Infection and Co-Infection in Critical Illness: Rapid Diagnosis by MT-PCR

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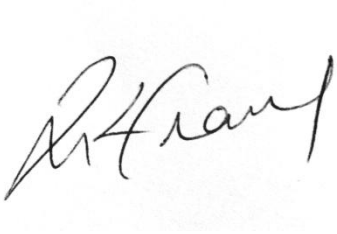
Declaration

In accordance with the by-laws of the University of Sydney, the work described herein is entirely the author‟s own work and responsibility, except where due acknowledgement has been made. This thesis contains no material previously published or written by any other person, except where duly referenced. The work outlined in this thesis was an original project undertaken at the Centre for Infection Diseases and Microbiology, Westmead Hospital, NSW, Australia under the supervision of Associate Professor Jonathan R. Iredell. The work was conducted in the degree of Master of Philosophy in Medicine in the Faculty of Medicine, Sydney Medical School at the University of Sydney. This thesis has not previously been submitted for examination, wholly or in part, for the award of any other degree or diploma at this or any other educational institution.



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Abstract

Sepsis and septic shock are the second highest cause of death in ICU after cardiovascular disease. The most common types of bacteria found in septic shock are Gram-negative bacteria followed by Gram-positive bacteria such as *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Enterococcus faecalis*.

A multiplexed-tandem polymerase chain reaction assay for the rapid and sensitive detection and identification of bacterial pathogens from the genera Staphylococci, Streptococci, Enterococci, was tested for the diagnosis of Gram-positive cocci signalling in an automated blood culture system. Additionally, blood culture broths, positive for *S. aureus* (MRSA) were spiked with *S. pneumoniae* to test the influence of contamination/co-infection.

The second part of the study involved the detection of bacterial co-infection in respiratory specimens using samples from the Australian influenza epidemic of 2009. Viral infections engender bacterial colonisation in the host by causing alteration in the innate immune response to common bacterial pathogens. These alterations may lead to severe bacterial pneumonia during influenza. Bacterial co-infection is believed to be common in influenza pneumonia and to increase morbidity and mortality. The most common pathogens that cause co-infection are *S. pneumoniae*, *S. pyogenes* and *S. aureus*.

A multiplexed quantitative PCR assay based on 16S rRNA consensus sequence of a family (*Enterobacteriaceae* species) and 4 genera (*Staphylococcus* sp., *Streptococcus* sp., *Enterococcus* sp., and *Pseudomonas* sp.) showed that Enterococci were commonly

found, perhaps only as colonizers, and that staphylococci and streptococci were important. Accurate speciation is necessary to make this assay more useful.

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Abbreviations Used in this Thesis

Organisms

AHS	α-haemolytic *Streptococcus*
B. parapertussis	*Bordetella parapertussis*
C. pneumoniae	*Chlamydia pneumoniae*
CoNS	Coagulase-negative *Staphylococcus*
E. avium	*Enterococcus avium*
E. casseliflavus	*Enterococcus casseliflavus*
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
E. faecium	*Enterococcus faecium*
E. gallinarum	*Enterococcus gallinarum*
E. hirae	*Enterococcus hirae*
H. influenzae	*Haemophilus influenzae*
K. pneumoniae	*Klebsiella pneumoniae*
L. longbeachae	*Legionella longbeachae*
L. pneumophila	*Legionella pneumophila*
M. catarrhalis	*Moraxella catarrhalis*
M. pneumoniae	*Mycoplasma pneumoniae*
MR-CoNS	Methicillin-resistant coagulase-negative *Staphylococcus*
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS-CoNS	Methicillin-sensitive coagulase-negative *Staphylococcus*
MSSA or *S. aureus*	Methicillin-sensitive *Staphylococcus aureus*
N. meningitidis	*Neisseria meningitidis*

P. aeruginosa	*Pseudomonas aeruginosa*
P. carinii	*Pneumocystis carinii*
P. jiroveci	*Pneumocystis jiroveci*
S. acidominimus	*Streptococcus acidominimus*
S. australis	*Streptococcus australis*
S. bovis	*Streptococcus bovis*
S. epidermidis	*Staphylococcus epidermidis*
S. milleri	*Streptococcus milleri*
S. parasanguinis	*Streptococcus parasanguinis*
S. pneumoniae	*Streptococcus pneumoniae*
S. pyogenes	*Streptococcus pyogenes*
VRE	Vancomycin-resistant *Enterococcus*

General Abbreviations

AIDS	Acquired immunodeficiency syndrome
AOM	Acute otitis media
ARDS	Acute respiratory distress syndrome
BA	Blood Agar plates
BAL	Bronchoalveolar lavage
BTS	British Thoracic Society
CAP	Community acquired pneumonia
CDC	Centers for Disease Control and Prevention
cDNA	complementary DNA
CIDMLS	Centre for Infectious Diseases and Microbiology laboratory Services
CO_2	Carbon dioxide
Ct	Cycle threshold
DIC	Disseminated intravascular coagulation
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ETT	Endotracheal tube
GluSE	Glutamic acid-specific serine protease
GPC 12	Gram-positive 12 assay
HIV	Human immunodeficiency virus
ICU	Intensive Care Unit
IFN	Interferon

IL	Interleukin
KH_2PO_4	Potassium Di-hydrogen Orthophosphate
LPS	Lipopolysaccharide
MALDI TOF	Matrix-assisted laser desorption/ionization time-of-flight
$MgCl_2$	Magnesium Chloride
MOSF	Multiple organ system failure
mPCR-RLB	Multiplex PCR-reverse line blot
mRNA	Messenger ribonucleic acid
MT-PCR	Multiplexed tandem-PCR
MV	Mechanical ventilation
Na_2HPO_4	Di-Sodium Hydrogen Orthophosphate
NaOH	Sodium hydroxide
NB	Nutrient broth
NH_4	Ammonium
NPA	Nasopharyngeal aspirates
NPV	Negative Predictive Value
PAI-1	plasminogen activator inhibitor-1
PB1-F2	Pro-apoptotic influenza A virus protein
PBP2	Penicillin binding protein
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
pH	The standard measurement of acidity
PPV	Positive Predictive Value
PVL	Panton-Valentine leukocidin
RNA	Ribonucleic Acid

rRNA	Ribosomal Ribonucleic Acid
RSV	Respiratory syncytial virus
RT-PCR	Reverse transcriptase-PCR
SCCmec	Staphylococcal chromosomal cassette
spp.	Species
SPS	Sodium polyanetholesulfonate
SPSS	Statistical Package for the Social Sciences
TBE	Tris-Borate-EDTA
TFPI	Tissue-factor-pathway inhibitor
TGF	Transforming growth factor
TLR	Toll-like receptor
*T*m	Melting temperature
TNF	Tumor necrosis factor
TNFR1	TNF receptor 1
TOC	Total organic carbon
TSST	Toxic shock syndrome toxin
WHO	World Health Organization

Units of measurement

°C	Degree celsisus
bp	Base pair
cfu	Colony forming units
cm	Centimetre
EU	ELISA units
L	Litre
M	Molar
min	Minutes
mL	Millilitre
mM	Millimolar
ng	Nanogram
ppb	Parts per billion
s	Second
V	Volt
v/v	Volume per volume
w/v	Weight per volume
µl	Microlitre
µM	Micromolar
Ω	Ohm

Chapter 1
Literature Review

1.1 Septic Shock

Sepsis, severe sepsis and septic shock are systemic inflammatory responses due to an uncontrolled infection (Bone *et al.* 1992). Patients with severe sepsis constitute approximately 11.8% of all patients in Intensive Care Unit (ICU) in Australia and New Zealand (Finfer *et al.* 2004). Sepsis and septic shock have a high rate of mortality, at around 38% (ARISE; *et al.* 2007), and are the second highest cause of death in ICU after cardiovascular disease (Brun-Buisson *et al.* 1995; Martin *et al.* 2003; Brun-Buisson *et al.* 2004).

Septic shock can be defined as sepsis with severe cardiovascular instability, acute organ dysfunction, hypotension and coagulopathy (Bone *et al.* 1992). Bacteraemia can trigger inflammatory cytokine storm which induces septic shock, and multiple organ dysfunctions which arise in septic shock are presumed to be the result of this inflammation rather than the actual infection (Pinsky and Matuschak 1990; Cursons *et al.* 1999).

1.1.1 Bacterial causes of septic shock

The most common types of bacteria found in septic shock are Gram-negative bacteria (47.9% of septic shock cases) such as *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, followed by Gram-positive bacteria (38.3%) such as *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Enterococcus faecalis* (Figure 1.1) (Kumar *et al.* 2006b). Although the amount of bacteria required to cause septic shock is unknown, the contribution of bacterial load and microbial virulence to septic shock is recognized (van der Poll and Opal 2008). Moreover, unidentified sepsis may result from translocation of bacteria from the gut and the frequency of polymicrobial

bacteraemia is not known (Kane *et al.* 1998; Ammori *et al.* 2003). The importance of Gram-negative bacteria in septic shock may be related to the influence of LPS from the Gram-negative envelope (Section 1.1.2)

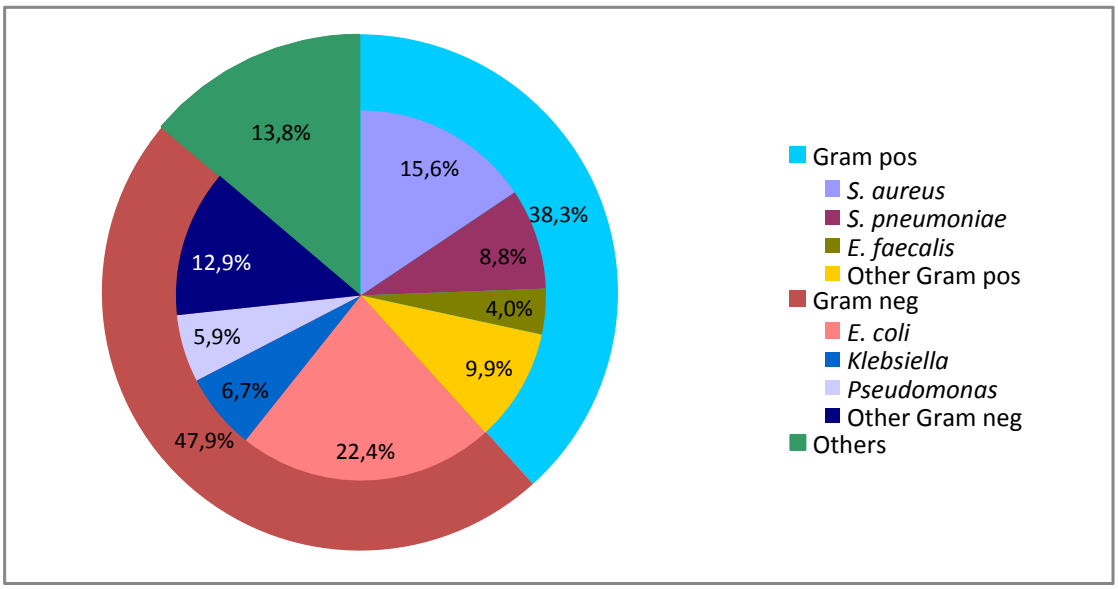

Figure 1.1 The percentage distribution of bacterial pathogens that cause septic shock; using data from Kumar *et al* (2006b)

Other Gram-positive organisms include: Group A *Streptococcus* (3.6%) Other β-hemolytic streptococci (2.2%), *Viridans streptococci* (1.9%), *E. faecium* (1.5%), *Bacillus* species (0.3%), *Corynebacterium jeijkeium* (0.3%), *Staphylococcus lugdunensis* (0.3%).

Other Gram-negative organisms include: *Enterobacter* species (4.1%), *Haemophilus influenzae* (2.2%), *Proteus* species (1.2%), *Acinetobacter* species (1.1%), *Serratia* species (1.0%), *Stenotrophomonas maltophilia* (0.8%), *Morganella morganii* (0.7%), *Citrobacter* species (0.7%), *Neisseria meningitidis* (0.3%), *Burkholderia cepacia* (0.2%), *Haemophilus parainfluenzae* (0.2%)

Others: yeast/fungi (8.2%), anaerobes (3.6%), *Legionella* species (0.4%), *Mycobacterium tuberculosis* (0.6%)

1.1.2 Inflammatory response

Both pro-inflammatory and anti-inflammatory cytokines constitute a part of the immune response mechanism. Although pro-inflammatory cytokines such as tumor necrosis factor (TNF), and Interleukins-1, -6 and -8 (IL-1, IL-6 and IL-8) appear in the early stages of infection, anti-inflammatory cytokines such as TNF receptor 1 (TNFR1), IL-1 receptor antagonist (IL-1ra) are more important in the continuation of the inflammatory process. Several inflammatory cytokines (TNF-α, IL-1β and IL-6) can increase the risk of severe sepsis and death through the inhibition of fibrinolysis and by activating coagulation, with thrombin known to stimulate the inflammatory process (Bevilacqua *et al.* 1986; Conkling *et al.* 1988; Esmon *et al.* 1991; Yan and Grinnell 1993; Stouthard *et al.* 1996; Fang *et al.* 1999; Kellum *et al.* 2007). Uncontrolled systemic inflammation may lead to multiple organ failure and lethal septic shock (Vervloet *et al.* 1998; Levi and Ten Cate 1999).

The three major anticoagulant proteins controlling blood clotting are tissue-factor-pathway inhibitor (TFPI), antithrombin and activated protein C (Figure 1.2) (Esmon 1992; Levi and van der Poll 2005). Reducing the level of activated protein C in patients with septic shock may decrease mortality rate (Taylor *et al.* 1987; Kylat and Ohlsson 2006). Activated protein C inhibits activation of fibrin by inhibiting plasminogen activator inhibitor-1 (PAI-1). The inactivation of thrombin is a significant modulator of coagulation and inflammation in septic shock (Taylor *et al.* 1987; Fourrier *et al.* 1992; Kylat and Ohlsson 2006).

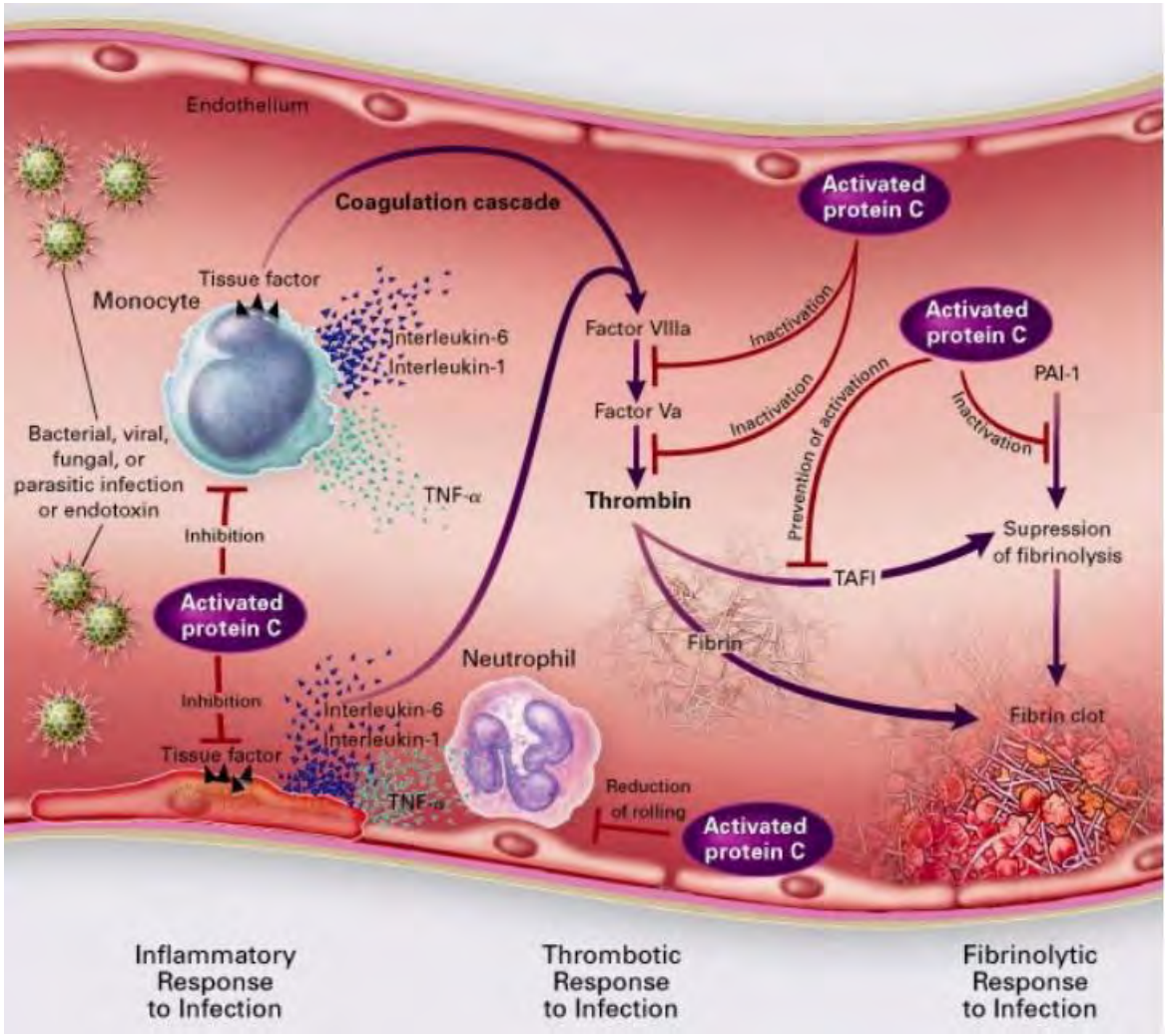

Figure 1.2 The role of thrombin and activated protein C in anticoagulation during the inflammatory and procoagulant host responses to infection in severe sepsis (from Bernard *et al* (2001)

1.1.2.1 The effect of endotoxin on coagulation and cytokine pathways

Lipopolysaccharide (LPS), also known as endotoxin, in the outer membrane of Gram-negative bacteria may drive the pathogenesis of septic shock. Endotoxin (LPS) consists of three main parts: Lipid A, O antigen (a long chain polysaccharide) and the LPS core, where Lipid A is the actual toxic moiety that plays the main role in septic shock (Bone 1993). LPS, especially Lipid A, activates the coagulation cascade and macrophages through the formation of a LPS-LPS binding protein complex. Activated macrophages

produce TNF-α, IL-1, IL-6 and IL-8, which lead to increased body temperature, vasodilation, hypotension and endothelial damage (Figure 1.3). Even if all pathogens are removed from the bloodstream, TNF and other cytokines continue to drive the process of septic shock (Salyers and Whitt 1994).

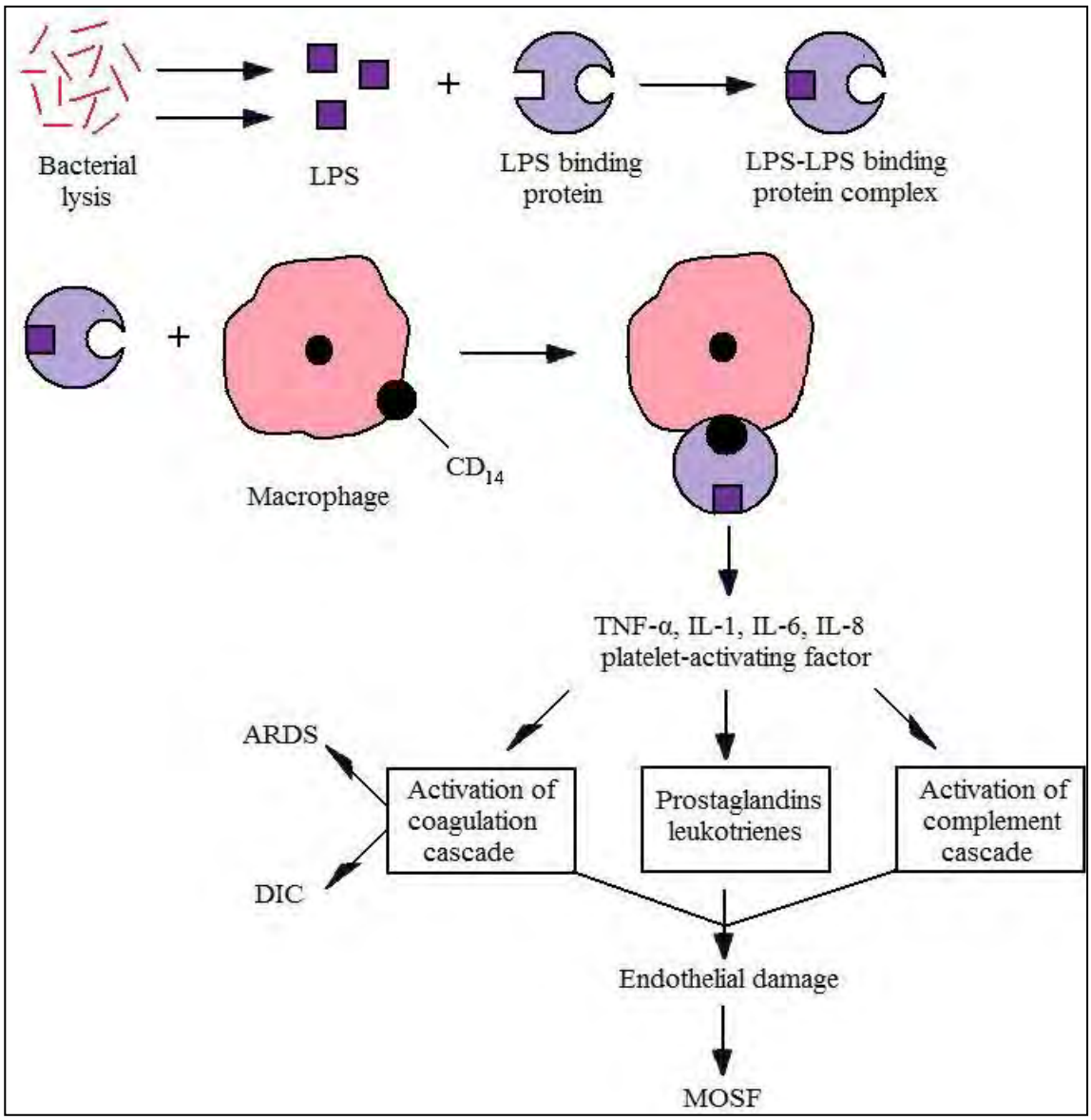

Figure 1.3 The role of Gram-negative LPS on the production of cytokines and endothelial damage in Septic Shock. (ARDS, acute respiratory distress syndrome; DIC, disseminated intravascular coagulation; MOSF, multiple organ system failure) Adapted from Salyers and Whitt (1994)

1.1.3 Antibiotic therapy in Septic Shock

Identification of the infective pathogen and relevant resistance can be crucial to the correct and adequate selection of antimicrobial therapy. The use of combination antibiotic therapy (especially broad-spectrum β-lactams) is preferred in the case of septic shock due to the high prevalence of polymicrobial sepsis (Kumar *et al.* 2010). Delays in antimicrobial therapy, incorrect or inadequate antibiotic choice, failure to control the source of sepsis in the early stage of septic shock, and resistance to "last line" ICU antibiotics increase the mortality rate of patients with septic shock (Bochud *et al.* 2004; Kumar *et al.* 2006b). There is a linear correlation between early antibiotic therapy and mortality rate in septic shock (Figure 1.4 and 1.5). The survival of patients treated for septic shock decreases by 7% for every hour the patient is not treated with antibiotics; and if the treatment is delayed 12 hours, the patient's survival rate has reduced to 25% (Figure 1.4) (Kumar *et al.* 2006b).

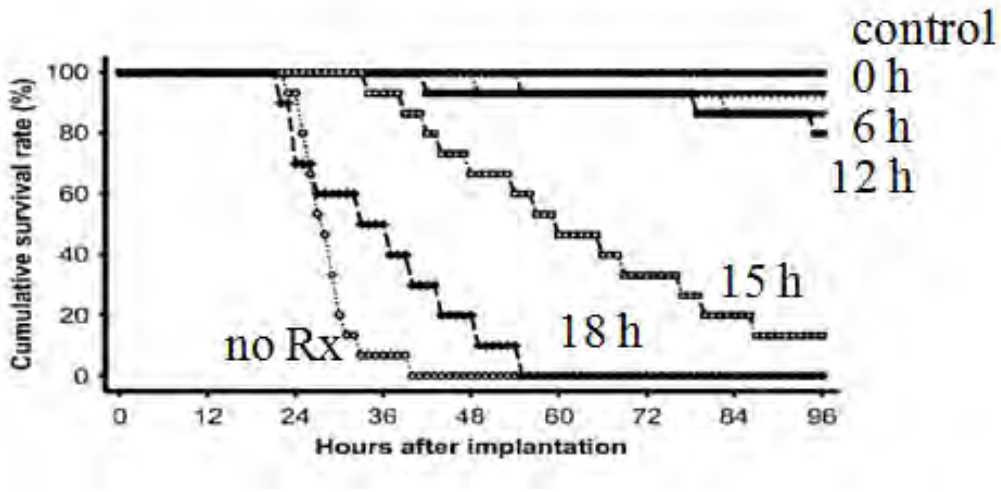

Figure 1.4 Survival rate in mice inoculated with 1000 cfu E. coli, with antibiotic treatment at 0, 6, 12, 15 and 18 hours after inoculation. No Rx = no antibiotic-treatment. Adapted from Kumar *et al*. (2006a)

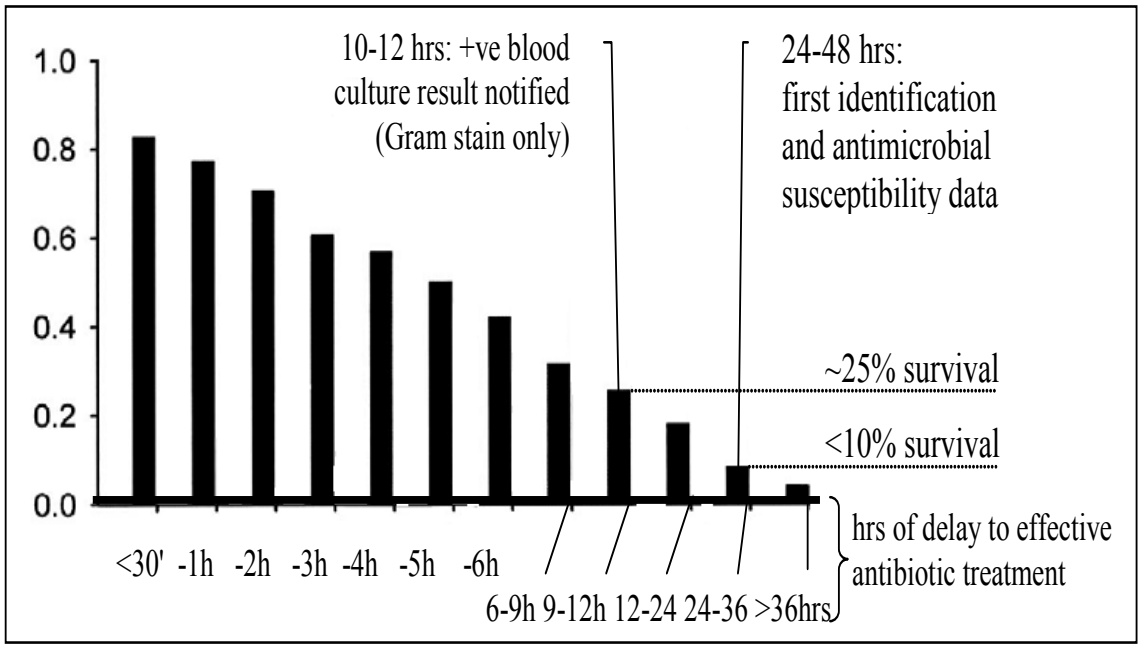

Figure 1: *likelihood of in-hospital survival from resuscitated septic shock* (0.0-1.0), as a function of delay in commencement of effective antibiotics (to >36 hours); adapted from (18)

Figure 1.5 Likelihood of in-hospital survival from resuscitated septic shock (0.0-1.0), as a function of delay in commencement of effective antibiotics. Adapted from Kumar *et al.* (2006b)

1.2 Community Acquired Pneumonia (CAP)

Community acquired pneumonia (CAP) is an acute infection of the pulmonary parenchyma. The common bacterial causes of mortality in lethal community acquired pneumonia are *S. pneumoniae* and *Legionella* species. In spite of appropriate initial antibiotic treatment, pathophysiological changes may still continue (Bartlett *et al.* 2000). An average of 4.5 million patients with CAP visits physicians, emergency departments and outpatient clinics annually at a cost of $4.8 billion for treatment of patients more than 65 years of age and less than $3.6 billion for younger patients. The average length of stay in hospital is 7.8 days (at a cost of USD $7166 per patient) and 5.8 days (USD $6042 per patient) respectively (Niederman *et al.* 1998). The evolution

of multidrug resistance in some pathogenic bacteria has made the management of pneumonia has more difficult (Bartlett *et al.* 2000).

In addition to influenza vaccination, the Advisory Council on Immunization Practices of the Centres for Disease Control and Prevention (CDC) suggests use of the pneumococcal vaccine for prevention of CAP. *S. pneumoniae* is the main pathogen in community-acquired pneumonia (CDC 2009). It is followed by other bacterial pathogens such as *Haemophilus influenzae, Mycoplasma pneumoniae, Chlamydia pneumoniae, S. aureus* (including methicillin-resistant *S. aureus*-MRSA), *Streptococcus pyogenes, Neisseria meningitidis, Moraxella catarrhalis, K. pneumoniae, Legionella* sp.; and viral pathogens such as influenza viruses, respiratory syncytial virus, adenovirus and parainfluenza virus (Bartlett *et al.* 2000).

The British Thoracic Society (BTS) states that the risk of mortality in CAP can be determined using four main clinical features (CURB score); mental confusion, respiratory rate≥ 30 / min., diastolic blood pressure ≤ 60 mm Hg and blood urea > 7 mmol/l. Neill et al modified the BTS prognosis rules to achieve 0.95 sensitivity and 0.71 specificity (Neill *et al.* 1996). BTS prognosis rules might not be accurate in patients with lower risk of death. Simple predictors such as age (≥65 years) and low serum albumin increase the accuracy of the CURB score in identifying CAP (Lim *et al.* 2003). Generally however, the lack of diagnostic tools and predictive markers are responsible for consumption of high levels of antibiotics and potentially inaccurate management of critically ill patients. Delays in appropriate initial antibiotic treatment increase risk of mortality during the first 24 hours of ventilator- associated pneumonia (Iregui *et al.* 2002). Correct antibiotic treatment in the first 8 hours of hospitalisation is

related to 15 % lower risk of mortality in the first 30 days and decreases treatment costs (Meehan *et al.* 1997; Niederman *et al.* 1998).

1.2.1 Pneumococcal pneumonia

Lower respiratory infection (primarily pneumonia) is the third leading cause of death (7.1%) after ischemic heart disease (12.2%) and cerebrovascular disease (9.7%) (WHO 2008). *S. pneumoniae* is the main cause of CAP in many studies, with high genomic loads of *S. pneumoniae* associated with an increased need for mechanical ventilation (MV), risk of septic shock and death (Mandell *et al.* 2007; Garau and Calbo 2008; Rello *et al.* 2009; Lin *et al.* 2010; Wispelwey and Schafer 2010).

Pneumococcal pneumonia is usually identified in older individuals and in patients who carry specific risk factors, such as alcoholism, severe diseases, specifically chronic lung disease, chronic obstructed airway disease, bronchial asthma, immunoglobulin deficiency, hematologic malignancy and HIV infection (Bartlett *et al.* 2000). *Pneumocystis jiroveci* (formerly known as *Pneumocystis carinii), S. pneumoniae C. pneumoniae H. influenzae* and *M. pneumoniae* are associated with acute pneumonia in AIDS and are commonly related to organ transplantation, renal deficiency and chronic lung disease (Keller and Breiman 1995; Boschini *et al.* 1996; Bartlett *et al.* 2000).

1.2.2 Viral community-acquired pneumonia

Influenza, respiratory syncytial virus, rhinoviruses, coronavirus and metapneumovirus, usually appears in winter. Even though pneumonia caused by *S. pneumoniae and H. influenzae* is commonly seen in the winter months, pneumonia caused by *C. pneumoniae* and *Legionella pneumophila* can appear during all seasons (Marston *et al.* 1994).

Respiratory viruses may be more common causes of CAP than pneumococcal pneumonia in adults (Jennings *et al.* 2008; Johnstone *et al.* 2008). Influenza A and respiratory syncytial virus (RSV) are the most common cause of viral pneumonia, while Influenza B, parainfluenza viruses (type 1, 2 and 3), rhinoviruses and adenoviruses are also capable of causing CAP (Tsolia *et al.* 2004; Johnstone *et al.* 2008).

Rhinovirus infection can be related to exacerbation of bronchiolitis in children, as well as asthma, chronic obstructive pulmonary disease and cystic fibrosis (Hayden 2004). In addition to causing trivial upper respiratory infection, rhinovirus and coronaviruses (specifically two serogroups 229E and OC43) can lead to pneumonia in adults (Marcos *et al.* 2006). Viral pneumonia is generally seen in older patients, though compared to pneumococcal pneumonia, is more commonly associated with cardiac disease. Chest pain, rigor and normal white blood cell are also traits of viral infections (Johnstone *et al.* 2008).

There are three main objectives to consider for the control of respiratory viruses: prevention of exposure, provision of immunity and administration of antiviral agents (Marcos *et al.* 2009). Besides using inactivated virus vaccines, some anti-influenza drugs are utilized for control of viral pneumonia, including adamantanes (amantadine and rimantadine; influenza A) and neuraminidase inhibitors (zanamivir and oseltamivir; Influenza A and B) (Abed and Boivin 2006; Jefferson *et al.* 2006). Treatment failure is becoming problematic, with some H1N1 strains with neuraminidase gene mutations being resistant to certain neuraminidase inhibitors (oseltamivir) (Hurt *et al.* 2009).

1.2.3 Influenza Infection

Influenza A viruses are single-stranded segmented RNA viruses of the Orthomyxoviridae virus family. This segmented genome structure facilitates exchange of gene segments between influenza A strains with several features of the virus enabling virus variability. The lack of proofreading mechanisms within RNA virus polymerases allow for variations to commonly occur during DNA replication. There are eight genetic segments that have specific functions in the genome. In addition to genetic recombination between segments, a new strain can evolve by transferring some of the segments between the two viruses during the infection of a cell. Meanwhile, the sharing of viral surface glycoproteins may also give rise to new strains possessing different glycoprotein combinations (Webster *et al.* 1997).

Both seasonal and sporadic pandemic influenza infections cause morbidity and mortality especially among high risk groups (very young, elderly or chronically ill patients) (WHO 2009a). The presence of influenza with bacterial co-infection increases morbidity and mortality in earlier stages of the disease (CDC 2009). *S. pneumoniae, S. aureus* or *H. influenzae* can trigger bacterial superinfections, between 4 and 14 days after resolution of the primary seasonal influenza infection, which appear with several symptoms such as a recurrence of fever, dyspnoea, cough and pulmonary consolidation (Hussell and Cavanagh 2009). In the case of more virulent influenza viruses, deaths may occur because of increased physiologic stress in the host, or the combined effects of viral infection and secondary bacterial infection (McCullers 2006).

1.2.3.1 Lung Pathology

Influenza is a cytolytic virus and may induce necrosis and desquamation of bronchial epithelial cells. Reparative responses of bronchial epithelial cells and formation of

hyaline membrane in alveoli and alveolar ducts are important signs in the diagnosis of the disease. Bacteria may invade the lung and be transferred to the bloodstream more readily than influnza. Influenza infection may influence respiratory epithelial cells in the lung by up-regulating cell surface molecules that promote bacterial adhesion (Hussell and Cavanagh 2009). Secondary bacterial pneumonia becomes more severe and frequent in mice in the presence of some viral proteins, such as pro-apoptotic influenza A virus protein (PB1-F2), that can enhance inflammation from the primary viral infection. At the same time, inflammatory cells in the lung increase production of inflammatory mediators such as IL-1α, IL-6, IL-10 and transforming growth factor-*β* (TGF-*β*) (McAuley *et al.* 2007).

1.2.3.2 Inflammatory Response

As a result of influenza infection, alveolar damage with pulmonary hemorrhage and necrotizing bronchiolitis trigger toll-like receptor-3 (TLR-3) expression while increased numbers of cytotoxic T-cells and an increase in Interferon-γ (IFN-γ) in lung tissues also occurs (Mauad *et al.* 2010). Altered innate immune responses related to severe influenza increase cytotoxic $CD8^+$ T-cell activity and enhance inflammation, and are also associated with increased granzyme B secretion, local production of IFN-γ, TLR-3 activation and hypercytokinemia (Mauad *et al.* 2010).

$CD8^+$ T cells play an important role in the inflammatory response to influenza by causing lysis of infected cells and production of inflammatory cytokines such as IFN-γ and tumor necrosis factor-α (TNF-α), which are found in high amounts in the first day of the infection (Mauad *et al.* 2010). However, tissue damage can also occur since these cytokines have the same inflammatory mechanism as that of the $CD8^+$ T cells (La Gruta *et al.* 2007).

1.2.3.3 Swine-Origin Influenza A/H1N1 Virus (S-OIV)

In 2009, more than 370,000 cases of pandemic influenza H1N1 (swine-origin influenza A) infection were identified worldwide with more than 4500 deaths reported to the World Health Organization (WHO) (WHO 2009b). During winter in 2009, 32.7% of H1N1 influenza cases admitted to ICU in Australia and New Zealand had respiratory risk factors, including asthma and other chronic pulmonary diseases. 48.8% of cases had acute respiratory distress syndrome or viral pneumonia, and 20.3% of cases had secondary bacterial pneumonia with 2009 H1N1 influenza infection in Australia and New Zealand ICU (ANZIC 2009).

1.2.3.4 H5N1 Influenza Infection

In contrast to most human influenza A viruses, avian influenza A virus (H5N1) strains may be highly pathogenic and cause very aggressive influenza infection (de Jong and Hien 2006; Hui 2008). H5N1 infection with is associated with a so-called "cytokine storm", especially with an increase in TNF-α (Cheung *et al.* 2002; Woo *et al.* 2010). This cytokine storm is produced by macrophages and lung epithelial cells, occurs in the first 12 to 24 hours and is much more significant than pandemic H1N1/09 and other seasonal influenza viruses (Woo *et al.* 2010).

1.2.4 Secondary Bacterial Pneumonia

S. pneumoniae and some other bacterial pathogens are common causes of lethal CAP and the most important cause of secondary bacterial pneumonia during influenza (McCullers 2004). Viral pneumonia, acute respiratory distress syndrome (ARDS) and bacterial co-infections have also been identified as common causes of death in several recent studies (Louie *et al.* 2009; Perez-Padilla *et al.* 2009).

Viral infections engender bacterial colonisation in the host by causing alteration in the innate immune response to common bacterial pathogens. These alterations may lead to severe bacterial pneumonia during influenza (Hussell and Cavanagh 2009). Lower respiratory infections are frequent complications of influenza A and influenza B infections. Furthermore, acute bronchitis, which can result from lower respiratory infections, is more common in those patients with influenza (Kaiser *et al.* 2003).

1.3 Polymerase Chain Reaction (PCR) diagnosis of severe infections

Various techniques have been used for bacterial diagnosis in the microbiology laboratories. Gram-staining, a basic technique, is still used for distinction of bacterial strains by detecting the shape and size of cell and basic cell wall characteristics, but it is not a highly discriminating method. More than 24 hours is required for identification and susceptibility testing of bacteria in blood cultures by routine phenotypic methods. Matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF) mass spectrometry is also used for detection of microorganisms by characterization of microbial proteins. However, it is dependent on protein characteristics and much less useful for recombinant bacteria and viruses (Lay 2001; Lasch *et al.* 2008). Meanwhile, four different methods are available for detection of respiratory viruses, such as viral culture, rapid antigen detection, serology and nucleic acid amplification (Marcos *et al.* 2009). In contrast to these techniques, quantitative PCR is becoming commonplace for the detection and identification of bacteria and viruses as a more rapid, specific and accurate technique (Stanley and Szewczuk 2005; Blyth *et al.* 2009; Szewczuk *et al.*

2010). Commonly targeted regions include species specific genes, genus specific (16S rRNA) or resistance markers (eg. *mecA*/*femA*, *vanA* and *vanB*).

1.3.1 Principles of PCR

PCR is a method for amplification of DNA *in vitro* which is commonly used in clinical microbiology laboratories; and was first developed by Harry A. Erlich, Kary Mullis and Randall K. Saiki in 1984 (Saiki *et al.* 1985; Mullis *et al.* 1986; Mullis and Faloona 1987; Saiki *et al.* 1988).

PCR is utilized for the diagnosis of genetic diseases, detection and identification of pathogenic organisms, genetic fingerprinting and identification of unknown DNA. This technique is also used to identify relationships between parent-child and siblings, to determinate evolution of organisms and to search the sequence specificity of DNA-binding proteins, the technique of which is called footprinting (Innis *et al.* 1988; Boehnke *et al.* 1989; Navidi and Arnheim 1991; Syvanen *et al.* 1993; van Belkum 1994; Schnitzler *et al.* 1998).

Previous techniques have also utilized combinations of PCR amplification and hybridization of sequence specific probes, either radioactively or fluorescently labeled, to increase specificity of laboratory identification, similar to that of southern hybridization, however time to result was close to 3 days. The use of enzyme-linked sequence specific hybridization probes allowed this time to be significantly reduced to around 1 day (Kumar *et al.* 1989; Cockerill 2003)

Conventional PCR protocols using open reaction systems increase the possibility of sample contamination. In the last decade, automated systems have been created for PCR amplification, with detection of the amplicons within this “closed” system (Uhl *et al.*

2002). Integrated thermocyclers and fluorimeters are found in automated systems. Faster, more accurate temperature ramping and „real time" fluorescence detection is now readily available (Cockerill 2003).

1.3.2 Reverse Transcriptase PCR (RT-PCR)

Reverse transcriptase PCR (RT-PCR) is used for amplification of complementary DNA (cDNA) from mRNA by using reverse transcriptase (RT) in the gene expression analysis. The RT step uses either oligonucleotide primers or random oligomers to convert mRNA into cDNA. Although adding primer is not necessary in this step, it may increase the efficiency of the PCR (Stahlberg *et al.* 2004). The subsequent steps are very similar to basic PCR and heat-stable polymerase, specific primers, dNTPs and appropriate buffer are needed in the RT-PCR reaction. The only difference is that cDNA is used in the second step of amplification instead of the target RNA (Hunt 2006).

Primers that hybridize to the poly(A) tail of eukaryotic mRNA trigger reverse transcription from the beginning of the 3" tail of mRNA. Random sequence primers are usually designed as six bases (random hexamers) or nine bases (random nonomers). Sequence specific primers are used for analyzing small amounts of mRNA and hybridization of the primers is target-specific (Kubista *et al.* 2006).

1.3.3 Real Time PCR

Real time PCR, developed in 1992, is a method for the detection and quantification of PCR amplification by monitoring fluorescent dyes or probes as a marker of amplicon produced (Higuchi *et al.* 1992; Lee *et al.* 1993; Livak *et al.* 1995). Although reverse transcriptase PCR is semi-quantitative, real time PCR is more quantitative in the

expression of mRNA and more effective for detection of small amounts of mRNA, due to the use of florescent reporters to monitor the amount of amplified DNA in "real-time" (Hunt 2006). A pre-defined level of fluorescence (threshold) is intersected by each curve at a cycle number (Ct) which is related to the efficiency of the reaction and the amount of starting template. For a reaction of given efficiency, the Ct value therefore relates directly to the amount of template at the start of the assay (Kubista *et al.* 2006). In contrast to conventional PCR, contamination by unwanted amplified DNA is decreased in real-time PCR process (closed systems) involving both amplification and detection of amplified DNA (Cockerill 2003).

Specific probes and non-specific labels are used to measure the fluorescence (Kubista *et al.* 2006). Although intercalating dyes such as ethidium bromide were used traditionally for fluorescence, asymmetric cyanine dyes, such as SYBR Green, have been more widely used as intercalators in recent years (Higuchi *et al.* 1992).

1.3.4 Nested PCR

Nested PCR is a two-step process involving either two distinct sets of primers or semi-nested primers (where one of the first step primers are utilised in the second stage of amplification) and is used to increase specificity of PCR In the first step of a nested PCR process, as in standard PCR, primer pairs are used to amplify a template. The second set of primers anneal to the fragment produced in the first step. The second amplified fragment is therefore shorter than the fragment which was produced in the initial step (Paradise 2002). The use of nested primers reduces the risk of non-specific amplifications. If the initial fragment was wrongly amplified, it is very unlikely that the unwanted locus can be amplified by the second set of specific primers.

1.3.5 Multiplexed Tandem PCR (MT-PCR)

Multiplexed tandem PCR (MT-PCR) is a rapid, sensitive, specific real-time PCR widely used for detection and identification of various pathogens (Stanley and Szewczuk 2005; Lau *et al.* 2010; Szewczuk *et al.* 2010). MT-PCR is the amplification of specific DNA sequence by using nested primers with low numbers of cycles in the first step. Within the linear phase of PCR amplification (the first 15-20 cycles; Figure 1.6) there is no significant amplicon produced, resulting in absence of competition between amplicons or production of inhibitors, meaning that this step may be highly multiplexed. The second step of this nested MT-PCR can subsequently result in highly specific and quantitative detection of target nucleic acids (Stanley and Szewczuk 2005).

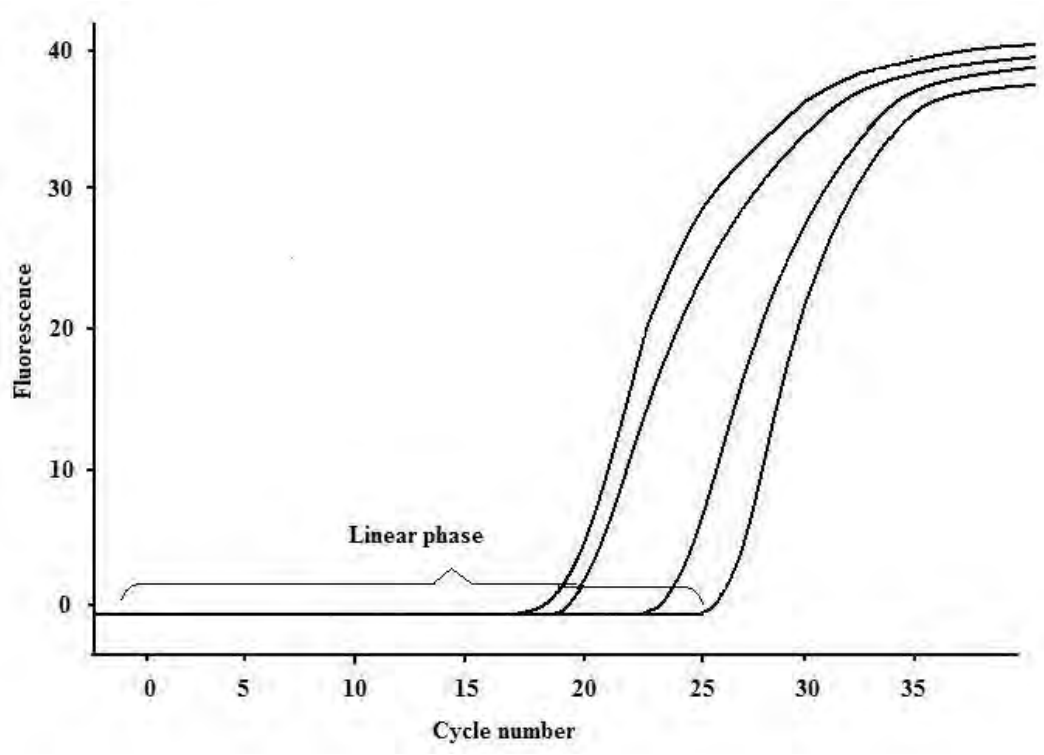

Figure 1.6 The main phases and the effect of cycle numbers and fluorescence level on DNA in PCR.

1.3.5.1 Gram-positive bacteria in blood culture

The Gram-positive 12 assay (GPC 12) is used for detection and identification of Gram-positive bacteria from the genera *Staphylococcus, Streptococcus* and *Enterococcus,* using targets including 16S rRNA consensus sequence and the *nuc, mecA, gseA, lytA, E. faecium ddl, E. faecalis ddl, vanA* and *vanB*.

The *nuc* gene is variably called the thermonuclease, heat-stable nuclease or micrococcal nuclease gene and is used for specific identification of both MRSA and MSSA. This gene was isolated firstly by Foggie and encodes SNase, characterized from *S. aureus*, a polypeptide either 149 or 168 amino acids long (SNase A and SNase B, respectively) (Shortle 1983; Wilson *et al.* 1991; Brakstad *et al.* 1992; Chesneau *et al.* 1993).

The *mecA* gene encodes methicillin resistance in *S. aureus* (MRSA) and *Staphylococcus epidermidis* (MRSE) (Geha *et al.* 1994; Barski *et al.* 1996; Cloney *et al.* 1999; Katayama *et al.* 2000; Louie *et al.* 2002; Fang and Hedin 2003; Cho *et al.* 2007; Goldmeyer *et al.* 2008). The presence of the *mecA* gene in *Staphylococcus* sp. is a more specific indicator of methicillin (oxacillin) resistance than phenotypic identification and susceptibility tests (Louie *et al.* 2000; Brown 2001). Methicillin resistance in *Staphylococcus* sp. is associated with a variant penicillin binding protein (PBP2) which is encoded by *mecA*. This *mecA* gene is carried by a mobile genetic element, the Staphylococcal chromosomal cassette (SCCmec) (Katayama *et al.* 2000; Katayama *et al.* 2003; Francois *et al.* 2004).

The *gseA* gene is used for identification of *S. epidermidis* and other CoNS. The *gseA* encodes glutamic acid-specific serine protease (GluSE) that might be one of the virulence factors in *S. epidermidis* (Ohara-Nemoto *et al.* 2002; Ikeda *et al.* 2004).

The *lytA* gene is usually used as a target gene for identification of *S. pneumoniae* in PCR assays (McAvin *et al.* 2001). Although there has been some controversy over whether the autolysin (*lytA*) is truly specific for *S. pneumoniae*, it is used as a marker for *S. pneumoniae* in various assays (Cherian *et al.* 1998; McAvin *et al.* 2001; Harris *et al.* 2008). Autolysin (*N*-Acetyl Muramoyl-L-Alanine Amidase) that is encoded by the *lytA* gene might be a virulence factor of *S. pneumoniae,* and it plays a main role in cellular autolysis and in cell division (Tuomanen and Tomasz 1990). Another virulence factor of *S. pneumoniae,* cytoplasmatic protein pneumolysin is encoded by the *ply* gene and is used for specific identification of *S. pneumoniae* (Virolainen *et al.* 1994; Lorente *et al.* 2000). When it has been directly compared, *lytA* appear to be more specific than *ply* for detection of *S. pneumoniae* (Messmer *et al.* 2004; Neeleman *et al.* 2004; Suzuki *et al.* 2006). Even though *lytA* may be present in other genetically related microorganisms, it can discriminate *S. pneumoniae* from *Streptococcus mitis* (Kawamura *et al.* 1999).

The identification of *Enterococcus faecium* and *E. faecalis* is effectively achieved by the target genes, $ddl_{faecium}$ and $ddl_{faecalis}$ respectively (Rice 2001; Palladino *et al.* 2003). Because of the synthesis of the peptidoglycan precursors which end in D-Ala-D-Lac (VanA, VanB and VanD) or D-Ala-D-Ser (VanC, VanE, and VanG) in place of D-Ala-D-Ala (D-Alanyl-D-Alanine synthesised by ddl ligase), Enterococci gains vancomycin resistance (Reynolds *et al.* 1994; Arthur *et al.* 1996; Depardieu *et al.* 2003a; Depardieu *et al.* 2003b). Moreover, the classification of *vanA, vanB* and *vanD* determines the level of the resistance to vancomycin and teicoplanin (Arthur *et al.* 1996; Depardieu *et al.* 2003a; Depardieu *et al.* 2003b). The *vanA* gene higher level of vancomycin resistance in VRE (vancomycin resistant Enterococci) than *vanB* (Appleman *et al.* 2004).

1.3.5.2 Respiratory Pathogens

The target genes, *nuc* gene, *mecA* and *lytA* are used for detection of *S. aureus,* MRSA and *S. pneumoniae* respectively. Methicillin resistance in *S. aureus* is mainly encoded by two different specific genes, including *femA* and *mecA* (Vannuffel *et al.* 1995). The *femA* encodes a 48-kDa protein and distinguishes *S. aureus* from coagulase-negative staphylococci (Unal *et al.* 1992). The *femA* gene product plays a role in the cell wall of the bacteria and in the expression of methicillin resistance in *S. aureus* (Berger-Bachi *et al.* 1989; Johnson *et al.* 1995). Unlike *mecA*, which is exclusively detected in methicillin resistant isolates, *femA* can also be found in methicillin-sensitive *S. aureus*. For this reason, detection of *femA* is not as effective a target as *mecA* for PCR detection of methicillin resistance in *Staphylococcus* species (Unal *et al.* 1992; Vannuffel *et al.* 1995).

H. influenzae can be efficiently detected by targeting the *H. influenzae* gyrase gene (*gyrA*). DNA gyrase unwinds double stranded DNA and is a member of the DNA topoisomerase enzyme family which play an essential role in regulation of genomic DNA supercoiling and in control of cell activities, including replication, transcription, recombination and repair (Bugreev and Nevinsky 2009). Mutations in *gyrA* are associated with antibiotic resistance (Perez-Vazquez *et al.* 2004).

For specific identification of *L. pneumophila* and *Legionella longbeachae,* the macrophage infectivity potentiator (Mip) protein, a major virulence factor for *Legionella* sp.*,* is a useful target. Mip analogues were detected in all *Legionella* sp. (Riffard *et al.* 1996). *L. pneumophila* and *L. longbeachae mip* gene sequence has 76% identity (Doyle *et al.* 1998). Mip protein is encoded by the *mip* gene and is important for invasion, optimal intracellular life cycle and survival of *Legionella* sp. Mip

contributes to the survival process of *Legionella* sp. in macrophages and protozoan cells (Cianciotto *et al.* 1989; Cianciotto *et al.* 1990; Cianciotto and Fields 1992).

For detection of *C. pneumonia,* the gene encoding the outer membrane protein A (*ompA*) is employed. Outer membrane proteins directly influence bacterial adaptation by facilitating interactions between the bacterial cell and its host cell (Yuan *et al.* 1989). Moreover, outer membrane proteins of *C. pneumoniae* may be highly conserved and that might be a cause of that *C. pneumoniae* does not have considerable amount of sequence homology (13.3-30%) with other *Chlamydia* species (Gaydos *et al.* 1992).

The matrix gene of Influenza A is used as a target in the MT-PCR assay for detection of Influenza A. The main role of the influenza matrix protein is in virus assembly and budding (Nayak *et al.* 2004). The matrix protein plays a major role in virus replication and in interactions between glycoproteins and ribonucleotide particles included in viral genome (Bui *et al.* 1996; Boulo *et al.* 2007).

Two Corona viruses, hCV-229E and hCV-OC43 are known to cause severe respiratory disease in humans (Siddell *et al.* 1983). For the detection of Corona virus strains hCV-229E and hCV-OC43, the specific genes encoding the Corona virus M (membrane) glycoprotein and the Corona virus N (nucleocapsid) protein are used in MT-PCR assay, respectively. The N protein gene is usually chosen as it is well conserved and large amounts of RNA are typically found in the infected cell (Van der Most and Spaan 1995). The corona virus N protein is a structural RNA-binding protein that plays a role in Corona virus RNA synthesis (Risco *et al.* 1996). In the expression of N protein by the vector RNA, replication of Corona virus-based vector RNA can be efficient and sustained (Schelle *et al.* 2005). On the other hand, M protein plays a multifunctional

role in Corona virus assembly in cooperation with virus nucleocapsid and virus particles (Escors *et al.* 2001).

1.3.5.3 Cytokine detection

Real-time PCR and intracellular immunohistochemistry can be used to detect the expression of cytokines at the RNA and intracellular protein level (Turlej 2009). To detect low amount of cytokines in media, there are two methods currently utilized: Enzyme-linked immunosorbent assay (ELISA) and planar array assays, such as Luminex, MULTI-ARRAY, Bio-Plex or FAST Quant. ELISA is an immunoenzymatic test, which measures the level of individual cytokine secretion (Fu *et al.* 2010; Rizzi *et al.* 2010). Cytokines can be detected by specific immunoreaction between the cytokines and antibodies (He *et al.* 2009). In contrast to the ELISA approach, planar array assays are multiplex immunoassay and can detect multiplex cytokines (Fu *et al.* 2010). Detection of cytokine mRNA by PCR is an effective alternative method and may be an important future tool in conjuction with specific microbial identification (Stanley and Szewczuk 2005).

1.4 Aims

The general aim was to explore the potential utility of MT-PCR in two of the most important clinical specimens coming to the diagnostic laboratory.

Specifically, this project seeks to investigate:

- The sensitivity and specificity of nucleic acid detection for positive blood cultures, using Gram-positive cocci as a model for study.
- Bacterial co-infection in respiratory specimens using samples from the Australian influenza epidemic of 2009 as a model for study.

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Chapter 2
Materials and Methods

2.1 Laboratory consumables

Laboratory consumables are listed in Table 2.1.

Table 2.1 List of laboratory consumables

Laboratory product	Manufacturer	
BACTEC Aerobic/F bottles	Becton Dickinson	Sparks, USA
BACTEC Anaerobic/Lytic bottles	Becton Dickinson	Sparks, USA
BBL Venting Unit	Becton Dickinson	Sparks, USA
Blood transfer device	Becton Dickinson	Sparks, USA
Blue loops (10 µl)	Interpath	Sydney, Australia
Cryotubes (2 mL)	Sarstedt	Mawson Lakes, Australia
Cotton swaps/wooden applicator sticks	Multigate Medical	NSW, Australia
EZ1 DNA Tissue Kit	Qiagen	Doncaster, Australia
Falcon polypropylene tubes (15, 50 mL)	Becton Dickinson	Sparks, USA
Filter-Tips (50 µl)	Qiagen	Doncaster, Australia
Flat cap PCR tubes (0.2 mL)	Corbett Research	Sydney, Australia
Flat cap PCR tubes (0.5 mL)	Astral	Gymea, Australia
Gene-Disc (72 well)	AusDiagnostics	Sydney, Australia
Gene-Disc 100 Sealing Film	AusDiagnostics	Sydney, Australia
Microcentrifuge tubes (1.5 mL)	Eppendorf	Hamburg, Germany
Neptune aerosol barrier tips (10,20,100, 200,1000 µl)	CLP (Continental Lab Products)	San Diego, USA
Parafilm M laboratory film	Pechiney Plastics	Menasha, USA
Phoenix Automated	Becton Dickinson	Sparks, USA

Laboratory product	Manufacturer	
Microbiology		
Pipette tips (200 and 1000 µl)	Sarstedt	Mawson Lakes, Australia
Polypropylene round bottom tubes (14 mL)	Becton Dickinson	Sparks, USA
Polystyrene spreader bars	Pacific Laboratory Products	Victoria, Australia
Thermal paper for video copy processor	Mitsubishi Electric	Kyoto, Japan
Skin Cleansing Alcohol Wipe	Tyco/ Healthcare	Gosport, UK
Syringe (10 mL)	Becton Dickinson	Sparks, USA
Weigh trays	Sarstedt	Mawson Lakes, Australia
Xtracta tool	Sigma-Aldrich	St. Louis, USA

2.2 Laboratory equipment

A list of equipment used in this study is shown in Table 2.2.

Table 2.2 Equipment used in this work

Equipment	Manufacturer	
BACTEC FX	Bactec Dickinson	Sparks, USA
BD Phoenix	Bactec Dickinson	Sparks, USA
Biorad Powerpac 300	Bio-Rad Laboratories	Hercules, USA
BioRobot EZ1	Qiagen	Doncaster, Australia
Cepheid Smart Cycler II®	Cepheid	Sunnyvale, USA
DensiChek densitometer	bioMérieux	Durham, USA
Ependorf Mastercycler	Crown Scientific	Sydney, Australia

Equipment	Manufacturer	
NucliSENS® easyMAG® Extraction Machine	bioMérieux	Durham, USA
Gene-Disc [72] Heat Sealer	Corbett Research	Sydney, Australia
Gene-Plex Robot	Corbett Research	Sydney, Australia
Mettler PB 300 Weight Scale	Mettler Instruments	Zürich, Switzerland
Mitsubishi P91 video copy processor	Mitsubishi Electric	Tokyo, Japan
Nuaire DH Autoflow CO_2 air-jacketed incubator	Nuaire	Plymouth, USA
Qik Spin	Edwards Instruments	Narellan, Australia
Rotor-Gene RG6000	Corbett Research	Sydney, Australia
Sub-cell® GT electrophoresis tank	Bio-Rad Laboratories	Hercules, USA
UviSave	UVItec	Cambridge, England
UviTec transilluminator	UVItec	Cambridge, England
Vortex mixer	Ratek Instruments	Boronia, Australia
Waterbath	Thermoline	Sydney, Australia

2.3 Chemicals and reagents

All chemicals and reagents used this work were of analytical or molecular biology grade.

Table 2.3 List of chemical and reagents

Chemical/Reagent	Source	
Agarose I	Amresco	Solon, USA

Chemical/Reagent	**Source**	
Blood agar base No. 2	Oxoid	Basingstoke, England
Boric acid	Amresco	Solon, USA
Bromophenol blue	Sigma-Aldrich	St. Louis, USA
Buffer G2 (Digestion buffer)	Qiagen	Doncaster, Australia
Ethidium bromide	Amresco	Solon, USA
Ethyl alcohol (Ethanol)	Merck	Kilsyth, Australia
Ethylenediaminetetraacetic acid (EDTA), disodium, dihydrate	Amresco	Solon, USA
Glycerol	Amresco	Solon, USA
Lysostaphin	Sigma-Aldrich	St. Louis, USA
Mueller Hinton agar base	Oxoid	Basingstoke, England
Nutrient broth base	Oxoid	Basingstoke, England
Sodium hydroxide (NaOH)	Amresco	Solon, USA
Tris (hydroxymethyl) aminomethane (Tris)	Amresco	Solon, USA
UltraPure Dnase/Rnase-Free Distilled Water	Gibco BRL	Grand Island, USA
Xylene cyanol	ICN Biomedicals	Aurora, USA

2.4 Preparation of buffers media and solutions

Buffers, media and solutions are listed in Table 2.4. Unless otherwise indicated, all solutions were prepared using water (<0.001 EU/mL, 1-5 ppb TOC, 18.2 MΩ•cm at 25°C) obtained from a Milli-Q Synthesis Ultrapure Water System (Millipore, Billerica,

MA, USA). When required, pH was measured using a Denver Instruments model 15 pH meter (Denver Instruments, Göttingen, Germany)

Table 2.4 List of buffers, media and solutions

Buffer/media/solution	**Composition**
5x Tris-Borate-EDTA (TBE) buffer	0.89 M Boric Acid 0.01 M EDTA, pH 8.0 0.45 M Tris
Blood Agar plates (BA)	20 g Blood Agar Base No. 2 Make up to 950 mL with distilled water Autoclave at 121ºC 15 min and cool to 50ºC before adding 5% Defibrinated Horse Blood
DNA loading dye solution (6×)	10mM Tris-HCl, pH7.6 60mM EDTA 0.03% (w/v) bromophenol blue 0.03% (v/v) xylene cyanol 60% (v/v) glycerol
Lysostaphin	2 mg Lysostaphin dissolved in 1 mL RNase free water
Nutrient broth (NB)	13 g Nutrient broth base to 1 L Autoclave at 121ºC 15 min
PBS (Phosphate Buffered Saline)	0.03 M Di-Sodium Hydrogen Orthophosphate (Na_2HPO_4) 0.03 M Potassium Di-hydrogen Orthophosphate (KH_2PO_4)

2.5 Bacterial Strains

The bacterial strains used as positive controls in this work are listed in Table 2.5.

Table 2.5 List of PCR-control strains

Sample Identification	Laboratory Identifier	Reference or source[a]	Utilized for
E. casseliflavus	906104014	Westmead	GPC 12 Disc
E. coli	ATCC25922	ATCC	Bacterial Load Disc
E. faecalis	ATCC29212	ATCC	Bacterial Load Disc
E. faecalis (VRE7)	RNSH 13334	Royal North Shore Hospital	Sensitivity of GPC 12 Disc
E. faecium (VRE38)	04 354 1687	Westmead	Sensitivity of GPC 12 Disc
E. gallinarium	825503963	Westmead	GPC 12 Disc
E. hirae	915604211	Westmead	GPC 12 Disc
MRSA	09-169-3530	Westmead	Sensitivity of GPC 12 Disc
MRSA	ATCC 25923	ATCC	Bacterial Load Disc[b]
MRSE	09-111-0021	Westmead	Sensitivity of GPC 12 Disc
P. aeruginosa	ATCC 27853	ATCC	Bacterial Load Disc
S. bovis	ATCC 33317	ATCC	GPC 12 Disc
S. pneumoniae	ATCC 49619	ATCC	Bacterial Load Disc[b]

Sample Identification	Laboratory Identifier	Reference or source[a]	Utilized for
S. pneumoniae	09-264-0745	Westmead	GPC 12 Disc
S. pneumoniae	09-260-2487	Westmead	GPC 12 Disc
S. pneumoniae	09-257-2504	Westmead	GPC 12 Disc
S. pneumoniae	09-254-1098	Westmead	GPC 12 Disc
S. pneumoniae	09-247-2394	Westmead	GPC 12 Disc
S. pneumoniae	09-272-0977	Westmead	GPC 12 Disc
S. pneumoniae	09-264-0990	Westmead	GPC 12 Disc
S. pneumoniae	09-266-1243	Westmead	GPC 12 Disc
S. pneumoniae	09-265-0645	Westmead	GPC 12 Disc
S. pneumoniae	09-266-0721	Westmead	GPC 12 Disc
S. pyogenes	ATCC 19615	ATCC	Sensitivity of GPC12 Disc
Vancomycin resistant-E. faecium Van A	817802882	Westmead	GPC 12 Disc
Vancomycin resistant-E. faecium Van A	817902411	Westmead	GPC 12 Disc
Vancomycin resistant-E. faecium Van A	819401052	Westmead	GPC 12 Disc
Vancomycin resistant-E. faecium Van A	828402852	Westmead	GPC 12 Disc
Vancomycin resistant-E. faecium Van A	900801430	Westmead	GPC 12 Disc
Vancomycin resistant-E. faecium Van B	09-319-1256	Westmead	GPC 12 Disc

Sample Identification	Laboratory Identifier	Reference or source[a]	Utilized for
Vancomycin resistant-E. faecium Van B	09-319-1257	Westmead	GPC 12 Disc
Vancomycin resistant-E. faecium Van B	09-319-1250	Westmead	GPC 12 Disc
Vancomycin resistant-E. faecium Van B	09-319-1265	Westmead	GPC 12 Disc
Vancomycin resistant-E. faecium Van B	09-320-0800	Westmead	GPC 12 Disc
Vancomycin sensitive-E. faecalis	09-262-0626	Westmead	GPC 12 Disc
Vancomycin sensitive-E. faecalis	07-212-4789	Westmead	GPC 12 Disc
Vancomycin sensitive-E. faecalis	08-080-1191	Westmead	GPC 12 Disc
Vancomycin sensitive-E. faecalis	08-038-4688	Westmead	GPC 12 Disc
Vancomycin sensitive-E. faecalis	ATCC 29212	ATCC	GPC 12 Disc

[a] Westmead refers to a clinical isolate from Westmead Hospital, Royal North Shore Hospital refers to a clinical isolate from Royal North Shore Hospital

[b] Also used in testing Sensitivity of GPC 12 Disc

2.5.1 Preparation of Bacterial Strains

2.5.1.1 Clinical isolates for GPC 12 assay

Blood culture broths that signalled positive in Aerobic (A) or Anaerobic/Lytic (L) bottles in the BACTEC FX Blood Culture System (Becton-Dickinson, Sparks, MD, USA) were used as clinical specimens. Either 15 µL blood culture broth was added to

185 µL lysostaphin or 20 µL blood culture broth was added to 180 µL G2 buffer by using 10 mL Syringe, BBL Venting Unit and Blood transfer device (Becton Dickinson,Sparks, MD, USA).

Culture-negative aerobic (A) and anaerobic (L) Bactec bottles that had been inoculated with human blood, were used for control samples of GPC 12 assay. Control strains were inoculated into culture-negative blood broth, and then treated immediately with lysostaphin or G2 buffer (Section 2.6.2).

2.5.1.2 Clinical isolates for Bacterial Load assay

Clinical isolates for Bacterial Load assay were from sputum, endotracheal tube (ETT) aspirate, bronchial washing, bronchoalveolar lavage (BAL), nose/throat swab and nasopharyngeal aspirates (NPA). 200 isolates were collected from patients admitted to Intensive Care Units with severe influenza during the 2009 Australian influenza epidemic (ANZIC 2009).

2.5.1.3 Culture of Control strains

Most bacterial strains were grown on Oxoid blood agar plates supplemented with 5 % defibrinated horse blood (BA) in humidified atmosphere at 37 °C and 5 % CO_2. Isolates cultured on BA were incubated overnight at 37 °C. Bacterial strains used as positive controls were sub-cultured on to fresh agar. After incubation, several colonies were collected from the agar plates and inoculated into distilled water by using sterile cotton swabs.

2.6 Nucleic acid Purification

Genomic DNA was extracted using the BioRobot EZ1 DNA Tissue Kit (Qiagen Pty Ltd, Doncaster, Victoria, Australia) or NucliSENS® easyMAG® extractor (bioMérieux, Durham NC, USA), according to the manufacturer‟s instructions (Table 2.6). BioRobot EZ1 Tissue Kit was used for isolation of genomic DNA from pure or mixed broth cultures of Gram positive bacterial strains. NucliSENS® easyMAG® extractor was used for isolation of genomic DNA from sputum, swab, fluid, ETT, NPA, aspirate, bronchial washing, BAL and nose/throat swab for Respiratory and Bacterial Load assays.

Table 2.6 DNA extraction Kits and protocols

Kit	Manufacturer	Use in this study	Protocol
BioRobot EZ1	Qiagen, Doncaster Victoria, Australia	Isolation of genomic DNA from pure or mixed broth cultures of Gram positive bacterial strains	200 µL samples eluted in final volume 100 µL
NucliSENS® easyMAG® generic extraction for DNA and RNA	bioMérieux, Durham NC, USA	Isolation of genomic DNA from sputum, swab, fluid, ETT, NPA, aspirate, bronchial washing, BAL and nose/throat swab for Bacterial Load assay	Final elution volume 100 µL

2.6.1 DNA extraction from blood culture broth

Blood culture broth was obtained separately from BD Bactec Plus Aerobic bottle and Bactec Lytic bottle. 15 µL was added into 185 µL Lysostaphin and incubated for 30 min

at 37 °C with CO_2, or 20 µL was added into 180 µL G2 Buffer and incubated for 30 min at room temperature. After incubation, they were extracted according to the manufacturer‟s protocol.

2.6.2 DNA extraction of positive controls for GPC 12 assay

Bacterial suspensions were prepared to obtain approximately 1.0 x 10^7 cfu/mL in RNase free water or PBS and serially diluted in NB (ten-fold dilutions to 10^0 cfu/mL). 20 µL from each dilution was added into 180 µL negative blood broth extracted from Lytic and Aerobic Bactec Blood bottles. 20 µL of each final solution was added into 180 µL G2 buffer and incubated at room temperature for 30 min. Bacterial samples were also treated with lysostaphin, where 15 µL of each blood culture was added into 185 µL lysostaphin and incubated for 30 min at 37 °C and CO_2. After incubation, samples were extracted using Qiagen EZ1 DNA Tissue Kit according to manufacturer‟s instructions.

2.6.3 DNA extraction of positive controls for Bacterial Load assay

Bacterial cultures on BA were suspended in PBS to achieve an optical density of 0.5 McFarland (~1.5 x 10^8cfu/mL) using a DensiChek densitometer. Serial dilutions were prepared in NB (ten-fold dilutions from 1.0 x 10^8cfu/mL to 1.0 x 10^4cfu/mL). Each diluted sample was extracted by using the NucliSENS® easyMAG® extractor (bioMérieux, Durham NC, USA) according to manufacturer‟s instructions.

2.7 Enumeration of positive controls

Serial dilutions of positive controls were plated onto appropriate agar plates using disposable polystyrene spreaders and incubated for 24 hours (Section 2.5.1.3) at 37°C

with 5% CO_2. After incubation, colony forming units (cfu) were calculated for each control strain.

2.8 Polymerase chain reaction

2.8.1 Conventional PCR

PCR amplification was performed in a 50 µl reaction volume (Table 2.7). Template DNA was replaced by MilliQ water in negative control reactions. Reactions were performed in a Eppendorf Mastercycler (Crown Scientific, Australia) under the following conditions: 94 °C for 5 min (initial denaturation), then 35 cycles of 94 °C for 1 min (denaturation), annealing for 30 secs, and 72 °C for extension; a final extension of 72 °C for 5 min and holding at 25 °C. The annealing temperature was generally set 2 °C below the T_m of the primers, while the extension time was determined using guidelines for *Taq* polymerase (30 s/500 bp). PCR amplified products were separated by gel electrophoresis in a Sub-cell® GT electrophoresis tank (Bio-Rad, USA) (Section 2.8.1.2).

Table 2.7 List of components for PCR reaction

PCR Component	Final concentration	Source
BioTaq DNA Polymerase	1 U	Bioline, London, England
10 × NH_4 PCR Buffer	1 ×	Bioline, London, England
dNTPs (dATP, dTTP, dCTP and dGTP)	200 µM	Bioline, London, England
$MgCl_2$	1.5 µM	Bioline, London, England
Primers	200 nM	Bioline, London, England

PCR Component	Final concentration	Source
MilliQ water	-	Bioline, London, England
Template DNA	2 µl genomic DNA	Bioline, London, England
Total	**50 µl**	

2.8.1.1 Oligonucleotide primers

Primer3 (version 0.4.0) was used to design oligonucleotide forward and reverse primers (http://frodo.wi.mit.edu/primer3/). Primer pairs were analysed by using Oligo-Analyzer (version 1.0.2) to check for self-complementarity and potential primer dimerization. Oligonucleotide primers were manufactured by Sigma Proligo (Sigma-Aldrich Australia Ltd), or Geneworks (Hindmarsh, Australia) using an automated DNA synthesiser. Oligonucleotides were provided lyophilised and were diluted with distilled MilliQ water to a concentration of 100 µM and stored -20 °C. Primers used in this work are listed in Table 2.8.

Table 2.8 List of oligonucleotide primers

Primer Name[a]	Sequence (5' - 3')	Init Time	Ann (°C)	Ext Time	Cycles	Reference/ Amplificon (bp)
nuc 1	GCG ATT GAT GGT GAT ACG GTT	1 min	55 for 30 secs	1.5 min	37	Brakstad et al.,1992)
nuc 2	AGC CAA GCC TTG ACG AAC TAA AGC					
nuc-OF	TGC AAC GAT TCA TGT TGT AG	1 min	51 for 30 secs	1 min	35	This study
nuc-OR	CCA CTA GCA GCA GTG ACA CTT					

[a]F denotes forward primer, R denotes reverse primer

2.8.1.2 Agarose gel electrophoresis

8 µL of samples containing PCR products were combined with 2 µL of 6 x DNA loading dye solution and separated by electrophoresis. PCR products with DNA loading dye solution were electrophoresed in a Biorad Sub-cell® GT electrophoresis tank containing 0.5 × TBE buffer using a 1-1.5 % agarose gel concentration at 200 V for a period of 35-40 min depending on the size and concentration of gel being electrophoresed. Hyperladder I (Bioline, London, UK) was run on the gel for size estimation of the PCR amplicons (Figure 2.1).

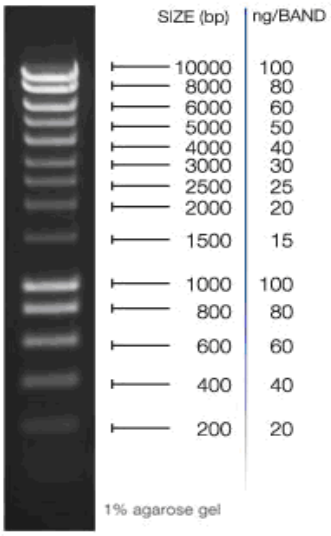


Figure 2.1 Hyperladder I. DNA ladder for quantitative analysis of linear double-stranded DNA fragments on agarose gel. Hyperladder I produces a pattern of 14 regularly spaced bands, ranging from 200 to 10,000 bp, each containing between 15 and 100 ng of DNA where the 1000 and 10,000 bp bands have the highest intensity (100 ng) to allow easy quantification. The featured gel: 720 ng (5 µl) of the ladder was loaded onto a 1-1.5 % agarose gel and stained with ethidium bromide.

2.8.1.3 Staining with ethidium bromide

After electrophoresis, agarose gels were stained in 1 µg/mL ethidium bromide in 0.5 × TBE for approximately 20 min. Gels were photographed under ultraviolet light using a UviSave and UviTec transilluminator apparatus (UVItec, Cambridge, England). When required, DNA bands of interest were excised from the gel using a clean Xtracta tool. The excised DNA was extracted and purified using the Quick Gel Extraction Kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer‟s instructions.

2.8.2 Multiplexed Tandem-PCR

MT-PCR consists of two main steps. The first step is a limited number of PCR cycles (10-20), designed to amplify without competition, at the linear stage of PCR. Oligonucleotide primers for specific ampification were designed by the company (Ausdiagnostics, Sydney, Australia). All primers have a T_m of 60.0 °C and were supplied with the discs. The second step involves amplification of target DNA using primers that are nested within the first step („outer‟) primers in a Corbett Rotor-Gene 6000 thermal cycler (Figure 2.2).

2.8.2.1 MT-PCR Amplification

2.8.2.1.1 First round amplification

In the first round amplification, template DNA was added to 0.2 mL tubes containing first step lyophilised outer primer (for all targets), then placed into the Gene-plex robot. Final primer concentration was 0.2 µM after addition of the first step reaction mixture (AusDiagnostics) and first step additive (Taq DNA polymerase and, where necessary, reverse transcriptase; AusDiagnostics). First step amplification proceeded using the following conditions; 55 °C for 2 min (reverse transcription), 95 °C for 5 min (RT

denaturation), followed by 10-20 cycles of 95 °C for 10 secs, 60 °C for 20 secs and 72 °C for 20 secs. These reactions products were subsequently utilised as template in the second round of amplification (Section 2.8.2.1.2).

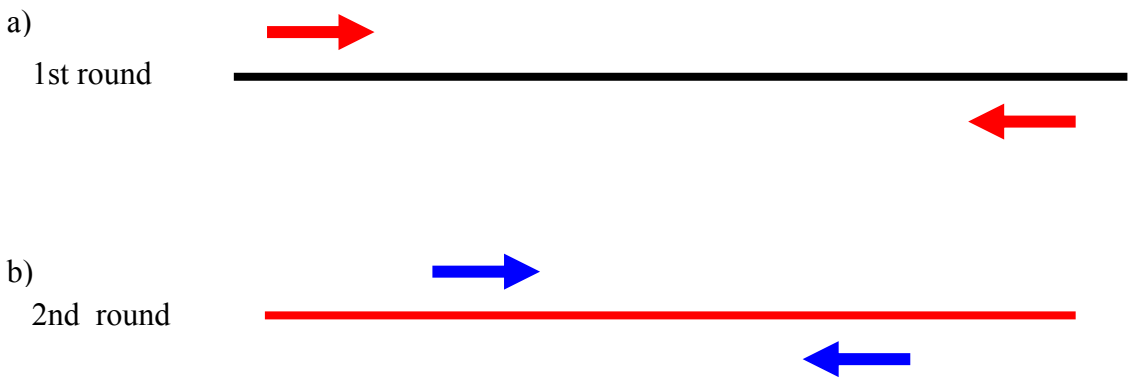


Figure 2.2 Fully nested primer arrangement utilised in MT-PCR.

Schematic diagram of primer arrangement for amplification of MT-PCR targets. The first round of amplification (a) utilises primers designed to amplify short target sequences (red arrows) in the template nucleic acid (black line). Products of this first round are used as template for the second round of amplification (b), where blue arrows indicate fully nested primers targeting the product of the first round of amplification (red line).

2.8.2.1.2 Second round amplification

After the first round of amplification, amplified product was diluted 1:75 in the Gene-plex robot by using second step mix (second step reaction master mix, containing Evagreen dye, and second step additive; AusDiagnostics) to obtain final primer concentration of 0.4 µM. An aliquot of 20 µl of this mixture was added to each position of a 72-well gene disc containing the lyophilised inner (nested) primers (one target per

well) by the Gene-plex robot. Discs were transferred from the Gene-plex robot to Gene-Disc Heat Sealer and sealed using Heat Sealing film (Corbett Research). Discs were placed in a Corbett Rotor-Gene 6000 for amplification using the following conditions: 95 °C for 10 min (initial denaturation), followed by 30 cycles of 95 °C for 30 secs, 60 °C for 10 secs and 72 °C for 10 secs, with acquisition after each extension step. Following the completion of cycling, a melting curve was analyzed using the following conditions; 75 °C for 90 secs (preconditioning) after which the temperature was gradually increased to 93 °C in 0.4 °C increments, with acquisition of fluorescence at each increment. Reaction results were analysed using the Rotor-Gene 6000 Series Software v 1.7 (Corbett Research).

2.8.2.1.3 Quantitative Analysis

Quantitative analysis of MT-PCR reactions was performed through comparison to standard curves generated from the extracted controls. Standard curves were prepared through ten-fold serial dilution of known quantities of extracted controls. Subsequent reactions were compared to standards to obtain quantitative values for DNA present in samples.

2.8.2.2 The discs used in the MT-PCR assays

2.8.2.2.1 Gram Positive 12 Disc

The Gram Positive 12 (GPC 12) disc (AusDiagnostics Pty. Ltd., Sydney, NSW) was used for detection and identification of the bacterial pathogens *S. pyogenes, S. pneumoniae, E. faecalis* (vancomycin sensitive and resistant), *E. faecium* (vancomycin sensitive and resistant), MRSA, MSSA and MRSE using the targets shown in (Table 2.9).

Table 2.9 The target genes and their description for Gram Positive 12 assay

Gene Symbol	Description
pan-*Staphylococcus*	*Staphylococcus* sp. 16S rRNA consensus sequence
pan-*Streptococcus*	*Streptococcus* sp. 16S rRNA consensus sequence
pan-*Enterococcus*	*Enterococcus* sp. 16S rRNA consensus sequence
nuc	*S. aureus* thermostable nuclease gene
mecA	Methicillin-resistance gene
gseA	*S. epidermidis* gene for glutamic acid-specific serine protease
lytA	*S. pneumoniae* autolysin gene
E. faecalis ddl	*E. faecalis* D-Ala:D-Ala ligase gene
E. faecium ddl	*E. faecium* D-Ala-D-Ala ligase gene
vanA	Vancomycin resistance gene A
vanB	Vancomycin resistance gene B
SPIKE	Artificial sequence for assay control

2.8.2.2.2 Bacterial Load Discs

The Bacterial Load assay (AusDiagnostics Pty. Ltd., Sydney, NSW) was used for detection and quantification of pathogens from a family *Enterobacteriaceae* species and the genera *Staphylococcus* sp., *Streptococcus* sp., *Enterococcus* sp., and *Pseudomonas* sp. using 16S rRNA consensus targets for each genus (Table 2.10).

Table 2.10 Target genes and their descriptions for Bacterial Load Assay

Gene Symbol	Description
pan-*Staphylococcus*	*Staphylococcus* sp. 16S rRNA consensus sequence
pan-*Streptococcus*	*Streptococcus* sp. 16S rRNA consensus sequence
pan-*Enterococcus*	*Enterococcus* sp. 16S rRNA consensus sequence
pan-*Enterobacteriaceae*	*Enterobacteriaceae* species 16S rRNA consensus sequences
pan-*Pseudomonas*	*Pseudomonas* sp. 16S rRNA consensus sequence
SPIKE	Artificial sequence for assay control

2.8.2.2.3 Statistical Analysis

Assays were performed on two or more separate occasions, each time in triplicate, and the results reported as mean values ± 1 standard deviation. Student‟s t-test was used for statistical analysis of combined species groups using SPSS v15 (SPSS Inc.), with a p-value of < 0.05 considered statistically significant.

Chapter 3

Detection of Bacterial Pathogens in Blood Culture

3.1 Introduction

Community-acquired infection is implicated in the majority (58.1%) of cases of septic shock (Aledo *et al.* 1998). Although Gram-negative bacteria are a major cause of septic shock (47.9%), Gram-positive bacteria are responsible for approximately 38.3% of cases. *S. aureus* is the major cause of Gram-positive septic shock at 40.7%, followed by *S. pneumonia* at 20.9% (Kumar *et al.* 2006b). Others have found *Streptococcus* to be the leading cause (22% of Gram-positive septic shock), followed by coagulase-negative *Staphylococcus* (CoNS) (16%) (Aledo *et al.* 1998). However, CoNS is commonly isolated from blood cultures as a result of contamination (pseudobacteremia) and can be a cause of false-positive blood cultures. Although CoNS bacteraemia may be clinically important in up to 24.7% cases, the majority are probably contamination (Souvenir *et al.* 1998). The amount of bacteria required to cause septic shock is unknown but varies with pathogen and it is known that bacterial load and microbial virulence contribute to septic shock (van der Poll and Opal 2008). Bacteraemia may also be polymicrobial, requiring the simultaneous recognition of multiple pathogens (Aledo *et al.* 1998).

The GPC 12 MT-PCR assay is designed to detect Gram-positive bacterial pathogens from the genera *Staphylococcus, Streptococcus* and *Enterococcus*. Principal pathogens sought in this assay are *S. aureus, S. epidermidis, S. pneumoniae, E. faecalis* and *E. faecium*.

S. aureus is a Gram-positive coccus and a leading cause of community-acquired and hospital-acquired bacteraemia. The annual incidence of *S. aureus* bacteremia has been estimated between 19- 40 cases per 100 000 population in the USA and Canada and 35

cases per 100 000 population annually in Australia (Collignon *et al.* 2005; Laupland *et al.* 2008; El Atrouni *et al.* 2009).

S. aureus causes a range of infections, including skin and soft tissue infections, necrotising pneumonia, uncommon meningitis, endocarditis, intravascular catheter-related bacteraemia and severe sepsis syndrome (Eady and Cove 2003; Mongkolrattanothai *et al.* 2003; Francis *et al.* 2005; Farley 2008; Aguilar *et al.* 2010). Toxin related diseases (e.g. staphylococcal scalded skin syndrome, staphylococcal foodborne disease and toxic shock syndrome) are associated with epidermolytic toxins, Panton-Valentine leukocidin (PVL), enterotoxins, and toxic shock syndrome toxin- 1 (TSST-1) (Lowy 1998; Murray 2005; Yamasaki *et al.* 2005; Labandeira-Rey *et al.* 2007; Cremieux *et al.* 2009; Herchline n.d.).

S. epidermidis is one of the CoNS. It is considered a relatively non-pathogenic microorganism and an uncommon cause of infection in immune compromised patients but is a very common blood-culture contaminant (Khatib *et al.* 1995; Ziebuhr *et al.* 2006). *S. epidermidis* normally colonizes the host‟s skin and mucous membranes. Although *S. epidermidis* is significantly less pathogenic than *S. aureus*, it is an important cause of native valve endocarditis; and is a major cause of infections associated with implanted medical devices (Caputo *et al.* 1987; Rupp and Archer 1994; Vuong and Otto 2002).

S. pneumoniae is a Gram-positive coccus that has more than 90 serotypes (Kaltoft et al. 2008). *S. pneumoniae* is a major cause of community-acquired bacterial pneumonia, upper respiratory tract infection, invasive disease and otitis media (Kaltoft et al. 2008; Murphy et al. 2009; Quintero et al. 2011). Pneumococcal pneumonia is associated with

high fever, cough, shortness of breath, rapid breathing and chest pains but none of these are specific for the pathogen itself (McChlery et al. 2009).

Enterococcus was first recognized in 1899 (Thiercelin 1899). *E. faecalis* is the major cause of enterococcal infections (85%-90%) followed by *E. faecium* with 5%-10% (Moellering 1992). Although Enterococci are commensal members in the gastrointestinal tract and vaginal vault, bacteraemia is the third most common infective manifestation of enterococcal infection. (Moellering 1992; Jett *et al.* 1994).

3.2 Aims

The purpose of this chapter is to:

- Analyse potential targets for nucleic acid based on detection of Gram-positive pathogens in blood culture.
- Test the inhibitory effect of blood culture broth on *S. pneumoniae* detection by MT-PCR, after extraction of DNA.

3.3 Results

3.3.1 Specific identification of Gram-positive cocci (including MRSA and MSSA)

A total of 159 positive blood cultures from 124 patients, including 31 MRSA, 53 MSSA, 30 MR-CoNS (Methicillin-resistant coagulase-negative *S. epidermidis*), 18 MS-CoNS (Methicillin-sensitive coagulase-negative *S. epidermidis*) , 11 Enterococci and 16 Streptococci strains, were extracted and tested by MT-PCR using the GPC-12 panel for MT-PCR and Lysostaphin for DNA extraction. A positive pan-*Staphylococcus* 16S signal was observed in all *S. aureus* and CoNS isolates (Table 3.1). 4 out of 53 MSSA

specimens, all from the same patient, were negative in the *nuc* gene assay (Table 3.1). The assay was repeated with the 4 discrepant samples to confirm the negative result. The final results give the GPC-12 assay 92.5 % sensitivity for MSSA. If this was considered by individual patient, there were 123/124 correct results, giving a sensitivity of 99.2% overall and a sensitivity for *S. aureus* of 98.1%.

Table 3.1 Results of MT-PCR compared with those of culture and susceptibility identification (Testing of Clinical samples by using Lysostaphin for extraction)

Isolate identification by culture and susceptibility testing	Number of Samples	Number of isolates identified by PCR			
		nuc+ *mecA*+	*nuc*+ *mecA*-	*nuc*- *mecA*+	*nuc*- *mecA*-
MRSA (n=26)	31	31	0	0	0
MSSA (n=36)	53	0	49	0	4
MR-CoNS (n=28)	30	0	0	30	0
MS-CoNS (n=15)	18	0	0	0	18
Other [a] (n=18)	27	0	0	0	27

[a] Nonstaphylococcal (*Streptococcus* sp., and *Enterococcus* sp.) (Appendix A)

Expected result: (*nuc*+ *mecA*+) = MRSA, (*nuc*+ *mec*A-) = MSSA, (*nuc*- *mecA*+) = MR-CoNS, (*nuc*- *mecA*-)=MS-CoNS Four blood cultures from 2 patients were identified as *Streptococcus* sp. and the MT-PCR identified these as *Enterococcus* sp. Left colum: number of patients in parentheses.

Cross-reactivity between genera using lysostaphin pre-treated extract 16S rRNA targets was trivial and was not a significant source of error (Appendix A). As seen in Table 3.2 the Ct values of *S. aureus* are significantly different from the Ct values of CoNS ($P < 0.001$).

Table 3.2 Statistical data for the Ct values of pan-*Staphylococcus* 16S target in *S. aureus* and CoNS

Strain	Mean	Std Deviation
S. aureus	3.6	1.8
CoNS	6.4	3.9

3.3.1.1.1 Conventional PCR for 4 missed nuc samples

Two different primers pairs were used for confirmation of the 4 MSSA specimens that were negative for the *nuc* gene in the MT-PCR assay (Section 2.8.1.1) , which one has been used in several studies and another pairs was designed for this study (Figure 3.1 and 3.2) (Brakstad *et al.* 1992; Barski *et al.* 1996; Saha *et al.* 2008). The identification of these 4 isolates was confirmed a second time as MSSA by automated identification (Phoenix, Becton Dickinson, Sparks, USA), but conventional PCR using these primers was unable to successfully amplify the *nuc* gene (Figure 3.1), despite nucleotide identity in multiple available sequences for both primer pairs (Figures 3.3-3.6). Further genotyping of this isolate is pending.

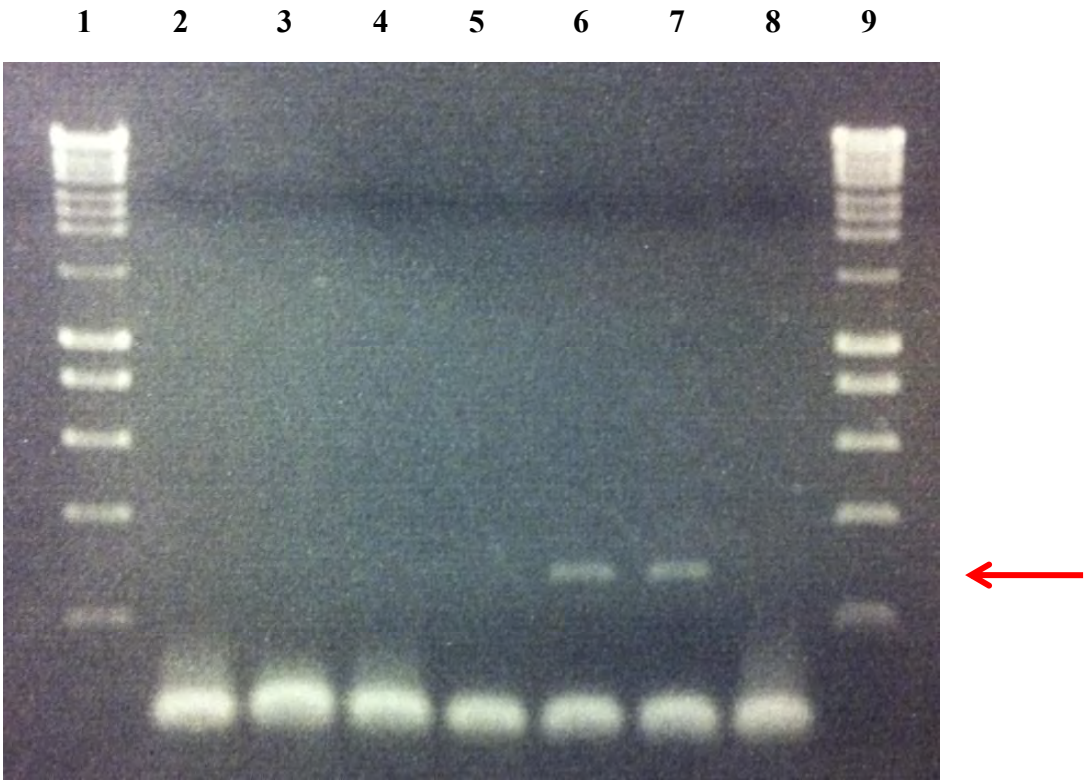


Figure 3.1 Gel electrophoresis of PCR by „nuc1“ and „nuc2“ primers.

Lane 1-Hyperladder I, Lanes 2-5 – Four *nuc* negative *S. aureus* DNA samples, Lane 6 and 7- Positive control (MRSA/ ATCC 25923), Lane 8- Negative control (RNase-free water), Lane 9-Hyperladder I. The red arrow indicates expected size.

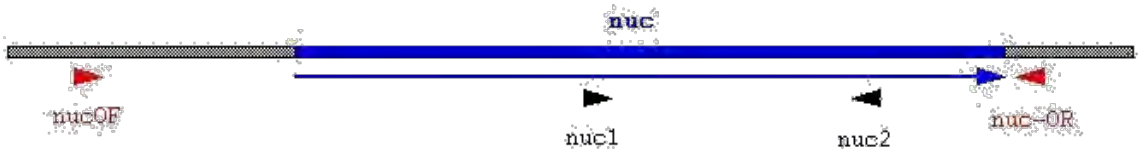


Figure 3.2 The positions of the *nuc* primers.

The blue line indicates the consensus sequence of the nuc gene in the Accession number NC_002951 (between 887710-888396 bp.). The red arrows indicate the position of primers nuc-OF and nuc-OR (outer primers) (between 887503-887522 bp. and 888409-888429 bp.); the black arrows indicate the position of primers nuc1 and nuc2 (inner primers) (between 887995-888015 bp. and 888250-888273 bp.). (Section 2.8.1.1)

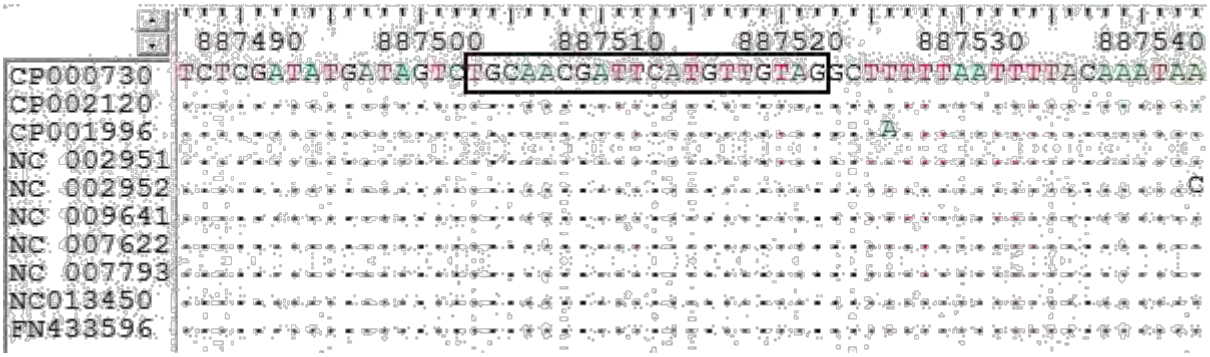


Figure 3.3 The position of the forward primer „nuc-OF" (numbering of the positions is according to Accession number NC_002951, between 887503-887522 bp.) (100% identity). The black box indicates the position of the primer.

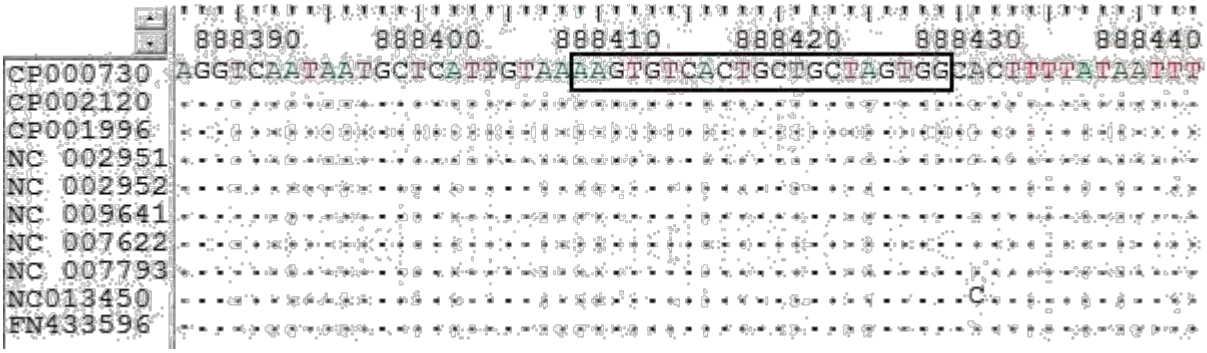


Figure 3.4 The position of the reverse primer „nuc-OR" (numbering of the positions is according to Accession number NC_002951, between 888409-888429 bp.) (100% identity). The black box indicates the position of the primer.

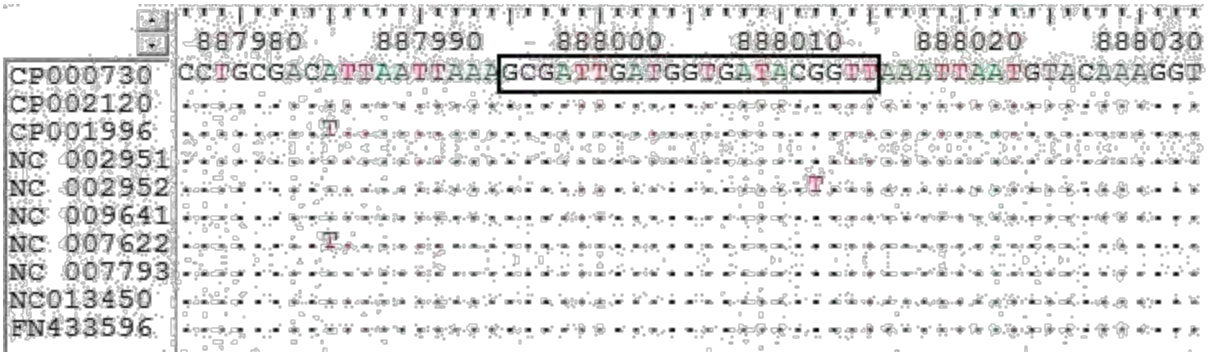


Figure 3.5 The position of the forward primer „nuc1" (numbering of the positions is according to Accession number NC_002951, between 887995-888015 bp.) (only 1 nucleotide in 1 sequnce is different) The black box indicates the position of the primer.

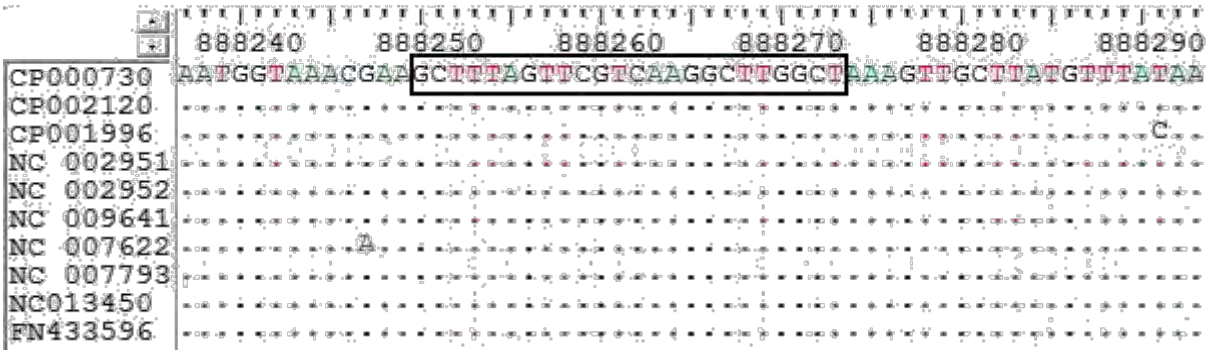


Figure 3.6 The position of the reverse primer „nuc2" (numbering of the positions is according to Accession number NC_002951, between 888250-888273 bp.) (100% identitiy) The black box indicates the position of the primer.

3.3.2 Increased cross-reactivity between 16S rRNA targets using a different extraction method

A total of 113 blood cultures from 91 patients were next tested using Lysis Buffer G2 from the manufacturer for extraction. These included 15 MRSA, 21 MSSA, 30 MR-CoNS, 17 MS-CoNS, 19 different types of Streptococci and 11 Enterococci. All results were consistent with the CIDMLS reference laboratory identification (Table 3.3).

Unlike the previous set of isolates studied after lysostaphin based extraction (Section 3.3.1 and Appendix A) there was some cross-reactivity apparent (Appendix B). 16 of the *S. aureus* samples also gave positive signals for pan-*Enterococcus* 16S target (less than 10^{-2} of primary signals) and 2 of them were positive for pan-*Streptococcus* 16S target (less than 10^{-1} of primary signals). Similarly, 24 of the CoNS samples were positive for pan-*Enterococcus* 16S target (less than 10^{-2} of primary signals) and 1 was positive for pan-*Streptococcus* 16S target at less than 10^{-1} of the level of the primary signal. Typically, compared to the primary positive target signal, cross-reaction was observed at less than 10% of primary signal intensity (Table 3.3). Only 1 of 113 tested samples were incorrect. In this case, an isolate identified as CoNS by Phoenix (Becton Dickinson, USA) yielded no staphylococcal PCR signal. If the automated Phoenix ID is accepted, the assay has 99.1% sensitivity with 112 positive results. The discrepant result has yet to be confirmed as *Staphylococcus* sp. by genotyping.

Table 3.3 The results of PCR for Testing of Clinical samples by using Lysis Buffer G2

Strains	Number of patients	Number of samples	Number of isolates positive by PCR		
			pan-*Staphylococcus*	pan-*Streptococcus*	pan-*Enterococcus*
MRSA	(n=12)	15	15	2 [a]	6 [a]
MSSA	(n=15)	21	21	-	10 [b]
MR-CoNS	(n=26)	30	30	1 [a]	14 [b]
MS-CoNS	(n=14)	17	16	-	10 [a]
E. faecium (*vanB*)	(n=1)	1	-	-	1
E. faecalis	(n=7)	10	4 [a,e]	-	10
S. pyogenes	(n=3)	3	-	3	1 [c]
S. parasanguinis	(n=1)	1	-	1	1 [c]
S. australis	(n=1)	1	-	1	1 [a]
S. acidominimus	(n=1)	1	1 [c]	1	-
S. pneumoniae	(n=1)	1	-	1	-
S. milleri	(n=1)	1	1 [d]	1	1 [d]
α-*hemolytic Streptococcus*	(n=5)	6	1 [b]	6	4 [a]
Group B/G *Streptococcus*	(n=3)	5	3 [a]	5	2 [a]

Positive for 16S targets less than: [a] 10^{-1}, [b] 10^{-2}, [c] 10^{-3}, [d] 5%, of primary signal intensity

[e] 2 of 4 blood culture samples were evidently contaminated with MR-CoNS, as subcultures grew both organisms.

Table 3.4 The combined results of PCR samples extracted with either G2 Lysis Buffer or Lysostaphin treatment

Clinical Samples	Number of patients	Number of samples	Correct results	Failed for
MRSA	(n=38)	46	46	-
MSSA	(n=51)	74	70	4 *nuc*[a] (n=1)
MR-CoNS	(n=54)	60	54	6 *mecA*[a,b] (n=5)
MS-CoNS	(n=30)	35	34	1 pan-*Staphylococcus* (n=1)
Enterococcus faecium (*vanB*)	(n=2)	2	2	-
Enterococcus faecalis	(n=11)	16	16[c]	1 pan-*Enterococcus* (n=1)
Enterococcus casseliflavus	(n=1)	2	2	-
Enterococcus sp.	(n=1)	2	2	-
Streptococcus pyogenes	(n=7)	9	9	-
Streptococcus parasanguinis	(n=1)	1	1	-
Streptococcus australis	(n=1)	1	1	-
Streptococcus acidominimus	(n=1)	1	1	-
Streptococcus pneumoniae	(n=5)	6	6	-
Streptococcus milleri	(n=1)	1	1	-
α-hemolytic *Streptococcus*	(n=6)	7	7	-
Group B and Group G *Streptococcus*	(n=3)	5	5	-
Streptococcus sp.	(n=2)	4	-	4 pan- *Streptococcus*[d] (n=2)
Total	(n=215)	272	257	

[a] Positive for pan-*Staphylococcus*

[b] 2 of 6 *mecA* negative samples were positive for *gseA*

[c] *E. faecalis* positive for *E. faecalis ddl*

[d] Four blood cultures were identified as *Streptococcus* sp. and the MT-PCR identified these as *Enterococcus* sp.

Of 272 clinical specimens tested, Positive Predictive Value (PPV) and Specificity was 100% for target genes, including pan-*Staphylococcus, nuc* and *mecA*. Negative Predictive Value (NPV) was 98.27%, 97.43% and 96.51%; Sensitivity was 99.53%, 96.66% and 94.64% for pan-*Staphylococcus, nuc* and *mecA,* respectively (Table 3.5). Of the 215 patients tested in both set (lysostaphin pre-treated and G2 Buffer Lysis pre-treated), the correct result was obtained for 205 (97.7%)

Table 3.5 The PPV, NPV, Sensitivity and Specificity for all 272 clinical specimens

Targets	Positive Predictive Value	Negative Predictive Value	Sensitivity	Specificity
pan-*Staphylococcus*	100%	98.27%	99.53%	100%
nuc	100%	97.43%	96.66%	100%
mecA	100%	96.51%	94.64%	100%

3.3.2.1 Cross reactivity

A colleague in this laboratory had previously shown that blood cultures signalling as positive in the BACTEC FX Blood Culture System (Becton-Dickinson, Sparks, MD, USA) were typically 10^6-10^8 cfu/mL when subcultured (Thomas 2007). Ten different isolates of *S. pneumoniae*, 4 isolates of Vancomycin resistant (*vanA*) *E. faecium*, a Vancomycin resistant (*vanA*) *E. faecalis* isolate, 5 isolates of Vancomycin resistant (*vanB*) *E. faecium*, 5 isolates of Vancomycin sensitive- *E. faecalis*, and a single isolate each of *Enterococcus gallinarum*, *Enterococcus casseliflavus*, *Enterococcus hirae*, *Enterococcus avium*, and *Streptococcus bovis* were therefore inoculated into blood

culture bottles at a final concentration of 10^6 cfu/mL, immediately extracted and tested by MT-PCR. Each strain gave a positive MT-PCR signal at the lowest concentration tested ($1.0x10^6$ cfu/mL) and cross-reacting fluorescence signals at less than 40% of the intensity of primary signals. *S. bovis* gave positive signals (approximately 40% of the dominant pan-*Streptococcus* 16S signals) for both the pan-*Staphylococcus* and pan-*Enterococcus* targets (Table 3.6).

Table 3.6 The results of low- concentration specimens in the testing threshold detection

Strains		No. of isolates identified by PCR		
		pan-*Staphylococcus*	pan-*Streptococcus*	pan-*Enterococcus*
Streptococcus pneumoniae	(n=20)	2 [a]	20	18 [a]
Streptococcus bovis	(n=2)	1 [b]	2	1 [b]
VRE faecium vanA	(n=10)	1 [c]	-	10
VRE faecium vanB	(n=10)	1 [d]	-	10
Enterococcus (non faecium-faecalis)	(n=8)	5 [e]	2 [c]	7[f]
Vancomycin sensitive-Enterococcus faecalis	(n=10)	1 [d]	-	10

Positive for 16S targets less than: [a] 5%, [b] 40%, [c] 7%, [d] 20%, [e] 10% of primary signal intensity.

[f] *Enterococcus* sp., which is negative for pan-*Enterococcus*, was positive for *vanA* target gene.

The sensitivity of the assay was then measured by inoculation of serial dilutions (from 10^6 down to 1 cfu/mL) in blood culture broth. *S. pyogenes, S. pneumoniae, E. faecalis* (*vanA*), *E. faecium* (*vanB*), MRSA, MSSA and MRSE were positive at $\geq 3.92 \times 10^5$ cfu/mL, 3.92×10^4 cfu/mL, 3.92×10^4 cfu/mL, 3.92×10^3 cfu/mL, 3.92×10^3 cfu/mL, 3.92×10^3 cfu/mL, 3.92×10^3 cfu/mL respectively (data not shown). This showed that

the lowest commonly found concentration of the major pathogens is reliably detected in this system, after extraction from blood culture broth.

3.3.3 The Inhibitory Effect of Blood Culture Broth

Several substances in blood culture broth are known to be inhibitory to PCR. These include SPS (sodium polyanetholesulfonate) (Fredricks and Relman 1998). Other important substances in the blood broth include heparin, haem, human plasma immunoglobulin G, and lactoferrin (Holodniy *et al.* 1991; Khan *et al.* 1991; Ahokas and Erkkila 1993; Akane *et al.* 1994; Al-Soud *et al.* 2000; Al-Soud and Radstrom 2001). In this study, the inhibitory effect of blood broth on MT-PCR detection of *S. pneumoniae* DNA was tested.

5 µL of extracted DNA taken from ten-fold dilutions (neat, 1:10, 1:100, and 1:1000) of blood cultures growing MRSA which had been identified as positive in the BACTEC FX Blood Culture System, were added to 5 µL of pure *S. pneumoniae* DNA and subjected to the MT-PCR assay. When the results of the *S. pneumoniae* with both of the two samples of MRSA (acquired separately from BD Bactec Plus Aerobic bottle and Bactec Lytic bottle) were compared, the greatest inhibitory influence on *S. pneumoniae* detection was observed when extracted MRSA in the Aerobic Bactec Bottle was added (Figure 3.7 and 3.8).

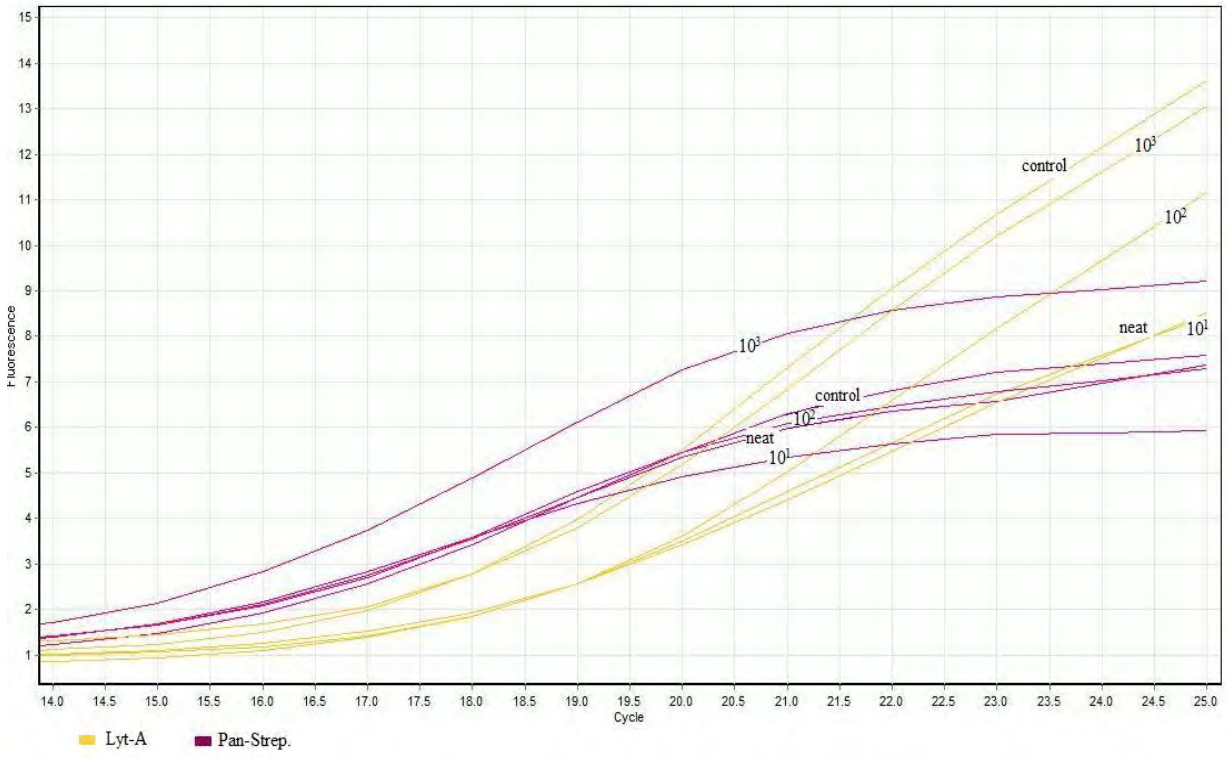


Figure 3.7 Cycling curve produced from *S. pneumoniae* seeded with serial dilutions of MRSA from the Aerobic Bactec bottle.

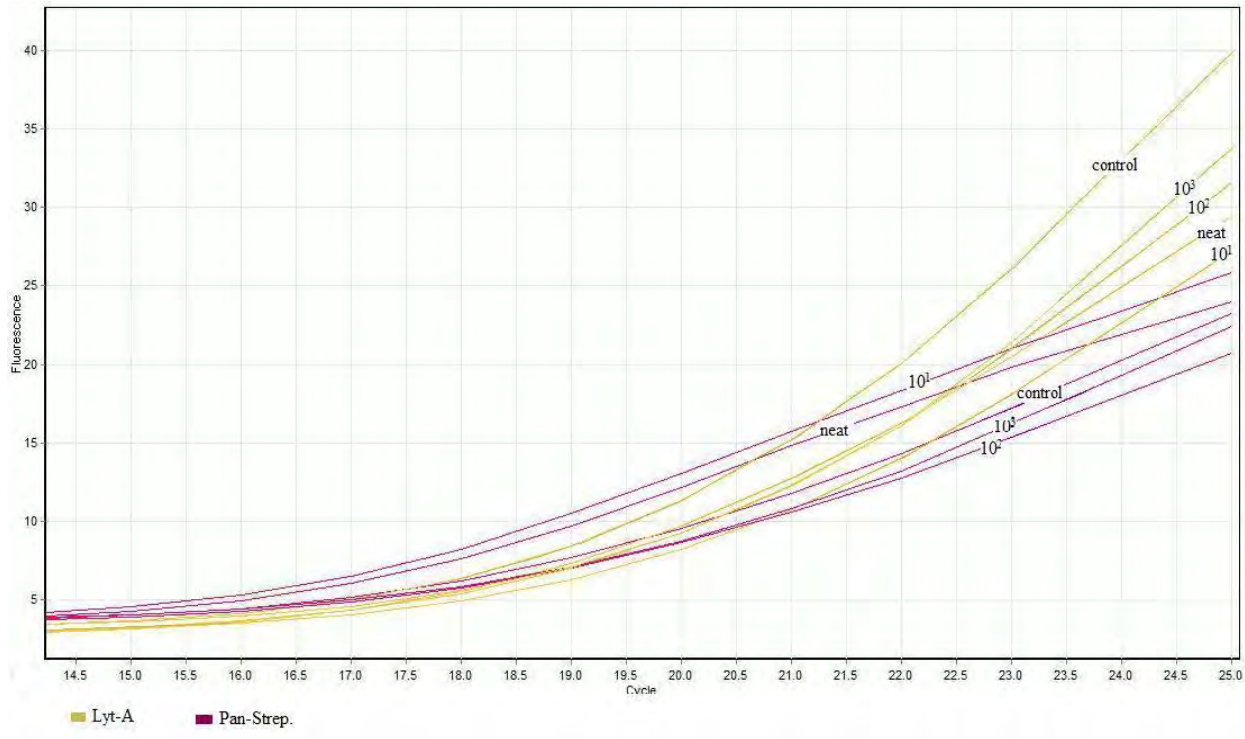


Figure 3.8 Cycling curve produced from *S. pneumoniae* seeded with serial dilutions of MRSA from the Lytic Bactec bottle.

The additional blood culture broth with MRSA appeared to delay signalling of the standardised amount of *S. pneumoniae* DNA, implying an inhibitory effect which persisted after extraction. The extract of positive MRSA from the lytic bottle appeared to less strongly influence the detection of pure *S. pneumoniae* template than the aerobic bottle (Figure 3.7 and 3.8)

3.4 Discussion

Multiplexed tandem-PCR may be utilized for the detection of specific targets up to 72 genes, and multiplexed PCR is known to be reliable, sensitive and efficient in detecting pathogenic bacteria (Mehrotra *et al.* 2000; Costa *et al.* 2005; Lau *et al.* 2008; Szewczuk *et al.* 2010). The GPC 12 MT-PCR assay appears to be very effective for identifying Gram-positive bacteria in blood culture broth. However, contamination is a common problem in microbiology laboratories and cross-reactivity between PCR targets add to the confusion. These results show that the signals from secondary or misprimed targets or genuine contaminants were low and easily recognized. Although definitive proof as to whether the CoNS isolates studied were culture contaminants, the relatively high Ct values for the Staphylococcal 16S target is suggestive of that, as this region is very highly conserved between *aureus* and *non-aureus* Staphylococci.

Cross-reaction between Streptococci, Enterococci and Staphylococci relates to similarity between 16S rRNA gene sequences of these three species. There are several potential consensus regions for cross reactivity in the 16S rRNA gene between *Streptococcus* sp. and *Enterococcus* sp., particularly including the regions between 296-388 bp., 679-733 bp., 878-1013 bp., 1058- 1129 bp., 1183-1256 bp., and 1351-1464 bp.

(Figure 3.9). Levels of relatedness at nucleic acid sequence level are 99.9%, 100%, 99.9%, 100%, 100% and 99.9% identity respectively.

Distinguishing between genera depends on the targeting of regions of sufficiently low similarity between the 16S rRNA sequences of *Enterococcus* and *Streptococcus*, with sufficient variation to provide the lowest risk of cross-reaction. The regions between 201-253 bp., 616-648 bp., 842-877 bp., 1257-1308 bp. have the lowest identity between the genera and levels of relatedness at nucleic acid sequence level are 71.7%, 57.6%, 75% and 73.1 % identity respectively (Figure 3.9 and 3.10).

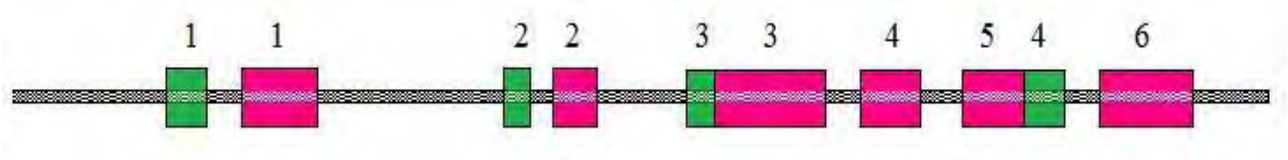


Figure 3.9 Potential regions for cross reaction and primer design in 16S rRNA gene aligned sequences of *Streptococcus* sp. and *Enterococcus* sp.

(Position 1 refers to position 1 of Accession number CP002121)

Green boxes (Potential regions for primer design, 71.7%, 57.6%, 75% and 73.1 % identity respectively) = 1/ 201-253 bp., 2/616-648 bp., 3/842-877 bp., 4/1257-1308 bp. respectively.

Pink boxes (Potential regions for cross reaction, 99.9%, 100%, 99.9%, 100%, 100% and 99.9% identity respectively) = 1/ 296-388 bp, 2/679-733 bp., 3/878-1013 bp., 4/1058-1129 bp., 5/1183-1256 bp., 6/1351-1464 bp. respectively.

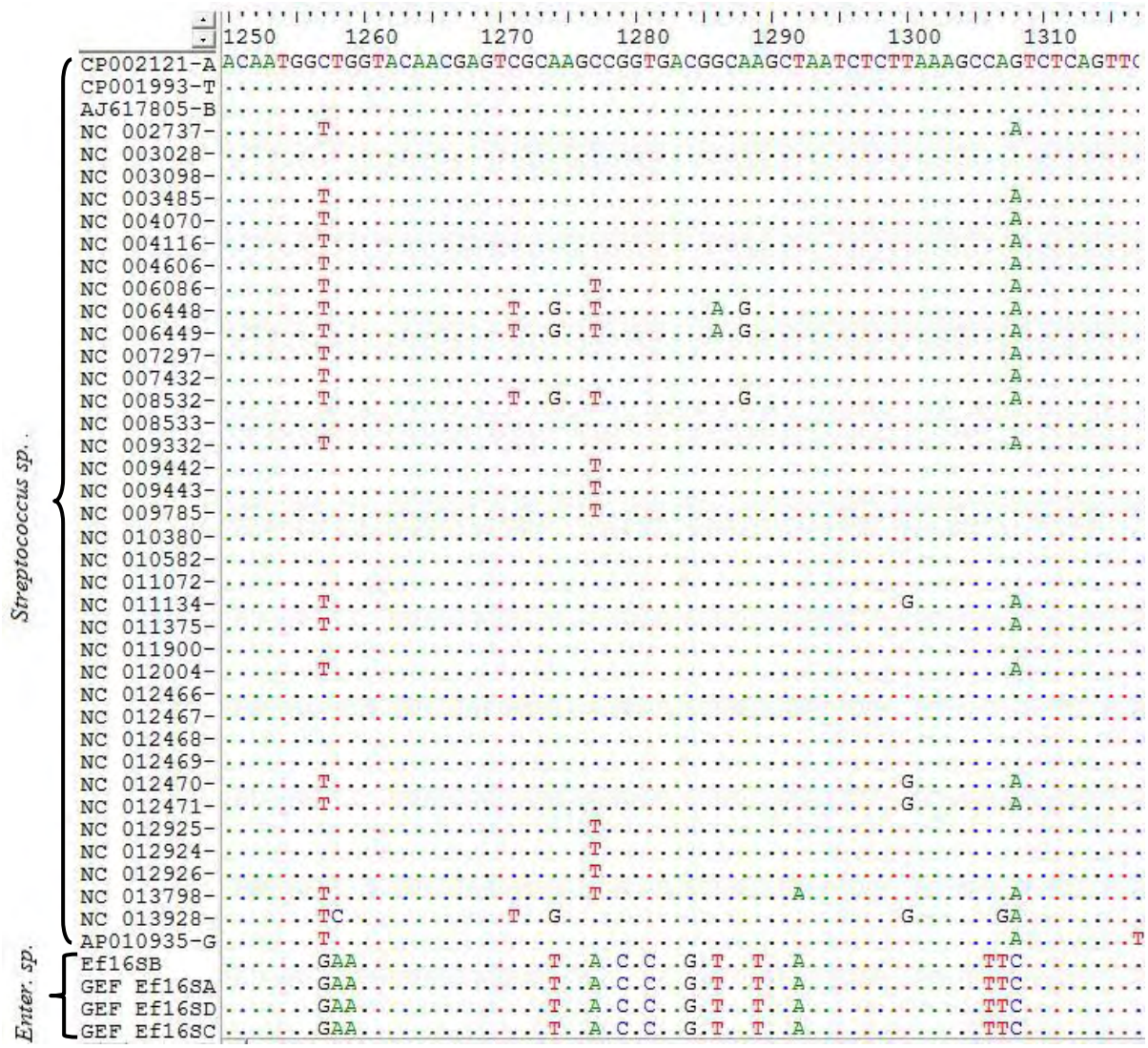


Figure 3.10 An example of a potential region for primer design (1257-1308 bp.) in *Streptococcus* sp. and *Enterococcus* sp. 16S rRNA gene aligned sequence (Position 1 refers to position 1 of Accession number CP002121). This region corresponds to the region (4th green box) in Figure 3.9 *Enter.* sp. stands for *Enterococcus* sp. (73.1% identity)

The GPC 12 MT-PCR assay was performed by two different extraction methods, but both were ultimately extracted by the BioRobot EZ1 extractor (Qiagen, Doncaster, Australia) and are expected to contain DNA only. An inhibitory effect from the added

blood culture broth, which has been previously reported, might be less important in the lytic bottle (Qian *et al.* 2001). If the aerobic and lytic blood culture bottles are compared, it can be seen that they include almost the same amount of hemin (0.00005% w/v) and SPS (0.035-0.04% w/v), suggesting that the other absorbing resin elements within the lytic bottle may specifically reduce inhibition.

This study revealed two isolates in which the expected MT-PCR results did not match the phenotypic results. In one, a CoNS was identified by the Phoenix BD automated system. In one other, an *E. faecalis* (on the basis of colony morphology and the Phoenix BD identification) was positive for *E. faecalis ddl* only. This would have been reported as correct although the 16S target failed. Blood culture isolates that are thought probable contaminants (eg. Signalling in BD Bactec >20 hours after inoculation, and only in a single bottle) and appear to be *non-aureus* Staphylococci may be repeated as "CoNS" without a full and accurate identification. 4 of 6 MR-CoNS isolates, which were negative for both *mecA* and *gseA,* were identified by phenotypic system and the results were not correct. Detailed genotyping is required.

Four negative *nuc* results (from one patient) also apparently reduced the sensitivity of the assay. An alternative primer set was employed (Fig 3.4, 3.5, 3.6 and 3.7), and this failed on two of three occasions, giving only a weak product on one occasion. This implies primer binding site mismatches and will requiring cloning of this region for sequencing. Alignments of several available sequences reveal approximately 100% identity over the region in which the MT-PCR primers and other two sets bind. The outer primer pair (Figure 3.3 and 3.4) binds at 887503-887522 bp. and 888409-888429 bp., in which sequence variation between the available *nuc* gene is 100%. If this is a true *S. aureus*, it may be that an alternative target should be added. Candidates include

femA, *femB, coaA* (Berger-Bachi *et al.* 1992; Kizaki *et al.* 1994; Jonas *et al.* 1999; Shopsin *et al.* 2000). Cloning of this gene and definitive subtyping of the presumed *S. aureus* is needed.

Chapter 4

Detection of Bacterial Co-infection in Patients with Severe Influenza

4.1 Introduction

Common cold and influenza (flu) are familiar syndromes and are the result of respiratory tract infections which can be both viral and bacterial. More than 200 different types of viruses have been discovered to cause the common „cold". 30–50% cases of respiratory tract infections are caused by rhinoviruses, followed by coronaviruses (10–15%), and then influenza viruses (5–15%) (Eccles 2005). Meanwhile, a global survey found that (excluding Australia), 41.8% of community acquired bacterial pneumonia is due to *S. pneumoniae*, followed by *M. pneumoniae* (19.1%), *C. pneumoniae* (10.1%), *H. influenzae* (8.6%), *S. aureus* (5.5%), *Haemophilus parainfluenzae* (4.4%), and *L. pneumophila* (3.5%). (Tanaseanu *et al.* 2008). Australia is expected to be similar, but with a significant number of Legionella cases being *L. longbeachae* (Stocks *et al.* 2004).

Pulmonary complications of influenza include primary influenza pneumonia, secondary bacterial pneumonia, pneumonia due to unusual pathogens in immunocompromised hosts and exacerbations of chronic pulmonary disease (Rothberg *et al.* 2008). Severe viral pneumonia is primarily caused by Influenza A and respiratory syncytial virus (RSV) followed by adenovirus, parainfluenza virus types 1, 2, and 3 and influenza B (Marcos *et al.* 2009).

Bacterial coinfection is common in influenza pneumonia (Lehtinen *et al.* 2006; Gupta *et al.* 2008). The most common pathogens that cause secondary bacterial pneumonia are *S. pneumoniae*, *S. aureus* and *H. influenzae (Marcos et al. 2009)*. It is difficult to distinguish whether pneumonia is viral or bacterial on the basis of clinical findings. In some studies of community-acquired pneumonia (CAP), the results showed that the

frequency of different types of microorganisms varies between populations. According to Johnstone et al., overall, only 39% of patients with CAP had their pathogens identified. 20% of these pathogens were bacteria followed by viruses (15%) and 4% of the patients have both. According to Jennings et al., overall, 58% of patients with CAP have their pathogens identified. 48 % of these pathogens were bacteria followed by viruses (29%) and 15% have both (Jennings *et al.* 2008; Johnstone *et al.* 2008).

M. pneumoniae was the first "atypical" pathogen identified as a cause of severe respiratory tract infection and was called the „Eaton agent", before it was renamed (Eaton *et al.* 1944; Chanock *et al.* 1961). Although there are more than 200 differrent *Mycoplasma* species, only a few of them are pathogenic to humans. *M. pneumoniae* triggers cytokine production such as tumor necrosis factor-α (TNF-α) and interleukins (IL-8 and IL-1β) by the activation of Toll-like receptors (TLR-1 and TLR-2) (Waites *et al.* 2008). Clinical symptoms of *M. pneumoniae* are not easily distinguished from other respiratory infections caused by respiratory viruses or other bacteria, including *C. pneumoniae* (Waites *et al.* 2008).

C. pneumoniae, an obligate Gram-negative intracellular bacteria, was first identified in 1986 and is a major respiratory pathogen that causes community acquired pneumonia (Grayston *et al.* 1986; Grayston *et al.* 1989). *C. pneumoniae* is also a common cause of upper respiratory tract infection, such as pharyngitis, sinusitis, and otitis; and lower respiratory tract infection such as acute bronchitis, exacerbations of chronic bronchitis, asthma, and CAP (Blasi *et al.* 2005). Elavated *C. pneumoniae* antibody titers were found in more than 60% of chronic bronchitis cases (Blasi *et al.* 1998).

Overall, Gram-negative bacteria are responsible for 1.2% of cases of CAP (Ruiz *et al.* 2010). *E. coli* and *P. aeruginosa* rarely cause CAP and are responsible for only 1.3% cases and 0.4% cases of CAP respectively (Ruiz *et al.* 2008; von Baum *et al.* 2010).

H. influenzae are Gram-negative rods and commonly coccobacilli, and may be encapsulated by a polysaccharide capsule. Six different capsular antigenic serotypes of *H. influenzae* have been recognized (a through to f). One of the most virulant serotypes, type b, causes more than 90 % of infections, including meningitis, epiglottitis, cellulitis, pneumonia, septic arthritis and sepsis (Marcon *et al.* 1984; McChlery *et al.* 2009; Hallstrom and Riesbeck 2010). Typically, *H. influenzae* is thought to responsible for 9.8% cases of Gram-negative CAP and a variety of other respiratory infections, attracting specific host complement regulators and causing upper and lower respiratory tract infection including bronchitis, acute otitis media (AOM) and sinusitis (Hallstrom and Riesbeck 2010; Ruiz *et al.* 2010).

More than 40 *Legionella* species and 70 serogroups have been identified as human pathogens. These Gram-negative aerobic bacilli may cause community acquired respiratory tract infections and a severe pneumonia with high mortality (Fields *et al.* 2002). *L. pneumophila* and *L. longbeachae* were recognized in 1977 and 1981 respectively (Fraser *et al.* 1977; McKinney *et al.* 1981). Legionellosis, a severe and potentially fatal form of pneumonia, was first described in Philadelphia in 1977 (Fraser *et al.* 1977; Fields *et al.* 2002). Although 90 % of legionellosis in the USA and Europe results from *L. pneumophila; L. longbeachae* is the leading cause of legionellosis in Australia and New Zealand with 30 % of cases and in South Australia and Thailand with 50 % of cases (Li *et al.* 2002; Phares *et al.* 2007; Ricketts and Joseph 2007; Neil and Berkelman 2008). In many patients, Legionellosis causes fever, kidney, liver,

gastrointestinal and central nervous system dysfunction, radiological features consistent with pneumonia, acute respiratory distress syndrome characterized by nonhydrostatic pulmonary edema, hypoxaemia, and oedematous pulmonary infiltrates on chest (Bernard *et al.* 1994; Cunha 1998). Clinical features and outcomes are similar between *L. pneumopila* and *L. longbeachae* (Amodeo *et al.* 2009).

4.2 Aim

The purpose of this chapter is to investigate the frequency of co-infection that were caused by Gram-negative and Gram-positive bacteria in patients with severe influenza during the 2009 Australian influenza epidemic using a multiplexed quantitative PCR assay.

4.3 Results

4.3.1 Bacterial Load assay

4.3.1.1 Testing of assay Validation

S. aureus, *E. facecalis, E. coli*, *S. pneumoniae* and *P. aeruginosa* strains (Table 2.5) were serially diluted in nutrient broth, extracted and tested in the MT-PCR Bacterial Load assay (Section 2.5). Standard curves were prepared for quantitative analysis (Appendix D)

4.3.1.2 Clinical specimens and performing PCR assay

A total of 200 clinical specimens from Intensive Care patients with influenza from the 2009 H1N1 epidemic were then evaluated (ANZIC 2009). These were from sputum,

endotracheal tube (ETT) aspirate, bronchial washing, bronchoalveolar lavage (BAL), nose/throat swab and nasopharyngeal aspirates (NPA) and were extracted (NucliSENS® easyMAG®, Durham, USA) and directly tested in the MT-PCR Bacterial Load assay. Of these, 37 (18.5%) of 200 specimens had no detectable bacterial signal, but 163 (81.5%) specimens had evidence of bacterial colonisation or co-infection, with 138 evidently polymicrobial and 25 evidently monobacterial in nature (9 streptococcal co-infection and 16 enterococcal co-infection) (Table 4.1, Appendix C).

Table 4.1 Bacterial colonisation or co-infection in patients with severe influenza

<table>
<tr><th>No bacteria detected</th><th colspan="3">Bacteria detected</th><th>TOTAL</th></tr>
<tr><td>37 (18.5%)</td><td colspan="3">163 (81,5%)</td><td>200 (100%)</td></tr>
<tr><td></td><td colspan="2">monobacterial</td><td>More than one bacterial type indicated</td><td></td></tr>
<tr><td></td><td colspan="2">25 (12.5%)</td><td>138 (69%)</td><td></td></tr>
<tr><td></td><td>Streptococcus sp.</td><td>Enterococcus sp.</td><td></td><td></td></tr>
<tr><td></td><td>9 (4.5%)</td><td>16 (8%)</td><td></td><td></td></tr>
</table>

In a total of 200 putative co-infection samples, *Enterococcus* sp. was apparently present in 139 samples (79.4%), being the dominant signal (any other signal was ≤10%) from 106 samples. *Streptococcus* sp. was next most commonly indicated with 137 positive results (78.3%), but was the dominant signal in 19 of 137. *Pseudomonas* sp. was not commonly identified with 17 positive results (9.7%), and being the dominant signal in only 1 of 17 (Table 4.2; Appendix C).

Table 4.2 The number and percentage of bacterial types identified in patients with influenza (n=200) and the number and percentage in which it was the dominant signal.

Signals*	***Staphylo-coccus* sp.**	***Strepto-coccus* sp.**	***Entero-coccus* sp.**	***Entero-bacteriaceae* species**	***Pseudomonas* sp.**
any	66/200 (37.7%)	137/200 (78.3%)	139/200 (79.4%)	45/200 (25.7%)	17/200 (9.7%)
dominant	31/66 (46.9%)	19/137 (13.8%)	106/139 (76.2%)	6/45 (13.3%)	1/17 (5.8%)

*37/200 (18.5%) had no bacterial signal detected

4.3.1.2.1 Comparation of specimens from upper and lower respiratory tract

45 out of 200 clinical specimens were not clearly identified by their origin, but 34 were identified lower respiratory tract specimens (eg. ETT aspirate, tracheal aspirate, bronchial washing). *Streptococcus* sp. is the leading cause of co-infection in the lower respiratory tract (19 positive results-19/44:55.9%). However, one of 19 positive signals for *Streptococcus* sp. appeared as the dominant signal and *Enterococcus* sp. (18 positive results- 18/34:52.9%) gave the highest number of dominant signals (12/34) (Appendix C). *Enterococcus* sp. was most commonly detected in upper respiratory tract specimens (92 positive results- 76%) and it was detected as the strongest signal in 73 samples of these 92 (Table 4.3 and Appendix C). In all cases discribed as the „dominant" signal, there was no other that was >10% in intensity normalized.

Table 4.3 The number of cases in which bacterial DNA targets were identified in the lower respiratory tract (LRT) and upper respiratory tract (URT).

	No	***Staphylo-coccus* sp.**	***Strepto-coccus* sp.**	***Entero-coccus* sp.**	***Entero-bacteriaceae* species**	***Pseudomonas* sp.**
LRT						
any	34[a]	15/34 (44.1%)	19/34 (55.9%)	18/34 (52.9%)	10/34 (29.4%)	4/34 (11.7%)
dominant		10/15 (66.6%)	1/19 (5.2%)	12/18 (66.6%)	1/10 (10%)	0/4 (0%)
URT						
any	121[b]	40/121 (33.1%)	88/121 (72.7%)	92/121 (76%)	26/121 (21.5%)	9/121 (7.4%)
dominant		15/40 (37.5%)	12/88 (13.6%)	73/92 (79.3%)	6/26 (23.1%)	0/9 (0%)
Total	155	55	110	110	36	13

[a] 10 of 34 samples have no bacterial signal

[b] 15 of 121 samples have no bacterial signal

In total, 85 of 110 positive signals for Enterococci appeared as the dominant signal. Of the 110 samples in which Enterococci were detected, 81 also yielded a Streptococcal signal (73.6%) which was much weaker, and was in all cases <10% of that for *Enterococcus* sp., suggesting some cross-reactivity (Appendix C). There were only 3 samples in which Streptococci was the dominant signal. There were 9 samples which the *Streptococcus* signal was strong and unique (Appendix C).

4.4 Discussion

In earlier studies, bacterial co-infection was detected in between 4 and 28% of influenza cases (Jennings *et al.* 2008; Johnstone *et al.* 2008; CDC 2009; Lee *et al.* 2010; Martin-Loeches *et al.* 2010; Shieh *et al.* 2010). *S. pneumoniae* is the major cause of bacterial co-infection in lung tissue, complicating approximately 13% of total cases of 2009 Pandemic Influenza A (H1N1) in US and 9.6% of influenza cases in the patients admitted to ICU in Spain (CDC 2009; Martin-Loeches *et al.* 2010). Accoding to Lee et al., *S. pneumoniae* is the leading cause of bacterial co-infection, infecting 17% of H1N1/09 influenza patients who died in New York (Lee *et al.* 2010). After *S. pneumoniae* co-infection, *S. pyogenes* has been reported as the second most important cause of co-infection, followed by *S. aureus*, *S. mitis* and *H.influenzae* (CDC 2009; Shieh *et al.* 2010).

In this study, 4 genera including *Staphylococcus* sp., *Streptococcus* sp., *Enterococcus* sp. and *Pseudomonas* sp. and a family, *Enterobacteriaceae* species, were investigated by using Bacterial Load MT-PCR assay. The Bacterial Load assay was performed on the NucliSENS® easyMAG® extractor (bioMérieux, Durham NC, USA) and is expected to contain total nucleic acid. No special efforts were made to preserve RNA and it is not likely that mRNA survived other parts of the handling processes. *Enterococcus* sp. is the most commonly detected genus, being found in 79.4%. *Streptococcus* sp. was just as common (78.3%) and this was followed by *Staphylococcus* sp. (37.7%), *Enterobacteriaceae* species (25.7%) and *Pseudomonas* sp. (25.7%). The high rate of *Enterococcus* sp. might be due to its ability to survive the antibiotic treatment used for severe pneumonia (eg. ceftriaxone), contamination, and/or cross reaction in the 16S

rRNA gene between *Enterococcus* sp. and *Streptococcus* sp. (Section 3.4) (Wang *et al.* 2010). *Enterococcus* sp. appear to be the most commonly encountered co-pathogen in severe influenza during the 2009 Australian influenza epidemic. Ceftriaxone is the recommended treatment for severe CAP (Segev *et al.* 1995; Norrby *et al.* 1998). Ceftriaxone is not active against *Enterococcus* sp. and can be expected to select for it as a coloniser of the respiratory tract. This assay does not distinguish between colonisation and infection, however, it may be that surrogate markers of inflammation are crucial (eg. cytokine assays, Gram-stain or other markers of neutrophil counts).

When comparing the number of strains that cause co-infection, there is a minor difference between the rate of strains identified in the lower respiratory tract and upper respiratory tract. The major genera and family identified were *Streptococcus* sp. (55.9%) ; followed by *Enterococcus* sp. (52.9%), *Staphylococcus* sp. (44.1%), *Enterobacteriaceae* species (29.4%) and *Pseudomonas* sp. (11.7%). There were dominant signals from *Enterococcus* sp. in the samples identified as BAL or other suitable deep specimens (n=34). Although *Streptococcus* sp. appeared in 19 of the lower respiratory tract samples, *Enteococcus* sp. has more primary signals (12 of 18 positive signals) than *Streptococcus* sp. (1 of 19 positive signals), and followed by *Staphylococcus* sp. (10 of 15 positive signals). This may be explained as a result of cross-reaction between the genera, and it would be important to try and obtain more specific identification (eg. *S. pneumoniae*) in future studies, using specific targets (eg. *lytA*, *ply*). It may be that true co-infection (eg. by *S. pneumoniae*) are more likely to be represented by dominant signals in the lower respiratory tract, but this is yet to be tested.

Cross reaction between *Staphylococcus* sp., *Streptococcus* sp., *Enterococcus* sp., *Enterobacteriaceae* species and *Pseudomonas* sp. might occur due to the similarity between 16S rRNA gene sequences. Levels of relatedness at nucleic acid sequence are high for *Streptococcus* sp. and *Enterococcus* sp., and this appears to be reflected to some extent in the results (Section 3.4). In relation to this, there are several potential consensus regions for cross reactivity in the 16S rRNA gene between *Enterobacteriaceae* species and *Pseudomonas* sp. particularly including the regions between 313-370 bp., 760-824 bp., 879-965 bp., 1371-1429 bp., (Figure 4.1). The relatedness at nucleic acid sequence level is 100% for all these regions.

On the other hand, there are several regions in 16 S rRNA gene sequences, with sufficient variation between *Enterobacteriaceae* species and *Pseudomonas* sp. to allow primer design and avoid cross-reaction including the regions between 123-167 bp., 457-479 bp., 639-683 bp., 706-759 bp., 825-860 bp., 1245-1296 bp. (Figure 4.1 and Figure 4.2). Levels of relatedness at nucleic acid sequence level are 64.4%, 34.8%, 64.4%, 75.9%, 50% and 50% identity respectively.

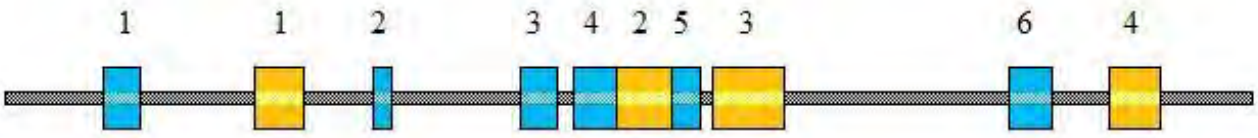


Figure 4.1 Potential regions for cross reaction and primer design in 16S rRNA gene aligned sequences of *Pseudomonas* sp. and *Enterobacteriaceae* species (Position 1 refers to position 1 of Accession number NC_002695)

*Blue boxes (*Potential regions for primer design 64.4%, 34.8%, 64.4%, 75.9 %, 50% and 50% identity respectively)= 1/123-167 bp., 2/457-479 bp., 3/639-683 bp., 4/706-759 bp., 5/825-860 bp., 6/1245-1296 bp. respectively.

*Yellow boxes (*Potential regions for cross reaction 100% identity)= 1/313-370 bp., 2/760-824 bp, 3/879-965 bp., 4/1371-1429 bp. respectively.

The art of primer design in multiplexed assays of this type is to identify regions that are sufficiently distinct between genera but highly conserved within them. The actual sequence of the primers used in the mPCR-RLB (multiplex PCR-reverse line blot) assays described are commercial-in-confidence but appear to have struck this balance reasonable well. Future work would logically be to confirm/subtype each positive signal with more specific PCR and sequencing, but that was beyond the scope of this work.

Figure 4.2 An example of a potential region for primer design (1245-1296 bp.) in *Pseudomonas* sp. and *Enterobacteriaceae* species 16S rRNA gene aligned sequence

(Position 1 refers to position 1 of Accession number NC_002695). This region corresponds to the region (6th blue box) in Figure 4.1. (50% identity)

Bacterial co-infection is a major determinant of the outcome of influenza patients as it is associated with inreased morbidity and mortality (Lee *et al.* 2010; Martin-Loeches *et al.* 2010). In contrast to a number of previous studies, this work shows that *Enterococcus* sp. the dominant member of the respiratory tract facultative/aerobic microflora in patients admitted to ICU with influenza. *Streptococcus* sp. and *Staphylococcus* sp. were next in importance numarically but are much more likely to include truly pathogenic species (eg. *S. pneumoniae*, *S. aureus*). It may be that the *Enterooccus* sp. signal related to colonization of airways in antibiotic-treated incubated patients, and it would be useful to examine a set of deep specimens known to have been collected on first intubation and without prior antibiotics.

Although a quantitative approach is feasible, these specimens vary greatly in their original quality and in subsequent handling, but a combination of greater specificity (eg. to species level) and quantitation would answer many of the questions about the significance of these signals. Even with all these limitations, this is a useful proof-of-principle.

Chapter 5
Final Discussion

About 11% of Intensive Care Unit (ICU) patients in Australia and New Zealand, around 27% of ICU patients in UK, and approximately 11% of ICU patients in USA contracted severe sepsis in the last few decades (Angus and Wax 2001; Padkin *et al.* 2003; Finfer *et al.* 2004). The mortality rate among hospitalized patients with sepsis increased over the last several decades in USA (Angus and Wax 2001; Martin *et al.* 2003) and ranges between 26% and 37% in Australia and New Zealand (Finfer *et al.* 2004).

Sepsis, severe sepsis and septic shock commonly result from infection by bacterial pathogens (Aledo *et al.* 1998; Kumar *et al.* 2006b; Lim *et al.* 2009; Welte and Kohnlein 2009; Woodhead 2009). Although inflammatory cytokine storm is thought to be the main cause of septic shock rather than the actual infection, the different frequencies of bacterial pathogens were investigated as the cause of sepsis in several studies (Pinsky and Matuschak 1990; Rangel-Frausto *et al.* 1995; Aledo *et al.* 1998; Cursons *et al.* 1999; Kumar *et al.* 2006b). The most common type of bacteria found in septic shock (83%) are Gram-negative bacteria such as *E. coli* and *K. pneumoniae* (Aledo *et al.* 1998). However, cases of Gram-negative infections became less common over time (Friedman *et al.* 1998), and the frequency of Gram-positive pathogens involved in septic shock have increased over the years, Gram-positive bacteria including *S. aureus* and *S. pneumoniae* are implicated in ~ 38% of all septic shock cases in North America (Kumar *et al.* 2006b).

In this study, it was found that the organism most commonly associated with sepsis was *S. aureus* with 89 of 215 patients (41%). Other organisms identified, in decreasing order of frequency, were CoNS (39%), *E. faecalis* (5%), *S. pyogenes* (3%), and *S. pneumoniae* (2%). The observed results were similar to previous studies and there is no significant different in the existing frequencies of the bacteria between these results and

the results of published studies (Aledo *et al.* 1998; Kumar *et al.* 2006b). It is important to note that contamination is not excluded as a source of positive cultures in this context, especially in those patients with coagulase-negative Staphylococci in blood cultures.

A PCR assay may be more sensitive than conventional blood culturing techniques for the detection of septicemia (Cursons *et al.* 1999). When compared with MT-PCR, conventional techniques require considerably more time (more than 24 hours). MT-PCR is used for the detection and identification of yeasts and viruses (Stanley and Szewczuk 2005; Lau et al. 2010; Szewczuk et al. 2010). The Gram-positive 12 (GPC 12) MT-PCR assay (chapter 3) appears to be sensitive, specific and reliable for the detection of Staphylococci, Enterococci and Streptococci, and may detect these major pathogens at relatively low concentrations. The assay can potentially be used in the diagnosis of bacteraemia, bloodstream infections, respiratory tract infections, pneumonia and severe sepsis.

The BACTEC FX blood culture system uses blood culture broths in Aerobic (A) or Anaerobic/Lytic (L) bottles. Although this is a reliable culturing technique with a low risk of contamination; this approach may require up to 24 hours after growth is signaled and the cost of culture and identification is approximately $30 (AUD) per sample (Wilson *et al.* 1993; Weinstein 1996; Chung *et al.* 2011). The MT-PCR Gram Positive 12 assay costs around $25 (AUD), and less than 3 hours is spent per test, once the initial 12-16 hour culture has signaled positive. For that reason, MT-PCR might be preferable for identification of bacterial pathogens.

Respiratory tract infections including severe CAP are most commonly caused by bacterial pathogens, and *S. pneumoniae* is the leading cause (Lim *et al.* 2009). Although bacterial co-infection with influenza is not usual (rates between 4 and 28%), (Jennings *et al.* 2008; Johnstone *et al.* 2008; CDC 2009; Lee *et al.* 2010; Martin-Loeches *et al.* 2010; Shieh *et al.* 2010), a very high rate of bacterial identification (163/200 - 81,5%) was observed in influenza patients in this study. Sample contamination in the laboratory is uncommon in automated "closed" systems (MT-PCR/ BACTEC FX) and is unlikely as an explanation. However, antibiotic resistance in bacterial pathogens is a growing problem in the last decade (Pallares *et al.* 1987; Appelbaum 1992; Carmeli *et al.* 1999; Weber *et al.* 1999; Davidson *et al.* 2002), and antibiotics might not sterilize the respiratory tract of patients, with even relatively susceptible pathogens growing in endotracheal tubes and pooled oropharyngeal secretions into which bacterial penetration is minimal. Many such respiratory tract contaminants are routinely dismissed when cultured in the laboratory from specimens of this type and go unreported. An unbiased nucleic acid-based method such as we employed identified these readily.

This study showed that *Enterococcus* sp. was the dominant bacterial pathogen identified in patients with influenza. This contrast with the typical reporting of *S. pneumoniae* in influenza, (CDC 2009; Lee *et al.* 2010; Martin-Loeches *et al.* 2010; Shieh *et al.* 2010). In contrast to reports of Streptococci dominating in the upper respiratory tract (Smith *et al.* 1989), in this study, Enterococci is the dominant bacteria in the upper respiratory tract of patients treated for severe pneumonia.

The greater representation of *Enterococcus* sp. among identified bacteria might therefore be due to the antibiotic resistance of this genus to drugs like ceftriaxone. Ceftriaxone is commonly used for treatment of severe pneumonia (Segev *et al.* 1995;

Norrby *et al.* 1998). This is expected to result in the proliferation of *Enterococcus* in the respiratory tract and does not imply a true invasive infection.

The Bacterial Load MT-PCR assay (chapter 4) allows for rapid, sensitive, and specific quantifications of the bacterial pathogens and it is also a quantitative assay for detection of the family *Enterobacteriaceae* and the genera *Staphylococcus, Streptococcus, Enterococcus*, and *Pseudomonas*. The assay is based on 16S rRNA consensus sequences, but the utility of genus-specific targets in sputum is questionable, and these need to be complemented by species-specific targets to get effective results (eg. *ltyA, femA, nuc, mecA*). *Staphylococcus* is one of the major causes of co infection after *Streptococcus*, and it is the leading cause of co-infection in some cases (CDC 2009; Williams *et al.* 2011). More specific targets developed for a more specific detection of *Staphylococcus* and *Streptococcus* may be very useful in this assay (eg. *ply*/*lytA* for *Streptococcus, nuc*/*mecA* for *Staphylococcus*).

In contrast to the Gram-positive bacteraemia assay samples, that are all DNA samples, the assays described for respiratory samples are all total nucleic acid samples (although with no special attempt made to preserve RNA). A quantitative result may be very important for respiratory assays. In addition, high levels of inflammatory cytokines such as IL-1α, IL-6, IL-10 and TNF-α, are associated with influenza and severe sepsis (Kellum *et al.* 2007; McAuley *et al.* 2007; Mauad *et al.* 2010). Measuring mRNA levels of cytokines in blood and respiratory samples by MT-PCR may also be useful (Turlej 2009; Vos *et al.* 2009), but require an RNA preserving approach such as collecting blood in guanidine isothiocyanate tubes (eg BD PaxGene, Becton Dickinson).

MT-PCR has significantly added to the standard (culture-based) methods and serology in the diagnostic microbiology laboratory and may be a cost-effective adjunct to standard methods.

Chapter 6
Appendices

Appendix A- Clinical samples tested using Lysostaphin for extraction

Table 6.1 MR-CoNS

Lab ID	**pan-*Staph***	**pan- *Strep***	**pan-*Entero***	***nuc***	***mecA***	***gseA***	***ply***	***E.faecium ddl.***	***E.faecalis ddl***	***vanA***	***van B***	**Spike**
09-115-1218	300189				26190	13784						10000
09-113-0309	1155518				33856	130321						10000
09-111-0401	441211				3150	24562						10000
09-112-4078	53059				179635							10000
09-112-2212	56576				1128	4629						10000
09-111-0021	1400886				341307	179635						10000
09-111-2663	1493751				534900	320088						10000
09-112=0528	786185				94545	41045						10000
09-111-2305	2496209				363932	10663						10000
09-111-0245	1083681				114621	60327						10000
09-111-2305	168467				872	7255						10000
09-110-0824	109378				8795	7736						10000
09-108-1978	7255				4326							10000
09-108-2018	570358				470459	281526						10000
09-110-2431	320088				53059							10000
09-107-2708	41045				7736	557						10000
09-107-2563	1155518				786185	41045						10000
09-115-1968	1155518				247610	191543						10000
09-115-1370	691471				6381	64326						10000
09-115-1179	648483				168467	232216						10000
09-113-3801	49760				46667							10000
09-115-1218	3150				53059	570358						10000
09-119-0293	648483				818	6804						10000
09-120-4072	264024				2954	4071						10000

Lab ID	**pan-*Staph***	**pan- *Strep***	**pan-*Entero***	***nuc***	***mecA***	***gseA***	***ply***	***E.faecium ddl.***	***E.faecalis ddl***	***vanA***	***van B***	**Spike**
09-123-0901 A	388057				20260	29777						10000
09-123-0901 L	77985				767	8249						10000
09-120-4072	94545				13784	12123						10000
09-119-4281 A	17819	127			2010	1768						10000
09-119-4281 L	570358				68590	114621						10000
09-122-0694	247610				23035	10000						10000

Table 6.2 MS-CoNS

Lab ID	**pan-*Staph***	**pan- *Strep***	**pan-*Entero***	***nuc***	***mecA***	***gseA***	***ply***	***E.faecium ddl.***	***E.faecalis ddl***	***vanA***	***van B***	**Spike**
09-110-3311	10397											10000
09-110-1276 A	691471					60227						10000
09-110-0276 L	12927					720						10000
09-104-5009	264024		293									10000
09-119-2825 L	4629					333						10000
09-119-2825 A	341307					767						10000
91361775	1768					2285						10000
91361281	157994											10000
91430253	11732					11370						10000
091431681 A	300189											10000
091431681 L	3150											10000
91450847	148172					13784						10000
91463627	737308											10000
91470197	33856					5984						10000
91474239	570358					53059						10000

Lab ID	**pan-*Staph***	**pan- *Strep***	**pan-*Entero***	***nuc***	***mecA***	***gseA***	***ply***	***E.faecium ddl.***	***E.faecalis ddl***	***vanA***	***van B***	**Spike**
91464538	4936											10000
91511742	1083681					247610						10000
91524781	100812					11370						10000

Table 6.3 MRSA

Lab ID	**pan-*Staph***	**pan- *Strep***	**pan-*Entero***	***nuc***	***mecA***	***gseA***	***ply***	***E.faecium ddl.***	***E.faecalis ddl***	***vanA***	***van B***	**Spike**
09-102-0402	534900			320088	534900							10000
09-097-4358 L	21603			1282	3581							10000
09-097-4358 A	501646			23035	992							10000
09-094-0600	953127			953127	953127	242						10000
09-092-4591	691471			648483	691471	17819						10000
09-090-4589	60327			3150	7255							10000
09-078-2122	953127			953127	953127							10000
09-078-3785	1930988			1930988	1930988							10000
09-061-3984	786185			786185	4071							10000
09-069-2251 A	648483			27926	14698							10000
09-069-2251 L	608167			26190	1282							10000
09-068-0678	6537495			6131066	6537495							10000
09=113-1764	1400886			1400886	1400886							10000
09-112-4353	1016309			281526	648483							10000
09-112-2862	1083681			1083681	1083681							10000
09-108-1500	1400886			12927	404							10000
09-109-0763	953127			14698	94545							10000

Lab ID	**pan-*Staph***	**pan- *Strep***	**pan-*Entero***	***nuc***	***mecA***	***gseA***	***ply***	***E.faecium ddl.***	***E.faecalis ddl***	***vanA***	***van B***	**Spike**
09-107-0189	953127			953127	953127							10000
91100483	953127			953127	953127							10000
91110539	3026266			3026266	3026266							10000
091361979 A	737308			737308	737308							10000
091361979 L	1232117			838301	441211							10000
91361859	1016309			1016309	1016309							10000
91371434	1493751			1493751	53059							10000
91693530	4447943			4447943	4447943							10000
091620296 A	300189			21603	49760							10000
091620296 L	4171419			2195484	4171419							10000
091624599 A	2838126			232216	534900							10000
091624599 L	4447943			413781	953127							10000
91671736	3668877			1083681	2195484							10000
91690164	6131066			6131066	6131066							10000

Table 6.4 MSSA

Lab ID	**pan-*Staph***	**pan- *Strep***	**pan-*Entero***	***nuc***	***mecA***	***gseA***	***lytA***	***E.faecium ddl.***	***E.faecalis ddl***	***vanA***	***van B***	**Spike**
09-108-0241 L	470459			470459								10000
09-108-0241 A	1400886			130321								10000
09-108-2047 A	786185			247610								10000
09-108-2047 L	893872			893872								10000
09-110-0374	1083681			191543								10000
09-110-3178	737308			60327	594							10000
09-110-2367 A	893872			191543								10000
09-110-2367 L	281526			1458								10000

Lab ID	**pan-*Staph***	**pan- *Strep***	**pan-*Entero***	***nuc***	***mecA***	***gseA***	***lytA***	***E.faecium ddl.***	***E.faecalis ddl***	***vanA***	***van B***	**Spike**
09-115-1267 A	953127			77985								10000
09-115-1267 L	570358			60327								10000
09-115-1874	470459			1658								10000
09-122-0717 A	present			10663								10000
09-122-0717 L	present			16711								10000
09-124-2141 A[a]	470459											10000
09-124-2141 L[a]	608167											10000
09-125-2732[a]	691471											10000
09-125-2733[a]	608167		154									10000
091242141 A[b]	2661683											10000
091242141 L[b]	4742797											10000
91252732[b]	5057198											10000
91252733[b]	2838126											10000
91263025	1083681			49760								10000
91343524	1155518			893872								10000
91344423	737308			737308								10000
091371814 A	838301			300189								10000
091371814 L	893872			232216								10000
091372091 A	893872			893872	293	404						10000
091372091 L	1155518			1155518								10000
91390053	341307			148172								10000
91424133	737308			737308								10000
91424036	1400886			893872								10000
91424526	1083681			1083681								10000
91440224	786185			786185								10000
91463308	1493751			1493751								10000

Lab ID	**pan-*Staph***	**pan- *Strep***	**pan-*Entero***	***nuc***	***mecA***	***gseA***	***lytA***	***E.faecium ddl.***	***E.faecalis ddl***	***vanA***	***van B***	**Spike**
91454348	691471			217780								10000
91463283 A	413781			441211								10000
91463283 L	737308			737308								10000
91463308	1493751			1493751								10000
91454731	1810940			1155518								10000
91471881	9378			5984								10000
091412900 A	1083681			73137								10000
091412900 L	6131066			5392440								10000
91412858	264024			19000								10000
091580512 A	2195484			1313794								10000
091580512 L	7925697			1810940								10000
091613850 A	413781			16711								10000
091613850 L	2661683			1698356								10000
91641309	2838126			179635								10000
91673002	7432965			648483								10000
091670513 A	3912087			608167								10000
091670513 L	3226877			2661683								10000
91683288	3912087			608167								10000
91683288	3668877			3226877								10000
91691145	5057198			1313794								10000
91692493	4447943			3026266								10000
091720787 A	5749905			4171419								10000
091720787 L	7432965			5392440								10000

[a] The samples that were negative for *nuc* gene

[b] The samples repeated for *nuc* gene

Table 6.5 *Streptococcus* sp. and *Enterococcus* sp.

Lab No	Phenotype	Found results	pan-*Staph*	pan-*Strep*	pan-*Enter*	*nuc*	*mecA*	*gseA*	*lytA*	*E.faecium ddl.*	*E.faecalis ddl*	*vanA*	*van B*	Spike
09-109-1154	*S.pneumoniae*	*S.pneumoniae*		737308					138960					10000
91580549	*S.pneumoniae*	*S.pneumoniae*		691471					53059					10000
091570174 A	*S.pneumoniae*	*S.pneumoniae*		341307					100812					10000
091570174 L	*S.pneumoniae*	*S.pneumoniae*		737308					737308					10000
091554149 A	*S.pneumoniae*	*S.pneumoniae*	320088	1016309			7255		1016309					10000
09-124-0076	*S.pyogenes*	*S.pyogenes*		14698										10000
09-121-2035	*S.pyogenes*	*S.pyogenes*		1282										10000
091453821 A	*S.pyogenes*	*S.pyogenes*	168467	122219										10000
091453821 L	*S.pyogenes*	*S.pyogenes*	1458	720										10000
091581652 A	*S.pyogenes*	*S.pyogenes*		363932	213									10000
091581652 L	*S.pyogenes*	*S.pyogenes*		29777										10000
09-119-3438 A	AHS	AHS		46667										10000
091564211 A	*Streptococcus* sp.	*Entrecoccus* sp.			648483									10000
091564211 L	*Streptococcus* sp.	*Entrecoccus* sp.			953127									10000
091561933 A	*Streptococcus* sp.	*E.faecalis*			388057						29777			10000
091561933 L	*Streptococcus* sp.	*E.faecalis*			893872						363932			10000
09-115-1473	*E. faecalis*	*E. faecalis*									227			10000
09-119-0105 A	*E. faecalis*	*E. faecalis*			247610						12927			10000
09-119-0105 L	*E. faecalis*	*E. faecalis*			2195484						138960			10000
09-127-4034	*E.faecalis*	*E.faecalis*			77985						12927			10000
091430456 A	*E.faecalis*	*E.faecalis*			73137						6804			10000
091430456 L	*E.faecalis*	*E.faecalis*			1400886						691471			10000
91264458	*E.faecium*	*E.faecium*			893872				786185	441211				10000
09-124-4773 A	*E.casseliflavus*	*E.casseliflavus*			953127									10000

Lab No	**Phenotype**	**Found results**	**pan-*Staph***	**pan-*Strep***	**pan-*Enter***	***nuc***	***mecA***	***gseA***	***lytA***	***E.faecium ddl.***	***E.faecalis ddl***	***vanA***	***van B***	**Spike**
09-124-4773 L	*E.casseliflavus*	*E.casseliflavus*			608167									10000
091562042 A	*Entrecoccus* sp.	*E.faecalis*			388057						31751			10000
091562042 A	*Entrecoccus* sp.	*Entrecoccus* sp.			838301									10000

AHS indicates α-haemolytic *Streptococcus*

Appendix B- Clinical samples tested using G2 Buffer Lysis for extraction

Table 6.6 MRSA

Lab ID	**pan-*Staph***	**pan- *Strep***	**pan-*Entero***	***nuc***	***mecA***	***gseA***	***ply***	***E.faecium ddl.***	***E.faecalis ddl***	***vanA***	***van B***	**Spike**
04093350427-L	23035	21		1367	1282							10000
04093350427-A	413781		49	8795	2143							10000
4093162018	5612	2954	1058	930								10000
30.09.288.2516	7432965		98	1155518	570358							10000
04.09.288.1952	179635		76	78390	10663							10000
04.09.288.2516	122219			20260	13784							10000
02.09.287.0459	5392440			441211	217780							10000
04.09.286.1086	4447943			648483	570358							10000
04.09.286.1085	6970866			2838126	2838126							10000
04.09.288.1952A	100812		81	43781	4936							10000
04.09.288.1952L	534900		164	29342	17819							10000
04.09.292.2937	300189			56576	5612							10000
04.09.293.2535	53059			11370	720							10000
04.09.292.2937	264024			43766	8248							10000
04.09.287.0459	17121498			2058993	157994							10000

Table 6.7 MSSA

Lab ID	**pan-*Staph***	**pan- *Strep***	**pan-*Entero***	***nuc***	***mecA***	***gseA***	***ply***	***E.faecium ddl.***	***E.faecalis ddl***	***vanA***	***van B***	**Spike**
3093350326	1493751			1313794								10000
03093351639-A	1400886			441211								10000
03093351639-L	179635			7255								10000
3093351635	3912087		49	232216								10000
04093361046-L	341307		40	33856								10000
04093361046-A	17819			8248								10000
04093172131L	17121498		633	441211								10000
04093172131A	50982707		818	1400886								10000
04-09-318-0422-A	57966037		557	2496209								10000
04-09-318-1356-A	79900669		1458	1592771								10000
2093172515	4171419			83155								10000
04093141219A	2496209			217780								10000
04093141219L	341307			122219								10000
04.09.285.2814A	1155518		52	83155								10000
04.09.285.2814L	737308			46667								10000
04.09.286.2973	53059			5263								10000
30.09.282.2503	1232117		81	60327								10000
30.09.283.1418	4742797		127	413781								10000
02.09.293.1178A	64326			2285								10000
02.09.293.1178L	17819		67	1658								10000
20.09.294.0228	22133149			786185								10000

Table 6.8 MR-CoNS

Lab ID	pan-*Staph*	pan- *Strep*	pan-*Entero*	*nuc*	*mecA*	*gseA*	*ply*	*E.faecium ddl.*	*E.faecalis ddl*	*vanA*	*van B*	Spike
3093312017[a]	10000											10000
4093311934	2436				20260							10000
4093342709	14698				2436	459						10000
02093341949-A	53059				992	557						10000
02093341949-L	46667		4		8248	5263						10000
2093342370	12123				930	1058						10000
03-09-331-2017 A[a]	2285		27			43						10000
03-09-331-2017 L[a]	5984											10000
2093330642[a]	38		35									10000
4093342702	470459		31		64326	26190						10000
4093353332[a]	11370											10000
4093362877	21603	19	16		522	767						10000
4093160108	79900669				1592771	1155518						10000
4093150195	15058824		1367		168467	232216						10000
1093152974[a]	264024		2285			3581						10000
4093160439	3226877				8795	19000						10000
3093143318	5057198		1768		12927	24562						10000
4093150302	172605541				1155518	3440787						10000
04-09-318-1054	88667				1930988							10000
02-09-319-0690	10245635				68590	100812						10000
01093132747-A	2010		1658		4936							10000
01093132747-L	247610		1658		3358	3818						10000
02093121272-A	85197289		992		534900	2058993						10000
02093121272-L	18256484		1458		83155							10000

Lab ID	pan-*Staph*	pan- *Strep*	pan-*Entero*	*nuc*	*mecA*	*gseA*	*ply*	*E.faecium ddl.*	*E.faecalis ddl*	*vanA*	*van B*	Spike
4093133101	10245635				49760	130321						10000
04.09.288.0935	6804				12123							10000
03.09.288.0670	2838126		71		388057	168467						10000
02.09.290.0696	87				5612							10000
02.09.286.0177	7432965		81		608167	441211						10000
04.09.284.0820	838301				24562	21603						10000

[a] The samples that were negative for *mecA* gene

Table 6.9 MS-CoNS

Lab ID	pan-*Staph*	pan- *Strep*	pan-*Entero*	*nuc*	*mecA*	*gseA*	*ply*	*E.faecium ddl.*	*E.faecalis ddl*	*vanA*	*van B*	Spike
1093312669	767											10000
1093311736-A	19000		431			633						10000
1093311736-L	17819				6804	6804						10000
4093312465-A	168467					2770						10000
4093312465-L	7255		29			154						10000
4093342709	26		15			15						10000
4093340216	300189					17819						10000
40093312804	4071					4071						10000
04093331332-A	675		27									10000
04093331332-L	1810940		19			94545						10000
04-09-332-0981[a]												10000
2093321380	953127		46			38493						10000
1093152227	3581		522		29777							10000
4093163193	570358		1658			5263						10000

Lab ID	pan-*Staph*	pan- *Strep*	pan-*Entero*	*nuc*	*mecA*	*gseA*	*ply*	*E.faecium ddl.*	*E.faecalis ddl*	*vanA*	*van B*	Spike
03.09.288.2999	388057					12123						10000
04.09.286.2354	5392440		81			893872						10000
04.09.285.2962	6131066		67			2838126						10000

[a]The sample that was negative for *Staphylococcus* 16 rRNA target gene

Table 6.10 *Streptococcus* sp.

Lab ID	Culture result	Pan-*Staph*	Pan-*Strep*	Pan-*Entero*	*nuc*	*mecA*	*gseA*	*ply*	*E.faecium ddl.*	*E.faecalis ddl*	*vanA*	*van B*	Spike
03093352948	*S. pyogenes*		1232117	31									10000
3093320329	*S. pyogenes*		29777										10000
4093321058	*S. pyogenes*		23035										10000
4093153007	*S. parasanguinis*		151811270	1367									10000
02093140295	*S. australis*		5057198	1768									10000
04.09.301.3026	*S. acidominimus*	1458	36986743										10000
03.09.299.2849	*S. pneumoniae*		584368202					54362354					10000
02.09.293.0851	*S. milleri*	71	1768	105									10000
04.09.293.1719	AHS		present	112									10000
04-09-332-1367-A	AHS	2770	281526	2010									10000
04-09-332-1367 L	AHS		10663										10000
3093152842	AHS		16057074	1367									10000
04.09.299.3101	AHS		present	2143									10000
04.09.302.0113	AHS		482014936										10000
4093312546-A	Group G *Strep.*	633	12421240	675									10000
4093312546-L	Group G *Strep.*		4171419										10000

Lab ID	Culture result	Pan-*Staph*	Pan-*Strep*	Pan-*Entero*	*nuc*	*mecA*	*gseA*	*ply*	*E.faecium ddl.*	*E.faecalis ddl*	*vanA*	*van B*	Spike
03093352279-	Group B *Strep.*		122219										10000
01093342364-A	Group B *Strep.*	16	9378										10000
01093342364-L	Group B *Strep.*	19	5263	22									10000

AHS indicates α-haemolytic *Streptococcus*

Table 6.11 *Enterococcus* sp.

Lab ID	Culture result	pan-*Staph*	pan-*Strep*	pan-*Entero*	*nuc*	*mecA*	*gseA*	*ply*	*E.faecium ddl.*	*E.faecalis ddl*	*vanA*	*van B*	Spike
2093132596	*E. faecium vanB*			22133149					3026266			691471	10000
02-09-319-0419-A	*E. faecalis*			25164826					1128	1232117			10000
02-09-319-0419-L	*E. faecalis*	1282		34687319						2058993			10000
04.09.301.1827	*E. faecalis*	79900669		223129080		157994	1493751			14122634			10000
04.09.309.3056	*E. faecalis*			786185						157994			10000
01.09.308.3074 A	*E. faecalis*	312		107495						6804			10000
01.09.308.3074 L	*E. faecalis*			7736						7736			10000
01.09.306.2115	*E. faecalis*			122219						6804			10000
04.09.290.0409	*E. faecalis*	633		1592771		87	67			88667			10000
04.09.288.1067 A	*E. faecalis*			3226877						138960			10000
04.09.288.1067 L	*E. faecalis*			2838126						138960			10000

Appendix C- MT-PCR Quantitative Results of Respiratory Samples

Table 6.12 Quantitative MT-PCR results of samples that were not clearly identified by origin

No	Sample Names	*Staphylo-coccus*	*Strepto-coccus*	*Entero-coccus*	*Entero-bacteriaceae*	*Pseudomonas*
1	MB09184410		91706	805109		
2	MB09188144		418329	37080351		
3	MB09176984		36	113033	118	
4	09K169602H		544081	527613		
5	92541018		55477	1965736		
6	178247					
7	179384			9752		
8	183702		413052	11422268		
9	182667					
10	91890641	818283	1361	27080	525	195915
11	91953054	16299643	12974	106349		
12	91850718	732238	1018	961605	18962	
13	92050933					
14	91871231	31682220		236628221		
15	91931776		10089984			0
16	92182582		50590831	21861481		
17	92070530		2358439			
18	92070438					
19	91871689					
20	91850206	2936950	1034	646505		
21	91943327					
22	91942998		10335621	312967721		
23	91881481	1513754	39974839	21006581		
24	92150662					
25	91903558					
26	92061518		11	262		
27	92223788		2	1194		
28	92240868		1	6		
29	09K154072C			67276464		
30	09R285631P		102	10746714		
31	09R275816T		8004724	38285640		
32	09R306831L	1214241			381	748
33	09R306832M					
34	09R305447N		368948939			
35	09K158392L	7526907		1007920	16908	5219
36	92071725					
37	91750227		403	1076939	121	

No	Sample Names	*Staphylo-coccus*	*Strepto-coccus*	*Entero-coccus*	*Entero-bacteriaceae*	*Pseudomonas*
38	91810268	539005	16707	9986324	296	
39	MB09177942		918666	244687819		
40	91772757					
41	92051176		3028517	1554439460		
42	92013352			704213424		
43	92061762		753127	87882576		
44	92122168					
45	09K166620E	36205	587	208114	29	

Table 6.13 Quantitative MT-PCR results of upper respiratory tract samples

No.	Sample Names	*Staphylo-coccus*	*Strepto-coccus*	*Entero-coccus*	*Entero-bacteriaceae*	*Pseudomonas*
1	MB09173156	141046238	899308	38807852		
2	MB09172330	3046756	45469	10039077	149592895	
3	MB09194801	177814	28	2572658	1502	
4	MB09189410	1086892	2573	1598216	2932	184558
5	MB09187006			274654821		
6	MB09184959	94733	98	3912459	2439	
7	MB09182501		1353156	268623806		
8	MB09181660		880652	9978811		
9	MB09179251		819823	22606087		
10	09R342481M		846860	549954544		
11	MB09175718		607850	32079010		
12	MB09179933		707134	1094795752		
13	MB09189409		1229	2388829	2831	2948143
14	09K169601G		37686	718076	12185017	1841774
15	92541018		55477	1965736		
16	162236 MOULD		677335	35885988		
17	91842911					
18	92190057					
19	92201406		216	264889		
20	91921545			113982219		
21	91842887		421750	60366163		
22	91951618	3196175		969854725		
23	92001378		7557736	70729430		
24	92420784		5670462	72127160		
25	92090518					
26	91841107		149355			
27	91843697	73049014		1516021335		

No.	Sample Names	*Staphylo-coccus*	*Strepto-coccus*	*Entero-coccus*	*Entero-bacteriaceae*	*Pseudomonas*
28	91921077					
29	91843075	2433901	100194	7079867		
30	92043303	5151749	734983	43519700	103179895	
31	92043767		11161079	215057095		
32	91921588					
33	91851179		4254467	25604880		0
34	92180633			2267254125		
35	92071380		42155600	15799169		
36	3700 arico			51019669		
37	91891255					
38	91842901	7572281	7471	1163748	54870	
39	91932051	21389276	393981215			
40	91851565	237129885	33656413	7010140513		
41	91940543			125745926		
42	91982842					
43	91874282		739168685	866804218		
44	91814036		110923132	20238354		
45	91821438					
46	92152054					
47	91911071	3413809	10582	1315151	2015	
48	92013999			33434815		
49	91861928		37	9350		
50	92040952	161040	0	2		
51	92220177	92266	0	0	2620	
52	92311615			8562		
53	92211580	1271499	1	5		
54	91821174	307289688	0	22648664		
55	92173360	95514474	0	5139157847		
56	91970823		0	763990945		
57	92071824	32769		456966	68	
58	92012667		418050	11277050		
59	09K158951M					
60	09K151460A	6584597		2973977062		
61	09R285468X	6576776	12949981	38851690		
62	09R282370M		2062055	340237640		
63	09R285626T	10543	61	773189	40	
64	09K158640H		19101866	485910546		
65	91740156			5529013	673	
66	91800206		302947			
67	91590131		58055976			

No.	Sample Names	*Staphylo-coccus*	*Strepto-coccus*	*Entero-coccus*	*Entero-bacteriaceae*	*Pseudomonas*
68	09R278458Y					
69	09R298694Z		131132345	5934802319		
70	09R311333E					
71	91872866			1341241149		
72	91841557			1252453188		
73	91740398	3018	4344	658413	43375	
74	91790132		1251405	5934356		
75	91740174		61377		78	
76	MB09166797			2276873260		
77	MB09172067		822291	131852045		
78	MB09160375	303289	72052			
79	MB09175319	2159779	1290787	38778655		
80	MB09179244			774791		
81	MB09171635	9353961	635			
82	MB09169794		1287234	2277730267		
83	MB09169497		117975	17208152		
84	MB09168173		1292783	1314754775		
85	MB09168170		507177	18030190		
86	MB09167708	607107	3064	767391	2554	
87	MB09166609	71946	6	116226	227	
88	MB09164872		912168	65158502		
89	MB09181161	1206	14		127	359
90	MB09179239		618734	4481777		
91	MB09179237	9233414	863410	456656214		
92	MB09175646			26377534		
93	MB09173573		920817	1545107978		
94	MB09173379	115824665	364112	30755027		
95	09R285468X	83248128	3063815	27658405	27609342	
96	9575209Y	4883471	12016	19681		
97	9116024L	574691	6196	21493	2410	
98	9121978E		1328	59981201		
99	8089404M		64	173656	133	
100	8090119H		21575	27132630		
101	912871OJ	76457343	130	551364		27946
102	9134444F		20147	364193		
103	9121070K		49199	4116673159		
104	92232961		5430		266257	
105	92232852	19963	743		249474	
106	92462624		2818			
107	MB09164094	3395312	789583	41371736		

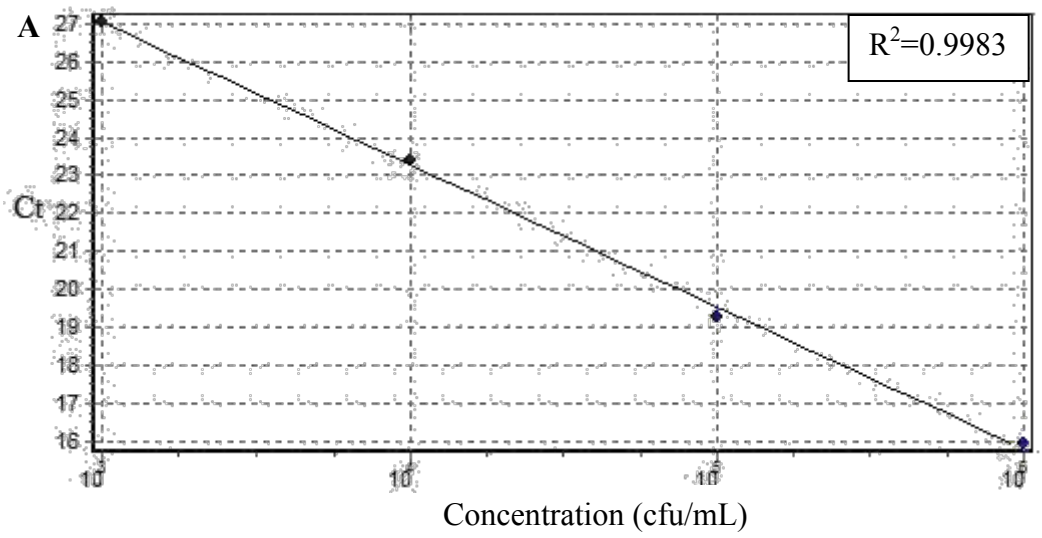
No.	Sample Names	*Staphylo-coccus*	*Strepto-coccus*	*Entero-coccus*	*Entero-bacteriaceae*	*Pseudomonas*
108	92460717		2523295			
109	09R299196O	519738	260	104583	365	89897
110	09K153573H					
111	09K165525H			54513290		
112	09R332350G	1151	176	68446	44	
113	09R290422J		4530239			91041
114	09R289245U		18952491	109888		1769344
115	09R275773V					
116	09R288232P	6142882	0	0	27174291	
117	8273474P		581579	612678937		
118	9123896C		1194623	1248688217		
119	9584731E		6907			
120	91861139		572997	70410743		
121	92232721					

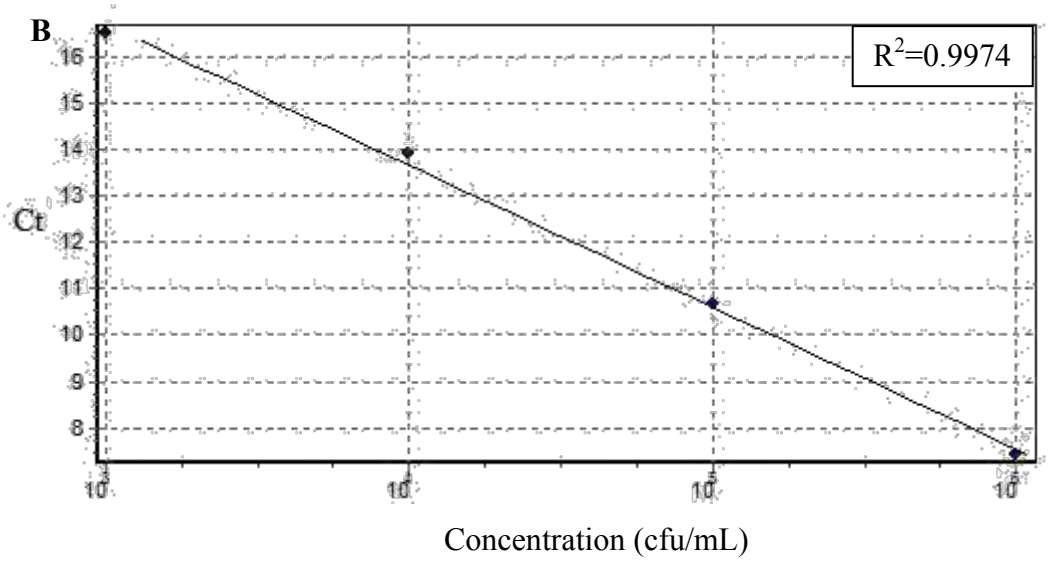
Table 6.14 Quantitative MT-PCR results of lower respiratory tract samples

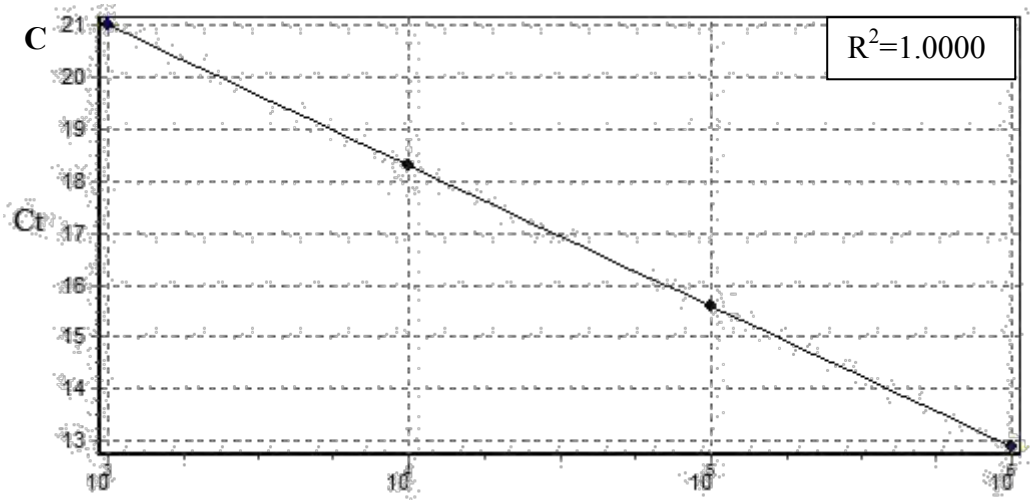
No	Sample Names	*Staphylo-coccus*	*Strepto-coccus*	*Entero-coccus*	*Entero-bacteriaceae*	*Pseudomonas*
1	MB09183509		190891	210240		
2	09-182-2005	59153	464		16458	362
3	09-197-0467		381674			
4	MB09178257		1723	22452		
5	MB09180513	9988	965	19083		1508
6	91951617	2400007	348929	30204467		
7	91951495		1890	606211		
8	92053473					
9	92093991	24288072	2865559	6872482		
10	91841880			15016557		
11	92044081	39914	75			
12	91850930		409891	195158408		
13	92191732					
14	92091165					
15	91952341					
16	92152053	1225			27	
17	91953777	18719133	868020			
18	92061767	24026	0	3		
19	92032861		0	14		
20	92233791		124	228817	86	
21	91823126	7416	12	81229	25	

No	Sample Names	*Staphylo-coccus*	*Strepto-coccus*	*Entero-coccus*	*Entero-bacteriaceae*	*Pseudomonas*
22	92193241	2666	0	195217	146	
23	91970536		30290	2559139	775	
24	92071593					
25	09K170825G	2593	14		583	
26	91841892					
27	91870906					
28	9589399G	78903	128	25078	10925	219
29	09R330393L	598246	166206	1332086		
30	9586680L	1151596	51	582286	87	7787
31	9121068U	258332	87	45433	31	
32	9584730Y					
33	9583976N					
34	91912172					

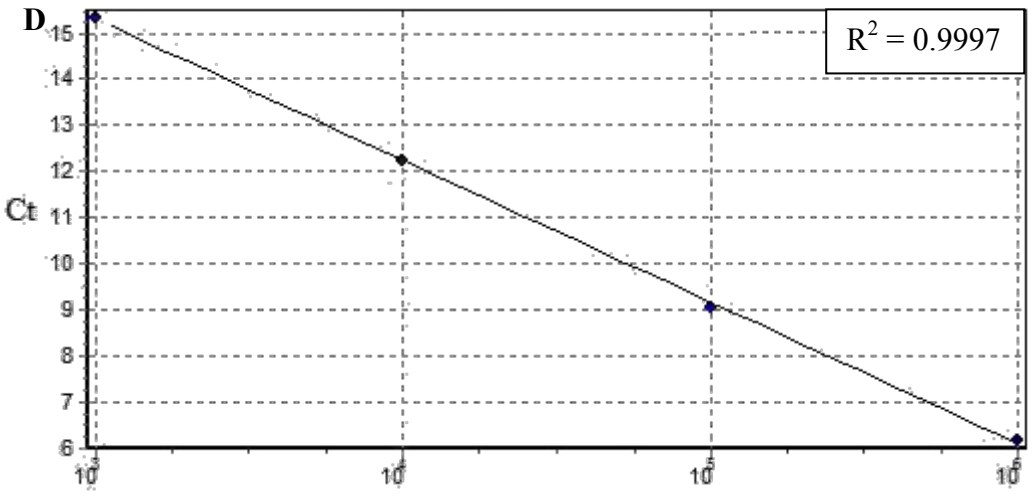
Appendix D- MT-PCR Standard Curves


A
R^2=0.9983
Ct
27
26
25
24
23
22
21
20
19
18
17
16
Concentration (cfu/mL)


B
R^2=0.9974
Ct
16
15
14
13
12
11
10
9
8
Concentration (cfu/mL)



Concentration (cfu/mL)



Concentration (cfu/mL)

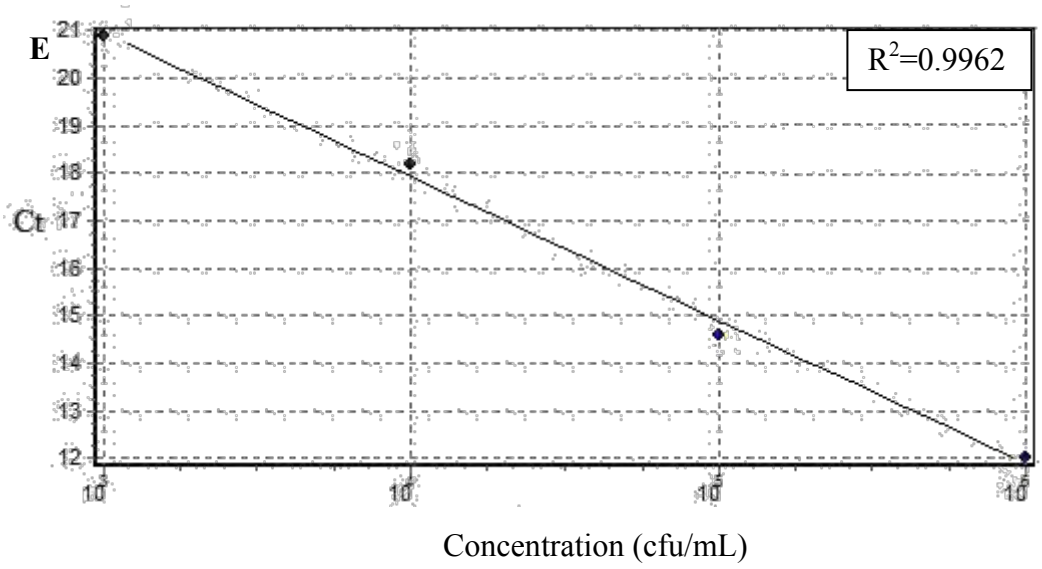


Figure 6.1 Standard curves generated by serial dilution of known amounts of control strains, presented as concentration (cfu/mL). The standard curve for each control strain was deemed acceptable providing four data points could be included and were produced using the Rotor-Gene 6000 Series software. A= *Staphylococcus* sp. B= *Streptococcus* sp. C= *Enterococcus* sp. D= *Enterobacteriaceae* species E= *Pseudomonas* sp.

Chapter 7
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