大韓獸醫學會誌(2009) 第49卷 第3號
Korean J Vet Res(2009) 49(3) : 231~236

Relationship between biofilm formation and the antimicrobial resistance in the *Staphylococcus spp.* isolated from animal and air

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(Accepted: September 14, 2009)

Abstract : Biofilm has been described as a barrier, which produced by microorganisms to survive and protect themselves against various environments, like antibiotic agents. *Staphylococcus spp*. is a common cause of nosocomial and environmental infection. Thirty-six and thirty-five Staphylococci were isolated from animals and air, respectively. Based on the biofilm forming ability of the bacterium reported in our previous report, relationship between biofilm formation and antibiotic-resistance was investigated in this study. Regarding antibiotics susceptibility, cefazolin was the most effective agent to the bacteria. Strong biofilm-forming *Staphylococcus spp*. isolates might have a higher antibiotic resistance than weak biofilm isolates regardless of the presence of antibiotic resistance genes ($p < 0.05$). This result suggested that the chemical complexity of the biofilm might increase the antibiotic resistance due to the decrease of antibiotic diffusion into cells through the extensive matrix.

Keywords : antibiotic susceptibility, biofilm, *Staphylococcus* spp.

Introduction

Biofilm was described simply as “a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface” [7]. Recently, the definition is changed to a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum of interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription [11].

Moreover, the ability of biofilm production can be considered to be a significant virulence factor for some strains of Staphylococci [1]. Thicker biofilm may suggest a role of slime in pathogenesis [4]. And biofilm is formed by a spectrum of microorganisms and provides a mean for these organisms to protect themselves against antimicrobial agents [12]. Microbial biofilms are notoriously hard to treat with antimicrobial compounds [13].

Biofilm-associated disease in humans has been increasing. Direct analysis of the surface of medical devices or of tissues that have been foci of chronic infections shows the presence of large numbers of bacteria surrounded by an exopolysaccharide matrix, which has been named the “biofilm” and can be considered as the role of bacterial biofilms in human persistent infections [19].

Of the microorganisms, *Staphylococcus* (*S.*) spp. has been studied because the bacterium is common cause of nosocomial and environmental infection [22]. Recently, *S. aureus* and *S. epidermidis* have been increased as one of the important causative agents in nosocomial infection [7]. The *Staphylococcus* spp. normally colonize skin and mucous membranes of human and animal, and can be easily isolated from environment, even in the air [6, 8, 24].

Staphylococcus spp. are generally susceptible to most antibiotics except those with purely anti-Gram-negative spectra. However, biofilm-forming coagulase-negative Staphylococci are more resistant to antibiotic agents when compared to planktonic cells [9], that protect bacteria against high antimicrobial concentrations and phagocytosis [8, 9], helping survival in hostile

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environments within the host [31]. This suggests that the biofilm may attribute to a decreased antibiotic diffusion through the extensive matrix [2, 17]. Recently, some investigators reported that bacteria in biofilm have attributed resistance or reduced susceptibility to the biofilm mode of growth [3, 13].

Based on the knowledges, to access the relationship between biofilm production and antibiotic resistance, antimicrobial susceptibility and presence of the related gene were investigated in the *Staphylococcus* spp. isolated from animals and air in the present study.

Materials and Methods

Bacterial strains

In this study, 36 and 35 *Staphylococcus* spp. isolated from animals and air, respectively, were used [26]. And five kinds of *Staphylococcus* spp. were used; *S. aureus*, *S. epidermidis*, *S. hominis*, *S. xylosus*, *S. auricularis* and *S. simulans*. Isolation and identification of the bacterium were described in our previous report [26].

Biofilm formation assay

Biofilm formation was investigated by tissue culture plate (TCP) methods. A TCP assay was performed with 96 well flat-bottomed tissue culture plates (Greiner bio-one, USA) as described by Stepanovic *et al.* [27]. The optical density (OD) of each well was measured by using a microplate ELISA reader (E max, USA) at A_{570}. This experiment was repeated three times. The adherence ability of the tested strains was classified into four categories [27]: non-adherent (0), weakly (+), moderately (++) and strongly (+++) adherent, based on the OD.

Antibiotic resistance test

Antibiotic resistance of the staphylococcal isolates was determined by disc diffusion method, determination of minimal inhibition concentration (MIC) and detection of antibiotic resistant genes. *Enterococcus fecalis* ATCC 51299 and *S. aureus* ATCC 25923 were used as reference strains.

Disc diffusion and MIC test

The disc diffusion test was performed by following the guideline of the Clinical and Laboratory Standards Institute (CLSI). Oxacillin, rifampin, cefazolin, ampicillin, erythromycin, tetracycline, cephalothin and penicillin (Becton Dickinson Microbiology System, USA) were used in this study. The discs were placed on Muller Hinton agar plates inoculated with 1×10^8 CFU/mL (0.5 McFarland) and cultured for 24 h at 37°C. After the incubation, the zones of inhibition surrounding the discs were measured and compared with the guideline provided by the manufacture and CLSI.

MICs of penicillin, erythromycin, oxacillin, and tetracycline against the isolates were measured by E-test (ABI Biodisk, Sweden). The E-test strips were placed on Muller Hinton agar plates inoculated with 1×10^8 CFU/mL (0.5 McFarland) and cultured for 16-24 h at 37°C. After incubation, the MIC was directly measured from the visible symmetrical inhibition ellipse of bacterial growth centered along the strips and compared with the guidelines provided by the manufacture (ABI Biodisk, Sweden) and CLSI.

Polymerase chain reaction for the antibiotic resistant genes

Six antibiotic-resistance related genes were analyzed from the staphylococcal isolates using PCR [22, 28]. The genes were *tet*K and M for tetracycline resistance, *erm*A and B for erythromycin resistance, *bla*Z for penicilline resistance and *mec*A for methicillin resistance. Multiplex PCR for *tet*K and M was followed by Strommenger *et al.* [28]. The PCR for *erm*B was followed by Martineau *et al.* [22]. PCR for *erm*A, *bla*Z and *mec*A was designed in this study. The following primer sequence were used: *erm*A 3'-TGA TGG AGG CTT ATG TCA AGT G-5', 5'-CAA TGG TTG ATG TCG TTC AAG-3', *bla*Z 3'-CAA ACA GTT CAC ATG CCA AAG-5', 5'-CTT ACC GAA AGC AGC AGG TG-3' and *mec*A 3'-ACG GTA ACA ATT GAT CGC AAC-5', 5'-TTGCCAACC TTT ACC ATC G-3'.

In *erm*A, *bla*Z and *mec*A, PCR reagent mixture in the 25 µl PCR reaction mixture contained ×1 PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 20 mM $MgCl_2$), 1.5 U *Taq* polymerase (iNtRon Biotechnology, Korea), 40 µM of each dNTP, and 100 pM of both primers and 2 µL of DNA samples. Initial denaturation at 94°C for 5 min was followed by 30 cycles of amplification cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. Final polymerization was conducted at 72°C for 7 min. PCR products were analyzed by electrophoresis in 1% agarose gel in ×0.5 TBE buffer and were visualized under a UV transilluminator.

After purification of the PCR products from agarose gel, the DNAs were sequenced using a MJ Research PTC-225 Peltier Thermal Cycler using a ABI PRISM BigDye Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (FS enzyme) (Applied Biosystems, USA). Identity of the PCR amplified products was confirmed by comparing of the sequence with those from GenBank of National Center for Biotechnology Information, USA (accession numbers of *tet*K and M, *erm*A and B, *bla*Z and *mec*A were S67449, X56353, X03216, U35228, U58139 and AB221124, respectively).

Statistical analysis

Chi-square analysis and logistic regression (SPSS 12.0 for Window; SPSS, USA) were used to analyze the correlation of our results obtained in this study.

Results

Classification of bacterial strain based on biofilm formation

Bacterial strains used in this study were the same strains reported in our previous study [26]. Adherence ability of the tested strains was classified into four categories as shown in our previous report [26]. About 80% of the isolates produced slime with various degrees in the slime production. Airborne isolates (82.9%) were slightly higher than animal isolates (77.8%) in the slime production. In the slime-producing strains, 2.8% of animal and airborne isolates were strong, 30.6% and 44.4% of animals isolates were intermediate and weak, respectively, while 37.1% and 42.9% of airborne isolates were moderate and weak, respectively [26].

Analysis of antibiotic resistance

Disc diffusion test

Antibiotic resistant patterns by the disc diffusion test were similar between animal isolates and airborne isolates. The most effective antibiotic against biofilm-forming *Staphylococcus* spp. isolates was cefazolin, which was 93% effective in animal isolates and 69% effective in airborne isolates (Fig. 1). Ampicillin, rifampin and cephalothine were also effective against the isolates while the isolates were resistant to oxacillin, erythromycin, penicillin and tetracycline (Fig. 1).

MIC test

MICs measured by E-test in all *Staphylococcus* spp. isolates were shown in Table 1. Although staphylococcal isolates were resistant to all antibiotics in this study

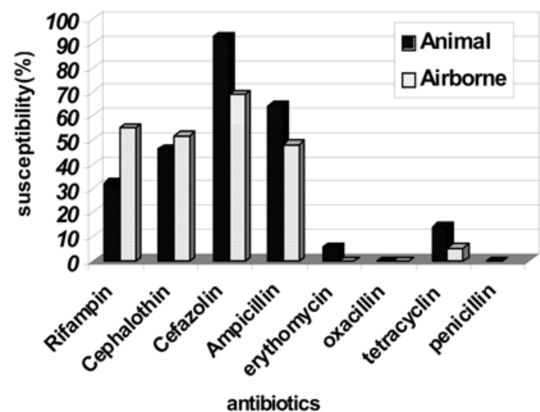

Fig. 1. Antibiotic susceptibility of biofilm-forming *Staphylococcus* spp. isolated from animals and air.

Table 1. The minimum inhibitory concentration of antibiotics against *Staphylococcus* (*S.*) spp. isolates related to biofilm formation

Biofilm formation	Origin	Minimal inhibition concentration (µg/mL)				
		Tetracyclin	Erythromycin	Penicillin	Rifampin	Oxacillin
Strong	Animals	> 256	> 256	> 256	64	> 256
	Airborne	> 256	> 256	> 256	64	> 256
Moderate	Animals	128	192	> 256	16	> 256
	Airborne	128	128	> 256	16	> 256
Weak	Animals	128	128	> 256	8	> 256
	Airborne	96	96	> 256	8	> 256
None	Animals	128	> 256	> 256	32	> 256
	Airborne	192	> 256	> 256	8	> 256

Table 2. Prevalence of antibiotic resistant genes determined by PCR

Bacteria	Origin	Prevalence of antibiotic resistant genes (%)					
		Penicillin	Methicillin	Erythromycin		Tetracycline	
		*bla*Z	*mec*A	*erm*A	*erm*B	*tet*K	*tet*M
S. aureus	Animal	80	10	20	0	20	10
	Airborne	100	9	9	9	9	9
S .epidermidis	Animal	86	14	0	57	57	57
	Airborne	100	25	0	50	0	50
S .hominis	Animal	40	0	60	20	0	0
	Airborne	40	40	40	40	0	20
S. xylosus	Animal	50	50	0	75	50	25
	Airborne	67	0	67	67	100	0
S. auricularis	Animal	75	0	75	50	0	0
	Airborne	50	17	33	33	0	0
S. simulans	Animal	67	0	50	33	67	17
	Airborne	100	0	20	80	80	60
Average	Total	71.3	13.8	31.2	42.8	31.9	20.7
	Animal	66.3	12.3	34.2	39.2	32.3	18.2
	Airborne	76.2	15.2	28.2	46.5	31.5	23.2

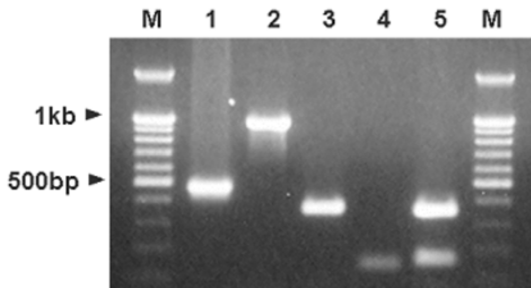

Fig. 2. Detection of antibiotic resistant genes in *Staphylococcus spp.* by PCR and agarose gel electrophoresis analysis. Lane M, 100 bp DNA ladder marker; Lane 1, *bla*Z (482 bp); lane 2, *mec*A (986 bp); lane 3, *erm*A (434 bp); lane 4, *erm*B (142 bp); lane 5, *tet*K (360 bp) and *tet*M (158 bp).

regardless of biofilm formation, the MICs of strong biofilm-forming staphylococcal isolates were higher than those of moderate and weak isolates ($p < 0.05$). There was no significant difference in MIC between moderate biofilm-forming isolates and weak biofilm-forming isolates ($p < 0.05$). Meanwhile, the MIC of biofilm-forming isolates was higher than that of non-biofilm-forming isolates against rifampin.

Detection of antibiotic resistant genes

PCR amplified antibiotic resistant genes were analyzed by agarose gel electrophoresis (Fig. 2). Identity of the PCR products was confirmed by sequencing and comparison with the GenBank sequences, ultimately showing 96-99% homology (data not shown). Also, the prevalence of the antibiotics resistant genes was analyzed based on the isolates' species and antibiotics (Table 2). The prevalence of the genes was similar between animal isolates and airborne isolates. About 70% of the isolates harbored the penicillin-resistant gene (Table 2). None of the *S. auricularis* isolates contained *tet*K or *tet*M (Table 2).

Discussion

Importance of biofilm formation has been described in the control of microbial infection in several areas because the biofilm can increase resistance to various physical and chemical agents, especially antibiotics [5, 7, 14, 15].

Therefore, the relationship of the antiboitic resistance with the formation of biofilm in the *Staphylococcus spp.* isolated from animals and air was investigated by

analysis of biofilm production and phenotype and genotype of antibiotic resistance in this study. In the disc diffusion test, the isolates showed higher resistance to erythromycin, oxacillin, tetracycline and penicillin. Based on the results, MIC was measured with the selected antibiotics against the isolates. Penicllin and oxacillin showed higher MICs against the isolates regardless of the degree of biofilm formation whereas the difference in MIC with of the rifampin was observed with regard to the biofilm formation. This result was similar with previous reports showing the antibiotics resistance related with biofilm formation [23]. Especially, the susceptibility of the isolates against cefazoline, cephalothin and rifampin in this study showed high similarity with previous reports which recommended the antibiotics as good candidates to control biofilm-forming bacteria in the chronic infection [2, 6, 16].

Based on the genetic analysis of the isolates using *mec*A PCR detection as the gold standard for methicillin resistance test [30], the resistance pattern of penicillin and oxacillin in this study showed difference with previous report [20]. The difference might be from the presence of heterogenous resistance genes in clinical isolates against the antibiotics [21, 25], or the formation of biofilm as an alternative strategy [5]. Interestingly, MIC of rifampin against the non biofilm-forming bacteria was higher MIC than moderate and weak biofilm-forming bacteria. This phenomenon might have emerged due to mutation of the *rpo*B gene resulting from cross resistance of another antibiotic used for veterinary purpose or from human handlers involved in the industry [32], even though further genetic analysis is required to elucidate this phenomenon.

In general, the effect on adherence inhibition of adherence was greater than the effect on inhibition of biofilm formation by antibiotics. Accordingly, adherence inhibition assays cannot fully predict the outcome in terms of biofilm formation [6]. As a result, to monitor and control biofilm formation of bacteria, a combination of genotypic and phenotypic assays should be performed.

Acknowledgments

This study was supported by the Research Project on Bio-Green 21 (No.20070401-034-009-007-01-00) RDA, BK21, KRF (2006-005-J502901) and the Research Institute for Veterinary Science, Seoul National University, Korea.

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