

## SCIENTIFIC OPINION

### Scientific Opinion on monitoring and assessment of the public health risk of “*Salmonella* Typhimurium-like” strains<sup>1</sup>

EFSA Panel on Biological Hazards (BIOHAZ)<sup>2,3</sup>

European Food Safety Authority (EFSA), Parma, Italy

#### ABSTRACT

Monophasic variants of *Salmonella* Typhimurium-like strains, lacking the fljB-encoded second phase H antigen – ie, with the antigenic structure 1,4,[5],12:i:- appear to be of increasing importance in many Member States. Such variants are referred to as ‘monophasic *S. Typhimurium*’. Strains lacking expression of the phase one or both flagellar antigens are also possible, but uncommon. Current standard methods are considered suitable for isolation of such monophasic *Salmonella* Typhimurium strains. For identification of the monophasic 1,4,[5],12:i:- variant, it is advisable to proceed with serotyping until a first negative result of agglutination after flagellar phase inversion, and then apply a PCR protocol in order to confirm the lack of the second phase antigen. To ensure complete consistency of reporting, all isolates of putative *Salmonella* should be fully serotyped and the full antigenic formula reported. If the full antigenic formula is not available but a phage type that is consistent with *S. Typhimurium* lacking phase two flagellar antigens has been confirmed, and the lack of the second phase flagellar antigen has been verified by PCR, then the term ‘monophasic *S. Typhimurium*’ is recommended for reporting purposes in the current situation. On the basis of genetic similarity and ability to obtain a recognised *Salmonella* Typhimurium phage type, these emerging epidemic monophasic strains with formula 1,4,[5],12:i:- are regarded as variants deriving from *S. Typhimurium*. Moreover, monophasic *S. Typhimurium* strains have been shown to have similar virulence and antimicrobial resistance characteristics to other strains of *S. Typhimurium*. Similar to what was observed in the past for epidemic clones of *S. Typhimurium*, recent studies in numerous countries worldwide confirm the rapid emergence and dissemination of such strains in food animals, companion animals and humans. The public health risk posed by these emerging monophasic *S. Typhimurium* strains is therefore considered comparable to that of other epidemic *S. Typhimurium* strains.

© European Food Safety Authority, 2010

#### KEY WORDS

*Salmonella* Typhimurium-like, monophasic, variant, strains, epidemicity.

1 On request from the European Commission, Question No EFSA-Q-2010-00055, adopted on 22 September 2010.

2 Panel members: Olivier Andreoletti, Herbert Budka, Sava Buncic, John D Collins, John Griffin, Tine Hald, Arie Havelaar, James Hope, Günter Klein, James McLauchlin, Christine Müller-Graf, Christophe Nguyen-The, Birgit Noerrung, Luisa Peixe, Miguel Prieto Maradona, Antonia Ricci, John Sofos, John Threlfall, Ivar Vågsholm and Emmanuel Vanopdenbosch. Correspondence: biohaz@efsa.europa.eu

3 Acknowledgement: The Panel wishes to thank the members of the Working Group on *Salmonella* Typhimurium-like strains: Robert Davies, Beatriz Guerra-Roman, Ida Luzzi, Kirsten Mooijman, Luisa Peixe, Antonia Ricci, John Threlfall and Therese Westrell for the preparatory work on this scientific opinion, and EFSA staff: Frank Boelaert, Ernesto Liebana-Criado and Pablo Romero Barrios for the support provided to this scientific opinion.

Suggested citation: EFSA Panel on Biological Hazards (BIOHAZ); Scientific Opinion on monitoring and assessment of the public health risk of “*Salmonella* Typhimurium-like” strains. EFSA Journal 2010;8(10):1826. [48 pp.] doi:10.2903/j.efsa.2010.1826. Available online: [www.efsa.europa.eu/efsajournal.htm](http://www.efsa.europa.eu/efsajournal.htm)

## SUMMARY

Following a request from the European Commission, the Panel on Biological Hazards (BIOHAZ) was asked to deliver a Scientific Opinion on the monitoring and assessment of the public health risk of “*Salmonella* Typhimurium-like” strains. In particular, the Panel was asked to evaluate the analytical methods currently used and to advise on the appropriateness for identifying these strains; to propose a harmonised terminology for reporting which allows trend-analyses, comparison between Member States and with humans isolates, as well as to indicate if these strains should be classified as variants of *Salmonella* Typhimurium or as a separate serotype. Finally, the Panel was asked to assess the public health risk posed by these emerging strains, in particular to advise whether the public health risk, when detecting these strains in animals or food, should be considered similar, more or less important than (other) *Salmonella* Typhimurium strains.

The BIOHAZ Panel concluded that, within *Salmonella* Typhimurium-like strains, monophasic variants lacking the second phase H antigen (1,4,[5],12:i:-), encoded by *fljB*, appear to be of increasing importance in many EU Member States (MSs) and have caused a substantive number of infections in both human and animals bred for food. Strains lacking expression of the phase one flagellar antigens (e.g. *S.* 4,[5],12:-:1,2) or both (*S.* 4,[5],12:-:-) are also possible, but have not commonly been reported to be associated with significant disease in animals or humans. Therefore, for the purposes of this Opinion, only the monophasic variants lacking second phase H antigens were considered. Such variants have been referred to as ‘monophasic *S.* Typhimurium’ in this document.

With regard to the analytical methods currently used and their appropriateness for identifying these strains, the current standard methods (ISO 6579 and Annex D of ISO 6579) were considered suitable for isolation of monophasic *Salmonella* Typhimurium strains. Various genetic and phenotypic characteristics have been described for the identification and subtyping of such strains. For identification of the monophasic 1,4,[5],12:i:- variant, it is advisable to proceed with serotyping until a first negative result of agglutination after flagellar phase inversion, and then apply a PCR protocol in order to confirm the lack of the second phase antigen. Other methods such as phage typing and genotyping are used to confirm relatedness to *S.* Typhimurium and/or to further subtype these isolates. Accurate characterisation of monophasic strains is deemed important, since misidentification of a non-*S.* Typhimurium-related strain could result in unnecessary regulatory action. Similarly, failure to confirm identity of a *S.* Typhimurium-like organism could have significant public health consequences.

The Panel found that it is currently difficult to monitor trends in the proliferation of monophasic strains because of the inconsistent way in which they are reported by different organisations within and between EU MSs and internationally. To ensure complete consistency of reporting, all isolates of putative *Salmonella* should ideally be fully serotyped in accordance with the White-Kauffman-Le Minor scheme, and the full antigenic formula reported, as recommended by the WHO Collaborating Centre for Reference and Research on *Salmonella* i.e., in the case of monophasic *S.* Typhimurium, 1,4,[5],12:i:-. It was suggested that, whenever possible, as much detail of the antigenic formula as determined by testing should be provided and reported. If the full antigenic formula is not available but a phage type that is consistent with *S.* Typhimurium lacking phase two flagellar antigens has been confirmed, and the lack of the second phase flagellar antigen has been verified by PCR, then the term ‘monophasic *S.* Typhimurium’ is recommended for reporting purposes in the current situation.

It was further concluded that, on the basis of genetic similarity and ability to obtain a recognised *Salmonella* Typhimurium phage type, these emerging epidemic monophasic strains with the basic antigenic formula 1,4,[5],12:i:- are regarded as variants deriving from *S.* Typhimurium. Moreover, monophasic *S.* Typhimurium strains have been shown to have similar virulence and antimicrobial resistance characteristics to strains of *S.* Typhimurium. Similar to what has been observed in the past

for epidemic clones of *S. Typhimurium*, recent studies in numerous countries worldwide confirm the rapid emergence and dissemination of such strains in food animals, companion animals and humans. The public health risk posed by these emerging monophasic *S. Typhimurium* strains is therefore considered comparable to that of other *S. Typhimurium* strains which have caused widespread epidemics of infection over the past four decades.

The BIOHAZ Panel made a series of further recommendations on typing, molecular methods, antimicrobial susceptibility testing and on monitoring the spread of these strains in EU MSs.

## TABLE OF CONTENTS

Abstract.....	1
Summary .....	2
Table of contents.....	4
Background as provided by the European Commission .....	5
Terms of reference as provided by the European Commission .....	5
Assessment.....	6
1. Introduction:.....	6
2. Hazard Identification.....	7
2.1. Identification and classification of <i>Salmonella</i> .....	7
2.2. Definition of <i>Salmonella</i> Typhimurium, ‘ <i>Salmonella</i> Typhimurium-like’ and monophasic Typhimurium strains .....	8
2.3. Emergence of monophasic <i>Salmonella</i> Typhimurium’ strains .....	10
2.4. Analytical methods for identification and subtyping of monophasic <i>Salmonella</i> strains .....	10
2.4.1. Primary isolation methods for <i>Salmonella</i> .....	10
2.4.2. Confirmation of identity of monophasic <i>S. Typhimurium</i> and/or further subtyping .....	11
2.5. Public health relevance.....	14
2.5.1. Human surveillance of salmonellosis .....	14
2.5.2. Epidemiological situation of salmonellosis in the EU .....	14
2.5.3. Cases of monophasic <i>S. Typhimurium</i> reported at the EU level .....	15
2.5.4. Outbreaks .....	15
2.5.5. Country-specific data .....	16
3. Hazard Characterisation .....	17
3.1. General molecular characteristics .....	17
3.2. Virulence factors .....	18
3.3. Antimicrobial resistance.....	19
3.4. Severity of human cases caused by monophasic <i>S. Typhimurium</i> .....	20
4. Exposure assessment.....	21
4.1. Monophasic <i>S. Typhimurium</i> reported in scientific literature .....	21
4.2. Monophasic <i>S. Typhimurium</i> in the EU zoonoses monitoring system .....	22
4.3. Monophasic <i>S. Typhimurium</i> in the <i>Salmonella</i> baseline studies conducted in EU.....	24
5. Risk characterisation - Considerations for human health .....	26
Conclusions.....	27
Recommendations.....	28
References.....	29
Appendices.....	39
A. Multiplex Polymerase Chain Reaction (PCR) for identification and differentiation of <i>S. Typhimurium</i> and monophasic 4,[5],12:i:-.....	39
B. <i>Salmonella</i> virulence attributes.....	42
C. Summary of monophasic <i>Salmonella</i> in Community zoonoses monitoring and baseline studies .....	44

## BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

Over the last 20 years a number of reports have been published on the isolation of “*Salmonella* Typhimurium-like” strains in food and animals. During the last few years an increasing number of cases and outbreaks have been reported in humans in Member States such as France, Germany, Austria, Ireland, Italy, Denmark, the Netherlands and Luxembourg. The French Food Safety Agency (AFSSA) recently provided an opinion on the increased number of isolates of these strains (Saisine n° 2009-SA-0182).

Monitoring of *Salmonella* is mandatory in humans in accordance with the provisions of Commission Decision 2000/96/EC on the communicable diseases to be progressively covered by the Community network, and in food and animals in accordance with the provisions of Directive 2003/99/EC on the monitoring of zoonoses and zoonotic agents.

An evaluation of trends and a comparison of the prevalence in food, animals and humans are often hindered by differences between the Member States in reporting these strains, sometimes considered as (variants of) *Salmonella* Typhimurium, sometimes reported as a separate serotype using different antigenic formulas. During a technical meeting with the MSs, the Commission collected information on the methods used for the monitoring and reporting of these strains. This information was gathered with the help of the Community Reference Laboratory for *Salmonella* and EFSA's unit on zoonoses monitoring.

In the Member States, a target for reduction must be achieved in different animal populations for certain serotypes, including *Salmonella* Typhimurium and trade restrictions may apply on the animals and their products in accordance with the provisions in Regulation (EC) No 2160/2003 on the control of *Salmonella* and other specified foodborne zoonotic agents.

In view of the above, there is a need to:

- Ensure that appropriate analytical methods are used across all Member States allowing the detection of the strain;
- Harmonise the way this strain is reported in order to compare reporting data;
- Assess the public health risk posed by these emerging strains.

## TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

EFSA is asked to issue a scientific opinion on the monitoring and risk of “*Salmonella* Typhimurium-like” strains and in particular:

- To evaluate the analytical methods currently used and advise on the appropriateness for identifying these strains;
- To propose a harmonised terminology for reporting which allows trends-analyses, comparison between Member States and with humans isolates;
- To indicate if these strains should be classified as variants of *Salmonella* Typhimurium or as a separate serotype;
- To assess the public health risk posed by these emerging strains, in particular to advise whether the public health risk, when detecting these strains in animals or food, should be considered similar, more or less important than (other) *Salmonella* Typhimurium strains.

## ASSESSMENT

### 1. Introduction:

Directive 2003/99 provides for the monitoring of zoonoses in animal populations in Europe. The purpose of this Directive is to ensure that zoonoses, zoonotic agents and related antimicrobial resistance are properly monitored, and that food-borne outbreaks receive proper epidemiological investigation, to enable the collection in the Community of the information necessary to evaluate relevant trends and sources (art. 1).

According to art. 4, monitoring shall be based on the systems in place in Member States (MSs). To make data easier to compile and compare, detailed rules for the monitoring of zoonoses and zoonotic agents listed in Annex I of the Directive may be laid down, when necessary.

Such detailed rules shall lay down minimum requirements for the monitoring of certain zoonoses or zoonotic agents. They may, in particular, specify:

- (a) the animal population or subpopulations or stages in the food chain to be covered by monitoring;
- (b) the nature and type of data to be collected;
- (c) case definitions;
- (d) sampling schemes to be used;
- (e) laboratory methods to be used in testing; and
- (f) the frequency of reporting, including guidelines for reporting between local, regional and central authorities.

The first indications on criteria for *Salmonella* monitoring have been laid down in Regulation 2160/2003, which in Annex II lists minimum requirements that food business operators have to respect having samples taken and analysed for the control of *Salmonella* in different animal species and categories. As far as flocks of *Gallus gallus*, turkeys and pigs are concerned, the Regulation requires all *Salmonella* strains with public health significance to be monitored, at different production stages. Annex III of Reg. 2160/2003 defines the criteria to be adopted to determine *Salmonella* serovars (also called serotypes) with public health relevance to which community targets will apply:

- the most frequent *Salmonella* serovars in human salmonellosis on the basis of data collected through EC monitoring systems;
- the route of infection (that is, the presence of the serovar in relevant animal populations and feed);
- whether any serovar shows a rapid and recent ability to spread and to cause disease in humans and animals;
- whether any serovar show increased virulence, for instance as regards invasiveness, or resistance to relevant therapies for human infections.

During the course of surveillance of zoonotic *Salmonella* there have been several examples of emergence of epidemic or pandemic strains that have spread widely in certain food animal and human populations (Folster et al., 2009; Guard-Petter, 2001; Kang et al., 2006; Threlfall, 2000). Some strains within certain serovars may have increased virulence for both humans (Molbak et al., 1999; Rabsch et al., 2002), and animals (Jones et al., 2008; Rabsch et al., 2002), or may be associated with certain exposure routes (Pires and Hald, 2010).

Since the late 1990s there have been increasing reports of the emergence, in many different countries worldwide, of a variety of *Salmonella* strains with antigenic structures similar to that of *Salmonella* Typhimurium but lacking certain flagellar antigens. Some of these strains appear to have been ecologically successful since they have spread rapidly in certain animal populations and have also caused numerous human infections. There is currently no legislative basis for control of such strains as they are not designated by a serovar name, even though they may behave in a similar way to *S.* Typhimurium and pose a similar or greater threat to food animal populations. Such strains are the subject of this Opinion.

## 2. Hazard Identification

### 2.1. Identification and classification of *Salmonella*

Various nomenclature systems have been used (or are still in use) to classify *Salmonella*. In general the nomenclature as specified in the White-Kauffman-Le Minor scheme, published by the WHO Collaborating Centre for Reference and Research on *Salmonella*, at the Pasteur Institute, Paris, France (Grimont and Weill, 2007) is used in Europe, but a slightly different system is used in the USA. According to the European nomenclature, the genus *Salmonella* belongs to the family of *Enterobacteriaceae* and consists of only two species: *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is divided into 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*. Within the species and subspecies, more than 2,600 different serovars have been identified (Guibourdenche et al., 2009), with most human and food-producing animal pathogens falling into the first subspecies, *Salmonella enterica* subsp. *enterica*.

The primary identification of *Salmonella* species and subspecies is based on the results of specific biochemical tests. The typing to serovar level is based on antigenic characteristics, supplemented in some cases by additional biochemical tests. To determine the antigenic formula, strains are tested for their O-antigens and H-antigens, and in some cases for their Vi-antigen, through the use of polyvalent and monovalent antisera.

The O-antigen (or somatic antigen) is the external component of the lipopolysaccharide located in the cell wall, consisting of a long linear polysaccharide comprising 50 to 100 saccharide units of four to seven sugars per unit. Historically, the O-antigens were classified in individual O-antigen groups and named with Arabic letters (A-Z). As there were more O-antigens than letters, it is now often preferred to designate each O group using the characteristic O factor, e.g. O:4 (previously called ‘group B’).

The H-antigen (or flagella antigen) correspond to the flagellin which is the major component of the flagella. The biphasic character of the flagella antigen of many salmonellas consists of its capacity to change its composition with a switch for the expression of two loci encoding the major flagellar protein (FliC, the phase 1 flagella and FljB of phase 2). This expression of two separate antigens was recognized before the nature of flagella was known and described as “phases”. Thus, *Salmonellae* possessing the capacity to express two antigens are termed “biphasic” and capable of “phase variation” with respect to their flagellar antigen. The expression of these two loci is regulated by a switch mechanism (*hin*) so that only one variety of flagellin protein is expressed at a time (Silverman

et al., 1979). Monophasic (producing only one type of flagellin), and less frequently triphasic or other complex (producing more than three flagellins) variants are also known. The first phase is indicated by a small type letter ‘a to z’. As with the ‘O’ antigens the number of letters is not sufficient for the number of H-antigens and new H-antigens are consequently indicated by the letters: z<sub>1</sub>, z<sub>2</sub>,...,z<sub>91</sub>, etc. The second phase of the H-antigens consists of many types and is indicated by numbers or letters. Non-motile *Salmonella* do not have H-antigens (i.e., they do not possess flagella). Most serovars possess two flagellar phases, and must be submitted to phase inversion in order to identify the second flagellar phase and determine the serovar (Jones et al., 2000).

The Vi-antigen is a surface (capsular) polysaccharide structure. The Vi-antigen occurs in only three *Salmonella* serovars (*Salmonella* Typhi, *Salmonella* Paratyphi C and *Salmonella* Dublin).

Serovars belonging to *S. enterica* subsp. *enterica* are isolated most frequently (more than 99.5 % of *Salmonella* isolates from humans and food producing animals). Such serovars are normally designated by a name, usually related to the geographical place where the serovar was first isolated or the animal from which it was isolated. Serovars belonging to other subspecies of *S. enterica* and those of *S. bongori* are designated by their antigenic formula.

Due to combinations of subspecies and many serovars, the full names are long (e.g. *Salmonella enterica* subsp. *enterica* serovar Typhimurium). It is therefore generally accepted to use a shorter format to indicate the names of the serovars of subspecies *enterica*, e.g. *Salmonella* Typhimurium (or *S.* Typhimurium).

The notation of the antigenic formulas is as follows:

O-antigens: H-antigens of first phase: H-antigens of second phase. Within each group of antigens, the individual antigens are separated by a comma.

For instance, the full antigenic formula of *Salmonella* Typhimurium is: 1,4,[5],12:i:1,2. Here the somatic O-antigens are: 1,4,[5],12. The underlined O factor 1 (1) means that this factor is determined by phage conversion. The factor is only present if the culture is lysogenized by the corresponding converting phage. The factor 5 between square brackets ([5]) means that the antigen may be present or absent without a relation to phage conversion. In summary both factors (1 and 5) can be present or absent.

## 2.2. Definition of *Salmonella* Typhimurium, ‘*Salmonella* Typhimurium-like’ and monophasic Typhimurium strains

In order to unambiguously determine whether isolates reported as “Typhimurium”, and also whether isolates of other serovars are, in fact, monophasic, the full antigenic formula needs to be considered. Strains defined as *S.* Typhimurium possess two phases of the H-antigens: in phase 1 this is H-antigen ‘i’ and in phase 2 they are H-antigens ‘1, 2’. These are universally regarded as ‘classic’ *S.* Typhimurium strains (antigenic formula: 1,4,[5],12:i:1,2). Antigenic variants that lack either the first or the second phase H antigen, or both, have been described (antigenic formulas respectively: 1,4,[5],12:-:1,2, or 1,4,[5],12:i:-, or 1,4,[5],12:-:-). Such variants have been termed ‘*Salmonella* Typhimurium-like’ strains.

Within these *Salmonella* Typhimurium-like strains, monophasic variants lacking the second phase H antigen (1,4,[5],12:i:-), encoded by *fljB*, appear to be of increasing importance. Strains lacking expression of the phase one flagellar antigens (e.g. *S.* 4,[5],12:-:1,2) or both (*S.* 4,[5],12:-:-) are also possible, but have not commonly been reported to be associated with significant disease in animals or humans. Therefore, for the purposes of this Opinion, only the monophasic variants lacking second



phase H antigens will be considered. Such variants will be referred to as ‘**monophasic *S. Typhimurium***’.

Examples of possible different antigenic formulae of monophasic *S. Typhimurium* strains and variations in how such strains have been reported by MSs are shown in Table 1 below. This variability makes it very difficult to be sure of the true identity of all reported isolates and to determine prevalence trends.

**Table 1:** Variability in antigenic formulas and terminology used to report possible monophasic *Salmonella* Typhimurium as reported to the EU-RL for *Salmonella*, EFSA and ECDC (TESSy);

Antigenic formulas reported
<u>1</u> ,4,[5],12:i:-
1,4,[5],12:i:-
4,[5],12:i:-
4,5,12:i:-
4,12:i:-
4,5:i:-
4:i:-
Names reported
<i>S.</i> Group B
<i>S.</i> Typhimurium DT120*
<i>S.</i> Typhimurium DT193*
<i>S.</i> Typhimurium
<i>S.</i> subsp. <i>enterica</i>
Subspecies 1
<i>S.</i> I 4,5:i:-
Monophasic variant
No name/ new serovar/ unknown

\*DT: Definitive phage type – i.e., that conforms to a specific and fully validated phage lysis pattern in the scheme of the Health Protection Agency, Centre for Infections, London, UK

## Conclusion

It is difficult to monitor trends in the proliferation of monophasic strains because of the inconsistent way in which they have been reported by different organisations within and between EU MSs and internationally. To ensure complete consistency of reporting, all isolates of putative *Salmonella* should ideally be fully serotyped and the full antigenic formula reported, as recommended by the WHO Collaborating Centre for Reference and Research on *Salmonella* according to the White-Kauffman-Le Minor scheme, i.e., in the case of monophasic *S. Typhimurium*, 1,4,[5],12:i:-. Whenever possible, as much detail of the antigenic formula as determined by testing should be provided and reported. The current practice of routinely reporting O antigens 1 and [5] when they are not tested for should be discontinued.

If the full antigenic formula is not available but a phage type that is consistent with *S. Typhimurium* has been confirmed, and the lack of the second phase flagellar antigen has been verified by PCR, then

the term ‘monophasic *S. Typhimurium*’ is recommended for reporting purposes in the current situation.

### **2.3. Emergence of monophasic *Salmonella* Typhimurium’ strains**

Scrutiny of the literature (Echeita et al., 1999; Switt et al., 2009) has revealed the emergence, in many countries, of a variety of apparently different monophasic *S. Typhimurium* of different phage types, genotypes and antimicrobial resistance profiles. Such studies indicate that certain monophasic *S. Typhimurium* isolates belong to multiple clones or clonal lines which have emerged through independent deletion events and which can only be further differentiated by highly sensitive molecular methods (Soyer et al., 2009).

Within EU countries and MSs two major clonal lines of monophasic *S. Typhimurium* have emerged over the last two decades. One such clonal line emerged in Spain in the late 1990s and exhibits plasmid-mediated resistance to a range of antimicrobials. The second clonal line has become particularly common in several MSs since 2000 and is characterized by chromosomally-encoded resistance to ampicillin (A), streptomycin (S), sulphonamides (Su) and tetracyclines (T) (= R-type ASSuT). In this respect, the antimicrobial resistance pattern (R-type) of ASSuT has often been used as a further characteristic to provisionally identify certain monophasic *Salmonella* Typhimurium isolates (Hauser et al., 2010b; Hopkins et al., 2010a; Lucarelli et al., 2010).

Monophasic *S. Typhimurium* strains have rapidly increased in prevalence in cases of human illness in the EU over a relatively short time period (see Section 5 for details) and appear to be derived from the *S. Typhimurium* genetic lineages. They therefore appear to behave exactly like a pathogenic strain of *S. Typhimurium* in terms of their ability to infect and cause disease in both animals and the human population.

### **2.4. Analytical methods for identification and subtyping of monophasic *Salmonella* strains**

#### **2.4.1. Primary isolation methods for *Salmonella***

For the isolation of *Salmonella* in samples from food, animal feed or from primary production, EN ISO standard methods are available. For the official monitoring of *Salmonella* in these matrices, the ISO procedures are prescribed in EU legislation. For the analyses of food and animal feed, ISO 6579 (Anonymous, 2002) should be followed. For the analyses of samples from primary production (e.g. animal faeces or dust), Annex D of ISO 6579 (Anonymous, 2007a) is prescribed. In both procedures the following culture steps are described: pre-enrichment in a non-selective broth, selective enrichment in/on one or two selective enrichment media, isolation on two selective agar media, confirmation.

The full ISO 6579 and Annex D of this ISO procedure differ only in the media for the selective enrichment culture step. For the food and animal feed samples two selective broths are prescribed: Rappaport-Vassiliadis broth with Soya (RVS) and Muller-Kauffmann Tetrathionate with novobiocin (MKTTn). For the analyses of the primary production samples (Annex D of ISO 6579) a selective semi-solid agar medium, Modified Semi-solid Rappaport-Vassiliadis agar (MSRV) is prescribed. The composition of this is similar to RVS, with the addition of a small amount of agar to provide ‘motility-enrichment’.

The isolation of *Salmonella* on MSRV is based on motility of the strain, meaning that (rare) non-motile variants of *Salmonella* may be more difficult to detect on this medium. It is therefore essential that any growth in MSRV is plated, not just growth that indicates motility in terms of turbidity that

spreads from the inoculation point. Non-spreading MSR/V is often taken to be non-*Salmonella* so may not be routinely sub-cultured to plating agars as an economy measure. In principle non-motile *Salmonella* strains might be expected to be more readily detected in the selective broths of ISO 6579, which must always be plated, but there have been no formal studies to compare isolation of non-motile strains. On the other hand, more background flora is able to grow in the broths (compared to the semi-solid agar medium), which may result in masking of the *Salmonella* strains by overgrowth of competing organisms.

The monophasic variants of *S. Typhimurium* (1,4,[5],12:i:- or 1,4,[5],12:-:1,2) are still motile and can be readily isolated on MSR/V. That this is indeed the case was shown by the fact that the monophasic variant 1,4,[5],12:i:- was regularly found in several EU MSs during the baseline surveys of slaughter pigs and breeding pigs (EFSA, 2008a, 2009; Mooijman et al., 2008; Mooijman et al., 2009), where the prescribed detection method was Annex D of ISO 6579 (EC, 2006, 2007b).

With the broths as described in ISO 6579 monophasic variants have also been detected, as shown in the baseline survey on broiler carcasses (EFSA, 2010) where the prescribed method was ISO 6579 (EC, 2007a).

Non-motile variants of *S. Typhimurium* (1,4,[5],12:-:- or 1,4,12:-:-) lacking both the first and the second phase H antigen may not be so easily detected with MSR/V unless non-spreading growth is routinely plated.

#### **2.4.2. Confirmation of identity of monophasic *S. Typhimurium* and/or further subtyping.**

As already described in section 2.1, the identification of the genus *Salmonella* is classically performed using biochemical tests. For this, the following tests are often used (between brackets the expected results for the majority of *Salmonella* serovars): fermentation of glucose (+), lactose (-) and sucrose (-), gas formation from glucose (+), production of hydrogen sulphide (+), urea hydrolysis (-), lysine decarboxylation (+), production of indole (-) and absence of enzyme  $\beta$ -galactosidase (Anonymous, 2002). By performing additional biochemical tests it is sometimes possible to further subtype the isolates to the level of species and subspecies. Once the genus is confirmed, isolates may be further subtyped. The classical procedure for this is serotyping, followed (for some serovars of epidemiological importance such as *S. Typhimurium* and *S. Enteritidis*) by phage typing. Additionally, several molecular procedures are available to test for the genotypic characteristics of strains within serotypes and/or phage types. The most commonly used methods for molecular typing are: Pulsed Field Gel Electrophoresis (PFGE), Multilocus Sequence Typing (MLST), Multiple-Locus Variable Number Tandem Repeat Analysis (MLVA/VNTR), DNA micro-arrays, and sequencing.

##### **2.4.2.1. Determination of serotype**

The principles underlying *Salmonella* serotyping have been briefly described in section 2.1. After confirming isolates to be ‘*Salmonella*’, serotyping is undertaken to confirm the serovar name. For this purified culture is first tested with O-antisera, followed by H-antisera (also see 2.1). An unknown type is mostly first tested with a pool of O-antisera, followed by tests with group antisera and/or single factor antisera relevant to the positive pool. A small amount of colony material of an isolate is mixed with a drop of antisera on a glass slide. The result can be read after a few seconds or minutes (depending on the antisera). The presence of granules in the suspension indicates a positive reaction. After agglutination with the O-antisera, the agglutination with H-antisera is performed. If one H-phase is found to be negative for biphasic strains (like for *Salmonella* Typhimurium), a phase-inversion method has to be used. The dominant H-phase is repressed with a phase-inversion method. By repressing the dominant H-phase the second H-phase can be expressed and identified. A frequently-used method for phase-inversion is one for which specific phase-inversion antiserum is

added to a swarming agar medium and the *Salmonella* strain is spot-inoculated on the plate. After overnight incubation, the swarmed strain can be used to identify the second, uninhibited H phase. If again no positive reaction is obtained with the second H-phase antisera but it is still positive with the first H-phase antisera, the phase-inversion need to be repeated. The number of times a phase-inversion should be repeated before a strain will be considered as monophasic has not been standardised (Jones et al., 2000). In order to unambiguously determine whether isolates are, in fact, monophasic *S. Typhimurium*, the full antigenic formula needs to be determined. There are differences in procedures for testing samples from food animals, humans and foodstuffs. In some cases isolates may not be serotyped or reported to national surveillance networks. This applies in particular to testing of food products by manufacturers, where rapid methods that do not produce an isolate or serogrouping/ partial serotyping only may be carried out.

#### 2.4.2.2. Phage typing.

##### *Used as a means for determining relatedness to S. Typhimurium*

There are a number of methods by which monophasic *S. Typhimurium* strains can be confirmed as “presumptive *S. Typhimurium*”. The simplest method for this is phage typing. Phage typing is an established method of detecting outbreaks of salmonellosis. The method is dependent on the lysis reactions of organisms with a panel of bacteriophages. The phage typing scheme most commonly used for Typhimurium is based on that originally described by Callow (1959) and expanded by Anderson (1977). In this phage typing scheme over 200 *definitive types* (DTs) have been defined, based on differences in the patterns of lysis. The phage typing of serovar Typhimurium is not dependent on the presence of the second phase H antigen and because of this, monophasic *S. Typhimurium* react with the same panel of phages (Amavisit et al., 2005; Echeita et al., 2001) (Mossong et al., 2007). All phage types that have been recognised so far within monophasic *S. Typhimurium* have also been found in normal di-phasic *S. Typhimurium*. The identification of a recognised phage type is characteristic of the derivation from *S. Typhimurium* and not the monophasic state.

##### *Used as a means for further subtyping within monophasic S. Typhimurium*

Phage typing is also used as a method of phenotypic subtyping within certain *Salmonella* serovars. For example, for monophasic *S. Typhimurium*, several phage types have been recognised. A range of definitive phage types – DTs 193, 120, 12, 18, 208, and as yet undefined phage types (PTs) - PTs U311 and 7 variant, have been associated with the monophasic ASSuT-resistant *S. Typhimurium* variant (Hopkins et al., 2010a; Rabsch, 2009). Phage type U302 has also been associated with the ‘Spanish’ *Salmonella* 4,5,12,i:– monophasic variant with plasmid-mediated antimicrobial resistance (de la Torre et al., 2003; Echeita et al., 2001; Walker et al., 2001). Similarly, the recently identified DT191A exhibiting resistance to tetracyclines is indicative of a different monophasic *S. Typhimurium* strain in USA and UK (Harker et al., 2011).

Although monophasic *S. Typhimurium* strains can be subtyped by phage typing, the resultant phage types are not of necessity a definitive indication of the monophasic state. For example, *S. Typhimurium* DT 193, a phage type commonly associated with the monophasic ASSuT-resistant clonal line, is a heterogenous phage type comprised of several different lineages (Rowe and Threlfall, 1984). Thus some isolates of *S. Typhimurium* DT 193 may be monophasic and others may not. Likewise *S. Typhimurium* DT 120 has been associated with monophasic isolates of R-type ASSuT (Rabsch, 2009), but some isolates of DT 120 of different R-types have been shown to have been derived from DT 104 (Lawson et al., 2002), and are not monophasic.

### 2.4.2.3. Genotyping

*Used as a means for identification of monophasic strains or for determining relatedness to S. Typhimurium*

A variety of molecular methods has been described for identifying *S. Typhimurium*. Most of these, except those that involve identification of the *fljB* genes that code for the phase 2 flagella antigens (Wattiau et al., 2008), do indicate that the monophasic strains are closely related to *S. Typhimurium*. Other molecular methods, including different polymerase chain reaction (PCR) and microarray-based methods have also been described (Cai et al., 2005; Cardona-Castro et al., 2009; Gurakan et al., 2008; Hackl et al., 2009; Kim et al., 2006; Lan et al., 2007; Leader et al., 2009; Yoshida et al., 2007). A simple method is to use a PCR that identifies a *S. Typhimurium*-specific fragment of the malic acid dehydrogenase gene (Lin and Tsen, 1999).

In order to overcome the need for submitting the strain to phase inversion several times (part of a normal serotyping routine), which increases dramatically the length of time necessary to obtain a final result, it is possible to use PCR to investigate the strains that are suspected to be monophasic. A PCR, in order to be suitable for this kind of confirmation, should ideally allow discrimination of *S. Typhimurium* from its monophasic variants, and also from other serovars that share the same O and H (first phase) antigens, such as:

*S. Lagos* (1, 4, [5], 12;i; 1,5)

*S. Agama* (4,12;i; 1,6)

*S. Farsta* (4,12;i; e,n,x)

*S. Tsevie* (4,12;i; e,n,z<sub>15</sub>)

*S. Gloucester* (1, 4,12, 27;i; 1,w)

*S. Tumodi* (1, 4,12;i; z<sub>6</sub>)

After a first negative phase inversion procedure, it is not possible to exclude the possibility of a negative or weak expression of a second flagellar phase other than 1,2.

To solve this problem, it is possible to apply a PCR to detect simultaneously the *fliB-fliA* intergenic region, and the phase 2 (*fljB*) flagellar gene. *Salmonella* Typhimurium and its monophasic variant possess an IS200 fragment of 1-kb in the *fliB-fliA* intergenic region, which is not detected in the other serovars listed above (apart from *S. Farsta*), which instead shows a 250-bp product. All these serovars except the monophasic variant 1,4,[5],12:-:- possess the *fljB* allele.

Thus, if submitted to PCR using a protocol such as described in Appendix A, the monophasic variant presents a single PCR product of 1-kb, whereas *S. Typhimurium* shows this fragment plus a heavier *fljB* one (1389 bp). Serovars Gloucester, Agama, Lagos, Tsevie and Tumodi present this *fljB* fragment and the 250-bp product in the *fliB-fliA* region (Tennant et al., 2010). The only inconsistent result in this procedure appears to be *S. Farsta*, which can be discriminated from *S. Typhimurium* only by serological or molecular identification of the second flagellar phase e,n,x.

In conclusion, for molecular identification of the monophasic 1,4,[5],12:-:- variant, it is advisable to proceed with serotyping until a first negative result of agglutination after phase inversion, and then apply a PCR protocol such as the one described in Appendix A in order to confirm the lack of the second phase antigen.

Other genotyping methods (MLST, PFGE, MLVA/VNTR, microarray analysis) have been used to confirm relatedness of monophasic strains to *S. Typhimurium* (de la Torre et al., 2003; Hauser et al., 2010b; Hoelzer et al., 2010; Soyer et al., 2009).

#### *Used as a means for further subtyping within monophasic S. Typhimurium*

Monophasic *S. Typhimurium* strains may also be subtyped by methods such as PFGE, MLST and MLVA. Within the monophasic ASSuT-resistant *S. Typhimurium* variants circulating widely within the EU, the predominant PFGE profiles are those of STYMXB.0131 and STYMXB.0083 (Best et al., 2009; Hopkins et al., 2010a; Lucarelli et al., 2010), the predominant MLST types are sequence type 34 and sequence type 19 (Ben-Darif et al., 2010; Hoelzer et al., 2010; Hopkins et al., 2010b), and the predominant MLVA types are 3-11-9-NA-211, 3-12-9-NA-211 and 3-13-10-NA-211 (Hauser et al., 2010a; Hopkins et al., 2010b). A new epidemic MLVA subtype of 3-13-15-NA-211 was identified in an outbreak in France in May 2010 using this sensitive methodology, which enabled discrimination of the outbreak strain from other monophasic MLVA types also circulating in that country (Bone et al., 2010).

### **Conclusion**

The current standard methods (ISO 6579 and Annex D of ISO 6579) are suitable for isolation of monophasic *Salmonella* Typhimurium strains. Various genetic and phenotypic characteristics have been described for the identification and subtyping of such strains. For identification of the monophasic 1,4,[5],12:i:- variant, it is advisable to proceed with serotyping until a first negative result of agglutination after flagellar phase inversion, and then apply a PCR protocol in order to confirm the lack of the second phase antigen. Other methods such as phage typing and genotyping methods (MLST, PFGE, VNTR, and virulotyping) are used to confirm relatedness to *S. Typhimurium* and/or to further subtype these isolates. Accurate characterisation of monophasic strains is important, since misidentification of a non-*S. Typhimurium*-related strain could result in unnecessary regulatory action. Similarly, failure to confirm identity of a *S. Typhimurium*-like organism could have significant public health consequences.

## **2.5. Public health relevance**

### **2.5.1. Human surveillance of salmonellosis**

Monitoring of *Salmonella* is mandatory in humans in accordance with the Commission Decision 2000/96/EC. Since 2007, EU and European Economic Area (EEA) countries report their surveillance data to The European Surveillance System (TESSy) at the European Centre for Disease Prevention and Control (ECDC). Surveillance systems for salmonellosis in the MSs differ between countries. In the majority of MSs, reporting of salmonellosis to the national level is compulsory. This is not the case in all MSs and in some not all regions report to the national level. Whether the data are reported by national reference laboratories, local laboratories, physicians and hospitals also varies (ECDC, 2009). For a case to be considered confirmed according to the EU case definition, it requires laboratory confirmation with isolation of the bacteria from stool or blood (EC, 2008).

### **2.5.2. Epidemiological situation of salmonellosis in the EU**

In 2008, a total of 135,335 non-typhoidal salmonellosis cases were reported to the ECDC, by all EU and EEA countries, of which 133,543 were confirmed (EFSA/ECDC, 2010). The overall notification rate in the EU was 26.4 per 100,000 population, which represents a significant decrease over the last five years. The notification rate of salmonellosis is high in children, in particular in the 0-4 year-olds,

where the rate is three times higher than in 5-14 year olds and more than five times higher than the remaining age groups.

*Salmonella* was also the most common causative agent reported in foodborne outbreaks in the EU in 2008, accounting for 1,888 outbreaks out of 5,332 reported (35%) (EFSA/ECDC, 2010). These 1,888 outbreaks involved 14,180 cases, 2,868 hospitalisations and 20 deaths. Pork and pork products were the most common sources for foodborne outbreaks of *S. Typhimurium* (EFSA/ECDC, 2010), although outbreaks associated with poultry and contaminated water have also occurred.

The two most common *Salmonella* serovars in the EU are Enteritidis and Typhimurium, representing in 2008 58% and 22%, respectively, of all known serovars (EFSA/ECDC, 2010). While the number of cases with *S. Enteritidis* is decreasing (most likely as a result of control measures implemented in poultry flocks), cases caused by *S. Typhimurium* are increasing. In 2008, 26,423 cases of *S. Typhimurium* were reported.

### 2.5.3. Cases of monophasic *S. Typhimurium* reported at the EU level

The ECDC has limited information on the presence of *Salmonella enterica* 1,4,[5],12:i:- in the EU as TESSy has only been in place for collecting data since 2007. Also, there is no harmonised way of reporting this variant and thus, cases can only be found in the database by analysing the antigenic formula, something which only ten out of 30 countries reported.

Seven countries out of the ten that reported the antigenic formula to TESSy reported monophasic *S. enterica* 1,4,[5],12:i:- (Denmark, Germany, Ireland, Italy, Luxembourg, the Netherlands and Spain). Although the numbers increased from 360 cases in 2007 to 1416 in 2009; the number of countries reporting this type also increased during this time period. In four countries, an increase over two or three years was observed, while in one country a decline in cases was observed for the two reported years. For one country there was no evident trend and the remaining country only provided data for one year. The first-line phenotypic method in several EU MSs for the primary subdivision of *S. Typhimurium* is phage typing (see above). In this respect, the most common phage type among the monophasic isolates was DT 193, representing 71% of monophasic isolates with known phage types, followed by DT 120 (19%).

### 2.5.4. Outbreaks

Several foodborne outbreaks caused by monophasic *S. Typhimurium* have been reported in Europe. In 2006, Luxembourg experienced two outbreaks of monophasic *S. Typhimurium* DT 193, involving 133 confirmed cases, 24 hospitalisations and one death (Mossong et al., 2007). A high proportion of cases were from institutions for the elderly and in day-care centres. Locally-produced pork meat was the suspected vehicle for the outbreaks (Mossong et al., 2007). The same variant monophasic, multidrug-resistant *S. 4,[5],12:i:-* DT 193 has been associated with large diffuse outbreaks in Germany since 2006, with increased need for hospitalisation reported (Trupschuch et al., 2010).

The most recent outbreak reported from Europe occurred in France in May 2010 involving 90 cases of *S. 4:12:i:-* (Bone et al., 2010). Twenty cases out of 54 interviewed had been temporarily hospitalised (37%). The outbreak was linked to consumption of a dried pork sausage sold in France and Belgium (so far no cases have been reported from Belgium).

Prolonged diffuse outbreaks with a tetracycline-resistant, corn snake-related, DT 191A strain involving significant numbers of cases have also occurred in UK since 2008 (HPS, 2010; Peters et al., 2010). This strain originated from infected frozen feeder mice imported into UK for feeding to exotic pets. In the USA several other *S. 4,5,12:i:-* outbreaks have occurred. One large multi-state outbreak

in 2007 was related to frozen chicken pies, and other cases involved exposure to turtles (CDC, 2007a, 2007b). Canada has also reported outbreak cases (Switt et al., 2009) and in São Paulo in Brazil, Chile and Costa Rica there have also been significant numbers of human cases (Switt et al., 2009).

### 2.5.5. Country-specific data

While the rare isolation of *Salmonella* 4,5,12:i:- before the 1990s may reflect a recent expansion and/or emergence of this serovar, it is important to acknowledge that *Salmonella* 4,5,12:i:- isolates have been and still may be classified as *S. Typhimurium*, possibly leading to underreporting of this variant of *S. Typhimurium*. *Salmonella* 4,5,12:i:- isolates also appear to have to often been reported as e.g. “Group B” or “untypable” or “*Salmonella enterica subspecies I*” (Switt et al., 2009).

#### 2.5.5.1. Europe

In England and Wales cases of *Salmonella* 4, [5], 12:i:- infections have risen from 47 in 2005 to 151 in 2009 (a 321% increase) against an overall decrease in the number of salmonellosis cases, with isolates of R-type ASSuT accounting for approximately 30% of these strains. These figures are probably not fully representative of the total number of infections of monophasic *S. Typhimurium*-like strains, as strains identified as Typhimurium by the isolation laboratory and referred to the reference laboratory for phage typing do not as a routine undergo full serotyping. For example in 2008 188 of 256 (42%) isolations of *S. Typhimurium* DT 193 from humans in England and Wales were of the ASSuT phenotype. It should also be noted that since 2008 there has been a substantive increase in isolations of *S. Typhimurium* DT 191A. The majority of these isolates were not serotyped but of those that were, over 80% were monophasic. In Scotland there was an increase in reports of *Salmonella* monophasic Group B (94 reports in 2009, compared to 37 in 2008).

In France the National Reference Laboratory for *Salmonella* reported a gradual increase in the isolation of *Salmonella* 4,5,12 :i :- from humans. The serovar moved from eleventh to third place in order of isolation between 2005 and 2008 (AFSSA, 2009) and a further significant increase was reported in the first five months of 2010 (Bone et al., 2010). Furthermore, numerous outbreaks of grouped cases corresponding to *Salmonella* 4,5,12 :i :- were identified in 2008 in France: 13 family epidemics, three collective infections and two infections in hospital (AFSSA, 2009).

In Germany, isolations of *Salmonella* 4,5,12:i:- have continuously increased in humans since 2000 (Hauser et al., 2010b). In 2006, a multi-drug resistant strain of this type was associated with large diffuse outbreaks and increased need for hospitalisation (isolates belonged mainly to DT 193 and exhibited at least tetra-drug resistance (Trupschuch et al., 2010). In 2008, the monophasic variant represented 42% of all *S. Typhimurium* isolates from humans analysed at the National Reference Centre (Hauser et al., 2010a).

In Italy, *S. 4,5,12 :i :-* represented the third most common serovar from cases of human infection from 2004 to 2008 (Dionisi et al., 2009) and the second most common in 2009 (ISS, 2010). Monophasic strains with the resistance pattern ASSuT (with or without additional resistances) represented 75% of the monophasic isolates from either 2008 or 2009. Forty-eight percent of the monophasic strains belonged to DT193 and 13% to U302. The most common PFGE profiles were STMXB 00131 (47%) and STMXB 0079 (37%).

In The Netherlands *S. 4,5,12 :i :-* was initially reported from humans in 2004. Subsequently, isolations have progressively increased in incidence, and it has represented the third most common serovar from human infections from 2005 to 2008 (van Pelt W., 2009).



In Spain a considerable number of *Salmonella* 4,5,12:i:- isolates has been reported since the first documented isolation in this country in 1997. Since then, this new serovar ranks fourth among the *Salmonella* serovars that are the most frequently isolated in Spain (de la Torre et al., 2003).

#### 2.5.5.2. Outside Europe

In the United States *Salmonella* 4,5,12:i:- has gone from the 18<sup>th</sup> most frequently reported serovar in human salmonellosis in 2002 to 14<sup>th</sup> in 2003 to seventh in 2004, to sixth in 2006 (CDC, 2008). *Salmonella* 4,5,12:i:- has also been reported in South and Latin America. In the Brazilian state Sao Paulo a five-fold increase of this serovar was observed in the 1990s, since its initial isolation in the late 1970s and it has been among the top five *Salmonella* serovars that have been isolated from humans and non-human sources associated with foodborne outbreaks in humans and extra-intestinal infections (Tavechio et al., 2009). In Thailand *Salmonella* 4,5,12:i:- has been found among the top five *Salmonella* serovars from cases of food-borne salmonellosis (Pornruangwong et al., 2008).

### 3. Hazard Characterisation

#### 3.1. General molecular characteristics

The monophasic *S. Typhimurium* strains that have been involved in recent international increases in human and food animal infections are typically very similar to *S. Typhimurium* at the molecular level (Amavisit et al., 2005; Echeita et al., 2001), but lack detectible flagella phase 2 antigens and associated genes (McQuiston et al., 2008; Parrenas et al., 2004; Switt et al., 2009; Zamperini et al., 2007). There are extensive genetic similarities with the *S. Typhimurium* LT2 type strain (Rabsch, 2009; Soyer et al., 2009). Moreover various phenotypic and molecular fingerprinting techniques have reported similarities with *S. Typhimurium*, e.g. specific monoclonal antibodies (Rementeria et al., 2009), biotyping (de la Torre et al., 2005), MLST (Alcaine et al., 2006; Soyer et al., 2009), PFGE (Dionisi et al., 2009; Hauser et al., 2010b; Vidal-Gallego, 2005; Zamperini et al., 2007), MLVA/Variable Number of Tandem Repeats (VNTR) typing (which also identifies the presence of some *S. Typhimurium*-specific loci) (Hauser et al., 2010b; Laorden et al., 2009; Torpdahl et al., 2009) and ribotyping. Random Amplified Polymorphic DNA (RAPD) analysis, and plasmid profile types (de la Torre et al., 2003; Sala, 2002) are also similar to *S. Typhimurium* (AFSSA, 2009; Garaizar et al., 2002) and different from *S. Lagos* (Soyer et al., 2009).

Characterization of American and Spanish *Salmonella* isolates by a MLST based on three genes (Sukhnanand et al., 2005) revealed, with exception of one isolate of sequence type (ST) 40, that monophasic strains were ST6, which is the predominant ST within *S. Typhimurium* isolates (Alcaine et al., 2006). ST6 is unique to serovar Typhimurium and its monophasic variants (Alcaine et al., 2006; Switt et al., 2009). Hopkins et al. (2010b) using a MLST typing according to the MLST Databases at the ERI, University College Cork (<http://mlst.ucc.ie/mlst/dbs/Senterica>) (based on 7 housekeeping genes) identified ST19 and ST34 (both ST defined by the alleles *aroC10-hemD12-hisD9-purE5-sucA9-thrA2*, and with *dnaN19* and 7, respectively), both STs also typical of *S. Typhimurium*. Hoelzer et al. also found ST19 for both monophasic and Typhimurium isolates from USA analysed in their study (2010).

Different mutations and deletions have been associated with the lack of phase 2 flagella expression in *Salmonella* 4,5,12:i:- isolates. Specifically, at least some of the *S. 4,5,12,i:-* isolates from Spain appear to be characterized by deletion of a large fragment, including *fljB* and *hin*, encoding a DNA invertase essential for *fljB* expression (Garaizar et al., 2002), although other variants have also been found (Laorden et al.; Laorden et al., 2010). Most of the isolates from the United States characterized thus far seem to be typified by deletions that eliminate *fljB* but maintain *hin* (Soyer et al., 2009;

Zamperini et al., 2007). These observations and other genomic characteristics suggest that *S.* 4,5,12,i:- has evolved through multiple independent emergence events involving different clonal groups during the last twenty years, during which the large increase in such strains has occurred (Garaizar et al., 2002; Laorden et al., 2009; Laorden et al., 2010). Nevertheless, the driver for this evolution is unknown, although for many epidemic strains antimicrobial resistance appears to be involved (Zaidi et al., 2007) and multi-drug resistance and virulence regulation mechanisms may overlap in some strains (Bailey et al., 2010).

### 3.2. Virulence factors

Factors contributing to the virulence of *Salmonella* are complex and vary hugely between both serovars and strains within a serovar. Some serovars, e.g. *S. Gallinarum* are host-specific and can only survive in macrophages of that host. Other serovars, e.g. *S. Choleraesuis* or *S. Dublin*, are host-adapted but can still occasionally infect other hosts and cause serious disease in humans. The majority of serovars can infect any host, but some, such as *S. Enteritidis*, are more likely to be found in a specific host because of certain special properties, e.g. ovarian transmission. Most salmonellas are either good survivors in the environment and able to multiply outside the host, or are able to exist long-term within carrier animals. Some strains have shown epidemic potential, e.g. *S. Typhimurium* DT 104, but others are endemic with more limited potential to spread. Infection of the host is normally by the faecal–oral route and the organism must survive the acidic conditions in the crop or stomach, and be able to compete with intestinal flora to multiply and invade lymphoid tissue. Colonisation, invasion and intracellular survival involves the complex interaction of numerous and variable virulence mechanisms that are still not fully understood despite decades of research (Stevens et al., 2009), but the adaptability and variability of *Salmonella* ensures that there is a suitable strain available to colonise any host given the opportunity. Additional details about the virulence of *Salmonella* are presented in detail in Appendix B.

With respect to the monophasic *S. Typhimurium* strains discussed in this Opinion, such strains have only relatively recently emerged and a variety of different strains exists (Soyer et al., 2008). Consistent data on virulence mechanisms are therefore limited. Nevertheless, where studies have been performed the occurrence of recognised virulence genes appears to correspond with that of ‘classic’ *S. Typhimurium* (Guerra et al., 2004; Hauser et al., 2010b; Rodriguez et al., 2008; Soyer et al., 2009). Indeed, virulence microarray analysis of isolates of *S. enterica* serovar 4,[5],12;i:- and ‘classic’ *S. Typhimurium* demonstrated that there were no differences except minor variations in single strains within and between isolates from pigs, pork and cases of human infection made in Germany during 2006 and 2007 (Hauser et al., 2010b).

Sequencing of the EU ASSuT-resistant DT 193 *Salmonella*.4,5,12:i:- strain shows virulence genes with some similarities to *Enterobacteriaceae* phage sequences that carry putative pathogenicity genes similar to those in *Shigella* and pathogenic *E. coli* strains (Rabsch et al., 2008) as well as carrying a characteristic 18-19 kb genomic island in the *thrW* transfer RNA gene locus. Most of the other tRNA regions investigated were identical to those found in *S. Typhimurium* (Rabsch and Laverde-Gomez, 2007; Trupschuch et al., 2009; Trupschuch et al., 2010). Other studies using a variety of PCR or microarray based methods have concluded that the virulence gene repertoire of monophasic *S. Typhimurium* and its variability is similar, sometimes identical or increased, compared with *S. Typhimurium*, depending on the individual strains examined (Gewirtz et al., 2001; Hauser et al., 2009; Hauser et al., 2010b; Ikeda et al., 2001). Studies by microarray techniques of strains of *S. Typhimurium* PT U302 with the antigenic structure 4,5,12:i:- that emerged in Spain in the late 1990s identified only very minor deletions in genetic content compared with *S. Typhimurium* (Garaizar et al., 2002) and homologous to virulence plasmid genes *spvC* (even in plasmid-free strains), as well as identical *invE* and *invA* invasion genes, *stn* enterotoxin genes, *slyA* cytolysin genes and genes associated with survival within macrophages (*pho*) to those typically found in *S. Typhimurium* (del

Cerro et al., 2003; Guerra et al., 2000). Although in many of these isolates the pSLT90 plasmid is lacking, *spv* genes have been found in some of these isolates, being located on plasmids belonging to different incompatibility groups (i.e. IncA/C or IncA/C plus with IncN) (Guerra et al., 2004). *S. Typhimurium* U302 is closely related to the pandemic Typhimurium DT 104 (Briggs and Fratamico, 1999; Walker et al., 2001) and the Spanish strain is highly clonal, which is another indication of virulence-related rapid expansion (Laorden et al., 2009). Virulence gene microarray studies of various monophasic *S. Typhimurium* strains that have arisen since 2000 in pigs and humans have demonstrated the same lineage as *S. Typhimurium*, although the monophasic strains that were prevalent in Germany and Spain differed from each other (Hauser et al., 2009; Hauser et al., 2010b). In most of these isolates (i.e. DT 193) the pSLT90 virulence plasmid and the *spv* genes are not present (Hauser et al., 2009; Hauser et al., 2010b; Hopkins et al., 2010a). Similar studies in Spain demonstrated overall similarities, as well as the presence of virulence plasmid genes homologous in plasmid-free strains (Garaizar et al., 2003; Garaizar et al., 2002). Poultry and cattle *S. 4,5,12:i:-* isolates from the USA clustered into at least four distinct clonal groups, each closely related in virulence gene content to *S. Typhimurium* (Zamperini et al., 2007).

### 3.3. Antimicrobial resistance

Over the last decades, the emergence and spread of antimicrobial-resistant zoonotic bacteria has become a serious public health problem. In the case of severe human *Salmonella* infections resistance may limit the options for treatment, particularly when strains exhibit resistance to critically-important antibiotics (Anonymous, 2007b; Collignon et al., 2009). Bacteria contain extremely efficient genetic transferable elements capable of exchanging and accumulating antimicrobial resistance genes (Guerra et al., 2004). Resistance genes can move between chromosomal and extra-chromosomal DNA elements, and they may move between bacteria of the same or different species or to bacteria of different genera by horizontal gene transfer. The most important vehicles for transfer of resistance genes in bacteria are mobile genetic elements, such as plasmids, transposons, and genomic islands. Different resistance determinants and mechanisms can be responsible for the same resistance phenotype. This observation has been confirmed in several studies on the molecular epidemiology of resistant bacterial clones (Guerra et al., 2000; Guerra et al. 2004). The presence of a gene does not always imply resistance of the strain to a particular antimicrobial, but reflects the potential to express resistance once a selective pressure is applied (Guerra et al., 2004).

In recent years there has been an overall decline in the level of resistance in serovar Typhimurium in several European countries, mainly as a result of a reduction in the number of isolates of penta-resistant *S. Typhimurium* DT 104 (Hopkins et al., 2010a; Meakins et al., 2008). To some extent this reduction has been counteracted by an increase in prevalence of *Salmonella* 4,[5],12:i:- isolates of R-type ASSuT (Dionisi et al., 2009; Hauser et al., 2010a; Hopkins et al., 2010a; Litrup et al., 2010). The antimicrobial resistance patterns of monophasic strains vary with the strain. In UK, strains of *Salmonella* 4,5,12:i:- (in this case designated and reported as *S. Typhimurium*, except in Scotland) have been seen as severe cases, particularly in humans that were exposed to corn snakes fed on specially-bred mice imported from the USA. These strain(s) are clearly virulent but are only resistant to tetracyclines (Harker et al., 2011). The newly emerged monophasic *S. Typhimurium* DTs 193 / 120 strains that have been reported from numerous EU countries are typically of R-type ASSuT, with the respective resistances encoded by chromosomal *bla*T EM, *strA-strB*, *sul2* and *tet*(B) genes (Hauser et al., 2010b; Hopkins et al., 2010a; Lucarelli et al., 2010), although a minority of strains may acquire additional resistances (i.e class 1 integron located *dfrA* genes encoding trimethoprim resistance). In the laboratory data from TESSy, 79% of isolates tested against these four antimicrobials were resistant compared to only 14% of all tested *S. Typhimurium*. Resistance in the Spanish *S. Typhimurium* PT U302-based monophasic strains was different. All these isolates were multiresistant (from 6-9 resistant determinants), and the most frequent phenotype found was that with resistance to ampicillin, choramphenicol, gentamicin, streptomycin, sulfamethoxazole, tetracyclines and

trimethoprim (Echeita et al., 1999; Guerra et al., 2000; Usera et al., 2002), mainly encoded by *bla*<sub>TEM-1</sub>, *cmlA1*, *aac(3)-IV*, *aadA2* and/or *aadA1*, *sul1*, *sul2*, *sul3*, *tet(A)* and *dfrA12* (Guerra et al., 2000; Guerra et al., 2001). Almost all isolates possessed large non self-transferable (but mobilizable) resistance plasmids (> 120-200 kb) with multireplicons for incompatibility groups IncN and/or IncA/C. In some of them, the *spv* genes have been captured (Guerra et al., 2004). These resistance plasmids often carry several class 1 integrons (up to three): one with a variable region of 1900bp with *dfrA12-orfX-aadA2* genes, a second one with the *estX-psp-aadA2-cmlA1-aadA1* gene cassettes associated to an unusual 3' sequence region carrying *qacH*, IS440 and *sul3*, and an empty one lacking of resistance genes in its variable region but with *qacEΔ1* and *sul1* in its 3' conserved segment (Antunes et al., 2010; Guerra et al., 2000; Guerra et al., 2001; Guerri et al., 2004).

Isolates from Thailand, and Korea had additional resistance to gentamicin, potentiated sulphonamides or chloramphenicol (Lee et al., 2009; Pornruangwong et al., 2008). In contrast US and Brazilian monophasic *Salmonella* strains were rarely multidrug resistant and often pan-susceptible (Agasan et al., 2002; Tavechio et al., 2004) but most strains had the 90 kb *S. Typhimurium* virulence plasmid (Tavechio et al., 2009).

*Salmonella* 4,5,12:i:- isolates carrying extended-spectrum β-lactamases (i.e. *bla*<sub>CTX-M-1</sub>) have been also detected in UK isolates from animals in 2009 (VLA, 2010).

In conclusion, the repertoire of antimicrobial resistance genes of monophasic *S. Typhimurium* is similar to that in *S. Typhimurium* and in other Enterobacteriaceae. The principal differences in the European monophasic strains are the presence of a unique chromosomal resistance island with the *bla*<sub>TEM</sub>, *strA-strB*, *sul2* and *tet(B)* genes in the epidemic ASSuT-resistant clonal line, and resistance genes linked to the serovar-specific plasmid in the Spanish monophasic *S. Typhimurium* U302 isolates.

### 3.4. Severity of human cases caused by monophasic *S. Typhimurium*

It is difficult to assess this from reported cases in the literature although some reports suggest increased severity compared with most *Salmonella* infections (Switt et al., 2009). In the UK, children have been commonly affected in outbreaks and incidents associated with monophasic tetracycline-resistant *S. Typhimurium* DT 191A and there has been an unusually high rate of hospitalisation, but no deaths up to 2009 (Harker et al., 2011). In Germany there have also been a large number of diffuse outbreaks involving an increased hospitalisation rate compared with normal *Salmonella* outbreaks and a particularly high attack rate in children and old people (Rabsch, 2009). In large outbreaks in New York involving a dulcitol-negative *S. 4,5,12:i:-* strain, 14% of cases involved haemorrhagic diarrhoea (Agasan et al., 2002). In two major outbreaks in Luxembourg in 2006 the most recent pandemic PFGE type STYMXB.0031 DT193-related monophasic strain of 133 reported cases involved 24 hospitalisations and one death (Mossong et al., 2007).

It is not clear if infections caused by monophasic *Salmonella* Typhimurium strains are more or less severe than with other *S. Typhimurium* infections since reports of severity appear to vary in different countries (Jones et al., 2008). The data reported to ECDC do not suggest infections with the monophasic variants to be more severe. This conclusion is based on the percentage of strains isolated from blood compared to faeces, as this ratio was in the same range or even lower, than for other *S. Typhimurium* (which had 1.8% isolated from blood and 95% from faeces).

As previously described, most of the emerging *Salmonella* 4,5,12:i:- strains have shown multiple antimicrobial resistance (Garcia-Feliz et al., 2008), although pan-susceptible and monoresistant (to tetracyclines only) strains have also featured in outbreaks in USA and UK (Harker et al., 2011; Zamperini et al., 2007). It would therefore appear that treatment failure is not as yet a feature of virulence in humans. Nevertheless, the development of resistance to critically important

antimicrobials such as fluoroquinolones and/or third generation cephalosporins, which seem to be emerging within monophasic *S. Typhimurium* isolates (Garcia-Feliz et al., 2008; Hauser et al., 2010b) could, in the case of need, lead to treatment failure. On the other hand, the regular or routine use of antimicrobials such as tetracyclines in pigs may promote higher levels of resistance and some multiple resistances, and the proliferation of resistant strains (Usera et al., 2002), thereby enhancing their colonisation and persistence in the porcine hosts.

## Conclusion

On the basis of genetic similarity and ability to obtain a recognised *Salmonella* Typhimurium phage type, we regard these emerging epidemic monophasic strains with formula 1,4,[5],12:i:- as variants derived from *S. Typhimurium*. Their virulence potential and their antimicrobial resistance characteristics are similar to those of *S. Typhimurium*. Consequently, they should be treated like *S. Typhimurium* and therefore be included in the *Salmonella* control programmes.

Antimicrobial susceptibility testing is important for epidemiological investigations. The antimicrobial resistance pattern should be determined and reported in a harmonised way for human, animal and food isolates, according to European guidelines (EFSA, 2008c; EUCAST, 2010).

## 4. Exposure assessment

### 4.1. Monophasic *S. Typhimurium* reported in scientific literature

Since the late 1990s the emergence of monophasic strains belonging to the serovar *S. 4,5,12:i:-* isolated from a wide variety of sources have been documented worldwide (Switt et al., 2009) as reported in the following table.

**Table 2:** Worldwide reports published on the isolation of *Salmonella enterica* serovar 4,5,12:i:- (Data obtained from Switt et al. 2009 and supplemented)

Years of isolation	Country	Source	Reference
1986-1987	Portugal	Chicken	Machado and Bernardo, 1990
1993-1994	Thailand	Human, chicken meat	Boonmar et al., 1998
1997	Spain	Human, food	Echeita et al., 1999
1991-2000	Brazil	Human, food, animals	Tavechio et al., 2009
1998-2000	United States	Human , raw chicken meat	Agasan et al., 2002
1998-2000	Spain	Swine	de la Torre et al., 2003
2000-2002	Spain	Swine	Vidal 2005
2000-2001	Thailand	Human, frozen meat, foods	Amavisit et al., 2005
2003-2004	Italy	Human, swine	Barone et al., 2008
2003-2004	Portugal	Pig carcasses	Vieira-Pinto et al., 2005
2003-2006	Thailand	Human, swine	Pornruangwong et al., 2008
2004	United States	Human, bovine	Alcaine et al., 2006
Not available	United States	Bovine, poultry, non domestic birds	Zamperini et al., 2007
2006	Luxembourg	Pork, pigs	Mossong et al., 2007
2004-2008	Germany	Human, food, swine, cattle, broiler	Trupschuch, 2010, Friedrich et al. 2010, Hauser et al. 2010

In Europe, a considerable number of *Salmonella* 4,5,12:i:- isolates have been reported:

Data from the French *Salmonella* network have demonstrated a gradual increase in the isolations of *S.* 4,5,12:i:- strains from animal and food samples. These isolations have been usually related to poultry production, but this is not always the case (AFSSA, 2009).

In addition, *S.* 4,5,12:i:- has continuously increased in pigs and humans in Germany since 2000 and this serovar resulted to be the second most common one from samples collected in the framework of the EU-monitoring study on the prevalence of *Salmonella* in slaughter pigs (Hauser et al., 2010a). Data collected from the German National Reference Laboratory on the prevalence of *Salmonella* spp. for the years 2004-2008 revealed that *S.* 4,5,12:i:- has represented the second most common serovar isolated from pig and the third from bovine samples (Friedrich et al., 2010).

In Italy, the monophasic *S.* Typhimurium R-type ASSuT strain has also ranked in the top five serovars isolated from non-human sources (animal, food and environment) during the last four years. In particular, it has been included among the first three most common serovars isolated from bovine samples in Italy since 2005 and from swine samples since 2002, the first year when the Italian Enterovet surveillance network was operational (ISS, 2010).

In The Netherlands, during the five-year period 2004-2008 *S.* 4,5,12:i:- was the fourth most common serovar isolated from pigs, and it moved up to the second position in 2009. Moreover, when considering strains from bovine samples *S.* 4,5,12:i:- was confirmed as the third serovar in 2009 as well as during the five previous years (MARAN, 2010).

In Spain *S.* 4,5,12:i:- has become the most common serovar in swine and the second most common in pork products (de la Torre et al., 2003).

In UK a first increase in isolations of *S.* 4,5,12:i:- from animal samples was observed in 1997-1998. During the following years the number of isolations of the monophasic strains went down but increased again, mainly in pigs and cattle but also in horses, pets and animal feed ingredients, during the last three years, as the monophasic *S.* Typhimurium R-type ASSuT DT193/DT120 clonal group emerged (2007-2009) (VLA, 2010).

Outside of Europe, this serovar also ranks in the top ten in both broiler and ground chicken samples in the United States, starting in 2004, the year in which the U.S. Department of Agriculture Food Safety Inspection Service began reporting this serovar (Zamperini et al., 2007). In addition, a highly virulent monophasic *S.* Typhimurium DT 193 strain has been reported in cattle and in wild birds (Phalen et al., 2010). In Thailand *S.* 4,5,12:i:- has become the most frequently encountered serovar in swine, and the second most common serovar in pork products (Pornruangwong et al., 2008).

#### **4.2. Monophasic *S.* Typhimurium in the EU zoonoses monitoring system**

In the EU, zoonoses data collection is based on the Directive 2003/99/EC (EC, 2003a). This Directive came into force into 2004, and since then EFSA has examined the zoonoses monitoring data collected by MSs and publishes the annual zoonoses Community summary report.

Summary results for the years 2004 to 2008 in food, animals and feed are presented in Table 3. Overall, 224 units were reported to be positive for the monophasic *S.* Typhimurium strains; 16 in food, 205 in animals and seven in feed. Eleven northern, central, western, and southern MSs have reported these serovars. The proportion of units reported positive for the various monophasic *S.* Typhimurium strains was very low (between 0.1% and 1%), except for *S.* 1,4,5,12:i:- in animals, *S.* 4,12:i:- in food and animals, and *S.* 4,5:i:- in animals. The specific proportion of positives was low

for these strains (between 1% and 10%). The first three variants *S.* 1,4,5,12:i:-, *S.* 4,12:i:- and *S.* 4,5:i:- were reported by more Member States and the numbers of reported positive units to these strains was higher compared to units positive to the other strains.

**Table 3:** Summary of reported monophasic *Salmonella* Typhimurium strains in food, animals and feed, Community zoonoses monitoring, EU, 2004-2008.

Possible Monophasic <i>Salmonella</i> Typhimurium strains	Origin	Number of positive units (N=233)	% of units with serovar	Number of countries reporting	Member States*
<i>S.</i> 1,4,5,12:i:-	food	4	0.8	2	EE, LU
	animals	124	6.2	4	EE, ES, IT, LU
<i>S.</i> 4,12:i:-	food	11	2.1	1	IT
	animals	20	1.0	5	BE, CZ, EE, IT, SK
	feed	2	0.9	2	FI, SK
<i>S.</i> 4,5:i:-	animals	38	1.9	2	IT, PT
<i>S.</i> 4,5,12:i:-	animals	11	0.6	1	IT
<i>S.</i> group B, monophasic strain	feed	1	0.5	1	AT
	animals	9	0.5	1	AT
<i>S.</i> group B H-	animals	2	0.1	1	AT
<i>S.</i> 1,4,5,12:-:1,2	food	1	0.2	1	EE
<i>S.</i> 4,5,12:-:1,2	animals	1	0.1	1	IT

\* See Glossary for the country names key

#### *Isolations from food, feed and food animals*

In food, 16 samples were reported positive for monophasic *S.* Typhimurium by three MSs. The specific proportion positive samples to these strains (out of the *Salmonella* spp.-positive samples in the EU) ranged between 0.2% and 0.8% except for *S.* 4,12:i:- for which one MS reported 11 isolates (2.1% of the *Salmonella* spp.-positive food samples). The food categories that were positive were fresh meat from bovine animals and pigs, meat products from pigs, minced meat from other animals or ‘not specified’.

In animals, 205 units (flocks, or herd, or slaughter batches) were reported positive for monophasic *S.* Typhimurium by ten MSs. The proportion of positive units (out of the *Salmonella* spp.-positive units in the EU) was very low (below 1%) except for *S.* 1,4,5,12:i:-, for which four MSs reported 124 isolates (6.2% of the *Salmonella* spp.-positive animal units); *S.* 4,12:i:- for which five MSs reported 20 strains (1.0% of the *Salmonella* spp.-positive animal units); *S.* 4,5:i:- for which two MSs reported 38 strains (1.9% of the *Salmonella* spp.-positive animal units). Almost all isolates, (92%) were reported to originate from pigs. Other reported incriminated animal species were poultry, cattle, pet animals and wild birds.

In feed, four units were reported positive for monophasic *Salmonella* Typhimurium by three MSs. The specific proportions of positive units (single sample or batch) ranged from 0.5% to 0.9%. The positive feed categories were pet food, final product - non-pelleted/meal and other feed material.

More detailed information about these strains from the *Salmonella* monitoring program in MSs is presented in Appendix C.

### 4.3. Monophasic *S. Typhimurium* in the *Salmonella* baseline studies conducted in EU

The presence of monophasic *S. Typhimurium* has also been reported previously in some of the *Salmonella* baseline studies conducted in the EU since 2004 in the context of Regulation EC/2160/2003 (EC, 2003b), which aims at reducing the incidence of food-borne diseases. A summary of these findings is presented in Table 4. While these strains were not found in baseline studies of laying hen (EFSA, 2007b) or broiler flocks (EFSA, 2007a) a single isolate was reported in the baseline study of turkey flocks carried out between 2006 and 2007 (EFSA, 2008b). Monophasic *S. Typhimurium* isolates were more commonly found in both baseline studies of the prevalence of *Salmonella* in slaughter pigs (EFSA, 2008a) and breeding pigs (EFSA, 2009), conducted in 2006-2007 and 2008 respectively.

Despite monophasic *S. Typhimurium* isolates not being found in poultry at farm level, they were reported in the baseline study of broiler carcasses conducted in 2008 (EFSA, 2010), which might indicate the more recent emergence of monophasic *S. Typhimurium* in poultry. Details of all the monophasic *S. Typhimurium* isolates identified in baseline studies can be found in Appendix C.

The hypothesis of the misclassification of *S. 4,5,12:i:-* and consequently the underreporting of this serovar because of the lack of harmonised typing and reporting procedures is demonstrated by the results of these EU baseline studies. These studies revealed a low prevalence of such isolates in pig production but pig meat is considered likely to be the most common link with human infections. Baseline studies in poultry holding were carried out before the widespread emergence of monophasic *S. Typhimurium* DT193 and no baseline studies have been undertaken in cattle or companion animals. Similarly the involvement of wild animals and environmental contamination in human infection has not been investigated.



**Table 4:** Summary of reported monophasic *Salmonella* Typhimurium strains in different food-producing species, Community baseline studies, EU, 2004-2008

Possible Monophasic <i>S. Typhimurium</i> strains	Baseline study	<i>Salmonella</i> spp positive units (total samples)	Positive for Monophasic <i>S. Typhimurium</i>	Member States* reporting	Comments
-	Laying hen flocks	-	-	-	No monophasic <i>Salmonella</i> reported
-	Broiler flocks	-	-	-	No monophasic <i>Salmonella</i> reported
Monophasic strain of Group B	Fattening turkey flocks	35 (202)	1	AT	-
-	Breeding turkey flocks	-	-	-	No monophasic <i>Salmonella</i> reported
<i>S.</i> 4,[5],12:i:-	Slaughter pigs (lymph nodes)	2,600 (19,071)	128	CY, DK, ES, GR, HU, NL, PT, UK	-
<i>S.</i> 4,[5],12:i:-	Slaughter pigs (swabs)	387 (5,736)	5	UK	-
<i>S.</i> 4,5,12:-:1,2	Slaughter pigs (swabs)	387 (5,736)	1	UK	-
<i>S.</i> 4,12:i:-	Swine breeding holdings	452 (1,530)	4	DE, ES, CH	-
<i>S.</i> 4,5,12:i:-	Swine breeding holdings	452 (1,530)	6	NL, PT, ES, UK	-
<i>S.</i> 4,12:i:-	Swine production holdings	950 (3,278)	6	CZ, DE, PL, ES, UK	-
<i>S.</i> 4,5,12:-:-	Swine production holdings	950 (3,278)	1	PT	-
<i>S.</i> 4,5,12:i:-	Swine production holdings	950 (3,278)	15	EE, DE, NL, PL, PT, ES, UK	-
<i>S.</i> 4,[5],12:i:-	Broiler carcasses	1,225 (10,035)	15	DE, MT, CH, NL	-

\* See Glossary for the country names key

## 5. Risk characterisation - Considerations for human health

The emergence in Europe of the monophasic strains of *S. Typhimurium* DTs 193 and 120 of R-type ASSuT since the late 1990's in pigs, humans, cattle and to a lesser extent poultry and companion animals in many countries (Hauser et al., 2010b; Hopkins et al., 2010a; Rabsch, 2009; Torpdahl et al., 2009) is evidence of its potential for enhanced spread (AFSSA, 2009; Barone et al., 2008; Boonmar et al., 1998; CDC, 2008; Garaizar et al., 2002; Soyer et al., 2009; Switt et al., 2009; Tavechio et al., 2004; Usera et al., 2002). In the UK the first animal isolate of the current pandemic strain of monophasic *S. Typhimurium* of R-type ASSuT and the characteristic 18.65 kb genomic island was detected in 2005 in a horse. Since 2006 the strain has increased dramatically in pigs, cattle and has occasionally been found in companion animals in the UK. In Scotland there has been a similar increase in monophasic isolates from clinical cases to around 80 cases in 2009, with 20 so far in 2010; in England and Wales the corresponding figures are 188 cases of *S. Typhimurium* DT 193 of ASSuT for 2009 and over 150 for the first seven months of 2010, indicating a substantive and on-going increase in isolations. In 2008, *S. 4,5,12:i:- Salmonella Typhimurium* DT 193 isolates represented over 40% of human *S. Typhimurium* isolates in Germany (Rabsch, 2009). The National Reference Centre for *Salmonella* in France (AFSSA, 2009) reported a rapid increase from rank 11 (99 reports) in 2005 to number three (410 reports) in 2008, with even more cases in 2009. In Spain, the *S. Typhimurium* PT U302-related monophasic strain was first detected in August 1997 (Echeita et al., 1999) and by 2007 represented 20.8% of all *Salmonella* in pigs and 2.5% in poultry (Soyer et al., 2009). In humans, monophasic *S. Typhimurium* was the fourth most common serovar in 1998 (Garaizar et al., 2002) and third in 1999 (Usera et al., 2002). In Italy, monophasic *S. Typhimurium* has rapidly emerged from the tenth to the second most common serovar since 2003 (ISS, 2010).

The emergence of monophasic *Salmonella 4,5,12:i:-* demonstrates its evolutionary success in rapidly becoming amongst the most prevalent serovars in humans in numerous countries worldwide (Switt et al., 2009). Such strains have also been associated with a wide range of animal species, from mice, snakes and turtles to poultry, pigs, cattle and horses.

Although outbreaks of infection of monophasic *S. Typhimurium* strains have been reported from several countries, in only one outbreak was a death reported, in an elderly patient (Mossong et al., 2007). To date, the accumulated evidence is not suggestive of increased virulence in these strains in humans in comparison with bi-phasic *S. Typhimurium* strains.

### Conclusion

Similar to what was observed in the past for epidemic clones of *S. Typhimurium*, recent studies in numerous countries worldwide confirm the rapid emergence and dissemination of such strains in food animals, companion animals and humans. We therefore regard the public health risk posed by these emerging monophasic *S. Typhimurium* strains as comparable to that of epidemic *S. Typhimurium* strains.

## CONCLUSIONS

Within *Salmonella* Typhimurium-like strains, monophasic variants lacking the second phase H antigen (1,4,[5],12:i:-), encoded by *fljB*, appear to be of increasing importance. Strains lacking expression of the phase one flagellar antigens (e.g. *S.* 4,[5],12:-:1,2) or both (*S.* 4,[5],12:-:-) are also possible, but have not commonly been reported to be associated with significant disease in animals or humans. Therefore, for the purposes of this Opinion, only the monophasic variants lacking second phase H antigens will be considered. Such variants were referred to as ‘**monophasic *S.* Typhimurium**’.

### Answer to the terms of reference

- *To evaluate the analytical methods currently used and advise on the appropriateness for identifying these strains;*

The current standard methods (ISO 6579 and Annex D of ISO 6579) are suitable for isolation of monophasic *Salmonella* Typhimurium strains. Various genetic and phenotypic characteristics have been described for the identification and subtyping of such strains. For identification of the monophasic 1,4,[5],12:i:- variant, it is advisable to proceed with serotyping until a first negative result of agglutination after flagellar phase inversion, and then apply a PCR protocol in order to confirm the lack of the second phase antigen. Other methods such as phage typing and genotyping methods (MLST, PFGE, VNTR, and virulotyping) are used to confirm relatedness to *S.* Typhimurium and/or to further subtype these isolates. Accurate characterisation of monophasic strains is important since misidentification of a non-*S.* Typhimurium-related strain could result in unnecessary regulatory action. Similarly, failure to confirm identity of a *S.* Typhimurium-like organism could have significant public health consequences.

- *To propose a harmonised terminology for reporting which allows trends-analyses, comparison between Member States and with humans isolates;*

It is difficult to monitor trends in the proliferation of monophasic strains because of the inconsistent way in which they have been reported by different organisations within and between EU MSs and internationally. To ensure complete consistency of reporting, all isolates of putative *Salmonella* should ideally be fully serotyped and the full antigenic formula reported, as recommended by the WHO Collaborating Centre for Reference and Research on *Salmonella* according to the White-Kauffman-Le Minor scheme, i.e., in the case of monophasic *S.* Typhimurium, 1,4,[5],12:i:-. Whenever possible, as much detail of the antigenic formula as determined by testing should be provided and reported.

If the full antigenic formula is not available but a phage type that is consistent with *S.* Typhimurium has been confirmed, and the lack of the second phase flagellar antigen has been verified by PCR, then the term ‘monophasic *S.* Typhimurium’ is recommended for reporting purposes in the current situation.

- *To indicate if these strains should be classified as variants of *Salmonella* Typhimurium or as a separate serotype;*

On the basis of genetic similarity and ability to obtain a recognised *Salmonella* Typhimurium phage type, we regard these emerging epidemic monophasic strains with the basic antigenic formula 1,4,[5],12:i:- as variants deriving from *S.* Typhimurium.

- *To assess the public health risk posed by these emerging strains, in particular to advise whether the public health risk, when detecting these strains in animals or food, should be considered similar, more or less important than (other) Salmonella Typhimurium strains.*

Monophasic *S. Typhimurium* strains have been shown to have similar virulence and antimicrobial resistance characteristics to strains of *S. Typhimurium*. Similar to what was observed in the past for epidemic clones of *S. Typhimurium*, recent studies in numerous countries worldwide confirm the rapid emergence and dissemination of such strains in food animals, companion animals and humans.

We therefore regard the public health risk posed by these emerging monophasic *S. Typhimurium* strains as comparable to that of other epidemic *S. Typhimurium* strains.

### **Additional conclusions**

Many of the clonal lines of monophasic *S. Typhimurium* described are multidrug resistant and exhibit resistance to long-standing and commonly used antimicrobials; a minority of isolates also present resistance to first-line antimicrobials used for therapy in humans, or carry virulence-resistance hybrid plasmids.

The cause of the recent emergence and rapid international spread of monophasic *S. Typhimurium* strains is unknown.

### **RECOMMENDATIONS**

In order to identify the emergence of new variants of *S. Typhimurium*, all *Salmonella* strains that could potentially be *S. Typhimurium* should be further typed by phage typing and /or molecular typing, referring the strains to a reference laboratory where necessary.

New molecular methods of identification are continuously under development and should be assessed in relation to the ability to characterise such strains as *S. Typhimurium*.

Antimicrobial susceptibility testing is important for epidemiological investigations. The antimicrobial resistance pattern should be determined and reported in a harmonised way for human, animal and food isolates, according to European guidelines.

It is important to monitor the further spread of such strains as much as possible, particularly in poultry breeding flocks, where to date they do not appear to be established in EU MSs.

## REFERENCES

- AFSSA, 2009. Opinion of the French Food Safety Agency concerning two draft amendments to Orders for controlling salmonellae in the *Gallus gallus* species. 2009-SA-0182, 22 pp.
- Agasan A, Kornblum J, Williams G, Pratt CC, Fleckenstein P, Wong M and Ramon A, 2002. Profile of *Salmonella enterica* subsp *enterica* (subspecies I) serotype 4,5,12 : i :- strains causing food-borne infections in New York City. *J Clin Microbiol*, 40, 1924-1929.
- Alcaine SD, Soyer Y, Warnick LD, Su WL, Sukhnanand S, Richards J, Fortes ED, McDonough P, Root TP, Dumas NB, Grohn Y and Wiedmann M, 2006. Multilocus sequence typing supports the hypothesis that cow- and human-associated *Salmonella* isolates represent distinct and overlapping populations. *Appl Environ Microbiol*, 72, 7575-7585.
- Amavisit P, Boonyawiwat W and Bangtrakulnont A, 2005. Characterization of *Salmonella enterica* serovar typhimurium and monophasic *Salmonella* serovar 1,4,[5],12 : i : - Isolates in Thailand. *J Clin Microbiol*, 43, 2736-2740.
- Anderson ES, Ward LR, Saxe MJ and de Sa JD, 1977. Bacteriophage-typing designations of *Salmonella typhimurium*. *J Hyg (Lond)*, 78, 297-300.
- Anonymous (International Organisation for Standardisation, Geneva, Switzerland), 2002. ISO 6579. Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp.
- Anonymous (International Organisation for Standardisation, Geneva, Switzerland), 2007a. EN ISO 6579: 2002/ Amd1. Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp. - Annex D: Detection of *Salmonella* spp. in animal faeces and in environmental samples from the primary production stage.
- Anonymous 2007b. Joint FAO/WHO/OIE Expert Meeting on Critically Important Antimicrobials. Report of a meeting held in Rome, Italy, 26-30 November 2007.
- Antunes P, Mourão J, Freitas A and Peixe L, 2010. Population structure of multidrug-resistant nontyphoidal *Salmonella enterica* isolates from Portugal. *Clin Microbiol Infect*, 16, S371-372.
- Bailey AM, Ivens A, Kingsley R, Cottell JL, Wain J and Piddock LJ, 2010. RamA, a member of the AraC/XylS family, influences both virulence and efflux in *Salmonella enterica* serovar Typhimurium. *J Bacteriol*, 192, 1607-1616.
- Barone dL, Dal Vecchio A, Pellissier N, Viganò A, Romani C and Pontello M, 2008. Emergence of *Salmonella* Typhimurium monophasic serovar: determinants of antimicrobial resistance in porcine and human strains. *Ann Ig*, 20, 199-209.
- Bedoui S, Kupz A, Wijburg OL, Walduck AK, Rescigno M and Strugnell RA, 2010. Different bacterial pathogens, different strategies, yet the aim is the same: evasion of intestinal dendritic cell recognition. *J Immunol*, 184, 2237-2242.
- Ben-Darif E, De Pinna E, Threlfall EJ, Bolton FJ, Upton M and Fox AJ, 2010. Comparison of a semi-automated rep-PCR system and multilocus sequence typing for differentiation of *Salmonella enterica* isolates. *J Microbiol Methods*, 81, 11-16.
- Best EL, Hampton MD, Ethelberg S, Liebana E, Clifton-Hadley FA and Threlfall EJ, 2009. Drug-resistant *Salmonella* Typhimurium DT 120: use of PFGE and MLVA in a putative international outbreak investigation. *Microb Drug Resist*, 15, 133-138.
- Biedzka-Sarek M and El Skurnik M, 2006. How to outwit the enemy: dendritic cells face *Salmonella*. *APMIS*, 114, 589-600.

- Bone A, Noel H, Le Hello S, Pihier N, Danan C, Raguenaud ME, Salah S, Bellali H, Vaillant V, Weill FX and Jourdan-da Silva N, 2010. Nationwide outbreak of *Salmonella enterica* serotype 4,12:i:- infections in France, linked to dried pork sausage, March-May 2010. *Euro Surveill*, 15,
- Boonmar S, Bangtrakulnonth A, Pornruangwong S, Samosornsuk S, Kaneko K and Ogawa M, 1998. Significant increase in antibiotic resistance of *Salmonella* isolates from human beings and chicken meat in Thailand. *Vet Microbiol*, 62, 73-80.
- Boyle EC, Bishop JL, Grassl GA and Finlay BB, 2007. *Salmonella*: from pathogenesis to therapeutics. *J Bacteriol*, 189, 1489-1495.
- Brabban AD, Hite E and Callaway TR, 2005. Evolution of foodborne pathogens via temperate bacteriophage-mediated gene transfer. *Foodborne Pathog Dis*, 2, 287-303.
- Briggs CE and Fratamico PM, 1999. Molecular characterization of an antibiotic resistance gene cluster of *Salmonella typhimurium* DT104. *Antimicrob Agents Chemother*, 43, 846-849.
- Bueno SM, Riedel CA, Carreno LJ and Kalergis AM, 2010. Virulence mechanisms displayed by *Salmonella* to impair dendritic cell function. *Curr Med Chem*, 17, 1156-1166.
- Cai HY, Lu L, Muckle CA, Prescott JF and Chen S, 2005. Development of a novel protein microarray method for serotyping *Salmonella enterica* strains. *J Clin Microbiol*, 43, 3427-3430.
- Callow BR, 1959. A new phage-typing scheme for *Salmonella typhi-murium*. *J Hyg (Lond)*, 57, 346-359.
- Cardona-Castro N, Sanchez-Jimenez M, Lavalett L, Munoz N and Moreno J, 2009. Development and evaluation of a multiplex polymerase chain reaction assay to identify *Salmonella* serogroups and serotypes. *Diagn Microbiol Infect Dis*, 65, 327-330.
- CDC 2007a. Investigation of Outbreak of Human Infections Caused by *Salmonella* I 4,[5],12:i:-. Centers for Disease Control and Prevention, Available from: <http://www.cdc.gov/Salmonella/4512eyeminus.html>
- CDC, 2007b. Turtle-Associated Salmonellosis in Humans - United States, 2006-2007. *MMWR*, 56, 649-652.
- CDC 2008. *Salmonella* Surveillance: Annual Summary, 2006. Atlanta, Georgia: US Department of Health and Human Services, CDC, 2008.
- Collignon P, Powers JH, Chiller TM, Aidara-Kane A and Aarestrup FM, 2009. World Health Organization ranking of antimicrobials according to their importance in human medicine: A critical step for developing risk management strategies for the use of antimicrobials in food production animals. *Clin Infect Dis*, 49, 132-141.
- Darwin KH and Miller VL, 1999. Molecular basis of the interaction of *Salmonella* with the intestinal mucosa. *Clin Microbiol Rev*, 12, 405-428.
- de la Torre E, Tello M, Mateu EM and Torre E, 2005. Use of biochemical kinetic data to determine strain relatedness among *Salmonella enterica* subsp. *enterica* isolates. *J Clin Microbiol*, 43, 43 (11) 5419-5424.
- de la Torre E, Zapata D, Tello M, Mejia W, Frias N, Pena FJG, Mateu EM and Torre E, 2003. Several *Salmonella enterica* subsp *enterica* serotype 4,5,12 : i: - Phage types isolated from swine samples originate from serotype typhimurium DT U302. *J Clin Microbiol*, 41, 2395-2400.
- del Cerro A, Soto SM and Mendoza MC, 2003. Virulence and antimicrobial-resistance gene profiles determined by PCR-based procedures for *Salmonella* isolated from samples of animal origin. *Food Microbiol*, 20, 20 (24) 431-438.

- Dionisi AM, Graziani C, Lucarelli C, Filetici E, Villa L, Owczarek S, Caprioli A and Luzzi I, 2009. Molecular Characterization of Multidrug-Resistant Strains of *Salmonella enterica* Serotype Typhimurium and Monophasic Variant (S. 4,[5], 12:i:-) Isolated from Human Infections in Italy. *Foodborne Pathog Dis*, 6, 711-717.
- Dong T and Schellhorn HE, 2009. Role of RpoS in virulence of pathogens. *Infect Immun*, 78, 887-897.
- Directive 2003/99/EC of the European Parliament and of the Council of 17 November 2003 on the monitoring of zoonoses and zoonotic agents, amending Council Decision 90/424/EEC and repealing Council Directive 92/117/EEC,
- Regulation (EC) No 2160/2003 of the European Parliament and of the Council of 17 November 2003 on the control of salmonella and other specified food-borne zoonotic agents,
- Commission Decision 2006/668/EC of 29 September 2006, concerning a financial contribution from the Community towards a baseline survey on the prevalence of *Salmonella* in slaughter pigs to be carried out in the Member States (notified under document number C(2006) 4306),
- Commission Decision 2007/516/EC of 19 July 2007 concerning a financial contribution from the Community towards a survey on the prevalence and antimicrobial resistance of *Campylobacter* spp. in broiler flocks and on the prevalence of *Campylobacter* spp. and *Salmonella* spp. in broiler carcasses to be carried out in the Member States (notified under document number C(2007) 3440),
- Commission Decision 2007/636/EC of 3 October 2007, concerning a financial contribution from the Community towards a survey on the prevalence of *Salmonella* spp. in herds of breeding pigs to be carried out in the Member States (notified under document number C(2007) 4434),
- Commission Decision of 28 April 2008 amending Decision 2002/253/EC laying down case definitions for reporting communicable diseases to the Community network under Decision No 2119/98/EC of the European Parliament and of the Council (notified under document number C(2008) 1589),
- ECDC 2009. Annual Epidemiological Report on Communicable Diseases in Europe 2009. Stockholm, European Centre for Disease Prevention and Control.
- Echeita MA, Aladuena A, Cruchaga S and Usera MA, 1999. Emergence and spread of an atypical *Salmonella enterica* subsp *enterica* serotype 4,5,12 : i :- Strain in Spain. *J Clin Microbiol*, 37, 3425-3425.
- Echeita MA, Herrera S and Usera MA, 2001. Atypical, fljB-negative *Salmonella enterica* subsp *enterica* strain of serovar 4,5,12 : i : appears to be a monophasic variant of serovar typhimurium. *J Clin Microbiol*, 39, 2981-2983.
- Eckmann L and Kagnoff MF, 2005. Intestinal mucosal responses to microbial infection. *Springer Semin Immunopathol*, 27, 181-196.
- EFSA, 2007a. Report of the Task Force on Zoonoses Data Collection on the Analysis of the baseline survey on the prevalence of *Salmonella* in broiler flocks of *Gallus gallus*, Part A. *The EFSA Journal*, 98, 1-85.
- EFSA, 2007b. Report of the Task Force on Zoonoses Data Collection on the Analysis of the baseline study on the prevalence of *Salmonella* in holdings of laying hen flocks of *Gallus gallus*. *The EFSA Journal*, 97,
- EFSA, 2008a. Report of the Task Force on Zoonoses Data Collection on the analysis of the baseline survey on the prevalence of *Salmonella* in slaughter pigs, Part A. *The EFSA Journal*, 135, 1-111.
- EFSA, 2008b. Report of the Task Force on Zoonoses Data Collection on the Analysis of the baseline survey on the prevalence of *Salmonella* in turkey flocks, Part A. *The EFSA Journal*, 134, 1-91.

- EFSA, 2008c. Working Group on Developing Harmonised Schemes for Monitoring Antimicrobial Resistance in Zoonotic Agents. Harmonised monitoring of antimicrobial resistance in *Salmonella* and *Campylobacter* isolates from food animals in the European Union. *Clin Microbiol Infect*, 14, 522-533.
- EFSA, 2009. Analysis of the baseline survey on the prevalence of *Salmonella* in holdings with breeding pigs, in the EU, 2008, Part A: *Salmonella* prevalence estimates. *The EFSA Journal*, 7, 93 pp.
- EFSA, 2010. Analysis of the baseline survey on the prevalence of *Campylobacter* in broiler batches and of *Campylobacter* and *Salmonella* on broiler carcasses in the EU, 2008, Part A: *Campylobacter* and *Salmonella* prevalence estimates. *The EFSA Journal*, 8, 99 pp.
- EFSA/ECDC, 2010. The Community Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from animals and food in the European Union in 2008. *The EFSA Journal*, 8, 261 pp.
- EUCAST 2010. The European Committee on Antimicrobial Susceptibility Testing - EUCAST. Available from: [www.eucast.org](http://www.eucast.org)
- Folster JP, Pecic G, Bolcen S, Theobald L, Hise K, Carattoli A, Zhao S, McDermott PF and Whichard JM, 2009. Characterization of extended-spectrum cephalosporin-resistant *Salmonella enterica* serovar Heidelberg isolated from humans in the United States. *Foodborne Pathog Dis*, 7, 181-187.
- Friedrich A, Dorn C, Schroeter A, Szabo I, Jaber M, Berendonk G, Brom M, Ledwolorz J and Helmuth R, 2010. [Report on *Salmonella* isolates in livestock, food and feed, received at the German national reference laboratory for *Salmonella* during 2004-2008]. *Berl Munch Tierarztl Wochenschr*, 123, 265-277.
- Garaizar, Porwollik S, Echeita A, Rementeria A, Herrera S, Wong RM-Y, Frye J, Usera MA and M M, 2003. DNA microarray-based typing of atypical monophasic *Salmonella* serovar (4,5,12:i:-) strains emergent in Spain. *Infect Genet Evol*, 2, 286-287.
- Garaizar J, Porwollik S, Echeita A, Rementeria A, Herrera S, Wong RMY, Frye J, Usera MA and McClelland M, 2002. DNA microarray-based typing of an atypical monophasic *Salmonella enterica* serovar. *J Clin Microbiol*, 40, 2074-2078.
- Garcia-Feliz C, Collazos JA, Carvajal A, Herrera S, Echeita MA and Rubio P, 2008. Antimicrobial resistance of *Salmonella enterica* isolates from apparently healthy and clinically ill finishing pigs in Spain. *Zoonoses and Public Health*, 55, 195-205.
- Garcia-Russell N, Elrod B and Dominguez K, 2009. Stress-induced prophage DNA replication in *Salmonella enterica* serovar Typhimurium. *Infect Genet Evol*, 9, 889-895.
- Gewirtz AT, Simon PO, Jr., Schmitt CK, Taylor LJ, Hagedorn CH, O'Brien AD, Neish AS and Madara JL, 2001. *Salmonella typhimurium* translocates flagellin across intestinal epithelia, inducing a proinflammatory response. *J Clin Invest*, 107, 99-109.
- Grimont PAD and Weill F-X (WHO Collaborating Centre for Reference and Research on *Salmonella*, Institute Pasteur, Paris, France), 2007. Antigenic formulae of the *Salmonella* serovars, 9th ed.
- Guard-Petter J, 2001. The chicken, the egg and *Salmonella enteritidis*. *Environ Microbiol*, 3, 421-430.
- Guerra B, Junker E, Miko A, Helmuth R and Mendoza MC, 2004. Characterization and localization of drug resistance determinants in multidrug-resistant, integron-carrying *Salmonella enterica* serotype typhimurium strains. *Microb Drug Resist*, 10, 83-91.
- Guerra B, Laconcha I, Soto SM, Gonzalez-Hevia MA and Mendoza MC, 2000. Molecular characterisation of emergent multiresistant *Salmonella enterica* serotype [4,5,12:i:-] organisms causing human salmonellosis. *FEMS Microbiol Lett*, 190, 341-347.



- Guerra B, Soto SM, Arguelles JM and Mendoza MC, 2001. Multidrug resistance is mediated by large plasmids carrying a class 1 integron in the emergent *Salmonella enterica* serotype [4,5,12 : i :-]. *Antimicrob Agents Chemother*, 45, 1305-1308.
- Guerri ML, Aladuena A, Echeita A and Rotger R, 2004. Detection of integrons and antibiotic-resistance genes in *Salmonella enterica* serovar Typhimurium, isolates with resistance to ampicillin and variable susceptibility to amoxicillin-clavulanate. *Int J Antimicro Ag*, 24, 327-333.
- Guibourdenche M, Roggentin P, Mikoleit M, Fields PI, Bockemuhl J, Grimont PA and Weill FX, 2009. Supplement 2003-2007 (No. 47) to the White-Kauffmann-Le Minor scheme. *Res Microbiol*, 161, 26-29.
- Gurakan GC, Aksoy C, Ogel ZB and Oren NG, 2008. Differentiation of *Salmonella typhimurium* from *Salmonella enteritidis* and other *Salmonella* serotypes using random amplified polymorphic DNA analysis. *Poult Sci*, 87, 1068-1074.
- Hackl E, Konrad-Koszler M, Kilian A, Wenzl P, Kornschöber C and Sessitsch A, 2009. Phage-type specific markers identified by Diversity Arrays Technology (DArT) analysis of *Salmonella enterica* ssp. *enterica* serovars Enteritidis and Typhimurium. *J Microbiol Methods*, 80, 100-105.
- Harker KS, Lane C, de Pinna E and Adak GK, 2011. An outbreak of *Salmonella* Typhimurium DT191a associated with reptile feeder mice. *Epidemiol Infect*, (in press),
- Hauser E, Huhn S, Junker E, Jaber M, Schroeter A, Helmuth R, Rabsch W, Winterhoff N and Malorny B, 2009. Characterisation of a phenotypic monophasic variant belonging to *Salmonella enterica* subsp. *enterica* serovar Typhimurium from wild birds and its possible transmission to cats and humans. *Berl Munch Tierarztl Wochenschr*, 122, 169-177.
- Hauser E, Tietze E, Blank K and Malorny B, 2010a. Comparative molecular typing of a monophasic *Salmonella enterica* serovar Typhimurium (4,[5],12:i:-) from the pig food chain to human. *ASM Conference on Salmonella: Biology, Pathogenesis and Prevention*, Aix-en-Provence, France,
- Hauser E, Tietze E, Helmuth R, Junker E, Blank K, Prager R, Rabsch W, Appel B, Fruth A and Malorny B, 2010b. *Salmonella enterica* serovar 4,[5],12:i:- contaminated pork is an emerging foodborne health risk for humans. *Appl Environ Microbiol*, AEM.02991-02909.
- Helaine S, Thompson JA, Watson KG, Liu M, Boyle C and Holden DW, 2010. Dynamics of intracellular bacterial replication at the single cell level. *Proc Natl Acad Sci U S A*, 107, 3746-3751.
- Hensel M, 2004. Evolution of pathogenicity islands of *Salmonella enterica*. *Int J Med Microbiol*, 294, 95-102.
- Hoelzer K, Soyer Y, Rodriguez-Rivera LD, Cummings KJ, McDonough PL, Schoonmaker-Bopp DJ, Root TP, Dumas NB, Warnick LD, Grohn YT, Wiedmann M, Baker KN, Besser TE, Hancock DD and Davis MA, 2010. The prevalence of multidrug resistance is higher among bovine than human *Salmonella enterica* serotype Newport, Typhimurium, and 4,5,12:i:- isolates in the United States but differs by serotype and geographic region. *Appl Environ Microbiol*, 76, 5947-5959.
- Hopkins KL, Kirchner M, Guerra B, Granier SA, Lucarelli C, Porrero MC, Jakubczak A, Threlfall EJ and Mevius DJ, 2010a. Multiresistant *Salmonella enterica* serovar 4,[5],12:i:- in Europe: a new pandemic strain? *Euro Surveill*, 15, 19580.
- Hopkins KL, Nair S, Kirchner M, Guerra B, Granier S, Lucarelli C, Porrero C, Jakubczak A, Threlfall EJ and Mevius D, 2010b. Genetic variation in emerging multidrug-resistant *Salmonella enterica* 4,[5],12:i:- from seven European countries. 110th General Meeting of the American Society for Microbiology, San Diego, CA, May 23-27,
- HPS, 2010. Gastro-intestinal and foodborne infections: Laboratory reports for common bacterial, protozoal and viral infections 2009. *HPS Weekly Report*, Volume 44 No.2010/05,

- Hueffer K and Galan JE, 2004. *Salmonella*-induced macrophage death: multiple mechanisms, different outcomes. *Cell Microbiol*, 6, 1019-1025.
- Ibarra JA and Steele-Mortimer O, 2009. *Salmonella*--the ultimate insider. *Salmonella* virulence factors that modulate intracellular survival. *Cell Microbiol*, 11, 1579-1586.
- Ikeda JS, Schmitt CK, Darnell SC, Watson PR, Bispham J, Wallis TS, Weinstein DL, Metcalf ES, Adams P, O'Connor CD and O'Brien AD, 2001. Flagellar phase variation of *Salmonella enterica* serovar Typhimurium contributes to virulence in the murine typhoid infection model but does not influence *Salmonella*-induced enteropathogenesis. *Infect Immun*, 69, 3021-3030.
- ISS 2010. ENTERNET *Salmonella*. Sorveglianza delle infezioni da *Salmonella*. Dati Storici dal 2007 al 2009. Available from: [www.iss.it/binary/salm/cont/4f\\_Dati\\_SALMONELLA\\_2007\\_2009.pdf](http://www.iss.it/binary/salm/cont/4f_Dati_SALMONELLA_2007_2009.pdf)
- Jones MA, Hulme SD, Barrow PA and Wigley P, 2007. The *Salmonella* pathogenicity island 1 and *Salmonella* pathogenicity island 2 type III secretion systems play a major role in pathogenesis of systemic disease and gastrointestinal tract colonization of *Salmonella enterica* serovar Typhimurium in the chicken. *Avian Pathol*, 36, 199-203.
- Jones TF, Ingram LA, Cieslak PR, Vugia DJ, Tobin-D'Angelo M, Hurd S, Medus C, Cronquist A and Angulo FJ, 2008. *Salmonellosis* outcomes differ substantially by serotype. *J Infect Dis*, 198, 109-114.
- Jones YE, McLaren IM and Wray C, 2000. Laboratory aspects of *Salmonella*. In: *Salmonella in Domestic Animals*. C Wray, A Wray. CABI Publishing, Wallingford, Oxon, UK,
- Kang MS, Besser TE, Hancock DD, Porwollik S, McClelland M and Call DR, 2006. Identification of specific gene sequences conserved in contemporary epidemic strains of *Salmonella enterica*. *Appl Environ Microbiol*, 72, 6938-6947.
- Kim S, Frye JG, Hu J, Fedorka-Cray PJ, Gautom R and Boyle DS, 2006. Multiplex PCR-based method for identification of common clinical serotypes of *Salmonella enterica* subsp. *enterica*. *J Clin Microbiol*, 44, 3608-3615.
- Kirk MD, Veitch MG and Hall GV, 2010. Gastroenteritis and food-borne disease in elderly people living in long-term care. *Clin Infect Dis*, 50, 397-404.
- Lan R, Stevenson G, Donohoe K, Ward L and Reeves PR, 2007. Molecular markers with potential to replace phage typing for *Salmonella enterica* serovar typhimurium. *J Microbiol Methods*, 68, 145-156.
- Laorden L, Bikandi J, Herrera-Leon S, Sanchez A, Echeita A, Rementeria A and Garaizar J, 2009. Characterisation and evolutionary study of *Salmonella enterica* [4,5,12:i:-] using PFGE, MLVA, PCR and sequencing techniques. ASM Conference on *Salmonella: Biology, Pathogenesis and Prevention*, Aix-en-Provence, France,
- Laorden L, Herrera-León S, Martínez I, Sanchez A, Kromidas L, Bikandi J, Rementeria A, Echeita A and Garaizar J, Genetic evolution of a Spanish multi-drug resistant *Salmonella enterica* 4,5,12:i:- monophasic variant. *J Clin Microbiol* (in press),
- Laorden L, Herrera-León S, Sanchez A, Bikandi J, Rementeria A, Echeita A and Garaizar J, 2010. Six types of deletions detected in monophasic *Salmonella enterica* 4, [5],12:i:- strains. International Meeting on Molecular Epidemiological Markers, Wernigerode, Germany,
- Lawson AJ, Dassama MU, Ward LR and Threlfall EJ, 2002. Multiply resistant (MR) *Salmonella enterica* serotype Typhimurium DT 12 and DT 120: a case of MR DT 104 in disguise? *Emerg Infect Dis*, 8, 434-436.
- Leader BT, Frye JG, Hu J, Fedorka-Cray PJ and Boyle DS, 2009. High-throughput molecular determination of *salmonella enterica* serovars by use of multiplex PCR and capillary electrophoresis analysis. *J Clin Microbiol*, 47, 1290-1299.

- Lee D, Kim S, Lee E, Park M, Oh H and Lee B, 2009. Prevalent and emerging *Salmonella* serovars from domestic residents and overseas travellers in Korea 2006-2008. Poster Abstract 77A. 109th General Meeting of the American Society for Microbiology, 18-19 May 2009, Philadelphia, Pennsylvania, USA,
- Lilic M and Stebbins CE, 2004. Re-structuring the host cell: up close with *Salmonella*'s molecular machinery. *Microbes Infect*, 6, 1205-1211.
- Lin JS and Tsen HY, 1999. Development and use of polymerase chain reaction for the specific detection of *Salmonella* Typhimurium in stool and food samples. *J Food Prot*, 62, 1103-1110.
- Litrup E, Torpdahl M, Malorny B, Huehn S, Christensen H and Nielsen EM, 2010. Association between phylogeny, virulence potential and serovars of *Salmonella enterica*. *Infect Genet Evol*, 10, 1132-1139.
- Lucarelli C, Dionisi AM, Torpdahl M, Villa L, Graziani C, Hopkins K, Threlfall J, Caprioli A and Luzzi I, 2010. Evidence for a second genomic island conferring multidrug resistance in a clonal group of strains of *Salmonella enterica* serovar Typhimurium and its monophasic variant circulating in Italy, Denmark, and the United Kingdom. *J Clin Microbiol*, 48, 2103-2109.
- Machado J and Bernardo F, 1990. Prevalence of *Salmonella* in chicken carcasses in Portugal. *J Appl Bacteriol*, 69, 477-480.
- MARAN 2010. Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in The Netherlands in 2008.
- McQuiston JR, Herrera-Leon S, Wertheim BC, Doyle J, Fields PI, Tauxe RV and Logsdon JM, Jr., 2008. Molecular phylogeny of the salmonellae: relationships among *Salmonella* species and subspecies determined from four housekeeping genes and evidence of lateral gene transfer events. *J Bacteriol*, 190, 7060-7067.
- Meakins S, Fisher IS, Berghold C, Gerner-Smidt P, Tschape H, Cormican M, Luzzi I, Schneider F, Wannett W, Coia J, Echeita A and Threlfall EJ, 2008. Antimicrobial drug resistance in human nontyphoidal *Salmonella* isolates in Europe 2000-2004: a report from the Enter-net International Surveillance Network. *Microb Drug Resist*, 14, 31-35.
- Molbak K, Baggesen DL, Aarestrup FM, Ebbesen JM, Engberg J, Frydendahl K, Gerner-Smidt P, Petersen AM and Wegener HC, 1999. An outbreak of multidrug-resistant, quinolone-resistant *Salmonella enterica* serotype typhimurium DT104. *N Engl J Med*, 341, 1420-1425.
- Mooijman KA, Veenman C and Maas HME (National Institute for Public Health and the Environment, Bilthoven, The Netherlands), 2008. Quality assurance of serotyping of *Salmonella* isolates obtained from the baseline study on slaughter pigs (2006-2007).
- Mooijman KA, Veenman C and Maas HME (National Institute for Public Health and the Environment, Bilthoven, the Netherlands), 2009. Quality assurance of serotyping of *Salmonella* isolates obtained from the baseline study on breeding pigs (2008).
- Moreira CG, Weinshenker D and Sperandio V, 2009. QseC mediates *Salmonella enterica* serovar typhimurium virulence in vitro and in vivo. *Infect Immun*, 78, 914-926.
- Mossong J, Marques P, Ragimbeau C, Huberty-Krau P, Losch S, Meyer G, Moris G, Strottner C, Rabsch W and Schneider F, 2007. Outbreaks of monophasic *Salmonella enterica* serovar 4,[5],12:i:- in Luxembourg, 2006. *Euro Surveill*, 12, 719.
- Parrenas R, Lockett J, Lysokowicz E, Fields P and McQuiston J, 2004. Molecular characterization of the fljB locus in monophasic *Salmonella* serotype I 4,5,12 : i : isolates. Abstracts of the General Meeting of the American Society for Microbiology, 104, 211-212.
- Patel JC, Rossanese OW and Galan JE, 2005. The functional interface between *Salmonella* and its host cell: opportunities for therapeutic intervention. *Trends Pharmacol Sci*, 26, 564-570.

- Peters T, Hopkins KL, Lane C, Nair S, Wain J and de Pinna E, 2010. Emergence and characterization of *Salmonella enterica* serovar Typhimurium phage type DT191a. *J Clin Microbiol*, 48, 3375-3377.
- Phalen DN, Drew ML, Simpson B, Roset K, Dubose K and Mora M, 2010. *Salmonella enterica* subsp. *Enterica* in Cattle Egret (*Bubulcus ibis*) chicks from central Texas: prevalence, serotypes, pathogenicity, and epizootic potential. *J Wildl Dis*, 46, 379-389.
- Pires SM and Hald T, 2010. Assessing the differences in public health impact of salmonella subtypes using a bayesian microbial subtyping approach for source attribution. *Foodborne Pathog Dis*, 7, 143-151.
- Pornruangwong S, Sriyapai T, Pulsrikarn C, Sawanpanyalert P, Boonmar S and Bangtrakulnonth A, 2008. The epidemiological relationship between *Salmonella enterica* serovar typhimurium and *Salmonella enterica* serovar 4,[5],12 : 1 :-isolates from humans and swine in Thailand. *Southeast Asian J Trop Med Publ Hlth*, 39, 288-296.
- Rabsch W, 2009. International emergence and trends of *S.Typhimurium* DT193. 2nd Annual Meeting of Food and Waterborne Diseases and Zoonoses Surveillance Network in Europe, 24-25 Sept 2009, Malta.,
- Rabsch W, Andrews HL, Kingsley RA, Prager R, Tschape H, Adams LG and Baumler AJ, 2002. *Salmonella enterica* serotype Typhimurium and its host-adapted variants. *Infect Immun*, 70, 2249-2255.
- Rabsch W, Ediberidze I, Trupschuch S and Laverde-Gomez J, 2008. Monophasic *Salmonella typhimurium* 4,5,12 : i :- of phage type DT193 carry a characteristic 18,65 kb island encoding 17 new open reading frames in the *thrW* locus. *Int J Med Microbiol*, 298, 72-72.
- Rabsch W and Laverde-Gomez J, 2007. Emergence of monophasic *Salmonella enterica* serovar 4,5,12 : i :-phage type DT193 in Germany with a characteristic 19kb island in the *thrW* locus. 59. Jahrestagung der Deutschen Gesellschaft fuer Hygiene und Mikrobiologie. *Int J Med Microbiol*, 297, 150.
- Rementeria A, Vivanco AB, Ramirez A, Hernando FL, Bikandi J, Herrera-Leon S, Echeita A and Garaizar J, 2009. Characterization of a Monoclonal Antibody Directed against *Salmonella enterica* Serovar Typhimurium and Serovar [4,5,12: i:-]. *Appl Environ Microbiol*, 75, 1345-1354.
- Rodriguez I, Rodicio MR, Herrera-Leon S, Echeita A and Mendoza MC, 2008. Class 1 integrons in multidrug-resistant non-typhoidal *Salmonella enterica* isolated in Spain between 2002 and 2004. *Int J Antimicro Ag*, 32, 158-164.
- Rowe B and Threlfall EJ, 1984. Drug resistance in gram-negative aerobic bacilli. *Br Med Bull*, 40, 68-76.
- Rychlik I, Gregorova D and Hradecka H, 2006. Distribution and function of plasmids in *Salmonella enterica*. *Vet Microbiol*, 112, 1-10.
- Sabbagh SC, Forest CG, Lepage C, Leclerc JM and Daigle F, 2010. So similar, yet so different: uncovering distinctive features in the genomes of *Salmonella enterica* serovars Typhimurium and Typhi. *FEMS Microbiol Lett*, 305, 1-13.
- Sala A, 2002. Tracing the origin of monophasic serovar *S. enterica* I 4, 5,12: I - - : Genetic similarity with *S. enterica* typhimurium. *Medicina (Ribeirao Preto)*, 35, 510-511.
- Santos RL, Raffatellu M, Bevins CL, Adams LG, Tukel C, Tsolis RM and Baumler AJ, 2009. Life in the inflamed intestine, *Salmonella* style. *Trends Microbiol*, 17, 498-506.
- Saroj SD, Shashidhar R, Karani M and Bandekar JR, 2008. Distribution of *Salmonella* pathogenicity island (SPI)-8 and SPI-10 among different serotypes of *Salmonella*. *J Med Microbiol*, 57, 424-427.

- Silverman M, Zieg J, Hilmen M and Simon M, 1979. Phase variation in *Salmonella*: genetic analysis of a recombinational switch. *Proc Natl Acad Sci U S A*, 76, 391-395.
- Soyer Y, Moreno A, Davis M, Maurer J, McDonough P, Warnick L, Grohn Y and Wiedmann M, 2008. Different Clones of *Salmonella* 4,5,12:I:- Isolates in Different Continents Causing Outbreaks. Abstracts of the General Meeting of the American Society for Microbiology, 108, 491.
- Soyer Y, Moreno Switt A, Davis MA, Maurer J, McDonough PL, Schoonmaker-Bopp DJ, Dumas NB, Root T, Warnick LD, Grohn YT and Wiedmann M, 2009. *Salmonella enterica* serotype 4,5,12:i:-, an emerging *Salmonella* serotype that represents multiple distinct clones. *J Clin Microbiol*, 47, 3546-3556.
- Stevens MP, Humphrey TJ and Maskell DJ, 2009. Molecular insights into farm animal and zoonotic *Salmonella* infections. *Philos Trans R Soc Lond B Biol Sci*, 364, 2709-2723.
- Sukhnanand S, Alcaine S, Warnick LD, Su WL, Hof J, Craver MP, McDonough P, Boor KJ and Wiedmann M, 2005. DNA sequence-based subtyping and evolutionary analysis of selected *Salmonella enterica* serotypes. *J Clin Microbiol*, 43, 3688-3698.
- Switt AI, Soyer Y, Warnick LD and Wiedmann M, 2009. Emergence, distribution, and molecular and phenotypic characteristics of *Salmonella enterica* serotype 4,5,12:i. *Foodborne Pathog Dis*, 6, 407-415.
- Tavechio AT, Fernandes SA, Ghilardi ACR, Soule G, Ahmed R and Melles CEA, 2009. Tracing lineage by phenotypic and genotypic markers in *Salmonella enterica* subsp *enterica* serovar 1,4,[5],12:i:- and *Salmonella* Typhimurium isolated in state of Sao Paulo, Brazil. *Memorias Do Instituto Oswaldo Cruz*, 104, 1042-1046.
- Tavechio AT, Ghilardi AC and Fernandes SA, 2004. "Multiplex PCR" identification of the atypical and monophasic *Salmonella enterica* subsp. *enterica* serotype 1,4,[5],12:i:- in Sao Paulo State, Brazil: frequency and antibiotic resistance patterns. *Rev Inst Med Trop Sao Paulo*, 46, 115-117.
- 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, 2010. Identification by PCR of non-typhoidal *Salmonella enterica* serovars associated with invasive infections among febrile patients in Mali. *PLoS Negl Trop Dis*, 4, e621.
- Threlfall EJ, 2000. Epidemic *Salmonella* Typhimurium DT 104--a truly international multiresistant clone. *J Antimicrob Chemother*, 46, 7-10.
- Threlfall EJ, Frost JA, Ward LR and Rowe B, 1994. Epidemic in cattle and humans of *Salmonella* typhimurium DT 104 with chromosomally integrated multiple drug resistance. *Vet Rec*, 134, 577.
- Tierrez A and Garcia-del Portillo F, 2005. New concepts in *Salmonella* virulence: the importance of reducing the intracellular growth rate in the host. *Cell Microbiol*, 7, 901-909.
- Torpdahl M, Litrup E and Nielsen EM, 2009. Characterisation and prevalence of *Salmonella* 4,5,12:i:- in Denmark, the monophasic variant of *Salmonella* Typhimurium. *ASM Conference on Salmonella: Biology Pathogenesis and Prevention*, Aix-en- Provence, France,
- Trupschuch S, Gomez JL, Ediberidze I, Flieger A and Rabsch W, 2009. Characterization of a novel 18.65 kb genomic island within the thrW tRNA locus of monophasic *Salmonella* typhimurium DT193 strains. *Int J Med Microbiol*, 299, 63-63.
- Trupschuch S, Laverde Gomez JA, Ediberidze I, Flieger A and Rabsch W, 2010. Characterisation of multidrug-resistant *Salmonella* Typhimurium 4,[5],12:i:- DT193 strains carrying a novel genomic island adjacent to the thrW tRNA locus. *Int J Med Microbiol*, 300, 279-288.
- Usera MA, Aladuena A, Gonzalez R, De la Fuente M, Garcia-Pena J, Frias N and Echeita MA, 2002. Antibiotic resistance of *Salmonella* spp. from animal sources in Spain in 1996 and 2000. *J Food Prot*, 65, 768-773.

- van Asten AJ and van Dijk JE, 2005. Distribution of "classic" virulence factors among *Salmonella* spp. *FEMS Immunol Med Microbiol*, 44, 251-259.
- van Pelt W. BMAH, Schimmer B., Stenvers O.F.J., Langelaar M.F.M. (National Institute for Public Health and the Environment), 2009. Staat van zoonosen 2007-2008. 64 pp.
- Vidal-Gallego AB, 2005. *Salmonelosis porcina: Monitorizacion y bases para su control*. Universidad de Leon, Leon.
- Vieira-Pinto M, Temudo P and Martins C, 2005. Occurrence of salmonella in the ileum, ileocolic lymph nodes, tonsils, mandibular lymph nodes and carcasses of pigs slaughtered for consumption. *J Vet Med B Infect Dis Vet Public Health*, 52, 476-481.
- VLA 2010. *Salmonella in Livestock Production in GB:2009*. VLA Publications, VLA Weybridge, Addlestone, UK.
- Vogel J, 2009. A rough guide to the non-coding RNA world of *Salmonella*. *Mol Microbiol*, 71, 1-11.
- Walker RA, Lindsay E, Woodward MJ, Ward LR and Threlfall EJ, 2001. Variation in clonality and antibiotic-resistance genes among multiresistant *Salmonella enterica* serotype typhimurium phage-type U302 (MR U302) from humans, animals, and foods. *Microb Drug Resist*, 7, 13-21.
- Wattiau P, Weijers T, Andreoli P, Schliker C, Veken HV, Maas HM, Verbruggen AJ, Heck ME, Wannet WJ, Imberechts H and Vos P, 2008. Evaluation of the Premi Test *Salmonella*, a commercial low-density DNA microarray system intended for routine identification and typing of *Salmonella enterica*. *Int J Food Microbiol*, 123, 293-298.
- Xu T, Maloy S and McGuire KL, 2009. Macrophages influence *Salmonella* host-specificity in vivo. *Microb Pathog*, 47, 212-222.
- Yoshida C, Franklin K, Konczy P, McQuiston JR, Fields PI, Nash JH, Taboada EN and Rahn K, 2007. Methodologies towards the development of an oligonucleotide microarray for determination of *Salmonella* serotypes. *J Microbiol Methods*, 70, 261-271.
- Zaidi MB, Leon V, Canche C, Perez C, Zhao S, Hubert SK, Abbott J, Blickenstaff K and McDermott PF, 2007. Rapid and widespread dissemination of multidrug-resistant bla<sub>CMY-2</sub> *Salmonella* Typhimurium in Mexico. *J Antimicrob Chemother*, 60, 398-401.
- Zamperini K, Soni V, Waltman D, Sanchez S, Theriault EC, Bray J and Maurer JJ, 2007. Molecular Characterization Reveals *Salmonella enterica* Serovar 4,[5],12:i:2-from Poultry Is a Variant Typhimurium Serovar. *Avian Dis*, 51, 958-964.

## APPENDICES

### A. MULTIPLEX POLYMERASE CHAIN REACTION (PCR) FOR IDENTIFICATION AND DIFFERENTIATION OF *S. TYPHIMURIUM* AND MONOPHASIC 4,[5],12:i:-

#### Introduction

In order to differentiate *Salmonella* strains belonging to the somatic antigen group “B” and sharing the same first flagellar antigen ‘i’, a multiplex PCR can be used to support the serological identification of the antigens.

This PCR uses two pairs of primers. Primers FFLIB and RFLIA amplify the *fliB*-*fliA* **intergenic region of the flagellin gene cluster. They amplify a 1-kb product from *S. Typhimurium* and *S. 4,[5],12:i:-* strains.** Primers Sense-59 and Antisense-83 amplify the *fljB* allele. These primers amplify a 1389 bp product from strains that possess a phase-2 flagellar antigen genetic determinant (*S. Typhimurium*) and no product from strains that lack these genes, such as *S. 4,[5],12:i:-*. So this multiplex PCR is able to discriminate between *S. Typhimurium*, for which it generates two amplicons, and monophasic strains, for which it generates only one amplicon. For the other serovars sharing the ‘i’ antigen, the PCR generates two amplicons: 250 bp and 1389 bp products (Figure 1). An example of each combination is represented in Figure 1.

#### Reference

Tennant S.M., Diallo S., Levy H., Livio S., Sow S.O., Tapia M., Fields P.I., Mikoleit M., Tamboura B., Kotloff K.L., Nataro J.P., Galen J.E. and Levine M.M. (2010) Identification by PCR of Non-typhoidal *Salmonella enterica* Serovars Associated with Invasive Infections among Febrile Patients in Mali. PLOS Neglected Tropical Diseases.

#### Methods

##### DNA Extraction

- Spread a strain of *Salmonella* spp. on a non selective agar tube
- Incubate the tube at 37°C ± 1°C for 18-24 hours
- Add 400µl of nuclease free water in a 1,5ml or 2ml dnase free tube and suspend the bacteria
- Mix the bacterial cells (e.g. using an agitator)
- Read absorbance at 600nm. Correct absorbance to be comprised between 1 and 2.
- Incubate the tube at 100°C for 15min
- Centrifuge at 20°C to 25°C for 5 min. at 10,000 g
- Collect the supernatant and store at -20°C
- Nucleic acid extracts that have been shown to cause PCR inhibition, indicated by appropriate controls, shall be purified before use in PCR.

##### Detection

- Prepare a PCR reaction using a Taq polimerase of high fidelity in a sterile tube as shown in Table 1:

**Table 1:** Reagents concentration for the PCR

Reagents	Final concentration
Buffer Taq	1X
MgCl <sub>2</sub>	2,5 mM
dNTPs	0.6 mM
Primer FFLIB	0.1 µM
Primer RFLIA	0.1 µM
Primer sense-59	1.0 µM
Primer antisense-83	1.0 µM
High fidelity Taq Polimerase	1 U
H <sub>2</sub> O	to adjust to a final 25 µl volume reaction

- Mix vigorously the DNA templates (e.g. using an agitator)
- Add 5µl of DNA and spin tubes.
- Prepare a negative control using all the PCR reagents with the exception of the DNA template
- The amplification is performed in a thermocycler using the following conditions:

Pre-denaturation: 95°C for 2 min;

Amplification (30 cycles): 95°C for 30 sec, 64°C for 30 sec, 72°C for 1min30 sec;

Post-extension: 72°C for 10 min.

**Table 2:** Sequences of primers (Tennant et al., 2010)

Primer FFLIB	5'-CTGGCGACGATCTGTCGATG-3'
Primer RFLIA	5'-GCGGTATACAGTGAATTCAC-3'
Primer Sense-59	5'-CAACAACAACCTGCAGCGTGTGCG-3'
Primer Antisense-83	5'-GCCATATTTTCAGCCTCTCGCCCG-3'

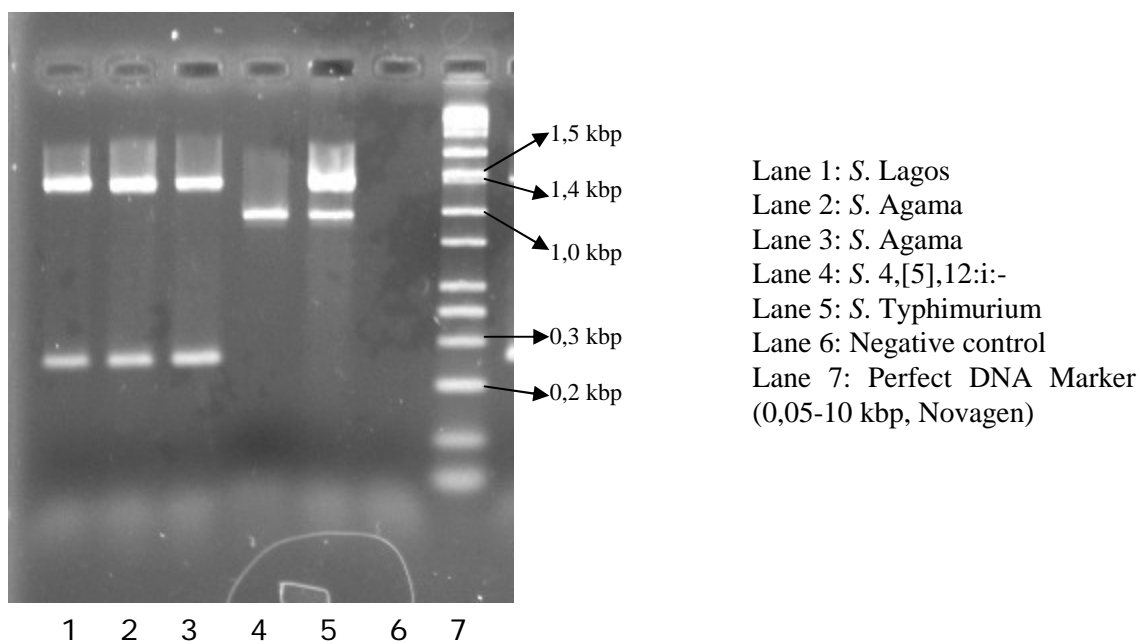
The PCR products are visualised by gel electrophoresis in 2% agarose gel after staining with DNA intercalating dyes (e.g. ethidium bromide solution) and under a UV light source. The amplicons sizes are estimated by comparison with bands of a DNA ladder.

The expected sizes of amplicons are:

- *S. Typhimurium*: 1389 bp and 1000 bp
- 4,[5],12:i:-: 1000 bp
- Other strains sharing the ‘i’ antigen: 1389 bp and 250 bp



**Figure 1:** Expected amplicons for different *Salmonella* serovars



## B. *SALMONELLA* VIRULENCE ATTRIBUTES

The severity and persistence of *Salmonella* infection is determined by characteristics of both the host and pathogen (van Asten and van Dijk, 2005). Host factors include those that affect the intestinal barrier, such as age (Kirk et al., 2010), stomach acid, diet, composition of the intestinal flora, immune status, and stress levels (Moreira et al., 2009), whereas the success of the pathogen relates to the dose of exposure and a strain-specific complex array of RNA-mediated molecular mechanisms, especially virulence factors (Boyle et al., 2007; Eckmann and Kagnoff, 2005; Stevens et al., 2009; Vogel, 2009) that are still not fully understood despite hundreds of studies and scientific papers on the subject.

Excretion of large numbers of organisms leading to rapid spread of infection has been a feature of various *S. Typhimurium* epidemics in cattle (Threlfall et al., 1994) leading to a high infective dose, often combined with mechanisms to assist survival in the protective gastric acid environment of the crop or stomach (Boyle et al., 2007). Once the gastric barrier has been breached *Salmonella* possesses an arsenal of molecular attributes that enable it to adhere to intestinal mucosa (Darwin and Miller, 1999; Patel et al., 2005) and subvert host cellular functions. *Salmonella* Typhimurium has a variety of fimbrial operons that code for different adhesion mechanisms, as well as 5-10 randomly positioned flagella, coded by *fliC* (phase 1 flagella) or *fliB* (phase 2 – absent in most monophasic strains). Flagella (Parrenas et al., 2004) are also thought to be important in pathogenesis of many strains, but some major pathogens, e.g. *S. Gallinarum* in poultry, are aflagellate. This variability appears to be typical of *Salmonella*, in which combinations of other virulence mechanisms can compensate for absence of specific factors (Jones et al., 2007; van Asten and van Dijk, 2005). Once an association with intestinal M cells has been achieved *Salmonella* exploits host cellular intestinal inflammatory responses to suppress competitive organisms (Santos et al., 2009) and induce its internalisation into intracellular *Salmonella*-containing vacuoles (Tierrez and Garcia-del Portillo, 2005). Six serovars typically harbour virulence plasmids of 60-95 kb that contain the *spv* locus that codes for proteins involved in intracellular multiplication. These are: Typhimurium, Gallinarum, Gallinarum biovar Pullorum, Enteritidis, Dublin, Choleraesuis and Abortusovis. Not all strains within these serovars contain a virulence plasmid but such strains may still be pathogenic (Rychlik et al., 2006). The typical virulence plasmid of *S. Typhimurium* (pSLT90), is about 90-95 kb, and belongs to the F<sub>II</sub> incompatibility group. Many monophasic ‘*S. Typhimurium*-like’ strains do not possess this virulence plasmid but are still highly represented in human disease (Switt et al., 2009). Virulence plasmids contain some of the genes that are involved in intracellular survival and multiplication as a facultative intracellular pathogen (Tierrez and Garcia-del Portillo, 2005) but an array of other mechanisms are also involved. In some monophasic *S. Typhimurium* isolates, although the virulence plasmid is lacking, genes belonging to it (i.i. *spv* genes) are present.

There are at least twelve chromosomally-encoded *Salmonella* Pathogenicity Islands (SPIs), the occurrence of which varies between serovars and strains (Hensel, 2004). Pathogenicity Islands can transfer between bacteria of different genera, leading to an accumulation of different virulence mechanisms in some strains (Saroj et al., 2008). SPI-1 codes for a type III secretion system that is present in all *Salmonella* and mediates cell invasion processes via proteins such as SptP and SopE; SPI-2 is only present in members of *S. enterica* and encodes other type III secretion systems that are involved in systemic pathogenesis; SPI-3 is conserved in *S. Typhi* and *S. Typhimurium*, but is variable in other serovars, and is involved in magnesium ion uptake systems that facilitate intracellular survival; SPI-4 has unknown functionality but a similar serovar distribution; SPI-5 is responsible for a varied range of characteristics that are not well characterised; SPI-6 (otherwise known as SCI in *S. Typhimurium*) is involved in maintenance of fimbriae and invasion genes; SPI-7 codes for the Vi toxin antigen that is only found in *S. Typhi*, *S. Paratyphi C* and *S. Dublin*, and SPI-8 is specific for *S. Typhi*; SPI-9 is present in *S. Typhi*, and as a pseudogene in *S. Typhimurium* and SPI-10 is responsible for *sef* fimbriae of different types in *S. Typhi* and *S. Enteritidis*. *Salmonella* Genomic Island 1 has been associated with some worldwide pandemic strains, especially *S. Typhimurium* DT104 and *S. Agona*. It has multiple functions and is usually linked with multiple antimicrobial resistance genes. Most

pathogenicity islands are associated with transfer RNA loci and acquisition of a new SPI has often been involved in the emergence of new multiple resistant epidemic strains (Brabban et al., 2005; Garcia-Russell et al., 2009; Hensel, 2004). Once inside the cell *Salmonella* subverts the host cellular process to avoid recognition (Biedzka-Sarek and El Skurnik, 2006) and slows down its own metabolic processes so that it can survive long term within macrophages (Bedoui et al., 2010; Bueno et al., 2010; Helaine et al., 2010; Hueffer and Galan, 2004; Ibarra and Steele-Mortimer, 2009; Lilibic and Stebbins, 2004; Xu et al., 2009). The generalised stress response RpoS, triggered by the stationary phase of growth, is also important for intracellular survival (Dong and Schellhorn, 2009). After invasion of epithelial cells and gut-associated lymphoid tissue *Salmonella* is carried to mesenteric lymph nodes, particularly ileo-caecal lymph nodes, where it may persist for long periods in some cases, even when no *Salmonella* can be found in intestinal samples. Pathogenic strains are able to multiply in host cells, escape from macrophage cells by inducing apoptosis and spread beyond the lymph nodes to cause septicaemia (Stevens et al., 2009). During multiplication of some strains pathogenicity is further enhanced by production of exotoxins, including an outer membrane cytotoxin in *S. Typhimurium*, and endotoxins, derived from the Lipid A component of the bacterial cell wall that may trigger a variety of host responses. Haemolysins are also produced by some strains of *S. Typhimurium*, *S. Enteritidis* and *S. Typhi*. It is clear that multiple virulence mechanisms are well represented in many strains of *S. Typhimurium* but full characterisation of individual strains is very time consuming and costly so each study only supplies a small piece of the virulence jigsaw for the particular strains involved in the study. Occurrence of genes is also only part of the picture as quantitative differences in the simultaneous or sequential expression of genes under different conditions is also vitally important and is very difficult to study in-vivo. High throughput full genome sequencing is likely to contribute considerably to the fund of knowledge of *Salmonella* virulence in the future (Sabbagh et al., 2010; Stevens et al., 2009).

To summarise, the virulence of *Salmonella* is very complex and varies hugely between serovars and strains within a serovar. Some serovars, e.g. *S. Gallinarum* are host-specific and can only survive in macrophages of that host. Other serovars, e.g. *S. Choleraesuis* or *S. Dublin*, are host adapted but can still occasionally infect other hosts and cause serious disease in humans. The majority of serovars can infect any host, but some, such as *S. Enteritidis*, are more likely to be found in a specific host because of certain special properties, e.g. ovarian transmission. Most *Salmonella* are either good survivors in the environment and able to multiply outside the host, or are able to exist long-term within carrier animals. Some strains have shown epidemic potential, e.g. *S. Typhimurium* DT 104, but others are endemic with more limited potential to spread. Infection of the host is normally by the faecal-oral route and the organism must survive the acidic conditions in the crop or stomach, and be able to compete with intestinal flora to multiply and invade lymphoid tissue. Colonisation, invasion and intracellular survival involves the complex interaction of numerous and variable virulence mechanisms that are still not fully understood despite decades of research (Stevens et al., 2009), but the adaptability and variability of *Salmonella* ensures that there is a suitable strain available to colonise any host, given the opportunity.

**C. SUMMARY OF MONOPHASIC *SALMONELLA* IN COMMUNITY ZOOSES MONITORING AND BASELINE STUDIES**

• **Community Zoonoses Monitoring**

Summary of reported monophasic *Salmonella* Typhimurium strains in food, animals and feed, Community zoonoses monitoring, EU, 2004-2008.

Serovar	Number of isolates	% of <i>Salmonella</i> spp. isolates	MSs	Non-MS
S. 1,4,5,12:i:-	128	4.7	EE, ES, IT, LU	
S. 4,12:i:-	40	1.5	BE, CZ, FI, EE, IT, SK, PT	CH
S. 4,5:i:-	38	1.4	IT, PT	
S. 4,5,12:i:-	11	0.4	IT	
S. group B, monophasic strain	10	0.4	AT	
S. 4,5,12:-:-	4	0.1	BE	
S. group B H-	2	0.1	AT	
S. 1,4,5,12:-:1,2	1	0	EE	
S. 4,5,12:-:1,2	1	0	IT	

• **Baseline studies**

1. **Report on the Analysis of the baseline study on the prevalence of *Salmonella* in holdings of laying hen flocks of *Gallus gallus* (2007).**

[www.efsa.europa.eu/en/scdocs/doc/97r.pdf](http://www.efsa.europa.eu/en/scdocs/doc/97r.pdf)

No monophasic *Salmonella* reported.

2. **Report of the Task Force on Zoonoses Data Collection on the Analysis of the baseline survey on the prevalence of *Salmonella* in broiler flocks of *Gallus gallus*, in the EU, 2005-2006 (2007). Part A: *Salmonella* prevalence estimates.**

[www.efsa.europa.eu/en/scdocs/doc/98r.pdf](http://www.efsa.europa.eu/en/scdocs/doc/98r.pdf)

No monophasic *S. Typhimurium* strains were reported by MSs. Cyprus and Denmark had one positive flock each to “*Salmonella* Group B” and Germany reported 20 flocks testing positive to “*Salmonella* Group B”.

3. **Report of the Task Force on Zoonoses Data Collection on the Analysis of the baseline survey on the prevalence of *Salmonella* in turkey flocks, in the EU, 2006-2007 (2008). Part A: *Salmonella* prevalence estimates.** [www.efsa.europa.eu/en/scdocs/doc/134r.pdf](http://www.efsa.europa.eu/en/scdocs/doc/134r.pdf)

(Data in Annex:

[www.efsa.europa.eu/en/scdocs/doc/zoon\\_report\\_ej134\\_finturkeys\\_annexes\\_en.pdf](http://www.efsa.europa.eu/en/scdocs/doc/zoon_report_ej134_finturkeys_annexes_en.pdf))

Frequency distribution of *Salmonella* serovars in fattening turkey flocks in EU and Norway, 2006-2007, by Member State

Country	Number of flocks		
	Sampled	Positive for <i>Salmonella</i>	Positive for “Monoph. strain of Group B”
Austria	202	35	1

Additionally, Cyprus and Germany had three positive flocks each to “*Salmonella* Group B”. Italy reported 5 positive flocks to *S. enterica subsp. enterica*.

Frequency distribution of *Salmonella* serovars in breeding turkey flocks in EU and Norway, 2006-2007: No monophasic *Salmonella* serovars were reported in this baseline study.

4. **Report of the Task Force on Zoonoses Data Collection on the analysis of the baseline survey on the prevalence of *Salmonella* in slaughter pigs, in the EU, 2006-2007 (2008). Part A: *Salmonella* prevalence estimates. [www.efsa.europa.eu/en/scdocs/doc/135r.pdf](http://www.efsa.europa.eu/en/scdocs/doc/135r.pdf) (Data in Annex: [www.efsa.europa.eu/en/scdocs/doc/135rax1.pdf](http://www.efsa.europa.eu/en/scdocs/doc/135rax1.pdf))**

Frequency distribution of isolated *Salmonella* serovars from lymph nodes in the slaughter pigs baseline survey, in the EU and Norway, 2006-2007

Serovar reported	Total number of lymph node samples	Positive for <i>Salmonella</i>	Positive for the monophasic serovar	No. countries where found
<i>S.</i> 4,[5],12:i:-	19,071	2,600	128	8

Frequency distribution of *Salmonella* serovars in lymph node samples of slaughter pigs, in the EU and Norway, 2006-2007, by Member State

Country	Total number of lymph node samples	Positive for <i>Salmonella</i>	Positive for the monophasic serovar	Serovar reported
Cyprus	359	47	1	<i>S.</i> 4,[5],12:i:-
Denmark	998	80	1	<i>S.</i> 4,[5],12:i:-
Spain	2,619	806	97	<i>S.</i> 4,[5],12:i:-
Greece	345	73	2	<i>S.</i> 4,[5],12:i:-
Hungary	658	76	4	<i>S.</i> 4,[5],12:i:-
The Netherlands	1,087	92	2	<i>S.</i> 4,[5],12:i:-
Portugal	658	156	17	<i>S.</i> 4,[5],12:i:-
UK	639	139	4	<i>S.</i> 4,[5],12:i:-

In addition, some countries had isolates under the following headings:

- Incomplete serotyping, *S. enterica*: Greece (1 isolate).
- Incomplete serotyping, *S. enterica subsp. enterica*: Bulgaria (4), Cyprus (3), Estonia (4), Greece (8), Italy (6), The Netherlands (2).
- Incomplete serotyping, *Salmonella* Group B: Belgium (1), Cyprus (2), Germany (64), Denmark (1), The Netherlands (1).

Frequency distribution of isolated *Salmonella* serovars from carcass swabs in the slaughter pigs baseline survey, in 13 MSs, 2006-2007

Serovar reported	Total number of carcass swab samples	Positive for <i>Salmonella</i>	Positive for the monophasic serovar	No. countries where found
<i>S.</i> 4,[5],12:i:-	5,736	387	5	1
<i>S.</i> 4,5,12:-:1,2	5,736	387	1	1

Frequency distribution of *Salmonella* serovars in carcass swabs of slaughter pigs in the 13-MSs group, 2006-2007, by Member State

Country	Total number of carcass swab samples	Positive for <i>Salmonella</i>	Positive for the monophasic serovar	Serovar reported
UK	641	97	5	<i>S.</i> 4,[5],12:i:-
UK	641	97	1	<i>S.</i> 4,5,12:-:1,2

In addition, some countries had isolates under the following headings:

- Incomplete serotyping, *S. enterica subsp. enterica*: Cyprus (1 isolate).
- Incomplete serotyping, *Salmonella* Group B: Cyprus (1).

5. Analysis of the baseline survey on the prevalence of *Salmonella* in holdings with breeding pigs in the EU, 2008 (2009). Part A: *Salmonella* prevalence estimates.

[www.efsa.europa.eu/en/scdocs/doc/1377.pdf](http://www.efsa.europa.eu/en/scdocs/doc/1377.pdf)

Breeding holdings:

Frequency distribution of isolated *Salmonella* serovars in breeding holdings, ranked by positive holdings, *Salmonella* EU baseline survey, 2008

Serovar reported	Number of holdings			Countries where serovar found
	Total sampled	Positive for <i>Salmonella</i>	Positive for monophasic serovar	
<i>S.</i> 4,12:i:-	1,530	452	4	3
<i>S.</i> 4,5,12:i:-	1,530	452	6	4

Frequency distribution of *Salmonella* serovars in breeding holdings, *Salmonella* EU baseline survey, 2008, by Member State

Country	Number of holdings			Serovar reported
	Total sampled	Positive for <i>Salmonella</i>	Positive for monophasic serovar	
Germany	46	13	1	<i>S.</i> 4,12:i:-
Spain	150	96	2	<i>S.</i> 4,12:i:-
Switzerland	71	11	1	<i>S.</i> 4,12:i:-
The Netherlands	109	63	1	<i>S.</i> 4,5,12:i:-
Portugal	33	15	2	<i>S.</i> 4,5,12:i:-
Spain	150	96	2	<i>S.</i> 4,5,12:i:-
UK	67	35	1	<i>S.</i> 4,5,12:i:-

In addition, Italy reported an unusually high number of isolates under the “*Salmonella* untypeable” heading (29/62 total *Salmonella* positive). Maximum was 2 for other MSs reporting them.

Production holdings:

Frequency distribution of isolated *Salmonella* serovars in production holdings, ranked by positive holdings, *Salmonella* EU baseline survey, 2008

Serovar reported	Number of holdings			Countries where serovar found
	Total sampled	Positive for <i>Salmonella</i>	Positive for monophasic serovar	
<i>S.</i> 4,12:i:-	3,278	950	6	5
<i>S.</i> 4,5,12:-:-	3,278	950	1	1
<i>S.</i> 4,5,12:i:-	3,278	950	15	7

Frequency distribution of *Salmonella* serovars in production holdings, *Salmonella* EU baseline survey, 2008, by Member State

Country	Number of holdings			Serovar reported
	Total sampled	Positive for <i>Salmonella</i>	Positive for monophasic serovar	
Czech Republic	160	25	1	S. 4,12:i:-
Germany	155	32	1	S. 4,12:i:-
Poland	157	17	1	S. 4,12:i:-
Spain	209	111	2	S. 4,12:i:-
UK	191	84	1	S. 4,12:i:-
Portugal	131	58	1	S. 4,5,12:-:-
Estonia	28	1	1	S. 4,5,12:i:-
Germany	155	32	2	S. 4,5,12:i:-
The Netherlands	212	118	7	S. 4,5,12:i:-
Poland	157	17	1	S. 4,5,12:i:-
Portugal	131	58	1	S. 4,5,12:i:-
Spain	209	111	1	S. 4,5,12:i:-
UK	191	84	2	S. 4,5,12:i:-

In addition, Italy reported an unusually high number of isolates under the “*Salmonella* untypeable” heading (92/213 total *Salmonella* positive). Maximum was 11 for other MSs reporting them.

6. **Analysis of the baseline survey on the prevalence of *Campylobacter* in broiler batches and of *Campylobacter* and *Salmonella* on broiler carcasses in the EU\*, 2008 (2010). Part A: *Campylobacter* and *Salmonella* prevalence estimates.**

[www.efsa.europa.eu/en/scdocs/doc/1503.pdf](http://www.efsa.europa.eu/en/scdocs/doc/1503.pdf)

Frequency distributions of *Salmonella* serovars detected on contaminated broiler carcasses in the EU, 2008

Serovar reported	Total number of carcasses sampled	Positive for <i>Salmonella</i>	Positive for the monophasic serovar	No. countries where found
S. 4,[5],12:i:-	10,035	1,225	15	4

Frequency distributions of *Salmonella* serovars detected on contaminated broiler carcasses, by country, 2008, by Member State

Country	Total number of samples	Positive for <i>Salmonella</i>	Positive for the monophasic serovar	Serovar reported
Germany	432	76	1	S. 4,[5],12:i:-
Malta	367	77	12	S. 4,[5],12:i:-
Switzerland	390	10	1	S. 4,[5],12:i:-
The Netherlands	429	43	1	S. 4,[5],12:i:-

\*Greece did not participate in the baseline survey

Additionally, Belgium, Italy, Lithuania and Malta reported a high number of isolates under the “*Salmonella* untypeable” heading (15/77, 13/66, 15/26 and 10/77 total *Salmonella* positive). Maximum was 1 for other MSs reporting them.

## GLOSSARY AND ABBREVIATIONS

### Member States of the European Union, 2010

Member State	ISO Country Abbreviations
Austria	AT
Belgium	BE
Bulgaria	BG
Cyprus	CY
Czech Republic	CZ
Denmark	DK
Estonia	EE
Finland	FI
France	FR
Germany	DE
Greece	GR
Hungary	HU
Ireland	IE
Italy	IT
Latvia	LV
Lithuania	LT
Luxembourg	LU
Malta	MT
The Netherlands	NL
Poland	PL
Portugal	PT
Slovakia	SK
Slovenia	SI
Spain	ES
Romania	RO
Sweden	SE
United Kingdom	UK