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Blind comparison of traditional serotyping with three multiplex PCRs for the identification of *Salmonella* serotypes

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Abstract

Salmonella serotypes are defined on the basis of somatic (O) antigens which define the serogroup and flagellar (H) factor antigens, both of which are present in the cell wall of *Salmonella*. Most *Salmonella* organisms alternatively express phase-1 or phase-2 flagellar antigens encoded by *fli*C and *flj*B genes, respectively. Our group previously published two multiplex PCRs for distinguishing the most common first- and second-phase antigens. In this paper we describe a third multiplex PCR to identify the most common serogroups (O:B; O:C1; O:C2; O:D and O:E). The combination of these three PCRs enabled us to completely serotype organisms belonging to the *Salmonella* species. This multiplex PCR includes 10 primers. A total of 67 *Salmonella* strains belonging to 32 different serotypes were tested. Each strain generated one serogroup-specific fragment ranging between 162 and 615 bp. Twenty-eight strains belonging to 21 serotypes, with a serogroup different from those tested in this work, did not generate any fragments. To compare molecular serotyping with traditional serotyping, 500 strains, received according to the order of arrival in the laboratory, were serotyped using both methods. The three multiplex PCRs were able to serotype 84.6% of the tested strains. This method was found to be very helpful in our laboratory as an alternative method for typing strains causing outbreaks, and it can be used to supplement conventional serotyping, since it is also applicable to motionless and rough strains.

Keywords: *Salmonella*; Molecular serotyping; Somatic antigens; Flagellins; PCR

1. Introduction

Salmonella species are recognized as major zoonotic pathogens of animals and humans. In many countries, including Spain, *Salmonella* is the leading cause of food-borne outbreaks and infections. The species *Salmonella enterica* is comprised of six subspecies: *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae* and *S. enterica* subsp. *indica*, or subspecies I, II, IIIa, IIIb, IV and VI, respectively [12]. *S. enterica* subsp. I is usually isolated from humans and warm-blooded animals and the majority of the serotypes isolated in clinical laboratories belong to this subspecies.

The Kauffmann–White serotyping scheme for designation of *Salmonella* serotypes is used by most laboratories for the characterization of *Salmonella* isolates. A serotype is determined on the basis of somatic (O) and flagellar (H) factor antigens present in the cell wall of *Salmonella* organisms. The O factors determine the grouping, while the H factors completely define the serotype identity of a *Salmonella* strain [11]. O-antigens are specific polysaccharides comprised in the lipopolysaccharide (LPS), an important component of Gram-negative bacteria. The genes for O-antigenic synthesis are normally grouped together on the chromosome in a gene cluster called *rfb*, which maps close to the gene *gnd* at 42 min on the *S. enterica* chromosome [1]. *rfb* Genes encode the nucleotide sugar

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biosynthesis pathways and the transferases necessary for assembly of the repeating unit. A gene named *wzx* (previously *rfb*X), which encodes a protein consisting of 12 potential transmembrane segments, is found in all of these *Salmonella* O-antigen gene clusters. The Wzx proteins from different O-antigen clusters are predicted to have a structural homology, but they have little similarity among them even at the amino acid sequence level [9]. It has been proposed that Wxz is responsible for transferring O-units from the cytoplasmic to the periplasmic side of the cytoplasmic membrane [13]. On the other hand, the flagellar antigens H1 and H2 are encoded by the *fli*C and *flj*B genes, respectively. With several monophasic exceptions, these two genes are present at two different locations on the chromosome, but only one of them is expressed by the cell at one time due to a mechanism regulated by the operon *flj*BA. To date, more than 2500 different serotypes have been described, of which 59% belong to *S. enterica* subsp. I. 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 [2].

Although serotyping offers a reliable method for differentiating *Salmonella* strains, identification by the slide agglutination method using a complete set of sera is a time-consuming process and requires the use of 167 anti-sera and well-trained technicians. Several molecular methods based on the amplification of DNA have been developed for the detection of *Salmonella* serotypes [6,8,14]. These molecular methods are highly sensitive, very specific, fast and reproducible. They permit a better lab-to-lab quality control, as they are fairly standard in most laboratories. Molecular serotyping also enables the preservation of the epidemiological information if the methods are based on the detection of the same antigen as the Kauffmann–White *Salmonella* scheme. The LNRSS has developed two multiplex PCRs for distinguishing first-phase and second-phase flagellar antigens of the most common serotypes of *Salmonella* [3,5]. In order to complete the Kauffmann–White scheme, we describe here a third multiplex PCR for differentiating the main serogroups of *Salmonella* spp. strains isolated in Spain.

The main objectives of this work are: (i) to describe a multiplex PCR that enables identification of O:B; O:C1; O:C2; O:D and O:E serogroups; (ii) to serotype 500 strains by molecular serotyping using the three multiplex PCRs developed in our laboratory; and (iii) to compare results obtained by traditional serotyping and molecular serotyping using a double-blind assay.

2. Materials and methods

2.1. Strains

For multiplex serogroup PCR assay, 69 strains expressing serogroups O:B (O:4); O:C1 (O:7); O:C2 (O:8); O:D (O:9; O:9,46; O:9,46,27) or O:E (O:3,10; O:1,3,19) were used as positive controls and 28 strains expressing O:A (O:2); O:11; O:13; O:14; O:17; O:18; O:28; O:30; O:38; O:43; O:45; O:48; O:50; O:51; O:53 or O:60 were used as negative controls (Table 1). All strains were from the LNRSS except for serotypes 48:z_{10}:z (Health Protection Agency, Colindale, UK), 45:g,z_{51}:− and 53:l,z_{28}:z_{39} (Institut Pasteur, France).

Table 1
Antigenic formula of *Salmonella* strains used for design of the multiplex PCR for serogroup identification and sizes of the amplified fragments

Serogroup	Serotype	Antigenic formula	No	Size of the amplicon
B	Agona	4,12:f,g,s:−	1	230 bp
	Brandenburg	4,12:l,v:1,6	1	
	Coeln	4,5,12: y:1,2	1	
	Indiana	4,12:z:1,5	1	
	Java	4,5,12:b:1,2	1	
	Typhimurium	1,4,[5],12:i:1,2	6	
C1	Infantis	6,7:r:1,5	1	486 bp
	Livingstone	6,7:d:l,w	1	
	Mikawasima	6,7:r:1,2	1	
	Ohio	6,7:b:l,w	3	
	Rissen	6,7:f,g:−	2	
	Tennessee	6,7:z_{29}:−	1	
	Thompson	4,9,12:i:1,2	2	
	Virchow	6,7:y:e,n,z_{15}	2	
C2	Altona	8,20:r:z_6	2	162 bp
	Blockley	6,8:k:1,5	2	
	Hadar	6,8:z_{10}:e,n,x	4	
	Kentucky	8,20:i:z_6	2	
	Manhattan	6,8:d:1,5	1	
	Muenchen	6,8:d:1,2	2	
	Newport	6,8:e,h:1,2	1	
	Tallahasee	6,8:z_4,z_{32}:−	1	
D	Enteritidis	9,12:g,m:−	8	615 bp
	Panama	9,12:l,v:1,5	1	
E	Anatum	3,10:e,h:1,6	6	344 bp
	Give	3,10:l,v:1,7	3	
	Goelzau	3,15:a:1,5	1	
	Lexington	3,10:z_{10}:1,5	2	
	London	3,10:l,v:1,6	3	
	Senftenberg	1,3,19; g,[s],t: −	1	
	Tambacounda	1,3,19:b:e,n,x	1	
	Tilburg	1,3,19:d:l,w	4	
Others	Cerro	18:z_4,z_{23}:1,5	2	–
	Friedrichsfelde	28:f,g:−	1	–
	Grumpensis	13,23:d:1,7	2	–
	Jangwani	17:a:1,5	1	–
	Nima	28:y:1,5	1	–
	Paratyphi A	1,2,12:a:[1,5]	5	–
	Pomona	28:y:1,7	2	–
	Urbana	30:b:e,n,x	1	–
	Veneziana	11:i:e,n,x	1	–
	Worthington	13,23:z:l,w	1	–
	6,14:z_4,z_{23}:− (subsp. IV)	6,14:z_4,z_{23}:−	1	–
	38:k:z_{53} (subsp. IIIb)	38:k:z_{53}	1	–
	43:z_4,z_{23}:− (subsp. IV)	43:z_4,z_{23}:−	1	–
	45:g,z51:− (subsp. IV)	45:g,z51:−	1	–
	48:z_{10}:z (subsp. IIIb)	48:z_{10}:z	1	–
	50:l,v:z_{35} (subsp. IIIb)	50:l,v:z_{35}	1	–
	50:l,w:z_{35} (subsp. IIIb)	50:l,w:z_{35}	1	–
	51:g,z_{51}:− (subsp. IIIa)	51:g,z_{51}:−	1	–
	53:l,z_{28}:z_{39} (subsp. II)	53:l,z_{28}:z_{39}	1	–
	60:k:z_{53} (subsp. IIIb)	60:k:z_{53}	1	–
	60:z_{41}:− (subsp. V)	60:z_{41}:−	1	–

No., number of strains tested.

2.2. DNA extraction

A 1-µl loop of bacterial cells was suspended in 1000 µl of distilled water to an OD_{600} of 1.0–2.0. The suspension was heated at 95 °C for 2 min and crude cell lysates were used as DNA templates in the PCRs.

2.3. Sequence analysis

DNA sequence assembly and analysis, and primers design were performed with the Lasergene software 6.0 (DNAstar, Madison WI, USA).

2.4. Serogroup multiplex PCR

Five primer pairs were designed based on previously published sequences of the *wzx* gene from serogroups B (accession number X56793), C1 (accession number M84642), C2 (accession number X61917), D1 (accession number M65054) and E1 (accession number X60665). The primers matching serogroup D were designed targeting the gene *tyv* (accession number M29682) (Table 2). The multiplex PCR was performed using Ready-To-Go PCR beads (GE Healthcare, UK). According to the manufacturer's instructions DNA amplification was performed in a reaction volume of 25 ml. Each reaction contained 1.5 mM $MgCl_2$ and 5 pmol of primers F-wzxB, R-wzxB, F-wzxC1, R-wzxC1, F-wzxC2, R-wzxC2, F-tyvD, R-tyvD, F-wzxE and R-wzxE. Five milliliters of a briefly centrifuged, boiled strain suspension was used as template. The PCR reaction was carried out under previously published conditions [5]. Fragments were separated in 2.5% agarose (MS8 type, Pronadise, Madrid, Spain) gel by unidirectional electrophoresis using TAE 1× buffer and visualized by staining with ethidium bromide. Fragment size was determined by comparison with 50 and 100 bp DNA ladders (GE Healthcare, UK).

2.5. Flagellar antigens

First-phase antigens and second-phase antigens were determined using the first-phase multiplex PCR and second-phase multiplex PCR, respectively, as previously reported [3,5].

2.6. Validation of the multiplex assay

Five-hundred isolates that were sent to the LNRSS during the first 2 months of 2004 were tested with the multiplex PCRs in comparison with traditional methods for *Salmonella* identification. Traditional serotyping was performed by slide agglutination with commercial anti-sera (BioRad, Spain).

About 74% of the Spanish clinical isolates belonged to serotypes Typhimurium and Enteritidis. In order to test the three PCRs in other serotypes, one person from the laboratory having no acquaintance with traditional serotyping or molecular serotyping processes selected 1 out of 3 Typhimurium strains, 1 out of 3 Enteritidis strains and 1 out of 3 non-Typhimurium or non-Enteritidis strains received according to order of arrival in the laboratory.

3. Results

3.1. Design of the serogroup multiplex PCR

The analysis of the *wzx* gene showed high diversity among the different serogroups under study. Thus, it was necessary to design a specific forward and a specific reverse primer for each serogroup. Each of the primer pairs was tested individually and in combination with the others by using strains of the positive control panel to ensure that a PCR product of the expected size was produced without generating additional or non-specific products. When strains of the negative control were tested, cross-reactivity was found between serogroups C1 and F (O:11). Sequencing of the generated amplicon of both serogroups (about 500 bp) showed high similarity. The two serogroups differed in 15 nucleotides and 5 amino acids. Primers for C1 identification were redesigned and the new *Salmonella* serogroup multiplex PCR was evaluated with 69 *S. enterica* subsp. *enterica* isolates expressing one of the following serogroups: O:B; O:C1; O:C2; O:D and O:E. These reactions gave unique and specific amplicons of 230, 483, 154, 615 and 345 bp. respectively. No amplification was seen from any of the 28 strains in the negative control panel (Fig. 1, Table 1).

3.2. Validation of the multiplex assay

Five-hundred isolates that were sent to the LNRSS during the first 3 months of 2004 were evaluated in multiplex PCRs in

Table 2
Characteristics of specific primers for identification of serogroups B, C1, C2, D and E

Target	Primer	Sequence (5′–3′)	Tm (°C)	Position on the gene	Amplicon size (bp.)
tyv-D	F-tyvD	GAGGAAGGGAAATGAAGCTTTT	63.4	−11 to 11	615
	R-tyvD	TAGCAAACTGTCTCCCACCATAC	63.9	582–604	
wzx-B	F-wzxB	GGCATATATTTCTGTATTCGCG	62.0	1051–1072	230
	R-wzxB	GCCTTAATTAAGTAAGTTAGTGGAAGC	61.4	1254–1280	
wzx-C1	F-wzxC1	CAGTAGTCCGTAAAATACAGGGTGG	65.2	356–380	483
	R-wzxC1	GGGGCTATAAATACTGTGTTAAATTCC	60.7	816–850	
wzx-C2	F-wzxC2	ACTGAAGGTGGTATTTCATGGG	63.7	697–718	154
	R-wzxC2	AAGACATCCCTAACTGCCCTGC	67.3	829–850	
wzx-E	F-wzxE1	TAAAGTATATGGTGCTGATTTAACC	59.0	150–174	345
	R-wzxE1	GTTAAAATGACAGATTGAGCAGAG	60.7	471–494	

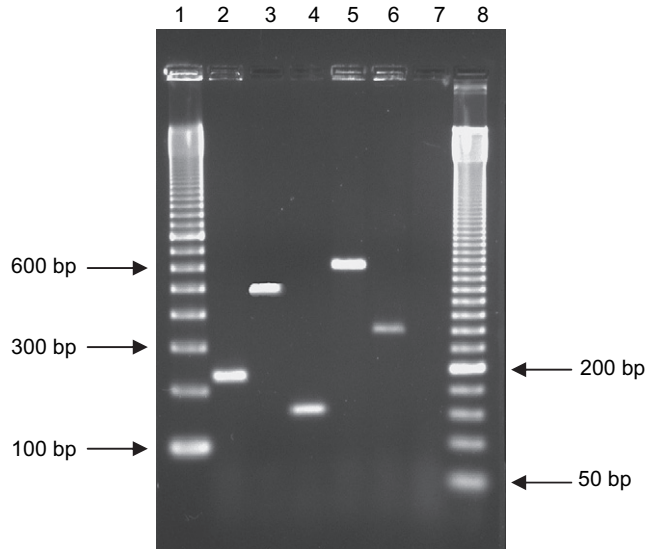

Fig. 1. Multiplex PCR amplification of *Salmonella* serogroups. Lane 1, 100 bp ladder (GE Healthcare, UK); lane 2, serotype Typhimurium (O:B); lane 3, serotype Ohio (O:C1); lane 4, serotype Hadar (O:C2); lane 5, serotype Enteritidis (O:D); lane 6, serotype Anatum (O:E); lane 7, serotype Cerro (O:18); lane 8, 50 bp ladder (GE Healthcare, UK).

comparison with the traditional method for *Salmonella* serotyping. The multiplex PCRs produced amplicons corresponding to all three antigen targets from 489 isolates (97.8%). Eleven isolates (2.2%) failed to amplify some of the amplicons. Traditional serotyping showed that all of these isolates had some antigens not included in the multiplex PCRs (Table 3). Four-hundred and twenty-three isolates (84.6%) were completely serotyped, and 66 (13.2%) showed ambiguous results, since the obtained antigenic formula matched with more than one serotype.

The multiplex PCRs were able to identify serotypes not identified by traditional serotyping. Thus, one autoagglutinable strain was identified by PCR as Enteritidis and four motionless strains were also serotyped as Enteritidis. Seven isolates had lost the capacity to express the second-phase flagellar antigen and were classified as 6:8:z10:− (1/7) and 4,5,12:i:− (6/7) by traditional serotyping. The multiplex PCRs showed they were monophasic variants of Hadar (6,8:z_{10}:e,n,x) or Istanbul (8:z_{10}:e,n,x) or Typhimurium (4,5,12:i:1,2) (Table 3).

Finally, excluding isolates giving ambiguous or incomplete results, the correlation between traditional and molecular serotyping was 99.2%. The discrepancies between the two techniques were as follows: four isolates were serotyped as Typhimurium by traditional serotyping and as Heidelberg (3/4) and B:i:− (1/4) by multiplex PCRs. In these cases, results achieved with the molecular approach were confirmed by repeating traditional serotyping.

4. Discussion

Serogroup-specific PCR assays have been described for serogroups A, B, C1, D and H [4,6,8]. However, because our overall goal was to combine a serogroup identification system with those previously described for phase-1 and phase-2 flagellar antigens, we developed a new multiplex PCR for the rapid identification of *Salmonella* serogroups O:B; O:C1; O:C2–C3; O:D and O:E, because serotypes of these serogroups are among the top 20 *Salmonella* serotypes isolated from humans in Spain [2]. The PCR developed by Luk et al. [8] is thus far the most comprehensive. Those authors designed primers targeting defined regions of the abequose and paratose synthase genes: *abe* of *Salmonella* serogroup B, *abe* of *Salmonella* serogroup C2 and *prt* of *Salmonella* serogroup D (also present in serogroup A). Serogroups A and D differ only at one nucleotide base between the two *tyv* genes, which creates an early codon stop in serogroup A, with the result that only paratose is incorporated in its O-antigen. This particular situation makes it mandatory to design primers in that particular position in order to distinguish the two serotypes. For the other serotypes, we designed primers targeting gene *wzx*. The Wzx protein is characteristic of the major class of the O-antigen cluster and encodes a flippase for O-unit translocation across the membrane. Wzx proteins from different O-antigen clusters are predicted to have a structural homology, but they have little similarity among them even at the amino acid sequence level, making this gene an excellent target for serogroup-specific primers [9]. In order to better differentiate among these major serogroups, the primers were designed not only to give precise specificity in priming but also to give DNA products with different sizes in polymerase chain reactions (PCRs) (615 bp for serogroup D, 483 bp for serogroup C1, 345 bp for serogroup E, 230 bp for serogroup B and 154 bp for serogroup C2). In a PCR assay utilizing these specific primers, all 69 strains expressing serogroups O:B; O:C1; O:C2; O:D or O:E were identified, whereas no amplification was obtained with strains of the negative control panel.

The combination of the three multiplex PCRs designed for identification of *Salmonella* spp. enabled serotyping of 423 out of 500 strains (84.9%). Sixty-six strains (13.2%) showed ambiguous results due to the fact that they belonged to a serotype differing from another one in only one of the minor antigens within the same serogroup. However, if the method here described is used as a first screening to serotype a limited number of isolates, then it can save time in the whole process, since not all polyvalent anti-sera need to be tested. In addition, one of the main advantages of the molecular method here described is the possibility of assigning an antigenic formula to non-typeable strains. Non-typeable strains originate when a strain loses the O-antigen and becomes rough or does not express the flagellar antigens and becomes monophasic or motionless. Monophasic or motionless *Salmonella* strains of well known serotypes could originate as mutants of biphasic strains which have lost the switching mechanism, or either *fli*C or *flj*B flagellar gene or the ability to express one of these genes. Thus, in our study, seven monophasic strains were further characterized as Typhimurium (6/7) and Hadar (1/6) by molecular serotyping, and four motionless strains were further characterized as Enteritidis.

Mutants displaying mismatches in either their flagellin gene sequences or in the *wzx* gene sequence near the 3′ position of the primers used in the assays could result in a loss of PCR

Table 3
Results of the comparison between molecular serotyping and traditional serotyping

No.	Traditional serotyping result	No.	Molecular serotyping result	Putative serotype(s) by PCR	Status
247	Enteritidis (9,12:g,m:−)	247	D:G:− plus specific band for Enteritidis[a]	Enteritidis	CS
110	Typhimurium (1,4,5,12:i:1,2)	106	B:i:1,2	Typhimurium	CS
		3	B:r:1,2	Heidelberg (4,5,12:r:1,2)	CS
		1	B:i:−	Typhimurium; Lagos (1,4,[5]12:i:1,5); Agama (4,12:i:−); Farsta (4,12:i:e,n,x); Tsevie (4,12:i:e,n,x); Gloucester (4,12,27:i:l,w); Tumodi (1,4,12:i:z6); II 4,12:i:z27	A
18	Infantis (6,7:r:1,5)	18	C1:r:1,5	Infantis	CS
17	Hadar (6,8:z_{10}:e,n,x)	17	C2:z_{10}:e,n,x	Hadar; Instanbul (8:z10:e,n,x)	A
14	Ohio (6,7: b:l,w)	14	C1:b:l,w	Ohio	CS
14	4,5,12:i:−	8	B:i:−	Typhimurium; Lagos (1,4,[5]12:i:1,5); Agama (4,12:i:−); Farsta (4,12:i:e,n,x); Tsevie (4,12:i:e,n,x) Gloucester (4,12,27:i:l,w); Tumodi (1,4,12:i:z6); II 4,12:i:z27	A
		6	B:i:1,2	Typhimurium	CS
10	Rissen (6,7,14:f,g:−)	10	C1:G:−	Rissen; Montevideo (6,7,14:g,m,[p],s:[1,2,7]); II 6,7:g,[m],s,t:[z42];Othmarschen (6,7,14:g,m,[t]−) Plumaugat (6,7:g,s,q:−); Menston (6,7:g,s,[t]:[1,6]); II 6,7:g,t,[e,n,x]:z42; Riggil (6,7;g,[t]:−); IV 6,7:g,z51:−; Oranienburg (6,7,14:m,t:[z57])	A
7	Livingstone (6,7:d:l,w)	7	C1:d:l,w	Livingstone	CS
6	Derby (1,4,12:f,g:−)	6	B:G:−	Derby; Agona (1,4,[5],12:f,g,s:[1,2]); Essen (4,12:g,m:−); Hato (1,4,[5],12:g,m,s:−); II 1,4,12,27:g,[m],t:[1,5]; California (4,12:g,m,t:[z67]); Budapest (1,4,12,27:g,t:-); II 4,12:g,z62:-	A
6	Agona (1,4,[5],12:f,g,s:[1,2])	6	B:G:−	Same as Derby	A
5	Bredeney (4,12:l,v:1,7)	5	B:l,v:1,7	Bredeney	CS
5	Virchow (6,7:r:1,2)	5	C1:r:1,2	Virchow	CS
4	Brandenburg (4,12:l,v:e,n,z_{15})	4	B:l,v:e,n,z_{15}	Brandenburg	CS
4	Anatum (3,10: e,h: 1,5)	4	E:e,h:1,6	Anatum; Hayindogo (1,3,19:e,h:1,6)	A
4	Indiana (1,4,12: z: 1,7)	4	B:−:1,7		IF
4	Mikawasima (6,7:y:enz15)	4	C1:−:enz15		IF
4	Motionless	4	D:G:− plus specific band for Enteritidis[a]	Enteritidis	CS
3	Muenchen (6,8: d: 1,2)	3	C2:d:1,2	Muenchen, Virginia 8:d:1,2:[z67]	A
2	Montevideo (6,7,14:g,m,[p],s:[1,2,7])	2	C1:G:−	Same as Rissen	A
2	Newport (6,8,20: e,h:1,2)	2	C2: e,h:1,2	Newport, Bardo (8:e,h:1,2)	A
2	Goldcoast (6,8: r: l,w)	2	C2:r:l,w	Goldcoast; Brikama (8,20: r: l,w)	A
1	Panama (9,12: l,v: 1,5)	1	D:l,v:1,5	Panama	CS
1	Give (3,10: l,v:1,7)	1	E:l,v:1,7	Give; Parkroyal	A
1	Mbandaka (6,7: z_{10}: e,n,z_{15})	1	C1:z_{10}:e,n,z_{15}	Mbandaka	CS
1	Bovismorbificans (6,8,20:i:1,5)	1	C2:i:1,5	Bovismorbificans; Hindmarsh (8,20:i:1,5)	A
1	Braenderup (6,7: e,h: e,n,z15)	1	C1:e,h:e,n,z15	Braenderup	CS
1	Oranienburg (6,7,14:m,t:[z57])	1	C1:G:−	Same as Rissen	A
1	Senftenberg (1,3,19:g,t:-)	1	E:G:−	Senftenberg; Regent (3,12:f,g,[s]:[1,6]); Suberu (3,10:g,m:−); Amsterdam (3,10,[*15*],[*15*,*34*]:g,m,s:−); Westhampton (3,10 [*15*],[*15*,*34*]:g,s,t:−); II 3,10:g,t:−; Rideau (1,3,19:f,g:−); Kouka (1,3,19:g,m,[t]:−); Cannstatt (1,3,19:m,t:−); Dessau (1,31,*15*,19:g,s,t:−)	A
1	Blockley (6,8:y:1,5)	1	C2:−:1,5		IF
1	Grumpensis (13,22:d:1,7)	1	d:1,7		IF
1	Kentucky (8:i:z6)	1	C2:i:−		IF
1	Autoagglutinable	1	D:G:− plus 333 bp band	Enteritidis	CS
1	Monophasic	1	C2:z_{10}:e,n,x	Hadar; Instanbul	A

No., Number of strains identified; CS, complete serotyping result; A, ambiguous results; IF, incomplete formula.
[a] Band of 333 bp obtained by using the PCR for identifying first-phase flagellar antigen (5).

reactivity. The genetic variation of *wzx*, *fli*C and *flj*B genes has been previously reported [3,5,9,10]. Thus, it is possible that specific primers designed in this work could fail to give amplification if a mutation affects a primer site, although we could unequivocally identify both somatic antigens and flagellar antigens of all 500 *Salmonella* strains tested.

A total of 72.8% out of 84.6% of the completely serotyped isolates belonged to serotypes Typhimurium and Enteritidis. In Spain, only 10 serotypes represent 89.8% of the clinical isolates studied in the LNRSS. These top 10 serotypes are: Enteritidis, Typhimurium, Hadar, 4,5,12:i:−, Virchow, Ohio, Brandenburg, Infantis, Anatum and Bredeney [2], together representing 87.8% of the isolates tested in this study. Serotypes Typhimurium and Enteritidis alone represented 72.8%.

Traditional serotyping is a time-consuming process and requires the use of 167 anti-sera and well-trained technicians. However, *Salmonella enteritidis* and *Salmonella typhimurium* constituted more than 80% of all *Salmonella* serotypes recorded in the Global Salm Surv database in 2000–2005 [7]. In the LNRSS, the incorporation of these molecular tools for the serotyping of *Salmonella* has enabled the combining of both molecular and traditional methods, and therefore has increased the capacity of serotyping. We have established a comprehensive DNA-based scheme for identification of the major *Salmonella* serotypes, without the need for serological testing. It is a rapid and convenient alternative for identification of *Salmonella* serotypes and it has proven to be very helpful as an alternative method for typing strains causing outbreaks that affect a large segment of the population and of supplementing conventional serotyping, for example when a strain becomes motionless or rough.

The molecular method described here cannot replace traditional serotyping, as only 15 of the 33 serotypes isolated during this period could be completely serotyped. It is necessary to find a high-through platform that would enable us to add more antigens and to analyze more samples in a shorter time. Finally, we would like to emphasize that the development of a molecular method for serotyping *Salmonella* spp. does not imply that we should rule out traditional serotyping. The two methods are complementary, and the molecular method simply enhances the available tools for successfully typing strains which cause health problems.

References

[1] D.A. Bastin, P.R. Reeves, Sequence and analysis of the O antigen gene (*rfb*) cluster of *Escherichia coli* O111, Gene 16 (1995) 17–23.

[2] M.A. Echeita, A.M. Aladueña, R. Diez, M. Arroyo, F. Cerdan, R. Gutierrez, M. de la Fuente, R. Gonzalez-Sanz, S. Herrera-Leon, M.A. Usera, Serotype and phage type distribution of human *Salmonella* strains isolated in Spain, 1997–2001, Enferm. Infecc. Microbiol. Clin 23 (2005) 127–134.

[3] M.A. Echeita, S. Herrera, J. Garaizar, M.A. Usera, Multiplex PCR-based detection and identification of the most common *Salmonella* second-phase flagellar antigens, Res. Microbiol. 153 (2002) 107–113.

[4] C. Fitzgerald, R. Sherwood, L.L. Gheesling, F.W. Brenner, P.I. Fields, Molecular analysis of the *rfb* O antigen gene cluster of *Salmonella enterica* serogroup O:6, 14 and development of a serogroup-specific PCR assay, Appl. Environ. Microbiol. 69 (2003) 6099–6105.

[5] S. Herrera-Leon, J.R. McQuiston, M.A. Usera, P.I. Fields, J. Garaizar, M.A. Echeita, Multiplex PCR for distinguishing the most common phase-1 flagellar antigens of *Salmonella* spp, J. Clin. Microbiol. 42 (2004) 2581–2586.

[6] K. Hirose, K. Itoh, H. Nakajima, T. Kurazono, M. Yamaguchi, K. Moriya, T. Ezaki, Y. Kawamura, K. Tamura, H. Watanabe, Selective amplification of *tyv* (*rfb*E), *prt* (*rfb*S), *via*B, and *fli*C genes by multiplex PCR for identification of *Salmonella enterica* serovars Typhi and Paratyphi A, J. Clin. Microbiol. 40 (2002) 633–636.

[7] http://www.who.int/salmsurv/en/.

[8] J.M. Luk, U. Kongmuang, P.R. Reeves, A.A. Lindberg, Selective amplification of abequose and paratose synthase genes (*rfb*) by polymerase chain reaction for identification of *Salmonella* major serogroups (A, B, C2, and D), J. Clin. Microbiol. 31 (1993) 2118–2123.

[9] C.L. Marolda, J. Vicarioli, M.A. Valvano, Wzx proteins involved in biosynthesis of O antigen function in association with the first sugar of the O-specific lipopolysaccharide subunit, Microbiology 150 (2004) 4095–4105.

[10] J.R. McQuiston, R. Parrenas, M. Ortiz-Rivera, L. Gheesling, F. Brenner, P.I. Fields, Sequencing and comparative analysis of flagellin genes *fli*C, *flj*B, and *flp*A from *Salmonella*, J. Clin. Microbiol. 42 (2004) 1923–1932.

[11] M.Y. Popoff, Antigenic formulas of the *Salmonella* serovars 8th revision, WHO Collaborating Centre for Reference Research on *Salmonella*, Institut Pasteur, Paris, 2001.

[12] M.W. Reeves, G.M. Evins, A.A. Heiba, B.D. Plikaytis, J.J. Farmer, Clonal nature of *Salmonella typhi* and its genetic relatedness to other salmonellae as shown by multilocus enzyme electrophoresis, and proposal of *S. bongori* comb. nov, J. Clin. Microbiol. 35 (1989) 2786–2790.

[13] G. Samuel, P.R. Reeves, Biosynthesis of O-antigens: genes and pathways involved in nucleotide sugar precursor synthesis and O-antigen assembly, Carbohydr. Res. 338 (2003) 2503–2519.

[14] D.H. Shah, J.H. Park, M.R. Cho, M.C. Kim, J.S. Chae, Allele-specific PCR method based on *rfb*S sequence for distinguishing *Salmonella* gallinarum from *Salmonella* pullorum: serotype-specific *rfb*S sequence polymorphism, J. Microbiol. Methods 60 (2005) 169–177.