Antimicrobial Resistance of Bacterial Isolates from Positive Urine Culture in Four Hundred Five Dogs Between 2013-2014

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ABSTRACT
We collected urine samples from dogs admitted to a South Korean referral animal hospital for diagnostic procedures. The samples were cultured and investigated for antimicrobial resistance. A total of 469 bacterial strains from 405 dogs were isolated and subjected to antimicrobial susceptibility testing. A total of 158 (33.7%) and 311 (66.3%) gram positive and negative bacterial strains, respectively, were identified. Bacterial strains identified included Escherichia coli (32.8%), Staphylococcus spp. (17.9%), Enterococcus spp. (12.4%), and Proteus mirabilis (11.9%). The multi-drug resistance rate for the entire bacterial population was 82.3%; the rates for E. coli and Staphylococcus spp. were 80.5% and 67.9%, respectively. Coagulase-negative Staphylococcus spp. accounted for 88.1% with multi-drug resistance rate of 68.9%. Imipenem, amikacin, chloramphenicol, amoxicillin/clavulanic acid, and norfloxacin (in decreasing order) had the greatest impact factors on the bacterial populations. Considering the impact factor of individual antimicrobial drugs and resistance rates for each bacterial strain in this study, amoxicillin-clavulanic acid may be recommended as a first-line drug for urinary tract infection. However, multi-drug resistance rates were high.; Therefore, bacterial culture and antimicrobial susceptibility tests should always be performed.

INTRODUCTION
Antimicrobial resistant bacterial strains have emerged as a worldwide concern for not only humans, but small animals as well. Under such circumstances, bacteria with resistance are being referred to as multidrug resistant organisms (MDROs), and while development of new antibiotics is slow going, the number of MDROs is increasing at a rapid pace. The problem at hand does not refer to the fact that MDROs are more virulent than non-MDROs, but that it is difficult to find the appropriate antimicrobials to use because of resistance to a wide variety of antimicrobials. Because animals use antimicrobials in human medicine, companion animals can act as reservoirs of MDROs.9,29 Recently, antibiotic surveillance programs have been carried out in small animals such as dogs and cats.16,20,21

Typical routes for transmitting bacteria from animal to human are bite, fecal-oral ingestion, inhalation, physical injuries, and urine.7 The causative agents of urinary tract infections (UTI) that are usually isolated in dogs include Escherichia (E.) coli, Staphylococcus spp., Proteus spp., Streptococcus spp., Klebsiella spp., and Enterococcus spp.14,23 However, the types and percentages of causative agents and MDROs or their antimicrobial resistance vary by region or veterinary hospital.3 Although such studies have been reported in various regions and hospitals,17,21,30,31 South Korea has no study using many individuals on this topic.

We conducted a retrospective study on the prevalence of MDROs and the antimicrobial susceptibility pattern of bacteria isolated from canine positive urine culture in a South Korean referral animal hospital aiming to compare results with other regions and hospitals, and subsequently find antibiotics that can be used as effective empirical therapy in dogs.

MATERIALS AND METHOD
Urine Samples
We obtained electronic record charts of patients admitted to Haemaru referral animal hospital in South Korea between January 2013 and December 2014. A total of 405 dogs with positive urine culture results were included in the study. The records included the patient’s signalments (age, breed, and sex), urine specific gravity, urine dipstick analysis, microscopic sediment analysis, bacterial species identification, and antimicrobial susceptibility analysis. Each patient had 5 mL of urine collected aseptically via ultrasound-guided cystocentesis and renal pelvic puncture.
Urine Culture and Antimicrobial Susceptibility Analysis

A sterile swab was used to inoculate 5 mL of urine with 5% sheep blood agar (Asanpharm, Korea), and all media were incubated for 24-48 hr at 37 °C. All bacteria were isolated and identified, then tested for susceptibility using antibiotics using an agar disk diffusion method. The test was performed by applying a bacterial inoculum of approximately 1–2×10⁸ CFU/mL to the surface of a large (150 mm diameter) Mueller-Hinton agar plate. Up to 12 commercially prepared, fixed concentration, paper antibiotic disks were placed on the inoculated agar surface. Plates were incubated for 16–24 h at 35°C prior to determination of results. The zones of growth inhibition around each of the antibiotic disks were measured to the nearest millimeter.

In the antimicrobial susceptibility test, azithromycin, amikacin, oxacillin, ampicillin, amoxicillin/clavulanic acid, ciprofloxacin, norfloxacin, cephalaxin, cephalothin, cefotaxime, cefovecin, trimethoprim-sulfamethoxazole, aztreonam, chloramphenicol, clindamycin, doxycycline, imipenem, vancomycin, polymyxin B, and tobramycin were used. Since the antibiotics panel was different according to gram positive and negative, and partially changed in 2014, antibiotics applied to some bacteria (azithromycin, aztreonam, cephalaxin, polymyxin B, and tobramycin) were excluded from the analysis (as shown in Table 2). All procedures were performed in Samkwang medical laboratories (South Korea), in accordance with the regulations set by the Clinical and Laboratory Standards Institute (CLSI).

The diameter of the clear zone in each antibiotic disk was measured and the bacterial isolates were classified as being susceptible, intermediate, or resistant to the antibiotic. Intermediate was regarded as resistant. Moreover, to help choose a reasonable selection of antibiotics, impact factors (FRAT) according to the type of major bacterial isolates (E.coli, Staphylococcus spp., Enterococcus spp., Proteus mirabilis, Klebsiella pneumoniae, Pseudomonas aeruginosa) were calculated [2]. Total impact factor (Fs) represents the sum of impact factor of each pathogen; \( P_{\text{pathogen}(i)} \) represents the percentage a specific pathogen accounts for among all pathogens; and \( S_{\text{antimicrobial}} \) represents the percentage of susceptibility that a specific pathogen has to a specific antimicrobial agent.

\[
F_s = \sum_{i=1}^{n} P_{\text{pathogen}(i)} \times S_{\text{antimicrobial}} \times 100
\]

Statistical Analysis

A computer software statistical package (Prism 6 Version 6.01; Graphpad) was used for statistical analysis. For data comparison between each group, a chi squared or Fisher’s exact test was performed. For all comparisons, a value of \( P < 0.05 \) was considered significant.

RESULTS

A total of 469 bacterial strains were isolated and identified through aerobic culturing from 405 dogs. The median age of the dogs was 10 years (range 0.4-19 years). There were:

- 105 intact females (25.9%)
- 161 neutered females (39.8%)
- 23 intact males (5.7%)
- 116 castrated males (28.6%)

The most common breeds were:

- Shih Tzu (n=91, 22.5%)
- Miniature Schnauzer (n=79, 19.5%)
- Maltese (n=78, 19.3%)
- Cocker Spaniel (n=28, 7%)
- Yorkshire Terrier (n=24, 6%), and
- Pekinese (n=20, 5%)

The dogs were divided into a younger dog group (0.4-7 years) and older dog group (≥ 8 years) for age-based MDRO infection rate analysis. However, the results did not show a statistically significant difference (\( P=0.88 \)). Sex-based MDRO infection rate did not show a significant difference (\( P=0.97 \)). Although there were no significant differences in MDRO rate between intact and neutered males (\( P=0.59 \)), neutered fe-
males had a significantly higher MDRO rate than intact females (P=0.03).

Among the 469 bacterial strains, 158 strains (33.7%) were gram- positive bacteria, while 311 strains (66.3%) were gram-negative bacteria. There was no statistical difference between gram-positive and negative bacteria with respect to the multi-drug resistance rate (P=0.31). Among the bacteria, *Escherichia coli* was the most prevalent with a total of 154 isolations (32.8%), followed in order by *Staphylococcus* spp. 17.9 (84), *Enterococcus* spp. 12.4 (58), *Enterococcus faecalis* was 8.7 (41), and *Enterococcus faecium* 3.4 (16). *Enterococcus gallinarum* 0.3 (1)

Among the total of 469 bacterial isolations, 444 strains (94.7%) showed resistance to at least one antibiotic. Cases involving resistance to three or more classes of antibiotics were defined as multi-drug resistant (MDR) and 82.3% of the 469 strains were identified as MDROs. The MDR rate of gram-positive and negative bacteria was 79.7% and 83.6%, respectively. Antimicrobial resistance analysis was performed on the six types of bacteria most commonly isolated and summarized in Table 2. The MDR rate of *Staphylococcus* spp. was 67.9%, however, the MDR rate of methicillin-resistant coagulase negative *Staphylococcus* (MRCoNS) reached 100%. *Enterococcus* spp. and *Pseudomonas aeruginosa* were all MDROs (100%). MDR rates of bacterial isolates are shown in Table 3.

Antibiotic impact factor calculated using the FRAT equation found imipenem to have the highest value (79), followed in order by amikacin (70.7), chloramphenicol (60.9), amoxicillin/clavulanic acid (55.6), and norfloxacin (52). Meanwhile, clindamycin (10.3) and oxacillin (16.8) showed the lowest values (Table 4). In gram-negative bacteria, the highest impact factor was found in imipenem in gram negative bacteria and vancomycin in gram positive bacteria.

**DISCUSSION**

The present study performed was a retrospective study on the antimicrobial resistance and identification of MDROs for positive urine culture in dogs. Comparison between the results from this study and other studies showed differences based on
<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Gram Positivity</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli</td>
<td>&lt; 0.0001</td>
<td>0.1387-0.2760</td>
</tr>
<tr>
<td>Staphylococcus spp.</td>
<td>0.0644</td>
<td>1.006-3.104</td>
</tr>
<tr>
<td>Enterococcus spp.</td>
<td>0.5963</td>
<td>0.3880-1.561</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>0.3024</td>
<td>0.3951-1.261</td>
</tr>
<tr>
<td>Klebsiella Pneumoniae</td>
<td>0.4729</td>
<td>0.7024-2.194</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>0.7488</td>
<td>0.6220-2.188</td>
</tr>
</tbody>
</table>

Table 2. Antimicrobial resistance of bacteria isolated from urine specimens in dogs (%). These 6 types of bacteria were classified as either Gram-positive or Gram-negative.
breed distribution. However, age and sex distributions were similar. The most common bacteria in this study were *E. coli*, *Staphylococcus* spp., and *Enterococcus* spp. These and are mostly similar to the findings in other studies for canine urine samples. Although *E. coli* was the most commonly isolated bacterial strain, other bacterial isolates varied in percentage and order. These studies may have shown different results due to differences in the study period, region, and dog population.

*E. coli* is a primary causative agent of UTI, and is a normal part of the intestinal flora of mammals. As they are key sources which can horizontally transfer antimicrobial resistance genes to other pathogens, *E. coli* serves as a good indicator of the

### Table 3. Multi-drug resistance (%) in bacterial isolations from urine specimens in dogs.

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Number of bacterial isolates (total n)</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>124 (154)</td>
<td>80.5</td>
</tr>
<tr>
<td><em>Staphylococcus</em> spp.</td>
<td>57 (84)</td>
<td>67.9</td>
</tr>
<tr>
<td><em>Enterococcus</em> spp.</td>
<td>58 (58)</td>
<td>100</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em></td>
<td>49 (56)</td>
<td>87.5</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>35 (44)</td>
<td>79.5</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>33 (33)</td>
<td>100</td>
</tr>
<tr>
<td><em>Enterobacter</em> spp.</td>
<td>10 (13)</td>
<td>76.9</td>
</tr>
<tr>
<td><em>Corynebacterium</em> spp.</td>
<td>4 (6)</td>
<td>66.7</td>
</tr>
<tr>
<td><em>Streptococcus</em> group G</td>
<td>2 (5)</td>
<td>40</td>
</tr>
</tbody>
</table>

### Table 4. Antibiotic impact factors calculated using the FRAT equation. Among all bacteria, 6 bacteria most commonly isolated were selected and used for impact factor calculation. *E. coli*, *Staphylococcus* spp., *Enterococcus* spp., *Proteus mirabilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>All organisms</th>
<th>Gram (+)</th>
<th>Gram (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>70.7</td>
<td>16.4</td>
<td>54.3</td>
</tr>
<tr>
<td>Amoxicillin/clavulanic acid</td>
<td>55.6</td>
<td>18.3</td>
<td>37.3</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>22.4</td>
<td>4.8</td>
<td>17.6</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>10.4</td>
<td>10</td>
<td>0.4</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>42.8</td>
<td>9.2</td>
<td>33.6</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>60.9</td>
<td>20.2</td>
<td>40.7</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>44.8</td>
<td>16.7</td>
<td>28.1</td>
</tr>
<tr>
<td>Cefovecin</td>
<td>40.8</td>
<td>11.4</td>
<td>29.4</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>28.6</td>
<td>11.1</td>
<td>17.5</td>
</tr>
<tr>
<td>Imipenem</td>
<td>79</td>
<td>18.9</td>
<td>60.1</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>16.8</td>
<td>9.8</td>
<td>7</td>
</tr>
<tr>
<td>Trimethoprim/Sulfamethoxazole</td>
<td>34.2</td>
<td>8.2</td>
<td>26</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>29.9</td>
<td>29.9</td>
<td>0</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>52</td>
<td>16.5</td>
<td>35.5</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>31.1</td>
<td>8.1</td>
<td>23</td>
</tr>
</tbody>
</table>
presence of an antimicrobial resistance reservoir.\textsuperscript{25} Other studies have reported the isolation of antimicrobial resistant \textit{E. coli} in dogs,\textsuperscript{22,23} and one study reported isolation of extended-spectrum \textbeta-lactamases (ESBLs)-producing \textit{E. coli} from both healthy and infected dogs.\textsuperscript{18}

The use of antibiotics in dogs over the past decade has been reported to be associated with the pattern of increased resistance of \textit{E. coli} to antibiotics.\textsuperscript{6,23} MDR \textit{E. coli} isolated from urine in dogs was
\begin{itemize}
  \item 15.3\% (England)\textsuperscript{27}
  \item 31.2\% (Taiwan)\textsuperscript{5}
  \item 52.6\% (USA)\textsuperscript{6}
  \item 81.2\% in the present study.
\end{itemize}

Patients and testing methods may have influenced the study results. However, regional differences and the level of exposure to the antibiotic could also be considered as influencing factors.

In the present study, a total of 84 \textit{Staphylococcus} spp. strains were isolated, which accounted for 17.9\% of isolated bacteria. Other studies that have investigated UTIs in dogs reported 14.7\%\textsuperscript{10} and 20.1\%\textsuperscript{24} However, the percentage of CoNS differed in these studies compared to our study. The proportion of CoNS were 3.7\%\textsuperscript{10} and 48.6\%\textsuperscript{24} in the other studies, respectively, and 88.1\% in this study. CoNS is a primary bacterium that forms the normal flora in dogs, as well as humans.\textsuperscript{14} Previously, CoNS was considered mostly as a bacterial contaminant\textsuperscript{26} and it did not receive much attention because of its weak virulence.\textsuperscript{12} However, there are increasing numbers of cases of opportunistic infections involving CoNS acting as MDR strains that are difficult to treat, especially in individuals with compromised immunity.\textsuperscript{32} As such, its significance as a human pathogen has grown, and it has become one of the bacteria that is now closely monitored. CoNS in dogs has started to receive attention in recent times and studies are beginning to emerge. In the present study, urine samples were obtained aseptically via cystocentesis, thus the possibility of CoNS being a bacterial contaminant is very low. In this study, among all CoNS, the rate of resistance to at least one antibiotic was 91.9\% and they were found to be especially highly resistant to ampicillin (91.4\%), cephalothin (57.1\%), cefotaxime (51.4\%), doxycycline (63.4\%), and trimethoprim-sulfamethoxazole (56.3\%).

Resistance of CoNS to oxacillin was found to be 36.5\%, and this is important because generally, cross-resistance to other antibiotics occurs more commonly in methicillin-resistant staphylococci (MRS) than in methicillin-sensitive staphylococci (MSS). Thus, higher rates of MDR is found among methicillin-resistant CoNS (MRCoNS).\textsuperscript{11} This presents an even bigger problem as one study reported that MRCoNS accounts for approximately 91\% of all clinical strains in humans.\textsuperscript{13} Methicillin resistance in staphylococci is mediated by the mecA gene, which encrypts penicillin binding protein 2a (PBP2a) and PBP2a mediates the role of reducing the affinity to beta-lactam antibiotics.\textsuperscript{4} A study from Nigeria reported that when MRCoNS was isolated from healthy dogs and tested, 81.3\% were found to be MDROs.\textsuperscript{3} MDR-CoNS in the present study was 74.3\%, while MDR-MRCoNS was 100\%. A high number of patients admitted to our hospital are elderly dogs, which have a lot of experience of being exposed to antibiotics and most have been infected with underlying diseases that can compromise their immunity. Therefore the possibility of opportunistic infection by CoNS is likely.

To determine the appropriate antimicrobial as a first-line drug or empirical therapy to be applied prior to bacterial culture, a FRAT equation was used to calculate the impact factor of each antibiotic (Table 4). The impact factor of an antimicrobial represents the treatment rate when that antimicrobial is used for a UTI. In the present study, imipenem, amikacin, and chloramphenicol had the highest impact factors. Besides these, those with an impact factor greater than 50 included amoxicillin/clavulanic acid and norfloxacin. Antimicrobials with the lowest impact factors included clindamycin (10.3),
penicillin class antibiotics, such as oxacillin (16.8) and ampicillin (22.4). In a consensus statement produced by the American College of Veterinary Internal Medicine (ACVIM), recommendations for antibiotic use involved 3 or 4 categories of classification. As empirical therapy for UTIs is used mostly in non-life-threatening conditions, prescribing first-line antibiotics for such cases is appropriate.

First-line drugs are older and often used in human medicine, having a narrower spectrum, and are typically considered less important for treating serious human infections or where development of resistance is of lesser concern. Antibiotics in this category include: penicillin, first- and second-generation cephalosporins, tetracyclines, and trimethoprim-sulfonamide. Considering these standards, along with the resistance rate and impact factor found in this study, amoxicillin-clavulanic acid can be recommended as the first-line drug for UTI in dogs. Although imipenem had the highest impact factor, it corresponds to a third-line drug. A third-line drug is one that must be used based on culture and susceptibility test results under situations involving serious, life-threatening infections and carbapenems such as imipenem fall into this category.

CONCLUSION
In this study, *E. coli*, *Staphylococcus* spp., and *Enterococcus* spp. were identified as major causative agents isolated from urine specimen in dogs. These bacteria have relatively high antimicrobial resistance and CoNS was also found to have high resistance rates. The findings in this study can be used as treatment guidelines for treating UTIs in animal hospitals. We recommend amoxicillin-clavulanic acid as a first-line drug. The predominant causative agents show high antimicrobial resistance, thus development of new antibiotics is important, but even more important is the proper use of antibiotics. Therefore, efforts should be taken to keep good antimicrobial stewardship in order to reduce resistance rates and increase treatment rate, while also decreasing the spread of resistant bacterial strains between animals and humans.

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CONFLICTS OF INTEREST
These is no conflict of interest.

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