56 *Shigella* (12 *S. flexneri*, 17 *S. dysenteriae*, 8 *S. sonnei*, and 19 *S. boydii*) from FDA were also included in the study.

Foodborne Bacteria v2 plate design

The layout of Foodborne Bacteria v2 plate (Abbott Molecular, Abbott Park, IL) is shown in Figure 1. The wells from rows A to E included primer pairs targeting *mutS* and *mdh*, and the wells in the last three rows contained primer pairs

targeting virulence genes: *stx1A* (*stx1* A subunit) and *stx2B* (*stx2* B subunit) in row F, *stx2A* (*stx2* A subunit) and EHEC *hlyA* in row G, and *eae* and *aggA* in row H. The 11 sets of forward/reverse primer sequences and the target gene segments are listed in Table 1. The Foodborne Bacteria v2 plate was preloaded with 0.2 mM deoxynucleoside triphosphates, 0.5 μ M PCR primers, 1 U of Immolase DNA polymerase in PCR buffer, and 100 copies of calibrant in each well.

PCR-MS

DNA template was extracted using the boiling method. Briefly, pure bacterial colonies grown overnight on freshly prepared LB agar were suspended in 500 μ L of sterile water. The bacterial suspension was heated at 100°C for 10 min in a dry heating block, and centrifuged at 10,000 $\times g$ for 5 min. A portion (10 μ L) of DNA supernatant was distributed into each well of the Foodborne Bacteria v2 plate for PCR assay (Eppendorf, Hauppauge, NY). The PCR conditions were as described previously (Pierce *et al.*, 2012). After amplification, the plate was loaded onto the Plex-ID system (Abbott Molecular) to determine the base composition of amplicons.

Data analysis

To build up the base composition database for target genes, 164 STEC isolates (59 from humans, 57 from animals, and 48 from food) with different serotypes and virulence gene profiles were characterized using the Foodborne Bacteria v2 plate, and the PCR amplicon data were analyzed and sorted. The isolates were differentiated based on base composition patterns of the PCR amplicons. The assay for STEC characterization was validated using an additional panel of 108 STEC isolates (40 from humans, 46 from animals and 22 from food) with known serotypes and virulence factors. The serotypes and virulence genes identified by PCR-MS were compared to those tested by conventional and

	Sample Lane s1	Sample Lane s2	Sample Lane s3	Sample Lane s4	Sample Lane s5	Sample Lane s6	Sample Lane s7	Sample Lane s8	Sample Lane s9	Sample Lane s10	Sample Lane s11	Sample Lane s12	
	1	2	3	4	5	6	7	8	9	10	11	12	
<i>mutS</i>	A	4769	4769	4769	4769	4769	4769	4769	4769	4769	4769	4769	<i>E. coli</i> <i>Shigella</i> <i>Salmonella</i> Detection And Serotyping
	B	4770	4770	4770	4770	4770	4770	4770	4770	4770	4770	4770	
	C	4771	4771	4771	4771	4771	4771	4771	4771	4771	4771	4771	
	D	4774	4774	4774	4774	4774	4774	4774	4774	4774	4774	4774	
<i>mdh</i>	E	4775	4775	4775	4775	4775	4775	4775	4775	4775	4775	4775	
<i>Salmonella</i> T2110	F	5635	5635	5635	5635	5635	5635	5635	5635	5635	5635	5635	<i>E. coli</i> toxin/ virulence markers + <i>Salmonella</i> -specific serotype primers
EHEC <i>stx1A</i>		3604	3604	3604	3604	3604	3604	3604	3604	3604	3604	3604	
EHEC <i>stx2B</i>		5639	5639	5639	5639	5639	5639	5639	5639	5639	5639	5639	
<i>Salmonella</i> <i>stbD</i>	G	5634	5634	5634	5634	5634	5634	5634	5634	5634	5634	5634	
EHEC <i>stx2A</i>		3608	3608	3608	3608	3608	3608	3608	3608	3608	3608	3608	
EHEC <i>hlyA</i>		3618	3618	3618	3618	3618	3618	3618	3618	3618	3618	3618	
<i>Salmonella</i> <i>mdh</i> /Isolation Control	H	5051/4437	5051/4437	5051/4437	5051/4437	5051/4437	5051/4437	5051/4437	5051/4437	5051/4437	5051/4437	5051/4437	
EHEC <i>eae</i> / <i>E. coli</i> O104 <i>aggA</i>		3611/5641	3611/5641	3611/5641	3611/5641	3611/5641	3611/5641	3611/5641	3611/5641	3611/5641	3611/5641	3611/5641	

FIG. 1. Layout of the Foodborne Bacteria v2 plate. This plate was designed for characterization of Shiga toxin-producing *Escherichia coli* (STEC) and *Salmonella*. Only STEC strains were tested in this study. Numbers in the wells represent primer ID. The genes they targeted are listed on the left and also in Table 1. Some wells have multiple primer pairs targeting multiple gene segments. Each plate can accommodate 12 isolates, one isolate per column.

TABLE 1. PRIMER SEQUENCES, THE GENES TARGETED, AND THE SIZE OF AMPLICONS ON FOODBORNE BACTERIA v2 PLATE

Primer ID	Forward (5'–3')	Reverse (5'–3')	Gene targeted	Size of amplicons (bp)
4769	TATCACCGAAGGTCGCCACCC	TCGCATATAGGTACTTTTACCGCCCA	<i>mutS</i>	139
4770	TGGGCGGTAAAAGTACCTATATGCG	TCGACGCGGGTAAAAATACGGTC	<i>mutS</i>	125
4771	TATTTTTACCCGCGTCGGCGCAGC	TACCGCGTCCGATTTTCATCCATCA	<i>mutS</i>	140
4774	TACGACAAAAACAACTGTTTGGCGT	TCAGAATAGTCACGCCGGAGTG	<i>mdh</i>	139
4775	TCGGATCGGCAACCCTTTCTATG	TGAGAGAAGAAGCGGGCATACTG	<i>mdh</i>	139
3604	TGGTTACATTGTCTGGTGACAGTAGC	TAGTCAACGAATGGCGATTATCTGC	<i>stx1A</i>	96
5639	TGGGGTAAAATTGAGTTGTCCAAGTATAATG	TCATTCTGTCAACTGAGCACTTTG	<i>stx2B</i>	130
3608	TGGCCGTTATACTGAATTGCCATCATC	TCGCCAGTTATCTGACATTCTGGTTG	<i>stx2A</i>	91
3618	TCACCACAACCTTGACAACTTCTGC	TGTAGTGCTGAGAGAACTGTTCCG	<i>hlyA</i>	119
3611	TGGTTACAACATTATGGAACGGCAGAG	TCATAGAACGGTAATAAGAAGTC	<i>eae</i>	92
		CAGTGAAC		
5641	TCCGTTACAAATGATTGTCCTGTTAC	TCATAACCAGACCCTTGCACC	<i>aggA</i>	144

molecular serotyping methods (Machado *et al.*, 2000; Beutin *et al.*, 2007; Zheng *et al.*, 2008; Xia *et al.*, 2010; Ju *et al.*, 2012).

Results

Analysis of virulence factors

The base compositions of virulence genes of the 164 STEC isolates are summarized in Table 2. Three base compositions

for Shiga toxin 1 A subunit were observed among STEC isolates carrying subtype *stx1a*, *stx1c*, and *stx1a + stx1c*, respectively (Table 2). STEC carrying *stx2b*, *stx2e*, and *stx2g* were separated from each other and from those carrying other *stx2* subtypes based on the base composition of Shiga toxin 2 B subunit. Although *stx2a* was not differentiated from *stx2c* and *stx2dact*, these three subtypes were separated from other *stx2* subtypes. Additionally, multiple alleles of *stx2* genes could be

TABLE 2. BASE COMPOSITIONS OF SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* (STEC) VIRULENCE FACTORS AND IDENTIFICATION OF THEM BY FOODBORNE BACTERIA v2 PLATE USING POLYMERASE CHAIN REACTION COUPLED TO ELECTROSPRAY IONIZATION MASS SPECTROMETRY (PCR-MS)

Virulence factor	Gene/subtype (Number isolates in database ^a)	Base compositions ^b	Identification result ^c
<i>stx1A</i>	<i>stx1a</i> (92)	[A23 G27 C19 T27]	82 (82)
	<i>stx1c</i> (10)	[A22 G28 C19 T27]	4 (4)
	<i>stx1a + stx1c</i> (1)	[A23 G27 C19 T27] + [A22 G28 C19 T27]	1 (1)
	Negative (61)	Negative	21 (21)
<i>stx2B</i>	<i>stx2a/2c/2dact</i> (67)	[A42 G37 C20 T31] / [A44 G35 C21 T30] / [A45 G34 C23 T28]	26 (25) ^d
	<i>stx2b</i> (6)	[A44 G35 C22 T29]	4 (4)
	<i>stx2e</i> (1)	[A44 G36 C16 T34]	1 (1)
	<i>stx2g</i> (2)	[A42 G37 C17 T34]	1 (1)
	negative (88)	Negative	76 (76)
<i>stx2A</i>	Positive (76)	13 different base compositions	32 (32)
	Negative (88)	Negative	76 (76)
Multiple alleles of <i>stx2</i>	<i>stx2a + stx2dact</i> (2)	[A42 G37 C20 T31] ^e + [A23 G27 C20 T21] ^f and [A23 G26 C20 T22] ^f	1 (1)
	<i>stx2a + stx2dact</i> (1)	[A42 G37 C20 T31] ^e and [A44 G35 C21 T30] ^e + [A23 G27 C20 T21] ^f	1 (1)
	<i>stx2a + stx2dact</i> (1)	[A44 G35 C21 T30] ^e + [A23 G27 C20 T21] ^f and [A23 G27 C21 T20] ^f	0 (0)
<i>eae</i>	<i>eae</i> positive (85)	[A25 G22 C15T 30]	81 (81)
	<i>eae</i> negative (79)	Negative	27 (27)
<i>aggA</i>	<i>aggA</i> positive (4)	[A46 G27 C31 T40]	2 (2)
	<i>aggA</i> negative (160)	Negative	106 (106)
<i>hlyA</i>	<i>hlyA</i> positive (98)	Four different base compositions	81 (79) ^g
	<i>hlyA</i> negative (66)	Negative	27 (25) ^h

^aNumber of isolates used to build the database for each virulence gene/subtype.

^bThe base compositions for virulence genes/subtypes from column 1, and they were obtained from the 164 STEC isolates in the database.

^cIdentification of the virulence factors using 108 STEC isolates. Numbers before parentheses represent number of isolates carrying respective virulence gene/subtypes in Column 2 by previous molecular methods. Numbers in parentheses represent the actual number of isolates that obtained the base compositions in Column 3, respectively.

^dOne *stx2a/2c/2dact* was not amplified and thus not identified by PCR-MS.

^eBase compositions of *stx2B*.

^fBase compositions of *stx2A*.

^gTwo *hlyA* were not amplified by PCR-MS and thus were false negatives.

^hThe PCR-MS detected *hlyA* in the two isolates, which were negative for *hlyA* by previous methods, and thus were false positives.

differentiated; for instance, all isolates that harbored *stx2a* + *stx2dact* had two different base compositions of *stx2B* or *stx2A*. All 85 *eae*-positive isolates had the same base composition of *eae*. Four O104:H4 strains linked to the German outbreak in 2011 exhibited base compositions of *aggA*, whereas the *aggA*-negative isolates did not. Four types of base compositions were obtained among 98 *hlyA*-positive isolates (Table 2).

In the assay validation study with 108 STEC isolates, the overall specificity was 100% for identifying *stx1*, *eae*, and *aggA*, 99% (107/108) for *stx2*, and 96% (104/108) for *hlyA* (Table 2). In comparison, the PCR-MS results were in agreement with those obtained by other molecular testing methods for *stx1* subtypes (*stx1a* and *stx1c*) and *stx2* subtypes (*stx2a*/*2c*/*2dact*, *stx2b*, *stx2e*, and *stx2g*), with a single exception that the sequence of one strain carrying *stx2a*/*2c*/*2dact* was not amplified and thus failed in identification by PCR-MS. A similar agreement between PCR-MS and previous testing methods was also found in one isolate carrying multiple alleles of *stx1* subtype (*stx1a* + *stx1c*) and two isolates carrying *stx2* subtype (*stx2a* + *stx2dact*). However, when PCR-MS was applied for *hlyA* gene identification, two previously positive isolates were tested negative by PCR-MS, and two negative isolates were reported positive (Table 2).

Differentiation of STEC isolates in the database

The Foodborne Bacteria v2 plate showed significant improvement over the previous FBP assay in discriminating STEC (Fig. 2). Based on the FBP assay, the 164 isolates were grouped to 24 base composition patterns, some of which were unique to serotypes O91:H14, O103:H25, O113:H21, O145:H28/NM, and O15:H27. In contrast, the new plate further classified the above 24 patterns into 81 new ones, due to additional information from the new virulence factor amplicon targets (Fig. 2). All O26:H11/NM, O111:H11/H8/NM, and O103:H11 belonged to patterns 1a–1e, all O45:H2/NM and O103:H2/NM to patterns 2a–2d, and O121:H19/NM to patterns 3a–3d. Isolates showing these patterns all had *eae*, and could be separated from *eae*-negative ones, like those belonging to patterns 1f–1r, 2e–2i, and 3e–3g, respectively. As the prevalence of *eae* was high in human and animal isolates but low in food isolates, most human (49/59) and animal isolates (38/57) belonged to *eae*-positive patterns (1a–1e, 1o, 2a–2d, 3a–3d, 4, 7, and 9), whereas only one food isolate (1/48) belonged to the same pattern group. Strains that caused severe diseases and outbreaks belonged to base composition patterns 1a, 1c, 1o, 2a, 2c, 3a, 3c, 4a, 4b, 4e, 13b, and 13c. It is noteworthy that pattern 1o was unique by including four O104:H4 German outbreak strains with both *stx2a* and *aggA*. Furthermore, isolates within a specific serotype could be further divided into several subgroups; for instance, isolates within serotypes O26:H11, O111:H11, O103:H2, O45:H2, O121:H19, and O128:H2 showed different base composition patterns, revealing difference in virulence potential of these subgroups.

Identification of serotypes/serogroups

The capability of PCR-MS assay for STEC serotyping is illustrated in Table 3. All isolates of serotypes O91:H14, O103:H25, O113:H21, O145:H28/NM, and O104:H4 were correctly identified according to its unique PCR-MS signature of each serotype. However, other single base composition

patterns may encompass multiple possible serotypes, as observed for isolates belonging to serotypes O26:H11/NM, O111:H11/H8/NM, O103:H11, O45:H2/NM, O103:H2/NM, and O121:H19/NM. All of the tested isolates belonging to serotypes O26:H11/NM, O111:H11/H8/NM, O103:H11, O45:H2/NM, O103:H2/NM, and O121:H19/NM were correctly identified as belonging to their respective serotype groups, though not uniquely. One exception was one O45:H2 isolate; it was misidentified as O128:H16 (pattern 3e) instead of patterns 2a–2d, which included all other O45:H2/NM isolates in the database. One O118:H16 isolate was identified as pattern 3g instead of pattern 1a, which included the other O118:H16 isolate in the database. One isolate of O104:H21, one O55:H7, and five isolates with unknown serotypes had new patterns and thus were not identified by PCR-MS based on the current database.

Identification of *Shigella* isolates

The PCR-MS results for all *Shigella* isolates indicated that they were not STEC, since they were negative for the virulence factors of STEC. Although PCR-MS identified three *stx1a* for three *S. dysenteriae* type 1 isolates, the *S. dysenteriae* type 1 isolates could be differentiated from STEC by distinct MS profile of *mdh* and *mutS* (data not shown). This indicated that the Foodborne Bacteria v2 plate could differentiate *Shigella* from STEC.

Discussion

Recent advances in PCR-electrospray ionization (ESI) using mass spectrometers have enabled the identification of bacterial species, subspecies, and even genotyping with a high degree of resolution (Sauer and Kliem, 2010). While the previous FBP assay could identify *E. coli* O157:H7 and its evolutionary precursor O55:H7, based on their unique MS profiles of *mutS* and *mdh* (Pierce *et al.*, 2012), the present study focused on non-O157 STECs. Unlike O157:H7, non-O157 STEC strains are highly diverse in genetic content and virulence. Currently, identification of non-O157 STEC, especially the pathogenic non-O157 STEC strains, is challenging. Among the numerous factors that are indicative of virulence, virulence gene and serotype are the two most important indicators. Thus, there is a need for the ability to determine the virulence genes/subtypes and serotypes of non-O157 STEC before more discriminative methods such as pulsed-field gel electrophoresis are applied for accurate typing.

The current plate was able to identify most of the virulence genes of non-O157 STECs successfully. The *stx1*, *eae*, and *aggA* genes were identified 100% of the time. The correct identification for *eae* has great clinical value, since *eae* carrying STEC isolates are considered to be highly pathogenic to humans (Werber *et al.*, 2003). One *stx2a*/*2c*/*2dact* was not identified by PCR-MS, and two false negatives (1.8%) and two false positives (1.8%) were observed for the *hlyA* gene. The false negatives may be due to the failed amplifications of gene targets in PCR-MS, and the false positives for *hlyA* may result from contamination, or because the primers used in this assay were different from those in previous methods. Detection of virulence genes using this plate has advantages in that it not only detected them, but also determined the subtypes for most of the targeted *stx* genes, but not for *eae*, for which primers were designed to target the conserved region. This is significant,

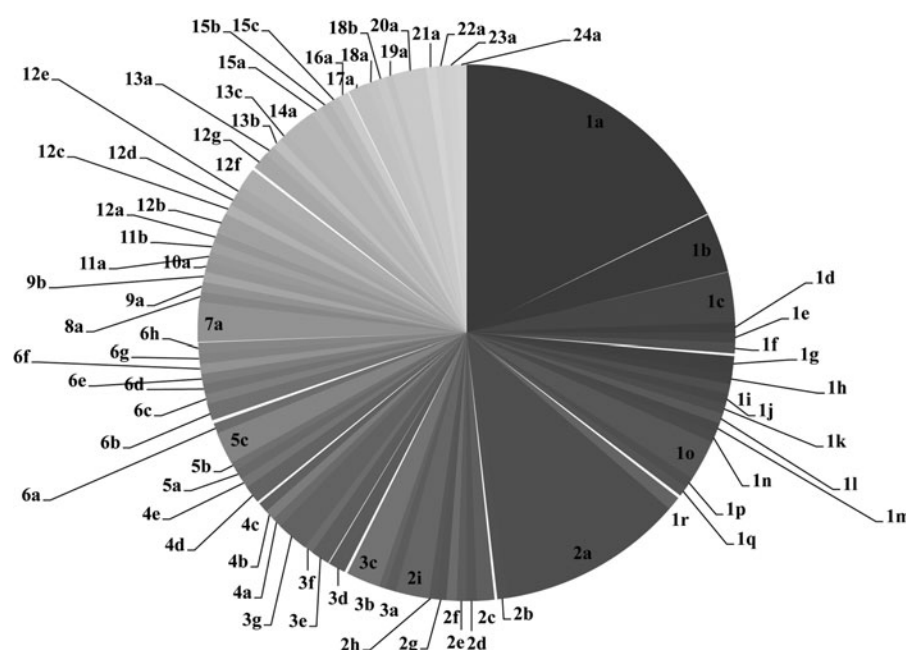


FIG. 2. The pie chart results of the 81 base compositions patterns of the 164 non-O157 Shiga toxin-producing *Escherichia coli* isolates by Foodborne Bacteria v2 plate. The patterns are obtained from the MS profiles of *mutS*, *mdh*, *stx1A*, *stx2B*, *stx2A*, *hlyA*, *eae* and *aggA*, and are shown by arabic numbers (1–24) correspond to the 24 foodborne pathogen assay (FBP) patterns that are based on the mass spectrometry profiles of *mutS* and *mdh*. Among them, each of the following serotypes—O91:H14 (Pattern 5), O103:H25 (Pattern 7), O113:H21 (Pattern 13), O145:H28/NM (Pattern 4), and O15:H27 (Pattern 18)—has its unique FBP pattern. The letters after the arabic numbers represent the subpatterns further divided by the additional virulence factors in the Foodborne Bacteria v2 plate. The area of each pie slice is proportional to the number of isolates within each pattern. The serotypes/serogroups and the number of isolates within each pattern are shown as follows. **Pattern 1a:** O26:H11 (13), O26:H2 (1), O26:NM (1), O103:H11 (1), O111:H11 (5), O111:H8 (6), O111:NM (1), O118:H16 (1); **Pattern 1b:** O111:H8 (1), O111:H11 (1), O26:H11 (4); **Pattern 1c:** O26:H11 (1), O111:H8 (3), O111:NM (1); **Pattern 1d:** O111:H8 (1); **Pattern 1e:** O26:H11 (1); **Pattern 1f:** O5:NM (1); **Pattern 1g:** O128:H2 (1), OU:NM (1); **Pattern 1h:** O174:H8 (1); **Pattern 1i:** O128:H2 (1); **Pattern 1j:** unknown (1); **Pattern 1k:** O8:H16 (1); **Pattern 1l:** O128:H2 (1); **Pattern 1m:** unknown (1); **Pattern 1n:** unknown (1); **Pattern 1o:** O104:H4 (4); **Pattern 1p:** O104:H? (1); **Pattern 1q:** unknown (1); **Pattern 1r:** O8:H30 (1), O104:H27 (1). **Pattern 2a:** O45:H2 (4), O45:NM (1), O103:H2 (11), O103:NM (1), O103:H6 (1); **Pattern 2b:** O103:H2(1); **Pattern 2c:** O45:H2 (1), O103:H2 (1); **Pattern 2d:** O103:H2 (1); **Pattern 2e:** O104:H6 (1); **Pattern 2f:** O116:H21 (1); **Pattern 2g:** unknown (1); **Pattern 2h:** unknown (1); **Pattern 2i:** O104:H6 (1), O104:H21 (1), O104:H? (1). **Pattern 3a:** O121:H19 (1); **Pattern 3b:** O121:H19 (1); **Pattern 3c:** O121:H19 (2), O121:NM (1); **Pattern 3d:** O50:H7 (1), O121:H19 (1); **Pattern 3e:** O128:H16 (2); **Pattern 3f:** unknown (1); **Pattern 3g:** O9(1), O28 (2). **Pattern 4a:** O145:H28(1); **Pattern 4b:** O145:H28 (1); **Pattern 4c:** O145:H28 (1); **Pattern 4d:** O145:H28 (1), O145:NM (1); **Pattern 4e:** O145:H28 (1). **Pattern 5a:** O91:H14 (1); **Pattern 5b:** O91:H14 (1); **Pattern 5c:** O91:H14 (3). **Pattern 6a:** O121:H7 (1); **Pattern 6b:** O22:H8 (1), O83:H8 (1); **Pattern 6c:** O104:H21 (1); **Pattern 6d:** O104:H21 (1); **Pattern 6e:** unknown (1); **Pattern 6f:** O22:H8 (1); **Pattern 6g:** O83:H8 (1); **Pattern 6h:** O83:H8 (1). **Pattern 7a:** O103:H25 (4). **Pattern 8a:** O22:H5 (1). **Pattern 9a:** O55:H7 (1); **Pattern 9b:** O55:H7 (1). **Pattern 10a:** O2:H27 (1). **Pattern 11a:** O111:H2 (1); **Pattern 11b:** O8:H2 (1). **Pattern 12a:** O88:H38 (1); **Pattern 12b:** O46:H38 (1), O91:H21 (1); **Pattern 12c:** O146:H21 (1); **Pattern 12d:** O88:H49 (1); **Pattern 12e:** O168:H8 (1); **Pattern 12f:** O91:H7 (1), O128:H2 (1); **Pattern 12g:** O174:H36 (1), O128:H2 (1). **Pattern 13a:** O113:H21 (1); **Pattern 13b:** O113:H21 (1); **Pattern 13c:** O113:H21 (1). **Pattern 14a:** O8:H28 (1), O8:H? (1), unknown (1). **Pattern 15a:** O128:H45 (1); **Pattern 15b:** O128:H45 (1); **Pattern 15c:** unknown (1). **Pattern 16a:** unknown (1). **Pattern 17a:** O5:NM (1), O125:NM (1). **Pattern 18a:** O15:H27 (1); **Pattern 18b:** O15:H27 (1). **Pattern 19a:** O126:H8 (1). **Pattern 20a:** O104:H7 (1), unknown (2). **Pattern 21a:** O36:H14 (1). **Pattern 22a:** O73:H18 (1). **Pattern 23a:** O2:H25 (1). **Pattern 24a:** unknown (1).

since these subtypes play an important role in causing human diseases. Although the assay was not able to discriminate *stx2a* from *stx2c* or *stx2dact*, it successfully identified and differentiated them from other *stx2* subtypes. This has important clinical value, because the three *stx2* subtypes are often associated with hemorrhagic colitis and hemolytic-uremic syndrome (Eklund *et al.*, 2002; Friedrich *et al.*, 2002; Kallquist *et al.*, 2010). Another advantage of this assay is the successful identification of multiple alleles of *stx1* and *stx2*. PCR/restriction fragment length polymorphism is currently a preferred tool for investigating multiple *stx* subtypes (Beutin *et al.*, 2007; Gobius *et al.*, 2003), but is time consuming and

labor intensive (Beutin *et al.*, 2007). PCR-MS is highly automated, fast, and high throughput, and thus could provide a more accurate, effective, and timely response in outbreaks of foodborne illness.

The plate was able to identify most of the non-O157 serotypes. The unique identification of serotypes O91:H14, O103:H25, O113:H21, and O145:H28/NM is significant, since these serotypes have been previously reported in humans and some have caused severe diseases including bloody diarrhea and hemolytic-uremic syndrome (Paton *et al.*, 1999; Karmali *et al.*, 2003; Rivas *et al.*, 2006; Frank *et al.*, 2011). The plate also successfully identified *E. coli* O104:H4 strains by recognizing

TABLE 3. IDENTIFICATION OF SEROTYPES OF THE 108 NON-O157 SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* (STEC) BY FOODBORNE BACTERIA v2 PLATE

	Number of isolates tested	PCR-MS ID ^a
O26:H11	20	1a(17); 1b(3)
O26:NM	1	1a(1)
O103:H11	1	1a(1)
O111:H11	7	1a(6); 1b(1)
O111:H8	13	1a(8); 1b(1); 1c(4)
O111:NM	2	1a(1); 1c(1)
O128:H2	1	1g(1)
O128:H2	1	1i(1)
O104:H4 ^b	2	1o(2)
O45:H2	4	2a(3); 3e(1)
O45:NM	1	2a(1)
O103:H2	20	2a(16); 2b(4)
O121:H19	3	3a(1); 3c(2)
O121:NM	1	3c(1)
O128:H16	2	3e(2)
O118:H16	1	3g(1)
O28	1	3g(1)
O145:H28 ^b	3	4a(2); 4d(1)
O91:H14 ^b	3	5b(1); 5c(2)
O83:H8	3	6b(2); 6h(1)
O104:H21	1	Unidentified
O103:H25 ^b	3	7a(3)
O113:H21 ^b	2	13b(1); 13c(1)
O55:H7	1	Unidentified
Unknown ^c	11	1j(1); 1m(1); 1k(1); 1r(2); 2g(1); unidentified (5)

^aPolymerase chain reaction coupled to electrospray ionization mass spectrometry identification (PCR-MS ID) is shown by the base composition pattern by Foodborne Bacteria v2 plate. The possible serotype(s) within each pattern are illustrated in the legend for Figure 2. The number of isolates identified to belong to each base composition pattern is shown in parentheses. Bold results indicate that these isolates were identified to the correct serotypes/serotype groups (base composition patterns) by PCR-MS.

^bThese serotypes were uniquely identified due to the unique FBP pattern (based on MS profiles of *mutS* and *mdh*) of each serotype.

^cThe serotypes of these 11 STEC isolates were unknown but did not belong to the several common clinically relevant O serogroups listed above. Their virulence gene information was known and therefore they were used mainly for identification of virulence genes/subtypes.

two co-existing virulence factors: the *aggA* and *stx* genes. As for the clinically relevant serotypes O26:H11/NM, O111:H11/H8/NM, O103:H11, O45:H2/NM, O103:H2/NM, and O121:H19/NM within the “top six” O serogroups, although it was common to observe more than one serotype listed as a possible identification, reducing the list from thousands down to a few possible serotypes is very useful. That information could easily be used to narrow focus during outbreak investigations. This assay has the potential to significantly reduce the time required to characterize STECs and greatly improve the response time for outbreaks. The misidentification of some serotypes indicates that expanding the current reference database will enhance the potential of this assay to provide accurate serotype information of STEC.

Most existing methods for the identification of pathogenic non-O157 STEC from food are based on primers and probes for specific clinically relevant O serogroups (Fratamico *et al.*, 2011; Lin *et al.*, 2011). Since the O and H antigen gene seg-

ments evolve rapidly, newly emerging clinically relevant serotypes might not be readily detected by this approach if the primer regions are different from those in the serotypes that have already been known to cause diseases (Moxon *et al.*, 1994; Sasaki 1994; Pierce *et al.*, 2012). However, the emerging clinically relevant serotypes can be screened out by the current assay, because it uses broad-range primers to target the housekeeping genes that which are present in all *E. coli* strains. Furthermore, this assay can divide STEC isolates into multiple groups that may have different levels of virulence potential. If unknown isolates were identified to be within the groups that included the outbreak strains or strains that have caused diseases, they may have high pathogenic potential.

Future plates that can include primers targeting extra housekeeping genes, and virulence factors of STEC would improve their discriminatory power and could become very promising if applied to molecular risk assessment of STEC strains, and to facilitate real-time monitoring and outbreak investigation. In addition, the expansion of the current reference library will also help improve the accuracy of calls from the instrument. The database will also facilitate rapid information sharing between clinical microbiology laboratories, public health laboratories, and biodefense agencies. The main drawback of the current PCR-MS is high equipment cost, but this could be reduced over time if large numbers of samples are tested. In addition, this technology continues to evolve, with yearly performance increases and cost reductions.

Conclusions

This work reports that the Foodborne Bacteria v2 plate could uniquely identify some clinically relevant STEC serotypes and most major virulence genes and subtypes. Although several possible serotypes were identified for an isolate, the assay helped to narrow down the isolates to a few possible serotypes. With the expansion of the current database and development of extra target genes in the future, the accuracy of identification will be improved. Furthermore, the method can help identify STEC isolates with pathogenic potential, and is very promising in becoming a tool for molecular risk assessment of STEC. The set of plates developed for identification of general foodborne pathogens (FBP for instance), and for particular pathogens (this Foodborne Bacteria v2 plate) may facilitate foodborne bacterial outbreak investigations. Most importantly, the data could be easily shared between microbiology laboratories around the world. Therefore, the PCR-MS assay is a reproducible, rapid, and high-throughput method for STEC detection and identification, and it is a very promising tool for food safety, clinical diagnosis, and epidemiological studies, among others.

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Disclosure Statement

No competing financial interests exist.

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