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High prevalence of *Enterococcus* spp. from dogs with otitis externa

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Abstract

Otitis externa (OE) is a frequent disease in the ear canals of dogs. To identify the pathogens causing OE in dogs and to determine their antimicrobial resistances, specimens were collected from animal hospitals in Daejeon. The isolates were examined by morphological and biochemical tests, 16S rRNA analysis and antimicrobial susceptibility tests. We analyzed correlation between the isolated pathogens and external factors of dogs such as breed, age, gender, ear mite, hair in ears and experience with antibiotic therapy. Thirty three strains of bacteria were isolated from 26 of the 68 heads of dogs with OE. The most isolated bacteria were *Enterococcus faecalis* (*E. faecalis*) followed by *Staphylococcus aureus* (*Sta. aureus*), *Sta. pseudointermedius*, *E. faecium*, *E. avium* and *Streptococcus canis* (*Strep. canis*) in order of frequency of occurrence. Isolation frequency of *Enterococcus* spp. and *Staphylococcus* spp. were 51.5% and 45.5%, respectively. *E. faecalis* and *E. faecium* isolates showed VanB phenotype, which is resistant to vancomycin but sensitive to teicoplanin were 58% and 25%, respectively. Nine isolates among total twelve isolates of *E. faecalis* were isolated from the dogs treated with antibiotics. There was no methicillin-resistant *Sta. aureus* (MRSA), but were MR-*Sta. pseudointermedius* (MRSP) (57.1%) and vancomycin-resistant (VR)-*Sta. pseudointermedius* (14.3%) (VRSP) showing VanB phenotype. However, *vanA*, *vanB* and *vanC* genes were not detected in VR isolates from the dogs. Taken together, VR-*Enterococcus* spp. (VRE) is one of the major pathogens in domestic animals, as well as community- and hospital-acquired infection.

Key words : Dog, Otitis externa, *Enterococcus* spp., Antimicrobial resistance, *van* gene

INTRODUCTION

Otitis externa (OE) is a frequent disease in the ear canal of a dog. Ear disease occupies 10～20% of dog disease and OE comprises 30% of them (Angus, 2004; Eom et al, 2000). OE is an inflammation in the ear canal with a multifactorial etiology. Various combinations of the primary, predisposing, and perpetuating causes are interacting in occurrences of OE. Predisposing factors are ear conformation, excessive moisture and excessive cerumen production. Primary factors are microorganisms, parasites, hypersensitivity diseases, keratinization disorders, autoimmune diseases and viral diseases. Frequent primary factors are allergenic dermatitis (43%), grass awns (12%) and ear mites (7%) in order of frequency of occurrence in terms of etiology of OE (Lilenbaum et al, 2000; Saridomichelakis et al, 2007). Perpetuating factors are bacteria, yeast and progressive pathological changes (Rosser, 2004). Eventually, the perpetuating factors prevent effective healing of OE. The most frequently isolated microorganism from dogs with OE is *Staphylococcus pseudintermedius* (*Sta. pseudintermedius*) (formerly referred to as *Sta. intermedius*), followed by *Malassezia pachydermatis*, *Sta. aureus*, *Pseudomonas aeruginosa*, *Streptococcus canis* (*Strep.*

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canis), *Proteus mirabilis* and *Escherichia coli* in order of frequency of occurrence (Lyskova et al, 2007; Oliveira et al, 2008; Zamankhan et al, 2010). Recently, antimicrobial resistances of these bacteria have been reported. Especially, some fluoroquinolone resistant bacteria are increasing (Oliveira et al, 2008; Yoo et al, 2010; Yoon et al, 2010). This might be due to excessive administering and unnecessary abuse. For this reason, there is no good response about OE treatment.

Enterococcus spp. is Gram-positive cocci and reclassified from Lancefield group D Streptococci. It is a normal flora in human intestines. In humans, isolation frequencies of *Enterococcus faecalis* (*E. faecalis*) and *E. faecium* are 90～95% and 5～10% each. *E. casseliflavus*, *E. gallinarum* and *E. raffinosus* have pathogenicity in animals (Kim et al, 2010a). These usually develop gastroenteritis in patients. It can be treated by ampicillin and vancomycin. Vancomycin is a glycopeptide antibiotic used to Gram-positive bacteria, and has been traditionally used after treatment with other antibiotics such as penicillin and methicillin had failed in clinics. Recently, vancomycin resistant bacteria have been increasing, and displaced by linezolid, daptomycin, and quinupristin/dalfopristin. Particularly, vancomycin-resistant *Enterococcus* spp. (VRE) has been trouble for hospital-acquired infection, and has to be treated with multiple antibiotic therapy due to multi-drug resistances (Kim et al, 2010a; Lai et al, 2011).

The purposes of this study were to identify the pathogens causing OE in dogs and to determine their antimicrobial resistances. Specimens were collected from the ears of dogs with OE. The isolates were examined by morphological, biochemical tests, 16S rRNA analysis and antimicrobial susceptibility tests. In addition, we analyzed correlation between the isolated pathogens and external factors of dogs such as breed, age, gender, ear mite, hair in ears and experience with antibiotic therapy.

MATERIALS AND METHODS

Samples

Samples were collected from 68 dogs with OE hospitalized at animal hospitals in Daejeon. The specimens were divided into several groups by external characteristics of dogs such as breed, age, gender, ear mite, hair in ears and experience with antibiotic therapy. The ear exudates of dogs were sampled with cotton swabs, stored to transport media, and transferred to laboratory to isolate bacterial pathogens from the samples.

Bacterial isolation and identification

Samples were cultured on 5% sheep blood agar (SBA, Komed, Korea) and Sabouraud dextrose agar (SDA, Difco, USA) at 37°C for 18 hrs. Then, isolated bacteria and fungi were primarily classified by morphological characters such as Gram stain patterns and catalase and oxidase tests. Mannitol salt agar (MSA, Difco, USA), 0.04% tellurite BHI (BHI-tel) media and MacConkey agar (Mac, Difco, USA) were used as selective media for the isolation of *Staphylococcus* spp., *Streptococcus* spp. and *Enterococcus* spp., and Gram-negative bacilli such as *E. coli*, respectively. The cultures were carried out under aerobic and anaerobic conditions. The isolates were identified using API kits (BioMérieux, France) or Vitek system (BioMérieux, France). The identified bacteria were confirmed through a more accurate genotyping test based on nucleotide sequences of 16S rRNA. For genotyping, the isolates were incubated in a brain-heart infusion broth (BHI-broth) at 37°C for 18 hrs. Their genomic DNAs were extracted using an Accuprep genomic DNA extraction kit (Bioneer, Korea). PCR using 16S rRNA primer sets was conducted as described previously (Kim et al, 2010b). After electrophoresis on agarose gels, individual amplified products were purified using an Accuprep gel purification kit (Bioneer, Korea). Then, the purified DNA was analyzed by DNA sequencing. The genotypes of the isolates were determined by BLAST analysis of the obtained base sequences. The sequences were identified through BLAST (http://www.ncbi.nlm.nih.gov) analysis. For analysis of *van* gene typing, PCR using *van* primer sets was conducted as described previously (Kim et al, 2010b).

Antimicrobial susceptibility

The antimicrobial susceptibility test of the isolates was performed using Vitek GPS-450 and 451 kits (BioMérieux, France) for Gram-positive bacteria and Vitek GPS-433 and 434 kits (BioMérieux, France), or the disk diffusion method, for Gram-negative bacteria. The media used for antibiotic tests were Mueller Hinton agar or Blood agar and the disk diffusion method was conducted according to the Clinical Laboratory Standards Institute (CLSI, 2009) guidelines. The bacteria were dissolved into tryptic soy broth (TSB) and cultured for 1～2 hrs to make a bacterial suspension with a turbidity of MacFarland No. 0.5. The suspension was evenly smeared on Mueller Hinton agar using swabs and an antibiotic disk was placed on it. The bacteria were cultured for 18～24 hrs at 37°C and their resistances to antibiotics were determined by measuring the diameter of the inhibition zone around the antibiotic disks. The isolation ratios of the isolates showing antibiotic resistance were statistically analyzed.

The selected antibiotics were clindamycin (5 μg), erythromycin (15 μg), linezolid (30 μg), oxacillin (1 μg), penicillin (10 U), rifampicin (5 μg), sulphamethoxazole-trimethoprim (25 μg), teicoplanin (30 μg), telithromycin (15 μg), tetracycline (30 μg), and vancomycin (30 μg) for *Staphylococcus* spp., ampicillin (10 μg), cefotaxime (30 μg), chlorampenicol (30 μg), ciprofloxacin (5 μg), clindamycin (2 μg), erythromycin (15 μg), gentamicin (10 μg), imipenem (10 μg), linezolid (30 μg), nitrofurantoin (300 μg), penicillin (10 U), quinupristin-dalfopristin (15 μg), streptomycin (10 μg), teicoplanin (30 μg), tetracyclin (30 μg), and vancomycin (30 μg) for *Streptococcus* spp., and ampicillin (10 μg), chlorampenicol (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), imipenem (10 μg), linezolid (30 μg), nitrofurantoin (300 μg), penicillin (10 U), quinupristin-dalfopristin (15 μg), streptomycin (10 μg), teicoplanin (30 μg), tetracyclin (30 μg), and vancomycin (30 μg), for *Enterococcus* spp.

Table 1. Isolation frequencies of pathogens isolated from dogs with otitis externa

Genus	No. of isolated species (%)	No. of isolated genus (%)
Enterococcus		17 (51.5)
E. faecalis	12 (36.4)	
E. faecium	4 (12.1)	
E. avium	1 (3.0)	
Staphylococcus		15 (45.5)
Sta. aureus	8 (24.2)	
Sta. pseudintermedius	7 (21.2)	
Streptococcus		1 (3.0)
Strep. canis	1 (3.0)	
Total	33 (100)	33 (100)

RESULTS

Isolation frequency of pathogenic bacteria from the dogs

Pathogenic bacteria were isolated from 26 of the 68 dogs. Thirty three bacterial stains were isolated from the dogs but no fungus. The most frequently isolated bacteria was *E. faecalis* (36.4%), followed by *Sta. aurues* (24.2%), *Sta. pseudintermedius* (21.2%), *E. faecium* (12.1%), *E. avium* (3.0%) and *Strep. canis* (3.0%) in order of frequency of occurrence. *Enterococcus* spp. (51.5%) was a more isolated strain than *Staphylococcus* spp. (45.5%). *E. faecalis* was the most isolated pathogen in *Enterococcus* spp. The isolation frequency of *Sta. aureus* and *Sta. pseudintermedius* was similar as shown in Table 1.

Emergence of vancomycin-resistant pathogen

Almost all of *E. faecalis* isolates were resistant to gentamicin, quinupristin-dalfopristin and streptomycin, and were resistant to penicillin but sensitive to imipenem, nitrofurantoin and teicoplanin. Likewise, most *E. faecium* isolates were resistant to ampicillin and tetracycline, and were sensitive to imipenem, nitrofurantoin and teicoplanin like those of *E. faecalis* isolates. *E. faecalis* and *E. faecium* isolates showed VanB phenotype which is resistant to vancomycin but sensitive to teicoplanin were 58% and 25%, respectively (Table 2), (Kim et al, 2010b). All of *Staphylococcus* spp. isolates were

Table 2. Antimicrobial resistance patterns of the *Enterococcus* spp. isolates

Antimicrobial drugs	*E. faecalis*	*E. faecium*
Ampicillin	3/12*	4/4
Chlorampenicol	4/12	1/4
Ciprofloxacin	1/12	1/4
Gentamicin	12/12	3/4
Imipenem	0/12	0/4
Linezolid	6/12	3/4
Nitrofurantoin	0/12	0/4
Penicillin	12/12	3/4
Quinupristin-Dalfopristin	12/12	3/4
Streptomycin	12/12	3/4
Teicoplanin	0/12	0/4
Tetracyclin	11/12	4/4
Vancomycin	7/12	1/4

*Numbers of the isolates showing antimicrobial resistance/numbers of the total isolates.

Table 3. Antimicrobial resistance patterns of the *Staphylococcus* spp. isolates

Antimicrobial drugs	*Sta. aureus*	*Sta. pseudintermedius*
Clindamycin	2/8*	4/7
Erythromycin	2/8	4/7
Linezolid	0/8	0/7
Oxacillin	0/8	4/7
Penicillin	0/8	0/7
Rifampicin	1/8	0/7
Sulphamethoxazole-Trimethoprim	4/8	4/7
Teicoplanin	0/8	0/7
Telithromycin	2/8	4/7
Tetracycline	6/8	7/7
Vancomycin	0/8	1/7

*Numbers of the isolates showing antimicrobial resistance/numbers of the total isolates.

sensitive to almost all of antibiotics, especially antibiotics that inhibit synthesis of cell walls. Susceptibilities of *Sta. aureus* isolates were higher than those of *Sta. pseudintermedius*. *Sta. pseudintermedius* isolates were resistant to tetracycline. There was no methicillin-resistant *Sta. aureus* (MRSA) isolates but were MR-*Sta. pseudointermedius* isolates (57.1%). Moreover, one strains of vancomycin-resistant (VR)-*Sta. pseudointermedius* (VRSP) (14.3%) showing VanB phenotype were isolated (Table 3). However, *vanA*, *vanB*, and *vanC* genes were not detected in VR isolates of both *Enterococcus* spp. and *Staphylococcus* spp. The antimicrobial susceptibility patterns of *E. avium* and *Strep. canis* isolates are shown in Table 4. *E. avium* was susceptible to vancomycin and teicoplanin. Therefore, 8 isolates (47.1%) among total 17 *Enterococcus* spp. isolates showed VanB phenotype without *van* gene.

Table 4. Antimicrobial resistance patterns of the *E. avium* and *Strep. canis* spp. isolates

Antimicrobial drugs	*E. avium*	*Strep. canis*
Ampicillin	S*	R
Chlorampenicol	S	R
Ciprofloxacin	S	I
Gentamicin	S	S
Imipenem	S	S
Linezolid	S	R
Nitrofurantoin	S	S
Penicillin	S	R
Quinupristin-Dalfopristin	R†	S
Streptomycin	S	S
Teicoplanin	I‡	S
Tetracyclin	R	R
Vancomycin	S	S
Cefotaxine	-	S
Clindamycin	-	S
Erythromycin	-	S

*Susceptibility, †Resistance, ‡Intermediate.

Table 5. Comparison of isolation frequencies of pathogens from the dogs with or without antibiotic therapy

Species	Treated group	Non-Treated group	Total
E. faecalis	9 (27.3)*	3 (9.1)	12 (36.4)
E. faecium	0 (0)	4 (12.1)	4 (12.1)
E. avium	1 (3.0)	0 (0)	1 (3.0)
Sta. aureus	4 (12.1)	4 (12.1)	8 (24.2)
Sta. pseudintermedius	3 (9.1)	4 (12.1)	7 (21.2)
Strep. canis	0 (0)	1 (3.0)	1 (3.0)
Total	17 (51.5)	16 (48.5)	33 (100)

*Numbers of the isolates (%).

High frequency of *E. faecalis* in dogs treated with antibiotics

In comparing the relationship between the frequency of specific pathogenic bacteria and external factors of dogs such as breed, age, gender, ear mite, hair in ears and experience with antibiotic therapy, 9 isolates (75%) among 12 isolates of *E. faecalis* were isolated in the

Table 6. Comparison of isolation frequencies of pathogens from the dogs by age

Species	Over 1 year	Under 1 year	Total
E. faecalis	11* (33.3)	1 (3.0)	12 (36.4)
E. faecium	0 (0)	4 (12.1)	4 (12.1)
E. avium	1 (3.0)	0 (0)	1 (3.0)
Sta. aureus	4 (12.1)	4 (12.1)	8 (24.2)
Sta. pseudintermedius	3 (9.1)	4 (12.1)	7 (21.2)
Strep. canis	0 (0)	1 (3.0)	1 (3.0)
Total	19 (57.6)	14 (42.4)	33 (100)

*Numbers of the isolates (%).

Table 7. Comparison of isolation frequencies of pathogens from the dogs by external factor of dog

Species	Male	Ear mite	Hair in ears
E. faecalis	5/12*	3/12	4/12
E. faecium	3/4	3/4	3/4
E. avium	1/1	1/1	0/1
Sta. aureus	5/8	4/8	5/8
Sta. pseudintermedius	4/7	3/7	3/7
Strep. canis	1/1	1/1	1/1

*Numbers of the positive isolates/numbers of the total isolates.

dogs treated with antibiotics. In contrast, all isolates of *E. faecium* were isolated in non-treated group (Table 5). Most of *E. faecalis* isolates were isolated from the over 1-year-old dogs (91.7%), but all *E. faecium* isolates were isolated from the under 1-year-old group (Table 6). There was no relationship between the external characteristics except for experience with antibiotic therapy and age (Table 7).

DISCUSSION

A dog is a companion animal and is a part of a number of families. When a dog is infected with microorganisms, the dog can become a carrier to infect a human. We isolated numbers of *Enterococcus* spp. (51.5%), which is a major pathogen in community and hospital-acquired infection. This is the first report studying *Enterococcus* spp. isolated from dogs with OE in Korea (Lyskova et al, 2007; Oliveira et al, 2008; Zamankhan et al, 2010). There are some studies referring *Enterococcus* spp. isolated from OE. Brothers et al (2002) isolated one *Enterococcus* spp. from OE. Nevertheless, they did not identify an exact species. De Graef et al (2001) isolated two *Enterococcus* spp. and identified them as *E. faecium* at first. After DNA analysis, those were conformed as *Enterococcus canis*. Hariharan et al (2006) isolated 9 *E. faecalis* among 44 *Enterococcus* spp. They suggested that increase of antibiotic resistance of *Enterococcus* spp. resulted from the treatment of otitis caused by *Staphylococcus* spp. However, they focused at *Staphylococcus* spp. and *Pseudomonas* spp.. We isolated 12 *E. faecalis* and 4 *E. faecium* to cause pathogenicity to human. This result suggests that natural acquired VRE without *van* gene has been increasing and is one of serious problems in public health.

Most of *Enterococcus* spp. isolates were *E. faecalis*. The *E. faecalis* were isolated from dogs over 1 year old. All *E. faecium* were isolated from dogs under 1 year old (Table 6). However, more information is needed to elucidate correlation with disease occurrence by each *Enterococcus* spp. and age of dog. In addition, *E. faecalis* are mostly isolated in dogs treated with antibiotics (75%). This suggests that appropriate treatment is not conducive to healing dogs with OE.

Occurrence of OE by *Enterococcus* spp. has not been fully understood. There are some reports about *E. faecalis* prevalence isolated from feces of other animals such as chickens and pigs (Hwang et al, 2011; Zou et al, 2011). Among the isolated *Enterococcus* spp., the isolation rate of VRE showing VanB phenotype was 47.1% in this study. High frequency of VRE isolates is a serious problem in public health as well as human and animal diseases (Vincze et al, 2010). Isolation frequency of *Staphylococcus* spp. was still high though is lower than that of *Enterococcus* spp. There was no MRSA but was MR-*Sta. pseudintermedius* (MRSP), which showed multidrug resistance (MDRSP). The increase of MRSP or MDRSP causes an increase of OE in a dog. Moreover, VRSP was isolated in this study. Recently, emergence of coagulase-negative *Staphylococcus* spp. (CNS) such as *Sta. pseudintermedius*, and MRCNS or MDCNS has been increasing and is a serious problem in humans and animals (Lilenbaum et al, 2000; Yoo et al, 2010; Yoon et al, 2010).

Although OE by fungi has still been occurring, patho-

genic fungi were not isolated from the samples in this study. To reduce the antibiotic resistance, prohibition of excessive administering and unnecessary abuse is required.

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