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Non-typhoidal *Salmonella* in Calabria, Italy: a laboratory and patient-based survey

Valentina Mascaro,[1] Claudia Pileggi,[1] Maria Crinò,[1] Yolande Therese Rose Proroga,[2] Maria Rosaria Carullo,[2] Caterina Graziani,[3] Fabio Arigoni,[4] Pasquale Turno,[4] Maria Pavia[1]

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[1]Department of Health Sciences, University of Catanzaro 'Magna Græcia', Catanzaro, Italy
[2]Department of Food Microbiology-Centro Pilota Tipizzazione Salmonelle, Istituto Zooprofilattico Sperimentale del Mezzogiorno, Portici, Naples, Italy
[3]Dipartimento di Sanità Pubblica Veterinaria e Sicurezza Alimentare, Reparto di Epidemiologia Veterinaria e analisi del rischio, Istituto Superiore di Sanità, Rome, Italy
[4]Dipartimento Tutela della Salute, Task Force per le Attività Veterinarie, Regione Calabria, Catanzaro, Italy

Correspondence to
Maria Pavia; pavia@unicz.it

ABSTRACT

Introduction Although there has been a decrease in the number of cases of salmonellosis in the European Union, it still represents the primary cause of foodborne outbreaks. In Calabria region, data are lacking for the incidence of human non-typhoid salmonellosis as active surveillance has never been carried out.

Objective To report the results of a laboratory and patient-based morbidity survey in Calabria to describe the incidence and distribution of *Salmonella* serovars isolated from humans, with a focus on antimicrobial resistance patterns.

Methods Positive cultures from human samples were collected from every laboratory participating in the surveillance, with a minimum set of information about each isolate. A questionnaire was then administered to the patients by telephone interview to assess the potential risk exposures. *Salmonella* isolates underwent biochemical identification, molecular analysis by PCR and antimicrobial susceptibility testing by the disk-diffusion method.

Results During a 2-year period, 105 strains of *Salmonella* spp were isolated from samples of patients with diarrhoea, with the highest isolation rate for children aged 1–5 years. The standardised rate was 2.7 cases per 1 00 000 population. The most common *Salmonella* isolates belonged to monophasic variant of *S.* Typhimurium (*S.* 4,[5],12:i:-) (33.3%), followed by *S.* Typhimurium (21.9%). 30.5% of the isolates were susceptible to all microbial agents tested and the most common pan-susceptible serotype was *S.* Napoli (100%). *S.* 4,[5],12:i:- was resistant to ampicillin, streptomycin, sulfonamides and tetracyclines in 42.9% cases, while resistance to quinolones was seen in 14.3% of the isolates.

Conclusions The results provide evidence that an active surveillance system effectively enhances *Salmonella* notifications. The high prevalence of antimicrobial resistance, including resistance to quinolones and multiresistance, enforces the need to strengthen strategies of surveillance and monitoring of antimicrobial use.

Strengths and limitations of this study

- ► This study reports for the first time the results of a laboratory and patient-based survey surveillance of human cases of salmonellosis in Calabria, a region with one of the lowest notification rates.
- ► All hospital and outpatient laboratories (public or private) were contacted and those who performed microbiological analyses for the detection of *Salmonella* spp were considered eligible in a surveillance network.
- ► As a surveillance system, the study inevitably underestimates diseases occurring in the community, analysing only a fraction of the total number of cases of illness in the population.

INTRODUCTION

Salmonella infections are among the most common foodborne diseases: they cause approximately 93.8 million illnesses and 1 55 000 deaths annually around the world.[1] Although there has been a steady decrease in the number of cases in the European Union (EU), salmonellosis represents the primary cause of foodborne outbreaks and the second most frequently reported zoonosis. Recently, it has been estimated that the overall incidence of human salmonellosis in the EU general population is approximately 20.4 cases per 1 00 000 population each year, while in Italy the incidence is 7.5 cases per 1 00 000 population each year.

In Italy, surveillance of acute infectious gastroenteritis and outbreaks of food-borne diseases is part of the activities of the Italian National Surveillance System of diseases (SIMI) whereas Enter-net (IT-ENTER-NET) is a laboratory-based surveillance system for enteropathogens based on a network of clinical microbiology diagnostic laboratories.[2 3] It is complementary to SIMI and collects microbiological information on *Salmonella* isolates from human cases each year.

The level of under-reporting of infectious diseases and laboratory surveillance, is expected to vary between countries, depending on differences in organisation and effectiveness of local systems.[4]

These surveillance systems vary greatly in their performance, representativeness and data quality in different Italian regions, since laboratories that participate in the surveillance are not homogeneously distributed nationally. There are differences in sensitivity among Italian regions; in particular, it has been shown that northern regions of Italy are generally more sensitive in detecting cases, leading to significantly higher notification rates in comparison with the national average.[5] In Calabria region, data for the incidence of human non-typhoid salmonellosis (NTS) are lacking.[5] Surveillance has never been carried out, but the information which could be obtained would help to guide clinicians in the treatment of those groups at risk, such as infants, the elderly and immunocompromised patients, who would not benefit from simple rehydration therapy, and would help local health authorities in the identification of appropriate control measures.[6]

Within the framework of a research project supported by the Ministry of Health, the study reports the results of a laboratory and patient-based morbidity survey in Calabria to describe the incidence and distribution of *Salmonella* serovars isolated from humans, with a focus on antimicrobial resistance patterns.

MATERIALS AND METHODS

A laboratory and patient-based survey for *Salmonella* spp infections has been carried out in Calabria (southern Italy), a region with 1 980 533 inhabitants (population estimates for 2014) living in three major cities and several smaller centres.

The five local health units provided a list of all clinical microbiology diagnostic laboratories of Calabria region. All hospital and outpatient laboratories (public or private) were contacted and those who carried out microbiological analyses for the detection of *Salmonella* spp were considered eligible and invited to participate. A healthcare worker was trained to carry out the survey for each laboratory agreeing to participate. The project took place between February 2013 and March 2015.

Surveillance system

Positive cultures from human samples were obtained from the different laboratories. Every laboratory sent the isolates to the coordinating unit with accompanying documentation. A minimum set of information about each isolate was collected: name of the laboratory that reached the microbiological diagnosis, isolation date, sample type (stools or blood), patient sex, age, residence and telephone number. The unit provided biochemical identification and collected the strains for dispatch to the reference laboratories (Veterinary Public Health Institute of Southern Italy and the National Institute of Health) for typing.

A questionnaire was then administered to patients by telephone interview by a trained physician after appropriate informed consent had been obtained. If a patient was aged <18 years, an adult member of the household responded to the questionnaire.

Review instrument

The questionnaire used in the survey (online supplementary data) was a validated Italian version[7] review instrument currently used in the surveillance of acute gastroenteritis, and was adapted from the Center for Disease Control and Prevention standardised foodborne disease outbreak questionnaire.[8]

Validation of the survey instrument was performed through assessment of internal and test–retest (external) reliability, in addition to face and content validity. Test–retest reliability was checked in a pilot study of a sample of patients to ensure clarity and ease of completion and to improve the validity of responses and the information included. Modifications were then made as necessary.

The questionnaire included 43 questions divided into five sections. Responses were obtained in a variety of formats: closed-ended questions with multiple answers possible, yes or no questions and open-ended questions.

The sections of the questionnaire were as follows: (1) sociodemographic characteristics of the patient; (2) details of whether the patients or their cohabitants had or had not any sign of illness, information about prescribed culture tests, health service use and eventual hospitalisation, drugs taken and travel in the previous 2 weeks; (3) information about food exposures and the origin of foodstuff; (4) other exposures (ie, contact with animals, outdoor activities, water sports); (5) information about the source of commonly used water and purpose of use.

Microbiological methods

Salmonella isolates were biochemically identified using conventional microbiological methods and the automated Vitek system (BioMérieux). Serotyping was performed by the slide agglutination method using commercial O and H antisera (the antisera were purchased from Statens Serum Institut, Denmark) according to Kauffmann-White-Le Minor scheme.[9]

Strains were definitively assigned to serovar Typhimurium or *S.* 4,[5],12:i:– on the basis of the presence or the absence of the *fljB* gene tested by PCR.[10]

Antimicrobial susceptibility was performed by the disk-diffusion method (Kirby-Bauer). The following antimicrobial agents and concentration (µg) were used: amikacin (30), amoxicillin/clavulanic acid (20/10), ampicillin (10), cefoxitin (30), ceftriaxone (30), cephalothin (30), chloramphenicol (30), ciprofloxacin (5), gentamicin (10), kanamycin (30), nalidixic acid (30), neomycin (30), nitrofurantoin (100), streptomycin (10), sulfamethoxazole/trimethoprim (23.75/1.25), trimethoprim (5) and tetracycline (30). Classification of the categories as susceptible, intermediate or resistant was based on the Clinical and Laboratory Standards Institute guidelines[11] and for the purpose of analysis, all readings classified as intermediate were considered as resistant where necessary.

The study protocol was ratified by the institutional ethics committee ('Mater Domini' Hospital of Catanzaro, Italy) (7/05/2013).

Data analysis

Data were stored and analysed using an appropriate database. Statistical analysis was performed using STATA software programme, version 11 (Stata Corporation. College Station, Texas, USA).

Age-specific and standardised incidence rates were calculated. Direct standardisation using the Italian population as standard was performed. Demographic data were obtained from the National Institute of Statistics (ISTAT).[12]

RESULTS

The health authorities listed a total of 245 regional microbiology diagnostic laboratories. After exclusion of those which did not perform microbiological analyses for the detection of *Salmonella* spp, the network comprised 114 laboratories. Of these, 110 agreed to participate, with a response rate of 96.5%. Among the participating laboratories, 25 were public laboratories (hospital or outpatient based) and 85 were private.

During the 2-year survey, 108 cultures from stool samples of patients with a clinical picture compatible with salmonellosis were collected from the participating laboratories; among these samples, 105 were confirmed as strains of *S. enterica*. Demographic data were available for 102 of the patients, and complete telephone interviews for 70/105 (66.7%) patients. Four patients (3.8%) were not directly interviewed owing to a lack of contact, and thus data were obtained from medical records.

Distribution of cases and age-specific rates are shown in table 1. The highest isolation rate was for children aged 1–5 years, followed by children aged 6–14 years and in those aged ≥65 years (table 1). The standardised rate was 2.7 cases per 100 000 population. Data from the phone interviews showed that, as expected, the three most frequently reported clinical symptoms were diarrhoea (100% of responders), fever (89.7%) and abdominal pain (65.4%).

At least one family member of the included patients had similar symptoms in 19 cases and, of these, only four were recommended by their general practitioner or community-based paediatrician to have a culture test for the diagnosis of salmonellosis: one of these was positive for *Salmonella* spp and was included in the study.

All 78 patients who answered the questionnaire sought medical assistance: 54 (69.2%) of them consulted a general practitioner or community-based paediatrician, while 24 (30.8%) went to the emergency department. Antimicrobial agents were used for 78% of the responders, for an average of 9.1 days (±3.9), and 82.3% of the patients from whom we could gather this information were hospitalised, with an average stay of 5.7 days (±3.4). Most frequently used antimicrobial agents were ceftriaxone (41%), sulfamethoxazole/trimethoprim (13.1%), amoxicillin/clavulanic acid (13.1%) and clavulanic acid (8.2%).

All responders referred to the consumption of food deemed to be potentially unsafe. In most cases food eaten within 24–48 hours before the onset of symptoms included milk and milk products (90% of cases); cooked meat (90.5% of cases); sausages (71.4% of cases); cooked eggs (64.3% of cases). Less common was the consumption of cooked (38.6%) and raw (34.3%) vegetables and raw eggs (20%).

Salmonella isolates belonged to 19 serotypes with monophasic variant of *S.* Typhimurium (*S.* 4,[5],12:i:-) being the most common (33.3% of the total isolates), followed by *S.* Typhimurium (21.9% of the total isolates), *S.* Enteritidis (13.3% of the total isolates), *S.* Napoli (9.5% of the total isolates) and *S.* Infantis (2.9% of the total isolates). Other serovars represented 19% of the total isolates (table 2).

Of all 105 NTS isolates, 30.5% were susceptible to all microbial agents tested. The most common serotypes of pan-susceptible isolates were *S.* Napoli (100%), *S.* Enteritidis (50%) and *S.* Infantis (33.3%). *S.* Typhimurium serovar 4,[5],12:i:- showed resistance to ampicillin,

Table 1 Number of *Salmonella* isolates for age group, year and average annual incidence in Calabria, Italy 2014–2015

Age groups	2014 total cases (N. of isolates)	2015 total cases (N. of isolates)	Total cases	Annual average incidence Calabria/100 000	Annual average incidence Italy/100 000
0–11 Months	6	3	9	27.4	13.5*
1–5 Years	36	14	50	28.4	32.5*
6–14 Years	13	7	20	5.9	8*
15–64 Years	6	3	9	0.5	1.6*
≥65 Years	9	5	14	1.8	2.8*
Total	70	32	105†	2.7‡	7.5§

*Distribution of the annual isolation rates of *Salmonella* spp serovars in Italy during the period between 2000 and 2011.[5]
†Number of total cases is different from the total cases number by age groups because of lack of information about age in three patients.
‡Standardised incidence rate of *Salmonella* spp isolation.
§Notification rate for confirmed cases in 2011.[14]

Table 2 Antimicrobial resistance patterns of the main *Salmonella* serovars isolated from human cases in Calabria, 2014–2015

Serovars	No of isolates	Antibiotic resistance patterns (%)						
		Sensitive	ASSuT	ASSuT +other	ACSSuT	ACSSuT+other	Quinolones	Other patterns
S. 4,[5],12:i:-	35	0	42.9	17.1	5.7	0	5.7	31.4
S. Typhimurium	23	30.4	13	21.7	0	4.4	17.4	21.7
S. Enteritidis	14	50	0	0	0	0	28.6	21.4
S. Napoli	10	100	0	0	0	0	0	0
S. Infantis	3	33.3	0	0	0	0	66.7	0
Other serovars	20	35	0	0	0	10	15	45
Total*	105	30.5	17.1	10.5	1.9	2.9	14.3	26.7

*Total rate exceeds 100% because *Salmonella* spp serotypes resistant to quinolones are also present in the categories ASSuT+other or ACSSuT+other.
ACSSuT, ASSuT + chloramphenicol; ASSuT, ampicillin, streptomycin, sulfamethoxazole, tetracycline; Quinolones, this category includes all patterns in which nalidixic acid or ciprofloxacin are present; *S.* 4,[5],12:i:- monophasic variant of *Salmonella* Typhimurium.

streptomycin, sulfonamides and tetracyclines (R-type ASSuT) with or without additional resistances in 60% of cases. Over the study period resistance to quinolones tested (nalidixic acid and ciprofloxacin) was seen in 15 (14.3%) of the total isolates and among these the most common serovar was *S.* Infantis (66.7% of cases) and *S.* Enteritidis (28.6% of cases) (table 2).

DISCUSSION

This study reports for the first time the results of surveillance for salmonellosis in the population in Calabria, a region with one of the lowest notification rates.[5 13]

The incidence rate (2.7 cases per 100 000 population) differs significantly from that published by the European Food Safety Authority, which collects data on salmonellosis cases reported in the EU; they reported in 2011 a higher Italian rate of 7.5 cases per 100 000 population.[14] Recently, there has been a significant reduction in reported cases rates across the EU.[14 15] We know that isolation rates are usually considerably lower in the southern part of Italy,[5] but in northern Italy surveillance systems are more sensitive in detecting cases of infectious gastroenteritis,[16] leading to relatively higher national notification rates of salmonellosis.

The total number of cases between the 2 years of our survey differs, showing a reduction of isolates that is only partly explained by the trend for reduction across the EU.[14 15] This might be due to under-reporting of cases.

In addition, any surveillance system inevitably underestimates diseases occurring in the community for different reasons. First of all many subjects do not seek medical attention and for salmonellosis, only a proportion of symptomatic patients submit stool specimens for investigation; moreover, the sensitivity of laboratory identification varies according to the pathogen and not all identified pathogens are reported to the surveillance centre.[15 16]

Isolation rates were highest in children aged 1–5 years. This finding is consistent with that of other studies that have shown that younger children are at greater risk of infection.[5 17] This might also be due to an overestimation of cases in certain age group. There is a greater proportion of symptomatic infections among children, who also are at risk of dehydration, and they are more likely to see a doctor and therefore to have a stool examination (ie, detection bias).[18]

Almost all the data in our report were collected from hospitalised patients. However, we know that hospitalisations represent only a fraction of the total number of cases of illness in the population: for a pathogen of moderate virulence, a relatively small proportion of those who seek medical attention will be hospitalised. Since our surveillance was extended also to outpatient laboratories, it seems that in non-hospitalised cases, probably with milder disease than in those admitted to hospital, microbiological analyses are less frequently performed. Moreover, asymptomatic and mild cases of disease are difficult to enumerate because of under-reporting by physicians and differences of diagnostic capabilities and protocols among laboratories.[15]

Seventy-eight per cent of patients received antibiotic therapy, even though there is no evidence for its benefit for NTS diarrhoea in otherwise healthy people, and routine antimicrobial therapy is not recommended for mild and moderate cases of NTS.[19] Therefore, such a high percentage might be because in the 2-year survey most of the antibiotic-treated patients were hospitalised and were 0–5-year-old children in more than 50% of cases, justifying the treatment for a severe disease that could not be treated by only electrolyte replacement and rehydration.

The most frequently isolated serovars in the 2 years survey were *S.* 4,[5],12:i:- (33.3% of all isolated) and *S.* Typhimurium (21.9% of all isolated). The monophasic variant of serotype Typhimurium, *S.* 4,[5],12:i:-, has only rarely been reported among *Salmonella* serovars isolated before 1993, whereas since 2000 it has been found in human clinical cases, different animal species and foods in different continents, including Europe, Asia

and South and North America.[5 20] In Europe, although *S.* Enteritidis still remains the most reported serovar, *S.* 4,[5],12:i:- showed a marked increase in foodborne infections associated with pig meat.[21] *S.* 4,[5],12:i:- has well-established reservoirs of livestock that are likely to have favoured its entrance into the food chain, and consequent rapid emergence and dissemination.[22] Our findings are consistent with the results of other Italian studies, with *S.* Typhimurium being the most reported in the period 2000–2011.[5 23] Moreover, a recent study in northeastern Italy reported that the most commonly isolated serotypes were *S.* Typhimurium and its monophasic variant, with *S.* Enteritidis being the third most isolated serovar.[24]

Evidence for the decrease in human salmonellosis in Italy since the late 1990s relates to specific serovars, namely, *S.* Enteritidis and *S.* Infantis, whereas there is evidence that other serovars have emerged (*S.* 4,[5],12:i:-, *S.* Derby and *S.* Napoli) or remained fairly stable (*S.* Typhimurium).[2 13] The decrease of *S.* Enteritidis is probably due to the implementation of control measures against *Salmonella*, especially within the poultry industry, such as the vaccination of laying hens, improved hygiene and education of food workers.[25 26]

In line with other studies in Italy, a substantial proportion of *S.* Napoli was found (9.5% of all isolated) during our 2-year survey. In Italy, there is a slight but constant increase of cases of *S.* Napoli[27] and a low association with foodborne exposure.[28] The number of cases of *S.* Napoli in Italy has been rising since 2000, but no factors accounting for this trend have been identified.[28] There is a possibility of the presence of *S.* Napoli reservoirs in the environment; results obtained applying different molecular typing techniques have shown the presence of *S.* Napoli in different settings, such as wildlife and surface waters.[29]

Rates of antimicrobial resistance varied according to serotype. All isolates belonging to serotype *S.* 4,[5],12:i:- were resistant to at least one antibiotic, while the other serovars were resistant to at least one antimicrobial agent in between 5.7% and 66.7% of cases. The only exception was *S.* Napoli isolates, which were susceptible to all the tested antibiotics. In Europe, *S.* Typhimurium has consistently exhibited the highest percentage of strains with multiple resistances, mainly of the ACSSuT type,[30] but the frequency of dissemination of this multiresistant clone has reduced since 2002.[31] On the other hand, in Italy strains with the ASSuT pattern have become increasingly common, both in *S.* Typhimurium and *S.* 4,[5],12:i:- since 2000,[14 29] and more recent data reported that this resistance pattern is present in 36.1% of *Salmonella* isolates, mainly in *S.* 4,[5],12:i:-. Moreover, *S.* 4,[5],12:i:- only rarely showed the ACSSuT pattern.[13]

The *Salmonella* serovar 4,[5],12:i:- resistant to ampicillin, streptomycin, sulfonamide and tetracycline (pattern ASSuT) is extensively circulating in Denmark, Italy, the United Kingdom and in Greece,[28 32 33] and it has been frequently isolated from animal sources,in particular, swine.[34] In agreement with previous studies, we found high rates of *S.* 4,[5],12:i:- that presented the ASSuT pattern with or without other resistances (60% of all *S.* 4,[5],12:i:- isolated).

Multidrug resistant *Salmonella* strains are indeed of worldwide interest and a serious public health concern, because it has been shown that outbreaks caused by multidrug resistant NTS are associated with an increased rate of hospitalisation compared with outbreaks caused by pan-susceptible NTS.[35 36]

Over the study period resistance to quinolones (nalidixic acid or ciprofloxacin) was seen in 14.3% of the isolates. Resistance to quinolones among *Salmonella* around the world was low until the 1990s, when it started to increase[21 37] and the serovar most involved was *S.* Enteritidis.[38] On the contrary, in Italy the resistance rate to quinolones and fluoroquinolones tends to be relatively low.[24]

Emergence of antibiotic resistance has been related to the massive use of antimicrobial agents both in human and in veterinary medicine. Especially in livestock animals, antimicrobial agents are used for treatment and prophylaxis of diseases and in the past have been used as growth promoters.[39] This particularly applied to the poultry industry, until European bans led to a substantial reduction in the amount of antibiotics used in animal production.[40] Indeed, the resistance to fluoroquinolones in *Salmonella* spp is a major concern, since fluoroquinolone is one of the preferred drugs for early empirical treatment of severe gastroenteritis in adults[41] and drug-resistant NTS is associated with increased severity.[42]

CONCLUSIONS

In conclusion, the results of the survey provide evidence of the effectiveness of an active surveillance system in enhancing the detection of cases of *Salmonella*, and identification of circulating strains associated with human infections. The high prevalence of antimicrobial resistance, including resistance to quinolones and multiresistance (R-type ASSuT), increases the need to strengthen strategies of surveillance and monitoring of antimicrobial use. It might be useful for the future to consider the sustainability of such a survey in order to enforce a true surveillance system for enteric diseases and related antimicrobial resistance problems.

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