5. Wey SB, Mori M, Pfaller MA, Woolson RF, Wenzel RP. Hospital-acquired candidemia: the attributable mortality and excess length of stay. *Arch Intern Med* 1988; 148: 2642–2645.
6. Falagas ME, Apostolou KE, Pappas VD. Attributable mortality of candidemia: a systematic review of matched cohort and case–control studies. *Eur J Clin Microbiol Infect Dis* 2006; 25: 419–425.
7. Ascioglu S, Rex JH, de Pauw B *et al.* Defining opportunistic invasive fungal infections in immunocompromised patients with cancer and hematopoietic stem cell transplants: an international consensus. *Clin Infect Dis* 2002; 34: 7–14.
8. Vardakas KZ, Samonis G, Michalopoulos A, Soteriades ES, Falagas ME. Antifungal prophylaxis with azoles in high-risk, surgical intensive care unit patients: a meta-analysis of randomized, placebo-controlled trials. *Crit Care Med* 2006; 34: 1216–1224.
9. Reagan DR, Pfaller MA, Hollis RJ, Wenzel RP. Characterization of the sequence of colonization and nosocomial candidemia using DNA fingerprinting and a DNA probe. *J Clin Microbiol* 1990; 28: 2733–2738.
10. Swoboda SM, Merz WG, Lipsetta PA. Candidemia: the impact of antifungal prophylaxis in a surgical intensive care unit. *Surg Infect (Larchmt)* 2003; 4: 345–354.
11. Blumberg HM, Jarvis WR, Soucie JM *et al.* Risk factors for candidal bloodstream infections in surgical intensive care unit patients: the NEMIS prospective multicenter study. The National Epidemiology of Mycosis Survey. *Clin Infect Dis* 2001; 33: 177–186.
12. Yamamura DL, Rotstein C, Nicolle LE, Ioannou S. Candidemia at selected Canadian sites: results from the Fungal Disease Registry, 1992–1994. Fungal Disease Registry of the Canadian Infectious Disease Society. *CMAJ* 1999; 160: 493–499.
13. Cheng MF, Yang YL, Yao TJ *et al.* Risk factors for fatal candidemia caused by *Candida albicans* and non-*albicans Candida* species. *BMC Infect Dis* 2005; 5: 22.
14. McKinnon PS, Goff DA, Kern JW *et al.* Temporal assessment of *Candida* risk factors in the surgical intensive care unit. *Arch Surg* 2001; 136: 1401–1408.

Occurrence of extended-spectrum β-lactamase-producing *Salmonella enterica* in northern Spain with evidence of CTX-M-9 clonal spread among animals and humans

I. Riaño[1], M. García-Campello[2], Y. Sáenz[1], P. Álvarez[2], L. Vinué[1], M. Lantero[3], M. Á. Moreno[4], M. Zarazaga[1] and C. Torres[1]

1) *Área de Bioquímica y Biología Molecular, Universidad de La Rioja, Logroño,* 2) *Servicio de Microbiología, Complejo Hospitalario de Pontevedra, Galicia,* 3) *Servicio de Microbiología, Hospital Universitario Central de Asturias, Oviedo and* 4) *Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid, Spain*

Abstract

Among the 1233 *Salmonella enterica* isolates obtained in two Spanish hospitals, five isolates (0.4%) (serovars: Virchow, four; Livingstone, one) had the phenotype of an extended-spectrum β-lactamase (ESBL) producer. The genetic characterization of the ESBL of *S. enterica* Livingstone revealed a bla_{SHV-2} gene. The $bla_{CTX-M-10}$ gene in a phage-related genetic environment was found in one *S. enterica* Virchow isolate, and the $bla_{CTX-M-9}$ gene within the In60 integron was found in the three remaining Virchow isolates. These three isolates presented indistinguishable or closely related pulsed-field gel electrophoresis patterns among themselves and also as compared with the two other $bla_{CTX-M-9}$-containing isolates previously obtained from animals. ESBL production is an emerging mechanism of resistance in *S. enterica* in the two studied hospitals.

Keywords: CTX-M-9, CTX-M-10, *Salmonella enterica*, SHV-2

Original Submission: 14 February 2008; **Revised Submission:** 9 July 2008; **Accepted:** 15 July 2008
Editor: L. Peixe

Clin Microbiol Infect 2009; **15:** 292–295
10.1111/j.1469-0691.2008.02673.x

Corresponding author and reprint requests: C. Torres, Área de Bioquímica y Biología Molecular, Departamento de Agricultura y Alimentación, Universidad de La Rioja, Madre de Dios 51, 26006 Logroño, Spain
E-mail: carmen.torres@unirioja.es

Salmonella enterica is a zoonotic bacterium, transmitted through the food chain, which causes gastroenteritis and other types of infection that require, in some cases, the use of antimicrobial agents. *S. enterica* isolates harbouring extended-spectrum-β-lactamases (ESBLs) have emerged worldwide during the last years, and ESBLs of the CTX-M type are increasingly reported in *Salmonella*, as well as in other members of the *Enterobacteriaceae* [1,2]. The occurrence of different genes encoding CTX-M enzymes within *sul1*-type integron structures that facilitate their dissemination has been previously reported [3,4], although other genetic environments have also been described for bla_{CTX-M} genes [5,6]. The objective of this study was to detect and characterize the ESBLs in *S. enterica* isolates recovered in two Spanish hospitals, and also to determine the genetic environment of ESBL genes and its possible location within integron structures.

During the period 2000–2004, a total of 1038 clinical isolates of *S. enterica* were recovered from unrelated

patients in the Complejo Hospitalario of Pontevedra (a 624-bed institution located in Pontevedra, Galicia, in the northwest of Spain) (2000–2002, 471 isolates; 2003, 310 isolates; and 2004, 257 isolates). For four of these isolates, all belonging to serovar Virchow, the MIC of cefotaxime and/or ceftazidime was ≥2 mg/L and the ESBL screening test was positive; they were included in this study for β-lactamase characterization. These isolates were obtained from ambulatory patients with acute gastroenteritis: one during the period 2000–2002, one in 2003, and two in 2004, representing 0.2%, 0.3% and 0.8%, respectively, of the total isolates in each of the studied periods in this hospital.

In addition, 195 isolates of *S. enterica* were recovered during 2003 in the Hospital Central of Asturias (a 1357-bed institution of Oviedo, Asturias, in the north of Spain). For one isolate (0.5%) (serovar Livingstone), which was recovered from a hospitalized patient with acute gastroenteritis, the MIC of cefotaxime and/or ceftazidime was ≥2 mg/L and the ESBL screening test was positive; it was also included in this study for β-lactamase characterization.

Antimicrobial susceptibility to amoxycillin, amoxycillin–clavulanic acid, cefotaxime, ceftazidime, cefepime, aztreonam, imipenem, nalidixic acid, ciprofloxacin, gentamicin, amikacin, tobramycin, streptomycin, fosfomycin, tetracycline, chloramphenicol, sulphonamide and trimethoprim–sulphamethoxazole was determined using disk diffusion and broth microdilution methods [7]. *Escherichia coli* ATCC 25922 was used as a quality control strain. The double-disk synergy test using amoxycillin–clavulanic acid and cefotaxime or ceftazidime disks was performed with all isolates to detect ESBL production [8].

The presence of genes encoding TEM-, SHV-, OXA-, CTX-M- and CMY-type β-lactamases was studied using specific PCRs [9]. All obtained amplicons were sequenced on both strands, and the sequences were compared with those included in the GenBank database and at the website http://www.lahey.org/Studies/, in order to ascribe the specific type of β-lactamase gene. The presence of the *sul1*, *sul2* and *sul3* genes, associated with sulphonamide resistance, and the *tet*(A) and *tet*(B) genes, associated with tetracycline resistance, were also determined by PCR in the five isolates [10]. Positive and negative controls from the University of La Rioja collection were used in all PCRs.

The presence of class 1 integrons as well as the characterization of the gene cassettes included in their variable regions was studied by PCR and sequencing [10]. The PCR 'primer-walking' strategy, with a wide variety of primers based on known sequences [6, 9], was used to amplify overlapping fragments, in order to determine the genetic environment of the $bla_{CTX\text{-}M}$ genes.

The five clinical *S. enterica* isolates included in this study represented 0.4% of the total *S. enterica* isolates obtained in both hospitals during the studied period. Table 1 shows the characteristics of the five ESBL-positive isolates. The genes encoding both CTX-M-9 and TEM-1b β-lactamases were detected in three *S. enterica* serovar Virchow isolates, those encoding CTX-M-10 and TEM-1b in another serovar Virchow isolate, and that encoding SHV-2 in the serovar Livingstone isolate.

The CTX-M-10 β-lactamase was initially described in an *E. coli* strain isolated in 1997 in a hospital of Madrid, Spain [6], and it was later reported in different species of *Enterobacter* and *Klebsiella* [1,11,12]. This enzyme was found in an *E. coli* isolate recovered in 2000 in the north of Spain, and recently also in an *S. enterica* serovar Virchow isolate from the same region [13,14]; no transconjugants of the described *S. enterica* strain were obtained after conjugation experiments, and the same result was found (data not shown) with the *S. enterica* isolate C683 (Table 1).

The genetic environment of the $bla_{CTX\text{-}M\text{-}10}$ gene in the *S. enterica* isolate C683, obtained by the PCR primer-walking

TABLE 1. Phenotypes, resistance genes and integron elements detected in the five extended-spectrum β-lactamase-producing *Salmonella enterica* isolates

S. enterica name/ serovar	MIC (mg/L)							Resistance phenotype (excluding β-lactams)	Antibiotic resistance genes detected		Class integron	
	AMX	AMC	CTX	CAZ	IPM	FEP	ATM		*bla*	Others[a]	*intI1*	Gene cassettes inside VR
C516/Virchow	>16	8/4	>128	1	<0.5	>32	4	STR–TET–SUL–SXT–NAL	CTX-M-9, TEM-1b	*tet*(A), *sul1*, *sul2*	–	*dfrA16*, *aadA2*
C650/Virchow	>16	16/8	>128	1	<0.5	8	2	STR–SUL–SXT–NAL	CTX-M-9, TEM-1b	*sul1*, *sul2*	–	*dfrA16*, *aadA2*
C651/Virchow	>16	8/4	>128	2	<0.5	8	8	STR–TET–SUL–SXT–NAL	CTX-M-9, TEM-1b	*tet*(A), *sul1*, *sul2*	–	*dfrA16*, *aadA2*
C683/Virchow	>16	<4/2	128	1	<0.5	4	4	SUL–NAL	CTX-M-10, TEM-1b	–	–	–
C493/Livingstone	>16	<4/2	64	8	<0.5	8	ND	STR–TET–SUL	SHV-2	*tet*(A), *sul1*	–	*aadA1*

AMX, amoxycillin; AMC, co-amoxiclav; CTX, cefotaxime; CAZ, ceftazidime; IPM, imipenem; FEP, cefepime; ATM, aztreonam; STR, streptomycin; TET, tetracycline; SUL, sulphonamide; SXT, trimethoprim–sulphamethoxazole; NAL, nalidixic acid; VR, variable region; ND, not determined.
[a]Antimicrobial resistance genes detected outside of the variable regions of class 1 integrons.

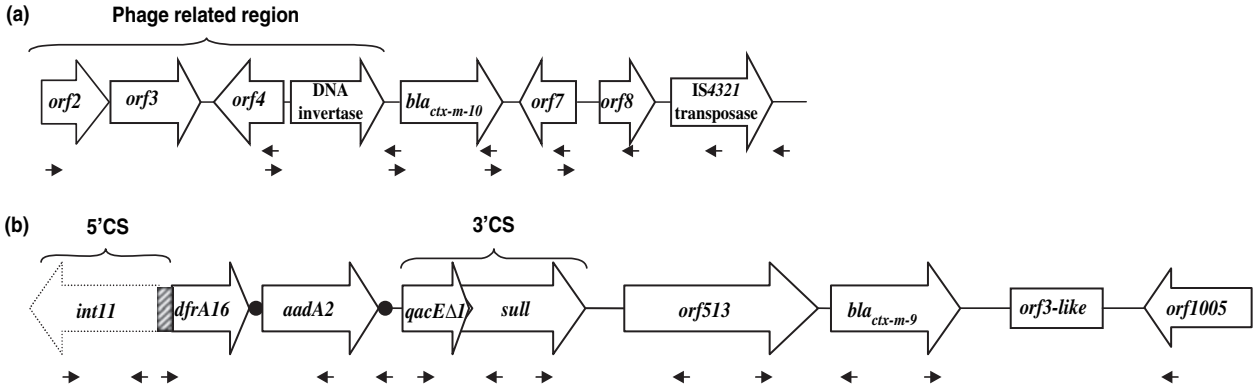

FIG. 1. Genetic structures detected surrounding: (a) the $bla_{CTX\text{-}M\text{-}10}$ gene in *Salmonella enterica* serovar Virchow isolate C683; and (b) the $bla_{CTX\text{-}M\text{-}9}$ gene in the three remaining *S. enterica* serovar Virchow isolates (C516, C650 and C651). The discontinued arrow indicates that the *intI1* gene was not detected; open box, the region that shows high similarity to *orf3* of *Kluyvera ascorbata*; ●, 59-be; diagonally striped box, the recombination site *attI*; ►, indicates the specific positions of the primers used for PCR primer-walking strategy.

strategy [6], is shown in Fig. 1. This structure is similar to that previously reported by Oliver *et al.* [6] in a $bla_{CTX\text{-}M\text{-}10}$-containing *E. coli* strain (GenBank accession number AY598759). This result reveals a linkage of this *bla* gene to a phage-related element, in contrast to the truncated IS*Ecp1* element upstream from the $bla_{CTX\text{-}M\text{-}10}$ gene that has been previously detected in the first $bla_{CTX\text{-}M\text{-}10}$-containing *E. coli* isolate in France [5]. This work provides another example of the influence of the genomic environment on local dissemination of resistance genes, even among different bacterial genera [6].

The CTX-M-9 group (mainly CTX-M-9 and CTX-M-14 enzymes) is highly represented in Spain and, in the case of CTX-M-9, is usually linked to class 1 integrons associated with an IS*CR1* element [2,4,15]. For that reason, a wide variety of primers based on the In60 integron structure (GenBank accession number AF174129) were used [9,10] (Fig. 1) in order to explore the genetic environment of the $bla_{CTX\text{-}M\text{-}9}$ gene in the three *S. enterica* serovar Virchow isolates. Curiously, the *intI1* gene was not found by PCR in any of these three CTX-M-9-producing isolates, even using other reported primers (data not shown). The genetic environment found in the present three clinical $bla_{CTX\text{-}M\text{-}9}$-containing isolates was identical to the surrounding region previously found in *S. enterica* serovar Virchow isolates of animal origin [9].

The clonal relationship among the $bla_{CTX\text{-}M\text{-}9}$-containing isolates was studied by pulsed-field gel electrophoresis, following the PulseNet Europe One-day protocol with *Xba*I as the restriction enzyme (http://www.pulsenet-europe.org/docs.htm), and the obtained patterns were found to be indistinguishable from those found in two $bla_{CTX\text{-}M\text{-}9}$-containing *S. enterica* serovar Virchow isolates previously obtained from animal samples [9].

All five isolates of human or animal origin showed indistinguishable or closely related patterns, indicating the clonal relationship among them. This observation is of interest and suggests a relationship among animal and human $bla_{CTX\text{-}M\text{-}9}$-containing *S. enterica* isolates that are emerging in both populations [16]. In addition, it is important to underline that the observed close clonal relationship of $bla_{CTX\text{-}M\text{-}9}$-containing *Salmonella* isolates is at variance with the high diversity among $bla_{CTX\text{-}M\text{-}9}$-containing *E. coli* isolates previously reported [17–19].

All three $bla_{CTX\text{-}M\text{-}9}$-containing isolates showed resistance to streptomycin, sulphonamide, trimethoprim–sulphamethoxazole and nalidixic acid, and two of them were also resistant to tetracycline. In addition to the resistance genes included in the In60 integron, these isolates also harboured the *sul2* gene, in addition to the *tet*(A) gene in the two tetracycline-resistant isolates.

The $bla_{SHV\text{-}2}$-containing *S. enterica* serovar Livingstone isolate harboured a class 1 integron that included the *aadA1* gene cassette, but the ESBL gene was not found to be associated with this class 1 integron.

S. enterica isolates producing ESBLs, mainly of the CTX-M group, seem to be an emerging problem in human medicine. The detection of closely related or indistinguishable pulsed-field gel electrophoresis patterns among CTX-M-9-producing *S. enterica* isolates of both human and animal origin could indicate the transfer of ESBL-producing *S. enterica* among animals and humans. The detection of the $bla_{CTX\text{-}M\text{-}10}$ gene in *S. enterica* isolates is interesting; this is the second time that it has been reported in the literature, and its genetic environment was linked to a phage-related element previously detected among *Enterobacteriaceae*. More studies should be performed in the future to track the evolution of ESBLs in *S. enterica* isolates from different environments.

Transparency Declaration

This work was supported in part by Project SAF2006-14207-C02. The authors have no conflict of interest to declare.

References

1. Livermore DM, Cantón R, Gniadkowski M *et al.* CTX-M: changing the face of ESBLs in Europe. *J Antimicrob Chemother* 2007; 59: 165–174.
2. Rossolini GM, D'Andrea MM, Mugnaioli C. The spread of CTX-M-type extended-spectrum β-lactamases. *Clin Microbiol Infect* 2008; 14: 33–41.
3. Sabaté M, Navarro F, Miró E *et al.* Novel complex *sul1*-type integron in *Escherichia coli* carrying $bla_{CTX-M-9}$. *Antimicrob Agents Chemother* 2002; 46: 2656–2661.
4. Poirel L, Naas T, Nordmann P. Genetic support of extended-spectrum β-lactamases. *Clin Microbiol Infect* 2008; 14: 75–81.
5. Lartigue MF, Poirel L, Nordmann P. Diversity of genetic environment of bla_{CTX-M} genes. *FEMS Microbiol Lett* 2004; 234: 201–207.
6. Oliver A, Coque TM, Alonso D *et al.* CTX-M-10 linked to a phage-related element is widely disseminated among Enterobacteriaceae in a Spanish hospital. *Antimicrob Agents Chemother* 2005; 49: 1567–1571.
7. Clinical and Laboratory Standards Institute. *Performance standards for antimicrobial susceptibility testing: seventeenth informational supplement M100-S17*. Wayne, PA: CLSI, 2007.
8. Jarlier V, Nicolas MH, Fournier G, Philippon A. Extended broad-spectrum beta-lactamases conferring transferable resistance to newer beta-lactam agents in Enterobacteriaceae: hospital prevalence and susceptibility patterns. *Rev Infect Dis* 1988; 10: 867–878.
9. Riaño I, Moreno MA, Teshager T *et al.* Detection and characterization of extended-spectrum β-lactamases in *Salmonella enterica* strains of healthy food animals in Spain. *J Antimicrob Chemother* 2006; 58: 844–847.
10. Sáenz Y, Briñas L, Domínguez E *et al.* Mechanisms of resistance in multiple-antibiotic-resistant *Escherichia coli* strains of human, animal and food origins. *Antimicrob Agents Chemother* 2004; 48: 3996–4001.
11. Cantón R, Oliver A, Coque TM *et al.* Epidemiology of extended-spectrum β-lactamase-producing *Enterobacter* isolates in a Spanish hospital during a 12-year period. *J Clin Microbiol* 2002; 40: 1237–1243.
12. Coque TM, Oliver A, Pérez-Díaz JC *et al.* Genes encoding TEM-4, SHV-2, and CTX-M-10 extended-spectrum β-lactamases are carried by multiple *Klebsiella pneumoniae* clones in a single hospital (Madrid, 1989 to 2000). *Antimicrob Agents Chemother* 2002; 46: 500–510.
13. Hernández JR, Martínez-Martínez L, Cantón R *et al.* Nationwide study of *Escherichia coli* and *Klebsiella pneumoniae* producing extended-spectrum β-lactamases in Spain. *Antimicrob Agents Chemother* 2005; 49: 2122–2125.
14. Cartelle M, Canle D, Pérez Llarena FJ *et al.* Characterisation of the first CTX-M-10-producing isolate of *Salmonella enterica* serotype Virchow. *Clin Microbiol Infect* 2006; 12: 285–298.
15. Cantón R, Novais A, Valverde A *et al.* Prevalence and spread of extended-spectrum β-lactamase-producing Enterobacteriaceae in Europe. *Clin Microbiol Infect* 2008; 14: 144–153.
16. Weill F-X, Lailler R, Praud K *et al.* Emergence of extended-spectrum-β-lactamase (CTX-M-9)-producing multiresistant strains of *Salmonella enterica* Serotype Virchow in poultry and humans in France. *J Clin Microbiol* 2004; 42: 5767–5773.
17. Briñas L, Moreno MA, Zarazaga M *et al.* Detection of CMY-2, CTX-M-14, and SHV-12 β-lactamases in *Escherichia coli* fecal-sample isolates from healthy chickens. *Antimicrob Agents Chemother* 2003; 47: 2056–2058.
18. Briñas L, Lantero M, de Diego I *et al.* Mechanisms of resistance to expanded-spectrum cephalosporins in *Escherichia coli* isolates recovered in a Spanish hospital. *J Antimicrob Chemother* 2005; 56: 1107–1110.
19. García A, Navarro F, Miró E *et al.* Characterization of the highly variable region surrounding the $bla_{CTX-M-9}$ gene in non-related *Escherichia coli* from Barcelona. *J Antimicrob Chemother* 2005; 56: 819–826.