ORIGINAL ARTICLE

Raccoons (Procyon lotor) in the Madrid region of Spain are carriers of antimicrobial-resistant Escherichia coli and enteropathogenic E. coli

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Abstract
The role of wildlife in the epidemiology of antimicrobial resistance is unclear. Raccoons in North America can carry a variety of enteric bacteria, with associated antimicrobial resistance, that could infect humans and livestock. The potential for raccoons to carry these bacteria in Europe, where they are an invasive species, has not been explored. Our objectives were to determine the prevalence of Escherichia coli with associated antimicrobial resistance in raccoons from the Madrid region of Spain and to determine whether they are carriers of potential human pathogens, including verotoxin-producing E. coli (VTEC) and enteropathogenic E. coli (EPEC). In total, we tested 237 E. coli isolates from the faeces of 83 euthanized raccoons for susceptibility to 14 antimicrobial agents and the presence of VTEC and EPEC. Antimicrobial resistance to at least one antimicrobial was detected in the faeces of 51% (42/83; 95% CI, 40.1–61.1) of the raccoons tested. A high percentage of raccoons carried, in their faeces, E. coli isolates resistant to ampicillin (33%), streptomycin (33%), tetracycline (30%), sulphafurazole (31%) and trimethoprim-sulphamethoxazole (23%). We detected one isolate of extended-spectrum β-lactamase-producing E. coli from the faeces of one raccoon. We detected VTEC in the faeces of 83 euthanized raccoons for susceptibility to 14 antimicrobial agents and the presence of VTEC and EPEC. Antimicrobial resistance to at least one antimicrobial was detected in the faeces of 51% (42/83; 95% CI, 40.1–61.1) of the raccoons tested. A high percentage of raccoons carried, in their faeces, E. coli isolates resistant to ampicillin (33%), streptomycin (33%), tetracycline (30%), sulphafurazole (31%) and trimethoprim-sulphamethoxazole (23%). We detected one isolate of extended-spectrum β-lactamase-producing E. coli from the faeces of one raccoon. We detected VTEC in the faeces of one raccoon, and EPEC in the faeces of 12% (10/83) of the raccoons. Of the raccoons that carried EPEC in their faeces, 60% (6/10) carried EPEC isolates that exhibited characteristics associated with pathogenicity in humans. Raccoons in Madrid can carry pathogenic and antimicrobial-resistant E. coli in their faeces and may be a risk to public health because of their potential to contaminate food and the environment with their faeces.

KEYWORDS
antimicrobial resistance, enteropathogenic E. coli, Escherichia coli, raccoons, verotoxin-producing E. coli
Antimicrobial resistance (AMR) inhibits the successful treatment of microbial infections in humans and livestock (Swift et al., 2019) and is a concern to human and veterinary medicine worldwide (Hofer, 2019). Antimicrobial resistance has become a complex ecological problem (McEwen & Collignon, 2018), and the role of wildlife in the epidemiology of AMR is unclear (Dolejska & Literak, 2019; Vittecoq et al., 2016). Antimicrobial-resistant enteric bacteria (e.g., Escherichia coli, Salmonella and Enterococcus) have been detected in faecal bacteria from a variety of wildlife, including wild birds and mammals; the occurrence and transmission of AMR in wildlife have been recently reviewed (Greig et al., 2015; Vittecoq et al., 2016; Wang et al., 2017). Although wildlife are not normally exposed to clinical antimicrobial agents, they may be exposed to resistant bacteria in the environment through natural and anthropogenic sources (Swift et al., 2019). Wildlife may acquire antimicrobial-resistant bacteria through direct or indirect contact with anthropogenic sources such as livestock and human waste, hospital effluent and pet faeces (Radhouani et al., 2014; Swift et al., 2019). They may also be exposed to bacteria with intrinsic resistance (Swift et al., 2019) from a natural source, such as the soil (D’Costa et al., 2011).

Once colonized with antimicrobial-resistant bacteria, wildlife have the potential to disseminate resistant bacterial pathogens and associated genetic determinants into the environment through their faeces (Wang et al., 2017). Humans and livestock may become infected with antimicrobial-resistant bacteria after consuming food and water that has been contaminated with wildlife faeces (Greig et al., 2015). E. coli is the most frequently studied bacteria in AMR studies of wildlife (Greig et al., 2015). E. coli is a commensal bacteria in the intestinal tracts of humans and many animals, and may constitute a reservoir of resistance genes for pathogenic bacteria (van den Bogard & Stobberingh, 2000). Extended-spectrum beta-lactamas (ESBL)-encoding genes, which confer resistance to many of the beta-lactams commonly used in human and veterinary medicine, can easily be transferred among different bacterial strains and co-selected with genes conferring resistance to other antimicrobial classes (Alonso et al., 2016; EFSA Panel on Biological Hazards (BIOHAZ), 2011). The genes of EBSL-producing E. coli (EBSL-EC) have been detected in several species of wild birds and mammals (Guenther et al., 2011; Wang et al., 2017), and their occurrence in wildlife has suggested to be from exposure to anthropogenic sources (Atterby et al., 2017).

Fewer studies have investigated the potential of wildlife to carry strains of E. coli that have the potential to be human pathogens, including verotoxin-producing E. coli (VTEC) and enteropathogenic E. coli (EPEC). Verotoxin-producing E. coli produce verotoxins, also known as Shiga toxins, and cause illnesses ranging from mild diarrhoea to haemorrhagic colitis and haemolytic uraemic syndrome in humans. Domestic ruminants, mainly cattle, have been implicated as the principal reservoir of VTEC infections in humans (Nataro & Kaper, 1998). In wildlife, VTEC have been detected in a variety of wildlife species, including wild ruminants, boars and birds (Alonso et al., 2017). It has been suggested that some wildlife species may play an important role in the maintenance of VTEC (Mora et al., 2012).

Enteropathogenic E. coli (EPEC) cause diarrhoea in humans and young domestic animal species (Holland, 1990; Nataro & Kaper, 1998). EPEC strains do not produce VT, but produce intimin, a protein which is encoded by the eae gene and involved in the production of attaching and effacing (A/E) lesions in the intestines (Nataro & Kaper, 1998). Typical EPEC (tEPEC, possessing the bfpA gene) are primarily found in humans, whereas atypical EPEC (aEPEC, lacking the bfpA gene) have been detected in humans, domestic animals and wildlife, including wild ruminants, boars and birds (Alonso et al., 2017; Blanco, Blanco, Dahbi, Mora, et al., 2006; Horcajo et al., 2012). The occurrence of tEPEC in wildlife has been associated with exposure to anthropogenic sources (Alonso et al., 2017). Raccoons (Procyon lotor) in North America can carry E. coli with associated antimicrobial resistance and enteric pathogens (e.g., Salmonella and Campylobacter) in their faeces (Bondo et al., 2016a, 2016b; Mutschall et al., 2020). Raccoons in North America have omnivorous diets (Rulison et al., 2012), can travel as far 45 km (Rosatte et al., 2010), can occur in high population densities in urban and suburban areas (37–94 raccoons/km²; Broadfoot et al., 2001), and often live in close association with humans and domestic animals (Prange et al., 2003), all of which have been suggested to increase the potential of a wildlife species to acquire and disseminate antimicrobial-resistant bacteria throughout the environment (Vittecoq et al., 2016). Raccoon populations that are non-native have become established in Alaska, the Antilles, Japan and Europe (Garcia et al., 2012; Hagiwara et al., 2009). Borna disease virus, Yersinia, Campylobacter and antimicrobial-resistant isolates of Salmonella have been detected in the faeces of raccoons in Japan (Hagiwara et al., 2009; Lee et al., 2011). A variety of zoonotic agents (viruses, protozoa, parasitic...
worns and mites) have been detected in raccoons from Germany (Stope, 2019). The potential of free-ranging raccoons in Europe to carry enteric pathogens and antimicrobial-resistant bacteria has not been explored.

Raccoons in the Madrid region of Spain have become widely distributed and live in close association with humans in a variety of habitats, including periurban areas and natural parks (García et al., 2012). These populations of raccoons were identified to be a potential ecological and public health risk (García et al., 2012), and urgent actions to control and eradicate the animals were recommended (García et al., 2012). After raccoons were included in a list of invasive alien species of European Union concern in 2016 (The European Commission, 2016), the government of the Madrid region authorized a control programme that involved the capture, removal and euthanasia of raccoons. This provided a unique opportunity to determine the prevalence of antimicrobial-resistant \( E. \) coli, VTEC and EPEC isolates carried by raccoons in the Madrid region of Spain and to characterize the isolates, so that the serotypes, phylogenetic groups and sequence types could be compared to those found in humans.

2 | MATERIALS AND METHODS

2.1 | Animals

The animal handling procedures were approved by the Forest and Wildlife Protection Area of the Community of Madrid, Spain (references 10/005505.9/17, 10/394116.9/17 and 10/008413.9/19), in accordance with law RD 630/2013 regarding invasive alien species in Spain. From October 2017 to March 2019, trapping sessions were carried out at nine sites in primarily periurban areas in the Madrid region, along the rivers Jarama and Henares, in a 225 km\(^2\) triangle with the limits framed by the vertex of 40°33′32.2″N 3°33′51.7″W and 40°19′13.8″N 3°30′42.5″W. In the areas studied, there are a low number of livestock or poultry farms and some of the areas are located in densely populated areas. The distance between sites ranged from 6 to 30 km. Capture of raccoons, animal handling, including euthanasia, and sampling were performed by Terra Naturalis and the Centro de Recuperación de Animales Silvestres de la Comunidad de Madrid (CRAS Madrid) as part of the control programme for raccoons in the Madrid region. The trapping method used in this study has been described previously (García et al., 2012). Briefly, trapping was conducted using homemade collapsible wire boxes constructed of galvanized wire mesh. Traps were set on major animal trails where signs of raccoon activity were found (e.g. tracks, latrines) and where raccoons had been previously sighted. Following capture, raccoons were weighted and then euthanized by veterinarians from the regional administration. Immediately after euthanasia, whole faecal samples were collected directly from the rectum. Samples were then placed in sterile plastic bottles and kept refrigerated until submitted to the laboratory the day after sampling.

2.2 | Isolation of \( E. \) coli

Faecal samples were plated on MacConkey agar to isolate \( E. \) coli. After overnight incubation, three colonies having the typical appearance of \( E. \) coli were randomly selected from each sample. Isolates were identified as \( E. \) coli by biochemical tests and mass spectrometry (Bruker Daltonik MALDI Biotyper).

2.3 | Antimicrobial susceptibility of \( E. \) coli

Antimicrobial testing was performed using the disc diffusion method and according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2019). The growth inhibition area of each isolate was measured, and then each isolate was classified as susceptible, intermediate or resistant based on the breakpoints provided by the CLSI (2019: Table S1). The following 14 antimicrobials belonging to 6 different classes were tested: ampicillin, amoxicillin-clavulanic acid, cefotaxim and ceftriaxime (β-lactams); streptomycin, kanamycin, amikacin and gentamicin (aminoglycosides); tetracycline (tetracyclines); chloramphenicol (phenicols); sulphafurazole and trimethoprim-sulphamethoxazole (inhibitors of the folic acid pathway); and nalidixic acid and ciprofloxacin (quinolones). All antimicrobial susceptibility discs were provided by Oxoid, Thermo Fisher Scientific. \( E. \) coli ATCC 25922 was used as a control strain. Isolates of EBSL-EC were detected using PCR and sequencing for the \( \text{bla}_{\text{TEM}}, \text{bla}_{\text{SHV}} \) and \( \text{bla}_{\text{CTX-M}} \) genes. The specific primers to sequence these genes have been described previously (Mamani et al., 2019).

2.4 | Detection and characterization of VTEC and EPEC isolates

All of the \( E. \) coli isolates were tested using PCR for the presence of the vt1, vt2 and eae genes (Table S2). The isolates that tested positive for the vt and eae genes were subsequently tested using PCR for other virulence genes associated with diarrhoeagenic and extraintestinal pathotypes of \( E. \) coli (Table S2). The intimin types of the eae-positive isolates were determined by sequencing as previously described (Blanco, Blanco, Dahbi, Mora, et al., 2006; Horcajo et al., 2012). Isolates positive to vt were classified as VTEC. Isolates positive to eae but negative to vt and bfpA were classified as aEPEC. Isolate positive to eae and bfpA but negative to vt were classified as tEPEC.

2.5 | ExPEC and UPEC status

The antimicrobial-resistant \( E. \) coli, VTEC and EPEC isolates were designated presumptively as extraintestinal pathogenic \( E. \) coli (ExPEC) if positive for ≥2 of 5 markers, including \( \text{papAH} \) and/or \( \text{papC}, \text{sfa}/\text{focDE}, \text{afa/draBC}, \text{kpsMII} \) and \( \text{iutA} \) (Johnson et al., 2015), or as uropathogenic
**E. coli** (UPEC) if positive for ≥3 of 4 markers, including *chuA*, *fyuA*, *vat* and *yfcV* (Spurbeck et al., 2012).

### 2.6 Molecular typing

ESBL-producing *E. coli*, VTEC, EPEC, ExPEC and UPEC isolates were serotyped using the agglutination method as described previously (Blanco, Blanco, Dahbi, Alonso, et al., 2006) with all available O (O1–O185) and H (H1–H56) antisera. The phylogenetic groups (A, B1, B2, C, D, E and F), sequence types (STs) and clonotypes were determined as described previously (Mamani et al., 2019).

### 3 RESULTS

#### 3.1 Isolation and antimicrobial susceptibility of *E. coli*

One faecal sample was collected from each of 83 raccoons (46 male and 37 female) that appeared clinical healthy upon capture during 2017–2019 (17 raccoons in 2017, 51 in 2018 and 15 in 2019). *E. coli* was detected in all of the raccoon faecal samples: altogether, 237 isolates were identified as *E. coli*. Antimicrobial-resistant *E. coli* was detected in 51% (42/83; 95% CI, 40.1–61.1) of the faecal samples from raccoons. Isolates resistant to at least one antimicrobial were detected in both sex of raccoons (22 male and 20 female), in each of the 3 years of sampling (6 raccoons in 2017, 26 in 2018 and 10 in 2019), and in seven out of nine sampled sites. Resistance to ampicillin, streptomycin, tetracycline, sulphafurazole and trimethoprim-sulphamethoxazole were the most common at the animal and isolate levels (Table 1).

Resistance to at least one antimicrobial was detected in 28% (66/237) of *E. coli* isolates. Multiclass resistance was detected in the faeces of 36% (30/83) of raccoons and 20% (47/237) of *E. coli* isolates: 18 isolates were resistant to 2 antimicrobial classes, 8 isolates to 3 antimicrobial classes, 5 isolates to 4 antimicrobial classes, 15 isolates to 5 antimicrobial classes and 1 isolate to 6 antimicrobial classes.

One ESBL-EC isolate was detected in the faeces from one male raccoon. This isolate was resistant to ceftriaxone and positive for the *bla*<sub>SHV-12</sub> gene, belonged to serotype ONT:H14 and to the new ST: *adk*-6, *fumC*-4, *gyrB*-5, *icd*-41, *mdh*-30, *purA*-8 and *recA*-2, but did not belong to the VTEC, EPEC, ExPEC or UPEC pathotypes.

#### 3.2 Detection and characterization of VTEC and EPEC isolates

Verotoxin-producing *E. coli* were detected in the faeces of one of 83 raccoons (Table 2). This raccoon was a female and carried three VTEC isolates, which all belonged to the O8:H19 serotype (Table 2). Sixteen EPEC isolates were detected in 12% (10/83) of the faecal samples of raccoons (Table 2). The EPEC isolates belonged to nine different O:H serotypes and exhibited six different intimin types (Table 2). EPEC isolates were detected in both sex of raccoons (six male and four female), in each of the 3 years of sampling (one raccoon in 2017, six in 2018 and three in 2019), and in six out of nine sampled sites. Fourteen of the 16 EPEC isolates were classified as aEPEC, and two as tEPEC (Table 2). Atypical EPEC isolates were detected in the faeces of nine raccoons and tEPEC isolates in the faeces of one raccoon (Table 2).

**TABLE 1** Number (percentage) of *Escherichia coli* isolates from the faeces of 83 raccoons in the Madrid region of Spain that were susceptible (S), intermediate (I) and resistant (R) to 14 antimicrobial agents

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Isolate level (n = 237)</th>
<th>Animal level (n = 83)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S (I%)</td>
<td>I (I%)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>162 (68.4)</td>
<td>35 (14.8)</td>
</tr>
<tr>
<td>Amoxicillin-clavulanic acid</td>
<td>198 (83.5)</td>
<td>28 (11.8)</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>236 (99.6)</td>
<td>1 (0.4)</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>228 (96.2)</td>
<td>8 (3.4)</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>148 (62.4)</td>
<td>53 (22.4)</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>233 (98.3)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Amikacin</td>
<td>235 (99.2)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>234 (98.8)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>199 (84.0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>223 (94.1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Sulphafurazole</td>
<td>134 (56.5)</td>
<td>68 (28.7)</td>
</tr>
<tr>
<td>Trimethoprim-sulphamethoxazole</td>
<td>210 (88.6)</td>
<td>1 (0.4)</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>221 (93.2)</td>
<td>4 (1.7)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>230 (97.0)</td>
<td>4 (1.7)</td>
</tr>
</tbody>
</table>
### Table 2: Characteristics of VTEC and EPEC isolates detected in the faeces of 1 out of 83 and 10 out of 83 raccoons, respectively, in the Madrid region of Spain

<table>
<thead>
<tr>
<th>Isolate type</th>
<th>Serotype</th>
<th>No. of isolates/no. of animals</th>
<th>vt type</th>
<th>Intimin type</th>
<th>Phylogenetic group</th>
<th>ST</th>
<th>Clonotype</th>
<th>UPEC</th>
<th>Resistance phenotype (no. of isolates)</th>
<th>Intermediate resistance phenotype (no. of isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VTEC</td>
<td>O8:H19</td>
<td>3/1</td>
<td>vt2</td>
<td>–</td>
<td>B1</td>
<td>ST201</td>
<td>CH65-32</td>
<td>–</td>
<td>–</td>
<td>SF (3)</td>
</tr>
<tr>
<td>aEPEC</td>
<td>O15:H18</td>
<td>1/1</td>
<td>–</td>
<td>NT</td>
<td>D</td>
<td>ST69</td>
<td>CH35-27</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>aEPEC</td>
<td>O21:H21b</td>
<td>3/1</td>
<td>–</td>
<td>B1</td>
<td>ST40c</td>
<td>CH4-31</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>aEPEC</td>
<td>O51:H49a</td>
<td>2/2</td>
<td>–</td>
<td>α1</td>
<td>B2</td>
<td>ST589d</td>
<td>CH39-135</td>
<td>+</td>
<td>AMP, SF, SXT (1)</td>
<td>AMC, S (1)</td>
</tr>
<tr>
<td>aEPEC</td>
<td>O106:H45</td>
<td>1/1</td>
<td>–</td>
<td>κ</td>
<td>B2</td>
<td>ST5965</td>
<td>CH18-NT</td>
<td>–</td>
<td>SF (1)</td>
<td>–</td>
</tr>
<tr>
<td>aEPEC</td>
<td>O123:H3g</td>
<td>1/1</td>
<td>–</td>
<td>ε2</td>
<td>B1</td>
<td>ST517</td>
<td>CH65-32</td>
<td>–</td>
<td>AMP, AMC, SF (1)</td>
<td>–</td>
</tr>
<tr>
<td>aEPEC</td>
<td>O171:H18</td>
<td>2/1</td>
<td>–</td>
<td>β2</td>
<td>E</td>
<td>ST117</td>
<td>CH45-97</td>
<td>+</td>
<td>AMP (2)</td>
<td>SF (2)</td>
</tr>
<tr>
<td>aEPEC</td>
<td>ONT:H4</td>
<td>1/1</td>
<td>–</td>
<td>β2</td>
<td>B2</td>
<td>ST28c</td>
<td>CH21-90</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>aEPEC</td>
<td>ONT:H14</td>
<td>3/1</td>
<td>–</td>
<td>o2</td>
<td>B2</td>
<td>ST3672c</td>
<td>CH13-5</td>
<td>+</td>
<td>SF (3)</td>
<td>–</td>
</tr>
<tr>
<td>tEPEC</td>
<td>O103:H8</td>
<td>2/1</td>
<td>–</td>
<td>α2</td>
<td>B1</td>
<td>ST327-likeg</td>
<td>CH4-like-25d</td>
<td>–</td>
<td>AMP, S, SF (2)</td>
<td>–</td>
</tr>
</tbody>
</table>

Abbreviations: aEPEC, atypical EPEC; AMC, amoxicillin-clavulanic acid; AMP, ampicillin; EPEC, enteropathogenic Escherichia coli; no., number; NT, not typeable; S, streptomycin; SF, sulphafurazole; ST, sequence type; SXT, trimethoprim-sulphamethoxazole; tEPEC, typical EPEC; UPEC, uropathogenic E. coli; VTEC, verotoxin-producing E. coli.

*a* Isolates positive to vt were classified as VTEC. Isolates positive to intimin but negative to vt and bfpA were classified as aEPEC. Isolates positive to intimin and bfpA but negative to vt were classified as tEPEC.

*b* Serotype-intimin combinations previously found in human-derived aEPEC from patients with diarrhoea (Blanco, Blanco, Dahbi, Alonso, et al., 2006; Nguyen et al., 2006; Xu et al., 2016).

*c* STs previously found in human-derived aEPEC from patients with diarrhoea (Afset et al., 2008; Xu et al., 2017).

*d* New ST and new clonotype not found in tEPEC strains in previous studies.
The VTEC and EPEC isolates belonged to four phylogenetic groups (with B1 and B2 being the most common), 10 STs and 10 clonotypes (Table 2). Eight aEPEC isolates from five animals were also classified as UPEC (Table 2). However, none of the VTEC and EPEC isolates found in this study were determined to be ExPEC, enteroinvasive *E. coli*, enteroaggregative *E. coli* or enterotoxigenic *E. coli*. When more than one VTEC or EPEC isolate was found in the faeces of the same animal, the isolates displayed the same characteristics (e.g. intimin type, serotype, phylogenetic group, ST, clonotype and presence or absence of virulence genes; Table 2).

### 3.3 Detection and characterization of antimicrobial-resistant ExPEC and UPEC isolates

Five antimicrobial-resistant *E. coli* isolates from the faeces of five raccoons showed ExPEC and/or UPEC status and were non-VTEC and non-EPEC (Table 3).

### 3.4 Co-infections of *E. coli* resistant to at least one antimicrobial, ESBL-EC, VTEC and EPEC isolates

Two raccoons were co-infected with EPEC and *E. coli* resistant to at least one antimicrobial isolates (one isolate resistant to ampicillin and one isolate resistant to sulphafurazole and trimethoprim-sulphamethoxazole, respectively). These raccoons were males and captured in different years (2017 and 2019) and sites. However, raccoons infected with ESBL-EC and VTEC isolates were not co-infected with other potentially pathogenic *E. coli* types.

### 4 DISCUSSION

Compared with other studies carried out in Spain, the percentages of *E. coli* isolates from raccoons resistant to the antimicrobials tested in this study were lower than those found in *E. coli* from livestock (Orden et al., 2000; Sáenz et al., 2001) and gulls (Stedt et al., 2014), similar to those found in *E. coli* from synanthropic birds (feral pigeon, hybrid ducks, house sparrows and spotless starlings) living in rural and urban environments (Sacristán et al., 2014), and higher than those reported for *E. coli* recovered from deer and small mammals (Alonso et al., 2016). These differences in the AMR among different animal species may be due to various factors. Exposure to the selective pressure associated with the widespread use of antimicrobials in farming and veterinary practice and direct contact with humans seems to be related to the high prevalence of antimicrobial-resistant bacteria in food-producing animals (Alonso et al., 2016). In addition, several host factors, including diet, might be expected to affect the dynamics of the normal gut microbiota and potentially affect the prevalence of AMR (Alonso et al., 2016).

The prevalence of antimicrobial-resistant *E. coli* in raccoon faeces from the Madrid region of Spain (50.6%; 95% CI, 40.1–61.1) was higher than those found in raccoon faeces from rural (16.7%; 95% CI, 7.3–33.6) and urban (15.0%; 95% CI, 5.2–36.0) areas in southern Ontario, Canada (Jardine et al., 2012). Although the use of different sampling and laboratory methods makes it difficult to directly compare studies, the differences may be due, at least partially, to the high levels of antimicrobial consumption and AMR in humans and food-producing animals in Spain (European Centre for Disease Prevention & Control, 2018; Sáenz et al., 2001; van de Sande-Bruinsma et al., 2008). The levels of AMR and antimicrobial consumption in Spain are among the highest in the European Union and in order to reduce the risk of selection and dissemination of AMR in the country a National Strategic Action Plan has been implemented (European Centre for Disease Prevention & Control, 2018). In addition, since AMR has increased during recent years (European Centre for Disease Prevention & Control, 2018), the higher levels of AMR detected among faecal *E. coli* isolates in this study compared to those found by Jardine et al. (2012) might be explained in part by the years in which the samples were collected (2007 in the study of Jardine et al., 2012 and 2017–2019 in this study).

The most common resistance phenotypes detected in raccoon faeces (ampicillin, streptomycin, tetracycline, sulphafurazole and...

### TABLE 3 Characteristics of non-VTEC and non-EPEC antimicrobial-resistant *Escherichia coli* isolates with extraintestinal pathogenic *E. coli* and/or uropathogenic *E. coli* status detected in the faeces of 5 out B3 raccoons in the Madrid region of Spain

<table>
<thead>
<tr>
<th>Serotype</th>
<th>No. of isolates/no. of animal</th>
<th>Phylogenetic group</th>
<th>ST</th>
<th>ExPEC</th>
<th>UPEC</th>
<th>Resistance phenotype</th>
<th>Intermediate resistance phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>O5:HNT</td>
<td>1/1</td>
<td>A</td>
<td>ST93&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+</td>
<td>–</td>
<td>AMP, S, TE, SF</td>
<td>AMC</td>
</tr>
<tr>
<td>O15:H16</td>
<td>1/1</td>
<td>D</td>
<td>ST69&lt;sup&gt;a&lt;/sup&gt;</td>
<td>–</td>
<td>+</td>
<td>AMP, TE, SF, SXT</td>
<td>S</td>
</tr>
<tr>
<td>O18:H31&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1/1</td>
<td>B2</td>
<td>ST372</td>
<td>+</td>
<td>+</td>
<td>AMP, S, SF, SXT</td>
<td>S</td>
</tr>
<tr>
<td>O75:H7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1/1</td>
<td>B2</td>
<td>ST80</td>
<td>+</td>
<td>+</td>
<td>S, SF, SXT</td>
<td></td>
</tr>
<tr>
<td>O83:H5</td>
<td>1/1</td>
<td>B2</td>
<td>ST83</td>
<td>+</td>
<td>+</td>
<td>AMP, TE</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>STs previously found in *E. coli* isolates causing human urinary tract and bloodstream infections (Flament-Simon et al., 2019).

<sup>b</sup>Serotype-phylogenetic group-ST combinations previously found in *E. coli* isolates causing human urinary tract and bloodstream infections (Flament-Simon et al., 2019).
trimethoprim-sulphamethoxazole) corresponded to the most widely used antimicrobials in humans and farm animals in Spain (Alonso et al., 2017; Orden et al., 2000; van de Sande-Bruinisma et al., 2008). In agreement with our data, Sacristán et al. (2014) found that the resistances to ampicillin, tetracycline and trimethoprim-sulphamethoxazole were among the most frequent resistances observed in E. coli from synanthropic birds. Our results suggest that raccoons may be exposed to antimicrobial-resistant bacteria through anthropogenic sources in the environment, which is consistent with studies of raccoons in North America and other wildlife (Jardine et al., 2012; Radhouani et al., 2014). In addition, since some bird species, including waterfowl, have shown high prevalences of antimicrobial-resistant E. coli (Sacristán et al., 2014; Stedt et al., 2014), it is possible that birds are a source of AMR for raccoons through the raccoon diet or water contamination with bird faeces.

The percentage of ESBL-EC isolates in the faeces of raccoons in the Madrid region was similar to those found previously in raccoons (Bondo et al., 2016a; Jardine et al., 2012) and deer (Alonso et al., 2016), but lower than those found in humans, livestock, and other wildlife species, such as gulls and boars (Atterby et al., 2017; EFSA Panel on Biological Hazards (BIOHAZ), 2011; Guenther et al., 2011; Mamaní et al., 2019). The small number of ESBL-EC isolates detected in raccoons may be due to the low number of studies carried out in this animal species. Another explanation could be that the occurrence of ESBL-EC is influenced by the host (Guenther et al., 2011). The only EBLS-EC isolate detected from the faeces of raccoons carried the blaSHV-12 gene. This gene is one of the most common ESBL genes in E. coli from humans and livestock (EFSA Panel on Biological Hazards (BIOHAZ), 2011), and its detection in wild animals probably reflects that fact (Guenther et al., 2011). The blaSHV-12 gene has been detected previously in E. coli from other wildlife species in several countries (Atterby et al., 2017; Guenther et al., 2011; Wang et al., 2017), but not in Spain or in E. coli from wildlife in North America.

As raccoons use and feed in aquatic habitats and may be exposed to resistant bacteria from water, sediment or biofilms from contaminated rivers, they may act as sentinels of AMR in the environment (Bondo et al., 2016a).

To our knowledge, this study is the first report of the presence of VTEC and EPEC in raccoon faeces. Only one raccoon carried VTEC in its faeces. Other studies carried out in wild boars and domestic and wild ruminants have found higher prevalence rates of VTEC (3%–48%; Alonso et al., 2017; Bardiau et al., 2010; Cortés et al., 2005; Mora et al., 2012; Orden et al., 2002, 2003; Sánchez et al., 2009). However, in agreement with our results, VTEC have rarely been detected in other wildlife species, such as birds (Alonso et al., 2017; Sacristán et al., 2014). This suggests that raccoons are occasional carriers of VTEC, probably because this E. coli pathotype is not well adapted for colonising the intestine of this host, so it is unlikely that they transmit VTEC to humans.

Twelve per cent of the raccoons sampled carried EPEC in their faeces. Previous studies carried out in wildlife species (mainly birds, boars and ruminants) and domestic ruminants (Alonso et al., 2017; Bardiau et al., 2010; Cortés et al., 2005; Orden et al., 2002) found lower prevalence rates of EPEC (3%–8%) than that found in this work. We detected a low level of antimicrobial resistance in EPEC isolates from raccoons, which is consistent with a previous study of wild birds, boars and ruminants (Alonso et al., 2017). In contrast, Medina et al. (2011) found that 204 of 226 EPEC and VTEC strains isolated from cattle, sheep and goats were resistant to at least one antimicrobial.

Most of the EPEC isolates from raccoons were aEPEC, which is consistent with previous studies of livestock and wild animals (Alonso et al., 2017; Horcajo et al., 2012; Sacristán et al., 2014). Two of the EPEC isolates found in this study were teEPEC, the new ST (ST327-like) and the new clonotype (CH4-like-25) of both teEPEC isolates found in this study have not been described, to our knowledge, in teEPEC in any previous studies of human, domestic animals, livestock and wildlife. Humans have been described as the only reservoir of teEPEC with few exceptions (Alonso et al., 2017). Thus, the finding in the present study of two teEPEC isolates in a raccoon could suggest the contact and acquisition from human sources (Alonso et al., 2017).

Three serotype-intimin combinations (O21:H21 eaeαβ, O51:H49 eaeα1 and O123:H19 eaeε2) were detected in the present study in 6 aEPEC isolates. These serotype-intimin combinations have been previously reported in human aEPEC strains isolated from patients with diarrhoea in Spain (Blanco, Blanco, Dahbi, Alonso, et al., 2006), Australia (Nguyen et al., 2006) and China (Xu et al., 2016). In addition, 4 (ST28, ST40, ST589 and ST3672) of the 9 STs detected in this study in aEPEC from raccoon faeces have been previously reported in human aEPEC strains isolated from patients with diarrhoea in Norway and China (Afset et al., 2008; Xu et al., 2017).

It is important to note that eight isolates from five raccoons were hybrid aEPEC/UPEC, which are likely capable of causing both intestinal and urinary tract infections in humans. Although hybrid E. coli pathotypes have been described previously (Cointe et al., 2018; Lara et al., 2017), this is the first report, to our knowledge, of a hybrid aEPEC/UPEC isolates occurring in a wild or domestic animal species. The remarkable genome plasticity displayed by E. coli allows the emergence of strains that possess virulence genes from different pathotypes (Lara et al., 2017).

We did not investigate the temporal dynamics of the E. coli isolates from the faeces of raccoons, so it is unknown if raccoons in this region of Spain are reservoirs or short-term carriers of antimicrobial-resistant E. coli and EPEC isolates. Raccoons were unlikely to carry specific E. coli serotypes, including antimicrobial resistance phenotypes, over multiple years in southern Ontario (Bondo et al., 2016a; Jardine et al., 2012). This suggests that raccoons are likely acquiring antimicrobial-resistant E. coli from their environment rather than maintaining them (Bondo et al., 2016a). Further studies, including longitudinal studies, are required to determine whether raccoons might be reservoirs of antimicrobial-resistant E. coli and EPEC isolates.

We detected five non-VTEC and non-EPEC antimicrobial-resistant E. coli isolates with ExPEC and/or UPEC status. Two (O18:H31-B2-ST372, O75:H7-B2-ST80) of the five serotype-phylogenetic group-ST combinations detected in these isolates have been found...
isolates in the study by Flament-Simon et al. (2019). Future studies, in addition, other two non-VTEC and non-EPEC antimicrobial-resistant isolates causing human urinary tract and blood-stream infections in Spain and France (Flament-Simon et al., 2019). In conclusion, raccoons can carry antimicrobial-resistant E. coli, including EBSL-EC and EPEC in their faeces. Because raccoons have the potential to contaminate food and environment with their faeces, raccoons may pose a risk to public health in the Madrid region of Spain. This provides further evidence that raccoons should be controlled in regions where they are not native. In addition, raccoons in Spain could be good sentinels of AMR in the environment.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

REFERENCES


SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.