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Molecular typing of epidemiologically unrelated *Staphylococcus epidermidis* recovered from dogs by pulsed-field gel electrophoresis

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Abstract : A total of 16 *Staphylococcus epidermidis* isolates collected from 14 dogs admitted to the Veterinary Medicial Teaching Hospital in Seoul National University over eleven months were examined for *in vitro* antibiotic susceptibility pattern with minimum inhibitory concentration (MIC) and slime production, a virulence-associated phenotype, and were genetically characterized by pulsed-field gel electrophoresis (PFGE). The frequency of resistance to antimicrobial agents tested was not high, with a susceptibility ranging from 56.3% to 100%. Three strains exhibited multiple drug resistance against amikacin (MIC, 32-64μg/ml), ampicillin (32μg/ml), fosfomycin (32-128μg/ml) and gentamicin (16μg/ml). Vancomycin, ciprofloxacin and rifampin were effective antibiotics against the isolates. All isolates were slime producers; strains isolated from dogs which died of bacteremia were more likely to produce slime than those isolated from dogs which survived. Chromosomal DNA fingerprinting of the isolates yielded 16 different genomic types with few common bands, indicating a variety of clones of *S epidermidis* were prevalent in the hospital. This study revealed that PFGE is an useful method for the genotype characterization of *S epidermidis* strains and this organism could probably be pathogenic in some dogs with severe disorders. Further works on a larger number of epidemiologically defined strains are required to assess these results.

Key words : *Staphylococcus epidermidis*, antimicrobial susceptibility, slime, pulsed-field gel electrophoresis, dog.

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Introduction

The coagulase-negative staphylococci (CNS), particularly *Staphylococcus epidermidis*, once considered only contaminants in bacterial culture, are increasingly recognized as true nosocomial pathogens in specific circumstances[1]. Several studies have provided evidence that CNS elaborate a variety of virulence factors including adherence factor, exotoxins[2,3], exopolysaccharides known as slimes[4], fibronectin and collagen[5].

In human beings, infections caused by CNS were almost always hospital-acquired and were mainly attributed to the adverse consequence of advances in invasive therapeutic procedures and prosthetic surgical implant devices including indwelling transcutaneous catheters[6,7], or severe underlying disease[8]. Among the species of CNS indigenous to humans, *S epidermidis* and *S saprophyticus* are consistent human pathogens, with the latter organism being a common urinary tract pathogen in non-hospitalized patients[9]. Bacteremia caused by CNS in immunocompromised patients can cause lethal illness, including septic shock. In contrast to humans, Staphylococcus spp other than *S intermedius* and *S aureus* have rarely been associated with specific disorders in cats and dogs.

Potentially many epidemiological typing methods for CNS have been suggested; biotyping[10], antimicrobial susceptibility testing profile[11], phage typing[11,12], plasmid analysis[13,14], immunoblotting[15], and restriction endonuclease analysis with probes[16,17]. Of these typing methods, restriction fragment length polymorphism analysis by pulsed-field gel electrophoresis (PFGE) has been shown to be superior to conventional typing methods[18].

This study was conducted to analyze the antibiotic resistant patterns of *S epidermidis* isolated from hospitalized dogs with severe disorders, to type the organisms genetically using PFGE, and to explore the clinical importance of these isolates by examining the production of slime which has been considered as one of the virulence factors of staphylococci species.

Materials and Methods

Bacterial strains and antimicrobial susceptibility : Bacterial culture was subjected to all dogs that had been admitted for more than 48h to the Veterinary Medical Teaching Hospital in Seoul National University. To avoid contaminants initially positive specimens (blood or intravenous catheters) were cultured repeatedly at least two consecutive days after the first isolation. These isolates were confirmed by using API-STAPH kit (bioMêrieux, Inc., France). The minimum inhibitory concentration (MIC) of individual antibiotics was determined by the macrodilution method in cation-supplemented Mueller-Hinton broth plus 2% NaCl at a final inoculum of 5×10^5CFU/ml from an overnight growth of the organism. MICs were read after 24h of incubation at 35℃ as the highest concentration yielding no visible growth. Susceptibility to each antimicrobial agent was defined using the breakpoint category of the National Committee for Clinical Laboratory Standards[19]. The antimicrobial agents tested were amikacin, ampicillin, cefamandole, cefazolin, chloramphenicol, ciprofloxacin, clindamycin, fosfomycin, gentamicin, imipenem, methicillin, rifampin, tetracycline, and vancomycin.

Quantitative assay for slime production : The quantitative microplate method was used for slime production, as described previously[20]. Optical density (OD) was measured at 492nm with a automated microplate reader (CERES UV 900, Bio-Tek Instrument Inc.)

Electron microscopy : To examine the polysaccharide-component materials in the isolates, after an overnight growth on blood agar supplemented with 10% milk whey, three pieces of 1mm cubes of surface of bacteria were scraped off, and fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4), for one day at 4℃. They were then post-fixed in 1% osmium tetroxide in the same buffered solution for 1 h, dehydrated in ethanol and transferred to propylene oxide for rinsing and embedded in epoxy resin (Polysciences, Inc., USA). Ultrathin (40-60nm) sections were stained with saturated aqueous uranyl acetate and lead citrate, and observed by the transmission electron microscope (Hitachi, H-600) at 75kV.

Preparation of chromosomal DNA : The protocol for the preparation of chromosomal DNA and electrophoresis

was carried out based on the methods of Goering and Winters[21] with some modifications. Briefly, the isolates were cultured overnight in 5ml of brain heart infusion broth at 37℃, and 0.7ml of this cultures was harvested by centrifugation at 7000rpm at 4℃ for 2 min in a microcentrifuge. The bacterial sediments were washed once in 1ml of autoclaved TEN buffer (0.1M Tris HCl, 0.15M NaCl, 0.1M EDTA, pH 7.6) and centrifuged again. The washed cells were resuspended in 0.3ml of sterilized EC lysis buffer (6mM Tris HCl, 1M NaCl, 0.1M EDTA, 0.5% Brij 58, 0.2% w/v sodium deoxycholate, 0.5% N-lauroylsarcosine, pH 7.6). 300μl of 2% SeaPlaque agarose was added to the lysostaphin-cell suspension. The suspension was quickly pipetted into a plug mold, and then solidified at 37℃ for 2 h. The plug was placed in a 3ml of lysis buffer supplemented with 1mg/ml of lysostaphin and 20μg/ml if RNase (Merck) and incubated at 37℃ for 2 h. The EC buffer was removed and replaced with 3ml of TE buffer (10mM Tris HCl, 5mM EDTA) and the cells in the plus were incubated 1 h at 55℃.

Pulsed field gel electrophoresis : For electrophoresis, the plug was cut into small slices and placed in a 125μl restriction enzyme mixture containing 20U of *Sma* I (New England BioLabs). After a 2 h incubation at 25℃ with shaking at 140rpm, chromosomal restriction fragment patterns were analyzed by loading the trimmed slices of the plug into a well of a 1% Seakem agarose running gel. Electrophoresis was performed with the CHEF-Mapper™ electrophoresis cell (BioRad). The lamda ladder DNA concatemers (New England BioLabs) were used as molecular weight standards. PFGE was carried out as follows: initial pulse = 5s, final pulse = 40s, voltage = 6V/cm, temperature = 12-14℃, time = 20h. The 312nm UV Transilluminator (TR-312A, USA) was used to visualize the DNA fragment bands.

Results

Epidemiologic data : During the eleven-month period, a total of 16 *S epidermidis* were isolated from 14 dogs (Table 1).

Table 1. Coagulase-negative *S epidermidis* isolates in 14 dogs

Dog No.	Age	Sex	Underlying disorders	Hospital stay (day)	Outcome
1	4	M	Parvovirus Inf.	4	S*
2	7	M	Parvovirus Inf.	7	S
3	16	F	Parvovirus Inf.	5	S
4	33	F	Parvovirus Inf.	9	S
5	48	M	Renal failure	3	D#
6	12	F	Distemper	9	D
7	37	M	Parvovirus Inf.	7	S
8	3	M	Parvovirus Inf.	6	S
9	23	F	Renal failure	10	S
10	28	M	Distemper	9	S
11	72	F	Renal failure	9	S
12	2	F	Parvovirus Inf.	5	D
13	110	F	Renal failure	8	S
14	2	F	Intes. Intus.[a]	5	S

[a]Intes. Intus., intestinal intussusception. *S, survived; #D, died.

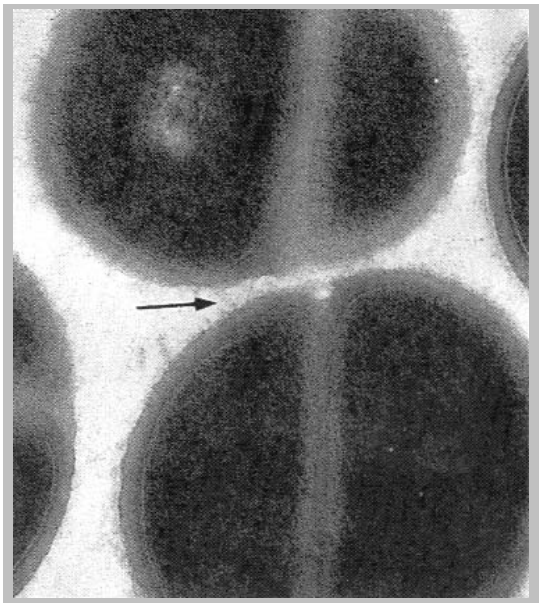

Fig 1. Transmission electron micrographs of *S epidermidis* grown on staphylococcus 110 medium supplemented with 100% (v/v) milk whey. Arrow shows exopolysaccharides, and bar represents 55μm.

Dogs had been hospitalized for an average of 4.6 days before the organism was first cultured, and the average duration of hospitalization was 6.9 days. They included 8 females and 6 males, with ages ranging from 2 to 110 months. The underlying disorders that led to hospitalization included parvovirus infection in seven dogs, renal failure in four, distemper in two, and intestinal intussusception in one. Two isolates were collected from dog 8 (Isolate no. 8 and 9; Fig 1) and 14 (Isolate no. 11 and 14; Fig 1) each during two possible episodes of bacteremia. The two strains had different PFGE patterns, with many variations in the position and size of the bands (Fig 1). The results of antimicrobial susceptibility testing were shown for 14 antibiotics in Table 2. All isolates were susceptible to vancomycin, rifampin and ciprofloxacin. The susceptibility to β-lactam antibiotics varied, ranging from 62.5% for ampicillin to 100% for methicillin. Gentamicin showed the least bactericidal effect with susceptibility of 56.3% Three strains (Isolate nos. 5, 6, and 12; Fig 1) expressed multiple drug resistance to amikacin, ampicillin, fosfomycin, and gentamicin (Table 3).

Slime production : All isolates were found to produce slime, with a OD range of 0.125-0.892 (median, 0.266). Of these, three isolates collected from dogs which died of bacteremia were strong producers (OD ≥0.582). The 14 remaining isolates were moderate producers (OD 0.125 to 0.48).

Typing of chromosomal DNA fingerprinting : Figure 1

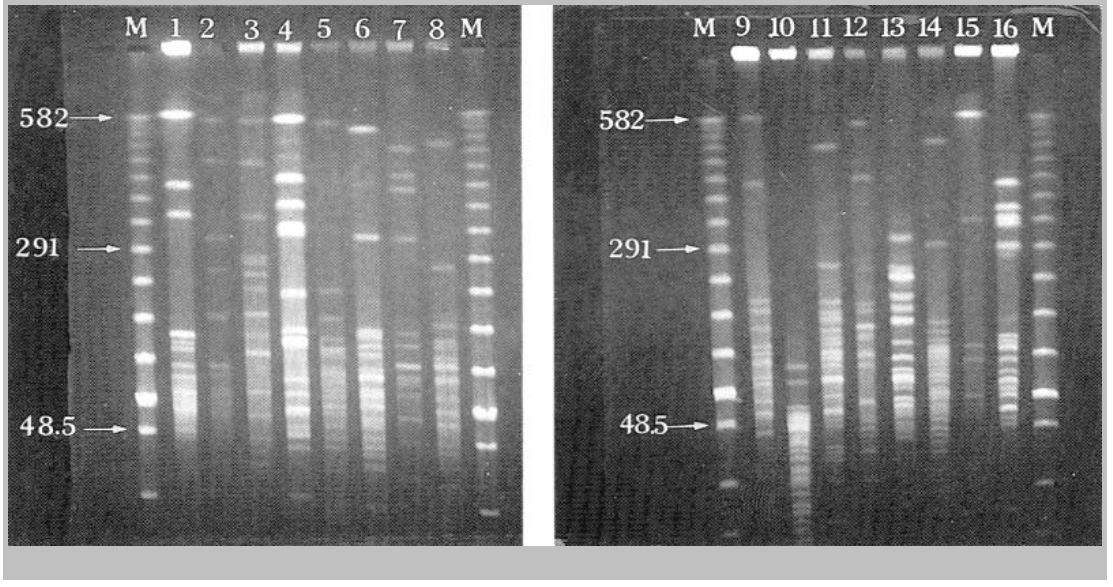

Fig 2. PFGE-mediated DNA pattern of *S epidermidis* digested with *Sma* I. Lanes: M, molecular weight standard (lamda ladder DNA concatemers); track number corresponds to isolate number. The arrows on the left indicate molecular sizes in kilobases.

Table 2. Range of MICs in *S epidermidis* to some selected antimicrobial agents

Antimicrobial agent	MIC (μg/ml)	Percent susceptible[a]
Amikacin	16~64	75.0
Ampicillin	4~32	62.5
Cefamandole	4~128	81.3
Cefazolin	2~64	81.3
Chloramphenicol	2~32	87.5
Ciprofloxacin	0.125~2	100
Clindamycin	0.25~8	87.5
Fosfomycin	8~128	75.0
Gentamicin	2~16	56.3
Imipenem	0.5~64	81.3
Methicillin	1~8	100
Rifampin	⟨ 0.015	100
Tetracycline	4~128	87.5
Vancomycin	⟨ 0.5~4	100

[a]Breakpoints for resistance were (in μg/ml): amikacin, ampicillin, cefamandole, cefazolin, chloramphenicol, fosfomycin, and vancomycin, ≥32; ciprofloxacin, clindamycin, and rifampin, ≥4; gentamicin, ≥8; and imipenem, methicillin, and tetracycline, ≥16.

shows the chromosomal DNA band digested with *Sma* I of 16 isolates. Each isolate was characterized by its own unique fingerprint; PFGE analysis revealed 16 different patterns consisting of an average of 12 bands (range, 6 to 16), and all isolates had few bands shared by all the strains.

Discussion

Because *S epidermidis* are one of natural inhabitants of animal skin, it has rarely considered necessary to link these organisms with specific disorders. In the clinical setting, however, the increasingly frequent and repeated isolation of the organism in the same patients have been reported in human medicine[7,18,22] and thus, necessitate genomic typing to confirm their relatedness. This procedure is especially important to identify nosocomial outbreaks. In this study, since dogs that had been hospitalized for more than 48h with severe disorders were subjected to bacterial surveillance all these cases were confirmed of being acquired their infection after admission. This finding suggests that routine microbiologic culture for medical surveillance purposes need to be established in the hospital to monitor nosocomial infection. Of 16 isolates, three were from intravenous catheters. These isolates may represent contaminants from the dog's cutaneous flora, but the possibility of true bacteremia must not be excluded. Archer[23] reported on the importance of *S epidermidis* in clinical settings by emphasizing that this organism may itself serve as a reservoir for resistance genes that are transferred to *S aureus*, so extensive use of antimicrobial agents in hospital, in turn, may provide selection pressure for the evolution and amplification of these genes.

It was reported that hospital-acquired isolates of *S epidermidis* are invariably resistant to multiple antibiotics by plasmid-mediated mechanisms[23,24]. In contrast, most strains in the study were susceptible to the antimicrobial agents employed. Of great interest was that gentamicin, the most frequently used antimicrobial agent in our hospital for nebulization showed the least bactericidal effect with 56.3% of susceptibility, while ciprofloxacin and vancomycin showed promising activity of 100% against the organisms, and thus, could be useful in therapy for multiresistant isolates of *S epidermidis*. Large degree of cross-resistance between penicillins and the cephalosporins was observed.

Table 3. MICs of 3 multidrug resistant *S epidermidis* isolates

Isolate no.	MIC (μg/ml)			
	amikacin	ampicillin	fosfomycin	gentamicin
5	64	32	128	16
6	32	32	64	16
12	64	32	32	16

One of the primary purpose of this study was to explore the potential pathogen of *S epidermidis* by examining slime production of the isolates. Of numerous factors that determine the virulence of *S epidermidis*, adherence factor has been investigated extensively. All strains were found to have characteristic of slime-producers with a variety range of its production. This finding is in agreement with previous studies[2,25]. In particular three strains isolated from dogs died of bacteremia were more likely to produce slimes, indicating that some strains of *S epidermidis* could have higher pathogenicity in dogs. Slimes prevent the organism from inhibiting phagocytosis[11] and protects the organism from the action of antibiotics[26]. This characteristic may probably resulted in extra mortality of hospitalized dogs in our study in spite of extensive therapy.

It is very important to distinguish relapse of infection with the same strain from re-infection with a different strain. Generally, one of the main guidelines to differentiate between bacteremia and contamination is the repeated isolation of the same strain from various blood cultures of the same patient as opposed to the isolation of different strains[27]. For this reason, in cases with multiple positive blood cultures, it is crucial to establish whether these isolates are related, suggesting true bacteremia, or unrelated, suggesting contamination [28,29]. Weinstein *et al*[30] were able to differentiate true pathogens from contaminants and considered 94% of the CNS to be clinically significant. With reference to this purpose PFGE has widely been used and proven to be highly effective technique for strain differentiation[31,33] and investigation of outbreaks[16]. In the current study although there were some unsheared DNA bands on the gel PFGE analysis classified the isolates into 16 different types, suggesting that a large degree of genomic diversity in *S epidermidis* were prevalent in the hospital. The similar findings on diversity of the strains were reported in human study[7,18]. In particular, Hu *et al*[22] reported that in human nares, four different types of *S epidermidis* were found to be colonized in a person. This molecular method aids in the clinical management of dogs with multiple blood cultures by alerting clinician as to whether the isolates are likely to be contaminants or true pathogens. Because of limited number of isolates the present study did not elucidate association between mortality of older animal with bacteremia and PFGE type. In the future it is worth studying this subject.

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