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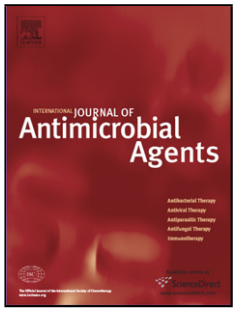

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Molecular characterisation of multidrug-resistant *Salmonella enterica* serotype Infantis from humans, animals and the environment in Italy

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ABSTRACT

During 2005–2006, *Salmonella enterica* serotype Infantis strains isolated from human and non-human sources and resistant to ampicillin (A), chloramphenicol (C), streptomycin (S), sulphonamide (Su), tetracycline (T), kanamycin (K) and trimethoprim/sulfamethoxazole (Sxt) emerged in Italy. The aim of this study was to analyse the molecular basis of antibiotic resistance and to evaluate the clonal origin of multiresistant *S*. Infantis strains isolated from different sources. Seventy *S.* Infantis strains, susceptible or resistant to antimicrobial drugs, were chosen for this study. Polymerase chain reaction (PCR) and conjugation experiments were performed to identify and localise the resistance genes in multidrug-resistant strains. PCR-based replicon typing was carried out for characterisation of conjugative plasmids. All strains were tested by pulsed-field gel electrophoresis (PFGE) according to the PulseNet protocol, and cluster analysis was performed using BioNumerics software. Strains with resistance (R)-type ACSSuTKSxt harboured $bla_{\text{TEM-1}}$, *strA-B*, *sul2*, *tet*(B), *catA1* and *aphA-1* resistance genes as well as a 2.2-kb class 1 integron containing *folA*, *catB3*, *aadA4* and *sul1* gene cassettes. A unique plasmid, belonging to the HI1 incompatibility group, harboured all the resistance genes. Cluster analysis showed that all ACSSuTKSxt-resistant strains belonged to a large cluster (A) with >90% genetic similarity. The presence of a plasmid harbouring all the resistance gene cassettes as well as molecular typing by PFGE demonstrated the circulation of <AF>a cluster of *S*. Infantis R-type ACSSuTKSxt during 2005–2006 in Italy. The presence of a plasmid conferring multidrug resistance could have facilitated the spread of a group of similar isolates through a variety of sources.

Introduction

Infections caused by the non-typhoidal *Salmonella* (NTS) serotypes continue to be a major public health burden worldwide. Although NTS usually causes self-limiting diarrhoea, invasive infections with bacteraemia and focal infections may occur [1]. In addition, in the last two decades a dramatic increase in multidrug-resistant (MDR) *Salmonella* has occurred, mainly due to the clonal spread of *S. enterica* serotype Typhimurium Definitive Type 104 (DT104) with the penta-resistance pattern ACSSuT [ampicillin (A), chloramphenicol (C), streptomycin (S), sulphonamide (Su) and tetracycline (T)] and of *S.* Typhimurium and its monophasic variant (*S.* 4,5,[12]:i:-) with the tetra-resistance pattern ASSuT [2–6]. Among NTS, *S. enterica* serotype Infantis is a cause of human salmonellosis in several countries [7,8] and represents the third most common serotype isolated in Europe (1.1%) after *S. enterica* serotype Enteritidis and *S.* Typhimurium (http://www.sva.se/upload/pdf/rapport/Zoonoses_CSR_2008.pdf).

In Italy, the surveillance system for enteric pathogens (EnterNet Italia), co-ordinated by the Istituto Superiore di Sanità (Rome), reported that the rate of isolation of *S.* Infantis from human infections ranged from 2% to 7% in the period 1980–2009, in several years representing the third or fourth most prevalent serotype after *S.* Typhimurium, *S.* Enteritidis and *S.* 4,5,[12]:i:- (http://www.iss.it/salm/arch/cont.php?id=1&lang=1&tipo=4). Regarding non-human sources, data from the Italian veterinary surveillance system (http://www.izsvenezie.it/images/stories/Pdf/Salmonelle/Enter_Vet_2007_2008.pdf) reported that this serotype is mainly isolated in pigs and poultry as well as in food derived from these animal species. Moreover, during the last years *S.* Infantis has

represented the third serotype among environmental samples (http://www.iss.it/binary/publ/cont/ONLINE_LINKABILE.pdf).

Antimicrobial resistance does not appear to be an important issue in this serotype; in fact, *S.* Infantis strains have always shown a low rate of antimicrobial resistance. In Italy in 2000, 0.5% and 2% of *S.* Infantis strains from human and animal sources, respectively, showed resistance to streptomycin (S), sulphonamide (Su) and tetracycline (T) [9]. However, in 2005 an increase in antimicrobial resistance among *S*. Infantis strains was reported by EnterNet Italia. In particular, 5.4% and 6.5% of strains isolated from human and non-human sources, respectively, showed multidrug resistance. Strains with an SSuT resistance pattern with additional resistance to ampicillin (A), chloramphenicol (C), kanamycin (K) and trimethoprim/sulfamethoxazole (Sxt) (ACSSuTKSxt) were the most frequent.

The aim of this study was to investigate the molecular basis of antibiotic resistance, to localise the genetic mechanism responsible for the MDR phenotype and to evaluate the genetic relationship of resistance (R)-type ACSSuTKSxt *S.* Infantis strains isolated in Italy from different sources between 2002 and 2008. Susceptible and resistant *S.* Infantis strains other than R-type ACSSuTKSxt have been included for comparison.

Materials and methods

2.1. Bacterial strains

Seventy *S.* Infantis strains isolated in Italy between 2002 and 2008, without any apparent epidemiological link, collected at the Istituto Superiore di Sanità in the framework of EnterNet Italia surveillance system (http://www.iss.it/ente), were

included in this study. The antimicrobial resistance pattern as well as the source of isolation of strains is shown in Table 1.

Serotyping of *Salmonella* spp. isolates was performed by slide agglutination according to the Kauffmann–White scheme [10]. Antimicrobial resistance was assessed by the disk diffusion method [11] using the EnterNet reference panel [6], which includes the following antibiotic disks (Becton Dickinson, Milan, Italy) and concentrations: nalidixic acid (Nx) 30 μg; ampicillin (A) 10 μg; cefotaxime (Ctx) 30 μg; ciprofloxacin (Cp) 5 μg; chloramphenicol (C) 30 μg; gentamicin (G) 10 μg; kanamycin (K) 30 μg; streptomycin (S) 10 μg; sulphonamides (Su) 0.25 μg; tetracycline (T) 30 μg; and trimethoprim/sulfamethoxazole (Sxt) 1.25/23.75 μg. *Escherichia coli* ATCC 25922 was used as a control strain in each experiment.

2.2. Detection of antimicrobial resistance genes

DNA samples were prepared using a Wizard Genomic DNA Purification Kit (Promega, Milan, Italy) and were analysed by standard polymerase chain reaction (PCR) using specific primers for the genes conferring resistance to ampicillin (bla_{TEM} and bla_{PSE}), chloramphenicol (*catA1* and *floR*) streptomycin (*strA–strB*), sulphonamides (*sul2*), tetracyclines [*tet*(A), *tet*(B), *tet*(C) and *tet*(G)] and kanamycin (*aphA-1*) and for detection of class 1 integrons (5'CS–3'CS) [12–14]. Reactions were performed with 2.5 U of *Taq* DNA Polymerase (Bioline, London, UK) according to the manufacturer's recommendations. PCR amplicons of the *bla* gene and class 1 integron fragment were fully sequenced.

2.3. Bacterial conjugation and plasmid characterisation

Conjugation experiments were performed to determine whether antimicrobial resistance markers were located on conjugative plasmids. An *S*. Infantis strain (n. 34/13/09) with R-type ACSSuTKSxt, isolated in Italy in 2005 from a surface water sample, was used as the donor in conjugation experiments. *Escherichia coli* CSH26Nx^{R} strain was used as the recipient, and the protocol of Maimone et al. [15] was followed with a few modifications. Briefly, bacterial pellets of overnight broth cultures of the donor and recipient strains were mixed at a ratio of 1:4 and were incubated overnight at 28 °C and 37 °C in nutrient agar. Transconjugants were selected on Luria–Bertani agar plates containing nalidixic acid (40 μg/mL) with chloramphenicol (30 μg/mL) or tetracycline (30 μg/mL). Transconjugant colonies were further purified by plating on selective agar media and were tested by PCR for antimicrobial resistance and for the presence of resistance genes.

Characterisation of plasmids was carried out following the PCR-based replicon typing protocol (PBRT) using positive controls kindly supplied by Dr A. Carattoli [16]. In the PBRT assay, MDR S. Infantis strains with R-type different from ACSSuTKSxt were included as controls.

2.4. Pulsed-field gel electrophoresis (PFGE) and cluster analysis

PFGE analysis was performed according to the PulseNet standardised protocol [17] using *Xba*I as the restriction enzyme (New England Biolabs, Ipswich, MA). *Salmonella enterica* serotype Braenderup H9812 strain was used as the molecular size marker [18]. Dendrogram and cluster analysis were performed using algorithms

available within the BioNumerics software package v.6.0 (Applied Maths, Sint-Martens-Latem, Belgium). Simpson's index was used to evaluate the discriminatory power of the *Xba*I enzyme [19]. Percent similarity between different chromosomal fingerprints was scored by the Dice coefficient. The unweighted pair group method with arithmetic means (UPGMA), with a 1.00% tolerance limit and 1.00% optimisation, was used to obtained the dendrogram. DNA profiles differing by one or more DNA fragments were considered as distinct patterns. Strains with a coefficient of similarity ≥90% were considered as genetically closely related.

Results

3.1. Antimicrobial resistance genes

PCR detection of resistance genes showed that all R-type ACSSuTKSxt strains carried the following resistance genes: bla_{TEM-1}, encoding ampicillin resistance; *strA-B*, encoding streptomycin resistance; *sul2*, encoding sulphonamide resistance; *tet*(B), encoding tetracycline resistance; *catA1*, encoding chloramphenicol resistance; and *aphA-1*, encoding kanamycin resistance (Table 2). Moreover, PCR for the detection of class 1 integrons produced a 2.2-kb amplicon encoding the *folA*, *catB3*, *aadA4 and sul1* gene cassettes conferring resistance to trimethoprim, chloramphenicol, streptomycin and sulphonamides, respectively. The same resistance genes were also found in the strains with R-type ACSuTKSxt, with the exception of *strA-B* genes, and in the strain with R-type ACSSuTSxt, with the exception of *aphA1*, as expected (Table 2). Four strains with R-type SSuTNx were positive for *tet*(A), encoding tetracycline resistance, and for a 1-kb class 1 integron containing *aadA1* and *sul1* gene cassettes conferring resistance to aminoglycosides and sulphonamide. Also, the strain with R-type ASSuTSxt was positive for the

presence of *bla*$_{TEM-1}$ and *tet*(A) genes and a 1-kb class1 integron (*aadA1–sul1*). Finally, the strain with R-type SSuTSxt was positive for the presence of *strA-B*, *tet*(A) and *sul2* genes (Table 2).

3.2. Conjugation and plasmid characterisation

Transconjugants from *S*. Infantis strain n. 34/13/09 were obtained from Luria–Bertani agar plates containing nalidixic acid both with chloramphenicol or tetracycline following incubation at 28 °C, whereas no growth was observed when plates were incubated at 37 °C. Antimicrobial susceptibility tests carried on transconjugants strains revealed that the R-type ACSSuTKSxt was completely transferred from donor to recipient by a conjugative plasmid. Also, PCR for the detection of resistance genes and class 1 integrons performed on transconjugant strains demonstrated that *bla*$_{TEM-1}$, *strA-B*, *sul2*, *tet*(B), *catA1* and *aphA-1* genes and a 2.2-kb class 1 integron encoding the *folA, catB3*, *aadA4* and *sul1* gene cassettes were on the conjugative plasmid. PBRT showed that the transconjugants and all *S*. Infantis strains with R-type ACSSuTKSxt harboured a plasmid belonging to the incompatibility group HI1 (p34/13/09). MDR S. Infantis strains with R-type SSuTNx and ASSuTSxt were positive for IncP group, whereas the strain with R-type SSuTSxt was positive for IncN group (Table 2).

3.3. Cluster analysis

The genetic relatedness among the 70 strains included in this study was evaluated by PFGE cluster analysis (Fig. 1). All strains were typeable by PFGE, and *Xba*I restriction produced 36 unique profiles. The discriminatory ability of the restriction

enzyme was 0.96, determined by the Simpson's index. Overall, two main clusters were distinguished (A and B), comprising restriction patterns with a genetic homology of 91.3% and 91.5%, respectively. Cluster A included all R-type ACSSuTKSxt strains [Int1(*folA–catB3–aadA4–sul1*); bla_{TEM}; *catA1*; *strA-B*; *sul2*; *tet*(B); *aphA-1*], 13 susceptible strains and 3 strains with R-type ACSuTKSxt [Int1(*folA–catB3–aadA4–sul1*); bla_{TEM}; *catA1*; *sul2*; *tet*(B); *aphA-1*] and 1 strain with R-type ACSSuTSxt [Int1(*folA–catB3–aadA4–sul1*); bla_{TEM}; *catA1*; *strA-B*; *sul2*; *tet*(B)]. Cluster B included 14 susceptible strains, 4 strains with R-type SSuTNx [Int1 (*aadA1–sul1*); *tet*(A)] and 2 strains with R-types SSuTSxt [*strA-B*; *sul2*; *tet*(A)] and ASSuTSxt [Int1(*aadA1–sul1*); bla_{TEM}; *tet*(A)], respectively.

Both clusters comprised isolates from different sources and in some cases they showed 100% genetic homology. In particular within cluster A (Fig. 1), one group included 8 strains, of which 5 were R-type ACSSuTKSxt isolated from human (3) and surface water (2); two other groups included 4 and 3 R-type ACSSuTKSxt strains isolated from human (2), surface water (1), processed poultry meat (1), processed pork (1) and human (2), respectively.

Finally, 10 strains, both sensitive or resistant to one to two drugs, showing a genetic homology <90% with respect to cluster A and B, were considered not closely related.

Discussion

An increased incidence of MDR strains of *S.* Infantis isolated from different sources, mainly showing the ACSSuTKSxt pattern, has been reported in Italy during 2005–2006. Molecular characterisation of resistance genes in ACSSuTKSxt strains

showed the presence of a 2.2-kb class 1 integron comprising *folA*, *catB3*, *aadA4* and *sul1* gene cassettes as well as bla_{TEM}, *strA-B*, *sul2*, *tet*(B), *catA1* and *aphA1* resistance genes. All strains with R-type ACSSuTKSxt harboured an IncHI1 plasmid, positive by PCR for the same resistance markers. PFGE cluster analysis showed that these strains belonged to a unique large cluster (A), which also included 13 susceptible strains, and were isolated from different sources. Moreover, it allowed strains with R-type ACSSuTKSxt to be distinguished from strains with other R-types and in particular from those with R-type SSuTNx, the prevalent pattern among MDR *S*. Infantis isolates until 2002 [9]. All ACSSuTKSxt strains belonged to cluster A, together with susceptible strains, thus leading to the hypothesis that MDR *S*. Infantis strains isolated in 2005–2006 could derive from susceptible strains by acquisition of the plasmid responsible for the whole antimicrobial drug resistance phenotype.

The possible derivation from other MDR patterns (SSuTNx, ASSuTSxt and SSuTSxt), all belonging to the PFGE cluster B, can be excluded by the presence of different resistance genes [a 1-kb class 1 integron including *aadA1* and *sul1* gene cassettes and *tet*(A), bla_{TEM}, *strA-B* and *sul2* resistance genes] as well as by the presence of plasmids belonging to different incompatibility groups (IncN and IncP) (Table 2).

The resistance pattern ACSSuT is widely diffused among Enterobacteriaceae and in particular in *Salmonella* spp. It was considered the phenotypic marker for recognising *S*. Typhimurium DT104 strains but was also found in other serotypes in association with the *Salmonella* genomic islands (SGIs) [20]. However, we can exclude the presence of SGIs in the strains in this study since they were negative for

the presence of *floR* and *tet*(G) resistance genes and for the presence of class 1 integrons containing the *aadA*2 and *bla*$_{PSE}$ gene cassettes.

Several studies reported the spread of MDR *S*. Infantis strains with R-types ACSSuT, with additional resistances, isolated from animals and humans in different countries. However, molecular characterisation, if performed, demonstrated that this phenotype was due to resistance genes different from those described in this paper or located on the chromosome [12,13,21,22].

The association of five resistance genes [*bla*$_{TEM-1}$, *strA-B, sul2*, *tet*(B) and *catA1*] on an IncHI1 plasmid has been previously described in a *S. enterica* serotype Paratyphi A strain isolated from a Pakistani patient in 2002 (pAKU_1; GenBank accession no. AM412236) [23], in a *S. enterica* serotype Typhi strain isolated in Vietnam in 1993 (pHCM1; GenBank accession no. AL513383) [24], in an *E. coli* strain isolated in Japan in 2001 (pO111_1; GenBank accession no. AP010961) [25] and in a *Salmonella choleraesuis* strain (pMAK1; GenBank accession no. NC009981). The presence of the 2.2-kb class 1 integron harbouring the genes *folA*, *catB3*, *aadA4* and *sul1* has been previously described only in the plasmid pAPEC-02-R (GenBank accession no. AY214164), belonging to IncF incompatibility group, found in an *E. coli* strain [26].

Besides the comparison between p34/13/09 and other IncHI1 plasmids based upon the incompatibility group and the identification of few resistance genes, this study reports for the first time the presence of a class 1 integron on a HI1 plasmid, thus suggesting the existence of a new plasmid construct [23–25]. The p34/13/09 plasmid

is characterised by the presence of pairs of genes conferring resistance to the same antimicrobial drug, such as *sul1–sul2*, *aadA4–strA-B* and *catB3–catA1*. These genes, apparently redundant, could be useful to maintain the resistance pattern in case one of the two genes is not expressed [26].

Plasmids belonging to the IncH family are temperature-sensitive for conjugative transfer. The H pilus is synthesised at an optimal temperature of 27 °C by extrusion from the cell surface; at 37 °C it remains stable while the formation of mating aggregates is inhibited [27]. For this reason, they could represent the potential vector for the dissemination of genes among bacterial species characteristic of environmental sources, where the temperature does not exceed 30 °C. Several studies observed the presence of resistance genes in DNA extracted from environmental samples owing to the large use of antibiotics in veterinary medicine and agriculture. Borjesson et al. [28] reported a high concentration of *tet*(B) and *tet*(A) genes in wastewater samples and demonstrated that *tet*(B) concentrations increased with increasing of tetracycline use at the feedlots. Moreover, because of the usual localisation of resistance genes on conjugative plasmids or transposons [29,14], high concentrations of tetracycline resistance genes can therefore indicate a potential risk for simultaneous spread of other genes encoding resistance. In this context, S. Infantis could represent a ubiquitous pathogen able to acquire antibiotic resistance genes from the environment and a potential reservoir of resistance for other human enteric pathogens.

In conclusion, this study demonstrates that during 2005–2006 there was the spread of an IncHI1 plasmid conferring the R-type ACSSuTKSxt among *S*. Infantis strains isolated from different sources in Italy. Further studies are needed to complete characterisation of the IncHI1 plasmid by sequence analysis of the whole structure or part of it to understand better its evolution and to compare it with plasmids of the same incompatibility group so far described.

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Competing interests

None declared.

Ethical approval

Not required.

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Fig. 1. Pulsed-field gel electrophoresis (PFGE) profiles and cluster analysis of 70 *Salmonella enterica* serotype Infantis strains included in this study. For each PFGE profile, the source, resistance genes, resistance pattern and year of isolation are reported. The dark bars highlight ACSSuTKSxt strains (resistant to ampicillin, chloramphenicol, streptomycin, sulphonamide, tetracycline, kanamycin and trimethoprim/sulfamethoxazole) with 100% genetic homology isolated from different sources.

Table 1

Antimicrobial resistance patterns and source of isolation of 70 *Salmonella enterica* serotype Infantis strains analysed in this study

No. of strains	Resistance pattern [a]	Source of isolation	Sampling year
18	ACSSuTKSxt	14 human, 2 food, 2 surface water	2005
4	ACSSuTKSxt	2 human, 2 surface water	2006
1	ACSSuTKSxt	Food	2003
2	ACSuTKSxt	1 human, 1 surface water	2006
1	ACSuTKSxt	Surface water	2005
1	ACSSuTSxt	Surface water	2006
3	SSuTNx	Food	2005
1	SSuTNx	Food	2004
1	ASSuTSxt	Food	2005
1	SSuTSxt	Food	2003
1	ST	Human	2003
1	ST	Human	2004
1	SSu	Surface water	2004
1	S	Human	2005
1	T	Human	2004
4	Susceptible	2 human, 1 animal, 1 food	2002
3	Susceptible	1 human, 1 food, 1 surface water	2003
3	Susceptible	1 human, 1 food, 1 surface water	2004
8	Susceptible	3 human, 3 surface water, 2 food	2005

10	Susceptible	5 animal, 3 human, 1 food, 1 surface water	2006
3	Susceptible	Human	2007
1	Susceptible	Human	2008

[a] A, ampicillin; C, chloramphenicol; S, streptomycin; Su, sulphonamide; T, tetracycline; K, kanamycin; Sxt, trimethoprim/sulfamethoxazole; Nx, nalidixic acid.

Table 2

Antimicrobial resistance patterns (R-type), class 1 integrons (*int1*), resistance genes, incompatibility (Inc) group of plasmid detected, and pulsed-field gel electrophoresis (PFGE) cluster group of 70 *Salmonella enterica* serotype Infantis strains analysed

No. of strains	R-type [a]	*int1*	Resistance genes	Inc group	PFGE cluster
23	ACSSuTKSxt	2.2 kb	Int1(*folA–catB3–aadA4–sul1*); bla_{TEM}; *catA1*; *strA-B*; *sul2*; *tet*(B); *aphA-1*	HI1	A
3	ACSuTKSxt	2.2 kb	Int1*(folA–catB3–aadA4–sul1*); bla_{TEM}; *catA1*; *sul2*; *tet*(B); *aphA-1*	HI1	A
1	ACSSuTSxt	2.2 kb	Int1(*folA–catB3–aadA4–sul1*); bla_{TEM}; *catA1*; *strA-B*; *sul2*; *tet*(B)	HI1	A
4	SSuTNx	1 kb	Int1(*aadA1–sul1*); *tet*(A)	P	B
1	ASSuTSxt	1 kb	Int1(*aadA1–sul1*); bla_{TEM}; *tet*(A)	P	B
1	SSuTSxt	Neg.	*strA-B*; *sul2*; *tet*(A)	N	B
2	ST	N/D	N/D	N/D	– [b]
1	SSu	N/D	N/D	N/D	–
1	S	N/D	N/D	N/D	–
1	T	N/D	N/D	N/D	–
32	Susceptible				A/B/–

N/D, not determined.

[a] A, ampicillin; C, chloramphenicol; S, streptomycin; Su, sulphonamide; T, tetracycline; K, kanamycin; Sxt, trimethoprim/sulfamethoxazole; Nx, nalidixic acid.

[b] – indicates strains that do not belong to either cluster A or B.

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Ethical approval: Not required