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Rapid Discrimination of *Salmonella* Isolates by Single-Strand Conformation Polymorphism Analysis

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ABSTRACT

A molecular typing technique was developed for the differentiation of *Salmonella* isolates based on single-strand conformation polymorphism (SSCP) analysis of amplicons generated by PCR. Amplicons from parts of the *fimA* (both the 5′ and 3′ ends), *mdh, invA,* and *atpD* genes were generated separately from a panel of *Salmonella* strains representing *Salmonella bongori,* and four subspecies and 17 serovars of *Salmonella enterica.* These amplicons were subjected to SSCP analysis for differentiation of the salmonellae on the basis of different conformational forms arising due to nucleotide sequence variations in the target genes. Several distinct SSCP banding patterns (a maximum of 14 each for *atpD* and *fimA* 3′ end) were observed with this panel of *Salmonella* strains for amplicons generated from each target gene. The best discrimination of *Salmonella* subspecies and serovar was achieved from the SSCP analysis of a combination of at least three gene targets: *atpD, invA,* and either *mdh* or *fimA* 3′ end. This demonstrates the applicability of SSCP analysis as an important additional method to classical typing approaches for the differentiation of foodborne *Salmonella* isolates. SSCP is simple to perform and should be readily transferable to food microbiology laboratories with basic PCR capability.

Members of the bacterial genus *Salmonella* are a leading cause of food- and waterborne illness worldwide. Infection occurs after the ingestion of contaminated food originating from a wide variety of sources *(2),* or after drinking sewage-contaminated water *(14).* Traditional techniques for the identification of *Salmonella* in foods, water, and clinical samples are based on their biochemical characteristics and serotyping, with more than 2,000 serovars having been identified *(3).* Most of the infections occurring in human and warm-blooded animals are caused by specific serogroups of *Salmonella enterica* subsp. *enterica (21).* The ability to differentiate *Salmonella* isolates derived from foods (and related samples) is an important element of risk-based inspection strategies aimed at controlling contamination of the food supply, and is especially useful in establishing the identity of individual isolates for trace back investigation purposes.

While very useful as a first step in the identification of salmonellae, serotyping has limited discriminatory power and requires the availability of an extensive assortment of high-quality antisera raised against specific O and H antigens. Other tools have been developed for the differentiation of salmonellae, including phage typing, ribotyping, random amplification of polymorphic DNA, restriction length polymorphism, and pulsed-field gel electrophoresis *(5–7, 23, 28).* Although phage typing can be useful in distinguishing different strains within serovars, this method does not always allow discrimination between outbreak-related and unrelated isolates *(19).* Discriminatory molecular typing techniques such as ribotyping and pulsed-field gel electrophoresis are labor-intensive, time-consuming procedures, and automated ribotyping requires a substantial investment in capital equipment and carries a high cost for consumable materials. Another molecular approach, multilocus sequence typing (MLST), involves the sequencing of strains for several key genes, each of which are amplified separately by PCR. Strains are often differentiated based on single-nucleotide mutations in DNA sequences. MLST studies that have used combinations of three to seven genes (e.g., *fimA, mdh, manB, aceK, gyrB, flicC,* and/or *atpD*) have shown that this molecular approach provides a high degree of discrimination between *Salmonella* strains *(9, 24, 27).* One limitation of MLST for the routine identification of isolates in the food microbiology laboratory is the requirement for sequencing multiple gene-specific amplicons for each test isolate, which are costly and time-consuming. Single-strand conformation polymorphism (SSCP) analysis represents a more cost- and time-effective approach than MLST to characterize and compare bacterial isolates *(11, 25, 29).*

For SSCP analyses, amplicons (ranging in size from 100 to 450 bp) are electrophoretically separated in nondenaturing gels, based on sequence-dependent secondary structural conformations arising in the DNA strands *(10, 11).* SSCP is simple to perform and does not require a substantial investment in capital equipment, providing rapid turnaround times for the generation of results in the identification and genetic characterization of pathogens *(25, 29).* This molecular technique has been used in the identification

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TABLE 1. *SSCP profiles of the different* Salmonella *isolates representing different species, subspecies, and serotypes*

Salmonella species, subspecies, or *S. enterica* serotype	Source[a]	SSCP profiles for:				
		fimA				
		(5′ end region)	(3′ end region)	*mdh*	*invA*	*atpD*
S. bongori	ATCC 43975	NA[b]	NA	2c	2d	1e
S. enterica subsp. *salamae*	ATCC 43972	1a	1b	1c	1d	1e
S. enterica subsp. *arizonae*	ATCC 13314	7a	10b	8c	10d	13e
S. enterica subsp. *indica*	ATCC 43976	NA	2b	3c	3d	2e
S. enterica Agona	OLC (poultry feed)	2a	3b	4c	4d	3e
S. enterica Braenderup	OLC (egg environmental)	3a	4b	5c	5d	4e
S. enterica Fresno	OLC (feed sample)	2a	5b	5c	6d	5e
S. enterica Newington	ATCC 29628	2a	6b	4c	7d	6e
S. enterica Thomasville	OLC (feed sample)	4a	7b	5c	7d	7e
S. enterica Senftenberg	ATCC 8400	2a	5b	4c	7d	8e
S. enterica Cerro	OLC (egg environmental)	2a	5b	4c	7d	9e
S. enterica Alachua	OLC (feed sample)	5a	8b	5c	8d	10e
S. enterica Johannesburg	OLC (feed sample)	2a	5b	5c	9d	11e
S. enterica Minnesota	ATCC 9700	2a	5b	5c	9d	4e
S. enterica Bleadon	OLC (feed sample)	6a	9b	6c	7d	12e
S. enterica Montevideo	ATCC 8387	2a	5b	7c	7d	6e
S. enterica Enteritidis	ATCC 13076	2a	11b	4c	8d	4e
S. enterica Enteritidis	LFZ (SE 010-12)	2a	11b	4c	8d	4e
S. enterica Enteritidis	LFZ (SE 016-13)	2a	11b	4c	8d	4e
S. enterica Enteritidis	LFZ SE 018-14	2a	11b	4c	8d	4e
S. enterica Enteritidis	LFZ (SE 052-16)	2a	11b	4c	8d	4e
S. enterica Enteritidis	LFZ (SE 183-19)	2a	11b	4c	8d	4e
S. enterica Poona	LFZ (LC-81-160-136)	5a	13b	5c	8d	14e
S. enterica London	OLC (feed sample)	2a	12b	4c	7d	10e
S. enterica Rubislaw	ATCC 10717	2a	5b	9c	11d	5e
S. enterica Typhimurium	ATCC 14028	5a	14b	5c	4d	7e

[a] ATCC, American Type Culture Collection; OLC, Ottawa Laboratory (Carling) (Canadian Food Inspection Agency); LFZ, Laboratory for Foodborne Zoonoses (Public Health Agency of Canada).
[b] NA, no PCR amplification.

of various bacteria *(1, 12, 26, 29)* and parasitic invertebrates *(13, 15).* Some *S. enterica* isolates have previously been analyzed by SSCP to examine the genetic variability of the *rpoS* gene *(16)* and to demonstrate mutations in the *gyrA* gene in a drug-resistant strain of *Salmonella* Typhi *(30).* The characterization of *Salmonella* strains, using SSCP analysis has previously been reported *(18),* and the potential of this technique to differentiate isolates at the serovar level has been described *(20).* These studies focused on the analysis of restriction length polymorphism fragments *(20)* and amplicons *(18)* from a single gene. In the present study, we adapted SSCP analysis to the separation of PCR-generated amplicons from the malate dehydrogenase (*mdh*), the invasion protein A (*invA*), the ATP synthase beta subunit (*atpD*), and two different regions of the fimbrial A (*fimA*) genes, and examined the use of combinations of amplicons from the different target genes to differentiate salmonellae at the species, subspecies, and serovar levels.

MATERIALS AND METHODS

Bacterial isolates. The *Salmonella* isolates used in this study (Table 1) were obtained from the American Type Culture Collection (Manassas, Va.); the culture collection of the Ottawa Laboratory (Carling), Canadian Food Inspection Agency (Ottawa, Ontario, Canada); and the culture collection of the Laboratory for Foodborne Zoonoses (Public Health Agency of Canada, Guelph, Ontario, Canada). Bacteria were grown in Trypticase soy broth cultures (ca. 10^9 CFU/ml; Difco, Becton Dickinson, Sparks, Md.), using conditions described previously *(31).* Genomic DNA (gDNA) was extracted from each bacterial culture, using the Wizard Genomic DNA purification kit (Promega, Madison, Wis.) according to the manufacturer's instructions. DNA concentration was quantified using a microplate fluorescence assay *(17).*

Primers design and PCR amplification. A 252- to 396-bp region of *fimA, mdh, invA,* and *atpD* genes were each selected as targets for PCR and SSCP. We designed primers for PCR, based on comparisons of DNA sequences for *Salmonella* deposited in GenBank for *fimA* (accession numbers EF113938, EF113937, EF113936, and AE008721), *mdh* (NC006905, AE008854, EF113946, and EF113947), and *atpD* genes (DQ838117, DQ838142, DQ838149, DQ095352, DQ09536, DQ095369, DQ095375, and DQ095382). For the *invA* gene, amplicons were initially generated and sequenced with primers described previously *(22).* Subsequently, new internal primers were designed to amplify a partial region (252 bp) of the *invA* gene. BLASTn searches (http://www.ncbi.nlm.nih.gov/BLAST/) were conducted to confirm sequence specificity of the designed primers for *Salmonella.* The sequences of the primers used for PCR are listed in Table 2. Primers fimA-1 forward and invA forward are those of Sukhnanand et al. *(24)* and Rahn et al. *(22),* respectively. All PCR reactions were conducted in 50-μl volumes containing 2 μl of

TABLE 2. *Primers and conditions used in the amplification of different genes in* Salmonella

Gene	Primer name: sequence 5′–3′	PCR conditions
fimA 5′ end	fimA-1 forward: TCAGGGGAGAAACAGAAAACTAAT[a] fimA-1 reverse: AAGCTGGCGGTACGGTATTG	94°C for 4 min; 30 cycles of 95°C for 30 s, 57.1°C for 30 s, and 72°C for 30 s; 72°C for 7 min
fimA	3′ end fimA-2 forward: CAATACCGTACCGCCAGCTT fimA-2 reverse: TGGCATTCCCTGACGGGA	94°C for 4 min; 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s; 72°C for 7 min
mdh	mdh forward: TTACCTTTCAGCTCTGCAAC mdh reverse: CGTTGATTTGAGCCACATCC	94°C for 4 min; 35 cycles of 95°C for 60 s, 53°C for 60 s, and 72°C for 60 s; 72°C for 7 min
invA	invA forward: TTATTGGCGATAGCCTGGCGGT invA reverse: TCATCGCACCGTCAAAGGAACC[b]	94°C for 4 min; 30 cycles of 95°C for 45 s, 60°C for 45 s, and 72°C for 45 s; 72°C for 7 min
atpD	atpD forward: TAGTTGACGTCGAATTCCCTCAGG atpD reverse: TCGATAACTTTGATACCGGTTTCC	94°C for 4 min; 30 cycles of 95°C for 45 s, 50°C for 45 s and 72°C for 45 s; 72°C for 7 min

[a] From Sukhnanand et al. *(24)*.
[b] From Rahn et al. *(22)*.

genomic DNA (50 to 100 ng), 1× GoTaq reaction buffer (Promega), 2.0 mM $MgCl_2$ (1.5 mM for *mdh*), 0.4 mM dNTP, 1.25 U of *Taq* DNA polymerase (Promega), and 0.2 μM of each primer. PCR reactions were performed in a GeneAmp PCR system 9700 thermocycler, using the conditions listed in Table 2. Amplicons were separated on 1.5% (wt/vol) agarose and 65 mM Tris-HCl–22.5 mM boric acid–1.25 mM EDTA (pH 8.7) gels and stained with SYBR Gold (Invitrogen, Burlington, Ontario, Canada) and photographed using an SPSS 2000 gel documentation system (Alpha-InnoTec, Kassendorf, Germany) with UV transillumination.

SSCP analysis and DNA sequencing. Each amplicon (10 μl) was mixed with 5 μl of tracking dye (Promega), denatured at 95°C for 5 min, then immediately snap cooled on ice for 1 min. A total of 5 μl of each sample was loaded into the well of a precast GMA Wide Mini S gel (Elchrom Scientific AG, Cham, Switzerland) and subjected to electrophoresis in a horizontal SEA 2000 electrophoresis apparatus containing 1× Tris-acetate-EDTA buffer (Elchrom Scientific) for 16 to 18 h at 74 V. A temperature-controlled circulating water bath was connected to the electrophoretic apparatus to maintain a constant temperature of 8°C. SSCP gels were then stained with SYBR Gold for 40 min and destained in H_2O for 20 min prior to examination with UV light. Selected amplicons from the *fimA, mdh* (10 *S. enterica* serotypes), and *invA* genes (all *Salmonella* strains) were purified using Wizard PCR Prep minicolumns (Promega). These were then subjected to automated sequencing using forward and reverse primers in separate reactions to verify that isolates with different SSCP profiles differed in DNA sequence for a specific gene region. For each gene, the sequences obtained were aligned manually.

RESULTS

Parts of the *fimA, mdh, invA,* and *atpD* genes were amplified from genomic DNA extracts of 26 *Salmonella* isolates representing two species (*Salmonella bongori* and *S. enterica*), four subspecies of *S. enterica,* and 17 serovars of *S. enterica* subsp. *enterica,* including several different poultry, egg, and environmental isolates of *S. enterica* subsp. *enterica* serovar Enteritidis (Table 1). Amplicons generated for the different target genes from the panel of *Salmonella* strains were analyzed by standard agarose gel electrophoresis. All strains produced amplicons of the following molecular sizes for each gene (exceptions noted in parentheses): *invA,* 252 bp; *fimA* (5′ end), 346 bp (352 bp for *S. enterica* subsp. *arizonae,* and no amplicon for *S. bongori* and *S. enterica* subsp. *indica*); *fimA* (3′ end), 396 bp (no amplicon for *S. bongori*); *mdh,* 362 bp; and *atpD,* 300 bp (not shown).

SSCP analyses of the PCR products for each gene revealed substantial variability in the banding patterns among the different *Salmonella* strains. The total number of distinct banding patterns observed for each gene was as follows: *fimA* (5′ end), 7 profiles; *fimA* (3′ end), 14 profiles; *mdh,* 9 profiles; *invA,* 11 profiles; and *atpD,* 14 profiles (Table 1). The different single-stranded DNA banding profiles obtained for the *invA* and *atpD* genes with amplicons from a selection of salmonellae are shown in Figures 1 and 2, respectively. The banding patterns for all of the *Salmonella* strains were reproducible upon reamplification of the target genes, which was then followed by SSCP analysis (not shown). *S. bongori* and the different subspecies of *S. enterica* examined gave unique SSCP patterns for most genes, allowing their differentiation on the basis of the pattern obtained for either of the individual genes (except for *atpD,* for which *S. bongori* and *S. enterica* subsp. *salamae* had the same banding pattern). The *fimA* (3′ end) and *atpD* genes displayed the highest degree of banding pattern diversity, with a total of 14 profiles occurring among the different *Salmonella* species, subspecies, and serovars, and 11 profiles among the different *S. enterica* subsp. *enterica* serovars (Table 1). Serovars not amenable to differentiation by either the *fimA* (3′ end) or *atpD* genes individually were for *fimA* (3′ end): Fresno, Senftenberg, Cerro, Johannesburg, Minnesota, Montevideo, and Rubislaw; and for *atpD*: Minnesota, Braenderup, Montevideo, Newington, Enteritidis, Fresno, and Rubislaw. The combined SSCP results for *fimA* (3′ end) and *atpD* provided greater discrimination between serovars, with only three (Minnesota, Montevideo, and Rubislaw) remaining indistinguishable. When the SSCP results from *fimA* (3′ end) and *atpD* were combined with either the *mdh* or the *invA* genes, all of the serovars could be differentiated from one another (Table 1). Thus, SSCP using a combination of three genes (*invA, atpD,* with either *mdh* or *fimA* 3′ end) resulted in the differentiation of all the *Salmonella* isolates examined at the species, subspecies, and serovar levels. No variation in profile was detected with any of the gene targets for several different isolates of

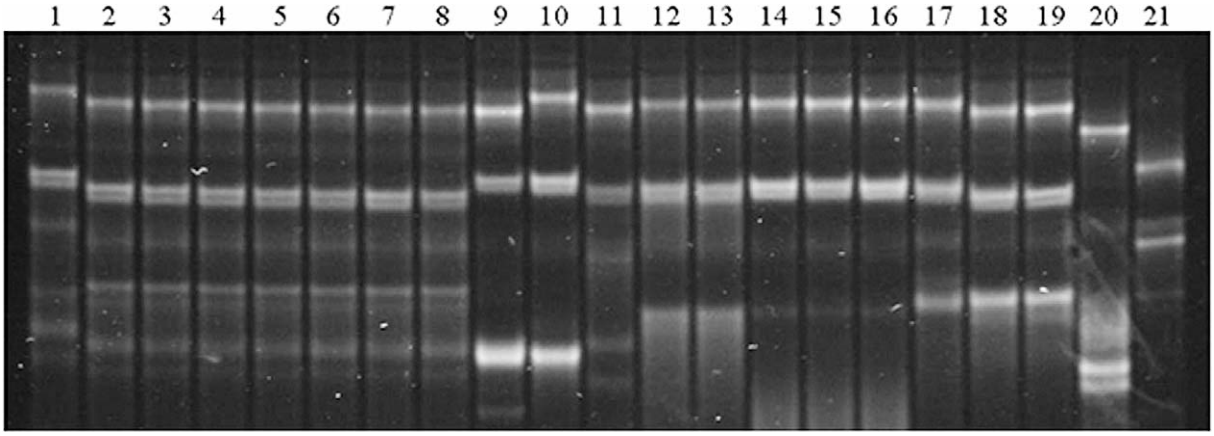

FIGURE 1. *SSCP profiles of* invA *amplicons from* Salmonella *isolates. Lane 1,* Salmonella *Braenderup; lane 2,* Salmonella *London; lane 3,* Salmonella *Senftenberg; lane 4,* Salmonella *Cerro; lane 5,* Salmonella *Bleadon; lane 6,* Salmonella *Newington; lane 7,* Salmonella *Thomasville; lane 8,* Salmonella *Montevideo; lane 9,* S. enterica *subsp.* arizonae*; lane 10,* Salmonella *Rubislaw; lane 11,* Salmonella *Fresno; lane 12,* Salmonella *Minnesota; lane 13,* Salmonella *Johannesburg; lane 14,* Salmonella *Enteritidis; lane 15,* Salmonella *Poona; lane 16,* Salmonella *Alachua; lane 17,* S. enterica *subsp.* indica*; lane 18,* Salmonella *Typhimurium; lane 19,* Salmonella *Agona; lane 20,* S. enterica *subsp.* salamae*; lane 21,* Salmonella bongori.

S. enterica Enteritidis, though the banding pattern for this serovar was distinguishable from the other serovars.

The sequence-dependent variability of the SSCP profiles is evidenced by a strong correlation with variations in the nucleotide sequence data obtained for the different target gene amplicons. Table 3 presents the nucleotide sequence data (and the corresponding SSCP profile designation) obtained for a portion of the *invA* gene of various salmonellae. As an example of the correlation between the SSCP profile and nucleotide sequence data for *invA,* the identical SSCP patterns displayed for Senftenberg and Cerro (Fig. 1, lanes 3 and 4, respectively) are matched by the lack of nucleotide sequence variability among these serovars (Table 3). Furthermore, the single-base divergence in the *invA* gene noted for Braenderup and London (Table 3) was reflected by differences in the SSCP patterns (Fig. 1, lanes 1 and 2, respectively). For the *atpD* gene, two different SSCP patterns were displayed for Agona and Braenderup, with a corresponding one-base difference occurring between these two serovars (based on a comparison of nucleotide sequence data for these serovars, GenBank accession numbers DQ838142 and DQ838149, respectively). On the other hand, the Braenderup and Enteritidis serovars produced the same unique SSCP pattern for the *atpD* gene, yet no nucleotide sequence difference has been detected between these two serovars (comparing sequence data from GenBank, accession numbers DQ838149 and DQ095352). The degree of resolution achievable by SSCP analysis on the basis of the nucleotide sequence variation among the different *Salmonella* isolates was high, with nucleotide variabilities of 0.3% (*fimA,* 5′ end), 0.25% (*fimA,* 3′ end), 0.27% (*mdh*), 0.35% (*invA*), and 0.33% (*atpD*), resulting in the generation of unique banding patterns.

DISCUSSION

Previous studies have explored the use of SSCP to discriminate *S. enterica* subsp. *enterica* serovars *(18, 20).* In the study by Nair et al. *(20),* a panel of *Salmonella* strains representing 10 different serovars was examined by SSCP analysis of restriction endonuclease-digested amplicons of the *groEl* gene. Using this approach, they were able to demonstrate the discriminatory power of SSCP analysis at both the inter- and intraserovar level. Lim et al. *(18)* examined the utility of SSCP targeting amplicons from a single region

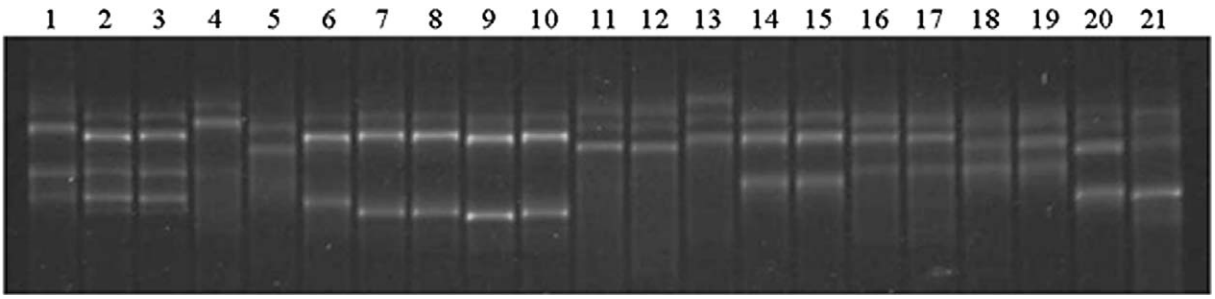

FIGURE 2. *SSCP profiles of* atpD *amplicons from* Salmonella *isolates. Lane 1,* Salmonella *Bleadon; lane 2,* Salmonella bongori*; lane 3,* S. enterica *subsp.* salamae*; lane 4,* Salmonella *Cerro; lane 5,* Salmonella *Senftenberg; lane 6,* Salmonella *Agona; lane 7,* Salmonella *Braenderup; lane 8,* Salmonella *Minnesota; lane 9,* Salmonella *Poona; lane 10,* Salmonella *Enteritidis; lane 11,* Salmonella *Thomasville; lane 12,* Salmonella *Typhimurium; lane 13,* Salmonella *Johannesburg; lane 14,* Salmonella *Rubislaw; lane 15,* Salmonella *Fresno; lane 16,* Salmonella *Alachua; lane 17,* Salmonella *London; lane 18,* Salmonella *Montevideo; lane 19,* Salmonella *Newington; lane 20,* S. enterica *subsp.* arizonae*; lane 21,* S. enterica *subsp.* indica.

TABLE 3. *Variable sites in the aligned sequences of the partial* invA *gene region for representative isolates of* Salmonella

Salmonella isolate	SSCP profile	Alignment position: 58	70	79	82	85	88	106	118	121	127	133	139	142	163	172	181	190	191	196	220	223	226	230	238	250	256
London	7d	T	C	C	C	G	C	C	A	C	G	C	C	A	C	G	T	T	T	G	C	G	C	C	C	G	C
Senftenberg	7d	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cerro	7d	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Bleadon	7d	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Newington	7d	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Thomasville	7d	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Montevideo	7d	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Braenderup	5d	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.
Rubislaw	11d	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	T	.	.
Fresno	6d	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Minnesota	9d	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.
Johannesburg	9d	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.
Enteritidis	8d	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
Poona	8d	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
Alachua	8d	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
Typhimurium	4d	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Agona	4d	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
enterica subsp. *indica*	3d	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	T	.	.	A	.	.	.
enterica subsp. *salamae*	1d	G	.	.	.	.	.	.	.	T	.	.	.	.	A	.	.	C	.	.	.	.	.	.	.	.	.
enterica subsp. *arizonae*	10d	.	.	.	T	.	T	.	.	.	.	.	A	T	.	A	A	.	.	.	.	A	.	.	.	.	T
bongori	2d	G	T	T	T	C	T	A	G	T	T	T	.	T	.	.	.	.	C	A	.	.	T	.	.	A	T

of the *invA* gene. They detected 11 different SSCP profiles among a panel of salmonellae representing 56 different serovars.

Our results build on the previous work and present further evidence of the utility of SSCP analysis in the discrimination of *Salmonella* strains at the species, subspecies, and serotype levels. In the present study, the power for distinguishing among isolates, using SSCP analysis was improved with a combination of several target genes. Using two species of *Salmonella,* four subspecies of *S. enterica,* and 17 serovars of *S. enterica* subsp. *enterica,* we developed an SSCP scheme based on the analysis of five different target regions associated with housekeeping (*atpD* and *mdh*) and virulence genes (*fimA* and *invA*). Housekeeping genes are known to be relatively conserved among *S. enterica* serovars *(8, 9),* and the *mdh* and *atpD* genes have been shown to provide a good basis for subtype discrimination by MLST when used together or in combination with other genes, such as the virulence gene *fimA (9, 24, 27).* In the latter studies, nucleotide sequence variability in well-characterized virulence genes (e.g., *invA*) has been found among different strains *(18, 27).* The *invA* gene provides a good target in any scheme for the identification of salmonellae since it has been shown to be specific for the genus *Salmonella* and is present in most *S. enterica* serovars examined *(4, 22).*

In the present study, *S. bongori* and the different subspecies of *S. enterica* exhibited distinct SSCP patterns for most of the gene targets (except *atpD*). Therefore, it was possible to differentiate all of the isolates at the species and subspecies levels, using any of the gene targets other than *atpD.* However, the differentiation of salmonellae at the serovar level required a combination of at least three gene targets. The *fimA* (3′ end) and *atpD* genes produced the highest number of different banding patterns (a total of 11 different patterns each for the 17 serovars tested), and provided the greatest resolution (e.g., for *fimA* [3′ end], a single nucleotide difference between serovars Enteritidis and London produced a detectable change in the SSCP pattern). When the SSCP results for the *fimA* (3′ end) and *atpD* genes were combined with either the *mdh* or the *invA* genes, all of the serovars could be distinguished. The inclusion of the *atpD* gene was crucial in obtaining full discrimination of all the serovars tested. For instance, SSCP analyses based on the combination of *fimA* (3′ end), *mdh,* and *invA* result in the delineation of most but not all of the *Salmonella* serovars, with the repartition of Senftenberg, Cerro, Johannesburg, and Minnesota into two groups. In contrast with the results reported by Lim et al. *(18),* the use of the *invA* gene in SSCP analysis did not enable the discrimination of Senftenberg and Cerro. Sequencing of the *invA* amplicons from these two serovars revealed a lack of sequence variability, which was consistent with the identical SSCP banding patterns observed. Based on the results obtained for the different serovars tested, it was possible to exclude the *fimA* (5′ end) from the present SSCP analysis scheme, since this region of the *fimA* gene did not provide any additional information, exhibiting a maximum of 7 unique SSCP patterns for the 17 serovars tested. It remains to be demonstrated whether the inclusion of *fimA* (5′ end) might be informative in the delineation of other *Salmonella* serovars. The use of a combination of several genes (up to

seven) to achieve better discrimination in molecular serotyping of *Salmonella* serovars has been established in MLST analysis *(9, 24, 27, 28).*

Subserovar discrimination could not be demonstrated in our study based on the isolates analyzed, as the six strains of *S. enterica* subsp. *enterica* serovar Enteritidis tested produced identical SSCP profiles for all of the target genes. This is due to a lack of nucleotide sequence variability in the *fimA, mdh, invA,* and *atpD* genes among the different strains belonging to this serovar, which was confirmed by sequencing the respective amplicons. Sequence variability in the *groEL* gene has been previously reported *(20)* among strains of Enteritidis serovar; however, this gene was not included in the present study, and only a limited number of different Enteritidis isolates (a total of six) was examined. Based on available nucleotide sequence information for the *atpD* gene, Tankouo-Sandjong et al. *(27)* demonstrated the occurrence of four Enteritidis subtypes within the same cluster in a neighbor-joining dendogram, while strains of the serovar Montevideo belonged to different clusters, suggesting the possible existence of sequence variation, which may be useful for discrimination at the subserovar level. The possibility of achieving subserovar discrimination by SSCP analysis was first suggested by Nair et al. *(20),* with the demonstration of three different SSCP profiles for 11 Enteritidis strains. Further studies will be necessary to more comprehensively demonstrate the ability of SSCP analysis to differentiate salmonellae at different levels of strain relatedness with panels of isolates representing a wide spectrum of different serovars (and strains), and utilizing PCR-generated amplicons from a variety of target genes (including *groEl*).

We suggest that SSCP analysis has the potential to serve as an adjunct to classical biochemical, serological, and molecular typing methods for the differentiation of salmonellae isolated from foods. However, no attempts have yet been made to develop standardized protocols, image analysis, or databases systems for SSCP banding patterns, in order to identify isolates per se. The SSCP technique was reproducible, simple to perform and cost-effective, making it a candidate for implementation in food microbiology testing laboratories to support regulatory compliance activities and trace back investigations.

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