

**Investigating Bacterial Biofilms in Chronic
Rhinosinusitis: An *In vitro* Study,
In vivo Animal Study and a
Examination of Biofilms in Human CRS**

A thesis submitted on 1st December 2008 for
The degree of Master of Surgery

Kien Rach Ha, M.B.B.S

Department of Surgery, Faculty of Health Sciences, The Queen Elizabeth Hospital
The University of Adelaide, South Australia



TABLE OF CONTENTS

Abstract	4
Declaration	6
Acknowledgements	7
Preface	9
List of Figures and Tables	10
Chapter 1	12
Aims	13
Chapter 2	14
Introduction	15
Chronic Rhinosinusitis.....	15
Definition and disease burden.....	15
Pathogenesis.....	15
Bacteria and Chronic Rhinosinusitis.....	16
<i>Staphylococcus aureus</i> and Chronic Rhinosinusitis.....	16
Bacterial Biofilms.....	17
Definition.....	17
Why Biofilms Form.....	18
Biofilm Characteristics.....	19
Extracellular Polymeric Substances (EPS) Matrix.....	19
Quorum Sensing.....	20
Antibiotic Resistance.....	20
Biofilm Dispersal.....	21
Microscopic Mucosal Biofilm Detection Techniques.....	21
Scanning Electron Microscopy.....	22
Transmission Electron Microscopy.....	22
Confocal Scanning Laser Microscopy.....	23
Fluorescence In Situ Hybridization.....	23
Biofilms and Chronic Disease.....	23
Biofilms and Chronic Rhinosinusitis.....	25
<i>In vitro</i> Biofilm Models.....	26
Growing and Analyzing Static Biofilms.....	26
<i>In vitro</i> CRS Biofilms Assays.....	28
Animal Sinusitis Models.....	29
Murinae Model.....	30
Canine Model.....	30
Porcine Model.....	30
Rabbit Model.....	31
Sheep Model.....	31

An Animal Biofilm Chronic Rhinosinusitis Model.....	32
Potential Biofilm Treatments in CRS.....	33
Arresting Biofilm Formation.....	33
Removal of Established Biofilms.....	34
Chapter 3.....	35
<i>In vitro</i> Activity of Mupirocin on Clinical Isolates of <i>Staphylococcus aureus</i> and its Potential Implications in Chronic Rhinosinusitis	
Chapter 4.....	54
A Sheep model for the study of Biofilms in Rhinosinusitis	
Chapter 5.....	79
Confocal Scanning Laser Microscopy Evidence of Biofilms in Patients With Chronic Rhinosinusitis	
Chapter 6.....	99
Summary.....	100
Conclusions.....	106
References.....	107

ABSTRACT

Introduction

Bacterial biofilms have been implicated in the pathogenesis of Chronic Rhinosinusitis (CRS). This thesis consists of a number of separate studies. The results of each study were designed to help provide an evolution of knowledge that could be applied to our subsequent investigations on the topic of bacterial biofilms and chronic rhinosinusitis.

In vitro studies were utilized to document the capacity of CRS bacteria to form biofilms as well as to investigate the efficacy of various antimicrobials at high concentrations.

Additionally, an in vivo sheep model was developed to examine different biofilm detection techniques. Finally, a study of CRS patients was conducted to investigate the incidence of biofilm related sinus disease.

Methods

Our *in vitro* studies used 96 well crystal violet microtiter plate assays to determine the biofilm growth characteristics of *S.aureus* isolated from patients with CRS. Established biofilms were then subjected various antimicrobial agents, and the degree of biofilm reduction calculated to examine their potential for sinus biofilm treatment. A sheep sinusitis model involved performing endoscopic sinus surgery, occlusion of frontal sinus ostia and the introduction of bacteria. Mucosal specimens were subsequently examined for the presence of bacterial biofilms using transmission electron microscopy (TEM), scanning electron microscopy (SEM) and confocal scanning laser microscopy (CSLM).

CSLM was also used in a prospective study to document the presence bacterial biofilms on the mucosa of patients with CRS compared to controls.

Results

The findings of in vitro experiments revealed that not all isolates were capable of forming biofilms. Of the antibiotics tested, only Mupirocin was capable of reducing biofilm mass by 90% in all isolates. The animal model showed considerable variation in biofilm detection rates. The CSLM biofilm detection rate was 100% in obstructed sinuses with bacteria introduced, whereas TEM detected only 66%. Both these objective measures failed to identify biofilms in control groups. SEM found biofilms in all experimental groups including controls. CSLM analysis of CRS patients found Bacterial biofilms in 44% and no biofilms in controls.

Conclusion

The demonstration of biofilms in the sheep model for sinusitis and biofilms on the mucosal specimens of patients with CRS, and the ability of bacteria in CRS to form biofilms in vitro, further supports the hypothesis that biofilms play a role in the pathogenesis of CRS. CSLM is the modality of choice in documenting the presence of bacterial biofilms on sinus mucosal surfaces due to the inherent flaws of sampling error and subjectivity of TEM and SEM. Finally, CRS is a multi-factorial disease, topical Mupirocin via nasal irrigation may be a therapeutic option in patients with likely S.aureus biofilms.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Kien Rach Ha

1st December, 2008

ACKNOWLEDGEMENTS

The work described in this thesis was performed at the Department of Surgery; Otolaryngology, Head and Neck Surgery, at the University of Adelaide and The Queen Elizabeth Hospital.

I wish to sincerely thank the following people for their assistance and involvement in this study.

Professor Peter-John Wormald,
my supervisor, for all his guidance and wisdom, encouragement and support.

Dr Alkisviadis Psaltis,
my co-researcher and best mate, for his friendship, enthusiasm, support, and
companionship through this journey.

Dr Lorwai Tan,
my co-supervisor, for her support, education, and ability to keep me focused in the right
direction.

Dr Andrew Butcher,
from the Department of Microbiology at The Queen Elizabeth Hospital for his assistance
and guidance on the microbiological aspects of the studies contained in this thesis.

The staff, at Adelaide Microscopy,
The University of Adelaide, for their education of the fine arts of microscopy.

My Fiancé Ruby,
the love of my life, for all her support and patience during my seemingly never ending
studies.

PREFACE

A portion of the work described within this thesis has been published, as listed below.

Ha, K.R., Psaltis, A.J., Butcher, A.R., Tan, L & Wormald, P.J. *In vitro* activity of mupirocin on clinical isolates of *Staphylococcus aureus* and its potential implications in Chronic rhinosinusitis. *Laryngoscope* **118**: 535-540 (2007).

Ha, K.R.#, Psaltis, A.J#., Butcher, A.R., Tan, L & Wormald, P.J. A Sheep Model for the Study of Biofilms in Rhinosinusitis. *Am J Rhinol* **21**: 339-345 (2007).

Ha, K.R.#, Psaltis, A.J#., Beule, A.G., Tan, L.W. & Wormald, P.J. Confocal scanning laser microscopy evidence of biofilms in patients with chronic rhinosinusitis. *Laryngoscope* **117**: 1302-1306 (2007).

Co-First Authors

LIST OF FIGURES AND TABLES

Chapter 3

Figure 1	In vitro biofilm formation of S.aureus ATCC 25923 and clinical isolates after 8 days.....	47
Figure 2	CSLM image of S.aureus isolate 1019 day 8 biofilm.....	48
Table 1	Minimum inhibitory concentrations for planktonic and biofilm clinical isolates.....	49
Table 2	Susceptibility of S.aureus isolates to antibiotics after 1 hour incubation	50
Table 3	Susceptibility of S.aureus isolates to antibiotics after 24 hours incubation.....	50

Chapter 4

Figure 3	Diagram illustrating endoscopic landmarks of sheep's left nasal cavity after resection of the middle and anterior ethmoid complex.....	63
Table 4	Summary of macroscopic and microscopic findings.....	66
Figure 4	CSLM image of sinus tissue from sheep control sinus (group1).....	68
Figure 5	CSLM image of sinus tissue from biofilm infected sinus (group 4)..	69
Figure 6	SEM photomicrograph of sinus tissue from control specimen.....	71
Figure 7	SEM photomicrograph of sinus tissue from biofilm specimen.....	71
Figure 8	SEM photomicrograph of sinus tissue from biofilm specimen.....	72
Figure 9	SEM high-powered photomicrograph of sinus tissue from biofilm specimen.....	73
Figure 10	TEM photomicrograph of sinus tissue from control specimen.....	74
Figure 11	TEM photomicrograph of sinus tissue from biofilm specimen.....	74

Chapter 5

Table 5	Comparison of demographics of CRS patients and control groups..	90
Table 6	Organisms isolated from CRS patients.....	91
Figure 12	CSLM image of sinus tissue from control specimen.....	92
Figure 13	CSLM image of CRS patient demonstrating a bacterial biofilm.....	93
Table 7	Comparison of demographic of CRS patients with biofilms and CRS patients without biofilms.....	96