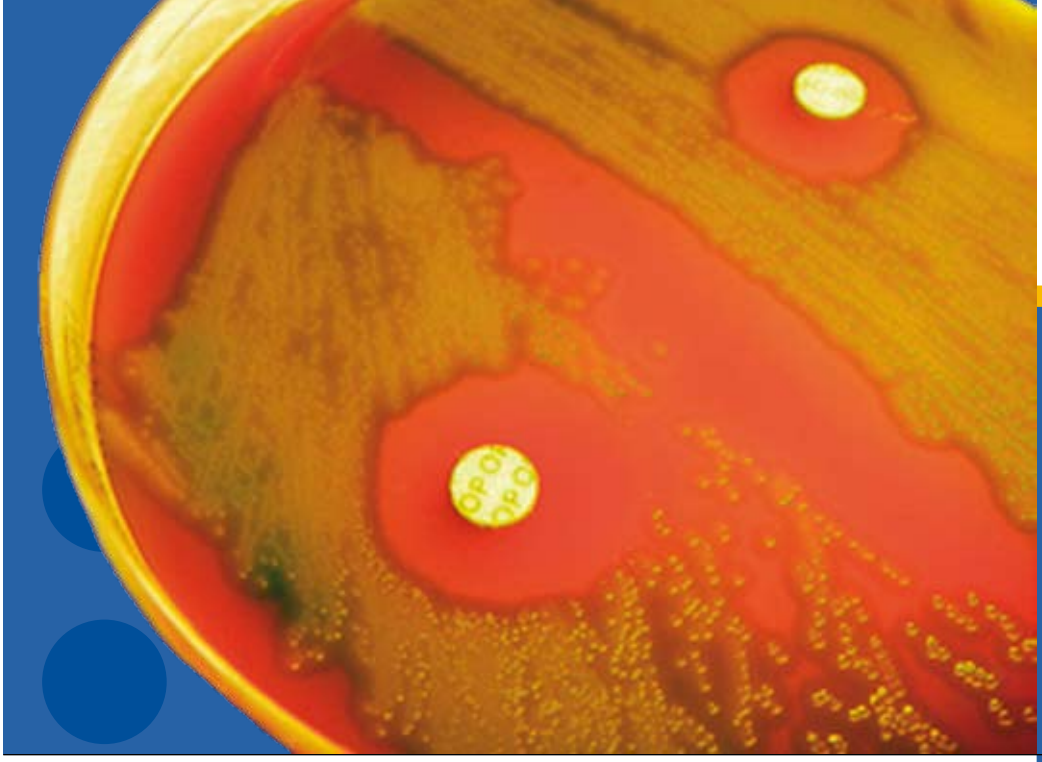

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Oulu 2006

Tarja Kaijalainen

THE IDENTIFICATION OF *STREPTOCOCCUS PNEUMONIAE*

A C A D E M I C D I S S E R T A T I O N

To be presented with the permission of the Faculty of Medicine, University of Oulu, for public examination in the Auditorium of Kastelli Research Center (Aapistie 1), on October 20th, 2006, at 12 noon.

National Public Health Institute, Department of Viral Diseases and Immunology, Oulu, Finland

and

University of Oulu, Faculty of Medicine, Department of Medical Microbiology

and

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ABSTRACT

Streptococcus pneumoniae, pneumococcus, is an important human pathogen that causes both serious invasive infections, such as septicaemia, meningitis and pneumonia, as well as mild upper respiratory infections. It also belongs to the normal nasopharyngeal microbial flora. The purpose of this study was to compare bacteriological phenotypic methods with genetechnological methods in the identification of pneumococci, especially among suspect pneumococcal isolates lacking one or more typical characteristics. In addition, we evaluated the usefulness of an STGG medium for the transport and storage of nasopharyngeal specimens and nasopharyngeal bacterial isolates.

α-hemolytic streptococcal isolates identified as pneumococcus and isolates suspected to be pneumococci were used in the comparisons of identification methods. *S. pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* were used to test the survival of these bacteria in the STGG medium. Several phenotypic (optochin sensitivity, bile solubility, capsular quellung reaction) and genetechnological (a PCR based on virulence genes *ply*, *psaA* and l*ytA*; a real-time *ply* PCR combined with melting curve analysis; AccuProbe™ and ARDRA) methods were used. A bacterial culture was used to detect bacteria in the STGG medium.

A typical *S. pneumoniae* isolate is an optochin-sensitive, capsulated or unencapsulated, bile-soluble α-hemolytic streptococcus. An optochin sensitivity test is a basic method for identifying pneumococcus, even from nasopharyngeal specimens. Genetechnological methods based on several pneumococcal virulence genes provide new possibilities for identifying *S. pneumoniae*, but their use may lead to misidentification of some α-hemolytic streptococci as pneumococcus. In the present study, a real-time *ply* PCR confirmed by melting curve analysis of amplification products could exclude false positive *ply* PCR results. The STGG medium was confirmed to be a suitable medium for deep-freeze storage and transport of nasopharyngeal specimens and isolates of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*.

In conclusion, an optochin sensitivity test is still the principal method for differentiating pneumococcus from other α-hemolytic streptococci. The presence of pneumococcus-like bacteria complicates identification. Differentiation between pneumococcus and suspected pneumococcal isolates was found to be problematic also when using genetechnological methods. A real-time *ply* PCR combined with melting curve analysis of amplification products differentiates pneumococcus from other α-hemolytic streptococci. The definition of *S. pneumoniae* and the search for a method to use as a "gold standard" for pneumococcal identification are under abundant research.

Keywords: *Streptococcus pneumoniae*, pneumococcus, identification, optochin sensitivity, real-time *ply* PCR combined with melting curve analysis, STGG medium

Kaijalainen, Tarja, Identification of *Streptococcus pneumoniae*

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TIIVISTELMÄ

Streptococcus pneumoniae, pneumokokki, aiheuttaa sekä vakavia invasiivisia infektioita kuten sepsistä (”verenmyrkytys”), aivokalvontulehdusta ja keuhkokuumetta että lieviä ylähengitysteiden tulehduksia. Pneumokokki kuuluu myös nenänielun normaaliin bakteerikasvustoon. Tässä tutkimuksessa verrattiin pneumokokin ilmiasuun perustuvia tunnistusmenetelmiä geeniteknologisiin menetelmiin. Lisäksi arvioitiin, soveltuuko STGG elatusaine nenänielunäytteiden ja niistä eristettyjen bakteerien kuljettamiseen ja säilyttämiseen.

Tutkimusaineisto koostui pneumokokiksi tunnistetuista ja pneumokokiksi epäillyistä (bakteerilta puuttuu yksi tai useampi pneumokokille tyypillisistä ominaisuuksista) α-hemolyyttisistä streptokokkikannoista. *S. pneumoniae*, *Haemophilus influenzae* ja *Moraxella catarrhalis* kantoja käytettiin tutkittaessa ko. bakteerien säilymistä STGG elatusaineessa. Tutkimuksessa käytettiin useita erilaisia bakteerin ilmiasuun perustuvia (optokiiniherkkyys, sappiliukoisuus, kapselin turpoaminen) sekä geeniteknologisia (virulenssigeenejä *ply*, *psaA* ja l*ytA* osoittavat PCR-menetelmät, reaali-aikainen *ply* PCR yhdistettynä sulamiskäyräanalyysiin, AccuProbe™ ja ARDRA) tunnistusmenetelmiä. Bakteerit osoitettiin STGG elatusaineesta bakteeriviljelyllä.

Tyypillinen pneumokokki on optokiinille herkkä, kapselillinen tai kapseliton, sappeen liukeneva α-hemolyyttinen streptokokki. Pneumokokki tunnistetaan useimmiten luotettavasti optokiiniherkkyystestillä. Molekyylimikrobiologiset menetelmät, jotka perustuvat pneumokokin virulenssigeenien osoittamiseen, tarjoavat uusia mahdollisuuksia tunnistamiseen. Muutkin α-hemolyyttiset streptokokit voivat siepata pneumokokin virulenssigeenejä, ja tämä voi johtaa näiden α-hemolyyttisten streptokokkien väärään tunnistamiseen pneumokokeiksi. Omien tutkimustemme mukaan α-hemolyyttisten streptokokkien ja pneumokokin *ply* geenit poikkeavat toisistaan ja väärät positiiviset *ply* PCR tulokset voitiin sulkea pois reaaliaikaisella *ply* PCR-menetelmällä yhdistettynä monistustuotteiden sulamiskäyräanalyysiin, jolla osoitettiin *ply* geenin mutaatiot. STGG elatusaine soveltui hyvin nenänielunäytteiden ja *S. pneumoniae*, *H. influenzae* ja *M. catarrhalis* kantojen kuljettamiseen ja säilyttämiseen.

Yhteenvetona voidaan todeta, että optokiinitesti on edelleen perusmenetelmä, jolla pneumokokki erotetaan muista α-hemolyyttisistä streptokokeista. Pneumokokkia läheisesti muistuttavien bakteerien tunnistaminen ja niiden erottaminen pneumokokista oli ongelmallista myös geeniteknologisilla menetelmillä. Reaaliaikainen *ply* PCR monistustuotteiden sulamiskäyräanalyyseineen erotti pneumokokin muista α-hemolyyttisistä streptokokeista. Pneumokokin määritelmä ja pneumokokin tunnistuksen “kultaisena standardina” toimivan menetelmän etsintä ovat edelleen tärkeitä tutkimuskohteita.

Avainsanat: *Streptococcus pneumoniae*, pneumokokki, tunnistaminen, optokiiniherkkyys, reaaliaikainen *ply* PCR yhdistettynä monistustuotteiden sulamiskäyräanalyysiin, STGG elatusaine

ABBREVIATIONS

AccuProbe™	identification kit for *S.pneumoniae*
AFLP	amplified fragment length polymorphism
ATCC	American Type Culture Collection
AOM	acute otitis media
API 20 STREP	commercial, biochemical identification kit
ARDRA	amplified rDNA restriction analysis
ATP	adenotriphosphate
bp	base pair
CIEP	counterimmunoelectrophoresis
CO_2	carbon dioxide
cpsA	capsule locus A gene
cpsB	capsule locus B gene
DE	Dorset egg medium
DNA	deoxyribonucleic acid
ETA	egg-thioglycolate-antibiotic medium
H_2O_2	hydrogen peroxide
Kb	kilobase pair (1000 base pairs)
kDa	kiloDalton
KTL	Kansanterveyslaitos, National Public Health Institute
LAP	leucine aminopeptidase
lytA	pneumococcal autolysin
MLEE	multilocus enzyme electrophoresis
MLST	multilocus sequence typing
NaCl	natriumsodium chloride
NE-1530	antiadhesive oligosaccharide 3'-sialyllacto-N-neotetraose
NP	nasopharyngeal

PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
psaA	pneumococcal surface antigen A
pspA	pneumococcal surface protein A
PBP	penicillin binding protein
ply	pneumolysin
Pnc	pneumococcus
PYR	pyrrolidonyl arylamidase
R6	genome-sequenced unencapsulated pneumococcal isolate
RapID STR system	commercial, biochemical identification kit
RFLP	restriction fragments length polymorphism
RLU	reflective light unit
rRNA	ribosomal ribonucleic acid
SGG	skim milk, glucose, glycerol
sodA	gene for MnSOD (manganese superoxide dismutase)
SpxB	pyruvate oxidase
SSI	Statens Serum Institut
STGG	skim milk, tryptone, glucose, glycerol
ST	sequence type
TIGR	genome-sequenced serotype 4 pneumococcal isolate
WHO	World Health Organization

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:

I Kaijalainen T, Rintamäki S, Herva E, and Leinonen M (2002) Evaluation of gene-technological and conventional methods in the identification of *Streptococcus pneumoniae.* J. Microbiol. Methods. 51: 111-118.

II Verhelst R, Kaijalainen T, De Baere T, Verschraegen G, Claeys G, Van Simaey L, De Ganck C, and Vaneechoutte M (2003) Comparison of five genotypic techniques for identification of optochin-resistant pneumococcus-like isolates. J. Clin. Microbiol. 41: 3521-3525.

III Kaijalainen T, Saukkoriipi A, Bloigu A, Herva E, and Leinonen M (2005) Real-time pneumolysin polymerase chain reaction with melting curve analysis differentiates pneumococcus from other α-hemolytic streptococci. Diagn. Microbiol. Infect. Dis. 53: 293-299.

IV Kaijalainen T, Ruokokoski E, Ukkonen P, and Herva E (2004) Survival of *Streptococcus pneumoniae, Haemophilus influenzae*, and *Moraxella catarrhalis* frozen in skim milk-tryptone-glucose-glycerol medium. J. Clin Microbiol. 42: 412-414.

Some previously unpublished data are also presented.

CONTENTS

1 INTRODUCTION

Streptococcus pneumoniae, pneumococcus, is an important pathogen that causes both serious invasive infections, such as septicaemia, meningitis and pneumonia, and mild upper respiratory infections. It belongs to the normal nasopharyngeal microbial flora that consists of bacteria with physiologic and genetic properties suitable for colonization and multiplication under certain conditions. These microbes are usually harmless and they even benefit human health by preventing the growth of more pathogenic bacteria.

Pneumococcus was described the first time over 130 years ago. In spite of the development of new possibilities to examine pneumococcus, the traditional phenotypic definition of *S. pneumoniae* has not changed. Pneumococcus is a gram-positive, α-hemolytic, bile-soluble and commonly capsulated streptococcus that is usually identified without problems. Identification is based on the bacterial colony morphology on a blood agar plate, optochin sensitivity, bile solubility and the presence of a capsule. So far, 90 different capsular serotypes have been identified (see White 1938, Kauffman *et al.* 1940, Mørch 1943, Lund 1970, Lund *et al.* 1972, Austrian *et al.* 1985, Henrichsen 1995). In addition, unencapsulated isolates are rather common in the nasopharynx (Finland & Barnes 1977, Carvalho *et al.* 2003).

S. pneumoniae belongs to the bacterial species that develop natural competence via transformation (Lorenz & Wackernagel 1994), which is an important mechanism allowing pneumococcus to adapt to environmental changes. Commensal species, especially other α-hemolytic streptococci, have a major role in the evolution of *S. pneumoniae*, and exchanges of virulence factors such as pneumolysin and autolysin occur among streptococcal species (Whatmore *et al.* 2000, Jado *et al.* 2001). Molecular taxonomic studies have increased information on bacterial relationships, and during the last decades have brought changes to the classification of the genus *Streptococcus* (van Belkum *et al.* 2001, Facklam 2002).

Identification of suspected pneumococcal isolates lacking one or more of the typical characteristics was found to be problematic in the National Reference Laboratory for Pneumococcus (National Public Health Institute, Oulu) that receives streptococcal isolates suspected of being pneumococci for confirmation of identification and serotyping. More specifically, the occurrence of optochin-resistant pneumococcal isolates (Kontiainen & Sivonen 1987, Pikis *et al.* 2001) and unencapsulated pneumococci, especially in the nasopharynx, creates a need to compare phenotypic identification methods with methods based on gene technology and to develop new identification methods. In addition, to analyze the clonal spread of bacteria during epidemics and to identify antibiotic-resistant or otherwise virulent clones, molecular typing methods are needed to characterize bacterial pathogens such as pneumococcus below the species level.

2 REVIEW OF THE LITERATURE

2.1 *Streptococcus pneumoniae*

2.1.1 Pneumococcal infections and epidemiology, treatment and prevention

Infections caused by *S. pneumoniae* can be divided into two categories. In invasive infections (e.g. meningitis, pneumonia and bacteraemia), pneumococcus can be isolated from blood or other normally sterile body fluids. In mucosal infections such as sinusitis, otitis media and conjunctivitis, pneumococcus can be isolated from mucosal excretions only (Musher 1992, Feldman & Klugman 1997, Bogaert *et al.* 2004).

The spectrum of pneumococcal diseases differs in different age groups and different populations (Musher 1992, O'Brien & Santosham 2004, Bogaert *et al.* 2004, Hausdorff *et al.* 2005). Several risk factors for pneumococcal infection, such as age, race, immunodeficiency, other illness, socio-economic status, previous antibiotic therapy and day-care attendance have been reported (O'Brien & Santosham 2004). Pneumococcus is a leading pathogen, causing infections with high mortality and morbidity (Austrian 1977, Scott *et al.* 1996, Hausdorff *et al.* 2000a, Hausdorff *et al.* 2000b, O'Brien & Santosham 2004). At least one million children die annually from pneumococcal diseases, and most of them are young children in developing countries (WHO 1999, Williams *et al.* 2002). Invasive pneumococcal infections also occur in industrialized countries, especially among children and elderly people (Eskola *et al.* 1992, Scott *et al.* 1996, Sankilampi *et al.* 1997, Hausdorff *et al.* 2005). In Finland, *S. pneumoniae* was among the six most common microbes isolated from invasive infections in all age groups during 1995-2004 (Kansanterveyslaitos 2005).

Acute otitis media (AOM) is one of the most frequent diagnoses in children under 15 years of age in the United States (Schappert 1992, Berman 1995, O'Brien & Santosham 2004). In Finland, the estimated number of otitis media episodes among Finnish children has increased from 200,000 per year in 1982 to 500,000 per year in 1997 (Kontiokari 1998). *S. pneumoniae* is the most common pathogen isolated from middle ear fluid in patients with AOM (Luotonen *et al.* 1981, Bluestone *et al.* 1992, Kilpi *et al.* 2001).

Quinine and its derivate, ethylhydrocupreine, was found to protect mice against pneumococcal infection, but unfortunately in therapeutic doses it was toxic to humans (see White 1938). Serum therapy used in the 1920s to treat pneumococcal infection was replaced by sulfonamides in the 1930s and later by penicillin and other antibiotics (Watson *et al.* 1993). Wide antibiotic use has led to the appearance of multidrug resistant pneumococcal isolates and difficulties in treating pneumococcal infections. In the United States, approximately 24 % of isolates of invasive pneumococcal infections have been reported to be penicillin-nonsusceptible (O'Brien & Santosham 2004). In Finland the corresponding number was 9.6 % in 2004, and multidrug resistance was 3.7 % (Kansanterveyslaitos 2005).

Pneumococcal vaccines have been developed to prevent pneumococcal infections. The first vaccination attempts were made among South African gold miners by using whole pneumococcal cells and later in young men by using capsular polysaccharides of types 1, 2, 5 and 7. In 1977, 14-valent and in 1983, 23-valent polysaccharide vaccines were introduced (Watson *et al.* 1993, WHO 1999, O'Brien & Santosham 2004). Unfortunately, polysaccharide vaccines were not immunogenic in young children, the elderly and immunocompromised patients, i.e. in people who are considered to be at a high risk for life-threatening pneumococcal infection. Later, new second-generation vaccines, 7-, 9- and 11-valent polysaccharide protein conjugate vaccines, have been developed (Klein & Eskola 1999, O'Brien & Santosham 2004, Peltola *et al.* 2004). These conjugate vaccines seem to work best in infants and high-risk groups (Klein & Eskola 1999, Whitney *et al.* 2003, O'Brien & Santosham 2004). According to Cutts and co-workers, pneumococcal conjugate vaccine is a valuable tool for preventing pneumonia, bacteraemia and mortality in African children (Cutts *et al.* 2005). Unfortunately, their effect against otitis media is limited (Eskola *et al.* 2001, O'Brien & Santosham 2004), and replacement into nonvaccine serotypes occurs (Dagan 2004, Bogaert *et al.* 2004). Next-generation vaccines, pneumococcal protein vaccines based on different pneumococcal virulence factors, such as pneumococcal surface protein A (PspA), pneumococcal surface antigen A (PsaA) and pneumolysin (Ply), are under development (Bogaert *et al.* 2004).

2.1.2 History of pneumococcus

The history of pneumococcus starts as early as 1875, when Klebs found nonmotile, sometimes linked “monads” when searching for the cause of pneumonia and examining fluids from the lungs of pneumonia patients with a microscope (reviewed by White 1938). This finding led to subsequent pneumococcal research during the following decades. In 1881, Sternberg in the USA and Pasteur in France independently inoculated rabbits with saliva and showed the pathogenicity of the organism (see Table 1). However, Pasteur was the first to report his finding, and he was also able to isolate the organism from infected rabbits by cultivation (see White 1938).

After the discovery of pneumococcus, its name has changed several times. It was named *Microbe septicemique du salive* by Pasteur and *Micrococcus pasteuri* by Sternberg. After having the names *Pneumococcus* and *Diplococcus pneumoniae*, the organism was given its present name, *Streptococcus pneumoniae*, in 1974 according to its characteristic property of growing as chains of cocci in a liquid media (Bergey *et al.* 1974).

Austrian (Austrian 1981) states in his review “Pneumococcus: The First One Hundred Years”: “It is not possible within the time allotted to review all that is known of the biology of the pneumococcus and of the diseases it causes in humans”. Pneumococcus has been involved in many historical findings concerning microbes, microbial pathogenesis and host defence. A new era in the research on pneumococcus started when the genome of pneumococcus was published (Hoskins *et al.* 2001, Tettelin *et al.* 2001). The most

significant findings related to identification of pneumococcus are summarized in Table 1. Two recently published handbooks, "The Pneumococcus" edited by Tuomanen, Mitchell, Morrison and Spratt (Tuomanen 2004) and "Streptococcus pneumoniae, Molecular Biology and Mechanism of Disease" edited by Tomasz (Tomasz 2000), present detailed facts on pneumococcal research in excellent chapters.

Table 1. *History of the identification and characterization of pneumococcus.*

Year	Description of phenomenon	Reference
1875	Nonmotile,linked "monads" in fluid in lungs of pneumonia patients.	Klebs (in White 1938)
1880	Nonmotile,slightly oval, nearly round "bodies" occuring singly but more often in pairs, considered as varieties of diphtheria or pyemia micrococci.	Eberth (in White 1938)
1880	Cocci found in sputum of pneumonia patients and in normal sputum, and named *Pneumoniekokken.*	Matray (in White 1938)
1881	Rabbits inoculated with Sternberg's saliva developed fatal septicemia. The first time pneumococcus was isolated through animal passage.	Sternberg (in White 1938)
1881	Rabbits inoculated with saliva of a child dead from rabies. "New virus", when transferred from infected to normal rabbits, caused infections each time.	Pasteur (in White 1938)
1881	Lance-shaped diplococci found from pneumonic lung tissue.	Koch (in White 1938)
1882	In stain preparations of lung puncture, cocci surrounded by an unstained rim. First observation of the capsule.	Günther (in White 1938)
1883	Micrococci in alveolar exudate stained by Gram stain exhibited the well-defined capsule. After cultivation on Koch`s coagulated blood serum, a colony description typical to pneumococcus. Coccus lost its capsule after several transfers and grew in "nail-form" colonies.	Friedländer (in White 1938)
1884	"Indispensable stain". Slightly elongated cocci with dark aniline-gentian violet (gram-positive) easier to find in lung tissues.	Gram (in White 1938)
1886	First complete description of pneumococcus and its etiological relationship with lobar pneumonia in humans.	Fraenkel (in White 1938)

1892	Inoculated virulent, heated pneumococci induced low immunity in rabbits. Obtained rabbit serum mixed with pneumococcal growth had different appearance compared with normal serum: the phenomenom is later called agglutination.	Mosny (in White 1938)
1897	Several "races" of pneumococci that behaved serologically as different microbes.	Bezançon and Griffon (in White 1938)
1900	Bile dissolves the pneumococcal cell	Neufeld (in White 1938)
1902	Specific reactions between pneumococcal antiserum and growth; macroscopic agglutination and microscopically visible, specific swelling of the capsule. Quellung reaction.	Neufeld (in White 1938)
1910	Isolates classified into two groups, I and II, according to protection of mice immunized with pneumococcal type I or II. Recognition of the existence of several types and recommendations for developing serums for all types. Determination of infection type before serum therapy suggested.	Neufeld and Haendel (in White 1938)
1911	Quinine and some of its derivates, especially optochin, have bactericidal property.	Morgenroth and Levy (in White 1938)
1913	Group I and II, group III consisted of organisms of *Pneumococcus mucosus* type, group IV is a heterogenous group.	Dochez and Gillespie (in White 1938)
1923	Letters S and R given to two forms of colonies. S colonies have a smooth surface, being virulent for laboratory animals, and R colonies have a rough surface and are avirulent.	Griffith (in White 1938)
1929	Simple, rapid "Stained Slide" for microscopic agglutination test.	Sabin (in White 1938)
1932	Separation of serotypes in group IV into 29 types. The total number of types was 30.	Cooper et al. (in White 1938)
1933	Quellung reaction method for typing.	Neufeld and Etinger-Tulczynska (in White 1938)
1940	20 new serotypes were described.	(Kauffman *et al.* 1940)
1942	18 new serotypes described. The total number of serotypes is 68.	(Mørch 1943)

1943	Optochin in low concentrations in blood agar inhibits pneumococcal growth.	(Mørch 1943)
1944	DNA	(Avery *et al.* 1944)
1954	Identification of pnemococcus by optochin sensitivity test.	(Bowers & Jeffries 1955)
1974	Pneumococcus was given its present name *S. pneumoniae.*	(in Bergey *et al.* 1974)
1978	The total number of serotypes is 83.	(Lund 1970, Lund *et al.* 1972)
1985	Serotype 16A	(Austrian *et al.* 1985)
1993	Identification of pneumococcus based on the amplification and detection of the pneumolysin gene	(Rudolph *et al.* 1993)
1995	6 new serotypes. The present number of serotypes is 90.	(Henrichsen 1995)
1999	Multilocus sequence typing (MLST) for pneumococci.	(Spratt 1999)
2001	Complete genome sequence of *S. pneumoniae.*	(Tettelin *et al.* 2001, Hoskins *et al.* 2001)
2006	Genetic analysis of the capsular biosynthetic locus of all 90 pneumococcal serotypes.	(Bentley *et al.* 2006)

2.1.3 Taxonomy of pneumococcus

The bacterial taxonomy of pneumococcus was earlier based on a description of the phenotypic, physiological and biochemical characteristics of bacterial species. The genomic definition of bacterial species became possible when DNA-based methods were developed and bacterial species were arranged into a phylogenetic tree based on a comparison of rRNA sequences (van Belkum *et al.* 2001, Vandamme 2003). Species in the genus *Streptococcus* have been classified in several ways over the years (Colman & Williams 1972, Facklam 1977, Schleifer & Kilpperbalz 1987, Kilian *et al.* 1989, Coykendall 1989, Facklam 2002). Despite the progress that has been made in recent years towards a better understanding of the roots of bacterial origin, identification of the viridans group of streptococci and the species within it has been problematic due to the close relationship and normal variation among strains. Genetic transformation (Lorenz & Wackernagel 1994, Fink 2005), exchanges of virulence factors among streptococcal species (Whatmore *et al.* 2000, Jado *et al.* 2001) and the appearance of new closely related streptococcal species, such as *S. sinensis* (Woo *et al.* 2002) and *S. pseudopneumoniae* (Arbique *et al.* 2004), confirm that problems in taxonomy and pneumococcal identification will continue in the future.

In the 1970s, the authorities Colman and Williams in the United Kingdom (UK) (Colman & Williams 1972) and Facklam in the United States (USA) (Facklam 1977) had a disagreement concerning the grouping and nomenclature of viridans streptococci. In 1986 Bergeys`s Manual of Systematic Bacteriology used the classification by Colman and Willison, but several commercial identification kits used the classification of Facklam (French *et al.* 1989). The next changes in the classification of streptococci were made after determining their phylogenetic relationship based on 16S rRNA sequences of the type strains of *S. mitis* and *S. gordonnii* among members of the genus *Streptococcus*. The genus *Streptococcus* was divided into the pyogenic and viridans groups, and the latter further into the anginosus group, the mitis group, the salivarius group, the bovis group and the mutans group, and into some separate species. *S. mitis* and *S. gordonnii* were clustered in the mitis group with *S. pneumoniae*, *S. oralis*, *S. sanguis* and *S. parasanguis*, and their sequence homology level was more than 96 %. The sequence homology of the *S. mitis*, *S. oralis* and *S. pneumoniae* group was more than 99 % with each other, although the DNA-DNA similarity values of their total chromosome DNAs were less than 60 % (Kawamura *et al.* 1995). The results of nucleic acid hybridization showed clearly that *Streptococcus pneumoniae* is a member of the oral streptococci and has a genetically close relationship with *S. oralis* (Schleifer & Kilpperbalz 1987).

The last decades have brought a lot of changes in the classification of the genus *Streptococcus*, as molecular taxonomic studies have produced more data on bacterial relationships (van Belkum *et al.* 2001, Facklam 2002). According to the Manual of Clinical Microbiology, 8th edition (Murray *et al.* 2003), pneumococcus is defined as follows: pneumococcus belongs to the genus *Streptococcus*. Among non-beta-hemolytic streptococcal strains, alpha-hemolytic strains can be separated into the species *S. pneumoniae* and the viridans division, which is composed of a number of species groups. *S. pneumoniae* has a role as a normal inhabitant and as a human pathogen. *S. pneumoniae* is considered as a member of the mitis species group (Ruoff *et al.* 2003). Nowadays this classification seems to be used in most publications.

2.1.4 Biology of pneumococcus

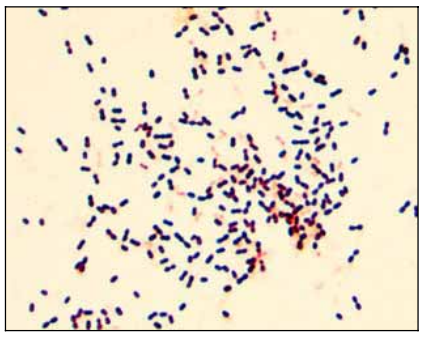

Fig. 1. ***Gram-stained pneumococci from blood culture.***

Over 130 years since the first discovery of pneumococcus, the traditional phenotypic definition of *S. pneumoniae* has not changed. In a Gram-stain, pneumococcus appears as an oval-shaped, gram-positive coccus, 1-2 μm in diameter, typically in pairs, sometimes singly or in short chains (Fig. 1). The gram-positive reaction of young cells may be lost when the culture is aged. Pneumococcus grows as α-hemolytic, centrally depressed colonies on blood agar, and generally upon primary isolation it is heavily encapsu-

lated. It is catalase-negative and facultatively anaerobic, but can grow aerobically (Bergey *et al.* 1974). However, 8 % of clinical pneumococcal isolates require an enriched carbon dioxide (CO_2) atmosphere if they are cultured on a solid medium (Austrian & Collins 1966), and thus it is recommended that cultures be incubated in a CO_2-enriched atmosphere. The nutritionally fastidious bacteria need blood or serum to grow, and they are unable to synthesize hemin. By the action of pyruvate oxidase (SpxB) under aerobic growth conditions, pneumococcus utilizes oxygen to form hydrogen peroxide. Hydrogen peroxide is toxic to cultured alveolar epithelial cells and other bacterial organisms of the upper respiratory tract, especially to *H. influenzae* (Pericone *et al.* 2000). Peroxide destroys the labile constituents of the pneumococcal cell and thus, pneumococcus may destroy itself.

Streptococci, and thus also pneumococcus, belong to the heterotrophic bacterial species that uses organic compounds as a source of carbon, and their energy-yielding metabolism is fermentative, yielding low levels of lactid acid. Glucose and other carbohydrates are fermented. Production of the leucine aminopeptidase (LAP) enzyme is a typical characteristic of all streptococci, whereas production of pyrrolidonyl arylamidase (PYR) is rare among streptococci, occurring only in *S. pyogenes* isolates and in some pneumococcal isolates (Ruoff et al. 2003). Published genome analysis suggests that *S. pneumoniae* has pathways for catabolism of pentitols as well as for cellobiose, fructose, fucose, galactose, galactitol, glucose, glycerol, lactose, mannitol, mannose, raffinose, sucrose, trehalose, and maltosaccharides. In addition, ten amino acids and N-acetylglucosamine can be used as nitrogen and carbon sources (Tettelin *et al.* 2001).

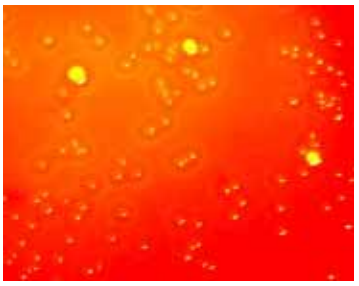

Fig. 2. ***Opaque and transparent colonies on a blood agar plate.***

So far, 90 different capsular serotypes have been identified (Henrichsen 1995), and they are grouped into 46 serotypes/groups based on antigenic similarities. Colony morphology may vary from small (e.g. unencapsulated) to clearly depressed and to very large and mucoid colonies, as in the case of serotypes 3 and 37. Colonies have been described as mucoid, ruffled and smooth (see White 1938), or opaque and transparent (Weiser *et al.* 1994) (Fig. 2). Appearance is dependent on surface proteins, capsule and cell wall composition (Watson *et al.* 1993). In the phenomenon called phase variation, variants have the same serotype and are named as transparent or opaque forms of colonies. Opaque variants are larger and whitish, whereas transparent colonies are smaller and bluish (Weiser *et al.* 1994). Opaque phenotypes have more capsular polysaccharide (Kim & Weiser 1998) and have been isolated commonly from blood (Weiser *et al.* 1994). Transparent colonies have more teichoic acid (Kim & Weiser 1998) and they are usually found in the nasopharynx (Weiser *et al.* 1994). Surprisingly, phase variation of the transparent phenotype increases invasion into human brain microvascular endothelial cells as much as six-fold, and this phenomenon might be important in the development of meningitis (Ring *et al.* 1998). In a comprehensive and

detailed review by van der Woude and Bäumler (van der Woude & Baumler 2004), the term phase variation in general means a reversible switch between on/off expressing phases, which results from variation in the level of expression of one or more proteins between individual cells of a clonal population.

2.1.5 Pneumococcal genetic transformation

Griffith found in 1928 that some heat-killed encapsulated (smooth form) pneumococci could transfer capsule production and mouse pathogenecity into unencapsulated (rough) strains (see White 1938). Later, a group at Rockefeller Institute studied the chemical composition of this "transforming principle", and in 1944 Avery, McCarty and MacLeod reported that " a nucleic acid of the deoxyribose type" (later named DNA) is the genetic material and is responsible for phenotypic changes during the transformation (Avery *et al.* 1944). Natural genetic transformation is a mechanism of horizontal gene transfer and depends on the function of several genes located on the bacterial chromosome. The ability to transform is mostly a transient and induced physiological property (Lorenz & Wackernagel 1994). The transformation of pneumococci is a complicated process in which several genes participate, and at least 28 genes are connected to the transformation of *S. pneumoniae* (Lacks 2004).

Natural competence transformation is an important mechanism allowing pneumococcus to adapt to changes in the environment. Commensal species, especially other α-hemolytic streptococci, have a major role in the evolution of *S. pneumoniae*. Exchanges of virulence factors among streptococcal species (Whatmore *et al.* 2000, Jado *et al.* 2001) as well as the appearance and spread of antibiotic resistance (Klugman 1990, Nissinen *et al.* 1995, Lister 1995, Whitney *et al.* 2000, Pihlajamäki *et al.* 2002) are well known. On the other hand, overestimation of penicillin resistance among nasopharyngeal pneumococcal isolates (Wester *et al.* 2002) may be caused by differentiation problems between *S. pneumoniae* and other α-hemolytic streptococci (Mundy *et al.* 1998, Messmer *et al.* 2004). Penicillin-binding protein (PBP) genes are responsible for the penicillin resistance of *S. pneumoniae* (Ubukata *et al.* 1996, Overweg *et al.* 2001). The connection between PBP genes, mosaic blocks and transformation (Hakenbeck 2000) is an interesting finding. The connection between penicillin resistance and pneumococcal serotype has also been demonstrated, and penicillin-resistant clones have been shown to switch their serotype (Coffey *et al.* 1991, Coffey *et al.* 1998). Since the capsular gene cluster is located immediately adjacent to the PBP genes, resistance and the new serotype could be transferred at the same time. Hakenbeck convincingly presents (Hakenbeck 2000) that nearly identical sequences of mosaic blocks in PBP genes between genetically distinct clones of resistant pneumococci are signs of a common ancestor but different transformation. The same class of mosaic PBP genes can be found in resistant strains of related streptococcal species, and penicillin-sensitive *S. mitis* and *S. oralis* strains have mosaic blocks that are highly related to the blocks of PBP genes of resistant *S. pneumoniae* (Hakenbeck 2000).

S. pneumoniae is able to express inter-strain heterogeneity, as the 90 different serotypes (Henrichsen 1995) and different phenotype variations (Weiser *et al.* 1994) demonstrate. Transparent variants, common among nasopharyngeal pneumococcal isolates (Kim & Weiser 1998), are transformed at 9-670 times higher rates than opaque variants and thus, genetic transformation selects a less-encapsulated subpopulation (Weiser & Kapoor 1999). Recent analysis of the genome-sequenced isolates TIGR 4 (Tettelin *et al.* 2001) and R6 (Hoskins *et al.* 2001) has revealed several signs of gene transfer events, such as the occurrence of insertion sequences and transposable elements (Bruckner *et al.* 2004).

In a comparison of pneumococcus with other transforming bacteria, homologs of the genes responsible for competence and DNA uptake have been found. These other species can be divided into four groups. Group I consists of closely related streptococcal species like *S. mitis* and *S. gordonnii*, and group II consists of more distantly related streptococcal species like *S. mutans* and *S. pyogenes.* Group III consists of bacterial species that are closely related to *Streptococcus*, but outside the genus *Streptococcus*, like *Lactococcus lactis* and *Entereococcus faecalis*. Group IV is formed from distantly related bacteria that are naturally transforming, among them *H. influenzae* and *B. subtilis* (Lacks 2004).

2.1.6 The capsule and serotypes/groups

Several human pathogenic bacteria, such as *Haemophilus influenzae, Neisseria meningitidis, Eschericia coli* and *S. pneumoniae*, have a polysaccharide capsule that surrounds the bacterial cell. Pneumococci may occur in two forms, unencapsulated and capsulated. The capsule and the amount of produced capsules are significant virulence factors (Paton *et al.* 1993, Kim & Weiser 1998). The capsule protects bacterial cells very effectively from phagocytosis and also inhibits complement activation. The capsule also protects against environmental effects, and the produced components help pneumococci compete with other bacteria, such as *H. influenzae*, *Moraxella catarrhalis* and *N. meningitidis*, for existence in the nasopharynx (Pericone *et al.* 2000).

Pneumococcal capsular polysaccharides are a diverse group of polyglycans that make the capsule acid (Jedrzejas 2004). These capsular polysaccharides are antigenic (Sørensen 1995) and they have the ability to induce specific antibodies (see White 1938, Austrian 1981).

In 1910 only two separate types of pneumococci were detected by inoculation of the isolates into previously immunized mice (see White 1938). In 1932 the number of identified serotypes/groups was 32 (see White 1938), and some fifty years later it was 84 (Lund & Henrichsen 1978, Austrian *et al.* 1985). In 1955 Henrichsen described (Henrichsen 1995) six new serotypes and thus, the number of serotypes is 90 today. The serotypes differ from each other by chemical differences in their capsular polysaccharides. Their ability to induce specific antibodies in rabbits is the basis for serotyping (Lund & Henrichsen 1978, Henrichsen 1999).

There are two different serotyping systems, namely the Danish (Kauffman *et al.* 1940, Lund 1970) and the American systems (Eddy 1944). The Danish system groups serotypes on the basis of antigenic similarities (for instance group 19 consists of 19F, 19A, 19B and 19C), whereas in the American system the serotype numbers are in the order in which they have been described (19, 57, 58 and 59, respectively) (Broome & Facklam 1981, Musher 1992). The Danish nomenclature for serotyping has proved to be functional and is commonly used both in pneumococcal reference laboratories and in research (Hall 1998).

Unencapsulated pneumococci are present in 0.5 % to 2 % of sterile site specimens (Broome & Facklam 1981, Carvalho *et al.* 2003), in 20 % of conjunctival specimens (Finland & Barnes 1977), and in up to 10 % of specimens obtained from nonsterile sites such as sputum, the oral pharynx or the nasopharynx (Carvalho *et al.* 2003).

Among the 90 different polysaccharide serotypes (Henrichsen 1995), some are more virulent than others. A small number of them, approximately 10 serotypes/groups, are common in pneumococcal infections (Scott *et al.* 1996, Hausdorff *et al.* 2000b, Hausdorff *et al.* 2000a, Hausdorff *et al.* 2005). According to a recent review, serotypes/groups 1, 3, 5, 6, 14, 19 and 23 are comprehensive types in invasive pneumococcal infections on several continents (Hausdorff *et al.* 2005). In the United States, serotypes 4, 6B, 9V, 14, 18C, 19F and 23F are commonly isolated from both invasive and middle-ear infections (O'Brien & Santosham 2004). In Finland, the most common serotypes isolated from invasive infections in 1995-2004 were 14, 4, 9V, 3, 23F and 7F, and in 2002-2004 these serotypes caused almost half of all invasive pneumococcal infections (Kansanterveyslaitos 2005).

The serotypes/groups 6A, 6B, 14, 15, 18C, 19F and 23F are commonly isolated from the nasopharynx of healthy children on every continent (Bogaert *et al.* 2004). The serotypes/groups 10, 11, 13, 15, 33 and 35 are also fairly common in the nasopharynx of healthy children (Bogaert *et al.* 2004, Hausdorff *et al.* 2005), whereas types 1, 5 and 46 are rare even in populations where they are isolated from invasive infections (Hausdorff *et al.* 2005). In Finland in 2001, the six most common serotypes isolated from nasopharyngeal specimens of healthy children under two years of age were 6B, 23F, 19F, 6A, 11 and 14 (Syrjänen *et al.* 2001) and 19F, 23F, 6A, 6B, 14 and 11 were isolated from specimens of acute otitis media (AOM) (Kilpi *et al.* 2001). This type of distribution is quite similar to that in an earlier AOM study (serogroups/types 19, 6, 3, 23, 11 and 18) in Finland (Luotonen *et al.* 1981).

Serotype changes due to recombinational exchanges at the capsular biosynthetic locus have been demonstrated among pneumococcal isolates (Coffey *et al.* 1998). However, Meats and coworkers did not find evidence of serotype changes when studying the nasopharyngeal pneumococcal isolates of children (Meats *et al.* 2003). Sequence analysis of the capsular biosynthetic genes of all 90 serotypes of *S. pneumoniae* (Bentley *et al.* 2006) will increase our knowledge of polysaccharide diversity, and may bring out new serotypes and new tools for serotyping.

2.1.7 Pneumolysin and other virulence factors

Pneumolysin (Ply) is an efficient intracellular toxin that can lyse any eukaryotic cell which has cholesterol in its membrane. Ply is a significant factor in pneumococcal virulence. Ply consists of a single 53 kDa polypeptide chain and is a pore-forming toxin, expressed during the late log phase of growth and produced by virtually all clinical isolates. The toxin has several functions, particularly in the early phase of pneumococcal infection (Paton *et al.* 1993, Jedrzejas 2001). Its presence was earlier considered to be characteristic only to *S. pneumoniae*, but later on it has been shown that *S. mitis* and other α-hemolytic streptococci can also harbor the *ply* gene (Whatmore *et al.* 2000).

Pneumococcal extracellular virulence factors can be divided into two groups: glycome-based and proteome-based factors (Jedrzejas 2004). The first group uses sugars as building blocks and consists mainly of capsule polysaccharides and teichoic and lipoteichoic acids. The second group includes numerous surface-attached proteins and enzymes. This group can be divided further into three categories: peptidoglycan-bound proteins (e.g. hyaluronate lyase and neuraminidase), choline-binding proteins (pneumococcal surface protein A, choline-binding protein A, autolysin) and cytoplasmic lipid bilayer attached macromolecules (pneumococcal surface antigen A, formerly called pneumococcal surface adhesion A) (Paton *et al.* 1993, Jedrzejas 2004).

Pneumococcal surface protein A (PspA) is the foremost studied surface protein among the numerous choline-binding proteins of *S.pneumoniae* (McDaniel *et al.* 1984, Novak & Tuomanen 1999). This highly variable protein (from 60 to 200 kDa) is produced by all pneumococci examined to date (Crain *et al.* 1990). PspA has an important role in protecting pneumococcus from the host immune system. It has been shown to be significant in the pathogenesis of disease. Its ability to bind lactoferrin may help it in the colonization of host mucosae. Recently it has been found that human lactoferrin has similar receptor-like properties as PspA, but the significance of the finding is still under research (Jedrzejas 2004).

Autolysins are a group of enzymes that degrade bacterial peptidoglycan. Their action leads to cell lysis. The major pneumococcal autolysin, *S. pneumoniae N*-acetylmuramoyl-L-alanine amidase, also known as LytA amidase, belongs to extra-cellular choline-binding proteins, and its size is 36 kDa. At the moment, the exact role of LytA in the virulence of pneumococcus is unclear. Mutations of the *lytA* gene reduce virulence. Moreover, LytA induces protective antibodies in mice (Jedrzejas 2001). The *lytA* gene is the first bacterial autolytic gene that was cloned (Garcia *et al.* 1985). Obregon and coworkers (Obregon *et al.* 2002) presented that atypical pneumococcal isolates had mutations in the *lyt A* gene, and the gene has also been found in closely related streptococci (Llull *et al.* 2006). Bile solubility, which tests the ability of deoxycholate to dissolve the cell wall of streptococci, depends on the presence of the autolysin gene *lytA* (Obregon *et al.* 2002).

Pneumococcal surface antigen A (PsaA) protein (37 kDa) is also an important virulence factor. This extracellular, lipid-attached protein is part of a bacterial transport system and provides for the transport of Mn^{2+} and Zn^{2+} into the cell (Jedrzejas 2004). Morrison and coworkers (Morrison *et al.* 2000) have detected by using a PCR that all 90 serotypes of S. *pneumoniae* contain the *psaA* gene. However, this gene has also been detected in other streptococcal species such as *S. mitis*, *S. oralis* and *S. anginosus* (Jado *et al.* 2001). Pneumococcal proteins are under abundant research as new vaccine candidates (Jedrzejas 2001, Bogaert *et al.* 2004). One recent publication demonstrates that intranasal immunization with a cholera toxin B subunit-PsaA fusion protein is able to protect mice against pneumococcal colonization (Pimenta *et al.* 2006).

Recent sequence analysis of the whole genome of the pneumococcal strains TIGR4 (Tettelin *et al.* 2001), R6 (Hoskins *et al.* 2001), serotype 23F strain and serotype 6B strain 670 (sequence information via the NCBI site at http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) has made it possible to study pneumococcal virulence factors in more detail. According to Jedrzejas (Jedrzejas 2004), sixty-six surface proteins have been identified, but only 56 % of them have been characterized or functionally worked out. New groups of virulence factors, including Pht family proteins and signal peptidase, have been described (Jedrzejas 2004). Approximately 2200 genes of *S. pneumoniae* are known, and future research will most likely bring out new virulence factors. The question remains why *S. pneumoniae* is a pathogen, but *S. mitis* and *S. oralis* are not (Hollingshead & Briles 2001).

2.2 Identification methods

In early times, the only way to detect and identify pneumococcus was to inoculate a small amount of sputum into the peritoneal cavity of white mice. After death, mouse blood was drawn and cultivated in broth and on blood agar plates and the colonies were examined (see White 1938). Later, a mouse virulence test was used as a "gold standard" in pneumococcal studies (Bowers & Jeffries 1955, Converse & Dillon 1977, Wasilauskas & Hampton 1984). In the 1880s, Gram made his staining experiments to visualize the bacteria from lung tissue specimens and found bacteria that were stained dark with aniline gentiane violet while others were not (White 1938). Pneumococcus was one of the first bacteria demonstrated with the Gram stain (Watson *et al.* 1993).

Pneumococcus is routinely identified by four phenotypic characteristics: colony morphology on a blood agar plate, bile solubility, optochin sensitivity and the presence of a capsule (Lund 1959, Austrian 1975, MacFaddin 1976, Lund & Henrichsen 1978, Mundy *et al.* 1998, Ruoff *et al.* 2003). Already very early, researchers found discrepancies between these characteristics, but still, identification based on them remained practical and cost-effective. A thorough comparison of the available biochemical and serological methods was published recently, pointing to the high sensitivity but limited specificity of most of the phenotypic tests (Kellogg *et al.* 2001). Later on, developed diagnostic molecular methods have made it possible to identify bacterial isolates through the presence of toxins

(Rudolph *et al.* 1993), antimicrobial resistance genes (Ubukata *et al.* 1996) and exact components of genes, e.g. *lyt A* (Pozzi *et al.* 1989, Fenoll *et al.* 1990, Hassan-King *et al.* 1994, Obregon *et al.* 2002). However, today most clinical laboratories still use the optochin sensitivity test or the bile solubility test as a screening test to differentiate pneumococcus from other α-hemolytic streptococcal isolates (Mundy *et al.* 1998, Ruoff *et al.* 2003). The molecular methods have provided important tools for epidemiological surveillance (Lefevre *et al.* 1993, Enright & Spratt 1999), too.

2.2.1 Optochin sensitivity test

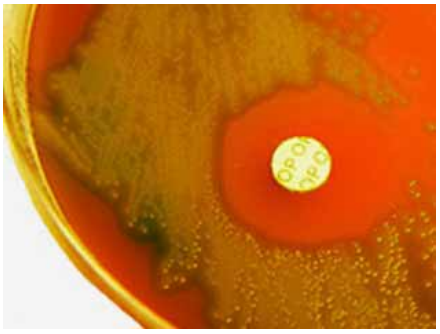

Fig. 3. ***Optochin sensitivity test.***

In 1911 Morgenroth and Levy found that quinine and some of its derivates have a bactericidal property for pneumococcus both *in vitro* and *in vivo*. Unfortunately, quinine proved to be toxic in doses that could be useful in the treatment of pneumococcal pneumonia. In 1915 Moore noticed that among streptococci, *S. pneumoniae* was most sensitive to a quinine derivate, ethylhydrocupreine (see White 1938). In 1943 Mørch demonstrated that ethylhydrocupreine hydrochloride (optochin) in low concentrations on blood agar would inhibit pneumococcal growth (Mørch 1943) (Fig. 3). Ten years later, the phenomenon was found to be suitable for differentiation of pneumococcus from other α-hemolytic streptococci. A paper disc with diluted optochin was placed on a cultured blood agar plate, and after incubation the inhibition zone was measured (Bowers & Jeffries 1955). The usefulness of the test was strengthened by Bowen and coworkers, who showed that inhibition was present with all the different serotypes (Bowen *et al.* 1957). Lund (Lund 1959) confirmed these findings and showed optochin sensitivity to be a more reliable method than bile solubility in the differentiation of pneumococcus and other streptococci. When smooth, capsulated, virulent pneumococci are changed into rough, unencapsulated, avirulent pneumococci, they keep their optochin sensitivity but lose their bile solubility (Lund 1959).

Optochin sensitivity is commonly used and often the only identification test for pneumococcus (Austrian 1975, MacFaddin 1976, Mundy *et al.* 1998). Incubation in the presence of carbon dioxide has been shown to influence the size of the inhibition zone (Ragsdale & Sanford 1971). Blood is essential for inhibition as the first finding showed (Mørch 1943), but the agar medium used may also have an influence on the size of the inhibition zone. A trypticase soy medium with sheep blood and incubation in a carbon dioxide atmosphere proved to be the most suitable conditions for testing optochin sensitivity (Gardam & Miller 1998).

Very early observations showed that pneumococcal cells could lose their sensitivity to optochin (see White 1938, Bowers & Jeffries 1955), and 30 years later optochin-resistant pneumococcal isolates were described. One of the earliest reports came from Finland (Kontiainen & Sivonen 1987). Later, in 1995-99 in Finland, the percentage of partly optochin-resistant invasive isolates was 2.5 % (59/2357), and fully resistant, 0.2 % (unpublished observation in the National Reference Laboratory for Pneumococcus, National Public Health Institute, Oulu). Most of the isolates grew as mixed cultures containing both optochin-sensitive and resistant colonies. Pikis and coworkers (Pikis *et al.* 2001) reported optochin resistance in only 0.5 % of the strains isolated from sterile specimens (blood, CSF) of children in Washington, USA, during 1992-1998. The optochin resistance of *S. pneumoniae* is a result of point mutations in either the ATP-ase a- or c-subunit (Cogne *et al.* 2000, Pikis *et al.* 2001). The origin of optochin-resistant variants is still unclear. An interesting discovery was the connection between optochin resistance and the modern increased treatment and prophylaxis of malaria; optochin is similar to quinine and mefloquine (Pikis *et al.* 2001).

2.2.2 Bile solubility

Neufeld first noticed in 1900 that bile has the property of being able to dissolve the pneumococcal cell. This property later became an important diagnostic property for differentiating pneumococcus from other streptococci. Neufeld also found that only freshly isolated and virulent pneumococcal strains were bile soluble. Levy (1907) used sodium taurocolate in 5 % and 10 % dilutions to dissolve pneumococcus and believed that the method could differentiate all pneumococci from other bacterial species (see White 1938, Lund 1959). Later studies demonstrated that bile solubility and optochin sensitivity have almost complete correlation, and strains with unclear results in a bile test were avirulent in a mouse virulence test (Bowers & Jeffries 1955). More evidence of the usefulness of the bile test came from observations that other streptococci were not soluble in bile (Lund 1959, Wasilauskas & Hampton 1984), but in 10 % of cases the interpretation was uncertain (Lund 1959).

2.2.3 Identification tests based on biochemical characteristics

Bacteria have several physiological and biochemical characteristics by which they can be differentiated from other species. Enzymatic activity or the fermentation of sugars by streptococci can be demonstrated in the laboratory using the procedures described earlier (Kilian *et al.* 1989, Facklam & Washington 1991). Commercial, standardized identification tests have been developed to make biochemical identification easier. In the 1970s, the authorities Colman and Williams in the UK (Colman & Williams 1972) and Facklam in the USA (Facklam 1977) had a disagreement concerning the grouping and nomenclature of viridans streptococci. The different conceptions of nomenclature have complicated the interpretation of identification results (French *et al.* 1989, Beighton *et al.* 1991) .

There are several studies on the physiological and biochemical differentiation of viridans streptococci in the literature, (Facklam 1977, Colman & Ball 1984, French *et al.* 1989, Coykendall 1989, Kilian *et al.* 1989, Beighton *et al.* 1991, Facklam 2002). *S. pneumoniae* is included in the viridans group. Pneumococcal isolates can easily be differentiated from the rest of the viridans group by means of their optochin sensitivity and bile solubility (Colman & Williams 1972, Facklam 1977). On the other hand, the difficulty in identifying *S. pneumoniae* using biochemical tests is known (Colman & Ball 1984). According to Norris (Norris 1968), if two bacterial strains carry out the same enzymic activities, it is difficult to say whether they produce exactly the same enzyme molecules or whether they produce similar enzymic activities produced by different molecules. In the latter situation there is no direct relationship at the genetic level.

Tillotson (Tillotson 1982) compared the improved API 20 STREP identification kit (bioMerieux, Marcy l′Etoile, France) with conventional physiological identification tests. The kit had occasional problems in differentiating some streptococcal groups, but identified viridans streptococci excellently. *S. pneumoniae* is included in the viridans group, but unfortunately no pneumococcal strain was included. However, the identification should be confirmed by colony morphology, optochin sensitivity and bile solubility according to the recommendation of the manufacturer (Tillotson 1982). Colman and Ball (Colman & Ball 1984) also recommended that the API system should be used together with other tests, e.g. Lancefield grouping and sensitivity tests. According to French (French *et al.* 1989), the API 20 STREP system may be more reliable than conventional biochemical test methods, although the identification codes need revision. The RapID STR system (Innovative Diagnostic Systems, Inc., Atlanta, Ga.) correctly identified 88.5 % of *S.pneumoniae* and 72.5 % of the viridans strains when an optochin sensitivity test was used simultaneously, as the manufacturer recommended for all α-hemolytic strains. Without optochin testing the correct identification of *S. pneumoniae* was 26.9 % and the viridans group 52.3 %, respectively. The RapID STR system requires only four hours of incubation, but the need for additional tests decreases the rapidity of identification (Appelbaum *et al.* 1986).

A review by Coykendall (Coykendall 1989) and a study by Kikuchi (Kikuchi *et al.* 1995) show how complicated and difficult the physiological and biochemical identification of viridans streptococci is, in spite of improved taxonomy based on molecular and genetic relationships. Pneumococcus has unfortunately been excluded from both studies, and thus biochemical and genetic descriptions of the bacterium are not available.

2.2.4 DNA hybridization (AccuProbe™)

In 1989 Pozzi and coworkers (Pozzi *et al.* 1989) described a DNA probe based on the *lytA* gene. In 1990 Fennoll and coworkers (Fenoll *et al.* 1990) reported an improved non-commercial pneumococcal DNA probe based on a 0.65 Kb fragment coding of the amino-terminal region of the major autolysin (*lytA* gene). They found the probe useful in identi-

fying atypical pneumococcal isolates and suitable for diagnostic use (Fenoll *et al.* 1990). Shortly after that a commercial probe kit became available.

AccuProbe ™ for *S. pneumoniae* is a commercial nucleic acid hybridization test that uses a single-stranded, chemiluminescent-labelled DNA complementary for ribosomal 16S RNA of *S. pneumoniae*. In 1992 Denys and Carey (Denys & Carey 1992) showed that the sensitivity and specificity of AccuProbe was 100 % compared with phenotypically identified pneumococcal and nonpneumococcal isolates. Geslin and coworkers (Geslin et al. 1997) correctly identified 100 % of typical optochin-sensitive and capsulated pneumocccal strains, but only 78 % of the optochin-sensitive, unencapsulated clinical strains were confirmed as pneumococci. In 1998 Mundy and coworkers (Mundy et al. 1998) reported that AccuProbe ™ for *S. pneumoniae* had 100 % sensitivity and 100 % specificity among phenotypically identified unequivocal pneumococcal and nonpneumococcal isolates. However, among 115 equivocal isolates that did not express all the typical pneumococcal characteristics, four (3 %) optochin-sensitive isolates were not identified as *S. pneumoniae* (Mundy et al. 1998). Kearns (Kearns *et al.* 2000) agreed with Mundy on the suitability of AccuProbe ™ in pneumococcal identification, although they identified as *S. pneumoniae* five phenotypically nonpneumococcal isolates. Later studies showed that the accuracy of AccuProbe ™ for *S. pneumoniae* is lower than it was earlier reported (Messmer et al. 2004), and according to Whatmore and coworkers (Whatmore et al. 2000), AccuProbe ™ identified many atypical pneumococcal isolates as pneumococci, although they probably represented organisms that are genetically rather divergent from typical pneumococci.

In 1991 Davis and Fuller described a flow chart for several bacterial species used in the direct identification of positive blood cultures with AccuProbe™. All 24 positive pneumococcal blood cultures were correctly identified as *S. pneumoniae* with AccuProbe™ (Davis & Fuller 1991). Later results confirmed the usefulness of the method, but the adjusted values for the reflective light units (RLU) of AccuProbe™ kits, calculated by the authors, improved the usability of direct identification (Lindholm & Sarkkinen 2004). For example, for pneumococcus the sensitivity was 97.9 % and the specificity was 100 % compared with conventional identification methods using a continuously monitoring blood culture system and Gram staining (Lindholm & Sarkkinen 2004).

2.2.5 Demonstration of the capsule

Fig. 4. ***Quellung reaction, detection of the capsule of serotype 7F.***

Pneumococcal capsular polysaccharides can be detected with several serological methods using specific pneumococcal antisera. The quellung reaction, first described by Neufeld in 1902 (see White 1938), continues to be the basic method used in the detection of a pneumococcal capsule (Fig. 4). This method has also been used for other bacterial species (Austrian 1976). The quellung reaction with polyvalent and specific antisera is the most specific identifying and serotyping method, but it requires much experience and a large panel of antisera. Therefore, it is not routinely used in clinical bacteriological laboratories, but mainly in reference and research laboratories. However, the method is used as a gold standard in comparisons with other methods. Other serological methods, such as latex agglutination (Severin 1972, Leinonen 1980), counterimmunoelectroforesis (CIEP) (Coonrod & Rytel 1972), coagglutination (Kronvall 1973) and dot blot assays (Fenoll *et al.* 1997), have been developed for serotyping.

A pneumococcal omniserum (Statens Serum Institut, Copenhagen, Denmark) contains antibodies against all 90 serogroups/types, and thus by using one reagent, the presence of a capsule can be detected in an isolate or a specimen. However, several streptococcus species with a carbohydrate capsule as well as other encapsulated bacteria, such as H. influenzae (Zepp & Hodes 1943), the klebsiella species (Heidelberger & Nimmich 1972) and E. coli (Robbins et al. 1972), and Bacillus with a polypeptide capsule (Tomcsik 1956), can cross-react with the pneumococcal antiserum. Reactions with other proteins that are not antibodies, such as C-reactive protein, may also cause a positive quellung reaction (Löfström 1944, Bornstein et al. 1968, Austrian 1976). Omniserum and antiserum pools contain low concentrations of antibodies against the pneumococcal cell wall (Henrichsen et al. 1980), and this has to be taken into account in interpreting the results. In one study as many as 68 % of α-hemolytic clinical streptococci showed cross-reactivity with the pneumococcal omniserum (Holmberg et al. 1985).

Modifications of the described methods have been used to detect pneumococcus from patient specimens (Merrill *et al.* 1973, Austrian 1976, Singhal *et al.* 1996), to detect a pneumococcal capsule in isolates, and thus as a part of the identification (Cima-Cabal *et al.* 1999, Chandler *et al.* 2000, Kellogg *et al.* 2001) in serotyping (Henrichsen *et al.* 1980, Lalitha *et al.* 1996, Lalitha *et al.* 1999, Arai *et al.* 2001, Slotved *et al.* 2004) and in a comparison with other methods (Wasilauskas & Hampton 1984, Fenoll *et al.* 1997, Mundy *et al.* 1998, Kearns *et al.* 2000). A molecular approach to serotyping is the PCR method based on polymorphisms within the *cpsA* and *cpsB* genes that are common to all

capsule loci (Lawrence *et al.* 2000). It has been modified for use as a multiplex PCR method and as a semiautomated method (Lawrence *et al.* 2003).

2.2.6 Demonstrations of virulence genes using a polymerase chain reaction (PCR)

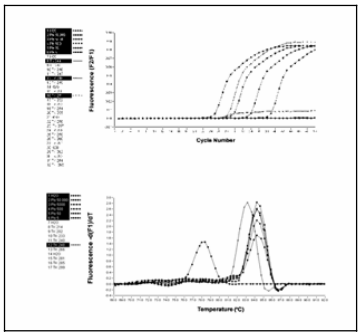

Fig. 5. ***Real-time ply PCR with melting curve analysis.***

The polymerase chain reaction (PCR) method was first described by Mullis in 1987 (Mullis & Faloona 1987). The method is based on the amplification of a fragment of the gene, where the amplified products can be detected by agarose gel electrophoresis (Norris 1968, Erlich *et al.* 1991, Bingen *et al.* 1994) or by using a microwell hybridization reaction, e.g. with an Europium-labelled probe (Dahlen *et al.* 1988). A real-time PCR, with amplification and detection of the target product in real time and simultaneously using fluorescent measurements and specific dyes, brought several advantages for gene technology. Confirmation of products and differentiation of a specific product from nonspecific products using melting temperature increases the sensitivity and specificity of the PCR (Wittwer *et al.* 1997b, Wittwer *et al.* 1997a). The amplification curves (top in the figure) and melting peaks of the amplification products (bottom in the figure) of a streptococcal strain, a pneumococcal standard and a negative control are presented in Figure 5.

Conventional and real-time PCR methods have been used to detect pneumococcal virulence factors from clinical specimens and from pneumococcal isolates. Pneumolysin is the most often used virulence factor in pneumococcal identification (Rudolph *et al.* 1993, Kearns *et al.* 2000, Neeleman *et al.* 2004b). A *ply* PCR has been used to demonstrate pneumococcus directly from blood (Rudolph *et al.* 1993, Toikka *et al.* 1999), from cerebrospinal fluid (Kearns *et al.* 1999), from serum (Salo *et al.* 1995, Salo *et al.* 1999) from middle ear fluid (Virolainen *et al.* 1994, Jero *et al.* 1996, Saukkoriipi *et al.* 2002) and from a nasopharyngeal specimen (Saukkoriipi *et al.* 2004).

PCR methods based on the demonstration of various other pneumococcal virulence factors, such as autolysin (*lytA*) (Rudolph *et al.* 1993, Ubukata *et al.* 1996, Whatmore *et al.* 2000, McAvin *et al.* 2001, Messmer *et al.* 2004, Neeleman *et al.* 2004a), pneumococcal suface antigen A (*psaA*) (Messmer *et al.* 2004), manganese-dependent superoxide dismutase (*sodA*) (Kawamura *et al.* 1999) and penicillin-binding protein (Zhang *et al.* 1995, Ubukata *et al.* 1996), have also been developed. Messmer and coworkers (Messmer *et al.* 2004) found that an *lytA* PCR was the most specific in differentiating pneumococcus from atypical streptococci compared with conventional, AccuProbe™-, *ply*, and *psaA* PCR methods. The number of new molecular methods for identifying pneumococcus and differenting pneumococcus from other streptococcal species is still increasing (Suzuki *et al.* 2005, Innings *et al.* 2005).

2.2.7 Genotyping methods

The characterization of bacterial pathogens below the species level is essential in analyzing the clonal spread of bacteria during epidemics and in identifying antibiotic-resistant or otherwise virulent isolates. Molecular typing methods are useful in studying a short-term (the spread of an isolate of a hospital or local community) as well as a longer-term (global spread of virulent isolates) epidemiological follow-up (Bingen *et al.* 1994, Hall 1998, Enright & Spratt 1999). For pneumococcal epidemiology, accurate typing methods, such as pulsed-field gel electrophores (PFGE) (Lefevre *et al.* 1993, Bingen *et al.* 1994, Hall *et al.* 1996), multilocus enzyme electrophoresis (MLEE) (Selander *et al.* 1986, Takala *et al.* 1996, Hall *et al.* 1996), restriction fragment length polymorfism (RFLP) (Bingen *et al.* 1994, Lawrence *et al.* 2000, Doit *et al.* 2002, Schlegel *et al.* 2003) and multilocus sequence typing (MLST) (Enright & Spratt 1998, Spratt 1999), have been developed. The amplified fragment length polymorphism (AFLP) method is one application of the RFLP –method, and it has also been used in pneumococcal serotyping (Neeleman *et al.* 2004a, Shaaly *et al.* 2005, Batt *et al.* 2005). In addition, amplified rDNA restriction analysis (ARDRA) has been used especially to identify bacteria growing slowly in a culture, such as a mycobacterial species (De Baere *et al.* 2002).

The PFGE method is based on the evaluation of total chromosomal DNA, and the obtained PFGE type is compared with similarity and diversity patterns. It is commonly used and considered as a gold standard (Lefevre *et al.* 1993, Shaaly *et al.* 2005). Neeleman and coworkers (Neeleman *et al.* 2004a) compared the AFLP method with the PFGE method and found that it differentiates pneumococci from other streptococci better than the "gold standard" PFGE method. Several modifications of the RFLP analysis have been used in the epidemiological follow-up of pneumococcus (Doit *et al.* 2002, Schlegel *et al.* 2003) and in the evaluation of DNA-based methods (Lawrence *et al.* 2000).

The multilocus sequence typing (MLST) system provides a new approach to the characterization of pneumococci, and it is described at http://www.mlst.net/: "Multilocus sequence typing (MLST) is an unambiguous procedure for characterizing isolates of bacterial species using the sequences of internal fragments of seven housekeeping genes. Approx. 450-500 bp internal fragments of each gene are used, as these can be accurately sequenced on both strands using an automated DNA sequencer. For each housekeeping gene, the different sequences present within a bacterial species are assigned as distinct alleles and, for each isolate, the alleles at each of the seven loci define the allelic profile or sequence type (ST)". In other words, in MLST, the internal fragments of the seven housekeeping genes *aroE*, *ddl*, *gdh*, *gki*, *recP*, *spi* and *xpt* are amplified, sequenced and after comparison with known alleles, their allelic profile is specified. This profile (ST) is compared with allelic profiles and defined in a database via the Internet (Enright & Spratt 1998, Enright & Spratt 1999).

2.3 Demonstration of pneumococcal carriage

2.3.1 Pneumococcus as a part of normal nasopharyngeal flora

Fig. 6. ***Culture from nasopharyngeal specimen and identified pneumococcus.***

Bacterial colonization starts immediately after birth and continues throughout life with small changes. Normal nasopharyngeal bacterial flora develops during the first year of life and the number of bacterial species varies much (Aniasson *et al.* 1992, Harrison *et al.* 1999). Normal flora has an important role in the prevention of infectious diseases. However, a major part of infections is caused by microbes like *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, *N. meningitidis* and S*taphylococcus aureus*, which originally belong to normal flora (Fig. 6). Pneumococcus has genetic properties allowing it to coexist with other bacteria and inhibit competing intruders, and by producing hydrogen peroxide it inhibits the growth of other bacteria such as *H. influenzae*, *M. catarrhalis* and *N. meningitidis* (Pericone *et al.* 2000). Alpha-hemolytic streptococci isolated from the nasopharynx of healthy children have been shown to inhibit the growth of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* (Tano *et al.* 1999, Bogaert *et al.* 2004).

Aniansson and coworkers studied (Aniasson *et al.* 1992) the development of nasopharyngeal bacterial colonization. The bacteria that were common in the nasopharynx of Swedish children at 2 months of age were diphteroid rods (39 %), *S. aureus* (39 %) and *M. catarrhalis* (20 %), *S. epidermidis* (15 %), α-hemolytic streptococci (13 %), *S. pneumoniae* (12 %) and *H. influenzae* (5 %). Their percentages changed to 35 %, 4 %, 40 %, 9 %, 20 %, 32 % and 13 %, respectively, at 10 months of age. The proportion of children carrying *S. pneumoniae*, *H. influenzae* or *M. catarrhalis* increased with age from 31 % at 2 months to 62 % at 10 months of age (Aniasson *et al.* 1992). Harrison and coworkers (Harrison *et al.* 1999) confirmed these findings when they studied nasopharyngeal bacterial flora in infancy and sleep position.

Pneumococcal carriage develops among children more rapidly in developing countries compared with industrialized countries, and more than one serotype can be carried simultaneously for several months (O'Brien & Nohynek 2003). For example, in Papua New Guinea, pneumococcal carriage was 36 % among children under 10 days old and increased to 100 % at 80 days of age (Gratten *et al.* 1986). On the other hand, in Costa Rica, pneumococcal carriage was low and increased from 3.1 % at one month of age to 19.4 % by the end of the first year (Vives *et al.* 1997). In Finland, pneumococcal carriage in healthy children increased from 9 % at 2 months of age to 22 % at 12 months and to 43 % at 24 months (Syrjänen *et al.* 2001). Respiratory infection with acute otitis media increased carriage up to 56 %, and with pneumococcal AOM it was 97-100 % (Syrjänen *et al.* 2001). Young children (< 6 months) are well protected from pneumococcal carriage,

but carriage in families raised the risk tenfold compared with children whose family members did not carry the bacteria (Leino *et al.* 2001).

People usually carry pneumococci without symptoms, but carriage can also contribute to respiratory or even systemic disease. Nasopharyngeal pneumococcal carriage has been studied much over the years, as recently published reviews show (Garcia-Rodriguez & Fresnadillo Martinez 2002, O'Brien & Santosham 2004, Bogaert *et al.* 2004). Several factors, such as age, geographical sites, socio-economic status, family size, number of siblings, day-care, the presence of upper respiratory tract infection and overcrowded living conditions, have considerable impact on pneumococcal carriage and its rates (Garcia-Rodriguez & Fresnadillo Martinez 2002, O'Brien & Santosham 2004, Bogaert *et al.* 2004). In addition, a comparison of pneumococcal carriage rates between studies is difficult due to the variable methodological factors, such as the number and frequency of specimen collection, the quality of specimens and culture techniques (Garcia-Rodriguez & Fresnadillo Martinez 2002). Generally, pneumococcal carriage is highest at the age of two years and it decreases over the years. Most of the carriage studies have been done among children, but some cover age groups up to adults (Christenson *et al.* 1997, Borer *et al.* 2001). Table 2. shows the pneumococcal carriage, the specimen, and the method used among different age groups.

Table 2. *Pneumococcal carriage among children, adolescents and adults.*

Country	Year	Studied population	Specimen	Serotyping	Age	Carriage rate
Sweden (Christenson *et al.* 1997)	1995	1129 day-care children, aged 2-7 years, and 308 adults	Nasopharyngeal swab in a transport medium, cultured within 24 hours.	CIEP (antisera from SSI)	2 years 4 years 7 years adult	50 % 42 % 21 % 3 %
Israel (Borer *et al.* 2001)	1998	152 children and adolescents and 244 adults	Nasopharyngeal swab in a modified Stewart transport medium, cultured within 1 hour.	Quellung reaction (antisera from SSI)	< 2 years 2-5 years 6-12 years 13-17 yrs adults	75 % 53 % 40 % 10 % 7 %

2.3.2 Transport of a specimen

The ideal situation, that a specimen would be cultured on plates immediately after sample collection, is usually impossible, as sample collection and isolation and identification of bacteria are carried out in separate places. The main requirement of a useful transport pack is to store the bacteria viably, uncontaminated and without variation or mutation, i.e. to keep the bacteria as nearly as possible in a state that corresponds to the original isolate. In early times of bacteriology (in the 1890s), cotton wool swabs were used to transport the clinical specimens, but transportation in a dry swab should happen without delay. A significant improvement in specimen transport was offered by the medium that Stuart, Toshach and Patsula described in 1954 (Stuart 1956). Agar jelly containing sodium thioglycollate to prevent oxidation, but no nutrient material, was found to be suitable for keeping such bacteria as *Neisseria* and *Shigella* viable during transport (Stuart 1956). This medium, with modifications, became the commonly used specimen transport method. Comparisons of commercial transport packs have shown that their ability to keep bacteria viable varies, and overgrowth of other nonpathogenic bacteria may cause problems (Human & Jones 1986). The dry swab without any medium gave similar results as many commercial packs if the specimen was plated within six hours. However, a delay of 24-48 hours illustrated the value of the transport medium (Human & Jones 1986).

In pneumococcal carriage studies, the number of collected specimens is high. In addition, the cost and limitations of commercial packs created a need to find a suitable medium for research purposes. In 2001 O´Brien and coworkers (O'Brien *et al.* 2001) showed that an STGG (skim milk-tryptone-glucose-glycerol) medium is excellently suited for nasopharyngeal carriage studies. Their results showed that low amounts of pneumococcus are recovered better from a nasopharyngeal swab in an STGG medium than from direct plating. Storage for nine weeks at temperatures of –70°C and –20°C did not decrease the recovery of pneumococcus, whereas storage for five days at + 4°C reduced pneumococcal growth (O'Brien *et al.* 2001). Later, it has been confirmed that samples in an STGG or SGG (skim milk-glucose-glycerol) medium could be stored at refrigerator temperature for up to three days without a significant loss of pneumococcal viability (Charalambous *et al.* 2003).

However, storage of specimens in commercial transport packs and also in STGG tubes has disadvantages, such as a need for rapid processing (24 hours), the high cost of a low-temperature freezer and an expensive component of the medium. Thus, other storage media for nasopharyngeal specimens have been studied. The SGG medium is more economical compared to the STGG medium and stored pneumococci survive well, but the STGG medium preserved pneumococci significantly better at + 21°C and + 30°C than did SGG (Charalambous *et al.* 2003). A modified Dorset egg (DE) medium stores pneumococcal colonies viably for at least 30 days without refrigeration, but further studies are needed to evaluate its suitability for nasopharyngeal swabs and other respiratory pathogens (Wasas *et al.* 1998). An egg-thioglycolate-antibiotic (ETA) medium was also used in nasopharyngeal swabs to store pneumococcus viably. The results showed that 94 % of the pneumococci

stayed viable at + 20°C for 7 days and 85 % for 14 days, and failure to recover pneumococcus was usually due to an overgrowth of α-hemolytic streptococci (Gray 2002).

In 2003 the WHO's Working Group published (O'Brien & Nohynek 2003) a standardization of the methods for detecting *S. pneumoniae* in nasopharyngeal carriage studies.

2.3.3 Bacterial preservation

An ultra-freeze method with a modified medium was used to study the survival of bacteria among 97 fastidious bacterial species (Gibson & Khoury 1986). The medium was composed of tryptone soya broth, glucose, skim milk and glycerol. Skim milk combined with the glycerol proved to be an excellent cryoprotectant, and transporting the tubes immediately into a freezer at –70°C increased bacterial viability. *S. pneumoniae* stayed viable for 40 months and *H. influenzae* for 28 months (Gibson & Khoury 1986). An STTG medium has been used successfully to store both bacterial isolates and nasopharyngeal specimens viably (O'Brien *et al.* 2001), and bacterial viability remains unchanged for several years (in Gray 2002 personal observations).

2.3.4 Detection of pneumococcus from an STGG medium

Pneumococcus can be detected from nasopharyngeal specimens by using direct plating (Syrjänen *et al.* 2001), but the method needs competent circumstances for isolating and identifying bacteria or special transportation to a bacterial laboratory. Direct plating is as sensitive as a culture from an STGG medium, but, however, in low bacterial concentrations pneumococcus could be detected better with a culture from an STGG medium (O'Brien *et al.* 2001). Charalambous and coworkers (Charalambous *et al.* 2003) showed that direct plating was not as sensitive as culturing from an STGG medium.

An STGG medium has several advantages compared with direct plating. Specimens collected in an STGG medium can be used not only for bacterial cultures, but also for other detection methods. Pneumococcus can be detected from nasopharyngeal specimens in an STGG medium also by using a real-time *ply* PCR (Saukkoriipi *et al.* 2004) that enables the detection of nonviable pneumococci, e.g. during antibiotic treatment. A recently published study shows that the medium can be used to simultaneously detect viral and bacterial pathogens (Pitkäranta *et al.* 2006), and several bacteriological studies (Ukkonen *et al.* 2000, Kristo *et al.* 2006, Herva *et al.* 2006) have shown its usefulness.

3 AIMS OF THE STUDY

The purpose of the study was to compare the conventional, phenotypic bacteriological and genetechnological methods of identifying *Streptococcus pneumoniae* and especially to evaluate their usefulness in the identification of suspected pneumococcal isolates lacking one or more of the typical characteristics of pneumococcus. The specific aims were:

1. To compare the phenotypic identification methods and a biochemical method (API 20 STREP), a hybridization method (AccuProbe™) and genetechnological methods with each other in the identification of *S. pneumoniae* (I, II, III).

2. To evaluate a real-time *ply* PCR combined with melting curve analysis of amplification products in the differentiation of *S. pneumoniae* from other α-hemolytic streptococci (III).

3. To evaluate the usefulness of an STGG medium for the transportation and storage of nasopharyngeal specimens and bacterial isolates at –20°C and –80°C (IV).

4 MATERIALS AND METHODS

4.1 Bacterial isolates

The study material in paper I consisted of 100 α-hemolytic streptococcal isolates suspected to be pneumococci. The isolates were divided into four groups according to their optochin sensitivity and capsular reaction. Figure 7. shows the pneumococcal isolates, their origin and classification. *S. pneumoniae* ATCC 6305 (ATCC CULTURES™) was used as a positive and *S. bovis* ATCC 9809 (ATCC CULTURES™) as a negative control strain.

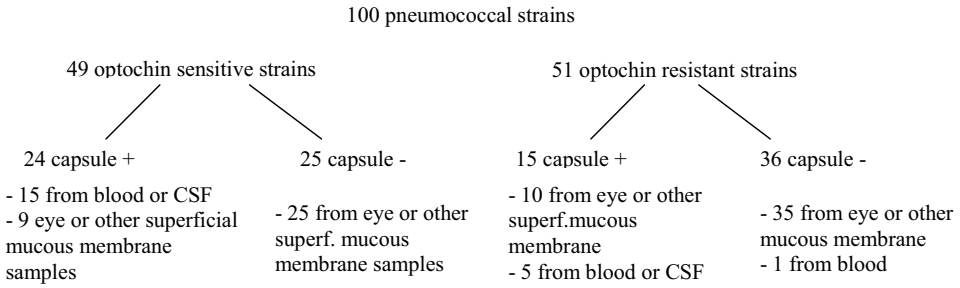

Fig. 7. ***Isolates used in study I.***

The reference strains of *S. pneumoniae*, *S. mitis*, *S. oralis* and *S. sanguinis* and 49 optochin-resistant α-hemolytic streptococcal pneumococcus-like isolates are presented in original paper II. The optochin-resistant clinical isolates are the same isolates that were used in paper I and had been sent to the Reference Laboratory (KTL, Oulu, Finland) for confirmation of identification and serotyping.

In paper III, the study material originally consisted of 100 α-hemolytic streptococcal strains isolated from consecutive nasopharyngeal swabs of young children. The children (N:507) participated in a study where the efficacy of 3´- sialyllacto-N-neotetraose in the prevention of otitis media was studied (Ukkonen *et al.* 2000). Isolated, α-hemolytic colonies, either optochin-sensitive or -resistant, were stored in 10 % skim milk (Difco 0032-17-3, USA) at −80 °C for further testing. Unfortunately, three optochin-resistant isolates were not viable after storage, and our final material thus consisted of 97 isolates. *S. pneumoniae* ATCC 6305 was used as a positive and *S. bovis* ATCC 9809 as a negative quality control strain.

In paper IV, *S. pneumoniae* (ATCC 6305), *M. catarrhalis* (ATCC 25238) and *H. influenzae* (a clinical isolate) strains were used to test the survival of these bacteria in an STGG

medium at –20°C and at –80°C. In addition, the survival of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* in nasopharyngeal specimens stored frozen at –80°C for one and a half year before subculturing was studied. Nasopharyngeal samples were collected in an STGG medium from children participating in an NE-1530 study (Ukkonen *et al.* 2000).

4.2 Ethical issues

The selected study material, pneumococci or streptococci suspected of being pneumococci (papers I and II), belong to the isolate collection of the National Infectious Diseases Register of the National Public Health Institute, Finland. The strains have been isolated from blood or cerebrospinal fluid or other sterile fluids or from other specimens with decreased penicillin sensitivity. Originally, the strains had been identified as pneumococcus in clinical microbiological laboratories and had been sent for confirmation of identification and for serotyping to the National Reference Laboratory for Pneumococcus. The Communicable Diseases Act (583/1986) with several regulations permit the use of isolates for research purposes.

The nasopharyngeal specimens and isolates used in papers III and IV were collected from children that were recruited from the Helsinki metropolitan area. Their age was 10-24 months and they were in good health, as confirmed by their medical history and complete physical examinations. In the study, the efficacy of antiadhesive oligosaccharide 3´-sialyllacto-N-neotetraose (NE-1530) was investigated as a prevention or treatment of infections of acute otitis media and for its effect on the nasopharyngeal carriage of bacteria (Ukkonen et al. 2000). The study protocol was approved by the National Agency for Medicines and the ethics committees of the Helsinki City Health Department and the National Public Health Institute, Helsinki.

The work was carried out according to good research practise at KTL. Good research practice, version 1.0 (Lankinen & Laitinen 1998) contains recommendations, guidelines and legislation concerning research at KTL and describes the practices to be followed.

4.3 Conventional identification methods

4.3.1 **Optochin sensitivity test (I, II, III, IV)**

Based on our findings in paper I, all optochin sensitivity tests in studies II and III were performed using heavy inoculum and optochin discs purchased from Biodisk. Thus, 5-10 colonies were touched with a disposable 1 µl loop and streaked on a blood agar plate and a Biodisk optochin disc was placed in the middle of the inoculated area. The plate was incubated in 5 % CO_2 at + 37 °C for 18-24 hours and the inhibition zone was measured (mm). Optochin sensitivity was interpreted as optochin-sensitive (≥ 12 mm) or optochin-resistant, using the manufacturer´s cutoff value.

4.3.2 **Tube bile solubility test (I, II, III)**

Bile solubility was tested according to the method described by Facklam and Washington (Facklam & Washington 1991). Fresh pneumococcal growth from a blood agar plate was suspended in 0.9 % NaCl to give 0.5-1.0 McFarland units. Equal 0.5 ml volumes were transferred into two tubes, and 0.5 ml of 2 % sodium deoxycholate (Merck 6504, Germany) or 0.5 ml of 0.9 % NaCl (control) was added. The tubes were incubated for 2 hours in 5 % CO_2 at + 37 °C. Disappearance of opacity in the deoxycholate tube, but not in the control tube, was interpreted as a positive result. Partial clearing was not accepted as a positive result.

4.3.3 **Demonstration of the presence of a capsule by quellung reaction (I, II, III)**

A capsular quellung test was performed as described by Austrian (Austrian 1976) to demonstrate the presence of a capsule in pneumococcal isolates. Fresh growth was transferred from a blood agar plate into 4 ml of brain heart infusion broth (Difco 0037-17-8, USA) with 10 % horse serum (Life Technologies™ 26050-047, England) and incubated in 5 % CO_2 at + 37 ^{o}C for 4 to 5 hours. On a glass slide, 5 µl of well-mixed broth culture and 5 µl of pneumococcal omniserum (Statens Serum Institut, Denmark) were mixed and examined immediately under a microscope (40X). Instead of omniserum, 0.9 % NaCl was used as a control. In a positive quellung reaction the organisms look light and swollen (the capsule becomes visible). The single cocci double or triple in size and agglutinate into clumps. In a negative reaction the organisms look like thin cocci in pairs and chains.

4.3.4. **Biochemical identification with API 20 STREP (III)**

The API 20 STREP system (bioMerieux, Marcy l′Etoile, France), based on the demonstration of enzymatic activity or the fermentation of sugars, was applied according to the manufacturer's instructions. Fresh bacterial growth from a blood agar plate was collected with a swab and immersed in the API medium and used in reactions. The metabolic end production of enzymatic activity comes into view either through spontaneous coloured reactions or by addition of reagents. Fermentation of carbohydrates is detected by a shift in the pH indicator. The reactions were interpreted after incubation at + 37 ^{o}C for 2 hours, after which the incubation was continued overnight. The reactions were interpreted according to the Reading Table and coded into a numerical profile after final interpretation of the reactions. The identification was obtained by comparing the achieved profile with the Analytical Profile Index.

4.4 Genetechnological identification methods

4.4.1 **Specific nucleic acid probe (AccuProbe™) (I, II, III)**

DNA hybridization was performed using the AccuProbe ™*Streptococcus pneumoniae* culture identification test (GEN-PROBE®, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, five to ten colonies were collected from a fresh blood agar plate, suspended in a lysis reagent tube, incubated and a hybridization buffer was added. After incubation a selection reagent was added. This reagent differentiated non-hybridized and hybridized probes, and labelled DNA-RNA hybrids could be measured with the Gen-Probe luminometer. The cutoff value of reflective light units (RLU) for a positive result was 50,000.

4.4.2 **Conventional *ply* PCR for detection of pneumococcal pneumolysin (I, II, III)**

A polymerase chain reaction, PCR, based on amplification of the *ply* gene fragment, as described originally by Virolainen (Virolainen *et al.* 1994), was performed in papers I, II and III with some modifications (Salo *et al.* 1995, Rintamäki *et al.* 2002) (Table 3). Briefly, one pneumococcal colony was suspended in 100 µl of PCR reaction mix. The primers that were used amplified a 209-bp region of the *ply* gene (IIa and Biotin labelled IIb). Amplification was done and the amplification products were identified using agarose gel electrophoresis with ethidium bromide staining and confirmed by solution hybridization with a Europium-labelled probe (Rintamäki *et al.* 2002).

4.4.3 **Detection of the pneumococcal surface antigen A (*psa*A) gene and the autolysin (*lyt*A*)* gene (II)**

To amplify the pneumococcal surface antigen A (*psa*A) gene and the autolysin (*lyt*A*)* gene, DNA from one colony was extracted by alkaline lysis and diluted with distilled water. To detect the presence of the *psa*A gene, the extracted DNA was amplified as designed by Morrison and coworkers (Morrison et al. 2000). The primers that were used amplified a part of the *psa*A gene and the resulting product was a fragment of 838 bp. To detect the presence of the *lyt*A gene, the extracted DNA was amplified as designed by McAvin and coworkers (McAvin *et al.* 2001). The primers amplified a 101 bp fragment of the *lyt*A gene (Table 3). The presence of amplification products of *psaA* and *lytA* was checked using electrophoresis on 2% agarose gels stained with ethidium bromide.

Table 3. *Primers and probes used in the PCR analyses to detect S. pneumoniae DNA.*

	Primer/probe (size of product)	Sequence	Reference
Conventional PCR	pneumolysin (ply) inner primers (209- bp) IIa	5′- CCCACTCTTCTTGCGGTTGA-3′	(Salo *et al.* 1995)
	IIb	5′-TGAGCCGTTATTTTTTCATACTG-3′	
Conventional PCR	ply (209-bp)		(Rintamäki *et al.* 2002)
	WO 506	5′-biotin-modC)$_1$CCCACTCTTCTTGCGGTTGA-3′	
	WO 507	5′-TGAGCCGTTATTTTTTCATACTG-3′	
	Eu hybridisation probe	5′-(Eu[a]-modC)$_{20}$ GAGAAAGCTATCGCTACT-3′	
Real-time PCR	ply (206-bp)		(Saukkoriipi *et al.* 2002)
	(forward) LCIIa,	5′-ACTCTTCTTGCGGTTGATCG-3′	
	(reverse) IIb	5′-TGAGCCGTTATTTTTTCATACTG-3′	
	FLU probe	5′-TCTCCAAGTGGAAGACCCCAGCAA-Flu[b]	
	LC probe	LCRed640c[c]-CAAGTGTTCGCGGAGCGGTAAAC-p[d]	
Conventional PCR	psaA1 (838-bp)	5′-CTTTCTGCAATCATTCTTG-3′	(Morrison *et al.* 2000)
	psaA2	5′-GCCTTCTTTACCTTGTTCTGC-3′	
Conventional PCR	lytAF (101-bp)	5′-ACGCAATCTAGCAGATGAAGC-3′	(McAvin *et al.* 2001)
	lytAR	5′-TGTTTGGTTGGTTATTCGTGC-3′	

a Europium. b Fluorescein. c LightCycler Red640. d Phosphate.

4.4.4 **ARDRA (II)**

Amplified rDNA restriction analysis (ARDRA), i.e. amplification of the 16S rRNA gene and subsequent restriction digestion with *Rsa*I followed by agarose electrophoresis, was carried out as described previously (De Baere *et al.* 2002). This analysis was carried out in the Department of Chemistry, Microbiology and Immunology, Ghent University Hospital, Belgium.

4.4.5 **Real-time pneumolysin PCR (III)**

For a real-time *ply* PCR, DNA was extracted from fresh growth and a real-time PCR was performed as follows. Five α-hemolytic streptococcal colonies were suspended in 100 µl

of distilled water and boiled. Amplification of a 206-bp fragment of the *ply* gene was performed as described earlier (Saukkoriipi *et al.* 2002) using the Light Cycler Instrument (Roche Diagnostics, Mannheim, Germany) and fluorescence resonance energy transfer (FRET) hybridization probes. Fluorescence was measured once after each annealing step.

4.4.6 **Melting curve analysis (III)**

Melting curve analysis was used to confirm the real-time *ply* results. For melting curve analysis, a real-time PCR was performed as described above, but instead of hybridization probes and a reaction mixture, a SYBRGreen dye kit (Roche Diagnostics) was used to detect the amplification products (Saukkoriipi *et al.* 2003). The PCR protocol with a few modifications was similar to the one described earlier, but continuous fluorescence data collection was performed. The fluorescence data gathered during the PCR were analyzed using the Second Derivative Maximum method of the Data Analysis program of the LightCycler software version 3.5.28 (Roche Diagnostics).

4.4.7 **Sequence analysis (III)**

The purified PCR products were dried and diluted in sterile aqua. Sequencing was performed on both strands using primers LCIIa and IIb. The sequences were assembled by using the ContigExpress™ program and aligned by using the AlignX® program, both included in the Vector NTI® Suite v.6 (Informax Inc., Bethesda, MD). This analysis was carried out by Macrogen (Seoul, South Korea).

4.5 Survival of bacteria in an STGG medium (IV)

To test the survival of *S. pneumoniae*, *M. catarrhalis* and *H. influenzae* isolates, STGG medium tubes were prepared. STGG tubes containing different amounts of these bacteria were prepared for storage at two temperatures, –20°C and –80°C, and subcultures for viability testing were performed on blood, blood and gentamicin, and chocolate agar plates 18 times during three years. To test the effect of the number of inoculated bacteria on the survival of *S. pneumoniae*, three STGG tubes were prepared for both temperatures (–20°C and –80°C) by inoculating them with: (1) five colonies lightly touched with a 1 µl loop, or (2) 1 µl loopful, or (3) plenty of growth. To test the survival of bacteria in nasopharyngeal swabs, 100 consecutive nasopharyngeal swabs in an STGG medium were chosen for repeat cultures. After one and a half years of storage at –80 °C, the specimens were subcultured and *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* were identified and the number of colonies was recorded. The culture results from the original and repeated cultures of the nasopharyngeal specimens were compared.

4.6 Statistical analysis

All the variables in paper I were dichotomous (+ or -), and cross-tabulation was used in the analysis. Because of the lack of a gold standard, the sensitivity, specificity and positive and negative predictive values were not included in the analysis. In paper II all the variables were dichotomous. In paper III sensitivity and specificity with 95 % confidence intervals were calculated using the optochin test as a gold standard. Kappa (κ) statistics were used to measure the agreement between the optochin test and the other methods. In paper IV cross-tabulation was used in the comparison of the culture results from the original and stored specimens.

5 RESULTS

5.1 Effect of inoculum size on optochin sensitivity and a comparison of different optochin discs (I)

The conditions that affect the results of the optochin sensitivity test, i.e. the amount of bacteria and different commercial optochin discs, were examined first. In the comparison between different inocula in the optochin tests, the light inoculum gave larger diameters than the heavy one, but the difference in the diameters was generally small: 0-4 mm in 95 % and 5-9 mm in 5 % of the strains tested. With the Biodisk product this difference occurred less frequently than with other products. In 23 (5.8 %) of the 400 comparisons, the optochin sensitivity results were different with light and heavy inocula. Generally, the optochin results were clearly sensitive or resistant. When the heavy inoculum was used, the inhibition zones were more clear-cut and measurement was easier. The effect of the inoculum was similar with all the optochin discs.

The comparison of four optochin discs classified by pneumococcal group is presented in Table 4. The isolates sensitive to optochin in the Biodisk test included three (6 %) isolates resistant according to BBL, seven (14 %) according to Oxoid and one (2 %) according to Rosco. On the contrary, the isolates resistant to optochin according to Biodisk included one (2 %) isolate sensitive according to BBL and four (8 %) according to Rosco.

Table 4. *Comparison of four optochin discs.*

Test disc		Optochin sensitive (+)		Optochin resistant (-)	
Manufacturer and result		Caps+ (N=24)	Caps- (N=25)	Caps+ (N=15)	Caps- (N=36)
BIODISK	+	24	25	0	0
	-	0	0	15	36
BBL	+	23	23	1	0
	-	1	2	14	36
OXOID	+	20	22	0	0
	-	4	3	15	36
ROSCO	+	23	25	2	2
	-	1	0	13	34

5.2 Comparison of genetechnological and conventional identification methods (I)

The isolates were divided into four groups according to their optochin sensitivity and the presence of a capsule: typical optochin-sensitive and encapsulated isolates (Opt S, Caps +, Group 1), typical optochin-sensitive but unencapsulated isolates (Opt S, Caps -, Group 2), atypical optochin-resistant, encapsulated isolates (Opt R, Caps +, Group 3), and equivocal optochin-resistant and unencapsulated isolates (Opt R, Caps -, Group 4). All the typical encapsulated isolates were bile-soluble, positive according to AccuProbe™ and had the *ply* gene according to the PCR, but discrepancies were seen between the methods in the other three groups. Detailed results of the identification tests are presented in Table 5. Discrepant results in bile solubility and nucleic acid tests were found in 24 % (6/25) of Group 2 (Opt S, Caps –), 13 % (2/15) of Group 3 (Opt R, Caps +) and 44 % (16/36) of Group 4 (Opt R, Caps -). Of the optochin-sensitive, unencapsulated isolates (Group 2), 92 % were positive in the AccuProbe™ test and 100 % in the *ply* PCR test. Of the atypical optochin-resistant but encapsulated isolates (Group 3), 80 % (12/15) were positive in the AccuProbe™ and *ply* PCR tests, but the results were not identical. In this group, four out of five blood isolates were positive in the AccuProbe™ and *ply* PCR tests; one was negative in both tests and weakly positive in the capsular reaction test. Of the equivocal optochin-resistant, unencapsulated isolates (Group 4), 22 % (8/36) were positive in the AccuProbe™ test and 42 % (15/36) in the *ply* PCR test. The only blood isolate in this group was negative according to both nucleic acid methods.

Table 5. ***Simultaneous results of the bile test, AccuProbe™ test and ply PCR test in four groups of isolates***.

	Group	N	Bile test (+/-)	AccuProbe™ (N)		*ply* PCR (N)	
				+	-	+	-
1	Optochin S [1] Capsule + [3]	24	+	24	0	24	0
			-	0	0	0	0
2	Optochin S Capsule – [4]	25	+	19	0	19	0
			-	4	0	4	0
			-	0	2	2	0
3	Optochin R [2] Capsule +	15	+	11	0	11	0
			+	0	1	1	0
			-	0	2[5]	0	2[5]
			-	1[5]	0	0	1
4	Optochin R Capsule -	36	+	2	0	2	0
			+	0	4	4	0
			+	0	2	0	2
			-	0	18	0	18
			-	5	0	5	0
			-	0	4	4	0
			-	1	0	0	1

1) sensitive, 2) resistant, 3) positive capsule reaction, 4) negative capsule reaction, 5) weak capsule reaction

5.3 Comparison of genetechnological methods in the identification of optochin-resistant pneumococcus-like isolates (II)

The reference strains that were used were identified correctly by all phenotypic and genetechnological methods, but the isolates of *S. pneumoniae* and *S. sanguinis* had a different *RsaI* restriction pattern compared with the mitis group-specific *RsaI* ARDRA pattern. Among the 49 optochin-resistant pneumococcus-like strains, 11 optochin-resistant but capsulated strains were identified as pneumococci by two phenotypic (bile solubility, the presence of a capsule) and genetechnological (PCR based on genes of *ply*, *lytA* and *psaA,* AccuProbe™ and ARDRA) techniques. Twenty of the 38 optochin-resistant, unencapsulated isolates were identified as non-pneumococci by all techniques, but due to the contradicting phenotypic and genotypic results, 18 of the 38 optochin-resistant, unencapsulated isolates could not be unequivocally identified. AccuProbe™ and *Rsa*I restriction were always in agreement and *psa*A PCR was always in agreement regarding encapsulation.

5.4 Comparison of phenotypic and nucleic acid-based methods in the identification of nasopharyngeal streptococcal isolates (III)

Ninety-seven α-hemolytic streptococcal isolates were divided into four groups according to their optochin sensitivity and the presence of a capsule for a comparison of the identification methods (Table 6). A positive capsular quellung reaction with omniserum was originally demonstrated in 59 isolates, but one of them proved to be false positive in later analysis. When all of the 65 (Groups 1 and 2) optochin-sensitive isolates were serotyped to the type/group level, 64 isolates were shown to have a capsule representing 16 different serotypes/groups and one isolate was unencapsulated. Thus, seven discrepancies were found between the capsular reactions with omniserum and serotyping. Of the seven omniserum-negative isolates (Group 2), however, six were serotypable and only one isolate was really unencapsulated and identified as pneumococcus by bile solubility and the AccuProbe™, conventional, and real-time *ply* PCR tests, but as *S. mitis* by API 20 STREP. All the optochin-sensitive isolates were bile-soluble, and two of the optochin-resistant isolates were also bile-soluble (2/32). The results of identification by API 20 STREP showed that 56 of the 97 strains were pneumococci, while 41 were identified as other streptococci: 23 *S. mitis*, 9 *S. sanguis*, 1 *S. salivarius* and 2 *Gemella morbillorum*. Six isolates remained without identification of species.

Table 6. *Results of tube bile solubility, API 20 STREP system, AccuProbe™, and conventional and real time ply PCR tests in four groups of isolates.*

	Group	N	Bile solubility (+/-)	API 20 STREP (+/-)	AccuProbe™ (+/-)	*ply* PCR (+/-)	Real-time *ply* PCR (+/-)
1	Optochin S [1)] Capsule + [2)]	58	58/0	47/11	58/0	58/0	58/0
2	Optochin S Capsule – [3)]	7	7/0	5/2	7/0	7/0	7/0
3	Optochin R [4)] Capsule +	1	0/1	0/1	0/1	0/1	0/1
4	Optochin R Capsule –	31	+ 2 –29	0/2 4/25	0/2 1/28	1/1 7/22	0/2 0/29

[1)] sensitive, [2)] positive capsule reaction with Pnc omniserum, [3)] negative capsule reaction with Pnc omniserum, [4)] resistant, +: positive/suggestive of Pnc, – : negative/not suggestive of Pnc

Identification results obtained with AccuProbe™ and a real-time *ply* PCR were almost identical. AccuProbe™ identified as pneumococcus one isolate in Group 4 (optochin-resistant, unencapsulated). The conventional *ply* PCR method detected the *ply* gene in all 65 optochin-sensitive isolates (Groups 1 and 2) and in eight optochin-resistant isolates (Group 4). All 65 isolates in Groups 1 and 2 were also positive according to the real-time *ply* PCR, and they had quickly rising amplification curves typical for pneumococcus. The only isolate in Group 3 as well as 22 of the 31 optochin-resistant isolates in Group 4 could not to be amplified at all.

The sensitivities, specificities, and agreement in κ-values of each test compared with the optochin test are found in Table 2 in the original publication. The strength of agreement between the optochin test and the other tests, measured by κ-statistics, was ‘very good’, except in the case pf API 20 STREP and conventional *ply* PCR, for which ‘good’ agreement was obtained.

5.5 Real-time *ply* PCR with melting curve analysis differentiates pneumococcus from other α-hemolytic streptococci

Nine isolates in Group 4 (Table 6.) produced some amplification products, but all these isolates had exceptionally low amplification curves compared with those of 65 optochin-sensitive isolates. The amplification curves of these nine isolates were similar to each other. In further analysis using a real-time *ply* PCR combined with melting curve analysis of all the amplification products, all nine of these isolates could be amplified in a PCR, but a slightly different product melting temperature was seen. A sequence analysis of the purified real-time *ply* PCR products of these nine isolates showed that the sequences closely resembled the *ply* sequence, with the exception that they all contained several point mutations in the 206-bp region studied. Three mutations were identical in all the isolates, and two of these (G→A and C→T) were located at the binding site of one of the hybridization probes. Four isolates also had a mutation (C→T) at the binding site of the other probe. The three optochin-sensitive isolates tested for comparison did not contain any mutations in the region studied.

5.6 Survival of *S. pneumoniae, H. influenzae* and *M. catarrhalis* in an STGG medium (IV)

S. pneumoniae and *M. catarrhalis* isolates survived freezing at –80°C and 18 cycles of thawing over a period of three years without any change in the number of colonies. The number of *H. influenzae* colonies decreased after 15 months. The number of colonies of all the bacteria decreased at –20°C, and only *S. pneumoniae* survived the whole study period. The number of pneumococci did not effect the survival of the bacteria. Also, in the mixture of the three species, *S. pneumoniae* and *M. catarrhalis* survived well at –80°C for three years with 18 cycles and *H. influenzae*, with a decreased number of colonies, for up to two years. The number of colonies decreased earlier at –20°C than at –80°C, and only *M. catarrhalis* survived the whole study period. Table 7. shows the growth of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* in nasopharyngeal swabs in an STGG medium cultured after one and a half years of storage at –80°C, compared with the original cultures. The results of the repeat and original cultures were almost identical.

Table 7. *Growth of S. pneumoniae, H. influenzae, and M. catarrhalis from 100 NP swabs in an STGG medium cultured after storage at – 80 ℃ for 1.5 years compared with original cultures.*

Original number of colonies	Number of NP swabs with *n* colonies after storage			
	0	<100	>100	Total
S. *pneumoniae*				
0	68	1		69
<100		8	3	11
>100			20	20
Total	68	9	23	100
H. influenzae				
0	91	1		92
<100	1	3		4
>100			4	4
Total	92	4	4	100
M. catarrhalis				
0	62	1		63
<100	2	8		10
>100		4	23	27
Total	64	13	23	100

6 DISCUSSION

The results of the present study show that the optochin sensitivity test is still a reliable and practical test for identifying pneumococcus from invasive as well as respiratory tract infections, and even from nasopharyngeal specimens that may contain high numbers of α-hemolytic streptococci. Nucleic acid methods such as the PCR method, based on amplification of the genes (*ply*, p*saA*, *lytA*) that encode pneumococcal virulence factors, and methods based on the detection of the 16S rRNA gene (AccuProbe ™*Streptococcus pneumoniae*, ARDRA) provide new possibilities for pneumococcal identification. However, the results of this study further show that these methods may misidentify as pneumococcus also those closely related streptococci that harbour elements of pneumococcal genes. The *ply* gene that is most commonly used in the detection of pneumococcus by means of PCR methods continues to be a suitable gene for identifying pneumococcus, since the real-time *ply* PCR confirmed by melting curve analysis can be used to exclude false positive *ply* PCR results.

Our study material included isolates that were optochin-sensitive, capsulated or unencapsulated and optochin-resistant, capsulated or unencapsulated isolates (in paper I and II). Thus, the study material contained a large proportion of difficult isolates sent to the reference laboratory for identification and serotyping, and does not represent typical isolates of either a clinical bacteriologic laboratory or a reference laboratory, and discrepant test results are therefore common in this study. The reason for this kind of selection was the need to test the methods used to identify "difficult" untypical pneumococci. The study material in paper III represents common nasopharyngeal isolates, although the proportion of capsulated isolates was higher than usual. However, a real-time *ply* PCR with melting curve analysis proved to be useful in differentiating pneumococcus from other α-hemolytic streptococci, and the method might be especially useful in certain circumstances like pneumococcal conjugate vaccine studies when very accurate pneumococcal identification is needed.

6.1 Conventional, phenotypic identification methods

According to our earlier experience in the bacteriological laboratory, the density of an inoculum (number of colonies) and the optochin discs that are used may influence the optochin sensitivity test result. Therefore, the conditions (inoculum, different commercial products) of the optochin test were investigated carefully to obtain a firm basis for the following classification of isolates according to optochin sensitivity. Wasilauskas and Hampton (Wasilauskas & Hampton 1984) have presented that inadequate numbers of colonies may lead to an erroneous result in optochin sensitivity, whereas our results suggest that the density of colonies has only a small effect on the result of the optochin sensitivity test. However, when a heavy inoculum is used, the diameter of the optochin sensi-

tivity test is smaller than when a light inoculum is used, and in borderline cases the result of the optochin sensitivity test should be interpreted as sensitive or the test should be repeated. When this study started there were at least four commercial manufacturers of optochin discs. In our experience the Biodisk disc seemed to be more reliable than the other products, and it was not too sensitive to the effect of inoculum size.

Although identification tests based on phenotypic characteristics of pneumococcus are reliable and commonly used to identify pneumococcus (Mundy *et al.* 1998, Ruoff *et al.* 2003), some identification problems exist. Optochin-resistant pneumococcal isolates were found already in the early days (see White 1938, Bowers & Jeffries 1955) as well as more recently (Kontiainen & Sivonen 1987, Pikis *et al.* 2001), unpublished data from the reference laboratory for pneumococcus). It is also well known that some typical pneumococci are unencapsulated (Finland & Barnes 1977, Broome & Facklam 1981, Carvalho *et al.* 2003), and on the other hand, several α-hemolytic streptococci (Austrian 1976, Holmberg *et al.* 1985) as well other bacteria (Zepp & Hodes 1943, Robbins *et al.* 1972, Heidelberger & Nimmich 1972) produce capsules which cross-react with *S. pneumoniae* serotypes (Lund & Henrichsen 1978). The quellung reaction, the most reliable method for demonstrating the capsule, is not commonly used in clinical bacteriological laboratories. Interpretation of the capsule reaction requires considerable experience, as also seen in our laboratory: three positive but weak reactions (3/100) were probably misinterpreted (paper I, II), and seven discrepancies were found between the capsular reactions with pneumococcal omniserum and actual pneumococcal serotyping (paper III).

Bile solubility and optochin sensitivity have shown to have almost complete correlation (Bowers & Jeffries 1955), but in 10 % of cases the interpretation was considered uncertain (Lund 1959). In our study (paper II), bile solubility results obtained in two laboratories were not similar, and therefore only the results of the reference laboratory were presented. The results of the present study suggest that bile solubility is a useful confirmatory test, but it could not be used as the only method to differentiate pneumococcus from other streptococci. However, Arbique and coworkers (Arbique *et al.* 2004) suggest that the bile solubility test differentiates pneumococcus from *S. pseudopneumoniae* (optochin-resistant, unencapsulated, bile-insoluble, AccuProbe™-positive and *ply* and *sodA*-positive PCR) and that bile solubility is more specific than optochin sensitivity in identifying pneumococcus.

Biochemical identification of pneumococcus has been proved to be quite difficult, and additional tests such as optochin sensitivity were recommended (Colman & Ball 1984, Appelbaum *et al.* 1986). If two bacterial strains carry the same enzymic activities, it is difficult to say whether they produce exactly the same enzyme molecules or whether they produce similar enzymic activities produced by different molecules (Norris 1968). In the latter situation there is no direct relationship at the genetic level. Many strains that were identified biochemically as *S. mitis* were found to be difficult to differentiate from *S. oralis* and *S. pneumoniae*, even with the quantitative DNA-DNA hybridization method,

because clinical strains strongly hybridized with both *S. mitis* and *S. oralis* and sometimes *S. pneumoniae* (Kawamura *et al.* 1995). These three species have more than 99 % sequence homology with each other, and thus they are closely related (Kawamura *et al.* 1995). In our first study (paper I), no biochemical tests were used in the characterization of the isolates, and no further classification of "nonpneumococcal" isolates could thus be given. Later, in paper III, we defined bacterial isolates also biochemically, and these results confirm our earlier experience that biochemical tests are not very useful in differentiating pneumococcus from other α-hemolytic streptococci.

6.2 Nucleic acid methods

The hybridization test, AccuProbe™ for pneumococcus, has been used and suggested as a "gold standard" for identifying pneumococcus (Denys & Carey 1992, Geslin *et al.* 1997, Mundy *et al.* 1998). Later studies have shown, however, that identification or definition of pneumococcus is a more complicated issue (Whatmore et al. 2000, Arbique et al. 2004, Hanage et al. 2005). The main results of our study are in agreement with those of Mundy and coworkers (Mundy et al. 1998) and Whatmore and coworkers (Whatmore et al. 2000). In our study, AccuProbe™ for pneumococcus correctly identified unequivocal pneumococci (optochin-sensitive, bile-soluble, quellung reaction-positive), but not all optochin-sensitive, unencapsulated isolates. Moreover, the results of Mundy as well our results show discrepancies among equivocal isolates (inconsistent or discordant combinations of optochin sensitivity, bile solubility and quellung reaction positivity). Mundy presented that almost 30 % were positive in the AccuProbe™ test, but only less than 3 % in the quellung reaction, and ten of the AccuProbe™ positive isolates had an intermediate optochin result as their only phenotypic characteristic. In our study, AccuProbe™ identified as pneumococci some optochin-resistant, unencapsulated isolates (papers I, II) as well as one optochin-resistant, unencapsulated, bile-insoluble isolate, which was identified biochemically as *S. mitis* (paper III). It is possible that these isolates represent a new streptococcal species such as *S. pseudopneumoniae* (Arbique et al. 2004). Although AccuProbe™ and *RsaI* digestion of the 16S rRNA gene appear to enable identification of all genuine *S. pneumoniae* isolates, they cannot exclude closely related nonpneumococcal *S. mitis* strains that have (nearly) identical 16S rRNA genes, resulting in "false positive" AccuProbe™ and ARDRA results.

The primers described by Morrison and coworkers (Morrison *et al.* 2000) that enabled amplification of the *psa*A gene from all 90 serotypes of *S. pneumoniae* and were used in this study appear to be very *S. pneumoniae*-specific. Only genuine pneumococci and the optochin-resistant but capsulated pneumococci gave amplification of *psa*A (see Table 1. in paper II). This *psa*A PCR positivity was always in agreement with capsulation. The specificity of the *lyt*A PCR was high as well. AccuProbe™ and ARDRA were considered to be somewhat less specific, but these approaches largely coincided with the presence of pneumococcal virulence genes and therefore retain their value.

Demonstration of Ply (Cima-Cabal *et al.* 1999) or the *ply* gene (Salo *et al.* 1995, Kearns *et al.* 2000) have been used as methods for detecting and identifying pneumococcus. In these studies, the presence of *ply* has been suggested to be specific for pneumococcus. However, in addition to typical optochin-sensitive, bile-soluble, capsulated pneumococci, these methods have also identified the *ply* gene in atypical pneumococci (Cima-Cabal *et al.* 1999, Kearns *et al.* 2000). Moreover, even other α-hemolytic streptococci and atypical "pneumococcus-like" bacteria sometimes had the *ply* gene (Whatmore *et al.* 2000). Our results (papers I, II and III) show that some isolates without any other characteristics of pneumococcus contained the *ply* gene in the conventional *ply* PCR test. However, the real-time *ply* PCR with melting curve analysis developed in our laboratory proved to be a specific method in discriminating the *ply* gene of pneumococcus from those in other α-hemolytic streptococci: the real-time *ply* PCR with melting curve analysis was able to exclude false positive *ply* findings, since isolates containing as few as two mutations in the target *ply* sequence of the probes produced aberrant amplification curves (paper III).

6.3 Storage and transport medium for pneumococcus

As both identification of problematic isolates and serotyping of pneumococci are usually done in specialized laboratories, the survival of three nasopharyngeal pathogens during storage and transport was also studied. Our results confirm and extend the findings of O'Brien and coworkers (O'Brien et al. 2001) by demonstrating that an STGG medium is excellent for deep-freeze storage of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* for long periods. It can also be used for storage at –20°C for shorter periods, even in conditions with a less than ideal electricity supply. The use of an STGG medium has several benefits, including enhanced recovery, long-term storage of original NP specimens at –70°C without any loss of CFU, and the possibility of testing NP specimens as batches. On the basis of the present study results, we fully agree with the conclusions of O'Brien and coworkers (O'Brien et al. 2001) on the usefulness of an STGG medium in the storage and transport of pneumococcus, extending them further to two other respiratory tract pathogens, *H. influenzae* and *M. catarrhalis*. Moreover, our observations over several years indicate that bacterial isolates stay viable in an STGG medium longer than the three studied years shown, and that an STGG medium is also suitable for transporting the isolates (Herva *et al.* 2006).

6.4 What is pneumococcus – does it need to be redefined?

On the basis of phenotypic identification methods, pneumococcus has been and still is defined as an optochin-sensitive, bile-soluble, usually encapsulated α-hemolytic streptococcus. In a Gram stain it appears as gram-positive diplococci that sometimes form short chains. Colony morphology on blood agar is typically smooth, glistening and doughnut-

like. With these conventional criteria, most pneumococcal isolates, especially when from blood or cerebrospinal fluid, can easily and reliably be identified and discriminated from other α-hemolytic streptococci that are generally considered to be less pathogenic and virulent than pneumococcus.

As a result of common genetic transformation - the exchanges of virulence factors among streptococcal species - untypical pneumococci and new, closely related streptococcal species such as *S. sinensis* (Woo *et al.* 2002) and *S. pseudopneumoniae* (Arbique *et al.* 2004) have emerged and led to problems in pneumococcal identification and, accordingly, to the development of molecular microbiological methods of identification. At least three main molecular approaches have been applied: 1) identification of pneumococcus by hybridization with a gene probe based on the 16S rRNA sequence, 2) identification of pneumococcus (actually pneumococcal clones) by sequencing housekeeping genes (MLST) and 3) demonstration of genes for pneumococcal virulence factors, such as *ply*, *psaA* and *lytA*. The question is: can these new molecular methods provide us with new definitions for pneumococcus?

Although a commercial gene probe test for identifying pneumococcus (AccuProbe™) on the basis of the 16S rRNA gene seems to enable identification of all genuine *S. pneumoniae* isolates, it cannot exclude closely related nonpneumococcal *S. mitis* or other streptococcal strains that have (nearly) identical 16S rRNA genes, resulting in "false positive" AccuProbe™ results. In these cases, conventional identification tests might be helpful. However, in the present study, AccuProbe™ misidentified several "difficult" atypical strains as pneumococcus. MLST, a rather time-consuming, complicated and expensive method can, in certain cases, be applied to confirm pneumococcal identification, even though it is most useful for identifying pneumococcal clones. Complete resolution of pneumococci from the sometimes closely related α-hemolytic streptococci is problematic due to recombination between them. However, a tree based on the concatenated sequences of the MLST loci in most cases unambiguously distinguishes whether an unencapsulated or otherwise atypical isolate is or is not a pneumococcus (Hanage *et al.* 2005).

Demonstration of the genes of pneumococcal virulence factors such as *psaA*, *lytA* and *ply* (Salo *et al.* 1995, Morrison *et al.* 2000, McAvin *et al.* 2001), e.g. by using a PCR, has been suggested to be helpful in identification. Several recent studies have, however, shown that these virulence factors can also be found in α-hemolytic streptococci and, thus, the presence of these genes cannot be used as the sole method for identifying pneumococcus. Luckily enough, both the *lytA* and *ply* genes, when present in α-hemolytic streptococci, seem to have mutations and can on this basis be distinguished from pneumococci: we showed in the present study that the melting points of the amplification products of the *ply* gene are different in α-hemolytic streptococci and pneumococcus due to minor mutations in the *ply* gene. Additionally, Llull and coworkers (Llull *et al.* 2006) recently showed that all the *S. pneumoniae* strains they tested harboured typical *lytA* alleles, whereas nonpneumococcal isolates belonging e.g. to the mitis group always carried

atypical alleles. However, further studies are needed to determine if these mutations are exclusively seen only in α-hemolytic streptococci.

Due to the common transfer of genetic material between pneumococcus and α-hemolytic streptococci, it is possible that no single perfect method for pneumococcal identification can be developed. In the case of equivocal pneumococci, molecular methods may help in identification, but in certain cases definite identification may still be missed.

7 CONCLUSIONS

The reason for starting this study was the practical need to solve the identification problem concerning some of the isolates that were sent to the National Reference Laboratory for Pneumococcus (National Public Health Institute, Oulu). Identification of suspected pneumococcal isolates lacking one or more of the typical characteristics of pneumococcus was found to be problematic. Carefully performed, the optochin sensitivity test is still the basic method used to differentiate pneumococcus from other α-hemolytic streptococci, even among nasopharyngeal pneumococcal isolates. Other phenotypic identification tests - the detection of a capsule and bile solubility - can be used as confirmation methods.

Genetechnological methods, for example based on the demonstration of pneumococcal genes, provide new possibilities for identifying *S. pneumoniae*, but their use may lead to misidentification of too many α-hemolytic streptococci as pneumococcus. The present study suggests that a real-time *ply* PCR with melting curve analysis, which discriminates *ply* genes with mutations, can be used to exclude false positive *ply* PCR findings among α-hemolytic streptococci. On the basis of this study, the following flow chart for identification is recommended (Fig. 8).

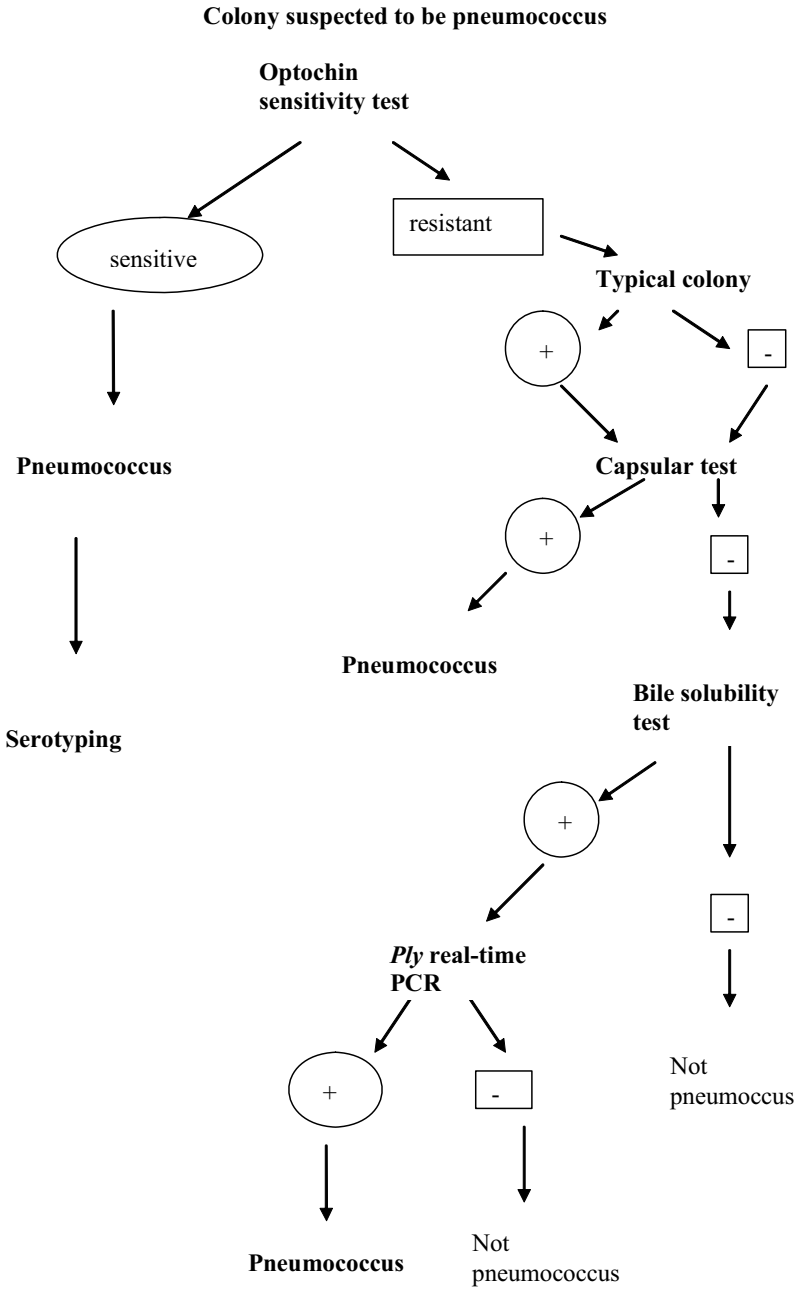

Fig. 8. Flow chart for the identification of pneumococcus

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