# Multiresistant Salmonella enterica serovar 4,[5],12:i:- in Europe: a new pandemic strain?

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A marked increase in the prevalence of *S. enterica* serovar 4,[5],12:i:- with resistance to ampicillin, streptomycin, sulphonamides and tetracyclines (R-type ASSuT) has been noted in food-borne infections and in pigs/pig meat in several European countries in the last ten years. One hundred and sixteen strains of *S. enterica* serovar 4,[5],12:i:- from humans, pigs and pig meat isolated in England and Wales, France, Germany, Italy, Poland, Spain and the Netherlands were further subtyped by phage typing, pulsed-field gel electrophoresis and multilocus variable number tandem repeat analysis to investigate the genetic relationship among strains. PCR was performed to identify the *fljB* flagellar gene and the genes encoding resistance to ampicillin, streptomycin, sulphonamides and tetracyclines. Class 1 and 2 integrase genes were also sought. Results indicate that genetically related serovar 4,[5],12:i:- strains of definitive phage types DT193 and DT120 with ampicillin, streptomycin, sulphonamide and tetracycline resistance encoded by $bla_{TEM}$, *strA-strB*, *sul2* and *tet*(B) have emerged in several European countries, with pigs the likely reservoir of infection. Control measures are urgently needed to reduce spread of infection to humans via the food chain and thereby prevent the possible pandemic spread of serovar 4,[5],12:i:- of R-type ASSuT as occurred with *S.* Typhimurium DT104 during the 1990s.

## Introduction

Infections with *Salmonella enterica* account for the second largest burden of bacterial gastrointestinal disease in the European Union (EU) [1]. The majority of *Salmonella* infections result in mild, self-limited illness and may not require treatment with antimicrobials. Nevertheless treatment with an appropriate antimicrobial can be life-saving in immunocompromised patients and in invasive disease, such as *Salmonella* bacteraemia and meningitis.

Serotyping according to the Kauffmann-White scheme is a widely used method for the initial characterisation of *Salmonella* isolates and is based on the antigenic variability of the somatic (O) and flagellar (H) antigens present in the cell wall of the organism [2]. Despite identification of more than 2,500 different serovars, the majority of cases of human infection are caused by a limited number of serovars. Most serovars are biphasic and express two distinct flagellar antigens encoded by *fliC* (phase-1 flagellin) and *fljB* (phase-2 flagellin). However, some serovars fail to express either the phase-1 or phase-2 flagellar antigen, therefore are classed as monophasic.

*S. enterica* serovar 4,[5],12:i:- is considered a monophasic variant of serovar Typhimurium (4,[5],12:i:1,2) due to antigenic and genotypic similarities between the two serovars [3,4]. Serovar Typhimurium is the second most common serovar associated with human cases of *Salmonella* infection in the EU [1]. In contrast isolates of serovar 4,[5],12:i:- were rarely identified before the mid-1990s but are now among the top 10 most common serovars isolated from humans in several countries [3-8]. According to Enter-net data this serovar was the fourth most common serovar in confirmed cases of human salmonellosis in the EU in 2006 [1]. Cases of infection with serovar 4,[5],12:i:- have reportedly been severe, with a 70% hospitalisation rate during an outbreak in New York City in 1998 [9], although a much lower rate of 21% was observed during an outbreak in Luxembourg in 2006 [6]. Infections have also been particularly associated with cases of septicaemia in Thailand and Brazil [7,10]. Overall, cases of infection have been linked to a number of sources, including

poultry and cattle, but particularly pigs and pork products [4,6,10-13]. Serovar 4,[5],12:i:- was among the top 10 most common serovars isolated from both pigs and pig meat in the EU in 2006 [1].

A marked increase in prevalence of *S. enterica* serovar 4,[5],12:i:- with resistance to ampicillin, streptomycin, sulphonamides and tetracyclines (R-type ASSuT) has been noted both in food-borne infections and in pigs/pig meat in several European countries over the last ten years [6,8,14,15]. In the baseline study from fattening pigs (Commission Decision 2006/668/EC), Spanish strains of *S. enterica* serovar 4,[5],12:i:- represented 14.3% of the isolates, 52.5% of which were of R-type ASSuT (VISAVET *Salmonella* database, unpublished data). In England and Wales cases of serovar 4,[5],12:i:- infection have risen from 47 in 2005 to 151 in 2009 (a 321% increase) against a backdrop of an overall decrease in the number of salmonellosis cases, with R-type ASSuT accounting for approximately 30% of these strains (Health Protection Agency (HPA) *Salmonella* database, unpublished data). In France isolations of serovar 4,[5],12:i:- increased from 99 to 410 between 2005 and 2008 to become the third most common serovar isolated from humans, with 62% of strains in 2007 being of R-type ASSuT [16]. In Italy cases of serovar 4,[5],12:i:- infection have risen from 59 in 2003 to 641 in 2009, with 75% of monophasic strains isolated in 2009 belonging to R-type ASSuT (with or without additional resistances) (Istituto Superiore di Sanità *Salmonella* database, unpublished data). A recent study described emergence of a clonal group of serovar Typhimurium and 4,[5],12:i:- R-type ASSuT strains in Italy, Denmark and the United Kingdom (UK) [17]. Resistance genes $bla_{TEM-1}$, *strA-strB*, *sul2* and *tet*(B) encoding resistance to ampicillin, streptomycin, sulphonamides and tetracyclines were localised on the bacterial chromosome. On the basis of resistance gene content and the lack of class 1 integrons these observations have suggested the existence of a new resistance island that differs from the *Salmonella* Genomic Island-1 [17].

In response to the rapid increase in the frequency of *S. enterica* serovar 4,[5],12:i:-, R-type ASSuT strains, isolates from England and Wales, Germany, France, Italy, Poland, Spain and the Netherlands were compared using phage typing, resistance gene characterisation, pulsed-field gel electrophoresis (PFGE) and multilocus variable number tandem repeat (MLVA) analysis to evaluate the possibility of clonal spread of this emerging multidrug-resistant (MDR) strain.

## Methods and Materials

### Isolate collection

The eight participating laboratories (the HPA Centre for Infections, London and the Veterinary Laboratories Agency, Weybridge in the UK, the Agence Française de Sécurité Sanitaire des Aliments in Maisons-Alfort, France, the Federal Institute for Risk Assessment in Berlin, Germany, the Istituto Superiore di Sanità in Rome, Italy, the National Institute of Public Health in Warsaw, Poland, the Health Surveillance Centre (VISAVET), University Complutense in Madrid, Spain and the Central Veterinary Institute of Wageningen in Lelystad, the Netherlands) were asked to submit a maximum of 10 isolates of *S. enterica* serovar 4,[5],12:i:- exhibiting resistance (according to local protocols) to ampicillin, streptomycin, sulphonamides and tetracyclines, and isolated from humans, pigs or pig meat between 2006-2008. In addition, laboratories were invited to send a maximum of 10 isolates of serovar 4,[5],12:i:- exhibiting other resistance phenotypes. All isolates were sent to the HPA.

### Strain characterisation

The *Salmonella* serotype was confirmed on the basis of the Kauffmann-White scheme and phage typing performed in accordance with HPA protocols [2,18]. In addition, isolates were screened using a duplex PCR targeting regions specific to serovar Typhimurium and to definitive phage type (DT) 104 and related strains of phage type (PT) U302 [19]. PCRs targeting the variable regions of the *fljB* genes encoding the phase-2 flagellar antigens H:1,2, H:1,5, H:1,6, H:l,7, H:e,n,x, H:e,n,z15 and H:1,w, were performed as previously described [20].

Susceptibility to a panel of 18 antimicrobials was determined by a breakpoint method in Isosensitest agar (Oxoid, Basingstoke, UK). The final plate concentrations (µg/mL) used routinely by the HPA on the basis of long-term studies were: ampicillin (A; 8), chloramphenicol (C; 8), gentamicin (G; 4), kanamycin (K; 16), neomycin (Ne; 8), streptomycin (S; 16), sulphonamides (Su; 64), tetracycline (T; 8), trimethoprim (Tm; 2), furazolidone (Fu; 8), nalidixic acid (Nx; 16), ciprofloxacin (low-level (Cpl) 0.125; high-level (Cp) 1.0), amikacin (Ak; 4), cephalexin (Cx; 16), cephradine (Cr; 16), cefuroxime (Cf; 16), ceftriaxone (Cn; 1) and cefotaxime (Ct; 1). Resistance genes $bla_{TEM}$, *strA-strB*, *sul2* and *tet*(B), and classes 1 and 2 integrase genes were sought by PCR using previous described primers [21,22].

### Molecular subtyping

PFGE was performed after digestion of genomic DNA with XbaI according to a standardised protocol [23]. The patterns were analysed using the Bionumerics software package (version 5.10; Applied Maths, Sint-Martens-Latem, Belgium) and resulting band profiles were submitted to the PulseNet Europe database for assigning profile names. Dendrograms were constructed using the Dice similarity coefficient and the unweighted pair group method with arithmetic averages (UPGMA) with optimisation and position tolerance set at 1.5%. Multilocus variable number tandem repeat (MLVA) analysis was performed according to a previously described protocol [24]. MLVA profiles were assigned based on the fragment size amplified from each locus, with 'NA' used to denote a locus not present [25].

## Results

Some 122 serovar 4,[5],12:i:- isolates were sent to the HPA Laboratory of Gastrointestinal Pathogens, of which 116 were confirmed as serovar 4,[5],12:i:-. These comprised 41 from England and Wales (20 from pigs and 21 from humans, including three from patients with a history of recent travel to Thailand, Greece and an undisclosed destination), 10 isolates from France (isolated from pig meat), 19 from Germany (12 from pigs, six from pig meat and one from a human), 23 from Italy (from humans), five from Poland (from humans), eight from Spain (from pigs) and 10 from the Netherlands (seven from human cases of infection; three from pigs). The H:1,2 phase-2 flagellar antigen could be serologically detected in the remaining six isolates.

Phage typing using the Typhimurium typing phages identified 16 different PTs (Table 1). The most commonly identified PTs were DT193 (51 isolates), DT120 (27 isolates) and RDNC (reacts but does not conform; 11 isolates). DT193 was the most common PT identified

**Table 1**

Phage type distribution among serovar 4,[5],12:i:- isolates from seven European countries, 2006-2008 (n=116)

| Country | Phage type (number of isolates) |
|---|---|
| England and Wales | 21 var (1), 120 (13[a]), 191 (1), 193 (21[b]), 208 (1), RDNC (2), U302 (2) |
| France | 68 var (1), 120 (2), 193 (5), U311 (1), UT (1) |
| Germany | 193 (13), 208 (1), 104b (2), RDNC (3) |
| Italy | 7 var (3), 18 var (2), 120 (6), 193 (3), RDNC (5), U311 (3), UT (1) |
| Poland | 120 (4), 104 (1) |
| Spain | 18 (1), 193 (4), RDNC (1), U302 (1), U311 (1) |
| The Netherlands | 12 (2), 120 (2), 193 (6) |

RDNC: isolates that react with the typing phages, but do not conform to a recognised pattern; UT: isolates that do not react with any of the typing phages; var: variant.

Phage type as determined by the scheme of Anderson *et al.* [18].

[a] Including two strains associated with foreign travel.

[b] Includes one strain associated with foreign travel.

**Table 2**

Comparison of phage type and R-type with PFGE profile of serovar 4,[5],12:i:- isolates from seven European countries, 2006-2008 (n=116)

| | PFGE profiles (STYMXB.) | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ASSuT | | | | | | ASSuT and other resistances[b] | | | | | | Other resistance patterns (not ASSuT)[c] | | | | | |
| PT | 0131 | 0083 | 0079 | 0010 | 0022 | Other[a] | 0131 | 0083 | 0079 | 0010 | 0022 | Other | 0131 | 0083 | 0079 | 0010 | 0022 | Other |
| 193 | 24 | 1 | | 1 | 2 | 7 | 2 | | | | 1 | 3 | 4 | | 1 | | 1 | 4 |
| 120 | 1 | 4 | 1 | 6 | 1 | 1 | | 4 | 1 | | | 1 | | 2 | 3 | 1 | | 1 |
| RDNC | 1 | | 2 | 1 | | 2 | | | | 1 | | 1 | | | | | | 2 |
| U311 | | | 1 | | 1 | | | | | | | 3 | | | | | | |
| U302 | | | 1 | | | | | | | | | 1 | | | | | | 1 |
| 7 var | | | 1 | | | | | | | | | | | | | | | 2 |
| 12 | | | | | | | | | | | | | | | | | | 2 |
| 208 | | | | | | | | | | | | | | | | | | 2 |
| 18 var | | | | | | | | | 1 | | | | | 1 | | | | |
| UT | | | | | | 1 | | | | | | 1 | | | | | | |
| 104B low | | | | | | | | | | | | | | 2 | | | | |
| 104 low | | | | | | | | | | | | 1 | | | | | | |
| 18 | | | | | | | | | | | | | | | | | | 1 |
| 191 | | | | | | | | | | | | | | | | | | 1 |
| 21 var | | | | | | 1 | | | | | | | | | | | | |
| 68 var | | | | | | | | | | | | | | | | | | 1 |
| **Total** | **26** | **5** | **6** | **8** | **4** | **14** | **2** | **4** | **2** | **1** | **1** | **11** | **4** | **5** | **4** | **1** | **1** | **17** |

PFGE: pulsed-field gel electrophoresis.

[a] Includes two untypable strains.

[b] Includes resistance patterns ACGNeKSSuTTmNxCpl (1 strain), ACKSSuT (1), ACSSuSpTTm (2), AGKNeSSuTTm (n=1), AGSSpSuT (n=1), AGSSuTTm (n=1), AKSSuT (n=1), ASSpSuTNxCpl (n=1), ASSuTNxCpl (n=2), ASSuTNxCpl (n=1) and ASSuTTm (n=9).

[c] Includes fully sensitive strains (n=6), AGST (n=1), AGSuT (n=1), AGT (n=1), ASSu (n=1), ASuT (n=1), SSuTm (n=1), SSuTTm (n=6), SuT (n=1), SuTTm (n=1) and T (n=9).

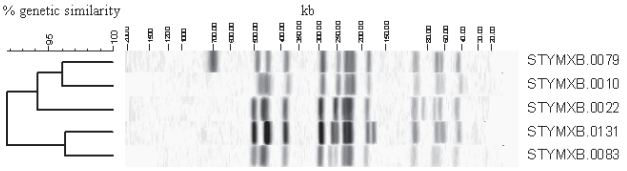
in England and Wales, France, Germany, Spain and the Netherlands, while DT120 predominated in Italy and Poland. All 116 isolates were PCR-positive for the Typhimurium-specific fragment of the malic acid dehydrogenase gene but only four isolates (one belonging to DT104, two to PT U302 and one untypable) gave a product with primers targeting the 16S to 23S spacer region specific to DT104 and the related PT U302 [19].

Overall, 94 of 116 isolates were PCR-negative for all variants of the *fljB* gene coding for the phase-2 flagellar antigen, including 48 of 51 DT193 and 17 of 27 DT120 isolates. H:1,2-specific amplicons were detected in the remaining 22 isolates.

Eighty-four isolates (72%) expressed resistance to ampicillin, streptomycin, sulphonamides and tetracyclines (R-type ASSuT), with or without additional

## Figure

Comparison of the five most common PFGE profiles identified in serovar 4,[5],12:i:- isolates from seven European countries, 2006-2008




PFGE: pulsed-field gel electrophoresis.

## Table 3

Comparison of common PFGE profiles with phage type, country of origin and sources of isolates, 2006-2008 (n=74)

| STYMXB. | Number of isolates | Phage type (number of isolates) | Country of origin (number of isolates.) | Source (number of isolates) |
|---|---|---|---|---|
| 0131 | 32 | DT193 (30)<br>DT120 (1)<br>RDNC (1) | France (2)<br>The Netherlands (6)<br>England and Wales (16)<br>Germany (8) | Humans (10)<br>Pigs/pig meat (22) |
| 0083 | 14 | DT120 (10)<br>DT104 (2)<br>18 var (1)<br>DT193 (1) | France (2)<br>England and Wales (9)<br>Germany (2)<br>Italy (1) | Humans (5)<br>Pigs/pig meat (9) |
| 0079 | 12 | DT120 (5)<br>RDNC (2)<br>18 var (1)<br>7 var (1)<br>U302 (1)<br>U311 (1) | England and Wales (2)<br>Spain (1)<br>Italy (9) | Humans (11)<br>Pigs/pig meat (1) |
| 0010 | 10 | DT120 (7)<br>RDNC (2)<br>DT193 (1) | France (1)<br>The Netherlands (1)<br>England and Wales (1)<br>Poland (3)<br>Spain (1)<br>Italy (1)<br>Germany (2) | Humans (5)<br>Pigs/pig meat (5) |
| 0022 | 6 | DT193 (4)<br>DT120 (1)<br>U311 (1) | France (2)<br>England and Wales (1)<br>Poland (1)<br>Spain (1)<br>Germany (1) | Humans (2)<br>Pigs/pig meat (4) |

PFGE: pulsed-field gel electrophoresis.

resistance(s) (Table 2). Six isolates were fully sensitive to all antimicrobials in the test panel. Eighty-three of 92 ampicillin-resistant iolates carried $bla_{TEM}$, 85 of 96 streptomycin-resistant isolates carried *strA-strB*,

**Table 4**

Subdivision of the five most common PFGE profiles using MLVA analysis, 2006-2008 (n=74)

| PFGE pattern | Number of strains | MLVA profile (based on number of tandem repeats at each locus)[a] | | | | | |
|---|---|---|---|---|---|---|---|
| | | SSTR9 | STTR5 | STTR6 | STTR10 | STTR3 | |
| | | | | | | 27 bp | 33 bp |
| STYMXB.0131 | 10 | 3 | 11 | 9 | NA | 2 | 11 |
| | 8 | | 13 | 10 | | | |
| | 5 | | 12 | | | | |
| | 3 | | 13 | | | | |
| | 3 | | | 10 | | | |
| | 1 | | | 12 | | | |
| | 1 | | | 14 | | | |
| | 1 | | | 8 | | | |
| STYMXB.0083 | 5 | 3 | 12 | 9 | NA | 2 | 11 |
| | 4 | | | 6 | | | |
| | 1 | | 11 | 11 | | | |
| | 1 | | 11 | 12 | | | |
| | 1 | | 13 | 10 | | | |
| | 1 | | 14 | | | | |
| | 1 | 13 | 13 | NA | | | |
| STYMXB.0079 | 2 | 3 | 12 | 11 | NA | 2 | 11 |
| | 2 | | 13 | 10 | | | |
| | 1 | | 11 | 8 | | | |
| | 1 | | 12 | 12 | | | |
| | 1 | | 12 | 9 | | | |
| | 1 | | 13 | 12 | | | |
| | 1 | | 13 | 9 | | | |
| | 2 | 11 | 15 | NA | | | |
| | 1 | 13 | 13 | NA | | | |
| STYMXB.0010 | 3 | 3 | 14 | 9 | NA | 2 | 11 |
| | 2 | | 12 | 10 | | | |
| | 1 | | 12 | 7 | | | |
| | 1 | | 12 | 9 | | | |
| | 1 | | 13 | 7 | | | |
| | 1 | | 14 | 10 | | | |
| | 1 | | 15 | 10 | | | |
| STYMXB.0022 | 2 | 3 | 12 | 9 | NA | 3 | 11 |
| | 1 | | | 13 | | | |
| | 1 | | 13 | 11 | | | |
| | 1 | | 14 | 9 | | 2 | |
| | 1 | | 15 | 10 | | 2 | |

NA: locus not present; PFGE: pulsed-field gel electrophoresis; MLVA: multilocus variable number tandem repeat analysis.

[a] Number of tandem repeats only listed where it differs from the most common repeat number in each PFGE profile.

88 of 99 sulphonamide-resistant isolates carried *sul2* and 93 of 105 tetracycline-resistant isolates carried *tet*(B) (data not shown). Of 84 R-type ASSuT strains, 68 possessed $bla_{TEM}$, *strA-strB*, *sul2* and *tet*(B) resistance genes. Eighty-two percent of RDNC isolates, 80% of DT193 and 74% of DT120 were of R-type ASSuT (with/without additional resistance(s)), with resistance encoded by genes $bla_{TEM}$, *strA-strB*, *sul2* and *tet*(B) in 78%, 75% and 56% of isolates respectively. Isolates of R-type ASSuT were negative for both class 1 and 2 integrase genes; these were found only in strains expressing resistance to aminoglycosides and/or trimethoprim. Among the remaining 16 R-type ASSuT strains from the present study that did not carry $bla_{TEM}$, *strA-strB*, *sul2* and *tet*(B), 11 strains lacked only one of *tet*(B), $bla_{TEM-1}$ or sul2, one strain each lacked $bla_{TEM-1}$ and *tet*(B) or *strA-strB* and *tet*(B), one strain lacked $bla_{TEM-1}$, *strA-strB* and *sul2* and one strain lacked all four genes. These strains belonged to phage types DT120 (five strains), DT193 (four strains), RDNC (two strains), and one each belonged to phage types DT104, DT18 variant, U302, U311 and UT.

PFGE analysis identified 36 unique banding profiles among 114 strains; two strains were untypable. These were grouped into 12 clusters of two or more strains and 23 patterns corresponding to a single isolate (data not shown). Sixty-five percent (74/114) of strains were represented by one of five banding patterns (STYMXB.0131, n=32, STYMXB.0083, n=14, STYMXB.0079, n=12, STYMXB.0010, n=10, and STYMXB.0022, n=6) that shared more than 90% similarity (Figure, Table 3). Strains from humans and pigs or pig meat were represented in each common PFGE pattern. The majority of strains with PFGE patterns STYMXB.0131 and STYMXB.0022 were phage type DT193, while patterns STYMXB.0083, STYMXB.0079 and STYMXB.0010 were dominated by phage type DT120 (Table 3). Some country-specific differences were noted within the distribution of PFGE patterns: nine of the 12 STYMXB.0079 strains were from Italy, three of the five Polish strains were STYMXB.0010 and six of 10 strains from the Netherlands were pattern STYMXB.0131 (Table 3). STYMXB.0010 was the only profile identified in all seven countries. However, larger numbers of strains need to be analysed to determine whether these country-specific distributions hold true. Patterns STYMXB.0131 and STYMXB.0010 were dominated by R-type ASSuT strains (representing 81% and 80% of strains, respectively), whereas resistance profiles of the other common PFGE profiles were more variable (Table 2).

MLVA typing identified 45 different profiles that differed by loss or addition of tandem repeats at loci STTR9, STTR5, STTR6 and STTR3, and was able to further subdivide the five most common PFGE profiles (Table 4). Ninety-one percent (105/116) of strains failed to amplify a fragment from the Typhimurium-specific virulence plasmid pSLT-bound locus STTR10. The five most common MLVA profiles (3-11-9-NA-211, n=12; 3-12-9-NA-211, n=13; 3-13-10-NA-211, n=12; 3-14-9-NA-211, n=7; and 3-13-9-NA-211, n=6) accounted for 43% of strains and differed by only one to three tandem repeats at locus STTR5 and one repeat at locus STTR6.

The most frequently occurring combination of phenotypic and genotypic characteristics was that 51 of 116 (44%) serovar 4,[5],12:i:- isolates belonged to phage type DT193. Of these 51, 48 were PCR-negative for *fljB*. Among the 51 DT193 isolates, 37 were of R-type ASSuT (plus additional resistance to chloramphenicol, aminoglycosides and/or trimethoprim in four isolates) encoded by $bla_{TEM}$, *strA-strB*, *sul2* and *tet*(B), and 36 exhibited PFGE profile STYMXB.0131, which could be further divided into eight related MLVA profiles. Isolates bearing these characteristics were isolated in England and Wales (including one isolate from a patient with history of recent travel to Thailand), France, Germany and the Netherlands.

## Discussion

Antimicrobial resistance is a serious public health problem limiting the therapeutic options available to clinicians treating complicated *Salmonella* infections. In recent years there has been an overall decline in the level of resistance in serovar Typhimurium in several European countries as a result of a reduction in the number of isolates of penta-resistant DT104 [14]. To some extent this reduction has been counteracted by an increase in prevalence of serovar 4,[5],12:i:- isolates expressing resistance to ampicillin, streptomycin, sulphonamides and tetracyclines [8,17].

One of the first reports of serovar 4,[5],12:i:- in Europe was of an isolate grown in the late 1980s from a chicken carcass in Portugal [26]. This serovar emerged in Spain in strains from humans and pork or pork products during 1997, and subsequently became the fourth most common *Salmonella* serovar identified from 1998 to 2000 [11]. All isolates belonged to phage type U302. These isolates were classed as monophasic variants of serovar Typhimurium due to presence of an IS*2000* fragment located in a Typhimurium-specific location within the *fliB-fliA* intergenic region and amplification of a Typhimurium DT104- and U302-specific region [3]. All 116 monophasic isolates in this study harboured the Typhimurium-specific fragment of the malic acid dehydrogenase gene, suggesting that these strains are monophasic variants of serovar Typhimurium. However, the majority (97%) were negative for the DT104- and U302-specific region, suggesting that these monophasic isolates may not be related to the serovar 4,[5],12:i:- strain(s) that emerged in Spain. This was confirmed by phage typing, which identified DT193 as the most common PT, followed by DT120, thereby adding to the diversity of phage types of serovar 4,[5],12:i:- linked to serovar Typhimurium. DT193 and DT120 have consistently fallen within the top five phage types of serovar Typhimurium from cases of human infection in England and Wales in recent years (HPA *Salmonella* database, unpublished data). It is plausible that at

least some of this increase may be attributed to the emergence of serovar 4,[5],12:i:- DT193 and DT120 strains. Putative Typhimurium isolates sent from primary diagnostic laboratories to the HPA *Salmonella* Reference Unit are only phage-typed and not routinely subjected to further serological examination. This may result in misclassification as serovar Typhimurium and under-reporting of this serovar in England and Wales, and in other countries where phage typing is used *in lieu* of full serotyping to identify strains as serovar Typhimurium. Serovar 4,[5],12:i:- DT193 strains have previously been isolated from human cases of infection and/or pigs in Luxembourg and Spain [6,13], while monophasic DT120 strains were identified in Italy [8].

The Spanish PT U302 serovar 4,[5],12:i:- strains were PCR-negative for H:1,2 [11], as were the majority (81%) of monophasic isolates in this study. Previous published work has shown that the lack of phase-2 flagellar expression may be due to different mutations (including point mutations) and partial or complete deletions in *fljB* and adjacent genes [4,27]. Monophasic strains in which the phase-2 flagellar antigen is not detected serologically but can be detected by PCR may contain deletions in a part of *fljB* that leave the H:1,2-specific PCR primer binding sites intact, or they may represent 'serotype inconsistent' strains [27]. These are serovar Typhimurium strains in which serological detection of the phase-2 flagellar antigen may be inconsistent. This may be due to problems with flagellar phase reversal, which is a time-consuming and technically demanding procedure that may result in misclassification of Typhimurium strains as serovar 4,[5],12:i:-. Alternatively, the invertible promoter controlling expression of *fljB* and *fliC* may have become locked in one position allowing only expression of *fliC* in these strains [4]. The range of mechanisms that can result in non-expression of the phase-2 flagellar antigen make definitive identification of serovar 4,[5],12:i:- problematic. It is possible that molecular serotyping could be used as a basis to define such strains as serovar 4,[5],12:i:- or Typhimurium, but as yet such methods lack standardisation, are not in place in most countries and may not be suitable for laboratories other than reference facilities. Given that there may be discrepancy in detection of the phase-2 flagellar antigen between classical and molecular serotyping, an international agreement both on the definition of monophasic strains and on detection methodology is required. Without reaching such a consensus the true incidence of such Typhimurium-like strains is difficult to assess; only the harmonisation and the sharing of methods will allow accurate comparison of reported data.

In contrast to the monophasic variants isolated in Thailand and Spain, which commonly expressed additional resistance to gentamicin and trimethoprim-sulphamethoxazole and/or chloramphenicol [10,11] and to serovar 4,[5],12:i:- strains isolated in Brazil and New York City, which were infrequently MDR [7,9], the countries participating in this study observed an increase in isolates of serovar 4,[5],12:i:- with resistance to ampicillin, streptomycin, sulphonamides and tetracyclines only. Characterisation of the resistance genes responsible for this phenotype identified $bla_{TEM}$, *strA-strB*, *sul2* and *tet*(B) in 81% of isolates. Such genes have also been identified in isolates of Typhimurium DT193 R-type ASSuT obtained during 2005 in England and Wales from raw beef and a human case of infection, although the majority of strains tested harboured *tet*(A) rather than *tet*(B) (unpublished data). Analysis of a 10 kb chromosomal region of a Typhimurium DT193 revealed the presence of an *strB-strA-sul2-repC-repA* region derived from plasmid RSF1010 located upstream of $bla_{TEM-1}$ and downstream of a class 1 integron [28]. The resistance genes encoding the tetra-resistant phenotype in isolates of serovars Typhimurium and 4,[5],12:i:- from Italy, Denmark and the UK were also identified as $bla_{TEM-1}$, *strA-strB*, *sul2* and *tet*(B), but all isolates were negative for class 1 integrons [17]. Transfer experiments were unsuccessful and probes specific for these genes bound to a 750 kb I-CeuI digest fragment, suggesting a chromosomal location and existence of a new resistance island. As in the present study, strains with other R-types than ASSuT, but with related PFGE profiles and harbouring one or more of $bla_{TEM-1}$, *strA-strB*, *sul2* and *tet*(B) were identified. This suggests that rearrangements or deletions may occur within the resistance island leading to partial resistance patterns [17]. In contrast, resistance to ampicillin, streptomycin, sulphonamides and tetracyclines was mediated by plasmid-borne $bla_{TEM-1}$ and *tet*(A), and a class 1 integron harbouring *aadA2* and *sul1* in the Spanish serovar 4,[5],12:i:- U302 isolates [29].

Thirty-six profiles were identified among the 114 strains typable by PFGE, thereby supporting previous observations that serovar 4,[5],12:i:- can demonstrate considerable diversity, even among strains from a single country [4,9,13,27]. However, serovar 4,[5],12:i:- strains have been reported to be less heterogenic than serovar Typhimurium strains [9,27,30]. Serovar Typhimurium demonstrates considerable diversity as evidenced by phage typing and molecular typing, but with certain clonal strains such as multidrug-resistant DT104 [31]. The most common PFGE profile identified in our study was STYMXB.0131, which, together with four other closely related banding patterns (STYMXB.0022, STYMXB.0079, STYMXB.0010 and STYMXB.0083), accounted for 65% of isolates. Previously submitted STYMXB.0131 patterns in the PulseNet Europe database belonged to serovar Typhimurium DT193 and PT507 (according to the Dutch phage typing scheme) strains isolated from human cases of infection in Finland, the Netherlands and England and Wales. Patterns STYMXB.0131 and STYMXB.0022 have also been identified in Typhimurium DT193 strains from humans, cattle and raw beef in England and Wales (unpublished data), while patterns STYMXB.0083 and STYMXB.0010 have been identified in Typhimurium DT120 isolates in England and Wales and in Denmark [32]. These observations are consistent with previous

studies that serovar 4,[5],12:i:- strains are genotypically closely related to serovar Typhimurium [4,7,8,27]. Patterns STYMXB.0079 and STYMXB.0010 represented 58% of serovar Typhimurium R-type ASSuT strains in Italy [8]. Pattern STYMXB.0131 has also been identified among Danish serovar 4,[5],12:i:- strains [17]. Serovar 4,[5],12:i:- R-type ASSuT strains belonging to profile STYMXB.0131 were responsible for two major outbreaks in Luxembourg in 2006 where pork meat was suspected as the vehicle for the outbreaks [6]. In Italy, profiles STYMXB.0079 and STYMXB.0010 represented 83% of serovar 4,[5],12:i:- R-type ASSuT strains [8]. However, the majority of strains were phage type U302 or untypable; only 8% of the isolates belonged to DT120 and none were DT193.

MLVA typing was also applied to the strain panel as the technique is reportedly more discriminatory than PFGE and provides unambiguous typing data that is free of the bias generated by differences in resistance genotype that reportedly affects PFGE [33]. Using the nomenclature of Larsson *et al.* allowed easy recognition of related profiles [25]. The five most common MLVA profiles identified in this study, and single locus variants thereof, have previously been identified in *S.* Typhimurium DT193 R-type ASSuT strains isolated from humans, pigs, cattle and beef products in England and Wales in 2005-2006 (unpublished data) and in isolates of Typhimurium DT120 R-type ASSuT associated with a putative outbreak in humans in the northeast of England in 2006 [32]. That all monophasic strains were typable by MLVA, using the Lindstedt *et al.* Typhimurium-specific scheme [24], and shared closely related profiles with these Typhimurium isolates provides tentative further evidence that monophasic 4,[5],12:i:- isolates derive from serovar Typhimurium.

The data presented here suggest that a serovar 4,[5],12:i:- DT193 R-type ASSuT clone with PFGE profile STYMXB.0131 has emerged from serovar Typhimurium and spread within several European countries, with pigs as a likely reservoir of infection. Isolates of serovar 4,[5],12:i:- DT120 R-type ASSuT with closely related PFGE profiles were identified in humans and pigs from five of the participating countries. The diversity of PFGE and MLVA profiles within serovar 4,[5],12:i:- DT193 and DT120 R-type ASSuT isolates, and the differences between these isolates and those previously described in Spain [30], suggests that serovar 4,[5],12:i:- is likely to represent several clones or strains that have emerged independently from serovar Typhimurium. Recent genotypic studies have shown that in addition to the Spanish 4,[5],12:i- clone, other 4,[5],12:i:- lineages exist [27].

In the first ten months of 2009, DT193 and DT120 accounted for 18% and 11% of Typhimurium isolates in England and Wales, respectively. In contrast, DT104 accounted for only 7% of Typhimurium isolates (HPA *Salmonella* database, unpublished data). Serovar 4,[5],12:i:- has already caused substantial outbreaks in several countries, with reports of severe infections and also deaths [6,7,9,10]. In order to prevent a global epidemic of these newly emerging clones or strains, as occurred with Typhimurium DT104, appropriate intervention strategies need to be put in place as soon as possible, particularly in pig husbandry throughout the EU.

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