

RESEARCH ARTICLE

INTERNATIONAL
MICROBIOLOGYINTERNATIONAL MICROBIOLOGY (2011) 14:173-181
DOI: 10.2436/20.1501.01.146 ISSN: 1139-6709 www.im.microbios.orgGenetic characterization of the mechanisms of resistance to amoxicillin/clavulanate and third-generation cephalosporins in *Salmonella enterica* from three Spanish hospitalsMaría de Toro,^{1,2} Yolanda Sáenz,¹ Emilia Cercenado,³ Beatriz Rojo-Bezares,¹ Marta García-Campello,⁴ Esther Undabeitia,⁵ Carmen Torres^{1,2*}

¹Molecular Microbiology Area, Center for Biomedical Research of La Rioja (CIBIR), Logroño, Spain. ²Biochemistry and Molecular Biology Area, University of La Rioja, Logroño, Spain. ³Servicio de Microbiología, Hospital General Universitario Gregorio Marañón, Madrid, Spain. ⁴Microbiology Service, Pontevedra Hospital Complex, Pontevedra, Spain. ⁵Microbiology Laboratory, San Pedro Hospital, Logroño, Spain

Summary. The mechanisms of antimicrobial resistance were characterized in 90 *Salmonella enterica* isolates either resistant or with intermediate resistance to amoxicillin/clavulanate (AMC^{R/I}) or resistant to third-generation cephalosporins (C3G^R). These isolates were recovered in three Spanish hospitals during 2007–2009. The C3G^R phenotype was expressed by three isolates that carried the following extended-spectrum β -lactamase genes: phage-associated $bla_{CTX-M-10}$ in *S. Virchow*, $bla_{CTX-M-14a}$ surrounded by *ISEcp1* and *IS903* in *S. Enteritidis*, and $bla_{CTX-M-15}$ linked to *ISEcp1* and *orf477* in *S. Gnesta* (first description in this serotype). The AMC^{R/I} phenotype was found in 87 isolates (79 *S. Typhimurim*, 7 *S. Enteritidis*, and one *S. Thompson*). The bla_{PSE-1} gene, followed by bla_{OXA-1} was mostly found among *S. Typhimurim*, and the bla_{TEM-1} gene among *S. Enteritidis*. Three different gene combinations [$bla_{PSE-1}+floR+aadA2+sul+tet(G)$; $bla_{OXA-1}+catA+aadA1/strA-strB+sul+tet(B)$ and $bla_{TEM-1}+cmlA1+aadA/strA-strB+sul+tet(A)/tet(B)$ genes] were associated with the ampicillin-chloramphenicol-streptomycin-sulfonamides-tetracycline phenotype in 68 AMC^{R/I} *S. enterica* isolates. Class 1 integrons were observed in 79% of the isolates and in most of them (45 isolates) two integrons including the *aadA2* and bla_{PSE-1} gene cassettes, respectively, were detected. The $bla_{OXA-1}+aadA1$ arrangement was detected in 23 isolates, and the $aac(6)-Ib-cr+bla_{OXA-1}+catB3+arr3$ in another one. Non-classic class 1 integrons were found in three isolates: $dfrA12+orfF+aadA2+cmlA1+aadA1$ (1 isolate), $dfrA12+orfF+aadA2+cmlA1+aadA1+qacH+IS440+sul3$ (1 isolate) and $dfrA12+orfF+aadA2+cmlA1+aadA1+qacH+IS440+ sul3+orfI+mef(B)\Delta-IS26$ (1 isolate). Taken together, these results underline the need for clinical concern regarding β -lactam resistance in *Salmonella* and thus for continuous monitoring. [*Int Microbiol* 2011; 14(3):173-181]

Keywords: *Salmonella enterica* · β -lactam-resistance · integrons · extended-spectrum β -lactamases (ESBL)

Introduction

Salmonella enterica is the second most frequent cause of zoonotic diseases in humans in Europe, and more than

150,000 cases of human salmonellosis were reported by The European Surveillance System during 2007 [8]. *Salmonella* Enteritidis and *Salmonella* Typhimurium are two of the ten most common serotypes confirmed in salmonellosis cases in humans, representing 81% of the isolates [8]. *S. Typhimurium* is frequently associated with multidrug resistance [3,26], in part due to the worldwide emergence of *S. Typhimurium* definitive phage type (DT) 104, which contains the chromosomal *Salmonella* genomic island type I (SGI-1). SGI-1 harbors genes that confer the ACSSuT phenotype (i.e., resistance to ampicillin, chloramphenicol, streptomycin, sul-

*Corresponding author: C. Torres

Área de Bioquímica y Biología Molecular
Departamento de Agricultura y Alimentación
Universidad de La Rioja
26006 Logroño, Spain
Tel. +34-941299750. Fax: +34-941299721
E-mail: carmen.torres@unirioja.es

fonamides, and tetracycline) [16]. Although *S. Typhimurium* DT104 is the main example of multiresistance in *S. enterica*, many antimicrobial resistance genes have been reported also in isolates of other serotypes [14].

Non-typhoidal *Salmonella* infections generally result in mild-to-moderate self-limiting gastroenteritis, and antimicrobial treatment is only required in severe cases occurring in vulnerable patient groups or to combat invasive infections. However, due to the increasing resistance of this bacterium to the conventional antimicrobial agents (ampicillin, and trimethoprim/sulfamethoxazole) used in the treatment of salmonellosis, amoxicillin/clavulanate, third-generation cephalosporins, and fluoroquinolones have become further treatment options. Resistance to β -lactams in *S. enterica* is mainly due to the production of acquired β -lactamases [14]. Among these, TEM-1, PSE-1, and OXA-1 have been described as the enzymes most frequently related to ampicillin and amoxicillin/clavulanate resistance [3,11]. The resistance of *Salmonella* to third-generation cephalosporins is primarily mediated by the production of extended-spectrum β -lactamases (ESBL) of the TEM, SHV, and CTX-M types, which are associated with different mobile genetic elements [11,14]. ESBL have been described not only in clinical *Salmonella* isolates but also in isolates from animals and food [6,21].

Mobile genetic elements such as plasmids and transposons, possibly containing integrons, are able to disseminate antimicrobial resistance by horizontal transfer in Enterobacteriaceae. Integrons are genetic elements that capture and incorporate gene cassettes by using a site-specific recombination mechanism [4]. Thus far, class 1 and, less frequently, class 2 integrons have been reported for *S. enterica* [4]. Class 1 integrons contain a 5'-conserved segment (5'-CS) that includes the integrase *intI1* gene, the *attI1* recombination site, and the Pc promoter. It is followed by a variable region where one or more gene cassettes are located. This class of integrons also contains a 3'-conserved segment (3'-CS) that includes the *sul1* and *qacE Δ 1* genes, which encode resistance to sulfonamides and ammonium quaternary compounds, respectively [4].

In recent years, resistance to amoxicillin/clavulanate among *S. enterica* isolated from different Spanish hospitals has become increasingly widespread, accompanied by the emergence of ESBL-producing isolates, detected in human samples. Consequently, there are fewer therapeutic options for the treatment of *S. enterica* infections, placing these patients at greater risk of serious morbidity and even death.

The aim of the present work was to characterize the mechanisms of resistance to β -lactams and other antimicrobial agents as well as the integrons in all amoxicillin/clavulanate-resistant, intermediately resistant (AMC^{R/I}), and third-generation cephalosporin-resistant (C3G^R) *S. enterica* isolates recovered in three Spanish hospitals during the period 2007–2009.

Materials and methods

Isolates and antimicrobial susceptibility testing. In this study, 90 *S. enterica* isolates with the AMC^{R/I} phenotype (87 isolates) or the C3G^R phenotype (3 isolates) were recovered in three Spanish hospitals located in geographically distinct areas: Hospital General Universitario Gregorio Marañón of Madrid (HGM, 39 isolates), Hospital San Pedro of Logroño (HSP, 36 isolates), and Complejo Hospitalario of Pontevedra (CHP, 15 isolates). AMC^{R/I} and C3G^R phenotypes were detected in 12–23% and <1%, respectively, of all *S. enterica* isolated in the three hospitals. The 90 isolates were recovered from fecal (73 isolates), blood (2 isolates), urine (1 isolate) and other (14 isolates) samples from different patients during 2007 (29 isolates), 2008 (34 isolates), and 2009 (27 isolates). The serotypes of these isolates were as follows: *S. Typhimurium* (79 isolates), *S. Enteritidis* (8 isolates, one of them C3G^R), *S. Virchow* (1 isolate, C3G^R), *S. Gnesta* (1 isolate, C3G^R), and *S. Thompson* (1 isolate).

Susceptibility testing to 20 antimicrobial agents (ampicillin, AMC, cefalotin, cefazolin, ceftazidime, cefotaxime, aztreonam, ceftiofur, gentamicin, tobramycin, kanamycin, amikacin, streptomycin, nalidixic acid, ciprofloxacin, tetracycline, chloramphenicol, sulfonamides, trimethoprim, trimethoprim/sulfamethoxazole) was performed by the disc-diffusion [5] and microdilution methods (MicroScan Combo Neg panels, Siemens, Sacramento, CA, USA) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. The AmpC phenotype was determined by comparison of the inhibition zone of ceftiofur discs (30 μ g) in the presence or absence of cloxacillin (200 μ g) [29]. The ESBL phenotype was determined using the double-disc synergy test with ceftiofur, ceftazidime, and aztreonam discs placed in the proximity of the AMC disc [13].

Detection of antimicrobial resistance genes. The presence of genes implicated in the resistance to β -lactams (*bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M}, *bla*_{OXA-1} and *bla*_{PSE-1}), and the *bla*_{CTX-M} genetic environment was detected by PCR and sequencing [7,17,31]. In addition, multiplex PCR for the detection of plasmidic AmpC-type β -lactamases was carried out [20].

Tetracycline [*tet*(A)-*tet*(E),*tet*(G)], aminoglycoside [*aadA*, *strA-strB*, *aac*(3)-I, *aac*(3)-II, *aac*(3)-IV, *ant*(2''), *aph*(3')-Ia, *aph*(3')-IIa, *rmtB*, *armA* and *aac*(6')-Ib], sulfonamides [*sul1*, *sul2* and *sul3*], trimethoprim [*dhfrA*], chloramphenicol [*cmlA*, *catA* and *floR*], and quinolone [*qnrA*, *qnrB*, *qnrS* and *qepA*] resistance genes were studied by PCR and sequencing [7,24,27]. The genetic environments of the *sul1*, *sul2*, and *sul3* genes were determined as previously reported [32].

Detection and characterization of integrons. The presence of class 1, 2, and 3 integrase-encoding genes and of the 3'-CS of class 1 integrons, *qacE Δ 1+sul1*, was analyzed by PCR. The variable regions of these integrons were PCR-amplified and subsequently sequenced to determine their gene cassette arrangements [24].

Results

Antimicrobial susceptibility in *Salmonella enterica* isolates. Table 1 shows the antimicrobial susceptibility of the 90 AMC^{R/I} or C3G^R *S. enterica* isolates included in this study. The *S. Typhimurium* isolates were highly resistant to sulfonamides (100%), tetracycline (91%), chloramphenicol (86%), and streptomycin (80%). Aminoglycosides resistance was found only among isolates of serotype *S. Typhimurium*. All isolates studied were susceptible to amikacin, ceftaxime and ciprofloxacin. A multiresistant phenotype (resistant to at least three different antimicrobial agent families) was observed among 100% of the *S. Typhimurium* and 12.5% of the *S. Enteritidis* isolates. Two *S. Typhimurium*

isolates had a heptaresistant phenotype that included the ACSSuT phenotype in addition to resistance to trimethoprim and gentamicin or nalidixic acid (ACGSSuTTm and ACSSuTTmN, respectively).

The AmpC phenotype was not identified among the isolates tested. However, the ESBL phenotype was determined in three of them and corresponded to one isolate each of *S. Enterica*, *S. Virchow*, and *S. Gnesta* serotypes (Table 1). All three were resistant to ceftaxime, while *S. Gnesta* isolate was also resistant to ceftazidime and aztreonam.

Antimicrobial resistance genes. Tables 2 and 3 list the resistance genes detected in the 90 *S. enterica* isolates, according to serotype. The most frequent β -lactamase gene identified among the AMC^{R/I} isolates was *bla*_{PSE-1}, detected in

Table 1. Number of AMC^{R/I} or C3G^R of *Salmonella enterica* isolates resistant to antimicrobial agents. The isolates were of different serotypes and obtained from three Spanish hospitals

Antimicrobial agent ^a	<i>S. Typhimurium</i> (n = 79)	<i>S. Enteritidis</i> (n = 8)	<i>S. Virchow</i> (n = 1)	<i>S. Gnesta</i> (n = 1)	<i>S. Thompson</i> (n = 1)	All <i>S. enterica</i> tested (n = 90)
AMC ^b	79	7	0	0	1	87
Cefalotin	7	1	1	1	0	10
Cefazolin	9	1	1	1	0	12
Ceftazidime	0	0	0	1	0	1
Ceftaxime	0	1	1	1	0	3
Aztreonam	0	0	0	1	0	1
Gentamicin	1	0	0	0	0	1
Tobramycin	1	0	0	0	0	1
Kanamycin	2	0	0	0	0	2
Streptomycin	63	0	0	0	0	63
Nalidixic acid	17	2	1	0	0	20
Tetracycline	72	1	1	0	1	75
Chloramphenicol	68	0	0	0	1	69
Sulfonamides	79	3	1	1	1	85
Trimethoprim	5	0	0	0	0	5
SXT ^c	5	1	0	0	0	6
ESBL phenotype	0	1	1	1	0	3

^aAll the isolates were resistant to ampicillin, but susceptible to ceftaxime, amikacin, and ciprofloxacin.

^bAMC: Amoxicillin/clavulanate.

^cSXT: Trimethoprim-sulfamethoxazole.

Table 2. Antimicrobial resistance genes and the resistance phenotype of *Salmonella enterica* isolates from three Spanish hospitals

Number of resistant isolates	Resistance genes	<i>S.</i> Typhimurium	<i>S.</i> Enteritidis	Other serotypes	Total (n = 90)
β-Lactams (n = 90)	<i>bla</i> _{PSE-1}	41	–	–	41
	<i>bla</i> _{OXA-1}	23	–	1	24
	<i>bla</i> _{TEM-1b}	9	6	–	15
	<i>bla</i> _{TEM-1c}	1	–	–	1
	<i>3bla</i> _{PSE-1} + <i>bla</i> _{OXA-1}	1	–	–	1
	<i>bla</i> _{PSE-1} + <i>bla</i> _{TEM-1b}	3	–	–	3
	<i>bla</i> _{CTX-M-10}	–	–	1	1
	<i>bla</i> _{CTX-M-14a}	–	1	–	1
	<i>bla</i> _{CTX-M-15} + <i>bla</i> _{TEM-1} ^c	–	–	1	1
	No studied <i>bla</i> genes	1	1	–	2
Tetracycline ^a (n = 82)	<i>tet</i> (A)	5	–	–	5
	<i>tet</i> (B)	29	–	1	30
	<i>tet</i> (G)	45	–	–	45
	No studied <i>tet</i> genes	–	1	1	2
Streptomycin ^b (n = 76)	<i>aadA1/aadA2</i>	66	–	–	66
	<i>strA-strB</i>	5	–	–	5
	<i>aadA1/aadA2</i> + <i>strA-strB</i>	4	–	–	4
	No studied genes	1	–	–	1
Gentamicin (n = 1)	<i>aac</i> (3)-IV	1	–	–	1
Kanamycin (n = 2)	<i>aph</i> (3')-Ia	1	–	–	1
	No studied genes	1	–	–	1
Chloramphenicol (n = 69)	<i>floR</i>	44	–	1	45
	<i>catA</i>	18	–	–	18
	<i>cmlA1</i>	3	–	–	3
	<i>floR</i> + <i>catA</i>	2	–	–	2
	<i>floR</i> + <i>cmlA</i>	1	–	–	1
Sulfonamides (n = 85)	<i>sul1</i>	54	–	–	54
	<i>sul2</i>	8	–	1	9
	<i>sul1</i> + <i>sul2</i>	14	–	1	15
	<i>sul2</i> + <i>sul3</i>	1	–	–	1
	<i>sul1</i> + <i>sul2</i> + <i>sul3</i>	1	–	–	1
	No studied <i>sul</i> genes	1	3	1	5
Trimethoprim (n = 5)	<i>dfrA12</i>	3	–	–	3
	<i>dfrA14</i>	2	–	–	2

^aSeven of the studied isolates with a phenotype of intermediate resistance to tetracycline harbored the *tet*(G) gene.

^bTwelve of the studied isolates with a phenotype of intermediate resistance to streptomycin harbored the *aadA1/aadA2* gene. Three of the isolates with a susceptibility to streptomycin harbored *strA-strB* genes.

^c*bla*_{TEM-1} variant showed a silent nucleotide change (T→C) at position 735 [28].

51.7% of the 87 AMC^{R/I} isolates including all those belonging to *S. Typhimurium*. In addition, the gene was associated with other *bla* genes in four of these isolates (*bla*_{TEM-1b} or *bla*_{OXA-1}). The *bla*_{OXA-1} gene was identified in 27.6% of the AMC^{R/I} isolates (23 *S. Typhimurium* and 1 *S. Thompson*), and only in one case in association with other *bla* genes. In addition, the *bla*_{TEM-1} gene was demonstrated in 21.8% of the AMC^{R/I} isolates (13 *S. Typhimurium* and 6 *S. Enteritidis*) and associated with other *bla* genes in three of them. As shown in Table 2, *bla*_{TEM-1} was the most frequent *bla* gene in *S. Enteritidis* isolates.

The β-lactamase genes identified among the three C3G^R isolates with an ESBL-positive phenotype were as follows: *bla*_{CTX-M-14a} (*S. Enteritidis*), *bla*_{CTX-M-15} (*S. Gnesta*), and *bla*_{CTX-M-10} (*S. Virchow*). In these isolates, the *ISEcp1-bla*_{CTX-M-14a}-IS903 and *ISEcp1-bla*_{CTX-M-15}-*orf477* structures were identified. The *bla*_{CTX-M-15}-positive *S. Gnesta* isolate also carried a new variant of the *bla*_{TEM-1} β-lactamase gene that showed a silent nucleotide change (T→C) at position 735 according to the Sutcliffe nomenclature [28]. Regarding the *bla*_{CTX-M-10} genetic environment, the gene's upstream region included a group of ORFs (*orf2*, *orf3* and *orf4*) and a phage-related DNA invertase. Downstream, *orf7* was identified. All of the *S. enterica* isolates tested were negative for the plasmid-mediated quinolone resistance genes *qnrA*, *qnrB*, *qnrS*, and *qepA*.

Integron detection and characterization. Seventy-one of the 90 isolates (79%) were positive for the *intI1* gene, and six different gene cassette arrangements were determined (Table 3, Fig.1). Class 2 and 3 integrons were absent. All 45 *bla*_{PSE-1}-positive *S. Typhimurium* isolates showed two integrons, with variable regions of 1000 and 1200 bp, harboring the *aadA2* and *bla*_{PSE-1} gene cassettes, respectively. The *bla*_{OXA-1} + *aadA1* gene array was found in most of the *bla*_{OXA-1}-positive isolates (23 of 25), whereas the *S. Thompson* isolate showed the *aac(6')*-Ib-cr+*bla*_{OXA-1}+*catB3*+*arr3* arrangement. Three non-classic class 1 integrons (lacking the 3'-CS) were found in three isolates (Fig. 1).

Genetic environment of *sul* genes. Of the 90 *S. enterica* isolates studied, 94.4% were resistant to sulfonamides. At least one *sul* gene was detected in 80 of them, and more than one *sul* gene in 17 of them (Table 2). The *sul1* gene was associated with class 1 integrons in all 70 *sul1*-positive isolates (Table 3).

The genetic environment of the *sul2* gene was determined in 11 of the 26 *sul2*-positive *S. enterica* isolates (42.3%). Four different structures were demonstrated (number of iso-

lates): *repC+sul2+strA-strB+tnpB* (6), *repC+sul2+strA-strB+IS26* (2), *repC+sul2+strA-strB* (1) and *sul2+strAΔdfrA14-strB* (2). In these two last isolates, the *strA* gene was truncated by the *dfrA14* gene, and a streptomycin-susceptibility phenotype was determined in both isolates. The *sul3* gene was associated with the above mentioned non-classic class 1 integrons (lacking the 3'-CS) in the two *sul3*-positive isolates.

In summary, an ACSSuT phenotype (including intermediate resistance) was confirmed in 68 *S. enterica* isolates (all of them *Typhimurium*), 15 of which were additionally resistant to nalidixic acid and three others to trimethoprim (Table 3). Three general gene profiles were mostly responsible for the ACSSuT multiresistant phenotype: (i) The *bla*_{PSE-1} and *aadA2* genes, located within two class 1 integrons (structure A, Fig. 1), were associated with the *floR*, *sul* and *tet(G)* genes in 45 of these isolates. In five of the 45 isolates, one non-classic class 1 integron (*dfrA12+orfF+aadA2+cmlA1+aadA1*), the *bla*_{TEM-1} gene, and the *bla*_{OXA-1} gene were additionally detected (one, three, and one isolate, respectively). (ii) The *bla*_{OXA-1} and *aadA1* (located within a class 1 integron of structure B, Fig. 1), *catA*, *sul*, and *tet(B)* gene profile occurred in 20 isolates. The *floR* gene was additionally found in two of them. (iii) An association between *bla*_{TEM-1b}, *cmlA1*, *aadA* or *strA-strB*, *sul*, and *tet(A)* or *tet(B)* genes was detected in three isolates. In one of them, the *aac(3)-IV* and *dfrA12* genes were additionally amplified, confirming this *S. Typhimurium* isolate's ACGSSuTTm phenotype (Table 3).

Discussion

Antimicrobial resistance in *S. enterica* is a cause of serious concern in human medicine. The drugs of choice for the treatment of complicated salmonellosis are usually ampicillin, amoxicillin/clavulanate, third-generation cephalosporins, or fluoroquinolones, but the increasing emergence of resistance to these antimicrobials limits the therapeutic choices [9,15,18]. In our study, the AMC^{R/I} phenotype was detected in 12–23% of all *S. enterica* isolates recovered from human samples obtained from three Spanish hospitals. The β-lactamase-related mechanisms implicated in this AMC^{R/I} phenotype were the production of the enzymes PSE-1, OXA-1 and TEM-1, as previously reported in other series [11]. The high prevalence of *bla*_{PSE-1} and *bla*_{TEM-1} observed among *S. Typhimurium* and *S. Enteritidis* isolates, respectively, was also previously reported [3,11,26]. The detection of more than one β-lactamase gene in the same isolate was infrequent in our study (4 isolates), in contrast to the data from other studies [3,11].

Table 3. Phenotypes and mechanisms of resistance detected in the 90 AMC^{R1} and C3G^R *Salmonella enterica* isolates

	Phenotype of resistance (number of isolates) ^{a,b}	Genotype of resistance (number of isolates) ^c	Class 1 integron ^d
<i>S. Typhimurium</i> (n = 79)	SUL+TET (1)	<i>bla</i> _{TEM-1c} + <i>sul2</i> + <i>tet</i> (B) (1)	–
	STR+SUL+TET (7)	<i>bla</i> _{OXA-1} + <i>aadA</i> + <i>sul1</i> + <i>tet</i> (B) (2)	(B)
		<i>bla</i> _{TEM-1b} + <i>strA-strB</i> + <i>sul2</i> + <i>tet</i> (B) (3)	–
		<i>bla</i> _{TEM-1b} + <i>strA-strB</i> + <i>sul2</i> + <i>tet</i> (A) (1)	–
		<i>tet</i> (B) (1)	–
	STR+SUL+TET+NAL (1)	<i>bla</i> _{OXA-1} + <i>aadA</i> + <i>sul1</i> + <i>tet</i> (B) (1)	(B)
	SUL+TET+TMP+SXT (1)	<i>bla</i> _{TEM-1b} + <i>strAΔdfrA14-strB</i> + <i>sul2</i> + <i>tet</i> (A) (1)	–
	CHL+STR+SUL+TET+KAN (2)	<i>bla</i> _{PSE-1} + <i>bla</i> _{OXA-1} + <i>floR</i> + <i>aadA</i> + <i>sul1</i> + <i>tet</i> (G) (1)	(A)
		<i>bla</i> _{PSE-1} + <i>floR</i> + <i>aadA</i> + <i>sul1</i> + <i>tet</i> (G) + <i>aph</i> (3')-Ia (1)	(A)
	CHL+STR+SUL+TET+NAL (14) ^f	<i>bla</i> _{PSE-1} + <i>floR</i> + <i>aadA</i> + <i>sul1</i> + <i>tet</i> (G) (10)	(A)
		<i>bla</i> _{OXA-1} + <i>floR</i> + <i>catA</i> + <i>aadA</i> + <i>sul1</i> + <i>tet</i> (B) (1)	(B)
<i>bla</i> _{OXA-1} + <i>catA</i> + <i>aadA</i> + <i>sul1</i> + <i>tet</i> (B) (3)		(B)	
SUL+TET+TMP+SXT+NAL (1)	<i>bla</i> _{TEM-1b} + <i>sul2</i> + <i>tet</i> (A) + <i>strAΔdfrA14-strB</i>	–	
CHL+STR+SUL+TET+TMP+SXT (1)	<i>bla</i> _{TEM-1b} + <i>cmlA1</i> + <i>aadA</i> + <i>strA-strB</i> + <i>sul2</i> + <i>sul3</i> + <i>tet</i> (A) + <i>dfrA12</i>	(E)	
CHL+STR+SUL+TET+TMP+SXT+NAL (1)	<i>bla</i> _{PSE-1} + <i>floR</i> + <i>cmlA1</i> + <i>aadA</i> + <i>sul1</i> + <i>tet</i> (G) + <i>dfrA12</i>	(A)+(D)	
CHL+STR+SUL+TET+GEN+TOB+TMP+SXT (1)	<i>bla</i> _{TEM-1b} + <i>cmlA1</i> + <i>aadA</i> + <i>sul1</i> + <i>sul2</i> + <i>sul3</i> + <i>tet</i> (A) + <i>aac</i> (3)-IV + <i>dfrA12</i>	(F) ^g	
<i>S. Enteritidis</i> (n = 8)	None (2)	<i>bla</i> _{TEM-1b}	–
	NAL (1)	– (1)	–
	TET (1)	<i>bla</i> _{TEM-1b}	–
	SUL (2)	<i>bla</i> _{TEM-1b}	–
	CTX+NAL (1)	<i>bla</i> _{CTX-M-14a}	–
	SUL+SXT (1)	<i>bla</i> _{TEM-1b}	–
<i>S. Gnesta</i> (n = 1)	ATM+CAZ+CTX+SUL (1)	<i>bla</i> _{CTX-M-15} + <i>bla</i> _{TEM-1}	–
<i>S. Thompson</i> (n = 1)	CHL+SUL+TET (1)	<i>bla</i> _{OXA-1} + <i>floR</i> + <i>catB3</i> + <i>sul1</i> + <i>sul2</i> + <i>aac</i> (6')-Ib-cr	(C)
<i>S. Virchow</i> (n = 1)	SUL+TET+CTX+NAL (1)	<i>bla</i> _{CTX-M-10} + <i>sul2</i> + <i>tet</i> (B) + <i>strA-strB</i>	–

^aAbbreviations: CAZ: ceftazidime, CTX: cefotaxime, ATM: aztreonam, GEN: gentamicin, TOB: tobramycin, KAN: kanamycin, STR: streptomycin, NAL: nalidixic acid, TET: tetracycline, CHL: chloramphenicol, SUL: sulfonamides, TMP: trimethoprim, SXT: trimethoprim/sulfamethoxazole.

^bACSSuT phenotype is marked in bold letters.

^cStreptomycin resistance genes *aadA* correspond to *aadA1* or *aadA2*.

^dIntegron structures A-F correspond to those shown in Fig. 1.

^eSix of these isolates had an intermediate phenotype with respect to tetracycline and nine isolates with respect to streptomycin.

^fOne of these isolates had an intermediate phenotype with respect to tetracycline and two with respect to streptomycin.

^gThis integron contained the putative macrolide efflux gene *mef*(B), truncated by IS26 such that only 256 bp of *mef*(B) remained.

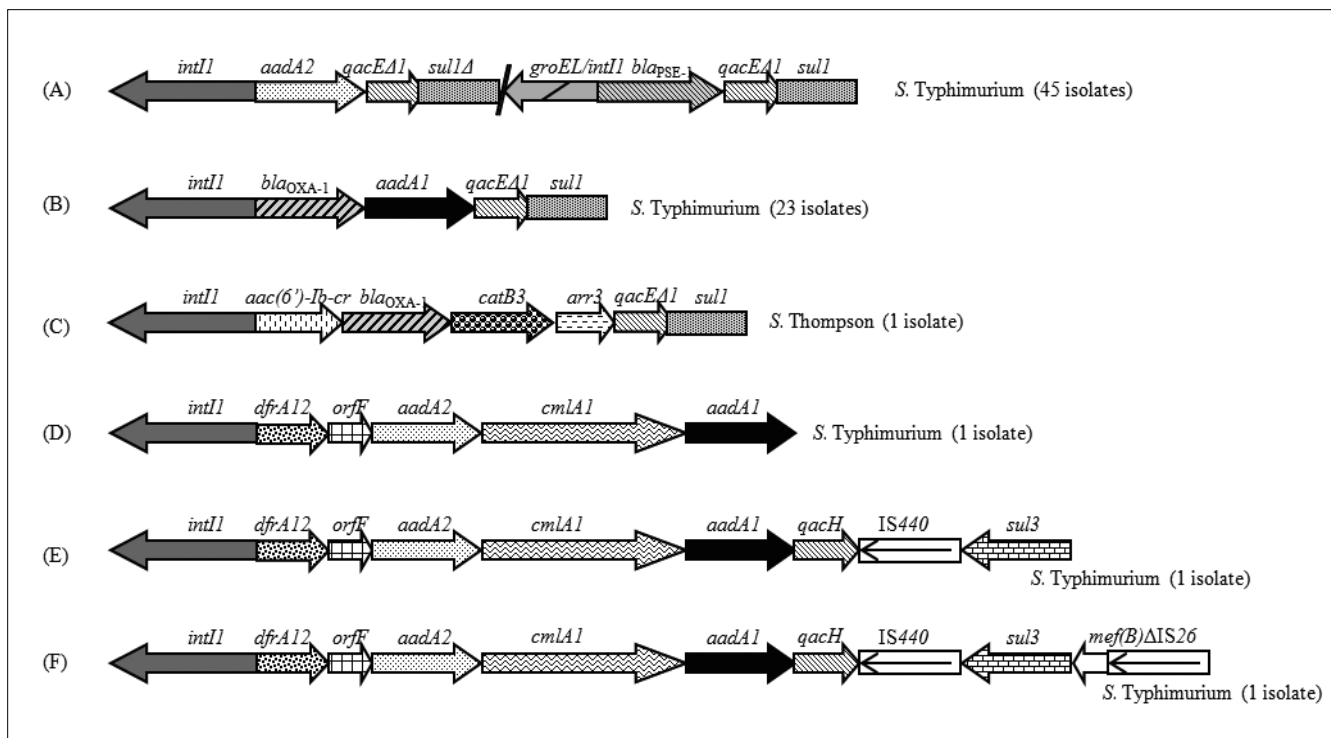


Fig. 1. Gene cassette arrangements among class 1 integrons detected in *Salmonella enterica* isolates.

The ESBL phenotype in human clinical isolates of *S. enterica* is of particular interest but in our study it was detected in <1% of the *S. enterica* isolates obtained from the three hospitals. ESBL are spreading very rapidly among *E. coli* and *Klebsiella* spp. isolates whereas their frequency among *S. enterica* isolates is much lower [2,15,18]. The diversity of ESBL detected among our three ESBL-positive *S. enterica* isolates (CTX-M-14, CTX-M-15, and CTX-M-10) is noteworthy as is the fact that these genes were identified in unusual serotypes, i.e., *S. Gnesta* and *S. Virchow*.

The CTX-M-14 β -lactamase-encoding gene, flanked by *ISEcp1* and *IS903* sequences, has been frequently detected in *E. coli* isolates of human and animal origin in Spain [6]. In *S. enterica*, the first description of this enzyme, in a clinical isolate of *S. Enteritidis* recovered in Spain, was that of Romero et al. [23]. However, the *bla*_{CTX-M-14} gene has been identified in *Salmonella* of different serotypes and in several countries [2,6,9,18]. The genetic element *ISEcp1* is a mobile and mobilizing element that may be implicated in the *bla*_{CTX-M-14} gene mobilization [19]. Similarly, the *bla*_{CTX-M-15} gene, flanked by *ISEcp1* and *orf477* elements, has been shown to be disseminated throughout the world and is mostly detected among *E. coli* and *Klebsiella* isolates [6]. In our study, this enzyme was identified in a *S. Gnesta* isolate. To our knowledge,

this is the first description of the presence of the CTX-M-15 β -lactamase in *S. Gnesta*, a serotype uncommonly associated with human salmonellosis.

The CTX-M-10 enzyme has been described in *E. coli*, *Enterobacter* spp., *Klebsiella* spp., and *S. Virchow* isolates in Spain [6,9,17,21]. In the present work, this enzyme was also found in a *S. Virchow* isolate, and the genetic environment of the *bla*_{CTX-M-10} gene was associated with a phage-related element, similar to one previously reported [17,21].

The ACSSuT multiresistance phenotype was detected in 68 of the *S. Typhimurium* isolates. Although this phenotype is usually associated with the widely distributed chromosomal SGI-1 (contains the *bla*_{PSE-1}, *floR*, *aadA2*, *sul*, and *tet(G)* genes) [16,30], other gene profiles have also been described [10,12,22]. Indeed, in our study different resistant genotypes were determined; the most common one was the SGI-1 linked profile. The association of the *bla*_{OXA-1}, *catA*, [*aadA1* / *strA-strB*], *sul*, and *tet(B)* genes, with the *bla*_{OXA-1}+*aadA1* arrangement included within a 2000-bp class 1 integron, was found among 20 *S. Typhimurium* ACSSuT-resistant isolates. In addition, the gene profile *bla*_{TEM-1}, *cmlA1*, [*aadA* / *strA-strB*], *sul* and [*tet(A)* / *tet(B)*] was identified in three *S. Typhimurium* isolates. In previous studies, these latter two resistance-gene profiles were shown to be located on hybrid self-

transferable plasmids, which also contain virulence genes, such as the pUO-StVR plasmids in *S. Typhimurium* and the recently reported pUO-SeVR1 in *S. Enteritidis* [10,12,22]. Further studies of our isolates are needed to determine the plasmid localization of these ACSSuT resistance genes and/or their possible association with virulence genes.

Class 1 integrons were present in 79% of the 90 isolates tested. Note the presence of a class 1 integron with the *aac(6)-Ib-cr+bla_{OXA-1}+catB3+arr3* structure in the *S. Thompson* isolate. While this arrangement has been previously described, it is usually associated with complex integrons containing the *ISCR1* elements, double copies of 3'-CS, and *qnr* genes, among others (e.g., GenBank accession numbers AJ971343 and AY259086). In addition, non-classical integrons (without *qacEΔ1+sul1* genes) were found in three isolates (4%). All three included the gene cassette organization *dfiA12+orfF+aadA2+cmlA1+aadA1*, in two of these three isolates in association with the *qacH+IS440+sul3* structure previously reported in *Salmonella* and *E. coli* [1,25].

In conclusion, *bla_{PSE-1}* and *bla_{OXA-1}* were the most frequent *bla* genes implicated in the AMC^{RI} phenotype in *S. Typhimurium*, and *bla_{TEM-1}* the most frequent in *S. Enteritidis*. ESBL-positive isolates, corresponding to non-*S. Typhimurium* serotypes, were identified in <1% of the *S. enterica* isolates obtained from the three hospitals. Among the three different ESBL variants detected, ours is the first description of CTX-M-15 in *S. Gnesta*. In addition, the frequent association of the β-lactamase production with nalidixic acid resistance (22%), which precludes the use of fluoroquinolones in the treatment of salmonellosis, is a cause for clinical concern and underlines the need to track the evolution of β-lactamases in *S. enterica* isolates.

Acknowledgements. We thank M. Aurora Echeita from the Centro Nacional de Microbiología, Instituto de Salud Carlos III, Madrid, Spain, for serotyping of the isolates. M. de T. is the recipient of a predoctoral fellowship from the Instituto de Salud Carlos III of Spain (MINCINN) (grant number FI08/00506).

Competing interests. None declared.

References

- Antunes P, Machado J, Peixe L (2007) Dissemination of *sul3*-containing elements linked to class 1 integrons with an unusual 3' conserved sequence region among *Salmonella* isolates. *Antimicrob Agents Chemother* 51:1545-1548
- Arllet G, Barrett TJ, Butaye P, Cloeckaert A, Mulvey MR, White DG (2006) *Salmonella* resistant to extended-spectrum cephalosporins: prevalence and epidemiology. *Microbes Infect* 8:1945-1954
- Biendo M, Laurans G, Thomas D, Canarelli B, Hamdad-Daoudi F, Rousseau F, Castelain S, Eb F (2005) Molecular characterisation and mechanisms of resistance of multidrug-resistant human *Salmonella enterica* serovar Typhimurium isolated in Amiens (France). *Int J Antimicrob Agents* 26: 219-229
- Cambray G, Guerout AM, Mazel D (2010) Integrons. *Annu Rev Genet* 44:141-166
- Clinical and Laboratory Standards Institute (2010) Performance standards for antimicrobial susceptibility testing: Eighteenth informational supplement M100-S20. CLSI, Wayne, PA, USA
- Coque TM, Baquero F, Cantón R (2008) Increasing prevalence of ESBL-producing *Enterobacteriaceae* in Europe. *Euro Surveill* 13:19044
- de Toro M, Rojo-Bezares B, Vinué L, Undabeitia E, Torres C, Sáenz Y (2010) *In vivo* selection of a *aac(6)-Ib-cr* and mutations in the *gyrA* gene in a clinical *qnrS1*-positive *Salmonella enterica* serovar Typhimurium DT104B strain recovered after fluoroquinolone treatment. *J Antimicrob Chemother* 65:1945-1949
- European Food Safety Authority (EFSA) (2009) The Community summary report on trends and sources of zoonoses and zoonotic agents in the European Union in 2007. *EFSA J* 223
- González-Sanz R, Herrera-León S, de la Fuente M, Arroyo M, Echeita MA (2009) Emergence of extended-spectrum β-lactamases and AmpC-type β-lactamases in human *Salmonella* isolated in Spain from 2001 to 2005. *J Antimicrob Chemother* 64:1181-1186
- Guerra B, Soto S, Helmuth R, Mendoza MC (2002) Characterization of a self-transferable plasmid from *Salmonella enterica* serotype Typhimurium clinical isolates carrying two integron-borne gene cassettes together with virulence and drug resistance genes. *Antimicrob Agents Chemother* 46:2977-2981
- Güerri ML, Aladueña A, Echeita A, Rotger R (2004) Detection of integrons and antibiotic-resistance genes in *Salmonella enterica* serovar Typhimurium isolates with resistance to ampicillin and variable susceptibility to amoxicillin-clavulanate. *Int J Antimicrob Agents* 24:327-333
- Herrero A, Mendoza MC, Threlfall EJ, Rodicio MR (2009) Detection of *Salmonella enterica* serovar Typhimurium with pUO-StVR2-like virulence-resistance hybrid plasmids in the United Kingdom. *Eur J Clin Microbiol Infect Dis* 28:1087-1093
- Jarlier V, Nicolas MH, Fournier G, Philippon A (1988) Extended broad-spectrum β-lactamases conferring transferable resistance to newer β-lactam agents in *Enterobacteriaceae*: hospital prevalence and susceptibility patterns. *Rev Infect Dis* 10:867-878
- Michael GB, Butaye P, Cloeckaert A, Schwarz S (2006) Genes and mutations conferring antimicrobial resistance in *Salmonella*: an update. *Microbes Infect* 8:1898-1914
- Miriagou V, Tassios PT, Legakis NJ, Tzouveleki LS (2004) Expanded-spectrum cephalosporin resistance in non-typhoid *Salmonella*. *Int J Antimicrob Agents* 23:547-555
- Mulvey MR, Boyd DA, Olson AB, Doublet B, Cloeckaert A (2006) The genetics of *Salmonella* genomic island 1. *Microbes Infect* 8:1915-1922
- Oliver A, Coque TM, Alonso D, Valverde A, Baquero F, Cantón R (2005) CTX-M-10 linked to a phage-related element is widely disseminated among *Enterobacteriaceae* in a Spanish hospital. *Antimicrob Agents Chemother* 49:1567-1571
- Pardos de la Gándara M, Seral C, Castillo García J, Rubio Calvo C, Weill FX (2011) Prevalence and characterization of extended-spectrum beta-lactamases-producing *Salmonella enterica* isolates in Saragossa, Spain (2001-2008) *Microb Drug Resist* 17:207-213

19. Partridge SR (2011) Analysis of antibiotic resistance regions in Gram-negative bacteria. *FEMS Microbiol Rev* 35:820-855
20. Pérez-Pérez FJ, Hanson ND (2002) Detection of plasmid-mediated AmpC β -lactamase genes in clinical isolates by using multiplex PCR. *J Clin Microbiol* 40:2153-2162
21. Riaño I, García-Campello M, Sáenz Y, Álvarez P, Vinué L, Lantero M, Moreno MA, Zarazaga M, Torres C (2009) Occurrence of extended-spectrum β -lactamase-producing *Salmonella enterica* in northern Spain with evidence of CTX-M-9 clonal spread among animals and humans. *Clin Microbiol Infect* 15:292-295
22. Rodríguez I, Guerra B, Mendoza MC, Rodicio MR (2011) pUO-SeVR1 is an emergent virulence-resistance complex plasmid of *Salmonella enterica* serovar Enteritidis. *J Antimicrob Chemother* 66:218-220
23. Romero L, López L, Martínez-Martínez L, Guerra B, Hernández JR, Pascual A (2004) Characterization of the first CTX-M-14-producing *Salmonella enterica* serotype Enteritidis isolate. *J Antimicrob Chemother* 53:1113-1114
24. Sáenz Y, Briñas L, Domínguez E, Ruiz J, Zarazaga M, Vila J, Torres C (2004) Mechanisms of resistance in multiple-antibiotic-resistant *Escherichia coli* strains of human, animal, and food origins. *Antimicrob Agents Chemother* 48:3996-4001
25. Sáenz Y, Vinué L, Ruiz E, Somalo S, Martínez S, Rojo-Bezares B, Zarazaga M, Torres C (2010) Class 1 integrons lacking qacEDelta1 and *sull* genes in *Escherichia coli* isolates of food, animal and human origins. *Vet Microbiol* 144:493-497
26. Soto SM, González-Hevia MA, Mendoza MC (2003) Antimicrobial resistance in clinical isolates of *Salmonella enterica* serotype Enteritidis: relationships between mutations conferring quinolone resistance, integrons, plasmids and genetic types. *J Antimicrob Chemother* 51:1287-1291
27. Sunde M, Norström M (2006) The prevalence of, associations between and conjugal transfer of antibiotic resistance genes in *Escherichia coli* isolated from Norwegian meat and meat products. *J Antimicrob Chemother* 58:741-747
28. Sutcliffe JG (1978) Nucleotide sequence of the ampicillin resistance gene of *Escherichia coli* plasmid pBR322. *Proc Natl Acad Sci USA* 75:3737-3741
29. Tan TY, Ng LS, He J, Koh TH, Hsu LY (2009) Evaluation of screening methods to detect plasmid-mediated AmpC in *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*. *Antimicrob Agents Chemother* 53:146-149
30. Targant H, Ponsin C, Brunet C, Doublet B, Cloeckert A, Madec JY, Meunier D (2010) Characterization of resistance genes in multidrug-resistant *Salmonella enterica* serotype Typhimurium isolated from diseased cattle in France (2002 to 2007). *Foodborne Pathog Dis* 7:419-425
31. Vinué L, Lantero M, Sáenz Y, Somalo S, de Diego I, Pérez F, Ruiz-Larrea F, Zarazaga M, Torres C (2008) Characterization of extended-spectrum β -lactamases and integrons in *Escherichia coli* isolates in a Spanish hospital. *J Med Microbiol* 57:916-920
32. Vinué L, Sáenz Y, Rojo-Bezares B, Olarte I, Undabeitia E, Somalo S, Zarazaga M, Torres C (2010) Genetic environment of sul genes and characterisation of integrons in *Escherichia coli* isolates of blood origin in a Spanish hospital. *Int J Antimicrob Agents* 35:492-496