Occurrence and characterization of extended-spectrum cephalosporin-resistant Enterobacteriaceae in healthy household dogs in Greece

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Abstract

Extended-spectrum cephalosporin- and/or carbapenem-resistant (ESC and/or Carb) Enterobacteriaceae constitute a public health hazard because of limited treatment options and are endemic among humans in Greece. Recently, ESC and Carb Enterobacteriaceae have been increasingly isolated from companion animals, stressing their potential role as a reservoir for humans. However, the presence of ESC bacteria in companion animals within Greek households has not been determined yet. Genes conferring the ESC and Carb phenotype were detected among canine isolates and their chromosomal or plasmid location was determined. Standard methods were applied for plasmid characterization. The clonal relatedness of the recovered isolates was examined by multilocus sequence typing (MLST). Here, we report the first findings on the presence of ESC Enterobacteriaceae in healthy Greek dogs. ESC Escherichia coli isolates were associated with different sequence types (STs), including the human pandemic ST131 clone. The occurrence of human-related ESBL/pAmpC genes, plasmid types and/or strain STS in this animal reservoir suggests possible bilateral transmission.

Since the first reports of extended-spectrum cephalosporin-resistant (ESC) Enterobacteriaceae in companion animals [1, 2], the significance of this new reservoir for human health has been discussed [3–5]. The prevalence of extended-spectrum β-lactamase and acquired AmpC β-lactamase (ESBL/AmpC)-producing bacteria in companion animals, dogs and cats in particular, has now been detected worldwide [6–10]. In addition, it has been demonstrated that human contact with pets increases the chance of being colonized with ESBL/AmpC-producing Escherichia coli by up to sevenfold and, together with travelling to endemic countries, this is one of the main risk factors for ESBL/AmpC carriage [11]. Recently, the emergence of carbapenem-resistant (Carb) Enterobacteriaceae among companion animals from different countries has also been documented [10, 12–14].

Although isolates from human and animals mainly belong to different sequence types (STs), certain ESBL/AmpC-producing E. coli STs can be found in both companion animals and humans [15, 16], with resistance genes being conveyed by mobile elements, i.e. plasmids, among isolates and reservoirs [17]. Therefore, humans may acquire antimicrobial resistance not only from food-producing animals, but also from their companion animals.

Carbapenem- and ESBL/AmpC-producing bacteria are endemic in Greece (http://atlas.ecdc.europa.eu/public/index.aspx?Instance=GeneralAtlas), and clinical infections attributed to these bacteria constitute a significant public health threat because of limited therapeutic options [18, 19]. However, there is a paucity of knowledge regarding Enterobacteriaceae from companion animals in Greece, including their

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Abbreviations: AMP, ampicillin; AmpC, AmpC β-lactamase; AZM, azithromycin; Carb, carbapenem-resistant; CAZ, ceftazidime; CC, clonal complex; CHL, chloramphenicol; CIP, ciprofloxacin; CLSI, Clinical and Laboratory Standards Institute; CST, colistin; CTX, cefotaxime; ESBL, extended-spectrum β-lactamase; ESC, extended-spectrum cephalosporin-resistant; EUCAST, European Committee on Antimicrobial Susceptibility Testing; FDA, Food and Drug Administration; GEN, gentamicin; IQR, interquartile range; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MEM, meropenem; MLST, multilocus sequence typing; NA, not applied; NAL, nalidixic acid; pCC, plasmid clonal complex; pST, plasmid sequence type; S1-PFGE, S1-nuclease pulsed-field gel electrophoresis; SMX, sulfamethoxazole; ST, sequence type; TET, tetracycline; TMP, trimethoprim; TGC, tigecycline; WT, wild-type.

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clinically relevant β-lactamase gene content, genetic environment and clonal relatedness. To address this shortcoming, we investigated the occurrence and the characteristics of ESCR and/or CarbR commensal bacteria among healthy dogs.

Between April and May 2014, freshly voided faecal samples were collected with owner consent from 49 non-duplicate consecutive healthy dogs. The median age of the dogs was 53 months [interquartile range (IQR) 82–20], with overall female/male ratio of approximately 1:1. The samples were collected during routine physical examinations, parasite screening or vaccination of the dogs in northern Greece (Thessaloniki: n = 32 and Thessaly: n = 17). Data regarding prior exposure to cephalosporins of the dogs colonized with ESCR Enterobacteriaceae were reviewed retrospectively.

Approximately 100 µg of each faecal sample was enriched in brain heart infusion broth (Becton–Dickinson, Franklin Lakes, NJ, USA) supplemented with 16 mg l–1 vancomycin for 18–24 h aerobically at 37 ºC, and subsequently inoculated on ChromID ESBL, ChromID CARBA and ChromID OXA-48 (bioMérieux, Solna, Sweden). Although no Enterobacteriaceae exhibiting the CarbR phenotype were recovered, we recovered 12 non-duplicate isolates exhibiting ESCR phenotype from 10 out of 49 dog faecal specimens (20.4 %) included in the study (Table 1). Our study revealed that the prevalence of ESCR Enterobacteriaceae in Greek household dogs was comparable to that indicated by previous studies in Turkey and Japan [7, 20], whereas it differed from that in other European and Mediterranean countries [21–25]. Distinctive colonial morphotypes from each faecal sample were identified as E. coli (n = 10) and Klebsiella pneumoniae (n = 2), based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Bruker, Coventry, UK).

The antibiotic susceptibility of these isolates was assessed using the broth microdilution method (EUCAST, Sensititre, Thermo Fisher, Basingstoke, UK) and interpreted using the epidemiological cut-off values recommended by EUCAST (http://mic.eucast.org), or, when those were not available, according to the CLSI [26] or FDA [27] guidelines. Most of the isolates exhibited non-wild type (WT) minimum inhibitory concentrations (MICs) to sulfamethoxazole (n = 10; 83.3 %); to ciprofloxacin and tetracycline (n = 11; 78.6 %); to azithromycin, gentamicin, nalidixic acid and trimethoprim (n = 9; 64.3 %); and to chloramphenicol (n = 6; 42.9 %). All of the isolates were resistant to ampicillin, cefotaxime and cefazidime, and exhibited WT MICs to colistin, meropenem and tigecycline (Table 1). The high occurrence of a multidrug-resistant phenotype among the ESCR isolates recovered in this study limits treatment options and decreases the likelihood of appropriate empirical therapy for canine and/or potential zoonotic human infections caused by these isolates. The median age of the dogs colonized by an ESCR Enterobacteriaceae was 40 months (IQR 69–17). Four of these dogs were female, which was a lower ratio than the overall female/male distribution of the dogs participating in the study. A retrospective review of the records of the dogs colonized by ESCR Enterobacteriaceae revealed that only one of the dogs (GR110.16) had prior exposure to cephalosporins. This finding suggests the successful dissemination of ESCR Enterobacteriaceae among household dogs in the absence of selective cephalosporin pressure. However, taking into consideration the high occurrence of multidrug resistance, we cannot exclude the possibility of co-selection and persistence of ESCR bacteria under the selective pressure of other antimicrobial agents.

Genes conferring ESCR were identified by microarray analysis using the Check-MDR CT-101 array platform (Check-Points, Wageningen, The Netherlands), followed by PCR amplification and DNA sequencing [28]. Five different ESBL/AmpC genes were identified (Table 1) with blaCTX-M-15 being predominant (n = 6; 50 %), as previously described among ESBL producers from companion animals worldwide [3]. The other genes associated with the ESCR phenotype were blaCMY-2 (n = 4), blaCTX-M-3 (n = 3), blaCTX-M-1 (n = 1) and blaDHA-1 (n = 1), highlighting the genetic heterogeneity conferring ESC resistance in dogs within Greek households. Two isolates were found to carry a combination of ESBL and AmpC resistance genes, either on different genetic vehicles (GR105.8) or the same genetic vehicle (GR110.16).

Transformation experiments were performed to assess the plasmid location of the ESBL/AmpC genes, while standard methods [PCR-based replicon typing, plasmid multi-locus sequence typing/plasmid double-locus sequence typing/repliscope sequence typing and S1-nuclease pulsed-field gel electrophoresis (S1-PFGE)] were applied for further plasmid analysis, as previously described [28]. The majority of the ESBL/AmpC genes were carried on plasmids belonging to seven different replicon types (Table 1). All blaCTX-M-15-bearing plasmids (n = 5) were assigned to the IncF incompatibility group and were subtyped as F1a: A4; B1 (n = 3) or F1: A6: B20 (n = 2), both frequently reported among humans [29–31], with the former also being reported from isolates of food products of animal origin [32]. The second most represented replicon type, IncK (n = 3), was associated with blaCMY-2 (n = 2) or blaCMY-3 and blaCTX-M-1 (n = 1), as previously described [31, 33, 34]. ESBL/AmpC genes were incidentally found on plasmids belonging to the HI2, II, K-P, L/M or R replicon types, or on the chromosome. According to S1-PFGE, the size of the plasmids varied from approximately 85 (IncLM) to 291 kb (IncHI2/pST1) (Table 1).

The genetic relatedness of the E. coli and K. pneumoniae isolates was determined by multilocus sequence typing (MLST), as previously described [35, 36]. The ESCR E. coli isolates were associated with eight different STs, namely ST10, ST127, ST155, ST131, ST361, ST429, ST744 and ST746, each comprising one to two isolates (Table 1). Several STs (ST10, ST127, ST131, ST361 and ST744) have previously been associated with E. coli isolates of canine origin
Table 1. Characteristics of ESC<sup>R</sup>-*Enterobacteriaceae* recovered from healthy household dogs in Greece

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Species</th>
<th>AMP</th>
<th>AZM</th>
<th>CAZ</th>
<th>CHL</th>
<th>CIP</th>
<th>CST</th>
<th>CTX</th>
<th>GEN</th>
<th>MEM</th>
<th>NAL</th>
<th>SMX</th>
<th>TET</th>
<th>TGC</th>
<th>TMP</th>
<th>ST/CC</th>
<th>ESBL/pAmpC gene(s)</th>
<th>Rep/Inc type</th>
<th>Size (kb)</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR4.2</td>
<td><em>E. coli</em></td>
<td>64</td>
<td>&gt;32</td>
<td>8</td>
<td>8</td>
<td>0.3</td>
<td>≥1</td>
<td>&gt;4</td>
<td>32</td>
<td>0.03</td>
<td>≤4</td>
<td>&gt;1024</td>
<td>≤2</td>
<td>≤0.25</td>
<td>&gt;32</td>
<td>155/155</td>
<td><em>bla&lt;sub&gt;CTX-M-15&lt;/sub&gt;</em></td>
<td>F</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GR126</td>
<td><em>E. coli</em></td>
<td>64</td>
<td>&gt;64</td>
<td>&gt;8</td>
<td>&gt;128</td>
<td>&gt;8</td>
<td>≤1</td>
<td>&gt;4</td>
<td>32</td>
<td>≤0.03</td>
<td>&gt;128</td>
<td>&gt;1024</td>
<td>≤2</td>
<td>≤0.25</td>
<td>&gt;32</td>
<td>744</td>
<td><em>bla&lt;sub&gt;CTX-M-15&lt;/sub&gt;</em></td>
<td>F (F31 : A4 : B1)</td>
<td>162</td>
<td>Thessaly</td>
</tr>
<tr>
<td>GR102.2</td>
<td><em>E. coli</em></td>
<td>64</td>
<td>&gt;64</td>
<td>&gt;8</td>
<td>&gt;8</td>
<td>≤1</td>
<td>&gt;4</td>
<td>32</td>
<td>0.06</td>
<td>&gt;128</td>
<td>&gt;1024</td>
<td>≤2</td>
<td>≤0.25</td>
<td>&gt;32</td>
<td>361</td>
<td><em>bla&lt;sub&gt;CMY-2&lt;/sub&gt;</em></td>
<td>F</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GR104.3</td>
<td><em>E. coli</em></td>
<td>64</td>
<td>4</td>
<td>&gt;8</td>
<td>&gt;8</td>
<td>≤1</td>
<td>&gt;4</td>
<td>32</td>
<td>≤0.03</td>
<td>&gt;128</td>
<td>≤8</td>
<td>&gt;64</td>
<td>≤0.25</td>
<td>0.5</td>
<td>131</td>
<td><em>bla&lt;sub&gt;CTX-M-15&lt;/sub&gt;</em></td>
<td>F (F31 : A4 : B1)</td>
<td>162</td>
<td>Thessaloniki</td>
<td></td>
</tr>
<tr>
<td>GR105.8</td>
<td><em>E. coli</em></td>
<td>64</td>
<td>&gt;64</td>
<td>&gt;8</td>
<td>16</td>
<td>0.03</td>
<td>≤1</td>
<td>&gt;4</td>
<td>1</td>
<td>0.06</td>
<td>≤4</td>
<td>&gt;1024</td>
<td>≤0.25</td>
<td>≤0.25</td>
<td>10</td>
<td><em>bla&lt;sub&gt;CMY-2&lt;/sub&gt;</em></td>
<td>F (F1 : A6 : B20)</td>
<td>97</td>
<td>Thessaloniki</td>
<td></td>
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<tr>
<td>GR110.16*</td>
<td><em>E. coli</em></td>
<td>64</td>
<td>&gt;64</td>
<td>&gt;8</td>
<td>&gt;128</td>
<td>&gt;8</td>
<td>≤1</td>
<td>&gt;4</td>
<td>0.5</td>
<td>0.06</td>
<td>&gt;128</td>
<td>&gt;1024</td>
<td>≤2</td>
<td>≤0.25</td>
<td>&gt;32</td>
<td>746</td>
<td>*bla&lt;sub&gt;CMY-2&lt;/sub&gt;, <em>bla&lt;sub&gt;CTX-M-3&lt;/sub&gt;</em></td>
<td>F</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GR111.19</td>
<td><em>E. coli</em></td>
<td>64</td>
<td>&gt;64</td>
<td>1</td>
<td>≥8</td>
<td>≤0.015</td>
<td>≤1</td>
<td>&gt;4</td>
<td>32</td>
<td>≤0.03</td>
<td>≤4</td>
<td>&gt;1024</td>
<td>≤2</td>
<td>≤0.25</td>
<td>&gt;32</td>
<td>127</td>
<td><em>bla&lt;sub&gt;CTX-M-3&lt;/sub&gt;</em></td>
<td>L/M</td>
<td></td>
<td></td>
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<tr>
<td>GR116.26</td>
<td><em>K. pneumoniae</em></td>
<td>64</td>
<td>&gt;64</td>
<td>&gt;8</td>
<td>&gt;8</td>
<td>≤1</td>
<td>&gt;4</td>
<td>32</td>
<td>≤0.03</td>
<td>&gt;128</td>
<td>&gt;1024</td>
<td>≤2</td>
<td>≤0.25</td>
<td>&gt;32</td>
<td>2544</td>
<td><em>bla&lt;sub&gt;CTX-M-3&lt;/sub&gt;</em></td>
<td>NA (Chromosome)</td>
<td>NA</td>
<td>Thessaloniki</td>
<td></td>
</tr>
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<td><em>E. coli</em></td>
<td>64</td>
<td>&gt;64</td>
<td>&gt;8</td>
<td>&gt;128</td>
<td>&gt;8</td>
<td>≤1</td>
<td>&gt;4</td>
<td>32</td>
<td>≤0.03</td>
<td>&gt;128</td>
<td>&gt;1024</td>
<td>≤2</td>
<td>≤0.25</td>
<td>&gt;32</td>
<td>744</td>
<td><em>bla&lt;sub&gt;CTX-M-3&lt;/sub&gt;</em></td>
<td>NA (pST3/pCC3)</td>
<td>97</td>
<td>Thessaloniki</td>
</tr>
<tr>
<td>GR126.39</td>
<td><em>E. coli</em></td>
<td>64</td>
<td>&gt;64</td>
<td>&gt;8</td>
<td>0.5</td>
<td>≤1</td>
<td>&gt;4</td>
<td>32</td>
<td>≤0.03</td>
<td>8</td>
<td>&gt;1024</td>
<td>64</td>
<td>≤0.25</td>
<td>≤0.25</td>
<td>429</td>
<td><em>bla&lt;sub&gt;CTX-M-3&lt;/sub&gt;</em></td>
<td>K-P</td>
<td>115</td>
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<td>GR128.41</td>
<td><em>E. coli</em></td>
<td>64</td>
<td>&gt;64</td>
<td>&gt;8</td>
<td>&gt;8</td>
<td>≤1</td>
<td>&gt;4</td>
<td>32</td>
<td>≤0.03</td>
<td>&gt;128</td>
<td>≤8</td>
<td>&gt;64</td>
<td>≤0.25</td>
<td>0.5</td>
<td>131</td>
<td><em>bla&lt;sub&gt;CTX-M-15&lt;/sub&gt;</em></td>
<td>F</td>
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<td></td>
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<tr>
<td>GR129.43</td>
<td><em>K. pneumoniae</em></td>
<td>64</td>
<td>&gt;64</td>
<td>&gt;8</td>
<td>16</td>
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<td>≤1</td>
<td>&gt;4</td>
<td>0.5</td>
<td>0.06</td>
<td>&gt;128</td>
<td>&gt;1024</td>
<td>≤2</td>
<td>≤0.25</td>
<td>&gt;32</td>
<td>709</td>
<td><em>bla&lt;sub&gt;DEH-1&lt;/sub&gt;</em></td>
<td>R</td>
<td>111</td>
<td>Thessaloniki</td>
</tr>
</tbody>
</table>

AMP, ampicillin; AZM, azithromycin; CAZ, ceftazidime; CHL, chloramphenicol; CIP, ciprofloxacin; CST, colistin; CTX, cefotaxime; GEN, gentamicin; MEM, meropenem; NAL, nalidixic acid; SMX, sulfamethoxazole; TET, tetracycline; TGC, tigecycline; TMP, trimethoprim.

NA, not applied; ST, sequence type; CC, clonal complex; pST, plasmid sequence type and pCC, plasmid clonal complex.

*Prior exposure to cephalosporins (cefazolin).
[37, 38], whereas ST131 and ST361 have been associated with ESCR E. coli recovered from human infections in Greece [39]. The two recovered ESCR K. pneumoniae belonged to ST2544 or ST709, with the latter having previously been associated with the ESCR and CarbR phenotypes among companion animals and human hospital infections, respectively [40–42].

There was no specific association between the plasmids and bacterial clones, indicating that ESBL- and/or AmpC-encoding plasmids are disseminated in genetically unrelated isolates representative of the canine gut flora. E. coli ST131 carrying bla\textsubscript{CTX-M-15} on IncFII plasmids has already been documented in dogs [43–45], suggesting possible transmission from a human reservoir. Nevertheless, this predominant E. coli human clone [46] contributed moderately to the ESBL/AmpC genes’ epidemiology among the Greek households dogs analysed here (n=2), in accordance with previous findings for companion animals from France [38], the Republic of Korea [47] and Switzerland [48].

In conclusion, to the best of our knowledge, these are the first data on the presence of ESCR Enterobacteriaceae in healthy dogs in Greece. Despite the small sample size, several E. coli clones contributed to the epidemiology of the ESBL/AmpC gene in dogs, including the human-related pandemic ST131, suggesting potential transmission between the two reservoirs. We documented that Greek household dogs act as a reservoir of diverse genes conferring the ESCR phenotype and plasmids associated with these genes. Plasmids belonging to F replicon type seem to have played an important role in the preservation and dissemination of the bla\textsubscript{CTX-M-15} gene. Considering the close contact between humans and their companion animals, we emphasize the need for further surveillance studies in countries with a high prevalence of antibiotic resistance in humans to determine the prevalence of ESCR Enterobacteriaceae among companion animals and their zoonotic potential.

References
17. Johnson JR, Clabots C, Kuskowski MA. Multiple-host sharing, long-term persistence, and virulence of Escherichia coli clones.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
All faecal samples were routine diagnostic samples collected with owner consent and without invasive techniques, for which ethical approval is not required, and became available for the research described in this paper. Therefore, approval by the Animal Care and Use Committee of Wageningen Bioveterinary Research was not necessary.


