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Phenotypic and genetic traits of *Salmonella enterica* subsp. serovar Typhimurium strains causing salmonellosis foci in rabbit farms from Southern Italy in 1999–2003

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ABSTRACT

In this study, we characterised the *Salmonella* Typhimurium strains responsible for four outbreaks which occurred in distinct rabbit farms (Southern Italy) from 1999 to 2003. Strains were typed by Pulsed Field Gel Electrophoresis (PFGE) and the genetic basis of antimicrobial resistance was established. A major group of clonally related isolates, pulsotype STYMXB.0061, accounted for three of the salmonellosis foci. Strains were resistant to streptomycin, chloramphenicol, tetracycline, ampicillin and sulphonamides encoded respectively by the *aadA2*, *floR*, *tetG*, bla_{PSE-1}, *sul1* gene cluster harboured by a Salmonella Genomic Island 1. The clonally related group of isolates included strains phage type DT104, DT12 or undefined type (NT). The fourth salmonellosis focus was caused by a strain pulsotype STYMXB.0147, resistant to sulphonamides (encoded by *sul2*) and phage type U302. Results provided first molecular characterisation of *S.* Typhimurium strains isolated from rabbit farms in Italy and highlighted the presence of the pulsotype STYMXB.0061 even before its wide detection among human clinical isolates collected in Italy in the mid 2000s from clinical cases.

1. Introduction

Rabbit (*Oryctolagus cuniculus*) is an important source of meat in many Mediterranean and Asian countries (Kohler et al., 2008). In the last decade, also due to its nutritional and dietary properties (Dalle Zotte and Szendro, 2011), the world rabbit production has been increasing with Italy enumerated among the major world producers (over 200,000 tons per year) (http://faostat.fao.org). However, intensive farmed rabbit are highly susceptible to gastrointestinal diseases among which salmonellosis causes serious concern in terms of economic loss and potential risks for public health. *Salmonella enterica* subsp. *enterica* serovar Typhimurium (hereafter referred to as *S.* Typhimurium) is one of the most common enteropathogen isolated from human intestinal diseases in Europe and US, along with *S. enterica* subsp. *enterica* serovar Enteriditis. (Weinberger and Keller, 2005). *S.* Typhimurium is also the most common serovar isolated from rabbit in Italy. The National Reference Centre for salmonellosis reported that the 76.9% of *S. enterica* isolates from rabbit during the period 2002–2009 were Typhimurium serovar (http://www.izsvenezie.it/index.php?option=com_content&view=article&id=193&Itemid=335, latest access 23rd March 2012).

S. Typhimurium infection in rabbit usually causes high mortality and morbidity both in does and in fattening animals, especially before and during weaning. The rapid diffusion of salmonellosis in rabbitry is promoted by the shedding of the pathogen with faeces whose dissemination greatly contaminate the surrounding environment. This in turn contributes to the persistence of *S.* Typhimurium even after sanitary breaks making the management of salmonellosis outbreaks very difficult to deal. In addition, the insurgence and spread of multidrug resistant (MDR) strains further complicates the control of the disease.

Antimicrobial resistance is usually acquired by point mutations in the bacterial genome or by horizontal transfer of genetic elements harbouring resistance genes. Plasmids, transposons and integrons (the last capable of integrate gene cassettes often encoding for antimicrobial resistance) are the genetic elements generally recognised as the main vectors in the dissemination of antimicrobial resistance (Stokes and Gillings, 2011). More recently, the wide diffusion of the Salmonella Genomic Island 1 (SGI1) extended the range of the genetic elements involved in the insurgence of

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antimicrobial resistance at least among *S. enterica* strains (Boyd et al., 2000). The SGI1 is an integrative mobilisable element integrated in the chromosome at the 3′ end of the *tdhF* gene (Boyd et al., 2001; Doublet et al., 2005). Within the 43 kbp SGI1 element lies a 13 kbp region harbouring the *floR* and *tetG* genes flanked by two class 1 integrons carrying one *aadA2* and one bla_{PSE-1} gene-cassette (this last followed by the *sul1* gene). The *aadA2*, *floR*, *tetG*, bla_{PSE-1}, *sul1* gene cluster encodes resistance for streptomycin (S), chloramphenicol (C), tetracycline (T), ampicillin (A) and sulphonamides (Su), respectively. SGI1 was first detected in a *S.* Typhimurium strain of the definitive phage type 104 (hereafter referred to as *S.* Typhimurium phage type DT104) isolated from UK in the mid 1980s (Ridley et al., 1998). Since then, MDR *S.* Typhimurium DT104 ACSSuT spread worldwide and nowadays it represents one of the most prevalent penta-resistant serovars isolated from animals (Glenn et al., 2011). The MDR resistance phenotype of SGI1 has also been detected in other *Salmonella* phage types and serovars. Variations in resistance pattern and encoding resistance genes (variant SGI1-like elements) have also been detected (Boyd et al., 2002).

Notwithstanding the increasing attention to the diffusion of MDR strains and to their genetic bases, current knowledge on *S.* Typhimurium strains isolated from rabbit farms is still poor.

In this study, we characterised the *S.* Typhimurium strains responsible for four outbreaks which occurred in distinct rabbit farms (Southern Italy) from 1999 to 2003. Strains were typed by Pulsed Field Gel Electrophoresis (PFGE) and the genetic basis of antimicrobial resistance was established.

2. Material and methods

2.1. Animal sources, bacterial strains, phage typing, antimicrobial susceptibility testing and conjugation assays

Four salmonellosis foci, which occurred in distinct rabbit farms in Southern Italy from 1999 to 2003 (Table 1), were investigated in this study. No less than 15 dead animals per focus were brought to our facilities for necropsy. *Salmonella* strains were isolated from gut, spleen and liver of the necropsied animals. The search was also conducted from lungs in animals with respiratory symptomatology. The identification was carried out according to the procedure described in ISO6579:2002 (ISO, 2002) and serotyping was performed by slide agglutination by standard sera (Bio Rad, Milan, Italy) according to the Kauffmann–White scheme (Popoff et al., 2004).

From each salmonellosis focus a number of *S.* Typhimurium strains (ranging from 20 to 30) were isolated from each focus. Ten randomly chosen strains, mainly isolated from gut, were initially screened for antimicrobial susceptibility by the disc diffusion method as described in the Clinical and Laboratory Standards Institute (CLSI) documents M2-A9 (2006). The antimicrobial discs were: ampicillin (A; 10 μg), chloramphenicol (C; 30 μg), gentamycin (CN; 10 μg), kanamycin (K; 30 μg), nalidixic acid (Nx; 30 μg), streptomycin (S; 10 μg), sulfamethoxazole (Su; 25 μg), tetracycline (T; 30 μg) and trimethoprim (W; 5 μg) (Oxoid, Milan, Italy). *Escherichia coli* ATCC 25922 was used as a quality control strain.

Each salmonellosis focus was characterised by *S.* Typhimurium strains exhibiting the same antimicrobial susceptibility, PFGE profile and resistance genes. A single isolate was then chosen as representative and included in this study.

Strains were phage typed according to the standard procedure (Ward et al., 1987).

Conjugation experiments were performed as described previously (Pugliese et al., 2009). Matings were also performed at 25 °C to detect any possible thermosensitive transfer of plasmids such as those of the IncH1 group (Sherburne et al., 2000). *E. coli* K-12 strain ZM46, a nalidixic acid-resistant mutant of CSH26 was used as recipient strains.

2.2. PCR detection of SGI1, class 1 integron and resistance genes

Primers for PCR detection of SGI1, class 1 integron and antimicrobial resistance genes are listed in Table S1 (supplementary data). Genomic DNA was extracted as previously described (Pugliese et al., 2009). PCR reactions were performed in a volume of 25 μL containing 50–100 ng of total DNA, 1.5 mM $MgCl_2$, dNTPs (Takara, Shiga, Japan) at the final concentration of 250 μM and 1 unit of Euro*Taq* polymerase (EuroClone S.p.A., Milan, Italy**).** PCR conditions were as follows: 94 °C for 5 min, then by 25 cycles of 94 °C for 30 s, 60 °C or 68 °C (according to selected primers, see Table S1) for 1 min and 72 °C for 5 min, followed by a final extension at 72 °C for 10 min. PCRs for detection of gene linkage and amplification of long DNA segments were performed as above mentioned with the following modifications: 2.5 mM $MgCl_2$, 2X GC buffer (Takara) and 1 unit of LA *Taq* polymerase (Takara); conditions were as follows: 98 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 68 °C for 5 min or 15 min and then by a final extension at 72 °C for 10 min.

Antimicrobial gene cassettes integrated into class 1 integrons were amplified with primers 5CS-F and 3CS-R and cloned into pGEM-T Easy vector (Promega, Milan, Italy) in accordance with the manufacturer's instructions. *E. coli* JM109 was used as a recipient strain. The cloned products were purified using the Pure Yield™ Plasmid Miniprep System (Promega) and sequenced by the Big Dye Terminator method (BMR Genomics, Padova, Italy). The resulting DNA sequences were analysed for similarity by using the BLAST program available on the NCBI BLAST homepage (http://www.ncbi.nlm.nih.gov/BLAST).

Table 1
Phenotypic and genetic traits of *S.* Typhimurium strains isolated from rabbit farms in Southern Italy from 1999 to 2003.

Place/Year	Resistance pattern (n. of strains)[a]	Phage type (n. of strains)[b]	PulseNet Europe nomenclature (Pulsotype)	SGI1[c]		Class 1 integron and resistance genes[c]		
				Left junction	Right junction	*intI1*	Gene cassette(s)	Resistance gene (s)
Apulia/2000	Su (1)	U302 (1)	STYMXB.0147	–	–	–	–	*sul2*
Basilicata/1999	ACSSuT (1)	DT104 (1)	STYMXB.0061	+	+	+	*aadA2*; bla_{PSE-1}	*sul1*; *floR*; *tet*(G)
Basilicata/2003	ACSSuT (2)	DT12 (1); NT (1)	STYMXB.0061	+	+	+	*aadA2*; bla_{PSE-1}	*sul1*; *floR*; *tet*(G)

[a] A, ampicillin; C, chloramphenicol; S, streptomycin; Su, sulfamethoxazole; T, tetracycline.
[b] NT, not typeable.
[c] Key: +, positive; –, negative.