

**Detection and characterization of extended-spectrum-betalactamases (ESBL) producing *Escherichia coli* in animals**

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## Abstract

The detection of multi-drug resistant bacteria is a growing problem, however, the role of domesticated animals in the propagation of antimicrobial resistance has barely been studied. The aim of this study was to identify ESBL-producing *Escherichia coli* strains in domestic animal feces in order to assess their antimicrobial resistance profile and carry out molecular characterization of the  $\beta$ -lactamases. A total of 325 samples were collected from 8 animal species. Of these, 34 bacterial isolates were identified as *E. coli*. The antibiotic resistance profile of the *E. coli* strains was as follows: 100% resistant to amoxicillin, aztreonam, and cephalosporines; 58.8% resistant to nalidixic acid, ciprofloxacin and trimethoprim/sulphamethoxazole; 41.2% resistant to gentamicin and tobramycin; 11.8% resistant and 32.4% intermediate to cefoxitin, 97.1% sensible and 2.9% intermediate to amoxicillin/clavulanate; and 100% sensible to ertapenem, minocycline, imipenem, meropenem, amikacin, nitrofurantoin, phosphomycin and colistin. All 34 *E. coli* strains met criteria for ESBL production. In total, 46  $\beta$ -lactamase genes were detected: 43.5% *bla*<sub>TEM</sub>, 30.4% *bla*<sub>CTX-M</sub> (23.9% *bla*<sub>CTX-M-1</sub> and 6.5% *bla*<sub>CTX-M-9</sub>) and 26.1% *bla*<sub>SHV</sub> (17.4% *bla*<sub>SHV-5</sub> and 8.7% *bla*<sub>SHV-12</sub>). All the  $\beta$ -lactamases were found in dogs except for 4 *bla*<sub>SHV</sub> found in falcons. No pAmpC genes were found. The high prevalence of ESBL-producing *E. coli* strains in animals could become a zoonotic transmission vector.

**Keywords:** ESBL;  $\beta$ -lactamases; Antimicrobial resistance; *Escherichia coli*;  
Domesticated animal

The increasing detection of multi-drug resistant bacteria is a growing problem, having become a real threat for the public health worldwide. Having bacteria become resistant to antibiotics implies that standard treatments are no longer effective, which make infections harder to control, increasing their morbidity as well as their mortality. The problem ahead is such that it threatens the achievements of modern medicine, making the idea of reaching a post-antimicrobial era in the 21<sup>st</sup> century possible (Suay-Garcia *et al.* 2014).

The role of domestic animals in the propagation of antimicrobial resistances is yet to be determined, possibly due to the fact that the administration of antibiotics in these animals is not as regulated as that of cattle (Guardabassi *et al.* 2004). This is a relevant fact, seeing as many of the antibiotics used in humans are also being used in animal therapy. Out of all the antimicrobial agents used by veterinarians to treat bacterial infections,  $\beta$ -lactams are the most frequently prescribed due to their wide therapeutic range, their pharmacokinetics and their broad spectrum of activity against pathogens (Prescott 2008). Furthermore, it is important to highlight that certain animals, such as dogs and cats, have frequent contact with humans and, therefore, may represent an important transmission vector of bacteria, their gut microbiota being a reservoir of  $\beta$ -lactam resistance genes (Hunter *et al.* 2010).

The main aim of this study was the detection and identification of ESBL or plasmidic AmpC (pAmpC)  $\beta$ -lactamase-producing *E.coli* in domesticated animal feces. Furthermore, the antimicrobial resistance profile of all isolated strains was assessed. Finally, the molecular characterization of these  $\beta$ -lactamases was carried out.

## **Materials and methods**

### **Sample collection**

A total of 325 fecal samples were randomly collected from different species of healthy animals with frequent human contact: 140 dogs, 46 cats, 35 monkeys, 35 horses, 25 sheep, 20 goats, 12 falcons and 12 pigeons. The samples were collected in different locations throughout Spain from February 2014 to February 2015, in veterinary diagnostics laboratories.

The Ethics and Animal Welfare Committee of the Universidad Cardenal Herrera-CEU approved this study. All animals were handled according to the principles of animal care published by Spanish Royal Decree 1201/2005 (BOE, 2005; BOE = Official Spanish State Gazette). The animal owners gave permission to take samples.

### **Bacterial Identification**

All samples were plated on selective and differential media (MacConkey and ChromID ESBL®) (Paterson *et al.* 2005). After 24/48h of incubation at 35-37°C, bacterial growth was determined along with colony colours.

Bacterial identification was verified by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS, Burker Daltonics Inc. Billerica, MA). Measurements were performed with Burker Microflex LT MALDI-TOF MS (Burker Daltonics Inc, Billerica, MA) using Flex Control software and a 60Hz nitrogen laser (337nm wavelength).

### **Antimicrobial resistance profile and phenotypic tests of *Escherichia coli* strains**

Susceptibility of the isolated strains identified as *E. coli* was assessed against

amoxicillin (AMX), aztreonam (AZT), cephalothin (CEF), ceftazidime (CAZ), cefotaxime (CTX), cefoperazone (CFP), cefoxitin (FOX), cefuroxime (CXM), nalidixic acid (NAL), ciprofloxacin (CIP), cotrimoxazole (SXT), gentamicin (GEN), tobramycin (TOB), amikacin (AMK), amoxicillin/clavulanic acid (AMC), colistin (COL), erythromycin (ERY), fosfomycin (FOS), imipenem (IMI), meropenem (MER), minocycline (MIN), and nitrofurantoin (NFT) using a commercial broth microdilution method (Wider panels, Soria-Melguizo, Spain). The criteria used for sensibility interpretation were those established by EUCAST (2013). Phenotypic tests using cefotaxime with/without clavulanate/cloxacillin disk-diffusion (Rosco Diagnostics, Denmark) and E-test (BioMérieux, France) were also performed.

### **Molecular characterization of $\beta$ -lactamases**

Once the strains that were possibly producing ESBL and/or pAmpC were detected and identified, their characterization was carried out using multiplex PCR. To identify the  $\beta$ -lactamase genes detected in the multiplex PCR assays, DNA sequence analysis of the amplicons was performed (Dallene *et al.* 2010). Firstly, bacterial DNA was extracted heating a colony suspended in 25 $\mu$ L of distilled water (99°C for 10min) to later centrifuge it, collecting the supernatant. That DNA was subduced to two multiplex PCR. The first one (Dallene *et al.*, 2010) was used to detect the following types of ESBL genes: *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>OXA-1</sub>, *bla*<sub>CTX-M</sub>. The process was carried out as follows: the extracted DNA (2 $\mu$ L) was added to a reaction mixture containing PCR buffer 1x (10mM Tris-HCl, 50mM KCl and 1.5mM MgCl<sub>2</sub>), dNTPs (200 $\mu$ M), primers (variable concentration) and *Taq* polymerase (1U). The total reaction volume was of 50 $\mu$ L. The amplification process consisted in an initial denaturalization at 94°C during 10min; 30 cycles at 94°C during 40s, 60°C during 40s and 72°C during 1min; and a final extension at 75°C during 7min.

In order to characterize the different types of pAmpC  $\beta$ -lactamases, a second multiplex PCR (Sundin 2009) was carried out for *bla*<sub>MOX</sub>, *bla*<sub>CIT</sub>, *bla*<sub>DHA</sub>, *bla*<sub>ACC</sub>, *bla*<sub>MIR-ACT</sub> and *bla*<sub>FOX</sub>. For this PCR, mixers with a total volume of 50 $\mu$ L containing: DNA (2 $\mu$ L), PCR buffer 1x (20mM Tris-HCl, 50mM KCl, 1.5mM MgCl<sub>2</sub>), dNTPs (0.2mM), primers (variable concentrations) and *Taq* polymerase (1.25U) were prepared. In this case, the process of amplification consisted in an initial denaturalization at 94°C during 3min followed by 25 cycles of denaturalization at 94°C during 3min, hybridization at 64°C during 30s and extension at 72°C during 1min to finish with a final elongation at 72°C during 7min.

All the PCRs were carried out in a 2720 Applied Biosystems thermocycler (Thermo Fisher Scientific, California, USA). Both, positive (well-characterized  $\beta$ -lactamases-producing strains) and negative (distilled water) controls were included in all series. Finally, the amplicons were sequenced to confirm their identification.

## **Results**

A total of 34 *E. coli* strains from healthy animals (25 dogs, 8 falcons and 1 monkey) were isolated and studied. The antimicrobial resistance profile of these 34 isolated *E. coli* strains was as follows: 100% resistant to amoxicillin, aztreonam, cefalothin, ceftazidime, cefotaxime, cefoperazone and cefuroxime; 58.8% resistant to nalidixic acid, ciprofloxacin and trimethoprim/sulphamethoxazole; 41.2% resistant to gentamicin and tobramycin; 11.8% resistant and 32.4% intermediate to ceftiofur, 97.1% susceptible and 2.9% intermediate to amoxicillin/clavulanate and 100% susceptible to erythromycin, minocycline, imipenem, meropenem, amikacin, nitrofurantoin, fosfomicin and colistin. All 34 *E. coli* strains met criteria for ESBL production.

In regard to the molecular characterization of the  $\beta$ -lactamases produced by the 34 isolated *E. coli* strains, we were able to identify ESBL in 29 (85.3%) of them. Of these 29 strains with ESBL (Table 1), *bla*<sub>TEM-21</sub> and *bla*<sub>CTX-M-1</sub> were detected in 6 (17.6%) strains; *bla*<sub>TEM-52</sub> and *bla*<sub>CTX-M-1</sub> in 5 (14.7%) strains; *bla*<sub>SHV-5</sub> in 5 (14.7%) strains; *bla*<sub>SHV-5</sub> and *bla*<sub>TEM-21</sub> in 3 (8.8%) strains; *bla*<sub>TEM-52</sub> and *bla*<sub>CTX-M-9</sub> in 3 (8.8%) strains; *bla*<sub>TEM-52</sub> in 3 (8.8%) strains and *bla*<sub>SHV-12</sub> in 4 (11.7%). In total, 46  $\beta$ -lactamases genes were detected in this study (Figure 1), of which 43.5% were *bla*<sub>TEM</sub> (19.6% *bla*<sub>TEM-21</sub> and 23.9% *bla*<sub>TEM-52</sub>), 30.4% *bla*<sub>CTX-M</sub> (23.9% *bla*<sub>CTX-M-1</sub> and 6.5% *bla*<sub>CTX-M-9</sub>) and 26.1% were *bla*<sub>SHV</sub> (17.4% *bla*<sub>SHV-5</sub> and 8.7% *bla*<sub>SHV-12</sub>). All  $\beta$ -lactamases were found in dogs except for 4 *bla*<sub>SHV-12</sub> found in falcons. No pAmpC genes were found.

As for the antibiotic resistance profile in relation to the detected  $\beta$ -lactamases, table 1 shows that most of the *E. coli* strains resistant to at least 7 antibiotics were detected in dogs. The presence of the following combinations of  $\beta$ -lactamase genes: *bla*<sub>TEM-52</sub>/*bla*<sub>CTX-M-9</sub> (8.8%), *bla*<sub>TEM-21</sub>/*bla*<sub>CTX-M-1</sub> (17.6%) and *bla*<sub>TEM-52</sub>/*bla*<sub>CTX-M-1</sub> (14.7%), has been detected in the *E. coli* strains that presented resistance to a higher number of antibiotics, 13 (AMX, AZT, CEF, CAZ, CTX, CFP, FOX, CXM, NAL, CIP, SXT, GEN and TOB) and 12 (AMX, AZT, CEF, CAZ, CTX, CFP, CXM, NAL, CIP, SXT, GEN and TOB) antibiotics, respectively, as it is specified in Table 1.

Table 2 compiles individual characteristics such as animal from which the sample was collected, antibiotic susceptibility MICs (Minimum Inhibitory Concentration) and ESBLs identified.

## **Discussion**

To our knowledge, this is the first study reporting the presence of ESBL-producing *E. coli* in falcons. Similar studies focused on wild birds such as those of Alcalá *et al.* (2016) and Parker *et al.* (2016) reported prevalence rates of 14% and 2.7% respectively. Coincidentally, both studies found *E. coli* strains containing *bla*<sub>SHV</sub>. Further studies should be carried out in order to determine the origin of these strains.

The study is focused on the molecular characterization of *E. coli* strains because ESBL production is the main mechanism of resistance to  $\beta$ -lactams in this species. This fact is corroborated by studies such as that of Mosquito *et al.* (2012), in which, after analyzing 369 samples of *E. coli* from pediatric patients with diarrhea, it was observed that the production of *bla*<sub>TEM</sub> represented the main mechanism of resistance against  $\beta$ -lactams (31%).

When carrying out the antimicrobial resistance profile of all 34 strains of *E. coli* isolated in this study, it was observed that 100% were resistant to amoxicillin, aztreonam, cephalothin (1<sup>st</sup> generation), cefuroxime (2<sup>nd</sup> generation), ceftazidime (3<sup>rd</sup> generation), cefotaxime (3<sup>rd</sup> generation) and cefepime (4<sup>th</sup> generation), which indicates that all of them are resistant to monobactams and cephalosporins belonging to all generations. This is a disturbing fact, seeing as some of these compounds, such as ceftazidime, were considered strategic molecules within the therapeutic array in case of bacterial infections, used exclusively in the hospital setting. The existence of bacteria with animal origin resistant to these compounds strengthens the idea that veterinarians might be making an indiscriminate use of antibiotics, without taking into account the impact this could have in the public health.



On the other hand, most ESBL-producing *E. coli* strains were susceptible to amoxicillin/clavulanate, which is consistent with the results obtained in a similar study by Rodrigues *et al.* (2002) in which, out of 104 strains of *E. coli* studied, only 1.9% were found to be resistant to amoxicillin/clavulanate. This fact is encouraging seeing as this combination is one of the most widely used in clinical practice, both in veterinary and human medicine.

According to the National Committee for Clinical Laboratory Standards (2003) it is recommended to investigate systematically the production of ESBL in any isolate of *Klebsiella* or *E. coli* that presents resistance to aztreonam, ceftazidime, ceftriaxone or cefotaxime. Three of these antibiotics, aztreonam, ceftazidime and cefotaxime, were included in the resistance profile of the strains isolated in this study and, as it was mentioned earlier, all 34 strains of *E. coli* showed resistance, which is why we proceeded to the molecular characterization of ESBLs in all of them.

The first case of an ESBL-producing *E. coli* strain from animal origin was detected in Spain in the year 2000 when analyzing a sample of a dog with recurring chronic cystitis, having found *bla*<sub>SHV-12</sub> (Teshager *et al.* 2000). It was only a few years later when Carattoli *et al.* (2005) detected the first cases of *bla*<sub>CTX-M</sub> producing bacteria, exactly *bla*<sub>CTX-M-1</sub>, in dogs and cats with and without pathology. As it was previously mentioned, the only genes detected were *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub> and *bla*<sub>CTX-M</sub>, which is not surprising seeing as other studies (Harada *et al.* 2011, Costa *et al.* 2008, Moreno *et al.* 2008) performed on animals with no pathology showed similar results. A study (Harada *et al.* 2011) performed in Japan isolated *bla*<sub>SHV-12</sub> producing *E. coli* strains in healthy puppies in two different breeding grounds. Likewise, two other studies performed in Portugal (Costa *et al.* 2008) and Chile (Moreno *et al.* 2008) identified *E. coli* strains producing *bla*<sub>CTX-M-1</sub> and *bla*<sub>CTX-M-9</sub> in fecal samples of

healthy dogs that had previously received antibiotic treatment.

The  $\beta$ -lactamase gene most frequently identified in this study has been *bla*<sub>TEM</sub>, representing a 43.5% of the total. As it was mentioned by Philippon et al. (1989) type *bla*<sub>TEM</sub>  $\beta$ -lactamases are responsible for most of the resistances to  $\beta$ -lactam antibiotics in enterobacteria.

*bla*<sub>CTX-M</sub> was the second most frequently detected  $\beta$ -lactamase, being present in 30.4% of the ESBL-producing *E. coli* strains. This was expected seeing as, since the year 2000, the presence of this ESBL has increased exponentially, with cases of *E. coli* strains producing *bla*<sub>CTX-M</sub> being detected in Europe, Asia, Africa and America, reaching a point at which the scientific community is considering it “the *bla*<sub>CTX-M</sub> pandemic” (Cantón *et al.* 2006). Even though this ESBL is predominantly found in *E. coli*, *bla*<sub>CTX-M</sub> has also been detected in other enterobacteria, having become the most extended and detected type of ESBL worldwide (Pitout *et al.* 2008). Moreover, the relevance of the presence of this  $\beta$ -lactamase is corroborated by similar studies, such as those of Schmiedel *et al.* (2014) and Schink *et al.* (2013). Both studies, carried out in Germany, analyzed fecal samples from dogs, cats and horses and concluded that *bla*<sub>CTX-M-1</sub> was a predominant subtype in animals with prevalence rates of 25.8% and 81.48% respectively.

Interestingly, out of the 8 animal species studied, ESBLs were only detected in dogs and falcons, whereas none were found in animals such as, horses, sheep, monkeys or goats which, while domesticated, have less contact with their owners. Along these lines, it is surprising that no ESBL-producing bacteria were found in cats, seeing as similar studies on domestic animals did report these strains in cat pets (Boagerts *et al.* 2015).

The increasing number of studies about the prevalence of ESBL-producing bacteria in domestic animals is contributing to a rise in the awareness of the scientific community in relation to what could become a serious public health issue. The potential zoonotic transmission of ESBL-producing bacteria is corroborated by studies such as that conducted by Rocha et al. (2015) in which, feces of healthy domestic dogs were analyzed, having found ESBL-producing *E. coli* strains in 6% of the samples. Furthermore, a study (Meyer *et al.* 2012) carried out on 231 volunteers in 2011 analyzing possible risk factors for colonization with ESBL-producing *E. coli* concluded that contact with pets increases by almost seven-fold the chance of being colonized.

### **Conclusions**

The results obtained in this study show that the animal feces analyzed presented a high prevalence of ESBL-producing *E. coli*. This fact could become a serious threat for the public health seeing as the presence of these microorganisms in pets should be considered a possible zoonotic transmission vector. Moreover, all the *E. coli* strains studied were resistant to, at least, 7 of the antibiotics studied, which reinforces the idea that there is a real need to implement control methods over veterinary antibiotic prescriptions in domestic animals. Finally, it is important to emphasize that this is the first report of ESBL-producing *E. coli* strains in falcons, suggesting that more studies should be carried out in order to determine its prevalence and the transmission route by which these falcons could have acquired drug-resistant bacteria.

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## **Author Disclosure Statement**

No competing financial interests exist.

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**Figure 1.**  $\beta$ -lactamase genes characterized from the isolated *E. coli* strains.

