

## Susceptibility of *Enterococcus faecalis* biofilm to antibiotics and calcium hydroxide

Wen L. Chai<sup>1)</sup>, Hassan Hamimah<sup>2)</sup>, Soo C. Cheng<sup>2)</sup>, Atiya A. Sallam<sup>3)</sup>  
and Mariam Abdullah<sup>4)</sup>

<sup>1)</sup>Department of General Dental Practice and Oral and Maxillofacial Imaging,  
Faculty of Dentistry, University of Malaya, Kuala Lumpur, Malaysia

<sup>2)</sup>Department of Medical Microbiology, Faculty of Medicine, University of Malaya,  
Kuala Lumpur, Malaysia

<sup>3)</sup>Department of Preventive and Social Medicine, Faculty of Medicine, University of Malaya,  
Kuala Lumpur, Malaysia

<sup>4)</sup>Department of Conservative Dentistry, Faculty of Dentistry, University of Malaya,  
Kuala Lumpur, Malaysia

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**Abstract:** The purpose of this study was to investigate the antimicrobial efficacy of six groups of antibiotics and calcium hydroxide against *Enterococcus faecalis* biofilm in a membrane filter model. Two-day-old *E. faecalis* (ATCC 29212) biofilm was exposed to ampicillin, co-trimoxazole, erythromycin, oxytetracycline, vancomycin, vancomycin followed by gentamicin, Ca(OH)<sub>2</sub>, and phosphate-buffered saline (control). After 1 h of exposure, the antimicrobial activity was neutralized by washing each disc five times in PBS, and then the colony-forming units of the remaining viable bacteria on each disc were counted. The results revealed that only erythromycin, oxytetracycline and Ca(OH)<sub>2</sub> showed 100% biofilm kill. An ANOVA with a Bonferroni post hoc test ( $P < 0.05$ ) detected significant differences among the test agents, except in the ampicillin group versus the co-trimoxazole group. It is concluded that erythromycin, oxytetracycline and Ca(OH)<sub>2</sub> are 100% effective in eliminating *E. faecalis* biofilm, whereas ampicillin, co-trimoxazole, vancomycin, and vancomycin followed by gentamicin are ineffective. (J. Oral Sci. 49, 161-

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Keywords: antibiotics; antimicrobial efficacy; biofilm; calcium hydroxide; *Enterococcus faecalis*.

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### Introduction

Biofilms are highly organized structures consisting of mushroom-shaped clumps of bacteria bound together by a carbohydrate matrix that contain water channels to deliver nutrients and remove wastes (1). Bacteria sequestered in biofilms are shielded and are often harder to kill than their free-floating or 'planktonic' counterparts (1,2). Biofilm bacteria are up to 1,000 times more resistant to phagocytosis, antibodies, and antibiotics (1). The dominant mechanisms of biofilm resistance are thought to be related to (a) delayed penetration of antimicrobial agents through the exo-polysaccharide matrix, (b) modified nutrient environments and suppression of growth rate within the biofilm, thus affording protection from antimicrobial killing, and (c) a subpopulation of microorganisms in a biofilm can develop into a spore state that is highly protected: a phenotypic state known as a persister (3). Most antimicrobial agents may only be effective on the superficial layer of microorganisms in a biofilm, as the matrix layer may prevent direct contact of the agents with the microorganisms (3,4). In the endodontic field, biofilm did not receive wide attention until it was

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Correspondence to Dr. Wen Lin Chai, Department of General Dental Practice and Oral and Maxillofacial Imaging, Faculty of Dentistry, University of Malaya, 50603 Kuala Lumpur, Malaysia  
Tel: +60-3-79674870/4895/4559  
Fax: +60-3-79674575  
E-mail: chailw@um.edu.my

reported by Sen et al. (5). Using scanning electron microscopy, they found that *Candida albicans* formed biofilms on root canal walls within 10 days after inoculation of tooth sections (5).

*E. faecalis* is one of the most common bacteria isolated from root-treated teeth with persistent periapical disease, but rarely appears in primary endodontic infections (6-8). It has been revealed that if this bacterium is isolated during failed endodontic treatment, and during the time of root filling, then the failure rate of retreatment will be higher (8). This bacterium appears to be highly resistant to the antibacterial effect of  $\text{Ca}(\text{OH})_2$  (9-11). Evans et al. (12) reported that *E. faecalis* was resistant to  $\text{Ca}(\text{OH})_2$  at a pH of 11.1, but unable to survive at a pH higher than 11.5. In radicular dentine, due to its buffer effect, the alkalinity of  $\text{Ca}(\text{OH})_2$  may only reach a pH of 10.3 after intracanal dressing (13). This could be one of the factors contributing to the resistance of *E. faecalis* to  $\text{Ca}(\text{OH})_2$ . This was confirmed in another study using scanning electron microscopy and scanning confocal laser microscopy, which showed that despite intracanal dressing with  $\text{Ca}(\text{OH})_2$ , *E. faecalis* formed biofilm in root canals (14). This biofilm phenotype may be another factor contributing to the resistance of *E. faecalis* to most antimicrobial agents.

The usage of antibiotics in root canal therapy was popular during the 1950s (15), but has since declined. However, with recent understanding of the role played by different root canal bacteria, and also reports on the ineffectiveness of  $\text{Ca}(\text{OH})_2$  against the *E. faecalis* biofilm (9-11), the usage of antibiotics warrants reconsideration (16). Several researchers have studied the antimicrobial efficacy of antibiotics such as clindamycin (16-19), metronidazole (18), tetracycline (19,20), erythromycin (20) and a mixture of ciprofloxacin, metronidazole with minocycline (21,22), against root canal bacteria. The antibiotics used in those studies were in different forms such as paste (16,19-22), gel (18) or impregnated in a delivery vehicle (17). To date, no study has been done on the antimicrobial efficacy of antibiotics in injection form against *E. faecalis* biofilm.

The purpose of this study was to evaluate the antimicrobial effectiveness of six groups of antibiotics and  $\text{Ca}(\text{OH})_2$  on *E. faecalis* biofilm in a cellulose membrane filter model.

## Materials and Methods

Biofilm of *E. faecalis* strain ATCC 29212 (American Type Culture Collection) was generated on sterile cellulose nitrate membrane filter discs (0.2  $\mu\text{m}$  pore size, 13 mm diameter; Whatman, Whatman International Ltd., Maidstone, UK). A bacterial suspension was prepared in

10 ml of sterile saline by suspending a loopful of *E. faecalis* colonies collected from an overnight culture of the bacteria grown on Columbia horse blood agar plate (Biomedica, Utas Maju Sdn. Bhd., Selangor Darul Ehsan, Malaysia). The density of the suspension was standardized by comparison with a 0.5 McFarland Standard to give an approximate count of  $10^8$  CFU/ml. Aliquots (50  $\mu\text{l}$ ) of the bacterial suspension were then inoculated on the sterile discs placed on the surface of Columbia horse blood agar and incubated at 37°C for 48 h aerobically.

After 48 h of incubation, the discs were removed aseptically from the agar plate and transferred carefully into a bottle containing 10 ml of phosphate-buffered saline (PBS) to remove loosely attached bacteria. Then, the discs were transferred to 10 ml of the following solutions and exposed for 1 hour in an aerobic incubator at 37°C. The test agents were:

- 1)  $\text{Ca}(\text{OH})_2$ : calcium hydroxide powder (Calcium hydroxide p.a., Merck, Darmstadt, Germany); saturated solution - pH = 12.3
- 2) ampicillin: 500 mg ampicillin sodium (Pamecil, Medochemie Ltd., Limassol, Cyprus) - pH = 8.5
- 3) erythromycin: 500 mg erythromycin lactobionate and 90 mg benzyl alcohol (Erythrocin Lactobionate IV, Abbott Laboratories, North Chicago, USA) - pH = 7.0
- 4) oxytetracycline: 500 mg oxytetracycline HCl and 200 mg lidocaine (Oxylim, Atlantic Laboratories Corp Ltd., Bangkok, Thailand) - pH = 7.0
- 5) co-trimoxazole: 32 mg sulfamethoxazole and 6.4 mg trimethoprim in 1.6% propylene glycol vehicle (Sulfamethoxazole and Trimethoprim Concentrate Injection BP, DBL, Mayne Pharma Pty Ltd., Mulgrave VIC, Australia) - pH = 10.0
- 6) vancomycin: 500 mg vancomycin hydrochloride lyophilized powder (Vancomycin DBL, Mayne Pharma Pty Ltd., Mulgrave VIC, Australia) - pH = 5.5
- 7) vancomycin: 500 mg vancomycin hydrochloride lyophilized powder (Vancomycin DBL, Mayne Pharma Pty Ltd., Mulgrave VIC, Australia) - followed by gentamicin: 400 mg gentamicin sulfate (Garasent, Duopharma (M) Sdn Bhd., Selangor Darul Ehsan, Malaysia) - pH = 5.5
- 8) control group: PBS (Dulbecco's A, Oxoid Ltd., Basingstoke, UK) - pH = 7.0

The antibiotics used in the experiment were commercially available and in injectable form, prepared according to the manufacturers' guidelines. The pH values of the test agents were measured using a pH meter (Istek Model 460CP, Istek Inc., Seoul, Korea). For each test group, from the same

incubation batch, five discs were exposed to the test agent, while another one disc was tested in PBS. This was performed to confirm the presence of bacteria growth on the discs prior to exposure to each test agent. In addition, another five discs were tested in PBS as a control group.

After 1 h of exposure, the activities of the test agents were terminated by washing the discs five times with 10 ml of sterile PBS each time. Subsequently, the remaining viable bacteria on the discs were removed by sonication for 5 min using an ultrasonic machine (BioSonic, Coltene/Whaledent Inc, NY, USA). This produced a 'neat' bacterial suspension. The suspension was then diluted 10-fold (up to  $10^{-7}$ ) and plated on Columbia horse blood agar plates. These plates were incubated for 24 h in an aerobic incubator at 37°C, and colony-forming units (CFU) per disc were then calculated.

The results were analyzed using one-way analysis of variance (ANOVA) and Bonferroni post hoc tests to compare the efficacy among the antimicrobial agents against *E. faecalis* biofilm. The type 1 error was preset at 0.05.

## Results

The mean CFU counts were transformed using the natural logarithm, and a value of '1' was added to the CFU counts as some data contained a value of '0' (no growth of bacteria). Thus, mean  $\log_{10}$  (CFU+1) was obtained, which provides a better normally distributed outcome. The mean  $\log_{10}$  (CFU+1) values of remaining viable bacteria recovered after exposure of *E. faecalis* biofilm to the test agents are shown in Fig 1.

The results revealed that Ca(OH)<sub>2</sub>, erythromycin and oxytetracycline were able to totally kill *E. faecalis* biofilm after 1 h of exposure. On the other hand, ampicillin, co-trimoxazole, vancomycin and vancomycin followed by gentamicin were unable to totally eradicate bacteria in the biofilm (Fig. 1). In detail, ampicillin and co-trimoxazole showed significant ( $P < 0.05$ ) reduction of the CFU count, i.e. about 1  $\log_{10}$ , when compared with the PBS positive control group. In contrast, it was noted that the vancomycin and vancomycin followed by gentamicin groups had higher mean  $\log_{10}$  (CFU+1) values than the PBS control group (Fig. 1). Ampicillin, co-trimoxazole, vancomycin and vancomycin followed by gentamicin are designated as 'ineffective antibiotics' in subsequent text. When the CFU counts were compared among these 'ineffective antibiotics', they were significantly different, except for the ampicillin group versus the co-trimoxazole group. The antimicrobial efficacy of 'ineffective antibiotics' in descending order was as follows: ampicillin, co-trimoxazole, vancomycin & gentamicin, and vancomycin (Fig. 1).

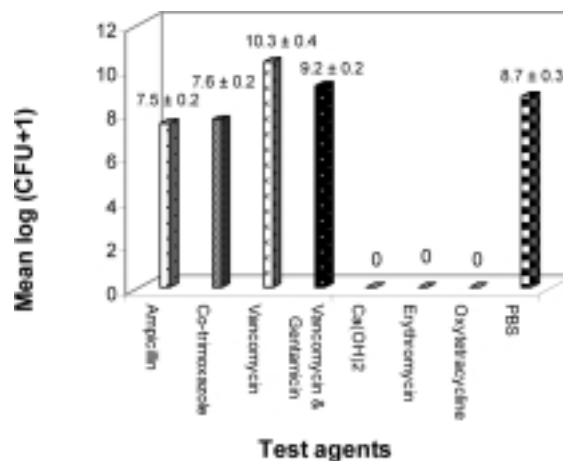


Fig. 1 Mean  $\log_{10}$  (CFU+1) value of *E. faecalis* following 1 h of exposure to test agents. PBS was used as a positive control.

## Discussion

The membrane filter disc model adopted in this study has been used by several researchers to compare antimicrobial efficacy of test agents on oral bacterial biofilm (2,18,23). It was reported that biofilm was generated after a 2-day incubation of *E. faecalis* on the discs (2,18,23). This model has the advantage of growing biofilm on standardized surfaces, thus allowing more accurate assessment of the efficacy of antimicrobial agents.

In this study, six antibiotics were selected based on the list of the National Committee for Clinical Laboratory Standards (NCCLS). From this list, it is evident that *E. faecalis* (ATCC29212) is susceptible to all of the six groups of antibiotics chosen. The susceptibility was reconfirmed by an agar diffusion test carried out prior to the actual experiment. To avoid the problem of insolubility of antibiotics in water, commercially available antibiotics in injectable form were used. The injectable antibiotics were in liquid form after preparation, thus allowing better exposure of *E. faecalis* biofilm to the antibiotics. Some of these commercially available antibiotics contain other chemical components such as benzyl alcohol in erythromycin, which by itself may have some antimicrobial effect. However, as the additional chemical components normally have weak antimicrobial effects, the main focus of this study was still the antibiotics concerned. In analyzing the results, the authors took into consideration the possible synergistic effect of the antibiotic and the solvents against *E. faecalis*.

In this study, a 1-hour exposure time was used, based on the results of a pilot study. At the end of exposure, it is imperative that the actions of the tested antimicrobials are terminated, to ensure that there is no residual activity

during the process of sampling. Other researchers have used neutralizing broth or Lethen broth to stop the activities of several antimicrobial agents such as chlorhexidine (2,18,23), NaOCl, povidone-iodine (2,23), Ca(OH)<sub>2</sub> (2), clindamycin and metronidazole (18). In this study, in order to terminate the antimicrobial actions of antibiotics as well as Ca(OH)<sub>2</sub>, each specimen was washed five times with 10 ml of sterile PBS each time. This procedure reduced the original concentration of the antibiotics to below the minimal inhibitory concentration (MIC) and the pH of Ca(OH)<sub>2</sub> to a neutral level (pH 7). Thus, it was expected that there would be no residual antimicrobial activity of antibiotics against *E. faecalis*, and that at neutral pH, Ca(OH)<sub>2</sub> would have no killing effect on the bacterium. However, this procedure may wash off some bacterial cells. These loosely attached cells were likely to be planktonic cells that had been detached from the discs. The volume of PBS (10 ml) and the duration of each wash were standardized throughout the study. Therefore, the loss of planktonic cells during the five washes was standardized for all test groups.

With regard to other studies involving tetracycline, Shabahang and Torabinejad have reported that MTAD (a mixture of a tetracycline isomer, an acid, and a detergent) was effective against *E. faecalis* (24). In contrast, Lin et al. (19) demonstrated that Ledermix (Lederle, Wolfratshausen, Germany), which contains demethyl-chlortetracycline, had poor antibacterial activity in reducing viable bacteria in dentinal tubules. This poor antibacterial activity may be due to the presence of a steroid compound (triamcinolone acetonide) in Ledermix, or inability of the antibiotic to penetrate the dentinal tubules. With regard to erythromycin, Molander and Dahlén (20) reported that it showed an overall superior performance to tetracycline. However, these studies made no mention about the presence of biofilm, and thus cannot be compared directly with the present study, as different models were used.

Most studies using the 'infected tooth' model have revealed that Ca(OH)<sub>2</sub> is ineffective against *E. faecalis* especially when it is used alone without combination with any other antimicrobial agents (9,11,14,25). In contrast, Ca(OH)<sub>2</sub> used in the present study was able to completely eradicate *E. faecalis* biofilm in a membrane filter model. This could be attributable to the fact that the pH of Ca(OH)<sub>2</sub> remained high in the membrane filter model. In contrast, when used in the 'infected tooth' model, the pH of Ca(OH)<sub>2</sub> was lower due to the buffering effect of root dentine (12), and thus it was less effective against *E. faecalis* in dentinal tubules. Another study that also used a membrane filter model (2) revealed that some viable bacteria were still present even after 1 hour of exposure. This different

outcome could have been attributable to the methodology used.

The biofilm phenotype could be the main reason for the resistance of *E. faecalis* biofilm to 'ineffective antibiotics'. The matrix in the biofilm may form a barrier that prevents diffusion of the antibiotics, thus retarding transportation of the antibiotic molecule into the biofilm or reaction of the antimicrobial within the matrix (4). Another possible mechanism could be related to alteration of the microorganism growth rate in the biofilm. The modified nutrient environment in a biofilm suppresses the growth rate of its constituent microorganisms (4). As ampicillin acts by inhibiting cell wall synthesis in actively dividing bacteria, it may not be effective against slow-growing *E. faecalis* biofilm. Thus the 1-hour exposure to ampicillin in this study may have been too short to affect cell wall synthesis of *E. faecalis* biofilm. However, in an earlier pilot study, it was noticed that even though five discs were exposed to ampicillin for 24 h, positive growth was still evident (data not shown). Therefore, the duration of exposure may not be the only factor responsible for the ineffective antimicrobial action of ampicillin against *E. faecalis* biofilm.

In this study, co-trimoxazole solution was diluted according to the manufacturer's instructions (1 ml in 25 ml of water for injection), but *E. faecalis* biofilm was not susceptible to the diluted co-trimoxazole. In an additional experiment, a similar antimicrobial test was carried out using a membrane filter model but with a full concentration of co-trimoxazole, and this showed that a 1-hour exposure totally eliminated the *E. faecalis* biofilm (data not shown). This finding concurred with the study done by Ceri et al. (26), who demonstrated that the minimum biofilm eradication concentration (MBEC) of an antibiotic may be 100 to 1000 times higher than the MIC for the same organism in planktonic form.

Lastly, vancomycin acts by inhibiting cell wall synthesis, whereas gentamicin is an irreversible inhibitor of protein synthesis. When vancomycin was combined with gentamicin, it significantly reduced ( $P < 0.05$ ) the number of colony-forming units of viable bacteria to a greater degree than vancomycin alone. This could be due to a synergistic effect of the two agents. In Fig. 1, it can be seen that the bacterial counts after treatment with vancomycin and vancomycin followed by gentamicin were higher than those in control group (PBS). This result may have been due to technical error, as duplicate plating was not carried out for these two groups. Nevertheless, this error did not alter the conclusion that these two antibiotics are ineffective against *E. faecalis* biofilm in a membrane filter model.

In conclusion, after 1 h of exposure to erythromycin,

oxytetracycline and calcium hydroxide, no bacterial growth on membrane filter discs was noted. On the other hand, viable bacteria remained after 1 h of exposure of *E. faecalis* biofilm to ampicillin, co-trimoxazole, vancomycin and vancomycin followed by gentamicin.

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### References

1. Costerton JW, Lewandowski Z, DeBeer D, Caldwell D, Korber D, James G (1994) Biofilms, the customized microniche. *J Bacteriol* 176, 2137-2142
2. Abdullah M, Ng YL, Gulabivala K, Moles DR, Spratt DA (2005) Susceptibilities of two *Enterococcus faecalis* phenotypes to root canal medications. *J Endod* 31, 30-36
3. Stewart PS, Costerton JW (2001) Antibiotic resistance of bacteria in biofilms. *Lancet* 358, 135-138
4. Donlan RM, Costerton JW (2002) Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* 15, 167-193
5. Sen BH, Safavi KE, Spangberg LSW (1999) Antifungal effects of sodium hypochlorite and chlorhexidine in root canals. *J Endod* 25, 235-238
6. Sirén EK, Haapasalo MP, Ranta K, Salmi P, Kerosuo ENJ (1997) Microbiological findings and clinical treatment procedures in endodontic cases selected for microbiological investigation. *Int Endod J* 30, 91-95
7. Molander A, Reit C, Dahlén G (1998) Microbiological status of root-filled teeth with apical periodontitis. *Int Endod J* 31, 1-7
8. Sundqvist G, Figdor D, Persson S, Sjogren U (1998) Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 85, 86-93
9. Haapasalo M, Ørstavik D (1987) In vitro infection and disinfection of dentinal tubules. *J Dent Res* 66, 1375-1379
10. Ørstavik D, Haapasalo M (1990) Disinfection by endodontic irrigants and dressings of experimentally infected dentinal tubules. *Endod Dent Traumatol* 6, 142-149
11. Siqueira JF Jr, de Uzeda M (1996) Disinfection by calcium hydroxide pastes of dentinal tubules infected with two obligate and one facultative anaerobic bacteria. *J Endod* 22, 674-676
12. Evans M, Davies JK, Sundqvist G, Figdor D (2002) Mechanisms involved in the resistance of *Enterococcus faecalis* to calcium hydroxide. *Int Endod J* 35, 221-228
13. Miñana M, Carnes DL Jr, Walker WA III (2001) PH changes at the surface of root dentin after intracanal dressing with calcium oxide and calcium hydroxide. *J Endod* 27, 43-45
14. Distel JW, Hatton JF, Gillespie MJ (2002) Biofilm formation in medicated root canals. *J Endod* 28, 689-693
15. Grossman LI (1951) Polyantibiotic treatment of pulpless teeth. *J Am Dent Assoc* 43, 265-278
16. Molander A, Reit C, Dahlén G (1990) Microbiological evaluation of clindamycin as a root canal dressing in teeth with apical periodontitis. *Int Endod J* 23, 113-118
17. Gilad JZ, Teles R, Goodson M, White RR, Stashenko P (1999) Development of a clindamycin-impregnated fiber as an intracanal medication in endodontic therapy. *J Endod* 25, 722-727
18. Lima KC, Fava LRG, Siqueira JF Jr (2001) Susceptibilities of *Enterococcus faecalis* biofilms to some antimicrobial medications. *J Endod* 27, 616-619
19. Lin S, Levin L, Peled M, Weiss EI, Fuss Z (2003) Reduction of viable bacteria in dentinal tubules treated with clindamycin or tetracycline. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 96, 751-756
20. Molander A, Dahlén G (2003) Evaluation of the antibacterial potential of tetracycline or erythromycin mixed with calcium hydroxide as intracanal dressing against *Enterococcus faecalis* in vivo. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 96, 744-750
21. Sato T, Hoshino E, Uematsu H, Noda T (1993) In vitro antimicrobial susceptibility to combinations of drugs on bacteria from carious and endodontic lesions of human deciduous teeth. *Oral Microbiol Immunol* 8, 172-176
22. Hoshino E, Kurihara-Ando N, Sato I, Uematsu H, Sato M, Kota K, Iwaku M (1996) In vitro antibacterial susceptibility of bacteria taken from infected root dentine to a mixture of ciprofloxacin, metronidazole and minocycline. *Int Endod J* 29, 125-130
23. Spratt DA, Pratten J, Wilson M, Gulabivala K (2001) An in vitro evaluation of the antimicrobial efficacy of irrigants on biofilms of root canal isolates. *Int Endod J* 34, 300-307

24. Shabahang S, Torabinejad M (2003) Effect of MTAD on *Enterococcus faecalis*-contaminated root canals of extracted human teeth. J Endod 29, 576-579
25. Gomes BP, Souza SF, Ferraz CC, Teixeira FB, Zaia AA, Valdrighi L, Souza-Filho FJ (2003) Effectiveness of 2% chlorhexidine gel and calcium hydroxide against *Enterococcus faecalis* in bovine root dentine in vitro. Int Endod J 36, 267-275
26. Ceri H, Olson ME, Stremick C, Read RR, Morck D, Buret A (1999) The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. J Clin Microbiol 37, 1771-1776