Recent Approaches in Vaccine Development against *Streptococcus pneumoniae*

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Key Words

Capsule · Cross-reacting material 197 · DNA vaccines · Pneumococcal conjugate vaccines · Proteins · Serotype coverage · *Streptococcus pneumoniae* · Vaccine delivery vehicles

Abstract

Streptococcus pneumoniae is a major cause of morbidity and mortality among children under 5 years of age worldwide. Vaccines have long been used for protection against pneumococcal infections. Capsular polysaccharides of pneumococci are main antigenic components of these vaccines. However, pneumococcal polysaccharide-based vaccines are not able to elicit appropriate immunological responses in young children and cannot induce the immune memory. Thus, pneumococcal conjugate vaccines were developed through chemical coupling of an immunogenic carrier protein to the capsule. The currently available pneumococcal conjugate vaccines elicited protection against the bacterium efficiently. However, these vaccines are expensive to manufacture and have limited serotype coverage. In this mini-review, therefore, we describe approaches attempted by researchers to circumvent the shortcomings of the conjugate vaccines including specifying appropriate cultivation conditions for the production of *S. pneumoniae* capsular an-

tigens, development of suitable expression systems for the frequently used carrier protein in the conjugate vaccines (cross-reacting material 197), construction of protein-based vaccines, whole-cell vaccines, DNA vaccines, and using antigen delivery vehicles. Future trends in this field are also discussed.

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Introduction

Streptococcus pneumoniae, a capsulated lancet-shaped gram-positive bacterium is a leading cause of morbidity and mortality among children all over the world and a major cause of diseases such as pneumonia, meningitis and sepsis [Mook-Kanamori et al., 2011]. World Health Organization (WHO) estimated that 476,000 deaths among children less than 5 years of age were caused by pneumococcal infections. Disease rates and mortality are higher in developing than in industrialized settings, with the majority of deaths occurring in Africa and Asia [WHO, 2012]. Antibiotic treatment of pneumococcal disease is being compromised by the worldwide emergence of multidrug-resistant strains [Aligholi et al., 2009; Appelbaum, 2002; Mera et al., 2005]. The capsule of *S. pneumoniae* has long been recognized as the major viru-

lence factor and consists of a high-molecular-weight polymer of repeated oligosaccharides. Currently, 94 different pneumococci serotypes have been identified, and each serotype corresponds to a different chemical composition of the capsule [Bentley et al., 2006; Calix and Nahm, 2010; Calix et al., 2012; Hyams et al., 2010; Jin et al., 2009; Melin et al., 2010; Park et al., 2007]. Seven serotypes including 1, 5, 6A, 6B, 14, 19F and 23F cause most invasive pneumococcal disease in children less than 5 years of age globally [Johnson et al., 2010]. Vaccines have been used to prevent pneumococcal disease for more than 30 years. The capsule is the main antigenic component of pneumococcal vaccines. However, pneumococcal polysaccharide vaccines are associated with poor or absent immunogenicity in young children and failure at any age to induce an anamnestic antibody response upon revaccination. Pneumococcal conjugate vaccines (PCVs) are constructed based on chemical coupling of *S. pneumoniae* polysaccharides to an immunogenic carrier protein. This enhances the antibody response and induces the immune memory. WHO recommends the inclusion of PCVs in childhood immunization programs worldwide. Three PCVs including PCV 7, PCV 10, and PCV 13 are currently available on the market, which are immunogenic and protective in children. The afforded protection of these vaccines is restricted to 7, 10, and 13 clinically relevant serotypes. PCV 7 protects against pneumococcal serotypes 4, 6B, 9V, 14, 18C, 19F and 23F. PCV 10 protects against PCV 7 serotypes plus serotypes 1, 5, and 7F. PCV 13 added serotypes 3, 6A, and 19A to the PCV 10 serotypes. The pneumococcal serotype distributions displayed differences by geographical locations. The PCV 7 serotype coverage percentage varied substantially by region (49–82%), with highest serotype coverage in North America and Europe. Therefore, the PCV 7 protection elicited against pneumococci depends on geographical locations. However, the geographical coverage of PCV 10 was similar to PCV 13 with less regional variability (70–84% and 74–88%, respectively) than with PCV 7 [Johnson et al., 2010].

PCVs are expensive to manufacture, and the emergence of pneumococcal disease caused by nonvaccine serotypes is of serious concern [Mook-Kanamori et al., 2011; WHO, 2012]. These issues reinforce the need for more affordable protective strategies against pneumococcal infections. In this mini-review, therefore, we describe recent approaches used in the vaccine construction against *S. pneumoniae* to circumvent the shortcomings of currently available PCVs.

Production of Pneumococcal Capsular Polysaccharides

Production of bacterial polysaccharides depends on the applied bacterial strains. Massaldi et al. [2010] observed different amounts of the capsule production in three different strains of *S. pneumoniae* serotype 14. Furthermore, capsule production of *S. pneumoniae* serotype 4 was dependent on the initial pH of the cultivation medium [Kim et al., 1996]. *S. pneumoniae* is a lactic acid bacterium, and lactate is its primary metabolic byproduct. Leal et al. [2011] showed that the glucose consumption ceased after a short time when *S. pneumoniae* serotype 14 was cultivated without pH control. The production of lactic acid caused a decrease in pH, which may have led to inhibiting the glucose uptake and the bacterial growth. However, when pH was kept constant during cultivation, all added glucose was consumed resulting in a remarkable increase in polysaccharide production relative to cultivation without pH control. Glucose feeding during pH-controlled fermentation resulted in a further increase in the capsule production. In contrast to the study by Leal et al. [2011], Carvalho et al. [2013] showed that *S. pneumoniae* serotype 2 cultivated under constant pH reached a maximal optical density value, which was slightly lower than that obtained under noncontrolled pH conditions. The central metabolic system of the bacterium and its regulatory compounds may contribute to the observed conditions. The capsule production of *S. pneumoniae* serotype 3 in stirred cultures was higher than that of unstirred cultures. However, at stirring rates exceeding 180 rpm, the capsule production decreased, perhaps due to elevated shear stress [Sheng-De et al., 2009]. Common cultivation media used for *S. pneumoniae* contain animal-derived components [Hathaway et al., 2012; Morona et al., 2003; Sheng-De et al., 2009]. Transmission of pathogenic agents such as bovine spongiform encephalopathy agent and animal viruses through contaminated animal-based compounds is considered as a risk to human health [WHO, 2003]. Therefore, WHO recommends to manufacturers of pneumococcal vaccines to avoid wherever possible the use of materials of animal origin in the production process [WHO, 2005]. Improved media for cultivation of *S. pneumoniae* containing few animal origin compounds or without these compounds such as modified Hoeprich media have been developed. Gonçalves et al. [2002] used modified Hoeprich media containing acid hydrolyzed casein (AHC) or enzymatically hydrolyzed soybean meal as nitrogen sources for cultivation of *S. pneumoniae* serotype 23. The best nitrogen source for the

capsule production was AHC. Using dialyzed yeast extract instead of Hoeprich's vitamin solution enhanced the capsule production. It was observed that the pneumococcus did not grow under aerobic conditions. Experiments under anaerobic conditions were performed using either CO₂ or N₂. When N₂ was used, higher capsule and biomass production were achieved, and an increase in the maximum specific growth rate was observed. On the other hand, CO₂ did not improve the process performance, increased foam formation during the cultivation and also increased sodium hydroxide consumption for pH control. These studies indicated the importance of designing appropriate production processes for pneumococcal capsules to obtain high yields.

Production of Cross-Reacting Material 197

Cross-reacting material 197 (CRM197) is a nontoxic mutant of diphtheria toxin that contains a single amino acid substitution from glycine to glutamate in position 52. This protein is a safe and effective T cell-dependent carrier for saccharides and is currently being used in PCVs. CRM197 is produced using *Corynebacterium diphtheriae* infected by the nontoxigenic phage $\beta 197^{tox-}$ created by nitrosoguanidine mutagenesis of the toxigenic corynephage- β [Giannini et al., 1984; Malito et al., 2012]. However, the single lysogens were not able to produce significant quantities of the CRM197 protein. Techniques have been developed to bolster the production of CRM197 using double lysogens of the nontoxigenic phage $\beta 197^{tox-}$. Rappuoli [1983] reported that yields of CRM197 from double lysogens of *C. diphtheriae* were up to three times higher than the single lysogens. In order to achieve higher yields of CRM197, the fermentation process requires multiple lysogenic *C. diphtheria* strains. However, introduction of multiple lysogens of the corynephage $\beta 197^{tox-}$ into *C. diphtheriae* requires a laborious screening process for identifying strains that can overproduce the CRM197 protein. Utilizing plasmid-based expression systems in which the plasmids are present in multiple copies in the cell represents an attractive solution to enhance the CRM197 production yield. Matcalf [1997] constructed a novel plasmid containing CRM197-encoding gene, which was capable of transforming strains of *C. diphtheriae* into strains expressing high levels of CRM197 without the use of multiple lysogens. A synthetic gene coding for CRM197, bearing a short histidine tag and optimized codons, was expressed at a high level in *Escherichia coli*. However, the recombinant protein was insoluble and al-

ways found inside protein aggregates. The expression of CRM197 devoid of the short tag always failed [Stefan et al., 2011]. Pfenex Inc. has developed a *Pseudomonas fluorescens* strain that generates high levels of CRM197. Results of intact mass analysis, LysC mapping and epitope density showed that the obtained CRM197 was identical to that made by native host *C. diphtheriae*. Moreover, in animal studies, conjugates made by chemically linking recombinant CRM197 produced in *P. fluorescens* to pneumococcal capsular polysaccharides induced antibody levels similar to that of the commercial PCVs [Brady et al., 2012]. These studies demonstrated the significance of selecting a suitable expression system for production of CRM197. In addition to the above-mentioned protein expression systems, various recombinant protein production systems are currently available [Terpe, 2006; Yin et al., 2007]. However, their abilities in the production of CRM197 have yet to be evaluated.

Protein Vaccines

Pneumococcal proteins that contribute to pathogenesis and are common to serotypes of *S. pneumoniae* represent an avenue for the development of pneumococcal vaccines with broad serotype coverage (table 1). Pneumococcal surface protein A (PspA) is a member of a family of structurally related choline-binding surface proteins, which interferes with fixation of complement component C3 on the pneumococcal cell surface, and thus inhibits complement-mediated opsonization. PspA is a highly variable molecule that based on the sequence of the N-terminus can be grouped into 3 families encompassing 6 different clades. Family 1 is comprised of clades 1 and 2, family 2 is comprised of clades 3, 4 and 5, and family 3 consists of clade 6. PspA provided protection against fatal bacteremia and sepsis caused by different pneumococcus serotypes [McDaniel et al., 1991, 1998]. PspA has three structural domains, and its N-terminal region is composed of repeated α -helices that protrude from the bacterial surface. Between the N-terminus and the C-terminal choline-binding region is a conserved proline-rich (PR) region of 60–80 amino acids. The α -helical region is variable in length and amino acid sequence. The antibodies directed against this region are cross-reactive and can be cross-protective, but the strongest protection in mice against challenge strains is often observed when the immunizing and challenge PspAs are of the same α -helical PspA family. These results point out the importance of including more than one PspA in any PspA-based vac-

Table 1. Pneumococcal serotype coverage of protein vaccines

Protein vaccine	Vaccine-derived pneumococcal serotype	Described vaccine serotype coverage	Reference
PspA	2	3, 6A	McDaniel et al. [1991]
PspA	4	2, 3, 6, 4	McDaniel et al. [1998]
PspA fusions	3, 14, 6B	3, 6B	Darrieux et al. [2007]
PspA	3, 4	3, 4	Roche et al. [2003]
PspC	2	2	Ogunniyi et al. [2001]
PR region	none described	3, 6A	Daniels et al. [2010]
PsaA	none described	3, 14, 19A	Talkington et al. [1996]; Whaley et al. [2010]
PsaA-derived peptides	none described	2, 4, 6B	Johnson et al. [2002]
PiuA, PiaA	2	2	Brown et al. [2001]
PppA	2	3	Green et al. [2005]
ZmpB	2	2, 3, 6B, 14, 19F	Gong et al. [2011]
Six identified proteins	4	6B	Wizemann et al. [2001]
Five identified proteins	4	6B	Moffitt et al. [2011]
RrgB321	4, 6B, 35B	4, 6B, 35B	Harfouche et al. [2012]
DnaJ	2	2, 3, 6B, 14, 19F	Cui et al. [2011]
ClpP	4	1, 2, 3, 4, 5, 6B, 7F, 9V, 18C, 19F, 23F	Cao et al. [2009]
PhtA	4	6A, 6B	Adamou et al. [2001]
PhtB	4	6B	Adamou et al. [2001]
PhtD	4	3, 4, 6A, 6B	Adamou et al. [2001]
PpmA	19	4, 6A, 9V, 14, 18C, 19F, 23F	Overweg et al. [2000]
PdB	2	1, 2, 3, 4, 6, 7F, 18C	Alexander et al. [1994]

PhtA, PhtB, PhtD = Pneumococcal histidine triad family proteins.

cines developed for human use [Darrieux et al., 2007; Roche et al., 2003]. Intranasal administration of PspA plus interleukin-12 (IL-12) to mice was found to augment the production of both secretory and systemic opsonizing antibodies and to confer increased protection against pneumococcal infection. Therefore, IL-12 can be an appropriate adjuvant for the PspA-based vaccine [Arulnandam et al., 2001]. *Vibrio vulnificus* flagellin (FlaB) is a Toll-like receptor 5 agonist, which has a strong mucosal adjuvant activity. Intranasal immunization of mice with recombinant fusion proteins consisting of PspA and FlaB is able to elicit more efficient protective mucosal immune responses against pneumococcal infection than immunization with PspA alone or a mixture of PspA and FlaB. Therefore, the genetic fusion of FlaB to PspA is required for the efficiency of the FlaB-adjuvanted pneumococcal vaccines [Nguyen et al., 2011]. A phase 1 clinical trial of a recombinant family 1 PspA was completed in man. PspA was observed to be safe and immunogenic. The immune human serum protected mice from infections with pneumococci expressing either of the major PspA fami-

lies (1 and 2) [Briles et al., 2000b]. Other pneumococcal protein that has shown potential as a vaccine candidate is pneumococcal surface protein C (PspC). PspC is also known as choline-binding protein A. This protein is supposed to bind secretory IgA and to interact with human epithelial and endothelial cells [Rosenow et al., 1997]. Vaccination with PspC has shown to be protective against sepsis in mice. In addition, antibodies directed against PspC have shown cross-reactivity against PspA [Ogunniyi et al., 2001]. The PR region is also present in PspC. In PR regions of PspA and PspC, there exists an almost invariant nonproline block (NPB) of about 33 amino acids. Immunization with recombinant PR molecules and passive immunization with monoclonal antibodies reactive with either NPB or PR epitopes are protective against pneumococcal infection in mice. Due to their conservation and cross-activity, the PR and NPB regions represent potential vaccine targets capable of eliciting cross-protective immunity against pneumococcal infection [Daniels et al., 2010].

Another important candidate is pneumococcal surface adhesin A (PsaA). This protein is the divalent metal ion-binding lipoprotein component of an ATP-binding cassette (ABC) transport system that has specificity for manganese. Recently, it was reported that anti-PsaA antibodies inhibited pneumococcal adherence to human nasopharyngeal epithelial cells [Romero-Steiner et al., 2003]. Immunization studies with PsaA resulted in protection against colonization but little to modest protection against invasive infections [Johnson et al., 2002; Talkington et al., 1996]. PsaA coadministered with PCV7 resulted in greater reduction of non-PCV7 serotype 19A carriage in mice compared to using each vaccine alone, indicating an expansion of the PCV7 vaccine serotype coverage [Whaley et al., 2010]. PiuA and PiaA are iron uptake ABC transporter lipoproteins of *S. pneumoniae*. Immunization of mice with PiuA and PiaA individually provided protection against systemic challenge with *S. pneumoniae*. Antibody responses generated against these proteins cross-reacted with each other and reacted with identical proteins from different *S. pneumoniae* serotypes. These data indicate that ABC transporters can be effective antigens for development of pneumococcal vaccines [Brown et al., 2001].

Pneumococcal protective protein A (PppA) is a small protein conserved antigenically among different serotype strains of *S. pneumoniae*. This protein has significant homology to a nonheme iron-containing ferritin protein from *Listeria innocua* and other bacto-ferritins. Intranasal immunization of mice with PppA and mucosal adjuvants elicited antibodies that were effective in reducing nasopharyngeal colonization in mice following intranasal challenge with a heterologous pneumococcal strain. Nasopharyngeal colonization is a prerequisite for otitis media. Therefore, these observations suggested that PppA may be considered as a promising candidate for inclusion in a vaccine against pneumococcal otitis media [Green et al., 2005].

Zinc metalloprotease B (ZmpB) is present in all isolated pneumococcal strains. The first 300–400 amino acids at the N-terminal region of ZmpB show 99% similarity between all pneumococcal serotypes examined, while the remaining part of this protein displays high sequence variation. Intranasal immunization of mice with ZmpB decreased pneumococcal lung colonization caused by *S. pneumoniae* serotypes 19F and 14 and increased mice survival times following invasive pneumococcal challenge with different pneumococcal strains [Gong et al., 2011].

Recently, Wizemann et al. [2001] proposed a whole genome-based approach for identification of novel vaccine proteins for protection against pneumococcal infection. This approach is based on identifying surface proteins from the genome sequence by searching for sequence motifs commonly related to their secretion or surface binding. From the 130 identified open reading frames with secretion motifs or similarity to predicted virulence factors, 108 proteins were used for immunization of mice, and six proteins conferred protection against disseminated pneumococcal infection. Each of the six proteins showed broad strain distribution and immunogenicity during human infection.

IL-17A-secreting CD4⁺ T cells (T_H17) mediate resistance to mucosal colonization by multiple pathogens including *S. pneumoniae*. Moffitt et al. [2011] identified antigens recognized by T_H17 cells from mice immune to pneumococcal colonization through screening an expression library containing >96% of predicted pneumococcal proteins. Immunization of mice with the identified antigens provided protection from pneumococcal colonization that was significantly diminished in animals treated with blocking CD4 or IL-17A antibodies.

The overall prevalence of the pilus islet-1 (PI-1) is approximately 25–30% in pneumococci, with no appreciable difference between carriage and disease isolates. Pneumococcal pilus 1 components, and in particular the pilus backbone RrgB demonstrated efficacy in protecting mice from lethal challenge. Since RrgB exists in three non-cross-protective variants, a fusion protein containing all three variants was generated (RrgB321). RrgB321 elicited an antibody response against each of the variants and protected mice against PI-1-containing pneumococcal strains. Moreover, the induced antibodies mediated a complement-dependent opsonophagocytic killing of strains representing RrgB variants at levels comparable to those elicited by antisera against the PCV. These data provide evidence that a vaccine composed of RrgB321 has the potential to cover 25% or more of all pneumococcal strains and support the inclusion of this fusion protein in a multicomponent vaccine against *S. pneumoniae* [Harfouche et al., 2012].

Heat shock proteins (HSPs) are a family of highly conserved proteins expressed during a number of stress conditions. These proteins are able to eliminate inactive or damaged proteins to help hosts to adapt to the environment and thus play an important role in the pathogenesis of infectious diseases. Intranasal immunization of mice with DnaJ of *S. pneumoniae*, a member of HSPs, could reduce nasal or lung colonization of pneumococcus and

elicit protection against invasive infections mediated by different pneumococcal serotypes. Moreover, intraperitoneal immunization with DnaJ could protect against invasive infections caused by different serotypes of pneumococcus, and this protection was antibody mediated [Cui et al., 2011]. Caseinolytic protease (ClpP) is another HSP whose gene sequence in different serotypes of *S. pneumoniae* is highly conserved. Immunization of mice with ClpP could effectively elicit serotype-independent protection against invasive pneumococcal infection [Cao et al., 2009]. Using pneumococcal proteins capable of eliciting protection against broad range of pneumococcal strains in the development of vaccines against *S. pneumoniae* decreases the vaccine manufacturing costs via decreasing necessary vaccine components and consequently increases the coverage of vaccination programs in developing countries.

Pneumococcal histidine triad (Pht) family includes homologous cell surface proteins, which contain histidine triad motifs. The Pht family members are able to elicit protection against different pneumococcal serotypes in a mouse model of systemic disease [Adamou et al., 2001].

Pneumococcal putative proteinase maturation protein A (PpmA) elicited species-specific opsonophagocytic antibodies that were cross-reactive with various pneumococcal serotypes. This cross-reactivity was in line with the limited sequence variation of *ppmA* gene [Overweg et al., 2000].

Pneumolysin is a thiol-activated pneumococcal toxin that oligomerizes to form large pores associated with pathogenesis in the membranes of target host cells. All clinical isolates of *S. pneumoniae* are known to produce pneumolysins and the absence of significant amino acid sequence variation between pneumolysins from different serotypes made this protein an attractive candidate for the development of pneumococcal vaccines. Although native pneumolysin is unsuitable for inclusion in a human vaccine because of its toxic nature, pneumolysin derivatives of reduced toxicity developed by site-directed mutagenesis could be used. PdB is a pneumolysin toxoid, which has a reduced hemolytic activity of 0.05% of the wild-type toxin, because of a Trp-433→Phe substitution. Differences in the degree of protection of mice immunized by PdB against invasive pneumococcal infection were noted between different strains of *S. pneumoniae* used in challenge studies [Alexander et al., 1994]. Oloo et al. [2011] applied a targeted genetic detoxification approach based on insights into the toxin's putative three-dimensional structure and functional mechanics to intro-

duce amino acid modifications into pneumolysin that completely detoxify the protein by arresting its transition to the pore-forming state. The mutant proteins retained their ability to elicit neutralizing antibodies against the native toxin. Pneumolysin neutralization titers of antisera raised against the majority of the mutants were similar to that of the control serum raised against wild-type pneumolysin.

In contrast to PsaA and PspA, immunization with PdB had no observable effect on nasopharyngeal carriage. Immunization with both PspA and PsaA, compared to immunization with either protein alone, provided better protection against nasal colonization by pneumococci [Briles et al., 2000a]. Following an intraperitoneal pneumococcal challenge, the median survival times for mice immunized with combinations of PdB and PspA, PdB and PspC, or PspA and PspC were significantly longer than those for mice immunized with any of the single antigens. The combination of PdB, PspA and PspC offered the best protection [Ogunniyi et al., 2007]. A combination vaccine composed of ClpP, a pneumolysin mutant, and putative lipote protein ligase conferred complete protection against intranasal infections of three of seven most common pneumococcal strains (serotypes 14, 19F, and 23F) and 80% protection for pneumococcal strain 6B. Moreover, the combination vaccine was as effective as PCV 7 in protection against the four pneumococcal serotypes.

Several functions have been assigned to pneumolysin with respect to modification of the immune response. These include induction of inflammatory responses and modification of cell signaling. Pneumolysin has recently been shown to bind to Toll-like receptor 4. Recognition of pathogen-associated molecular patterns through such receptors has been shown to result in changes in antigen presentation and cellular activation [Malley et al., 2003]. Using PsaA from *S. pneumoniae*, it was shown that genetic fusion to pneumolysin is essential to ensure high levels of PsaA-specific IgG and IgA in the serum and at mucosal surfaces. Therefore, in addition to the protective role of pneumolysin against *S. pneumoniae*, this study confirms the utility of pneumolysin to act as an adjuvant to substantially increase the local and systemic humoral response to the genetically fused pneumococcal protein antigen [Douce et al., 2010].

Immunization with a combination of both PiuA and PiaA resulted in additive protection and was highly protective against systemic infection with *S. pneumoniae* [Wu et al., 2010].

Whole-Cell Vaccines

The use of killed cells of unencapsulated pneumococci maximizes the exposure of a variety of species to common subcapsular antigens, thus potentially providing synergistic immunity to multiple pneumococcal targets. These whole-cell vaccines can provide the advantage of low cost of production. Currently, injection is the recommended method for administration of pneumococcal vaccines [WHO, 2008]. Thus, in a study by Lu et al. [2010a], whole-cell pneumococcal vaccine was examined as an injectable vaccine using the currently approved aluminum adjuvant. A pneumococcal strain was engineered to be capsule negative, autolysin negative, and to express a non-toxic mutant pneumolysoid. The adjuvant $\text{Al}(\text{OH})_3$ strongly increased immune responses in mice injected with the chemically inactivated cells. The immunized mice were protected against nasal colonization and sepsis with different pneumococcal serotypes. However, not all the antigens presented by killed bacteria may contribute to protection, and some may even interfere with protection. Moreover, a particular combination of immunogenic surface antigens might prove difficult to reproduce from lot to lot in whole-cell preparations.

The mucosal, needle-free administration of vaccines can significantly decrease the need for syringes with their inherent added cost and risk of disease transmission, and it can increase compliance and consequently the coverage of vaccination programs [Giudice and Campbell, 2006]. Therefore, vaccine developers are encouraged to explore these routes of administration [WHO, 2008]. Unencapsulated killed whole-cell pneumococci administered intranasally with cholera toxin (CT) as an adjuvant can protect against nasopharyngeal colonization and invasive disease, using two different serotypes unrelated to the capsular type from which the vaccine strain was derived [Malley et al., 2001]. Lu et al. [2010b] demonstrated protection with a vaccine strain of *S. pneumoniae* expressing a nonhemolytic derivative of pneumolysin. Retention of soluble antigens released from the cells during the chemical killing procedure contributed to the protection. Intranasal administration of the vaccine with CT as an adjuvant protected mice against nasopharyngeal challenge with pneumococci. Neither CT nor *E. coli* heat-labile toxin (LT) is suitable for human use, which promoted the evaluation of nontoxic mutants of LT. Nontoxic single and double mutants of LT were effective as mucosal adjuvants for this vaccine. However, a recent study raised concerns over the safety of detoxified mutants of LT when given by the intranasal route and pointed a possible as-

sociation between their use and the development of Bell's palsy [Lewis et al., 2009]. Therefore, alternative routes were explored for the vaccination. Protection was induced by sublingual, buccal routes, albeit requiring larger doses than when given intranasally. Protection was also induced transdermally with sonicates of the killed cell preparation. These results indicated that the whole cell vaccine can be made and administered in different ways to suit the manufacturer and the vaccination program. A preparation of heat-inactivated *S. pneumoniae* was immunogenic when applied to mucosal surfaces and the nasal mucosa was the preferred site for the vaccine delivery with regard to the induction of serum and saliva antibody responses. Mice immunized intranasally were protected against both systemic infection and death following an intraperitoneal pneumococcal challenge [Hvalbye et al., 1999]. Furuya et al. [2010] demonstrated the superior immunogenicity of γ -ray inactivated influenza A virus compared with formalin or UV-inactivated virus. Moreover, in a study by Cryz et al. [1982], *Vibrio cholera* was inactivated with heat and chemical inactivants (phenol or formalin) alone or in combination. The antigenicity and immunogenicity of *V. cholera* are greatly affected by the inactivation conditions employed. These studies, therefore, indicated the importance of selecting an appropriate method for inactivation of microorganisms while preparing vaccines based on whole killed microorganisms.

Roche et al. [2007] constructed live attenuated mutants of *S. pneumoniae* containing deletions in genes encoding three of major virulence determinants including capsular polysaccharides (*cps*), pneumolysin (*ply*), and *pspA*. The attenuated strains were not able to cause disease but retained the ability to colonize the upper respiratory tract. Nasal colonization by live attenuated vaccine strains was used to immunize mice. A single intranasal administration of live attenuated vaccine without adjuvant induced both systemic and mucosal protection from intranasal challenge with a high dose of the parent strain. Immunization with *cps* mutants demonstrated cross-protective immunity following challenge with a distantly related *S. pneumoniae*. The antibody responses were required for the protection. Thus, colonization by live attenuated *S. pneumoniae* is an effective vaccine strategy that may offer broad protection against pneumococci. These vaccines do not require inactivation process and pharmacological adjuvants. Therefore, the downstream processing of these vaccines is simpler and less costly than that of whole-cell killed vaccines. However, the possibility of reversion of live attenuated vaccines to their pathogenic states is a cause for concern.

Pep27, an autolysis-inducing factor of *S. pneumoniae* is known to mediate pneumococcal major autolysin-dependent and -independent lysis. When the *pep27* gene was disrupted, the obtained pneumococcal mutant showed major decrease in virulence. Virulence attenuation by the mutant strain seems to be due to inhibition of bacterial autolysis and release of pneumococcal inflammatory mediators. Intranasal immunization of mice twice with the live *pep27* mutant in the absence of any adjuvant protected them from subsequent lethal intranasal challenge in a capsular serotype-independent manner. Moreover, intranasal immunization was more protective than intraperitoneal immunization following an intraperitoneal lethal challenge. This result may be attributed to the more rapid clearance of the intraperitoneally injected bacteria compared to the intranasally administered bacteria and indicated the importance of selecting an appropriate route for the immunization [Kim et al., 2012].

Despite some success in reducing the clinical impact of *S. pneumoniae* in children through vaccination, the burden of pneumococcal acute otitis media remains significant. Rosch et al. [2014] generated a novel live attenuated pneumococcal vaccine that was able to protect against acute otitis media. The attenuation strategy was focused on microbial adaptation physiology while maintaining a full set of virulence determinants. Mutants in the signal recognition particle pathway show heightened sensitivity to environmental stresses and are greatly attenuated. FtsY is a central component of the signal recognition particle pathway, which is responsible for delivering membrane and secretory proteins to the proper cellular destination. A live vaccine constructed based on deletion of the *ftsY* gene induced serotype-independent protection against otitis media. In addition, the vaccine was protective against sinusitis, pneumonia and invasive pneumococcal disease. Protection against pneumonia was maintained in animals coinfecting with influenza virus. The live vaccine generated strong antibody responses reactive against pneumococci, which required the presence of CD4+ T cells during vaccination.

DNA Vaccines

DNA vaccines are easy to manufacture, have a low cost, and are stable in transportation. These properties make DNA vaccines ideal for implementation in developing countries. Vectors encoding the complete N-terminal regions of PspAs elicited humoral responses in mice, and cross-reactivity was mainly restricted to the same family.

Mice immunized intramuscularly with the DNA vaccines were protected only against intraperitoneal challenge with a strain expressing PspA from the same clade [Miyaji et al., 2002]. The DNA vaccine expressing the C-terminal region of PspA elicited protection in mice vaccinated intramuscularly against intraperitoneal challenge with a virulent strain of *S. pneumoniae*. When a more virulent pneumococcal strain was used in the challenge, the DNA vaccine expressing the N-terminal- α -helical region of PspA elicited protection in mice at a level similar to that of PspA purified from the challenge strain, whereas the DNA vaccine expressing the C-terminal region of PspA was not protective [Miyaji et al., 2003]. These results indicated the importance of an appropriate vaccine design for the effective protection of DNA vaccines against *S. pneumoniae*.

Nasal delivery of naked plasmid DNA induces only weak immune responses, which may be due to the degradation of naked DNA at mucosal surfaces [Hobson et al., 2003]. Therefore, for construction of effective DNA vaccines, an appropriate DNA delivery system is demanded. Chitosan is a natural biodegradable polysaccharide derived from chitin that possesses biocompatibility and mucoadhesion properties. The positively charged chitosan has great potential for complexation with negatively charged DNA and forming nanoparticles to improve the mucosal delivery of DNA [Aspden et al., 1997]. Mice were immunized intranasally with chitosan-DNA nanoparticles expressing PsaA (chitosan-*psaA*). Compared to levels in control mice immunized with naked DNA containing *psaA* gene or chitosan-backbone vector without *psaA* gene, systemic and mucosal immune responses against PsaA were elevated in mice immunized with chitosan-*psaA*. In addition, fewer pneumococci were recovered from the nasopharynx of mice vaccinated with chitosan-*psaA* than for the control groups following intranasal pneumococcal challenge. These findings suggested that chitosan-DNA nanoparticles expressing pneumococcal major antigens could be developed to prevent pneumococcal infections [Xu et al., 2011].

Antigen Delivery Vehicles

PspA fusion proteins comprised of PspA fragments from families 1 and 2 delivered by a recombinant attenuated *Salmonella* vaccine (RASV) orally elicited serum IgG and mucosal IgA responses against both families of PspA in mice and protected the animals against challenge by pneumococcal strains from both families. Low-cost vac-

cine production, needle-free delivery, and induction of strong mucosal immunity are advantages associated with using RASV for antigen delivery. The obtained results indicated that the RASV synthesizing a PspA fusion protein representing both PspA families was an effective anti-pneumococcal vaccine, extending and enhancing protection against multiple strains of *S. pneumoniae* [Xin et al., 2009].

Outer membrane vesicles (OMVs) are released by most Gram-negative bacteria into the surrounding environment during growth. During formation of these vesicles, periplasmic and outer membrane proteins are entrapped, while components from the inner membrane and cytoplasm are excluded. This property has been exploited as a method to derive immunogenic vesicle preparations for use as vaccines. Localization of pneumococcal PspA into the *Salmonella* periplasm by fusion to the β -lactamase secretion signal led to its packaging and release in OMVs. Intranasal immunization of mice with OMVs containing PspA provided complete protection against a lethal dose challenge of *S. pneumoniae*, which was more than that elicited with PspA or empty vesicles. Enhancement of anti-PspA serum antibody responses after using OMVs for the antigen delivery demonstrated the ability of these vesicles to act as mucosal adjuvants. Results of this study establish that OMVs can be used to mucosally deliver an antigen from a Gram-positive organism and induce a protective immune response [Muralinath et al., 2011].

Arevalo et al. [2009] constructed replication-defective adenoviral vectors encoding PsaA, the N-terminal fragment of PspA, and PdB. Nasal vaccination of mice with the adenoviral vectors elicited antibody responses against the pneumococcal antigens. Mice vaccinated with combinations of two or three of the constructed vectors showed reduced pneumococcal colonization in the lung after intranasal challenge with *S. pneumoniae*, compared with the control group immunized with physiological saline or the group of mice immunized only with the PdB-encoding vector. Adenoviral vectors are attractive as gene transfer vectors for gene-based vaccination in mammalian systems for a number of reasons. The adenoviral genome is well characterized and easy to manipulate through molecular biology techniques. Most adenoviruses cause mild diseases in immunocompetent human adults and by deletion of crucial regions of the viral genome the vectors can be rendered replication defective, which increases their predictability and reduces unwanted side effects. These viruses can be grown to high titers in tissue culture and their relative thermostability facilitates their clinical use

[Tatsis and Ertl, 2004]. These adenoviral vectors, when delivered to mice through the nasal mucosal route, could direct endogenous antigen expression in the host and mobilize host immune responses against the antigen. Results of the study by Arevalo et al. [2009] demonstrated the feasibility of developing a mucosal vaccine against *S. pneumoniae* using recombinant adenoviruses for antigen delivery.

Lactic acid bacteria (LAB) are used for the fermentation and preservation of food products, particularly dairy products, fermented meat, and vegetables. Consequently, these bacteria have a long record of safe association with humans and human foodstuffs. LAB, therefore, are attractive antigen delivery vehicles. Nasal immunization of mice with *Lactobacillus casei* expressing the N-terminal region of PspA intracellularly resulted in induction of anti-PspA IgG in the sera. The elicited anti-PspA IgG was able to bind to native PspA on the cell surface of *S. pneumoniae*, inducing complement deposition on the cell surface. The vaccinated mice were protected against systemic pneumococcal challenge, whereas no survival was observed in the control group of mice immunized with *L. casei* cells carrying the plasmid vector without the antigen gene. These results suggested the applicability of *L. casei* cells as antigen delivery vehicles in the development of mucosal vaccines against *S. pneumoniae* [Campos et al., 2008]. Intranasal vaccination of mice with *Lactococcus lactis* expressing the N-terminal region of PspA intracellularly afforded protection against respiratory challenge with *S. pneumoniae*, which was higher than that elicited with the purified antigen administered intranasally or by injection with alum. This finding was attributed to a shift towards a Th1 response. In an intraperitoneal challenge with *S. pneumoniae*, the lactococcal nasal vaccine afforded resistance to the infection similar to that obtained with the injected purified antigen, and both vaccines produced equivalent mean survival times, whereas the survival time of mice in the control group was shorter. This investigation indicated that mucosal immunization with a recombinant *L. lactis* vaccine expressing a pneumococcal protein immunogen was an alternative approach to an injected protein vaccine. In addition, the survival time of mice vaccinated with an inactivated lactococcal vaccine following respiratory pneumococcal challenge was less than that of mice vaccinated with live lactococcal vaccine. The reduced efficacy of the inactivated vaccine may be explained by a shift towards a Th2 response [Hanniffy et al., 2007]. In another study, PsaA was expressed in different strains of LAB in a cell wall-associated form. *Lactobacillus plantarum* and *Lactobacillus helveticus* were found

to be more effective at inducing mucosal and systemic anti-PsaA immune responses than *L. casei* following intranasal vaccination of mice. Because all three *Lactobacillus* strains expressed almost the same amount of PsaA and were also recovered from mice nasal mucosa in the same period, the observed differences among their respective antibody responses may reflect their different intrinsic adjuvant properties. On the other hand, all the recombinant *Lactobacillus* strains showed a significant reduction of *S. pneumoniae* colonization when compared with the saline group. However, only *L. helveticus* expressing PsaA showed a reduction of *S. pneumoniae* colonization in relation to control *L. helveticus* carrying the empty plasmid without the antigen [Oliveira et al., 2006]. Selection of LAB strains for use as antigen carriers depends on their persistence in the host, capacity to express foreign antigens, and intrinsic adjuvanticity. It has been reported that several LAB strains – particularly strains from the genera *Lactobacillus* – are able to act as adjuvants. This aspect should be considered when selecting a vaccine strain as it is a natural way to potentiate the immune reaction against heterologous antigens produced by recombinant LAB. It might be speculated that a high level of the antigen expression will not be necessary when using immunostimulatory LAB strains [Pouwels et al., 1998; Wells et al., 1996]. Nasal immunization of mice with recombinant lactococci expressing PppA on the cell surface (*L. lactis* PppA+) induced strong mucosal and systemic immune responses. The vaccinated mice showed increased resistance to both systemic and respiratory infections with different pneumococcal serotypes [Medina et al., 2008]. Nasal immunization of mice with inactivated *L. lactis* PppA+ in association with oral administration of a probiotic *L. casei* strain was able to prevent lung colonization by *S. pneumoniae* in a respiratory infection model. This result demonstrated the applicability of probiotic-inactivated vaccine association as a valuable tool for protection of health against infectious diseases, which is valuable especially in at-risk populations [Vintini et al., 2009].

Audouy et al. [2006] used killed nonrecombinant *L. lactis* particles obtained by chemical pretreatment of whole bacteria with hot acid, referred to as Gram-positive enhancer matrix (GEM) particles as delivery vehicles for pneumococcal antigens. These particles are bacterial-shaped peptidoglycan spheres that lack other cell wall components and intracellular materials. Antigens are attached to the surface of GEM particles noncovalently using the peptidoglycan-binding domain (PA) of lactococcal peptidoglycan hydrolase. Chimeric antigen-PA genetic constructs were prepared with three antigens of *S.*

pneumoniae including PpmA, the streptococcal lipoprotein rotamase A (SlrA), and the immunoglobulin A1 protease. Following nasal immunization of mice with the antigen displaying GEM particles, antibody responses were induced at systemic and local levels. Only background levels of SlrA-specific IgG were measured when free SlrA was administered to mice intranasally in the absence of any adjuvants. When SlrA was bound to or mixed with GEM particles, levels of elicited serum IgGs were higher than that obtained with free SlrA. These results indicated the adjuvant properties of GEM particles. In addition, different GEM-based vaccines can be used consecutively in the same mice without adverse effects or loss of activity. Utilizing GEM-based vaccines can circumvent the drawbacks associated with the use of genetically modified bacterial delivery systems.

Conclusions

The rapid increase in antibiotic resistance, high cost, and limited serotype coverage of the currently available PCVs encourage the search for novel vaccine candidates that would be more affordable and elicit protection against a broader range of pneumococcal strains. Determining suitable bacterial strains and cultivation conditions for *S. pneumoniae* can improve the pneumococcal capsule production yield and thus can decrease the capsule production cost. Furthermore, utilizing a suitable expression system for production of CRM197 can make the production process more economical. Currently, in our lab we are performing screening of serotype 19 pneumococcal strains to determine high-producer strains of the capsular polysaccharide.

Pneumococcal proteins common to *S. pneumoniae* serotypes can contribute to the development of the vaccines with broad serotype coverage. Using these proteins in combinations or as fusions can enhance the vaccine efficiency. Applying suitable adjuvants can further improve the performance of the protein-based vaccines. However, more completed studies regarding the serotype coverage of protein-based vaccines are required. Coadministration of the conjugate vaccines with the protein-based vaccines can broaden the vaccine serotype coverage. Other developments involve determination of appropriate combinations of conjugate and protein-based vaccines.

Inactivated and live attenuated whole cell pneumococcal vaccines are low-cost vaccines, and the protection induced by these vaccines is affected by the route of vaccine administration. However, when using live attenuated

S. pneumoniae strains in vaccination, the risk for reversion of the bacterium to the pathogenic state should be considered.

DNA vaccination is also an attractive approach for the vaccine construction against *S. pneumoniae*. Encapsulation of DNA vaccines with appropriate compounds can prevent the degradation in the host body and thus improve the elicited protection.

Several antigen delivery vehicles have been used for pneumococcal antigens. A comparison of performance of these delivery vehicles should be made for pneumococcal antigens desired to be used in the pneumococcal vaccine design.

The discovery of *Campylobacter jejuni* N-linked glycosylation system combined with its functional expression in *E. coli* marked the dawn of a new era in glycoengineering. PglB, an oligosaccharyltransferase is the key enzyme of the *C. jejuni* N-glycosylation system, which transfers O polysaccharide from a lipid carrier to an acceptor protein. The relaxed specificity of PglB toward the glycan structure was exploited to create novel protein glycan structures. This technology may have enormous implications for the design of glycoconjugate vaccines that will be inexpensive to manufacture. Investigating the application of this technology in the construction of PCVs represents an attractive subject for future studies [Feldman et al., 2005; Terra et al., 2012].

References

- Adamou JE, Heinrichs JH, Erwin AL, et al: Identification and characterization of a novel family of pneumococcal proteins that are protective against sepsis. *Infect Immun* 2001;69:949–958.
- Alexander JE, Lock RA, Peeters CCAM, et al: Immunization of mice with pneumolysin toxoid confers a significant degree of protection against at least nine serotypes of *Streptococcus pneumoniae*. *Infect Immun* 1994;62:5683–5688.
- Aligholi M, Emaneini M, Jabalameli F, et al: Antibiotic susceptibility pattern of Gram-positive cocci cultured from patients in three university hospitals in Tehran, Iran during 2001–2005. *Acta Med Iran* 2009;47:329–334.
- Appelbaum PC: Resistance among *Streptococcus pneumoniae*: implications for drug selection. *Clin Infect Dis* 2002;34:1613–1620.
- Arevalo MT, Xu Q, Paton SK, et al: Mucosal vaccination with a multicomponent adenovirus vectored vaccine protects against *Streptococcus pneumoniae* infection in the lung. *FEMS Immunol Med Microbiol* 2009;55:346–351.
- Arulnandam BP, Lynch JM, Briles DE, Hollingshead S, Metzger DW: Intranasal vaccination with pneumococcal surface protein A and interleukin-12 augments antibody mediated opsonization and protective immunity against *Streptococcus pneumoniae* infection. *Infect Immun* 2001;69:6718–6724.
- Aspden TJ, Mason JD, Jones NS, Lowe J, Skaugrud O, Illum L: Chitosan as nasal delivery system: the effect of chitosan solutions on in vitro and in vivo mucociliary transport rates in human turbinates and volunteers. *J Pharm Sci* 1997;86:509–513.
- Audouy SAL, van Roosmalen ML, Neef J, et al: *Lactococcus lactis* GEM particles displaying pneumococcal antigens induce local and systemic immune responses following intranasal immunization. *Vaccine* 2006;24:5434–5441.
- Bentley SD, Anaensen DM, Mavroidi A, et al: Genetic analysis of the capsular biosynthetic locus from all 90 pneumococcal serotypes. *PLoS Genet* 2006;2:e31.
- Brady C, Killeen K, Taylor W, Patkar A: Carrier protein outsourcing. *Bioproc Int* 2012;10:50–55.
- Briles DE, Ades E, Paton JC, et al: Intranasal immunization of mice with a mixture of the pneumococcal proteins PsaA and PspA is highly protective against nasopharyngeal carriage of *Streptococcus pneumoniae*. *Infect Immun* 2000a;68:796–800.
- Briles DE, Hollingshead S, Brooks-Walter A, et al: The potential to use PspA and other pneumococcal proteins to elicit protection against pneumococcal infection. *Vaccine* 2000b;18:1707–1711.
- Brown JS, Ogunniyi AD, Woodrow MC, Holden DW, Paton JC: Immunization with components of two iron uptake ABC transporters protect mice against systemic *Streptococcus pneumoniae* infection. *Infect Immun* 2001;69:6702–6706.
- Calix JJ, Nahm MH: A new pneumococcal serotype, 11E has a variably inactivated *wcjE* gene. *J Infect Dis* 2010;202:29–38.
- Calix JJ, Porambo RJ, Brady AM, et al: Biochemical, genetic and serological characterization of two capsule subtypes among *Streptococcus pneumoniae* serotype 20 strains: discovery of a new pneumococcal serotypes. *J Biol Chem* 2012;287:27885–27894.
- Campos IB, Darrieux M, Ferreira DM, et al: Nasal immunization of mice with *Lactobacillus casei* expressing the pneumococcal surface protein A: induction of antibodies, complement deposition and partial protection against *Streptococcus pneumoniae* challenge. *Microbes Infect* 2008;10:481–488.
- Cao J, Li D, Gong Y, et al: Casinolytic protease: a protein vaccine which could elicit serotype independent protection against invasive pneumococcal infection. *Clin Exp Immunol* 2009;156:52–60.
- Carvalho SM, Kuipers OP, Neves AR: Environmental and nutritional factors that affect growth and metabolism of the pneumococcal serotype 2 strain D39 and its nonencapsulated derivative strain R6. *PLoS One* 2013;8:e58492.
- Cryz SJ, Furer E, Germanier R: Effect of chemical and heat inactivation on the antigenicity and immunogenicity of *Vibrio cholerae*. *Infect Immun* 1982;38:21–26.
- Cui Y, Zhang X, Gong Y, et al: Immunization with DnaJ (hsp40) could elicit protection against nasopharyngeal colonization and invasive infection caused by different strains of *Streptococcus pneumoniae*. *Vaccine* 2011;29:1736–1744.
- Daniels CC, Coan P, King J, Hale J, Benton KA, Briles DE, Hollingshead SK: The proline rich region of pneumococcal surface protein A and C contains surface accessible epitopes common to all pneumococci and elicits antibody mediated protection against sepsis. *Infect Immun* 2010;78:2163–2172.
- Darrieux M, Miyaji EN, Ferreira DM, Lopes LM, Lopes APY, Ren B, Briles DE, Hollingshead SK, Leite LCC: Fusion proteins containing family 1 and family 2 PspA fragments elicit protection against *Streptococcus pneumoniae* that correlates with antibody mediated enhancement of complement deposition. *Infect Immun* 2007;75:5930–5938.
- Douce G, Ross K, Cowan G, Ma J, Mitchell TJ: Novel mucosal vaccines generated by genetic conjugation of heterologous proteins to pneumolysin from *Streptococcus pneumoniae*. *Vaccine* 2010;28:3231–3237.

- Feldman MF, Wacker M, Hernandez M, et al: Engineering N-linked protein glycosylation with diverse O antigen lipopolysaccharide structures in *Escherichia coli*. *Proc Natl Acad Sci USA* 2005;102:3016–3021.
- Furuya Y, Regner M, Lobigas M, et al: Effect of inactivation method on cross protective immunity induced by whole killed influenza A viruses and commercial vaccine preparations. *J Gen Virol* 2010;91:1450–1460.
- Giannini G, Rappuoli R, Ratti G: The amino acid sequence of two non toxic mutants of diphtheria toxin: CRM45 and CRM197. *Nucleic Acids Res* 1984;12:4063–4069.
- Giudice EL, Campbell JD: Needle free vaccine delivery. *Adv Drug Deliv Rev* 2006;58:68–89.
- Gonçalves VM, Zangirolami TC, Giordano RL, Raw I, Tanizaki MM, Giordano RC: Optimization of medium and cultivation conditions for capsular polysaccharide production by *Streptococcus pneumoniae* serotype 23F. *Appl Microbiol Biotechnol* 2002;59:713–717.
- Gong Y, Wenchun X, Cui Y, et al: Immunization with a ZmpB based protein vaccine could protect against pneumococcal diseases in mice. *Infect Immun* 2011;79:867–878.
- Green BA, Zhang Y, Masi AW, et al: PspA, a surface exposed protein of *Streptococcus pneumoniae* elicits cross reactive antibodies that reduce colonization in a murine intranasal immunization and challenge model. *Infect Immun* 2005;73:981–989.
- Hanniffy SB, Carter AT, Hitchin E, Wells JM: Mucosal delivery of a pneumococcal vaccine using *Lactococcus lactis* affords protection against respiratory infection. *J Infect Dis* 2007;195:185–193.
- Harfouche C, Filippini S, Gianfaldoni C, et al: RrgB321, a fusion protein of the three variants of the pneumococcal pilus backbone RrgB, is protective in vivo and elicits opsonic antibodies. *Infect Immun* 2012;80:451–460.
- Hathaway LJ, Brugger SD, Morand B, et al: Capsule type of *Streptococcus pneumoniae* determines growth phenotype. *PLoS Pathog* 2012;8:e1002574.
- Hobson P, Barnfield C, Barnes A, Klavinskis LS: Mucosal immunization with DNA vaccines. *Methods* 2003;31:217–224.
- Hvalbye BKR, Aaberge IS, Lovik M, Haneberg B: Intranasal immunization with heat inactivated *Streptococcus pneumoniae* protects mice against systemic pneumococcal infection. *Infect Immun* 1999;67:4320–4325.
- Hyams C, Yuste J, Bax K, Camberlein E, Weiser JN, Brown JS: *Streptococcus pneumoniae* resistance to complement mediated immunity is dependent on the capsular serotype. *Infect Immun* 2010;78:716–725.
- Jin P, Kong F, Xiao M, et al: First report of putative *Streptococcus pneumoniae* serotype 6D among nasopharyngeal isolates from Fijian children. *J Infect Dis* 2009;200:1375–1380.
- Johnson HL, Deloria-Knoll M, Levine RS, et al: Systematic evaluation of serotypes causing invasive pneumococcal disease among children under five: the pneumococcal global serotype project. *PLoS Med* 2010;7:e1000348.
- Johnson SE, Dykes JK, Jue DL, et al: Inhibition of pneumococcal carriage in mice by subcutaneous immunization with peptides from the common surface protein pneumococcal surface adhesin A. *J Infect Dis* 2002;185:489–496.
- Kim E-H, Choi S-Y, Kwon M-K, et al: *Streptococcus pneumoniae* pep27 mutants as a live vaccine for serotype independent protection in mice. *Vaccine* 2012;30:2008–2019.
- Kim S-N, Min K-K, Choi I-H, Kim S-W, Pyo S-N, Rhee D-K: Optimization of culture conditions for production of pneumococcal capsular polysaccharide type IV. *Arch Pharm Res* 1996;19:173–177.
- Leal MM, Pereira DSG, Jessouroun E, Couto MAPG, Pereira N Jr: Investigation of cultivation conditions for capsular polysaccharide production by *Streptococcus pneumoniae* serotype 14. *Electron J Biotechnol* 2011;14:1.
- Lewis DJ, Huo Z, Barnett S, et al: Transient facial nerve paralysis (Bell's palsy) following intranasal delivery of a genetically detoxified mutant of *Escherichia coli* heat labile toxin. *PLoS One* 2009;4:e6999.
- Lu Y-J, Leite L, Gonçalves VM, et al: GMP grade pneumococcal whole cell vaccine injected subcutaneously protects mice from nasopharyngeal colonization and fatal aspiration-sepsis. *Vaccine* 2010a;3:7468–7475.
- Lu Y-J, Yadav P, Clements JP, et al: Options for inactivation, adjuvant, and route of topical administration of a killed unencapsulated pneumococcal whole cell vaccine. *Clin Vaccine Immunol* 2010b;17:1005–1012.
- Malito E, Bursulaya B, Chen C, et al: Structural basis for lack of toxicity of the diphtheria toxin mutant CRM 197. *Proc Natl Acad Sci USA* 2012;109:5229–5234.
- 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.
- Malley R, Lisitch M, Stack A, et al: Intranasal immunization with killed unencapsulated whole cells prevents colonization and invasive disease by capsulated pneumococci. *Infect Immun* 2001;69:4870–4873.
- Massaldi H, Ines Bessio M, Suarez N, Texeira E, Ross S, Ferreira F: Feature of bacterial growth and polysaccharide production of *Streptococcus pneumoniae* serotype 14. *Biotechnol Appl Biochem* 2010;55:37–43.
- Matcalf BJ: Plasmid for production of CRM protein and diphtheria toxin. US Pat No 5614382. Washington, US Patent and Trademark Office, 1997.
- McDaniel LS, McDaniel DO, Hollingshead SK, Briles D: Comparison of the PspA sequence from *Streptococcus pneumoniae* EF5668 to the previously identified PspA sequence from strain Rx1 and ability of PspA from EF5668 to elicit protection against pneumococci of different capsular types. *Infect Immun* 1998;66:4748–4754.
- McDaniel LS, Sheffield JS, Delucchi P, Briles DE: PspA, a surface protein of *Streptococcus pneumoniae*, is capable of eliciting protection against pneumococci of more than one capsular type. *Infect Immun* 1991;59:222–228.
- Medina M, Villeno J, Vintini E, Herbert EM, Raya R, Alvarez S: Nasal immunization with *Lactococcus lactis* expressing the pneumococcal protective protein A induces protective immunity in mice. *Infect Immun* 2008;76:2696–2705.
- Melin M, Trzcinski K, Antonio M, et al: Serotype related variation in susceptibility to complement deposition and opsonophagocytosis among clinical isolates of *Streptococcus pneumoniae*. *Infect Immun* 2010;78:5252–5261.
- Mera RM, Miller LA, Daniels JJ, Weil JG, White AR: Increasing prevalence of multidrug resistant *Streptococcus pneumoniae* in the US over a 10-year period: Alexander project. *Diagn Microbiol Infect Dis* 2005;51:195–200.
- Miyaji EN, Dias WO, Tanizaki MM, Leite LCC: Protective efficacy of PspA-based DNA vaccines: contribution of both humoral and cellular immune responses. *FEMS Immunol Med Microbiol* 2003;37:53–57.
- Miyaji EN, Ferreira DM, Lopes APY, Brandileone CC, Dias WO, Leite LCC: Analysis of serum cross reactivity and cross protection elicited by immunization with DNA vaccines against *Streptococcus pneumoniae* expressing PspA fragments from different clades. *Infect Immun* 2002;70:5086–5090.
- Moffitt KL, Gierahn TM, Lu Y-J, et al: T_H17-based vaccine design for prevention of *Streptococcus pneumoniae* colonization. *Cell Host Microbe* 2011;9:158–165.
- Mook-Kanamori BB, Geldhoff M, van der Poll T, van de Beek D: Pathogenesis and pathophysiology of pneumococcal meningitis. *Clin Microbiol Rev* 2011;24:557–591.
- Morona JK, Morona R, Miller DC, Paton JC: Mutational analysis of the carboxy terminal (YGX)₄ repeat domain of CPSD, an autophosphorylating tyrosine kinase required for capsule biosynthesis in *Streptococcus pneumoniae*. *J Bacteriol* 2003;185:3009–3019.
- Muralinath M, Kuehn MJ, Roland KL, Curtiss R III: Immunization with *Salmonella enterica* serovar Typhimurium-derived outer membrane vesicles delivering the pneumococcal protein PspA confers protection against challenge with *Streptococcus pneumoniae*. *Infect Immun* 2011;79:887–894.
- Nguyen CT, Kim SY, Kim MS, Lee SE, Rhee JH: Intranasal immunization with recombinant PspA fused with a flagellin enhances cross protective immunity against *Streptococcus pneumoniae* infection in mice. *Vaccine* 2011;29:5731–5739.

- Ogunniyi AD, Grabowicz M, Briles DE, Cook J, Paton JC: Development of a vaccine against invasive pneumococcal disease based on combinations of virulence proteins of *Streptococcus pneumoniae*. *Infect Immun* 2007;75:350–357.
- Ogunniyi AD, Woodrow MC, Poolman JT, Paton JC: Protection against *Streptococcus pneumoniae* elicited by immunization with pneumolysin and CbpA. *Infect Immun* 2001;69:5997–6003.
- Oliveira MLS, Areas APM, Campos IB, et al: Induction of systemic and mucosal immune response and decrease in *Streptococcus pneumoniae* colonization by nasal inoculation of mice with recombinant lactic acid bacteria expressing pneumococcal surface antigen A. *Microbe Infect* 2006;8:1016–1024.
- Oloo E, Yethan JA, Ochs MM, Carpick B, Oomen R: Structure guided antigen engineering yields pneumolysin mutants suitable for vaccination against pneumococcal disease. *J Biol Chem* 2011;286:12133–12140.
- Overweg K, Kerr A, Sluiter M, et al: The putative proteinase maturation protein A of *Streptococcus pneumoniae* is a conserved surface protein with potential to elicit protective immune responses. *Infect Immun* 2000;68:4180–4188.
- Park IH, Pritchard DG, Cartee R, et al: Discovery of a new capsular serotype (6C) within serogroup 6 of *Streptococcus pneumoniae*. *J Clin Microbiol* 2007;45:1225–1233.
- Pouwels PH, Leer RJ, Shaw M, et al: Lactic acid bacteria as antigen delivery vehicles for oral immunization purposes. *Int J Food Microbiol* 1998;41:155–167.
- Rappuoli R: Isolation and characterization of *Corynebacterium diphtheriae* nontandem double lysogens producing CRM 197. *Appl Environ Microbiol* 1983;46:560–564.
- Roche AM, King SJ, Weiser JN: Live attenuated *Streptococcus pneumoniae* strains induce serotype independent mucosal and systemic protection in mice. *Infect Immun* 2007;75:2469–2475.
- Roche H, Ren B, McDaniel LS, Hakansson A, Briles DE: Relative roles of genetic background and variation in PspA in the ability of antibodies to PspA to protect against capsular type 3 and 4 strains of *Streptococcus pneumoniae*. *Infect Immun* 2003;71:4498–4505.
- Romero-Steiner S, Pilshvili T, Sampson JS, et al: Inhibition of pneumococcal adherence to human nasopharyngeal epithelial cells by anti-PsaA antibodies. *Clin Diagn Lab Immunol* 2003;10:246–251.
- Rosch JW, Iverson AR, Humann J, et al: A live attenuated pneumococcal vaccine elicits CD4+ T cell dependent class switching and provides serotype independent protection against otitis media. *EMBO Mol Med* 2014;6:141–154.
- Rosenow C, Ryan P, Weiser JN, et al: Contribution of novel choline binding proteins to adherence, colonization and immunogenicity of *Streptococcus pneumoniae*. *Mol Microbiol* 1997;25:819–829.
- Sheng-De J, Kim Y-M, Kang H-K, Jung S-J, Kim D: Optimization of capsular polysaccharide production by *Streptococcus pneumoniae* type 3. *J Microbiol Biotechnol* 2009;19:1374–1378.
- Stefan A, Conti M, Rubboli D, Ravagli L, Presta E, Hockoeppel A: Overexpression and purification of the recombinant diphtheria toxin variant CRM197 in *Escherichia coli*. *J Biotechnol* 2011;156:245–252.
- Talkington DF, Brown BG, Thrapp JA, Koenig A, Russell H: Protection of mice against fatal pneumococcal challenge by immunization with pneumococcal surface adhesin A (PsaA). *Microb Pathog* 1996;21:17–22.
- Terpe K: Overview of bacterial expression systems for heterologous protein production: from molecular and biochemical fundamentals to commercial systems. *Appl Microbiol Biotechnol* 2006;72:211–222.
- Terra VS, Mills DC, Yates LE, Aboulhadid S, Cucui J, Wren BW: Recent developments in bacterial protein glycan coupling technology and glycoconjugate vaccine design. *J Med Microbiol* 2012;61:919–926.
- Vintini E, Villena J, Alvarez S, Medina M: Administration of a probiotic associated with nasal vaccination with inactivated *Lactococcus lactis*-PppA induces effective protection against pneumococcal infection in young mice. *Clin Exp Immunol* 2009;159:351–362.
- Wells JM, Robinson K, Chamberlain LM, Schofield KM, Le Page RWF: Lactic acid bacteria as vaccine delivery vehicles. *Antonie Van Leeuwenhoek* 1996;70:317–330.
- Whaley MJ, Sampson JS, Johnson SE, et al: Concomitant administration of recombinant PsaA and PCV7 reduces *Streptococcus pneumoniae* serotype 19A colonization in a murine model. *Vaccine* 2010;28:3071–3075.
- WHO: Report of a WHO consultation on medicinal and other products in relation to human and animal transmissible spongiform encephalopathies. Geneva, WHO, 2003. <http://www.who.int/biologicals/publications/en>.
- WHO: Recommendations for the production and control of pneumococcal conjugate vaccines. WHO Tech Rep Ser 2005;927:66–86.
- WHO: Target product profile (TPP) for the advanced market commitment (AMC) for pneumococcal conjugate vaccines. Geneva, WHO, 2008.
- WHO: Pneumococcal vaccines, WHO position paper. *Wkly Epidemiol Rec* 2012;87:129–144.
- Wizemann TM, Heinrichs JH, Adamou JE, et al: Use of a whole genome approach to identify vaccine molecules affording protection against *Streptococcus pneumoniae* infection. *Infect Immun* 2001;69:1593–1598.
- Wu K, Zhang X, Shi J, et al: Immunization with a combination of three pneumococcal proteins confers additive and broad protection against *Streptococcus pneumoniae* infections in mice. *Infect Immun* 2010;78:1276–1283.
- Xin W, Li Y, Mo H, Ronald KL, Curtiss R III: PspA family fusion proteins delivered by attenuated *Salmonella enterica* serovar Typhimurium extend and enhance protection against *Streptococcus pneumoniae*. *Infect Immun* 2009;77:4518–4528.
- Xu J, Dai W, Wang Z, Chen B, Li Z, Fan X: Intranasal vaccination with chitosan-DNA nanoparticles expressing pneumococcal surface antigen A protects mice against nasopharyngeal colonization by *Streptococcus pneumoniae*. *Clin Vaccine Immunol* 2011;18:75–81.
- Yin J, Li G, Ren X, Herrler G: Select what you need: a comparative evaluation of the advantages and limitations of frequently used expression systems for foreign genes. *J Biotechnol* 2007;127:335–347.