Université de Montréal

Implication des biofilms dans la rhinosinusite chronique et l'évaluation des traitements avec un modèle *in vitro*

Par

Zohra Bendouah

Département de sciences biomédicales

Faculté de médecine

Thèse présentée à la Faculté des études supérieures

en vue de l'obtention du grade de maîtrise en sciences biomédicales

option recherche clinique

Août, 2008

Université de Montréal

Faculté des études supérieures

Cette thèse intitulée :

Implication des biofilms dans la rhinosinusite chronique et l'évaluation des traitements avec un modèle *in vitro*

Présentée par :

Zohra Bendouah

a été évaluée par un jury composé des personnes suivantes :

Président-rapporteur : John S. D. Chan

Directeur de recherche : Martin Desrosiers, MD, FRCSC

Codirecteur : Jean Barbeau Ph D

Membre du jury : Marc C. Lavoie

Résumé

Introduction : La chronicité de la rhinosinusite, sa résistance aux antibiotiques, et ses exacerbations aiguës laissent croire que les biofilms sont impliqués dans la rhinosinusite chronique. **Objectifs :** Nous avons évalué la capacité des bactéries *Pseudomonas aeruginosa*, staphylocoques à coagulase négative et *Staphylococcus aureus* à former des biofilms par un essai *in vitro,* et si cette capacité de formation a un lien avec l'évolution de la maladie. Nous avons évalué *in vitro* l'effet de la moxifloxacine, un antibiotique utilisé dans le traitement de la rhinosinusite chronique sur des biofilms matures de *Staphylococcus aureus*. **Méthodes :** Trent et une souches bactériennes ont été isolées de 19 patients atteints de rhinosinusite chronique et qui ont subit au moins une chirurgie endoscopique des sinus. L'évolution de la maladie a été notée comme "bonne" ou "mauvaise" selon l'évaluation du clinicien. La production de biofilm a été évaluée grâce à la coloration au crystal violet. Nous avons évalué la viabilité du biofilm après traitement avec la moxifloxacine. Ces résultats ont été confirmés en microscopie confocale à balayage laser et par la coloration au LIVE/DEAD BacLight. **Résultat et Conclusion :** Vingt deux des 31 souches ont produit un biofilm. La production d'un biofilm plus importante chez *Pseudomonas aeruginosa* et *Staphylococcus aureus* était associée à une mauvaise évolution. Ceci suggère un rôle du biofilm dans la pathogenèse de la rhinosinusite chronique. Le traitement avec la moxifloxacine, à une concentration de 1000X la concentration minimale inhibitrice réduit le nombre des bactéries viables de 2 à 2.5 log. Ces concentrations (100 µg/ml - 200 µg/ml) sont faciles à atteindre dans des solutions topiques. Les résultats de notre étude suggèrent que l'utilisation de concentrations supérieure à la concentration minimale inhibitrice sous forme topique peut ouvrir des voies de recherche sur de nouveaux traitements qui peuvent être bénéfiques pour les patients atteints de forme sévère de rhinosinusite chronique surtout après une chirurgie endoscopique des sinus.

Mots-clés : Biofilm, rhinosinusite chronique, *Pseudomonas aeruginosa*, *Staphyloccocus aureus,* chirurgie endoscopique des sinus, Microscope confocal à balayage laser, LIVE/DEAD BacLight, moxifloxacine, concentration minimal inhibitrice.

Abstract

Introduction: The role of biofilms in chronic diseases is increasingly recognized. Chronic rhinosinusitis, with its chronic indolent course, resistance to antibiotics, and acute exacerbations, has an evolution that parallels that of other biofilm-related diseases. **Objectives:** 1-To develop an *in vitro* method to assess the biofilm formation capacity. 2- To determine whether biofilm-forming capacity of bacteria demonstrated in chronic rhinosinusitis has an impact on persistence of the disease following endoscopic sinus surgery. 3- To determine the *in vitro* activity of moxifloxacin against *Staphyylococcus aureus* in biofilm form. **Method:** Thirty-one bacterial strains recovered from 19 patients with chronic rhinosinusitis at least one year post-endoscopic sinus surgery. Evolution of disease was assessed by questionnaire and endoscopy as favorable or unfavorable. The bacteria were cultured on a 96-well culture plaque and a semi-quantitative method using crystal violet to quantify biofilm production was used. Confirmation of the effect of the antimicrobial agents on viability was performed with confocal laser microscopy, using a LIVE/DEAD BacLight staining. **Results:** Twenty-two of 31 samples produced a biofilm thicker or equal to the positive control. Biofilm formation was associated with a poor evolution for *Pseudomonas aeruginosa* and *Staphylococcus aureus*, but not for coagulase-negative staphylococci. Biofilm treated with moxifloxacin at 1000X (0.1mg/ml – 0.2 mg/ml) gave a 2 to 2.5 log reduction in number of viable bacteria. **Conclusion:** We have shown that Crystal violet method is able to detect biofilm formation. There is a correlation between *in vitro* biofilm production by *Pseudomonas aeruginosa* and *Staphylococcus aureus* and unfavorable evolution after endoscopic sinus surgery, suggesting a role for biofilm in chronic rhinosinusitis. Increased concentrations of moxifloxacin, easily attainable in topical solutions have a potential role in the management of biofilm infections.

Key words: Biofilm, chronic rhinosinusitis, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, endoscopic sinus surgery, confocal laser scanning microscopy, LIVE/DEAD BacLight, moxifloxacin, minimal inhibitory concentration.

Table des matières

Résumé ……..…….……**i**

Abstract …….……..….**ii**

Liste des abréviations …….....**v**

Remerciements ……...**vi**

Introduction…….…....**1-14**

Objectifs..…….…..…..**15**

Résultats sous forme d'articles

1. Bendouah Z, Barbeau J, Hamad W, Desrosiers M. Use of an in vitro assay for determination of biofilm-forming capacity of bacteria in chronic rhinosinusitis. Am J Rhinol. 2006 Sep-Oct;20(5):434-8…….....**16-35**
2. Bendouah Z, Barbeau J, Hamad WA, Desrosiers M. Biofilm formation by *Staphylococcus aureus* and *Pseudomonas aeruginosa* is associated with an unfavorable evolution after surgery for chronic sinusitis and nasal polyposis. Otolaryngol Head Neck Surg. 2006 Jun;134(6):991-6….....**36-57**
3. Desrosiers M, Bendouah Z, Barbeau J. Effectiveness of topical antibiotics on *Staphylococcus aureus* biofilm *in vitro*. Am J Rhinol. 2007 Mar-Apr ; 21(2):149-53…….....……..**58-75**

Résultats non publiés

4. Bendouah Z, Desrosiers M, Barbeau J. Bacterial killing in *Staphylococcus aureus* biofilm doesn't improve with increasing concentration of moxifloxacin beyond 1000XCMI. (soumis).....…….....**76-92**

Discussion générale…….…….....**93-101**

Conclusion …….**102-103**

Références ……...……...**104-110**

La liste des abréviations

CES : chirurgie endoscopique des sinus

CMI : concentration minimal inhibitrice

CV : crystal violet

DO : densité optique

EPS : substances polymériques extracellulaires

FISH : Fluorescence *in situ* hybridization

MCBL : microscope confocal à balayage laser

MEB : microscope électronique à balayage.

MOXI : moxifloxacine

P. aeruginosa : *Pseudomonas aeruginosa*

RSC : rhinosinusite chronique

***S. aureus* (Sa)** : *Staphylococcus aureus*

SCN : Staphylocoques à coagulase négative

UFC : unités formant des colonies

Les remerciements

Je voudrais exprimer ma gratitude aux personnes suivantes :

Un grand merci à mon directeur de recherche monsieur Martin Desrosiers ainsi que mon codirecteur monsieur Jean Barbeau pour leur soutien tout au long de ma maîtrise et pour tout ce qu'ils m'ont appris.

Annie Leduc et Jacynthe Séguin pour leur précieuse assistance au laboratoire.

Je voudrais remercier également monsieur Onder Agbaba pour avoir isolé et identifié les souches bactériennes qui ont servies à notre étude.

Je remercie madame Sylvista Archana de m'avoir bien formée pour l'utilisation de la microscopie confocale.

En fin, je remercie le support financier de la fondation Antoine Turmel.

Introduction

Les biofilms

Depuis l'époque de Koch, les bactériologistes et les cliniciens ont focalisé leurs travaux sur les bactéries planctoniques (sous forme libre), relativement faciles à manipuler au laboratoire car détectables et prédominantes dans les infections aiguës. Par contre, comme on le sait maintenant, seulement une très petite fraction de bactéries se présente sous la forme planctonique ; la majorité des bactéries existent dans la nature sous forme attachée à une surface (biofilm) [1, 2].

Les fossiles retrouvés démontrent que tôt dans l'évolution, les microorganismes ont acquis une capacité à s'organiser en biofilm. La morphologie des biofilms a été identifiée dans des roches sédimentaires qui dataient de 3.5 milliards d'années [3] et dans des dépôts volcaniques de sulfure [4].

La capacité de formation de biofilm chez les microorganismes représente une adaptation acquise durant l'évolution. Une protection était nécessaire pour survivre contre les conditions extrêmes qui régnaient sur terre il y a des milliards d'années (températures extrêmes, pH, rayons UV) [5].

En 1978, John William Costerton a proposé le terme de biofilm en suggérant que ce serait le mode de vie naturel de la plupart des microorganismes. Il l'a définit comme une communauté organisée de microorganismes collaborant entre eux, contenus dans une substance hydratée polymérique extracellulaire (EPS) ou matrice autoproduite, adhéra à une surface inerte ou vivante. Cette matrice qui représente 85% du volume total du biofilm

est constituée essentiellement de polysaccharides, d'acides nucléiques et de protéines [6,7] (Figure 1).

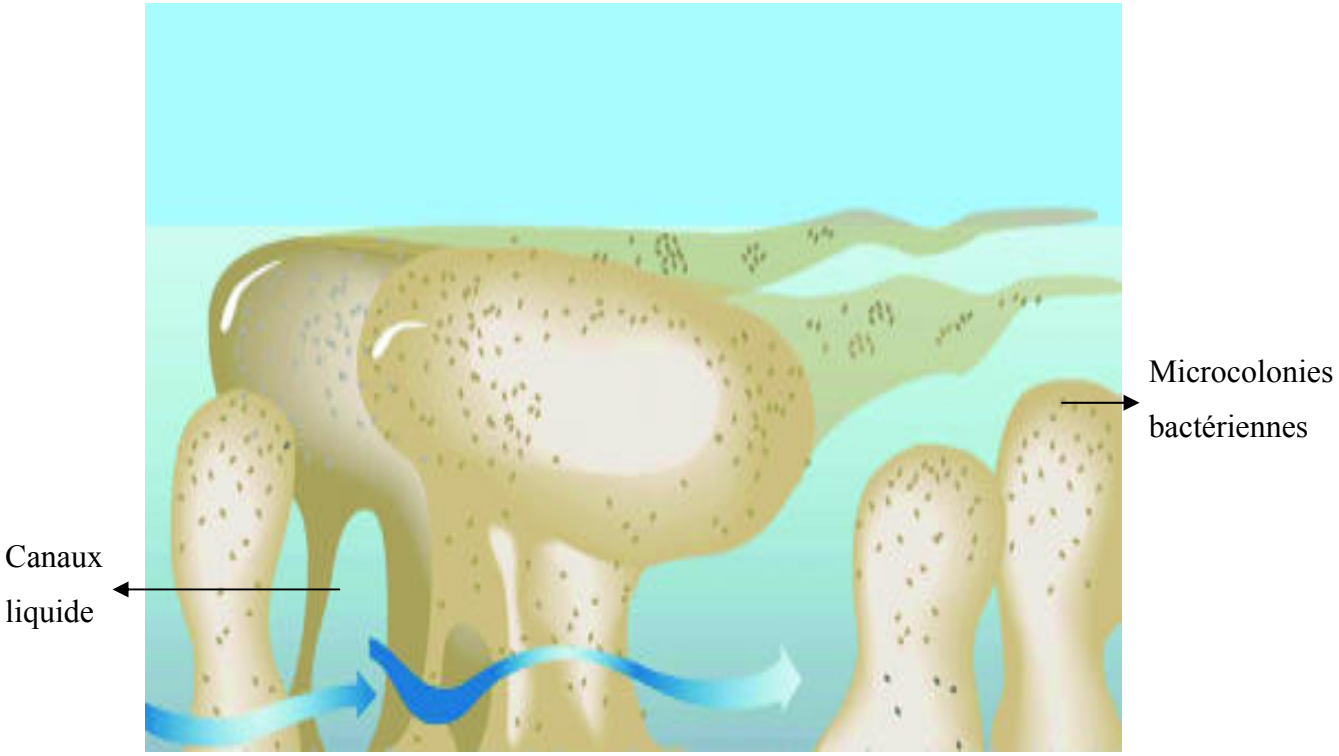


Figure 1. Représentation schématique d'un biofilm [8].

La définition des biofilms a beaucoup évoluée depuis. Elle inclut maintenant l'ensemble des phénomènes où les bactéries adhérent à une surface. La surface d'adhésion des organismes sessiles peut être abiotique (les matériaux inertes : des implants médicaux, des tubes en acier, le sol... etc) ou biotique (les tissus ou cellules vivantes, des cellules épithéliales par exemple).

La définition de biofilms qui a généralement été appliquée aux interfaces solide-liquide a évolué pour inclure des interfaces air-liquide, ou pas d'interface définie comme dans le cas des agrégats de bactéries en suspension.

Il n'est cependant pas très clair si le type de surface sur laquelle se développe un biofilm joue un rôle au niveau de son métabolisme et sa physiologie. Par exemple; est ce qu'un biofilm de *Pseudomonas aeruginosa* attaché à une lame de verre a les mêmes propriétés qu'un biofilm attaché à des cellules épithéliales ou résidant dans un poumon d'un patient atteint d'une fibrose kystique?

La physiologie du biofilm est complexe, on peut le considérer comme un microcosme dans lequel les bactéries se multiplient et s'organisent en microcolonies (Figure 2).

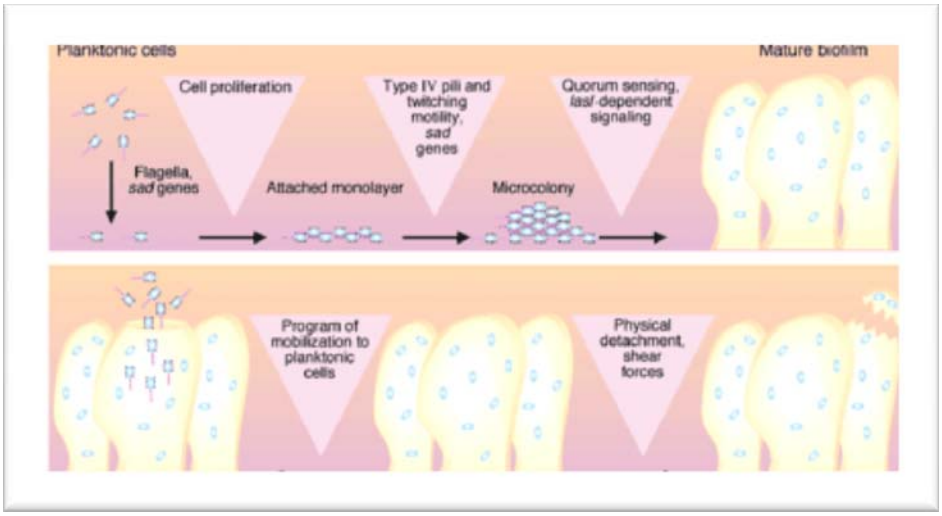


Figure 2. Représentation générale des étapes de formation de biofilm[10].

Le microenvironnement joue un rôle important dans le développement ainsi que la structure du biofilm. En effet, le groupe de Stoodley a pu démontrer que des changements hydrodynamiques ainsi que la composition du milieu de culture peuvent influencer la biomasse du biofilm ainsi que plusieurs processus impliqués dans le développement de ce dernier[9].

Un des défis concernant la recherche sur les biofilms consiste à comprendre pourquoi les bactéries capables de former un biofilm adhèrent spécifiquement à un certain type de surface, et comment ces bactéries résistent aux différents agents antimicrobiens.

À l'état naturel, les biofilms sont des communautés au sein desquelles on peut trouver plusieurs espèces bactériennes différentes qui sont capables d'échanger leur matériel génétique.

La communication entre les microorganismes dans un biofilm se fait grâce à la détection du quorum, appelée *quorum sensing* en anglais. C'est un mécanisme de régulation qui contrôle l'expression de certains gènes bactériens. Les bactéries qui utilisent la détection du quorum produisent des signaux moléculaires dits auto-inducteurs, qui contribuent a l'expression (ou la répression) de ces gènes au sein d'une population bactérienne en fonction de la densité de celle-ci.

Les différents mécanismes de résistance du biofilm

La forme planctonique des bactéries peut permettre leur dissémination dans le sang et les tissus. Ce phénomène est plutôt associé aux infections aiguës[1].

Cependant, sous forme de biofilm les bactéries agissent en communauté organisée, ce qui augmente leur résistance et parfois leur virulence [5].

Les biofilms sont très résistants aux défenses immunitaires, ils ne sont pas susceptibles aux macrophages et aux anticorps, et sont très résistants aux antibiotiques[11, 12]. Cette résistance est dûe à plusieurs facteurs.

La matrice d'exopolysaccharide

La matrice d'EPS représente une barrière physique et confère une résistance au biofilm. Le groupe de Stewart suggère qu'elle peut retarder la diffusion d'antibiotiques [13] et diminuer leur diffusion due au gradient osmotique. Elle empêche aussi les neutrophiles ainsi que les anticorps d'accéder aux bactéries [14] et les protège contre les radicaux libres comme les dérivés toxiques de l'oxygène [15].

Plusieurs souches de *Staphylococcus aureus* produisent le PIA (polysaccharide intercellular adhesin) [16]. Ce polysaccharide est constitué de longues chaînes de beta-1,6-glucosamines. Sa synthèse semble être sous le contrôle d'un locus nommé *ica* (intercellular adhesin). En plus de son rôle dans l'adhésion intercellulaire et l'augmentation de l'adhésion à des surfaces hydrophiles, le PIA est responsable de l'hémagglutination des globules rouges [17, 18].

De nombreuses souches de *Pseudomonas aeruginosa* produisent une substance muqueuse appelée alginate, c'est un exopolysaccharide produit par le gène *algc*. L'expression de ce dernier est quatre fois plus élevée en biofilms [19]. L'alginate a un rôle protecteur et confère une résistance très élevée au biofilm.

Les bactéries vivant dans un biofilm ont une résistance sensiblement différentes de celles des bactéries planctoniques de la même espèce (Figure 3).

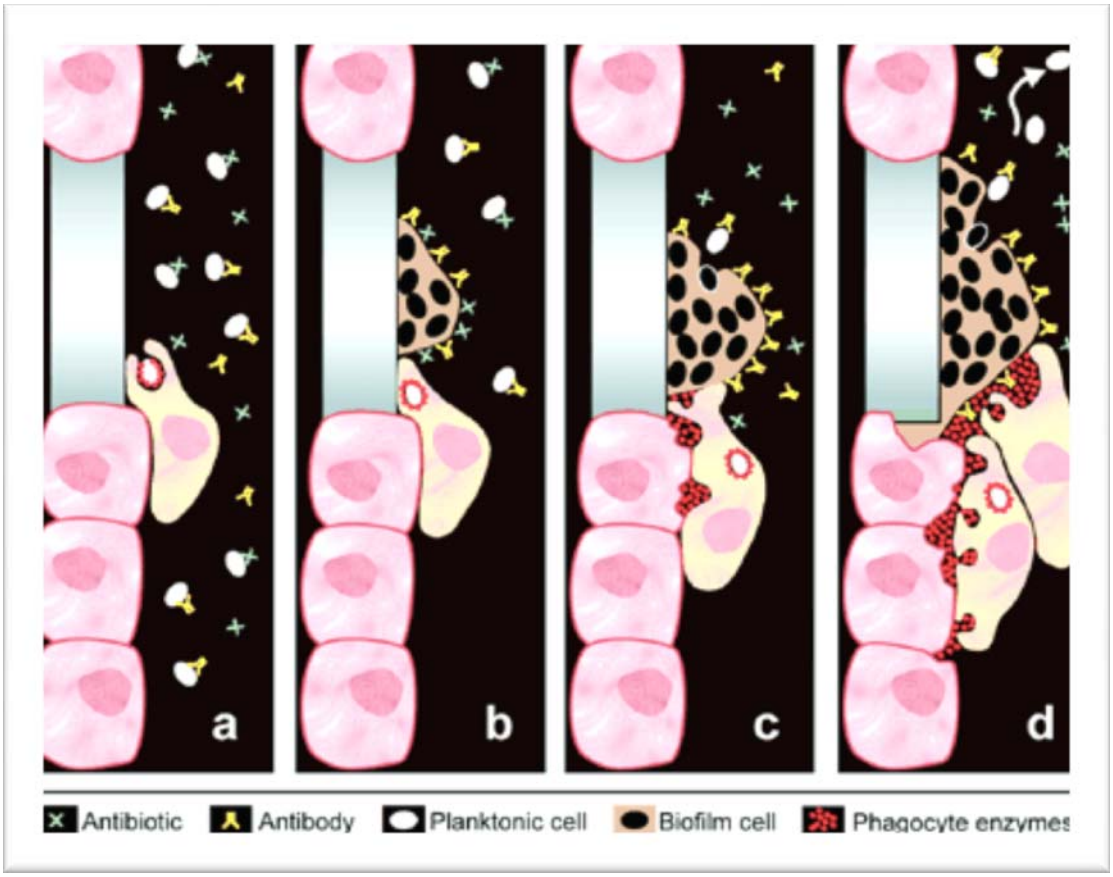


Figure 3. Diagramme d'un biofilm médical.

(a) Les bactéries planctoniques peuvent être éliminées par les anticorps, les phagocytes et sont sensibles aux antibiotiques. (b) Les bactéries adhérentes forment une communauté de biofilm résistante aux anticorps, phagocytes et antibiotiques. (c) Les phagocytes attaquent le biofilm, mais leurs tentatives échouent et les enzymes phagocytaires sont libérées. (d) Les enzymes phagocytaires endommagent les tissus entourant le biofilm et des bactéries peuvent être libérées du biofilm, menant potentiellement à une infection aiguë dans les tissus avoisinants [10].

Des études de phénotype ont démontré une différence de métabolisme entre les bactéries inclues dans le biofilm et les bactéries planctoniques. Les bactéries sous un phénotype de biofilm peuvent exprimer plusieurs gènes qui ne sont pas exprimés par les bactéries de forme planctonique[20].

À l'intérieur d'un biofilm toutes les bactéries n'ont pas la même activité métabolique.

Les bactéries situées dans les couches les plus profondes du biofilm ont une activité métabolique réduite; certaines bactéries peuvent désactiver leur métabolisme et adopter un état de survie. La division cellulaire est 5 à 15 fois plus lente à l'intérieur d'un biofilm que dans les conditions planctoniques [21].

L'accès limité à ces bactéries au sein du biofilm, ainsi que les modifications de leur activité métabolique, augmentent leur niveau de résistance aux attaques des défenses naturelles et aux antibiotiques.

Les persisteurs

Les antibiotiques qui en général sont efficaces contre les bactéries planctoniques montrent une efficacité réduite envers les infections chroniques[5].

L'expérience clinique a montré que lorsque les défenses de l'organisme, ainsi que les traitements antibiotiques, sont insuffisants pour éliminer un biofilm mature [11, 22], la chirurgie est parfois inévitable pour éradiquer l'infection.

Les bactéries d'un biofilm peuvent survivre à des concentrations très élevées d'agents antimicrobiens [53]. Ce phénomène est dû à la présence de persisteurs. Ces bactéries sont

génétiquement identiques à la population du biofilm, et sont capables de survivre même après une période prolongée de traitements avec des fortes concentrations d'antibiotiques. Les persisteurs représentent généralement 1% ou moins du total de la population bactérienne [49].

L'hypothèse des persisteurs est la plus récente explication de la résistance des biofilms aux agents antimicrobiens. Il est cependant très difficile de les étudier, à cause de leur nombre très petit d'une part, ainsi que le manque de compréhension au niveau de leur physiologie.

Le rôle des biofilms dans les maladies chroniques est maintenant très reconnu. De récentes publications estiment qu'au moins 65% des infections bactériennes humaines impliquent les biofilms [23].

Les biofilms s'avèrent responsable de plusieurs maladies chroniques respiratoire telles que : la fibrose kystique, l'otite séreuse, le cholestéatome et l'adénoïdite chronique [1, 24]. Les biofilms sont aussi impliqués dans les infections reliées aux implants médicaux [10].

La rhinosinusite chronique

La rhinosinusite chronique (RSC) est une maladie inflammatoire qui persiste plus de trois mois dans la région des muqueuses nasales et les cavités aérées de la face appelées sinus paranasaux [25]. Elle se caractérise aussi par une colonisation bactérienne et une infection.

On distingue la rhinosinusite chronique de la rhinosinusite aiguë par la durée des symptômes (Figure 4).

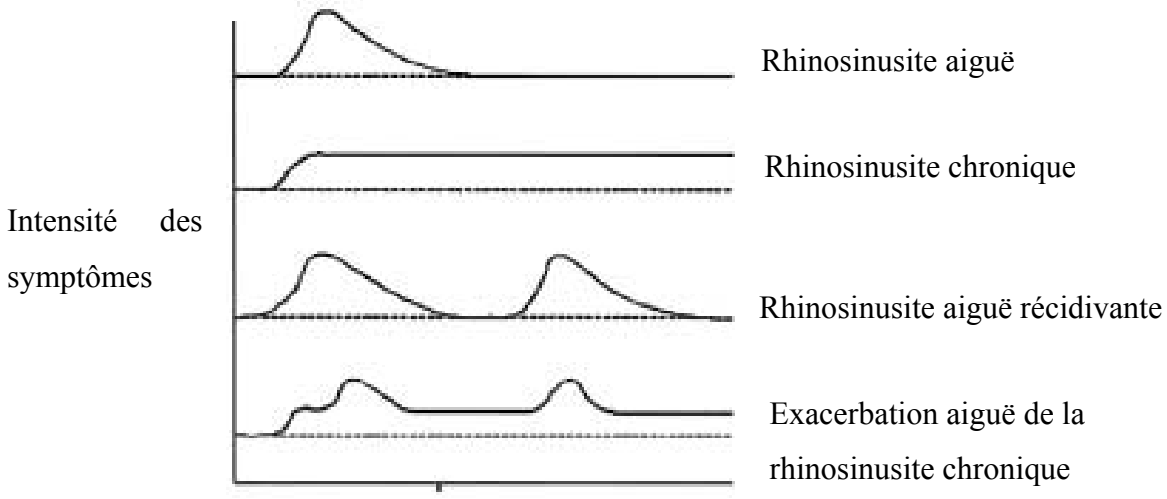


Figure 4. Classification de la rhinosinusite[26].

La RSC est parmi les maladies chroniques les plus fréquentes, causant un inconfort chez les patients ainsi que la morbidité [27].

Quatorze pour cent des américains disent souffrir de RSC. Il n'y a pas de statistique indiquant la prévalence de cette maladie au Canada.

Un élément clef dans le développement de la RSC est la dysperméabilité du complexe ostioméatal. C'est une région anatomique située sous le cornet moyen où sont localisés les ostia de drainage des sinus maxillaires, des cellules ethmoïdales antérieures et des sinus frontaux. L'obstruction du complexe ostioméatal entraîne une diminution de la ventilation et du drainage des sinus paranasaux avec comme conséquence une stase de mucus, un risque infectieux et une inflammation de toutes les cavités rhino-sinusiennes.

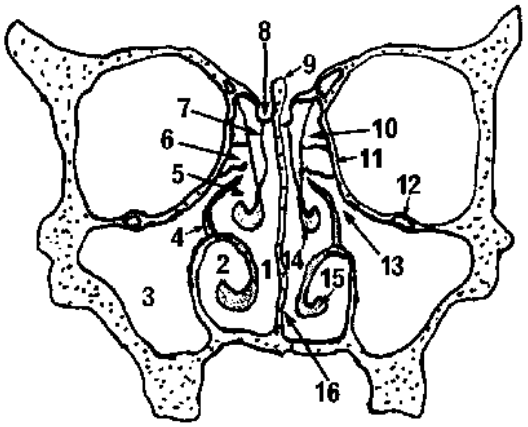


Figure 5. Schéma d'une coupe tomodensitométrique frontale du massif facial passant par les ostia des sinus maxillaires. 1. Cavité nasale, 2. Méat inférieur, 3. Sinus maxillaire, 4. Processus unciforme, 5. Région du méat moyen, 6. Cellule de la bulle, 7. Racine d'attache verticale du cornet moyen, 8. Lame criblée, 9. Processus crista galli, 10. Cellule supra-bullaire, 11. Os planum, 12. Canal sous-orbitaire, 13. Ostium du sinus maxillaire et gouttière uncibullaire, 14. Cornet moyen, 15. Cornet inférieur, 16. Septum nasal [28].

Les individus avec des sinusites réfractaires souffrent de symptômes persistants de rhinosinusite chronique même après une chirurgie endoscopique des sinus. La qualité de vie de ces patients est compromise, ils souffrent fréquemment de douleurs faciales et d'écoulement nasal avec des exacerbations qui nécessitent généralement un traitement antibiotique ou même une chirurgie. Le traitement chez ces patients est très difficile dû au manque de compréhension au niveau de la pathophysiologie entourant cette maladie [25].

L'étiologie de la RSC est très probablement multifactorielle. Elle inclut l'asthme, des dysfonctions ciliaires, des déficiences immunitaires, l'obstruction du complexe ostioméatal, des infections virales, bactériennes ou fongiques, la présence de superantigènes (exotoxines) et des facteurs environnementaux [26, 29].

La présence de biofilms dans les sinus pourrait expliquer l'échec des thérapies conventionnelles et de la chirurgie[25]. En effet, la présence du biofilm au niveau de la muqueuse sinusale chez les patients atteints de RSC fournit une présence continuelle des antigènes (surface des bactéries, éléments fongiques, exotoxines ect.), pouvant résulter en une inflammation chronique au niveau du sinus.

L'inflammation chronique de la muqueuse sinusale génère des dommages tissulaires qui pourraient favoriser la création de surfaces où le biofilm peut adhérer [10]. Ce profil inflammatoire de la RSC concorde avec celui d'une infection reliée à la présence de biofilms.

Traitements de la rhinosinusite chronique :

Le traitement de la RSC comprend l'utilisation d'antibiotiques, de décongestionnants, de mucolytiques et de corticostéroïdes topiques [30]. Lorsque la RSC ne répond pas aux traitements courants, on a recours à la chirurgie endoscopique des sinus pour enlever les tissus infectés et rétablir l'ouverture et le drainage au niveau des sinus (Figure 6).

Les bactéries sous forme planctonique montrent généralement une sensibilité aux antibiotiques *in vitro,* ce qui explique l'efficacité de l'antibiothérapie par voie orale dans le traitement de la rhinosinusite maxillaire bactérienne aigue. Cependant, ce même traitement

est particulièrement inefficace chez des patients atteints de rhinosinusite chronique qui ont subi une chirurgie endoscopique des sinus.

L'échec de l'antibiothérapie chez ces patients, l'inflammation chronique des muqueuses sinusales et le taux élevé des cultures stériles laissent croire que les biofilms sont impliqués dans la pathophysiologie de la RSC.

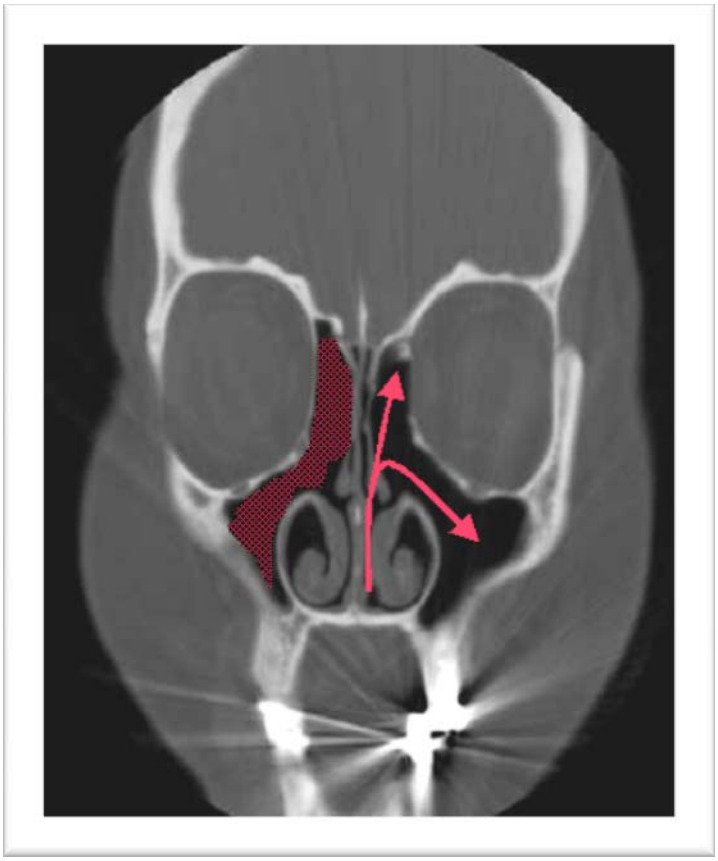


Figure 6. Les changements effectués après la chirurgie endoscopique des sinus facilitent la pénétration des solutions à l'intérieur des cavités sinusales.

Les bactéries impliquées dans la RSC

Pour mieux comprendre cette maladie il est crucial d'évaluer la flore bactérienne au niveau des sinus des patients atteints de rhinosinusite chronique.

Les cultures provenant de patients atteints de RSC et qui ne répondent pas à la chirurgie endoscopique des sinus ont démontré une incidence élevée de *Staphylococcus aureus*, *Pseudomonas aeruginosa,* staphylocoques à coagulase négative, *Streptococcus pneumoniae* et *Haemophilus influenzae* [31, 32].

Pseudomonas aeruginosa est un agent pathogène opportuniste, responsable d'infections nosocomiales et d'infections irréversibles et mortelles chez les malades souffrant de mucoviscidose. Les staphylocoques sont des coques à Gram positif, et ils sont responsables d'infections nosocomiales. L'espèce *Staphylococcus aureus* se distingue généralement des autres staphylocoques appelés staphylocoques à coagulase négative (SCN) par la présence d'une coagulase. Les SCN sont responsables de l'infection des implants médicaux.

Évidences de l'implication des biofilms dans la rhinosinusite chronique

La présence des biofilms au niveau de la muqueuse sinusale des patients atteints de RSC a été suggérée par plusieurs chercheurs qui ont utilisé différentes techniques.

Le groupe de Palmer a analysé des biopsies de muqueuses sinusales provenant de 16 patients atteints de RSC. Grâce au microscope électronique à balayage (MEB), ils ont remarqué la présence de biofilm de *P. aeruginosa* dans 4 des spécimens [33].

Dans une autre étude menée par le groupe de Ferguson, quatre biopsies de muqueuses ont été analysées par un MEB. Des évidences morphologiques de la présence de biofilms ont été remarquées dans 2 des spécimens analysées [34].

Ramadan et al [35] ont aussi démontré grâce à la MEB la présence de biofilms sur des biopsies de muqueuses sinusales chez 5 patients atteints de RSC.

Le groupe de Hunsaker a utilisé le microscope confocale à balayage laser (MCBL) et l'hybridation fluorescente in situ (FISH) [36]. L'avantage du FISH est qu'il permet l'identification des bactéries tout en gardant la structure du biofilm. Dans cette étude 18 spécimens ont été prélevés au moment de la chirurgie endoscopique des sinus de patients atteints de RSC. La présence de biofilms a été démontrée dans 14 des 18 spécimens. Les espèces prédominantes dans ces biofilms étaient : *Haemophilus influenzae, Streptococcus pneumoniae* et *Staphylococcus aureus*. Parmi les 5 spécimens contrôles utilisés, 2 ont démontré la présence d'un biofilm d' *Haemophilus influenzae.*

Dans un modèle animal, le groupe de Perloff a analysé 22 lapins avec une sinusite maxillaire, infectés par *Pseudomonas aeruginosa*. En utilisant le microscope électronique à balayage, des évidences morphologiques de présence de biofilms ont été remarquées chez tous les lapins. Aucun biofilm n'a été remarqué dans les 22 contrôles dans cette étude [37].

Objectifs

Notre projet d'étude sur l'implication des biofilms dans la RSC s'est étalé sur 3 étapes :

- Tout d'abord nous avons testé la capacité de *Pseudomonas aeruginosa*, des staphylocoques à coagulase négative et *Staphylococcus aureus,* des bactéries isolées de patients atteints de RSC, à former un biofilm *in vitro*.
- La prochaine étape était d'évaluer si la capacité de formation de biofilm par ces bactéries a un lien avec l'évolution clinique de la maladie.
- Finalement nous avons évalué *in vitro* l'effet de la moxifloxacine, un antibiotique utilisé dans le traitement de la RSC sur des biofilms matures de souches cliniques de *Staphylococcus aureus*.

Résultats déjà publiés

1. Bendouah Z, Barbeau J, Hamad W, Desrosiers M. Use of an *in vitro* assay for determination of biofilm-forming capacity of bacteria in chronic rhinosinusitis. Am J Rhinol 20(5):434-4388, Sep-Oct 2006.

Abstract

Introduction: Bacterial biofilms are increasingly implicated in the pathogenesis of chronic disease and have been demonstrated in several chronic ear, nose and throat (ENT) conditions, including chronic sinusitis. However, this relies upon specialised imaging methods not widely available. Objectives: We wished to assess the capacity of an easily performed, inexpensive *in vitro* test to assess biofilm production by bacteria recovered from individuals with chronic rhinosinusitis with or without nasal polyposis. Setting: Academic tertiary rhinology practice. Method: Biofilm formation was determined with an *in vitro* staining method using crystal violet. Ten isolates of *Pseudomonas aeruginosa*, *Staphylococcus aureus* and 11 of coagulase negative staphylococcus from patients with chronic rhinosinusitis having previously undergone endoscopic sinus surgery greater than one year previously were assessed. Samples were cultured 24 hours at 37ºC on 96 well plates in TSB 0.5% glucose medium. After staining with crystal violet, optical density at 570 nm was measured to quantify biofilm production. Biofilm-forming capacity was

compared with positive and negative controls for each species obtained from commercial sources.

Results: Positive controls all grew biofilms, with a tendency to lesser biofilm formation at higher dilutions. Twenty-two of 31 clinical samples produced a biofilm greater or equal to the positive control. Biofilm was recovered consistently for all 3 species studied. Conclusion: This *in vitro* assessment method is capable of detecting biofilm-forming capacity in bacteria isolated from individuals with chronic rhinosinusitis. This simple assay may be useful complement to existing techniques for clinical research.

Keywords: Chronic rhinosinusitis, bacterial biofilms, endoscopic sinus surgery

Funding: Internal funding. Conflict of interest statement: None

Introduction

Biofilms are defined as an organized community of bacteria collaborating between themselves, adherent to a surface and contained in an extracellular polymeric substances made of exopolysaccharides, nucleic acids and proteins [1].

This matrix represents 98% of the volume of the biofilm [2, 3]. Biofilms represent an ancient prokaryotic survival strategy, and constitute the most defensive life strategy that can be adopted by prokaryotic cells [4]. Biofilms are highly resistant to host defence mechanisms, both innate and specific immunity. Because of their exopolysaccharide matrice and reduced metabolic rate, they are less susceptible to phagocytic macrophages and are resistant to antibiotics that attack only dividing cells [5, 6, 7]. These local conditions encourage

persistence of bacteria for periods of months to years, with intermittent occurrence of acute exacerbations.

Bacterial biofilms are increasingly implicated in the pathogenesis of human chronic disease [8]. While initially understood to be responsible for dental plaque leading to the development of caries and later to the development of catheter-borne infections, their presence has been documented in both and their presence has been demonstrated in several ENT conditions including chronic otitis media, cholesteatomas and chronic adenoiditis [9].

Chronic rhinosinusitis, with its chronic indolent course, acute exacerbations and resistance to antibiotics, has an evolution that parallels that of other biofilm diseases and thus seems an ideal candidate for a biofilm disease. In support of this, the presence of biofilms on the mucosa of subjects with chronic sinusitis has been demonstrated by at least two previous authors. Using scanning electron microscopy, Cryer et al, showed presence of biofilm on the mucosa of a limited number of subjects with persistent chronic rhinosinusitis [10]. In a study of biopsies of the mucosa of the ethmoid sinus taken at the time of endoscopic sinus surgery (ESS), Sanclement demonstrated presence of biofilms in 24/30 affected individuals and 0/4 controls [11].

Current methods for biofilm detection include scanning electron microscopy (SEM), transmission electron microscopy (TEM), confocal laser microscopy and detection of

presence of genes identified with biofilm forming capacity [12]. However, biofilm detection by confocal or electron microscopy or genetic methods is expensive and not routinely available. Specialised skills are required for performance and interpretation. As well, SEM and TEM require tissue sample for use, obviating its utility in clinical practice.

As well, while these methods can help identify the presence of a biofilm, they are unable to identify the agent(s) involved. While this can be addressed by fluorescent *in situ* hybridisation probes identified via laser confocal microscopy, this expertise is currently unavailable outside a limited number of specialised centers.

Methods for assessment of biofilm-forming capacity of bacteria by means of an *in vitro* analysis exist [13, 14]. While they may have a higher false-negative rate that genetic tests [15], these techniques may nevertheless offer simple and inexpensive means for assessment of biofilm forming capacity of different species of bacteria, allowing *in vitro* study and facilitating research.

Objective

We wished to determine the capacity of an easily performed, low cost, *in vitro* detection method of biofilm-forming capacity in bacteria commonly identified in chronic rhinosinusitis.

Materials

Bacterial isolates were recovered from patients consulting in a tertiary academic-based rhinology practice for routine follow up care. All had previously undergone endoscopic sinus surgery for a diagnosis of chronic sinusitis and/or nasal polyposis according to 2003 AAO-HNS guidelines [16], and are refractory to maximal medical therapy over 12 months previously. Patients were not required to be symptomatic at the time of study.

Patients with cystic fibrosis or underlying immunosuppressive disorders were not included. Treatment with topical intranasal corticosteroids with or without nasal irrigations was allowed, however patients having taken antibiotics or oral prednisone within a 1-month period previous were not allowed.

All cultures were performed by the senior author (M.D.) under endoscopic guidance as described by Nadel et al [17]. Briefly, after topical anaesthesia, the nasal ala was retracted and the endoscope was used to visualise the middle meatus and sinus cavities. A thin, flexible calcium alginate swab (Starswab Microorganism Collection and Transport system (Starplex Scientific, Etobicoke, Ontario). was then inserted under direct endoscopic control, and directed to the site of maximal purulence. Where no purulence was seen, the surface of the maxillary sinus was swabbed for a fifteen second period. Care was taken at all times to avoid contact with the lateral nasal wall or the nasal vestibule. These were rapidly transported to the hospital laboratory for culture and identification of the pathogens according to standard hospital protocol, and then frozen until the time of study.

Bacterial species selected for study were pathogens previously identified as those most frequently recovered from individuals with chronic sinusitis refractory to medical and surgical therapy: *Staphylococcus aureus*, coagulase-negative staphylococci and *Pseudomonas aeruginosa*.

This study was approved by the Ethical review board for Human Subejcts of the Centre Hospitalier de l'Université de Montréal.

Methods

Crystal violet staining was adapted from the method previously described by Stepanovic et al [14].

Growth

Previously frozen strains were initially inoculated on blood Agar (TSA 0.5% of sheep blood). After culture for 24 hours, one to four colonies per strain were recultured on TSA (Typtic Soy Agar). These were incubated at 37ºC for 24 h in order to condition them to the TSB/TSA medium and to ensure non-contamination. The colonies grown on TSA solid medium were amplified in 5 ml of TSB medium (Typtic soy broth) with 0.5% glucose [18] and incubated at 37ºC for 24 h.

After the incubation, the optical density of the cultures was standardized in the following manner:

The amplified culture was centrifuged at 3000 RPM during 10 minutes, the supernatant removed, and the aliquot washed with 10 ml of TSB. The centrifugation was repeated and the aliquot again suspended in 5 ml of TSB with 0.5 % glucose.

Serial dilutions of the bacterial suspension were distributed in 96 well plates. Optical density (O.D) at 630 nm was measured using a spectrophotometer (Dynatech MR5000). The dilution corresponding to the D.O of 0.1 at 0.15 was determined and served as a starting point for the dilutions.

Using the bacterial suspension producing an O.D of 0.1 to 0.15, the suspensions were then distributed in the microplaques. Serial dilutions with TSB 0.5 % glucose were performed in order to obtain concentrations varying from 10^0 to 10^{-4} (1:1 to 1: 10 000). 4 replicates were used for each sample. The plates were incubated at 37ºC without agitation for 24 h. O.D at 630 nm was measured at times 0 and 24 hours in order to assess growth by evaluation of the cellular density. The supernatant was then withdrawn and the plate rinsed under running tap water. After drying, staining for adherent biofilm was performed using crystal violet.

Staining for biofilm

Crystal violet (Fisher Scientific Co) was applied during 10 minutes. The dye fixes itself to the attached biofilm giving it a characteristic purple colour. Following the 10 min of staining, the plate was again rinsed with running water and left to dry. After drying, semi-quantitative assessment of biofilm formation was obtained by extracting the crystal violet

with 100 µl per well of the following bleaching solution: 200 ml methanol, 50 ml glacial acetic acid, 250 ml H_2O. This dissolved the bound crystal violet and produced a violet coloured solution in each well. The intensity of coloration was determined by measuring the absorbance at 570nm. The average value of O.D. was determined by calculating the average O.D for the four replicates.

Controls:

Commercially obtained slime producing and non-slime producing strains for each of the tested species are used as reference strains to confirm biofilm production (Table I). For *Staphylococcus aureus*, no slime – negative control could be obtained.

Determination of biofilm-production was established in reference to positive controls for each of the species. An example of biofilm formation across the serial dilutions in the 96 well plate is shown in figure I.

Results

Thirty-one strains isolates were recovered from 19 patients: 10 *Staphylococcus aureus* (Sa), 10 *Pseudomonas aeruginosa* (Pa) and 11 coagulase-negative staphylococci (CNS). Patient population was 9 women and 10 men, with an average age of 52 years (range 29 to 68 years).

Biofilm formation was recorded for positive controls and several of the isolates from each of the three species studied. Results for each of the separate organisms studied are presented in graphical form in Figures II, III, and IV. While growth was usually maximal for the undiluted culture, for several of the *S. aureus* species it was maximal at the 10^{-1} dilution. Thus, for comparison purposes of biofilm-forming capacity with control, the O.D. at the 10^{-1} dilution was used.

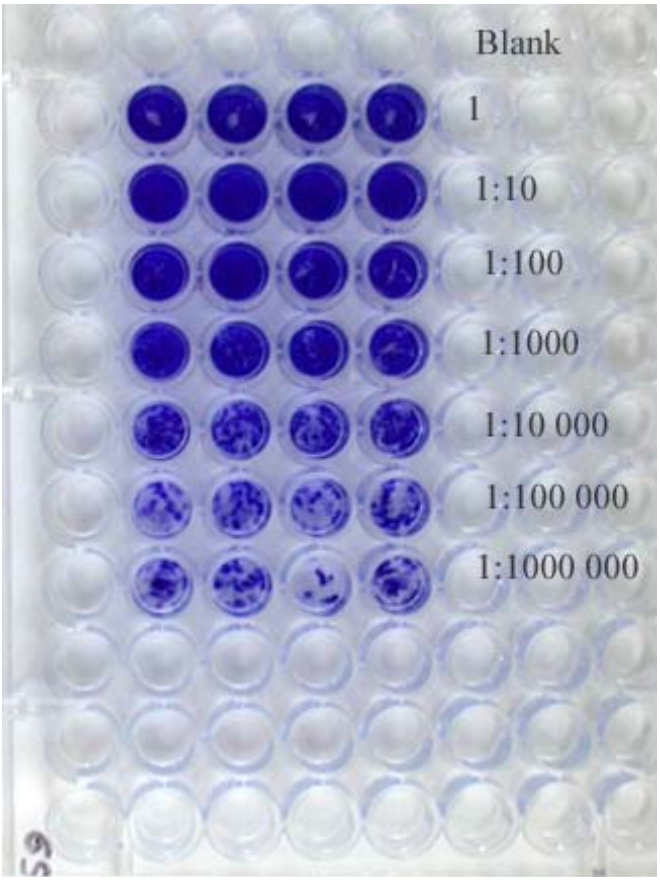
Biofilm-forming capacity was detected in 22 of the 31 isolates studied. For *P. aeruginosa*, biofilm formation was noted in 6/10, with some samples having quite marked biofilm production. For *S. aureus*, 8/10 samples produced a biofilm definitely higher than positive controls. For coagulase negative staphylococci 6/11 samples produced a biofilm greater than positive control. Of note is that the negative control for biofilm formation for CNS produced a biofilm with our method. This may however be due to the methodology used in that study (production of exopolysaccharides on Congo red agar), which differs from ours[19].

It can be seen that staining intensity varies between species and according to dilution. For intensity, *Staphylococcus sp*. stains more intensely, despite the strong amount of slime production by the *Pseudomonas sp.* This is related to the site of slime production in the 96 well plate. For the *Staphylococcus sp*, slime production occurs over the entire surface of the floor of the well, however, for *P. aeruginosa*, slime tends to form mainly at the air-liquid interface on the side of the well, limiting the amount of biofilm available for binding the crystal violet after washing of the plate.

For all three species studied growth is maximal at the 10^{-1} dilution, it persists over a number of subsequent dilutions. For comparison purposes of biofilm-forming capacity between different isolates, the O.D at this single dilution could also be used.

Legends for Illustrations

Figure I. Example of biofilm formation the 96 well plate. A biofilm producing strain of *Staphylococcus aureus* is shown. Note that intensity of staining is actually maximal in the 10^{-1} dilution near the top.



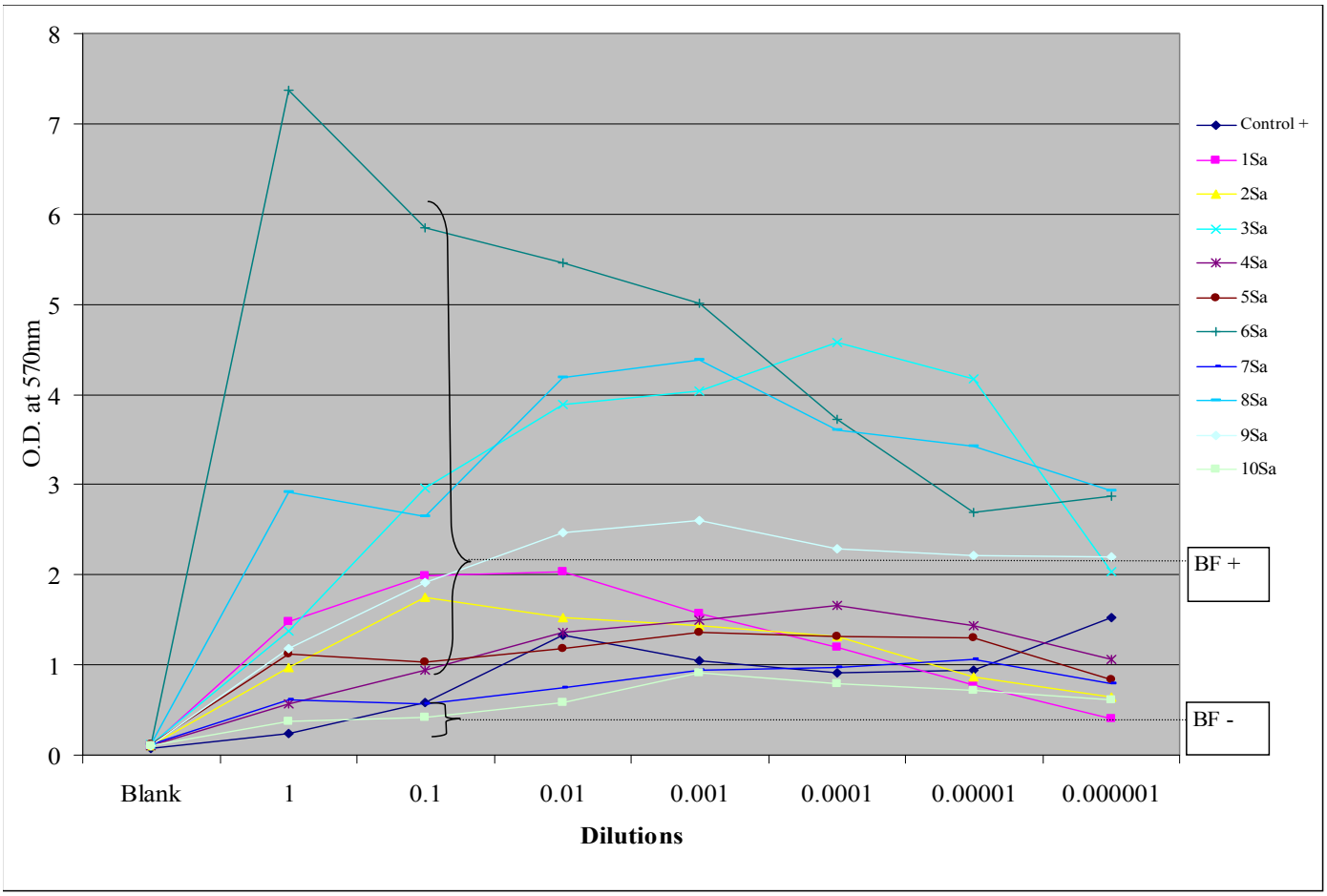


Figure II. Biofilm-forming capacity for *Staphylococcus aureus*. The average O.D. is plotted for the different serial dilutions.

BF+: Biofilm production.

BF-: No biofilm production.

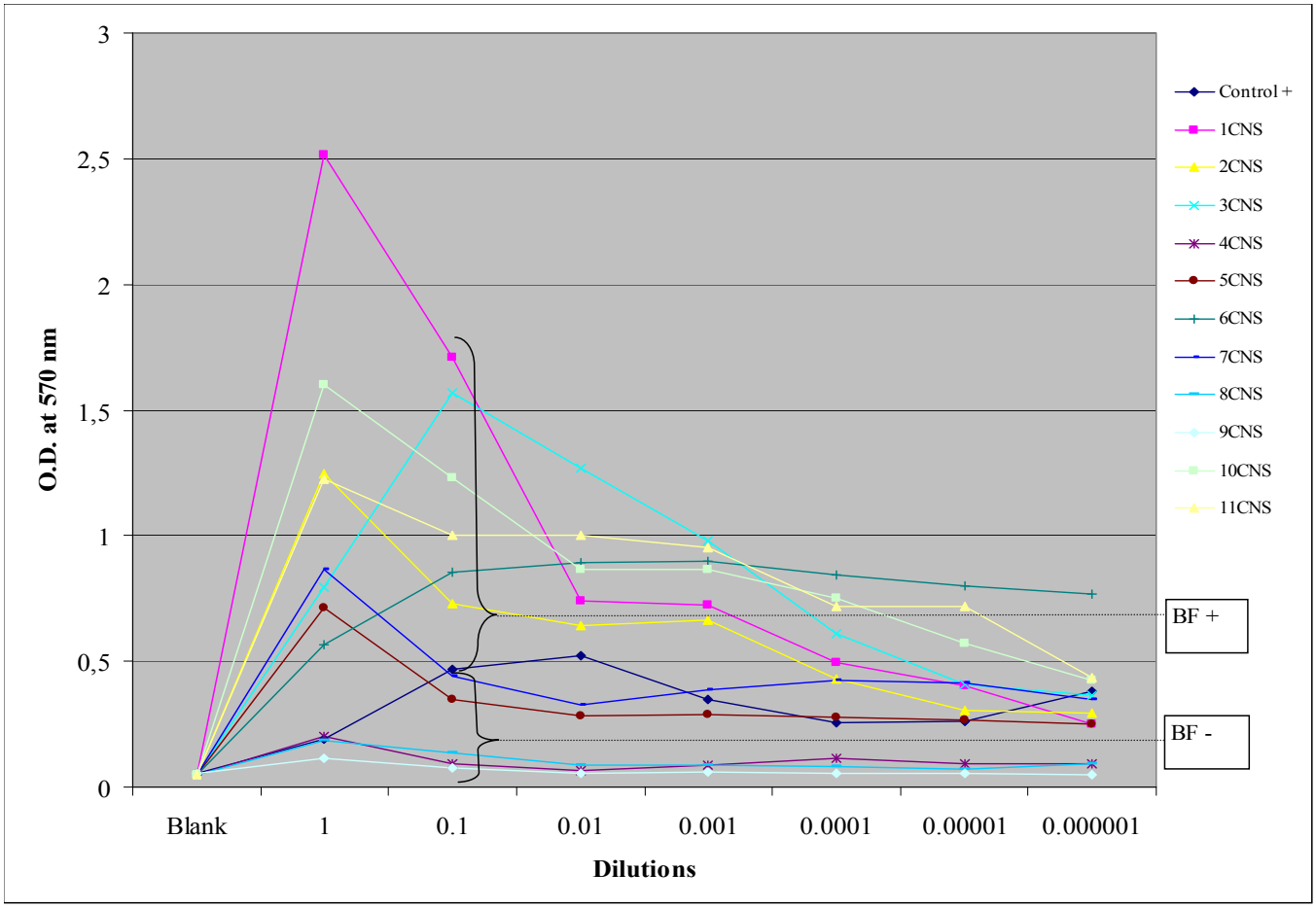


Figure III. Biofilm-forming capacity for Coagulase negative staphylococci. The average O.D. is plotted for the different serial dilutions.

BF+: Biofilm production.

BF-: No biofilm production.

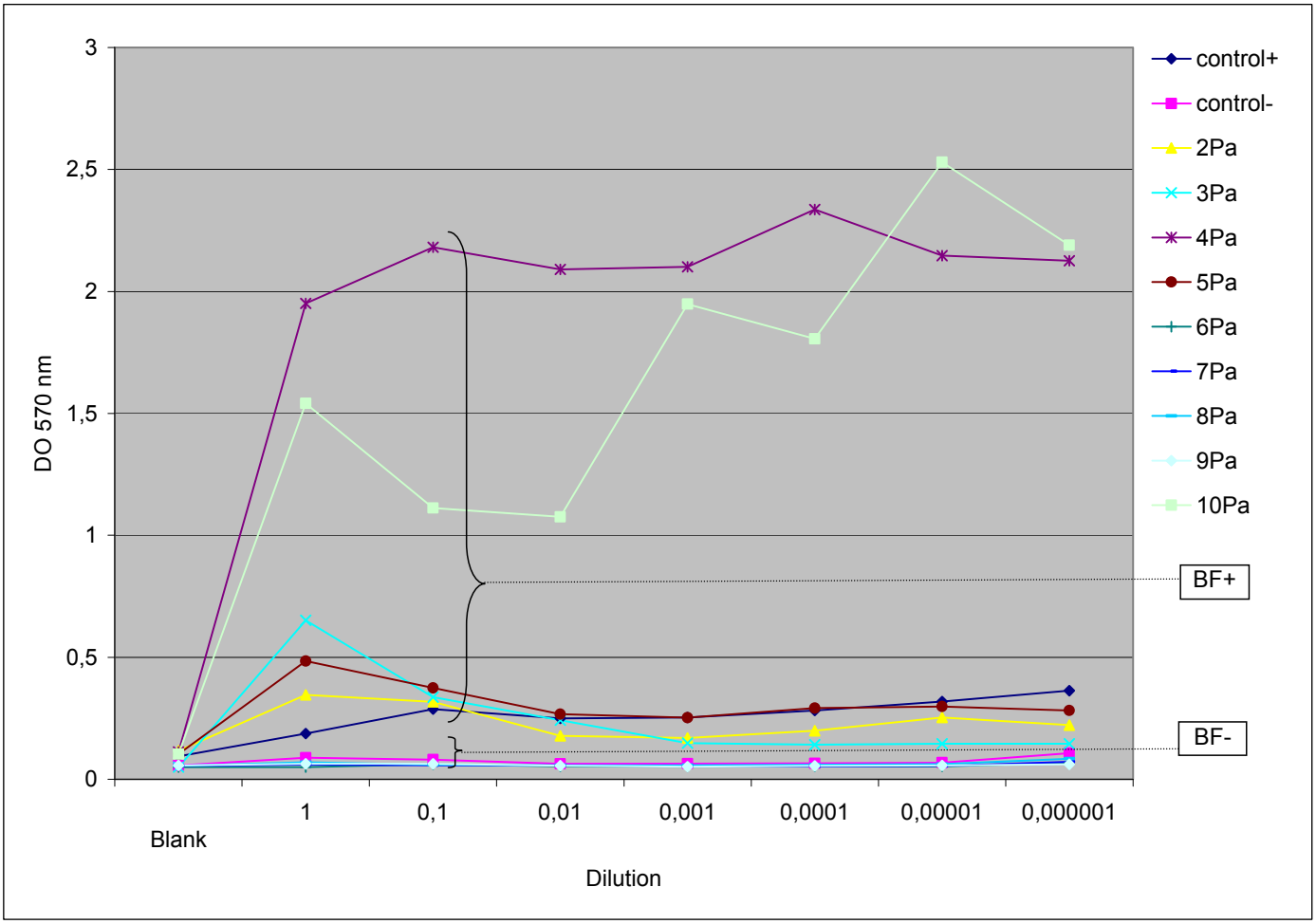


Figure IV. Biofilm-forming capacity for *Pseudomonas aeruginosa*. The average O.D is plotted for the different serial dilutions. Sample 1, a strong biofilm producer, has been removed to improve clarity. BF+: BF+: Biofilm production.

BF-: No biofilm production.

Table I. Control strains for biofilm formation (ATCC: American Type Culture Collection, LSPQ: Laboratoire de Santé Publique du Québec)

Strain	Biofilm formation
Staphylococcus aureus LSPQ 2520	Positive
Staphylococcus epidermidis ATCC 12228	Negative
Pseudomonas aeruginosa LSPQ 3332	Positive
Pseudomonas aeruginosa ATCC 10145	Negative
Staphylococcus epidermidis LSPQ 3027	Positive

Discussion

We evaluated the capacity of a simple, *in vitro* method to detect biofilm-forming capacity in commonly identified species of bacteria (*S. aureus*, CNS, and *P. aeruginosa*) recovered from individuals with chronic rhinosinusitis with or without nasal polyposis (CRS +/- NP) having previously undergone endoscopic sinus surgery for disease unresponsive to maximal medical management.

Using this method, biofilm-forming capacity could be established in 22 of 31 of the specimens, a rate comparable with the results reported by Ramadan *et al* using scanning electron microscopy [22]. This high recovery rate in our study suggests that this technique is capable of detecting biofilm-forming capacity in chronic rhinosinusitis, and that it may offer a complement to existing methods. It is obvious that biofilm-forming capacity *in vitro* does not necessarily correlate to *in vivo* biofilm production. Certainly favourable local conditions are required to favor the expression of the biofilm forming phenotype. Establishment of the biofilm with subsequent chronic bacterial infection may help maintain the chronic course experienced by these individuals[20,21].

It is interesting to note that biofilm-forming capacity is noted for all three bacterial species assessed, even CNS. What contribution this makes to disease in our patient population remains to be determined. The questions of whether all bacterial species have the same pathogenic potential remain unanswered.

Conclusion

This *in vitro* assessment method demonstrates biofilm-forming capacity in a majority of bacterial samples recovered from individuals with chronic rhinosinusitis with or without nasal polyposis. This suggests that this easily performed; inexpensive technique may offer a complement to other modalities currently used to study biofilms in chronic rhinosinusitis. Further experience with its use in relation to impact of biofilm-forming capacity on clinical disease and *in vivo* studies of biofilm modulation will offer additional information on its potential as a clinical and research tool.

Acknowledgements

The support of the Fondation Antoine Turmel is greatly appreciated for its support.

References

1. Costerton JW, Stewart PS, and Greenberg EP. Bacterial biofilms: A common cause of persistent infections. Science 284: 1318–1322, 1999.
2. Donlan RM. Biofilms: Microbial life on surfaces. Emerg Infect Dis 8:881–890, 2002.
3. Fleming H-C, Wingender J, Griebe T, and Mayer C. Physicochemical properties of biofilms. In Biofilms: Recent Advances in Their Study and Control. Evans LV (Ed.). Amsterdam: Harwood Academic Publishers, 19–34, 2000.
4. Stoodley P, Sauer K, Davies DG, and Costerton JW. Biofilms as complex ifferentiated communities. Annu Rev Microbiol 56:187–209, 2002.
5. Potera C. Forging a link between biofilms and disease. Science 283:1837–1839, 1999.
6. Amorena B, Gracia E, Monzon M, et al. Antibiotic susceptibility assay for *Staphylococcus aureus* in biofilms developed *in vitro*. J Antimicrob Chemother 44:43–55, 1999.
7. Monzon M, Oteiza C, Leiva J, et al. Biofilm testing of *Staphylococcus pidermidis* clinical isolates: Low performance of vancomycin in relation to other antibiotics. Diag Microbiol Infect Dis 44(4):319–324, 2002.
8. Costerton W, Veeh R, Shirliff M, et al. The application of biofilm sciences to the study and control of chronic bacterial infections. J Clin Invest 112:1466–1477, 2003.
9. Post JC, Stoodley P, Hall-Stoodley L, and Ehrlich GD. The role of biofilms in otolaryngologic infections. Curr Opin Otolaryngol Head Neck Surg 12:185–190, 2004.
10. Cryer J, Schipor I, Perloff J, and Palmer JN. Evidence of bacterial biofilms in human chronic sinusitis. ORL 66:155–158, 2004.

11. Sanclement J, Webster P, Thomas J, and Ramadan H. Bacterial biofilms in surgical patients with chronic rhinosinusitis. Laryngoscope 115: 578–582, 2005.

12. Cramton SE, Gerke C, Schnell NF, et al. The intercellular adhesion (ica) locus is present in *Staphylococcus aureus* and is required for biofilm formation. Infect Immun 67:5427–5433, 1999.

13. Arciola CR, Campoccia D, Gamberini S, et al. Search for the insertion element IS256 within the *ica* locus of *Staphylococcus epidermidis* clinical isolates collected from biomaterial-associated infections. Biomaterials 25:4117–4125, 2004.

14. Stepanovic S, Vukovic D, Dakic I, et al. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J Microbiol Methods 40:175–179, 2000.

15. Cramton SE, Gerke C, and Gotz F. *In vitro* methods to study staphylococcal biofilm formation. Methods Enzymol 336:239–255, 2001.

16. Benninger MS, Ferguson BJ, Hadley JA, et al. Adult chronic rhinosinusitis: Definitions, diagnosis, epidemiology, and pathophysiology. Otolaryngol Head Neck Surg 129(suppl): S1–S32, 2003.

17. Nadel DM, Lanza DC, and Kennedy DW. Endoscopically guided cultures in chronic sinusitis. Am J Rhinol 12:233–241, 1998.

18. Gotz F. *Staphylococcus* and biofilms. Mol Microbiol 43:1367–1378, 2002.

19. Arciola CR, Baldassarri L, and Montanaro L. Presence of *icaA* and *icaD* genes and slime production in a collection of staphylococcal strains from catheter-associated

infections. J Clin Microbiol 39:2151–2156, 2001. 20. Bachert C, Gevaert P, and van Cauwenberge P. *Staphylococcus aureus* superantigens and airway disease. Curr Allergy

20. Bachert C, Gevaert P, and van Cauwenberge P. *Staphylococcus aureus* superantigens and airway disease. Curr Allergy Asthma Rep 2:252–258, 2002.

21. Tripathi A, Conley DB, Grammer LC, et al. Immunoglobulin E to staphylococcal and streptococcal toxins in patients with chronic sinusitis/nasal polyposis. Laryngoscope 114:1822–1826, 2004.

2. Bendouah Z, Barbeau J, Hamad WA, Desrosiers M. Biofilm formation by *Staphylococcus aureus* and *Pseudomonas aeruginosa* is associated with an unfavorable evolution after surgery for chronic sinusitis and nasal polyposis. Otolaryngol Head Neck Surg 2006 June ;134(6):991-6.

Abstract

Objectives: To determine whether biofilm-forming capacity of bacteria demonstrated in chronic rhinosinusitis (CRS) has an impact on persistence of the disease following endoscopic sinus surgery (ESS).

Method: Thirty-one bacterial strains recovered from 19 patients with CRS at least one year post-ESS. Evolution of disease was assessed by questionnaire and endoscopy as favorable of unfavorable. The bacteria were cultured on a 96-well culture plaque and a semi-quantitative method using crystal violet to quantify biofilm production was used.

Results: Twenty-two of 31 samples produced a biofilm thicker or equal to the positive control. Biofilm production was noted in 6/10 *Pseudomonas aeruginosa* isolates, 8/10 *Staphylococcus aureus* and 8/11 coagulase-negative staphylococci. Biofilm formation was associated with a poor evolution for *Pseudomonas aeruginosa* and *Staphylococcus aureus*, but not coagulase-negative staphylococcus.

Conclusion: There is a correlation between in vitro biofilm-producing capacity by *Pseudomonas aeruginosa* and *Staphylococcus aureus* and unfavorable evolution after ESS, suggesting a role for biofilm production in chronic sinusitis.

Keywords: Chronic rhinosinusitis, bacterial biofilms, endoscopic sinus surgery, *Staphylococcus aureus*, *Pseudomonas aeruginosa.*

Running head: Biofilm formation is associated with a poor evolution following ESS

Funding: Internal funding. Conflict of interest statement: None.

Presented at the 2005 AAO-HNS annual meeting, Los Angeles, CA

Introduction

Biofilms are defined as an organized community of bacteria, adherent to a surface and contained in an extracellular polymeric substances made of exopolysaccharides, nucleic acids and proteins [1]. Biofilms are highly resistant to both innate and specific host defense mechanisms. In part because of their exopolysaccharide matrix and reduced metabolic rate, they are less susceptible to phagocytic macrophages and are resistant to antibiotics that attack only dividing cells[2, 3]. These local conditions encourage persistence of bacteria for periods of months to years, with intermittent occurrence of acute exacerbations.

The role of the biofilms in the chronic diseases is increasingly recognized [4]. The frequency is such that infectious disease experts at the Centers for Disease Control and Prevention (CDC) estimate that 65% of human bacterial infections involve biofilms[5]. In the upper respiratory tract, the presence of biofilms has been demonstrated in several chronic diseases including chronic otitis media, cholesteatoma and chronic adenoiditis [5].

Chronic rhinosinusitis (CRS), with its chronic indolent course, resistance to antibiotics and acute exacerbations has an evolution that parallels that of other biofilm-related diseases. In support of this, the presence of biofilms on the mucosa of subjects with chronic sinusitis

has been demonstrated by at least two previous authors. Using scanning electron microscopy (SEM), Cryer et al, showed presence of biofilm on the mucosa of a limited number of subjects with persistent chronic rhinosinusitis [6]. Using SEM to study biopsies of the mucosa of the ethmoid sinus taken at the time of ESS, Sanclement et al demonstrated the presence of biofilms in 24/30 affected individuals and 0/4 controls [7]. However, the authors did not identify the organisms present within these biofilms nor did they establish whether the presence of biofilms plays a role in the pathophysiology of CRS.

Objectives

We wished to determine whether the biofilm-forming capacity in bacteria commonly recovered from individuals with chronic sinusitis/sino-nasal polyposis has an impact on persistence of the disease following endoscopic sinus surgery.

Materials and methods

This study was approved by the Ethical Review Board for Human Subjects of the Centre Hospitalier de l'Université de Montréal.

Bacterial species identified for study were pathogens previously identified as those most frequently recovered from individuals with chronic sinusitis refractory to medical and surgical therapy: *Staphylococcus aureus*, coagulase-negative staphylococci and *Pseudomonas aeruginosa* [8].

Bacterial isolates were recovered from a consecutive series of patients seen for sinus disorders in the tertiary academic-based rhinology practice of the senior author (M.D.). Consecutive patients were sampled until a representative number of organisms for each species were obtained, for a total of ten each samples of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and coagulase-negative staphylococci. Both good and poor outcome patients were included.

All patients had previously undergone technically successful endoscopic sinus surgery (ESS) (absence of synechiae, frontal sinus obstruction or technical problems such as retained uncinate process) for a diagnosis of chronic sinusitis and/or nasal polyposis according to 2003 AAO-HNS guidelines [9], and refractory to maximal medical therapy over 12 months previously. Evolution of disease was assessed by evaluation of symptoms of CRS and rigid sinonasal endoscopy.

A favorable evolution was defined as absence of symptoms or no more than one mild symptom (defined as a score of no more than 1 on a scale 0-3) of symptoms of facial pain/ pressure, nasal discharge or nasal obstruction on an ongoing basis. This had to be accompanied by an adequate technical result documented by endoscopy defined as presence of patent cavities and absence of purulent secretions. All others were deemed to have an unfavorable evolution.

Patients with cystic fibrosis or underlying immunosuppressive disorders (HIV, insulin-dependent diabetes mellitus, and renal disease) were not included. Treatment with topical intranasal corticosteroids with or without nasal irrigations was allowed, however patients having taken antibiotics or oral prednisone within a 1-month period previous were excluded.

All cultures were performed by the senior author (M.D.) under endoscopic guidance as described by Nadel et al [10]. Briefly, after topical anesthesia, the nasal ala was retracted and the endoscope used to visualize the middle meatus and sinus cavities. A thin, flexible calcium alginate swab (Starswab Microorganism Collection and Transport system (Starplex Scientific, Etobicoke, Ontario). was then inserted under direct endoscopic control, and directed to the site of maximal purulence. Where no purulence was seen, the surface of the maxillary sinus was swabbed for a fifteen second period. Care was taken at all times to avoid contact with the lateral nasal wall or the nasal vestibule.

Samples were rapidly transported to the hospital microbiology laboratory for Gram staining, culture, and identification. Clinical specimens were identified after plating and incubation according to standard procedures. Correct speciation of microorganisms was done using the Vitek2 system (Biomerieux, Marcy l'Étoile, France) for gram-positive and gram-negative strains.

Antibiotic susceptibilities were tested according to standard procedures using an automated microbroth dilution system (Vitek 2, Biomerieux, France). All procedures were conformed to NCCLS standards.

Staining for biofilm:

Crystal violet staining was adapted from the method previously described by Stepanovic et al [11].

The person performing the assay of biofilm forming capacity (ZB) was blinded to the patient outcome.

Growth

Previously frozen strains were initially inoculated on blood Agar (TSA 0.5% of sheep blood). After culture for 24 hours, one to four colonies per strain were cultured on TSA (Typtic Soy Agar). These were incubated at 37ºC for 24 h in order to condition them to the TSB/TSA medium and to ensure non-contamination. The colonies grown on TSA solid

medium were amplified in 5 ml of TSB medium (Typtic soy broth) with 0.5% glucose [12] and incubated at 37ºC for 24 h.

After the incubation, the optical density of the cultures was standardized in the following manner:

The amplified culture was centrifuged at 3000 RPM during 10 minutes, the supernatant removed, and the aliquot washed with 10 ml of TSB. The centrifugation was repeated and the aliquot again suspended in 5 ml of TSB with 0.5 % glucose.

Serial dilutions of the bacterial suspension were distributed in 96 well plates, Optical density (O.D) at 630 nm was measured using a spectrophotometer (Dynatech MR5000). The dilution corresponding to the D.O of 0.1 to 0.15 was determined and served as a starting point for the dilutions.

Using the bacterial suspension producing an O.D of 0.1 to 0.15, the suspensions were then distributed in the microplaques. Serial dilutions with TSB 0.5 % glucose were performed in order to obtain concentrations varying from 10^0 to 10^{-4} (1:1 to 1: 10 000). Four replicates were used for each sample. The plates were incubated at 37ºC without agitation for 24 h. O.D. at 630 nm was measured at times 0 and 24 hours in order to assess growth by evaluation of the cellular density. The supernatant was then withdrawn and the plate rinsed under running tap water. After drying, staining for adherent biofilm was performed using crystal violet.

Staining for biofilm

Crystal violet (Fisher Scientific Co) was applied during 10 minutes. The dye fixes itself to the attached biofilm giving it a characteristic purple color. Following the 10 min of

staining, the plate was again rinsed with running water and left to dry. After drying, semi-quantitative assessment of biofilm formation was obtained by extracting the crystal violet with 100 µl per well of the following bleaching solution: 200 ml methanol, 50 ml glacial acetic acid, 250 ml H_2O. This dissolved the bound crystal violet and produced a violet colored solution in each well. The intensity of coloration was determined by measuring the absorbance at 570nm. The average value of O.D was determined by calculating the average O.D. for the four replicates.

Net O.D was calculated by subtracting the O.D of the blank (no specimen) from the one under study. For comparison purposes between isolates, the net O.D. at the 10^{-1} dilution were used as it best represented maximal biofilm formation for all species studied (Data not shown). Determination of biofilm-production was established in reference to positive controls for each of the species.

Controls:

Control biofilm-producing commercially obtained slime-producing and non-slime producing strains for each of the tested species were obtained from the American Type culture collection (ATCC) and the Laboratoire de Santé Publique du Québec (LSPQ) used as reference strains to confirm biofilm production.

As no well-categorized, standardized biofilm-producing agents are available (catalog listings of organisms available from central supply sources use the term ‘slime-producing’ rather than ‘biofilm-producing’ in their description), slime-production was used as a surrogate for biofilm-producing capacity when noted.

Slime producing organisms used were: LSPQ 3332 (*Pseudomonas aeruginosa*), LSPQ 3027 (*Staphylococcus epidermidis*) and LSPQ 2520 (*Staphylococcus aureus*). Slime negative organisms used were: ATCC 10145 (*Pseudomonas aeruginosa*) and ATCC 12228 (*Staphylococcus epidermidis*). For *Staphylococcus aureus*, no commercial source for slime negative control could be obtained.

Results

Patient population was 9 women and 10 men, with an average age of 52 years (range 29 to 68 years). Five were deemed to have a favorable evolution while 14 to have an unfavorable evolution. Thirty-one isolates were recovered from 19 patients: 10 *Staphylococcus aureus* (Sa), 10 *Pseudomonas aeruginosa* (Pa) and 11 coagulase-negative staphylococci (CNS).

Biofilm-forming capacity greater than control was detected in 16/19 patients or in 22 of the 31 isolates (Table I). There were marked differences between the three species studied. For *P. aeruginosa*, biofilm formation was noted in 6/10, with some samples having quite marked biofilm production. For *S. aureus*, 8/10 samples produced a biofilm definitely higher than positive controls. For coagulase-negative staphylococci 6/11 samples produced a biofilm greater than positive control.

The quantitative results of biofilm formation by *Pseudomonas aeruginosa* seem lower than those produced by *Staphylococcus aureus* or coagulase-negative staphylococci. This is

explained by the fact that *Pseudomonas aeruginosa* produces a biofilm limited to the air-liquid interface, thus after washing, only the biofilm formed around the wall remains available for staining. For staphylococci, biofilm production is distributed evenly over the well surface.

Of note is that the negative control for biofilm formation for CNS produced a biofilm with our method. This may however be due to the methodology used in that study (production of exopolysaccharides on Congo red agar), which differs from ours [13].

Isolates from each species were grouped according to clinical evolution. A poor evolution was associated with biofilm formation in-vitro for both *S. aureus* and *P. aeruginosa*, but not for CNS (Figures III, IV, and V).

Legends for Illustrations:

Figure I. Examples of biofilms in the 96 well plate for *Staphylococcus aureus* and coagulase-negative staphylococci after crystal violet staining. Light, medium and heavy growth are illustrated. .

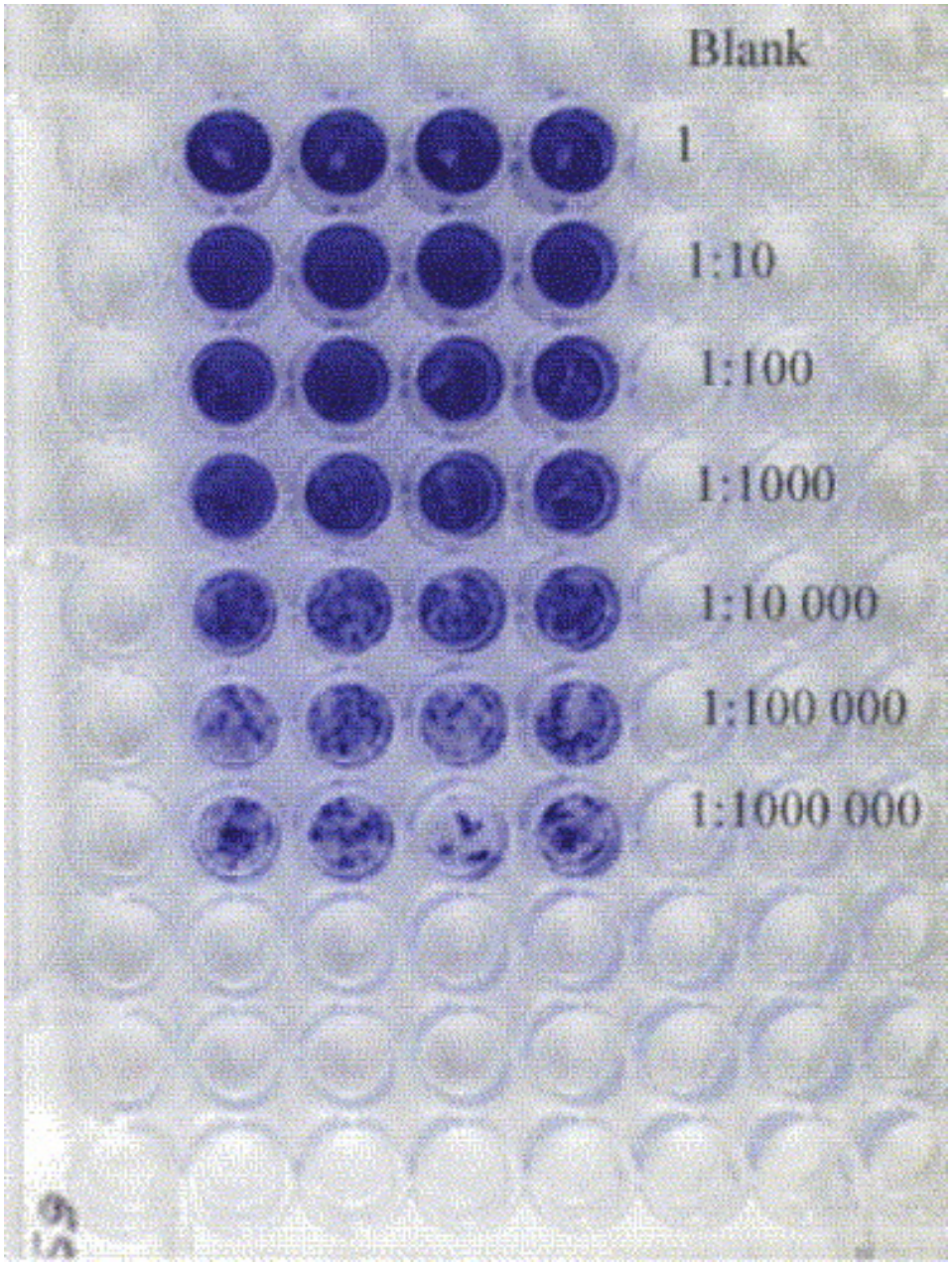


Figure II. Biofilm-forming capacity for *Staphylococcus aureus*. Biofilm formation is reported in relation to control strain at 10^{-1} dilution.

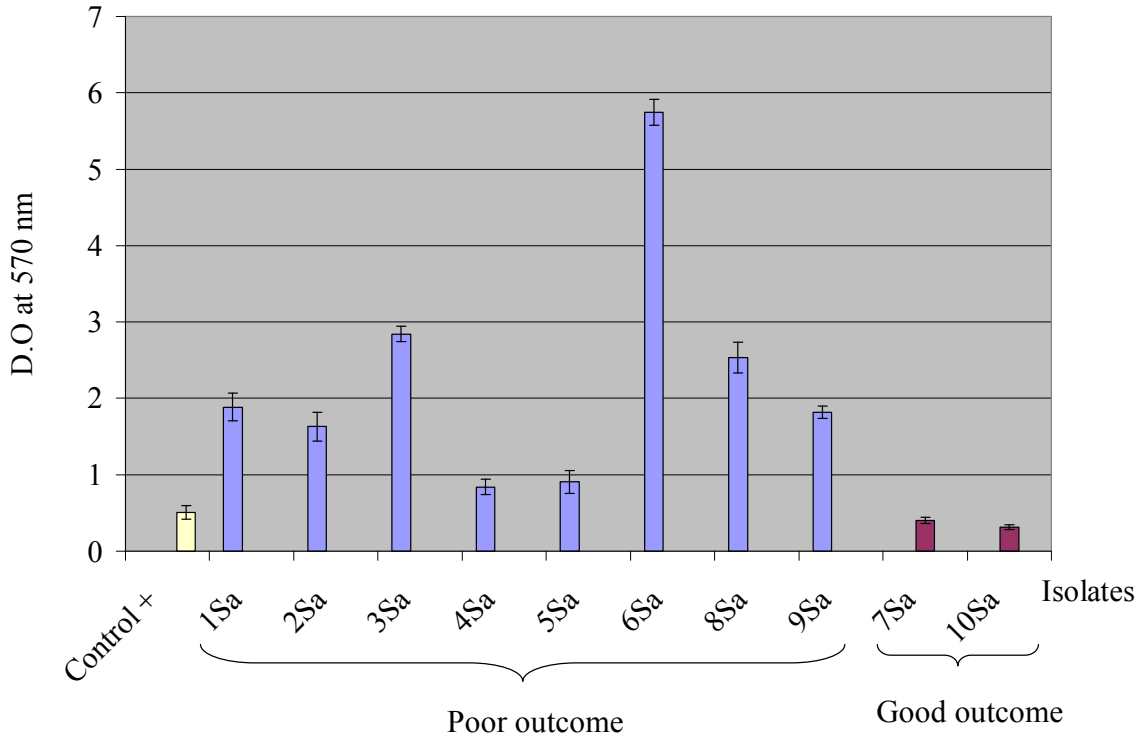


Figure III. Biofilm-forming capacity for Coagulase negative staphylococci. Biofilm formation is reported in relation to control strain at 10^{-1} dilution.

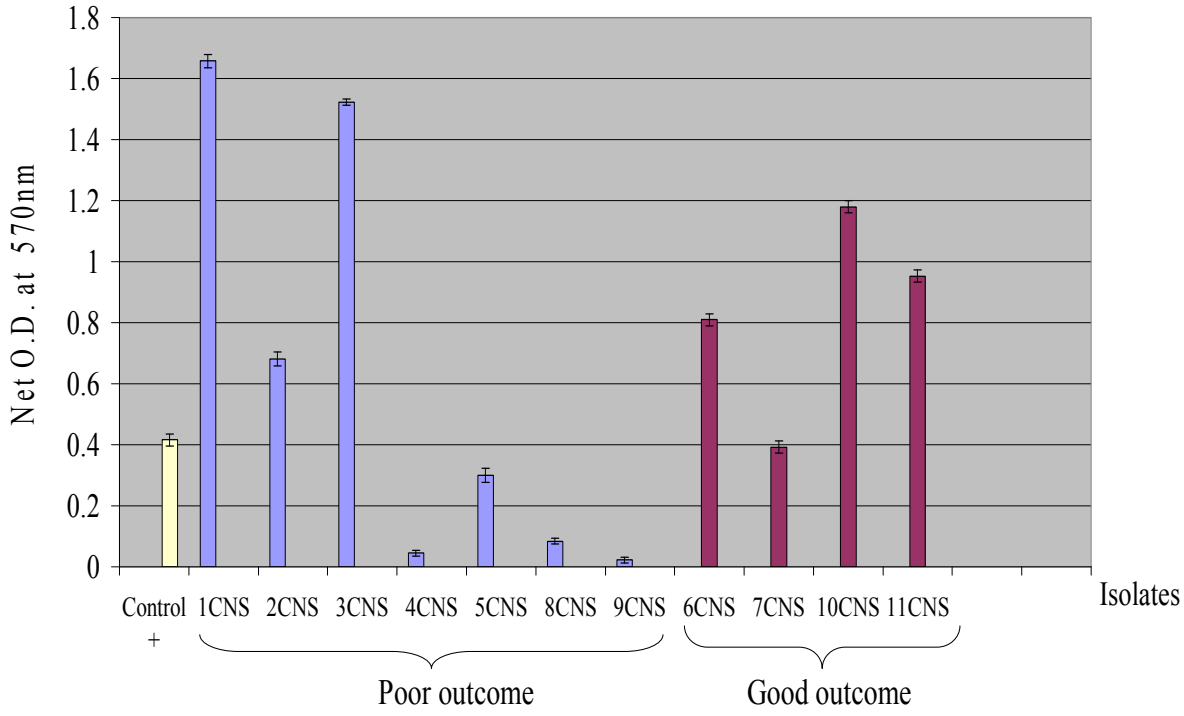


Figure IV. Biofilm-forming capacity for *Pseudomonas aeruginosa*. Sample 1Pa, a strong biofilm producer, has been removed to improve clarity. Biofilm formation is reported in relation to control strain at 10^{-1} dilution.

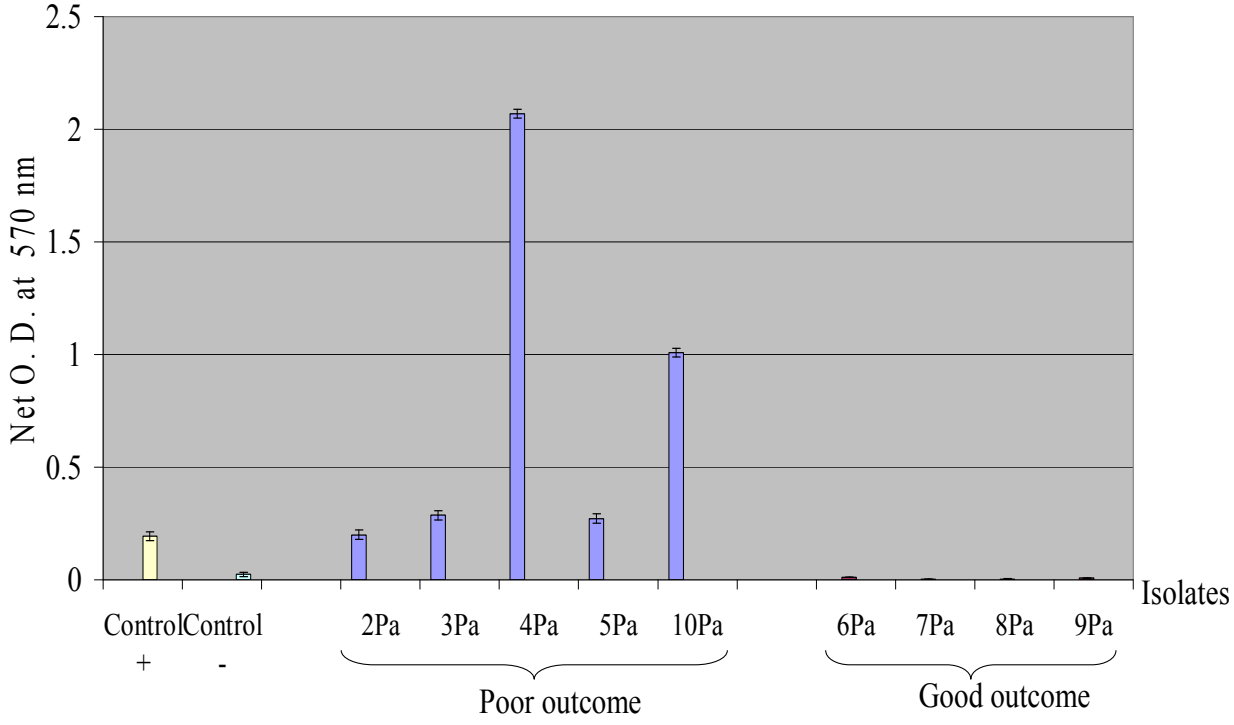


Table I. Clinical evolution, bacteriology and biofilm-forming capacity in subjects studied.

ID	Sex	Age	Diagnosis	Biofilm-forming capacity			
				P. aeruginosa	*S. aureus*	Coagulase-negative staphylococci	Evolution
1	M	68	CRS w/NP	BF-			Good
2	F	52	CRS w/ o NP		BF+		Poor
3	M	54	CRS w/ o NP			BF+ , BF+ *	Good
4	F	52	CRS w/ NP		BF+		Poor
5	M	68	CRS w/ NP	BF-	BF-	BF+	Good
6	F	66	CRS w/ o NP	BF-	BF-		Good
7	M	40	CRS w/ NP	BF-		BF+	Good
8	M	57	CRS w/ NP	BF+	BF+		Poor
9	F	44	CRS w/ NP		BF+		Poor
10	M	75	CRS w/ NP		BF+		Poor
11	M	42	CRS w/ o NP	BF+		BF- , BF+	Poor
12	F	43	CRS w/ NP	BF+			Poor
13	M	44	CRS w/ NP		BF+		Poor
14	M	68	CRS w/ o NP	BF+			Poor
15	M	36	CRS w/ NP			BF- , BF-	Poor
16	F	47	CRS w/ NP			BF+ , BF+	Poor
17	F	54	CRS w/ o NP	BF+			Poor
18	F	29	CRS w/ NP	BF+	BF+		Poor
19	F	68	CRS w/ o NP		BF+	BF+	Poor

* More than one isolate may be recovered from the same subject

CRS: chronic rhinosinusitis

NP: nasal polyposis

Discussion

In this preliminary study, we have shown that biofilm-forming capacity by *S. aureus* and *P. aeruginosa* but not CNS is associated with a poor clinical evolution in individuals with chronic rhinosinusitis with or without nasal polyposis (CRS +/- NP) having previously undergone endoscopic sinus surgery. This suggests that biofilm-producing capacity by *S. aureus* and *P. aeruginosa* influences the clinical evolution and may help explain the persistence of disease in CRS.

S. aureus and *P. aeruginosa* have both been implicated as pathogens in respiratory disease and in CRS. *S. aureus* is believed to exert a role in CRS by toxin production with superantigenic stimulation of specific immunity, and possible sensitization to the toxin [14, 15]. *P. aeruginosa* is a Gram-negative, frequently associated with long-term respiratory tract disease. Inflammation and tissue destruction from natural enzymes secreted by *P. aeruginosa* is enhanced by products of the systemic defence mechanisms, such as products of neutrophil degradation [16]. However, there is a paucity of information explaining why these bacterial species remain difficult to eradicate with conventional antibiotic therapy. The presence of biofilms may help explain this and open the door to novel means of therapeutic intervention.

It is interesting to note that there is no relation between the production of biofilm by coagulase-negative staphylococci and the evolution of the patients. Coagulase negative staphylococci are frequently regarded as mere contaminants of the nasal flora and are the

agent the most frequently recovered in healthy, asymptomatic post-ESS cavities. However, in other areas CNS can be potent pathogens, and are frequently responsible for infection and failure of a variety of medical implants [17]. In this study, no conclusion can be drawn on their role as a potential pathogen as results for CNS may reflect inherent difficulties in culture of bacteria from biofilms.

In this study, we have used a simple, in vitro method to detect biofilm-forming capacity instead of other methods for in situ biofilm detection such as scanning electron microscopy (SEM), transmission electron microscopy (TEM), confocal laser microscopy or detection of presence of genes identified with biofilm-forming capacity [18]. However, these methods are expensive, may require tissue sample for use, and are not routinely available. These factors limit their utility in clinical practice. In addition, while these methods can help identify the presence of a biofilm, they do not permit to identify the agent(s) involved, limiting the study to specific bacterial species. Though this limitation may be addressed by use of fluorescent in situ hybridization (FISH) probes combined with laser confocal microscopy, this expertise is currently unavailable outside of a limited number of specialized centers. One of the principal limitations of in vitro staining techniques is that they have a higher false-negative rate for specimens with weak biofilm formation [19]. The organisms we studied are mostly strong producers and this technique appears to perform well in our model.

It is obvious that biofilm-forming capacity in vitro does not necessarily correlate to in vivo biofilm production. Certainly favorable local conditions are required to favor expression of the biofilm-forming phenotype. It may be that the microenvironment of the inflamed sinuses in patients with CRS favors this, even in the absence of a foreign body. Changes brought about at the level of the sinus mucosa by the inflammatory process create areas of damaged epithelium with impaired local defenses [20], favoring the attachment of certain types of bacteria [21] with possible biofilm formation. Establishment of the biofilm with subsequent chronic bacterial infection may help maintain the chronic course experienced by these individuals. Once biofilm is installed, persistent infection and host response to biofilm-bound and free forms of the bacteria may contribute to continue tissue damage.

The presence of bacterial biofilms in the cavities of symptomatic post-ESS patients may help explain previously noted contradictory findings in studies of topical antibiotic therapy for post-ESS sinusitis. While topical antibiotic therapy was reported beneficial in individuals with acute exacerbations of symptomatology in post-ESS sinusitis [22], in another trial it was of no benefit in patients with chronic disease with stable symptomatology [23]. In these situations, bacteria organized in biofilms may have been responsible for chronicity, with exacerbations caused by increased presence of planktonic (free) forms responsive to antibiotic therapy.

Despite the potential interest of biofilms in CRS, it is probable that biofilms do not represent the entire explanation for CRS. The initial events leading to inflammation remain to be explained, and host factors predisposing to CRS need to be better characterized. The occurrence of poor outcome in patients where cultures revealed lack of biofilm forming capacity may be due to other phenomenon responsible for clinical disease.

Conclusion

This preliminary work suggests that biofilm-forming capacity by *S. aureus* and *P. aeruginosa* is associated with a poor evolution in individuals having previously undergone ESS for CRS and that biofilm formation by these two organisms may play a role in the chronicity of this disorder. Before drawing definite conclusions, confirmation of these preliminary results using both larger, better powered series and more sophisticated detection methods are warranted. If biofilms are found to be implicated in CRS, therapies modulating biofilm formation and detachment may be incorporated into strategies for management of this disorder.

Acknowledgements

We thank Onder Agbaba for his excellent technical assistance with isolation and identification of the pathogens. The financial support of the Fondation Antoine Turmel is greatly acknowledged.

References

1. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science 1999; 284:1318–22.

2. Potera C. Forging a link between biofilms and disease. Science 1999; 283(5409):1837–1839.

3. Amorena B, Gracia E, Monzon M, et al. Antibiotic susceptibility assay for *Staphylococcus aureus* in biofilms developed *in vitro*. J Antimicrob Chemother 1999;44(1):43–55.

4. Costerton W, Veeh R, Shirliff M, et al. The application of biofilm science to the study and control of chronic bacterial infections. J Clin Invest 2003;112:1466 –77.

5. Post JC, Stoodley P, Hall-Stoodley L, et al. The role of biofilms in otolaryngologic infections. Curr Opin Otolaryngol Head Neck Surg 2004;12:185–90.

6. Cryer J, Schipor I, Perloff JR, et al. Evidence of bacterial biofilms in human chronic sinusitis. ORL J Otorhinolaryngol Relat Spec 2004;66: 155–8.

7. Sanclement J, Webster P, Thomas J, et al. Bacterial biofilms in surgical patients with chronic rhinosinusitis. Laryngoscope 2005;115:578–82.

8. Bhattacharyya N, Kepnes LJ. The microbiology of recurrent rhinosinusitis after endoscopic sinus surgery. Arch Otolaryngol Head Neck Surg 1999;125(10):1117–20.

9. Benninger MS, Ferguson BJ, Hadley JA, et al. Adult chronic rhinosinusitis: definitions, diagnosis, epidemiology, and pathophysiology. Otolaryngol Head Neck Surg 2003;129(3 Suppl):S1–32.

10. Nadel DM, Lanza DC, Kennedy DW. Endoscopically guided cultures in chronic sinusitis. Am J Rhinol 1998;12(4):233– 41.

11. Stepanovic S, Vukovic D, Dakic I, et al. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J Microbiol Methods 2000;40(2):175–9.

12. Gotz F. *Staphylococcus* and biofilms. Mol Microbiol 2002;43(6): 1367–78.

13. Arciola CR, Baldassarri L, Montanaro L. Presence of *icaA* and *icaD* genes and slime production in a collection of staphylococcal strains from catheter associated infections. J Clin Microbiol 2001;39(6):2151–6.

14. Bachert C, Gevaert P, van Cauwenberge P. *Staphylococcus aureus* superantigens and airway disease. Curr Allergy Asthma Rep 2002; 2(3):252– 8.

15. Tripathi A, Conley DB, Grammer LC, et al. Immunoglobulin E to staphylococcal and streptococcal toxins in patients with chronic sinusitis/nasal polyposis. Laryngoscope 2004;114(10):1822– 6.

16. Ratjen F, Doring G. Cystic fibrosis. Lancet 2003;22;361(9358):681–9.

17. Arciola CR, Campoccia D, Gamberini S, et al. Search for the insertion element IS256 within the *ica* locus of *Staphylococcus epidermidis* clinical isolates collected from biomaterial-associated infections. Biomaterials 2004;25(18):4117–25.

18. Arciola CR, Campoccia D, Montanaro L. Detection of biofilm-forming strains of *Staphylococcus epidermidis* and *Staphylococcus aureus*. Expert Rev Mol Diagn 2002;2(5):478–84.

19. Knobloch JK, Horstkotte MA, Rohde H, et al. Evaluation of different detection methods of biofilm formation in *Staphylococcus aureus*. Med Microbiol Immunol (Berl) 2002;191(2):101– 6. Epub 2002 Jun 29.

20. Ponikau JU, Sherris DA, Kephart GM, et al. Features of airway remodeling and eosinophilic inflammation in chronic rhinosinusitis: is the histopathology similar to asthma? J Allergy Clin Immunol 2003; 112(5):877– 82.

21. De Bentzenmann S, Plotkowski C, Puchelle E. Receptors in the *Pseudomonas aeruginosa* adherence to injured and repairing airway epithelium. Am J Respir Crit Care Med 1996;154:S155–62.

22. Vaughan WC, Carvalho G. Use of nebulized antibiotics for acute infections in chronic sinusitis. Otolaryngol Head Neck Surg 2002; 127(6):558–68.

23. Desrosiers MY, Salas-Prato M. Treatment of chronic rhinosinusitis refractory to other treatments with topical antibiotic therapy delivered by means of a large-particle nebulizer: results of a controlled trial. Otolaryngol Head Neck Surg 2001;125(3):265–9.

3. Desrosiers M, Bendouah Z, Barbeau J. Effectiveness of topical antibiotics on *Staphylococcus aureus* biofilm *in vitro*. Am J Rhinol 21(2):149-53, Mar-Apr 2007.

Abstract

Introduction: *In vitro* biofilm producing capacity in isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa* collected from the sinus cavities after ESS are associated with a poor outcome in patients with chronic rhinosinusitis (CRS). However, conventional oral antibiotic therapy is frequently ineffective in eradicating bacteria in the biofilm form. Increasing the concentration of antibiotics may offer a means of countering this resistance.

Objectives: To determine the *in vitro* activity of moxifloxacin (MOXI) against *Staphylococcus aureus* (Recovered from patients with CRS at least 1 year post–endoscopic sinus surgery) in biofilm form.

Setting: Research microbiology laboratory.

Method: Five isolates of *S. aureus* with known biofilm-forming capacity were cultured in TSB 0.5% glucose in 96 well plates at 37°C for 24h. After visual confirmation of biofilm formation, plates were incubated in PBS with MOXI at minimal inhibitory concentration (MIC) or concentrations of 0.1X, 1X, 100X and 1000X MIC for a further 24h. Biofilm from 3 wells of each concentration were collected, sonicated and the number of viable bacteria determined by serial dilution and plating.

Results: After incubation, the number of viable bacteria was similar for non-treated and MOXI treated biofilms at MIC and sub-MIC levels. However, MOXI at 1000X (0.1mg/ml – 0.2 mg/ml) gave a two to 2.5 log reduction in number of viable bacteria.

Conclusion: *In vitro* results show that increased concentrations of antibiotics, easily attainable in topical solutions, are effective in killing bacteria in bacterial biofilms. This suggests a role for topical antibiotic therapies in the treatment of biofilm infections.

Running head: Topical antibiotics: Effect on *Staphylococcus aureus* biofilm

Keywords: Chronic rhinosinusitis, endoscopic sinus surgery, *Staphylococcus aureus,* bacterial biofilm, moxifloxacin, topical antibiotic therapy.

Funding: Internal funding.

Conflict of interest statement: Drug was furnished free of charge by Bayer Canada however no financial support was received.

Introduction

Chronic rhinosinusitis is a chronic disease of the paranasal sinuses characterised by persistent inflammation with bacterial colonisation and infection. Currently available therapy centers on medical management with antibiotics and topical or oral corticosteroids. For individuals failing to respond to these therapies, endoscopic sinus surgery is recommended for removing diseased tissue and improving drainage.

Cultures of individuals with chronic sinusitis not responding to surgery have shown higher than usual incidences of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus pneumonia* and *Haemophilus influenzae* [1, 2]. On sensitivity testing, these are usually susceptible to available oral agents. However, oral antibiotic therapy is frequently unsuccessful in clearing CRS, particularly after ESS. The failure of antibiotic therapy in CRS is somewhat surprising, given the high success rate of antibiotics in the treatment of acute bacterial maxillary rhinosinusitis.

Biofilms have been implicated in CRS and may help explain a portion of these phenomenons. The presence of bacterial biofilms on the mucosa of patients with chronic rhinosinusitis and its role in sinus disease has now been demonstrated using a variety of techniques. Using scanning and transmission electron microscopy (SEM), Palmer et al [3], Ferguson et al [4] and Ramadan et al [5] have all shown the presence of biofilm on the sinus mucosa. Using confocal laser microscopy with FISH technique for identification of bacterial species, Hunsaker et al [6] have shown the presence of *Haemophilus influenzae*,

Streptococcus pneumoniae and *Staphylococcus aureus*. Our group has demonstrated a correlation between *in vitro* biofilm-producing capacity by *Staphylococcus aureus* and unfavourable evolution after endoscopic sinus surgery (ESS) [7], suggesting that biofilm producing capacity may play a role in the pathogenesis of chronic rhinosinusitis.

The presence of bacterial biofilms may help explain the lack of effectiveness of oral antibiotics. By virtue of their nature, bacteria in biofilms are much less susceptible to antibiotics. Minimally inhibitory concentrations (MIC) effective on an organism in planktonic form are ineffective against the same organism when present in the biofilm form. Increasing the concentration of antibiotics may offer a means of countering this resistance [8]. While high concentrations of antibiotics are difficult to attain in serum because of toxicity risks, antibacterials can be delivered safely in high concentrations when applied topically. Given that as after sinus surgery, the mucosa of the sinus cavities is accessible to topical therapy, it seems reasonable to evaluate the effect of topical antibiotics on bacteria from sinus isolates in the biofilm form.

While topical antibiotic therapy has previously been attempted as therapy, newer broad-spectrum agents may offer an alternative to agents such as topical tobramycin previously attempt for topical therapy. Moxifloxacin is a new enantiomerically pure 8-methoxy quinolone that was first synthesized by Petersen and colleagues [9] with a broad-spectrum of anti-bacterial activity against Gram-positive and Gram-negative bacteria, anaerobes and

atypical organisms [10]. Compared to first-generation quinolones, it has higher potency and pharmacodynamic activity [11, 12].

We wished to explore the efficacy of topical moxifloxacin on mature bacterial biofilms of *Staphylococcus aureus* using isolates obtained from individuals with chronic rhinosinusitis.

Objectives

The aim of the present study was to assess the in vitro activity of moxifloxacin against clinical isolates of *S. aureus* in biofilm form.

Materials and methods

This study was approved by the ethical review board for human subjects of the Centre Hospitalier de l'Université de Montréal.

Organisms

Four isolates previously recovered from patients with bad outcome after ESS for CRS with or without sinonasal polyposis and an isolate of *S. aureus* (LSPQ 2520) obtained from the provincial reference laboratory were used. This isolates have been used in our previous studies of biofilm forming-capacity of sinus isolates and were selected for biofilm-forming capacity. This population and collection method has been previously described [13].

Antimicrobial agent :

Moxifloxacin (MOXI) was supplied as dry powder for laboratory use by Bayer Canada.

MIC determination

Minimally inhibitory concentration (MIC) to MOXI was determined for each of the organisms studied. MIC's are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. MICs were determined by a standard macro dilution method [14]. In previous published studies of acute bacterial sinusitis, MIC90 of MOXI for isolates of *S. aureus* = 0.12 µg/ml [15, 16].

Biofilm formation

Mature biofilm formation was carried out on 96-well culture plaque in TSB 0.5% glucose at 37°C for 24h as previously described [13]. After visual confirmation of biofilm formation, the wells were washed three times with sterile phosphate-buffered saline (PBS) under aseptic conditions to eliminate nonadherent bacteria [17]. Plates were then incubated for a further 24 hours at 37°C in either sterile PBS or with PBS/MOXI solution at concentrations of 0.1X, 1X 100X and 1000X MIC. Wells with sterile PBS free of MOXI were used as controls. After incubation, wells were washed again three times with PBS. The biofilm was then collected to determine viable bacteria. Biofilms from 3 wells of each concentration were collected by scraping the well with a swab, and placed in 1 ml of PBS. The swab was sonicated for 2 minutes to free bacteria, and the biofilm form the 3 wells pooled together. The number of viable bacteria was determined by serial dilution and plating. All experiments were performed in triplicate.

Analysis

Average colony forming units (CFU) for the three samples tested were expressed as log of CFU per ml. Variability is expressed as standard error of the mean (SEM).

Results

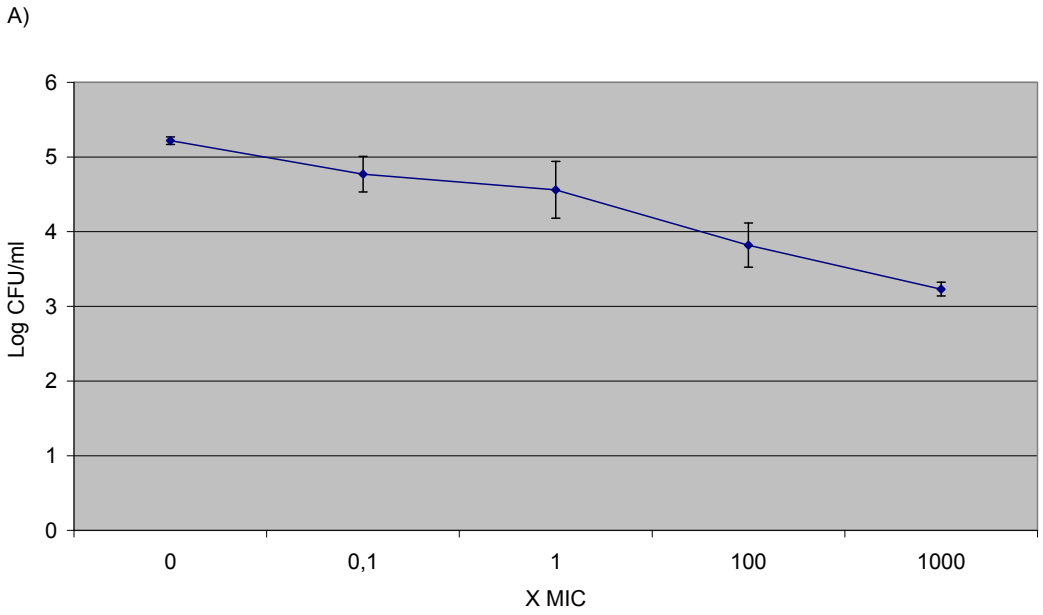
The susceptibilities (MIC) to Moxi of the bacterial strains tested are shown in table 1. These range from 0.0937 to 0.1875 mcg/ml. These MIC levels are comparable to those previously reported for isolates of *S. aureus* recovered in trial of acute bacterial maxillary sinusitis.

Results for each species are expressed in graphical form in Figure 1. For all strains tested, MOXI at sub MIC and MIC levels had no effect on established biofilm. At concentrations above MIC, there appeared to be a dose-related reduction in number of CFU. Maximal effect was noted with MOXI at 1000X (100 μg/ml – 200 μg/ml), which gave a two to 2.5 log reduction in number of viable bacteria within the biofilm.

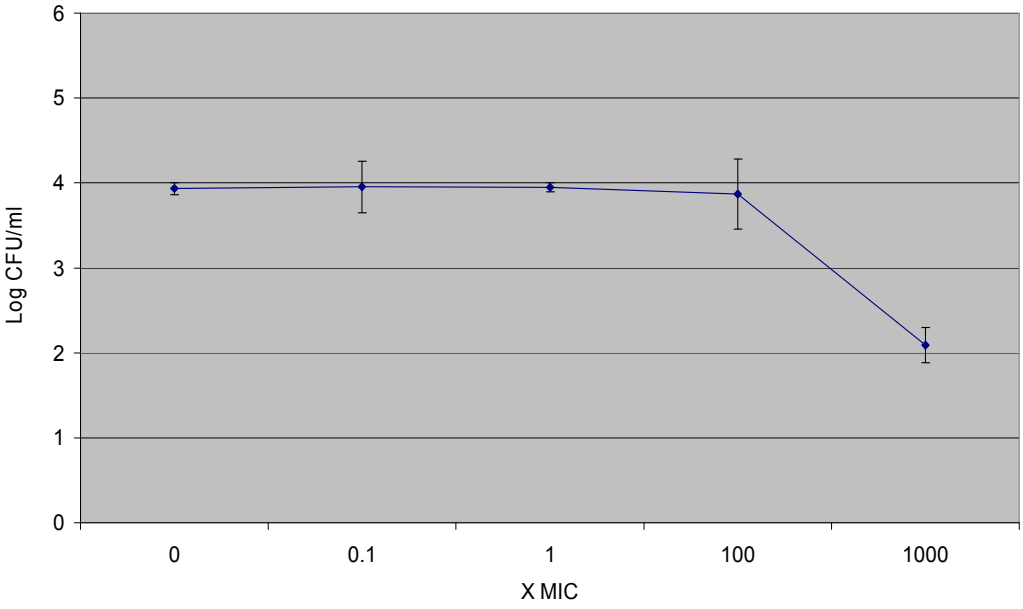
Despite this reduction, MOXI was incapable of eradicating completely viable bacteria in the mature biofilms.

Legends for Illustrations:

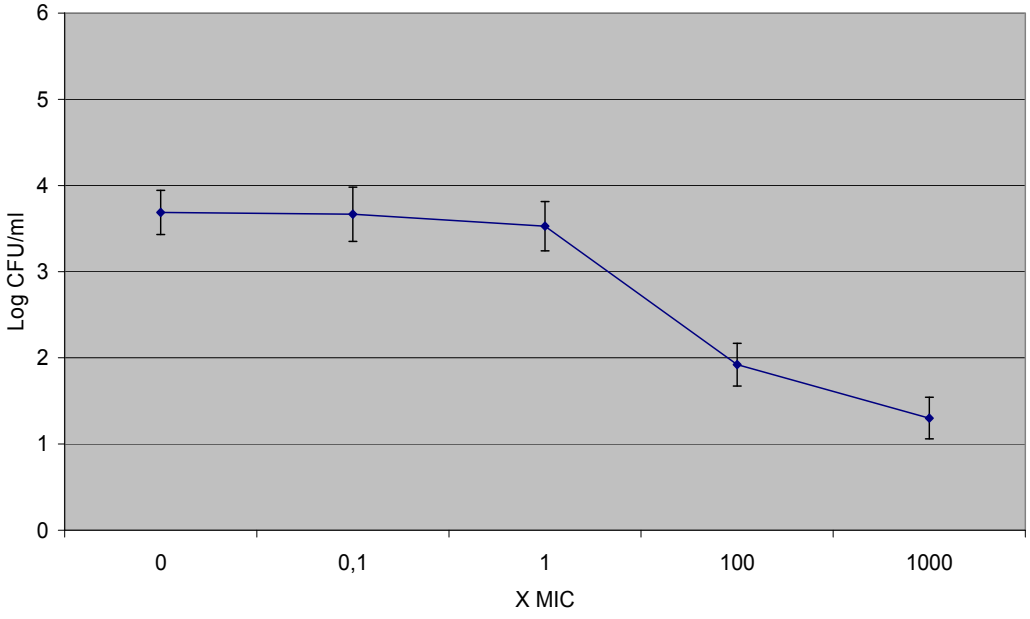
Fig 1. Bacterial viability (log_{10} CFU/ml) after exposure of a mature *Staphylococcus aureus* biofilm to different concentrations of moxifloxacin for 24 h. A) 2Sa, B) 3Sa, C) 8Sa, D) 9Sa, E) LSPQ 2520.



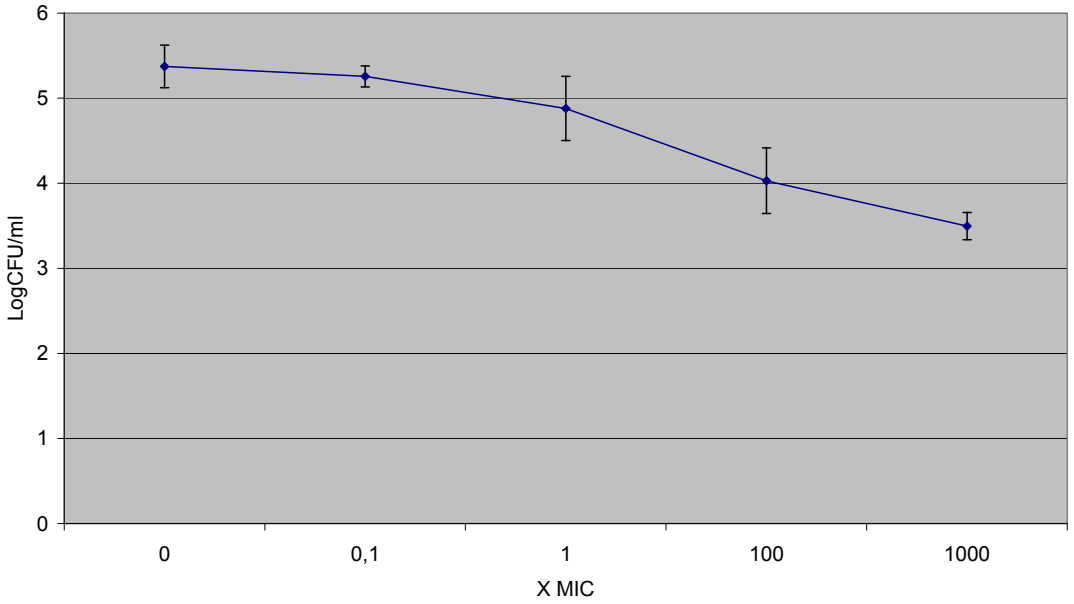
B)


6
5
4
3
2
1
0
Log CFU/ml
0
0.1
1
100
1000
X MIC

C)


6
5
4
3
2
1
0
Log CFU/ml
0
0,1
1
100
1000
X MIC

D)


6
5
4
3
2
1
0
LogCFU/ml
0
0,1
1
100
1000
X MIC

E)

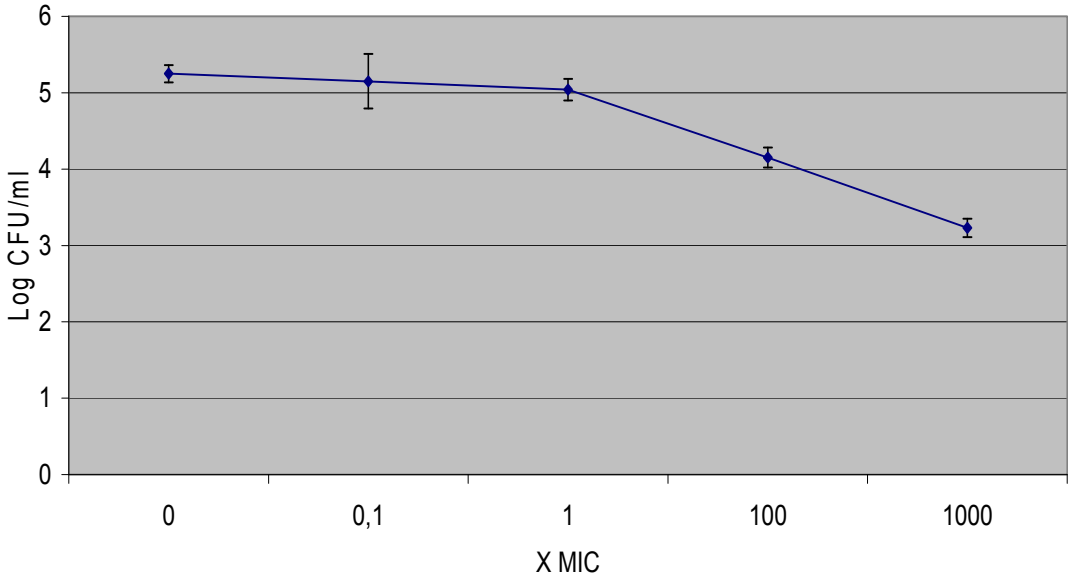

6
5
4
3
2
1
0
Log CFU/ml
0
0,1
1
100
1000
X MIC

Fig 2. Post-ESS changes facilitate penetration of solutions into the sinus cavities. Shaded area shows sinus mucosa that can be reached by topically instilled medications.

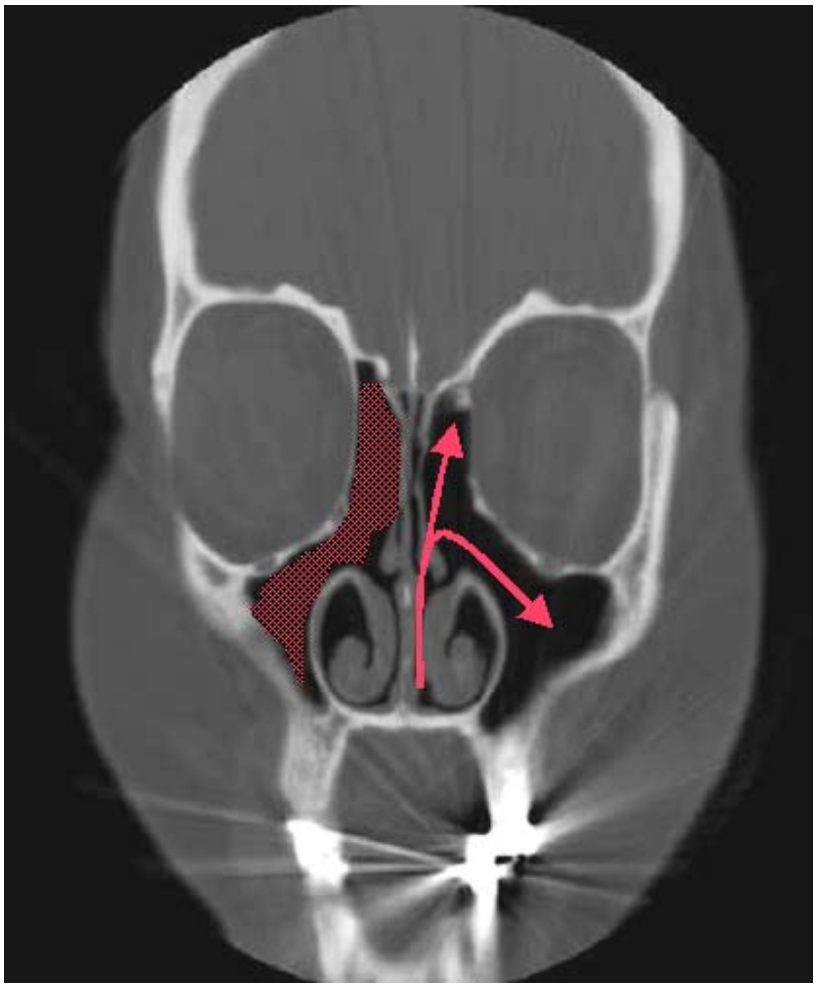


Table 1. Minimal inhibitory concentrations (MIC) to MOXI of the *S. aureus* strains tested.

Strain	MIC (µg/L)
LSPQ 2520	93.7
2 Sa	93.7
3 Sa	187.5
8 Sa	93.7
9 Sa	187.5

Discussion

We have performed an in-vitro assessment of the effectiveness of different concentrations of topical moxifloxacin in killing *S. aureus* contained in a bacterial biofilm. Sub-MIC and MIC levels of MOXI have no effect on *S. aureus* contained in mature biofilms, confirming previous reports [17].

Our results do however suggest that above-MIC concentrations of MOXI have some effectiveness in reducing the number of viable bacteria in mature biofilms of *S. aureus*, suggesting that antibiotic therapy with supra-MIC levels of MOXI and other antibiotics may actually have a role in the management of chronic sinusitis. Delivery of supra-MIC levels of MOXI to the sinus cavities via an oral route will however be difficult. Following administration of a standard 400mg dose, concentrations of MOXI in the sinus mucosa, even though approximately double that of serum levels, do not exceed 10 μg/ml at peak. For the isolates in our study, this would be approximately 50-100X MIC [18].

Delivery via a topical route may represent an option to oral administration, particularly after ESS. After ESS, the sinuses passages freely communicate with the nasal passages, allowing solutions applied within the nose to penetrate the sinus cavities (Figure 2). Topical administration offers a potential for deposition of medication at higher concentrations directly on the surface of the biofilm.

Topical therapies have previously been recommended for post-ESS patients but have met with varying rates of success. A sole prospective, placebo controlled, trial of nebulised tobramycin for the treatment of chronic sinusitis refractory to medical and surgical therapy did not show any benefit of tobramycin over placebo [19], while a retrospective case series

suggested effectiveness in certain cases [20]. Criticisms to these trials include differences in patient populations treated (stable chronic refractory patients vs. acute exacerbations), the use of an agent (tobramycin) with limited efficacy against gram-positive agents such as *S. aureus*, and mainly, the absence of precise measure of the quantity and concentration of antibiotic delivered to the sinus cavity via the apparatus used for topical administration.

It is to note that MOXI at these concentrations did not completely eradicate bacteria in biofilms. Further work to evaluate the post-antibiotic effect on the biofilm remains to be determined. Nevertheless, our results suggest that supra MIC levels of antibiotics represent an interesting area of study as we seek alternate means of improving the management of severe CRS.

Conclusion

In vitro results show that increased concentrations of antibiotics, easily attainable in topical solutions, are effective in killing bacteria in mature bacterial biofilms. This suggests a potential role for topical antibiotic therapies in the management of biofilm infections, and that further exploration of this area is warranted.

Acknowledgements

We thank the Fondation Antoine Turmel for their generous support of our research activities.

References

1. Bhattacharyya N, Kepnes LJ. The microbiology of recurrent rhinosinusitis after endoscopic sinus surgery. Arch Otolaryngol Head Neck Sur Oct;125(10):1117-20, 1999.

2. Al-Shemari H, Abou-Hamad W, Libman M, Desrosiers M. Bacteriology of Sinus Cavities of Asymptomatic Individuals after Endoscopic Sinus Surgery Journal of Otolaryngology (in press).

3. Cryer J, Schipor I, Perloff JR, Palmer JN. Evidence of bacterial biofilms in human chronic sinusitis. ORL J Otorhinolaryngol Relat Spec 66: 155-158, 2004.

4. Ferguson BJ, Stolz DB. Demonstration of biofilm in human bacterial chronic rhinosinusitis. Am J Rhinol 19(5):452-7, 2005.

5. Ramadan HH, Sanclement JA, Thomas JG. Chronic rhinosinusitis and biofilms. Otolaryngology - Head and Neck Surgery Vol. 132, Issue 3, Pages 414-417, 2005.

6. Sanderson AR, Leid JG, Hunsaker D. Bacterial biofilms on the sinus mucosa of human subjects with chronic rhinosinusitis. Laryngoscope 116(7):1121-6, 2006.

7. Bendouah Z, Barbeau J, Hamad WA, Desrosiers M. Biofilm formation by *Staphylococcus aureus* and *Pseudomonas aeruginosa* is associated with an unfavorable evolution after surgery for chronic sinusitis and nasal polyposis. Otolaryngol Head Neck Surg 134(6):991-6, 2006.

8. Ghnnoum M, O'Toole GA, Microbial Biofilms, 2004.

9. Dalhoff A, Petersen U and Endermann R. In vitro activity of BAY 12-8039, a new 8-methoxyquinolone. Chemotherapy (Basel) 42:410-425, 1996.

10. Wolfson JS and Hooper DC. Fluoroquinolone antimicrobial agents. Clin. Microbiol Rev. 2, 378–424, 1989.

11. Woodcock JM, Andrews JM, Boswell F, et al. In vitro activity of BAY 12-8039, a new fluoroquinolone. Antimicrobial Agents and Chemotherapy 41, 101–6, 1997.

12. Fass RJ. In vitro activity of Bay 12-8039, a new 8-methoxyquinolone. Antimicrobial Agents and Chemotherapy 41, 1818–24, 1997.

13. Bendouah Z, Barbeau J, Hamad W, Desrosiers M. Use of an *in vitro* assay for determination of biofilm-forming capacity of bacteria in chronic rhinosinusitis. Am J Rhinol 20(5):434-4388, 2006.

14. Andrews JM. Determination of minimum inhibitory concentrations. Journal of Antimicrobial Chemotherapy 48, Suppl. S1, 5-15, 2001.

15. Blondeau JM. A review of the comparative in-vitro activities of 12 antimicrobial agents, with a focus on five new respiratory quinolones. J Antimicrob Chemother 43(suppl B): 1–11, 1999.

16. Ackermann G, Schaumann R, Pless B et al. Comparative activity of moxifloxacin in vitro against obligately anaerobic bacteria. Eur J Clin Microbiol Infect Dis 19(3):228-32, 2000.

17. Perez-Giraldo C, Gonzalez-Velasco C, Sanchez-Silos RM et al. Moxifloxacin and biofilm production by coagulase-negative staphylococci. Chemotherapy 50 (2):101-4, 2004.

18. Gehanno P, Darantiere S, Dubreuil C et al. A prospective, multicentre study of moxifloxacin concentrations in the sinus mucosa tissue of patients undergoing elective surgery of the sinus. Journal of Antimicrobial Chemotherapy 49, 821-826, 2002.

19. Desrosiers MY, Salas-Prato M. Treatment of chronic rhinosinusitis refractory to other treatments with topical antibiotic therapy delivered by means of a large-particle nebulizer: results of a controlled trial. Otolaryngol Head Neck Surg 125(3):265-9, 2001.

20. Vaughan WC, Carvalho G. Use of nebulized antibiotics for acute infections in chronic sinusitis. Otolaryngol Head Neck Surg 127(6):558-68, 2002.

4-Bacterial killing in *Staphylococcus aureus* biofilm doesn't improve with increasing concentration of moxifloxacin beyond 1000XCMI (soumis)

ABSTRACT

Objective: To determine whether increasing the concentration of antibiotics beyond 1000XMIC would improve bacterial kill rates.

Study Design: Prospective study.

Subject and methods: Four isolates of *Staphylococcus aureus* with known biofilm-forming capacity were cultured in 24 well plates. After visual confirmation of biofilm formation, plates were incubated in PBS only or with moxifloxacin at concentrations of 1X, 1 000X, 5 000X and 10 000X MIC for a further 24h. Viable bacteria in biofilm was determined by serial dilution and plating. Confirmation of these results was performed with confocal laser microscopy, using a BacLight stain.

Results: After incubation, the number of viable bacteria was similar for non-treated and MOXI treated biofilms at MIC. MOXI at 1 000X MIC (0.1mg/ml – 0.2 mg/ml) gave a 2 to 2.5 log reduction in number of viable bacteria. However, application of MOXI at 5 000X and 10 000X MIC (1mg/ml – 2 mg/ml) did not show additional reduction in the number of viable bacteria over the 1 000X MIC MOXI treatment.

Conclusion: *In vitro* results show that concentrations of moxifloxacin beyond 1000X MIC do not improve bacterial killing within the biofilm beyond log 2 or 99%. This suggests a persister phenomenon.

Keywords: Chronic rhinosinusitis, endoscopic sinus surgery, *Staphylococcus aureus,* bacterial biofilm, moxifloxacin, topical antibiotic therapy.

Funding: Internal funding.

Conflict of interest statement: Drug furnished by Bayer Canada.

Introduction

Chronic rhinosinusitis (CRS) is a chronic disease of the paranasal sinuses characterised by persistent inflammation with bacterial colonisation and infection. For individuals failing to respond to antibiotics and topical or oral corticosteroids, endoscopic sinus surgery is recommended for removing diseased tissue and improving drainage. Even after surgery, certain individuals may nevertheless progress to a severe, crippling evolution characterised by continued inflammation and frequent infections of the paranasal sinuses. Cultures of individuals with chronic sinusitis not responding to surgery have shown higher than usual incidences of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae* and *Haemophilus influenzae* [1, 2]. On sensitivity testing, these are usually susceptible to available antibiotics. However, oral antibiotic therapy is frequently unsuccessful in clearing CRS, particularly after endoscopic sinus surgery (ESS).

The presence of bacterial biofilm may help to explain the lack of effectiveness of oral antibiotics.

Biofilms are structured, specialised communities of adherent micro-organisms embedded in a complex extra-cellular polymeric substance [3]. Recent evidence demonstrating biofilm of micro-organisms living within the paranasal sinuses has been published [4, 5, 6, 7]. These biofilms may provide a continual chronic inflammation of the mucosa of the affected sinuses.

Our previous work suggests that *in vitro* biofilm-forming capacity by *S. aureus* and *P. aeruginosa* recovered from sinuses of individuals with CRS is associated with a poor evolution in individuals having previously undergone ESS for CRS [8].

Despite the potential importance of bacterial biofilms in the pathogenesis of CRS, currently no biofilm-specific strategy is employed in current management. Current therapy of CRS centers on oral or intravenous antibiotics for control of bacterial infection and combinations of topical and oral corticosteroids for control of the inflammatory component. An important insight to modifying therapy may be suggested by the fact that bacterial susceptibility in biofilm form is more dependant on the antimicrobial concentration than is planktonic killing. Thus, biofilms may be better controlled by using brief but relatively high concentrations of antimicrobial agents rather than prolonged doses of lower concentrations [9].

Topical antibiotic therapy offers the opportunity to deliver higher local concentrations of antibiotics than does oral administration and may thus offer an improvement over oral antibiotics in the management of biofilm disease. Our previous *in vitro* results support this, showing that increasing the concentrations of moxifloxacin to 1000X MIC (0.1mg/ml – 0.2 mg/ml) is required to significantly reduce the bacterial population in biofilm [10]. This suggests that topical antibiotic solutions delivered directly to the sino nasal mucosa may have a role in managing biofilm infection in CRS. However, the optimal dosage remains to be determined.

Objectives

We wished to explore the efficacy of supra-1000X MIC concentrations of moxifloxacin on mature bacterial biofilms of *Staphylococcus aureus* grown from clinical isolates obtained from individuals with chronic rhinosinusitis.

Materials and methods

This study was approved by the ethical review board for human subjects of the Centre Hospitalier de l'Université de Montréal.

Organisms

Four isolates previously recovered from patients with poor outcome after ESS for CRS with or without sinonasal polyposis and a single isolate of *S. aureus* (LSPQ 2520) obtained from the provincial reference laboratory were used. This isolates have been used in our previous studies of biofilm forming-capacity of sinus isolates and were selected for biofilm-forming capacity. This population and collection method has been previously described [11].

Antimicrobial agent

Moxifloxacin (MOXI) was generously supplied as dry powder for laboratory use by Bayer Canada and was dissolved in PBS to attain the appropriate concentration.

MIC determination

Conventional culture and serial dilution techniques were used to determine the minimally inhibitory concentration (MIC) to MOXI for the planktonic form of each of the *S. aureus* organisms [12]. MIC's are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. In previous published studies of acute bacterial sinusitis, MIC90 of MOXI for isolates of *S. aureus* = 0.12 μg/ml, which are similar to ours [13, 14].

Biofilm formation

Mature biofilm formation was carried out on 24-well culture plates in TSB 0.5% glucose at 37°C for 24h as previously described. After visual confirmation of biofilm formation, the wells were washed three times with sterile phosphate-buffered saline (PBS) under aseptic conditions to eliminate non-adherent bacteria. Plates were then incubated for a further 24 hours at 37°C in either sterile PBS (control) or with PBS/MOXI solution at concentrations of 1X, 1000X, 5000X and 10 000X MIC. Wells with sterile PBS free of MOXI were used as controls. After incubation, wells were washed again three times with PBS. The biofilm was then collected to determine viable bacteria. Biofilm from each well was collected by scraping with a swab, and placed in 1 ml of PBS. The swab was treated with ultrasounds for 2 minutes to disperse bacteria. The number of viable bacteria was determined by serial dilution and plating. All experiments were performed in triplicate.

Non-specific bacterial staining

In order to visualise the bacteria within the biofilm, cell population was stained using the BacLight Bacteria Viability Kit (Molecular Probes, Eugene, OR, USA). This kit consists of two fluorescent dyes, Syto-9 (S-9) and propodium iodide (PI), pre-aliquoted in sealed plastic pipettes. Both of these dyes have a high affinity for DNA. All viable bacteria stain green with the S-9 dye but the red dye (PI) will displace the S-9 dye if the bacterial cell wall is damaged. Consequently, dead or dying bacteria are stained red.

The BacLight solution was added to the specimen according to manufacturer instructions. After 15 minutes of incubation at room temperature the excess of the BacLight solution was removed. ProLong Gold antifade reagent was added to ensure color stability and the specimen was incubated for 24 hours at room temperature in the dark.

Analysis

Average colony forming units (CFU) for the three samples tested were expressed as log of CFU per ml. Variability is expressed as standard error of the mean (SEM).

Results and discussion

The *in vitro* susceptibilities (MIC) to MOXI of the planktoic forms of the bacterial strains tested are shown in table 1. These are all susceptible to MOXI, with MIC's ranging from 0.0937 to 0.1875 µg/ml, and are comparable to those previously reported for isolates of *S. aureus* recovered in trial of acute bacterial maxillary sinusitis.

Results for each strain are expressed in graphical form in Figure 1. For all strains tested, maximal effect was noted with MOXI at 1000X MIC (0.1 mg/ml – 0.2 mg/ml), which gave a two to 2.5 log reduction in number of viable bacteria within the biofilm. MOXI at 5000X and 10 000X MIC did not increase bacterial killing comparing to the 1000X MOXI treatment.

These results are confirmed visually by BacLight staining confirming persistence of viable bacteria within the biofilm even after treatment with 1000X MOXI (Figure 2).

Despite this reduction, MOXI was incapable of eradicating completely the bacteria in the biofilm with approximately 1% of bacteria still viable after treatment with MOXI at 10 000X MIC. This suggests an important insight: More than simple killing of bacteria may be required for management of biofilms. For example, in another series of *in vitro* experiments, we showed that significant reduction of viable bacteria in biofilm could be attained by treatment with a citric acid/zwitterionic surfactant solution, with an enhanced result when pressurised irrigation was used. Evaluation of the biofilm architecture with CSLM in those experiments shows that while viable bacteria nevertheless remain present, very few non viable bacteria are present in the images, suggesting that the biofilm matrix has been removed successfully [15].

This suggests that optimal therapy will require a means for biofilm dispersal as well as bacterial killing.

The resistance of biofilms to drug therapy has been one of the most elusive problems in microbiology. The phenomenon of bacteria persisting after tharapy, or persisters, has increasingly been reported as an important cause of biofilm resistance to killing by antimicrobial agents. Persister cells have probably been generated to increase chances of survival of bacterial populations and constitute a relatively small fraction of the population, but these few cells have entered a highly protected state [16, 17].

Our *in vitro* experiments suggest that antibiotic treatment will kill most biofilm cells leaving persisters alive. This phenomenon may partially explain the persistence of the biofilm in the sinus of patients with CRS since *in vivo*, the remaining persister cells cannot be killed by the immune system because of the exopolymer matrix [18, 19].

Persister cells that are contained in the biofilm can survive both the antibiotic treatment and the immune system attack. When the concentration of antibiotic reduces, persisters can repopulate the biofilm, producing the acute exacerbation in our patients with CRS. Targeting the persisters may offer an additional option for the treatment of biofilm disease.

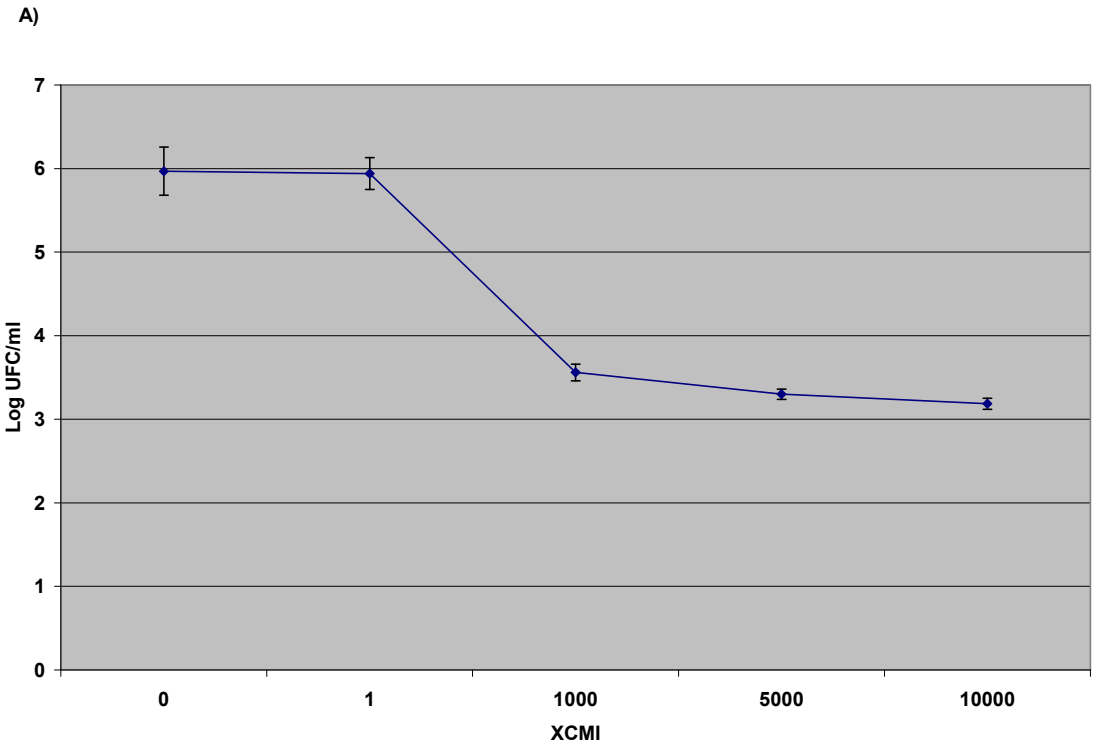
Conclusion

Supra-MIC concentrations of MOXI are more effective in killing bacteria in the biofilm form in clinical isolates of *Staphyloccocus aureus* taken from patients with CRS. Optimal killing occurs at 1000X MIC, with no additional benefit to increasing the concentration to 5 000X or 10 000X MIC.

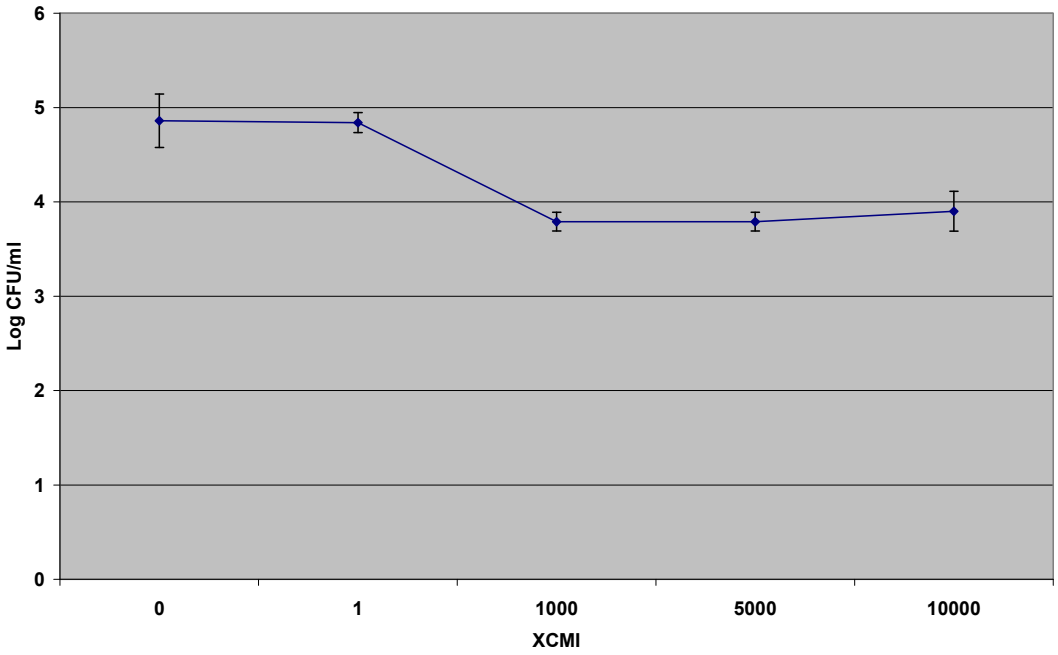
Persistence of large quantities of non-viable bacteria in the biofilm matrix suggests that optimal therapy will ultimately combine a therapy with bactericidal effects with a biofilm dispersing technique.

Legends for Illustrations:

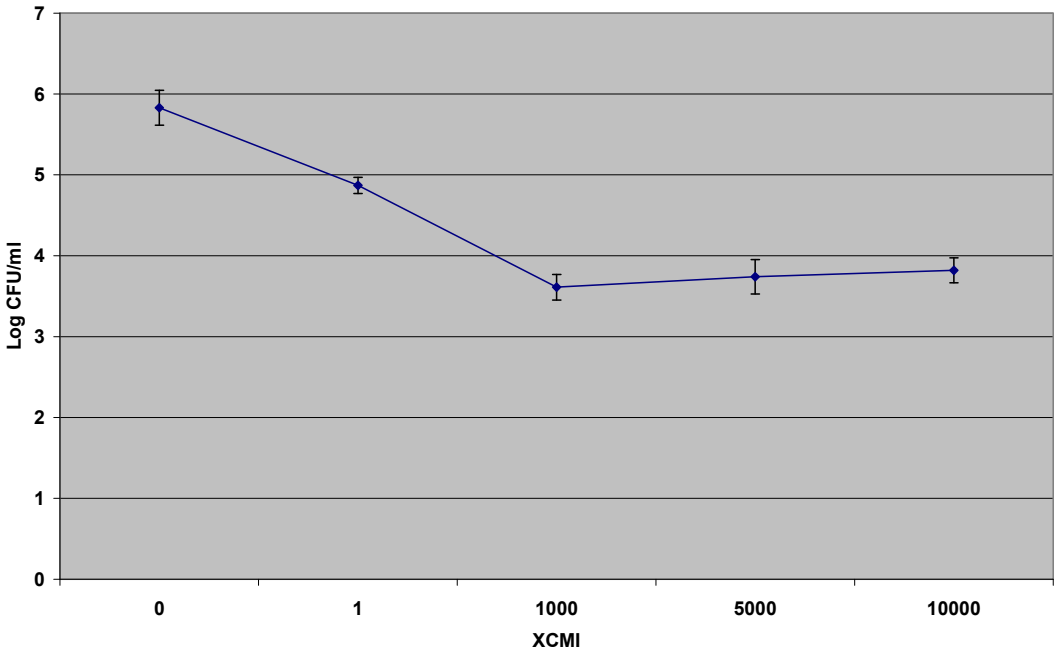
Figure 1. Bacterial viability (log_{10} CFU/ml) after exposure of a mature *S. aureus* biofilm to different concentrations of MOXI for 24 h. A) 3Sa, B) 8Sa, C) 9Sa, D) LSPQ 2520. Note that MOXI at MIC has little or no effect on bacterial viability. Increasing concentration to 1000X MIC enhances killing, but is not improved by increasing the concentration beyond 1000X MIC.



B)


6
5
4
3
2
1
0
Log CFU/ml
0
1
1000
5000
10000
XCMI

C)


7
6
5
4
3
2
1
0
Log CFU/ml
0
1
1000
5000
10000
XCMI

D)

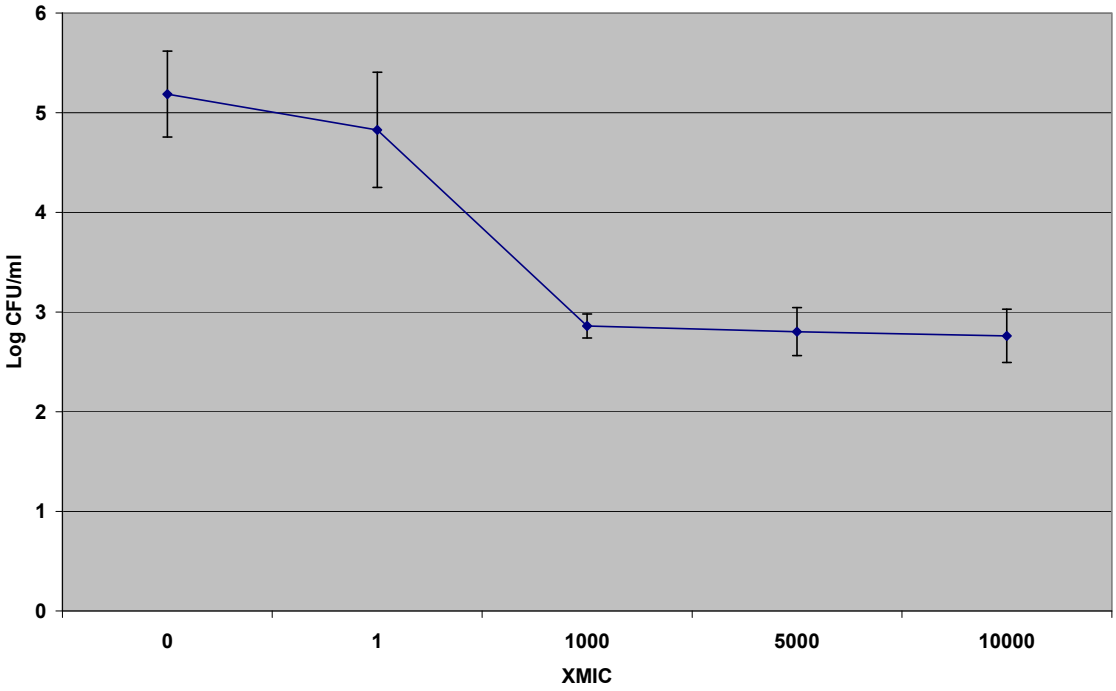
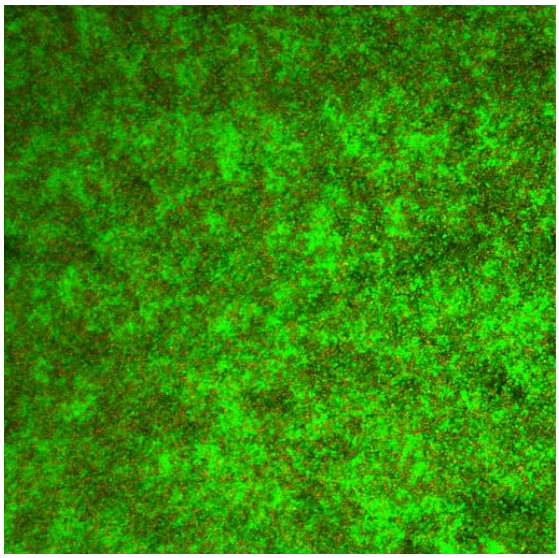


Figure 2. Representative section of *in vitro* mature biofilms from isolate 9 Sa after treatment with either A) PBS only or B) with MOXI 1000XMIC. Confocal laser microscopy; 63x with BacLight stain (green= viable, red = dead) Note that despite significant bacterial killing in the treated sample, the biofilm matrix persists with a number of persistent viable bacteria.

A)



B)

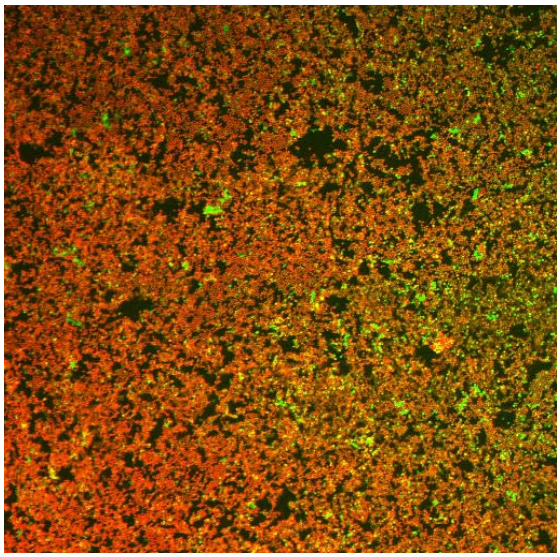


Table 1. Minimal inhibitory concentrations (MIC) to MOXI of the *Staphylococcus aureus* strains tested

Strain	**MIC (mg/L)**
LSPQ 2520	0.0937
3 Sa	0.1875
8 Sa	0.0937
9 Sa	0.1875

References

1. Bhattacharyya N, Kepnes LJ. The microbiology of recurrent rhinosinusitis after endoscopic sinus surgery. Arch Otolaryngol Head Neck Surg. 1999 Oct;125 10:1117-20.

2. Al-Shemari H, Abou-Hamad W, Desrosiers M J et al. Bacteriology of Sinus Cavities of Asymptomatic Individuals after Endoscopic Sinus Surgery. J Otolaryngol. 2007 Feb;36 1:43-8.

3. Hall-Stoodley L, Stoodley P. Biofilm formation and dispersal and the transmission of human pathogens. Trends Microbiol. 2005 Jan;13 1:7-10.

4. Cryer J, Schipor I, Perloff JR et al. Evidence of bacterial biofilms in human chronic sinusitis. ORL J Otorhinolaryngol Relat Spec. 2004;66 3:155-8.

5. Ferguson BJ, Stolz DB. Demonstration of biofilm in human bacterial chronic rhinosinusitis. Am J Rhinol. 2005 Sep-Oct;19 5:452-7.

6. Ramadan HH, Sanclement JA, Thomas JG. Chronic rhinosinusitis and biofilms. Otolaryngol Head Neck Surg. 2005 Mar;132 3:414-7.

7. Sanderson AR, Leid JG, Hunsaker D. Bacterial biofilms on the sinus mucosa of human subjects with chronic rhinosinusitis. Laryngoscope. 2006 Jul;116 7:1121-6.

8. Bendouah Z, Barbeau J, Desrosiers M et al. Biofilm formation by *Staphylococcus aureus* and *Pseudomonas aeruginosa* is associated with an unfavourable evolution after surgery for chronic sinusitis and nasal polyposis. Otolaryngol Head Neck Surg. 2006 Jun; 134 (6):991-6.

9. Ghnnoum M, O'Toole GA, Microbial Biofilms.

10. Desrosiers M, Bendouah Z, Barbeau J. Effectiveness of topical antibiotics on *Staphylococcus aureus* biofilm in vitro. Am J Rhinol. 2007 Mar-Apr; 21 2:149-53.

11. Bendouah Z, Barbeau J, Desrosiers M et al. Use of an *in vitro* assay for determination of biofilm-forming capacity of bacteria in chronic rhinosinusitis. Am J Rhinol. 2006 Sep-Oct;20 5:434-8.

12. Andrews JM. Determination of minimum inhibitory concentrations. J Antimicrob Chemother. 2001 Jul;48 Suppl 1:5-16.

13. Blondeau JM. A review of the comparative in-vitro activities of 12 antimicrobial agents, with a focus on five new respiratory quinolones. J Antimicrob Chemother. 1999 May;43 Suppl B:1-11.

14. Ackermann G, Schaumann R, Pless B et al. Comparative activity of moxifloxacin in vitro against obligately anaerobic bacteria. Eur J Clin Microbiol Infect Dis. 2000 Mar;19 3:228-32.

15. Desrosiers M, Myntti M, James G. Methods for removing bacterial biofilms: in vitro study using clinical chronic rhinosinusitis specimens. Am J Rhinol. 2007 Sep-Oct;21 5:527-32.

16. Lewis K. Riddle of biofilm resistance. Antimicrob Agents Chemother. 2001 Apr;45 4:999-1007.

17. Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. Lancet. 2001 Jul 14;358 9276:135-8.

18. Leid JG, Shirtliff ME, Costerton JW et al. Human leukocytes adhere to, penetrate, and respond to *Staphylococcus aureus* biofilms. Infect Immun. 2002 Nov;70 11:6339-45.

19. Vuong C, Voyich JM, Fischer ER et al. Polysaccharide intercellular adhesin (PIA) protects Staphylococcus epidermidis against major components of the human innate immune system. Cell Microbiol. 2004 Mar;6 3:269-75.

Discussion générale

Nous présentons un travail qui évalue le rôle et les traitements possibles de l'infection par biofilm dans la rhinosinusite chronique (RSC).

Notre projet d'étude sur l'implication des biofilms dans la RSC comprend plusieurs étapes :

1. Méthode d'évaluation de la capacité d'une bactérie à produire un biofilm *in vitro* : technique du crystal violet

Nous avons développé une méthode simple et fiable pour évaluer la capacité de *P. aeruginosa*, des staphylocoques à coagulase négative, et *S. aureus,* des bactéries retrouvées chez des patients atteints de RSC, à former un biofilm *in vitro*.

Nous avons démontré que la méthode employée dans ce projet ; technique du crystal violet, est capable de détecter la production de biofilms chez les patients atteints de RSC.

Nous avons comparé la capacité de production de biofilm à des souches de référence provenant de banques de données et qui sont bien connues dans la littérature pour leur capacité à produire ou non un biofilm.

Dans la collection des 31 souches cliniques étudiées, seulement 7 n'ont pas produit de biofilm *in vitro*. Nous verrons par la suite que cette production ou non de biofilm est liée à l'évolution de la maladie chez les patients atteint de RSC.

Notre méthode à des limitations. Nous avons développé un biofilm dans les conditions les plus idéales à sa croissance. Cependant, ces conditions sont différentes de celles retrouvées chez nos patients atteint de RSC. De plus, notre protocole suggère une production de

biofilm simple, mais dans le sinus d'un patient le biofilm est mixte. À cause du risque de contaminations, l'étude d'un biofilm simple s'est avérée plus faisable.

Il existe d'autres méthodes *in situ* pour la détection de biofilm comme la microscopie électronique et la microscopie confocale à balayage laser[38].

Cependant, ces méthodes ont un coût très élevé et nécessitent des spécimens qui ne sont pas faciles à obtenir cliniquement. Un des désavantages de la microscopie électronique est que le spécimen subit une déshydratation qui peut altérer la structure du biofilm.

Aussi, ces techniques ne permettent pas l'identification des espèces bactériennes présentes dans le biofilm; pour cela il faudrait utiliser la technique d'hybridation FISH sur des spécimens de muqueuse sinusale et les visualiser à l'aide de micoscopie confocale à balayage laser. Le FISH est une technique de biologie moléculaire d'hybridation *in situ* utilisant des sondes marquées à l'aide d'un marqueur fluorescent. Ces sondes peuvent être utilisées sur de l'ADN ou de l'ARN, ou sur des protéines (sonde anticorps). Cette technique n'est disponible que dans certains centres spécialisés, ce qui y limite la facilité d'accès.

L'étape suivante dans notre étude était de vérifier si cette capacité de production de biofilm était associée à l'évolution clinique de la maladie.

2. Évaluation du lien entre la formation du biofilm par *Staphylococcus aureus,* staphylocoques à coagulase négative et *Pseudomonas aeruginosa,* et l'évolution de la maladie

Avec les bactéries *P. aeruginosa* et *S. aureus,* nous avons constaté que la capacité des bactéries à produire un biofilm important se retrouve chez les patients avec une mauvaise évolution de la maladie. Avec les SCN l'inverse est retrouvé : la mauvaise évolution est reliée à une plus faible production de biofilm.

Ces résultats suggèrent que la capacité de production de biofilm par *S. aureus* et *P. aeruginosa* peut avoir une influence sur l'évolution clinique ainsi que sur la persistance de l'infection.

S. aureus et *P. aeruginosa* sont impliquées dans plusieurs pathologies respiratoires dont la RSC[33]. *S. aureus* est un coque à Gram positif qui cause des infections nosocomiales. On pense qu'il agit au niveau de la RSC en produisant des toxines qui ont un effet superantigénique capable de stimuler le système immunitaire [39, 40].

P. aeruginosa est un agent pathogène opportuniste, bacille à Gram négatif, et est relié aux infections nosocomiales et à des infections mortelles chez les malades souffrant de mucoviscidose [41].

L'inflammation et la destruction de la muqueuse sinusale par les enzymes secrétées par *P. aeruginosa* peuvent être accentuées par les mécanismes de défense. On cite comme exemple les produits de dégradation des neutrophiles [42].

Il est intéressant de noter la corrélation inverse que nous avons obtenue dans notre étude entre la production de biofilm par les staphylocoques à coagulase négative et l'évolution

des patients. Cette espèce bactérienne est plutôt considérée comme un contaminant de la flore nasale. C'est l'agent le plus fréquemment isolé des cavités nasales de patients sains et de patients asymptomatiques qui ont subi une CES. Cependant les SCN sont aussi responsables de l'infections des implants médicaux [43].

Il existe plusieurs facteurs pouvant expliquer la RSC mais aucun n'est capable d'expliquer la persistance des bactéries au niveau des sinus de ces patients ainsi que l'échec dans certains cas des thérapies antibiotiques conventionnelles.

La présence de biofilms pourrait être une explication valable. L'inflammation de la muqueuse sinusale de ces patients peut créer un microenvironnement favorable au développement du biofilm. Suite à l'endommagement de l'épithélium sinusal, des surfaces sont créées favorisant ainsi l'adhérence de certaines espèces bactériennes et éventuellement la formation de biofims.

L'épithélium recouvrant les voies respiratoires est normalement protégé de la colonisation bactérienne par une élimination mucociliaire. En effet, les mucines présentes dans le mucus possèdent des récepteurs de carbohydrate capables de reconnaître les bactéries, qui sont par la suite éliminées avec le mucus. Cependant, ce mécanisme de protection peut subir des défaillances. L'épithélium peut subir des dommages, exposant ainsi non seulement les récepteurs cellulaires mais aussi la matrice extracellulaire (MEC) à l'adhésion bactérienne et la formation de biofilms. Les laminines et le collagène de types I et IV, des composantes de la MEC, représentent des sites auxquels *Pseudomonas aeruginosa* peut adhérer. Lors de la réparation tissulaire dans la fibrose kystique, les cellules expriment les récepteurs asialo GM1, qui sont reconnues par *Pseudomonas aeruginosa*. Une meilleure compréhension des

récepteurs reconnus par ces bactéries se montre ainsi une stratégie thérapeutique dans la prévention des infections de la voie respiratoire [44].

La présence de biofilm dans la cavité nasale des patients peut expliquer les résultats de certaines études contradictoires sur l'utilisation de l'antibiothérapie après la CES.

En effet, le groupe de Vaughan a démontré l'efficacité de l'antibiothérapie topique chez les patients avec des exacerbations aiguës [45].

Cependant une autre étude conduite par le groupe du docteur Desrosiers a démontré l'échec du même traitement chez des patients atteints de sinusite chronique[46].

Dans cette dernière situation, la présence de bactéries sous forme de biofilm peut expliquer la chronicité de la maladie ainsi que l'échec de la thérapie; inversement, dans la première étude, nous avons plutôt affaire à des patients avec des exacerbations aiguës (figure 4), donc les bactéries présentes dans la cavité sinusale sont plutôt sous forme planctonique qui en conséquence est une cible plus facile pour l'antibiothérapie.

La présence des biofilms au niveau de la muqueuse sinusale des patients atteints de RSC peut expliquer l'échec de l'antibiothérapie orale. Par sa nature, un biofilm est plus résistant aux antibiotiques comparé aux bactéries planctoniques qui sont plus susceptibles. En clinique, des concentrations d'antibiotiques sous les concentrations minimales inhibitrices (CMI) sont généralement utilisées. Nous voulions évaluer ces mêmes concentrations sur un biofilm mature. Ce qui nous amène à la troisième étape de notre projet.

3. Évaluation de l'effet de la MOXI sur des biofilms matures de *Staphylococcus aureus in vitro* :

Nous avons évalué *in vitro* l'effet de différentes concentrations topiques de MOXI sur un biofilm mature de *S. aureus*.

Des concentrations égales ou inférieures à la CMI n'ont aucun effet sur la viabilité des bactéries dans le biofilm. Cependant, les résultats de notre étude suggèrent que des concentrations supérieures à la CMI réduisent le nombre des bactéries viables dans le biofilm mature. Nos résultats concordent avec d'autres études [47].

Ces résultats suggèrent que l'utilisation de l'antibiothérapie avec des concentrations supérieures à la CMI peut avoir un rôle dans le traitement de la RSC.

L'administration topique de ces concentrations peut offrir une alternative aux traitements par voie orale, particulièrement après une chirurgie endoscopique des sinus. En effet, après la chirurgie, les cavités sinusales communiquent librement avec le passage nasal, et les solutions de lavage peuvent accéder via le nez aux cavités sinusales.

L'avantage de cette thérapie topique est qu'elle permet le dépôt de concentrations élevées d'antibiotiques directement dans la cavité sinusale où le biofilm serait présent.

Plusieurs études ont été menées afin d'évaluer l'efficacité des thérapies topiques chez des patients qui ont subi une chirurgie endoscopique des sinus.

Dans une étude prospective menée chez des patients atteints de RSC et qui ne réagissaient ni aux traitements antibiotiques ni aux chirurgies, il n'y a eu aucune différence entre le groupe traité topiquement avec la tobramycine et le groupe placebo[46]. Ces résultats peuvent être dûs à l'utilisation de la tobramycine, un antibiotique qui a un effet limité contre les

bactéries à gram positif comme le *S. aureus*, ainsi qu'au manque de précision au niveau des doses administrées topiquement aux cavités sinusales.

Dans notre étude, il est important de noter que même des concentrations de 1000X la CMI étaient incapables de tuer complètement les bactéries dans le biofilm. Une des explications plausibles serait la présence de persisteurs qui représenteraient une petite fraction (1%) de la population du biofilm et qui serait capable par un mécanisme inconnu de survivre à des conditions extrêmes.

De récentes études confirment cette hypothèse. En effet, le groupe de Singh a vérifié l'effet de plusieurs antibiotiques (oxacilline, cefotaxime, amikacine, ciprofloxacine et vancomycine) sur une souche de *Staphylococcus aureus* sous une forme planctonique et sous forme de biofilm. Après 48 heures de traitement antibiotique, des bactéries persisteurs sont toujours viables. Ce même traitement a tué toutes les bactéries planctoniques de la même espèce [48].

Les résultats de notre étude suggèrent que l'utilisation de concentrations supra CMI sous forme topique peut ouvrir des voies de recherche sur de nouveaux traitements des infections par biofilm, traitements qui peuvent être bénéfiques chez les patients atteints de forme sévère de RSC, surtout après une chirurgie endoscopique des sinus. Cependant, en clinique il y a des risques de toxicité au niveau de l'organisme. Afin de contrer cet inconvénient, des agents anti-bactériens peuvent être administrés de manière topique, surtout après la chirurgie. Ce qui nous mène à la quatrième partie de notre projet.

4. Évaluation de l'effet de concentrations de MOXI supérieurs à 1000X la CMI sur des biofilms matures de *Staphylococcus aureus in vitro*

Suite aux résultats de notre dernière expérience nous avons voulu augmenter la concentration de la MOXI pour voir si cela réduit davantage la viabilité des bactéries au niveau du biofilm mature de *S. aureus* isolé de patients atteints de RSC.

L'effet maximal de la MOXI a été noté à une concentration de 1000X CMI (0.1 mg/ml – 0.2 mg/ml). L'exposition du biofilm à cette concentration réduit le nombre de bactéries viables de 2.0 à 2.5 de log. Des concentrations de MOXI à 5000X et 10 000X la CMI ne réduisent pas davantage le nombre des bactéries viables dans le biofilm comparé à l'effet noté avec le traitement avec 1000X CMI.

Nous avons confirmé ces résultats grâce à la coloration au LIVE/DEAD BacLight. Nous avons remarqué la persistance de bactéries viables dans le biofilm, même après le traitement avec la MOXI à 1 000 X CMI [49]. Le traitement avec 10 000 X CMI n'était pas capable de tuer toutes les bactéries viables dans le biofilm; en effet 1% des bactéries ont persisté.

Dans une autre séries d'expériences, il a été démontré que l'utilisation d'une solution d'acide citrique (citric acid/zwitterionic surfactant solution) réduit de manière considérable le nombre de bactéries viables dans le biofilm. L'évaluation de l'architecture du biofilm grâce à MCBL a révélé que la matrice du biofilm a été réduite [50].

Ces résultats très importants suggèrent qu'un traitement optimal contre le biofilm serait de cibler les bactéries ainsi que la matrice du biofilm.

Les persisteurs constituent une petite fraction de la population du biofilm et leur survie suite à un stress subi confère a cette communauté une forme de survie [51, 52]. Les résultats de notre étude suggèrent que des concentrations très élevées d'antibiotiques utilisées sont capables de tuer la majorité des bactéries dans le biofilm, mais laissent néanmoins les persisteurs en vie. Ce phénomène peut expliquer la persistance du biofilm dans les sinus de patients atteints de RSC même après le traitement antibiotique.

Les persisteurs contenus dans le biofilm sont capables de survivre aux traitements antibiotiques ainsi qu'aux défenses immunitaires. *In vivo*, quand la concentration des antibiotiques diminue au niveau du sinus, les persisteurs peuvent repeupler le biofilm; ils peuvent ensuite se détacher sous une forme planctonique produisant ainsi des exacerbations aiguës chez les patients atteints de RSC.

Le groupe de De Groote a effectué une vaste étude sur la souche de *P. aeruginosa* PA14 afin d'identifier des gènes impliqués dans le phénomène de persistance. Neuf mutants au total ont été identifiés, dont quatre ont démontré un phénotype de faible persistance et cinq de forte persistance. Ces gènes peuvent servir comme cible pour les traitements des infections par *P. aeruginosa* [53].

Cibler les persisteurs peut offrir une nouvelle option thérapeutique très intéressante dans le traitement des infections par biofilm.

Conclusion

Nous avons pu utiliser une méthode d'évaluation de la production de biofilm par des souches cliniques, qui est reproductible et simple à exécuter *in vitro*.

Notre première étude suggère que la capacité importante de formation de biofilm par les bactéries *P. aeruginosa* et *S. aureus* se retrouve chez les patients avec une mauvaise évolution de la maladie qui sont atteints de rhinosinusite chronique et qui ont déjà subit une chirurgie endoscopique des sinus. La formation de biofilm par ces deux bactéries peut jouer un rôle très important dans la chronicité de la RSC.

Nos travaux ajoutent une nouvelle dimension à la compréhension de la pathogenèse de la RSC et suggèrent que les biofilms représentent une cible importante dans le traitement de cette malade. L'antibiothérapie est fréquemment utilisée dans le traitement de la RSC. Nous avons pu démonter que des concentrations supra CMI de moxifloxacine sont plus efficaces à réduire la viabilité au niveau d'un biofilm mature des souches cliniques de *S. aureus*. La réduction maximale qu'on a pu obtenir était autour de 1000X la CMI laissant une fraction de bactéries persisteurs au niveau du biofilm traité. Ces résultats *in vitro* nous démontrent un rôle potentiel de l'antibiothérapie topique dans le traitement de la RSC en utilisant des concentrations d'antibiotiques qui peuvent être atteignables dans des solutions de lavage topiques.

La persistance des bactéries dans les sinus même après le traitement antibiotique pourrait expliquer l'échec de l'antibiothérapie ainsi que la CES chez certains de nos patients qui sont atteints de RSC.

Les thérapies conventionnelles utilisées dans le traitement de la RSC ne sont pas toujours efficaces pour combattre le biofilm. Cela suggère qu'une thérapie optimale serait de combiner des traitements qui visent à tuer les bactéries inclues dans le biofilm avec d'autres qui altéreraient la structure du biofilm.

Une meilleure compréhension du mécanisme avec lequel les biofilms colonisent la muqueuse sinusale nous aidera à mieux traiter cette maladie. Plusieurs recherches sont amorcées pour essayer de trouver des molécules qui peuvent interférer avec la communication entre les bactéries au sein du biofilm. Une inhibition directe des voies de signalisation du quorum sensing par des antagonistes s'est avérée une solution prometteuse dans la prévention des infections par biofilm [54, 55].

Toutes les substances qui pourraient soit empêcher l'adhésion du biofilm, soit inhiber le quorum sensing ou altérer la matrice du biofilm, ou encore cibler des gènes spécifiques au niveau des persisteurs, peuvent être testées sur des modèles animaux ou encore humains. Ces traitements pourront constituer une voie d'avenir dans le traitement de la rhinosinusite chronique.

Références

1- Costerton W, Veeh R, Shirliff et al. The application of biofilm sciences to the study and control of chronic bacterial infections. J. Clin. Invest. 2003; 112:1466-1477.

2- Costerton JW, Geesey GG, Cheng KJ. How bacteria Stick. Sci Am. 1978;238:86-95.

3- Westall F, Wit MJ, Dann J et al. Early archean fossil bacteria and biofilms in hydrothermally-influenced sediments from the Barberton green-stone belt, South africa. Precambrian Res. 2001; 106 :93-116.

4- Rasmussen B. Filamentous microfossils in a 3,235- million-year-old volcanogenic massive sulphide deposit. Nature. 2000; 405:676-679.

5- Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms : from the natural environment to infectious diseases. Nat Rev Microbiol. 2004; 2:95-108.

6- Costerton JW, Lewandowski Z, Caldwell DE et al. Microbial biofilms. Annu. Rev. Microbiol. 1995; 49:711-745.

7- Sutherland IW. The biofilm matrix, an immobilized but dynamic microbial environment. Trends Microbial. 2001; 9:222-227.

8- Potera C. Studying slime. Environ Health Perspect. 1998; 106(12):A604-6.

9- Stoodley P, Dodds I, Boyle JD et al. Influence of hydrodynamics and nutrients on biofilm structure. J. Appl. Microbiol. 1999; 85:19S-28S.

10- Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms : a common cause of persistance infections. Science. 1999; 284:1318-1322.

11- Amorena B, Gracia E, Monzon M et al. Antibiotic susceptibility assay for *Staphylococcus aureus* in biofilms developed in vitro. J Antimicrob Chemother. 1999; 44(1):43-55.

12- Monzón M, Oteiza C, Leiva J. Biofilm testing of *Staphylococcus epidermidis* clinical isolates: low performance of vancomycin in relation to other antibiotics. Diagn Microbiol Infect Dis. 2002; 44:319-324.

13- Stewart PS. A review of experimental measurements of effective diffusive permeabilities and effective diffusion coefficients in biofilm. Biotechnol Bioengineer. 1998; 59:261-272.

14- Stewart PS, Roe F, Rayner J al. Effect of catalase on hydrogen peroxide penetration into *Pseudomonas aeruginosa* biofilm. Appl Environ Microbiol. 2000; 66:836-838.

15- Jensen ET, KharazmiA, Lam K et al. Human polymorphonuclear leukocyte response to *Pseudomonas aeruginos* grown in biofilms. Infect Immun. 1990; 58:2383-2385.

16- Götz F. *Staphylococcus* and biofilms. Mol Microbiol. 2002 Mar; 43(6):1367-78.

17- Fey PD, Ulphani JS, Götz F et al. Characterization of the relationship between polysaccharide intercellular adhesin and hemagglutination in *Staphylococcus epidermidis*. J Infect Dis. 1999 Jun; 179(6):1561-4.

18- Mack D, Riedewald J, Rohde H et al. Essential functional role of the polysaccharide intercellular adhesin of *Staphylococcus epidermidis* in hemagglutination. Infect Immun. 1999 Feb; 67(2):1004-8.

19- Garett ES, Perlegas D, Wozniak DJ. Negative control of flagellum synthesis in *Pseudomonas aeruginosa* is modulated by the alternative sigma factor AlgT (AlgU). J Bacteriol. 1999; 181:7401-4.

20- Sauer K, Camper AK, Ehrlich GD et al. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. J Bacteriol. 2002; 184:1140-1154.

21- Elder MJ, Stapleton F, Evans E et al. Biofilm-related infections in ophthalmology. Eye. 1995;9:102-109.

22- Walters MC, Roe F, Bugnicourt A. Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. Antimicrob Agents Chemother. 2003; 47:317-323.

23- Potera C. Forging a link between biofilm and disease. Science. 1999; 283:1837-9.

24- Post JC, Stoodley P, Hall-Stoodley L et al. The role of biofilms in otolaryngologic infections. Current Opinion in Otolaryngology & Head and Neck Surgery. 2004 ; 12:185-190.

25- Desrosiers MY. Refractory chronique rhinosinusitis : pathophysiology and management of chronic rhinosinusitis persisting after endoscopic sinus surgery. Current Allergy and Asthma Reports. 2004 May; 4(3):200-7.

26- Fokkens W, Lund VJ, Bachert C et al. European position paper on rhinosinusitis and nasal polyposis. Rhinology. 2005; 43(Suppl 18): S1–88.

27- Meltzer EO, Hamilos DL, Hadley JA et al. Rhinosinusitis: establishing definitions for clinical research and patient care. J Allergy Clin Immunol. 2004; 114(6 Suppl):155-212.

28- Vivarrat-Perrin L, Veillon F. Radioanatomie du crane. Hopitale de Hautepierre. Strasbourg.
http://www.med.univ-rennes1.fr/cerf/edicerf/RADIOANATOMIE/002.html

29- Benninger MS, Ferguson BJ, Hadley JA et al. Adult chronic rhinosinusitis : definitions, diagnosis, epidemiology, and pathophysiology. Otolaryngol Head Neck Surg. 2003;129 (suppl) S1-32.

30- Benninger ME, Anon J, Mabry RL. The medical management of rhinosinusitis. Otolaryngol Head Neck Surg. 1997; 117:S41-9.

31- Bhattacharyya N, Kepnes LJ. The microbiology of recurrent rhinosinusitis after endoscopic sinus surgery. Arch Otolaryngol Head Neck Sur. 1999 Oct; 125(10):1117-20.

32- Al-Shemari H, Abou-Hamad W, Libman M et al. Bacteriology of Sinus Cavities of Asymptomatic Individuals after Endoscopic Sinus Surgery. J Otolaryngol. 2007; 36(1):43-8.

33- Cryer J, Schipor I, Perloff JR et al. Evidence of bacterial biofilms in human chronic sinusitis. ORL J Otorhinolaryngol Relat. Spec 2004; 66:155-158.

34- Ferguson BJ, Stolz DB. Demonstration of biofilm in human bacterial chronic rhinosinusitis. Am J Rhinol. 2005; 19(5):452-457.

35- Ramadan HH, Sanclement JA, Thomas JG. Chronic rhinosinusitis and biofilms. Otolaryngology - Head and Neck Surgery. 2005 ; Vol. 132, Issue 3, Pages 414-417.

36- Sanderson AR, Leid JG, Hunsaker D. Bacterial biofilms on the sinus mucosa of human subjects with chronic rhinosinusitis. Laryngoscope. 2006; 116(7):1121-6.

37- Perloff JR, Palmer JN. Evidence of bacterial biofilms in a rabbit model of sinusitis. Am J Rhinol. 2005; 19:1-6.

38- Arciola CR, Campoccia D, Montanaro L. Detection of biofilm-forming strains of *Staphylococcus epidermidis* and *Staphylococcus aureus*. Expert Rev Mol Diagn. 2002; 2(5):478–84.

39- Bachert C, Gevaert P, van Cauwenberge P. *Staphylococcus aureus* superantigens and airway disease. Curr Allergy Asthma Rep. 2002; 2(3):252– 8

40- Tripathi A, Conley DB, Grammer LC et al. Immunoglobulin E to staphylococcal and streptococcal toxins in patients with chronic sinusitis/ nasal polyposis. Laryngoscope. 2004;114(10):1822– 6.

41- Filloux A, Vallet I. Biofilm : Mise en place et organisation d'une communauté bactérienne. Médecine sciences. 2003; Vol 19 no 1.

42- Ratjen F, Doring G. Cystic fibrosis. Lancet. 2003 ; 22 ; 361 (9358) : 681–9.

43- Mack D, Haeder M, Siemssen N et al. Association of biofilm production of coagulase-negative staphylococci with expression of a specific polysaccharide intercellular adhesin. J Infect Dis. 1996 ; 174(4):881-4.

44- De Bentzenmann S, Plotkowski C, Puchelle E. Receptors in the *Pseudomonas aeruginosa* adherence to injured and repairing airway epithelium. Am J Respir Crit Care Med. 1996; 154:S155–62.

45- Vaughan WC, Carvalho G. Use of nebulized antibiotics for acute infections in chronic sinusitis. Otolaryngol Head Neck Surg. 2002; 127(6):558–68.

46- Desrosiers MY, Salas-Prato M. Treatment of chronic rhinosinusitis refractory to other treatments with topical antibiotic therapy delivered by means of a large-particle nebulizer: results of a controlled trial. Otolaryngol Head Neck Surg. 2001;125(3):265–269.

47- Perez-Giraldo C, Gonzalez-Velasco C, Sanchez-Silos RM et al. Moxifloxacin and biofilm production by coagulase-negative staphylococci. Chemotherapy. 2004; 50(2):101-104.

48- Singh R, Ray P, Das A et al. Role of persisters and small-colony variants in antibiotic resistance of planktonic and biofilm-associated *Staphylococcus aureus*: an in vitro study. J Med Microbiol. 2009 Aug; 58(Pt 8):1067-73.

49- Bendouah Z, Desrosiers M, Barbeau J. Bacterial killing in *Staphylococcus aureus* biofilm doesn't improve with increasing concentration of moxifloxacin beyond 1000XCMI. (soumis)

50- Desrosiers M, Myntti M, James G. Methods for removing bacterial biofilms: in vitro study using clinical chronic rhinosinusitis specimens. Am J Rhinol 2007 ; (5):527-32.

51- Lewis K. Riddle of biofilm resistance. Antimicrob Agents Chemother. 2001 ; 45(4):999-1007.

52- Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. Lancet. 2001 ; 14;358(9276):135-138.

53- De Groote VN, Verstraeten N, Fauvart M et al. Novel persistence genes in *Pseudomonas aeruginosa* identified by high-throughput screening. FEMS Microbiol Lett. 2009 May 21.

54- Hentzer M, Eberl L, Nielsen J et al. Quorum sensing : a novel target for the treatment of biofilm infections. BioDrugs. 2003;17(4):241-50.

55- Stephenson K, Hoch JA. Developing inhibitors to selectively target two-component and phosphorelay signal transduction systems of pathogenic microorganisms. Curr Med Chem. 2004 Mar;11(6):765-73.