New Robbins dispositive to evaluate antimicrobials activity against bacterial biofilms on central venous catheters

Un nouveau dispositif de Robbins pour l'évaluation d'activité des agents antimicrobiens contre les biofilms bactériens formés sur les cathéters veineux centraux

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RÉSUMÉ

Prérequis: L'usage fréquent des implants médicaux a été étroitement associé avec l'émergence de Staphylococcus epidermidis comme cause majeure d'infections nosocomiales. Cette émergence est principalement associée avec l'habilité de cette bactérie à s'organiser dans une structure appelé biofilm sur la surface de l'implant. Différents modèles d'étude de biofilm in-vitro ont été développés.

Le but de ce travail a été de développer un modèle d'étude dynamique de biofilm basé sur le réacteur Robbins à partir de dispositifs disponibles dans la pratique médicale courante et peu couteux.

Méthodes : L'évaluation de ce modèle a été faite par la détermination du nombre de Reynolds et l'énumération des cellules bactériennes sur boîtes de pétri et par amplification PCR en temps réel lors de la simulation d'élimination du biofilm de Staphylococcus epidermidis par la daptomycine.

Résultats : Le calcul hydrodynamique a estimé nombre de Reynolds à 26.62 correspondant à un flux laminaire adapté à une croissance dynamique. Le dénombrement cellulaire a noté que la colonisation bactérienne du segment de cathéter testé a été réduite de manière significative après 24 et 48 heures de traitement avec la daptomycine ($P < 0,01$).

Conclusion : le dispositif développé a montré des propriétés dynamiques acceptables.

Mots-clés

Dispositif ; Antimicrobiens ; Biofilm ; Cathéters

SUMMARY

Background: Layouts of biomedical devices were tightly related with the emergence of Staphylococcus epidermidis as a major cause of nosocomial infections because of its ability to form biofilm on the biomaterial surfaces. This fact led researchers to develop in-vitro models to simulate what is really happening during biofilm formation process in order to have a better understanding of this phenomena and then to control it and to resolve the associated problems.

The aim of this paper was to develop a homemade dynamic device based on instruments used in clinical practice, easy to mount, with low coast and with no sophisticated features.

Methods : used to evaluate this dispositive were hydrodynamic calculation and enumeration of bacterial cells on petri dishes and with real time polymerase chain reaction during simulation of Staphylococcus epidermidis biofilm eradication with daptomycin.

Results: With hydrodynamic calculation, Reynolds number was estimated to be 26.62 corresponding to a perfect laminar flux giving suitable dynamic growth environment for such experiment. Data recovered from cell enumeration with the two methods showed that bacterial colonization of the tested catheter segment was significantly reduced after 24 and 48h of treatment with daptomycin ($P<0.01$) reflecting a considerable reliability of this device.

Conclusion: the simple dispositive developed in this work has shown acceptable hydrodynamic proprieties and good reliability making research on biofilm easy to reach.

Key-words

Dispositive; Antimicrobials; Biofilm; Catheters.

Outside Petri dishes, bacteria lives in structured communities called biofilms [1] formed by one or more bacterial species [2]. The emergence of bacterial biofilms in medical field as an important leading cause of indwelling devices associated infections especially those related to central venous catheter [3] and the special attitude of bacteria inside biofilm [4], is behind the needs to study those communites as they are in nature and to develop laboratory biofilm reactors as in vitro experimentation tool used to simulate the real growth conditions [5].
Biofilm reactors are now often used to study bacterial population dynamics, interaction between cells and response to several environmental stresses [5]. In biomedical research, such devices are used in two major applications; the first one is to assess the bacterial adhesion to engineered polymers of modified surfaces used in medical implants technology [6], the second application is the evaluation of anti-biofilm compounds efficiency and that's by adding chemicals in the growth environment and then monitoring the real time response at cell or biofilm scale [7].
Biofilm reactors technology has evolved in last twenty years and a lot of devices were described and shaped [8], from simple devices as the one of Robbins [9] to high-tech microscopic scale reactors used for real time biofilm monitoring [10]. All described reactors are characterized with constant elements: inflow dispositive, biofilm growth chamber and outflow dispositive. More and more expensive reactors make studying biofilm harder and harder and so will contribute to a slower progress in this research field since only laboratories with huge budget will be able to have necessary materials.
The aims of this work was to develop a low coast and simple to use biofilm reactor and to validate it for biofilm in vitro studies.

METHODS

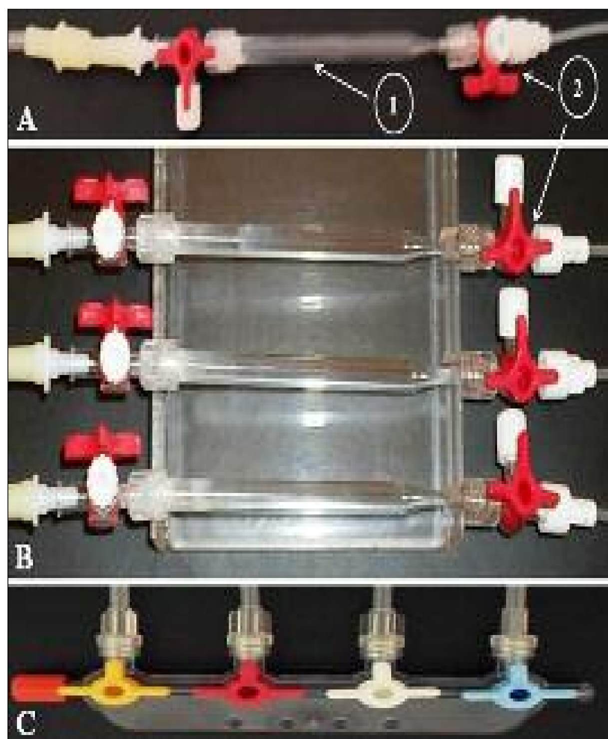
Bacterial strain, medium, antimicrobial agent, and equipments:
The reference strain RP62A [11] was used as biofilm forming strain. The culture medium were the Tryptocasein soja broth (TSB) and Muller-Hinton agar (MHA) (Biorad, Marnes – La Coquette, France) supplemented with 0.25% of glucose. The antimicrobial agent used in the present study was the lipopeptides daptomycin (Sigma aldrich, Steinheim, Germany).
Equipments needed for dispositive assembly are: serum bag (Bieffe Medital, Lyon, France), Perfusion set (Multimedical, Viadana, Italy), three way stopcock (Nubenco, Paramus, New Jersey, USA), Pipette Pasteur (CML, Nemours France), polyethylene extension tube (Ethycath, adhe-els labs, Sousse, Tunisia) and four gang manifold (CAIR LGL, Cirvieux d'Azergues France)

Dispositive assembly

Experimental procedure:

- Connect the reactor components as it is demonstrated in figure 1A (but do not connect the drainer P1 to the TSB carboy.
- Maintain the TSB carboy and the reactor at 2 m and 0.5 m of height respectively.
- Connect the drain1 to the TSB carboy with a locked fluid flow regulator.
- Open the flow regulator entirely to have a high flow rate in order to fill up the dispositive with TSB.

Figure 1 : Biofilm growth chamber
(1): Pipette Pasteur, (2): Three ways stopcock
(A) Single cell of the biofilm growth chamber, (B) Three cells used for simultaneous experimentation of different chemicals, (C) Four gang manifold used for same solution simultaneous delivery to different cells.



- Once the TSB is cooling from the drainer P2, adjust the flow to seven drops / minute (corresponding to 20ml / hour).
- At the starting time of the anti-biofilm test, the tape T1 was deviated to simultaneously stop the TSB flow and to liberate the second entry of the tape in which a syringe containing 10 ml of bacterial suspension was already connected.
- The bacterial suspension was injected in the cell culture throw the tape 1, which will remain, locked for further two hours allowing the bacterial adhesion to the catheter segment.
- After two hours, the syringe was disconnected and the tape 1 was deviated to its originally position allowing the TSB flow to pass from the TSB carboy to the cell culture through drainer 1, and from the cell culture to the waste carboy through drainer P2.

Troubleshooting verification

To verify dispositive sealing, TSB is left to fill all tubulation with high flow rate, then tapes were adjusted one by one to block liquid through up. Dispositive was ready for experimentation only if there was no drops falling down from the TSB carboy in the opened fluid flow adjuster. If there were any leaks, industrial silicon might be used to correct any disconnection between the dispositive parts. To verify sterility of dispositive, 1 ml of cooling TSB was pipetted with a sterile syringe from the tape 2 before and after the experiment and then cultured in MHA dishes.

Dispositive validation

Hydrodynamic calculation of the running flow inside the reactor was performed to identify the flow nature. The efficiency of the reactor was evaluated with simulation of biofilm eradication experiment using already described antibiotic, and then comparing results obtained with those already reported in literature.

Hydrodynamics: Reynolds number calculation

Flow nature was defined using Reynolds number [12, 13] according to the following formula:

Re = □□

where: Re = Reynolds Number (non-dimensional)

ρ = density (kg/m3)

u = velocity based on the actual cross section area of the pipe (m/s)

μ = dynamic viscosity (N.s/m2)

L = characteristic length (m)

The velocity "u" of TSB which is estimated to have same physical proprieties as water at 20° is calculated as follows:

u =

where:

Qv: Volumetric flow rate (m3/s)

D: diameter of pipe (m)

According to Re values [5], flow was categorized as Stokes regimen (Re <1), laminar regimen (1< Re < 2320), transitory regimen (2320 < Re < 50000), turbulent regimen (50000< Re).

Cell enumeration after exposure to daptomycine

Cell enumeration on petri dishes

Catheter segment were incubated for 24h with antibiotic free TSB, then for 24 or 48h with TSB containing 15µg/ml of daptomycine. Once catheter segments were recovered from the dispositive and washed with sterile PBS, they were subjected to alternative cycle of sonification and vortexing as it was described previously [14], briefly, a cycle of 30 seconds vortexing at 1200 rpm, 1min sonification at 40Hz/s and again 30 seconds vortexing at 1200 rpm was performed in a total volume of 1 ml of sterile PBS in order to collect the maximum of viable cells. Twenty microliters were serially diluated and cultred in MHA plates for colony forming units (CFU) enumeration.

Cell enumeration with Real Time PCR (RT-PCR)

For absolute enumeration, 100µl of bactrail suspension obtained after sonification was subjected to DNA extraction for RT-PCR targeting sodA gene [15] using following primers Forward 5'-TCA GCAGTTGAAGGGACAGAT-3' and Reverse 5'-CCAGAACAATGAATGGTTAAGG-3', as described by Iwase et al (2). Real-time PCR was performed in the Exicycler 96 ™ RT-PCR system with SYBR GreenI premix kit (Bioneer, Alameda, California, USA), Individual RT-PCR reactions were carried using the default thermocycler program furnished with the kit.

Data analysis of cell enumeration experiments were carried out with one-way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences Software (SPSS V19.0, Inc., Chicago). All tests were performed with a confidence level of 95%.

RESULTS

Observation on the reactor

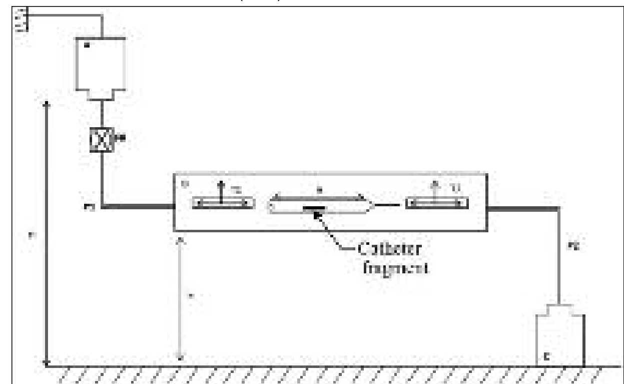
After connection of different reactor parts, TSB flow was controlled in order to detect any leaks. There were few problems only in the connection between the tape1 and the biofilm growth chamber. This problem was definitively corrected using silicon to adjust connection between those components. Sterility of dispositive was confirmed since cultured specimen collected before experiment showed no bacterial growth and the one after experiment showed only S. epidermidis colonies.

Reynolds number

With formula application using dimensions mentioned in the schematic presentation of the dispositive (figure 2), we found Re of 26,62. According to classification mentioned above [5], the flow running in the biofilm growth chamber was a laminar flow.

Figure 2 : Shematic design of the biofilm reactor

(A) Medium (TSB) carboy, (B) Biofilm growth chamber, (C) Waste carboy, (FR) Flow Regulator Height (H): 2m, Height (h): 0,5m, P1: prolongator 1, P2: prolongator 2, T1: three ways stopcock, T2: three ways stopcock, a: lengh of the biofilm cell culture (95 mm), b: section of the biofilm cell culture (5mm)

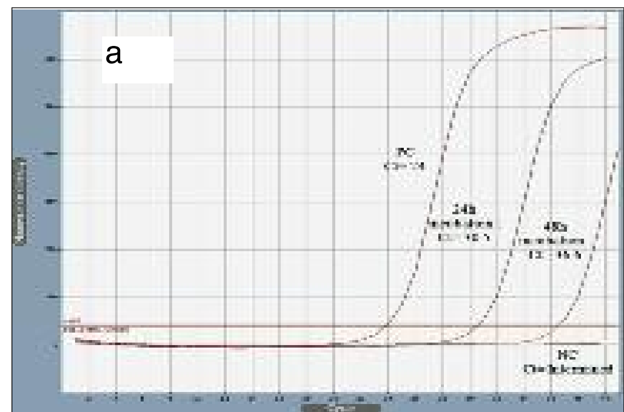


Cell enumeration

Bacterial cell enumeration with colonies counting on agar plates (figure 3a) or RT-PCR assay (figure 3b) has shown that after incubation for 24h and 48h, in the presence of daptomycin, the number of bacterial cells collected from treated catheters was significantly lower when compared to the number of bacteria recovered from the non-treated catheter segment (P<0.01).

Figure 3: Bacterial enumeration after 24 and 48h of treatment with daptomycin using real time polymerase chain reaction method (fig 3a) or absolute Colony Forming Unit quantification on agar plate (fig3b)

(Ct): Cycle threshold, PC: positive control (non treated sample), NC: Negative Control (sample without biofilm)



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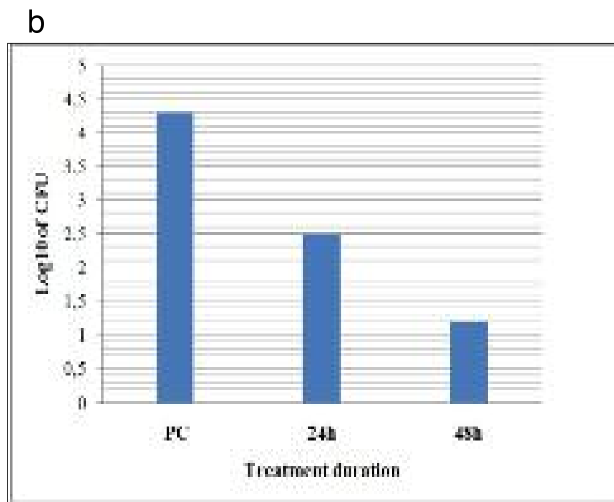

DISCUSSION

As in classic cell flow biofilm reactors [8], Robbins reactor allows providing a continuous flow of nutrient to a biofilm growing on abiotic surface and of course the evacuation of wastes and detached cell clusters throw different tabulation. Several modifications were introduced to renew this dispositive [16]. Here in the present work, modifications were done as described in the inpout flow delivering and in the biofilm growth chamber. Sharp nutriment flow in cell flow biofilm reactor model is insured by an adjustable peristaltic pump injecting the broth into the cell and so insuring the reactor dynamism [17]. Therefore, medium cooling in such reactors is going step by step with air penetration into the medium carboy for volume replacement. In our model, a suspended deformable broth container is used with no pumps, indeed medium cooling flow is insured by gravity action and there is no need for air penetration into the medium container because of its deformability making its volume equal to the medium volume along the experiment time. So, if we compare our model to the classic one, we can conclude that we are using less equipment with a maximum of aseptic experimental conditions. Another point to consider in our dispositive is the nature of the medium flow in the biofilm growth chamber. According to Reynolds number calculation, flow was laminar like for other dispositives [17].This kind of flow is suitable for such experiment since it applies medium shear forces in the same time simulating realty and keeping the biofilm architecture safe [13]. In addition, a very important point making our reactor more suitable for use is the low coast of the dispositive equipment if compared to the Drip Flow Reactor used in several studies [17,18].

Besides, some weakness points are noted, since our dispositive is done especially to study biofilm on catheter surfaces and not on any other abiotic surface. However, using dispositive parts (figure 1B or figure 1C), we can test the impact of several catheter polymers toward bacterial adhesion or the impact of different chemicals on bacterial biofilm respectively.

Daptomycin was described to have high penetration ability through S. epidermidis biofilm [19] and considerable ability to abolish it [20]. Here by, we used this antibiotic not for testing its ability to eradicate S. epidermidis biofilm, but to evaluate how reliable is our dispositive in such experiment. Compared to another study in which the same antibiotic was used in a similar dispositive [19], our reported observations were near of those described in the mentioned study, hence reflecting a trustful experimental layout.

CONCLUSION

The founding of this work came in the simplification of the experimental procedure which was based on easy-to-purchase clinic material making possible biofilm experimenting in any laboratory.

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