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Recent Advances in Pathogenic *Streptococcus* Vaccine Development

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Abstract

Streptococcus pneumoniae (Spn) and *Streptococcus pyogenes* (Spy) cause many invasive and noninvasive diseases responsible for high morbidity and mortality worldwide. Safe, efficacious and affordable vaccines could have a significant, positive impact on the global infectious disease burden. Since the implementation of pneumococcal vaccine in the 1980s, the incidence of Spn infection has decreased significantly. Still so, these currently used multivalent polysaccharides and conjugated pneumococcal vaccines have some limitations. For Spy, there are even no vaccines available yet. There is an urgent need of new vaccines against Spn and Spy. Encouragingly, with the hard work of many investigators worldwide, a number of new vaccines candidates are developed with promising results. Of them, many have already entered the clinical trial stage. This review will describe the current status of Spn and Spy vaccine development, with particular focus on protein-based strategy.

Introduction

Human beings can be infected by many streptococcal species that are called pathogenic streptococci. Among them, *Streptococcus pneumoniae* (Spn) and *Streptococcus pyogenes* (also known as Group A streptococci, GAS) are major causes of many invasive and noninvasive diseases responsible for high morbidity and mortality worldwide. For these infectious diseases, safe and effective vaccines are the best way to prevent infections. In this review, first we would like to briefly summarize the weaknesses of current licensed serotype-specific Spn vaccines, then discuss recent progress in the development of pneumococcal and GAS vaccines, and share our opinions on vaccinal strategies to overcome Spn- and GAS-caused diseases in different situations.

1. Spn Vaccine

As a Gram-positive diplococcus and a part of commensal flora in the upper respiratory tract in human, Spn opportunistically results in various invasive and non-invasive diseases including meningitis, bacteremia, pneumonia, otitis media, sinusitis, etc(Centers for Disease Control and Prevention, 2015). Known to be the leading cause of bacterial pneumonia in children under the age of 5 (O'Brien et al., 2009; WHO, 2016), Spn is also a huge disease burden of the elderly mainly by causing community-acquired pneumonia (Drijkoningen and Rohde, 2014), posing a great threat on the health of humans (Bridy-Pappas et al., 2005) and raising a global economic concern (Boccalini et al., 2017; De Graeve and Beutels, 2004; Huang et al., 2011; Porchia et al., 2017). A World Health Organization (WHO) report showed that pneumonia accounts for 16% of all deaths of children under 5 years old, killing 920,136 children in 2015(WHO, 2016). Moreover, drug-resistance has been observed in Spn over decades worldwide (Cherazard et al.,

2017). Recently, WHO released a list of bacteria for which new antibiotics are urgently needed, and Spn made its mark (WHO, 2017). Since vaccines (including pneumococcal conjugate vaccines, PCVs) have a good reputation for solving the antimicrobial resistance problem (Laxminarayan et al., 2013; Laxminarayan et al., 2016; Lipsitch and Siber, 2016), the needs of Spn vaccines are strongly addressed.

Given that the encapsulated, instead of non-capsulated, pneumococcus accounts for Spn-related diseases (Centers for Disease Control and Prevention, 2015), scientists focused on pneumococcal capsule polysaccharides (CPs), with which Spn is typed due to their immunological distinction, providing the basis of existing pneumococcal vaccines (Bogaert et al., 2004b; Smit et al., 1977)(Figure 1).

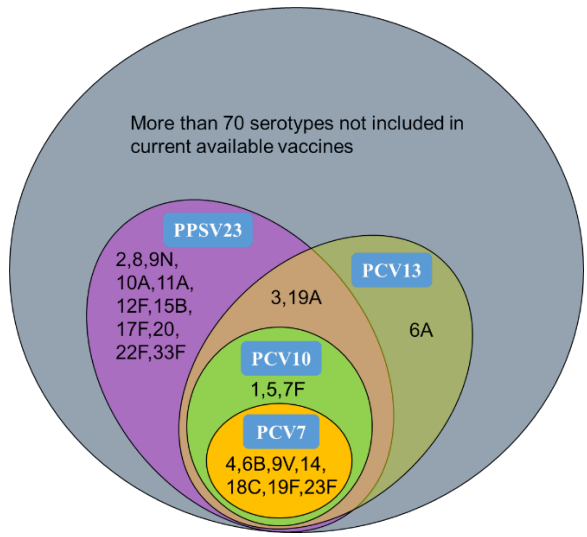

Figure 1. Serotypes contained in existing Spn vaccines. Among the over 90 serotypes of Spn, 24 were chosen to develop pneumococcal vaccines including one polysaccharide type (PPSV23) and three polysaccharide conjugate types (PCV7, PCV10 and PCV13) (WHO Publication, 2012).

Albeit with their great success in preventing serotype covered Spn diseases, serotype-based vaccines have several limitations, addressing the needs of improvement on their designing, research and development of alternative strategies such as protein-based vaccine.

1.1 Limitations of existing Spn vaccines

The limitations of current available pneumococcal vaccines were summarized in Figure 2, and each was briefly introduced as following.

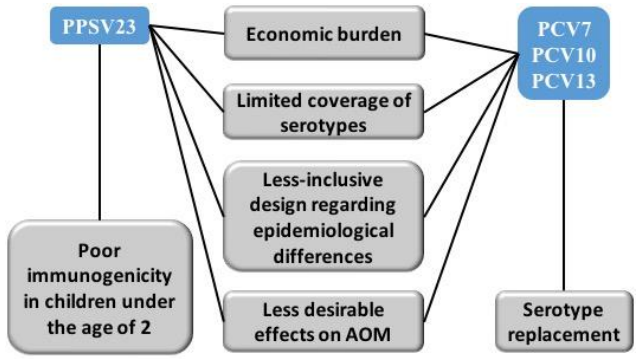

Figure 2. Limitations of current available pneumococcal vaccines.

1.1.1 Poor immunogenicity in children younger than 2

Licensed in 1983, PPSV23, containing 23 serotypes responsible for 85-90% invasive pneumococcal diseases (IPD) in the U.S, served well as being effective to protect against 56% diseases caused by serotype-covered Spn (Shapiro et al., 1991). However, this vaccine failed to elicit an immunogenic response in children under the age of 2, as it requires a T-cell-independent immunity of B cells (Heilmann, 1990), which is lacking in those younger than 2. As a result, vaccines with capsular polysaccharide conjugated to a protein carrier, diphtheria toxin (CRM197), were developed.

1.1.2 Limited coverage of serotypes, less inclusive design in terms of epidemiological differences and less economically friendly

This is an inherent disadvantage of a CPs-based vaccine, as its protection against pneumococcal infection is serotype-specific, which means that among the almost 100 types CPs of Spn, those included in current vaccines covered less than 1/3 out of all serotypes potentially leading to diseases (Figure 1). In addition, the distribution of prevalent strains varied epidemiologically in region, age and medical condition (Hausdorff et al., 2005) while it is technically irrational to wrap all CPs antigens in a single dose. Combined with the intrinsic need of increasing numbers of serotypes by PCVs, current vaccine manufacturing production made cost an issue of concern (Hanage, 2008; Josefsberg and Buckland, 2012; Ray, 2002), especially for developing countries.

1.1.3 Serotype replacement and poor performance on AOM-controlling

Serotype replacement, a phenomenon that vaccine-excluded serotypes (including drug-resistant strains) become more dominant than the pre-vaccination period, emerged after the administration of PCVs (Kaur et al., 2016; Miller et al., 2011), and is primarily attributed by reducing the carriage of strains which the serotypes were included in the vaccines. The interference on the asymptomatic carriage of respiratory tract commensal could also influence human health by breaking the equilibrium of the microbiota (Man et al., 2017). Moreover, the administration of CPs vaccines shows an undesirable effect on Spn-causing acute otitis media (AOM) (Jambo et al., 2010), which is partially thought to be the outcome of the replacement by other organisms such as *Staphylococcus aureus* (SA) (Fortanier et al., 2014).

1.2 Alternative strategies for the research and development of pneumococcal vaccine

Designing an effective pneumococcal vaccine largely depends on a better understanding of structures of Spn and how each component works as a whole during the interplay within the host (Kadioglu et al., 2008). In particular, protein-based pneumococcal vaccines have long been receiving attention (Daniels et al., 2016; Pichichero et al., 2016; Principi and Esposito, 2018) and are thought to be the most promising tactics with making up for the shortfalls of existing vaccines. In addition, some adjuvants such as pFL (a dendritic cell-targeting DNA-based adjuvant) showed potency in mucosal immunity enhancement, providing a new strategy to boost vaccinal function (Kataoka et al., 2017). Moreover, whole cell vaccines (WCVs), live attenuated or inactivated, have also been used as an alternative strategy by virtue of manufacturing convenience (Minor, 2015) and presenting a great number of antigens at once (Moffitt and Malley, 2011). Finally, modifications or adaptions of current CPs conjugate vaccines have been made by using conserved pneumococcal proteins as carriers (Table 2) to achieve WHO/GAVI target product profile (Moffitt and Malley, 2011).

1.2.1 Pneumococcal protein-based vaccine

1.2.1.1 Promising pneumococcal proteins utilized in vaccinal research and development

Due to their pathogenesis and antigenic characteristics, over 10 proteins (Table 1) of pneumococcus have been employed in many alternative strategies on vaccinal research and development (Rigden et al., 2003).

(1) Choline-binding proteins (CBPs)

Phosphorylcholine (ChoP), a sort of bacterial adhesin, constitutes lipoteichoic acids and cell wall teichoic acids of Spn (Cundell et al., 1995), and is strongly associated with colonization in the upper respiratory tract (Hammerschmidt, 2006). A group of proteins(Table 1), such as PspA, PspC, PcpA and LytA, being non-covalently anchored to ChoP, can bind to receptors on the epithelial cells of the host (Bergmann and Hammerschmidt, 2006).

PspA is expressed by all clinical pneumococcal isolates (Khan and Jan, 2017). By interfering the binding of C-reactive protein to phosphocholine, PspA inhibits complement-mediated opsonization (Mukerji et al., 2012), which in turn assists the immune evasion of Spn. Moreover, as a lactoferrin-binding protein, PspA can also protect Spn from killing by apolactoferrin (Shaper et al., 2004). PspC is another well-studied protein, also known as choline binding protein A (CbpA) as was found to be the major component of CBPs (Rosenow et al., 1997); or referred to as secretory pneumococcal surface protein A (SpsA) as binding to the ectodomain of polymeric immunoglobulin (Ig) receptor transporting secretory IgA on host respiratory epithelial cells, aiding to bacterial colonization and invasion (Dave et al., 2004; Zhang et al., 2000). PspC can also promote pneumococcal colonization by binding to C4b-binding protein, an inhibitor of the classical pathway (Dieudonne-Vatran et al., 2009). Moreover, PspC has been reported to inhibit C3b formation via binding to the heparin-binding FH domain of complement factor H (Cao et al., 2011; Dave et al., 2004), protecting Spn from complement-mediated opsonization (Quin et al., 2005). Existing on nearly all pathogenic pneumococci, PcpA is considered one of the potential candidate antigens for pneumococcal vaccine. To date, the ligand of PcpA is uncertain, while a study showed that CodY, a global nutritional repressor in bacteria, can activate PcpA,

favoring bacterial adaptation to nutrients, and regulating the adherence to nasopharyngeal and lung epithelial cells (Hendriksen et al., 2008; Khan et al., 2012). Additionally, the expression of this protein is found to be inversely correlated to the concentration of manganese (Manzoor et al., 2015). LytA, also referred to as autolysin, is an enzyme cleaving the pneumococcal cell wall, promoting the release of pneumolysin (Ply) and other bacterial components such as peptidoglycan and teichoic acids (Mellroth et al., 2012; van der Poll and Opal, 2009). Recently, it has also been reported to inhibit C3 convertase formation (Andre et al., 2017).

(2) ATP-binding cassette transporter

The ATP-binding cassette (ABC) transport system provides energy for binding and transporting the solute (mainly metal) through the cell membrane by ATP hydrolysis (Jedrzejas, 2001), and includes a group of proteins such as PsaA, PiaA and PiuA(Table 1).

PsaA was thought to be a kind of adhesin due to its sequence homology to other adhesins of Spn(Sampson et al., 1994), but was shown by other studies that it is a lipoprotein of an ABC transport system, with the property of divalent metal-ion-binding(Lawrence et al., 1998; McAllister et al., 2004) and manganese-transporting(Dintilhac et al., 1997), preventing from oxidative stress(Kadioglu et al., 2008; Tseng et al., 2002). PiaA (pneumococcal ion acquisition A) and PiuA (pneumococcal ion uptake A) are two lipoprotein components of Spn for ion-acquisition and uptake, which is associated with bacterial growth and virulence (Brown et al., 2001a; van der Poll and Opal, 2009). Immunization with PiaA and PiuA protected mice from systemic infection of Spn (Brown et al., 2001b). SP2108

and SP0148 are solute-binding components of ABC transport systems, specifically binding to aromatic amino acids and maltose (Paton and Ogunniyi, 2011), respectively, enhancing bacterial growth and colonization (Shelburne et al., 2008). In recent years, they have been considered as potential vaccinal candidates due to their high conservation and mucosal immunity-triggering (Paton and Ogunniyi, 2011).

(3) LPXTG motif-binding proteins

The most universal mechanism for Gram-positive bacteria to anchor a set of surface proteins is that sortase transpeptidase recognizes an amino-acid sequence, LPXTG, harbored on the surface of these proteins (including NanA, RrgA, RrgB and PrtA), and cleaves the precursor between threonine and glycine of the LPXTG motif (Kadioglu et al., 2008; Navarre and Schneewind, 1994). Most LPXTG-containing proteins are anchored by StrA (Table 1), a pneumococcal virulence factor, which has been found to play an important role in aiding bacterial colonization and pathogenesis (Paterson and Mitchell, 2006).

(4) Neuraminidase

Known for its pre-invasive property (Brittan et al., 2012), neuraminidase (NA), an enzyme expressed on all pneumococci, shows potential to be an ideal vaccine target. There are at least 3 forms of NA: NanA, NanB and NanC(Table 1), out of which NanA is the most essential and well-studied.

NanA is encoded by all strains of Spn, and contains the LPXTG motif, covalently binding to the peptidoglycan (Camara et al., 1994). By cleaving the terminal sialic acid of glycoproteins of the epithelial cells in the host respiratory tract, NanA and

NanB expose more decoy receptors for Spn to bind, enhancing the pneumococcal attachment and invasion (Kadioglu et al., 2008). Recently, NanA was found to help pneumococcus escape the complement pathway in host (Andre et al., 2017). Research on the role of NanC in the pneumococcal pathogenesis is still lacking, though the finding that NanC is more commonly seen in isolates from cerebrospinal fluid than those from carriage suggested its tissue-specificity characteristic (Pettigrew et al., 2006).

(5) Pili proteins

Pili are strongly associated with pneumococcal adherence. There are 7 pilus-encoding genes (*rrgA*, *rrgB*, *rrgC, srtB, srtC, srtD, rlrA*) on the pathogenic island. Of them, *rrgA*, *rrgB* and *rrgC* respectively encode the subunits of pneumococcal pili 1, RrgA, RrgB and RrgC (Telford et al., 2006), which are anchored on the cell surface with the help of sortase (LeMieux et al., 2006).

RrgA has been found to act as the dominant adhesive element other than RrgB (Nelson et al., 2007), also known as pilus backbone (Gentile et al., 2011). RrgB is the most abundant subunit of pili 1 (Gentile et al., 2011), and has been reported to elicit a host inflammatory response (Gianfaldoni et al., 2007). Both RrgA and RrgB have been studied for vaccinal purposes for over a decade (LeMieux et al., 2006), while the structure and functional information of RrgC was rarely known until recent years after RrgC had been structurally described (Gianfaldoni et al., 2007), in which RrgC was found to bind the preformed pilus to the peptidoglycan with the catalytic activity of SrtA.

(6) Polyhistidine triad family

Polyhistidine triad (Pht) family includes a group of pneumococcal surface-expressed proteins, PhtA, PhtB, PhtD and PhtE, which are well-conserved and contain a histidine triad motif, playing an important role of the attachment of Spn to respiratory epithelial cells (Plumptre et al., 2013). Moreover, Pht proteins are considered to regulate metal homeostasis, especially the zinc storage, providing ion for pneumococcal invasion during early stages (Godfroid et al., 2011). Of the four proteins, PhtD and PhtE are mostly studied due to their prevalence on 97~100% Spn strains (Khan and Pichichero, 2012; Rioux et al., 2011).

(7) Cholesterol-dependent cytolysin family

Cholesterol-dependent cytolysin (CDC) is a family consisting of a group of proteins, such as pneumolysin (Ply), that lead to cell death by forming pores on cell membrane (Tilley et al., 2005). Ply, a transmembrane pore-forming oligomer, is a crucial pneumococcal virulence factor and expressed by all clinical isolates of Spn (Vernatter and Pirofski, 2013). Pneumococcal Ply can damage the host respiratory cells by binding to cholesterol-containing membranes, forming ring-shaped pores (van Pee et al., 2016), reinforcing the invasion of Spn. Within the hosts, Ply is able to trigger an inflammatory response through interaction with Toll-like receptors (TLRs) and activating NLRP3 inflammasomes (Vernatter and Pirofski, 2013), contributing to pneumococcal pathogenesis, including host-to-host transmission (Zafar et al., 2017). As the original form of Ply is highly toxic, it has been genetically modified to keep the property of immunogenicity instead of antigenicity (dPly). The immunization with dPly on mice successfully induced serotype-independent protection against pneumococcal diseases and colonization (Alexander et al., 1994).

Table 1. Pathogenesis-related proteins of Spn-potential targets for protein-based vaccine

Location	Protein family	Representative(s)
Cell-surface	Choline-binding	PspA
		PspC
		PcpA
		LytA
		LytB/C
	ATP-binding cassette (ABC) transporter	PsaA
		PiaA
		PiuA
		SP2108
		SP0148
	LPXTG motif-binding	StrA
	Neuraminidases	NanA
		NanB
		NanC
	Pili	RrgA
		RrgB
		RrgC
	Polyhistidine triad family	PhtA
		PhtB
		PhtD
		PhtE
From cell matrix to outside cell	Cholesterol-dependent cytolysin (CDC) family	dPly (Genetically modified)

1.2.1.2 The forefront of Spn protein-based vaccines

Monovalent vaccine mainly focused on PspA. As one of the most abundant surface proteins expressing on all pneumococci, PspA becomes one of the forefront antigens of candidate vaccine since its immunization can elicit broadly protective serotype-independent serum antibodies in both humans and mice, regardless of its immunogenic variants (McCool et al., 2003; Nabors et al., 2000). Due to the low sequence homology between PspA and human cardiac myosin,

PspA is presumed to cause an autoimmune response (Ginsburg et al., 2012), whereas no evident proof has yet been found to confirm this possibility. Hence, PspA is still undergoing a series of research into animal experiments and clinical trials (Converso et al., 2017b; Entwisle et al., 2017; Goulart et al., 2017) with an avoidance of the involvement of the homology sequence to human cardiac myosin. A recombinant PspA has been tested in adults in a phase I clinical trial and has proved to be both safe and highly immunogenic (Briles et al., 2000). Thus, cross-reactivity with human should be tested and filtered for a rationale vaccine development.

In order to increase antibody-accessibility, vaccines involving multiple components have been springing out for decades (Moffitt and Malley, 2011) (Table 2) and have been well-reviewed elsewhere (Darrieux et al., 2015; Kim et al., 2017b; Odutola et al., 2017; Pichichero, 2017; Pichichero et al., 2016; Singh and Dutta, 2017). Among them, dPly, PhtD, PspA and PsaA are highly employed. Here we introduce two protein-based vaccines lately passed the clinical trial this year. ImmunoBiology Ltd. completed the phase I clinical trial on adults with PnuBioVax, a multi-valent recombinant vaccine, containing PspA, RrgA, RrgB, PsaA and dPly (Entwisle et al., 2017) (Table 2). This randomized clinical trial proved the safety and immunogenicity of PnuBioVax in all dosage levels, and more well-tolerant than its first investigation in humans where severe injection site reactions occurred. Presently, PnuBioVax is awaiting further tests on the pediatric population of whom the natural immunity is immature. In the same year, GSK tested the efficacy of PhiD-CV/dPly/PhtD-30, based on its well-tolerance showed in the first part study, on infants for a phase II clinical trial (Odutola et al., 2017) (Table 2). In this randomized, controlled, observer-blind trial, the nasopharyngeal

carriage of Spn was particularly investigated, and the results showed that antibodies against PhtD and dPly were elicited by PhiD-CV/dPly/PhtD-30 without interfering the prevalence of pneumococcal carriage in the nasopharynx.

There are numerous recently developed protein-based vaccines undergoing a series of preclinical research. Of them, quite a few focused on mucosal immunity, which we particularly discussed later. Here we introduce research on adjuvant for pneumococcal vaccine development conducted by *Cibelly Goulart* et al. earlier this year, who used BCG as an adjuvant to deliver an infusion protein, rPspA-PdT, containing a fragment of PspA and a genetically detoxified Ply. In this prime-boost mice study, the administration of rBCG PspA-PdT followed by a boost of rPspA-PdT provided favorable effects on anti-lethal pneumococcal disease, reinforcement of complement deposition to Spn and inhibition of cytolysis by Ply (Goulart et al., 2017).

There are at least two facts that prompt scientists to look for a vaccine that boosting a strong mucosal immune response, i) Spn vaccines on the market are less effective against AOM which is the most common Spn-caused disease in children under the age of 5; ii) colonization on the mucosal surface is the primary step for Spn invasion. Since mucosal defense is critical in preventing AOM (Bergenfelz and Hakansson, 2017), recent years of experimental studies on pneumococcal vaccines illustrated a mucosal immunity-oriented trend, with the employment of adjuvant-assistance, pneumococcal surface proteins, whole cell mutants, etc. PspA, PhtD, PcpA, PlyD1 and PotD have been selected to develop vaccines focusing on mucosal immunity (Converso et al., 2017a; Kuipers et al., 2017; Xu et al., 2017). PPrV, a trivalent protein vaccine containing PhtD, PcpA

and PlyD1, was shown to successfully elicit both serum and mucosal antibodies (IgG) that protect infant mice from pneumococcal bacteremia and AOM with the challenge of heterotypic strains (Xu et al., 2017). In another mice study, immunization with recombinant PotD can decrease the nasopharyngeal colonization by inducing immune response including production of IL-17 (Converso et al., 2017a). Additionally, mucosal immunity can be employed by other strategies. Similar to other adjuvants such as BCG, as mentioned above, dendritic cell-targeting DNA-based nasal adjuvants have shown their potential in immune response enhancement, especially for mucosal immunity promotion (Kataoka et al., 2017). Furthermore, innate and adaptive IL-17 responses in mice can be achieved by using γ-irradiated RX1 LytA/PdT and attenuated SPY1 strain of Spn, respectively (Babb et al., 2016; Gao et al., 2016).

1.2.2 WCVs

There are at least two prominent advantages of using whole cells of the specific organism as vaccine: cost-effectiveness and broad-coverage of antigens (Minor, 2015). WCVs are classified as the live attenuated and the inactivated. To date, two types of WCVs for Spn have been under clinical trial stage. An avirulent *Salmonella Typhi* strain was used as a vector to delivery PspA for enhancing immune responses in mice with high efficiency (Wang et al., 2010), promising results from this study drove further investigations of its potential use in humans. A few years later, 09-RASV-Sp-01, attenuated recombinant *Salmonella Typhi* vectors (RASV) expressing PspA, was used for a phase I dose-escalation trial to test its safety and immunogenicity (Frey et al., 2013) (Table 2). In this study, three RASV vaccines synthesizing PspA-RX1 antigen were employed to immunize adults, and the results proved that all three RASV pneumococcal vaccines were

safe and well-tolerated, but with limited immunogenicity which could be a result from pre-existing immunity. Another pneumococcal WCV is an inactivated non-encapsulated whole cell vaccine, RX1 LytA/PdT, which was employed in a phase I clinical trial on adults, and showed safety, well-tolerance, and immunogenicity with high level of IgG antibodies against Ply and PspA (Alderson et al., 2014) (Table 2).

Preclinical efforts have also been made these years. *Eun-Hye Kim* et al. successfully constructed Spn *pep27* mutant (EHpep27), a live attenuated vaccine with deletion of pneumococcal virulent gene, *pep27* (Kim et al., 2012). Mice were intranasally immunized with EHpep27 at various dose levels, in which no virulent effect was detected, IgG antibody was elicited without the help of adjuvant, and serotype-independent protection against lethal pneumococcal challenge was achieved. EHpep27 was then tested for its immunogenicity and protection against non-typeable strains (Kim et al., 2016). In this study, EHpep27 showed a wide range of protection and long-lasting immunity, making itself a very potent vaccine candidate.

Table 2. Alternative strategies for research and development of pneumococcal vaccines under clinical trial

Vaccines		Institute	Status	References
Type	**Name (Components)**			
Protein	PspA	Sanofi-Pasteur	phase I complete	(Darrieux et al., 2015; Pichichero et al., 2016)
	PspA and PsaA	Sanofi-Pasteur/CDC	phase I complete	(Darrieux et al., 2015; Pichichero et al., 2016)

	PhpA and PhtB	GSK	phase II complete	(Pichichero, 2017; Pichichero et al., 2016)
	IC47 (PsaA, PcsB and StkP)	Valneva Austria GmbH/PATH	phase I complete	(Darrieux et al., 2015; Kim et al., 2017a; Pichichero et al., 2016)
	PhtD/PcpA and PlyD1	International Centre for Diarrhoeal Disease Research, Bangladesh/Sanofi-Pasteur	phase I complete	(Darrieux et al., 2015; Kim et al., 2017a; Pichichero, 2017; Pichichero et al., 2016)
	dPly/PhtD w/PHiD-CV	GSK	phase II complete	(Darrieux et al., 2015)
	PhiD-CV/dPly/PhtD-30	GSK	phase II complete	(Odutola et al., 2017; Pichichero, 2017)
	w/DTPa-HBV-IPV/Hib	GSK	phase II complete	(Darrieux et al., 2015; Pichichero et al., 2016)
	SP0148,1912,2108	Genocea	phase IIa complete	(Pichichero, 2017; Pichichero et al., 2016)
	PnuBioVax (PspA, RrgB, RrgA, PsaA and dPly)	ImmunoBiology Ltd.	phase I complete	(Entwisle et al., 2017)
CPs-protein conjugate	V114 (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F and 33F and CRM197 protein)	Merck Sharp & Dohme Corp	phase II complete	(Kim et al., 2017a)
	V114 w/Alum (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F and 33F and CRM197 protein)	Merck Sharp & Dohme Corp	phase II complete	(Kim et al., 2017a)
	PhtD/dPly w/or w/o PCV10	GSK	phase I complete	(Darrieux et al., 2015; Pichichero, 2017; Pichichero et al., 2016)
	PhtD/dPly/w/PCV10	GSK	phase II complete	(Darrieux et al., 2015; Pichichero, 2017; Pichichero et

				al., 2016)
	PHiD-CV w/PPV 23 (1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23F and non-typeable H.influenzae protein D conjugate vaccine)	GSK	phase III complete	(Pichichero et al., 2016)
	PHiD-CV (1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23F and non-typeable H.influenzae protein D conjugate vaccine)	GSK	phase III complete	(Darrieux et al., 2015)
Whole-cell	RX1 LytA/ PdT (killed non-encapsulated whole cell vaccine)	Boston Children's Hospital Instituto Butantan/PATH	phase I complete	(Darrieux et al., 2015; Kim et al., 2017a; Pichichero, 2017; Pichichero et al., 2016)
	09-RASV-Sp-01 (Attenuated Salmonella typhi expressing PspA)	Arizona State University/Biodesign Institite	phase I complete	(Darrieux et al., 2015; Kim et al., 2017a)

2. GAS Vaccine Development

Gram positive bacteria GAS, which is responsible for substantial worldwide morbidity and mortality, can cause a wide range of invasive and noninvasive diseases, including pharyngitis, impetigo, necrotizing fasciitis (NF), and immune mediated diseases such as acute rheumatic fever (ARF), rheumatic heart disease (RHD) and acute post-streptococcal glomerulonephritis (APSGN). GAS can also cause toxin-mediated diseases including scarlet fever and streptococcal toxic shock syndrome (STSS) (Sims Sanyahumbi et al., 2016). Although GAS infections can be treated by one of the cheapest and oldest antibiotics known—penicillin, the global burden of GAS disease is substantial, and most infections, especially those in severe cases, are in low- and middle-income countries (LMIC). There is an urgent need for a safe, efficacious and affordable GAS vaccine to reduce the prevalence and the sequelae caused by this organism.

2.1 Feasibility for GAS vaccine development

A clear understanding of the mechanisms of protective immunity to GAS infection and identification of immune correlates of protection are essential for the rational design of a GAS vaccine. Although accurate protection mechanisms are needed to figure out with extra efforts, a large amount of serologic data from natural history studies and pre-clinical studies suggests good feasibility for GAS vaccine development and encourages investigators throwing themselves into this field. The phenomenon that peak incidence of GAS infections occurs in childhood and declines in adulthood indicates that natural infection with GAS would lead to a protective immunity. This is because of accumulation of protective bactericidal antibodies in serum following multiple GAS infections during childhood. According to the research of Lancefield, immunity against GAS infections has been believed to be type specific (Dale et al., 2016; Lancefield, 1959). Recently, a body of evidences indicate that mucosal immunity plays important roles in the protection against GAS infection, especially in the pharyngitis cases (Chen et al., 2016; Ghaffar et al., 2016; Loh et al., 2017; Marasini et al., 2017; Marasini et al., 2016; Mortensen et al., 2017; Schulze et al., 2017). These findings suggest that systemic IgG and local IgA are involved in the protection against GAS infection and provide implications in vaccine strategies.

2.2 The challenges of GAS vaccine development

Many investigators have been working on GAS vaccine development for more than 90 years, nevertheless, no licensed vaccines are currently available to prevent GAS infection (Dale et al., 2016; Excler and Kim, 2016; Gandhi et al., 2017; Schodel et al., 2017). Vaccine development is impeded for several reasons.

2.2.1 The epidemiology of GAS is extremely complex.

As mentioned previously, the presence of type-specific antibodies were responsible for protection against the homologous serotype of GAS infection. This made serotyping to be the first method to categorized different GAS strains, also known as “M typing”. This time-consuming method has been progressively replaced by “*emm* typing” which is based on PCR and sequencing of the hypervariable region of the M gene (Beall et al., 1996). This *emm* typing method is widely used in molecular epidemiology investigation of GAS currently and through this method, 223 *emm* types have been identified to date. The *emm* type distribution represents considerable variation at both the country and global regional level. Unfavourably for vaccine development, limited data on *emm* type distribution in LIMC is available and the distribution patterns are completely different between LIMC and industrialized countries. In industrialized countries, the most common circulating types are *emm* 1, 4, 6 and 12, but in LIMC the diversity of *emm* types is much greater and no particular *emm* type is highly dominant (Sims Sanyahumbi et al., 2016). This complexity makes vaccine development very difficult because it is hard to cover all the GAS strains, at least, most of them.

2.2.2 There are some autoimmune epitopes in GAS and this makes the vaccine safety a major concern.

GAS infection can cause autoimmune diseases such as acute rheumatic fever (ARF) (Karthikeyan and Guilherme, 2018), this fact implies GAS vaccines must be designed very meticulously and it does make great obstacles for investigators.

Lots of studies indicate both cross-reactive antibodies and T cells have roles in ARF pathogenesis (Cunningham, 2000; Tandon et al., 2013). Fortunately, the autoimmune epitopes were found mainly existing in the M protein, precisely speaking, in the B-repeats region of M protein. This led to the strategy using the full length of M protein as antigen in the vaccine design being abandoned.

2.2.3 The socio-economy factor can influence GAS vaccine development.

Most GAS infections occur in developing countries and some specific populations in industrialized countries. (Chang, 2012; Gandhi et al., 2017; Zuhlke et al., 2017). From the perspective of investment-return, this is very hard to attract biological companies to invest GAS vaccine development.

2.2.4 The correlation of protection for GAS vaccine needs to be researched in-depth.

Establishing a standardized immunoassay that correlates with immune protection is essential in vaccine development. However, despite decades of research, currently used immunoassays to assess antibodies in GAS research are ELISA and functional bactericidal assays. ELISA gives a purely quantitative measurement of antibodies which include non-functional antibodies. The latter protocol can measure functional antibodies, but whole human blood must be used and this makes it labor-intensive and inter-assay variability. Recently, to overcome these limitations, Lorenz and colleagues developed a high-throughput opsonophagocytic assay for the determination of functional antibody activity against GAS using bioluminescence (Lorenz et al., 2017). This assay is believed to replace the two methods currently used and to make clinical trials easier.

2.3 Current status of GAS Vaccine Candidates

GAS vaccine development started in the early 1920s with whole heat-killed GAS, cell wall or M proteins (Dale et al., 2016). Although protective effectivity was observed, these relatively crude preparations had severe problems including reactogenicity caused by contaminating antigens, and risk of leading to ARF because of the autoimmune epitopes. With the advance in purification technology and understanding of the GAS related autoimmune mechanism, great progress has been made in GAS vaccine development. Many vaccine candidates have been developed to preclinical and clinical stages. Generally, these vaccine candidates can be classified into M protein based vaccines and other antigen based vaccines and summarized in Table 3.

2.3.1 M protein based vaccines

As a major surface-associated virulence factor and protective antigen, the M protein has been extensively researched for many years. The M protein is a coiled-coil protein consisting of A-C 3 domains. A-repeat/N-terminal domain is highly variable and used for *emm* typing; B-repeat domain contains autoimmune but not protective epitopes; C-repeat domain is highly conserved. Up to now, most notably promising vaccine candidates are based on the M protein. Early studies indicated antibodies against M protein could opsonize the homologous type strains and promote C3-mediated phagocytosis (Lancefield, 1962). The protective epitopes were found mainly existing in the N-terminal hypervariable region of M protein. These findings resulted in a vaccine design strategy that use only the N-terminal M peptides from different *emm* type strains in multivalent vaccines to devoid of potential autoepitopes (Dale et al., 2011).

2.3.1.1 Multivalent M protein based vaccines

Just like multivalent pneumococcal vaccines, theoretically, multivalent GAS vaccines could also be developed using the N-terminal M peptides linked in tandem based on the *emm* typing epidemiological data. The first multivalent GAS vaccine using this strategy was reported by Dale et al. in 1999 (Dale, 1999), in which protective M protein peptides from serotypes 1,3,5,6,19, and 24 of GAS were selected to form a hexavalent vaccine. This vaccine evoked high titers of antibodies against all six serotypes of GAS included in the vaccine. In the subsequent phase I trial, the hexavalent vaccine showed good tolerance and immunogenicity in humans, which represents a critical step in the development of a multivalent vaccine using hybrid fusion protein (Kotloff et al., 2004). Shortly afterwards, the multivalent vaccine was expanded to a 26-valent vaccine (Hu et al., 2002; McNeil et al., 2005) and later to a 30-valent vaccine (Dale et al., 2011; Dale et al., 2013). Both vaccines underwent phase I clinical trials in human adult volunteers and were shown to be safe and immunogenic. Compared to the 26-valent vaccine, the 30-valent vaccine has an increased coverage of circulating *emm* types worldwide, mainly in North America and Europe (Dale et al., 2011; Dale et al., 2013). Imperfectly, these two vaccines provide good coverage of circulating strains of GAS in industrialized countries but poor coverage in LIMC. A recent epidemiological study in Western Australia shows the new 30-valent M-protein GAS vaccine's potential coverage is 70% (Speers et al., 2017). Interestingly, cross-reactive immune responses were observed and raised new opportunities to optimize multivalent vaccines with broader coverage. This cross-protection can be partly explained by the *emm*-cluster typing system which classifies the 223 *emm* types into 48 functional *emm* clusters (McMillan et al., 2013). Given the cross-protection mechanism, the combination of selected *emm*

types included in multivalent GAS vaccines could be further optimized to remove some redundant types within the same *emm* cluster and add some new cluster representative types which are absent in current multivalent vaccine candidates.

2.3.1.2 Conserved region of M-protein based vaccines

To overcome the imperfect coverage of multivalent vaccines, vaccines were designed using the highly conserved C-repeat portion of the M protein and believed to protect against all GAS strains. Bessen and colleagues used the entire C-terminal region of M protein conjugated to the mucosal adjuvant cholera toxin B subunit to construct a recombinant vaccine (Bessen and Fischetti, 1988). The results demonstrated that immune response evoked by conserved portions of M protein reduced GAS infection at the nasopharyngeal mucosa in the mouse model and highlighted the role of conserved region peptide-specific local mucosal Ig in controlling GAS colonization of the throat.

However, low levels of antibodies to selected peptides were observed to bind to cleaved or denatured myosin (Vashishtha and Fischetti, 1993), thus raising safety concerns on this strategy. This led to define minimal epitopes for GAS vaccine design to eliminate the hidden danger.

According to the minimal epitope strategy, P145 was identified in the C-repeat region of the M-protein exhibiting immunogenicity and no cross-reactive to human tissue (Pruksakorn et al., 1992). Furthermore, J8 and J14 vaccines were derived from P145 containing shorter single minimal B cell epitopes. J8 was a 28-mer synthetic peptide which contained a central 12 amino acids B cell epitope (J8i) with the flanking sequences derived from the yeast DNA binding protein GCN4 to

fold J8i as a helix (Relf et al., 1996). To enhance the immunogenicity of this poor immunogenic one-epitope peptide, J8 was conjugated to the diphtheria toxoid (DT) to form J8-DT (Batzloff et al., 2003). This vaccine was highly immunogenic and the induced opsonic IgG had significant protection following intraperitoneal GAS challenge (Batzloff et al., 2003; Pandey et al., 2009; Pandey et al., 2013). Virus-like particles (VLPs) was used to carry J8 peptide as a GAS vaccine J8-VLPs. Results of the sublingual immunization indicated effective immune responses in both systemic and mucosal compartments. The saliva isolated from mice immunized with J8-VLPs demonstrated opsonizing activity against GAS in vitro. This suggests the vaccine's potential prevention of pharyngitis (Seth et al., 2016). Furthermore, J8-DT has completed a double-blinded Phase I pilot trial with no adverse events and a good antibody response (Dale et al., 2016). Furthermore, J8 was also conjugated to CRM197 to form the J8-CRM197 vaccine and shown immunogenic in mice and non-human primates (Caro-Aguilar et al., 2013). Subsequently, vaccine efficacy was further improved by incorporation of SpyCEP (Pandey et al., 2017). An alternative strategy using rational sequence modification to improve immunogenicity of p145 was recently reported (Nordstrom et al., 2017). After modification, the peptide can elicit higher titer antibodies with significantly higher affinity.

Another promising conserved M-protein based vaccine candidate is StreptInCor, which composed of 55 amino acid residues of the C-terminal region of the M5 protein containing both T and B cell protective epitopes (Guilherme et al., 2011; Guilherme et al., 2006; Guilherme et al., 2009). A molecular epidemiology study in Sao Paulo, Brazil and in silico analysis of vaccine coverage capacity indicated that StreptInCor could provide 71.0 % coverage in this area with high diversity of

GAS strains (Freschi de Barros et al., 2015). Studies demonstrated that StreptInCor is effective against several GAS strains and can prevent infection without causing autoimmune reactions (De Amicis et al., 2014; Freschi de Barros et al., 2015; Guerino et al., 2011; Guilherme et al., 2011; Guilherme et al., 2013; Guilherme et al., 2009; Postol et al., 2013). Moving forward on the basis of these immunogenicity and safety data, this vaccine has entered into phase I of clinical trials (Steer et al., 2016).

Table 3. Current status of promising GAS M protein-based vaccines

Vaccine candidates	Current status	Significant findings	References
6-valent M protein-based vaccine	completed phase I clinical trials	Safe, immunogenic, elicits antibodies to all homologous serotypes.	(Dale, 1999; Hall et al., 2004; Kotloff et al., 2004)
26-valent M protein-based vaccine	phase II clinical trials	Safe, immunogenic, elicits antibodies to all homologous serotypes; good coverage in industrialized countries but poor in LIMC.	(McNeil et al., 2005)
30-valent M protein-based vaccine	phase I clinical trials	Safe, immunogenic, elicits antibodies to all homologous and some heterologous serotypes; better coverage than the 26-valent vaccine.	(Dale et al., 2011; Dale et al., 2013)
J8/J14/P145	phase I clinical trials	Safe, immunogenic, elicits antibodies to different serotypes; no cross-reactive antibodies; vaccines were optimized based on this candidate to improve immunogenicity significantly.	(Pandey et al., 2017) (Nordstrom et al., 2017)
StreptInCor	phase I clinical trials	Safe, immunogenic, elicits antibodies to different serotypes; no cross-reactive	(Freschi de Barros et al., 2015; Guerino et al., 2011; Guilherme et al.,

	antibodies.	2011; Guilherme et al., 2013; Postol et al., 2013)

2.2.2 Non-M protein based vaccines

Since multivalent vaccines cannot provide full coverage against all GAS strains, and that there are lessons of serotype replacement from the multivalent pneumococcal vaccines implementation, highly conserved non-M protein GAS antigens received more and more attention. Several promising candidates were discovered with some encouraging results, such as GAS carbohydrate, C5a peptidase (Park and Cleary, 2005; Shet et al., 2003), Streptococcal pyrogenic exotoxin B (extracellular cysteine protease) (Kapur et al., 1994), streptococcal fibronectin-binding proteins, Combo vaccine (composed of Streptolysin O, SpyCEP and Spy0269) (Bensi et al., 2012), Pili (T antigen) (Koller et al., 2010), Streptococcal Heme Binding Protein (Shp) (Zhang et al., 2017), M-related protein (Mrp) (Courtney et al., 2017), but none of these candidates had entered clinical trials to date. The status of these vaccine candidates is summarized in Table 4.

Table 4. Current status of promising GAS non-M protein based vaccines

Vaccine candidates (antigen)	Current status	Significant findings	References
GAS carbohydrate	Preclinical studies	Conjugated to tetanus toxoid can protect mice from GAS challenge; no cross-reactivity was observed.	(Sabharwal et al., 2006)
GAS C5a peptidase (SCPA)	Preclinical studies	Highly conserved in all GAS serotypes; a virulence factor; not associated with autoimmune reactivity.	(Park and Cleary, 2005; Shet et al., 2003)
Fibronectin-Binding Protein	Preclinical studies	11 Fn-binding proteins were found, including protein F1 (PrtF1)/SfbI, protein F2 (PrtF2)/PFBP, FbaA (formerly Fba), FbaB, SfbII/serum opacity factor (SOF), SfbX, Fbp54, M1 protein, GAPDH/Plr, Shr and Scl1. These proteins have different coverage and	(Courtney et al., 2003; Kawabata et al., 2001; Schulze et al., 2006)

		some can elicit protective immune response.	
spy0469, spy1228 and spy1801	Preclinical studies	The antigens were tested for both antibody recognition and T cell responses in human adults and children; provide protection in a mice model.	(Mortensen et al., 2016)
M-related protein (Mrp)	Preclinical studies	The antisera opsonized GAS strains representing each Mrp family; also opsonized *emm* types not covered by the 30-valent M protein–based vaccine and can be combinated into the 30-valent vaccine improve the efficacy.	(Courtney et al., 2017)
Pili (T antigen)	Preclinical studies	Recombinant pilus proteins was shown to protect mice from GAS challenge; GAS FCT region displays considerable genetic diversity, with nine different FCT variants identified to date.	(Koller et al., 2010)
Combo vaccine (composed of Streptolysin O, SpyCEP and Spy0269)	Preclinical studies	Identified using 3 high throughput technologies; broad protection against a panel of four different GAS strains; SpyCEP was conjugated to J8 to improve the J8 efficacy.	(Bensi et al., 2012; Pandey et al., 2017; Zingaretti et al., 2010)
Streptococcal Heme Binding Protein (Shp)	Preclinical studies	Immunization elicited a robust IgG response, enhanced GAS clearance and reduced systemic dissemination.	(Zhang et al., 2017)

3. Issues worth of consideration during vaccinal design for Spn and GAS

3.1 Epidemiology--- essential parameter for both protein- and serotype-based strategy

The study of pneumococcal epidemiology is of particular importance because the efficacy varies by region, age and medical state of vaccinees. Pneumococcal proteins that are highly conserved and more widely distributed in human would be more likely to prevent Spn-related on a larger scale (Cornick et al., 2017). In addition, a recombinant multi-valent vaccine is recommended, preferably with adjuvants if needed. Similarly, overall and accurate epidemiological data is needed to optimize GAS vaccine design for better coverage and safety.

3.2 Whether Spn should be thoroughly eliminated is of discrete concern

Spn is a member of microbial commensals in human nasopharynx. Although colonization of Spn is the key step of bacterial invasion (Bogaert et al., 2004a), the balance of the microbiota in the respiratory tract plays an important role of immunity enhancement (Man et al., 2017). The serotype replacement is seen as the emergence of vaccine-excluded serotypes which becomes dominant following the use of PCVs. The "replacement phenomenon" was also thought to be observed as inverse correlate with other bacteria such as SA (Reiss-Mandel and Regev-Yochay, 2016), whilst whether the replacement by SA is a result of PCVs administration remains controversial (Bergenfelz and Hakansson, 2017; Fortanier et al., 2014; Reiss-Mandel and Regev-Yochay, 2016). Overall, it is paramount that the impact on pneumococcal carriage should be taken into consideration during the assessment on the efficacy of a vaccine, of which the original intention is to protect hosts from diseases rather than leading to possible side effects by unnecessary overtreatment. Although mucosal immunity is the best way to "shut out" Spn, vaccines that keep the colonized pneumococci below a pathogenic inoculum is suggested. Thus, a rationale endpoint setting is suggested during the efficacy assessment. Additionally, selectively targeting disease-causing factors of Spn could also be able to take the interests of the whole into account (McDaniel and Swiatlo, 2016), so as to achieve the goal of no interference to the asymptomatic pneumococcal carriage in human respiratory tract. As such, using genomic approach for identifying potential microbial targets can provide a comprehensive view to support rationale design of the future pneumococcal vaccines (Wizemann et al., 2001).

3.3 Using a systematic approach to optimize GAS vaccine design

Neither the multivalent vaccine nor the vaccine candidate based on a single conserved antigen can provide perfect coverage for all the serotypes, and serotype replacement might be developed under the selective pressure of vaccines. This prompts the optimization of vaccine design to increase the coverage and decrease the potential risk such as autoimmune responses. With an increasing number of antigens being discovered, a systematic approach can be employed to combine several highly conserved, immunogenic and non-autoimmune inducible antigen fragments to develop combination GAS vaccines which could potentially overcome the limitations of the current vaccine candidates.

Conclusions

As summarized in this review, there are a number of GAS vaccine candidates which have been proven to be promising. But the fact that must be faced is that there is no GAS vaccine currently on the market because of several impediments. This means there is no roadmap for GAS vaccine development. Unlike GAS, feasible Spn vaccines using novel tactics are much more fruitful, especially protein-based strategies which are considered the most prospective for developing next generation pneumococcal vaccines. As research moves along, the impediments are becoming largely solvable and the first GAS vaccine will come into being eventually.

Conflicts of interests

The authors declare no conflicts of interests.

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