Antigenic cross reactivity between the pneumococcal polysaccharide vaccine and the *Streptococcus pneumoniae* 7F one of the prevalent serotypes in Bangladesh

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DEDICATED TO

ALL OF MY IDOLS

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APS	Ammonimum persulfate
AOM	Acute Otitis Media
ATCC	American Type Culyure Collection
BCIP	5-bromo 4-chloro 3-indolyl phosphate
^{0}C	Degree centigrade
CDC	Center for disease control and prevention
CFU	Colony forming unit
C3	Third component of complement
CBP	Cholin-Binding Protein
CbpA	Cholin-Binding Protein A
CI	Confidence Interval
CMIS	Common Mucosal Immune System
CPS	Cell Wall Polysaccharide
CSF	Cerebrospinal fluid
CV	Coefficient of variation
EIA	Enzyme Immunoassay
et al.	And others
Fig.	Figure
FBS	Fetal Bovine Serum
FinOM	Finnish Otitis Media
GM	Geometric mean
GMC	Geometric mean concentration
gm	Gram
Hr.	Hour
Hic	Factor H-binding inhibitor of complement
HIV	Human Immunodeficiency Virus
ICDDR, B	International Center for Dirrhoeal Diseases Research, Bangladesh
Ig	Immunoglobin
IgA	Immunoglobin class A
IgG	Immunoglobin class G
IgM	Immunoglobin class M
IL	Interleukin
KDa	Kilo Dalton
KTL	Kansanterveyslaitos (Ntional Publich Health Institute)
LytA	Major pneumococcal autolysin

lbs	Pound
LPS	Lipopolysaccharide
M	Molar
mg	Milligram
ml	Milliliter
min	Minute
mM	Milimolar
mA	Milliampere
mm	Millimeter
MALT	Mucosa-associated lymphoid tissue
MEF	Middle ear fluid
MW	Molecular weight
NBT	Nitro Blue Tetrazolium Salt
N	Normal
NP	Nasopharyngeal swab
NPA	Nasopharyngeal aspirate
OD	Optical density
OMPC	Outer membrane protein complex
OR	Odds ratio
PAF	Platelet-activating factor
PbcA	C3-binding protein A
PBP	Penicillin-binding protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Ply	Pneumolysin
Pnc	Pneumococcal
PncCRM7	Pneumococcal capsular polysaccharide-CRM_{197} conjugate vaccine
PncOMPC	Pneumococcal capsular polysaccharide-meningococcal outer membrane protein complex conjugate vaccine
PS	Polysaccharide
PS14	Capsular polysaccharide of pneumococcal serotype 14
PsaA	Pneumococcal surface adhesin A
PspA	Pneumococcal surface protein A
PspC	Pneumococcal surface protein C
R_f	Relative mobility

RPM	Revolution per minute
RR	Relative risk
SC	Secretory component
S. pneumoniae	*Streptococcus pneumoniae*
SDS	Sodium Dodecyl Sulfate
SD	Standard deviation
sIg	Secretory immunoglobulin
SpsA	Streptococcus pneumoniae secretory IgA binding protein
ST	Serotypoe
TD	T cell dependent, thymus-dependent
TI	T cell independent, thymus-independent
TEMED	N,N,N',N' Tetramethyl-ethylenediamine
U/ml	Units per milliliter
µg	Microgram
µl	Microliter

ABSTRACT

Streptococcus pneumoniae (the pneumococcus) is a human pathogen that causes life threatening, invasive disease such as pneumonia, bacteremia, meningitis with high morbidity and morbidity throughout the world; young children and the elderly are particularly susceptible. In developing countries as estimated five million children under the age of five years died each year from pneumonia, with *S. pneumoniae* bring the single most common causative agent. In Bangladesh it is a common cause of pneumonia and the second leading cause of meningitis with high mortality. The present study was carried out to demonstrate antigenic cross reactivity between the pneumococcal polysaccharide vaccine and the *Streptococcus pneumoniae* 7F. Here the serotype 7F was used because it is the rank 1 serotype of *S. pneumoniae* in Bangladesh. In this present study SDS-PAGE was done for the detection of protein bands of the water extracted surface materials of *S. pneumoniae* 7F and pneumococcal polysaccharide vaccine. To ensure whether the proteins were really immunogenic or not, they were subjected to Western blot analysis. In case of Western blot analysis, 41, 15 and 17 KDa proteins were found that ensure the antigenic cross reactivity between the vaccine and the whole cell extract of *S. pneumoniae* 7F, one of the prevalent serotypes in Bangladesh.

INTRODUCTION

Streptococcus pneumoniae (pneumococcus) continues to be a leading cause of morbidity and mortality worldwide. Along with a number of invasive infections such as pneumonia, meningitis and sepsis, pneumococcus causes local infections such as sinusitis and acute otitis media (AOM). Although usually a self-healing infection, AOM is a marked health problem among infants and young children resulting in a burden on health services and respectable inconvenience for the young patients and their families.

The vaccine development against pneumococcus begun a long time ago and it still continues. The Pneumococcal (Pnc) 23-valent capsular polysaccharide (PS) vaccine is efficacious among healthy adults, but it induces poor antibody responses in young children. Covalent conjugation of Pnc PS antigens to a protein carrier has improved their immunogenicity in children. However, the serotype selection of Pnc conjugate vaccines is restricted and the protection against overall Pnc AOM incidence is therefore modest. These realities have stimulated an interest in the development of vaccines based on common Pnc protein antigens. The crucial information still lacking, is whether human mucosal antibodies to Pnc protein vaccine candidates would have a role in the prevention of Pnc diseases.

1. *Streptococcus pneumoniae*

1.1. The causative agent for pneumonia

S. pneumoniae is one of the major causes of bacterial pneumonia in children worldwide, particularly in light of rapidly developing bacterial resistance to commonly used antibiotics. It is a general term used to describe inflammation of the lungs, in which fluid fills the alveoli, or air sacs, severely restricting breathing.

The symptoms of acute lower respiratory tract infection appear fairly suddenly and include a fever, localized pain in the chest, rapid, shallow and wheezy breathing with grunting and flared nostrils, the drawing in of the body below the chest and a cough which often produces yellow-green mucus. Babies with severe pneumonia may be cyanosed (that is, have blue lips and fingers), often cannot feed and will probably have an enlarged liver. These symptoms are the result of the body's reaction to an invasion of the lungs by one or more of a number of bacteria (Ian *et al.*, 2002).

S. pneumoniae, the leading cause of childhood pneumonia and meningitis, accounts for 20 to 40% of the estimated annual global burden of 2.7 million childhood death from pneumonia per year in developing countries (Mulholland *et al.*, 1999). It is also a common cause of pneumonia and meningitis, with high mortality in Bangladesh (Rahman *et al*, 1990). Other than meningitis, *S. pneumoniae* is a major cause of commonly acquired pneumonia of pediatric patients in the developing and developed countries, which carries a high rate of mortality (Frederikson *et al.*, 1988) other than these diseases, the organism also causes them diseases like parulentorifis media, sepsis etc. (Chowdhury, 1983).

S. pneumoniae is an antigenically diverse species in which more than 90 serotypes have been identified. However, the prevalence with which the serotypes are recovered from patients with invasive disease varies greatly, presumably because some serotypes have a much greater propensity to cause invasive disease than others. (Hausdorff *et al.*, 2000). The geographical distribution and prevalence of serotypes differ among the United States, Europe, and some Asian countries (Lee, 1987). Twenty serotypes are responsible for about 90% of all reported infections in United States and Europe, whereas, for instance, the 23-valent whole polysaccharide vaccine, which contains these serotypes, is effective against less than 70% of the pneumococcal infections in Asia (Lee, 1987; Van Dam *et al.*, 1990).

Population based studies conducted over the past two decades have suggested that the incidence of pneumococcal bacteremia and meningitis are on the rise. Investigations conducted during the past 10 years have generally found 2 to 3 fold higher rates of bacteremia among adults compared to rates found in earlier studies (Chi and Lee, 1998). Incidence rates due to *S. pneumoniae* are particularly high in the developing countries compared to the rates in industrialized nations. The rates of incidence in the United States are estimated at 25% of all community-acquired pneumonia. Septicemia can develop independently or as a complication of pneumonia and is associated with a mortality rate of 17% to 25% (Shann, 1995).

1.2. General description

S. pneumoniae is an exclusively human pathogen, which was isolated for the first time over 120 years ago, in 1881. Before the era of antibiotics, Pnc diseases, particularly Pnc pneumonia, were common causes of death. Nowadays, pneumococcus continues to be an important cause of both mucosal and systemic diseases worldwide. Pneumococcus has been the subject of intensive investigation for many decades. This has generated many scientific discoveries,

including the comprehension of DNA being a carrier of genetic information (Avery *et al.*, 1931), the therapeutic efficacy of penicillin (Abraham *et al.*, 1941; Keefer *et al.*, 1943), the role of the bacterial capsule in resistance to phagocytosis (Issaef, 1893), and the ability of bacterial polysaccharides (PS) to induce protective antibodies (Felton *et al.*, 1955; Baker, 1990). Despite all intensive investigation, many questions concerning the mechanisms of the Pnc pathogenesis and the immunology against Pnc infections still remain to be answered. Furthermore, a new challenge is now being faced by the appearance and spread of Pnc strains, which are resistant to one or more antibiotic drugs.

Pneumococcus is a gram-positive, encapsulated, facultative anaerobic, lancet-shaped coccus, which usually grows in pairs or in short chains. The three major surface layers that can be distinguished on the surface of pneumococci are plasma membrane, cell wall, and PS capsule. On the basis of the differences in the structure of the PS capsule, pneumococci can be divided into more than 90 different serogroups or serotypes. Two nomenclature systems have been used to classify these types. In the American nomenclature, the serogroups or serotypes are designated in the order of their discovery. In contrast, in the Danish nomenclature, the serogroups or serotypes are designated according to the structural and antigenic characteristics of the capsule, e.g., the serologically cross-reactive serotypes 6A and 6B are placed in the same serogroup 6 (Lund *et al.*, 1978). Nowadays, the Danish nomenclature is more widely adopted. The Danish nomenclature was used in the present study.

The complete DNA sequence of *S. pneumoniae* (serotype 4 from a child with meningitis) was published for the first time in November 1997. The opportunity to exploit the whole genome sequence of pneumococcus should allow the rapid discovery of regulatory networks and therapeutic targets for this pathogen, as well as new candidates for vaccine development (Hoskins *et al.*, 2001; Wizemann *et al.*, 2001).

1.3. Identification of pneumococcus

The laboratory culture of pneumococci requires multiple nutritional factors, carbon dioxide (CO_2) and an ideal pH of 7.2 to 7.4. On solid media pneumococci grow characteristically as flat round colonies with depressed centers. When grown on media containing blood, partial α-hemolysis of surrounding erythrocytes is detected. The identification of isolates with appropriate colonial morphology can be performed with a number of conventional biochemical and/or immunochemical tests, and in most cases the identification of pneumococci is rather

straightforward. At the moment, however, no "gold standard" method is available for the identification of pneumococci.

The conventional methods for the identification of pneumococcus are based on optochin sensitivity and bile solubility of the organism. Sensitivity to optochin (ethylhydrocupreine: a derivative of quinine) is the most important identification criterion for pneumococcus (Lund & Henrichsen, 1978; Ruoff *et al.*, 1999). On the plate inoculated with pneumococci, an inhibition zone appears around the optochin disk. This test is the most frequently used method to identify pneumococci in clinical laboratories (Kaijalainen *et al.*, 2002). As an additional test, the bile solubility test can be used to confirm the result. Addition of bile salts on a broth culture of pneumococci results in prompt dissolution of the bacteria due to the activation of peptidoglycan degrading autolysin enzyme. The other α-hemolytic streptococci are generally resistant to optochin and bile insoluble.

The presence and type of the Pnc PS capsule can be determined by using several immunochemical tests. The capsular swelling reaction (Neufeld's Quelling reaction) is an old-time, but still useful method to identify Pnc isolates rapidly and with high certainty. Equal volumes of bacterial suspension, methylen blue and antiserum are mixed on a glass slide, and the bacteria are examined under a light microscope.

Appearance of capsular swelling identifies genus, species and serotype. The presence of capsular PS can also be established by immunochemical methods, e.g. latex agglutination and counter immuno electrophoresis (CIEP). Furthermore, several rapid tests are also available for the identification of Pnc antigens directly in clinical samples, e.g., in blood, sputum, urine and cerebrospinal fluid (CSF).

In the 1990s, new possibilities for the identification of pneumococcus were introduced by the development of novel gene amplification methods for detection of pneumococci directly from the clinical samples (Virolainen *et al.*, 1994; Murdoch *et al.*, 2003). For example, demonstration of the gene for the Pnc virulence protein pneumolysin (Ply) by polymerase chain reaction (PCR) method has been used to detect and identify pneumococci in reference laboratories (Salo *et al.*, 1995; Kearns *et al.*, 2000). The PCR analysis for the gene of Pnc surface protein A (psaA) has shown to be a sensitive tool for diagnosis of Pnc pneumonia (Scott *et al.*, 2003). Hybridization methods have also been used for the identification of pneumococcus (Pozzi *et al.*, 1989). Furthermore, the molecular characterization of Pnc isolates

by multi locus sequence typing (MLST) has now become available (Enright *et al.*, 1998; Meats *et al.*, 2003). The above-mentioned methods, however, have not yet been introduced in routine use for the identification of Pnc isolates.

1.4. Characteristics of *S. pneumoniae*

1.4.1. Morphology and cultural characteristics of *S. pneumoniae*

S. pneumoniae are gram-positive lance-shaped 0.5 to 1.25 µm in diameter. They are encapsulated cocci, oval or spherical in shape. It occurs in singly, in pairs and in short chains. In diplococci, they are ovoid or lanceolate in shape with their distal narrow ends (Mackie and Mc.cartney, 1996). Continued laboratory cultivation, especially on unfavorable media, leads to the formation of larger chains. Gram positive reaction of young cells may be lost as culture ages and subsequently stains gram negative. They are non-spore forming, non-motile and non-acid fast. On blood agar, they produce typical a (alpha) haemolytic colonies. Autolysis of *S. pneumoniae is* greatly enhanced by surface-active agents.

The pneumococci have complex nutritional requirements. It can be grown on chemically defined synthetic media. But for primary isolation and routine culture, enriched infusion agar and broth such as tryptic soy brain heart infusion enriched with 5% defibrinated sheep or goat blood is recommended.

S. pneumoniae is fastidious bacterium, growing best in 5% carbon dioxide. Nearly 20% of fresh clinical isolates require fully anaerobic condition. The optimum pH and temperature for growth is 7.4 to 7.8 and 37°C respectively. A Candle Extinction jar or CO_2 incubator should be used for this purpose. The bacteria have a doubling time of 20 to 30 minutes under optimum condition. Normally, *S. pneumoniae* produce small, smooth and semi-transparent colonies about 1.0 mm in diameter with a haemolysis on blood agar. All pneumococci do not always produce large, flat and wet colonies with depressed centers (Converse and Dillon, 1977).

Colonies on blood agar plates are small, smooth and transparent, low convex while tiny, they become flattened or depressed centrally, showing the "Draughtsman form", as they grow older. Some strains e.g. of type 3, which form very large capsule, tends to remain convex (Mackie and Mc.cartney, 1996). A partial clearing of blood and a greenish discoloration, (α-haemolysis) is

produced underneath. A narrow zone around the colonies formed when they are incubated aerobically (Mackie and Mc.cartney, 1996).

Anaerobic incubation results in p-haemolysis due to pneumolysin '0' activity. Unlike other streptococci, the *S. pneumoniae* requires chlorine for growth in defined media. Ethanolamine replaces choline but need a molar basis.

1.4.2. Physiology

Different reducing agents are essential for *S. pneumoniae.* Most strains require at least 4 of the 13 vitamins for growth as well as adenine, guanine and uracil and 7 to 10 amino acids.

S. pneumoniae is an aero tolerant anaerobe and its energy-yielding metabolism-is fermentative producing primarily low levels of lactic acid. Under aerobic condition, a significant amount of hydrogen peroxide (H_2O_2) accumulates, with some degree of acetic and formic acids (Begey's Manual of Systemic Bacteriology, 1988).The growth requires a source of catalase (e.g. blood) to neutralize large amount of H_2O_2 produced by the bacteria.

S. pneumoniae does not produce catalase or peroxidase and the accumulation of hydrogen peroxide kills the organism unless culture is provided by addition of RBC to the culture media. The organism tends to die fairly quickly in cultures in course of a day or two, particularly in aerobic cultures in media without blood. The dead organisms tend to undergo autolysis (Mackie and McCartney, 1996).

1.5. Risk factors of developing pneumonia

Predisposing factors for pneumococcal pneumonia include immunodeficiency, crowded living situations, chronic lung diseases/ sickle cell disease, nephrotic syndrome, certain hematologic malignancies, previous or current serious illness, chronic inhalation of smoke and malnutrition. Certain racial groups such as American Indians, Native Alaskans and Australian Aboriginals have a higher incidence (6 to 34 times greater) of pneumococcal respiratory disease than do Caucasians. Socioeconomic conditions found in the developing world predispose to illness and mortality. It is estimated that in developing countries, 1 million deaths per year among children. <5 years of age (Ian *et al.*, 2002).

The problem of pneumococcal pneumonia is a multifaceted one. It occurs most frequently in patients least well equipped to handle the effects of the disease, namely, the very young or the very old, the immunosuppressed or chronically ill, in certain ethnic groups and in groups who are economically disadvantaged. In addition certain penicillin resistant and multidrug-resistant clones are rapidly gaining dominance as the major serotypes globally. The over all effect is that in the United States alone there are > 53 000 cases of pneumococcal pneumonia annually in all age groups. The greatest risk of pneumococcal pneumonia is usually among:

- Anyone with chronic lung or heart disease (not asthma, unless indicated by their doctor).
- Imrnunocompromised adults and children.
- Those diagnosed with chronic illnesses including HIV, sickle cell, asplenia (those without a spleen), lyrnphoma, multiple myeloma, diabetes, kidney or liver disease, and alcoholism.
- Special groups or environments such as nursing homes and long-term care facilities.
- Person with age 65 or older.

1.6. Pathogenesis of *S. pneumoniae*

The bacteria invade and grow primarily due to their resistance to the host phagocytic response. The cell wall components directly activate multiple inflammatory cascades including the alternative pathway of complement activation, the coagulation cascade, and the cytokine cascade, inducing interleukin-1, interleukin-6 and tumor necrosis factor from macrophages and other cells. It is now recognized that the pneumococcus colonizes the nasopharynx of up to 40% of healthy adults, and carriage of up to four serotypes for several months has been documented. Virtually all humans are colonized by pneumococci at some stage, and in certain population nasopharyngeal carriage rates -at any given time may exceed 70%.

S. pneumoniae is the leading cause of bacterial pneumonia beyond the neonatal period. Pleural effusion is the most common and empyema (pus in the pleural space) one of the most serious complications of *S. pneumoniae.* Dissemination from a respiratory focus results in serious disease: outpatient bacteremia in children, meningitis, and occasionally acute septic arthritis and bone infections in patients with sickle cell disease and, more rarely, peritonitis (especially in patients with nephritic syndrome) or endocarditis (Velasco *et al.*, 1995).

1.7. Pneumococcal Virulence Factors

Certain proteins or enzymes displayed on the surface of gram-positive organisms significantly contribute to pathogenesis and might be involved in the disease process caused by these pathogens. Often, these proteins are involved in direct interactions with host tissues or in concealing the bacterial surface from the host defense mechanisms. *S. pneumoniae* is not an exception in this regard. Recent studies, have suggested that certain pneumococcal proteins, including hyaluronate lyase (Hyl), pneumolysin (Ply), two neuraminidases (NanA and NanB), major autolysin (LytA), choline binding protein A (CbpA) (Rosenow *et al.*, 1997), pneumococcal surface antigen A (PsaA), and pneumococcal surface protein A (PspA), could be used as potential vaccine candidates. If antibodies to these proteins could offer better protection to humans, they could provide the source of a pneumococcal vaccine to be used in conjunction with or in place of the more traditional capsular polysaccharide vaccine or the conjugate vaccine that is under development (Mcdaniel *et al.*, 1991). Characteristics of major virulence factors of *S. pneumoniae* are listed in table 1.

1.7.1. Capsule

The capsule is highly prominent in virulent strains and its carbohydrate antigens vary greatly in structure among strains. The capsule is anti-phagocytic and immunization is primarily against the capsule. Apparently, resistance to phagocytosis is brought about by interference with binding of complement C3b to the cell surface. For many years, the polysaccharide capsule was considered to be the "sine qua non" of pneumococcal virulence. This was based on the observation that all fresh clinical isolates of *S. pneumoniae* were encapsulated, and all spontaneous non-encapsulated (rough) derivatives of such strains, were almost completely avirulent (Velasco *et al.*, 1995).

During invasion of the mucosal surface, encapsulated strains are 10^5 times more virulent than unencapsulated strains. The polysaccharide is nontoxic and non-inflammatory, and the capsule does not appear to engage any host defenses except for the induction of antibody-mediated immunity.

1.7.2. Cell Wall Components

The pneumococcal cell wall is a collection of potent inflammatory stimuli. The phosphorylcholine decorating the teichoic acid and the lipoteichoic acid is a key molecule enabling invasion, and acts both as an adhesin and as a docking site for the choline-binding proteins (CBPs). Two host-derived elements that recognize choline are platelet activating factor (PAF) receptor and the C-reactive protein. Since respiratory pathogens may be recognized and cleared by the C-reactive protein response as part of the constitutive defenses, respiratory pathogens may share this invasive mechanism to subvert the signaling cascade of endogenous PAF.

The peptidoglycan/teichoic acid complex of the pneumococcus is highly inflammatory. Smaller components of peptidoglycan progressively lose specific inflammatory activity. The cell wall directly activates the alternative pathway of the complement cascade, generating chemotaxins for leukocytes, and the coagulation cascade, which promotes a "procoagulant state" favoring thrombosis.

1.7.3. Hyaluronate Lyase

The full-length Hyaluronate lyase (Hyl) has a molecular mass of 107 kDa when expressed in *E. coli*. Hyl is another major surface protein of *S. pneumonias* with potential antigenetically variable properties that might be essential for full pneumococcal virulence. Thus, it might represent an alternative for a pneumococcal vaccine or drug target, especially when combined with other pneumococcal virulence factors such as PspA or pneumolysin (Berry *et al.*, 2000).

Hyaluronate lyase is part of a broader group of enzymes called hyaluronidases. The hyaluronidase enzyme-mediated facilitation of tissue invasion by breaking down the extra cellular matrix (ECM) components was first suggested. Increased tissue permeability caused by the action of hyaluronidase on the ECM appears to play a role in wound infections, pneumonia, and other sepses such as bacteremia and meningitis.

1.7.4. Autolysin

Autolysins are members of a widely distributed group of enzymes that degrade the peptidoglycan backbone of bacterial organisms. These enzymes are located in the cell envelope

and play roles in a variety of physiological cell functions associated with cell wall growth an example of one such enzyme is the *S. pneumoniae* N-acetylmuramoyl-L-alanine amidase, also known as LytA amidase (Lopez *et al.*, 1992). Pneumococcal LytA amidase has a molecular mass of -36 kDa and has a modular organization; it is composed of two distinct domains. The mutations of *lytA* gene in the *S. pneumoniae* chromosome lead to significantly decreased virulence of this organism compared to wild-type strain in mouse intraperitoneal challenge (Berry *et al.*, 2000). Such behavior clearly indicates that the *lytA* gene is important to pneumococcal pathogenesis. Experiments showed that *lytA* negative mutants are less virulent than wild type pneumococci, and immunization with autolysin confers some protection against pneumococcal challenge in mice (Velasco *et al.*, 1995).

1.7.5. Pneumolysin

Pneumolysin, one of the best-characterized virulence factors of the pneumococci that penetrates the physical defenses of the host. Pneumolysinis a 53-kDa protein produced by all clinical isolates of *S. pneumoniae* (Paton *et al.*, 1993). It is a cytoplasmic enzyme that is released due to the action of surface pneumococcal autolysin. The virulence properties of Ply are therefore directly dependent on the action of autolysin. Ply functions especially in the early pathogenesis of pneumococcal infection, which is crucial to the pneumococcal colonization of a host. The cytotoxic effects of pneumolysin can directly inhibit phagocyte and immune cell function, which leads to suppression of the host inflammatory and immune responses.

1.7.6. Neuraminidase

The precise role of *S. pneumoniae* neuraminidase in pathogenesis has not been clearly established, but it probably enhances colonization due to its action on glycans. The enzyme cleaves terminal sialic acid from cell surface glycans such as mucin, glycolypids, and glycoproteins, which probably causes significant damage to host cell glycans as well as to the host. This action changes the glycosylation patterns of the host and probably exposes more of the host cell surface.

There appear to be at least two forms of the pneumococcal neuraminidase enzymes, NanA and NanB, with NanA having a molecular mass of ~108 kDa and NanB having a mass of ~75 kDa. The structural genes for NanA and NanB have been cloned and sequenced (Berry *et al.*, 1996).

Table 1: Characteristics of major virulence factors of *S. pneumoniae* (Adapted from Velasco *et al.*, 1995).

Virulence factor	Proposed mechanism of virulence
Capsule	(1) Lack of activation of alternative complement pathway. (2) Resistance of phagocytosis (3) Deposition of opsonically inactive complement components. (4) No or low immunogenecity of some serotypes.
Cell wall or CWPS	(1) Inflammatory effects: a) Activation of the alternative complement pathway, resulting in anaphylatoxin production. b) Enhancement of vascular permeability mast cell degranulation. PMN activation. c) IL-1 production increased cytopathic for endothelium (2) Mediator of attachment of endothelial cells.
Pneumolysin	(1) Cytolytic at high concentration (2) Cytotoxic at lower concentration a) Inhibition of ciliary movement and disruption of epithelium b) Inhibition of bactericidal activity of PMN c) Inhibition of lymphocyte proliferation d) Inhibition of Ab synthesis (3) Complement activation (4) IL-1 B and TNF-a production by monocytes Increased (5) Binding of Fc fragment of Ab
PspA	Inhibition of complement activation
Complement factor H binding component	(1) Inhibition of complement activation (2) Inhibition of phagocytosis
Autolysin	Release pneumolysin and cell was products
Peptide permeases	Enhancement of adhesion
Neuraminidase	Exposure of "receptors" for pneumococci
H_2O_2	Lung injury
IgAl	Counteracts mucosal defense mechanism

CWPS = Cell wall capsular polysaccharide, PspA = Pneumococcal surface protein antigen, H_2O_2 = Hydrogen Peroxide, IgAl = Immunoglobulin Al.

1.7.7. Surface proteins

Various proteins were suggested to be involved in pathogenecity of *S. pneumoniae*. However only a few of them have been confirmed as virulence factors (Velasco *et al.*, 1995).

1.7.7.1. Pneumococcal Surface Protein A (PspA)

Antibody studies have shown that Pneumococcal Surface Protein A (PspA), protective antigen for pneumococci, is found on every *S. pneumoniae* strain discovered to date. PspA is a surface protein with variable molecular size ranging from 67 to 99 kDa. The function of PspA appears to be protecting against host complement system (Yother *et al.*, 1994).

Biological evidence of the anti-complementary properties of PspA has been observed and has shown that PspA reduced the complement-mediated clearance and phagocytosis of *S. pneumoniae*. The conformational analysis suggests the coiled-coil structure of the N-terminal module of PspA. PspA can attach itself to *S. pneumoniae* by non-covalent binding to the choline of both lipoteichoic and teichoic acids via its C-terminal end, consisting of the repeat region, also called the choline binding region (CBR) (Yother *et al.*, 1994).

Analysis of the genomic sequences to identify similar proteins to the functional part of PspA yielded PspC, a PspA-like molecule with similar structure and function and the only homologous molecule which is closely related in its properties to PspA (Briles *et al.* 1992b and Talkington *et al.*, 1991) have purified a truncated derivative of PspA (the 43-kDa N-terminal half) from the culture medium of a pneumococcus in which the gene had been interrupted by insertion-duplication mutagenesis. Mice immunized with this antigen were protected against challenge with a virulent strain of *S. pneumoniae* that produced a closely related PspA type.

1.7.7.2. Pneumococcal Surface Adhesin A (PsaA)

Pneumococcal surface antigen A (PsaA) has a molecular weight of 34,539. It elicits protective properties in mice against *S. pneumoniae* (Talkington *et al.*, 1996), and the PsaA- mutants of pneumococci were also avirulent in a mouse model (Berry *et al.*, 1996). The gene encoding the 37-kDa antigen has also been cloned in *Escherichia coli*. The likely function of PsaA is the transport of Mn^{2+} and Zn^{2+} into the cytoplasm of the bacteria. The protein is thought to be anchored to *S. pneumoniae* via the bacterial cell membrane and a lipid component that is covalently attached to the protein. The initial adhesin features of PsaA deduced based on

analysis of PsaA pneumococcal cells may have been due to a secondary effect causing the absence of another adhesin molecule like choline binding protein A (CbpA) modulated by the presence or absence (in PsaA- mutant pneumococci) of Mn^{2+} or Zn^{2+}. Genomic sequence analysis shows that PsaA belongs to an ATP binding cassette (ABC)-type transport system (Berry *et al.*, 1996). The ABC-type transport system is characteristic of an integral membrane part responsible for transport of the solute through the cell membrane.

2. PNEUMOCOCCAL INFECTIONS

S. pneumoniae is frequently present in the upper respiratory tract of healthy children and adults as a member of the normal nasopharyngeal bacterial flora. The bacterial spread from person to person in droplets of respiratory secretions (Figure 1). Pneumococci adhere to their receptors on the surface of the mucosal cells of the recipient and subsequently colonize the nasopharyngeal epithelium. The balance between the virulence factors of the colonizing pathogen and the defense mechanisms of the host leads to an asymptomatic carriage state, which is the prevailing outcome of Pnc encounters. This is presumably how *S. pneumoniae* manages to persist as a human parasite. Pnc disease is rarely associated with prolonged nasopharyngeal carriage of a particular Pnc type. Instead, Pnc disease is usually caused by recently acquired strains. It has been suggested that a prolonged carriage of one Pnc type may even by beneficial to the individual in preventing colonization by other types (Gwaltney *et al.*, 1975). Multiple Pnc serotypes may be carried concomitantly (Loda *et al.*, 1975).

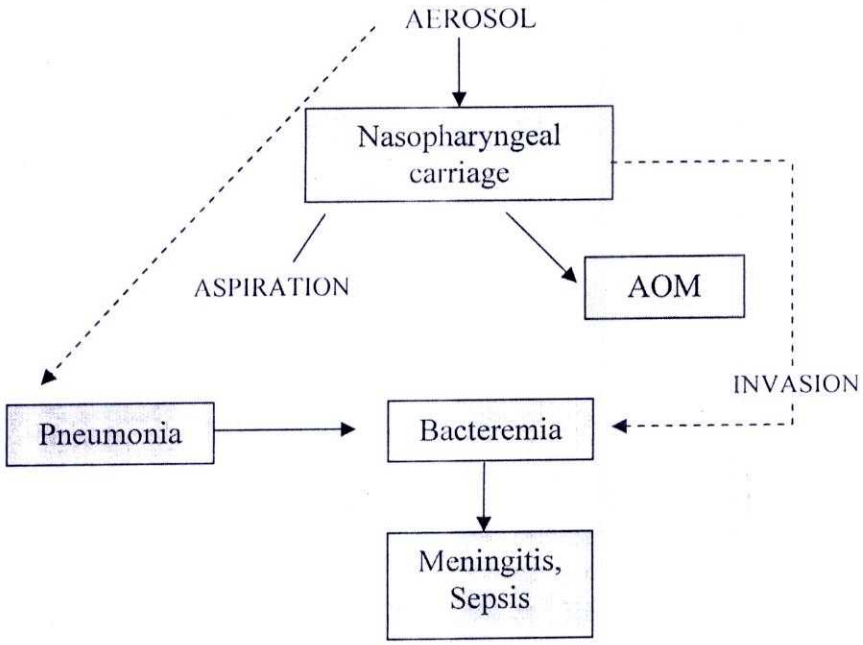

Figure 1: National history of diseases caused by *Streptococcus pneumoniae*.

3. Mode of Infection and the Immune Response

Pneumococcal meningitis is caused by direct spreading from the nasopharynx to the meninges or by hematogenous spread. In hematogenous spread the bacteria usually initially infect the lungs, causing pneumonia. Colonization involves pneumococcal surface adhesins which bind those epithelial cell receptors which display glycoconjugates with the disaccharide GlcNAcl-4Gal.

Pneumococci adhere to platelet activating factor (PAF) receptors on cytokine activated cells by the phosphorylcholine in the teichoic acid component of their cell walls. The bacteria bind epithelia, endothelia and leukocytes and trigger production of interleukin-1 (IL-1), a key cytokine in the inflammatory response (Tuomanen *et al.*, 1995). IL-1 increases vascular permeability and stimulates platelet production (Kuby, 1997). Cell wall components also stimulate cytokine production, activate the procoagulant cascade, damage neurons and affect cerebral blood flow and vascular-perfusion pressure. Thus, due to the strong response created to the pneumococcal cell wall, the alternative complement pathway is activated prior to the production of specific anti-capsular antibody.

The cell wall components which result from degradation by the body's own defenses are much more effective chemoattractants than the intact cell walls. If the concentration of cell wall components exceeds 10^5 particles per milliliter, a rapid inflammatory response is initiated (Tuomanen *et al.*, 1995). If a patient is able to survive this event, the decline in bacterial products will decrease the inflammatory response.

4. Pneumococcal infections in Bangladesh

S. pneumoniae is the most prevalent cause of community acquired bacterial pneumonia and of other acute respiratory infections throughout the world and Bangladesh, being a developing country, is not an exception. Although not much work has been done on this important pathogen, the limited data suggest that it is the common cause of child mortality and morbidity due to acute respiratory infections.

4.1. As a cause of pneumonia and acute lower respiratory infection (ALRI)

In rural areas of Bangladesh, a child >5 years old experiences two to three episodes of ALRI (Rahman *et al.*, 1990). The major respiratory pathogen that had been identified was *S. pneumoniae* (47%) (Rahman *et al.*, 1990) in Bangladesh it is the most common cause of pneumonia (Saha *et al.*, 1997).

4.2. As a cause of bacteremia

Pneumococcal bacteremia among children in Bangladesh is the commonest of all ALRI diseases. In the 34th Annual meeting of Infectious Disease Society of America in May 1996 had reviewed the epidemiological and clinical features of 156 episodes of pneumococcal bacteremia, occurring among 13,796 patients who were admitted to the ICDDR, B from 1990-1993. They showed that incidence did not vary by year/ and occasionally was marked with 87%. Episodes, occurring in winter and early spring, patients of pneumococcal bacteremia had a median age of 10 months. All but 6 patients received an antimicrobial agent effective against *S. pneumoniae* on admission.

Thirty one percent of patients with pneumococcal bacteremia died. The scientists also indicated that *S. pneumoniae* bacteremia is a potentially preventable cause of death among patients in

Bangladesh; Prevention of such death requires earlier identification and these facilities are unavailable in Bangladesh.

4.3. As a cause of meningitis

S. pneumoniae is the 2nd most common cause of bacterial meningitis in Bangladesh. A laboratory-based study of diagnosed bacterial meningitis in the National Pediatric Hospital identified 587 culture positive cases from 862 cases of meningitis in 8-year period (Saha *et al.*, 1997). According to the scientists *H. influenzae* was the most frequent cause (47%) for meningitis followed by *S. pneumoniae* (32%). Study also indicated that *S. pneumoniae* was the predominant organisms for meningitis until 1987. After 1987, a 700% increase in the *H. influenzae* isolate was obtained among patients with acute bacterial meningitis (Saha *et al.*, 1997). *S. pneumoniae* in the leading cause of childhood pneumonia in respiratory tract.

4.4. Serotypes predominant in Bangladesh

In a study in Bangladesh, which was carried out at Dhaka Shishu (Children) Hospital (DSH), a total of 165 invasive pneumococcal strains were isolated from children under five years. Ninety-four (57%) of the strains were isolated from 412 pyogenic CSF specimens, and seventy-one (43%) were from the blood of 531 pneumonia patients. Eighty-nine percent (146 of 165) of the strains were isolated from patients in the 2 to 24 months age group, and 56% (93 of 165) were from patients aged 6 to 24 months. Only 3.0% (5 of 165) of the isolates were from patients in the neonatal age group (0 to 30 days), 8.5% (14 of 165) were from patients of 2 years old or older (Saha *et al.*, 1997).

In Bangladesh, the most prevalent serotypes were 7F, 12F, 14, and 15B (Saha *et al.*, 1997). These four serotypes were found in 54% of the cases, other serotypes were 4, 23F, 18, 5, and 22A. Serotypes of infrequent occurrence made up 23.5% of the isolates. Predominant serotypes in meningitis and pneumonia cases were similar (Saha *et al.*, 1997). The serotype distribution found in Bangladesh is distinctively different from that found in children in other parts of the world. The two predominant serotypes in Bangladesh were 7F and 12F, which together made up 33% of all isolates. Predominant serotypes in pneumonia cases were summarized in Table 2.

Table 2: Groups and types of invasive *S. pneumoniae* strains isolated from Bangladeshi children with pneumonia:

Serotypes	% of total (cumulative %)
7F	16.9 (16.9)
15B	11.3 (28.2)
12F	9.9 (38.1)
14	9.9 (58.0)
23F	7.0 (65.0)
4	7.0 (72.0)
1	5.6 (77.6)
16F	4.2 (81.8)
6A	4.2 (86.0)
Others	8.4 (94.4)
Untypeable	5.6 (100)

5. Pneumococcal diseases

Pneumococci cause a wide variety of diseases, ranging from mild mucosal to life-threatening invasive diseases. Pneumococci cause diseases primarily near by their normal residence that is the upper respiratory tract. Clinical illness follows the spread of pneumococci to surrounding tissues from the nasopharynx. Pneumococci are a primary cause of pneumonia, meningitis and bacteremia in children and pneumonia in adults. The symptoms of all Pnc diseases are primarily due to the ability of the bacteria to evoke an intense inflammatory response, either locally or systemically. Pneumococci reach their target within the body either by direct extension from colonized mucosal surfaces causing sinusitis, AOM (Tuomanen, 2000), and pneumonia (Tuomanen *et al.*, 1995), or by hematogenous spread causing sepsis and meningitis (Sande *et al.*, 1999). Despite the availability of effective antimicrobial drugs, the mortality in serious Pnc diseases remains high.

Certain Pnc serogroups have been shown to be preferentially associated with specific disease manifestations. For instance, serogroups 1 and 14 are most commonly isolated from blood in both children and adults. Serogroups 6, 10 and 23 are most commonly isolated from CSF and serogroups 3, 19 and 23 from middle ear fluid (MEF). The pneumococci causing disease may need different strategies to remain at the sites of infection and overcome host defense mechanisms (Andersson *et al.*, 1981) have shown that strains of serotypes 6A, 14, 19F and 23F adhere strongly when isolated from the nasopharynx of patients with AOM, but less strongly when isolated from the blood or CSF. Thus, strongly and poorly adhering strains may be found within the same capsule type, and the avidity of adhesion of a Pnc strain may correlate with the type of infection that results.

5.1. Acute otitis media (AOM)

AOM is a mild, but extremely common disease during childhood. It is the most common reason for the prescription of antibiotics to children. In Finland, 0.5 million attacks of AOM have been calculated to occur each year (Eskola *et al.*, 2000). The incidence of AOM among children is highest before 2 years of age showing a peak between 6 and 18 months (Teele *et al.*, 1989; Kilpi *et al.*, 2001). The annual incidence rate of AOM in FInnish children during the first two years of life is around 50% (Pukander *et al.*, 1982).

AOM may be of bacterial, viral, or both bacterial and viral origin. *S. pneumoniae* causes approximately 35% of the AOM episodes, nontypeable *H. influenzae* 25% and *M. catarrhalis* 15% (Luotonen *et al.* 1981; Karma *et al.* 1985; Pelton 1998). In the FinOM Cohort Study, *S. pneumoniae, H. influenzae* and *M. catarrhalis* were isolated in 26%, 23% and 23% of AOM events, respectively (Kilpi *et al.* 2001). In a number of studies, Pnc groups 6, 14, 19 and 23 have been reported to cause the most causes of Pnc AOM (Gray *et al.* 1980; Klein 1980; Prellner *et al.* 1984b; Kilpi *et al.* 2001; Hausdorff *et al.* 2002).

Most cases of AOM are preceded by a viral upper respiratory tract infection. The viral infection predisposes the child to the development of AOM by causing Eustachian tube dysfunction and enhancing nasopharyngeal carriage of the middle ear pathogens (Syrjanen *et al.*, 2002). Rhinovirus, respiratory syncytial virus (RSV), adenovirus, influenza and para-influenza viruses are detected in middle ear effusions of 17 to 24% of children with AOM, either alone or in combination with bacteria (Sung *et al.*, 1993; Chonmaitree *et al.*, 2000). A clear association between RSV- epidemics and AOM has been demonstrated (Ruuskanen *et al.*, 1989).

Furthermore, AOM associated with a viral respiratory tract infection is prolonged in comparison to AOM in the absence of a viral infection (Heikkinen, 2000). AOM is predominantly a winter disease and the pattern seen with AOM is consistent with the appearance of winter respiratory viruses (Faden *et al.*, 1997).

5.2. Pneumococcal pneumonia and invasive diseases

5.2.1. Pneumococcal pneumonia

S. pneumoniae is the most frequent cause of community-acquired pneumonia among patients requiring hospitalization in the various geographical areas. The attack and case fatality rates of Pnc pneumonia are highest in elderly patients (Feldman, 2001). Pnc pneumonia causes about three million deaths of children less than 5 years of age each year, nearly all of which are in developing countries (Greenwood, 1999). The distinctive symptoms of Pnc pneumonia are cough and sputum production, which reflect the proliferation of bacteria and the inflammatory response in the alveoli, and fever, which results from the release of cytokines and other pyrogenic substances both locally and systemically (Musher, 1992).

In most cases Pnc pneumonia results from the aspiration of pneumococci resident in the upper respiratory tract. The lower respiratory tract is protected by several specific and nonspecific defense mechanisms. Failure of these defenses may facilitate access of pneumococci to the bronchi and the lungs. However, progression to pneumonia requires more than the simple association of pneumococci with alveolar cells (Musher, 1992).

Persons with underlying conditions or altered clearance mechanisms (such as cigarette smokers and persons having chronic bronchitis, asthma, chronic obstructive pulmonary disease, or lung cancer) are at a high risk of getting pneumonia (Musher, 1992). Viral upper respiratory tract infections may play a particularly important role in facilitating Pnc invasion by compromising the nonspecific defense mechanisms of the lung and causing epithelial cell damages. Sequelae of the common viral upper respiratory tract infection (such as excessive mucus production, disruption of the normal epithelium and dampening of ciliary function) have been shown to predispose to Pnc pneumonia (Gray *et al.*, 1989). The association between influenza and Pnc pneumonia is well documented. Prior influenza virus infection enhances the adherence of pneumococci to tracheal epithelial cells. This enhancement is thought to be mediated by viral

neuraminidase, which cleaves sialic acid from glycolipids in human lung tissue. This way, viral neuraminidase may expose other structures that can function as receptors for Pnc adherence.

Bacteria that colonize the lungs may gain access to the bloodstream. Bloodstream infections are a common complication of bacterial pneumonia; bacterial invade the alveolar spaces and cause enough tissue damage to disrupt the barriers between alveoli and blood vessels. Blood cultures are positive in 15-30% of cases of Pnc pneumonia, depending upon the population under study and, to a lesser extent, the Pnc serotype (Musher, 1992).

5.2.2. Pneumococcal septicemia

Septicemia is a systemic disease in which micro-organisms multiply in the blood or are continuously seeded into the bloodstream. Pnc septicemia occurs frequently as a complication of Pnc pneumonia. Septicemia may occur also as a primary bacteremia (bacteria present in bloodstream) in the absence of a clinically evident focus of infection. This phenomenon has been recorded in 15% of bacteremias, most commonly in children (Balakrishnan *et al.*, 2000). Epithelial damage caused by previous viral upper respiratory tract infections can increase the opportunity to pneumococci to reach the bloodstream.

A highest incidence of Pnc bacteremia has been repeatedly documented in infants up to two years of age. The incidence is low among teenagers and young adults, increases in patients of middle age, and reaches a high level among population over 65 years. Invasive Pnc diseases are more common among men than women in all age groups (Sankilampi *et al.*, 1997). In a Finnish study, the incidence of invasive Pnc disease was 45.3 per 100 000 among children less than 2 years of age, and 24.2 per 100 000 among children less than 5 years of age (Eskola *et al.*, 1992). The overall incidence of invasive Pnc diseases for all Finnish adults is 9.1 per 100 000, but 27.1 or more per 100 000 in those aged 65 or over (Sankilampi *et al.*, 1997). Various studies performed between 1974 and 1987 in the U.S. show an overall incidence of Pnc invasive diseases between 16 and 82 per 100 000 children in the first 5 years of life (Breiman *et al.*, 1990).

In developing countries the incidence of invasive Pnc diseases is several times higher than in the industrialized world. Among Gambian infants a minimum incidence of 185 per 100 000 per year has been reported (Usen *et al.*, 1998). An extremely high incidence, 297 per 100 000 persons, has been observed among the Australian aboriginals. High incidence figures have also

been reported from the native populations in Alaska. Several factors may contribute to these differences in incidence rates between regions, including country-specific epidemiologic surveillance systems, differing blood culturing practices, living conditions, genetic factors, climate and age.

The incidence of different Pnc serotypes in invasive Pnc disease varies by age and region. In children of industrialized countries, the most frequent Pnc groups/ types to cause Pnc bacteremic diseases are 6,14,18,19 and 23 (Riely *et al.*, 1991). The distribution is different in developing countries, where groups/ types 1,2,3,5,7,12 and 46 are found more frequently in children (Barker *et al.*, 1989). In adults, the most frequent groups associated with Pnc bacteremia are 3, 1, 14, 7, 4 and 8 (Nielsen *et al.*, 1992).

Pnc septicemia in children seems to be associated with low risk of death, while increasing age, an extra pulmonary site of infection, the presence of chronic disease, or infection with certain serotypes (particularly type 3) contribute to a higher risk of death. The mortality of Pnc septicemia over decades has remained stable between 25 and 29%. The overall case fatality rate in Finland has ranged from 21 to 34% during the last decades. The case fatality rate in children is lower than in adults ranging from 2 to 15% (Dagan *et al.*, 1992; Takala *et al.*, 1992).

5.2.3. Pneumococcal meningitis

The most severe form of Pnc disease is meningitis. Pnc meningitis is of exceptional severity and it is associated with a higher mortality than meningitis caused by other common meningeal pathogens (Goetghebuer *et al.*, 2000). The Pnc serogroups most often isolated from CSF included groups 6, 10 and 23 at all ages.

Meninges are a set of membranes that cover the brain and spinal column, protecting them from harmful substances in blood. Pnc meningitis usually occurs in relatively few individuals as a result of seeding of the meanings during high-grade bacteremia or a head trauma. The actual mechanisms and route used by pneumococci to migrate to the meninges are not clear. It has been suggested that local inflammation caused by pneumococci breaches the blood-brain barrier and admits entry of bacteria and phagocytes to this fragile area. The inflammatory reaction, rather than the pathogen itself, is largely responsible for the damage that results from bacterial meningitis. Neurological squeal such as hearing loss, neurological deficits and neuropsychological impairment are common among survivors (Van de Beek *et al.*, 2002).

The rates of morbidity and mortality of Pnc meningitis in children of industrialized countries are approximately 30% and 10%, respectively. In developing countries the figures are dramatically higher: about 50% of children with Pnc meningitis die while in hospital (Goetghebuer *et al.*, 2000).

6. Treatment of pneumococcal diseases

Penicillin, a cheap and safe antimicrobial drug, has been the standard choice for treatment of Pnc diseases for decades. However, due to the emergence of antibiotic-resistant strains, Pnc diseases have become more difficult to treat. The first Pnc strain resistant to penicillin was isolated in 1965 and ten years later the first multi-resistant pneumococci were reported (reviewed in: Appelbaum 1992). Penicillin acts by binding to and thereby blocking the action of cell membrane transcarboxypeptidase-enzymes (also called penicillin-binding proteins, PBPs), which are responsible for the bacterial cell wall synthesis. Resistance to penicillin results from changes in PBPs that decrease their affinity for penicillin without altering their functions in cell wall synthesis. At the moment, the proportion of Pnc strains resistant to penicillin is steadily increasing in all parts of the world. The foci of high levels of resistance have been detected particularly in parts of Southern and Eastern Europe. In some areas up to 35% of Pnc isolates are resistant to penicillin (Whitney *et al.*, 2000).

Epidemiologic studies have shown that frequent antibiotic use and the use of prophylactic antibiotics are the risk factors for spread of drug-resistant Pnc strains. This is true particularly in institutional settings, such as child care centers and hospitals, where person to person transmission of respiratory pathogens may be facilitated. Young children are often treated with antibiotics due to the high frequency of upper respiratory tract infections. Thus, this is probably the reason why antimicrobial resistance has developed primarily in Pnc serogroups prevalent in young children, namely 6, 14, 19 and 23 (Dagan *et al.*, 1994).

The rapid spread of penicillin- and multi-resistant strains has required switching to more costly antibiotics. A frightening characteristic of penicillin-resistant strains of *S. pneumoniae* is that their rate of resistance also to other commonly used antibiotics such as erythromycin, tetracycline and trimethoprim-sulfamethoxazole is much higher than in penicillin-susceptible strains. Acquisition of high-grade resistance is thought to originate via horizontal transfer of genetic material, probably from another bacterial species. Some multi-resistant Pnc isolates are

susceptible only to vancomycin. However, the appearance of vancomycin tolerance (i.e., the ability of bacteria to survive but not grow in the presence of an antimicrobial drug) in pneumococci has now also emerged. To date, five penicillin-resistant and vancomycin tolerant clinical strains of pneumococcus have been reported: three of these strains have been invasive isolates from the nasopharynx (Hidalgo *et al.*, 2003).

6.1. Antimicrobial Therapy

Prior to the 1990s, *S. pneumoniae* was uniformly sensitive to penicillin but a recent abrupt shift in the usefulness of penicillin has occurred, penicillin had reduced the severe morbidity and mortality associated with *S. pneumoniae.* The emergence of resistance has now forced re-evaluation of empiric therapy. Penicillin susceptible organisms show MIC's 0.06 mg/ml, intermediate strains 0.1-1.0 mg/ml and high level resistant strains 2 mg/ml.

The treatments for *S. pneumoniae* infections need suitable antibiotics like procaine penicillin, amoxicillin, and co-trimoxazole at a minimal cost. An intramuscular dose of procaine penicillin of 50 μg/g once a day, results in a serum concentration of penicillin that is continuously >1.0 μg/ml in most children, and thus is likely to be effective against susceptible strains of *S. pneumoniae* (MIC < 0.1 μg /ml) (Shann *et al.*, 1987). The WHO ARI programmer recommends four first-line antibiotics for the outpatient treatment of pneumonia in children 2 months up to 5 years of age: cotrimoxazole (trimethoprim- sulphamethoxazole), amoxycillin, ampicillin, and procaine penicillin, benzylpenitillin, chloramphenicol, oxacillin or (flu) cloxacillin, and gentamycin (WHO, 1994). Co-trimoxazole is cheaper than amoxicillin. Therefore, it is the preferred drug for children, with *S. pneumoniae* in most developing countries. Amoxicillin administered to irnmunocompetent rats with pneumonia caused by pneumococcus strains for which amoxicillin MJCs range from ≤0.015 to 4 μg/ml, and achieving a $T_{SAC\ >MIC}$ higher than 46%, was able to reduce lung damage by all strains to a significant degree.

6.2. Pneumococcal Vaccines

The search for an efficient vaccine against pneumococcus has continued for a long time. The vaccine development began originally at the beginning of the 20th century (year 1914), when attempts to induce protective immunity against pneumococcus in humans by vaccination with whole-cell killed pneumococci turned out to be successful. In the 1930s, the immunogenicity of purified capsular PSs was demonstrated and the first Pnc PS vaccine was developed. However, along with the demonstration of the therapeutic efficacy of antibiotics, the enthusiasm for Pnc vaccine development ceased for some decades. Despite the use of antibiotics, the mortality rate of systemic Pnc diseases remained high. This, with the emergence of the first penicillin-resistant pneumococci in 1965 led to renewed efforts to develop improved Pnc vaccines. At the moment, increasing antibiotic-associated resistance complicated disease management and highlights the importance of effectively preventing Pnc diseases.

The clinical efficacy of the current Pnc vaccines is based on the production of opsonizing anti-capsular antibodies, which have proved to be important in the host defense against Pnc disease. The data on the pathogenesis of Pnc injection and development of new vaccination strategies in animal models (including immunization with Pnc proteins and mucosal immunization) have accumulated during the past few years. This has opened up new possibilities for the prevention of Pnc inspections by immunization. Furthermore, clinical trials on combination vaccines including Pnc vaccine are underway with the aim of decreasing the number of shots administered during early childhood.

6.2.1. Systemic immunization

6.2.1.1. Pneumococcal polysaccharide vaccines

Purified Pnc capsular PSs used as a vaccine can induce type-specific anti-capsular antibodies, which are protective against Pnc disease in healthy adults. The efficacy of the first Pnc PS vaccine against bacteremic Pnc disease was demonstrated in the 1930s and 1940s. In 1977, a 14-valent vaccine containing 50μg of capsular PSs of each 14 serotypes was licensed. In 1983, the valency was increased to 23 serotypes. The vaccine contains 25μg of each capsular PS of the 23 serotypes most frequently causing disease in the U.S. The 23- valent vaccine remains the current preparation of Pnc PS vaccine. Pneumococcal PS vaccine protects healthy non-elderly, immunocompetent adults against pneumonia, invasive disease and death (reviewed in: French 2003).

Although safe and efficacious among healthy adults, the current Pnc PS vaccine has some apparent shortcomings. First, young children respond PS vaccines. A number of clinical trials on the Pnc capsular PS vaccines have demonstrated limited or no evidence of efficacy among children less than 2 years of age. Second, the PS molecules are of TI nature and the important features of these antigens are poor immunogenicity, as well as a lack of ability to induce affinity maturation, isotypes witch and immunologic memory (Stein, 1992). Thus, revaccination with a PS vaccine does not result in booster responses but instead leads to a similar or even reduced serological response in comparison to the response to primary vaccination. Consequently, the antibody concentrations and eventually the protection diminish in the course of time. Third, the Pnc PS vaccine does not protect certain high-risk groups such as immunologically incompetent individuals and elderly against Pnc pneumonia or invasive disease. Finally, the Pnc vaccine does not provide significant protection against mucosal Pnc diseases, such as AOM in young children, or against the spread of resistant strains from person to person (Eskola *et al.*, 1999).

6.2.1.2. Pneumococcal conjugate vaccines

In order to obtain improved vaccines for infant use, Pnc capsular PSs of the epidemiologically most important Pnc serotypes have been covalently coupled with various carrier proteins, such as diphtheria toxoid, tetanus toxoid and the outer membrane protein carrier converts the nature of the vaccine into TD and increases the immunogenicity of the hapten molecule. The antigen-presenting cells take up the conjugated PS-protein vaccine molecule, internalize it via the membrane-bound IGg and present the peptides of the protein to the helper T-cells in association with the major histocompatibility complex class II (MHC II) molecules. This induces the helper T-cells to stimulate PS-specific B-cells to mature either into antibody-producing plasma cells or into memory cells (Siber, 1994).

T-cell help associated with Pnc conjugate vaccines leads to several benefits. Long-lived memory B-cells are produced, which leads to the induction of a strong antibody response after revaccination (Ahman *et al.*, 1998). Additionally, T-cell signaled rearrangements of the Ig variable region leads to affinity maturation of the antibody response, improved antibody-antigen 'fit' and an increased opsonising function (Goldblatt, 1998). Finally, mucosal immune responses are enhanced with the production of mucosally active IgG (Nurkka *et al.*, 2001b).

The first Pnc conjugate vaccine, a 7-valent vaccine PncCRM7, was licensed in the U.S. in 2000 (Prevnar, Wyeth-Ayerst Laboratories, Philadelphia, USA) and in Europe in 2001 (Prevenar). This vaccine includes Pnc serotypes 4, 6B, 9V, 14, 18C, 19F and 23F conjugated to a nontoxic mutant diphtheria toxin known as CRM_{197}. Another investigational 7-valent vaccine, Pnc OMPC, contains same serotypes as PncCRM7, but the carrier protein is the meningococcal OMPC.

Current information is insufficient to suggest whether Pnc conjugate vaccines would be indicated to other target groups than infants. Although Pnc conjugate vaccines are able to induce better antibody responses than a Pnc PS vaccine in healthy adults (Wuorimaa *et al.*, 2001), they do not seem to offer any significant advantages to the PS vaccine in an adult population (Ahmed *et al.*, 1996). With the substantially greater cost of the Pnc conjugate vaccines in comparison with the Pnc PS vaccine, it will be important to clearly demonstrate efficacy of the conjugate vaccine in the target populations along with safety and acceptability. Future clinical trials of Pnc conjugate vaccines will include elderly to find out whether conjugates prove to be efficacious in protection against Pnc pneumonia (Pelton *et al.*, 2003).

The main problem with the Pnc conjugate vaccines is that only a limited number of types may be included in the conjugated formulation due to logistic difficulties in the manufacturing process, and the attendant high cost. Thus, the choice of antigens to be included in a Pnc conjugate vaccine has to be based primarily on the predominant serotypes causing disease in the target population. It is apparent that a vaccine, which is based on the most prevalent serotypes among children in one country, may not be appropriate for adults in the same region or children in a different region of the world. This is because of the differences in Pnc serotype prevalence in various age groups and localities. In accordance with this, the serotypes included in the 7-valent conjugate vaccine provide different levels of coverage in different geographic regions. Adding serotypes may increase coverage, especially in developing countries, where serotypes not included in PncCRM7 are more common (e.g. serotypes 1 and 5). It appears that especially the 9-valent conjugate vaccines might have the potential to prevent a large portion of the cases of Pnc pneumonia and meningitis in the developing world. However, when the number of serotypes is increased, production costs of the vaccine also increase. Regardless of its efficacy, an expensive vaccine may be unlikely to be used on a large scale in the developing world.

6.2.1.3. Pneumococcal protein vaccine candidates

The problems with the Pnc PS and conjugate vaccine have stimulated an interest in alternative Pnc vaccination strategies. A promising complementary or alternative approach for prevention of Pnc infections is to develop vaccines directed against an antigenic moiety common to all Pnc serotypes, such as a Pnc protein antigen. The use of Pnc proteins would have several advantages. As TD antigens they are expected to be immunogenic even in young children and to induce immunological memory. Pnc proteins would provide protection against pneumococci regardless of the serotype. Thus, the development of Pnc protein vaccines could be used to fill the gaps in protection provided by the Pnc PS or conjugate vaccines and, if highly successful, they might be even able to act as stand-along vaccines. They have potential to cover the high-risk target groups who may not be covered by the current conjugate vaccine formulations, such as young infants in the developing countries and the elderly. Protein antigens can be produced with low expenses by recombinant technology. However, before Pnc protein antigens can be considered for large-scale human trails, their protective efficacy has to be clearly established in animal models.

Diversity among protein antigens is an important consideration in the selection of vaccine candidates. Non-variable protein antigens could potentially protect against a whole population of bacteria, provided that they have a critical function. However, many of the proteins required for critical bacterial functions show diversity among the strains. Despite the diversity, the proteins may still offer cross-protection. A number of proteins that act at different stages of the pathogenic process contribute to the virulence of *S. pneumoniae*. It has been suggested that vaccination with a mixture of different Pnc virulence proteins might provide a higher degree of protection than any antigen alone (Ogunniyi *et al.*, 2001).

Several Pnc proteins have been considered as potential vaccine candidates, including PspA, PsaA and inactivated Ply (Paton, 1998). These proteins may be used either as a pure protein, conjugated with Pnc PSs (Michon *et al.*, 1998) or as fusion proteins combined with immuno modulating molecules (Wortham *et al.*, 1998). Immunization of mice with inactivated or recombinant Ply toxoid induced enhanced survival against intra-peritoneal challenge with several Pnc strains of different serotypes. Similarly, mice immunized with PspA or with truncated PspA molecule were protected against challenge with virulent pneumococci. Also, administration of PsaA together with an appropriate adjuvant protected mice against Pnc challenge (Talkington *et al.*, 1996).

Human trials on immunization with Pnc proteins are sparse at the moment (Nabors *et al.*, 2000) immunized healthy adults with a single recombinant PspA variant and were able to stimulate broadly cross-reactive antibodies to heterologous PspA molecules. These antibodies have been shown to protect mice passively from fatal infection with *S. pneumoniae* strains bearing heterologous PspAs.

6.3. Problems associated with pneumococcal vaccines

Vaccine production for the streptococci presents several formidable problems. In case of polysaccharide vaccine, certain cohorts respond poorly to the PPV (Robertson *et al.*, 2000). Although young adults have the strongest responses to pneumococcal polysaccharide vaccine but the response is reduced at the extremes of age and in a range of underlying conditions. Almost no response is seen in individuals with leukemia, lymphoma, or Hodgkin's disease. Even infants below one year of age will respond to few serotypes but the antibody does not persist (McIntyre *et al.*, 1997).

Besides this, polysaccharide-based vaccines impart type-specific protection. For this, a formulation of serotypes, which is effective for one population, may not be as effective for another if serotype prevalence differ significantly. For example studies on pneumococcal isolates from Asian populations show that only 63-64% belong to serotypes included in the current 23-valent vaccine compared with 88-93% for the US population. Implementation of immunization recommendation has been poor with only 20% of patients at risk for pneumococcal disease including older adults receiving the vaccine. Approximately 50% of patients develop mild local side effects, such as redness at the site of injection/ muscle aches and several local reactions are rare. Revaccination may lead to increased incidence of adverse reaction (Saha *et al.*, 1997).

The pneumococcal surface protein A (PspA), a protective antigen for pneumococci, functions to protect against host complement system (Cundell *et al.*, 1995a). Although different investigators have described the vaccine efficacy of autolysin, pneumolysin, neuraminidase, etc. in mice. PspA reduced the complement-mediated clearance and phagocytosis of *S. pneumoniae*. Antibody against PspA has been found to be partially protective against pneumococcal infection in mice (Alexander *et al.*, 1994).

6.4. Mucosal immunization

Pneumococci enter the body via mucosal surfaces of the upper respiratory tract and mucosal immunization represents an attractive alternative for current systemic immunization strategies. The protection against Pnc acquisition or carriage and local Pnc diseases, such as AOM, are thought to depend on mucosal antibodies. Already in early studies, intranasal immunization of rabbits with pneumococci was shown to produce resistance to a subsequent challenge with the live organism in the absence of a detectable serum antibody. Following nasal immunization with killed pneumococci, the rabbits were also resistant to an intravenous challenge with a live organism, suggesting that local immunization may induce also systemic immunity.

It appears that nasal-associated lymphoid tissue (NALT) and upper airway mucosal epithelium are able to process and present antigens and mount a specific immune response locally, as well as in distant mucosal sites, via distinct homing mechanisms. Available evidence based on the vaccine antigens appropriate for the preventing AOM has suggested that oral immunization can induce effective immune responses in the middle ear cavity and nasopharyngeal tissues. Immunization via the nasal route appears to be as effective as the oral route, may require a smaller antigen dose, and can be effective even with non-replicating agents. Human experience with intranasal immunization is limited at this time, but recent studies with live attenuated influenza virus vaccine have been encouraging.

Several advantages have been linked to mucosal immunization. Mucosal immune responses induced by systemic immunization with the Pnc conjugate vaccines have been modest in infants and children. However, immunization by mucosal (e.g. intranasal) route might possibly induce substantially stronger mucosal secretory IgA responses. Based on animal studies, mucosal immunization may induce antibody responses simultaneously in mucosal surfaces and serum (Seong *et al.*, 1999). Local administration of Pnc vaccines would also be attractive due to its easiness, particularly in the developing world. Further more, the sIgA immune system has a potent immunological memory that is stimulated repeatedly by renewed contact with the antigen; this leads to a high level of production of specific IgA (McGhee and Mestecky, 1990). Finally, mucosal immune responses have been suggested to develop early in life and still function well in the elderl. The possibility of exploiting the potential of young infants to respond to PS antigens by the early production of sIgA should be considered in the design of vaccines, particularly because of the high risks associated with infection by encapsulated bacteria. Similarly, elderly people are more susceptible to various infections, among this Pnc

pneumonia. Aging has a generally negative effect on the immune system. The mucosa-associated lymphoid system, however, is suggested to remain immunologically vigorous even during old age have shown in a mouse model that the response to a 23-alent PS-vaccine in the mucosa-associated mesenteric lymph nodes does not decline with age, but remains constant over the entire age range.

Encouraging results regarding mucosal vaccination against pneumococcus have been obtained in animal models. Both oral and intranasal immunization of mice with PspA has elicited protective immunity against Pnc carriage and systemic disease. Intranasal immunization of mice with a mixture of the PsaA and PspA has proved to be highly protective against Pnc carriage. Mucosal immunization of mice with the Pnc capsular PS antigens or conjugate vaccine and an appropriate mucosal adjuvant induce both mucosal and systemic antibody responses and can protect against intranasal challenge with live bacteria. These data suggest that the mucosal vaccination is able to reduce Pnc carriage and disease.

The experience with mucosal vaccination in humans is so far largely restricted to the use of the oral attenuated live viral vaccine against polio and bacterial vaccines against cholera and typhoid fever. At the moment, a viral intranasal vaccine against the influenza virus and an oral vaccine against the rotavirus are subjects of active investigation. Before a mucosal vaccination against Pnc disease can be considered in humans, more basic work will be necessary.

6.5. Other immunization strategies

Other options of immunization to induce antibody responses against *S. pneumoniae* include the delivery of Pnc protein antigens either in recombinant carrier bacterial or in a form of nucleic acid vaccine (DNA vaccine). Protective responses against Pnc challenge have been obtained in mice with a recombinant Bacilli Chalmette-Guerin (rBCG) vaccine (Langermann *et al.*, 1994) and an oral recombinant *Salmonella* vaccine (Nayak *et al.*, 1998), both expressing PspA. In addition, protection against fatal Pnc infection has been elicited in mice after intramuscular injection of a plasmid expressing PspA.

7. PNEUMO 23® (PNEUMOCOCCAL VACCINE POLYVALENT)

7.1. Description

PNEUMO 23® (Pneumococcal Vaccine Polyvalent) is a sterile, liquid vaccine for intramuscular or subcutaneous injection. It consists of a mixture of highly purified capsular polysaccharides from the 23 most prevalent or invasive pneumococcal types of *Streptococcus pneumoniae*, including the six serotypes that most frequently cause invasive drug-resistant pneumococcal infections among children and adults in the United States (Table 3). The 23-valent vaccine accounts for at least 90% of pneumococcal blood isolates and at least 85% of all pneumococcal isolates from sites which are generally sterile as determined by ongoing surveillance of U.S. data (Robbins *et al.*, 1983).

Each PNEUMO 23® has a 0.5 ml dose of vaccine containing 25 µg of each polysaccharide type in isotonic saline solution containing 0.25% phenol as a preservative.

Table 3

23 Pneumococcal Capsular Types Included in PNEUMO 23®

Nomenclature	Pneumococcal Types
Danish	**1 2 3 4 5 6B** 7F 8 9N 9V** 10A 11A 12F 14** 15B 17F 18C 19F** 19A** 20 22F 23F** 33F**
****These serotypes most frequently cause drug-resistant pneumococcal infections**	

7.2. Clinical pharmacology

Pneumococcal infection is a leading cause of death throughout the world and a major cause of pneumonia, bacteremia, meningitis, and otitis media.

Strains of drug-resistant *S. pneumoniae* have become increasingly common in the United States and in other parts of the world. In some areas as many as 35% of pneumococcal isolates have been reported to be resistant to penicillin. Many penicillin-resistant pneumococci are also

resistant to other antimicrobial drugs (e.g., erythromycin, trimethoprim-sulfamethoxazole and extended-spectrum cephalosporins), therefore emphasizing the importance of vaccine prophylaxis against pneumococcal diseases.

7.3. Immunogenicity

It has been established that the purified pneumococcal capsular polysaccharides induce antibody production and that such antibody is effective in preventing pneumococcal disease. Clinical studies have demonstrated the immunogenicity of each of the 23 capsular types when tested in polyvalent vaccines.

Studies with 12-, 14-, and 23-valent pneumococcal vaccines in children two years of age and older and in adults of all ages showed immunogenic responses (Borgono *et al.*, 1978; Hilleman *et al.*, 1978). Protective capsular type-specific antibody levels generally develop by the third week following vaccination.

Bacterial capsular polysaccharides induce antibodies primarily by T-cell-independent mechanisms. Therefore, antibody response to most pneumococcal capsular types is generally poor or inconsistent in children aged < 2 years whose immune systems are immature.

7.4. Efficacy of the vaccine

The protective efficacy of pneumococcal vaccines containing 6 or 12 capsular polysaccharides was investigated in two controlled studies of young, healthy gold miners in South Africa, in whom there was a high attack rate for pneumococcal pneumonia and bacteremia.13 Capsular type-specific attack rates for pneumococcal pneumonia were observed for the period from 2 weeks through about 1 year after vaccination. Protective efficacy was 76% and 92%, respectively, in the two studies for the capsular types represented.

In similar studies carried out by Dr. R. Austrian and associates (Austrian *et al.*, 1976), using similar pneumococcal vaccines prepared for the National Institute of Allergy and Infectious Diseases, the reduction in pneumonia caused by the capsular types contained in the vaccines was 79%. Reduction in type-specific pneumococcal bacteremia was 82%.

A prospective study in France found pneumococcal vaccine to be 77% effective in reducing the incidence of pneumonia among nursing home residents.

In the United States, two post licensure randomized controlled trials, in the elderly or patients with chronic medical conditions, who received a multivalent polysaccharide vaccine, did not support the efficacy of the vaccine for non-bacteremic pneumonia. However, these studies may have lacked sufficient statistical power to detect a difference in the incidence of laboratory-confirmed, non-bacteremic pneumococcal pneumonia between the vaccinated and non-vaccinated study groups.

A meta-analysis of nine randomized controlled trials of pneumococcal vaccine concluded that pneumococcal vaccine is efficacious in reducing the frequency of nonbacteremic pneumococcal pneumonia among adults in low-risk groups but not in high-risk groups (Fine *et al.*, 1994). These studies may have been limited because of the lack of specific and sensitive diagnostic tests for non-bacteremic pneumococcal pneumonia. The pneumococcal polysaccharide vaccine is not effective for the prevention of common upper respiratory disease in children

More recently, multiple case-control studies have shown pneumococcal vaccine is effective in the prevention of serious pneumococcal disease, with point estimates of efficacy ranging from 56% to 81% in immunocompetent persons.

Only one case-control study did not document effectiveness against bacteremic disease possibly due to study limitations, including small sample size and incomplete ascertainment of vaccination status in patients (Forrester *et al.*, 1987). In addition, case-patients and persons who served as controls may not have been comparable regarding the severity of their underlying medical conditions, potentially creating a biased underestimate of vaccine effectiveness.

A serotype prevalence study, based on the Centers for Disease Control pneumococcal surveillance system, demonstrated 57% overall protective effectiveness against invasive infections caused by serotypes included in the vaccine in persons ≥6 years of age, 65-84% effectiveness among specific patient groups (e.g., persons with diabetes mellitus, coronary vascular disease, congestive heart failure, chronic pulmonary disease, and anatomic asplenia) and 75% effectiveness in immunocompetent persons aged ≥65 years of age. Vaccine effectiveness could not be confirmed for certain groups of immuno-compromised patients:

however, the study could not recruit sufficient numbers of unvaccinated patients from each disease group.

In an earlier study, vaccinated children and young adults aged 2 to 25 years who had sickle cell disease, congenital asplenia, or undergone a splenectomy experienced significantly less bacteremic pneumococcal disease than patients who were not vaccinated.

7.5. Duration of Immunity

Following pneumococcal vaccination, serotype-specific antibody levels decline after 5-10 years. A more rapid decline in antibody levels may occur in some groups (e.g., children).1 Limited published data suggest that antibody levels may decline in the elderly > 60 years of age (Konradsen *et al.*, 1995).

The Advisory Committee on Immunization Practices (ACIP) states that these findings indicate that revaccination may be needed to provide continued protection.

The results from one epidemiologic study suggest that vaccination may provide protection for at least nine years after receipt of the initial dose. Decreasing estimates of effectiveness with increasing interval since vaccination, particularly among the very elderly (persons aged ≥85 years) have been reported (Shapiro *et al.*, 1991).

7.6. Indications and Usage

PNEUMO 23® is indicated for vaccination against pneumococcal disease caused by those pneumococcal types included in the vaccine. Effectiveness of the vaccine in the prevention of pneumococcal pneumonia and pneumococcal bacteremia has been demonstrated in controlled trials in South Africa, France and in case-control studies.

PNEUMO 23® will not prevent disease caused by capsular types of pneumococcus other than those contained in the vaccine.

8. PURPOSE OF THE PRESENT STUDY

Purified Pnc capsular PSs used as a vaccine can induce type-specific anti-capsular antibodies, which are protective against Pnc disease in healthy adults. The efficacy of the first Pnc PS vaccine against bacteremic Pnc disease was demonstrated in the 1930s and 1940s. In 1977, a 14-valent vaccine containing 50μg of capsular PSs of each 14 serotypes was licensed. In 1983, the valency was increased to 23 serotypes. The vaccine contains 25μg of each capsular PS of the 23 serotypes most frequently causing disease in the U.S. The 23- valent vaccine remains the current preparation of Pnc PS vaccine. Pneumococcal PS vaccine protects healthy non-elderly, immunocompetent adults against pneumonia, invasive disease and death.

Although safe and efficacious among healthy adults, the current Pnc PS vaccine has some apparent shortcomings. First, young children respond PS vaccines. A number of clinical trials on the Pnc capsular PS vaccines have demonstrated limited or no evidence of efficacy among children less than 2 years of age. Second, the PS molecules are of TI nature and the important features of these antigens are poor immunogenicity, as well as a lack of ability to induce affinity maturation, isotypes witch and immunologic memory. Thus, revaccination with a PS vaccine does not result in booster responses but instead leads to a similar or even reduced serological response in comparison to the response to primary vaccination. Consequently, the antibody concentrations and eventually the protection diminish in the course of time. Third, the Pnc PS vaccine does not protect certain high-risk groups such as immunologically incompetent individuals and elderly against Pnc pneumonia or invasive disease. Finally, the Pnc PS vaccine does not provide significant protection against mucosal Pnc diseases, such as AOM in young children, or against the spread of resistant strains from person to person.

S. pneumoniae (pneumococcus) considered a leading cause of morbidity and mortality worldwide. Along with a number of invasive infections such as pneumonia, meningitis and sepsis, pneumococcus causes local infections such as sinusitis and acute otitis media (AOM). Childhood pneumonia and meningitis, account for 20 to 40% annual child mortality on the estimated annual global burden of 2.7 million childhood death from pneumonia in developing countries.

S. pneumoniae is also an antigenically diverse species in which more than 90 serotypes have been identified. However, the prevalence with which the serotypes are recovered from patients with invasive disease varies greatly, presumably because some serotypes have a much greater

propensity to cause invasive disease than other. The geographical distribution and prevalence of serotypes differ among the United States, Europe, and some Asian countries. Twenty serotypes are responsible for about 90% of all reported infections in United States and Europe, whereas, for instance, the 23-valent whole polysaccharide vaccine, which contains these serotypes, is effective against less than 70% of the pneumococcal infections in Asia. In Bangladesh, the most prevalent serotypes were 7F, 12F, 14, and 15B, which were found in 54% of the cases. Serotypes of infrequent occurrence made up to 23.5% of the isolates. Predominant serotypes in meningitis and pneumonia cases were similar. The serotype distribution found in Bangladesh is distinctively different from that found in children in other parts of the world. Among the predominant serotypes in Bangladesh 7F and 12F together made up 33% of all isolates.

Despite convincing reports in certain cohorts, controversy still exists over the effectiveness of the pneumococcal polysaccharide vaccine in older subjects. Observational studies consistently indicate 50 to 70% aggregate effectiveness in preventing invasive pneumococcal disease in elderly persons among serotypes found in the pneumococcal polysaccharide vaccine. Antibody response to pneumococcal polysaccharide vaccine have been studied as surrogate markers of vaccine protection, which is also serotype specific. In this study, we tried to demonstrate the cross reactive antigen between the 23 valent pneumococcal polysaccharide vaccine and the surface components of the *Streptococcus pneumoniae* 7F one of the most prevalent serotypes in Bangladesh.

MATERIALS AND METHODS

2.1. Collection of Vaccine:

The PNEUMO 23® (Pneumococcal Polysaccharide Vaccine) was collected from Aventis maintaining the cold chain.

2.2. Collection of model animals:

Four healthy rabbits (2 - 2.5 kg each) were collected from Animal Resources Division of ICDDR, B, Mohakhali, Dhaka.

2.3. Strain collection

The *S. pneumoniae* serotype 7F used in this study were obtained from the stock culture of the department of Microbiology, Dhaka Shishu Hospital. Originally the *S. pneumoniae* strain was collected from the out-and-inpatients of Dhaka Shishu Hospital and was serotyped by capsular swelling procedure (quelling reaction) with type specific anti-pneumococcal pool, type or group, and factor sera. However, identity of this strain was reconfirmed by the Gram staining. Optochin susceptibility and Bile solubility tests.

2.3.1 Gram staining

The *S. pneumoniae* strains were grown on gentamycin blood agar plate and a gram staining was performed and observed under a microscope for Gram-positive diplococci.

2.3.2. Optochin susceptibility

The bacterial strains were grown on gentamycin blood agar and a colony was touched with a sterile loop and streaked onto a fresh blood agar plate. After the loop was streaked across the plate, an Optochin disk (Ethylhydrocupreine hydrochloride, and diameter of 6 mm containing 5 µg, BBL, USA) was placed aseptically at the end of the streak where the loop was first placed. The plates were incubated at 37°C for 24 hrs and an α-haemolytic growth greater than 14 mm in diameter was identified as *S. pneumoniae* (Gardam and Miller, 1998).

2.3.3. Bile solubility test

The bile solubility test was performed by taking several loops of the bacterial strains from the growth on a gentamycin blood agar plate and making a suspension in a 1.0 ml of sterile saline. The suspension of cells was divided into two equal volumes (0.5 ml per tube). An amount of 0.25 ml of saline was added into one tube and 0.25 ml of 2% sodium deoxycholate (bile salts) was added to the other. The tubes were gently shaken and incubated at 37°C for up to two hours. Then the tubes were periodically examined for lysis of the cells in the tube containing bile salts. A clearing of the tube or loss of turbidity was considered as a positive result (Chuard and Reller, 1998).

2.3.4. Slide agglutination test

Slide agglutination test is a very rapid, direct and specific test to detect any bacterial strain. The collected strain was reacted with the antisera of the specific strain by the test.

2.4. Extraction of surface proteins

Water extraction procedure was applied for the isolation of surface materials from the *S. pneumoniae* strains following the method described by (Oaks *et al.*, 1986).

1. A fresh plate was subcultured from the stock culture.
2. A clearly isolated colony was inoculated and the organism was grown in Brain Heart Infusion (BHI) broth at 37°C for 24 hrs in a shaker.
3. The whole culture medium was harvested by centrifugation (10,000 x g for 20 min) and the supernatant was discarded.
4. The pellet was taken and washed three times in normal saline. (Pellet was suspended in 5-10ml normal saline and centrifuged at 10000 x g for 10 min. The procedure was repeated for three times.)
5. Cells were then re-suspended in 20 volumes of distilled water and were shaken (100 oscillations per min) for 6 hrs at room temperature.
6. The suspensions were centrifuged at 10000 x g for 30 min and supernatants were collected, filtered through 0.45 µm Millipore membrane filters and were stored at -20°C in aliquots until used.
7. Sterility was maintained at every step and one drop (before Millipore filtration) of each of the antigenic preparation was taken on a blood agar plate, spread over and incubated at 37°C overnight.

2.5. Estimation of protein concentration

The concentrations of the surface proteins were estimated by Bio-Rad protein assay, which is based on the Bradford method. It is a simple technique to determine concentration of the solubilised protein. It involves the addition of diluted (1:4) acidic dye solution and subsequent measurement of absorbance at 595 nm with a spectrophotometer. Comparison to a standard curve provides a relative measurement of protein concentration.

2.6. Immunization process of the model animals:

The PNEUMO 23® vaccine was injected intra-muscularly in two of the healthy rabbits, respectively and surface antigens of *S. pneumoniae* 7F was injected intra muscularly in another two of the healthy rabbits, respectively and continuously undertaken observation for one month starting from the vaccination, because of the development of antibody, referred by the official literature. Approximately 5 ml of blood was collected from each rabbits before injection and used as negative control.

2.7. Collection of blood from model animals:

1. 5 ml of blood samples were collected from the two 2.5 kg healthy rabbit respectively.
2. 30 ml of blood samples were collected from both the vaccinated rabbit and both the hyper immunized rabbits from their heart respectively.

2.8. Procedure for the blood serum separation and storage:

After collection of the blood samples into the centrifuge tube, the samples were kept stand by at 37^0C for 1 hour and then stand still at 4^0C over night. Then the samples were centrifuged by 4000 rpm for 10 minutes and collect the supernatant (upper reddish clear solution) with the aid of a micropipette and stored at -20^0C to -70^0C in a refrigerator with proper labeling until use.

2.9. Sodium Dodecyl Sulfate - Polyacrylaznide Gel Electrophoresis (SDS-PAGE) analysis of the surface proteins of *S. pneumoniae* and PNEUMO 23® vaccine

To determine the protein profile of the surface materials from *S. pneumoniae* and PNEUMO 23® vaccine, they were subjected to SDS-PAGE analysis (Laemmli, 1970).

2.9.1. Preparation of separating the gel

1. Clean and dry glass plates were assembled with spacer in *the* gel-casting chamber of the Mini protein two (Bio- Rad).
2. A 10% separating gel was constructed by gently mixing the distilled water/ lower gel buffer along with 10% SDS and acrylamide-bis acrylamide solution according to the table 4.

Table 4: Preparation of the separating gel (10%)

Composition	Amount
Distilled water	2 ml
Lower gel buffer (P^{H} 8.8)	1.25 ml
30% Acrylamide-bis acrylamide solution	1.70 ml
10% SDS	0.05 ml
10% Ammonium per sulfate	20µl
TEMED	4µl

3. This step was followed by rapid addition of the 10% freshly prepared ammonium per sulfate (APS) and N, N, N', N^{1} Tetramethyl-ethylenedi amine (TEMED)
4. As soon as APS and TEMED were added to the mixture, the freshly mixed solution was carefully poured into the glass plate chamber with a pipette. The gel mixture was poured to a level of about 3 cm below the top edge of the glass plates. Precaution was taken during pouring the gel mixture to avoid bubble formation.
5. The water overlaying the gel was poured out and excess water and any unpolymerized acrylamide were rinsed from the gel plates and extraneous moisture was removed with a piece of 1 mm Whatman filter paper.
6. It was then left undisturbed for about 2 hour to allow polymerization of the gel. A very sharp liquid-gel interface was visible with naked eye when the polymerization was completed.

2.9.2. Construction of the stacking gel

After the polymerization of the separating gel had been completed, a 5% stacking gel mixture was prepared.

1. Just like the separating gel, stacking gel mixture was also prepared by primarily mixing the distilled water, upper gel buffer along with 10% SDS and acrylamide- bis acrylamide solution according to the table 5.

Table 5: Preparation of the stacking gel (5%)

Composition	Amount
Distilled water	1.4 ml
Upper gel buffer (P^H 6.8)	600 µl
30% Acrylamide-bis acrylamide solution	400 µl
10% SDS	50 µl
10% Ammonium per sulfate	10 µl
TEMED	2.5 µl

2. A stacking gel solution was prepared by mixing the first three reagents from the above. But APS and TEMED were added respectively just before pouring.
3. The gel mixture was then rapidly poured above the previously constructed separating gel. Before pouring the stacking gel solution the over layered water was poured off.
4. A 10 well comb was then pressed between the two glass plates carefully so that no bubbles can take place inside the comb channels.
5. The gel was then allowed to sit undisturbed for 1-1.5 hr for polymerization.

2.9.3. Sample Preparation

1. Bacterial protein preparation and vaccine were thawed just before loading.
2. The protein sample was mixed with sample loading buffer at a ratio of 1: 1 and was then boiled in water bath for 2-3 minutes. During boiling precaution was taken so that the sample did not bump up.
3. 5 µl tracking dye (0. 1% Bromophenol blue) was then added to the boiled mixture.

2.9.4. Sample Loading

1. After the polymerization of the stacking gel has been completed, it is ready for sample loading.
2. Before loading the sample the comb was removed from the glass chamber with soft hand so that the well divider does not cracks.
3. The glass chamber was then fixed in the electrophoresis unit and gently was placed in the buffer reservoir.
4. The wells were filled with the running buffer by pouring running buffer inside two-glass chamber of the electrophoresis unit up to the top edge of the glasses.
5. Running buffer was also poured in to the main buffer reservoir up to 1/3rd of the height of the reservoir.
6. 25 µl of sample was then added to six wells with a micropipette from the right side of the gel.
7. The first column from the left was loaded with 5 µl of Molecular Weight Standard (Invitrogen, Mark 12^{TM} Standard, Catalog no. LC5677).

2.9.5. Running the gel

1. After the loading process was done carefully the electrophoresis unit was connected with the power pack adjusting at 20 mA current keeping the voltage supply free.
2. The power supply was kept on for 2-2.5 hour.
3. As soon as the tracking dye reached the bottom level of the gel, the power supply was turned off.

2.9.6. Staining and destaining of the gel

1. After the run had been completed the gel was released from the glass plates and immersed in a fresh staining solution (0.1% Coomassie Brilliant Blue R250).
2. The gel was then shaken on a rotary shaker for approximately 2 hour.
3. The gel was then taken out of the staining solution and flooded with destaining solution (10% Acetic acid) and placed overnight in 4°C or placed on a rotary shaker for more 2 hours.
4. When the gel background become transparent, it was taken out of the destaining solution and immersed in distilled water.

2.10. Western Blot analysis

2.10.1. Protein transfer from gel to nitrocellulose membrane

1. The polyacrylamide gel was prepared according to the procedure described in section 2.9.
2. The gel was washed in distilled water,
3. Each component of the sandwich cassette was soaked in the transfer buffer before assembly. The sandwich components were assembled in the following order:
   Red side of the cassette (facing positive electrode) / Sponge (smooth in side) / 2 pieces of whattman blotting paper / Nitrocellulose membrane / Gel / Sponge (smooth out side)/ black side of the cassette (facing negative electrode).
4. The tank was filled with transfer buffer to a level that covered the electrode panels and the cassette was inserted in the proper orientation (red side facing the positive electrode).
5. Power supply was connected and proteins were transferred to the membrane at a constant volt of 10 for overnight.

2.10.2. Immuno detection of antigens

1. The membrane was removed from the cassette by using a forceps and was stained in the amido black stain for 1 minute with moderate shaking in the hand.
2. After a little rinsing with distilled water sharp bands of protein were revealed on the membrane.

3. The whole paper was then cut into 4 strips: the strip labeled M, VR, HR, IR are transferred to a plastic box and was incubated for at least 1 hour in 2% skim milk on a rotary shaker.
4. Strip labeled M was kept undisturbed for the calibration of the molecular weight of the proteins that are immunogenic.
5. 100µl of the three different rabbit serum (VR, HR, IR) was taken in three falcon tubes containing 5 ml of 2% skim milk thus providing primary antibody in a concentration of 1:50 dilution and were shaken at room temperature on a rotary shaker for 1.5 hour.
6. After that, the membrane strips were washed two times in 0.1% Tween 20 in PBS for 5 min each.
7. The membrane was then transferred to a solution of secondary antibody (Alkaline phosphate conjugated Goat Antirabbit Antibody, whole molecule; Sigma, USA) diluted at 1:5000 in 2% skim milk and was shaken for 1 hour.
8. The membrane was washed once more as the same way it was previously done at step 6.
9. After that, strips were further washed for 1 min in 10 ml Tris-HCl buffer (pH 9.14)
10. Substrate was prepared by dissolving 10 mg AS-MX Napthol Phosphate (Sigma, USA) and 20 mg Fast Red TR (Sigma, USA) into 10 ml of 50 mM Tris-HCl.
11. The strips were then shaken in the substrate preparation until the bands had appeared. Washing the membrane several times in the demonized water stopped the reaction.
12. The membrane strips were then air dried and stored away from light.

2.11. Determination of protein molecular weights

1. To determine the relative mobility (R_f) of a protein, migration distance of the protein was divided by the migration distance of the tracking dye.

Relative mobility (R_f) = Distance of protein migration / Distance of tracking dye migration

2. The R_f values were plotted (x-axis) against the known molecular weights (y-axis) on a semi-logarithmic graph paper.
3. A calibration curve was prepared by using the molecular weight standards (Kaleidoscope prestained standards, Catalog No: 161-0324, BIO-RAD, USA).
4. The molecular weights of unknown proteins were estimated from linear calibration curve.

RESULTS

3.1. Gram Staining

Streptococcus pneumoniae serotype 7F was collected from the stock culture of the Microbiology Department of Dhaka Shishu (Children) Hospital. However, identity of the strain was again reconfirmed by the Gram staining, Optochin susceptibility and bile solubility tests (Table 6).

Table 6: Confirmatory tests of the *S. pneumoniae* strains used in this study

Tests	Results
Microscopic examination	Gram positive diplococci
Optochin susceptibility test	Inhibition zone >14 mm
Bile solubility test	Positive

3.2. Preparation of Antigens

Surface antigen was prepared by water extraction method and the protein preparation was stored at -20^{0}C refrigerator. Each and every step was performed aseptically to prevent any type of contamination.

3.3. Determination of soluble protein concentration

The soluble protein concentration of the surface materials of *S. pneumoniae* were calculated from the standard curve (Fig.2) and the concentration of the protein was 1.0 mg/ml. This concentration was sufficient for SDS-PAGE analysis and subsequent Western blot experiment.

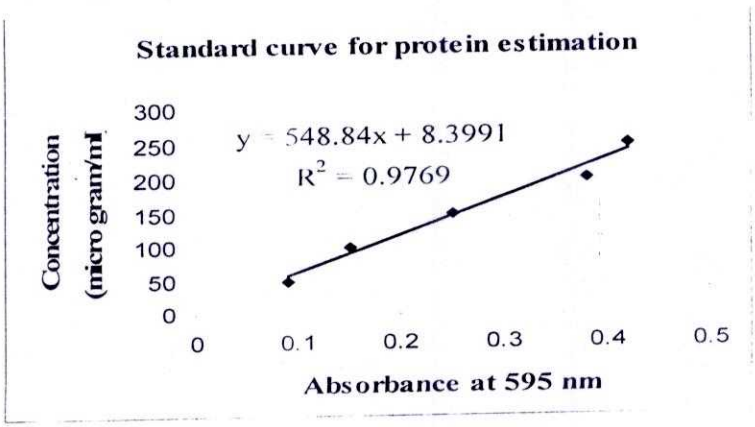

Figure 2: Standard curve for protein estimation. Soluble protein concentration of surface materials was determined using bovine serum albumin as the standard

3.4. SDS-PAGE analysis

The proteins collected from the whole cells of *S. pneumoniae* 7F and PNEUMO 23® vaccine were analyzed by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis and band of certain molecular weight were shown to be prominent than others. From the SDS-PAGE, about 16 protein bands from the whole cells of *S. pneumoniae* 7F and about 02 bands from PNEUMO 23® vaccine were found. To detect the proteins that were antigenic, Western Blot analysis was performed. Gel documentation was done to the PAGE and the picture has been attached below (Fig. 3).

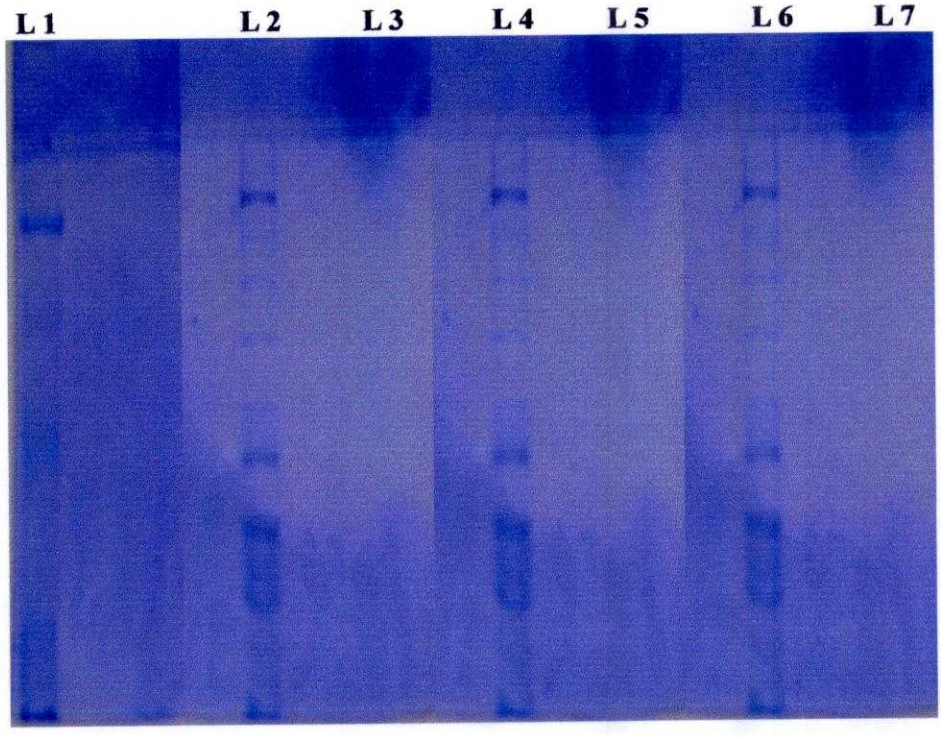

Figure 3: SDS-PAGE analysis for the whole cell extracts of *S. pneumoniae* 7F and PNEUMO 23® vaccine

Lane 1 = Molecular Weight Marker
Lane 2, 4 and 6 = Whole cell extracts of *S. pneumoniae*
Lane 3, 5 and 7 = PNEUMO 23® vaccine

3.5. WESTERN BLOT analysis

To study the immunogenicity of the antigenic fragments of *S. pneumoniae* serotype 7F and PNEUMO 23® vaccine the proteins were transferred from the gel to the Nitrocellulose membrane and immunodetection was done by a series of immune reaction. In fact 06 proteins were shown to be immunogenic from which 03 proteins were prominent in the immunogenic reaction while the others were less immunogenic (Fig. 4).

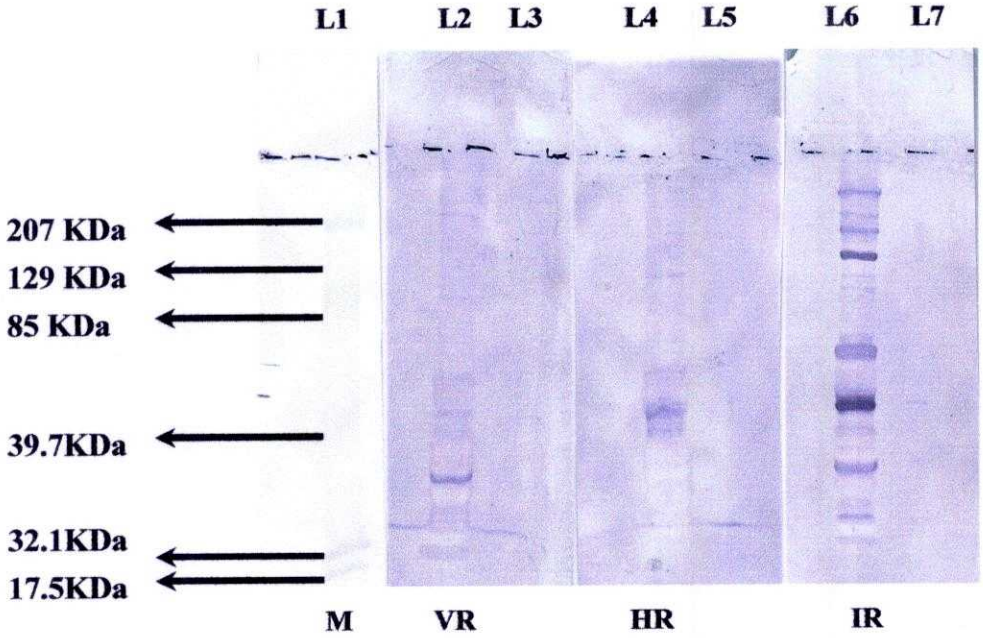

Figure 4: Western Blot analysis for the *S. pneumoniae* 7F whole cell extract and PNEUMO 23® vaccine against 2 different types of immunized rabbit serum

L 1 (M) = molecular weight marker

VR = Rabbit immunized by PNEUMO 23® vaccine

HR = Control

IR = Rabbit immunized by *S. pneumoniae* 7F

L 2, 4 and 6 = Whole cell extracts of *S. pneumoniae* 7F

L 3, 5 and 6 = PNEUMO 23® vaccine

3.6. Molecular weight determination of the proteins

3.6.1. Relative mobility of the proteins

Relative mobility of the marker proteins were determined out and listed in the table 7.

Table 7: Relative mobility of the protein molecular weight marker

Proteins	Color of bands	Relative mobility (R_f)	Molecular weight (Dalton)
Myosin	Blue	0.0592	207,000
β-Galactosidase	Magenta	0.1764	129,000
Bovine serum albumin	Green	0.3921	85,000
Carbonic anhydrase	Violet	0.5579	39,700
Soybean trypsin inhibitor	Orange	0.7058	32,100
Lysozyme	Red	0.9410	17,500

3.6.2. Standard curve for the molecular weight marker

Standard curve was constructed by plotting the molecular weight of the marker protein against the relative mobility of the proteins in a semi-logarithmic curve. From this curve the molecular weight of the unknown immunogenic proteins were calibrated (Fig. 5).

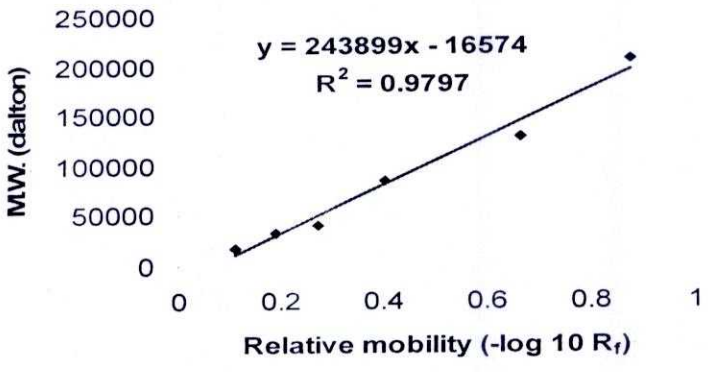

Figure 5: Standard curve for the determination of the molecular weight of the unknown immunogenic proteins isolated from the *S. pneumoniae* 7F whole cell extract and PNEUMO 23® vaccine

3.6.3. Relative mobility of the immunogenic proteins and PNEUMO 23® vaccine against Vaccinate rabbit, Healthy rabbit and Immunized rabbit serum and the corresponding molecular weight.

Table 8: Molecular weight of the whole cell protein extracts of *S. pneumoniae* 7F and PNEUMO 23® vaccine treated with antibody against vaccinated rabbit serum

Protein (Top to bottom)	*S. pneumoniae* 7F		PNEUMO 23® vaccine	
	Relative mobility (R_f)	Molecular Weight (KDa)	Relative mobility (R_f)	Molecular Weight (KDa)
Band 01	0.050	200	0.625	41
Band 02	0.100	177	0.875	15
Band 03	0.150	155	---	---
Band 04	0.200	137	---	---
Band 05	0.350	92	---	---
Band 06	0.550	51	---	---
Band 07	0.625	41	---	---
Band 08	0.675	34	---	---
Band 09	0.725	29	---	---
Band 10	0.750	26	---	---
Band 11	0.825	19	---	---
Band 12	0.875	15	---	---
Band 13	0.925	12	---	---

Table 9: Molecular weight of the whole cell protein extracts of *S. pneumoniae* 7F and PNEUMO 23® vaccine treated with antibody against Healthy rabbit serum

Protein (Top to bottom)	*S. pneumoniae* 7F		PNEUMO 23® vaccine	
	Relative mobility (R_f)	Molecular Weight (KDa)	Relative mobility (R_f)	Molecular Weight (KDa)
Band 01	0.075	188	0.600	44
Band 02	0.175	146	0.900	13
Band 03	0.275	112	---	---
Band 04	0.350	92	---	---
Band 05	0.425	74	---	---
Band 06	0.500	60	---	---
Band 07	0.600	44	---	---
Band 08	0.650	37	---	---
Band 09	0.725	29	---	---
Band 10	0.775	24	---	---
Band 11	0.850	17	---	---
Band 12	0.900	13	---	---

Table 10: Molecular weight of the whole cell protein extracts of *S. pneumoniae* 7F and PNEUMO 23® vaccine treated with antibody against *S. pneumoniae* 7F immunized rabbit serum

Protein (Top to bottom)	*S. pneumoniae* 7F		PNEUMO 23® vaccine	
	Relative mobility (R_f)	Molecular Weight (KDa)	Relative mobility (R_f)	Molecular Weight (KDa)
Band 01	0.100	177	0.600	44
Band 02	0.150	155	0.725	29
Band 03	0.200	137	0.850	17
Band 04	0.250	120	---	---
Band 05	0.300	105	---	---
Band 06	0.325	98	---	---
Band 07	0.450	69	---	---
Band 08	0.475	64	---	---
Band 09	0.575	48	---	---
Band 10	0.600	44	---	---
Band 11	0.650	37	---	---
Band 12	0.675	34	---	---
Band 13	0.750	26	---	---
Band 14	0.825	19	---	---
Band 15	0.850	17	---	---
Band 16	0.900	13	---	---

DISCUSSION

S. pneumoniae continues to be a major cause of life threatening invasive diseases such as pneumonia, meningitis, and bacteremia as well as other highly prevalent albeit less serious infections, such as otitis media and sinusitis (Garenne *et al.*, 1992). Pneumococcal infections are prevalent throughout the world, and children under the age of five years, the elderly and immunocompromised individuals are particularly susceptible. Mortality from pneumococcal disease is particularly high in developing countries, where pneumococcal pneumonia has been estimated to account for 20 to 25% of all deaths in children under the age of five years (Yother *et al.*,1992). These organisms are very difficult to handle in terms of vaccine production as they present more than 90 serotypes. 90 different capsular types of pneumococci have been identified and from the basis of antigenic serotyping of the organism.

The limitations of the currently available polyvalent vaccine formulations comprising purified pneumococcal capsular polysaccharide (PS) are well documented. These include the fact that the PS vaccines confer strictly serotype-specific protection and that the present formulation contain only 23 of the 90 known serotypes. PS are also T-cell-independent antigens and are poorly immunogenic in children under 2 years of age Protein-PS conjugate vaccines that are currently undergoing clinical trials, although highly immunogenic have limited serotype coverage. Moreover, they are likely to be expensive and this may limit their development in developing countries where they are needed most (Kuo *et al.*, 1995).

To overcome the limitations of PS vaccines, Protein-PS conjugate are being evaluated (Lee *et al.*, 1994). The conjugate PS appears to active T-helper cells, thereby eliciting T-cell-dependent responses that provide a long-term immunological memory. *Haemophilus influenzae* type b (Hib) vaccines with this conjugate approach have been enormously successful. They induce high, boostable and protective antibody levels in infants and have dramatically reduced Hib-associated disease in countries where they are in general use. Although Protein-PS conjugate vaccines were effective in eliminating nasopharyngeal carriage of vaccine serotypes. they increased carriage of non-vaccine serotypes causing invasive disease (Obaro *et al.*, 1996). Therefore, conjugate vaccines seem to be less effective in reducing the overall incidence of pneumococcal disease than expected.

Pneumococcal proteins eliciting cross-protective immunity might provide alternative approaches (Paton, 1998). Several pneumococcal proteins, such as pneumolysin, neuraminidase, autolysin, pneumococcal surface protein A (PspA) and pneumococcal surface adhesion A (PsaA) are known to elicit protective immunity. Recently, it has been also reported

that combinations of pneumococcal virulence proteins, pneumolysin, PsaA and PspA can elicit enhanced protection (Ogunniyi *et al.*, 2000). Among the protein antigens, pneumolysin appears to satisfy the essential criteria for inclusion as a vaccine antigen. Virtually all clinical isolates of *S. pneumoniae* produce pneumolysin and its primary structure apparently varies little. Native pneumolysin is, of course, Unsuitable for inclusion in a vaccine for humans because of its toxicity. However, site-directed mutagenesis studies have resulted in the development of pneumolysin derivatives (pneumolysoids) deficient in either cytolytic activity or complement activation or both (Paton *et al.*, 1998).

The use of the surface proteins as vaccine agents is not complicated by toxic side effects. Immunization of mice with an purified truncated durative of pneumococcal surface protein (PspA) provides a high degree of protection from challenge with virulent pneumococci producing a similar surface-protein type (Talkington *et al.*, 1991), but antigenic variability between strains may complicate the use of PspA in human vaccines. Polyclonal antibody studies have identified PspA epitopes that are conserved among numerous strains (Crain *et al.*, 1990), but whether these are protective remains to be determined. However, the functions of the PspA are, however, still largely unknown, its structures varies among different pneumococcal strains, and no data on the T-cell-activating capacity of PspA are available (Briles *et al.*, 1992a).

The diagnosis of pneumococcal pneumonia is difficult. The isolation of the pneumococci from blood is definitive proof of the disease's presence (Marrie *et al.*, 1989), but it is estimated that only 20 to 25% of the cases of pneumococcal pneumonia are bacteremic (Mufson, 1990; Musher, 1992). The demonstration of pneumococci in sputum or in the nasopharynx is of little diagnostic value, as *S. pneumoniae* can be found as a commensal organism in the upper respiratory tracts of the healthy individuals (Musher, 1992).

The present study was carried out to demonstrate the immunogenicity and the protective efficacy of the PNEUMO 23$^{(R)}$ vaccine against hyper immunized rabbit by *S. pneumoniae* 7F and vaccinated rabbit by PNEUMO 23$^{(R)}$ vaccine. Here the serotype 7F was used because it is the rank 1 serotype of *S. pneumoniae* in Bangladesh (Saha *et al.*, 1997).

For the isolation of surface materials from the bacterial strains, water extraction procedure was applied. This simple low ionic strength extraction by the use of distilled water has been used previously to release surface protein components from other sources (Oaks *et al.*, 1986). As

such extraction procedure does not contain any chemical treatment or drastic conditions, there is less possibility of altered immunogenic specificity of the product and the proteins may remains in this native undissociated conformation, which has been confirmed by the Sodium Dodecyl Sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the surface materials.

Before analysis of the surface proteins by SDS-PAGE, concentrations of the proteins were estimated using Bio-Rad protein assay. Concentration of proteins in the solution obtained was 1 mg/ml which was sufficient for SDS-PAGE analysis and subsequent Western blot experiment.

In the SDS-PAGE analysis, the water extracted surface materials of *S. pneumoniae* 7F and PNEUMO 23® vaccine showed few bands. Some bands in gel were prominent than others in comparison to size and color intensity. This strategy gives information that some proteins from the surface extract were present in higher concentration and amount than others. To ensure whether the proteins were really immunogenic or not, they were subjected to Western blot analysis.

The Western blot experiment showed that the antibody against vaccinated rabbit serum showed 02 prominent immunogenic protein bands in case of PNEUMO 23® vaccine and about 13 immunogenic protein bands were found in case of *S. pneumoniae* 7F whole cell protein extract. The antibody against healthy rabbit (control) serum showed 02 immunogenic protein bands in case of PNEUMO 23® vaccine and about 12 immunogenic protein bands were detected against *S. pneumoniae* 7F surface antigen. Finally the antibody against *S. pneumoniae* 7F immunized rabbit serum showed 03 immunogenic protein bands were detected for PNEUMO 23® vaccine and about 16 bands were found in case of *S. pneumoniae* 7F surface antigen.

From this study 41 and 15 KDa immunogenic protein bands that were detected in PNEUMO 23® vaccine against vaccinated rabbit serum was unique. In healthy rabbit (control) serum and *S. pneumoniae* 7F immunized rabbit serum 44, 13, 29 and 17 KDa immunogenic protein bands were detected but the band for 44 KDa protein was minimized because of the presence of the band in both healthy rabbit (control) serum and *S. pneumoniae* 7F immunized rabbit serum. However, 13 and 17 KDa protein bands were detected in both the *S. pneumoniae* 7F surface antigen of antibody against healthy rabbit (control) serum and *S. pneumoniae* 7F immunized rabbit serum but the 17 KDa protein band was also detected in PNEUMO 23® vaccine treated with antibody against *S. pneumoniae* 7F immunized rabbit serum and 13 KDa protein band was

also detected in PNEUMO 23® vaccine treated with antibody against healthy rabbit serum and therefore this band was considered as non-immunogenic one. Again 29 KDa protein was detected antibody against *S. pneumoniae* 7F immunized rabbit serum and did not match *S. pneumoniae* 7F surface antigen of the same study although it matched *S. pneumoniae* 7F surface antigen of antibody against healthy rabbit (control) serum. Therefore the 29 KDa protein was also considered as less-immunogenic.

Finally 41, 15 and 17 KDa proteins were considered the 03 prominent immunogenic proteins produced by the PNEUMO 23® vaccine in this very present study and ensure the antigen show reactivity between the vaccine and the whole cell extract of *S. pneumoniae* 7F, one of the prevalent serotypes in Bangladesh.

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APPENDICES

Appendix 1: Apparatus

Apparatus	Company
Autoclave	Model H42 AE, Hirayama Manufacturing corp., Tokyo, Japan.
Centrifuge	SORVO
Deionizer	Ultraflow, USA
Disposable micropipette tips	Eppendrof, Ireland
Distilled water plant	Fistreen water purification, UK
Electronic balance	Adventurer, OHAUS corp, USA
Electrophoresis power pack	Biometra, Germany
Eppendrof tube	Microcentrifige tube, 1.5 ml, labsystem, Ireland.
Incubator	SLI-600-D, EYELA, Tokyo, Japan.
Lainar airflow	Gelair, Flow Laboratories, Model HF48, Italy
Magnetic stirrer	Gallenkamp, UK
Sonicator	Virsonic 100, ultrasonic, USA
Spectrophotometer	UV- Visible, Model M302 CampSpec, UK.
Sterilizer	NDS-600D, EYELA, Tokyo, Japan
SDS-PAGE apparatus	Bio-Rad, USA
Vortex	WhirliMixer, Fisons, England
Western blot apparatus	Neido, Japan

Appendix 2: Solutions and buffers

SDS-PAGE analysis

1) 30% acrylamide-bis acrylamide solution:

Acrylamide	29.0 gm
Bis-acrylamide	1.0 gm
Distilled water	100 ml

2) 10% ammonium per sulfate

Ammonium per sulfate (APS)	1 gm
Distilled water	10 ml
Store in 4^0C	

3) 0.1% BMB (Bromophenol blue solution) or Tracking dye

Bromophenol blue	0.1 gm
Distilled water	100 ml

4) Destaining solution (10% acetic acid)

Glycial acetic acid	10 ml
Distilled water	90 ml

5) Staining solution

Coommassie brilliant blue G-250	0.20 gm
10% acetic acid	100 ml

6) Sample loading buffer

0.5 M Tris-cl (pH 6.8)	10 ml
10% SDS	10 ml
2-mercaptoethanol	1 ml
Glycerol	10 ml
Distilled water	19 ml

7) Electrophoresis buffer (pH 8.3)

Tris	3.0 gm
Glycine	14.4 gm
10% SDS	10 ml
Distilled water	1000 ml

8) Upper gel buffer (pH 6.8)

Tris-base	6.1 gm
SDS	0.4 gm
pH adjusted to 6.8 by adding HCL	
distilled water	up to 100 ml

9) Lower gel buffer (pH 8.8)

Tris base	18.17 gm
SDS	0.4 gm
pH adjusted to 8.8 by adding HCL	
Distilled water	up to 100 ml

Immunoblot analysis

1) 0.1% Amido Black

Amido Black	0.1
1% acetic acid	100 ml

2) Blocking solution

Skim milk	2 gm
PBS	100 ml

3) 10% Na-azide (NaN_3) solution

NaN_3	1 gm
Distilled water	10 ml

4) 0.1% Tween 20

Tween 20	0.1 ml
Distilled water	100 ml

5) PBS (pH 7.2)

NaCl	8.56 gm
Na_2HPO_4	0.18 gm
KH_2PO_4	0.23 g
KCl	0.20 g
Distilled water	1 liter

6) Transfer buffer (Blot buffer)

Deionized water	1 litre
Methanol	250 ml
Tris	3.6 gm
Glycine	18 gm

7) 50mM Tris-HCl (pH 9.14)

Tris base	605 mg
Distilled water	100 ml

pH adjusted to 9.4 by adding few drops of Hcl.

8) Substrate preparation

A.	AS-MX Napthol phosphate	10 mg
	Fast red TR	20 mg
	50 mM Tris-HCl (pH 9.14)	10 ml
B.	0.084M $MgCl_2.7H_2O$	12 ml
	0.5M Tris-Base	4 ml
	0.5M NaCl	4 ml
	NBT	88 ul
	BCIP	66 ul

Appendix 3: Media

Media	Components
1) Brain Heart infusion broth supplemented with 5% FBS (pH 7.2)	
BHIB dehydrated media	3.7 gm
FBS	5 ml
Distilled Water	100 ml
2) Gentamycin blood agar media	
Nutrient broth	100 ml
Sheep blood	5-7 ml
Gentamycin powder (Antibiotic)	0.035 gm

Appendix 4: Chemicals

Chemicals	Company
Acetone	Loba chemic, Bombay, India
Acrylamide, monomer	Nacalai Tesque, Inc., Kyoto, Japan
Ammonium persulfate (APS)	Serva, Germany
Anti-Human polyvalent Antibody (Alkaline phosphattase conjugates)	Sigma, USA
Anti-Mouse IgG Antibody (Alkaline phosphatase conjugate)	Sigma, USA
AS-MX Naphtohol Phosphate	Sigma, USA
BCIP	Sigma, USA
Bile salts	Oxoid, England
Bovine Serum Albumin	Sigma, USA
B-Mercaptoethanol	Sigma, USA
bromophenol blue indicator	Loba chemic, Bombay, India
Coomassie Brilliant Blue R250	Serva, Germany
Ethylhydrocupreine, hydrochloride	Optochin disk, BBL, USA
Fast Red TR	Sigma, USA
Glycial acetic acid	BDH Laboratory Supply, England
Glycerol	BDH Laboratory Supply, England
Glycine	Nacalai Tesque, Inc., Kyoto, Japan
Hydrochloric acid	BDH Laboratory Supply, England
MgC12,7h2O	Merck, Germany
Methanol	BDH Laboratory Supply, England
Molecular weight marker	Bio-Rad, USA
N,N' methylene-bis-acrylamide	Sigma, USA
Na_2HP0_4	Merck, Germany
Nitro Blue Tetrazolium (NBT)	Sigma, USA

Chemicals	**Company**
Nitrocellulose membrane	Hybond, USA
Sodium azide (NaN_3)	Sigma, USA
Sodium bi-carbonate	Merck, Germany
Sodium Carbonate	Merck, Germany
Sodium chloride	Merck, Germany
Sodium dodecyl sulfate (SDS)	Sigma, USA
TEMED (N, N, N', N' Tetramethyl-ethylenediamine)	Serva, Germany
Trizma base (Tris[hydroxy-methyl] amino methane)	Sigma, USA
Tween 20 (Polyoxyethylene sorbitan monolaurate)	Sigma, USA