Streptococcus pneumoniae virulence factors and their clinical impact: an update

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The morbidity and mortality rates associated with *Streptococcus pneumoniae* remain very high worldwide. The virulence of this bacterium is largely dependent on its polysaccharide capsule, which is quite heterogeneous and represents a serious obstacle for designing effective vaccines. However, it has been demonstrated that numerous protein virulence factors are involved in the pathogenesis of pneumococcal disease. An important related finding from experimental animal models is that non-capsulated strains of pneumococci are protective against capsulated ones. Hence, new vaccine designs are focused on the surface proteins (e. g., PspA and PspC) and on the cytolysin, pneumolysin. Moreover, several virulence factors have potential value for pneumococcal diagnosis by urinalysis. In this paper, we review the virulence factors involved in bacteria-host interactions, and the new developments in vaccines and diagnostic methods.

***Key words: Streptococcus pneumoniae*. Virulence factors. Vaccines. Diagnosis.**

Una revisión actualizada de los factores de virulencia de *Streptococcus pneumoniae* y su impacto clínico

Las tasas de morbimortalidad por *Streptococcus pneumoniae* permanecen muy elevadas en todo el mundo. La cápsula polisacarídica es esencial para la virulencia y, por su heterogeneidad, es un serio obstáculo en la generación de una vacuna más eficaz. Sin embargo, se ha demostrado que múltiples factores de virulencia proteicos están implicados en la patogénesis de la enfermedad neumocócica. Un importante hallazgo es el hecho de que cepas no capsuladas de neumococo ofrezcan protección frente a cepas capsuladas, en modelos animales de experimentación. Por ello, el diseño de nuevas vacunas se ha centrado en proteínas de superficie, como PspA y PspC, y en la citolisina neumolisina. Además, varios factores de virulencia tienen valor potencial para el diagnóstico del neumococo en muestras de orina. En este trabajo, revisamos los factores de virulencia implicados en la interacción bacteria-huésped, y en el desarrollo de nuevas vacunas y métodos de diagnóstico.

***Palabras clave: Streptococcus pneumoniae*. Factores de virulencia. Vacunas. Diagnóstico.**

Introduction

In humans, *Streptococcus pneumoniae* typically colonizes the nasopharynx asymptomatically[1,2]. The innate and adaptive immune system generally prevents colonization from progressing to disease. However, alterations of the host-pathogen homeostasis is associated with life-threatening, invasive diseases such as meningitis, septicaemia, and pneumonia[3]. Pneumococcus is also the leading cause of acute otitis media and sinusitis. Pneumococcal infections continue to be highly prevalent all over the world. Pneumococcus-related morbidity and mortality remains high, particularly in developing countries[4]. In Spain, the incidence of invasive pneumococcal infections in children $\leq$ 2 years was 90 in 100,000[2]. Illness rates increase in the elderly and in patients with predisposing factors, particularly AIDS[4]. Although resistance to antibiotics is a problem worldwide[5-7], there is some evidence that it can be reduced by decreasing the use of these agents[8]. The 23-valent polysaccharide vaccine is scantly immunogenic in high-risk groups and in children under the age of two. The 7-valent conjugate pneumococcal vaccine is more effective in this latter group. In developing countries, although the efficacy vaccination is lower in HIV-infected when compared with uninfected children, a substantial proportion of children will be protected[4]. New 9- and 11-valent conjugate vaccines that provide more optimal serotype coverage are currently undergoing clinical trials[1]. At present, conjugate vaccines are too expensive for developing countries. The fact that standard vaccination is incapable of changing the frequency of carriage of drug-resistant strains is of concern; furthermore, the initially dominant vaccine serotypes can be replaced by serotypes not covered by the vaccine[9]. Higher rates of *S. aureus*-related acute otitis media have been reported in children following vaccination[10]. For all these reasons, the development of new and improved diagnostic tools and therapies to combat pneumococcal disease are necessary.

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TABLE 1. **Virulence factors of *S. pneumoniae* involved in vaccine development**

Virulence factor	Bacterial function	Mechanism of virulence	Protection[a]	Comments	References
Capsule	Isolation from external medium	Inhibition of phagocytosis	High[b]	Switching serotype. Phenotypic change	22, 23
C-polysaccharide and lipoteichoic acids	Binding of surface proteins	Proinflammatory (?)	Low	Number of acyl groups is associated with species-specific responses	24, 25
Peptidoglycan	Protection of osmotic pressure	Macrophage activation. Cytotoxic	ND	Nod 1/Nod 2 are sensors of unique muropeptides	26, 27
PspA	Stabilization of capsular charge	Inhibition of complement	Good	Clades and families	28, 29, 30, 31
PspC	Adhesion, binding pIgR, secretory IgA, C3, and factor H	Inhibition of complement	Good	Also known as SpsA, CbpA, and Hic	32
FBA and GAPDH	Glycolytic enzymes	Immunogenic in children	Good	Immunoproteomics	33
LytA	Cell wall lysis and growth	Proinflammatory (?)	Low	Allolysis	31, 34
PsaA	Mn^{2+} transporter	Inhibition of complement	Low	Good protection in otitis media	30, 35
PiuA and PiaA	Iron uptake ABC transporters	Mutants had reduced virulence	Good	Protection against intraperitoneal infection	36
PpmA	Peptidyl-prolyl cis/trans isomerase	Mutants *ppmA*-deficient reduced virulence	Low	Controversial accessibility of antibodies	30, 37
Hyl	Spreading	Mutants had no decrease in virulence	None	Inhibitors could be useful pharmacological tools	31
NanA	Adhesion. Spreading	Mutants had no decrease in virulence	Very low	Good protection in otitis media	31, 38
PLY	Cytolysin. Spreading	Cytotoxic. Proinflammatory	Good	Cytoplasmatic	31, 32, 39

[a]Protection in animal models.
[b]Protection with conjugate-polysaccharide in humans.
Nod1 and Nod 2: nucleotide-binding oligomerization domain; PspA: pneumococcal surface protein A; PspC: pneumococcal surface protein C; pIgR: polymeric immunoglobulin receptor; FBA: fructose-bisphosphate aldolase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; LytA: autolysin; PsaA: pneumococcal surface adhesion A; PiuA and PiaA: pneumococcal iron uptake ABC transporter; PpmA: Putative proteinase maturation protein A; Hyl: hyaluronidase; NanA: neuraminidase; PLY: pneumolysin; ND: not determined.

Pathogenicity and new vaccine development

The polysaccharide capsule has been considered the primary virulence factor of *S. pneumoniae* and is a major determinant in antibody accessibility to surface antigens. The pneumococcal capsule has a modular structure which facilitates the exchange of specific genes between serotypes. The quorum-sensing system, which is a chemically-mediated alert, exist in many bacteria and help them to monitor their population densities, genetic transformation, and regulate several cellular functions. Inhibiting this cell-to-cell communication may provide a means of treating pneumococcal infections[11]. The phenomenon of capsular switching is seen in capsular types isolated from invasive disease, as well as in serogroups carried in the human nasopharynx[12]. The potential replacement of most common serotypes is of great clinical importance (especially when it takes place between antibiotic-resistant strains) and has implications for long-term efficacy of conjugate pneumococcal vaccines. Each serotype typically includes a number of genetically divergent clones with a different invasive potential[13,14]. Depending on the site of infection the combination of capsule type and genetic background is important in determining virulence[15]. The pneumococcal intra-strain phenotypic variants (opaque and transparent) are evident in colonies growing in solid transparent media –as TSA–, and are associated with both virulence and capsule expression. The transparent phenotype has less capsular polysaccharide, which exposes adhesive molecules; hence, the pathogen is able to bind closely to epithelial cells[16]. In contrast, the opaque phenotype is more virulent and common in systemic infections. Both phenotypic variants have recently been detected in nasal mucosal tissues. The opaque pneumococci probably penetrate into the nasal tissues, creating a reservoir that is not affected by mucus flow or by competition from normal nasal flora and, over time, may repopulate the nasal surface[17]. In addition to the capsule, interactions between bacteria and host involve extracellular and intracellular virulence factors that are expressed by the pneumococci[18]. Current research is focused on developing vaccines based on protein antigens common to all pneumococcal types[1,19-21] (table 1). Pneu-

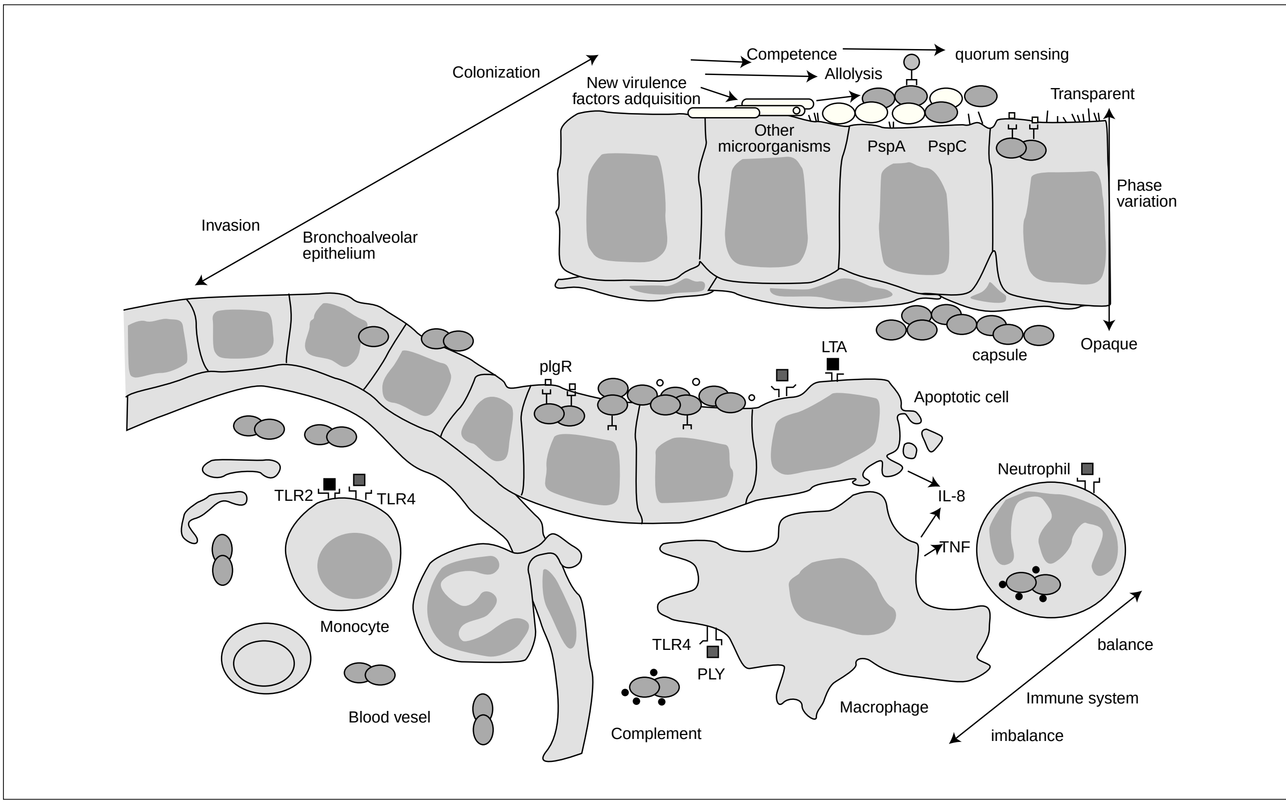

Figure 1. Model of pneumococcal invasion into bronchoalveolar epithelium and inflammatory changes induced by virulence factors.

mococcal surface protein A (PspA) displays high-quality protection in animal models[29-31] and is immunogenic in humans[28]. This protein has a high polymorphism attributable to immunological selection because it is readily accessible to antibodies[30]. In contrast, pneumolysin (PLY) is a highly conserved antigen capable of stimulating protective immunity and is an excellent vaccine candidate[40]. It is likely that any future vaccine will turn out to be a combination of pneumococcal antigens generated by recombinant proteins that display the highest protective immunity and are common to all pneumococcal strains.

Carriage and immunity

The colonization of mucosal surfaces in the human respiratory tract is a dynamic process in which bacteria are acquired, eliminated, and reacquired many times over the course of a lifetime[13]. In the nasopharynx, genetic exchange takes place by means of processes such as transformation with foreign DNA, bacterial intra- and interspecies conjugation, and phage transduction[22]. In fact, the replacement of strains susceptible to antimicrobial agents by resistant ones is considered to occur during carriage and fostered by widespread and excessive use of antibiotics. Asymptomatic carriers comprise the reservoir of *S. pneumoniae* in humans; consequently, carriage rates must be lowered and by that, the incidence of pneumococcal infections should be decreased. Natural immunity to *S. pneumoniae* is thought to be induced by exposure to pneumococci or cross-reactive antigens and is initiated upon recognition of conserved pathogen-associated molecular patterns by various host cells expressing pattern recognition receptors[41]. Recognition of bacterial components by the innate immune system, more specifically the interaction of bacterial components with Toll-like receptors (TLRs) has been recognized as an effective method by which to protect the host against several pathogens[42]. TLRs are a family of 12 types of transmembrane proteins that recognize pathogens and are expressed on various immune cells including macrophages, dendritic cells, B cells, specific types of T cells, and even on nonimmune cells such as fibroblasts and epithelial cells[42]. Phagocyte activation by inflammatory cytokines and apoptosis of infected phagocytes and other cells play an important role in clearing the pathogen (fig. 1). Distinct TLRs may differentially regulate innate versus adaptive immunity to intact *S. pneumoniae*. PLY is involved in the innate immune response to pneumococci, and triggers their proinflammatory and proapoptotic properties by interacting with TLR4[42]. The humoral response to some virulence factors such as PspA, PLY, and pneumococcal surface adhesin A (PsaA) has been studied extensively in asymptomatic carriers[43-45]. Recent findings have demonstrated differential and specific mucosal, humoral, and T helper cell cytokine responses to PsaA, PspA, pneumococcal surface protein C (PspC), and PLY during pneumococcal carriage[46]. In addition to systemic immunity, mucosal immunity may also play an important role in local protection against pneumococcal carriage and in preventing invasive infection. Mucosal immunization of mice with PsaA is known to be highly protective against pneumococcal carriage[47]. On the

TABLE 2. **Diagnostics, identification, and typing methods for *S. pneumoniae* that involve virulence factors**

Tools	Target	Performance	Comments	Reference
Diagnostic				
Binax NOW[a]	C-polysaccharide	86% sensitivity, 94% specificity in urine	False-positive with upper respiratory tract colonization	Binax, Inc.
ELISA assay	PLY	100% sensitivity in urine	False-positive with *S. suis*	54
	Capsular polysaccharide	90% sensitivity, 99% specificity in urine	Only detects 13 serotypes	55
Immunosensor	PLY	Simpler, cheaper, and faster than ELISA	Doesn't improve chemiluminescent ELISA	56
PCR	*lytA*	78% sensitivity in pleural fluid	False-positive with upper respiratory tract colonization	57
Nested-PCR	*ply*	78% sensitivity, 93% specificity in pleural fluid	4% false-positive rate	58
Real-time PCR	*ply*	90% sensitivity, 80% specificity in sputum	13% false-negative rate	59
		96% sensitivity in nasopharyngeal samples	Potential false-positive with upper respiratory tract colonization	60
Identification				
Binax NOW	C-polysaccharide	100% sensitivity, 81% specificity in BCB	False-positive due to the *S. mitis* group	53
Agglutination	PLY	95% sensitivity, 100% specificity. Easy, fast, cost-effective	False-negatives correspond to low PLY-producing isolates	51
ELISA assay	PLY	Sensitivity 100%	No false-positive results. Potential false-negatives	61
LAMP	*lytA*	Simple equipment, visible results with the naked eye	Differentiates *S. pneumoniae* vs. *S. mitis* and *S. oralis*	62
Typing				
PCR-RFLP	Capsular polysaccharide locus	Serotype/serogroup identification. Fully portable, cost-effective	Laboratory with sufficient molecular experience	63
Multiplex PCR	Capsular polysaccharide locus	Fast and far more cost-effective	Only determines seven serotypes	64
MCT	Capsular polysaccharide locus	Serotype/serogroup identification. Specific, objective	Genechip microarrays would make more practical for routine use	65
MLST + *ply* sequencing	*aroE, gdh, gki, rec, spi, xpt, ddl, ply*	Serotypeable/nonserotypeable identification	*ply* alleles different. Useful to resolve difficult cases	66

[a]Used in clinical samples in diagnostic microbiological laboratories.
PLY: pneumolysin; *lytA:* autolysin gene; *ply:* pneumolysin gene; BCB: blood culture bottles; LAMP: Loop-mediated isothermal amplification; RFLP: Restriction fragment length polymorphism; MCT: molecular capsular typing; MLST: multilocus sequence typing; *aroE:* shikimate dehydrogenase; *gdh:* glucose-6-phosphate dehydrogenase; *gki:* glucose kinase; *recP:* transketolase; *spi:* signal peptidase I; *xpt:* xanthine phosphoribosyltransferase; *ddl:* D-alanine-D-alanine ligase.

other hand, acquired immunity to pneumococcus has long been assumed to depend on the presence of anticapsular antibodies. However, the age-specific incidence of pneumococcal disease in humans declines simultaneously with and parallel to a wide range of serotypes, long before natural acquisition of anticapsular antibodies, which suggests a common and probably capsular serotype-independent mechanism of protection[48]. Additionally, intranasal administration of unencapsulated pneumococci whole-cell vaccine in mice prevents colonization by pneumococci of various capsular serotypes, supporting the possibility that other components of the immune response, independently of anticapsular antibodies, play an important role in this process. In fact, recent studies reveal that protection against pneumococcal colonization is CD4T cell-dependent and is possibly acquired independently of antibodies[49,50].

New perspectives on diagnostics, identification, and typing

S. pneumoniae is identified in clinical microbiology laboratories by colony morphology, bile solubility, and optochin sensitivity. However, a number of isolates shows resistance to one or both compounds, leading to misinterpretation in their characterization. On the other hand, the amount of nonserotypeable pneumococcal strains is low (7%) when recovering from respiratory tract, blood and usually sterile sites, but this percentage could reach 20% in conjunctival and nasopharyngeal exudates[51]. In these conditions, DNA techniques are needed for accurate species identification. In 1999, a simple test based on PLY immunodetection for fast, reliable species identification with good sensitivity and specificity was developed[51]. Pneumococcal serotyping by capsular swelling (Quellung reaction) is labour intensive and requires a certain degree of expertise; thus, its use has been restricted to specialized reference or research laboratories. A ready-to-perform latex agglutination kit (Pneumotest-Latex, Statens Serum Institut, Copenhagen, Denmark) is available for more general use[52].

Low recovery rate of *S. pneumoniae* from clinical samples could be difficult if patient received previous antimicrobial therapy. Therefore, it is often hard to correlate pneumococci in non-invasive clinical samples (especially from the upper respiratory tract) with clinical infection. Detection of C-polysaccharide in urine samples and cerebrospinal fluid with Binax NOW *S. pneumoniae* test (Binax, Inc., Portland; Maine) represents a major step forward in the diagnosis of pneumococcal infections[53], avoiding the need to obtain representative clinical samples by means invasive procedures. However, C-polysaccharide can be found in the urine of healthy children carriers as well as in patients recovering from pneumococcal infections. Several tools for microbial diagnostics, identification, and typing are now based on the detection of virulence factors (table 2). Recent observations have demonstrated the presence of PLY-derived peptides in the urine of patients with pneumococcal pneumonia confirmed by blood culture[54]. This finding opens a new line of research to determine how PLY can be used as a diagnostic and prognostic marker. In general, cross-reactions with usual nasopharyngeal flora have been a serious handicap, in particular with viridans group *streptococci*. In fact, *Streptococcus mitis* may cross-react with the pneumococcal C polysaccharide antigen[53] and pneumococcal virulence genes, such as *lyt* and *ply* have been detected in some members of the viridans group. New techniques have not yet reached laboratories at a scale suitable for assisting clinicians because of the time and skill needed to perform the assays, although progress is being made in enhancing performance and convenience.

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