Extraction and purification of capsular polysaccharide from streptococcus pneumoniae and Escherichia coli for conjugate vaccines preparation

Thesis submitted in partial fulfillment for the requirement of
the degree of
Master of Technology

By
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Certificate

This is to certify that the thesis entitled **"Extraction and purification of capsular polysaccharide from streptococcus pneumoniae and Escherichia coli for conjugate vaccines preparation"** by **Mr. Jagannath Mallick** submitted to the National Institute of Technology, Rourkela for the Degree of Master of Technology is a record of bonafide research work, carried out by him in the Department of Biotechnology and Medical Engineering under my supervision. I believe that the thesis fulfils part of the requirements for the award of master of Technology. The results submitted in the thesis have not been submitted for the award of any other degree.

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Jagannath Mallick
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IV. ABBREVIATIONS

CPS	Capsular polysaccharide
LPS	Lipopolysaccharide
DNA	Deoxy riobo nucleic acid
RNA	Ribo nucleic acid
Ig	Immunoglobin
EC	Escherichia coli
SP	Streptococcus pneumoniae
TD	Thimos dependant
TI	Thimos independent
ETEC	Entero toxigenic Escherichia coli
EPEC	Entero pathogenic Escherichia coli
EHEC	Entero haemorrhagic Escherichia coli
EAEC	Entero aggregative Escherichia coli
STEC	Shige toxin producing E. coli
UPEC	Uro pathogenic Escherichia coli
UTI	Urinary tract infection
IBC	Intracellular bacterial communities
MHC	Major histocompatibility
NMR	Nucleo magnetic resonance
Hib	Himophylus influanja type- b

ABSTRACT

Glycoconjugate vaccines, in which a cell surface carbohydrate from a micro-organism is covalently attached to an appropriate carrier protein, are proving to be the most effective means to generate protective immune responses to prevent a wide range of diseases (1). In this work we extracted the capsular polysaccharides from S. pneumoniae and E. coli by culturing in soybean casein digest medium and terrific broth respectively. The polysaccharides extracted were purified using tris-magnesium sulphate, DNAse and RNAse for removal of nucleic acid. The removal was confirmed spectrophotometrically by measuring $A_{260/280}$ ratio. The protein contamination was removed by precipitation of proteins with phenol acetal solution. The residual amount of protein was detected using Bradford assay, the residual amount of protein left in the extracted polysaccharide was found to be 0.5% and 0.45% in case of S. pneumoniae and E. coli respectively. The polysaccharides extracted and purified in this work was finally quantified using sialic acid assay and glucuronic acid assay and was confirmed using FTIR analysis. These polysaccharides extracted can be further used for conjugate vaccine preparation by conjugating along with carrier protein.

Chapter.1

INTRODUCTION

1. INTRODUCTION

Bacterial infections remain major killers of infants and children (Table-1), particularly in developing countries. Several million children die each year due to such infections. The most important pathogens are *Streptococcus pneumoniae, Haemophilius influenzae* type b, *Neisseria meningitidis, Salmonella entericus* subspecies *typhi, Staphylococcus aurous*, and diarrhoea-causing organisms such as *Shigella, Salmonella* and *Vibrio cholerae*. The disease profile can be endemic or epidemic, as occurs with Group A meningococcal disease in sub-Saharan Africa. Disease and mortality are concentrated amongst children in developing countries. Each of these pathogens possesses a cell surface capsular polysaccharide (CPS) or lipo polysaccharide (LPS) shell, or both, which helps the pathogen to establish an infection. The CPS hides cell surface components of the bacterium from the immune system of the host, preventing complement activation by cell surface proteins (Roitt 1997) and inhibiting phagocytosis. If the bacterium is phagocytosed, the CPS helps prevent bacterial killing. The role of LPS as a virulence factor is less well defined. In some cases it has been demonstrated, and in other cases it is suspected, that antibodies against a CPS or LPS O-chain will protect against infection (2) express CPSs or LPSs of different structures, resulting in a number of different serotypes or serogroups. Virulence and pathogenicity may be serotype or serogroups dependent, or there may be geographic differences in the clinically relevant serotypes. Whilst *Haemophilus influenzae* disease is caused overwhelmingly by a single serotype, the type b, pneumococcal disease is caused by a very large number of the ninety known serotypes. Meningococcal disease in developed countries is principally Groups B and C, although Groups W135 and Y are becoming increasingly important, and Group A disease is virtually unknown (3). Meningococcal Group A strains are, however, responsible for the regular meningitis epidemics which plague sub- Saharan Africa. Practically therefore, most of the saccharide-derived vaccines must contain multiple carbohydrates structures to provide adequate coverage against the disease causing strains. In some cases there is immunological cross reactivity between related structures which can provide partial protection. The optimal choice of polysaccharide to include in the vaccine is therefore a complex epidemiological problem (Robbins et al. 1983). Pneumococcal Types 1 and 5, for example, are important

pathogens in South America, but much less important in North America or Europe. For this reason, these serotypes were not included in the first 7-valent glycol conjugate vaccines licensed (Wyeth's Prevenar®), but have been included in subsequent 9- and 11-valent glycol conjugate vaccines under development. A number of these pathogens, including *Neisseria meningitidis* and *Streptococcus pneumoniae*, can leave surviving infants with severe neurological damage. This may affect as many infants as actually die from the infection. This has profound social and economic impacts.

Once it became clear that antibodies against CPSs protect against infection, it was a logical step to attempt to use these polysaccharides as immunogens. The first attempts were made in the late 1940s (McLeod et al. 1945), but the seemingly miraculous potential of antibiotics to control disease postponed development of this field until the late 1960s, when antibiotic resistance and the potential for neurological damage in "antibiotic-cured" infants became apparent (Cochi et al. 1985). CPS vaccines clearly work, and vaccines of this type are licensed and used in many countries, but this approach has several severe limitations. Repeating polysaccharides are T cell-independent type 2 (TI-2) immunogens: without the involvement of T cells they do not induce immunological memory, avidity maturation and isotype switching do not occur, and the antibodies induced, largely IgM and IgG2 (Musher et al. 1990, Lortan et al. 1993), are not good activators of complement. Crucially, vaccines of this type fail to induce immune responses in infants below the age of about two years, who are the major group at risk for these infections, because this aspect of the immune system develops relatively late. Repeat vaccination does not lead to increased antibody levels, so only one dose is given, but without immunological memory re-vaccination is required at regular intervals as antibody levels decline. This is typically every five years. Whilst the specificity of the immune response depends upon the structure of the CPS, the magnitude of the response depends critically upon its molecular weight. Only very high molecular weight polysaccharides are immunogenic and product development focused at first on the isolation of material of sufficiently high molecular weight. For this reason, LPS O-chains and low molecular weight CPSs, such as those from *Staphylococcus aureus*, are not effective as vaccines.

Chapter.2

LITERATURE REVIEW

2. LITERATURE REVIEW

2.1. STREPTOCOCCUS PNEUMONIAE

Although a common and serious pathogen of humans, the highly host-adapted *S. pneumoniae* is seldom isolated from clinical disease in animals. However, a unique clone of capsular serotype 3 is found in the respiratory tract of normal horses and has been associated with lower airway disease in combination with other bacteria and respiratory viruses (31,32), (fig2). Different case of pneumonias in a neonatal foal has also been reported (33). Equine isolates of *S. pneumoniae* are remarkable because they exhibit deletions in the *lytA* and *ply* genes for the virulence factors autolysin and pneumolysin and are genetically almost identical to each other. Moreover, they are genetically distinct from isolates of *S. pneumoniae* serotype 3 from humans (34). Experimental intratracheal inoculation of ponies is followed by fever, cough, ocular and nasal discharge and lesions of lobar pneumonia (35).

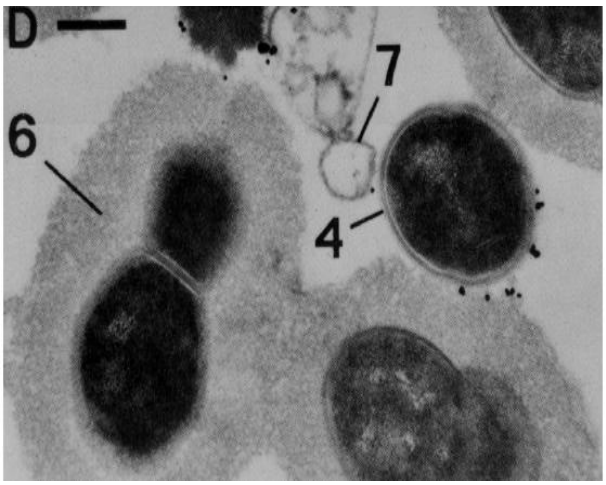

Fig. 1 – Electron micrograph of *Streptococcus pneumoniae* and the associated pneumococcal capsular polysaccharide (labelled 6). The bacteria shows the typical diplococcus morphology of the pneumococcus. Figure reproduced from Skov Sørensen et al. (1988) Infect Immun 56: 1890-1896 (copyright American Society for Microbiology), with permission

2.1.1. Virulence factors

The well studied virulence factors of *S. pneumoniae* of human origin as observed in mouse models include the capsular polysaccharide, pneumolysin, autolysin, neuraminidase, hyaluronidase, cell wall peptidoglycan, teichoic acid and phosphorylcholine (35). A large number of surface anchored proteins are also expressed including zinc metalloproteases involved in IgA proteolysis and in processing and export of other proteins, peptide permeases AmiA and PlpA, neuraminidases NanA and NanB, glycolytic enzymes, fibronectin binding enolase, an array of 12 choline binding proteins including PspA, LytA, a protective antigen, an autolysin, and Cpp A, an adhesin. A notable feature of the capsular polysaccharide is the presence of D-glucose, glucosamine, galactose and sialic acid as repeating unit (65).

2.1.2. *Pathogenesis*

Adhesion of *S. pneumoniae* to epithelium of the tonsil and soft palate of ponies has been noted following experimental infection (35). Invasion may trigger a number of host responses including the coagulation cascade with thrombus formation, the complement cascade with accumulation of leucocytes, and the chemokine/ cytokine cascade that ultimately leads to increased vascular permeability and leukocyte recruitment. Resistance to phagocytosis is mediated by a complex polysaccharide capsule that forms a hydrophilic gel on the surface of the organism. This gel shields the bacterium from antibodies and complement proteins. In addition, capsular sialic acid contributes to the anti phagocytic effect by inhibiting complement amplification and alternative pathway activation. Intrinsic complement inactivation mechanisms, which degrade C3b bound to the bacterial surface and prevent further C3 deposition, are also facilitated by capsular sialic acid. Capsular material has, however, been noted in the alveolar macrophages of ponies experimentally infected with *S. pneumoniae*, indicating that successful phagocytosis does take place. It is unclear how this relates to the clinically mild self-limiting nature of the naturally occurring respiratory disease of young horses. Alveolar necrosis has also been observed in experimentally produced lesions in ponies (35). Toxin involvement in pneumococcal pneumonia in humans is suggested by the acute fulminating and toxic clinical character of the disease. Neuraminidase may act both to decrease the viscosity of mucus and to

alter oligosaccharides of mucosal cells by removing Nacetyl neuraminic acid residues and thus expose receptors for bacterial attachment. Increased numbers of

S. pneumoniae are associated with the stress of race training and with lower respiratory tract inflammatory disease suggesting that the host/parasite interaction is opportunistic. Increased respiration during intense exercise may result in aspiration of *S. pneumoniae* from the tonsil and soft palate (36). At the same time, impairment of the mucociliary escalator mechanism and fluid accumulation may contribute to failure to clear aspirated organisms. Bacteria that proliferate in the highly cellular exudates will generate highly inflammatory streptococcal cell wall products. The significance in lesion development of the large numbers of *S. zooepidemicus* often found with *S. pneumoniae* in tracheal aspirates is unknown. It is possible that IgA protease produced by *S. pneumoniae* may destroy protective antibodies that control proliferation of *S. zooepidemicus* (36).

2.1.3. Immuniy

Much of the information on protective immunity to *S. pneumoniae* must be interpreted with caution since it is based on mouse models. Type-specific capsular antibody produced during convalescence is opsonizing and protective. However, capsular polysaccharaide is often poorly immunogenic. Protein antigens including PspA, pneumolysin, PsaA, autolysin, the neuraminidases, NanA and B, and at least six other surface proteins reactive with human convalescent serum and mouse protective (36) may have potential as vaccine components. But the conjugation of polysaccharide with a carrier protein shows significant development of antibody.

2.2. ESCHERICHIA COLI

Many types of bacteria produce extracellular polysaccharides (EPSs). Some are secreted polymers and show only limited association with the cell surface, whereas others are firmly attached to the cell surface and form a discrete structural layer, the capsule, which envelopes the cell and allows the bacteria to evade or counteract the host immune system(42). EPSs have critical roles in bacterial colonization of surfaces, such as epithelia and medical implants; in addition some EPSs have important industrial and biomedical applications in their own right. A

resolution structure of the 340 kDa octamer of Wza, an integral outer membrane lipoprotein, which is essential for group 1 capsule export in Escherichia coli(fig.3). The transmembrane region is a novel alpha-helical barrel. The bulk of the Wza structure is located in the periplasm and comprises three novel domains forming a large central cavity. Wza is open to the extracellular environment but closed to the periplasm. The route and mechanism for translocation of the capsular polysaccharide.

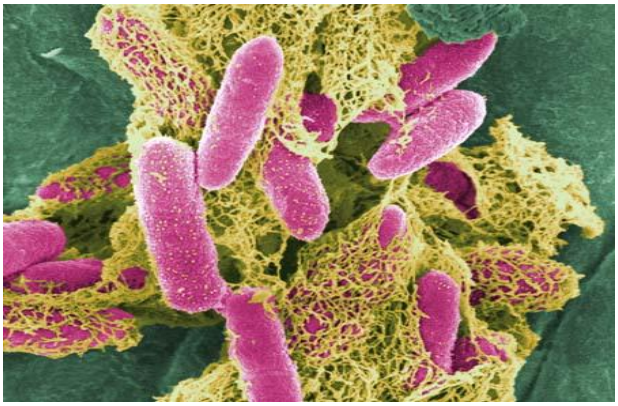

Fig.2: Pictorial Representation of an E.coli bacteria strain (66).

The late steps in assembly of capsular polysaccharides (CPS) and their translocation to the bacterial cell surface are not well understood. The Wza protein was shown previously to be required for the formation of the prototype group 1 capsule structure on the surface of Escherichia coli serotype K30. Wza is a conserved outer membrane lipoprotein that forms multimers adopting a ringlike structure, and collective evidence suggests a role for these structures in the export of capsular polymer across the outer membrane. Wza was purified in the native form and with a C-terminal hexahistidine tag. WzaHis6 was acylated and functional in capsule assembly, although its efficiency was slightly reduced in comparison to the native Wza protein (42). Ordered two-dimensional crystals of WzaHis6 were obtained after reconstitution of purified multimers into lipids. Electron microscopy of negatively stained crystals and Fourier filtering revealed ringlike multimers with an average outer diameter of 8.84 nm and an average central cavity diameter of 2.28 nm. Single particle analysis yielded projection structures at an estimated resolution of 3 nm, favoring a structure for the WzaHis6 containing eight identical subunits (43). A derivative of Wza (Wza*) in which the original signal sequence was replaced

with that from OmpF showed that the native acylated N terminus of Wza is critical for formation of normal multimeric structures and for their competence for CPS assembly, but not for targeting Wza to the outer membrane. In the presence of Wza*, CPS accumulated in the periplasm but was not detected on the cell surface. Chemical cross-linking of intact cells suggested formation of a transmembrane complex minimally containing Wza and the inner membrane tyrosine autokinase Wzc (43).

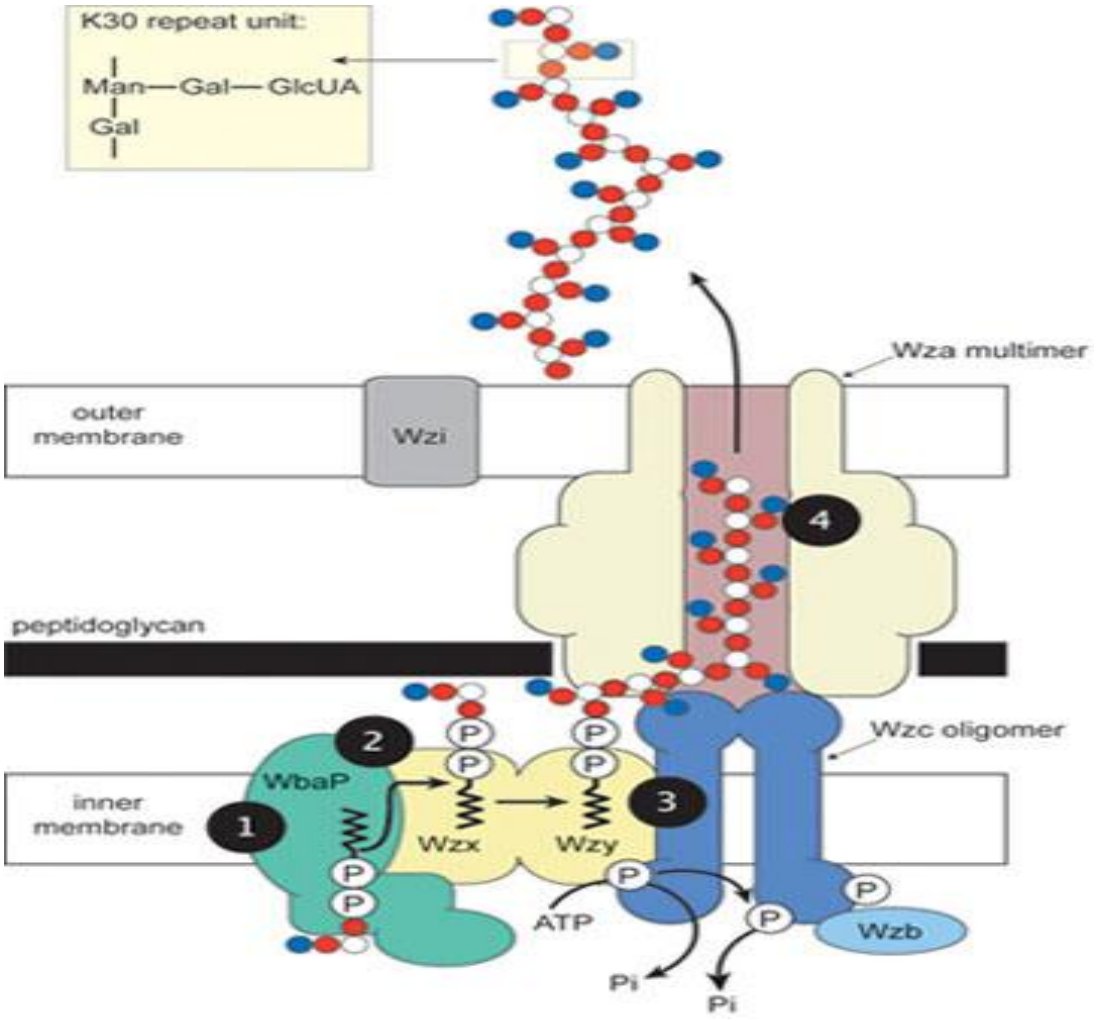

Fig.3 A model for the envelope-spanning enzyme complex involved in group 1 capsule assembly (67).

2.2.1. Role in disease

Virulent strains of *E. coli* can cause gastroenteritis, urinary tract infections, and neonatal meningitis. In rarer cases, virulent strains are also responsible for hæmolytic-uremic syndrome (HUS), peritonitis, mastitis, septicemia and Gram-negative pneumonia

2.2.2. Virulence properties

The virulence properties of E. coli are classified in table.1

Table1. Enteric *E.coli* (EC) classification on the basis of serological characteristics and virulence properties.

Name	Hosts	Description
Enterotoxigenic *E. coli* (ETEC)	Causative agent of diarrhea (without fever) in humans, pigs, sheep, goats, cattle, dogs, and horses	ETEC uses fimbrial adhesins (projections from the bacterial cell surface) to bind enterocyte cells in the small intestine. ETEC can produce two proteinaceous enterotoxins: • The larger of the two proteins, **LT enterotoxin**, is similar to cholera toxin in structure and function. • The smaller protein, **ST enterotoxin** causes cGMP accumulation in the target cells and a subsequent secretion of fluid and electrolytes into the intestinal lumen. ETEC strains are non-invasive, and they do not leave the intestinal lumen. ETEC is the leading bacterial cause of diarrhea in children in the developing world, as well as the most common cause of traveler's diarrhea. Each year, ETEC causes more than 200 million cases of diarrhea and 380,000 deaths, mostly in children in developing countries.
Enteropathogenic *E. coli* (EPEC)	Causative agent of diarrhea in humans, rabbits, dogs, cats and	Like ETEC, EPEC also causes diarrhea, but the molecular mechanisms of colonization and etiology are different. EPEC lack fimbriae, ST and LT toxins, but they utilize

	horses	an adhesin known as intimin to bind host intestinal cells. This virotype has an array of virulence factors that are similar to those found in *Shigella*, and may possess a shiga toxin. Adherence to the intestinal mucosa causes a rearrangement of actin in the host cell, causing significant deformation. EPEC cells are moderately-invasive (i.e. they enter host cells) and elicit an inflammatory response. Changes in intestinal cell ultrastructure due to "attachment and effacement" are likely the prime cause of diarrhea in those afflicted with EPEC.
Enteroinvasive *E. coli* (EIEC)	Found only in humans	EIEC infection causes a syndrome that is identical to Shigellosis, with profuse diarrhea and high fever. EIEC are highly invasive, and they utilize adhesin proteins to bind to and enter intestinal cells. They produce no toxins, but severely damage the intestinal wall through mechanical cell destruction.
Enterohemorrhagic *E. coli* (EHEC)	Found in humans, cattle, and goats	The sole member of this virotype is strain O157:H7, which causes bloody diarrhea and no fever. EHEC can cause hemolytic-uremic syndrome and sudden kidney failure. It uses bacterial fimbriae for attachment (E. coli common pilus, ECP is moderately-invasive and possesses a phage-encoded Shiga toxin that can elicit an intense inflammatory response.
Enteroaggregative *E. coli* (EAEC)	Found only in humans	So named because they have fimbriae which aggregate tissue culture cells, EAEC bind to the intestinal mucosa to cause watery diarrhea without fever. EAEC are non-invasive. They produce a hemolysin and an ST enterotoxin similar to that of ETEC.

2.2.3. Neonatal meningitis

It is produced by a serotype of *Escherichia coli* that contains a capsular antigen called K1. The colonisation of the new born's intestines with these stems that are present in the mother's vagina, lead to bacteriemia, which leads to meningitis. And because of the absence of the igM antibodies from the mother (these do not cross the placenta because they are too big), plus the fact that the body recognises as self the K1 antigen, as it resembles the cerebral glicopeptides, this leads to a severe meningitis in the neonates(51).

*2.2.4. Gastrointestinal in*fection

Transmission of pathogenic *E. coli* often occurs via fecal-oral transmission.(50-52) Common routes of transmission include: unhygienic food preparation,[24] farm contamination due to manure fertilization,(53) irrigation of crops with contaminated greywater or raw sewage,(54) feral pigs on cropland,(55) or direct consumption of sewage-contaminated water.(56) Dairy and beef cattle are primary reservoirs of *E. coli* O157:H7, and they can carry it asymptomatically and shed it in their feces.(57) Food products associated with *E. coli* outbreaks include raw ground beef, raw seed sprouts or spinach, raw milk, unpasteurized juice, and foods contaminated by infected food workers via fecal-oral route

According to the U.S. Food and Drug Administration, the fecal-oral cycle of transmission can be disrupted by cooking food properly, preventing cross-contamination, instituting barriers such as gloves for food workers, instituting health care policies so food industry employees seek treatment when they are ill, pasteurization of juice or dairy products and proper hand washing requirements

Shiga toxin-producing *E. coli* (STEC), specifically serotype O157:H7, have also been transmitted by flies,(59-61) as well as direct contact with farm animals, petting zoo animals,[37] and airborne particles found in animal-rearing environments.(62-65)

2.2.5. Urinary tract infection

Uropathogenic *E. coli* (UPEC) is responsible for approximately 90% of urinary tract infections (UTI) seen in individuals with ordinary anatomy. In ascending infections, fecal bacteria colonize the urethra and spread up the urinary tract to the bladder as well as to the kidneys (causing pyelonephritis),(56) or the prostate in males. Because women have a shorter urethra than men, they are 14-times more likely to suffer from an ascending UTI.

Uropathogenic *E. coli* utilize P fimbriae (pyelonephritis-associated pili) to bind urinary tract endothelial cells and colonize the bladder. These adhesins specifically bind D-galactose-D-galactose moieties on the P blood group antigen of erythrocytes and uroepithelial cells. Approximately 1% of the human population lacks this receptor, and its presence or absence dictates an individual's susceptibility to *E. coli* urinary tract infections. Uropathogenic *E. coli* produce alpha- and beta-hemolysins, which cause lysis of urinary tract cells.

UPEC can evade the body's innate immune defenses (e.g. the complement system) by invading superficial umbrella cells to form intracellular bacterial communities (IBCs). They also have the ability to form K antigen, capsular polysaccharides that contribute to biofilm formation. Biofilm-producing *E. coli* are recalcitrant to immune factors and antibiotic therapy and are often responsible for chronic urinary tract infections.(57) K antigen-producing *E. coli* infections are commonly found in the upper urinary tract.

2.3. CAPSULAR POLYSACCHARIDE.

2.3.1. Polysaccharides as T lymphocyte independent antigens

Immunologically, an antigen can be classified either as T lymphocyte dependent (TD) or T lymphocyte independent (TI). Proteins and peptides are usually TD antigens since they require stimulation from helper T lymphocytes in order to elicit an immune response. The TD antigen is presented to T lymphocytes by the Major Histocompatibility Complex (MHC) molecules present on macrophages, B lymphocytes or dendritic cells. TD antigens induce an immune response that is long lasting due to formation of memory B and T lymphocytes. The

antibodies against TD antigens are of high affinity and of multiple isotypes (IgA, IgM, IgG1, IgG2a, IgG2b, IgG3). The affinity of an antibody is a thermodynamic parameter that quantifies the strength of the association between the antibody and the antigen and depends on the structural complementarity of the binding site on the antibody and the binding site on the antigen. In contrast to TD antigens, the TI antigens do not give rise to immunological memory neither do they require T lymphocytes to induce an immune response. Memory responses are characterized by the production of high-avidity antibody, i.e., antibodies strongly binding to the antigen. A majority of carbohydrates are categorized as TI antigens in nature. The TI antigens are further divided into TI type 1 and TI type 2 based on their interaction with B lymphocytes. TI type 1 antigens are defined as antigens capable of inducing proliferation and differentiation of both native and mature B lymphocytes.(20) These antigens activate B lymphocytes and may induce immune responses in neonates, adults and in mice with an X-linked B lymphocyte defect (xid).(14,19 - 21) Common examples of the TI type 1 antigens are the bacterial LPS(14,20). Conversely, TI type 2 antigens are of high molecular mass repetitive polysaccharide structures that exhibit no intrinsic B lymphocyte stimulating activity(20) These antigens are also characterized by their poor in vivo degradability and inability to stimulate MHC class II restricted T lymphocyte help(22,23) TI type 2 antigens will activate only mature B lymphocytes and most likely act by cross linking the cell surface immunoglobulin (Ig) of specific, mature B lymphocytes. This results in the production of antigen-specific antibodies. However, the TI-type 2 antigens are not suitable as vaccines for children below 2 years of age and for adults above 65 years of age since these populations do not respond. CPS from S. pneumoniae, N. meningitidis and H. influenzae are some examples of TI type 2 antigens.

It was recognized early last century that small molecules, known as haptens, can be made immunogenic after conjugation to carrier proteins ***(4)***. This principle has since been applied successfully to improve the immunogenicity of (poly) saccharides ***(5,6)***. We now know that the carrier proteins ensure the involvement of T-helper lymphocytes in the activation of the hapten- or polysaccharide-specific antibody-producing B lymphocytes (**Fig. 2**). In contrast to small molecules or haptens, polysaccharides (or other macromolecules with a repeating

structure) are able to induce an immune response, most likely by directly activating B-lymphocytes. Antigens that are able to induce an immune response without the involvement of T-helper lymphocytes are referred to as TI (thymus-independent) antigens ***(7)*** (**Table-2**). TI-2 antigens, such as plain polysaccharides, are not able to activate relatively immature B-cells. This is in contrast to TI-l antigens, which can activate immature B-cells because of their mitogenic activity. Lipopolysaccharides (LPS) are examples of TI-l antigens. Conventional T-cells recognize peptide sequences in association with the major histocompatibility complex (MHC). Recently, unconventional T-cells were found to recognize (glyco) lipids in a CD1-restricted way, γδT-cells were shown to respond to non-proteinaceous microbial ligands (that may include carbohydrates) in a virtually MHC-unrestricted way ***(8)***. The findings of T-cell regulation of the immune response against polysaccharides ***(9-11)*** without biochemical demonstration of the specificity of the molecular interactions can best be explained by assuming a role for anti-idiotypic antibodies and T-cells specific for the idiotopes (carbohydrate mimotopes) or via the newly discovered unconventional T-cells.

The characteristics of polysaccharides described here are reflected in the antibody responses found in humans. Plain polysaccharides induce a poor response in infants, and at later ages of life the responses are of short duration and cannot be boosted ***(12-14)***, and the affinity does not mature. To overcome these problems, polysaccharides must be conjugated to carrier proteins in order to create effective vaccines. The *Haemophilus influenzae* type-b capsular polysaccharide conjugate vaccine has been successfully introduced in many national childhood vaccination programs ***(15-18)***. *N. meningitidis* serogroup C polysaccharide conjugate vaccines have now been developed. Clinical trials for these vaccines proved successful ***(19)*** and, as a result, a number of these vaccines were introduced into the UK vaccination schedules in 1999. These conjugate vaccines have already had a significant impact on the incidence of meningococcal serogroup C disease in immunized groups ***(20-21)***. This has led to a better definition of important criteria needed for potent conjugate vaccines. Finally, in recent clinical trials, pneumococcal conjugate vaccines have been shown to be effective at preventing pneumococcal disease in children ***(22)***. In this chapter, we describe

details of the preparation of a pneumococcal type 19F polysaccharide-protein conjugate vaccine ***(23)***.

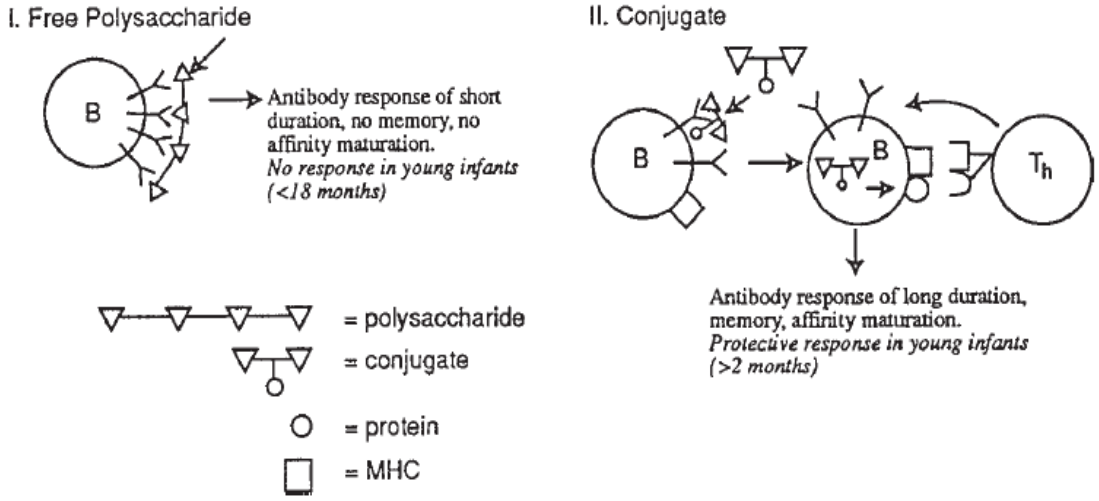

Fig.4: Polysaccharides are poor in activating B-cells to the production of antibodies in children younger than 2 yr of age. If antibodies are formed, they are of short duration. For conjugate vaccines T-cells are involved in the activation of B-cells. Presumably, the conjugate is taken up by polysaccharide-specific B-cells, processed, and presented to carrier-specific Tcells. The involvement of T-cells results in the activation of B-cells to production of antibodies and induction of memory in children younger than 2 yr of age.

Table.2: Characteristics of T-Cell Independent Antigens present in Streptococcus pneumoniae strain important for vaccine preparations.

Type 1	**Type 2**
Bacterial cell-wall components Mitogenic or polyclonal B-cell activator Stimulate antibody responses in neonates Stimulate antibody responses in CBA/N mice Examples: lipopolysaccharide and hapten derivatives; *Brucella abortus*	Polysaccharides, polypeptides, polynucleotides High mol wt, multiple repeating antigenic determinants Slowly metabolized Tolerogenic in large doses or soluble form Activate alternative complement pathway (some) Generate few (if any) memory B-cells Restriction of isotypes induced Lack of affinity maturation Lack of T-cell memory

	Fail to stimulate antibody responses in neonates Fail to stimulate antibody responses in CBA/N mice Examples: Pneumococcal polysaccharides; *Haemophilus influenzae* type b polysaccharide; Meningococcal polysaccharides

2.3.2. Polysaccharide structure

CPSs and LPS O-chains have strict repeating structure, which may consist of either a single sugar unit or oligosaccharide units, containing as many as seven or eight sugar residues (Kamerling 2000). The repeat units can either be linear or branched and contain non carbohydrate substituents such as *O*-acetyl, glycerol phosphate, or pyruvate ketals. Structural heterogeneity may occur as a result of the loss of or migration of labile *O*-acetyl groups between

sites. Bacterial polysaccharides may contain unusual sugar residues including diamino deoxy and branched chain sugars (Lindberg 1990a). As a general rule, CPSs tend to be anionic in character whilst LPS O-chains are neutral. The structures of some the repeat units of the capsular polysaccharides of clinically important bacteria are shown in Table 3. Whilst these vaccines elicit a strong antibody response, it is likely that protection depends upon a relatively small proportion of high avidity antibodies, with those directed against the saccharide backbone perhaps most important. Whilst antibodies against substituents such as *O*-acetyl groups may predominate, they may be of relatively low avidity and not clinically important (Michon et al. 2000).

*2.3.3. The molecular mechanism of generation of immune responses against polysaccharid***e**

The molecular mechanisms by which TI-2 immunogens with repeating structures, such as bacterial polysaccharides, stimulate an antibody response have been revealed by the work of Snapper and coworkers (24). In brief, the polysaccharide crosslinks approximately 15-20 surface immunoglobulin molecules (sIg) molecules present on a B cell of appropriate

specificity, leading through a series of intermediate protein phosphorylation steps to an increase in free intracellular calcium. Such a cell is primed to secrete antibody, but a second signal is also required. The nature of this second signal has not been well defined, and may be different in the case of a natural infection than when a vaccine is used. When this second signal is received, the B cells mature into plasma cells and secrete antibodies. There appears to be no direct interaction between B cells and T cells. The necessity to crosslink many sIg molecules would seem to be the reason why only high mass CPSs are immunogenic. The mechanism by which glycoconjugate vaccines elicit an immune response is significantly different and is discussed in more detail below, but it is this difference which explains why glycoconjugate are so much more effective as vaccines, and why they can be used to stimulate an immune response against a much wider variety of carbohydrate immunogens. Processing of zwitterionic capsular polysaccharides by an MHC II pathway has very recently been suggested (Cobb et al. 2004).

2.3.4. Carrier Protein

A variety of proteins, including bacterial pili, outer membrane proteins (OMPs), and excreted toxins of pathogenic bacteria, preferably in toxoid form, have been employed as carriers for carbohydrate antigens. Most popular as carrier proteins are tetanus and diphtheria toxoids, which are readily available and accepted for human use. However, the use of detoxified bacterial toxins as carrier proteins has some disadvantages. The process of chemically detoxifying produces lot-to-lot variations. Thus, physical and
chemical properties of the toxin can be substantially modified, which can affect the conjugation efficiency. Conjugation of these proteins with large amounts of saccharide may further affect protein conformational features and inactivate T- and/or B-cell epitopes. This might limit the amount of saccharide to be coupled to the protein, since a precondition of the conjugation is to maintain the T-cell activating properties of the
carrier protein. Bacterial toxins offer advantages over their corresponding toxoids if cytotoxic effects can be reduced by the conjugation itself. Alternative carrier proteins have been developed, such as CRM197, a nontoxic analog of diphtheria toxoid ***(37-38)***. These proteins have the same advantages as native toxins—light or heavy loading of saccharide is possible without influencing the carrier characteristics. Although diphtheria and tetanus toxin-

derivatized proteins have proven to be successful carrier proteins, both in animal and human studies, such problems as hypersensitivity or suppression of anti-carbohydrate response caused by the pre-existence of anti-carrier antibodies may still be a matter of concern, especially when a broad range of saccharides is analyzed ***(39-43)***. These negative effects could become more evident when conjugated carrier proteins are tested in polyvalent or combination vaccine formulations. The use of a carrier protein derived from the homologous bacterial species from which the polysaccharide was obtained would avoid possible problems. Furthermore, a homologous carrier protein may afford protection by itself that would enhance the protective action of the anti-polysaccharide immunity through synergistic action. In addition, homologous T help will be boosted on infection. It may be necessary to develop and use multiple carrier proteins as an approach to reduce interference when more than one conjugate vaccine is used; alternative carrier proteins, such as *Bordetella pertussis* fimbriae, have been used experimentally ***(44-45)***. Alternatively, the use of synthetic peptides corresponding to T-cell of proteins may be a viable approach to circumvent the phenomena described previously ***(46-48)***. However, to be able to induce an immune response across HLA barriers in the human population, synthetic polypeptides containing multiple epitopes from proteins would have to be used.

2.3.5. Coupling Chemistry (Linkage)

To confer a TD character to a saccharide, it must be coupled to a carrier protein through a covalent bond. Other strong, noncovalent couplings or associations with proteins have not proven to be as effective in reaching that goal. There are numerous existing or potential techniques available for the conjugation of bio-organic molecules, including saccharides and proteins ***(74–81)***. A large variety of these, often derived from pioneering research in affinity chromatography, have been used for the preparation of conjugate vaccines ***(24)***, including mainly reductive amination, amidation, and etherification reactions, but also the formation of disulfide, thiocarbamoyl, *O*-alkylisourea, or diazo couplings, among others. Conjugates obtained by reductive amination ***(82–84)***, amidation ***(85)***, the formation of a thioether bond ***(21,86–88)***, or a combination of these ***(89,90)*** have been shown to be highly stable. However, at present, it is uncertain whether some other types of linkage (e.g. disulfide bonds) have

enough stability in vivo. New techniques, sometimes adapted from other areas of biochemical research, constantly broaden the choice already available ***(91–99)***. Because of the lability of some saccharide components, coupling conditions should be as mild as possible. Accordingly, reaction parameters (pH, temperature, reaction time, and chemical reagents), should be chosen with the goal of avoiding the denaturation of protein or unwanted hydrolysis of saccharides. The stability of the linkage formed by conjugation is also of paramount importance. Significant decoupling during storage could lead to loss of immunogenicity or of the TD character of the conjugate. The choice of a coupling chemistry is also largely driven by the model of conjugate that is needed, namely a well-defined neoglycoprotein or a crosslinked lattice ***(24,25)***. The former is obtained by activation of a single end of the saccharide, and is generally more efficient for the coupling of oligosaccharides or short polysaccharides ***(100,101)***. The latter is obtained by random activation at several points on the saccharide chain, and is the more practical approach for the coupling of large polysaccharides. This model appears also to be the most appropriate for reducing the TI character of a polysaccharide, by controlling the length of continuous chains of intact RU in the conjugate. It should be noted that, in some rare instances, a well-defined crosslinked lattice can be obtained when a saccharide is amenable to specific activation of both ends of the chain—e.g. by periodate-induced depolymerization ***(82,83)***. When conjugating oligosaccharides, it is often desirable to use a spacer arm (e.g., adipic acid dihydrazide, diaminobutane, or 6-aminohexanoic acid) as a linker between the saccharide and the protein in order to avoid the shielding of important saccharide epitopes by the secondary structure of the carrier protein. A spacer can also provide greater efficiency of coupling with polysaccharides by reducing steric hindrance of activated moieties ***(86,102–104)***. In turn, a spacer can create a neo-antigenic structure that may be either harmless or toxic (e.g. aromatic spacer), or may lead to the unnecessary production of large amounts of nonprotective antibodies on immunization with the conjugate. Other considerations that influence the choice of a coupling chemistry include the availability of active groups on both the saccharide and the carrier protein, or the practical feasibility of introducing new ones by chemical or enzymatic modifications. Moreover, unrelated groups must be deactivated after

conjugation, to avoid uncontrolled reactions within the conjugate itself and with body tissues after immunization.

2.3.6. Saccharide-to-Protein Ratio

The spacing and density of the saccharide on the protein are likely to have major impacts on the ability of the conjugate to induce an immune response. Once the saccharide antigen is coupled to the carrier protein, measuring the relative ratio of those two moieties will provide some information about the conjugate structure. To obtain accurate data, it is essential that all free (uncoupled) material is removed from the final reaction mixture. This can be accomplished by ultrafiltration, liquid chromatography, electrophoresis, or differential precipitation. In some instances, it may be difficult to separate native polysaccharides from conjugates, since both components have high mol wts that are not always amenable to chromatographic separation. If a conjugate contains appreciable amounts of free polysaccharide, dose calculations for animal experiments become unreliable. In addition, the presence of a comparatively large amount of the TI form of the saccharide antigen, together with its coupled TD form, can have adverse effects on the immune response ***(105–107)***. Long-term storage of a conjugate can equally lead to partial depolymerization or decoupling of the saccharide antigen, which in turn will affect the saccharide-to-protein ratio. For a neo-glycoprotein model, the saccharide-to-protein ratio provides valuable information about the number of attachment points on the carrier. It then becomes possible to compare several conjugates with different ratios ***(25,41,108)***, and to evaluate the importance of the shielding of protein epitopes. With high saccharide-to-protein ratios, essential carrier epitopes may be hidden from the immune system, preventing recognition of the conjugate as a TD antigen ***(24–26,102)***. For a lattice model, since activation points are randomly distributed on the saccharide, information about linkage points to the protein is not readily available. Additional tests are needed, such as the titration of remaining activated groups on the carrier. Attachment points can also be measured when amino acids become covalently modified in such a way that physical (e.g., NMR) or chemical analysis is able to detect a change in their structure ***(86,109)***. It is equally difficult to measure the extent of crosslinking or the length of intact saccharide chains between attachment points. Other characteristics, such as the actual

mol wt of the conjugate, particularly if physical aggregation has taken place, certainly play a role in the way the conjugate is processed by the immune system. Finally, it should be emphasized that an optimal conjugation scheme must be determined for each particular saccharide and protein combination ***(25)***.

2.3.7. Polysaccharide vaccines in clinical use today

Three families of CPS vaccines are in widespread clinical use at present, whilst a fourth against *Haemophilus influenzae* type b (Hib) infection was used as a short term measure before the introduction of Hib conjugates. The simplest CPS vaccine, against typhoid, contains the so-called Vi (for virulence) antigen as its sole component, but with, typically, lactose present as a stabiliser. Clinical trials of the *Salmonella typhi*Vi polysaccharide in Nepal indicated an efficacy of approximately 70%, which is similar to older whole cell vaccines against typhoid but the side effects of the polysaccharide vaccine are much less severe (**25**). Vaccines containing two (Groups A and C), three (Groups A, C and W135) or four meningococcal (Groups A, C, Y and W135) CPSs are licensed. In developed countries they are currently used for control of outbreaks, but vaccination is required by Muslims undertaking the Hajj pilgrimage to Mecca. These vaccines are also used to control epidemic Group A meningitis in sub-Saharan Africa (Anonymous 2002a).

The pneumococcal polysaccharide vaccine is a blend of 23 serotype-specific polysaccharides, and is used in developed countries to protect the elderly from pneumonia. There is active discussion about exactly how effective these vaccines are for that purpose: some recent metastudies have cast doubt upon its efficacy (**26**). It is known that, for genetic reasons, some vaccinees are incapable of generating an immune response against some of the serotypes (Musher et al. 1998). This appears to be linked to the very limited genetic diversity of the immune response to polysaccharides. The elderly population appear to produce antibodies of lower avidity (Romero-Steiner et al. 1999). Despite their limitations, polysaccharide vaccines

are available, have moderate efficacy in appropriate populations, are generally cheap (Fleck 2003, Plans 2002) and have an excellent safety record. Glycoconjugate vaccines against meningococcal Group C and against seven pneumococcal serotypes have been licensed,

whilst glycoconjugate vaccines against other meningococcal CPSs, more pneumococcal serotypes and typhoid are in development

2.3.8. Glyco conjugate vaccines

The means to increase the immunogenicity of polysaccharides was first discovered by Avery and Goebel in 1931 (Avery and Goebel 1931) – covalent attachment of the polysaccharide to an appropriate protein carrier, to form a conjugate. Such conjugates provide T cell-dependent immunogenicity against the saccharide hapten. With the involvement of T cells, immunological memory is invoked, avidity maturation and isotypes switching occurs, to generate complement-activating antibody isotypes such as IgG1 (Wuorimaa et al. 2001). The avidity of the antibodies elicited is much higher than those from polysaccharide vaccines. Crucially, since a different arm of the immune system is involved, that used to process protein immunogens, glycoconjugate vaccines are effective in young infants. Multiple immunizations are necessary to provide the required immune response, but not regular revaccination. In the UK the vaccination regime for Hib conjugate vaccines is at two, three and four months, and a booster at 18 months has recently been introduced to ensure long term protection. The mechanism by which glycoconjugates stimulate an immune response involves an initial binding of the conjugate to the surface immunoglobulin (sIg) of B cell with appropriate specificity for the saccharide hapten (Siber 1994). This complex is internalised and the carrier protein degraded by proteolytic enzymes. Suitable peptides are trans- ported to and displayed by MHC II complexes. The peptide-loaded MHC II complex is recognised by T cells, which then provide appropriate signals through direct interactions of cell surface proteins and through cytokine signalling processes, to induce maturation of the B cell into an antibody secreting plasma cell. The role of dendritic cells in the process is not yet defined, and the process is probably different in adults who have already been exposed to the saccharide immunogens – glycoconjugate vaccines typically invoke an antibody response in adults after a single dose (**27**). Since crosslinking of surface immunoglobulin molecules is not required, glycoconjugate vaccines can be produced from small saccharide chains. In many cases, the glycans attached in the conjugate are oligosaccharides prepared by degradation of the original polysaccharide (**28**). In addition, glycoconjugates can be produced

from relatively low molecular weight oligosaccharides related to the repeating polymers (**29**), or the short glycans of LPS O-chain (Gupta et al. 1998), or low molecular weight capsular polysaccharides such as those expressed by *Staphylococcus aureus* (Fattom et al. 2004). It has been shown possible to make effective glycoconjugate immunogens from low molecular weight oligosaccharides such as those present on the lipo-oligosaccharides of pathogens such as a *Neisseria meningitidis* (Mieszala et al. 2003). The same glycoconjugate technology has been used to prepare immune therapeutics to slow the redevelopment of cancer following chemotherapy, prepared from the glycan chains of glycolipids overexpressed by tumour cells (Musselli et al. 2001). Further discussion of cancer immunotherapeutics is outside the remit of this review. The first glycoconjugate vaccines against *Haemophilus influenzae* type b were licensed in the late 1980s. They arose from the academic work of Porter Anderson and of others (**30**). The Anderson approach involved reductive amination of periodate-generated aldehyde-terminated oligosaccharides to a carrier protein. In modern preparations, CRM197, a genetically toxoided variant of diphtheria toxin is used. The resulting glycoconjugate is approximately 90 kDa in size, is approximately 30% carbohydrate and contains an average of six glycan chains per carrier protein. It is similar in size and saccharide content to many serum proteins, and can be termed a "neo-glycoconjugate" vaccine. Another approach, originally developed by Hilleman (Tai et al. 1987) and commercialised by Aventis Pasteur and GSK, involves random activation of the polysaccharide with cyanogen bromide, addition of linker such as 6-aminocaproic acid or adipic acid dihydrazide linker, and attachment to an appropriate carrier protein – typically tetanus toxoid. As there are multiple activation points within each polysaccharide and multiple linkage points on each carrier protein, the resulting conjugate is a crosslinked network of polysaccharide and protein with a molecular weight of, on average, 5×106 Da. Such a vaccine can be described as a "crosslinked network". The third approach uses conjugation of size-reduced polysaccharide to LPS-depleted vesicles of outer membrane proteins – a "vesicle vaccine". Thus there are three fundamentally different structures for these conjugates, which are illustrated as cartoons in Figure- 3. The immune responses elicited by these different structural variants are generally similar, all are T cell-dependent immunogens, although the vesicle-based vaccines seem to be characterized by a stronger antibody response following the first immunisation, a less pronounced booster effect

on subsequent immunisations, and that the antibodies produced tend to be of lower avidity to than those produced by the other two structural types (Schlesinger and Granoff 1992), and different light chain V regions are used (Granoff et al. 1993). The time course for the development of an antibody response following administration of different Hib conjugate vaccines. Usually only two doses of the vesicle vaccines and a booster dose are given. These conjugate vaccines proved extremely effective at preventing disease in those countries which have adopted them as part of mass vaccination programmes, so that Hib meningitis, which had been the most common form of neonatal meningitis in developed countries, has been almost completely eradicated. The startling effectiveness of these vaccines stimulated a demand that their usage be expanded to other countries, with a WHO target of global coverage (Anonymous 1998). Trial introduction of Hib vaccination into some developing countries highlighted the fact that the disease burden due to this organism had been seriously underestimated. It had also become clear that glycoconjugate vaccines were an effective generic technology which could be used to protect against a wide variety of other pathogens, if the conjugates were made.

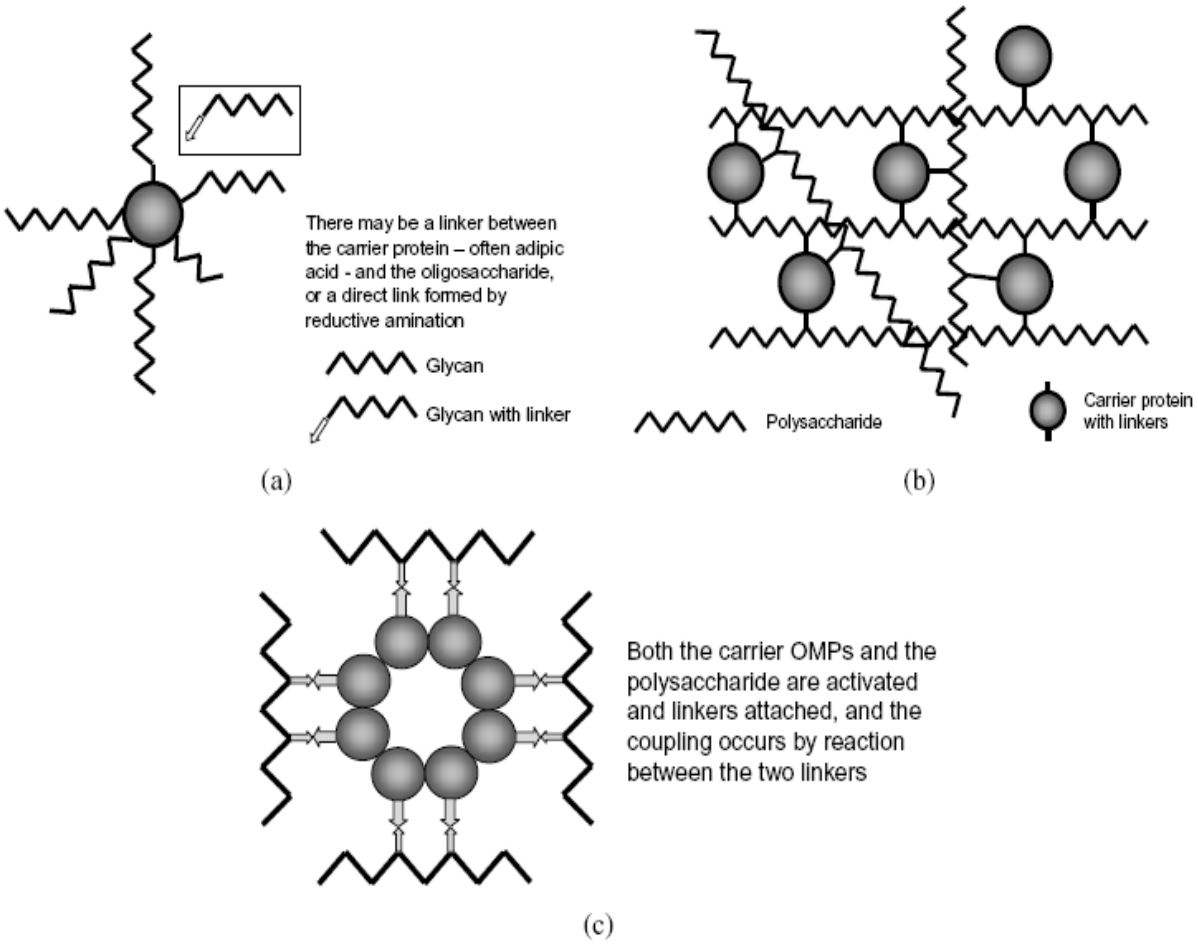

Fig.5: Pictorial representations of different structural types of glycoconjugate vaccines.

As shown in Figure 5(a) neoglycoconjugate vaccine is produced by coupling of oligosaccharides to an appropriate carrier protein such as CRM197. Typical CRM197 conjugates contain an average of six chains per carrier protein. Whilst monofunctional activated oligosaccharides such as those produced by active ester chemistry are incapable of crosslinking protein, bifunctional oligosaccharides produced by periodate oxidation may lead to occasional crosslinks. In Figure 5 (b) a crosslinked network conjugate vaccine is showed. Random multiple activation of the polysaccharide and coupling to a carrier protein leads to multiple crosslinks between the macromolecules to form a network of very high molecular weight. In Figure 5(c) a vesicle-based vaccine is shown, in which size-reduced polysaccharide is coupled to a LPS-depleted vesicle comprised of outer membrane proteins. There are multiple linkages between the saccharide chain and the “carrier protein”.

Capter.3

MATERIALS AND METHODS

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3. MATERIALS AND METHODS

3.1. General

This chapter describes materials used and outlines the experimental design for culture of S.pneumoniae and E. coli, extraction and purification of capsular polysaccharide.

3.2 Glassware and Apparatus

All glass wares (Conical flasks, Measuring cylinders, Beakers, Petri plates and Test tubes etc.) are purchased from Mr Vinoy of San Medico Ltd (Kolkata, India) under the name Borosil. The equipment and apparatus used throughout the experiment are listed in Annexure-I.

3.3 Chemicals and Reagents

Thimerosal (Himedia), terrific broth , formaldehyde , PBS, sodium deoxycholate , ethanol, Tris-$MgSO_4$ buffer , hydroxymethylamino-methane (Himedia) , tryptic soy broth, phenol-acetate solution , ribonuclease-A 0.75 mg (Sigma-Type 1-AS, R-5503), hydrochloric acid, methyl- Cellosolve , acidified butan-1-ol , ethylene glycol, dimethyl sulphoxide, dimethyl formamide, thiobarbituric acid, periodic acid, sodium arsenite, *m*-hydroxydiphenyl solution, sodium tetraborate, sulfamic acid.

*3.3. Culture Organis*m

The microbes Strepococcus pneumoniae and E. coli was purchased from IMTECH Chandigarha. These organisms are and subsequently sub cultured in soybean casein digest and terrific broth respectively.

Table.3: Composition of Soybean casein digest broth(pH 7.3)for S. pneumoniae culture

Ingredients	Grams/litre
Casein peptone	17.0
Soy peptone	3.0
Sodium chloride	5.0
Dipotassium hydrogen phosphate	2.5
Glucose	2.5

Table.4:Composition of Terrific Broth for Escherichia coli culture

Ingredients	**Grams/Litre**
Protein hydrolyzate NZ amine	12.0
Yeast extract	24.0
Dipotassium hydrogen phosphate	9.4
Potassium dihydrogen phosphate	2.2

3.5. Extraction and purification of PS

The extraction and subsequent purification of the PS is carried out using the following procedure:

CPS was prepared from *S. pneumoniae and E. coli* strains (obtained from IMTECH Chandigarh) in 5-liter flasks containing 2 liters of tryptic soy broth and terrific broth respectively and incubated at 37°C for 18 to 24 h. Growth was stopped by adding formaldehyde to a final concentration of 0.2% (wt/vol); then the cells are separated by centrifugation at 4^{0} c for 15 min min at 6000rpm and washed 2 to 3 times with PBS, then cells were lysed with sodium deoxycholate (0.1%, wt/vol) . The mixture was centrifuged for 15 minutes at 17,000g in the cold (4°C). The supernatant is collected and ethanol was added to a concentration of 25%. This material was then centrifuged for 2 hours at 17,000 xG (4°C) and the supernatant was collected. Ethanol, at four times the volume of the supernatant was added and was incubated at 4° C overnight.

3.6. Removal of Nucleic Acids

The material is centrifuged for 5 minutes at 2800 xG (4° C.). The sediment is collected and

resuspended in Tris-$MgSO_4$ buffer at one-fourth the volume originally used to extract the paste.

The composition of the Tris buffer is as follows per liter of distilled water: tris-

hydroxymethylamino-methane (Himedia) 6 gm $MgSO_4.7H_2O$ 246 mg thimerosal (Himedia)

50 mg. The pH is adjusted to 7.0±0.2 with concentrated hydrochloric acid.

Deoxyribonuclease I 1.5 mg (Sigma D-0876) and ribonuclease-A 0.75 mg (Sigma-Type 1-AS, R-5503) per 100 gm of original wet paste are added and incubated for 18 hours at 37° C.

3.7. Removal of Proteins

The material is further processed to remove protein components by adding an equal volume of phenol-acetate solution (135 ml of 10 percent (w/v) sodium acetate combined with 454 gms of phenol). The material is then shaken for 30 minutes (4° C.), centrifuged for 15 minutes at 17,000 xG and the aqueous phase collected. Two additional phenol extractions are conducted.

The material at this stage constitutes the bulk liquid capsular polysaccharide (PS) and is stored at -20° C. until further processing.

3.8. Sialic acid assay of polysaccharide

Among the colorimetric methods in use for the quantification of sialic acid (N-acetylneuraminic acid) the thiobarbituric acid test gives the highest molar extinction coefficient and thus is the most sensitive. However, the fading character of the chromophore and the necessity to extract it with acidified butan-1-ol are inconvenient. In addition the thiobarbituric acid assay lacks the uniformity of colour development with the different sialic acid analogues (N-acetyl-, N-glycollyl-neuraminic acid and their O-acetylsubstituted forms). We investigated the effect of the water-miscible organic solvents ethylene glycol, dimethyl sulphoxide, dimethyl formamide and methyl- Cellosolve on the thiobarbituric acid chromophore.

3.9. Uronic acid assay.

Uronic acid in the form of galacturonic acid is a major component of the capsular polysaccharide. Quantitative measurement of total uronic acid is commonly done using colorimetric methods after first hydrolyzing the polysaccharides in sulfuric acid and extrapolating from the standard glucuronic acid curve

3.10. FTIR analysis of the samples.

Finally, the presence polysaccharide was confirmed by detecting the functional groups in the repeating monomer unit characteristic for the polysaccharide.

Chapter.4

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

4.1. Culture and extraction

The microbes Strepococcus pneumoniae and E. coli was subsequently sub cultured in soybean casein digest and terrific broth respectively. The growth curve are studied after 18 hours of culture the growth are stopped by formaldehyde and centrifuged. After centrifugation

The PS are extracted by adding sodium deoxycholate

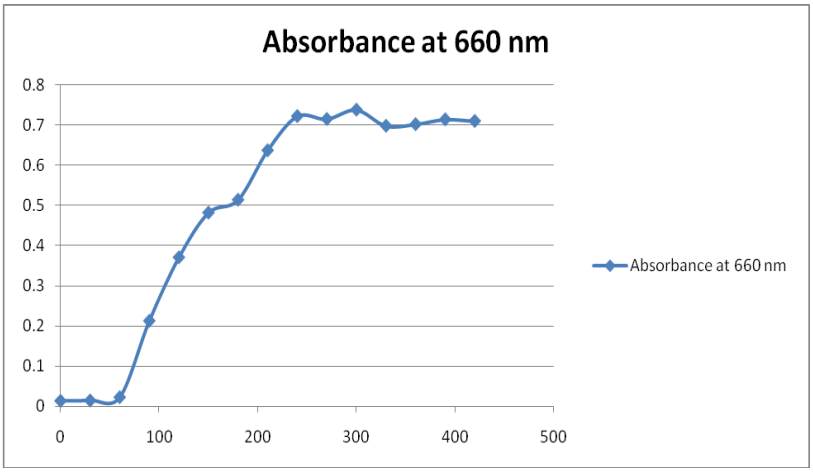

Fig.6: Characteristic growth curve of Escherchia coli observed as absorbance at 660nm as a function of time.

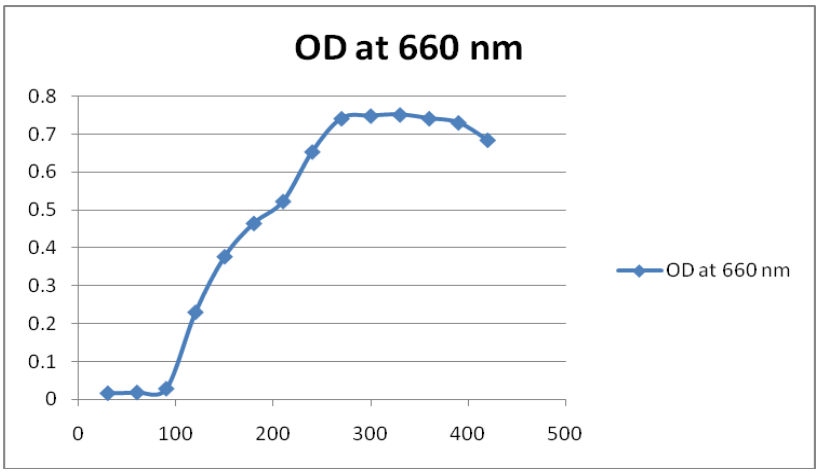

Fig.7: Characteristic growth curve of Streptococcus pneumoniae observed as absorbance at 660nm as a function of time.

4.2 Purification of PS

4.2.1. Bradford assay

The Bradford assay, a colorimetric protein assay, is based on an absorbance shift in the dye Coomassie when the previously red form coomassie reagent changes and stabilizes into coomassie blue by the binding of protein. The (bound) form of the dye has an absorption spectrum maximum historically held to be at 595 nm. The cationic (unbound) forms are green or red while binding of the dye to protein stabilizes the blue anionic form. The increase of absorbance at 595 nm is proportional to the amount of bound dye, and thus to the amount (concentration) of protein present in the sample.

4.2.2. Removal of nucleic acid

Before removing the nucleic acid the solution of polysaccharide, nucleic acid and protein are analyzed for protein and nucleic acid content. The Bradford assay graph shows the concentration of protein in the solution. The graph shows the concentration of protein in polysaccharide solution of S. pneumoniae and E. coli as approximately 36µg and 48µg per ml of the extracted PS mixture respectively

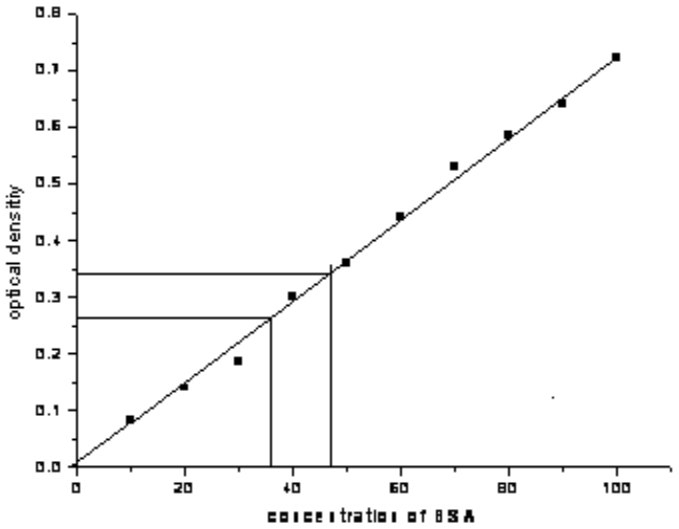

Fig.8: The concentration of protein in polysaccharide extract of Streptococcus pneumoniae and Escherichia coli extra plotted from BSA standard curve.

4.2.3. Nucleic acid contamination

The ratio of absorbance at 260:280 is commonly used to assess the purity of protein solution with respect to nucleic acid contamination, since protein (in particular, the aromatic amino acid) tends to absorb at 280nm. After solution becomes nucleic acid free it is assayed for the nucleic acid contamination shown in table.5

Table.5: Results of the nucleic acid contamination in different polysaccharide extracts of S. pneumoniae and E.coli detected by absorbance ratio at 260nm and 280nm.

Wave length in nm	absorbance		260:280	
	S. pneumoniae	E.coli	S. pneumoniae	E.coli
250	0.56	0.52	1.355	1.619
260	0.61	0.68		
270	0.42	0.40		
280	0.45	0.42		
290	0.34	0.38		

Table.6: Values of standard of nucleic acid contamination and their 260:280 absorbance ratio.

% protein	%nucleic acid	260:280 ratio
100	0	0.57
95	5	1.06
90	10	1.32
70	30	1.73

After removal of nucleic acid the sample are further processed for nucleic acid contamination and the result are shown in the table.7 and compared with the standard 260:280 ratio table

Table 7: Results of nucleic acid contamination in the polysaccharide extracts after nucleic acid removal using Nucleases

Wave length in nm	Absorbance		260:280 ratio	
	S. pneumoniae	E.coli	S. pneumoniae	E.coli
250	0.251	0.214	0.5548	0.572
260	0.253	0.242		
270	0.372	0.325		
280	0.456	0.423		
290	0.339	0.397		

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4.2.4. Removal of protein

The mixture when treated with tris $MgSO_4$ and DNAse and RNAse the nucleic acid are dissolved and the concentration of protein subsequently increased due to DNAse and RNAse

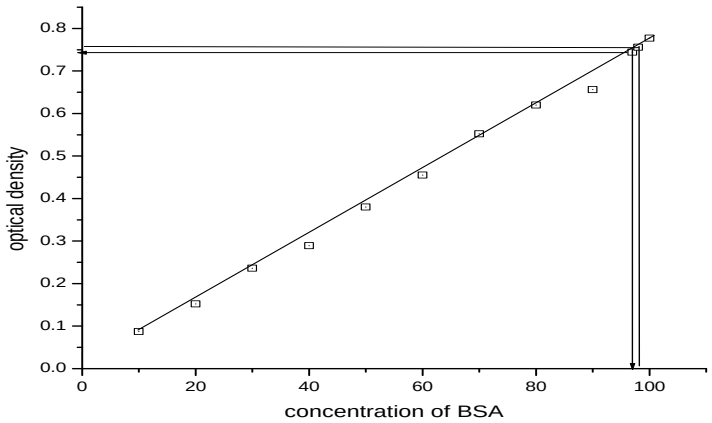


Fig.9: Increased concentration of protein after removal of nucleic acid by DNAse and RNAse plotted from BSA standard curve

The material is further processed to remove protein components by adding an equal volume of phenol-acetate solution (135 ml of 10 percent (w/v) sodium acetate combined with 454 gms of phenol). The material is then shaken for 30 minutes (4° C.), centrifuged for 15 minutes at 17,000 xG and the aqueous phase collected. Two additional phenol extractions are conducted. The material at this stage constitutes the bulk liquid capsular polysaccharide (PS) and is stored at -20° C. until further processing.

Table.8: Concentration of protein obtained using Bradford assay after protein removal using phenol acetal precipitation.

Conc. µg	5	5.5	6	6.5	7	7.5	8	8.5	9	9.5	10	S.p 0.18	E.coli 0.22
Abs	.039	.042	.047	.049	.053	.057	.061	.067	.07	.073	.78	.0043	.0062

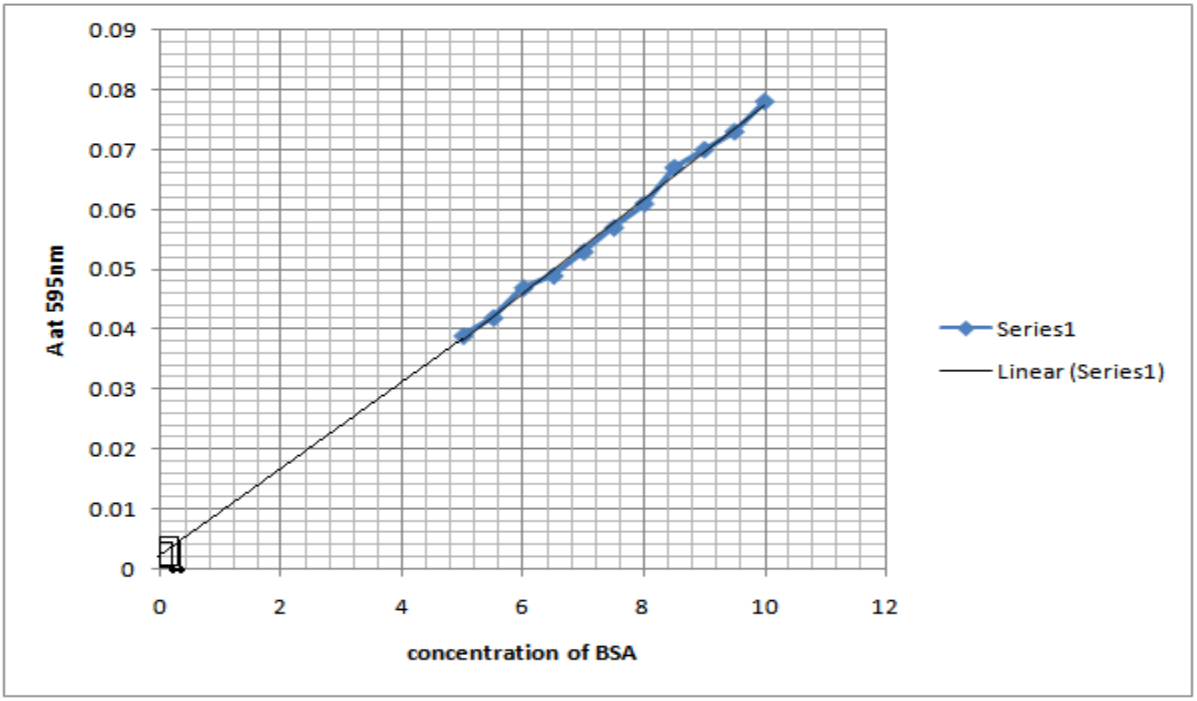


Fig.10: Concentration of protein obtained using Bradford assay after protein removal using phenol acetal precipitation.

4.3. Sialic acid assay for PS

The quantification of sialic acid (N-acetylneuraminic acid) the thiobarbituric acid test is the most sensitive. However, the fading character of the chromophore and the necessity to extract it with acidified butan-1-ol are inconvenient. In addition the thiobarbituric acid assay lacks the uniformity of colour development with the different sialic acid analogues (N-acetyl-, N-glycollyl-neuraminic acid and their O-acetylsubstituted forms). We investigated the effect of the water-miscible organic solvents ethylene glycol, dimethyl sulphoxide, dimethyl formamide and methyl- Cellosolve on the thiobarbituric acid chromophore. Although both the Warren (1959) and Aminoff (1961) procedures are able to measure the sialic acid with an accuracy of ±1 %, they react only with free sialic acid, which must therefore be released by acid hydrolysis or neuraminidase digestion. To extend the usefulness of the test, several scaled-down versions of the standard Warren (1959) assay were developed (Bretscher, 1971; Hahn et al., 1974), which used 0.05-0.025 times the sample and reagent volumes. Although the fluorimetric assay of Hess & Rolde (1964) is capable of detecting submicrogram

concentrations of sialic acid, it requires acid hydrolysis for 24h at 100°C for maximum efficiency. The alkaline condition is necessary to convert thiobarbituric acid-resistant O-acetylated sialic acid variants into reactive neuraminates.

4.3.1. Acid hydrolysis for the liberation of sialic acid

Samples of polysaccharide with sialic acid were hydrolysed in 0.025 or 0.05M-H_2S0_4, the pH being rigorously maintained between 1.6 and 2.0. Hydrolysis was performed at 80°C for 60 minutes.

4.3.2. Thiobarbituric acid assay for sialic acid

Periodate/thiobarbituric acid assay is a modification of the Aminoff (1961) assay, and covers a range of 0.3-20µg of sialic acid. The final volume varies according to the sialic acid content of the test material. If sialic acid content of the test material is low(less than 2 %), the acid-hydrolysed material, usually 1 mg/ml, containing up to 3 µg of sialic acid in sample volumes of 0.2 ml, can be measured by the micro-method. The sample (0.2ml) is oxidized by addition of 0.05 ml of 25µM-periodic acid/62.5mM-H_2SO_4 at 37^0C for 30min. Oxidation is terminated by the addition of 0.05 ml of 2% (w/v) sodium arsenite/0.5M-HCI. This is followed by the addition of 0.1 ml of 6% (w/v) thiobarbituric acid, adjusted to pH 9.0 with NaOH, to give a final concentration of at least 1 %. The chromophore is developed by heating the reaction mixture in a boiling-water bath for 7.5min. The colour is intensified by the addition of an equal volume of dimethyl sulphoxide, bringing the total volume to 0.8 ml.

For PS with high sialic acid content the method may be used. As shown in Figure 11 the effect of organic solvents on the spectrum of thiobarbituric acid chromophore of N-acetylneuraminic acid was investigated using Ethylene glycol, dimethyl sulphoxide; dimethylformamide (water-miscible solvents); acidified butanol ('water-immiscible' solvent). Solvent volume exceeded that of the sample mixture by a factor of 10, because at this proportion butanol becomes miscible with water and does not give phase separation, allowing direct comparison of spectra. N-Acetylneuraminic acid (100µg) in 2ml of water reacted with 0.5ml of periodate, 0.5ml of arsenite and 1.0 ml of thiobarbituric acid reagents;

O.5ml of this test mixture was added to 5ml of the respective organic solvents. The inset shows yield of chromophore as a function of the percentage of solvent/total test volume. Therefore, the we deduced that the volume used in spectrophotometry can be increased by the addition of more dimethyl sulphoxide (4ml or more; see Fig. 11), to obtain a final range of 1-2, µg of sialic acid/ml. In manipulations requiring several pH adjustments, as in alkaline de-O-acetylation, it is most convenient to use the macro-method. In this, 1.0ml batches of the processed material, 0.25ml of periodate, 0.25ml of arsenite, 0.5ml of thiobarbituric acid reagent and 2.5ml of dimethyl sulphoxide are used. The A_{549} and A_{532} were measured. Other steps in the procedure, including preparation of the reagents, followed the original procedure of Aminoff (1961).

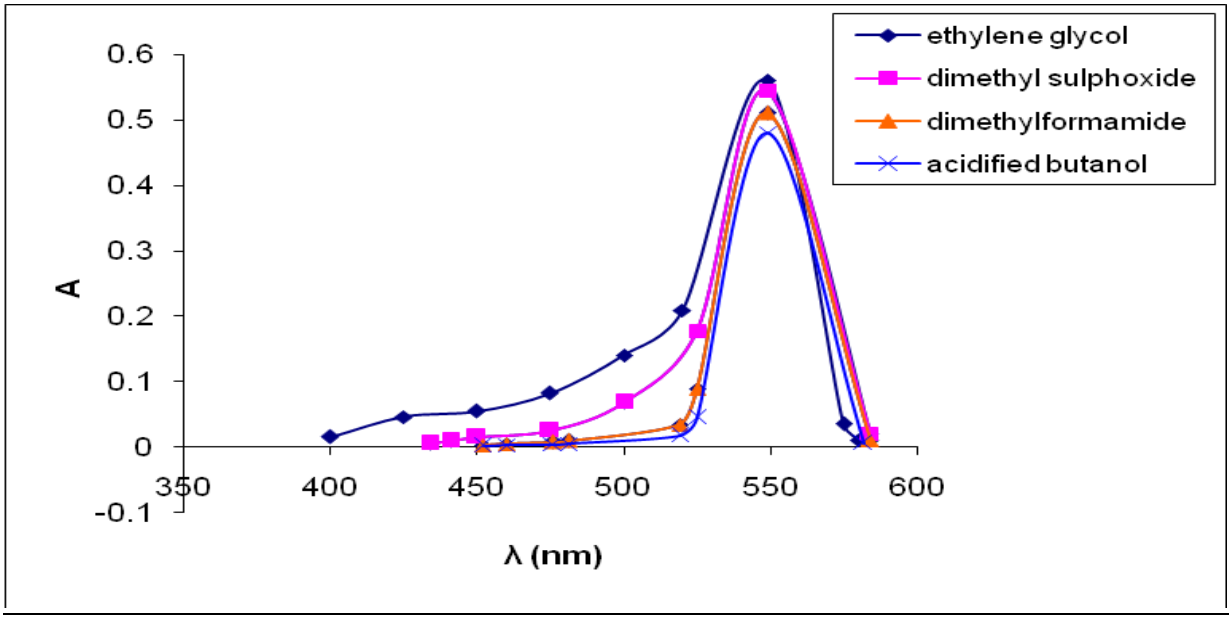


Figure 11 Effect of organic solvents on the spectrum of thiobarbituric acid using Ethylene glycol, dimethyl sulphoxide; dimethylformamide (water-miscible solvents); acidified butanol ('water-immiscible' solvent).

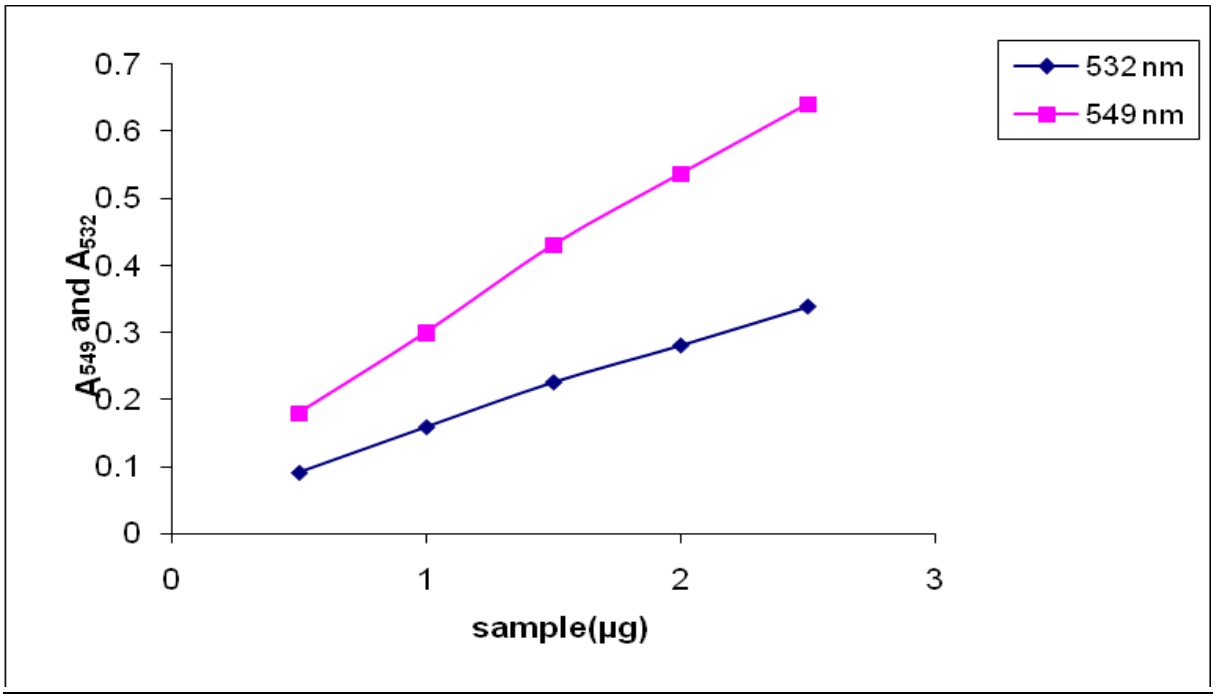


Fig.12: Calibration plot of N-acetylneuraminic acid S. pneumoniae by modified thiobarbituric acid micro-assay

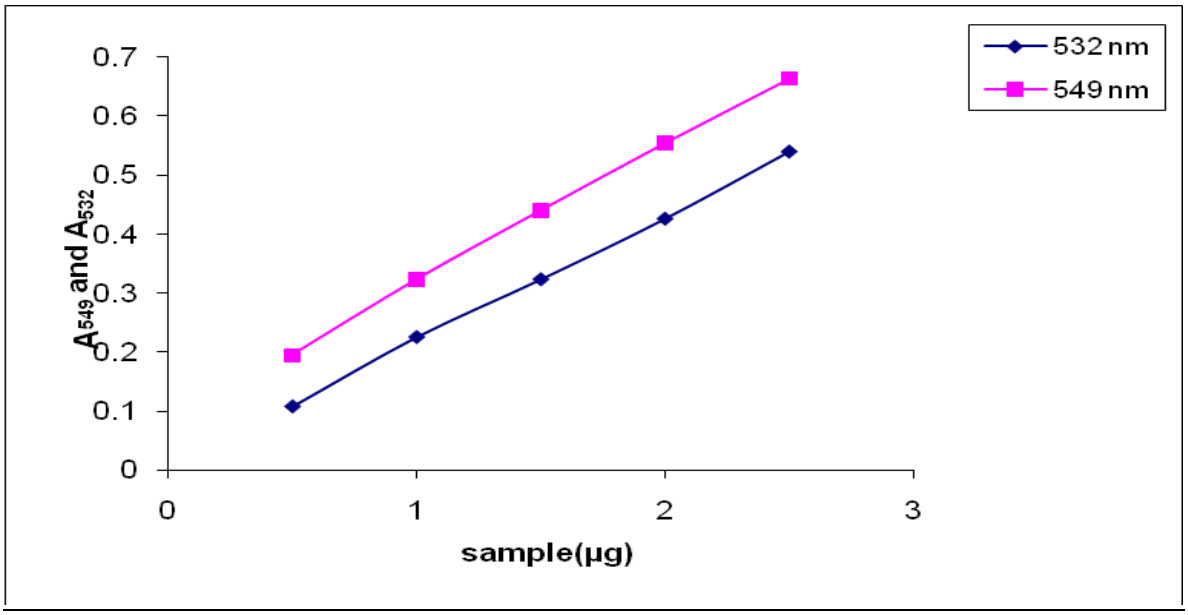


Fig.13: Calibration plot of N-acetylneuraminic acid of E.coli by modified thiobarbituric acid micro-assay

Fig.12 and 13 shows the calibration plot of N-acetylneuraminic acid of S. pneumoniae (fig.12) and E. coli (fig.13) by our modified thiobarbituric acid micro-assay. For this 0.2ml of sample, 0.05ml of periodic acid, O.05ml of sodium arsenite, 0.1 ml of thiobarbituric acid reagent and 0.4ml of dimethyl sulphoxide were used. Each test mixture was read at the absorption maximum for sialic acid (549nm).

4.3.3. Alkaline treatment for analytical de-O-acetylation

A sample of PS in water is mixed with an equal volume of 0.1M-NaOH and kept for 20min at room temperature (21°C). To terminate the reaction, the pH is adjusted to 1.6-2.0 with 0.1M-H_2SO_4. If 0.1M-NaOH is used (0.05M final concentration), the incubation period is extended to 60min (see Fig. 14). After alkaline treatment, the sialic acid of the sample is liberated by acid hydrolysis at 80°C for 1 h. Samples of the thus processed material, containing approx. 1.75 or 8.8µg of sialic acid, are used in our micro- or macro-modification of the thiobarbituric acid assay. Alkaline treatment after the acid hydrolysis is similar, but requires initial neutralization of the acid and adjustment to acidic pH for the assay.

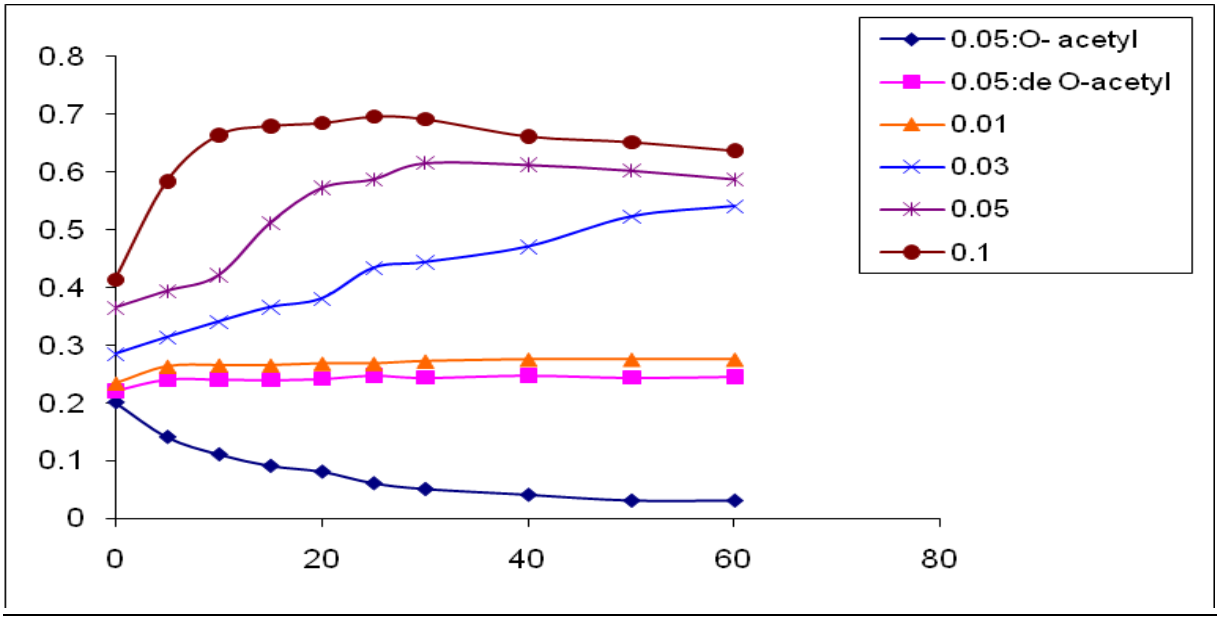


Fig.14: Progress curve of de-O-acetylation of sialic acid under different alkaline conditions.

4.3.4. Application of water-miscible organic solvents

To circumvent the precipitation and the necessity for partitioning, and to stabilize the chromophore, attempts were made to use the following water miscible organic solvents: ethylene glycol, dimethyl sulphoxide, dimethylformamide and methyl-Cellosolve. Fig. 4 indicates that the above solvents can substitute successfully for acidified butanol, since nearly identical absorption spectra are obtained. Methyl-Cellosolve has been applied for the extraction of the chromophore by Saifer & Gerstenfeld (1962); however, we found it inferior to the other listed solvents, because it yields some precipitate, and thus centrifugation is still necessary.

This reagent is an excellent solvent of most PS and also of the reagents used in the thiobarbituric acid assay. Because there is no phase separation or precipitation, no centrifugation is needed. Since the chromophore is distributed in the total reaction volume, the concentration of dye is only 50% of that obtained with an equal volume of acidified butanol. The inset in Fig.11 indicates that for optimal colour yield with dimethyl sulphoxide at least 50% of the total volume must consist of the organic solvent. However, a great advantage is that the colour is stable for days. This eliminates the uncertainties associated with the fading character of the chromophore of acidified butanol extraction and allows dilution with additional dimethyl sulphoxide and accurate measurement if a particular sample gives too high absorbance.

4.3.5. Analytical aspects of alkaline de-O-acetylation by using our dimethyl sulphoxide/thiobarbituric acid method

Another deficiency of the routine thiobarbituric acid spectrophotometric assay is its lack of uniformity of colour yield with the different sialic acids (N-acetyl-, N-glycollyl-neuraminic acid and their O-acetyl-substituted forms) (49). The O-acetyl groups of N-acetyl-O-acetylneuraminic acids are cleaved under very mild alkaline conditions (Neuberger & Ratcliffe, 1972), converting these sialic acids into their thiobarbituric acid-sensitive N-acetyl forms.

Fig.14 illustrates the progress of alkaline de-O-acetylation at various concentrations of NaOH, followed by acid hydrolysis and thiobarbituric acid reaction. As Fig.14 indicates,

without alkaline treatment, only 56% of the sialic acid was reactive in the thiobarbituric acid assay, whereas at completion of the alkaline reaction(20minfor 0.1 M-NaOH or 50min for 0.05M-NaOH), the de-O-acetylated neuraminic acid reacted in the thiobarbituric acid assay with an efficiency equal to that of the N-acetyl form, resulting in 100% recovery. Total recovery was confirmed by the resorcinol assay, a test capable of detecting both the N-acetyl and the O-acetyl forms of both free and bound sialic acids. The gradual disappearance of the O-acetyl content on alkaline saponification was followed by the hydroxamic acid method of Hestrin (1949) for acetyl ester groups. The decrease of acetyl esters was proportional to the thiobarbituric acid-colour increment. Both reactions followed first-order kinetics. With 0.05M-NaOH, both reactions reached one-half completion in 12.5 min.

By using 0.05M-H_2SO_4 the time-curve of acid hydrolysis of PS with N-acetylneuraminic acid alone indicates that the amount of N-acetylneuraminic acid liberated from the PS is maximal within 30min and declines thereafter (Gibbons, 1963). Acid hydrolysis under the same conditions gave a thiobarbituric acid maximum only after 2h. Alkaline treatment with 0.1 M-NaOH before the acid hydrolysis allows all the sialic acid to react and peak efficiency to be achieved in 30min, when destruction of free N-acetylneuraminic acid is still negligible. Nearly complete recovery of all sialic acids was confirmed by the resorcinol method. Alkaline treatment after acid hydrolysis also yielded the same alkaline increment, suggesting that the acidhydrolysed fraction already contained the non chromogenic O-acetylneuraminic acid in a free form that became fully chromogenic (de-O-acetylated) on the subsequent alkaline treatment. Alkaline treatment preceding acid hydrolysis de-O-acetylates in situ and provides more N-acetylneuraminic acid for acid hydrolysis and direct thiobarbituric acid reaction. Thus the 'alkali' curves in Fig. 8 are a composite of release of N-acetylneuraminic acid, release of the O-acetylated (alkaline post-treatment) or de-Oacetylated (alkaline pre-treatment) neuraminic acid and of decay. Results with PS under identical conditions indicated that liberation of O-acetylneuraminic acid by acid hydrolysis is not universal. PS also yielded an abnormal hydrolysis curve, which could be corrected by preceding alkaline treatment, probably removing the O-acetyl group while the sialic acid is still PS bound. However, alkaline treatment after acid hydrolysis did not give the increment. This aberrant

behaviour suggests that the O-acetyl derivative of neuraminic acid in PS is not liberated by acid hydrolysis.

In contrast with our short and mild alkaline treatment, de-O-acetylation for extended time or at elevated temperature may lead to a false thiobarbituric acid-positive reaction, unrelated to sialic acid. Gibbons (1963) used 0.05M-Na_2CO_3 at 100°C for 80min. Under these harsher conditions alkaline treatment produces isosaccharinic acid from 1-4- linked aldoses (such as lactose or maltose) if present. These, like sialic acid, yield formylpyruvic acid on periodate oxidation and give the same chromophore (at 549 nm) with thiobarbituric acid (Barkeretal., 1967). However, under the mild alkaline conditions used in our tests, these sugars did not give the red chromophore and could not lead to false results.

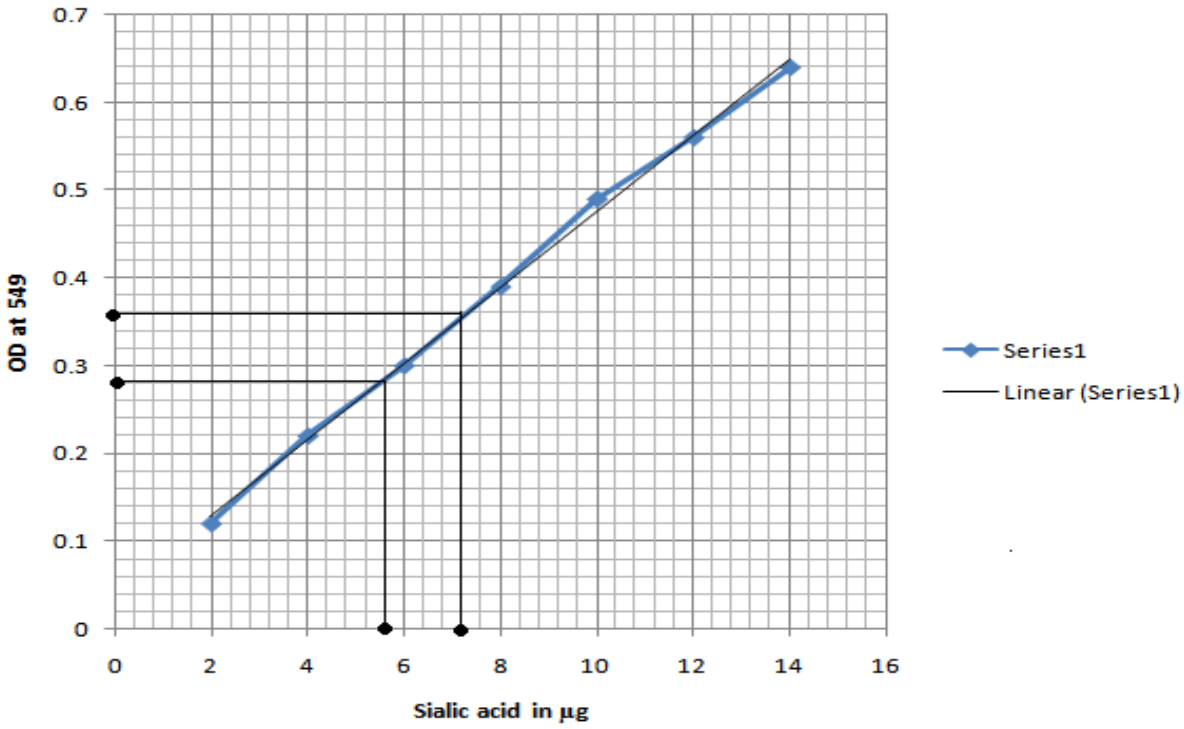


Fig.15: Standard curve of sialic acid showing concentration of sialic acid. The final concentrations of sialic acid in the sample are 7.2µg and 5.8µg for S. pneumoniae and E. coli respectively.

4.4. Determination of uronic acid

Uronic acid in the form of galacturonic acid is a major component of the capsular polysaccharide. Quantitative measurement of total uronic acid is commonly done using colorimetric methods after first hydrolyzing the polysaccharides in sulfuric acid (Ahmed and Labavitch, 1977; Selvendran et al., 1979). However, there is a major problem with the older methods of determining uronic acid content. Neutral sugars and their degradation products from acid hydrolysis can interfere in the colorimetric determination of uronic acids. The procedure developed by Filisetti-Cozzi and Carpita (1991) solves the problem, allowing uronic acids to be determined in up to ten times their weight of neutral sugars.

4.4.1. Hydrolyze polysaccharide

Add 1 ml concentrated sulfuric acid to the tube containing PS and cap. Set up a reagent control tube containing only 1 ml concentrated sulfuric acid and carry this through all the procedures. Place the tubes in an ice bath and stir the contents for 5 min. Add another 1 ml concentrated sulfuric acid to the tube and stir on ice for 5 min. Then add 0.5 ml water and stir for 5 min on ice.

Add another 0.5 ml water and stir for 5 min on ice. Dilute the contents of each tube with water to 10 ml in a 10-ml volumetric flask. Transfer to a 15-ml centrifuge tube and centrifuge for 10 min at 2000 × *g*, room temperature, to pellet any unhydrolyzed material.

4.4.2. Perform colorimetric assay

Set up three 15-ml borosilicate glass tubes. For the reagent control, set up two tubes. Take aliquots of 400 µl from each hydrolysate supernatant and reagent control and place in the respective tubes. Add 40 µl of 4 M sulfamic acid/potassium sulfamate solution, pH 1.6, to all the tubes. Vortex contents of the tubes. Then add 2.4 ml of 75 mM sodium tetraborate in sulfuric acid solution to all the tubes. Vortex vigorously. Place the tubes in a 100°C water bath (boiling) for 20 min, then cool by plunging tubes into an ice bath for 10 min. Add 80 µl *m*-hydroxydiphenyl solution to 2 tubes of each sample and the 2 reagent control tubes. Add to the third tube of each sample 80 µl of 0.5% NaOH (this is the sample control). Vortex the contents of the tubes three times; ensure they are mixed well. Between 10 min and 1 hr after complete mixture, read the absorbances at 525 nm against the reagent control. Subtract the values for the sample controls from their corresponding sample absorbances

4.4.3. D-Galacturonic acid standards

Make up a stock standard solution and generate a standard curve from this. Prepare a stock solution of 20 mg/ml of galacturonic acid by weighing 20 mg of the dried D-galacturonic acid into a vial and adding 1 ml of water to dissolve (this stock solution can be stored frozen). Then prepare a 200 µg/ml solution by taking 100 µl of the stock solution and adding 9900 µl of water. Use this solution to prepare the dilution series in Table

Table.9: Concentration and absorbance table of standard glucoronic acid and test samples showing the concentration of uronic acid in the polysaccharide extract of Streptococcus pneumoniae and Escherichia coli

Concentration of galacturonic acid (µg/400 µl)	Volume of 200 µg/ml galacturonic acid solution (µl)	Volume of water (µl)	Absorbance at 525 nm	Absorbance of test sample		Concentration of uronic acid in µg	
				S.pneumoniae	E.coli	s.pneumoniae	E. coli
5.0	125	1875	0.14	0.2	0.26	12	18
10.0	250	1750	0.18				
15.0	375	1625	0.23				
20.0	500	1500	0.27				
30.0	750	1250	0.36				
40.0	1000	1000	0.43				

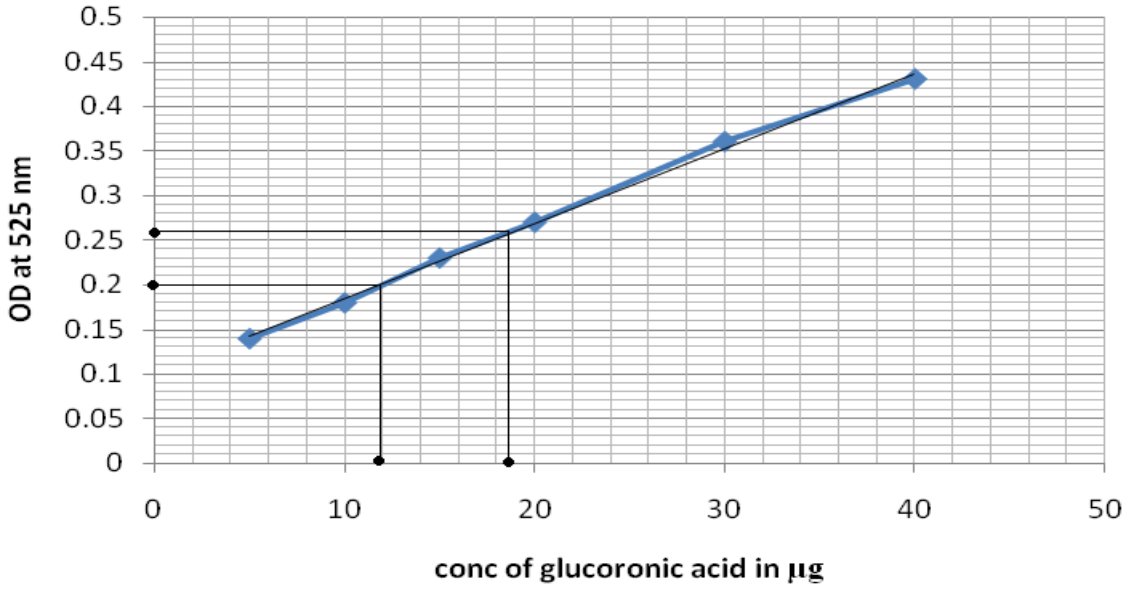


Fig.16: Standard curve of glucoronic acid showing concentration of uronic acid in the samples of Streptococcus pneumoniae and Escherichia coli polysaccharide.

4.4.4. m-Hydroxydiphenyl solution

Weigh out 0.15 g of 3-phenylphenol into a 100-ml volumetric flask, dissolve in <100 ml of 0.5% (w/v) sodium hydroxide (see recipe) then adjust the final volume to 100 ml with 0.5% sodium hydroxide. Store in a dark bottle (or wrap the bottle in aluminum foil) at 4°C. The solution is stable for 1 month

4.4.5. Sodium hydroxide, 5% (w/v)

Place 0.5 g of NaOH into a 100-ml volumetric flask. Add 20 ml water to dissolve the pellets and then adjust the volume to 100 ml with water. The reagent was prepared freshly.

4.4.6. Sodium tetraborate solution, 75 mM

To prepare 100 ml of 75 mM solution, weigh out 1.501 g of sodium tetraborate (mol. wt., 201.2) into a 100-ml volumetric flask and add ~90 ml concentrated sulfuric acid, and place a stopper in the flask. Stir until dissolved then adjust the final volume to 100 ml with sulfuric acid.

4.4.7. Sulfamic acid/potassium sulfamate solution, 4 M (pH 1.6)

Weigh out 38.84 g of sulfamic acid (mol. wt., 97.09) and stir vigorously in 50 ml of water. Add saturated KOH dropwise until the sulfamic acid has dissolved. Allow the sulfamic acid solution to cool and then carefully adjust the pH to 1.6 with saturated KOH. Adjust the volume to 100 ml with water to give a final concentration of 4 M. Store at room temperature

4.5. *FTIR analysis*

It is an acronym of Fourier Transfer Infrared spectroscopy. It may also called Infrared spectroscopy which is a chemically- specific analysis used to identify chemical compound and substituent groups**.**

The application of traditional infrared spectroscopy to low concentration measurements, such as ambient air measurements, is limited by several factors. First is the significant presence of water vapour, CO_2 and methane, which strongly absorb in many regions of the infrared (IR) spectrum. Consequently, the spectral regions that can easily be used to search for pollutants are limited to 760-1300cm^{-1}, 2000-2230 cm^{-1}, and 2390-3000 cm^{-1}. Another problem is that the sensitivity is not enough to detect very small concentrations in the sub-ppm level. Finally, spectral analysis was difficult since subtraction of background spectra had to be carried out manually.

The degree of absorption/transmission of infrared radiation at each wavelength is quantitatively related to the number of absorbing/transmitting molecules in the sample . Since there is a linear relationship between the absorbance/transmittance and the number of absorbing/transmitting molecules, multicomponent quantitative analysis of mixtures is feasible.

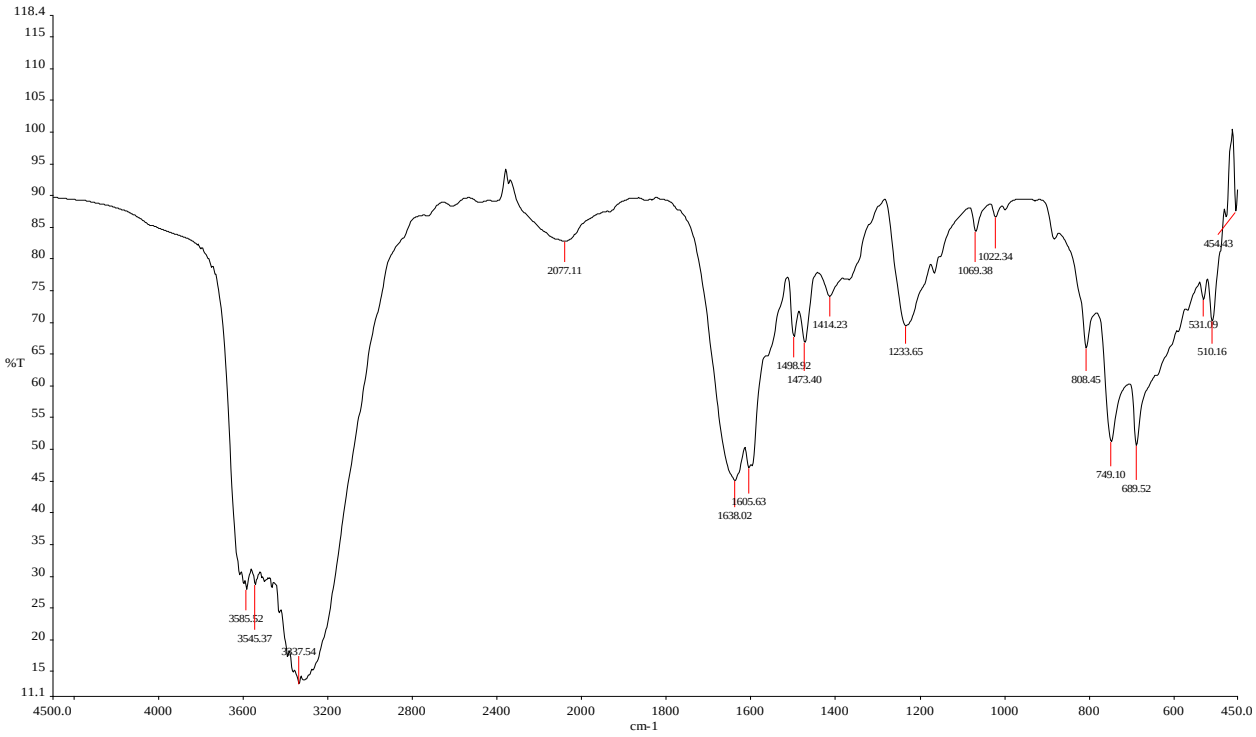


Fig. 17: FTIR spectroscopy analysis of Streptococcus pneumoniae polysaccharide extracted showing the transmittance trough at different wave number.

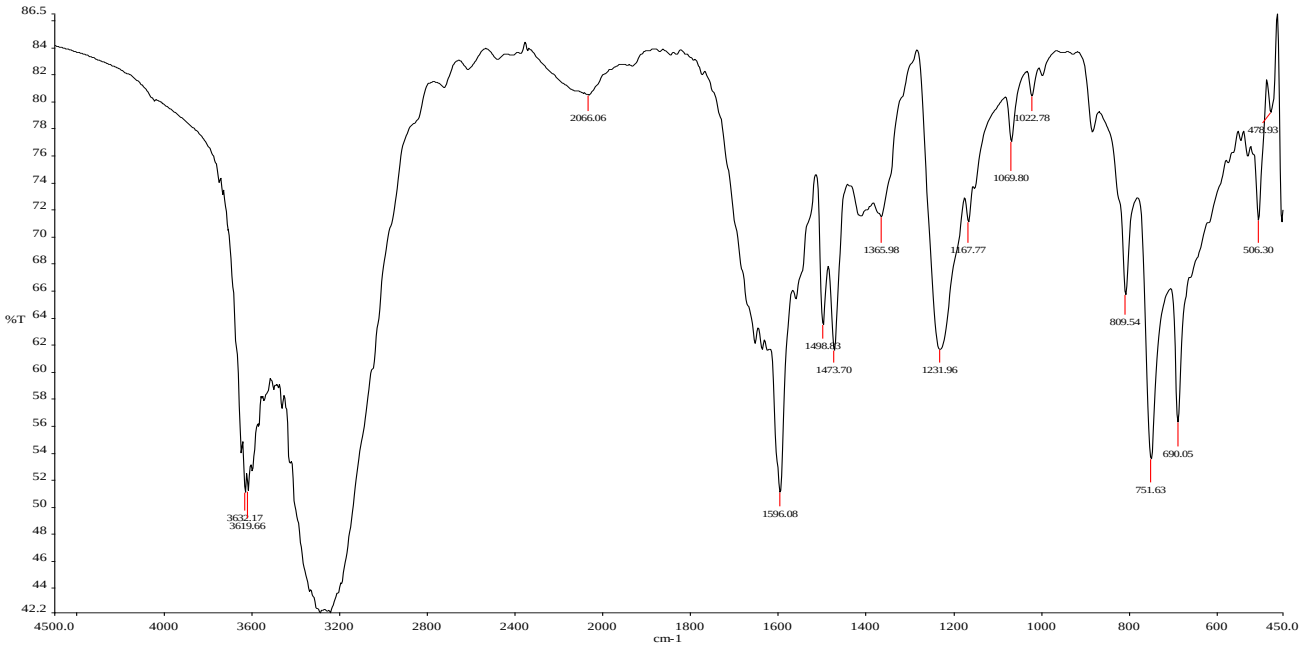


Fig. 18: FTIR spectroscopy analysis of Escherichia coli polysaccharide extracted showing the transmittance trough at different wave number

The FTIR results of S. pneumoniae showed in figure, has transmittance at wave numbers 3585, 3545 cm^{-1} for O – H stretch of alcohol, 3337 cm^{-1} N - H of amines, 1638, 1605 cm^{-1} of carboxylate and 1200–850 cm^{-1} of carbohydrate which is characteristic of capsular polysaccharides to be extracted in this work.

Also, the FTIR results of E. coli showed in figure. 17, has transmittance at wave numbers 3632,3619cm -1 for O – H stretch1596 cm-1 for carboxylate, 1498,1473 for N – O stretch and 850 – 1200cm-1 for carbohydrate.

4.6. Discussion

Streptococcus pneumoniae and Escherichia coli are responsible for various invasive and non invasive diseases in the humans especially in children of age group of two to eight. The main antigenic part that causes the pathogenicity is the capsular polysaccharide. The large structure of polysaccharide hides the cell surface antigen and also protects the bacteria from phagocytosis by macrophages. This polysaccharide is T-cell independent so unable to activate the B–cell for antibody against it (36).

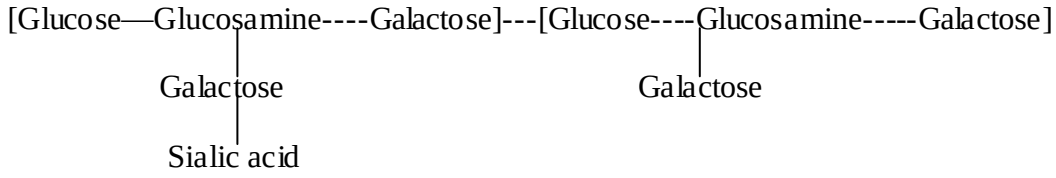
The structure of this bacterial antigen is similar to some part of the brain due to which the host immunity is unable to detect the antigen as it considers it as a part of the human body. So to activate the antigens and make it T-cell dependent, it has to be conjugated with a carrier protein such as tetanus toxoid which has a number of activating sites (37).

A number of methods are employed for the extraction and purification of capsular polysaccharide from different bacteria. In this thesis work the extraction and purification of capsular polysaccharides from Streptococcus pneumoniae and Escherichia coli was done using simple methods so that the preparation of conjugate vaccines becomes less tedious and cost effective.

In this method the extracted polysaccharides were purified by removing the nucleic acid using Nucleases and protein content by precipitation of the proteins present. The final concentration of nucleic acid contamination was determined by 260:280 absorbance ratios and it was found that no nucleic acid contamination was present in both the extracts. In the following step of protein removal by precipitation it was found that the protein contamination in the final sample was negligible. Finally, the presence of the specific polysaccharides was confirmed by sialic acid assay and uronic acid assay which showed the presence of polysaccharide at a concentration of 12 and 18µg for Streptococcus pneumoniae and Escherichia coli respectively.

Further FTIR analysis was used in this work for the confirmation of the extracted compound and the respective transmittance at the specific wave number characteristic for the functional groups documented in the composition of the polysaccharides of the two strains was confirmed to be present in the compound extracted through this thesis work.

Below the repeating unit in case of S. pneumoniae and E.coli polysaccharide and the respective molecular weight of the compounds which form the repeating units is shown (62).



Chemical Compound	Molecular Weight
Glucose	180
Glucosamine	179.17
Galactose	180.16
Sialic acid	309.27
Acetamido-2deoxy D mannose	221.21
D-Glucuronic acid	194.14

Using the above values we got the total molecular weight of the repeating unit in the polysaccharides as 1748.25. The fraction of sialic acid present in one repeating unit was found to be 0.1769 (309.27/1748.25). As earlier shown, the amount of sialic acid quantified in the final obtained polysaccharide was 7.2 µg and 5.8 µg in case of S. pneumoniae and E.coli respectively (Fig.15). By simple mathematical calculation one can find out the amount of total polysaccharide from the amount of sialic acid present in the extract.

Total amount of Polysaccharide:
In case of S. pneumoniae = 7.2/0.1769 = 40.70 µg/ml
In case of E.coli = 5.8/0.1769 = 32.78 µg/ml

Similarly, from the amount of glucoronic acid one can calculate the amount of total polysaccharide in the extract.

The repeat sequence of the poly saccharide in the sample where glucoronic acid is present is:

D-Glucos -----Galactose-----D-Glucuronic acid----Acetamido-2deoxy D mannose
|
D-Glucose

Using the above values we got the total molecular weight of above shown repeating unit in the polysaccharides as 1135.67. The fraction of glucuronic acid present in one repeating unit was found to be 0.1709 (194.14/1135.67). As earlier shown, the amount of glucuronic acid quantified in the final obtained polysaccharide was 12µg and 18µg in case of S. pneumoniae and E.coli respectively (Table.9). By similar mathematical calculation the total polysaccharide from the amount of glucuronic acid present in the extract is thus calculated.

Total amount of Polysaccharide:

In case of S. pneumoniae = 122/0.1709 = 70.22 µg/ml

In case of E.coli = 18/0.1709 = 105 µg/ml

Therefore we report that the concentration of polysaccharides in the final purified extract of S. pneumoniae and E.coli respectively is much higher than the amount of protein contamination quantified (0.18 µg/ml and 0.22 µg/ml respectively, (Table.8).

Results indicate that the polysaccharide of interest is extracted in sufficient amount and can be considered enough for proceeding toward conjugate vaccine preparation after due lyophilization or concentration of the polysaccharide.

Capter.5

CONCLUSION

5. CONCLUSION

In conclusion, extraction and purification of capsular polysaccharide from Streptococcus pneumoniae and Escherichia coli was accomplished successfully. The polysaccharide was quantified using sialic acid and uronic acid assays. A qualitative analysis using Infrared spectroscopy confirmed the presence of polysaccharide in the sample. This method would be useful for obtaining antigens from the capsule of any organism that has capsular polysaccharide. The polysaccharide prepared using this method is expected to be avirulent; however, a successful conjugate preparation using multivalent protein will enable the polysaccharide to increase its antigenicity.

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ANNEXURE - I

LIST OF EQUIPMENTS USED

Instruments	Make
Analytical Balance	Afcoset ER-200A
pH meter	Systronics
Ultra Low Temperature freezer	Remi-RQFP 265
Ultra pure water system	Millipore
Spectrophotometer(UV/Vis)	Systronics 2203 Double beam
Refrigerator	Whirlpool
Ultra Centrifuge	Remi-C24BL
Water bath	LAUDA Ecoline-staredition RE-104
Vortex Mixer	Genie
Magnetic stirrer	Spint