



A pivotal role for pneumolysin in the immunopathogenesis, treatment and prevention of pneumococcal disease

Charles Feldman, Ronald Anderson

The pneumococcus remains an extremely important cause of morbidity and mortality worldwide. While the micro-organism has a number of significant virulence mechanisms, many recent studies have focused on pneumolysin, which is recognised to be one of the most important virulence factors of the organism. The purpose of this manuscript is to review recent developments with respect to the role of pneumolysin in pneumococcal disease. It describes how the cytotoxic and other pro-inflammatory activities of this toxin contribute to disease pathogenesis and pathology, as well as various immunological and pharmacological strategies that may target these deleterious effects of the toxin.

Streptococcus pneumoniae remains a major cause of disease and death in both the First World and in developing countries, and is the commonest cause of pneumonia, meningitis and otitis media. A number of host factors are known to predispose to pneumococcal infections including extremes of age (the very young and the elderly), disturbances of local immune defence mechanisms, such as those caused by previous viral infections and cigarette smoking, as well as those such as alcoholism, splenectomy, immunosuppression, and metabolic disorders, which disturb systemic immune responses.^{1,2} At least one of these factors tends to be found in patients with invasive pneumococcal disease.

More recently, a number of studies of pneumococcal disease have investigated the pathogenesis of these infections and in particular the role played by the major pneumococcal virulence factors. Pneumolysin, a 53-kDa, thiol-activated, protein toxin that is produced by almost all clinically relevant isolates of pneumococci, is considered to be one of the most important virulence factors of this micro-organism (extensively reviewed elsewhere³⁻⁹). This toxin is able to bind to cholesterol in cell membranes of eukaryotic cells, where it

oligomerises, ultimately creating trans-membrane pores. As a pore-forming toxin, pneumolysin is cytotoxic to a variety of mammalian cells, including respiratory and ependymal epithelium, as well as neuronal cells. In addition to its cytolytic domain, the pneumolysin molecule also has a complement-activating domain that can activate complement directly in the absence of anti-pneumolysin antibody. Moreover, this toxin also has important effects on the host's cellular and humoral immune system. Recently, we reviewed the potential role of pneumolysin in the pathogenesis and prevention of pneumococcal infections and the current article updates that manuscript,³ focusing almost exclusively on major developments which have occurred during the past 5 years.

The role of pneumolysin in the pathogenesis of pneumococcal infection

Pro-inflammatory activities

Pneumococcal colonisation is mostly asymptomatic, but is the first step in the pathogenic route of pneumococci to invasive disease.¹⁰ Pneumococcal disease will not occur without preceding nasopharyngeal colonisation with a homologous strain. Colonisation also plays an important part not only in the horizontal spread of virulent pneumococcal strains, but may also play a crucial role in the prevention of pneumococcal infections. The subject of pneumococcal colonisation has been extensively reviewed elsewhere.¹⁰

There is considerable understanding of the mechanisms by which both Gram-positive and Gram-negative micro-organisms interact with the human host. While the immune response almost certainly plays an important role in preventing progression from colonisation by micro-organisms such as the pneumococcus to invasive disease, it is also recognised that part of the morbidity and mortality associated with pneumococcal disease is mediated through the host response to cellular components of the pneumococcus, resulting in excessive release of inflammatory mediators. A similar situation exists with other Gram-positive and Gram-negative pathogens.

Pneumolysin, by virtue of cholesterol-binding, pore-forming activity, as well as its more-recently described interactions with Toll-like receptor 4 (TLR4),¹¹ may either protect against or exacerbate pneumococcal infection, depending on the local density of toxin-producing bacteria in the airways. There are

Division of Pulmonology, Department of Medicine, Johannesburg Hospital and University of the Witwatersrand, Johannesburg

Charles Feldman, MB BCH, PhD

Medical Research Council Unit for Inflammation and Immunity, Department of Immunology, Faculty of Health Sciences, University of Pretoria, and Tshwane Academic Division of the National Health Laboratory Service

Ronald Anderson, PhD

Corresponding author: C Feldman (feldmanc@medicine.wits.ac.za)



currently 11 recognised members of the TLR family, these being the prototype pattern recognition molecules which function primarily as the sentinels of the innate immune system.

Exposure to small numbers of pneumococci in the airways results in the production of low, sub-cytolytic concentrations of pneumolysin, which induce production of the potent neutrophil chemoattractant, interleukin-8 (IL-8), by airway epithelium via a mechanism dependent on Ca^{2+} influx and activation of p38 mitogen-activated protein kinase and nuclear factor- κ B.¹²⁻¹⁴ In addition, interaction of pneumolysin with TLR4 on or in airway epithelium and other resident cells of the innate immune system in the airways, results in the production not only of IL-8, but also that of other proinflammatory cytokines such as IL-1 β , IL-6, and tumour necrosis factor- α , all of which co-operate to facilitate transendothelial migration of neutrophils.¹¹ In this setting, the consequent influx of neutrophils is protective, resulting in clearance of *S. pneumoniae* from the airways, and prevention of colonisation.^{13,14}

However, hyperactivation of these same mechanisms occurs when colonisation of the airways with the pneumococcus is poorly controlled, resulting in excessive production of pro-inflammatory cytokines and over-exuberant inflammatory responses. These, in turn, cause inflammation-mediated disruption of the bronchoalveolar-epithelial barrier, which favours extrapulmonary dissemination of the pneumococcus.^{5,15} In addition to its pore-forming and TLR-4-mediated pro-inflammatory activity, pneumolysin also directly activates the complement cascade, a strategy used by the pneumococcus to evade host defences, which also amplifies inflammation-mediated tissue damage. Vulnerability to this onslaught is intensified by the direct, cytotoxic effects of pneumolysin on airway epithelium.

Cytotoxic activity

Acute respiratory failure is a potentially serious complication of severe pneumococcal pneumonia. Factors that may support the role of pneumolysin in its pathogenesis include the fact that the toxin facilitates penetration of pneumococci from alveoli to the interstitium and then into the bloodstream, and that various soluble factors, predominantly pneumolysin, are cytotoxic to pulmonary endothelial and alveolar epithelial cells. Furthermore, pneumolysin, as mentioned above, has a wide range of pro-inflammatory activities even at sub-cytolytic concentrations. With these factors in mind, and having previously demonstrated that significant microvascular leakage occurs early in the course of pneumococcal infection, when neutrophil influx was still absent, Witznarth and colleagues¹⁶ studied the possible role of pneumolysin in the pathogenesis of this acute lung injury. In an experimental model, they provided evidence for the role of pneumolysin in the acute lung injury due to rapid-onset action on the alveolar-capillary membrane and extracapillary vessels. Lung microvascular

leakage was evident 6 hours after transnasal infection in mice before neutrophil influx into the lungs. Recombinant pneumolysin aerosolisation or infusion into the lung caused significant vascular leakage and pulmonary oedema, and pneumolysin infusion into the pulmonary circulation induced pulmonary hypertension, both these manifestations being important features of acute respiratory distress syndrome.

In the brain, the ventricular surfaces and aqueducts are lined by ciliated ependymal cells which form a barrier between the infected CSF of patients with meningitis and the neuronal tissue.¹⁷ A direct link between severity of meningitis and pneumolysin has been shown in experimental models. Pneumolysin has been demonstrated in *in vitro* experiments to cause both inhibition of ependymal ciliary beat frequency, as well as ependymal cell disruption. Furthermore, wild-type pneumococci, as well as pneumococci deficient in pneumolysin, also cause ciliary slowing. Most importantly, penicillin lysis of wild-type, but not pneumolysin-deficient pneumococci, increases the extent of ciliary inhibition. This effect, as well as the effect on ependymal cells of recombinant pneumolysin alone, is blocked by anti-pneumolysin monoclonal antibody. While the outcome of pneumococcal meningitis has been improved by the use of appropriate antibiotics, supplemented more recently by the addition of corticosteroid therapy in the form of dexamethasone, based on these studies it is possible that addition of anti-pneumolysin antibodies may further improve prognosis and warrants further investigation.

Immunogenicity of pneumolysin

Pneumolysin evokes a T-cell-dependent antibody response, leading to the production of secretory immunoglobulin (Ig) A and both circulating and mucosal antibodies of the IgG class in animals and humans. The antibody response to pneumolysin in humans involves activation and proliferation of antigen-specific CD4+ T-lymphocytes, with resultant generation of the cytokines interferon-gamma (IFN- γ) and tumour necrosis factor- α (TNF- α) by circulating lymphocytes, and IFN- γ /IL-10 by adenoidal cells.¹⁸ The antibody response to pneumolysin is dependent on TLR4, compatible with a toxin-capture function for TLR4 expressed on antigen-presenting cells. Moreover, several members of the TLR family, including TLR4, are expressed by T-lymphocytes, and appear to function as co-stimulatory receptors, enhancing both proliferation and cytokine production.^{18,19}

Role of pneumolysin antibodies in host defence in humans

Antibodies to pneumolysin have been implicated in prevention of both extrapulmonary dissemination of the pneumococcus, and nasopharyngeal carriage. In the case of the former, Mushner and colleagues reported that circulating antibodies to



pneumolysin measured in adult patients with pneumococcal pneumonia at the time of hospital admission were highest and lowest in patients with non-bacteraemic and bacteraemic disease respectively.²⁰ More recently, Huo *et al.* reported that in a small group of patients with documented pneumococcal disease, the concentrations of circulating IgG antibodies to both pneumolysin and pneumococcal capsular polysaccharide (PPS) measured at the time of hospital admission were significantly lower than those of a group of age-matched control subjects, while IgM levels were significantly higher in the group of patients, suggesting that pre-existing immunity to these antigens may be a determinant of susceptibility to pneumococcal infection.²¹ Interestingly, in the healthy control subjects, salivary IgA levels to PPS, and to pneumolysin in particular, were significantly higher than those of IgG and IgM, apparently underscoring the vaccine potential of pneumolysin as an inducer of mucosal immunity to *S. pneumoniae*.

In a longitudinal study designed to investigate the relationship between nasopharyngeal carriage and acquisition of circulating IgG antibodies to both serotype-specific capsular polysaccharide and pneumococcal proteins, Goldblatt and colleagues observed that carriage of *S. pneumoniae* was accompanied by significant increases in capsule-specific IgG, as well as by small but significant increases in antibodies to both pneumolysin and pneumococcal surface adhesin A (PsaA), but not to pneumococcal surface protein A (PspA), with higher levels of anti-capsular antibodies at the outset being associated with a reduction in carriage.²²

Using a study design apparently uncomplicated by prior immunisation with PPS, Holmlund *et al.* recently investigated the potential of natural, maternal antibodies to pneumococcal proteins to protect against pneumococcal colonisation in early life.²³ To do this, they measured the concentrations of anti-pneumolysin, anti-PsaA and anti-PspA IgG antibodies in maternal and cord blood, as well as in serial blood specimens taken from infants during the 7 - 48-week period after birth, and correlated these with pneumococcal carriage. Interestingly, they found that transplacentally acquired anti-pneumolysin antibodies were associated with a significantly decreased risk of pneumococcal carriage during the first 3 months of life.²³ The findings of a similar type of study, designed to investigate the relationship between nasopharyngeal carriage of the pneumococcus and serum and mucosal antibodies to pneumolysin, choline-binding protein A (CbpA), and PspA, were recently reported by Zhang *et al.* in a group of children aged 2 - 12 years undergoing adenoidectomy.²⁴ These investigators observed that carriage rates declined with age in the setting of increased serum levels of antibodies to all three pneumococcal proteins. Anti-pneumolysin and anti-CbpA serum and salivary IgG antibody levels were found to be higher in children who were culture-negative than in those who were colonised.²⁴

Taken together, these various studies underscore the immunogenicity of pneumococcal proteins such as pneumolysin and CbpA, both of which are candidate immunogens for inclusion in pneumococcal protein vaccines.

Pneumolysin as a vaccine candidate

Notwithstanding their improved efficacy, particularly in children, the major limitation of pneumococcal polysaccharide-protein conjugate vaccines is that they only elicit protective antibodies against the limited range of serotypes contained in the vaccine. The validity of this concern is underscored by the findings of a recent study conducted in Alaskan native children younger than 2 years and immunised with heptavalent conjugate pneumococcal vaccine, which reported that these children are experiencing replacement invasive pneumococcal disease with non-vaccine serotypes.²⁵ Importantly, protein-based immunisation strategies represent an attractive alternative, because they are likely to provide broader protection than the currently available conjugate vaccines. Pneumococcal protein vaccines may utilise individual proteins, or mixtures of different proteins; alternatively, individual pneumococcal proteins or protein cocktails could be used as carriers for pneumococcal polysaccharides, eliciting immunity against the vaccine serotypes, while protecting, albeit to a lesser extent, against non-vaccine serotypes. In addition to pneumolysin, other pneumococcal, protein virulence factors, CbpA, PsaA, and surface proteins A, B and C (PspA, PspB, PspC), have also been identified as potential candidates for protein vaccine development.²⁶

Pneumolysin represents a particularly attractive antigen on which to base the development of a protein vaccine for the following reasons: (i) it is produced by almost all clinically relevant strains of the pneumococcus; (ii) it induces T-lymphocyte-dependent immune responses resulting in protective, antibody-mediated systemic and mucosal immunity as alluded to above; and (iii) inactivated pneumolysin (toxoid) has been demonstrated to function not only as a protective immunogen *per se*, but also as an effective immunogenic carrier of pneumococcal polysaccharide in murine models of conjugate vaccine efficacy, being equivalent or superior to conventional carriers such as tetanus toxoid.²⁷

Pneumolysin-based vaccines

Several studies using murine models of pulmonary infection have documented the immunogenicity and protective efficacy of inactivated pneumolysin, used as a mono-component protein vaccine, or as a carrier of polysaccharide antigens in conjugate vaccines.^{26,27} Interestingly, glycoconjugate vaccines consisting of pneumococcal polysaccharide coupled to pneumolysin have been reported to promote broader protective immunity in the setting of production of high avidity



antibodies with opsonophagocytic activity than conjugates which use non-pneumococcal carriers.²⁷

In spite of its clear potential as a pneumococcal vaccine candidate, concerns have been expressed in respect of the residual toxicity of the inactivated pneumolysin (pneumolysoid) used as vaccine *per se* or as a carrier. This issue has been addressed recently by Kirkham *et al.*, who have developed a modified pneumolysin molecule with a single amino acid deletion that completely attenuates pore-forming activity.²⁸ This modified pneumolysin not only retains its immunogenicity, but was also found to be protective in a murine model of experimental lung infection, suggesting that it may be an ideal contender for inclusion in pneumococcal protein vaccines.²⁸

More recently, Ogunniyi *et al.*, using an invasive disease model, evaluated the protective efficacy of immunisation of mice with pneumolysin toxoid, CbpA, PspA, and the pneumococcal histidine triad proteins (Pht) B and E, individually and in combination.²⁹ While the protective effects of the individual protein vaccines were variable, immunisation with the combination of pneumolysoid, CbpA, and PspA, was found to offer the best protection.²⁹

To our knowledge, however, no pneumococcal protein vaccines, either monocomponent or multicomponent, or as carriers in glycoconjugate vaccines, have been evaluated in humans.

Pneumolysin – targeted pharmacological strategies for the treatment of pneumonia

The two most promising approaches are: (i) anti-inflammatory agents, specifically the use of corticosteroids to attenuate the harmful pro-inflammatory activities of the toxin; and (ii) antimicrobial agents which effectively antagonise the synthesis of pneumolysin.

Corticosteroids

Two lines of evidence support the role of early corticosteroid adjunctive therapy in the management of severe pneumococcal disease. Firstly, several, admittedly small, studies have reported beneficial effects of early administration of corticosteroids on outcome of patients with pneumococcal meningitis or community-acquired pneumonia.³⁰ Secondly, several recently conducted trials have demonstrated significant clinical benefit of low-dose corticosteroids in patients with septic shock/acute respiratory distress syndrome.³⁰ Although we concluded in a recent review that there is currently insufficient evidence to enable firm recommendations to be made in respect of the use of corticosteroids in patients with severe pneumococcal disease,³⁰ the emerging evidence with regard to corticosteroid use is very promising.

Antimicrobial agents

Although beta-lactam antibiotics are the agents of choice in the treatment of severe pneumococcal disease and are generally highly effective, the rate of treatment failure in severe disease remains unacceptably high. Recently, it has been proposed that combining a beta-lactam with a macrolide antimicrobial agent leads to a reduction in the mortality associated with bacteraemic pneumococcal pneumonia. Notwithstanding the possible involvement of the well-documented, secondary anti-inflammatory properties of macrolides, the precise mechanisms which underpin the therapeutic efficacy of the beta-lactam/macrolide combination remain to be established. The added benefits of the macrolide may be related to inhibition of the synthesis of protein virulence factors, particularly pneumolysin, which is released during beta-lactam-mediated disintegration of the pneumococcus.

We have recently reported that the production of pneumolysin is attenuated by exposure of the pneumococcus to sub-inhibitory concentrations of the macrolide, clarithromycin.³¹ Interestingly, clarithromycin-mediated inhibition of the production of pneumolysin was observed not only with macrolide-susceptible strains of the pneumococcus, but also with macrolide-resistant strains which harboured either the ribosomal methylase (*erm* gene)- or efflux pump (*mef* gene)-based mechanisms of macrolide resistance.³¹ These observations are in agreement with those of Fukuda *et al.*, who reported that clarithromycin and azithromycin inhibited the production of pneumolysin by macrolide-resistant strains of *S. pneumoniae in vitro*, as well as in the lungs of experimentally infected mice.³² In a recent study, we observed that macrolides (clarithromycin, erythromycin), azalides (azithromycin), ketolides (telithromycin), and lincosamides (clindamycin), at sub-inhibitory concentrations, effectively attenuate the production of pneumolysin by both macrolide-susceptible and macrolide-resistant strains of the pneumococcus. Other types of antimicrobial agent commonly used in the treatment of infections caused by this microbial pathogen, including amoxicillin, ceftriaxone, ciprofloxacin, moxifloxacin, and tobramycin did not affect pneumolysin production.³³

These apparently selective inhibitory effects of macrolides on the production of pneumolysin by both macrolide-susceptible and macrolide-resistant strains of the pneumococcus may provide an explanation for the benefit of combining these agents with beta-lactams on the outcome of bacteraemic pneumococcal pneumonia.

Conclusions

Infections with *S. pneumoniae* continue to be a major cause of patient morbidity and mortality worldwide. Several recent studies have underscored the involvement of pneumolysin, one of the major virulence factors of the organism, in the pathogenesis of severe infections caused by the pneumococcus.



In addition to playing a significant role in the disease process, the toxin is also highly immunogenic and represents not only an ideal protein carrier for a future polysaccharide conjugate vaccine, but can also serve as a major component of a pneumococcal protein vaccine. Furthermore, strategies which improve the outcome of severely ill patients with pneumococcal infection, such as the use of corticosteroids, or the addition of a macrolide to standard beta-lactam therapy appear to target pneumolysin, by respective attenuation of its pro-inflammatory activities, or by preventing its synthesis.

References

1. Spreer A, Lis A, Gerber J, et al. Differences in clinical manifestations of *Streptococcus pneumoniae* infection are not correlated with *in vitro* production and release of the virulence factors pneumolysin and lipoteichoic and teichoic acids. *J Clin Microbiol* 2004; 42: 3342-3345.
2. Nuorti JP, Butler JC, Farley MM, et al. Cigarette smoking and invasive pneumococcal disease. *N Engl J Med* 2000; 342: 681-689.
3. Cockeran R, Anderson R, Feldman C. The role of pneumolysin in the pathogenesis of *Streptococcus pneumoniae* infection. *Curr Opin Infect Dis* 2002; 15: 235-239.
4. Cockeran R, Anderson R, Feldman C. Pneumolysin in the immunopathogenesis and treatment of pneumococcal disease. *Expert Rev Anti Infect Ther* 2003; 1: 231-239.
5. Cockeran R, Anderson R, Feldman C. Pneumolysin as a vaccine and drug target in the prevention and treatment of invasive pneumococcal disease. *Arch Immunol Ther Exp* 2005; 53: 189-198.
6. Tuomanen EI. Pathogenesis of pneumococcal inflammation: otitis media. *Vaccine* 2001; 19: S38-S40.
7. Jedrzejak MJ. Pneumococcal virulence factors: structure and function. *Microbiol Molec Biol Rev* 2001; 65: 187-207.
8. Gillespie SH, Balakrishnan I. Pathogenesis of pneumococcal infection. *J Med Microbiol* 2000; 49: 1057-1067.
9. Mitchell TJ. Virulence factors and the pathogenesis of disease caused by *Streptococcus pneumoniae*. *Res Microbiol* 2000; 151: 413-419.
10. Bogaert D, de Groot R, Hermans PWM. *Streptococcus pneumoniae* colonisation: the key to pneumococcal disease. *Lancet Infect Dis* 2004; 4: 144-154.
11. Malley R, Henneke P, Morse SC, et al. Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. *Proc Natl Acad Sci USA* 2003; 100: 1966-1971.
12. Fickl H, Cockeran R, Steel HC, et al. Pneumolysin-mediated activation of NF B in human neutrophils is antagonized by docosahexaenoic acid. *Clin Exp Immunol* 2005; 140: 274-281.
13. Van Rossum AM, Lysenko ES, Weisner JN. Host and bacterial factors contributing to the clearance of colonization of *Streptococcus pneumoniae* in a murine model. *Infect Immun* 2005; 73: 7718-7726.
14. Ratner AJ, Hippe KR, Aguilar JL, et al. Epithelial cells are sensitive detectors of bacterial pore-forming toxins. *J Biol Chem* 2006; 281: 12994-12998.
15. Garcia-Suarez M del M, Florez N, Astudillo A, et al. The role of pneumolysin in mediating lung damage in a lethal pneumococcal pneumonia murine model. *Respir Res* 2007; 8: 3.
16. Witzenth M, Gutbier B, Hocke AC, et al. Role of pneumolysin for the development of acute lung injury in pneumococcal pneumonia. *Crit Care Med* 2006; 34: 1947-1954.
17. Hirst RA, Mohammed BJ, Mitchell TJ, et al. *Streptococcus pneumoniae*-induced inhibition of rat ependymal cilia is attenuated by antipneumolysin antibody. *Infect Immun* 2004; 72: 6694-6698.
18. Zhang Q, Bernatoniene J, Bagrade L, et al. Regulation of production of mucosal antibody to pneumococcal protein antigens by T-cell-derived gamma interferon and interleukin-10 in children. *Infect Immun* 2006; 74: 4735-4743.
19. Cairns B, Maile R, Barnes CM, et al. Increased Toll-like receptor 4 expression on T cells may be a mechanism for enhanced T cell response late after burn injury. *J Trauma* 2006; 61: 293-298.
20. Musher DM, Phan HM, Baughn RE. Protection against bacteremic pneumococcal infection by antibody to pneumolysin. *J Infect Dis* 2001; 183: 827-830.
21. Huo Z, Spencer O, Miles J, et al. Antibody response to pneumolysin and to pneumococcal capsular polysaccharide in healthy individuals and *Streptococcus pneumoniae* infected patients. *Vaccine* 2004; 22: 1157-1161.
22. Goldblatt D, Hussain M, Andrews N, et al. Antibody responses to nasopharyngeal carriage of *Streptococcus pneumoniae* in adults: a longitudinal household study. *J Infect Dis* 2005; 192: 387-393.
23. Holmlund E, Quiambao B, Ollgren J, et al. Development of natural antibodies to pneumococcal surface protein A, pneumococcal surface adhesin A and pneumolysin in Filipino pregnant women and their infants in relation to pneumococcal carriage. *Vaccine* 2006; 24: 57-65.
24. Zhang Q, Bernatoniene J, Bagrade L, et al. Serum and mucosal antibody responses to pneumococcal protein antigens: relationships with carriage status. *Eur J Immunol* 2006; 36: 46-57.
25. Singleton RJ, Hennessy TW, Bulkow LR, et al. Invasive pneumococcal disease caused by nonvaccine serotypes among Alaska native children with high levels of 7-valent pneumococcal conjugate vaccine coverage. *JAMA* 2007; 297: 1784-1792.
26. Tai S. *Streptococcus pneumoniae* protein vaccine candidates: properties, activities and animal studies. *Crit Rev Microbiol* 2006; 32: 139-153.
27. Lee C-J, Wang TR, Frasch CE. Immunogenicity in mice of pneumococcal glycoconjugate vaccines using pneumococcal protein carriers. *Vaccine* 2001; 19: 3216-3225.
28. Kirkham LA, Kerr AR, Douce GR, et al. Construction and immunological characterization of a novel nontoxic protective pneumolysin mutant for use in future pneumococcal vaccines. *Infect Immun* 2006; 74: 586-593.
29. Ogunniyi AD, Grabowicz M, Briles DE, et al. Development of a vaccine against invasive pneumococcal disease based on combinations of virulence proteins of *Streptococcus pneumoniae*. *Infect Immun* 2007; 75: 350-357.
30. Feldman C, Anderson R. Controversies in the treatment of pneumococcal community-acquired pneumonia. *Future Med* 2006; 1: 271-281.
31. Anderson R, Steel HC, Cockeran R, et al. Clarithromycin alone and in combination with ceftriaxone inhibits the production of pneumolysin by both macrolide-susceptible and macrolide-resistant strains of *Streptococcus pneumoniae*. *J Antimicrob Chemother* 2007; 59: 224-229.
32. Fukuda Y, Yanagihara K, Higashiyama Y, et al. Effects of macrolides against pneumolysin of macrolide-resistant *Streptococcus pneumoniae*. *Eur Respir J* 2006; 27: 1020-1025.
33. Anderson R, Steel HC, Cockeran R, et al. Comparison of the effects of macrolides, amoxicillin, ceftriaxone, doxycycline, tobramycin and fluoroquinolones, on the production of pneumolysin. *J Antimicrob Chemother* 2007; Epub ahead of print: doi:10.1093/jac/dlem338.