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Masterproef

Value of pneumococcal PCR in diagnosis of parapneumonic pleural effusion

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CLINICAL BOTTOM LINE

Streptococcus pneumoniae is the most common causative bacterial pathogen of community-acquired pneumonia in children. Pleural empyema is an increasingly reported complication of pneumonia in children. A definitive diagnosis requires the isolation of *S. pneumoniae* from normally sterile body sites, such as pleural fluid. However, because of previous administration of antibiotics, routine bacterial culture remains often negative. In this study, the clinical value of the pneumococcal autolysin gene (*lyt*A gene) polymerase chain reaction (PCR) for the diagnosis van pneumococcal pneumonia and empyema was evaluated with 31 pleural fluid samples. *Streptococcus pneumoniae* is divided into 93 serotypes, only a few of which are responsible for most cases of invasive pneumococcal disease (IPD). Serotyping of pneumococcal isolates is important for the development of future conjugate vaccines and to evaluate their efficacy. Currently, pneumococcal serotyping is dependent on isolation of the organism, followed by serological determination by quellung reaction (capsular swelling). However, the quellung reaction is labor-intensive, and several new approaches have appeared. In this study, we evaluated a serial multiplex PCR approach for identification of serotypes of pneumococci, performed on culture isolates and on pleural fluid samples.

CLINICAL/DIAGNOSTIC SCENARIO

Streptococcus pneumoniae is one of the major bacterial pathogens causing severe infections with high morbidity and mortality[1]. The organism causes more than 1,2 million deaths each year in children due to sepsis, meningitis and pneumonia (CDC, 1997). The frequency of empyema in children has increased worldwide over the last decade. This increase could be related to IPD caused by emergent nonvaccine replacement serotypes, particularly serotypes 1,3,7 and 19A after the introduction of the 7-valent pneumococcal conjugate vaccine (PCV7; Prevenar 7^{TM})[2].

The gold standard for diagnosis of IPD is currently based on culture methods, which needs up to 48-72h to confirm the diagnosis and may have a low sensitivity, especially after antibiotic therapy. Pretreatment of children with antibiotics can prevent the growth of *Streptococcus pneumoniae* in cultures[3]. The low rate of pathogen detection complicates clinical care and selection of appropriate antibiotics. Polymerase chain reaction is a new diagnostic method that offers many potential advantages: results are positive early in the course of infection, it is less influenced by previous antibiotic therapy or unsatisfactory conditions of transport and storage, and it is not dependent on a host response[4].

To investigate the apparent failure of PCV7 in reducing the incidence of pneumonia with empyema, it is important to investigate an ecological shift in non-vaccine types associated with parapneumonic pleural effusions obtained after universalization of PCV7[5]. Serotype surveillance will continue to be necessary in vaccinated and non-vaccinated populations for evaluation of the impact and suitability of current multivalent vaccines[6].

Currently, serotype distribution is monitored by culture of the organism followed by serological determination of the capsular type by the Quellung reaction[7]. Binding of specific monoclonal antibodies with the capsule induces a capsular swelling visualized under the microscope[8]. The Quellung reaction remains the gold standard for pneumococcal serotyping, but presents major limitations: the high cost of antisera, subjectivity in interpretation of results and technical expertise requirements[7]. The immunochemistry of the capsular polysaccharide differentiates into 93 distinct serotypes, but only about 15 serotypes cause the majority of IPD worldwide[9].

A major limitation of pneumococcal serotyping, is that most samples remain culture negative due to widespread use of antibiotics prior to presenting to the hospital[10]. Direct detection of selected serotypes in clinical samples could be a valuable aid in surveillance, particularly in situations where culture is insensitive[11]. Multiplex PCR-based methods for determining capsular serotypes of *S. pneumoniae* offer an alternative approach for the surveillance of pneumococcal disease[9].

The purpose of our study was to redevelop a sequential multiplex PCR procedure, modified on the method of Pai et al.[11], to identify the most frequent serotypes of *S. pneumoniae* isolated in Belgium.

QUESTION(S)

The objectives of this study were:

1) **Evaluate a rapid quantitative real-time PCR for the detection of *Streptococcus pneumoniae* in pleural fluids**
2) **Usefulness of pneumococcal antigen detection (Binax NOW®) in pleural fluids for rapid diagnosis of infection by *Streptococcus pneumoniae***
3) **Capsular serotyping of *S. pneumoniae* by molecular techniques, directly on cultures**
4) **Capsular serotyping of *S. pneumoniae* by molecular techniques, directly on pleural fluids**
5) **Comparison of cost-effectiveness and time-effectiveness between Quellung reaction and Multiplex PCR reaction for capsular serotyping**

SEARCH TERMS

1) *MeSH Database (PubMed): MeSH term: "Streptococcus pneumoniae, capsular serotyping, pleuropneumonia "*
2) *PubMed Clinical Queries (from 1966; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi): Systematic Reviews; Clinical Queries using Research Methodology Filters (diagnosis + specific, diagnosis + sensitive, prognosis + specific)*
3) *Pubmed (Medline; from 1966), SUMSearch (http://sumsearch.uthscsa.edu/), National Guideline Clearinghouse (http://www.ngc.org/), Institute for Clinical Systems Improvement (http://www.icsi.org), The National Institute for Clinical Excellence (http://www.nice.org.uk/), Cochrane (http://www.update-software.com/cochrane, Health Technology Assessment Database (http://www.york.ac.uk/inst/crd/htahp.htm)*
4) *National Committee for Clinical Laboratory Standards (NCCLS; http://www.nccls.org/), International Federation of Clinical Chemistry (IFCC; http://www.ifcc.org/ifcc.asp), American Diabetes Association (ADA; http://www.diabetes.org/home.jsp), National Diabetes Information Clearinghouse (NDIC; http://diabetes.niddk.nih.gov/), Westgard QC (http://www.westgard.com), Clinical Laboratory Improvement Amendments (CLIA; http://www.cms.hhs.gov/clia/)*
5) *UpToDate Online version 12.2 (2004)*

1) Evaluation of a rapid quantitative real-time PCR for the detection of *Streptococcus pneumoniae* in pleural fluids

Patients and methods

We retrospectively studied samples from 31 children and adolescents (less than 18 years of age) with parapneumonic pleural effusions who attended the UZ Leuven hospital, between April 2008 and November 2010. Molecular testing for *Streptococcus pneumoniae*, targeting the autolysin gene (*lyt*A), was performed on archived pleural fluid from these children. Children were included in this study if they had confirmed parapneumonic pleural effusion, bacterial cultures were performed and a residual pleural fluid specimen was available.

Clinical findings among patients with parapneumonic pleural effusions

The median age of 31 children tested was 5,8 years (range: 9 months till 17 years). Only 9,7% had a preexisting medical condition (chromosomal disorder, kidneytransplant, mental retardation with epileptic insults). Patients had a median length of illness prior to presentation of 8,8 days (range: 3-60 days) and 80,6% had been pretreated with antibiotics when their pleural fluid was sampled. The children had a median length of hospital stay of 17 days (range: 4-52 days) and three patients required care in the intensive care unit (ICU). Two children died of their infection.

Extraction of DNA+ PCR reaction

Bacterial genomic DNA was extracted from samples using the NucliSENS® easyMAG® (bioMérieux) according to the manufacturer's instructions. TaqMan fluorescent probes and specific primers for the *lyt*A gene of *Streptococcus pneumoniae* were used[12].
The real-time amplification was performed in 40 µl reaction volumes containing 10 µL *lyt*A primer/probe mix, primers at a concentration of 250nM, a FAM/TAMRA-labeled probe was used at a concentration of 200nM. Ten µL of DNA extract was used for each reaction. DNA was amplified in a an ABI 7500 Sequence Detection System device (Applied Biosystems) using the following cycling parameters: 50°C for 2 min, 95 °C for 10 min, followed by 45 cycles of a two-stage temperature profile of 95°C for 15s and 60°C for 1 min.

Results

Pleural fluid specimens from 31 children were available for culture and PCR. All cultures, except two, were negative. *Streptococcus pyogenes* was isolated from pleural fluid in 1 patient, *Proteus mirabilis* from pleural fluid in another patient. Compared with culture, PCR testing significantly increased detection of *S. pneumoniae (22/31 samples, 71%).* Two samples showed inhibition of the PCR reaction. One of these inhibited samples had a positive culture for *S. pyogenes*. Five of thirty (16,6 %) pleural fluids remained negative by PCR, probably due to infection by other pathogens.

Table 1. Comparison of diagnostic results of culture and PCR for S. pneumoniae in samples of pleural fluid (n=31)

	Positive PCR	Negative PCR	Inhibition PCR	Total
Positive Culture	0	0	0	0
Negative Culture	23	5	3	30
Total	23	5	3	**31**

To determine if pleural fluid samples of patients with *S. pneumoniae* detected by PCR were associated with a high percentage of neutrophils in pleural fluid samples, we made a comparison of WBC differentiation and PCR results (table 2).

Table 2. Comparison of WBC differentiation and PCR results(n=31)

	Differentiation				
	Neutrophils (>80%)	**Mixed**	**Lympho-monocytes (>80%)**	**No differentation**	**Total**
Positive PCR	16	2	1	4	23
Negative PCR	**1 (PRMI)**	2	0	2	5
Inhibition PCR	**1 (SRPY)**	2	0	0	3
Total	18	6	1	6	**31**

The Gram stain of pleural fluid is useful because it rapidly detects gram-positive cocci suggestive of *S. pneumoniae*. However, direct examination is a poorly sensitive test for the diagnosis of pneumococcal empyema, but has a good positive predictive value. In our study none of the PCR-positive samples were positive for gram-positive cocci on Gram stain (table 3).

Table 3. Comparison of gram stain and PCR results (n=31)

	Gram stain				
	Negative	**PMN cells ++**	**PMN++, GPC++**	**No gram stain**	**Total**
Positive PCR	8	6	0	9	23
Negative PCR	2	0	0	3	5
Inhibition PCR	1	1	1 (SRPY)	0	3
Total	11	7	1	12	**31**

Figure 1 demonstrates a comparison of pleural fluid WBC count and PCR results for *S. pneumoniae*. Although a high WBC count is helpful for the diagnosis of pneumococcal pneumonia, a low count does not rule out the presence of pneumococci. In contrast, some pleural fluid WBC counts were high (> $5*10^9$/L) in patients with negative or inhibited PCR results. This may suggest that other pathogens are responsible for the high rate of WBCs in these samples. The red colored squares in figure 1 represent the identification and isolation of other organisms, *Proteus mirabilis* and *Streptococcus pyogenes* in the pleural fluids with negative and inhibited PCR results, respectively.

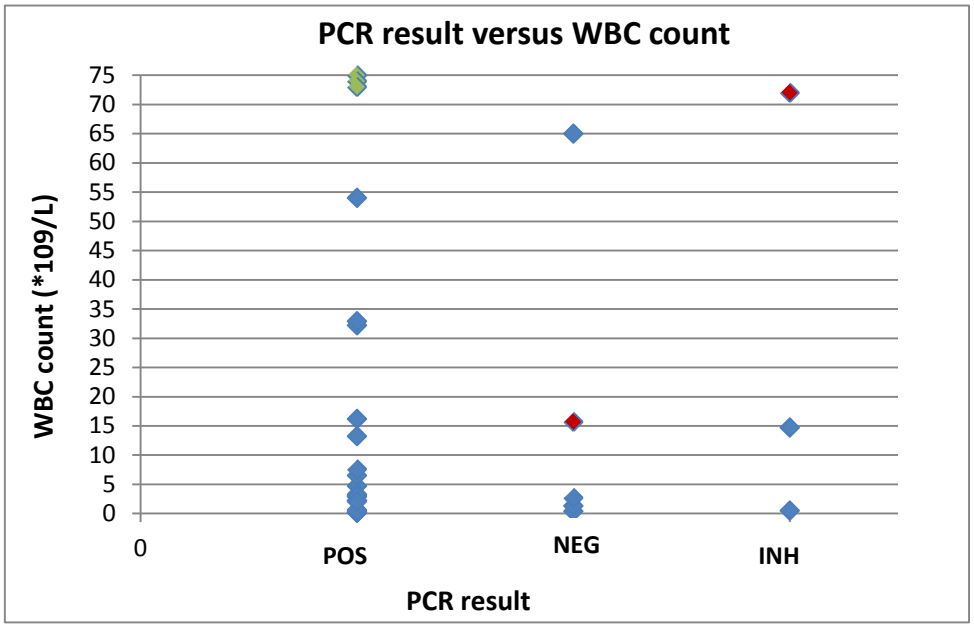


Figure 1. Correlation lytA PCR result and total WBC count

Discussion

There is a significant increase of detecting pneumococcal pneumonia and empyema in children when adding real-time PCR for diagnosis to traditional culture-based methods. PCR testing of pleural fluids for *S. pneumoniae* significantly increases pathogen identification compared with culture of blood and pleural fluid. Unfortunately, the bacteriologic study of pleural fluid, based on Gram Stain and cultures, is limited by the lack of sensitivity. All pleural fluid cultures remained negative for *S.pneumoniae*.

Because of the high percentage of patients who received antibiotics upon admission, the rate of microbiological diagnosis is low. Although culture is still the golden standard for diagnosis of *S. pneumoniae* infection, a comparison of culture and PCR is not possible because of the low culture rate. In previous studies, the presence of some false positive results has been reported, with a prevalence between 4% and 17%[4]. In our study it's not possible to evaluate the amount false positives.

Our results are in agreement with other published studies who found PCR of pleural fluid for *S. pneumoniae* to be more sensitive than culture in children and adolescents[3,4,13,14,15,16]. In these studies, different pneumococcal targets were used for real-time PCR detection. Messmer et al. determined the accuracy of 4 conventional PCR assays (*lytA*, *psaA*, and two primer sets from the *ply* gene) in the discrimination of nontypeable *Streptococcus pneumoniae* from closely related atypical streptococci (table 4)[17]. The *lyt*A primers were the most specific with 100% specificity for all strains tested.

Carvalho et al. developed three novel real-time PCR assays for the detection of specific sequence regions of the *lyt*A, *ply* and *psa*A genes[12]. The assays targeting the *lyt*A and *psa*A genes were the most specific for the detection of isolates confirmed to be *S. pneumoniae*, with *lyt*A showing the greatest specificity. The use of the *ply* gene for the detection of pneumococci can lead to false-positive reactions in the presence of pneumococcus-like viridans group streptococci. The three assays performed on 15 culture-positive cerebrospinal fluid samples showed a 100% sensitivity. Because of the best specificity of the *lyt*A assay, we implemented the *lyt*A real-time PCR as assay of choice for the detection of pneumococcal DNA.

Table 4. Specificity of PCR assays (Messmer et al.)

	Encapsulated pneumococci	**Nontypeable pneumococci**	**Atypical *Streptococci***	**Closely related viridans and *D. pigrum***
lytA	40/40 (100%)	4/4 (100%)	0/16 (100%)	0/35 (100%)
psaA	40/40 (100%)	4/4 (100%)	1/16 (96%)	0/35 (100%)
IAIB *ply*	40/40 (100%)	4/4 (100%)	8/16 (50%)	0/35 (100%)
IIAIIB *ply*	40/40 (100%)	4/4 (100%)	16/16 (0%)	0/35 (100%)

2) Usefulness of pneumococcal antigen detection (Binax NOW®) in pleural fluids for rapid diagnosis of infection by Streptococcus pneumoniae

Binax NOW *Streptococcus pneumoniae* test (Inverness Medical) is a rapid immunochromatographic membrane assay; which detects the C polysaccharide cell wall antigen of *S. pneumoniae.* The results are available within 15 min. The test has been validated for rapid diagnosis of pneumococcal pneumonia, based on urine samples and of *S. pneumoniae* meningitis, based on cerebrospinal fluid samples. This test has not been routinely used for testing pediatric pleural fluid, because it has not been validated for this indication. In our study, we evaluate the Binax NOW test on the same pleural fluid samples collected from 31 children admitted to UZ Leuven hospital for thoracic empyema, by comparison of the *lyt*A PCR.

The Binax NOW antigen test was applied to pleural fluid samples according to the manufacturer's instructions for urine and CSF samples. Binax NOW uses a rabbit anti-*S. pneumoniae* antibody adsorbed onto nitrocellulose membrane (the Sample Line). Goat anti-rabbit immunoglobulin G (control antibody) is adsorbed onto the same membrane as a second stripe. The rabbit anti-*S. pneumoniae* antibody are conjugated to visualizing particles to bind soluble pneumococcal antigen (C polysaccharide) present in the sample. To perform the test, a swab is dipped into the specimen, removed and the inserted into the test device; a buffer solution is added and the device is closed. A positive test result, read in 15 minutes, will include the detection of both a sample and a control line. Color on the control line alone indicates a negative test. Absence of color on the control line indicates an invalid test. Each kit contains positive and negative control swabs.

Le Monnier et al. studied 40 children with pneumococcal empyema and compared pleural fluid culture and 16S rDNA to the Binax NOW test[18] (Table 5).They found an overall sensitivity of 90% and specificity of 95% in pleural fluid. Similarly, Ploton et al. found high sensitivity and false positive results due to cross-reactions with *Streptococcus oralis* and *Streptococcus salivarius* in 69 samples of pleural effusion[19]. As PCR has significant advantages over culture, as stated above, but the technique is not available in all hospitals or throughout the day. Thus, a rapid, reliable bedside test for diagnosis of pleural effusion with a possible pneumococcal origin, is an attractive alternative in the clinical setting.

Table 5. Literature review: Binax NOW testing in pleural fluids

Binax NOW in pleural fluids						
Author	**Year**	**Country**	**No of samples**	**PCR**	**Sensitivity**	**Specificity**
Le Monnier et al.[18]	2006	France	40	16S rDNA PCR	97,5%	95%
Ploton et al.[19]	2006	France	69	16S rDNA PCR	100%	83,3%
Hernandez-Bou et al.[20]	2009	Spain	59	pneumolysin RT-PCR	100% (culture) 87,8% (PCR)	100% (culture/PCR)
Flores et al[21].	2009	Spain	73	pneumolysin RT-PCR	88%	71%
Strachan et al.[2]	2011	Australia	130	*lyt*A PCR	83,8%	93,5%

One aim of this study was to compare Binax NOW with PCR for identification of *S. pneumoniae* in pleural fluid from children hospitalized with pleural effusion. Binax NOW was used for the same 31 patients, of whom 22 had a positive PCR result for *S. pneumoniae.* We used these defrosted pleural fluid samples to test retrospectively the performance of *the S. pneumoniae* antigen test. Because of insufficient sample, one patient was excluded for Binax NOW testing. Among the 30 pleural fluid samples, 21 were positive for *S. pneumoniae* by immunochromatographic antigen detection (table 6). The sensitivity of the test was 95,5%, the specificity 100%, with a positive predictive value of 100%,

when PCR was used as the gold standard (table 7). The samples with inhibited test results by PCR reaction were excluded for this calculation. No false positive results were obtained. We only found one false negative result. The PCR reaction of this sample, however, shows a Cycle threshold (Ct)-value of 36,4, which indicates a very weak response. In conclusion, detection of pneumococcal antigen in pleural fluid by Binax NOW is a rapid and easy test for diagnosis of *Streptococcus pneumoniae* thoracic empyema.

Table 6. Binax NOW testing in pleural fluids

Nr	EMD	Pleura PCR	Ct PCR	BinaxNOW
1	040528B101	**pos**	**19,3**	**pos**
2	011208G020	neg	neg	neg
3	021202B118	**pos**	**26**	**pos**
4	040522G014	neg	neg	neg
5	030801G090	**pos**	**19,6**	**pos**
6	050101B002	**pos**	**24,1**	**pos**
7	010731B087	**pos**	**26,8**	**weak pos**
8	930702V128	INH	INH	neg
9	050424B044	**pos**	**25,6**	**pos**
10	070405G016	**pos**	**22,8**	**pos**
11	080310B010	**pos**	**18,7**	**pos**
12	001121B019	**pos**	**39,3**	**weak pos**
13	050417G025	**pos**	**24,7**	**insufficient sample**
14	050522B006	**pos**	**22,6**	**pos**
15	910710V080	INH	INH	neg
16	040529B007	**pos**	**26,7**	**weak pos**
17	020920B156	**pos**	**27,3**	**weak pos**
18	080520B006	**pos**	**19,7**	**pos**
19	071128B009	**pos**	**21,7**	**pos**
20	080324B004	**pos**	**26,4**	**weak pos**
21	980414V166	neg	neg	neg
22	060502B057	**pos**	**28,7**	**pos**
23	070808G013	**pos**	**27**	**pos**
24	081126G003	**pos**	**25,3**	**pos**
25	020320B166	neg	neg	neg
26	970405M011	neg	neg	neg
27	930702V128	**pos**	**36,4**	**neg**
28	070814G013	**pos**	**23**	**pos**
29	991206V102	INH	INH	neg
30	081208G003	**pos**	**22**	**pos**
31	010427B102	**pos**	**23,5**	**pos**

Table 7. Binax NOW in pleural fluids in patients with pleural empyema

		PCR RESULT		
		Positive	Negative	Total
BINAX	Positive	21	0	21
	Negative	1	5	6
Total		22	5	27

Sensitivity, 95,5%; specificity, 100%; PPV, 100%; and NPV, 83,3%

3) Capsular serotyping by molecular techniques, directly on cultures

The development of PCR-based serotyping systems has the potential to overcome some of the difficulties associated with the standard capsular test (Quellung reaction). Recently, the Centers for Disease Control and Prevention (CDC), United States, established a multiplex PCR protocol for the identification of pneumococcal serotypes that can be applied to clinical or research use[7]. The capsular polysaccharides synthesis pathway is encoded by the cps (capsular polysaccharide synthesis) locus[8]. The sequences of the cps loci from all the 93 known serotypes were completed recently (http://www.sanger.ac.uk/Projects/S_pneumoniae/CPS/), providing the molecular basis for identifying most commonly occurring serotypes. The first sequential multiplex PCR assay for pneumococcal serotyping on IPD isolates was developed by Pai and colleagues [11]. They have developed a seven-step sequential multiplex PCR assay that is capable of distinguishing 29 pneumococcal serotypes, and this scheme has now been expanded to 33 serotypes. This initial design was adapted by several other groups based on the IPD epidemiology in their geographical area (Table 8).

Table 8. Utilization of the multiplex PCR in different geographical areas on bacterial isolates

Bacterial Isolates							
Author	**Year**	**Country**	**Isolates**	**No of samples**	**No of primer pairs**	**PCR reaction**	**Concordance****
Pai et al.[11]	2006	USA	IPD (blood)	421	29	multiplex PCR	100%
Morais et al.[22]	2007	Mozambique	IPD (blood, CSF)/AOM*	153	29	multiplex PCR	97%
Dias et al.[6]	2007	Brazil	IPD (blood, CSF, pleural fluid)	147	30	multiplex PCR	95%
Iraurgui et al.[9]	2010	Spain	IPD	257	29	multiplex PCR	95,70%
Jourdain et al.[8]	2011	Belgium	Nasopharyngeal aspirates	332	30	multiplex PCR	95,13%
Yun et al.[7]	2011	Korea	All clinical specimens	77	30	multiplex PCR	98,70%
Miernyk et al.[23]	2011	Alaska	Nasopharyngeal samples	1135	30	multiplex PCR	94%
Vickers et al.[24]	2011	Ireland	AOM	144	11	multiplex PCR	96,90%

*AOM: acute otitis media, CSF: Cerebrospinal Fluid
** Concordance between conventional and PCR-based serotyping

We aimed to compare pneumococcal typing using PCR with the conventional quellung reaction. At first, we tried to develop a SYBR Green-based real-time quantitative PCR for detection of different capsular serotypes. SYBR Green chemistry is an alternate method used to perform real-time PCR analysis. Selection of primer pairs was performed to detect the eight most predominant serotypes in Belgium. Serotype distribution of invasive isolates (blood, cerebrospinal fluids, pleural fluids, AOM) demonstrated a large preponderance of serotype 1, followed by serotypes 19A en 7 (figure 2). Based on these serotype distribution, serotypes 1, 3, 6A/B/C/D, 7F/A, 12F/(A), 15A/F, 19A and 22F/A were chosen for PCR reaction. These 8 serotypes count for 70% of all serotypes, responsible for invasive infections in our region. In 2010, 22 out of 28 (79%) submitted pleural fluids were positive for one of these 8 serotypes. In 2011, 20 out of 25 (80%).

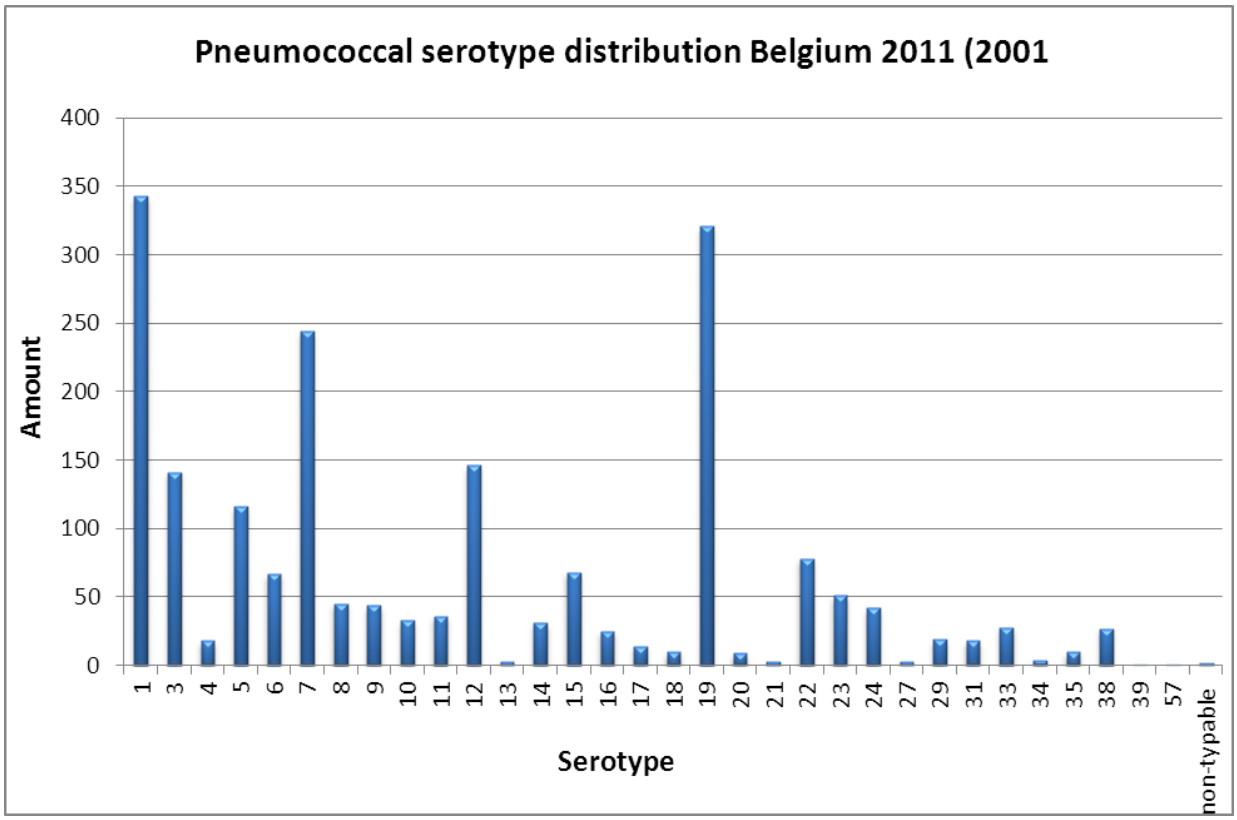


Figure 2. Pneumococcal serotype distribution in Belgium in 2011

EXPERIMENT 1: SYBR GREEN® BASED DETECTION

A SYBR-Green based detection uses SYBR Green I dye, a highly specific, double-stranded DNA binding dye, to detect PCR products as it accumulate during PCR cycles. It enables you to monitor the amplification of any double-stranded DNA sequence. No probes are required, which reduces your assay setup and running costs. Since the SYBR Green dye binds to all double-stranded DNA, an increase in fluorescence intensity is proportionate to the amount of PCR product produced[25]. A fluorometer integrated within a thermal cycler was used to acquire DNA melting curves during PCR by fluorescence monitoring of the double-stranded DNA specific dye SYBR Green. Plotting fluorescence as a function of temperature as the thermal cycler heats through the dissociation temperature of the product gives a DNA melting curve. Throughout a temperature cycle, product denaturation can be observed as a rapid loss of fluorescence near the denaturation temperature. The melting temperature (T_m) is a function of both the fragment length and the GC content and can be used to differentiate amplification products separated by less than 2°C in melting temperature[26]. Desired products can be distinguished from undesired products, in many cases eliminating the need for gel electrophoresis. Unlike gel electrophoresis, melting curve analysis can distinguish products of the same length but different GC/AT ratio. They can quantify PCR products with greater reproducibility while eliminating the need for post-PCR processing, thus preventing carryover contamination[27]. The primary disadvantage of the SYBR Green dye chemistry is that it may generate false positive signals, because the SYBR Green dye binds to any double-stranded DNA, it can bind to nonspecific double-stranded DNA sequences.

Extraction

DNA was isolated form pure cultures of bacterial growth on blood agar plates. Pneumococcal colonies were suspended in nuclease-free water and the turbidity was adjusted to that of MacFarland standard 0,5. Preliminary serotyping was performed by the latex agglutination test with capsular typing sera prepared by the CDC. All serotypes were confirmed using the Quellung reaction.

Material and methods

PCR was performed in 30 µL volumes, with each reaction mixture containing: 15 µL SYBR Green™ mix, 1.5 µL forward and 1.5 µL reverse primers for the eight capsular serotypes 1, 3, 6A/B/C/D, 7F/A, 12F/A, 15A/F, 19A and 22F/A, 2 µL H_2O MBG and 10 µL DNA extract (table 9).

Table 9. PCR reaction mixture

	1 sample (µL)
f-primer (5µM → 0.25µM)	1.5
r-primer (5µM → 0.25 µM)	1.5
Sybr green mix	15
H_2O MBG	2
Total	**20**
+ 10 µL DNA extract	**30**

Reactions were run in a ABI 7900 Sequence Detection System device (Applied Biosystems), according to the fast PCR program: 20sec at 95°C, 45 cycles of 1sec at 95°C, and 20s at 60 °C. After PCR amplification, T_m curve analysis was performed using the Dissociation Curve Analysis Software v1.0 (Applied Biosystems). The PCR products were heated to 95 °C during 15s, cooled at 60°C for 15 s and then slowly heated back to 95 °C. A negative control without target DNA was included with each run. Obtained fluorescence signals were continuously monitored during the slow warming-up gradient and showed a decreasing curve with a sharp fluorescence drop near the denaturation temperature. Plotting the negative derivate of the fluorescence over temperature versus the temperature generated peaks from which the T_m of the product were calculated.

Results

DNA melting was observed as a sudden decrease in the fluorescence of the dsDNA dye SYBR GREEN ER™ as a sample was heated through the T_m of the product. Melting curves were acquired for different purified PCR products and for primer oligomers formed after 45 cycles of amplification in the absence of template (attachment 2 and 3).

Table 10. Serotypes and their melting temperatures

Serotype	**Melting temperature (°C)**
1	73,3 - 76,3
3	78,4
6A/B/C/D	78,6
7F/A	74,3
12F/A	78,9
15A/F	76,2
19A	78
22F/A	76,8 - 78,2

The design of T_m multiplex analysis requires that each different amplified DNA melts at a distinguishable temperature. Amplified DNA fragments with T_m means differing by only 1.2 °C or less produced single peak melting curves and thus are not suitable for use in multiplex reactions. PCR products with T_m's differing by 2°C can be distinguished within mixtures. In this study however, the melting temperatures of most frequent serotypes 3, 6, 12 and 19 are almost the same (±78 °C) (table10). According to these results, a SYBR Green based melting curve determination multiplex assay is not usable for the simultaneous identification of three or more PCR products, because the T_m of these products are not distinguishable.

Afterwards, PCR products of the eight serotypes were analyzed by polyacrylamide gel electrophoresis. Gels were stained with GELRED, and gel images were recorded (figure 3). The amplified products displayed the expected size for each of the serotypes (table 11).

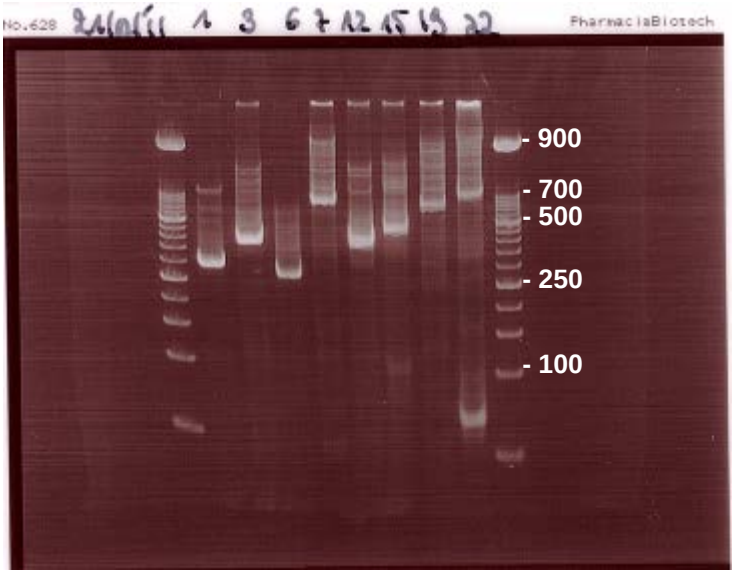


Figure 3. The amplified products of the 8 serotypes on gel electrophoresis

Table 11. Serotypes and the size of their amplified products

Serotype	Product size (bp)
1	280
3	371
6 A/B/C/D	250
7F/A	599
12F/(A)	376
15A/F	434
19A	566
22F/A	643

To optimize the PCR, all serotype-specific primers were first tested with individual isolates of the targeted serotypes in different concentrations (undiluted, 10^{-1} and 10^{-2} DNA extract)(table 12).

Table 12. PCR reaction of the different serotypes in different concentrations

Extract	Primers mix	Ct value
SP* type 1	SP type 1	18,76
SP type 1 10^{-1}		22,62
SP type 1 10^{-2}		26,5
SP type 3	SP type 3	17,48
SP type 3 10^{-1}		20,95
SP type 3 10^{-2}		24,65
SP type 6	SP type 6	20,56
SP type 6 10^{-1}		23,83
SP type 6 10^{-2}		27,32
SP type 7	SP type 7	19,32
SP type 7 10^{-1}		22,77
SP type 7 10^{-2}		26,64
SP type 12	SP type 12	18,65
SP type 12 10^{-1}		22,13
SP type 12 10^{-2}		25,79
SP type 15	SP type 15	16,27
SP type 15 10^{-1}		21,41
SP type 15 10^{-2}		25,15
SP type 19	SP type 19	17,87
SP type 19 10^{-1}		21,55
SP type 19 10^{-2}		24,51
SP type 22	SP type 22	18,72
SP type 22 10^{-1}		21,46
SP type 22 10^{-2}		24,93

*SP: *Streptococcus pneumoniae* DNA extract

Table 13 shows Ct values of 8 serotypes in different PCR mixes containing primers of 8 different serotypes. This experiment was done to exclude cross-reaction between one serotype and the PCR mix specific for other serotypes. *Streptococcus pneumoniae* serotype 3 showed cross-reaction with the PCR mix of *Streptococcus pneumoniae* type 1. The high Ct-value of 42,13, however, is suggestive for a non-specific reaction. A similar high Ct-value was seen for the PCR reaction of SP serotype 22 and the negative control sample with the PCR mix for SP type 19. Another problem was observed for the PCR mix of serotype 22. For this PCR mix, a positive Ct-value was observed for all samples. This is suggestive for a contamination of the samples, the reaction mix or the molecular biological water. Further research gave evidence for contamination of PCR reaction mix with serotype 22 DNA.

Table 13. PCR reaction of the 8 serotypes in different PCR mixes containing primers of each serotype.

Sample (10^{-1})	Mix	Ct	Mix	Ct	Mix	Ct	Mix	Ct	Mix	Ct	Mix	Ct	Mix	Ct	Mix	Ct
SP* type 1	SP 1	**22,38**	SP 3	-	SP 6	-	SP 7	-	SP 12	-	SP 15	-	SP 19	-	SP 22	27,11
SP type 3		42,13		**21,33**		-		-		-		-		-		23,77
SP type 6		-		-		**23,85**		-		-		-		-		29,11
SP type 7		-		-		-		**23,26**		-		-		-		28,62
SP type 12		-		-		-		-		**22,29**		-		-		25,13
SP type 15		-		-		-		-		-		**21,72**		-		28,93
SP type 19		-		-		-		-		-		-		**22,26**		27,41
SP type 22		-		-		-		-		-		-		43,56		**21,61**
NK		-		-		-		-		-		-		44,27		26,71

*SP: *Streptococcus pneumoniae* DNA extract

EXPERIMENT 2: CONVENTIONAL MULTIPLEX-SEQUENTIAL PCR REACTION

Because of non-distinguishable melting temperatures of the different serotypes, SYBR Green PCR reaction was not a possibility for capsular serotyping. So we tried to develop a schematic approach to detect 8 serotypes, using 8 primer pairs divided among 2 multiplex PCRs. In the future, we will try to expand this analysis to more serotypes by using more multiplex PCRs. These 2 multiplex reactions are designed to sequentially include the most frequently occurring serotypes (70%), based on the epidemiology of serotypes in Belgium. The primers were grouped into two multiplex reactions as showed in table 14. Each reaction was designed to include four primer pairs targeting four different serotypes. Primers were combined in each reaction to yield differences of more than 70 bp in PCR fragment size for clear interpretation. Reaction 1 contained primers for serotypes 1, 12F/(A), 19A and 22F/A while reaction 2 could detect serotypes 3, 6A/B/C/D, 7F/A and 15A/F.

Table 14. Determination of reaction based on the size of amplified products

Reaction 1				
Serotypes	1	12F/(A)	19A	22F/A
Product Size	280	376	566	643
Reaction 2				
Serotypes	3	6A/B/C/D	7F/A	15A/F
Product Size	371	250	599	434

Material and methods

This study aimed to develop a molecular method for pneumococcal serotyping by multiplex PCR assays. Sixty invasive pneumococcal isolates of 8 different serogroups (1, 3, 6, 7, 12, 19 and 22) were used to validate the multiplex PCR reaction. The PCR reaction was performed in 50 µL volumes, with each reaction mixture containing 10 µL DNA extract, 25 µL Express Q PCR mixture, 5 µL molecular water, 1,25 µL forward en reverse primers of each serotype with a concentration of 0.5 µM.

Table 15. PCR reaction mixture

	1 sample (µL)
f-primer 1 (20µM → 0,5µM)	1,25
r-primer 1 (20µM → 0,5µM)	1,25
f-primer 2 (20µM → 0,5µM)	1,25
r-primer 2 (20µM → 0,5µM)	1,25
f-primer 3(20µM → 0,5µM)	1,25
r-primer 3 (20µM → 0,5µM)	1,25
f-primer 4 (20µM → 0,5µM)	1,25
r-primer 4 (20µM → 0,5µM)	1,25
Express Q PCR	25
H_2O MBG	5
Total	**40**
+ 10 µL DNA extract	50

Reaction mixture 1: serotypes 1 – 12F/A – 19A – 22F/A

Reaction mixture 2: serotypes 3 – 6 A/B/C/D – 7F/A – 15A/F

PCR mix

40 µL mix 1 10 µL **SP*1** (10^{-1})	40 µL mix 1 10 µL **SP12** (10^{-1})	40 µL mix 1 10 µL **SP19** (10^{-1})	40 µL mix 1 10 µL **SP22** (10^{-1})	40 µL mix 1 10 µL MB H2O

40 µL mix 2 10 µL **SP3** (10^{-1})	40 µL mix 2 10 µL **SP6** (10^{-1})	40 µL mix 2 10 µL **SP7** (10^{-1})	40 µL mix 2 10 µL **SP15** (10^{-1})	40 µL mix 2 10 µL MB H2O

*SP: Streptoccus pneumonia DNA extract

Thermal cycling was performed in GeneAmp 9700 (Applied Biosystems) under the following conditions: 94°C for 5 min followed by 45 amplification cycli of 94°C for 15s, 60°C for 45s and 60°C for 5 min.

Results

The products were analyzed by polyacrylamide gel electrophoresis at 200 V for 55 min. Gels were stained with GELRED and gel images were recorded. The sizes of the PCR products were determined by comparison with the molecular size standard (50 bp-ladder)

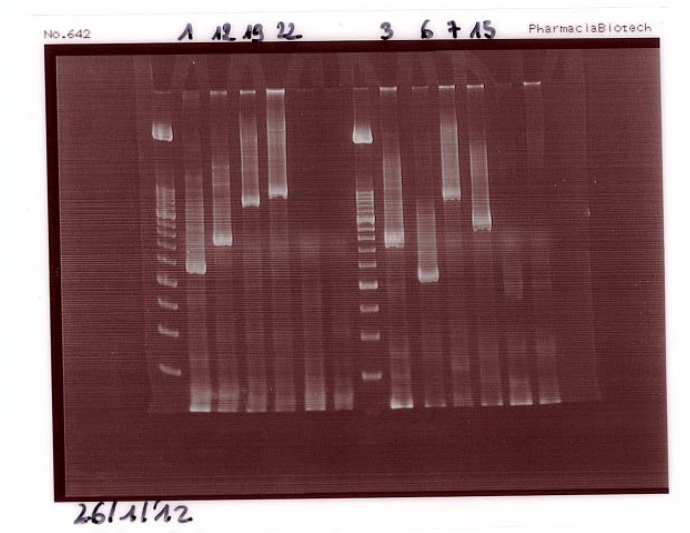


Figure 4. Multiplex reaction 1 and reaction 2. The serotypes of four strains tested are written above their respective lanes

We were able to identify the serotype correctly of 57 out of 60 pneumococcal isolates for which serotypes were previously determined by the quellung reaction (attachment 4). Serotypes were retested by the Quellung reaction when no serotype was identified by multiplex PCR. For the 3 discrepant results, subtyping of serotypes revealed subtypes not included in our primer set, more specifically 15B, 15C and 19F. Additionally, we used a *lyt*A-targeted real-time PCR for pneumococcal detection (attachment 5). *Lyt*A PCR was positive for all tested 60 pneumococcal isolates.

4) Capsular serotyping by molecular techniques, directly on pleural fluids

Literature

PCR-based approaches were superior overall to the corresponding culture-based methods in detecting pneumococcal serotypes. Conventional multiplex-sequential PCR (MS-PCR) for serotyping directly on clinical samples was used in 6 out of 10 studies (table 16). Five authors evaluate the use of a real-time PCR for diagnosis and serotyping of IPD. Carvalho et al. showed that broth enrichment prior to a PCR-based methods appeared to improve the detection of mixed serotypes and low-density pneumococcal carriage[28].

Table 16. Utilization of the multiplex PCR in different geographical areas directly on clinical samples

Clinical samples							
Author	**Year**	**Country**	**Isolates**	**No of samples**	**No of primer pairs**	**PCR reaction**	**Typeable**
Tarrago et al.[29]	2008	Spain	Pleural fluid	88	35	RT-PCR	77,60%
Saha et al.[10]	2008	Bangladesh	CSF + isolates	358	56	multiplex PCR on isolates and directly on CSF	94,00%
Antonio et al.[30]	2009	Gambia	Nasopharyngeal samples	279	29	multiplex PCR	65,90%
Njanpop et al.[31]	2009	Burkina Faso/Togo	CSF + isolates	194	29	multiplex PCR on isolates and directly on CSF	79,30%
Carvalho et al.[28]	2010	USA	Nasopharyngeal samples	100	40	broth enrichment + RT-PCR + multiplex PCR	NA
Azzari et al.[32]	2010	Italy	Blood samples	67	MS-PCR: 31 RT-PCR: 21	multiplex PCR vs RT-PCR	M-PCR (64,2%) RT-PCR (91%)
Resti et al.[15]	2010	Italy	Blood samples	80	21	RT-PCR	91,20%
Yu et al. [33]	2011	USA	Pleural fluid	49	7	multiplex immunoassay (latex beads) + 19A PCR	73,50%
Strachan et al.[34]	2011	Australia	IPD (pleural fluid)	43	NA	multiplex PCR/RLB	65,10%
Marchese et al.[35]	2011	Italy	Blood samples	46	35	RT-PCR	80,40%

Azzari et al concluded that both sequential multiplex-PCR and Real-time PCR can be used for pneumococcal serotyping of most serogroups directly on clinical samples from culture-negative patients, but Real-time PCR appears more sensitive[32]. Figure 5 demonstrates that the percentage of cases which remained non-typeable with MS-PCR is low or null when cerebrospinal fluids or pleural fluids are used, but it significantly increases if other biological samples, such as blood or nasopharyngeal swabs are used.

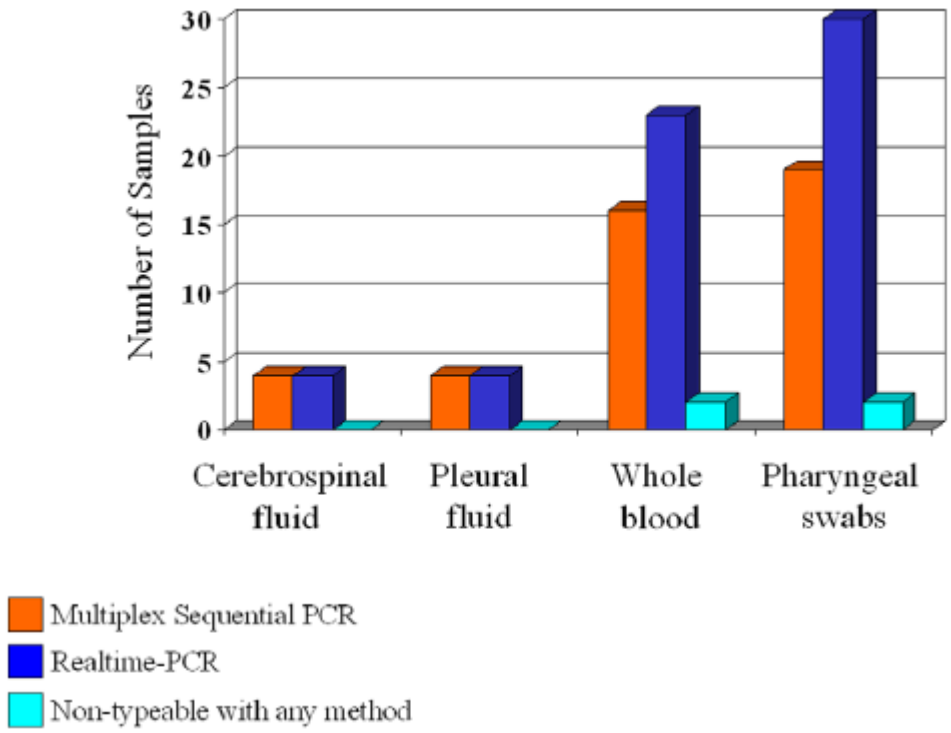


Figure 5. Number of typeable or non-typeable samples obtained from patients with culture-negative invasive pneumococcal infections as evidenced by Multiplex Sequential PCR or Realtime- PCR

Results

The 31 pleural samples were directly analyzed for capsular serotyping with the multiplex PCR. The amount of bacterial DNA (DNA load) was deducted from the C_t value.

Table 17. Serotyping results directly on pleural fluids

No	EMD	Pleura *lytA* PCR	*lytA* C_T PCR	PCR 1/12/19A/22	PCR 3/6/7/15A
1	040528B101	**pos**	**19,3**	negative	negative
2	011208G020	neg	neg	negative	negative
3	021202B118	**pos**	**26**	1 (weak)	negative
4	040522G014	neg	neg	negative	negative
5	030801G090	**pos**	**19,6**	1	negative
6	050101B002	**pos**	**24,1**	1	negative
7	010731B087	**pos**	**26,8**	1 (weak)	negative
8	930702V128	INH	INH	negative	negative
9	050424B044	**pos**	**25,6**	1	negative
10	070405G016	**pos**	**22,8**	negative	3
11	080310B010	**pos**	**18,7**	19	negative
12	001121B019	**pos**	**39,3**	negative	negative
13	050417gG25	**pos**	**24,7**	1	negative
14	050522B006	**pos**	**22,6**	negative	7
15	910710V080	INH	INH	negative	negative
16	040529B007	**pos**	**26,7**	1	negative
17	020920B156	**pos**	**27,3**	negative	negative
18	080520B006	**pos**	**19,7**	19A	negative
19	071128B009	**pos**	**21,7**	negative	3
20	080324B004	**pos**	**26,4**	negative	negative
21	980414V166	neg	neg	negative	negative
22	060502B057	**pos**	**28,7**	negative	negative
23	070808G013	**pos**	**27**	1	negative
24	081126G003	**pos**	**25,3**	negative	negative
25	020320 B166	neg	neg	negative	negative

26	970405M011	neg	neg	negative	negative
27	930702V128	**pos**	**36,4**	negative	negative
28	070814G013	**pos**	**23**	1	negative
29	991206V102	INH	INH	negative	negative
30	081208G003	**pos**	**22**	negative	3
31	010427G102	**pos**	**23,5**	negative	negative

When the 31 pleural samples were directly analyzed for capsular serotyping with a multiplex PCR assay, serotypes could be identified for 15 samples (table 17). The most common serotype was serotype 1, accounting for 9 samples. The next most common serotypes were serotype 3 and 19, accounting for 3 and 2 samples, respectively. Since our multiplex PCR tests only for 8 serotypes, the 8 not identified samples may express one of the remaining serotypes that were not included in our multiplex PCR. Alternatively, this 8 samples may contain *Streptococcus pneumonia*e below the limit of detection of our PCR assay. Figure 6 shows a comparison between the possibility to serotyping and the Ct-value of the *lyt*A PCR. Sixty-five (15/23) percent of all *lyt*A PCR positive samples were determined within these reactions.

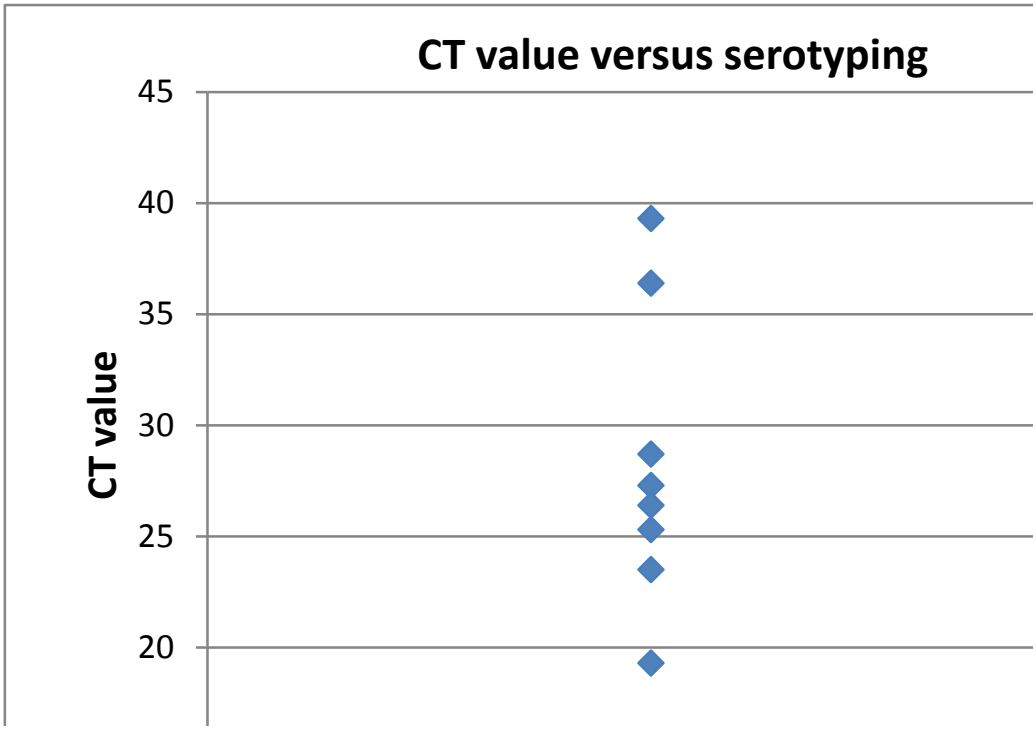


Figure 6. Correlation between C_T value and serotyping result

Our findings are in agreement with other studies suggesting over-representation of serotypes 1, 3 and 19A in the pleural fluid from children with empyema. Previous studies have shown that pneumococcal pneumonia with pleural effusion is associated with a limited number of serotypes. Yu et al. published studies of parapneumonic pleural effusions and lists the five most common pneumococcal serotypes isolated in culture (table 18). It is possible that these serotype distribution data may be biased toward serotypes that express antibiotic resistance and thus are more likely to be recovered in culture[5].

Table 18. Summary of 11 published reports showing the five most prevalent pneumococcal serotypes isolated from pleural effusions

TABLE 3. Summary of 11 published reports showing the five most prevalent pneumococcal serotypes isolated from pleural effusions

No. of samples	Collection date (yr)	Serotype (% of total) by ranking:					Sum (%)	Location of study	Reference
		1	2	3	4	5			
10	1975–1978	1 (50)	3 (37.5)	7F (12.5)	X[a]	X	100	Pennsylvania	24
26	1993–1999	1 (50.0)	14 (15.4)	9V (15.4)	19F (3.8)	18C (3.8)	88.5	Utah	6
133	1993–2000	14 (29.1)	1 (24.4)	19 (9.0)	3 (8.4)	6 (8.4)	79.3	U.S., multiple centers	26
24	1996–2000	1 (45.8)	14 (12.5)	6B (8.3)	19F (8.3)	6A (4.2)	79.2	Utah	7
27	1997–2001	1 (53.1)	14 (15.6)	3 (9.4)	X	X	78.1[b]	UK	10
11	1990–2002	1 (62.5)	4 (25.0)	5 (12.5)	X	X	100.0	Israel	13
35	2000–2003	14 (26.3)	3 (23.7)	1 (21.1)	6B (7.9)	9V (5.3)	84.2	Canada	17
30	2002–2004	19A (26.7)	1 (23.3)	14 (13.3)	3 (10)	23F (6.7)	100	France	3
27	2003–2004	1 (66.7)	4 (11.1)	3 (7.4)	7F (3.7)	9V (3.7)	92.6	UK	12
50	2001–2005	1 (34.0)	3 (20.0)	19A (14.0)	19F (6.0)	7 (4.0)	78.0	Utah	7
51	2001–2007	1 (33.3)	3 (27.5)	19A (25.5)	7F (3.9)	17 (2.0)	92.2	Utah	6

[a] "X" indicates the absence of reported serotypes.
[b] Three samples (11.9%) were negative for the 13 serotypes tested.

5) Comparison of cost-effectiveness and time-effectiveness between Quellung reaction and Multiplex PCR reaction for capsular serotyping

The multiplex PCR system described in this work is more labor-intensive than the Quellung reaction (total test-time: 420 min versus 10 minutes for the Quellung). The Quellung reaction has an hands-on time of 10 minutes for 1 sample, the PCR reaction of 200 minutes for 15 samples. The multiplex PCR reaction is also more expensive than the Quellung reaction. The estimated average cost per strain by a combination of latex agglutination (for pools) and Quellung reaction is 10,1 €, and this increases up to 38,4 € for nontypeable strains. The cost per isolate when characterized by multiplex PCR would be 18 € if only 2 multiplex reactions (8 serotypes) are performed.

Table 19. Test-time Quellung reaction versus Sequential Multiplex PCR reaction

Test time					
PROCEDURE	**SECTION**		**Time (min)**	**Total time (min)**	**Sample No**
QUELLUNG	**AVERAGE 8 test reactions**		**10**	**10**	**1**
SEQUENTIAL MULTIPLEX PCR REACTION	**EXTRACTION**		**90**	**420**	**15**
	PCR REACTION	**Preparation**	**90**		
		PCR reaction	**120**		
	GELELECTROPHORESIS		**120**		

Table 20. Hands-on time Quellung reaction versus Sequential Multiplex PCR reaction

Hands-on time					
PROCEDURE	**SECTION**		**Time (min)**	**Total time (min)**	**Sample No**
QUELLUNG	**AVERAGE 8 test reactions**		**10**	**10**	**1**
SEQUENTIAL MULTIPLEX PCR REACTION	**EXTRACTION**		**50**	**200**	**15**
	PCR REACTION	**Preparation**	**90**		
		PCR reaction	**0**		
	GELELECTROPHORESIS		**60**		

Hands-on time for 1 sample (min)	
QUELLUNG	**10**
SEQUENTIAL MULTIPLEX PCR REACTION	**13**

Table 21. Costing Quellung reaction

QUELLUNG REACTION (1 sample)			
Cost-effectiveness	**Cost (€)**	**Number**	**Total cost (€)**
1 test	0,45	8	3,6
Hands-on time	38/hour	0,17	6,5
		Total	**10,1**

Table 22. Costing multiplex PCR reaction

	Multiplex PCR (1 reaction, 15 samples)			
		Cost (€)	**Number**	**Total (€)**
EXTRACTION	EasyMAG	7,86	15	117,9
PCR REACTION	Primers	0,02/primer	8	0,16
	PCR Express Mix	0,07/µL	375	26,25
	PCR Plate 7500	4,74/plate	0,16	0,79
	Tip 10 µL	0,05	15	0,75
	Tip 20 µL	0,05	10	0,5
	Tip 1000µL	0,07	4	0,28
GELELECTROPHORESIS	Gelelectrophoresis	2,31 +0,06/sample	15	3,21
HANDS-ON TIME	MLT	38€/hour	3,3	126,7
			Total (15)	**277**
			1 sample	**18**

COMMENTS

Major advantage of molecular serotyping include the potential to type isolates directly on culture-negative clinical samples. PCR has the potential to bring out a different distribution of serotypes circulating in the population compared to conventional non-molecular methods[10] . However, it is uncertain PCR for pneumococcal serotyping adds value to surveillance by standard culture alone. Accurate serotyping of *Streptococcus pneumoniae* is of critical importance as vaccine development presently relies on serotype prevalence data[11]. The recent implementation of the 13-valent pneumococcal conjugate vaccine (PCV13), which includes PCV7 serotypes plus serotypes 1,3,5,6A,7F and 19A may reduce IPD caused by these added serotypes. As a consequence however, the possibility of replacement of PCV 13 serotypes by nonvaccine type in the future exists. Therefore, geographical continuous monitoring for serotype distribution remains important for the introduction and development of new pneumococcal conjugate vaccines[7].

In our study, the most common serotype was serotype 1, accounting for 9 out of 31 samples (29%). It is perhaps not surprising that large numbers of serotype 1 were isolated in our pleural fluid study as it is the predominant serotype responsible for parapneumonic pleural effusions because of its predilection for the pleural space[34]. Serotype 3 is also a well-documented cause of empyema. Serotype 3 has a thicker polysaccharide capsule, allowing protection against host immunity and making it more resistant to killing by phagocytosis.

The sequential multiplex PCR reaction suffers some difficulties:

1) It's difficult to validate a PCR-based detection method on culture-negative samples.

2) A sequential multiplex PCR cannot detect more than 29 serotypes compared with 91 from the conventional Quellung methods. The current PCR approach has the inability to determine all types due to the absence of some type specific-PCR primers. The currently available assays cannot identify all known capsular types.

3) The amount of genetic variability that exists among different isolates expressing the same serotype is unknown. The key limitation with a molecular-based assay is the plasticity of the pneumococcus, as capsular transformation or point mutations could easily result in serotype non-classification.

4) Another disadvantage is possibility to misreading of the PCR gel. In the first reaction of our assay (serotyping 1, 12,19 and 22), the bands were sometimes too close together to be resolved accurately. To overcome this problem, we created positive-control mixtures for each reaction, used them as molecular weight markers.

The genes for pneumococcal capsular polysaccharides synthesis are located at the same chromosomal locus (cps)[36,37]. We didn't include the internal control (cps locus) primer set in our multiplex assay, because every sample was tested by *lyt*A PCR to confirm *S. pneumoniae* identification before serotyping. All samples containing the *lyt*A gene were thought to be serotypeable and continued through the multiplex PCR.

TO DO/ACTIONS

- The use of cpsA primers as internal positive control. The inclusion of positive-control primers (cpsA) in each reaction will reliably identify sample preparation problems so that reactions that fail to amplify a product can be repeated.

- The implementation of the next multiplex reaction based on the geographical epidemiology : serotypes (5,8,9N and 24)

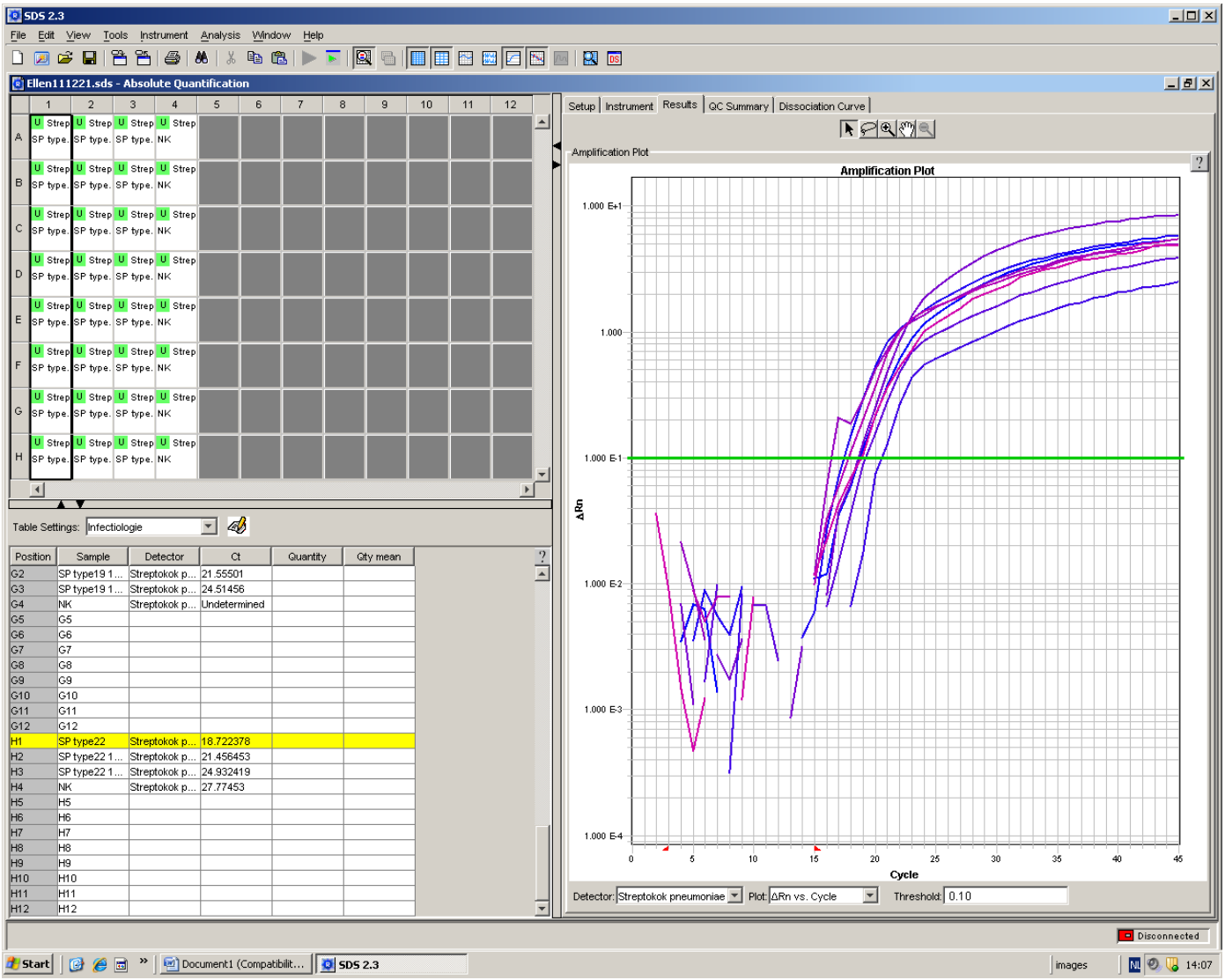
- Additional clinical isolates of viridans group streptococci should be assessed to ensure that no cross-reactivity occur (*lyt*A PCR and capsular serotyping).

ATTACHMENTS

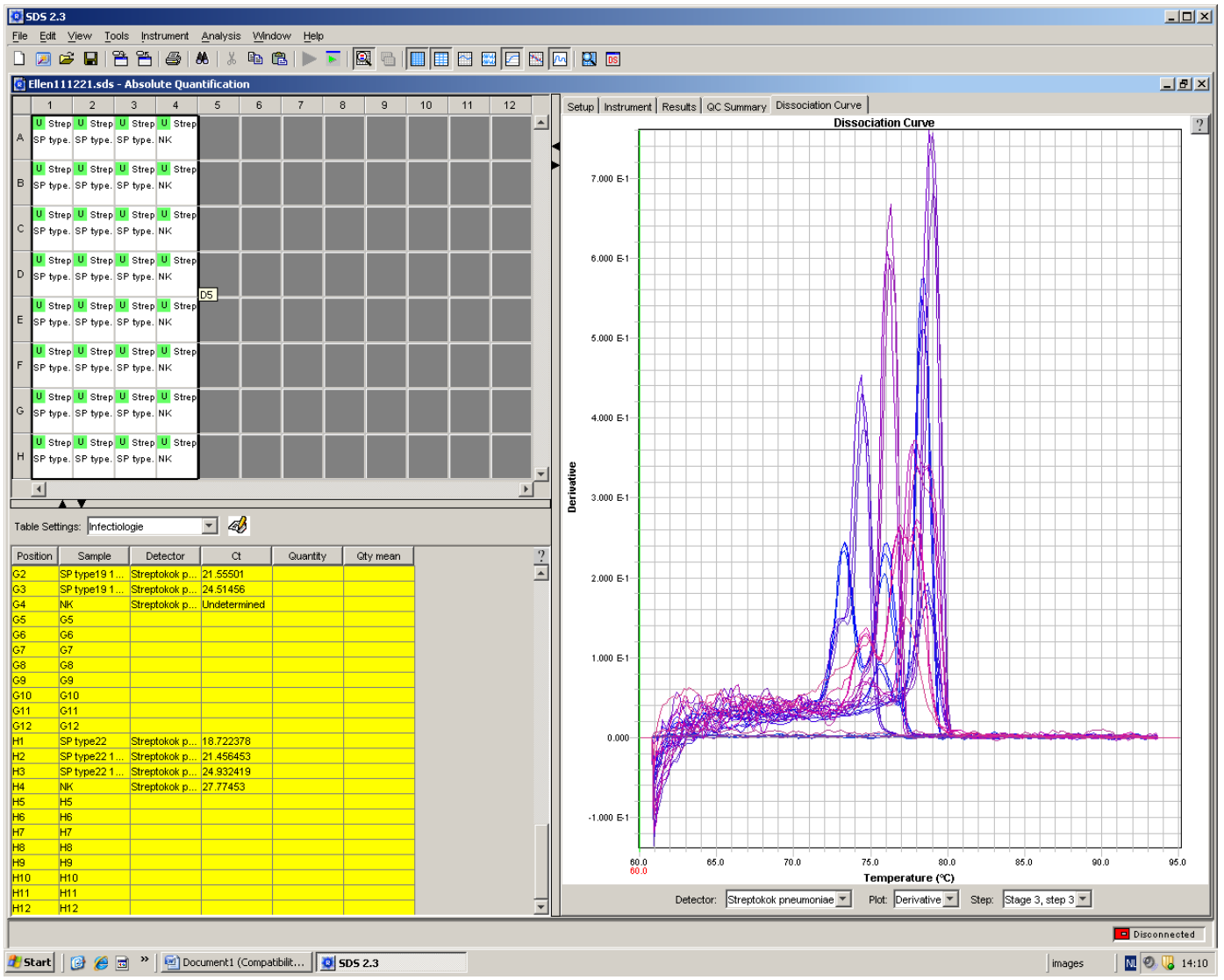
Attachment 1: List of oligonucleotide primers used for pneumococcal serotype deduction

Primer Pair	Primer sequence (5'→3')	Product size (bp)
1-f	CTC TAT AGA ATG GAG TAT ATA AAC TAT GGT TA	280
1-r	CCA AAG AAA ATA CTA ACA TTA TCA CAA TAT TGG C	
3-f	ATG GTG TGA TTT CTC CTA GAT TGG AAA GTA G	371
3-r	CTT CTC CAA TTG CTT ACC AAG TGC AAT AAC G	
6A/B/C/D-f	AAT TTG TAT TTT ATT CAT GCC TAT ATC TGG	250
6A/B/C/D-r	TTA GCG GAG ATA ATT TAA AAT GAT GAC TA	
7F/7A-f	TCC AAA CTA TTA CAG TGG GAA TTA CGG	599
7F/7A-f	ATA GGA ATT GAG ATT GCC AAA GCG AC	
12F/(12A/44/46)-f	GCA ACA AAC GGC GTG AAA GTA GTT G	376
12F/(12A/44/46)-r	CAA GAT GAA TAT CAC TAC CAA TAA CAA AAC	
15A/15F-f	ATT AGT ACA GCT GCT GGA ATA TCT CTT C	434
15A/15F-r	GAT CTA GTG AAC GTA CTA TTC CAA AC	
19A-f	GAG AGA TTC ATA ATC TTG CAC TTA GCC A	566
19A-r	CAT AAT AGC TAC AAA TGA CTC ATC GCC	
22F/22A-f	GAG TAT AGC CAG ATT ATG GCA GTT TTA TTG TC	643
22F/22A-f	CTC CAG CAC TTG CGC TGG AAA CAA CAG ACA AC	

Attachment 2: Fluorescent amplification curves of real-time SYBR Green PCR products using primer sets of different capsular serotypes



Attachment 3: SYBR Green melting curves for the different capsular serotypes

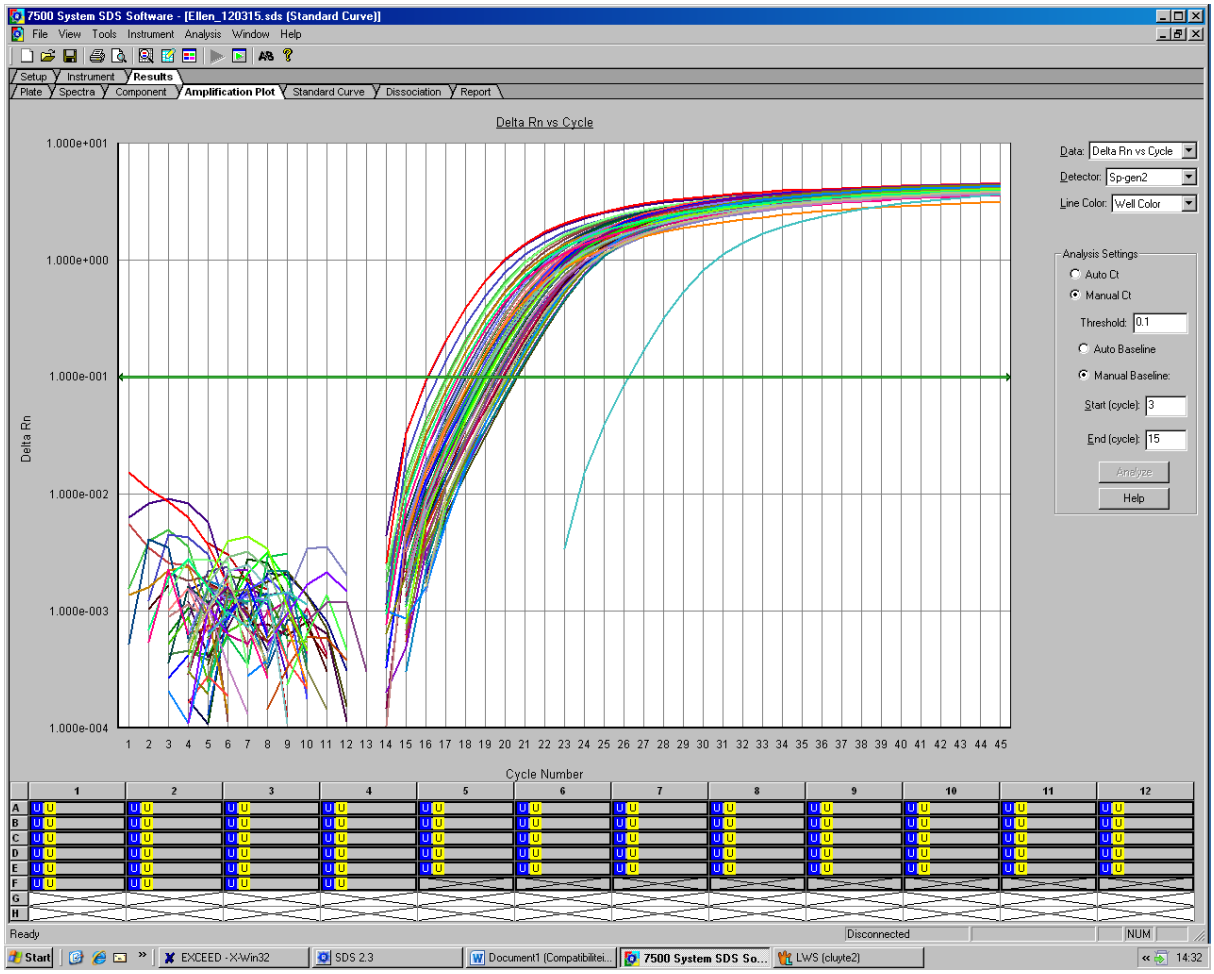


Attachment 4: Multiplex PCR reaction performed on 60 bacterial isolates of S. pneumoniae

S. pneumoniae	Sample	Multiplex PCR	Retesting Quellung
Type 1	11-1671	SP 1	
	12-0155	SP 1	
	12-0164	SP 1	
	12-0171	SP 1	
	12-0174	SP 1	
	12-0366	SP 1	
	12-0359	SP 1	
	12-0342	SP 1	
Type 3	11-1659	SP 3	
	12-0111	SP 3	
	12-0144	SP 3	
	12-0154	SP 3	
	12-0158	SP 3	
	12-0362	SP 3	
	12-0331	SP 3	
	12-0315	SP 3	
Type 6	11-1693	SP 6	
	11-1993	SP 6	
	12-0034	SP 6	
	12-0118	SP 6	
	12-0157	SP 6	
	12-0354	SP 6	
	12-0223	SP 6	
	12-0191	SP 6	
Type 7	11-1690	SP 7	
	12-0084	SP 7	
	12-0119	SP 7	
	12-0129	SP 7	
	12-0165	SP 7	
	12-0365	SP 7	
	12-0343	SP 7	
	12-0332	SP 7	
Type 12	11-1470	SP 12	
	12-0125	SP 12	
	12-0135	SP 12	
	12-0163	SP 12	
	12-0156	SP 12	
	12-0360	SP 12	
	12-0358	SP 12	
Type 15	11-1465	SP 15	
	12-0061	SP 15	
	12-0124	**NA**	**15B**
	12-0127	SP 15	
	12-0143	SP 15	
	12-0344	**NA**	**15C**
	12-0307	SP 15	
Type 19	11-1694	SP 19	
	12-0152	SP 19	
	12-0160	SP 19	
	12-0168	SP 19	
	12-0170	SP 19	
	12-0357	SP 19	
	12-0339	**NA**	**19F**
Type 22	11-1683	SP 22	
	12-0099	SP 22	
	12-0109	SP 22	

	12-0159	SP 22	
	12-0145	SP 22	
	12-0352	SP 22	
	12-0314	SP 22	

Attachment 5: LytA PCR reaction performed on 60 bacterial isolates of S. pneumoniae



Strachan RE, Cornelius A, Gilbert GL et al. Bacterial causes of empyema in children, Australia, 2007-2009. *Emerg Infect Dis* 2011 Oct;17(10):1839-45

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