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Rapid Genoserotyping Tool for Classification of *Salmonella* Serovars▽†

Kristyn Franklin,[1]* Erika J. Lingohr,[1] Catherine Yoshida,[1] Muna Anjum,[2] Levente Bodrossy,[3]‡ Clifford G. Clark,[4] Andrew M. Kropinski,[1] and Mohamed A. Karmali[1]

Public Health Agency of Canada, Laboratory for Foodborne Zoonoses, 110 Stone Road West, Guelph, ON, Canada N1G 3W4[1]*; Animal Health and Veterinary Laboratories Agency, New Haw, Addlestone, Surrey, United Kingdom*[2]*; Department of Bioresources, Austrian Institute of Technology, A-2444 Seibersdorf, Austria*[3]*; and Enteric Diseases Program, Bacteriology and Enteric Diseases Program, National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, MB, Canada R3E 3R2*[4]

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We have developed a *Salmonella* genoserotyping array (SGSA) which rapidly generates an antigenic formula consistent with the White-Kauffmann-Le Minor scheme, currently the gold standard for *Salmonella* serotyping. A set of 287 strains representative of 133 *Salmonella* serovars was assembled to validate the array and to test the array probes for accuracy, specificity, and reproducibility. Initially, 76 known serovars were utilized to validate the specificity and repeatability of the array probes and their expected probe patterns. The SGSA generated the correct serovar designations for 100% of the known subspecies I serovars tested in the validation panel and an antigenic formula consistent with that of the White-Kauffmann-Le Minor scheme for 97% of all known serovars tested. Once validated, the SGSA was assessed against a blind panel of 100 *Salmonella enterica* subsp. I samples serotyped using traditional methods. In summary, the SGSA correctly identified all of the blind samples as representing *Salmonella* and successfully identified 92% of the antigens found within the unknown samples. Antigen- and serovar-specific probes, in combination with a *pepT* PCR for confirmation of *S. enterica* subsp. Enteritidis determinations, generated an antigenic formula and/or a serovar designation consistent with the White-Kauffmann-Le Minor scheme for 87% of unknown samples tested with the SGSA. Future experiments are planned to test the specificity of the array probes with other *Salmonella* serovars to demonstrate the versatility and utility of this array as a public health tool in the identification of *Salmonella*.

Food-borne salmonellosis is an important public health concern worldwide and continues to be one of the leading causes of gastroenteritis in North America. Since salmonellae are primarily found in the intestinal tracts of animals, most infections are the result of drinking contaminated water or eating improperly prepared foods of animal origin, including meat, poultry, eggs, and dairy products (45). *Salmonella* can also be found on fresh produce, including tomatoes (1, 3), and on dry foods such as pet food (4). *Salmonella* infections commonly present with watery diarrhea, abdominal cramps, fever, headache, nausea, and vomiting. In approximately 1 to 4% of immunocompetent patients, bacteremia occurs, and in 5 to 10% of those individuals, other extraintestinal complications, including central nervous system infections, endocarditis, reactive arthritis, and urinary tract infections, may occur (26).

It is estimated that, in the United States, 1.2 million nontyphoidal *Salmonella* infections occur annually, resulting in 19,336 hospitalizations and 378 deaths (56). The annual cost of these infections, including medical expenses and loss of productivity, has been estimated to range between $0.5 and $2.3 billion dollars (19). Salmonellosis is significantly underreported; therefore, it is very difficult to precisely determine the actual public health burden of *Salmonella* worldwide (59). Canadian studies suggest that the ratio of salmonellosis infections per reported case ranges from 13 to 37, highlighting the need to develop rapid, accessible, and economical assays to facilitate clinical diagnosis and reporting strategies (62).

Currently, *Salmonella* isolates are typed using the White-Kauffmann-Le Minor scheme. This classification scheme is utilized by public health organizations worldwide and is considered the gold standard for the determination of *Salmonella* serotypes. The White-Kauffmann-Le Minor scheme subtypes *Salmonella* into serotypes on the basis of surface antigen identification using polyclonal antiserum to determine the O (somatic) and H (flagellar) antigenic epitopes (20). Serotyping is essential for human disease surveillance and outbreak detection, as both the virulence and host range of *Salmonella* isolates can be serotype specific (15, 67).

Many of the genes required for the biosynthesis of the O antigen are organized in a large regulon called the *rfb* cluster, which is located between the *galF* and *gnd* genes in both *Salmonella* and *Escherichia coli* (53, 55). Differences among the 46 *Salmonella* O serogroups described in the White-Kauffmann-Le Minor scheme are mainly due to genetic variations in their respective *rfb* clusters. While the sequences of sugar transferase genes within the *rfb* cluster are relatively conserved (68), the O-antigen flippase (*wzx*) and polymerase (*wzy*) genes are highly variable and are considered specific with respect to the serogroup (2).

* Corresponding author. Mailing address: Laboratory for Foodborne Zoonoses, Public Health Agency of Canada, 110 Stone Road West, Guelph, Ontario, Canada N1G 3W4. Phone: 519-826-2638. Fax: 519-822-2280. E-mail: Kristyn.Franklin@phac-aspc.gc.ca.

‡ Present address: CSIRO Marine and Atmospheric Research, Hobart, Tasmania, Australia.

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There are 114 H antigens used to serotype this bacterium. The antigenic portion of the flagellar structure is encoded by two genes, *fliC* (phase 1 flagellin) and *fljB* (phase 2 flagellin). These genes are typically highly conserved at their 5′ and 3′ ends, whereas the central region is generally quite variable (39, 43). Flagellar antigens that are immunologically related are known as antigen complexes, and antigens within these complexes often exhibit very homologous gene sequences (43). Most *Salmonella* serotypes exhibit diphasic flagellar antigen expression, alternately expressing *fliC* and *fljB* genes; however, serovars that express only one flagellar antigen are considered monophasic. This genetic switching mechanism is regulated by the invertible element *hin* (58).

Despite its usefulness, traditional serotyping is labor-intensive and expensive and can take up to 5 days to complete. It requires specialized expertise and a set of more than 250 stringently quality-assured reagents to characterize the more than 2,500 *Salmonella* serovars. Many hospital and private laboratories rely on the use of a limited number of commercially available antisera, covering only a restricted number of serotypes (35). These laboratories are forced to ship isolates to reference laboratories for full serotyping, causing delays in isolate identification that ultimately impede progress in outbreak investigations and containment.

Drawbacks to traditional *Salmonella* serotyping have prompted many groups to investigate alternative molecular methods. In recent years, molecular typing assays have been developed based upon multiplex real-time PCR (31, 49), primer extension (5), microarrays (61, 66, 69), DNA sequence-based approaches (47), and bead-based suspension arrays (16, 41). To date, many of the molecular techniques have been able to type only a very small subset of the thousands of *Salmonella* serovars. Some do not provide an antigenic formula that mimics the globally understood White-Kauffmann-Le Minor scheme, and others are considered too expensive to be implemented in public or private diagnostic laboratories. In 2007, we described the development of a fluorescence-based glass slide microarray for the classification of prevalent *Salmonella* serovars (69). Shortly thereafter, we switched to the ArrayTube platform (2) (Alere Technologies [formerly Clondiag], Germany), which offered a rapid and more economical alternative to the expensive and time-consuming glass slide array system.

Here we describe an ArrayTube-based *Salmonella* genoserotyping array (SGSA) that generates an antigenic formula. Validation and testing of the array was completed with 287 *Salmonella* strains representative of 133 *Salmonella* serovars, including the most prevalent *Salmonella* serovars from human and nonhuman isolates within North America, the United Kingdom, and Austria, to ensure the development of a comprehensive assay with an international scope.

MATERIALS AND METHODS

Bacterial strains and culture methods. *Salmonella enterica* strains used in this study were obtained primarily from animal isolates submitted to the *Salmonella* OIE Reference Laboratory at the Laboratory for Foodborne Zoonoses (LFZ; Guelph, Ontario, Canada) and were serotyped using conventional methods. In brief, serotyping at the LFZ utilizes slide agglutination for the determination of somatic antigens (14) and a mechanized microtechnique for flagellar antigenic determination (57). In order to designate serotypes based on an antigenic formula, the White-Kauffmann-Le Minor classification scheme was utilized (20).

The *Salmonella* strains in the blind study were provided by the Animal Health and Veterinary Laboratories Agency (AHVLA; Addlestone, Surrey, United Kingdom). All *Salmonella* strains were grown overnight at 37°C on Luria-Bertani agar (BD Canada, Mississauga, ON, Canada).

TABLE 1. Probe target list

Control	Capsular or O antigen	Flagellar phase 1 antigen(s)	Flagellar phase 2 antigen(s)	Additional characteristics
Biotin	A (Paratyphi) (O:2)	a	1,2	RHS-E
invA	B (O:4)	b	1,2_1,5_1,2,7	f subcomplex
	C1 (O:6,7)	c	1,5	RHS-GP
	C2 (O:8)	d	1,5-2	*S*. Pullorum
	D (O:9)	e,h	1,5-4	
	E (O:3)	f,g	1,5 (Kottbus)	
	G (O:13)	f,g,s	1,5_1,2,7	
	H (O:6,14)	f,g,t_f,g_g,m,t	1,6	
	J (O:17)	f,g,t	1,7	
	K (O:18)	g,m,s_g,m,p,s	e,n,x	
	L (O:21)	g,m,q_g,q	e,n,x,z_{15}	
	M (O:28)	gp	e,n,z_{15}_e,n,x,z_{15}	
	O (O:35)	g,p_g.p.s	l,w	
	P (O:38)	g,s,t_g,t		
	V (O:44)	g,z_{51}		
	Y (O:48)	i		
	O:58	k		
	O:61	k (O:61)		
	Vi	l,z_{13}_l,v		
		l,z_{28}		
		m,t_g,m,t		
		r_r,i		
		y		
		z		
		z_4,z_{23}		
		z_6_z_{67}		
		z_{10}		
		z_{29}		
		z_{38}		

Array layout and design. All 66 oligonucleotide probes (Table 1) (18 to 35 bp in length) were designed using PrimerSelect (DNASTAR, Madison, WI), and sequence specificity was assessed using GenBank's Basic Local Alignment Search Tool (BLASTN). Probes were synthesized and printed in triplicate onto ArrayTube strips by Alere Technologies (Jena, Germany). Biotinylated oligonucleotides were spotted on the microarray as staining controls and for use as reference spots by the image analysis software. The *Salmonella*-specific gene *invA* is utilized as a positive *Salmonella* control (52).

Multiplex PCR. Genomic DNA was isolated from *Salmonella* grown overnight at 37°C using LB agar (BD Canada) and an EZ1 DNA tissue kit and BioRobot (Qiagen Ltd., Mississauga, ON, Canada) according to the manufacturer's instructions and with the addition of 100 μg of lysozyme (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) (10 mg/ml) in the cell lysis incubation. DNA was assessed for quality and quantified spectrophotometrically (Nanodrop ND-1000; Nanodrop Technologies Inc., Wilmington, DE). A minimum recovery of 60 ng/μl of DNA was required for subsequent use as a PCR template.

A multiplex PCR was developed to amplify targeted somatic genes within the *Salmonella rfb* cluster, the capsular Vi antigen encoded by the *viaB* gene, and unique sequences within *rhs* and *rhs*-like genes (Table 2). A phase 1 flagellar gene (*fliC*) and a *Salmonella*-specific gene (*invA*) were coamplified in a second reaction, and the phase 2 flagellar gene (*fljB*) antigen was amplified separately for optimum performance (Table 2). Additional somatic genes within the *Salmonella rfb* cluster were also amplified and tested in the SGSA; however, they were not amplified as part of the multiplex PCR described above (Table 2). All of the gene targets tested on the array were amplified using a Qiagen multiplex PCR kit according to the manufacturer's instructions. Each 25-μl PCR mixture contained 1× multiplex master mix, 0.2 μM each primer, and 1.75 μl (approximately 100 ng/μl) of genomic DNA. Amplification conditions were 15 min at 95°C, 35 cycles of 30 s at 95°C, 90 s at 57°C, and 90 s at 72°C, with a final elongation of 5 min at 72°C performed using a T Gradient thermocycler (Biometra, Montreal Biotech Inc., Montreal, QC, Canada). For validation purposes only, the presence of appropriately sized bands was verified using 1.2% agarose Flashgel DNA cassettes (Lonza, Rockland, ME).

Processing of SGSA. In a 20-μl reaction mixture containing 2-μl aliquots of each of the three multiplex PCRs with 4 μl of shrimp alkaline phosphatase (SAP) buffer 1 (Roche Diagnostics, Indianapolis, IN), 3 μl (3 U) of SAP was added to dephosphorylate the remaining nucleotides. After incubation for 10 min at 37°C,

TABLE 2. Primer sequences for *Salmonella* genoserotyping array (SGSA) target amplification

Target	Gene	Accession no.	Primer	Primer sequence (5′–3′)	Amplicon size (bp)	Multiplex	Reference or source
A (O:2)/D (O:9)	*prt*	NC_006511	rfbS (D) rfbS (D)	TCACGACTTACATCCTAC CTGCTATATCAGCACAAC	720	1	Luk et al. (38)
B (O:4)	*rfbJ*	X56793	B_rfbJ_F B_rfbJ_R	TGAAAGAATATGTAATTGTCAGTGG TTTCATTATCTCTTTGCTCTATCG	789	1	This study
C1 (O:6,7)	*wbaA*	M84642	C1_wbaA_F3 C1_wbaA_R	TTGGCAGACTGGTACTGATTGG GCAGGAATCCGTGTAAAAATTC	976	1	This study
C2 (O:8)	*rfbJ*	X61917	C2_rfbJ_F C2_rfbJ_R	GAACCCCTATATCTGAACAAT CTCGGCACTCCAACCTAATC	593	1	This study
E1/E4 (O:3)	*wzx*	X60665	E_wzx_F E_wzx_R	ATGCAAGTATATCCCCTGAAAATC CCGATTTAAGGGCATTTTTGTA	1,000	1	This study
G (O:13)	*wzx*	EF204526	G_wzx_F G_wzx_R	CTGAAAAATGGTTTAGATTG ACCATTGGATACTGTAACTG	502	1	This study
H (O:6,14)	*wzy*	AY334017.1	H_wzy_F H_wzy_R	GTCTCCGCTAAGCTATTTCGGTTTGTA CCCTTGTGATTGAATTATTGCGGTA	501	na[a]	Fitzgerald et al. (18)
J (O:17)	*wzy*	EF032635	J_wzy_F J_wzy_R	GGCTGGGTTGTGGCTTTTT CTTCCGAAATCAATAGAAAAATCAA	565	na	Fitzgerald et al. (17)
K (O:18)	*wzx*	EF032634	K_wzx_F K_wzx_R	CTCTAGGATCAACTGAAGGTGGTC CAACCCAGCAATAAAGCAGAA	370	1	Fitzgerald et al. (17)
L (O:21)	*wzx*	HQ291553	L_wzx_F2 L_wzx_R2	AAGGATGGGACTACCGTAAG ATTCCCCAATGTAATAACCA	810	na	This study
M (O:28)	*wzx*	HQ291554	M_wzx_F M_wzx_R	GCTGGCTATGCTAGGACTTA ACCCAGATACTTCCCAAGAT	704	na	This study
O (O:35)	*wzy*	AF285969	O_wzy_F O_wzy_R	ATGTCTATTGATTTTCTTT CAACTTGTAATAATAATAAAC	684	na	This study
P (O:38)	*wzx*	HQ291552	P_wzx_F P_wzx_R	AGGGAAAGTAACGCTCAGTA CAAGTGCAGGAATCAACATA	707	na	This study
V (O:44)	*wbcM*	HQ416970	V_wbcM_F V_wbcM_R	ACGATCTAAGTAATTCAGGTGGTA TCATAGTAAAACCTCGTCCAGTA	1,031	na	This study
Y (O:48)	*wzx*	HQ291555	Y_wzx_F Y_wzx_R	TTTTTCGAGCATTTATCACA TCGCATAGCATATAGAGCAA	708	na	This study
O:58	*wfbE*	EU825757	O58_wfbE_F O58_wfbE_R	AGTTAGTGTTTGTATTATTTCGTA ACAAGTCAATGAGTTTATCCA	746	na	This study
O:61	*wzx*	HQ416969	O61_wzx_F_2 O61_wzx_R_2	CGAGAGCAATGGGGATGGATG AGGAAAAGCGAAAGAAATAACAAT	599	1	This study
Vi	*viaB*	X67785	ViaBF ViaBR	CACGCACCATCATTTCACCG AACAGGCTGTAGCGATTTAGG	738	1	Kumar et al. (31)
RHS-GP	SG1045	NC_011274	RHS_GP_F RHS_GP_R3	GACGACCAGAGAAATGAG CGTACCACGTCACTTCC	437	1	This study
RHS-E	SEN0272	NC_011294	E_RHSfam_F E_RHSfam_R	GTGCTGTATGAAGTGTGC CAGGTGTAGTAATACCGTTC	569	1	This study
H1-*fliC*	*fliC*	AY353389	fliC_13 fliC_14	GCGCGGAATAATGAGGCATAAAGC GCTTTCGCTGCCTTGATTGTGT	1,700	2	McQuiston et al. (43)
Salmonella-specific	*invA*	M90846	invA_F1 invA_R1	CTGCTTTCTCTACTTAACAGTGCTCG CGCATCAATAATACCGGCCTTC	495	2	Yoshida et al. (69)

Continued on following page

TABLE 2—*Continued*

Target	Gene	Accession no.	Primer	Primer sequence (5′–3′)	Amplicon size (bp)	Multiplex	Reference or source
H2-*fljB*	*fljB*	AY353269	Ph2_8_F	GAAAAGATCATGGCACAAGTAATCAA CACT	1,500	3	Yoshida et al. (69)
			AllRev3_R	GGAATCTTCGATACGGCTACG			
pepT	*pepT*	NC_011294	pepT_F2	GTTTGCCATATTGCTGCGAGGC	2,061[b]/150[c]	na	This study
			pepT_R2	GCGCTATCTCGGCGGCTG			

[a] na, currently used only in singleplex PCRs.
[b] Amplicon size for *Salmonella* Enteritidis.
[c] Amplicon size for *Salmonella* Nitra.

the enzyme was denatured by 5 min of incubation at 75°C in a T Gradient thermocycler (Biometra).

The SAP-treated PCR products were then biotin labeled by a sequence-specific end labeling of oligonucleotides (SSELO) method modified from Kostić et al. (30). The labeling method uses reverse-complement oligonucleotides lacking the 3′ terminal nucleotide. Each 20-μl reaction mixture consisted of 1× PCR buffer (Applied Biosystems, Branchburg, NJ), 1.5 mM $MgCl_2$, 0.5 pmol of each reverse complement primer/μl multiplexed with primers corresponding to each of the ArrayTube probes, 0.8 pmol of each biotinylated ddCTP (dideoxy-CTP), ddTTP, and ddGTP/μl (Perkin Elmer Life and Analytical Sciences, Boston, MA), 8 pmol of ddATP/μl (Roche), 0.125 units of AmpliTaq Gold DNA polymerase/μl (Applied Biosystems), and 5 μl of SAP-treated PCR product. The cycling conditions were 15 min at 95°C and then 25 cycles of 30 s at 95°C and 75 s at 60°C in a T Gradient thermocycler (Biometra). The dephosphorylation and SSELO reactions were optimized for robustness, eliminating the need for standardized DNA concentrations. Once labeled, the samples were used directly on the ArrayTubes without the need for further purification.

Samples were hybridized to the SGSA using a hybridization kit (Alere Technologies) and processed according to the manufacturer's instructions, except the hybridization was carried out for 1 h at 60°C and bound with D1 substrate reagent for 15 min at room temperature.

Signal intensities were detected using an ArrayMate ArrayStrip reader (Alere Technologies). Biotin signal values must be greater than 0.7 for the experiment to be considered valid. Positive signal values correspond to spot intensities above a minimum cutoff value of 0.2. This value was determined after analyzing the signal intensity range, based on the 95% central interval for each of the probes designed to detect the serovars in subset 1 of the validation panel (see Table S1 in the supplemental material). After the probe patterns of the validation strains were tested and confirmed, it was determined that, in the case of antigenic complexes with high sequence homology, the probe with the highest signal intensity was to be used for antigenic formula determinations. A Microsoft Excel macro was developed to automate data analysis and generate an antigenic formula for determination of simple and nonsubjective results. Each *Salmonella* sample was characterized by a unique probe pattern based on the identification of an O antigen and of phase 1 and phase 2 flagellar antigens. The antigenic formula was then used to designate serotype according to the White-Kauffmann-Le Minor scheme (20).

Validation of the array. A strain set of 82 serovars was selected as our validation panel, which was composed of three subsets. The first subset contained a group of 43 *S. enterica* serovars representing the most prevalent human and nonhuman *S. enterica* isolates identified from North America, the United Kingdom, and Austria. The second subset comprised 33 subspecies I serovars used to validate additional probes. The third subset of six serovars was used solely to analyze the detection of additional O serogroups in the SGSA. Flagellar antigens were not tested for the third subset of serovars, as flagellum-specific array probes were designed using only subspecies I *Salmonella* sequence data and the probe set does not represent subspecies sequence variability (47). The remaining 76 serovars were analyzed based on their full antigenic formulas. The full panel of 82 serovars served as the fundamental strain set to test the specificity and the repeatability of the SGSA probe and probe pattern results. This panel was utilized to evaluate expected probe patterns, and the results were based solely on the known samples within the panel (Table 3 and Table 4). Hybridization of the 82 serovars was performed in triplicate, and antigenic formulas were derived using the SGSA and compared to those obtained by traditional serotyping.

In order to further validate the specificity of the SGSA, 20 five monophasic serovars and eight rough strains were selected to assess the genoserotyping results compared to those obtained by traditional antibody-based methods. *E. coli* EDL 933 and *Campylobacter jejuni* NCTC 11168 were also tested as negative controls.

Amplification and detection *of pepT*. The genome sequences of *S. enterica* subsp. Enteritidis and *S. enterica* subsp. Nitra are highly homologous and currently cannot be differentiated on the SGSA. Whole-genome sequence alignments revealed a large deletion of the *pepT* gene in *Salmonella* Nitra (JN081866) compared to *Salmonella* Enteritidis (NC_011294). Based on the sequence of the latter, forward and reverse primers flanking *pepT* were designed (Table 2). These primers amplified a 2,061-bp target in *Salmonella* Enteritidis and a 106-bp fragment in *Salmonella* Nitra. The target was amplified using a Qiagen multiplex PCR kit according to the manufacturer's instructions. Each 25-μl PCR mixture contained 1× multiplex master mix, 0.2 μM each primer, and 1.75 μl (approximately 100 ng/μl) of genomic DNA. Amplification conditions matching those of the multiplex reactions were 15 min at 95°C, 35 cycles of 30 s at 95°C, 90 s at 57°C, and 90 s at 72°C, with a final elongation of 5 min at 72°C performed using a T Gradient thermocycler (Biometra). The amplicons were sized using 0.8% SeaKem LE agarose (Cambrex, Rockland, ME) gels with 1× Tris-borate buffer (Invitrogen, Carlsbad, CA). Sixty *Salmonella* Enteritidis strains and three *Salmonella* Nitra strains, isolated from human, animal, and environmental samples of multinational origin, were used as a validation panel for the *pepT* PCR. All 63 samples were correctly differentiated using the *pepT* assay. The validated *pepT* PCR was utilized to confirm the serovar identification of all unknown samples that were typed by the SGSA as representing either *Salmonella* Enteritidis or *Salmonella* Nitra.

Blind study. Once validated, the SGSA was assessed against a blind panel of 100 *S. enterica* subsp. I strains (see Table S2 in the supplemental material) obtained from and serotyped using traditional methods of the AHVLA (36). Serovar designations were determined according to serovars identified in the 2007 antigenic formulae of the *Salmonella* serovars, 9th ed. (WHO Collaborating Centre for Reference and Research on *Salmonella*) (20).

RESULTS

***Salmonella* genoserotyping array design and layout.** Probes were designed to detect each of the antigens required to characterize the 82 serovars within the validation panel. Unique sequences were identified based on alignments of homologous sequences performed using SeqMan software (Lasergene 8; DNASTAR Inc.). Somatic probes were designed based on publicly available sequences and on sequences determined in-house for gene targets (Table 2) within the *Salmonella rfb* cluster. The probes printed on the current array are capable of detecting 18 somatic serogroups: A (O:2); B (O:4); C1 (O:6,7); C2 (O:8); D (O:9); E (O:3); G (O:13); H (O:6,14); J (O:17); K (O:18); L (O:21); M (O:28); O (O:35); P (O:38); V (O:44); Y (O:48); O58; and O61. Serogroup A and D *Salmonella* serovars cannot currently be differentiated with a single somatic probe due to the high level of sequence homology between their *rfb* loci (63). *Salmonella* Paratyphi A, which belongs to serogroup A, can be differentiated from serogroup A and D serovars by the use of a probe [A (Paratyphi) (O:2)] designed to target a 2-bp mismatch within the *prt* gene (16). In order to differen-

TABLE 3. *Salmonella* enterica strains used to validate the *Salmonella* genoserotyping array (SGSA) probes and probe patterns

Salmonella serovar	Serogroup as determined by traditional serotyping	Antigenic formula as determined by traditional serotyping	SGSA-identified antigenic formula	SGSA antigenic formula correlation with traditional serotyping	Correct SGSA serovar designation (based on known samples)
Subset 1					
1,4,5,12:i:-	B	4,5,12:i:-	B:i:-	Yes	Yes
Abony	B	4,12:b:e,n,x	B:b:e,n,x	Yes	Yes
Agona	B	4,12:f,g,s:-	B:f,g,s:-	Yes	Yes
Anatum	E1	10:e,h:1,6	E:e,h:1,6	Yes	Yes
Orion var. Binza	E1	3,15:y:1,5	E:y:1,5	Yes	Yes
Braenderup	C1	C1 6,7:e,h:e,n,z_{15}	C1:e,h:e,n,z_{15}	Yes	Yes
Cerro	K	18:z_4,z_{23}:-	K:z_4,z_{23}:-	Yes	Yes
Corvallis	C2	8,20:z_4,z_{23}:-	C2:z_4,z_{23}:-	Yes	Yes
Derby	B	4,12:f,g:-	B:f,g:-	Yes	Yes
Dublin	D1	9,12:g,p:-	A/D:g,p:-	Yes	Yes
Enteriditis	D1	9,12:g,m:-	A/D:-:-, RHS-E	Partial	Yes
Gallinarum	D1	9,12:-:-	D:-:-, RHS-GP	Yes	Yes
Give	E1	10:l,v:1,7	E:l,v:1,7	Yes	Yes
Hadar	C2	6,8:z_{10}:e,n,x	C2:z_{10}:e,n,x	Yes	Yes
Heidelberg	B	4,12:r:1,2	B:r:1,2	Yes	Yes
Indiana	B	4,12:z:1,7	B:z:1,7	Yes	Yes
Infantis	C1	6,7,14:r:1,5	C1:r:1,5	Yes	Yes
Javiana	D1	9,12:l,z_{28}:1,5	D:l,z_{28}:1,5	Yes	Yes
Kedougou	G	13,23:i:l,w	G:i:l,w	Yes	Yes
Kentucky	C2	8,20:i:z_6	C2:i:z_6	Yes	Yes
Kiambu	B	4,12:z:1,5	B:z:1,5	Yes	Yes
Kottbus	C2	6,8:e,h:1,5	C2:e,h:1,5	Yes	Yes
Mbandaka	C1	6,7,14:z_{10}:e,n,z_{15}	C1:z_{10}:e,n,z_{15}	Yes	Yes
Mississippi	G	13,23:b:1,5	G:b:1,5	Yes	Yes
Montevideo	C1	6,7:g,m,s:-	C1:g,m,s:-	Yes	Yes
Muenchen	C2	6,8:d:1,2	C2:d:1,2	Yes	Yes
Newport	C2	6,8,20:e,h:1,2	C2:e,h:1,2	Yes	Yes
Oranienburg	C1	6,7:m,t:-	C1:m,t:-	Yes	Yes
Paratyphi A	A	2,12:a:1,5	A:a:1,5	Yes	Yes
Paratyphi B var. Java	B	4,12:b:1,2	B:b:1,2	Yes	Yes
Pullorum	D1	9,12:-:-	D1:-:-, P	Yes	Yes
Rissen	C1	6,7,14:f,g:-	C1:f,g:e,n,x,z_{15}	Partial	Yes
Saintpaul	B	4,5,12:e,h:1,2	B:e,h:1,2	Yes	Yes
Schwarzengrund	B	4,12,27:d:1,7	B:d:1,7	Yes	Yes
Senftenberg	E4	3,19:g,s,t:-	E:g,s,t:-	Yes	Yes
Stanley	B	4:d:1,2	B:d:1,2	Yes	Yes
Stanleyville	B	4:z_4,z_{23}:-	B:z_4,z_{23}:-	Yes	Yes
Tennessee	C1	6,7:z_{29}:-	C1:z_{29}:-	Yes	Yes
Thompson	C1	6,7,14:k:1,5	C1:k:1,5	Yes	Yes
Typhi	D1	9,12,Vi:d:-	D,Vi:d:-	Yes	Yes
Typhimurium	B	4,5,12:i:1,2	B:i:1,2	Yes	Yes
Virchow	C1	6,7,14:r:1,2	C1:r:1,2	Yes	Yes
subsp. IIIb 61:k:1,5,(7)	O:61	61:k:1,5,7	61:k:1,5	Yes	Yes
Subset 2					
Alachua	O	35:z_4,z_{23}:-	O:z_4,z_{24}:-	Yes	Yes
Amsterdam	E1	15,34:g,m,s:-	E:g,m,s:-	Yes	Yes
Berlin	J	17:d:1,5	J:d:1,5	Yes	Yes
Berta	D1	9,12:f,g,t:-	D:f,g,t:-	Yes	Yes
Blegdam	D1	9,12:g,m,q:-	D:g,q/g,m,q:-	Yes	Yes
Blijdorp	H	1,6,14,25:c:1,5	H:c:1,5	Yes	Yes
Blockley	C2	6,8:k:1,5	C2:k:1,5	Yes	Yes
Brandenburg	B	4,12:l,v:e,n,z_{15}	B:l,v:e,n,z_{15}	Yes	Yes
Bredeney	B	4,12,27:l,v:1,7	B:l,v:1,7	Yes	Yes
Breukelen	C2	6,8: l,z_{13}:enz$_{15}$	C2:l,z_{13}:e,n,z_{15}	Yes	Yes
Budapest	B	4,12:g,t:-	B:g,t/g,s,t:-	Yes	Yes
California	B	4,12:g,m,t:-	B:g,m,t	Yes	Yes
Carrau	H	6,14:y:1,7	H:y:1,7	Yes	Yes
Choleraesuis	C1	6,7:c:1,5	C1:c:1,5	Yes	Yes
Cubana	G	1,13,23:z_{29}:-	G:z_{29}:-	Yes	Yes
Ealing	O	35:g,m,s:-	O:g,m,s:-	Yes	Yes
Inverness	P	38:k:1,6	P:k:1,6	Yes	Yes
Kiel	A	1,2,12:g,p:-	A/D:g,p:-	Yes	Yes

Continued on following page

TABLE 3. *Salmonella* enterica strains used to validate the *Salmonella* genoserotyping array (SGSA) probes and probe patterns

Salmonella serovar	Serogroup as determined by traditional serotyping	Antigenic formula as determined by traditional serotyping	SGSA-identified antigenic formula	SGSA antigenic formula correlation with traditional serotyping	Correct SGSA serovar designation (based on known samples)
Lansing	P	38:i:1,5	P:i:1,5	Yes	Yes
Lille	C1	6,7,14:z_{38}:-	C1:z_{38}:-	Yes	Yes
Manhattan	C2	6,8:d:1,5	C2:d:1,5	Yes	Yes
Minnesota	L	21:b:e,n,x	L:b:e,n,x	Yes	Yes
Morotai	J	17:l,v:1,2	J:l,v:1,2	Yes	Yes
Moscow	D1	9,12:g,q:-	D:g,q/g,m,q:-	Yes	Yes
Naestved	D1	9,12:g,p,s:-	D:g,p,s:-	Yes	Yes
Ohio	C1	6,7,14:b:l,w	C1:b:l,w	Yes	Yes
Panama	D1	9,12:l,v:1,5	A/D:l,v/l,z_{13}:1,5	Yes	Yes
Pomona	M	28:y:1,7	M:y:1,7	Yes	Yes
Poona	G	13,22:z:1,6	G:z:1,6	Yes	Yes
Reading	B	4,12:e,h:1,5	B:e,h:1,5	Yes	Yes
Ruiru	L	21:y:e,n,x	L:y:e,n,x	Yes	Yes
Uganda	E	10:l,z_{13}:1,5	E:l,v/l,z_{13}:1,5	Yes	Yes
Westhampton	E1	3,10:g,s,t:-	E;g,s,t:-	Yes	Yes

tiate many of the serogroup A and D *Salmonella* serovars, alternative probes outside the *rfb* cluster have been designed (Table 1). Three alternative probes have been designed to differentiate *Salmonella* Enteritidis (RHS_E), *Salmonella* Gallinarum (RHS_GP), and *Salmonella* Pullorum (Pullorum), as they are genetically indistinguishable on the basis of the *fliC* allele. A probe was designed to identify a unique sequence within an *rhs*-like gene in *Salmonella* Enteritidis (SEN0271) due to the absence of an H1:g,m-specific probe on the array. The unique sequence within the *rhs*-like gene was discovered by comparing the whole-genome sequence of *Salmonella* Enteritidis (NC_011294) to sequences of other *Salmonella* serovars by the use of PanSeq software (32). Similarly, a unique *rhs*-like gene sequence (SG1045) of *Salmonella* Gallinarum (NC_011274) was used to design a serovar-specific probe. A *Salmonella* Pullorum-specific probe was designed using sequences within the *prt* gene (38).

Flagellar probes were designed using unique antigenic sequences within the phase 1 (*fliC*) and phase 2 (*fljB*) flagellar genes. Some probes have been designed to identify multiple antigens; thus, the SGSA has 42 flagellar probes which identify 41 antigens. The following flagellar antigens can be identified on the SGSA: a; b; c; d; e,h; e,n,x; e,n,z_{15}; e,n,x,z_{15}; f,g; f,g,t; f,g,s; g,m,s; g,m,t; g,t; g,m,q; g,q; g,p; g,p,s; g,s,t; g,t; g,z_{51}; i; k; l,w; l,v; l,z_{13}; l,z_{28}; m,t; r; [r]; y; z_{10}; z_{29}; z_{38}; z_4,z_{23}; z_6; z_{67} and 1,2; 1,5; 1,6; and 1,7 from the 1 complex.

An additional three probes were present for further identification, including the control *invA* probe that confirms the identity of *Salmonella* species, a probe for the Vi capsular antigen, and a probe that detects G-complex antigens containing the H1:f epitope (Table 1).

TABLE 4. Additional *Salmonella* serovars used to validate serogroup probes on the *Salmonella* genoserotyping array (SGSA)

Somatic serogroup of interest	Somatic antigen of interest	*Salmonella* serovar tested	Antigenic formula	Successful serogroup detection
V	O:44	Subsp. IV	44:z_4,z_{23}:-	Yes
V	O:44	Subsp. IIIa	44:z_4,z_{23}:-	Yes
Y	O:48	Subsp. IV	48:g,z_{51}:-	Yes
Y	O:48	Subsp. IIIb	48:k:e,n,x,z_{15}	Yes
O:58	O:58	Subsp. II	58:d:z_6	Yes
O:58	O:58	Subsp. II	58:l,z_{13},z_{28}:-	Yes

Validation of the array. A set of 82 *Salmonella* serovars of known serotype were assembled to validate the array for specificity and reproducibility. The strain set included a subset of 43 serovars representing a combination of the 15 most prevalent human and nonhuman serovars isolated in North America, the United Kingdom, and Austria. The remaining 39 *Salmonella* serovars are significantly less prevalent and were included to (i) confirm the efficacy and specificity of the antigens covered in the above-described subset (as 11 probes could detect multiple antigens), (ii) test potential cross-reactivity of probes with similar sequences, and/or (iii) test the detection of 10 additional serogroups and three flagellar antigens to increase the number of serovars detected by the SGSA. These samples included *Salmonella* subspecies II, IIIa, IIIb, and IV, which were tested only for determination of serogroup probe efficacy. Prior to being tested using the SGSA, each strain was serotyped using classical antibody-based methods and characterized using the White-Kauffmann-Le Minor scheme by the LFZ *Salmonella* OIE Reference Laboratory. Each serovar was tested a minimum of three times using the SGSA to ensure consistent results and to confirm the identification of the unique and reproducible serovar-specific probe patterns used in the macro design. Probe signals required for the detection of subset 1 *Salmonella* serovars were analyzed, and the signal mean, median, and 95% central interval range for each probe were calculated (see Table S1 in the supplemental material). Whereas most of the probe signal ranges differed only slightly, some of the ranges displayed a larger variation. For example, probe d-2 had a minimum signal intensity of 0.21 and a maximum intensity of 0.87. This variation in probe signal intensity may have been due to the efficiency of DNA isolation and target amplification, as these parameters were not standardized within the array protocol due to the robustness of the assay. Large variations within probe signal intensity ranges may also have been dependent on the efficiency of labeling and hybridization of individual samples. That said, the minimum

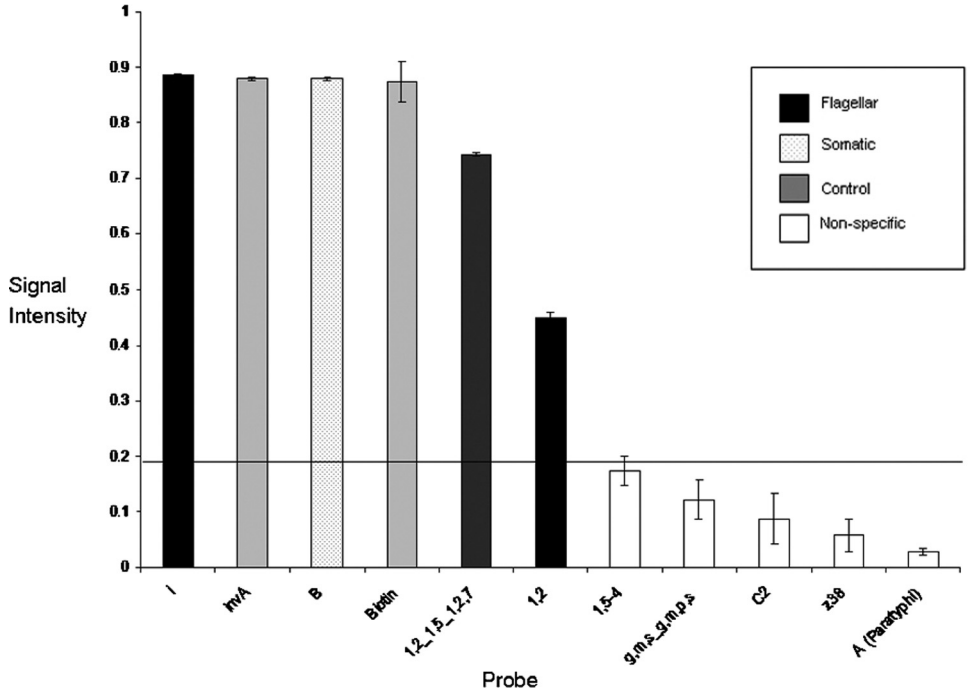

FIG. 1. Probe patterning and signal intensities for *Salmonella* Typhimurium. The graph depicts average signal intensities generated from triplicate probes used to identify a unique probe pattern for *Salmonella* Typhimurium on the SGSA array. The standard deviations of the means of the probe signal have been depicted using error bars. The assay cutoff for positive signals has been set at 0.2 (horizontal line), allowing clear differentiation of positive probe signals from background and nonspecific hybridization.

value for all signal ranges was above the positive signal cutoff value of 0.2; therefore, regardless of the range, all probe signals measuring above 0.2 are considered to represent a positive result.

The SGSA generated the correct serovar designations for the 76 known serovars in subset 1 and subset 2 of the validation panel and an antigenic formula consistent with the White-Kauffmann-Le Minor scheme for 74 of the 76 known serovars (Table 3). Complete antigenic formulas were not generated for *Salmonella* Enteritidis because of the absence of an H1:g,m probe on the array. Designation of serovars of *Salmonella* Enteritidis required the use of a probe pattern rather than positive confirmation of the presence of H1:g,m. Furthermore, the array identified the H2:e,n,z_{15}/e,n,x,z_{15} probe for *Salmonella* Rissen, a result which was not consistent with the monophasic formula generated with traditional serotyping; however, the presence of the H2 allele was confirmed by sequencing.

The majority of the antigenic formulas generated were identified using a combination of two to three probe signals representative of the serogroup and flagellar antigen(s). Exceptions occurred in some instances where additional probes were utilized for the identification of particular antigens as part of a serovar pattern. In the example of *Salmonella* Typhimurium, two probes were used for positive identification of the H2:1,2 antigen (Fig. 1), one unique to the H2:1,2 antigen and one shared with the H2:1,5 and H2:1,2,7 antigens. In the case of *Salmonella* Typhi, a positive signal for the Vi probe encoding the capsular virulence antigen was required as part of its pattern. Other exceptions included *Salmonella* serovars Pullorum, Gallinarum, and Enteritidis, whose serovar identification is based on the detection of additional probes in combination with their unique serovar probe patterns to differentiate them from each other.

E. coli EDL 933 and *C. jejuni* 11168 as negative controls were also tested using the SGSA. The *Salmonella*-specific control, *invA*, did not produce any signal when hybridized with either sample (data not shown).

Monophasic and rough *Salmonella*. Monophasic and rough *Salmonella* strains were tested to examine the correlation between traditional serotyping methods and the genoserotyping results. SGSA results classified 16 of the 25 monophasic samples as diphasic; therefore, only 36% of the serovar designations were consistent with those generated using traditional serotyping (data not shown). Furthermore, the SGSA identified serogroups from five of the eight rough mutants assessed, thus generating an antigenic formula consistent with the White-Kauffmann-Le Minor scheme for only 38% of samples tested. Antigens characterized as phenotypically absent using traditional serotyping methods but detected on the array were sequenced to confirm the presence of the probe sequence. In all cases, the probe sequence was present and therefore accounted for the difference between the genotypically and the phenotypically derived antigenic formulas (data not shown).

Blind study. Once validated, the SGSA was used to genoserotype a blind panel of 100 *Salmonella enterica* subsp. I samples provided by the AHVLA. Table S2 in the supplemental material illustrates the antigenic formula generated using the SGSA and the serovar designation for each sample based on antigen-specific probes and probe patterns.

In summary, the results from the 100 blind samples tested

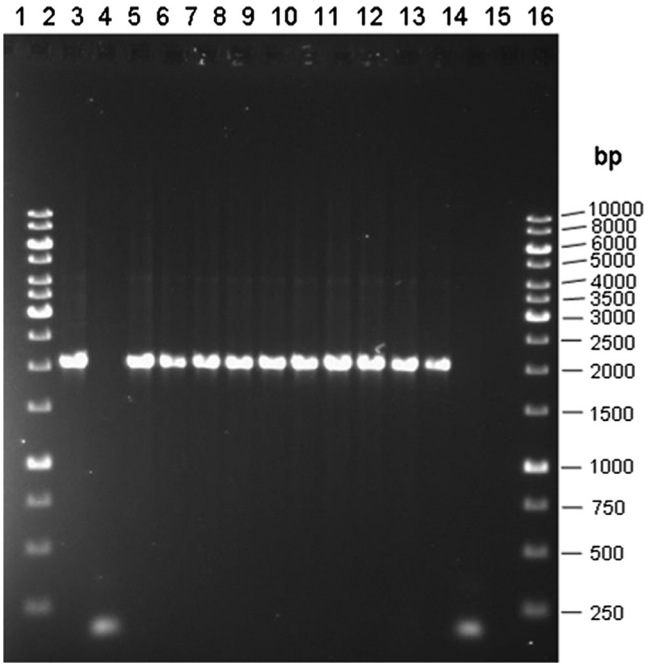

FIG. 2. Agarose gel of *pepT* amplicons from PCR used to differentiate blind samples identified as either *Salmonella* Enteritidis or *Salmonella* Nitra on the basis of SGSA results. Lanes 1 and 16 represent 1-kb DNA ladders (Fermentas, Burlington, ON). Lanes 2 (blind sample 20), 4 (blind sample 21), 5 (blind sample 22), 6 (blind sample 23), 7 (blind sample 24), 8 (blind sample 25), 9 (blind sample 26), 10 (blind sample 27), 11 (blind sample 28), and 12 (blind sample 29) show the 2,061-bp *pepT* band corresponding to *Salmonella* Enteritidis. Lane 3 (blind sample 60) shows the 150-bp band corresponding to *Salmonella* Nitra. Lane 13 represents the *Salmonella* Enteritidis control strain, and lane 14 represents the *Salmonella* Nitra control strain. Lane 15 is a negative PCR control.

revealed that the SGSA correctly identified all of the samples as *Salmonella* and was successful in identifying 92% of the antigens found within the blind samples. The SGSA generated an antigenic formula consistent with the White-Kauffmann-Le Minor scheme for 76 out of the 100 blind panel samples. The antigenic formula generated by the SGSA consists of a serogroup designation and does not include the detection of individual somatic factors. Thus, in instances in which the serogroup designation generated by the SGSA matched the serogroup identified using traditional serotyping methods, antigenic formulas were considered correct when analyzing the blind panel samples.

The inability of the array to differentiate *Salmonella* Enteritidis from the rarely isolated *Salmonella* Nitra accounted for 11 of the 24 samples not assigned a complete antigenic formula. All 11 samples were further analyzed using the *pepT* PCR for serovar confirmation. The *pepT* PCR was able to correctly identify 10 of the samples as *Salmonella* Enteritidis and 1 as *Salmonella* Nitra, thus increasing the number of correctly identified blind samples to 87 out of 100 (Fig. 2).

Partial antigenic formulas were generated for 13 of the 100 blind panel samples. Two of the blind panel samples could not be fully characterized due to the absence of antigen-specific probes on the array required to classify the O:47 antigen present in *Salmonella* Bergen and the H1:g,p,u antigen required to identify *Salmonella* Rostock.

The remaining 11 blind panel samples produced antigenic formulas indicative of two or more serovars due to single probes targeting multiple antigens and/or the inability of the array to detect single somatic factors. The remaining two samples were only partially characterized. The SGSA was not able to differentiate between *Salmonella* Blegdam and *Salmonella* Moscow, because there is currently only a single probe on the array that targets both H1:g,m,q and H1:g,q. Furthermore, the SGSA was unable to differentiate the two *Salmonella* Panama samples from *Salmonella* Houston, *Salmonella* India, *Salmonella* Itami, and *Salmonella* Koessen. In order to generate definitive designations for these serovars, the SGSA would need to incorporate the ability to target individual serogroup D somatic factors and the ability to differentiate between serogroups A and D and would also require individual probes specific for H1:l,v and H1:1,z_{13}. The four *Salmonella* Senftenberg samples tested as part of the blind panel could not be differentiated from *Salmonella* Westhampton or *Salmonella* Dessau because of a shared H1:g,t/g,s,t probe and the inability of the array to target individual serogroup E somatic factors. Three of the blind panel samples (two *Salmonella* Dublin samples and one *Salmonella* Kiel sample) were only partially identified due to the lack of a probe able to differentiate between serogroups A and D.

DISCUSSION

A *Salmonella* genoserotyping array (SGSA) has been developed for utilization as a rapid and economical tool to serotype *Salmonella.* Surveillance tools such as these are needed to help identify outbreaks and raise awareness among health professionals, food producers, and consumers. The implementation of a simple, robust, and cost-effective genoserotyping array may prompt those in the food industry, clinicians, and reference and small private laboratories to perform more testing, in turn increasing the reporting of *Salmonella* and enhancing surveillance data (25).

The SGSA is an attractive alternative to traditional serotyping, because it benefits from a simple, less expensive protocol employing a variable platform of single tubes or a 96-well plate format with automated data analysis for nonsubjective serovar designation. The Arraytube platform (Allere, Inc.) requires inexpensive equipment and reagents that can be easily incorporated into both diagnostic and research laboratories; however, the major cost benefit to using the SGSA comes from the reduction of the technician time required to process a sample. With the use of the SGSA, *Salmonella* isolates involved in outbreaks can be genoserotyped in 1 day versus the minimum of 3 to 4 days required for traditional serotyping, expediting downstream subtyping with methods such as pulsed-field gel electrophoresis (PFGE) and phage typing to aid in source attribution and widespread tracking. Other methods that are alternatives to traditional serotyping have been developed; however, many are not amenable to high-throughput platforms, some are much less cost-effective, and most do not generate an antigenic formula consistent with that of the globally recognized White-Kauffmann-Le Minor scheme.

To further optimize the assay for speed and simplicity, the

use of cell lysates in place of purified genomic DNA as a template for the multiplex target amplification reaction was tested (data not shown). The use of cell lysates is considered advantageous, as they are amenable to high-throughput laboratories and substantially decrease the cost of the protocol. Our results indicated that genomic DNA generated target amplification of greater consistency and, subsequently, SGSA results of greater reliability. Although the cell lysates were not included in the final protocol, the results showed promise, and since several groups have demonstrated the success of optimization of multiplex PCRs for use in *Salmonella* molecular typing methods, the use of lysates in investigations of future layouts is planned (7, 22, 23).

A study describing a multiplex PCR assay designed to detect all of the *Salmonella* subspecies, including the species *S. bongori*, was recently reported (33). Adaptation of this method is planned to be investigated as an additional multiplex reaction in the SGSA protocol, and subspecies-specific probes are to be added to the array for verification of all six distinct subspecies. Additionally, the next layout will have an *shdA*-specific probe for use in further confirmation of *Salmonella* subspecies I serovars (29).

The SGSA described here assesses mainly subspecies I *Salmonella* isolates due to their prevalence in human clinical infections (50). As an exception, a subspecies IIIb O61:k:1,5,(7) isolate was examined since it represented the third-most-prevalent animal isolate in the United Kingdom in 2007 (54). In order to test this serovar, our array also includes unique probes specific to subspecies IIIb H1:k and H2:1,5,(7).

The subspecies I *Salmonella* serovars tested in this study represent only a small subset of the 1,532 *Salmonella* subsp. I serovars classified in the White-Kauffmann-Le Minor scheme; however, the 59 targets detected by the array are theoretically capable of identifying 985 of the subspecies I *Salmonella* serovars. Further testing of *Salmonella* subsp. I serovars should result in a more accurate depiction of the array capabilities on the basis of the allelic variation within the gene targets.

Currently, 41 of 114 flagellar antigens are represented on the array, along with serogroup-specific probes for 18 of 46 *Salmonella* O serogroups. Publically available sequence data were used for 12 of the 21 serogroups: A (O:2) (37), B (O:4) (27), C1 (O:6,7) (34), C2 (O:8) (6), D1 (O:9) (37), E (O:3) (65), G (O:13) (17) H (O:6,14) (18), J (O:17) (17), K (O:18) (17), O (O:35) (64), and O58 (9). Six *rfb* cluster sequences were obtained by our laboratory: L (O:21), M (O:28), P (O:38), V (O:44), Y (O:48) and O61. Additionally, five serogroups [F (O:11), I (O:16), R (O:40), U (O:43), and Z (O:50)] have been selected for sequencing for inclusion on the next SGSA layout. The SGSA is based on the genes responsible for O-antigen and flagellar biosynthesis; thus, in most cases, it provided antigenic formulas and subsequent serovar designations comparable to those determined by traditional methods. *Salmonella* serovars Enteritidis, Gallinarum, and Pullorum all required the use of additional genes outside the traditional typing scheme as part of their unique pattern to ensure concise serovar identification. Other genes included in the SGSA are the *invA* gene (52), used as a *Salmonella*-specific control to ensure species detection, and the Vi antigen encoded by the *viaB* gene used in the detection of some serovars of *Salmonella* Typhi (44) and, in rare cases, *Salmonella* Dublin (46) and *Salmonella* Paratyphi C (10). In most instances, the SGSA produced an antigenic formula that corresponds to a unique serovar classified within the White-Kauffmann-Le Minor scheme. However, the inability of the array to discriminate between single somatic factors resulted in the generation of an antigenic formula common to two or more serovars in a few instances. Table S2 in the supplemental material details the antigenic formulas generated by the SGSA and the alternative serovar possibilities. For example, the table illustrates that the SGSA generated an antigenic formula of G:z:1,6, which represents both *Salmonella* Poona (1,13,22:z:1,6) and *Salmonella* Farmsen (13,23:z:1,6). Due to the lack of probes specific for single O-antigen factors defined within serogroup G (1, 13, 22, and 23), the SGSA was not able to differentiate between the two serotypes. Blind samples were found to be in agreement with traditional serotyping results as long as the serogroup designation was correct; however, all of the possible serovars based on somatic factors are listed in Table S2 in the supplemental material. This limitation is shared by all other molecular serotyping methods that rely on serogroup detection rather than individual factors (16, 49).

Four of the blind panel samples, *Salmonella* Bovismorbificans, *Salmonella* Hadar, *Salmonella* Manhattan, and *Salmonella* Newport, were unable to be differentiated from *Salmonella* Hindmarsh, *Salmonella* Istanbul, *Salmonella* Yovokome, and *Salmonella* Bardo, respectively, on the basis of the serogroups. According to the World Health Organization (WHO), these serovars, while regarded as distinct in the White-Kauffmann-Le Minor scheme, are considered to be correctly identified, as colonial form variations (the variable expression of minor antigens by different single-colony picks from the same strain) may occur in some serogroup C2 serovars (21).

Salmonella serovars Enteritidis (serogroup D) and Nitra (serogroup A) have antigenic formulas that differ only in their serogroups. The *rfb* region of *Salmonella* Paratyphi A, a representative of serogroup A, has been shown to differ from serogroup D *rfb* regions by only a minor modification resulting from a frameshift mutation (37). Together with *Salmonella* Paratyphi A, *Salmonella* serovars Nitra, Kiel, and Koessen represent the only serogroup A serovars. A DNA sequence alignment of the *prt* gene from *Salmonella* Paratyphi A, *Salmonella* Nitra, and four serogroup D sequences highlighted a 2-bp mismatch within the CDP-paratose synthase (*prt*) gene of *Salmonella* Paratyphi A (16). This region was targeted with a *Salmonella* Paratyphi A-specific probe to differentiate *Salmonella* Paratyphi A from the remaining serogroup A serovars and all serogroup D serovars. Currently, there is not a serogroup probe able to distinguish the rest of the serogroup A and D isolates. *Salmonella* Nitra and *Salmonella* Kiel are rarely isolated and have been shown by PFGE cluster analysis to demonstrate molecular similarities of between 81% and 100% to the related serogroup D serovars, whereas the *Salmonella* Paratyphi A isolates showed only 68% similarity (16). These results are in agreement with other microarray data that have shown that serogroup A isolates are variants of serogroup D (40, 51). The SGSA generated the antigenic formula A/D:-:-RHS-E for two of the blind samples and was not able to decipher the data further to produce a single serovar designation. The combination of our inability to differentiate most serogroup A and D serovars, the lack of a g,m-positive probe, and the fact that the alternative *Salmonella* Enteritidis gene

target is also found in *Salmonella* Nitra left these two serovars indistinguishable. In instances such as this, prevalence data can be examined that may support the likelihood of the serovar designation through the comparison of isolation frequency statistics. According to Canadian data from the National Enteric Surveillance Program that were collected between 2004 to 2009, *Salmonella* Enteritidis accounted for 27.55% of all Canadian *Salmonella* isolates, whereas *Salmonella* Nitra isolates were not reported during that same time period (11, 12, 13). The use of prevalence data would suggest that the 11 samples designated A/D:-:- RHS-E all represent *Salmonella* Enteritidis; within the blind panel, however, one of the samples designated A/D:-:- RHS-E was in actuality *Salmonella* Nitra. We propose that having prevalence data for geographic location can be of interest; however, this is not used as part of the final serovar designation derived from the SGSA or its macro, as it can lead to misidentification. Although the positive predicted value is extremely high when using the prevalence data to predict a serovar, it is important that, in critical situations, samples be sent for traditional serotyping to confirm the serovar designation. Fitzgerald et al. published a similar result, stating that serogroup A isolates other than *Salmonella* Paratyphi A tested positive with their serogroup D probe on their bead-based suspension array but noting that they were extremely rare serovars (16). Although the SGSA was unable to discriminate between these two serovars, a newly designed *pepT* PCR was used to confirm that 10 of the 11 samples were *Salmonella* Enteritidis and one of the blind samples was serovar Nitra. The *pepT* PCR is to be added to the current sample preparation protocol, and the next layout of the SGSA is to be expanded to include *pepT* probes to provide direct differentiation of *Salmonella* Enteritidis from *Salmonella* Nitra.

We are currently subjecting *Salmonella* serovars Kiel and Koessen to whole-genome pyrosequencing in order to identify serovar-specific genes for definite identification of these closely related serovars on the array.

In other instances, the SGSA was unable to definitively derive a single serovar designation because a small number of probes on the array are specific for multiple antigens. For example, the array was unable to differentiate between *Salmonella* Blegdam and *Salmonella* Moscow, as currently there is only a single probe that detects both the H1;g,q and H1:g,m,q antigens, which have highly homologous gene sequences. These results align with the sequencing results generated in a previous study by Sonne-Hanson and Jenabian in 2005 (60). Sequence identity and high sequence similarity have often been reported as obstacles to the development of probes for the differentiation of antigens within the g-complex, as their sequences are highly homologous and sequence variation is often seen among single alleles (48). Currently, both *Salmonella* Blegdam and *Salmonella* Moscow are sequenced in order to identify serovar specific-probe targets. More sequence data from *Salmonella* serogroup D serovars would also aid in the development of an SGSA scheme to more easily differentiate serogroup A from serogroup D serovars.

The SGSA currently utilizes shared probes to identify H1: m,t/g,m,t, H1:g,s,t/g,t, H1:l,v/l,z_{13}, H1:g,q/g,m,q, and, finally, H1:z_6/z_{67}.

Antigens present in the blind samples but not represented on the array are H1:g,p,u and serogroup O:47, and their absence resulted in an only partial antigenic formula identification (see Table S2 in the supplemental material). With the addition of these antigens to the array, it should be possible to identify serovars *Salmonella* Rostock and *Salmonella* Bergen along with other serovars sharing these antigens. Ongoing sequencing continues to reveal additional single nucleotide polymorphisms among H1 and H2 alleles and will be highlighted with newly designed probes on future layouts. Lastly, the SGSA generated an incorrect antigenic formula (C1:f,g: e,n,x,z_{15}) for *Salmonella* Rissen in the validation panel, which included H2:e,n,z,x_{15}, and another incorrect antigenic formula (D:g,p:1,5) for *Salmonella* Dublin in the blind panel, which included H2:1,5 (Table 3 and Table S2 in the supplemental material, respectively). The *fljB* gene from the *Salmonella* Rissen strain tested on the SGSA array was sequenced, and the results confirmed the presence of our e,n,z,x_{15} probe sequence. These results supported data from a 2010 study done by Jong et al. in which amplification of an *fljB* gene from *Salmonella* Rissen yielded a positive PCR fragment (28). Similarly, the *fljB* gene from the *Salmonella* Dublin confirmed the presence of our 1,5 probe sequence.

Several monophasic and rough *Salmonella* serovars were tested on the array in order to compare the genetically derived antigenic formula generated by the SGSA to the antigenic formula identified by traditional antibody-based methods. The SGSA identified alleles that were not phenotypically expressed in 64% of monophasics and 62% of rough serovars. Note that the lack of Phase 2 flagellar expression of some serologically monophasic strains can be due to a variety of mechanisms, ranging from point mutations to partial or complete deletions in *fljB* and adjacent genes (42), and therefore may be missed by the use of a single probe on the array. Although the SGSA generated antigenic formulas that did not always correlate with traditional serotyping results, this ability could be advantageous, as it identifies the uncharacterized allele (8).

The SGSA has 66 probes printed in triplicate, and the platform can accommodate an additional 156 probes in triplicate or up to 268 probes if printed in duplicate. Future layouts are planned to include newly sequenced O serogroup-specific probes, somatic factor probes, new phase 1 and phase 2 flagellum-specific probes (41), and new serovar-specific probes to aid in finite designation of serovars. Moreover, future layouts may also include subspecies-specific probes and relevant *Salmonella* virulence and antimicrobial resistance markers for additional surveillance information (24). Further studies are planned to include large-scale multiple site validation, together with a comparison to traditional serotyping as an assessment of the feasibility of implementing the SGSA as a public health tool to aid in *Salmonella* outbreak identification and surveillance.

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