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Detection and characterization of extended-spectrum β-lactamases in *Salmonella enterica* strains of healthy food animals in Spain

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***Objectives*: To carry out the characterization of the genes encoding extended-spectrum β-lactamases (ESBLs) and their genetic environments in four expanded-spectrum cephalosporin-resistant *Salmonella enterica* isolates (serovars: two Virchow, one Enteritidis, one Rissen) recovered during the monitoring programmes performed in Spain by the VAV Network from faecal samples of pigs, poultry and laying hens at the slaughterhouse level.**

***Methods*: The presence and characterization of ESBL genes as well as their genetic environments in the four *S. enterica* isolates were investigated by PCR and sequencing. The presence of other resistance genes was also analysed by PCR and sequencing.**

***Results*: Three avian *S. enterica* isolates (two Virchow and one Enteritidis) harboured the $bla_{CTX\text{-}M\text{-}9}$ gene combined with $bla_{TEM\text{-}1b}$. The $bla_{CTX\text{-}M\text{-}9}$ gene was included in these three isolates in a class 1 integron with the following 5′→3′ structure: integron 1 variable region (*dfrA16-aadA2* gene cassettes)-*qacEΔ1-sul1-orf*513-$bla_{CTX\text{-}M\text{-}9}$-*orf*3-like-*orf*1005. The *sul2* gene was also detected in these three $bla_{CTX\text{-}M\text{-}9}$-containing isolates and *tet*(A) in one of them. The two serovar Virchow isolates showed an indistinguishable PFGE pattern, although they were recovered from different animal species (broiler and laying hen). A porcine ESBL-positive isolate (serovar Rissen) harboured the $bla_{SHV\text{-}12}$ gene combined with $bla_{TEM\text{-}1b}$. This $bla_{SHV\text{-}12}$-containing isolate also harboured the *tet*(A), *aadA* and *sul1* genes.**

***Conclusions*: The emergence of ESBL-producing *S. enterica* isolates among food animals is described for the first time in Spain, with those of the CTX-M group being the predominant ESBLs detected.**

Keywords: *S. enterica*, ESBLs, CTX-M-9, integrons, SHV-12

Introduction

Salmonella enterica is a zoonotic bacteria transmitted through the food chain. *Salmonella* isolates harbouring extended-spectrum β-lactamases (ESBLs) have emerged worldwide during the last decade, with the CTX-M group being particularly important.[1] In addition, the occurrence of different genes encoding CTX-M enzymes within integron structures that facilitate its dissemination in different environments has been reported.[1,2] A large number of studies have investigated the presence of ESBLs in *Salmonella* strains from human patients,[3] but only a few studies have been carried out in strains of food-producing animals or food products,[4–6] and none of them in Spain. The characterization of genes encoding ESBLs and their genetic environments has been carried out in the present study in four *S. enterica* isolates recovered from faecal samples of food-producing animals in Spain.

Materials and methods

The Spanish Veterinary-Antimicrobial-Resistance-Surveillance Network (VAV) is monitoring antimicrobial resistance of *S. enterica* obtained from faecal samples of healthy food animals from 1999 at the slaughterhouse level in Spain (pigs, 1999–2004: $n = 436$; laying hens, 2003: $n = 44$; and broilers, 2003–2004: $n = 76$). Four of these 556 *S. enterica* isolates showed a resistant phenotype for

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expanded-spectrum cephalosporins (cefotaxime and/or ceftazidime) and also a positive ESBL synergy test (synergy between clavulanic acid and cefotaxime or ceftazidime), and were included in the study for ESBL genetic characterization. These four isolates were recovered as follows: one (serovar Rissen) porcine isolate from 2003 (n = 129); one (serovar Virchow) laying hen isolate from 2003 (n = 44); and two broiler isolates (serovars Virchow and Enteritidis) from 2004 (n = 36).

Susceptibility testing to 26 antimicrobials (ampicillin, amoxicillin, amoxicillin/clavulanic acid, ticarcillin, cefalotin, cefoxitin, cefotaxime, cefotaxime/clavulanic acid, ceftazidime, imipenem, aztreonam, gentamicin, apramycin, tobramycin, amikacin, streptomycin, neomycin, nalidixic acid, ciprofloxacin, chloramphenicol, florfenicol, tetracycline, fosfomycin, sulphonamides, trimethoprim and trimethoprim/sulfamethoxazole) was determined in the four ESBL-positive isolates by agar dilution and/or disc diffusion methods (NCCLS).

The presence of genes encoding TEM (forward, 5′-TTCTTGAAGACGAAAGGGC-3′; reverse, 5′-ACGCTCAGTGGAACGAAAAC-3′), SHV (forward, 5′-CACTCAAGGATGTATTGTG-3′; reverse, 5′-TTAGCGTTGCCAGTGCTCG-3′), OXA-1 (forward, 5′-ACACAATACATATCAACTTCGC-3′; reverse, 5′-AGTGTGTTTAGAATGGTGATC-3′), OXA-2 (forward, 5′-TTCAAGCCAAAGGCACGATAG-3′; reverse, 5′-TCCGAGTTGACTGCCGGGTTG-3′), OXA-10 (forward, 5′-CGTGCTTTGTAAAAGTAGCAG-3′; reverse, 5′-CATGATTTTGGTGGGAATGG-3′), CTX-M-9 group (forward, 5′-GTGACAAAGAGAGTGCAACGG-3′; reverse, 5′-ATGATTCTCGCCGCTGAAGCC-3′), CTX-M-3 group (forward, 5′-GTTACAATGTGTGAGAAGCAG-3′; reverse, 5′-CCGTTTCCGCTATTACAAAC-3′), CTX-M-10 (forward, 5′-CCGCGCTACACTTTGTGGC-3′; reverse, 5′-TTACAAACCGTTGGTGACG-3′), CMY-type (forward, 5′-GATTCCTTGGACTCTTCAG-3′; reverse, 5′-TAAAACCAGGTTCCCAGATAGC-3′) and PSE (forward, 5′-TGCTTCGCAACTATGACTAC-3′; reverse, 5′-AGCCTGTGTTTGAGCTAGAT-3′) β-lactamases was analysed by PCR and sequencing.[7,8] The sequences were compared with those included in the GenBank database in order to ascribe the specific type of β-lactamase gene.

The presence of class 1 and class 2 integrons as well as the characterization of their gene cassettes were studied by PCR and sequencing of the variable regions in the four ESBL-positive *S. enterica* isolates.[8–10] In addition, other genes associated with tetracycline [*tet*(A) and *tet*(B)] or sulfamethoxazole resistance (*sul1*, *sul2* or *sul3*) were also analysed by PCR and sequencing.[8]

The clonal relationship among the isolates was studied by PFGE using *Xba*I enzyme for chromosomal DNA restriction.

Results and discussion

The characteristics of the four ESBL-positive *S. enterica* isolates are shown in Table 1. The three avian *S. enterica* isolates harboured the $bla_{\text{CTX-M-9}}$ gene combined with $bla_{\text{TEM-1b}}$ and showed higher MIC values of cefotaxime (≥256 mg/L) than of ceftazidime (1–2 mg/L). They also presented resistance to ampicillin, ticarcillin, amoxicillin, cefalotin, trimethoprim, trimethoprim/sulfamethoxazole, streptomycin, sulphonamides and nalidixic acid, and one of them also to tetracycline.

The variable regions of class 1 integrons were amplified by PCR in the three *S. enterica* isolates harbouring a $bla_{\text{CTX-M-9}}$ gene and the sequences of the obtained amplicons showed the combination of the *dfrA16* plus *aadA2* gene cassettes in the three isolates (Table 1). In order to know the possible inclusion of the $bla_{\text{CTX-M-9}}$ gene into this integron structure, a wide variety of

Table 1. Phenotypes and antibiotic resistance genes detected in four multiresistant ESBL-harbouring *S. enterica* isolates recovered from food-producing animals

S. enterica isolate	Serovar	Origin of strain	MIC (mg/L)		Phenotype of resistance to non-β-lactams	ESBL synergy test	Resistance genes detected		Class 1 integron genes		
			CTX	CAZ			*bla* genes	others	*intI1*	variable region	genes included in variable region
P3S569570S3	Rissen	pig	256	>256	STR-TET-SUL	+	$bla_{\text{TEM-1b}}$, $bla_{\text{SHV-12}}$	*tet(A)*, *aadA*, *sul1*	–	–	–
A4S1002100 4S1	Virchow	broiler	>256	1	STR[a]-SUL-SXT-NAL	+	$bla_{\text{TEM-1b}}$, $bla_{\text{CTX-M-9}}$	*dfrA16*, *aadA2*, *qacE*Δ*1*, *sul1*, *sul2*	–	+	*dfrA16*, *aadA2*
G3S188190S1	Virchow	laying hen	>256	2	STR-SUL-SXT-NAL	+	$bla_{\text{TEM-1b}}$, $bla_{\text{CTX-M-9}}$	*dfrA16*, *aadA2*, *qacE*Δ*1*, *sul1*, *sul2*	–	+	*dfrA16*, *aadA2*
A4S934936S3	Enteritidis	broiler	256	1	STR-TET-SUL-SXT-NAL	+	$bla_{\text{TEM-1b}}$, $bla_{\text{CTX-M-9}}$	*dfrA16*, *aadA2*, *tet(A)*, *qacE*Δ*1*, *sul1*, *sul2*	–	+	*dfrA16*, *aadA2*

CTX, cefotaxime; CAZ, ceftazidime; STR, streptomycin; TET, tetracycline; SUL, sulphonamides; SXT, trimethoprim/sulfamethoxazole; NAL, nalidixic acid.
[a]Intermediate category according to the NCCLS standards.

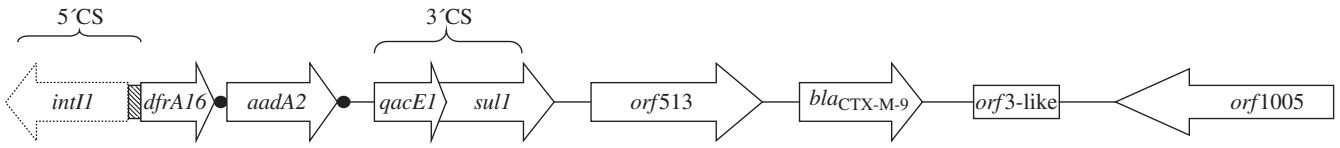

Figure 1. Schematic presentation of the integron carrying the $bla_{\text{CTX-M-9}}$ gene in the three *S. enterica* strains of our study. Open box, the region that shows high similarity to *orf*3 of *Kluyvera ascorbata*; black circle, 59-be; diagonally striped box, the recombination site *attI1*; discontinued arrow, the *intI1* gene not detected.

primers based on the In60 structure (GenBank accession number AF174129) were used,[8–10] to amplify the upstream and the downstream regions of the $bla_{\text{CTX-M-9}}$ gene. All the obtained amplicons were sequenced. Figure 1 shows the confirmed structure found in these three strains after the comparison of the sequences obtained with those included in the GenBank database. The variable region of class 1 integrons (containing the *dfrA16* and *aadA2* gene cassettes) followed by the 3′ conserved region (including the *qacEΔ1* and *sul1* genes) and *orf*513 were detected upstream of the $bla_{\text{CTX-M-9}}$ gene in these three *S. enterica* isolates (Figure 1). The *orf*3-like sequence followed by the *orf*1005 sequence were detected downstream of the $bla_{\text{CTX-M-9}}$ gene. Curiously, the *intI1* gene was not found by PCR in any of these three CTX-M-9-harbouring isolates; in addition, negative PCR results were also obtained when degenerate primers for integrases of classes 1, 2 and 3 were used (forward, 5′-TGCGGGTYAARG-ATBTKGATTT-3′; reverse, 5′-CARCACATGCGTRTARAT-3′).

The sequence of both the variable region of class 1 integrons and the open reading frame *orf*513 in our three $bla_{\text{CTX-M-9}}$-harbouring *S. enterica* isolates was 100% identical to the corresponding sequence of the In36 integron (GenBank accession no. AY259085) and 99% identical to the corresponding sequence of In60 (GenBank accession no. AF174129). Similar sequences have been previously found in *S. enterica* serovar Virchow strains from poultry isolates.[6] Nevertheless, the sequence of the downstream region of $bla_{\text{CTX-M-9}}$ in our strains was 100% identical to In60, first reported by Sabaté *et al.*[2] in an *Escherichia coli* strain harbouring the $bla_{\text{CTX-M-9}}$ gene. In36 and In60 belong to the In6–In7 integron family and both contain a second copy of the 3′-conserved region. Both integrons have the same structure in their 5′ region that includes the genes *intI1-dfrA16-aadA2-qacEΔ1-sul1-orf*513, but they differ in their 3′ region.[2]

Both Virchow isolates showed an indistinguishable PFGE pattern, although they were recovered from different animal species (broiler and laying hen), indicating the presence of the same clone from different sources.

The porcine ESBL-positive isolate (serovar Rissen) harboured the $bla_{\text{SHV-12}}$ gene combined with $bla_{\text{TEM-1b}}$ (Table 1). This isolate presented a high MIC value of both cefotaxime and ceftazidime (≥256 mg/L), also showing resistance to ampicillin, ticarcillin, amoxicillin, aztreonam, cefalotin, streptomycin, sulphonamides and tetracycline. The *sul1*, *tet*(A) and *aadA* genes were detected in this isolate, although it did not harbour class 1 or class 2 integrons.

The first β-lactamase of the CTX-M group was detected in a cefotaxime-resistant *E. coli* strain in Japan in 1986 and was designated as FEC-1, followed by the detection of CTX-M-1 in a clinical *E. coli* strain in Germany in 1989.[1] In 1992, the first *Salmonella* strain harbouring a CTX-M β-lactamase (CTX-M-2) was reported in South America, and later this type of resistance was detected in other countries of different continents.[1] The first report of the β-lactamase CTX-M-9 was in 1996 in a clinical *E. coli* strain in Spain, being first found in *S. enterica* in 2000.[3] To our knowledge, there are only three previous reports (carried out in the Netherlands, Greece and France) in which genes encoding CTX-M β-lactamases were detected in *Salmonella* strains of animal origin,[4–6] the genes detected being $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-9}}$ and $bla_{\text{CTX-M-32}}$. This is the first time that *S. enterica* of animal origin, harbouring genes encoding ESBL, have been found in Spain.

In the present study, we report the emergence of ESBL-producing *S. enterica* isolates, mainly of the CTX-M group, in food-producing animals at the slaughterhouse in Spain. It is important to underline that the $bla_{\text{CTX-M-9}}$ gene in these isolates is incorporated in the structure of an integron of class 1, associated with genes that confer resistance to antimicrobials that could be extensively used among animals and humans (such as trimethoprim, sulfamethoxazole or streptomycin) and that could be an important factor for selection of multiresistant *S. enterica* strains harbouring $bla_{\text{CTX-M}}$ genes. More studies should be carried out in the future in order to track the evolution of this type of resistance among *S. enterica* living in different ecosystems (humans, animals and food).

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Transparency declarations

Nothing to declare.

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