

Study of Biofilm forming capacity of pathogens involved in Chronic Rhinosinusitis



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Master of Philosophy

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Dedicated to my loving husband Antony

“To climb steep hills requires slow pace at first.”

William Shakespeare

Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person, nor material which to a substantial extent has been accepted for the award of any other degree or diploma of a university or other institution of higher learning, except where due acknowledgement has been made in the text.

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ABSTRACT

The purpose of this study is to evaluate biofilm formation by the bacteria involved in chronic rhinosinusitis. Mixed cultures of the pathogens in chronic rhinosinusitis including coagulase negative *Staphylococcus epidermidis*, *Hemophilus influenzae*, *Pseudomonas aeruginosa*, *Moraxella catarrhalis* and *Streptococcus pneumoniae* were obtained. The microbial attachment and biofilm formation was measured by a crystal violet based microtitre assay. In order to grow biofilms under flowing conditions, a CDC laboratory biofilm reactor system was used. Pure, then mixed species biofilms were examined as a preliminary study for a larger project in which innovative treatments will be tested *in vitro*. *P. aeruginosa* has the maximum biofilm forming capacity. This pathogen showed a steady growth rate in pure species as well as in mixed species biofilm formation. The pure culture of *S. epidermidis* was able to grow biofilms, but it decreased in numbers from the initial level when it was combined with *P. aeruginosa*. *M. catarrhalis*, also has the capacity to form biofilms, but showed an increased biofilm cell density when it was grown in mixed culture. *S. pneumoniae* and *H. influenzae* were not good biofilm formers in pure culture, but these pathogens showed some attachment when they were combined in mixed culture. The crystal violet based microtitre assay and the CDC laboratory biofilm reactor system, are suitable for *in vitro* study, but represents a very artificial condition. The next stage is to study mixed films in a flowing system and ultimately on sinus tissues.

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LIST OF ABBREVIATIONS

ALI	-	Air Liquid Interface
AIDS	-	Acquired Immune Deficiency Syndrome
BHI	-	Brain Heart Infusion
CBD	-	Calgary Biofilm Device
CDC	-	Centre for Disease Control
CFU	-	Colony forming units
CRS	-	Chronic rhinosinusitis
CSLM	-	Confocal Scanning Laser Microscopy
CV	-	Crystal Violet
DNA	-	Deoxy-ribo Nucleic Acid
ENT	-	Ear Nose Throat
EPS	-	Extra cellular polymeric substances
ESS	-	Endoscopic sinus surgery
FESS	-	Functional Endoscopic Sinus Surgery
OD	-	Optical Density
QS	-	Quorum sensing

RFC	-	Radio frequency electric current
RIP	-	Ribonucleic acid-III-inhibiting peptide
SEM	-	Scanning Electron Microscope

CHAPTER 1

INTRODUCTION

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INTRODUCTION

Chronic rhinosinusitis (CRS) is now recognized as a common and more often a debilitating form of sinusitis (Aukema & Fokkens, 2004). This disease affects more than 14% of the world's population (Kalinier, 1998). It is characterized by sinonasal mucosal inflammation which lasts for at least 12 weeks (Lane & Lee, 2011). CRS presents chronic symptoms such as rhinorrhea (runny nose), hyposmia (reduced ability to detect and to smell odours), facial pain and nasal obstruction, which have a considerable impact on quality of life, and health care expenditure (Tomassen et al., 2011). Currently, chronic rhinosinusitis pathogenesis is widely described as multifactorial. Microbial entities including fungi, bacterial enterotoxins and biofilms have been concerned as inflammatory stimuli, along with airborne irritants and allergens (Lane & Lee, 2011).

A biofilm is a structured association of bacteria embedded in a self-produced polymer matrix consisting of polysaccharide, protein and extracellular DNA (Hoiby et al., 2011). Studies indicate that the majority of bacteria in their ecological niche are growing as biofilms (Costerton & Donlan, 2002). Biofilms are very difficult to remove, and thus they are a matter of great concern in medicine. Gradients of nutrients and oxygen exist from the top to the bottom of biofilms and the bacterial cells situated in nutrient deprived areas have decreased metabolic activity and increased doubling times. These more or less dormant cells are therefore responsible for some of the tolerance to antibiotics (Hoiby et al., 2011). More important to this thesis is the increasing evidence that at least some diseases of the respiratory tract are biofilm-mediated (Singh et al., 2000).

This study comprises the analysis of the biofilm forming capacity of the main pathogens involved in CRS, which includes *Pseudomonas aeruginosa*, *Moraxella catarrhalis*, Coagulase negative *Staphylococcus epidermidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae*. Mixed species biofilm formation was also examined. Growth rates of the individual members of the biofilm community were estimated. Methods were developed to follow the growth of individual members of the community in a developing biofilm. The first few chapters describe the use of a crystal-violet (CV) assay to examine biofilm formation. Later chapters detail use of the CDC Biofilm reactor system to study biofilm formation.

CHAPTER 2

REVIEW OF LITERATURE

CHAPTER 2

REVIEW OF LITERATURE

2.1 SINUSITIS

The chambers in the bones of the skull and face that are lined normally with a thin membrane (called mucosa) which produces mucus, can be termed “sinuses” (Brook, 2007). The sphenoid, frontal, ethmoid and maxillary sinuses are the four paired paranasal sinuses (Rachelefsky & Shapiro, 1992). The *sphenoid sinuses* are located behind the ethmoids in the upper region of the nose and behind the eyes, the *frontal sinuses* over the eyes in the brow area, the *ethmoid sinuses* just behind the bridge of the nose and between the eyes and *maxillary sinuses* inside each cheek bone (Melen, 1994). The nasal cavity communication with these paranasal sinuses is via narrow openings (Gliklich & Metson, 1995). Through these openings the air and mucus can enter and exit the sinuses (Pankey et al., 2000). The blockage of these small openings from swelling, which is caused by allergy, infections and other causes, results in sinusitis (Derebury, 1993). Air trapped inside a blocked sinus together with pus or other discharges can cause pressure on the sinus wall, where the end result is the severe pain of a sinus attack (Kennedy & Lanza, 1997). In the same way, when air is prevented from entering a paranasal sinus by a swollen membrane at the opening, a vacuum can be formed that also causes pain (Reuler et al., 1995).

2.1.1. DEFINITIONS

The literal meaning of the word *sinusitis* is “inflammation of sinus cavities” (Rachelefsky & Shapiro, 1992). The inflammation of the nasal mucosal linings can be referred to as *rhinitis* (Baraniuk, 1997). Because the two go together, today the

Ear Nose and Throat (ENT) specialists commonly use the term *rhinosinusitis* (Kennedy & Lanza, 1997). On the other hand the terms *rhinitis*, *sinusitis* and *rhinosinusitis* are frequently used interchangeably (Melen, 1994). The Rhinosinusitis Task Force of the American Rhinologic Society has defined rhinosinusitis as a state manifested by an inflammatory reaction concerning the mucous membranes of the nasal cavity, paranasal sinuses and fluids within the cavity or underlying bone (Brook, 2003).

2.1.2 CLASSIFICATION

On the basis of duration of the disease, sinusitis can be classified into four different types which include acute sinusitis, sub acute sinusitis, chronic sinusitis and recurrent sinusitis (Jackson, 2005).

Acute Sinusitis

A sudden onset of cold like symptoms such as runny, stuffy nose, and facial pain that does not go away after 7 to 10 days is the common condition of acute sinusitis. Acute sinusitis typically lasts four weeks or less (Torpy, 2009)

Sub acute Sinusitis

The diseased condition in which the inflammation lasts for 4 to 8 weeks is termed as sub acute sinusitis (Byers et al., 1989).

Chronic Sinusitis

The condition which is characterized by the sinus inflammation symptoms lasting for 8 weeks or longer can be defined as chronic sinusitis (Becker, 2003).

Recurrent Sinusitis

When several sinus attacks occur within a year, it can be termed recurrent sinusitis (Hagaman & Wise, 2007)

2.2 CHRONIC RHINOSINUSITIS (CRS)

A group of disorders which is characterized by the inflammation of paranasal sinuses and the mucosal lining of the nasal cavity which lasts for at least 12 weeks is termed chronic rhinosinusitis (Jackson, 2005). Dental infections, cystic fibrosis, cigarette smoke, abuse of nasal sprays etc. can worsen chronic rhinosinusitis (Anand, 2004). Malfunction of the micro hairs, thick secretions and blockage of natural sinus openings are the major problems identified which ultimately lead to the symptoms of chronic rhinosinusitis, such as post nasal drip, nasal obstruction and facial pressure (Reuler, 1995). The presentation of this disease will be unique in each patient (Eric, 2003); depending on the age of the patient and the severity of the problem, there is a regular variation in the presentation of the condition (Collins, 1997). It is a very common disease that affects up to 17% of the world population (Anand, 2004). Functional endoscopic sinus surgery (FESS) has now become the most widely used treatment for chronic rhinosinusitis (Khalil, 2006).

2.2.1 AETIOLOGICAL/PATHOGENIC FACTORS

The exact aetiology and pathogenic factors of CRS still remain unclear (Baraniuk & Maibach, 2005). A single unifying cause for this condition has not been identified yet (Porter et al., 2011). So CRS is now considered as a multifactorial disease (Lane & Lee, 2011). These factors can be commonly categorised into intrinsic or host related factors and extrinsic or non host related factors. Intrinsic

factors are thought to include anatomic or structural abnormalities, genetic abnormalities such as cystic fibrosis and disorders of innate and cell mediated immune system (Asero & Bottazzi, 2001). Extrinsic factors include environmental factors such as air pollution, allergens as well as microbial infections (Wolf, 2002). Both intrinsic and extrinsic factors, trigger infection that causes damage to the cells of the sinus lining, which ultimately leads to inflammation (Gelfand, 2005). The sinus lining thickens with fluid that obstructs the nasal passages. This passage connects to the sinuses and the obstruction disrupts the process that removes the bacteria normally present in the nasal passages (Margolis et al., 2010). The bacteria begin to multiply and invade the lining of the sinus, which causes the infection symptoms (Baroody, 2007).

2.2.2 EPIDEMIOLOGY

CRS remains the single most common self-reported chronic health condition affecting adults in the western world (Anand, 2004). It is a highly frequent condition affecting 16% of the US population. Its occurrence resembles that of hypertension. The financial burden of CRS is far reaching, with direct annual US health care costs in excess of \$ 5.8 million US (Ray et al., 1996). Aside from the enormous financial implications of CRS, a number of quality life and disability index studies have repeatedly confirmed the significant negative psycho-social impact that this condition has on the sufferer. The disability and the distress caused by the CRS has been shown to be similar to that of other chronic diseases such as asthma and lower back pain (Gliklich & Metson, 1995).

2.2.3 RISK FACTORS

Some hospitalized patients are at higher risk of sinusitis, mostly those with head injuries, conditions requiring insertion of tubes through the nose, usage of mechanical ventilators for breathing (these patients are at higher risk for maxillary sinusitis) and also for those patients with weak immune system (Chow et al., 2010). Some other medical conditions also put people at a possible risk for sinusitis. These conditions include, nasal polyps, septal deviation, oral steroid treatment, diabetes, AIDS and other immune system disorders, diabetes, hypothyroidism, and cystic fibrosis (Honigberg & Rubin, 1990).

Dental problems are the other main risk factor. Anaerobic bacteria are associated with infections from dental problems and procedures (Brook, 2007). This is estimated to be the reason for 10% of the cases of maxillary sinusitis (Brook, 2006). There is an increased chance of developing sinusitis by sinus blockage for those people who experience change in atmospheric pressure while diving, climbing to higher altitudes or while flying (Baroody, 2007). Cigarette smoke poses an increased risk for sinusitis. Smoke can damage cilia responsible for moving mucus through sinuses (Duse et al., 2007). Patients with severe asthma and allergies combined with nasal polyps are also at possible risk for chronic rhinosinusitis (Luong & Pakdaman, 2011). There is a tendency which is commonly seen in elderly people whose nasal passages dry out with age. Also the cartilage which supports the nasal passage weakens and there by results in air flow changes (Tomooka et al., 2000). Smaller nasal passages makes infants more vulnerable to chronic sinusitis than older children and adults (Criddle et al., 2008). Ear infections, such as otitis media, are also associated with chronic sinusitis (Pagella et al., 2010).

2.2.4 SYMPTOMS

Most of the symptoms of the chronic sinusitis and acute sinusitis are similar, where as only the duration of the symptoms differ (Hamilos, 2007). Facial pain, pressure and nasal congestion, pain around the eyes, yellowish to yellow green thick discharge from the nose or down the back of the throat, swelling around the cheeks, nose or forehead, ear pain, bad breath (halitosis), cough which may be worse at night, sore throat, upper jaw and tooth-ache, fatigue, nausea, swollen forehead, severe head-ache, shortness of breath, stiff neck, double vision or other vision changes, symptoms spreading to both side of the face, confusion, mild personality or mental change which indicates the spread of the infection to the brain etc. are the main symptoms associated with chronic rhinosinusitis. Current research studies have shown that most of the symptoms used to study chronic rhinosinusitis often do not predict prognosis or response to antibiotic treatment (Christopher, 2008).

2.2.5 COMPLICATIONS

A serious complication arising from ethmoid chronic sinusitis is the infection of eye socket (Busaba et al., 2004). This results in swelling and subsequent drooping of eyelid. In this particular condition, the patient loses movements in the eye. The pressure on the optic nerve can lead to vision loss. Fever and severe illness are most commonly present (Paterson et al., 1994). Another dangerous rare condition from frontal chronic sinusitis is blood clot. The symptoms are more or less similar to orbital infection when a blood clot forms in the sinus area which is around the front and top of the face. The symptoms usually begin on one side of the head and spread to the other side (Kuczkowsky et al., 2005).

The most life threatening complication of sinusitis is brain infection (Wolff, 1914). In frontal and sphenoid sinusitis, the anaerobic bacteria spread the infection to the brain (Brook, 2006), either through the blood vessels or through bones. This results in abscesses and meningitis in which the patient experiences visual problems, head aches, personality changes and finally coma and death (Yildirim et al., 2004). Frontal chronic sinusitis sometimes leads to infection of the bones of the forehead and other facial bones. This condition is known as osteomyelitis. In such cases the patient experiences swelling over the bone and head-ache (Hamilos, 2007). In this way chronic sinusitis symptoms and conditions of this disease cause emotional distress and alter normal activity which significantly affects the quality of life (Kaliner et al., 1997).

2.3 ROLE OF BACTERIA IN CHRONIC RHINOSINUSITIS

The significance of bacteria in the aetiology of CRS remains the subject of debate (Doyle & Woodham, 1991). Summarising the literature pertaining to the bacteriological evaluation of CRS patients is difficult because of practical differences between studies. Such differences include: (1) the characteristics of the patients studied (age, gender and immune state) (2) severity, extent and duration of the disease (3) use of anti microbials and anti inflammatory agents (4) location and sinus of sampling (5) methods used for sampling (irrigation or aspiration) (6) handling and processing of specimens prior to analysis and (7) methods used to detect bacteria (Brook, 2007). A low-oxygen environment is created inside the obstructed sinus, which is perfectly suited for both anaerobic bacteria and microaerophilic bacteria (Frazier, 2004). The most commonly isolated microorganism from CRS patients is coagulase negative *Staphylococcus* (Patrick, 1990). *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterobacter spp* are the

most important Gram negative species commonly isolated in CRS (Benninger et al., 2003). *Prevotella*, *Peptostreptococcus* and *Fusobacterium* are other organisms which are later identified and isolated from CRS patients (Stevens et al., 1970). *Moraxella catarrhalis* has also been found, but it was not found as frequently as coagulase negative *Staphylococcus* (Benninger et al., 2003). It is not clear whether all of these microorganisms are involved in pathogenesis in CRS. A direct culture of the involved sinus is widely used for the proper identification of the involved bacterial agents in CRS. Gram stain quantification as well as aerobic and anaerobic culture is the most common techniques used for the identification of any microorganism in CRS (Frazier, 2004).

2.4 BIOFILMS

Biofilm can be defined as a “microbially derived sessile community, characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of self produced extracellular polymeric substances, and exhibit an altered phenotype in terms of growth rate and genotype” (Costerton & Donlan, 2002).

2.4.1 BIOFILM STRUCTURE

The introduction of new imaging modalities helped in the conceptual understanding of biofilms. In early light and transmission electron microscopic studies, biofilms were observed as unstructured, homogenous, planar accretions of bacterial cells embedded within the exopolysaccharide matrices. This misperception about the ultra structure of biofilms arose from the inherent faults associated with the new imaging techniques (Fejerskov & Nyvad, 1997). The most significant advancement in our understanding of biofilms came after the invention

of Confocal Scanning Laser Microscopy (CSLM). Using CSLM, the advanced model of biofilms which resembles intense, confluent mushroom-type structures, which are separated from other micro colonies by interstitial voids, has been evolved (Sutherland, 2001).

Biofilms are primarily composed of microbial cells, an extracellular slime layer, fluid channels and primitive communication systems. As the bacteria attach to a surface and to each other they form clustered sessile micro colonies where as each micro colony is an independent community containing thousands of compatible bacteria (Kolter & Watnick, 2000). All these different micro colonies may contain different combinations of bacterial species. The biofilm structure provides a range of customized living environments within which the bacteria with different physiological needs can survive.

2.4.2 BIOFILM CHARACTERISTICS

The major characteristics of biofilm include extracellular polymeric substances (EPS), quorum sensing and antibiotic resistance.

2.4.2.1 EPS

EPS is a complex matrix of excreted polymeric compounds which holds the biofilm microbes together and protects them (Donlan, 2002). The structural composition of EPS and biofilm will vary as it develops in a vast array of differing environments (Flemming et al., 2007). These polysaccharides can either be anionic, such as in the case of Gram negative bacteria, neutral or cationic as in the case of some Gram positive bacteria. Besides polysaccharides and water, a wide variety of glycoproteins, glycolipids and extracellular DNA are present (Sutherland, 2001).

2.4.2.2 QUORUM SENSING

Quorum sensing (QS) is defined as a cell density-dependent bacterial intercellular communication, involved in gene expression (e.g. virulence genes for exoenzymes, exopolysaccharides) and the consequent changed behavior of the biofilm cells, including the resistance to stress conditions (Xie, 2000). Quorum sensing allows the bacteria to display a unified response that benefits the population (Davies, 1998). Quorum sensing can occur within a single species as well as between diverse species, where it essentially serves as a single communication network (Shiner et al., 2005).

2.4.2.3 ANTIBIOTIC RESISTANCE

Established biofilms can withstand antibiotic concentrations of 10 -1000 times that needed to kill planktonic counterparts (Costerton et al., 2005). Biofilms also display an inherent resistance to phagocytosis (Lewis, 2001). The actual mechanism behind antibiotic resistance still remains unclear (Stewart, 2002). However it is likely to be multifactorial. The EPS secreted by the biofilms restricts the penetration of antibodies and antibiotics (Szomolay et al., 2005). Negatively charged molecules present within the EPS matrix are capable of binding to antimicrobial agents (Mah & O'Toole, 2001). The inactivation of antibiotics can occur either on the surface of biofilms or within the EPS matrix (Potera, 1999). Also, the bacteria that exist deep inside the matrix express a lower metabolic rate and reduced growth (Mah & Zhang, 2008). There may be subpopulations of drug resistant, phenotypically and genetically different bacteria within the biofilms, as the close knit community provides an ideal niche for the exchange of extra-chromosomal DNA (Stewart, 1996).

2.4.3 BIOFILM LIFE CYCLE

Proteomic studies of *Pseudomonas* species have established 5 main steps in the biofilm life cycle (Sauer et al., 2002). The stages are described as follows:

Reversible Attachment

During this process individual microbial cells become reversibly associated with the surface and exhibit several species specific behaviours, such as rolling and aggregate formation (Stoodley et al., 2002). They don't form biofilms at this stage and so can easily detach.

Irreversible Attachment

At this stage microbes produce different multiple adhesins (appendages of bacteria that facilitate bacterial adhesion). This enables molecularly mediated binding of adhesins to the surface (Wang et al., 1995). The exopolysaccharides produced by the microbe's complex with the surface molecules located on the pili. Attachment is now irreversible, making these structures extremely difficult to remove (Dunne, 2002).

Aggregation and Maturation

There is an increase in the overall density and complexity of the biofilm as the surface bound micro-organisms actively replicate (Davies et al., 1998). The microbial colonies and the extracellular substances interact with each other which results in the generation and maturation of the biofilm architecture (Sauer et al., 2002). According to proteomic studies, the biofilm bacteria showed different levels of genetic and protein expression compared with the planktonic counterparts (Whiteley et al., 2001).

Detachment

When biofilms reach their critical mass the peripheral layer of growth re-differentiates into planktonic organisms that can embolise (Allison et al., 1998). This phenomenon can explain the periodic spikes in fever associated with device related biofilm infections (McLean et al., 1997).

2.4.4 INTERACTION OF BIOFILM WITH TISSUE

At the time of post-mortem examination and also from biopsies, microbial biofilms have been identified on the epithelial tissue surfaces (Costerton et al., 1999). The initial adhesion of bacteria to a tissue surface can be mediated by the presence of specific ligands on the bacteria and the receptor sites on tissues. Once a bacterium becomes attached to the tissue surface, it continues to divide, forming a biofilm. It can also invade to form micro colonies within the tissue (Sanderson et al., 2006). The initial phase of biofilm formation on tissue surfaces is susceptible to many defense mechanisms, such as ciliary clearance, mucus flow and phagocytes of the host (Caldwell et al., 1995). However, once the biofilm is established on the tissue surface, the structure is resistant to elimination by certain host defense cells.

2.4.5 MICROBIAL BIOFILMS RESIST HOST DEFENCE

One of the main reasons for the failure in the immune response has been attributed to the composition of the biofilm (Donlan, 2002). Phagocytes are incapable of phagocytising the biofilm effectively because of the size of the microcolony, the masking of binding sites by the exopolysaccharides and inhibition of activity to destroy phagocytised cells through an oxidative burst (Buret et al., 1991). Both planktonic and sessile bacteria are able to induce complement activation, but biofilm bacteria are able to survive the lytic action of the complement cascade.

Bacterial biofilms are able to absorb complement fragments, thereby inhibiting the complement activation (Donlan, 2002).

2.4.6 ROLE OF BIOFILM IN CHRONIC RHINOSINUSITIS

Cryer *et al.* (2004) first suggested the presence of biofilms on the sinus mucosa of CRS patients. This short study of 16 CRS patients, who had failed in both surgical and medical treatments, utilised scanning electron microscopy to analyse the specimens of sinus mucosa. In their experiments, they found four specimens with a thicker coating than that of the normal mucociliary-blanket. This finding led the authors to suspect the presence of biofilms in CRS patients (Cryer *et al.*, 2004). Using electron microscopy similar pilot studies were published. Staphylococcal-type biofilm was reported in CRS patients using SEM examination (Ramadan *et al.*, 2005). Other early electron microscopy research also revealed that biofilms were not limited to the sinus mucosa. Multicellular syncytia coated with extracellular matrix on all frontal stents were removed post-operatively from CRS patients (Palmer & Perloff, 2005). The similarity of these structures to known images of biofilms, as well as to structures visible on sterile stents cultured *in vitro* with known biofilm forming organisms, suggested that they may represent biofilms. The absence of these structures on stents that did not undergo *in vitro* culture with bacteria supported the authors' hypothesis that frontal sinus stents may serve as a reservoir for biofilms. Stoodley *et al.* (2009) demonstrated that *H. influenzae*, *P. aeruginosa*, *S. pneumoniae* and *S. aureus* which are responsible for chronic rhinosinusitis are able to develop a biofilm. The most important virulence factor of coagulase negative *Staphylococcus* is its ability to form a biofilm. Further research revealed the prevalence of specific genes associated with biofilm formation in coagulase negative *Staphylococcus* (Patrick, 1990).

2.4.7 GROWING BIOFILMS

A large number of *in vitro* systems for the examination of bacterial biofilms have been reported. As a result of the *in vitro* examination of this mode of bacterial growth, an extensive knowledge concerning every aspect of biofilm morphology, physiology and pathology has been made possible (Friedman, 1997). One of the major challenges in working with biofilms is to select an appropriate method for growing them.

One of the first laboratory methods used for growing biofilms was the Robbins device, which consists of a circular tubing with inlets for bacterial inoculum, fresh growth media and an outlet for the effluent (Nickel et al., 1985). It is also equipped with a sampler which has evenly spaced ports into which the test surfaces may be inserted during biofilm growth (McCoy, 1982). Another commonly employed method for biofilm growth is the flow cell, which is an open channel which is supplied by a peristaltic pump with inoculated media flowing through (Kuehn et al., 1998). The constant depth film fermenter is another method which has been used to study biofilms (Lawrence et al., 1991). Lack of standardisation is one of the major drawbacks to all of the above mentioned devices. Another major issue is that most of these techniques are time consuming, so it is not possible to screen many samples simultaneously. So scientists developed a device called the Calgary Biofilm Device (CBD), which consists of a 96-well plate fitted with a lid with 96 pegs, which is placed on an oscillating platform. Biofilms can form on each peg. By sonicating the biofilms from each peg, followed by dilution plating, viable counts can be enumerated (Ceri et al., 1999).

A large number of methods have also been documented for the cultivation and quantification of static biofilms *in vitro* (Harraghy, 2006). Growth parameters,

such as media used, incubation time and washing forces can be adjusted (O'Toole, 2003). A direct method to enumerate the number of viable bacteria in a static biofilm mass has also been described (Phelan, 1996). Using a 96 well microtiter plate, biofilms are propagated. Wells are then sonicated to remove the adherent bacteria. The resulting bacterial suspension is plated on an agar medium to enumerate the bacteria by determining the number of colony forming units (cfu). This is the most common method by which anti-biofilm treatments are tested (Berthaud & Desnottes, 1997). An air liquid interface (ALI) assay model which allows for the microscopic analysis of biofilm formation over a time range of 4 to 48 hours has also been described (Caiazza et al., 2004). A 24-well flat bottomed plate is placed at an angle of 45 degrees to horizontal, diluted bacterial cultures are then inoculated into the wells such that the upper edge of each culture aliquot is positioned in the centre of a well's bottom. Bacteria are grown for the desired period of time. The wells can then be washed to remove the planktonic bacteria and the remaining biofilms can be viewed with various microscopic techniques. Many bacteria prefer aerobic growth and will therefore only form biofilms at the air-liquid interface (Wijman et al., 2007).

The colony biofilm system works by the same principle of air-liquid interface (ALI) model. These systems involve attachment of bacteria on to a surface supported with nutrient medium (Stewart & Zheng, 2002). Biofilms are propagated on a semi-permeable membrane that sits on a nutrient medium of either agar or nutrient broth, thus allowing a passage for bacteria to attain nutritional needs. Supply of nutrients can be changed or alternatively drug treatment may be administered without the need to disrupt the biofilms mass by washing (Fanucchi et al., 1999). It is thought that changes in the cell number are more attributable to cell death rather than detachment and thus these systems have been applied to the

assessment of biocides. An adaptation of this model is the application of biological tissues such as a respiratory epithelium above a support semi-permeable membrane. This membrane rests on a nutrient medium and allows the maintenance of viable tissue, whilst allowing biofilm to be propagated on biological surface (Jang et al., 2005).

2.4.8. IN VITRO ASSAYS IN CRS BIOFILMS

A Crystal Violet (CV) assay has been widely used to measure the biofilm formation (Christensen, 1985). By the addition of CV, bacterial attachment to a polystyrene surface can be visualised, where crystal violet binds to negatively charged molecules within the biofilms, including nucleic acids and acid polysaccharides (Burmolle, 2006). Bacteria are usually grown on a 96-well microtiter plate for a desired period of time and then the wells are washed thoroughly to remove all planktonic bacteria. The remaining adherent cells are stained with CV which allows visualization of the biofilm mass (O'Toole et al., 1998). Ethanol can be added subsequently to extract the crystal violet from adherent bacteria, thus allowing a semi-quantitative assessment using a standard laboratory plate reader or spectrophotometer (Burmolle, 2006). Stepanovic described a detailed overview of this protocol using staphylococci (Stepanovic, 2007). Bendouah et al. (2006) recently described an *in vitro* CV staining method where the isolates of *S.aureus*, *P. aeruginosa* and coagulase negative *Staphylococcus* from patients with CRS were examined for their biofilm forming capacity. This showed a positive association between the biofilm forming capacity of these organisms and a poor clinical evolution in patients who had undergone Endoscopic Sinus Surgery (ESS) (Bendouah et al., 2006).

2.4.9 POTENTIAL BIOFILM TREATMENTS IN CRS

2.4.9.1 ARRESTING BIOFILM FORMATION

A wide area of research has been conducted for the manipulation of different stages of biofilm development and also for the identification of genes required for biofilm formation (Jefferson, 2004). These approaches are mainly based on interfering with the biofilm quorum sensing and also on the utilisation of signalling molecules to block adhesion processes (Jones et al., 2005). Recently, researchers have succeeded in modulating the quorum sensing using QS inhibitors (Rasmussen, 2005).

Ribonucleic acid-III-inhibiting peptide (RIP) has been shown to have the ability to block *S.aureus* and *S.epidermidis* biofilm formation (Balaban, 2005). The role of iron uptake genes associated with biofilm formation in *P. aeruginosa* has also been reported (Dell'Acqua, 2004).

2.4.9.2 REMOVAL OF ESTABLISHED BIOFILMS

High concentrations of Moxifloxacin, obtainable in topical solutions were effective in killing biofilm bacteria *in vitro* (Desrosiers et al., 2007). The mechanical hydrodynamic disruption of biofilm matrix with the use of saline irrigation and sprays are found to have a role in biofilm removal (Harvey & Lund, 2007). Additives such as soap-like surfactant and calcium sequestering agents are also found to be effective (Desrosiers et al., 2007).

In vitro experiments have shown that electric current can enhance the activity of some antimicrobial agents against certain bacteria in biofilms; this has been termed the bioelectric effect (Del Pozo et al., 2008). A Radio frequency electric current (RFC) will vibrate polar molecules, charged particles, and polar parts of large

molecular chains. A molecular structure that is exposed to an imposed vibration can have its fluidity increased and its structure weakened. This could increase the exchanges between the bacterial cells in the biofilm and the surrounding liquid (Caubet et al., 2004). The possibility that the RFC could produce a mechanical effect upon the EPS matrix should be compared to the fact that the use of ultrasound at frequencies between 70kHz and 10 MHz to vibrate a biofilm gives rise to a synergy phenomenon with antibiotics that is very similar to the bioelectric effect (Qian et al., 1997). The proposed explanation was that the phenomenon was due to the increased fluidity of the matrix, which allowed a better penetration of the antibiotic (Peterson & Pitt, 2000).

CHAPTER 3

MATERIALS & METHODS

CHAPTER 3

MATERIALS & METHODS

3.1 BACTERIAL STRAINS USED FOR THE STUDY

- (i) *Pseudomonas aeruginosa*
- (ii) *Moraxella catarrhalis*
- (iii) *Staphylococcus epidermidis*
- (iv) *Haemophilus influenzae*
- (v) *Streptococcus pneumoniae*

All these isolates were obtained from a medical laboratory. Their identities were then initially confirmed by standard microbiological techniques.

Isolation Media

➤ **Pseudomonas isolation agar:**

Pseudomonas Isolation Agar, (Sigma Aldrich, New South Wales, Australia) is a modified medium based on the formulation of Medium A by King et al., (1954) which was used to differentiate *P. aeruginosa*, from other Pseudomonas strains based on pigment formation.

Forty five grams of dehydrated medium were suspended in 1 litre deionised water containing 20 ml glycerol and boiled to dissolve the medium completely. It was then sterilized by autoclaving at 121°C for 15 minutes.

➤ Baird–Parker agar:

Baird-Parker agar (Sigma Aldrich, New South Wales, Australia) is used for the selective isolation of Gram-positive *Staphylococcus* species (Baird-Parker, 1962). Staphylococci produce black, shiny, convex colonies with entire margins and clear zones, with or without an opaque zone.

Sixty grams of dehydrated medium were suspended in 1 litre of deionised water and heated with frequent agitation and boiled for one minute to completely dissolve the medium. It was then autoclaved at 121°C for 15 minutes. 50 mL of Egg yolk tellurite emulsion was added when it was cooled to 45-50°C and mixed thoroughly before dispensing.

➤ Hemophilus isolation agar with bacitracin

Haemophilus Isolation Agar with Bacitracin, (Sigma Aldrich, New South Wales, Australia) is a primary plating medium used for the selective isolation of *Haemophilus* species (Doern, 1983). Members of the genus *Haemophilus* are fastidious microorganisms that require the addition of the growth factor haemin (Source: - Bovine, Sigma Aldrich, New South Wales, Australia). The antimicrobial agent bacitracin is incorporated to inhibit the growth of bacteria that could mask the presence of *Haemophilus* species. Bacitracin is frequently utilized in enriched media as a selective agent to increase the recovery of *Haemophilus* species from the upper respiratory tract (Rennie, 1992). *H. influenzae* produces pale gray, smooth, glistening and slightly convex colonies.

Thirty seven grams of Brain-heart infusion powder, (Sigma Aldrich, New South Wales, Australia) were dissolved in 1 litre of distilled water and autoclaved at 121°C for 15 minutes. It was then cooled to room temperature and 10 ml of haemin stock (1 mg/ mL) was added. It is then supplemented with bacitracin (0.3 g/ L) which is filter-sterilized.

➤ Streptococcus isolation agar

It is a selective media used for the isolation of Streptococcus species. Haemin supplemented brain heart infusion media with the antibiotics neomycin, (Sigma Aldrich, New South Wales, Australia) and polymyxin, (Sigma Aldrich, New South Wales, Australia) were used to prepare the Streptococcus isolation agar (Ellner et al., 1966). Neomycin and polymyxin inhibit the growth of all other bacteria *S. pneumoniae* produces small, round, mucoid colonies.

Thirty seven grams of Brain-heart infusion powder was dissolved in 1 litre of distilled water. It was then autoclaved at 121°C for 15 minutes. When it was cooled to room temperature, 10 ml of haemin stock (100 mg/ 100 mL) was added. It is then supplemented with neomycin (3 mg/ L) and polymyxin (24 mg/ L) which is filter-sterilized.

➤ Moraxella isolation agar

Moraxella-isolation agar is a selective media used for the isolation of Gram-negative *Moraxella* species. Brain heart infusion media supplemented with acetazolamide (10 µg/ mL) was used to prepare the Moraxella isolation agar. Acetazolamide, (Sigma Aldrich, New South Wales, Australia) was added to inhibit the growth of other bacteria. *M. catarrhalis* produces opaque colonies, with rough surface and friable consistency.

37 g of Brain-heart infusion powder was dissolved in 1 litre of distilled water. It was then autoclaved at 121°C for 15 minutes and cooled it to room temperature. It was then supplemented with filter-sterilized acetazolamide (10µg/ mL).

3.2 CRYSTAL VIOLET ASSAY

- **Biofilm Assay**

All these pure species bacterial strains involved in CRS were tested for their ability to form biofilms by means of a 96 well microtitre plate assay based on the method described by Oh, Chen & Kang (2007). Tissue culture treated plates were used in this study as this will improve adhesion of hydrophilic cells, including tissue cells (Stepanovic, 2007).

Protocol

200µL of uninoculated Brain heart infusion (BHI) broth supplemented with haemin (suitable substitute for blood) was used as a negative control. Other wells were filled with 184µL of sBHI broth. 16µL of overnight grown cultures of the microorganism were added to the wells. The plate was incubated at 35°C for 24 hours (the pure culture plate of *H. influenzae* was incubated at 37°C in a CO₂ - enriched incubator set at 5% CO₂). The contents of the wells were removed after incubation. Wells are then washed 3 times with 250µL of distilled water. The attached cells were fixed for 15 minutes with 200µL of methanol per well. The methanol was discarded and the plates were left to air dry in a 35°C incubator. The wells were stained for 5 minutes with 200µL of 0.05% (w/v) crystal violet. The stain was rinsed off under a gentle stream of running deionised water and the plates air-dried. The stain in the attached cells was resolubilized with 200µL of 33% (v/v)

glacial acetic acid per well. The OD of each well was measured at 550nm and 595nm using the microplate reader (Omega Fluostar, BMG Labtech GmbH, Offenberg, Germany).

Attachment Assay

The attachment assay was carried out with the same protocol as above, except that the incubation time, was reduced to 4 hours.

3.2.1 DUAL COMBINATION TRIALS

Pairs of bacterial species were assayed for both attachment and biofilm formation using the CV assay as described above. The inoculum volume was 8µL from each species. The dual species combinations were as follows:

1. *P. aeruginosa* & *S. epidermidis*
2. *P. aeruginosa* & *M. catarrhalis*
3. *P. aeruginosa* & *H. influenzae*
4. *P. aeruginosa* & *S. pneumoniae*
5. *S. epidermidis* & *M. catarrhalis*
6. *S. epidermidis* & *H. influenzae*
7. *S. epidermidis* & *S. pneumoniae*
8. *M. catarrhalis* & *H. influenzae*
9. *M. catarrhalis* & *S. pneumoniae*
10. *H. influenzae* & *S. pneumoniae*

3.3 GROWTH OF BIOFILMS USING CDC BIOFILM REACTOR

3.3.1 Biofilm Growth Protocol

The Centre for Disease Control (CDC) biofilm reactor consisted of a one litre glass vessel with an effluent spout positioned to provide approximately 350 mL operational fluid capacity. An ultra high molecular mass polyethylene top supported eight independent and removable polypropylene rods. Each rod held 3 removable polycarbonate coupons (biofilm growth surfaces) for a total of 24 sampling opportunities. The reactor, filled with 300mL of sBHI broth and with the coupons held in the coupon holder, was sterilized by autoclaving for <time-temp conditions>. The glass vessel was placed on a digitally controlled heated stirrer plate to provide constant rotation of the baffled magnetic stir bar at a speed of 60 rpm. Rotation of the baffle provided constant mixing and consistent shear to the coupon surface. The intensity of the shear experienced by the coupons was a function of the speed at which the baffle rotated and the distance from the outer edge of the baffle to the coupon faces. A thermocouple, sterilized by treatment with 70% ethanol and 1% formaldehyde solution, was aseptically inserted through the vessel headplate and connected to the stir plate. Temperature was maintained at 35°C by a digital proportional temperature controller and heating element. 0.1 mL of the 12 hour culture was inoculated into the reactor. At the end of the 24 hour incubation period (or in later experiments, at 4 hourly intervals), the biofilm coated coupons were removed and sampled

3.3.2 Biofilm Sampling Protocol

Coupons were aseptically removed from the rod, gently dipped in 10 ml of sterile peptone water to remove planktonic cells and placed on a sterile glass slide for

sampling with the inward side facing up. The top of the coupon was scraped for 30 seconds with the edge of a sterile stainless steel spatula held perpendicular to the surface and then the spatula was rinsed by swirling in 9ml of peptone water. This process was repeated. After scraping, the coupon was held over the dilution tube and the scraped surface was rinsed with 1ml of peptone water to remove any remaining biofilm resulting in a final volume of 10 ml in the dilution tube. The sample was then vortexed for 1 minute to disaggregate biofilm clumps. It was then serially diluted, and plated on isolation agar (specific isolation media for each micro organism).

After plating into the specific isolation agar, it was then incubated at 37°C. The colony forming units (CFU) on the plates were counted and a biofilm cell density for each coupon was calculated

Biofilms were sampled at time intervals of 4, 8, 12, 16, 20 and 24 hours. Two coupons were removed at each time interval and analysed using the above protocol. Biofilm cell density for each coupon at each specific time interval was calculated and the growth curve was plotted as mean count per coupon.

Both pure species and mixed species trials were carried out using the CDC Biofilm reactor to analyse the biofilm formation.

3.4 Fluorescence Microscopy

Biofilm coated coupons were removed from the biofilm reactor and gently rinsed in 10mL peptone water. Each coupon was then treated with 1% formalin for 1-2 minutes and the excess formalin was rinsed with distilled water. It was then stained with Acridine Orange (Fluka Analytical, Sigma Aldrich, Standheim) (20 µg/mL) for 2-3 minutes. The excess stain was gently rinsed off with deionised water. It was

then viewed at 1000X magnification under the fluorescence microscope (Meiji Techno MX 5300H, Meiji Techno Co. Ltd, Saitama, Japan).

CHAPTER 4

CRYSTAL VIOLET–BASED ASSAY TO CHARACTERIZE

BIOFILM FORMATION BY BACTERIA INVOLVED IN

CHRONIC RHINOSINUSITIS

CHAPTER 4

CRYSTAL VIOLET– BASED ASSAY TO CHARACTERIZE BIOFILM FORMATION BY BACTERIA INVOLVED IN CHRONIC RHINOSINUSITIS

4.1 INTRODUCTION

In order to screen several different pathogens for their ability to form biofilms, it became important to identify a convenient method for biofilm study. The crystal violet based assay seemed an ideal choice for biofilm research which was first reported by O'Toole and Kolter (1998).

Initially, pure cultures of the five major species involved in CRS, *P. aeruginosa*, *S. epidermidis*, *M. catarrhalis*, *S. pneumoniae* and *H. influenzae*, were tested for their biofilm forming capacity.

4.2 RESULTS AND DISCUSSION

4.2.1. BIOFILM FORMATION BY PURE SPECIES OF CRS BACTERIA

The results of these assays showed that, these five species vary in their ability to form biofilms, some forming very poor biofilms under these *in vitro* conditions. The physical appearance of the biofilms also differed between species. Three species, *P. aeruginosa*, *S. epidermidis* and *M. catarrhalis* formed firmly adhered biofilms in an approximately uniform density on the tissue culture wells. The remainder formed very ragged-appearing biofilms that could be easily detached from the plastic. *P. aeruginosa*, *S. epidermidis*, and *M. catarrhalis* consistently yielded the highest level of staining in the crystal violet assay.

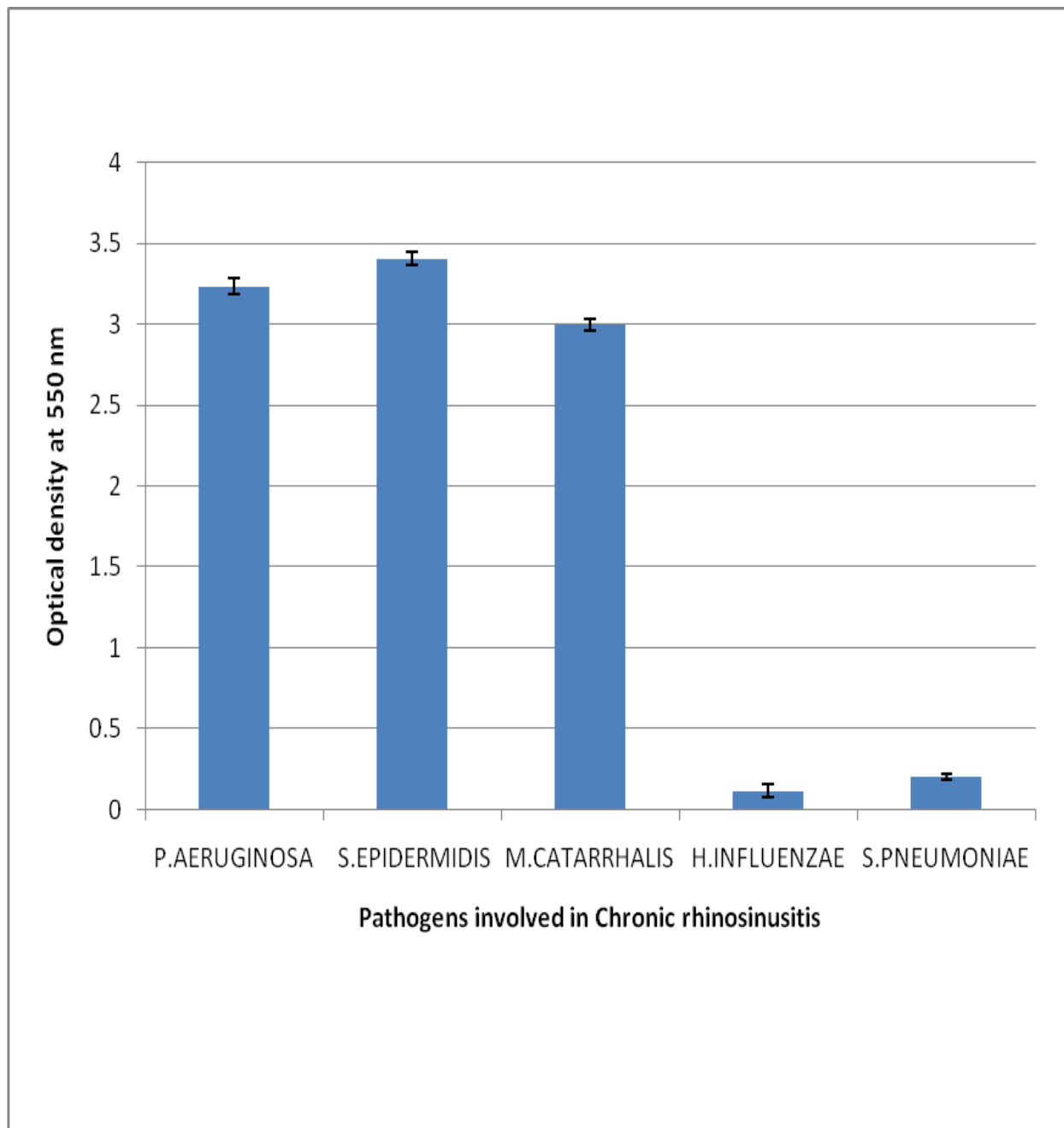


Fig. 1. Biofilm formation by pure species of CRS bacteria. Bacteria were grown in 96 well tissue culture plates. Each assay was performed with eight replicates.

4.2.2. ATTACHMENT OF PURE SPECIES OF CRS BACTERIA

The results of attachment assay also showed varying levels of attachment by these bacteria, after an incubation time of 4 hours.

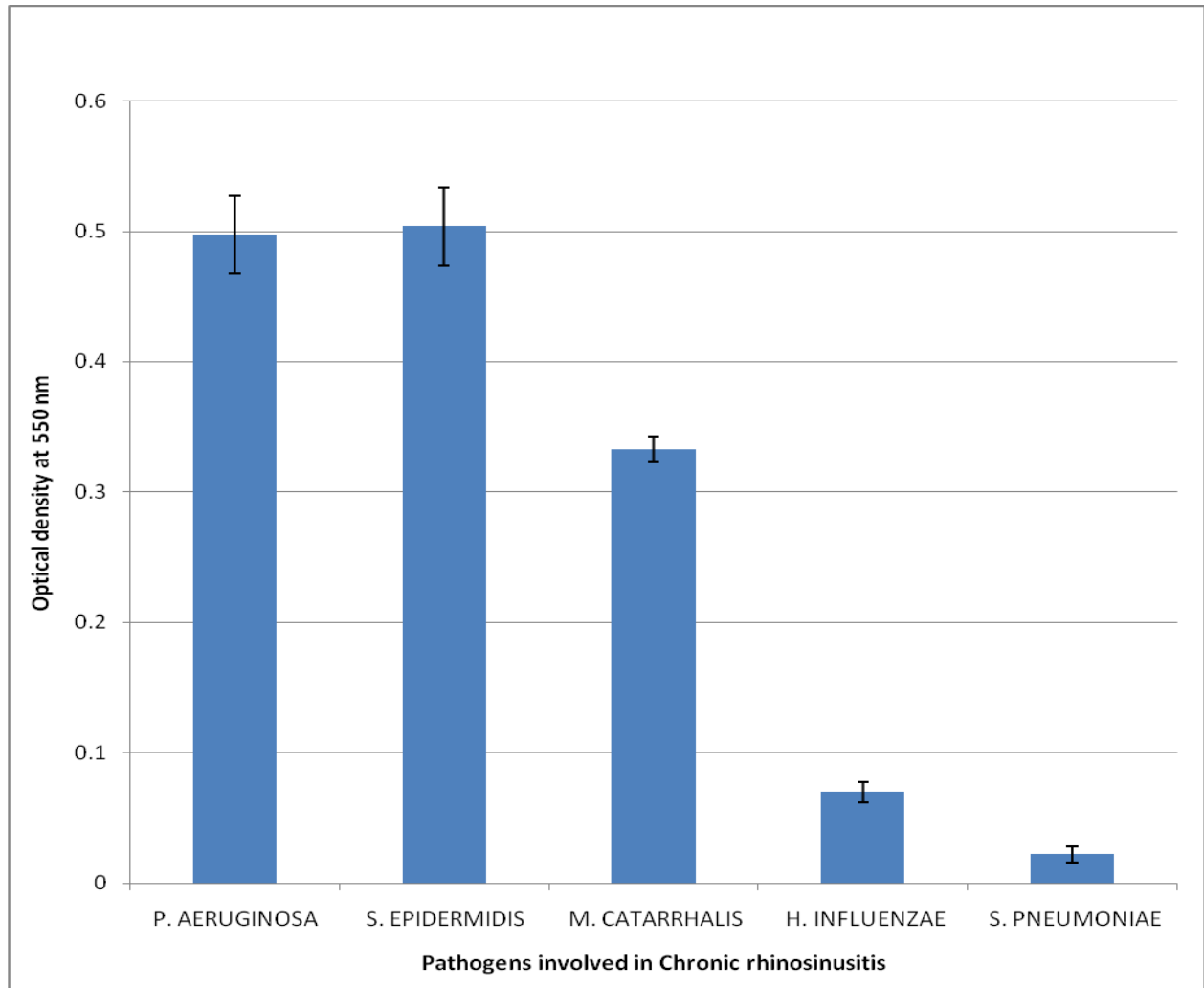


Fig. 2. Attachment of pure species of CRS bacteria to 96 well microtiter plate after 4 hours incubation. Each assay was performed with eight replicates.

The results from the crystal violet-based microtitre plate assay were based on the absorbance at 550nm. The results showed that *P. aeruginosa*, *S. epidermidis* and *M. catarrhalis* form firm attachment to the surface and develop good biofilms (by correlation of observations with CV assay results), whereas *H. influenzae* and *S. pneumoniae* showed poor attachment and biofilm formation (Fig.1 & Fig.2).

4.2.3. MIXED SPECIES BIOFILM FORMATION BY CRS BACTERIA

The results of mixed species assays indicated some variations in the absorbance values when compared with the pure species assays. Dual combinations of the CRS bacteria showed that the presence of one or more species in a biofilm might influence the growth of other micro organisms.

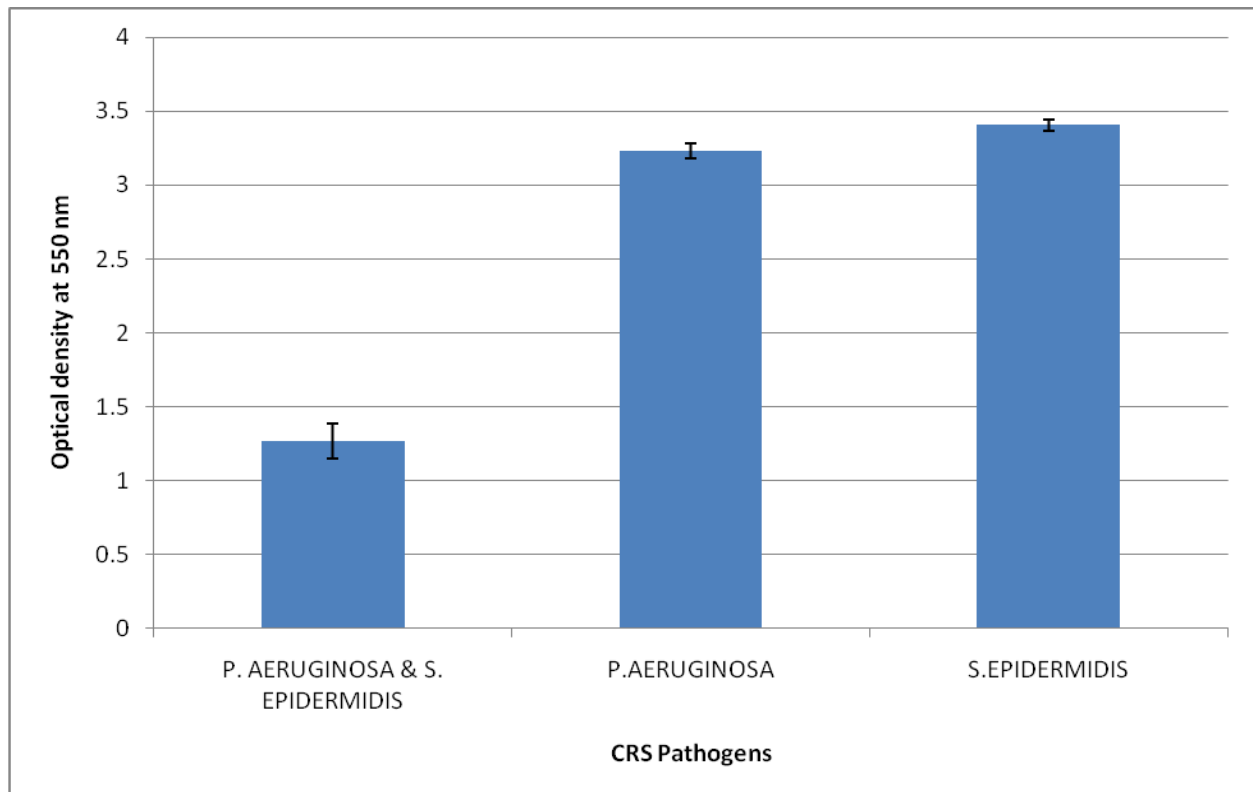


Fig.3. Comparison of biofilm formation after 24 hours by a combination of *P. aeruginosa* and *S. epidermidis* with the pure species biofilm formation, using the absorbance value at 550nm obtained from crystal violet assay.

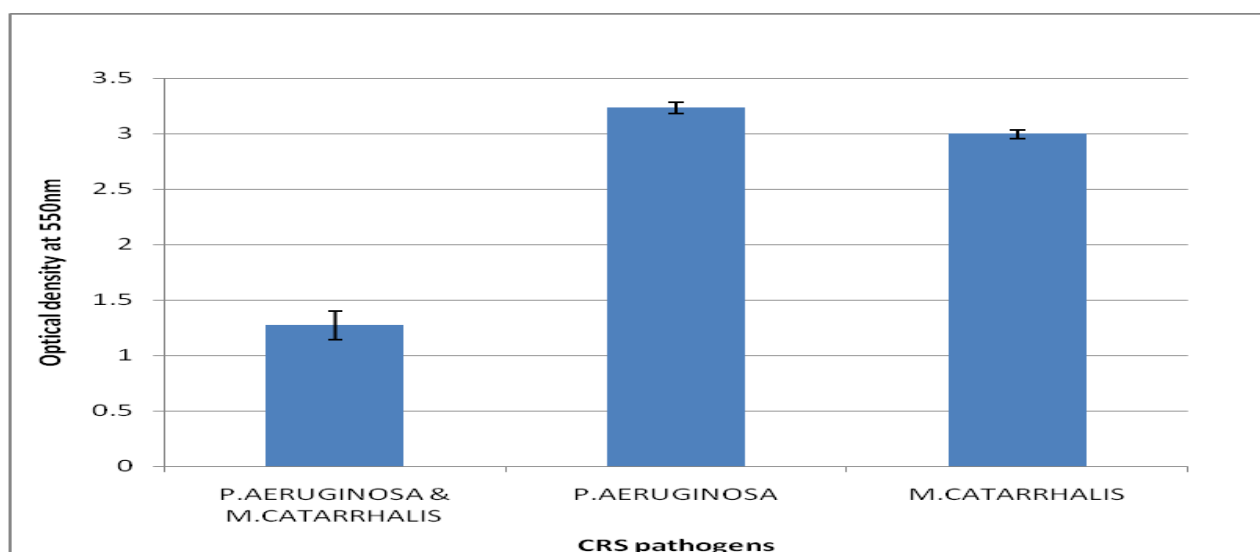


Fig.4. Comparison of biofilm formation by a combination of *P. aeruginosa* and *M. catarrhalis* with the pure species biofilm formation, using the absorbance value at 550nm obtained from crystal violet assay.

When the results of mixed species combination assays were analyzed, variations were observed in the absorbance values. During the first combination trial of *P. aeruginosa* and coagulase negative *S. epidermidis*, the absorbance value at 550nm was reduced when compared with the absorbance values obtained for each of the pure species trials (Fig. 3). Quin et al., (2009) have suggested that this behavior is the result of the action of the extracellular products released by the *P. aeruginosa*, which can inhibit the staphylococcal growth and disrupt the established biofilms produced by *S. epidermidis*. However, this dual combination still showed an absorbance value >1 at 550 nm, which indicates the formation of a very good biofilm. The dual combination of *P. aeruginosa* with *M. catarrhalis* (Fig.4) showed similar results.

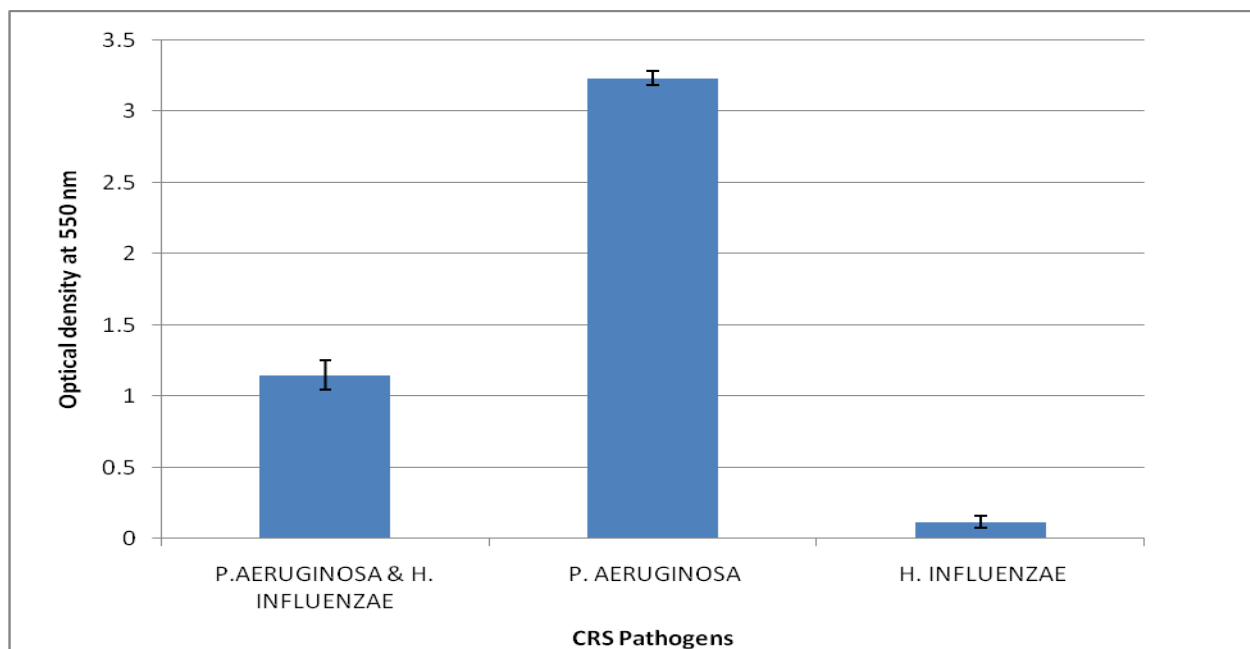


Fig.5. Comparison of biofilm formation by a mixed combination of *P. aeruginosa* and *H. influenzae* with the result of pure species biofilm formation, using the absorbance value at 550nm obtained from crystal violet assay.

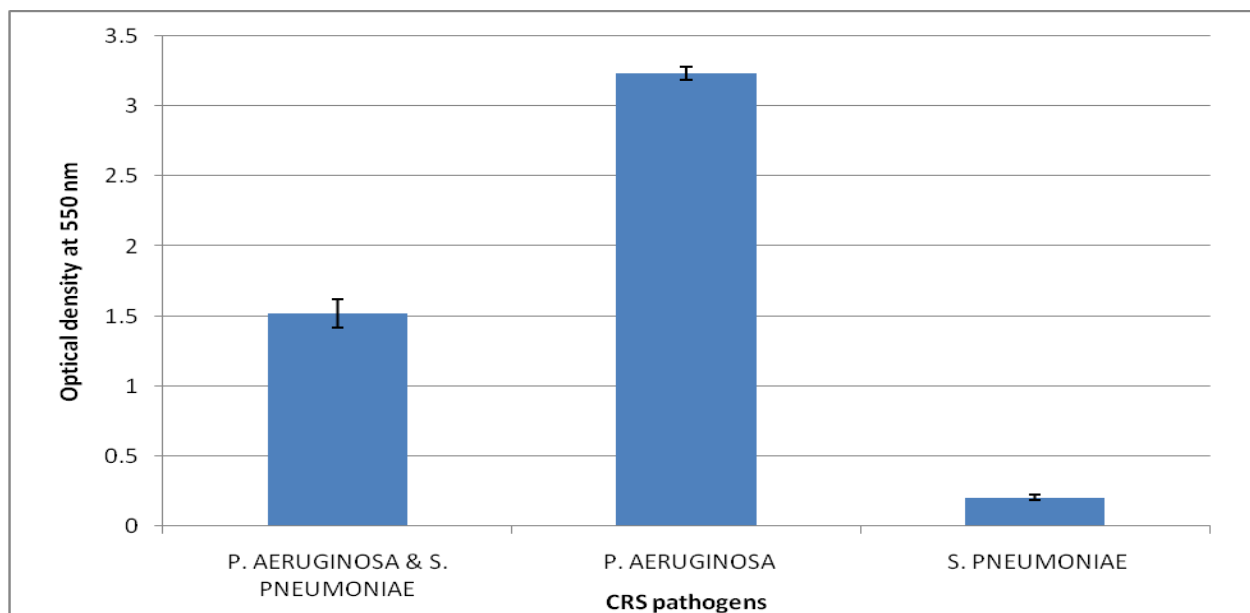


Fig. 6. Comparison of biofilm formation by a combination of *P. aeruginosa* and *S. pneumoniae* with the pure species biofilm formation, using the absorbance value at 550nm obtained from crystal violet assay.

P. aeruginosa was then combined with the pathogens having poor biofilm forming capacity, *S. pneumoniae* and *H. influenzae*. But the dual combination results showed an absorbance value > 1 at 550nm, which shows a strong biofilm formation in these combinations (Fig 5 & Fig. 6). I believe (Brooks, personal communication) that in mixed culture, the dominant micro organism would release dissolved carbon dioxide, which will allow growth of *H. influenzae* without exogenous carbon dioxide.

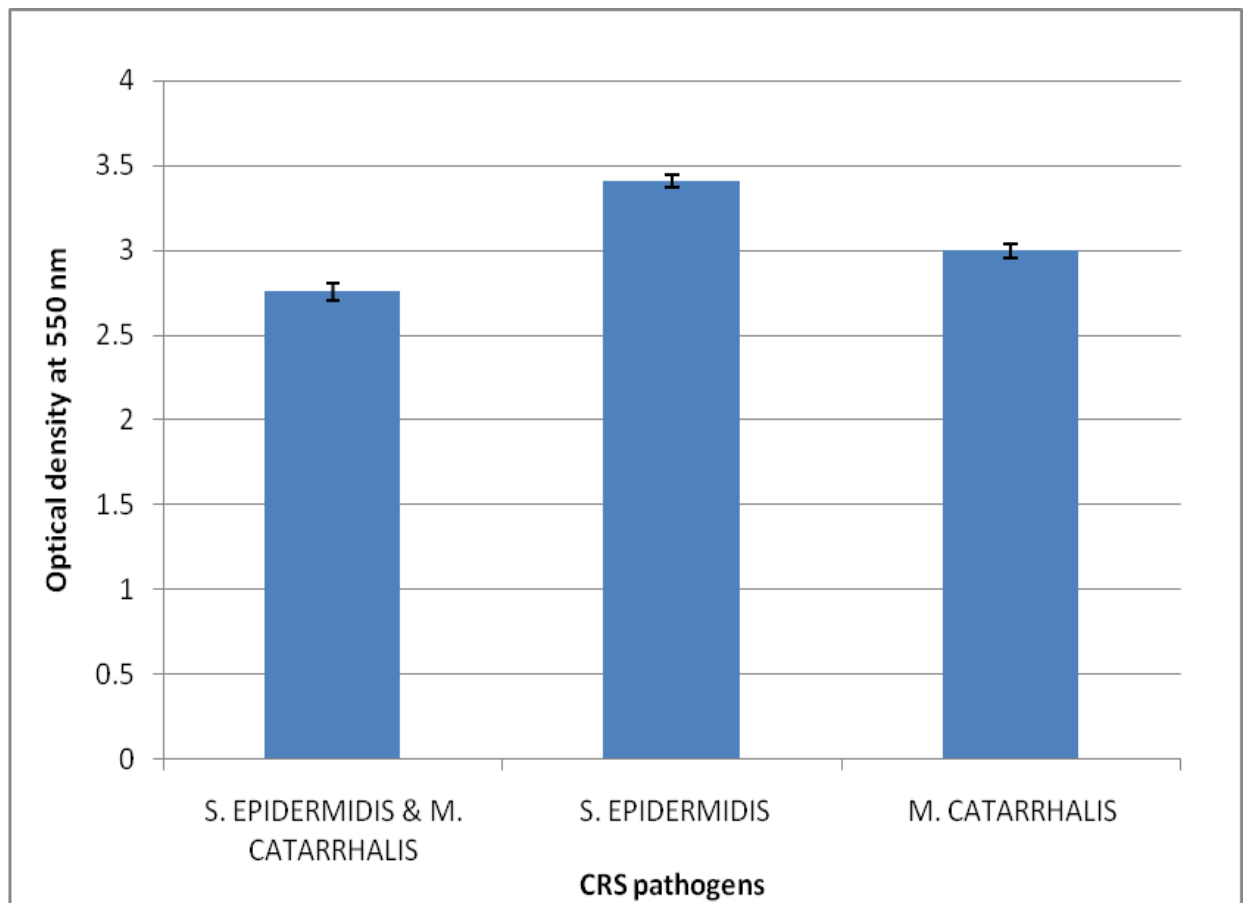


Fig. 7. Comparison of biofilm formation by a combination of *S. epidermidis* and *M. catarrhalis* with the pure species biofilm formation, using the absorbance value at 550nm obtained from crystal violet assay.

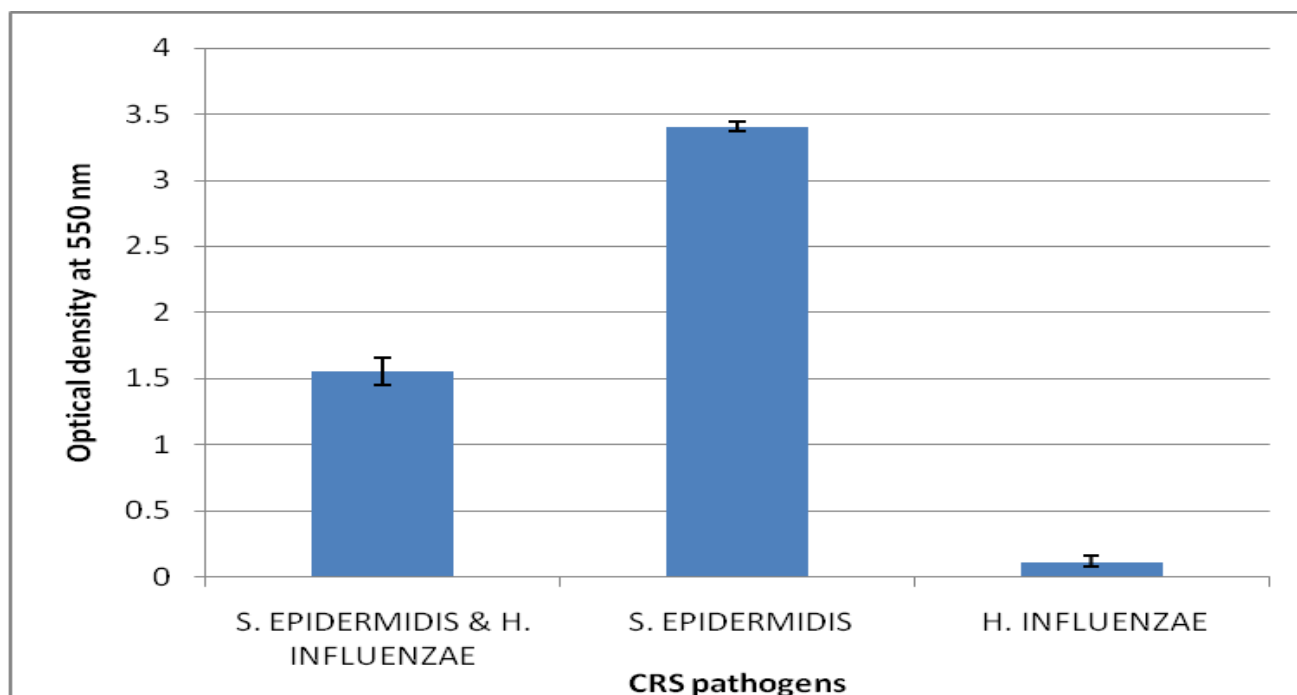


Fig. 8. Comparison of biofilm formation by a combination of *S. epidermidis* and *H. influenzae* with the pure species biofilm formation using the absorbance value at 550nm obtained from crystal violet assay.

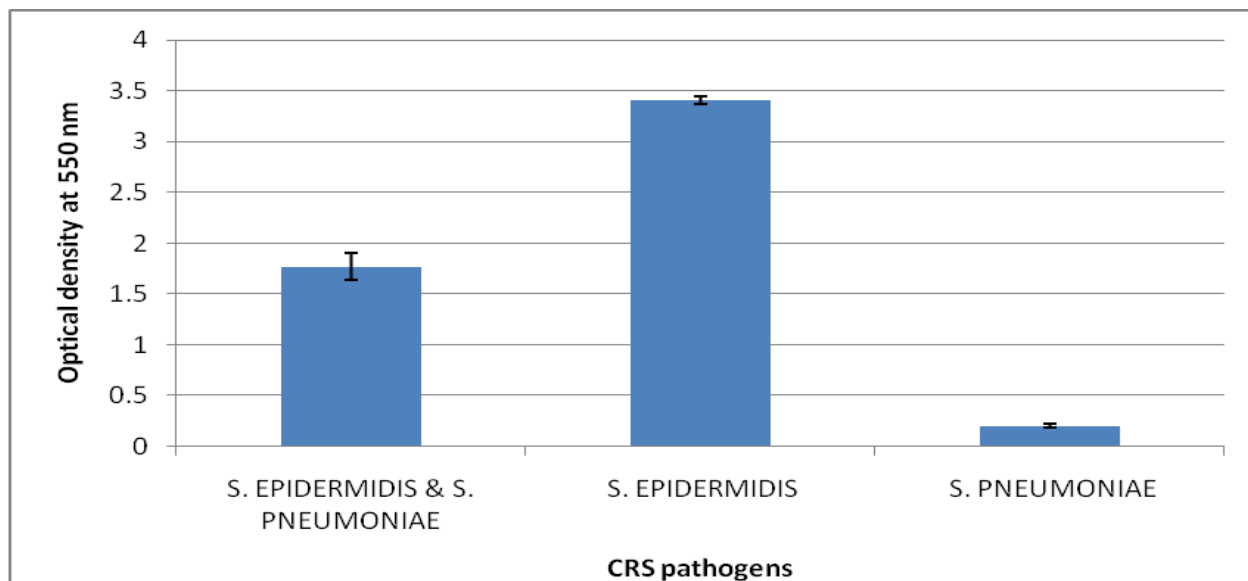


Fig. 9. Comparison of biofilm formation by a combination of *S. epidermidis* and *S. pneumoniae* with the pure species biofilm formation, using the absorbance value at 550nm obtained from crystal violet assay.

Coagulase negative *S. epidermidis* and *M. catarrhalis*, which possess a strong biofilm forming capacity as individual species, also showed a good biofilm formation in combination, with an absorbance value > 2 at 550 nm. Coagulase negative *S. epidermidis*, which is a good biofilm forming bacterium, was then combined with the pathogen *S. pneumoniae* and then with *H. influenzae* (Fig. 8 & Fig. 9).

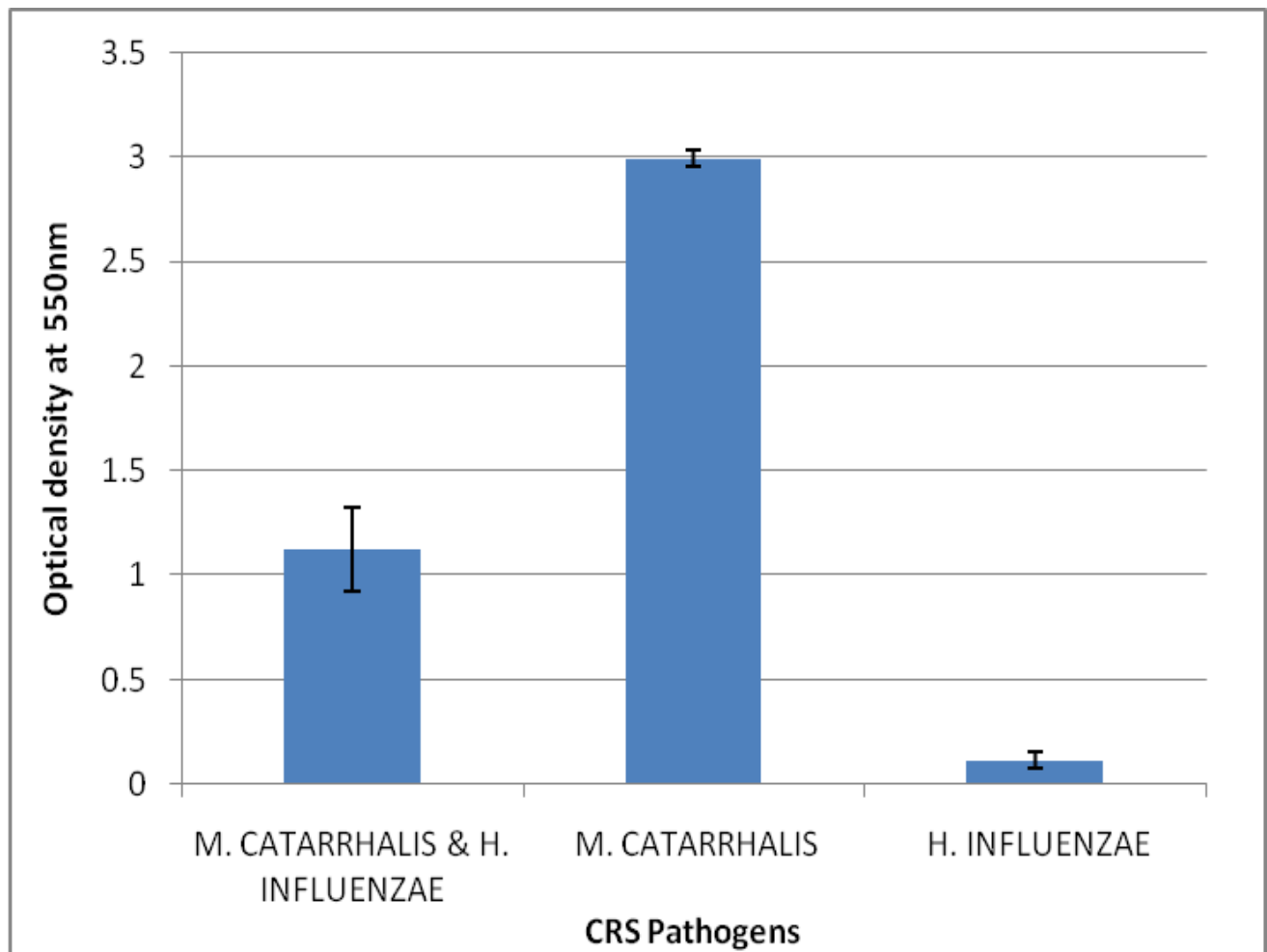


Fig. 10. Comparison of biofilm formation by a combination of *M. catarrhalis* and *H. influenzae* with the pure species biofilm formation, using the absorbance value at 550nm obtained from crystal violet assay.

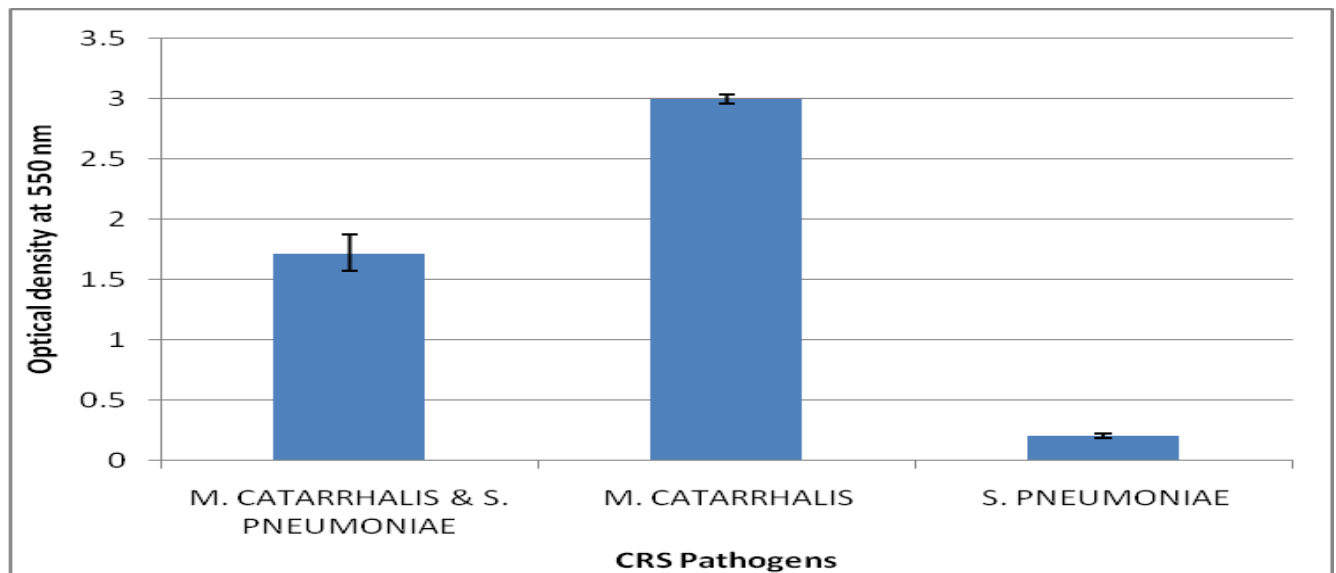


Fig. 11. Comparison of biofilm formation by a combination of *M. catarrhalis* and *S. pneumoniae* with the pure species biofilm formation, using the absorbance value at 550nm obtained from crystal violet assay.

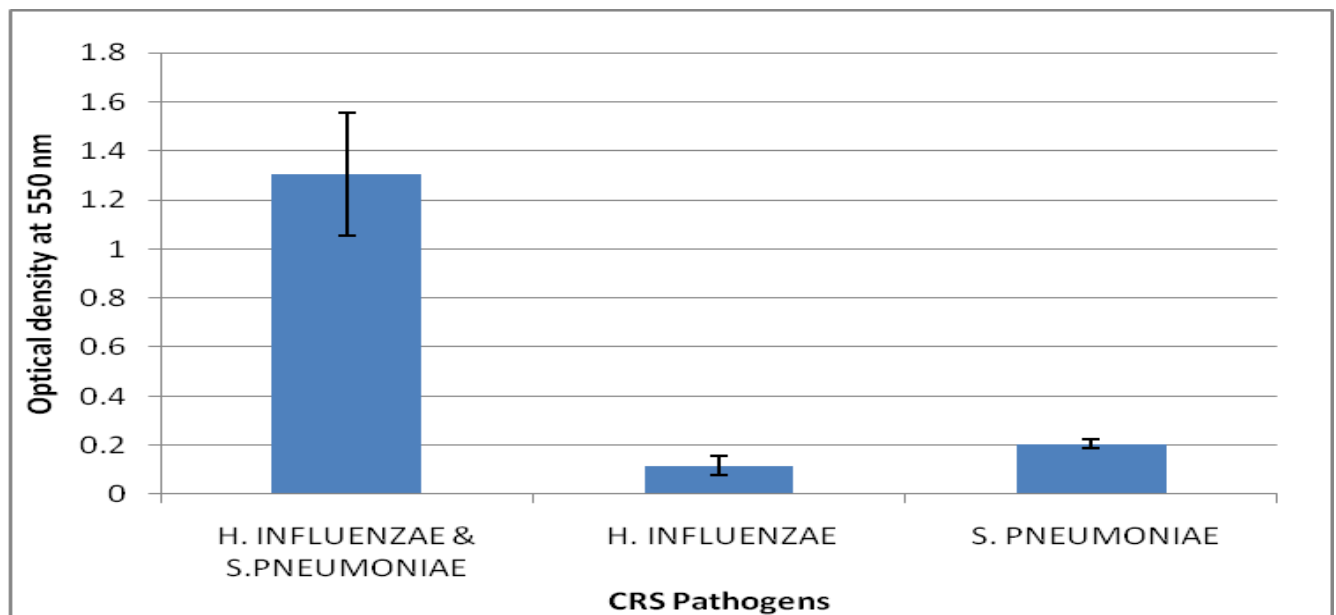


Fig.12. Comparison of biofilm formation by a combination of *H. influenzae* and *S. pneumoniae* with the pure species biofilm formation using the absorbance value at 550nm obtained from crystal violet assay

The dual combination results showed an absorbance value > 1 at 550nm, which showed a dense biofilm formation. *M. catarrhalis* was also combined with *S. pneumoniae* and then with *H. influenzae* (Fig. 10 & Fig.11).

Finally a combination trial was made with *H. influenzae* and *S. pneumoniae*, both of which have poor biofilm forming capacity. The combination showed an absorbance value >1 at 550 nm, which is a good indication of biofilm formation when they exist as pairs (Fig.12).

Crystal violet assay was used only for the initial screening of the biofilm formation by these bacteria, where as it doesn't show the proportions of each strain in the film and that work in the following chapter to elucidate.

CHAPTER 5

GROWING BIOFILMS

USING

CDC BIOFILM REACTOR SYSTEM

CHAPTER 5

GROWING BIOFILMS USING CDC BIOFILM REACTOR SYSTEM

5.1. INTRODUCTION

In laboratories, biofilms are engineered in growth reactors, which are designed to produce a specific biofilm type (Harraghy, 2006). The Centre for Disease Control (CDC) used a biofilm reactor system that is also suitable for efficacy testing (Goeres et al., 2005). The reactor is versatile and can also be used for growing and characterizing biofilms of varying species. The biofilm cell density is expressed as the log colony forming units per coupon (Phelan, 1996)

5.2. RESULTS AND DISCUSSION

5.2.1. GROWING PURE SPECIES BIOFILMS OF CRS BACTERIA

P. aeruginosa, *M. catarrhalis* and *S. epidermidis*, which showed the highest absorbance value at 550 nm during the initial CV screening of the biofilm forming capacity, were selected for the measurement of growth rate in a CDC Biofilm reactor. The remaining two species were not included at this stage because of their poor ability to form biofilms and the fact that *H.influenzae* requires an atmosphere enriched with carbon dioxide for growth. The results of these assays showed that all three species are capable of forming biofilms on coupons in pure culture.

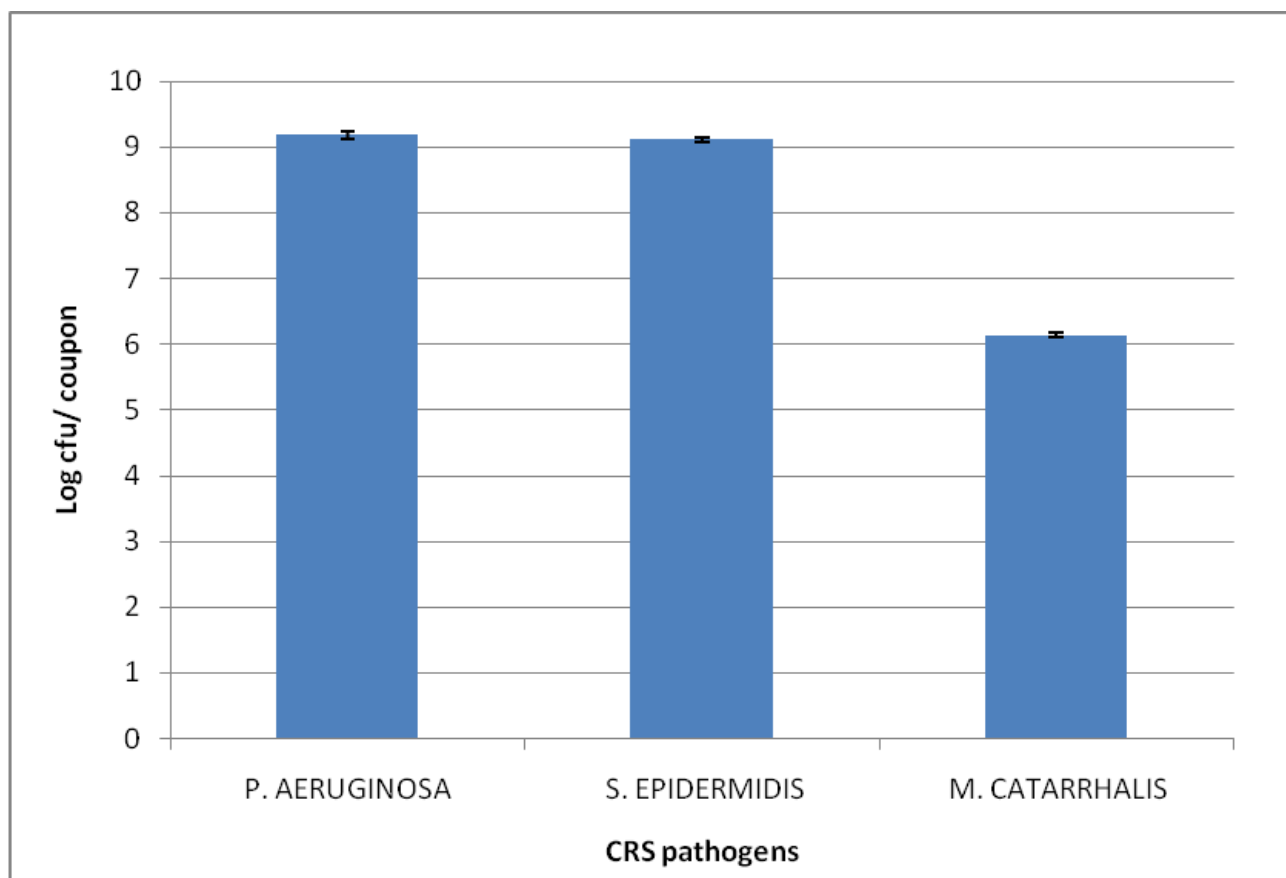


Fig. 13. Biofilm formation of major species involved in CRS after 24 hours incubation using CDC biofilm reactor system. Six replicates were measured after 24 hours. Biofilm cell density is expressed in terms of log colony forming units per coupon.

This chapter clearly demonstrates the biofilm forming capacity of the major bacterial species involved in chronic rhinosinusitis using the CDC biofilm reactor system. From the initial screening using crystal violet assay, the results showed the biofilm forming capacity of the pure species of *P. aeruginosa*, *S. epidermidis* and *M. catarrhalis*. These three pathogens were then allowed to grow in a CDC biofilm reactor to measure the biofilm cell density. The results showed that all these three pathogens are good biofilm formers (Fig.13). The biofilm cell density was expressed in terms of log colony forming units per coupon.

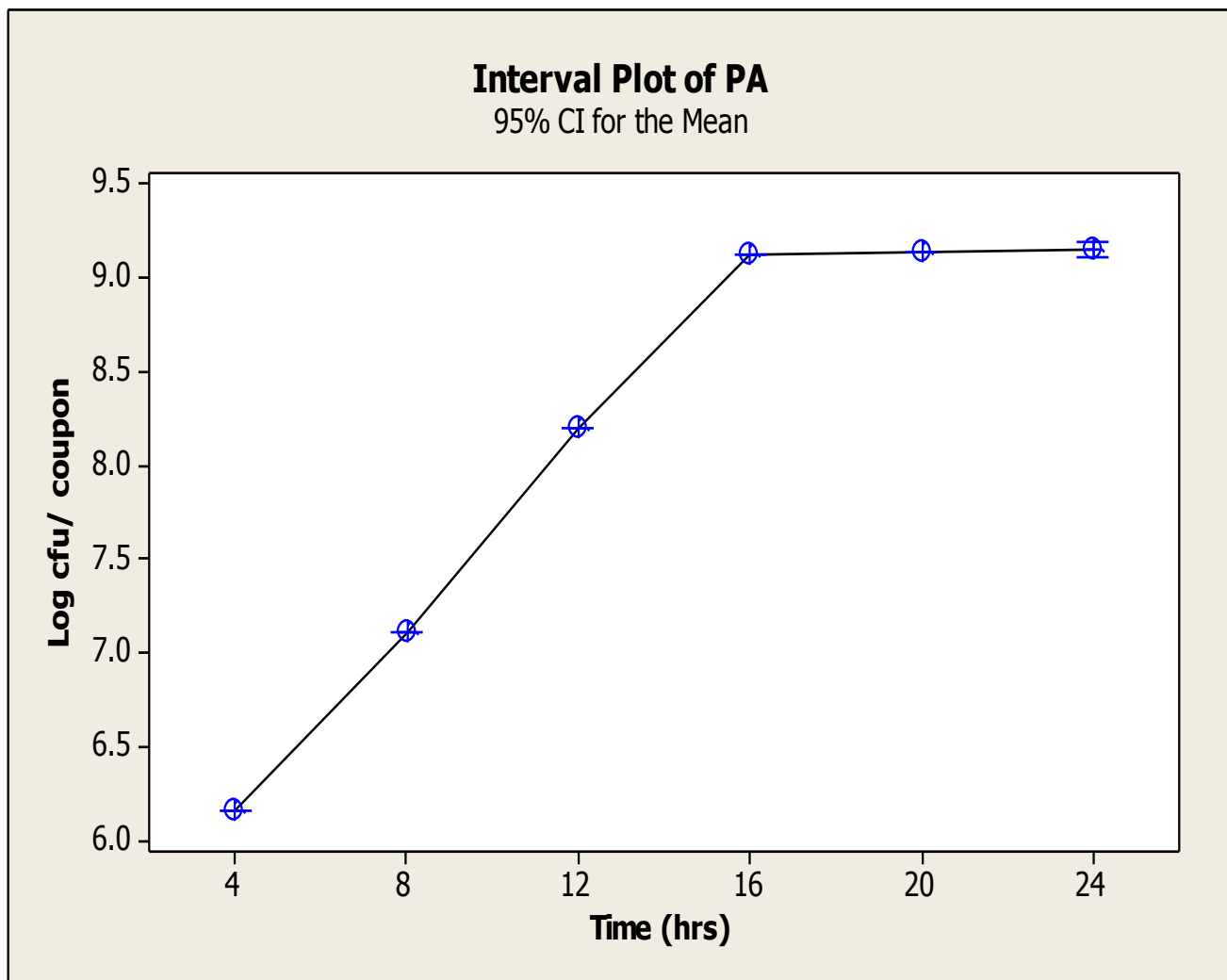


Fig. 14. Growth of *P. aeruginosa* in a CDC biofilm reactor system. The coupons were sampled at 4, 8, 12, 16 and 24 hours to calculate the biofilm cell density. Sampling at each time interval was performed with two replicates. Mean log colony forming units per coupon was plotted. Six replicates were measured after 24hours. Error bar is 95% confidence limit.

The growth rate of each of these pure species was calculated by sampling the coupons at 4, 8, 12, 16, 20 and 24 hour time intervals. The initial growth experiment with the pure species of *P. aeruginosa* indicated that the bacteria achieved a density of approximately 9.2 log cfu/ coupon within 24 hours (Fig. 14).

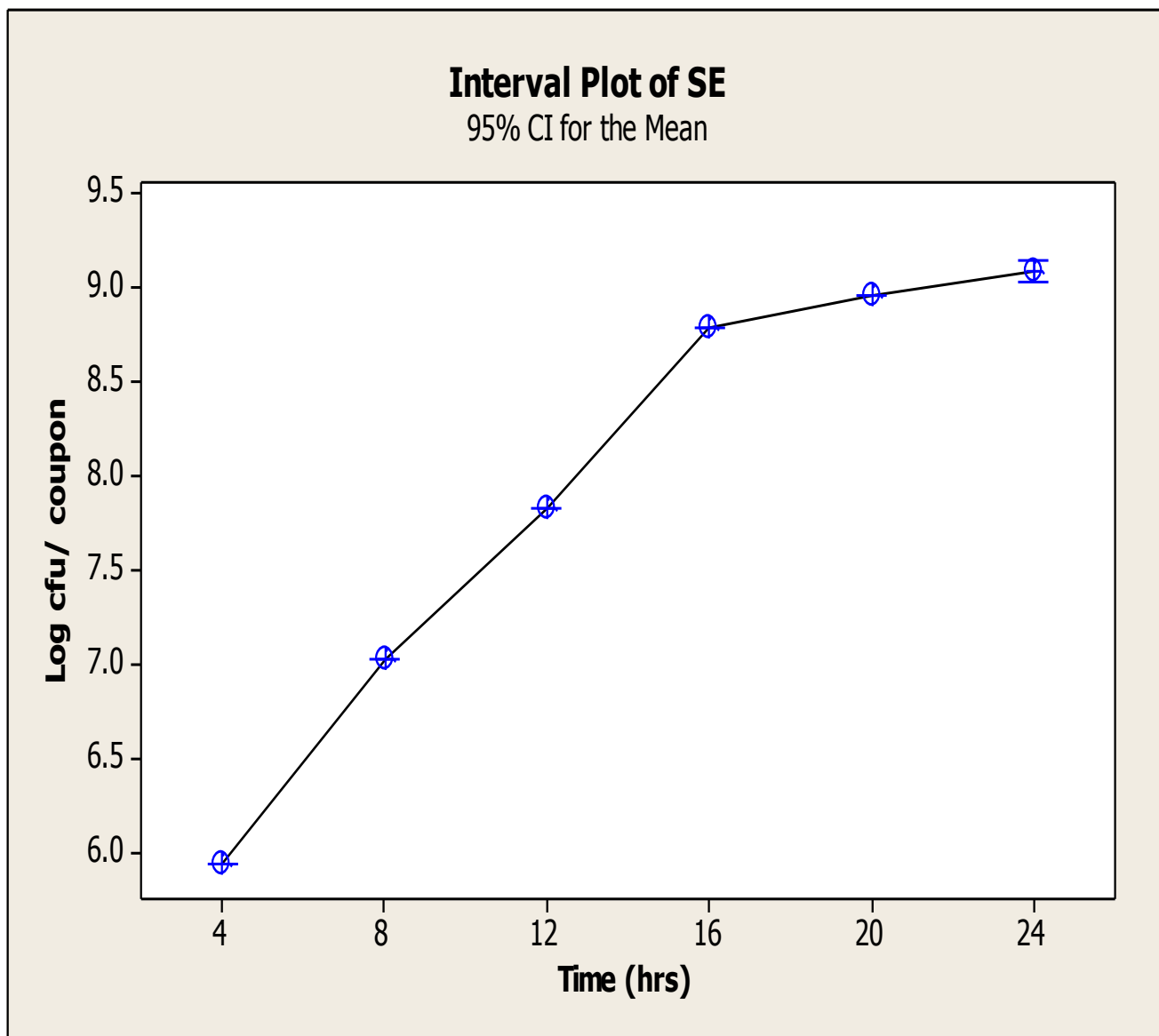


Fig.15. Growth of coagulase negative *S. epidermidis* in a CDC biofilm reactor system. The coupons were sampled at 4, 8, 12, 16 and 24 hours. Six replicates were measured after 24hours.

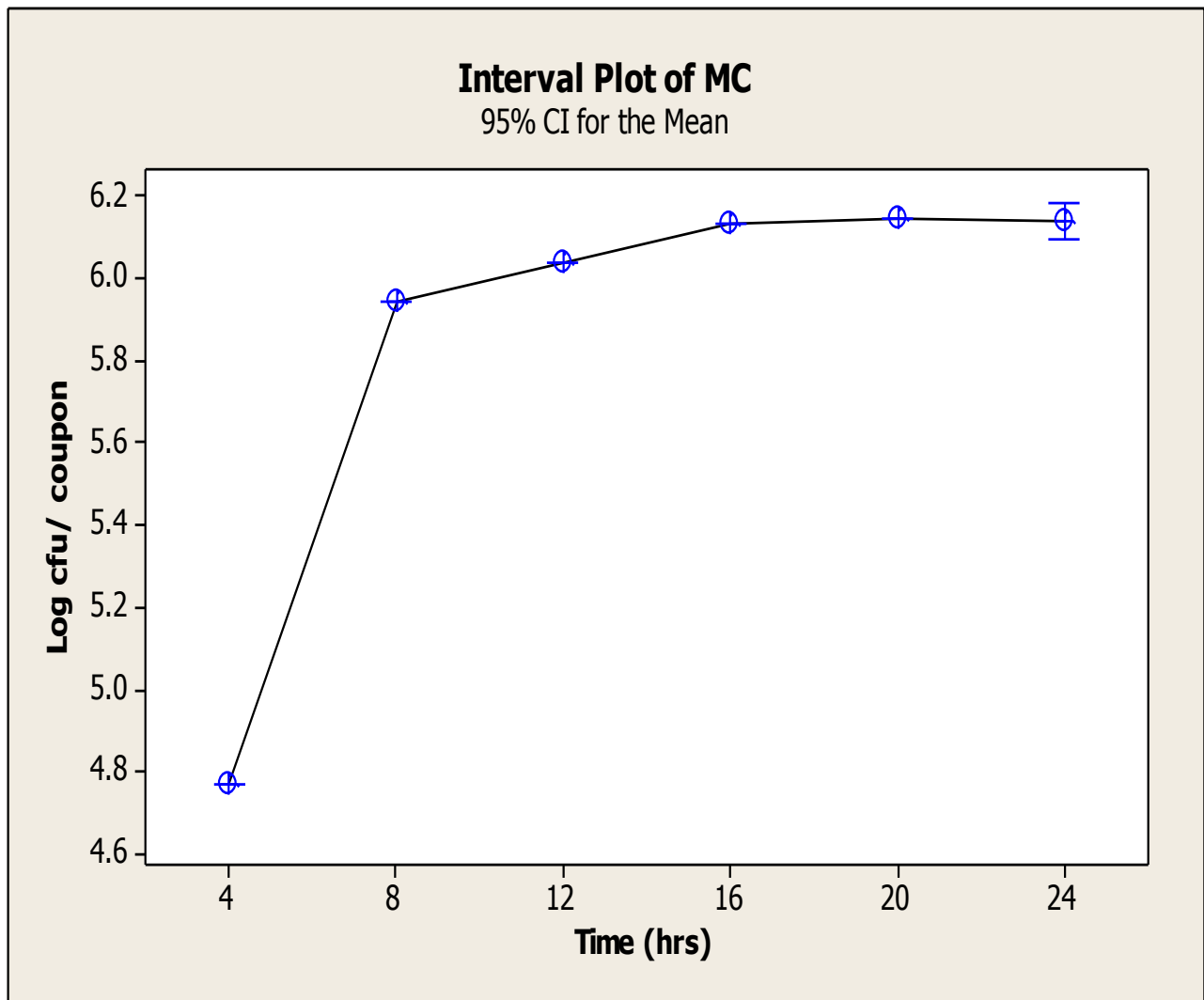


Fig.16. Growth of *M. catarrhalis* in a CDC biofilm reactor system. The coupons were sampled at 4, 8, 12, 16 and 24 hours. Six replicates were measured after 24 hours.

This experiment was followed by the pure species trials of coagulase negative *S. epidermidis* and *M. catarrhalis*, which showed a density of 9.1 log cfu/ coupon and 6.1 log cfu/ coupon within 24 hours (Fig. 15 & Fig. 16). This experiment showed that *P. aeruginosa* formed the most dense biofilms in the pure species trials of CRS bacteria. These 24 hour coupons of the pure species of these three major pathogens were then viewed under the fluorescence microscope by staining with the dye Acridine Orange (Fig. 17, Fig.18 & Fig.19).



Fig.17. Coupon coated with *P. aeruginosa* biofilm, stained with Acridine Orange and viewed under fluorescence microscope at 1000X magnification

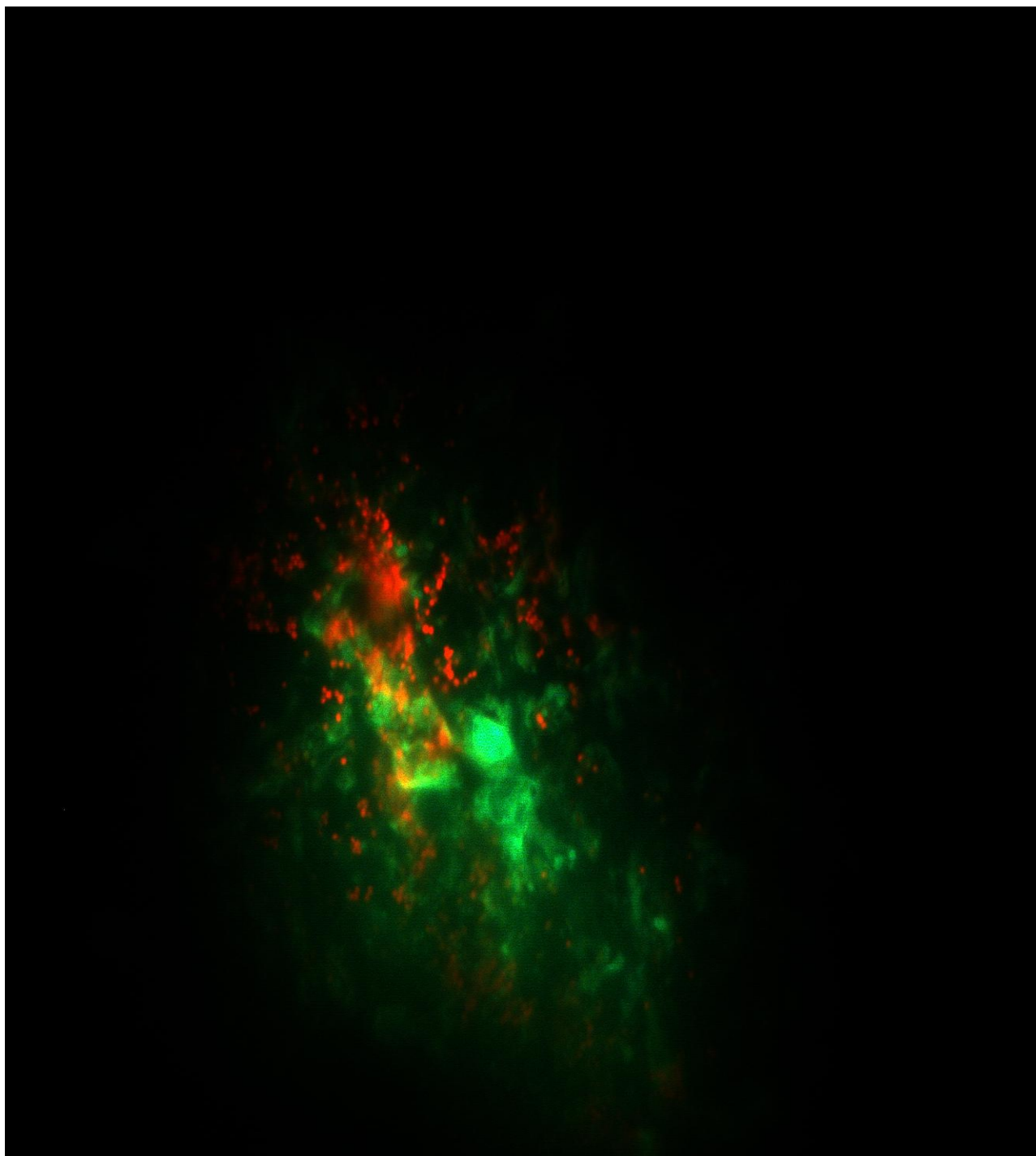


Fig.18. Coupon coated with *S. epidermidis* biofilm stained with Acridine Orange and viewed under fluorescence microscope at 1000X magnification.

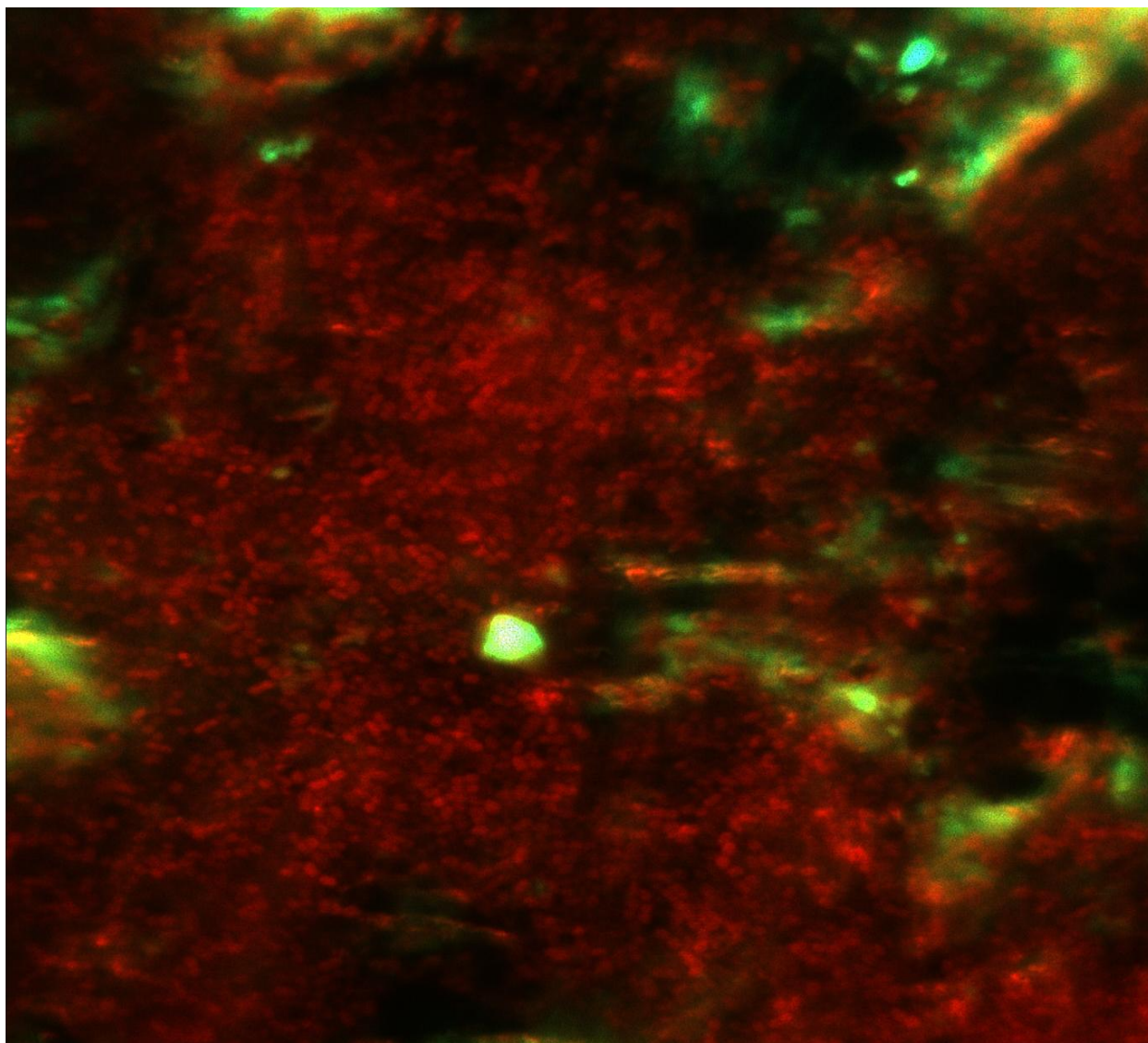


Fig. 19. Coupon coated with *M. catarrhalis* biofilm stained with Acridine Orange and viewed under fluorescence microscope at 1000X magnification.

5.2.2. GROWING MIXED SPECIES BIOFILMS OF CRS BACTERIA

Different combination trials were conducted. The three major bacteria which showed maximum absorbance value for initial screening using CV assay were selected for the dual species combinations: *P. aeruginosa* & *S. epidermidis*, *P. aeruginosa* & *M. catarrhalis*, *S. epidermidis* & *M. catarrhalis*. These major

bacteria were then combined with *H. influenzae* and *S. pneumoniae*. A final mixed species trial was conducted with another pathogen, *S. aureus* replacing *S. epidermidis* to analyse the activity of *Staphylococcus* species with *P. aeruginosa* (Bartley, personal communication). Some species showed an altered growth rate in the combinations when compared with the pure species trials. The results indicated a wide variation in the values for log colony forming units per coupon of the individual members of the biofilm population. The trend line was fitted to the linear portion of the graph and the slope of the trend line was measured to estimate the growth rate (Tables 1 and 2).

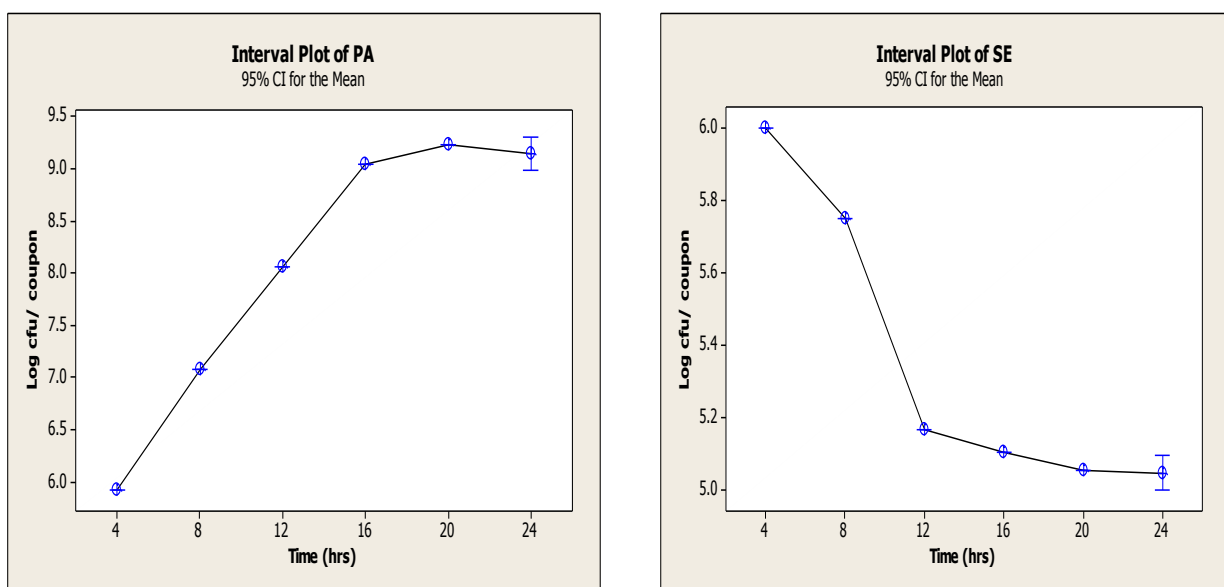


Fig. 20. Dual species combination trial of *P. aeruginosa* and *S. epidermidis* in CDC biofilm reactor system. Pseudomonas isolation agar and Baird-Parker agar were used for isolating the above bacteria from mixed species biofilm. Log colony forming units per coupon were analyzed at each 4 hours time interval up to 24 hours. Mean log colony forming units per coupon were plotted. Four replicates were measured after 24 hours. Error bar is 95% confidence limit.

The growth experiment on pure species was then carried over to the dual species combinations. Combinations as pairs were made of the three major species *P. aeruginosa*, *S. epidermidis* and *M. catarrhalis* as an initial experiment for mixed species biofilm formation. The first dual species combination was composed of *P. aeruginosa* and *S. epidermidis* (Fig. 20). In this experiment the *P. aeruginosa* showed a similar rate of increase as in the pure species trial, but numbers of *S. epidermidis* actually decreased during the first 12 hours of the experiment, after which the rate of decrease slowed. The trend suggested that, had the experiment been prolonged, the two species would have come to equilibrium. Based on the work of Qin et al., (2009) this might be explained by inhibition of staphylococcal growth by extracellular products of *P. aeruginosa*.

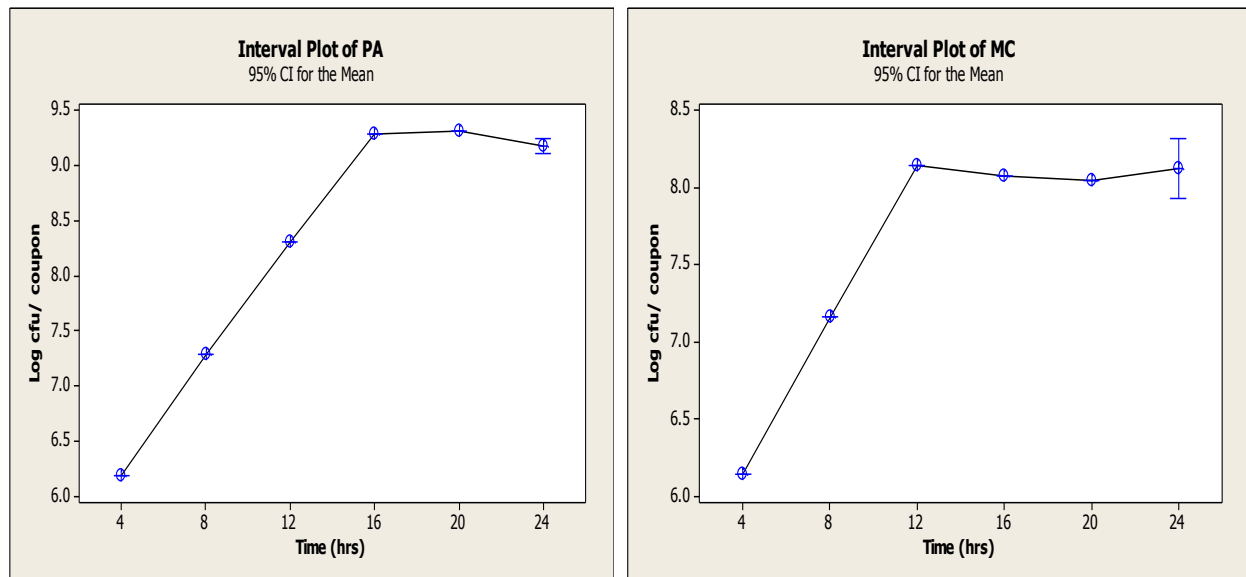


Fig. 21. Dual combination trial of *P. aeruginosa* and *M. catarrhalis* in CDC biofilm reactor system. Pseudomonas isolation agar and Moraxella isolation agar were used for isolating the above bacteria from mixed species biofilm. Log colony forming units per coupon were analyzed at each 4 hours time interval till 24 hours. Four replicates were measured after 24 hours. Error bar is 95% confidence limit.

P. aeruginosa was then combined with *M. catarrhalis*, where the *P. aeruginosa* showed the same density as for the pure species trial. However, in this combination, there was an increase in the growth of *M. catarrhalis* on the coupon as its density reached 8.0 log cfu/ coupon, which is actually higher than the result of its pure species trial (Fig.21). This suggests some interaction between *P. aeruginosa* and *M. catarrhalis* in the, mixed species biofilm.

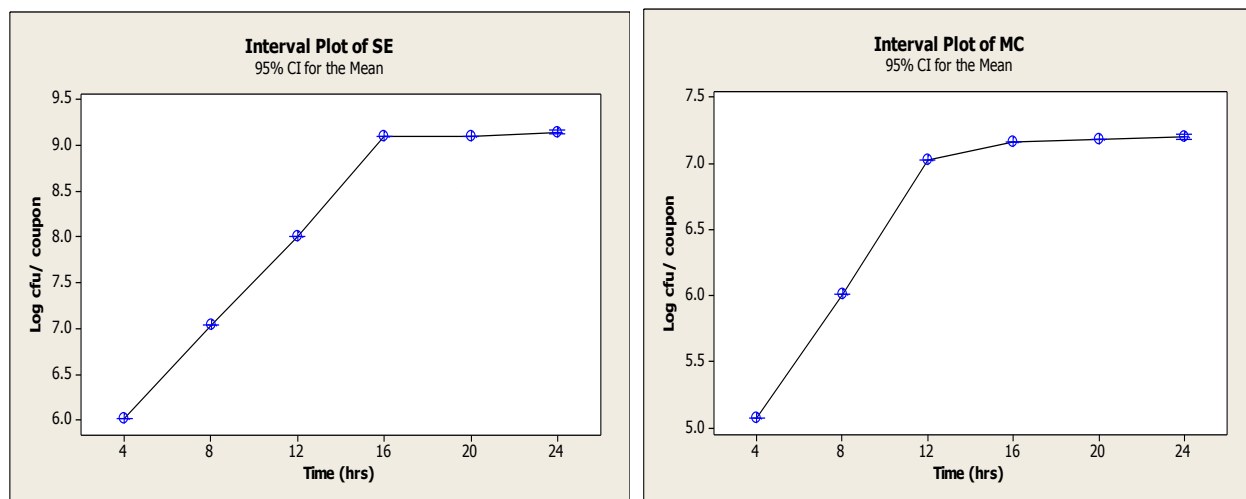


Fig. 22. Dual species combination trial of *S. epidermidis* and *M. catarrhalis* in CDC biofilm reactor system. Baird-Parker agar and Moraxella isolation agar were used for isolating the above bacteria from mixed species biofilm. Log colony forming units per coupon were analyzed at each 4 hours time interval till 24 hours. Four replicates were measured after 24 hours. Error bar is 95% confidence limit.

The last dual species combination trial was between *S. epidermidis* and *M. catarrhalis*. Again, in this trial, *S. epidermidis* showed a similar growth to that of the pure species trial, where as *M. catarrhalis* reached a density of 7.2 log cfu/ coupon (Fig. 22), which is higher than its pure species density. The maximum density for *M. catarrhalis* was observed when it was grown along with *P. aeruginosa*.

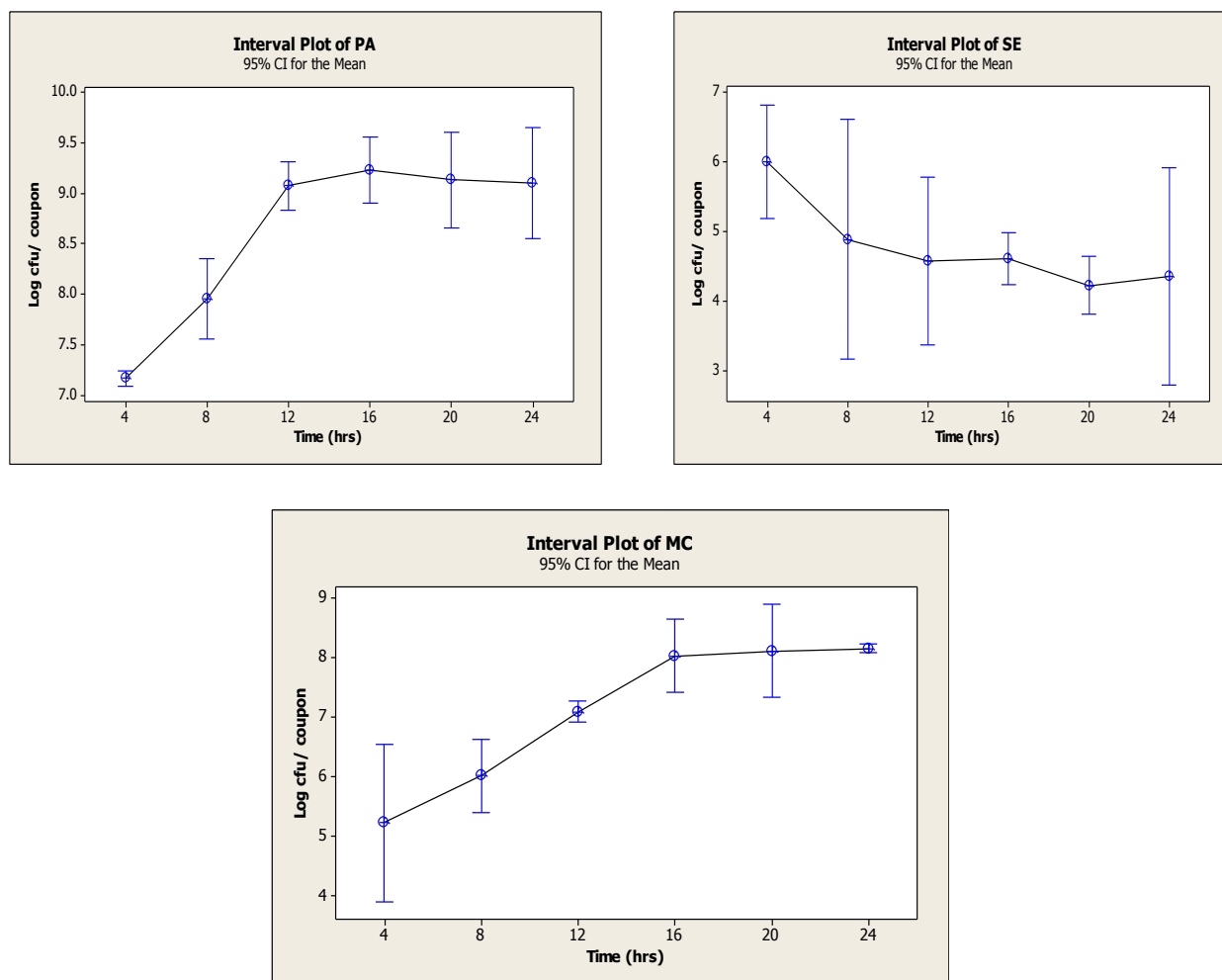


Fig. 23. Three-species combination trial of *P. aeruginosa*, *S. epidermidis* and *M. catarrhalis* in CDC biofilm reactor system. Pseudomonas isolation agar, Baird-Parker agar and Moraxella isolation agar were used for isolating the above bacteria from mixed species biofilm. Sampling at each time interval was performed with two replicates. Log colony forming units per coupon were analyzed at each 4 hours time interval till 24 hours. Error bar is 95% confidence limit.

The major three pathogens *P. aeruginosa*, *S. epidermidis* and *M. catarrhalis* were grown together in the CDC biofilm reactor (Fig.23). In this trio combination, *P. aeruginosa* and *M. catarrhalis* showed a stable growth as in the above trials, whereas the density of *S. epidermidis* in the biofilms was again reduced to 4.4 log cfu/ coupon.

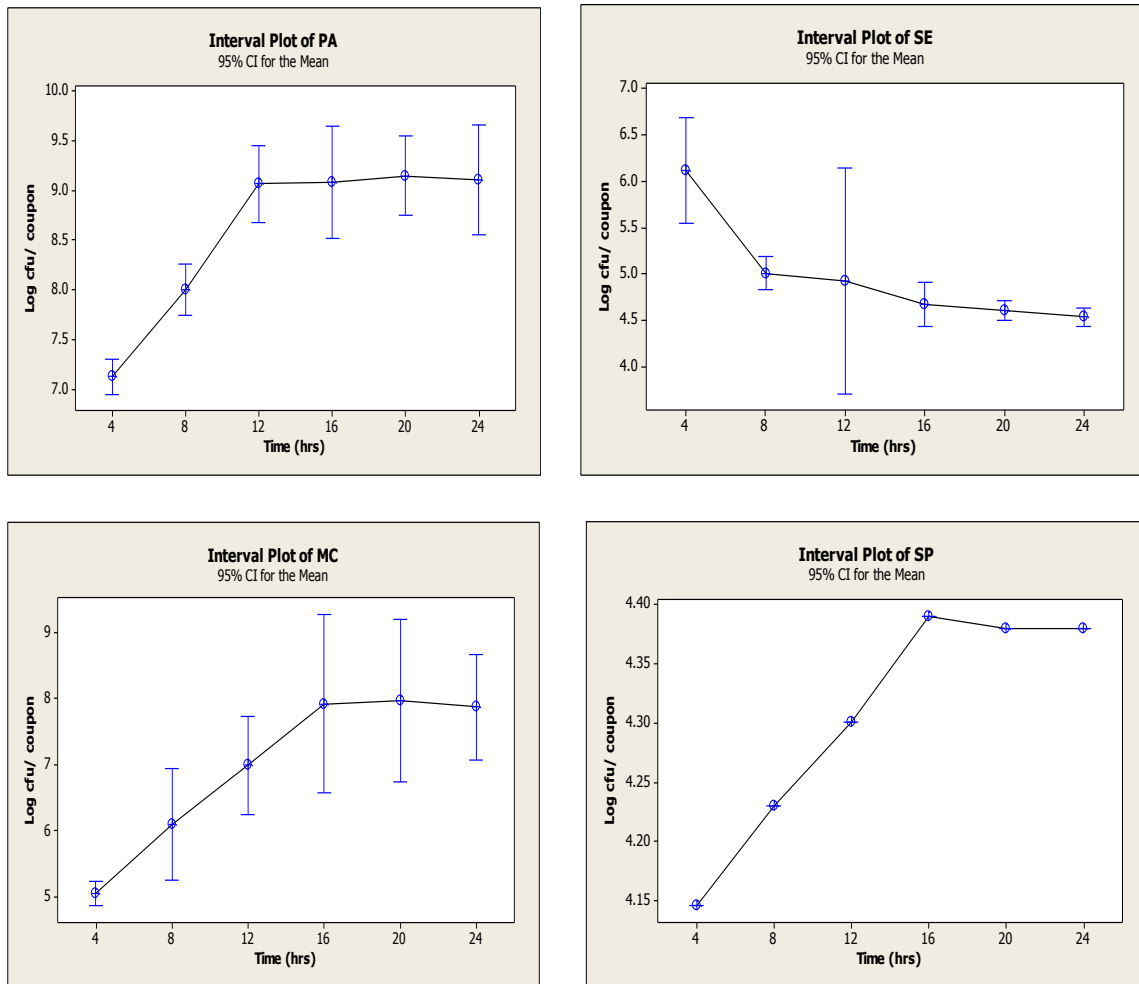


Fig.24. Four-species combination trial of *P. aeruginosa*, *S. epidermidis*, *M. catarrhalis* and *S. pneumoniae* in CDC biofilm reactor system. Pseudomonas isolation agar, Baird-Parker agar, Moraxella isolation agar and Streptococcus isolation agar were used for isolating the above bacteria from mixed species biofilm. Sampling at each time interval was performed with two replicates. Log colony forming units per coupon were analyzed at each 4 hours time interval till 24 hours.

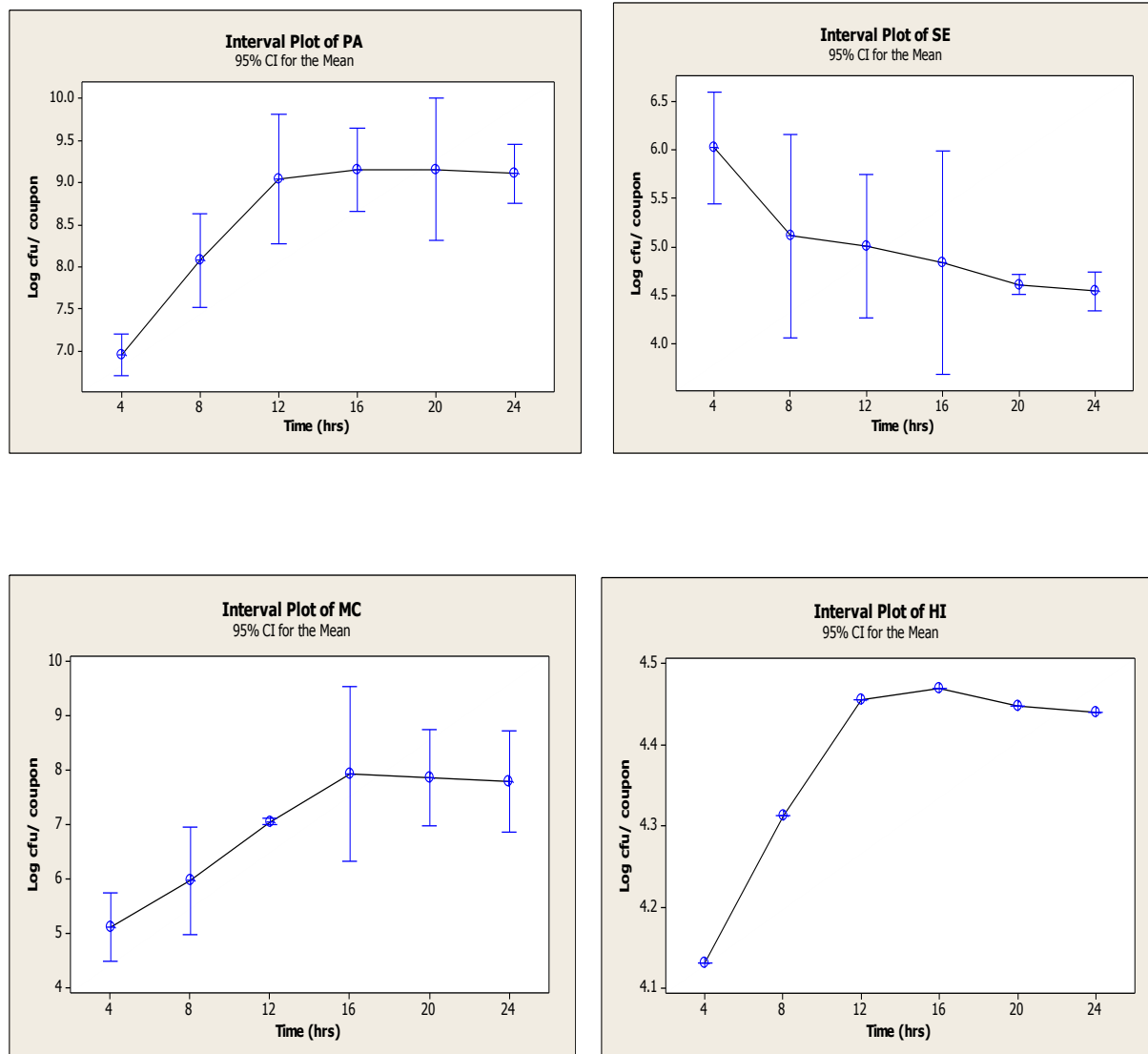


Fig.25. Four-species combination trial of *P. aeruginosa*, *S. epidermidis*, *M. catarrhalis* & *H. influenzae* in CDC biofilm reactor system. Pseudomonas isolation agar, Baird-Parker agar, Moraxella isolation agar and Haemophilus isolation agar were used for isolating the above bacteria from mixed species biofilm. Sampling at each time interval was performed with two replicates. Log colony forming units per coupon were analyzed at each 4 hours time interval till 24 hours.

These major species were then combined with the pathogens *S. pneumoniae* & *H. influenzae*, which have a lower relative ability to form biofilms. In this combination experiment, *P. aeruginosa*, *S. epidermidis* and *M. catarrhalis* were first combined with *S. pneumoniae*, where the *S. pneumoniae* reached a density of 4.4 log cfu/ coupon after 24 hours (Fig. 24). Then the three major species were combined with *H. influenzae*, where it reached a density of 4.4 log cfu/ coupon within 24 hours (Fig.25).

Finally, all the five pathogens involved in this study of CRS were combined together, where the results showed the biofilm forming capacity of all pathogens in a mixed species biofilm (Fig.26). In another trial *S. epidermidis* was replaced by *S. aureus* to compare the growth rate (Fig.27). *S.aureus* also showed similar growth pattern as *S. epidermidis*. This shows the inhibited growth of *Staphylococcus* species in the presence of *Pseudomonas* species (Qin et al., 2009)

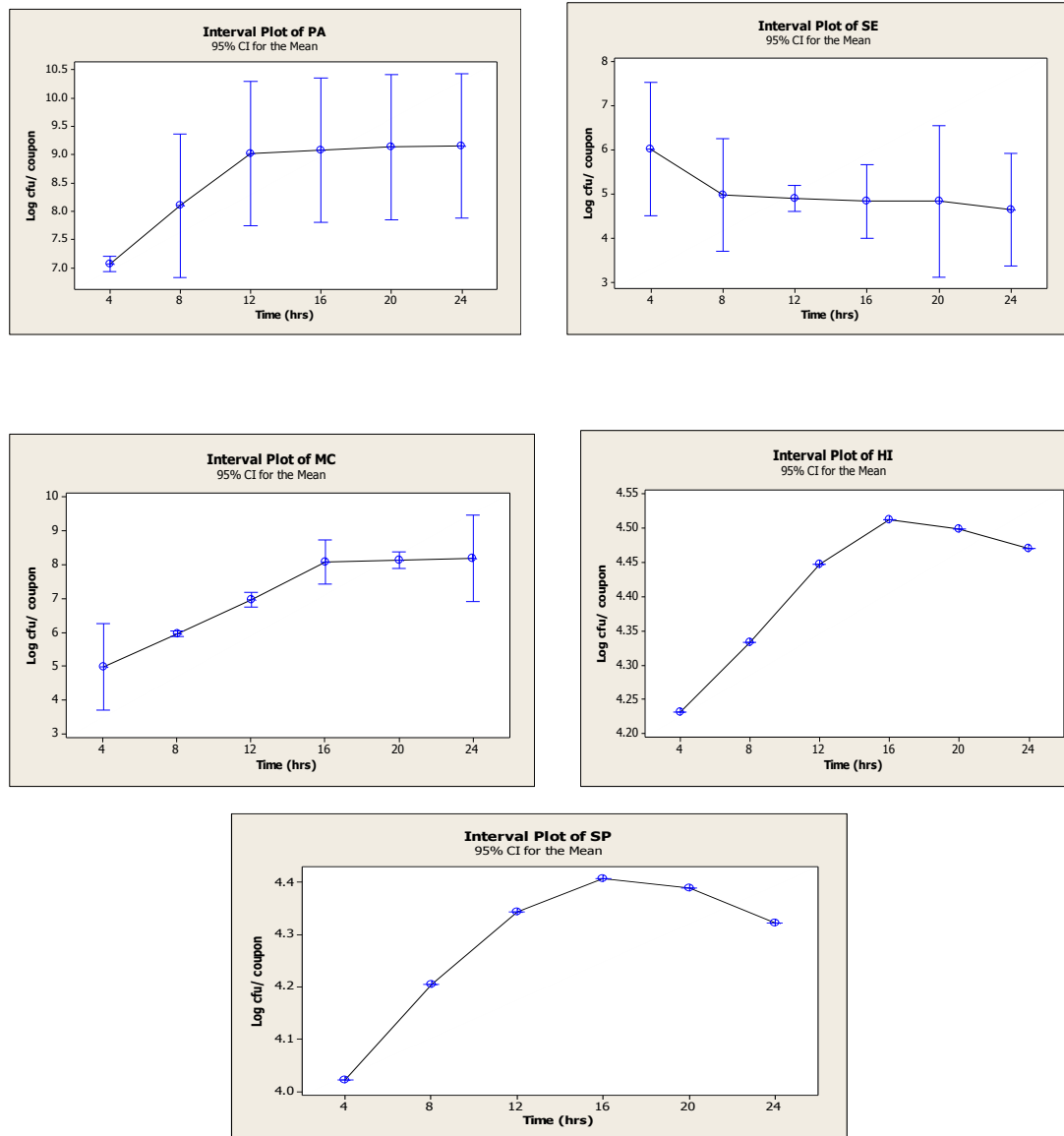


Fig.26. Five-species combination trial of *P. aeruginosa*, *S. epidermidis*, *M. catarrhalis*, *H. influenzae* and *S. pneumoniae* in CDC biofilm reactor system. Pseudomonas isolation agar, Baird-Parker agar, Moraxella isolation agar, Haemophilus isolation agar and Streptococcus isolation agar were used for isolating the above bacteria from mixed species biofilm. Sampling at each time interval was performed with two replicates. Log colony forming units per coupon were analyzed at each 4 hours time interval till 24 hours.

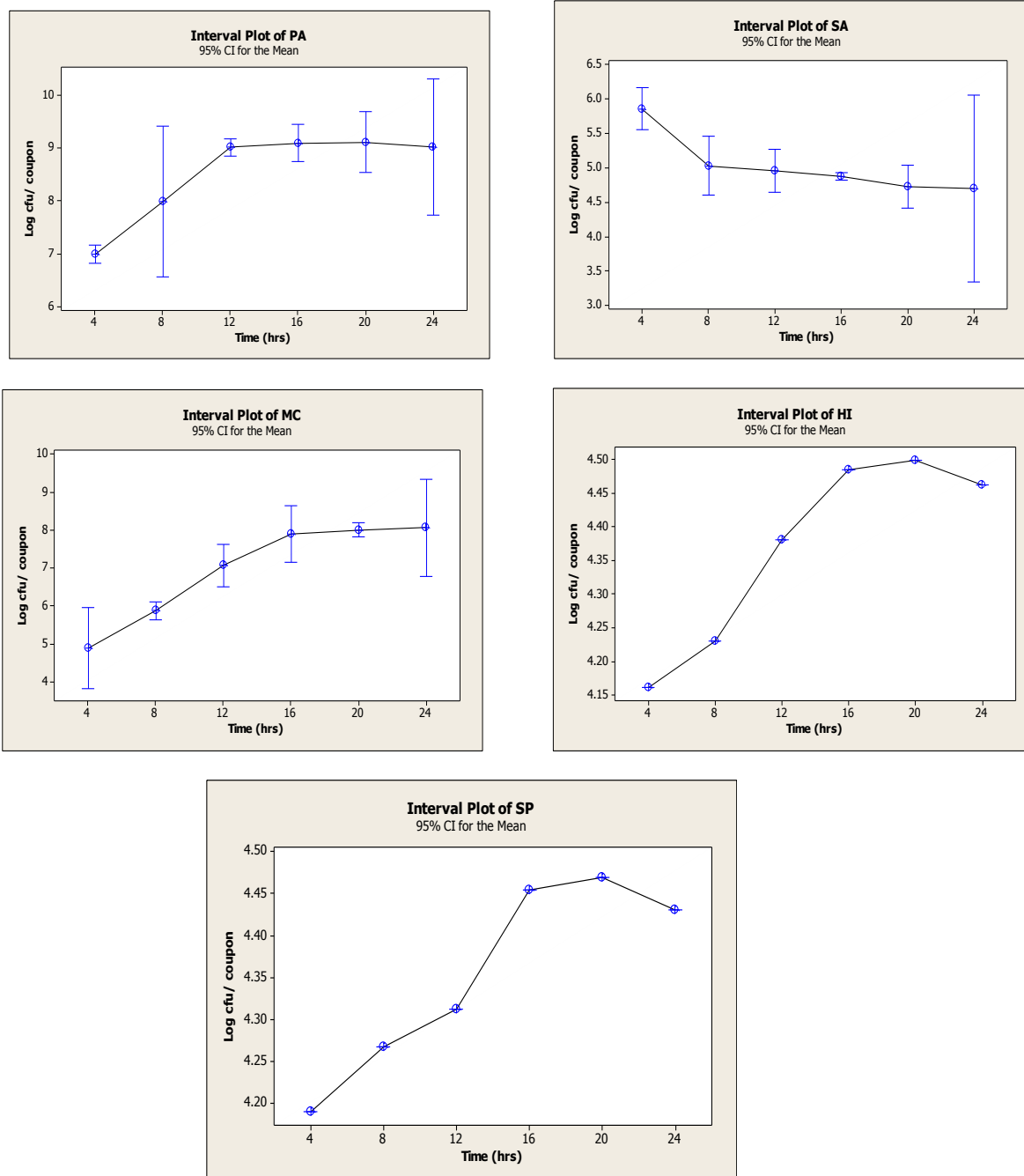


Fig. 27. Five species combination trial of *P. aeruginosa*, *S. aureus*, *M. catarrhalis*, *H. influenzae* and *S. pneumoniae* in CDC biofilm reactor system. Pseudomonas isolation agar, Baird-Parker agar, Moraxella isolation agar, Haemophilus isolation agar and Streptococcus isolation agar were used for isolating the above bacteria from mixed species biofilm. Sampling at each time interval was performed with two replicates. Log colony forming units per coupon were analyzed at each 4 hours time interval till 24 hours.

Micro Organism	μ (h ⁻¹)
<i>P. aeruginosa</i>	0.56
<i>S. epidermidis</i>	0.54
<i>M. catarrhalis</i>	0.26

Table 1. Growth rates of bacteria in pure species trials.

Mixed Species Combination	μ (h ⁻¹)
<i>P. aeruginosa</i>	0.58
<i>S. epidermidis</i>	0.16
<i>P. aeruginosa</i>	0.56
<i>M. catarrhalis</i>	0.57
<i>S. epidermidis</i>	0.58
<i>M. catarrhalis</i>	0.55
<i>P. aeruginosa</i>	0.55
<i>S. epidermidis</i>	-0.15
<i>M. catarrhalis</i>	0.53
<i>P. aeruginosa</i>	0.56
<i>S. epidermidis</i>	-0.07
<i>M. catarrhalis</i>	0.55
<i>S. pneumoniae</i>	0.04
<i>P. aeruginosa</i>	0.59
<i>S. epidermidis</i>	-0.09
<i>M. catarrhalis</i>	0.54
<i>H.influenzae</i>	0.06
<i>P. aeruginosa</i>	0.57
<i>S. epidermidis</i>	-0.02
<i>M. catarrhalis</i>	0.59
<i>H.influenzae</i>	0.05
<i>S. pneumoniae</i>	0.10
<i>P. aeruginosa</i>	0.58
<i>S. aureus</i>	-0.03
<i>M. catarrhalis</i>	0.57
<i>H.influenzae</i>	0.06
<i>S. pneumoniae</i>	0.04

Table 2. Growth rates of bacteria in mixed species combination trials.

Growth rates were estimated for both pure species and mixed species trials (Tables 1 & 2). *P. aeruginosa* showed a constant growth rate of approximately 0.5 h^{-1} in both pure species and mixed species trials. *S. epidermidis* exhibited a negative growth rate in combination with *P. aeruginosa*, whereas it showed a positive growth rate in the pure species trial and also in the combination with *M. catarrhalis*. *S. aureus* also showed a negative growth rate, in the five-species combination trial.

The growth rate of *M. catarrhalis* increased from 0.2 h^{-1} in the pure species trial to 0.5 h^{-1} in the mixed species experiment. Both *H. influenzae* and *S. pneumoniae* showed a poor growth rate in all trials.

One of the major limitations of this *in vitro* study of CRS is the fact that the cells were grown on polycarbonate coupons rather than tissue and there is no flow washing out planktonic cells. This represents a very artificial condition.

Future Work

CRS Biofilms are composed of complex (often) multi-species bacterial organizations, and so preparing DNA probes for one species and performing FISH (fluorescence in situ hybridization) with this probe allows one to visualize the distribution of this specific species within the biofilm. Preparing probes (in two different colors) for two species allows visualizing and studying the co-localization of these two species in the biofilm, and can be useful in determining the fine architecture of the biofilm. Also, fluorescent antibodies can be used in confocal laser scanning microscope to view the spatial arrangements throughout the biofilm.

CHAPTER 6

CONCLUSION

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CONCLUSION

Chronic rhinosinusitis is one of the most prevalent chronic diseases in the world, affecting people of all age groups (Aukema & Fokkens, 2004). Biofilm formation is now commonly seen in almost all of the chronic infections caused by bacteria (Anand, 2004). The experiments described in this thesis were designed to characterize the biofilm forming capacity and to measure the biofilm cell density achieved by the major species involved in CRS.

The crystal violet (CV) assay was a convenient method for the initial screening of the attachment and biofilm formation by bacteria involved in CRS (Christensen, 1985). This technique helped to determine which of the strains have the ability to form biofilms. The CDC biofilm reactor system appears to allow biofilm growth by all these bacteria (Goeres et al., 2005). Also this technique allowed enumeration of the viable numbers of bacteria present in the biofilm community by analyzing the coupons treated in, the biofilm reactor.

According to the crystal violet micro-titer plate assay, *P. aeruginosa*, *S. epidermidis*, and *M. catarrhalis* showed maximum absorbance values >1 at 550 nm, which indicated a high level of biofilm forming capacity. All these bacteria were grown in a CDC biofilm reactor. Several combination trials were carried out to analyze the growth rate of the pure species and the mixed species biofilms by sampling the coupons at 4, 8, 12, 16, 20 and 24 hour time intervals.

According to these experiments, *P. aeruginosa* has the maximum biofilm forming capacity. This pathogen showed a steady rate in pure species as well as in mixed

species biofilm formation. The pure culture of *S. epidermidis* was able to grow biofilms, but it decreased in numbers from the initial level when it was combined with *P. aeruginosa*. *M. catarrhalis*, also has the capacity to form biofilms, but showed an increased biofilm cell density when it was grown in mixed culture.

S. pneumoniae and *H. influenzae* were not good biofilm formers in pure culture, but these pathogens showed some attachment when they were combined in mixed culture.

This work completes a preliminary study of the biofilm forming capacity of bacteria involved in CRS and provides the necessary methods and preliminary work for studies in vitro of novel treatment methods. However, the CDC reactor runs represent a very artificial environment – they do not approximate to the conditions in the sinus membranes. The next stage is to study the behavior of the mixed cultures in a continuous flowing system and ultimately on tissues or tissue cultures.

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APPENDICES

APPENDIX I

CRYSTAL VIOLET BIOFILM ASSAY					
	Absorbance at 550 nm				
Wells	<i>P.aeruginosa</i>	<i>S.epidermidis</i>	<i>M.catarrhalis</i>	<i>S.pneumoniae</i>	<i>H.influenzae</i>
1	3.302	3.466	2.896	0.136	0.179
2	3.138	3.466	3.053	0.255	0.050
3	3.159	3.275	2.907	0.111	0.058
4	3.050	3.175	3.103	0.183	0.041
5	3.164	3.466	2.930	0.226	0.081
6	3.154	3.466	2.796	0.138	0.066
7	3.469	3.466	3.225	0.283	0.075
8	3.440	3.466	3.064	0.313	0.383
Mean	3.2345	3.40575	2.99675	0.205625	0.116625

APPENDIX II

ATTACHMENT ASSAY					
	Absorbance at 550 nm				
Wells	<i>P.aeruginosa</i>	<i>S.epidermidis</i>	<i>M.catarrhalis</i>	<i>S.pneumoniae</i>	<i>H.influenzae</i>
1	0.416	0.557	0.337	0.025	0.105
2	0.558	0.659	0.370	0.024	0.090
3	0.432	0.593	0.350	0.006	0.058
4	0.389	0.450	0.343	0.016	0.052
5	0.550	0.464	0.280	0.067	0.069
6	0.456	0.394	0.260	0.012	0.036
7	0.462	0.406	0.342	0.008	0.091
8	0.716	0.505	0.380	0.018	0.058
Mean	0.497375	0.5035	0.33275	0.022	0.069875

APPENDIX III

DUAL SPECIES BIOFILM ASSAY										
	Absorbance at 550 nm									
Wells	<i>P.aeruginosa</i> & <i>S.epidermidis</i>	<i>P.aeruginosa</i> & <i>M.catarrhalis</i>	<i>P.aeruginosa</i> & <i>H.influenzae</i>	<i>P.aeruginosa</i> & <i>S.pneumoniae</i>	<i>S.epidermidis</i> & <i>M.catarrhalis</i>	<i>S.epidermidis</i> & <i>H.influenzae</i>	<i>S.epidermidis</i> & <i>S.pneumoniae</i>	<i>M.catarrhalis</i> & <i>H.influenzae</i>	<i>M.catarrhalis</i> & <i>S.pneumoniae</i>	<i>H.influenzae</i> & <i>S.pneumoniae</i>
1	0.992	0.657	0.729	1.099	2.578	1.023	1.796	0.465	1.629	0.351
2	0.909	1.841	0.786	1.471	2.679	1.584	1.405	0.502	1.147	0.875
3	1.065	1.468	1.110	1.249	2.980	1.606	2.166	0.740	2.054	0.542
4	1.254	1.323	1.402	1.503	2.610	2.070	1.269	0.658	1.152	1.076
5	1.548	1.171	1.151	1.626	2.781	1.836	1.897	1.045	1.639	1.380
6	1.275	1.665	1.604	1.876	2.589	1.877	1.827	1.104	2.042	1.808
7	1.957	1.176	1.121	2.008	2.946	1.430	1.459	1.945	1.663	2.270
8	1.135	0.859	1.280	1.318	2.851	1.017	2.293	2.514	2.430	2.139
Mean	1.2668	1.27	1.1478	1.5187	2.75175	1.55537	1.764	1.12162	1.7195	1.3051

APPENDIX IV

Pure Species Biofilm growth of major pathogens involved in CRS				
Using CDC Biofilm reactor				
Time Interval		Log cfu/ coupon		
hrs		<i>P. aeruginosa</i>	<i>S. epidermidis</i>	<i>M. catarrhalis</i>
4		6.1537	5.9395	4.7671
8		7.1139	7.0294	5.9395
12		8.1972	7.8293	6.0394
16		9.1221	8.7889	6.1287
20		9.1382	8.9614	6.1461
24	i)	9.1875	9.0899	6.1477
	ii)	9.0969	9.1658	6.1945
	iii)	9.2041	9.0737	6.1399
	iv)	9.1732	9.1255	6.0773
	v)	9.1139	9.0473	6.1004
	vi)	9.1583	9.0253	6.1553
	Mean	9.1556	9.0879	6.1358

APPENDIX V

Dual Species Biofilm growth of major pathogens involved in CRS							
Using CDC Biofilm reactor							
Time Interval		Log cfu/ coupon					
hrs		<i>P. aeruginosa</i> & <i>S. epidermidis</i>		<i>P. aeruginosa</i> & <i>M. catarrhalis</i>		<i>S. epidermidis</i> & <i>M. catarrhalis</i>	
		PA	SE	PA	MC	SE	MC
4		5.9186	5.9978	6.1832	6.1461	6.0193	5.0717
8		7.0788	5.7499	7.2805	7.1613	7.0348	6.0057
12		8.0651	5.1628	8.3155	8.1461	8.0071	7.0241
16		9.0446	5.1003	9.2955	8.0792	9.1058	7.1643
20		9.2345	5.0510	9.3162	8.0511	9.1066	7.1817
24	i)	9.2041	5.0792	9.2175	8.0212	9.1553	7.2148
	ii)	9.0212	5.0212	9.1903	8.0881	9.1287	7.1903
	iii)	9.1903	5.0414	9.1761	8.3010	9.1461	7.1931
	iv)	9.1732	5.0607	9.1173	8.0773	9.1628	7.2082
	Mean	9.1472	5.0506	9.1753	8.1219	9.1482	7.2016

APPENDIX VI

Mixed species biofilm formation in CDC Biofilm reactor												
Multiple combinations of CRS pathogens												
T. I	Cp	<i>P. aeruginosa</i> , <i>S. epidermidis</i> & <i>M. catarrhalis</i>			<i>P.aeruginosa</i> , <i>S. epidermidis</i> , <i>M. catarrhalis</i> & <i>S.pneumoniae</i>				<i>P.aeruginosa</i> , <i>S.epidermidis</i> , <i>M. catarrhalis</i> & <i>H.influenzae</i>			
hrs		Log cfu/ coupon			Log cfu/ coupon				Log cfu/ coupon			
		PA	SE	MC	PA	SE	MC	SP	PA	SE	MC	HI
4	I	7.1673	5.9445	5.1189	7.1351	6.1613	4.9777		6.9731	5.9708	5.0626	
	II	7.1553	6.0737	5.3263	7.1072	6.0719	5.0414		6.9345	6.0607	5.1613	
	Mean	7.1613	6.0091	5.2226	7.1211	6.1166	5.0095	4.1461	6.9538	6.0157	5.1119	4.1303
8	I	7.9845	4.7482	5.9638	7.9801	4.9934	6.0294		8.0273	5.0253	5.8865	
	II	7.9217	5.0212	6.0607	8.0212	5.0212	6.1613		8.1139	5.1903	6.0414	
	Mean	7.9531	4.8847	6.0122	8.0071	5.0073	6.0953	4.2304	8.0731	5.1078	5.9639	4.3117
12	I	9.0531	4.4771	7.0701	9.0354	4.8293	6.9243		8.9754	4.9445	7.0531	
	II	9.0916	4.6674	7.0969	9.0969	5.0212	7.0414		9.0969	5.0607	7.0607	
	Mean	9.0723	4.5722	7.0835	9.0699	4.9252	6.9828	4.3010	9.0253	5.0026	7.0569	4.4548
16	I	9.2041	4.6385	8.0607	9.1303	4.6857	8.0212		9.1121	4.7482	8.0607	
	II	9.2553	4.5798	7.9638	9.0414	4.6483	7.8095		9.1903	4.9294	7.8062	
	Mean	9.2297	4.6091	8.0122	9.0858	4.6670	7.9153	4.3902	9.1512	4.8221	7.9334	4.4698
20	I	9.1717	4.1903	8.1628	9.1803	4.6128	7.8663		9.0846	4.6483	7.7889	
	II	9.0969	4.2553	8.0414	9.1173	4.5966	8.0607		9.2175	4.6674	7.9294	
	Mean	9.1343	4.2228	8.1406	9.1459	4.6047	7.9635	4.3802	9.1544	4.6578	7.8591	4.4471
24	I	9.1477	4.4771	8.1351	9.0588	4.5250	7.8028		9.0755	4.5854	7.7283	
	II	9.0607	4.2304	8.1461	9.1461	4.5563	7.9294		9.1303	4.5051	7.8750	
	Mean	9.1042	4.3537	8.1406	9.1024	4.5406	7.8661	4.3800	9.1029	4.5452	7.8016	4.4393

APPENDIX VII

Mixed species biofilm formation in CDC Biofilm reactor											
Multiple combinations of CRS pathogens											
T. I	Cp	<i>P. aeruginosa, S. epidermidis, M. catarrhalis, S. pneumoniae, H. influenzae</i>					<i>P. aeruginosa, S. aureus, M. catarrhalis, S. pneumoniae & H. influenzae</i>				
hrs		Log cfu/ coupon					Log cfu/ coupon				
		PA	SE	MC	SP	HI	PA	SA	MC	SP	HI
4	I	7.0719	5.9021	4.8701			7.0021	5.8808	4.8095		
	II	7.0511	6.1403	5.0715			6.9754	5.8325	4.9777		
	Mean	7.0615	6.0212	4.9708	4.0212	4.2304	6.9887	5.8566	4.8936	4.1903	4.1613
8	I	7.9971	4.8843	5.9445			7.8721	4.9934	5.8543		
	II	8.1969	5.0847	5.9566			8.0969	5.0607	5.8921		
	Mean	8.0970	4.9845	5.9505	4.2041	4.3324	7.9786	5.0270	5.8732	4.2672	4.2304
12	I	8.9123	4.8836	6.9445			8.9961	4.9777	7.1139		
	II	9.1129	4.9294	6.9777			9.0212	4.9294	7.0253		
	Mean	9.0126	4.9065	6.9611	4.3424	4.4471	9.0090	4.9535	7.0696	4.3117	4.3802
16	I	8.9761	4.9031	8.0273			9.1161	4.8750	7.8357		
	II	9.1763	4.7708	8.1303			9.0607	4.8663	7.9542		
	Mean	9.0762	4.8369	8.0788	4.4065	4.5119	9.0866	4.8706	7.8949	4.4548	4.4843
20	I	9.0281	4.9777	8.1072			9.1513	4.6990	7.9934		
	II	9.2297	4.7076	8.1461			9.0607	4.7482	8.0212		\
	Mean	9.1289	4.8426	8.1266	4.3891	4.4983	9.1080	4.7236	8.0073	4.4698	4.4983
24	I	9.0450	4.5530	8.0901			8.9129	4.5857	7.9601		
	II	9.2458	4.7534	8.2905			9.1151	4.7994	8.1613		
	Mean	9.1454	4.6532	8.1903	4.3222	4.4698	9.0634	4.6857	8.0607	4.4313	4.4624