

(Matsumura et al., 1998; Raimondi et al., 2001; Mammeri et al., 2004b; Hidri et al., 2005), and even in *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Rodriguez-Martinez et al., 2009, 2010). ESACs were considered an emerging mechanism of resistance to beta-lactams and their prevalence was estimated around 0.2%, almost identical to that of plasmidic cephalosporinases (Mammeri et al., 2008). Among the modifications of the *ampC* gene in ESAC-producing *E. coli*, certain nucleotides substitutions were more frequently identified, such as the S287N replacement. Other replacements were found in the coding sequence of plasmid-borne AmpCs (Kim et al., 2006), which also contribute to the extension of their hydrolysis spectrum. Finally, ESAC *E. coli* producers were predominantly found to belong to the Clonal Complex (CC) 23 (Cremet et al., 2010), and this raises the question on the selection scheme of those isolates.

To date, ESACs were reported in human isolates only. The purpose of this study was thus to detect and characterize the very first ESAC producers in animals worldwide.

MATERIALS AND METHODS

BACTERIAL ISOLATES

A total of 6765 non-replicate *E. coli* isolates collected from cattle between February 2005 and December 2010 in France were included in this study. They were mostly recovered from fecal samples and from diseased animals ($n = 6158$), in particular from calves severely affected with gastro-enteritidis. Those isolates were recovered through the Resapath, the long-term surveillance network for antimicrobial resistance in pathogenic bacteria in France (www.resapath.anses.fr). An additional set of *E. coli* isolates ($n = 607$) collected from healthy French cattle (carriage) was also included. These latter isolates were recovered during a unique sampling program at slaughterhouse in 2006–2007. All isolates originated from various districts throughout the country and, when originating from the same district, came from different and widely distant farms. As the definition of AmpC over-producers may vary among studies (in particular with regard to resistance or not to expanded-spectrum cephalosporins in line with the strength of the *ampC* promoter), inclusion criteria for AmpC over-production were defined as follows, i.e., resistance to amoxicillin and amoxicillin-clavulanic acid, resistance to narrow-spectrum cephalosporins, reduced susceptibility to cefoxitin [diameter <22 mm, using the criteria of the Antibiogram Committee of the French Society for Microbiology (CA-SFM); (www.sfm-microbiologie.fr)] and resistance to ceftazidime (MIC ≥ 6 $\mu\text{g/ml}$) with a negative double-disk synergy test. For presumptive ESAC production, a reduced susceptibility to cefepime (MIC ≥ 0.5 $\mu\text{g/ml}$) was further added as an inclusion criterion.

ANTIMICROBIAL SUSCEPTIBILITY TESTING

Resistance to beta-lactams and non-beta-lactams was determined by the disc diffusion method according to the guidelines of the CA-SFM. *E. coli* ATCC 25922 was used as the quality control strain. The inhibitory effect of cloxacillin on AmpC production was observed on plates supplemented with 200 mg/L cloxacillin (AES Chemunex, Bruz, France). MICs to cefoxitin, ceftazidime, cefotaxime, and cefepime were determined by *E*-test (BioMérieux, Marcy l'Etoile, France) on all presumptive ESACs,

and MICs to cefoxitin, ceftazidime, cefepime, and imipenem were also determined by *E*-test on the recombinant *E. coli* clones. As *E*-tests were not available for ceftiofur, MICs to ceftiofur were determined by broth dilution method on the recombinant *E. coli* clones.

PHYLOGROUP ANALYSIS AND MOLECULAR TYPING OF *E. COLI* ISOLATES

All *E. coli* isolates with reduced susceptibility to cefepime were assigned to a phylogenetic group (A, B1, B2, or D) using the PCR described by Clermont et al. (2000). Genetic relatedness of these isolates was determined by Pulse-Field Gel Electrophoresis (PFGE) after digestion with the *BlnI* restriction enzyme. Multi-Locus Sequence Typing was performed according to the scheme described on the *E. coli* MLST website (<http://mlst.ucc.ie/mlst/dbs/Ecoli>).

CHARACTERIZATION AND SEQUENCE ANALYSIS OF THE β -LACTAMASE AND *ampC* GENES

The *bla*_{SHV}, *bla*_{TEM}, *bla*_{OXA}, and *bla*_{CTX-M} genes were searched by PCR as previously described (Shibata et al., 2006; Dierikx et al., 2010). The plasmidic AmpC *bla*_{CMY-2} gene was sought using previously published primers (Mammeri et al., 2010). Chromosomal *ampC* promoter mutations were detected by PCR and sequencing of a 271 bp amplicon encompassing the -35 box, the -10 box and the attenuator (Caroff et al., 2000). The entire *ampC* gene of *E. coli* including its own promoter sequence was analyzed by PCR and sequencing using the Int-B2 and Int-H1 primers (Mammeri et al., 2006).

CLONING OF THE *ampC* GENES

The coding region (without the promoter) of *ampC* genes from *E. coli* with a reduced susceptibility to cefepime was amplified with primers Int-B1 and Int-HN, as previously described (Mammeri et al., 2006). These 1120 bp products were cloned into pCR BluntII TOPO (Invitrogen) and the recombinant plasmids were transformed into *E. coli* strain TOP10. Transformants were selected on plates containing kanamycin (50 $\mu\text{g/ml}$) and ceftazidime (1 $\mu\text{g/ml}$). The orientation of the insert was confirmed by PCR with the SP6 and T7 primers in order to only keep transformants with the *ampC* gene under the transcriptional control of the *lacZ* promoter.

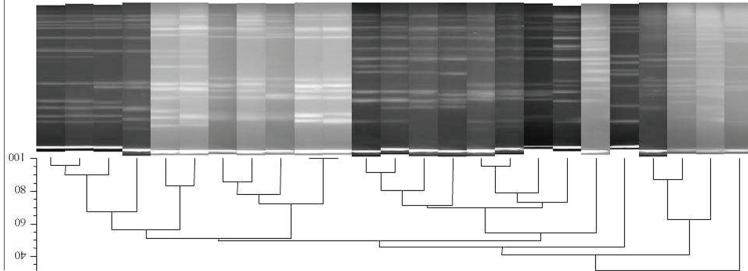
RESULTS

PREVALENCE OF PRESUMPTIVE ESAC PRODUCERS

Amongst the 6765 *E. coli* isolates studied, a total of 80 isolates (1.18%) met the criteria of AmpC over-production, of which 71 were clinical *E. coli* isolates (diarrheic calves) and 9 from healthy animals (carriage). Of them, 28 *E. coli* isolates harbored an additional reduced susceptibility to cefepime (MICs ranging from 0.5 to 12 $\mu\text{g/ml}$) and were investigated as presumptive ESACs producers (Table 1). MICs for ceftazidime for those 28 isolates ranged from 6 to 96 $\mu\text{g/ml}$, without a positive synergy test. Twenty-three presumptive ESACs producers (23/28, 82.1%) were from diseased cattle and 5 (5/28, 17.9%) were from healthy ones. None of the 28 *E. coli* had a plasmid-mediated *bla*_{CMY} gene, whereas 10 had a *bla*_{TEM-1} gene, 3 a *bla*_{OXA-1} gene, and 2 a *bla*_{CTX-M-1} gene. The 28 cattle were from different geographic

Table 1 | Characteristics of the 28 *E. coli* strains presenting a reduced susceptibility to cefepime.

Isolate	Pathology	Date	District	MIC (mg/ml)			Acquired β -lactamase	Phylogenetic group	ST (CC) ^c	<i>ampC</i> promoter mutations	Overproduced AmpC type (mutation)
				CAZ ^b	CTX	FOX					
16290	Carriage	2006	35	12	6	32	-	A	23 (88)	-88, -82, -42, -18, -1, +58	none
19023	Carriage	2007	03	64	8	48	-	A	23 (88)	-88, -82, -42, -18, -1, +31, +58	H296P
18976	Carriage	2007	57	16	4	16	1.5	A	23 (88)	-88, -82, -42, -18, -1, +58	S287N
15816	Diarrhea	2006	53	12	4	8	2	A	23 (88)	-88, -82, -42, -18, -1, +58	S287N
24288	Diarrhea	2009	71	6	3	128	2	A	23 (88)	-88, -82, -42, -18, -1, +58	none
24290	Diarrhea	2009	03	12	1.5	16	1	A	23 (88)	-88, -82, -42, -18, -1, +58	H296P
21928	Diarrhea	2008	38	32	6	16	3	A	23 (88)	-88, -82, -42, -18, -1, del ^c (II), +34, +58	H296P
23156	ND ^a	2008	16	24	6	12	1.5	A	ND (1615)	-88, -82, -42, -18, -1, del (I), +34, +58	S287N
22096	Diarrhea	2008	88	16	3	6	2	A	ND (1615)	-88, -82, -42, -18, -1, +58	H296P
25011	Diarrhea	2010	18	64	6	32	4	A	ND (1615)	-88, -82, -42, -18, -1, del (I), +34, +58	H296P
25016	Diarrhea	2010	17	48	6	16	3	A	ND (1615)	-88, -82, -42, -18, -1, del (II), +34, +58	H296P
14610	Diarrhea	2005	53	24	8	32	2	A	23 (88)	-88, -82, -42, -18, -1, +20, +58	S287N
16157	Diarrhea	2006	53	32	6	32	2	A	23 (88)	-88, -82, -42, -18, -1, +20, +58	S287N
15228	Diarrhea	2006	22	32	8	32	2	A	23 (88)	-88, -82, -42, -18, -1, +20, +58	S287N
15084	Diarrhea	2006	50	32	8	32	2	A	23 (88)	-88, -82, -42, -18, -1, +20, +58	S287N
15190	Diarrhea	2006	76	32	8	32	2	A	23 (88)	-88, -82, -42, -18, -1, +20, +58	S287N
16159	Diarrhea	2006	76	48	8	32	3	A	23 (88)	-88, -82, -42, -18, -1, +20, +58	S287N
20818	Diarrhea	2007	61	32	8	48	3	A	23 (88)	-88, -82, -42, -18, -1, +20, +58	S287N
16249	Carriage	2006	35	32	12	32	3	A	23 (88)	-88, -82, -42, -18, -1, +20, +58	S287N
22603	Diarrhea	2008	50	48	8	48	2	A	23 (88)	-88, -82, -42, -18, -1, +20, +58	S287N
20098	Diarrhea	2007	54	8	128	3	8	Bl	155 (68)	-88, -82, -42, -18, -1, +58	R309C
15166	Diarrhea	2006	54	32	6	16	4	A	ND (783)	-88, -82, -42, -18, -1, +20, +58	H296P
25456	Diarrhea	2010	50	6	24	24	3	A	ND (783)	-88, -82, -42, -18, -1, +58	none
25025	Diarrhea	2010	49	24	12	24	1.5	A	ND (783)	-88, -82, -42, -18, -1, +58	S287C
25451	Diarrhea	2010	50	64	24	128	12	D	ND (648)	-88, -82, -42, -18, -1, ins ^d (33), +22, +58	S287N
18991	Carriage	2007	35	16	12	24	1	A	ND (783)	-88, -82, -42, -18, -1, +32, +58	S287C
20819	Diarrhea	2007	61	16	12	48	0.75	A	23 (88)	-88, -82, -42, -18, -1, +23, +58	none
25448	Death	2010	50	96	8	48	6	A	23 (88)	-88, -82, -42, -18, -1, +23, +58	H296P



^aND, not determined.
^bCAZ, ceftazidime; CTX, cefotaxime; FOX, ceftoxitin; FEP, cefepime.
^cST, sequence type; CC, clonal complex.
^ddel, deletion, ins, insertion.

origin in France, and *E. coli* were genetically unrelated (using PFGE, **Table 1**). However, most of them (18/28, 64.2%, **Table 1**) were of sequence type (ST) 88 belonging to the CC23. ST783 and ST1615 were also found four times each, respectively.

MUTATIONS IN THE *ampC* PROMOTER/ATTENUATOR AND IN THE *ampC* GENE

Compared with the *E. coli* ATCC 25922, sequencing of the *ampC* promoter revealed highly conserved mutations at position -88, -82, -42, -18, -1, and +58. The number of nucleotides between the novel -35 and -10 sequences was 17 bp in all isolates. Mutations in the *ampC* gene attenuator (from +17 to +37) were frequent, particularly at position +20, but also at +23 or +34 (**Table 1**). Nucleotides deletion or insertion (at positions +1 or +33, respectively) were occasionally identified. Full sequencing of the *ampC* gene showed that amino acid substitutions of the AmpC beta-lactamase were mainly detected inside or near the H-10 helix (**Figure 1**) at positions 287 ($n = 16$; mostly S287N, but also S287C), 292 ($n = 2$, A292V) and 296 ($n = 8$; H296P), but also outside this hot spot at positions 89 ($n = 1$, A89T), 194 ($n = 1$, P194A), 215 ($n = 4$; A215V), 220 ($n = 1$; A220T), and 232 ($n = 1$, R232C) (**Table 1** and **Figure 1**). The majority of the isolates presented one mutation in the coding sequence ($n = 23$), but three isolates presented two mutations and one isolate even presented four of them. Finally, one isolate which additionally harbored a CTX-M enzyme presented no mutation of the *ampC* gene compared the control.

CHARACTERIZATION OF THE *ampC* RECOMBINANT *E. COLI* CLONES

A subset of 7 *E. coli* isolates representative of the various amino acid substitutions found alone or in combination (A215V, A220T, S287N, S287C, A292V, H296P) and one control (15829, strain susceptible to cefepime) were chosen for cloning experiments (**Table 2**). PCR products of 1120 bp corresponding to the *ampC* coding sequence without its promoter were cloned into pCR BluntII TOPO. The seven recombinant plasmids were successfully transformed into *E. coli* strain TOP10, giving rise to clones TF-15166, TF-19023, TF-15816, TF-18991, TF-25456, TF-16290, TF-20098, and TF-15829 (susceptible control). In all recombinant plasmids, the insert was proved to be under the transcriptional control of the *lacZ* promoter. MICs of beta-lactams determined for the recombinant clones showed that the S287N substitution had a greater impact on resistance than S287C, H296P, and A292V (**Table 2**). TF-15166 and TF-19023 shared the same H296P mutation, but TF-15166 additionally harbored an A215V mutation that did not further increase the resistance to cefepime (MIC = 2 mg/L vs. MIC = 4 mg/L for TF-19023). The A215V had also weak impact on the susceptibility to cefepime when tested alone (TF-25456). Isolates 20098 displayed four different mutations (A89T, P194A, A220T, R232C), which do not increase resistance to cefepime (MIC = 0.19 mg/L) and were not found alone in the other tested isolates. TF-15829 (susceptible control) showed high MICs to ceftazidime (MIC = 24 mg/L) and ceftaxime (>256 mg/L) but a similar MIC value to cefepime (MIC = 0.125 mg/L) that TF-25456 or TF-20098. No mutation in the coding sequence of the *ampC* gene was found in TF-15829 compared to the reference strain.

DISCUSSION

In this study, we report the first ESAC producers in animals worldwide. Contrary to humans (Barnaud et al., 2001; Mammeri et al., 2004b), ESACs were exclusively searched and found in the *E. coli* species. This results from the low proportion of animal infections due to other Gram negative bacteria expressing a chromosomal *ampC* gene, such as *S. marcescens*, *E. cloacae*, *E. aerogenes*, *A. baumannii*, or *P. aeruginosa* (Nordmann and Mammeri, 2007). Also, all ESAC-producing *E. coli* were from cattle as a result of our study design, and further investigations are surely needed to explore other animal sectors. The prevalence of ESAC-producing *E. coli* in cattle in France was investigated over a 6-year period of 2005–2010 and estimated at 0.37% (23/6158) in clinical isolates. Of note, this prevalence is similar to that found at a French hospital in the Paris suburb, where six ESAC producers were reported out of 2800 *E. coli* isolates (0.21%) (Mammeri et al., 2008). This prevalence is however four times higher than that found at another French hospital, where 41 AmpC-overproducing *E. coli* isolates accounted for 0.16% of all clinical *E. coli* isolates studied, of which only 11 produced ESACs (Cremet et al., 2010). However, in that study, inclusive criteria for ESAC producers included a MIC superior or equal to 64 mg/L of ceftazidime, which may have been a factor of underestimation.

Here, ESAC producers were mostly detected in diarrheic calves, which are also known as the main reservoir of ESBL genes in cattle (Madec et al., 2008, 2012; Valat et al., 2012). This may reflect the overall high antibiotic pressure with beta-lactams in infections of the enteric tract of young animals. However, a subset of 607 *E. coli* isolates from healthy cattle was also investigated, which revealed an even higher ESAC prevalence compared to diseased ones (5/607, 0.82%). This mirrors the situation in humans where ESAC producers were most likely found in *E. coli* belonging to the less pathogenic phylogroups A or B1 (Mammeri et al., 2006; Corvec et al., 2007). Moreover, the cattle ESAC-producing *E. coli* isolates predominantly belonged to the CC23, similarly to what has been reported in humans (Cremet et al., 2010). Further studies would be needed to understand why this evolution of the chromosomal *ampC* gene of *E. coli* seems restricted to the same genetic lineages in humans and animals despite different antimicrobial pressures and a rather host-specific distribution of the *E. coli* clones.

This study also provides an overview of 23 *ampC* promoter/attenuator sequences in ceftaxime-resistant *E. coli* from cattle. Contrary to humans, reports on mutations in the *ampC* promoter/attenuator regions of *E. coli* from animal sources are scarce (Brinas et al., 2002; Guillouzoic et al., 2009). The polymorphism at positions -88, -82, -42, -18, -1, +58 found in strong *ampC* promoters (Olsson et al., 1983; Guillouzoic et al., 2009) was highly conserved in our collection. In particular, this includes the -42 mutation shown to increase *ampC* gene expression (Nelson and Elisha, 1999; Caroff et al., 2000), and the association of the -42 and -18 changes, which creates two displaced new -35 and -10 boxes with an optimal 17-pb spacer region. The -18 mutation without the -42 mutation was rare (one isolate only) but had already been reported, in particular in *E. coli* isolates which do not over-express their chromosomal AmpC (Brinas et al., 2005). Other reported changes were not detected here, such as mutations -32, -11, or +6 (Caroff et al.,

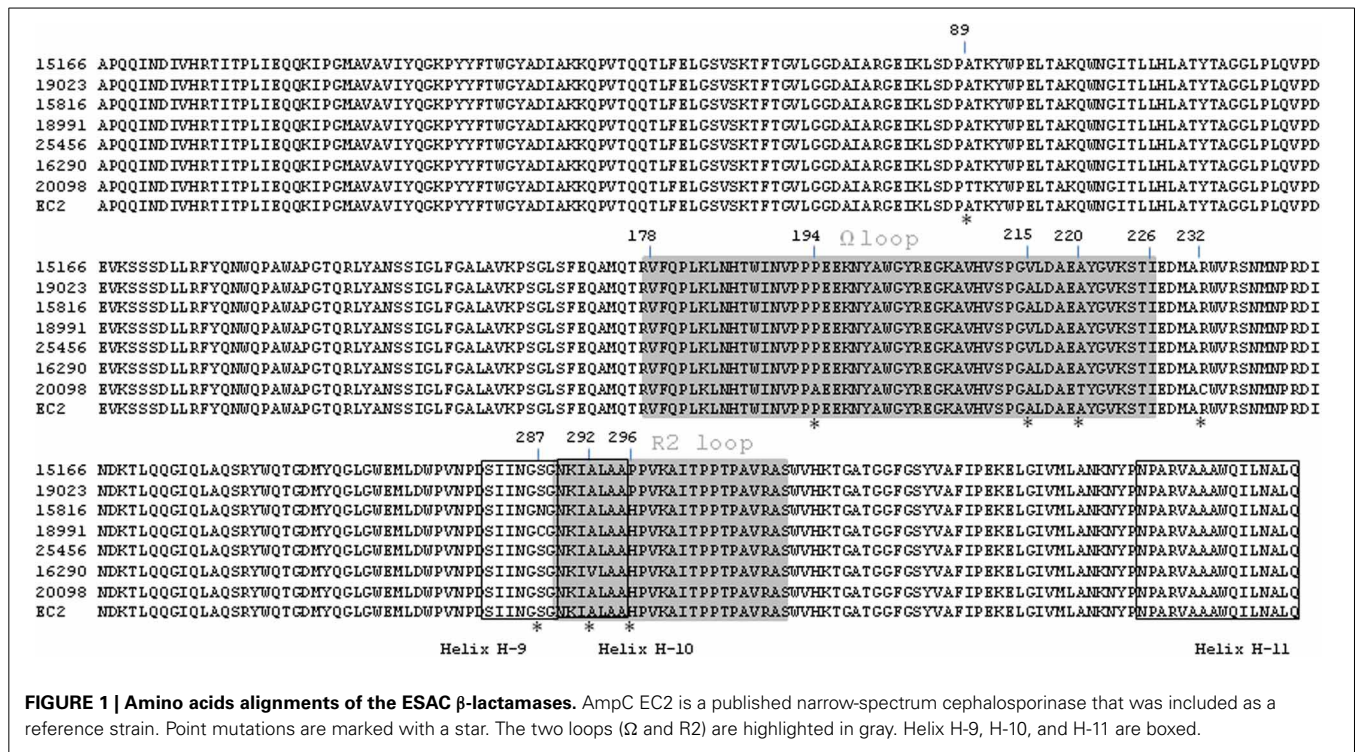


FIGURE 1 | Amino acids alignments of the ESAC β-lactamases. AmpC EC2 is a published narrow-spectrum cephalosporinase that was included as a reference strain. Point mutations are marked with a star. The two loops (Ω and R2) are highlighted in gray. Helix H-9, H-10, and H-11 are boxed.

Table 2 | Characteristics of the 7 *E. coli* transformants representative of the strains presenting a reduced susceptibility to cefepime.

Isolate	Date	MIC (mg/L) in transformants					Mutations in the <i>ampC</i> sequence
		CAZ ^a	FOX	FEP	IMI	XNL	
REDUCED SUSCEPTIBILITY TO CEFEPIME							
TF-15166	2006	48	24	2	0.25	16	H296P, A215V
TF-19023	2007	>256	32	4	0.25	32	H296P
TF-15816	2006	>256	64	8	0.38	128	S287N
TF-18991	2007	96	32	1	0.25	64	S287C, A215V
TF-25456	2010	16	>256	0.125	0.25	32	A215V
TF-16290	2006	32	34	0.38	0.125	16	A292V
TF-20098	2007	8	48	0.19	0.5	16	A89T, P194A, A220T, R232C
SUSCEPTIBLE CONTROLS							
TF-15829	2006	24	>256	0.125	0.25	64	none
TOP 10	–	0.19	4	0.023	0.19	1	none

^aCAZ, ceftazidime; FOX, cefoxitin; FEP, cefepime; IMI, imipenem; XNL, ceftiofur.

2000; Corvec et al., 2002; Mulvey et al., 2005; Tracx et al., 2007). However, these mutations might be more frequent in *E. coli* isolates of B2 or D phylogroups, which are not well represented here (Mammeri et al., 2008). The deletion of the entire attenuator was not observed either (Tracx et al., 2005).

In this study, all relevant amino acid replacements found in the *ampC* gene (namely S287N, S287C, A292V, and H296P) were single substitutions, confirming that cumulating substitutions in the catalytic site is not a preferential evolutionary scheme for AmpCs, contrary to class A beta-lactamases, as previously demonstrated (Le Turnier et al., 2009). A new A215V replacement was reported here in four *E. coli* isolates, either as a single

or an associated mutation. This mutation, which occurs close to the DAEX motif in the Ω loop, has obviously little if no impact on the cefepime susceptibility. Indeed, in the corresponding native isolates, reduced susceptibility to cefepime may result from decreased permeability and/or oxacillinase production. Also, in the recombinant clones, the wild-type *ampC* gene of the cefepime susceptible control conferred the same MIC to cefepime than the A215V replacement. The main structural alteration found was the S287N replacement, which occurred in the H-9 helix close to the R2 loop. This mirrors the situation in humans where the S287N substitution has been shown to widely contribute to the ESAC phenotype (Mammeri et al., 2006). Similarly, other substitutions

were also found, such as the S287C mutation, or both the H296P or the A292V mutations inside the R2 loop. Comparison of MICs of cefepime also confirmed that the S287N substitution has greater impact on resistance than the others (Mammeri et al., 2006).

Altogether this study is, to our best knowledge, the very first report of ESACs in animals. In humans, those enzymes have likely evolved from wild-type AmpCs by modifications in the vicinity of the R1 or R2 active sites. While the ESACs described here strongly resemble the human ones in both their genetic backgrounds and the responsible mutations, the driving forces for the selection of ESACs in animals are still unknown. In humans, the ESAC phenotype frequently occurs after cefepime therapy. On the contrary, different ESCs are used in animals, whose possible impact needs to be considered. In particular, ceftiofur is an ESC which is widely used in animals, and elevated MICs values to ceftiofur were found here in the recombinant *E. coli* clones. Hence, the use of veterinary cephalosporins in the selection of ESAC producers in animals might be a plausible hypothesis. Of note, most human and animal ESAC *E. coli* reported so far belong to the CC23—a well-adapted *E. coli* clone in the two hosts –, so that the transfer of ESAC producers from humans to animals may have contributed as well. Alternately, this may result from a favorable genetic background of the *ampC* genes in *E. coli* isolates of phylogroup A, irrespective of their human or animal origin (Mammeri AAC 2009). Even though data in humans report similar ranges of ESACs vs. plasmidic AmpCs (Mammeri et al., 2008), the true prevalence of ESACs in animals should be further investigated since this emerging mechanism surely contributes to the global burden of resistance to ESCs in animals.

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