BIOFILM FORMATION AND IMMUNOMODULATION BY *ACINETOBACTER BAUMANNII* ON ENDOTRACHEAL TUBES: *IN VITRO* STUDY

Dissertation Report

Submitted in partial fulfilment for the requirement of the degree of

Master of Philosophy

in

Biomedical Technology

By

Shrikant Nema

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Sree Chitra Tirunal Institute for Medical Science and Technology,
Trivandrum 695012, Kerala, India

DECLARATION

I **Shrikant Nema** hereby declare that I had personally carried out the work depicted in the thesis entitled, "**Biofilm formation and Immunomodulation by *Acinetobacter baumannii* on endotracheal tubes: *In vitro* study**" under the supervision of Dr A Maya Nandkumar, Scientist G, Division of Microbial Technology, BMT WING SCTIMST. , No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

Date Signature:

Name of the candidate: (Shrikant Nema)

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES & TECHNOLOGY
TRIVANDRUM – 695011, INDIA
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CERTIFICATE

This is to certify that the dissertation entitled "**Biofilm formation and Immunomodulation by *Acinetobacter baumannii* on endotracheal tubes: *In vitro* study**" submitted by **Mr. Shrikant Nema** in partial fulfilment for the Degree of Master of Philosophy in Biomedical Technology to be awarded by this Institute. The entire work was done by him under my supervision and guidance at **Division of Microbial Technology**, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Thiruvananthapuram-695012.

Thiruvananthapuram Signature

Date ***Dr. A Maya Nandkumar***

Thesis entitled

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Evaluated and approved

By

Dr. A Maya Nandkumar	*Dr. Rekha MR*
(Name of Guide)	**(Name of thesis examiner)**

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Synopsis

Acinetobacter baumannii is a gram negative pleomorphic non-motile bacillus, an opportunistic bacterial pathogen primarily associated with hospital-acquired infections, specifically in the intensive care units. Despite increased number of cases of *A. baumannii* infections, the immunological responses that confer resistance to disease development are largely understudied. In the limited studies on the immune response mounted against *A. baumannii* there has been little investigation into the role of cytokines gene expression. They cause serious infections ranging from pneumonia, septicaemia, wound infections etc. *Acinetobacter* is often resistant to many commonly used antibiotics. Intubation of the respiratory tract (using endotracheal tube – ETT) would lead to the development of ventilator associated pneumonia (VAP). This is facilitated by the adhesion and subsequent biofilm formation by bacteria, which forms the hub of infection. Mortality rate associated with VAP is estimated to be 47.5% in hospital patients. Studying biofilm dynamics and immunomodulation is crucial in understanding the pathogenicity of this opportunistic pathogen. Biofilm formation is a mechanism adopted by most organisms at interphases and is the primary mode of survival. Therefore we in our study evaluated biofilm formation by clinical isolates and modulation of various cytokine gene expressions profiling by *A. baumannii* biofilms.

Methodology: Both qualitative and quantitative methods were used. Biofilm formation by ATCC and clinical isolates were quantified by crystal violet assay. Qualitative evaluation was carried out by microscopic evaluation of biofilms formed on ETT materials using crystal violet and Acridine orange staining. Biofilm architecture was confirmed using ESEM analysis. Immune modulations by *A. baumannii* biofilms were studied by challenging THP1 monocyte cell-line with biofilm loaded endotracheal tube and endotracheal tube alone, at

different time points (0, 2, 4, 8 hrs) the total RNA was isolated and the mRNA was used to evaluate the gene expression using real time PCR.

Result: Biofilm formation was assayed quantitatively and qualitatively**.** We have observed immune-modulations by *A. baumanii* biofilms on endotracheal tubes *vis ~a ~vis* endotracheal tube alone. Regulation of IL- 1β at all time points was observed, and it was up regulated. IL-8 was up regulated with 2 fold change in expression at 4 and 8 hours. TNF-α level indicates a significant up regulation at 8 hours.

Conclusion: It demonstrates that *A. baumannii* biofilm is capable of up regulating inflammatory cytokines –IL8 and TNF- α promoting macrophage phagocytosis. In conclusion, *A. baumannii* biofilm develop and are sustained on endotracheal tubes and inspite of up regulation of IL8 and TNF-α, leading to the assumption that other mechanism are at work in the persistence of biofilm on endotracheal tube that leads to development of ventilator associated pneumonia.

Key words: *Acinetobacter baumannii*, ventilator associated pneumonia, biofilm, Gene expression

Chapter 1

Introduction

1.1 Introduction

Ventilator-associated pneumonia (VAP) is the most frequent device related infection in intensive care units. VAP is categorized as pneumonia having a microbial origin that generally occurs within 48-72 hours following endotracheal intubation, with symptoms of fever, altered white blood cell count, changes in sputum characteristics etc,. The onset of VAP and the nature of the causative pathogen depend on the time of infection subsequent to intubation. Ideally, early onset of VAP i.e. within the first 100hrs is caused by pathogens that are susceptible to antibiotics, while late onset VAP is brought about by multi-drug resistant and more difficult to treat bacteria (Kalanuria *et al.* 2014; American Thoracic Society and Infectious Diseases Society of America 2005). Depending on the diagnostic criteria used rate of occurrence of ventilator-related pneumonia vary across institutions. As reported in the study done by Koenig S. et al, 2006, 9.3% of patients on mechanical ventilators developed pneumonia, and alarmingly, about 250,000 to 300,000 cases occur every year in the United States alone (Koenig and Truwit 2006). Evidently, VAP is a challenging disease to diagnose and treat, and is associated with high morbidity and mortality rates. Rello J. et al, 2006 has tried to bring in perspective on accuracy of diagnosing techniques, with survey of the post-mortem examinations, and found that only 69% of patients with VAP were accurately identified (Rello *et al.* 2006).

Endotracheal tubes (ETT) are disposable devices for keeping the airways patent and facilitate mechanical ventilation. Intubation is the single critical factor responsible for pneumonia with a mortality ranging from 0 to 50%. A number of bacteria such as *Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, Acinetobacter baumannii, Stenotrophomonas maltophila, S. epidermidis* play key role in the VAP. Recently, In United States of America and European centres, *Acinetobacter* species accounted for 7.9 % of bronchoscopically acknowledged ventilator associated pneumonia (Chastre and Fagon 2002;

Hurley 2016). *Acinetobacter baumannii* species are opportunistic gram negative bacteria which are typically associated with outbreaks in the hospital setting which can survive adverse conditions such as desiccation, nutrient starvation, antimicrobial treatments (Gaddy and Actis 2009) and has major antimicrobial resistance issues. There is evidence for (Falagas *et al.* 2006) and against (Garnacho *et al.* 2003) and increase in attributable mortality in association with *Acinetobacter baumannii* infections in the ICU. The presence of Hospital acquired pneumonia increases hospital stay by an average of 7–9 days per patient (Chastre and Fagon 2002; Rello *et al.* 2002) and in addition, imposes an extra financial burden on the hospital/individual. *A. baumannii* forms biofilms on abiotic surfaces such as polystyrene and glass as well as biotic surfaces such as epithelial cells and fungal filaments. Pili assembly and production of the Bap surface-adhesion protein play a role in biofilm initiation and maturation after initial attachment to abiotic surfaces (Gaddy and Actis 2009). It's been recently, reported that *Acinetobacter baumannii* was responsible for 29.4% of VAP in intensive care unit (ICU), after *Pseudomonas aeruginosa* (Chaari *et al.* 2013). Evidence suggests that the number of multiple-drug-resistant *A. baumannii* infections in intensive care unit (ICU) patients is on the rise, not only in North America but also in Europe and South America (Breslow *et al.* 2011; Peleg *et al.* 2008).

Over the three decades, a shocking increment in the antibiotic resistance of *A. baumannii* has been accounted for, a circumstance that prevents effective treatment. So as to create successful treatments against *A. baumannii* it is significant to comprehend the basis of host–bacterium interactions, especially those concerning the immune response of the host (García-Patiño et al., 2017a). *A. baumannii* is an emerging pathogen responsible for the cause of nosocomial infections in many hospitals. Incorporation of an ETT could produce injury and inoculate endogenous oropharyngeal bacteria in the low airway tract (Rello et al., 1996). Formation of biofilm on the surface of ETT is an almost universal phenomenon and it has

been related to the pathogenesis of ventilator-associated pneumonia (VAP) (Pneumatikos et al., 2009). Perotin et al. shown that *Acinetobacter baumannii* and *Pseudomonas aeruginosa* has been most frequently isolated in the 56% of the cases from endotracheal aspirates (ETA) (Gil-Perotin et al., 2012). A number of virulence traits of *A. baumannii*, such as biofilm formation in the Endotracheal tube (Breij et al., 2010; Lee et al., 2008), adherence and invasion to host cells (Choi et al., 2008; Lee et al., 2006) have been characterized. Endotracheal tubes (ETT) are disposable devices for keeping the airways patent and facilitate mechanical ventilation. Intubation is the single critical factor responsible for pneumonia with a mortality ranging from 0 to 50% (Koenig and Truwit, 2006). Recently, reported that *Acinetobacter baumannii* was responsible for 29.4% of VAP in intensive care unit (ICU), after *Pseudomonas aeruginosa* (Chaari et al., 2013). Evidence suggests that the number of multiple-drug-resistant *A. baumannii* infections in intensive care unit (ICU) patients is on the rise, not only in North America but also in Europe and South America (Breslow et al., 2011; Peleg et al., 2008). However, none of the literature covered the knowledge regarding immune responses to *A. baumannii* biofilm in or on endotracheal tube material that are critical to ventilator associated pneumonia development. Mortality rate was 47.5 % of VAP in the 90-day in-hospital patients (Heredia-Rodríguez et al., 2016). Despite of increase cases of *A. baumannii* infections, the immune systems that regulate infection are largely understudied. Limited studies on the immune response mounted against *A. baumannii;* there has been little investigation into the role of cytokines gene expression. Moreover, immune system has ability to recognize pattern-associated molecular patterns through pattern recognition receptor such as TLR, for example TLR-2 and TLR-4 found on the cell surface have been widely explored in the context of *A. baumannii* infection for example *baumannii* employs TLR2 and TLR4 to activate the expression of IL-8 and that sCD14 contributes to the recognition of this pathogen (March et al., 2010). Study clearly demonstrates that *A. baumannii* OMVs are

potent stimulators of pro-inflammatory cytokines, including IL-1β, IL-6, IL-8, in epithelial cells. Interleukin-1 receptor antagonist (IL-1ra) has been considered a requirement for host immune defence in pneumonia and it has been proved by the studies that IL-1ra polymorphism was associated with risk of multi drug resistance *A. baumannii* related pneumonia. (Hsu et al., 2012) similarly, It's reported that the there was rapid recruitment of neutrophils at the site of infection, as early as 4 h, which peaked at 24 h postinfection. Increased lethality and severity of infection was observed in neutrophil depleted hosts, together with delayed production of cytokines involved in neutrophil recruitment, including Tumour necrosis factor (TNF-α) (García-Patiño et al., 2017b) in case of *Pseudomonas aeruginosa* (Hawn et al., 2007) and *Cryptococcus neoformans* (Fuse et al., 2007) in different infections models. However, such studies have not been done in case of *baumannii* infection.

In this study with the aim to decipher the mechanisms of biofilm persistence and development of VAP, we sought to determine the role of various cytokines in the immune response cascade when challenged with *Acinetobacter baumanii* biofilms. For this monocyte response which forms part of the early immune response was looked at.

Chapter 2

Review of literature

2.1 Review of literature

Acinetobacter baumannii: An emerging hospital based pathogen

Acinetobacter baumannii is one of the most common causes of ventilator-associated pneumonia in intensive care units. *Acinetobacter baumannii* is a Gram-negative bacillus that is, aerobic, pleomorphic and non-motile. An opportunistic pathogen, *A. baumannii* infection has a high frequency among immunocompromised individuals, especially those who have had prolonged (>90 d) hospital stay (Montefour et al. 2008). It colonizes the skin as well as the respiratory and oropharynx of infected individuals (Sebeny et al. 2008). Recently it has been assigned as a "red alert" human pathogen, creating alert among the medical fraternity, emerging to a great extent from its broad anti-infection resistance spectrum (Cerqueira et al. 2011).

Domain:	Bacteria
Kingdom:	Eubacteria
Phylum:	Proteobacteria
Class:	Gammaproteobacteria
Order:	Pseudomonadales
Family:	Moraxellaceae
Genus:	Acinetobacter
Species:	A. baumannii
Binomial name	*Acinetobacter baumannii*

Table1: Scientific classification of Genus *Acinetobacter*

The Dutch microbiologist Beijerinck first isolated the organism in 1911 from soil using minimal media enriched with calcium acetate (Beijerinck et al. 1911). Originally described as *Micrococcus calco-aceticus*, the genus Acinetobacter (coming from the Greek “akinetos,” meaning non-motile) was proposed some 43 years later by Brisou and Prevot (Brisou et al. 1954) to differentiate it from the motile organisms within the genus Achromobacter. The genus *Acinetobacter* was widely accepted by 1968 after Baumann et al. published a comprehensive study of organisms such as *Micrococcus calco-aceticus*, *Alcaligenes*

hemolysans, *Mima polymorpha*, *Moraxella lwoffi*, *Herellea vaginicola* and *Bacterium anitratum*, which concluded that they belonged to a single genus and could not be further sub-classified into different species based on phenotypical characteristics. (Baumann et al. 1968) In 1971, the sub-committee on the Taxonomy of Moraxella and Allied Bacteria officially acknowledged the genus Acinetobacter based on the results of Baumann's 1968 publication (Lessel et al. 1971).

The genus Acinetobacter, as currently defined, comprises Gram-negative, strictly aerobic, non-fermenting, non-fastidious, non-motile, catalase-positive, oxidase-negative bacteria with a DNA G + C content of 39% to 47% (Peleg et al. 2008). Following DNA-DNA hybridization studies performed by Bouvet and Grimnot in 1986, the Acinetobacter genus now consists of 26 named species and nine genomic species (Nocera et al. 2011). Four species of *Acinetobacters* (*A. calcoaceticus*, *A. baumannii*, *Acinetobacter* genomic species 3 and Acinetobacter genomic species 13TU) have such phenotypic similarities that they are difficult to differentiate, and as such are often referred to as the *A. calcoaceticus*-complex. (Gerner et al. 1991) This nomenclature can be misleading as the environmental species *A. calcoaceticus* has not been implicated in clinical disease, while the other three species in the *A. calcoaceticus*-complex are perhaps the most clinically significant species, being implicated in both community-acquired and nosocomial infections (Seifer et al. 1997).

2.1.1 Species

Acinetobacter may be identified presumptively to the genus level as Gram-negative, catalase-positive, oxidase-negative, non-motile, non-fermenting coccobacilli. However, the organisms are often difficult to de-stain and, as such, are often incorrectly identified as Gram-positive. There is no definitive metabolic test that can distinguish *Acinetobacter* from other non-fermenting Gram-negative bacteria (Seifer et al. 1997). A method which is often used to identify to the genus level relies on the ability of the mutant *A. baylyi* strain BD413 trpE27 to

be transformed by crude DNA of any *Acinetobacter* species to a wild-type phenotype (i.e., the transformation assay of Juni17). While for species level identification, the 28 available phenotypic tests have proven to be 95.6% effective in identifying human skin-derived *Acinetobacter* (Vaneechoutte et al. 1995). However, phenotypic tests alone have proven to be ineffective in identifying more recently discovered genomic strains of *Acinetobacters* (Seifer et al. 1997).

More advanced molecular diagnostic methods have been developed for identification of Acinetobacter to the species level, these include:

- Amplified 16S rRNA gene restriction analysis (ARDRA) (Ehrenstein et al. 1996)
- High-resolution fingerprint analysis by amplified fragment length polymorphism (AFLP)20 N Ribotyping21 N tRNA spacer fingerprinting (Dolzani et al. 1995)
- Restriction analysis of the 16S–23S rRNA intergenic spacer sequences(Chang et al. 2005)
- Sequence analysis of the 16S–23S rRNA gene spacer region (Scola et al. 2004)
- Sequencing of the rpoB (RNA polymerase β-subunit) gene and its flanking spacers (Fournier et al. 2006)

2.1.2 Genome structure

A. baumannii is characterized by a single circular chromosome that contains 3,976,747 base pairs in which 3,454 are used for protein coding. One strain of *A. baumannii* called AYE contains an 86kb resistance island, called AbaR1, which is made up of 45 resistance genes and is currently the largest island known to date. Resistance Island is a section on a chromosome that contains genes necessary to code for antibiotic resistance. Of those 45 resistance genes, 25 genes code for resistance against many antibiotics such as: tetracycline, aminoglycosides, cotrimoxazole, and chloramphenicol. Not only does the resistance island code against antibiotics, but also for operons for arsenic and mercury resistance. There are 14

resistance genes that code for class 1 integrons, which are sections of the chromosome capable of recombination, expression, and integration. Mobility elements, such as transposase were found on 22 ORFs (open reading frames). The *A. baumannii* AYE has three plasmids, but none contain resistance markers. Not only does the strain AYE contain resistance genes, but also a common amino acid sequence with other organisms, which demonstrates genetic exchange, where "39 genes (44%) are likely to have originated from *Pseudomonas spp*., 30 (34%) from *Salmonella spp*., 15 (17%) from *Escherichia spp*., and four (4%) from other microorganisms".

2.1.3 Hospital based infection

A hospital-acquired infection (HAI), also known as a nosocomial infection, is an infection that is acquired in a hospital or other health care facility. To emphasize both hospital and nonhospital settings, it is sometimes instead called a health care–associated infection (HAI or HCAI). Such an infection can be acquired in hospital, nursing home, rehabilitation facility, outpatient clinic, or other clinical settings. Infection is spread to the susceptible patient in the clinical setting by various means. Health care staff can spread infection, in addition to contaminated equipment, bed linens, or air droplets. The infection can originate from the outside environment, another infected patient, staff that may be infected, or in some cases, the source of the infection cannot be determined. In some cases the microorganism originates from the patient's own skin microbiota, becoming opportunistic after surgery or other procedures that compromise the protective skin barrier. Though the patient may have contracted the infection from their own skin, the infection is still considered nosocomial since it develops in the health care setting.

2.1.4 Biofilm formation on endotracheal tube

Biofilms consist of microorganisms and their self-produced extracellular polymeric substances (Exopolysaccharide). A fully developed biofilm contains many layers including a matrix of exopolysaccharide with vertical structures, and a conditioning film. Vertical structures of microorganisms sometimes take the form of towers or mushrooms, and are separated by interstitial spaces.

Formation of biofilms is rather complex, but can be generalized in four basic steps: 1) deposition of the conditioning film which alter the surface properties of the substratum and allow microorganisms to adhere to the surface. 2) Microbial (planktonic) attachment to the conditioning film. 3) Growth and bacterial colonization, where production of polysaccharides that anchor the bacteria to the surface allow colonies to grow (Hjortso et al. 1995, Lennox et al. 2011) and 4) biofilm formation, where a fully developed biofilm will contain an EPS matrix and vertical structures separated by interstitial spaces.

Some of the cells are adsorbed to the surface for only a finite time, before being deadsorbed, in a process called “reversible adsorption” (Marshall et al. 1992) This initial attachment is based on electrostatic attraction and physical forces, but not due to any chemical attachments. Some of these reversibly adsorbed cells begin to make preparations for a lengthy stay by forming structures which may then permanently bind then to the surface within the next few hours, the pioneer cells proceed to reproduce and the daughter cells, form microcolonies on the surface and begin to produce a polymer matrix around the microcolonies, in an irreversible steps (Marshall et al. 1992)

Biofilms are permeated at all levels by a network of channels through which water, bacterial garbage, nutrients, enzymes, metabolites and oxygen move to and from, with gradients of chemicals and ions between micro-zones providing the power to shunt the substances around the biofilms. (Paraje et al. 2011) In a mature biofilm, more volume is occupied by the loosely

organized glycocalyx matrix (75-95%) than by bacterial cells (5-25%) (Prakash et al. 2003, Hjortso et al. 1995, Lennox et al. 2011) In most cases, the base of the biofilm is a bed of dense, with thickness up 5 to 50 μm, composed of a sticky mix of polysaccharides, other polymeric substances and water, all produced by the bacteria. (Costerton et al. 1999) Soaring 100 to 200 μm upwards are colonies of bacteria, shaped like mushrooms or cones. The development of a mature biofilm may take from several hours to several weeks, depending on the system. (Mittelman et al. 1996)

2.1.5 Regulation of Gene expression

Acinetobacter baumannii interacts with epithelial cells through the binding of a 34-kDa protein referred as outer membrane protein A (OmpA), as well as a TonB-dependent copper receptor (an energy transducer) to fibronectin (De Yang et al., 2000). One of the consequences of this interaction is the production of antimicrobial peptides. *In vitro* studies using skin and oral epithelial cells exposed to *A. baumannii* reported bacterial-induced expression of the human β-defensins (hBDs) hBD-2 and hBD-3 with antibacterial activity against *A. baumannii* (Moffatt et al., 2013). Interestingly, hBD-2 is also produced by airway epithelial cells during *A. baumannii* pneumonia, suggesting a conserved protective mechanism independent of the epithelial origin during an extracellular infection (March et al., 2010). The importance of the expression of hBDs for host protection is also observed during intracellular infections, where signalling dependent on the cytosolic pattern recognition receptors (PRRs), nucleotide-binding oligomerization domain (NOD) NOD1 and NOD2, results in hBD-2 production (Bist et al., 2014). Therefore, the use of antimicrobial peptides produced during the early stages of the infection with efficient bactericidal activity may be a therapeutic option. Besides the essential role of neutrophils in resolving *A. baumannii* infections, other immune cell types have been shown to be activated in response to this opportunistic pathogen. Monocytes and macrophages are among the first responding cells to

be recruited and/or activated by *A. baumannii*. Tissue-resident macrophages, such as alveolar macrophages, would be present at the site of infection before the recruitment of neutrophils. This situation confers an advantage for the early response against *A. baumannii*, so that macrophages can phagocyte and limit bacteria while neutrophils are recruited. *In vivo*, phagocytosis of *A. baumannii* by macrophages can be observed as early as 4 h postinfection, by then, neutrophils get recruited, and phagocytosis is underway. Phagocytosis by macrophages *in vitro* can be detected as early as 10 min after macrophage interaction with *A. baumannii* (Qiu et al., 2012). In addition to phagocytosis, macrophages produce high amounts of MIP-2, IL-6, and TNF-α in response to *A. baumannii* infection. Early production of MIP-2 by macrophages might be relevant for neutrophil recruitment but has not been formally proven. Upto extended period's postinfection (approximately 48 h), high levels of the cytokines and chemokine are maintained by macrophages, together with an increment in the production of other cytokines, including IL-10 and IL-1β. Even though macrophages take longer to kill equivalent amounts of bacteria than neutrophils do, the macrophages are capable of killing more than 80% of the phagocytosed bacteria within the first 24 h. A confirmed mechanism used by macrophages to kill bacteria is the production of nitric oxide (Qiu et al., 2012). Depletion of macrophages in an *in vivo* model of pneumonia resulted in a higher bacterial burden in comparison with control mice; however, unlike depletion of neutrophils (Rice, 2010) the lack of macrophages does not increase infection lethality (Tsuchiya et al., 2012) (Qiu et al., 2012). Similar results, showing an increased bacterial burden, were observed in a bacteremia model where macrophages were also depleted (Bruhn et al., 2014). These findings suggest that macrophages may be dispensable for the resolution of *A. baumannii* infection, but they might help to control bacterial replication at early phases of the pathogen–host interactions. Natural killer cells (NKs) represent another immune cell type acting during the early defense response against *A. baumannii*. Depletion of NKs in a

pneumonia model interferes with bacterial clearance and hence resolution of the infection. The mechanism through which NKs contribute to control *A. baumannii* pneumonia is indirect and relies on the production of the chemoattractant KC, which in turn recruits neutrophils to the site of infection (Tsuchiya et al., 2012). Finally, dendritic cells (DCs), the bridge between innate and adaptive immune responses, have been shown to become activated in response to *A. baumannii* LPS. Moreover, OmpA activates DCs' signaling via mitogen-activated protein kinases (MAPKs) and nuclear factor kappa B (NFκB), thus resulting in high expression of molecules involved in antigen presentation and production of the inflammatory cytokine IL-12. As a consequence, DCs are prone to polarize T cells into TH1 effectors (Lee et al., 2007).

2.1.6 Hypothesis

In this study with the aim to decipher the mechanisms of biofilm persistence and development of VAP, we hypothesised that immune mechanisms may be impaired at various levels and this could lead to development of VAP. So we sought to determine the role of various cytokines in the immune response cascade when challenged with *Acinetobacter baumanii* biofilms. For this monocyte response which forms part of the early immune response was looked at.

Chapter 3

Objectives

3.1 Objectives

1. Biochemical characterization of *A. Baumannii* (Clinical isolate)
2. Study of dynamics of *A. baumannii* Biofilm formation by microtiter plate method (Clinical isolate and ATCC strain)
3. Study of *A. Baumannii* (Clinical isolate alone) Biofilm formation on endotracheal tubes by
    - ESEM
    - Microscopy (AO staining)
    - Viable counting
4. Study antibiotic sensitivity pattern of *A. baumanii* (clinical isolate) by disk diffusion method
5. Immunomodulation by *A. baumanii* biofilms on endotracheal tubes.

Chapter 4

Materials and Methods

4.1 Materials and Methods

Materials: Endotracheal tube (ETT) manufactured by Teleflex Medical Sdn. Bhd., Malaysia was used in this study. ETT's were cut aseptically using sterile scissors and tweezers into 1cm long pieces followed by ethylene oxide (ETO) sterilization.

Sodium acetate was purchased from Merck, India. Strains used at various times were *A. baumannii* strains (ATCC BAA 747) and clinical isolates of *A. baumannii*. Tryptic soya broths (TSB), Tryptic soya agar (TSA), Muller Hinton media were acquired from Hi-Media, India. McFarland standard 1 (HiMedia, India), Deionised water (DI/W) was used throughout this study.

4.1.1 Biochemical Characterization of *A. baumannii*

Sugar utilisation by *A. baumannii* was done using Hugh-Leifson medium, Change in colour with production of gas confirms the utilisation of sugars (Glucose, mannose, xylose, and mannitol) by fermentation.

Indole test was done by growing cultures in Trypton water and adding Kovac's reagent and mixing. Indole production was confirmed by the presence of cheery red coloured ring at the interphase.

Christensen's Urea agar was used to confirm capability of organism to produce Urease. Simmons citrate agar was used to confirm the capability of the organism to use citrate as sole carbon source which gives it blue colour.

Oxidase test was done to confirm the capability of the organism to produce cytochrome C, when present; the cytochrome c oxidase converts the reagent (tetramethyl-p-phenylenediamine) to (indophenols) purple colour end product.

Catalase test was done by treating the culture to substrate hydrogen peroxide and its presence was confirmed by production of brisk effervescence.

4.1.2 Dynamic Bacterial Adhesion Study

1 cm × 1 cm pieces of endotracheal tube were cut and sterilized by ETO. *Acinetobacter baumannii* (ATCC and clinical isolates) was inoculated into TSB and allowed to grow at 37.5 ± 2.5 °C and 100 rpm in a shaker incubator. Culture was harvested at the log phase and brought to 10^8 CFU/mL using McFarland's standard 1. Dilution was made to get a final bacterial count of 10^5 CFU/mL. ETT test material in triplicates was placed into 20 mL of TSB with 10^5 CFU/mL of bacteria. These were incubated for 20−24 h in a shaking incubator at 35.5 ± 2.5 °C and 100 rpm. Each ETT was taken and washed thrice with normal saline to remove loosely adhered bacteria and then placed into a sterile tube with 1 mL of normal saline and sonicated for 1 min followed by 30 second of vortexing; this was repeated thrice to extract bacteria adhered to the ETT. The bacteria thus collected were diluted and inoculated onto Tryptic soya agar (TSA) plates in triplicate. Plates were incubated overnight at 37.0 ± 1.0 °C allowing bacteria to grow. Colonies were counted and extrapolated to CFU/ cm^2 of ETT. Experiments were repeated thrice, and an average of at the least 9 plates was taken for test ETT respectively, for concluding CFU/cm^2 of the ETT.

Culture characteristics: MacConkey agar (MA) and Blood agar (BA) were used to determine purity and colony characteristics. On MacConkey agar non lactose fermenting pale colonies were observed and there was no contamination. On Blood agar (BA) non-hemolytic colonies in pure culture were observed.

4.1.3 Biofilm formation assay by Crystal violet staining: ETT with biofilm were washed thrice with normal saline to remove loosely adhered bacteria and transferred into sterile test tube. They were fixed in 2.5% glutaraldehyde for 1 hour at 22 °C, washed in normal saline,

and stained with 1% crystal violet for 5 minutes. The excess stain was rinsed off by washing with normal saline. Later the ETT were air dried and observed under light microscope.

4.1.4 Acridine Orange staining

Biofilm was formed on ETT for 24 hours, ETT were transferred into sterile test tube and washed thrice with normal saline to remove loosely adhered bacteria and then placed into a sterile tube and fixed in 2.5% glutaraldehyde for 1 hour at 22 °C and washed with normal saline, and stained with Acridine orange (0.1M, pH 7.2) for 2 minutes. The excess of the stain was rinsed off by washing with normal saline. Later the ETT was air dried and seen under fluorescence microscope.

4.1.5 ESEM

Environmental scanning electron microscopy (ESEM) (FIE, Quanta 200) was used for studying bacteria biofilm architecture on endotracheal tubes. Biofilm was formed on ETT for 72 hours, ETT were transferred into sterile test tube and fixed overnight in 2.5% glutaraldehyde at 22^0C and washed with phosphate buffer followed by dehydration in series of increasing concentrations of alcohol (30, 50, 70, 90, and 100%) and air-dried. After that, the ETT were coated with gold for ESEM examination.

4.1.6 Biofilm assay

Clinical strain and ATCC strain were inoculated in Tryptic soy broth (TSB) and adjusted to McFarland standard 1. Three wells, sterile 96-well round bottomed dishes were filled with 200 µL of bacterial suspension. Negative controls contained only TSB. Then, plates were covered and aerobically incubated for 24, 48. 72, 96 hours at 4°C, 30°C and 37°C. Afterward, the content of each well was aspirated, rinsed three times with 250 µL of sterile normal saline, emptied and left to dry. Then, the plates were stained for 20 minutes with 0.2 mL of

1% crystal violet (Merck, Germany). The excess of the stain was rinsed off by washing with normal saline. Later the plates were air dried; the dye bound to the adherent cells was resolubilized with 95% ethanol. By using a multimode reader (BioTek), the OD of each well was measured at 620 nm.

4.1.7 Disk diffusion Test

Antibiotic sensitivity was assayed by disk diffusion assay. Clinical strain was inoculated on Mueller Hinton (MH) agar plates to form a uniform lawn. The different antibiotic discs were placed on the plates using sterile forceps and the plates were incubated overnight, the diameter of zone of inhibition was measured.

4.1.8 Cell culture maintenance

A human monocytic cell line (THP-1) derived from the blood of a male with acute monocytic leukaemia was obtained from ATCC. THP-1 was always cultured in RPMI-1640 containing, 10% fetal bovine serum (FBS), L-glutamine and antibiotics gentamicin (1%) and amphotericin (0.1%) at 37°C and in an atmosphere of 5% CO_2. The culture was maintained in 25cm^2 T-flask.

4.2 Assay of Cytokine gene modulations by biofilm

For this 10^3 cell/mL were inoculated into 24 well plates in HEPES containing RPMI-1640 medium and challenged with bacterial biofilm loaded endotracheal tube (clinical strain E1603), and endotracheal tube alone. At different time points (0, 2, 4, 8 hrs) the cells were harvested and total RNA extracted by Trizol method to evaluate the gene expression.

4.2.1 Isolation of RNA from THP1 cell

RNA was extracted from the cell using TRIzol reagent (Ambion) Protocol of the kit followed strictly. In brief, cells were collected from the dish using TRIzol reagent and kept on ice. For every 1ml of trizol 200µl of chloroform (merck) was added and mixed for 15 seconds. It was then incubated at room temperature for 5-10 minutes followed by a spin of 12000 rpm for 15 minutes at 4 °C. Three layers were formed – upper aqueous phase (colourless), interphase and lower phase (pink). The upper aqueous phase was transferred in to a fresh tube and 500µl of isopropanol was added. RNA was precipitated by incubation at room temperature for 5-10 minutes and then centrifuged at 12000g for 8 minutes at 4°C. The pellet was washed with 1 ml of 75% ethanol, air dried for 3 minutes and resuspended in 10-15 µl of RNase free autoclaved water and stored at -20° C. It was quantified using NanoVue™ Plus Spectrophotometer (GE Healthcare UK), ratio between 1.8 and 2.0 at 260nm/280 nm (A260/A280) absorbance were considered suitable for quantitative mRNA analysis using real time PCR (qRT-PCR).

4.2.2 Complementary DNA synthesis

Total of 1 µl volume is used for the cDNA synthesis. Components of the cDNA synthesis are given below in the table 2, using the C1000 Touch Thermal cycler (Bio Rad).

Components	Volume (µl)	Concentration
10X buffer	1	1X
2.5 mM $MgCl_2$	2	5mM
2.5 mM Dntp	2	500 µM
Nanomer	1	2.5 µM
RNase Inhibitor	0.2	0.4U/ µl
Euro script RT	0.25	1.25U/ µl
RNA template	1	
Water	2.55	

Table 2: Components used in the cDNA synthesis

4.2.3 Gene expression analysis using real time PCR

Cytokine genes analyzed in the study were IL-1β, IL-8, TNF-α, IL-6. β-actin was used as housekeeping gene and differential gene expression was calculated using the expression below

Delta CT = C(t)1 – C(t)2, where [$C(t)_2$ = β-actin] [$C(t)_1$ = Gene of interest]

Delta Delta CT = DC(t)1 – DC(t)2, where [DC(t)1= Ct of test , DC(t)2 = Ct of control]

All real-time PCR amplifications, data acquisition, and analysis were performed using an iQ5 real-time PCR system (Bio-Rad).

Components	Volume (µl)
Reaction mixture (Takyon)	10
Forward primer	2
Reverse primer	2
Water	3.5
DNA template	2.5

Table 3: Components used in RT-PCR

Genes	Forward and Reverse primers sequence
IL-1β	F:ATAAGCCCACTCTACAGCT R:ATTGGCCCTGAAAGGAGAGA
IL-6	F: CAGCCACTCACCTCTTCAGAAC R:TGCAGGAACTGGATCAGGAC
IL-8	F: GCTTTCTGATGGAAGAGAGC R:GGCACAGTGGAACAAGGACT
TNF- α	F: CCG TCT CCT ACC AGA CCA AGG 3’ R: CTG GAA GAC CCC TCC CAG ATA G 3’
β– Actin	F: GCG TGT GTG TGT GTG TGT GT-3’ R: CCT CCC TCC TCC CTA TGT GT-3’

Table 4: Cytokine primers used for qRT-PCR

Chapter 5

Result and Discussion

5.1 Results & Discussion

Acinetobacter baumannii is an opportunistic pathogen which has been recently notified as an emerging pathogen by WHO. This is because of the wide spectrum of antibiotic resistance exhibited by the organism and its ability to evade the host immune mechanism and persist and give rise to infections that are recalcitrant to treatment leading to increased mortality.

The infectious process and persistence has been least understood specifically in relation to medical device related infections and it is the leading cause of Ventilator associated pneumonia (VAP).

The immune system has evolved to protect the host from infection by either development of innate immunity or adaptive immunity. Innate immunity is the ability to produce response within minutes or hours after infection by recognizing certain ligands on pathogens triggering signalling cascades within these cells. The epithelial layer is the first line of attack in any pathogenic infection with the macrophages mounting phagocytosis. Previous work form out laboratory has shown that phagocytosis is impaired in case of biofilms and our intention was to look at the next step in the immune response – the role of monocytes. In patients when the endotracheal tube is implanted we have observed that there is development of biofilm by72 hours. We hypothesized that this would lead to immune modulations in monocyte responses and this was analysed.

Morphological analysis: Morphological analysis was done by gram staining and viewing under 1000X oil immersion microscopy. The organism was gram negative pleomorphic form usually seen as cocobacillary [Figure 1].

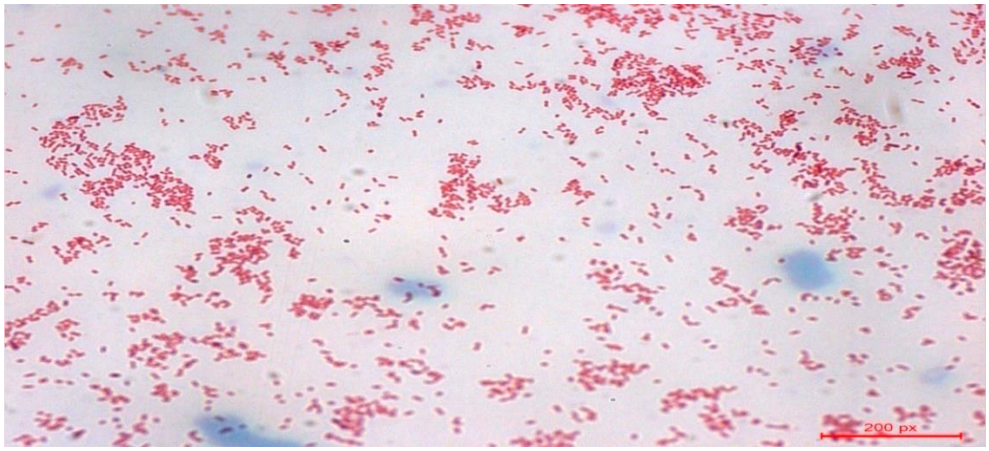

Figure 1: Gram staining (100x) *A. baumannii* clinical strain E1603 *(Gram negative)*

Growth and purity of cultures were determined by culture on MacConkey agar and Blood agar. On MacConkey agar it's formed pale coloured, Non lactose fermenting colonies and on Blood Agar it's formed non-hemolytic colonies. There was only one type of colonies attesting to its purity.

Antibiotic sensitivity assay for clinical isolate was done by disc diffusion assay. The clinical isolate was sensitive to the Ciprofloxacin, Gentamicin, Amikacin, Co-trimoxazole and Colistin tested [Figure 2] but it was resistant to Ceftazidime. The measurement of diameter of zone sizes is given in table 5.

5.1.1 Antibiotic sensitivity pattern analysis by Disk diffusion assay

Figure 2: E1603 (Clinical strain) was used for Antibiotic sensitivity pattern analysis by Disk diffusion assay *A. baumannii* is susceptible to Ciprofloxacin, Gentamicin, Amikacin, Co-trimoxazole and colistin while Ceftazidime is resistant to *A. Baumannii*

Antibiotics	Zone of Inhibition
Co-trimoxazole	36mm
Colistin	10mm
Amikacin	27mm
Gentamicin	23mm
Ciprofloxacin	38mm
Ceftazidime	No zone of Inhibition

Table 5: Antibiotics with zone of inhibition

5.1.2 Biochemical characterization of *A. baumannii*

Biochemical capabilities of organisms were analysed using different media for utilisation of amino acids and Sugars and enzyme production. The standard strain ATCC strain *A. baumannii* (ATCC® BAA- 747™) and Clinical isolate *A. baumanii* were used. Indole, oxidase [Figure 4] and catalase tests [Table 6] were done using standard microbiological procedures to understand presence of enzymes oxidase and catalase. Indole test was done to understand utilisation of amino acid tryptophan. They are listed in Table 6

Parameters	Clinical Isolates E1603	ATCC
Carbohydrate utilisation		
• Glucose (C6)	+	+
• Sucrose (C6)	-	-
• Mannitol (C12)	-	-
• Xylose (C5)	+	+
Simmon's Citrate	+	+
Urea	+	+
Motality	Non motile	Non motile
Indole test	-	-
Oxidase	-	-
Catalase	+	+
Mackonkey agar	Pale colonies	Pale colonies
Blood agar	Non hemolytic	Non hemolytic

Table 6: Biochemical characterization of *A. baumannii*

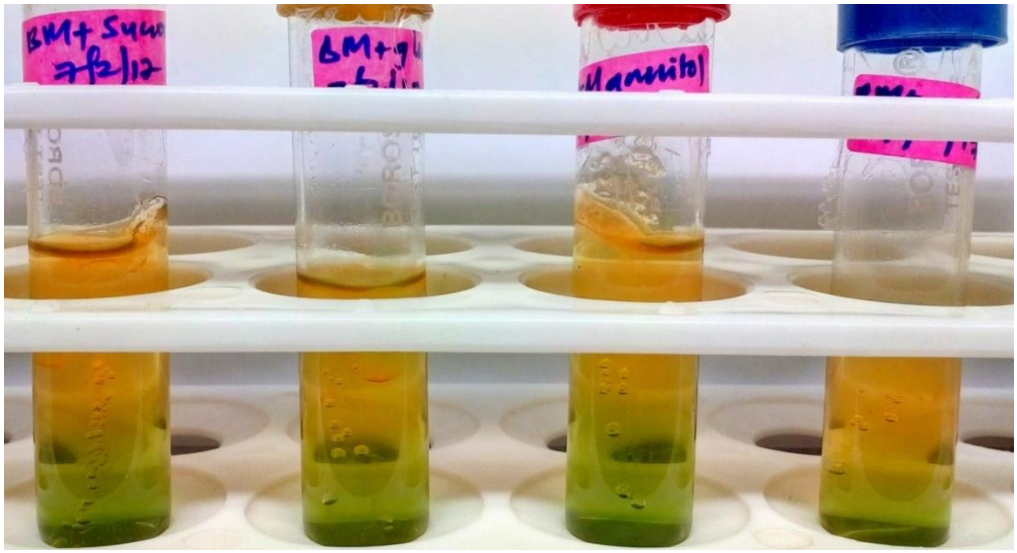

Figure 3: Test showing the ability of *A. baumannii* Clinical strain (E1603) to use by oxidation the given Carbohydrate (Sugar) Sucrose, Glucose, Mannitol and Xylose (From right to left)

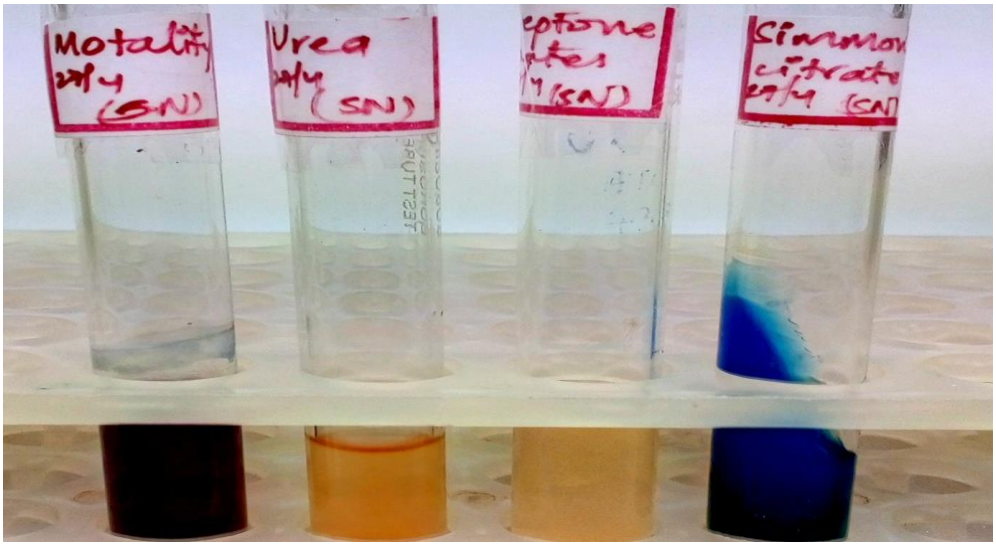

Figure 4: Test showing the Motility test, utilisation Urea test, Indole test, Simmons's citrate test of clinical strain (E1603) (From right to left)

5.1.3 Bacterial adhesion study: To understand adhesion and biofilm formation on ETT by *A baumannii* using clinical isolate both qualitative and quantitative methods were used.

Qualitative assay was done by Microscopy light, fluorescent and scanning electron microscopy. Figure 5 shows crystal violote staining of *Acinetobacter* biofilm formed on ETO sterilised ETT at 24 hours. Fluorescent micorscopy using acridiene orange staining also

was done to understand the initial biofilm formation. Figure 6 shows bacterial adhesion and initiation of biofilm formation on endotracheal tube by 24 hours.

To undertand the three dimensional microarchitecture of biofilm formation environmental scanning elctron mciroscopy was done after developing biofilm on ETT for 72 hours. Here in figure 7 the pleomorphic nature of *A. baumanni* is evident and the red arrow points to it. By 72 hours the biofilm archietcture was also evident with mushroom like growth, with water channel pointed out by the white arrow in figure 7.

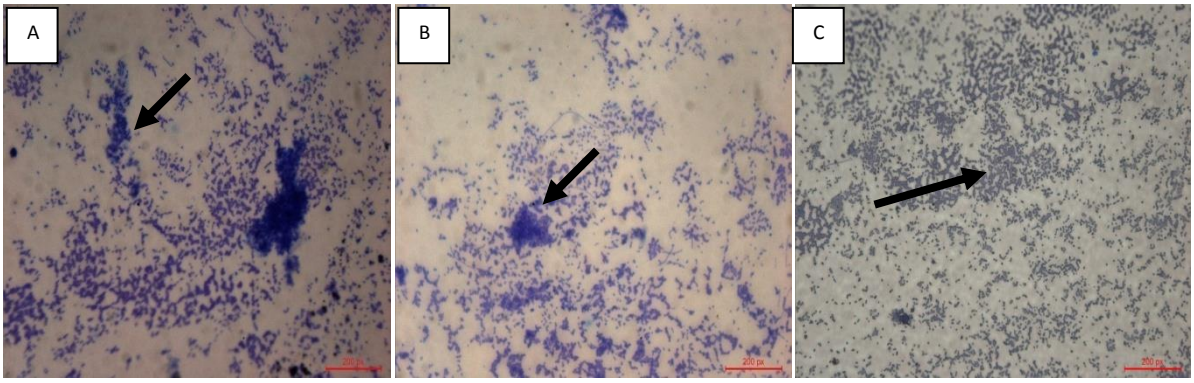

Figure 5: Formation of *A. baumannii* biofilm E1603 (clinical strain) on the surface of endotracheal tube (A) and (B) shown the biofilm formation by black arrow (c) aggregate formation by crystal violet staining

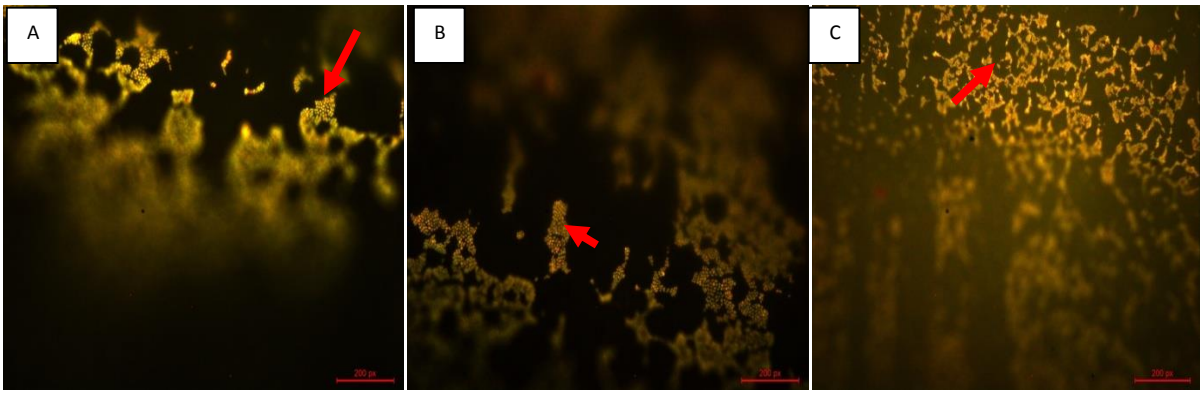

Figure 6: Formation of *A. baumannii* biofilm E1603 (clinical strain) on the surface of endotracheal tube (A) and (B) shown the biofilm formation by red arrow (c) aggregate formation by Acridine orange staining

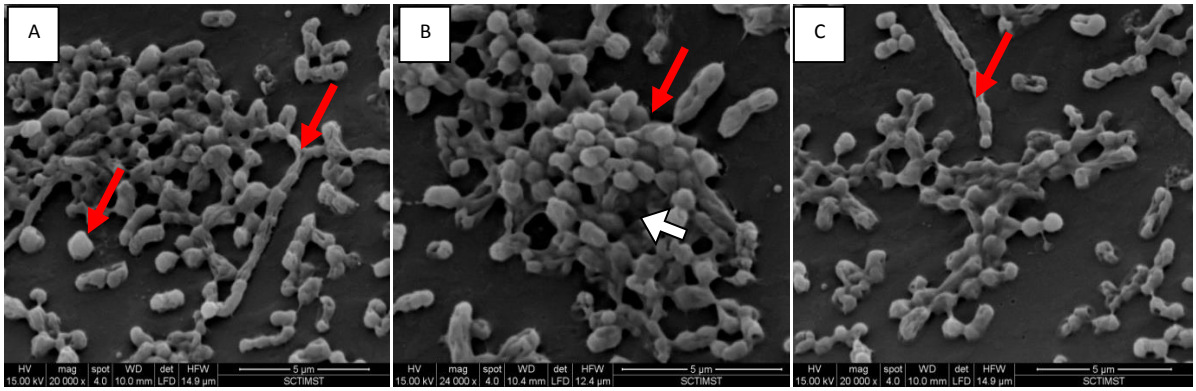

Figure 7: Formation of *A. baumannii* biofilm E1603 (clinical strain) on the surface of endotracheal tube by 72 hrs. Environmental scanning electron microscopy (ESEM) (A) showing the pleomorphic nature of bacteria by red arrow (B) white arrow showing the micro channel in biofilm (C) showing the cocci shape of bacteria on endotracheal tube

5.1.4 Bacterial adhesion Qualitative asaay: . Here bacterial adhesion was allowed to occur overnight in TSB inoculated with the clinical isolate at a concentration of $1x10^5$cfu/mL. Figure 8 shows the experimental setting and figure 5 shows the 1cm long ETT pieces cut for ETO sterilisation for performance of the assay.

Figure 8: Flask showing negative control (clear suspension) and positive control (clinical strain E1603) of TSB suspension (turbid due to bacterial growth)

Figure 9: 1cm long pieces have been cut from endotracheal tube (Teleflex Medical Sdn. Bhd., Malaysia) using sterile scissors and ETO sterilised

Figure 10 shows the viable number of bacteria that adhered to 1cm long ETT pieces within 18 hrs (Overnight) of culture in TSB. Here control was the planktonic form in the culture supernatant. The number of bacteria present in 5 and 10 µl volumes were counted. The figure 10 shows that the amount of bacteria that had adhered co-related the planktonic forms showing that *Acinetobacter* had preponderance for biofilm formation, adherence being the first step.

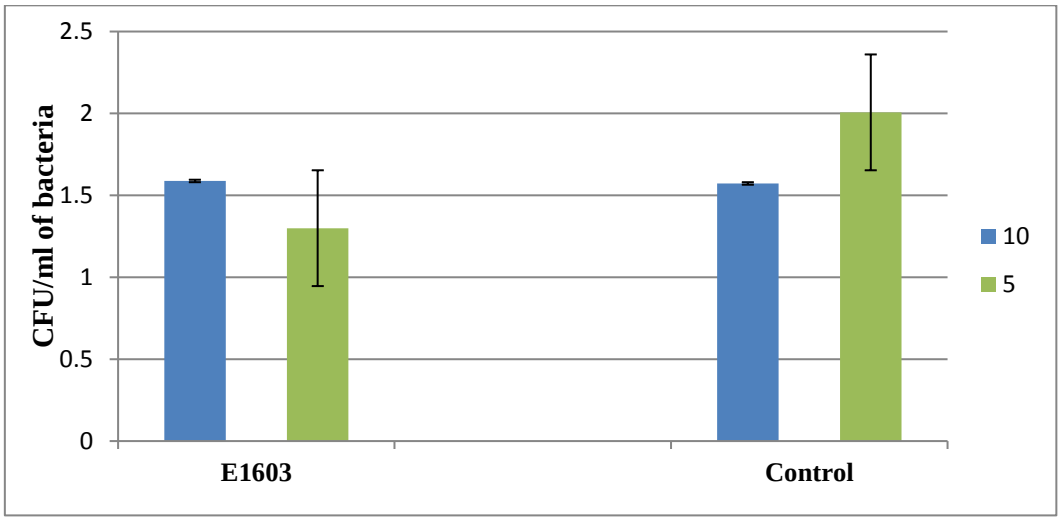

Figure 10: Bacterial adhesion study on endotracheal tube at 24 hours. E1603 (clinical strain) was used and Positive control (Bacterial suspension)

5.1.5 Biofilm assay:

Biofilm formation assay was done using the crystal violet microtiter plate assay at two temperatures of 37 ^{0}C which is normal body temperature in humans and 30 ^{0}c which is the environmental temperature in Kerala for ATCC strain and clinical strain (E1603).

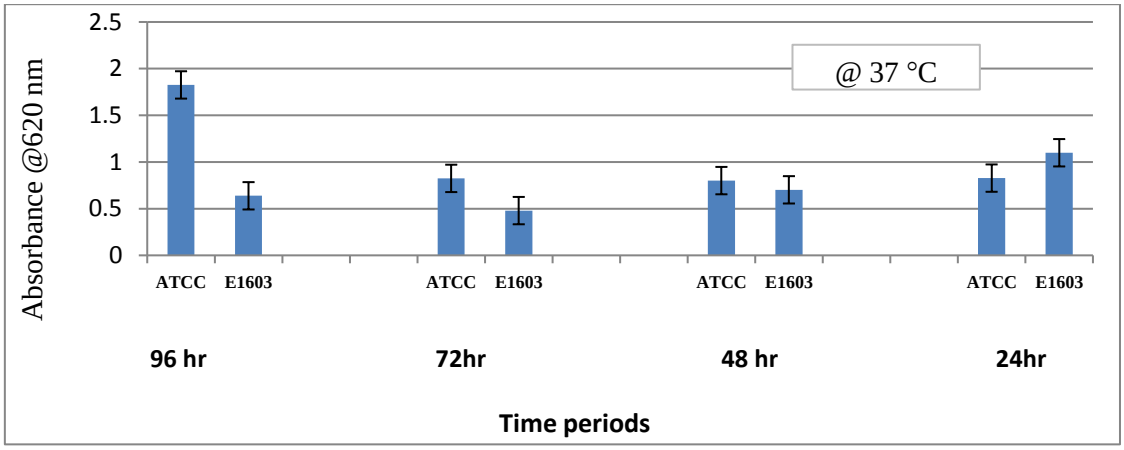

Figure 11: Showing the maximum biofilm formation shown at 37^{0}C (Body temperature) by ATCC strain in 96 hours, whereas E1603 (Clinical strain) shown maximum biofilm formation in 24 hours

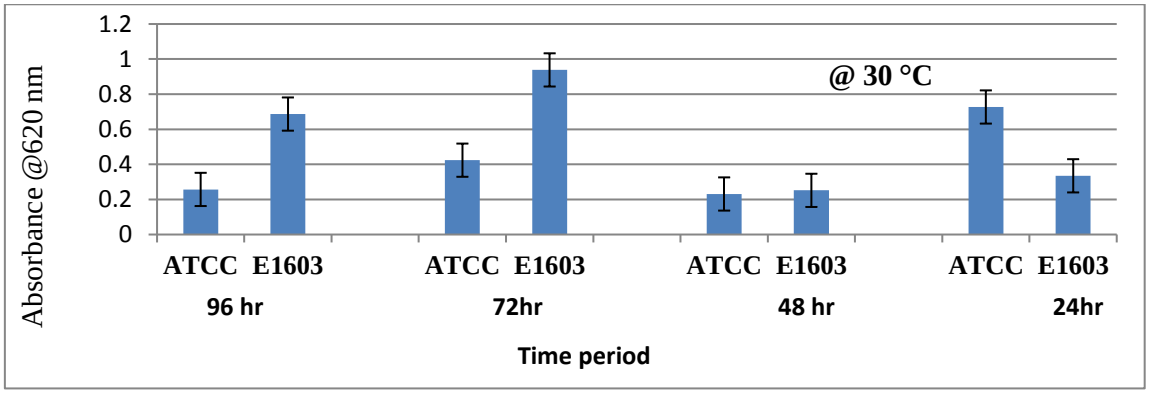

Figure 12: Showing the maximum biofilm formation shown under environmental conditions of 30^0 C in 72 hours by clinical isolates and ATCC strain in 24 hours.

5.1.6 Cytokine gene expression profiling

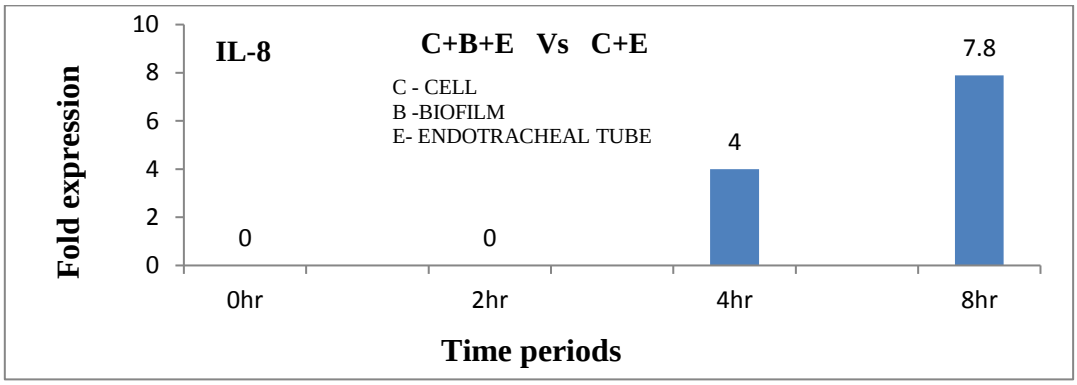

Figure 13: Comparison of IL-8 gene expression in THP1 challenged with biofilm coated endotracheal tube and endotracheal tube alone.

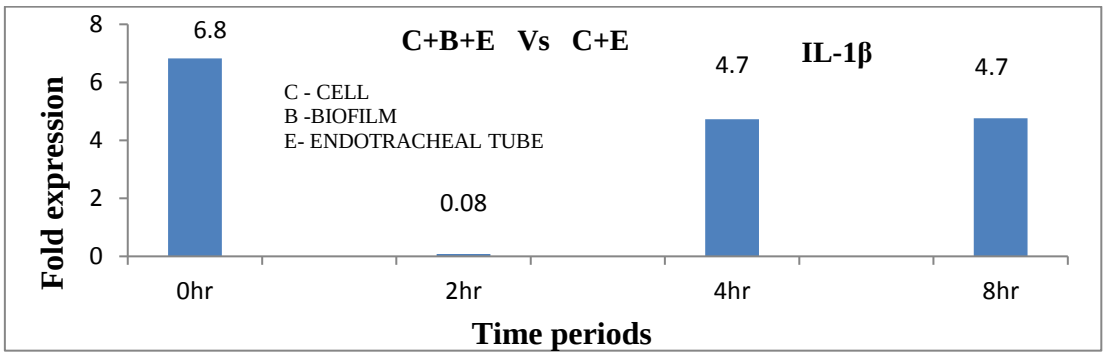

Figure 14: Comparison of IL-1β gene expression in THP1 challenged with biofilm coated endotracheal tube and endotracheal tube alone.

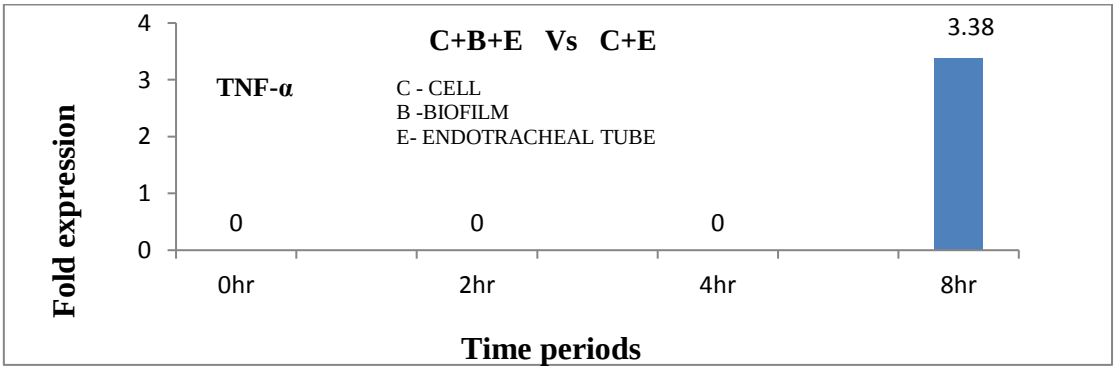

Figure 15: Comparison of TNF-α gene expression in THP1 challenged with biofilm coated endotracheal tube and endotracheal tube alone.

5.1.7 Biofilm formation and Cytokine gene expression at various time points in THP1 challenged with biofilm (E1603) coated endotracheal tube, endotracheal tube alone.

The main findings of our studies are the ability of *A. baumannii* to produce biofilms on ETT by 24 hours and this biofilm matured by 72 hours (Figure 11, 12). Other authors have previously shown a high prevalence of biofilm on ETT, even at short permanence times (Feldman et al., 1999). We confirmed these results and were able to assess these observations in the case of multi-resistant gram-negative bacteria. Resistances to harsh environmental conditions by *A. baumannii* strains seems to be directly related to its capacity to form biofilm, as has been shown (Ioanas et al., 2004). Biofilm formation on ETTs is a virulence mechanism and provides a bacterial reservoir for VAP among mechanically ventilated patients. In order to understand the mechanism of biofilm persistence it was mandatory to understand immune response in the host to Acinetobacter biofilms. For this we used an *in vitro* system consisting of monocyte cell line THP-1 and challenged it to ETT alone and ETT with biofilm and looked at the modulation of various cytokine mRNAs by qRT PCR. The chemistry used was SYBR Green chemistry and housekeeping gene β-actin was the internal calibrator. In our system, THP-1 did not produce IL-6 but produced and IL-8 by 4hours and the production was sustained up to 8 hours clearing indicating that pro-inflammatory stimuli was there with the biofilm vis a vis ETT alone [Figure 13] Knapp et al showed in an *in vivo* model that *A. baumannii* strain RUH2037 in planktonic phase induced the release of pro-inflammatory cytokines and chemokine resulting in clearance of bacteria from the lungs of experimentally infected mice (Knapp et al., 2006). Airway colonization by nosocomial bacteria is a common phenomenon and many investigations recognize a direct relationship between colonization and nosocomial pneumonia (Ewig et al., 1999). A total of 87% of patients were colonized, most frequently by *A. baumannii* (45%). In more than half of the patients (56%), the same bacteria could be found in endotracheal aspirate (ETA) and ETT biofilm, 69% in the case of Gram-negative bacteria. Despite the high prevalence of airway colonization and biofilm on

ETT, clinical isolates formed maximum biofilm in 72 and 96 hours at 30°C also indicating a mode of survival in the hospital environment [Figure 12]. Therefore, biofilm formation and airway colonization were necessary and sufficient for VAP development. Clinical isolates on one side, showed maximum biofilm formation at 37 °C shown in 24 hours and ATCC strain showed maximum biofilm formation in 96 hours. Expression analysis of proinflammatory cytokine such as IL-1β, IL-6, chemokine such as IL-8, and cell signaling protein such as TNF-α in monocytic cell challenged with *A. baumannii* biofilm loaded endotracheal tube at various time periods such as 0h, 2h, 4h, and 8h was done. We have observed the regulation of IL- 1β which has fever producing property at all time points, but down regulated by 2nd hour onwards and persists up to 8 hours. [Figure 14], this cytokine is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis. IL-8 has shown 2 fold change expression and is up regulated at 4 and 8 hours, *vis a vis* expression when challenged with ETT alone, IL-8 induce chemotaxis in target cells, primarily neutrophils causing them to migrate toward the site of infection [Figure 13]. TNF-α level indicates a significant up regulation at 8 hours [Figure 15] and clearly indicating the immune suppression by *A. baumannii* biofilm. Large amounts of TNF are released in response to lipopolysaccharide. THP-1 cells were incubated with LPS, all supernatants significantly decreased TNF-α production (Aoudia et al., 2016).

Chapter 6

Conclusion

6.1 Conclusion

In summary, our study supports the idea of a dynamic relationship among airway colonization, biofilm and VAP development. Adhesiveness and biofilm-forming capacity in *A. baumannii* presume a vital part in the host-pathogen communications and in medical device related infection. *A. baumannii* is pleomorphic which is typically rod-shaped during rapid growth but forms coccobacilli during stationary phase. It also demonstrates that *A. baumannii* biofilm is capable of up regulating anti-inflammatory cytokine and preventing macrophage phagocytosis contributing to biofilm and persistence. *A. baumannii* biofilm develop and are sustained on endotracheal tube and the persistence of biofilm on endotracheal tube leads to development of ventilator associated pneumonia

Chapter 7

Reference

7.1 Reference

American Thoracic Society and Infectious Diseases Society of America (2005). Guidelines for the management of adults with hospital-acquired, ventilator-associated, and healthcare-associated pneumonia. American Journal of Respiratory and Critical Care Medicine 171:388–416.

Aoudia, N., Rieu, A., Briandet, R., Deschamps, J., Chluba, J., Jego, G., Garrido, C., Guzzo, J., 2016. Biofilms of Lactobacillus plantarum and Lactobacillus fermentum: Effect on stress responses, antagonistic effects on pathogen growth and immunomodulatory properties. Food Microbiol., 19th Meeting of the Club des Bactéries Lactiques 53, 51–59. doi:10.1016/j.fm.2015.04.009

Baumann P, Doudoroff M, Stanier RY. A study of the Moraxella group. II. Oxidative-negative species (genus Acinetobacter). J Bacteriol 1968; 95:1520-41; PMID: 5650064

Beijerinck MW. Über Pigmentbildung bei Essigbakterien. Centr Bakteriol Parasitenk Abt 1911:167-76.

Bist, P., Dikshit, N., Koh, T.H., Mortellaro, A., Tan, T.T., Sukumaran, B., 2014. The Nod1, Nod2, and Rip2 Axis Contributes to Host Immune Defense against Intracellular *Acinetobacter baumannii* Infection. Infect. Immun. 82, 1112–1122. doi:10.1128/IAI.01459-13

Breij, A. de, Dijkshoorn, L., Lagendijk, E., Meer, J. van der, Koster, A., Bloemberg, G., Wolterbeek, R., Broek, P. van den, Nibbering, P., 2010. Do Biofilm Formation and Interactions with Human Cells Explain the Clinical Success of *Acinetobacter baumannii*? PLOS ONE 5, e10732. doi:10.1371/journal.pone.0010732

Breslow, J.M., Meissler, J.J., Hartzell, R.R., Spence, P.B., Truant, A., Gaughan, J., Eisenstein, T.K., 2011. Innate Immune Responses to Systemic *Acinetobacter baumannii* Infection in Mice: Neutrophils, but Not Interleukin-17, Mediate Host Resistance. Infect. Immun. 79, 3317–3327. doi:10.1128/IAI.00069-11

Brisou J, Prevot AR. [Studies on bacterial taxonomy. X. The revision of species under Acromobacter group]. Ann Inst Pasteur (Paris) 1954; 86:722-8; PMID: 13197842

Bruhn, K.W., Pantapalangkoor, P., Nielsen, T., Tan, B., Junus, J., Hujer, K.M., Wright, M.S., Bonomo, R.A., Adams, M.D., Chen, W., Spellberg, B., 2014. Host Fate is Rapidly Determined by Innate Effector-Microbial Interactions During *Acinetobacter baumannii* Bacteremia. J. Infect. Dis. doi:10.1093/infdis/jiu593

Cerqueira GM, Peleg AY. Insights into *Acinetobacter baumannii* pathogenicity. IUBMB Life 2011; 63:1055-60; PMID:21989983; http://dx.doi.org/10.1002/iub.533

Chaari, A., Mnif, B., Bahloul, M., Mahjoubi, F., Chtara, K., Turki, O., Gharbi, N., Chelly, H., Hammami, A., Bouaziz, M., 2013. *Acinetobacter baumannii* ventilator-associated pneumonia: epidemiology, clinical characteristics, and prognosis factors. Int. J. Infect. Dis. IJID Off. Publ. Int. Soc. Infect. Dis. 17, e1225-1228. doi:10.1016/j.ijid.2013.07.014

Chang HC, Wei YF, Dijkshoorn L, Vaneechoutte M, Tang CT, Chang TC. Species-level identification of isolates of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex by sequence analysis of the 16S-23S rRNA gene spacer region. J Clin Microbiol 2005; 43:1632-9; PMID:15814977; http://dx.doi.org/10.1128/JCM.43.4.1632-1639.2005

Chastre, J. and Fagon, J.-Y. (2002). Ventilator-associated pneumonia. American Journal of Respiratory and Critical Care Medicine 165:867–903.

Choi, C.H., Lee, J.S., Lee, Y.C., Park, T.I., Lee, J.C., 2008. *Acinetobacter baumannii* invades epithelial cells and outer membrane protein A mediates interactions with epithelial cells. BMC Microbiol. 8, 216. doi:10.1186/1471-2180-8-216

Costerton J, Stewart P, Greenberg E (1999) Bacterial biofilms: A common cause of persistent infections. Science 284: 1318–22.

De Yang, null, Chen, Q., Schmidt, A.P., Anderson, G.M., Wang, J.M., Wooters, J., Oppenheim, J.J., Chertov, O., 2000. LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide

receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. J. Exp. Med. 192, 1069–1074.

Di Nocera PP, Rocco F, Giannouli M, Triassi M, Zarrilli R. Genome organization of epidemic Acinetobacter baumannii strains. BMC Microbiol 2011; 11:224; PMID:21985032; http://dx.doi.org/10. 1186/1471-2180-11-224

Dolzani L, Tonin E, Lagatolla C, Prandin L, MontiBragadin C. Identification of Acinetobacter isolates in the *A. calcoaceticus-A. baumannii* complex by restriction analysis of the 16S-23S rRNA intergenic-spacer sequences. J Clin Microbiol 1995; 33:1108-13; PMID:7542263.

Ehrenstein B, Bernards AT, Dijkshoorn L, GernerSmidt P, Towner KJ, Bouvet PJ, et al. Acinetobacter species identification by using tRNA spacer fingerprinting. J Clin Microbiol 1996; 34:2414-20; PMID: 8880491

Ewig, S., Torres, A., El-Ebiary, M., Fábregas, N., Hernández, C., González, J., Nicolás, J.M., Soto, L., 1999. Bacterial colonization patterns in mechanically ventilated patients with traumatic and medical head injury. Incidence, risk factors, and association with ventilator-associated pneumonia. Am. J. Respir. Crit. Care Med. 159, 188–198. doi:10.1164/ajrccm.159.1.9803097

Falagas, M.E., Bliziotis, I.A. and Siempos, I.I. (2006). Attributable mortality of *Acinetobacter baumannii* infections in critically ill patients: a systematic review of matched cohort and case-control studies. Critical Care 10:R48.

Feldman, C., Kassel, M., Cantrell, J., Kaka, S., Morar, R., Goolam Mahomed, A., Philips, J.I., 1999. The presence and sequence of endotracheal tube colonization in patients undergoing mechanical ventilation. Eur. Respir. J. 13, 546–551.

Fournier, P. E., D. Vallenet, V. Barbe, S. Audic, H. Ogata, L. Poirel, H. Richet, C. Robert, S. Mangenot, C. Abergel, P. Nordmann, J. Weissenbach, D. Raoult, and J. M. Claverie. "Comparative genomics of multidrug resistance in *Acinetobacter baumannii*". PLOS Genet. 2006. Volume 2:e7.

Fuse, E.T., Tateda, K., Kikuchi, Y., Matsumoto, T., Gondaira, F., Azuma, A., Kudoh, S., Standiford, T.J., Yamaguchi, K., 2007. Role of Toll-like receptor 2 in recognition of Legionella pneumophila in a murine pneumonia model. J. Med. Microbiol. 56, 305–312. doi:10.1099/jmm.0.46913-0

Gaddy, J.A. and Actis, L.A. (2009). Regulation of *Acinetobacter baumannii* biofilm formation. Future microbiology 4:273–278.

García-Patiño, M.G., García-Contreras, R., Licona-Limón, P., 2017a. The Immune Response against *Acinetobacter baumannii*, an Emerging Pathogen in Nosocomial Infections. Front. Immunol. 8. doi:10.3389/fimmu.2017.00441

Garnacho, J., Sole-Violan, J., Sa-Borges, M., Diaz, E. and Rello, J. (2003). Clinical impact of pneumonia caused by *Acinetobacter baumannii* in intubated patients: a matched cohort study. Critical Care Medicine 31:2478–2482.

Gerner-Smidt P, Tjernberg I, Ursing J. Reliability of phenotypic tests for identification of Acinetobacter species. J Clin Microbiol 1991; 29:277-82; PMID: 2007635

Gil-Perotin, S., Ramirez, P., Marti, V., Sahuquillo, J.M., Gonzalez, E., Calleja, I., Menendez, R., Bonastre, J., 2012. Implications of endotracheal tube biofilm in ventilator-associated pneumonia response: a state of concept. Crit. Care 16, R93. doi:10.1186/cc11357

Hawn, T.R., Berrington, W.R., Smith, I.A., Uematsu, S., Akira, S., Aderem, A., Smith, K.D., Skerrett, S.J., 2007. Altered inflammatory responses in TLR5-deficient mice infected with *Legionella pneumophila*. J. Immunol. Baltim. Md 1950 179, 6981–6987.

Heredia-Rodríguez, M., Peláez, M.T., Fierro, I., Gómez-Sánchez, E., Gómez-Pesquera, E., Lorenzo, M., Álvarez-González, F.J., Bustamante-Munguira, J., Eiros, J.M., Bermejo-Martin, J.F., Gómez-Herreras, J.I., Tamayo, E., 2016. Impact of ventilator-associated pneumonia on mortality and epidemiological features of patients with secondary peritonitis. Ann. Intensive Care 6, 34. doi:10.1186/s13613-016-0137-5

Hjortsø, Martin A, Joseph W (1995) Cell Adhesion: Fundamentals and Biotechnological Applications. New York, USA.

Hsu, M.-J., Lu, Y.-C., Hsu, Y.-C., Liu, W.-S., Wu, W.-T., 2012. Interleukin-1 receptor antagonist gene polymorphism in patients with multidrug-resistant *Acinetobacter baumannii*-associated pneumonia. Ann. Thorac. Med. 7, 74–77. doi:10.4103/1817-1737.94523

Hurley, J.C. (2016). World-wide variation in incidence of Acinetobacter associated ventilator associated pneumonia: a meta-regression. BMC Infectious Diseases 16:577.

Ioanas, M., Ferrer, M., Cavalcanti, M., Ferrer, R., Ewig, S., Filella, X., de la Bellacasa, J.P., Torres, A., 2004. Causes and predictors of nonresponse to treatment of intensive care unit-acquired pneumonia. Crit. Care Med. 32, 938–945.

Kalanuria, A.A., Zai, W. and Mirski, M. (2014). Ventilator-associated pneumonia in the ICU. Critical Care 18:208.

Knapp, S., Wieland, C.W., Florquin, S., Pantophlet, R., Dijkshoorn, L., Tshimbalanga, N., Akira, S., van der Poll, T., 2006. Differential roles of CD14 and toll-like receptors 4 and 2 in murine *Acinetobacter* pneumonia. Am. J. Respir. Crit. Care Med. 173, 122–129. doi:10.1164/rccm.200505-730OC

Koenig, S.M. and Truwit, J.D. (2006). Ventilator-Associated Pneumonia: Diagnosis, Treatment, and Prevention. Clinical Microbiology Reviews 19:637–657.

La Scola B, Raoult D. *Acinetobacter baumannii* in human body louse. Emerg Infect Dis 2004; 10:1671-3; PMID:15498175

Lee, H.-W., Koh, Y.M., Kim, J., Lee, J.-C., Lee, Y.-C., Seol, S.-Y., Cho, D.-T., Kim, J., 2008. Capacity of multidrug-resistant clinical isolates of *Acinetobacter baumannii* to form biofilm and adhere to epithelial cell

surfaces. Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis. 14, 49–54. doi:10.1111/j.1469-0691.2007.01842.x

Lee, J.C., Koerten, H., van den Broek, P., Beekhuizen, H., Wolterbeek, R., van den Barselaar, M., van der Reijden, T., van der Meer, J., van de Gevel, J., Dijkshoorn, L., 2006. Adherence of *Acinetobacter baumannii* strains to human bronchial epithelial cells. Res. Microbiol. 157, 360–366. doi:10.1016/j.resmic.2005.09.011

Lee, J.S., Lee, J.C., Lee, C.-M., Jung, I.D., Jeong, Y.-I., Seong, E.-Y., Chung, H.-Y., Park, Y.-M., 2007. Outer membrane protein A of *Acinetobacter baumannii* induces differentiation of CD4+ T cells toward a Th1 polarizing phenotype through the activation of dendritic cells. Biochem. Pharmacol. 74, 86–97. doi:10.1016/j.bcp.2007.02.012

Lennox J (2011) Biofilm Development. Biofilms: The Hypertextbook.

Lessel EF. International Committee on Nomenclature of Bacteria Subcommittee on the Taxonomy of Moraxella and Allied Bacteria: Minutes of the Meeting, 11 August 1970. Room Constitution C, Maria-Isabel Hotel, Mexico City, Mexico. Int J Syst Bacteriol 1971; 21:213-4; http://dx.doi.org/10.1099/ 00207713-21-2-213

March, C., Regueiro, V., Llobet, E., Moranta, D., Morey, P., Garmendia, J., Bengoechea, J.A., 2010. Dissection of Host Cell Signal Transduction during *Acinetobacter baumannii* – Triggered Inflammatory Response. PLoS ONE 5, e10033. doi:10.1371/journal.pone.0010033

Marshall KC (1992) Biofilms: an overview of bacterial adhesion, activity, and control at surfaces. ASM News 58: 202–7.

Mittelman M (1996) Biological fouling of purified-water systems: Part 3, Treatment of Micro-contamination 4: 30-40.

Moffatt, J.H., Harper, M., Mansell, A., Crane, B., Fitzsimons, T.C., Nation, R.L., Li, J., Adler, B., Boyce, J.D., 2013. Lipopolysaccharide-Deficient *Acinetobacter baumannii* Shows Altered Signaling through Host Toll-Like

Receptors and Increased Susceptibility to the Host Antimicrobial Peptide LL-37. Infect. Immun. 81, 684–689. doi:10.1128/IAI.01362-12

Montefour K, Frieden J, Hurst S, Helmich C, Headley D, Martin M, et al. *Acinetobacter baumannii*: an emerging multidrug-resistant pathogen in critical care. Crit Care Nurse 2008; 28:15-25, quiz 26; PMID: 18238934

Peleg AY, Seifert H, Paterson DL. *Acinetobacter baumannii*: emergence of a successful pathogen. Clin Microbiol Rev 2008; 21:538-82; PMID:18625687; http://dx.doi.org/10.1128/CMR.00058-07

Pneumatikos, I.A., Dragoumanis, C.K., Bouros, D.E., 2009. Ventilator-associated pneumonia or endotracheal tube-associated pneumonia? An approach to the pathogenesis and preventive strategies emphasizing the importance of endotracheal tube. Anesthesiology 110, 673–680. doi:10.1097/ALN.0b013e31819868e0

Qiu, H., KuoLee, R., Harris, G., Van Rooijen, N., Patel, G.B., Chen, W., 2012. Role of Macrophages in Early Host Resistance to Respiratory *Acinetobacter baumannii* Infection. PLoS ONE 7, e40019. doi:10.1371/journal.pone.0040019

Rello, J., Kollef, M., Diaz, E., Sandiumenge, A., del Castillo, Y., Corbella, X. and Zachskorn, R. (2006). Reduced burden of bacterial airway colonization with a novel silver-coated endotracheal tube in a randomized multiple-center feasibility study. Critical Care Medicine 34:2766–2772.

Rello, J., Soñora, R., Jubert, P., Artigas, A., Rué, M., Vallés, J., 1996. Pneumonia in intubated patients: role of respiratory airway care. Am. J. Respir. Crit. Care Med. 154, 111–115. doi:10.1164/ajrccm.154.1.8680665

Rice, L.B., 2010. Progress and challenges in implementing the research on ESKAPE pathogens. Infect. Control Hosp. Epidemiol. 31 Suppl 1, S7-10. doi:10.1086/655995

Sebeny PJ, Riddle MS, Petersen K. *Acinetobacter baumannii* skin and soft-tissue infection associated with war trauma. Clin Infect Dis 2008; 47:444-9; PMID: 18611157; http://dx.doi.org/10.1086/590568

Seifert H, Dijkshoorn L, Gerner-Smidt P, Pelzer N, Tjernberg I, Vaneechoutte M. Distribution of *Acinetobacter* species on human skin: comparison of phenotypic and genotypic identification methods. J Clin Microbiol 1997; 35:2819-25; PMID:9350741

Tsuchiya, T., Nakao, N., Yamamoto, S., Hirai, Y., Miyamoto, K., Tsujibo, H., 2012. NK1.1+ cells regulate neutrophil migration in mice with *Acinetobacter baumannii* pneumonia: NK1.1+ cells in *Acinetobacter* pneumonia. Microbiol. Immunol. 56, 107–116. doi:10.1111/j.1348-0421.2011.00402.x

Vaneechoutte M, Dijkshoorn L, Tjernberg I, Elaichouni A, de Vos P, Claeys G, et al. Identification of *Acinetobacter* genomic species by amplified ribosomal DNA restriction analysis. J Clin Microbiol 1995; 33: 11-5; PMID:7699025