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Repeated Occurrence of Diverse Extended-Spectrum β-Lactamases in Minor Serotypes of Food-Borne *Salmonella enterica* subsp. *enterica*

L. Politi,[1] P. T. Tassios,[1] M. Lambiri,[2] A. Kansouzidou,[3] M. Pasiotou,[4] A. C. Vatopoulos,[2] K. Mellou,[5] N. J. Legakis,[1] and L. S. Tzouvelekis[1]*

Department of Microbiology, Medical School, National and Kapodistrian University of Athens,[1] Salmonella and Shigella Reference Centre, National School of Public Health,[2] and Department of Epidemiological Surveillance and Intervention, Hellenic Centre for Infectious Disease Control (KEEL),[5] Athens, Salmonella Reference Centre for Macedonia and Thrace, Thessaloniki,[3] and State Veterinary Laboratory, Halkida,[4] Greece

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Screening of Greek nontyphoid salmonellae from 2000 to 2002 yielded three extended-spectrum β-lactamase (ESBL)-producing human isolates. *Salmonella enterica* serotype Brandenburg harbored a multiresistant SHV-5 gene-carrying plasmid. *S. enterica* serotype Blockley and *S. enterica* serotype Hadar harbored a TEM-52 gene-carrying plasmid. An *S. enterica* serotype Virchow strain producing plasmid-mediated CTX-M-32 was isolated twice from poultry end products. All ESBL plasmids were self-transferable and carried by clones currently common in Greece.

Infection with nontyphoidal salmonellae (NTS) usually results in self-limiting diarrhea not requiring treatment. However, for bacteremia and severe focal infections, expanded-spectrum cephalosporins (ESC) are indicated (7). Emergence of 3GC-resistant NTS strains producing potent β-lactamases was first noticed in North Africa in the late 1980s. So far, virtually all β-lactamase types found in ESC-resistant nosocomial enterobacteria have also been encountered in NTS. High-prevalence areas appear to be North Africa, Latin America, and various parts of Asia (12). A rise in the incidence of ESC-resistant NTS is also apparent in developed countries, the most notable example being the spread of CMY-producing NTS in the United States (5). Isolation of ESC-resistant NTS in Europe is still uncommon, though the number of relevant reports is increasing (12). ESC-resistant, extended-spectrum β-lactamase (ESBL)-producing *Salmonella enterica* serotype Typhimurium strains apparently associated with travel to or immigration from Eastern Europe have been sporadically isolated in Greece since 1994 (19, 21). In the present study, we describe domestic ESC-resistant NTS isolates derived from humans as well as animal products in Greece during the period from 2000 to 2002.

Salmonella spp. clinical isolates were referred from the national hospitals to three reference centers, those in Athens, Thessaloniki, and Herakleion, Greece, where serotyping and susceptibility tests were performed. Animal, animal product, and animal feed isolates were similarly collected and analyzed by the State Veterinary Laboratory in Halkida, Greece. Further susceptibility testing, analysis of 3GC resistance mechanisms, and molecular typing were carried out in the Department of Microbiology, Medical School of Athens, Athens, Greece, on isolates from the Athens, Thessaloniki, and Halkida centers. These laboratories constitute the National Network for Enteric Pathogens, coordinated by the Hellenic Center for Infectious Disease Control (KEEL), which also collects data on the isolates, including recent travel of their hosts. The above-mentioned activities are part of the continuous surveillance of salmonellosis.

Species identification was performed using API 20E (BioMerieux, Marcy l'Etoile, France), and serotyping was performed using commercial antisera (BioMerieux). For human strains (3,142 isolates were serotyped during the period from 2000 to 2002; 2,751 [88%] were from the Athens and Thessaloniki centers), randomly selected isolates of the two dominant serotypes, Enteritidis (292 [15%] of the total of 1,956 serotype Enteritidis isolates) and Typhimurium (110 [18%] of the total of 612 serotype Typhimurium isolates), were studied further. Additionally, 252 isolates (48 to 93% of the total number of isolates of each serotype) of the following minor serotypes—those of which five or more isolates were collected during a given year—were also studied, with isolates from previous years used as controls: Abony, Blockley, Brandenburg, Bredeney, Hadar, Heidelberg, Infantis, Kottbus, Montevideo, Newport, and Virchow. Ninety-four animal isolates (22 to 100% of the total number of isolates of each serotype) of serotypes Blockley, Hadar, Infantis, Montevideo, and Virchow collected from 2000 to 2001 (1,148 isolates serotyped in total) were also studied.

Susceptibility to the panel of 11 antibiotics (ampicillin, streptomycin, kanamycin, gentamicin, tetracycline, chloramphenicol, trimethoprim, sulfonamides, nalidixic acid, ciprofloxacin, and cefotaxime) recommended by the international network for enteric pathogens, Enter-net (20), was determined by disk diffusion. The cefotaxime inhibition zone was used to screen for ESBL producers (13). The ESBL screening test was positive for three human isolates (serotypes Blockley, Brandenburg, and Hadar) and two serotype Virchow isolates from chicken meat. Etest (AB Biodisk, Solna, Sweden) containing cefotaxime or ceftazidime plus clavulanate confirmed ESBL

* Corresponding author. Mailing address: Department of Microbiology, Medical School, National and Kapodistrian University of Athens, M. Asias 75, Athens 11527, Greece. Phone: 00302107462150. Fax: 302107462124. E-mail: ltzouvel@cc.uoa.gr.

TABLE 1. Characteristics of nontyphoid salmonella isolates producing ESBLs

Isolate no.	Serotype	Isolation date (day-mo-yr)	Place of isolation	Origin	Resistance pattern[a]	ESBL type	Plasmid[b] size (kb)	Cotransferred resistances
2000/222	Brandenburg	22-08-00	Ioannina	Human	Amp, Ctx, Kan, Sul, Tmp	SHV-5	120	Kan, Sul, Tmp
2002/860	Blockley	26-10-02	Xanthi	Human	Amp, Ctx, Str, Tet, Nal	TEM-52	100	
2002/844	Hadar	18-10-02	Drama	Human	Amp, Ctx, Str, Tet, Nal	TEM-52	100	
2001/1	Virchow	26-01-01	Halkida	Poultry	Amp, Ctx, Kan, Str, Tet, Sul, Tmp, Chl	CTX-M-32	50	
2001/2	Virchow	29-01-01	Halkida	Poultry	Amp, Ctx, Kan, Str, Tet, Sul, Tmp, Chl	CTX-M-32	50	

[a] Amp, ampicillin; Ctx, cefotaxime; Sul, sulfonamides; Tmp, trimethoprim; Kan, kanamycin; Str, streptomycin; Tet, tetracycline; Chl, chloramphenicol; Nal, nalidixic acid

[b] All plasmids were found to be transferable.

production. ESBL-positive isolates were subjected to further studies. Conjugative transfer of ESC resistance was performed in mixed broth cultures as described previously (22) by using an *Escherichia coli* K-12 (strain 20R764; lac^+ Rif^r) recipient. Transconjugant clones were selected on Mueller-Hinton agar containing rifampin (250 μg/ml) plus ampicillin (50 μg/ml) or cefotaxime (1 μg/ml). ESBL gene-carrying plasmids were extracted with the Nucleobond BAC 100 kit (Macherey-Nagel, Duren, Germany) and digested with the endonucleases EcoRI and BamHI. Restriction fragments were electrophoresed through 1% agarose gels. β-Lactamase crude extracts were obtained by ultrasonic treatment of bacterial cell suspensions and clarified by centrifugation. Isoelectric points of β-lactamases were determined by electrofocusing of cell extracts in polyacrylamide gels containing ampholytes (pH range, 3.5 to 9.5). Identity of β-lactamase genes was confirmed by PCR using plasmid DNA preparations and primers specific for TEM-, SHV-, and CTX-M-encoding genes (9, 11, 17), followed by sequencing of the respective amplicons using an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, Calif.). Results are summarized in Table 1.

Isolates were subtyped by pulsed-field gel electrophoresis (PFGE) as described previously (18), and the resulting XbaI DNA fingerprints were compared using the GelCompar software (Applied Maths, Sint-Martens-Latem, Belgium). Patterns differing by one to four DNA fragments were assigned to different subtypes of the same type (6).

S. enterica serotype Brandenburg was isolated from stool and blood samples from a 2-month-old infant with gastroenteritis admitted in August 2000 to the General Hospital of Ioannina, Ioannina, Greece. The isolate was resistant to aminoglycosides and co-trimoxazole. All resistance traits were transferred to *E. coli* via a 120-kb bla_{SHV-5}-carrying plasmid. Restriction analysis confirmed that this plasmid was similar to those from SHV-5-producing nosocomial enterobacteria in Greece (10, 22). The isolate was one of eight PFGE subtype A1 strains; of the three types observed, type A (subtypes A1 through A3) was predominant (10 strains) among the 13 serotype Brandenburg isolates from humans (1997 to 2002) (data not shown).

S. enterica serotype Blockley was isolated in October 2002 from a stool sample from a 69-year-old woman treated in the General Hospital of Xanthi, Xanthi, Greece. It was also resistant to various non-β-lactam drugs, including nalidixic acid. A 100-kb plasmid produced a TEM β-lactamase with an isoelectric point of 6.0, encoded by bla_{TEM-52}. Transconjugants exhibited resistance only to β-lactams. Since 1998, serotype Blockley has emerged in Greece as one of the five most frequent serotypes among NTS isolated from humans (18) and an important serotype among poultry isolates. This was one of the two PFGE type C (subtypes C1 and C2) isolates observed among the 99 human and 25 animal isolates tested (1996 to 2002). Overall, seven types, with A (16 subtypes) being predominant, have been characterized (data not shown).

S. enterica serotype Hadar was isolated in October 2002 in Drama, Greece, from a 2-year-old patient with gastroenteritis. It was resistant to multiple drugs, including nalidixic acid. A conjugative plasmid (100 kb), conferring resistance to β-lactams only, carried a TEM-52-encoding gene and exhibited a restriction pattern indistinguishable from that of the serotype Blockley plasmid. This isolate belonged to the PFGE subtype, A2 (29 isolates; 26 human and 3 animal), that was most common among 59 human and 19 animal serotype Hadar isolates (1998 to 2002). Overall, seven PFGE types (A, with eight subtypes, to G) have been observed (data not shown).

The *S. enterica* serotype Virchow isolates were derived from two batches of poultry end products submitted by an industrial unit in central Greece over the space of 4 days; it is therefore possible that they were epidemiologically linked. Both displayed identical multidrug resistance patterns. Conjugation experiments and plasmid DNA analysis showed that resistance to ESC only was conferred by a single 50-kb plasmid. Sequencing, following PCR using CTX-M gene-specific primers, showed that these isolates produced CTX-M-32, a recently identified variant of the CTX-M-1 subgroup (3). The isolates belonged to two of the six subtypes of PFGE type A (17 isolates) predominant (68%) among the nine types observed in 18 human and 7 animal isolates (1998 to 2002) (data not shown).

Our results add to the growing evidence of worldwide emergence of salmonellae producing β-lactamases able to hydrolyze ESC. ESBL production in serotypes Brandenburg and Hadar is, to our knowledge, documented here for the first time.

This study also illustrates the interplay among the several factors contributing to the emergence and spread of 3GC-resistant salmonellae. Resistance gene "abduction" by animal flora from various species, such as *Citrobacter freundii* for bla_{CMY} (14) and *Kluyvera* spp. for bla_{CTX-M} (8), can be envisaged as a possible first step. This would result in single-resistance-gene plasmids, such as the serotype Virchow CTX-M-32 plasmid in the present study or the serotype Typhimurium CTX-M plasmid described previously (19). In this scenario,

salmonellae can be the primary ESBL gene recipients, as transient or persistent members of both animal and human intestinal flora, or secondary recipients, since they can exchange plasmids with other enterobacteria in the mammalian gut. Salmonellae could also act as secondary gene recipients within the hospital environment, this time acquiring a multidrug resistance plasmid, as in the serotype Brandenburg SHV-5 case presented here, from nosocomial flora colonizing the human intestine. However, given that fecal matter eventually contaminates the environment, i.e., the natural habitat of several salmonella serotypes, these initial acquisition events can also take place in the environment. Therefore, at least with respect to the development of salmonella drug resistance, there is a continuum between human hospitals and the community, animal salmonella hosts, and the environment. Finally, where resistance emerges, strain selection and clonal expansion can also occur, since antibiotic usage operates as a selective pressure within the human community, in the hospital, and among animals.

Epidemiological information suggests that the ESBL producers described here were acquired domestically. This idea was supported by the PFGE typing results showing that—with the exception of serotype Blockley—they belonged to strain types currently dominant in Greece. Nevertheless, the observed diversity of ESBLs emphasizes the multiplicity of *bla* gene acquisition events among different salmonella serotypes.

A multidrug-resistant SHV-5 gene-carrying plasmid, commonly found in nosocomial enterobacteria, was isolated from the rare serotype Brandenburg, raising the possibility of previous transfer from hospital flora. This hypothesis is in line with the isolation of SHV-producing NTS in North Africa, Romania, Italy, and India, where this ESBL type predominates in nosocomial isolates also (12).

On the other hand, two emerging serotypes, Blockley and Hadar, harbored a single plasmid carrying the TEM-52 gene only. Emergence of domestically acquired TEM-52-producing NTS human isolates of serotypes Typhimurium, Enteritidis, Blockley, and Panama has also been reported recently in France and the United Kingdom (23, 25). Additionally, TEM-52-producing *E. coli* strains have been isolated recently from healthy and sick animals in Spain and Portugal (2, 4). These data indicate the possible spread of TEM-52-encoding determinants among animal flora in Europe.

A similar contamination event, of equal public health importance, was shown by the repeated isolation of a CTX-M-producing *S. enterica* serotype Virchow strain from poultry end products. Although contamination from a human or environmental source cannot be ruled out, this finding is in line with previous reports on the establishment of CTX-M-producing enterobacteria in food-producing animals and the increasing implication of these microorganisms in community-acquired infections in Greece as well as other European countries (1, 15, 24). Similarly, there is evidence that livestock is the main reservoir of CTX-M-producing enterobacteria in Japan (16). In this situation, the use of modern cephalosporins, such as ceftiofur, in animal rearing may have played a role.

In conclusion, our study showed 3GC resistance genes incorporated into the dominant clones of three different minor serotypes (and a minor clone of a fourth serotype), but only sporadically (5 out of 748 isolates tested; 0.7%). Even though dissemination of these ESC-resistant strains has not yet occurred, the opportunities for their expansion, as outlined above, emphasize the need for continued alertness in both the clinical and veterinary contexts.

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