Case report of clinical salmonellosis by *Salmonella* Typhimurium that occurred in Portuguese children

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Keywords
antimicrobial resistance, children, pulsed-field gel electrophoresis, *Salmonella* Typhimurium.

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Abstract

Aims: Aim of this study is to characterize clinical isolates of *Salmonella* Typhimurium that occurred in Portuguese children on the basis of their virulence and antimicrobial resistance profiles and pulsed-field gel electrophoresis typing and to analyse possible strain relatedness.

Methods and Results: Different *Salmonella* serotypes were isolated from clinical cases of salmonellosis that had occurred in two Portuguese hospitals (a total of 259 isolates). All *Salm.* Typhimurium strains, with the age of the patients known, (total of 26 isolates) were selected for this study. These isolates were characterized for their virulence gene profiles (*agfA*, *iroB*, *slyA*, *hin/H2*, *spv*), antimicrobial resistance profiles and investigated for the occurrence of multidrug-resistant *Salm.* Typhimurium DT 104 by PCR. *Salmonella* isolates showed high rates of resistance to four or more antibiotics, 100% resistance to sulfadiazine and a high percentage of strains with the resistance profile of *Salm.* Typhimurium DT 104, two of them with this phage type (determined by PCR). A relationship between some clusters and their resistance and virulence profiles was detected, each cluster having the same profile.

Conclusions: This study showed high-antibiotic resistance of the *Salmonella* strains investigated, and the presence of multidrug-resistant *Salm.* Typhimurium DT104 in infections of Portuguese children.

Significance and Impact of the Study: Study is based on regarding the increase in antibiotic resistance by *Salmonella* strains isolated from infections in Portuguese children and on the presence of *Salm.* Typhimurium DT 104 circulating in Portugal.

Introduction

Nontyphoidal *Salmonella* (NTS) are major foodborne pathogens and the leading cause of hospitalization and death because of foodborne infections in industrialized countries (European Food Safety Authority, 2009). In the European Union, *Salmonella* Enteritidis and *Salmonella* Typhimurium are the serovars most frequently associated with human illness (European Food Safety Authority, 2009). Most cases are self-limiting, lasting 5–7 days with no antibiotic treatment required. However, antimicrobial treatment may be necessary for invasive infections, particularly in younger, older and immuno-compromised individuals (Solghan *et al.* 2010). Urinary tract infections caused by *Salmonella* are rare (oscillates between 0·015 and 0·118%), but occur mainly in children <10 years old and adults older than 60 years (Geffken *et al.* 1996; Tena *et al.* 2007). It is unclear whether the increased rate of salmonellosis among infants result from greater susceptibility, or whether infants are simply more likely than persons in other age groups to presented for medical care or have stool cultures performed for diagnosis of the infection. However, infants are more likely to experience severe illness and death from salmonellosis, and infants with immuno-compromising conditions are particularly vulnerable (World Health Organization and Food and Agriculture Organization 2006). The emergence of *Salm.* Typhimurium showing multidrug-resistance in

general, and the definitive phage type 104 (DT 104) in particular exhibiting the penta-resistant profile ACSSuT (resistant to at least ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline), have prompted global concern because of a link with more invasive disease and a higher case fatality rate (Greene *et al.* 2008; Solghan *et al.* 2010). Increased levels of multidrug-resistant NTS in food products have corresponded to increased use of antibiotics in food animals for therapeutic purposes, disease prevention and growth promotion (Solghan *et al.* 2010). Virulence genes may also play an important role in disease. Examples are *agf*A (thin aggregative fimbriae), *sly*A (encoding a transcriptional regulator of virulence genes required for survival within macrophages, initially identified as salmolysin), *spv*C (*Salmonella* plasmid virulence, important for systemic infection), *iro*B (regulation by iron) and *hin/*H2 (flagellar phase variation) (del Cerro *et al.* 2003; Rodríguez *et al.* 2006).

The aim of this study was to characterize *Salm.* Typhimurium isolates from Portuguese children with urinary and gastrointestinal tract infections, based on virulence genes (*agfA*, *iroB*, *slyA*, *hin/H2*, *spv*) and antimicrobial resistance profiles (ampicillin, chloramphenicol, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfadiazine, trimethoprim-sulfamethoxazole and tetracycline). Further aims included investigation for occurrence of the multidrug-resistant *Salm*. Typhimurium DT 104 by PCR, and pulsed-field gel electrophoresis (PFGE) typing to analyse strain relatedness.

Material and methods

Bacterial isolates

Different *Salmonella* were isolated from clinical cases of salmonellosis that had occurred in two hospitals from the North of Portugal (Hospital de São Marcos and Hospital de São João), in a total of 259 isolates (Table 1), between March 2003 and September 2007. All *Salm.* Typhimurium strains, with the age of the patients known (total of 26 isolates), were selected for this study. Serotyping of the isolates was performed at the hospitals. Two of these isolates were collected from urine samples of urinary tract infection and 24 from faeces of gastrointestinal infection. Isolates were stored at −20°C in Tryptone Soy Broth (TSB; Lab M, Lancashire, UK) with glycerol (30% v/v) and, when necessary, were streaked on Tryptone Soy Agar (TSA; Lab M) plates and incubated at 37°C for 24 h.

Table 1 Frequency of *Salmonella* serotypes isolated from clinical cases of salmonellosis that had occurred in two hospitals from the North of Portugal

Serotype	No. isolates (%)
Enteritidis	174 (67·2)
Typhimurium	30 (11·6)
Paratyphi A	1 (0·4)
Unidentified	54 (20·8)

Antimicrobial susceptibility testing

The minimal inhibitory concentration (MICs) of eight different antimicrobial agents: streptomycin (S), kanamycin (K), gentamicin (G), ampicillin (A), nalidixic acid (Nx), chloramphenicol (C), tetracycline (T) and sulfadiazine (Su) were determined by the agar dilution method as recommended by the Clinical Laboratory Standards Institute (CLSI 2007). The breakpoints used were those defined by the CLSI for *Enterobacteriaceae*. *Staphylococcus aureus* ATCC 29213 was used as the control strain. For each antibiotic susceptibility determination, at least duplicate experiments were performed.

PCR-based methods

Extraction of DNA was performed as previously described by Salehi *et al.* (2005).

The detection of the virulence genes *agf*, *hin/H2*, *slyA*, *spvC*, *iroB* and the identification of the DT 104 phage type was performed as previously described by del Cerro *et al.* (2003) and Pritchett *et al.* (2000), respectively. The primers are described in Table 2. In all experiments, a negative control without template DNA and a positive control with the DNA of *Salm.* Typhimurium ATCC 14028 (*hin/H2*, *slyA*, *spvC*, *iroB*) or *Salm.* Enteritidis ATCC 13076 (*agf*) were used.

For the identification of the DT 104 phage type, *Salm.* Typhimurium DT 104 FSL W1-032, kindly provided by Prof. Martin Wiedmann (Department of Food Science, Cornell University) was used as a positive control. All the PCR experiments were made in triplicate.

Gels were stained with ethidium bromide, and bands visualized by UV light illumination (Gel Doc 2000; Bio-Rad, Richmond, CA, USA).

Pulsed-field gel electrophoresis

Clonality among isolates was assessed by PFGE following *Xba*I and *Avr*II digestion of genomic DNA according to the standard 1-day PulseNet protocol of the Centers for Disease Control and Prevention with some modifications: agarose-embedded DNA plugs were cut (2·0 mm) and restricted with 50 U of *Xba*I (MBI Fermentas, Mundolsheim, France) or 30 U of *Avr*II (MBI Fermentas) for 5 h

Table 2 Primer sequences for PCR assays used in the study

Target	Oligonucleotide primers Sequence (5′–3′)	Reference
iroB gene	[TGCGTATTCTGTTTGTCGGTCC/TACGTTCCCACCATTCTTCCC]	Baümler *et al.* (1997)
agfA gene	[TCCGGCCCGGACTCAACG/CAGCGCGGCGTTATACCG]	Doran *et al.* (1993)
hin/H2 gene	[CTAGTGAAATTGTGACCGCA/CCCATCGCGCTACTGGTATC]	Way *et al.* (1993)
spvC gene	[ACTCCTTGCACAACCAAATGCGGA/TGTCTTCTGCATTTCGCCACCATCA]	Chiu and Ou (1996)
slyA gene	[GCCAAAACTGAAGCTACAGGTG/CGGCAGGTCAGCGTGTCGTGC]	Soto *et al.* (2000)
DT 104	[GTCAGCAGTGTATGGAGCGA/AGTAGCGCCAGGACTCGTTA]	Pritchett *et al.* (2000)

at 37°C (CDC 2004). Genomic DNA from *Salmonella enterica* serotype Braenderup H9812 was also restricted with *Xba*I and used as a molecular size marker. Gel images were obtained using Gel Doc 2000 under UV transillumination. Banding patterns were analysed with Gelcompar software (Applied Maths, Sint-Martens-Latem, Belgium) using the Dice similarity coefficient and clustering was created using the unweighted-pair group method using average linkages (UPGMA).

Results

Antimicrobial susceptibility testing and identification of *Salmonella* DT 104

A high percentage of resistance was observed for ampicillin (92·3%), chloramphenicol (69·2%), streptomycin (84·6%), tetracycline (80·7%) and sulfadiazine (100%). Most of the isolates were sensitive to trimethoprim-sulfamethoxazole, nalidixic acid, gentamicin and kanamycin. Most of the isolates (92·3%) were resistant to more than one antibiotic, and 80·7% of the isolates were resistant to four or more antibiotics. The resistance profile of the strains investigated is presented in Table 3. Simultaneous resistance to ampicillin, chloramphenicol, streptomycin, sulfadiazine and tetracycline (ACSSuT) was the most common (50·0%) profile among the isolates. From the thirteen multi-resistant clinical isolates (ACSSuT), two isolated in different years (2003 and 2005) were positive in the DT 104 PCR test.

Table 3 Frequency of *Salmonella* Typhimurium resistance patterns

Resistance profile	Number of strains (%)
Su	2 (7·7)
ASu	2 (7·7)
ASSu	1 (3·8)
ASSuT	2 (7·7)
ACSSuT	13 (50·0)
ASSuTNx	1 (3·8)
ACSSuTNx	5 (19·2)

A, ampicillin; C, chloramphenicol; S, streptomycin; T, tetracycline; STX, trimethoprim/sulfamethoxazole; Nx, nalidixic acid; Su, sulfadiazine.

PCR-based methods

The virulence genes profile was determined by conventional PCR using specific primers (*agfA*, *iroB*, *hin/H2*, *slyA*, *spvC*). Results showed that the genes *iroB* (regulation by iron), *slyA* (salmolysin), *hin/H2* (flagellar phase variation) and *spvC* (*Salmonella* plasmid virulence) were present at different frequencies (Table 4).

Pulsed-field gel electrophoresis

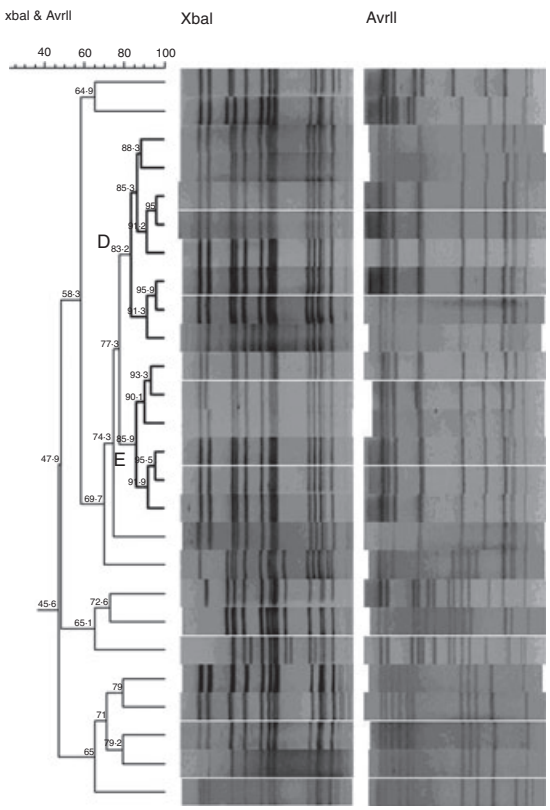
Clusters were defined using an 80% similarity according to Foley *et al.* (2006). After digestion of DNA with *Xba*I, two clusters were found. Following the digestion of DNA with *Avr*II, only one cluster was found (data not shown). The dendrogram produced by the combination of the two enzymes (Fig. 1) resulted in two clusters (D and E).

Discussion

Thirty of 259 isolates were classified as *Salm.* Typhimurium, the objective of this study. Four isolates were not included because of lack of information on patients' age. Interestingly, all the 26 isolates were recovered from children (age range between 9 months and 9 years). This represents 8·9% of all the isolates, which is in agreement with the report of EFSA for EU MS (European Union Member States) in 2005 for the serotype *Salm.* Typhimurium (9·1%) (European Food Safety Authority, May,

Table 4 Frequency of *Salmonella* Typhimurium virulence genes

Virulence gene	No. isolates (%)
agfA	0 (0)
iroB	25 (96·2)
hin/H2	26 (100)
spvC	20 (76·9)
slyA	26 (100)



Isolate n°	Sample	Date of Isolation	Hospital	Resistance Profile	Virulence Genes Profile				
					agfA	*iroB*	*hin/H2*	*spvC*	*slyA*
2	Urine	22-08-2007	HSJ	Su	−	+	+	+	+
9	Faeces	21-12-2004	HSM	Su	−	+	+	+	+
10	**Faeces**	**20-09-2003**	**HSM**	**ACSSuT**	**−**	**+**	**+**	**+**	**+**
18	**Faeces**	**05-12-2003**	**HSM**	**ACSSuTNx**	**−**	**+**	**+**	**+**	**+**
23	**Faeces**	**29-09-2005**	**HSM**	**ACSSuTNx**	**−**	**+**	**+**	**+**	**+**
7	**Faeces**	**29-09-2006**	**HSJ**	**ACSSuTNx**	**−**	**+**	**+**	**+**	**+**
26	**Faeces**	**11-03-2006**	**HSM**	**ACSSuT**	**−**	**+**	**+**	**+**	**+**
21	**Faeces**	**29-03-2005**	**HSM**	**ACSSuT**	**−**	**+**	**+**	**+**	**+**
5	**Faeces**	**30-05-2005**	**HSM**	**ACSSuT**	**−**	**+**	**+**	**+**	**+**
4	**Faeces**	**18-01-2005**	**HSM**	**ACSSuT**	**−**	**+**	**+**	**+**	**+**
13	**Faeces**	**15-06-2005**	**HSM**	**ASuT**	**−**	**+**	**+**	**+**	**+**
28	**Faeces**	**18-07-2005**	**HSM**	**ASu**	**−**	**+**	**+**	**+**	**+**
27	**Faeces**	**17-08-2005**	**HSM**	**ACSSuTNx**	**−**	**+**	**+**	**+**	**+**
14	**Faeces**	**23-06-2006**	**HSM**	**ACSSuT**	**−**	**+**	**+**	**+**	**+**
24	**Faeces**	**20-07-2007**	**HSM**	**ACSSuTNx**	**−**	**+**	**+**	**+**	**+**
12	**Faeces**	**15-01-2005**	**HSM**	**ACSSuT**	**−**	**+**	**+**	**+**	**+**
20	Faeces	19-03-2007	HSM	ACSSuT	−	+	+	+	+
1	Urine	15-03-2005	HSM	ACSSuT	−	+	+	+	+
11	Faeces	20-04-2006	HSM	ASSu	−	−	+	+	+
16	Faeces	05-02-2006	HSM	ASSuTNx	−	+	+	−	+
8	Faeces	13-06-2007	HSM	ASSuT	−	+	+	−	+
15	Faeces	01-06-2007	HSM	ACSSuT	−	+	+	+	+
17	Faeces	13-06-2003	HSM	ACSSuT	−	+	+	−	+
19	Faeces	12-01-2004	HSJ	ACSSuT	−	+	+	−	+
6	Faeces	05-08-2003	HSM	ASSuT	−	+	+	−	+
25	Faeces	21-09-2003	HSM	ACSSuT	−	+	+	+	+

Figure 1 Dendrogram of similarity illustrating the genetic relationships between the *Xba*I and *Avr*II profiles using the dice coefficient, and clustering was by UPGMA. Bold cluster D and E. (HSM: Hospital de São Marcos and HSJ: Hospital de São João).

2007). In Portugal, as far as we know, no data have been published about the prevalence of *Salm.* Typhimurium in children and resistance profiles of these foodborne isolates have never been reported in our country. The only data reported are about salmonellosis cases: 47% in children (0–4 years old) in 2006 (DGS 2007).

All of the isolates investigated were resistant to sulfadiazine, which was the antibiotic with a higher percentage of resistance (59·1%) for *Salm.* Typhimurium in 2006 in EU MS (European Food Safety Authority, December, 2007). The high percentage of resistance to sulfonamides might be due to the high use of this antibiotic in food-producing animals, as had already been verified in Portugal by Pena *et al.* (2004). The percentage of resistance to trimethoprim-sulfamethoxazole, nalidixic acid, gentamicin and kanamycin, are in agreement with the EFSA report for the EU MS (European Food Safety Authority, December, 2007). The percentage of the isolates resistant to four or more antibiotics was 80·7%; this value is considerably higher than the 39·7% reported by EFSA for EU MS in 2006 (European Food Safety Authority, December, 2007). ACSSuT resistance profile is commonly described for *Salm.* Typhimurium DT 104 (Biendo *et al.* 2005). Although being expected, the presence of *Salm.* Typhimurium DT 104 detected in our study in two children is always of concern, although expected. *Salmonella* Typhimurium DT 104 has caused outbreaks of infection in Ireland, Denmark, Germany, Austria, France, Czech, Italy, Sweden, Trinidad, South Africa, Netherlands, United Arab Emirates, Philippines and Israel (Threlfall 2000). This phage type has become a matter of concern because of its rapid international dissemination in the 1990s and its ability to readily acquire additional resistance traits to other clinically important antimicrobial drug classes, such as quinolones, trimethoprim and cephalosporins (Helms *et al.*, 2005).

The lack of virulence gene *agfA* (specific fimbrial) (Table 4) in all isolates is in agreement with previous observations by del Cerro *et al.* (2003) and Rodríguez *et al.* (2006). Seventy-three percent of the isolates were positive for *iroB*, *hin/H2*, *slyA*, *spvC*, an already founded profile for *Salm.* Typhimurium as observed by del Cerro *et al.* (2003) and Rodríguez *et al.* (2006).

In this study, PFGE was used to investigate any relationship between the 26 isolates. Two restriction enzymes were used because if genetic variation does not

significantly impact the size or electrophoretic mobility of a restriction fragment, then the change may not be identified as a separate pulsotype. This limitation can be mitigated to some extent by using a second enzyme for PFGE analysis, which has been shown to further increase the discriminatory power for differentiating several bacterial pathogens, including *Salmonella* (Foley *et al.* 2006). Observing the dendrogram of Fig. 1, the isolates no. 12 and 14 in cluster E such as no. 4, 5, 21, 26, 10 in cluster D, showed the same virulence and resistance profile and a high percentage of similarity (91·9 and 83·2% respectively). The same occurred with another resistance profile in isolates no. 7, 23 and 18 in different years (2006, 2005 and 2003, respectively) demonstrating being part of the same cluster with high similarity. Two isolates, no. 13 and no. 28, showed the same unusual resistance profile (that represents 7·7% of resistance profile frequency). These isolates also showed the same virulence profile, 93·3% of similarity with two combined enzymes and isolated within a month of difference, which may indicate the same source. The isolate no. 27 is the only one in these clusters that showed the lack of *spv*C gene which could be explained by the known fact that the plasmids can be lost, and thereby its inclusion in the clusters.

This study showed that *Salm.* Typhimurium isolates investigated had high rates of resistance to four or more antibiotics, 100% resistance to sulfadiazine, a high percentage of strains with the resistance profile of *Salm.* Typhimurium DT 104 and two with this phage type. Therefore, our study reinforces the need for attention regarding antibiotic resistance of *Salm.* Typhimurium.

Acknowledgements

Grateful acknowledgements to Maria Dolores Pinheiro (Hospital de São João, Porto), Maria Alberta Faustino and Maria Carmen Iglesias (Hospital de São Marcos, Braga) for supplying strains and epidemiological information.

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