FOODBORNE PATHOGENS AND DISEASE
Volume 8, Number 6, 2011

DOI: 10.1089/fpd.2010.0776

A Rapid and Sensitive Method to Identify and Differentiate *Salmonella enterica* Serotype Typhimurium and *Salmonella enterica* Serotype 4,[5],12:i:- by Combining Traditional Serotyping and Multiplex Polymerase Chain Reaction

Lisa Barco, Antonia Anna Lettini, Elena Ramon, Alessandra Longo, Cristina Saccardin, Maria Cristina Dalla Pozza, and Antonia Ricci

Abstract

Salmonella enterica subspecies *enterica* serotype 4,[5],12:i:- is an emerging serovar considered as a monophasic variant of *Salmonella enterica* serotype Typhimurium. The antigenic and genetic similarity between *Salmonella* 4,[5],12:i:- and *Salmonella* Typhimurium suggests that they may behave in a similar way and represent a comparable threat to public health. As serotyping alone does not necessarily provide for identification of *Salmonella* 4,[5],12:i:- and its differentiation from *Salmonella* Typhimurium, a method that combines traditional serotyping and a multiplex polymerase chain reaction has been tested on 208 strains serotyped as *Salmonella* 4,[5],12:i:-, *Salmonella* Typhimurium, and similar serovars of serogroup B sharing the same phase-1 antigen "i." For 191 strains, the combined method fully confirmed the results provided by traditional serotyping, whereas for 17 strains of *Salmonella* 4,[5],12:i:- and *Salmonella* Typhimurium some inconsistencies emerged between the two methods. The combined method resulted in a more accurate and faster identification of these two relevant serovars.

Introduction

Salmonella enterica subspecies *enterica* serotype 4,[5],12:i:-, considered a monophasic variant of *Salmonella* Typhimurium, emerged in Europe in the mid-1990s and, within the last years, has been increasingly implicated in human diseases worldwide (Echeita *et al.*, 1999, 2001; Hauser *et al.*, 2010). It is difficult to have a clear picture of the spread of this emergent serovar because it appears to be underreported owing to the lack of a harmonized and easily interpretable analytic method to identify this serovar and to differentiate it from *Salmonella* Typhimurium.

Salmonella serotyping is based on the antigenic variability of lipopolysaccharides (O antigen) and flagellar proteins (phase-1 and phase-2 antigens). Most serovars, including *Salmonella* Typhimurium, are biphasic and express two flagellar antigens encoded by *fljC* (phase-1 flagellin) and *fljB* (phase-2 flagellin), whereas *Salmonella* 4,[5],12:i:- is classified as monophasic because it lacks the *fljB* gene (Hopkins *et al.*, 2010).

To identify *Salmonella* 4,[5],12:i:- strains, according to traditional serotyping, somatic antigens of serogroup B and phase-1 flagellar antigen "i" must be identified, and repeated phase inversion assays must not provide evidence for phase-2 flagellar antigen production. There is not a general agreement on how many times phase inversion should be repeated to ensure that the strain is truly monophasic and that the inability to detect phase-2 antigen was not due to low-level expression of the antigen. For this reason, several days will be necessary to confirm the identification of *Salmonella* 4,[5],12:i:-, hampering the timely application of consumers' protection measures. Therefore, polymerase chain reaction (PCR)-based serotyping could represent an appealing alternative to identify these serovars, because phase inversion to detect phase-2 flagellar antigen is not necessary at the genetic level.

The present study addresses the need of identifying *Salmonella* 4,[5],12:i:- and differentiating it from other closely related strains by investigating the usefulness of a PCR assay, previously designed by Tennant *et al.* (2010) and partly modified, combined with traditional serotyping.

Materials and Methods

A total of 29 isolates of *Salmonella* Typhimurium, 154 of *Salmonella* 4,[5],12:i:-, and 25 of other antigenically similar

OIE, National Reference Laboratory for Salmonella, Istituto Zooprofilattico Sperimentale delle Venezie, Viale dell'Università, Padova, Italy.

Salmonella serovars (Lagos, Agama, and Gloucester) were tested. Then, a panel of 16 strains belonging to genera different from *Salmonella* was used for exclusivity test without obtaining any amplicons.

The isolates were serotyped according to the Kauffmann-White scheme by slide agglutination. After having identified the somatic antigen and the phase-1 flagellar antigen, if the second one was negative, the phase inversion method was used to allow the expression of the second flagellar phase. When the phase inversion was repeated at least three times without getting expression of the phase-2 flagellar antigen, a strain was considered monophasic.

A multiplex PCR protocol that allows simultaneous amplification of a fragment between the genes *fljB* and *fljA* and the phase-2 flagellar gene (*fljB*) was used. This PCR was based on the method previously described by Tennant *et al.* (2010), which was partly modified (concentrations of some components of the master mix and number of cycles of the amplification profile) to guarantee the amplification of all fragments. Template DNA was prepared by boiling of pure bacterial culture for 10 min. The PCR assay was performed in a total volume of 30 μL containing 2.5 mM $MgCl_2$, 0.6 mM of dNTPs, 1×Buffer-Taq, 1 U of AmpliTaq GoldTM DNA Polymerase (Applied Biosystems, Roche), 0.1 μM of primers specific for fliB-fliA intergenic region, 1 μM of primers specific for *fljB* gene, and 5 μL of template DNA. The amplification profile was denaturation (95°C for 2 min), amplification (30 cycles: 95°C for 30 sec, 64°C for 30 sec, 72°C for 90 sec), and final extension (72°C for 10 min).

Results and Discussion

In the PCR assay, *Salmonella* 4,[5],12:i:- generates only one amplicon of 1 kb, corresponding to fliB-fliA intergenic region, whereas *Salmonella* Typhimurium shows the same fragment plus another one of 1389 bp, corresponding to *fljB*. For all the other serovars, two amplicons are visualized: one of 1389 bp and another, corresponding to fliB-fliA intergenic region, of 250 bp, because of the lack of a fragment (IS200) in this region (Table 1).

One hundred thirty nine of 154 strains tested, serotyped as *Salmonella* 4,[5],12:i:-, were confirmed by the PCR (90.3%). For 14 strains, serotyped as monophasic, the PCR profile classified them as *Salmonella* Typhimurium (9.1%). These strains, in which phase-2 flagellar antigen is not detected serologically but can be detected by PCR, may have deletions in part of *fljB* that, nevertheless, leave intact the specific PCR primer-binding sites. As an alternative, for these strains, serological detection of phase-2 flagellar antigen may be inconsistent because of problems with phase inversion caused by the invertible promoter controlling the expression of *fljB* and *fljC*, which may be in "locked" position allowing the expression of *fljC* and curtailing the transcription of *fljB* (Zamperini *et al.*, 2007). The remaining strain, serotyped as *Salmonella* 4,[5],12:i:-, did not generate the amplicon of 1 kb (0.6%). This could be attributed to polymorphisms within the primer-binding sites or complete loss of the IS200 fragment. Twenty-seven strains (93.1%), serotyped as *Salmonella* Typhimurium were confirmed by PCR, whereas the two remaining isolates (6.9%) generated the smaller amplicon of 250 bp corresponding to the fliB-fliA intergenic region, and hence, it is supposed that they were neither *Salmonella* Typhimurium nor *Salmonella* 4,[5],12:i:-. This finding could be due to an incorrect serotyping. Additionally, for the 25 strains sharing somatic antigen and phase-1 flagellar antigen with *Salmonella* Typhimurium and *Salmonella* 4,[5],12:i:-, the serotyping was completely consistent with the PCR results.

According to the procedure suggested in this article, traditional serotyping is performed until the first phase inversion gives negative result and then a PCR assay is applied to differentiate among *Salmonella* Typhimurium, *Salmonella* 4,[5],12:i:-, and other serovars sharing the same somatic and phase-1 flagellar antigen. Not having to perform repeated phase inversion assays for suspected monophasic *Salmonella* Typhimurium variants reduces the time to obtain definitive strain identification.

Moreover, using this PCR, it is possible to differentiate these two serovars and other antigenically similar strains according to specific PCR results, whereas traditional serotyping only identifies monophasic strains by repeated negative outcomes of phase inversion assays.

Table 1. Combined Protocol (Serotyping and Multiplex PCR) to Identify *Salmonella* Typhimurium and *Salmonella* 4,[5],12:i:- and to Differentiate Them from Other Strains Sharing the Same Somatic and Phase-1 Flagellar Antigens

Pure culture of Salmonella *spp.*			
Traditional serotyping	First step	Identification of O antigen	Positive agglutination with "Group B" antisera
	Second step	Identification of the phase-1 flagellar antigen	Positive agglutination with "i" antisera
	Third step	Phase inversion to allow the expression of the phase-2 flagellar antigen	Negative reaction
			PCR amplicons
PCR assay	Fourth step	Multiplex PCR to differentiate among *Salmonella* Typhimurium, *Salmonella* 4,[5],12:i:-, and other serovars	*Salmonella* Typhimurium: 1 kb (fliB-fliA intergenic region) +1389 bp (*fljB*) *Salmonella* 4,[5],12:i:-: 1 kb (fliB-fliA intergenic region) Other serovars: 250 bp (fliB-fliA inter genic region) +1389 bp (*fljB*)

PCR, polymerase chain reaction.

A range of different mechanisms that result in nonexpression of the phase-2 flagellar antigen have been described (Hopkins *et al.*, 2010), and consequently, definitive differentiation between *Salmonella* Typhimurium and *Salmonella* 4,[5],12:i:- by traditional serotyping is problematic. This PCR assay represents a reliable method to avoid possible incorrect results in the differentiation among these serovars, especially in case of false negative or weak expression of the phase-2 "1,2" flagellar antigen.

In this protocol, standard serotyping is combined with PCR when traditional serotyping is not conclusive. This approach is rapid, provides reliable results, and improves correct identification of this emerging strain that is already causing severe infections worldwide (Mossong *et al.*, 2007; Switt *et al.*, 2009).

Acknowledgments

The authors thank Rob Davies from Veterinary Laboratories Agency (Weybridge, United Kingdom) and Kirsten Mooijman from the CRL-*Salmonella* at the National Institute for Public Health and the Environment (Bilthoven, The Netherlands) for providing some of *Salmonella* strains used as controls in this study.

Disclosure Statement

No competing financial interests exist.

References

Echeita MA, Aladuena A, Cruchaga S, and Usera MA. Emergence and spread of an atypical *Salmonella enterica* subsp. *enterica* serotype 4,[5],12:i:- strain in Spain. J Clin Microbiol 1999; 37:3425–3425.

Echeita MA, Herrara S, and Usera MA. Atypical *fljB*-negative *Salmonella enterica* subsp.*enterica* strain of serovar 4,[5],12:i:- appears to be a monophasic variant of *Salmonella* Typhimurium. J Clin Microbiol 2001;39:2981–2983.

Hauser E, Tietze E, Helmuth R, Junker E, Blank K, Prager R, Rabsch W, Appel B, Fruth A, and Malorny B. Pork contaminated with *Salmonella enterica* serovar 4,[5],12:i:-, an emerging health risk for humans. Appl Environ Microbiol 2010;76:4601–4610.

Hopkins KL, Kirchner M, Guerra B, Granier SA, Lucarelli C, Porrero MC, Jakubczak A, Threlfall EJ, and Mevius DJ. Multiresistant *Salmonella enterica* serovar 4,[5],12:i:- in Europe: a new pandemic strain? Euro Surveill 2010;15:pii = 19580.

Mossong J, Marques P, Ragimbeau C, Huberty-Krau P, Losch S, Meyer G, Moris G, Strottner C, Rabsch W, and Schneider F. Outbreaks of monophasic *Salmonella* enterica serovar 4,[5],12:i:- in Luxembourg, 2006. Euro Surveill 2007;1;12:156–158.

Switt AI, Soyer Y, Warnick LD, and Wiedmann M. Emergence, distribution, and molecular and phenotypic characteristics of *Salmonella enterica* serotype 4,[5],12:i:-. Foodborne Pathog Dis 2009;6:407–415.

Tennant SM, Diallo S, Levy H, Livio S, Sow SO, Tapia M, Fields PI, Mikoleit M, Tamboura B, Kotloff KL, Nataro JP, Galen JE, and Levine MM. Identification by PCR of non-typhoidal *Salmonella enterica* serovars associated with invasive infections among febrile patients in Mali. PLos Negl Trop Dis 2010;4: e621.

Zamperini K, Soni V, Waltman D, Sanchez S, Theriault EC, Bray J, and Maurer JJ. Molecular characterization reveals *Salmonella enterica* serovar 4,[5],12:i:- from poultry is a variant Typhimurium serovar. Avian Dis 2007;51:958–964.

Address correspondence to:
Antonia Ricci, D.V.M.
OIE
National Reference Laboratory for Salmonella
Istituto Zooprofilattico Sperimentale delle Venezie
Viale dell'Università 10
35020 Legnaro, Padova
Italy

E-mail: aricci@izsvenezie.it