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Tetracycline resistance determinants in *Staphylococcus intermedius* isolated from dogs in Jeju

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ABSTRACT

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One hundred-two *Staphylococcus intermedius* were isolated from diseased dogs and healthy dogs to investigate the antimicrobial resistant rates on 15 commonly used drugs and tetracycline resistant gene (*tet* gene) was analysed on 78 tetracycline resistant isolates. Fifty-four *S. intermedius* isolates were recovered from oral cavity, nasal cavity and/or cranial hair coat cultures of 20 clinically healthy dogs. *S. intermedius* was colonized at more than one sites, including 18 (90.0%) cranial hair coat, 10 (50.0%) oral cavity, and 8(40.0%) nasal cavity of healthy dogs. Antibiograms of these commensal isolates were compared to antibiograms from 48 historical clinical isolates (2003-2006) obtained from cases of canine pyoderma (24), otitis externa (8), nasal discharge (12), pyometra (2) or cystitis (2). Antimicrobial resistant test were performed by disk diffusion test of CLSI and final resistance decisions on oxacillin and vancomycin were

made by minimum inhibitory concentration. All isolates from both healthy and diseased dog were susceptible to vancomycin and only those from the former were sensitive to amoxicilline/clavulanic acid, and cefazolin, while 8 % of those from the latter were resistant to both antibiotics. Among *S. intermedius* isolates recovered from diseased dogs, resistance was most often seen to penicillin (85%), ampicillin (81%) tetracycline (79%), erythromycin (52%), trimethoprim/sulfamethoxazole (48%), kanamycin (44%), norfloxacin and ciplofloxacin (38%), and gentamicin (35%). Resistance was also noted, but to a lesser degree, to neomycin (23%) and cloramphenicol (17%). Among the *S. intermedius* isolates recovered from healthy dogs, resistance was most often observed to penicillin (80%), ampicillin (80%), tetracycline (78%), kanamycin (65%), and erythromycin (44%), trimethoprim/ sulfamethoxazole (43%) and gentamicin (33%). Resistance was also noted, but to a lesser degree, to chloramphenicol (28%), norfloxacin (22%), ciprofloxacin (20%) and neomycin (24%). The commensal isolates were lesser resistant to most antimicrobials than those of diseased dogs, exception with chloramphenicol, kanamycin, and neomycin. The data from this study might serve as a guideline in selecting drugs to be used for treating dogs with staphylococcal infections.

S. intermedius harboring *tet*(M) and *tet*(K) were 62 (79.5%) and 3 (3.8%) strains, respectively and 3 (3.8%) and 4 (5.1%) isolates were harboring both *tet*(M) and *tet*(K), and *tet*(M) and *tet*(L), respectively.

Keywords: *Staphylococcus intermedius*, Antimicrobial resistance, Tetracycline resistant determinents, Dog

CONTENTS

Chapter Ⅱ. Tetracycline resistance determinants

Chapter I. Antimicrobial Resistance of *Staphylococcus intermedius* Isolates

Introduction

Staphylococcus intermedius is a coagulase-positive zoonotic pathogen found in pigeons, dogs, foxes, mink, and horses [16]. Initially, all coagulase-positive staphylococci were identified as *S. aureus*, until Hajek in 1976 established the unique identity of a group of organisms, originally identified as *S. aureus* biotypes E and F, as *S. intermedius* [16]. *S. intermedius* is a common commensal of oral, nasal, and skin flora in healthy dogs, where it can also be that the predominant pathogen isolated from dogs with cutaneous infections and is an important cause of ocular disease, otitis externa, cystitis, respiratory and wound infections [6, 16, 30, 35, 41, 45]. There are no vaccines to control these diseases [44]. This organism is increasingly reported to be resistant to many antibiotics [2, 19, 25, 27, 30, 34, 38, 39, 40, 46,47] and failures in treatment are a cause of problems in small animal practices. Moreover, *S. intermedius* can transfer occasionally from dogs to humans [12. 17] and the risk of owners being infected by resistant strains must be considered [14, 15]. However, little was known about the study of the antimicrobial susceptibility patterns of theses isolates from dogs in Korea. For these reasons, a survey of trends in the susceptibility of canine *S. intermedius* strains to antimicrobial drugs appeared to be great importance in the selective use of chemotherapeutics, the evaluation of new antimicrobial agents and the development of drug resistance

through continuous use of antimicrobial agents against field isolates.

The present study was designed to evaluate the degree of *in vitro* activity of different antimicrobial agents against *S. intermedius* strains recently isolated in Jeju from clinically healthy and diseased dogs, such as pyoderma, otitis externa, respiratory disease, pyometra, and cystitis, and to provide a progress report on antimicrobial resistance patterns in this bacterial species.

MATERIALS AND METHODS

Bacterial isolation and identification

Fifty-four *S. intermedius* isolates were collected between April 2006 to July 2006 from oral cavity, nasal cavity and/or cranial hair coat cultures of 20 clinically healthy dogs visiting in private veterinary practice clinics in the Jeju-si, the south Korea for the vaccination. The specimens were collected by the method of the previous study [9]. A sterile BBL Culture Swab (Becton, Dickinson and CO., Franklin Lakes, NJ) was used to sample the tonsilar areas of the oral cavity. The cranial hair coat was sampled using a sterile BBL Culture-Swab (Becton, Dickinson and CO., Franklin Lakes, NJ) moistened in Stuart's medium and rubbed vigorously against the grain over the hair and skin of the shoulder area followed by the top of the head. Swabs were immediately placed in Stuart's transport medium (Becton, Dickinson and CO., Franklin Lakes, NJ) and kept at 4℃. Swabs were inoculated within 24 h by spread plating onto trypticase blood agar base supplemented with 5% sheep blood and Columbia CNA agar supplemented with 5% sheep blood. A single representative colony of each different morphological type resembling *S. intermedius* was isolated and identified from each culture using standard identification procedures [24]. A commercial identification system (ATB 32 Staph-system, BioMerieux, France) was used to further speciate the isolates. In addition, 48 *S. intermedius* isolates recovered between May 2003 and July 2006 from clinical cases of canine pyoderma(11) nasal discharge (n=12), otitis externa (n=6), cystitis (n=2), pyometra (n=2) were included in the study. These pyoderma isolates were

recovered from veterinary schools in Cheju National University, Jeju-si, Jeju-do, South Korea. All isolates were stored at -80℃ until analysis.

Antimicrobial susceptibility test

Once samples were identified, the staphylococcal strains were tested for susceptibility to antibiotics by disc agar diffusion in accordance with NCCLS guidelines [31]. Discs of antibiotics commonly used in clinical veterinary medicine for suppurative diseases were tested: Ampicillin (AM), Amoxicillin/Clavulanic acid (AMC), Chlorampenicol (C), Ciprofloxacin (CIP), Cephazolin (CZ), Erythromycin (E), Gentamicin (GM), Kanamycin (KM), Neomycin (N), Norfloxacin (NOR), Oxacillin (OX), Penicillin (P), Trimethoprim/Sulfamethoxazole (SXT), Tetracycline (T), Vancomycin (Va). After measuring the zones of inhibition, the strains were classified as sensitive, intermediate or resistant to the drug according to the literature [21] and manufacturer [BD, Franklin Lakes, NJ, USA].

Oxacillin agar screening test

For the agar screening test, strains were plated on tryptic soy agar with 5% sheep blood and a 0.5 McFarland standard suspension was prepared for each sample. All isolates were plated on Mueller-Hinton agar supplemented with 4% (w/v) NaCl and Mueller-Hinton agar supplemented with 4% NaCl containing oxacillin at a concentration of 6 ㎍/ml according to National Committee for Clinical Laboratory Standard guidelines [32]. Oxacillin resistance was conferred by

bacterial growth after 24 and 48 h of incubation at 35 ℃ on both plates. The minimum inhibitory concentration (MIC) for oxacillin was determined in the staphylococcal strains growing onto the plate containing 6 ㎍/ml of oxacillin.

Vancomycin agar screeing test

For the agar screening test, strains were prepared as oxacilline agar screening test. All isolates were plated on braine heart infusion agar (BHIA) and BHIA containing vancomycin at a concentration of 6 ㎍/ml according to Centers for Disease Control and Prevention guidelines [4]. Vancomycin resistance was conferred by bacterial growth after 24 h of incubation at 35 ℃ on plates. The minimum inhibitory concentration (MIC) for vancomycin was determined in the staphylococcal strains growing onto the plate containing 6 ㎍/ml of vancomycin.

Results

Bacterial strains and isolation

A total of 48 *Staphylococcus intermedius* were isolated from 24, 12, 8, 2 and 2 samples collected from pyoderma, nasal discharge, otitis externa, cystitis and pyometra, respectively (Table 1).

Table 1. Numbers of *Staphylococcus intermedius* isolated from 48 diseased dog.

Disease	No. of *Staphylococus intermedius* isolated
Pyoderma	24
Nasal discharge	12
Otitis externa	8
Cystitis	2
Pyometra	2
Total	48

Among 20 healthy dogs, the *S. intermedius* were recovered from one or more sites of 19 dogs (95%). A total of 54 strains of *S. intermedius* were recovered from 10 (50%, 12 strains), 8 (40%, 12 strains) and 18 (90%, 30 strains) of each 20 samples taken from the oral and nasal cavities, and cranial hair coats, respectively (Table 2).

Table 2. Isolation rates of *Staphylococcus intermedius* from nasal cavity, oral cavity and skin of 20 healthy dog.

Sampling sites	No. of dogs positive (%)	No. of *Staphylococcus intermedius isolated*
Oral cavity	10 (50)	12
Nasal cavity	8 (40)	12
Cranial hair coat	18 (90)[a)]	30
Total	19 (95)	54

[a)] one sample was not able to isolate any bacteria for the overgrowth of *Proteus* sp.

Amtimicrobial resistance

All 102 *S. intermedius* isolates from both healthy (54 strains) and diseased dog (48 strains) were susceptible to vancomycin and only those from the former were sensitive to amoxicilline/clavulanic acid, and cefazolin, while 8 % of those from the latter were resistant to both antibiotics. Among *S. intermedius* isolates recovered from diseased dogs, resistance was most often seen to penicillin (85%), ampicillin (81%) tetracycline (79%), erythromycin (52%), trimethoprim/sulfamethoxazole (48%), kanamycin (44%), norfloxacin and ciplofloxacin (38%), and gentamicin (35%). Resistance was also noted, but to a lesser degree, to neomycin (23%) and cloramphenicol (17%). Among the *S. intermedius* isolates recovered from healthy dogs, resistance was most often observed to penicillin (80%), ampicillin (80%), tetracycline (78%), kanamycin (65%), and erythromycin (44%), trimethoprim/sulfamethoxazole (43%) and gentamicin (33%). Resistance was also noted, but to a lesser degree, to chloramphenicol (28%), norfloxacin (22%), ciprofloxacin (20%) and

neomycin (24%). The commensal isolates were lesser resistant to most antimicrobials than those of diseased dogs, exception with chloramphenicol, kanamycin, and neomycin (Fig. 1).

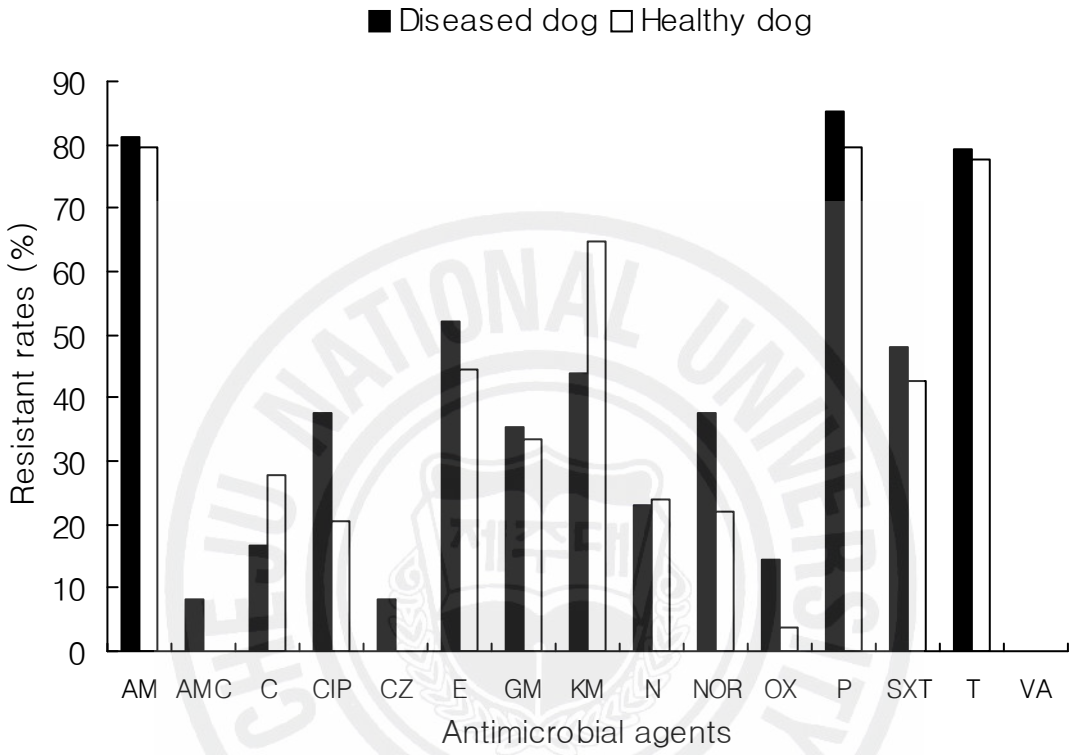

Fig. 1. Antimicrobial resistant rates of *Staphylococcus intermedius* isolated from healthy and diseased dogs. Ampicillin(AM), Amoxicillin/Clavulanic acid (AMC), Chlorampenicol (C), Ciprofloxacin (CIP), Cephazolin (CZ), Erythromycin (E), Gentamicin (GM), Kanamycin (KM), Neomycin (N), Norfloxacin (NOR), Oxacillin (OX), Penicillin (P), Trimethoprim/Sulfamethoxazole (SXT), Tetracycline (T), Vancomycin (Va).

Antimicrobial resistance patterns

A total of 31 resistance patterns were observed in 48 *S. intermedius* strains from diseased dogs (Table 3). Fourteen antimicrobial agents, except for vancomycin were affected by resistance. Overall 47 of the 48 strains (98.0%) were resistant to two or more antimicrobial drugs and 43 strains (90%) were resistant to three or more antimicrobial drugs (multiresistance). Five oxacillin resistant strains were resistant to six to fourteen antibiotic agents and AM, P, T-mutiresistant strains were most prominent as 7 strains (14.6%).

A total of 37 resistance patterns were observed in 54 *S. intermedius* strains from healthy dogs (Table 4). As like as the results of diseased dogs, fourteen antimicrobial agents, except for vancomycin were affected by resistance. Overall, all 54 strains were resistant to one or more antimicrobial drugs and 45 strains (83.3%) were resistant to three or more antimicrobial drugs (multiresistance). Two oxacillin resistant strains were resistant to AM,CIP,E,GM,KM,NOR or AM,AMC,C,CIP,CZ,E,GM,KM,N,NOR,P,SXT,T.

S. intermedius with same antibiograms in mouth, nose and/or cranial hair coat were isolated from only 4 healthy dogs and other isolates showed totally different antibiograms, indicating that *S. intermedius* colonized in the dogs as transient, intermittent, or persistent might be different strains in same time.

Table 3. Antimicrobial resistance patterns of *Staphylococcus intermedius* strains isolated from diseased dogs

Antimicrobial resistance patterns	Total, *n* (%)
No resistance	1 (2.1)
Resistance to	
SXT,T	1 (2.1)
AM,P	3 (6.3)
AM,C,T	1 (2.1)
AM,P,SXT	1 (2.1)
AM,P,T	7 (14.6)
C,E,KM	1 (2.1)
P,SXT,T	1 (2.1)
AM,AMC,E,P	1 (2.1)
AM,E,P,T	3 (6.3)
C,E,GM,KM	1 (2.1)
E,GM,KM,T	1 (2.1)
AM,CIP,NOR,P,T	2 (4.2)
AM,C,KM,P,T	1 (2.1)
AM,KM,P,SXT,T	2 (4.2)
AM,AMC,CZ,E,N,P	1 (2.1)
AM,CIP,CZ,NOR,P,T	1 (2.1)
AM,GM,KM,P,SXT,T	1 (2.1)
AM,E,GM,P,SXT,T	1 (2.1)
CIP,NOR,OX,P,SXT,T	1 (2.1)
AM,E,GM,KM,P,SXT,T	1 (2.1)
C,CIP,E,GM,N,NOR,R,T	1 (2.1)
CIP,E,GM,KM,NOR,P,SXT,T	1 (2.1)
AM,C,CIP,E,GM,KM,N,NOR,P	1 (2.1)
AM,CIP,E,GM,KM,NOR,P,SXT,T	1 (2.1)
AM,CIP,E,KM,N,NOR,P,SXT,T	2 (4.2)
AM,CIP,E,KM,NOR,OX,P,SXT,T	1 (2.1)
AM,CIP,E,GM,KM,N,NOR,P,SXT,T	2 (4.2)
AM,AMC,C,CZ,E,GM,KM,P,SXT,T	1 (2.1)
AM,CIP,E,GM,KM,NOR,OX,P,SXT,T	1 (2.1)
AM,CIP,E,GM,KM,N,NOR,OX,P,SXT,T	3 (6.3)
AM,AMC,C,CIP,CZ,E,GM,KM,N,NOR,OX,P,SXT,T	1 (2.1)
Total	48 (100)

Ampicillin(AM), Amoxicillin/ Clabulanic acid (AMC), Chlorampenicol (C), Ciprofloxacin (CIP), Cephazolin (CZ), Erythromycin (E), Gentamicin (GM), Kanamycin (KM), Neomycin (N), Norfloxacin (NOR), Oxacillin (OX), Penicillin (P), Sulfamethoxazole/Trimethoprim (SXT), Tetracycline (T), Vancomycin (Va).

Table 4. Antimicrobial resistance patterns of *Staphylococcus intermedius* strains isolated from healthy dogs

Antimicrobial resistance patterns	Total, *n* (%)
Resistance to	
AM	1 (1.9)
E	1 (1.9)
T	5 (9.3)
GM,KM	1 (1.9)
N,T	1 (1.9)
AM,P,T	4 (7.4)
AM,NOR,P,SXT	1 (1.9)
AM,KM,P,T	1 (1.9)
AM,KM,P,SXT	1 (1.9)
AM,P,SXT,T	2 (3.7)
AM,C,E,KM,P	1 (1.9)
AM,C,GM,P,T	1 (1.9)
AM,C,KM,P,T	1 (1.9)
AM,C,N,P,T	1 (1.9)
AM,GM,KM,P,SXT	1 (1.9)
AM,GM,KM,P,T	1 (1.9)
AM,KM,P,SXT,T	1 (1.9)
CIP,KM,NOR,P,T	1 (1.9)
CIP,E,NOR,SXT,T	1 (1.9)
AM,C,E,KM,N,P	5 (9.3)
AM,C,GM,KM,P,T	1 (1.9)
AM,CIP,KM,NOR,P,T	1 (1.9)
AM,E,GM,P,SXT,T	1 (1.9)
AM,E,KM,P,SXT,T	3 (5.6)
AM,GM,KM,P,SXT,T	1 (1.9)
AM,C,E,GM,KM,P,T	1 (1.9)
AM,C,E,KM,N,P,SXT	1 (1.9)
AM,CIP,E,GM,KM,NOR,OX	1 (1.9)
AM,CIP,GM,KM,NOR,P,T	2 (3.7)
AM,E,KM,N,P,SXT,T	2 (3.7)
AM,C,E,GM,KM,N,PT	2 (3.7)
C,CIP,E,GM,KM,NOR,SXT,T	1 (1.9)
AM,C,E,KM,N,P,SXT,T	1 (1.9)
AM,CIP,GM,KM,NOR,P,SXT,T	1 (1.9)
AM,CIP,E,GM,KM,NOR,P,SXT,T	1 (1.9)
AM,C,CIP,E,GM,KM,NOR,P,SXT,T	1 (1.9)
AM,CIP,E,GM,KM,NOR,OX,P,SXT,T	1 (1.9)
Total	54 (100)

Ampicillin(AM), Amoxicillin/ Clabulanic acid (AMC), Chlorampenicol (C), Ciprofloxacin (CIP), Cephazolin (CZ), Erythromycin (E), Gentamicin (GM), Kanamycin (KM), Neomycin (N), Norfloxacin (NOR), Oxacillin (OX), Penicillin (P), Sulfamethoxazole/Trimethoprim (SXT), Tetracycline (T), Vancomycin (Va).

Discussion

The present study confirms the occurrence of *Staphylococcus intermedius* strains in various sites of healthy dogs as well as variety diseases of dogs, due to the isolation of 54 strains of *S. intermedius* from oral and nasal cavities and cranial hair coats of 20 healthy dogs and 48 isolates from dogs with pyoderma, otitis externa, raspiratory disease, cystitis, and pyometra of 48 dogs. This is expected results, since privious study usually reports *S. intermedius* is a normal inhabitant of canine skin; however, it becomes the primary or secondary agents in certain diseases, such as the bacterial dermatitis, otitis externa [1, 5, 29, 43].

S. intermedius may function as a super-antigen and regulate the immune system [7, 18]. *S. intermedius* can produce numerous toxins and readily demonstrates antibiotic resistance [7]. In our study, of 102 *S. intermedius* isolates from both healthy and diseased dog were susceptible to vacomycin but 101 strains (99%) were resistant to one or more antimicrobial agents.

The antimicrobial resistance for *S. intermedius* is well documented in the literature outside of Korea [13, 33, 40, 43]. Most importantly the penicillins and tetracyclines are described virtually useless, as most strains are resistant to these compounds [7, 43]. Amoxicillin with clavulanic acid, cephalosporins, potentiated sulfa drugs, erythromycin and fluoroquinolones are usually effective [33, 36, 43].

Since the introduction of penicillin, methicillin and oxacillin into clinical use, staphylococci have obtained resistance to β-lactam antibiotics [38]. Resistant to penicillin in *Staphylococcus* spp. is a well-known problem in human and veterinary medicine. The secretion of β-lactamase is very frequent in *S. intermedius* strains from dogs and leads to the non-prescription of penicillin, ampicillin and amoxicillin [38].

The previous studies have been reported a high percentage of resistance to penicillin in staphylococci isolated from pyoderma in

dogs: 50 to 74%. According to Lloyd et al. [27] the resistance to penicillin increased from 69% in 1980 to 89% in 1996. According to Reedy [42], the resistance to ampicillin increased from 41% to 67% in the United States, between 1982 and 1994. In this study, data revealed high both ampicillin and penicillin-resistant *S. intermedius*; around 80%, comparing with the previous reports of other countries [25, 26, 37, 38]. An increase over time of resistance to penicillin and ampicillin in staphylococci may be explained by the selective pressure exerted by considerable use of penicillin and ampicillin in treating many diseases in dogs.

The methicillin-resistant *S. aureus* strains are usually resistant to all the group M; penicillins, including oxacillin and cloxacillin. Methicillin has never been used in animal antibiotherapy in Korea, but the other two drugs are widely used in *S. aureus* cow mastitis treatment and in *S. intermedius* dog pyodermitis treatment [38]. Very different from the hospital methicillin-resistant *S. aureus* strains, is the situation in *S. intermedius* strains. Only few group M-penicillin resistance strains were found in dog *S. intermedius* strains. In this study, 3.7% (2/54) of healthy dog origins and 14.6% (7/48) of diseased dog origins were resistant to oxacillin. Investigations conducted in several other countries also revealed oxacillin resistant *S. intermedius*, but usually low occurrence (0 to 3.3%) were reported [25, 26, 37, 38].

Only 0 to 2.5% of the strains were resistant to cefalexine in the UK [27], in the United States [26], in Norway [25], in France [38], and in Sweden [37]. We found the relatively higher cefazolin resistant *S. intermedius* (8%) isolated from the diseased dogs, whereas did not from the healthy dogs. We also found the same situation on amoxycillin with clavulanic acid (8% resistant from the diseased dogs and 100% sensitive from the heathy dogs). This indicates that the dog continue administrated with antibiotics might have more resistant strains because our bacteria tested were isolated from dog failed to treat in the local animal hospital. However, oxacillin, amoxycillin/calvulanic acid or cefazolin is one of the best

qualities for the treatment of dog staphylococcal disease.

According to Pellerin et al. [38], the resistance to Kanamycin increased from 18.9% in 1987-88 to 22% in 1992, and Kunkle[26] has been reported 50% of the strains isolated in dogs with prior antibiotic therapy were resistant to kanamycin. According to Pellerin et al. [38], kanamycin should be replaced by gentamicin, but 44% of the strains from the diseased dogs and 65% from the healthy dogs were resistant to kanamycin, and both originated *S. intermedius* were moderately resistant (33 and 35%) to gentamicin. We do not have any explanation for this difference between them, but it should be replaced by other aminoglycosides, such as amikacin, tobramycin because both antibiotics, kanamycin and gentamicin have been used for long time without prescription by veterinarian in Korea.

Although chloramphenicol has rarely been used as therapy for *S. intermedius* infections, it has been used for the treatment of many bacterial disease and *S. intermedius* is commonly sensitive to the antibiotics (80 to 90%). According to Pellerin et al. [38] chloramphenicol is no longer of interest for the treatment of dog pyodermas, as the frequency of chloramphenicol resistance is increased with time. However, the resistant rates were around 25% in our results, indicating that the antimicrobial resistant rates may depend on the regions and countries.

The frequency of tetracycline resistance bacteria is well-known. As previously reported by others [25, 26, 30, 33]. Tetracycline shows a high percentage (35-60%) of resistant strains. According to Reedy, the resistance to tetracycline was, in the United States, at the level of 49% of the stains in 1982, and according to Pellerin et al. [38], the resistance to tetracycline increased from 19% to 40% of the strains between 1988 and 1993, and then was stable. However, our results showed much higher resistance rates to tetracycline in accordance to 90% resistant to the drugs of the previous report in Korea [23].

The striking increase in resistance to the lincosamides, lincomycin and clindamycin and to the macrolide erythromycin between the two

periods was the most significant finding by Kruse et al. [25] in 1996. Accoding to the Pellerin et al. [38], these drugs in canine dermatology has increased steadily over the last decade and a stable and relatively high frequency of resistance for 23% to 30% of the strains tested. However, 44% and 52% of the strains isolated from the healthy and the diseased dogs were respectively found in this study. This finding is in accordance to the study of Kunkle [25] with some difference, where nearly all the strains of *S. intermedius* isolated in dogs without prior antibiotic therapy are susceptible to macrolides, as compared to nearly half of the strains isolated in dogs with prior antibiotic therapy being resistant to macrolides.

Trimethoprim-sulphonamides combinations have been used extensively in most cases of first-time pyoderma in dogs [3, 8]. An interesting observation was to increase number of resistant strains to trimethoprim-sulphonamides with time, from 5% in 1987-88, to 20% in 1992 and to 36% in 1995-96 [38]. Some studies have revealed relatively low frequencies of resistance to trimethoprim or trimethoprim-sulphonamides [19, 25, 27, 30], but others have reported resistance rates reaching 70% [2]. In this study, 43% of healthy dog origins and 48% of diseased dog origins were resistant to trimethoprim-sulphonamides combinations.

Fluoroquinolones are recommended principally for antimicrobial therapy in mixed infection, in recurrent pyoderma, in chronic, deep pyoderma with extensive scar tissue, or when canine pyoderma has proved to be refractory to 'first-line' antibiotics [20]. The increasing use of fluoroquinolones over the past years has given rise to an increase in resistance to fluoroquinolones [10, 20, 28, 40, 46]. Ciprofloxacin and norfloxacin have been little used for the treatment of dog diseases in Korea, but in this study, 25% of healthy dog origins and 35% of diseased dog origins were resistant to ciprofloxacin and/or norfloxacin.

The many different resistance patterns observed confirm other studies, reporting a great variety of resistance patterns in staphylococci. However, any comparison between the proportions of

multidrug-resistant organisms and those referred to in other veterinary reports in confounded by inconsistencies in defining multiple drug resistance, and different reporting techniques. During this study, most of strains were resistant to three or more antimicrobial drugs. Multiple antimicrobial-resistant *S. intermedius* isolates from canine pyodermas [11] have been reportedly transmitted from infected dogs to their owners [15, 16]. Also, there was a report of a mastoid cavity infection in the owner caused by *S. intermedius* transmitted from his dog's saliva [22]. Therefore, high occurrence of multi-antimicrobial resistant strains in dogs could increase the implications of zoonotic transmission of multi -antimicrobial resistant *S. intermedius*.

Conclusion

Staphylococcus intermedius strains isolated form dogs, were most highly resistant to penicillin, ampicillin, and tetracycline, and also high resistance were observed on erythromycin, kanamycin, fluoroqiunolons, trimethoprim/sulfamethoxazole, and gentamicin. Resistance is very rare to amoxicillin-clavulanic acid, cefazolin, and oxacillin. This must provide guidelines for the dog veterinary practitioner's choice of antibacterial compound. These results also show that there are many multidrug-resistance *S. intermedius* in dogs, including oxacillin-resistant strains, human potential pathogen.

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Chapter Ⅱ. Tetracycline resistance determinants

Introduction

Tetracyclines were the first major group of antibiotics to which the term 'broad-spectrum' was used [16]. Because of the spectrum of activity, the relative safety and low cost, tetracyclines have been widely used throughout the world and are second after penicillins in total tons used each year [4]. During many years, the therapeutic use of tetracyclines in human medicine has been reduced as bacterial resistance has become more widespread [3, 16, 24]. Oxytetracycline has been used to treat certain bacterial diseases which effect field crops and fruit trees and in subtherapeutic levels as food additives for growth promotion in billions of animals raised for food each year. Use of oxytetracycline in food production ranges from chickens, cows, honeybees, salmon, and catfish [16]. Tetracyclines are also used for therapy in food animals and in pets such as dogs, cats, and horses [16]. The bacteria which cause disease in animals and plants can be of the same species as those found in man, may belong to related species found within the same or related genera as those found in man, or more distantly related. Unique plasmids and antibiotic resistance genes first described in animal specific bacteria have made their way into human bacteria and vice versa [16, 24]. The reason this happens is due to the fact that bacteria exchange antibiotic resistance genes. Thus, a bacterium unique to a food animal or fruit tree disease which acquires tetracycline resistance as a result of treatment with oxytetracycline may pass that gene to other species

and genera and ultimately influence the antibiotic resistance carriage of strictly human bacterial species [16, 24]. The result is that antibiotic use anywhere in the world, regardless of its original purpose, can effect the antibiotic resistance genes in bacteria in other ecosystems including those that are pathogenic for man. Thus, tetracycline resistance determinants are wide-spread among bacterial species and have been identified in as many as 32 Gram negative and 22 Gram positive organisms and are often found in multi-drug resistant bacteria [3,14, 23].

Staphylococcal infections are frequently treated with antimicrobial agents, but treatment is problematic because of the limited number of effective antimicrobial agents available. The frequent occurrence of antimicrobial resistance has previously been reported among *S. intermedius* in different countries [20, 32, 33, 35]. Strains used for these previous studies were multi-resistant to antimicrobials; mostly to ampicillin, chloramphenicol, gentamicin, erythromycin, penicillins, sulphonamides and tetracyclines.

Resistance to tetracycline occurs by three mechanisms: the use of an energy-dependent efflux of tetracycline, altering the ribosome to prevent effective binding of the tetracycline, and producing tetracycline-inactivating enzymes [23]. The tetracycline resistant genes associated with an efflux mechanism are *tet*(A), (B), (C), (D), (E), (G), (I), (M) and (K). The tetracycline resistance genes associated with a ribosomal protection mechanism and/or efflux mechanism are *tet*(K), (L), (M), (O), (S), (P), (Q), (B), (D), (H) and (C). The only example of a tetracycline resistance gene causing the enzymatic alteration of tetracycline is *tet*(X) [23]. Among those *tet* genes, *tet*(A)

to *tet*(G) genes are usually found in Gram negative bacterial species and are associated with efflux mechanism. The *tet*(L), *tet*(M) and *tet*(Q) genes, associated with a ribosomal protection mechanism and/or efflux mechanism, are also carried by Gram negative bacteria. Gram positive bacteria carry commonly *tet*(M) gene associated with efflux mechanism and also take *tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(S) and *tet*(Q) genes associated with a ribosomal protection mechanism and/or efflux mechanism.

Several studies have determined the occurrence of different genes encoding antimicrobial resistance in *S. intermedius* of canine origin [5, 12, 26] Four genes, such as *tet*(K), *tet*(L), *tet*(M) and *tet*(O) encoding tetracycline resistance have been identified in *Staphylococcus* species [26, 32]. Nevertheless, there is limited information about the distribution of the *tet* genes in *S. intermedius* of canine origin in Jeju, Korea. This study describes the distribution of tetracycline resistance genes in *S. intermedius* of canine origin in Jeju, Korea.

Materials and Methods

Bacterial strains and growth conditions

A total 78 tetracycline resistant *Stapylococcus intermedius* were used for this study and included 40 and 38 strains originated from healthy and diseased dogs. The organisms were subcultured from the frozen stock (-80℃) onto Trypticase blood agar base containing 5% sheep blood.

DNA extraction

Bacterial DNA was extracted by the modification of the method of Richard et al [21]. *S. intermedius* isolates were grown onto Colombia blood agar base containing 5% sheep blood at 37℃ for 18-24 hr and one loopful bacterial cells were mixed with 1.0 ml of PBS (pH 7.2) in 1.7 ml microfuge tube and 0.25 g of Chelex-100 (Bio-Rad, Hercules, Calif.) was suspended into 0.5 ml of 10 mM TE buffer (pH 8.4). in another microfuge tube. The bacterial and 0.6 ml of Chelex-100 suspension were added to 2 ml screw-caped tube containing 1 g of 0.1-mm-diameter glass beads (Biospec Products, Inc., Bartlesville, Okla.). The samples were mixed and processed in the bead beater (Biospec Products; Inc.) at three-quarters speed for 5 min and then boiled for 5 min. The samples were then centrifuged for 5 min at 13,000g, and the supernatants were removed to clean 1.7-ml Eppendorf tubes. All DNA samples were measured for concentration using a DNA/RNA calculator (Pharmacia Biotech, Piscataway, N.J.).

Table 1. Tetracycline-resistant PCR primers

Tetracycline resistance gene	PCR primer sequence 5'-3'	Amplicon size(bp)
Tet(A)	GCT ACA TCC TGC TTG CCT TC	210
	CAT AGA TCG CCG TGA AGA GG	
Tet(B)	TTG GTT AGG GGC AAG TTT TG	659
	GTA ATG GGC CAA TAA CAC CG	
Tet(C)	CTT GAG AGC CTT CAA CCC AG	418
	ATG GTC GTC ATC TAC CTG CC	
Tet(D)	AAA CCA TTA CGG CAT TCT GC	787
	GAC CGG ATA CAC CAT CCA TC	
Tet(E)	AAA CCA CAT CCT CCA TAC GC	278
	AAA TAG GCC ACA ACC GTC AG	
Tet(Ga)	GCT CGG TGG TAT CTC TGC TC	468
	AGC AAC AGA ATC GGG AAC AC	
Tet(Gb)	CAG CTT TCG GAT TCT TAC GG	844
	GAT TGG TGA GGC TCG TTA GC	
Tet(K)	TCG ATA GGA ACA GCA GTA	169
	CAG CAG ATC CTA CTC CTT	
Tet(L)	TCG TTA GCG TGC TGT CAT TC	267
	GTA TCC CAC CAA TGT AGC CG	
Tet(M)	GTG GAC AAA GGT ACA ACG AG	406
	CGG TAA AGT TCG TCA CAC AC	
Tet(O)	AAC TTA GGC ATT CTG GCT CAC	515
	TCC CAC TGT TCC ATA TCG TCA	
Tet(S)	CAT AGA CAA GCC GTT GAC C	667
	ATG TTT TTG GAA CGC CAG AG	
Tet(P)	CTT GGA TTG CGG AAG AAG AG	676
	ATA TGC CCA TTT AAC CAC GC	
Tet(Q)	TTA TAC TTC CTC CGG CAT CG	904
	ATC GGT TCG AGA ATG TCC AC	
Tet(X)	CAA TAA TTG GTG GTG GAC CC	468
	TTC TTA CCT TGG ACA TCC CG	

Primers

Primers used for PCR amplification of 14 tetracycline resistance genes *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(S), *tet*A(P), *tet*(Q) and *tet*(X) in accordance with published information [18]. Size of PCR product for each primer pair, reference plasmid cultures and appropriate restriction enzyme for confirmation

of amplicon are listed in Table 1.

Multiplex PCR conditions

Multiplex PCR was performed by the modification of the method of Ng et al. [18]. following the determination of DNA concentration using a ultraviolet spectrophotometer at A_{260}. The PCR reaction mix (total 50 ㎕) included 0.5 ㎍ template DNA, 1×PCR buffer, 2.5 U DNA Taq polymerase (Intron), 2.5 mM of each of the deoxynucleotides dNTP (Gibco-BRL, Burlington, Ontario, Canada) and ddH2O. Group I contained primers for *tet*(B) (0.25 μM), *tet*(C) (0.25 μM) and *tet*(D) (2.0 μM) each (4.0mM MgCl2). Group II contained primers for *tet*(A) (1.0 μM), *tet*(E) (1.0 μM) and *tet*(G) (1.0 μM) each (3.0 mM MgCl2). Group III contained primers for *tet*(K) (1.25 μM), *tet*(L) (1.0 μM), *tet*(M) (0.5 μM), *tet*(O) (1.25 μM) and *tet*(S) (0.5 μM) each 3.0 mM MgCl2). Group IV contained primers for *tet*A(P) (1.25 μM), *tet*(Q) (1.25 μM) and *tet*(X) (1.25 μM) each (4.0 mM MgCl2). DNA amplification was carried out in a PCR Thermal cycler DICE Gradient Model TP600 (TaKaRa, Tokyo, Japan) using the following conditions: a 5 min initial denature at 94°C followed by 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1.5 min. PCR products were analysed by gel electrophoresis (1% (w/v) agarose in 1×TAE buffer). DNA bands were stained with ethidium bromide and then visualized by u.v. transillumination. The sizes of the PCR products were determined by comparing them with the migration of 100-bp plus DNA ladder (Intron).

Results

Of the 102 *S. intermedius* isolated from dogs 78 (76.5%) was resistant to tetracycline. Among the 78 tetracycline resistance bacteria, 65 (83%) were also resistant to penicillin 62 (80%) to ampicillin, 42 (54%) to kanamycin, 38 (49%) to trimethoprim/sulfamethoxazole, 35 (45%) to erythromycin, 29 (37%) to gentamicin, 27 (35%) to both ciprofloxacin and norfloxacin, and 16 (21%) to neomycin. Of the 24 tetracycline sensitive bacteria, 19 (79%) were also resistant to ampicillin, 17 (71%) to penicillin, 15 (63%) to kanamycin, 14 (58%) to erythromycin, 12 (50%) to chloramphenicol, 7 (29%) to neomycin, 6 (25%) to gentamicin, and 5 (21%) to trimethoprim/sulfamethoxazole (Data not shown).

Table 1. Tetracycline resistant gene types of *Staphylococcus intermedius* isolated from dogs.

Origins	Tet gene type with					
	K	M	KM	LM	NT	Total
Diseased dog	0 (0.0)	33 (86.8)	1 (2.6)	2 (5.3)	2 (5.3)	38
Healthy dog	3 (7.5)	29 (72.5)	2 (5.0)	2 (5.0)	4 (10.0)	40
Total	3 (3.9)	62 (79.5)	3 (3.9)	4 (5.1)	6 (7.7)	78

* NT, non-typed

Tetracycline resistance genes were detected by the muliplex PCR method to amplify all 14 genes on 78 *S. intermedius* isolates,

containing 38 strains from the diseased dogs and 40 strains from the healthy dogs (Table 1). Group III multiplex PCR was initially tested individually, and the template DNA of samples with ambiquous PCR products were re-tested using another 3 multiplex PCR groups. Overall, 62 (79.5%) were found to carry only *tet*(M), 3 (3.9%) carried only *tet*(K), 3 (3.9%) carried both *tet*(K) and *tet*(M), and 4 (5.1%) carried both *tet*(L) and *tet*(M). Seven strains (7.7%) did not carry any tetracycline resistance genes (Fig 1 and 2).

Fig. 1 PCR products representing *tet*(M) gene amplified by group III multiplex PCR. Lane M, 100 bp plus DNA maker (Intron); lane 1, *tet*^{S} *S. intermedius;* lane 2-8, *tet*^{R} *S. intermedius;* lane 9, non-typed *S. intermedius.*

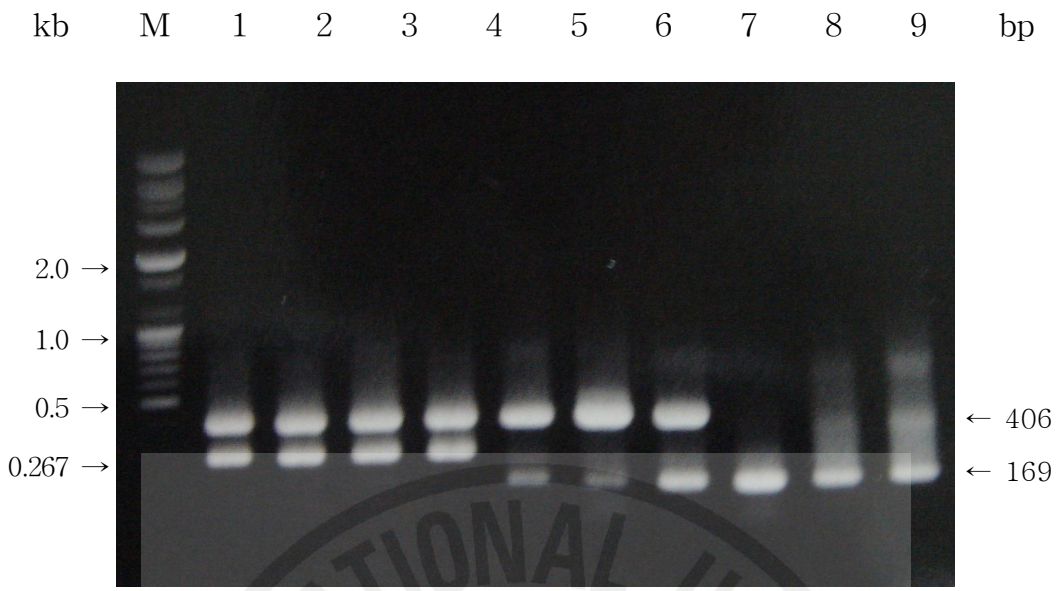

Fig. 2 PCR products amplified by group III multiplex PCR. Lane M, 100 bp plus DNA maker (Intron); lane 1-4, *tet*(L) and *tet*(M); lane 5-7, *tet*(K) and *tet* (M); lane 8-10, *tet*(K).

Discussion

Tetracyclines are broad-spectrum antimicrobial agents with activity against a wide range of Gram positive and Gram-negative anaerobic and aerobic bacteria, cell-wall free mycoplasmas, chlamydiae, mycobacterium, rickettsia, *Helicobacter, Listeria* and protozoan parasites such as *Entamoeba histolytica, Giardia lamblia,* and *Plasmodium falciparum* [3, 7, 13, 15, 16, 24]. They have been used extensively for therapy in man for bacterial respiratory and urogenital tract diseases, periodontal, Lyme, and rickettsial diseases. In addition, Tetracyclines have non-antibacterial properties which include antiinflammatory and immunosuppressive properties [11]. Studies have linked tetracycline with suppression of antibody production in lymphocytes, reduction in phagocytic function of polymorphonuclear leukocytes, reduction of leukocyte and neutrophil chemotaxis, as a sclerosising agent, as an inhibitor of lipase and collagenase activity and as an enhancer of gingival fibroblast cell attachment [11, 34]. These additional properties have encouraged the use of tetracycline in non-infectious conditions such as resistant rheumatoid arthritis, rosacea, pyoderma gangrenosum, prurigo pigmentosa, pleural effusions, recurrent pneumothorax, recurrent thyroid cysts, and biliary-cutaneous fistula. These uses unfortunately expose the patient's normal flora to active tetracycline which can select for tetracycline-resistant bacteria as does tetracycline used for treatment of an infectious disease.

In 1953, the first tetracycline-resistant bacterium isolated was *S. dysenteriae*, which causes bacterial dysentery [6]. The first

multi-resistant *Shigella* was isolated in 1955 and was resistant to tetracycline, streptomycin, and chloramphenicol [1, 6, 31] and came from a total of 5327 (0.02%) isolates tested. By 1960, multi-resistant *Shigella* represented almost 10% of the strains tested in Japan [1, 6, 31], a dramatic increase in five years. The increase in multi drug resistant *Shigella* has continued to the present as illustrated by a recent study [17] where over 60% of the *S. flexneri* isolated between 1988-1993 were resistant to tetracycline, streptomycin, and chloramphenicol. This is the same combination of antibiotic resistance determinants found in the 1953 *S. dysenteriae* isolate: Subsequently, it was demonstrated that these antibiotic-resistant bacteria could transfer their antibiotic-resistant phenotypes to susceptible isolates by cocultivation. This transfer was dependent upon direct contact of the bacteria [31]. We now know that the Japanese studies were the first reports of tetracycline resistance genes carried on conjugative R-plasmids. These tetracycline genes coded for the efflux of tetracycline out of the cell and were the first of the three different tetracycline resistance mechanisms found in bacteria [24]. Multi-drug resistance, which includes tetracycline resistance, has also been seen in Gram-positive species. A recent 1994 study [8] indicated that approximately 90% of methicillin-resistant *Staphylococcus aureus*, 70% of *Streptococcus agalactiae*, 70% of multi-drug resistant *Enterococcus faeca!is* and: 60% of multi-drug resistant *S. pneumoniae* now are tetracycline-resistant. This suggests that tetracycline resistance has become widespread in pathogenic Gram-positive species as well as among pathogenic Gram-negative species and is often found in multi-drug resistant bacteria. It has been found that

extended use of tetracycline may select for both tetracycline and multi-drug resistant bacteria [16]. This situation applies equally to animals given low dose tetracycline for growth promotion and patients given long term tetracycline for control of acne [16]. Since 1960, investigators have found that bacterial resistance to tetracyclines is primarily due to acquisition of tetracycline resistance determinants rather than by mutation of existing chromosomal genes [22, 24]. There have been 16 different tetracycline resistant determinants characterized.

Of the 102 canine *S. intermedius* investigated in this study 78 (76.5%) were resistant to tetracycline and the resistant rate was much higher than that reported in studies conducted in the UN [19], Canada [10] and USA [26], but slightly lower than that reported in the previous study in Korea [12].

The *tet*(M) genes have been most commonly detected in tetracycline-resistant *S. intermedius* [26]. In this study, 69 strains (88.5%) of 78 tetracycline resistance isolates carried also *tet*(M) gene with or without other genes. The *tet*(K) gene was present in 3.9%, there are no strains with only *tet*(L) gene, and the *tet*(O) gene, including other *tet* genes were not found in any of these isolates.

All isolates carrying *tet*(M) and *tet*(O) genes are tetracycline-resistant by a ribosome protecting protein [30], and also exhibit resistance to minocycline, whereas *tet*(K) and *tet*(L) bearing isolates are sensitive to minocycline [26]. Tetracycline resistance in these latter isolates is based on a membrane-associated efflux system. *S. intermedius* strains carry both efflux and ribosomal protection genes in combination [23]. The *tet*(K) genes, which were previously known as plasmid-borne, are thought to be indigenous in most

Staphylococcus spp., but not in *S. intermedius* [26].

The *tet*(L) genes, which are commonly located on small plasmids, have rarely been detected in *Staphylococcus* spp. from animals [25] and *S. intermedius* harboured *tet*(L) gene independendly have been reported in 24% or the isolates investigated [12]. However, in this study, there were no strains harboured *tet*(L) in accordance with the report of Schwarz et al. (1998). The *tet*(K) and *tet*(L) genes can be found in single isolates of streptococci [2], though not in isolates in this study.

The observation that *tet*(M) genes are detected in most tetracycline-resistant *S. intermedius* isolates independently of their animal origin suggests that they are most readily acquired by these bacteria [26]. Most *tet*(M) genes are located on conjugative transposons [29], and are usually found in the chromosomal DNA rather than on plasmids [5]. The reason for the preferential acceptance of transposon-encoded resistance genes in *S. intermedius* is still unknown [26]. However, *S. intermedius* isolates differ from other staphylococcal species by their high number of chromosomally located insertion elements [9]. These may play a role in the development of chromosomal multi-resistance in this species. In the majority of Gram-positive species, the *tet*(M) determinant is found in the chromosome, most often on conjugative elements [26]. It has been reported that the presence of *tet*(O) genes in chromosomal DNA are uncommon in staphylococci [26] in accordance with the result of this study while there are some reports the *tet*(O) genes occur frequently in streptococci [2, 27], where both plasmid and chromosomal locations of *tet*(O) genes have been described [2, 28].

Conclusion

Of the 102 *S. intermedius* isolated from dogs 78 (76.5%) was resistant to tetracycline. Among the 78 tetracycline resistance bacteria, 65 (83%) were also resistant to penicillin 62 (80%) to ampicillin, 42 (54%) to kanamycin, 38 (49%) to trimethoprim/ sulfamethoxazole, 35 (45%) to erythromycin, 29 (37%) to gentamicin, 27 (35%) to both ciprofloxacin and norfloxacin, and 16 (21%) to neomycin. *S. intermedius* harboring *tet*(M) and *tet*(K) independently were 62 (79.5%) and 3 (3.8%) strains, respectively and 3 (3.8%) and 4 (5.1%) isolates were harboring both *tet*(M) and *tet*(K), and both *tet*(M) and *tet*(L), respectively.

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