Antimicrobial resistance among canine enteric *Escherichia coli* isolates and prevalence of attaching-effacing and extraintestinal pathogenic virulence factors in Spain

Running title: EPEC, ExPEC and MDR *E. coli* in dogs

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Abstract

The aim of this study was to estimate the prevalence of antimicrobial resistance (AMR) in *Escherichia coli* from a dog population in Spain and assess specific virulence factors. Susceptibility to 22 antimicrobials was tested along with the production of extended-spectrum β-lactamases (ESBLs) and AmpC in faecal isolates from 100 dogs. Virulence-related genes associated with attaching and effacing *E. coli* (*eae*, *Stx1*, *Stx2*) and extraintestinal pathogenic *E. coli* –ExPEC- (*papC*, *hlyA* and *cnf1*) were detected by PCR. At least one kind of AMR was observed in 73% of the isolates. The highest prevalences corresponded to penicillin (45%), aminoglycoside (40%), and non-extended spectrum cephalosporin (39%) classes. Multidrug resistance (MDR) was observed in 53.4% of the resistant isolates. No resistance to colistin was found. Production of ESBL/AmpC enzymes was detected in 5% of *E. coli*. Shiga toxin-producing *E. coli* were not observed, enteropathogenic *E. coli* were identified in only 12% of them, and ExPEC were found in 25%. Dog faeces can be a source of *E. coli* strains potentially presenting a threat to humans through their virulence factors or AMR. The non-hygienic keeping of animals may increase the risk of colonisation of such pathogens in humans.

Key words: Dogs, *Escherichia coli*, Drug resistance, Virulence factors, Spain

Introduction

Antimicrobial resistance (AMR) is a world-wide problem of great concern. High levels of AMR have been increasingly observed, including a rise in resistant bacteria from companion animals (Marques et al., 2018). *Escherichia coli* is considered an important member of the intestinal microbiota of a wide variety of animal species. Because of its genetic plasticity, commensal character and ubiquity, it is regarded as an important source of resistance genes, and therefore a useful indicator of AMR (Szmolka and Nagy, 2013).

Pet animals may be considered a potential reservoir of AMR due to the extensive use of antimicrobials in dog and cats and the close contact between them and humans (Pomba et al., 2017). In fact, it has been shown as resistant *E. coli* strains from companion animals were clonally related to human strains, suggesting a bidirectional transmission (Ewers et al., 2010; Platell et al., 2011). Besides, a recent study reported that contact with dogs (or dog faeces) was associated with an increased risk of urinary tract infections (UTI) in humans caused by multidrug resistant (MDR) *E. coli* (Ukah et al., 2018). Consequently, the role that companion animals play in the dissemination of AMR should be of concern.

Pathogenic *E. coli* can cause either enteric or extraintestinal disease through the acquisition of several virulence genes. In dogs, two main pathotypes have been associated with enteric disease, namely, enterotoxigenic *E. coli* (ETEC) and attaching and effacing *E. coli* (AEEC) (DebRoy and Maddox, 2001). The latter is characterized by the presence of the pathogenicity island termed LEE (locus of enterocyte effacement) and includes enteropathogenic *E. coli* (EPEC) and Shiga toxin-producing *E. coli* (STEC). EPEC and STEC represent potential causes of diarrhoea in dogs and both have been reported to occur in healthy and diarrhoeic dogs (Beutin, 1999).

Apart from enteric pathotypes, extraintestinal pathogenic *E. coli* (ExPEC), characterized by specific adhesins and toxins, are considered the most common cause of UTI in dogs (Thompson et al., 2011). Canine faeces may be an important source of ExPEC (Johnson et al., 2001a). Several studies have shown pathotypic and phylogenetic similarities between canine and human *E. coli* isolates, including AEEC and ExPEC, suggesting their zoonotic potential (Johnson et al., 2001b; Nakazato et al., 2004; Osugui et al., 2014).

Thus, the aim of this study was to characterize *E. coli* isolates from a dog population from Spain with regard to AMR patterns and virulence factors associated with the AEEC and ExPEC pathotypes, both scarcely described in companion animals in this country.

Materials and methods

Sample collection

Canine faecal samples were collected by private veterinary practitioners in Spain on a voluntary basis between 2012 and 2017. Samples belonged either to healthy dogs on admission to the hospital for routine clinical examination (checkups, etc.), or to dogs presenting digestive disorders (i.e. diarrhoea) according to the attending practitioner. Samples were taken before any antibiotic treatment was established. A total of 100 canine faecal samples were analysed, 50 from each group. Data regarding date of sampling, gender, breed, age and location were collected. Considering the geographical distribution of the samples, they were grouped in three major geographical locations: northwest (NW), northeast (NE) and Centre/South (CS) of Spain.

E. coli isolation

Faecal specimens were collected using rectal sterile swabs and immediately refrigerated and submitted to the Laboratory of Microbiology, Faculty of Veterinary Medicine at the University of Zaragoza (Spain). Samples were enriched in Buffered Peptone Water (Panreac, Barcelona, Spain) and incubated aerobically at 37°C for 24 h. After enrichment, samples were inoculated on MacConkey agar plates (Panreac), at 37°C for 24 h. Three identical colonies with typical *E. coli* appearance were selected from each sample and tested for Gram staining and indole production. Once *E. coli* was confirmed, colonies were stored at -30°C until further analysis.

Antimicrobial susceptibility testing

One *E. coli* strain from each faecal sample was tested against a total of 22 antimicrobial agents grouped in 15 antimicrobial classes (Table 1 and Figure 1) and selected based on their frequent use in clinical practice or because of its importance in human medicine, i.e. ceftriaxone, ciprofloxacin, colistin and imipenem.

Susceptibility testing was performed by the Kirby-Bauer disk diffusion method, being each isolate classified as susceptible, intermediate or resistant (CLSI, 2017). Isolates presenting intermediate susceptibility results were categorized as resistant for statistical analysis. Since the disk diffusion method is not recommended for colistin (http://www.eucast.org/), the Minimum Inhibitory Concentration (MIC) was determined in this case by the broth microdilution method (ISO 20776-1:2006) and the interpretive breakpoint value of >2 mg/L was applied. Phenotypic detection of extended-spectrum β–lactamase (ESBL) and AmpC production was investigated through the Total ESBL + AmpC Confirm kit (Rosco Diagnostica, Taastrup Denmark), and results interpreted following manufacturer's instructions. *E. coli* ATCC 25922 was used as a reference strain in all performed assays.

A MDR isolate was considered as one displaying resistance to at least one agent in three or more antimicrobial classes. AMR levels (rare, low, moderate, very high and extremely high) were defined according to EFSA and ECDC (2019). A summary measure for AMR describing the percentage of resistance (PR) to all antimicrobial agents was calculated as described by Poppe et al. (2001).

Virulence genes detection

The same *E. coli* strains tested for AMR were screened for the presence of virulence-related genes, including those specific of AEEC, i.e. *eae* (intimin), *Stx1* (Shiga toxin 1) and *Stx2* (Shiga toxin2), and some of the most prevalent ones associated with ExPEC in dog and human strains, i.e. *papC* (P fimbriae assembly), *hlyA* (α-haemolysin) and *cnf1* (cytotoxic necrotizing factor type 1) (Osugui et al., 2014).

DNA was extracted by boiling, and conventional PCR was used for detection of the virulence factors as described elsewhere (Olsvik and Strockbine, 1993; Blanco et al., 1997; Oswald et al., 2000). Positive *E. coli* controls used were CECT 4783 (eae+, VT1+, VT2+) and C136b (Hly+, CNF1+), kindly provided by Dr. J.A. Orden, University Complutense of Madrid, Spain. One of our strains (Pe8) displayed a positive amplification for *papC* (GenBank accession number MK034302) and was used as control for further analysis.

Statistical analysis

Basic prevalence estimates with their 95% Confidence Intervals (95% CI) were calculated. Simple comparisons among categories within a factor (i.e. gender, age, geographical origin, etc.) were made using the Fisher's exact test. The Mantel–Haenszel *Chi*-square test was used to assess potential trends (i.e. age). A difference was considered statistically significant for a *P*-

value ≤0.05. All the analyses were performed using MedCalc v. 18.10 (MedCalc, Ostend, Belgium).

Results

Antimicrobial resistance (AMR)

Overall, *E. coli* strains displaying phenotypic resistance to at least one antimicrobial were detected in 73% (95%CI: 63.6-80.7) of the isolates. AMR to at least one antimicrobial was more common in healthy dogs compared to diseased dogs (86% *vs*. 60%; *P*=0.006). AMR levels were high for ampicillin (45%), followed by cephalothin (39%), streptomycin (37%), sulfamethoxazole-trimethoprim (26%) and tetracycline (25%). No AMR was observed against imipenem, fosfomycin, amikacin and colistin (Table 1). Occurrence of ESBL and/or AmpC production was detected in 5% (95%CI: 2.1-11.1) of isolates, of which three possessed ESBL, one was AmpC positive and another isolate produced ESBL+AmpC. None of these five isolates was neither EPEC nor ExPEC, but all were isolated from diseased dogs.

The prevalence of AMR according to antimicrobial classes is presented in Figure 1. AMR was more prevalent for penicillin (45%), aminoglycoside (40%), non-extended spectrum cephalosporin (39%), sulphonamide and pyrimidine (26%) and tetracycline (25%) classes. Among the resistant strains, 53.4% (95%CI: 42.1-64.4) displayed MDR. Susceptibility to all antimicrobials was found in 27% (19.3-36.4) of the *E. coli* strains.

Among the numerous MDR profiles identified the most common ones were A-S-Su-T and A-NSC-S (10.3% each of them), followed by A-S-Na-Su-T (7.7%). Interestingly, within the group of resistant *E. coli* isolates from diarrhoeic dogs the proportion of MDR strains was significantly higher than that in the group coming from healthy dogs (76.7% *vs*. 37.2%; *P*=0.001). The most

common profiles in MDR isolates from diseased dogs were A-S-Su-T (17.4%) and A-S-Na-Su-T (13%).

The percentage of resistance (PR) to the antimicrobial agents tested for the whole population of dogs was 12.1% (95%CI: 7.1-19.9). This value was slightly higher, but not statistically significant, for the group of diseased dogs (14.9%, 95%CI: 7.5-27.2) compared to the healthy ones (9.4%, 95%CI: 3.9-20.5).

Virulence factors in *E. coli* isolates

Attaching and effacing *E. coli* (AEEC)

The *eae* gene that characterizes EPEC was detected only in 12% (95%CI: 7-19.8) of the isolates, but its presence was not associated with dogs with digestive disorders (10% *vs*. 14% in healthy dogs; P=0.54). EPEC was neither related to other factors except age and geographical location (Table 2). The prevalence of EPEC decreased somewhat as age increased (χ^2(for trends) =3.36; P=0.06). Although only 6 dogs originated from Centre-South Spain, the prevalence of EPEC was significantly higher in this group compared to dogs from North (East and West) of Spain (50% *vs*. 10.6%; P=0.03), after controlling by age and breed. No significant differences were observed with regard to prevalence of MDR between EPEC and non-EPEC strains (25% *vs*. 40.9%; P=0.29).

None of the 100 *E. coli* strains analysed carried any of the Shiga toxin genes (*Stx1* and *Stx2*), thus, no STEC were present in this study.

Extraintestinal pathogenic *E. coli* (ExPEC)

188 Strains harbouring at least one of the studied extraintestinal virulence-related genes were
189 considered ExPEC (Osugui et al., 2014). They were more commonly detected (25%; 95%CI:
190 17.5%-34.3%) than EPEC. The *papC* gene was identified in 24% of the canine isolates, *hlyA*
191 gene was found in 19%, and the *cnf1* gene in 18%. Overall, no significant associations were
192 observed between ExPEC and the factors considered (Table 2), but *cnf1* gene was more
193 commonly detected in *E. coli* strains from healthy dogs than in those from dogs with diarrhoea
194 (26% *vs*. 5%; *P*=0.042). Within the ExPEC isolates, 68% (17/25) encoded simultaneously the
195 three studied virulence factors related to this pathotype, and 70% of them belonged to healthy
196 dogs. Among the ExPEC isolates, a total of six (24%) were classified as MDR, with five of
197 them being isolated from healthy dogs. MDR strains were somewhat more common in non-
198 ExPEC compared to ExPEC strains (44% *vs*. 24%; *P*=0.08).
199
200 **Discussion**
201 This work analysed a collection of faecal samples from a population of diseased (i.e. evidence
202 of diarrhoea) and healthy dogs collected by practitioners from several locations of Spain and
203 voluntarily submitted to our laboratory. Thus, although the design of the study precludes
204 considering the results representative of the dog population in Spain, it may provide useful
205 information on AMR in *E. coli* from this animal species as well as on two pathotypes scarcely
206 studied in this country. Despite the interest of using *E. coli* as indicator bacteria, there are few
207 descriptive studies on AMR among enteric *E. coli* strains collected from dogs in Spain and, in
208 particular, on AMR against critically important antibiotics in human medicine such as colistin,
209 or on the occurrence of ESBL/AmpC-producing *E. coli*.
210
211 Overall, a high AMR prevalence was observed as only 27% (95%CI: 19.3-36.4) of the isolates
212 were susceptible to all antimicrobials tested. This figure appeared to be slightly lower than the

overall prevalence of full susceptibility observed in a previous study (43.2%; 95%CI: 27.3-59.2) performed in Spain on *E. coli* isolated between 2008 and 2013 from dogs and cats with UTI (Marques et al., 2016). AMR was more prevalent for the following antimicrobial classes: penicillins (45%), aminoglycosides (40%), non-extended spectrum cephalosporins (39%), sulphonamide and pyrimidine (26%) and tetracyclines (25%), and MDR was observed in 39% (95%CI: 30-48.8) of the isolates, which was also somewhat higher than Marques´ study. However, comparison between both studies is impaired by the different study design, since in the latter study the number of isolates (around 50) and the antimicrobial classes tested (5) were smaller, which may explain the lower overall level of resistance.

AMR was also higher than that in other studies where only populations of healthy dogs were considered (Costa et al., 2008; Wedley et al., 2011). However, when these figures were compared to those from studies carried out on dogs visiting veterinary hospitals, then the AMR prevalence was similar (Thungrat et al., 2015; Wedley et al., 2017) or even lower (Leite-Martins et al., 2014), reflecting the likely impact of antibiotic treatments on the development of AMR. In any case, the high level of AMR in *E. coli* from dogs was in accordance with the overall higher resistance frequencies found in European Southern countries (Marques et al., 2016). The fact that overall sales of antimicrobial agents for veterinary use in Spain are the highest among those of European countries (EMA, 2018) may have contributed to this situation.

None of the isolates was resistant to colistin, a last-resort antibiotic against MDR Gram-negative bacteria in humans, suggesting that plasmid-mediated colistin resistance genes (*mcr*) had not yet spread on these dogs despite they were present in other type of samples (i.e. food-producing animals and sewage water) at that time in Spain (Carattoli et al., 2017; Ovejero et al., 2017). In contrast, ESBLs and AmpC β-lactamases were detected in 5% of the *E. coli*

strains, all of them coming from diseased dogs. Bacteria producing ESBL/AmpC enzymes are usually resistant to third generation cephalosporins, which are critically important antimicrobials in human medicine (Paterson and Bonomo, 2005). Thus, to prevent the spread of this type of resistance, a careful selection of antibiotics should be carried out by practitioners when facing to diarrhoeic dogs.

A great proportion of isolates from healthy dog (86%) showed resistance to at least one antimicrobial, which was significantly higher than that for the group of diseased dogs (60%; P=0.006). In addition, no associations were observed between AMR or MDR and the pathotypes included in this study. Although it may be a link between resistant *E. coli* and virulence (da Silva and Mendonça, 2012), it seemed that, in this study, AMR may be more associated with other, likely commensal, *E. coli* that may have acquired resistance genes from elsewhere (Szmolka and Nagy, 2013). However, it would be required to search for other pathotypes, such as ETEC, before reaching any conclusion on this matter.

When only the group of resistant *E. coli* isolates was considered, MDR was significantly more prevalent in those coming from diarrhoeic dogs compared to those from healthy dogs (76.7% *vs*. 37.2%; P=0.001). This result was supported by a somewhat higher PR value for the group of diseased dogs (14.9% *vs*. 9.4% in the healthy group). Resistance genes are usually included within genetic mobile elements, such as plasmids, which may also carry virulence determinants (Carattoli, 2013). It is likely that MDR *E. coli* harbours either a greater number of plasmids or larger plasmids, and therefore presenting a higher probability of carrying genes of virulence, other than those considered in this study.

According to the assessment of virulence factors, the *eae* gene that characterizes EPEC was found in 12% (95%CI: 7%-19.8%) of them, a prevalence similar to that observed in other studies (Nakazato et al., 2004; Puño-Sarmiento et al., 2013), but a relationship between EPEC and diarrhoea could not be observed. Indeed, 14% and 10% of the isolates from healthy and diarrhoeic individuals, respectively, were characterized as EPEC, showing even a somewhat higher proportion of EPEC+ within the group of healthy dogs. Although the prevalence of EPEC in diarrhoeic dogs is rather variable, EPEC may represent a significant cause of diarrhoea in this animal species (Nakazato et al., 2004; Puño-Sarmiento et al., 2013). Since EPEC causes diarrhoea mostly in young animals (Beutin, 1999), our results may be likely biased by the low proportion of young (<4 months old) dogs in this study (11.8% among dogs with a known age).

STEC have been isolated from dog faeces but, in general, their presence is usually low, i.e. ≤6%, in healthy dogs (Sancak et al., 2004; Puño-Sarmiento et al., 2013), even when they live close to STEC-infected cattle (Hancock et al., 1998). Although their role in canine diarrhoea is not yet well known, some studies report a significant higher prevalence in dogs with acute or chronic diarrhoea (Sancak et al., 2004). In the present study STEC could not be found neither in diarrhoeic or healthy dogs, which was in line with the low prevalence observed in previous studies and suggested the limited importance of this pathotype in this population.

With regard to virulence factors linked to ExPEC, in 25% (95%CI: 17.5%-34.3%) of this dog population at least one of the ExPEC-related genes was detected, being the *papC* gene the most prevalent (24%). This prevalence was rather consistent with that observed in other studies based on faecal canine isolates (Mateus et al., 2013; Tramuta et al., 2014). Virulence factors associated with ExPEC are not as well defined as those related to enteric pathotypes, since very different combinations of virulence factors have been described in strains causing similar

pathologies (Bélanger et al., 2011). The assessment of other extraintestinal virulence factors in this analysis, such as CNF2 or CDT (cytolethal distending toxin), could have hence contributed to know a more detailed virulence repertoire of these isolates. Nevertheless, the three virulence factors included in this study have been found in *E. coli* strains causing extraintestinal disease in both human and companion animals (Johnson et al., 2001b). Veterinarians should be aware of the high proportion of ExPEC-positive dogs in this population and their potential as a reservoir of this pathotype for humans (Johnson et al., 2001a). No association was found between ExPEC and digestive disorders in these dogs, but neither with dogs presenting clinical signs compatible with UTI. Indeed, 68% of the ExPEC isolates displayed the three extraintestinal virulence-related genes in combination, and most of them (70%) belonged to healthy dogs. Since *E. coli* strains may be host-specific with regard to their ability to cause a disease (Ewers et al., 2007), it could be possible these strains had a non-canine origin, and perhaps a human origin (Johnson et al., 2008; Platell et al., 2011). The three extraintestinal virulence factors appeared together in most of the ExPEC isolates, which was in line with the likely presence of a possible pathogenicity island (Diard et al., 2010).

In conclusion, MDR was widely distributed among *E. coli* isolates from this population of dogs, and therefore dogs may be regarded as important carriers of AMR. Some isolates from diarrhoeic dogs showed resistance to critically important antibiotics (i.e. ceftiofur, ceftriaxone and ciprofloxacin) and some also produced ESBLs and AmpC β-lactamases. Practitioners should be aware of this type of resistance to prevent its further spread. Practical guidelines on antimicrobial use and AMR testing are advised for the treatment of companion animals. Regarding pathotypes, EPEC was present in an expected frequency, but was not associated with gastrointestinal disorders. ExPEC was more common, suggesting that faeces from both healthy and diarrhoeic dogs may constitute a relevant reservoir of *papC*, *hlyA*, and *cnf1* genes.

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Conflict of interest

No competing interest.

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465 **Table 1.** Prevalence of antimicrobial resistance in *E. coli* isolates and categorization of AR

466 according to EFSA levels.

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469 **Table 2.** Distribution of EPEC and ExPEC strains among factors analysed.

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472 **Figure 1.** Percentage of resistant *E. coli* isolates according to antimicrobial class categorization.