High-Throughput Molecular Determination of *Salmonella enterica* Serovars by Use of Multiplex PCR and Capillary Electrophoresis Analysis[▽]

Brandon T. Leader,¹ Jonathan G. Frye,² Jinxin Hu,¹ Paula J. Fedorka-Cray,² and David S. Boyle^{1*}

Washington State Department of Health, Public Health Laboratories, Shoreline, Washington,¹ and Bacterial Epidemiology and Antimicrobial Research Unit, U.S. Department of Agriculture, Athens, Georgia²

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Salmonella enterica is a leading cause of food-borne illness worldwide and is also a major cause of morbidity and mortality in domestic and wild animals. In the current study, a high-throughput molecular assay was developed to determine the most common clinical and nonhuman serovars of *S. enterica* in the United States. Sixteen genomic targets were identified based on their differential distribution among common serovars. Primers were designed to amplify regions of each of these targets in a single multiplex PCR while incorporating a 6-carboxyfluorescein-labeled universal primer to fluorescently label all amplicons. The fluorescently labeled PCR products were separated using capillary electrophoresis, and a *Salmonella* multiplex assay for rapid typing (SMART) code was generated for each isolate, based upon the presence or absence of PCR products generated from each target gene. Seven hundred fifty-one blind clinical isolates of *Salmonella* from Washington State, collected in 2007 and previously serotyped via antisera, were screened with the assay. A total of 89.6% of the isolates were correctly identified based on comparison to a panel of representative SMART codes previously determined for the top 50 most common serovars in the United States. Of the remaining isolates, 6.2% represented isolates that produced a new SMART code for a previously determined serotype, while the final 8.8% were from serotypes not screened in the original panel used to score isolates in the blinded study. This high-throughput multiplex PCR assay allowed simple and accurate typing of the most prevalent clinical serovars of *Salmonella enterica* at a level comparable to that of conventional serotyping, but at a fraction of both the cost and time required per test.

Infection with *Salmonella* in humans and animals primarily causes self-limiting gastrointestinal infections with mild to moderate symptoms, including fever, abdominal cramps, and diarrhea (26). More severe clinical outcomes, including death, may occur in cases of bacteremia or enteric fever (typhoid), which is often characterized by severe headaches and high fever but no diarrhea (5). Humans with typhoid may also become asymptomatic carriers who are capable of spreading the disease either through direct human contact or via fecal contamination of food. However, the most common mode of transmission for the majority of *Salmonella* infections in humans occurs through the consumption of contaminated food-stuffs and water. Recent estimates from the Food-Borne Diseases Active Surveillance Network of the Centers for Disease Control and Prevention (CDC) suggest that approximately 1.4 million cases of salmonellosis occur annually in the United States, resulting in 15,000 hospitalizations and 400 deaths (47). Furthermore, estimates of the economic impact of *Salmonella* infections in the United States suggest that annual expenditures due to lost productivity and medical care may be up to \$2.3 billion (22).

Salmonella bongori and *S. enterica* are the two species that comprise the genus *Salmonella*. *S. enterica* is further divided

into six subspecies, namely, *enterica* (I), *salamae* (II), *arizonae* 5 (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI). *S. enterica* subsp. *enterica* strains are of the greatest clinical relevance and are typically isolated from humans and warm-blooded animals. Strains belonging to one of the other five *S. enterica* subspecies and *S. bongori* are associated with environmental or reptilian sources (10, 23a). Serologic classification of *Salmonella* strains based upon properties of various surface polysaccharide (O) and flagellar (H) antigens is the reference method for epidemiologic surveillance. This method involves the characterization of over 150 unique O and H antigens to produce an antigenic formula that can be scored using the Kauffman-White scheme to determine a serovar for an isolate (7, 23a). Currently, serotyping classifies over 2,500 serovars of *Salmonella*, of which over 1,400 belong to *S. enterica* subsp. *enterica* (7, 12). Although serotyping using the Kauffman-White scheme remains the standard for serovar determination through its longstanding and widespread use, it is not without significant deficiencies. Aside from being labor-intensive and expensive, serotyping is also time-consuming to perform, often taking three or more days after receipt of a specimen for a highly trained laboratory technician to produce a result. Incomplete or incorrect serologic classification may occur due to atypical expression of an isolate's surface O or H antigen as in the case of mucoid strains in which the O antigen is obscured or for nonmotile and/or monophasic isolates for which only one flagellar phase antigen can be determined. Recent comparative genomic studies of common clinical serotypes have also revealed evidence of a high level of intraserovar variation

* Corresponding author. Present address: Program for Appropriate Technologies Diagnostics Group, 1455 NW Leary Way, Seattle, WA 98107. Phone: (206) 788-2032. Fax: (206) 285-6619. E-mail: dboyle@path.org.

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among isolates of some serovars (16, 29, 38). In other cases, some serovars have been shown to be highly similar genetically, which suggests that a "genovar" classification be adopted, based upon genetic relatedness. It is also proposed that genovar classification may be more appropriate than serovar classification (4, 39).

The deficiencies of conventional serotyping combined with the wealth of genomic information now available for *Salmonella* have led to the development of alternative molecular strategies to replace or complement conventional serotyping. A number of recent strategies have employed PCR-based approaches to determine different O and H antigens as a means to replace serologic identification of these antigens (14, 20, 25, 32). Others have proposed alternative strategies examining genetic differences as a means of identifying serovars, including ribotyping (17), pulsed-field gel electrophoresis (PFGE) (28), multiplex PCR (3, 4, 29), IS200 analysis (18, 46), random amplification of DNA polymorphisms (45), and DNA microarray analysis (38, 42).

For this study, a rapid, high-throughput multiplex PCR-based method was developed that allows for discrimination of the majority of common serotypes that are reported in the United States based upon their genetic differences. The gene targets are present in some serotypes but not others, as demonstrated by previous comparative genomic studies (19, 35, 38, 39). Primers for all 16 gene targets were designed for use in a single multiplex PCR, thus allowing for a reduction in costs, a decrease in turnaround time per test, and a lower rate of laboratory error. Additionally, the scoring of resulting amplicons was adapted for high-throughput testing by fluorescently labeling PCR products and employing capillary electrophoresis for separation and analysis. Aside from offering high sensitivity and accurate sizing of the PCR amplicons, DNA sequencing equipment is robust and already common to a large number of public health laboratories. The use of this typing method, especially in conjunction with serogrouping or PFGE, allows for serovar determination of *Salmonella* isolates at a level comparable to that of conventional serotyping, with considerable time and cost savings.

MATERIALS AND METHODS

Bacterial strains and DNA extraction. All *Salmonella* isolates examined in this study were from the Washington State Public Health Laboratory (WAPHL) strain collection or the U.S. Department of Agriculture (USDA) strain collection. Serologic determination according to the modified Kaufmann-White scheme was performed for all isolates (10, 12, 23a). A DNA template was prepared from each isolate by boiling 15 μ l of a colony suspension in a total volume of 75 μ l of 1 $\times$ TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) for 20 min at 100°C. Alternatively, DNA was extracted by melting agarose gel slices (1 mm by 2 mm) from PFGE plugs in 200 μ l 1 $\times$ TE buffer for 30 min at 100°C.

Primer design and PCR amplification. The method for selecting genomic targets used in the assay was described previously (30). Briefly, genetic loci were selected based upon their variability among isolates from different clinical *S. enterica* serovars but relative stability within isolates of the same serovar, according to the results of a previous study comparing genomic contents of different *S. enterica* serovars by use of a microarray containing the entire genomic complements of serovar Typhimurium LT2 (STM gene names) and serovar Typhi CT18 (STY gene names) (38). Primers were designed to amplify 12 discriminatory regions previously identified by Kim et al. (30). An additional gene target, STM3518, whose presence was also variable among different serovars by comparative microarray analysis, was added to the panel to improve the discriminatory power (38). Two universal *Salmonella*-specific genomic regions, STM1608 and STM0171, were identified in this study and used as internal DNA ampli-

cation controls. These targets were selected based upon their presence in all common *Salmonella enterica* serovars, as determined by comparative genomics using microarray analysis (38) and by BLAST sequence comparison of all available *Salmonella*-specific genome sequences (2). *Salmonella*-specific primers contained limited sequence similarity to non-*Salmonella* gene sequences by BLAST sequence analysis. Primers for amplification of the genomic region representing the phase 2 flagellin gene (*fljB*) were added later in the study as a means to discriminate between *Salmonella* serovar Typhimurium and *Salmonella* 4[5]12:i:- strains (1, 15).

muPlex primer software (40) was used to design primers amplifying all genomic targets in a single multiplex PCR (Table 1). For each primer pair, either the forward or reverse primer included a universal sequence complementary to a 6-carboxyfluorescein-linked primer used to label all amplicons (Table 1). A 10 $\times$ primer master mix was prepared to contain 1 μ M of each primer and the labeling probe. All PCRs were carried out in a final volume of 25 μ l containing 12.5 μ l of Immolase DNA polymerase 2 $\times$ master mix (Bioline, USA Inc., Randolph, MA), 2.5 μ l of 10 $\times$ primer master mix, 3 mM MgCl₂, and either 1 μ l of template DNA from boiled culture preparations or 2.5 μ l of DNA from heated agarose plugs. Thermocycling parameters were 94°C for 10 min; 25 cycles of 94°C for 30 s, 57°C for 90 s, and 72°C for 30 s; 72°C for 5 min; 15 cycles of 94°C for 30 s, 68°C for 90 s, and 72°C for 30 s; and 72°C for 5 min. Control reaction mixtures containing no template or genomic DNA from *Salmonella* serovar Typhimurium LT2, serovar Typhi CT18, or serovar Enteritidis PT4 were included with each sample run.

Capillary electrophoresis, sample scoring, and construction of a genovar database. PCR products were diluted 1:5 in molecular-grade water (Millipore, Billerica, MA), and 1 μ l of diluted PCR product was then added to 9 μ l of sequencing-grade formamide (Applied Biosystems, Foster City, CA) containing a carboxy-X-rhodamine-labeled GeneFlo 625 DNA ladder (CHIMERx, Milwaukee, WI) to make a final dilution of 1:50 (vol/vol). Samples were separated by capillary electrophoresis in an ABI 3130XL gene analyzer according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Genemapper software v3.5 (Applied Biosystems, Foster City, CA) was used to analyze the sizes of resulting PCR products. Scoring was based upon the presence of a PCR product that corresponded to the predicted amplicon size, as detected in control reactions with DNA from *Salmonella* serovar Typhimurium, *Salmonella* serovar Typhi, or *Salmonella* serovar Enteritidis. Each PCR product detected was given a number (1 through 16) based upon the size of the amplicon (Table 1). The amplicons detected for an isolate were then combined to create a *Salmonella* multiplex assay for rapid typing (SMART) code.

SMART codes were determined for the top 50 *Salmonella* serovars, which represented all serovars contained in both the top 30 clinical and nonhuman *Salmonella* serovars reported in the United States (Table 2) (12). Four hundred eighty-nine isolates from WAPHL and USDA, previously serotyped by conventional methods, were assayed via the PCR method and assigned SMART codes. For each serovar, a minimum of five isolates were screened, with the exception of *Salmonella* serovar Cerro (four isolates). All isolates within a serovar that were used for the database were nonclonal by PFGE analysis whenever possible.

Analysis of 751 blinded clinical isolates. The effectiveness of using a high-throughput multiplex typing method to determine the serovars of unknown isolates was assessed by screening all 751 *Salmonella* specimens submitted to the WAPHL in 2007. The results of serologic typing using the Kauffmann-White scheme were assumed to be 100% correct for the purpose of evaluating the accuracy of the PCR method. The representative SMART codes generated with the panel of the 50 most common serovars (described above and listed in Table 2) were used to designate putative serovars for *Salmonella* isolates by use of the PCR method. In some cases, new SMART codes were identified and provisionally assigned as described below. In addition, eight isolates which were only partially typed were also screened by this method.

PFGE analysis. PFGE was used to subtype each isolate, using a standard method with XbaI digestion previously described for *Escherichia coli* (23). For isolates for which the PCR result matched an amplicon code generated by multiple serovars, BioNumerics v 4.1 (Applied Maths, Austin, TX) was used to compare the similarity of the PFGE pattern of the isolate with other patterns of isolates within those serovars. Relatedness was calculated using the Dice similarity coefficient, with optimization and band position tolerance each set to 1.5%. In each case, the PFGE pattern of the isolate with an amplicon code matching multiple serovars was used as the reference pattern for comparison against the banding pattern of isolates from each of the possible serovars. Using more stringent criteria for scoring relatedness, patterns with a similarity of 100% were considered indistinguishable, patterns with 99 to 90% similarity were considered probably related, those with 89 to 80% similarity were considered possibly related, and those with 79% or less similarity were considered unrelated (44).

TABLE 1. Primers and probes used for high-throughput multiplex PCR determination of *Salmonella enterica* serotypes using capillary electrophoresis

SMART code no.	NCBI accession no.	Primer name ^a	Primer sequence (5'→3')	Amplicon size (±0.5 bp) ^b
		Universal probe sequence	CAGGAGCAGCTATGCCAGGACAGCTTGCGG	
1	AE008771	STM1608F	GTCGGCGAGCTTCACC	90
		STM1608 UR	CAGGAGCAGCTATGCCAGGACAGCTTGCGGTCTGTAGCAAAGCGCAGTT	
2	AE008735	STM0839UF	CAGGAGCAGCTATGCCAGGACAGCTTGCGGTCTGTAGCAAAGCGCAGTT	105
		STM0839UR	GCACTGGTTGCCCGTA	
3	AL627273	STY2296F	CTCTGTTGCAAACAGATGTTA	116
		STY2296UR	CAGGAGCAGCTATGCCAGGACAGCTTGCGGGATGGAGACGATAAGTTTACCAGTAT	
4	AL627273	STM2349F2	GGCGAGTTCTACGCAGAGATAA	120
		STM2349UR	CAGGAGCAGCTATGCCAGGACAGCTTGCGGCCAGCGAAATGTCACAGTGA	
5	AE008758	STM1350F	GGAACACATCTTGCCAGGT	137
		STM1350UR	CAGGAGCAGCTATGCCAGGACAGCTTGCGGCCGGCACAGAAGGAATG	
6	AE008913	STM4538F	CGGAATGATGGAAGCCTA	146
		STM4538UR	CAGGAGCAGCTATGCCAGGACAGCTTGCGGCTTCTCCGTACCAAACGTA	
7	AE008729	STM0716F	GGAAAGAAACCGCTGCTTA	151
		STM0716UR	CAGGAGCAGCTATGCCAGGACAGCTTGCGGTTGAGGCGCCGGATAT	
8	AE008879	STM3845F	GTGTTTGAAGATGATATAGCCAGTAT	168
		STM3845UR	CAGGAGCAGCTATGCCAGGACAGCTTGCGGTTCCGCTGAAGCAACAAT	
9	AL627273	STY0345F	GGTATGTCGTTCAAACAGGAAT	181
		STY0345UR	CAGGAGCAGCTATGCCAGGACAGCTTGCGGCACTGCCGAGCAGTATGAG	
10	AL627266	STY0311F	GGGCTTGCCGAGACAC	195
		STY0311UR	CAGGAGCAGCTATGCCAGGACAGCTTGCGGAAGGAGAGTCATAGCCCACAC	
11	AE008702	STM0171 F	GACCCCGGATTTTGTAGAA	206
		STM0171 UR	CAGGAGCAGCTATGCCAGGACAGCTTGCGGACCACGGAGAGACAGTTTTCAGAT	
12	AE008913	STM4525F	CAGGAGCAGCTATGCCAGGACAGCTTGCGGGAAGTCGTCCGCGGAT	221
		STM4525UR	CCAGGATCAGATGCAGTTCTAC	
13	AE008795	STM2150UF	CAGGAGCAGCTATGCCAGGACAGCTTGCGGAGCCTGCATAATCGCAAAGG	228
		STM2150UR	CATCAGCGACACGATAGTGAGA	
14	AE008862	STM3518F	CTAACCAAGTTGCGCATGA	244
		STM3518UR	CAGGAGCAGCTATGCCAGGACAGCTTGCGGCAAGATAGCCACTTTCGGTTG	
15	AF370716	SdfIF	GGCGATATAAGTACGACCATCATGG	257
		SdfIUR	CAGGAGCAGCTATGCCAGGACAGCTTGCGGGCACGCGGCACAGTTAAAA	
16	AE008826	STM2771F	CCATTGGATGTCTCACACC	264
		STM2771UR	CAGGAGCAGCTATGCCAGGACAGCTTGCGGGCAATTCTTGAAGAATTATCAGG	

^a U denotes a primer with the universal sequence.^b Based on ≥10 independent size measurements of the amplicon.

RESULTS

Discrimination of *Salmonella* serovars Typhimurium and 4[5]12:i:–. Due to the recent increase in the prevalence of *Salmonella* serovar 4[5]12:i:– (12) and studies indicating the absence of *fljB* in monophasic strains (1, 15), primers for the *fljB* gene (STM2771) were belatedly included in the multiplex PCR assay. Forty-eight clinical isolates of both *Salmonella* serovar Typhimurium and *Salmonella* serovar 4[5]12:i:– were tested with the multiplex PCR. An amplicon of *fljB* was observed for 100% of *Salmonella* Typhimurium isolates, while 98% (47 of 48 isolates) of *Salmonella* serovar 4[5]12:i:– isolates produced no amplification. The *Salmonella* serovar 4[5]12:i:– strain from which *fljB* was amplified was tested multiple times by conventional serotyping but never expressed the second flagellar antigen. This isolate was also capable of fermenting galactitol (formerly called dulcitol), which is common for *Salmonella* serovar Typhimurium but not *Salmonella* serovar 4[5]12:i:– (1), which could indicate that the isolate has a different mutation leading to the loss of the second-phase flagellar antigen (50).

Determination of multiplex PCR amplicon codes for common clinical human and animal *Salmonella* serovars. To develop the assay, 489 isolates identified by conventional serotyping were used to determine SMART codes for the top 50 most common *Salmonella* serovars (Table 2). For 43 of the 50 (86%) serovars tested, a single SMART code was produced for each serovar. Isolates of *Salmonella* serovars Agona, Paratyphi

B [including biovar *d*-tartrate (+)], Stanley, and Javiana each produced two different SMART codes, three codes were generated from serovar Reading, and serovars Poona and Havana each had four different SMART codes.

Twenty-nine of the 50 (58%) serovars screened in the panel resulted in completely unique SMART code identifiers, including *Salmonella* serovars Typhimurium, Enteritidis, 4[5]12:i:–, Montevideo, St. Paul, Heidelberg, and Javiana, which are among the top 10 most common clinical serovars isolated in the United States (12). Two of the typhoid-causing serovars, *Salmonella* serovars Typhi and Paratyphi A, also produced unique SMART codes. Twelve of the remaining 21 serovars in the panel shared a SMART code with one additional serovar, and 3 serovars shared a common SMART code with two other serotypes (Table 2). The SMART code 156-11-13 was shared among four isolates (*Salmonella* serovars San Diego, Reading, Brandenburg, and Chester).

Blinded comparison of high-throughput multiplex PCR typing with conventional serotyping. A total of 751 serotyped isolates of *Salmonella enterica* were screened in a blinded study to determine the ability of the multiplex PCR assay to correctly determine their serotypes from the SMART code database (Table 2). Five hundred isolates (66.6%) generated SMART codes that corresponded to the codes listed in Table 2 and correctly matched the conventional serotyping results (Tables 3 and 4). A further 139 of the remaining 251 isolates (18.5%) were putatively identified to the correct serotype, but with

TABLE 2. Representative PCR amplicon codes for most common U.S. clinical and veterinary serovars of *Salmonella enterica* subsp. *enterica*

O group	Serotype	SMART code	Alternative SMART code	No. of isolates	O antigen	H antigen phase 1	H antigen phase 2
O:2 (A)	Paratyphi A	1379-10-11		10	[1],2,12	a	[1,5]
O:4 (B)	Agona	125-11-13	1267-11-14	8	[1],4,[5],12	f,g,s	[1,2]
	Brandenburg	157-11 ^a		8	4,[5],12	l,v	e,n,z15
	Chester	157-11 ^a		8	[1],4,[5],12	e,h	e,n,x
	Derby	12567-11-14		8	[1],4,[5],12	f,g	[1,2]
	Heidelberg	15679-11-12-13-14		8	[1],4,[5],12	r	1,2
	I 4[5]12:i:–	125678-11-12-13-14		48	4,[5],12	i	
	Paratyphi B	1567-11-13 ^b	15-11-13-14 ^c	16	[1],4,[5],12	b	1,2
	Reading	157-10-11	157-11, ^a 1567-11-12-13-14	9	[1],4,[5],12	e,h	1,5
	Saint Paul	12567-11-12-13-14		8	[1],4,[5],12	e,h	1,2
	San Diego	157-11 ^a		7	4,[5],12	e,h	e,n,z15
	Schwarzengrund	1567-11 ^d		9	[1],4,12,[27]	d	1,7
	Stanley	1567-11-13-14	1567-11 ^d	8	[1],4,[5],12,[27]	d	1,2
	Typhimurium	125678-11-12-13-14-16		48	[1],4,[5],12	i	1,2
O:6,7 (C1)	Bareilly	15-11-13-14 ^c		10	6,7,[14]	y	1,5
	Braenderup	156-11-13 ^e		8	6,7,[14]	e,h	e,n,z15
	Choleraesuis	156-11-13 ^e		5	6,7	c	1,5
	Hartford	156-11-13 ^e		5	6,7	y	e,n,x
	Infantis	159-11		8	6,7,[14]	r	1,5
	Mbandaka	1256-11-14		8	6,7,[14]	z10	e,n,z15
	Montevideo	168-11		8	6,7,[14]	g,m,[p],s	[1,2,7]
	Ohio	156-11		8	6,7,[14]	b	l,w
	Oranienburg	1568-11		8	6,7,[14]	m,t	[z57]
	Thompson	1256-11 ^f		8	6,7,[14]	k	1,5
	Virchow	1257-11-13		10	6,7,[14]	r	1,2
O:8 (C2-C3)	Hadar	1256-11-13		8	6,8	z10	e,n,x
	Kentucky	1569-11		8	8,[20]	i	z6
	Litchfield	12567-11 ^g		7	6,8	l,v	1,2
	Muenchen	1567-11-13 ^b		8	6,8	d	1,2
	Newport	12567-11 ^g		8	6,8,[20]	e,h	1,2
O:9 (D1)	Berta	12356-11-13-14		8	[1],9,12	[f],g,[t]	
	Dublin	12356-11-14		8	1,9,12,[Vi]	g,p	
	Durban	135679-11		8	[1],9,12	a	e,n,z15
	Enteritidis	12356-11-14-15		16	[1],9,12	g,m	
	Javiana	13457-11	134579-11	8	[1],9,12	l,z28	1,5
	Panama	13567-11		7	[1],9,12	l,v	1,5
	Typhi	13479-10-11-13		8	9,12,[Vi]	d	
O:3,10 (E1)	Anatum	12567-11-12		8	3,10,[15],[15,34]	e,h	1,6
	Meleagridis	1567-11-13 ^b		7	3,10,[15],[15,34]	e,h	l,w
	Muenster	15679-11		8	3,10,[15],[15,34]	e,h	1,5
	Uganda	1579-11-13		7	3,10,[15]	l,z13	1,5
	Weltevreden	15678-10-11		8	3,10,[15]	r	z6
	Westhampton	12578-10-11-13 ^h		8	3,10,[15],[15,34]	g,s,t	
O:1,3,19 (E4)	Senftenberg	12578-10-11-13 ^h		8	1,3,19	g,[s],t	
O:13 (G)	Havana	1245-11	12456-11, 1256-11, ^f 125-10-11	8	[1],13,23	f,g,[s]	
	Mississippi	167-11 ⁱ		9	[1],13,23	b	1,5
	Poona	1679-10-11	1679-11, 1579-11, 1579-10-11	8	[1],13,22	z	1,6
	Worthington	1256-10-11		10	[1],13,23	z	l,w
O:18 (K)	Cerro	15-11		4	[6],[14],18	z4,z23	[1,5]
O:40 (R)	Johannesburg	167-11 ⁱ		5	[1],40	b	e,n,x

^a Code shared between serotypes San Diego, Reading, Brandenburg, and Chester.^b Code shared between serotypes Paratyphi B, Muenchen, and Meleagridis.^c Code shared between serotypes Paratyphi B and Bareilly.^d Code shared between serotypes Stanley and Schwarzengrund.^e Code shared between serotypes Braenderup, Hartford, and Choleraesuis.^f Code shared between serotypes Thompson and Havana.^g Code shared between serotypes Newport and Litchfield.^h Code shared between serotypes Senftenberg and Westhampton.ⁱ Code shared between serotypes Mississippi and Johannesburg.

another serotype or serotypes sharing the same SMART code. Within this shared-code data set, there were 83 isolates which were scored as being one of two serotypes and 56 isolates with a SMART code that was shared by three serotypes. Serotypes with shared SMART codes are described in Table 2.

The remaining 112 blinded isolates were incorrectly typed

according to the SMART code database listed in Table 2. These formed five subcategories (Table 4). The smallest group contained five isolates (0.7%) from serotypes that had been screened in the construction of the SMART code database and which gave new SMART codes that matched existing SMART codes from other serotypes. These were *Salmonella* serovar

TABLE 3. Comparison of serovar determinations using conventionally derived serotypes and SMART codes

O group	Serotype	SMART code	Serovar from SMART code	No. of isolates					Total no. of isolates
				Matched	Matched and shared	Unique code for tested serotype	Shared code for tested serotype	Shared cod for new serotype	
O:2 (A)	Paratyphi A	1379-10-11	Paratyphi A	1					1
	Agona	125-11-13	Agona	12					12
O:4 (B)	Ball	12567-11-12-13-14	Saint Paul					1	1
	Brandenburg	157-11	San Diego/Brandenburg/Chester	4					4
	Chester	157-11	San Diego/Brandenburg/Chester	1					1
	Clackamas	146-10-11				1			1
	Heidelberg	15679-11-12-13-14	Heidelberg	38					38
	I 4,[5],12:i:--	125678-11-12-13-14	I 4,[5],12:i:--	56					56
	Kiambu	15-11				1			1
	Paratyphi B	1567-11-13	Paratyphi B/Muenchen/Meleagridis		14				14
	Saint Paul	15-11-13-14	Paratyphi B/Bareilly	27	11				38
	San Diego	12567-11-12-13-14	Saint Paul			4			4
	Schwarzengrund	157-11	San Diego/Brandenburg/Chester		1				1
	Typhimurium	1567-11	Reading		1		4		5
O:6,7 (C1)	Bareilly	1567-11-13-14	Stanley/Schwarzengrund						
	Braenderup	1567-11-13-14	Stanley	18					18
	Choleraesuis	125678-11-12-13-14-16	Typhimurium	121					121
	Daytona	156-10-11-13-14				10			10
	Infantis	159-11	Paratyphi B/Bareilly		1				1
	Mbandaka	1256-11-14	Braenderup/Hartford/Choleraesuis		10				10
	Montevideo	168-11	Braenderup/Hartford/Choleraesuis		1				1
	Ohio	156-11	Infantis	10					10
	Oranienburg	1256-11-13	Mbandaka	7					7
	Oslo	1256-11-13	Montevideo	28					28
	Potsdam	125-11-13	Ohio			5			5
	Richmond	1256-11-13	Oranienburg			2			2
O:8 (C2-C3)	Singapore	156-11-14	Hadar						
	Tennessee	1256-10-11-13	Hadar						
	Thompson	1256-11-13	Thompson		9				9
	Virchow	1257-11-13	Virchow	1					1
	Albany	12456-11	Havana						
	Corvallis	1256-11-13	Hadar						
	Duesseldorf	1256-11	Thompson						
	Hadar	1256-11-13	Hadar	6					6
	Kentucky	1569-11	Hadar	1					1
	Kottbus	1567-11-13	Paratyphi B/Muenchen/Meleagridis		1				1
	Litchfield	12567-11	Newport/Litchfield						
	Manhattan	157-11-13	Newport/Litchfield						
O:9 (D1)	Muenchen	1567-11-13	Paratyphi B/Muenchen/Meleagridis		12				12
	Newport	12567-11	Newport/Litchfield		37				37
	Dublin	12567-11-13	Dublin	6					6
	Durban	1356-11-14	Durban	1					1
	Enteritidis	135679-11	Enteritidis	122					122
	Javiana	12356-11-14-15	Enteritidis			1			1
	Manhattan	13567-11-14	Javiana	6					6
	Muenchen	13457-11	Javiana	4					4
	Newport	134579-11	Panama	4					4
	Oslo	1256-11-13	Oslo						
	Potsdam	1256-11-13	Potsdam						
	Richmond	1256-11-13	Richmond						
	Singapore	156-11-14	Singapore						

[illegible]

TABLE 4. Summary of results for serovar determination of 2007 WAPHL *Salmonella* isolates by SMART

Result	No. (%) of isolates
Matched unique SMART code for serotype	500 (66.6)
Matched SMART code shared by multiple serotypes	139 (18.5)
Total correct	639 (85.1)
New shared SMART code for previously determined serotype ^a	5 (0.7)
Unique SMART code for previously determined serotype	41 (5.5)
Unique SMART code for new serotype ^b	26 (3.5)
Shared SMART code for new serotype ^b	28 (3.7)
Total incorrect	100 (13.3)
Non-subgenus I ^b	12 (1.6)
Total tested	751

^a Represents isolates belonging to serotypes for which SMART codes were determined in original panel of common serotypes.

^b Serotypes which were not screened in the original panel representing the top 50 most commonly reported clinical and veterinary serotypes in the United States.

Reading (four isolates) and *Salmonella* serovar Poona (one isolate); *Salmonella* serovar Reading matches *Salmonella* serovar San Diego, which emerged in this study to also be a highly variant serotype (Table 2). A previous study identified *Salmonella* serovar Poona as being the most variant serotype when screened by a multiplex PCR-based assay (30). The second subset of data was from 41 isolates (5.5%) of serotypes that had previously been screened but which gave new unique SMART codes that were different from the original codes assigned to the serotypes (Table 4). Of these, 40 isolates were represented by only four serotypes, namely, *Salmonella* serovar Newport (19 isolates), *Salmonella* serovar Typhimurium (10 isolates), *Salmonella* serovar Montevideo (7 isolates), and *Salmonella* serovar Saint Paul (5 isolates) (Table 3).

There were 54 isolates that corresponded to rare serotypes not screened in the original panel of serotypes that were analyzed for creation of the SMART code database used in the blinded study (Table 4). Twenty-six of these isolates (3.5% of the total tested) produced unique SMART codes, of which isolates of *Salmonella* serovar Wandsworth, *Salmonella* serovar Tennessee, and *Salmonella* serovar Manhattan were the most frequent, with six, four, and three isolates, respectively (Table 3). The remaining 28 of these isolates (3.7% of the total tested) produced SMART codes that matched codes already assigned to other more common serotypes in the database (Table 4). The most common code was 1256-11-13, which was initially scored for *Salmonella* serovar Hadar. A further six isolates representing five rare serotypes shared this code. It is notable that they also shared the same serogroup, O28 (M). *Salmonella* serovar Apapa also shared the same code but was from serogroup O45 (W). The last subset of blinded samples included 12 isolates (1.6%) that were *S. enterica* strains other than *S. enterica* subsp. I. Interestingly, all specimens produced one of two short codes, i.e., 17-11 and 1-11 (Table 4).

Although the overall sensitivity of the assay with the panel

was 85.1%, the data suggest that a larger database encompassing the SMART codes for more isolates and rarer serotypes would be beneficial. The addition of the data from this blinded study will aid in the improvement of the overall sensitivity of the assay. In this study, 9% of the isolates screened had unique SMART codes relative to those in the current database, and these data will be added to further develop the database. Similarly, it was observed that all non-subgenus I isolates screened gave a unique two-digit or very rare three-digit SMART code. The control amplicons 1 and 11 were present in all of these codes, with amplicon 7 being the third amplicon. These data suggest that these can also be discriminated in future screening using the SMART assay. Only *Salmonella* serovar Lexington gave a 17-11 SMART code, one shared by 10 of the 12 non-subgenus I isolates screened in this study. The other two isolates that were subgenus III had the SMART code 1-11.

PFGE pattern analysis as a tool to further discriminate serotypes for isolates which share SMART codes. Serovars that share SMART codes include isolates of *Salmonella* serovars Newport, Senftenberg, Paratyphi B [including biovar *d*-tartrate (+)], Muenchen, Braenderup, and Thompson (Table 2). Isolates representing these six serovars accounted for 125 of 139 (89.9%) isolates in the blinded study that produced shared SMART codes (Table 3). To provide further discrimination, the XbaI PFGE pattern of each of these putatively typed isolates was compared to existing databases containing the PFGE patterns of each serovar. For instance, the PFGE patterns for the nine isolates that generated a SMART code of 1256-11 were compared to the PFGE patterns of isolates from *Salmonella* serovars Thompson and Havana. PFGE patterns of 129 isolates (92.8%) had >90% similarity to PFGE patterns of isolates representing the serovar identified by conventional serotyping. Eight of the remaining isolates had a similarity of at least 84% with patterns of the correct serovar, suggesting the possibility of relatedness. A PFGE result for one isolate of *Salmonella* serovar Choleraesuis showed a similarity of 77.4% to the patterns of other *Salmonella* serovar Choleraesuis isolates in the PulseNet database but was scored as unrelated, using the 80% similarity cutoff value. The similarity to *Salmonella* serovar Choleraesuis via PFGE was still higher than that to the other potential serovars, Braenderup and Hartford, which share a SMART code with *Salmonella* serovar Choleraesuis. In this study, the only isolates with shared SMART codes which could not be discriminated further by PFGE pattern analysis were *Salmonella* serovar Senftenberg and *Salmonella* serovar Westhampton, which share the SMART code 12578-10-11-13. An isolate of *Salmonella* serovar Bareilly produced only a smear (data not shown) after XbaI digestion, and thus a PFGE pattern could not be determined for comparison. In total, the use of PFGE pattern comparison allowed the determination of the serotype identified by conventional serotyping for 103 of these 139 isolates (74.1%).

Serovar determination using SMART and PFGE for isolates unidentified by conventional serotyping. The absence or masking of surface antigens is a limitation of conventional serotyping and is usually observed in single-phase, nonmotile, rough, or mucoid isolates. The serotypes of eight isolates could not be determined definitively using conventional methods. The XbaI PFGE pattern was available for all of these isolates. SMART codes were obtained for the isolates, and serovar designations

were assigned to six of them. One isolate produced a SMART code that matched *Salmonella* serovar Mbandaka (antigenic formula 6,7:z10:z15) and had a PFGE pattern with 97% similarity to other *Salmonella* serovar Mbandaka patterns in the PFGE database. While serologic classification of the first flagellar phase matched that of *Salmonella* serovar Mbandaka, the serogroup of the isolate was O:42, which is different from that of *Salmonella* serovar Mbandaka (O:6,7). The strong molecular evidence along with the partial antigenic match suggests that the isolate may be a variant of serovar Mbandaka, although a definitive classification could not be made here due to the discrepancy with the serogroup classification. A second isolate was identified as *Salmonella* serovar Berta via its SMART code. However, it was serogrouped as O:47, while *Salmonella* serovar Berta belongs to serogroup O:9. While the possibility of incorrect classification of its O antigen by serotyping cannot be excluded, it seems plausible that the isolate represents a variant of *Salmonella* serovar Berta, given the 100% similarity of the PFGE pattern of this isolate to the patterns produced by multiple isolates of *Salmonella* serovar Berta.

Four of the isolates with partial antigenic formulae gave SMART codes that matched two or more serotypes (Table 3). Use of PFGE pattern comparison showed similarities of >90% for patterns of three of these isolates with the PFGE patterns of isolates from one of the putative serovars identified by the SMART code. The combined results of SMART assays and PFGE comparison along with the observation that only one phase of the H antigen could be identified, even with multiple attempts at serotyping, suggest these three isolates may represent monophasic variants of *Salmonella* serovar Newport, *Salmonella* serovar Mississippi, and *Salmonella* serovar Choleraesuis (Table 3). The fourth isolate produced the SMART code shared between *Salmonella* serovar Stanley and *Salmonella* serovar Schwarzengrund but did not produce a PFGE pattern of significant similarity (>80%) to the PFGE patterns of other isolates representing either of these serotypes. A unique SMART code, 1456-11, was generated for one isolate belonging to serogroup O:43, but a serovar could not be designated based upon SMART codes from the original panel in Table 2. Finally, a SMART code of 17-11 was generated for the one isolate for which a *Salmonella* subgenus classification could not be determined using conventional serotyping. This reduced SMART code was the predominant code observed among non-subgenus I isolates and one isolate of *Salmonella* serovar Lexington (Table 3).

DISCUSSION

Reference laboratories that serotype large numbers of *Salmonella enterica* subsp. *enterica* isolates incur significant costs associated with reagents, laboratorian training, and the time taken to accurately serotype each isolate and to report and store the data. Additional concerns with conventional serotyping include the maintenance of a large collection of antisera and quality control issues that are associated with the continued use of expired antisera in the event of new lots not being readily available. SMART allows for the determination of many *S. enterica* serotypes from cultured isolates within 1 day. This would be highly beneficial to outbreak control of food-

borne diseases, since the rapid screening of isolates permits the triage of specimens for subtyping by PFGE. The limited numbers of reagents required for the SMART assay are available from many commercial vendors, and thus it is straightforward to maintain an effective supply and quality control regimen for the assay in the laboratory.

Unlike other multiplex PCR-based methods for serotyping *S. enterica* subsp. *enterica* isolates, the relevant targets in this assay were amplified in a single reaction, and the assay was developed by testing 489 isolates representing the top 50 U.S. clinical serotypes of *Salmonella*. The use of fluorescently labeled PCR amplicons permits automatic sampling via a capillary electrophoresis machine that also automates data collection and data analysis. The use of capillary electrophoresis increases the throughput of the assay and also allows the introduction of a quality control regimen based on the predetermined correct amplicon sizes and a threshold fluorescence of amplicons to predict effective labeling of the amplified DNA targets. The transfer of large analyzed data sets is simple via computer, and the creation of binary scores is rapid and reduces errors in scoring, using software such as Excel (Microsoft, Redmond, WA). The principle behind this technology could easily be applied to other bacterial species, and other forms of binary scoring have been applied already to *Salmonella*, *E. coli*, and group B *Streptococcus* (4, 43, 51).

In this study, we demonstrated that the incorporation of a sensitive detection system permits the pooling and expansion of discriminatory targets for a single multiplex PCR targeting 16 genomic targets. A previous study by Kim et al. demonstrated a series of multiplex reactions and single PCRs that could discriminate the common serotypes of *Salmonella enterica* subsp. I (30). The versatility of the assay was tested by adding a new target to the assay, with the belated addition of *fljB* after a 15-plex assay had been designed. This helped to identify an emerging subgroup of monophasic isolates, including some members of *Salmonella* serovar 4[5],12:i:–, that may contain deletions of *fljB* (1, 15). The current work was based upon advances in *Salmonella enterica* genomics by several groups in the field, most notably from the laboratory of Michael McClelland (9, 13, 16, 38, 39). Recent publications by Arrach et al. and Scaria et al. have described new typing techniques based on analysis of these comparative genomic hybridizations (4, 30, 42). Arrach et al. designed an array of 384 PCR assays based on the most informative genes found in *Salmonella enterica* subsp. I. From this data set, they designed an assay consisting of 12 multiplex PCRs, each with eight targeted amplicons, that was capable of identifying 11 serotypes, with at least two discriminatory amplicons to confirm each result. However, the assay requires 12 eight-plex PCRs, analysis by agarose electrophoresis, and result scoring by hand. The complexity of each of these processes is very prone to operator error during reaction setup, sample analysis, and data interpretation. By designing a single multiplex PCR with built-in quality controls and an automated system for sample analysis, data preparation, and scoring, we have developed a rapid assay that is amenable for high-throughput screening of large numbers of specimens, which can be experienced by a reference laboratory during peak testing months for enteric diseases.

To create the SMART database, 489 isolates representing the top 50 clinical and veterinary serovars were screened by the

assay, and the majority of the serotypes gave a unique code. The blinded testing of a further 751 isolates revealed that 66.6% were correctly identified from the database and that isolates representing serotypes which share SMART codes (18.5%) were also partially identified. The use of a partially identified isolate's SMART code to direct which serotypes to use to conduct initial PFGE pattern comparison permitted the correct identification of 74.1% of isolates with shared codes. This finding reflects the current need to utilize a parallel subtyping system in conjunction with the SMART method to maximize overall sensitivity. However, the assay was originally designed for use in large local, regional, or national reference laboratories where PFGE is a routine procedure. The PulseNet USA guidelines require state laboratories to subtype each *Salmonella enterica* isolate submitted by PFGE with XbaI-digested DNA. The resulting pattern is then compared with state and national databases for matching patterns for the monitoring of local and national outbreaks of food-borne diseases (41). Consequently, the use of PFGE complements the confirmation of the serotype and also allows mistyped isolates to be identified when very dissimilar patterns are observed. A further benefit of using this assay is that unique SMART codes can be identified prior to PFGE, and therefore outbreak-related serotypes are identified earlier, allowing for preferential PFGE analysis of these isolates in order to expedite ongoing epidemiologic investigations.

The isolates that were not identified by the assay were comprised mainly of serotypes that had not been screened by the SMART assay, and this reflects a need for a more comprehensive screening with a greater number of rare serotypes. A limiting factor in the development of the SMART database was the number of isolates in the WAPHL strain collection. However, the USDA has over 70,000 archived isolates of *Salmonella enterica*, and the low cost, simplicity, and speed of the assay will allow for further mass screening to expand the existing database. The assay has demonstrated its potential to identify some monophasic or rough isolates of *Salmonella enterica*. With monophasic isolates, the assay can identify *fljB* deletion phenotypes, but other factors known to prevent flagellar antigen expression are not detected by this target. One observation from this study was that *Salmonella* serovars other than those in subgenus I were discriminated from those in subgenus I, with the sole exception of a single isolate of *Salmonella* serovar Lexington, of which only 20 isolates have been reported in the United States in the preceding 14 years (12).

Other molecular methods for serovar determination, such as DNA microarrays and liquid microsphere arrays, have great potential for molecular serotyping of *Salmonella enterica* subsp. *enterica* due to their sensitivity and specificity (20, 38, 42). However, these methods require extra time for processing due to additional hybridization and washing steps following target amplification or labeling, and microarrays are not well suited for high throughput testing. Currently, these methods are prohibitively expensive for routine testing due to the high costs associated with the required acquisition and maintenance of new instrumentation, consumables, and reagents; in particular, fluorescent dyes and array chips for microarrays and fluorescently labeled microspheres for liquid arrays are very expensive. Most reference laboratories have access to capillary electrophoresis machines due to their widespread use in DNA

sequencing-based identification and subtyping of microorganisms (6, 8, 21, 24, 27, 33, 34, 49). A basic cost analysis of the SMART method, including the current costs of reagents, primers, probes, and consumables used for DNA preparation, PCR, and capillary electrophoresis, showed an estimated cost reduction as high as 95% per test versus conventional serotyping (\$1.50 per test versus \$40.00 per test for serotyping).

The further incorporation of new target sequences with the aim to increase the specificity of the assay is under development. This will reduce the need for PFGE patterns as discriminators and thus increase the suitability of the method for wider use by noncentralized reference laboratories involved with serotyping of *Salmonella* isolates. A notable feature is the development of other multiplex PCR assays which incorporate a range of different fluorescent dyes into the reaction products. For example, the ABI 3130 system has the capability to simultaneously detect five spectra from different fluorescent dyes, and thus it may be possible to screen up to four separate multiplex PCRs pooled into the same analysis and run with standard size markers. This approach may generate very large amounts of epidemiologic data while keeping the cost per test comparatively low. Examples of this future potential include developing further targets to discriminate serotypes that currently share the same SMART code (30), using molecular markers to replace phage typing of *Salmonella* serovar Typhimurium (31), subtyping by variable-number tandem repeats (8), and screening for the presence/emergence of clinically significant extended-spectrum beta-lactamases and/or R plasmid markers (11, 36, 37). The cost-effectiveness of this approach not only is of great benefit to public health laboratories involved in food-borne disease surveillance but also has the potential to aid other clinical research studies by screening large sample sets of untyped *Salmonella enterica* isolates.

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