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Molecular confirmation method for monophasic and non-motile variant strains of *Salmonella* serovar Typhimurium

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***Salmonella* remains the primary cause of confirmed foodborne illness outbreaks in France. Among the 2600 serovars identified in the *Salmonella* genus, some are isolated more frequently in human health, food hygiene and/or animal health.**
Over the past five years, salmonellae known as "Typhimurium-like variants" have emerged in humans and are found in many areas of the food chain and livestock sector.
This article presents a molecular characterisation method developed and applied since 2010 for surveillance purposes. The method meets the on-going need to change laboratory analyses to comply with regulatory requirements and to implement control measures for the prevention of the microbiological hazards associated with the possible presence of salmonellae in food.

Abstract
Since 2008, the French Reference Laboratories tasked with monitoring salmonellae in human health or in the food and veterinary sectors have observed the emergence of strains with the *S.* 1,4,[5],12:i:- antigenic formula, known as "monophasic Typhimurium variants". Emergence of these strains has also been demonstrated at the European level and, in 2010, led the European Food Safety Authority (EFSA) to issue recommendations concerning characterisation and surveillance of these isolates throughout the food chain. Detection of these variants in regulated poultry sectors has led Europe to implement control measures identical to those required for *S.* Typhimurium.
French regulations are more stringent and cover two types of monophasic variants, *S.* 1,4,[5],12:i:- and *S.* 1,4,[5],12:-:1,2, and the non-motile variant *S.* 1,4,[5],12:-:-.
In order to confirm the presence of variants of the serovar Typhimurium, a conventional multiplex polymerase chain reaction (PCR) method has been developed. This makes it possible to monitor changes in isolation trends for these variants throughout the food chain.
Overall analysis of the range of strains collected by the *Salmonella* network for the 2011-2012 period has demonstrated the emergence of strains with the *S.* 1,4,[5],12:i:- antigenic formula, confirmed as monophasic variants of the Typhimurium serovar, within several animal production sectors.
This PCR method can be used in conjunction with the conventional serotyping method by slide agglutination and provides rapid confirmation of the identity of these variants. It is also a useful tool in determining the epidemiological picture, in monitoring trends related to strain isolation, and in assessing risks and adjusting control measures in the various sectors.

Background
Internationally, monitoring data from recent years have shown a considerable increase in the occurrence of strains with an antigenic formula (*S.* 1,4,[5],12:i:-) very similar to that of *Salmonella* Typhimurium (*S.* 1,4,[5],12:i:1,2) (EFSA, 2010; ANSES, 2013; Mulvey, 2013). These strains are flagellar variants of the serovar Typhimurium, called monophasic because they lack expression of the second flagellar phase, encoded by the *fljB* gene. Strains that have lost antigen expression of the first flagellar phase or of both phases (*S.* 1,4,[5],12:-:1,2 and *S.* 1,4,[5],12:-:-, respectively), are also found but far less commonly (EFSA, 2010; ANSES, 2013; Mulvey, 2013).
Considering, on the one hand, the emergence of monophasic variant strains of *S.* Typhimurium at the European level, and on the other, the risk that they pose to public health, thought to be similar to serovar Typhimurium, EFSA recommended full serotyping of all strains suspected of being salmonellae, followed by PCR confirmation of absence of the *fljB* gene for strains with the *S.* 1,4,[5],12:i:- antigenic formula (EFSA, 2010).
In France, given that there have been several foodborne illness outbreaks associated with *Salmonella* strains known as "variants of serovar Typhimurium", the scope of Ministerial Orders has been extended beyond European regulations to include the three existing flagellar variants of serovar Typhimurium (*S.* 1,4,[5],12:i:-, *S.* 1,4,[5],12:-:1,2 and *S.* 1,4,[5],12:-:-). These Orders[1] stipulate that flocks contaminated with a variant of serovar *S.* Typhimurium are now to be treated as positive flocks for *S.* Typhimurium.
Depending on the type of farm involved, these measures require slaughter of the contaminated flock, transfer of eggs to establishments producing egg products, or heat treatment of positive meat following tests in muscle.
In view of emergence of these strains and the associated regulations, since 2010, the Directorate General for Food[2] has required that first-line veterinary and agro-food analysis laboratories forward the strain without delay to the *Salmonella* network of the Laboratory for Food Safety, along with the specific identification sheet of the network, whenever they isolate a variant with one of the above-mentioned antigenic

1. Both Ministerial Orders of 26 February 2008 concerning control of *Salmonella* infections (in the egg-laying and broiler sectors); Ministerial Order of 4 December 2009 concerning control of *Salmonella* infections in breeding turkey flocks; Ministerial Order of 22 December 2009 concerning control of *Salmonella* infections in flocks of broiler chickens and meat turkeys.
2. Guidance note DGAL/SDSSA/N2010-8059 of 04 March 2010, amending Guidance note DGAL/SDSSA/N2010-8026 of 27 January 2010.

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formulas. The *Salmonella* network ensures surveillance after confirming the identity of the so-called "Typhimurium-like variants" using an in-house method based on molecular tests defined earlier by EFSA and described below.
Strains suspected of being variants of serovar Typhimurium may in fact be found to be variants of other less frequently identified serovars, given the antigenic formula detected. In this way, for the *S.* 1,4,[5],12:i:- antigenic formula, it is possible to identify 6 serovars. For *S.* 1,4,[5],12:-:1,2, and *S.* 1,4,[5],12:-:-, 16 and 148 serovars, respectively, can be identified (ANSES, 2013).

Principle of the method

The method used to confirm the identity of the variants of serovar Typhimurium is based on EFSA recommendations (2010) concerning solely confirmation of the emerging monophasic *S.* 1,4,[5],12:i:- variant, and on studies carried out by Bugarel *et al.* (2012). French regulations concern all monophasic and non-motile variants. As a result, additional markers were included in this method to cover all the confirmation needs for these variants.
The method uses the principle of polymerase chain reaction (PCR) and is applied after conventional serotyping detection of a strain with one of the following antigenic formulas: *S.* 1,4,[5],12:i:-, *S.* 1,4,[5],12:-:1,2 or *S.* 1,4,[5],12:-:-. It aims to amplify four genes through two multiplex PCRs. The first targets the *fljB* gene, coding for the second flagellar phase, and the *fliA-fliB* intergenic region. The presence of an *IS200* sequence of 1000 bp in this region is specific to the serovar Typhimurium and its variants, since it is not detected in the other serovars for which the corresponding amplicon is 250 bp in size. The second PCR targets the *mdh* gene, marker of the serovar Typhimurium and the *fliC* gene coding for the first flagellar phase. The sequences of the primers used to detect these markers are listed in **Table 1**.

Analytical procedure

The molecular method described in this article is applied using a pure culture of a *Salmonella* strain for which the antigenic formula has been determined by slide agglutination serotyping. This conventional serotyping method uses specific antisera against cell wall ("O") or flagellar ("H") antigens (Danan, 2009). The steps in the molecular confirmation method for variants are as follows:

- Culture of strains on TSAYE agar, 18 - 24h at 37°C;
- Extraction of DNA from isolated colonies on TSAYE agar using a standard kit;
- Measurement of the concentration of DNA extract using a spectrophotometer at 260 nm;
- Dilution of the extract to adjust its concentration to 50 - 100 ng/µl;
- Two multiplex PCRs for fliA-fliB + fljB and mdh + fliC, as per the conditions presented in **Table 2**;
- Migration of the amplification products on 2% agarose gel;
- Visualisation of EtBr-labelled amplicons by fluorescence under a UV lamp;
- Reading of the gel (see **Figure 1**) and interpretation of results.

The method requires use of control strains: *Salmonella* Typhimurium LT2 reference strain (positive control) and a *Salmonella* Brandenburg strain (field strain and negative control). A negative control without DNA is also included in each experiment.

Table 1: Sequences of PCR primers used

Target gene	Function	Name of primer	Sequence (5'-3')	Reference
mdh	Malate dehydrogenase	MDH F MDH R	TGCCAACGGAAGTTGAAGTG CGCATTCCACCACGCCCTTC	[Amavisit, 2005]
fliC	Phase 1 flagellar antigen	Anti-sense-i Sense-60	ATAGCCATCTTTACCAGTTCC ACTCAGGCTTCCCGTAACGC	[Herrera-Leon, 2004] [Bugarel, 2012]
fljB	Phase 2 flagellar antigen	Sense-59 Anti-sense-83	CAACAACAACCTGCAGCGTGTGCG GCCATATTTCAGCCTCTCGCCCG	[EFSA, 2010]
fliA-fliB	Intergenic region of variable size depending on whether it contains an IS200 insertion sequence	FFLIB RFLIA	CTGGCGACGATCTGTCGATG GCGGTATACAGTGAATTCAC	[EFSA, 2010]

Table 2: Description of operating conditions for the two multiplex PCRs (*fliA-fliB* + *fljB* and *mdh* + *fliC*)

PCR 1 Preparation of mix		PCR 2 Preparation of mix	
Buffer without MgCl2	1 X	Buffer without MgCl2	1 X
MgCl2	2 mM	MgCl2	2 mM
dNTPs	0.2 mM	dNTPs	0.2 mM
Anti-sense 83 primer	0.8 µM	MDH-F primer	0.4 µM
Sense 59 primer	0.8 µM	MDH-R primer	0.4 µM
FFLIB primer	0.4 µM	Anti-sense I primer	0.4 µM
RFLIA primer	0.4 µM	Sense-60 primer	0.4 µM
Taq polymerase	1 unit	Taq polymerase	1 unit
Total PCR reaction volume 25 µl (24 µl or 23 µl of reaction mix / tube + 1 µl of DNA at a concentration of 100 ng/µl or 2 µl of DNA at 50 ng/µl)			
Amplification conditions		**Amplification conditions**	
3 min	94°C	3 min	94°C
35 cycles:		35 cycles:	
30 sec	94°C	30 sec	94°C
40 sec	64°C	40 sec	58°C
1 min 30 sec	72°C	1 min 30 sec	72°C
7 min	72°C	7 min	72°C

Expression of results

Interpretation of results is carried out according to predefined rules presented in **Table 3**. The strain is considered to be a non-motile or monophasic variant if the amplicons corresponding to the *fliC* and/or *fljB* genes are absent.
A variant of serovar Typhimurium is confirmed if the amplicons corresponding to the *mdh* gene and to the *fliA-fliB* intergenic region are detected and if the amplicon of the intergenic region has the expected length of 1000 bp.
A variant of a serovar other than Typhimurium is confirmed if the amplicon corresponding to the *mdh* gene is absent, and if that of the *fliA-fliB* intergenic region is 250 bp in length.

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Inconsistent variants are also identified (as per Hopkins *et al.* (2010)) for *S.* 1,4,[5],12:i:- variants, and this term can be extrapolated to the two other antigenic formulas if the genes coding for the flagellar phases (*fliC* and *fljB*) are detected but not expressed (non-detection of the antigens by conventional agglutination serotyping).

Table 3: Interpretation of results obtained by the method of confirmation for *Salmonella* strains, variants of serovar Typhimurium.
[+: detection of the specific amplicon for the marker of expected length; -: absence of detection of the specific amplicons for the marker; bp: DNA base pairs]

Serovar by agglutination	Target markers				Interpretation
	fliC	*fliA-fliB*	*fljB*	*mdh*	
S. 1,4,[5],12:i:-	+	1000 bp	–	+	Confirmed monophasic variant of Typhimurium
	+	1000 bp	+	+	Inconsistent monophasic variant of Typhimurium
	+	250 bp	–	–	Monophasic variant of a serovar other than Typhimurium
S. 1,4,[5],12:-:1,2	-	1000 bp	+	+	Confirmed monophasic variant of Typhimurium
	+	1000 bp	+	+	Inconsistent monophasic variant of Typhimurium
	-	250 bp	+	–	Monophasic variant of a serovar other than Typhimurium
S. 1,4,[5],12:-:-	-	1000 bp	-	+	Non-motile variant of Typhimurium
	+	1000 bp	+	+	Inconsistent non-motile variant of Typhimurium
	-	250 bp	-	–	Non-motile variant of a serovar other than Typhimurium
S. 1,4,[5],12:i:1,2	+	1000 bp	+	+	Typhimurium

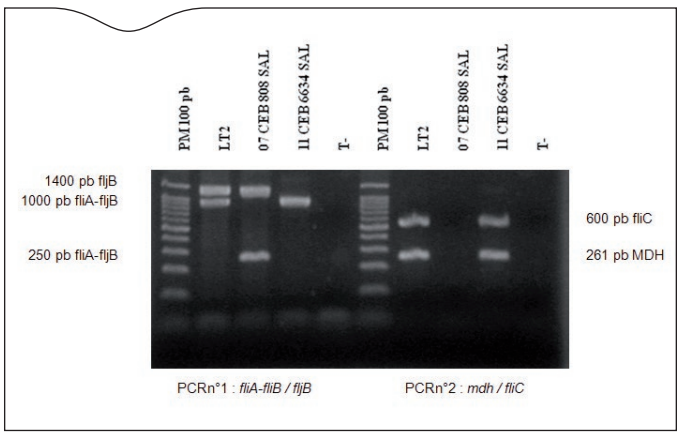

Figure 1: Illustration of the results of amplification obtained for confirmation of *Salmonella* strains, variants of serovar Typhimurium.
[PM100bp: molecular mass marker; LT2: serovar Typhimurium; 07CEB808SAL: *S.* Brandenburg; 11CEB6634SAL: monophasic variant *S.* 1,4,[5],12:i:- of serovar Typhimurium; T-: negative control without DNA]

Summary of results using the confirmation method

During the 2011 to 2012 period, a total of 703 "*Salmonella* Typhimurium-like" strains of various origins (see **Table 4**) were analysed using this method (Lailler, 2013). Within this group, 690 strains had the *S.* 1,4,[5],12:i:- antigenic formula, of which 650 strains (94.2%) were identified as monophasic variants of serovar Typhimurium, 38 strains as "inconsistent variants" by the presence of the *fljB* gene, and only two strains as monophasic variants of serovars other than Typhimurium (see **Table 5**).
Analysis of the eight strains with the *S.* 1,4,[5],12:-:1,2 antigenic formula showed the presence of the *fliC* gene and confirmed their status as monophasic variants of serovar Typhimurium.
Of the five non-motile strains with the *S.* 1,4,[5],12:-:- antigenic formula, only one strain was confirmed as a monophasic variant, the others were variant strains of other serovars.

Table 4: Distribution of sources for the 703 "*Salmonella* Typhimurium-like" strains collected in 2011 and 2012 by the *Salmonella* network, coordinated by the Maisons-Alfort Laboratory for Food Safety (ANSES).

Serovars	*S.* 1,4,[5],12:i:-	*S.* 1,4,[5],12:-:1,2	*S.* 1,4,[5],12:-:-
Feed	23	/	2
Ecosystem	27	4	1
Animal health and production… *including* *cattle* *poultry* *swine*	233 *48* *160* *13*	4 *4* 	2 *1*
Food … *including* *beef* *Poultry meat ?* *pork*	407 *39* *13* *133*	/	/
Total	**690**	**8**	**5**

Table 5: Results obtained by multiplex PCR on the range of strains collected in 2011 (n=312) and in 2012 (n=391) by the *Salmonella* network, coordinated by the Maisons-Alfort Laboratory for Food Safety (ANSES).

markers \ serovar				*S.* 1,4,[5],12:i:-	*S.* 1,4,[5],12:-:1,2	*S.* 1,4,[5],12:-:-
fliC	*fliA-fliB*	*fljB*	*mdh*			
+	1000 bp	–	+	650	/	1
+	1000 bp	+	+	38	8	/
+	250 bp	–	–	2	/	4
Total				**690**	**8**	**5**

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Discussion / Conclusion

The confirmation method for monophasic and non-motile variants presented in this article is a qualitative test based on the absence or presence of the amplicon of expected length, detected by multiplex PCR for genotyping, as described in Chapter 8 of French Standard XP U47-600-2.

This method is based on the protocol recommended by EFSA, and on studies carried out by Bugarel *et al* (2012). As part of these studies, three different markers, known to be specific for the serovar Typhimurium, were tested in a series of known strains belonging to the serovar Typhimurium or confirmed variant of *S.* Typhimurium.

The markers are the *fliA-fliB* intergenic sequence proposed in EFSA recommendations and the *mdh* gene. *Mdh* was systematically detected in all strains of serovar Typhimurium and variants. This marker, known to be present in many *Salmonella* strains, was also tested for in a series of 937 strains of various serovars (more than 230 different serovars), enabling determination of its extrinsic specificity. No cross-reaction was detected, with the exception of one strain of serovar Kibusi (*S.* 28:r:e,n,x) and one of serovar Newmexico (*S.* 9,12:g,z_{51}:1,5) (Bugarel, 2012). These two serovars do not belong to the O:4 group, unlike Typhimurium and its variants.

Inclusion of the *mdh* gene in the series of tested markers makes it possible to exclude any false positive or false negative result (100% detection in strains expected to be positive).

As EFSA recommends in its opinion (EFSA, 2010), confirmation of the identity of these "*Salmonella* Typhimurium-like" strains by accurate and complete characterisation is important in terms of surveillance. Regular updates will be used to assess the suitability of regulatory measures in view of public health objectives in France and in Europe.

Concerning variants classified as inconsistent (*fljB+, fliC+, fliA-fliB+ at 1000 bp and mdh+)*, these strains have all the genetic material required to be identified as belonging to the serovar Typhimurium. When considering only the results of the PCR tests applied, these strains cannot be distinguished from strains of *S.* Typhimurium. Only characterisation by conventional serotyping can demonstrate the absence of expression of one or both flagellar phases. This lack of expression could also be reversible (Soyer, 2009). Identification of these inconsistent strains by the method described here could also be useful in detecting new genes involved in the inversion mechanism of the flagellar phase.

This method cannot be used for complete identification of variants of other serovars. Additional geno-serotyping methods could help to counter this limitation. One of the currently available methods, that can be used for this molecular serotyping, was used in part to complement molecular confirmation with the described method. This sometimes enabled identification of other serovars such as *S.* Coeln and *S.* Schwarzengrund, which were the sources of the monophasic and non-motile variants in the study carried out in 2011-2012. However, this approach to geno-serotyping is still experimental and needs to be validated more generally.

As part of surveillance carried out by laboratories, it is important that the epidemiological situation concerning salmonellae be evaluated regularly in order to adjust monitoring, and if necessary, control measures in the various sectors, to changes in serovars (particularly emerging ones such as the recently identified Kentucky serovar) and to changes in antibacterial resistance profiles.

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