STREPTOCOCCUS PNEUMONIAE

SSI Diagnostica

Textbook in Diagnosis, Serotyping, Virulence Factors and Enzyme-linked Immunosorbent Assay (ELISA) for Measuring Pneumococcal Antibodies

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INTRODUCTION

Streptococcus pneumoniae (S. pneumoniae), or pneumococcus, is a Gram-positive aerotolerant anaerobe, alpha-haemolytic, bile soluble diplococcus member of the genus *Streptococcus* [1].

It is a significant human pathogenic bacterium, and was recognized as a major cause of pneumonia in the late 19th century. Hence it has been the subject of many humoral immunity studies.

The pneumococcus causes many types of pneumococcal infections other than pneumonia, including meningitis, bacteremia, sepsis, osteomyelitis, septic arthritis, acute sinusitis, otitis media, endocarditis, peritonitis, pericarditis, cellulitis, and brain abscess [2].

Thus the pneumococcus is the most common cause of bacterial meningitis in industrialized countries, and also often found in otitis media. Pneumonia caused by *S. pneumoniae* is more common in the very young and the very old age groups [3].

As *S. pneumoniae* is optochin sensitive, it can be differentiated from *Streptococcus viridans*, which is also alpha-haemolytic, using an optochin test. The encapsulated, Gram-positive coccoid bacteria have a distinctive morphology on Gram stain, the "lancet shape". It has a polysaccharide capsule that acts as a virulence factor for the organism, and so far 92 different serotypes are known [4]. The serotypes differ in virulence, prevalence, and extent of antibiotic resistance [77, 78].

Pathogenesis

S. pneumoniae is normally found in the nasopharynx of 5-10% of healthy adults, and 20-40% of healthy children [79]. It can be carried more frequently in certain crowded environments (e.g. day care centers, army barracks etc.) [1].

The pneumococcus attaches to the nasopharyngeal cells through interaction of bacterial surface adhesins. This normal colonization can become infectious if the organism is carried into areas such as the eustachian tube or nasal sinuses, where it can cause otitis media and sinusitis, respectively.

Pneumonia occurs if the organism reaches the lungs. Factors such as very old or very young age, previous occurrence of viral infection, or smoking-induced ciliary paralysis might be predisposing factors. Once the organism makes its way to a site where it is not normally found, it activates the complement system, stimulates the production of cytokines, and attracts white blood cells (specifically neutrophils) [80]. The surrounding polysaccharide capsule makes it resistant to phagocytosis, and if there is no pre-existing anticapsular antibody, alveolar macrophages cannot adequately kill the pneumococci. The organism may spread to the blood stream (bacteremia) and can be carried to the meninges, joint spaces, bones, and peritoneal cavity, resulting in serious invasive diseases, such as meningitis, brain abscesses, septic arthritis or osteomyelitis [5].

The risk of pneumococcal infection is much increased in people with impaired IgG synthesis, impaired phagocytosis or defective clearance of pneumococci. In particular, the absence of a functional spleen, through congenital asplenia, splenectomy, or sickle-cell disease predisposes to a more severe course of infection (overwhelming post-splenectomy infection) and preventive measures such as vaccination are indicated. Also, certain ethnic groups such as native americans (indians), eskimos and aborigines have a predisposition to acquire pneumococcal infections [13, 54].

Pneumonia

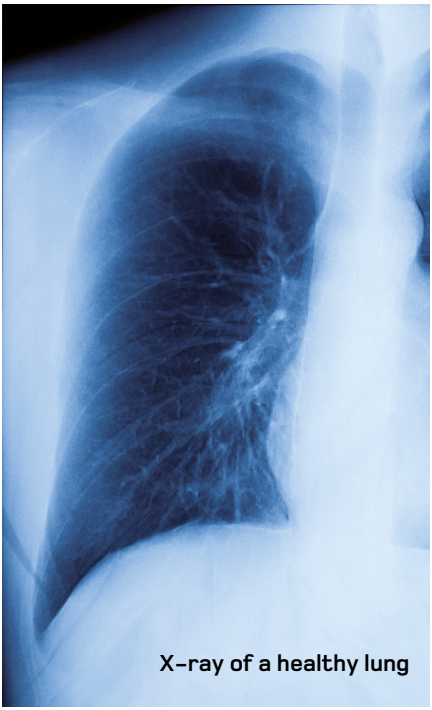
Pneumonia is the most common clinical presentation for pneumococcal disease among adults (although pneumonia limited to the lung parenchyma is not considered to be an "invasive" disease). The incubation period of pneumococcal pneumonia is short, about 1 to 3 days.

Typical symptoms of pneumonia include a cough, chest pain, fever, and breathing difficulties. The physical examination may reveal fever, low oxygen saturation and crackles in the affected area [55]. The diagnostic of pneumonia is challenging, and often based on clinical findings, changes on the X-rays, and culture of lower respiratory tract samples and blood culture.

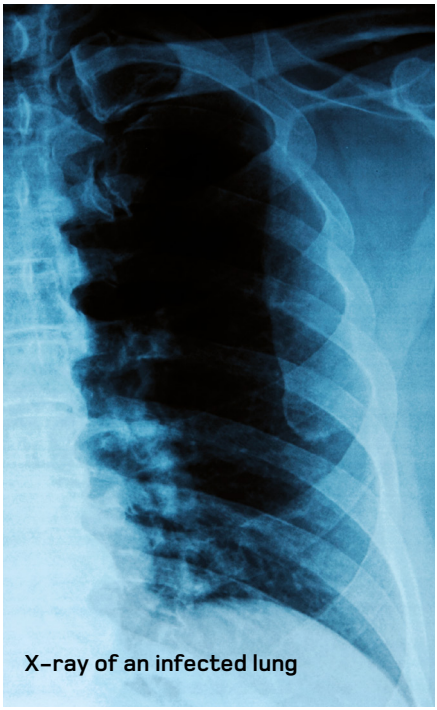
X-ray presentations of pneumonia can be classified as lobar pneumonia, bronchopneumonia (also known as lobular pneumonia), and interstitial pneumonia [60]. Bacterial community-acquired pneumonia classically shows lung consolidation of one lung segmen-

tal lobe, which is known as lobar pneumonia. However, findings vary, and other patterns are common in other types of pneumonia. Aspiration pneumonia may show bilateral opacities primarily in the bases of the lungs and on the right side [61].

When hospitalized for severe pneumonia, both lower respiratory tract samples and blood cultures should be taken, as well as testing the urine for antigens to *Legionella* spp. and pneumococcus [59, 63]. Other etiologies such as viral and fungal infections can be confirmed via detection of either the virus or its antigens with culture or polymerase chain reaction (PCR), or other techniques [64].



X-ray of a healthy lung



X-ray of an infected lung

Blood Culture

The blood stream is usually a sterile environment. Blood culture is a microbiological culture of blood under appropriate conditions that will optimize the growth of microorganisms. It is used to detect bacterial infections that are spreading through the bloodstream (such as bacteremia, and sepsis).

Method
Blood is taken through venipuncture, and injected into two or more "blood culture collection bottles" with specific media for aerobic and anaerobic organisms. Two major commercial blood culture systems exist: Bactec™ (Becton Dickinson) and Bact/Alert (bioMériux).

The blood should be collected using an aseptic technique. This requires that both the top of the culture bottles and the venipuncture site of the patient are cleaned with swabs of 70% isopropyl alcohol prior to collection [74].

To maximize the diagnostic sensitivity of blood cultures (increase the probability of discovering a pathogenic organism from contaminants), multiple sets of cultures are performed (each set consisting of aerobic and anaerobic vials filled with ~ 10 mL).

After inoculating the blood culture collection bottles, they are sent to the clinical microbiology department, where they are entered into a blood culture machine for incubation of specimens at body temperature.

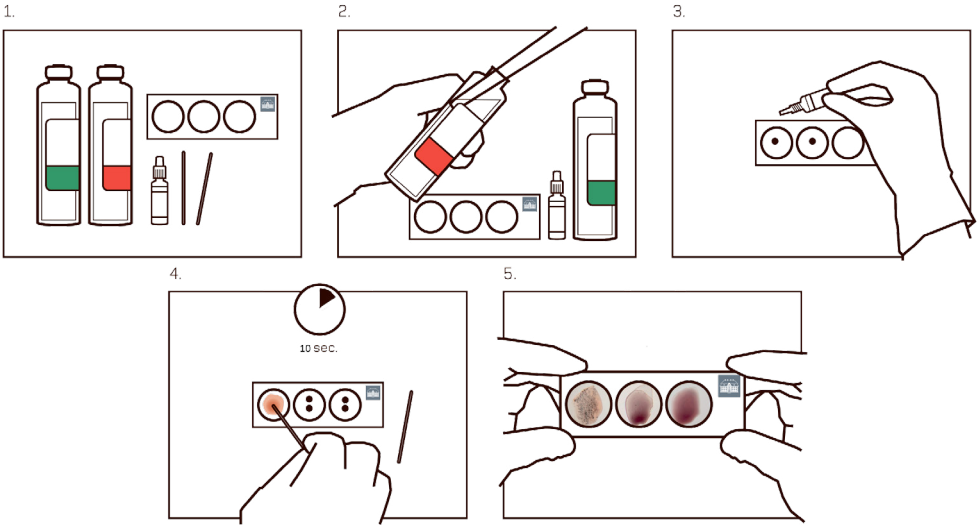
The blood culture instrument reports positive blood results when there is a detectable bacterial growth from the culture media. Most blood cultures are monitored for 5 to 6 days, after which negative vials are removed.

If a vial is positive, a microbiologist will perform a Gram stain on the blood for a rapid, general identification of the bacteria, which will be reported to the attending physician of the bacteremic patient. Furthermore, a quick *S. pneumoniae* latex agglutination can be performed directly on 10 µL of the blood culture sample. SSI Diagnostica's ImmuLex™ *S. pneumoniae* Omni test will provide an answer within 10 seconds.

Procedure for ImmuLex™ *S. pneumoniae* Omni

1. Contents: One bottle of latex particles coated with pneumococcal antiserum, a positive and negative control, reaction card and mixing sticks.
2. Take the blood culture bottle where growth has been detected. Bring the bottle with latex suspension to room temperature and shake well.
3. For each reaction add a drop (approximately 10 µL) of latex suspension in the circle on the reaction card. Apply a drop (approximately 10 µL) of positive blood culture medium next to the drop of latex suspension.

4. Important: Read the result while mixing the two drops for maximum 10 seconds. Use a separate stick for each reaction.



ImmuLex™ *S. pneumoniae* Omni	Positive Blood Culture	Negative Blood Culture
Sensitivity: 98%	182	4
Specificity: 96%	3*	66

The blood is also subcultured or "subbed" onto blood agar plates to isolate the pathogenic organism for identification and antimicrobial susceptibility testing (AST) , and to inform clinicians on appropriate antibiotics for treatment.

Urinary Antigen Detection

Pneumococcal antigen detection dates back to the work done by Dochez and Avery in 1917 when they demonstrated capsular polysaccharides in urine from patients with lobar pneumonia [13, 66]. The procedure was to mix clear urine with serum, and to let it incubate at 37°C for one hour. If there was no reaction, the urine was concentrated, and potential carbohydrate was precipitated with ethanol, collected and dissolved in water and mixe- with serum again.

*Three crossreactions to *Streptococcus haemolyticus C* (n=2) and *Pseudomonas aeruginosa/Bacteroides thethaiothaomicron* (n=1) have been observed in only the anaerobic blood culture bottle

In the study by Dochez and Avery serotypes from 112 cases were studied, showing a sensitivity of 62.5%.

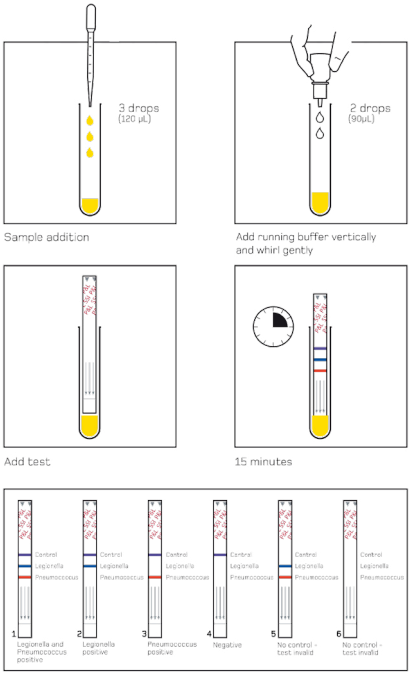
In 1999, the first *S. pneumoniae* urinary antigen test was developed and commercialized. The test was a lateral flow assay, which could give a positive or negative answer within 15 minutes.

In 2014, Statens Serum Institut started a project to make a combined *S. pneumoniae* and *L. pneumophila* urinary antigen test. The project was initiated because many guidelines recommend performing both assays in community-acquired pneumonia hospitalized patients [75, 76]. The combined assay ImmuView® *S. pneumoniae* and *L. pneumophila* Urinary Antigen Test was commercialized and launched in September 2014.

Procedure for ImmuView® *S. pneumoniae* and *L. pneumophila* Urinary Antigen Test
The procedure of the assay is to take three drops of urine and two drops of running buffer and mix it. Subsequently, insert the test strip in the vial and wait for 15 minutes before reading the result.

The urinary antigen test can be used in the early stages of pneumonia, as the antigen will be excreted within the first week after onset of symptoms.

Urinary antigen tests cannot be used on toddlers, as children might carry pneumococcus in the nasopharynx and might excrete pneumococcal antigens in the urine without having a pneumococcal pneumonia.



Results Note: Three grey/purple test lines do not indicate a positive result

The sensitivity and specificity of ImmuView® *S. pneumoniae* and *L. pneumophila* Urinary Antigen Test is:

S. pneumoniae

	ImmuView® *S. pneumoniae* and *L. pneumophila* Urinary Antigen Test	Commercial Rapid Test
Sensitivity (n=71)	85%	78%
Specificity (n=76)	99%	

L. pneumophila

	ImmuView® *S. pneumoniae* and *L. pneumophila* Urinary Antigen Test	Commercial Rapid Test
Sensitivity (n=99)	89%	54%* (72%)
Specificity (n=76)	100%	

S. pneumoniae* and *L. pneumophila

	ImmuView® *S. pneumoniae* and *L. pneumophila* Urinary Antigen Test	Combination of two Commercial Rapid tests for *S. pneumoniae* and *L. pneumophila*
Sensitivity (n=170)	87%	64%* (74%)
Specificity (n=76)	99%	

* Sensitivity when very faint/weak test lines are scored negative.

Acute Otitis Media

The highest incidence of acute otitis media is seen in children aged 6 to 12 months. 70% of all 3 year old children have had at least one episode, and 30% of all 3 year olds have had three episodes of acute otitis media [82, 83]. Acute otitis media is an inflammation of the middle ear, which occurs when the bacteria from the nasopharynx passes into the middle ear via the Eustachian tube. This is done in the context of viral infections in which the bacteria are more easily trapped in the middle ear, and can cause an inflammatory condition.

Acute otitis media occurs mainly in connection with upper viral respiratory infections (such as respiratory syncytial virus (RSV) , rhinovirus (cold virus), adenoviruses, enteroviruses, and influenza). Acute otitis media is commonly caused by the bacterium *S. pneumoniae*, causing between 30% and 60% of all cases. Other common bacteria are *Haemophilus influenzae*, *Branhamella catarrhalis* and *Streptococcus pyogenes*. The course of pneumococcal acute otitis media has been described to be more severe and has more complications than *H. influenzae* and *B. catarrhalis* [67].

Symptoms of acute otitis media are pain in the middle ear, and often fever. The pain may disappear if the eardrum perforates (due to high pressure in the middle ear), and pus will come out through the ear canal. Acute otitis media in children often goes with a cold, and conjunctivitis and in many cases hearing is reduced [68].

There are a number of risk factors for acute otitis media. Young age at first episode of acute otitis media increases the risk of having repeated episodes of acute otitis media. Early and massive colonization of nasopharynx with pathogenic bacteria , frequent upper respiratory infections , attending day care and the absence of breastfeeding are all important risk factors for acute otitis media. Furthermore, use of pacifier and passive smoking in the home increases the risk of developing acute otitis media. Anatomical factors such as lip cleft palate and dysfunction of the Eustachian tube are other risk factors. Conditions like heritage, race, sex, premature birth and nutritional status have also proven to affect the risk for acute otitis media [69].

Acute otitis media is diagnosed with regular medical examination with an otoscope. If the child has the disease, the eardrum and its surroundings will often be red, and clear pus can often be seen in a build-up behind the eardrum so that it bulges out.

If the eardrum is punctuated, there will be pus in the ear canal that can be collected for microbiological examination.

Often the child will be very sore to the touch of the front ear cartilage (tragus soreness).

Meningitis

Bacterial meningitis is an inflammation of the brain's protective membranes, caused by a variety of bacteria and virus. The bacteria that most often produce meningitis are the pneumococcus and meningococcus [70, 71]. Bacterial meningitis is a serious life-threatening disease that rapidly can progress and lead to death if untreated.

Bacteria that cause meningitis are often found naturally in the mucous membranes of the respiratory tract. As part of other inflammatory conditions, such as acute otitis media, bacteria can penetrate into the blood and move into the meninges where it establishes inflammation. Furthermore, bacteria and viruses can in some cases penetrate directly from the nose into the meninges if for example nasopharynx has defects.

Meningitis can develop in a matter of hours. Typical symptoms are severe headache, nausea and vomiting, and high fever (39-40°C, 102-104°F). Additional severe signs are: neck stiffness, pain and difficulties bending the neck, altered consciousness, small bleedings (the size of a pinhead (petechiae)). The bleeding does not disappear when putting pressure to the skin [72, 73].

It is often impossible to assess based only on symptoms whether the patient's meningitis is caused by bacteria or virus. The main diagnostic tool is a lumbal puncture, where a small amount of cerebrospinal fluid is collected from the spinal canal, and examined for inflammatory markers and different etiologies.

If the patient has received antibiotic treatment before samples are collected for laboratory testing and cultures, results might be negative. When this is the case, the diagnosis has to be made by PCR (i.e. methods based on DNA amplification).

VIRULENCE FACTORS

The pneumococcus has several virulence factors including polysaccharides, surface proteins, excreted proteins and cytoplasmic proteins. Figure 1 shows a schematic illustration of a pneumococcus and its virulence factors. Among others, these factors define the characteristics of the bacteria, and serotype.

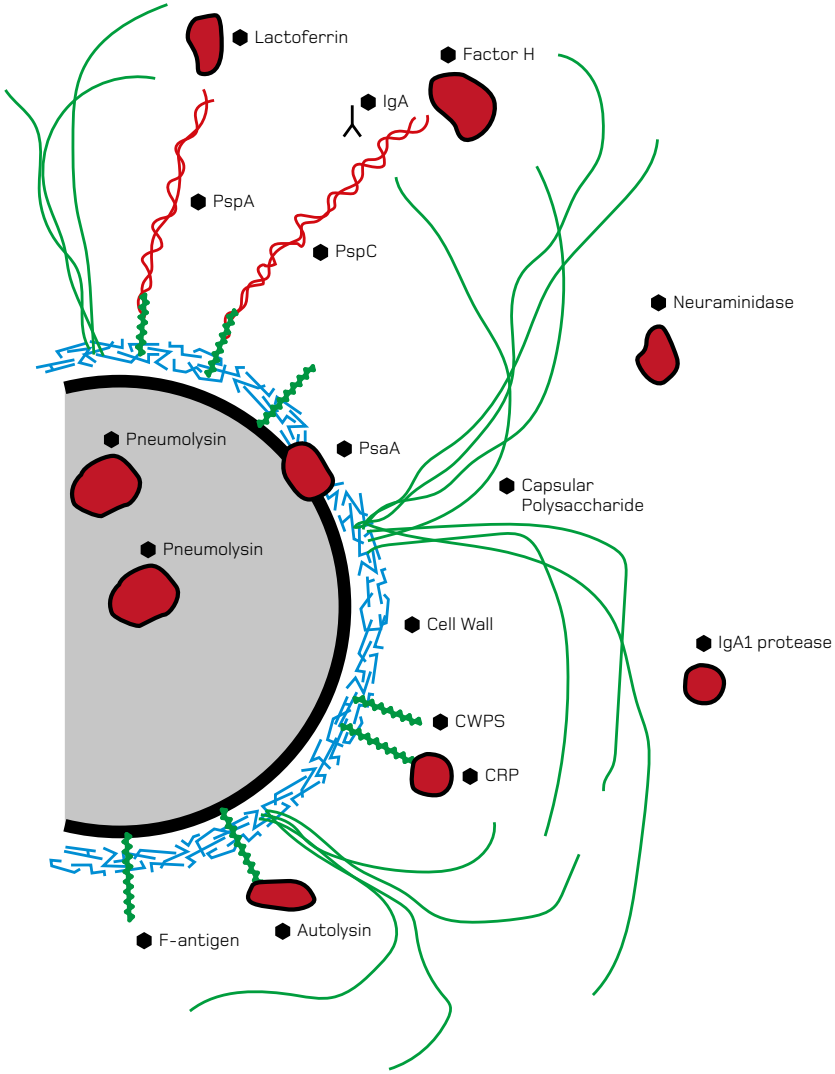


Figure 1. Schematic illustration of a pneumococcus and its virulence factors

Protein Virulence Factors

Pneumococcal surface protein A (PspA)

PspA is expressed with much variability. It is exposed on the surface of the pneumococcus and the size of the protein varies between 67-99 kDa. This variability occurs because the gene for PspA has a mosaic gene pattern which emerges due to recombination followed by horizontal gene transfer. PspA is bound to the cell surface though cell wall polysaccharide (CWPS) as choline attachment anchors the protein. The major role of PspA is to block the binding of complement component C3, thereby inhibiting both opsonisation and phagocytosis of the bacteria. Furthermore, PspA binds lactoferrin [7, 8].

Pneumococcal surface protein C (PspC)

PspC is also called CbpA, SpsA and PbcA. PspC is another choline attached protein and is 75 kDa. PspC recognises sialic acid on the epithelial cells and thereby mediates adherence to the host. In addition, PspC binds IgA and factor H [9].

Autolysin (LytA)

LytA is 36 kDa and situated in the cell envelope. LytA is an enzyme, N-acetylmuramoyl-L-alanine amidase, which degrades the peptidoglycan in the cell wall which leads to pneumococcal lysis. The cell destruction releases the cytoplasmic pneumococcal toxin, pneumolysin. LytA is activated by bile giving the reason to bile solubility [10].

Pneumolysin

Pneumolysin is 53 kDa intracellular toxin which is liberated by LytA lysis. Pneumolysin binds cholesterols in all cells of the host and forms transmembrane pores which lead to lysis of the host cells [11].

Neuraminidase

S. pneumoniae expresses two neuraminidases, NanA (108 kDa) and NanB (75 kDa), of 108 kDa and 75 kDa respectively. Neuraminidase is an enzyme (sialidase) which cleaves the terminal sialic acid from glycolipids, glycoproteins and oligosaccharides in the mucus and on cell surfaces [12].

Pneumococcal surface adhesin A (PsaA)

PsaA is an extracellular cell membrane bound lipoprotein which transports magnesium ions into the cells [14].

IgA1 protease

IgA1 protease is a secreted zinc metalloprotease that specifically targets human immunoglobulin A1 (IgA1) which constitutes more than 90% of the IgA in the human airway [6].

Polysaccharides Virulence Factors

Capsular Polysaccharides (CPS)

The polysaccharide capsule forms the outermost layer of *S. pneumoniae*, and is approximately 200-400 nm thick [15]. With the exception of serotypes 3, 37, rough types, and possibly others, the capsule is covalently attached to the outer surface of the cell wall peptidoglycan. More than 92 structurally and serologically distinct CPS types have been recognized [4]. Capsular production is indispensable for pneumococcal virulence and is strongly anti-phagocytic in non-immune hosts. Although non-encapsulated strains ("rough" strains) have been associated with superficial infections, clinical isolates from other sterile sites are encapsulated, and non-encapsulated variants of these strains are largely avirulent [16]. However, non-encapsulated strains may cause invasive infections in immuno-compromised patients. Most CPS serotypes are highly charged at physiological pH, and this can directly interfere with interactions with phagocytes [17]. The most simple CPS is a linear polymer with repeating units consisting of two or more monosaccharides. More complicated CPS are branched, and the repeating unit backbones consist of one to six monosaccharides and have additional side chains [18].

The serotypes are distinguished by chemical difference in their CPS and on the ability of the immune system of rabbits to recognize these structural differences and to respond with specific antibodies against the antigens of each different type.

Two different systems of nomenclature exist for the pneumococcal serotypes, the Danish system and the American system. For instance, the Danish serotype 10A is equal to the US type 34. The Danish system is based on cross-reactions between different types, so that serologically cross-reaction types are assigned to a common serogroup, with individual serotypes within each group distinguished by the trailing letter [65]. In the American system, serotypes are numbered sequentially (connected with the order of their discovery); it does not recognize antigenic cross-reactivity among types. In the key schemes on pages 27-32, the serotypes are presented in the generally used Danish system. They are presented together with the antigenic formulas that represent arbitrary designation of cross-reactions as observed by the capsular reaction (Quellung) [19].

Cell Wall Polysaccharide (CWPS)

All pneumococci, both virulent and avirulent strains possess a common polysaccharide, CWPS (**C**ell **W**all **P**oly**S**accharide, teichoic acid). The CWPS is composed of a tetrasaccharide joined together through ribitol phosphate diester. In addition, CWPS contains either one or two phosphocholine substituents. CWPS and CPS are linked covalently to the cell wall peptidoglycan, which in the purification of the CPS makes it impossible to remove the CWPS [15, 20].

F-antigen

All pneumococci (both virulent and avirulent strains) possess a common polysaccharide, the F-antigen. It is a lipoteichoic acid, with a polysaccharide part that has the same repeat as CWPS. This part is then linked to a diacylated glycerol residue via a glucose residue [15].

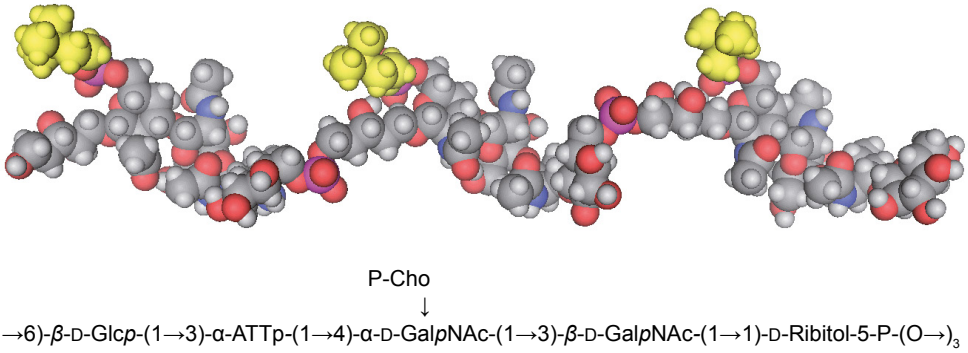


Figure 2. 3D illustration of repeating sugar units and chemical structure [53].

Before the availability of antimicrobials, more than 70% of patients hospitalized with pneumococcal bacteremia died [21]. In 1911, Wright et al. developed a crude whole-cell pneumococcal vaccine to immunize South African gold miners, a group with an extremely high incidence of serious pneumococcal infections [22, 23]. Subsequently, a number of other investigators conducted clinical trials on the safety and efficacy of polysaccharide vaccines against pneumococci of various serotypes [24]. The validity of the results from many of these trials was questionable because of methodological pitfalls, such as the lack of randomization, and inadequate clinical follow-up of subjects. However, controlled trials of bi-valent, tri-valent, and quadric-valent polysaccharide vaccines conducted in the 1940s provided stronger evidence that these vaccines were efficacious [25, 26]. Two hexa-valent vaccines were later commercially produced and marketed.

At about the same time, antimicrobials effective against pneumococci became available, and the outcome of patients with pneumococcal infections improved substantially. The seemingly miraculous efficacy of penicillin led to the widespread belief that pneumococcal infections were entirely curable, and clinicians, researchers, and public health officials lost interest in the prevention of this previously feared pathogen. By the 1950s, the pneumococcal vaccines had been withdrawn from the market.

However, in 1964, complacency over pneumococcal disease ended when Robert Austrian and Jerome Gold presented clinical descriptions of some 2,000 cases of pneumococcal pneumonia diagnosed at Kings County Hospital in Brooklyn between 1952 and 1962 [27]. Despite the substantial impact of antimicrobials on reducing mortality, nearly 25% of the patients admitted with pneumococcal bacteremia died. Mortality was highest among the elderly and nearly half of the patients aged 60 years or older who had bacteremia died. Deaths were also more common among people with certain chronic medical conditions. Accordingly, Austrian and others worked together to redevelop an effective polyvalent pneumococcal polysaccharide vaccine [28].

To evaluate the vaccine expeditiously and at relatively low cost, double-blinded randomized controlled trials were conducted in a population with a high rate of pneumococcal infections. As in earlier decades, young gold miners in South Africa were recruited. These well-designed trials produced conclusive evidence of the efficacy of the vaccines for preventing invasive infection and pneumonia in this population. The estimates of protective efficacy were 76%-92% [29, 30]. In 1977, these findings led to licensure of a 14-valent polysaccharide vaccine in the United States. This was replaced by a 23-valent vaccine in 1983.

Pneumococcal Polysaccharide Vaccines

Currently available pneumococcal vaccines, manufactured by Merck and Co. (Pneumovax 23; West Point, Pennsylvania) and Lederle Laboratories (Pneu-Immune 23; Wayne, New Jersey), contain 25 µg of each of 23 purified capsular polysaccharide antigens of *S. pneumoniae* (serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F). Based on data from surveillance conducted by the Center for Disease Control and Prevention (CDC), these 23 capsular types represent at least 85%-90% of the serotypes that cause invasive pneumococcal infections among children and adults in the United States. Protection against additional serotypes may also be provided as they are serologically related to types in the vaccine (e.g. serotype 6A).

Conjugate Pneumococcal Vaccines

Rates of invasive *S. pneumoniae* infection are highest during the first 2 years of life. Unfortunately, pneumococcal capsular polysaccharides are T-cell–independent antigens that induce limited antibody responses in children under 2 years. Clinical trials of pneumococcal capsular polysaccharide vaccines among young children have demonstrated limited efficacy or no evidence of efficacy as the immune responses are poor in children under 5 years [31, 32, 33]. By conjugating polysaccharide antigens to a carrier protein, an immunologic response elicits. The protein becomes T-cell-dependent and induces higher antibody concentrations in infants. Additionally, memory B-cells are produced and primed for booster responses.

At present three Pneumococcal conjugate vaccines (PCV) are available:

Synflorix® is a 10-valent vaccine (GSK). It contains the following serotypes of pneumococcus (1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23F). The vaccine was put on the market in March 2009.

Prevenar 13 is a 13-valent vaccine (Phizer Inc.) which contains the following serotypes of pneumococcus (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F). This vaccine was approved for release February 2010 and replaced the previously existing Prevenar 7, which only included serotypes 4, 14, 6B, 19F, 18C, 23F and 9V.

Figure 3. Since year 2000 vaccination for pneumococcus has been part of the childhood vaccination programme in USA. In 2007 the vaccine was included in the Danish vaccination program.

TYPING OF STREPTOCOCCUS PNEUMONIAE

Early on, researchers using various immune antisera realized that different isolates of pneumococci could be divided into different categories, groups, or types through agglutination methods and the mouse protection model. Little by little it became clear, as noted in White's monograph, that "Whatever benefit is to be derived from the use of anti-pneumococci serum depends on the rapid and accurate determination of the type of pneumococcus causing the infection" [34]. The question thus became one of devising ways of shortening the time elapsing between the collection of the specimen and the identification of the serologic type of the infecting pneumococci. The following were among the most important methods White described [34]:

- Mouse protection test (1910)
  Early works leading to the detection of the pneumococcus and eventually to the appreciation that isolates differed in agglutination and that antisera differed in their capacity to protect against pneumococcal infection in the mouse protection test [62].

- Culture agglutination
  In 1918, Avery inoculated washed sputum in broth containing 1% glucose; after 5 hours of incubation, the culture was used as an agglutinating antigen with three types of antisera.

- Urine precipitation test
  This was based on the 1917 finding by Dochez and Avery that pneumococci *in vivo* as well as *in vitro* produce a soluble-specific substance that is easily detected with an appropriate immune serum. See chapter "Urinary Antigen Detection" page 8 for more.

- Stained slide microscopic agglutination test
  Sabin introduced this method in 1929. Sputum is injected into the peritoneal cavity of a mouse, and a few hours later, a small amount of peritoneal exudate is withdrawn and mixed with diagnostic antisera of the various types in separate drops on the same slide. The drops are then smeared and the slide fixed, stained, and examined under the microscope.

- Neufeld test
  First described by Neufeld and Etinger-Tulczynska in 1933, but based on the Neufeld test described in 1902 and also known as the capsular reaction test. Until recently, this was probably the fastest, most specific, and easiest of all methods. It continues to be carried out essentially as described by Austrian [35].

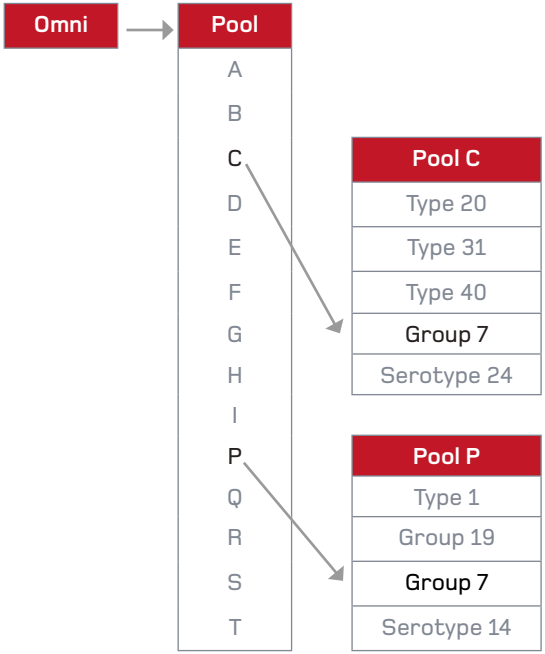
In most instances it is sufficient to know if a given strain is a pneumococcus or not. If the strain is sensitive to optochin (ethylhydrocuprein hydrocholide) and bile (sodium deoxycholate) it is very likely a pneumococcus. The strain may be confirmed using Omni serum which is a pooled polyvalent, purified pneumococcal serum giving a capsular reaction in a Neufeld test. This antiserum was first introduced in 1966 by Erna Lund [36]. Today, it is still available and reacts with all known serotypes of pneumococci. The Neufeld test is not a swelling of the capsule but a reaction between the type-specific antiserum and the capsular polysaccharide rendering the capsule visible.

However, not all type-specific antibodies of Omni serum, are present in a concentration high enough to allow the capsule to become visible, but produce agglutination, which must sometimes be accepted as the sole criterion of a positive reaction [37].

Neufeld Test (Quellung reaction)

Procedure with a phase contrast microscope:
Approximately 2 µL of pneumococcus (liquid, broth or from a single colony) is mixed with an equal amount of antiserum. It is preferable to have relative few organisms per microscope field. The cover slip is inverted over the wet preparation and blotted lightly. A drop of immersion oil is applied to the cover clip and the preparation is examined under the oil immersion (magnification x100).
(For video demonstration please visit ssidiagnostica.com and search for Neufeld).

Procedure with a normal microscope:
A loop full of emulsified broth culture is spread over an area of 0.5 to 1 cm in diameter on a glass slide and allowed to dry at room temperature. In a similar fashion, the cells from a single colony of pneumococcus may be emulsified in a drop of saline on a slide and allowed to dry. A loop full of 1% aqueous methylene blue is placed on the cover slip, and a loop full of typing serum is then applied directly to the dried spot on the slide. The residual antiserum adhering to the loop is next mixed with the methylene blue on the cover slip. Then it is applied to and mixed with a droplet of antiserum on the slide which previously was spread over the area of the dried preparation. The cover slip is inverted over the wet preparation and blotted lightly. A drop of immersion oil is applied to the cover slip and the preparation is examined under the oil immersion lens of a microscope with x10 ocular lenses [35].



Factor Sera				
	7b	7c	7e	7f
7F	+	-	-	-
7A	+	+	-	-
7B	-	-	+	-
7C	-	-	-	+

Figure 4. Example of serotyping using the Neufeld test.

Figure 4 shows an example of serotyping using the Neufeld test. Omni serum comprising antibodies against all known serotypes is positive. The chessboard is positive in pool C and P indicating a group 7. Using factor sera 7b, 7c, 7e and 7f, the type is confirmed. If e.g. factor sera 7e is positive then the strain is serotype 7B.

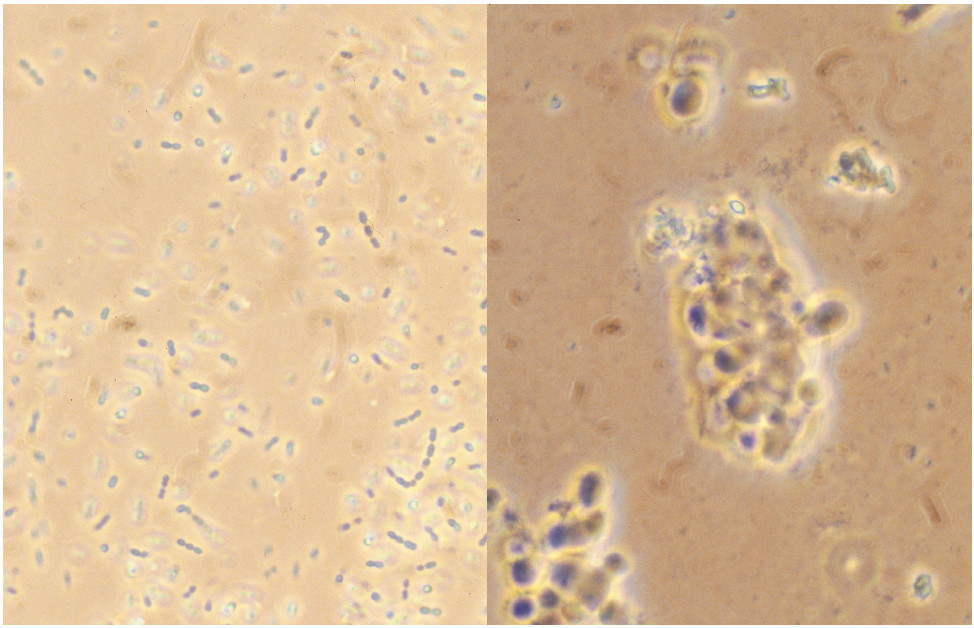


Figure 5. The Neufeld test. Negative (left) and positive reaction (right).

Latex Agglutination Test

Two bacteriologists, Herbert Edward Durham (1866-1945) and Max Von Gruber (1853–1927), discovered specific agglutination in 1896 [38]. The clumping became known as the Gruber-Durham reaction. Gruber introduced the term agglutinin (from Latin) for any substance that caused agglutination of cells.

The French physician Fernand Widal (1862–1929) put Gruber and Durham's discovery to practical use later in 1896, using the reaction as the basis for a test for typhoid fever. Widal found that blood serum from a typhoid carrier caused a culture of typhoid bacteria to clump, whereas serum from a typhoid-free person did not. This Widal test was the first example of serum diagnosis [39].

In 1900 the Austrian physician Karl Landsteiner found another important practical application of the agglutination reaction. His discovery of ABO blood groups was the start of the science of blood transfusion and serology [40].

The first description of a test based on latex agglutination was the rheumatoid factor test proposed by Singer and Plotz in 1956. Since then, tests to detect microbial and viral infections etc. have been developed [41].

In 2004, SSI Diagnostica introduced the ImmuLex™ Pneumotest [41].
(For video demonstration please visit ssidiagnostica.com and search for Pneumotest Latex Kit).

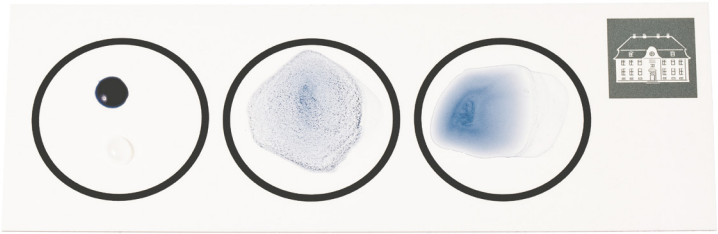


Figure 6. Latex agglutination – To the left a drop of latex and bacterial suspension. In the middle a positive agglutination, and a negative agglutination to the right.

Serotyping *S. pneumoniae*

Serotyping of *S. pneumoniae* is performed in the following order. First the strain is tested in either the Pneumotest Kit (Neufeld test) or in the ImmuLex™ Pneumotest, where the type/group/pool of serotypes are determined, Thereafter further typing is made with single type sera, group sera and factor sera (see Figure 4) [42,43].

For a full serotyping the following antisera or kit is needed:

- Omni serum, reacting with all known types
- Pool antisera, for typing or grouping most pneumococci (A to I and P to T) or Pneumotest kit
- Type sera, reacting with a single type
- Group sera, reacting with all the types within one group
- Factor sera for differential typing within a group

Some of the above mentioned products can also be purchased for latex agglutination:

- ImmuLex™ *S. pneumoniae* Omni
- ImmuLex™ Pneumotest Kit
- ImmuLex™ Pneumococcus 7-10-13-Valent Kit
- ImmuLex™ Pneumococcus 7-Valent Kit

Pool	P	Q	R	S	T	Non-vaccine groups/types
A	**1**	**18** (18F, 18A, 18B, **18C**)	**4**	**5**	**2**	
B	**19** (**19F**, **19A**, 19B, 19C)	**6** (**6A**, **6B**, 6C, 6D)	**3**	**8**		
C	**7** (**7F**, 7A, 7B, 7C)				**20**	**24** (24F, 24A, 24B) **31**, **40**
D			**9** (9A, 9L, **9N**, **9V**)		**11** (11F, **11A**, 11B, 11C, 11D)	**16** (16F, 16A) **36**, **37**
E			**12** (**12F**, 12A, 12B)	**10** (10F, **10A**, 10B, 10C)	**33** (**33F**, 33A, 33B, 33C, 33D)	**21**, **39**
F				**17** (**17F**, 17A)	**22** (**22F**, 22A)	**27** **32** (32F, 32A) **41** (41F, 41A)
G						**29**, **34** **35** (35F, 35A, 35B, 35C) **42** **47** (47F, 47A)
H	**14**	**23** (**23F**, 23A, 23B)		**15** (15F, 15A, **15B**, 15C)		**13** **28** (28F, 28A)
I						**25** (25F, 25A) **38**, **43**, **44**, **45**, **46**, **48**

Table 1. Pneumotest Kit. Chessboard for identification of pneumococcal groups/types.

Boldface indicates that the group/type is included in the 23-valent pneumococcal polysaccharide vaccine.

( ) states types within a group.

Key to *S. pneumoniae* Types and Pneumococcal Diagnostic Antisera

S. pneumoniae	*S. pneumoniae* type	Positive reaction in:				Antiserum/antisera recommended for identification of type
		Pool serum	Type serum	Group serum	Factor serum	
Type 1	**1**	A, P	1			Type serum 1
Type 2	**2**	A, T	2			Type serum 2
Type 3	**3**	B, R	3			Type serum 3
Type 4	**4**	A, R	4			Type serum 4
Type 5	**5**	A, S	5			Type serum 5
Group 6	6A	B, Q		6	6b	Factor serum 6b
	6B	B, Q		6	6c	Factor serum 6c
	6C	B, Q		6	6d	Factor serum 6d
	6D	B, Q		6	6c, 6d	Factor serum 6c + 6d
Group 7	**7F**	C, P		7	7b	Factor sera 7b + 7c
	7A	C, P		7	7b, 7c	Factor serum 7c
	7B	C, P		7	7e	Factor serum 7e
	7C	C, P		7	7f	Factor serum 7f
Type 8	**8**	B, S	8			Type serum 8
Group 9	9A	D, R		9	9d	Factor sera 9d + 9g
	9L	D, R		9	9b	Factor sera 9b + 9e
	9N	D, R		9	9b, 9e	Factor serum 9e
	9V	D, R		9	9d, 9g	Factor serum 9g
Group 10	10F	E, S		10	10b	Factor sera 10b + 10d + 10f
	10A	E, S		10	10d	Factor sera 10d + 10b
	10B	E, S		10	10b, 10d	Factor sera 10b + 10d
	10C	E, S		10	10b, 10f	Factor serum 10f
Group 11	11F	D, T		11	11b, 11g	Factor sera 11g + 11f
	11A	D, T		11	11c	Factor sera 11c + 11b
	11B	D, T		11	11b, 11f, 11g	Factor sera 11f + 11g
	11C	D, T		11	11b, 11c, 11f	Factor sera 11c + 11f
	11D	D, T		11	11b, 11c	Factor sera 11b + 11c + 11f
Group 12	**12F**	E, R		12	12b	Factor sera 12b + 12c
	12A	E, R		12	12c	Factor sera 12c + 12b
	12B	E, R		12	12b, 12c, 12e	Factor serum 12e
Type 13	13	H	13			Type serum 13
Type 14	**14**	H, P	14			Type serum 14

S. pneumoniae	*S. pneumoniae* type	Positive reaction in:				Antiserum/antisera recommended for identification of type
		Pool serum	Type serum	Group serum	Factor serum	
Group 15	15F	H, S		15	15b, 15c	Factor sera 15b + 15c
	15A	H, S		15	15c	Factor sera 15c + 15b
	15B	H, S		15	15b, 15e, 15h	Factor serum 15h
	15C	H, S		15	15e	Factor sera 15e + 15h
Group 16	16F	D		16	16b	Factor serum 16b
	16A	D		16	16c	Factor serum 16c
Group 17	**17F**	F, S		17	17b	Factor serum 17b
	17A	F, S		17	17c	Factor serum 17c
Group 18	18F	A, Q		18	18c, 18e, 18f	Factor serum 18f
	18A	A, Q		18	18d	Factor serum 18d
	18B	A, Q		18	18e	Factor sera 18e + 18c
	18C	A, Q		18	18c, 18e	Factor sera 18c + 18f
Group 19	19F	B, P		19	19b	Factor serum 19b
	19A	B, P		19	19c	Factor serum 19c
	19B	B, P		19	7h	Factor sera 7h + 19f
	19C	B, P		19	19f, 7h	Factor serum 19f
Type 20	**20**	C, T	20			Type serum 20
Type 21	21	E	21			Type serum 21
Group 22	**22F**	F, T		22	22b	Factor serum 22b
	22A	F, T		22	22c	Factor serum 22c
Group 23	**23F**	H, Q		23	23b	Factor serum 23b
	23A	H, Q		23	23c	Factor serum 23c
	23B	H, Q		23	23d	Factor serum 23d
Group 24	24F	C		24	24d	Factor serum 24d + 24c
	24A	C		24	24c, 24d	Factor serum 24c
	24B	C		24	24e	Factor serum 24e
Group 25	25F	I		25	25b	Factor serum 25b
	25A	I		25	25c	Facor serum 25c
Type 27	27	F	27			Type serum 27
Group 28	28F	H		28	28b	Factor serum 28b
	28A	H		28	28c	Factor serum 28c
Type 29	29	G	29			Type serum 29
Type 31	31	C	31			Type serum 31
Group 32	32F	F		32	32a	Factor sera 32a + 32b
	32A	F		32	32a, 32b	Factor sera 32b

S. pneumoniae	*S. pneumoniae* type	Positive reaction in:				Antiserum/antisera recommended for identification of type
		Pool serum	Type serum	Group serum	Factor serum	
Group 33	**33F**	E, T		33	33b	Factor sera 33b + 20b
	33A	E, T		33	33b, 20b	Factor serum 20b
	33B	E, T		33	33f	Factor sera 33f + 33e + 6a
	33C	E, T		33	33e, (33f)	Factor serum 33e
	33D	E, T		33	33f, 6a	Factor serum 6a
Type 34	34	G	34			Type serum 34
Group 35*	35F	G		35	35a, 35b	Factor sera 35a + 35b
	35A	G		35	35a, 35c	Factor sera 35a + 35c + 29b + 42a
	35B	G		35	35a, 35c, 29b	Factor sera 35a + 29b
	35C	G		35	35a, 35c, 42a	Factor sera 35a + 42a
Type 36	36	D	36			Type serum 36
Type 37	37	D	37			Type serum 37
Type 38	38	I	38			Type serum 38
Type 39	39	E	39			Type serum 39
Type 40	40	C	40			Type serum 40
Group 41	41F	F		41	41a, 41b	Factor serum 41b
	41A	F		41	41a	Factor sera 41a + 41b
Type 42	42	G	42			Type serum 42
Type 43	43	I	43			Type serum 43
Type 44	44	I	44			Type serum 44
Type 45	45	I	45			Type serum 45
Type 46	46	I	46			Type serum 46
Group 47	47F	G		47	47a	Factor sera 47a + 43b
	47A	G		47	47a, 43b	Factor serum 43b
Type 48	48	I	48			Type serum 48

( ) indicates a weak positive reaction.

*All types within *S. pneumoniae* group 35 have to be identified by means of Factor serum 35a, because of cross-reactions to *S. pneumoniae* types 42 and 47F in Group antiserum 35.

S. pneumoniae types included in the 23-valent vaccine are indicated by **boldface**.

All types of *S. pneumoniae* will give a positive reaction in Omni-serum and Anti C-polysaccharide serum.

Key to Pneumococcal Factor Serum

Type[1]	Reaction in factor serum				Antigenic form
	6b	6c	6d		
6A	+	-	-		6a, 6b
6B	-	+	-		6a, 6c
6C	-	-	+		6a, 6d
6D	-	+	+		6a, 6c, 6d
	7b	7c	7e	7f	
7F	+	-	-	-	7a, 7b
7A	(+)	+	-	-	7a, 7b, 7c
7B	-	-	+	-	7a, 7d, 7e, 7h
7C	-	-	-	+	7a, 7d, 7f, 7g, 7h
	9b	9d	9e	9g	
9A	-	+	-	-	9a, 9c, 9d
9L	+	-	-	-	9a, 9b, 9c, 9f
9N	+	-	+	-	9a, 9b, 9e
9V	-	+	-	+	9a, 9c, 9d, 9g
	10b	10d	10f		
10F	+	-	-		10a, 10b
10A	-	+	-		10a, 10c, 10d
10B	+	+	-		10a, 10b, 10c, 10d, 10e
10C	+	-	+		10a, 10b, 10c, 10f
	11b	11c	11f	11g	
11F	+	-	-	+	11a, 11b, 11e, 11g
11A	-	+	-	-	11a, 11c, 11d, 11e
11B	+	-	+	+	11a, 11b, 11f, 11g
11C	+	+	+	-	11a, 11b, 11c, 11d, 11f
11D	+	+	-	-	11a, 11b, 11c, 11e
	12b	12c	12e		
12F	+	-	-		12a, 12b, 12d
12A	-	+	-		12a, 12c, 12d
12B	+	+	+		12a, 12b, 12c, 12e

Type[1]	Reaction in factor serum				Antigenic form
	15b	15c	15e	15h	
15F	+	+	-	-	15a, 15b, 15c, 15f
15A	-	+	-	-	15a, 15c, 15d, 15g
15B	+	-	+	+	15a, 15b, 15d, 15e, 15h
15C	-	-	+	-	15a, 15d, 15e
	16b	16c			
16F	+	-			16a, 16b, 11d
16A	-	+			16a, 16c
	17b	17c			
17F	+	-			17a, 17b
17A	-	+			17a, 17c
	18c	18d	18e	18f	
18F	+	-	+	+	18a, 18b, 18c, 18e, 18f
18A	-	+	-	-	18a, 18b, 18d
18B	-	-	+	-	18a, 18b, 18e, 18g
18C	+	-	+	-	18a, 18b, 18c, 18e
	19b	19c	19f	7h	
19F	+	-	-	-	19a, 19b, 19d
19A	-	+	-	-	19a, 19c, 19d
19B	-	-	-	+	19a, 19c[2], 19e, 7h
19C	-	-	+	+	19a, 19c[2], 19f, 7h
	22b	22c			
22F	+	-			22a, 22b
22A	-	+			22a, 22c
	23b	23c	23d		
23F	+	-	-		23a, 23b, 18b
23A	-	+	-		23a, 23c, 15a
23B	-	-	+		23a, 23b2, 23d
	24c	24d	24e		
24F	-	+	-		24a, 24b, 24d, 7h
24A	+	+	-		24a, 24c, 24d
24B	-	-	+		24a, 24b, 24e, 7h

Type[1]	Reaction in factor serum					Antigenic form
	25b	25c				
25F	+	-				25a, 25b
25A	-	+				25a, 25c, 38a
	28b	28c				
28F	+	-				28a, 28b, 16b, 23d
28A	-	+				28a, 28c, 23d
	32a	32b				
32F	+	-				32a, 27b
32A	+	+				32a, 32b, 27b
	33b	33e	33f	6a	20b	
33F	+	-	-	-	-	33a, 33b, 33d
33A	+	-	-	-	+	33a, 33b, 33d, 20b
33B	-	-	+	-	-	33a, 33c, 33d, 33f
33C	-	+	(+)	-	-	33a, 33c, 33e
33D	-	-	+	+	-	33a, 33c, 33d, 33f, 6a
	35a	35b	35c	29b	42a	
35F	+	+	-	-	-	35a, 35b, 34b
35A	+	-	+	-	-	35a, 35c, 20b
35B	+	-	+	+	-	35a, 35c, 29b
35C	+	-	+	-	+	35a, 35c, 20b, 42a
	41a	41b				
41F	+	+				41a, 41b
41A	+	-				41a
	47a	43b				
47F	+	-				47a, 35a, 35b
47A	+	+				47a, 43b

Vaccine types are indicated by **boldface**.

[1]*Streptococcus pneumoniae* type.

[2]Factor positive with some antisera, but do not react with the currently distributed antisera.

(+) Weak positive reaction.

ELISA

Measuring of antibodies against pneumococcal capsules is preformed to evaluate vaccine trails, science purpose or for evaluation potential re-vaccination. Several publications indicates that the 23-valent polysaccharides vaccines do not produce memory cells and antibody levels disappear over time [81]. ELISA is an abbreviation for Enzyme-Linked ImmunoSorbent Assay. The original assay used to quantify the level of circulating antibodies to pneumococcal capsules in humans was based on the Farr assay (a radioimmunoassay that measured antibody binding to radio labelled capsular CPS) [44]. However, the Farr assay was impractical to perform on large numbers of antiserum samples associated with for instance clinical trials. It consumed large volumes of antisera for each serotype, used radioactive isotopes, and was not informative relative to the isotype being elicited by the vaccine. In addition, it was not clear whether the Farr assay provided the necessary specificity [45, 46].

In the early 1980s, ELISA became the preferred method for estimating antibody concentrations, but it had to undergo several developments to end up as the method that is used today.

In studies using ELISA, results showed a poor correlation between antibody concentration and the efficacy of the vaccine.

The first generation ELISAs were later found to overestimate the true anti-capsular CPS antibody concentration. The primary reason was that the assay measured antibodies to pneumococcal cell wall polysaccharides (CWPS, teichoic acid), as well as anti-capsular CPS antibodies. This occurred because purified capsular CPS contains up to 5% (by weight) CWPS, which is covalently bound to the serotype-specific CPS via a peptidoglycan moiety [47]. In addition, most people have antibodies to CWPS, perhaps in response to pneumococcal carriage, previous infection or due to close related CWPS on *S. mitis* and *S. oralis* [48][49].

Once the problems with antibodies to CWPS were recognized, a second-generation pneumococcal ELISA was developed by taking steps to neutralize CWPS antibodies in the test antiserum samples prior to ELISA measurement [50]. Two different approaches with preadsorption were used, either with CWPS or with a cruder cell wall preparation from a non-encapsulated serotype [51]. This simple alteration resulted in better quantization of serotype-specific pneumococcal CPS antibodies and also improved the correlation of the antiserum antibody concentration with immune protection.
However, the second-generation ELISA was found to have insufficient specificity when antiserum samples from unimmunized adults were investigated.

Following the discovery that ELISA specificity could be further improved when the test antisera were preadsorbed with CWPS and an irrelevant pneumococcal capsular CPS, a third-generation ELISA was developed.

For the third-generation ELISA, the test antiserum is preadsorbed with CWPS and pneumococcal type 22F capsular CPS [52]. Serotype 22F was chosen for this purpose because the capsular PS is available and is not likely to be included in any future conjugate vaccine. After the discovery that the active part in the 22F CPS was a CWPS variant (amount of choline) a new adsorption product CWPS Multi was launched by SSI Diagnostica, which made the preadsorption step easier. [53]. Today, SSI Diagnostica has 92 CPS available in 10 mg freeze-dried vials as well as the CWPS and CWPS Multi.

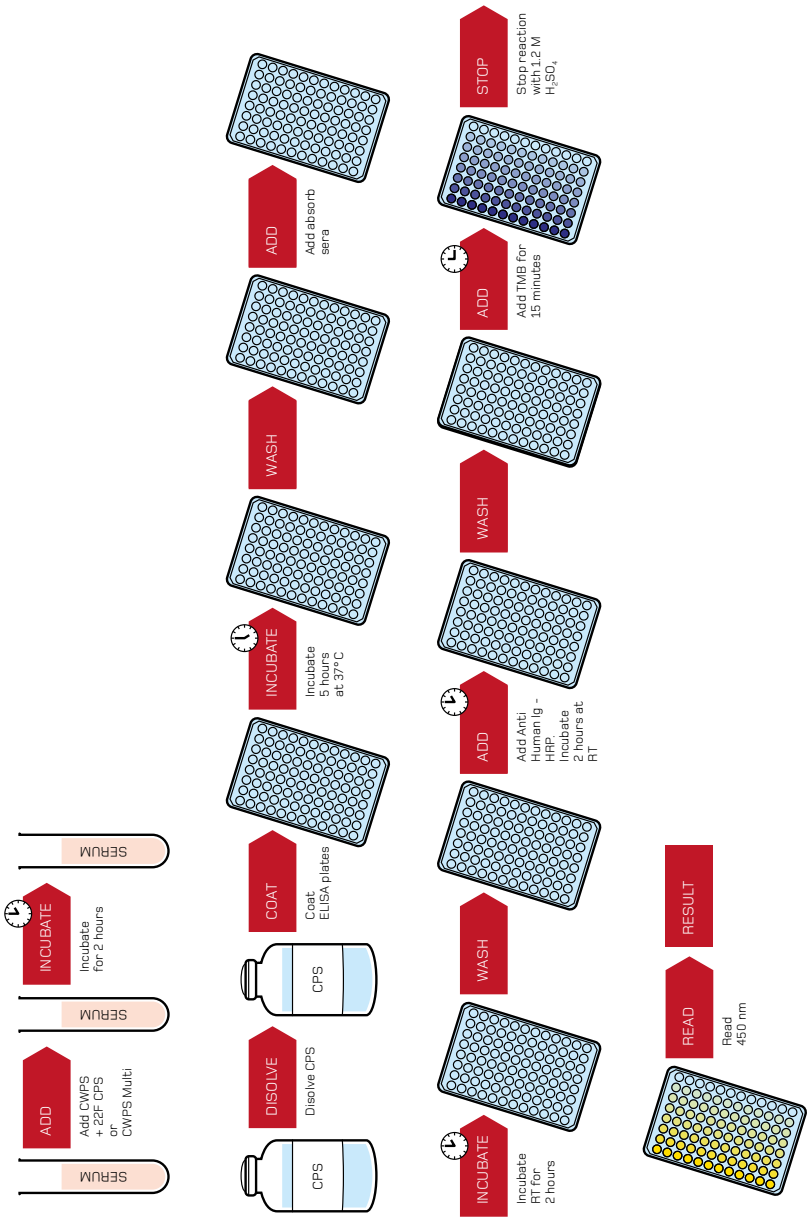


Figure 7. Overview of the third-generation ELISA.

For a more detailed protocol and WHO training manual please visit:
http://www.vaccine.uab.edu

Guidance protocol for pneumococcal antibody ELISA

The guidance procedure for the pneumococcal CPS ELISA suggests coating each well of a medium-binding microtiter plate with 100 µL of serotype specific pneumococcal CPS antigen and incubate it at 37 ºC for five hours in a humidified chamber. The coated plates are washed by soaking for 30 seconds with 1x Tris-buffered saline-0.01% Brij 35 solution (pH 7.2). After preadsorption (either with CWPS and 22F CPS or CWPS Multi) for 30 minutes, the serum specimens are serially diluted and added to the microtiter plates (50 µL per well) following a predetermined template. Some wells in the microtiter plates have no serum specimens in order to monitor nonspecific background binding in the assay. Sera are incubated in the CPS-coated microtiter plates for 2 hours at room temperature. The plates are washed as described above, and 50 µL of diluted goat anti human IgG (horseradish peroxidase or alkaline phosphatase) conjugate is added to each well.

The plates are again incubated for 2 hours and washed as previously described. Finally, the substrate is added (100 µL TMB or 100 µL of 1-mg/mL p-nitrophenyl phosphate) and the mixture is incubated for 15 minutes at room temperature. The reaction is stopped by adding 1.5M H_2SO_4 to all the wells, and the optical density is measured. Optical density data are converted to antibody concentration with a computer program like CDC ELISA, which uses a four-parameter logistic-log method to perform a curve-fitting procedure. In general, the detection limit is about 0.01 mg/L, and interassay coefficient of the variation is about 30%.

For pneumococcal standard serum "007sp" please contact:

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REFERENCES

1. Ryan KJ, Ray CG (editors) (2004). Sherris Medical Microbiology.

2. Siemieniuk, Reed A.C., Gregson, Dan B., Gill, M. John (2011). "The persisting burden of invasive pneumococcal disease in HIV patients: an observational cohort study."BMC Infectious Diseases 11: 314.

3. Dagan R (2000). "Treatment of acute otitis media—challenges in the era of antibiotic resistance." Vaccine 19 Suppl 1: S9–S16.

4. Park IH, Pritchard DG, Cartee R, Brandao A, Brandileone MC, Nahm MH. (2007) "Discovery of a new capsular serotype (6C) within serogroup 6 of *streptococcus pneumoniae*." J Clin Microbiol. 45(4):1225-33.

5. Bogaert D, De Groot R, Hermans PW. (2004) "*Streptococcus pneumoniae* colonisation: the key to pneumococcal disease." Lancet Infect Dis. 4(3):144-54.

6. Kilian M, Reinholdt J, Lomholt H, Poulsen K, Frandsen EV (1996) " Biological significance of IgA1 proteases in bacterial colonization and pathogenesis: critical evaluation of experimental evidence " APMIS. 104(5):321-38.

7. Hollingshead SK, Becker R, Briles DE. (2000) " Diversity of PspA: mosaic genes and evidence for past recombination in *streptococcus pneumoniae*." Infect Immun. 68(10):5889-900.

8. Hammerschmidt S, Bethe G, Remane PH, Chhatwal GS. (1999) " Identification of pneumococcal surface protein A as a Lactoferrin-binding protein of *streptococcus pneumoniae*." Infect Immun. 67(4):1683-7.

9. Dave S, Carmicle S, Hammerschmidt S, Pangburn MK, McDaniel LS. (2004) "Dual roles of PspC, a surface protein of *streptococcus pneumoniae*, in binding human secretory IgA and factor H." J Immunol. 1;173(1):471-7.

10. Mosser JL, Tomasz A. (1970) "Choline-containing teichoic acid as a structural component of pneumococcal cell wall and its role in sensitivity to lysis by an autolytic enzyme." J Biol Chem. 25;245(2):287-98.

11. Gilbert RJ, Jiménez JL, Chen S, Tickle IJ, Rossjohn J, Parker M, Andrew PW, Saibil HR. (1999) "Two structural transitions in membrane pore formation by pneumolysin, the pore-forming toxin of *streptococcus pneumoniae*." Cell. 28;97(5):647-55.

12. Berry AM, Lock RA, Paton JC. (1996) "Cloning and characterization of NanB, a second *streptococcus pneumoniae* neuraminidase gene, and purification of the NanB enzyme from recombinant Escherichia coli." J Bacteriol. 178(16):4854-60.

13. Said MA, O'Brien KL, Nuorti JP, Singleton R, Whitney CG, Hennessy TW. (2011) "The epidemiologic evidence underlying recommendations for use of pneumococcal polysaccharide vaccine among American Indian and Alaska native populations." Vaccine. Jul 26;29(33):5355-62.

14. Dintilhac A, Alloing G, Granadel C, Claverys JP. (1997) "Competence and virulence of *streptococcus pneumoniae*: Adc and PsaA mutants exhibit a requirement for Zn and Mn resulting from inactivation of putative ABC metal permeases." Mol Microbiol. 25(4):727-39.

15. Skov Sørensen UB, Blom J, Birch-Andersen A, Henrichsen J. (1988) "Ultrastructural localization of capsules, cell wall polysaccharide, cell wall proteins, and F antigen in pneumococci." Infect Immun. 56(8):1890-6.

16. Martin M, Turco JH, Zegans ME, Facklam RR, Sodha S, Elliott JA, Pryor JH, Beall B, Erdman DD, Baumgartner YY, Sanchez PA, Schwartzman JD, Montero J, Schuchat A, Whitney CG. (2003)."An outbreak of conjunctivitis due to atypical *streptococcus pneumoniae*." N. Engl. J. Med. 348, 1112–1121.

17. Lee CJ, Banks SD, Li JP. (1991) "Virulence, immunity, and vaccine related to *streptococcus pneumoniae*" Crit Rev Microbiol.18(2):89-114.

18. Paton JC, Morona JK (2000) "*Streptococcus pneumoniae* capsular polysaccharide." In: Rood, JI (ed) Gram-positive pathogens. America Society for Microbiology, Washington, D.C., 201-213.

19. Henrichsen J (1999) "Typing of *streptococcus pneumoniae*: past, present, and future." Am J Med. 26;107(1A):50S-54S.

20. Sørensen UB, Henrichsen J. (1984) "C-polysaccharide in a pneumococcal vaccine." Acta Pathol Microbiol Immunol Scand C. 92(6):351-6.

21. Sutliff WD, Findlan M. (1934) "Type 1 pneumococcal infections with especial reference to specific serum treatment". N. Engl. J. Med. 337:970-976.

22. Wright AE, Morgan W, Colbrook L, Dodgson RW (1914) "Observations on prophylactic inoculations against pneumococcus infections, and on the results which have been achieved by it." Lancet 1:1-10, 87-95.

23. Maynard GD (1913) "An enquiry into aetiology, manifestations and prevention of pneumonia amongst natives on the rand recruited from tropical areas" Publ. S. Afr. Inst. Med. Res. 1:1-101.

24. Felton LD (1938) "Studies on immunizing substances in pneumococci. VII. Response in human beings to antigenic pneumococcus polysaccharides, type I and II" Public Health Rep. 53:2855-2877.

25. Macleod CM, Hodges RG, Heidelberger M, Bernhard WG (1945) "Prevention of pneumococcal pneumonia by immunization with specific capsular polysaccharides." J Exp Med. 30;82(6):445-65.

26. Kaufman P (1947) "Pneumonia in old age; active immunization against pneumonia with pneumonococcus polysaccharide; results of a six year study." Arch Intern Med (Chic). 79(5):518-31.

27. Austrian R, Gold J. (1964) "Pneumococcal bacteremia with especial reference to bacteremic pneumococcal pneumonia" Ann Intern Med. 1964 May;60:759-76.

28. U.S. Congress, Office of technology assessment. A review of selected federal vaccine and immunization policies. Washington, DC: U.S. Government printing office, 1979.

29. Austrian R, Douglas RM, Schiffman G, Coetzee AM, Koornhof HJ, Hayden-Smith S, Reid RD. (1976) "Prevention of pneumococcal pneumonia by vaccination." Trans Assoc Am Physicians. 89:184-94.

30. Smit P, Oberholzer D, Hayden-Smith S, Koornhof HJ, Hilleman MR. (1977) "Protective efficacy of pneumococcal polysaccharide vaccines." JAMA. 12;238(24):2613-6.

31. Douglas RM, Paton JC, Duncan SJ, Hansman DJ. (1983) "Antibody response to pneumococcal vaccination in children younger than five years of age." J Infect Dis. 148(1):131-7.

32. Koskela M, Leinonen M, Häivä VM, Timonen M, Mäkelä PH. (1986) "First and second dose antibody responses to pneumococcal polysaccharide vaccine in infants." Pediatr Infect Dis. 5(1):45-50.

33. Leinonen M, Säkkinen A, Kalliokoski R, Luotonen J, Timonen M, Mäkelä PH. (1986) "Antibody response to 14-valent pneumococcal capsular polysaccharide vaccine in preschool age children." Pediatr Infect Dis. 5(1):39-44.

34. White B (1979) "The biology of pneumococcus" Harvard University press.

35. Austrian R. (1976) "The quellung reaction, a neglected microbiologic technique." Mt Sinai J Med. 43(6):699-709.

36. Lund E, Rasmussen P. (1966) "Omni-serum. A diagnostic pneumococcus serum, reacting with the 82 known types of pneumococcus." Acta Pathol Microbiol Scand. 68(3):458-60.

37. Lund E, Henrichsen J (1978) "Laboratory diagnosis, serology and epidemiology of *streptococcus pneumoniae*" Methods in Microbiology (1978) Vol: 12, Issue: 12, 241-262.

38. Gruber M, Durham HE.(1896) "Eine neue methode zur raschen erkennung des choleravibrio und des typhusbacillus." Münchener medicinische Wochenschrift, 1896, 43: 285-286.

39. Widal F (1896) "Sérodiagnostic de la fièvre typhoide. " Bulletins et Mémoires de la Société Médicale des Hôpitaux de Paris, 13: 681-682.

40. Landsteiner K (1900) "Bur kennie red argumentativeness, fleischer undo agglomeration workings eds blusters fund deer nymphet." Centralblatt f. Bakteriologie, Parasitenkunde u. Infektionskrankheiten, vol. 27 357-362.

41. Plotz CM, Singer JM (1956) "The latex fixation test. I. Application to the serologic diagnosis of rheumatoid arthritis."Am J Med. 21(6):888-92.

42. Slotved HC, Kaltoft M, Skovsted IC, Kerrn MB, Espersen F. (2004) "Simple, rapid latex agglutination test for serotyping of pneumococci (Pneumotest-Latex)." J Clin Microbiol. 42(6):2518-22.

43. Sørensen UB.(1993) "Typing of pneumococci by using 12 pooled antisera." J Clin Microbiol. 31(8):2097-100.

44. Schiffman G, Douglas RM, Bonner MJ, Robbins M, Austrian R. (1980) "A radioimmunoassay for immunologic phenomena in pneumococcal disease and for the antibody response to pneumococcal vaccines. I. Method for the radioimmunoassay of anticapsular antibodies and comparison with other techniques." J Immunol Methods. 33(2):133-44.

45. Musher DM, Watson DA, Baughn RE. (1990) "Does naturally acquired IgG antibody to cell wall polysaccharide protect human subjects against pneumococcal infection?" J Infect Dis. 161(4):736-40.

46. Nahm MH, Siber GR, Olander JV. (1996) "A modified farr assay is more specific than ELISA for measuring antibodies to *streptococcus pneumoniae* capsular polysaccharides." J Infect Dis. 173(1):113-8.

47. Sørensen UB, Henrichsen J, Chen HC, Szu SC. (1990) "Covalent linkage between the capsular polysaccharide and the cell wall peptidoglycan of *streptococcus pneumoniae* revealed by immunochemical methods." Microb Pathog. 8(5):325-34.

48. Frasch CE, Concepcion NF. (2000) "Specificity of human antibodies reactive with pneumococcal C polysaccharide." Infect Immun. 68(4):2333-7.

49. Koskela M. (1987) "Serum antibodies to pneumococcal C polysaccharide in children: response to acute pneumococcal otitis media or to vaccination." Pediatr Infect Dis J. 6(6):519-26.

50. Konradsen HB, Sørensen UB, Henrichsen J. (1993) "A modified enzyme-linked immunosorbent assay for measuring type-specific anti-pneumococcal capsular polysaccharide antibodies." J Immunol Methods. 26;164(1):13-20.

51. Quataert SA, Kirch CS, Wiedl LJ, Phipps DC, Strohmeyer S, Cimino CO, Skuse J, Madore DV. (1995) "Assignment of weight-based antibody units to a human antipneumococcal standard reference serum, lot 89-S." Clin Diagn Lab Immunol. 2(5):590-7.

52. Concepcion NF, Frasch CE. (2001) "Pneumococcal type 22f polysaccharide absorption improves the specificity of a pneumococcal-polysaccharide enzyme-linked immunosorbent assay. " Clin Diagn Lab Immunol. 8(2):266-72.

53. Skovsted IC, Kerrn MB, Sonne-Hansen J, Sauer LE, Nielsen AK, Konradsen HB, Petersen BO, Nyberg NT, Duus JØ. (2007) "Purification and structure characterization of the active component in the pneumococcal 22F polysaccharide capsule used for adsorption in pneumococcal enzyme-linked immunosorbent assays." Vaccine. 25(35):6490-500.

54. Torzillo PJ, Hanna JN, Morey F, Gratten M, Dixon J, Erlich, SourceDepartment of Respiratory Medicine, Royal Prince Alfred Hospital, Camperdown, NSW. (1995) "Invasive pneumococcal disease in central Australia." J.Med J Aust. 1995 Feb 20;162(4):182-6.

55. Nair GB, Niederman MS (2011). "Community-acquired pneumonia: an unfinished battle". The Medical clinics of North America 95 (6): 1143–61.

56. Murray and Nadel (2010). Chapter 32. Mason: Murray and Nadel's Textbook of Respiratory Medicine, 5th ed. Copyright © 2010 Saunders, An Imprint of Elsevier

57. Hoare Z, Lim WS (2006). "Pneumonia: update on diagnosis and management" (PDF). BMJ 332 (7549): 1077–9.

58. Singh V, Aneja, S (March 2011). "Pneumonia — management in the developing world". Paediatric respiratory reviews 12 (1): 52–9.

59. Lim WS, Baudouin SV, George RC, Hill AT, Jamieson C, Le Jeune I, Macfarlane JT, Read RC, Roberts HJ, Levy ML, Wani M, Woodhead MA, Pneumonia Guidelines Committee of the BTS Standards of Care, Committee (2009). "BTS guidelines for the management of community acquired pneumonia in adults: update 2009". Thorax 64 (Suppl 3): iii1–55.

60. Helms, editors, William E. Brant, Clyde A. Fundamentals of diagnostic radiology (4th ed.). Philadelphia: Wolters Kluwer/Lippincott Williams & Wilkins. p. 435. ISBN 9781608319114.

61. Sharma S, Maycher B, Eschun G (May 2007). "Radiological imaging in pneumonia: recent innovations". Current Opinion in Pulmonary Medicine 13 (3): 159–69. doi:10.1097/MCP.0b013e3280f3bff4. PMID 17414122.

62. Fennel EA. Prophylactic inoculation against pneumonia: a brief history and the present status of the procedure. JAMA 1918; 71: 2115–2130.

63. Mandell LA, Wunderink RG, Anzueto A, Bartlett JG, Campbell GD, Dean NC, Dowell SF, File TM Jr, Musher DM, Niederman MS, Torres A, Whitney CG, Infectious Diseases Society of America; American Thoracic Society (2007). "Infectious Diseases Society of America/American Thoracic Society consensus guidelines on the management of community-acquired pneumonia in adults". Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 44 (Suppl 2): S27–72.

64. Ruuskanen O, Lahti E, Jennings LC, Murdoch, DR (2011-04-09). "Viral pneumonia". Lancet 377 (9773): 1264–75. doi:10.1016/S0140-6736(10)61459-6. PMID 21435708 11) Leach, Richard E. (2009). Acute and Critical Care Medicine at a Glance (2nd ed.). Wiley-Blackwell. ISBN 1-4051-6139-6. Retrieved 2011-04-21.

65. Henrichsen, J. "The pneumococcal typing system and pneumococcal surveillance." Journal of Infection,Volume 1, Supplement 2, September 1979, Pages 31–34, IN1, 35–37.

66. Dochez, A. R and Avery O.T. J Exp Med., 26, 477 (1917)

67. Donaldson, John D. "Middle ear, acute otitis media, medical treatment." eMedicine. Eds. Carol A. Bauer, et al. 28 Sep. 2009

68. Lieberthal AS, Carroll AE, Chonmaitree T, Ganiats TG, Hoberman A, Jackson MA, Joffe MD, Miller DT, Rosenfeld RM, Sevilla XD, Schwartz RH, Thomas PA, Tunkel, DE (Feb 25, 2013). "The diagnosis and management of acute otitis media.". Pediatrics 131 (3): e964–99

69. Owen MJ, Baldwin CD, Swank PR, Pannu AK, Johnson DL, Howie VM (1993). "Relation of infant feeding practices, cigarette smoke exposure, and group child care to the onset and duration of otitis media with effusion in the first two years of life". J. Pediatr. 123 (5): 702–11

70. Sáez-Llorens X, McCracken GH (June 2003). "Bacterial meningitis in children". Lancet 361 (9375): 2139–48

71. Tunkel AR, Hartman BJ, Kaplan SL et al. (November 2004). "Practice guidelines for the management of bacterial meningitis". Clinical Infectious Diseases 39 (9): 1267–84

72. Van de Beek D, de Gans J, Spanjaard L, Weisfelt M, Reitsma JB, Vermeulen M (October 2004). "Clinical features and prognostic factors in adults with bacterial meningitis" .The New England Journal of Medicine 351 (18): 1849–59

73. Attia J, Hatala R, Cook DJ, Wong JG (July 1999). "The rational clinical examination. Does this adult patient have acute meningitis?". Journal of the American Medical Association 282 (2): 175–81

74. Leach, Richard E. (2009). "Acute and critical care medicine at a glance (2nd ed.). Wiley-Blackwell. ISBN 1-4051-6139-6. Retrieved 2011-04-21.

75. Mandell LA et al. (2007) "Infectious diseases society of america/american thoracic society consensus guidelines on the management of community-acquired pneumonia in adults." Clinical Infectious Diseases 44: S27–72.

76. Woodhead et al. (2014) "NICE guideline – pneumonia: diagnosis and management of community- and hospital-acquired pneumonia in adults." DRAFT FOR CONSULTATION.

77. Harboe ZB, Benfield TL, Valentiner-Branth P, Hjuler T, Lambertsen L, Kaltoft M, Krogfelt K, Slotved HC, Christensen JJ, Konradsen HB. (February 2010). "Temporal trends in invaise pneumoccocal disease and pneumococcal serotypes over 7 decades." Clin Infect Dis. 50(3): 329.37.

78. Harboe ZB, Thomsen RW, Riis A, Valentiner-Branth P, Christensen JJ, Lambertsen L, Krogfelt KA, Konradsen HB, Benfield TL. (2009). "Pneumococcal serotypes and mortality following invasive pneumococcal disease: a populaton-based cohort study." PLoS Med. 6(5):e100081. doi: 10.1371/journal.pmed.1000081. Epub 2009 May 26.

79. Harboe ZB, Slotved HC, Konradsen HB, Kaltoft MS. (May 2012). "A pneumococcal carriage study in danish pre-school children before the introduction of pneumococcal conjugate vaccination." Open Microbiol J. 6;40-4. Epub 2012 May 4.

80. J. G. Liese, S. A. Silfverdal, C. Giaquinto, A. Carmona, J. H. Larcombe, J. Garcia-Sicilia, A. Fuat, M. Garces-Sanchez, M. L. Arroba Basanta, E. Muñoz Hiraldo, L. Cantarutti, W. Kroeniger, J. Vollmar, K. Holl, J. Y. Pircon and M. R. Rosenlund (2014). "Incidence and clinical presentation of acute otitis media in children aged <6 years in European medical practices." Epidemiol Infect. 142(8): 1778-1788.

81. Konradsen HB. "Quantity and avidity of pneumococcal antibodies before and up to five years after pneumococcal vaccination of elderly persons." Clin Infect Dis. 1995 Sep;21(3):616-20.

82. Colin J. McWilliams, Ran D. Goldman. "Update on acute otitis media in children younger than 2 years of age." Can Fam Physician. 2011 November; 57(11): 1283–1285.

83. Colin J. McWilliams, Christine H. Smith, Ran D. Goldman. "Acute otitis externa in children." Can Fam Physician. 2012 November; 58(11): 1222–1224.

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DIAGNOSTICS		
ImmuView® *S. pneumoniae* and *L. pneumophila* Urinary Antigen Test		Lateral Flow Test
Omni Antiserum	92 serotypes	Capsular Reaction Test (Neufeld)
ImmuLex™ *S. pneumoniae* Omni	92 serotypes	Latex Agglutination
TYPING MATERIAL		
Pneumococcus Pool Antisera	A-I, P-T	Capsular Reaction Test (Neufeld)
Pneumococcus Pool Antisera	Latex Pool A-I, P-T	Latex Agglutination
Pneumococcus Group Antisera	Group 6, 7, 9-12, 15-19, 22-25, 28, 32, 33, 35, 41, 47	Capsular Reaction Test (Neufeld)
Pneumococcus Type Antisera	1-5, 8, 13, 14, 20, 21, 27, 28, 29, 31, 34, 36, 37, 38, 39, 40, 42, 43, 44, 45, 46, 48	Capsular Reaction Test (Neufeld)
Pneumococcus Type Antisera	Latex Type 1, 3, 4, 5, 14	Latex Agglutination
Pneumococcus Factor Antisera	Latex Factor 6b, 6c, 7b, 9g, 18c, 18f, 19b, 19c, 23b	Latex Agglutination
Pneumococcus Factor Antisera	Factor 6a, 6b, 6c, 6d, 7b, 7c, 7e, 7f, 7h, 9b, 9d, 9e, 9g, 10b, 10d, 10f, 11b, 11c, 11f, 11g, 12b, 12c, 12e, 15b, 15c, 15e, 15h, 16b, 16c, 17b, 17c, 18c, 18d, 18e, 18f, 19b, 19c, 19f, 20b, 22b, 22c, 23b, 23c, 23d, 24c, 24d, 24e, 25b, 25c, 28b, 28c, 29b, 32a, 32b, 33b, 33e, 33f, 35a, 35b, 35c, 41a, 41b, 42a, 43b, 47a	Capsular Reaction Test (Neufeld)
Pneumococcus Anti CWPS		
ImmuLex™ Pneumotest Kit		Latex Agglutination
ImmuLex™ Pneumococcus 7-10-13-Valent Kit		Latex Agglutination
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