Ann Microbiol (2010) 60:135–141
DOI 10.1007/s13213-010-0021-8

ORIGINAL ARTICLE

Two five-plex PCRs methods for identification of common *Salmonella* spp. serotypes

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Received: 12 October 2009 / Accepted: 8 January 2010 / Published online: 10 February 2010

Abstract In Tunisia, *Salmonella* is the most common bacterial agent responsible for childhood diarrhoea. Currently, isolation of the bacterium by microbiological and biochemical methods and confirmation of the serotype by serological method remain as the "gold standard". This study aimed to differentiate among the most common serotypes of *Salmonella* spp. via two rapid five-plex PCRs assay (MPCR) to evaluate the molecular serotyping method compared with the gold standard serotyping technique. The two five-plex PCRs assays were designed for the simultaneous detection of six genetic loci from *Salmonella enterica* serovar Typhimurium and four from *S. enterica* serovar Typhi. Sixty-one Tunisian strains (46 collected from patients and 15 from food) were isolated during the period 2002–2007. The STM and STY primers were able to discriminate all tested *Salmonella* serotypes that represent the most common clinical and food strains of *S. enterica* subsp. *enterica* in our laboratory. All strains belonged to 19 different serotypes: 15 serotypes gave unique amplification patterns compared each other and the other 4 serotypes were grouped into two pairs that gave the same molecular profile. We resolved this problem through the addition of a monoplex PCR. *Salmonella typhimurium* ATCC 14028 consistently produced the same molecular profile as *S. typhimurium* laboratory isolates. Interestingly, seven strains of Anatum serovar produced two different PCR profiles with these primers: five strains had the same amplification pattern STM 2,4,5 and STY 2; however, two strains had another molecular profile STM 2,3,5 and STY 2; so the reproducibility of this method was reduced to 93%. The MPCR system is a rapid, specific, and cost-effective molecular method that gas been proved to have efficient discrimination in serotyping of the most common isolates of *S. enterica* subsp. *enterica*.

Keywords *Salmonella* spp. · Serotypes · Somatic O and flagellar H antigens · Molecular serotyping

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Introduction

Salmonellosis is one of the most common causes of foodborne infections in humans all over the world. The genus *Salmonella* is extremely polymorphic. It has been classified into more than 2,400 different known serovars (Nair et al. 2002; Martinez-Urtaza et al. 2004) of which 1,478 are *Salmonella enterica* subsp. *enterica* (Bopp et al. 2003; Perch et al. 2003).

Many *Salmonella* serovars cause gastroenterites that are accompanied by diarrhoea, abdominal pain,s and fever. Among the *Salmonella enterica* serovars commonly implicated in most major outbreaks are serovars Enteritidis, Anatum, Newport, Cerro, Montevideo, Infantis, and Saint-Paul (Bopp et al. 2003; Perch et al. 2003).

Salmonella enterica subsp. *enterica* shows different disease syndromes and host specificities according to their antigenic profiles. Therefore, it is necessary and important to discriminate *Salmonella* serovars in order to ensure that each pathogen is correctly recognized. The differences within *Salmonella* serovars are based on the surface antigen

differences of O and H antigens. The O antigens are derived from the polysaccharide domain of lipopolysaccharide (LPS) in the cell wall, while the H antigens are derived from flagellin protein in the flagella. O and H antigens are used for the identification of *Salmonella* serovars by agglutination tests using O and H antigen-specific anti-sera, then *Salmonella* serovars are determined by the combination of O and H antigen types using the Kauffmann-White scheme which is annually updated by the World Health Organization (Popoff et al. 1992; Bopp et al. 2003).

The serological method has been used to identify *Salmonella* serovars. However, this method is complex, time consuming, and 5–8% of isolates are partially typed or untyped. This can be due to capsular polysaccharides in mucoid strains and in "rough" strains that produce partially formed O antigens that can cross-react with different O antisera (Bopp et al. 2003). So these types of isolates can be partially typed by their H antigens. With H-antigen typing, both flagellar phases must be assayed. Non-motile isolates can be partially typed by their O antigens.

At present, alternative strategies for the identification of serotypes, such as ribotyping (Esteban et al. 1993), IS200 analysis (Ezquerra et al. 1993; Uzzau et al. 1999), real-time PCR, and DNA microarrays (Chan et al. 2003; Porwollik et al. 2004; O'Regan et al. 2008) are under development in many laboratories, but these methods require specialized equipment and there are problems with the reproducibility of results. For these reasons, an easier and simpler method is needed to identify *Salmonella* serovars.

In this study, we establish two simple five-plex PCRs methods (MPCR) to type the most common *Salmonella enterica* subsp. *enterica* serovars. This method is based on detection of genes present in specific serotypes. These genes were selected from analysis of previous work including whole-genome sequencing (Porwollik et al. 2004, 2005). Reproducibility of the assay was also evaluated. As our results show, the method described here is specific, fast, and cost-effective and can be applied in the clinical microbiology laboratory for the serotyping of *Salmonella* serovars.

Materials and methods

Bacterial strains and DNA extraction The 19 most common serotypes of *Salmonella* spp. strains used in this study were collected from 61 Tunisian strains (46 collected from patients and 15 from food) isolated during the period 2002–2007. These serovars that represent more than 90% of all serotypes commonly identified by our clinical laboratory were selected according to their frequency and importance. *Salmonella typhimurium* ATCC 14028 was also tested as standard strain (Table 1).

Table 1 Molecular detection of common serotypes of *Salmonella enterica* subsp. *enterica* using STM and STY primers by multiplex PCR

Serotype	Amplification pattern	
	STM	STY
Typhimurium (ATCC 14028)	1,3,5	2,4
Typhimurium	1,3,5	2,4
Bovis-morbificans	2,3	5,2
Nikolaifleet	5	6
Mbandaka	2,3,5	2,4
Newport	1,2,3,5	0
Braenderup	2,5	0
Cerro	5	4
Muenster	3,5	2
Heidelberg	1,2,4,5	2
Corvallis	3,5	6
Zanzibar	2,5	2,4
Derby	1,2,3,5	6
Amsterdam	2,3	2,4
Carnac	2,3	2,4
Montevideo	5	2,4
Altona	2,3	6
Infantis	2	2
Anatum	2,4,5	2
Enteritidis	2,3,5	3

Each isolate was previously serotyped according to the Kauffmann-White scheme based on slide agglutination to identify the somatic O antigens and flagellar H antigens using commercial antisera (Bio-Rad, Marnes-la-Coquette, France) in Centre de Référence des *Salmonella*, *Shigella* et *Vibrions cholériques*, Institut Pasteur Tunis, Tunisia. All strains were cultured on nutrient agar plates with overnight incubation at 37°C. DNA was prepared from each isolate by boiling (Agarwal et al. 2002).

Primer design and PCR amplification program Genomic regions chosen for this serotyping study were derived from previous work that used microarray technology to compare the genomic complement of *S. enterica* serovar Typhimurium LT2 (STM) and *S. enterica* serovar Typhi CT18 (STY) to common serotypes of *S. enterica* subsp. *enterica* (Table 2; Porwollik et al. 2004). These loci were chosen for their ability to give unique results and so to differentiate the clinically most common serotypes of *Salmonella*. The biological functions and phylogenetic implications of these loci have been previously discussed (Shangkuan and Lin 1998; Chan et al. 2003; Porwollik et al. 2002, 2004).

Suitable primers for two five-plex PCRs, each amplifying five different regions, were designed using Primer Express v2.0 (Applied Biosystems, Foster City, CA) and were synthesized by MWG-Biotech (High Point, NC).

Table 2 Chromosomal regions of *Salmonella enterica* serovars Typhimurium LT2 and Typhi CT18 used to create primers for multiplex PCR (Porwollik et al. 2004)

Assay	NCBI accession no.	Primer sequence (5′ →3′)	Amplicon size (bp)
STM 1	AE008729	5′-AACCGCTGCTTAATCCTGATGG-3′ 5′-TGGCCCTGAGCCAGCTTTT-3′	187
STM 2	AE008758	5′-TCAAAATTACCGGGCGCA-3′ 5′-TTTTAAGACTACATACGCGCATGAA-3′	171
STM 3	AE008735	5′-TCCAGTATGAAACAGGCAACGTGT-3′ 5′-GCGACGCATTGTTCGATTGAT-3′	137
STM 4	AE008913	5′-TGGCGGCAGAAGCGATG-3′ 5′-CTTCATTCAGCAACTGACGCTGAG-3′	114
STM 5	AE008913	5′-TGGTCACCGCGCGTGAT-3′ 5′-CGAACGCCAGGTTCATTTGT-3′	93
STY 1	AL627266	5′-TGGTATGGTTAAGCGGAGAATGG-3′ 5′-GAGAGTCATAGCCCACACCAAAG-3′	301
STY 2	AL627273	5′-GGCTGGAGCAGCCTTACAAAA–3′ 5′-AAGAGTTGCCTGGCTGGTAAAA-3′	262
STY 3	AL627273	5′-AATCCCCCCCCCTCAAAAA-3′ 5′-GGTACACGTTTACTGTTTGCTGGA-3′	220
STM 6	AE008879	5′-ATATCTCATCGTCTCCTTTTCGTGT-3′ 5′-GAAGGTCCGGATAGGCATTCT-3′	181
STM 7	AE008795	5′-CATAACCCGCCTCGACCTCAT-3′ 5′-AGATGTCGTGAGAAGCGGTGG-3′	101

The other discriminatory monoplex PCR used primer set STM7, which was derived from serovar Typhimurium LT2 (Porwollik et al. 2005).

For all PCRs, primer sequences (5′→3′) are provided in Table 2 (Porwollik et al. 2004). The first five-plex PCR assay (named STM) incorporated loci from serovar Typhimurium. The second assay (named STY) was based on four loci in serovar Typhi C18 and one from serovar Typhimurium LT2.

All reactions were performed in a final volume of 34 µl containing 5 µl of template DNA, 0.2 mM dNTPs, 2 mM $MgCl_2$, 5.0 units of Taq polymerase, 50 ng of each primer and deionised water to make up the volume. All assays used the same cycling parameters under the following conditions: enzyme activation at 94°C for 5 min and then an additional 40 cycles with heat denaturation at 94°C for 30 s, primer annealing at 62°C for 30 s, and DNA extension at 72°C for 1 min. After the last cycle, samples were maintained at 72°C for 5 min to complete the synthesis of all strands.

Each PCR products (10 µl) were separated by electrophoresis and analyzed on 2% Tris-acetate EDTA agarose gel stained with ethidium bromide, visualized with UV induced fluorescence, and photographed.

Results

Optimization strategies

The optimization of MPCR is more tedious and difficult to achieve than monoplexes so the correct size of each primer is first established in a monoplex PCR rather than in multiplex PCR.

A monoplex PCR for each primer set was initially carried out based on a previous mixture protocol and PCR amplification program. Initial attempts are to amplify equally all the five STM and STY genes using a monoplex rather than a multiplex PCR reaction. The monoplex PCRs were successful and all STM and STY primer sets gave a single and a specific amplification product of a required gene except for STY 1 that cannot be detected in the tested *Salmonella* serovars (Fig. 1).

Reproducibility

Reproducibility for the detection of STM and STY genes assayed in the MPCR, to confirm our PCR

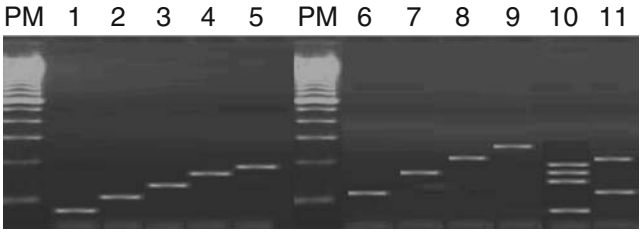

Fig. 1 Ethidium bromide-stained agarose gel of PCR products. *PM* 1,000-bp DNA ladder size standard; *lanes 1, 3, 4, and 5 Salmonella* Newport DNA amplified with STM 1, STM 2, STM 3, and STM 5 primers, respectively; *lane 2 Salmonella* Anatum DNA amplified with STM 4 primer; *lanes 6 and 9 Salmonella* Zanzibar DNA amplified STY 2 and STY 4 primers, respectively; *lane 7 Salmonella* Altona DNA amplified with STM 6 primer; *lane 8 Salmonella* Enteritidis DNA amplified with STY 3 primer; *lane 10* a MPCR product obtained with *Salmonella* Newport DNA amplified with STM 1, STM 2, STM3, and STM 5 primers, respectively; *lane 11*a MPCR product obtained with *Salmonella* Zanzibar DNA amplified with STY 2 and STY 4 primers, respectively

Table 3 Antigenic formulas of *Salmonella* strains and results of numbers of tested isolates using the STM and STY assays to predict their serotype by PCR

Serotype	Antigenic formula			No. of isolates	
	Somatic (O) antigen	Flagellar (H) antigen		Tested	Failed[a]
		Phase 1	Phase 2		
Typhimurium	4,[5]	[i]	1,2	5	0
Bovis-morbificans	6,8,20	r,[i]	1,5	1	0
Mbandaka	6,7	z_{10}	e,n,z_{15}	1	0
Newport	6,8	e,h	1,2:[z_{67}]	1	0
Braenderup	6,7	e,h	e,n,z_{15}	1	0
Cerro	18	z_4,z_{23}	[1,5]	5	0
Nikolaifleet	16	g, m, s	_	1	0
Muenster	3,10[15]	e,h	1,5	4	0
Corvallis	8,20	z_4,z_{23}	[z_6]	1	0
Zanzibar	3,10	k	1,5	2	0
Derby	4	f, g	_	1	0
Amsterdam	3,10	g,m,s	–	1	0
Montevideo	6, 7	g,m,[p],s	–	6	0
Heidelberg	4,[5]	r	1, 2	2	0
Carnac	18	z_{10}	z_6	1	0
Altona	8 20	r,[i]	z_6	3	0
Infantis	6,7	r	1,5	1	0
Anatum	3,10	e,h	1,6	7	2
Enteritidis	9	g,m	–	17	0
Typhimurium (ATCC 14028)				1	0

[a] Number of isolates that produced different amplicon patterns with the same tested serotypes

results, was 100% if we except *Salmonella* serovar Anatum (Table 1). In this way, all *Salmonella*-specific tested serotypes gave the same amplification profiles with the same *Salmonella* serotypes (Table 3) but *Salmonella* serovar Anatum produced two different STM amplicon patterns (Table 3).

Detection of amplified products with STM primers to discriminate between different serotypes by multiplex PCR

To identify *Salmonella* serovars using multiplex PCR, five primer sets were used in the same reaction mixture (STM 1, STM 2, STM 3, STM 4, and STM 5) (Table 2); the 19 most common serotypes of *Salmonella* spp. strains and the ATCC strain were tested (Table 1).

To confirm our results, we mostly tried to test more than one strain from each serotype (Table 3). For a total of 61 tested isolates, all *Salmonella* serotypes produced at least one amplicon. We can note that the amplified product obtained with STM 5 primer was produced with the most *Salmonella* serovars (80% of *Salmonella* serovars). Using these five STM primers with the 19 *Salmonella* serovars, we can identify four distinct groups: seven serotypes produced two amplicons; four serotypes produced one amplified product; five serotypes produced three amplified STM products, but the most common STM

Fig. 2 Ethidium bromide-stained agarose gel of Multiplex PCR products from extracted *Salmonella* serovars DNA amplified with STM 1, STM 2, STM 3, STM 4, and STM 5 primers. *PM* 1,000-bp DNA ladder size standard; *lane 1* negative control; *lanes 2 to 12 Salmonella* serovars Corvallis, Mbandaka, Muenster, Bovis-morbificans, Braenderup, Infantis, Cerro, Typhimurium, Typhimurium ATCC 14028, Nikolaifleet, and Montevideo, respectively

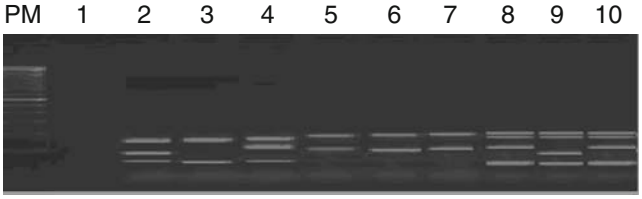

Fig. 3 Ethidium bromide-stained agarose gel of Multiplex PCR products from extracted *Salmonella* serovars DNA amplified with STM 1, STM 2, STM 3, STM 4, and STM 5 primers. *PM* 1,000-bp DNA ladder size standard; *lane 1* negative control; *lanes 2 to 10 Salmonella* serovars Anatum, Zanzibar, Enteritidis, Amsterdam, Carnac, Altona, Newport, Heidelberg, and Derby, respectively

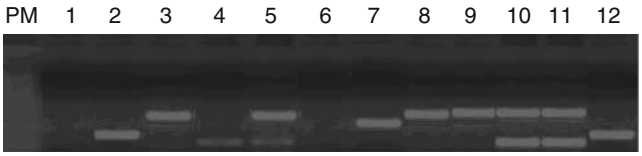

Fig. 4 Ethidium bromide-stained agarose gel of Multiplex PCR products from extracted *Salmonella* serovars DNA amplified with STY 1, STY 2, STY 3, STY 4, and STM6 primers. *PM* 1,000-bp DNA ladder size standard; *lane 1* negative control; *lanes 2 to 12 Salmonella* serovars Corvallis, Muenster, Cerro, Mbandaka, Braenderup, Enteritidis, Bovis-morbificans, Infantis, Typhimurium, Typhimurium ATCC 14028, and Nikolaifleet, repectively

multiplex PCR amplicon pattern were 2,3,5; the last group was obtained with three serotypes that produced four amplified PCR products. Interestingly, *Salmonella* serovar Anatum produced either STM profile 2,4,5 or 2,3,5 (Table 1; Figs. 2 and 3).

Finally, we noted that *Salmonella typhimurium* ATCC 14028 consistently produced the same molecular profile compared with *Salmonella typhimurium* laboratory isolates.

Detection of amplified products with STY primers to discriminate between different serotypes by multiplex PCR

In a second approach, we validated the MPCR for *Salmonella* serovars detection by using STY primers: STY 1, STY 2, STY 3, and STY 4 genetic regions of *S. enterica* serovar Typhi; we also used STM 6 primer that designed the genomic region of *S. enterica* serovar Typhimurium (Table 2). The 19 different tested *Salmonella* serovars could be classified into three groups on the basis of scoring the presence or absence of appropriately size amplicons (Table 1): the first group contained the largest number of *Salmonella* serovars (58%) identified by the presence of only a single product, either STY 2, STY 3, STY 4, or STM 6 patterns, of which 45 and 36% of serotypes were obtained, respectively, with STY 2 and STM 6 primers; the second group contained six serotypes that produced two amplicons which were 100% STY 2 and STY 4 profiles; and the third group contained the two serovars Newport and Braenderup and was negative for all STY primers (Figs. 4, 5).

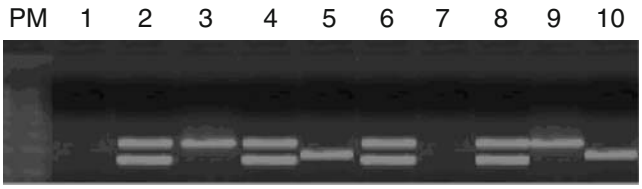

Fig. 5 Ethidium bromide-stained agarose gel of Multiplex PCR products from extracted *Salmonella* serovars DNA amplified with STY 1, STY 2, STY 3, STY 4, and STM6 primers. *PM* 1,000-bp DNA ladder size standard; *lane 1* negative control; *lanes 2 to 10 Salmonella* serovars Zanzibar, Anatum, Amsterdam, Altona, Carnac, Newport, Montevideo, Heidelberg, and Derby, respectively

Table 4 Molecular detection of four serotypes of *Salmonella enterica* subsp. *enterica* using STM7 primer by a monoplex PCR

Serotype	Amplification pattern STM7
Bovis-morbificans	Positive
Amsterdam	Positive
Carnac	Negative
Anatum	Negative

Combining the STM and STY primers to better discriminate between different *Salmonella* serotypes

To further evaluate the discriminatory method for *Salmonella* serotyping and to increase identified serovars, we combined both the STM and STY primers. With five STM primers (STM 1, STM 2, STM3, STM 4, STM 5), four STY primers (STY 1, STY 2, STY 3, STY 4) and STM 6 primer, it was possible to discriminate 15 different serotypes. However, four serotypes (*Salmonella* Anatum, *Salmonella* Bovis-morbificans, *Salmonella* Carnac and *Salmonella* Amsterdam) were grouped into two pairs that had the same amplification pattern (Table 1); for this reason, and to be able to discriminate between these four serotypes, we made a monoplex PCR using STM 7 primer set designed from *S. enterica* serotype Typhimurium (Table 4).

Using the STM 7 primer that creates an amplicon of 101 bp, we were able to discriminate between Anatum and Bovis-morbificans and also between Carnac and Amsterdam serotypes (Fig. 6). In this way, and using these panels of primers, we could type all of the tested *Salmonella* serotypes.

Comparison between the two approaches: serotyping standard method and MPCR serotyping for identification of *Salmonella* serotypes

The results of the two five-plex PCRs were converted to amplification products and then compared to traditional

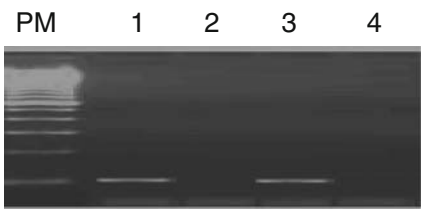

Fig. 6 Ethidium bromide-stained agarose gel of simple PCR products from extracted *Salmonella* serovars DNA amplified with STM 7 primer for discrimination of paired serotypes. *PM* 1,000-bp DNA ladder size standard; *lanes 1 to 4* amplification products of *Salmonella* serovars Bovis-morbificans, Anatum, Amesterdam, and Carnac, respectively

serotyping. We noted that all tested *Salmonella* serovars gave the same PCR profile results with each specific tested serotype (Tables 1 and 3). In this way, there was 100% correlation between traditional serotyping and molecular serotyping. However, one exception was found with *Salmonella* Anatum serotype: two isolates gave the molecular combination named STM 2,4,5 STY 2 and the others gave the molecular combination STM 2,3,5 STY 2 (Table 1).

Discussion

Salmonella serotypes were determined on the basis of antigenic variability at lipopolysaccharide moieties (O antigen), the flagellar protein (H antigen), and the capsular polysaccharide (Vi antigen).

Salmonella isolates are diphasic with respect to the flagellar antigens, with several monophasic exceptions. In this way, *Salmonella* serovars were determined by the combination of O and H antigen types (phase-1 and phase-2) using commercial antisera (Bio-Rad). This serological method has been used to identify *Salmonella* serovars and specifically more than 1,478 different serovars of *Salmonella enterica* subsp. *enterica* (Bopp et al. 2003; Perch et al. 2003).

However, when using the serological method, antibodies must be produced for each serovar, and this is extremely complex and time consuming. So easier and simpler methods were needed for identification of *Salmonella* serovars. Recently, different molecular techniques were developed for detection of *Salmonella* serovars such as pulsed-field gel electrophoresis (PFGE) (Uzzau et al. 1999; Torpdahl and Ahrens 2004), ribotyping (Altwegg et al. 1989; Pang et al. 1992), and random amplification of polymorphic DNA (RAPD) (Shangkuan and Lin 1998), as well as the recent development of amplified fragment length polymorphism (AFLP) (Nair et al. 2000) and the real-time multiplex PCR (O'Regan et al. 2008).

In this context, different PCR protocols for specific detection of *S. enterica* subsp. *enterica* were developed, but they were limited to the number of strains that can be typed (Echeita et al. 2002; Herrera-Leon et al. 2004).

In this study, using suitable primers for the two five-plex PCRs and the monoplex PCR methods for molecular *Salmonella* serotyping, we could easily discriminate all the tested *Salmonella* serotypes that represented 100% of all *Salmonella* isolates in our regional Tunisian laboratory during the period 2002–2007. These results have been found elsewhere (Perch et al. 2003). The STM 7 primer was used in a monoplex PCR to differentiate between the four serotypes (*Salmonella* Anatum, *Salmonella* Bovismorbificans, *Salmonella* Carnac, and *Salmonella* Amsterdam) that gave the same STM and STY profiles (Table 4). The same molecular serotyping profiles were also reported with other serovars (Kim et al. 2006). The reasons for this resemblance in molecular amplicon code can be explained by the presence of a very similar region in these serovars. It can also be explained by deletion problems that can concern a specific region and so the absence of appropriately sized amplicons with specific primers (Garaizar et al. 2002). A secondary discrimination problem that was interesting to note was that the serovar Anatum gave more than one amplicon code with STM primers which may reflect intraserovar variation.

To further discriminate each serovar, we can associate to this multiplex PCR serotyping the PFGE analysis, the enterobacterial repetitive intergenic sequence PCR (ERIC2-PCR), or the 16 S\23 S r RNA ribotyping. These methods provided a high degree of intraserovar discrimination. In this way, we describe the MPCR as a rapid, specific, and cost-effective molecular method that has demonstrated its efficient discrimination in serotyping of the most common clinical and food isolates of *S. enterica* subsp. *enterica* in our region. This method can be used as an alternative method of standard serotyping in many clinical laboratories.

References

Agarwal A, Makker A, Goel SK (2002) Application of the PCR technique for a rapid, specific and sensitive detection of *Salmonella* spp. in foods. Mol Cell Probes 16:243–250

Altwegg M, Hickman-Brenner FW, Farmer JJ (1989) Ribosomal RNA gene restriction patterns provide increased sensitivity for typing *Salmonella typhi* strains. J Infect Dis 160:145–149

Bopp CA, Brenner FW, Fields PI, Wells JG, Stockbine NA (2003) *Escherichia*, *Shigella*, and *Salmonella*. In: Murray PR, Baron EJ, Jorgensen JH, Pfaller MA, Yolken RH (eds) Manual of clinical microbiology, vol 1, 8th edn. ASM Press, Washington D.C,, pp 654–671

Chan K, Baker S, Kim CC, Detweiler CS, Dougan G, Falkow S (2003) Genomic comparison of *Salmonella enterica* serovars and *Salmonella bongori* by use of an *S. enterica* serovar Typhimurium DNA microarray. J Bacteriol 185:553–563

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

Esteban E, Snipes K, Hird D, Kasten R, Kinde H (1993) Use of ribotyping for characterization of *Salmonella* serotypes. J Clin Microbiol 31:233–237

Ezquerra E, Burnens AP, Frith K, Costas M, Stanley J (1993) Molecular genotype analysis of *Salmonella bovismorbificans*. Mol Cell Probes 7:45–54

Garaizar J, Porwollik S, Echeita A, Rementeria A, Herrera S, Wong RM, Frye J, Usera MA, McClelland M (2002) DNA microarraybased typing of an atypical monophasic *Salmonella enterica* serovar. J Clin Microbiol 40:2074–2078

Herrera-Leon S, McQuiston JR, Usera MA, Fields PI, Garaizar J, Echeita MA (2004) Multiplex PCR for distinguishing the most

common phase 1 flagellar antigens of *Salmonella* spp. J Clin Microbiol 42:2581–2586

Kim S, Frye JG, Hu J, Fedorka-Cray PJ, Gautom R, Boyle DS (2006) Multiplex PCR-based method for identification of common clinical serotypes of *Salmonella enterica* subsp. *enterica*. J Clin Microbiol 44:3608–3615

Martinez-Urtaza J, Liebana E, Garcia-Migura L, Perez-Pineiro P, Saco M (2004) Characterization of *Salmonella enterica* serovar Typhimurium from marine environments in costal waters of Galicia (Spain). Appl Environ Microbiol 70:4030–4034

Nair S, Schreiber E, Thong KL, Pang T, Altwegg M (2000) Genotypic characterization of *Salmonella typhi* by amplified fragment length polymorphism fingerprinting provides increased discrimination as compared to pulsed-field gel electrophoresis and ribotyping. J Microbiol Methods 41:35–43

Nair S, Lin TK, Pang T, Altwegg M (2002) Characterization of *Salmonella* serovars by PCR-single-strand conformation polymorphism analysis. J Clin Microbiol 40:2346–2351

O'Regan E, McCabe E, Burgess C, McGuinness S, Barry T, Duffy G, Whyte P, Fanning S (2008) Development of a real-time multiplex PCR assay for the detection of multiple *Salmonella* serotypes in chicken samples. BMC Microbiol 8:156

Pang T, Altwegg M, Martinetti G, Koh CL, Puthucheary S (1992) Genetic variation among Malaysian isolates of *Salmonella typhi* as detected by ribosomal RNA gene restriction patterns. Microbiol Immunol 36:539–543

Perch M., Braden C.R., Bishop R., Fields P., Plikaytis R., Tauxe R.V. (2003). *Salmonella* surveillance summary, 2003. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, Atlanta, Ga.

Popoff MY, Bockemuhl J, McWhorter-Murlin A (1992) Supplement 1991 (no. 35) to the Kauffmann-White scheme. Res Microbiol 143:807–811

Porwollik S, Wong RM, McClelland M (2002) Evolutionary genomics of *Salmonella*: gene acquisitions revealed by microarray analysis. Proc Natl Acad Sci USA 99:8956–8961

Porwollik S, Boyd EF, Choy C, Cheng P, Florea L, Proctor E, McClelland M (2004) Characterization of *Salmonella enterica* subspecies I genovars by use of microarrays. J Bacteriol 186:5883–5898

Porwollik S, Santiviago CA, Cheng P, Florea L, McClelland M (2005) Differences in gene content between *Salmonella enterica* serovar Enteritidis isolates and comparison to closely related serovars Gallinarum and Dublin. J Bacteriol 187:6545–6555

Shangkuan YH, Lin HC (1998) Application of random amplified polymorphic DNA analysis to differentiate different strains of *Salmonella typhi* and other *Salmonella* species. J Appl Microbiol 85:693–702

Torpdahl M, Ahrens P (2004) Population structure of *Salmonella* investigated by amplified fragment length polymorphism. J Appl Microbiol 97:566–573

Uzzau S, Hovi M, Stocker BA (1999) Application of ribotyping and IS200 fingerprinting to distinguish the five Salmonella serotype O6, 7:c:1, 5 groups: Choleraesuis sensu stricto, Choleraesuis var. Kunzendorf, Choleraesuis var. Decatur, Paratyphi C, and Typhisuis. Epidemiol Infect 123:37–46