Detection of CMY-2, CTX-M-14, and SHV-12 β -Lactamases in *Escherichia coli* Fecal-Sample Isolates from Healthy Chickens

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Genes encoding the CMY-2, CTX-M-14, and SHV-12 β -lactamases were detected in three of five *Escherichia coli* isolates from fecal samples from healthy chickens which showed resistance or diminished susceptibility to extended-spectrum cephalosporins. A –42 mutation at the promoter region of the *ampC* gene was detected in the other two isolates.

Escherichia coli is an important pathogen of animals and humans, and it is also a common inhabitant of their intestinal tracts. An increase in the rate of β -lactam resistance in *E. coli* isolates has been observed in the last few years, β -lactamase synthesis being the main mechanism of resistance. Class A extended-spectrum β -lactamases (ESBLs) (CTX-M or enzymes derived from the classic TEM-1 and SHV-1) and plasmidic class C β -lactamases (FOX and CMY, among others) have been described for *E. coli*, and this fact is a significant cause of concern (5, 21). Several studies show the dissemination in Spain of ESBLs among human clinical isolates from hospitalized patients as well as from those in the community (4, 10; C. Sanchez, B. Padilla, C. Perez, E. Cercenado, M. Sanchez, J. Gomez, and E. Bouza, Abstr. 42nd Intersci. Conf. Antimicrob. Agents Chemother., abstr. K-1947, 2002). Very few studies on the characterization of β -lactamases in *E. coli* isolates from sick animals (6, 7, 12, 27; T. Teshager, L. Dominguez, M. A. Moreno, Y. Sáenz, M. Zarazaga, C. Torres, and S. Cardeñosa, Letter, Antimicrob. Agents Chemother. 44: 3483–3484, 2000) or from healthy animals (8) exist to date.

In this study, β -lactamase characterization of five *E. coli* isolates recovered from fecal samples of healthy chickens which showed a cefotaxime and/or a ceftazidime MIC of ≥ 4 $\mu\text{g/ml}$ was carried out. These isolates belong to a collection of 120 recovered (between November 2000 and February 2001) at the slaughterhouse level from fecal samples of healthy chickens as part of the Spanish antimicrobial resistance surveillance program (17). MICs of 18 different antibiotics were determined for these isolates by the agar dilution method (18); the disk diffusion method was used for 3 additional antibiotics. Results are shown in Table 1. The ranges of the MICs of cefotaxime and ceftazidime were 0.25 to 128 $\mu\text{g/ml}$ and 0.5 to 32 $\mu\text{g/ml}$, respectively. Three of the isolates showed resistance to nalidixic acid, ciprofloxacin, tetracycline, and trimethoprim-sulfamethoxazole, and two of them were also resistant to chloramphenicol, gentamicin, kanamycin, and tobramycin. All five isolates were susceptible to amikacin and fosfomycin.

The presence of genes encoding different types of β -lactamases (TEM, SHV, OXA-1, CMY, CTX-M, FOX, and the mutation in the promoter of *ampC*) in the five isolates was studied. The detection of the *bla*_{TEM}, *bla*_{SHV}, and *bla*_{OXA-1} genes was carried out by PCR (Table 2). The *bla*_{TEM} and *bla*_{SHV} amplicons obtained were sequenced (both strands) using the same set of primers as for the PCRs. The DNA and the deduced amino acid sequences were compared with those previously described (GenBank database and G. Jacoby and K. Bush website [<http://www.lahey.org/studies/webt.htm>]). The nomenclature used for the *bla*_{TEM} variants and promoters was as previously proposed (13, 15).

The detection of plasmidic class C β -lactamases was carried out using specific primers for the amplification of *bla*_{CMY} and *bla*_{FOX} genes (Table 2). The complete sequence (approximately 1,800 bp) of the amplified fragment obtained with *bla*_{CMY} primers was determined on both strands by applying a primer-walking strategy to obtain the whole *bla*_{CMY} sequence as well as the upstream and downstream regions. The detection of plasmidic CTX-M-9-type and CTX-M-10-type (class A β -lactamases) was also analyzed by PCR and by subsequently sequencing the amplicons (Table 2). Positive and negative controls were used in all reactions.

A positive *bla*_{TEM} PCR result was obtained for three of the five *E. coli* isolates, and a positive *bla*_{SHV} PCR result was obtained for an additional isolate (Table 3). Each of the three *bla*_{TEM} amplicons was identified as a *bla*_{TEM-1b} gene (the weak P3 promoter was identified in two of the isolates). The *bla*_{SHV} amplicon was identified as a *bla*_{SHV-12} gene and was obtained in an *E. coli* isolate with an antibiotic resistance phenotype typical of an ESBL. The SHV-12 β -lactamase had been previously found in human (19, 28) and animal *E. coli* clinical isolates (Teshager et al., letter, 2000) but never in healthy animal isolates.

A β -lactamase CMY-2 was found in one *E. coli* isolate under study that also harbored a *bla*_{TEM-1b} gene (Table 3). The sequence of the 1,800-bp amplicon, which included the *bla*_{CMY-2} gene, showed a genetic organization that enclosed (upstream of the *bla*_{CMY-2} gene) a partial region of the *tnpA* gene (encoding a transposase) and the right inverted repeat sequence of ISEcpI and (upstream of the *bla*_{CMY-2} gene) a partial region of an outer membrane lipoprotein gene. This genetic organi-

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TABLE 1. MIC values for five *E. coli* isolates recovered from healthy chickens with diminished susceptibility or resistance to cefotaxime and/or ceftazidime

Antibiotic	MIC ($\mu\text{g/ml}$) ^a				
	AOEC117120	AOEC273276	AOEC221224	AOEC477480	AOEC429432
Ampicillin	>256	>256	128	64	>256
Ticarcillin	>128	>128	32	>128	>128
Amoxicillin-clavulanic acid	64	64	64	1	4
Cefazolin	64	256	32	16	>256
Cefoxitin	64	128	16	4	8
Ceftazidime	8	32	8	4	0.5
Cefotaxime	2	8	1	0.25	128
Ceftriaxone	2	16	1	2	64
Imipenem	0.25	0.25	0.25	0.06	0.125
Aztreonam	8	16	4	16	2
Nalidixic acid	>256	>256	4	2	>256
Ciprofloxacin	16	8	0.125	0.125	8
Gentamicin	>256		1	1	1
Streptomycin	16	>256	4	64	>256
Amikacin	2	4	1	4	2
Tetracycline	>256	128	1	2	128
Chloramphenicol	256	256	4	32	8
Fosfomicin	4	8	4	4	2
Kanamycin	R	R	S	S	S
Tobramycin	R	R	S	S	S
Trimethoprim-sulfamethoxazole	R	R	S	S	R

^a Disk diffusion method was used for three of the antibiotics in susceptibility testing (kanamycin, amikacin, tobramycin, and trimethoprim-sulfamethoxazole). S, susceptible; R, resistant.

zation was similar to that previously described by Stapleton et al. (25; P. D. Stapleton, Abstr. InterSci. Conf. Antimicrob. Agents Chemother., abstr. 1457, 1999) in an *E. coli* plasmid carrying the *bla*_{CMY-4} gene and also to that found by Briñas et al. (7) in two *E. coli* isolates from sick animals (a chicken and a pig) which harbored the *bla*_{CMY-2} gene. The CMY-2 β -lactamase has been previously detected in clinical bovine and porcine *E. coli* isolates (27) but never in healthy animals.

TABLE 2. Primers and conditions used to amplify different β -lactamase genes by the PCR technique

Gene	Primers	Size (bp)	Reference
<i>bla</i> _{TEM}	F: ATT CTT GAA GAC GAA AGG GC R: ACG CTC AGT GGA ACG AAA AC	1,150	3
<i>bla</i> _{SHV}	F: CAC TCA AGG ATG TAT TGT G R: TTA GCG TTG CCA GTG CTC G	885	22
<i>bla</i> _{OXA-1}	F: ACA CAA TAC ATA TCA ACT TCG C R: AGT GTG TTT AGA ATG GTG ATC	813	26
<i>ampC</i>	F: GAT TCC TTG GAC TCT TCA G R: TAA AAC CAG GTT CCC AGA TAG C	191	9
<i>bla</i> _{CMY} ^a	F: GAT TCC TTG GAC TCT TCA G R: TAA AAC CAG GTT CCC AGA TAG C	1,807	25
<i>bla</i> _{FOX}	F: CAC CAC GAG AAT AAC CAT R: ATG TGG ACG CCT TGA ACT	1,192	2
<i>bla</i> _{CTX-M-9 type}	F: GTG ACA AAG AGA GTG CAA CGG R: ATG ATT CTC GCC GCT GAA GCC	857	10
<i>bla</i> _{CTX-M-10 type}	F: CCG CGC TAC ACT TTG TGG C R: TTA CAA ACC GTT GGT GAC G	944	10

^a The *bla*_{CMY} gene and the flanking region were amplified with the indicated set of primers.

The only isolate (*E. coli* AOEC429432) for which cefotaxime had a MIC (128 $\mu\text{g/ml}$) higher than that of ceftazidime (0.5 $\mu\text{g/ml}$) gave a positive PCR result with the primers to detect *bla*_{CTX-M-9}-like genes. This product was sequenced, and the deduced amino acid sequence revealed a CTX-M-14 β -lactamase. This isolate also harbored a *bla*_{TEM-1b} gene (Table 1). The nucleotide sequence of a 162-bp region upstream of the *bla*_{CTX-M-14} gene in this isolate was determined and analyzed with the BLASTN program at the National Center for Biotechnology Information. The sequence was 100% identical to the upstream region of the *bla*_{CTX-M-9} gene that was included in the In60 integron (23) (GenBank accession no. AF74129). Furthermore, our sequence included the 28-bp segment that had been proposed as a recognition site for the *orf513* putative transposase (1). These results suggested that the *bla*_{CTX-M-14} gene in our isolate was located in an integron, as had been reported for other CTX-M β -lactamases (1, 23). The CTX-M-14 β -lactamase had been previously detected in human clin-

TABLE 3. Plasmidic β -lactamase genes and mutations in the promoter of the chromosomal *ampC* gene in five *E. coli* isolates recovered from healthy chickens with diminished susceptibility or resistance to cefotaxime and/or ceftazidime

<i>E. coli</i> isolate	Plasmidic <i>bla</i> gene detected	Positions of mutations in the promoter of the <i>ampC</i> gene ^c
AOEC117120	<i>bla</i> _{TEM-1b}	-42, -18, -1, +58
AOEC273276	<i>bla</i> _{TEM-1b} (P3) ^a , <i>bla</i> _{CMY-2}	No mutation
AOEC221224	None detected ^b	-42, -18, -1, +58
AOEC477480	<i>bla</i> _{SHV-12}	-18, -1, +58
AOEC429432	<i>bla</i> _{TEM-1b} (P3) ^a , <i>bla</i> _{CTX-M-14}	No mutation

^a The structural gene and the promoter are indicated.

^b Negative results were obtained in all of the PCR experiments for detection of plasmidic *bla* genes in this isolate.

^c Compared to *E. coli* K-12 sequence (14).

ical *E. coli* isolates (4, 11, 16, 20, 24) but never before in animal isolates.

Negative results were obtained for the five *E. coli* isolates analyzed when the CTX-M-10-like, FOX, and OXA-1 sets of primers were used in the PCR assay.

The regulatory region of the chromosomal *ampC* gene was amplified by PCR (Table 2) with the five isolates and sequenced. The mutations in the promoter and attenuator region were studied by comparing the sequence with the same region in the *E. coli* K-12 *ampC* gene (14). No mutations were found in the two *E. coli* isolates which harbored the CMY-2 or CTX-M-14 β -lactamases. Mutations at positions -18, -1, and +58 were detected in the *E. coli* isolate with the *bla*_{SHV-12} gene. Nevertheless, mutations at positions -42, -18, -1, and +58 were detected in the other two isolates (Table 1). Mutations at the -42 position were associated with hyperproduction of the chromosomal AmpC β -lactamase and caused resistance to cefoxitin and a diminished susceptibility to extended-spectrum cephalosporins, as has been previously reported (9). The *E. coli* isolates which harbored *bla*_{CMY-2}, *bla*_{CTX-M-14}, or *bla*_{SHV-12} genes did not show a mutation at -42 position (Table 3).

To our knowledge, this is the first time that genes encoding SHV-12, CMY-2, and CTX-M-14 β -lactamases have been detected in *E. coli* isolates from healthy animals. The use of extended-spectrum cephalosporins in chickens is very unusual, and the possibility of cross-selection with other antimicrobials used in poultry (such as sulfonamides and tetracyclines, among others) might explain this discovery and should be further analyzed in the future.

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