A periodontitis-associated multispecies model of an oral biofilm

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Purpose: While single-species biofilms have been studied extensively, we know notably little regarding multispecies biofilms and their interactions. The purpose of this study was to develop and evaluate an in vitro multispecies dental biofilm model that aimed to mimic the environment of chronic periodontitis.

Methods: Streptococcus gordonii KN1, Fusobacterium nucleatum ATCC23726, Aggregatibacter actinomycetemcomitans ATCC33384, and Porphyromonas gingivalis ATCC33277 were used for this experiment. The biofilms were grown on 12-well plates with a round glass slip (12 mm in diameter) with a supply of fresh medium. Four different single-species biofilms and multispecies biofilms with the four bacterial strains listed above were prepared. The biofilms were examined with a confocal laser scanning microscope (CLSM) and scanning electron microscopy (SEM). The minimum inhibitory concentrations (MIC) for four different planktonic single-species and multispecies bacteria were determined. The MICs of doxycycline and chlorhexidine for four different single-species biofilms and a multispecies biofilm were also determined.

Results: The CLSM and SEM examination revealed that the growth pattern of the multispecies biofilm was similar to those of single-species biofilms. However, the multispecies biofilm became thicker than the single-species biofilms, and networks between bacteria were formed. The MICs of doxycycline and chlorhexidine for four different single-species biofilms and a multispecies biofilm were also determined.

Conclusions: To mimic the natural dental biofilm, a multispecies biofilm composed of four bacterial species was grown. The 24-hour multispecies biofilm may be useful as a laboratory dental biofilm model system.

Keywords: Biofilms, Microbial sensitivity tests, Microbiology, Periodontitis.

INTRODUCTION

Dental plaque is a complex biofilm composed of a consortium of multiple species. For a long time, the removal of dental plaque has been a key step in attempts to overcome periodontal disease. There are various combinations of nearly 700 bacterial species that are postulated to colonize the surfaces of the oral cavity [1,2]. Oral biofilms have a biodiverse, heterogeneous, organized structure that changes over time [3]. The internal structure of
the dental biofilm could be the result of inter- and intraspecies coaggregation and coadhesion interactions aided by specific adhe-
sin-receptor molecules [4,5]. Biofilm-embedded bacteria are less
susceptible to antimicrobial agents than are planktonic bacteria.
The biofilm structure serves as a physical barrier that limits the dif-
fusion of agents, thereby severely limiting the effect of antibacte-
rial treatment compared with the efficacy in planktonic bacteria
[6,7].
Because in vivo studies of natural dental biofilms are limited and
highly variable, the interpretation of the results of these in vivo
studies is difficult. Difficulties with in vivo studies of the dental
biofilm and its complex, heterogeneous structures have led to the
development of laboratory biofilm model systems [8]. Although
laboratory models cannot completely reproduce the complexity
of the oral environment, they have certain advantages. An in vitro
dental biofilm model circumvents the ethical conflicts that arise in
clinical studies and can be used to analyze a variety of important
in vivo processes in a highly reproducible fashion. These models
have contributed considerably to elucidating the role of microbial
interactions in biofilm development and the effect and working
mechanisms of periodontitis-preventing agents [9].
Bacteriological studies revealed that gram-positive bacteria are
early colonizers, including Streptococcus gordonii, Streptococcus
oralis, Streptococcus sanguinis and other viridans group strepto-
cocci. As gingival inflammation progresses, gram-negative bacteria
develop in dental biofilms, including Fusobacterium nucleatum,
which plays a crucial role as a bridging microorganism between
early and late colonizers, and Porphyromonas gingivalis, Treponema
denticola, and Tannerella forsythia, late colonizers present as a
portion of the climax community in the biofilms at sites that ex-
hbit progressing periodontitis [2,10]. Most biofilms in their natural
environments are likely to consist of a consortium of species that
influence one another synergistically or antagonistically. However,
time is little knowledge of their structure, characteristics (includ-
ing community dynamics), or response to antimicrobial agents.
While single-species biofilms have been studied extensively, we
know notably little concerning multispecies biofilms and their in-
teractions. The purpose of this study was to develop and evaluate
an in vitro multispecies dental biofilm model that aimed to mimic
the environment of chronic periodontitis.

MATERIALS AND METHODS

Bacterial strains and culture conditions
S. gordonii KN1, F. nucleatum ATCC 23726, A. actinomycetem-
comitans ATCC 33384, and P. gingivalis ATCC 33277 were obtained
from the culture collection of the Department of Oral Microbiolo-
y, Gangneung-Wonju National University Dental College. All bac-
teria were grown in trypticase soy broth (Becton Dickinson and
Company, Sparks, MD, USA) containing 1 mg/mL of yeast extract
(Becton Dickinson and Company), 5 μg/mL of hemin (Sigma Chem-
ical Co., St. Louis, MO, USA), and 1 μg/mL of menadione (Sigma
Chemical Co., St. Louis, MO, USA), and 1 μg/mL of menadione (Sigma

Preparation of biofilms
The bacteria were grown in 12-well plates with a glass slip
(round, 12-mm diameter). For four different single-species bio-
films, the concentration of bacteria (S. gordonii, F. nucleatum, A.
actinomycetemcomitans, and P. gingivalis) was adjusted by spec-
trophometry (OD660) after incubation for 24 hours and then
standardized by dilution with phosphate buffered saline (PBS) (pH
7.4) to 1 x 10^7 colony forming unit (CFU)/mL each. The glass slips
were placed in the wells of a 12-well tissue culture plate. Next, the
12 wells were filled with 4 mL of culture medium and 25 μL of sin-
gle-species bacteria, and incubated in anaerobic conditions at 37°C
for up to 48 hours, with a supply of fresh medium. For multispecies
biofilms, we used the same method as for a single-species biofilm.
However, 25 μL of medium was pooled with 4 different single-spe-
cies bacteria. The glass slip was completely submerged in a 12-well
plate. The growth kinetics was evaluated by generating growth
curves for each experiment in triplicate. Briefly, the glass slip with
the biofilm was harvested in the specific intervals (5, 24, 29, and
48 hours) and gently washed with sterile PBS to remove nonadher-
ent bacteria. Next, 1,000 μL of crystal violet solution was added to
the glass slip for 1 minute. Excess stain was removed with the ad-
ministration of 2 mL of sterile PBS three times. A total of 1,000 μL
of 80% ethanol and 20% acetone were added into the well and
mixed on a shaker for 10 minutes. Next, 100 μL of prepared solu-
tion was transferred into new 96-well plates, and the absorbance
was measured at 595 nm (ELx 800TM Absorbance microplate read-
er, BioTek, Winooski, VT, USA) to count the bacteria attached on a
glass slip.

Confocal laser scanning microscope
Before the confocal laser scanning microscope (CLSM) analysis,
the glass slip was gently washed with 2 mL of sterile PBS to re-
move nonadherent bacteria. After that, the glass slip was carefully
mounted on a slide glass. The live/dead BacLight bacterial viability
kit (Molecular Probes, Eugene, OR, USA) was used to view live or
active cells (fluorescent green) and dead or inactive cells (fluores-
cent red) simultaneously. A total of 3 μL of SYTO 9 and 3 μL of
propidium iodide were diluted in 1 mL of distilled water for the
living/dead stain. A total of 100 μL of prepared solution
was transferred into new 96-well plates, and the absorbance
was measured at 595 nm (ELx 800TM Absorbance microplate read-
er, BioTek, Winooski, VT, USA) to count the bacteria attached on a
glass slip.

Scanning electron microscopy
The glass slips with single-species and multispecies biofilm after
24 hours incubation were observed by scanning electron microscopy (VP-SEM, CarlZeiss, Oberkochen, Germany). The glass slips with single-species and multispecies biofilms were washed twice with PBS. Glass slips with attached bacteria were fixed in 2.5% glutaraldehyde in PBS (pH 7.4) for 1 hour at room temperature. The fixed samples were washed three times with PBS for 10 minutes and dehydrated for 30 minutes in a graded series of ethanol. After critical point drying, the samples were mounted on stubs, coated with gold, and observed with SEM. Glass slips with single- and multispecies biofilms were observed by SEM (×10,000, ×30,000).

**Determination of the minimal inhibitory concentration for planktonic and biofilm bacteria**

Planktonic susceptibility tests of different single-species and multispecies bacteria were performed by a broth microdilution assay according to the Clinical and Laboratory Standards Institute M07-A9 (2012) [11]. Antimicrobial agents were prepared with doxycycline (Sigma Chemical Co.) and chlorhexidine (Daewoongpharm Seoul, Korea). Overnight cultures of each bacterium were adjusted to 1 x 10^7 CFU/mL with bacterial culture broth, and 100 μL of broth was added to 96-well plates containing 1 mg/mL of doxycycline or 1,110 μM of chlorhexidine, and serial two-fold dilutions were prepared from working solutions. The bacteria were inoculated into serially diluted doxycycline or chlorhexidine in 96-well round bottom microtiterplates for final concentrations of 5 x 10^6 CFU/mL. The final volume was 100 μL in a microtiter plate well. The plates were read for turbidity after 18 hours for streptococci and 48 hours for *F. nucleatum*, *A. actinomyctecomitans*, and *P. gingivalis*.

The biofilms were grown in 12-well plates with a glass slip for 24 hours. The glass slips with biofilm were transferred into new 48-well plates after washing with PBS. Approximately 1 mg/mL of doxycycline or 1,110 μM of chlorhexidine and serial two-fold dilutions from working solutions were added. The MIC was determined after 48 hours under anaerobic conditions. The MIC of the antibiotics was defined as the lowest concentration without turbidity of the broth. The tests were repeated at least twice.

**RESULTS**

**Biofilm growth curves**

Fig. 1 represents the bacterial count of the biofilms over time. Comparison of the OD obtained at 595 nm indicates that *S. gordonii* and multispecies bacteria counts reached the stationary phase after 24 hours. *S. gordonii*, which is an early colonizer, and multispecies bacteria showed rapid growth patterns until 24 hours. *F. nucleatum*, the intermediate colonizer, showed a steady state until 48 hours. *P. gingivalis* and *A. actinomyctecomitans*, which are late colonizers, showed a slow increase after 29 hours.

**Structural analysis of biofilms**

Each biofilm was collected at 24 hours and analyzed by CLSM and SEM. Fig. 2 represents confocal micrographs describing two-dimensional maximum projection images of the biofilms. Fig. 2 shows the vitality and thickness of biofilms. Multispecies biofilms were thicker than single-species bacteria. *S. gordonii* and multispecies biofilms showed a higher vitality than other species.

Fig. 3 represents SEM photographs of biofilms after 24 hours. *F. nucleatum* did not form a biofilm at 24 hours. Multispecies biofilms showed a multilayered structure compared to single-species biofilms. The CLSM and SEM examination revealed that the growth patterns of multispecies biofilms were similar to those of single-species biofilms. However, multispecies biofilms became thicker than the single-species biofilms, and networks between bacteria were formed.

**Antibacterial activity**

The MICs of bacteria used in this study are listed in Tables 1 and 2. In the multispecies model, the MIC value of planktonic bacteria for doxycycline was 0.0001 μg/mL, and the MIC value for the biofilm was 0.125 μg/mL. The MIC value of planktonic bacteria for chlorhexidine was 8.67 μM, and the MIC value for the biofilm was >1110 μM. The MICs of doxycycline and chlorhexidine were higher in the biofilm state than in planktonic bacteria. The MIC of doxycycline for the multispecies biofilm was higher than those for single-species biofilms of *P. gingivalis*, *F. nucleatum*, or *A. actinomyctecomitans*. The MIC of chlorhexidine for the multispecies biofilm was higher than those for single-species biofilms of *P. gingivalis* or *F. nucleatum*.

**DISCUSSION**

Oral biofilms are a main focus of research in periodontal disease. In the present study, we attempted to develop an *in vitro* multispecies biofilm model that aimed to mimic the environment of chronic periodontitis, and we sought to evaluate the difference be-
tween single-species biofilms and multispecies biofilms using CLSM, SEM, and MICs.

The bacteria used in this study are known periodontal pathogens, and they were composed of early, intermediate and late colonizers [10,12]. We observed a biofilm growth curve over time (Fig. 1). The multispecies biofilm seemed to enter the stationary phase after 24 hours; therefore, we selected 24-hour models in this study. However, the incubation period of a single-species biofilm with P. gingiva-

Table 1. Minimal inhibitory concentration of doxycycline for four different single-species and multispecies bacteria.

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Doxycycline (μg/mL)</th>
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<tbody>
<tr>
<td></td>
<td>Planktonic state</td>
<td>Biofilm state</td>
<td></td>
</tr>
<tr>
<td>P. gingivalis</td>
<td>0.000005</td>
<td>0.0002</td>
<td></td>
</tr>
<tr>
<td>F. nucleatum</td>
<td>0.000025</td>
<td>0.00097</td>
<td></td>
</tr>
<tr>
<td>S. gordonii</td>
<td>0.00002</td>
<td>0.125</td>
<td></td>
</tr>
<tr>
<td>A. actinomycetemcomitans</td>
<td>0.00003</td>
<td>0.00048</td>
<td></td>
</tr>
<tr>
<td>Multispecies</td>
<td>0.0001</td>
<td>0.125</td>
<td></td>
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</table>

Table 2. Minimal inhibitory concentration of chlorhexidine for four different single-species and multispecies bacteria.

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Chlorhexidine (μM)</th>
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<tbody>
<tr>
<td></td>
<td>Planktonic state</td>
<td>Biofilm state</td>
<td></td>
</tr>
<tr>
<td>P. gingivalis</td>
<td>2.775</td>
<td>277.5</td>
<td></td>
</tr>
<tr>
<td>F. nucleatum</td>
<td>5.550</td>
<td>555</td>
<td></td>
</tr>
<tr>
<td>S. gordonii</td>
<td>4.336</td>
<td>&gt;1,110</td>
<td></td>
</tr>
<tr>
<td>A. actinomycetemcomitans</td>
<td>1.084</td>
<td>&gt;1,110</td>
<td></td>
</tr>
<tr>
<td>Multispecies</td>
<td>8.670</td>
<td>&gt;1,110</td>
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lis, A. actinomycetemcomitans, and F. nucleatum might be necessary for longer than 48 hours. Considering previous studies, Prates et al. [13] used a 48-hour biofilm model of A. actinomycetemcomitans to investigate the bactericidal effect of photodynamic therapy with malachite green and red lasers, and Wright et al. [14] used a 72-hour biofilm model of P. gingivalis to compare the in vitro effect of metronidazole between the planktonic state and a single-species biofilm. However, we postulated the 24-hour multispecies biofilm model would minimize the possibility of contamination and increased the reproducibility.

In this study, we took CLSM and SEM images to observe the characteristics of biofilm structure. The CLSM and SEM examination revealed that the growth pattern of a multispecies biofilm was similar to those of single-species biofilms except F. nucleatum. With time, however, the multispecies biofilm became thicker than the single-species biofilms, and networks between bacteria were formed. This biofilm behavior was in agreement with previous studies [15,16]. Sanchez et al. [15] demonstrated that F. nucleatum was not detected in the subgingival biofilm model until 24 hours of incubation. P. gingivalis and A. actinomycetemcomitans could be measured inside the biofilm after 48 hours.

In the SEM image, the 24-hour biofilm model showed less adhesion of bacteria compared to previous studies. This study used a smooth glass slip to form biofilms. Conversely, in the previous studies, bacteria were grown on the disks with a rough surface such as ceramic calcium hydroxyapatite [15,17]. The difference in bacterial adhesion was most likely due to surface roughness and incubation time. However, it was postulated that the smooth glass slip was similar to the tooth surface in oral bacterial adhesion compared to disks with rough surfaces.

The MIC has been used as the gold standard for determining the antibacterial sensitivity for pathogenic bacteria [11,18]. Previous publications have reported that most bacteria embedded in various biofilms are far less sensitive to antibacterial treatment than are planktonic bacteria [7,14,19]. In this study, the MICs of doxycycline and chlorhexidine were observed to be higher in the biofilm state than in planktonic bacteria, as in previous studies (Tables 1 and 2). We observed that the antibiotic resistance in the biofilm state was increased approximately 100 to 10,000 times of resistance in planktonic bacteria regardless of the bacterial species. In the planktonic state, however, the antibiotic resistance of multispecies bacteria was higher than those of single-species bacteria by 10 to 100 times. In the biofilm state, we did not find differences in the antibiotic resistance between multispecies bacteria and S. gordonii bacteria. These results may be used in this experimental model, which is a 24-hour cultured biofilm model. S. gordonii, which is an early colonizer, showed a rapid growth pattern until 24 hours (Fig. 1). However, Figs. 2 and 3 showed that the multispecies biofilm became thicker than the S. gordonii biofilm, and networks between bacteria were formed.

This study is limited in that we did not clearly observe changes in the anaerobic bacteria inside the biofilm after 24 hours. However, the multispecies biofilm closely mimicked the condition of the oral cavity compared to single-species biofilms. The results obtained in this study suggested that a 24-hour multispecies biofilm has some advantages. First, it would minimize the possibility of contamination and increase reproducibility. Second, it would form a thicker biofilm and more complex networks than a single species of bacteria. Third, it showed a higher antibiotic resistance compared to a single-species biofilm. Therefore, a 24-hour multispecies biofilm can be easily applied in many experiments that use biofilms.

In conclusion, our results demonstrated that to mimic the natural dental biofilm, a multispecies biofilm composed of four bacterial species was grown. The 24-hour multispecies biofilm may be useful as a laboratory biofilm model system.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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