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Comparison of *Salmonella enterica* Serovar Typhimurium LT2 and Non-LT2 *Salmonella* Genomic Sequences, and Genotyping of Salmonellae by Using PCR†

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Genes of *Salmonella enterica* serovar Typhimurium LT2 expected to be specifically present in *Salmonella* were selected using the Basic Local Alignment Search Tool (BLAST) program. The 152 selected genes were compared with 11 genomic sequences of *Salmonella* serovars, including *Salmonella enterica* subsp. I and IIIb and *Salmonella bongori* (V), and were clustered into 17 groups by their comparison patterns. A total of 38 primer pairs were constructed to represent each of the 17 groups, and PCR was performed with various *Salmonella* subspecies including *Salmonella enterica* subsp. I, II, IIIa, IIIb, IV, VI, and V to evaluate a comprehensive DNA-based scheme for identification of *Salmonella* subspecies and the major disease-causing *Salmonella* serovars. Analysis of PCR results showed that *Salmonella enterica* subsp. I was critically divided from other subspecies, and *Salmonella* strains belonging to *S. enterica* subsp. I were clustered based on their serovars. In addition, genotypic relationships within *S. enterica* subsp. I by PCR results were investigated. Also, *Salmonella* signature genes, *Salmonella enterica* serovar Typhimurium signature genes, and *Salmonella enterica* subsp. I signature genes were demonstrated based on their PCR results. The described PCR method suggests a rapid and convenient method for identification of *Salmonella* serovars that can be used by nonspecialized laboratories. Genome sequence comparison can be a useful tool in epidemiologic and taxonomic studies of *Salmonella*.

Salmonellae are divided taxonomically into two species, *Salmonella enterica* and *Salmonella bongori* (V). *Salmonella enterica* comprises 6 subspecies: *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV), and *S. enterica* subsp. *indica* (VI). *Salmonella* is classified into more than 2,500 serovars using the Kauffmann-White scheme (25). *Salmonella enterica* subsp. I consists of almost 1,500 serovars (24), and most infections in warm-blooded animals are caused by *Salmonella enterica* subsp. I. Among *S. enterica* subsp. I, only a small number of *Salmonella* serovars (e.g., *Salmonella enterica* serovar Typhimurium, *Salmonella enterica* serovar Enteritidis, *Salmonella enterica* serovar Newport, *Salmonella enterica* serovar Typhi, *Salmonella enterica* serovar Paratyphi A, *Salmonella enterica* serovar Paratyphi C, and *Salmonella enterica* serovar Choleraesuis) account for most human and domestic animal *Salmonella* infections (26, 27). The different host ranges, diseases, and virulence potentials demonstrated by the various serovars belonging to *S. enterica* subsp. I (4, 13) are thought to be caused by genetic variation.

The serology of *Salmonella* is based on the Kauffmann-White scheme, which differentiates *Salmonella* serovars by the surface antigen differences of somatic (O) and flagellar (H) antigens (24). This serological method, which is a convenient and epidemiologically useful method of categorizing *Salmonella*, has been used to identify *Salmonella* serovars. At the same time, this method is labor-intensive, expensive, complicated, and time-consuming. Also, it does not provide a basis for investigating evolutionary genetic relatedness among strains. Molecular characterization of each *Salmonella* serovar has been reported using multilocus enzyme electrophoresis (MLEE), and many serovars were found to be represented by two or more electrophoretic types. Evolutionary trees constructed by MLEE data classified *Salmonella* serovars as monophyletic or polyphyletic, and these results found genotypic differences between the same *Salmonella* serovars and genetic distance between serovars (8). Recently, microarray methods were applied to evaluating the gene contents of *Salmonella* serovars. Comparative genomic hybridization using microarrays suggested that *Salmonella* strains of the same serovars are not always genotypically closely related and that differences are characterized at single-gene resolution. Also, a new term, "genovars," was proposed to describe groups of strains with genetic similarity, distinguishing them from traditional *Salmonella* serovar classification (26).

Recently, genomic DNA sequencing projects of various *Salmonella* strains have been in process or completed for some serovars (12, 20, 22). These genome projects incorporate fast-capacity screening technology, such as microarray analysis, and are expected to reveal more information about *Salmonella* genotyping. Comparative genomics in *Salmonella* biology have also been initiated by genome sequencing of other related *Salmonella* serovars and will provide more efficient ways of identifying all of the genetic differences between closely related bacteria (13). Also, this sequence comparison will provide more information about characteristics of *Salmonella* serovars. PCR has become a potentially powerful alternative in microbiological diagnostics due to its simplicity, rapidity, reproducibility, and accuracy (5, 23, 32). PCR is relatively easy to

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† Supplemental material for this article may be found at http://aem.asm.org/.

TABLE 1. *Salmonella* strains used in this study

Salmonella subspecies and serovar	Serogroup	Source	Strain(s)
S. enterica subsp. I			
Typhimurium	B	ATCC 19585	LT2
		ATCC 13311	
		ATCC 14028	
Typhi	D1	ATCC 33459	
Choleraesuis	C1	ATCC 13312	
Enteritidis	D1	ATCC 4931	
Gallinarum	D1	ATCC 9184	
Pullorum		ATCC 9120	
Paratyphi C	C1	ATCC 13428	
Paratyphi B	B	ATCC 10719	
Typhimurium	B	KCPB[a]	S9, S15, S17, S21
		BFR[b]	G02
		FDA[c]	DT-104
Enteritidis	D1	KCPB	S25, S26, S27, S29, S32, S34, S35, S38, S39, S40, S41, S53, S54, S56, S57, S63, S64, S65, S66
		BFR	G01
		FDA	3512H, H3353, Benson-1, ME-13, Me-14
Haardt	C2-C3	KCPB	S30, S31, S33, S36, S37
Virginia	C2-C3	KCPB	S3, S5, S6, S7, S8
Heidelberg	B	BFR	G06
		FDA	3390H, UN-L
Infantis	C1	KCPB	S22
		BFR	G05
		FDA	1232H
Agona	B	KCPB	S12, S28
		BFR	G10
Bredeney	B	BFR	G13
		FDA	1370H
Derby	B	FDA	1591H
		BFR	G14
Hadar	C2-C3	KCPB	S2
		BFR	G03
Georgia	C1	KCPB	S4, S18
Litchfield	C2-C3	BFR	G20
		FDA	3483H
Montevideo	C1	BFR	G17
		FDA	1231H
Schwarzenground	B	KCPB	S16, S19
Agona B		FDA	4000H
Anatum	E1	FDA	1904H
Barcilly		FDA	1955H
Blockley	C2-C3	BFR	G11
Bovismorbificans	C2-C3	BFR	G12
Braenderup	C1	FDA	10N
Brandenburg	B	BFR	G08
California	B	FDA	3515H
Cerro	K	FDA	1325H
Dublin	D1	BFR	G15
Edinburg	C1	KCPB	S10
Give E1	E1	FDA	1432H
Illinois		FDA	2386H
Istanbul	C2-C3	KCPB	S20
Java B		FDA	2234H
Javiana	D1	FDA	2080H
Joal	E1	KCPB	S23
Kentucky	C2-C3	FDA	2035
Livingstone	C1	BFR	G16
Madelia	H	FDA	22N
Manhattan	C2-C3	FDA	1293H
Mbandlaka	C1	FDA	37N
Meleagridis	E1	FDA	1054H
Mhenohen		FDA	2761H
Mississippi	G	FDA	2883H
Muenster	E1	FDA	1250H
Newington		FDA	3144H
Newport	C2-C3	BFR	G07
Ohio	C1	FDA	2060H
Oranienburg	C1	FDA	1410H
Paratyphi A	A	KCPB	S11
Poona	G	FDA	3417H
Saintpaul	B	BFR	G09
Sandow	C2-C3	KCPB	S13
Senftenberg	E4	BFR	G19
Tennessee	C1	KCPB	S24
Virchow	C1	BFR	G04
S. enterica subsp. II			
S. enterica subsp. *salamae*		ATCC 15793	
42:r:-	T	BFR	G22
9,12:z:z39	D	BFR	G23
48:d:z6	Y	BFR	G24
42:b:e,n,x,z15	T	BFR	G25
30:l,z28:z6	N	BFR	G26
S. enterica subspecies IIIa			
S. enterica subsp. *arizonae*		ATCC 13314	
21:g,z51:-	L	BFR	G27
47:r:-	X	BFR	G28
18:z4,z32:-	K	BFR	G29
S. enterica subspecies IIIb			
S. enterica subsp. *diarizonae*		ATCC 43973	
50:z:z52	Z	BFR	G30
47:l,v:z	X	BFR	G31
18:i,v:z	K	BFR	G32
S. enterica subsp. IV			
S. enterica subsp. *houtenae*		ATCC 43974	
16:z4,z32:-	I	BFR	G33
48:g,z51:-	Y	BFR	G34
11:z4,z23:-	F	BFR	G35
S. enterica subsp. VI			
S. enterica subsp. *indica*		ATCC 43976	
45:a:e,n,x	W	BFR	G39
1,6,14,25:a:e,n,x	H	BFR	G40
41:b:1,7	S	BFR	G41
S. bongori (V)			
S. bongori		ATCC 43975	
44:r:-	V	BFR	G36
66:z65:-		BFR	G37
48:z35:-	Y	BFR	G38

[a] KCPB, Korea Consumer Protection Board (11).
[b] BFR, Federal Institute for Risk Assessment (18).
[c] FDA, U.S. Food & Drug Administration (CFSAN/OPDFB) (30).

perform with simple equipment in the laboratory compared to microarray analysis or other molecular methods. Also, genomic sequence comparison can be a powerful tool for probe searching (marker gene searching) and characterizing the gene contents of closely related bacterial species.

In this study, genes of *S. enterica* serovar Typhimurium LT2 that were expected to be specific to the *Salmonella* genus were selected using genomic sequence comparison. The selected gene sequences were compared with genomic sequences of 11 *Salmonella* strains. Primer pairs of these selected genes were constructed and used to evaluate the genomic DNA of various *Salmonella* serovars, including all subspecies. First, we confirmed that genomic sequence comparison patterns and PCR result patterns were comparable to determine the acceptability of applying genomic sequence comparison to a substantial experiment. Second, genomic sequence comparison results were used to identify genes that were *Salmonella* specific, *S. enterica* subsp. I specific, and *S. enterica* serovar Typhimurium

TABLE 2. *Salmonella* genomic sequences used in this study

Strain	Subspecies	Reference sequence	Genome size (kb)	Status of genome project[a]	Contributor	Reference	Source
S. enterica serovar Typhimurium LT2	I	NC_003197	4,857	Finished (4,451)	R. K. Wilson (Washington University, GSC)	20	http://www.ncbi.nlm.nih.gov/
S. enterica serovar Typhimurium DT104	I	STmDT104.dbs (NC_004513)	5,020	Finishing/gap closure	Sanger Institute		http://www.sanger.ac.uk/Projects/Salmonella/
S. enterica serovar Typhimurium SL1344	I	STmSL1344.dbs (NC_004509)	5,091	Finishing/gap closure	Sanger Institute		http://www.sanger.ac.uk/Projects/Salmonella/
S. enterica serovar Typhi CT18	I	NC_003198	4,809	Finished (4,949)	B. G. Barrell (Sanger Institute)	22	http://www.ncbi.nlm.nih.gov/
S. enterica serovar Typhi Ty2	I	NC_004631	4,791	Finished (4,639)	F. R. Blattner	12	http://www.ncbi.nlm.nih.gov/
S. enterica serovar Paratyphi A ATCC 9150	I	SparatyphiA.txt (NC_006511)	4,585	Finished	Washington University (GSC)		http://www.ncbi.nlm.nih.gov/
S. enterica serovar Enteritidis PT4	I	SePT4.dbs	4,686	Finishing/gap closure	Sanger Institute		http://www.sanger.ac.uk/Projects/Salmonella/
S. enterica serovar Gallinarum 287/91	I	SG.dbs	4,869	Finishing/gap closure	Sanger Institute		http://www.sanger.ac.uk/Projects/Salmonella/
S. enterica serovar Dublin	I	Sdu.dbs.txt (NC_002961)		Incomplete	University of Illinois		http://www.salmonella.org/genomics/
S.enterica serovar Pullorum	I	Spu.dbs.txt		Incomplete	University of Illinois		http://www.salmonella.org/genomics/
S. enterica subsp. diarizonae serovar 61:1,v:1,5,(7)	IIIb	Diarizonae.txt	3,600	Sequence is now in shotgun	Washington University (GSC)		http://genome.wustl.edu/projects/bacterial/
S. bongori 12419	V	SB.dbs (NC_004548)	4,460	Finished	Sanger Institute		http://www.sanger.ac.uk/Projects/Salmonella/

[a] Numbers in parentheses indicate numbers of coding genes from the complete genome sequence.

specific to establish a comprehensive DNA-based scheme for identification of *Salmonella* subspecies and the major disease-causing *Salmonella* serovars without the need for serological testing. Also, we suggest genotypic relationships between *Salmonella* serovars on the basis of PCR results. These results suggest a rapid and convenient method for identification of the *Salmonella* serovars attainable by nonspecialized laboratories.

MATERIALS AND METHODS

Bacterial strains. *Salmonella* strains used in this study are listed in Table 1. Sixteen type strains of *Salmonella* were collected from the American Type Culture Collection (ATCC). Forty-seven *Salmonella* strains were provided by Y. H. Jung of the Korea Consumer Protection Board (KCPB) (11). Thirty-nine *Salmonella* strains were provided by Reiner Helmuth of the Federal Institute for Risk Assessment (BFR, Molecular Biology, National *Salmonella* Reference Laboratory, Germany) (18). Thirty-five *Salmonella* strains were donated by K. H. Seo of the U.S. Food and Drug Administration (FDA, CFSAN/OPDFB) (30). *Salmonella* strains were inoculated in Luria-Bertani broth medium and cultured at 37°C with vigorously shaking. Non-*Salmonella* strains, including food-borne pathogens and *Enterobacteriaceae*, were collected from the ATCC and are listed in Table S4 in the supplemental material.

Genomic DNA extraction. Cultured media of *Salmonella* strains were harvested in microtubes, and genomic DNA from *Salmonella* strains was extracted using the DNEasy tissue kit (QIAGEN, Hilden, Germany) according to the manufacturer's manual. Concentrations of extracted DNA were measured using a UV spectrophotometer (model UV-1700; Shimadzu, Tokyo, Japan), and genomic DNA with a 1.8 to 2 ratio (A_{260}/A_{280}) was used. Genomic DNA from *Salmonella* strains was diluted in distilled water to 25 ng/μl and stored at 4°C until PCR.

Genomic sequences of *Salmonella* species. Table 2 lists the 12 genomic sequences of *Salmonella* strains used in this study and their sources. The genomic sequencing projects of *S. enterica* serovar Typhimurium LT2, *S. enterica* serovar Typhi CT18, and *S. enterica* serovar Typhi Ty2 are complete (12, 20, 22), and their genomic sequences were obtained from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). An additional 9 genomic sequencing projects of *Salmonella* strains were not completed, but raw sequence data were obtained from the Sanger Institute, Washington University, and the University of Illinois. Genomic sequences of *Salmonella* serovar Typhimurium DT104, *Salmonella* serovar Typhimurium SL1344, *Salmonella* serovar Enteritidis PT4, *Salmonella enterica* serovar Gallinarum 287/91, and *S. bongori* 12419 were obtained from the Sanger Institute (http://www.sanger.ac.uk/Projects/Salmonella/). Genomic sequences of *Salmonella enterica* serovar Dublin and *Salmonella enterica* serovar Pullorum were obtained from the University of Illinois (http://www.salmonella.org/genomics/). Genomic sequences of *S. enterica* subsp. *diarizonae* serovar 61:1,v:1,5,(7) and *Salmonella* serovar Paratyphi A ATCC 9150 were obtained from the Genome Sequencing Center (GSC) at Washington University (http://genome.wustl.edu/home.cgi).

Comparative genomics between *Salmonella* serovars. A total of 4,451 gene sequences (NC_003197.ffn) of *Salmonella* serovar Typhimurium LT2 were submitted to the nonredundant (nr) DNA sequence NCBI database using the Basic Local Alignment Search Tool (BLAST) program (version 2.2.5) (2). BLAST outputs that matched the *Salmonella* genus were eliminated and the highest scored output of each 4,451 genes was selected from BLAST outputs of each gene. Based on BLAST outputs, *Salmonella* specific expected genes that had an nr database match score of less than 40.14 and had a matched length less than 21 bp were selected and compared to the genomic sequence of 11 *Salmonella* strains using the BLAST program (version 2.2.5). Each highest matched output of *Salmonella* specific expected genes with each *Salmonella* genome sequence were defined as high homology, moderate homology, and low homology, and *Salmonella* specific expected genes were grouped based on homology patterns with each *Salmonella* genomic sequence.

Primer construction and PCR conditions. A total of 38 oligonucleotide primer pairs were constructed representing each group. Each 25 μl contained 1× EX *Taq* buffer (Mg^{2+} plus), 0.4 μM primer, 200 μM concentrations of each dNTP, 0.5 U of EX *Taq* DNA polymerase (TaKaRa, Shiga, Japan), and 25 ng/μl template DNA from various *Salmonella* serovars. PCR amplification was performed in a thermal cycler (model PC 808; ASTEC, Fukuoka, Japan) with an initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 45 s, annealing at the temperature listed in Table 3 for each primer pair for 30 s, 72°C for 30 s, and finishing with a final extension at 72°C for 3 min and storage at 4°C thereafter. Amplified products were electrophoresed on 1.5% agarose gels in 0.5× Tris-acetate-EDTA buffer, stained with ethidium bromide, visualized under

TABLE 3. Constructed primer pairs used in this study

Group	Target gene (synonym)	PCR product size (bp)	Annealing temp (°C)	Primer	Sequence	Product
1	STM3098	423	65	STM3098-f2	5′-TTTGGCGGCGCAGGCGATTC	Putative transcriptional regulator
				STM3098-r2	5′-GCCTCCGCCTCATCAATCCG	
	STM4444	253	65	STM4444-f	5′-ATGCCGACTGGTCGTTCCCG	Putative inner membrane protein
				STM4444-r	5′-CCCACGCCGGTCTGAATTGC	
2	STM0349	249	65	STM0349-f	5′-TCGTCGGCTTGGCTTTAACC	Putative outer membrane lipoprotein
				STM0349-r	5′-CTGCACGCTGGGTTAACAGG	
	STM4030	310	65	STM4030-f	5′-AAACCGTCCCACTGATGGGG	Putative cytoplasmic protein
				STM4031-r	5′-CGTTAGTGCTCTGCGGCCAT	
3	STM1550	187	67	STM1550-f	5′-AGCTAAGGGAACGGCTTGAA	Putative cytoplasmic protein
				STM1550-r	5′-CGTGTCATTTTGTAGACGGC	
	STM2235	177	65	STM2235-f	5′-TGCAGTCAGTGGCAATAACG	Putative phage protein
				STM2235-r	5′-CGTCACCTTTAGCCATCCCA	
	STM2630	115	65	STM2630-f	5′-CTGCCGCAAATCCATTGATG	Hypothetical protein
				STM2630-r	5′-GTATTCAGCGCACTGCCTGG	
	STM2744	141	65	STM2744-f	5′-CCGAAAGCGGCAACGTGCGT	Putative cytoplasmic protein
				STM2744-r	5′-CCGCAGCATCGAAGACCACC	
	STM2752	203	65	STM2752-f	5′-TTATTCCTCCCGGTCCCGGC	Putative glucitol-specific PTS enzyme III
				STM2752-r	5′-CCCGGCGCAGTTAATCACCA	
	STM2755	187	65	STM2755-f	5′-AGCTGCTTTTCGACGCCGGG	Putative hexulose 6 phosphate synthase
				STM2755-r	5′-ACCGCCAGCATATCTGCCCC	
	STM4203	316	65	STM4203-f	5′-CTGCCTTGCAACGTCCTGAA	Putative phage baseplate protein
				STM4203-r	5′-CGCCATAACACCTCCGTTGA	
	STM4214	109	65	STM4214-f	5′-ACGCTCGCCGACGGTCAGGA	Putative cytoplasmic protein
				STM4214-r	5′-CTGGCACCAGGTGACGGCGG	
	STM4497	310	63	STM4497-f	5′-AACAACGGCTCCGGTAATGA	Putative cytoplasmic protein
				STM4497-r3	5′-TGACAAACTCTTGATTCTGA	
	STM4571	154	65	STM4571-f	5′-TTTGTGCAGGCCTCAGCGGG	Putative outer membrane protein
				STM4571-r	5′-GGGCACTGTCATTGGGAGCA	
4	STM2453	270	65	STM2453-f	5′-TTGTATGCCCTGCGTCCAGG	Putative cytoplasmic protein
				STM2453-r	5′-GCTTCCTCCTGCCATCCGGA	
	STM2624	284	65	STM2624-f	5′-CTGGTGAAAGAGCAGGGGCG	Hypothetical protein
				STM2624-r	5′-GCTCCCCCCTTGTTGATGCT	
5	STM1269	425	65	STM1269-f	5′-GTGCAGCACCACTTTTGCCG	Putative chorismate mutase
				STM1269-r	5′-GCGCTCTCAGCCACACCATA	
	STM1277	289	65	STM1277-f	5′-AAGCGCGTCTATTTCCCGGC	Putative cytoplasmic protein
				STM1277-r	5′-GGCGAGTATCTTTAGCGGCG	
6	STM0538	288	65	STM0538-f	5′-TCTCTTCCACAGTCCCCGCT	Putative outer membrane protein
				STM0538-r	5′-CTGTCGCCGCTGTTTAGCCC	
	STM3532	233	65	STM3532-f	5′-TCCGCCAGTTCCGACCATTG	Putative dihydrodipicolinate synthetase
				STM3532-r	5′-CCTGCGTGCTGGTGCTGCTA	
7	STM4509	312	65	STM4509-f	5′-TGGCGTTCCGTCCTTGTCAG	Putative cytoplasmic protein
				STM4509-r	5′-TTGCGCCCTTATCACGACGG	
8	STM0305	155	65	STM0305-f	5′-CGGAAACAGGACGGGGCTGT	Putative cytoplasmic protein
				STM0305-r	5′-CCGAAGGCGCAATGGAGGAT	
	STM1408	187	65	STM1408-f	5′-TCCTCTGCAGAACCGAGCCA	Type III secretion system apparatus protein
				STM1408-r	5′-TAAGCGCTTGCGATGCTGCG	
	STM1859	115	65	STM1859-f	5′-AACACGATGCCATTTTCAAT	Putative cytoplasmic protein
				STM1859-r	5′-TTGAGGTCAGTGTGCAATTC	
	STM2056	342	65	STM2056-f	5′-TGATGTTTATCGGCCCCAGC	Propanediol utilization protein
				STM2056-r	5′-CCAGCCTGCGTAAGCCACTC	
	STM3690	268	65	STM3690-f	5′-GAAGTCGTTGGCCGCGTTGA	Putative inner membrane lipoprotein
				STM3690-r	5′-GGAGTTGTTTCCAGCGAGGC	
	STM4057	137	65	STM4057-f	5′-GGTGGCCTCGATGATTCCCG	Putative inner membrane protein
				STM4057-r	5′-CCCACTTGTAGCGAGCGCCG	
	STM4071	167	65	STM4071-f	5′-AAGCGGTGAAGTGTGCCTGT	Putative mannose-6-phosphate isomerase
				STM4071-r	5′-CGGGGTGGCTGTCATTTTCC	
	STM4317	220	65	STM4317-f	5′-GCGAAACCCTGAACCTGCGT	Hypothetical protein
				STM4317-r	5′-CGCAGTGCGGCATTAGGTGA	
	STM4457	215	65	STM4457-f	5′-AGCAACAGCACGCTCCGTCG	Putative transposase
				STM4457-r	5′-GATATGCGACGAAAGCGGCG	
9	STM0339	232	65	STM0339-f	5′-GCCCTACCCGCCACAGCATC	Putative fimbrial chaperone
				STM0339-r	5′-CCTGGCCTGCTTTGGGTTGA	
10	STM1006	165	65	STM1006-f	5′-TCTGATTGCGGTTACCGGGC	Excisionase
				STM1006-r	5′-TGCGCCTCGATCCACTGATC	
11	STM3752	165	65	STM3752-f	5′-CGGCTTGGCGTATACAGCGA	Putative cytoplasmic protein
				STM3752-r	5′-GCCTCCCTCCAGATACACGG	

Continued on following page

TABLE 3—*Continued*

Group	Target gene (synonym)	PCR product size (bp)	Annealing temp (°C)	Primer	Sequence	Product
12	STM0287	328	65	STM0287-f	5′-CGTATTTGCCTGGGGCGGAA	Putative periplasmic protein
				STM0287-r	5′-CGCCAGCTTCTGATCCCGTA	
13	STM2434	103	65	STM2434-f	5′-AGATATCTGCGTGGCGCGAG	Putative cytoplasmic protein
				STM2434-r	5′-ATCCGGGCCACTCTCCAGCA	
14	STM4596	573	65	STM4596-f	5′-ATGAAGCAGTTAAACGGCGG	Putative inner membrane protein
				STM4596-r	5′-GCTGCGTGAAAGCCCGGTTC	
15	STM2955S	186	65	STM2955-f	5′-CTTGGCGATGAACTGCGCGA	Putative transcriptional regulator
				STM2955-r	5′-CTTTTCCCAGGCCTGCGGCT	
16	STM0409	170	65	STM0409-f	5′-TCGGGAAACCATGGATGGGG	Hypothetical protein
				STM0409-r	5′-CACCGGCAAGGACGACACGT	

UV irradiation, and photographed with a digital camera (COOLPIX 4300; Nikon, Tokyo, Japan).

Analysis of PCR results. PCR results were scored 1 for positive results (amplified band with expected size) and 0 for negative results. Numerical taxonomy analysis of PCR results for each *Salmonella* strain was carried out using similarity matrices of SIMQUAL (similarity for qualitative data) and unweighted-pair group method using arithmetic means cluster analysis by the NTSYS-pc (Numerical taxonomy system using multivariate statistical program, version 2.02j; Exeter Software, Setauket, NY) program (31).

RESULTS

BLAST sequence comparison of *Salmonella* serovar Typhimurium LT2 genes. A total of 4,451 genes of *Salmonella enterica* serovar Typhimurium LT2 (NC_003197.ffn) were submitted to the nonredundant database of NCBI using the BLAST program. One hundred fifty-two putative *Salmonella*-specific genes were selected from 4,451 genes (5 genes, no hits found; 147 genes, match score less than 40.14 and matched length less than 21 bp with nr database of NCBI) (see Tables S1 and S2 in the supplemental material). The 152 genes included some of the *Salmonella* pathogenicity island 1 and 2 genes but not *rfbJ*, *fliC*, and *fljB* of O antigen or H antigen, which were related with the Kauffmann-White scheme of *Salmonella* serovar Typhimurium (17, 33).

Sequence comparison of selected genes with various *Salmonella* genome sequences. The selected 152 genes of *Salmonella* serovar Typhimurium LT2 were compared using BLAST with each genomic sequence of 11 *Salmonella* strains including *S. enterica* subsp. I, IIIb, and V. The 152 genes were divided into 17 groups by the BLAST output pattern of each of the 152 genes as shown in Table 4 and Table S2 in the supplemental material.

Several groups of 17 groups showed a subspecies- or serovar-specific expected comparison pattern as shown in Table 4.

TABLE 4. Groups of 152 genes of *Salmonella enterica* serovar Typhimurium LT2 based on comparison patterns between various *Salmonella* serovars

Group	No. of genes	Homology with[a]:											
		S. enterica subsp. I serovar Typhimurium			*S. enterica* subsp. I serovar Typhi		*S. enterica* subsp. I serovar Enteritidis PT4	*S. enterica* subsp. I serovar Gallinarum 287/91	*S. enterica* subsp. I serovar Pullorum	*S. enterica* subsp. I serovar Dublin	*S. enterica* subsp. I serovar Paratyphi A ATCC 9150	*S. enterica* subsp. IIIb serovar Diarizonae 611,v1,5,(7)	*S. bongori* subsp. V 12419
		LT2	DT104	SL1344	CT18	Ty2							
1	31	+	+	+	+	+	+	+	+	+	+	+	+
2	9	+	+	+	+	+	+	+	+	+	+	+	−
3	10	+	+	+	− or =	− or =	− or =	−	− or =	− or =	−	−	−
4	3	+	+	+	+	+	−	−	−	− or =	−	−	−
5	12	+	+	+	+	+	+	+	+	− or =	+	+	+
6	8	+	+	+	−	−	+	+	+ or =	+	−	−	−
7	8	+	+	+	+	+	+	+	− or =	+	+	+	+
8	9	+	+	+	+	+	+	+	+	+ or =	+	− or =	−
9	5	+	+	+	+	+	+	+	− or =	+	+	+	−
10	3	+	+	+	+	+	−	−	−	+	−	−	−
11	4	+	+	+	+	+	+	+	+	− or =	+	−	− or =
12	2	+	+	+	+	+	+	− or =	− or =	+	− or =	− or =	− or =
13	3	+	+	+	+	+	+	− or =	+	− or =	+	+	+
14	4	+	+	+	+	+	+	+	+	+	+	−	+
15	3	+	+	+	+	+	−	−	−	−	+	−	−
16	3	+	+	+	+	+	+	+	−	− or =	+	− or =	− or =
17[b]	35												
Total	152												

[a] +, high homology, highest matched sequence size is more than 50% of query gene; =, moderate homology, highest matched sequence size is between 20 and 50% of query gene; −, low homology, highest matched sequence size is less than 20% of query gene.

[b] The genes of group 17 showed various comparison patterns. The data are shown in Data S2 in the supplemental material.

First, the 31 genes of group 1 were expected to be present in all *Salmonella* subspecies, and the 9 genes of group 8 were expected to specifically to be present in each *S. enterica* subsp. I strain. The 10 genes of group 3 were expected to be specific to *Salmonella* serovar Typhimurium. Also, the 9 genes of group 2 were expected to be present in all *Salmonella* subspecies except *Salmonella bongori* (i.e., *Salmonella enterica* signature genes). The genes in group 17 showed various comparison patterns with various *Salmonella* strains.

Almost 152 genes of serovar Typhimurium LT2 shared their sequences with the genomic sequence of *S. enterica* subsp. I, and a small number of genes were shared with *S. enterica* subsp. *diarizonae* and *S. bongori* and were considered genetically distant from *S. enterica* subsp. I, as previously reported (10).

Primer construction and PCR results. A total of 38 primer pairs, representing each group of 152 genes, were constructed as shown in Table 3. PCR was performed with genomic DNA of various *Salmonella* serovars, as seen in Table S3 in the supplemental material, and the concordance of PCR results with comparison patterns of Table 4 was confirmed. Primer pairs STM3098 and STM4444 belong to group 1 and were candidate genes to amplify PCR product in all *Salmonella* serovars based on the results of sequence comparison. Primer pair STM3098 amplified PCR products from all *Salmonella* serovars from *S. enterica* subsp. I to VI at the expected size. Primer pair STM4444 amplified PCR products in all except *S. enterica* subsp. *arizonae* ATCC 13314. These results imply that the 31 genes of group 1 are suitable candidate genes for *Salmonella* signature genes. STM3098 was suggested as a specific target gene of *Salmonella* in this study.

The genes of group 2 were expected to be specific to *S. enterica* subsp. I and IIIb, and the primer pair STM0349 amplified specific PCR products with *S. enterica* subsp. I, II, and IIIb. Primer pair STM4030 amplified PCR product with *S. enterica* subsp. I and IIIb, except in some *S. enterica* subsp. I serovars.

The genes of group 3 were expected to be specific to *S. enterica* serovar Typhimurium. Among 10 genes, the primer pair STM4497 was highly specific to *Salmonella* serovar Typhimurium. Other primer pairs of group 3 were also relatively highly specific to *Salmonella* serovar Typhimurium. STM4203 and STM4214 were reported to be present in *Salmonella* serovar Paratyphi C and *Salmonella* serovar Choleraesuis by microarray results, and in this study, these genes were detected by PCR in these two serovars (10). Two primer pairs of group 4 (STM2624 and STM2453) amplified PCR products from *Salmonella* serovar Typhimurium and *Salmonella* serovar Typhi, as expected, as well as from some *Salmonella* serovars of *S. enterica* subsp. I and II.

The genes of group 8 were specific to *S. enterica* subsp. *enterica* (*S. enterica* subsp. I) by sequence comparison, and 9 primer pairs were constructed. Among the 9 primer pairs, STM4057 and STM0305 showed PCR products with *S. enterica* subsp. I, except for a few *Salmonella* serovars, including *S. enterica* subsp. I. Among 9 candidate genes for the *S. enterica* subsp. I signature, only the primer pair STM4057 showed specific results with *S. enterica* subsp. I. These PCR results demonstrated the possibility of detecting *S. enterica* subsp. I using primer pairs STM4057 and STM0305.

Thirty-eight primer pairs were constructed with genomic DNA of non-*Salmonella* strains, including food-borne pathogens and *Enterobacteriaceae*, and showed negative results (see Table S4 in the supplemental material).

Acceptance of sequence comparison among *Salmonella* serovars. The PCR results of 38 primer sets are shown in Table S3 in the supplemental material. PCR results showed constant and reproducible results by *Salmonella* serovars and comparison patterns as shown in Table 4. With some primer pairs, PCR result patterns did not match the pattern of genomic sequence comparison in Table 4. For example, primer pair STM2056, which included group 8, expected to be present in *S. enterica* subsp. I, as shown in Table 4, was negative for *Salmonella* serovar Typhi and *Salmonella* serovar Paratyphi A. Also, with some primer pairs, PCR results were not consistent for serovar or subspecies. For example, primer pair STM2630 yielded different PCR results with 6 strains of *S. enterica* subsp. II (positive result, 2 strains; negative result, 4 strains). Primer pair STM2453 PCR results differed with the same serovar (*Salmonella enterica* serovar Heidelberg; positive result, 2 strains; negative result, 1 strain).

Subtyping of *Salmonella* using PCR result patterns. The relationship between serovars was analyzed using the NTSYS-pc program, and positive or negative PCR results were generated with the 38 primer pairs. A phylogenetic tree of various *Salmonella* serovars based on PCR results is shown in Fig. 1. *Salmonella* serovars, including *S. enterica* subsp. I to VI, were grouped into two clusters. One cluster (cluster A) is *S. enterica* subsp. I, and the other cluster (cluster B) consists of the rest of the *Salmonella* subspecies. These data mean that *S. enterica* subsp. I, which causes infections in warm-blooded animals, could be detected by PCR, which would be useful for epidemiology. Only *S. enterica* subsp. VI was strictly subdivided in cluster B; other subspecies were not strictly discriminated. Nine strains of *S. enterica* serovar Typhimurium were clustered in a narrow portion of the phylogenetic tree shown in Fig. 1. Strains in the same serovar, such as serovar Enteritidis (26 strains), serovar Heidelberg (3 strains), *Salmonella enterica* serovar Schwarzenground (2 strains), *Salmonella enterica* serovar Haardt (5 strains), *Salmonella enterica* serovar Virginia (5 strains), *Salmonella enterica* serovar Agona (3 strains), and *Salmonella enterica* serovar Infantis (3 strains), were genetically similar, as shown in Fig. 1. However, some strains within the same serovar, such as *Salmonella enterica* serovar Bredeney (2 strains), *Salmonella enterica* serovar Derby (2 strains), *Salmonella enterica* serovar Georgia (2 strains), *Salmonella enterica* serovar Litchfield (2 strains), and *Salmonella enterica* serovar Montevideo (2 strains) were not clustered. Interestingly, PCR results of certain strains in the same *Salmonella* serovar were not always the same patterns. This result provides further evidence that there are many genotypes in the same serovar (8, 26).

The phylogenetic tree in Fig. 1 was similar to microarray and MLEE results for *Salmonella* in previous studies (6–10, 16, 26–28). *Salmonella* serovars Enteritidis, Gallinarum, and Pullorum, which are considered avian-adapted serovars, were closely clustered, but serovar Enteritidis and serovar Pullorum were not discriminated in this study. Also, other strains were closely clustered, such as *Salmonella* serovar Choleraesuis with serovar Paratyphi C, serovar Heidelberg with serovar Typhimurium, serovar Montevideo with *Salmonella enterica* serovar Oranienburg, and serovar Typhi with serovar Paratyphi A.

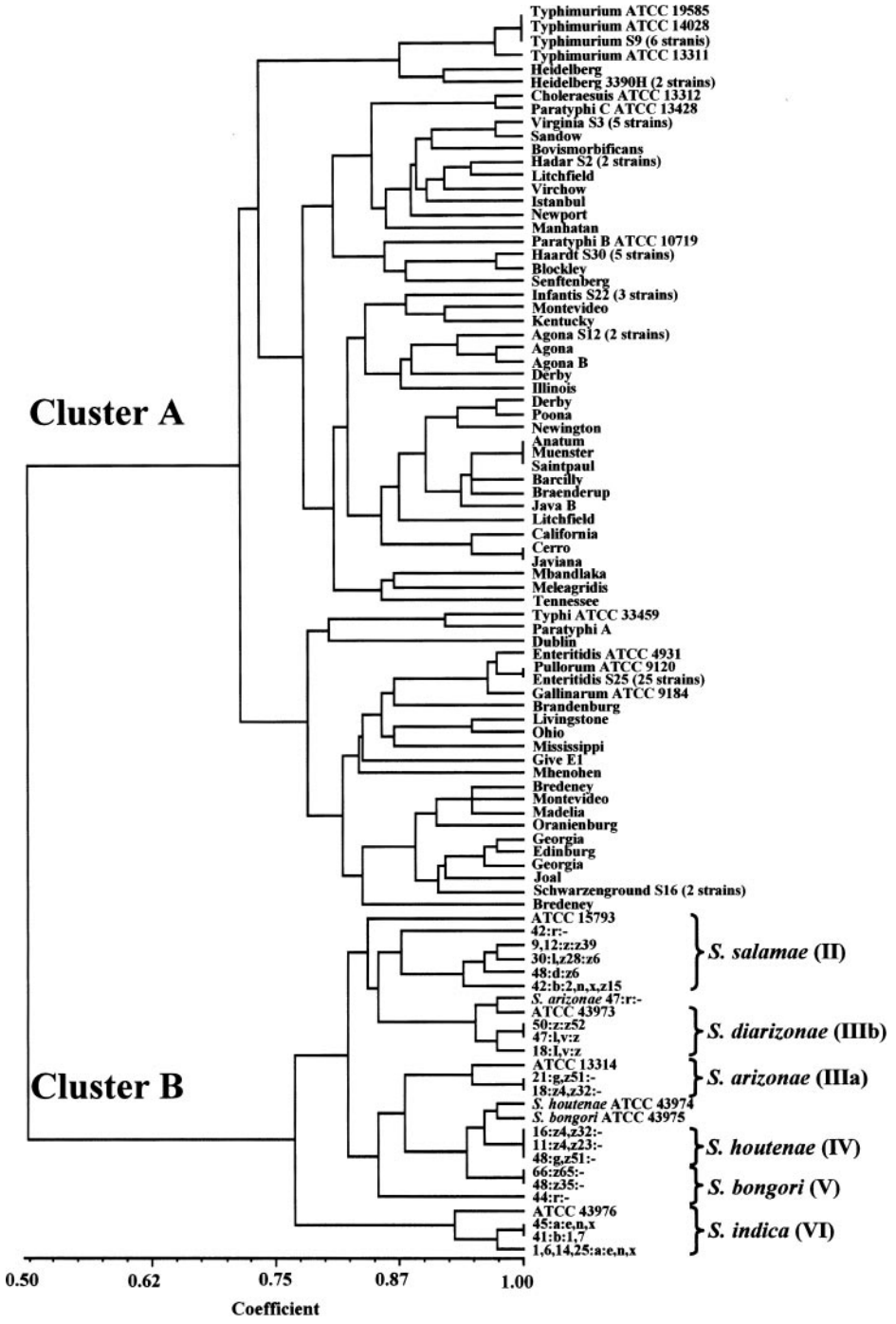

FIG. 1. Phylogenetic tree of various *Salmonella* strains, including *S. enterica* subsp. I to VI, constructed using PCR results from 38 primer pairs by the NTSYS-pc program.

DISCUSSION

Comparative genomics is an efficient way to identify all of the genetic differences between closely related bacteria (13). The *Salmonella* genus is a suitable model bacteria for genomic sequence comparison because more than 2,500 *Salmonella* serovars are very closely related and genome sequencing projects including various *Salmonella* serovars have been completed or are in progress. Also, the sequence data of various *Salmonella* serovars are publicly available. *Salmonella* genome sequences have been anticipated to usher in a new era of comparative genomics in *Salmonella* biology and are expected to provide a valuable resource to explore how and why differences arose between *Salmonella* serovars with different host specificities and virulence (10).

Until now, various DNA-based methods have been applied to find marker genes for specific detection of *Salmonella*, *Sal-*

monella serovar Typhimurium, serovar Enteritidis, and serovar Typhi, including suppression subtractive hybridization, microarray analysis, and PCR (1, 15, 17, 18, 27). In the case of suppression subtractive hybridization and microarray analysis, specific probes or genes could be highlighted, but these methods are also labor-intensive, expensive, complicated, and time-consuming. In this study, an in silico method with genome sequences of *Salmonella* was used to find genes specific to *Salmonella*. Constructed primers of selected genes were evaluated to compare the results between in silico and substantive experiments using PCR. Specific genes of *Salmonella*, *S. enterica* subsp. I, and *Salmonella* serovar Typhimurium were identified using genomic sequence comparison, and their specificities were evaluated across various *Salmonella* genomic DNAs. In addition, PCR results were accordant with genomic sequence comparison, demonstrating the effectiveness of genomic sequence comparison (Table 4; see Tables S3 and S4 in the supplemental material).

At the same time, this method has a limitation in that genomic sequence comparison is only possible when a database of genomic sequences is available. In the case of *Salmonella*, 12 genome sequences were used in this study. But only 3 genomic sequences (*Salmonella* serovar Typhimurium LT2, serovar Typhi CT18, and serovar Typhi TY2) were completed, and the genomic sequences of the other 9 strains were only available as raw sequence data. The inconsistency of these PCR results may be attributed to the fact that, in this study, genomic sequence comparison of *Salmonella* serovars was limited to only a few genome sequences (including *S. enterica* subsp. I, IIIb, and V). In addition, numerous cases of isolates of the same serovar with markedly different chromosomal genotypes have been reported (26, 29). It is impossible to characterize more than 2,500 *Salmonella* serovars with 12 genomic sequences. Nonetheless, efficient comparisons of 12 genomic sequences are expected to provide not only marker genes of *Salmonella* but also an easier approach to finding biological characteristics of *Salmonella*.

From the sequence comparison, the 31 genes of group 1 were expected to be present in all *Salmonella* subspecies. This group did not include previously reported specific target genes of *Salmonella* such as the *invA* and *ompC* genes (18, 21), as these genes were present not only in *Salmonella* but also in other closely related bacteria, such as *Escherichia coli* O157:H7 or *Citrobacter freundii*, and were eliminated through sequence comparison due to high homology with other related bacteria. In previous reports, 56 genes were suggested as *Salmonella* signature genes using comparison of genomic sequences with the four other enterobacteria and microarray analysis of PCR-amplified whole open reading frames of *Salmonella* serovar Typhimurium LT2 with 22 *Salmonella* strains (27). Compared with the results of genome comparison, only 4 genes (STM0699, STM2064, STM2549, and STM3098) from 31 genes of group 1 overlapped with previously reported *Salmonella* signature genes from microarray data. The different result of *Salmonella* signature genes between previous reports and this study comes from the different genome sequences used in each study. As a result, criteria for evaluating gene presence and absence were different.

In addition, the genes of group 8 were specifically expected to be present in each *S. enterica* subsp. I strain. In previous reports, 31 genes were suggested as a signature to *S. enterica* subsp. I, as they were found in strains belonging to *S. enterica* subsp. I but not in strains of the other subspecies using cDNA microarray analysis (26, 27). The 9 genes of group 8 were suggested as candidate *S. enterica* subsp. I signature genes, and only STM0305 was included in the 31 previously reported genes (26, 27). The 10 genes of group 3 were expected to be specific to *Salmonella* serovar Typhimurium. STM4203 and STM4214 were reported to be present not only in serovar Typhimurium but also in serovar Paratyphi C and serovar Choleraesuis. STM4497 was included in *Salmonella* serovar Typhimurium signature genes of STM4488 to STM4497 in a previous report (10). The 9 genes of group 2 were expected to be present in all *Salmonella* subspecies except *Salmonella bongori* (i.e., *Salmonella enterica* signature genes). In this group, STM1406 and STM1407 of *Salmonella* pathogenicity island 2 were included which were related with the type III secretion system of *Salmonella* (19). STM2773 (*iroB*), which was known as a *Salmonella enterica*-specific target gene, was not included in group 2 (3). The genes in group 17 showed various comparison patterns with various *Salmonella* strains. Among group 17, STM0894 and STM0902 (Fels-1 prophage) were only specific to the *S. enterica* serovar Typhimurium LT2. Fels-1 prophage was previously reported as specific to *S. enterica* serovar Typhimurium LT2 using microarray analysis (26). Fels-2 prophage genes (STM2716, STM2718, STM2721, STM2698, and STM2710) of group 17 were also present in other serovars, including serovar Typhimurium SL1344, but absent in serovar Typhimurium DT104, as reported previously. STM2344 and STM3736 of group 7 were previously reported as *Salmonella* signature genes by microarray analysis, but in this study, these genes were excluded because of low homology results with serovar Pullorum (27).

Comparative genomics between *Salmonella* species would provide not only genotyping and identification of *Salmonella* subspecies but also more information about the host specificity of *Salmonella* between subspecies and serovars. For example, *S. enterica* subsp. I signature genes might be target genes which differentiate *S. enterica* subsp. I that infect warm-blooded animals (including humans) from other *Salmonella* subspecies. Also, gene profile differences between host-specific serovars and host general serovars may give clues as to how and why the differences arose.

In an attempt to improve on serological typing using the Kauffmann-White scheme, many molecular methods have been applied to type or characterize *Salmonella*. But these methods have not provided enough discriminative power to resolve all *Salmonella* serovars. Also, these methods have been available only in a few reference laboratories. Recently, the onset of microarray and genomic sequencing technology has allowed for differences among *Salmonella* strains to be characterized at single-gene resolution (10, 14, 26). Microarrays appear to subdivide *Salmonella* with accuracy, but this method is too expensive to profile the 2,500 serovars of *Salmonella* and is only possible in a specialized laboratory. Further, microarray methods have disadvantages in sensitivity and scale that prevent application to field identification and detection of *Salmonella* and *Salmonella* serovar Typhimurium in the food industry. In contrast, PCR has the potential to become a powerful alternative in microbiological diagnostics due to its simplicity, rapidity, and accuracy.

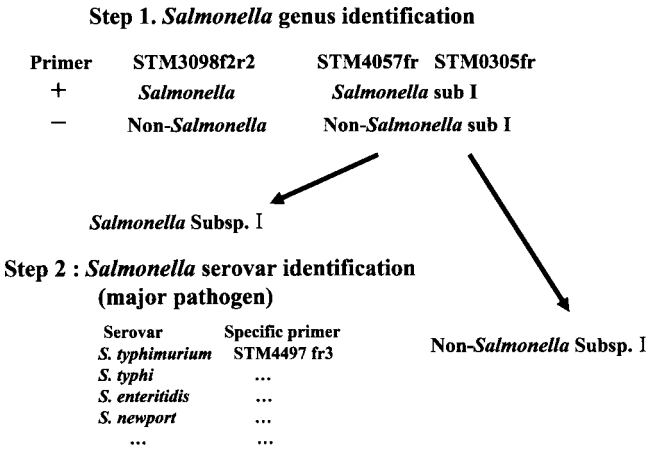

FIG. 2. New identification scheme of major pathogenic *Salmonella* strains and *Salmonella* spp. based on PCR results without serological testing. +, positive result; −, negative result.

In this study, 38 primer pairs were evaluated by PCR to subtype and characterize *Salmonella*. The phylogenetic tree in Fig. 1 generally agrees with the results of microarray analysis from several previously published reports (6–8, 26), supporting the successful application of genome sequence comparison for characterization of *Salmonella* strains using PCR. There were some discrepancies between this study and previous reports. For example, *Salmonella* serovar Enteritidis was previously observed as close to serovar Dublin with serovar Pullorum and serovar Gallinarum by MLEE and microarray results. In this study, serovar Dublin and serovar Enteritidis were in the same node but not closely clustered as shown in Fig. 1 (10). It is difficult to define a *Salmonella* serovar as monophyletic or polyphyletic by PCR results because of the limited number of serovars and primer pairs used in this study. However, several serovars might be monophyletic, including serovars Typhimurium, Enteritidis, Heidelberg, Virginia, Agona, and Haardt. Based on the results of this study, genomic sequence comparison can inform new microarray design to minimize the number of target genes and spots for effective genotyping and detection of bacteria. Also, in this study, we suggest target signature genes of *Salmonella*, *Salmonella* serovar Typhimurium, and *S. enterica* subsp. I by PCR results, providing a rapid and accurate protocol for epidemiological studies.

From PCR results of 38 primer pairs, we suggest a new identification scheme of the major pathogenic *Salmonella* strains in Fig. 2. This identification scheme consists of 2 PCR steps. The first step is *Salmonella* genus identification, including *S. enterica* subsp. I, by three primer pairs. At the results of step 1, samples are discriminated as *S. enterica* subsp. I or not. In the case of a positive test for *S. enterica* subsp. I, several sets of primers specific to major pathogens are evaluated to identify serovars. We are designing the specific primer pair of each pathogenic *Salmonella* serovar using genomic sequences in our laboratory. This *Salmonella* identification scheme needs to be evaluated with a greater variety of *Salmonella* serovars, including blind tests by consortium with laboratories in the other countries to examine the accuracy of *Salmonella* identification in epidemiological and taxonomical studies. Also, genotypic diversity within the *Salmonella* serovars must be considered. We are currently exploring multiplex PCR to allow simple identification of specific *Salmonella* serovars that cause disease.

In conclusion, the coding sequence region of *Salmonella enterica* serovar Typhimurium LT2 was compared with various *Salmonella* serovars, and selected genes were applied to genotyping and identification of *Salmonella* species. These results imply that genome sequence comparison can be successfully applied as a powerful tool for genotyping of *Salmonella* and can provide an easier means to detect and characterize *Salmonella*. In addition, we suggest target genes to differentiate between *Salmonella* subspecies and serovars, although these require further investigation. These methods and results can be used to expand investigations into different host ranges, distinct disease symptoms in different hosts, and specific detection of *Salmonella* serovars.

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