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SHORT COMMUNICATION

Characterization of antimicrobial resistant Salmonella enterica serovars Enteritidis and Typhimurium isolates from animal and food in Southern Italy

Anna Giannina Perugini · Maria Rosaria Carullo · Assunta Esposito · Vincenzo Caligiuri · Federico Capuano · Giorgio Galiero · Giuseppe Iovane

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Abstract In the last two decades, the emergence and spread of antimicrobial-resistant pathogens, among them Salmonella, has become a serious health hazard worldwide, and specifically the high incidence of multidrug resistance has been encountered widely in many European countries. This study examines the antimicrobial supsceptibility of Salmonella enterica strains Typhimurium and Enteritidis isolated in Campania and Calabria region (Southern Italy) from animal and food of animal origin. The relationship of antibiotic resistance phenotype and the presence of some resistance genes has been also investigated. As espected, our results showes that resistance to ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracycline is common, although resistance to other antibiotics (i.e.:nalidixic acid) and other resistance patterns occur. The genetic resistant patterns have been partially described for this food-borne pathogen but efforts are needed to realize the complete characterization of antimicrobial resistance genes.

Keywords Salmonella · PCR · Gene · Antibiotic resistance

Introduction

Salmonella is one of the most important genus that encompasses pathogenic serovars responsible for gastrointestinal infections in humans, and contaminated food from livestock (poultry, swine, ovine and buffaloes) is one of the best known source of infection. The transmission of these bacteria from food of animal origin to humans has been largely demonstrated (Swartz 2002; Haeghebaert et al. 2003; O'Brien and De Valk 2003), specifically for Salmonella enterica serovar Enteritidis and Typhimurium, consider as the two major etiologic agents of food-borne salmonellosis in human.

A. G. Perugini (✉) · A. Esposito · V. Caligiuri · G. Galiero · G. Iovane
Istituto Zooprofilattico Sperimentale del Mezzogiorno, Portici, Italy
e-mail: annagiannina.perugini@cert.izsmportici.it

M. R. Carullo · F. Capuano
Centro pilota per la tipizzazione delle Salmonelle nella Regione Campania,
Istituto Zooprofilattico Sperimentale del Mezzogiorno, Portici, Italy

In the latest years, the routine practice of giving antimicrobial agents to domestic livestock as a means of preventing and treating diseases, as well as promoting growth, is an important factor in the emergence of antibiotic-resistant bacteria that are subsequently transferred to humans through the food chain (i.e.: meat and animal products during harvest and processing are contaminted because of improperly preparation), so most infections with antimicrobial-resistant Salmonella are acquired by eating contaminated foods of animal origin (Gomez et al. 1997; Fey et al. 2000).

The use of antimicrobial agents in any environment creates selection pressures that favor the survival of antibiotic-resistant pathogens.

Antibiotic resistance increased dramatically in *S. Typhimurium*, specifically the high incidence of multidrug resistance (MDR) strains in Europe was due to a clonal and international spread of Salmonella enterica subsp. enterica serovar Typhimurium phage type DT104, initially linked to bovine farms (Akkina et al. 1999; Chaslus-Dancla et al. 2000; Threlfall et al. 2000a; Threlfall 2000; Cloeckaert and Schwarz 2001).

Non-typhoidal Salmonella enterica strains with multidrug resistance (MDR) have been encountered widely in many European countries, with a variety of food products being implicated in their spread (Threlfall et al. 2000b, 2003); other MDR Salmonella have been isolated from bovine products that did not belong to DT104 phage type (Gupta et al. 2003; Threlfall et al. 2003).

The aim of this study was to examine the antimicrobial supsceptibility of the most frequent Salmonella isolates collected in Campania and Calabria regions from animal and food, Salmonella enterica serovars Enteritidis and Typhimurium isolates (2004–2006), analyzed also for the carriage of some antibiotic resistance genes.

Materials and methods

Origin and isolation of bacterial strains

Samples collected for this study comprised organs/carcasses' swabs and, food (bovine, ovine, swine and chicken meat, shellfish, eggs, bovine ad bubaline milk).

Presumptive Salmonella colonies isolated on xylose–lysine desoxycholate–4 (XLT4, (Oxoid) and Rambach medium (VWR International, Italy), were identified through biochemical test, such as BBL Crystal E/NF (BD, USA). Strains confirmed as *S. Enteritidis* or *Typhimurium* by conventional biochemical test were serotyped with respect to cell wall (O) (Statens Serum Institut, Dk) and flagella (H) antigens (Difco, USA). Strains were also phage typed by the Centro di Referenza Nazionale per le Salmonellosi (IZSVE) according to a standard procedure.

Antibiotic sensitivity testing

The Bauer–Kirby standard procedure (Bauer et al. 1966) using Mueller Hinton (CM337: Oxoid, Basingstoke) agar was followed to test the antibiotic sensitivity of the Salmonella isolates. The following antibiotics were used: ampicillin (Amp)-10 μg; cephalothin (Cef)-30 μg; colistin sulphate (Co)- 10 μg; streptomycin (Strep)-10 μg; tetracycline (Tet)- 30 μg; gentamicin (Gen)-10 μg; chloramphenicol (Clo)-10 μg; nalidixic acid (Nal)-30 μg; sulphamethoxazole (Stx)- 25 μg; sulphonamide (Sul)-300 μg; amoxicillin (Amox)-30 μg; kanamicin (Kan)-30 μg; ceftazidime (Caz)-30 μg; ciprofloxacin (Cip)-5 μg;

enrofloxacine (Enr)-5 μg. Inhibition diameters were measured and interpreted as resistant intermediate or susceptible according to manufacturer recommendations (Oxoid).

PCR

For DNA isolation, 2–3 individual colonies were suspended in 0.2 mL of distilled water. They were boiled for 5 min, and centrifuged at 8,000× g. The supernatant was used as DNA and stored at −20°C until use.

Four pairs of primers were used for the amplification of four individual target genes simultaneously as described by Yang et al. (2001). Specific primers for sul1, sul2 and sul3 were also used to detect sulphonamide resistance genes, as described by Grape et al. (2003). Amplifications were performed with a GenAmp PCR System 2700 (Applied Biosystems, USA).

PCR products of both multiplex and simplex PCR were separated using 2% agarose gels, stained with ethidium bromide (0.5 μg/mL), and imaged with UV illumination; all amplicons obtained from Enteritidis strains and they obtained from 20 Typhimurium strains, representing different animal origins, were purified (QIAquick Gel Extraction Kit; Qiagen Inc., Hilden, Germany) and directly sequenced using fluorescent-dye-labeled dideoxynucleotides and a model 310 automatic DNA sequencer (Applied Biosystems, USA).

Sequences obtained were BLAST analysed and compared with those registered in GenBank.

Results

A total of 249 Salmonella enterica strains were isolated from food, feed and carcasses from 2004 to 2006. Different serotypes were identified, but only isolates belonging to the most frequent serotypes observed, such as Typhimurium (37) and Enteritidis (7), were tested for the presence of resistance genes. Of the selected isolates, 14 Thyphimurium and 3 Enteritidis, not reacting with any of the typing phages were considered nontypeable (NT). The most representative Typhimurium phage types were DT104, DT124, DT12 and U302 (10.8% each), whereas for Enteritidis the four phage types identified (PT13A, PT 14B, PT4, PT22) were observed at the same frequency.

At all, only one (Enteritidis) strain isolated from seafood (mussels or clams) was susceptible to all antibiotic tested, instead 39 (39/44) isolates were resistant to at least two antimicrobials, and 32 (32/44) to a least three antimicrobials.

The most predominant group was that of isolates resistant to sulphonamide (85.71% Enteritidis and 86.41% Typhimurium), followed by isolates resistant to streptomycin, tetracycline and ampicillin in Typhimurium (75.68, 70.27 and 67.57%, respectively) and resistant to colistin sulphate and nalidixic acid (both 28.57%) for S. Enteritidis. The multiple resistance (to five or more antimicrobials) was never observed in Enteritidis serotype, whereas it occurred in 24 (65%) of Typhimurium isolates.

According to the results of antimicrobial susceptibility testing, tree types of antimicrobial resistance were detected: the most predominant group was that of isolates resistant to ampicillin and tetracycline (AmpTet, 59.46%), followed by isolates resistant to ampicillin, tetracycline, and chloramphenicol (AmpTetClo, 34%) and finally strains resistant to five drugs (AmpCloSulTetStx, 14%). None of the isolates was resistant to ceftriaxone.

When analyzed by source, meat Salmonella isolates displayed the highest rate of resistance to at least one antimicrobial (59.09%), followed by those recovered from organs (18.2%), eggs (6.82%), shellfish and milk (both 4.5%). Meat Salmonella isolates also displayed the highest rate of resistance to at least 3 antibiotics (47.73%), followed by those recovered from organs (15.91%), milk (4.5%) and shellfish (2.3%). Isolates resistant to more then 9 antimicrobials were isolated only from meat (9.1%) and organs (2.3%).

When analyzed by species, swine isolates displayed the highest rate of resistance to at least one antimicrobial (36.4%), followed by those recovered from chicken (11.36%), bovine, buffalo and canary (9.1%), shellfish (4.5%) and ovine (2.3%). Isolates resistant to more then 8 antimicrobials were isolated only from swine (6.82%), chicken and canary (2.3%).

Detection of various antimicrobial resistance types by PCR was compared with detection with disk diffusion method and only few discrepancies were found: en effect; because of the little number of investigated genes, not always we could identified the genetic marker associated to antimicrobial resistance(s), although disk diffusion sensitivity was always associated to negative amplification of resistance(s) gene(s) investigated.

In particular, no one Salmonella eEnteritidis resistant to ampicillin could be found and no amplification products were obtained for the associated genes investigated. However, when amplification was carried out using as template a DNA isolated from some of the identified Typhimurium phage types, PSE and TEM amplicons where obtained, and in such cases both genes were found in the same isolates (in one DT104 and two U302 isolates). Moreover, cmla/tetR amplicon was observed only in isolates identified as S. Typhimurium, once again in different phage typed isolates (DT104, DT12, U310, U302, DT120, NT and RDNC isolates).

Even though S. Enteritidis isolates resistant to sulphomanide were identified after disk diffusion agar test, no amplicons for sul genes were obtained for any isolate belonging to this serovar. Amplification of sul1 and sul2 genes was observed only in Typhimurium isolates, specifically sul1 in isolates identified as phage type DT104, DT12, DT20, U302, U310, and sul2 in DT193, DT194, RDCN and NT isolates. Only 2 Typhimurium isolates amplified for both sul1 and sul2 genes, but the phage type of these isolates could not be identified. No one amplicons was obtained for sul3.

Discussion

Because antimicrobial-resistant bacteria from food animals may colonize the human population via the food chain, contact through occupational exposure, or waste runoff from animal production facilities (van den Bogaard and Stobberingh 1999; Witte 1998) it is possible that resistant bacteria may be readily transferred from food animals to humans. It is known that swine-associated phage types have been reported to be common causes of human salmonellosis (Baggesen and Wegener 1994; Schiellerup et al. 2001; Wegener et al. 1994) so the high prevalence of multidrug-resistant Salmonella contamining pork products could represent a substantial risk for the acquisition of strains with this type of multiresistance by humans.

Animal used in petting zoos and educational programs, when carry enteric organisms pathogenic to humans, can also increase the probability of disease transmission. En effect, it has been shown that pet, in particular rodents, snakes, terrestrial and aquatic turtles, cats probably are an underrecognized source of human salmonella infection (Ebani et al. 2005).

In the collection of Salmonella isolates analyzed it has been observed that in addition to DT104 isolates, also phage type U302, DT12, DT120 and DT19, obtained from pork meat, exhibited the AmpCloStrSulTet pattern resistance, and the genetic characterization also revealed that these isolates carried the same resistance genes.

Different U302 isolates, with the AmpCloStrSulTet pattern resistance, and carrying the same resistance genes, had been also isolated from bovine meat and from died canaries, supporting the thesis of food and animal contact as possible way of trasmission of multidrug resistant enteric pathogens.

Despite the fact that it is not yet clear to what extent the use of antibiotics in animals contributes to the resistance problems in human medicine, it cannot be disputed that it is a definite factor. Because we are now encountering in human medicine some microorganisms that are so multiresistant that it is difficult and may be soon impossible to fight these with the clinically available antibiotics, every source of resistance must be controlled as well as possible.

Efforts are needed to reduce the prevalence of resistant bacteria in food, including the adoption of guidelines for the prudent use of antimicrobial agents in animals used for food, the passage of new food safety regulations, and a reduction in the number of pathogens present on farms and in slaughterhouses.

The characterization of antimicrobial resistance genes as well as their location and diversity seems to be important in identifying factors involved in resistance, understanding the diversity of MDR strains, identifying genetic linkages among markers, understanding potential transfer mechanisms, and developing efficient detection methods.

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