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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).

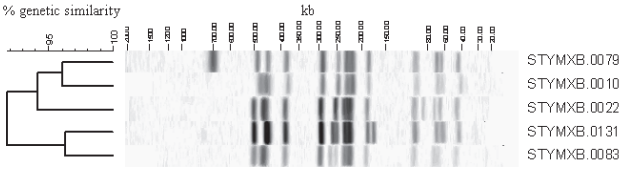
in England and Wales, France, Germany, Spain and the Netherlands, while DT120 predominated in Italy and Poland. All 116 isolates were PCR-positive for the Typhimurium-specific fragment of the malic acid dehydrogenase gene but only four isolates (one belonging to DT104, two to PT U302 and one untypable) 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) DT120 (1) RDNC (1)	France (2) The Netherlands (6) England and Wales (16) Germany (8)	Humans (10) Pigs/pig meat (22)
0083	14	DT120 (10) DT104 (2) 18 var (1) DT193 (1)	France (2) England and Wales (9) Germany (2) Italy (1)	Humans (5) Pigs/pig meat (9)
0079	12	DT120 (5) RDNC (2) 18 var (1) 7 var (1) U302 (1) U311 (1)	England and Wales (2) Spain (1) Italy (9)	Humans (11) Pigs/pig meat (1)
0010	10	DT120 (7) RDNC (2) DT193 (1)	France (1) The Netherlands (1) England and Wales (1) Poland (3) Spain (1) Italy (1) Germany (2)	Humans (5) Pigs/pig meat (5)
0022	6	DT193 (4) DT120 (1) U311 (1)	France (2) England and Wales (1) Poland (1) Spain (1) Germany (1)	Humans (2) 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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Risk of Salmonella infection with exposure to reptiles in England, 2004-2007

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Non-typhoidal *Salmonella* infections are a common cause of gastroenteritis in England. Non-Enteritidis, non-Typhimurium *Salmonella* serotypes have gained in relative importance in recent years, but their modes of transmission are poorly understood. In a large case-case study in England between 2004 and 2007, the association between exposure to reptiles and *Salmonella* illness was investigated using multivariable logistic regression. Recent reptile exposure was associated with *Salmonella* illness with an odds ratio of 2.46 (95% confidence interval: 1.57-3.85, p<0.001), with much stronger effects among children under five years of age. The exposure was rare, and a population attributable fraction was estimated as 0.9%. Among the *Salmonella* serotypes found in people exposed to reptiles, several non-Enteritidis, non-Typhimurium serotypes were strongly associated with exposure. Reptile exposure is a rare but significant risk factor for *Salmonella* illness in England, with much higher risk in children.

Introduction

Non-typhoidal *Salmonella* is the second most common bacterial cause of gastrointestinal infection in England and Wales. It was estimated to account for 116,000 cases of illness, 3,400 hospitalisations and 268 deaths in 1995 [1]. In recent years, there has been a decline in notified infections in England and Wales of the most common *Salmonella* serotype, *S.* Enteriditis, due to improved control of *Salmonella* in chicken flocks [2], meaning that non-Enteritidis non-Typhimurium serotypes of *Salmonella* are becoming of greater relative importance in the United Kingdom (UK) – see Figure 1 [3].

Epidemiological associations of *Salmonella* infections are mainly inferred from investigation of outbreaks [3], although these account for only a small proportion of notified cases. Furthermore, it is thought that as little as one in six cases of gastrointestinal illness are notified to public health authorities in the UK [4]. Therefore, understanding of the causes of *Salmonella* illness outside of recognised outbreaks is limited. Food- [5] and travel-related exposures [3] are believed to be the dominant causal factors. The role of other rarer modes of transmission at a population level is less well understood.

Salmonella are among the flora naturally found in the gastrointestinal tract of many reptiles [6]. Human infection with *Salmonella* acquired from contact with reptiles is a well-recognised phenomenon and a recent review article summarised recent reports of reptile-associated salmonellosis in Europe [7]: Some European countries (Belgium, Finland, France, Germany, Ireland, Latvia) had reports of confirmed or likely reptile-associated *Salmonella* cases. In the Netherlands, serotype attribution techniques based on past identifications were used to estimate the fraction of human isolates that could be accounted for by exposure to reptiles. It was concluded that although less than 1% of *Salmonella* isolates were attributable to exposure to reptiles and amphibians between 2000 and 2007, this proportion was increasing in recent years [7]. Other European countries reported no known cases of *Salmonella* associated with reptiles, although information on this kind of exposure might not have been available in notification data. In the United States (US), reptile-associated salmonellosis is well known: there are documented outbreaks of salmonellosis related to pet reptiles [8,9] and two case-control studies [10,11] have described contact with reptiles or amphibians as important risk factors for salmonellosis in children. We are not aware of any previous studies describing the population-wide effect of reptile-associated salmonellosis in the UK.

Salmonella taxonomy and nomenclature is complex. This study employs the standard Kaufmann-White serology-based naming system for serotypes described here. Serotypes are referred to by abbreviated versions of the full name: the formal title of *Salmonella* enterica serotype Enteritidis as is here abbreviated to *S.* Enteritidis.

The Co-ordinated Local Authority Sentinel Surveillance of Pathogens (CLASSP) study was conducted by the Health Protection Agency (HPA) to investigate the effects of a wide variety of exposures on the acquisition

of gastrointestinal illness in the general population in England. Among these exposures pet ownership in general and exposure to reptiles in particular were investigated. The CLASSP study used a case-case format [12] which is a variation of the standard case-control methodology where cases of another disease (here *Campylobacter* infections) are used as control cases for comparison with the disease cases under investigation (here *Salmonella*).

The main theoretical advantages of the case-case methodology are that it should be able to avoid introduction of notification bias and to minimise recall bias. The main disadvantages are that no apparent effect may be observed if the exposure under investigation is associated with both diseases, and that the control group will differ from the ideal study base (here the general population).

The most commonly described epidemiological associations of *Campylobacter* infection are with handling or consumption of inadequately cooked chicken meat and foreign travel, particularly to developing countries [13]. Consumption of some other foods has been described as a risk factor (RF) for *Campylobacter* illness [14,15]. *Campylobacter* is not among the commensal bacteria known to be carried by reptiles [6] and none of the many large epidemiological studies looking at RFs associated with *Campylobacter* (including those referenced above) have cited reptiles or amphibians as significant associations with this disease.

The aims of our study were to test the hypothesis that recent exposure to a reptile is associated with development of a *Salmonella* illness after accounting for all important confounding effects and to calculate a population attributable fraction (PAF) for reptile ownership on all *Salmonella* infections occurring in England.

Methods

Data collection

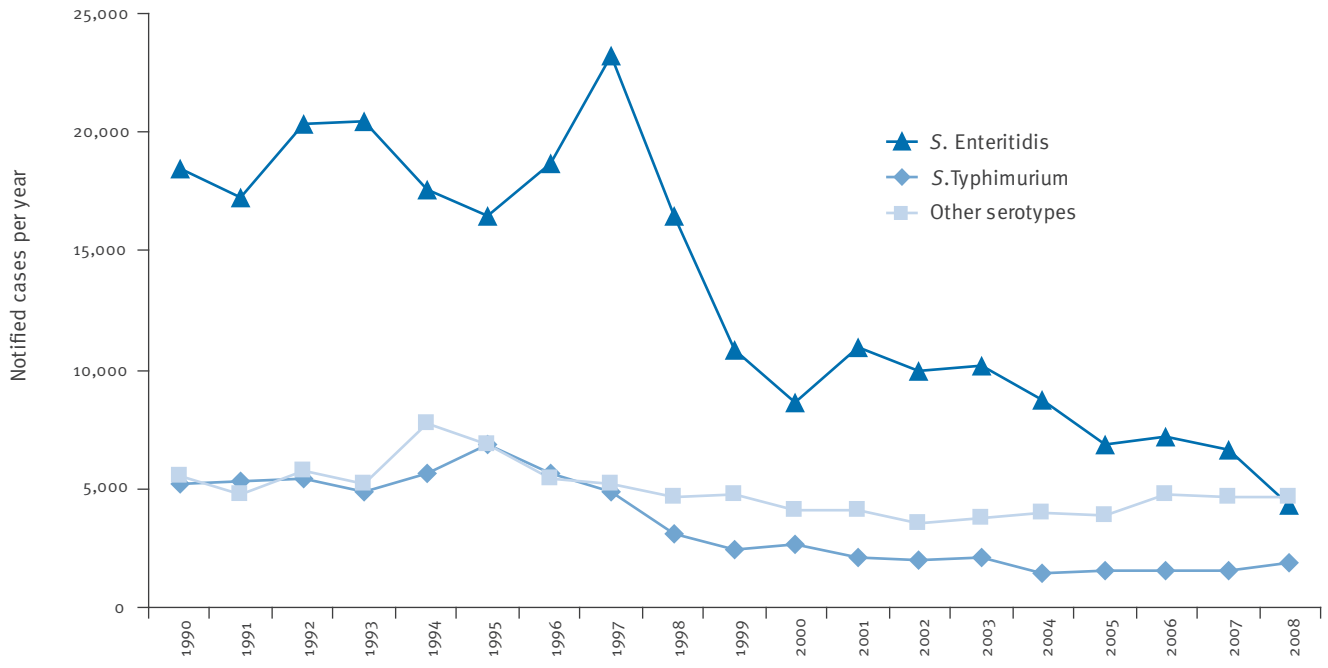
Health Protection Units of Local Authorities in England participated in the CLASSP study on a voluntary basis. Any individual resident in areas covered by these Local Authorities who had a microbiological isolate of either *Salmonella* or *Campylobacter* during the study period was eligible for inclusion. Information on exposures under investigation was collected using a standard study questionnaire covering a wide variety of plausible risk factors for acquisition of either *Salmonella* or *Campylobacter*. Questionnaires were filled in by Environmental Health Officers (who were unaware of the serotype of *Salmonella* isolates and to the hypothesis under investigation here) or were posted to the participants. Data entry and microbiological procedures were performed according to standard methods at HPA

Outcome and exposure variables

The outcome variable for this analysis was 'type of infection': either *Campylobacter* or *Salmonella*. Cases of *Campylobacter* were used as the control cases for this analysis with no matching of cases to controls. Questionnaires relating to infections with organisms other than non-typhoidal *Salmonella* or *Campylobacter* infections were excluded, as were records missing data for age, sex or cultural background.

Figure 1

Notified *Salmonella* infections in England and Wales, 1990-2008



Source: Health Protection Agency surveillance data.

A binary variable for the main exposure (ownership of reptiles) was derived by extraction of a variety of synonyms from the free text section of the CLASSP questionnaire relating to recent exposure to animals. The synonyms used for extraction were REPTILE, SNAKE, LIZARD, TORTOISE, TURTLE, TERRAPIN, DRAGON. Additionally, a manual search through all records was performed.

Other variables in the CLASSP questionnaire which represented potential RFs for acquisition of either *Salmonella* or *Campylobacter* infection were extracted from the study database These included variables relating to food and drink consumption, food handling, travel, pets (other than reptiles), visits to farms or zoos, recreational water activities, eating outside of the home. All of these study exposures were related to contact with the particular factor in the five days before development of illness. Age, sex and self-reported ethnicity were also included as study variables. Binary variables were created for each of these exposures, except for age and ethnicity which were categorical.

Missing data

For all binary exposures studied, we compared 'unexposed' individuals (those with no positive report of exposure) against 'exposed' individuals (reported exposure in questionnaire). Thus missing or unknown exposures were grouped into the 'no exposure reported' (baseline) group for each variable for the purposes of this analysis.

Statistical methods

All statistical analyses were performed using STATA v10.1.

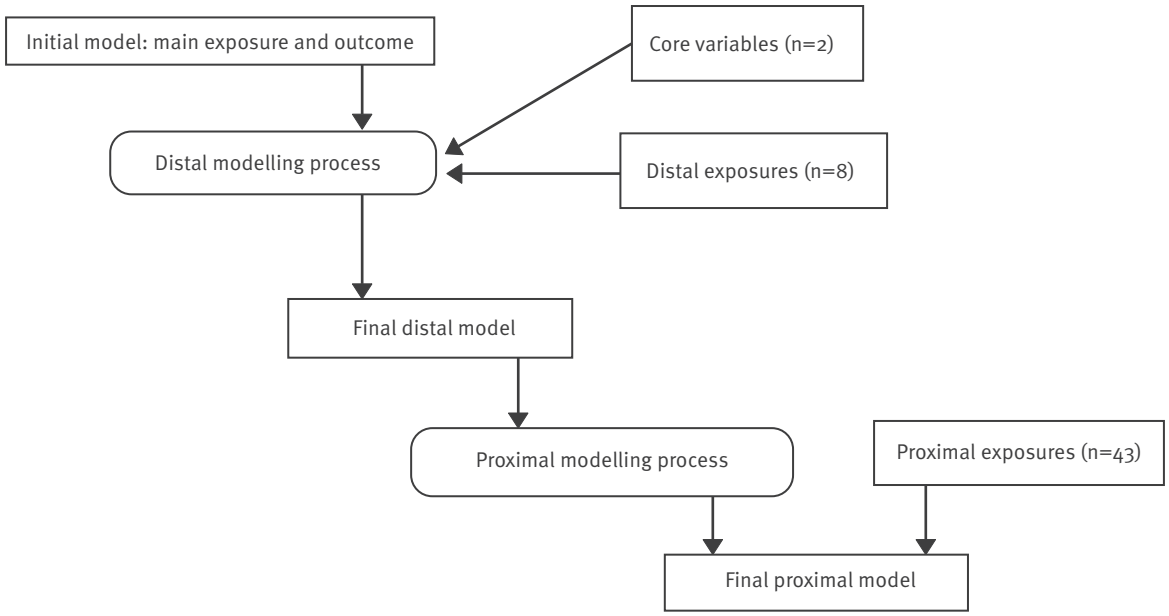
We described the demographic characteristics of study participants using Chi-square tests and Fisher's exact tests for association and Student's t-test for continuous variables. All exposure variables associated with the outcome with $p \leq 0.2$ in the bivariate analysis were included in the multivariable modelling process. Variables with $p > 0.2$ were considered not to have a direct effect on outcome, but were tested as potential confounders of the main exposure-outcome relationship.

We used a multivariable logistic regression model to determine the effect of reptile ownership on outcome and whether other exposure variables (i) provided an alternative explanation for outcome or (ii) confounded the main exposure-outcome relationship. We formulated a simple hierarchical framework to describe the causal relations of exposure variables [16]. We thus divided variables into a main exposure variable (ownership of a pet reptile), core variables (age and sex) and potential distal (n=8) and proximal (n=43) exposure variables. Distal exposure variables were those that might alter the likelihood of pathogen acquisition through a wide variety of end transmission vehicles, such as travel outside the UK or eating outside of the home. Proximal exposure variables were those relating to a specific method of pathogen acquisition, e.g. exposure to a particular foodstuff (e.g. eating or handling chicken) or type of animal (e.g. contact with a dog) or particular high-risk activities (e.g. watersports). Questions regarded a wide variety of exposures covering all known or suspected vehicles of transmission of these infections.

Variables were progressively added to the initial model as shown in Figure 2. First the distal exposure variables were introduced to the core model using a step-wise

Figure 2

Flow diagram of multivariable modelling process



process if they were significantly associated with the outcome (p≤0.05) based on evaluation of the p value in the likelihood ratio test. This generated the preliminary distal model. All excluded distal variables were then tested one by one to see if their inclusion resulted in significant confounding (>10% alteration) of the odds ratio (OR) for the main exposure-outcome relationship. This gave a final distal model.

Following this, proximal exposure variables were then introduced to the final distal model by the same process. The preliminary proximal model was examined to

Table 1

Descriptors of study participants, CLASSP study 2004-2007

	Salmonella (n=2,310)	*Campylobacter* (n=11,204)	Chi-square value	p value
Demographic variables				
Mean age (years)	35.1	44.0	-	<0.001 (t-test)
Sex				
Male	1,113 (48.2%)	5,412 (48.3%)	0.01	0.91
Female	1,197 (51.8%)	5,792 (51.7%)		
Ethnicity				
White	2,130 (92.2%)	10,600 (94.6%)		
Asian	105 (4.6%)	393 (3.5%)	23.6	<0.001
Other	75 (3.3%)	211 (1.9%)		
Data collection variables				
Questionnaire method				
Personal interview	469 (20.3%)	397 (3.5%)		
Telephone interview	452 (19.6%)	941 (8.4%)	>1,000	<0.001
Posted	318 (13.8%)	5,112 (45.6%)		
Unknown	1,071 (46.4%)	4,754 (42.4%)		

Table 2

Main multivariable results, CLASSP study, 2004-2007

	Distal model		Proximal model	
	OR (95% CI)	p value	OR (95% CI)	p value
Main exposure				
Reptile as pet	2.49 (1.61-3.83)	<0.001	2.46 (1.57-3.85)	<0.001
Distal exposure variables with OR>1.0				
Travel abroad	4.02 (3.63-4.45)	<0.001	–[1]	
Eating out at parties or buffets	1.18 (1.05-1.33)	0.005	–[2]	
Proximal exposure variables with OR>1.0				
Eggs eaten at home			1.19 (1.05-1.35)	0.006
Eggs eaten outside the home			1.60 (1.39-1.83)	<0.001
Bacon eaten at home			1.30 (1.15-1.48)	<0.001
Cold meats eaten outside the home			1.23 (1.07-1.42)	0.005
Poultry other than chicken eaten outside the home			1.41 (1.18-1.69)	<0.001
Contact with an ill person			1.15 (1.01-1.30)	0.037
Swimming			1.22 (1.07-1.39)	0.003
Fishing			1.59 (1.05-2.42)	0.029
Total N	13,514		13,514	
Degrees of freedom[2]	16		35	

CI: confidence interval; OR: odds ratio.

[1] Variables from the distal model are included in the proximal model, but are not shown in the proximal model column as their effects are intended to be analysed in the distal model.

[2] Age, sex, ethnicity and any variables with a modelled OR of <1.0 are not shown in this table.

see if inclusion of any of the excluded proximal variables had a confounding effect (>10% alteration of OR) on the effect of the main exposure. We tested for interaction between the main exposure variable and age category, sex and history of travel abroad in the final model.

Results

Participating subjects

The CLASSP study took place in England between November 2004 and October 2007. There were 66 participating Local Authorities (or County Councils), covering approximately 20% of the English population.

Cases with mixed infections (both *Salmonella* and *Campylobacter*, or involving other organisms) were excluded from this analysis (n=140, 0.9% of questionnaires). All individuals with missing data for age, sex or ethnicity were also excluded (n=777, 5.4%). The remaining 13,514 questionnaires formed the basis for this analysis. There were completed questionnaires from 2,310 individuals with non-typhoidal *Salmonella* isolates (17.1%) and 11,204 with *Campylobacter* isolates (82.9%).

Questionnaires were completed by personal interview, telephone interview or by postal questionnaire. The method of data collection was only known for 7,689 of the 13,514 questionnaires (56.9%). In general terms, data for *Campylobacter* cases were more frequently collected by postal questionnaire (overall 46% *Campylobacter* versus 14% *Salmonella*), whilst questionnaires for *Salmonella* infections were more often collected by personal or telephone interview

Table 3

Interaction of main exposure and age in final multivariable model, CLASSP Study, 2004-2007

Age group (years)	*Salmonella* cases in reptile owners	*Campylobacter* cases in reptile owners	Multivariable OR	95% CI	p value
0	9	3	17.3	4.50-66.25	<0.001
1-4	6	1	44.6	5.17-385	<0.001
5-19	8	4	12.1	3.52-41.7	<0.001
20-49	9	43	1.23	0.56-2.68	0.61
50+	2	23	0.65	0.15-2.85	0.57
Total	**34**	**74**			

CI: confidence interval; OR: odds ratio.

Table 4

Salmonella serotypes in subjects with and without exposure to reptiles, CLASSP study, 2004-2007

	Number of cases (% of *Salmonella* cases)			
	Reptile ownership		Univariate OR	
Organism/serotype	No	Yes	(95% CI)	p value[1]
Campylobacter	11,130	74	1.0 (baseline)	-
Salmonella Arizonae	1 (0)	2 (5.7)	300 (26-3,400)	<0.001
Salmonella Blockley	3 (0.1)	1 (2.9)	50 (5.1-490)	0.027
Salmonella Chester	6 (0.2)	1 (2.9)	25.1 (3.0-210)	0.046
Salmonella Ealing	0 (0)	1 (2.9)	infinite	0.007
Salmonella Enteritidis	1,211 (53.2)	5 (14.3)	0.62 (0.25-1.54)	0.3[b]
Salmonella Java	12 (0.5)	2 (5.7)	25.1 (5.5-114)	0.004
Salmonella Kentucky	22 (0.9)	1 (2.9)	6.8 (0.91-51)	0.14
Salmonella Muenchen	4 (0.1)	3 (8.6)	112 (24.5-520)	<0.001
Salmonella Oranienburg	4 (0.1)	3 (8.6)	112 (24.5-520)	<0.001
Salmonella Panama	4 (0.1)	1 (2.9)	37 (4.1-341)	0.033
Salmonella Senftenberg	14 (0.6)	1 (2.9)	10.7 (1.4-82.8)	0.096
Salmonella Stanley	33 (1.4)	1 (2.9)	4.56 (0.62-33.8)	0.204
Salmonella Tel-El-Kebir	0 (0)	2 (5.7)	infinite	<0.001
Salmonella Typhimurium	311 (13.6)	2 (5.7)	0.97 (0.24-3.96)	0.96[b]
Unnamed serotypes	136 (5.9)	8 (23.5)	8.85 (4.18-18.74)	<0.001
Other named serotypes	515 (22.6)	0(0)	0	-
Total (all *Salmonella*)	**2,276**	**34**	**2.24 (1.49-3.38)**	**<0.001[2]**

CI: confidence interval; OR: odds ratio.

[1] p value for Fisher's exact test unless otherwise specified.

[2] p value for Chi-square test.

(20% *Salmonella* versus 4% *Campylobacter*), see Table 1. The study questionnaire was administered (or sent by post) on the same day as the case notification was received. The interval between reported onset of illness and administration of questionnaire was thus generally short (median interval: 9 days, interquartile range (IQR): 6-13 days).

Key demographic characteristics of the study population are shown in Table 1. In both pathogen groups, infections occurred most frequently in children under the age of five years, with reduced frequency between the ages of five years and 20 years and a plateau among adults over 20 years. Of all 13,514 included participants, 49.1% were male (49.3% of the *Campylobacter* cases, 48.2% of the *Salmonella* cases, chi-square: 0.1, p=0.91).

Risk factors for disease

Main exposure

A total of 34 of the 2,310 individuals (1.5%) experiencing a *Salmonella* illness reported ownership of a pet reptile, compared with 74 of the 11,204 (0.66%) individuals experiencing *Campylobacter* illness. Using *Campylobacter* as control cases, we calculated a crude OR for exposure to a pet reptile as 2.25 (95% confidence interval (CI): 1.49-3.38, p<0.001).

Types of reptiles were tortoises (n=51), snakes (n=30, various types), lizards (n=31, various types), turtles or terrapins (n=5), with some participants reporting exposure to more than one of these.

Multivariable analysis

The results of the multivariable modelling process are presented in Table 2 below. All exposure variables with an OR>1.0 are shown here, whilst age, sex, ethnicity and variables with a modelled OR<1.0 are included in the model but not shown. The main exposure was associated with the outcome with an OR of 2.46 (95% CI: 1.57-3.85, p<0.001) in the final proximal model. We identified 10 other exposures with association with *Salmonella* infection independent of the main exposure. These were consistent with known risk factors for *Salmonella* [3,5]. None of the identified risk factors related to pets, although fishing was identified as weakly associated with *Salmonella* infections. None of the variables investigated as potential confounders were found have a major confounding effect (>10%) on the main exposure-outcome association.

In the final multivariable model, there was evidence of interaction between the effect of the main exposure and age category (likelihood ratio (LR) test p=0.03) and between the main exposure and sex (LR test p=0.01). Children under the age of five years were at much greater risk when exposed to reptiles than other age groups. For infants (under one year old) the OR was 17.3 (95% CI: 4.50-66.25) and for young children (between one and four years old) the OR was 44.6 (95% CI: 5.17-385). The age-stratified effects of the main exposure are shown in Table 3. Males appeared to be at higher risk of *Salmonella* infection when exposed to reptiles than females.

Salmonella serotypes

Numbers of cases of *Salmonella* serotypes with one or more isolates among people with reported reptile exposure are shown in Table 4. Odds ratios are calculated in comparison to *Campylobacter* control-cases. There was a clear indication that the overall pattern of serotypes of *Salmonella* seen among people with exposure to reptiles was different to that seen among people without this exposure (chi-square: 654, p<0.001, data not shown). *S.* Enteriditis and *S.* Typhimurium were no more common among reptile owners than would be expected by chance, whilst several other serotypes appeared to have some degree of association with exposure to reptiles.

Population attributable fraction

A PAF is defined as "the proportional reduction in average disease risk (...) that would be achieved by eliminating the exposure(s) of interest from the population" [17]. To estimate PAF for *Salmonella* disease caused by reptile exposure, we needed a proportion of the (general) population with this exposure (ppe). We used the proportion of *Campylobacter* cases reporting reptile ownership to estimate this: 74/11,204=0.66%. OR was used as an approximation of risk ratio as this was a rare exposure. Using the formula

$$\text{PAF} = \frac{\text{ppe}(\text{OR}-1)}{\text{ppe}(\text{OR}-1)+1}$$

and the OR from the final multivariable model, we obtained a PAF value of 0.95% for reptile exposure on *Salmonella* infections in England. If such a PAF were calculated for under five-year-olds only, it would be significantly larger than this: note the very high multivariable OR for these age groups in Table 3. However, we feel it is not appropriate to actually calculate such a figure as it would be unreliable due to the small numbers of individuals in these groups.

Discussion

Main findings

In this large case-case study of the exposures associated with *Salmonella* acquisition, we hypothesised that contact with reptiles was associated with development of illness after adjustment for alternative modes of acquisition and confounding factors. In our final multivariable model, there was a strong association of reported exposure to reptiles with *Salmonella* illness with an OR of 2.46 (95% CI: 1.57-3.85, p<0.001). The risk of exposure to reptiles was strongly influenced by age: children under the age of five years with this exposure were at much greater risk of developing *Salmonella* infection whilst individuals over the age of twenty years with this exposure did not experience significantly elevated risk.

These findings are unlikely to have occurred by chance, although the precise size of the effects could be subject to minor variation due to the small number of exposed individuals. None of the other variables of acquisition of infection in the multivariable model explained or negatively confounded this effect, and the effect of travel abroad acted as a positive confounder on the effect of reptile exposure. The effect of exposure to reptiles is unlikely to be confounded by unmeasured aspects of pet ownership in general as none of the seven other types of animal exposure examined in this analysis (dogs, cats, fish, poultry, other birds, other pets and farm animals) showed an independent association with *Salmonella* illness.

These findings are consistent with two case-control studies of risk factors associated with *Salmonella* infections in children in the United States (US) [10,11] where the odds of *Salmonella* illness in children were increased in association with recent contact with reptiles or amphibians.

There was clear indication from analysis of *Salmonella* serotypes that they were of different relative importance among people with and without exposure to reptiles. In those without recent reptile exposure, *S.* Enteritidis and *S.* Typhimurium predominated, in line with prevalent patterns of illness in the UK. Among those with exposure to reptiles, these two serotypes were much less common – they are known to be rare in poikilotherms [18] – and a variety of unusual *Salmonella* serotypes predominated. Many of these serotypes are known to be mainly found in reptiles (*S.* Arizonae [19]) or have previously been reported in cases or outbreaks of reptile-associated salmonellosis (*S.* Tel-el-Kebir [20], *S.* Java [21]). The analysis of serotypes was based on small numbers, so some of these associations may be chance effects.

We calculated a PAF for reptile exposure on all *Salmonella* infections in England during the study period as being 0.95%. We believe that this is the first such estimation made for such a PAF in England. This is consistent with an observation of 0.7% of *Salmonella* cases being of reptile-associated serotypes in the Netherlands [7], but less than a PAF estimate of 6% in a study specifically investigating reptile-associated salmonellosis in the US [10]. Although this PAF for reptile-associated salmonellosis in England is small, it represents a part of a sizeable disease burden – approximately 12,000 cases of salmonellosis were reported in England and Wales in 2007 [22], and this may underestimate the true community incidence by as much as threefold [4]. Furthermore, reptile-associated *Salmonella* appears to predominantly affect infants and children and could represent an amenable target for public health interventions [10].

Strengths and weaknesses

An important strength of the case-case format adopted for the CLASSP study was that it should have minimised bias due to case notification [23] as both cases and control cases came through the same notification process. The median interval from illness to interview was similar for *Salmonella* (11 days) and *Campylobacter* (nine days), indicating that a significant degree of recall bias was unlikely. As interviewers and participants were blind to the main hypothesis of this analysis, report of exposure to reptiles is unlikely to have been affected by interviewer bias or purposeful misreporting.

We are not aware of any association between *Campylobacter* illness and reptile ownership. Therefore whilst this case-case methodology may not have detected all exposures conferring risk for either *Salmonella* or *Campylobacter*, we are confident that it has accurately assessed the risk associated with the main exposure.

An important limitation of this analysis is the method of ascertainment of exposures, including the main exposure. Study participants were questioned on a wide variety of exposures and there were no objective validations of such exposure. Recall and report of pet ownership is likely to be more accurate than food recall, particularly as this concerned a period (on average) 9-14 days earlier. We felt that accuracy of exposure classification was likely to be adequate for the purposes of this analysis, and any resulting bias would be more likely to lead to underestimation than overestimation of the true effect size for the main exposure. The CLASSP study did not investigate RFs relating to susceptibility to disease (except age and sex) – factors such as recent antibiotic usage [24] may have had an effect on the development of illness.

Some element of bias may have been introduced to this study by use of different questionnaire methods between pathogen types: *Salmonella* cases were more likely to have a personal interview and *Campylobacter* cases were more likely to have a posted questionnaire. If there was differential accuracy in report of exposure by different interview methods this could have led to over or underestimation of effect sizes. We believe that such influences are unlikely to affect our main findings.

We analysed this study by comparing people with positive report of exposure against those with no reported exposure, such that people with unknown exposure status were included with the baseline group. This was done as a high proportion (>70%) of study participants had an unknown value for ≥1 exposure. The tick-box format of the questionnaire makes it likely that some participants omitted to tick for negative responses, which would lead to the data being Missing Not At Random (MNAR). The effects of bias introduced by this pragmatic compromise are limited: Other analyses of this dataset using different strategies (complete-case only and missing-indicator approaches) both suggested very similar sizes of effect for the main exposure-outcome relationship [25].

Some caution is required for the interpretation of the PAF estimate. The estimate of exposure to pet reptiles in the general population (0.66%) was obtained from the *Campylobacter* control cases in this study. The age distribution of *Campylobacter* cases did not match the general population – children were over-represented. Precise information on reptile ownership in the UK is difficult to obtain. A conference presentation in 2008 indicated there were approximately one million households in the UK with one or more pet reptiles, based on estimates from pet food sales [26], suggesting the calculated PAF may be an underestimate.

Conclusions

Reptile ownership is an important risk factor for *Salmonella* illness, with the effect being much stronger among infants and children. Although this exposure is rare in the general population, it may account for approximately 1% of *Salmonella* infections currently occurring in the UK. The calculated effect of exposure to reptiles is supported by the serological data on specific *Salmonella* serotypes seen among people self-reporting this exposure – these individuals are much more likely to be infected with unusual serotypes of *Salmonella* known to occur in conjunction with reptiles. Public health measures to minimise the risks of reptile-associated salmonellosis have been discussed elsewhere [10]. The HPA has published a leaflet outlining risks associated with reptiles [27]. Ownership of reptiles represents a serious risk to children.

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Nationwide outbreak of Salmonella serotype Kedougou associated with infant formula, Spain, 2008

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On 5 August 2008, the National Centre of Microbiology in Madrid, Spain, notified an increase in *Salmonella* Kedougou isolations compared to 2007, with 21 cases including 19 children under one year of age. Active case finding and a matched case-control study were carried out to confirm this increase, identify source, transmission mode and risk factors in order to implement control measures. Cases were defined as any child under one year of age with *S.* Kedougou isolated since 1 January 2008, and were matched for age, sex, medical practitioner and diagnosis week with controls who were selected among patients of the cases' medical practitioners. An *ad hoc* questionnaire was completed for cases and controls and a univariate analysis was conducted to identify risk factors. We found 42 isolates from 11 of the 19 Spanish Regions. Completed questionnaires were available for 39 of 42 patients identified; 31 were children under one year of age and fulfilled the case definition. The median age of the 31 cases was 4.3 months and 13 were male. Main symptoms were diarrhoea (n=31) and fever (n=13). Ten cases required hospitalisation. All 31 cases had consumed infant formula milk of Brand A which was associated with illness in the univariate analysis (exact matched odds ratio: 74.92; 95% confidence interval: 12.89-∞). All patient isolates showed indistinguishable pulsed-field gel electrophoresis and antimicrobial susceptibility patterns. Five milk samples from three cases' households were negative for *Salmonella*. Our results suggest that Brand A was the transmission vehicle of *S.* Kedougou in the outbreak that occurred in Spain between January and August 2008. Food safety authorities recalled five batches of Brand A milk on 26 August 2008. No further cases have been detected as of 15 September 2009.

Background

Salmonella Kedougou belongs to serogroup G *Salmonella* and is one of the nearly 2,000 *Salmonella* serotypes that can cause illness in humans, but it is a rare serotype identified in Spain. The National Centre of Microbiology (NCM) in Madrid isolated a mean of three *S.* Kedougou strains from humans per year between 2002 and 2007 (unpublished data). We only found two outbreaks involving this serotype in the literature: one in Norway in 2006 linked to consumption of Salami [1] and one in the United Kingdom in 1992 linked to cooked meat [2].

On 5 August 2008, the NCM notified an increase in number of *S.* Kedougou isolates during the first half of 2008: 21 isolates from seven Spanish regions, compared to six isolates in 2007 and two in 2006. Nineteen of these 21 isolates were from children under one year of age. The widespread distribution and the cases' age suggested a commercial infant food product as the likely vehicle of transmission in this *Salmonella* outbreak.

On 6 August 2008, the National Centre of Epidemiology (NCE) in collaboration with NCM, regional epidemiologists and microbiologists of the Spanish epidemiological surveillance network began an epidemiological study and sent an alert to the Spanish Food Safety and Nutrition Agency (SFSNA) and to the Ministry of Health. The alert was also sent to the European Food and Waterborne Diseases Network, asking for *S.* Kedougou increases during 2008. Eleven member countries answered but did not report any increase in *S.* Kedougou isolates.

The objectives of our study were to confirm the increase in number of cases and to identify the source of infection, the transmission mode and associated risk factors in order to implement appropriate control measures.

Materials and methods

Epidemiological investigation

An active case finding and a matched case-control study were conducted by the NCE in collaboration with NCM and regional and local epidemiologists to test the hypothesis that consumption of commercial infant food product was associated with the illness.

Active Case Finding

An outbreak case was defined as any person with an isolate of *S.* Kedougou identified during 2008. NCE sent a request to all regions in Spain through the Spanish Epidemiological Surveillance Network, to notify any case from whom *Salmonella* Group G was isolated in 2008.

We collected information on the cases with confirmed *S.* Kedougou infection using a structured questionnaire. They were filled in by regional and local epidemiologists in interviews with the cases or their parents. We asked for demographic information (age, sex, place of residence), clinical information (date of onset, main symptoms, severity, hospitalisation) microbiological information, human and/or animal contact, food consumed in the 72 hours before the onset of symptoms (including brands and batch numbers of infant food consumed), and information about the way of preparation and disinfection as well as the time from preparation to consumption.

The epidemiological data and food history of the first identified cases (see below) raised the hypothesis that consumption of infant formula could be the cause of infection and we started an analytical study.

Analytical study

A case was defined as any child under one year of age with *S.* Kedougou isolated between 1 January 2008

Figure 1

Cases of *Salmonella* Kedougou by region, Spain 2008 (N=42)

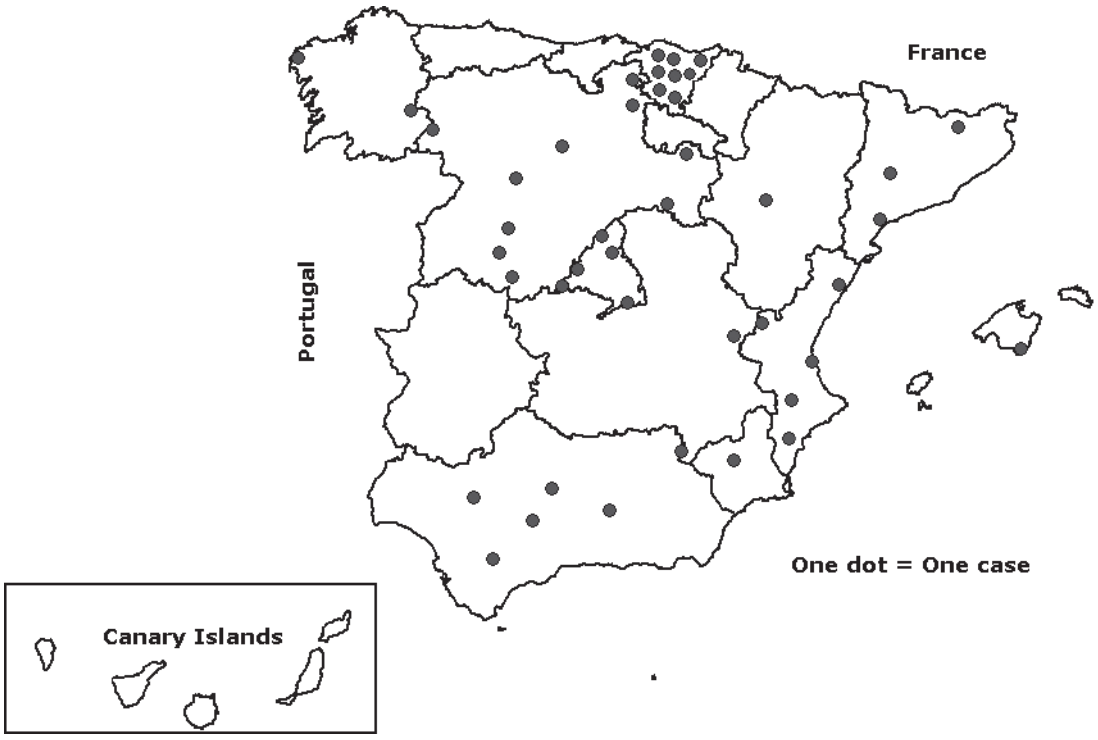
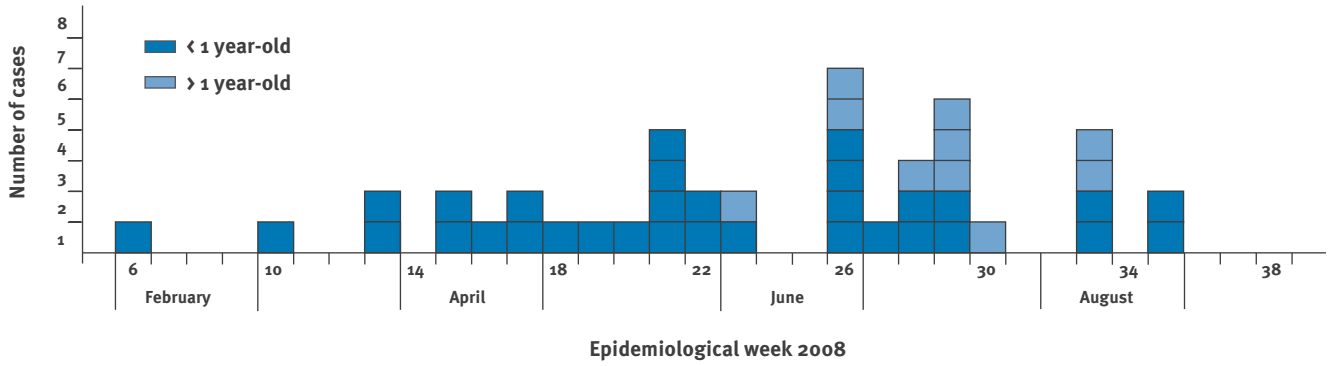


Figure 2

Cases of *Salmonella* Kedougou by week of isolation and age, Spain, 2008 (N=42)



and 31 August 2008. For each case, four controls were selected and matched for age (±one month), sex, same medical practitioner and week of diagnosis (±one week), without gastrointestinal symptoms and non-exclusive breastfeeding. The same questionnaire was applied to cases and controls, asking for information on their food intake during the three days previous to the onset of symptoms of the case.

Statistical analysis

Odds ratios (OR) and their 95% confidence intervals (CI) for the association between risk factors and disease were estimated using exact conditional logistic regression [3]. Maximum likelihood estimates (MLE) were applied when possible, and median unbiased estimates (MUE) when MLE could not be calculated. All analyses were carried out using STATA 10.0 [4-5].

Microbiological Investigation

Strains isolated from cases at regional hospital laboratories were sent to NCM for serotyping, and comparison of pulsed-field electrophoresis (PFGE) profiles and susceptibility patterns. The strains were tested for susceptibility to ampicillin, cefalotin, cefotaxime, amoxicillin/clavulanic acid, chloramphenicol, gentamicin, kanamycin, nalidixic acid, ciprofloxacin, tetracycline and trimethoprim/sulfamethoxazole.

Samples of infant foods (opened or unopened) provided by cases' households were also collected and sent to the laboratory of the SFSNA and to the regional laboratories for *Salmonella* testing.

Results

A total of 42 isolates from 42 patients were identified from January to August 2008. Sixteen patients were male and 32 were children under one year of age. Ten children under one year of age and a pregnant woman required hospitalisation. Patients were from 11 of the 19 regions in Spain (Figure 1).

The first isolate of *S.* Kedougou was identified on 4 February 2008 and the last one on 29 August 2008 (Figure 2).

Questionnaires for the children were answered by the parents. Completed questionnaires were available for 39 of 42 patients identified. For further analysis, we only considered cases under the age of one year. These were 31 of the 39 respondents, with a median age of 4.3 months, and 13 were male. Main symptoms were diarrhoea (n=31), fever (n=13) and vomiting (n=7). Blood was present in the stools of 20 of the patients. Ten children were hospitalised; none of them had a history of immunosuppression.

Five children had a mixed diet (breast feeding and infant formula) and 26 used infant formula exclusively. All the infants had consumed the same milk, Brand A, in the 72 hours before the onset of symptoms. Table 1 shows products consumed by the patients in the 72 hours before onset of symptoms, as well as other exposures.

The eight patients over one year of age, for whom a completed questionnaire was available, had a median age of 28 years (range: 1-84 years of age). Two patients were parents of cases under one year of age. Three patients had consumed powder infant formula of Brand A in the 72 hours before the onset of symptoms.

We included 22 cases and 70 controls in the matched case-control study (12 cases were matched with four controls, two cases with three controls and eight cases with two controls).

All cases included in this analytical study consumed infant formula of Brand A in the 72 hours before onset of symptoms compared with seven (10%) of the controls. Because all cases consumed an infant formula of Brand A, the maximum likelihood estimation for the OR of association with illness was infinite. The median unbiased estimate for the Mantel-Haenszel OR (OR_{M-H}) and the lower limit of the CI were thus calculated (exact OR_{M-H}: 74.92; 95% CI: 12,89–$^{\infty}$). Other food products, food preparation, or preservation and disinfection habits were not associated with the disease (Table 2).

Antimicrobial susceptibility tests and PFGE were done on all the strains. All PFGE pattern were indistinguishable (SAL-XBA-KDG-1) and all strains had the same sensitivity profile to all antibiotics tested.

We tested five samples of milk consumed before the occurrence of symptoms and provided by three cases' families. *Salmonella* was not detected in any of them.

Table 1

Distribution of exposures during the 72 hours before onset of symptoms in children under one year of age with isolation of *Salmonella* Kedougou, Spain, 2008 (N=31)

Product	N
Breast feeding	5
Milk of Brand A	31
Water	
Tap water	5
Bottled water	24
Baby cereal	11
Baby puree	
Fruit (homemade)	7
Fruit (commercial food)	5
Vegetables and chicken (homemade)	8
Baby bottle disinfection	
Boiling bottles	10
Using steriliser	11
Consumption of formula milk, cereal or puree immediately after preparation	28
Animal contact	9

No further cases' families were able to provide the batch number of the product consumed.

The infant formula Brand A distributed in Spain up to the day of the study had been produced in a local production plant. The company had closed this production plant in March 2008, five months before the outbreak alert. The results of factory quality control tests provided by the producers from raw materials and incriminated end products were negative for *Salmonella*, but positive for *Enterobacteriaceae* in some batches of end product.

On 26 August, the Spanish food safety authorities recalled five batches of infant formula of Brand A. This product was distributed only in Spain. A press release was issued informing people to avoid the use of these batches of milk Brand A and a contact telephone help line was set up to provide information to consumers.

Discussion

Our results suggest that the consumption of an infant formula of Brand A was associated with *S.* Kedougou infection. In our analytical study, 100% of the cases had consumed this milk compared with only 10% of the controls.

Outbreaks associated with infant powder formula are not uncommon because this is not a sterile product. This type of feeding is now usual because of many reasons such as the increased survival of premature babies and newborns with low birth weight, maternal illnesses in which breastfeeding is not recommended, early return of women to work after giving birth, or difficulties in breastfeeding [6].

We are not aware of any outbreak of *S.* Kedougou associated with infant formula. However, other serotypes of *Salmonella* had been associated with infant formula as a vehicle of transmission in many outbreaks in the world, such as in France (*S.* Agona, 2005), Korea (*S.* London, 2000), Spain (*S.* Virchow, 1994), Canada and the United States (*S.* Tennessee, 1993) [7-16]. One of the latest outbreaks occurred in France at the time of our study (*S.* Give, 2008) [17].

Outbreaks associated with commercial products like infant formula tend to have a low epidemic profile (small number of cases spread over long periods of time) because of the low bacterial load usually contained in the product [11]. However, continuous exposure to the factor for several months increases the probability of infection. The current Codex Alimentarius specification for *Salmonella* considers food products as fit for consumption when 60 samples of 25 gr are free from microorganisms [18]. Data provided by the infant food industry and inspection authorities indicate that *Salmonella* is rarely detected in powder infant products; nevertheless the microorganism can survive in powdered formula milk for up to 15 months and the method of detection can fail [19].

The increase in *S.* Kedougou isolations in Spain in 2008 was detected by the NCM because of the low expected frequency of this serotype in our country. This highlights the crucial role of the microbiology laboratories detecting outbreaks involving rare serotypes of microorganisms. Laboratory networks with a role in early detection of alert signals can complement surveillance systems in detecting uncommon microorganisms that otherwise might go unnoticed.

The higher attack rate in children under the age of one year, the identical PFGE pattern, the wide geographical distribution in Spain and the consumption of a particular brand of infant formula by the first cases identified lead to the hypothesis that the milk could be the vehicle of infection. Moreover, almost all cases older than one year could be explained by consumption of Brand A milk or by epidemiological link with younger cases.

In our study, most adult cases were probably secondary cases. Those cases for whom no contact with children under the age of one year could be established had consumed Brand A formula milk. By restricting our study to cases under one year of age we increased the specificity of the case definition because only primary cases were included. In case-control studies recall bias usually differs between cases and controls; parent cases tend to recall better than parent controls. In our study, we minimised this bias by restricting the

Table 2

Matched univariate analysis between *Salmonella* Kedougou infection and different exposures, Spain, 2008

Variable	Matched OR	95% CI
Tap water	2.11	0.43–10.22
Fruit baby food	0.8	0.08–7.51
Baby puree (vegetables and chicken)	0.34	0.01–26.07
Infant formula milk Brand A	**74.92[a]**	**12.89–∞**
Boiled water for baby bottle	5.77	0.95–35.02
Disinfection: water and detergent	0.48	0.12–1.92
Disinfection: boiled water	2.07	0.54–7.85
Animal contact	0.98	0.3–3.12

CI: confidence interval; OR: odds ratio.

[a] Median unbiased estimates; maximum likelihood estimation= infinite.

analysis to cases under the age of one year, for whom food consumption patterns are usually constant and thus less prone to recall bias. However, some bias could be present given the delay between the interview and the onset of symptoms (mean: 108 days, range: 9-222 days).

The matched case-control study design chosen [20] included matching for medical practitioner as a way to facilitate the control search and selection. This could have lead to overexposure among controls because doctors could tend to recommend the same infant formula to their patients. Nevertheless this assumption was not confirmed by our data, as only few controls consumed the involved milk brand compared with 100% of the cases.

In the case-control questionnaires, up to five varieties of Brand A milk were reported, but the small number of exposed controls did not allow further analysis to identify a specific variety associated with the outbreak. Except in two cases, it was not possible to obtain details on the batch of formula milk consumed by the patients.

The number of cases confirmed at the laboratory (42 cases) might be an underrepresentation of the real number of infections since only a small proportion of people with gastroenteritis seeks medical assistance and provide a sample for laboratory testing. This might be also applicable for the age group at risk (under one year) because even when parents seek medical assistance for their children, gastrointestinal illness might be frequently misdiagnosed as milk/food intolerance as initially happened in two of the cases identified.

This is the first outbreak of *S.* Kedougou associated with the consumption of infant formula in Spain. The results of the investigation involving epidemiological services of all Spanish regions and the NCE support the hypothesis that the Brand A formula milk was the vehicle of the *S.* Kedougou gastroenteritis outbreak, occurring between February and August 2008.

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