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Multiplex PCR for Distinguishing the Most Common Phase-1 Flagellar Antigens of *Salmonella* spp.

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Most *Salmonella* serotypes alternatively express either phase-1 or phase-2 flagellar antigens, encoded by the *fliC* and *fljB* genes, respectively. Flagellar phase reversal for the identification of both flagellar antigens is not necessary at the genetic level. Variable internal regions of the *fliC* genes encoding the H:i, H:r, H:l,v, H:e,h, H:z_{10}, H:b, and H:d antigens have been sequenced; and the specific sites for each antigen in selected *Salmonella* serotypes have been determined. These results, together with flagellar G-complex variable internal sequences obtained by the Foodborne and Diarrheal Diseases Branch at the Centers for Disease Control and Prevention in Atlanta, Ga., have been used to design a multiplex PCR to identify the G-complex antigens as well as the H:i, H:r, H:l,v, H:e,h, Hz_{10}, H:b, and H:d first-phase antigens. These antigens are part of the most common *Salmonella* serotypes possessing first-phase flagellar antigens. *Salmonella enterica* serotype Enteritidis is identified by adding a specific primer pair published previously (P. G. Agron, R. L. Walker, H. Kinde, S. J. Sawyer, D. C. Hayes, J. Wollard, and G. L. Andersen, Appl. Environ. Microbiol. 67:4984-4991, 2001). This multiplex PCR includes 13 primers. A total of 161 *Salmonella* strains associated with 72 different serotypes were tested. Each strain generated one first-phase-specific antigen fragment ranging from 100 to 500 bp; *Salmonella* serotype Enteritidis, however, generated two amplicons of 500 bp that corresponded to the G complex and a 333-bp serotype-specific amplicon, respectively. Twenty-three strains representing 19 serotypes with flagellar genes different from those targeted in this work did not generate any fragments. The method is quick, specific, and reproducible and is independent of the phase expressed by the bacteria when they are tested.

Salmonella enterica is believed to be responsible for the majority of cases of food-borne disease worldwide (14). Serotyping is widely used as an epidemiological typing method to divide *Salmonella* strains into groups on the basis of surface antigenic variability. Phage typing, pulsed-field gel electrophoresis, and antibiogram analysis can then be used for further subdivision of the strains within serotypes. In most countries, *Salmonella* isolates are typed by the Kauffman-White (K-W) scheme. A serotype of *Salmonella* is determined on the basis of antigenic variability at lipopolysaccharide moieties (O antigen), the flagellar protein (H antigen), and the capsular polysaccharide (Vi antigen). To date, 2,501 *Salmonella* serotypes have been identified (15). *Salmonella* isolates are diphasic with respect to the flagellar antigens, with several monophasic exceptions (4). *Salmonella* is unique among the members of the family *Enterobacteriaceae*, as it commonly has two distinct flagellar antigens, phase 1 and phase 2, that are coordinately regulated so that only one flagellar antigen is expressed at any time. In Spain, only 10 serotypes, all of subspecies I, represent 89.8% of the clinical isolates studied in the Spanish National Reference Laboratory for *Salmonella* and *Shigella* (LNRSS). Serotypes Typhimurium and Enteritidis alone represent 73.9% of the clinical isolates (16).

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Although serotyping offers a reliable method for differentiating *Salmonella* strains, identification by the slide agglutination method with a complete set of sera is a time-consuming process that requires the use of 167 specific antisera and well-trained technicians. At present, alternative methods for the identification of serotypes, such as DNA-based serotyping, or "molecular serotyping," are under development in many laboratories around the world, including LNRSS (5, 6, 12). These molecular methods are highly sensitive, very specific, and reproducible. These methods also allow for better laboratory-to-laboratory quality control, as they are fairly standard in most laboratories. Molecular serotyping schemes that are based on the K-W *Salmonella* serotyping scheme allow the continuation of surveillance data analysis based on this scheme. Thus, the information that has been collected over many decades could not only be maintained but also continue to be collected.

The *fliC* and *fljB* genes encode the phase-1 and phase-2 flagellins, respectively. These genes are found at two different locations on the chromosome. The *fljBA* operon contains *hin*, which encodes the Hin recombinase; the *fljB* gene, which encodes the phase-2 flagellin; and the *fljA* gene, which encodes a repressor for the *fliC* gene. The Hin recombinase gene catalyzes the reversible inversion of a 993-bp segment of the chromosome containing a promoter for the *fljBA* operon. In one orientation, the promoter directs the transcription of the *fljB* and *fljA* genes, inducing repression of the *fliC* gene. In the other orientation of *hin*, *fljB* and *fljA* are not expressed, the phase-2 flagellin is turned off, and *fliC* is derepressed, allowing

TABLE 1. Antigenic formulas for the *Salmonella* strains used to sequence the first-phase flagellar antigens

Serogroup	Serotype	Antigenic formula	N^a	Consensus sequence	GenBank accession no.
C-1	Livingstone	6,7,14:d:l,w	2	ICS^{d-2}	AY434707
E-1	Madjorio	3,10:d:e,n,z_{15}	1	ICS^{d-1}	AY434706
G	Tilburg	1,3,19:d:l,w	2	ICS^{d-3}	AY434708
B	Typhimurium	1,4,[5],12:i:1,2	2	ICS^{i-1}	AY429608
C-1	Garoli	6,7:i:1,6	1	ICS^{i-1}	AY429609
C-2	Kentucky	8,20:i:z_6	1	ICS^{i-2}	AY429612
C-2	Poeseldorf	8,20,54:i:z_6	1	ICS^{i-2}	AY429613
F	Aberdeen	11:i:1,2	1	ICS^{i-1}	AY429610
X	Bergen	47:i:1,5	1	ICS^{i-1}	AY429611
C-1	Mbandaka	6,7,14:z_{10}:e,n,z_{15}	2	ICS^{z10-1}	AY434692
C-2	Hadar	6,8:z_{10}:e,n,x	4	ICS^{z10-2}	AY434693
E-1	Lexington	3,10[15][15,34]:z10: 1,5	2	ICS^{z10-3}	AY434694
B	Wien	1,4,12,27:b:lw	3	ICS^{b-2}	AY434697
C-1	Ohio	6,7,14:b:lw	3	ICS^{b-1}	AY434695
N	Urbana	30.b:e,n,x	1	ICS^{b-1}	AY434696
B	Saintpaul	1,4,[5],12:e,h:1,2	1	$ICS^{e,h-2}$	AY434699
C-1	Braenderup	6,7,14:e,h:e,n,z_{15}	2	$ICS^{e,h-1}$	AY434698
E-1	Anatum	3,10[15][15,34]:e,h:1,6	3	$ICS^{e,h-3}$	AY434700
B	Heidelberg	1,4,[5],12:r.1,2	1	ICS^{r-1}	AY434701
C-1	Infantis	6,7,14:r:1,5	2	ICS^{r-1}	AY434702
C-1	Virchow	6,7,14:r:1,2	3	ICS^{r-2}	AY434705
C-2	Bovismorbificans	6,8,20:ri:1,5	1	ICS^{r-1}	AY434703
C-2	Goldcoast	6,8:r:lw	1	ICS^{r-1}	AY434704
B	Brandenburg	4,[5],12:l,v:e,n,z_{15}	2	$ICS^{l,v-1}$	AY434709
B	Bredeney	1,4,12,27:l,v:1,7	2	$ICS^{l,v-1}$	AY434710
D-1	Panama	1,9,12:l,v:1,5	1	$ICS^{l,v-2}$	AY434711
E-1	Give	3,10[15],[15,34]:[d]l,v:1,7	1	$ICS^{l,v-3}$	AY434712

[a] *N*, number of strains sequenced.

phase-1 flagellin to be expressed (9, 19). Some of these alleles are defined by a single factor (antigen i, d, or r); others are defined by several subfactors (e.g., antigens l,v, g,m, and e,n,x) (15). Comparison of the amino acid sequences of *Salmonella* flagellins has led to the definition of eight variable regions. The amino- and carboxy-terminal sequences (regions I and II and region VIII, respectively) are conserved and are thought to be important for polymerization and transportation. The central region, which comprises regions IV, V, and VI, is highly variable in both sequence and length between flagellar antigen genes and is generally believed to determine the epitope of the H antigen (13, 18).

In the past year, LNRSS has developed a PCR method that can identify second-phase flagellar antigens (complex H1, H:l,w, H:e,n,x, and H:e,n,z_{15}) belonging to the most common serotypes isolated in Spain (5, 6). The aim of the project described here was to develop a multiplex PCR method that can detect the most common phase-1 flagellar alleles by an approach similar to that developed for the phase-2 flagellar alleles described previously (5, 6).

MATERIALS AND METHODS

Strains. The *fliC* gene was sequenced from 47 strains belonging to 27 different serotypes of *S. enterica* subsp. *enterica* and expressing H:i, H:r, H:l,v, H:e,h, H:z_{10}, H:b, and H:d first-phase flagellar antigens (Table 1). For the multiplex PCR assay, 137 strains expressing the antigens listed above were used as positive controls and 23 strains expressing H:a, H:c, H:k, H:y, H:z, H:z_{29}, H:z_{38}, H:z_4,z_{23}, H:z_4,z_{24}, and H:z_4,z_{32} were used as negative controls (Table 2). All strains were from LNRSS, except for serotypes Rhone (Centers for Diseases Control and Prevention, Atlanta, Ga.); Tallase (Central Public Health Laboratory, London, United Kingdom); and Arechavaleta, Budapest, Chester, Kamoru, Kiel, Moscow, Naestved, Neumuenster, Pensacola, Rostock, and Wayne (World Health Organization International Laboratory for Serotyping, Pasteur Institute, Paris, France). Sequences from G-complex alleles were obtained from the Centers for Diseases Control and Prevention. These included the sequences of the alleles H:f,g (serotype Derby), H:f,g,s (serotype Agona), H:g,m and H:g,m,p (serotype Enteritidis), H:g,m,p,s (serotype Montevideo), H:g,m,s,t (serotypes Namib and Mosselbay), H:g,m,t (serotype Pensacola), H:g,p (serotype Dublin), H:g,p,s (serotype Naestved), H:g,p,u (serotype Rostock), H:g,q (serotype Moscow), H:g,s,t (serotypes Missouri and Senftenberg), H:g,t (serotypes Agodi and Merseyside), and H:m,t (serotype Oranienburg).

DNA sequencing. The *fliC* genes from the 47 strains shown in Table 1 were amplified by PCR with primer 1 and primer 2, as described previously (8). PCR amplification was performed with Ready-To-Go PCR beads (Amersham Biosciences, Piscataway, N.J.). Five microliters of a strain suspension that had been boiled for 10 min and briefly centrifuged was used as the template. PCR amplification was performed as follows: denaturation at 95°C for 5 min; 30 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 2 min; and a final extension cycle at 72°C for 7 min. The amplification products were purified and sequenced by using primers sense-60 (17) and Reverse-*fliC* (ACTTCGGTTTTGCCGTCTGC GCC). Sequencing was performed on an ABI PRISM 377 DNA sequencer (Applied Biosystems, Applera Hispania, S.A., Barcelona, Spain) and the Taq Dye Deoxy Terminator cycle sequencing kit (Applied Biosystems/Perkin-Elmer).

Other sequences from GenBank were also used. These included sequences with GenBank accession numbers Z15064 (serotype Berta $fliC^{f,g,t}$), Z15065 (serotype Budapest $fliC^{g,t}$), Z15066 (serotype Derby $fliC^{f,g}$), Z15067(serotype Dublin $fliC^{g,p}$), D78639 (serotype Naestved $fliC^{g,p,s}$), Z15068 (serotype Enteritidis $fliC^{g,m}$), Z15069 (serotype Montevideo $fliC^{g,m,s}$), Z15070 (serotype Oranienburg

TABLE 2. Antigenic formulas for the *Salmonella* strains used for multiplex PCR identification of the first-phase flagellar antigens

Serogroup	Serotype	Antigenic formula	N[a]	Amplicon size (bp)
A	Kiel	1,2,12:g,p:−	1	500
B	Derby	4,[5],12:f,g:1,2	2	500
B	Agona	4,12:f,g,s:−	3	500
B	California	4,12:g,m,t:−	1	500
C-1	Rissen	6,7:f,g:−	4	500
C-1	Montevideo	6,7:g,m,s:−	3	500
C-1	Oranienburg	6,7,14:m,t:[z_{57}]	1	500
D-1	Berta	1,9,12:[f],g,[t]:−	1	500
D-1	Budapest	1,4,12,27:g,t:−	1	500
D-1	Moscow	1,9,12:g,q:−	1	500
D-1	Naestved	1,9,12:g,p,s:−	1	500
D-1	Rostock	1,9,12:g,p,u:−	1	500
D-1	Pensacola	9,12:m,t:−	1	500
E-4	Senftenberg	1,3,19:g,s,t:−	2	500
N	Wayne	30:gz_{51}:−	1	500
D-1	Enteritidis	9,12:g,m:−	7	500 and 333
C-2	Hadar	6,8:z_{10}:e,n,x	11	400
C-1	Mbandaka	6,7,14:z_{10}:e,n,z_{15}	3	400
E-1	Lexington	3,10,[15][15,25]:z_{10}:1,5	2	400
H	Nessa	1,6,14,25:z_{10}:1,2	1	400
B	Brandenburg	4,[5],12:l,v:e,n,z_{15}	5	330
B	Bredeney	1,4,12,27:l,v:1,7	4	330
D-1	Panama	1,9,12:l,v:1,5	7	330
D-1	Kapemba	9,12:l,v:1,7	1	330
E-1	Give	3,10[15][15,34]:[d],l,v:1,7	3	330
D-1	London	3,10[15]:l,v:1,6	2	330
C-1	Infantis	6,7,14:r:1,5	3	275
C-1	Virchow	6,7,14:r:1,2	5	275
E-1	Weltevreden	3,10[15]:r:z_6	1	275
F	Rubislaw	11:r:e,n,x	1	275
B	Typhimurium	4,12:i:1,2	8	250
F	Veneciana	11:i:e,n,x	4	250
C-2	Kentucky	8,20:i:z_6	2	250
B	Chester	1,4,5,12:e,h:e,n,x	1	200
C-1	Braenderup	6,7,14:e,h:e,n,z_{15}	3	200
E-1	Anatum	3,10[15][15,34]:e,h:1,6	6	200
C-2	Newport	6,8,20:e,h:1,2	4	200
C-2	Kottbus	6,8:e,h:1,5	1	200
B	Abony	1,4,[5],12,27:b:e,n,x	1	150
B	Wien	1,4,12,27:b:l,w	2	150
B	Paratyphi B	1,4,[5],12:b:1,2	1	150
B	Paratyphi B var. Java	1,4,[5],12:b:1,2	1	150
B	Wagenia	1,4,12,27:b:e,n,z_{15}	4	150
C-1	Ohio	6,7,14:b:l,w	4	150
E-4	Tambacounda	1,3,19:b:e,n,x	1	150
N	Urbana	30:b:e,n,x	1	150
C-1	Livingstone	6,7,14:d:l,w	2	100
C-2	Manhattan	6,8:d:1,5	2	100
C-2	Muenchen	6,8:d:1,2	3	100
E-1	Madjorio	3,10:d:e,n,z_{15}	1	100
E-4	Tilburg	1,3,19:d:l,w	2	100
D-1	Ndolo	9,12:d:1,5	1	100
G	Grumpensis	12,23:d:1,7	2	100
B	Miami	1,9,12:a:1,5	1	—[b]
B	Sendai	1,9,12:a:1,5	1	—
B	Arechavaleta	4,[5],12:a:1,7	1	—
B	Tinda	1,4,12,27:a:e,n,z_{15}	1	—
B	Neumuenster	1,4,12,27:k:1,6	1	—
B	Kamoru	1,4,12,27:y:z_6	1	—
B	Coeln	1,4,[5],12:y:1,2	1	—
C-1	Mikawasima	6,7,14:y:e,n,z_{15}	3	—
C-1	Lille	6,7,14:z_{38}:−	1	—
C-1	Thompson	6,7,14:k:1,5	2	—
C-1	Choleraesuis	6,7:c:1,5	1	—
C-1	Tennessee	6,7,14:z_{29}:[1,2,7]	1	—
C-2	Albany	8,20:z_4,z_{24}:−	1	—
C-2	Tallahassee	6,8:z_4,z_{32}:−	1	—
G	Worthington	1,13,27:z:l,w	2	—
K	Cerro	6,14,18:c:z_4,z_{23}:[1,5]	1	—
L	Rhone	21:c:e,n,x	1	—
M	Nima	28:y:1,5	1	—
O:51	Treforest	1,51:z:1,6	1	—

[a] *N*, number of strains.
[b] —, no PCR products were obtained.

fliC^{m,t}), Z15071(serotype Rostock *fliC*^{g,p,u}), Z15072 (serotype Senftenberg *fliC*^{g,s,t}), and Z15086 (serotype Moscow *fliC*^{g,q}) (10).

Sequence analysis. Sequence analysis was performed with Lasergene (version 5.0) software (DNA-Star, Madison, Wis.). The variable internal region (VIR) sequences of each allele were aligned and compared to determine the internal consensus sequence (ICS). To avoid cross-reactions among the antigens belonging to the same complex, i.e., H:E (e,h, e,n,x, and e,n,z_{15}) and H:L (l,v and l,w), the sequences of previously identified alleles *fljB*^{enz15}, *fljB*^{enx}, *flj*B^{lw}, and *fliC*^{lw} (5) were included in the alignment. The specific priming sites were determined for each allele.

Multiplex PCR development. To develop a multiplex PCR for the H:i, H:r, H:e,h, H:l,v, H:b, H:z_{10}, and H:d first-phase flagellar antigens, 11 primers were designed with particular concern for the genetic stability of the target sequence, the presence of sequences specific for consensus antigen-specific sites, and the lengths of the amplicons. Primers Forward-G and Reverse-G were designed in order to detect antigens belonging to the G-complex alleles (g,m, g,m,s, g,m,t, g,p, g,p,s, g,m,q, g,q, g,m,p, g,m,p,s, g,m,s,t, g,s,t, m,t, m,p,t,u, f,g,s, f,g,m,t, g,z_{51}, and g,z_{63}). Sdf primer pairs (1) were added in order to detect serotype Enteritidis (9,12:g,m:−). The primers used are shown in Table 3.

The multiplex PCR was performed with Ready-To-Go PCR beads (Amersham Biosciences). According to the instructions of the manufacturer, DNA amplifications were performed in a reaction volume of 25 μl. Each reaction mixture contained 1.5 mM $MgCl_2$ and 5 pmol each of primers P60, Reverse-r, Reverse-i, Reverse-l,v, Forward-G, Reverse-G, and Reverse-z_{10} and the Sdf primer pairs (1). Also, 7 pmol each of primers Reverse-eh, Reverse-b, Forward-d, and Reverse-d was included. To improve the efficiency of the multiplex reaction and to reduce cross-reactivity, the buffer concentration was raised to 1.6× (7). Three microliters of a briefly centrifuged, boiled strain suspension was used as the template. The PCR was carried out under the conditions published previously (5): one initial denaturation cycle at 95°C for 5 min, followed by 30 cycles of 95°C for 40 s, 58°C for 20 s, and 72°C for 20 s and one final extension cycle of 72°C for 7 min. The fragments were separated in 2.5% agarose gels by unidirectional electrophoresis and were visualized by staining with ethidium bromide. Fragment size was determined by comparison with 50- and 100-bp DNA ladders (Amersham Biosciences).

Nucleotide sequence accession numbers. All ICSs were deposited in GenBank with accession numbers AY429608 to AY429613 and AY434692 to AY434712.

RESULTS

Two consensus sequences for the *fliC*^{i} 600-bp VIR were determined. These sequences differed at 2 nucleotides, which gave only one change in the amino acid sequences (Thr-195^{ACG}→Ala-195^{GCG}).

In the case of *fliC*^{r}, two consensus sequences were also

TABLE 3. Primers used for multiplex PCR identification of the most frequent *Salmonella* first-phase flagellar antigens

Primer	Sequence (5′-3′)	5′ position in *fliC* gene[a]	Reference or source
Sense-60	GCAGATCAACTCTCAGACCCTGGG	481–504	17
Antisense-i	ATAGCCATCTTTACCAGTTCC	714–734	This work
Antisense-z_{10}	CGTCGCAGCTTCTGCAACC	911–929	This work
Antisense-b	CGCACCAGTCYWACCTAAGGCGG	628–650	This work
Antisense-eh	AACGAAAGCGTAGCAGACAAG	658–678	This work
Antisense-lv	CCTGTCACTTTCGTGGTTAT	790–809	This work
Antisense-r	AAGTGACTTTTCCATCGGCTG	741–761	This work
Forward-d	CCCGAAAGAAACTGCTGTAACCG	539–561	This work
Reverse-d	TGGATATCAGTATTGCTCTGGGC	625–647	This work
Forward-G	GTGATCTGAAATCCAGCTTCAAG	547–569	This work
Reverse-G	AAGTTTCGCACTCTCGTTTTTGG	1055–1078	This work
Forward-Sdf-l	TGTGTTTTATCTGATGCAAGAGG	—	1
Reverse-Sdf-l	CGTTCTTCTGGTACTTACGATGAC	—	1

[a] Dashes indicate that primers do not target the *fliC* gene.

determined. Three polymorphic sites were found within the sequences, but these resulted in synonymous substitutions.

Three consensus sequences for the *fliC*^{z10} VIR were determined, ICS$^{z10\text{-}1}$, ICS$^{z10\text{-}2}$, and ICS$^{z10\text{-}3}$, within which there were seven polymorphic sites. Four of the substitutions were nonsynonymous. ICS$^{z10\text{-}1}$ and ICS$^{z10\text{-}2}$ differed at only 1 nucleotide, which gave a change in the amino acid sequence (Ala-204^{GCT}→Thr-204^{ACT}). ICS$^{z10\text{-}3}$ and ICS$^{z10\text{-}1}$ differed at 7 nucleotides and 3 amino acids (Thr-204^{ACC}→Ala-204^{GCT}, Ser-205^{AGT}→Gly-205^{GGT}, and Thr-348^{ACT}→Ser-348^{AGT}). ICS$^{z10\text{-}3}$ and ICS$^{z10\text{-}2}$ differed at 6 nucleotides. These changes were translated into two changes in the amino acid sequence (Ser-205^{AGT}→Gly-205^{GGT} and Thr-348^{ACT}→Ser-348^{AGT}, respectively).

Three consensus sequences for *fliC*^{l,v} were established: ICS$^{lv\text{-}1}$, ICS$^{lv\text{-}2}$, and ICS$^{lv\text{-}3}$. Only two polymorphic sites were found, but they were not involved in amino acid sequence changes.

Three consensus sequences for *fliC*^{e,h} were determined: ICS$^{eh\text{-}1}$, ICS$^{eh\text{-}2}$, and ICS$^{eh\text{-}3}$. There were four polymorphic sites among them, and all of the sites were nonsynonymous: Ala-185^{GCT}→Asp-185^{GAT}, Ala-215^{GCG}→Thr-215^{ACG} and Ala-215^{GCG} →Lys-215^{AAG}, and Thr-277^{ACA}→Lys-277^{AAA}, respectively.

Three consensus sequences for *fliC*^{d} were established: ICS$^{d\text{-}1}$, ICS$^{d\text{-}2}$, and ICS$^{d\text{-}3}$. There were five polymorphic sites. One of the sites (Lys-262^{AAA}→Thr-262^{ACA}) was nonsynonymous.

Finally, two consensus sequences were determined for *fliC*^{b}. There were 27 polymorphic sites. Sixteen of them gave 11 changes in the amino acid sequence: Ala-201^{GCA}→Thr-201^{ACA}, Asp-204^{GAC}→Thr-204^{ACC}, Ala-207^{GCA}→Glu-207^{GAA}, Ala-210^{GCC}→Thr-210^{ACC}, Thr-214^{ACG}→Leu-214^{TTG}, Ser-218^{TCG}→Thr-218^{ACG}, Ala-261^{GCA}→Ser-261^{TCA}, Val-274^{GTT}→Ile-274^{ATT}, Thr-281^{ACG}→Ala-281^{GCA}, and Ala-357^{GCA}→Thr-357^{ACA}. Furthermore, ICS$^{b\text{-}2}$ (corresponding to serotype Wien) had a GCT insertion (Ala-239).

G-complex analysis. The complete gene sequence was used to study the G complex. We used only one sequence for the *fliC*^{g,m,p}, *fliC*^{g,m,t}, and *fliC*^{m,p,t,u} alleles. The sequences obtained for the *fliC*^{g,m}, *fliC*^{g,m,s}, *fliC*^{g,p}, *fliC*^{g,p,u}, *fliC*^{g,q}, and *fliC*^{m,t} alleles were identical to those published previously (10).

Two consensus sequences were found for *fliC*^{g,m,s,t}. Both sequences were different at 15 nucleotides. Three of them gave changes in the amino acid sequence: Ala-254^{GCT}→Thr-254^{ACT}, Ala-314^{GCC}→Gly-314^{GGC}, and Val-383^{GTC}→Ile-383^{ATC}.

The *fliC*^{g,p,s} sequence was different from the previously published sequence (10) for the same serotype (serotype Naestved) by one nonsynonymous nucleotide change (Asn-348^{AAC}→Ser-348^{AGC}).

Two consensus sequences were determined for *fliC*^{g,s,t}. They were different at 7 nucleotides, although the amino acid sequences were identical. The sequence obtained for serotype Senftenberg was identical to that published previously (10).

The sequences obtained for *fliC*^{g,t} were different in the two serotypes studied: Agodi and Merseyside. There were 44 polymorphic sites. Eight of them gave changes in the amino acid sequence: Asn-246^{AAC}→Asp-246^{GAT}, Asp-258^{GAT}→Ala-258^{GCT}, Ser-265^{AGT}→Gly-265^{GGT}, Asn-319^{AAT}→Asp-319^{GAT}, Asp-322^{GAT}→Ala-322^{GCT}, Ala-380^{GCG}→Thr-380^{ACG}, Val-384^{GTC}→Ile-384^{ATC}, and Ser-421^{TCA}→Ala-421^{GCA}. The sequence of *fliC*^{g,t} obtained from serotype Agodi was different from the previously published *fliC*^{g,t} sequence (10) from serotype Budapest at 9 nucleotides, which resulted in one change in the amino acid sequence Ser-265^{AGT}→Gly-265^{GGT}. Finally, the sequence of *fliC*^{g,t} obtained for serotype Merseyside was different from the previously published sequence (10) at 40 nucleotides, which gave six different amino acids: Asn-246^{AAC}→Asp-246^{GAT}, Asp-258^{GAT}→Ala-258^{GCT}, Asp-322^{GAT}→Ala-322^{GCT}, Arg-380^{CGC}→Thr-380^{ACG}, Val-384^{GTC}→Ile-384^{ATC}, and Ser-421^{TCA}→Ala-421^{GCA}.

Position numbers are equivalent to those previously reported for the *fliC* flagellin with antigen i (11).

Primer design. By comparison of both the DNA sequences and the deduced amino acid sequences, we chose the common forward primer sense-60 for antigens H:i, H:r, H:z_{10}, H:l,v, H:e,h, and H:b and six reverse primers specific for each of these alleles.

In the case of the E complex, the differences were diverse enough that we were able to design a reverse primer specific for the e,h antigen.

The alleles belonging to the L complex showed high degrees of similarity when the sequences within the complex were compared. There were only five nonsynonymous sites in the se-

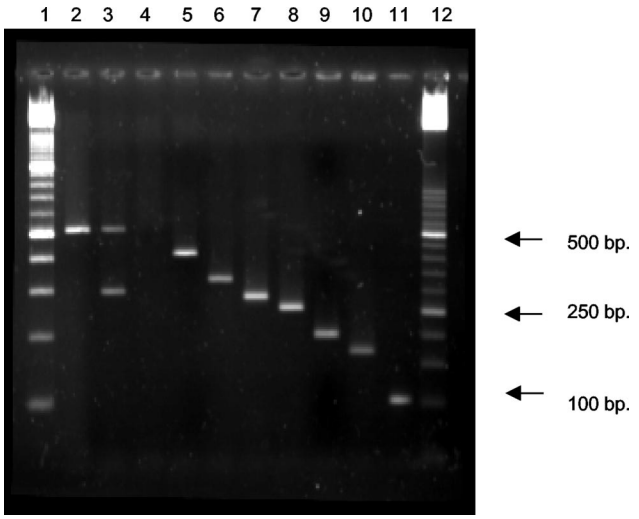

FIG. 1. Multiplex PCR amplification of *Salmonella* first-phase flagellar antigens. Lane 1, 100-bp ladder (Amersham Biosciences); lane 2, serotype Derby (H:f,g); lane 3, serotype Enteritidis (H:g,m); lane 4, serotype Mikawasima (H:y); lane 5, serotype Hadar (H:z_{10}); lane 6, serotype Brandenburg (H:l,v); lane 7, serotype Infantis (H:r); lane 8, serotype Typhimurium (H:i); lane 9, serotype Anatum (H:e,h); lane 10, serotype Ohio (H:b); lane 11, serotype Grumpensis (H:d); lane 12, 50-bp ladder (Amersham Biosciences).

quence. However, a reverse primer specific for l,v could be designed.

The sequences of the antigens belonging to the G complex showed high degrees of homology. Because of this homology, it was not possible to design a primer specific for the m antigen. A highly conserved region was used to design a universal forward primer (primer Forward-G) and a universal reverse primer (primer Reverse-G). In order to identify *Salmonella* serotype Enteritidis (9,12:g,m:−), the serotype reported most frequently in Spain, Sdf primer pairs, previously described by Agron et al. (1) to be specific for this serotype, were added to our multiplex PCR mixture to distinguish *Salmonella* serotype Enteritidis from other serotypes with H:6 in *fliC*.

The *fliC*^{d} sequences were diverse enough with respect to the sequences of the other alleles sequenced and did not match the sense-60 sequence. Consequently, we have designed *fliC*^{d}-specific forward and reverse primers.

Multiplex PCR sensitivity and specificity. Each of the reverse primers was tested individually and in combination with its specific forward primer by using a panel of known serotypes to ensure that a PCR product of the expected size was produced and that no additional or nonspecific products were generated. Once the specificity was determined, the PCR conditions, buffers, and primer concentrations were optimized (7) to establish conditions in which the primers could be combined into a single PCR mixture (see Material and Methods).

This new *Salmonella* phase-1 multiplex PCR developed with the newly designed and previously published (1, 3, 17) primers (Table 3) was evaluated with 161 *S. enterica* subsp. *enterica* isolates expressing one of the following flagellar antigens: H:G, H:z_{10}, H:l,v, H:r, H:i, H:e,h, H:b, and H:d. These reactions gave unique and specific amplicons of 500, 400, 300, 275, 250, 200, 150, and 100 bp, respectively.

Two amplicons were identified for *Salmonella* serotype Enteritidis: one of 500 bp that corresponds to the G complex and one of 333 bp that is specific for this serotype (Fig. 1). No cross-reactions with any of the 12 strains expressing the second-phase H:l,w flagellar antigen or 22 strains expressing the first-phase H:l,v flagellar antigen were found. In addition, no cross-reactivity with 41 *Salmonella* strains expressing second-phase H:e,n,x and H:e,n,z_{15} flagellar antigens or 15 *Salmonella* strains expressing first-phase H:e,h flagellar antigens was found. Finally, no amplification of flagellar antigen alleles was seen from any of the strains in the negative control panel. The correlation of the PCR assay results with the results of traditional serotyping was 100%.

DISCUSSION

Traditional serotyping of *S. enterica* has identified 2,501 unique serotypes based on antigenic surface structures of the lipopolysaccharide (O antigen), the phase-1 flagellar protein (H1), and the phase-2 flagellar protein (H2) (15). In reality, though, each year only a limited number of serotypes are reported to LNRSS. For example, the 10 most common serotypes constitute 89.8% of the clinical isolates reported to LNRSS. *Salmonella* serotypes Typhimurium and Enteritidis alone represent 73.9% of the clinical isolates (16).

However, serotyping by the traditional K-W scheme is time-consuming, requires well-trained technicians, and uses large amounts of high-quality sera. For these reasons, the use of DNA methods, such as the multiplex PCR for H-antigen identification described here, is an attractive alternative to the more traditional techniques.

During the past decade, many studies have demonstrated the extremely high capacity of PCR to detect specific genes of interest. These studies have shown that PCR can be a powerful tool in clinical microbiology. Echeita and colleagues (5, 6) showed that a multiplex PCR method which identifies the specific second-phase flagellar antigens found in the most common *Salmonella* serotypes reported in Spain could be developed. We illustrate here that the use of a similar method to identify the first-phase flagellar antigens and the use of a PCR approach to O-antigen typing could identify the major *Salmonella* serotypes, leaving conventional serotyping to be used for identification of the less common serotypes. The use of these methods would dramatically reduce the reliance on the traditional time-consuming methods and reduce the amount of sera needed for *Salmonella* identification.

The analysis of first-phase alleles encoding H:i, H:r, H:l,v, H:e,h, H:b, H:z_{10}, and H:d antigens showed the high degree of variability among phase-1 antigens. *fliC* VIR sequences were variable enough to allow the design of primers specific for each antigen. The presence of more than one primer pair in a multiplex PCR increases the possibility of obtaining spurious amplification products, primarily due to primer-dimer formation (2). For this reason, previously designed primer sense-60 (17) was chosen as a common forward primer specific for alleles *fliC*^{i}, *fliC*^{r}, *fliC*^{z10}, *fliC*^{e,h}, *fliC*^{b}, *fliC*^{d}, and *fliC*^{l,v}. The remaining primers were designed for genetic stability of the target sequence, specificity for consensus antigen-specific sites, and the lengths of the amplicons.

For detection of the L and E complexes, previously pub-

lished sequences of *fljB*^{e,n,x}, *fljB*^{e,n,z15}, *fljB*^{l,w}, and *fliC*^{l,w} (5) were also included in order to design primers specific for the l,v and e,h antigens. The previously reported high degrees of homology within these complexes led us to include these sequences in our alignment in order to detect and eliminate future nonspecific reactions.

E-complex sequence analysis demonstrated that first-phase-antigen amino acid sequences (represented by *fliC*^{e,h}) and second-phase-antigen amino acid sequences (represented by *fljB*^{e,n,x} and *fljB*^{e,n,z15}) were very different. Second-phase allelic differences implicated only three amino acid changes: Ala^{224} and Ser^{237}, which are probably implicated in the e,n,x epitope conformation, while Gly^{237} is probably at least partially responsible for the e,n,z15 epitope conformation.

The L-complex sequences were more homogeneous, with only five polymorphic sites among their amino acid sequences. All amino acid substitutions had the same hydrophilic properties. Thr^{264} and Thr^{269} are probably implicated in l,v epitope specificity; similarly, Ala^{197}, Thr^{202}, and Ala^{206} are responsible for l,w antigen specificity.

A high degree of homology existed among the H:G complex (g,m, g,m,s, g,m,t, g,p, g,p,s, g,m,q, g,q, g,m,p, g,m,p,s, g,m,s,t, g,s,t, m,t, m,p,t,u, f,g,s, f,g,m,t, g,z_{51}, and g,z_{63}) VIR sequences, and the sequences diverged from all other gene sequences studied. The *fliC*^{g,m} allele was found to be so highly conserved that we have been unable to find a region that was not shared with at least one of the other alleles belonging to the H:G complex. Consequently, we designed specific forward and reverse primers that generate an amplicon of 500 bp common to all G-complex antigens. We could not distinguish *Salmonella* serotype Enteritidis (9,12:g,m:−) phase-1 alleles from other G-complex alleles by this approach. Because this serotype is the most frequent cause of salmonellosis in Spain, as well as in most countries, we believed that it was important to design a multiplex PCR that could also distinguish this serotype from the other G-complex serotypes. For this reason, the Sdf primer pairs that Agron et al. (1) described as being specific for serotype Enteritidis were included in the multiplex PCR.

We report here on a set of both known and novel primers, comprising four forward primers and nine reverse primers, that are used together in a unique multiplex reaction. Using this single PCR assay, we can now rapidly identify the most common first-phase flagellar antigens of *S. enterica* isolates found in Spain. As our results showed, the method described here is a specific, fast, and cost-effective method that can be applied in a clinical microbiology laboratory for the serotyping of the first-phase flagellar antigens commonly expressed by *Salmonella* strains. The PCR described in this work, together with the multiplex PCR specific for the second-phase flagellar antigen reported by Echeita et al. (5), could identify the first- and second-phase flagellar antigens of up to 80% of the *Salmonella* strains isolated in Spain.

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