

SALMONELLA SER. TYPHIMURIUM ISOLATED FROM RABBIT FARMS: CHARACTERIZATION AND EPIDEMIOLOGICAL IMPLICATIONS

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ABSTRACT

Salmonella enterica infections are not common in rabbits, but, whenever they occur, they induce high morbidity and mortality and they also raise concern in terms of public health impact. Moreover, the worldwide spread of multidrug resistant strains complicates the treatment of infection in both rabbits and humans. Despite those premises, knowledge about salmonellosis in rabbits is still poor. In this study four *S. Typhimurium* strains were isolated from as many rabbit farms, and they were characterized for antimicrobial susceptibility, resistance genes, class 1 integrons and Pulsed-Field Gel Electrophoresis (PFGE). The results showed that a group of strains were circulating in Basilicata, and that it was indistinguishable from human isolates for PFGE profile, multidrug resistance pattern and genetic features, as they harbored the Salmonella Genomic Island 1 (SGI1) which characterized the *S. Typhimurium* strains which are widespread among humans all over the world. Our data suggest that molecular characterization is a useful tool to promptly recognize *Salmonella* strains which are potentially harmful to rabbits or humans.

Key words: Salmonella Typhimurium, PFGE, Resistance genes.

INTRODUCTION

Salmonellosis may represent a serious threat for rabbit farms. *Salmonella* spp. infections may cause significant economic losses due to its deleterious effects on animal health, and to the relevant implications in terms of public health. In fact, other than being a major cause of food-borne disease, salmonellosis also induces high morbidity and mortality in rabbit farms, especially in young rabbits (Borrelli *et al.*, 2011).

Among the about 2,500 known *Salmonella* spp. serovars, Typhimurium is considered one of the most threatening for both rabbits and humans. It induces a severe symptomatology in rabbits and it has the highest prevalence among humans (Borrelli *et al.*, 2011, Dionisi *et al.*, 2009), being the most common serovar isolated in Europe and US along with Enteritidis (Weinberger and Keller, 2005). In the last three decades, medical and veterinary treatments of salmonellosis have been complicated by the wide spread of multidrug resistant (MDR) strains. In particular, major concern has been raised by the worldwide diffusion of MDR phage type DT104 strains, which harbor a Salmonella Genomic Island (SGI1) encoding resistance genes (Glenn *et al.*, 2011)

Despite the increasing concern, the current knowledge on strains identified in rabbit farms is still poor. This study was aimed to the molecular characterization of four *S. Typhimurium* strains isolated from rabbit farms in the South Italy from 1999 to 2003. The phage types, the Pulsed Field Gel Electrophoresis (PFGE) profiles, the antimicrobial susceptibility patterns were determined, the presence of SGI1 was assessed and the antimicrobial resistance genes were identified.

MATERIALS AND METHODS

Strains, phage typing, antimicrobial susceptibility testing and conjugation assays

Four salmonellosis foci, which occurred in different rabbit farms in Southern Italy (Apulia and Basilicata) from 1999 to 2003, were investigated in this study (Table 1). From each focus no less than ten dead rabbit were necropsied and *S. Typhimurium* strains were isolated and biochemically identified according to standard bacteriological procedures. Serotyping was performed by slide agglutination according to the Kauffmann-White scheme with standard antisera (BioRad, Milan, Italy) and phage type was performed according to Anderson *et al.* (1977). The antimicrobial susceptibility of each strain was tested by the disk diffusion method according to the CLSI recommendations (2006). The antimicrobial disks used were: ampicillin (AMP; 10 µg), chloramphenicol (CHL; 30 µg), enrofloxacin (ENR, 5 µg), gentamicin (GEN, 10 µg), kanamycin (KAN; 30 µg), streptomycin (STR; 10 µg), sulfamethoxazole (SMX; 25 µg), tetracycline (TET; 30 µg), and trimethoprim (TMP; 5 µg) (Oxoid, Milan, Italy).

Escherichia coli ATCC 25922 was used as a quality control strain. Since the isolates which were identified in each focus showed the same phage type and resistance pattern, we selected a representative strain for each farm, for the subsequent molecular characterization.

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.

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

Place/Year of isolation	Resistance pattern (n. of strains) ^a	Phage type (n. of strains) ^b	Pulsetype according to the PulseNet Europe nomenclature	SGI1 ^c	Class 1 integrons ^c	Resistance genes
Apulia/2000	SMX (1)	U302 (1)	STYMXB.0147	-	-	<i>Sul2</i>
Basilicata/1999	AMP-CHL-STR-SMX-TET (1)	DT104 (1)	STYMXB.0061	+	+	<i>aadA2, floR, tetA(G), bla_{PSE-1}, sul1</i>
Basilicata/2003	AMP-CHL-STR-SMX-TET (2)	DT12 (1); NT (1)	STYMXB.0061	+	+	<i>aadA2, floR, tetA(G), bla_{PSE-1}, sul1</i>

^a AMP, ampicillin; CHL, chloramphenicol; STR, streptomycin; SMX, sulfamethoxazole; TET, tetracycline;

^b NT: not typeable

^c Key: +, positive; -, negative

PCR detection of SGI1, class 1 integrons and resistance genes.

Genomic DNA was extracted as previously described (Pugliese *et al.*, 2009). SGI1, class 1 integrons and antimicrobial resistance genes were detected by PCR with specific primers.

PCR reactions for detection of gene linkage were also performed as mentioned above according to the protocol for long amplification products.

The amplicons yielded from PCRs amplifying resistance genes and gene cassettes were purified and cloned into pGEM-T Easy Vector (Promega, Milan, Italy), according to 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 analyzed for similarity by using the BLAST program available online (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Pulsed-field gel electrophoresis (PFGE)

Genetic relatedness was determined by PFGE analysis after digestion of genomic DNA with *Xba*I (Roche, Milan, Italy) and according to the PulseNet standardized protocol (Ribot *et al.*, 2006). The *Salmonella* Braenderup H9812 strain was used as a molecular standard.

The PFGE agarose gels were stained with ethidium bromide (40 µg/mL) and the DNA band images were acquired by the Gel Doc 2000 photo documentation system (Bio-Rad). Digital images of the

PFGE profiles were analyzed using algorithms available in the BioNumerics software package (v. 6.1, Applied Maths, Sint-Martens-Latem, Belgium). DNA profiles differing in one or more DNA fragments were considered as distinct patterns (Tenover *et al.*, 1995). Strains with a coefficient of similarity $\geq 80\%$ were considered as genetically closely related. The analyzed profiles of strains were uploaded to the international database established at the Health Protection Agency (HPA; Colindale, London, United Kingdom) compared with the profiles of the PulseNet Europe and then named conventionally with a six-letter code followed by four-digit numerical identifier, for example: STYMXB.0006 (Lukinmaa *et al.*, 2006).

RESULTS AND DISCUSSION

The main features of the characterized strains are reported in Table 1. The strains exhibited heterogeneity in terms of phage types, as they were DT104, DT12, U302 and not typable (NT). However, the DT104, DT12 and NT strains showed the same PFGE profile, which, in turn, was 100% identical to the STYMXB.0061. The three strains came from farms of the same region, the Basilicata. By contrast the other strain, from Apulia, was phage type U302 and exhibited the PFGE profile STYMXB.0147. According to the interpretative criteria of PFGE profiles proposed by Tenover *et al.* (1995), the two groups of strains should be considered as unrelated.

The strains from Basilicata also shared the resistance pattern AMP-CHL-STR-SMX-TET, confirming their relatedness, while the U302 strain was only resistant to SMX. All strains were susceptible to ENR and GEN. The conjugation experiments did not evidence transfer of resistance determinants (frequency detection limit 10^{-9}). The STYMXB.0061 strains resulted PCR positive for class 1 integrons and SGI1. The strains were also PCR positive to the *floR*; *sul1*; *tetA(G)*, *aadA2*; *bla_{PSE-1}* genes, which confer the resistances to CHL, SMX, TET. STR and AMP, respectively. The array of such genes was also verified by PCR and it was *aadA2*, *floR*, *tetA(G)*, *bla_{PSE-1}*, *sul1*, indistinguishable to that reported for the SGI1 by Boyd *et al.* (2001). The STYMXB.0147 strain was negative for PCR detection of class 1 integron and SGI1, while its sulphonamide resistance was conferred by the *sul2* gene.

In conclusion, our findings suggest a close relatedness of rabbit strains with human strains. While there is no epidemiological data for the pulsetype STYMXB.0147, the pulsetype STYMXB.0061 has already been reported in Europe. Interestingly, between 2000 and 2004, it was one of the prevalent profiles in North Europe, but it was under-represented in Italy. (Gatto *et al.*, 2006).

However, a few years later, it characterized 30% of *S. Typhimurium* isolates from humans in Italy. (Dionisi *et al.*, 2009). The other features of the Basilicata strains such as the presence of SGI1, and the array of the resistance genes strongly matches with those of the pandemic MDR DT104 strain, which is worldwide spread. Therefore it is possible to hypothesize that between the end of 1990s and the beginning of the next decade, three salmonellosis foci in rabbit farms in Basilicata were due to a clonally related group of strains, which, in turn, was part of a wider pandemic group. Furthermore, it is noteworthy that the STYMXB.0061 group circulated among Basilicata rabbits few years before it became predominant among humans in Italy.

The resistance pattern AMP-CHL-STR-SMX-TET was also broadly diffused among isolates from human sources, and it is remarkable that the Basilicata strains were susceptible to ENR and GEN, largely used in veterinary practice and, especially the latter, in rabbitries.

Those data highlight the close relatedness among human and rabbit isolates and also let us hypothesize a bilateral transmission pathway, from human to rabbit and vice versa. Even if further data should be provided to support the hypothesis, it should be tempting to speculate that rabbit infections were caused by strains selected among humans and then introduced, directly or indirectly, in the rabbit farms. Hence, rabbit may not only represent a source of infection for humans, as stated by Vieira-Pinto *et al.* (2011) and Borrelli *et al.* (2011) but it can be infected by humans, even though by an indirect way.

Therefore, we strongly advise for the need of a deeper characterization of *Salmonella* spp. strains responsible for salmonellosis in rabbit, in order to prevent wider outbreaks among rabbits and to quickly reveal the insurgence of any dangerous variant for humans or rabbits.

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