

Antibiotic resistance in *Salmonella enterica* serotypes Typhimurium, Enteritidis and Infantis from human infections, foodstuffs and farm animals in Italy

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SUMMARY

The antimicrobial susceptibility of isolates of *Salmonella enterica* serotypes Typhimurium, Enteritidis, and Infantis isolated from humans, foodstuffs and farm animals in Italy between 1999 and 2001 was examined. All the isolates were susceptible to cefotaxime and ciprofloxacin, but high rates of resistance were observed for several other drugs, especially for *S. Typhimurium*. The rates of resistance and multiresistance were generally higher among animal and food isolates than in human strains; conversely, no significant difference was observed between animal and food isolates. Among *S. Typhimurium*, multiresistance was more common in bovine, poultry and rabbit strains than in swine isolates, and was rare in strains from pigeon. Resistance to trimethoprim–sulphamethoxazole was mainly found in isolates of swine and human origin. This study confirms the role of livestock as a reservoir of drug-resistant *Salmonella* spp. and underlines the need for integrated surveillance systems of antibiotic resistance that consider isolates not only from human disease but also from the animal reservoirs and the food vehicles.

INTRODUCTION

Emergence of antibiotic-resistant bacteria has become an increasing problem all over the world, favoured by overuse or misuse of antibiotics not only in human medicine but also in veterinary medicine and agriculture. In farm animals, antibiotics are used for therapy and prophylaxis of diseases, and have also been widely used as growth promoters. Food animals represent a large reservoir of resistant bacteria and

resistance genes, and foodstuffs of animal origin can be the vector for their transmission from the animal ecological niche to humans [1].

Salmonella enterica is one of the most important foodborne zoonotic pathogens, and in recent years, antibiotic multiresistant strains have been isolated with increasing frequency from human infections, farm animals, and foodstuffs [2]. For *Salmonella enterica* serotype Typhimurium, this phenomenon has been associated with the spread among farm animals of a single clone, referred to as definitive phage-type (DT) 104 [3, 4]. However, multiresistance is not an exclusive feature of *S. Typhimurium*, as it

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characterizes other serotypes, such as *S. Hadar*, *S. Blockley* and *S. Virchow* [3–6], and has also been detected in *S. Enteritidis* [6, 7].

Surveillance systems for salmonella infections and the associated antibiotic resistance have been developed in many countries. In the European Union, an international surveillance network, designated Enter-net, has been active since 1994 [6]. Italy participates in the Enter-net Surveillance system, and information on approximately 10 000 salmonella isolates from different sources is collected every year. *S. Typhimurium*, *S. Enteritidis* and *S. Infantis*, usually account for more than 70% of the strains responsible for human infections [8].

In this paper we describe the antimicrobial susceptibility of strains of *S. Typhimurium*, *S. Enteritidis* and *S. Infantis* isolated in Italy from humans, animals, foodstuffs, and environmental sources between 1999 and 2001, and submitted to the medical and veterinary Salmonella Reference Laboratories for further characterization. The resistance rates and profiles observed for these serotypes were compared, taking into account the different origin of the isolates.

MATERIALS AND METHODS

Bacterial strains

Strains were isolated in Italy from January 1999 to December 2001 from human cases, diseased and healthy animals, foodstuffs, mainly of animal origin, and environmental sources, mainly sewage and surface waters. Isolates were sent to the National Reference Laboratory for Enteric Pathogens and to the Veterinary Reference Laboratory for Salmonella for characterization. Serotyping was performed by the slide agglutination method using commercial O and H antisera (Statens Serum Institut, Copenhagen, Denmark). Phage-typing was performed on 512 of the *S. Typhimurium* isolates, mainly of animal origin, by using standard methods [9] and phages provided by the International Phage-typing Reference Laboratory (Health Protection Agency, London, UK).

Antimicrobial susceptibility testing

Susceptibility was determined by the disk diffusion method, following the National Committee for Clinical Laboratory Standard (NCCLS) recommendations [10]. Isolates were plated onto Mueller–Hinton agar (Becton Dickinson, Franklin Lakes, NJ, USA) and

the following antibiotic disks (Becton Dickinson) and concentrations (μg) were used: nalidixic acid (Nx) 30, ampicillin (A) 10, cefotaxime (Ctx) 30, ciprofloxacin (Cp) 5, chloramphenicol (C) 30, gentamicin (G) 10, kanamycin (K) 30, streptomycin (S) 10, sulphonamides (Su) 300, tetracycline (T) 30, trimethoprim–sulphamethoxazole (SXT) 1.25/23.75. Animal and food isolates were also tested for amoxicillin–clavulanic acid (AMC) 30, cephalothin (Cf) 30, and amikacin (An) 30. *Escherichia coli* ATCC 25922 was used as control strain in each experiment. Susceptibility was assessed following the NCCLS criteria, and intermediate resistant strains were considered as a separate group. Strains resistant to four or more antibiotics were considered as multiresistant.

Data analysis and significance testing of differences in proportions was performed with Epi-Info version 6.04b (CDC, Atlanta, GA, USA) using the χ^2 test or Fisher's exact test when appropriate.

PFGE analysis

An agreed protocol for PFGE was performed using the Bio-Rad CHEF[®] system (Bio-Rad, Hercules, CA, USA). This involved proteinase K lysis of cells overnight at 54 °C, a series of washes at 50 °C followed by digestion with 50 U *Xba*I (overnight, 37 °C). Electrophoresis conditions were as follows: RAMP initial 2 s; final 64 s; 6 V/cm, angle 120°; 14 °C for 24 h (CHEF Mapper[®]; Bio-Rad).

DNA macrorestriction fragments were resolved on 1% agarose gels [Seakem Gold Agarose (BMA); Rockland, ME, USA] with a *S. Braenderup* strain (kindly supplied by PulseNet US, CDC) as a molecular reference marker. Gel was stained with ethidium bromide solution (0.5 $\mu\text{g}/\text{ml}$). Gel image was taken in tag image file format (TIFF files). PFGE patterns were analysed with BioNumerics software using Dice coefficient and the unweighted pair group method of averages (UPGMA) with a 1.5% tolerance limit and 1.5% optimization [11].

RESULTS

A total of 1871 salmonella isolates were examined, and the rates of resistance to the antibiotics tested are shown in Table 1, according to the serotype and the source of isolation. All the isolates were susceptible to Ctx and Cp and a low prevalence of resistance, less than 10%, was observed for G, K, An and Cf, the last two were only tested for food and animal isolates.

Table 1. Occurrence of antimicrobial drug resistance in *Salmonella enterica* by serotype and source of isolation, in Italy, 1999–2001

| Serotype | Source (no. of strains) | % resistant to antibiotic* | | | | | | | | | | | % resistant to (no. of drugs) | | | | | | | |
|-----------------------|-------------------------|----------------------------|-----|----|-----|----|-----|-----|------|----|-----|----|-------------------------------|-----|----|----|----|----|----|----|
| | | A | AMC | Cf | Ctx | S | G | K | An | Su | SXT | Nx | Cip | C | T | 0 | 1 | 2 | 3 | ≥4 |
| <i>S. Typhimurium</i> | Total (1033) | 57 | 19 | 4 | 0 | 57 | 2 | 3 | 5 | 63 | 12 | 14 | 0 | 44 | 64 | 25 | 13 | 8 | 4 | 50 |
| | Human (424) | 49 | nt† | nt | 0 | 42 | 3 | 0.5 | nt | 51 | 10 | 4 | 0 | 33 | 56 | 32 | 16 | 8 | 3 | 41 |
| | Food (292) | 61 | 15 | 4 | 0 | 61 | 2 | 3 | 5 | 66 | 11 | 16 | 0 | 47 | 70 | 16 | 16 | 9 | 4 | 55 |
| | Animals (296) | 61 | 22 | 4 | 0 | 70 | 2 | 7 | 0 | 73 | 15 | 26 | 0 | 54 | 68 | 21 | 5 | 8 | 4 | 62 |
| <i>S. Enteritidis</i> | Environment (21) | 81 | nt | nt | 0 | 81 | 0 | 5 | nt | 81 | 5 | 44 | 0 | 71 | 86 | 14 | 5 | 0 | 0 | 81 |
| | Total (629) | 8 | 3 | 9 | 0 | 4 | 0.5 | 2 | 5 | 5 | 2 | 10 | 0 | 1 | 6 | 75 | 21 | 3 | 0 | 1 |
| | Human (525) | 7 | nt | nt | 0 | 2 | 0 | 1 | nt | 3 | 1 | 10 | 0 | 1 | 5 | 76 | 21 | 2 | 0 | 1 |
| | Food (54) | 22 | 6 | 17 | 0 | 12 | 4 | 2 | 3 | 13 | 4 | 10 | 0 | 5 | 12 | 58 | 32 | 6 | 0 | 4 |
| <i>S. Infantis</i> | Animals (50) | 4 | 0 | 2 | 0 | 16 | 2 | 6 | 12.5 | 17 | 6 | 19 | 0 | 0 | 10 | 67 | 14 | 14 | 2 | 3 |
| | Total (209) | 4 | 0 | 2 | 0 | 17 | 1 | 4 | 9 | 19 | 4 | 6 | 0 | 1.5 | 10 | 67 | 19 | 5 | 6 | 3 |
| | Human (43) | 0 | nt | nt | 0 | 14 | 0 | 0 | nt | 2 | 2 | 0 | 0 | 0 | 9 | 84 | 7 | 9 | 0 | 0 |
| | Food (126) | 6 | 0 | 2 | 0 | 14 | 0 | 2 | 9.5 | 21 | 2 | 6 | 0 | 2 | 9 | 65 | 22 | 4 | 4 | 5 |
| <i>S. Infantis</i> | Animals (40) | 5 | 0 | 3 | 0 | 27 | 5 | 1.5 | 0 | 27 | 12 | 10 | 0 | 2 | 15 | 54 | 26 | 3 | 15 | 2 |

* Abbreviations are given in the Materials and Methods section.
† nt, Not tested.

As far as other drugs are concerned, resistance rates were higher in *S. Typhimurium* than in the other two serotypes. In particular, high rates of resistance (from 44 to 64%) were observed for A, C, S, Su, T, and to a lesser extent (19 and 12% respectively) to AMC and SXT. Resistance to Su was also common in *S. Infantis*, while resistance to Nx ranged between 6–14% in the three serotypes.

S. Typhimurium strains isolated from humans showed resistance rates for A, C, S, Su, T, and Nx significantly lower than those observed for isolates from non-human sources ($P < 0.01$). The highest rates of resistance were observed among the isolates of environmental origin. The differences between strains of food and animal origin were not statistically significant.

Among *S. Enteritidis* strains, resistances to S and Su were significantly more frequent among non-human strains ($P = 0.01$), while resistance to A was significantly more common in food isolates ($P < 0.01$).

Among *S. Infantis* strains, the only significant differences were observed for K and Su: K resistance was more common in strains from animals than in strains from humans and food ($P < 0.01$), and Su resistance was more frequent in animal and food isolates than in human strains ($P < 0.01$).

The distribution of the number of resistances within the three serotypes is also reported in Table 1, according to the source of the isolates. As expected, multiresistance was more common in *S. Typhimurium* than in *S. Enteritidis* and *S. Infantis*. For the three serotypes, the proportion of sensitive isolates was higher in human strains ($P < 0.02$ for human vs. both animal and food isolates for *S. Typhimurium* and *S. Infantis*; $P < 0.01$ for human vs. food isolates for *S. Enteritidis*) whereas multiresistance was generally more common among non-human isolates ($P < 0.02$ for animal and food vs. human isolates for *S. Typhimurium*; $P < 0.01$ for animal vs. human isolates for *S. Enteritidis*).

The most frequently observed pattern in *S. Typhimurium* was ACSSuT, often with Nx, SXT or AMC as additional resistances. Most of the isolates showing this pattern were phage-type DT104 (results not shown). The resistance patterns of *S. Enteritidis* and *S. Infantis*, were usually limited to a few antibiotics and often included S and Su for *S. Enteritidis*, in addition to Nx and T for *S. Infantis*.

For *S. Typhimurium* strains isolated from animals and meat products, the resistance rates were analysed according to the animal species, considering

Table 2. Resistance rates in *Salmonella Typhimurium* isolates from animals and products, by species of origin

| Origin (no. of strains) | % resistant to antibiotic* | | | | | | | | | | | % resistant to (no. of drugs) | | | | | |
|----------------------------|----------------------------|-----|----|----|---|---|----|-----|----|-----|----|-------------------------------|----|----|----|----|----|
| | A | AMC | Cf | S | G | K | Su | SXT | Nx | Cip | C | T | 0 | 1 | 2 | 3 | ≥4 |
| Swine (231) | 63 | 20 | 8 | 64 | 4 | 7 | 72 | 30 | 11 | 0 | 41 | 73 | 15 | 12 | 4 | 11 | 58 |
| Rabbit (92) | 88 | 33 | 1 | 91 | 0 | 3 | 88 | 0 | 51 | 0 | 71 | 84 | 8 | 2 | 14 | 3 | 73 |
| Cattle (63) | 87 | 22 | 3 | 86 | 0 | 4 | 86 | 0 | 38 | 0 | 84 | 94 | 3 | 6 | 6 | 0 | 85 |
| Poultry (40) | 60 | 18 | 8 | 85 | 3 | 3 | 77 | 0 | 29 | 0 | 62 | 78 | 15 | 7 | 3 | 0 | 75 |
| Pigeon (34) | 3 | 0 | 0 | 12 | 0 | 9 | 6 | 0 | 9 | 0 | 0 | 9 | 80 | 7 | 3 | 3 | 7 |

* Abbreviations are given in the Materials and Methods section.

altogether the isolates from an animal species and the derived meat products (Table 2). The highest rates of resistance were usually observed in strains of bovine and rabbit origin, and the lowest in isolates from pigeons. Resistance to SXT was observed in 30% of the isolates of swine origin, whereas it was absent among the isolates from other animal species. Multi-resistance was more common in bovine, poultry and rabbit strains than in swine isolates, and was rare in strains from pigeons. The ACSSuT DT104-related pattern was observed in 43% of bovine, 28% of rabbit, 22% of poultry, and 19% of swine isolates, but it was never found in isolates from pigeon. It was also present in 28% of the isolates of human origin and in 38% of the strains from the environment, mainly isolated from surface water. The same pattern complemented by resistance to Nx was common among rabbit (34%), bovine (30%) and poultry (20%) isolates. Conversely, it was found in only 6% and 2% of swine and human isolates respectively. The ASSuT profile was observed in 79 (7.6%) of the *S. Typhimurium* isolates, and 33 and 32 of them were of swine and human origin respectively. Most of these isolates were not typable with the phages panel used. When analysed by PFGE, human and swine isolates showed similar profiles (Fig.), which were clearly different from those of the strains showing the classical DT104-related ACSSuT profile.

DISCUSSION

Salmonellae are zoonotic pathogens, widespread in nature, and can infect a variety of domesticated and wild animals ranging from mammals to birds and reptiles. Farm animals are frequently colonized by *Salmonella* spp., and infections are often transmitted to human beings through the consumption of food of animal origin [12]. A dramatic increase in the occurrence of multiple drug-resistant strains of *Salmonella*

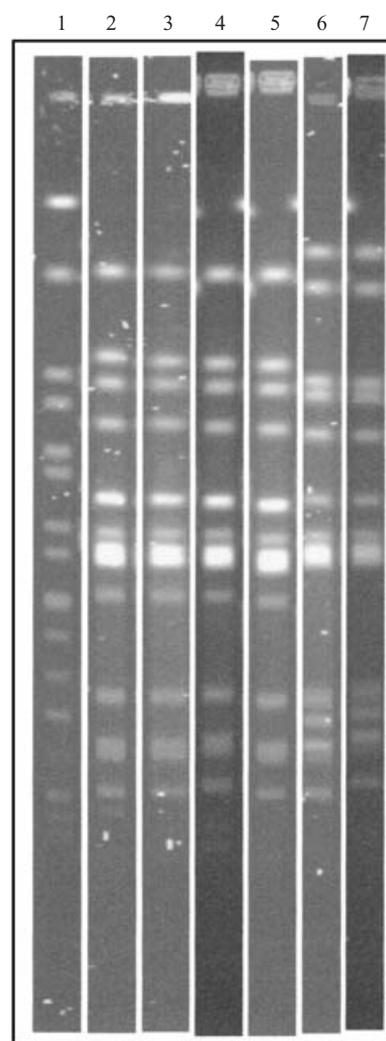


Fig. PFGE analysis of *S. Typhimurium* isolates of human and swine origin untypable by phage typing (NT) with ASSuT resistance profile. Lane 1, *S. Braenderup* as control. Lanes 2, 3, *S. Typhimurium* NT from humans with ASSuT resistance profile. Lanes 4, 5, *S. Typhimurium* NT from swine with ASSuT resistance profile. Lane 6, *S. Typhimurium* DT104 from humans with ACSSuT resistance profile included for comparison. Lane 7, *S. Typhimurium* DT104 from swine with ACSSuT resistance profile included for comparison.

spp. has occurred during the last decade [4]. In this study we have examined the antibiotic susceptibility of strains of *S. Typhimurium*, *S. Enteritidis* and *S. Infantis*, that usually accounts for more than 70% of human infections in Italy. Resistance rates and patterns of human isolates have been compared with those of strains isolated from animals, foodstuffs of animal origin, and from the environment.

For all the three serotypes considered in the study, both the prevalence of resistance to single drugs and the frequency of multiresistant strains were generally higher among animal and food isolates than in human strains. Conversely, no significant difference was observed in the resistance rates of animal and food isolates. This observation appears to be consistent with the hypothesis that farm animals and the derived foodstuffs represent the source of most human infections due to multiresistant strains of *Salmonella* spp. [1, 13].

Fluoroquinolones and expanded-spectrum cephalosporins have become the recommended drugs for invasive salmonella infections and current concerns focus on the emergence of resistance to these drugs [4, 13–15]. These resistances were not found in the sample of isolates investigated in this study. However, resistance to first-generation cephalosporins and Nx were both observed. In particular, Nx resistance was found in all three serotypes with frequencies around 10% and was the most common resistance in *S. Enteritidis*, as also reported by Threlfall et al. [5]. Nx resistance was more common among non-human isolates and this could be related to the frequent use of quinolones in livestock production [16]. A very high rate of Nx resistance (65.1%) has recently been reported for *S. Enteritidis* strains of poultry origin in Spain [17] and has been related to the authorization of the use of several quinolones in that country. Both resistance and multiresistance was more common in *S. Typhimurium* than in the other two serotypes, with 75% of isolates drug resistant and 50% multiresistant. This feature is mainly related to the frequent occurrence of the clonally related strains of *S. Typhimurium* DT104 [3, 4] that carry a chromosomally located gene cassette [4, 18, 19] accounting for multiple resistance to ACSSuT with several isolates showing additional resistance to SXT and Nx.

As for the human isolates, the resistance rates observed in this study for *S. Enteritidis* were similar to those reported in Spain [20], in England and Wales [21], and in a multicentre European study [6].

Conversely the resistance rates of *S. Typhimurium* were generally much lower than those of the Spanish isolates [20], and also lower than those reported in the English [21] and European [6] studies. Also the *S. Typhimurium* isolates of animal origin had a rate of multiresistance lower than that reported for strains from animal sources in Spain [17]. Comparing the data obtained in this study for human isolates with those of an old study carried out in Italy during 1977–1978 [22], it can be seen that resistance to C and A in *S. Typhimurium* increased from 8 to 33% and from 13 to 49% respectively. Nx resistance also increased from 0 to 4% in *S. Typhimurium* and from 0 to 10% in *S. Enteritidis*. These increases clearly reflect the spread of *S. Typhimurium* DT104 which occurred in Europe and other countries during the last decade [3, 4] and the increased use of quinolones in livestock production during this period [16, 23].

S. Typhimurium is the serotype of salmonella with the most ubiquitous host range, and in our series of strains we had isolates from several animal species and from the derived meat products. It was, therefore, possible to compare the resistance rates in strains from different animal species. The highest rates of resistance were observed in isolates of bovine and rabbit origin, in particular for A and C. This result probably reflects the high prevalence of *S. Typhimurium* DT104 among bovine and rabbit isolates (results not shown) and is in good agreement with other studies that reported the frequent occurrence of *S. Typhimurium* DT104 and other multiresistant phage types in cattle [2, 4, 17]. On the contrary, the lowest resistance rates were observed among isolates from pigeon. This result is not surprising since most of the isolates were from feral pigeons, which are not expected to be as exposed to antibiotics as farm animals usually are.

A high level of resistance to SXT (30%) was observed in porcine isolates, while this resistance was almost absent in *S. Typhimurium* isolates from the other animal species. SXT resistance was also observed, but to a lesser extent, in approximately 10% of the human isolates of *S. Typhimurium* and it could represent a useful index of the role of swine as a source of human salmonellosis and a valid phenotypic marker for epidemiological investigation. A high level of SXT resistance in *S. Typhimurium* of swine origin has also been reported in Spain [17] whereas porcine isolates of *S. Typhimurium* from the United States were all susceptible to this drug [24].

Multiresistance was more common in bovine, poultry and rabbit strains than in swine isolates, and was rare in strains from pigeons. The DT104-related pattern also added to the resistance to Nx was common among rabbit, bovine and poultry isolates. The DT104-related pattern was also common in human and swine isolates but additional resistance to Nx was rare in isolates from these two species. The ASSuT pattern was observed in 79 (7.6%) *S. Typhimurium* strains, mainly of swine and human origin. This pattern has been associated with *S. Typhimurium* DT193 strains from human infection in England and Wales [21] and Spain [20] and has been previously associated with pigs in the United Kingdom [25, 26]. However, most of our isolates examined in this study were untypable by phage typing. PFGE analysis showed that these untypable strains are highly related, regardless of their origin being human or swine.

In conclusion, this study indicates that in Italy: (i) strains of salmonella of animal origin are in general more resistant than human strains; (ii) resistance to Nx is frequent in strains of animal origin but quite uncommon in human isolates; (iii) among *S. Typhimurium* strains from farm animals, multiresistance was more common in bovine, poultry and rabbit strains than in swine isolates; (iv) SXT resistance and the pattern ASSuT appear to be associated with *S. Typhimurium* strains of swine origin and are also found in human infections.

These results confirm the role of livestock as a reservoir of drug-resistant salmonella and underline the need of integrated surveillance systems of antibiotic resistance in zoonotic pathogens that consider isolates not only from human disease but also from the animal reservoirs and the food vehicles [26–28].

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