Characterization of Extended-Spectrum β-Lactamase-Producing and AmpC β-Lactamase-Producing *Enterobacteriales* Isolated from Companion Animals in Korea

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Abstract: The emergence of extended-spectrum cephalosporin (ESC)-resistant Gram-negative bacteria is of great concern in both human and veterinary medicine. The aim of this study was to investigate ESC-resistant bacterial isolates from companion animals in South Korea between 2017 and 2019. Isolates with ESC resistance genes, which were identified by PCR, were assessed for genetic relatedness by multi-locus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE). In total, 91 ESC-resistant *Escherichia coli*, *Klebsiella* spp., *Serratia* spp., and *Enterobacter cloacae* isolates harbored the blaTEM gene. Among other ESC resistance genes, blaCTX-M-15, blaCTX-M-1 and blaCTX-M-55 were predominantly detected in *E. coli* isolates, whereas blaSHV and blaDHA were more frequently detected in *Klebsiella pneumoniae* isolates. In addition, all blaESC-positive isolates were classified as *E. cloaca*. From the MLST results, blaCTX-M-9-carrying ST131, blaCTX-M-carrying ST405, and blaCTX-M-1-carrying ST3285 strains were dominant among *E. coli* isolates. ST273 and ST275 strains harboring blaSHV were frequently detected in *K. pneumoniae* isolates. Various sequence types were obtained in *E. cloaca* and *Klebsiella oxytoca* isolates. All isolates demonstrated unique PFGE profiles (<57–98% similarity) and were unlikely to be derived from a single clone. The present study reveals the presence and wide genetic distribution of ESC-resistant bacterial species in South Korean companion animals.

Keywords: extended-spectrum cephalosporins; extended-spectrum β-lactamases; AmpC β-lactamases; antimicrobial resistance; Gram-negative bacteria; companion animal

1. Introduction

The emergence and prevalence of β-lactam resistance in Gram-negative bacteria has increased consistently over the past few decades [1,2]. Resistance to β-lactams is mostly caused by bacterially produced β-lactamases that hydrolyze and inactivate extended-spectrum cephalosporins (ESCs), such as third and fourth generation cephalosporins [1]. ESC resistance is mainly caused by the expression of extended-spectrum β-lactamase (ESBL) and AmpC β-lactamase (AmpC) genes that are normally encoded on mobile genetic elements, mostly plasmids [1].

The first ESBLs have evolved from the native β-lactamases TEM and SHV via genetic mutations [3]. CTX-M β-lactamases, a new group of plasmid-mediated ESBLs, were first reported in Japan in 1986 [4]. However, since 2000, CTX-M β-lactamases have increasingly been reported in both human and animal populations and are now the dominant type of ESBL, replacing classical TEM- and SHV-type ESBLs in most areas of the world [5]. Currently, there are >120 different CTX-M β-lactamases that are clustered into five groups...
(CTX-M-1, 2, 8, 9, and 25) [6]. Among CTX-M-type enzymes, the presence of CTX-M-15 and CTX-M-14 has increasingly been reported in most areas of the world, including South Korea [5,7].

Since the detection of CMY-1, the first reported AmpC type β-lactamases, in 1989 [6], various types of AmpCs have been identified in clinical isolates of Enterobacterales around the world [8]. Among AmpCs, CIT- and DHA-type enzymes are the most prevalent [2]. Especially, DHA-producing Klebsiella spp. isolates and CIT-producing Escherichia coli isolates have been repeatedly reported for Enterobacterales in South Korea [2,9]. Despite many studies on ESBL- and AmpC-producing bacteria from human or livestock isolates, studies concerning antimicrobial resistance (AMR) bacteria associated with companion animals are lacking, especially with respect to Serratia spp. and Enterobacter spp. [10–12].

The popularity of companion animals in South Korea has been growing, which provides a potential reservoir of AMR bacteria, as pets are closely associated with humans, living in their homes and near their food [9]. Thus, the importance of profiling AMR bacteria was emphasized in the “One Health” initiative, which integrates veterinary medicine, human health, animal-production systems, and the environment [13]. Systematic control and prevention, through implementation of a national AMR surveillance program, is greatly needed and should be applied in both human and veterinary clinical medicine. The goal of the current study was to investigate AMR among bacterial isolates belonging to Enterobacterales in companion animals within the province of South Korea, with an emphasis on ESC resistance genes in E. coli, Klebsiella spp., Serratia spp., and Enterobacter cloacae.

2. Results

2.1. ESC Resistance Gene Detection

Among the 91 ESC-resistant Enterobacterales isolates analyzed in this study, all of the isolates harbored the blaoem gene. blaCTX-M (n = 42, 82.4%) was abundantly detected in E. coli isolates, whereas blashv (n = 16, 94.1%) was mainly detected in Klebsiella pneumoniae isolates (Table 1). None of the Serratia spp. and E. cloacae isolates, except one S. marcescens isolate from Serratia spp. that harbored blashv, were positive for blashv or blaCTX-M (Table 1). Among 55 blaCTX-M-positive Enterobacterales isolates, blaCTX-M-15 (n = 23, 41.8%) and blaCTX-M-35 (n = 12, 21.8%) were the most commonly detected, followed by blaCTX-M-14 (n = 7, 12.7%) (Table 2). Enterobacterales isolates carrying genes of blaCTX-M-3, blaCTX-M-61, blaCTX-M-62, and blaCTX-M-65 were also identified (Table 2). blaCTX-M-2, blaCTX-M-8, and blaCTX-M-25 gene clusters were not detected in any isolates. Among blaAmpC genes, blacit was prevalent in E. coli isolates, bladha was predominantly detected in Klebsiella spp. isolates, and blaebb was common in E. cloacae isolates (Table 3). No isolates carrying genes for blamo or bblaacc were detected.

2.2. Multi-Locus Sequence Typing (MLST)

Various sequence types (STs) were revealed among E. coli, K. pneumoniae, K. oxytoca, and E. cloacae isolates. STs of Serratia spp. isolates were not defined due to the lack of an MLST scheme for this genus. Among E. coli isolates, five ST131 and two ST3285 strains were detected from hospital-admitted dogs, with one of each of the following STs: ST372, ST457, ST648, ST1981, ST2179, ST2505, ST4616, ST5150, ST5667, ST8451, ST8885, ST8908, ST10207, ST10220, and ST11000. Accordingly, five ST405, three ST354, two each of ST3285, ST410, ST448, and ST457, and one each of ST68, ST38, ST648, ST1193, ST2541, ST7644, and ST10459 strains were detected in stray dogs. Among six E. coli isolates from hospital-admitted cats, two ST31, two ST156, and one each of ST1262 and ST6105 strains were obtained. In a comprehensive analysis of 19 Klebsiella spp. isolates from hospital-admitted dogs, one ST285, eight ST275, and six ST273 strains were identified among K. pneumoniae isolates, whereas one each of ST34, ST145, ST273, and ST293 strains were identified among K. oxytoca isolates. All Klebsiella spp. isolates from hospital-admitted and stray cats were identified as ST273 strains. Among seven E. cloacae isolates, two ST114 and one each of ST110, ST171, ST198, ST1252, and ST1303 strains were identified (Table 4).
Table 1. Distribution of extended-spectrum β-lactamase (ESBL) genes among 91 Enterobacterales isolates from companion animals.

| Organism       | Hospital-Admitted Dogs (n = 56) | Stray Dogs (n = 23) | Hospital-Admitted Cats (n = 11) | Stray Cats (n = 1) | Total |
|----------------|---------------------------------|---------------------|---------------------------------|-------------------|-------|
|                | bla<sub>CTX-M</sub> | bla<sub>SHV</sub> | bla<sub>TEM</sub> | bla<sub>CTX-M</sub> | bla<sub>SHV</sub> | bla<sub>TEM</sub> | bla<sub>CTX-M</sub> | bla<sub>SHV</sub> | bla<sub>TEM</sub> | bla<sub>CTX-M</sub> | bla<sub>SHV</sub> | bla<sub>TEM</sub> | bla<sub>CTX-M</sub> | bla<sub>SHV</sub> | bla<sub>TEM</sub> |
| E. coli (n = 51) | 21 | – | 22 | 15 | – | 23 | 6 | – | 6 | – | – | 42 | (82.4%) | – | 51 | (100%) |
| K. pneumoniae (n = 17) | 7 | 15 | 15 | – | – | – | 1 | 1 | 1 | 1 | – | 9 | (17.6%) | – | 16 | (100%) |
| K. oxytoca (n = 5) | 4 | 1 | 4 | – | – | – | – | – | – | 1 | – | – | 4 | (80.0%) | – | 2 | (40.0%) |
| S. marcescens (n = 4) | – | 1 | 3 | – | – | – | – | – | – | 1 | – | – | 1 | (45.0%) | – | 4 | (100%) |
| S. liquefaciens (n = 7) | – | – | 7 | – | – | – | – | – | – | – | – | – | 7 | (100%) | – | – | – |
| E. cloacae (n = 7) | – | – | 5 | – | – | – | – | 2 | – | – | – | – | 7 | (100%) | – | – | – |

Table 2. CTX-M subtype detection of 55 bla<sub>CTX-M</sub>-positive Enterobacterales isolates.

| Organism       | Sample Origin                      | CTX-M-1     | CTX-M-3 | CTX-M-61 | Total | CTX-M-9 | CTX-M-65 | Total | Unidentified |
|----------------|------------------------------------|-------------|---------|----------|-------|---------|----------|-------|--------------|
|                |                                    | CTX-M-15    | CTX-M-55 | CTX-M-9  | Total |         |          |       |              |
| E. coli (n = 42) | Hospital-admitted dogs (n = 21)    | 7           | 6       | 1        | 15    | 3       | 1        | 4     | 2            |
|                | Stray dogs (n = 15)                | 8           | 3       | –        | –     | 11      | 3        | 1     | 4            |
|                | Hospital-admitted cats (n = 6)     | 2           | 2       | –        | –     | 4       | –        | –     | 0            |
| K. pneumoniae (n = 9) | Hospital-admitted dogs (n = 7)    | 4           | 1       | –        | –     | 5       | –        | 1     | 1            |
|                | Hospital-admitted cats (n = 1)     | –           | –       | 1        | –     | 1       | –        | –     | 0            |
|                | Stray cats (n = 1)                 | –           | –       | –        | 0     | –       | –        | –     | 0            |
| K. oxytoca (n = 4) | Hospital-admitted dogs (n = 4)     | 2           | –       | –        | –     | 2       | 1        | –     | 1            |
| Total          |                                    | 23 (41.8%)  | 12 (21.8%) | 2 (3.6%) | 1 (1.8%) | 38 (69.1%) | 7 (12.7%) | 3 (5.5%) | 1 (1.8%) | 11 (20.0%) | 6 (10.9%) |
Table 3. Distribution of AmpC β-lactamase genes among ESBL-producing Enterobacterales isolates.

| Organism   | Sample Origin                  | AmpC β-Lactamases Gene |
|------------|--------------------------------|------------------------|
|            |                                | MOX  | CIT  | DHA  | ACC  | EBC  | FOX  | EBC+CIT | EBC+DHA | Total |
| E. coli    | Hospital-admitted dogs (n = 22) | −    | 5    | −    | −    | −    | −    | −        | −        | 5     |
|            | Stray dogs (n = 23)             | −    | 13   | −    | −    | −    | 1    | −        | −        | 14    |
| K. pneumoniae | Hospital-admitted dogs (n = 15) | −    | 1    | 4    | −    | 1    | −    | −        | −        | 6     |
|            | Hospital-admitted cats (n = 1)  | −    | −    | 1    | −    | −    | −    | −        | −        | 1     |
| K. oxytoca | Hospital-admitted dogs (n = 4)  | −    | −    | 2    | −    | −    | −    | −        | −        | 2     |
| E. cloacae | Hospital-admitted dogs (n = 5)  | −    | −    | 1    | 2    | −    | −    | −        | −        | 3     |
|            | Hospital-admitted cats (n = 2)  | −    | −    | −    | −    | −    | −    | −        | −        | 2     |
| Total      |                                | −    | 19   | 8    | −    | 3    | 1    | 1        | 1        | 33    |

Table 4. Multi-locus sequence typing (MLST) results based on the spread of β-lactamase resistance genes.

| Organism   | bla<sub>CTX-M</sub> Clusters | ESC Resistance Gene | No. Isolation |
|------------|------------------------------|---------------------|---------------|
|            |                             |                     | ESBLs         | AmpCs         | ST Type |
|            |                             |                     | Hospital-Admitted Dogs | Stray Dogs | Hospital-Admitted Cats | Stray Cats |
| CTX-M-1    | CTX-M-15                     | −                   | −             | −             | 648 (n = 1)  | 354 (n = 1) | 131 (n = 1) | −         | −         |
|            |                             |                     | −             | −             | 1981 (n = 1) | 2179 (n = 1) | 7644 (n = 1) | −         | −         |
| CTX-M-55   | −                           | −                   | +             | + CIT        | 4616 (n = 1) | 2505 (n = 1) | 410 (n = 1)  | −         | −         |
| CTX-M-9    | CTX-M-61                     | 3285 (n = 1)        | −             | + CIT        | 2505 (n = 1) | 410 (n = 1)  | 156 (n = 1)  | −         | −         |
|            |                             | 8908 (n = 1)        | −             | + CIT        | 3285 (n = 1) | 3285 (n = 1) | −             | −         | −         |
| CTX-M-27   | −                           | −                   | +             | −            | 10,220 (n = 1) | 10,220 (n = 1) | 131 (n = 2)  | −         | −         |
| CTX-M-27   | −                           | −                   | +             | −            | 10,220 (n = 1) | 10,220 (n = 1) | 131 (n = 2)  | −         | −         |
| CTX-M-27   | −                           | −                   | +             | −            | 1193 (n = 1)  | 1193 (n = 1)  | 156 (n = 1)  | −         | −         |
| CTX-M-27   | −                           | −                   | +             | −            | −             | −             | −             | −         | −         |
| Unidentified | −                         | 131 (n = 2)         | −             | −            | 156 (n = 1)  | 156 (n = 1)  | 156 (n = 1)  | −         | −         |
| Negative   | −                           | 372 (n = 1)         | −             | −            | 2541 (n = 1) | 2541 (n = 1) | 6105 (n = 1) | −         | −         |
| Total      |                                | 22                  | 23            | 6             | 0            | 0             | 0             | −         | −         |

K. pneumoniae

| CTX-M-1    | CTX-M-15                     | −                   | −             | −             | 273 (n = 2)  | 273 (n = 1)  | −             | −         | −         |
| CTX-M-55   | +                           | + EBC              | 285 (n = 1)  | −             | −             | −             | −             | −         | −         |
| CTX-M-3    | +                           | + DHA              | 275 (n = 1)  | −             | −             | −             | −             | −         | −         |
| CTX-M-9    | CTX-M-27                     | + DHA              | 275 (n = 1)  | −             | −             | −             | −             | −         | −         |
| CTX-M-65   | +                           | + DHA              | 275 (n = 1)  | −             | −             | −             | −             | −         | −         |
| Unidentified | −                         | −                   | −             | −             | 273 (n = 2)  | 273 (n = 1)  | −             | −         | −         |
| Negative   | −                           | +                   | −             | −             | 273 (n = 1)  | 273 (n = 1)  | −             | −         | −         |
| Total      |                                | 15                 | 0             | 1             | 1             | 1             | 1             | −         | −         |
Table 4. Cont.

| Organism     | bla<sub>CTX-M</sub> | bla<sub>SHV</sub> | bla<sub>TEM</sub> | ESC Resistance Gene | No. Isolation | ST Type |
|--------------|---------------------|-------------------|-------------------|---------------------|---------------|---------|
|              |                     |                   |                   |                     | Hospital-Amitted Dogs | Stray Dogs | Hospital-Amitted Cats | Stray Cats |
| **K. oxytoca** |                     |                   |                   |                     | 293 (n = 1)                  | –         | –                  | –         |
| CTX-M-1      | CTX-M-15            | –                 | +                 | DHA                 | 273 (n = 1)              | –         | –                  | –         |
| CTX-M-9      | CTX-M-14            | –                 | +                 | +                   | 145 (n = 1)              | –         | –                  | –         |
| Unidentified | –                   | –                 | +                 | DHA                 | 34 (n = 1)               | –         | –                  | –         |
| **Total**    | –                   | –                 | +                 | –                   | 4                         | 0         | 273 (n = 1)         | –         |
| **S. liquefaciens** | –           | –                 | +                 | –                   | ND (n = 7)               | –         | –                  | –         |
| **S. marcescens** | –             | –                 | +                 | –                   | ND (n = 2)               | –         | ND (n = 1)          | –         |
| **Total**    | –                   | –                 | +                 | –                   | 10                        | 0         | 1                  | 0         |
| **E. cloacae** |                     |                   |                   |                     | 196 (n = 1)              | –         | –                  | –         |
| Negative     | –                   | –                 | +                 | –                   | 114 (n = 1)              | 110 (n = 1) | –                  | –         |
| –            | –                   | +                 | –                 | DHA + EBC            | –                         | –         | –                  | 171 (n = 1) |
| –            | –                   | +                 | –                 | –                   | 1252 (n = 1)             | –         | –                  | –         |
| –            | –                   | +                 | –                 | –                   | 114 (n = 1)              | –         | –                  | –         |
| **Total**    | 5                   | 0                 | 2                 | 0                   |                           |           |                    |           |

* ND, non-defined.

2.3. Pulsed-Field Gel Electrophoresis (PFGE)

PFGE analysis was only conducted for 46 E. coli, 13 K. pneumoniae, five K. oxytoca, and six E. cloacae isolates; no or few banding patterns were obtained for five E. coli, four K. pneumoniae, and one E. cloacae isolates. Using a >85% similarity cut-off, 31 pulsotypes in E. coli (e1 to e31), nine pulsotypes in K. pneumoniae (kp1 to kp9), four pulsotypes in K. oxytoca (ko1 and ko4), and four pulsotypes in E. cloacae (ec1 to ec4) were identified. Generally, all isolates demonstrated unique PFGE profiles (57–95% similarity), indicating genetic heterogeneity in ESBL- or AmpC-producing strains (Figure 1).

2.4. Genetic Relatedness

In E. coli PFGE analysis, the e1 group consisted of three ST3285 strains containing bla<sub>CTX-M-55</sub>, bla<sub>TEM</sub>, and bla<sub>CIT</sub> from hospital-admitted and stray dogs and showed high similarity (>90%) (Figure 1a). Seven ST131 strains of E. coli isolates from five hospital-admitted dogs and two hospital-admitted cats were identified. PFGE results involved only five hospital-admitted dogs and one hospital-admitted cat, because the banding pattern for one hospital-admitted cat isolate was not defined. Two of them, which belonged to the e18 group, showed more than 93% similarity, compared with the remaining isolates in which low similarity was observed (<85%) (Figure 1a). e3 and e25 groups contained two ST448 strains with bla<sub>CTX-M-55</sub> and bla<sub>TEM</sub> and two ST457 strains with bla<sub>TEM</sub> and bla<sub>CIT</sub> from the same shelter and showed high similarity (>90%) (Figure 1a).

All bla<sub>TEM</sub>-positive K. pneumoniae isolates from hospital-admitted dogs harbored the bla<sub>SHV</sub> gene and exhibited 60–95% similarity (Figure 1b). One K. pneumoniae ST273 strain from stray cats belonged to the kp8 group and showed 87% similarity with hospital-admitted dog isolates (Figure 1b). Two ST275 strains co-carrying bla<sub>SHV</sub> and bla<sub>TEM</sub>, which belonged to the kp5 group, showed high similarity (>90%) (Figure 1b). The same pulsotype belonged to both ST273 and ST275, which were single-locus variants at the tonB allele; for example, kp1 and kp8. The remaining strains were independent of the groups obtained. Various STs and PFGE profiles were obtained for K. oxytoca and E. cloacae isolates, and genetic relatedness was not revealed for those strains (Figure 1c,d).
isolates demonstrated unique PFGE profiles (57–95% similarity), indicating genetic heterogeneity in ESBL- or AmpC-producing strains (Figure 1).

Figure 1. Cont.
Figure 1. Dendrogram of pulsed-field gel electrophoresis (PFGE) patterns showing the genetic relatedness of ESBL-producing Enterobacterales isolates. (a) Escherichia coli, (b) Klebsiella pneumoniae, (c) K. oxytoca, and (d) Enterobacter cloacae.

3. Discussion

This study presents the characteristics of 91 Enterobacterales isolates harboring ESC resistance genes, including E. coli, Klebsiella spp., Serratia spp., and E. cloacae, collected from South Korean companion animals between 2017 and 2019. All isolates harbored the blαTEM gene and demonstrated unique PFGE profiles. Similarly, a study by Shin et al. revealed that all E. coli isolates from beef cattle harbored the blαTEM gene [12]. Recent reports have identified CTX-M-type β-lactamases as the most widespread ESBL type, replacing classical TEM and SHV-type ESBLs [14]; however, TEM-type β-lactamases remained the most prevalent ESBL type identified in the current study. We observed varying predominant β-lactamase gene types in different Enterobacterales species, summarized as follows: blαCTX-M and blαCT in E. coli isolates, blαSHV and blαDHA in K. pneumoniae isolates, and blαEBC in E. cloacae. β-Lactamase gene distribution for each Enterobacterales species was similar to that described in a previous study with human samples [2]. These findings reveal that ESC resistance gene variants are not limited to certain hosts, emphasizing the need for coordinated control in both humans and animals.
In *E. coli* isolates in this study, *bla*<sub>TEM</sub> and *bla*<sub>CTX-M</sub> were most frequently detected, followed by *bla*<sub>CIT</sub>. Among *bla*<sub>CTX-M</sub>-positive isolates, *bla*<sub>CTX-M-15</sub> was the most commonly detected gene followed by *bla*<sub>CTX-M-55</sub> in both dogs and cats. A previous study investigating *E. coli* isolates from dogs reported that *bla*<sub>CTX-M-15</sub>, *bla*<sub>CTX-M-14</sub>, and *bla*<sub>CIT</sub> were the most prevalent β-lactamase genes, whereas *bla*<sub>CTX-M-55</sub> was rarely detected in South Korea [15]. However, *bla*<sub>CTX-M-55</sub>-carrying *E. coli* has become increasingly prevalent in dogs in South Korea [9]. The present study revealed that *bla*<sub>CTX-M-55</sub> was predominantly detected rather than *bla*<sub>CTX-M-14</sub> in *E. coli* from companion animals, which concurred with the results of the study by Hong et al. [9]. All five *E. coli* ST405 strains investigated in the current study harbored both *bla*<sub>TEM</sub> and *bla*<sub>CIT</sub> and were collected from stray dogs in the same shelter. The spread of *bla*<sub>CIT</sub>-carrying *E. coli* ST405 was described in a previous study, which suggested the possibility of direct transmission between humans and companion animals [9]. The spread of *E. coli* ST405 is usually described in humans harboring *bla*<sub>CTX-M-15</sub> [16]. However, *E. coli* ST405 did not harbor *bla*<sub>CTX-M-15</sub> in the current study. The increasing prevalence of *E. coli* ST131 carrying *bla*<sub>CTX-M-15</sub> has been described in humans and animals [15,17]. Unexpectedly, only one *bla*<sub>CTX-M-15</sub>-carrying *E. coli* ST131 strain was detected from hospital-admitted cats in this study. From *E. coli* PFGE results, the two ST3285 strains showing the same PFGE pattern were both from the same shelter and isolated on the same date (Figure 1a). In this case, it could be the result of contaminated samples during sampling or transmission of a same clone between the two stray dogs in a shared place.

Among the seven *K. pneumoniae* and four *K. oxytoca* isolates harboring *bla*<sub>CTX-M</sub> from hospital-admitted dogs in this study, the CTX-M-15 genotype accounted for a large proportion. A recent study also reported that *K. pneumoniae* isolates from companion animals producing CTX-M-15 either alone or in combination with DHA were frequently detected in South Korea [9]. The present MLST results revealed that ST275 and ST273 strains carrying both *bla*<sub>SHV</sub> and *bla*<sub>TEM</sub> were most commonly identified among *K. pneumoniae* isolates from hospital-admitted dogs. ST275 and ST273 are differentiated by one allele of the seven housekeeping genes, indicating that they are genetically close sequence types. Recently, the *bla*<sub>SHV</sub>- and *bla*<sub>TEM</sub>-co-carrying *K. pneumoniae* ST273 strain has emerged in human patients in Italy and is being disseminated, whereas ST273 and ST275 *Klebsiella* spp. isolates carrying both *bla*<sub>SHV</sub> and *bla*<sub>TEM</sub> have not yet been reported in South Korea [18,19]. Moreover, ST11, ST15, ST307, and ST392 strains have been globally identified as β-lactamase-producing *Klebsiella* spp. [9,20,21]. β-Lactamase-producing ST275 or ST273 strains among *Klebsiella* spp. isolates were newly discovered in South Korean companion animals in the current study.

All *E. cloacae* isolates in this study harbored *bla*<sub>TEM</sub> and some carried *bla*<sub>EBC</sub>, whereas none carried *bla*<sub>CTX-M</sub> and *bla*<sub>SHV</sub>. *E. cloacae* isolates from companion animals combined with more than two ESBL-type genes were described in Germany [22]. Meanwhile, *E. cloacae* isolates investigated in the present study harbored only one type of ESBL gene. In *Serratia* spp. isolates, only *bla*<sub>TEM</sub> was detected, except one *S. marcescens* isolate that harbored both *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub>. An *S. marcescens* isolate carrying *bla*<sub>TEM</sub> was previously identified in South Korea that caused urinary infections in humans [23]. However, in many countries including South Korea, the status of emerging AMR among *Serratia* spp. and *E. cloacae* in companion animals remains unknown. To our knowledge, this is the first report of ESC-resistant *Serratia* spp. and *E. cloacae* isolates from companion animals in South Korea.

In conclusion, we illustrated the presence and genetic heterogeneity of ESC-resistant Gram-negative bacteria in companion animals in South Korea, providing a potential reservoir of ESC-resistant bacteria and a transmission pathway. More organized surveillance is required to prevent and control the spread of ESC-resistant bacteria between companion animals and humans, in accordance with the “One Health” initiative.
4. Materials and Methods

4.1. Bacterial Characterization

Sampling, isolation, identification, antimicrobial susceptibility tests, and phenotypic characterization of Enterobacterales were previously studied [24,25]. In total, 91 Enterobacterales isolates carrying ESC resistance genes (51 E. coli, 17 K. pneumoniae, five K. oxytoca, four S. marcescens, seven S. liquefaciens, and seven E. cloacae) were collected from companion animals (56 hospital-admitted dogs, 23 stray dogs, 11 hospital-admitted cats, and one stray cat, Table 5). The antimicrobial resistance profile of the 91 bacterial isolates used in this study were summarized against cephalosporins (Table 5).

| Organism         | Origin                          | Cephalexin | Cefoxitin | Ceftiofur | Ceftriaxone | Cephalothin | Reference |
|------------------|---------------------------------|------------|-----------|-----------|-------------|-------------|-----------|
| E. coli (n = 51) | Hospital-admitted dogs (n = 22) | 95.5       | 27.3      | 95.5      | 95.5        | 100         | [25]      |
|                  | Stray dogs (n = 23)             | 100        | 47.8      | 100       | 100         | 100         |           |
| K. pneumonia (n = 17) | Hospital-admitted dogs (n = 15) | 53.3       | 60.0      | 33.3      | 40          | 53.3        | [25]      |
|                  | Hospital-admitted cats (n = 1)  | 100        | 100       | 100       | 100         | 100         | [24]      |
| K. oxytoca (n = 5) | Hospital-admitted dogs (n = 4)  | 75.0       | 50.0      | 100       | 100         | 100         | [25]      |
|                  | Stray cat (n = 1)               | 100        | 100       | 0         | 0           | 100         | [24]      |
| S. marcescens (n = 4) | Hospital-admitted dogs (n = 3)  | 100        | 100       | 0         | 0           | 100         | [25]      |
|                  | Hospital-admitted cat (n = 1)   | 100        | 100       | 0         | 0           | 100         | [24]      |
| S. liquefaciens (n = 7) | Hospital-admitted dogs (n = 5)  | 100        | 100       | 60.0      | 80.0        | 100         | [25]      |
|                  | Hospital-admitted cats (n = 2)  | 100        | 100       | 100       | 100         | 100         | [24]      |

4.2. Characterization of β-Lactamase Genes

PCR amplification of entire \( \text{bla}_{\text{CTX-M}}, \text{bla}_{\text{TEM}}, \) and \( \text{bla}_{\text{SHV}} \) genes was performed as previously described [24,25]. For \( \text{bla}_{\text{CTX-M}} \)-positive isolates, PCR and DNA sequencing were carried out for CTX-M-subtype detection. \( \text{bla}_{\text{CTX-M}} \) group-specific primers for five clusters (CTX-M-1, 2, 8, 9, and 25) were used following Kor-GLASS (Korea Global Antimicrobial Resistance Surveillance System) guidelines and previously published protocols [26,27]. DNA sequencing was performed by Intron Biotechnology (Seongnam, South Korea) and homologous sequences were searched against the GenBank database using the BLAST tool of the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST (accessed on 15 December 2020)). In ESBL-positive strains, six groups of AmpC β-lactamases (MOX, CIT, DHA, ACC, EBC, and FOX) were screened by PCR amplification [28].

4.3. Multi-Locus Sequence Typing

Multi-locus sequence types (STs) were based on the allelic profile of seven housekeeping genes. For E. coli, K. pneumoniae, K. oxytoca, and E. cloacae isolates, MLST for each bacterial strain was carried out in reference to previous studies [29–31]. PCR was performed using primers for the following: \text{adk}, \text{fumC}, \text{gyrB}, \text{idc}, \text{purA}, \text{mdh}, \text{and recA} (E. coli); \text{gapA}, \text{infB}, \text{mdh}, \text{pgi}, \text{phoE}, \text{rpoB}, \text{and tonB} (K. pneumoniae and K. oxytoca); \text{danA}, \text{fusA}, \text{gyrB}, \text{leuS}, \text{pyrG}, \text{rplB}, \text{and rpoB} (E. cloacae). Allelic profile and ST determinations were performed according to web-based MLST databases (https://pubmlst.org/databases/ (accessed on 15 December 2020) and https://bigsdb.pasteur.fr/klebsiella/klebsiella.html (accessed on 15 December 2020)).

4.4. Pulsed-Field Gel Electrophoresis

PFGE of XbaI (Takara Bio Inc., Shiga, Japan)-digested genomic DNA was carried out for E. coli, K. pneumoniae, K. oxytoca, and E. cloacae isolates according to the CDC PulseNet standardized procedure using the Chef Mapper system (Bio-Rad Laboratories, Hercules, CA, USA) [32]. PFGE analysis for Serratia spp. was ignored because different digested genomic DNA samples were employed for Serratia spp. Similarities between restriction frag-
ment length polymorphisms were analyzed using GelCompar II software v. 6.5 (Applied Maths NV, St-Martens-Latem, Belgium) to produce a dendrogram. The unweighted-pair group method using average linkages (UPGMA) cluster analysis was conducted based on an 85% similarity cut-off with 0.5% optimization and 2.0% band tolerance.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

AmpC: AmpC β-lactamase; AMR, antimicrobial resistance; ESBL, extended-spectrum β-lactamase; ESC, extended-spectrum cephalosporin; MLST, multi-locus sequence typing; PFGE, pulsed-field gel electrophoresis; ST, sequence type.

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