Molecular typing of Salmonella enterica *subspecies* enterica *serovar Typhimurium isolated in Abruzzo region (Italy) from 2008 to 2010*

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Veterinaria Italiana 2014, **50** (1), 31-39. doi: 10.12834/VetIt.1304.07
Accepted: 08.02.2013 | Available on line: 31.03.2014

Keywords
Antibiotic resistance, *floSt*, *int*, *invA*, phage type, *spvC*, *Salmonella enterica* subspecies *enterica* serovar Typhimurium (ST), *Salmonella enterica* subspecies *enterica* serovar Typhimurium monophasic variant (mST).

Summary
In this study, 47 antibiotic-resistant strains of *Salmonella enterica* subspecies *enterica* serovar Typhimurium (ST) were characterised, including 15 monophasic variants 1, 4, [5], 12:i:-, (STm) isolated from different matrices. They were all selected from 389 *Salmonella enterica* subspecies *enterica* strains isolated during 2008-2010 in Abruzzo region (Italy). Thirty-seven strains showed to be resistant to more than 1 antibiotic. Among 47 isolates, phage type U311 and DT104 were identified. The ASSuT resistance pattern was predominant in mST strains and ACSSuT in ST DT104 and U302. A multiplex Polimerase Chain Reaction (PCR) method was used to investigate 4 genes: fluorfenicol (*floSt*), virulence (*spvC*), invasine (*invA*) and integrase (*int*). All ST the strain were positive for invA gene and 28,32% of strains were positive for *spvC* gene. PFGE analysis revealed a large number of small clonal populations, however not ascrivable to outbreaks.

Tipizzazione molecolare di ceppi di Salmonella enterica *subspecies* enterica *serovar Typhimurium isolati in Abruzzo (Italia) dal 2008 al 2010*

Parole chiave
Antibioticoresistenza, fagotipo, *floSt*, *int*, *invA*, *spvC*, *Salmonella enterica* subspecies *enterica* serovar Typhimurium (ST), *Salmonella enterica* subspecies *enterica* serovar Typhimurium variante monofasica (STm).

Riassunto
Nel presente studio sono stati caratterizzati 47 ceppi di *Salmonella enterica* subspecies *enterica* serovar Typhimurium (ST) resistenti agli antibiotici, tra cui 15 varianti monofasiche 1,4,[5],12:i:-; (STm) isolate da varie matrici. Essi sono stati selezionati da 389 ceppi di *Salmonella enterica* subspecies *enterica* isolati in Abruzzo (Italia) nel periodo 2008-2010. Trentasette ceppi sono risultati resistenti a più di un antibiotico. Nei ceppi STm, il pattern di resistenza predominate è stato ASSuT, mentre nei ceppi ST DT104 and U302 il pattern ACSSuT. La Polimerase Chain Reaction (PCR) multiplex è stata utilizzata per valutare la presenza di quattro geni: fluorfenicolo (*floSt*), virulenza (*spvC*), invasione (*invA*) e l'integrone (*int*). Tutti i ceppi ST sono risultati positivi per il gene *invA* e il 28,32% per il gene *spvC*. L'analisi della PFGE ha rivelato un gran numero di piccole popolazioni conali, non ascrivibili tuttavia a focolai.

Introduction

Salmonella enterica subspecies *enterica* serovar Typhimurium (ST) is one of the most common causes of non-typhoid salmonellosis in the world, with both the biphasic and monophasic variants being implicated (CDC 2007a, CDC 2007b, EFSA Panel on Biological Hazards 2010). Between 2000 and 2010, this was one of the serotypes most frequently isolated in Europe, from animals and foods and it was responsible, together with *Salmonella enteritidis* and *Salmonella infantis*, for 14.1% of cases of food poisoning, with the epidemic strain ST DT104 mainly involved (Barco *et al.* 2011, EFSA Panel on Biological Hazards 2010, Health Protection Agency 2010).

The high number of antibiotic resistant *Salmonella* strains is principally due to the horizontal transfer of mobile DNA elements such as plasmids, transposons and integrons (Barnaud *et al.* 1998, Carattoli *et al.* 2002). In some serotypes, the resistance genes are collected in a chromosome area known as *Salmonella* Genomic Island 1 (SGI1), which was first identified in a multi-resistant epidemic strain of ST, DT104 (Carattoli *et al.* 2002, Doublet *et al.* 2005). These genes confer resistance to ampicillin (A), chloramphenicol/florfenicol (C/Fl), streptomycin/spectinomycin (S/Sp), sulphonamide (Su) and tetracycline (T). The most widespread resistant pattern is ACSSuT, which may be associated with additional antibiotic resistances patterns (Borrego *et al.* 1992, Boyd *et al.* 2001, Boyd *et al.* 2002, Busani *et al.* 2004, Doublet *et al.* 2005, Doublet *et al.* 2008).

Recently a monophasic variant of *Salmonella* Typhimurium (mST), serotype 1, 4, [5], 12:i:- has rapidly spread (Bone *et al.* 2010, CDC 2007a, CDC, 2007b, EFSA Panel on Biological Hazards 2010). Between the end of the 90s and 2008, the prevalence of this variant increased from 0.1% to 8.3% in samples of animal origin and from 0.1% to 14% in samples of human origin (Health Protection Agency 2010). This variant was isolated from several matrices, including foods and pets, and was one of the serotypes most often implicated in cases of human infections. Its virulence and antibiotic resistance, common to all strains of ST, make it a high risk for public health (Echeita *et al.* 2001, Folster *et al.* 2009, Guard-Petter 2001, Threlfall 2000). It is worthwhile stressing that the outbreaks of food poisoning in which it was implicated were often associated with ASSuT resistance pattern and different phage types (AFSSA 2009, Borrego *et al.* 1992, De la Torre *et al.* 2003, Echeita *et al.* 1999, Harker *et al.* 2011, Hauser *et al.* 2010, Rabsch 2009, Trupschuch *et al.* 2010, Walker *et al.* 2001).

It Italy, like in other countries, mST has been one of the most common serotypes found in human infections since 2004 (Dionisi *et al.* 2009) and it was the second most isolated in 2009. While prevalence of ACSSuT pattern remained constant (29.6%), ASSuT (with or without additional resistances) increased from 7.6% to 34.1% (Busani *et al.* 2004), with a prevalence for phage types DT193 (48%) and U302 (13%) (Lucarelli *et al.* 2010) (Istituto Superiore Sanità 2010). However, phage types do not indicate whether the variant is monophasic or biphasic (Echeita *et al.* 1999).

The aim of this study was to characterize the local population of ST and mST, by PFGE, phage typing and antibiotic resistance patterns in order to reveal any relationship among the strains circulating in Italy between 2008-2010. Polymerase chain reaction (PCR) was used to detect SGI1 gene conferring resistance to chloramphenicol/florfenicol (*floSt*), int coding integrase, *invA* gene for invasiveness, and *spvC* gene for virulence.

Materials and methods

Phenotype identification and antibiogram

This study examined 47 strains of ST and mST selected from 389 strains of *Salmonella* isolated from different matrices in the period 2008-2010. The strains were stored at –80°C, revitalised in Brain Infusion broth B H I broth and subcultured in BHI agar (Thermo Scientific, Basingstoke, UK).

Biochemical identification was conducted using the automated Vitek 2 system (Biomerieux, Marcy l'Etoile, France). Serological identification was carried out using the Kauffmann-White method with commercial antisera (Statens Serum Institute, Copenhagen, Denmark) and using a PCR to detect *fljB* gene (Barco *et al.* 2011). Phage typing was accomplished within the National Reference Centre for Salmonellosis (Istituto Zooprofilattico Sperimentale delle Venezie, Italy - IZSVE).

Antibiotic resistance patterns were identified using the disk diffusion method (Kirby-Bauer) in Mueller

Table I. *Primers used for the investigation of* floSt, int, invA *and* spvC *genes.*

Primer	Target Gene	5-3' sequence
FloF	*floSt*	5'- ACCCGCCCTCTGGATCAAGTCAAG -3'
FloR		5'- CAAATCACGGGCCACGCTGTATC -3'
VirF	*spvC*	5'- GGGGCGGAAATACCATCTACA -3'
VirR		5'- GCGCCCAGGCTAACACG -3'
InvF	*invA*	5'- CGCGGCCCGATTTTCTCTGGA -3'
InvR		5'- AATGCGGGGATCTGGGCGACAAG -3'
IntF	*int*	5'- GCCCTCCCGCACGATGAT -3'
IntR		5'- ATTGGCGCCCTTGCTGTTCTTCTA -3'

Hinton agar (Basingstoke Scientific, UK), testing for the following compounds: ampicillin (10 µg), amoxicillin + clavulanic acid (20/10 µg), cefazolin (30 µg), gentamicin (10 µg), kanamycin (30 µg), enrofloxacin (5 µg), trimetoprim + sulfamethoxazole (1.25/23.75 µg), tetracycline (30 µg), ceftazidime (30 µg), colistin (10 µg), sulfisoxazole (300 µg), nalidixic acid (30 µg), streptomycin (10 µg), cloramphenicol (30 µg), cefalotin (30 µg) and ciprofloxacin (5 µg) (Becton Dickinson, Italy). The results were interpreted according to Clinical and Laboratory Standard Institute criteria[1].

Table II. *ST and mST strains and matrices.*

	ST (4, 5, 12:i:1, 2)	STm (4, 5:i:-;)
Pork	8	12
Human faeces	3	1
Chicken meat	8	1
Eggs	4	
Beef	2	
Pigeon faeces	2	
Mulluscs/fish	4	
Animal feed	1	
River water		1
	32	15

Polymerase Chain Reaction

Resistance and pathogenicity genes *floSt, int, invA* and *spvC* were investigated by using PCR (Khan *et al.* 2000) and reported in Table I; while DNA extraction was performed using the UltraClean™ Microbial DNA Isolation Kit (Mo BIO, Carlsbad, CA, USA). The PCR was structured using Master Mix 2X according to the manufacturer's instructions (Promega, Madison, WI, USA). The PCR products were identified with Qiaxcel (Qiagen, Hilden, Germany); ST DT104 (IZSVE) was used as reference strain. PCR for *fljB* gene detection was carried out according to Barco (Barco *et al.* 2011).

[1] Clinical and Laboratory Standard Institute (CLSI) 2008. Performance Standards for Antimicrobial disk and dilution susceptibility. Test for bacteria isolated from animals: Approved Standard Third Edition. 2008. Document M31-A3. Vol. 24 No. 1, Wayne PA. USA.

Table III. *Phage type, antibiotic resistance patterns and genes identified in ST strains of* Salmonella enterica *subspecies* enterica *strains isolated during 2008-2010 in Abruzzo region (Italy).*

Number	Sample type	Phage type	Resistance pattern	Genes
1	Pork	RNDC		*invA*
3	Pork	DT104	ACSSuT + AMC	*int+invA+spvC+floSt*
1	Pork	DT104	ACSSuT + AMC	*int+invA+spvC*
1	Pork	DT20	ACST + AMC	*int+invA+spvC*
2	Pork	U311	ASSuT	*invA*
1	Human faeces(CH)	NT		*invA*
1	Human faeces (PE)	U302	ACSSuT + AMC + CL	*int+invA+spvC+floSt*
1	Human faeces (TE)	NT	ACSSuT	*int+invA+spvC+floSt*
1	Chicken meat	NT	A	*invA*
6	Chicken meat	U311	ASSuT + CZ + SXT + NA + CF	*int+invA*
1	Chicken meat	U311	ASSu + ENO + NA	*invA*
2	Eggs	DT2		*invA*
1	Eggs	DT3		*invA*
1	Eggs	DT99		*invA*
1	Pigeon faeces	DT3		*invA*
1	Pigeon faeces	U302		*invA*
1	Animal feed	DT99		*invA*
1	Beef	DT99		*invA*
1	Beef	U302	ACSSuT + SXT	*int+invA+spvC*
1	Molluscs	DT104	ACSSuT	*int+invA+spvC+floSt*
1	Molluscs	NT		*invA*
1	Molluscs	U311	ASSuT	*invA*
1	Fish	NT	ASSuT	*invA*
32				

NT = strain could not be typed; RNDC = stable unidentified reading.

Table IV. *Phage type, antibiotic resistance patterns and genes identified in mST strains of* Salmonella enterica *subspecies* enterica *strains isolated during 2008-2010 in Abruzzo region (Italy).*

Number	Sample type	Phage type	Resistance pattern	Genes
4	Pork	DT120	ASSuT	*invA*
	Pork	U302	ASSuT	*invA*
	Pork	U311	ASSuT	*invA+floSt*
	Pork	U311	ASSuT	*invA*
1	River water	DT193	ASSuT	*invA*
1	Pork	RNDC	ASSu	*invA*
2	Pork	U311	ASSu	*invA*
2	Pork	DT193 U311	ACSSuT	*invA*
2	Pork	NT, DT193	T	*invA*
1	Pork	DT193	T	*invA*
1	Chicken	DT7	AST+STX+Na	*int+invA*
1	Human	U311		*invA*
15				

A = ampicillin; S = streptomycin; Su = sulfisoxazole; T = tetracycline; C = chloramphenicol; AMC = amoxicillin + clavulanic acid; CL = colistin; SXT = trimetoprim + sulphamethoxazole; Eno = enrofloxacin; NA = nalidixic acid; Cz = cefazolin; CF = cefalotin.

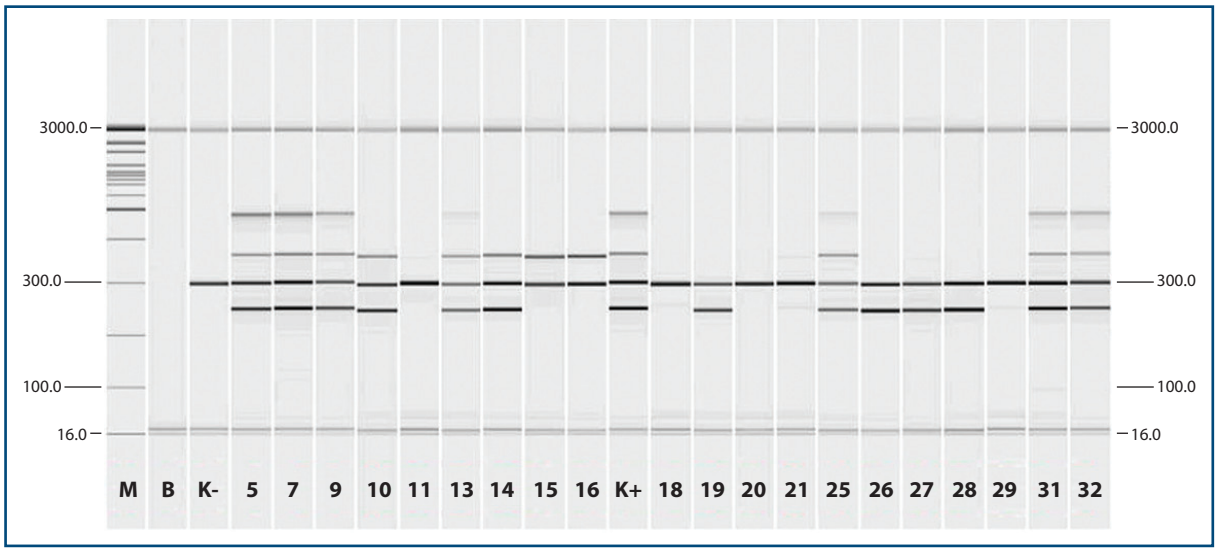

Figure 1. *PCR results of strains of* Salmonella enterica *subspecies* enterica *strains isolated during 2008-2010 in Abruzzo region (Italy).*
Line 1: molecular weight markers; line 2: blank; line 3: negative control (*S. branderup*); line 13: positive control (STm DT104); from 5 to 16 and from 18 to 32: multi-resistant samples.

Pulsed Field Gel Electrophoresis

Pulsed Field Gel Electrophoresis (PFGE) was carried out according to the Pulsenet protocol (PulseNet USA 2010) after digestion with restriction enzymes *Xba1* and *Bln1* (Promega, Madison, WI, USA). The gel was stained with Sybr Safe DNA Gel Stain (Life Technologies, Paisley, UK) and the images were taken using Chemilmager 5500 v. 3.04 (Alpha Innotech Corporation, San Leandro, CA, USA) and analysed with Bionumerics 6.0 programm (Applied Math, Sint-Martens-Latem, Belgium). For the analysis, 1% tolerance and optimisation criteria were imposed. The dendrogram was calculated by using the computer program UPGMA (Unweighted Pair Group Method using Arithmetic averages). The discriminating power was measured with Simpson's index, considering the set of strains with a similarity above 98% as the subpopulation (Hunter and Gaston 1998).

Results

Serotyping, phage typing and antibiotic resistance patterns

Thirty-two of the 47 strains tested were confirmed as ST (4, 5, 12; i:1, 2) and 15 as mST (4, 5, 12;i:-.). Table II

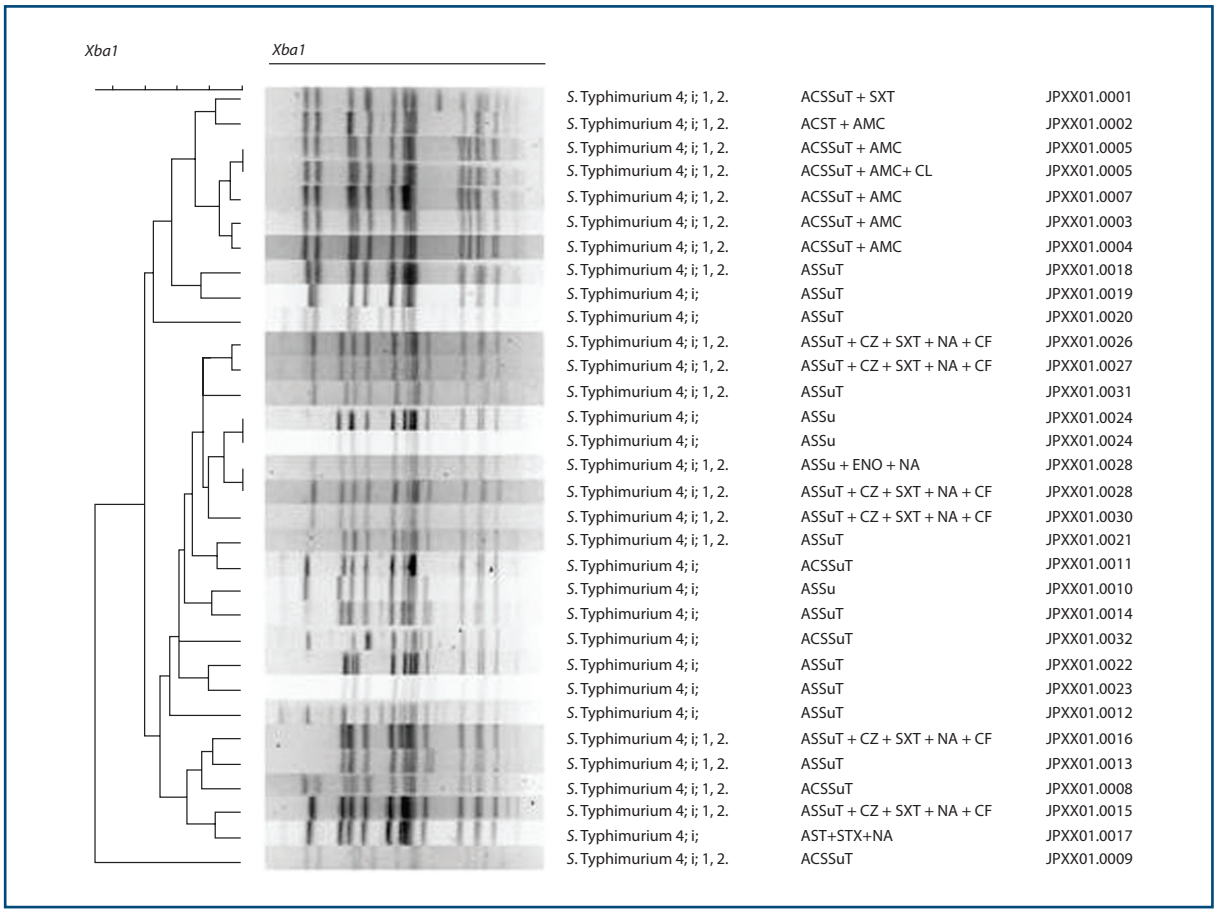

Figure 2. Xba1 *PFGE profile of strains of* Salmonella enterica *subspecies* enterica *strains isolated during 2008-2010 in Abruzzo region (Italy).*

reports the matrices from which the 2 variants ST and mST were isolated. Table III and Table IV describe the results of phage typing, antibiotic resistance patterns and PCR for ST and mST. Of the strains tested, 10.6% were characterized as phage type DT104 and 36.2% as phage type U311. A total of 68% of the analysed strains showed to be resistant to at least 2 antibiotics (Tables III and IV). Six strains isolated from chicken were resistant to 9 different antibiotics, with resistence pattern ACSSuTCzSxtNaCf. ACSSuT resistance pattern was the most common in ST strains, while ASSuT was the most common in mST strains (Tables III and IV).

Polymerase Chain Reaction

The polymerase chain reaction confirmed the absence of *fljB* gene for all 15 strains identified as mST by serotyping. All ST and mST strains analyzed were positive for *invA* gene, regardless of whether they were sensitive (S) or resistant (R) to the tested antibiotics (Tables III and IV). Figure 1 depicts the PCR results showing the bands corresponding to the investigated genes: *floSt* (584 bp), *spvC* (392 bp), *invA* (321 bp) and *int* (265 bp). Of the tested ST strains, 18.75% were positive for the association *int+invA+spvc+floSt* in addition to ACSSuT resistance pattern (with or without additional resistance), while the association *int+invA+spvc* was found in 9.37% of strains (2 from pork and 1 from beef) with ACSSuT+AMC, ACST+AMC, ACSSuT+SXT resistance patterns association. Six mST strains isolated from chicken were positive for *int+invA* genes associated with a multiple resistance pattern, with ACSSuT associated with CzSxtNaCf. Only 1 mSTM strain showed the association *invA+flo* and ASSuT and 1 *int+invA* (Tables III and IV).

Pulsed Field Gel Electrophoresis

Analysis of the images revealed numerous small clone populations but none with characteristics ascribable to outbreaks. The analytical method we endorsed was found to be highly discriminating, with a Simpson's index (D) of 0.99.

Figure 2 shows the dendrogram with the restriction profiles of the tested strains after digestion with *Xba1* and their comparison with the resistance patterns. A clear distinction among samples with ACSSuT and ASSuT profiles can be observed, regardless of the phage type. Figure 3 illustrates the comparison of strains after digestion with *Xba1* and *Bln1*.

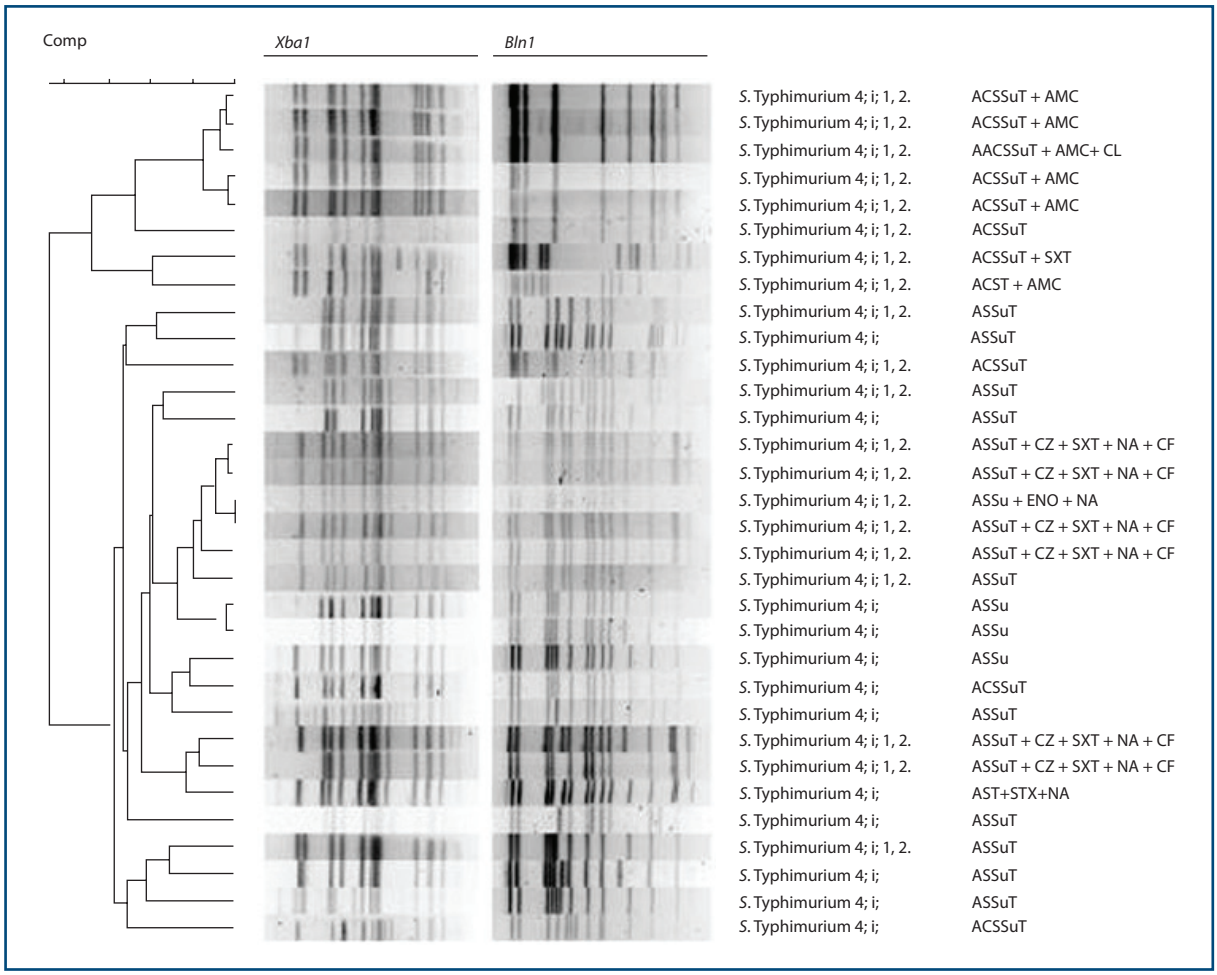

Figure 3. Xba1 *and* Bln1 *PFGE profile of* Salmonella enterica *subspecies* enterica *strains isolated during 2008-2010 in Abruzzo region (Italy).*

Discussion

The widespread occurrence of multi drug resistant strains of both monophasic and biphasic *S.* Typhimurium and the constant increasing of human infections are becoming a big concern for public health in a large part of the world. As already occurred in other Italian regions (Graziani *et al.* 2008), also in Abruzzo there is a predominance of resistant strains with ASSuT pattern (with or without additional resistance), in 31.23% of ST and 33.3% of mST strains. In 6 strains isolated from chicken, resistance was found to other 4 antibiotics: CzSXTNaCf. ACSSuT pattern (with or without additional resistance) was found in 25% of ST and in 6.66% of mST strains. Some of the strains were also resistant to AMCCl and/or SXT.

The increasing growth of ASSuT resistance pattern, which predominates in monophasic *S.* Typhimurium, could be related to the postulated ability of mST to evade the immune system, while the association of ACSSuT with the phage type DT104 could be linked to the latter's predisposition to combine both horizontal and vertical resistance transfer phenomena (Cloeckaert and Chaslus-Dancla 2001, Bolton *et al.* 1999). Integrons express mobile genes known as cassettes, which in many cases are antibiotic resistance genes. The integrase gene *int* is an essential part of all integrons and codes for a site-specific recombinase that catalyses the insertion of the gene into the integron. This gene was found in 46.9% of the strains. The invasiveness gene *invA* is considered essential for the complete virulence of *Salmonella*. It is thought to activate the interiorisation wich is necessary for the invasion of deeper lying tissues (Galan and Curtiss 1989).

In addition to the chromosome genes described above, a highly conserved 8 kb plasmid region, called *spv* (*Salmonella* plasmid virulence), was also observed, which is usually associated with a particularly serious form of disease. This region is more common in strains isolated from extraintestinal human infections than in those isolated from stool samples or environmental samples and includes a regulatory gene, *spvR*, and 4 structural genes, *spv* ABCD. The *spv* genes seem to promote the macrophagic stage of the disease, preventing neutrophils from destroying the bacteria and facilitating the proliferation of

extraintestinal *Salmonella* strains. *spvC* in particular interacts with the host's immune system and it is responsible for the hight growth rate in the host cells (Gulig *et al.* 1993).

All ST strains were positive for *invA*, as reported in literature (Khan *et al.* 2000), and 28.32% were positive for *spvC* constantly associated with ACSSuT resistance profile and phage type DT104 in strains isolated from pigs, cattle and molluscs. The presence of less common phage types in ST and mST reflected the trend reported in literature, which also highliths that the ST phage type DT193 could have a new pathogenicity island, which seems to increase its virulence and whose functions on metabolic activity are still under study (Threlfall 2000). The distribution of resistance genes in relation to the various phage types and serotypes is particularly complex, *floSt* was found in only 1 case of mST U311 with ASSuT profile, while it was always present in DT104 and was also found in one strain whose phage type could not be determined (NT). No particular distribution of resistance genes was found with respect to other phage types.

The analysis of PFGE data revealed large groups with a common resistance profile, one with ASSuT and the other with ACSSuT, but neither was suspected as responsible of the outbreak, as it could be deduced from the extreme spatiotemporal variety of the test samples. This partition reflects the literature evidence on this type of *Salmonella*. In fact, previous studies (Dionisi *et al.* 2009, Lucarelli *et al.* 2010) already highlighted the different genomic origin of *Salmonella* strains with one resistance profile rather than another. Only ST strains with an ACSSuT profile formed part of the ASSuT group; however these strains did not have integrase. The combination of these events suggests that they derive from the clonal line ASSuT, although they developed resistance to chloramphenicol through other routes.

Interestingly, phage type U311 was common in strains with ASSuT profile. Differently from DT193, it is not one of the most widespread in Europe (Hopkins *et al.* 2010) but seems to be particularly present in Italy. The prevalence of ST and mST in Abruzzo over the period of this study reflects the general trend for Italy and Europe, with a prevalence of phage type U311 and ASSuT resistance profile over ACSSuT (both with and without additional resistance).

Finally, bacteria with multiple antibiotic resistance, especially those newly resistant to quinolones, sulphonamides and cephalosporin, are becoming more and more widespread, with a growing socioeconomic impact. There is a need to intensify campaigns for reducing use of antibiotics and common therapeutic protocols. Constant monitoring of pathogens with an impact on public health through phenotyping and molecular techniques is also necessary in order to enable the competent authorities to activate effective, specific surveillance and control plans.

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