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Title:

Mucosal immunisation with novel *Streptococcus pneumoniae* protein antigens enhances bacterial clearance in an acute mouse lung infection model.

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Abstract

*Streptococcus pneumoniae* contains many proteins that have not been evaluated as potential protective vaccine antigens. In this study we isolated proteins from a serotype 3 strain of *S. pneumoniae* for use in mouse immunisation studies. Separation of the protein mix was achieved by SDS-PAGE electrophoresis followed by electro-elution to isolate individual proteins. This procedure successfully separated 21 fractions from which six proteins were selected based on purity and quantity and were initially denoted by their molecular masses: 14-, 34-, 38-, 48-, 57- and 75kDa. The immunogenicity of these proteins was investigated in a mucosal immunisation model in mice involving a primary inoculation to the intestinal Peyer’s patches followed by an intra-tracheal boost two weeks later. The immune response was assessed by enhancement of pulmonary clearance of infection, recruitment of phagocytes to the lungs and induction of an antibody response. Two of the proteins, the 14kDa identified as a L7/L12 ribosomal protein, and the 34kDa identified as glyceraldehyde-3-phosphate dehydrogenase resulted in up to 99% and 94%, respectively, enhanced clearance of infection within 5 hours following pulmonary challenge with *S. pneumoniae*. This study has shown that novel pneumococcal proteins have the potential to be vaccine candidates to enhance clearance of an acute mucosal *S. pneumoniae* infection.
Introduction

A major component of the *Streptococcus pneumoniae* cell wall is a polysaccharide capsule, which is serotype specific. Embedded throughout this cell wall is a mixture of proteins of which some play a role in the pathogenesis of the disease associated with this bacterium. Clinical infection with this bacterium results when host factors allow the organism to access locations ordinarily free of bacteria such as the middle ear and the lung. From these sites the bacterium may also reach the bloodstream leading to more serious disease, such as sepsis and meningitis [1,2]. The current licensed vaccines are based on the polysaccharide component of this bacterium.

There are two major drawbacks associated with capsular-based vaccines. First, the requirements to conjugate a number of polysaccharide types to a protein carrier to give efficacy in young children who are unable to mount protective immune responses to polysaccharides [3]; and secondly, conjugation restricts the number of polysaccharide types that can be included in any given formulation [4] and as a result serotype replacement is occurring in vaccinated subjects [5]. Hence, it is desirable to identify either a single or small number of protein antigens which, when formulated into a vaccine, will induce protective immunity against the diverse range of *S. pneumoniae* serotypes that exist.

Initial searches for conserved pneumococcal antigens focused on proteomic approaches. Of those proteins so far identified, PspA [6], PsaA [7] and pneumolysin [8] are the major candidates for development of a protein-based vaccine. The development of PspA as a vaccine candidate has progressed to clinical phase I trials which have demonstrated that this protein was immunogenic in humans [4,9]. Recently technology has advanced the search to genomic approaches [10-12]. The
complete genome sequence of distinct strains of *S. pneumoniae* [13,14], which can be used to predict conserved surface exposed proteins, are now available. However, one of the disadvantages of this approach for vaccine development in particular, is that the investigation of large numbers of genes associated with immunologically irrelevant proteins is time consuming and costly.

The present study used electrophoretic techniques to separate proteins from a cell wall extract in the search for candidate protein antigens that could be used for immunisation against pneumococcal infections. The rationale for this approach was to specifically focus only on novel proteins that were expressed by the bacteria and to use bacterial clearance following vaccination with the native protein as the screening tool.
Materials and methods

Growth and bacterial strains. All the experiments were performed by using serogroup 3 *S. pneumoniae* (NTCC 49619). The bacteria were grown overnight on blood agar plates at 37°C, in 5% CO₂ or cultured in tryptic soy broth in a shaker incubator. The bacteria were washed 4 times with phosphate buffered saline (PBS) by centrifugation. For killed bacteria preparations, *S. pneumoniae* were killed by suspension in 1% (wt/vol) formaldehyde in PBS and incubated at 37°C for 2 hours. The bacteria were washed 3 times in PBS, killing was checked by overnight culture onto blood agar, and the concentration was adjusted to 5x10⁸ bacteria per ml as estimated by determining the optical density at 405nm. For use in bacterial challenge in mice, live washed bacteria cultured on blood agar plates were used and the concentration estimated by measuring the optical density at 405nm. The accuracy of the bacterial counts was confirmed by counting colony forming units (CFU) from titration of overnight cultures.

Protein extraction and separation. An extract of proteins in the cell wall was prepared essentially as described [15]. In summary, following washing twice in PBS, the cell walls were disrupted by resuspending in 0.2% (wt/vol) sodium deoxycholate in PBS and stirred for 1 hour at room temperature. The cell wall proteins were precipitated by the gradual addition of ammonium sulfate, to a final concentration of 70% (wt/vol). The precipitated proteins were resuspended in 10mM sodium phosphate pH 7.0 and clarified by centrifugation for 20 minutes at 4°C and 15000g. The extract was lyophilised to adjust the concentration to 2.5-3.0 mg per ml. The proteins in the extract were separated by SDS-PAGE using the large protean II xi cell (Bio-Rad) according to the standard protocol for reducing condition separation using a discontinuous 12% acrylamide gel with a 4% stacking gel (1mm thickness).
**Purification of proteins by electro-elution.** The proteins separated by SDS-PAGE were recovered by electro-elution using the BioRad protean II electro-eluter according to the manufacturer's protocol. Elution time was 1 hour at constant 250mA and the eluted fractions were harvested under vacuum into 30 fractions. SDS was removed from the fractions using a potassium phosphate solution to precipitate the detergent, as described [16]. Protein concentration was determined with the Pierce Micro BCA protein assay. The fractions were concentrated by lyophilization and assessed for protein content by analytical SDS-PAGE and stained with Coomassie Blue.

**Immunisation.** The choice of procedure for mucosal immunisation and bacterial challenge was based on previous studies by our group for efficacy in clearance for *S. pneumoniae* (unpublished) and the other respiratory pathogens. The procedures were performed as described [17]. Specific pathogen free male BALB/c mice aged between 6-10 weeks were obtained from the Animal Resources Centre (Perth, Australia). Mice were immunised on days 0 and 14 and 5 mice per group were used. The non-immune groups consisted of a mix of untreated and sham-immunised animals (receiving incomplete Freund's adjuvant (IFA) and PBS only). On day 0 the mice were immunised by sub-serosal inoculation to the intestinal Peyer's patches as described [17] under sedation by subcutaneous injection of 0.25ml ketamine/xylazine in PBS (5 mg/ml ketamine hydrochloride {Troy Laboratories, Smithfield, NSW, Australia}; 2 mg/ml xylazine hydrochloride {Bayer, Pymble, NSW, Australia}). The protein or killed *S. pneumoniae* was prepared for inoculation by emulsification in a 1:1 ratio with IFA. A total concentration of 10μg of protein or 1x10⁷ CFU of killed bacteria was administered to each animal per dose in a total volume of 10μL. The mice were kept warm while recovering. On day 14 the mice received an immunisation boost with the same concentration of protein or killed *S. pneumoniae*.
but delivered intra tracheally and solubilised in PBS only. The mice were sedated with approximately 0.12ml saffan administered intravenously (20 mg alphadone in PBS/kg body weight; Pitman-Moore, Nth Ryde, NSW, Australia) and received 10µg of protein or 1x10^7 CFU killed *S. pneumoniae* in 20µL delivered as described [17] by oral insertion of a paediatric cannula into the trachea and aspiration of the inoculum with two 0.3 ml volumes of air.

**Bacterial challenge.** On day 21 the mice were sedated with saffan (as described above) and a 20µL bolus of live *S. pneumoniae* was introduced into the lungs via the trachea as for the IT boost. The exact live CFU count for the inoculum in each challenge is shown in Table 1. Five hours following the challenge, the mice were euthanased by an intraperitoneal injection of 0.2ml pentobarbital sodium (60mg/ml; Rhône Mérieux, Australia). Blood was collected by heart puncture, serum separated by centrifugation and aliquots stored at −20°C for subsequent assay. Lungs were subjected to lavage with 0.5ml of PBS via the trachea, which had been exposed through an incision in the neck, and the bronchoalveolar lavage (BAL) fluid was assessed for bacteria by plating of ten-fold serial dilutions of the washings for CFU determination. After obtaining the BAL, the lungs were removed, the heart and connective tissues excised, the lungs placed in 2ml of sterile PBS and homogenised (9500 rpm; Heidolph DIAX 600, Electro GmbH & Co, Kelheim, Germany). The lung homogenate was assessed for the presence of bacteria by plating ten-fold serial dilutions onto Blood agar for CFU determination. The BAL was assessed for the total number and differential white cell counts as previously described [17].

**Antigen-specific ELISA.** Antibody titres were measured by enzyme linked immunoassays (ELISA). 96-Well maxisorb microtitre plates (Nunc, Roskilde, Denmark) were coated with 40µg of a cell wall extract (this extract was prepared
separately to the extracts used in the purification) in 50\(\mu\)l of coating buffer (15mM NaCO\(_3\), 35mM NaHCO\(_3\), pH 9.6) overnight at 4\(^\circ\)C. The wells were washed five times in PBS-Tween (6 mM disodium hydrogen phosphate, 2 mM sodium dihydrogen phosphate, 0.27 M sodium chloride, pH 7.2 and containing 0.05% v/v Tween 20), blocked with 100\(\mu\)L 2.5% (w/v) skim milk in PBS-Tween for 90 minutes at room temperature and washed in PBS-Tween. The wells were incubated with serum diluted between 1/50 to 1/500 for IgG, 1/10 to 1/100 for IgA or BAL diluted 1/2 to 1/8 in 1% w/v skim milk in PBS-Tween and incubated for 90 minutes at room temperature. The plates were washed as above and incubated for 90 minutes with horseradish peroxidase-conjugated anti-mouse IgG (1:3000) or IgA (1:250) (Sigma). Following washing, the wells were developed with TMB (3',3',5',5'-tetramethylbenzidine in 0.05 M citric acid, 0.1 M sodium hydrogen phosphate, 0.05% (v/v) hydrogen peroxide) for 10 min and the reaction stopped with 0.5 M H\(_2\)SO\(_4\). The plates were read at 450 nm on a microplate reader (BioRad, model 3550) and analysed using the microplate reader data analysis software (Microplate Manager, BioRad). IgG and IgA antibody titres were quantitated against a standard curve generated by serial dilutions of standards for mouse IgG or IgA (Calbiochem-Novabiochem) ranging from 63-1000 ng/ml or 16-250 ng/ml, respectively.

**Sequencing of proteins.** A fraction of the protein of interest used in the immunisation studies was run on a 12% homogenous SDS-PAGE gel. The N-terminal sequence of the proteins was determined from the excised band following SDS-PAGE. N-terminal sequencing was carried out by the Biomolecular Resource Facility, Centre for Molecular Structure and Function, at the Australian National University, using the SDS-PAGE compatible S-2-carboxamidoethylation method. The data obtained was subjected to BLAST search (Basic Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov/BLAST).
Statistical analysis. Data were expressed as the mean and standard error of the mean (SEM). Data for comparison of the immunised and the control groups were analysed for significance using independent t-tests (Macintosh Systat).
RESULTS

Profile of proteins extracted using electro-elution. A total of six protein extractions were performed yielding an average of 13mg of protein per 1L culture and ranging between 7mg – 31mg/L. Following preparation of the extracts, the protein profile for each extraction was assessed using SDS-PAGE. The profiles showed no significant quantitative differences between extractions. Factors that did influence electrophoretic separation of proteins for electro-elution included the relative density of a protein, the degree of separation of the individual proteins within the SDS-PAGE gel, and the linearity of the gel for alignment across the elution channels. Numerous proteins were separated by electrophoresis yielding many proteins of molecular masses ranging between 10kDa to 90kDa (Fig. 1). Subsequent electro-elution isolated the pneumococcal proteins into 21 fractions contain detectable protein and according to molecular masses. Six fractions had sufficient yield and purity of a distinct protein band to be assessed in animal immunisation studies. Purity was confirmed by either one or a combination of the following methods: silver staining of SDS-PAGE, 2 dimensional electrophoresis, lack of interfering amino acids in the N-terminal sequence analysis and/or immunoblotting techniques (data not shown). These corresponded to proteins of molecular masses of approximately 14-, 34-, 38-, 48-, 57-, and 75kDa. In the representative gel in Fig. 1, these correspond to lanes C9-10, B8, B5, B3 and A8, respectively (although the 75 kDa had a low yield in this particular purification).

Effect of immunisation on pulmonary clearance of S. pneumoniae. The ability of these proteins to enhance clearance of a S. pneumoniae infection was investigated. The degree of clearance was compared to non-immune mice as well as mice
immunised with whole killed *S. pneumoniae*. Table 1 shows the bacterial clearance from mice mucosally immunised with two 10µg doses of purified protein. All mice were challenged 21 days post-immunisation with live *S. pneumoniae*.

Immunisation with a whole killed cell inoculum resulted in significant clearance of *S. pneumoniae* from both the BAL (p<0.001) and lung homogenate (p<0.05) (Table 1). The reduction in bacterial load in the immunised animals compared to the non-immune group was 68% and 68% in the BAL and lungs, respectively (Table 1). Immunisation with the 38kDa, 48kDa, and 75kDa proteins resulted in no significant reduction in the bacterial load. Immunisation with the 57kDa protein showed a decrease in the bacterial load of 60% in the BAL and 37% in the lung homogenate, though this did not reach statistical significance. Immunisation with the 34kDa protein resulted in variation in the clearance in the BAL within the immunised group (Table 1) with individual animals within the group exhibiting different abilities to clear the bacteria. The mean clearance of 79% of the bacteria, therefore, was not statistically significant. In contrast, the 94% clearance measured in the lung tissue was significant (p<0.05). Immunisation with the 14kDa protein significantly enhanced clearance with a 94% reduction (p < 0.05) in the lung homogenate and >99% (p < 0.001) in the BAL. The level of bacterial clearance measured in both BAL and lung tissue as a result of immunisation with the 34kDa and 14kDa proteins was greater than that of the whole killed cell immunised mice.

**The recruitment of phagocytic white cells.** The BAL was assessed for both the total number of cells and the differential cell population (Table 2). The total numbers of cells recruited to the lungs were not significantly different between any of the non-
immune and immune groups (data not shown). Those proteins that appeared to enhance clearance from the BAL and lungs tended to have a greater percentage of macrophages present in the BAL. The exception was the 57kDa protein that had a greater percentage of PMNs.

**Specific antibody titres in the serum and BAL fluid.** Antibody responses following mucosal immunisation were assessed by ELISA against the extract from which the proteins had been isolated. The data in Table 3 shows significant increases in anti-14kDa, anti-34kDa, anti-38kDa and anti-48kDa IgG titres in the serum of the immunised group compared with the non-immunised group (p < 0.001). Further analysis of the specificity, cross-strain presence of the protein and cell surface location for some of these antigens is under investigation (manuscript in preparation). IgA titres in the BAL and the serum were not detected following any of the immunisations (data not shown).

**Identification of proteins by N-terminal sequence analysis.** The identity of the 14-, 34- and 57kDa proteins that showed the most enhanced clearance was achieved by N-terminal sequence analysis. For the 14kDa protein, a result for the first 15-amino acids was obtained and an electronic search of GeneBank sequences (http://www.ncbi.nlm.nih.gov/BLAST) revealed a 100% homology to a S. *pneumoniae* 12kDa ribosomal protein L7/L12 (accession number P80714, *Streptococcus pneumoniae* TIGR4) [18] (Table 4). The first 7 amino acids were obtained for the 34kDa protein this short sequence had homology to a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the general database, with a predicted molecular mass of 38.8kDa that is highly conserved across species. These N-terminal sequences corresponded with the N-terminus of the homologous proteins identified by
the database searches. N-terminal sequencing of the 57kDa protein did not reveal any identity for the first 9-amino acids (Table 4) using several algorithms.
Discussion

Pneumococcus remains an important human pathogen in both developed and developing countries. In the light of increasing antibiotic resistance [19] over recent years there has been a considerable effort to develop efficacious vaccines against pneumococcal disease.

In developed countries the availability of a 7-valent conjugate vaccine, for use in infants and young children, and a 23-valent polysaccharide vaccine, for use in adults and institutional settings, provide significant protection against invasive disease [4]. However, the efficacy of protein-conjugated vaccines for treatment of acute otitis media is low, primarily due to the fact that the middle ear is largely dependent on the induction of a mucosal immune response [20], and the significant number of non-vaccine serotypes which cause mucosal disease [21,22]. In developing countries, pneumococcus is responsible for significant invasive disease and mucosal infections, and is a significant disease burden in these communities. The World Health Organization reports that 1 million children, mostly young and living in developing countries, die from pneumococcal disease annually [23]. Access to the vaccine and different serotype distributions in many countries, including developing countries, are the primary reasons affecting the disease burden in these communities [24].

An added complication with the current restricted serotype conjugated 7-valent vaccine is the replacement of vaccine types with non-vaccine types, and the potential for these non-vaccine types to also cause invasive disease [4,24]. In Australia, the emergence of significant carriage rates for serotype 16F, which is absent from both
the 7-valent conjugate and the 23-valent polysaccharide vaccines, has been reported [22]. Findings on serotype distribution and vaccine coverage in children younger than 2 years of age in the Czech Republic have shown that serotypes 1 and 3, not present in the 7-valent vaccine formulation, account for 25% of both acute otitis media and invasive pneumococcal diseases [25]. Serotype 1 is an uncommon isolate from children in the USA and Canada, whereas it is a frequent cause of meningitis in Africa and India [26]. In addition to this, increase in Staphylococcus aureus related acute otitis media has been reported following vaccination with the 7-valent pneumococcal conjugate vaccine [27], which certainly extends replacement issues to pathogens other than non-vaccine serotypes of pneumococcus.

Alternative vaccine strategies based on protein formulations to provide protection across a wide range of serotypes have been considered now for a number of years. The advantages of such strategies are that vaccines based on universal pneumococcal protein antigens would reduce the incidence of non-vaccine type replacement and be efficacious in young children and the elderly. However, the clinical application of such a vaccine would need to investigate the impact of protein formulations on the microbial ecology of the nasopharynx. Several studies performed with animals have demonstrated the ability of protein-based vaccines to protect against experimental pneumococcal disease, but new protective proteins are still being reported. The surface exposed BVH-3 protein has recently been shown to elicit protection in a mouse model of pneumococcal sepsis and pneumonia and the authors report that phase I clinical trials of BVH-3 are in progress [28]. Immunisation with PspA DNA or protein elicits protection against pneumococcal challenge. PiuA and PiaA, two ABC transporter proteins, enhanced survival in a mouse model when immunised
singly or in combination [29] and PiaA elicits immunity partially by anti-PiaA antibodies promoting opsonophagocytosis [30]. In addition, vaccination route remains the focus of studies, with a prime boost immunisation regime with an alpha-helical domain of PspA now reported to elicit protective immunity [31]. In this study, we examined a number of previously untested pneumococcal antigens in a murine model of mucosal immunisation and acute lung challenge. Of those proteins studied, a 14kDa protein and a 34kDa protein were of most interest.

The 14kDa protein was identified as a pneumococcal 50s ribosomal protein L7/L12 that had previously been described by Kolberg et al [18] (accession number P80714). The identity of this protein was 86% homologous to a B. subtilis ribosomal protein BL-9, that also corresponded to an E. coli ribosomal protein L7/L12 in the first 42 residues identified (accession number P02394) [32]. This protein is conserved across species, including other respiratory pathogens such as Haemophilus influenzae [18]. Mucosal immunisation with the 14kDa protein resulted in significantly enhanced clearance of S. pneumoniae as measured in both the BAL and lung homogenate with >99% reduction in bacterial load within 5 hours of infection.

The 34kDa protein was identified as a glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH functions as both a transferrin binding protein [33] and an ADP-ribosylated enzyme [34]. It is ubiquitous in both bacterial and eukaryotic cells. Kolberg and Sletten (1996) also identified a 40kDa protein as a GAPDH from S. pneumoniae [35], and in Group A Streptococci, GAPDH has been reported to be a major surface expressed protein [36]. Mice immunised with the 34kDa protein demonstrated a significant reduction in bacterial load (94%) in the lung following
infection with *S. pneumoniae*. GAPDH from different species, including mammalian, have significant functional homologies and as such may encounter some resistance for use as a component of a human vaccine. However, alignment of the *S. pneumoniae* sequence with GAPDH for *Homo sapiens* reveals only a 15% identity and this is distributed randomly through the amino acid sequence alignments. Since there was no significant decrease in clearance from the BAL our results would suggest that immunization with GAPDH prevented invasive disease but was not as effective in inducing immune mechanisms to enhance clearance from the bronchial tree.

N-terminal sequencing of the 57kDa protein failed to reveal any identity to other known proteins in the databases. Immunisation with this protein resulted in over 60% decrease in bacterial load in the BAL fluid of these animals although this trend was not significantly different from non-immunised animals. Perhaps this is a protein that is encoded by a gene that is unique to this or a limited number of strains of *S. pneumoniae*.

IgG serotype specific antibodies to capsular polysaccharides have been shown to be both opsonic and bactericidal [37-40]. Similar functional antibody studies have not been reported for pneumococcal protein antigens, however, specific IgG to the streptococcal protein enolase, an ubiquitous surface-expressed protein, has been shown to effectively opsonise and enhance phagocytosis of both serotype 6 and 14 of group A streptococci [41]. This data supports the concept that antibodies specific to proteins are capable of providing protection across multiple serotypes. An increase in IgG antibodies observed in this study related to increased clearance of the bacteria from the lungs of mice immunised with both the 14kDa and the 34kDa protein.
Increases in serum IgG antibody observed in this study were not associated with increased specific IgA antibodies in the BAL fluid or the serum of the immunised mice. These findings are consistent with other observations reported in the literature [42,43]. These observations do not rule out a possible role that specific IgA antibodies may play in protection against colonisation and infection with potentially pathogenic serotypes. Local secretory IgA has been known to prevent both the colonisation of *S. pneumoniae* at the mucosal tissues and the spread of infection [44]. In addition, pneumococcal carriage and infections induce salivary and serum antibodies to a number of pneumococcal proteins including PsaA, pneumolysin and PspA in children [45,46].

In this study the total number of white blood cells recruited to the site of infection, in this case the lungs, was not greater in the immune groups and did not correlate with enhanced clearance. Effective host defence against lung bacterial infection is primarily dependent on rapid bacterial clearance mediated by macrophages and neutrophils. In the current study the number of macrophages present in the phagocytic cell count at the site of infection was significantly higher at 5 hours post-infection and was a consistent trend in animals with enhanced clearance. This data supports the concept that induced mucosal immunity results in the early recruitment of such cells to the infection site and that this is important in the enhanced clearance of bacteria from respiratory tract.

In conclusion, this study successfully purified and identified a number of proteins from *S. pneumoniae* that had not been assessed as potential vaccine candidates. Extraction and purification techniques were successfully used in providing sufficient
quantities of several proteins for study in the mucosal immunisation, acute lung
challenge murine model. Attention was drawn in particular to two proteins, a 34kDa
GAPDH and a 14kDa ribosomal protein as potential vaccine protein candidates
against pneumococcal infection.

Acknowledgements

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of Canberra.


protect mice from fatal infection with *Streptococcus pneumoniae* bearing heterologous PspA. J. Infect. Dis 182, 1694-1701.


communities throughout the Northern Territory of Australia: a before and after study. Proceedings of the 4th International Symposium of pneumococci and pneumococcal disease, Helsinki, Finland PSV-37.


Table 1: Live *S. pneumoniae* recovered from the BAL fluid and lung homogenate of mice following immunisation with *S. pneumoniae* proteins or whole killed cells.

<table>
<thead>
<tr>
<th>Antigen(^b)</th>
<th>Bacterial recovery(^a) BAL</th>
<th>% clearance(^b)</th>
<th>Bacterial recovery(^a) LUNG</th>
<th>% clearance(^b)</th>
<th>Challenge Dose(^d) (log(_{10}) CFU/mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-immune(^c)</td>
<td>Immune</td>
<td>Non-immune(^c)</td>
<td>Immune</td>
<td>Non-immune(^c)</td>
</tr>
<tr>
<td>14kDa</td>
<td>5.1 ± 0.3</td>
<td>2.5 ± 0.2**</td>
<td>99</td>
<td>4.8 ± 0.4</td>
<td>3.6 ± 0.4*</td>
</tr>
<tr>
<td>34kDa</td>
<td>5.1 ± 0.3</td>
<td>4.6 ± 0.5</td>
<td>79</td>
<td>4.8 ± 0.4</td>
<td>3.6 ± 0.2*</td>
</tr>
<tr>
<td>38kDa</td>
<td>4.1 ± 0.2</td>
<td>3.7 ± 0.5</td>
<td>60</td>
<td>3.2 ± 0.5</td>
<td>4.4 ± 0.6</td>
</tr>
<tr>
<td>48kDa</td>
<td>4.6 ± 0.1</td>
<td>4.5 ± 0.2</td>
<td>21</td>
<td>4.5 ± 0.2</td>
<td>4.4 ± 0.3</td>
</tr>
<tr>
<td>57kDa</td>
<td>5.5 ± 0.2</td>
<td>5.1 ± 0.2</td>
<td>60</td>
<td>5.3 ± 0.3</td>
<td>5.1 ± 0.1</td>
</tr>
<tr>
<td>75kDa</td>
<td>4.1 ± 0.9</td>
<td>3.8 ± 0.6</td>
<td>50</td>
<td>4.1 ± 0.9</td>
<td>4.0 ± 0.6</td>
</tr>
<tr>
<td>WKC(^e) S. pneumoniae</td>
<td>5.6 ± 0.1</td>
<td>5.1 ± 0.2**</td>
<td>68</td>
<td>5.5 ± 0.1</td>
<td>5.0 ± 0.1*</td>
</tr>
</tbody>
</table>

\(^a\) The values shown represent the mean± standard error of the mean (SEM) for the BAL fluid and lung homogenate. Values are expressed as log\(_{10}\) CFU for n = 4-5 mice per group

\(^b\) % clearance compared with bacteria recovered in non-immune group

\(^c\) Non-immune mice were either untreated or sham immunised

\(^d\) Live bacterial challenge dose given per animal on day 21

\(^e\) WKC=Whole killed cell inoculum

\(*\) p < 0.05 compared with non-immune mice

\(**\) p < 0.001 compared with non-immune mice
Table 2: Percentage phagocytic cell counts in the BAL 5 hours post-pulmonary challenge with live *S. pneumoniae*.

<table>
<thead>
<tr>
<th>Group</th>
<th>Percentage differential cell count&lt;sup&gt;b&lt;/sup&gt;</th>
<th>PMN</th>
<th>Macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-immune</td>
<td></td>
<td>78.3 ± 9.9</td>
<td>21.9 ± 9.8</td>
</tr>
<tr>
<td>14kDa</td>
<td></td>
<td>60.7 ± 22.1</td>
<td>38.7 ± 21.4*</td>
</tr>
<tr>
<td>34kDa</td>
<td></td>
<td>43.3 ± 12.8</td>
<td>56.7 ± 12.8*</td>
</tr>
<tr>
<td>38kDa</td>
<td></td>
<td>49.7 ± 22.5</td>
<td>50.2 ± 22.5</td>
</tr>
<tr>
<td>48kDa</td>
<td></td>
<td>83.5 ± 2.8</td>
<td>16.5 ± 2.9</td>
</tr>
<tr>
<td>57kDa</td>
<td></td>
<td>95.5 ± 0.5</td>
<td>4.5 ± 0.5</td>
</tr>
<tr>
<td>75kDa</td>
<td></td>
<td>81.8 ± 7.1</td>
<td>18.3 ± 7.1</td>
</tr>
<tr>
<td>WKCe</td>
<td></td>
<td>72.4 ± 3.5</td>
<td>29.4 ± 4.2**</td>
</tr>
</tbody>
</table>

<sup>a</sup> Immunisation group with n = 4-5 mice per group

<sup>b</sup> The values shown represent the mean ± SEM of 3 fields per slide per mouse

<sup>c</sup> Whole killed cell inoculum

* p < 0.05 compared with non-immune mice

** p < 0.001 compared with non-immune mice
Table 3: Specific antibody to the protein extract in serum following immunisation.

<table>
<thead>
<tr>
<th>Mouse group&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Mean IgG titres in serum (ng/ml)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non immune</td>
<td>10&lt;sup&gt;p&lt;/sup&gt;</td>
</tr>
<tr>
<td>14kDa</td>
<td>1300 &lt;sup&gt;** p&lt;/sup&gt;</td>
</tr>
<tr>
<td>34kDa</td>
<td>2474 ± 132&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>38kDa</td>
<td>364 &lt;sup&gt;** p&lt;/sup&gt;</td>
</tr>
<tr>
<td>48kDa</td>
<td>3180 &lt;sup&gt;** p&lt;/sup&gt;</td>
</tr>
<tr>
<td>57kDa</td>
<td>ND&lt;sup&gt;p&lt;/sup&gt;</td>
</tr>
<tr>
<td>75kDa</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> The values shown represent the mean ± standard error of the mean (SEM)

<sup>b</sup> The immunisation group of mice from which the serum was obtained.

<sup>*</sup> P < 0.05 compared with non-immune mice

<sup>**</sup> P < 0.001 compared with non-immune mice

<sup>p</sup> Sera was pooled from mice within the group

ND Not detected
Table 4: N-terminal sequence results of significant proteins in this study.

<table>
<thead>
<tr>
<th>Estimated Molecular Mass (kDa)</th>
<th>Amino-terminal acid sequence</th>
<th>Homology identity&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>ALNIE NIIAEIKEAS</td>
<td>Ribosomal protein L7/L12</td>
</tr>
<tr>
<td>57</td>
<td>KKKEVYVAKK</td>
<td>unknown</td>
</tr>
<tr>
<td>34</td>
<td>VxxVGINT</td>
<td>GAPDH</td>
</tr>
</tbody>
</table>

<sup>a</sup> Homology identity determined using BLAST search
Figure Legends

Figure 1.

SDS-PAGE analysis of fractions collected after electroelution of the extracted *S. pneumoniae* proteins. Proteins were separated on a 12% acrylamide gel and Coomassie stained. Gel A represents eluted fractions 4-12, gel B are fractions 13-21 and gel C are fractions 22-30. Molecular mass markers are in lanes 1, 2 and 3 on gels A, B and C, respectively, and the values expressed in kDa are shown on the left.
Figure 1, Jomaa et al