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A HIGHLY PROTECTIVE STREPTOCOCCUS PNEUMONIAE VACCINE CANDIDATE BASED ON PILUS PROTEINS

Direttore della Scuola : Ch.mo Prof. Giuseppe Zanotti
Coordinatore d'indirizzo: Ch.mo Prof. Cesare Montecucco
Supervisore: Ch.mo Prof. Cesare Montecucco

Dottoranda: Claudia Gianfaldoni

ABSTRACT

Streptococcus pneumoniae is one of the most important human pathogens and a major cause of morbidity and mortality worldwide, causing several pathologies including pneumonia, meningitis, sepsis, otitis media, sinusitis and bronchitis.
Pneumococcal infection can be treated with antibiotics such as penicillin and erythromicin, however the increase of strains resistant to these antibiotics hampers the disease treatment.

The current 23-valent polisaccharide vaccine is not effective in children under 2 years of age, while the 7- and 13-valent conjugates are effective but only against the included serotypes, which in the long term may be replaced by serotypes not included in the vaccine (serotype replacement).
New strategies for the development of pneumococcal vaccine should include surface protein antigens that may provide a broad range of coverage against the >90 pneumococcal serotypes.

The discovery that *S. pneumoniae* isolates possess pili, reported to influence virulence and elicit host inflammatory responses, led us to investigate their potential use as a protein vaccine candidate.

The three pilus subunits (RrgA, RrgB, RrgC) were expressed in *E. coli* and purified, and were found to be highly immunogenic in mice.
The pilus subunits were then found to exert protective efficacy in mouse models of sepsis (intravenous or intraperitoneal challenge), with RrgB affording the best protective efficacy overall.
However, RrgB exists in three different variants (clade I, II, and III), with limited protein sequence identity and scarce cross-immunoreactivity.
Recombinant RrgB belonging to each of the three clades were expressed and purified, and tested for cross-protective ability. In particular, the absence of cross-protective ability between clade I and II was observed.
Thus, fusion proteins encompassing the three clades in different order were designed, expressed in *E. coli* and purified.

Among the RrgB fusion proteins, RrgB321 was selected as the best candidate, mainly because it was better expressed and purified than the other fusion proteins. RrgB321 fusion protein was found to be highly protective against each of the three *S. pneumoniae* strains representative of each of the RrgB clades.

The high protective efficacy of RrgB321 makes it a good vaccine candidate against piliated *S. pneumoniae* strains. RrgB321 can also be proposed as part of a multi-component vaccine against *S. pneumoniae*.

RIASSUNTO

Streptococcus pneumoniae è uno dei principali patogeni umani e rappresenta una delle maggiori cause di morbilità e mortalità in tutto il mondo. È infatti responsabile di molte patologie fra cui polmonite, meningite, sepsi, otite media, sinusite e bronchite.
L'infezione da pneumococco può essere trattata con antibiotici come la penicillina e l'eritromicina; tuttavia l'aumento di ceppi resistenti a questi antibiotici ostacola gravemente il trattamento della malattia.

Il vaccino polisaccaridico 23-valente attualmente in commercio non è efficace nei bambini sotto i 2 anni di età, mentre i vaccini coniugati 7- e 13-valente sono efficaci, ma solamente contro i sierotipi inclusi all'interno del vaccino stesso, il che può dar luogo, a lungo termine, al fenomeno noto come *serotype replacement* da parte dei sierotipi non inclusi.
Nuove strategie per lo sviluppo di un vaccino contro lo pneumococco dovrebbero rivolgersi all'utilizzo di antigeni proteici, esposti superficialmente, in grado di fornire una copertura ad ampio raggio contro i >90 sierotipi circolanti.

La scoperta che *S. pneumoniae* possiede pili in grado di influenzare la virulenza e di stimolare una risposta infiammatoria, ci ha spinto a studiare il loro potenziale utilizzo come vaccino proteico.

Le tre subunità del pilo (RrgA, RrgB, RrgC) sono state espresse in *E. coli* e purificate, e si sono dimostrate altamente immuniogeniche nel topo.
È stato quindi osservato che le subunità del pilo hanno efficacia protettiva in modelli murini di sepsi (infezione intraperitoneale o endovenosa). Nel complesso, RrgB è risultata la più efficace fra le tre subunità.
RrgB tuttavia esiste in tre diverse varianti (*clade* I, II and III) con una limitata identità di sequnza amminoacidica e scarsa cross-immunoreattività.
Sono state perciò espresse e purificate le RrgB ricombinanti appartenenti a ciascuna delle tre *clade* ed è stata analizzata la loro capacità cross-protettiva. In particolare è stata osservata la mancanza di cross-protezione fra *clade* I e II.

Pertanto sono state progettate delle proteine di fusione contenenti le tre *clade* in differente ordine, quindi espresse in *E. coli* e purificate.
La proteina di fusione RrgB321 è stata selezionata perchè meglio espressa e purificata rispetto alle altre proteine di fusione. RrgB321 è risultata altamente protettiva contro i tre ceppi di pneumococco rappresentativi delle tre varianti di RrgB.

L'elevata efficacia protettiva della proteina di fusione RrgB321 la rende una buona candidata come vaccino contro ceppi piliati di *S. pneumoniae*. RrgB può anche essere proposta come parte di un vaccino multi-componente contro *S. pneumoniae*.

CONTENTS

1. INTRODUCTION

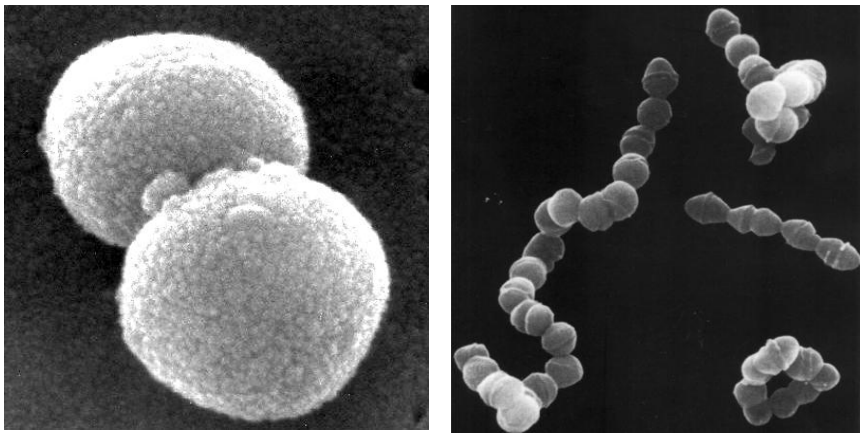
1.1 Generalities on *Streptococcus pneumoniae*

1.1.1 Historical Aspects and Microbial Characteristics

In 1881 the microorganism, then named pneumococcus for its role as an etiologic agent of pneumonia, was first isolated simultaneously and independently by the U.S. Army physician George Sternberg [1] and the French chemist Louis Pasteur [2] (who recovered pneumococci from rabbits which developed fatal septicemia after inoculation with human saliva from patients with pneumococcal desease). The organism was termed *Diplococcus pneumoniae* from 1926 because of its characteristic appearance in Gram-stained sputum. It was renamed *Streptococcus pneumoniae* in 1974 because of its growth in chains in liquid media [3].

S. pneumoniae played a central role in demonstrating that genetic material consists of DNA. In 1928, Frederick Griffith demonstrated transformation of live, harmless pneumococcus into a lethal form by co-inoculating the live pneumococci into a mouse along with heat-killed, virulent pneumococci. In 1944 Oswald Avery, Colin MacLeod, and Maclyn McCarty demonstrated that the transforming factor in Griffith's experiment was DNA, not protein as was widely believed at the time [4]. Avery's work marked the birth of the molecular era of genetics.

Streptococcus pneumoniae (the pneumococcus) is a Gram-positive, lancet-shaped coccus. As aforementioned, usually, pneumococcal cells are seen as pairs of cocci (diplococci), but they may also occur singly and in short chains. When cultured on blood agar, they form macroscopic colonies characterized by a surrounding greenish halo, derived from partial degradation of erythrocytes due to alpha-hemolytic activity. Individual cells are between 0.5 and 1.25 micrometers in diameter. They do not form spores, and they are non-motile. Like other streptococci, they lack catalase and ferment glucose to lactic acid. Unlike other streptococci, they do not display an M protein, they hydrolyze inulin, and their cell wall composition is characteristic both in terms of their peptidoglycan and their teichoic acid. *S. pneumoniae* is also sensitive to optochin and is lysed by bile salts.



1. www.nmpdr.org/images/SpneumoniaeCDC263.png
2. genome.microbio.uab.edu/strep/info/strep5.gif

Figure 1. elctron microscopy appearance of *S. pneumoniae* in the form of diplococcus (left) or of chains of variable length (right)

Pneumococci are surrounded by a polysaccharide capsule, comprised of repeating oligosaccharide units of sugars. Capsule represents the major virulence factor since it protects the pathogen from phagocytosis. Different strains show a high variation of their capsule composition and the corresponding serotype can be determined by capsule-specific antisera. At least 90 different serotypes have been identified [5]. Epidemiological studies, indicating the spread and occurrence of individual serotypes in certain geographic regions and over time, are abundant.

1.1.2 Pathogenesis

S. pneumoniae is a member of the human commensal flora and is known to asymptomatically colonize the nasopharynx of up to 60% of healthy children and 30% of healthy adults [6], a non-pathological status defined as carriage (Fig. 2).

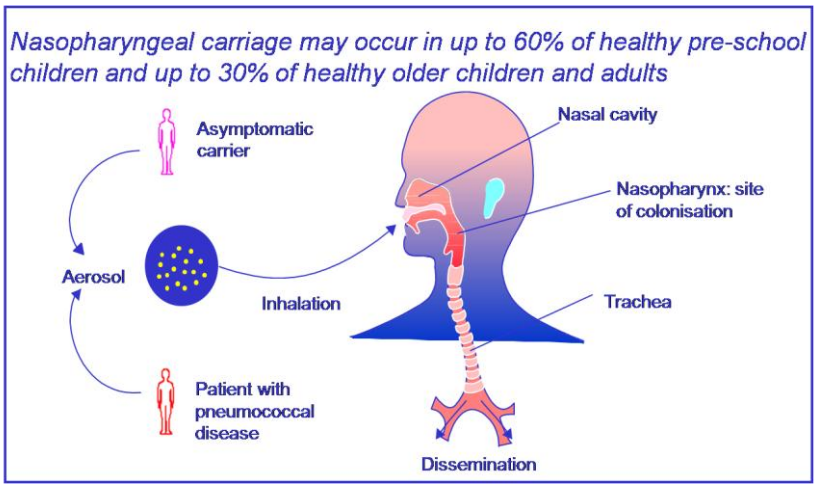


Figure 2. Schematic illustration of pneumococcal trasmission and colonization processes in the human host. Modified from D. Bogaert *et al.*, Lancet Infect Dis 2004; 4:144.

Children typically acquire a succession of serotypes early in life and are the primary vector for transmission to vulnerable population [7]. Factors associated with increased carriage include winter season, overcrowding, and day-care attendance. Persistence of colonization varies according both to age and to serotype. In infants, colonization may persist for a mean of 4 months [7], but is much shorter in adults: usually 2-4 weeks [8].

In contrast to this asymptomatic carriage the *S. pneumoniae* is also an important human pathogen, representing a major public health problem worldwide. Serious diseases that are often caused by pneumococci include pneumonia (*S. pneumoniae* is one of the most common causes of community-acquired pneumonia), meningitis, and bacteremia; otitis media, sinusitis and bronchitis are common but less serious manifestations of infection. WHO estimates that 1.6 million people die of pneumococcal disease every year in the world; this estimate includes the deaths of at least 0.7 million children aged <5 years, mostly living in developing countries [9] (Fig. 3).

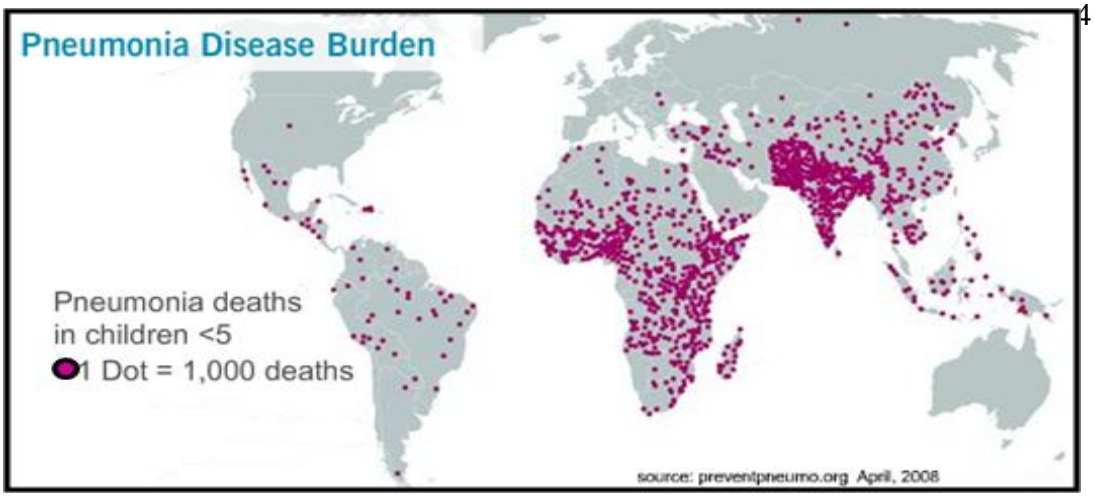


Figure 3. Graphic representation of pneumonia disease burden in children with less than 5 years of age, based on WHO estimates**.**

Pneumococci are transmitted from person to person by respiratory droplets. Of the >90 known serotypes, only a minority is frequently associated with pneumococcal disease, with patterns often temporal or specific for certain geographical regions. Different serotypes show different abilities to cause disease: for example types 1, 2, 3 and 5 are less often isolates from carriers and seem to have a greater tendency to spread epidemically, while types 14, 6, 19 and 23 are more prevalent in young children who are often asymptomatic nasopharyngeal carriers [10]. The development of the disease and its outcome is affected also by host factors such as socio-economic origin (e.g. crowding), age and underlying disease (chronic bronchitis, splenic dysfunctions and heart failure).

Pneumococcal infection can be treated with antibiotics such as penicillin and erythromicin, however the increase of strains resistant to these first-line antibiotics [11] hampers the disease treatment.

1.1.3 Pneumococcal Cell Surface Structure

The pneumococcal surface is composed as shown in Fig. 4. On top of the cytoplasmic membrane a layer of peptidoglycan (PG) is present. PG is a heteropolymer of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) and forms a rigid cell wall around the bacterium. Furthermore polymers of teichoic acids (TA), anchored to the peptidoglycan, and of lipoteichoic (LTA) acids, anchored to the lipids in the membrane, are involved in the control of cell shape and autolytic enzyme activity [12]. TA and LTA with their exposed

phosphorylcholine residues are also involved in non-covalent binding of pneumococcal surface proteins, so-called choline-binding proteins (CBPs).

Externally to the peptidogycan cell wall the polysaccharide capsule is present. Capsular polysaccharide (CPS) is one of the most important virulence factors of *S. pneumoniae*. The capsule protects pneumococci from phagocytosis by physically shielding the inner structures of the bacterium from antibodies and complement, separating bound opsonins from receptors on phagocytes and serving as a barrier to the deposition of complement. Pneumococci that lack a capsule can cause conjunctivitis [13], but are normally avirulent [14].

Several surface proteins have been described, and some of them proposed as potential vaccine candidates, including those composing the pneumococcal pilus, which will be described further and represents the rationale for the studies presented in this Thesis.

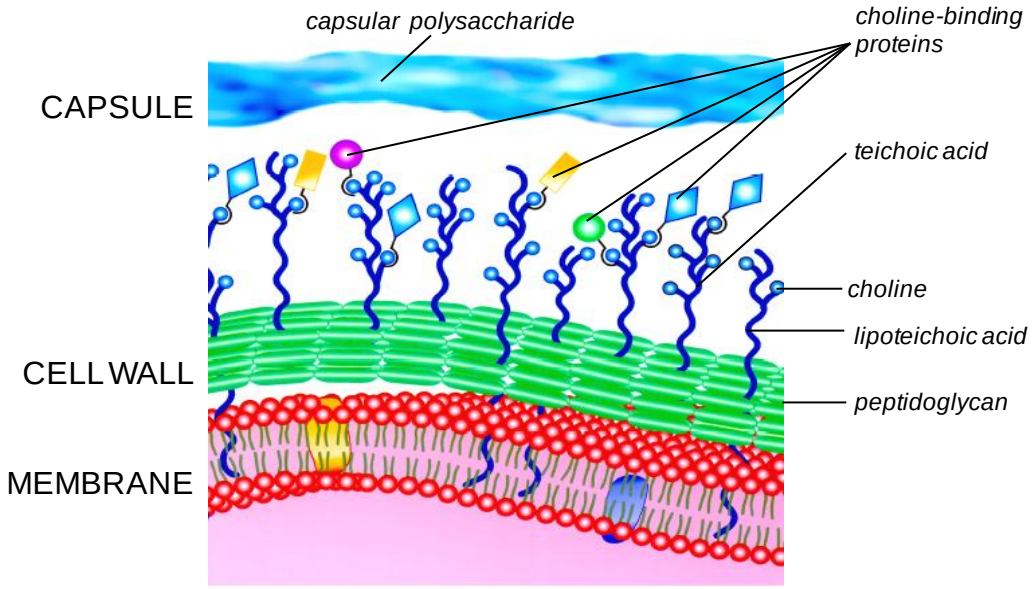


Figure 4: schematic representation of the cell membrane, cell wall and capsule of *S. pneumoniae***.** Image taken from: Khoosheh G, Tuomanen E (2000). *Streptococcus pneumoniae*: invasion and inflammation. In "Gram-positive pathogens" (VA Fischetti *et al.* Eds.), pp. 214-224. American Society for Microbiology, Washington D.C.

1.2 Pneumococcal Vaccines

1.2.1 History

Vaccines to prevent pneumococcal infection were first evaluated in the early 1900s. Several heat- or formalin- inactivated whole cell vaccines were initially studied [15] followed by the development and evaluation of more purified capsular polysaccharide vaccines [16]. Two hexavalent pneumococcal capsular polysaccharide vaccines were licensed in the United States shortly after World War II, but the promise of newly available antimicrobials effective against pathogenic bacteria led to diminished interest in the use of vaccines and those two vaccines were eventually withdrawn from the market. In the 1960s, recognition of continued morbidity and mortality from pneumococcal infections despite the use of appropriate antibiotics [17] led to renewed interest in prevention of disease by vaccination. A 14-valent vaccine, which contained 50µg of each capsular polysaccharide per dose, was licensed in 1977 and in 1983 this vaccine was replaced by 23-valent vaccine containing 25µg of each polysaccharide per dose.

1.2.2 Existing Polysaccharide-based vaccines

Current pneumococcal vaccines are exclusively targeted at the capsular polysaccharide (CPS) of *S. pneumoniae* and these vaccines provide strictly serotype-specific protection.

The current 23-valent formulation (serotypes 1 to 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F) is effective against approximately 90% of disease-causing serotypes in the United States and Europe. Nevertheless, CPSs are T-cell-independent antigens and do not induce memory B cells. Therefore, they are uneffective in children younger than 2 years [18] and in immunocompromised subjects. Moreover, 23-valent polysaccharide vaccine does not appear to be effective in preventing non-bacteremic pneumonia [19].

The poor immunogenicity of CPS antigens has been overcome by conjugation to protein carriers, as already done for other microbial vaccines; this converts them into T-cell-dependent antigens, which are considerably more immunogenic. The 7-valent polysaccharide-protein conjugate vaccine (PCV-7), licensed in 2000, includes capsular polysaccharide of 7 serotypes (4, 6B, 9V, 14, 18C, 19F, 23F) most

frequently circulating in North America and Europe conjugated to CRM197, a non-toxic variant of diphtheria toxin. PCV-7 is highly effective in infants and children, however protection is still serotype specific and, because of the high cost, the number of serotypes that are targeted has been reduced to seven.

A 13-valent conjugate vaccine (PCV-13) covering 6 additional serotypes (1, 3, 5, 6A, 7F, 19A) has been recently licensed.

In spite of the good safety record of pneumococcal conjugate vaccines and their demonstrated high efficacy against invasive infections, several limitations remain.

One of the issues is that conjugate vaccines are only capable of protecting against infection with bacteria that express polysaccharide capsule types that are included in the vaccine.

Another problem is that the potential for replacement disease with non-vaccine serotypes, as already experienced after the introduction of the PCV-7, may attenuate the overall benefit seen from reduction in disease due to vaccine serotypes [20, 21].

The third major issue is the complexity of conjugate vaccines production. For this reason only a limited number of companies are capable of manufacturing conjugate vaccines and in part explains their relatively high prices that also represent a limit for their use in developing countries, where the highest burden of disease and poor economical resources exist.

Therefore other types of immunogens are being evaluated as vaccine candidates. In particular, the research on conserved proteins has been intense during the past decades. Protein-based vaccines in fact are attractive for several reasons. They are expected to be immunogenic in the early infancy and in the elderly due to their T-cell-dependent nature and their coverage could be, at least in theory, broader than that of conjugate vaccines being serotype-indipendent.

1.3 Protein Antigens

1.3.1 *Virulence factors*

Besides the polysaccharide capsule, many proteins displayed on the surface of pneumococcus significantly contribute to pathogenesis and might be involved in the disease process caused by these pathogens. Often, these proteins are involved in direct interaction with host tissues or in concealing the bacterial surface from the host defense mechanisms.

In detail, three major groups of pneumococcal cell-surface proteins have been identified so far: 1) choline-binding proteins (Cbps), 2) lipoproteins and 3) proteins that are covalently linked to the bacterial cell wall by a carboxy C-terminal sortase motif (LPXTG: in which X denotes any amino acid).

S. pneumoniae is the only human-pathogenic bacterium known to express surface proteins specifically binding to choline as a mechanism of surface attachment. Interestingly, almost all the known pneumococcal choline-binding proteins are somehow recognized to exploit a role in the virulence: LytA, LytB and LytC are three autolysins [22, 23]; the pneumococcal surface protein A (PspA) plays an important role during systemic infection at least in part through the inhibition of complement deposition on the pneumococcal surface, a mechanism of evasion from the immune system [24, 25]; CbpA, CbpD, CbpE and CbpG are bacterial factors implicated in colonization processes and in the binding to Factor H [26, 27, 28]. Notably, some of the most reviewed candidates have been already selected for clinical trials: pneumolysin, a secreted protein mostly associated with the inflammation induced by *S. pneumoniae*; the choline binding proteins PspA and PspC; a family of four LPXTG surface anchored proteins rich in HxxHxH motifs (PhtA, PhtB, PhtD and PhtE) thought to act as zinc and manganese scavengers, that might be able to store these metals and to release them when the bacterium faces ion-restricted environment [29, 30]. However, the current lack of pneumococcal protein-based vaccine indicates that these vaccine candidates presented problems that limited the development of a vaccine.

1.3.2 Pili in Gram-positive bacteria

The discovery that Gram-positive pathogens possess pili has opened a new area of research into their function in pathogenesis and their role as protective antigens.

Pilus-like surface structures in Gram-positive bacteria were first identified in *Corynebacterium diphtheriae* by electron microscopy and have also been characterized genetically and biochemically [31, 32]. They were subsequently found in other species including *Streptococcus parasanguis* [20], *Streptococcus salivarius* [33] and *Streptococcus sanguis* [34]. Finally, in the past years, pili were also characterized in all three of the principal streptococcal pathogens that cause invasive disease in humans: group A *Streptococcus* (GAS, *Streptococcus pyogenes*) [35], group B *Streptococcus* (GBS, *Streptococcus agalactiae*) [36], and *Streptococcus pneumoniae* [37].

In Gram-positive bacteria two types of pilus-like structure have been identified by electron microscopy.

Certain Gram-positive bacteria (for example *S. gordonii* and *S. oralis*) are decorated with short, thin rods or fibrils that extend between 70 and 500 nm from the bacterial surface [38, 39]. Much longer (up to 3µm) pilus-like structure that appear as flexible rods have been described in the Gram-positive oral pathogens *Corynebacterium* species and pathogenic streptococci [20, 23, 25, 40].

A general feature of these latter rod-like pili is that they comprise three covalently linked protein subunits, each of which contains an LPXTG amino-acid motif (where X denotes any amino-acid) or a variant of this motif which is the target of sortase enzymes. During pilus formation, specific sortases catalyse the covalent attachment of the pilin subunits to each other and to the peptidoglycan cell wall [20]. This structural organization seems to be a peculiar characteristic of Gram-positive bacteria, because covalent bonds have not yet been detected between the subunits of the pili of Gram-negative bacteria.

Immunogold electron microscopy using antisera specific for the pilus revealed that the pilus is generally composed by one main component (the backbone component) of the structure and other two components considered ancillary proteins.

1.3.3 Streptococcus pneumoniae *pili*

Two kind of pili have been described for *S. pneumoniae*: 1) pilus 1, present in about 30% of the isolates, which will be further described in the present Thesis and represents the target of the vaccine candidate; 2) pilus 2, present in about 16% of the isolates, involved in adherence to host respiratory cells [41], which will be no longer discussed in the present Thesis.

The pneumococcal pilus 1, hereafter referred to as "pilus", is a multimeric structure consisting of three proteins (RrgA, RrgB, RrgC) polymerized by three sortases (SrtB, SrtC, SrtD) through the formation of covalent intermolecular isopeptide bonds [42, 43]. In particular, multiple copies of RrgB are polymerized to form the scaffold of the pilus, whereas the major adhesin, RrgA, and the putative anchor, RrgC, are localized at the tip and at the base of the pilus respectively [44, 45]. All these proteins are encoded by genes contained in a 14-kb pathogenicity island known as *rlr*A islet.

The function of pneumococcal pili is currently an area of investigation. To date, pili are known to be involved in adhesion to lung epithelial cells *in vitro*, as well as in colonization in a murine model of infection [25, 46].

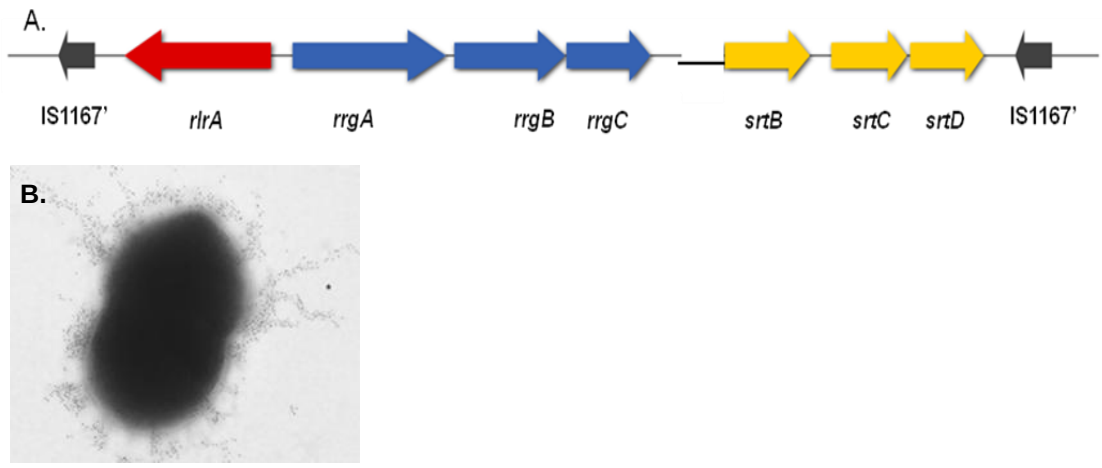


Figure 5: (**A**) Genome organization of the *rlrA* islet in serotype 4 strain TIGR4.; (**B**) electron micrograph of *S. pneumoniae* stained with anti-RrgB (immunogold)

The presence of the pilus among different pneumococcal isolates has been studied. Moschioni *et al* showed that in a panel of 424 isolates, the *rlr*A islet was present in about 30% of strains [47]. Data on protein sequence similarity revealed RrgC to be the most conserved pilus subunits (98-99%) followed by RrgA (84-98%). Conversely, RrgB is the least conserved (49-67%), existing in 3 different variants which correspond to 3 different clades (I, II, III) of the *rlr*A operon. Moreover, in the

same study, clade I resulted to be the most common (62.2%), followed by clade II (26.8%) and by clade III (10.7%).

2. AIM OF THE STUDY

- The limits of the current polysaccharide-based vaccines (CP-23: uneffective in children <2 years; PCV-7 and PCV-13: limited serotype coverage, serotype replacement) could be overcome by a protein-based vaccine.

- In the frame of the investigation on a pneumococcal protein-based vaccine, the identification of *S. pneumoniae* pilus led to focus the research on pilus antigens as potential component of a pneumococcal, protein based, vaccine.

- The purpose of the present Thesis was to investigate the protective efficacy of the *S. pneumoniae* pilus subunits, identifying and characterizing the most promising vaccine candidate. The protective efficacy was tested in two mouse models of *S. pneumoniae* infection: the intravenous challenge and the intraperitoneal challenge. Both models generate bacteremia and lethality that were used to estimate the levels of protection.

3. MATERIALS AND METHODS

3.1 Bacterial strains and growth conditions

S. pneumoniae strains were routinely grown at 37°C in 5% CO_2 on Tryptic Soy Agar plates (TSA) (Becton Dickinson) supplemented with colistine 10 mg/l, oxolinic acid 5 mg/l and 5% defibrinated sheep blood (vol/vol) or in liquid media: Tryptic Soy Broth (TSB) (Difco) and Todd Hewitt Broth supplemented with 0.5% (w/w) yeast extract (THYE) (Becton Dickinson). For the animal experiments, bacteria grown over night (ON) on the plates were inoculated in TSB and grown until exponential phase A_{600} = 0.25. Bacteria were then harvested by centrifugation, resuspended in TSB 20% glycerol (vol/vol) 10% Fetal Bovine Serum (vol/vol), and frozen in aliquots at -80°C. The frozen stock was titrated by plating culture aliquots at serial dilutions and counting CFUs. Immediately prior intraperitoneal challenge, frozen aliquots were thawed and diluted in saline to reach the working concentration. For intravenous challenge, bacteria were freshly harvested from THYE liquid cultures at A_{600} = 0.5, and brought to the working concentration before administration. The challenge input was titrated by plating bacterial suspensions as above immediately after challenge.

3.2 Cloning, protein expression and purification

Standard recombinant DNA techniques were used to construct plasmids expressing the three pilus subunits RrgA, RrgB, RrgC (~ 93, 66, 40 kDa, respectively), the single RrgB domains (~ 18, 15, 13 and 20 kDa for D1, D2, D3 and D4 respectively), and the RrgB fusion proteins (~180 kDa) encompassing the three RrgB clades. The fusion proteins consisted of the three variants in a head-to-tail organization and separated by a six aminoacid linker (Gly-Ser-Gly-Gly-Gly-Gly). Briefly, pilus proteins open reading frames (nucleotides corresponding to the N-terminal signal sequence and C-terminal cell wall sorting signal motif were excluded from the cloning) were amplified by PCR from chromosomal DNAs of *S. pneumoniae* TIGR4 (*rrgA* clade I and *rrgB* clade I), 6B-SPEC (*rrgA* clade II and *rrgB* clade II) and 23F-Taiwan15 (*rrgA* clade II and *rrgB* clade III). The obtained PCR fragments were digested

with the appropriate restriction enzymes and ligated into the C-terminal 6xHis-tag expression vector pET21b+ (Novagen). The resulting plasmids were confirmed by DNA sequencing and then transformed into competent *E. coli* BL21 DE3 star (Invitrogen). Protein expression was induced by adding IPTG (isopropyl-β-d-thiogalactopyranoside, Sigma) 1mM final concentration to a bacterial culture at an A_{600} of 0.4-0.5 (LB medium supplemented with ampicillin 100 μg/ml) and the proteins purified by metal chelate affinity chromatography on His-Trap HP columns (GE Healthcare). Pooled fractions containing the purified protein were dialyzed overnight against phosphate-buffered saline (PBS) and stored at -80°C until further use. Purified recombinant proteins were subsequently used to immunize animals either for antibody generation or evaluation of their protective efficacy.

3.3 Antibody titer evaluation

Quantification of immunoglobulin G (IgG) was made by an enzyme-linked immunosorbent assay (ELISA) of mouse sera. Single sera were analyzed. Serial dilutions of sera were dispensed in Maxisorp 96-well plates (Nalge Nunc International) coated with recombinant pilus protein at 0.2 μg/well. Antibody binding was detected by alkaline phosphatase-conjugated anti-mouse IgG (Southern Biotechnology Associates), followed by the substrate *p*-nitrophenyl-phosphate (Sigma). Absorbance was measured at 405 nm. Sera were titrated by comparison with the curves obtained with reference sera using a reference line calculation program. Reference sera consisted of pooled mouse anti-RrgA, -RrgB, or -RrgC sera, to which the titer of 50,000 was assigned.

3.4 SDS-PAGE and Western Blot analysis

Bacteria were grown on blood agar plates for up to 16 hours. 30 mg bacteria (wet weight) were resuspended in 1 ml 50 mM Tris-HCL pH 6.8, containing 400 units Mutanolysin (Sigma) and incubated 2 hours at 37°C, shaking at 350 rpm. After 3 cycles of freeze and thaw, cellular debris was removed by centrifugation at 13.000 rpm for 15 min. Samples were concentrated with 100MWC Centicon . 50 μl of the supernatant was treated with NuPage Sample Buffer for 5 min at 90°C and 20 μl were loaded on 3-8% NuPage Novex Bis-

Tris Gel. Hi-Mark^{TM} pre-stained HMW protein standard (Invitrogen) served as protein standard. Gels were processed for Western Blot analysis by using standard protocols. Rabbit antisera raised against recombinant RrgA, RrgB, RrgC and RrgB321 chimera were used at 1:10,000 dilution. Secondary goat anti-rabbit IgG alkaline phosphatase-conjugated antibodies (Promega) were used at 1:5,000 and the signal developed by using Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega).

3.5 Flow Cytometry on entire bacteria

Bacteria were grown in THYE to an exponential phase (A_{600} = 0.25), stained with rabbit primary antibodies (final dilution 1:300), and then with a FITC conjugated secondary antibody (final dilution 1:100) (Jackson Laboratories). Finally, bacteria were fixed with 2% formaldehyde and bacterial staining was analyzed by using a FACS-Calibur cytometer (Becton Dickinson). Sera from animals immunized with PBS plus adjuvant served as negative control.

3.6 Formulation

Formulation of proteins was performed in sterile conditions; 0.1 mg/ml protein recombinant protein was adsorbed onto 3 mg/ml Aluminum Hydroxide in 9 mg/ml NaCl, 10 mM Histidine pH 6.5. The antigen was added and left for 15 minutes under stirring at RT, and then stored overnight at 4°C before the immunization. Final formulations were isotonic and at physiological pH.

All formulations were characterized soon after immunization, antigen adsorption was $\geq$ 95% and adsorption profile was similar for all antigens tested.

3.7 Antisera

To generate sera against the specific proteins, purified recombinant proteins were used to immunize either BALB/c or CD1 mice (as detailed in Animal Experiments paragraph) or New Zealand White rabbits (Charles River Laboratory) of 2.5 kg body weight. Rabbits received three doses of 100 µg of protein along with Freund's adjuvant, administered subcutaneously on day 0, 21 and 35, and immune serum was obtained two weeks after the last immunization.

3.8 Animal experiments

Animal studies were done in compliance with the current law, approved by the local Animal Ethics Committee and authorized by the Italian Ministry of Health.

Female, 6-week-old, specific pathogen-free BALB/c or CD1 mice (Charles River) received three intraperitoneal (i.p.) immunizations, two weeks apart. Each dose was composed by 20 μg of recombinant protein, along with either Freund's adjuvant or 400 μg of aluminium hydroxide, in a final volume of 200 μl of saline. Negative controls received the same course of saline plus adjuvant. Ten days after the third immunization, samples of sera were obtained for serological and functional studies. Two weeks after the third immunization, BALB/c mice were challenged intraperitoneally (i.p.), while CD1 mice were challenged intravenously (i.v.) via the tail vein. The pneumococcal strains and the challenge doses are reported in Table 1. Bacteremia was evaluated in blood samples taken 24 (i.p. challenge) or 48 hrs (i.v. challenge) post-challenge and plated on blood-agar plates at serial dilutions. After 24 hrs of culture, CFU were counted and the CFU/ml of blood calculated. Bacteremia was expressed as log10 (Log) of the CFU/ml value. After challenge, the animals were monitored for 10 (i.p. challenge) or 15 (i.v. challenge) days. Mice were euthanized when they exhibited defined humane endpoints that had been pre-established for the study in agreement with Novartis Animal Welfare Policies, and the day recorded. Survival rates were calculated according to the following formula: survival rate (%) = [1 – (% dead vaccinated / % dead controls)] x 100.

For the passive protection experiments, 15 min before TIGR4 i.p. challenge, each mouse received i.p. 50 μl of pooled mouse specific antisera. Controls received 50 μl of pooled mouse sera obtained from the negative controls immunized with saline plus adjuvant. The challenge dose was 140 CFU/mouse.

3.9 Statistical analysis

GraphPad Prism Software (version 5.0) was used for statistical analyses. The following one-tailed tests were applied: Mann-Whitney U test to analyze data

of bacteremia and of survival courses. Fisher's exact test or chi square test were used to analyze survival rates, according to the low or high number of animals per group, respectively. Values of $P \leq 0.05$ were considered and referred to as significant.

4. RESULTS

4.1 Pilus subunits are immunogenic in mice

The recombinant pilus subunits RrgA, RrgB, RrgC, corresponding to the sequence of the *S. pneumoniae* TIGR4 strain, were expressed, purified and tested for their immunogenicity.

Serum IgG response was quantified by ELISA in mice immunized intraperitoneally with recombinant pilus subunits. Individual pilus antigens (20µg each) elicited high IgG response (Fig. 6) with sera becoming titrable at >1:50,000 dilution.

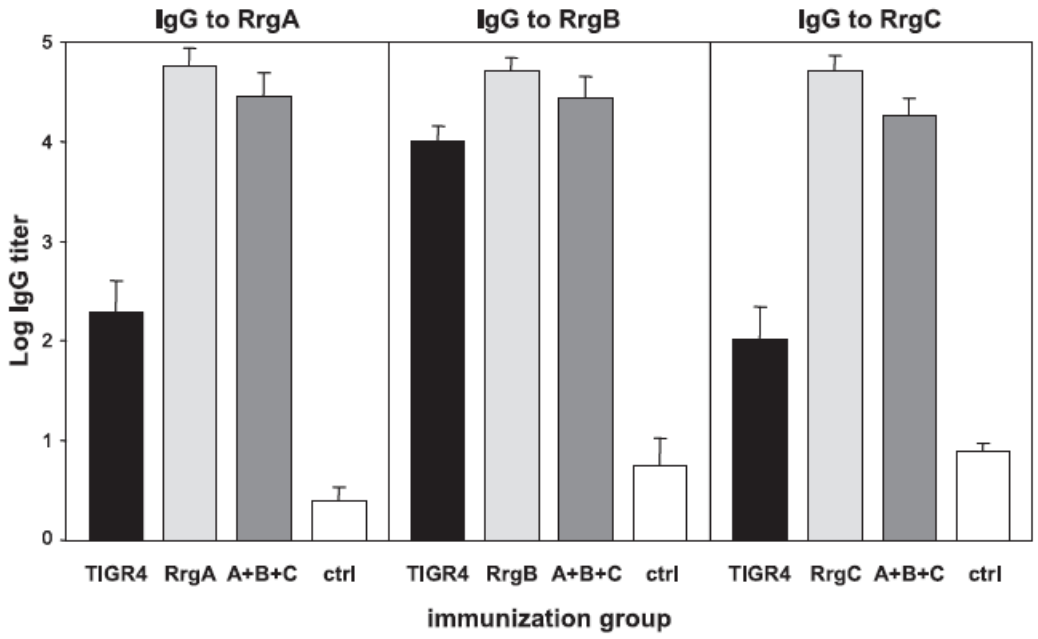


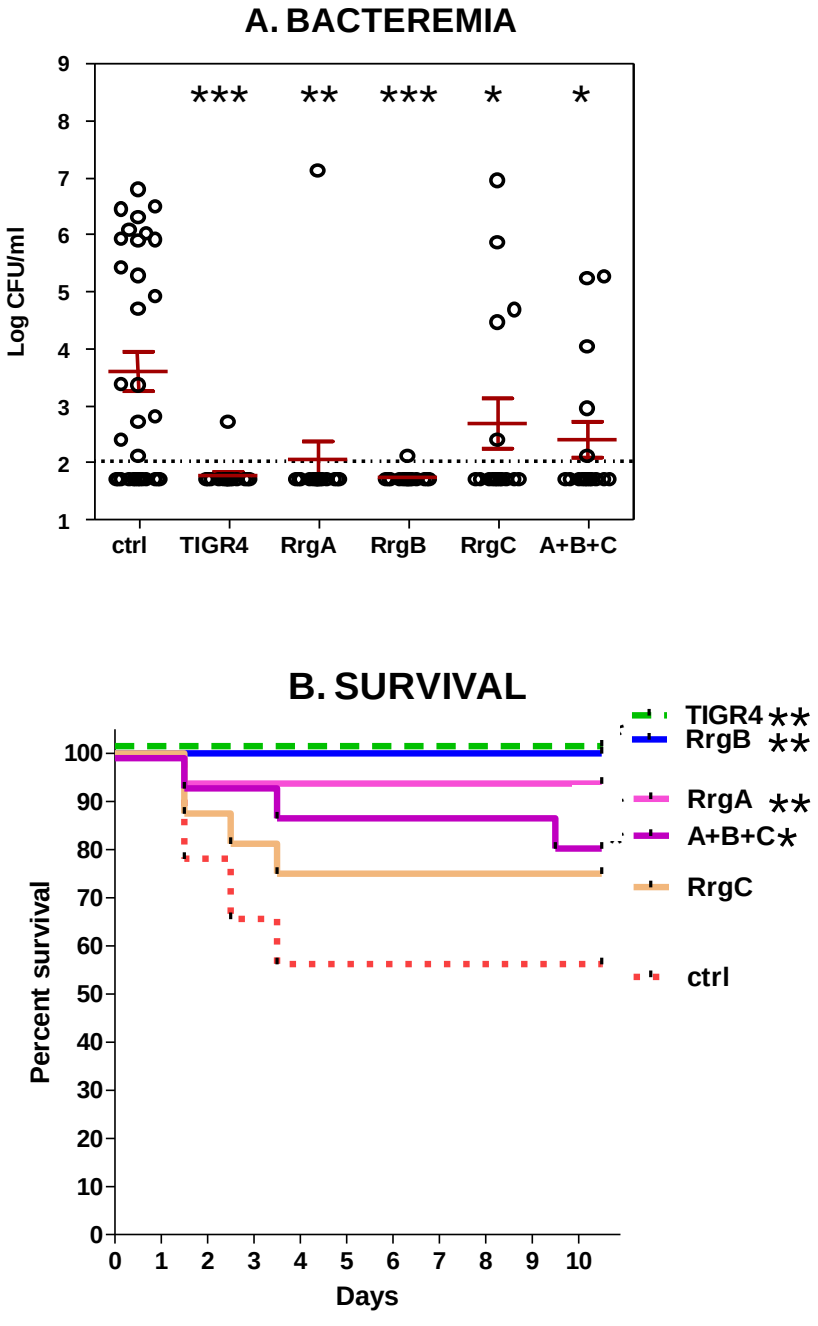
Figure 6: Immunogenicity of pilus subunits in mice. Enzyme-linked immunosorbent assay (ELISA) quantification of specific IgG titers against recombinant RrgA, RrgB, or RrgC in sera of immunized mice. Eight mice were used for each group, with the exception of the control (ctrl) group, in which 16 mice were used. *P* was <0.001 (one-tailed Mann-Whitney U test) for each immunized group in comparison to the corresponding control. Columns represent the means for each of the groups; A+B+C, the combination RrgA+RrgB+RrgC; bars, standard deviations.

Immunization with the combination RrgA+RrgB+RrgC also elicited high IgG levels against each of the three antigens, with titers slightly reduced consistently with the lower antigen dose used (10µg each, instead of 20µg). Moreover mice vaccinated with heat-inactivated TIGR4, containing native pilus structures, generated serum antibodies able to detect the recombinant pilus antigens. Interestingly, the most evident response to immunization with heat-inactivated

TIGR4 was directed against RrgB (Fig. 6), consistently with the fact that RrgB constitutes the pilus backbone and therefore is the most abundant subunit in the native structure.

4.2 Immunization with recombinant pilus antigens is protective in mice

The protective efficacy of recombinant pilus subunits RrgA, RrgB, RrgC was then tested in mice. The results are summarized in Figure 7.



C

P values

	bacteremia	survival course	survival rate
TIGR4	0.0003	0.0031	0.0001
RrgA	0.0010	0.0070	0.0074
RrgB	0.0003	0.0041	0.0014
RrgC	0.0369	0.1124	0.1719
RrgA+B+C	0.0153	0.0313	0.0806

Figure 7: Protective efficacy of pilus subunits in mice.
16 mice for vaccinated groups and 32 for control group were used. (**A**) Bacteremia at 24 h post-challenge. Circles = Log CFU per ml of blood for single animals; horizontal bars = the mean value of Log CFU/ml ± SEM; dashed line = detection limit (*i.e.*, no CFU were detected in blood samples below the dashed line). (**B**) Survival course (Kaplan-Meier curve). Percentage of survival at each observation is indicated. Mice were controlled for ten days; ctrl = mice receiving adjuvant plus saline; *, ** and *** = *P* values of <0.05, <0.01 and <0.001 respectively for comparison with the corresponding control groups. In the survival panel only the *P* value for survival course is indicated. (**C**) *P* values for bacteremia, survival course (one tailed Mann-Whitney U test) and survival rate (one tailed Fisher's exact test) for each group in comparison with the control group.

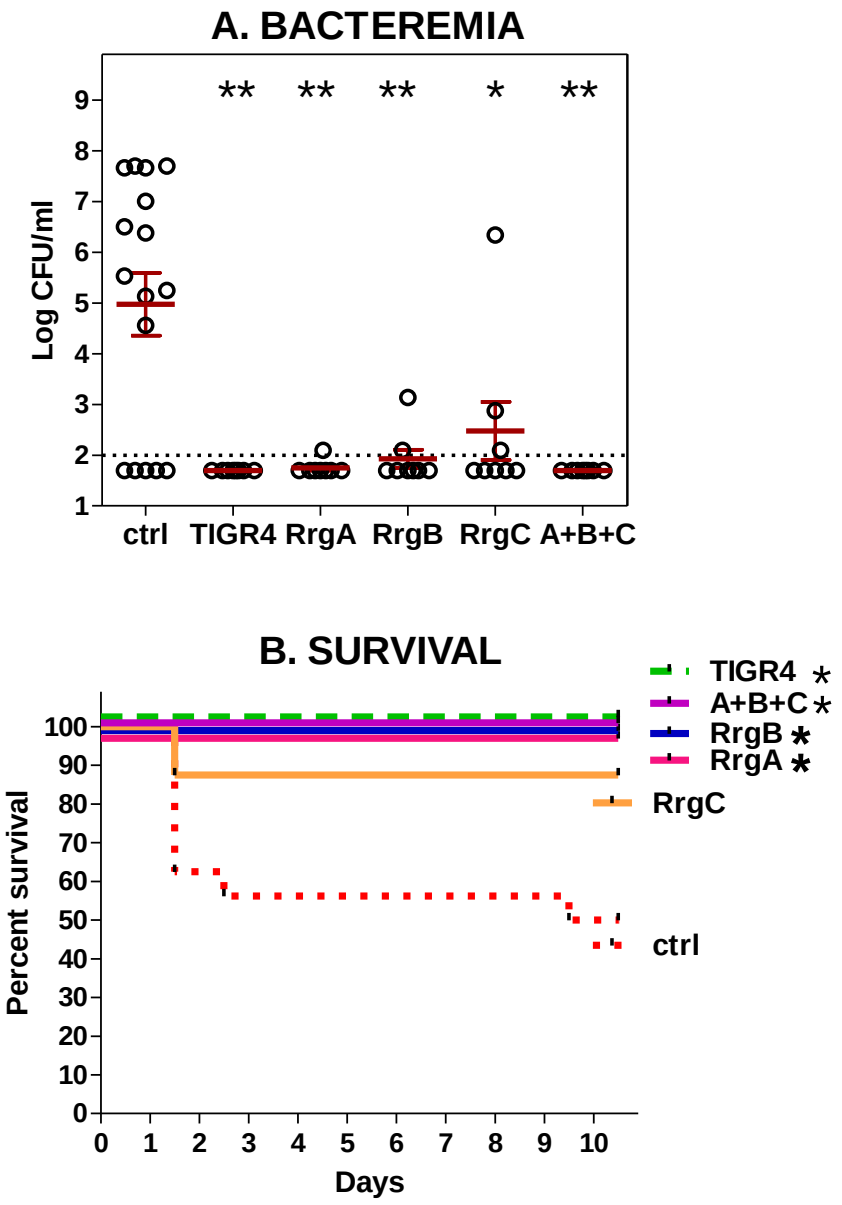
Mice immunized intraperitoneally with single pilus subunits, combination of the three subunits and heat-inactivated bacteria (positive control) were challenged intraperitoneally with 100 CFU of TIGR4, a dose previously observed to correspond to a 50% lethal dose in Freund's adjuvant-treated mice.

All groups vaccinated with recombinant pilus antigens showed lower bacteremia levels and higher survival course than controls.

In fact, control group had a mean Log CFU/ml of 3.6 and a survival of ~ 55%. Immunization with RrgA and RrgB afforded almost complete protection, with only one bacteremic mice (>1.5 Log reduction) and a survival rate of 86 and 100% respectively. RrgC elicited ~ 1 Log reduction in bacteremia and a non significant increase of survival rate (43%) compared to the control group. The bacteremia levels and the survival courses for the groups immunized with pilus antigens were not statistically different (P > 0.1) from those for the group vaccinated with heat-inactivated bacteria. The combination RrgA+RrgB+RrgC afforded protection against bacteremia (> 1 Log reduction) and 57% as survival rate.

4.3 Passive transfer of sera to recombinant pilus antigens is protective in mice.

In order to further investigate whether the protective efficacy of pilus subunits involves antibodies, we tested mouse antisera raised against recombinant pilus antigens for their protective ability by passive serum transfer. For this purpose, 10-week-old mice received intraperitoneally 50 µl each of immune serum 15 min before challenge with 10^2 CFU of TIGR4.



C

P values

	bacteremia	survival course	survival rate
TIGR4	0.0036	0.0058	0.0175
RrgA	0.0036	0.0058	0.0175
RrgB	0.0066	0.0058	0.0175
RrgC	0.0210	0.0537	0.0875
RrgA+B+C	0.0036	0.0058	0.0175

Figure 8: Protection afforded by immune sera passive transfer. 8 mice were used in vaccinated group and 16 in the control group. Mice received 50µl of immune sera about 15 minutes before challenge. (**A**) Bacteremia at 24 h post-challenge; (**B**) survival course. Serum specificity is indicated in the graph. (**C**) *P* values for bacteremia, survival course and survival rate. Symbols are explained in Fig 7 legend.

The results, reported in Figure 8, were consistent with those obtained with active immunization: all groups receiving antisera against recombinant pilus antigens showed reduced bacteremia levels and increased survival times compared to the control group.

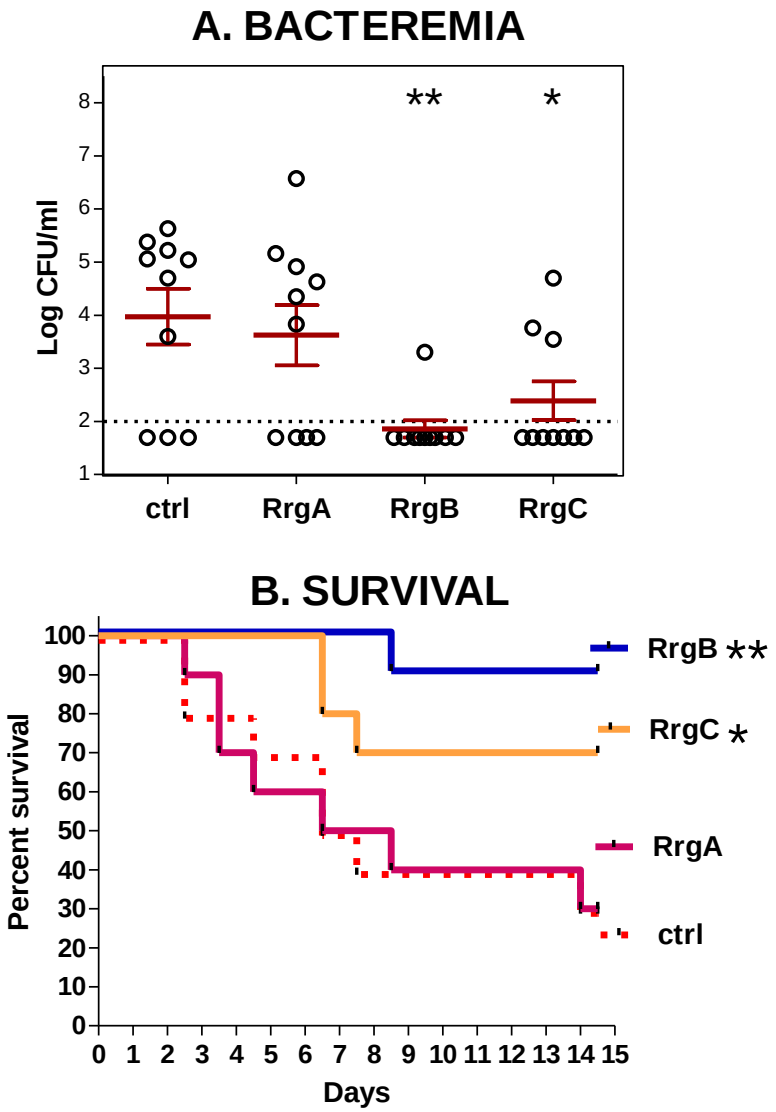
At 24 h post-challenge, control group presented a mean Log CFU/ml of 5 and, at 10 days postchallenge, 50% of control mice were alive. The passive transfer of anti-RrgA+RrgB+RrgC serum resulted in undetectable bacteremia at 24 h for all eight mice and 100% survival at the endpoint. After passive transfer of either anti-RrgA or anti-RrgB serum, only one or two mice, respectively, were found bacteremic at 24 h post-challenge, and 100% of mice survived lethal challenge. Passive transfer of anti-RrgC serum resulted in 5/8 mice having no detectable bacteremia showing a mean Log CFU/ml of ~2.5 and 87% of survival rate.

Again, bacteremia levels and survival rates in groups receiving antisera to pilus antigens were not statistically different ($P > 0.1$) from those for the group that received anti-TIGR4 serum.

These results indicate that antibodies are involved in the protection elicited by pilus subunits.

3.4 RrgA does not afford protection in the intravenous model of infection

The protective efficacy of pilus antigens was also tested using the intravenous challenge model. The challenge was performed with $2x10^6$ CFU of TIGR4.



C

P values

	bacteremia	survival course	survival rate
RrgA	0.2316	0.5000	0.5000
RrgB	0.0024	0.0031	0.0099
RrgC	0.0131	0.0339	0.0894

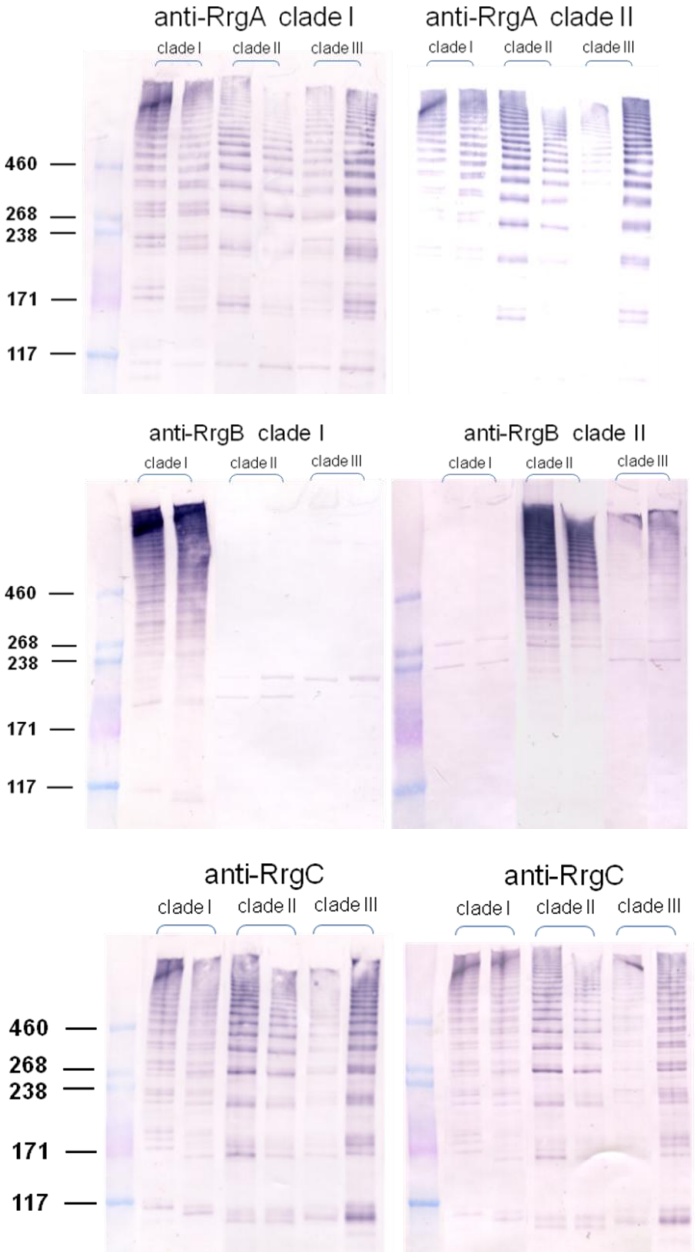
Figure 9: Protective efficacy of pilus subunits in intravenous (i.v.) model of infection. Ten mice in each group were used. (**A**) Bacteremia at 48h post-challenge; (**B**) survival course. (**C**) *P* values for bacteremia, survival course and survival rate . Symbols are explained in Fig. 7 legend.

As shown in Fig. 9, in the negative control group 7/10 mice were bacteremic with a mean Log CFU/ml of 4; at the 15th day 30% of mice survived. RrgB resulted to be highly protective with only one out of 10 mice becoming bacteremic and a survival rate of 86%. RrgC afforded good protection presenting in the blood a mean Log CFU/ml of 2.5 (1.5 Log reduction) and a survival rate of 57%.
In contrast with the results of intraperitoneal challenge, upon intravenous challenge RrgA was not effective in reducing bacteremia and increasing survival, in fact it showed an infection level and a mortality course similar to that observed in the negative control group.

4.4 RrgB clades present scarce cross-immunoreactivity

Since the three pilus subunits were found to have different degrees of amino-acid identity among the different stains sequenced, we aimed at evaluating the cross-protective efficacy of pilus antigens. The lowest amino acid identity observed was 84% for RrgA, 49% for RrgB, and 99% for RrgC. In particular, three clades were identified for RrgB (I, II, III) and two for RrgA (clade I associated with RrgB clade I and III, and clade II associated to RrgB clade II). The following strains were initially selected as representative of RrgB clades: TIGR4 (clade I), 6BSpain2 or 6BFinland12 (clade II), and 23FTaiwan15 or 35BSME15 (clade III). Thus, besides the recombinant pilus antigens designed on the sequence of TIGR4 already used in the previous experiments, those based on the 6B strain and the 23F strain sequences were expressed and purified.
We analyzed the surface exposure and cross-reaction of pilus proteins *in vitro* by western blot and FACS.

A.


anti-RrgA clade I
clade I
clade II
clade III
anti-RrgA clade II
clade I
clade II
clade III
460
268
238
171
117
anti-RrgB clade I
clade I
clade II
clade III
anti-RrgB clade II
clade I
clade II
clade III
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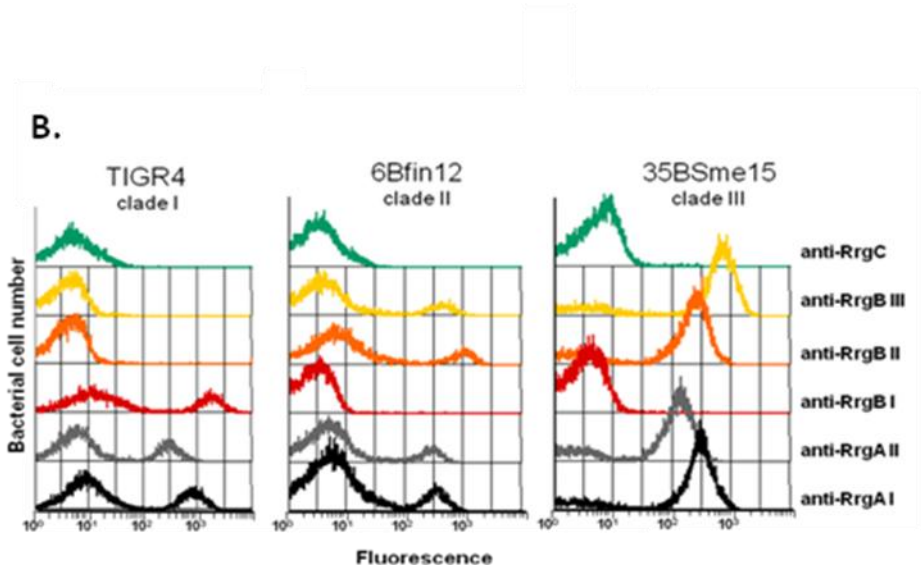


Figure 10: CROSS-immunoreaction of pilus subunits. (**A**) WB analysis performed on whole bacterial lysates of TIGR4 and 19FTaiwan14 expressing RrgB clade I, 19AHungary6 and 6BSpain2 expressing RrgB clade II, 23FTaiwan15 and 35BSme15 expressing RrgB calde III. Rabbit antibodies raised against recombinant RrgA clade I and II, RrgB clade I, II and III and RrgC were used. (**B**) Three pneumococcal strain representative for the three clades (TIGR4, 6BFin12, 35BSme15) were incubated with rabbit serum directed against pilus subunits followed by FITC-conjugated goat anti-rabbit IgG. Bacterial staining was analyzed by flow cytometry (FACS-Calibur).

Western Blot analysis (Fig. 10A) revealed that rabbit serum raised against RrgA and RrgC were able to detect pilus structure (ladder of HMW) belonging to the 3 clades accordingly to their high level of similarity. Conversely, serum raised against RrgB clade I recognized only pilus structure containing homologous protein, while serum anti RrgB clade II was able to slightly detect also clade III RrgB in addition to the homologous RrgB, accordingly to the higher degree of similarity between RrgB clade II and III.

The cross-immunoreactivity of pilus subunits was also analyzed by FACS (Fig. 10B). For RrgA and RrgB, FACS confirmed the results obtained by WB. In fact antisera raised against RrgA clade I and II recognized strains containing not only the homologous protein, but also the heterologous one. For RrgB the cross-reaction was observed between clade II and III, but not between clade I and II or clade I and III. RrgC was not detectable by FACS analysis in the native pilus structure in any pneumococcal strain tested.

4.5 Pilus antigens are no or scarcely cross-protective

After the *in vitro* cross-immunoreactivity analysis of pilus subunits, we tested their cross- protection ability *in vivo*.

In Fig. 11 are shown the active and passive immunization experiments performed to test cross-protection between clade I and II.

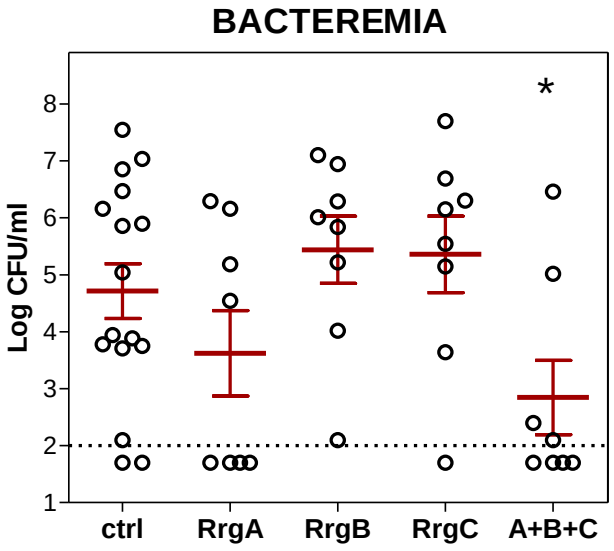
In the active immunization experiment (Fig. 11A) we used 6BSpain2 pilus antigens (clade II) to immunize mice and TIGR4 (clade I) as challenge strain. No protection was afforded by RrgB and RrgC; RrgA showed a not significant trend toward reducing bacteremia and the combination RrgA+B+C elicited significant reduction of bacteremia and a non significant trend in increasing survival.

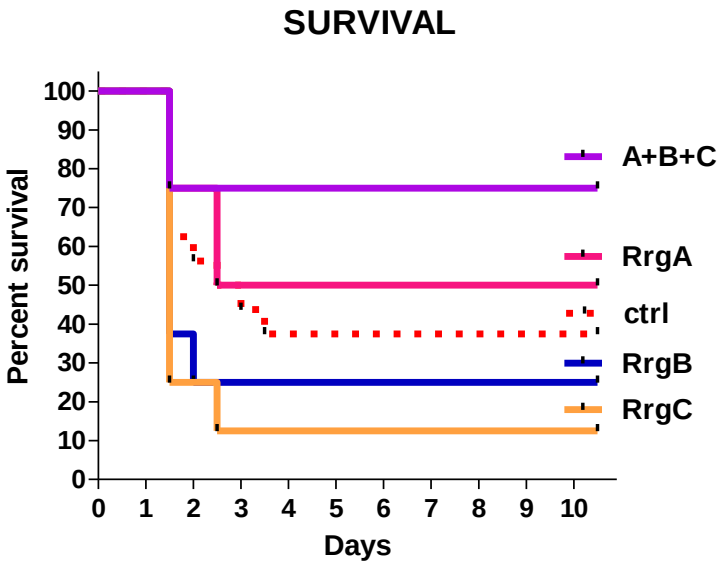
In the passive immunization experiment (Fig. 11B) mice received intraperitoneally immune sera raised against 6B pilus antigens (clade II) and challenge with TIGR4 strain (clade I). No protection was observed in the groups immunized with serum directed against single pilus subunits but, as observed in the active immunization experiment, significant protection against bacteremia and mortality was achieved in the group receiving the RrgA+B+C immune sera.

These *in vivo* results indicate that cross-protection is difficult to achieve with single pilus subunits.

A. ACTIVE IMMUNIZATION

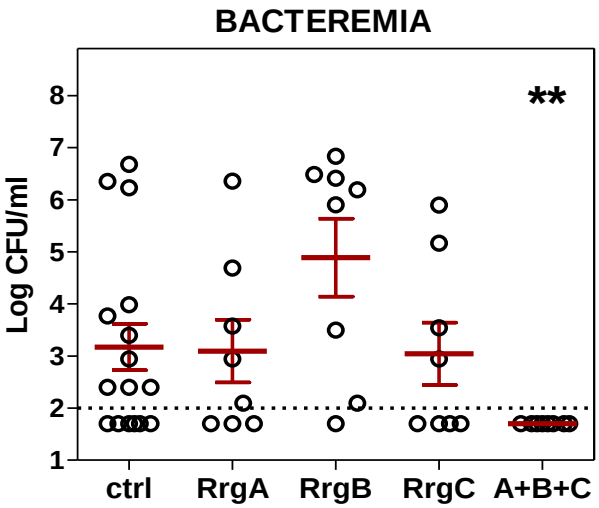
clade II antigens *vs.* clade I challenge strain

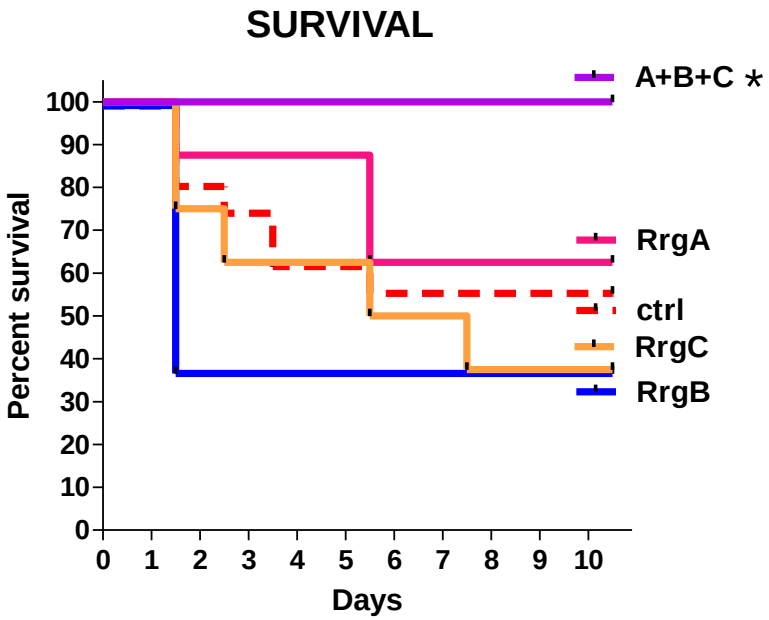




B. PASSIVE IMMUNIZATION

clade II antisera vs. clade I challenge strain





C

P values – ACTIVE IMMUNIZATION

	bacteremia	survival course	survival rate
RrgA	0.1144	0.2906	0.4389
RrgB	0.1635	0.1464	0.8581
RrgC	0.2802	0.0485(1)	0.9669
RrgA+B+C	0.0179	0.0962	0.0965

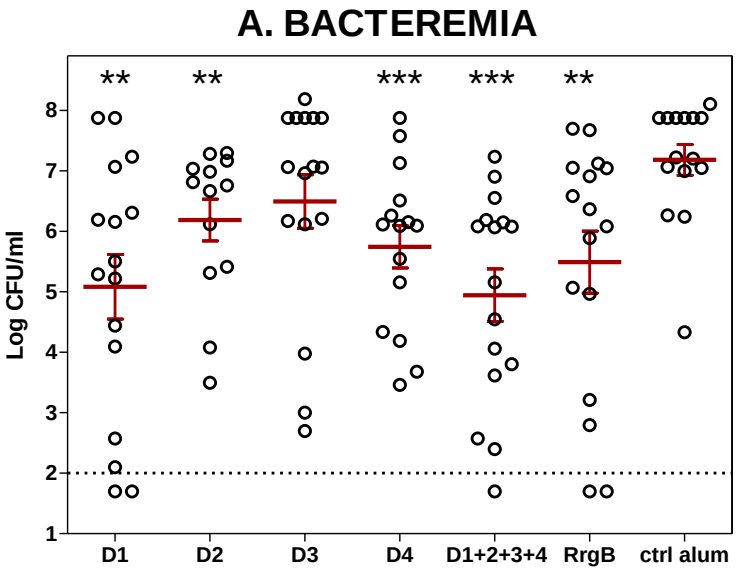
P values – PASSIVE IMMUNIZATION

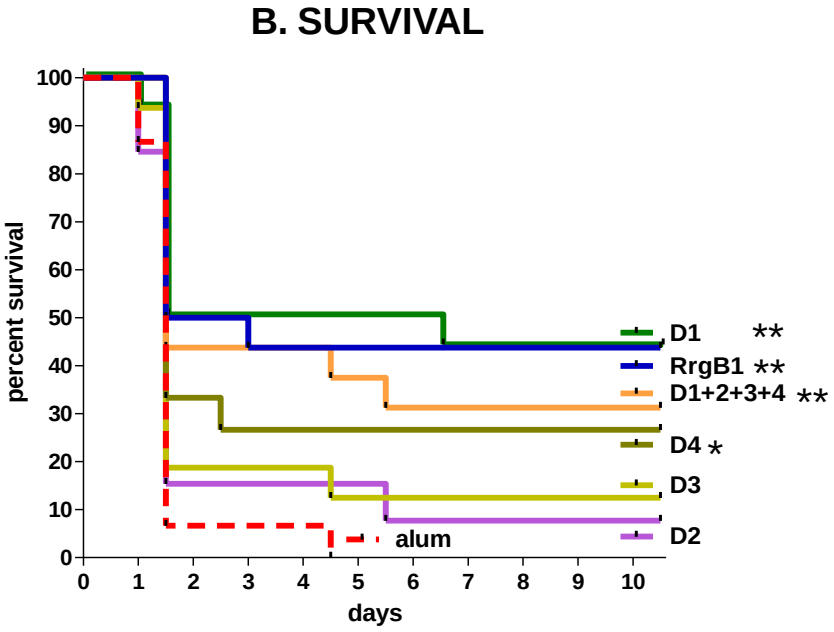
	bacteremia	survival course	survival rate
RrgA	0.5000	0.3033	0.5611
RrgB	0.0440(1)	0.0440(1)	0.9035
RrgC	0.3752	0.2768	0.9035
RrgA+B+C	0.0074	0.0097	0.0331

FIG. 11: Single pilus subunits do not afford cross-protection. 8 mice for vaccinated groups and 16 mice for control group were used. (**A**) Active immunization experiment with 6B pilus antigens (clade II) against TIGR4 challenge (clade I). (**B**) Immune sera passive transfer. Mice immunized with antisera raised against 6B pilus antigens were challenged with TIGR4 strain. (**C**) *P* values for bacteremia, survival course and survival rate are reported.
(1) values almost significantly worse than those for the control group were observed. Symbols are explained in Fig. 7 legend.

4.6 RrgB domains are protective in active immunization

Recently, the structure of a major portion of RrgB (residues 184–627) was solved at a 1.6 Å resolution [**48**] and revealed an organization into three independently folded IgG-like domains (D2, D3, and D4, residues 184–326, 326–446, and 446–627, respectively), stabilized by intramolecular isopeptide bonds. On the contrary, the structure of the RrgB N-terminal region (D1, residues 1–184), likely constituting a fourth independently folded domain, remained unsolved due to the failure to obtain the crystals of the full-length (FL) RrgB. D1 structure was then solved by NMR [**49**]. The spectra analysis revealed that D1 has many flexible regions, does not contain any intramolecular isopeptide bond and shares with the other domains an Ig-like fold.





C

P values

	bacteremia	survival course	survival rate
D1	0.0014	0.0054	0.0043
D2	0.0048	0.3578	0.4643
D3	0.1331	0.1283	0.2581
D4	0.0009	0.0144	0.0498
D1+D2+D3+D4	<0.0001	0.0037	0.0257
RrgB	0.0014	0.0018	0.0043

FIG. 12: RrgB domains show protective efficacy. 16 mice in each group were used. The immunizations were performed using Alum as adjuvant. The positive control of full-length RrgB was also included. (**A**) Bacteremia (**B**) Survival course. (**C**) *P* values for

bacteremia, survival course and survival rate are reported. Symbols are explained in Fig. 7 legend.

We investigated the protective ability of the single recombinant D1, D2, D3, and D4 domains of RrgB in a mouse model of sepsis. The results obtained are shown in Fig. 12. Among the single RrgB domains, D1 and D4 showed the most significant protective efficacy both in terms of reducing bacteremia and increasing survival. D2 showed a partial trend of protection with a significant reduction of bacteremia, but not a significant increase of survival. The combination D1+D2+D3+D4, as expected, afforded high protection similar to that afforded by full-length RrgB.

4.7 Five RrgB fusion proteins were created with similar protective efficacy

To generate an antigen able to elicit an immune response directed against each of the three RrgB clades, five fusion proteins that included the full length three variants in different reciprocal positions (RrgB123, RrgB231, RrgB321, RrgB312 and RrgB213) were expressed and purified. The sixth possible combination (RrgB132) was not expressed by the expression system used, thus it was excluded from the study.

All the five RrgB chimeras were tested for their in vivo protective efficacy.

					RrgB 123	**RrgB 312**	**RrgB 231**	**RrgB 321**	**RrgB 213**
challenge strain (pilus clade)	imm. route	chall. route	adj.	challenge dose (CFU)	survival	survival	survival	survival	survival
TIGR4 (I)	**I.P.**	**I.P.**	FA	~1E+02	**+++**	**+++**	**++**	**+++**	**+++**
	I.P.	**I.V.**	FA	4,0E+06	**+++**	**+++**	**+++**	**+++**	**++**
6B-FinI.12 (II)	**I.P.**	**I.P.**	FA	1,8E+04	**-**	**-**	**-**	**-**	**-**
	I.P.	**I.V.**	FA	8,0E+07	**+++**	**+++**	**+++**	**++**	**+++**
35B-SME15 (III)	**I.P.**	**I.P.**	FA	~1E+04	**+**	**++**	**+**	**+**	**+**
	I.P.	**I.V.**	FA	6,0E+07	**+++**	**+++**	**+++**	**+++**	**++**

TABLE 1: protective efficacy of the 5 fusion proteins in the intraperitoneal (I.P.) or intravenous (I.V.) challenge model. +, ++ and +++ indicates *P* values respectively <0.01, <0.001 and <0.0001.

In Table 1 is summarized the protection obtained in the intraperitoneal and intravenous challenge models, in terms of survival course, against the three strains selected as representative for the 3 RrgB clades. We couldn't observe any striking difference in the protective ability among the 5 chimeras. In the intraperitoneal infection model a moderate protection was observed against 35BSME15 strain and no protection was observed against 6BFinland12 strain .

Among the five chimeras, RrgB321 was selected for further studies mainly for reasons other than the efficacy (expression level, yield and purity).

4.8 RrgB321 antisera recognize the three RrgB variants

To evaluate whether the RrgB321 antisera recognized the native pilus as efficiently as the recombinant proteins, the same sera were probed on entire bacteria by FACS analysis and on whole bacterial lysates by Western Blot. Strains TIGR4, 6B SPEC and 35B SME15, expressing a pilus containing RrgB variant I, II and III, respectively, were tested by FACS with sera raised against RrgA, the three RrgB variants and RrgB321 (Fig 13A).

The serum raised against the chimera recognized the pili expressed by the three stains to similar extent; in addition, for each strain the fluorescence intensity was comparable to that obtained with homologous RrgB clade specific antibodies. Strains lacking expression of pili were consistently negative by FACS analysis when probed with the same panel of sera (data not shown). Consistently with these data, RrgB321 antiserum recognized by WB analysis (performed on whole bacterial lysates) the typical High Molecular Weight pilus ladder of all the three pilus backbone variants (Fig. 13B).

Taken together, these data indicate that antibodies raised against the RrgB321 chimera are able to efficiently recognize the recombinant RrgB variants as well as the pilus backbone once incorporated into the pilus fiber.

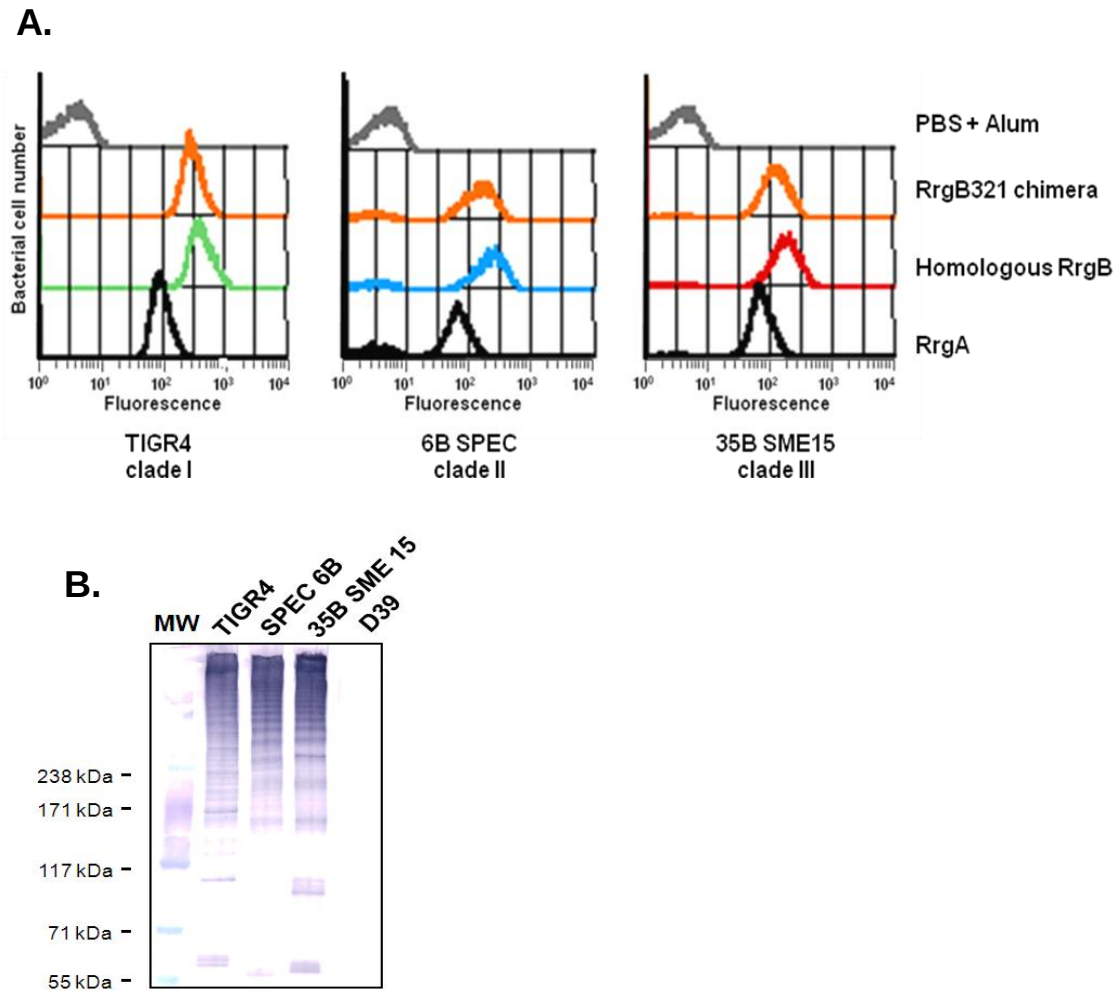


Figure 13: (A) Rabbit polyclonal antisera raised against RrgB of clade I, clade II and clade III and RrgB321 chimera were tested by FACS analysis on TIGR4, SPEC 6B and 35B SME15 strains expressing pilus of clade I, II and III, respectively. RrgA and PBS+alum antisera were used as positive and negative controls. **(B)** WB analysis on TIGR4, SPEC 6B and 35B SME15 whole lysates performed with rabbit RrgB321 antisera (1:10000 dilution) (D39, which is not expressing pilus, is used as negative control).

4.9 RrgB321 is protective against strains representative of the three pilus clades

To assess the protective efficacy of the RrgB321 chimera, the protein was tested in mouse models of sepsis where mice were immunized intraperitoneally with the recombinant protein and then challenged either intraperitoneally or intravenously with strains representative of the three pilus clades.

challenge			*N*	bacteremia		survival			
strain [RrgB clade]	dose (CFU/mouse)	route [a]	immunized / controls	Log reduction	*P* [b]	mean survival increase (days)	*P* [b]	% survival	*P* [d]
active immunization									
TIGR4 [1]	1,5E+02	IP	72 / 71	3,20	< 0.0001	4,4	< 0.0001	60	< 0.0001
TIGR4 [1]	3,1E+06	IV	66 / 62	2,26	< 0.0001	3,5	< 0.0001	21	0,0021
6B-Finland12 [2]	1,0E+08	IV	103 / 98	2,06	< 0.0001	3,7	< 0.0001	15	0,0336
35B-SME15 [3]	4,8E+07	IV	98 / 94	3,09	< 0.0001	6,7	< 0.0001	28	< 0.0001
passive immunization, mouse serum									
TIGR4 [1]	1,4E+02	IP	7 / 8	3,20	0,0019	6,6	0,0079	71	0,0019

[a] IP. = intraperitoneal; IV = intravenous
[b] Mann-Whitney U-test
[d] chi-square

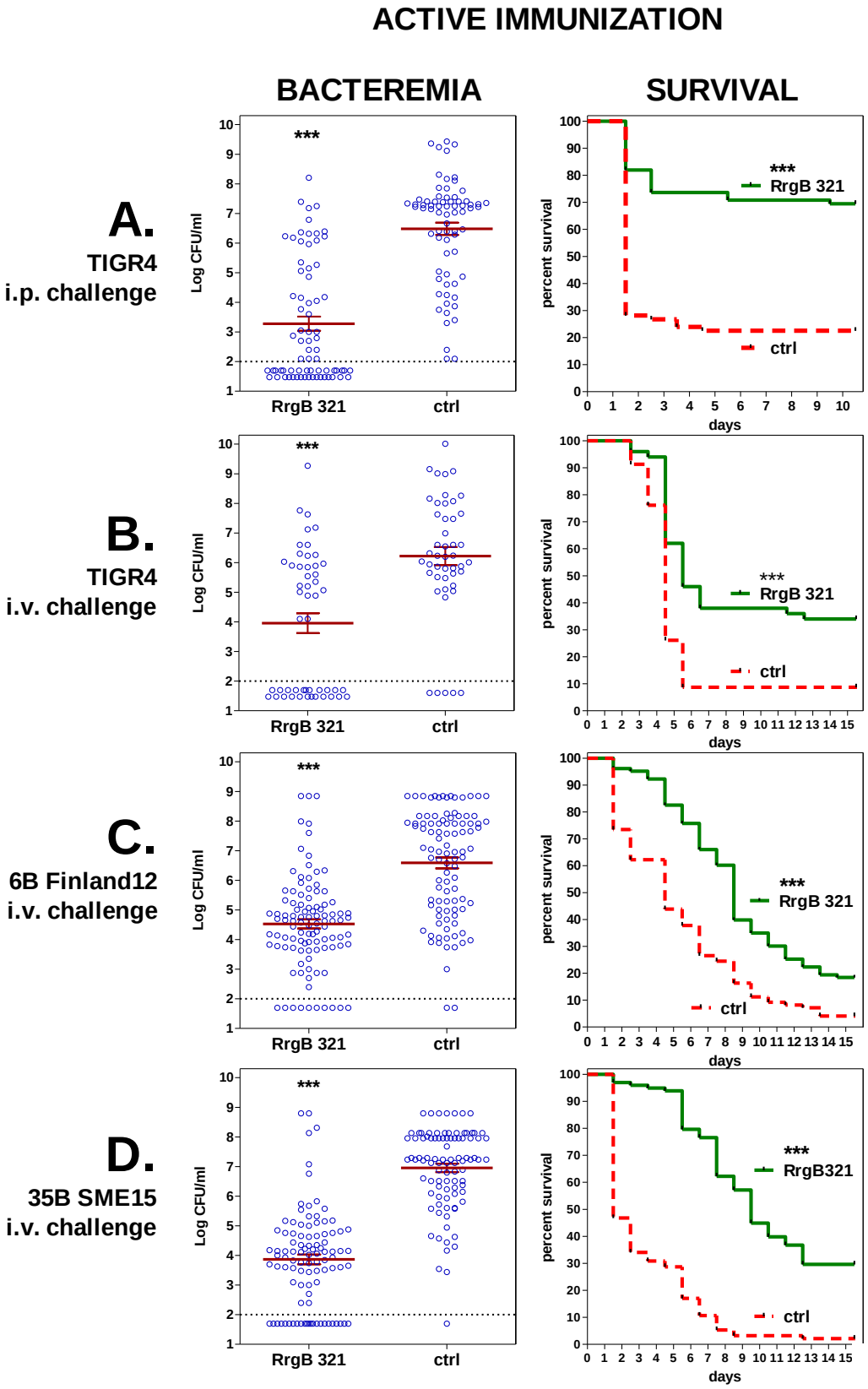
Table 2. *In vivo* results with RrgB321. The data originated from the combination of the results of three to seven independent experiments performed under the same conditions are shown. The challenge strains used were TIGR4, 6B Finland12 and 35B SME15, which express pilus of clade I, II and III, respectively.

As summarized in Table 2, RrgB321 immunization elicited protection against intraperitoneal challenge with TIGR4: the bacteremia for immunized group was > 3 Logs lower than that of the control group (3.28 vs. 6.48, $P < 0.0001$), the mean survival was 8.1 vs. 3.6 days ($P < 0.0001$), and a highly significant survival rate was found at the end of the observation ($P < 0.0001$).

Good protective efficacy was exerted by RrgB321 immunization also against intravenous challenge (Fig). Highly significant reduction of bacteremia ($P < 0.0001$) was obtained for each of the three strains tested. A mean Log CFU of 3.96 for the immunized group *vs.* 6.22 for the control group was found with TIGR4 challenge, 4.53 *vs.* 6.59 with 6B Finland12, and 3.87 *vs.* 6.96 with 35B SME15. Consistently with the reduced bacteremia, the mean survival was significantly increased ($P < 0.0001$) in immunized *vs.* the corresponding control groups: 8.8 *vs.* 5.3 days with TIGR4 challenge, 9.1 *vs.* 5.4 for 6B Finland12, and 10.2 *vs.* 3.4 for 35B SME15. Immunization with RrgB321 also resulted in significant ($P < 0.05$) survival rates at the end of the observation.

To determine whether the immunization with RrgB321 generates functional antibodies, experiments of passive serum transfer were performed. The administration to mice of mouse serum anti-RrgB321 before TIGR4 challenge resulted in significant reduction of bacteremia of > 3 Logs (mean Log CFU 3.20 vs. 6.41, $P < 0.01$), an increase of mean survival time by 6.3 days (7.9 vs 1.6 days, $P < 0.01$), and a significant survival rate ($P < 0.01$) (Table 2). These results indicate that specific antibodies are sufficient to achieve protection under the experimental conditions tested.

The protective efficacy of RrgB321 against the three strains TIGR4, 6B Finland12 and 35BSME15 is also showed in Fig. 14 where the results of active (panel A, B, C, D) and passive (panel E) immunization are presented.



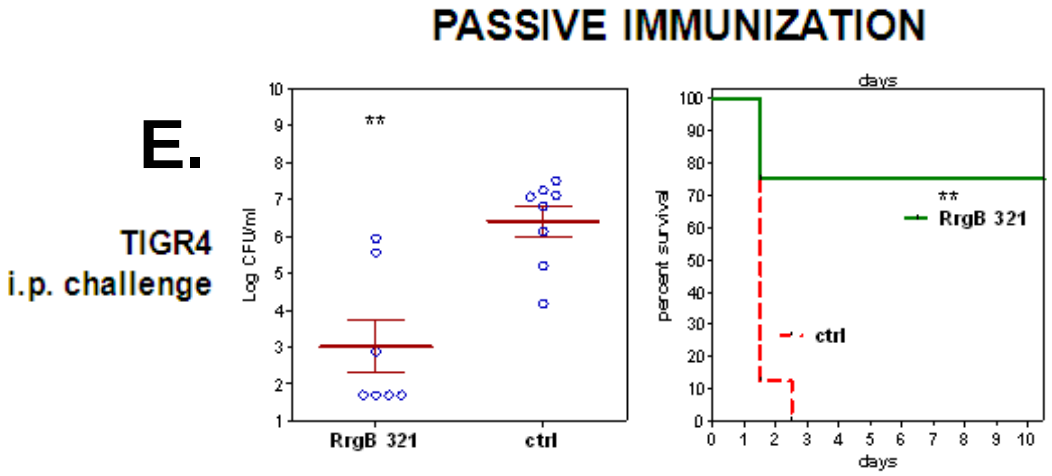


Figure 14: Protective efficacy afforded by active (panels **A-D**) or passive (panel **E**) RrgB321 immunization against intraperitoneal (IP, panels **A** and **E**) or intravenous (IV, panels **B-D**) challenge with the indicated pneumococcal strain. For active immunization mice were either immunized with RrgB321 or received alum plus saline (ctrl). For passive immunization mice received mouse anti-RrgB321 serum before challenge; the corresponding control groups received serum from mice immunized with alum plus saline. In the left panels values of bacteremia are shown, and in the right panels the survival course is represented. Symbols are explained in the Figure 7 legend.

5. DISCUSSION

The discovery that *S. pneumoniae*, like other Gram-positive bacteria such as Group A and Group B streptococci (GAS and GBS) [35, 36], possesses pili, led us to investigate the possible use of the pilus proteins as vaccines candidates. Indeed, both GAS and GBS pilus antigens were found to be protective against the corresponding pathogen [35, 50].

Pneumococcal pilus1 is composed by three proteins: RrgA, the pilus adhesin, RrgB, which constitutes the backbone, and RrgC, the putative anchor between the pilus and the bacterial membrane. RrgB is estimated to represent about the 90% of the pilus protein, while RrgA and C, defined as ancillary proteins, represent the remaining 10% [44].

To investigate the protective efficacy of pilus antigens, recombinant RrgA, RrgB, RrgC corresponding to the TIGR4 strain sequence were expressed, purified and tested as a vaccine in mouse models of *S. pneumoniae* infection. The 3 subunits raised comparable IgG levels and resulted to be highly immunogenic. Interestingly, also mouse serum directed against whole TIGR4 bacteria recognized recombinant pilus antigens, but in this case differences could be observed among the three subunits. The highest response was directed against RrgB, consistently with the fact that RrgB is more abundant than the ancillary subunits in the native pilus structure and indicating that it is well exposed.

The protective efficacy of the recombinant pilus antigens was then tested in mouse models of sepsis using intraperitoneal (i.p.) or intravenous (i.v.) challenge route. Protection was achieved in both models of infection; in particular, RrgB elicited a protection similar to that obtained with heat-inactivated bacteria, which represented our positive control. RrgA resulted protective in the i.p. infection model, but not in the i.v. infection model. RrgC afforded significant protection in the i.v. challenge model, but only a non-significant protective trend in the i.p. challenge model. Protection was also observed in passive immunization experiments followed by i.p. challenge, suggesting an important role of antibodies in the mechanism of protection. In the passive protection experiments the sera directed against RrgA, RrgB, RrgC showed similar protective ability.

Considering the *in vivo* results, the pilus backbone protein RrgB was preferred because it was able to reproducibly confer significant protection in both sepsis

models and both by active and passive immunization. Conversely, although generally protective, RrgA or RrgC showed lower efficacy than RrgB.

When the surface exposure of the three pilus subunits was investigated by FACS analysis, we observed that, while RrgB and RrgA were recognized on the bacterial surface, RrgC was not, meaning that RrgC is not accessible to antibodies in the native pilus structure. This observation, together with the *in vivo* results, allowed us to de-prioritize RrgC as vaccine candidate.

According to all the results obtained, RrgB was still considered the best vaccine candidate among the pilus proteins. However, among the pilus subunits, RrgB presented the disadvantage of having the highest level of variability. In fact the three subunits present different levels of sequence conservation: RrgB is the most variable subunit existing in three different variants (clade I, II and III), RrgA exists in two major variants and RrgC is well conserved. Thus, we selected strains able to infect mice expressing each of the different pilus clades, and we investigated whether cross-immunoreaction and cross-protection could be obtained.

FACS together with WB analysis revealed, consistently with the percentage of homology, that sera directed against RrgB clade I could not recognize strains expressing RrgB clade II or clade III and *vice versa*, while a weak cross-reaction could be observed between sera directed against RrgB clade II and RrgB clade III expressing strain. The two clades of RrgA were cross-reactive both in WB and FACS. RrgC was fully cross-reactive in WB being conserved.

In vivo cross-protective experiments between clade I and II were performed and revealed that significant protection could be afforded only by the combination RrgA+B+C, whereas no cross-protection could be observed with RrgB and RrgC. A protective trend was instead achieved with RrgA.

At this stage, despite RrgA showed better cross-protective ability than RrgB, the latter was finally selected as potential vaccine candidate for the reasons already mentioned, *i.e.* 1) RrgB is the most abundant subunit; 2) RrgB elicited the highest antibody response upon immunization with inactivated bacteria; 3) RrgB was well detectable on the bacterial surface; 4) in both the mouse sepsis models used RrgB reproducibly afforded high protection. We considered that the lack of cross-protective ability among the RrgB clades could be overcome by the construction of a fusion protein encompassing the sequences of the three RrgB clades.

In order to design the fusion protein, we aimed at determining if the protective efficacy of RrgB was restricted to a limited domain of the protein. The structure of RrgB was solved and it resulted to be composed by four domains: D1, D2, D3, and D4. The protective efficacy of each of the domains was tested and it was found that it was not restricted to a well-defined region. In fact, most of the efficacy was retained by D1 and D4 domains, but also D2 contributed to the protective efficacy. Based on these results, we decided to include all four domains in the chimera construction.

Six possible constructs resulted from the combination of the three clades in different reciprocal positions. Cloning and expression in *E. coli* was successful for five of the constructs. Due to similar protective efficacy afforded by the 5 fusion proteins, the final candidate RrgB321 (*i.e.* containing RrgB clade III, II, and I in this order from N- to C-terminal of the fusion protein) was selected on the basis of the productivity, yield and final purity of the recombinant protein.

It was found by FACS and WB that antibodies raised against RrgB321 recognized the native pilus of each of the three clades. RrgB321 was then tested for its protective efficacy against three piliated strains belonging to each of the three RrgB clades and was found highly protective overall, both in active and passive immunization. These observations supported the rationale for the construction and the use of this fusion protein as a vaccine.

In conclusion, we demonstrated that the fusion protein RrgB321 is able to afford high levels of protection in mouse models of *S. pneumoniae* infection. Moreover, immunization with RrgB321 has been recently shown to elicit potent opsonophagocytic activity in mouse sera, to an extent comparable to that obtained with sera raised against conjugated polysaccharides [**51**], reinforcing the data of protection obtained *in vivo*. Further data have been recently published providing evidence that RrgB321 can afford protection against piliated strains also in the mouse intranasal challenge model [**52**]. The high protective efficacy of RrgB321 makes it a good vaccine candidate against pneumococcal isolates possessing pili. RrgB321 can also be proposed as part of a multi-component vaccine against S. pneumoniae, following a strategy that has been already proposed for S. pneumoniae [**29**] as well as successfully adopted for other pathogens [**53**].

6. REFERENCES

1. Stenberg G. A fatal form of septicemia in the rabbit, produced by the subcutaneous injection of human saliva. *Natl Board Health Bull* 2:781-783, 1881

2. Pasteur L. Note sur la maladie nouvelle provoquée par la salive d'un enfant mort de la rage. *Bull Acad Med* 10:94-103, 1881

3. Buchanan RE, Gibbons NE. Bergey's manual of determinative bacteriology, 8th edition, Williams and Wilkins Co., Baltimore, MD, 1974

4. Avery OT, MacLeod CM, and McCarty M. Studies on the chemical nature of the substance inducing transformation of pneumococcal types.; *J Exp Med* 79:137-158, 1944.

5. Henrichsen J. Six newly recognized types of *Streptococcus pneumoniae*. *J Clin Microbiol* 33(10):2759-2762, 1995

6. Bridy-Pappas AE *et al*. *Streptococcus pneumoniae*: description of the pathogen, disease epidemiology, treatment, and prevention. *Pharmacotherapy* 25(9):1193-212, 2005

7. Gray BM, Dillon Jr HC. Epidemiological studies of *Streptococcus pneumoniae* in infants: antibody to types 3, 6, 14, and 23 in the first 2 years of life. *J Infect Dis* 158(5):948-55, 1988

8. Ekdahl K *et al*. Duration of nasopharyngeal carriage of penicillin-resistant *Streptococcus pneumoniae*: experiences from the South Swedish pneumococcal intervention project. *Clin Infect Dis* 25:1113-17, 1997

9. WHO: Pneumococcal vaccines. World Health Organization Wkly Epidemiol Record 12:93-140, 2007

10. Kalin M. Pneumococcal serotypes and their clinical relevance. *Thorax* 53(3):159-162, 1998

11. MacCormick AW *et al*. Geographic diversity and temporal trends of antimicrobial resistance in *Streptococcus pneumoniae* in the United States. *Nat Med* 9(4):424-30, 2003

12. Neuhaus FC, Baddiley J. A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in gram- positive bacteria. *Microbiol Mol Biol Rev* 67(4):686-723, 2003

13. Martin M *et al*. An outbreak of conjunctivitis due to atypical *Streptococcus pneumoniae. N Engl J Med* 348(12):1112-1121, 2003

14. Watson DA, Musher DM. Interruption of capsule production in *Streptococcus pneumoniae* serotype 3 by insertion of transposon Tn916. *Infect Immun* 58(9): 3135-3138, 1990

15. Wright AE *et al*. Observations on prophylactic inoculations against pneumococcus infection and on the results which have been achieved by it. *Lancet* 1:1-10, 87-95, 1914

16. MacLeod CM *et al*. Prevention of pneumococcal pneumonia by immunization with specific capsular polysaccharides. *J Exp Med* 82:445-465, 1945.

17. Austrian R, Gold J. Pneumococcal bacteremia with especial reference to bacteremic pneumococcal pneumonia. *Ann Intern Med* 60:759-776, 1964.

18. Cowan MJ *et al*. Pneumococcal polysaccharide immunization in infants and children. *Pediatrics* 62:721-727, 1978.

19. Huss A *et al*. Efficacy of pneumococcal vaccination in adults: a meta-analysis. CMAJ 180(1):48-58, 2009

20. Hange WP. Serotype-specific problems associated with pneumococcal conjugate vaccination. *Future Microbiol*. 3:23-30, 2008

21. Dagan R. Serotype replacement in perspective. *Vaccine* 27(Suppl. 3):C22-C24

22. Frias MJ, Melo-Cristino J, Ramirez M. The autolysin LytA contributes to efficient bacteriophage progeny release in *Streptococcus pneumoniae*. *J Bacteriol*, 191(17):5428-40, 2009

23. Eldholm V *et al*. Fratricide in *Streptococcus Pneumoniae*: contributions and role of the cell wall hydrolases CbpD, LytA and LytC. *Microbiol* 155:2223-34, 2009

24. Briles DE *et al*. PspA and PspC: their potential for use as pneumococcal vaccines. *Microb. Drug Resist.* 3:401-408, 1997

25. Jedrzejas MJ, Lamani E and Becker RS. Characterization of selected strains of pneumococcal surface protein A. *J. Biol. Chem.* 276:33121-33128, 2001

26. Dave S *et al*. Dual role of PspC, a surface protein of *Streptococcus pneumoniae*, in binding human secretory IgA and factor H. *J Immunol* 173(1):471-7, 2004.

27. Jedrzejas MJ. Pneumococcal virulence factors: structure and finction. *Microbiol Mol Biol* 65(2):187-207, 2001

28. Hakenback R *et al*. Versatility of choline metabolism and choline-binding proteins in *Streptococcus pneumoniae,* and commensal streptococci. *FEMS Microbiol* 33(3):572-86, 2009.

29. Giefing C, Meinke AL, Hanner M *et al*. Discovery of a novel class of highly conserved vaccine antigens using genomic scale fingerprinting of pneumococcus with human antibodies. J Exp Med 205(1):117-31, 2008

30. Di Guilmi AM and Dessen A. New approaches towards the identification of antibiotic and vaccine targets in *Streptococcus pneumoniae. EMBO reports* 3(8):728-734, 2002.

31. Gaspar AH and Ton-That H. Assembly of distinct pilus structures on the surface of *Corynebacterium diphtheriae. J Bacteriol* 188:1526-33, 2006

32. Thon That H and Schneewind O. Assembly of pili on the surface of *Corynebacterium diphtheriae*. *Mol Microbiol* 50:1429-38, 2003

33. Levesque C *et al*. *Streptococcus salivarius* fimbriae are composed of a glycoprotein containing a repeated motif assembled into a filamentous nondissociable structure. *J Bacteriol* 183:2724-2732, 2001

34. Fives-Taylor PM and Thompson DW. Surface properties of *Streptococcus sanguis* FW213 mutants nonadherent to saliva-coated hydroxyapatite. *Infect Immun* 47:752-759, 1985

35. Mora M *et al.* Group A *Streptococcus* produce pilus-like structures containing protective antigens and Lancefield T antigens. *Proc Natl Acad Sci USA.* 102:15641-15646, 2005

36. Lauer P *et al*. Genome analysis reveals pili in Group B *Streptococcus*. Science 309:105, 2005

37. Barocchi MA *et al*. A pneumococcal pilus influences virulence and host inflammatory responses. *Proc Natl Acad Sci USA* 103:2857-2862, 2006

38. McNab R *et al*. Cell wall-anchored CshA polypeptide (259 kilodaltons) in *Streptococcus gordonii* forms surface fibrils that confer hydrophobic and adhesive properties. *J Baceriol* 181:3087-3095, 1999

39. Willcox MD and Drucker DB. Surface structures, co-aggregation and adherence phenomena of *Streptococcus oralis* and related species. *Microbios* 59:19-29, 1989

40. Yanagawa R and Otsuki K. Some properties of the pili of *Corynebacterium renale. J Bacteriol* 101:1063-1069, 1970

41. Bagnoli F *et al*. A second pilus type in *Streptococcus pneumoniae* is prevalent in emerging serotypes and mediates adhesion to host cells. *J Bacteriol* 190:5480-5492, 2008

42. Telford JL *et al.* Pili in Gram-positive pathogens. *Nat Rev Microbiol* 4:509-519, 2006

43. El Mortaji L *et al*. Stability and assembly of pilus subunits of *Streptococcus pneumoniae*. *J Biol Chem* 285:12405-12415, 2010

44. Hilleringmann M *et al*. Pneumococcal pili are composed of protofilaments exposing adhesive clusters of RrgA. *PLoS Pathog* 4(3):e1000026, 2008

45. Hilleringmann M *et al*. Molecular architecture of *Streptococcus pneumoniae* TIGR4 pili. *EMBO J* 28:3921-3930, 2009

46. Nelson AL *et al*. RrgA is a pilus-associated adhesin in *Streptococcus pneumoniae*. *Mol Microbiol* 66:329-40, 2007

47. Moschioni M *et al*. *Streptococcus pneumoniae* contains 3 *rlr*A pilus variants that are clonally related. *J Infect Dis* 197:888-896, 2008

48. Spraggon G *et al.* Supramolecular organization of the repetitive backbone unit of the *Streptococcus pneumoniae* pilus. *PLoS One* 5(6):e10919, 2010

49. Gentile MA *et al.* Structural and functional characterization of the *Strptococcus pneumoniae* RrgB pilus backbone D1 domain. *J Biol Chem* 286(16):14588-97, 2011

50. Margarit I *et al*. Preventing bacterial infections with pilus-based vaccines: the group B streptococcus paradigm. *J Infect Dis* 199:108-15, 2009

51. Harfouche C *et al*. RrgB321, a fusion protein of the three variants of the pneumococcal pilus RrgB, is protective in vivo and elicits opsonic antibodies. *Infect Immun,* in press

52. De Angelis G *et al*. The *Streptococcus pneumoniae* pilus-1 display a biphasic expression pattern. *PLoS One* 6:e21269

53. Maione D *et al*. Identification ofa universal group B *Streptococcus* vaccine by multiple genome screen. *Science* 309:148-150