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Short communication: Extended-spectrum AmpC–producing *Escherichia coli* from milk and feces in dairy farms in Brazil

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ABSTRACT

The AmpC enzyme normally is expressed constitutively in *Escherichia coli*, and its overproduction confers resistance to cefoxitin. A newly reported AmpC, the extended-spectrum AmpC (ESAC), is related to resistance to cefepime, a fourth-generation cephalosporin. This enzyme presents more flexibility in the active site due to insertions, replacements, and deletions on AA sequences. Many isolates producing ESAC were reported in human clinical isolates, but *E. coli* ESAC producers were reported in animals only in France. The animal *E. coli* strains can produce this enzyme and possibly disseminate it to human and production environments. In our study, 3 strains of *E. coli* from milk and feces bovine samples, collected in Rio de Janeiro, Brazil, were suspected to produce ESAC. After excluding other mechanisms of resistance, the gene was sequenced to verify ESAC characteristics. These strains presented replacement of AA in omega and R2 loops, suggesting ESAC production. This is the first report to study ESAC *E. coli* in dairy farms in Brazil.
Key words: AmpC, cefepime, chromosomal gene

Short Communication

The AmpC enzyme is able to hydrolyze β-lactams and clavulanic acid, an inhibitor of extended-spectrum β-lactamase (**ESBL**), but normally cannot hydrolyze fourth-generation cephalosporins. Cefoxitin is used to differentiate AmpC from other β-lactamase production in Enterobacteriaceae (Jacoby, 2009; Haenni et al., 2014; Al-Bayssari et al., 2015). However, extended-spectrum AmpC (**ESAC**) has alarmed health organizations worldwide because it can cause difficulty in human treatments (Rodríguez-Martínez et al., 2012; Pires et al., 2015). Naturally, AmpC is expressed constitutively at a low level in *Escherichia coli* and confers resistance to cefoxitin only when it is overproduced, in the case of ESAC production or plasmidic AmpC genes (Peter-Getzlaff et al., 2011). The production of ESAC was first detected in human *Enterobacter cloacae* in 1992. It is characterized by resistance to cefepime, ceftazidime, cefotaxime, and aztreonam, and some hydrolytic activity has been observed on imipenem (Nukaga et al., 1995). Insertions, replacements, or deletions on the 10 or 11 helix, R2 loop, or omega loop can modify the spectrum of enzymes (Barnaud et al., 2001; Mammeri et al., 2004; Nordmann and Mammeri, 2007). These alterations in the AmpC gene lead to conformational changes responsible for more flexibility in the region of the enzyme active site, thereby affecting hydrolytic activity (Nordmann and Mammeri, 2007; Jørgensen et al., 2010). The dissemination of this resistant *E. coli* may occur between animals and humans by direct transmission or through the food chain (e.g., consumption of unpasteurized cheese; Dierikx et al., 2013; Hammerum et al., 2014; García-Cobos et al., 2015; Wohlwend et al., 2015). *Escherichia coli* ESAC producers from animal samples were detected in a herd in France (Haenni et al., 2014); there are no data about this strain in production animals in Brazil.

Ten dairy herds from Rio de Janeiro and 20 dairy herds from Mato Grosso, Brazil, were visited between 2009 and 2015 using ethical standards parameters approved by the ethics committee and institutional biosafety committee (protocol no. CEUA-3664040915, Federal Rural University of Rio de Janeiro, Brazil). A total of 185 and 41 *E. coli* strains were obtained from fecal and milk bovine samples, respectively. All isolates were identified by routine biochemical tests and matrix-assisted laser desorption/ionization time-of-flight MS assay (Rodrigues et al., 2017; Santiago, 2017). The disk diffusion using amoxicillin, amoxicillin-clavulanic acid, cefoxitin, ceftazidime, cefotaxime, cefepime, aztreonam,

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Table 1. *Escherichia coli* from milk and feces suspected to produce extended-spectrum AmpC (ESAC) and controls used in this study

Strain[1]	Sample/origin	Relevant properties	Source
F8	Feces/Rio de Janeiro	Cefoxitin and cefepim resistance, ESAC suspect	This study
F21	Feces/Rio de Janeiro	Cefoxitin and cefepim resistance, ESAC suspect	This study
N18	Milk/Rio de Janeiro	Cefoxitin and cefepim resistance, ESAC suspect	This study
ATCC[2] 25922	—	Cefoxitin susceptible, control strain	ATCC

[1]The nucleotide sequences F8, F21, and N18 have been submitted to the GenBank nucleotide database and have been assigned accession numbers MF361841, MF361842, and MF361843, respectively.

[2]American Type Culture Collection (Manassas, VA).

and imipenem antibiotics was performed on all 226 *E. coli* strains. The suspected production of ESAC in these isolates was based on cefoxitin and cefepime susceptibly testing according to the Clinical and Laboratory Standards Institute (CLSI, 2013, 2014). To exclude ESBL producers, the synergism between amoxicillin-clavulanic acid and third-generation cephalosporins was screened in these strains. The *ampC* plasmidic genes were studied by PCR (Pérez-Pérez and Hanson, 2002) and by conjugation in suspected ESAC isolates to observe the ability of the isolates to transmit the gene to other bacteria (Gonçalves, 2008). To detect the mutation in *ampC* genes and confirm the production of ESAC, the entire coding region of *ampC* was amplified with the primers IntB2 (5′-TTCCTGATGATCGTTCTGCC-3′) and IntHN (5′-AAAAGCGGAGAAAAGGTCCG-3′), yielding a 1,315-bp amplification product (Mammeri et al., 2006). The PCR reactions were performed with a final volume of 25 μL containing 20 m*M* Tris-HCl (pH 8.4), 50 m*M* KCl, 0.2 m*M* deoxynucleoside triphosphates, 1.5 m*M* $MgCl_2$, and 0.6 μ*M* each primer; 1.25 U of Taq DNA polymerase (Kapa Biosystems, Fermentas, Brazil) and template DNA (2 μL) were added to 23 μL of the master mixture. The PCR program consisted of an initial denaturation step at 95°C for 5 min followed by 30 cycles of 95°C for 1 min, primer annealing at 60°C for 1 min, and primer extension at 72°C for 1 min. After the last cycle, a final extension step at 72°C for 5 min was performed using a T100 Thermal Cycler (Bio-Rad, São Paulo, Brazil). The amplification products were separated by electrophoresis on 2% agarose gel (Bio-Rad) stained with SYBR Green (Invitrogen, São Paulo, Brazil) and visualized using the photo documentation system L-PIX EX (Loccus Biotecnologia, Cotia, Brazil). The products were purified with Exo-Sap (USB 39 Corp., Cleveland, OH), and the sequencing was performed in ABI 3130xl (Applied Biosystems, São Paulo, Brazil) and edited by DNA Sequence Assembler version 4 (Heracle BioSoft, Arges, Romania) and Mega software version 7 (Caspermeyer, 2016). *Escherichia coli* ATCC 25922 was used as the control in this study.

Three strains of *Escherichia coli* from milk (N18) and feces (F8, F21) presented resistance to cefoxitin and cefepime (Table 1). Curiously, these strains were collected from the same farm in Rio de Janeiro in the winter of 2014. Cefoxitin and cefepime resistance is observed in *E. coli* producing ESAC or coproducing plasmidial AmpC and ESBL (Nordmann and Mammeri, 2007; Jørgensen et al., 2010). To elucidate this, the strains were submitted to conjugation and PCR. The conjugation was evaluated first to detect conjugative AmpC genes, and transconjugants were not found. The PCR excluded the possibility of plasmidial AmpC in these *E. coli*. After disk diffusion test and interpretative test with third- and fourth-generation cephalosporins and amoxicillin-clavulanic acid disks, no strain demonstrated synergism between β-lactam and enzyme inhibitors, also excluding the possibility of ESBL producers (CLSI, 2017). Finally, *ampC* gene sequences were evaluated to confirm the suspicion of ESAC. *Escherichia coli* ATCC 25922 was used as a negative control to ESAC to compare *ampC* gene sequences from strains.

Mammeri et al. (2004, 2008), Sohn et al. (2008), and Bogaerts et al. (2015) reported replacements in the *E. coli AmpC* gene codifying a different enzyme in human strains. These replacements cause AA substitution in R2, H-9 (close to the R2 loop), and H-10 of AmpC, leading to conformational changes on the enzyme. These loops are considered a catalytic site of AmpC (Mammeri et al., 2004, 2008; Haenni et al., 2014). These modifications cause conformational and flexibility changes in AmpC. Thus, the enzyme can hydrolyze fourth-generation cephalosporins. Only one report describes *E. coli* from animals presenting mainly substitutions in the H-9 helix in the 287 position, causing ESAC production (Haenni et al., 2014). Other substitutions were described by Haenni et al. (2014) but were not related to enzyme modification.

In our study, the relevant AA replacements were in the omega and R2 loops of ESAC suspected strains (Figure 1). All 3 strains showed substitution in the 191(omega), 209 (omega), and 300 (R2) positions. The omega loop is an important region for enzymes, and 2 substitutions on the omega loop were observed in our study. The first substitution was in the 191 position, and the other was in the 209 position. In the 191 position lysine was sub-