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► To cite this version:

Kanchana Chathoth, Bénédicte Martin, Martine Bonnaure-Mallet, Christine Baysse. Method for screening antimicrobial gels against multi-species oral biofilms. *Journal of Microbiological Methods*, 2021, 187, pp.106253. 10.1016/j.mimet.2021.106253 . hal-03282834

HAL Id: hal-03282834

<https://hal.science/hal-03282834>

Submitted on 8 Sep 2021

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Method for screening antimicrobial gels against multi-species oral biofilms

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Keywords: oral biofilm, antibacterial gels, *Porphyromonas gingivalis*, *Treponema denticola*, *Streptococcus gordonii*, hyaluronic acid, oxygen, MBEC^{TM} assay.

Running headline: *In-vitro* assay to evaluate gels against oral biofilm.

Abstract

We described a microtiter plate-based method that was effectively tailored for testing gel formulations against oral multispecies biofilms established on peg-lids. This method lifts the limitations imposed mainly by the anaerobic nature of the targeted bacterial species and the viscous properties of the targeted treatments.

Periodontitis is a polymicrobial chronic inflammatory disease of the periodontium caused by the accumulation of dental plaque (Pihlstrom *et al.*, 2005). It is regarded as the second most common disease worldwide, with severe forms affecting up to 10 to 15% of adults (Petersen and Ogawa, 2012) resulting in tooth loss. Gel formulations of antibacterial agents are a promising delivery system for the treatment of periodontitis *via* topical administration. Their advantages

include the ease of use, increased retention time at the site of application and controlled drug release. Several authors have demonstrated the effectiveness of gel formulations in reducing microbial content or plaque index (Gomes *et al.*, 2006; Noyan *et al.*, 1997; Paquette *et al.*, 1997; Sauvêtre *et al.*, 1993) in human, animal-based or *in vitro* studies. Similar improvement in probing depth and/or bleeding was reported (Esposito *et al.*, 1996; Graça *et al.*, 1997) on use of gel formulations alone or in conjunction with other modes of treatment. Our objective is to develop a process for high-throughput screening of the efficacy of gel-based formulations on a biofilm of oral bacteria.

The protocol is based on the model biofilm we previously established (Martin *et al.*, 2018) which includes three key periodontal biofilm species. *Streptococcus gordonii* is a representative species of peroxygenic oral streptococci and produces hydrogen peroxide that can influence the pathogenic switch of the oral subgingival biofilm (Chathoth *et al.*, 2020; Kreth *et al.*, 2008). Whereas, *Porphyromonas gingivalis* and *Treponema denticola*, co-exist in deep periodontal pockets (Kigure *et al.*, 1995; Kumawat *et al.*, 2016) display a coaggregation reaction (Grenier, 1992) and are associated with severe forms of periodontitis. A recent theory proposed that the peroxygenic Streptococci (initial colonizers of dental plaque) and the sulfidogenic species such as *P. gingivalis* and *T. denticola* (pathogenic late colonizers) may act as the key players in a dysbiosis process (Chathoth *et al.*, 2020). We therefore choose to implement an assay and test the effect of antimicrobial gels on this biofilm, considering the limitations imposed by both the anaerobic nature of the targeted bacterial species which prevent the use of classical Live/Dead stain or viability-PCR using PMA and the viscosity of the gels. The antimicrobial activity is evaluated by analysis of biofilm development after treatment by qPCR. The conversion of genomic DNA quantity to cells ml⁻¹ was made possible by considering specific genome weights (2.58x10⁻⁶ ng for *P. gingivalis*,

3.12x10⁻⁶ ng for *T. denticola* and 2.41x10⁻⁶ ng for *S. gordonii*) as described by Ammann *et al.*, (2013). In this study, the primers used to distinguish between species were (5' → 3'): 16S *S. gordonii* F, AAG-CAA-CGC-GAA-GAA-CCT-TA; 16S *S. gordonii* R, GTC-TCG-CTA-GAG-TGC-CCA-AC; 16S *P. gingivalis* F, TGG-GTT-TAA-AGG-GTG-CGT-AG; 16S *P. gingivalis* R, CAA-TCG-GAG-TTC-CTC-GTG-AT; 16S *T. denticola* F, CGC-GTC-CCA-TTA-GCT-AGT-TG and 16S *T. denticola* R, TTC-TTC-ATT-CAC-ACG-GCG-TC.

The mixed biofilm was grown for 6 or 24 hours on saliva-coated pegs of Minimum Biofilm Elimination Concentration (MBEC™) Assay System (formerly the Calgary Biofilm Device) (Ceri *et al.*, 1999) and challenged for 1 hour with two gels of similar viscosity already used for the treatment of oral infections. The method is described in Figure 1. Hyalugel®-ADO (Ricerfarma, Milan, Italy) mainly consists of hyaluronic acid (0.2 %, w/w); blue®m oral gel (blue®m Europe B.V., Netherlands) has the oxygen donor sodium perborate (1.72 %, w/w) and the iron chelator lactoferrin (0.2 %, w/w) as main active ingredients (Shibli *et al.*, 2021). A prior experiment using the agar diffusion method indicated that both gel formulations exhibited antibacterial activity against *S. gordonii* and *P. gingivalis*, with blue®m oral gel having better antibacterial activity than Hyalugel®-ADO (Table S1).

In a preliminary assay to implement the method, the antimicrobial gels were tested on mature biofilms, grown for 24 hours. However, no significant change was observed in the ability of *P. gingivalis* and *T. denticola* to develop in the biofilm after the treatment for a further 24-hour incubation in comparison to the control treated with the growth medium MMBC-3 (Martin *et al.*, 2018). Meanwhile, the biofilm growth of *S. gordonii* was severely affected by blue®m oral gel, resulting in a dysbiosis with an increase in the ratio of anaerobic pathogens and a decreased total biomass in comparison to the control (Table S2). This indicated that the treatment with these gels

could be effective as a preventive measure on a thinner biofilm. Therefore, we run the assays on 6-hour biofilms.

The results corresponding to the treatment of a 6-hour biofilm are shown in Figure 2. Confocal fluorescent microscopy imaging of biofilms was performed as a qualitative test to ensure the presence of bacterial biofilm all over the pegs surface (Figure S1). The concentration of the total bacteria (biofilm + planktonic) was significantly reduced from 4.11×10^9 cells ml⁻¹ in MMBC-3 control to 5.15×10^6 cells ml⁻¹ and 1.5×10^7 cells ml⁻¹ for Hyalugel®-ADO and blue®m oral gel respectively after treatment of the biofilms for one hour (Figure 2A). The samples treated with Hyalugel®-ADO and blue®m oral gel displayed a biofilm reduction of 96.5 % and 97.6 % respectively, in comparison to untreated control (taken as 0 % of reduction). This decrease in biofilm concentration due to Hyalugel®-ADO and blue®m oral gel is also evident in the microscopic images (Figure S1). Similarly, a decrease of over 99.5 % was observed in the concentration of detached/planktonic cells on treatment with either of the gels in comparison to control (MMBC-3-treated samples) (Figure 2A). The biofilms on the peg surface (Figure 2B) and the corresponding planktonic (unattached or detached) (Figure 2C) cells contained a higher proportion of *S. gordonii* in comparison to *P. gingivalis* and *T. denticola* cells. The concentration of *S. gordonii* and *T. denticola* in the biofilm decreased significantly between the control samples and the samples treated with Hyalugel®-ADO and blue®m oral gel, while the concentration of *P. gingivalis* remained unchanged. Hyalugel®-ADO decreased the concentration of planktonic *S. gordonii* and *P. gingivalis* compared to controls while no effect was observed on *T. denticola*. blue®m oral gel induced a significant decrease in the concentration of planktonic *S. gordonii* cells and an increase in planktonic forms of *P. gingivalis* and *T. denticola*. These results indicate that both gels are effective in reducing biofilm development after a 1-hour treatment and that blue®m

oral gel is additionally effective in inducing detachment of anaerobic pathogenic species. The method will then allow the number of product applications and the duration of the treatment to be refined in order to optimise the beneficial effects.

In conclusion, this method appears suitable to evaluate the antimicrobial effects of gels against a biofilm whose composition is relevant to predict the evolution of periodontitis. It allows high-throughput testing and is easy to use since antimicrobial activity is assessed by the ability of bacterial strains to grow after treatment. The use of qPCR allows monitoring of both the evolution of the total biofilm and the behaviour of each partner in the biofilm. The modulation of biofilm growth times and treatment exposure times will make it possible to refine the method on a case-by-case basis.

Acknowledgements

We thank blue®m Europe B.V., Netherlands for sending us their product blue®m oral gel, for this study and for product-related information. We also thank the manufacturer of Hyalugel®-ADO, Ricerfarma (Milan, Italy) and the distributor, Laboratoire COOPER, Melun, France, for providing the product-related information. Our sincere gratitude to the Microscopy Rennes Imaging Center (Biosit) for their technical assistance for the microscopy experiments.

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Legends of figures

Figure 1. The different steps of the method for screening the effectiveness of gels on a biofilm of oral bacteria. Strains of *Streptococcus gordonii* Challis DL1 (Chen *et al.*, 2004), *Porphyromonas gingivalis* TDC60 (Watanabe *et al.*, 2011) and *Treponema denticola* ATCC35405 (Chan *et al.*, 1993) were used for the study. The lid with pegs (Nunc-TSP, polystyrene) was placed over the microtiter plate and incubated in filter-sterilised saliva that was diluted twice in sterile distilled water (Normal Saliva Human Fluid, Pool Human Donors, MyBioSource) for 30 minutes. The saliva-coated pegs were immersed in 200 µl of inoculum consisting of *S. gordonii* (OD_{600nm}:0.05), *P. gingivalis* (OD_{600nm}:0.1) and *T. denticola* (OD_{600nm}:0.1) and incubated in anaerobic conditions for 6 or 24 hours at 37°C. The 6-hour or 24-hour three-species biofilms or adherent cells present on the surface of the pegs were subjected to 150 µL of either MMBC-3 or Hyalugel®-ADO or blue®m oral gel for 1 hour in anaerobic condition at 37°C. The pegs were then

incubated in a new microtiter well containing 200 μ l of MMBC-3 for 24 hours in anaerobic conditions at 37°C. The detached/planktonic cells were collected from the microtiter plate and the biofilm were collected from the surface of the pegs by sonication (30 min) and were quantified by qPCR as previously described (Martin *et al.*, 2017). Six pegs (two per condition) were also kept for microscopic visualisation.

Figure 2. qPCR quantification of the number of bacteria (cells ml⁻¹) in the biofilm (collected from the pegs) and in planktonic form (collected from the 96-well microtiter plate). The 6-hour three-species biofilm on the pegs were subjected to MMBC-3 or Hyalugel®-ADO or blue®m oral gel for 1 hour and further incubated in MMBC-3 for 24 hours. The planktonic cells were collected from the 96-well plate while the biofilm was collected from the pegs and quantified. (A) Total concentration (cells ml⁻¹) of bacteria (planktonic/detached + biofilm), bacteria in biofilm and bacteria detached or in planktonic form after each treatment. (B) Concentration of each species in the biofilm after each treatment. (C) Concentration of each species in planktonic/ detached cells for each treatment. White bars = MMBC-3, grey bars = Hyalugel®-ADO and black bars = blue®m oral gel. p-value < 0.05 = *, p-value < 0.01 = **, p-value < 0.001 = ***

Figure S1. Representative microscopic images of the 6-hour three-species oral biofilm on the surface of the pegs. Post 1-hour treatment (with MMBC-3, Hyalugel®-ADO or blue®m oral gel) of the 6-hour biofilms on the surface of the pegs, the pegs were incubated in MMBC-3 for 24 hours. The pegs were further broken from the lid using pliers and stained using Styo®9. The stained pegs were visualized using the Leica TCS-SP5 confocal laser scanning microscope. To compare

between the three treatments, maximum z-production of the Z stack were taken using 10X oil immersion objective lens and numerical zoom of 2: (A), (B), (C). Magnified images were captured using the 63X oil immersion objective lens, numerical zoom of 5.05: (D), (E), (F).

Figure 1

Figure 2