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DEVELOPMENT OF MURINE MONOCLONAL ANTIBODIES TO PNEUMOCOCCAL POLYSACCHARIDE ANTIGEN

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Abstract: Streptococcus pneumoniae is a major cause of mortality in infants, children and the elderly. Despite the availability of excellent antimicrobial therapy and adequate care systems, respiratory diseases and invasive infections caused by pneumococci still comprise a major health problem. Thus to overcome this, murine monoclonal antibodies were developed using pneumococcal polysaccharide as the antigen. Polyclonal MAb was developed against different serotypes namely 4, 6B, 9V, 14, 18C, 19F, 23F. Hybridoma cell line developed against Serotype 4 showed maximum fluorescence absorbance units when detected, using Rapid multiplex assay. Single cell cloning against Serotype 4 hybridoma cell line was done by Limiting dilution. A stable single hybridoma cell line was developed producing Monoclonal antibody against pneumococcal polysaccharide Type 4 serotype. The expression of the murine monoclonal antibody was assayed using SDS- PAGE (Reducing). Presence of heavy chain at 50kDa and light chain at 25kDa in reducing condition confirmed presence of MAb.
Keywords: murine monoclonal antibody, rapid multiplex assay, pneumococcal polysaccharide antigen

1. INTRODUCTION

Streptococcus pneumoniae, a gram- positive bacteriumis well known human pathogen and a major etiologic agent associated with various human infections. It is an important zoonotic agent causing severe invasive infections such as pneumonia, meningitis, sepsis and also mucosal infections (e.g. sinusitis and otitis media), arthritis, pericarditis, peritonitis primarily among young children and older adults. In developing countries, the prevalence of carriage approaches 95% in healthy children under the age of 3 years and 40% in adults[(1) (2)]. The process involved in translocation of the pneumococcus from the nasopharynx to other sites, including lungs are probably multifunctional and are poorly understood [(19)].It has been generally accepted from several decades that pneumonia results from the aspiration of the pneumococci from the upper respiratory track, although a blood borne route of dissemination from the upper respiratory tract is also possible [(3)]. The surface of S. Pneumoniae bacterial species is covered by Polysaccharide. These can be in the form ofcapsules, glycoprotein or glycolipids. The capsular polysaccharides (CPS) are made up of either monosaccharidemaking a homopolymer like α-(2-8) - linkedsialic acid or from repeating units normally consisting of two to six sugar residues. It is well established that an immune response against the surface polysaccharides confers protection against the disease [(4)]. The pneumococcal outer surface consists of cell wall covered by a polysaccharide capsule. Capsule polysaccharides are highly heterogeneous and, thus far almost divided into 90 serotypes, based on its expression of serologically distinct carbohydrate capsules. The polysaccharide capsule is the most important virulence factor of the pneumococcus as it protects the bacteria from phagocytosis. Capsular polysaccharides are highly immunogenic and antibodies to these polysaccharides protect against infection with the homologous serotype. The antigenicity of the capsule is type specific, but cross reactions occur because of shared polysaccharides [(5)]. An antigen can be classified as T lymphocyte dependent (TD) or T lymphocyte independent (TI). Polysaccharides are TI antigens whereas proteins and peptides are usually TD antigens. The TI antigens stimulate mainly M (IgM) antibody with weak immunological memory and readily induce tolerance.

Whereas TD immune responses are characterized by high titer IgG antibody synthesis and memory. The antibodies against TD antigens are of high affinity and of multiple isotypes (IgA, IgM, IgG1, IgG2a, IgG2b and IgG3). Thus, such responses to TI antigen can be produced by conjugating the antigen to protein carriers.

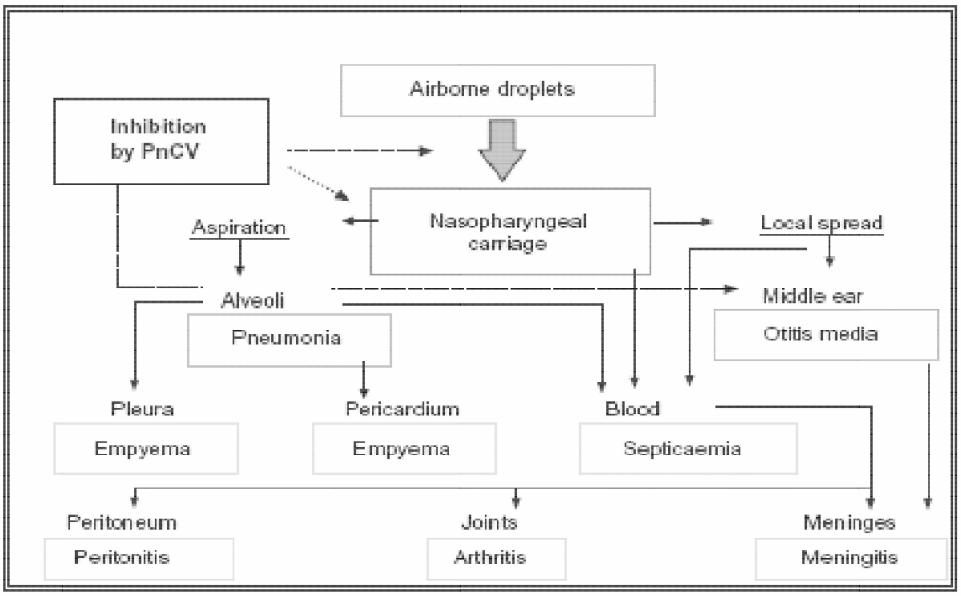


Figure 1: Pathogenesis of invasive pneumococcal disease.

The immunogenicity of the pneumococcal immunogen made by conjugation of whole polysaccharides or oligosaccharides to protein carriers have been extensively studied and implemented [(4) (6)]. The recently developed type of polysaccharide protein conjugate contains capsular PS from 7 to 11 serotypes that are conjugated to a protein molecule. The polysaccharide protein conjugate contains seven serotypes (4, 6B, 9V, 14, 18C, 19F, 23F) [(2)]. The use of polysaccharide protein conjugate has been potentially important because the isotype of IgG antibody differ in protective efficacy for many infections. The IgG2a and IgG3 isotype have been reported to be particularly effective in conferring protection against *S. Pneumoniae* [(17)]. The invasion by a foreign substance into humans and many other animals often leads in a response to eliminate and clear the invasive substance by the host. One such response is production of proteins known as antibodies (Ab) [(7)].Ab are protein molecules which bind non–covalently and specifically to the substance that caused their production called the antigen (Ag), usually another protein or polysaccharide of viral or bacterial origin. The most prevalent type of Ab is the immunoglobulin G molecule (IgG).

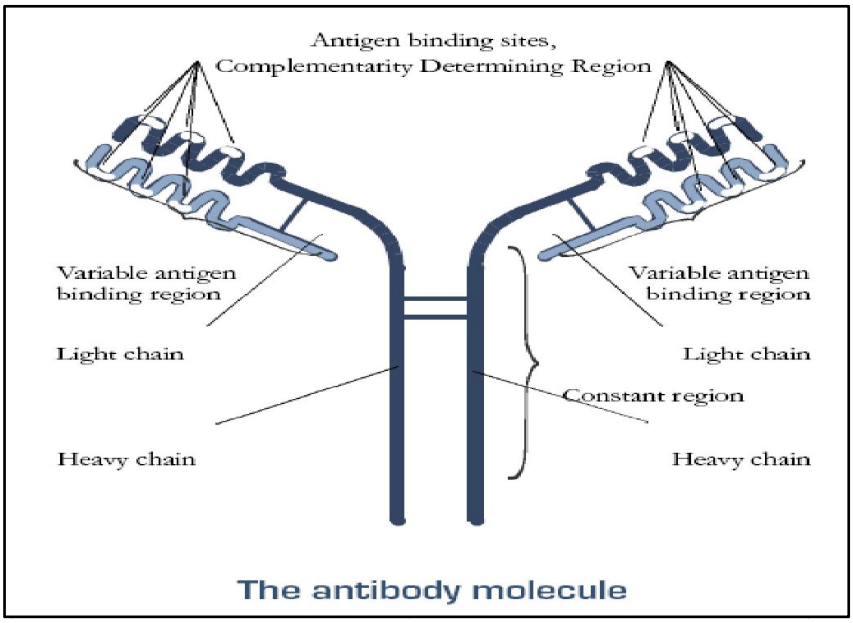


Figure 3: Structure of Antibody

A single IgG molecule is composed of four polypeptide chains: two identical heavy chains (M.W.-50kDa each) and two identical light chains (M.W.-25kDa each) which are linked through disulfide bridges to form the Y- shaped structure [(8)].

The two arms of the IgG are referred to as the Fab regions and contain the amino acid residues responsible for Ag recognition and binding. Thus each IgG molecule contains two identical sites which can form an associationwith Ag. The stem region of the IgG is known as the Fc region and is directly involved in the number of effectorfunctions common to many immunoglobulins, including Fc-mediated phagocytosis, antibody-dependent cell-mediated cytotoxicity and complement activation [9]. There are five classes of antibodies: IgM, IgD, IgG, IgE, and IgA. The class to which an antibody belongs, and thus the function that it carries out, is determined by the structure of its heavy chain constant region (i.e., the structure of its effector function domain). For example, IgD antibodies usually remain bound to the surface of the cells in which they are synthesized, whereas IgG antibodies are usually secreted and circulated through the body in the bloodstream. The light chains of antibodies are of two types, *kappa* and *lambda*, with type being determined by the structure of the light chain constant region [8]. While an Ab may have binding specificity for a particular Ag (a protein, for example), the physical association occurs only within a specific region on the Ag molecule, this physical association is known as an epitope of the Ag. Because an Ag in principle has many epitopes within its molecular structure, a single Ag can elicit the production of many different Ab. Each one having specificity for a different epitope on the same Ag. The set of all Ab produced in response to a particular Ag is known as polyclonal Ab against the Ag.

Monoclonal antibody

The normal mammalian response to presentation of a single Ag is the production of polyclonal Ab, containing Ab which differs both with respect to epitope specificity and the strength of Ab-Ag association. This is due to the fact that each individual contains an enormous number (possibly > 10^9 in humans) of different clones of Ab producing lymphocytes, each of which produces an Ab unique in its precise amino acid sequence and therefore in its Ag specificity and affinity. A monoclonal Ab (mAb) is the product of a unique cell line known as a Hybridoma cell, the development of which was first reported by Köhler and Milstein in 1976 [10]. A Hybridoma cell line is produced by the fusion of a mouse spleen cell (responsible for the particular Ab which is produced) to a mouse myeloma cell (allowing for unlimited cell growth and enhanced Ab secretion). Because each fused cell line obtained is derived from a single precursor B lymphocyte, the Ab produced by a particular cell line will be molecularly homogeneous. Therefore a mAb, unlike polyclonal Ab, can be characterized by a unique amino acid sequence, and can generally be characterized interms of a specific epitope for Ag binding and a single association constant. Monoclonal antibodies offer reproducible and potentially inexhaustible supply of antibody with exquisite specificity. Monoclonal antibodies help the development of standardized and secure immunoassay systems. They serve a powerful tool for investigation of macromolecules and cells and have proved effective reagents in terms of specificity for clinical diagnostic tests. The development of monoclonal antibodies has led to a large number of applications involving their use. Monoclonal can be developed to a variety of protein Ag and can be used in novel ways to probe the protein structure and function. When a humoral immune response is provoked by an immunogen, a plethora of antibodies are produced in an individual against different parts or regions of this foreign substance. These are termed antigenic determinants or epitopes, which usually comprise six to eight amino acids [12]. It should be appreciated that most antibodies recognize and interact with a three dimensional shape composed of “discontinuous” residues brought into juxtaposition by the folding of a molecule. Alternatively, antibodies can also recognize linear stretches of amino acids or “continuous” epitopes [11]. Of course, an important concept to bear in mind is that each antibody molecule is specific for a single epitope, and that each antibody is the product of a single B cell clone. Thus, an antibody of unique specificity, derived from a single B cell clone, is termed a monoclonal antibody. The propagation of an isolated B cell clone would produce antibody of single specificity. However, a problem arises is that in tissue culture medium, B cells die within a few days of their isolation (for example, from a mouse spleen). Consequently, methods of conferring immortality on to B cells have been investigated. Indeed, immortality has been accomplished by means of viral transformation (for example, using Epstein-Barr virus) and/or fusion to cancerous cells to generate hybrids or “hybridomas” [20]. In general, the former technique is used for the immortalization of peripheral blood B cells (and production of human monoclonal antibodies), whereas myeloma cells have mainly been used in the production of murine monoclonal antibodies. Let us start with a working definition: A monoclonal antibody is regarded as an antibody of single specificity, generated from the immortalization of a plasma B cell in vitro. The process of demystifying monoclonal antibodies is best illustrated by the generation of murine monoclonal reagents.

In essence, four main stages are highlighted:

(1) Immunization, (2) Fusion and selection, (3) Screening, (4) Characterization

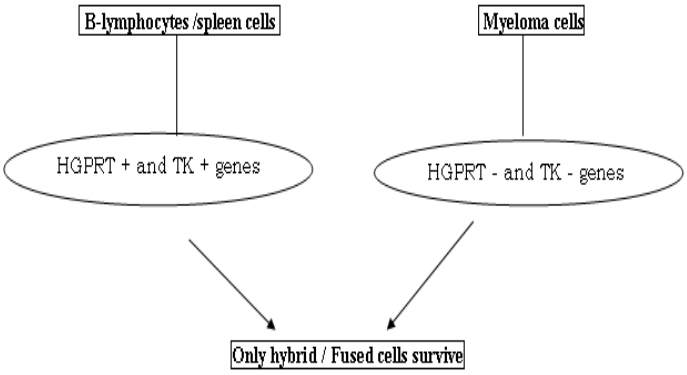
Stage 1: Immunization

Substances that induce an immune response are usually foreign to the individual and are termed immunogen. In general, protein (50– 100 μg), cells ($1*10^7$), multiple antigenic synthetic peptides or a short peptide (6–18 amino acids) linked to a carrier protein can be used for the primary immunization of BALB/c mice [13]. An immunogen will be delivered in conjunction with an adjuvant, which is regarded as a non-specific immune enhancer. Regular boosting is needed to augment a polyclonal response, which can be monitored indirectly using tail or eye bleeds. These offer sufficient serum to ascertain the antibody titer to a desired antigen usually in an assay system—for example, Rapid multiplex assay—that is ultimately required for the monoclonal reagent.

Stage 2: Fusion and Selection

The hybridization process centers on the fusion of murine splenic B cells with Histocompatibility myeloma cells, such as SP2/0 in presence of fusogenic agent, PEG (polyethylene glycol) [18]. The fused cells are generally grown onto feeder cells. Peritoneal cells contain lot of macrophage so also called as macrophage feeder layer. Macrophage, which secretes cytokines, supports cell signaling and helps in proliferation of cells. Since they are easily accessible and they mimic the in vivo environment, feeder cells promote the growth of hybridomas. SP2/0 cell line is preselected for a deficiency in the enzyme hypoxanthine guanine phosphoribosyltransferase (HGPRT)—for example, by culturing in medium containing 8-azaguanine. In essence, this enzyme is fundamental to the post-fusion hybridoma selection process. To understand this process it should be noted that cells possess two pathways of nucleotide biosynthesis: the de novo pathway and the salvage pathway, which uses HGPRT. Consequently, myeloma cells that are HGPRT negative are unable to use the salvage or "alternative" pathway for purine biosynthesis and are thus entirely reliant on the de novo pathway for survival. In the fusion process, splenic B cells are mixed with HGPRT negative myeloma cells and a fusing agent, such as Polyethylene Glycol. Hopefully, the mixing and centrifugation steps generate myeloma–splenic B cell hybridomas. Once these hybrid cells are formed and plated into tissue culture wells, the priority shifts towards removing unfused myeloma cells. This is necessary because the latter have the potential to outgrow other cells, particularly weakly established hybridomas. This situation is resolved by using a selective medium containing Hypoxanthine, Aminopterin and Thymidine, otherwise known as "HAT". Of importance, is the fact that Aminopterin blocks the de novo pathway—the only one available to HGPRT negative cells, and as a consequence all unfused myeloma cells will die. Of course, newly formed hybridomas survive this selection process because the salvage pathway enzyme is provided by its splenic B cell counterpart. Unfortunately, some hybridomas are unstable and regress. Hence, meticulous attention should be given to the visual examination of hybridomas using an inverted microscope. A record of poorly growing, newly emerging or established hybridomas provides credibility to immunoassay screening data. Once established, a given hybridoma colony will continually grow in culture medium (such as DMEM-F12 with antibiotics and fetal bovine serum) and produce antibody. Twenty to 30 days post fusion, Hybridomas can be propagated in "HT" medium (Hypoxanthine and Thymidine only) because Aminopterin is no longer required.

2. Principle of selection:



Stage 3: Screening

This stage focuses on identifying and selecting those hybridomas that produce antibody of appropriate specificity. Here clonality testing is performed i.e. to determine the percentage of cells in the culture producing Ab of similar antibody titer. Antibody titer (It is a test that measures the presence and amount of antibodies in blood, serum, or in culture supernatant). These Ab may be against a particular type of tissue, cell, bacteria, virus or external substances at a specified wavelength and dilution. The selection process must be ruthless otherwise numerous unwanted hybridomas will compete for your time and incur unnecessary expense in terms of culture plates and medium.
Obviously, it is important to screen supernatants with some degree of equity and, therefore, it might be wise to test hybridomas when at least three quarters confluent. Hybridoma can initially be grown in multiwell plates and then, once selected, expanded to larger tissue culture flasks.

Stage 4: Characterization

Further analysis of a potential monoclonal antibody producing hybridoma in terms of reactivity, specificity and cross-reactivity can be achieved using culture supernatant. However, before any further work it is often necessary to reclone hybridoma(for example, by limiting dilution) because when we isolate the spleen cells, it is a pool of all the B-lymphocytes secreting Ab against the different epitopes of Ag. The Ab secreted by them may be polyclonal or monoclonal in nature so we go for cloning by limiting dilution method [14]. It is an assumption that if a suspension of cells is diluted with enough culture medium, a concentration of cells will be produced such that an accurately measured volume of the diluted suspension will contain 1 cell i.e. each well should receive 1 cell.

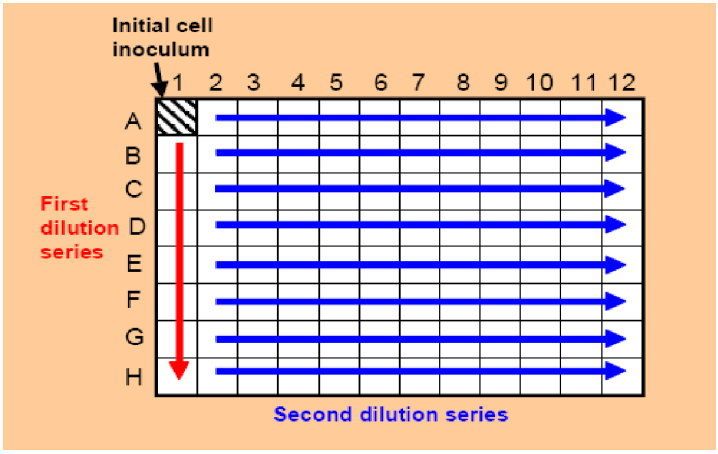


Figure 4: Single cell cloning by Serial Dilution.

As the selected cell will further grow to form clone, the antibodies secreted would be against one particular serotype i.e. monoclonal antibody will be produced.

TECHNIQUES USED:

Rapid Multiplex Assay:

To facilitate the development of new pneumococcal vaccines, an accurate assessment of vaccine induced immune response is important. However the assessment of pneumococcal antibody response is not simple. Antibodies to PS antigens are often of low avidity and may not be so functional, yet assays for pneumococcal antibodies vary in their capacity to detect antibodies with very low avidity. Although there are numerous serotyping methods, the ones that are currently used are inadequate. The assays use polyclonal antibodies, which may not be able to distinguish closely related serotype easily. To overcome these problems, a Rapid Multiplex Assay based method has been described [2]. Rapid multiplex assay is widely ascribed to be a recent innovation, but description of this assay can be found in the literature as far back as 1977. This method provides quantitative measurement of large number of analytes using an automated 96- well plate format. Even though ELISA has long been the standard for quantitative analysis, they are not well suited for high throughput multiplex analysis [15].

The potential advantages of the assay are:

- It has the ability to independently and quantitatively assay multiple analytes simultaneously in small volumes of material and the collection of data from numerous beads for each ligand to provide statistical rigor.
- The potential cost and time saving can be accured by use of this assay in comparison to other methods, as it provides a strong impetus for the routine use in, research and clinical laboratories.
- The assay is most often compared to ELISA (where an immobilized antibody is used to capture a soluble ligand with subsequent detection of the captured ligand by second 'reporter' antibody). Whereas Rapid multiples assay, uses fluorescence as reporter system. But in case of ELISA, it uses enzyme amplification of a colorimetric substrate.
- Rapid multiplex assay captures ligands onto spherical latex beads.
- Most importantly, Rapid multiplex assay by their very nature, are multiplexed and therefore may be subject to any perturbations that arise from analyzing multiple analytes simultaneously, such as cross- reactivities [16].

Analysis of MAb Expression by SDS- PAGE:

SDS- PAGE, sodium dodecyl sulphate polyacryalamide gel electrophoresis, is a technique used to separate proteins according to their electrophoretic mobility (a function of length of polypeptide chain or molecular weight as well as higher order protein folding post- translational modification and other factors). The solution of proteins to be analyzed is first mixed with SDS, an anionic detergent which denatures secondary and non- disulphide linked tertiary structure, and applies a negative charge to each protein in the proportion to its mass. Without SDS, different proteins with same molecular weights would migrate differently due to differences in mass charge ratio, as each protein has an isoelectric point and molecular weight to its primary structure. This is known as Native PAGE. Adding, SDS solves this problem, as it binds to and unfolds the protein, giving a near negative charge along the length of the polypeptide. SDS binds in a ratio of approximately 1.4 gm / 1 gm protein (although binding ratios vary from 1.1- 2.2 per gram protein), giving an approximately uniform mass: charge ratio for most proteins, so that the distance of migration through the gel can be assumed to be directly related to only the size of the protein. A tracking dye may be added to the protein solution to allow the experimenter to track the progress of the protein solution through the gel during the electrophoretic run. As molecular weight of Antibody is 150kDa (50kDa of heavy chain and 25kDa of light chain), the expression of the MAb can be correlated accordingly.

3. REVIEW OF LITERATURE

Sternberg and Pasteur were the first to identify *S.pneumoniae*, initially described as the pneumococcus [21].

Effective therapy against pneumococcal disease was reported only a few years later by Klemperer and Klemperer [22], who discovered the protective potential of patient serum against homologous organisms. The first preventive strategy was introduced by Sir Almroth E. Wright in 1911, who suggested that inoculation of killed, whole pneumococci might induce a protective effect against pneumococcal infections [23]. Unfortunately, this pioneering vaccine failed because only two serotypes discovered that time were included, and the maximum applicable vaccine dosage was insufficient because of the relatively large inocula required. In 1926, Felton and Bailey [24] for the first time isolated pneumococcal capsular polysaccharide. This directly led to the first capsular polysaccharide vaccine, which proved its effectiveness by successfully aborting an outbreak of pneumonia at a state hospital in Worchester, MA in 1931[25]. Because of subsequent development of successful antibiotic therapy that could more effectively deal with pneumococcal disease, the vaccine's popularity decreased and finally the PS vaccine was withdrawn from the market [26] [27]. However, despite the development of new classes of antibiotics, morbidity and mortality stopped declining [28]. An additional problem was the rapidly emerging penicillin and multidrug resistance. The first report of resistance to penicillin was reported in the 1930s in Australia and New Guinea [29]. It took until 1977 before highly resistant pneumococci were reported in South Africa. In addition, these strains were also resistant to other penicillin's and cephalosporins. In the years following, penicillin resistant pneumococci rapidly spread throughout the world. The cause of this international spread was mostly due to a few multidrug resistant clones with serotypes 6B, 9V, 14, 19A, 19F and 23F [30]. These problems have lead to renewed interest in vaccine strategies, resulting in 1977 in the production of a 14- valent pneumococcal PS vaccine by Robert Austrian and coworkers [31]. This vaccine was expanded in 1983 to a 23- valent vaccine and a theoretical coverage of more than 80% of the pneumococci causing infection in adults. Unfortunately, the vaccine was less immunogenic in small children and immunocompromised patients [32]. To overcome this, the most recently developed type of vaccine (called conjugate vaccine) containing capsular PS from 7-11 serotypes that are conjugated to a protein molecule. A 7- valent conjugate vaccine was introduced in 2000 for routine immunization in the United States and has reduced the incidence of invasive pneumococcal diseases in children [2]. But researchers have known the potential of use of monoclonal antibody as a tool of research. Since antibody binds to specific probe to elicit an immune response as compared to vaccine, which takes time to elicit response. Two monoclonal antibodies against serotype 6B and 19F have already been developed, which is showing good response against children and adults. So the need to develop monoclonal antibodies against other serotypes is being considered [17].

4. RESEARCH MATERIALS AND METHODS

MATERAILS:

LIST OF CHEMICALS

S.NO.	CHEMICALS	MFG.	LOT NO.
1.	Dulbecco's modified eagles medium : Nutrient mixture f-12 (HAM)1:1	GIBCO	12500-096
2.	HAT media supplement (50X)Hybri-Max , γ irradiated	Sigma	H0262
3.	HT media supplement (50X)Hybri-Max , γ irradiated	Sigma	H0137
4.	HEPES >99.5 %	Sigma	H4034
5.	Fetal bovine serum ,sterile filtered	Moregate	17300102
6.	Dimethyl sulfoxide for mol. biology.	Sigma	D8418
7.	Trypan blue soln. (0.4 %)	Sigma	T8154
8.	Albumin from bovine serum min 98% electrophoresis	Sigma	A7906
9.	Sodium phosphate dibasic for mol. biology min 98.5%	Sigma	S3264
10.	Sodium phosphate monobasic >99 %	Sigma	S5011
11.	Casein digest	BD Difco	211610
12.	3,3'5,5'-tetramethyl-benzidine	Sigma	T2885
13.	Glycine for electrophoresis min. 99%	Sigma	G8898
14.	Acrylamide , for mol. biology min 99 %	Sigma	A9099
15.	N,N'-methylene-bis-Acrylamide	Sigma	M7279
16.	Ammonium per sulfate	Sigma	A3678
17.	N,N,N',N'-Tetramethyl ethylenediamine	Sigma	T7024
18.	2-Mercapto Ethanol	Sigma	M7154
19.	Tween- 20	Sigma	P1379
20.	Sodium Azide	Sigma	S2002- 100G
21.	Latex bead coated with serotype	-	-
22.	Goat anti- mouse IgG phycoerythrin conjugate	-	-
23.	Prestained protein Mol.wt marker	Fermentas	SM0441
24.	Unstained protein Mol.wt marker	Fermentas	SM0431
25.	Polysaccharide protein conjugate	-	-

LIST OF EQUIPMENTS & INSTRUMENTS

S. NO.	INSTRUMENT	MANUFACTURER
1.	Gel Doc	Alpha innotech corporation
2.	Inverted microscope	Zeiss
3.	Centrifuge	Eppendorf
4.	pH meter	Thermo Orion 550A
5.	Magnetic stirrer	Remi Equipments
6.	CO_2 jacketed incubator	Nuaire
7.	Analytical balance (0.01mg-210gm)	Sartorius
8.	Analytical balance (10 mg-210 gm)	Mettler Toledo
9.	Ultra centrifugal filter devices 10KDa (50 ml)	Millipore
10.	Sterilized Centrifugation tubes (15 & 50 ml)	Nunc
11.	Microfuge tubes (1.5 & 2 ml)	Eppendorf
12.	Ultra filtration disk membranes (0.22 & 0.45 µm)	Millipore
13.	Filter assembly with vacuum pump	Millipore
14.	Luminex System	Biorad
15.	96 well filter plate	Nunc
16.	SDS-PAGE apparatus	Biorad
17.	Shaker incubator	Barnstead
18.	Syringe driven filter unit (0.22 µm)	Millipore, Pall
19.	Sterile cell culture flasks	Nunc
20.	Sterile pipettes	Corning
21.	Water bath	Lauda ecoline 011
22.	Micropipettes (2-20, 10-100, 20-200, 100-1000, 500-5000 µl)	Eppendorf
23.	Pipetting aid	Steripette

PREPARATION OF REAGENTS:

- Incomplete cell culture medium: D-MEM/F12, (Dulbecco's modified eagle medium: Nutrient mixture F-12 (HAM) 1:1 powder with L-glutamine and pyridoxine hydrochloride) containing 20 mM of HEPES and $NaHCO_3$ was prepared by adding 12 grams/l in WFI. The pH of the medium was adjusted to 7.2.The medium was filtered aseptically using Stericup and Steritop filtration system with 0.2 µm filter.
- Complete cell culture medium: Incomplete medium + 10 % FBS +2 % HAT/HT was prepared.
- 5 X Electrode running buffer: 15 grams of Tris base + 72 grams of Glycine + 5 grams of SDS were dissolved in WFI to make 1000 ml. The stock was diluted 5 times with WFI before using.
- Sample buffer (SDS reducing buffer) : 3.8 ml of deionised water, 1 ml of 0.5 M Tris –HCl, pH 6.8, 0.85 ml Glycerol, 1.6 ml 10 % w/v SDS. 0.4 ml 2-mercaptoethanol, 0.4 ml 1 % w/v bromophenol blue. The sample was diluted at least 1:4 with sample buffer. SDS non reducing buffer contain same reagent without 2 mercaptoethanol.
- Resolving buffer (1.5 M Tris-HCl) : 18.15 g Tris base dissolved in 100 ml of WFI .The pH was adjusted to 8.8 with 6 N HCl.
- Stacking Buffer (0.5 M Tris HCl): 6 g Tris base dissolved in 100 ml of WFI. The pH was adjusted to 6.8 with 6 N HCl.
- 10% SDS: Dissolve 1 gm of SDS in 10 ml of WFI.
- 30 % Acrylamide Soln: 29.2 g of Acrylamide +0.8 g of N'N'-bis –methylene –Acrylamide, was dissolved in 100 ml of WFI.
- 10% Ammonium persulphate: Dissolve 100mg of ammonium persulphate in 1 ml of WFI.
- Destaining Solution: Glacial Acetic Acid, Methanol and water were added in a ratio of 1:4:5n respectively.
- Staining Solution: 0.25% brilliant blue R in the Destaining soln.
- 10 mM Phosphate buffer : 10 mM phosphate buffer containing 1.09 grams/l of Na_2HPO_4 and 0.32 gram/l of NaH_2PO_4 was prepared. The pH of the buffer was adjusted to 7.4.
- Luminex buffer: 10 mM phosphate buffer with 100 mM NaCl and 0.05 % Tween-20 (PBST) +1 % BSA + 0.05% sodium azide.

ANIMALS: Six-to-eight weeks old female, Balb/c mice were used in this study. The care and use of the animals were according to the Institutional Animal Ethics committee guidelines. A total of 6 mice were used.

CELL LINE: The myeloma cell line SP2/0-AG14 was grown in Dulbecco's modified minimal essential medium supplemented with 10 % fetal calf serum, 2 mM L-glutamine and 10 mM sodium pyruvate. Hybridomas were grown in same medium with 0.1 mM Hypoxanthine, 0.4 M Aminopterin and 16 mM Thymidine.

5. METHODS

5.1) Immunization of mice:

- Four mice were immunized subcutaneously with 0.5 ml of Pneumococcal conjugate vaccine using 22-G needle.
- 21days later, booster dose was give subcutaneously.
- 7 days after second immunization blood samples were collected using retro- orbital bleeding.100 to 200µl of blood was collected.
- The sera samples were analyzed for presence or absence of antibodies against pneumococcal polysaccharide using Rapid multiplex assay.
- Booster dose was again given twice.
- When the antibody titer was found to be satisfactory, final booster dose was given intraperitoneally.
- Cell fusion was performed 3 days after the final immunization.

5.2) Myeloma cell preparation:

- Frozen SP2/ 0, Ag 14 cells were recovered from liquid nitrogen storage.
- SP2/ 0 cells were grown in DMEM/ F12 medium in tissue culture flasks incubated at 37^{0}C with 5 % CO_2 incubator and 95 % humidity.
- The cells were examined properly under inverted microscope and the culture flasks were again incubated at 37^{0}C with 5 % CO_2 incubator and 95 % humidity for continuation of cell growth.
- SP2 /0 cells were treated with 8- Azaguanine to ensure that they remain Aminopterin sensitive for the selection process after fusion. The complete culture medium was supplemented with 8-Azaguanine one week prior to fusion.
- Cell count was taken using Trypan blue exclusion method, as prior to cell fusion the flask should be 95% confluent.

5.3) Spleenectomy and Preparation of Single Cell Suspension:

- The mouse was killed by dislocating the neck and the body was immersed in 70 % ethanol. The skin of the abdomen was cut open. Exposed tissues were cleaned with 70 % ethanol.
- The peritoneal cavity was cut open and the spleen was removed. It was transferred into a Petri dish containing 10 ml of DMEM/F12 incomplete medium.
- Spleen was washed thrice with DMEM/F12 incomplete medium. DMEM/F12 incomplete filled syringe was used to poke 30-50 holes in spleen. Spleen was then perfused with DMEM/F12 incomplete medium. Single cell suspension was transferred into a 15 ml centrifuge tube.
- The spleen cells were centrifuged at 1200rpm for 10 minutes.
- The red blood cells were removed by resuspending the pellet in 10 ml of DMEM/F12 incomplete medium.
- The number of spleen cells were counted under inverted microscope using Neubaur's counting chamber using 0.4 % Trypan blue dye.
- The myeloma cells grown in DMEM-F12 +10 % FBS medium were centrifuged at 1200rpm for 10 minutes at room temperature. The cells were washed twice with DMEM/F12 incomplete medium. Cells were resuspended in 10 ml of DMEM/F12 incomplete medium and cell count was taken.

5.4) Fusion of Myeloma cells and Immune spleen cells:

- Myeloma cells and the spleen cells were mixed in a ratio of 1:5. The myeloma cells and spleen cells were centrifuged together at 1200rpm for 10 minutes in a 50 ml centrifuge tubes. The supernatant was discarded and the tube was gently tapped to spread the pellet.
- 1 ml of PEG was added over a period of 1 minute and mixed gently. The tube was incubated in water bath maintained at 37^{0}c for 1 minute. Fusion was stopped by adding DMEM/F12 complete medium with constant stirring.
- 2 ml of medium was added over 1 minute.
- 4 ml of medium was added over 1 minute.
- 8 ml of medium was added over 1 minute.
- The suspension was centrifuged at 1200 rpm for 5 minutes. The supernatant was discarded and the cells were resuspended in 40 ml of DMEM+15 % FBS.
- 100µl of the cell suspension was distributed into each well of 96- well plates. The plates were incubated at 37^{0}C for 24 hours with 5 % CO_2 incubator and 95 % humidity.

5.5) Post fusion cells cultured in Hybridoma selection medium (HAT):

- 100µl of DMEM +15% FBS+ HAT, one fold concentrate was added to all the 96- well plates which contained fused cell suspension, after 24 hours of incubation at 37^{0}C with 5 % CO_2 incubator and 95% humidity.
- 100 µl of supernatant was replaced with 100 µl of fresh DMEM +15 % FBS + HAT after every alternate day.
- This step was repeated till the time the hybrid cell growth covered 50% to 60% of the well.
- Once the cell growth was satisfactory, culture supernatant was screened for presence of mAb's with desired specificity using Rapid multiplex assay.

- The hybrids were grown in HAT medium for nearly 2 weeks after fusion.
- After that HAT medium was replaced with HT medium, one fold concentrate.

5.6) Single Cell Dilution / Cloning / Limiting dilution:

- The Hybridoma cells which showed highest antibody titer were chosen for Limiting dilution.
- Cell viability count was performed using Trypan blue exclusion method.
- Limiting dilution was performed using 2 methods:
  - 1 cell/ well.
  - Serial dilution using Checker board method.
- According to the cell count taken, dilutions were made so, that only 1 cell is present per well.
- In case of serial dilution, 200µl of cell suspension was added in well A1. Then using single channel pipettor 100µl was transferred from A1 to B1. Gentle mixing was done using pipette.
- Using same tip, the 1:2 dilutions were repeated down the entire column, discarding 100µl of from well H1, so that all the wells ended up with the same volume.
- With the multichannel pipettor additional 100µl was added to all the wells of column 1 (giving a final volume of cells and medium of 200µl/ well).
- Then a multichannel pipettor was used to transfer 100µl of medium from the wells in the first column (A1 through H1) to those in the second column (A2 through H2). Gentle mixing was done.
- Using the same tips, the 1:2 dilutions were repeated across the entire plate, discarding 100µl from each of the wells in the last column (A12 through H12) so that all the wells end up with 100µl of cell suspension.
- The final volume was brought to 200µl by adding 100µl medium to each well.
- Plates were incubated at 37^0C with 5 % CO_2 incubator and 95% humidity.

5.7) Cryopreservation of cells:

- Freezing mixture used for cryopreserving cells contained 90% FBS + 10% Dimethyl- sulfoxide.
- The culture supernatant of the 12- well and 6- well plate was collected. 5 ml of fresh DMEM/ F12 complete medium was added.
- The cells were centrifuged at 1200 rpm for 10 minutes.
- The supernatant was discarded and pellet was collected and gradually diluted using Freezing mixture.
- Each clone was aliquoted in 2 ml cryovial.
- The cryovial was labeled properly with the name of the sample on the top, name of clone and date of cryopreservation.
- The cryovials were placed immediately at -20^0 C for 24 hours and then transferred to liquid canister maintained at -196^0 C till further use.

5.8) Rapid Multiplex Assay:

- Multiplex assay was performed using Luminex Bioplex 200 System.
- The 96- well filter plate was prewetted with 50µl of Luminex buffer. The plate was incubated at room temperature for 2 minutes.
- 96- well filter plate was coated with 50µl aliquot/ well (aliquot: luminex buffer containing beads coated with serotype). The aliquot was vortexed properly. Plate was incubated at room temperature for 2 minutes.
- 50µl of sample (to be assayed) was added to wells. Plate was incubated at 37^0C for 1 hour with continuous shaking at 100 rpm.
- Plate was washed 3 times with 100µl luminex buffer. Aspiration was done using vacuum pump.
- 50µl of Goat anti mouse IgG conjugated with phycoerythrin was added to all the wells. Plate was incubated at 37^0 C for 30 minutes with continuous shaking at 100 rpm.
- Plate was washed 3 times with 100µl luminex buffer. Aspiration was done using vacuum pump.
- 130µl of luminex buffer was added and the plate was incubated at 37^0C for 2 minutes with continuous shaking at 100 rpm.
- The plate was vortexed slowly.
- Fluorescence absorbance units were measured using Luminex Bioplex 200 System (Biorad).

5.9) SDS-Polyacrylamide Gel Electrophoresis:

- Gel electrophoresis of mAb was performed using Biorad electrophoresis unit.
- Proteins were separated under Reducing condition by means of gradient gels.
- In gradient gels, the nominal pore size within the gel decreases continuously throughout. Thus under the same electric field, proteins can be separated on the basis of hydrodynamic size due to hindrance effects.
- The discontinuous SDS Polyacrylamide gel contains Resolving and Stacking gel.
- Resolving gel was prepared of 12 % conc. by adding 2.4 ml WFI, 4 ml Resolving buffer, 3.3 ml 30% acryl amide, 100 µl 10 % SDS, 100 µl 10 % APS and 5 µl TEMED.
- Stacking gel was prepared of 5 % conc. by adding 2.5 ml WFI, 640 µl Stacking buffer, 840 µl 30% acryl amide, 50 µl 10 % SDS, 50 µl 10 % APS and 4 µl TEMED.

- Assay samples were dissolved in sample lysis buffer (125 mM Tris –HCL, 4.6 % SDS, 10% 2-mercaptoethanol, 0.005% bromophenol blue and 20 % glycerol.
- In Reducing PAGE proteins are boiled for 3 minutes in 1 X sample lysis buffer with 2-β-mercapto ethanol.
- Samples applied to discontinuous gel system were separated according to standard program constant voltage 200 for 50 min.
- One lane of Fermentas molecular weight standards (containing each of the following: Lysozyme, M.W. 14.4 kDa; β-lacto globulin, M.W. 18.4 kDa; REaseBSP981, M.W. 25 kDa; Lactate dehydrogenase, M.W. 35 kDa; Ovalbumin , M.W. 45 kDa; BSA, M.W. 66.2 kDa; β-galactosidase, M.W. 116 kDa) were included in each gel to determine relative mobility of sample protein. Protein bands were visualized by staining with 0.25 % Coomassie brilliant blue R (sigma).

5.10) Expansion of clones:

- The clones which showed maximum fluorescence absorbance units were further transferred from 96- well plate to 12- well plate.
- Plate was incubated at 37^{0}C with 5 % CO_2 incubator and 95% humidity.
- After the well were 60-70% confluent, clones were then transferred to 6- well plate.
- The clones were observed under inverted microscope.
- After the clones had occupied 75% of the well i.e. the well was confluent, the clone was transferred into T-25 cm^2 flask.
- Flask was incubated at 37^{0}C with 5 % CO_2 incubator and 95% humidity.

6. RESULTS

6.1) SCHEDULE OF IMMUNIZATION:

Table 1: Result of Immunization

Mice used	Conc/ Mice in µg	Antigen	dose/ mice in µl	Adjuvant	Route	Date	No. of mice died
4	160	Pneumococcal conjugate.	500	Aluminium phosphate.	SC	31.01.08	0
4	160	Pneumococcal conjugate.	500	Aluminium phosphate.	SC	21.02.08	0
4	160	Pneumococcal conjugate.	500	Aluminium phosphate.	IP	10.03.08	0
4	160	Pneumococcal conjugate.	500	Aluminium phosphate.	IP	26.03.06	1

Above table shows immunization schedule. Animals were observed for eight weeks and deaths were reported daily.

6.2) RESULT AFTER 1ST IMMUNIZATION

Table 2: Result of Day 6.

Type	Description	Type 4 (31)	Type 6B (4)	Type 9V (26)	Type 14 (62)	Type 18C (92)	Type 19F (5)	Type 23F (55)
X1	M1 (1:100)	0	0.5	0	0	0	-0.5	0
X2	M1 (1:200)	0	0.5	0	0	0	-0.3	0
X3	M1 (1:400)	0	0	0	0	0	-0.5	0
X4	M2 (1:100)	0	-0.5	0	0	0	0	0
X5	M2 (1:200)	0	-0.5	0	0	0	0	0
X6	M2 (1:400)	0	-0.5	0	0	0	-0.5	0

Above table shows result after 1st immunization i.e. Day 6. The sera samples were assayed for the presence or absence of antibodies to Pneumococcal polysaccharide using Rapid Multiplex Assay. It showed absence of cross reactive antibodies.

6.3) RESULT AFTER 2ND IMMUNIZATION

Table 3: Result of Day 28.

Type	Type 4 (31)	Type 6B (4)	Type 9V (26)	Type 14 (62)	Type 18C (92)	Type 19F (5)	Type 23F (55)
Control	1	1	-1	-3	0	1	-1
Control	1	1	-1	-3	0	3	-1
RH	1668.3	201	5.5	9835.8	1.8	5039.3	46.5
RT	11312	14460.5	6202.5	10510.5	8963.3	13886.3	1
GH	18116	6	2589.8	8565.5	27.5	11060.5	0
GT	1149.3	2	1348.8	1669.5	223.5	1451.8	-1.5

Above table shows the result after 2nd immunization i.e. Day 28. The sera samples were analyzed for the presence or absence of antibodies to the serotypes (4, 6B, 9V, 14, 18C, 19F, 23F, CRM) of Pneumococcal polysaccharide using Rapid Multiplex Assay. Here the sera samples were diluted 1:50 times to check the antibody titer.
Animal labeled RT (Red Tail) showed satisfactory antibody titer.

6.4) RESULT AFTER 3RD IMMUNIZATION

Table 4: Result of Day 56.

Type	Dilution	Type 4 (31)	Type 6B (4)	Type 9V(26))	Type 14 (62)	Type 18C(92)	Type 19F(5)	Type 23F(55)	CRM (25)
Blank		2	2	2	2	4	2	2	2
Control 1	1:100	1	1	0	2	1	3	0	13
Control 1	1:1k	1	0	0	0	1	0	0	1
Control 1	1:10k	2	1	1	2	1	2	2	2
Control 1	1:100k	1	0	-1	0	0	0	0	0
Control 2	1:100	1	1	0	1	1	1	0	15
Control 2	1:1k	1	0	0	0	0	0	0	1
Control 2	1:10k	1	0	1	1	1	0	0	1
Control 2	1:100k	1	-1	0	0	0	0	0	0
GT	1:100	22991	7	3193	6031	228	5605	2	9190
GH	1:1k	8730	0	601	842	21	681	0	11590
GH	1:10k	1315	0	70	121	2	72	0	5291
GH	1:100k	151	-1	2	11	1	3	0	860
GT	1:100	7137	1	6255	2039	980	2546	0	8420
GT	1:1k	861	1	1036	307	126	316	1	4446
GT	1:10k	94	0	125	43	14	31	0	776
GT	1:100k	7	0	5	4	1	1	0	80
RH	1:100	2526	20	3	3841	1	942	20	10712
RH	1:1k	287	0	0	550	0	74	0	7663
RH	1:10k	28	0	0	77	0	4	1	1782
RH	1:100k	2	0	-1	7	0	0	0	197
RT	1:100	15783	15970	1635	13670	6246	12060	2	7019
RT	1:1k	12401	7274	169	3133	1430	1972	1	7510
RT	1:10k	1709	1119	11	458	163	231	0	1812
RT	1:100k	206	148	0	61	14	18	0	235

Above table shows the result after 3rd Immunization i.e. Day 56. The sera sample were analyzed for the presence of antibodies to the serotypes (4, 6B, 9V, 14, 18C, 19F, 23F, CRM) of Pneumococcal polysaccharide using Rapid multiplex assay. Here the sera samples were diluted until 1:100k (starting from 1:1k, 1:10k, 1:100k). Animal labeled RT (Red Tail) showed satisfactory antibody titer. That is why, it was used for fusion.

6.5) RESULTS OF FUSION

Table 5: Results of fusion of spleen cells with SP2/0 myeloma cells

1	Number of spleen cells	$8.4*10^6$ cells/ml
2	Number of myeloma cells	$6.8*10^6$ cells/ml
3	Number of macrophage cells	$2.5*10^6$ cells/ml
4	Total no. of wells used for fusion	360
5	Number of hybrid cells producing specific activity for Pnemococcal polysaccharide	31
6	Number of hybrid cells propagated for large scale production	1

Above table shows spleen cells (obtained from animal labeled RT i.e. Red Tail) were fused with myeloma cells in a ratio of 5:1 in the presence of Polyethylene glycol (PEG). After fusion, cells were grown in the presence of selective medium i.e. HAT medium and also Macrophage feeder cells. 96- well plates were screened for the presence of hybrid cells. Screening was done on basis of that secreting maximum mouse antibody and that secreting specific pneumococcal polysaccharide antibody against serotypes (4, 6B, 9V, 14, 18C, 19F, 23F, CRM). Cross reactivity of clone secreting Ab with other proteins were also checked.

6.6) SCREENING AFTER FUSION

Table 6: Result of screening done on Day 13 after fusion.

Type	CRM (25)	Type 4 (31)	Type 6B (4)	Type 9V (26)	Type 14 (62)	Type 18C (92)	Type 19F (5)	Type 23F (55)
Blank	6.3	5.4	5	4.9	4.9	6.7	5	5.7
89SF	5004.7	12167.1	14508	17559.1	23419.6	9675.8	12188.5	15614.8
89SF	3442.7	7835.6	9317	11634.1	22627.1	6141.3	8122	10165.8

89SF	2426.7	4468.1	5522	7146.6	15221.1	3617.8	5075	6394.3
89SF	1400.7	1676.6	2424	2907.6	6576.1	1510.3	2203.5	2905.3
89SF	765.2	915.6	1341	1512.1	3619.6	792.8	1183	1601.3
89SF	469.2	770.6	1026.5	1280.6	2930.1	697.3	904	1382.3
89SF	234.2	279.1	490	476.6	1188.1	257.3	369	564.3
P1 B2	2.7	39.1	112	2.1	203.1	1.3	8	0.3
P1 G6	-2.3	19.6	69	-2.9	145.1	-1.7	-2	-3.7
P2 D5	12.5	55	548	2	29	2	9	2
P3 B3	44.5	55	30	32	40	29	33	34
P3 E9	7	8	5	1	135	4	2	1
P4 E2	464	6	6	4	10	6	6	5
P6 E9	1099	30	3	2	14	6	4	2
P1 D5	-3.3	9.6	10	-2.9	-0.9	36.3	-3	-3.7
P1 G10	-2.3	2005.6	0	-2.9	0.1	-2.7	-2	-3.7
P2 C8	16	100	284	54	9	109	176.5	29.5
P3 B10	5	18	3	1	4	50	3	2
P4 B5	54	71	27	25	25	24	27.5	26
P4 F3	421	3	2	2	2	4	2	2
P6 G5	3	19	3	1	1087	5	2	2
P1 D6	1.7	578.6	138.5	-2.9	-0.9	-1.7	-3	-3.7
P1 G11	-1.3	43.6	335	-2.9	88.6	12.3	0	-3.7
P2 F7	4	197	28	2	11	54	8	2
P3 C11	88	23167.5	11	7	10	10	8.5	7.5
P4 B11	27	33	19	22	106.5	22	23	22
P4 F11	3	707	14	2	4	5	6	2
P1 G3	8.7	2.6	-1	-2.9	0.1	2.3	77	1.3
P2 C6	3	44	408.5	2	9	7	3	2
P2 F8	3	90	28	2	168.5	6	2	2
P3 D10	6	23	16739	1	31	4	2	2
P4 D4	4	80	15	2	6	6	76	2
P4 G3	135	30	6	2	3	4	2	2

Above table shows result of screening done after fusion i.e. Day 13. The samples were analyzed for the presence of antibodies to the serotypes (4, 6B, 9V, 14, 18C, 19F, 23F, CRM) of Pneumococcal polysaccharide using Rapid multiplex assay. Among the 360 samples analyzed, 31 clones showed satisfactory fluorescent absorbance units. For each serotype four clones were chosen. The selected clones were picked and transferred to 12- well plate. After 3 days they were transferred to 6- well plate. Here the clones were grown in presence of HT medium.

6.7) SCREENING AFTER FUSION

Table 7: Result of screening done on Day 19 after fusion.

Type	CRM (25)	Type 4 (31)	Type 6B (4)	Type 9V (26)	Type 14 (62)	Type 18C (92)	Type 19F (5)	Type 23F (55)
Blank	2.2	1.6	1.6	1.7	2	4	1.8	2
89SF	3318.3	6758.9	832.4	9817.3	17544	5057	6934.2	8376
89SF	2939.3	5071.9	6930.4	8003.3	16601.5	4343	5613.2	7036
89SF	1984.8	3952.4	4649.4	6424.3	12595	3451	4136.2	5527
89SF	1153.3	1453.9	2021.9	2618.3	5217	1362.5	1845.7	2629.5
89SF	630.3	812.4	1085.4	1275.3	2875	723	948.2	1384
89SF	321.8	420.4	601.4	685.8	2875	388	506.2	756
89SF	168.3	245.4	352.4	406.3	1514	243	283.2	467
P1 B2	-0.2	0.4	-0.1	-0.7	0.5	0	-0.3	0
P1 G6	-0.2	0.4	0.9	0.3	2.5	0	-0.3	0
P2 D5	-0.2	0.9	16.9	-0.2	1	0	0.2	0
P3 B3	-0.2	-0.1	-0.6	-0.2	0	0	0.2	0
P3 E9	-0.2	0.4	-0.1	0	3	0	0.2	0

P4 E2	8.8	-0.1	-0.1	-0.2	0	0	0.2	0
P6 E9	229.1	0.4	-0.1	0.3	0.5	0	0.2	-0.3
P1 D5	-0.2	0.4	-0.6	0.3	0	1	0.2	0
P1 G10	-0.2	87.9	-0.6	-0.2	0	0	-0.3	-0.3
P2 C8	-0.2	1.9	9.9	0.8	0	2.8	6.7	1
P3 B10	-0.2	0.4	0.4	-0.2	0	1	0.2	-0.5
P4 B5	-0.2	0.4	-0.4	0.3	0	0	0.2	0
P4 F3	15.8	-0.1	-0.6	0.3	0	0	-0.3	-0.5
P6 G5	-0.2	0.4	-0.1	-0.7	27	0	0.2	-0.5
P1 D6	-0.2	24.6	2.4	0.3	0	0	0.2	0
P1 G11	-0.2	0.4	3.9	-0.2	0.5	0	0.2	0
P2 F7	-0.2	4.4	0.4	-0.2	0	1.5	0.2	0
P3 C11	90.8	23261.9	-0.6	-0.2	0	0	0.2	2
P4 B11	-0.2	-0.1	-0.6	-0.2	0.5	0	0.2	0
P4 F11	-0.7	20.4	0.4	-0.2	0	0	0.2	0
P1 G3	-0.2	-0.1	-0.1	-0.2	0	0	1.7	0
P2 C6	-0.7	1.4	13.9	-0.2	0	0	0.2	0
P2 F8	-0.2	1.9	0.4	-0.2	5	0	0.2	0
P3 D10	-0.2	0.9	811.4	-0.2	0	0	0.2	0
P4 D4	-0.2	1.4	0.4	-0.7	0	0	0.2	0
P4 G3	0.3	0.4	-0.6	0.3	0	0	0.2	0

Above table shows result of screening done after fusion i.e. Day 19. The samples were analyzed for the presence of antibodies to the serotypes (4, 6B, 9V, 14, 18C, 19F, 23F, CRM) of Pneumococcal polysaccharide using Rapid multiplex assay. Out of the 31 clones, only one clone i.e. P3C11 which was against serotype 4 showed maximum fluorescent absorbance units. So this clone was selected for Limiting dilution whereas other clones were cryo preserved.

Limiting dilution for clone P3C11 was done as follows:

- 1 cell present in 1 well i.e. 10 cells/ ml.
- Serial dilution using checker board method.

A clone which was had developed from single cell was observed. After the clone had occupied nearly 60% of the well, it was analyzed for the presence of antibodies to specific serotype.

6.7) SCREENING AFTER LIMITING DILUTION

Table 7: Result of screening done on Day 11 after Limiting dilution.

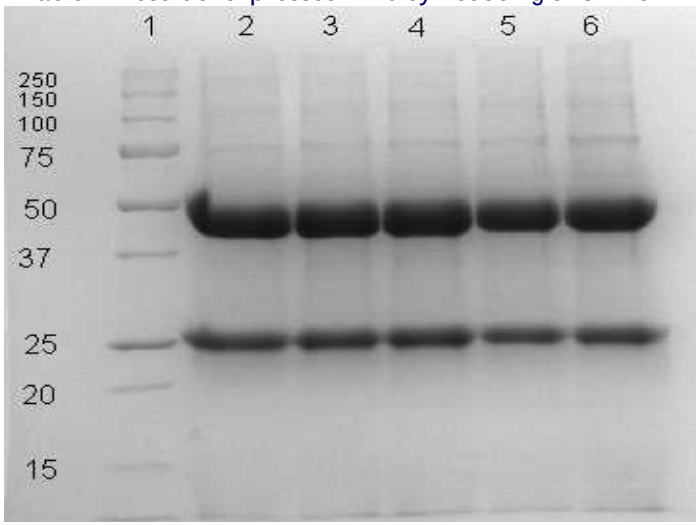
Type	CRM (25)	Type 4 (31)
Blank	2	19
P1 B6	69.5	23030.5
P1 C9	78	16993.5
P1 D4	82.5	23114
P3 C2	65	23702
P3 C3	79	22300
P3 D3	65	23829.5
P3 E2	70	21920
P3 E3	67	23758
P3 E4	73	18462
P3 E6	36	23506
P3 G4	82.5	16675
P3 C10	80	16100
P3 D9	79	16932
P3 F10	85	17659
P4 C4	84.5	22807
P4 E5	78.5	23395
P4 E7	31	20727
P4 F7	32	22860.5
P4 G4	67	17929

P4 D10	66	22475
P4 E9	80.5	15491.5
P4 G10	74.5	16877.5
P5 B9	76	18973
P5 G3	84.5	23152
P5 G4	62	23389.5
P6 B7	76	18570
P6 D7	77	16187.5
P6 D8	45	23073
P6 E5	43.5	20595.5
P6 E7	64.5	15829
P6 F6	74.5	20391.5
P6 G4	76	20665
P6 H3	79	22884
P6 A10	73.5	19042
P6 A9	50	16165

Above table shows result of screening done after limiting dilution i.e. Day 11. As per earlier result, it was seen that clone P3C11 showed cross reactivity only for Type 4 and CRM, so assay was done against only these two serotypes. The samples were analyzed for the presence of antibodies to Type 4 serotype and CRM of Pneumococcal polysaccharide using Rapid multiplex assay. The result shows that florescence absorbance units were obtained maximally against Type 4 serotype, as compared to CRM. This means that clone P3 C11 has developed monoclonal antibodies against Type 4 serotype. As per the result, six clones showed maximum fluorescence absorbance units against Serotype 4 and minimum against CRM. So these, six clones were chosen for expansion. These clones were first transferred to 12- well plate, then to 6- well plate and finally T-25 cm^2 flask.

6.7) EXPRESSION OF DEVELOPED MAb BY SDS- PAGE

Table 7: Result of expressed MAb by Reducing SDS-PAGE.



Lane 1: Protein marker.
Lane 2-6: Supernatant of Pneumococcal polysaccharide MAb.
Above figure shows expression of pneumococcal polysaccharide MAb run on 12% gel in reducing condition. Appreance of band at 25kDa (light chain) and 50kDa (heavy chain) indicates presence of MAb. As the MAb is not purified, thick band can be seen, due to presence of Fetal bovine serum.

7. CONCLUSION

Murine monoclonal antibody development was done against pneumococcal polysaccharide, keeping in mind the number of deaths caused due to the infection of the bacteria. Antibodies developed against polysaccharide causes protection against from the disease. Polysaccharide protein conjugate containing serotypes 4, 6B, 9V, 14, 18C, 19F, 23F were used. The polysaccharide protein conjugate is conjugated with protein, as polysaccharide is a T cell independent antigen. The immune response against polysaccharide is not specific, has weak immunological memory and readily induces tolerance. So the polysaccharide has been conjugated with protein. Mice were immunized subcutaneously with polysaccharide protein conjugate. Antibody titer was assayed at regular intervals using Rapid multiplex assay, till the time the antibody titer was above thousand fluorescence absorbance units. It took nearly 8 weeks to obtain the required antibody titer. In addition, myeloma cells were also revived and grown for fusion.

The final booster was given intraperitonially 3 days before isolation of spleen. Feeder macrophage cells were isolated from mice, by injecting high sucrose solution in the abdominal cavity. At the time of fusion, spleen cells were isolated and fused with myeloma cells at a ratio of 5:1. Fusion was carried out in presence of PEG. The fused as well as unfused cells were seeded in 96- well plates, which were incubated at 37^0C with 5 % CO_2 incubator and 95 % humidity. After 24 hours DMEM/ F12 + 20% FBS complete medium was replaced with HAT selective medium. It was used, so as to eliminate the unfused cells. HAT selective medium was used for nearly 2 weeks i.e. till the time the hybrid cell growth was nearly 50%- 60%. The culture supernatant was analyzed to check the antibody titer against the different serotypes, using Rapid multiplex assay. Nearly 31 hybrid clones, showed good fluorescence absorbance units. These hybrid clones were then transferred to 12 well plates and further to 6 well plates. After assaying by Rapid multiplex assay, it was observed that the clone P3C11 against Serotype 4, showed 23,167.5 fluorescence absorbance units. This was the highest antibody titer as compared to other clones. The P3C11 clone was selected for Single cell cloning/ Limiting dilution. The dilutions were made in such a way that there was only 1 cell per well. The clones were growing rapidly. The culture supernatant was finally analyzed using Rapid multiplex assay. The results showed that the clones which were developed showed maximum fluorescence absorbance unit against Serotype 4 only. This means that monoclonal antibodies were developed against Type 4 Serotype. The expression of the monoclonal antibody developed against Serotype 4 was assayed by SDS- Polyacrylamide gel electrophoresis. Reducing SDS- PAGE was performed. Presence of heavy chain at 50 kDa and light chain at 25 kDa in reducing condition confirmed the presence of MAb. The P3 C11 clone against serotype 4 was further expanded in 12- well plate, 6- well plate and then finally in T-25 cm^2 flask.The study that was conducted will primarily help to fight against pneumococcal pneumonia amongst children by helping develop a pediatric pneumococcal vaccine in near future.

REFERENCES

1. Marcelo Gottschalk, Jan Kolberg,. Streptococcus Pneumoniae Types 19A and 19F and Streptococcus suis capsular type 8 share common capsular epitopes. Journal of clinical microbiology: 1995; 33: 2492-2495.
2. Jingui Yu, Jisheng Lin, William H. Benjamin Jr., Ken B. Waites, Chen- hung Lee, Moon H. Nahm. Rapid multiplex assay for serotyping Pneumococci with monoclonal and polyclonal antibodies. Journal of clinical microbiology 2005; 43: 156- 162.
3. Steven Obario, Richard Adegbola. The pneumococcus: carriage, disease and conjugate vaccines. Journal of medical microbiology. 2002; 51: 98- 104.
4. Andrej Weintraub. Immunology of bacterial polysaccharide antigens. Carbohydrate Research 2003; 338: 2539-2547.
5. Catherine M. Wernette. Enzyme linked immunosorbent assay for quantitation of human antibodies to pneumococcal polysaccharides. Clinical and diagnostic laboratory immunology: 2003; 10: 514-519.
6. J.H.H.M Van De Wijgert, A.F.M Verheul, H. Snippe, I.J Check. Immunogenicity of Streptococcus pneumoniae Type14 capsular polysaccharide: Influence of carriers and adjuvants on isotype distribution. Infection and immunity. 1991; 59: 2750-2757.
7. Baldwin RW and Robbins RA. Circulating immune complexes in cancer. Cancer Markers: Diagnostic and Developmental Significance. *Humana Press, Clifton, New Jersey* 1980; 19:507-531.
8. Spiegelberg HL. Biological activities of immunoglobulins of different classes and subclasses. *Advances in Immunology* .197; 19:259-294.
9. D. R. Burton DR and Woof JM. Human antibody effector function. *Advances in Immunology. Academic Press, Inc.*1992; 51:1-84.
10. Kőhler G and Milstein C. Continuous culture of fused cells secreting antibody of predefined specificity. *Nature*.1975; 259:495-497.
11. Nelson PN, Westwood OM, Jeffers R, et al. Characterization of anti-IgG monoclonal antibody A574 by epitope mapping. Biochem. Soc. Trans. 1997; 25:375.
12. Nelson PN, Reynolds GM, Wards E. Monoclonal antibodies. Journal of clinical pathology 2003; 53:111-117.
13. Monoclonal antibody production. A report of the committee on manuals of producing Monoclonal Antibodies, Institute for Laboratory Animal Research. National research council. National Academy Press 1999; 1-47.
14. Lafon M. Techniques for the production, screening and characterization of monoclonal antibodies. In: Laboratory techniques, WHO, Geneva. 1996; 4:133-144.
15. Mohamed F. Elshal, Ph.D. and J. Philip McCoy, Jr., PhD.Multiplex Bead Array Assays: Performance Evaluation and Comparison of Sensitivity to ELISA. PubMed Central.2006; 38(4): 317-323.
16. Horan PK, Wheeless LL. Quantitative Single Cell Analysis and Sorting. Science. 1977; 198:149–157.
17. D.Bograert, P.W.N. Hermans, P.V. Adrian, H.C. Rumke, R.de. Groot. Pneumococcal vaccines: an update on current stratergies.Vaccines 2004; 22: 2209-2220.
18. Fatima Y. Production of monoclonal antibody specific for progesterone. Tr. Journal of Biology 1999; 23:393-399.
19. E. alonosodevelasco, A.F.M. Verueul, H. Snipppe. Streptococcus pneumoniae: virulence factors, pathogenesis and vaccines. Microbiological reviews. 1995; 59: 591- 60.

20. Cragg MS, French RR, Glennie MJ. Signaling antibodies in cancer therapy. Current opinion in Immunology 1999; 11:539-540.
21. Austrian r. The pneumococcus at the millennium: not down, not out. J Infect Dis: 1999; 179: 338- 341.
22. Klemperer and Klemperer F. versuche uber Immunisierung und Heilungbei der Pneumokokkeninfection. Berlklinwochenstr: 1891; 28: 5869-5874.
23. Watson DA, Musher DM. a brief history of the pneumococcus in biomedical research. Semin respire Infect: 1999; 14(3): 198-208.
24. Felton L, Baily G. Biologic significance of the soluble specific substance of pneumococci. J Infect Dis. 1926; 38: 131-144.
25. Smillie W, White h. A study of a type I pneumococcus epidemic at the state hospital in Worchester, MA. Am J public health. 1938; 28:293-302.
26. Austrian R. Pneumococcus: the first one hundred years. Rev Infect Dis.1981; 3(2): 183-189.
27. Austrian R. Some observations on the pneumococcus and on the current status of pneumococcal disease and its prevention. Rev Infect Dis.1981; 3: s1- S17.
28. Austrian R, Gold J. Pneumococcal bacteremia with especial reference to bacteremic pneumococcal pneumonia. Ann Intern med.1964; 60: 759-776.
29. Meis JF, Neeleman C. Problems of resistance in Streptococcus Pneumoniae. Resistentieproblem Ned tijdsclrgeneeskd. 1996; 140(3): 141-144.
30. Appelbaum PC, Bhamjee A. Streptococcus Pneumoniae resistant to penicillin and chloramphenicol. Lancer JT.1977; 2 (8046): 995-997.
31. Kroon FP, Zwinderman. Antibody response after influenza vaccination in HIV infected individual: a consecutive 3 year study. Vaccine 2000; 18 (26): 3040-3049.
32. Prevention of pneumococcal disease: recommendations of the advisory committee on immunization practices (ACIP). MMWR Morb mortal Wkly Rep.1997; 46 (RR-8): 1-24.

