HYBRID PEPTIDES DERIVED FROM NATURAL ANTIMICROBIAL PEPTIDES, INDOLICIDIN AND RANALEXIN, EXHIBIT POTENT ANTIMICROBIAL ACTIVITIES AGAINST *STREPTOCOCCUS PNEUMONIAE IN VITRO* AND *IN VIVO*

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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: **Hassan Mahmood Kzar Jindal**

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Title of Thesis: **Hybrid peptides derived from natural antimicrobial peptides, indolicidin and ranalexin, exhibit potent antimicrobial activities against** *streptococcus pneumoniae in vitro* **and** *in vivo***.**

Field of Study: **Medical Microbiology**

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ABSTRACT

Streptococcus pneumoniae is one of the leading causes of morbidity and mortality in both children and adults. This pathogen is responsible for invasive and noninvasive diseases. Moreover, pneumococcus is the most leading cause of community-acquired pneumonia (CAP), meningitis, and bacteremia worldwide. According to WHO, this bacterial pathogen is responsible for 1.6 million deaths each year. Like other gram-positive bacteria, *S. pneumoniae* is increasingly difficult to treat due to the inappropriate use of antibiotics. At present, *S. pneumoniae* has developed resistance to conventional drugs including novel antibiotics such as vancomycin. Therefore, finding a new class of antibacterial agents to overcome this serious issue is a top priority worldwide. One of the promising alternatives to today's antibiotics is antimicrobial peptides (AMPs). AMPs are produced by almost all living organisms as the first line of defense in their innate immune system against microbial infection. In this study, novel synthetic peptides were designed based on two natural templates indolicidin and ranalexin. Out of thirteen newly designed peptides, five hybrid peptides (RN7-IN10, RN7-IN9, RN7-IN8, RN7-IN7, and RN7-IN6) showed the strongest *in vitro* antibacterial activity against thirty pneumococcal clinical isolates. These four hybrid peptides also showed broad spectrum antibacterial activity against *S. aureus*, methicillin-resistant *S. aureus* (MRSA), and *E. coli*. Moreover, the killing kinetics of peptides demonstrated that the hybrid peptides were able to eliminate pneumococci within 150 min of treatment which is faster than the standard drugs erythromycin and ceftriaxone. The hybrid peptides produced synergism when combined with each other and with standard antibiotics erythromycin and ceftriaxone. *In vitro* toxicity assessment revealed that none of the designed peptides displayed and toxic effects against human erythrocytes, WRL-68, and NL-20 cell lines at their MIC levels. *In silico* molecular docking showed that all five hybrid peptides revealed strong binding affinity toward three pneumococcal virulence factors autolysin, pneumolysin, and

pneumococcal surface protein A (PspA). Mechanism of action of peptides exhibited that the hybrid peptides kill pneumococci by attacking and damaging the integrity of their cellular membranes. Moreover, DNA binding assay showed that at 62.5 µg/ml all hybrid peptides were able to bind effectively to genomic DNA and to prevent it from migrating through the agarose gel, these results suggesting that hybrid peptides could possess another mechanism of action besides their ability to disrupt the cell membrane. Two hybrid peptides RN7-IN10 and RN7-IN8 were selected to test their *in vivo* therapeutic efficacy. At 20 mg/kg, the peptides were able to protect 30% and 50% of the mice from lethal systemic infection by resistant pneumococcal strain. The combination of both peptides at 10 mg/kg for each was able to protect 60% of the mice. Interestingly, a combination of RN7-IN8 (20 mg/kg) and ceftriaxone (20 mg/kg) were able to provide 100% protection to mice infected with a virulent strain of *S. pneumoniae*. Infections caused by antibiotic-resistant *S. pneumoniae* remain a serious threat to human life. The present study demonstrated that AMPs represent a promising new class of antibacterials either as standalone or in combination with traditional antibiotics.

ABSTRAK

Streptococcus pneumoniae adalah salah satu punca utama morbiditi dan kematian di kalangan kanak-kanak dan orang dewasa. Patogen ini bertanggungjawab dalam jangkitan invasif dan tidak invasif. Selain itu, pneumokokus adalah punca yang paling utama bagi pneumonia dalam masyarakat (CAP), meningitis dan bakteremia di seluruh dunia. Menurut WHO, 1.6 juta kematian adalah disebabkan oleh jangkitan pneumokokal setiap tahun dengan purata 0.7-1.000.000 pada kanak-kanak berusia di bawah lima tahun dan kebanyakannya ditemui di Asia dan Afrika. Seperti bakteria gram-positif lain, *S. pneumoniae* semakin sukar untuk dirawat kerana penggunaan antibiotic yang salah dan tidak bertepatan. Pada masa ini, *S. pneumoniae* telah menyebabkan berlaku rintangan kepada ubat-ubatan konvensional termasuk antibiotik novel seperti vancomycin. Oleh itu, mencari kelas baru agen anti-bakteria untuk mengatasi isu yang serius ini menjadi perkara utama yang dilakukan di seluruh dunia. Salah satu alternatif yang membantu kepada isu antibiotik hari ini adalah peptida anti-mikrob (AMPS). Penguat dihasilkan oleh hampir semua organisma hidup sebagai barisan pertama pertahanan dalam sistem imun semula jadi terhadap jangkitan mikrob. Dalam kajian ini, peptida sintetik novel direka berdasarkan dua templet indolicidin semula jadi dan ranalexin. Daripada tiga belas peptida yang baru direka, lima peptida hibrid (RN7-IN10, RN7-IN9, RN7-IN8, RN7-In7 dan RN7-IN6) menunjukkan vitro aktiviti anti-bakteria yang kuat terhadap tiga puluh pencilan klinikal pneumokokal. Keempat-empat peptida hibrid juga menunjukkan spektrum antibakteria yang luas terhadap S. aureus, methicillin tahan S. aureus (MRSA), dan E. coli. Selain itu, kinetik pembunuhan peptida menunjukkan bahawa peptida hibrid dapat menghapuskan pneumococci dalam masa 150 min rawatan lebih cepat daripada ubat-ubatan biasa seperti erythromycin dan ceftriaxone. Peptida hibrid dihasilkan oleh sinergi apabila ia digabungkan dengan antibiotic erythromycin dan ceftriaxone. Dalam penilaian ketoksikan vitro menunjukkan bahawa tiada satu pun daripada peptida yang

direka mempunyai kesan toksik terhadap eritrosit manusia, WRL-68, dan NL-20 bahagian sel pada tahap MIC mereka. Dalam silico molekul telah menunjukkan bahawa kesemua lima peptida hibrid mendedahkan pertalian pengikat yang kuat terhadap tiga kebisaan pneumokokal faktor seperti autolysin, pneumolysin, dan permukaan pneumokokal protein A (PspA). Mekanisma tindakan peptida yang dipamerkan menunjukkan peptida hibrid membunuh pneumococci dengan menyerang dan merosakkan integriti membran selular mereka. Selain itu, DNA mengikat assay menunjukkan bahawa pada 62.5 µg / ml semua peptida hibrid dapat mengikat secara berkesan kepada DNA genomik untuk mengelakkan ia daripada berhijrah melalui gel agarose, keputusan ini menunjukkan bahawa peptida hibrid boleh mempunyai satu lagi mekanisma tindakan selain keupayaan mereka mengganggu membran sel. Dua peptida hibrid RN7-IN10 dan RN7-IN8 telah dipilih untuk menguji keberkesanan terapeutik mereka. Pada 20 mg / kg, peptida dapat melindungi 30% hingga 50% tikus dari jangkitan sistemik maut oleh strain pneumokokus. Gabungan kedua-dua peptida pada 10 mg / kg dapat melindungi 60% tikus dari jangkitan. Menariknya, gabungan RN7-IN8 (20 mg / kg) dan ceftriaxone (20 mg / kg) mampu memberi perlindungan 100% kepada tikus yang dijangkiti dengan strain virulen S. pneumoniae. Jangkitan yang disebabkan oleh antibiotik tahan S. pneumoniae kekal menjadi ancaman serius kepada kehidupan manusia. Kajian ini menunjukkan bahawa amp mewakili kelas baru menjanjikan antibacterials sama ada sebagai berdiri sendiri atau kombinasi bersama antibiotik tradisional.

ACKNOWLEDGEMENT

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°C	Degree celsius
%	Percent
α	Alpha
β	Beta
hr	Hour
Kcal/mol	Kilocalorie/mole
pM	Picomole
kDa	Kilodalton
L	Liter
mg	Milligram
μg	Microgram
ng	Nanogram
μL	Microliter
mM	Millimolar
μm	Micrometer
nm	Nanometer
mL	Milliliter
kg	Kilogram
kb	Kilo base
min	Minute
v/v	Volume/volume
G	Gauge
U	Unit
g	Gram
$	Dollar
ABC	ATP-binding cassette
ACIP	Advisory Committee on Immunization Practices
Ala	Alanine
ALP	Alkaline phosphate
ALT	Alanine transaminase
AMPs	Antimicrobial peptides
APD	Antimicrobial peptide database
Arg	Arginine
AST	Aspartate aminotransferase
ATCC	American type culture collection
ATP	Adenine triphosphate
ATS	American thoracic society
BBB	Blood-brain barrier
BHI	Brain heart infusion broth
BTS	British thoracic society
CAMHB	Cationically-adjusted Müeller-Hinton broth
cAMP	Cyclic adenosine monophosphate
CAP	Community-acquired pneumonia
CbpA	Choline binding protein A
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
CL	Cardiolipin
CLSI	Clinical and Laboratory Standards Institute
CPS	Capsular polysaccharide
CSF	Cerebrospinal fluid

DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthetase
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylene diamine tetraacetic acid
ERS	European respiratory society
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FICI	Fractional inhibitory concentration index
GC	Guanine-cytosine
GRAVY	Grand average hydropathy value
HCT	Hematocrit
Hgb	Hemoglobin
HIV	Human immunodeficiency virus
hLF	Human lactoferrin
HMM	High molecular mass
HR	Hydrophobicity ratio
HSV	Herpes simplex virus
Hyl	Hyaluronate lyase
IACUC	Institutional Animal Care and Use Committee
IDSA	Infectious Diseases Society of America
IL1β	Interleukin 1 beta
IN	Intranasal
IP	Intraperitoneal
IPD	Invasive pneumococcal disease
IT	Intrathoracic
LMM	Low molecular mass
Lys	Lysine
LytA	Autolysin
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MDR	Multidrug resistance
MIC	Minimum inhibitory concentration
MMPs	Matrix metalloproteinases
Mn	Manganese
MRSA	Methicillin-resistant *Staphylococcus aureus*
NetC	Net charge
NO	Nitric oxide
NOS	Nitric oxide synthase
OD	Optical density
PAF	Platelet-activating factor
PBPs	Penicillin-binding proteins
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PCV	Pneumococcal conjugate vaccine
PCV-10	10-valent pneumococcal conjugate vaccine
PCV-13	13-valent pneumococcal conjugate vaccine
PCV-7	7-valent pneumococcal conjugate vaccine
PDB	Protein databank
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol

Phe	Phenylanine
PLT	Platelet Counts
Ply	Pneumolysin
PPV	Pneumococcal polysaccharide vaccine
PPV23	23-valent pneumococcal polysaccharide vaccine
PS	Phosphatidylserine
PsaA	Pneumococcal surface antigen A
PspA	Pneumococcal surface protein A
RBCs	Red blood cells
RNA	Ribonucleic acid
RP-HPLC	Reverse phase high-performance liquid chromatography
rpm	Rotation per minute
rRNA	Ribosomal ribonucleic acid
SC	Subcutaneous
SM	Sphingomyelin
TGFβ1	Transforming growth factor beta one
TMP-SMX	Trimethoprim-sulfamethoxazole
TNFα	Tumor necrosis factor alpha
tRNA	Transfer ribonucleic acid
Trp	Tryptophan
Tyr	Tyrocine
UK	United kingdom
UMMC	University of Malaya Medical Centre
US	United states
WBCs	White blood cells
WHO	World health organization

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 *Streptococcus pneumoniae* an overview

Streptococcus pneumoniae (S. pneumoniae) or pneumococcus was first discovered by Louis Pasteur (1881) and George Miller Sternberg (1881). Pasteur found this pathogen in the saliva of a patient with rabies while Sternberg isolated the organism from his saliva (D. a Watson *et al.*, 1993). Both recovered the organism from the blood of rabbits after injecting the saliva into them. Three years later Carl Friedlander (1883) highlighted the association of the bacterium with labor pneumonia (Austrian, 1999; López, 2006). *S. pneumoniae* is a gram-positive, lancet-shaped, facultatively anaerobic, α-haemolytic, extracellular pathogen belonging to the genus *Streptococcus* and naturally colonises the human upper respiratory tract as part of the microflora. Typically, this microorganism appears in pairs (diplococci), single cells or short chains (Figure 1.1) (AlonsoDeVelasco *et al.*, 1995). Pathogenic isolates of *S. pneumoniae* are encapsulated with polysaccharide capsule and to date more than 91 pneumococcal serotypes have been classified based on the chemical composition of their capsular polysaccharides (Hackel *et al.*, 2013; Staples *et al.*, 2014). The capsule coats the cell wall which is composed of peptidoglycan and teichoic acid. The peptidoglycan consists of glycan chains of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid residues, cross-linked to each other through peptide side chains. The teichoic acid contains phosphorylcholine and is connected to peptidoglycan via *N*-acetylmuramic acid. The phosphorylcholine serves as a signal for autolysin an enzyme engaged in the process of cell division by cleaving the peptidoglycan. Another important component of the surface of *S. pneumoniae* is lipoteichoic acid or Forrsman antigen. This antigen inhibits the activity of autolysin during the stationary phase of growth. Lack of this inhibitor followed by uncontrolled activity of autolysin will cause the disruption of the cell wall and subsequently cell death (AlonsoDeVelasco *et al.*, 1995).
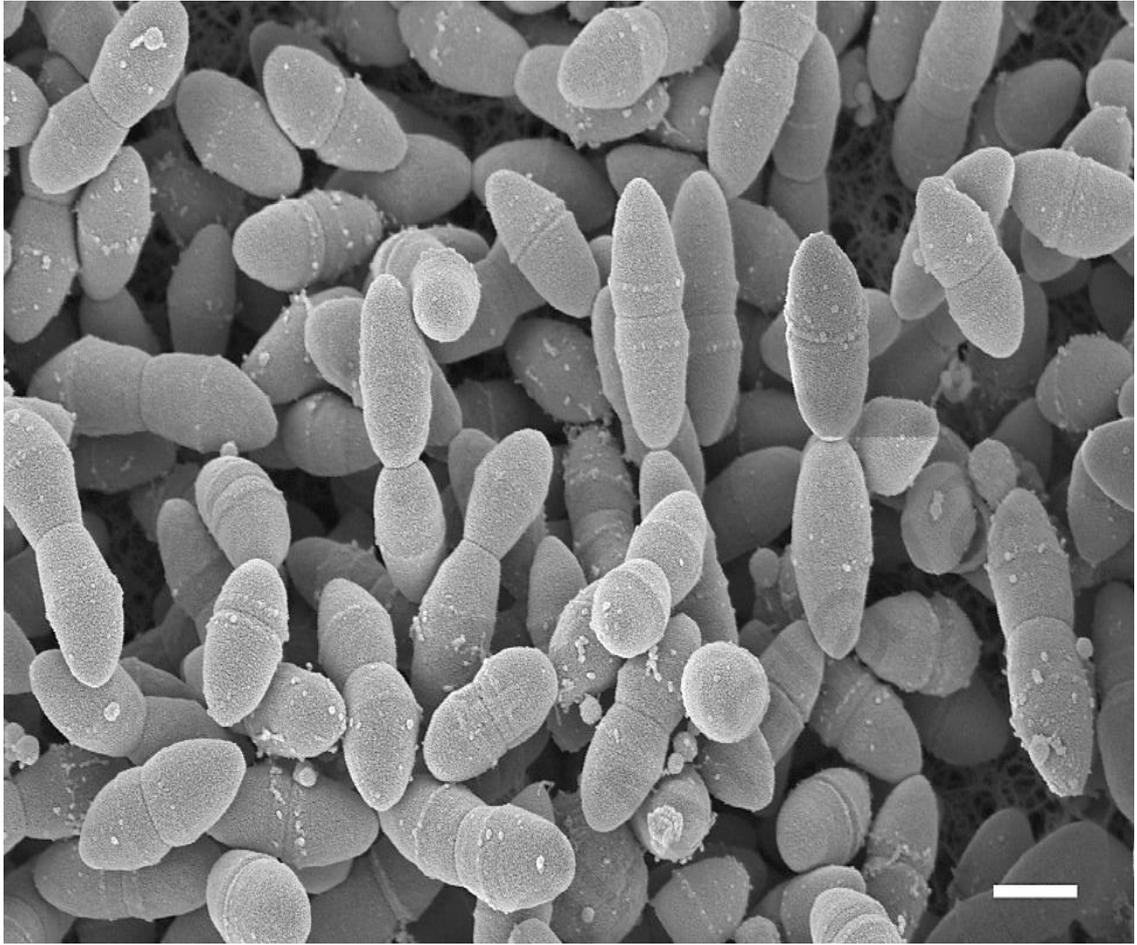
Figure 1.1: *Streptococcus pneumoniae* under scanning electron microscopy. Bar = 500 nm (source: Muhsin Özel, Gudrun Holland and Rolf Reissbrodt / Robert Koch Institut http://www.rki.de/EN/Home/homepage_node.html).

Pneumococcus is naturally transformable, allowing DNA uptake to take place for the whole pneumococcal population at one time in a greatly controlled pattern. The genome of *S. pneumoniae* contains between 2 million and 2.1 million base pairs with the guanine-cytosine content of 40% which is lesser than that of various bacterial microbes. The circular DNA is regularly accompanied by small cryptic plasmids. The lack of citric acid (TCA) cycle makes this microorganism dependent on fermentation of carbohydrates for energy and it can catabolize 14 different sugars (Henriques-Normark & Tuomanen, 2013; van der Poll & Opal, 2009). Strain TIGR4 was one of the first pneumococcal genomes to be sequenced. It consists of 2236 predicted coding regions with about 64% genes assigned a functional role. Sixteen percent of the predicted proteins have unknown

function and the remaining 20% exist only in *S. pneumoniae* (Tettelin *et al.*, 2001). TIGR4 genome comprises 1553 genes that are crucial for cell viability. A further 154 bacterial genes contribute to virulence (the virulome) and 176 genes actively retain a non-invasive phenotype (Tettelin *et al.*, 2001). Four rRNA operons, 58 tRNAs, and three structural RNAs were identified in TIGR4 genome (Figure 1.2).
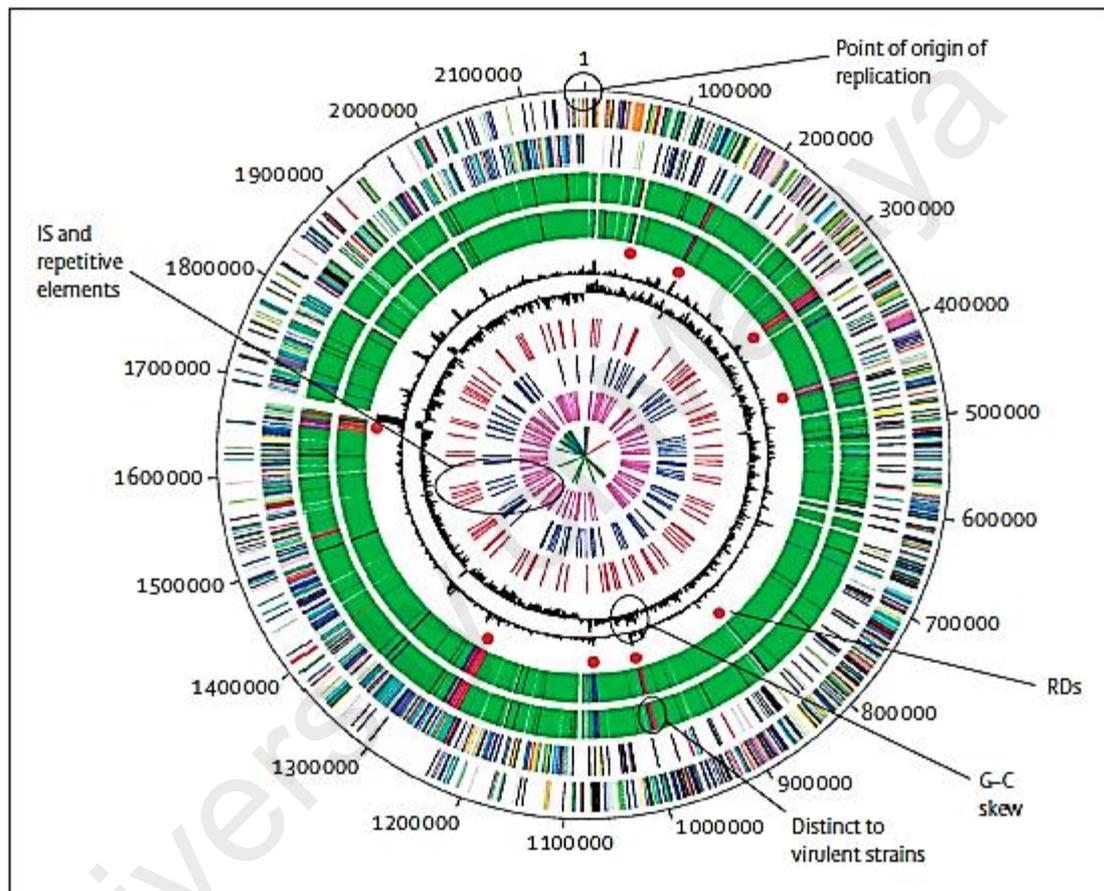
Figure 1.2: Genomic assembly of pneumococcal strain TIGR4. RDs=regions of diversity. G–C skew=difference in guanine (G)–cytosine (C) ratio from G–C average of the pneumococcal genome. IS=insertion sequence elements (source: Tettelin *et al*., 2001).

Nearly 5% of the pneumococcal genome consists of insertion sequences that may participate in genome reformations via uptake of foreign DNA (Tettelin *et al.*, 2001). Pneumococcus has the highest number of DNA repeat regions among all available genomes and this probably contributes to plasticity of the genome. The pneumococcal genome has at least 13 two-component sensor kinase signal systems that spot

environmental signals like cell density, microbial contestants and substrate availability and give a response by altering expression of the genome (Paterson *et al.*, 2006).

1.2 Pneumococcal virulence factors

1.2.1 Capsular polysaccharide (CPS)

The polysaccharide capsule covers the vast majority of pneumococcal clinical isolates recovered from the sterile sites. The CPS is approximately 200-400 nm in thickness (Hammerschmidt *et al.*, 2005) and is the most crucial virulence determinant of *S. pneumoniae.* Pneumococcal isolates lacking the capsule were found to be at least 10^5 less virulent than encapsulated strains (AlonsoDeVelasco *et al.*, 1995; D. A. Watson & Musher, 1990). As mentioned previously, according to the chemical composition of their capsules, pneumococci can be classified into more than 91 distinct serotypes. Some of these serotypes are antigenically-related and therefore they are grouped in one group called serogroup (for example 9A, 9L, 9N, and 9V). On the other hand, serotypes with no antigenic relationship are given numbers only (for example 1, 2, 3, and 4) (KALIN, 1998). The virulence of *S. pneumoniae* and its ability to cause invasive disease is governed by the chemical structure of the CPS and to a lesser degree its thickness (Knecht *et al.*, 1970). The key role of the capsule is to form a shield to protect *S. pneumoniae* from phagocytosis by leukocytes. CPS is highly negatively charged, and this prevents the interaction between the complement component iC3b on the pneumococcal surface and phagocytic complement receptor 3 (CR3) and the interaction between Fcγ receptor and Fc component of IgG attached to pneumococci (Hammerschmidt *et al*., 2005; Mitchell & Mitchell, 2010). The capsule is also critical for bacterial colonization, as it avoids mechanical removal by mucus and hence helps pneumococci spread to the epithelial surface (Nelson *et al.*, 2007). Moreover, CPS limit autolysis and decrease exposure to antibiotics (Kadioglu *et al.*, 2008).

In 2006, Bentley *et al*. (2006) identified the sequences of the genes responsible for the synthesis of capsular polysaccharide of all the pneumococcal serotypes and these genes are located at the same locus (*cps*) between *dexB* and *aliA*. Except for serotypes 3 and 37, the first four genes of *cps* locus (CpsA-D) can be found in all pneumococcal serotypes. These four genes encode proteins that affect the level of CPS expression (Morona *et al*., 2000). The central segment of the *cps* locus consists of genes that are responsible for the synthesis of specific glycosyltransferases that assemble the serotype-specific oligosaccharide repeat unit on a lipid carrier. The terminal segment of this locus consists of genes that encode the synthesis of activated sugar precursors. Except for serotypes 3 and 37, the CPS for all pneumococcal serotypes are synthesized by transmitting a monosaccharide phosphate from a nucleotide diphosphate sugar to a membrane-associated lipid carrier. Further monosaccharide molecules will be transferred sequentially to form the lipid-linked repeat unit. This unit will be redeployed to the outer surface of the membrane by an enzyme called Wzx flippase, polymerized to produce the mature CPS and finally linked to the peptidoglycan (Sorensen *et al.*, 1990). Figure 1.3 illustrates the steps of capsule biosynthesis in *S. pneumoniae*.
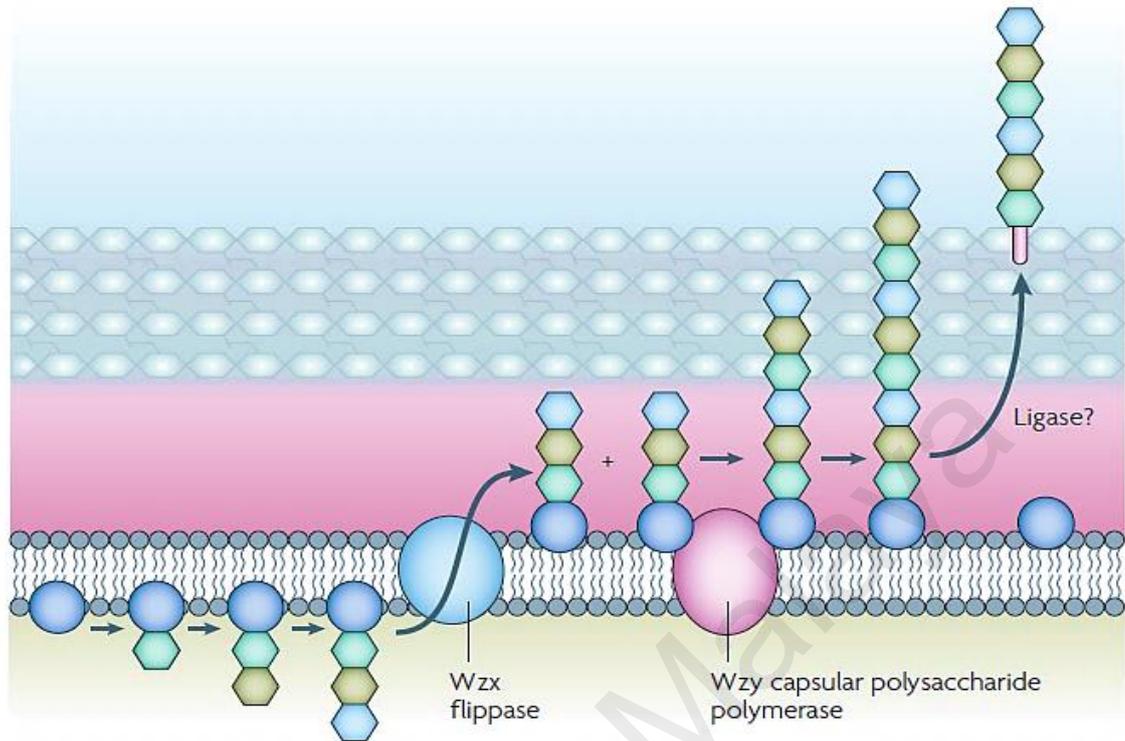
Figure 1.3: The regulation of capsular polysaccharides by *cps* locus in *S. pneumoniae* (source: Kadioglu *et al*., 2008).

Although CpsA has no impact on the transcription of CPS in *S. pneumoniae*, a mutant of pneumococcus lacking CpsA has been shown to produce a reduced level of CPS (Bender *et al*., 2003). CpsB is a manganese-dependent phosphotyrosine-protein phosphatase. It has been shown that CpsB is necessary for the dephosphorylation of CpsD. Mutants with CpsB deletion tend to have increased levels of phosphorylated CpsD which lead to a significant decrease of CPS production (Morona *et al*., 2003). CpsC is a membrane protein required for CpsD tyrosine autophosphorylation. It has been identified recently that CpsC play a unique role in the attachment of CPS to the pneumococcal cell wall (Morona *et al*., 2006). CpsD is an autophosphorylating protein-tyrosine kinase. Alterations in CpsD distressing the ATP-binding domain will eliminate CPS synthesis. Therefore, CpsB, CPsC, and CpsD work together to manage CPS biosynthesis (Morona *et al*., 2003; Morona *et al*., 2006).

1.2.2 Cell wall

The pneumococcal cell wall is covered by the capsule and is typically composed of peptidoglycan. Unlike the capsular polysaccharide, purified cell wall components have the capacity to increase inflammation similarly to that induced after infection with whole pneumococcal cells (Tuomanen *et al*., 1987). The pneumococcal cell wall is responsible for the activation of the alternative pathway of complement. The components of pneumococcal cell walls have several important roles; they boost the permeability of cerebral endothelia and pulmonary alveolar epithelia; induce leukocyte recruitment into the lung and subarachnoid space; stimulate the synthesis of platelet-activating factor (PAF); enhance cytokine production; initiate the procoagulant cascade; cause direct injury to neurons and alter cerebral blood flow and vascular-perfusion pressure (Tuomanen *et al*., 1995). The cell wall of *S. pneumoniae* is also involved in attaching the unencapsulated *S. pneumoniae* to human endothelial cells and has cytopathic effects on these cells (Geelen *et al.*, 1993).

1.2.3 Pneumococcal pili

The pneumococcal pili were first discovered by Barocchi and his colleagues in 2006 (Barocchi *et al.*, 2006). These organelles can extend beyond the capsule and they play a role in enhancing the adhesion of pneumococci and subsequently the ability to cause invasive disease. These pili are encoded by two loci or islets called PI-1 and PI-2. Pili encoded by the pneumococcal *rlrA* locus or PI-1 have been shown to enhance adherence to lung epithelial cells, virulence, and host inflammatory response. This pilus consists of three structural proteins and three sortase enzymes involved in linking structural subunits (Barocchi *et al.*, 2006). Another type of pili has been recognized in *S. pneumoniae* and is engaged in adherence of bacteria to epithelial cells. Not all

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pneumococcal strains are capable of expressing pili, but some could express both types (Bagnoli *et al.*, 2008).

1.2.4 Autolysin (LytA)

Autolysin (LytA) is a pneumococcal virulence factor belonging to a set of enzymes that degrade the peptidoglycan of the bacterial cell wall which eventually leads to cell lysis (Howard & Gooder, 1974). This enzyme is located in the cell envelope and plays several roles related to cell wall development and its turnover and cell separation (Berry *et al.*, 1989). The contribution of LytA to the pathogenicity of pneumococci is still unclear. One hypothesis suggests that the virulence effect of pneumococcal autolysin is mediated by the release of other virulence factors such as pneumolysin (Martner *et al.*, 2008). An alternative hypothesis proposes that LytA is released to lyse nearby non-competent pneumococcal cells in a fratricidal manner and subsequently the released DNA can be taken up by the attacker cells (Eldholm *et al.*, 2009). A third theory suggests that autolysin facilitates lysis so as to release proteins such as cell envelope components that may hinder the host immune response (Martner *et al.*, 2009).

Mutant strains of *S. pneumoniae* lacking LytA have been shown to be less virulent than wild-type strains when induced in murine models (Canvin *et al.*, 1995). LytA is also known to participate in the penicillin and vancomycin-induced lysis of *S. pneumoniae*, as the penicillin and vancomycin resistance among clinical isolates is partially associated with the activity of LytA (Mellroth *et al.*, 2012). Structurally, the pneumococcal autolysin consists of two domains, the N-terminal *N*-acetylmuramoyl L-alanine amidase domain and the C-terminal choline binding domain. The choline binding domain allows the enzyme to attach to phosphocholine residues present on the teichoic acids of the cell wall. The attachment to cell wall through the choline binding domain is necessary for the

enzyme activity as choline concentrations that prevent cell wall binding also inhibit autolysis (Mellroth *et al.*, 2012).

1.2.5 Pneumolysin (Ply)

Pneumolysin is an intracellular toxin synthesized by Gram-positive bacteria and belongs to the family of cholesterol-dependent cytolysins. It is a 53-kDa soluble protein produced during the late log phase of growth by all pneumococcal clinical isolates (Benton *et al.*, 1997). Previously, it was thought that Ply could only be released when pneumococcal cells undergo cell lysis. However, it can also be released independently as a major autolysin (Balachandran *et al.*, 2001). At high concentrations Ply converts from soluble monomer to a membrane-inserted oligomer by oligomerization of up to 50 monomers. The oligomers bind to membrane cholesterol of target cells causing large pores (approximately 30 nm in diameters). At sublytic concentrations, Ply has several roles, including stimulation of apoptosis, induction of proinflammatory reactions in immune cells and initiation of host complement pathway. Moreover, Ply has a major role in reducing the ability of human respiratory epithelial cells to clear the mucus from the lower respiratory tract by inhibiting the beating of the cilia presented on the surfce of those (Hirst *et al*., 2004). Ply also participate considerably in the virulence of *S. pneumoniae*. It has been shown that Ply-negative strains are less virulent than the wild pneumococcal strains. Alexander and his co-workers (1994) found that immunization of mice with pneumolysin significantly protected them against different pneumococcal serotypes (Alexander *et al.*, 1994). A previous study has reported that pneumolysin has no effect on the inflammation in the brain of rabbits injected with Ply-negative mutants or wild-type strains. However, other recent reports have been in disagreement with this conclusion. A recent study has shown that injecting the mice with Ply-deficient pneumococci directly into the brain caused attenuated meningitis. The same study also

revealed that injecting the mice with Ply-negative mutants caused reduced sepsis (Hirst *et al.*, 2004).

1.2.6 Pneumococcal surface protein A (PspA)

Pneumococcal surface protein A is another surface protein located on the pneumococcal cell wall and is expressed by all pneumococci clinical isolates. PspA is a 67 to 99 kDa protein consists of five distinct domains: an α helical domain, a signal peptide, a choline-binding domain, a proline-rich region and a C-terminal domain (Cao *et al.*, 2007). PspA plays a significant role for pneumococci as a defensive antigen against host complement system. In addition, PspA can bind to lactoferrin providing a significant protection for pneumococci against the bactericidal activity of this protein. PspA-negative pneumococcal strains were shown to be more sensitive to killing by apolactoferrin and according to the same study, anti-PspA antibodies improve killing of *S. pneumoniae* by apolactoferrin (Shaper *et al.*, 2004). The *in vivo* effect of this protein is still unclear but several studies have shown that PspA is necessary for *in vivo* growth (Hava & Camilli, 2002; McDaniel *et al.*, 1987).

1.2.7 Choline-binding protein A (CbpA)

Choline-binding protein (also known as pneumococcal surface protein C, PspC) is a multifunctional virulence factor of *S. pneumoniae*. This surface antigen has a molecular mass of 75 kDa and consists of 663 amino acid (Rosenow *et al.*, 1997). It has two individual domains: a C-terminal domain that shares more than 95% homology with PspA and an N-terminal domain that consists of α helical coiled-coil structure similar to that of PspA. CbpA plays a significant role in the attachment of pneumococcal cells to the nasopharyngeal and lung epithelial cells and the brain microvascular endothelium. (Rosenow *et al.*, 1997).

CbpA-knockout mutants were shown to be less effective in binding to epithelial cells and sialic acid *in vitro* and revealed decreased efficacy in colonizing the nasopharyngeal cells in comparison with wild-type pneumococci (Rosenow *et al.*, 1997). Besides, CbpA binds to the polymeric immunoglobulin receptor that usually transports secretory IgA. Thus this factor is also known as SpsA (secretory pneumococcal surface protein A) (Zhang *et al.*, 2000). This activity allows the translocation of pneumococcal cells across the respiratory epithelium (Kadioglu *et al.*, 2008). Another property of this antigen is the ability to bind to complement regulatory factor H, which prevents the formation of C3b and thus blocks the activation of the alternative pathway of the complement system (Dave *et al.*, 2001). It has been suggested that CbpA has an important role in pneumococcal meningitis by allowing the bacteria to cross the blood-brain barrier (Cao *et al.*, 2007).

1.2.8 Pneumococcal surface antigen A (PsaA)

PsaA is another virulence antigen expressed on the surface of all pneumococcal serotypes. This antigen has a molecular mass of 37 kDa and 309 residues. Deleting PsaA from pneumococcal strains results in mutant pneumococci avirulence in murine models of pneumonia, bacteremia, and colonization (Berry & Paton, 1996; Johnson *et al.*, 2000). Originally, this antigen was thought to be a pneumococcal adhesion molecule due to the similarities between its sequence and sequences of adhesins from other streptococci. Several studies supported this assumption and showed that PsaA-knockout mutants were less efficient in binding to mammalian cells. Moreover, it was stated that antibodies against PsaA were able to inhibit pneumococcal adherence (Kadioglu *et al.*, 2008). PsaA is part of the ATP-binding cassette (ABC) transport system in which PsaA is the divalent metal-ion binding lipoprotein with specificity for manganese (Mn^{2+}). Knocking out PsaA

revealed the absolute requirement of adding Mn^{2+} for normal growth of pneumococcal mutants (McAllister *et al.*, 2004).

1.2.9 Hyaluronate lyase (Hyl)

Hyl is a major surface enzyme of *S. pneumoniae* which belongs to a family of enzymes called hyaluronidases. These enzymes facilitate pneumococcal envision by breaking down connective tissue (Jedrzejas, 2001). The primary substrate of this lysing enzyme is hyaluronan, an essential structural component of the extracellular matrix (ECM) (Meyer *et al.*, 1941). Chondroitin sulfates are a secondary substrate for Hyl, where chondroitin sulfates binds to significant amounts of water to protect the bordering structures and limits the freedom of diffusion of other macromolecules (Jedrzejas, 2001). The action of Hyl on the host tissues is involved in wound infection, pneumonia, and bacteremia. Additionally, it seems that Hyl allows *S. pneumoniae* to penetrate the blood-brain barrier more efficiently. Reports have revealed that pneumococcal isolates from patients with meningitis have significantly higher hyaluronate activity than that from strains causing otitis media, which specify the importance of this protein in the pathogenicity of pneumococcal meningitis (Gillespie & Balakrishnan, 2000). The molecular mass of this enzyme is of 107 kDa and the it has four domains, an N-terminal carbohydrate binding domain, a spacer region, a catalytic domain and a C-terminal tail, which controls access to the catalytic cleft of the enzyme transversing the catalytic domain (Rigden & Jedrzejas, 2003).

1.2.10 Neuraminidase

Neuraminidase (also known as sialidase) cleaves the N-acetylneuraminic acid from glycoproteins, glycolipids and cell-surface oligosaccharides, thus producing severe damage to the host (Camara *et al.*, 1994). The action of neuraminidase on cell glycan may serve to unmask more of the host cell receptors for pneumococcal adhesion, enhancing both colonization and invasion (Gillespie & Balakrishnan, 2000). Three pneumococcal genes encoding neuraminidase enzymes are NanA, NanB, and NanC. NanA is expressed by all pneumococcal strains and NanB present in most of them. On the other hand, only 50% of pneumococci strains encode NanC (Pettigrew *et al.*, 2006).

Although all neuraminidases are exported proteins, only NanA contains cell-surface anchorage domain (LPXTGE), suggesting that these proteins have different *in vivo* roles as NanA and NanB have different pH optimum and different molecular mass (108 kDa and 74.5 kDa respectively) (Berry *et al.*, 1996). Experiments with mutant strains have shown that both NanA and NanB are significant for the survival of pneumococci in the respiratory tract and blood stream (Manco *et al.*, 2006). To date, there is no clear evidence of a biological function for NanC. However, analyzing the distribution of NanC among pneumococcal strains revealed that NanC was more common in strains isolated from cerebrospinal fluid compared to carriage strains (Pettigrew *et al.*, 2006).

In summary, the pneumococcal virulence factors can be classified into two sets. One set of virulent factors present on the surface of integral pneumococcal cell, which appears to be activated at the beginning of the infection, primarily by blocking phagocytosis through complement inhibition. The second set of factors consists of those that act at the phase of pneumococcal breakdown and lysis. At this step, complement activation increases the inflammation and this appears to be the point of no return of pneumococcal infection (AlonsoDeVelasco *et al.*, 1995). Figure 1.4 represents the most important virulence determents of *S. pneumoniae*.
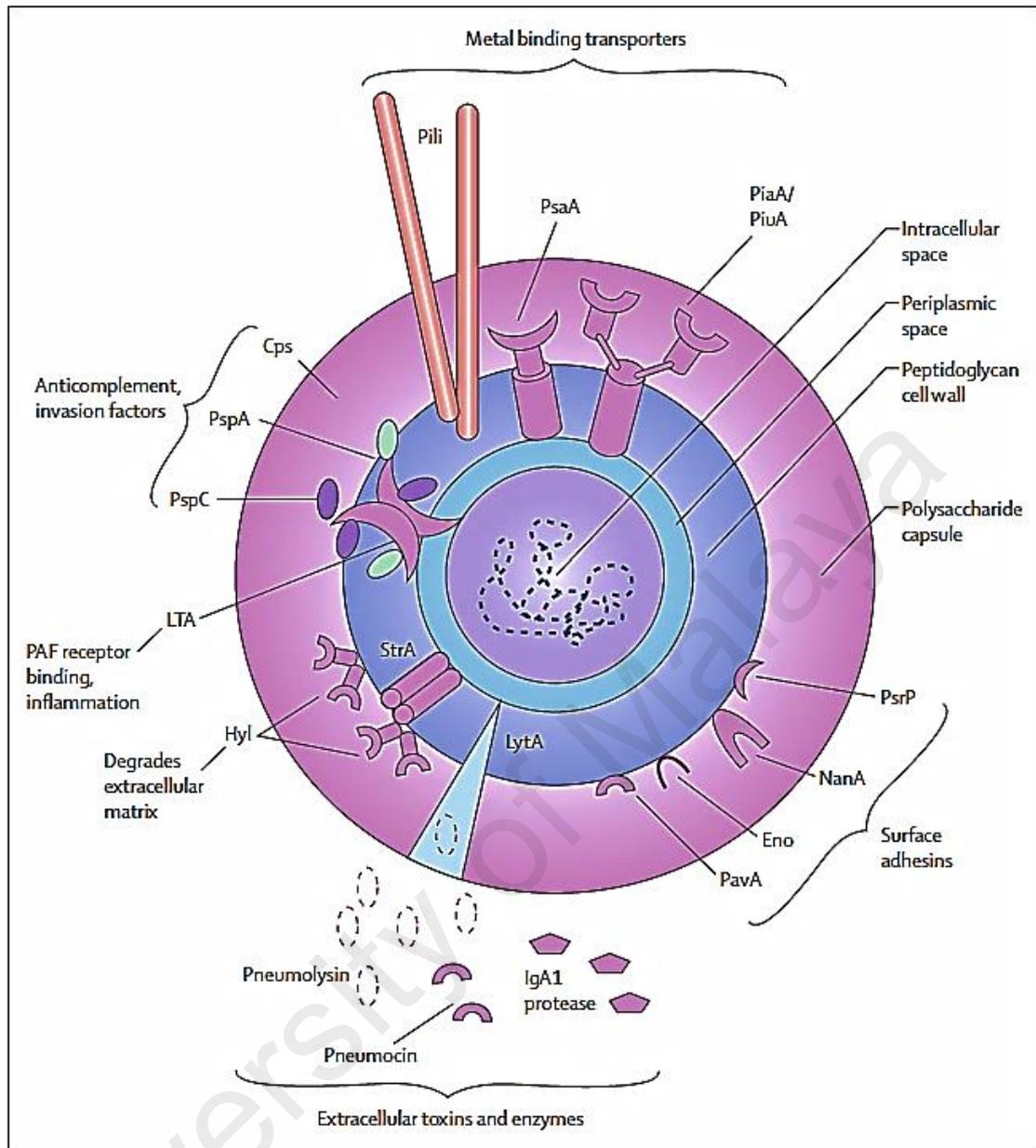
Figure 1.4: Pneumococcal virulence factors (source: van der Poll & Opal, 2009).

1.3 Epidemiology

S. pneumoniae naturally inhabits the upper respiratory tract as a commensal microorganism along with other bacterial species such as *Neisseria meningitidis, Moraxella cattarrhalis*, *Staphylococcus aureus, Haemophilus influenzae* and various α-haemolytic streptococci. Pneumococcus can be easily isolated from the nasopharynx of humans and sometimes from large mammals that live in contact with humans. In general, nasopharyngeal colonization by pneumococcus is asymptomatic, but in certain cases,

colonization is followed by infection if the microorganism gains entry to the sterile parts of the airway (Weinberger *et al.*, 2008). It is well known that without nasopharyngeal colonization, pneumococcal disease will not occur in humans. Moreover, nasopharyngeal colonization is a major source of horizontal spreading of this bacterium within the community. Crowded places such as day care centers, hospitals, and prisons increase the rate of transmission of pneumococcal strains (Donkor, 2013; Lynch & Zhanel, 2010; Sá-Leão *et al.*, 2008). The rates of pneumococcal colonization and carriage vary with age, environment, genetic background and the presence of upper respiratory infection (Bogaert, De Groot, & Hermans, 2004; Debby Bogaert *et al*., 2001; Conklin *et al*., 2016; Simell et al., 2012). About 10% of healthy adults are carriers, the carriage rate in healthy children is 20–40% and about 60% of infants and children in day-care centers can carry pneumococcus in their nasopharynx (van der Poll & Opal, 2009). After colonization by one of the pneumococcal serotypes, the new strain eradicates other competing serotypes and stays as a colonizer (for weeks in adults or months in children). After colonization, pneumococci can transmit into other body parts such as the ears, sinuses or even to the lung through the bronchi and from the lung it can breach the mucosal barrier to enter the blood stream or penetrate the blood-brain barrier leading to meningitis (Henriques-Normark & Tuomanen, 2013). Figure 1.5 demonstrates the progression of the pneumococcal disease.
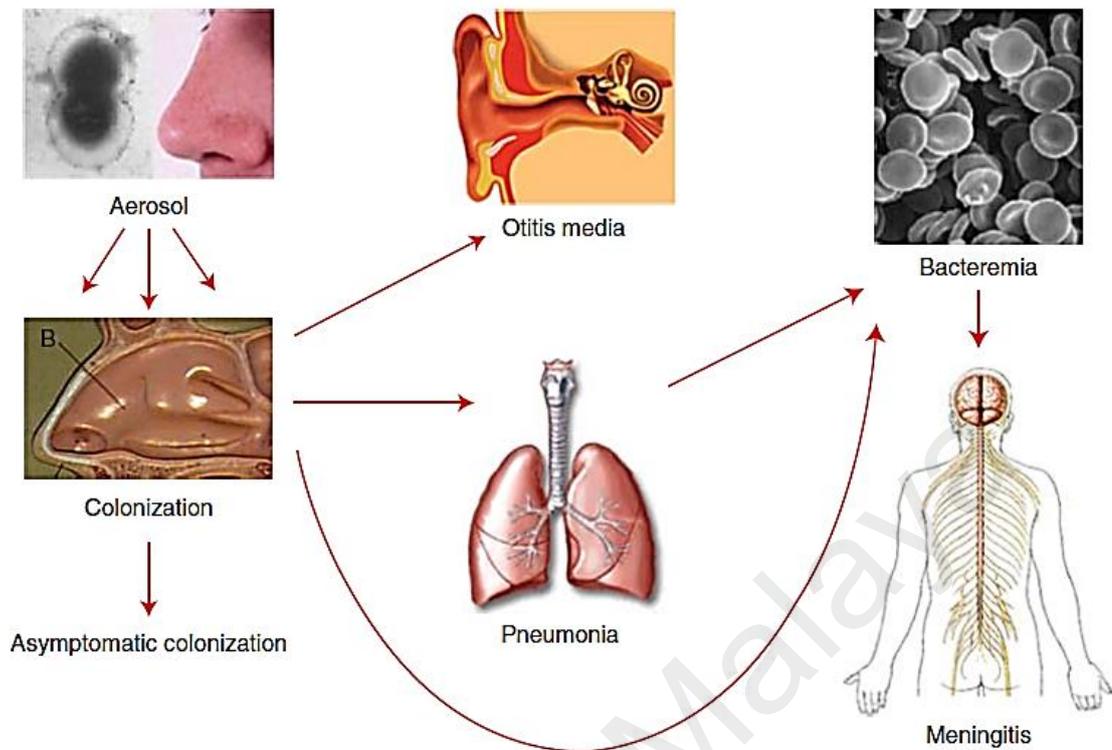
Figure 1.5: The spread of *S. pneumoniae* by aerosol from the nasopharynx to other organs (source: Henriques-Normark & Tuomanen, 2013).

The main way of transmission for this pathogen is via direct contact with contaminated aerosol/droplets between family members, newborns, and children. Although this pathogen is commonly not considered as highly infectious and incidence of outbreaks of invasive pneumococcal disease (IPD) are infrequent, a widespread community outbreak of pneumococcal serotype five has been reported in Vancouver, Canada during the winter months of 2006-2007 (Romney *et al.*, 2008). It is known that the incidence of IPD varies according to regional and ethnical differences. IPD is more common among Australian Aborigines, indigenous peoples of Alaska and Canadian Arctic, African Americans and Bedouins of Israel, Native Americans and Maoris in New Zealand, (Lynch & Zhanel, 2009). Furthermore, the rate of pneumococcal infections is higher among patients with immunodeficiency (e.g. sickle cell disease, organ transplant recipients, and HIV infection), alcohol abuse, recent influenza infection, asthma and exposure to cigarette smoke (Lynch & Zhanel, 2010).

Globally, pneumococcus is a primary cause of morbidity and mortality in both children and adults. This pathogenic bacterium can cause both invasive and noninvasive infections, such as pneumonia, meningitis, bacteremia, peritonitis, acute otitis media and sinusitis. Moreover, *S. pneumoniae* is the main cause of community-acquired pneumonia (CAP), meningitis, and bacteremia worldwide (Chaïbou *et al*., 2014; Hung *et al*., 2013; Jones *et al*., 2010). As stated by World Health Organization (WHO), this bacterium causes 1.6 million deaths each year including 0.7 to 1 million in children below five years predominately in Asian and African countries (Lin *et al*., 2010; O'Brien *et al*., 2009) (Figure 1.6). According to US Centers for Disease Control and Prevention (CDC), this bacterium is responsible for about 4 million disease episodes and 22,000 death incidence every year in the United States alone (http://www.cdc.gov/drugresistance/threat-report-2013/). In a study conducted in 2000, it was reported that five of ten countries with the highest number of pneumococcal deaths in children under the age of five were in Asia (India, China, Bangladesh, Pakistan, and Afghanistan) (O'Brien *et al.*, 2009). In a study done in Denmark in 2009 on 18,000 IPD cases, it has been documented that the mortality rates are influenced by age and comorbidities (Harboe *et al.*, 2009).

In Malaysia, pneumonia is the most clinical presentation of *S. pneumoniae* with morbidity and mortality being highest in children below two years (Bravo, 2009). *S. pneumoniae* along with *H. influenzae* were the most causative agents of meningitis in children below five years old. After the introduction of *H. influenzae* type b (Hib) vaccine to the country in 2002, it is assumed that *S. pneumoniae* is now the most leading cause of meningitis in Malaysia (Bravo, 2009). In terms of mortality, the rates of deaths caused by pneumococcal pneumonia are below 3% in children and ranging between 10 and 30% in adults. Mortality rates caused by meningitis range between 1 to 3% in children and from 16 to 37% in adults (Lynch & Zhanel, 2010).
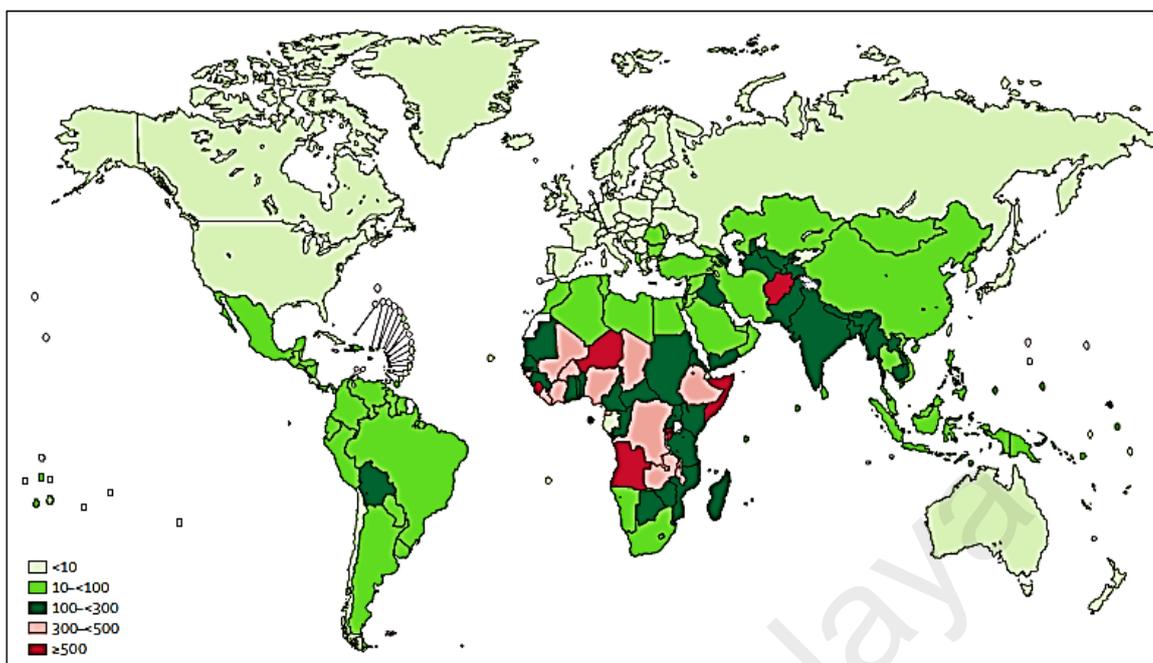
Figure 1.6: Mortality caused by Pneumococcal infection in children of 1-59 months old per 100000 children below five years (source: O'Brien *et al*., 2009).

Although all pneumococcal serotypes have the ability to cause disease, 20 serotypes only account for 80% of IPD worldwide. The most frequent serotypes responsible for IPD worldwide are 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F (Azzari *et al.*, 2016; Nhantumbo *et al.*, 2016). In young children under the age of years serotypes 6B, 14, 19F, 19A and 23F are the most common pneumococcal strains associated with IPD and mortality globally (Lindstrand *et al.*, 2016). Internationally, the most common serotypes inducing acute otitis media (AOM) are 3, 6A, 6B, 9V, 14, 19A, 19F and 23F. On the other hand, serotypes 1, 5, and 7F are seldom associated with AOM. (Rodgers *et al.*, 2009). The distribution of pneumococcal serotypes differs according to the geographical regions. Unlike USA and Europe serotypes 1 and 5 are more common in developing countries. Different serotypes differ in their virulence, ability to colonize and the potential to cause disease. In a survey of IPD conducted in Taiwan in 2007, it has been found that serotypes 14 and 19A had the greatest potential for causing IPD (Hsieh *et al.*, 2009). In the Netherlands, a study done from 2004 to 2006 showed that Serotypes

3, 19F, 23F, 16F, 6B, 8N, and 18C were associated with higher mortality rates than other serotypes (Jansen *et al.*, 2009). In Malaysia, the most common vaccine serotypes were 19F, 14, 6B, 1 and 19A. In children below five years old, serotypes 6A and 6B were more common, while serotypes 19F, 1 and 6B were more common in patients more than five years old (Jauneikaite *et al.*, 2012).

1.4 Invasive pneumococcal disease (IPD)

1.4.1 Pneumonia

When pneumococci enter the lower respiratory tract, they are ejected by several mechanisms such as cough, mucociliary clearance, antimicrobial peptides and local innate immune defenses. If all these systems fail to eradicate the bacteria, migration to the alveolus will occur to initiate pneumonia (Kadioglu *et al.*, 2008; Siegel & Weiser, 2015). The infection process can be divided into five phases. In the first phase (0-4hr) the infection is established due to the ineffective phagocytosis as the pneumococcal capsule and pneumolysin will inhibit the alveolar macrophages. The dominant cytokines in this stage are tumor necrosis factor (TNF), interleukin-6 (IL-6) and nitric oxide (NO) in bronchial lavage fluid, TNF, IL-6 and interleukin-1 (IL-1) in lung tissue and IL-6 in serum. In the second phase (4-24hr) multiplication of pneumococcal cells begins in the alveoli and the levels of TNF, IL-6, IL-1, and leukotriene B_4 increase in both lung tissue and bronchial lavage fluid. Serum IL-1 levels also increase temporarily (Gillespie & Balakrishnan, 2000). The third phase (24-48hr) is marked by lung damage along with alveolar injury and interstitial oedema due to the cytolytic effect of pneumolysin. Regeneration also occurs at this phase. Type II pneumocytes proliferate to regenerate both type I and type II cells. Also, progression from the pre-septicaemic to the septicaemic phase will occur in this phase as the bacteria shifts from alveoli through lung tissue into the blood stream and this is associated with a decrease in IL-1 and TNF levels in the lung.

In the fourth phase (48-72hr) the activity of the alveolar monocytes and lymphocytes is sharply increased, accompanied by nitric oxide (NO) release in lung tissue and alveolar spaces. Finally, the last phase (72-96hr) is marked by further pneumococcal proliferation, high NO levels, huge tissue injury, lipid peroxidation and high fatality (Gillespie & Balakrishnan, 2000). Typically, Pneumonia starts with sudden severe illness and develops with a shaking chill, fever, malaise, cough and dyspnoea. The cough becomes prolific with purulent sputum, sometimes with brownish or blood-tinged sputum with respirophasic chest pain and progressive dyspnoea. Without treatment, this lethal disease could develop into acute respiratory failure, septic shock, multiorgan failure and subsequently death within several days from beginning (van der Poll & Opal, 2009).

1.4.2 Bacteremia

Bacteremia is the most common clinical syndrome of invasive pneumococcal disease (IPD) together with pneumonia and meningitis. In a study performed in Geneva from 1988 to 2004 on 185 consecutive cases of IPD caused by pneumococci in children, it has been found that bacteremia was present in 50% of the cases, 27% for pneumonia and 16% for meningitis (Myers & Gervaix, 2007). Kaplan et al. (1998) have reported similar results in a 3-year multicentre surveillance study done in the US. The nasopharyngeal carriage is necessary but not adequate to produce bacteremia. The mechanisms used by pneumococci to cause bacteremia are poorly understood, but most likely it caused by breakdown in nasal mucosal integrity and serotype-specific virulence factors (Joffe & Alpern, 2010). Some pneumococcal serotypes are much more aggressive than the other. In a study performed before routine pneumococcal conjugate vaccine (PCV-7) immunization, it was revealed that the seven serotypes included in that vaccine accounted for 98% of pneumococcal bacteremia cases (Alpern *et al.*, 2001). The pneumococcal polysaccharide capsule is the most significant factor in the development

of high titer bacteremia. The steric retardation and negative charge define the capacity of capsule type to hinder surface deposition of complement, mannose binding proteins and antibodies that bind to receptors on the phagocytes (Briles *et al.*, 1992). In addition to the pneumococcal capsule, pneumococcal surface protein A (PspA) blocks classic component deposition and choline binding protein A (CbpA) binds C3 and factor H to block component depostion (Henriques-Normark & Tuomanen, 2013).

1.4.3 Meningitis

With high titer bacteremia in the bloodstream, *S. pneumoniae* comes in contact with blood-brain barrier (BBB) vascular endothelial cells. Inflammatory activation is critical for the migration of pneumococci through the cerebral microvascular endothelial cells. Penetrating the tight junctions of this endothelium allows the bacteria to enter the cerebrospinal fluid and the brain parenchyma (Ring *et al.*, 1998). Bacterial meningitis is predominantly caused by three bacterial pathogens, *Streptococcus pneumoniae, Haemophilus influenzae, and Neisseria meningitidis*. These three pathogens share a common mechanism of adhesion to cerebral vascular endothelium, which includes adhesion to the same endothelial 37/ 67-kDa laminin receptor (LR) (Orihuela *et al.*, 2009). In the case of pneumococcus, the corresponding LR-binding adhesin is CbpA. Pneumococcal CbpA binds to LR via a surface-exposed loop structure different from that which binds mucosal receptor pIgR in the nasopharynx (Orihuela *et al.*, 2009). Once pneumococcal cells bind to the vascular wall, they resist the blood flow and use the phosphorylcholine on their surface to bind to the human platelet-activating factor receptor (PAFr). Phosphorylcholine on the pneumococcal surface is thought to serve as a molecular mimic of the chemokine PAF (Gould & Weiser, 2002; Radin *et al.*, 2005). Binding of pneumococcal phosphorylcholine to PAFr will activate the β-arrestin–mediated uptake of the bacteria into BBB cells, which is considered a critical stage for

the progression of pneumococcal meningitis. It has been shown that mice deficient in PAFr were significantly impaired in their ability to translocate bacteria from blood to brain (Radin *et al.*, 2005).

Once the bacteria reach the cerebrospinal fluid (CSF) space, the multiplication occurs rapidly and with a significant severity as there is no immune defense in this part of the body. Pneumococcal components responsible for the stimulation of CSF inflammation are the cell wall components (including teichoic acid and peptidoglycan) and the surface protein pneumolysin. The pneumococcal capsular polysaccharide has no role in the CSF inflammation as its main function is to protect bacteria against killing by phagocytosis (Tuomanen *et al.*, 1985a; Tuomanen *et al.*, 1985b). Once released by autolytic activity, pneumococcal cell wall components stimulate the release of pro-inflammatory cytokines (such as TNF-α, IL-1, and IL-6) from mononuclear macrophages. The release of cytokines activates the opening of the tight junctions between brain capillary endothelial cells, permitting an inflow of serum components especially chemotactic complement factors. Cytokines also upregulate adhesion molecules (e.g., intercellular adhesion molecule ICAM-1) on cerebral vascular endothelial cells and attract neutrophils across the BBB. As a result, neutrophil penetration is evident in the CSF within 8-12 hr of inoculation (Tuomanen *et al.*, 1995). Increased intracranial pressure is possibly the most significant consequence of the inflammatory reaction in the CSF, which results from cerebral oedema, increased cerebral blood volume and alterations of CSF hydrodynamics. Increased intracranial pressure together with the weakness of autoregulation of cerebral blood flow plays a major role in decreasing cerebral blood flow, causing ischaemic necrosis and neuronal loss. It has been shown in experimental meningitis that cerebral blood flow increases as a result of vasodilation which is mediated by nitric oxide (NO). Inhibition of inducible nitric oxide synthase (iNOS), which is responsible in part of NO production during meningitis has led to an

increase in cerebral ischemia in animal models with advanced meningitis and subsequently increase in neural damage (Sande & Tauber, 1999). Several vasoconstrictive mediators like endothelin and oxidative radicals (such as peroxide, hydrogen peroxide and others) act against the vasodilatory effects of the increased NO production in meningitis and further contribute to cerebral ischemia. The other implications of these mediators on cells and macromolecules include lipid peroxidation, DNA damage, and protein oxidation (Scheld *et al.*, 2002). Direct neurotoxicity is another critical mechanism that contributes to brain damage. Excitatory amino acids (such as glutamate) released at higher levels from neurons under stress such as ischemia or hypoglycemia. Their increased concentrations in the extracellular fluid stimulates the activation of various cellular pathways including production of NO by neurons containing neural NOS and production of oxidative radicals, which eventually leads to cell death. Nitric oxide (NO) can be toxic by itself or in combination with superoxide (together form peroxynitrite (ONOO), a highly cytotoxic compound (Tuomanen *et al*., 1995). Figure 1.7 illustrates the mechanism used by *S. pneumoniae* to cross the blood-brain barrier.

The mortality rates of pneumococcal meningitis differ dramatically by age. While the rate is 8.4% in children (Tan, 2012), it is about 11-25% in adults (Tan, 2012; Wall *et al.*, 2013). The risk of death from meningitis is highest in people with neurologic complications during the severe illness (such as hydrocephalus, cerebrovascular insults, brain edema and envelopment of large intracranial blood vessels
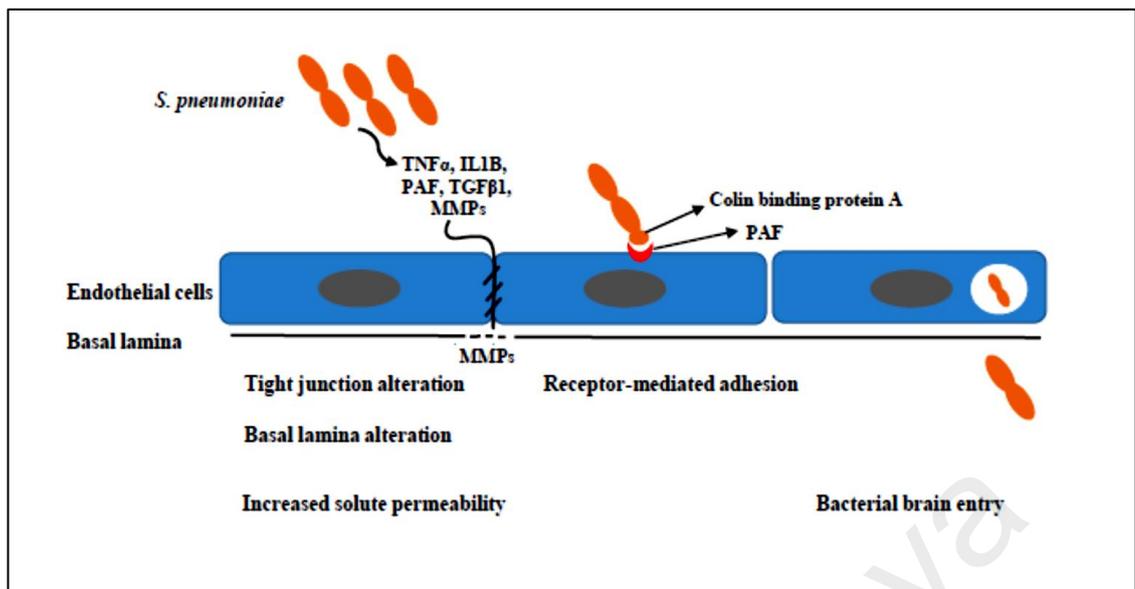
Figure 1.7: *S. pneumoniae* crossing blood-brain barrier (BBB). *S. pneumoniae* interacts by the cholin binding protein A to PAF on endothelial cells (ECs). In addition to secreting multiple cytokines and other inflammatory components (TNFα, IL1 β, PAF, MMPs, TGF β1) which are able to increase trans-endothelial migration, *S. pneumoniae* secretes pneumolysin, a hemolysin forming transmembrane pores in brain ECs and LPS inducing EC apoptosis.

1.5 Treatment

Since 1943, penicillin was the antibiotic of choice for the management of infection by *Streptococcus pneumoniae*. Austrian and Gold (1964) revealed that the fatality caused by pneumococcal pneumonia reduced from 80% in untreated patients to 17% amongst patients who given treatment with penicillin. The mortality rate of serum therapy recipients was intermediate at 45%. Penicillin continued to be the favorable drug against pneumococci during the 1960s and 1990s. Other agents such as first-generation cephalosporins, macrolides and tetracyclines were replaced for hypersensitive subjects. However, the rise of antibiotic-resistant pneumococcal strains has resulted in noticeable shifts away from penicillin to other antibacterial agents for the empirical therapy of community acquired pneumonia (CAP) and other pneumococcal invasive diseases (IPD) (Chiou, 2006). Therapeutic recommendations for the treatment of CAP and others IPD are available from several organizations. These including the guidelines provided by

European Respiratory Society (ERS) originally published in 2005 and updated in 2011 (Woodhead *et al.*, 2011), the Infectious Diseases Society of America in collaboration with the American Thoracic Society (IDSA/ATS) published in 2007 and updated in 2016 (Kalil *et al.*, 2016), and the British Thoracic Society (BTS) in 2009 (Lim *et al.*, 2009). According to their website, the guidelines have been reviewed BTS in 2014 and are still valid (https://www.brit-thoracic.org.uk/standards-of-care/guidelines/). The key aims of effective therapy for CAP and other invasive diseases are the rapid killing of the infectious pathogen and decreasing associated morbidity and mortality. Therefore, choosing the initial appropriate antimicrobial drug is critical (Jones *et al*., 2010).

High dose of β-lactams (penicillins, or second or third generation cephalosporins) can be used to treat non-meningeal pneumococcal strains with intermediate or high levels of resistance to penicillin. Meningitis caused by pneumococcal strains with intermediate-level of resistance requires the use of other antibacterial agents to assure an effective outcome (van der Poll & Opal, 2009). The initial therapy in most patients with community acquired pneumonia remains empirical as many sputum cultures collected from patients with CAP do not grow any bacteria, especially if antibacterial treatment has already been directed. For that reason, the choice of antimicrobial agent to be used for treatment should be directed by estimation of the most likely bacteria, understanding of local susceptibility patterns and utilization of treatment recommendations (Jones *et al*., 2010). Also, infection severity, the presence of co-morbid factors, and differences in routes of administration available to inpatients versus outpatients should be taken into consideration (Jones *et al*., 2010). Compared to monotherapy in patients with bacteraemic pneumococcal pneumonia, several studies propose that combination therapy could be associated with lower fatality rates (Weiss *et al.*, 2004). Combination therapy could be used to treat some unidentified co-pathogens not covered by β-lactams (e.g., *Chlamydophila* spp, *Mycoplasma pneumoniae* or other untypical pathogens) (Baddour *et al.*, 2004; Martínez *et al.*, 2003).

In a previous study, researchers stated that using combination of inhibitors of bacterial protein synthesis (azithromycin or clindamycin) with ampicillin decreased lung inflammation and enhanced the outcomes for patients with post-influenza pneumococcal pneumonia (Karlström *et al.*, 2009).

Respiratory fluoroquinolones (moxifloxacin, gemifloxacin and levofloxacin) remain effectual for the management of infection caused by pneumococci, although monotherapy with these antibacterial drugs has not been established for severe CAP (Jones *et al*., 2010). These antimicrobials are easy to use and have a wide range of antibacterial activity against pathogens responsible for typical and atypical pneumonia. So, the importance of maintaining the efficiency of fluoroquinolones for treatment of multidrug-resistant (MDR) *S. pneumoniae* infections cannot be overemphasized. Increased use of these agents should be associated with surveillance efforts to monitor resistance expansion. Even though levofloxacin-nonsusceptible *S. pneumoniae* strains are still comparatively rare, the increased number of pneumococcal fluoroquinolone-resistant isolates has been reported previously (Deshpande *et al*., 2006; Jones *et al*., 2010). Table 1.1 displays the current recommended therapy routines and comparisons between the British, European, and American recommendations for pneumococcal infections.

Table 1.1: Treatment recommendations for patients with invasive pneumococcal diseases (IPD). Recommendations based on guidelines from the British Thoracic Society (BTS) 2009, Infectious Diseases Society of America/American Thoracic Society (IDSA/ATS) 2007, and European Respiratory Society (ERS) 2011. PCN=penicillin. S=susceptible. I=intermediate. R=resistant. TMP SMX=trimethoprim-sulfamethoxazole. Fluoroquinolone=respiratory fluoroquinolones (moxifloxacin or levofloxacin) (source: van der Poll & Opal, 2009).

Community-acquired pneumonia with PCN-S or PCN-I *S pneumoniae*	BTS: 500 mg ampicillin every 6 h or benzylpenicillin 1–2 g every 6 h; ATS; penicillin G 6–10 million units per day; ERS: penicillin, or second or third generation cephalosporin, with or without macrolide	Second or third generation cephalosporin; if β-lactam allergy give clarithromycin, azithromycin, or fluoroquinolone
Community-acquired pneumonia with PCN-R *S pneumoniae*	BTS: high-dose penicillin or second or third generation cephalosporin or alternative therapy; ATS: high-dose second or third generation cephalosporin or fluoroquinolone; ERS: fluoroquinolone, vancomycin, or linezolid	Vancomycin, fluoroquinolones, linezolid, carbapenems if susceptible
Pneumococcal bacteremia with sepsis or meningitis, PCN-S/I *S pneumoniae*	High-dose penicillin or ampicillin, with or without macrolide or fluoroquinolone; add dexamethasone for pneumococcal meningitis	High-dose second or third generation cephalosporin; if β-lactam allergy give vancomycin, fluoroquinolones
Pneumococcal bacteremia with sepsis or meningitis, PCN-R *S pneumoniae*	High-dose third-generation cephalosporin with or without macrolide or fluoroquinolone; if meningitis add vancomycin with or without rifampin+dexamethasone	If β-lactam allergy give vancomycin with or without rifampin; consider TMP-SMX or chloramphenicol in meningitis if susceptible
Suspected pneumococcal infection or overwhelming post-splenectomy infection	Immediate treatment with oral amoxicillin and clavulanate, seek immediate attention	If β-lactam allergy, immediate treatment with oral clarithromycin, seek medical attention
Invasive pneumococcal disease and immunoglobulin deficiency	Standard antimicrobial therapy	Consider adding intravenous immunoglobulin 1–2g/kg with antimicrobial therapy

1.6 Antibiotic resistance in *S. pneumoniae*

In 1967, Hansman and his colleagues isolated the first clinically significant strain of resistant pneumococcus (MIC, 500ng/ml). Since the last 30 years, there has been an enormous increase in the incidence of pneumococcal strains with reduced sensitivity to penicillin and other antibacterial drugs (Charpentier & Tuomanen, 2000). The massive increase in the overuse of antibiotics internationally has strongly contributed to the development of multidrug-resistant *S. pneumoniae*. The epidemic of IPD occurred in South Africa (1977) which caused by multidrug-resistant isolates was the dramatic event in the epidemiology of multidrug-resistant *S. pneumoniae* (Tomasz, 1997). Unlike the first resistant isolate, which was still susceptible to erythromycin, tetracycline, chloramphenicol and sulfa drugs, the multidrug-resistant strains responsible for the outbreak in South Africa were shown to have significantly increased resistance, not only to penicillin but also to tetracycline, erythromycin, chloramphenicol, clindamycin, streptomycin and for some isolates, rifampin (Tomasz, 1997).

Emergence of resistant pneumococcal strains to various antibacterial drugs has been recognized internationally in surveillance studies, including the SENTRY Antimicrobial Surveillance Program. A total number of 2379 pneumococcal isolates were collected from patients in 60 geographically different medical centers in North America, Latin America, and Europe in 2003 (Johnson *et al*., 2006). Similarly, a subset of 592 multidrug-resistant pneumococcal strains defined as being non-susceptible to all five antimicrobial classes including penicillin, erythromycin, clindamycin, tetracycline and Trimethoprim-sulfamethoxazole were collected from 1999 to 2003. The prevalence of penicillin resistance (MIC ≥2mg/L) in pneumococci was 12.7% in Latin America, 14.7% in Europe and 15.9% in North America. In North America, the rate of MDR *S. pneumoniae* increased from 5.7% in 1999 to 6.3% in 2003 (Johnson *et al*., 2006). In the United States, a total of 18,911 *S. pneumoniae* isolates collected from 1998 to 2011

showed that 18.9% were resistant to amoxicillin/clavulanate, 14.8% were resistant to penicillin (MIC of ≥4μg/mL), and 11.7% to ceftriaxone (Jones *et al*., 2013). In South America particularly Brazil, 829 pneumococcal isolates collected from 1998 to 2004 revealed that there has been an increment in the resistance to penicillin from 2.9% to 11.0% during the seven years of surveillance (Castanheira *et al.*, 2006). Data collected from 20 European sentinel hospitals as part of the SENTRY Program showed that 21% of pneumococcal strains were intermediate to penicillin, while 7% of them were resistant. Among those isolates resistant to penicillin; 35% and 55% showed also resistance to clindamycin and erythromycin, respectively (Fluit *et al.*, 1999). Data collected from multi-country studies of antibiotic resistance in *S. pneumoniae* indicated that the levels of antibacterial resistance were very high in Asian countries (Kang & Song, 2013). Pneumococcal strains isolated from China, Hong Kong, South Korea, Taiwan, Thailand, and Vietnam revealed the highest rates of resistance to antibiotics rates among all Asian countries (Hung *et al.*, 2013). In a recent study done in China, 91 pneumococcal clinical isolates were collected from patients of diverse ages from 2009 to 2010. The data indicated that 10%, 30%, 70%, 25%, and 40% of the isolates were resistant to penicillin, amoxicillin, cefuroxime, ceftriaxone, and meropenem, respectively and 96% of the isolates exhibited resistance to both erythromycin and clindamycin. The majority of those isolates also showed resistance to tetracycline (84%) and co-trimoxazole (74%) (Zhang *et al*., 2012). In Malaysia, previous surveillance studies have showed that the resistance rate to penicillin among pneumococcal isolate is ranging from 9 to 39%. Furthermore, the incidence of pneumococcal resistance against erythromycin has increased from 3% during 1996-1997 to 37% during 1998-2001 *(Lin et al*., 2010).

1.6.1 β-lactam resistance

β-lactam antibacterial agents are the most extensively used and efficacious of all antibiotics (Hakenbeck *et al.*, 1999). The mechanism of action of these compounds is based on targeting the cell wall synthesizing enzymes (penicillin-binding proteins, PBPs). Binding to these enzymes interferes with the biosynthesis and remodeling of the bacterial peptidoglycan, leading to growth inhibition or cell lysis (Charpentier & Tuomanen, 2000). Penicillin-binding proteins (PBPs) possess a significant role in catalyzing several reactions involved the process of peptidoglycan synthesis. They catalyze the polymerization of the glycan strand (transglycosylation) and the cross-linking between glycan chains (transpeptidation) of the bacterial cell wall. Some of these enzymes can hydrolyze the last D-alanine of stem pentapeptides (DD-carboxypeptidation) or hydrolyze the peptide bond linking two glycan strands together (endopeptidation) (Sauvage *et al.*, 2008). The penicillin-binding domains (PB domains) of PBPs are transpeptidases or carboxypeptidases participate in peptidoglycan metabolism. PB domains consist of three specific motifs: SXXK, (S/Y)XN, and (K/H)(S/T)G. these motifs define the active-site serine penicillin-recognizing enzymes family (for ASPRE) which also includes the class A and C b-lactamases (Zapun *et al.*, 2008). The serine of the SXXK motif is essential to the catalytic mechanism. This serine targets the carbonyl of the penultimate D-Ala amino acid of the stem peptide allowing the release of the last D-Ala amino acid from the 'donor' peptide to form a covalent acyl–enzyme complex. In transpeptidases, the carbonyl of the D-Ala amino acid forms an ester linkage with the active site serine; this complex will undergo an attack from a primary amine linked in different ways to the third residue of a second 'acceptor' stem peptide. A peptide bridge is then formed between two stem peptides, creating a link between glycan strands (Zapun *et al.*, 2008).

Bacterial pathogens have developed several mechanisms to resist the action of antibiotics. Bacterial resistance toward β-lactams involved two major strategies: the

synthesis of β-lactamases that inhibit the antibiotic before it reaches the penicillin-binding proteins (PBPs) and modifying PBPs to reduce their attraction to the antibiotic (Grebe *et al.*, 1997). PBPs have been classified into two different sets based on their molecular mass, the high molecular mass (HMM) PBPs and the low molecular mass (LMM) PBPs. PBPs of high molecular mass are multimodular enzymes responsible for polymerizing the peptidoglycan and inserting it into the pre-existing cell wall. These high molecular mass proteins consist of cytoplasmic tail, a transmembrane anchor and two domains linked together by a β-rich linker. Based on the structure and the catalytic activity of their N-terminal domain, HMM PBPs can be further classified into either class A or class B PBPs (Sauvage *et al*., 2008). The C-terminal of both class A and B has a transpeptidase activity, catalyzing peptide cross-linking between two adjacent glycan chains. The N-terminal domain in class A PBPs controls their glycosyltransferase activity, catalyzing the elongation of uncross-linked glycan chains. In class B, the N-terminal domain is thought to be engaged in cell morphogenesis by interaction with other proteins involved in the cell cycle (Sauvage et al., 2008). LMM PBPs are frequently described as class C of PBPs and their role is to alter the peptidoglycan by making the peptide side chains unapproachable for crosslinking or by cleaving crosslinks. Deleting these PBPs will result in producing bacterial cells with random length shapes and diameters. For that reason, the majority of these PBPs are most probably significant for perfecting the peptidoglycan synthesis (Den Blaauwen *et al.*, 2008).

Six PBPs have been described in *S. pneumoniae*. Three belong to the class A high molecular mass PBPs (PBP1a, PBP1b and PBP2a) and two represent class B PBPs (PBP2x and PBP2b) (Hoskins *et al.*, 1999) and one low molecular mass PBP (PBP3) (Morlot *et al.*, 2005). In pneumococcus, the mechanism of β-lactam resistance is based on altering the sequence and structure of its PBPs to decrease their affinity for the antibiotic (Hakenbeck *et al.*, 1999). Mutations in the genes encoding PBP2x and PBP2b

allow low-level resistance toward β-lactams and required for high-level resistance mediated by alterations in other PBPs, such as PBP1a. In several clinical strains of *S. pneumoniae*, resistance to β-lactams arises from mutations in only these three proteins. Besides its main part in giving pneumococci high-level of resistance to penicillins, it appears that PBP2b play a part in reducing the affinity of pneumococci to cephalosporins due to the fact that these antibiotics do not interact with PBP2b (Grebe & Hakenbeck, 1996). In addition to PBP2x, PBP2b and PBP1a, PBP2a plays also a role in penicillin resistance. However, PBP2a's role seems to improve pre-existing resistance rather than conferring resistance alone as this protein has low affinity to penicillin and thus probably is not a principal target for β-lactams (Zhao *et al.*, 2000). The genes encoding PBPs in resistant isolates of *S. pneumoniae* are referred to as mosaic genes. The nucleotide sequences of mosaic PBP genes differ from non-mosaic PBP genes by up to 21%. The level of difference between those two groups of genes suggests that the mosaic blocks are non-pneumococcal in origin (Cornick & Bentley, 2012). The root of these blocks remains unknown; however, similar fragments of PBPs sequences have been identified in other commensal streptococci such as *Streptococcus oralis and Streptococcus mitis* suggesting that these strains have developed resistance through point mutations by exposure to β-lactam treatment for various diseases. As a result, segments of genes encoding resistant PBPs were swapped between related streptococcal species, including *S. pneumoniae* (Zapun *et al.*, 2008).

Beside low-affinity PBPs, two other mechanisms of resisting penicillin have been described *in vitro* in *S. pneumoniae*. Both mechanisms involve mutations in non-PBPs genes particularly ciaH and cpoA (Charpentier & Tuomanen, 2000). Mutated CiaH or CpoA increase resistance to β-lactam antibiotics when introduced to sensitive pneumococcal strains with non-altered PBP genes (Hakenbeck *et al.*, 1999). CpoA may participate in the biosynthesis of teichoic acid by shifting carbohydrate molecules to the

lipid intermediate. histidine kinase protein which encoded by the gene CiaH is also used by *S. pneumoniae* to resist β-lactams. The Cia system is believed to play a role in sensing and counteracting cell wall damage caused by treatment with β-lactams. It has been shown previously that Cia system is necessary for maintaining the stationary growth phase and preventing autolysis caused by many different conditions, proposing a key role for this system in ensuring cell wall integrity (Mascher *et al.*, 2006). To date, none have reported pneumococcal resistance to penicillin using β-lactamase (Charpentier & Tuomanen, 2000).

1.6.2 Fluoroquinolone resistance

Fluoroquinolones were first introduced in the 1980s for treating Gram-negative bacterial pathogens. The emergence of β-lactam resistance amongst pneumococcal clinical isolates urged the development of new fluoroquinolones (such as sparfloxacin, trovafloxacin and moxifloxacin) effective against gram-positive bacteria (Janoir *et al.*, 1996). The antibacterial activity of fluoroquinolones is principally based on targeting the type II topoisomerase A_2B_2 complex, also known as DNA gyrase (GyrA, GyrB), which catalyzes DNA supercoiling during replication. In addition, fluoroquinolones target the topoisomerase IV complex C_2E_2 (ParC, ParE) which is critical for chromosome separation. Fluoroquinolones prevent pneumococcal DNA synthesis by binding to the target sites of DNA gyrase and topoisomerase IV. The main target for moxifloxacin is DNA gyrase (subunit GyrA), while ciprofloxacin and levofloxacin basically target topoisomerase IV (subunit ParC) (Li *et al*., 2002).

Pneumococcal resistance to fluoroquinolone is based on mutating the fluoroquinolone binding site which involves alterations in the gyrase genes and/or the topoisomerase IV genes (Charpentier & Tuomanen, 2000; Piddock, 1999). Previous reports have shown that mutations modifying parC result in low-level resistance to

ciprofloxacin and subsequent gyrA alterations leading to greater levels of resistance (Janoir *et al.*, 1996). The alterations in parC involve switching of Ser-79 to Tyr/Phe, Asp-84 to His, Ser-80 toTyr, or Asp-83 to Gly. The alterations in gyrA involve substitutions of Ser-84 to Tyr/Phe or Glu-88 to Gln/Lys (Janoir *et al*., 1996; Jones *et al*., 2000; Piddock, 1999; Tankovic *et al*., 1996). Unlike ciprofloxacin, resistance to sparfloxacin results primarily from mutations in gyrA followed by additional mutations in parC. A mutation in gyrA leading to the substitution of Ser-83 to Tyr/Phe and mutations in parC resulting in switching of Ser-79 to Tyr and Asp-83 to Asn were identified in pneumococcal clinical isolates resistant to sparfloxacin (Jones *et al*., 2000; Pan & Fisher, 1997). Multiple mutations in gyrA and parC are required for conferring high-level resistance against clinafloxacin (Xiao *et al*., 1998). It has been described previously that one alteration in parE of *in vitro* mutants of *S. pneumoniae* leading to a single amino acid substitution of Asp-435 to Asn was responsible for low-level resistance to fluoroquinolones. However, higher levels of resistance require serial acquisitions of mutations in parE and gyrA (Perichon *et al.*, 1997).

1.6.3 Macrolide-lincosamide-streptogramin B (MLS$_B$) resistance

MLS$_B$ are microbiostatic antibiotics that share similar mode of action by targeting the ribosomal 50S subunit of *S. pneumoniae* (Edelstein, 2004). Two mechanisms of resistance are used by pneumococcal strains to avoid inhibition by MLS agents: modifying the target site or efflux of the antimicrobial agents from the bacterial cell (Cattoir *et al.*, 2007). The mechanism of altering the target site involves the acquisition of the gene erm which is responsible for encoding an S-adenosylmethionine-dependent methylase that methylates an adenine residue in the peptidyl transferase domain of the 23S rRNA. The expression of the erm gene leads to a modification in the ribosome, hence decreasing the affinity of MLS$_B$ agents for the 23S rRNA binding site (Cattoir *et al.*,

2007). The majority of pneumococcal isolates that encode erm(B) display the (MLS$_B$)

phenotype (macrolide, lincosamide, and streptogramin B resistance). In *S. pneumoniae*

the erm(B) gene is carried on a member of the Tn916 family of transposons (Tn3872,

Tn6002, Tn6003, and Tn1545). These transposons also carry the tet(M) gene conferring

tetracycline resistance. Therefore there is a high prevalence of tetracycline resistance

among pneumococci resistant to macrolides (Jacobs, 2004).

The second mechanism of MLS resistance is mediated by acquisition of mef genes

encoding an efflux pump that pumps out MLS antibiotics from inside the cell. Most of

mef positive pneumococcal strains exhibit the M resistance phenotype (macrolide-

resistant, lincosamide-susceptible). The M phenotype isolates exhibit moderate resistance

in comparison with the MLS$_B$ phenotype isolates (Charpentier & Tuomanen, 2000). Three

mef variants have been identified in *S. pneumoniae*: mef(A), mef(E), and a rare variant

mef(I) which has only been described in two Italian clinical isolates (Cochetti *et al.*,

2005). The incidence of mef(A) and mef(E) differs greatly according to the geographical

region, mef(A) dominates in Europe, while mef(E) dominates in the United States, Asia,

and South Africa (Daly *et al.*, 2004).

1.6.4 Chloramphenicol resistance

Chloramphenicol is a bacteriostatic agent that stops bacterial protein synthesis by

targeting the peptidyltransferase during translation (Charpentier & Tuomanen, 2000).

Resistance to chloramphenicol by *S. pneumoniae* is mediated by synthesis of the

chloramphenicol acetyltransferase (CAT) enzyme catalyzing the alteration of

chloramphenicol to derivatives. These derivatives lack the ability to bind to the ribosomal

50S subunit and thus are no longer capable of neutralizing the peptidyltransferase (Dang-

Van *et al.*, 1978; Widdowson *et al.*, 2000). A 65.5-kb conjugative transposon Tn5253 has

been identified in pneumococcal clinical isolates, which carries the CAT gene responsible

for chloramphenicol acetyltransferase synthesis (Vijayakumar *et al.*, 1986). Tn5253 is a composite transposon that consists of the tetracycline resistance transposon Tn5251 and Tn5252, a 47-kb transposon carrying the chloramphenicol resistance factor (Ayoubi *et al.*, 1991). In 1970, the first clinical strain showed resistance to chloramphenicol was reported in Poland, however, since that date resistance to chloramphenicol has not become a critical issue worldwide. In Spain, 30–50% of clinical pneumococcal strains have been described to have resistance against chloramphenicol, but fewer than 5% of pneumococci collected from other countries exhibited resistance to chloramphenicol (Charpentier & Tuomanen, 2000).

1.6.5 Tetracycline resistance

Tetracycline antibiotics avert bacterial protein synthesis by attaching to either the acceptor site (A-site) or the peptidyl-donor site (P-site) of the 30S subunit of the bacterial ribosome, hence inhibiting the attachment of the aminoacyl-tRNA to the A-site (Charpentier & Tuomanen, 2000; Chopra & Roberts, 2001). Resistance to tetracycline in *S. pneumoniae* occurs through ribosomal protection mediated by the tet genes namely tet(M) and tet(O). These genes are often located on conjugative transposons or associated with small plasmids and therefore easily transmitted between bacteria (Widdowson *et al.*, 1996). These two genes code for membrane-associated proteins that protect ribosomes within the cells from the action of tetracyclines by pumping them out of the pneumococcal cell and thus reducing the intracellular drug concentration (Chopra & Roberts, 2001; Taylor & Chau, 1996).

The widespread use of tetracyclines has caused resistance development in pneumococcal infections. In February 1963, the first clinical strain exhibited resistance to tetracycline was isolated from the CSF of a 10-month-old child in New South Wales, Australia (Klugman, 1990). In the same year in the United Kingdom, pneumococcal

isolates resistant to tetracycline were isolated from the sputa of four elderly patients with chronic bronchitis (Klugman, 1990). Since that date, reports on pneumococci isolates resistant to tetracycline have been described in the literature. In the United States, pneumococcal resistance to tetracycline has been increasing from 7.6% in 1994-1995 to 17% in 2000 (Starr *et al.*, 2008).

1.6.6 Trimethoprim-sulfamethoxazole resistance

Due to their attractive cost and efficacy, combination of Trimethoprim-sulfamethoxazole (TMP-SMX) has been used broadly to treat lower respiratory tract infections in developing countries (Maskell *et al.*, 1997). Trimethoprim and sulfamethoxazole act by interfering with the folic acid biosynthesis. Trimethoprim stops bacterial growth by inhibiting dihydrofolate reductase (DHFR) hence preventing the reduction of dihydrofolate to tetrahydrofolate. Sulfamethoxazole interferes with paraaminobenzoate for dihydropteroate synthetase (DHPS), averting the production of 7,8-dihydropteroate and so inhibiting DNA synthesis (Charpentier & Tuomanen, 2000; Maskell *et al*., 1997).

Pneumococcal resistance toward Trimethoprim results from a single amino acid alteration (Ile- 100 to Leu) in the chromosomal-encoded DHFR. This amino acid substitution would possibly interrupt the hydrogen bonding of the DHFR to the 4-amino group of trimethoprim thus changing the DHFR function (Adrian & Klugman, 1997). Resistance to Sulfonamide antibiotic in *S. pneumoniae* is based on duplication of either three or six base pairs within sulA, a chromosomal gene coding dihydropteroate synthase (Maskell *et al*., 1997). In 1972, the first pneumococcal isolate showed resistance to a combination of these to antibiotics was isolated from a patient with chronic bronchitis (Charpentier & Tuomanen, 2000). In the United States, pneumococcal resistance to Trimethoprim-sulfamethoxazole has increased between 1990 and 2000 from 9.1% to

35.9% (Doern *et al.*, 2001). In a study conducted Taiwan, 200 pneumococcal isolates were collected in a period of two years and the results revealed that 87% of the clinical isolates were resistant to trimethoprim-sulfamethoxazole (Hsueh *et al.*, 1999).

1.6.7 Glycopeptides resistance

Glycopeptides such as vancomycin and teicoplanin act against a wide-range of Gram-positive bacteria by inhibiting both the transglycosylation and transpeptidation reactions that mediate the latter steps in cell wall synthesis (Finch & Eliopoulos, 2005; Yim *et al.*, 2014). These glycopeptide antibiotics is considered to be the last line of defense against infections caused by penicillin-resistant strains. Up to now, no pneumococcal resistance to glycopeptides has been recorded. However, there is a great concern that the vancomycin-resistance genes found in enterococci might be transmitted to pneumococci. These enterococcal genes encode enzymes for the synthesis of altered cell wall precursors with reduced affinity to vancomycin and eradication of the high-affinity precursors usually produced by the host (Courvalin, 2005). These resistant genes could give great levels of vancomycin-resistance and are carried by infectious elements (Arthur *et al.*, 1996).

The market for antibiotic drugs is massive. In 2009, global sales of antibiotics totaled US$42 billion, and 5% of the international pharmaceutical market. However, unlike antiviral drugs and vaccines, the antibiotics market is growing. It showed an average yearly expansion of 4% over the past five years, in comparision to a growth of 16.7% and of 16.4% for antivirals and vaccines, respectively. The most widely consumed antibitoics are penicillins and cephalocporins. Macrolide antibiotics are also vastly used for treatment of common infections specially those cuased by respiratory and uriny tract pathogens (Hamad, 2010). However, in the past ten years, the rate of antibiotics usage has been declined significantly in a number of countries. Moreover, only two completely

new antibiotics the oxazolidinone and daptomycin have been introduced to the market over the past three decades. One of the reasons for this decline in antibiotics consumption and development is the problem of antibiotic resistance. In several countries, it is recommended to identifiy the causative pathogen with labrotory tests before using antibiotics (Hamad, 2010). In addition, economic factors have had an vital role, the cost and complexities of drug research and development (R&D) have moved the investment interest away from the developing drugs targeting short-term presicrptions for acute diseases towards long-term therapies of chronic conditions (Projan, 2003). Even with success in antimicrobial development, nearly every antibacterial drug on the market today is subject to development of resistance to its efficacy by bacterial transformation (Hamad, 2010). As a result, there is an serious need to identify and develop a new class of anti-infectives that can overwhelm the emergence of bacterial resistance to antibiotics following extensive clinical, veterinary, and animal agriculture usage (Gordon *et al.*, 2005; Shea, 2003).

In the 20th century, antibiotic drug discovery progressed with natural compounds with small molecular size. However, in the latter part of the 20th century, new classes of drugs that were of a very different size regime to small molecule therapeutics began to appear (Craik *et al.*, 2013). These new classes are protein-based drugs (referred to as biologics) and involve molecules such as growth factors, insulin and engineered antibodies. An important reason which brings more attraction to those biologics is their higher target specificity, which is mostly driven by their larger size compared to small molecule drugs. The higher specificity of the protein-based drugs leads to the potential for fewer (off-target) side-effects, a phenomenon that is probably the main disadvantage of small molecule drugs. Of these biologics, peptides offer a promising alternative class of antibacterial agents that have the specificity and effectiveness of larger biologics, but at the same time are smaller in size and more attainable and can be manufactured using

low-cost chemical methods (Craik *et al*., 2013). In addition to their advantages over small molecules, peptides have some advantages over other biologics such as proteins and antibodies. Owing to their smaller size, peptides have the ability to penetrate deep into tissues. Peptides have higher activity per unit mass than antibodies and proteins. Also, therapeutic peptides, including synthetic ones, are normally less immunogenic than recombinant proteins and antibodies (Vlieghe *et al.*, 2010). Thus, in several ways, these peptides combine some of the advantages of small molecules with those of proteins. Considering their fundamental properties and attractive pharmacological profile, peptides represent an excellent starting point to design advanced therapeutic agents and their specificity can be converted into excellent safety, tolerability, and efficiency profiles in humans (Fosgerau & Hoffmann, 2015).

1.7 Vaccination

Spread and extensive virulence of *S. pneumoniae*, as well as the massive resistance to antibacterial treatment, has stimulated an interest in developing vaccines against pneumococci. Currently, two types of vaccines are available to prevent infections caused by pneumococci, the pneumococcal polysaccharide vaccine (PPV) and the pneumococcal conjugate vaccine (PCV). Both of these vaccines are designed to produce antibodies against pneumococcal capsular polysaccharide (CPS) (Wang & Curtiss, 2014). The PPV is a 23-valent vaccine which contains 23 purified capsular polysaccharides of commonly encountered serotypes (PPV23) and is recommended for the elderly (>65 years) and children ≥2 years. There are several limitations associated with this vaccine, purified capsular vaccines could cause hyporesponsiveness that reduces the immune response to following doses as a result of the initial dose. Also, the capsular polysaccharide is a T-cell-independent immunogen. Vaccination with capsular polysaccharide does not lead to isotype switching and generation of memory B-cell

responses which just produces temporary protection (Wang & Curtiss, 2014). It has been noted that PPV23 limited efficacy in infants and children below two years (a group that is highly susceptible to infection) is due to the poor immunogenicity of polysaccharide in children. Thus, this vaccine is not recommended for children under the age of 2 years (Feldman & Anderson, 2014). Several meta-analyses indicated that PPV23 is effective in preventing IPD in low-risk adults (particularly healthy adults), but not in high-risk groups (Huss *et al.*, 2009; Sudarsanam & Tharyan, 2014).

The immunological limitations of PPV23 led to the development of the pneumococcal conjugate vaccines (PCVs) (Hung *et al.*, 2013). The pneumococcal conjugate vaccine has been used for almost a decade and the outcomes were very positive. PCVs consist of pneumococcal capsular polysaccharides conjugated to different protein carriers. These vaccines were capable of inducing long-term protection, immunological memory and herd immunity in both infants and children. The introduction of PVCs has significantly reduced the rate of IPD and nasopharyngeal carriage by vaccine serotypes in children. In 2000, the conjugate vaccine PCV7 was licensed for use primarily in children below five years old, and for children younger than five years with high-risk conditions. PCV7 targets the pneumococcal serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F. These seven serotypes are accountable for 80% of invasive pneumococcal diseases in children in developed countries (van der Poll & Opal, 2009). PCV7 has decreased the rate of pneumococcal infection in children below one year in the USA by 82% (Centers for Disease Control and Prevention, 2008; Pilishvili *et al*., 2010). In February 2010, PCV7 was replaced by PCV13 to be used to vaccinate children in the United States. In December 2011, the Food and Drug Administration (FDA) licensed PCV13 for use as a single dose alone in adults more than 50 years old for the prevention of pneumococcal pneumonia and other pneumococcal infections (Feldman & Anderson, 2014). In June 2012, the Advisory Committee on Immunization Practices (ACIP) advised routine use of this

vaccine for adults older than19 years with immunocompromising conditions, functional or anatomic asplenia, cerebrospinal fluid (CSF) leaks and cochlear implants. In addition, ACIP specified that PCV13 should be given followed by PPV23 (Centers for Disease Control and Prevention, 2012). In East Asia and Southeast Asia, PCV7 and other PCVs has been gradually introduced. The effectiveness of PCV in these regions have not been reported but estimated based on the predicted vaccine serotype coverage (Tai, 2016). Several studies have recommended the use of PCV13 over PPV23 for adults. It has been documented that PCV13 induces a greater functional immune response than PPV23 for most serotypes covered by PCV13, in vaccine-naive adults aged between 60 and 64 years (Jackson *et al*., 2013). Besides, in adults aged 50 to 64 years, initial vaccination with PCV13 seemed to result in an immune state that results in recall antibody responses upon the following vaccination with either conjugate or free polysaccharide vaccines. On the other hand, initial vaccination with PPV23 results in an immune state whereby vaccination led to lower responses (Jackson *et al*., 2013).

Regardless of the efficiency of the PCVs, alarming patterns are emerging in the epidemiology of pneumococcal infection that threatens the long-term benefits of this vaccine. Pneumococcal infections caused by non-vaccine serotypes of pneumococci has significantly increased. The efficacy of a vaccine may be impacted by the pathogen's ability to switch the serotype of its capsule. Capsular switching possibly arises by transformation and recombination of capsular genes from one pneumococcal serotype to another. Vaccine-to-nonvaccine serotype switching could arm a naturally virulent pneumococcal strain previously encapsulated with a vaccine serotype with a new antigenic polysaccharide capsule to escape vaccine-induced immune recognition (Tan, 2012).

1.8 Antimicrobial peptides (AMPs)

Antimicrobial peptides (AMPs) are small molecules produced by almost all living things from bacteria to human beings as the first line of defense in their innate immune system (Brogden & Brogden, 2011). In addition to their ability to kill microorganisms directly (endogenous antibiotics), AMPs possess several additional features. AMPs can prevent biofilm formation and prompt the degradation of existing biofilms. Many AMPs are chemotactic, being able to attract phagocytes and mediate non-opsonic phagocytosis. Moreover, several AMPs participate in epithelialization, regulation of cell proliferation, angiogenesis, wound healing or adaptive immunity (Beisswenger & Bals, 2005).

Generally speaking, AMPs share common features. They are amphipathic, about 12 to 50 amino acid residues in length, have net positive charge ranging from +2 to +9 due to the excess content of arginine and lysine residues over acidic residues and about 50% or more of the amino acid residues are hydrophobic (Hancock, 2001). AMPs possess several advantages over conventional antibiotics. They have a wide-range of activity against viruses, parasites, fungi, Gram-positive bacteria and Gram-negative bacteria (Lin *et al*., 2013). AMPs can interact with their *in vivo* target with a high level of selectivity, resulting in high degree of potency and relatively few side effects (Craiket *et al*., 2013). Additionally, AMPs have less tendency to be resist by microbes. Several ways can be utilized by bacteria to resist conventional drugs including suppression of the drug-target interaction, efflux of the drug from target cells and alteration of the drug-binding site in target proteins. However, AMPs can avoid bacterial resistance due to their unique mode of action. With their amphipathic nature, most AMPs act directly on the microbial membrane. Due to their positive charge, AMPs can interact selectively with the negatively charged surfaces of microbial membranes. Once the AMPs are accumulated on the membrane surface, their hydrophobic composition interacts with the hydrophobic

components of the membrane causing membranolytic effects. This distinctive mechanism enables AMPs to avoid microbial resistance (Seo *et al.*, 2012).

1.8.1 Structure of antimicrobial peptides

Thus far, more than 7000 naturally occurring peptides have been discovered with several activities including antimicrobial peptides, anticancer peptides, insecticidal peptides, chemotactic peptides, antioxidant peptides and protease-inhibitor peptides (Fosgerau & Hoffmann, 2015; Wang *et al*., 2016). Based on their structure, AMPs are classified into four distinct groups: α-helical peptides, β-sheet peptides, extended peptides, and loop peptides. The class of α-helical peptides is categorized by their α-helical conformation, and frequently comprise a slight bend in the center of the molecule. α-helical peptides such as cecropins and magainins are considered to be the most well-established AMPs in structure-activity relationships. This class of AMPs is generally formless in aqueous solution and forms amphipathic helices in membranes or membrane-mimicking environments (Seo *et al*., 2012). The α-helical cecropins are representative of this structural group. Cecropins were first discovered in the hemolymph of the giant silk moth, *Hyalophora cecropia*. All cecropins have the tendency to form an amphipathic helix in certain organic cosolvents such as trifluoroethanol (Reddy *et al.*, 2004). Previous studies revealed that cecropin A (37 residues) displayed potent antimicrobial activities against bacteria (Andreu *et al.*, 1992). Magainins are another group of AMPs with α-helical conformation. Magainin 1 and 2 peptides were isolated for the first time from the skin of the African clawed frog, *Xenopus laevis*. Like cecropins, NMR studies showed that these peptides adopt amphipathic α -helical structures in the presence of dodecylphosphocholine (DPC) and sodium dodecylsulfate (SDS) micelles (Powers & Hancock, 2003). Magainins possess antimicrobial activities against a wide – spectrum of pathogenic microorgansims including Gram-positive and Gram-negative bacteria, fungi,

and protozoa and even cancer cells. It's widely accepted that these peptides act basically on lipids of the membrane as the main target. Magainins permeabilize the phospholipids of bacterial membranes forming spanning pores which eventually lead to membrane disruption (Matsuzaki *et al.*, 1999).

β-Sheet peptides such as α-defensins, β-defensins and tachyplesins are a group of peptides classified by the presence of an antiparallel β-Sheet usually stabilized by disulfide bridges (Nguyen *et al.*, 2011). Tachyplesins were obtained from the blood cells of the Japanese horseshoe crab, *Tachypleus tridentatus*. Tachyplesin I is a β-Sheet peptide consists of 16-18 amino acid residues forming antiparallel β-sheet stabilized by two disulfide bonds (residues 3 and 16 and residues 7 and 12). Tachyplesin peptides exhibit antimicrobial activity against fungi, bacteria and certain viruses, including extracellular HIV-1 (Powers & Hancock, 2003). α- and β-defensins are peptides that belong to the class of β-Sheet AMPs. α-defensins consist of 29-35 residues including six cysteines linked to each other forming three disulfide bridges in a 1-6, 2-4, 3-5 pattern. In humans, six defensin peptides have been isolated. Four α-defensins (HNP 1–4) have been found in the cytoplasmic granules of neutrophils, while the other two peptides (HD 5-6) were isolated from Paneth's cells of the small intestine (Beisswenger & Bals, 2005). β-defensins are 36-42 amino acids in length with six cysteines that form three disulfide bonds in a 1-5, 2-4, 3-6 alignment. Human β-defensin 1 (HBD-1) is expressed in epithelial cells of the urinary and respiratory tract. HBD-2 is produced by epithelia of the inner or outer surfaces of the human body like the skin and the respiratory and gastrointestinal tract. α- and β-defensins possess antimicrobial activity against many bacteria, yeast and some enveloped viruses (Beisswenger & Bals, 2005; Pinheiro da Silva & Machado, 2012).

Extended AMPs such as tritrpticin and indolicidin are largely rich in certain amino acids specifically arginine, tryptophan, proline and histidine. Due to their high content of proline and/or glycine, this group of AMPs does not have regular secondary structure. Several peptides of this group are not membrane active. Instead, they penetrate across the membrane and exert their antimicrobial activity by interacting with intracellular components (Nguyen *et al*., 2011). Tritrpticin peptide belongs to the cathelicidin group of peptides and consists of thirteen amino acids with a high proportion of arginine and tryptophan. This peptide is isolated from the porcine leucocytes, can cause membrane leakage and exhibits a broad – range of activity against several kinds of bacteria (Schibli *et al.*, 2006). Indolicidin is another thirteen amino acid peptide rich in arginine and tryptophan. Isolated from the cytoplasmic granules of bovine neutrophils, indolicidin contains five tryptophans making it the peptide with the highest proportion tryptophan residues. Indolicidin displays antimicrobial activities against several microbial organisms including bacteria, viruses (HSV and HIV), fungi, and parasites (Hancock, 2001).

Loop peptides such as thanatin and bactenecin are classified by having a loop structure imparted by the presence of a single bond (Powers & Hancock, 2003). Thanatin is a loop peptide with 21 residues in length. This peptide is isolated from the spined soldier bug, *Podisus maculiventris*, possesses antimicrobial activity against Gram-positive bacteria and fungi (Fehlbaum *et al.*, 1996). Bactenecin is a 12 amino acid looped antimicrobial peptide with two cysteine molecules forming an intracellular disulfide bridge. Bactenecin was originally isolated from the granules of bovine neutrophils and displayed antibacterial activities against both Gram-positive and Gram-negative bacteria (Wu & Hancock, 1999). Figure 1.8 illustrates the four different structural classes of antimicrobial peptides.
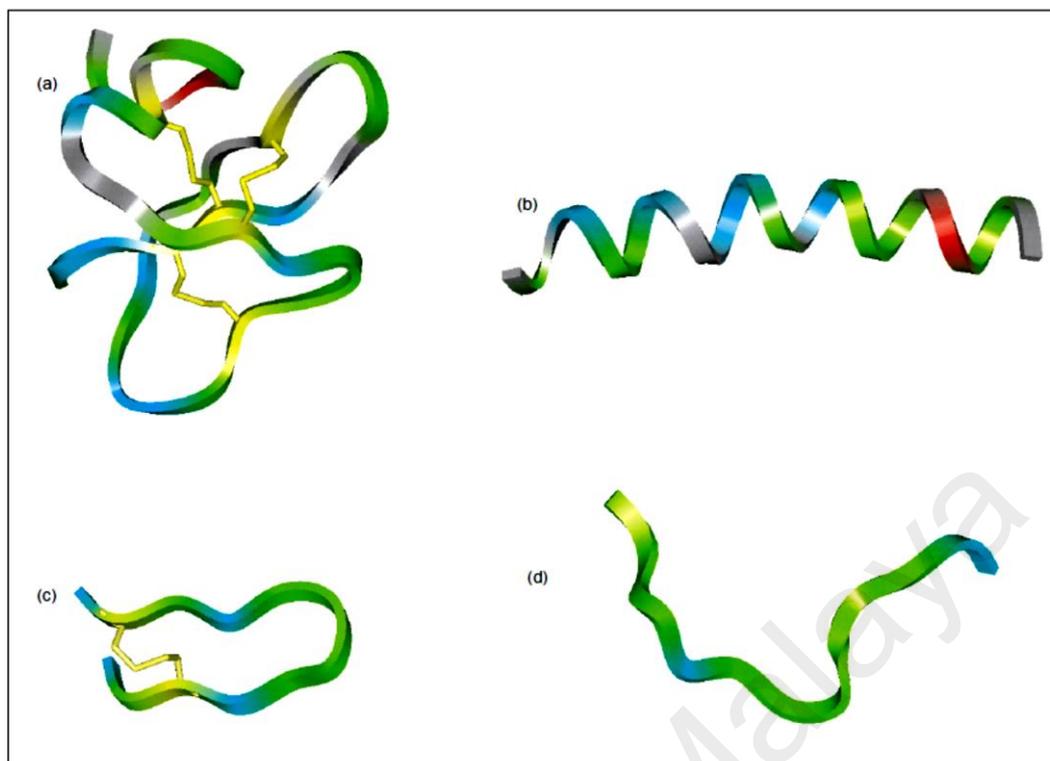
Figure 1.8: Molecular models of the four structural classes of antimicrobial peptides (taken from the NMR structural database). (a) HBD-2 represents a β-sheet structure, (b) magainin 2 represents an α-helical structure), (c) bovine bactenecin represents a loop structure, and (d) bovine indolicidin represents an extended structure (source: Hancock, 2001).

1.8.2 Mechanisms of action of AMPs

1.8.2.1 Membrane-active peptides

The mode of action of cationic antimicrobial peptides have been studied extensively. The main target for AMPs is the microbial membrane. Whether they are acting on the membrane or not, antimicrobial peptides must interact with microbial membranes as the first step. This interaction will lead to membrane perturbation, disturbance of membrane-associated physiological events such as cell wall biosynthesis or cell division and/or translocation across the membrane to interact with intracellular components (Fjell *et al.*, 2012). Several models have been proposed to illustrate the mechanism of interaction between peptides and the membrane of target cells including the toroidal pore model, the barrel-stave model and the carpet-like mechanism (Bahar & Ren, 2013).

1.8.2.1.1 The toroidal-pore model

The toroidal-pore model hypothesizes that peptide and lipid together create well-defined pores, with the target membrane also bending inside to create a hole with the head groups facing towards the center of the pore while the peptides line this hole. This model predicts that the target membrane will exhibit a positive curvature as a result of the membrane curving around to form the toroidal hole with the peptides within (Jenssen *et al.*, 2006). This model explains the activity of peptides such as melittin, protegrins and magainins. Unlike the barrel-stave model, this model indicates that the peptides are always linked to the lipid head groups even when they are vertically introduced in the lipid bilayer (Brogden, 2005; Yang *et al.*, 2001) (Figure 1.9).
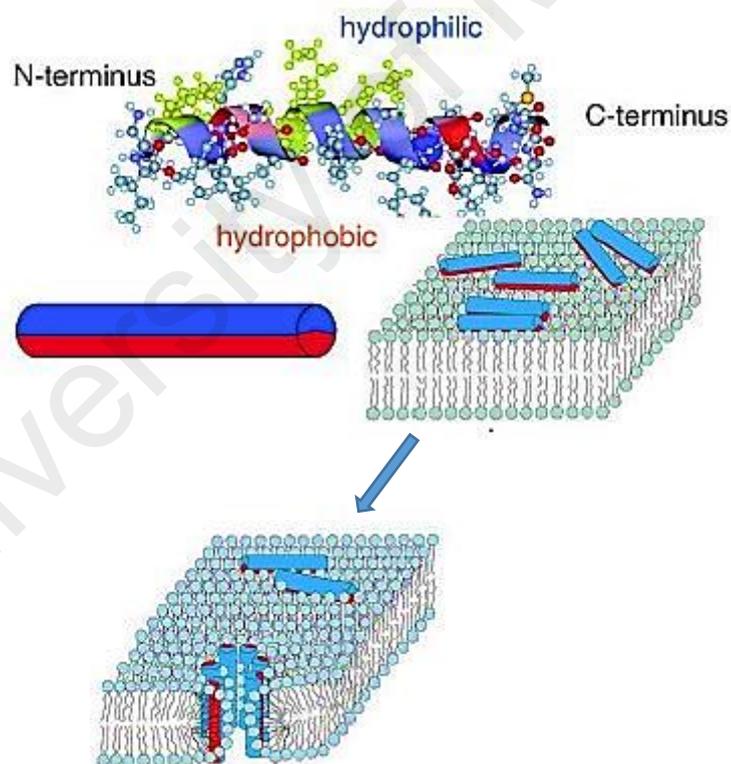
Figure 1.9: The toroidal-pore model. In this model, the attached peptides assemble and stimulate the lipid monolayers to bend constantly through the pore so that the water core is lined by both the inserted peptides and the lipid head groups (source: Salditt *et al.*, 2006)

1.8.2.1.2 The barrel-stave model

The barrel-stave model illustrates the creation of transmembrane channel/pores by AMPs via the interaction of their hydrophobic layers with the lipid part of the microbial membrane, and their hydrophilic layers point inside the membrane creating an aqueous pore. In this model, the peptide monomers attach to the membrane in an α-helical manner followed by localization of the helices into the hydrophobic core of the cell membrane. Once the helices insert themselves into the hydrophobic core, a continuous enrolment of further monomers increases the pore size leading to leakage of cytoplasmic contents and as a result death of the target cell (Reddy *et al*., 2004) (Figure 1.10). This type of transmembrane pore model explains the activity of AMPS such as alamethicin. The pores formed by this peptides can contain 3–11 parallel helical molecules, and the outer and inner diameters have been calculated as ~4.0 nm and ~1.8 nm, respectively (Spaar *et al.*, 2004)
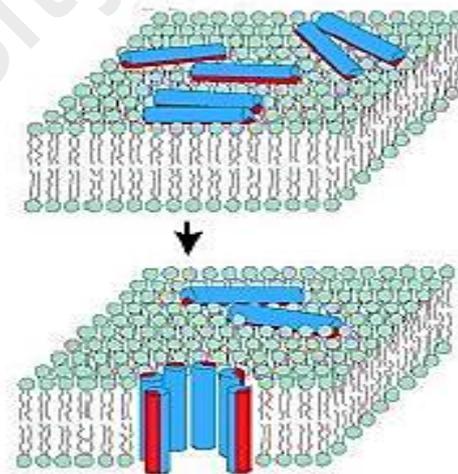
Figure 1.10: The barrel-stave model. Peptides insert themselves into the membrane perpendicularly. The hydrophobic core regions of the peptides align with the lipid core region of the bilayer and the hydrophilic peptide regions form the interior region of the pore (source: Salditt *et al.*, 2006).

1.8.2.1.3 The carpet-like model

        Unlike the other membrane permeabilization models, the carpet-like model suggests that aggregates of peptide align parallel to the lipid bilayer of the membrane and cover it in a carpet-like manner. In this model, peptide monomers preferentially bind to the phospholipid head groups. This step is followed by the orientation of the peptide monomers on the membrane surface allowing the hydrophilic residues to face the phospholipid head groups. Then the peptides rearrange themselves towards the hydrophobic core of the cell membrane. Finally, the peptides break down the membrane by disrupting the bilayer curvature (Shai & Oren, 2001) (Figure 1.11). This model elucidates the activity of AMPs such as ovispirin (Yamaguchi *et al.*, 2001).
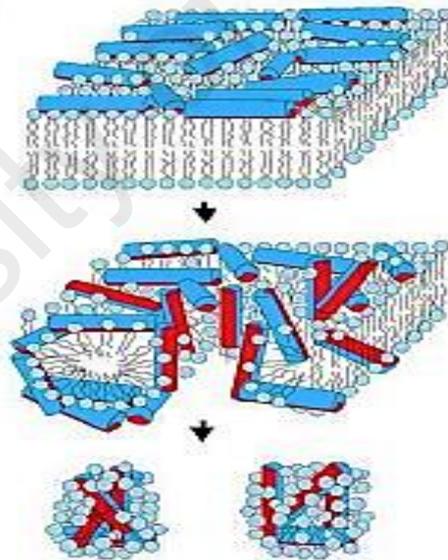
Figure 1.11: The carpet-like model. In this model, the peptides orientate parallel to the surface of the lipid bilayer and cover it in a carpet-like manner (source: Salditt *et al.*, 2006)**.**

1.8.2.2 Intracellular peptides

As mentioned above, majority of antimicrobial peptides acts directly on the lipid bilayer of the cell membrane. However, several AMPs exert their activity intracellularly by interacting with cytoplasmic components. Several reports have suggested that some peptides permeate through the cell membrane and accumulate inside the cell inhibiting a variety of intracellular processes including protein synthesis, enzymatic activity, nucleic acid synthesis and cell wall synthesis (Bahar & Ren, 2013; Brogden, 2005). Buforin II, a 21 amino acid frog-derived AMP translocate across the cell membrane without lysing it and inhibits cellular processes by binding to DNA and RNA of *E. coli* (Park, Kim, & Kim, 1998). α-helical peptides such as pleurocidin and dermaseptin are able to block nucleic acid and protein synthesis without injuring the *E. coli* plasma membrane (Patrzykat *et al.*, 2002). Proline-rich peptide PR-39 prevents protein synthesis and stimulates degradation of some proteins that are necessary for DNA replication (Brogden, 2005). On the other hand, other short proline-rich AMPs such as Pyrrhocoricin, drosocin, and apidaecin kill bacteria by binding specifically to DnaK, a 70-kDa bacterial heat-shock protein, and nonspecifically to GroEL, a 60-kDa bacterial chaperonin (Otvos *et al.*, 2000). It has been reported previously that histatin 5, a member of a group of low-molecular-weight salivary proteins exerts its bacterial activity by strongly inhibiting trypsin-like protease produced by *Bacteroides gingivalis* (Gusman *et al.*, 2001). Other peptides like the lantibiotic mersacidin can prevent peptidoglycan biosynthesis via interfering with membrane-associated transglycosylation. By combining with lipid II, mersacidin stops peptidoglycan precursors from forming the nascent polymeric peptidoglycan and thus inhibits cell wall synthesis (Brötz *et al.*, 1998). Antimicrobial peptides can also several mechanisms of action, for instance, disruption of the cell membrane together with inhibition of one or more intracellular components (Jenssen *et al*., 2006). Some antimicrobial peptides can activate non-membrane external targets such as autolysins and

phospholipases. In *Staphylococcus simulans*, the addition of Pep 5 and nisin stimulated the activity of the N-acetylmuramoyl-L-alanine amidase (an autolysin). The presence of teichoic and teichuronic acids of the cell wall inhibit the amidase, however, adding Pep 5 and nisin reversed the inhibition and reactivated the enzyme and this might explain the lysis of treated cells (Bierbaum & Sahl, 1987). Figure 1.12 displays the mode of action of intracellular peptides.
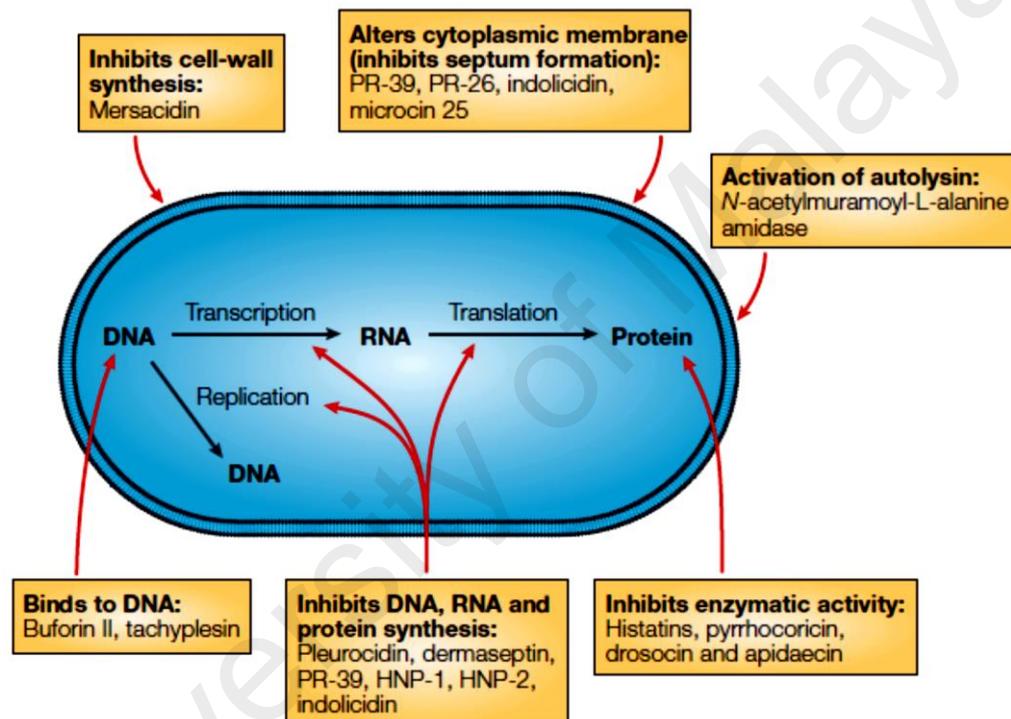
Figure 1.12: Mechanism of action of intracellular antimicrobial peptides (source: Brogden, 2005).

1.8.3 Antimicrobial peptides as potential therapeutic agents

Throughout the past ten years, peptides have involved in a variety of applications in medicine and biotechnology. For instance, the peptide-based drug Lupron developed by Abbott Laboratories for treating prostate cancer, has reached universal sales of more than US$2.3 billion in 2011. As well, Lantus another peptide-based medicine from Sanofi achieved sales of US$7.9 billion in 2013 (Kaspar & Reichert, 2013). In terms of research,

there are about 140 peptide drugs currently in clinical trials and more than 500 therapeutic peptides in preclinical development. Globally, the peptide-drug market has been expected to grow from US\$14.1 billion in 2011 to about US\$25.4 billion in 2018 (Fosgerau & Hoffmann, 2015).

Despite the efforts to develop antibiotics based on natural AMPs over the last two decades, no AMP has yet been approved by Food and Drug Administration (FDA) for therapeutic use (Fjell *et al.*, 2012; Midura-Nowaczek & Markowska, 2014). Even with their several considerable advantages over conventional antibiotics, AMPs have some boundaries for pharmaceutical development. Naturally occurring AMPs are vulnerable to proteolytic degradation and pH, the high cost of AMPs production and their potential toxicity when administered systemically. In general, Many peptides are less toxic to eukaryotes when tested *in vitro* and they are effective *in vivo* only at high doses. Furthermore, since peptides share features with eukaryotic nuclear localization signal peptides, they are able to translocate into the cells causing more subtle toxicities including apoptosis and mast-cell degranulation (Hancock & Sahl, 2006).

To address those obstacles, many techniques have been suggested. For instance, the high cost of manufacturing peptides can be reduced by synthesizing smaller AMPs. Few attempts have been successful in producing peptides such as plectasin by recombinant DNA techniques instead of solid-phase chemical synthesis (Hancock & Sahl, 2006; Zhang *et al*., 2011). The liability to proteolytic degradation can be addressed by using unusual amino acids (primarily D-form amino acids or non-natural amino acid analogues) or altering the terminal regions (acetylation or amidation) (John *et al.*, 2008; McPhee *et al.*, 2005). Additionally, the use of effective drug delivery systems like liposome encapsulation can improve stability and reduce potential toxicity. Peptides toxicology can be reduced by making a plethora of highly active peptide sequences and testing them for the absence of toxicity *in vivo.* Another way to reduce toxicity is by using

formulations that encapsulate the peptides, for instance, liposomal formulations (Fjell *et al.*, 2012; Samad, Sultana, & Aqil, 2007). Table 1.2 shows selective antimicorbial peptides in preclinical and clinical development.

Table 1.2: Selected AMPs in commercial development (source: Fjell *et al.*, 2012).

Peptide	Sequence	Company	Description	Application	Trial phase
Omiganan/ (MBI-226)	ILRWPW WPWRRK	Migenix/ BioWest therapeutics	Synthetic cationic peptide derived from indolicidin	Topical antiseptic, prevention of catheter infections	III
hLF1–11	GRRRRS VQWCA	AM-Pharma	Cationic peptide fragment comprising amino-terminal amino acids 1–11 of human lactoferricin	Bacteraemia and fungal infections in immuno-compromised haematopoetic stem cell transplant recipients	I/II
PAC-113	AKRHHG YKRKFH	Pacgen Biopharmaceuticals	Synthetic 12-mer peptide derived from histatin 3 and histatin 5	Oral candidiasis	IIb
CZEN-002	(CKPV)2	Zengen	Dimeric octamer derived from α-melanocyte-stimulating hormone	Vulvovaginal candidiasis; anti-inflammatory	IIb
HB1345	Decanoyl-KFKWPW	Helix BioMedix	Synthetic lipohexapeptide	Acne; broad-spectrum antibiotic	Pre-clinical
OP-145	IGKEFK RIVERIK RFLREL VRPLR	OctoPlus; Leiden University, The Netherlands	Synthetic 24-mer peptide derived from LL-37 for binding to lipopolysaccharides or lipoteichoic acid	Chronic bacterial middle ear infection	II (com-pleted
Plectasin	GFGC$_1$NG PWDEDD MQC$_2$HNH C$_3$KSIKGYK GGYC$_1$AKG GFVC$_2$KC$_3$Y)	Novozymes	Fungal defensin; candidate in development is an amino-acid substitiution variant	Bacterial diseases	Pre-clinical
Ghrelin	GSSFLSPE HQRVQQ RKESKKPP AKLQPR	University of Miyazaki, Japan; Papworth Hospital, Cambridge, UK	Endogenous host-defence peptide	Airway inflammation, chronic respiratory infection and cystic fibrosis	II
IMX942	KSRIVPA IPVSLL	Inimex	Synthetic cationic peptide derived from IDR1 and bactenecin	Nosocomial infection, febrile neutropenia	Ia

1.8.4 Bacterial resistance to AMPs

Microorganisms use several mechanisms to counter killing by antimicrobial peptides. These mechanisms can be divided into two different kinds of resistance: constitutive resistance and inducible resistance. Constitutive or passive mechanisms of resistance involve electrostatic shielding, alterations in membrane potential during different stages of cell growth and biofilm formation. The inducible or adaptive resistance mechanisms consists of alteration of extracellular structures, proteolytic enzymes activation, modification of intracellular targets and efflux pumps (Bahar & Ren, 2013; Yeaman & Yount, 2003). *Staphylococcus aureus* is able to express membrane with reduced negative charge. Through products of the dlt operon (dltA, dltB, dltC and dltD genes), *S. aureus* can transport D-alanine from the cytoplasm to the surface teichoic acid, which leads to a reduction in net negative charge and reduces affinity to AMPs (Peschel *et al.*, 1999). The activity of many AMPs is influenced by the target cell growth phase or transmembrane potential. *S. aureus* can resist some types of AMPs through constitutive reduction of its membrane potential. Altering membrane energetics will result in the ability to subvert peptide-induced membrane dysfunction and cell death (Lehrer & Ganz, 1996). Several virulent pathogens that colonize or infect the gastrointestinal mucosa, mammalian bloodstream, and respiratory tract use capsules or biofilms to avoid AMPs. For instance, virulent strains of *P. aeruginosa* can produce alginic acid (highly anionic capsular exopolysaccharide), which is able to seize AMPs present in mucosal secretions before they reach the pseudomonal membrane and thus prevent cell death (Friedrich *et al.*, 1999). *Klebsiella pneumoniae* with Capsule polysaccharide showed more resistance to HNP-1, HBD1, lactoferrin, protamine sulphate, and polymyxin B. on the other hand, *K. pneumoniae* strain 52K10; an acapsular mutant was more susceptible to peptides than the capsulated clinical strain (Campos *et al.*, 2004).

Many microbial pathogens have developed a range of inducible manoeuvres to defeat or subvert the effects of host defense mechanisms including AMPs. Some microbes can modify their extracellular structures to prevent initial binding of peptides to those structures. Gram-negative bacteria can reduce their sensitivity to AMPs by altering the moiety of the lipid A of the LPS or by decreasing the fluidity of the outer membrane by increasing the number of hydrophobic interactions between the increased number of Lipid A acyl tails (Brogden, 2005). Other pathogens resist the action of antimicrobial peptides by efflux mechanism. In *S. aureus*, the plasmid responsible for encoding qacA confer staphylococcal resistance to tPMP-1 using a proton motive force-dependent efflux pump (Kupferwasser *et al.*, 1999). Protease-mediated resistance is an inducible mechanism used by microorganisms to counter antimicrobial peptides. Proteases such as protease OmpT of *E. coli* and PgtE of *Salmonella* have been shown to confer resistance to AMPs. Likewise, the heat-shock serine protease DegP reduce the sensitivity of *E. coli* to lactoferricin B *in vitro* (Yeaman & Yount, 2003). Modifying intracellular targets is another complex mechanism by which microbes can resist antimicrobial peptides. Mutation in gyrB gene significantly reduce susceptibility of *E. coli* to microcin B17 (antimicrobial peptide believed to prevent DNA replication). In this mutation, tryptophan 751 replaced by arginine in the GyrB polypeptide leading to a reduction of microcin B17 targeted inhibition of DNA gyrase (del Castillo *et al.*, 2001).

In terms of *S. pneumoniae*, several tactics are utilized by this pathogen to avoid killing by AMPs. For instance, previous study has showed that *S. pneumoniae* uses it anionic capsule to bind both polymyxin B and the α-defensin HNP-1 before they can bind to the underlying cell structures. Furthermore, both polymyxin B and HNP-1 stimulate release of the capsule from *S. pneumoniae* without loss of cell viability. Thus, the anionic capsule of *S. pneumoniae* acts as a decoy attracting AMPs and preventing them from reaching the bacterial cell membrane (Llobet *et al.*, 2008). Decorating the outer surface

with autolysin (LytA), which confers highly charged choline is another strategy used by pneumococci to decrease attraction of AMPs (LaRock & Nizet, 2015). In addition, previous study has exhibited that *S. pneumoniae* encodes an efflux pump which confers resistance not only for macrolides, but also against the human cathelicidin LL-37 (Zähner *et al.*, 2010).

1.8.5 Designing novel synthetic AMPs

In order to design synthetic AMPs for therapeutic application, several objectives must be met. A designed peptide must be active against the pathogen of interest, cost effective, must maintain stability, and have low toxicity at the therapeutic dose. Fjell et al. (2012) pointed out three distinguished approaches to design novel synthetic AMPs including template-based studies, biophysical modeling, and virtual screening. In the template-based method, the amino acid sequence of the template peptide is modified by adding, removing, or substituting one or more amino acid residues to improve their activity and/or reducing their toxicity. Hybrid peptides constructed from the active segments of two or more naturally peptides is another approach to design novel AMPs based on template peptides. These chimeric peptides can be assembled by direct chemical synthesis or as fusion protein expressed using an appropriate recombinant technique (Brogden & Brogden, 2011). The template-based approach has been widely used by researchers as it involves limited computational input to improve the antimicrobial activity of natural AMPs.

Unlike template-based techniques, the biophysical studies attempt to understand the activity of a peptide and design enhanced analogs by examining peptide structures in hydrophobic environments or by modeling peptides at the atomic level. This kind of studies include methods such as molecular modeling based on free energy perturbation and molecular dynamics simulation of the interactions of peptides with membranes.

Molecular dynamics simulation has been successfully applied to increase the activity as well as decreasing the toxicity of naturally occurring peptides (Fjell *et al.*, 2012).

Virtual screening methods can be applied when peptide synthesis and testing are too costly, and biology-assisted approaches such as phage display cannot be applied. The advantage of these techniques compared to the computational simulation studies, is that they only need a few assumptions to assign peptide structure based on primary sequences. Instead of creating models with an immediate outcome, virtual screening studies use numerical methods to define quantifiable properties of peptides like charge and hydro-phobicity from the primary structure and physicochemical features of the peptides, and then relate these properties to biological activities of peptides using SAR models (Fjell *et al.*, 2012).

1.9 Justification and objectives of the study

Pneumococcal resistance to traditional antibiotics represents a major public health concern. The rates of pneumococcal resistance to various antibiotics are the highest in Asian countries. According to the Asian Network for Surveillance of Resistant Pathogens (ANSORP), more than 70% of pneumococcal isolates were fully resistant to erythromycin. Moreover, 59.3% of isolates from Asian countries were multidrug-resistant (MDR). Therefore, a new class of antibacterial agents is desperately needed. AMPs represent a promising next-generation antimicrobial candidates owing to their broad – range and rapid antimicrobial activity which can overwhelm the antibiotic-resistance issue. The market for peptides is steadily increasing, up to now, about 100 therapeutic peptides are on the market in the United States, Europe and Japan. With their advantages over small molecules, peptides represent a strong candidates to contribute to the expansion of the future pharmaceutical industry. The main objective of the current study was to design novel synthetic antimicrobial peptides with stronger and more rapid activity than currently available antibiotics. The therapeutic effectiveness of the newly designed peptides was evaluated using *in vivo* murine pneumococcal infection models. Accordingly, the study was designed with the following objectives:

1. To design novel antimicrobial peptides with potent antibacterial activity against *Streptococcus pneumoniae.*

    -   Template-based approach will be used to design novel peptides using to natural peptides indolicidin and ranalexin as templates.

2. To assess the *in vitro* antibacterial activity and cell toxicity of the newly designed peptides.

    -   Broth microdilution assay, time killing assay and synergism activity will be used for antibacterial assessment.

    -   Hemolytic and cytotoxicity assays will be used for toxicity assessment.

- Molecular docking will be used to investigate the affinity of the designed peptides toward three pneumococcal virulent factors: autolysin, pneumolysin and pneumococcal surface protein A (PspA).

3. To investigate the mechanisms of action of the synthetic antimicrobial peptides.

- Transmission and scanning electron microscopy and ATP release assay will be used to measure the damaging effect of peptides on bacterial membrane.

- DNA binding assay will be used to assess the effect of peptides on genomic DNA.

4. To evaluate the toxicity, antibacterial efficacy, and therapeutic synergism activity of these antimicrobial peptides *in vivo* using murine models.

- ICR male mice will be used to assess the toxicity and therapeutic efficacy of peptides *in vivo*.

CHAPTER 2: MATERIALS AND METHODS

2.1 Design of synthetic antimicrobial peptides

In the present study, the template-based method was used to design novel synthetic peptide analogs against *Streptococcus pneumoniae* based on modifying the sequences of two naturally occurring AMPs. Upon searching the Antimicrobial Peptide Database (APD) (http://aps.unmc.edu/AP/main.php) (Wang *et al*., 2016), several peptides have selected as potential template candidates for designing novel analogs. The selection was based on three main criteria: first, the peptides should not exceed 20 amino acids in length to minimize the cost of production as one of our goals is to design active peptides which are cost effective. The second criterion is that the template peptides should possess activity against Gram-positive bacteria as reported in previous literature. Finally, the third criterion is that the parent peptides must be positively charged, as the cationicity plays a crucial role in the initial electrostatic attraction of the positively charged peptides to the negatively charged bacterial membrane.

Two naturally occurring peptides indolicidin (Selsted et al., 1992) and ranalexin (Clark *et al*., 1994) were selected as template peptides. The sequences of both peptides were modified by substituting one or more amino acid residues in order to design two groups of novel analogs based on each parent peptide, taking into consideration two important parameters hydrophobicity and net charge (see section 3.1.1 for reason of choice). The third group of synthetic peptides was designed by fusion fragments from parent peptides to produce hybrid peptides with different length, charge, and hydrophobicity. The C-terminal for all peptides were modified by adding amide group (NH$_2$). All the newly designed AMPs were named accordingly, and the amino acid sequence of each peptide was searched online to determine the novelty of the AMPs.

2.1.1 AMPs physicochemical analytical tools

The physicochemical parameters including total net charge (NetC), total hydrophobicity ratio (HR), and the grand average hydropathy value (GRAVY) of each newly designed peptide were calculated using the ExPASy ProtParam tool (http://www.expasy.org/tools/protparam/) (Gasteiger *et al.*, 2005). The total net charge of each AMP was calculated by substracting the sum of positively charged amino acids (Arg, Lys, His) from the sum of negatively charged amino acids (Asp, Glu). The C-terminal amidation of each peptide was given one positive charge (+1). The total hydrophobicity ratio (HR) was defined by calculating the percentage of total hydrophobic amino acids residues (Ile, Val, Leu, Phe, Cys, Met, Ala, Trp) in the peptide sequence. The Grand Average hydropathy score was defined as the mean hydrophobicity value of the AMP and calculated by dividing the sum of hydrophobicity values of all amino acids by the number of residues.

2.1.2 AMPs synthesis and storage

All the designed and template AMPs were synthesized using 9-fluorenylmethoxycarbonyl by the peptide manufacturer company Mimotopes Pty Ltd ABN (Clayton, Victoria, Australia). Reverse phase high-performance liquid chromatography (RP-HPLC) and mass spectrometry were used to validate the quality analyses of all the antimicrobial peptides. AMPs used for *in vitro* assays were synthesized as a white powder to > 90% purity. All peptides used for *in vivo* toxicity and antibacterial efficacy were synthesized to > 95% purity. Sterilized ultrapure water was used to dissolve the peptides, all peptides were stored at -20 °C until use.

2.2 *In vitro* antipneumococcal activity assessment

2.2.1 Bacteria and growth conditions

Thirty clinical strains of *S. pneumoniae* were collected from University of Malaya Medical Centre (UMMC) and used in this study. Columbia agar with 5% sheep blood (Isolab, Malaysia) was used to culture pneumococcal isolates. Nutrient agar (Oxoid, UK) was used for *Staphylococcus aureus* ATCC 25923, *Acinetobacter baumanii* ATCC 15308, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 15442, and one clinical strain of each *Enterococcus cloacae,* methicillin-resistant *S. aureus* (MRSA), *Citrobacter spp.* and *K. pneumoniae*. Müeller-Hinton agar (MHA) and cationically-adjusted Müeller-Hinton broth (CAMHB) were used for antibacterial assays. All the bacterial strains were stored in Brain Heart Infusion Broth (BHI) (Oxoid, UK) supplemented with 10% (v/v) glycerol at -80 °C. All freeze-stocked bacterial strains were passaged two times before any experiment.

2.2.2 Broth microdilution method

The biological activities of the designed AMPs against 30 pneumococci strains were evaluated by identifying minimum inhibitory concentration (MIC) as described in the guidelines of CLSI (Clinical and Laboratory Standards Institute, 2012). Briefly, Columbia agar with 5% sheep blood was used to culture pneumococcal isolates for 24hr at 37°C in the presence of 5% CO_2. Few bacterial colonies were transferred to cationically-adjusted Müeller-Hinton broth (CAMHB) to prepare the bacterial inoculum. The inoculum was spectrophotometrically adjusted to OD_{625} 0.08 - 0.1 which equivalents to $1 \sim 2 \times 10^8$ colony forming unit (CFU)/ml, the incoculum was then serially diluted to give an inoculum of 1×10^6 CFU/ml supplemented with 10% (v/v) laked horse blood (LHB) (Oxoid, UK). Fifty µl of bacterial suspension was then added to U-bottomed 96-well microtiter plates (Corning, USA) containing 50 µl of serially diluted peptides with a

range of (1.95 - 250 µg/ml). The 96-well plates were incubated for 24hr at 37°C under 5% CO_2. The MIC was defined as the least concentration of peptide that totally prevents visible growth of the bacterial pathogen. The MIC of each peptide was specified from three independent experiments and each time in duplicate. Bacterial suspension without treatment was used as negative control.

The broad spectrum antibacterial activity of all AMPs was further evaluated by testing them against eight clinical bacterial isolates namely *Staphylococcus aureus* ATCC 25923, methicillin-resistant *S. aureus* (MRSA), *E. coli* ATCC 25922, *P. aeruginosa* ATCC 15442, *Acinetobacter baumanii* ATCC 15308, *Enterococcus cloacae*, *Citrobacter spp.* and *K. pneumoniae*.

2.2.3 *In vitro* Synergy assay

Combinations of peptide-peptide and peptide-standard antibiotic (ceftriaxone and erythromycin) were assessed for their synergistic effects using the chequerboard titration method previously described with minor modification (Bajaksouzian *et al.*, 1996). Two pneumococcal isolates (resistant and susceptible to erythromycin and ceftriaxone) were grown overnight on Columbia agar with 5% sheep blood at 37 °C under 5% CO_2. Bacterial suspensions (5×10^5 CFU/ml) were prepared according to CLSI guidelines using cation-adjusted Mueller-Hinton broth. In order to prepare a range of concentrations allowing detection of synergism, indifference/additive and antagonism, the experiment was designed in such a way that each column of the U-bottomed 96-well microtiter plate contained 50µl of a fixed 0.25×MIC of the first peptide and eight serial two-fold dilutions of the second peptide (50µl) beginning at 4×MIC. One hundred µl of bacterial suspension were then added to each well. The fractional inhibitory concentration index (FICI) of each antimicrobial drug combination (drugs A and B) was calculated according to the equation:

$$\text{FICI} = \frac{\text{MIC of drug A in combination}}{\text{MIC of drug A alone}} + \frac{\text{MIC of drug B in combination}}{\text{MIC of drug B alone}}$$

FIC index values were interpreted as follows: Two drugs have synergy if FIC≤ 0.5, additive or indifference if $0.5 < \text{FIC} \leq 4.0$, and antagonism if FIC > 4.0.

2.2.4 Time kill kinetics assay

The time required for the designed AMPs to kill pneumococcal cells was evaluated as previously described (Giacometti *et al.*, 1999b). One susceptible and one resistant pneumococcal isolate were overnight cultured and prepared at 1×10^6 CFU/ml. Bacterial isolates and peptides at their individual MICs were incubated in Müeller-Hinton broth (MHB) at 37 °C under 5% CO_2. Ten μl of the bacterial inoculums treated with peptides were removed at different time points (0, 30, 60, 90, 120, 150, 180, 210 and 240 min), serially diluted in phosphate-buffered saline (PBS) on ice to prevent further bacterial growth and plated on Columbia agar with 5% sheep blood for 24 hr at 37 °C in under 5% CO_2 to obtain feasible colony counts. Results were collected from three different experiments. Erythromycin and ceftriaxone were used in this assay as positive controls to compare their killing kinetics with the designed AMPs.

2.3 *In vitro* toxicity assessment

2.3.1 Hemolytic activity assay

The biological effects of the designed and template AMPs on human red blood cells (RBCs) was assessed as described by the protocol of Xi *et al*. (2013) with slight changes. Firstly, Human erythrocytes were freshly collected from a healthy donor and washed triplicate with sterile phosphate buffered saline (PBS) and centrifuged for 10 min at 2000 rpm until the upper solution became clear. The centrifuged erythrocytes were diluted to a final concentration of 4%, and 100 μl of peptides at graded concentrations

(1.95 – 250 μg/ml) were added to 100 μl of RBC suspensions in U-bottomed 96 well polystyrene plates (Corning, USA) and incubated for 1 hr at 37°C. After that, the RBCs were centrifuged at 2000 rpm for 10 min and GloMax Multi Detection System (Promega, USA) was used to measure the absorbance of the supernatants at 560 nm. 0.1% Triton X-100 and PBS were used as positive and negative controls, respectively. The test was performed in triplicate, and percentage of hemolysis was calculated using the following equation:

Hemolysis (%) = ($A_{peptide}$-A_{PBS}) / (A_{triton}-A_{PBS}) × 100%

Blood samples were obtained from the Blood Bank of University Malaya Medical Centre and ethical clearance for this work was approved by the Scientific and Ethical Committee of UMMC (Ethics Committee/IRB Reference No: 321.4). Written informed consent was obtained for each collection.

2.3.2 Cytotoxicity against normal human cell lines

In order to measure the cytotoxic effect of the designed peptides, the CellTiter 96 AQ$_{ueous}$ Non-Radioactive Cell Proliferation assay (Promega, USA) was performed following the protocol described by Sánchez-Vásquez *et al*. (2013). Human normal liver cell line WRL-68 (ATCC® CL-48™) and human normal lung bronchial epithelial cell line NL-20 (ATCC® CRL-2503™) were purchased from American Type Culture Collection (ATCC, USA). Dulbecco's modified Eagle's medium (DMEM) (Lonza, USA) was used to grow WRL-68 cell line, while Ham's F-12 medium (Lonza, USA) was used to grow NL-20 cell line. Both cell cultures mediums were supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco, USA) in T75 flasks and incubated at 37 °C under 5% CO_2. One hundred μl of WRL-68 Cells at a density of 1×10^4 were seeded into 96-well cell culture-treated flat bottom microtiter plates (Corning, USA), while 100 μl of NL-20 cells at 3×10^4 were seeded into each well. Hundred μl of Peptides at graded concentrations

(1.95 – 250μg/ml) were added to the wells and the 96-well plates were incubated at 37°C in the presence of 5% CO_2 for 24, 48, and 72 hr. Medium with and without cells was served as positive and negative controls, respectively. After incubation, 20μl of the reagent were added to each well and incubated for 1 hr at 37 °C under 5% CO_2.

CellTiter 96 AQ$_{ueous}$ Non-Radioactive Cell Proliferation assay consists of a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine methosulfate; PMS). The dehydrogenase enzymes found in metabolically active cells will convert the yellow MTS to soluble purple formazan product, allowing for monitoring of the reaction. GloMax Multi Detection System (Promega, USA) was used to measure the absorbance at 490 nm. The percentage of viable cells was calculated according to the following equation:

100% survival = (A$_T$-A$_B$/A$_C$-A$_B$) × 100

Where A$_T$ and A$_C$ are the absorbance of treated and untreated cells (100% survival), respectively. A$_B$ is the absorbance of medium alone. The experiment was done in triplicate.

2.4 *In silico* molecular docking study

Molecular docking study was conducted to evaluate the basis interaction of the designed AMPs with three pneumococcal virulent factors: autolysin (LytA), pneumolysin (Ply) and pneumococcal surface protein A (PspA). The molecular docking was carried out by Dr. Vannajan Sanghiran Lee's Lab, Department of Chemistry, Faculty of Science, University of Malaya. These three proteins are well known to play a significant role in the pathogenicity of *S. pneumoniae* (see discussion for reason of selection). Choline-binding domain of major pneumococcal autolysin with high resolution crystal structure (PDB ID: 1GVM) was obtained from the RCSB protein data bank (http://www.pdb.org).

The homodimer in chain A and B was used for autolysin while pneumolysin was homology modeled with those deposited in SWISS-MODEL repository (UniProt: Q04IN8) (Kiefer *et al.*, 2009; Kopp & Schwede, 2004). Automated comparative modeling of three-dimensional (3D) protein structures for PspA (ALA453-VAL653) was built using SWISS-MODEL (Schwede *et al.*, 2003) server (http://swissmodel.expasy.org). The water and ligands were removed from the original crystal structures. The initial structures were modified according to the CHARMm force field with partial charge Momany-Rone (Momany & Rone, 1992), and short minimizations of the structures were performed with RMS gradient tolerance of 0.1000 kcal/(mol x Angstrom). PROCHECK (Laskowski *et al.*, 1993) for evaluating Ramachandran plot quality was used to assess the overall quality of the minimized model. PROSA (Sippl, 1993) was performed to test the interaction energy and VERIFY3D (Lüthy *et al.*, 1992) was used to assess the compatibility of each amino acid residue. The NMR structure of an indolicidin peptide analog with enhanced activity against gram-positive bacteria (PDB ID: 1HR1 model 1) was initially used and further modeled by using Build Mutant Protocol in Discovery Studio ("Discovery Studio, version 2.5.5; Accelrys Inc.: San Diego, CA, USA, 2009) to change all three alanine to proline as the sequence of natural indolicidin substrate (Table 2.1). The conformation of the mutated residues and their neighbors were optimized using MODELER (Sali *et al.*, 1995) was used to optimize the conformation of the altered amino acids and their adjacent residues. Peptide tertiary structure prediction server (http://www.imtech.res.in/raghava/pepstr/) (Kaur *et al.*, 2007) with short minimizations was used to model all five hybrid peptides and the parent template ranalexin. AUTODOCK VINA (Trott & Olson, 2010) was used to dock the antimicrobial peptides into the target proteins with rigid docking and the low interaction complex structures were further minimized. The binding site for autolysin is at Chain B: LYS258-ALA277, for pneumolysin at ARG426-ARG437, and for PspA at GLY577-LEU588. The binding site

of pneumolysin and PspA has been predicted from prosite (http://prosite.expasy.org/). Detailed interaction energy was investigated by using calculate binding energies protocol in Discovery Studio (Discovery Studio, version 2.5.5; Accelrys Inc.: San Diego, CA, USA, 2009). By using this protocol, we were able to estimate the interaction energy between the target protein and designed peptides within 3 Å.

Table 2.1: Molecular docking of parent and hybrid peptides using Autodock Vina.

Receptor	Peptide	Sequence	aa [a]
Autolysin	Indolicidin(natural peptide)	ILAWKWAWWAWRR-NH$_2$	13
	Indolicidin (lab)	ILPWKWPWWPWRR-NH$_2$	13
	Ranalexin (natural)	FLGGLIKIVPAMICAVTKKC-OH	20
	RN7IN10	FLGGLIKWKWPWWPWRR-NH$_2$	17
	RN7IN9	FLGGL IKKWPWWPWRR-NH$_2$	16
	RN7IN8	FLGGLIKWPWWPWRR-NH$_2$	15
	RN7IN7	FLGGLIKPWWPWRR-NH$_2$	14
	RN7IN6	FLGGLIKWWPWRR-NH$_2$	13
Pneumolysin	Indolicidin (natural peptide)	ILAWKWAWWAWRR-NH$_2$	13
	Indolicidin (lab)	ILPWKWPWWPWRR-NH$_2$	13
	Ranalexin (natural)	FLGGLIKIVPAMICAVTKKC-OH	20
	RN7IN10	FLGGLIKWKWPWWPWRR-NH$_2$	17
	RN7IN9	FLGGL IKKWPWWPWRR-NH$_2$	16
	RN7IN8	FLGGLIKWPWWPWRR-NH$_2$	15
	RN7IN7	FLGGLIKPWWPWRR-NH$_2$	14
	RN7IN6	FLGGLIKWWPWRR-NH$_2$	13
PspA	Indolicidin (natural peptide)	ILAWKWAWWAWRR-NH$_2$	13
	Indolicidin (lab)	ILPWKWPWWPWRR-NH$_2$	13
	Ranalexin (natural)	FLGGLIKIVPAMICAVTKKC-OH	20
	RN7IN10	FLGGLIKWKWPWWPWRR-NH$_2$	17
	RN7IN9	FLGGL IKKWPWWPWRR-NH$_2$	16
	RN7IN8	FLGGLIKWPWWPWRR-NH$_2$	15
	RN7IN7	FLGGLIKPWWPWRR-NH$_2$	14
	RN7IN6	FLGGLIKWWPWRR-NH$_2$	13

[a] Number of amino acids.

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2.5 Mechanisms of action of designed peptides

2.5.1 Transmission electron microscopy (TEM)

Five hybrid peptides were chosen for investigation of mechanisms of action (see section 3.3 for reason of selection). Bacteria were prepared for TEM according to the guidelines of the Electron Microscopy Unit at the Faculty of Medicine, University of Malaya. *S. pneumoniae* cultures were grown overnight on Columbia agar with 5% sheep blood and suspended in CAMHB at 10^8 CFU/ml. Pneumococcal suspensions were incubated with hybrid peptides at $8 \times$ MIC for one hour at 37°C under 5% CO_2. Cells in CAMHB were used as an untreated control. After one hour of incubation, the pneumococcal cells were washed three times with 10 mM phosphate buffer saline at pH 7.3 and fixed overnight in 4% (v/v) glutaraldehyde. All samples were washed for two times with cacodylate buffer, incubated for 2 hr in osmium tetroxide buffer (OsO4 1: 1 cacodylate), washed twice with cacodylate buffer and then overnight incubated in cacodylate buffer. All The samples were washed with distilled water for two times and incubated for 10 min with uranyl acetate. After incubation with uranyl, all samples were then washed twice with distilled water and dehydrated in an ascending series of ethanol: 35% (10 min), 50% (10 min), 70% (10 min), 95% (15 min), and three times in 100% ethanol (15 min). After dehydration, Samples were incubated with propylene oxide (15 min), propylene oxide 1:1 Epon (1 hr), propylene oxide 1:3 Epon (2 hr) and finally overnight incubated with Epon. All the samples were embedded in agar 100 resin at 37°C for 5hr and maintained at 60°C until viewing. Reichert Ultramicrotome copper grids 3.05 mm (300 square mesh) (Agar Scientific) were used to prepare Ultrathin sections. Ethanol-based uranyl acetate and lead citrate were used to stain the samples for 5 min. Transmission electron microscope (Leo Libra 120) was used to capture the images.

2.5.2 Scanning electron microscopy (SEM)

S. pneumoniae with a starting inoculum of 1×10^8 cells/mL in CAMHB medium was exposed to $8 \times$ MIC of respective peptides and incubated for 1hr at 37°C for under 5% CO_2. After incubation, 20 µl of the untreated and treated cell suspensions were transferred onto the membrane filters and processed as described by the standard guidelines provided by Electron Microscopy Unit, Faculty of Medicine, University of Malaya. Briefly, the bacterial samples were overnight fixed with 4% glutaraldehyde at 4°C and then washed twice with sodium cacodylate buffer for 10 min each. In the second fixation, 1% osmium tetroxide was used to fix the samples for 1 hr at 4°C and then washed two times with distilled water for 10 minutes each. All the samples were then dehydrated through a serially graded ethanol (30%, 50%, 70%, 80%, 90%, 95% and twice in 100%) for 15 minutes each, followed by dehydration in ethanol:acetone mixtures (3:1, 1:1 and 1:3) for 15 min each and three rounds of pure acetone for 20 min each. The samples were then dried for 1 hr and kept in a desiccator before the examination. The samples were then mounted on stubs, coated with gold in sputter coater and viewed under the FEI-Quanta 650 Scanning Electron Microscope.

2.5.3 ATP efflux assay

The amount of ATP released from pneumococcal cells incubated with hybrid peptides was measured as previously described with slight modification (Tanida *et al.*, 2006) using an ATP determination kit (Molecular Probes, USA) based on the luciferin/luciferase method according to the manufacturer's instructions. Briefly, pneumococcal cells (1×10^7 CFU/mL) were incubated with hybrid peptides at $8 \times$ MIC for 1, 2 and 3 hr in Eppendorf tubes. The samples were then centrifuged at 5000 rpm for 5 min, and ATP efflux was subsequently estimated using an ATP standard curve. Values

were obtained from three independent experiments. Ceftriaxone and erythromycin were used as positive controls.

2.5.4 Gel retardation assay

This assay was carried out as previously described with minor modifications (Li *et al.*, 2013). Genomic DNA was isolated from pneumococcal cells using DNeasy Blood & Tissue Kit (Qiagen). The optical density ratio of 260 and 280nm (OD260/OD280 = 1.83) was used to measure the purity of the DNA. Genomic DNA (250 ng) was incubated with hybrid peptides at various concentrations in 12 μl at room temperature for 10 min. Two μl of loading buffer were added to the mixture and the migration of DNA through 1% agarose gel was evaluated by electrophoresis in 1× Tris borate–EDTA buffer (45mM Tris–borate and 1mM EDTA at pH8.0) and spotted by the fluorescence of gel stain (Gel Red, BIOTIUM).

2.6 *In vivo* assessment of peptides

2.6.1 Mice and environmental conditions

In this study, 4-week old male, pathogen free ICR (CD-1) mice were purchased from InVivos (Singapore) and used the entire animal study to assess the *in vivo* toxicity and antibacterial efficacy of the novel peptides as these animals by far are the most commonly used model for the study of pneumococcal disease (Chiavolini *et al.*, 2008). All mice were kept in ventilated polycarbonate cages (12 hr light/dark cycle, 20±2 °C and 55 % relative humidity). All mice were familiarized for 7 days before any experimental procedure and were given unlimited pellets and water *ad libitum*. All animal experimentations were conducted according to the guidelines approved by Faculty of Medicine Institutional Animal Care and Use Committee (FOM IACUC), University of Malaya (ethics Reference no. : 2013-07-15/MMBTR/SDS).

2.6.2 Peptides administration procedures

Myjector 1 ml U100 29G insulin syringe (BD bioscience, USA) was used to perform all the injection procedures. In the intraperitoneal (IP) administration mouse was held in a supine position with its head lower than his body and injection was given to the lateral side at lower-left quadrant of the abdomen. Subcutaneous (SC) administration was given into the loose skin of the left thigh and followed by the right one if repeated injections were required. The intrathoracic (IT) injection was administered to the lateral side of the right thorax through the intercostal muscles between ribs. Intranasal administration route was performed using a micropipette with anesthetized mouse held in supine position and the head is elevated.

2.6.3 *In vivo* toxicity assessment

In order to evaluate the possible toxic effects correlated to peptides administered in mice. Four hybrid peptides RN7-IN10, RN7-IN9, RN7-IN8 and RN7-IN6 were chosen for *in vivo* toxicity assessment due to their promising *in vitro* antibacterial activity (Jindal *et al.*, 2015). Mice were separated into 4 groups (each with 4 mice) and were injected with respective peptides at 1 hr, 12 hr, and 24 hr (three-dose regimen) via IP, SC, and IN administration routes. The hybrid peptides were firstly administered at maximum doses (100 mg/kg for IP route, 100 mg/kg for SC routes and 20 mg/kg for IN route). Doses administered via IN route were limited by the low volume (20 µl) deliverable through the nasal cavity of the mouse and thus the highest dose given was 20 mg/kg. Any abnormal behavior was recorded and survival of mice was noted as well. In the case of adverse effects such as high physical stress, highly lethargic, physical inactiveness, and/or death were detected, lower graded doses were given. All the administered mice were monitored for 7 days or until death occurs. At day seven post administration, all animals were sacrificed and blood and organs were collected. Mice injected with PBS only were used

as the control group. For whole blood analysis, the parameters were number of red cells (RBC), number of white cells (WBC), lymphocytes, monocytes, eosinophil, granulocytes, haemoglobin (Hgb), mean corpuscular volume (MCV), hematocrit (HCT), platelet Counts (PLT), Mean corpuscular haemoglobin (MCH) and corpuscular haemoglobin concentration (MCHC). For biochemistry analysis, the parameters were alanine transaminase (ALT), creatinine, alkaline phosphatase (ALP), aspartate aminotransferase (AST), total bilirubin and urea. The following tissues were obtained from all animals: lung, kidney, brain, spleen and liver. All tissues were fixed in 10 % (v/v) buffered formalin and processed for paraffin embedding. The histological sections were stained with hematoxylin–eosin (HE) at the histopathology laboratory at Veterinary Laboratory Service Unit, University Putra Malaysia (UPM).

2.6.4 *In vivo* antipneumococcal activity assessment

Two pneumococcal infection models developed previously in our lab (Le *et al.*, 2015) were used to assess the therapeutic efficacy of peptides *in vivo*. The systemic infection model was used to mimic pneumococcal bacteremia in humans and the pneumococcal pneumonia model was used to mimic pneumococcal pneumonia in humans. A highly virulent strain was used in both models. The bacterial isolate was grown overnight on Columbia agar with 5% sheep blood at 37 °C under 5% CO_2. The bacterial suspension was adjusted to OD_{625} 0.08-0.1 ($1 \sim 2 \times 10^8$ CFU/ml). Mice tested for lethal systemic infection were inoculated with 1.5×10^2 CFU/mouse (100μl) via IP route. Mice used to assess the Pneumococcal pneumonia model were inoculated with pneumococcal cells of 5×10^3 CFU/mouse (50μl) via the intrathoracic route. Both of the infection models caused 100% death within 2 to 4 days post-infection.

After 1hr of inoculation, Mice receiving treatment were randomized and divided into six groups. RN7-IN10 and RN7-IN8 were tested at three different doses for each (5 mg/kg,

10mg/kg and 20mg/kg) using a group of 10 mice. Graded dosages of ceftriaxone (5 mg/kg, 10 mg/kg, 20 gm/kg, 40 mg/kg and 80 mg/kg) were also tested to evaluate the *in vivo* antibacterial activity of this antibiotic. Mice injected with PBS only were served as uninfected control. Mice injected with bacterial inoculum were used as untreated control group and given sterile distilled water only. Survival of mice was documented for seven days or until death. After seven days, the experiment was ended, the blood of the survived mice were plated on Columbia agar with 5 % sheep blood to detect the presence of pneumococcal cells.

2.6.5 *In vivo* synergy assessment of peptide/peptide and peptide/ceftriaxone

After evaluating the *in vivo* antibacterial activity of the hybrids in standalone mode, the *in vivo* efficiency of the hybrid peptides in combination with each other and with the standard antibiotic ceftriaxone was carried out. Graded doses of peptide and ceftriaxone were chosen and prepared at 2X the desired concentration separately in 1 ml tubes, and the volume was 0.1 ml. Immediately before injection, both drugs were combined giving the final desired concentration at a volume of 0.2 ml. The synergetic effect was then performed in infection models (n = 10).

2.6.6 Anesthesia and necropsy

Mice used to evaluate the *in vivo* toxicity and antibacterial activity of the hybrid peptides using the subcutaneous (SC) and intranasal (IN) administration routes were anesthetized using a combination of standard dose of xylazine (ilium xylazil-20, 10 mg/kg) and ketamine (Narketan®-10, 100 mg/kg) through intraperitoneal (IP) injection. After 7 days of treatment, the *in vivo* toxicity and antibacterial effecacy experiments were ended and survived mice were anesthetized. Blood samples for Hematological and biochemical analysis were collected via cardiac puncture using a 25G syringe (BD

bioscience, USA). Whole blood for heamatological analysis was collected in 500 µl dipotassium EDTA microtainer tubes (BD Bioscience, USA). About 500 µl of blood collected an eppedorff tube and centrifuged at 8000 rpm for 5 min and then the serum was transferred into a new 1.5 ml tube for biochemistry analysis. The mice were then euthanized by cervical dislocation, dissected and the following organs were collected for histopathology evaluation (lung, kidney, brain, liver and spleen).

2.7 Statistical analysis

GraphPad Prism 5 was used to perform the Statistical analysis. The results were expressed as mean ± standard deviation. Two-way ANOVA with Bonferroni post-test was used to analyze the significant difference between treated and control groups in time killing assay and ATP assay. One-way ANOVA with *post-hoc* Dunnett-t test was used to assess the statistical difference between the blood haematogram and blood serum biochemistry parameters of the treated and the untreated control groups in the *in vivo* toxicity assay. Kaplan-Meier analysis with log-rank test (Mantel-Cox) was used to generate the survival curve for each treated group versus untreated control for both *in vivo* antibacterial activity and *in vivo* synergy assays.
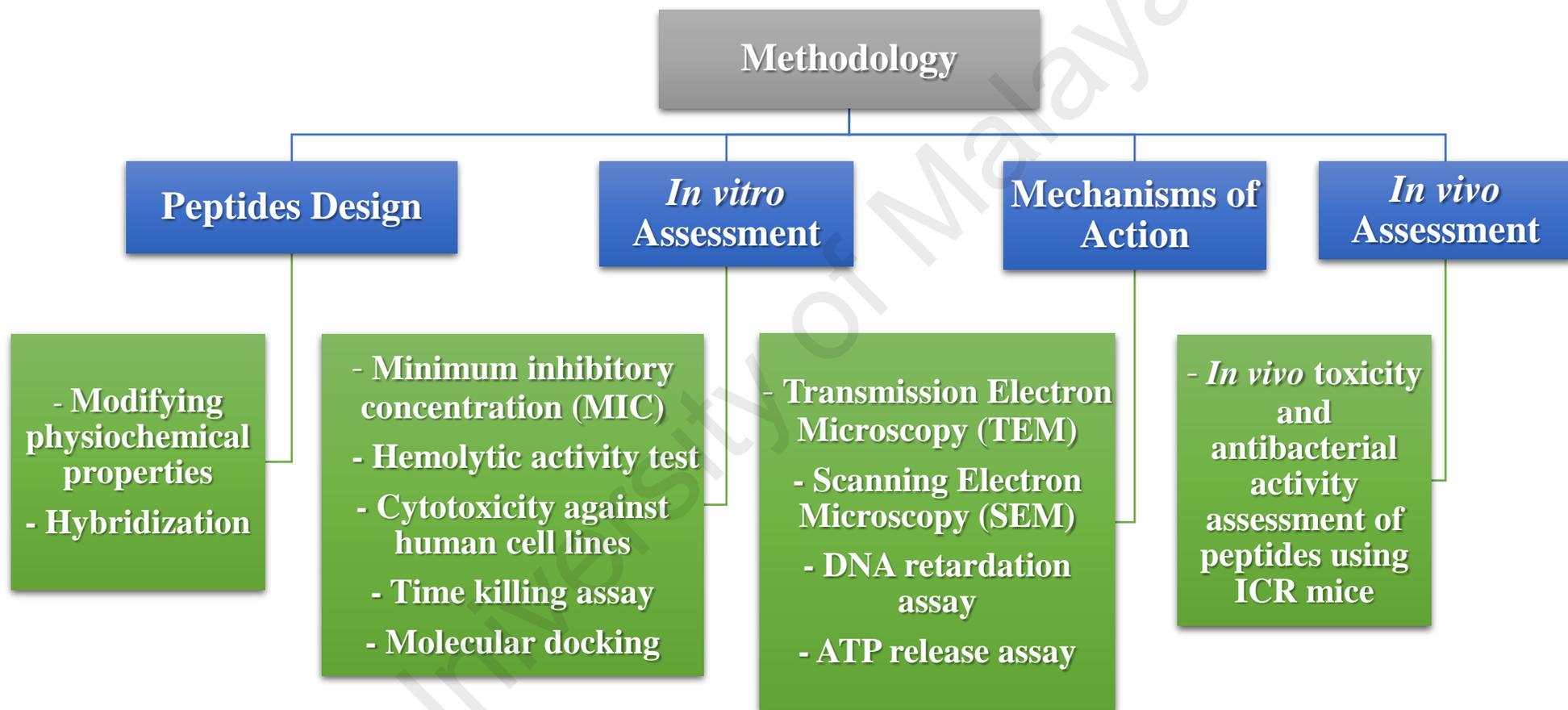
Figure 2.1: Overall flowchart of the methodology for the current study.

CHAPTER 3: RESULTS

3.1 Peptides' design

3.1.1 Selection of template peptides

In this study, indolicidin and ranalexin were selected to serve as two natural templates. Indolicidin is a naturally occurring peptide purified from the cytoplasmic granules of bovine neutrophils (Selsted *et al.*, 1992). According to antimicrobial peptide database (APD), indolicidin (APD ID: AP00150) is an extended peptide composed of 13 amino acid residues and a unique composition of 39% tryptophan and 23% proline (ILPWKWPWWPWRR-NH$_2$). This peptide shows a total net charge and hydrophobic residue% of +3 and 53% respectively, and the peptide is amidated at carboxyl terminus in nature. In terms of the antimicrobial activity, Selsted *et al*, (1992) evaluated the bactericidal activity of indolicidin by incubating it with Gram-negative (*E. coli*) and Gram-positive (*S. aureus*). Their results showed that after two hours of incubation both bacteria were eliminated by indolicidin at low concentrations (25 µg/ml). Besides its antibacterial activity against both Gram-negative and Gram-positive bacteria, indolicidin possesses potent antimicrobial activity against a variety of microorganisms including fungi (Ahmad *et al.*, 1995), parasites (Aley *et al.*, 1994), and viruses (Albiol Matanic & Castilla, 2004).

The second template peptide, ranalexin, is another natural AMP obtained from the skin of the bullfrog, *Rana catesbeiana* (Clark *et al*., 1994). According to APD, ranalexin (APD ID: AP00513) consists of 20 amino acids (FLGGLIKIVPAMICAVTKKC) with a total net charge and hydrophobic residue% of +3 and 65% respectively. This peptide is α-helical and has a single intramolecular disulfide bond which forms a heptapeptide ring within the molecule (Aleinein *et al.*, 2013). Previous studies have shown that ranalexin is active against Gram-negative and Gram-positive bacteria, especially *S. aureus* (Giacometti *et al*., 1998; Giacometti *et al*., 1999b). Furthermore, ranalexin displayed

antimicrobial activity against Cryptosporidia alone or in combination with other peptides (Giacometti *et al.*, 1999a).

3.1.2 Design of synthetic peptides

The two template peptides were used to design three groups of novel peptides. The first group of peptides has been developed based on a single amino acid substitution approach, whereby the cationicity of indolicidin increased the hydrophobicity decreased by swapping the hydrophobic amino acids of indolicidin with the cationic amino acids (lysine, K and arginine, R). The first indolicidin analog was generated by replacing proline (P) at 10th position with lysine (K) residue, and changing hydrophobic amino acid isoleucine (I) at the 1st position to the lesser hydrophobic leucine (L). This substitution generated an increase in the total net charge by +1. Following the same strategy, the second analog was designed by substituting isoleucine (I) (1st position) and leucine (2nd position) with positively charged arginine (R) residue, and lysine (K) (5th position) with arginine (R). As a result, the total net charge was increased from +3 to +5, and the hydrophobic% was decreased from 53% to 38%. The third analog peptide was designed by changing isoleucine (I) (1st position) and leucine (2nd position) to arginine (R), and by replacing lysine (K) and tryptophan (W) (5th position and 8th position, respectively) with arginine (R). This analog has a total net charge of +7 and hydrophobic% of 30%. For the fourth analog, isoleucine (1st position), lysine (5th position), and two tryptophan residues (8th position and 9th position, respectively) were changed to arginine (R). This alteration increased the positive net charge of the fourth analog from +3 to +7 and reduced the hydrophobicity from 53% to 30%. As a result, four indolicidin analog peptides (IN1, IN2, IN3, and IN4) were designed (Table 3.1).

Table 3.1: Design of indolicidin analogs

Peptide name	Sequence
Indolicidin	ILPWK^{5}WPWWP^{10}WRR
IN1	<mark>L</mark>LPWK^{5}WPWW<mark>K</mark>^{10}WRR
IN2	<mark>RR</mark>PW<mark>R</mark>^{5}WPWWP^{10}WRR
IN3	<mark>RR</mark>PW<mark>R</mark>^{5}WP<mark>R</mark>WP^{10}WRR
IN4	<mark>R</mark>LPW<mark>R</mark>^{5}WP<mark>RR</mark>P^{10}WRR

Amino acid residues highlighted in yellow represent replaced residue
with the original one in the parent peptide

Similarly to the first group, the second group of peptides were designed based on the second template peptide ranalexin. Modification of ranalexin previously performed by Clark et al. (1994) revealed that the first four amino acids in the peptide sequence are critical for antimicrobial activity. For that reason, those four amino acid residues that originally exist in ranalexin were unchanged. The first analog peptide of this group was generated by altering threonine (T) residue at 17th position to arginine (R), which increased the net charge by +1. The second analog was designed by replacing isoleucine (I) at 8th position with proline (P), and threonine (T) residue at 17th position to arginine (R). This alteration decreased the hydrophobic% from 65% to 60%, at the same time the total net charge remain +4. In the third analog, isoleucine (I) at 8th position and threonine (T) at 17th position were both changed to arginine (R), resulted in rising the net charge by +2 and reducing the hydrophobicity to 60%. The last analog of this group was generated by swapping isoleucine (8th position), valine (V) at 9th position, and threonine (17th position) with arginine (R), proline (P), and arginine (R) respectively. This modification increased the net charge by +2 and decreased the hydrophobicity to 55%. Accordingly, four analog peptides (RN1, RN2, RN3, and RN4) were generated based on the parent peptide ranalexin (Table 3.2).

Table 3.2: Design of ranalexin analogs

Peptide name	Sequence
Ranalexin	FLGGL^{5}IKIVP^{10}AMICA^{15}VTKKC^{20}
RN1	FLGGL^{5}IKIVP^{10}AMICA^{15}VRKKC^{20}
RN2	FLGGL^{5}IKPVP^{10}AMICA^{15}VRKKC^{20}
RN3	FLGGL^{5}IKRVP^{10}AMICA^{15}VRKKC^{20}
RN4	FLGGL^{5}IKRPP^{10}AMICA^{15}VRKKC^{20}

Amino acid residues highlighted in yellow represent replaced residue with the original one in the parent peptide

The third group of synthetic peptides was designed based on forming hybrids of fragments from parent peptides indolicidin and ranalexin (Table 3.3). five hybrid peptides were designed and named RN7-IN1, RN7-IN9, RN7-IN8, RN7-IN7, and RN7-IN6. The first hybrid peptide RN7-IN10 was generated by trimming the first seven residues at the N-terminus (FLGGLIK) of ranalexin which was then attached to the 4th to 13th residual fragment (WKWPWWPWRR) of indolicidin. Likewise, RN7-IN9, RN7-IN8, RN7-IN7, and RN7-IN6 were also designed by trimming the first seven amino acid residues of ranalexin and fusing it with 5th to 13th, 6th to 13th, 7th to 13th and 8th to 13th residual fragments of indolicidin (Table 3.3). Amino acid sequences and physicochemical properties of all the new peptides are provided in Table 3.4. The lengths of the hybrid peptides were ranging from 13 to 17 amino acids. The net charge for RN7-IN10 and RN7-IN9 was +5, while it was +4 for RN7-IN8, RN7-IN7 and RN7-IN6. Using CELLPPD tool (http://crdd.osdd.net/raghava/cellppd/multi_pep.php), all the peptides showed amphipathicity of more than 0.5 which means that all peptides have good balance between hydrophilic and hydrophobic phases. All indolicidin analogs, RN7-IN8, RN7-IN7 and RN7-IN6 showed high stability like the two parent peptides. On the other hand, ranalexin

analogs, RN7-IN10 and RN7-IN9 displayed normal stability suing the HLP tool (http://www.imtech.res.in/raghava/hlp/pep_both.htm).

Table 3.3: Design of hybrid peptides

Peptide name	Sequence
Ranalexin	**FLGGL^{5}IKIVP^{10}AMICA^{15}VTKKC^{20}**
Indolicidin	**ILPWK^{5}WPWWP^{10}WRR**
RN7-IN10	FLGGLIKWKWPWWPWRR
RN7-IN9	FLGGLIKKWPWWPWRR
RN7-IN8	FLGGLIKWPWWPWRR
RN7-IN7	FLGGLIKPWWPWRR
RN7-IN6	FLGGLIKWWPWRR

Amino acid residues highlighted in red and blue represent fragment from N- terminus of Ranalexin and C-terminus of indolicidin, respectively.

Table 3.4: Sequences and physicochemical properties of the template and designed antimicrobial peptides.

Peptide	Sequence	aa[a]	MW[b]	Q[c]	A[d]	S[e]	Pho%[f]	GRAVY[g]
Indolicidin analogs								
IN1	LLPWKWPWWKWRR-NH2	13	1926.35	+5	0.94	High	53 %	-1.300
IN2	RRPWRWPWWPWRR-NH2	13	2003.371	+6	0.94	High	38 %	-2.446
IN3	RRPWRWPRWPWRR-NH2	13	1973.346	+7	1.13	High	30 %	-2.723
IN4	RLPWRWPRRPWRR-NH2	13	1900.293	+7	1.13	High	30 %	-2.362
Ranalexin analogs								
RN1	FLGGLIKIVPAMICAVRKKC-OH	20	2154.824	+4	0.67	Normal	65 %	1.210
RN2	FLGGLIKPVPAMICAVRKKC-OH	20	2132.781	+4	0.67	Normal	60 %	0.905
RN3	FLGGLIKRVPAMICAVRKKC-OH	20	2197.852	+5	0.80	Normal	60 %	0.760
RN4	FLGGLIKRPPAMICAVRKKC-OH	20	2189.836	+5	0.80	Normal	55 %	0.470
Hybrid peptides								
RN7-IN10	FLGGLIKWKWPWWPWRR-NH2	17	2300.791	+5	0.72	Normal	52 %	-0.612
RN7-IN9	FLGGLIKKWPWWPWRR-NH2	16	2114.578	+5	0.76	Normal	50 %	-0.594
RN7-IN8	FLGGLIKWPWWPWRR-NH2	15	1986.408	+4	0.57	High	53 %	-0.373
RN7-IN7	FLGGLIKPWWPWRR-NH2	14	1800.195	+4	0.61	High	50 %	-0.336
RN7-IN6	FLGGLIKWWPWRR-NH2	13	1709.078	+4	0.66	High	53 %	-0.238
Template peptides								
Indolicidin	ILPWKWPWWPWRR-NH2	13	1907.30	+4	0.66	High	53%	-1.069
Ranalexin	FLGGLIKIVPAMICAVTKKC-OH	20	2105.70	+3	0.55	High	65%	1.400

[a]Number of amino acids.

[b]Molecular weight.

[c]Net charge. Lys (K), Arg (R), and C-terminal amidation (NH$_2$) was assigned with +1 charge.

[d]Amphipathicity.

[e]Stability.

[f]hydrophobic residues%.

[g]Grand Average hydropathy value of the peptide.

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3.2 *In vitro* antimicrobial activity of peptides

3.2.1 Determination of peptides' MICs against *S. pneumoniae*

The antibacterial activity of the templates (indolicidin and ranalexin), four indolicidin analogs (IN1, IN2, IN3, and IN4), four ranalexin analogs (RN1, RN2, RN3, and RN4), and five hybrid peptides (RN7-IN10, RN7-IN9, RN7-IN8, RN7-IN7, and RN7-IN6) were assessed against 30 pneumococcal clinical isolates using the broth microdilution assay.

Among all the newly designed peptides, four hybrid peptides (RN7-IN10, RN7-IN9, RN7-IN8, and RN7-IN6) showed the most potent antipneumococcal activity against all 30 pneumococcal isolates with MICs ranging from 7.81 to 15.62 μg/ml. All four hybrid peptides revealed enhanced antipneumococcal activity as compared to their template peptides. The MIC values were one-fold lower than indolicidin (MIC of 15.62-31.25 μg/ml) and four-fold lower than ranalexin (MIC of 62.5 μg/ml) (Table 3.5, highlighted in blue). On the other hand, *S. pneumoniae* isolates were less sensitive to hybrid peptide RN7-IN7 with MIC value ranging from 31.25 to 62.5 μg/ml) (Table 3.5, highlighted in blue).

In terms of indolicidin analogs, increasing the net charge did not improve the antibacterial activity against *S. pneumoniae* when compared to their parent peptide indolicidin (MIC of 15.62-31.25 μg/ml). Analog peptides IN1 and IN2 displayed MIC values of 31.25-62.5μg/ml against all 30 pneumococcal isolates. The third indolicidin analog IN3 exhibited MIC value of 62.5 μg/ml. On the other hand, IN4 peptide failed to display any antipneumococcal activity up to a concentration of 250 μg/ml when tested against all the 30 clinical isolates (Table 3.5).

Unlike their parent peptide, ranalexin analogs revealed the weakest antipneumococcal activity when tested against all the clinical isolates. Peptide analog RN1 was the only ranalexin analog that possessed antipneumococcal activity but at very high concentration (250 μg/ml). On the contrary, RN2, RN3, and RN4 failed to display any activity against all the thirty clinical isolates of *S. pneumoniae* up to a concentration of 250 μg/ml (Table 3.5).

Table 3.5: Antipneumococcal activity of all AMPs against 30 clinical isolates of *Streptococcus pneumoniae*.

Isolate	MIC^a (µg/ml)														
	IN1	IN2	IN3	IN4	RN1	RN2	RN3	RN4	RN7-IN10	RN7-IN9	RN7-IN8	RN7-IN7	RN7-IN6	Indolicidin	Ranalexin
SP01	31.25	62.5	62.5	250	250	>250	>250	>250	**15.62**	**15.62**	**15.62**	**31.25**	**7.81**	15.625	62.5
SP02	62.5	31.25	62.5	250	250	>250	>250	>250	**15.62**	**7.81**	**15.62**	**31.25**	**15.62**	15.625	62.5
SP03	62.5	31.25	62.5	250	250	>250	>250	>250	**7.81**	**7.81**	**7.81**	**62.5**	**7.81**	15.625	62.5
SP04	31.25	31.25	62.5	250	250	>250	>250	>250	**7.81**	**7.81**	**7.81**	**31.25**	**7.81**	31.25	62.5
SP05	62.5	62.5	62.5	250	250	>250	>250	>250	**7.81**	**15.62**	**7.81**	**31.25**	**7.81**	31.25	62.5
SP06	31.25	31.25	62.5	250	250	>250	>250	>250	**7.81**	**7.81**	**7.81**	**62.5**	**7.81**	31.25	62.5
SP07	62.5	62.5	62.5	250	250	>250	>250	>250	**7.81**	**15.62**	**15.62**	**31.25**	**7.81**	15.625	62.5
SP08	31.25	62.5	62.5	250	250	>250	>250	>250	**15.62**	**15.62**	**15.62**	**62.5**	**15.62**	31.25	62.5
SP09	31.25	31.25	62.5	250	250	>250	>250	>250	**15.62**	**15.62**	**15.62**	**62.5**	**15.62**	31.25	62.5
SP10	31.25	62.5	62.5	250	250	>250	>250	>250	**7.81**	**15.62**	**15.62**	**62.5**	**7.81**	15.625	62.5
SP11	31.25	31.25	62.5	250	250	>250	>250	>250	**15.62**	**7.81**	**7.81**	**31.25**	**15.62**	15.625	62.5
SP12	31.25	62.5	62.5	250	250	>250	>250	>250	**7.81**	**15.62**	**15.62**	**62.5**	**7.81**	15.625	62.5
SP13	62.5	62.5	62.5	250	250	>250	>250	>250	**15.62**	**15.62**	**7.81**	**62.5**	**15.62**	15.625	62.5
SP14	62.5	31.25	62.5	250	250	>250	>250	>250	**15.62**	**15.62**	**7.81**	**31.25**	**7.81**	31.25	62.5
SP15	31.25	31.25	62.5	250	250	>250	>250	>250	**15.62**	**7.81**	**7.81**	**62.5**	**15.62**	15.625	62.5
SP16	62.5	62.5	62.5	250	250	>250	>250	>250	**7.81**	**15.62**	**15.62**	**31.25**	**15.62**	31.25	62.5
SP17	31.25	31.25	62.5	250	250	>250	>250	>250	**7.81**	**7.81**	**15.62**	**31.25**	**15.62**	15.625	62.5
SP18	62.5	31.25	62.5	250	250	>250	>250	>250	**7.81**	**7.81**	**7.81**	**62.5**	**7.81**	31.25	62.5
SP19	62.5	62.5	62.5	250	250	>250	>250	>250	**7.81**	**15.62**	**15.62**	**31.25**	**7.81**	31.25	62.5
SP20	62.5	62.5	62.5	250	250	>250	>250	>250	**7.81**	**7.81**	**7.81**	**31.25**	**7.81**	31.25	62.5
SP21	31.25	31.25	62.5	250	250	>250	>250	>250	**15.62**	**7.81**	**15.62**	**62.5**	**15.62**	15.625	62.5
SP22	62.5	31.25	62.5	250	250	>250	>250	>250	**15.62**	**15.62**	**7.81**	**62.5**	**15.62**	31.25	62.5
SP23	31.25	31.25	62.5	250	250	>250	>250	>250	**7.81**	**15.62**	**15.62**	**31.25**	**7.81**	31.25	62.5
SP24	31.25	62.5	62.5	250	250	>250	>250	>250	**7.81**	**15.62**	**15.62**	**62.5**	**15.62**	15.625	62.5

Table 3.6, continued

SP25	62.5	62.5	62.5	250	250	>250	>250	>250	**15.62**	**7.81**	**15.62**	**31.25**	**15.62**	15.625	62.5
SP26	31.25	62.5	62.5	250	250	>250	>250	>250	**15.62**	**15.62**	**7.81**	**62.5**	**15.62**	31.25	62.5
SP27	31.25	31.25	62.5	250	250	>250	>250	>250	**15.62**	**7.81**	**7.81**	**31.25**	**7.81**	15.625	62.5
SP28	62.5	62.5	62.5	250	250	>250	>250	>250	**7.81**	**15.62**	**7.81**	**31.25**	**7.81**	31.25	62.5
SP29	62.5	31.25	62.5	250	250	>250	>250	>250	**15.62**	**15.62**	**15.62**	**31.25**	**15.62**	31.25	62.5
SP30	62.5	31.25	62.5	250	250	>250	>250	>250	**15.62**	**15.62**	**15.62**	**62.5**	**15.62**	31.25	62.5

[a] MIC: Minimum inhibitory concentration.

Highlighted in blue: MIC of the hybrid peptides against 30 pneumococcal isolate

3.2.2 Determination of broad spectrum antibacterial activity of peptides

The broad range antibacterial activity of all the designed AMPs was further tested using broth microdilution technique against eight different bacterial species frequently encountered in the clinical setting. The bacterial isolates included *Staphylococcus aureus* ATCC 25923, methicillin-resistant *S. aureus* (MRSA), *E. coli* ATCC 25922, *P. aeruginosa* ATCC 15442, *Acinetobacter baumanii* ATCC 15308, *Citrobacter spp*, *Enterococcus cloacae* and *K. pneumoniae*.

In terms of hybrid peptides, four of them (RN7-IN10, RN7-IN9, RN7-IN8, and RN7-IN6) revealed the most potent antibacterial activity among all the tested peptides against three bacterial pathogens namely *E. coli* (7.81 μg/ml), *S. aureus* (7.81 μg/ml) and methicillin resistant *S. aureus* (MRSA) (7.81 μg/ml). However, they displayed moderate activity against *P. aeruginosa* (MIC value of 32.25 μg/ml) and *Acinetobacter baumanii* (31.25 μg/ml). RN7-IN7 was the weakest among all the hybrid peptides, the MIC value ranged from 31.25-62.5 μg/ml against *E. coli, S. aureus,* MRSA and *P. aeruginosa* and 125 μg/ml against *Acinetobacter baumanii* (Table 3.6)

Indolicidin and its analog peptides IN1, IN2, and IN3 revealed modest antibacterial activity against *E. coli* (31.25 μg/ml), *S. aureus* (31.25 μg/ml), methicillin resistant *S. aureus* (MRSA) (31.25 μg/ml) and *P. aeruginosa* (31.25 μg/ml). On the other hand, IN4 presented the weakest antibacterial activity with MIC of 250 μg/ml against *E. coli* and MRSA and 125 μg/ml against *S. aureus* and *P. aeruginosa* (Table 3.6).

Ranalexin analogs were inactive and failed to exhibit any antibacterial activity against *E. coli*, *S. aureus*, (MRSA) and *P. aeruginosa* up to a concentration of 250 μg/ml (Table 3.6). All the newly designed peptides along with parent peptides failed to reveal any antibacterial activity against *citrobacter spp, Enterococcus cloacae* and *K. pneumoniae* up to a concentration of 250 μg/ml (Table 3.6).

Table 3.6: MICs of all the designed AMPs against eight isolates of common human bacterial pathogens.

Peptide	MIC[a] (µg/ml)							
	S. aureus	(MRSA)	*E. coli*	*P. aeruginosa*	*Acinetobacter baumanii*	*Enterococcus cloacae*	*citrobacter spp*	*K. pneumoniae*
Indolicidin	31.25	31.25	31.25	31.25	>250	>250	>250	>250
IN1	31.25	31.25	31.25	31.25	>250	>250	>250	>250
IN2	31.25	31.25	31.25	31.25	>250	>250	>250	>250
IN3	31.25	62.5	31.25	31.25	>250	>250	>250	>250
IN4	125	250	250	125	>250	>250	>250	>250
Ranalexin	31.25	31.25	62.5	125	>250	>250	>250	>250
RN1	125	250	250	>250	>250	>250	>250	>250
RN2	>250	>250	>250	>250	>250	>250	>250	>250
RN3	>250	>250	>250	>250	>250	>250	>250	>250
RN4	>250	>250	>250	>250	>250	>250	>250	>250
RN7-IN10	7.81	7.81	7.81	31.25	31.25	>250	>250	>250
RN7-IN9	7.81	7.81	7.81	31.25	31.25	>250	>250	>250
RN7-IN8	7.81	7.81	7.81	31.25	62.5	>250	>250	>250
RN7-IN7	31.25	31.25	31.25	62.5	125	>250	>250	>250
RN7-IN6	7.81	7.81	7.81	31.25	31.25	>250	>250	>250

[a]MIC, minimum inhibitory concentration.

3.2.3 *In vitro* synergism activity of peptides

The antibacterial effects of peptide/peptide and peptide/antibiotic (erythromycin and ceftriaxone) combinations were evaluated against two pneumococcal strains (susceptible and resistant to standard drugs) using the checkerboard assay as described in section 2.2.3. Fractional inhibitory concentration index (FICI) of ≤ 0.5 represents synergism, $> 0.5 - \leq 4$ represents indifference, and > 4 represents antagonism. Based on the MIC results, only AMPs with MIC of 62.5 µg/ml or less were verified for their synergism activity. The results showed that among all the peptide-peptide combinations, synergism activity was noted when the hybrids paired with each other with FICI of less than 0.5 (Table 3.7 highlighted in blue) irrespective of antibiotic susceptibility of the isolates. In contrast, combinations of indolicidin analogs with each other and with hybrid peptides did not reveal any synergism activity against both susceptible and resistant isolates with FICI ranging from $> 0.5 - 2.25$ (Table 3.7). In terms of peptide-antibiotic combinations, all five hybrid peptides showed synergism when paired with erythromycin and ceftriaxone and tested against both pneumococcal strains (Table 3.7 highlighted in yellow). In the same way, parent peptide indolicidin and three indolicidin analogs namely IN1, IN2, and IN3 displayed synergism when combined with standard drugs against both *S. pneumonaie* strains (Table 3.7, highlighted in yellow). No antagonism (FICI > 4.0) was detected in any of the combinations. Our results revealed that all the tested peptides were able to enhance the antibacterial activity of standard drugs when used in combination.

Table 3.7: FIC index of various combinations of hybrid peptides with each other and with standard antibiotics against susceptible and resistant *S. pneumoniae*.

Combination		Susceptible *S. pneumoniae*		Resistant *S. pneumoniae*	
Drug A	Drug B	FIC index[a]	Interpretation	FIC index[a]	Interpretation
RN7-IN10	RN7-IN9	0.50	Synergy	0.37	Synergy
	RN7-IN8	0.28	Synergy	0.28	Synergy
	RN7-IN7	0.37	Synergy	0.50	Synergy
	RN7-IN6	0.26	Synergy	0.31	Synergy
	Indolicidin	0.75	Indifference	0.75	Indifference
	IN1	1.25	Indifference	0.75	Indifference
	IN2	0.75	Indifference	1.25	Indifference
	IN3	1.25	Indifference	0.75	Indifference
	Ceftriaxone	0.37	Synergy	0.31	Synergy
	Erythromycin	0.26	Synergy	0.28	Synergy
RN7-IN9	RN7-IN8	0.37	Synergy	0.50	Synergy
	RN7-IN7	0.50	Synergy	0.50	Synergy
	RN7-IN6	0.28	Synergy	0.37	Synergy
	Indolicidin	2.25	Indifference	0.75	Indifference
	IN1	0.75	Indifference	2.25	Indifference
	IN2	2.25	Indifference	1.25	Indifference
	IN3	1.25	Indifference	1.25	Indifference
	Ceftriaxone	0.31	Synergy	0.37	Synergy
	Erythromycin	0.28	Synergy	0.26	Synergy
RN7-IN8	RN7-IN7	0.50	Synergy	0.50	Synergy
	RN7-IN6	0.31	Synergy	0.37	Synergy
	Indolicidin	0.75	Indifference	1.25	Indifference
	IN1	0.75	Indifference	0.75	Indifference
	IN2	1.25	Indifference	2.25	Indifference
	IN3	1.25	Indifference	2.25	Indifference
	Ceftriaxone	0.31	Synergy	0.37	Synergy
	Erythromycin	0.28	Synergy	0.26	Synergy

Table 3.7, continued

RN7-IN7	RN7-IN6	0.50	Synergy	0.50	Synergy
	Indolicidin	1.25	Indifference	1.25	Indifference
	IN1	0.75	Indifference	1.25	Indifference
	IN2	2.25	Indifference	0.75	Indifference
	IN3	1.25	Indifference	2.25	Indifference
	Ceftriaxone	0.50	Synergy	0.50	Synergy
	Erythromycin	0.37	Synergy	0.37	Synergy
RN7-IN6	Indolicidin	1.25	Indifference	2.25	Indifference
	IN1	1.25	Indifference	0.75	Indifference
	IN2	0.75	Indifference	1.25	Indifference
	IN3	2.25	Indifference	0.75	Indifference
	Ceftriaxone	0.37	Synergy	0.31	Synergy
	Erythromycin	0.28	Synergy	0.28	Synergy
Indolicidin	IN1	1.25	Indifference	0.75	Indifference
	IN2	2.25	Indifference	0.75	Indifference
	IN3	2.25	Indifference	1.25	Indifference
	Ceftriaxone	0.37	Synergy	0.50	Synergy
	Erythromycin	0.31	Synergy	0.50	Synergy
IN1	IN2	1.25	Indifference	1.25	Indifference
	IN3	2.25	Indifference	0.75	Indifference
	Ceftriaxone	0.37	Synergy	0.50	Synergy
	Erythromycin	0.31	Synergy	0.31	Synergy
IN2	IN3	2.25	Indifference	1.25	Indifference
	Ceftriaxone	0.50	Synergy	0.28	Synergy
	Erythromycin	0.31	Synergy	0.31	Synergy
IN3	Ceftriaxone	0.37	Synergy	0.31	Synergy
	Erythromycin	0.26	Synergy	0.31	Synergy

[a]FIC index ≤ 0.5 represents synergy; > 0.5 – ≤4.0 represents indifference; > 4.0 represents antagonism.

Highlighted in green: peptide-peptide combination with synergistic effect.

Highlighted in yellow: peptide-antibiotic combination with synergistic effect.

3.2.4 Killing kinetics of peptides against *S. pneumonaie*

The bactericidal effects of the designed peptides was further evaluated by assessing the time course taken to kill pneumococcal cells of susceptible and resistant clinical strains at concentrations of 1× the respective MIC. Two antibiotics erythromycin and ceftriaxone were also included as standard drug control to compare the antipneumococcal activity of peptides. Surviving colony forming units (CFU) at several time points post treatments were enumerated and expressed as mean log decrease (CFU/ml).

Like the MIC results, Hybrid peptides presented robust and rapid killing kinetics as compared to standard antibiotics erythromycin and ceftriaxone. Among all the hybrid peptides, RN7-IN10 was the most effective candidate eradicating 10^6 CFU/ml of both susceptible and resistant *S. pneumoniae* at a concentration of 1×MIC over a period of 30 min post-treatment (Figure 3.1A and C). RN7-IN8 was able to eliminate 10^6 CFU/ml of bacterial suspension of both isolates after 90 min post-treatment (Figure 3.1A and C). RN7-IN7 achieved maximum killing of susceptible and resistant strains after 120 and 150 min respectively (Figure 3.1A and C). Similarly, peptide RN7-IN9 was able to eliminate susceptible pneumococcal suspension (10^6 CFU/ml) after 150 min post treatment. Nevertheless, it was able to achieve maximum clearance of resistant bacteria after 240 min of incubation (Figure 3.1A and C). RN7-IN6 showed the weakest killing kinetic among all the hybrid peptides, taking 240 min to eradicate the bacterial suspension of susceptible pneumococci. However, this peptide failed to eliminate the suspension of resistant cells up to 240 min post treatment (Figure 3.1A and C).

In terms of Indolicidin analogs, the killing kinetics of both IN1 and IN2 were slower than their template peptide. They required 240 min to completely kill susceptible pneumococci, whereas the parent peptide indolicidin and IN3 required 180 min to eradicate 10^6 CFU/ml of pneumococcal suspension (Figure 3.1B). Unlike their parent

peptide, none of the indolicidin analogs was able to totally clear bacterial suspension of resistant pneumococci, but they were able to reduce it by two logs (Figure 3.1D). With regard to the standard antibiotics, erythromycin and ceftriaxone totally eliminated pneumococcal cells of the sensitive isolate at 120 and 150 min post-treatment respectively and failed to completely eradicate the cells of the resistant isolate up to 240 min post incubation (Figure 3.1C and D).
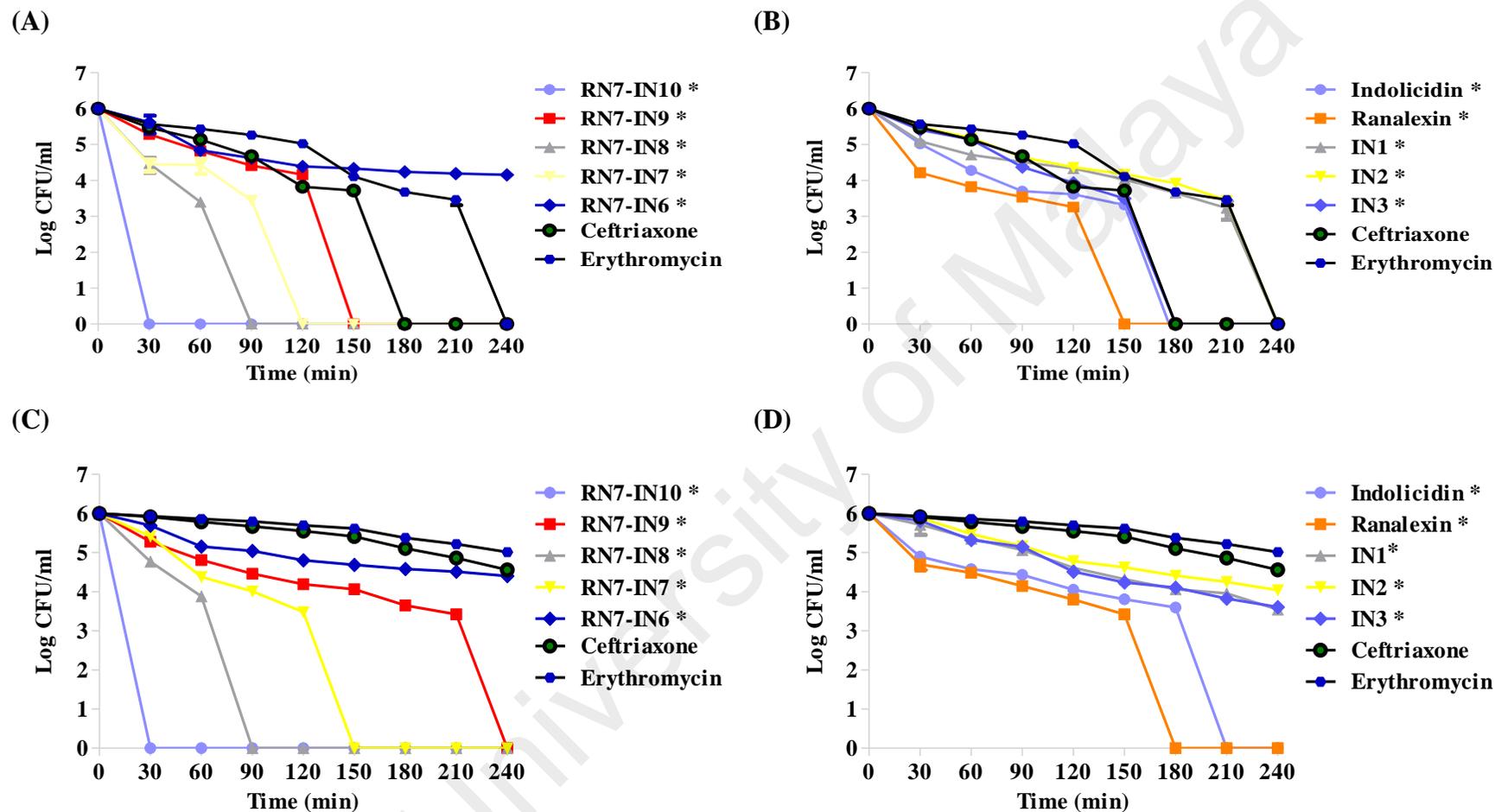
Figure 3.1: Killing kinetics of the designed AMPs at 1× their respective MIC. Killing kinetics of AMPs against susceptible isolate to both erythromycin and ceftriaxone (A and B, respectively). Killing kinetics of AMPs against resistant isolate (C and D). All the designed AMPs displayed rapid bactericidal activity than conventional antibiotics at their MIC values (Two-way ANOVA with Bonferroni post-test was used to analyze the significance of difference). An asterisk (*) adjacent to AMP name specifies statistical analysis significance ($P < 0.0001$).

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3.3 *In vitro* toxicity assessment

3.3.1 Hemolytic effects of the designed AMPs against human erythrocytes

The hemolytic assay was used to assess the lysing effect of the designed AMPs (with MIC values of ≤ 62.5 mg/ml) against freshly collected human erythrocytes at varying concentrations (1.95-250 μg/ml). All the tested peptides did not exhibit any hemolytic effect at their respective MIC values. RN7-IN7 was the least hemolytic peptide among all the hybrids, it did not show any hemolytic effect up to a concentration of 250μg/ml (Figure 3.2A). Hybrid peptides RN7-IN10, RN7-IN9, RN7-IN8, and RN7-IN6 displayed no or vey minimum effects at their MICs. At a concentration of 15.62 μg/ml their hemolytic effects were 5.9 %, 0.0 %, 0.0 %, and 0.0 % respectively (Figure 3.2A). However, at 250 μg/ml RN7-IN10 and RN7-IN8 were the most hemolytic of the hybrid peptides with effect of 93.7 % and 96.91 %, respectively (Figure 3.2A). The hemolytic effects of RN7-IN9 and RN7-IN6 were 39.57 % and 76.8 %, respectively (Figure 3.2A)

In terms of indolicidin analogs, IN1, IN2 and IN3 did not reveal any hemolytic effects toward human RBCs at their respective MIC values (Figure 3.2B). At 250 μg/ml, the toxic effects of IN1 and IN3 were 3.6 and 2.39% respectively. About Fifteen percent (15.39 %) of RBCs were lysed when treated with indolicidin analog IN2 at a concentration of 250 μg/ml. In contrast, the parent peptide indolicidin exhibited a relatively high hemolytic effect of 56.98 % at 250 μg/ml, while 14.3 % of human RBCs were lysed when treated with parent peptide ranalexin at 250 μg/ml (Figure 3.2B).

(A)
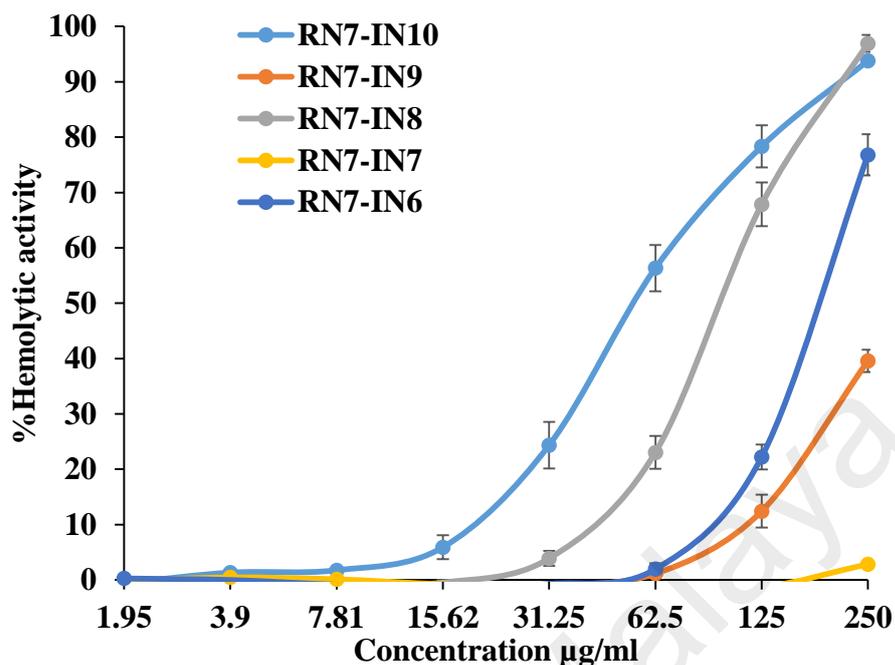
(B)
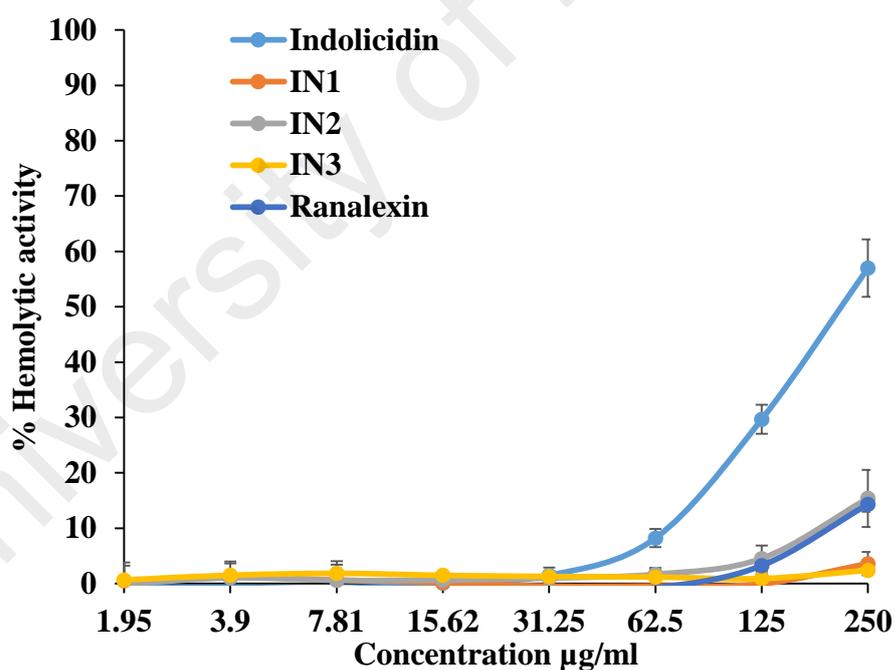
Figure 3.2: Hemolytic activity of the designed peptide against freshly collected human RBCs. (A) hybrid AMPs and (B) indolicidin analogs along with parent peptides. Hemolysis was determined by measuring hemoglobin absorbance at 560 nm in the supernatant presented as percentage hemolysis achieved with 0.1% Triton X-100. The data plotted are average values ± SD of three independent experimental trials.

3.3.2 Cytotoxicity of the designed AMPs against human cells

In addition to their effect on human erythrocytes, the cytotoxic effects of the designed peptides (MIC value of 62.5 µg/ml or less) on the viability of two human cell lines NL-20 normal human lung cells and WRL-68 normal human liver cells after 24, 48, and 72 hr of treatment were assessed. The results demonstrated that up to 72 hr of incubation none of the tested peptides displayed any toxic effect with both cell lines at their MIC values. The viability of NL-20 cell line was not affected (< 50 %) by incubation with hybrid peptides at a concentration of 62.5 µg/ml, yet, over 50 % cell death was remarked upon incubation with the hybrids at a concentration of 125-250 µg/ml (Figure 3.3A-C). RN7-IN7 was the least toxic peptide among all the hybrid peptides, it did not reveal any toxic effect against NL-20 cells up to a concentration of 125 µg/ml (Figure 3.3A-C). In terms of WRL-68 cell line, the hybrids exhibited strong cytotoxicity (> 50 %) at a concentration of 62.5 µg/ml which is 3-4 times higher than the MIC values of these peptides (Figure 3.4A-C). Hybrid peptide RN7-IN7 was the least toxic among them, it didn't reveal any toxic effect up to a concentration of 62.5 µg/ml (Figure 3.4A-C).

In terms of indolicidin analogs, IN1, IN2 and their parent peptide did not show any toxic effects toward both NL-20 and WRL-68 cells up to a concentration of 62.5 µg/ml (Figures 3.3D-F and 3.4D-F). however, they displayed a high level of cytotoxicity at a concentration of ≥ 125 µg/ml. Among all the indolicidin analogs, IN3 was the least toxic analog against both cell lines. IN3 did not exhibit any toxicity up to a concentration of 125 µg/ml. (Figures 3.3D-F and 3.4D-F).
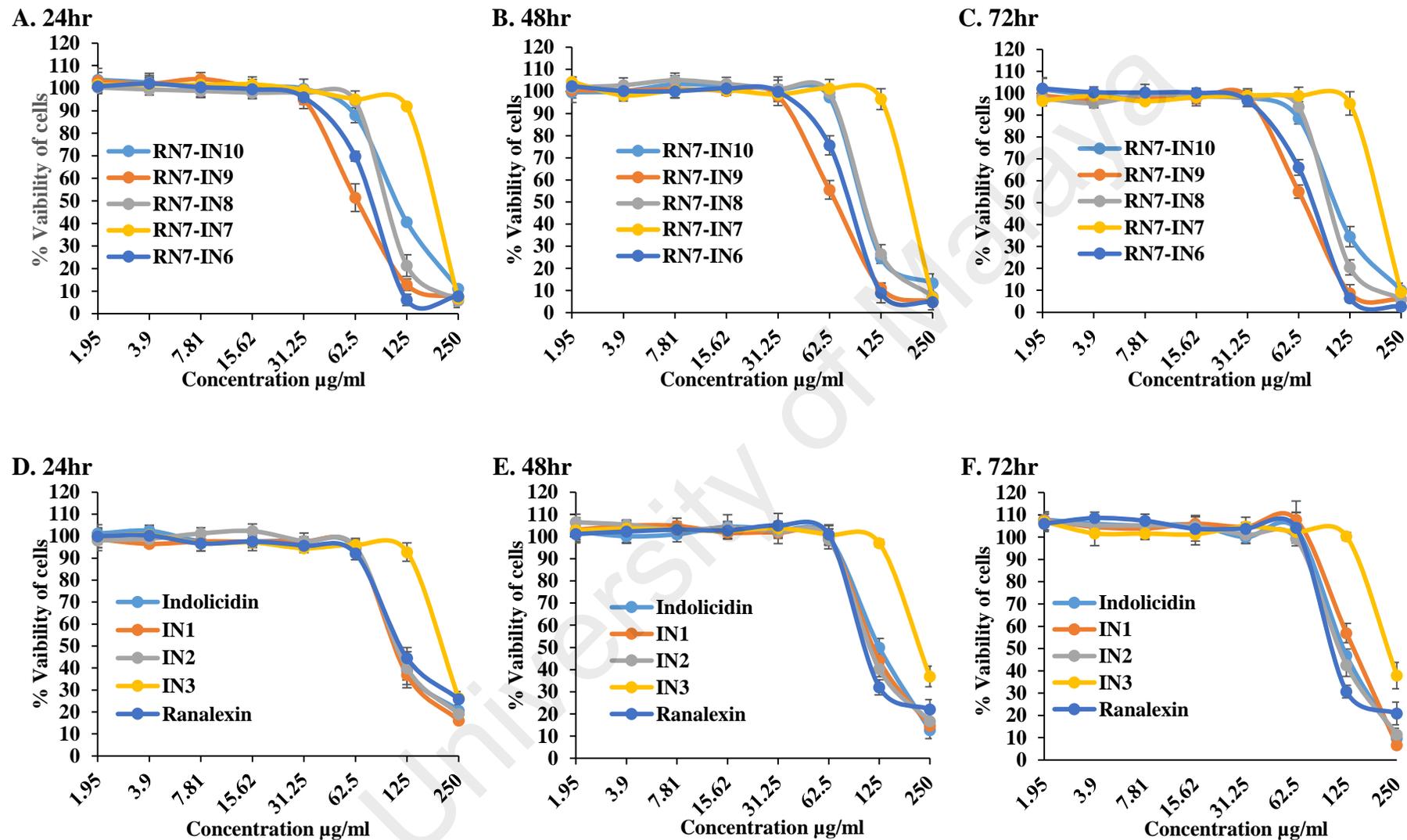
Figure 3.3: Viability of NL-20 cell line incubated with designed peptides at various concentrations for 24, 48, and 72 hr. (A, B, and C) represent Hybrid peptides, (D, E, and F) represent indolicidin and its analog peptides. The data plotted are average values from three independent experimental trials.

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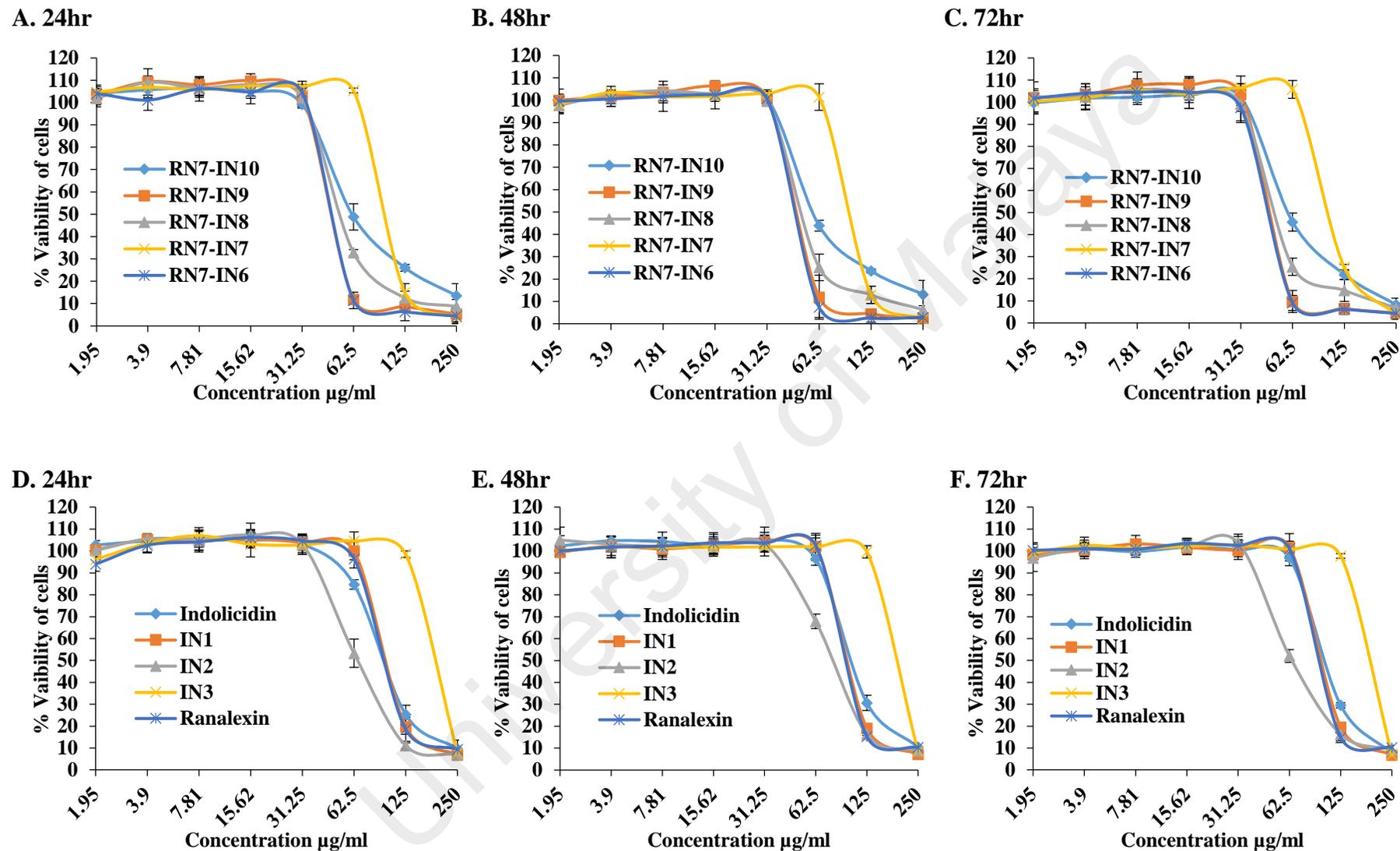
Figure 3.4: Viability of WRL-68 normal liver cell line incubated with designed AMPs at various concentrations for 24, 48 and 72 hr (A, B, and C) represent hybrid peptides, (D, E, and F) represent indolicidin and its analog peptides. The data plotted are average values from three independent experimental trials.

3.4 *In silico* molecular docking of peptides

Molecular docking was performed to evaluate the affinity of the designed AMPs toward three major pneumococcal virulent factors Lyta, Ply and PspA. The x-ray structure of homodimer in chain A and B was used for autolysin while pneumolysin and pneumococcal surface protein A (PspA) were homology modeled using SWISS-MODEL. The results obtained from rigid docking using Autodock Vina software showed that all hybrid peptides have strong affinity toward virulence proteins autolysin, pneumolysin and PspA and were within the negative binding affinity range. The binding affinity ranged from -7.0 to (-3.7), -8.9 – (-3.9), and -9.4 – (-3.6) for the target proteins, respectively (Table 3.8).

Table 3.8: Binding affinity of hybrid peptides with pneumococcal virulent proteins by autodock Vina.

Peptide	Binding affinity (kcal/mol)		
	Autolysin	**Pneumolysin**	**PspA**
Indolicidin(natural peptide)	-7.0 – (-5.6)	-8.9 – (-6.9)	-8.7 – (-6.2)
Indolicidin (lab)	-6.4 – (-4.3)	-8.5 – (-5.6)	-9.3 – (-6.5)
Ranalexin (natural)	-6.5 – (-3.7)	-7.7 – (-5.2)	-6.5 – (-3.6)
RN7IN10	-4.9	-6.4 – (-3.9)	-7.3
RN7IN9	-5.1 – (-4.3)	-7.7 – (-5.5)	-8.5 – (-5.9)
RN7IN8	-7.4 – (-5.3)	-7.3 – (-6.0)	-9.4 – (-6.6)
RN7IN7	-7.1 – (-4.2)	-8.4 – (-6.3)	-7.6 (-5.9)
RN7IN6	-8.7 – (-5.7)	-8.5 – (-7.1)	-7.8 – (-5.6)

Among all the hybrids, RN7-IN6 with shortest amino acid sequence, revealed the most potent binding affinity toward autolysin (-8.7) and pneumolysin (-8.5) (Table 3.8). The more negative and lower the value of binding affinity the stronger the bonds between receptor and peptide. In addition, the length of the designed AMPs has impact on the

stability and binding affinity. The minimized of lowest docking energy complexes of peptides with CHARMm force field against autolysin, pneumolysin, and PspA were visualized in discovery studio (Figures 3.5, 3.6, and 3.7 respectively). Since all the hybrid peptides showed a similar binding affinity, two peptides RN7-IN8 and RN7-IN6 were further analyzed. In the representation, peptide a- indolicidin (lab), b- ranalexin, c- RN7-IN6, and d- RN7-IN8 in blue, purple, yellow and orange, respectively. A close view of interactions has been depicted; whereas green dotted lines represented the hydrogen bonds. The details of van der Waal (VDW) and electrostatic with amino acids in 3 Å vicinity of the peptides, and total interaction energy (IE) value were tabulated (Tables 3.9, 3.10, and 3.11).

Among all the test peptides, the template peptide ranalexin displayed the strongest affinity -266.78, -232.72 and -206.52 kcal/mol toward autolysin, pneumolysin and PspA respectively. The contribution of the interaction mainly comes from the electrostatic interaction than van der Waals. For pneumolysin and PspA, RN7-IN6 exhibited stronger binding interaction (-177.23 and -202, respectively) compared to indolicidin (Table 3.10 and 3.11). Likewise, hybrid peptide RN7-IN8 revealed stronger binding affinity (-186.60) for PspA than its parent peptide indolicidin (Table 3.11).

Only one hydrogen bonding interaction (Figure 3.6) at ILE6 of RN7-IN6 between RN7-IN6:ILE6:HN and A:ALA370:O was found, and strong contributions (<-10 kcal/mol, Table 3.9) are from the interaction with ALA370, TYR371, THR405, CYS428, THR429, GLY430, LEU431, ALA432 and TRP435 of pneumolysin. Several hydrogen bond interactions of the N-terminal of RN7-IN6 with PspA are illustrated in Figure 3.7. All the in vitro activity results along with molecular docking have been published in PLoS ONE journal (Jindal *et al.*, 2015).
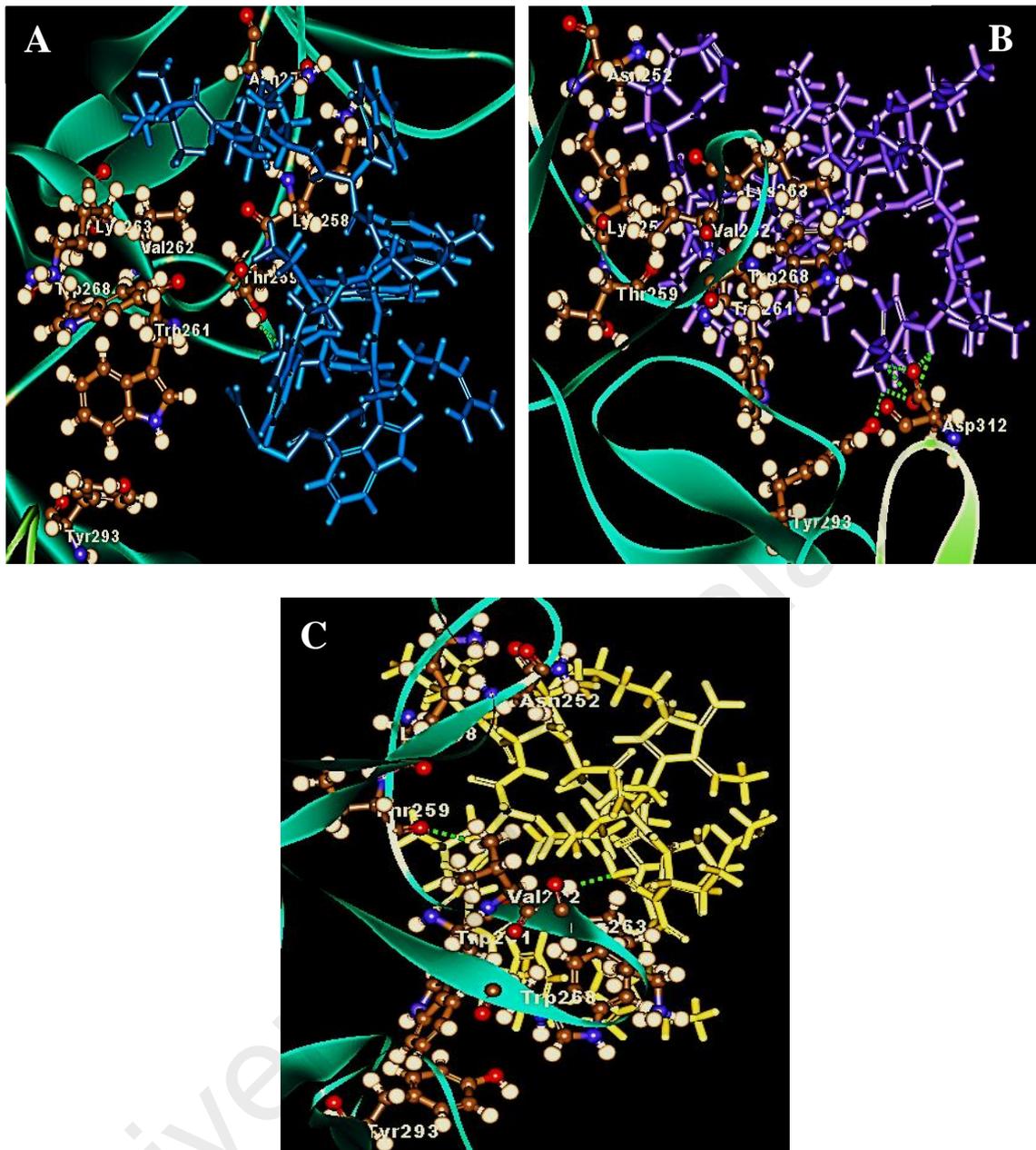
Figure 3.5: The interaction of autolysin with three peptides indolicidin, ranalexin, and RN7-IN6. (A) Hydrogen bonding interaction of indolicidin lab (blue). (B) Interaction of hydrogen bonds of ranalexin (purple). (C) Hydrogen bonds of RN7IN6 (yellow).
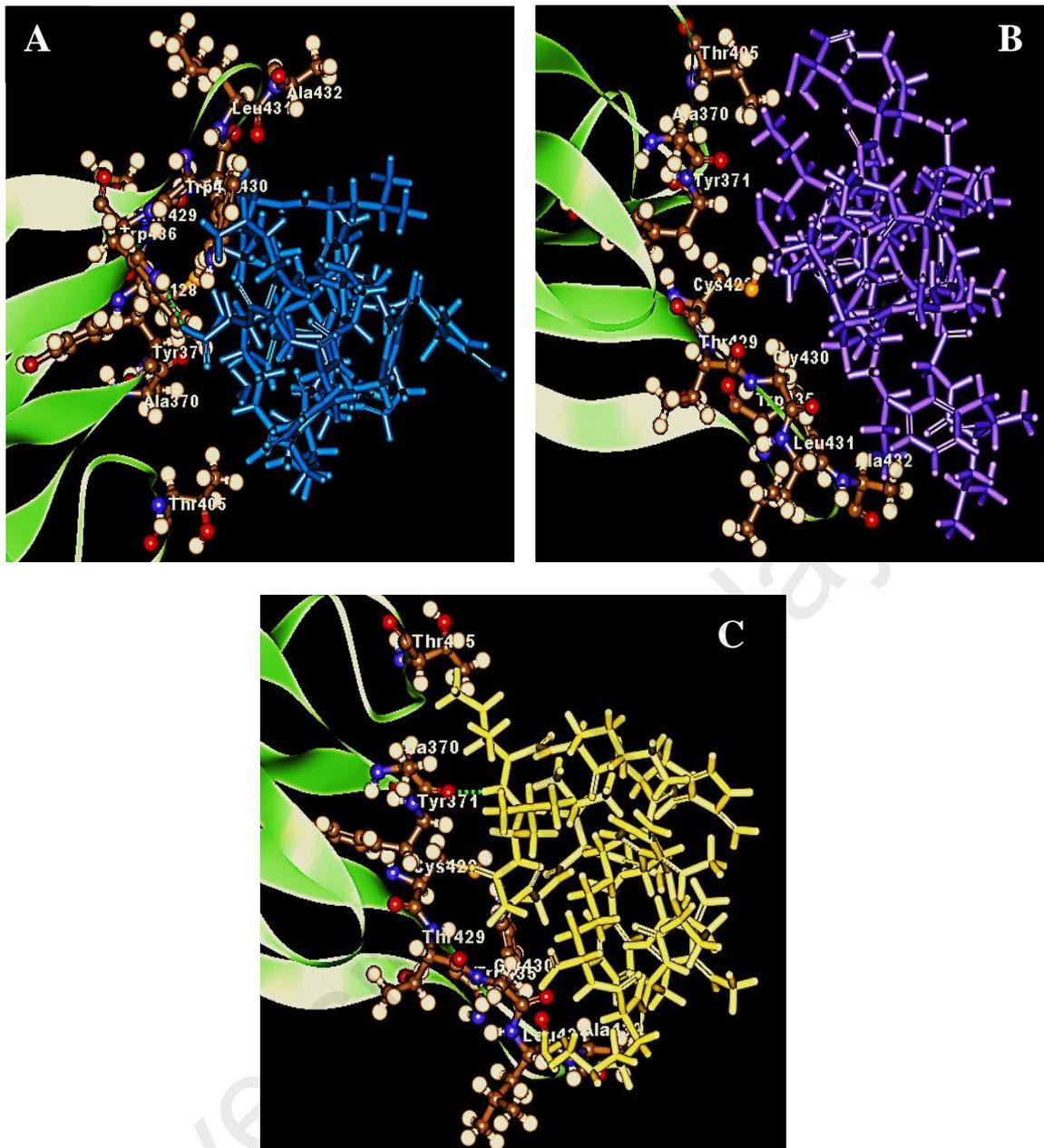
Figure 3.6: The interaction of pneumolysin and peptides. (A) Indolicidin lab (blue) showed one hydrogen bonding interaction at A:TRP436:HE1 and indolicidin lab:ARG13:OXT. (B) No hydrogen bond interaction for ranalexin (purple). (C) Hydrogen bond between RN7IN6 (yellow) and pneumolysin at RN7IN6:ILE6: HN-A:ALA370:O.
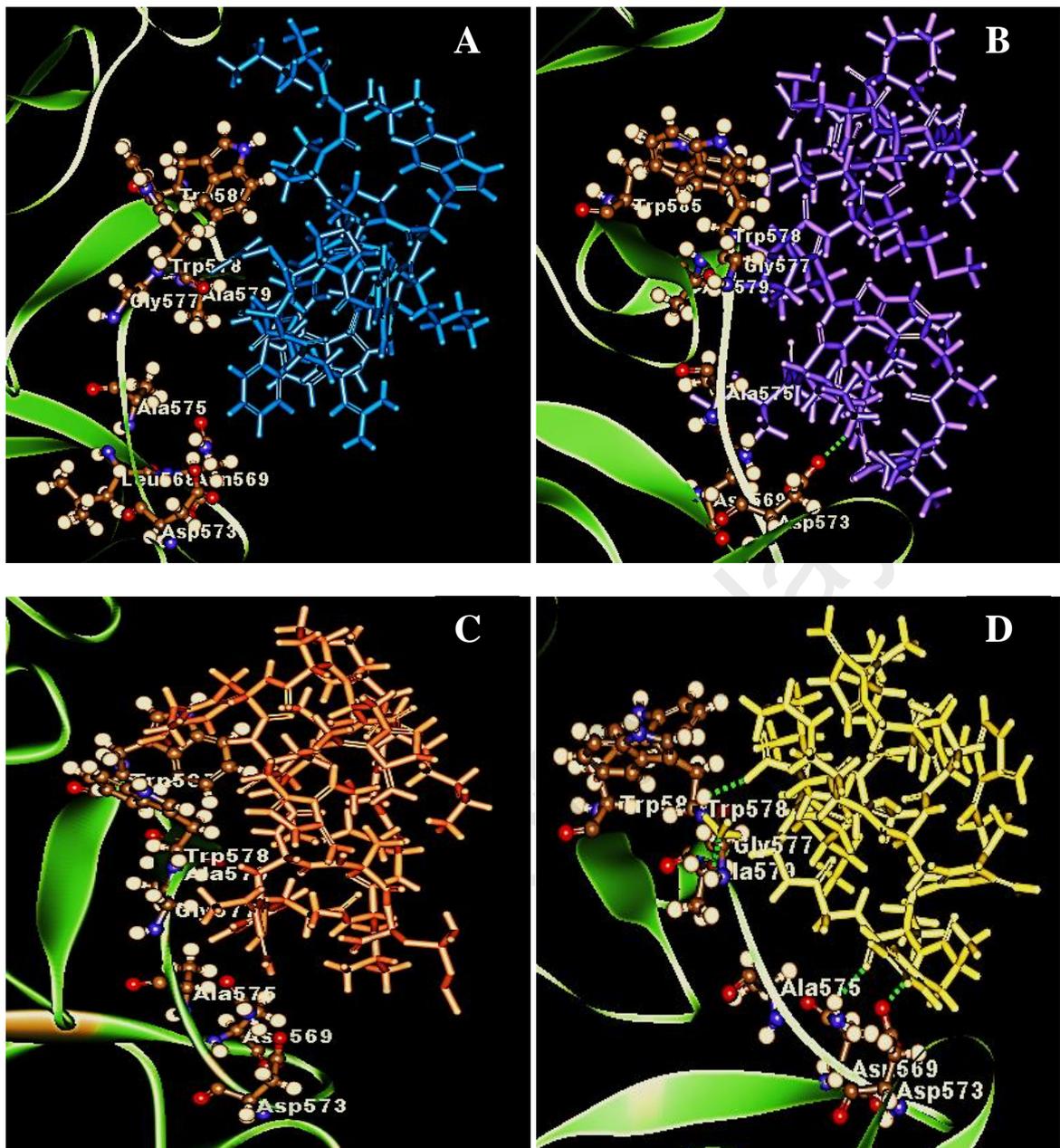
Figure 3.7: The interaction of pspA and peptides. (A) No hydrogen bonding interaction of indolicidin lab (blue) with pspA. (B) Hydrogen bond was observed at ranalexin:LYS7:HZ3-A:ASP573:OD2 for ranalexin (purple). (C) No hydrogen bond showed in RN7IN8 (orange). D RN7IN6 (yellow) has several hydrogen bonding interaction at A:ASN569:HD21–RN7IN6: LEU2:O, A:GLY577: HN–RN7IN6: NH214:N1, A:TRP578: HN–RN7IN6: ARG13:OXT and RN7IN6: LEU2: HN–A:ASP573:OD2.

Table 3.9: Contribution of the interactions energy in kcal/mol of the autolysin binding residues in the 3 Å from peptides.

Residue	Interaction Energy (IE)	VDW	Electrostatic	Residue	Interaction Energy (IE)	VDW	Electrostatic	Residue	Interaction Energy (IE)	VDW	Electrostatic
Indolicidin (lab) ILP**WKWPWWPWRR-NH$_2$**				Ranalexin **FLGGLIK**IVPAMICAVTKKC-OH				RN7IN6 FLGGLIK**WWPWRR-NH$_2$**			
B_TYR250	-25.35	-2.02	-23.33	A_ASP312	-193.44	1.39	-194.83	B_ASN252	-31.03	-1.68	-29.35
B_ASN252	-8.09	-2.17	-5.92	B_ASN252	-23.74	-1.26	-22.48	B_LYS258	-19.64	-5.97	-13.67
B_GLU253	-61.26	-1.14	-60.12	B_LYS258	-2.35	-6.31	3.96	B_THR259	-33.85	-1.99	-31.86
B_LYS258	-11.28	-3.98	-7.31	B_TRP261	-27.68	-3.52	-24.16	B_GLY260	8.58	-2.13	10.71
B_THR259	-13.85	-4.78	-9.07	B_VAL262	-16.49	-1.52	-14.96	B_TRP261	-26.87	-6.92	-19.95
B_GLY260	3.41	-2.37	5.77	B_LYS263	36.97	-1.97	38.94	B_VAL262	-14.95	-2.78	-12.17
B_TRP261	-22.07	-1.27	-20.80	B_TRP268	-10.34	-4.89	-5.45	B_LYS263	-31.42	0.10	-31.53
B_LYS263	7.75	-2.00	9.75	B_TYR293	-29.71	-1.13	-28.58	B_TRP268	-14.69	-4.77	-9.92
B_TYR264	-0.65	-0.92	0.27					B_TYR293	-14.90	-1.64	-13.25
B_TYR270	-15.30	-2.01	-13.29								
B_LEU271	-23.34	-0.63	-22.71								
B_ALA273	-12.43	-3.51	-8.92								
Total IE	-182.48	-26.80	-155.68	Total IE	-266.78	-19.23	-247.56	Total IE	-178.76	-27.76	-151.00

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Residue	Interaction Energy (IE)	VDW	Electrostatic	Residue	Interaction Energy (IE)	VDW	Electrostatic	Residue	Interaction Energy (IE)	VDW	Electrostatic
Indolicidin (lab) ILP**WKWPWWPWRR-NH₂**				Ranalexin **FLGGLIK**IVPAMICAVTKKC-OH				RN7IN6 FLGGLIK**WWPWRR-NH₂**			
A_GLN374	-29.91	-1.22	-28.69	A_ALA370	-19.43	-2.75	-16.68	A_ALA370	-17.76	-3.85	-13.90
A_TYR376	-9.97	-1.69	-8.28	A_TYR371	-22.13	-2.12	-20.01	A_TYR371	-10.00	-3.07	-6.93
A_ARG426	46.94	-3.27	50.20	A_VAL372	-8.38	-5.03	-3.35	A_VAL372	-9.50	-2.39	-7.11
A_LEU431	-6.80	-1.06	-5.74	A_ASP403	-67.39	-3.31	-64.08	A_THR405	-13.17	-1.79	-11.38
A_TRP433	1.05	-2.72	3.77	A_CYS428	-16.55	-1.46	-15.09	A_ALA406	-2.78	-0.11	-2.68
A_GLU434	-105.07	-7.07	-98.00	A_GLY430	-6.85	-2.21	-4.64	A_CYS428	-24.18	-1.50	-22.68
A_TRP435	-21.68	-2.42	-19.26	A_ALA432	-31.90	-1.67	-30.23	A_THR429	-13.30	-1.47	-11.83
A_TRP436	-18.76	-4.19	-14.57	A_TRP435	-19.21	-5.27	-13.94	A_GLY430	-23.40	-3.36	-20.04
A_ARG437	18.99	-5.21	24.20	A_TRP436	-23.80	-2.25	-21.55	A_LEU431	-12.73	-2.08	-10.65
A_THR438	-22.35	-3.50	-18.86	A_THR459	-17.08	-2.02	-15.06	A_ALA432	-29.16	-2.72	-26.45
								A_TRP435	-13.58	-3.37	-10.21
								A_THR459	-7.68	-1.49	-6.19
Total IE	-147.56	-32.34	-115.22	Total IE	-232.72	-28.09	-204.63	Total IE	-177.23	-27.18	-150.04

Table 3.11: Contribution of the interactions energy in kcal/mol of the PspA binding residues in the 3 Å from peptides.

Residue	Interaction Energy (IE)	VDW	Electrostatic	Residue	Interaction Energy (IE)	VDW	Electrostatic	Residue	Interaction Energy (IE)	VDW	Electrostatic	Residue	Interaction Energy (IE)	VDW	Electrostatic
Indolicidin (lab) ILP**W**K**W**P**WW**P**W**RR-NH₂				Ranalexin **FLGGLIK**IVPAMICAVTKKC-OH				RN7IN8 FLGGLIK**W**P**WW**P**W**RR-NH₂				RN7IN6 FLGGLIK**WW**P**W**RR-NH₂			
A_ASN569	-14.66	-1.63	-13.03	A_GLY543	2.25	-0.74	2.99	A_TYR567	-7.65	-1.28	-6.37	A_SER544	-7.56	-1.44	-6.12
A_TRP578	-14.97	-4.04	-10.93	A_SER544	1.21	-1.12	2.33	A_ASN569	-15.43	-1.16	-14.27	A_ASN569	-17.96	-1.39	-16.57
A_ALA579	0.54	-2.41	2.95	A_ASP573	-124.71	-2.37	-122.34	A_ASN571	-15.57	-0.90	-14.67	A_ASN571	-3.07	-1.89	-1.18
A_LYS580	7.26	-7.68	14.94	A_TRP578	-23.99	-1.77	-22.21	A_ALA575	-12.63	-1.45	-11.18	A_ASP573	-67.42	-0.81	-66.61
A_VAL581	-12.36	-4.74	-7.62	A_ALA579	-2.16	-1.58	-0.58	A_THR576	-23.96	-2.19	-21.77	A_ALA575	-26.33	-1.57	-24.76
A_HIS582	-10.20	-1.79	-8.41	A_LYS580	-38.34	-5.43	-32.91	A_GLY577	-7.43	-2.48	-4.95	A_GLY577	-10.09	-1.16	-8.93
A_GLY583	-18.75	-2.54	-16.21	A_VAL581	-8.74	-1.15	-7.60	A_TRP578	-19.56	-7.54	-12.02	A_TRP578	-30.05	-3.86	-26.20
A_ALA616	-9.98	-1.15	-8.82	A_TRP585	-12.04	-4.29	-7.75	A_LYS580	-8.77	-3.57	-5.19	A_ALA579	-13.30	-1.32	-11.98
A_GLY631	-9.86	-1.87	-7.99					A_TRP585	-20.10	-3.77	-16.33	A_LYS580	-8.66	-4.93	-3.73
								A_ASP601	-24.83	-1.75	-23.08	A_TRP585	-17.56	-1.62	-15.95
								A_TYR606	-16.29	-0.78	-15.51				
								A_LEU632	-14.38	-2.83	-11.56				
Total IE	-82.99	-27.86	-55.13		-206.52	-18.45	-188.07		-186.60	-29.71	-156.89		-202.00	-19.98	-182.03

3.5 Mechanisms of action of designed peptides

3.5.1 Effects of hybrid peptides on cell morphology

Transmission electron microscopy (TEM) studies were conducted to examine the effect of the designed peptides on the pneumococcal cell morphology. According to *in vitro* activity results, five hybrid peptides were selected to investigate their effects of action as these peptides showed the strongest antimicrobial activity. As shown in Figure 3.8A, the untreated cells appeared with complete cell wall and plasma membrane and therefore preserved the normal integral shape of *S. pneumoniae*. The pneumococcal capsular polysaccharide appeared as a thin layer sheltering the whole cell and the cytoplasm of the cell was compactly packed and occupied the entire space (Fig. 1A, arrow a). On the contrary, incubation of pneumococcal cells with hybrid peptides has led to an extreme damage to the bacterial surface. (Figure 3.8B – F). These damages manifested as changes in the morphology of the treated cells which were clearly visible. After 1hr of incubation, the hybrid peptides were able to breach the intact cell wall and/or plasma membrane causing membrane breakages and fragmentation (Figure 3.8B-F, arrow b). Additionally, TEM results revealed that treatment with hybrid peptides led to the leakage of the cytoplasmic components to the outer environment through the disruption of the cell wall. As a result, large halos were detected in the inner space of all these treated cells leading to cell wall collapse and death (Figure 3.8B-F, arrow c). The TEM results also revealed partial disconnection of the cell wall from the cell membrane in pneumococci treated with hybrid peptides especially those treated with RN7-IN9, RN7-IN8, RN7-IN7 and RN7-IN6 (Figure 3.8C-F, arrow d).

Figure 3.8: TEM images of pneumococcal strain belongs to serotype 1, resistant to both erythromycin and ceftriaxone incubated with hybrid peptides. Control cells without treatment appeared with normal shape (Fig1A, arrow a). Fig B – F display the damage of pneumococcal cells after 1hr incubation in presence of (B) RN7-IN10, (C) RN7-IN9, (D) RN7-IN8, (E) RN7-IN7 and (F) RN7-IN6. (Arrow b) Breakage and loss of cell wall/membrane fragments. (Arrow c) Leakage of cytoplasm and halo formation. (Arrow d) detachment of cytoplasmic membrane from pneumococcal cell wall. Bar indicates 200 nm.

Scanning electron microscopy (SEM) was used to visualize any potential surface damage of *S. pneumoniae* cells induced by the hybrid peptides after one hour of treatment. Untreated *S. pneumoniae* cells displayed a normal smooth surface with no fragments observed (Figure 3.9A, arrow a), whereas *S. pneumoniae* cells treated with hybrid peptides at 8×MIC for one hour displayed a disrupted surface likely to represent pore formation (Figure 3.9B – F, arrow b). All the treated cells appeared rough with bulging surfaces. Numerous fragments were observed in treated cells which a clear indication of the strong interaction of the hybrid peptides with their target pneumococcal membrane (Figure 3.9B – F, arrow b). The results from SEM suggest that the principal target of hybrid peptides is the cell membrane in which they could interrupt the integrity and result in permeabilization of the bacterial membrane, which is in agreement with the results obtained from the TEM study.
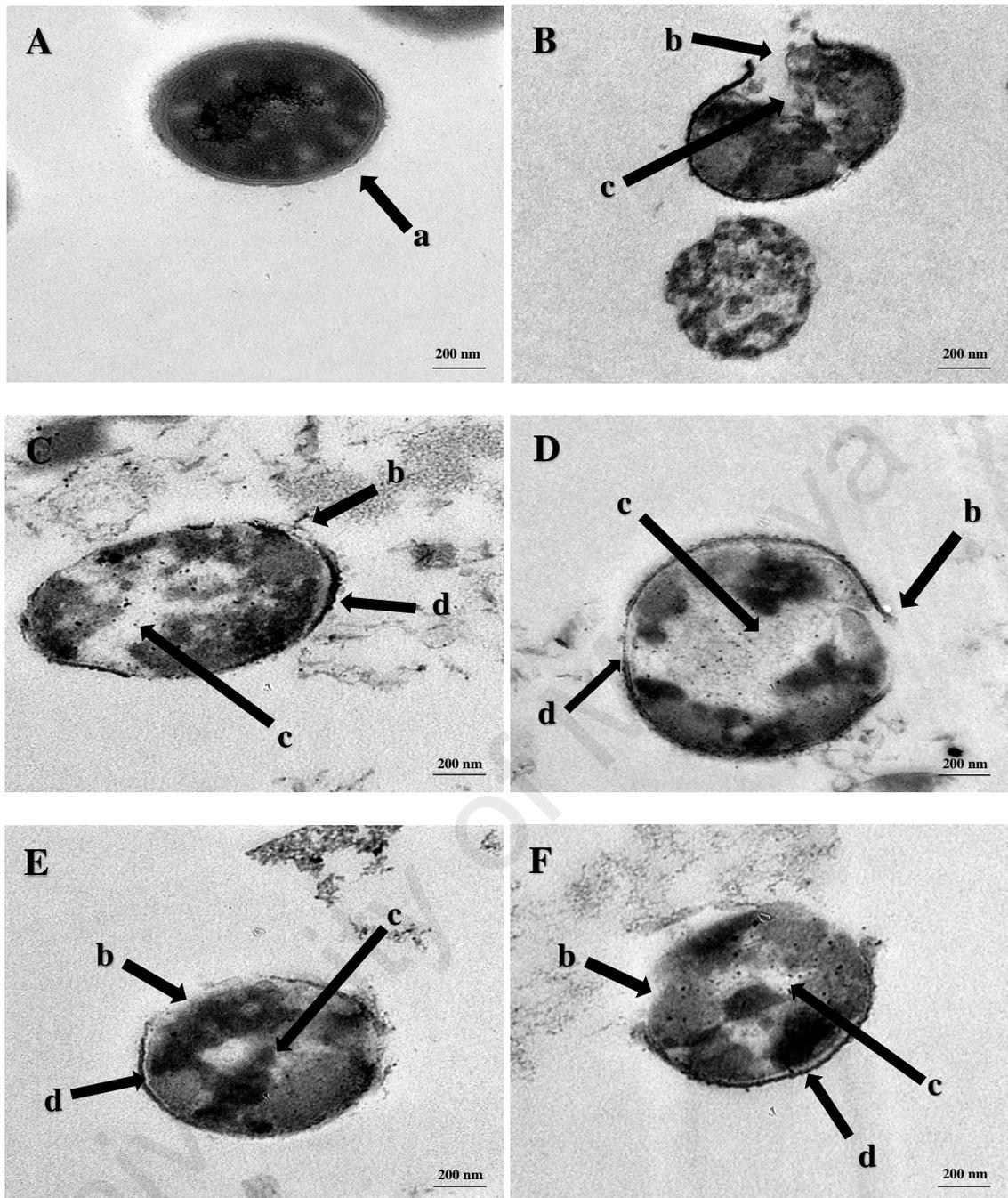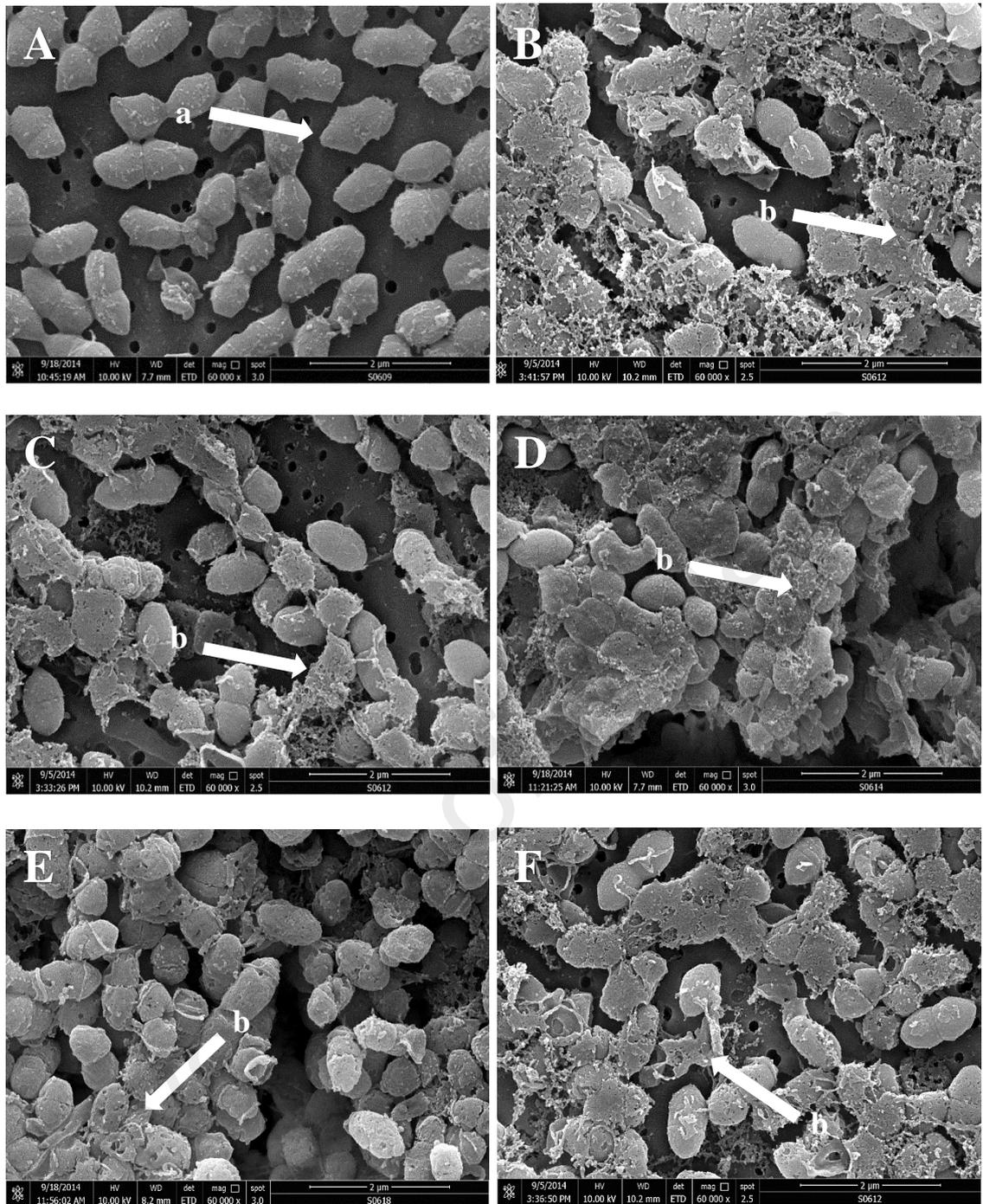
Figure 3.9: SEM images of pneumococcal strain belongs to serotype 1, resistant to both erythromycin and ceftriaxone treated with hybrid peptides. (A) Control cells without treatment appeared with normal shape and smooth surface (arrow a). Fig B – F show the severe morphological changes and surface disruption (arrow b) of pneumococcal cells following 1hr incubation in presence of (B) RN7-IN10, (C) RN7-IN9, (D) RN7-IN8, (E) RN7-IN7, and (F) RN7-IN6. Bar indicates 2 µm.

3.5.2 Effects of the hybrid peptides on membrane permeability

In order to evaluate the permeability of membrane and leakage of intracellular components upon treatment with hybrid peptides, the level of ATP in the supernatant following contact of the pneumococcal cells with hybrid peptides was determined using the ATP release assay. After 1h of treatment with hybrid peptide, the levels of ATP released after 1hr of treatment with RN7-IN10 and RN7-IN9 were the highest among the five hybrid peptides tested (54.5±4.7 and 42.27±92pM respectively) (Figure 3.10). The ATP efflux steadily decreased, and the ATP release reached 25.42±3.51 and 22.9±3.22pM after 3hr of treatment with RN7-IN10 and RN7-IN9 (Figure 3.10). The quantities of ATP released from pneumococcal cells upon incubation with RN7-IN8, RN7-IN7 and RN7-IN6 after 1hr were 39.03±0.2, 22.35±0.9 and 14.8±0.35. The levels of ATP release from pneumococcal cells treated with RN7-IN8, RN7-IN7 and RN7-IN6 were 20.54±1.03, 13.02±2.26 and 11.47±0.32pM respectively after 3hr of treatment (Figure 3.10). However, all the hybrid peptides showed better capacity in efflux ATP from pneumococcal cells in comparison with standard antibacterial drugs ceftriaxone and erythromycin. The efflux levels of ATP by ceftriaxone and erythromycin treated cells after 1hr of incubation were 5.24±1.43pM and 0.49±0.004pM, respectively (Figure 3.10).
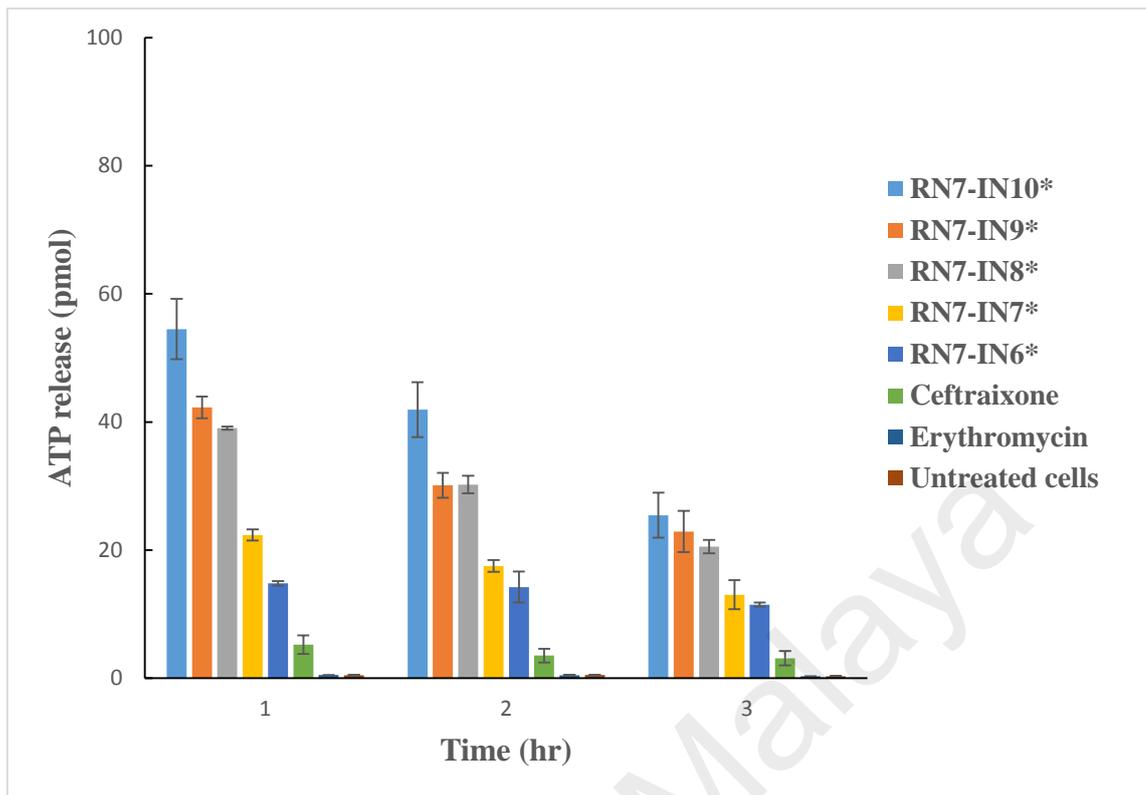
Figure 3.10: The influence of peptides on ATP release. Pneumococcal cells belong to serotype 1 resistant to both erythromycin and ceftriaxone were treated with hybrid peptides or standard drugs for the indicated times. All Hybrid peptides presented stronger ATP efflux activity than erythromycin and ceftriaxone. Two-way ANOVA with Bonferroni post-test was used to perform the statistical analysis. An asterisk (*) adjacent to peptide name directs statistical significance ($P < 0.0001$).

3.5.3 DNA binding activity

In order to clarify the effect of the hybrid peptides on the intracellular components of the pneumococcal cell, the DNA-binding affinity of the peptides was assessed by analyzing electrophoretic mobility of genomic DNA bands at various peptide/DNA weight ratios on an agarose gel (1%, w/v). The results clearly indicate that all the hybrids were capable of inhibiting DNA migration at a concentration of 62.5 µg/ml (Figure 3.11A – E). On the other hand, standard drugs ceftriaxone and erythromycin could not prevent the migration of DNA band through the agarose gel up to a concentration of 250 µg/ml (Figure 3.11F & G). These results suggest that the hybrid peptides could possess another intracellular mechanism of inhibiting the bacteria besides their main mechanism of targeting the bacterial membrane.
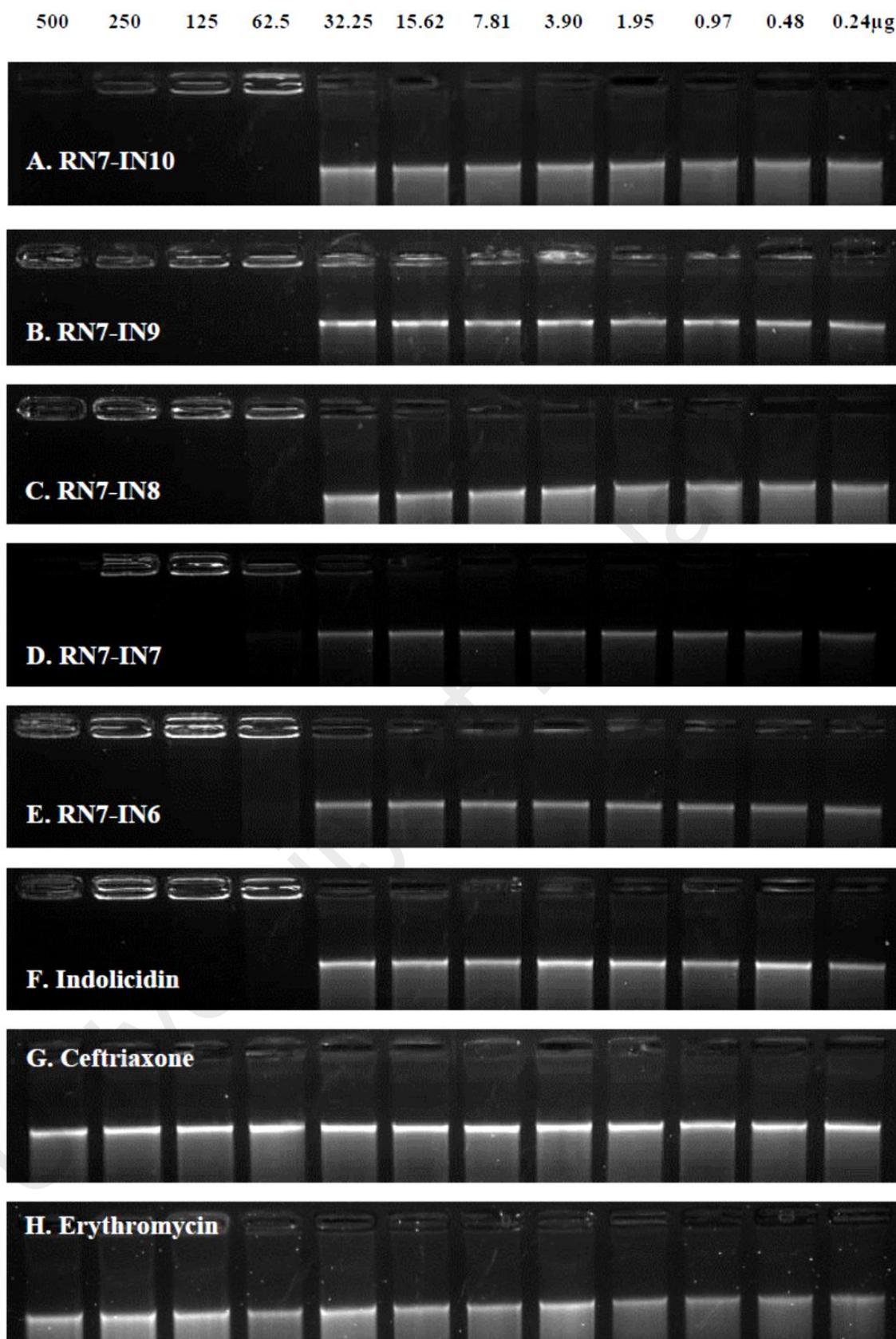
Figure 3.11: The impact of the hybrid peptides on the migration of genomic DNA. All hybrid peptides and parent template indolicidin prevented the migration of the DNA through the gel at 62.5 µg/ml. RN7-IN10 (A), RN7-IN9 (B), RN7-IN8 (C), RN7-IN7 (D), RN7-IN6 (E) and indolicidin (F). While ceftriaxone (F) and erythromycin (G) failed to stop the migration of genomic DNA up to a concentration of 500 µg/ml.

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3.6 *In vivo* toxicity assessment of peptides

The efficiency of four hybrid peptides (RN7-IN10, RN7-IN9, RN7-IN8 and RN7-IN6) were further assessed for their *in vivo* toxicity in ICR male mice. Each of the four peptides were given 100mg/kg subcutaneously, 100mg/kg intraperitonealy and 20mg/kg intranasaly at 1hr, 12hr, and 24hr. The immediate behavioral and physical abnormalities in mice were recorded for the observations of physical stress, shortness of breath, lethargy, and physical inactiveness (Table 3.12). Any dose that caused death or high stress to the mice was ignored and lower graded dose was attempted until an appropriate dose which caused minimum/no significant stress or inactiveness to the mice was selected. At day 7 post treatment, the experiment was terminated and all mice were euthanized. Whole blood was collected for heamatogram analysis: the parameters were number of red cells (RBC), number of white cells (WBC), lymphocytes, monocytes, eosinophil, granulocytes, haemoglobin (Hgb), mean corpuscular volume (MCV), hematocrit (HCT), platelet Counts (PLT), Mean corpuscular haemoglobin (MCH) and corpuscular haemoglobin concentration (MCHC). For serum biochemistry analysis, the parameters were alanine transaminase (ALT), creatinine, alkaline phosphatase (ALP), aspartate aminotransferase (AST), total bilirubin and urea. The five major organs (lung, kidney, brain, spleen, and liver) were harvested and processed for histological examination.

Mice injected with hybrid peptides via the IP route at the highest dose (100 mg/kg) displayed a number of behavioral and physical abnormalities (Table 3.12). The treated mice appeared highly stressed, lethargic and became inactive. Death was noted for mice treated with RN7-IN10 at 100 mg/kg (4/4 mice died) and at 80 mg/kg (2/4 mice died). For mice treated with RN7-IN9, death was noted at a concentration of 100 mg/kg and 80 mg/kg (4/4) and at 60 mg/kg (3/4 mice died). For mice treated with RN7-IN8, death was noted at 100 mg/kg and 80 mg/kg (4/4 mice died). Death also noted for mice treated with RN7-IN6 at a concentration of 100 mg/kg and 80 mg/kg (4/4) and at 60 mg/kg (2/4)

(Table 3.12). Lower graded doses were then attempted, and it was noted that mice treated with RN7-IN10 (20 mg/kg), RN7-IN9 (10 mg/kg), RN7-IN8 (20 mg/kg) and RN7-IN6 (10 mg/kg) did not display signs of physical stress and no death occurred in any of the treated groups (Table 3.12, highlighted in blue). Statistical analysis of serum biochemistry and blood heamatogram parameters displayed that mice injected with RN7-IN10 (20 mg/kg) and RN7-IN8 (20 mg/kg) did not reveal significant difference in any of the parameters as compared to the control group (Table 3.13). For mice treated with RN7-IN9 (10 mg/kg) significantly lower lymphocytes ($p = 0.0445$) and lower ALP ($p = 0.0187$) (Table 3.13, highlighted in yellow) were noted. RN7-IN6 treated mice had lower ALT ($p = 0.0425$) when compared to control group (Table 3.13, highlighted in blue). None of the five major organs of the treated mice revealed any significant histological abnormality as compared to the control group (Figure 3.12).

No death or allergic reaction reactions were seen with mice injected with hybrid peptides via subcutaneous (SC) route at the maximum dose (100mg/kg) up to seven days post-treatment. Mice treated with RN7-IN9 and RN7-IN8 showed no significant difference ($p > 0.05$) in all the parameters of blood haematogram and serum biochemistry as compared to the control group (Table 3.14). Mice treated with RN7-IN10 displayed significantly lower granulocytes ($p = 0.0172$) and ALP ($p = 0.0037$) (Table 3.14, highlighted in yellow), while mice treated with RN7-IN6 displayed significantly higher platelet counts ($p = 0.0487$) as compared to the control group (Table 3.14, Highlighted in blue). No histological abnormalities were detected in the organs of any group as all the tissue sections were normal and did not display differences with the control group (Figure 3.13).

Mice administrated with peptides at the highest deliverable dose of 20 mg/kg via IN route showed no physical and behavioral abnormality and no death occurred up to seven days post-treatment. Statistical analysis for whole blood haematogram and serum

biochemistry of mice treated with RN7-IN10 and RN7-IN8 showed no significant difference ($p > 0.05$) as compared to the control group (Table 3.15). Mice treated with RN7-IN9 displayed significantly lower MCV ($p = 0.001$) (Table 3.15, highlighted in yellow). Whereas RN7-IN6 treated mice revealed significantly lower percentage of granulocytes ($p = 0.0482$) as compared to the control group (Table 3.15, highlighted in blue). Histological examination of the organs collected from all the treated and control groups did not expose any histopathological changes (Figure 3.14).

Table 3.12: The physical and behavioral abnormalities observed in mice following administration of four hybrid peptides via three different routes.

Route	Peptide	Dose (mg/ml)	Observations	No. of dead mice
IP	RN7-IN10	100	Highly stressed and lethargic, highly inactive	4/4
		80	Highly stressed and lethargic, highly inactive	2/4
		60	moderately stressed and lethargic, moderately inactive	-
		40	slightly stressed and lethargic (resolved within 1h), physically active	-
		20	Slight discomfort resolved in 20 min, no signs of stress and lethargy, physically active	-
		10	Slight discomfort resolved in 20 min, no signs of stress and lethargy, physically active	-
	RN7-IN9	100, 80	Highly stressed and lethargic, highly inactive	4/4
		60	Highly stressed and lethargic, highly inactive	3/4
		40	moderately stressed and lethargic, moderately inactive	-
		20	slightly stressed and lethargic (resolved within 1h), physically active	-
		10	Slight discomfort resolved in 20 min, no signs of stress and lethargy, physically active	-
	RN7-IN8	100, 80	Highly stressed and lethargic, highly inactive	4/4
		60	moderately stressed and lethargic, moderately inactive	-
		40	slightly stressed and lethargic resolved within 1h), physically active	-
		20	Slight discomfort resolved in 20 min, no signs of stress and lethargy, physically active	-
		10	Slight discomfort resolved in 20 min, no signs of stress and lethargy, physically active	-
	RN7-IN6	100, 80	Highly stressed and lethargic, highly inactive	4/4
		60	Highly stressed and lethargic, highly inactive	2/4
		40	moderately stressed and lethargic, moderately inactive	-
		20	slightly stressed and lethargic resolved within 1h), physically active	-
		10	Slight discomfort resolved in 20 min, no signs of stress and lethargy, physically active	-
SC	RN7-IN10 RN7-IN9 RN7-IN8 RN7-IN6	100	No signs of stress and lethargy, physically active	-
IN	RN7-IN10 RN7-IN9 RN7-IN8 RN7-IN6	100	No signs of stress and lethargy, physically active	-

Highlighted in blue: highest deliverable dose of each hybrid peptide injected via IP route to the mice.

Abbreviations: IP, intraperitoneal; SC, subcutaneous; IN, intranasal.

Table 3.13: Whole blood heamatogram and serum biochemistry of mice injected with four hybrid peptides via IP route.

Parameter	IP treatment[a]				
	Control	RN7-IN10 (20 mg/kg)	RN7-IN9 (10 mg/kg)	RN7-IN8 (20 mg/kg)	RN7-IN6 (10mg/kg)
Whole blood					
Erythrocytes, RBC (10^6/mm^3)	8.895±0.86	9.16±0.92	8.06±1.04	8.14±0.34	8.71±0.44
Hemoglobin, Hgb (g/dl)	15.5±1.11	15.85±1.27	13.9±1.67	14.55±0.17	15.02±0.59
Mean corpuscular volume, MCV (μm^3)	52±3.16	50.75±2.06	51.5±2.64	52.75±0.95	50.5±1.29
Mean corpuscular haemoglobin concentration, MCHC (g/dl)	33.33±0.96	33.85±0.60	33.63±0.34	34.17±1.13	33.42±0.62
Mean corpuscular haemoglobin, MCH (pg)	17.28±0.78	17.6±0.46	17.25±0.78	17.92±0.56	16.67±1.07
platelet Counts, PLT (10^3/mm^3)	673.75±211.42	535±150.11	674±118.72	472.5±100.52	540.75±107.41
Hematocrit, HCT %	46.2±4.69	47.67±5.6	41.42±5.25	42.73±1.66	44.97±1.75
White blood cells, WBC (10^3/mm^3)	5.33±0.46	4.2±0.55	3.7±1.54	4.18±0.36	4.1±0.72
Lymphocytes %	62.12±6.9	51.03±16.69	43.25±4.07	53.7±8.22	54.6±3.8
Monocytes %	2.77±0.47	2.8±0.5	2.47±0.25	2.77±0.88	2.53±0.33
Granulocytes %	56.1±7.02	46.27±16.41	53.02±4.07	53.38±12.35	57.87±11.66
Eosinophil %	3.28±0.71	2.23±1.61	3.95±2.56	2.34±0.63	3.22±1.47
Serum biochemistry					
Aspartate aminotransferase, AST	168±23.33	181.66±38.27	172.33±26.35	148.9±21.51	176±19.31
Alanine transaminase, ALT	58.37±3.37	52.76±12.22	51.54±5.83	62.1±23.84	34.36.4±1.7
Alkaline phosphatase, ALP	144.5±17.29	124.33±10.6	89.76±10.7	115.4±34.9	114.97±24.9
Creatinine	39.85±2.67	38.1±6.97	37.3±3.51	39.2±3.81	41.47±4.2
Urea	9.86±1.67	9.6±2.11	10.56±5.2	10.34±2.36	8.97±1.17
Total bilirubin	2.05±0.1	1.82±0.03	1.9±0.02	1.98±0.11	2.1±0.1

[a] Given for three doses (1hr, 12hr, and 24hr).

One-way ANOVA with *post hoc* Dunnett-t test was used to find Statistical difference between treated mice with different peptides vs untreated control.

Mean value (s) showing significant difference ($p \leq 0.05$) as compared to the control group was highlighted:

Highlighted in yellow: RN7-IN9 treated mice (lymphocytes, $p = 0.0445$; ALP, $p = 0.0187$).

Highlighted in blue: RN7-IN6 treated mice (ALT, $p = 0.0425$).

Figure 3.12: Histology of kidney harvested from mice treated with hybrid peptides via IP route for *in vivo* toxicity assessment. No significant abnormality was observed between (A) control group (injected with PBS) and mice treated with maximum non-toxic dose of (B) RN7-IN10 (20mg/kg), (C) RN7-IN9 (10mg/kg), (D) RN7-IN8 (20mg/kg), and (E) RN7-IN6 (10mg/kg). All kidney sections showed normal structure with normal glomerulus, proximal convoluted tubules and distal convoluted tubules. Hematoxylin and eosin stain. Bar indicates 100 µm.

Figure 3.12 (continued): Histology of liver harvested from mice treated with hybrid peptides via IP route for *in vivo* toxicity assessment. No significant abnormality was observed between (A) control group (injected with PBS) and mice treated with maximum non-toxic dose of (B) RN7-IN10 (20mg/kg), (C) RN7-IN9 (10mg/kg), (D) RN7-IN8 (20mg/kg), and (E) RN7-IN6 (10mg/kg). All liver sections of the treated groups showed normal hepatic structure with central vain and hepatocytes. Hematoxylin and eosin stain. Bar indicates 100 µm.
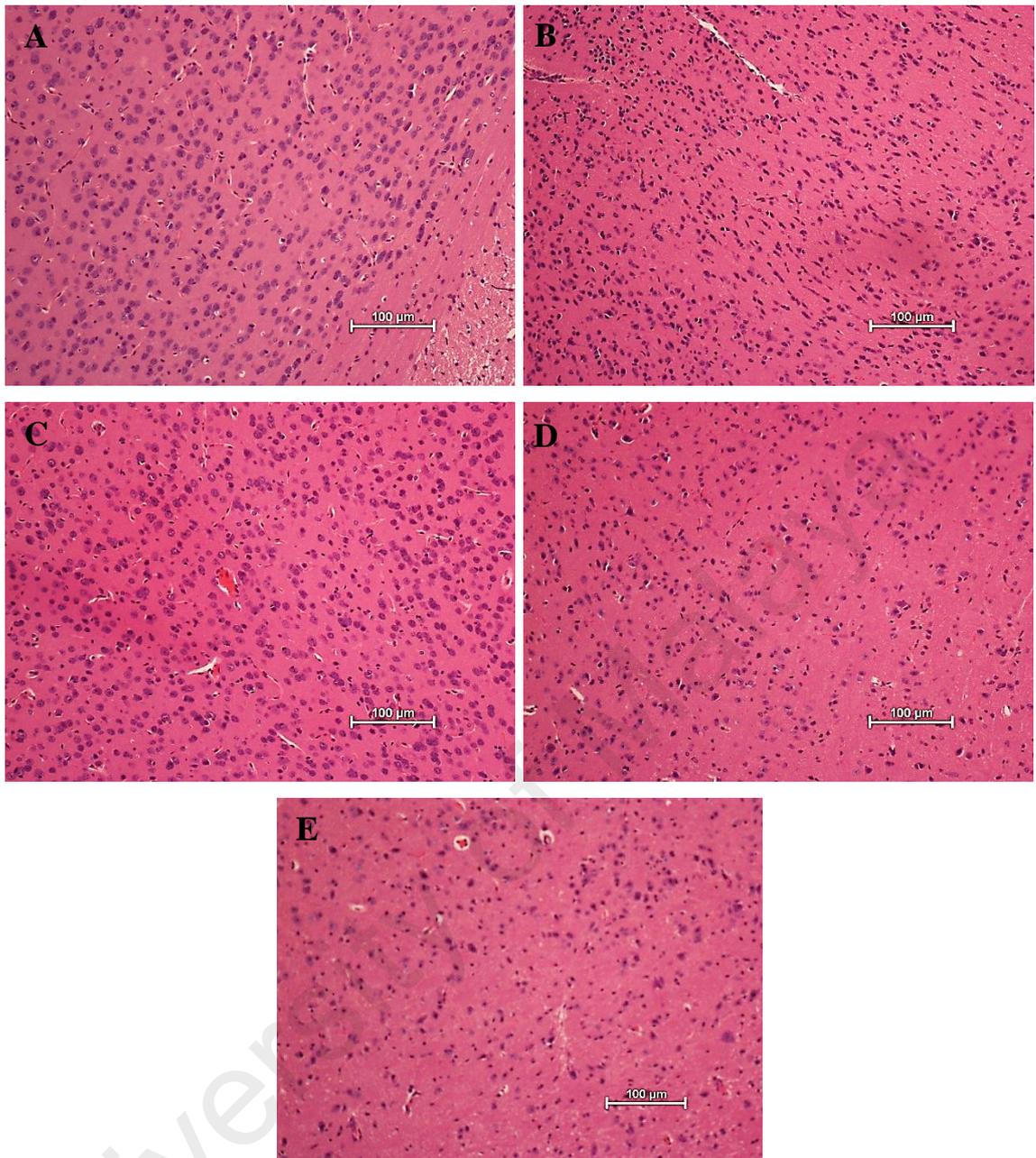
Figure 3.12 (continued): Histology of brain harvested from mice treated with hybrid peptides via IP route for *in vivo* toxicity assessment. No significant abnormality was observed between (A) control group (injected with PBS) and mice treated with maximum non-toxic dose of (B) RN7-IN10 (20mg/kg), (C) RN7-IN9 (10mg/kg), (D) RN7-IN8 (20mg/kg), and (E) RN7-IN6 (10mg/kg). All brain sections showed normal structure with no sign of inflammation or neuronal injury. Hematoxylin and eosin stain. Bar indicates 100 µm.
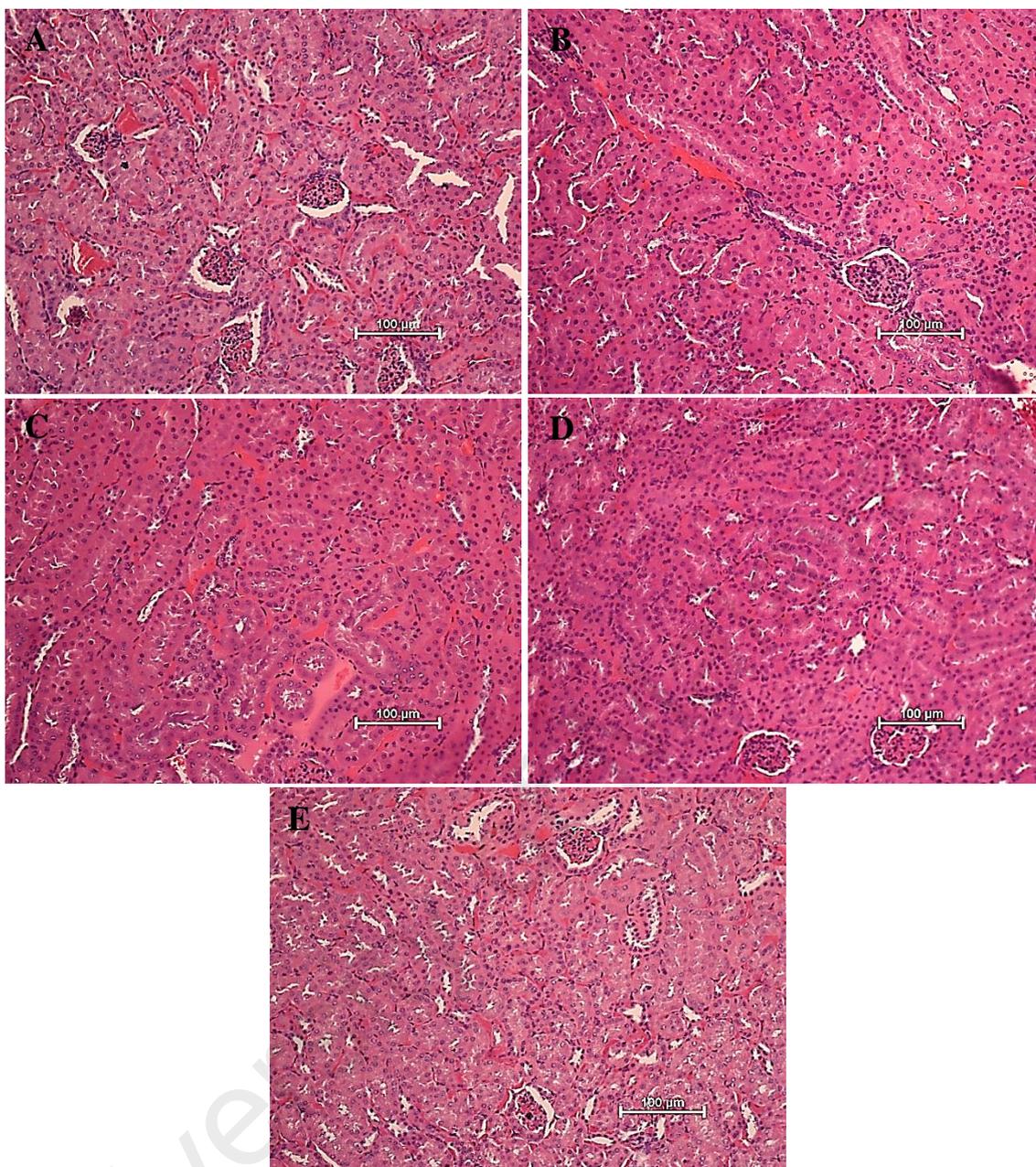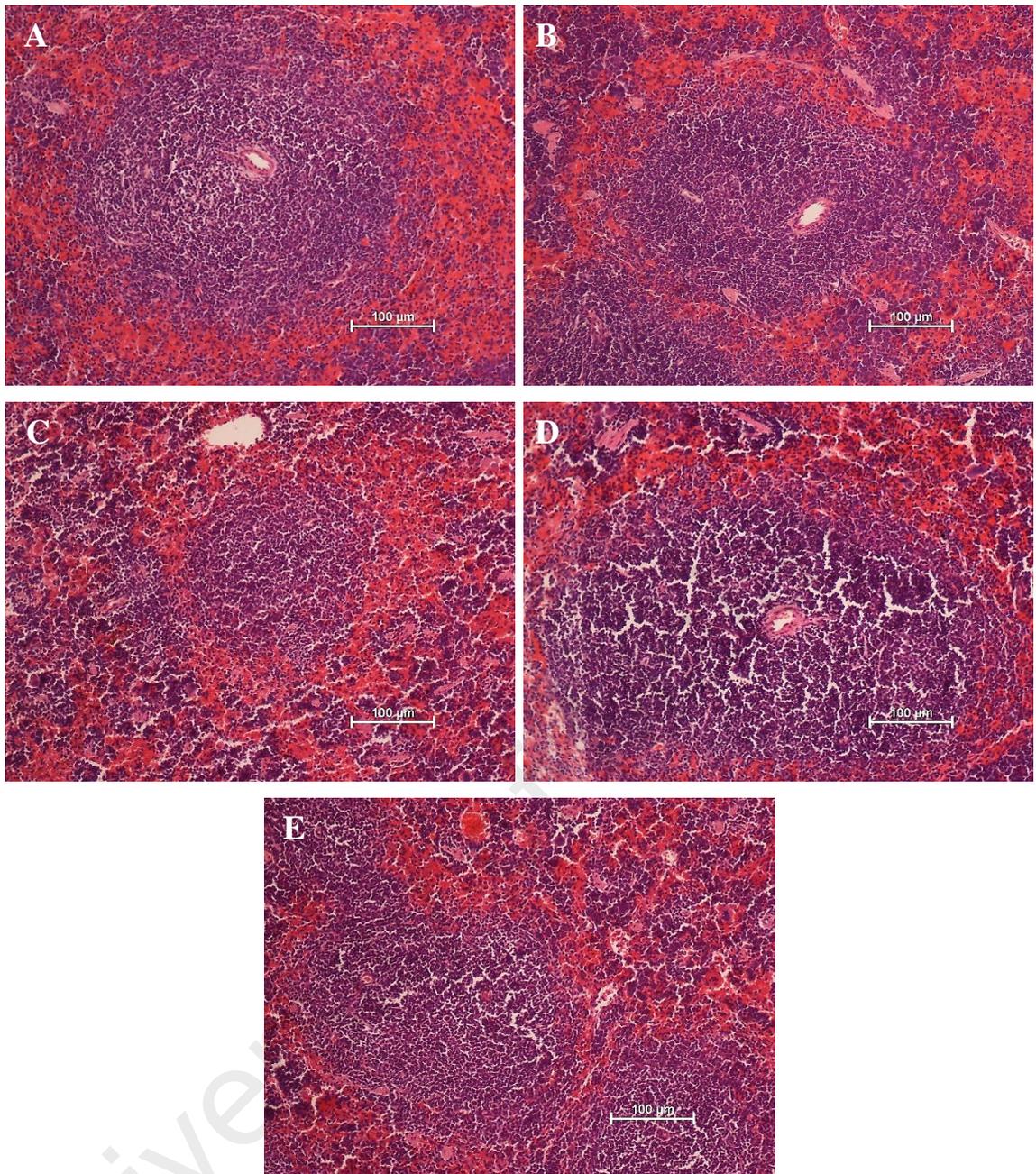
Figure 3.12 (continued): Histology of spleen harvested from mice treated with hybrid peptides via IP route for *in vivo* toxicity assessment. No significant abnormality was observed between (A) control group (injected with PBS) and mice treated with maximum non-toxic dose of (B) RN7-IN10 (20mg/kg), (C) RN7-IN9 (10mg/kg), (D) RN7-IN8 (20mg/kg), and (E) RN7-IN6 (10mg/kg). All spleen sections showed normal structure with normal white and red pulps. Hematoxylin and eosin stain. Bar indicates 100 µm.
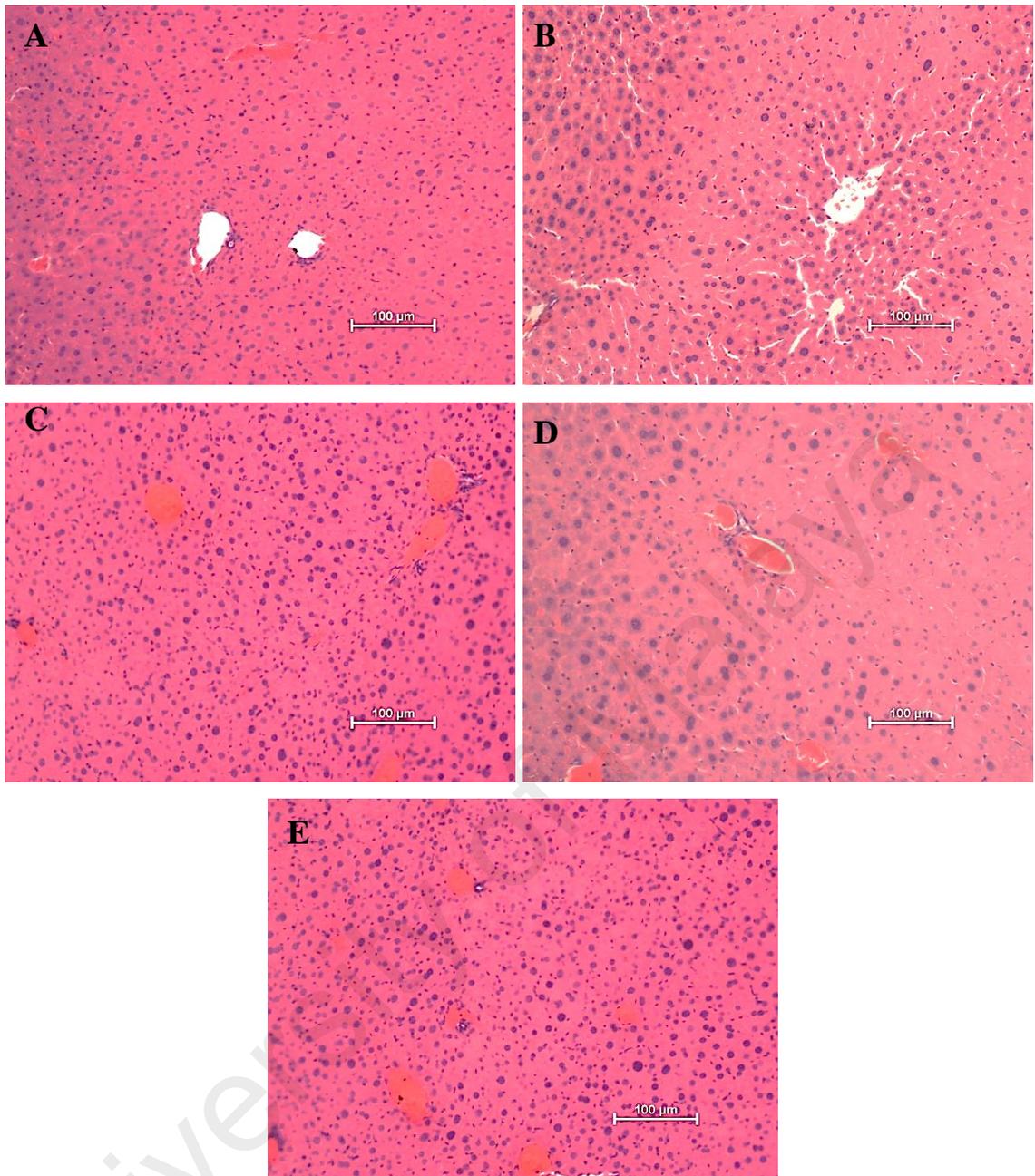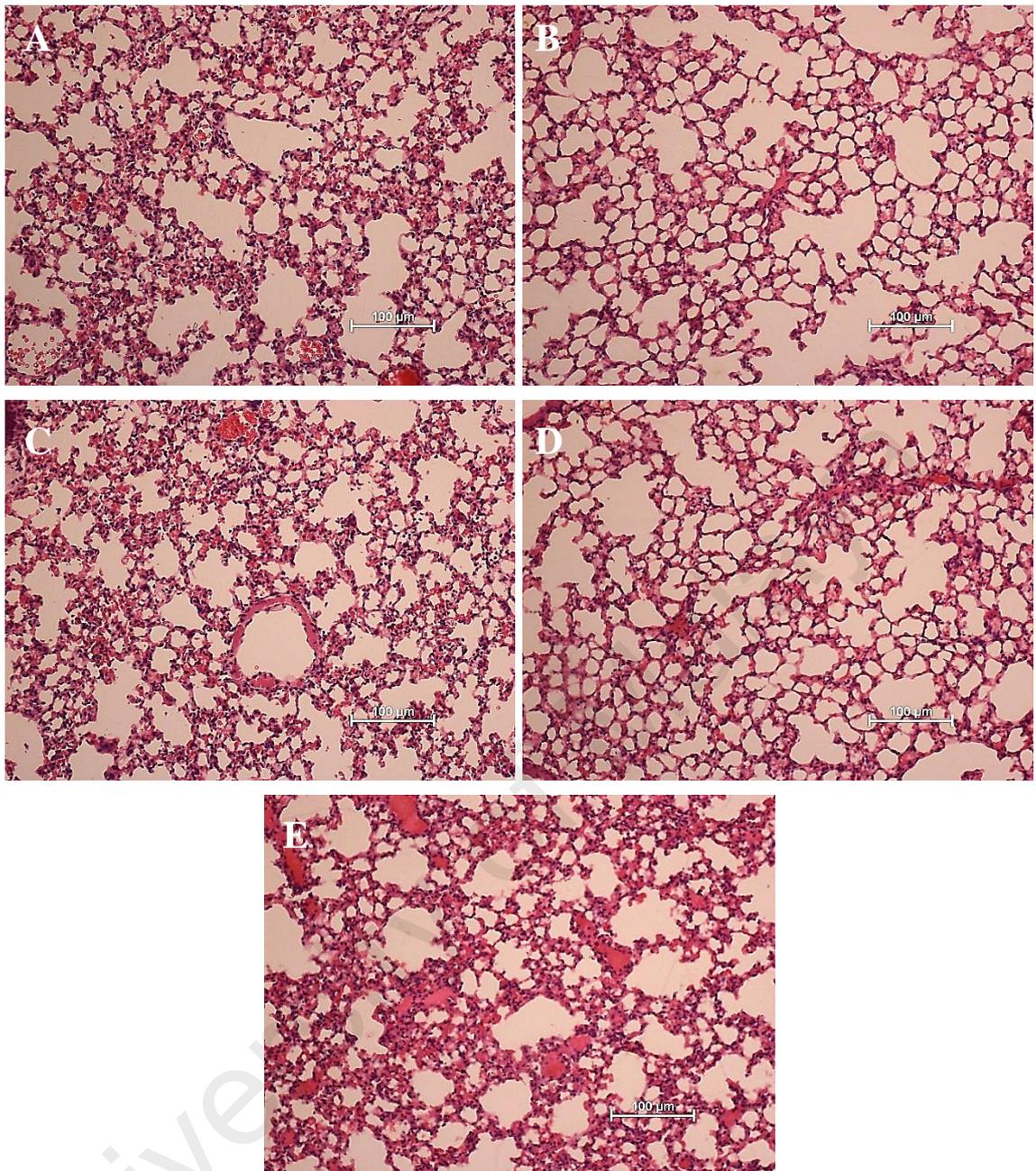
Figure 3.12 (continued): Histology of lung harvested from mice treated with hybrid peptides via IP route for in vivo toxicity assessment. No significant abnormality was observed between (A) control group (injected with PBS) and mice treated with maximum non-toxic dose of (B) RN7-IN10 (20mg/kg), (C) RN7-IN9 (10mg/kg), (D) RN7-IN8 (20mg/kg), and (E) RN7-IN6 (10mg/kg). All lung sections from treated groups showed normal structure with thin alveolar septa. Hematoxylin and eosin stain. Bar indicates 100 µm.

Table 3.14: Whole blood heamatogram and serum biochemistry of mice injected with four hybrid peptides via SC route.

Parameter	SC treatment[a]				
	Control	RN7-IN10 (20 mg/kg)	RN7-IN9 (10 mg/kg)	RN7-IN8 (20 mg/kg)	RN7-IN6 (10mg/kg)
Whole blood					
Erythrocytes, RBC (10^6/mm^3)	8.31±0.4	8.04±1.18	9.22±0.98	9.17±0.83	9.59±0.92
Hemoglobin, Hgb (g/dl)	14.3±0.82	13.75±1.77	15.75±1.4	15.82±1.11	16.17±1.44
Mean corpuscular volume, MCV (µm^3)	51.5±0.57	50±2.16	51±1.82	50.25±0.95	50±1.82
Mean corpuscular haemoglobin concentration, MCHC (g/dl)	33.55±0.91	34.45±1.35	34.2±1.37	34.38±0.52	32.87±0.42
Mean corpuscular haemoglobin, MCH (pg)	17.02±0.53	17.08±0.4	17.1±0.53	17.32±0.49	16.85±0.3
platelet Counts, PLT (10^3/mm^3)	545±98.88	568.75±81.73	449.75±100.23	445.75±47.21	273±43.55
Hematocrit, HCT %	42.67±2.5	41.5±3.7	46.15±5.19	46.15±3.69	49.2±4.43
White blood cells, WBC (10^3/mm^3)	4.4±0.52	3.8±0.2	4.32±0.55	3.47±0.48	3.86±0.34
Lymphocytes %	49.75±11.3	52.2±3.3	46.47±5.48	43.85±6.28	51.85±3.3
Monocytes %	2.3±0.6	2.2±0.28	1.95±0.1	1.92±0.15	2.8±0.25
Granulocytes %	66.25±7.6	46.6±7.47	55.55±4.52	58.62±9.82	51.12±5.16
Eosinophil %	2.5±0.6	2.82±0.25	2.77±0.54	2.42±0.61	2.7±0.71
Serum biochemistry					
Aspartate aminotransferase, AST	173.8±13.1	175±25.41	188.33±11.22	170.75±14.52	158.25±18.9
Alanine transaminase, ALT	43.5±11.45	51.25±11.9	43.17±13.81	50±5.49	43.32±10.3
Alkaline phosphatase, ALP	130.25±6.9	89.87±8.07	131.25±19.7	134±8.24	106.56±20.1
Creatinine	39.6±0.61	37.8±3.43	35±1.27	43.4±0.8	34.89±0.25
Urea	11.13±3.24	9.2±0.8	8.84±1.04	9.61±3.02	9.07±2.02
Total bilirubin	2.1±0.1	2.1±0.35	2.2±0.13	1.84±0.12	2.03±0.14

[a] Given for three doses (1hr, 12hr, and 24hr).

One-way ANOVA with *post hoc* Dunnett-t test was used to find Statistical difference between treated mice with different peptides vs untreated control.

Mean value (s) showing significant difference ($p \leq 0.05$) as compared to the control group was highlighted:

Highlighted in yellow: RN7-IN10 treated mice (granulocytes, $p = 0.0172$; ALP, $p = 0.0037$).

Highlighted in blue: RN7-IN6 treated mice (platelet counts, $p = 0.0487$).

Figure 3.13: Histology of kidney harvested from mice treated with hybrid peptides via SC route at 100 mg/kg for *in vivo* toxicity assessment. No significant abnormality was observed between (A) control group and mice treated with (B) RN7-IN10, (C) RN7-IN9, (D) RN7-IN8, and (E) RN7-IN6. All kidney sections showed normal structure with normal glomerulus, proximal convoluted tubules and distal convoluted tubules. Hematoxylin and eosin stain. Bar indicates 100 µm.

Figure 3.13 (continued): Histology of liver harvested from mice treated with hybrid peptides via SC route at 100 mg/kg for *in vivo* toxicity assessment. No significant abnormality was observed between (A) control group and mice treated with (B) RN7-IN10, (C) RN7-IN9, (D) RN7-IN8, and (E) RN7-IN6. All liver sections of the treated groups showed normal hepatic structure with central vain and hepatocytes. Hematoxylin and eosin stain. Bar indicates 100 µm.

Figure 3.13 (continued): Histology of brain harvested from mice treated with hybrid peptides via SC route at 100 mg/kg for *in vivo* toxicity assessment. No significant abnormality was observed between (A) control group and mice treated with (B) RN7-IN10, (C) RN7-IN9, (D) RN7-IN8, and (E) RN7-IN6. All brain sections showed normal structure with no sign of inflammation or neuronal injury. Hematoxylin and eosin stain. Bar indicates 100 µm.

Figure 3.13 (continued): Histology of spleen harvested from mice treated with hybrid peptides via SC route at 100 mg/kg for *in vivo* toxicity assessment. No significant abnormality was observed between (A) control group and mice treated with (B) RN7-IN10, (C) RN7-IN9, (D) RN7-IN8, and (E) RN7-IN6. All spleen sections showed normal structure with normal white and red pulps. Hematoxylin and eosin stain. Bar indicates 100 μm.

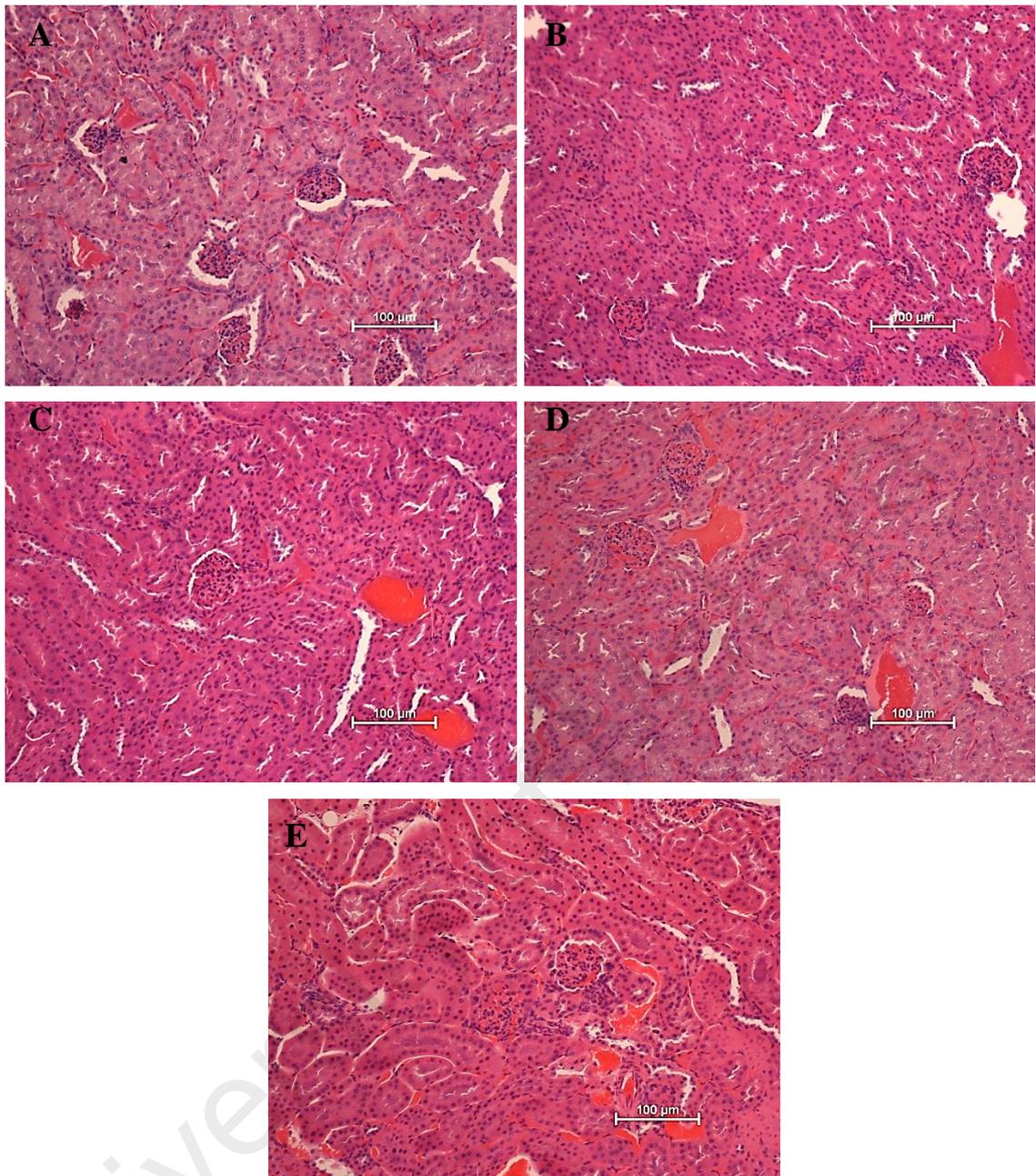**Figure 3.13 (continued):** Histology of lung harvested from mice treated with hybrid peptides via SC route at 100 mg/kg for *in vivo* toxicity assessment. No significant abnormality was observed between (A) control group and mice treated with (B) RN7-IN10, (C) RN7-IN9, (D) RN7-IN8, and (E) RN7-IN6. All lung sections from treated groups showed normal structure with thin alveolar septa. Hematoxylin and eosin stain. Bar indicates 100 μm.

Table 3.15: Whole blood heamatogram and serum biochemistry of mice administered with four hybrid peptides via IN route.

Parameter	IN treatment[a]				
	Control	RN7-IN10 (20 mg/kg)	RN7-IN9 (10 mg/kg)	RN7-IN8 (20 mg/kg)	RN7-IN6 (10mg/kg)
Whole blood					
Erythrocytes, RBC (10^6/mm^3)	9.42±1.42	9.62±0.79	9.36±0.9	8.95±0.53	9.12±0.25
Hemoglobin, Hgb (g/dl)	15.5±2.35	15.47±1.07	14.9±1.32	14.42±0.76	15±0.28
Mean corpuscular volume, MCV (μm^3)	51.5±0.57	50.75±1.7	42±6.05	50.25±1.25	52.66±1.24
Mean corpuscular haemoglobin concentration, MCHC (g/dl)	32.42±0.43	28.3±8.15	31.85±0.17	32.22±0.4	31.7±0.21
Mean corpuscular haemoglobin, MCH (pg)	16.67±0.27	20±7.87	15.95±0.33	16.12±0.33	16.5±0.17
platelet Counts, PLT (10^3/mm^3)	578.5±94.77	619.5±100.42	656.75±76.9	612±130.37	597.66±123.23
Hematocrit, HCT %	48.37±7.83	47.95±3.18	46.82±4.23	44.8±2.9	47.43±0.61
White blood cells, WBC (10^3/mm^3)	4.2±0.87	5.2±1.44	5.2±2.35	3.95±0.54	3.6±0.35
Lymphocytes %	54.82±9.23	58.5±7.53	41.27±10.56	45.6±14.31	47.67±12.2
Monocytes %	3±0.43	2.2±0.37	2.52±0.27	3.22±0.68	2.7±0.57
Granulocytes %	57.18±4.95	66.6±21.64	56.2±10.81	52.9±9.54	37.67±3.8
Eosinophil %	2.7±1.05	2.7±0.97	3.2±1.2	4.3±3.6	3.5±0.9
Serum biochemistry					
Aspartate aminotransferase, AST	174.75±12.6	172±8.78	141.2±23.08	176.3±31.91	140±7.2
Alanine transaminase, ALT	49.9±6.48	66.9±17.02	41.22±15.75	44.5±18.9	43.2±7.56
Alkaline phosphatase, ALP	149.3±2.62	133.3±10.1	127.75±6.07	128.5±21.7	138.33±5.5
Creatinine	40.15±2.7	40.94±0.83	37.66±1.26	40.56±1.74	40.15±3.6
Urea	9.95±1.63	9.3±1.68	9.6±1.02	8.89±1.30	10.14±1.52
Total bilirubin	2.04±0.12	2.15±0.14	2.01±0.27	2.21±0.18	1.95±0.08

[a] Given for three doses (1hr, 12hr, and 24hr).

One-way ANOVA with *post hoc* Dunnett-t test was used to find Statistical difference between treated mice with different peptides vs untreated control.

Mean value (s) showing significant difference ($p \leq 0.05$) as compared to the control group was highlighted:

Highlighted in yellow: RN7-IN9 treated mice (MCV, $p = 0.001$).

Highlighted in blue: RN7-IN6 treated mice (granulocytes, $p = 0.0482$).

Figure 3.14: Histology of kidney harvested from mice treated with hybrid peptides via IN route at 20 mg/kg for *in vivo* toxicity assessment. No significant abnormality was observed between (A) control group and mice treated with (B) RN7-IN10, (C) RN7-IN9, (D) RN7-IN8, and (E) RN7-IN6. All kidney sections showed normal structure with normal glomerulus, proximal convoluted tubules and distal convoluted tubules. Hematoxylin and eosin stain. Bar indicates 100 µm.
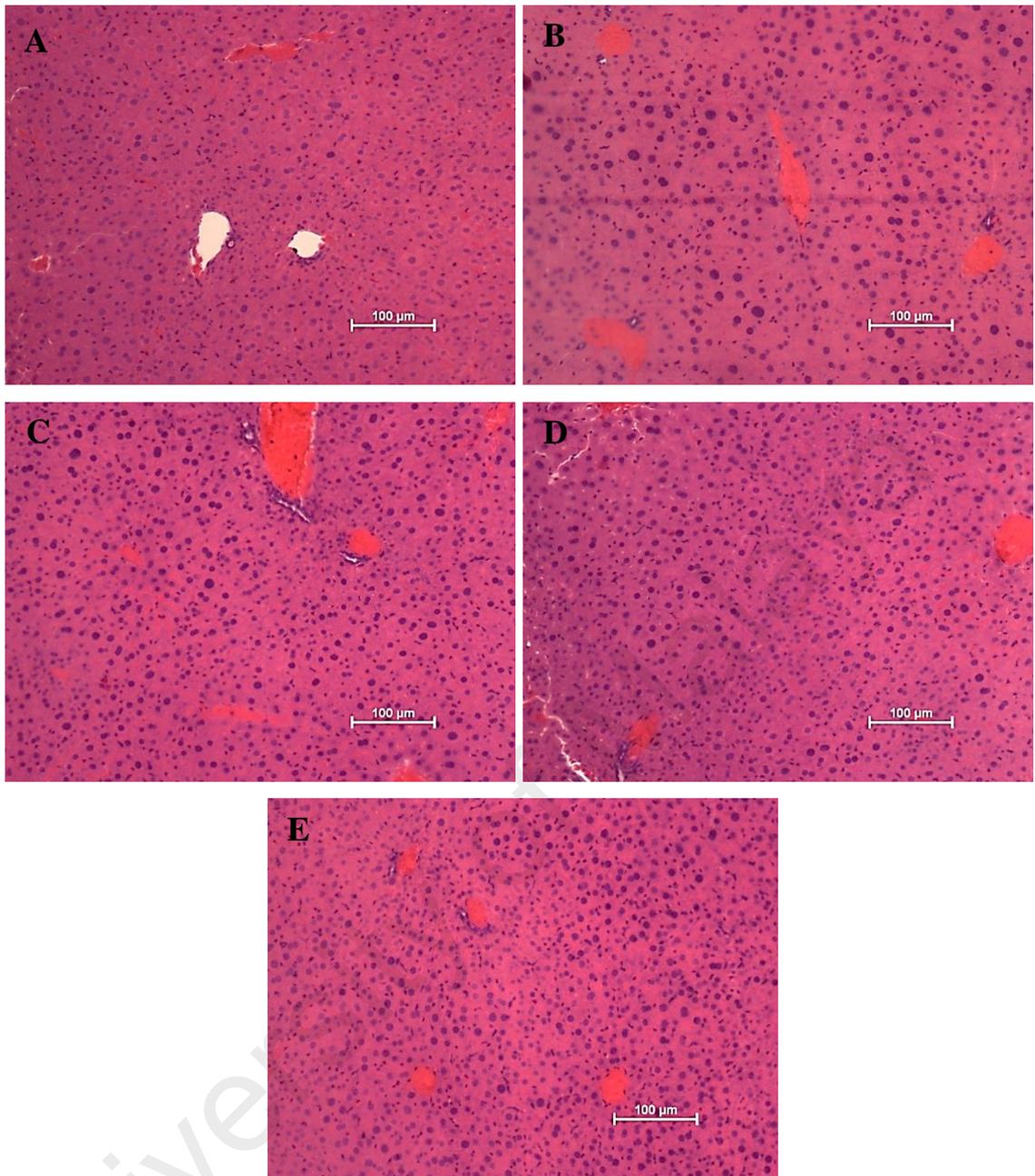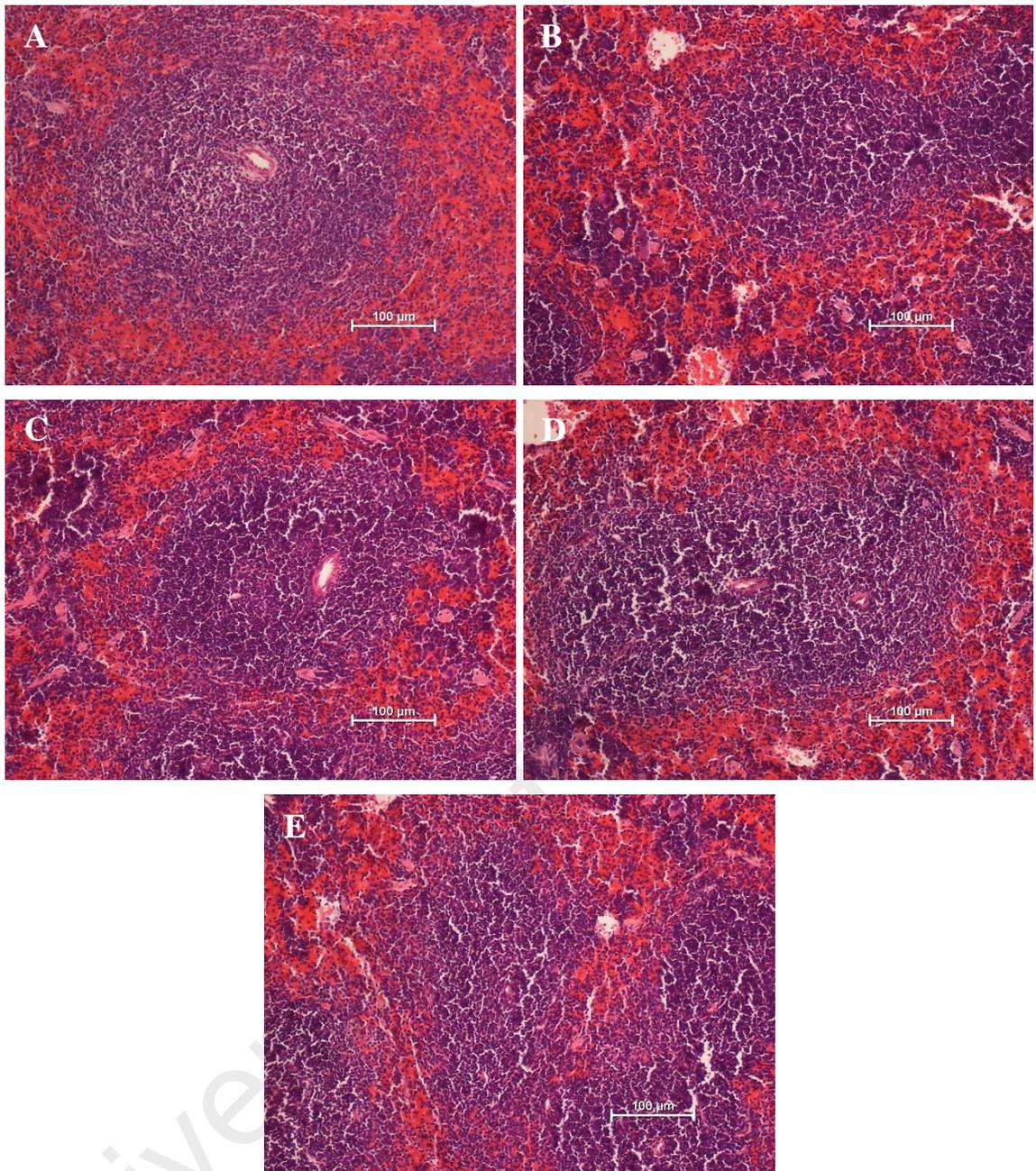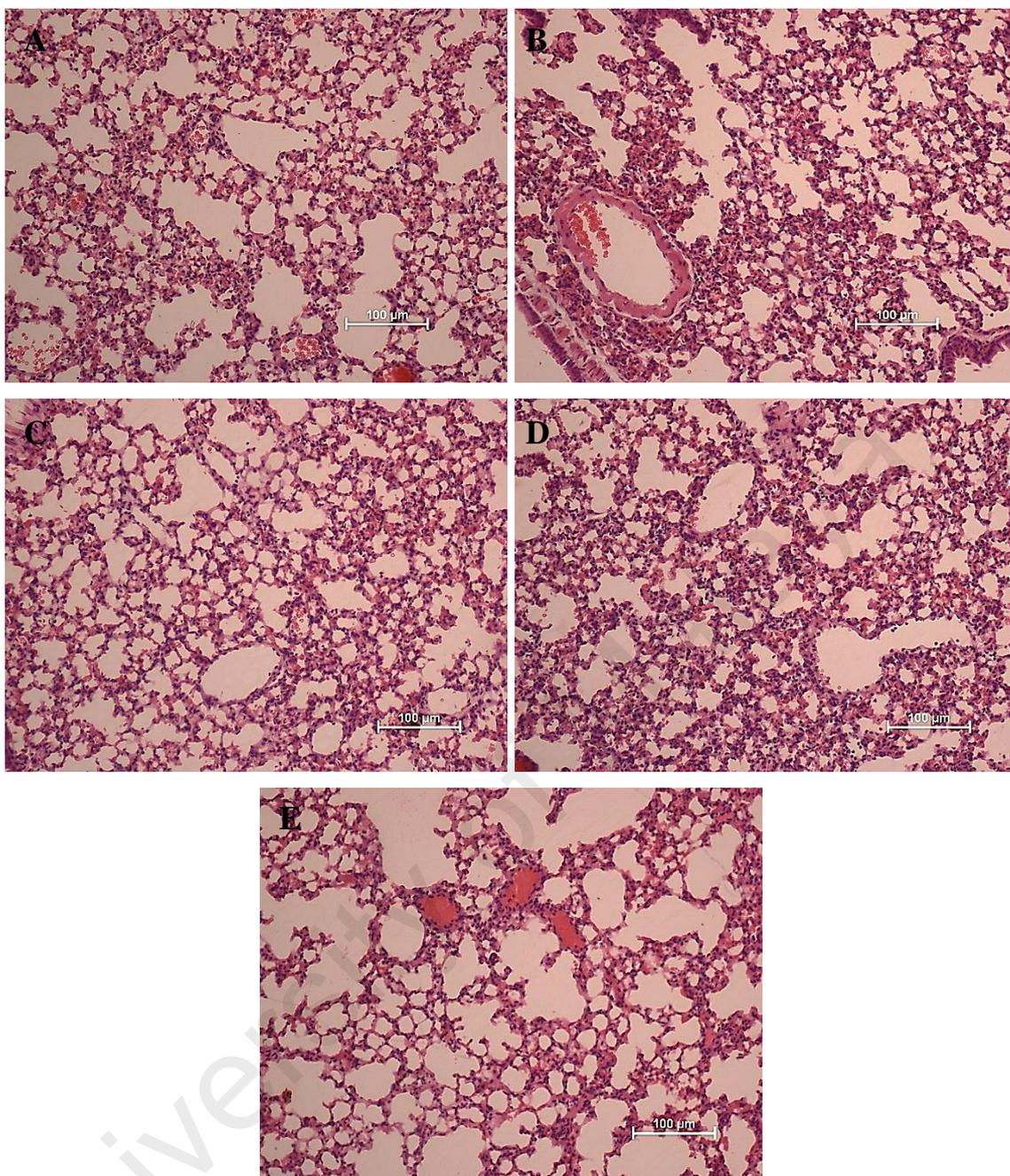
Figure 3.14 (continued): Histology of liver harvested from mice treated with hybrid peptides via IN route at 20 mg/kg for *in vivo* toxicity assessment. No significant abnormality was observed between (A) control group and mice treated with (B) RN7-IN10, (C) RN7-IN9, (D) RN7-IN8, and (E) RN7-IN6. All liver sections of the treated groups showed normal hepatic structure with central vain and hepatocytes. Hematoxylin and eosin stain. Bar indicates 100 µm.

Figure 3.14 (continued): Histology of brain harvested from mice treated with hybrid peptides via IN route at 20 mg/kg for *in vivo* toxicity assessment. No significant abnormality was observed between (A) control group and mice treated with (B) RN7-IN10, (C) RN7-IN9, (D) RN7-IN8, and (E) RN7-IN6. All brain sections showed normal structure with no sign of inflammation or neuronal injury. Hematoxylin and eosin stain. Bar indicates 100 µm.

Figure 3.14 (continued): Histology of spleen harvested from mice treated with hybrid peptides via IN route at 20 mg/kg for *in vivo* toxicity assessment. No significant abnormality was observed between (A) control group and mice treated with (B) RN7-IN10, (C) RN7-IN9, (D) RN7-IN8, and (E) RN7-IN6. All spleen sections showed normal structure with normal white and red pulps. Hematoxylin and eosin stain. Bar indicates 100 µm.

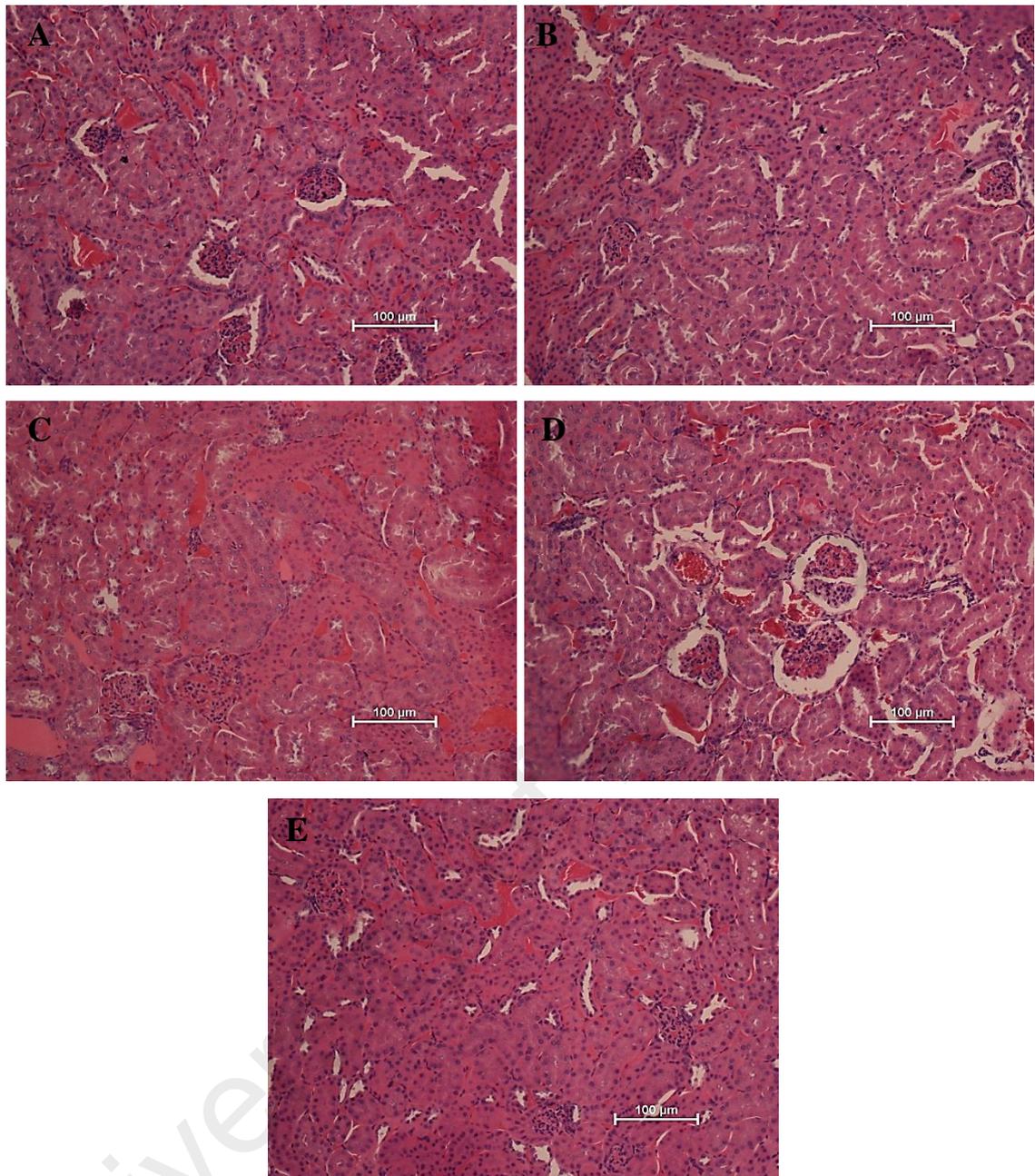**Figure 3.14 (continued):** Histology of lung harvested from mice treated with hybrid peptides via IN route at 20 mg/kg for in vivo toxicity assessment. No significant abnormality was observed between (A) control group and mice treated with (B) RN7-IN10, (C) RN7-IN9, (D) RN7-IN8, and (E) RN7-IN6. All lung sections from treated groups showed normal structure with thin alveolar septa. Hematoxylin and eosin stain. Bar indicates 100 µm.

3.7 *In vivo* antibacterial activity assessment of hybrid peptides

In the present study, two lethal pneumococcal infection models induced by a virulent pneumococcal strain were used to evaluate the antibacterial efficacy of hybrid peptides *in vivo*. The lethal pneumococcal systemic infection model was developed by infecting the ICR male mice with a pneumococcal inoculum of 1.5×10^2 CFU/mouse via IP route to mimic pneumococcal bacteremia. On the other hand, pneumococcal pneumonia was induced using inoculum of 5×10^3 CFU/mouse via the intrathoracic route. Both infection models induced 100% mortality within two to four days post-infection. Both models were developed using highly virulent pneumococcal clinical isolate belonged to serotype 1. *S. pneumoniae* serotype 1 is highly invasive and ranked among the top four pneumococcal serotypes that infected children below five years old (von Mollendorf *et al.*, 2016).

In both infection models, infected mice showed several symptoms such as bending of body posture, ruffled fur, less responsive to physical sensitization, lethargy, loss of body temperature and difficulty in defecation (Figure 3.15A & B). All the infected mice died within two to four days post-infection. Upon necropsy, the lungs of infected mice appeared with redness and swelling indicating severe inflammation (Figure 3.15C, arrow a) as compared to that of uninfected mice which appeared normal and were in pink color (Figure 3.15D, arrow b). In addition, leakage of body fluid into the peritoneal cavity and thoracic cavity, swelling and loss of the arrangement and compaction of the digestive tract were observed (Figure 3.15C). Cultures of the blood of the dead/moribund mice following infection with *S. pneumoniae* via IP and IT routes detected pneumococci.

Since all hybrid peptides displayed strong antipneumococcal activity *in vitro*, two hybrid peptides namely RN7-IN10 and RN7-IN8 were selected to evaluate their *in vivo* therapeutic efficacy. These two peptides showed the fastest killing kinetics (see section 3.2.4) and both exhibited less toxic effects *in vivo* (see section 3.6). For IP route, both

hybrid peptides were tested at three different doses 5 mg/kg, 10 mg/kg and 20 mg/kg for three treatment regimens 1 hr, 12 hr and 24 hr post-infection. In the pneumococcal bacteremia model, both RN7-IN10 and RN7-IN8 failed to treat any of the infected mice at 5mg/kg. At 10 mg/kg, 10 % of the infected mice survived after treatment with RN7-IN10 ($p = 0.005$), whereas 30% of the mice were able to survive after treatment with RN7-IN8 ($p < 0.001$). the results showed that 30% of the mice treated with RN7-IN10 at 20mg/kg were able to survive ($p < 0.001$). On the other hand, hybrid peptide RN7-IN8 at 20mg/kg displayed the highest protection efficacy with a 50 % survival rate up to 7 days post-infection ($p < 0.001$) (Figure 3.16). Treatment via SC and IN routes had no impact on infected mice up to seven days post-infection and none of the mice survived death. In addition to our designed peptides, ceftriaxone as a standard drug was tested in stand alone to evaluate its efficacy in treating infected mice via IP route at 5 mg/kg, 10 mg/kg, 20 mg/kg, 40 mg/kg, and 80 mg/kg and the survival was 10 %, 30 %, 40 %, 70 % and 90 % up to seven days post-infection (Figure 3.17). None of the survived mice showed presentation of illness as compared to the untreated group which was severely ill and inactive.

Combinations of peptide-peptide were used in present study to assess the ability of hybrid peptides RN7-IN10 and RN7-IN8 to treat infected mice with pneumococcal bacteremia in combination with each other. Two combinations were used, 5mg/kg + 5mg/kg and 10 mg/kg + 10 mg/kg to treat mice via IP route at three regimens 1 hr, 12 hr and 24 hr. The results displayed that combination of the two peptides at (5 mg/kg + 5 mg/kg) was able to treat 40 % of the mice and protected them from death up to 7 days post-infection ($p = 0.003$). On the other hand, combination of peptides at (10 mg/kg + 10 mg/kg) was able to protect 60 % of the infected mice ($p < 0.001$) (Figure 3.18) . In all experiments, mice that survived did not show any presentation of illness as compared to the untreated group which was severely ill and inactive. Unlike untreated mice which

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showed pneumococcal growth uppon culturing blood on agar plates, no pneumococci were detected from the blood of the survived mice. In the pneumococcal pneumonia model, mice treated with both peptides via IP, SC and IN routes showed no survival up to seven days post-infection
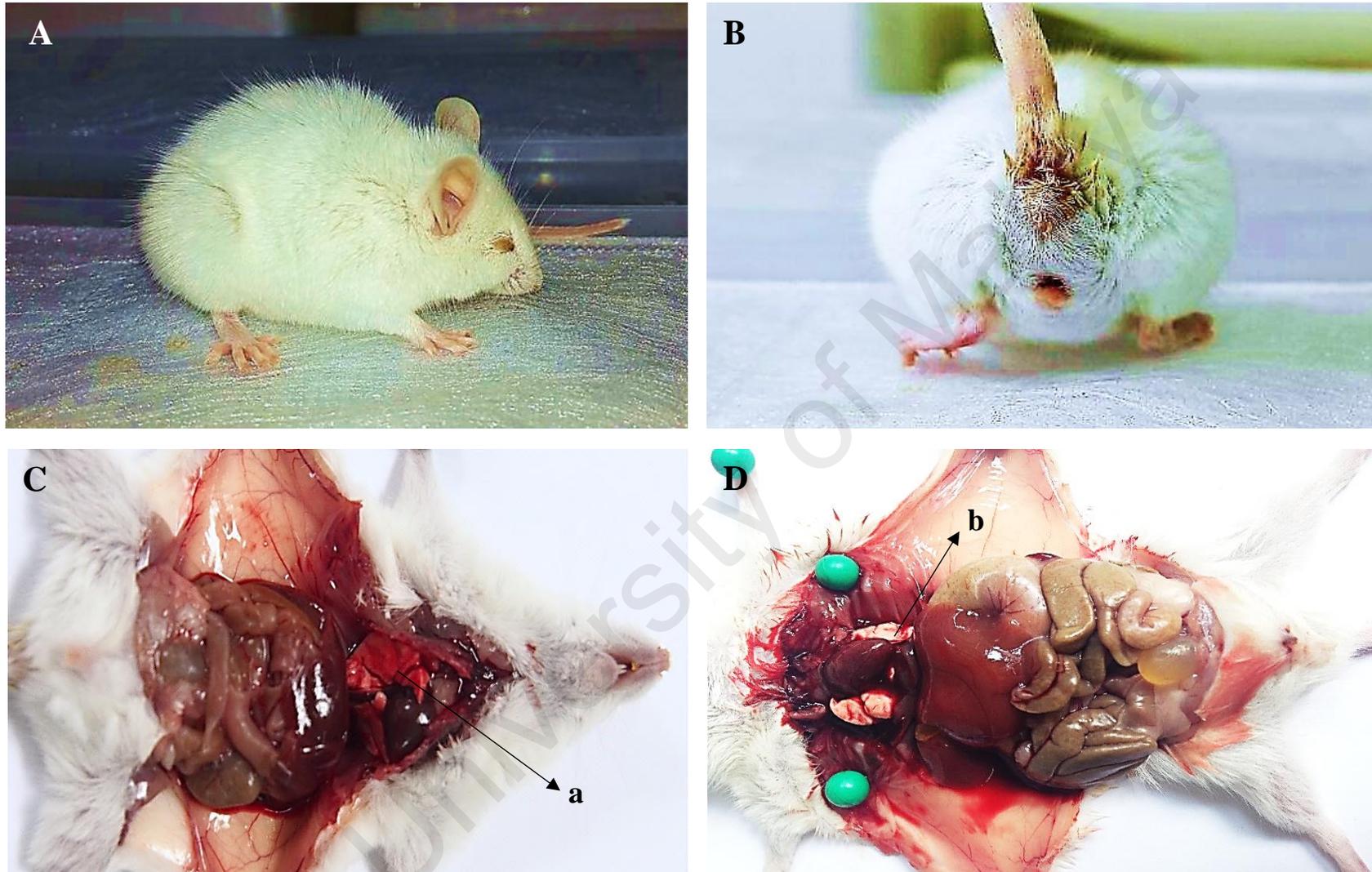
Figure 3.15: The physical abnormalities observed in mice infected with pneumococcal bacteremia infection model. (A) Infected mouse with ruffled fur, hunched posture, the presence of fluid around the orbital regions, and (B) difficulty in defecation. The organs of (C) the infected mouse especially lungs were presented with exudates, swelling and redness (arrow a) indicating severe inflammation as compared to the lung (arrow b) of the uninfected control (D).

Figure 3.16: Survival curve of mice infected with serotype 1 isolate of *S. pneumoniae* resistant to ceftriaxone and treated with RN7-IN10 and RN7-IN8. Kaplan-Meier with log-rank test (Mantel-Cox) was used to perform the statistical for all treated groups and the untreated control using. Treatment with RN7-IN8 at 20 mg/kg displayed the highest survival rate of 50 % up to 7 days post-infection ($p < 0.001$).
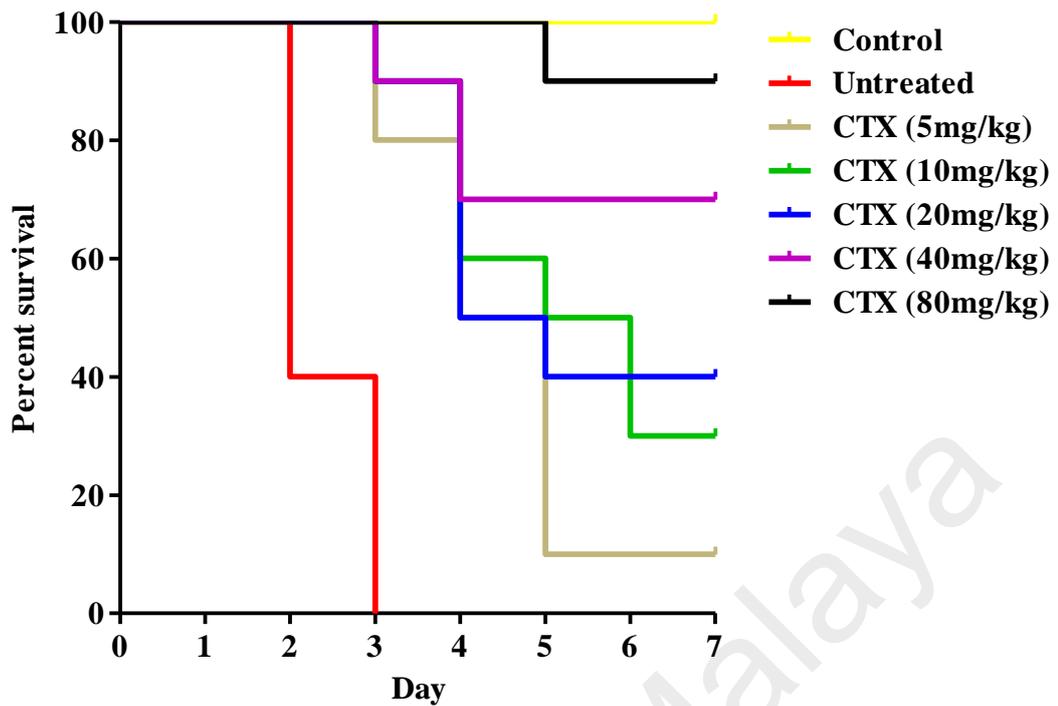
Figure 3.17: Survival curve of mice infected with serotype 1 isolate of *S. pneumoniae* resistant to ceftriaxone and treated with ceftriaxone (CTX) in a standalone form. Kaplan-Meier with log-rank test (Mantel-Cox) was used to perform the statistical analysis for all treated group and the untreated control.
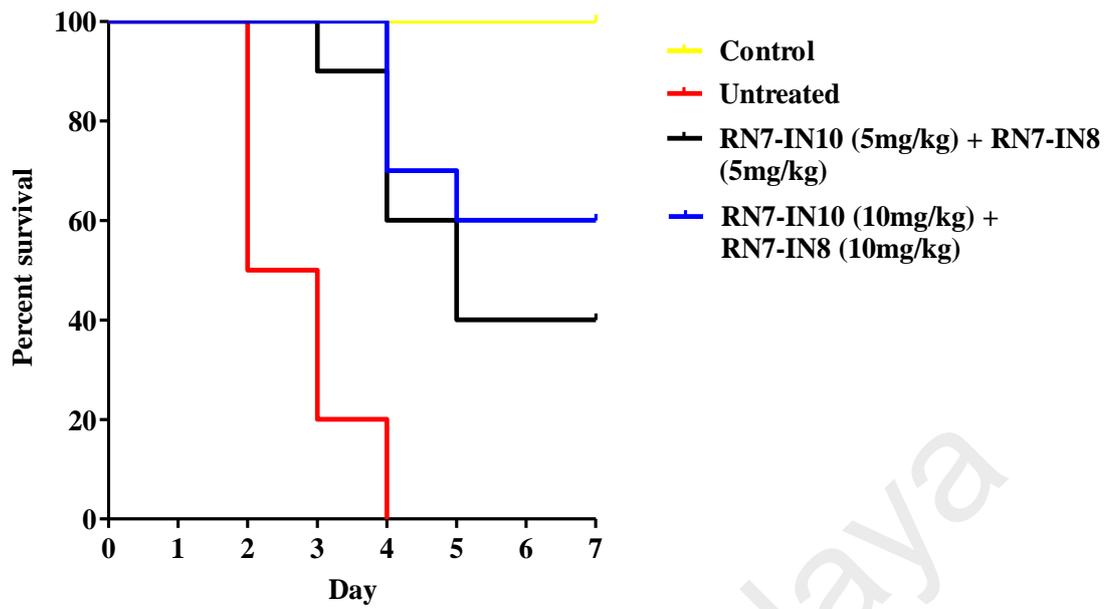
Figure 3.18: Survival curve of mice infected with serotype 1 isolate of *S. pneumoniae* resistant to ceftriaxone and treated with combinations of RN7-IN10 and RN7-IN8. Kaplan-Meier with log-rank test (Mantel-Cox) was used to perform the statistical analysis for all treated group versus the untreated control.

3.8 *In vivo* synergism of RN7-IN8 in combination with ceftriaxone

Among the hybrid peptides RN7-IN10 and RN7-IN8, standalone treatment with RN7-IN8 at 20mg/kg was found to confer significant survivability to mice infected by a highly virulent pneumococcal clinical isolate via IP route. In order to assess the synergistic effect of RN7-IN8 in combination with the standard drug ceftriaxone (CTX), three different doses of RN7-IN8 (5 mg/kg, 10 mg/kg and 20 mg/kg) and ceftriaxone (5 mg/kg, 10 mg/kg and 20 mg/kg) were tested using the same bacteremia infection model in three treatment formulation: $RN7\text{-}IN8_5 - CTX_5$ (5 mg/kg of RN7-IN8 and 5 mg/kg of CTX), $RN7\text{-}IN8_{10} - CTX_{10}$ (10 mg/kg of RN7-IN8 and 10 mg/kg of CTX) and $RN7\text{-}IN8_{20} - CTX_{20}$ (20 mg/kg of RN7-IN8 and 20 mg/kg of CTX). Using groups of 10 mice, the combinations of RN7-IN8 and ceftriaxone $RN7\text{-}IN8_5 - CTX_5$ (5 mg/kg of RN7-IN8 and 5 mg/kg of CTX), $RN7\text{-}IN8_{10} - CTX_{10}$ (10 mg/kg of RN7-IN8 and 10 mg/kg of CTX) and $RN7\text{-}IN8_{20} - CTX_{20}$ (20 mg/kg of RN7-IN8 and 20 mg/kg of CTX) displayed 60 %, 80 % and 100 % survival in mice infected with highly virulent pneumococcal strain up to seven days post-infection ($p < 0.0001$) (Figure 3.19). Our results displayed that that treatment using combinations of peptide-antibiotics conferred higher survival rate than peptide and antibiotic in their stand-alone form. In addition, all treated mice survived from infection appeared physically active and none of them showed sign of abnormal behavior.
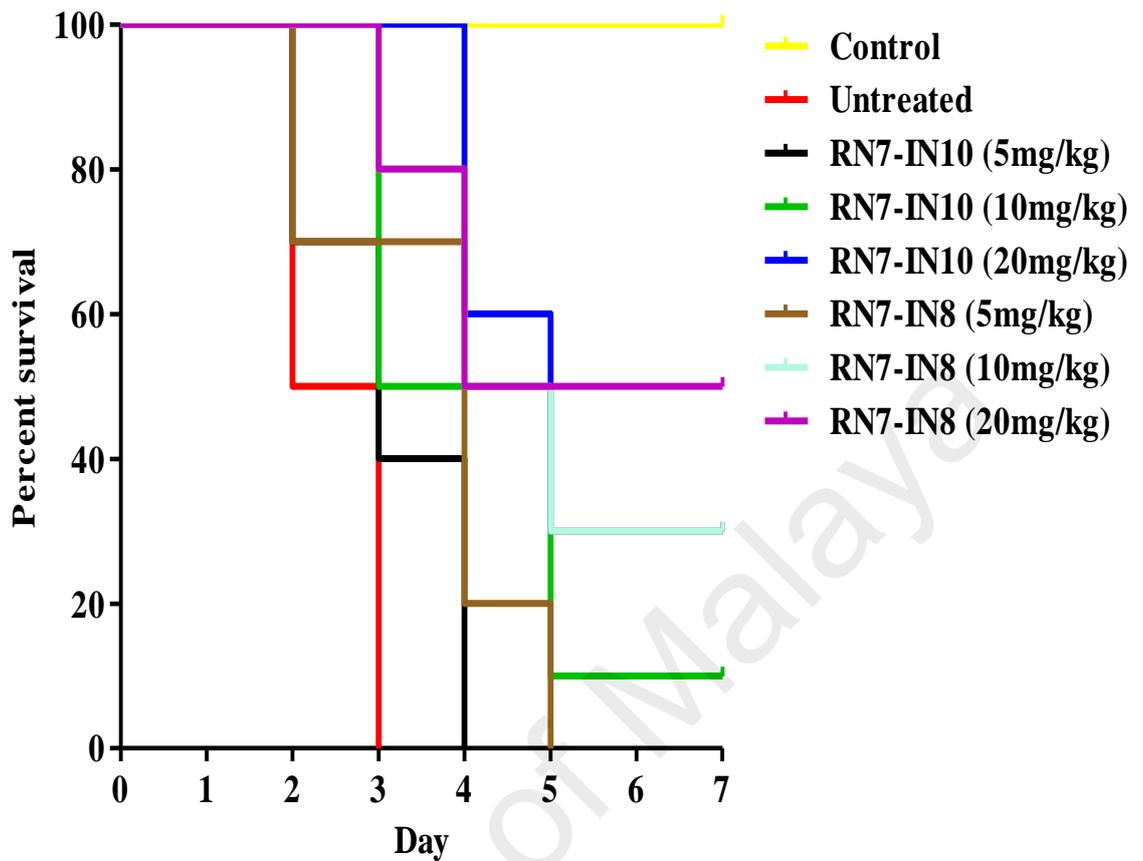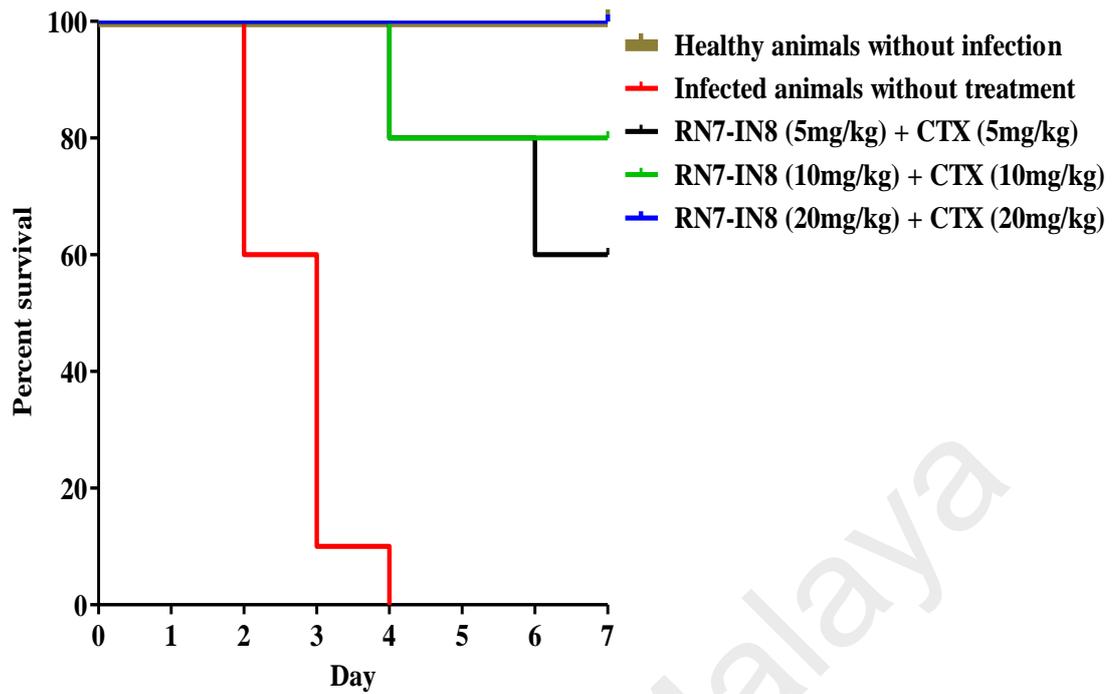
Figure 3.19: Survival curve of mice infected with serotype 1 isolate of *S. pneumoniae* resistant to ceftriaxone and treated with combinations of RN7-IN8 and ceftriaxone (CTX). Kaplan-Meier with log-rank test (Mantel-Cox) was used to perform the statistical analysis for all treated group and the untreated control. Combination of RN7-IN8 and ceftriaxone (CTX) at 20mg/kg + 20mg/kg displayed the highest survival rate 100% (p < 0.0001).

3.9 Histopathological evaluation

All the histopathological examinations of the mice infected with bacteremia model with and without treatment are presented in Figure 3.20 and Figure 3.21. Out of five major organs examined, the lung and spleen of the infected animals were the most severely affected. A number of histopathological changes were observed in the lung of the infected mice. As compared to the uninfected control group, the lung of the infected and untreated group exhibited extensive vascular congestion with foci consolidation. Heavy permeation of the red blood cells into the alveolar spaces strongly denotes pulmonary hemorrhage (Figure 3.20A, arrow a). The greatly congested lung appeared with little alveolar spaces (Figure 3.20A, arrow b).This is in contrast to the uninfected group, where the normal lung displayed greatly aerated alveolar spaces with a thin layer of the alveolar wall (Figure 3.20B). Severe tissue injury was also noticed in the spleen of the infected group (Figure 3.21A). Unlike the normal spleen which showed normal red and white pulps (Figure 3.21B, arrow b), the infected spleen demonstrated depleted splenocytes with no white matter/germinal center (Figure 3.21A, arrow a). No significant histopathological lesions were observed in other organs such as brain, liver, kidney and heart.

For the respective treatments of infected mice including hybrid peptide RN7-IN8 at 20mg/kg, combination of hybrid peptides RN7-IN10 and RN7-IN8 (10mg/kg + 10mg/kg) and combination of RN7-IN8 and ceftriaxone (5mg/kg + 5mg/kg, 10mg/kg + 10mg/kg and 20mg/kg + 20mg/kg), it was noticed that although lesions, inflammatory events and the degree of tissues damage can be found in the organs, however, the degree and severity of the damage were much lower than the infected control group. Unlike the lung of the untreated mice which exhibited severe inflammation and the alveolar spaces were about 90% congested (Figure 3.20A), all the lungs harvested from the treated mice revealed only low level of congestion and minor thickening of the alveolar wall even

though these histological changes were still noticeable in the mice (Figure 3.20C-G). Treatment of infected mice with a combination of hybrid peptide RN7-IN8 and ceftriaxone at three different dosages (5mg/kg - 5mg, 10mg/kg - 10mg and 20mg/kg - 20mg/kg) showed a gradual decrease in the degree of congestion and damage (Figure 3.20E-G). Lungs harvested from mice treated with a combination of RN7-IN8 and ceftriaxone at 20mg/kg + 20mg/kg (Figure 3.20G) presented 100% mice survival. Likewise, all the spleens of treated mice displayed none or minimum damage with the white and red pulps clearly observed (Figure 3.21C-G) as compared to the infected mice (Figure 3.21A). No significant tissue damage was observed in brain, kidney and liver in both treated and untreated mice.
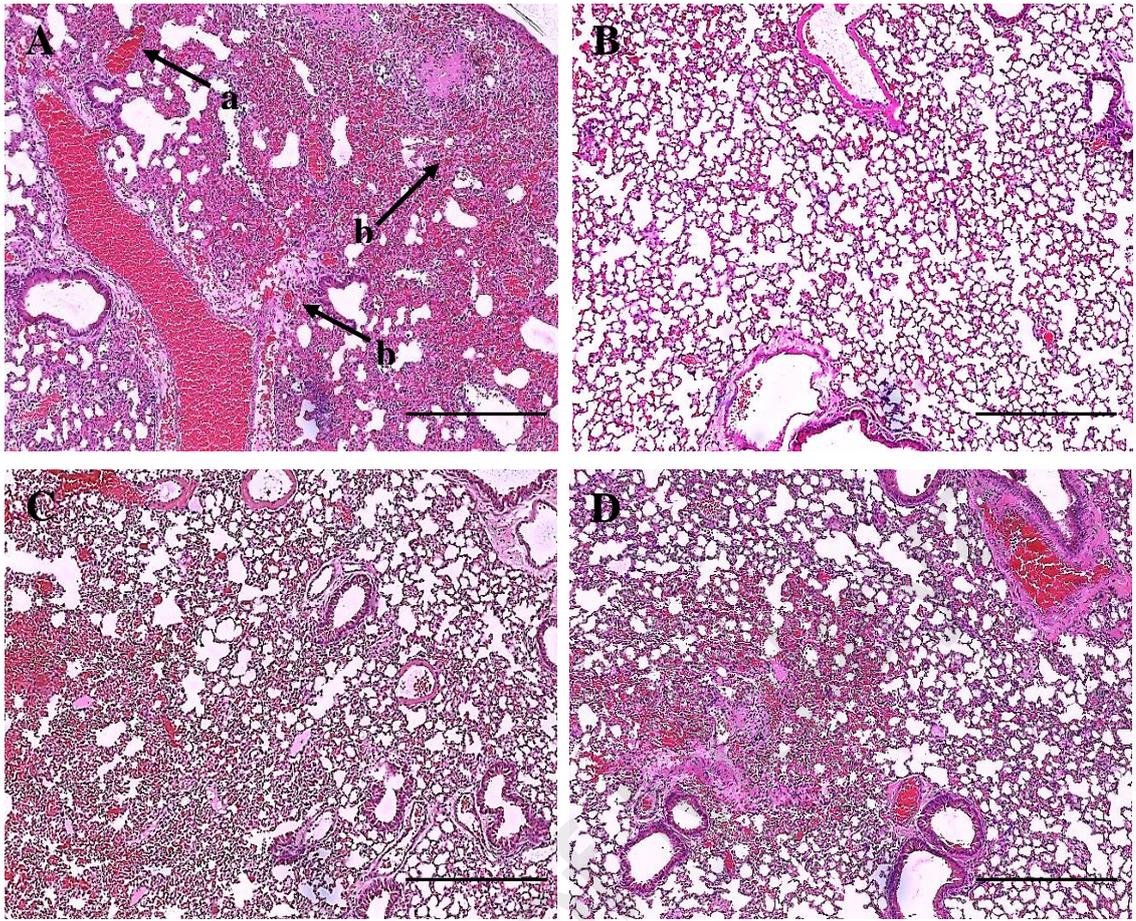
Figure 3.20: Histology of lungs harvested from mice infected with *S. pneumoniae* receiving treatments. (A) infected mice, (B) uninfected mice (control), (C) mice treated with RN7-IN8 (20mg/kg), (D) mice treated with combination of RN7-IN10 and RN7-IN8 (10mg/kg + 10mg/kg), (E) mice treated eith combination of RN7-IN8 and CTX (5mg/kg + 5mg/kg), (F) mice treated with combination of RN7-IN8 and CTX (10mg/kg + 10mg/kg), (G) mice treated with combination of RN7-IN8 and CTX (20mg/kg + 20mg/kg). Bar indicates 500 µM.

Figure 3.20 (continued): Histology of lungs harvested from mice infected with *S. pneumoniae* receiving treatments. (A) infected mice, (B) uninfected mice (control), (C) mice treated with RN7-IN8 (20mg/kg), (D) mice treated with combination of RN7-IN10 and RN7-IN8 (10mg/kg + 10mg/kg), (E) mice treated eith combination of RN7-IN8 and CTX (5mg/kg + 5mg/kg), (F) mice treated with combination of RN7-IN8 and CTX (10mg/kg + 10mg/kg), (G) mice treated with combination of RN7-IN8 and CTX (20mg/kg + 20mg/kg). Bar indicates 500 µM.

Figure 3.21 Histology of spleens harvested from mice infected with *S. pneumoniae* receiving treatments. (A) infected mice, (B) uninfected mice (control), (C) mice treated with RN7-IN8 (20mg/kg), (D) mice treated with combination of RN7-IN10 and RN7-IN8 (10mg/kg + 10mg/kg), (E) mice treated eith combination of RN7-IN8 and CTX (5mg/kg + 5mg/kg), (F) mice treated with combination of RN7-IN8 and CTX (10mg/kg + 10mg/kg), (G) mice treated with combination of RN7-IN8 and CTX (20mg/kg + 20mg/kg). Bar indicates 500 µM.

Figure 3.20 (continued): Histology of spleens harvested from mice infected with *S. pneumoniae* receiving treatments. (A) infected mice, (B) uninfected mice (control), (C) mice treated with RN7-IN8 (20mg/kg), (D) mice treated with combination of RN7-IN10 and RN7-IN8 (10mg/kg + 10mg/kg), (E) mice treated eith combination of RN7-IN8 and CTX (5mg/kg + 5mg/kg), (F) mice treated with combination of RN7-IN8 and CTX (10mg/kg + 10mg/kg), (G) mice treated with combination of RN7-IN8 and CTX (20mg/kg + 20mg/kg). Bar indicates 500 μM.

CHAPTER 4: DISCUSSION

Streptococcus pneumoniae 'the captain of the men of death' as described by Sir William Osler still the most causative agent of community-acquired pneumonia (CAP), bacteremia and meningitis in children and adults as well as the leading cause of otitis media in infants and young children. Globally, this bacterium causes death in children beneath five years old more than any other pathogen. Furthermore, this bacterial pathogen is the most common cause of death among vaccine-preventable diseases (Kim *et al.*, 2016). The increased incidence of invasive pneumococcal diseases (IPD) and the global emergence of multidrug-resistant *Streptococcus pneumoniae* not only to β – lactams but also to other antibiotics such as macrolides, tetracycline, chloramphenicol, fluoroquinolones and cotrimoxazole complicate the control of IPD and may lead to treatment failure (Crowther-Gibson *et al.*, 2012; Gómez-Barreto *et al.*, 2000; Ziglam & Finch, 2002). A previous study showed that pneumococcal resistance to antibiotics (erythromycin, penicillin and fluoroquinolones) had led to 32,398 additional outpatient visits and 19,336 additional hos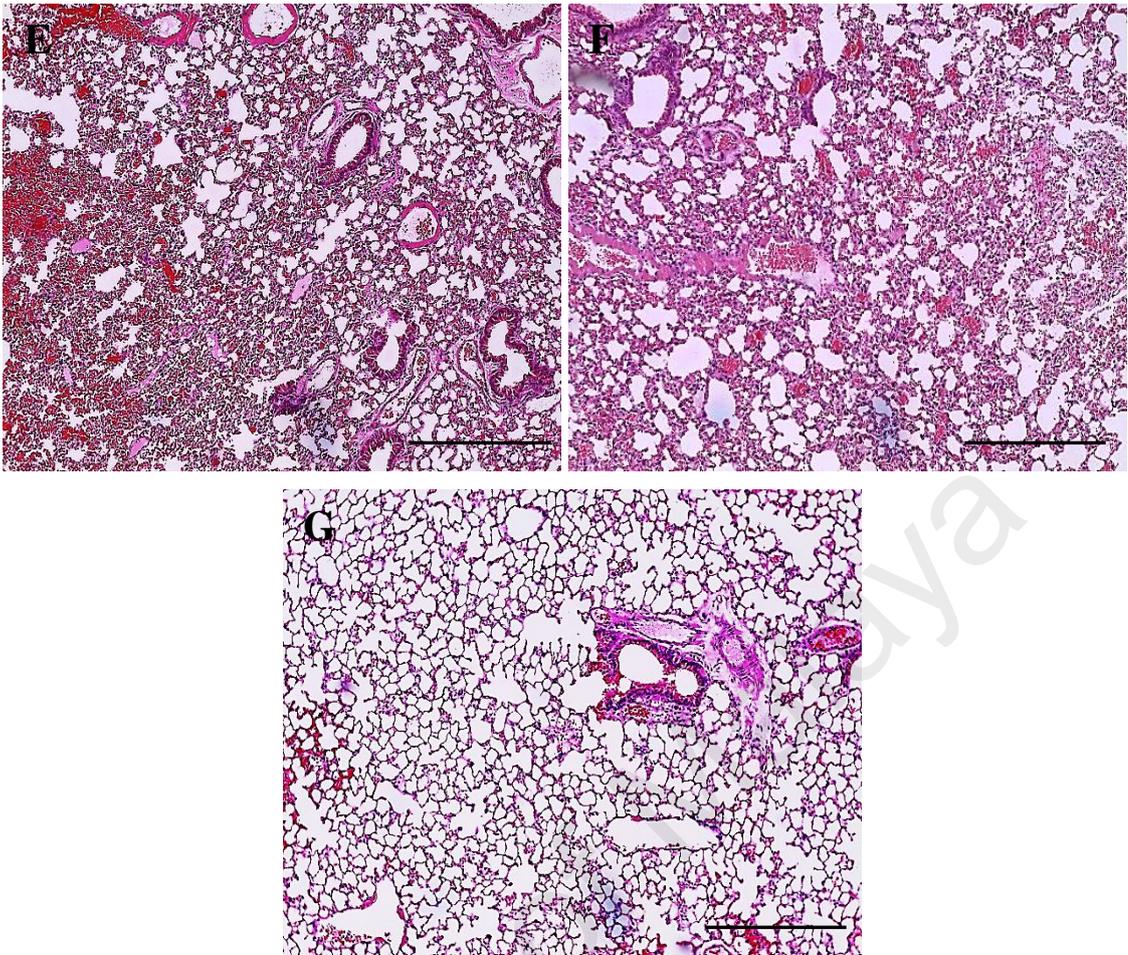pitalizations (Reynolds *et al.*, 2014). The incremental cost of antibacterial drug resistance accounted for $91 million (4%) of direct medical fees and $233 million (5%) of total fees including work and productivity losses (Reynolds *et al.*, 2014). Although Pneumococcal conjugate vaccine PCV served as a great tool against antibiotic resistance by *S. pneumoniae* and helped decrease the frequency of vaccine serotypes, there has been a considerable rise in the disease induced by non-vaccine serotypes (Reynolds *et al.*, 2014). Due to their broad-spectrum antimicrobial activity and rapid killing, antimicrobial peptides (AMPs) can serve as promising candidates for the development of a novel class of antibacterials to overcome the drug – resistance issue. In the present study, the template-based approach which relies on systematic manipulation of known peptide sequences with minimal computational input was utilized to design novel synthetic antipneumococcal peptides with improved antibacterial activity compared

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to their parent peptides. This strategy represents a straightforward yet effectual method for designing novel peptides with improved activity through systemic modifications of particular amino acid residues and the physicochemical properties of template peptides (Zelezetsky & Tossi, 2006).

Indolicidin, a cationic 13-amino acid antimicrobial peptide purified from the cytoplasmic granules of bovine neutrophils (Selsted *et al.*, 1992) while ranalexin, a cationic 20-amino acid peptide was isolated from the skin of the bullfrog, *Rana catesbeiana* (Clark *et al*., 1994). They were used as templates to design novel synthetic peptides with potent activity against *S. pneumoniae*. Indolicidin and ranalexin were selected for this study as both peptides are positively charged and have more than 50% hydrophobic residues. Cationicity and net charge are unquestionably crucial for the antibacterial activity of peptides, as the first step for cationic AMPs to exert their activity is to interact with the negatively charged bacterial membrane (Zelezetsky & Tossi, 2006). The differences between prokaryotic and eukaryotic membranes enable selective targeting of AMPs against the microbes but not the host cells (Yeaman & Yount, 2003). In general, electrostatic interaction represents the main attraction force driven the first contact between AMPs and negatively charged membranes of bacteria and other microbes (Brogden, 2005; Dathe & Wieprecht, 1999; Vaara, 1992). All biological membranes are composed of proteins and phospholipids. The net charge of any biomembrane is based on the composition of its phospholipid. Unlike eukaryotic cell membranes, bacterial membranes contain up to 30% highly electronegative lipids such as phosphatidylserine (PS), cardiolipin (CL) or phosphatidylglycerol (PG), a characteristic property favored by cationic AMPs. These acidic phospholipids tend to make the bacterial membrane highly negative in charge and thus attract the positively charged antimicrobial peptides to attach to the bacterial membranes and make them preferred by AMPs over eukaryotic membranes (Matsuzaki, 1999). Quite the opposite, the membrane of the eukaryotic cell

is enriched with zwitterionic phospholipids which are neutral in net charge such as sphingomyelin (SM), phosphatidylethanolamine (PE) or phosphatidylcholine (PC). These substances prevent the amalgamation of peptide molecules into membranes and the formation of pores (Shai, 1999). Moreover, sterols such as cholesterol and ergosterol which can be found in eukaryotic cytoplasmic membranes but rarely in prokaryotic membranes can decrease the activity of AMPs either by stabilizing the lipid bilayer of the membrane or by directly interact with peptides and neutralizing them (Giuliani *et al.*, 2007).

The impact of cationicity on the biological activity of AMPs has been documented in several studies. Ringstad *et al*. (2007) have reported that the ability of peptide CNY21 to disrupt microbial membranes increased with increasing net charge and hydrophobicity, and vice versa. Increasing the net charge of CNY21 variant CNY21K from +3 to +5 resulted in enhanced bactericidal effect of CNY21K for both *P. aeruginosa* and *B. subtilis*. In another study, the effect of net charge on the biological activity of peptide V13K analogs with a net charge ranging from -5 to +10 were investigated. The results revealed that decreasing the net charge to -5 increased the MIC value to >500µg/ml against *P. aeruginosa,* while increasing the net charge to +10 decreased the MIC to 4 µg/ml (Jiang *et al.*, 2008). However increasing the net charge to more than +8 has dramatically increased the hemolytic activity of the analog peptide by 32 fold (Jiang *et al.*, 2008). Similarly, Matsuzaki *et al*. (1997) have studied the role of cationicity in the antimicrobial activity of Magainin 2, an AMP isolated from the amphibian *Xenopus laevis* skin. Four magainin 2 analogs MG0, MG2+, MG4+, and MG6+, were designed with net charges ranging from 0 to +6. Their results showed that peptides with higher positive charges were correlated with enhanced binding affinity for the negatively charged artificial membranes, suggesting that net charge has a great impact on the antimicrobial activity of cationic peptides. However, there is a limit beyond which increasing net charge

no longer confers improved activity. For instance, increasing the net charge of magainin peptides to +6 or +7 has caused an increase in hemolytic tendency and reduction of antimicrobial activity (Dathe *et al.*, 2001).

Besides being cationic, both template peptides indolicidin and ranalexin have a high content of hydrophobic amino acids (53% and 65%, respectively). Hydrophobicity can be defined as the percentage of hydrophobic amino acid residues within a peptide sequence. Peptide hydrophobicity is another critical property that governs the attraction of AMPs toward bacterial membrane, as it directs the level to which an AMP can penetrate into the lipid bilayer (Yeaman & Yount, 2003). Nevertheless, high-level hydrophobicity would result in self-association or even precipitation of the peptide in water and would thus stop its transport to the microbial target. In contrast, a peptide with a very low hydrophobicity has an insufficient lipid affinity (Dathe & Wieprecht, 1999). Several studies have investigated the relation between hydrophobicity and antibacterial activity of peptides. Chen *et al*. (2007) have studied the impact of hydrophobicity on antibacterial activity of synthetic peptide V13K$_L$. They systematically reduced or improved the hydrophobic content by changing leucine residues with less hydrophobic alanine residues or switching alanine residues with more hydrophobic leucine residues on the nonpolar face of the helix, respectively. Their results revealed that reducing the hydrophobic content of the peptide was accompanied by reduced biological activity. Improving the antimicrobial activity of the V13K$_L$ by increasing the hydrophobicity was achievable, however, only up to a certain level. As there was an optimum hydrophobicity window in which high antimicrobial activity could be achieved. Deviation from the specific hydrophobicity window would result in a significant loss of antimicrobial activity. Likewise, the lower hydrophobic variant 6K-F17 designed by Yin *et al*. (2012) was found to possess a four-fold stronger antipseudomonal activity at 4 µM as compared to the Ala-substituted analog 6K-F17-4L. An added advantage with 6K-F17 was the

minimal hemolytic activity up to 320 µM over the 40 – 80% hemolysis exhibited by 6K-F17-4L at the same concentration. Furthermore, their findings proposed that AMPs with higher ratio of hydrophobic amino acids have greater self-association and aggregation tendencies as compared to those peptides with lower levels of hydrophobicity. Thus, when modifying peptide hydrophobicity careful consideration should be taken into count.

Two sets of synthetic analogs were designed in this study, four indolicidin analogs (IN1, IN2, IN3, and IN4) and four ranalexin analogs (RN1, RN2, RN3, and RN4) based on the amino acid substitution strategy to increase or decrease the net charge and hydrophobicity. Apart from these two sets, another group of five hybrid peptides (RN7-IN10, RN7-IN9, RN7-IN8, RN7-IN7, and RN7-IN6) were designed based on trimming and hybridizing two fragments from the parent peptides indolicidin and ranalexin. Hybridization is one of the efficient methods to design novel synthetic peptides with enhanced properties than their templates (Brogden & Brogden, 2011). Several studies have reported the design of hybrid peptides such as cecropin A-melittin (Andreu *et al.*, 1992), LFB15(W4,10) - HP(2-20) - cecropin A (Tian *et al.*, 2009), and cecropin A-magainin 2 (Lee *et al.*, 2004) using similar strategies. Their results demonstrated that the hybrid candidates possessed an enhanced antimicrobial activity as compared to their respective parent peptides.

In term of *in vitro* antibacterial activity, four hybrid peptides (RN7-IN10, RN7-IN9, RN7-IN8, and RN7-IN6) displayed the strongest antipneumococcal activity against 30 pneumococcal strains, with MIC values ranging between 7.81 and 15.62µg/ml, which are less than their parent peptides indolicidin (31.25µg/ml) and ranalexin (62.5µg/ml). These four hybrids exhibited the strongest activity against *E.coli, S. aureus* and MRSA with MIC values of 7.81µg/ml (Table 3.5 and 3.6). The four hybrids have a high hydrophobic residues content and positively charged amino acids which enable the hybrid peptides to interact more efficiently with the negatively charged bacterial membranes and

consequently destroy the bacterial pathogen. They retain the hydrophobic amino-terminal residues (Phe-Leu-Gly-Gly) of the template peptide ranalexin. The previous study done by Clark *et al*. (1994) revealed that deletion of these four amino acid residues has significantly reduced the antibacterial activity of ranalexin against *E. coli* (the MIC increased from 32 to 256µg/ml) and *S. aureus* (MIC increased from 4 to 256µg/ml). Also, like their parent peptide indolicidin, these hybrid AMPs have a high content of tryptophan (Trp) and arginine (Arg) residues (Table 3.3). AMPs with more than one Trp and Arg residues have been gathered in the Trp/Arg-rich family of antimicrobial peptides (Chan *et al.*, 2006). Trp exists in about 34% of all antimicrobial peptide sequences identified to date (Waghu *et al.*, 2014). It is widely known that Trp has a significant role in membrane-spanning proteins, as this amino acid residue strongly prefer the interfacial regions of lipid bilayers. In certain cases, Trp is considered hydrophobic due to its uncharged side-chain. However, it is observed that Trp residues do not reside in the hydrocarbon region of lipid bilayers and accordingly it is placed towards the more hydrophilic side of the scale (Chan *et al.*, 2006). Another key factor is the extensive π–electron system of the aromatic indole sidechain that gives rise to a significant quadrupole moment. Cation–π interactions occur between the cationic sidechains of the basic amino acids arginine (Arg) or lysine (Lys) and the aromatic side-chains of the aromatic amino acids tryptophan (Trp), tyrosine (Tyr) or phenylalanine (Phe) (Gallivan & Dougherty, 1999). Cation–π interactions are significant for peptide self-association inside membranes and enable deeper insert into membranes by sheltering the cationic side chains (Torcato *et al.*, 2013). Earlier reports have shown that Trp is the most likely of aromatic residues to be involved in a cation- π interaction as its sidechain best-suited for cation- π interactions (Gallivan & Dougherty, 1999; Mecozzi *et al.*, 1996). Consequently, the Trp residue assists the insertion of the cationic amino acid arginine (Arg) into the membrane hydrophobic region through cation–π interactions. Several reports have acknowledged the significance of Trp

in the disruption of the microbial membrane and antimicrobial activities of AMPs. In a previous study, the effect of replacing Trp residues on the biological activity of puroidoline (puroA) derived peptides was assessed. The data showed that decreasing the number of Trp by two residues had led to an increase in the MIC of the analog peptide puroB1 from 5.3µM against *E. coli* to >125 µM and from 20 µM against *S. aureus* to >125 µM (Haney *et al.*, 2013). Besides this, a previous study has demonstrated that replacing Trp with Phe in tritrpticin (another Trp/Arg rich peptides) has led to a peptide analog with comparable antimicrobial activity but decreased membrane disruption capacities (Schibli *et al.*, 2006), while substituting Trp with Tyr or alanine (Ala) produced varients with less antimicrobial activity (Arias *et al.*, 2016). Arginine is another crucial amino acid for the antimicrobial activity of the peptides. The importance of this residue comes from its guanidinium sidechain which is able to create strong bidentate H-bonds with phosphate moieties from two lipid head groups of the bacterial membranes (Torcato *et al.*, 2013). Moreover, the guanidinium sidechain can form almost as many hydrogen bonds with the surrounding water molecules even when it is engaged in cation–π interactions with an aromatic residue (Chan *et al*., 2006). The effect of arginine on the activity of antimicrobial peptides has been studied previously. Replacing lysine residues in BP100 with arginine in its analog R-100BP resulted in decreased MIC of the new peptide against resistant *S. pneumoniae* from 16µM to 4µM (Torcato *et al.*, 2013). Altogether, this study proposes that all these characteristics have probably boosted the antibacterial activity of the newly designed hybrids over their template peptides.

Four indolicidin analogs (IN1, IN2, IN3, and IN4) and four ranalexin analogs (RN1, RN2, RN3, and RN4) were designed, and their positive charge was increased while their hydrophobicity was decreased gradually in the hope of improving their activity and at the same time reducing their possible toxicity (Table 3.4). Although they have an increased positive charge as compared to their parent peptides, they demonstrated lower

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antibacterial activity than their template peptides against all the pneumococcal clinical isolates. Their reduced activity could possibly be due to the reduction in their hydrophobicity and hence reducing their affinity to the lipid bilayer of the bacterial membrane. As mentioned above, increasing or decreasing the hydrophobicity beyond the optimum window will affect the peptide activity. On the other hand, increasing the net charge of the analog peptides even up to +7 in the case of IN3 and IN4 did not have any impact on their antibacterial activity as none of the analogs were more potent than its parent peptide (Table 3.5 and 3.6). Thus, further studies are needed to determine whether increasing the net charge beyond +7 or decreasing it below +3 would improve the attraction of the peptides to the negatively charged bacterial membrane and to identify the optimal degree of cationicity for these particular analog peptides.

Combinations of antimicrobial agents are often used to expand the antimicrobial spectrum, reduce cytotoxicity and combat multi-drug resistant isolates (Novy *et al.*, 2011). Several studies reported the synergistic effects of combinations of AMPs with standard antibiotics. The hybrid peptide LHP7 revealed a synergistic effect against a clinical isolate of methicilin-resistant *S. aureus* (MRSA) when combined with ampicillin (Xi *et al.*, 2013). Ranalexin exhibited synergism activity against Gram-positive bacteria *S. aureus* when combined with doxycycline and clarithromycin, individually (Zhou & Peng, 2013). Similarly, a combination of the A3 peptide with chloramphenicol showed synergistic act against *E. coli, P. aeruginosa* and *S. aureus* (Park *et al*., 2004). In the current study, the checkerboard MIC technique was utilized to assess drug-drug interaction. The results revealed that all the five hybrid peptides showed synergistic effects (FICI ≤0.5) against both susceptible and resistant pneumococcal clinical isolates when combined with each other and with conventional drugs erythromycin and ceftriaxone (Table 3.7). Combinations of indolicidin analogs with each other demonstrated indifference activity (0.5< FICI ≤4), however, they exhibited synergistic

activity with conventional drugs. One possible explanation of the synergistic effects of peptides-drugs combinations is that the breakdown of peptidoglycan by β-lactam antibiotics increase the access of the of hybrid peptides to the cytoplasmic membrane (Zhou & Peng, 2013). Hybrid peptides may also increase membrane permeability by interacting with the bacterial cell wall/membrane, allowing conventional drugs to easier act on their targets. Previous reports have shown that β-lactam antibiotics like ceftriaxone exert higher antimicrobial activity when combined with membranolytic peptides such as nisin, as these AMPs cause changes in cell morphology by forming pores allowing antibiotics to enhance their act and generate a greater disruption within the cell wall (Singh *et al.*, 2013; Tong *et al.*, 2014). Another possible mechanism of synergistic combinations is that antimicrobial peptides alter the efflux pump systems allowing intracellular antibiotics such as macrolides to act more efficiently on their intracellular targets (Ruhr & Sahl, 1985; Soren *et al.*, 2015). Moreover, antimicrobial peptides and erythromycin may act synergistically by inhibiting sequential steps in protein biosynthesis (Park *et al*., 2004).

One of the important features which favor AMPs over traditional antibiotics is that they are able to kill microbial pathogens rapidly. As reported here, results obtained from time killing assay of the peptides revealed that at 1×MIC all five hybrid peptides were able to show rapid antipneumococcal activity and their killing kinetics were far superior to those of the standard drugs erythromycin and ceftriaxone (Figure 3.1A and C). Among all the designed peptides, RN7-IN10 and RN7-IN8 exhibited the most active killing kinetics (30 min and 90 min, respectively) and were able to eliminate both susceptible and resistant pneumococci (Figure 3.1A and C). The rapid reduction of pneumococcal cell number by 6 $\log_{10}$ after 30 min of treatment (RN7-IN10) and 3 $\log_{10}$ after 60 min (RN7-IN8) indicates a bactericidal nature of these peptides and usually occurs within membrane acting compounds (Bauke Albada *et al.*, 2012; Pag *et al.*, 2008).

Thus, the robust and fast killing kinetics of the hybrid peptides correlates with their cationic and hydrophobic properties, which gives them the ability to interact more efficiently and rapidly with the pneumococcal membrane. As mentioned above, the high content of tryptophan and arginine allows the peptides to bind more efficiently and rapidly to the bacterial membrane. Fast killing kinetics is a vital feature for the antibacterial agent development as the rapid killing prevents the selection for resistance and consequently reduces any possibility for resistant phenotypes development during antimicrobial peptide treatment (Deslouches *et al.*, 2013). The killing kinetics of natural and synthetic peptides against Gram-positive bacteria have been assessed previously. The killing kinetics of plectasin, a 40 amino acid peptide, was tested against *S. pneumoniae*. After 5hr of incubation, plectasin was able to reduce the number of pneumococcal cells ($\log_{10}$(CFU/ml)) from 6.5 to 3 (Mygind *et al.*, 2005). Another study showed that the synthetic peptide FBRW-15 (21 amino acid, MIC = 4 - 16 µg/ml) at 25µg/ml was able to eliminate more than 3 $\log_{10}$ of *S. mutuns* cells (1×10^6 CFU/ml) within 30 min of treatment (He *et al.*, 2007).

The results obtained from assessing the effects of peptides on human erythrocytes (RBCs), NL20 and WRL68 cell lines demonstrated that all the hybrid peptides did not display any hemolytic (Figure 3.2) or cytotoxic effects (Figure 3.3 and 3.4) at their MIC levels. However, RN7-IN10, RN7-IN9, RN7-IN8, and RN7-IN6 showed high toxic effects at concentrations of ≥ 62.5µg/ml (WRL-68 cell line) and ≥ 125µg/ml (NL-20 cell line) (Figure 3.3 and 3.4). The high toxicity of the tested peptides against human cells is probably due to their high content of hydrophobic residues, as peptides with greater hydrophobic content lean towards penetrating deeper into the hydrophobic core of the electrically neutral membranes. The high content of tryptophan residues within the peptides could be another reason for their toxicity at high concentrations (Podorieszach & Huttunen-Hennelly, 2010). It is worth noting that complex biological environments

such as human serum and whole blood may markedly decrease the potential toxicity of synthetic peptides to eukaryotic cells (Deslouches *et al.*, 2013). However, a number of strategies could be used to reduce the toxic effects of AMPs without affecting their antimicrobial activity. A previous study showed that replacing proline residues with lysine increased the antimicrobial activity of tritrpticin-amide peptide analog and decreased its toxicity to mammalian cells (Zhu *et al.*, 2006). Shifting the position of tryptophan residues within the hydrophobic part of the amphipathic helix is another way to decrease the toxic effect of the peptide without affecting its antimicrobial activity (Rekdal *et al.*, 2012). Toxicity of AMPs toward mammalian cells can also be minimized by cycling linear peptides. Cyclization of the linear peptide CKLLLKWLLKLLKC with an intramolecular disulfide bond improved its activity against Gram-negative bacteria and at the same time decreased its toxicity to erythrocytes (Matsuzaki, 2009). Cyclization of melittin and magainin2 analogs reduced their selective toxicity to toward erythrocytes (Unger *et al.*, 2001).

Apart from studying the antibacterial activity of the designed peptides, this study aimed to investigate the ability of peptides to neutralize pneumococcal virulence factors. The protein-peptide interaction has a great effect on structural based drug design. The molecular docking study was conducted to evaluate the ability of peptides to bind effectively to three virulent factors; autolysin (LytA), pneumolysin (Ply), and pneumococcal surface protein A (PspA). These three enzymes play an important role in the pathogenicity of pneumococci, and they seem to be the best candidates for drug or vaccine development (Jedrzejas, 2001). A previous study demonstrated that CSF infection of rats with wild-type pneumococcal strain caused meningitis within 26hr, while mutant pneumococcal isolate that does not express autolysin or pneumolysin produced very mild or no disease (Hirst *et al*., 2008). In the same way, the PspA-negative mutant was less virulent in a mouse model than the wild-type (Ren *et al*., 2012). The purpose of

the current study was to assess the capacity of the novel hybrid peptides to bind to these virulence factors as part of their mechanism of action. The results from the molecular docking indicated that all the hybrids have negative binding energy which indicates the favorable binding of peptides with all three receptors. All the test peptides bound well with the target enzymes since they demonstrated negative binding affinities to the target proteins (Table 3.7). Detailed amino acids involved in binding of the minimized lowest docking complexes are analyzed in term of the van Der Waal (VDW) and electrostatic with amino acids in 3 Å vicinity of the peptides and total interaction energy (IE) value CHARMm force field. Among all the five hybrid peptides, RN7-IN6 displayed the strongest binding by docking and correlated with our experimental results possibly due to the inhibition of autolysin and pneumolysin receptor. For PspA, RN7-IN8 had the lowest binding energy due to the more number of amino acids in the binding interaction. RN7-IN6 peptide are involved in forming several hydrogen bonds in both end chain with several protein residues at RN7-IN6:GLY4:HN − B:THR259:O, RN7-IN6:ILE6:HN - A:ALA370:O, A:ASN569:HD21 − RN7-IN6:LEU2:O, A:GLY577:HN − RN7-IN6:NH214:N1, A:TRP578:HN − RN7-IN6:ARG13:OXT and RN7-IN6:LEU2:HN − A:ASP573:OD2.

Microscopic techniques like transmission and scanning electron microscopy have been used comprehensively for the elucidation of the interaction of membrane-active peptides (Hyde *et al.*, 2006; López-Expósito *et al.*, 2008). TEM and SEM were used to evaluate the morphological changes induced by hybrid peptides. Results obtained from TEM give strong evidence that targeting the bacterial cell wall and or plasma membrane seems to be the main mechanism used by the hybrid peptides to kill pneumococcal cells. Unlike the untreated cells, pneumococcal cells treated with hybrid peptides resulted in breakage and fragmentation of the bacterial cell wall/membrane indicating a strong interaction between the negatively charged membrane and the hybrid peptides due to their

positive charge and high hydrophobic content (Jindal *et al.*, 2015). Also, the detachment of the cytoplasmic membrane from the cell wall was observed in pneumococcal cells upon treatment with hybrid peptides is a possible indication of the capability of hybrid peptides to interpolate themselves between pyrophosphate-linked cell-wall anchors and the cell membrane. Subsequently, this act would pull out the isoprenyl anchor chains away from the cell membrane and weaken cell-wall adhesion. The results obtained by TEM were similar to that reported on the mechanism of nisin against *B. subtilis* (Hyde *et al.*, 2006) and *E. faecalis* (Tong *et al.*, 2014). Likewise, chicken CATH-2 was able to disrupt and detach the plasma membrane from the cell wall of *C. albicans* (Ordonez *et al.*, 2014). Another possible explanation is that the breakage of the cell wall will allow the insertion of water from the medium between the two membranes and detach them (López-Expósito *et al.*, 2008). Likewise, in SEM studies changes detected were swelling and aggregation in contrast to the smooth surface of untreated bacterial cells. Besides, the numerous fragments observed in *S. pneumoniae* cultures treated with hybrid peptides point to a cell wall breakage and cell lysis. Membrane disruption could be associated with leakage of ions and metabolites, depolarization and eventually cell death. Adenosine triphosphate (ATP) is one of most significant molecules for all living cells as it used as an intracellular source of energy for many biological processes (Mempin *et al.*, 2013). In normal conditions, bacterial membranes are impervious to ATP and other intracellular constituents as membrane destabilization might lead to the release of normally impervious substances. Therefore, ATP has been used as a tool to measure the integrity of living cells. Since most of the ATP is found within the cells, any cell injury will result in a prompt reduction in the cytoplasmic ATP. The results of the current study revealed that ATP efflux was not increased by incubation of pneumococcal cells with standard drugs ceftriaxone and erythromycin but was increased by hybrid peptides. The ATP efflux results suggest that our positively charged hybrid peptides have high affinity to bind to

the negatively charged bacterial membrane, disrupting its integrity and allowing a significant amount of ATP to be released to the surrounding environment. However, a reduction in the amount of ATP released to the medium was noticed after 1hr of incubation; this might be due to the rapid degradation of ATP by enzymes released to the medium as a result of membrane damage which subsequently leads to rapid cell death. Such results were also observed when *Candida albicans* was treated with CATH-2 peptide, the measurable ATP after 5min of incubation were higher than the levels of ATP after 1hr of incubation (Ordonez *et al.*, 2014). Also, the synthetic peptide Tet052 was capable of causing a significant leakage of ATP from *S. aureus* after 30min of treatment (Hilpert *et al.*, 2009). Altogether, results from TEM, SEM and ATP release assay point out that the hybrid peptides destabilize the cell envelope of the pneumococcal cells. Hence it can be hypothesized that the disturbance of the bacterial surface must activate an autolytic and/or cell death mechanism. However, pore-forming and membrane damage do not preclude hybrid peptides from other mechanisms of action due to the fact that antimicrobial peptides could be membrane-disruptive when present at high concentration but shift to attack intracellular components when present at low concentration, or both (Friedrich *et al*., 2000; Friedrich *et al*., 2001).

It is well known that the cell membrane is not the only target for antimicrobial peptides. AMPs may also attack other cell components such as DNA, RNA and proteins (Li *et al*., 2013). For instance, buforin II showed the ability to translocate to the inner leaflet of the cytoplasmic membrane and target intracellular DNA after penetrating the cell membranes resulting in rapid cell death (Park *et al*., 1998). This study examined the ability of hybrid peptides to bind to genomic DNA through gel retardation assay which provides qualitative and quantitative information that evaluates the DNA-binding capabilities of hybrid peptides. The results from the DNA retardation assay displayed that all the hybrid peptides were capable of binding to DNA efficiently and preventing it from

moving down through the agarose gel. These results suggest that hybrid peptides could possess another mechanism of bacterial killing by inhibiting intracellular functions via interference with DNA function. Peptides interaction with DNA can prevent or hinder gene expression which is an efficient tactic to suppress and inhibit normal enzyme and receptor synthesis, disrupt the components required for the life cycle of the bacterial cell and consequently cell death (Li *et al*., 2013).

Based on the *in vitro* antimicrobial activity findings, four hybrid peptides RN7-IN10, RN7-IN9, RN7-IN8 and RN7-IN6 were selected to evaluate their *in vivo* toxicological profile using mice models. The results showed that at 100mg/kg and 20mg/kg all the peptides appeared to be well tolerated when injected via SC and IN route, respectively. All the treated mice showed normal physical behavior, the heamatogram and blood serum profiles were similar to that of the untreated group. Histopathological studies were performed with the lung, liver, spleen, kidney and brain of control and treated animals. No histological changes were detected in the five organs of any group. On the other hand, when injected via IP route, hybrid peptides displayed high toxic effects and death was occurred. Reduced doses were injected into the mice until the maximum non-toxic dose was identified which is 20mg/kg for both RN7-IN10 and RN7-IN8, and 10mg/kg for RN7-IN9 and RN7-IN6. These findings are in match with the *in vitro* toxicity results, which showed that at 62.5µg/ml both RN7-IN10 and RN7-IN8 were less toxic to NL-20 and WRL-68 human cell lines than RN7-IN9 and RN7-IN6.

Towards evaluating the *in vivo* antipneumococcal activity of peptides in living organisms, two hybrid peptides RN7-IN10 and RN7-IN8 were selected to treat mice models infected with highly virulent pneumococcal strain. At 5mg/kg, both hybrid peptides could not treat infected mice. However, even at low concentration they were able to delay pneumococcal spread and subsequently mice death as compared to untreated mice that died within three days post-infection. Four out of ten mice received RN7-IN10

at 5mg/kg were able to survive up to day four post-infection, while two mice were able to survive up to day five post-infection when received RN7-IN8 at 5mg. Among all the doses assessed, RN7-IN8 at 20mg/kg showed the greatest therapeutic potential with 50% of the infected mice cured up to seven days after treatment ($p < 0.001$) as compared to the untreated mice. Two combinations RN7-IN10 and RN7-IN8 at 5mg + 5mg and 10mg + 10mg were tested for their synergistic effect. The survival rates of infected mice receiving the combination treatment of RN7-IN10 and RN7-IN8 at varying dosages were significantly increased by 40% and 20% as compared to the sum of the survival rates of the standalone treatment. These findings are consistent with our *in vitro* synergism result, which showed that hybrid peptides could act synergistically when combined with each other.

The use of two or more antibacterial drugs in combination therapy has been the alternative strategy to enhance treatment outcome in the clinical setting (Caballero & Rello, 2011). This is especially valuable in patients with severe pneumococcal infections. For instance, combination antibiotic therapy with both β-lactam and macrolide had a significantly lower case-mortally rate as compared with those given single antibiotic therapy (Mufson & Stanek, 2006). Extended-spectrum cephalosporins such as ceftriaxone are important antibiotics in the treatment of invasive infections caused by penicillin-resistant *S. pneumoniae*. However, the rate of pneumococcal strains resistant to ceftriaxone has been increased significantly (Chiu *et al.*, 2007). Hence, RN7-IN8 which showed significant therapeutic efficacy to the infected mice in its standalone form was further assessed for the *in vivo* therapeutic synergism in combination with ceftriaxone. Based on the *in vitro* results, RN7-IN8 has been found to produce a synergistic effect when combined with standard antibiotic ceftriaxone. Interestingly, combinations of RN7-IN8 and ceftriaxone also produced a synergistic effect when tested *in vivo* using mice infected with pneumococcal bacteremia model. The survival rates in mice treated with

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combinations of RN7-IN8 and ceftriaxone at varying dosages were dramatically increased as compared to the sum of the survival rates of standalone treatment. Adding the hybrid peptide RN7-IN8 at 5mg, 10mg and 20mg to ceftriaxone has improved the efficacy of the standard drug to treat infected mice as compared to its standalone form by 50%, 50% and 60% respectively. Hence, reducing the dose of antibiotic needed to treat pneumococcal infection and at the same time minimize the chance of developing bacterial resistance. Unlike untreated mice that died within four days after infection, 100% of mice treated with a combination of RN7-IN8 and ceftriaxone at 20mg – 20mg survived at day seven post-infection and none of them showed signs of sickness or abnormal behavior. The severe tissue damages especially in lung and spleen caused by pneumococci in the infected mice confirm the spread and establishment of pneumococcal infection following injection of the bacterial inoculum. Remarkably, such histological changes in the organs of mice received treatments with RN7-IN8 in standalone form and in combination with CTX were minimal and significantly lower than those of the untreated group. These findings indicated that the current treatments particularly the combination of RN7-IN8 and CTX at 20mg-20mg were able to rapidly clear the invaded bacteria and reduce the infection to the minimal level. This result proposes that both the peptide and antibiotic were able to work synergistically and enhance the act of each other as each drug has its own mechanism of action and thus makes it harder for the bacteria to survive and resist.

CHAPTER 5: CONCLUSION

The inappropriate use of antibiotics for the last decades has led to the rise of antibiotic-resistant bacteria including the major human pathogen *Streptococcus pneumoniae*. This phenomenon made pneumococcus harder to treat and complicates empirical therapy and patient management. Hence, a newer class of antibacterial drugs to overcome this serious issue is desperately in demand. In the present study, thirteen novel peptides have been designed based on modifying the sequences of two naturally occurring templates indolicidin and ranalexin. Out of these thirteen novel peptides, five hybrid peptides namely RN7-IN10, RN7-IN9, RN7-IN8, RN7-IN7 and RN7-IN6 showed potent and rapid antibacterial activity when tested *in vitro*. Another advantage of these novel peptides is that they are able to act synergistically when combined with the standard drug. Thus, reducing the ability of pneumococci to resist treatment. Several experiments have revealed that the main mechanism of action of these hybrid peptides is by attacking and disrupting the integrity of the cell membrane. Their ability to bind genomic DNA and prevent it from migration through the gel give another possibility that these hybrids could possess another intracellular mechanism of action.

From the therapeutic efficacy assessment in mice infected with highly virulent pneumococcal strain, RN7-IN8 at 20mg/kg protected 50% of the mice from death. None of the survived mice showed apparent symptoms of infection. Notably, combinations of RN7-IN10 and RN7-IN8 enhanced the survival rate of the infected mice in a synergistic manner. Similarly, combinations of hybrid peptide RN7-IN8 and ceftriaxone showed synergistic effects when tested on infected mice confirming the results of the *in vitro* synergism assay. In current study, the antimicrobial activity and toxicity of RN7-IN8 have been investigated using several *in vitro* and *in vivo* experiments and according to our results we believe that this peptide has a great potential to be further developed into a therapeutic drug either as a standalone agent or in combination with conventional

antibiotics to improve the treatment outcomes especially against antibiotic-resistant *S. pneumoniae*.

However, there are several limitations encountered with this study. Although RN7-IN8 showed promising therapeutic outcome, there are some limitations in its efficacy. Primarily, the peptide had no antibacterial effect when tested in the pneumococcal pneumonia model. This perhaps because the bacterial suspension was injected directly to the thoracic cavity to infect the lungs whereas the peptide was given at distant site (IP route) indicating that the peptide could not diffuse effectively to the site of infection. Probably due to the degradation of the peptide by blood or cellular components. On the other hand, the effectiveness of the peptide in the bacteremia model is probably due to both infection and treatment being carried out in the same site and thus the peptide has more time to interact with the bacterial cells.

Although the TEM and SEM results clearly showed that the hybrid peptides are damaging the cell membrane, the effects of these peptides on pneumococcal gene expression are still unclear and have to be investigated via microarray or transcriptome analysis to understand the mechanisms of action of the hybrid peptides in altering different pathways leading to cell death. Synthesizing the hybrid peptides using recombinant techniques would be of great interest to reduce the cost of synthesis and thus reducing the cost of therapy. Redesigning the hybrid peptides especially RN7-IN8 as a circular AMP rather than linear AMP is one of the significant strategies to reduce its toxicity. Moreover, evaluating the pharmacokinetics/pharmacodynamics of the hybrid peptides will be significant to understand the various changes that take place upon injecting the peptide inside the animal or human body. These aspects are critical and shall be studied in the future towards developing the hybrid peptides as potential novel antibiotics.

In summary, antimicrobial peptides (AMPs) represent the most favorable candidates for the development of novel antibiotics to fight in the war against resistant microbes considering their several advantages over traditional antimicrobial drugs. The simplicity of the primary amino acid sequence and the secondary structure would allow the design of new AMPs with the less computational burden. With the rapid expansion in the strategies of designing new AMPs and the use of recombinant DNA techniques, AMPs have a huge potential to play a crucial role in the combat of resistant bacteria either as standalone therapeutics or in combination with other drugs.

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Shahadat, H., **Jindal, H. M.,** Rasid, S. M., Raju, C. S., Sekaran, S. D.,…. Wiart, C. (2017). Antibacterial effects of 18 medicinal plants used by the Khyang tribe in Bangladesh against human pathogenic bacteria determined by disc diffusion, broth microdilution, time-killing and synergistic interaction assays. (Submitted to Phytotherapy Research).

Jindal, H. M., Ramanathan, B., Le, C. F., Gudimella, R., Razali, R., Manikam, R., & Sekaran, S. D. (2017). Comparative genomic analysis of ten clinical Streptococcus pneumoniae collected from Malaysian hospital reveal 31 new unique drug resistant SNPs using whole genome sequencing. (Submitted to Journal of Biomedical Science).

Jindal, H. M., Zandi, K., Ong, K. C., Velayuthan, R. D., Raju, C. S., & Sekaran, S. D. (2017). Mechanisms of action and in vivo antibacterial efficacy assessment of five novel hybrid peptides derived from Indolicidin and Ranalexin against Streptococcus pneumoniae. *PeerJ, 5*, e3887.

Ramanathan, B., **Jindal, H. M.,** Le, C. F., Gudimella, R., Anwar, A., Razali, R., Iskandar, J., Manikam, R., & Sekaran, S. D. (2017). Next generation sequencing reveals the antibiotic resistant variants in the genome of Pseudomonas aeruginosa. *PLoS ONE, 12(8),* e0182524.

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Ali, Z. A., Ismail, W. A., Le, C. F., **Jindal, H. M.,** Yahya, R., Devi Sekaran, S., & Puteh, R. (2014). Antibacterial coating for elimination of Pseudomonas aeruginosa and escherichia coli. Journal of Nanomaterials. Article ID 523530.

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APPENDIX A: PRESENTATIONS AND WORKSHOPS

A1) ORAL PRESENTATION

A1.1 **Hassan Mahmood Jindal**, Kaivan Zandi, Rukumani Devi, and Shamala Devi Sekaran. *In vitro* and *in vivo* antimicrobial activity assessment of novel synthetic peptides against *Streptococcus pneumoniae.*

Presented at the 26th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID 2016), 25th - 28th April 2016, Amsterdam – The Netherlands.

ABSTRACT
Background: According to WHO, 1.6 million deaths are caused by pneumococcal infections every year with 0.7 to 1 million in children younger than 5 years mostly in Asia and Africa. Like other gram positive bacteria, *Streptococcus pneumoniae* is increasingly difficult to treat due to the irrational use of antibiotics. Antimicrobial peptides (AMPs) represent a possible alternative for current antibiotics against drug resistant pathogens.

Methods & Materials: In this study, thirteen antimicrobial peptides were designed based on two natural peptides indolicidin and ranalexin. The *in vitro* activity of these peptides was investigated using broth microdilution assay, hemolytic activity assay, time killing assay, and toxicity assay against two cell lines WRL-68 and NL-20. Mechanisms of action of peptides were assessed using transmission electron microscopy (TEM), scanning electron microscopy (SEM), DNA binding assay, and in silico molecular docking against three virulent factors.

Results: Our results revealed that four hybrid peptides RN7-IN10, RN7-IN9, RN7-IN8, and RN7-IN6 possess potent antibacterial activity against 30 pneumococcal clinical isolates (MIC 7.81-15.62µg/ml). These four hybrid peptides showed broad spectrum antibacterial activity (7.81µg/ml) against *S. aureus*, methicillin resistant *S. aureus* (MRSA), and *E. coli*. Furthermore, the time killing assay results indicated that the hybrid peptides were able to eliminate *S. pneumoniae* within less than one hour which is faster than the standard drugs erythromycin and ceftriaxone. The cytotoxicity was tested against human erythrocytes, WRL-68 normal liver cell line, and NL-20 normal lung cell line. The results revealed that none of the thirteen peptides have cytotoxic or hemolytic activities at their MICs. TEM and SEM results showed that these four peptides are killing the bacteria by destroying the integrity of their membranes. DNA binding assay revealed that the hybrid peptides were able to bind to DNA at 62.5µg/ml preventing it from migration through the agarose gel.

A1.2 **Hassan Mahmood Jindal**, Kaivan Zandi, Rukumani Devi, and Shamala Devi Sekaran.
Novel synthetic antimicrobial peptides against *Streptococcus pneumoniae*.

Presented at the International Postgraduate Research Awards Seminar (inPRAS 2016), 7th - 8th March, University of Malaya, Kuala Lumpur – Malaysia.

ABSTRACT

Antimicrobial peptides (AMPs) represent promising alternatives to conventional antibiotics in order to defeat multidrug-resistant bacteria such as Streptococcus pneumoniae. In this study, thirteen antimicrobial peptides were designed based on two natural peptides indolicidin and ranalexin. Our results revealed that four hybrid peptides RN7-IN10, RN7-IN9, RN7-IN8, and RN7-IN6 possess potent antibacterial activity against 30 pneumococcal clinical isolates (MIC 7.81-15.62µg/ml). These four hybrid peptides also showed broad spectrum antibacterial activity (7.81µg/ml) against *S. aureus*, methicillin resistant *S. aureus* (MRSA), and *E. coli*. Furthermore, the time killing assay results showed that the hybrid peptides were able to eliminate *S. pneumoniae* within less than one hour which is faster than the standard drugs erythromycin and ceftriaxone. The cytotoxic effects of peptides were tested against human erythrocytes, WRL-68 normal liver cell line, and NL-20 normal lung cell line. The results revealed that none of the thirteen peptides have cytotoxic or hemolytic effects at their MIC values. The in silico molecular docking study was carried out to investigate the binding properties of peptides with three pneumococcal virulent targets by Autodock Vina. RN7IN6 showed a strong affinity to target proteins; autolysin, pneumolysin, and pneumococcal surface protein A (PspA) based on rigid docking studies. Our results suggest that the hybrid peptides could be suitable candidates for antibacterial drug development.

A2) WORKSHOPS AND COURSEWORKS

A2.1 Digital Image Processing – Fundamental Concepts & Techniques in MATLAB. 25 & 26 February 2014. University of Malaya.

A2.2 Drug Discovery & Designing: Insights of Protein-Ligand Interaction. Conducted by Panoply Consultancy, Malaysia & BioDiscovery Group Life Sciences, India. 7th – 11th April 2014. University of Malaya.

A2.3 Basic and Clinical Immunology Course. Faculty of Medicine, University of Malaya. 23rd – 27th July 2013.

A2.4 Introductory Bioinformatics workshop. Perdana University. 1st – 9th July 2013.