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MOLECULAR SEROTYPING OF *STREPTOCOCCUS PNEUMONIAE*

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science.

Johannesburg, 2012

DECLARATION

The experimental work described in this dissertation was conducted under the supervision of Dr. Nicole Wolter and Dr. Anne von Gottberg, in the Centre for Respiratory Diseases and Meningitis of the National Institute for Communicable Diseases, Johannesburg.

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

_____

Victoria Magomani

_____ day of _____2012

DEDICATION

To my baby Vutshila Golele

LIST OF PRESENTATIONS

1. Victoria Magomani, PCR-based serotyping of *Streptococcus pneumoniae in* South Africa. Oral presentation, NICD Scientific Forum, National Institute for Communicable Diseases, Johannesburg, 08 September 2010.
2. Victoria Magomani, Nicole Wolter, Mignon du Plessis, Linda de Gouveia, Keith Klugman and Anne von Gottberg. Molecular serotyping of *Streptococcus pneumoniae* from isolates and culture-negative clinical specimens in South Africa. Poster presentation, University of the Witwatersrand Faculty of Health Sciences Research Day and Postgraduate Expo, University of the Witwatersrand, Johannesburg, 22 September 2010.
3. Victoria Magomani, Nicole Wolter, Mignon du Plessis, Linda de Gouveia, Keith Klugman and Anne von Gottberg. Molecular serotyping of *Streptococcus pneumoniae* from isolates and culture-negative clinical specimens in South Africa. Poster presentation, University of the Witwatersrand, Johannesburg 3rd Cross Faculty Symposium, 26-29 October 2010.
4. Victoria Magomani, Mignon du Plessis, Nicole Wolter, Linda de Gouveia, Keith P. Klugman and Anne von Gottberg for GERMS-SA (Group for Enteric, Respiratory and Meningeal Disease Surveillance in South Africa). Multiplex PCR serotyping of invasive *Streptococcus pneumoniae* from culture-negative clinical specimens and non-viable transport medium samples, 2010. Poster presentation, National Institute for Communicable Diseases, Johannesburg. NICD Academic Day, 23 November 2010.
5. Victoria Magomani, Mignon du Plessis, Nicole Wolter, Linda de Gouveia, Keith P. Klugman and Anne von Gottberg for GERMS-SA (Group for Enteric, Respiratory and Meningeal Disease Surveillance in South Africa). Multiplex PCR serotyping of invasive *Streptococcus pneumoniae* from culture-negative clinical specimens and non-viable

transport medium samples, 2010. Oral presentation, Laboratory Medicine Congress, Sandton Convention Centre, Sandton, Johannesburg. 31 August-04 September 2011.

6. Victoria Magomani, Nicole Wolter, Mignon du Plessis, Linda de Gouveia, Keith P. Klugman and Anne von Gottberg for GERMS-SA (Group for Enteric, Respiratory and Meningeal Disease Surveillance in South Africa). PCR detection of invasive pneumococcal serotypes from culture-negative samples, South Africa, 2010. Oral presentation (best abstracts), 4th FIDSSA congress, The Elangeni Hotel, Durban, South Africa. 8-11 September 2011.
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8. Nicole Wolter, Cheryl Cohen, Stefano Tempia, **Victoria Magomani**, Michelle Groome, Jocelyn Moyes, Sibongile Walaza, Babatyi Kgokong, Marthi Pretorius, Marietjie Venter, Halima Dawood, Kathleen Kahn, Ebrahim Variava, Shabir A Madhi, Keith Klugman and Anne von Gottberg for the SARI (Severe Acute Respiratory Illness) surveillance group. Carriage of high pneumococcal load is associated with an increased risk of developing invasive pneumococcal disease. Oral presentation, ISPPD-8, Iguaçu Falls, Brazil. 11-15 March 2012.

ABSTRACT

Streptococcus pneumoniae, known as the pneumococcus, remains the leading cause of mortality and morbidity worldwide in children <5 years due to pneumonia, with the highest burden of disease in developing countries. The pneumococcus is responsible for a variety of diseases, from localised infections such as otitis media and sinusitis, to life-threatening diseases such as meningitis and pneumonia.

The polysaccharide capsule, exterior to the cell wall, is the major virulence factor of the organism. To date, 93 different capsular types, called serotypes, have been described. These differ in their chemical structures, and <15% of serotypes are responsible for majority of pneumococcal disease worldwide. Some serotypes have a higher invasive potential than others, while some are associated with more severe outcome than others. There is also a geographic and age-specific variation in the distribution of serotypes. Pneumococcal diseases are largely vaccine preventable. The current vaccine formulations contain serotypes that are most prevalent across regions. The licensed polysaccharide and polysaccharide conjugate vaccines (PCV) contain at least 23 and 10 or 13 of most prevalent serotypes, respectively. Serotypes that were historically not commonly associated with invasive pneumococcal disease (IPD) are increasing in prevalence due to the reduction of vaccine serotypes. Thus, it is important to monitor serotype distribution for future vaccine formulations and to determine the vaccine effectiveness in the region.

The "gold standard" for serotyping, the Quellung reaction, is dependent on the viability of the organism, and hence it is problematic in settings where a culture cannot be obtained due to insensitivity of blood cultures, incorrect culturing techniques, initiation of antibiotic therapy

before the specimen is taken or autolysis of the pneumococcus. Although molecular methods have been established to identify pneumococcus as the disease-causative agent, serotyping was not possible on culture-negative samples until recently. Sequencing of the capsular biosynthetic loci of >90 serotypes revealed conserved regions and serotype-specific genes, which serve as targets for the PCR-based assays.

In this study, PCR-based serotyping assays were established in South Africa (SA) for culture-negative samples initially by using isolates of known serotypes. The conventional PCR assay (C-PCR) showed 100% sensitivity (801/801) and specificity (29/29) for serotypes included in the assay when compared with the Quellung reaction. However, specificity of the real-time PCR serotyping assay (RT-PCR) was 95% (89/94) compared to the Quellung reaction and the C-PCR assay, due to cross-reactions between genetically related serotypes/serogroups. The prevalent disease-causing serotypes were detected in the first three reactions of the C-PCR assay and first four reactions of the RT-PCR serotyping assay, allowing for the majority of isolates to be assigned a serotype in a time-efficient manner.

While both C-PCR and RT-PCR serotyping assays had comparable sensitivities on samples with sufficient DNA concentration, RT-PCR had an increased sensitivity in assigning serotypes for clinical specimens and non-viable transport medium samples (NVTMs). These samples often had a *lytA* cycle threshold (C_t) values of >26, indicative of low bacterial loads/DNA concentration. In 2010, 4201 cases of IPD were reported to the national IPD surveillance laboratory in SA. Viable isolates were not available for 10% (n=440) of all cases reported and 395 were available for serotyping. Of these, 80% (173/217) of NVTMs and 91% (162/178) of culture-negative clinical

specimens could be assigned a serotype using the PCR-based methods. The PCR-based assays added 8% (335/4201) of serotyping data to the national surveillance database for 2010. The newly discovered serotype 6D was not identified in SA in 2010.

The establishment of PCR-based serotyping assays was useful in the monitoring of serotype-specific disease burden. The prevalent serotypes in all SARI (severe acute respiratory illness) cases were 19A, 1 and 6A/B. This data is useful for determining the effectiveness of the 7-valent pneumococcal conjugate vaccine (PCV7) effectiveness in children with known vaccination status. The PCR-based serotyping assays could assign a serotype to 95% (18/19) of the pleural fluids positive for *lytA* received as part of an empyema study. Serotypes 6A/B (n=8, 44%), 19A (n=3, 17%), 23F (n=3, 17%), 14 (n=2, 11%) and 1 (n=2, 11%) were prevalent in these specimens.

PCV13 serotypes 19A and 1 were prevalent in cultures and culture-negative samples included in this study, suggesting the potential increased positive public health impact offered by introduction of 13-valent PCV in SA in 2011. PCR provided important serotyping data on a significant proportion of culture-negative samples. A number of culture-negative samples were positive for PCV7 serotypes, and therefore these data are useful for monitoring vaccine impact on serotype distribution. Thus, the burden of disease and the serotype distribution can be monitored effectively for vaccine failures and future vaccine developments, in a cost-effective and timeous manner

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NOMENCLATURE

ABCs	Active Bacterial Core surveillance
AIDS	Acquired immune deficiency syndrome
BHI	Brain heart infusion
bp	base pair
β	beta
c	concentration
CAP	Community-acquired pneumonia
CDC	Centers for Disease Control and Prevention
CI	Confidence interval
CLSI	Clinical and Laboratory Standards Institute
CSF	Cerebrospinal fluid
CPS	Capsular polysaccharide synthesis
cps	Capsular polysaccharide synthesis gene
CRDM	Centre for Respiratory Diseases and Meningitis
C-PCR	Conventional PCR
˚C	Degrees Celsius
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dATP	deoxy-adenosine-5´-triphosphate
dGTP	deoxy-guanosine-5´-triphosphate
dTTP	deoxy-thymidine-5´-triphosphate
dCTP	deoxy-cytidine-5´-triphosphate

dH_20	deionised water
g	gram
HIV	Human immunodeficiency virus
mg	milligram
IPD	Invasive pneumococcal disease
$MgCL_2$	Magnesium Chloride
min	minutes
ml	millilitre
mM	millimolar
µl	microlitre
µM	micromolar
nM	nanomolar
NP	Nasopharyngeal
NVTMs	Non-viable transport medium samples
PCR	Polymerase chain reaction
PCV7	7-valent pneumococcal conjugate vaccine
PCV9	9-valent pneumococcal conjugate vaccine
PCV10	10-valent pneumococcal conjugate vaccine
PCV11	11-valent pneumococcal conjugate vaccine
PCV13	13-valent pneumococcal conjugate vaccine
PPSV23	23-valent pneumococcal polysaccharide vaccine
RT-PCR	Real-time PCR
RPM	Revolutions per minute

SA	South Africa
SARI	Severe Acute Respiratory Illness
sec	seconds
SNP	single nucleotide polymorphism
S. pneumoniae	*Streptococcus pneumoniae*
TAE	Tris-Acetate-EDTA
WHO	World Health Organization
US	United States
V	volts
v	volume

1 Introduction

1.1 Background

Streptococcus pneumoniae, or the pneumococcus, is a commensal of the human upper respiratory tract with the highest colonisation rates observed in children <5 years of age [1]. It is an aerotolerant anaerobic Gram-positive diplococcus appearing in chains in a liquid medium. It is soluble in bile salts, α-haemolytic on blood agar medium and susceptible to optochin (ethyl hydrocuprein). However, resistance to optochin and other microbial agents have been observed in rare strains of the pneumococcus [2;3]. Haemolysis on blood agar medium is due to the breakdown of haemoglobin in blood agar resulting in greenish colonies.

This organism has historically played a central role in the field of microbiology. The identification of DNA as being the genetic carrier was first discovered through the pneumococcus [4]. Avery *et al* discovered that the pneumococcus can take up foreign DNA and incorporate it into its genome, thereby conferring a new phenotype with the characteristics of the foreign piece of DNA [4]. This concept is known as bacterial transformation and is now applied globally in many cloning experiments and other scientific research. The concept of antibiotic resistance was first observed in the pneumococcus in 1912 using optochin [5;6]. The principle of the Gram stain was discovered in 1881 using the pneumococcus and is the most important bacterial characterisation test used in microbiology [5;6].

1.2 Pneumococcal diseases

Apart from colonising the nasopharynx of healthy individuals, *S. pneumoniae* is a significant human pathogen. The reason for changing from the colonisation state to the invasive state remains unclear [7]. *S. pneumoniae* can cause invasive diseases such as meningitis and bacteraemia, as well as non-invasive diseases such as non-bacteraemic pneumonia, otitis media, sinusitis and bronchitis [1;8] (Figure 1). The pneumococcus is a leading cause of community-acquired pneumonia (CAP) [9-11] and meningitis. The pneumococcus is also the leading cause of empyema, described as an accumulation of pus in the pleural cavity. The organism is responsible for more than 1 million deaths per year among children <5 years of age worldwide, with the highest burden in developing countries such as in African and Asian countries [12]. Pneumonia causes more deaths than malaria, AIDS and measles combined [11].

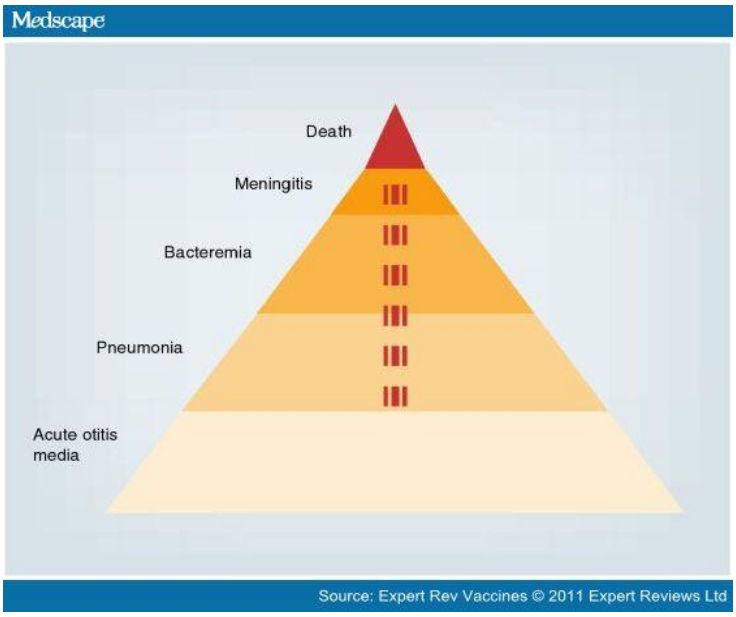


Figure 1. Clinical syndromes of pneumococcal disease classified according their frequency, with IPD being represented by a small fraction as bacteraemia and meningitis. The red vertical bars indicate syndromes that could result in death [13].

1.2.1 Risk factors for pneumococcal disease

S. pneumoniae is transmitted through inhalation of aerosols from an asymptomatic carrier or diseased individual [14]. IPD affects mostly children <2 years of age and the elderly [1;15;16]. Individuals who have underlying conditions e.g. cardiovascular diseases or chronic liver diseases, or are immunocompromised, are at increased risk of IPD and disease in such patients is associated with greater severity and complications [17-19]. Due to reduced clearance of encapsulated bacteria in persons with anatomical or functional asplenia (absence of spleen or non-functional spleen) [18;20], people with this condition are at greatest risk of pneumococcal infections associated with high mortality rates. Children attending day-care centres [21;22], persons in prisons, old-age homes or people living in clustered and disadvantaged communities are at higher risk for IPD and carriage [23;24]. In vaccinated children, risk factors for both carriage and IPD due to vaccine and non-vaccine serotypes includes absence of breastfeeding, exposure to smoke, presence of underlying sickness and male gender [17].

1.3 Diagnosis of pneumococcal diseases

Culture is the diagnostic gold standard; however, it is dependent on the growth of the organism. For culture-negative clinical specimens, PCR-based detection of pneumococcal DNA can be used for diagnosis. PCR has shown to be useful in diagnosing pneumococcal disease in patients who have already started antibiotic treatment [25]. Antibiotic usage reduces the yield of blood cultures, which are dependent on viable bacteria. PCR is not dependent on the viable bacteria, and can detect DNA from a non-viable microorganism following antibiotic treatment. Regions of the *lytA* gene, coding for autolysin, are specific to the pneumococcus and can be used for detection of the pneumococcus in a clinical specimen [26-28]. The quantitative real-time PCR

(RT-PCR) assay can also be used to determine the bacterial load, which is associated with the severity of disease [25;29;30]. Thus patients with high pneumococcal bacterial loads are more likely to have more severe disease at time of specimen collection than patients with low bacterial loads.

Aetiological diagnosis of the causative agent of bacterial pneumonia, also known as severe acute respiratory illness (SARI), remains a challenge due to inadequate diagnostic tests. A new method being investigated for the diagnosis of pneumococcal pneumonia in patients with respiratory illness is the RT-PCR detection of *lytA* in blood specimens of patients presenting with symptoms of pneumonia [31]. Molecular methods detecting pneumococcal DNA in the blood specimens of patients has been shown to serve as a useful marker for pneumococcal pneumonia [29;32]. Azzari *et al* [33;34] employed a technique in which the diagnostic *lytA* RT-PCR and the conventional serotyping PCR were aimed to target two different genes for specificity purposes i.e. the *lytA* gene in RT-PCR and the *cps*A gene in the serotyping assay. Thus, if both reactions were positive, then the confidence of the diagnosis was enhanced [33;34].

1.4 The polysaccharide capsule

The pneumococcal capsule is one of the major virulence factors of the organism [7]. The capsule is a polysaccharide structure made up of repeating sugar units. The structure is external to the cell wall and it provides resistance to phagocytosis by the host cells and aids in the evasion of the host immune system [35]. The capsule was first discovered in 1880, by Sternberg and Pasteur, who also were the first to isolate this micro-organism, and named it *Diplococcus pneumoniae* [36].

The pneumococcal polysaccharide capsule is highly diverse. A number of genetic events have resulted in the diversity of the polysaccharide synthesis loci such as point mutations, deletions,

insertions, duplication, and divergent evolution [37]. Pneumococci can produce at least 93 distinct capsules that differ in their chemical structure; and are called serotypes [38-40]. The capsular polysaccharide synthesis genes (*cps)* encode for different saccharides that makes up the repeating units. More than one serotype can be found within the same capsular group (serogroup) with differences within the biosynthetic locus due to recombinational events. Historically, serotypes were numbered as types according to how commonly they were isolated in disease episodes i.e. Serotype 1, 2 and serotype 3 were named as such because it was the most prevalent serotype isolated in disease episodes and associated with high mortality [41].

1.4.1 The capsular polysaccharide synthesis (*cps*) locus

In 2006, the capsular biosynthetic loci of all 90 serotypes available then were genetically analysed [38]. All the genes within the polysaccharide capsular locus were sequenced for the first time to determine the diversity between serotypes. The *cps* loci of all serotypes are flanked by *dex*B and *ali*A genes on the 5` and 3` ends respectively, and all other genes are oriented in the same direction as the *dex*B and *ali*A genes. The first four genes of the biosynthetic loci i.e. *wzg*, *wzh*, *wzd* and *wze* are conserved in all serotypes, whereas the central genes are serotype-specific genes [38] (Figure 2). The *cps* loci key enzymes were found to be highly diverse, consisting of highly diverse transferases, 40 homology groups for polymerases, and 13 homology groups for flippases [38]. Serotype 1 is the only case where the *cps* loci lack initial transferases encoding genes [38;42].

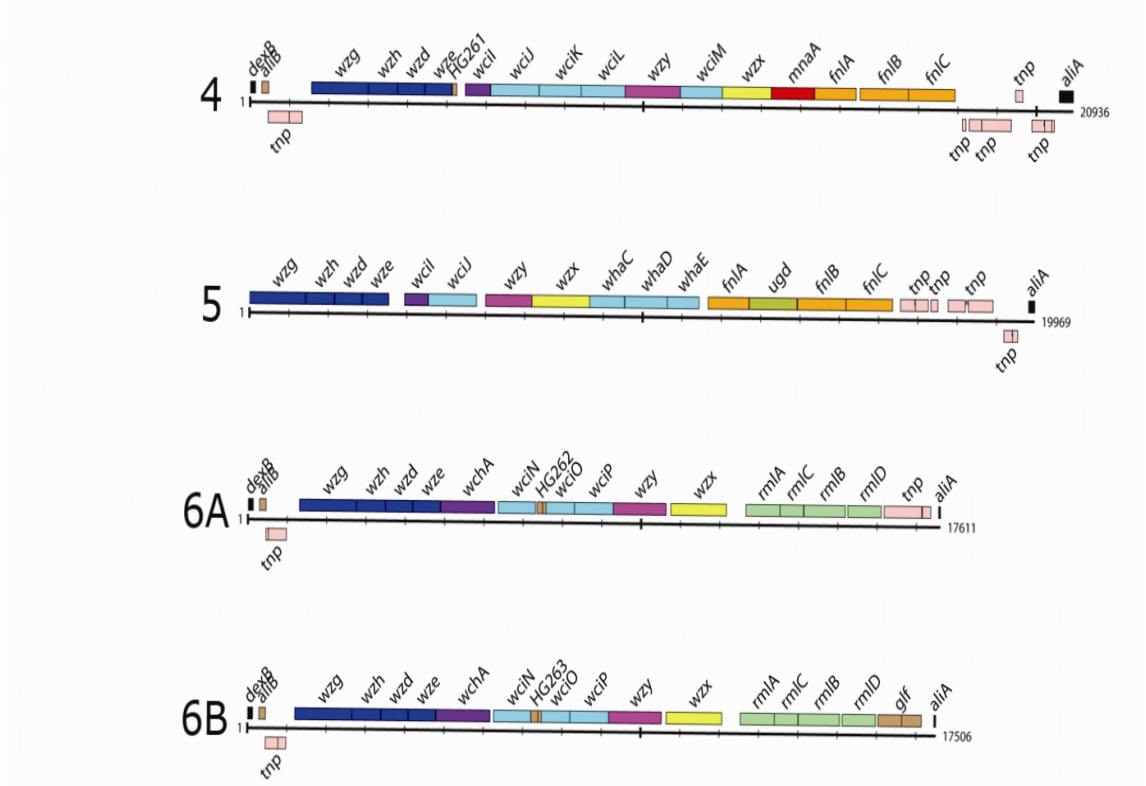


Figure 2. An example of the *cps* loci from four selected serotypes showing the conserved flanking regions at the ends, the four conserved genes in blue and the hypervariable regions in the middle of the capsular locus [38].

The availability of the *cps* sequences and the polysaccharide structures (CPS structures) enabled studies of genetic relatedness between the *cps* loci to be performed [42]. Eight pneumococcal clusters and 21 sub-clusters from 88 serotypes were assigned based on their genetic similarities. Serotypes within the same serogroup were grouped into the same cluster except for serotypes within serogroups 7, 16, 17, 33, 35 and 47. Serotypes were classified in the same cluster or sub-cluster if they shared a dominant epitope, irrespective of other major differences in other parts of the *cps* [38]

I. Cluster 1

This cluster consists of one sub-cluster comprising of serotypes 12F, 44, 12B, 12A and 46. Serotypes 12F and 12A are highly similar, differing only by an initial sugar and a side branch. Serotypes 44 and 46 have the same synteny as serogroup 12, differing only in the transposase genes. The relatedness between these serotypes suggests a common ancestry. Serotypes 44 and 46 cross-react with factor sera 12b/12d and 12d respectively. Serotypes 4 and 5 are also grouped in this cluster, although they show minor similarities to the other serotypes in this cluster, but share a dominant epitope [38;42].

II. Cluster 2

This cluster is comprised of 40 serotypes grouped into 12 sub-clusters, all containing the rhamnose biosynthesis genes as the dominant epitope, with an exception of serotype 1 and 24B due to mutations.

Serogroup 6

Serotypes 6A and 6B are highly similar, differing by a sugar linkage due to a point mutation. Serotype 6C, which was discovered in 2007 [43], differs from serotype 6A by the presence of glucose residue as the second sugar in the repeating unit compared to a galactose residue in serotype 6A as the second sugar of the repeating unit [42].

Serogroup 7

This serogroup is comprised of two sub-clusters. Serotypes 7A and 7F are grouped into the same sub-cluster, while serotypes 7C, 7B and 40 form the second sub-cluster. Serotype 40 is closely related to serotypes 7C and 7B than to serotypes 7A and 7F. Serotypes 7A and 7F differ only by the side branch available in 7F, but not 7A due to frame-shift mutations [38;42]

Serogroup 17

Serotypes 17A and 17F are found in this serogroup. Apart from sharing a dominant epitope, these serotypes are less similar, clustered into two different sub-clusters. Serotype 17F is sub-clustered with serogroups 24 and 48. There is a high similarity between serotypes 24B and 24F, as compared to serotype 24A. Two unique genes are only found in the *cps* loci of serogroups 24, 17F and 48 [42]. Serogroup 17 serotypes are grouped together because of sharing the most important genes e.g. initial transferase [and the central genes [42], although belonging to different sub-clusters.

Serogroup 18

All serogroup 18 serotypes fall into one sub-cluster. Serotypes 18B and 18C differ only by two base substitutions [38]. Serotype 18F has an additional acetyltransferase gene compared to serotypes 18B and 18C, and in serotype 18A, the acetyltransferase gene is absent. Apart from the differences in the linkage structures, the CPS structures of these serotypes are almost identical, with the major difference being in serotype 18A [42].

Serogroup 19

Serotypes 19A, 19B, 19C and 19F belong into the same sub-cluster and the CPS structures of all serogroup 19 serotypes have been studied intensively. Serotypes 19F and 19A are closely related, differing only in the polymerisation linkage between the glucose and rhamnose sugars [42;44]. Switching from serotype 19F to serotype 19A capsule production is highly possible by replacing only two genes within serotype 19A *cps* locus [44]. Serotype 19C has an additional glucose side chain compared with all other serogroup 19 serotypes. The gene rearrangements within the central region of the serogroup 19 *cps* loci have led to the emergence of four highly distinguishable serotypes (Figure 3).

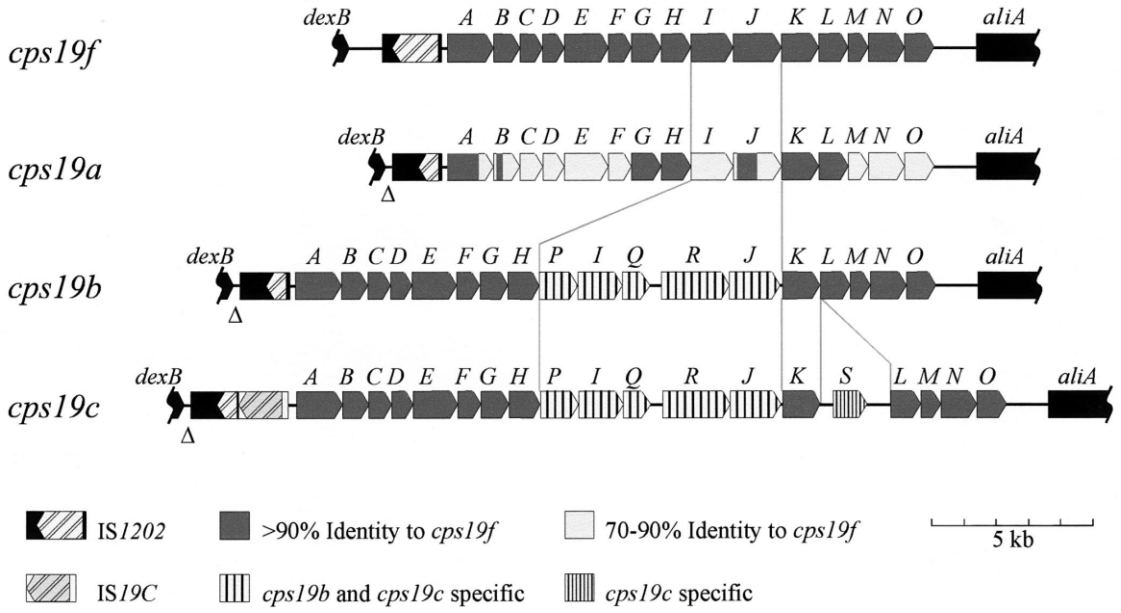


Figure 3. Capsular polysaccharide synthesis loci of serogroup 19 serotypes showing the central hyper-variable regions responsible for divergence of serogroup19 serotypes [44].

Serogroup 22

Serotypes 22A and 22F are highly similar, forming one sub-cluster. Although serologically distinguishable, the chemical differences on the CPS structures between the two serotypes is yet to be determined [42].

Serogroup 23

All serogroup 23 serotypes belong to one sub-cluster. Serotypes 23A, 23B and 23F differ in their polymerisation linkages [42].

Serogroup 28

Due to the cross-reactions in the immunological assay, serotypes 28F, 28A and serotype 16F are grouped into the same sub-cluster [42]. The *cps* locus of serotype 16F is highly similar to serogroup 28, and less similar to serotype 16A, which belongs to a different sub-cluster [42].

Serogroup 32

Serotypes 32F, 32A and 27 capsular biosynthetic loci are classified under one sub-cluster. Serotypes 27 and 32 *cps* loci only differ at the central regions, while the 5` and the 3` ends are highly similar. Serogroup 32 cross-reacts with factor sera 27b. Serotypes 32A and 32F differ only in the acetylation patterns [42].

Serogroup 41

Serotypes 41A, 41F, 31, and 17A, are closely related, forming a sub-cluster. Serotypes 41F and 41A differ only at the 3` end by having two different non-functional genes. Serogroups 31 and 41 *cps* loci differ only at the acetylations and initial transferases genes. Serotypes 17A and 41F are highly similar at both ends, forming similar linkages at the 5` and the 3` ends.

III. Cluster 3

The immunological cross reactivity between serotypes 25A, 25F and 38, and the similarities in their *cps* loci warranted a single sub-cluster for these serotypes. Serotypes 25A and 25F loci are highly similar, and only differ to serotype 38 in one gene which is truncated in serogroup 25 and not truncated in serotype 38. Serotypes 25F and 38 cross-react with factor sera 25b, while factor sera 38a cross-reacts with serotypes 25A and 38 in the immunological reactions [42].

IV. Cluster 4

Four sub-clusters are found in this cluster, constituting 23 serotypes. These serotypes are mostly similar at the 3` end of the *cps* loci

Serogroup 10

Serogroup 10 consists of serotypes 10A, 10B, 10C and 10F, which are all sub-clustered together. Serotypes 10A and 10B are more similar to each other, lacking an acetyltransferase gene, while the gene is present in serotypes 10C and 10F [42].

Serogroup 33

Serogroup 33 serotypes form three syntenic pairs, which are not found in the same sub-cluster. Serotypes 33A and 33F differ only by a frame-shift mutation in the acetyltransferase gene, and they belong to one sub-cluster. Serotypes 33B and 33D form a separate sub-cluster and are similar. Serotype 33C is not similar to any of the serogroup 33 serotypes, and it is classified alone in a separate sub-cluster [42].

Serogroup 35

Serotypes of serogroup 35 are classified into three different sub-clusters. Factor sera 35a cross-reacts with all serotypes of serogroup 35 in immunological reactions. Serotypes 35A, 35C and 42 are found in the same sub-cluster, and factor sera 35c cross-reacts with the three serotypes found in this sub-cluster. There is a strong resemblance in the central region of the *cps* locus of serotype 35B and serotype 29, forming a separate sub-cluster, and factor sera 29b cross-reacts with these 2 serotypes. Serotype 35F is highly similar to serotype 47F, constituting a third sub-cluster with a difference in the phosphate transferase gene [42].

Serogroup 47

Apart from serotype 47F, serotype 47A is found in a different sub-cluster. Serotype 47A is more similar to serotype 43 than to serotype 47F [42].

V. Cluster 5

The serotype 8 biosynthetic locus is highly divergent, and it is the only serotype found in this sub-cluster. Previous studies showed that serotype 8 might have originated from serotype 33F, due to the sequence similarities at the 3` end of the cps *loci* [45].

VI. Cluster 6

All serogroup 9 serotypes falls within this cluster, forming one sub-cluster with two branches. Serotypes 9V and 9A have the same synteny while serotypes 9L and 9N are highly similar [42] Although these serotypes are an example of divergence from a single *cps* locus, the sequences of the two pairs are highly dissimilar [38]. Serotypes 9A and 9V differ only in the acetylation patterns, with serotype 9V having an O-acetylation not present in the other serogroup 9 serotypes. In serotype 9A, the gene responsible for the O-acetylation was disrupted by a frame-shift mutation. Serotype 9N differs from 9L by having a glucose rather than galactose as residue 3. Serotypes 9A and 9V have an insertion element, which is not present in serotypes 9N and 9L [38].

VII. Cluster 7

The five serotypes of serogroup 11 i.e.11A, 11B, 11C, 11D and 11F form one sub-cluster, grouped into two syntenic groups. Serotypes 11F, 11A and 11D are grouped together, while serotypes 11B and 11C belong to the second group. As in serogroup 9, these serotypes are an example of divergence from a common ancestor. The main difference between the five serotypes is in the acetyltransferase genes. Serotypes 11F, 11A, and 11D have two extra acetyltransferase genes, while 11F and 11A genes only possesses one extra acetyltransferase gene [42].

VIII. Cluster 8

This cluster contains two sub-clusters comprised of serogroup 15 serotypes in one cluster, and serotype 14 forming a second sub-cluster [42]. The CPS structures of serogroups 15 and 14 are highly similar, differing at the 3` end of the capsular biosynthetic loci. However, no cross-reaction between the two serogroups had been observed in the serological reactions. Serotypes 15B and 15C form linear structures, while serotypes 15A and 15F are branched due to the

polymerisation patterns [38]. Serotype 14 resembles serotypes 15B and 15C more than serotypes 15A and 15F [42].

1.4.2 Role of polysaccharide capsule in invasive disease

The capsular type of each individual strain plays a role in its ability to cause invasive disease. Heavily encapsulated isolates e.g. serotype 19F and 23F are more resistant to clearance by the host and are mostly involved in carriage. Thus, there is an inverse relationship between heavy encapsulation and invasiveness [46-48]. However, heavily encapsulated serotypes may be highly lethal should they invade. In addition, some serotypes e.g. serotypes 4 and 5 are rarely isolated from carriage compared to disease episodes [47]. Some serotypes are associated with a higher case-fatality ratios than others e.g. serotypes 3, 6A, 6B, 9N and 19F whereas serotypes 1 and 14 are associated with low case-fatality ratios [48]. Pneumococcal clones of the same serotype can have different invasion capacities, indicating that the polysaccharide capsule is not the only factor contributing to virulence [49].

Although there are 93 serotypes described to date, only a small fraction of these cause most cases of invasive disease worldwide [50]. There is geographical variation in the distribution of pneumococcal serotypes, although the most common serotypes are found globally e.g. serotype 6B. Serotype 1 is predominant in Africa and in older children [15;50-52]. Certain serotypes are associated predominantly with disease in children or adults while others are associated with a specific syndrome. Serotype 14 and serotype 1 are more commonly isolated from bacteraemia cases whereas serotypes 6A/6B, 10A/B and 23F are more commonly associated with meningitis [53].

1.5 Phenotypic serotyping

Pneumococcal serogroup and serotype identification can be performed by various phenotypic antibody-antigen based methods, mostly involving large panels of antisera, such as the Quellung reaction, latex agglutination, co-agglutination, the dot blot assay, enzyme-linked immunosorbent assay, and counterimmunoelectrophoresis [27;54;55].

1.5.1 The Quellung reaction

The Quellung reaction ("swelling" in German), which is considered the "gold standard" for serotyping of the pneumococcus was first described by Neufeld in 1902. It refers to the refractive property of the pneumococcal capsule when exposed to homologous antibodies [56]. The method involves mixing equal amounts of the antiserum and pneumococcal suspension and fixing it on a glass slide. The cells are then viewed under a phase-contrast microscope to detect swelling of the capsule. For clear visualisation, methylene blue dye is added on the cover slip before covering the slides. A positive agglutination will result in the capsule doubling in its size, and this swelling is visible under the phase-contrast light microscope (Figure 4) [56]. A simplified method for the Quellung reaction was developed, whereby a group to which an isolate belongs is detected by pooled antisera, and then if positive for a certain group, the isolate is tested for all serotypes belonging to that group. The most prevalent serotypes responsible for IPD are included in one group, and in this manner, the most commonly occurring serotypes can be detected first [27;57].

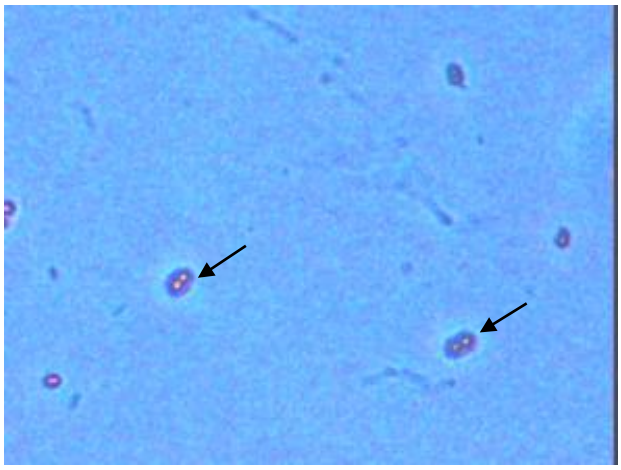


Figure 4. A phase-contrast microscopic image at 100X magnification illustrating the positive Quellung reaction for pneumococcus with the swollen capsule indicated by the purple colour around the diplococcus, using methylene blue stain.

To date, the Quellung method uses sera including the omni-, pool-, type-, and factor sera, which cover >90 capsular types of pneumococci [58]. The omni serum contains all the antibodies for >90 serotypes, and is used to confirm encapsulation. An omni-negative isolate implies a non-typeable pneumococcus or that the isolate is not *S. pneumoniae.* In the latter case, other biological tests such as solubility in bile salts and Gram stain, for identification of the pneumococcus are used.

The major disadvantages of the Quellung reaction method are the high cost of antisera, the requirements for technical skills, the complexity in interpretation of the results and the unsuitability for large-scale serotyping. Another major limitation of this method is that it is dependent on the viability of the organism. It is therefore not useful in cases of culture-negative clinical specimens. Specimens may be culture negative due to a number of reasons, such as the

use of antibiotics prior to the patient presenting to the hospital and incorrect handling/storage and transportation of the specimens.

1.5.2 Latex agglutination

Latex agglutination, developed in 2004 [27], was shown to be promising as an alternative to the Quellung reaction because of its simplicity and the fact that the results could be obtained rapidly. The method involves mixing the pneumococcal broth culture with the pooled antisera on a glass slide and within seconds, the results are readable by the naked eye. The disadvantage of this method is that it can only detect pooled serogroups, and the Quellung reaction is still needed to determine serotype within the serogroup [54].

1.5.3 Monoclonal antibody-based serotyping

Monoclonal antibody-based serotyping e.g. the multibead assay, employs flow cytometry using monoclonal antibodies and latex beads with pneumococcal polysaccharides covalently linked to the beads. The method was the basis for discovery of serotypes 6C and 11E [39;43]. The technique poses a challenge in the development of antibodies, technical experience required and the cost involved [54]. However, it is very specific and with the generation of more antibodies, new serotypes that are misclassified by the available typing methods could be discovered.

1.6 Molecular serotyping

The sequences of the *cps* loci from 90 serotypes have been completed by the Sanger Institute (UK) in 2006 [59], thereby providing the opportunity to develop a sequence-based scheme for identifying pneumococcal serotypes. PCR primers designed from the central variable regions of the capsular biosynthetic loci are the basis of molecular serotyping.

1.6.1 The conventional sequential multiplex PCR assay

A conventional sequential multiplex PCR, referred to as conventional PCR (C-PCR) assay was developed at the Centers for Disease Control and Prevention (CDC) in Atlanta, US [60]. The initial assay detected only 29 serogroups/types while more recently the protocols have been updated to detect at least 40 serogroups/types [61;62]. The reactions are arranged such that the most commonly occurring serotypes in the US and African regions are detected within the first few reactions. At least five or six serotypes are detected in each reaction and an internal control for pneumococcal encapsulation is included in all reactions in the assay (Figure 5). The serotypes selected cover a wide range of IPD-causing serotypes, and different protocols are available for regions with varying serotype distributions.

Pai *et al* were able to serotype 96% (401/421) of their isolates using the protocol for targeting only 29 serogroups/types. The results were in agreement with the results of the Quellung reaction. The remaining 20 isolates could not be assigned a serotype either because they were a rare serotype not included in the assay or they were non-typeable [60]. The method has been used successfully in many countries with minor adaptations to the multiplex reactions used at the CDC [63-65]. The method was used directly on clinical specimens from a DNA-containing supernatant of clinical specimens (after centrifugation) and the yield of serotypes determined was 90% compared to 40% from the Quellung reaction [66]. Multiplex PCR-based serotyping has proven to be cost effective, rapid, and less laborious than the Quellung reaction and can be employed successfully for culture-negative specimens [67]. The Quellung reaction can be used to supplement the multiplex PCR for serotypes not included in the PCR assay.

To limit the number of multiplex PCR reactions, only the most prevalent serotypes are included in the assay. Thus, one limitation is that the rarely occurring serotypes are not detected. Another limitation is that for some serotypes which have highly similar sequences, the primer sequences cannot distinguish individual serotypes, and only a serogroup can be obtained e.g. serogroup 18B/C or serotype 38/25F [60]. The internal control (*cps*A) that is currently used to confirm encapsulation is also not reliable for <2% of encapsulated pneumococci especially serotypes 38 and 25, so the absence of the *cps*A band does not equate to a non-typeable pneumococcus [62]. The presence of the serotype band without a *cps*A control band is considered a positive serotype. However, the problem arises in culture-negative samples where the Quellung reaction cannot be used due to lack of a culture, and therefore the serogroups would remain unresolved. Identification of a serotype within a serogroup or a cluster can be epidemiologically important, in cases where the one serotype is in one of the conjugate vaccine formulations and the other is not, e.g. 18C is in the PCV7 formulation, while 18B is not. The inability to differentiate these serotypes is an epidemiological challenge for vaccine effectiveness studies. However, the complexity and the cost of the Quellung reaction still make PCR a useful alternative.

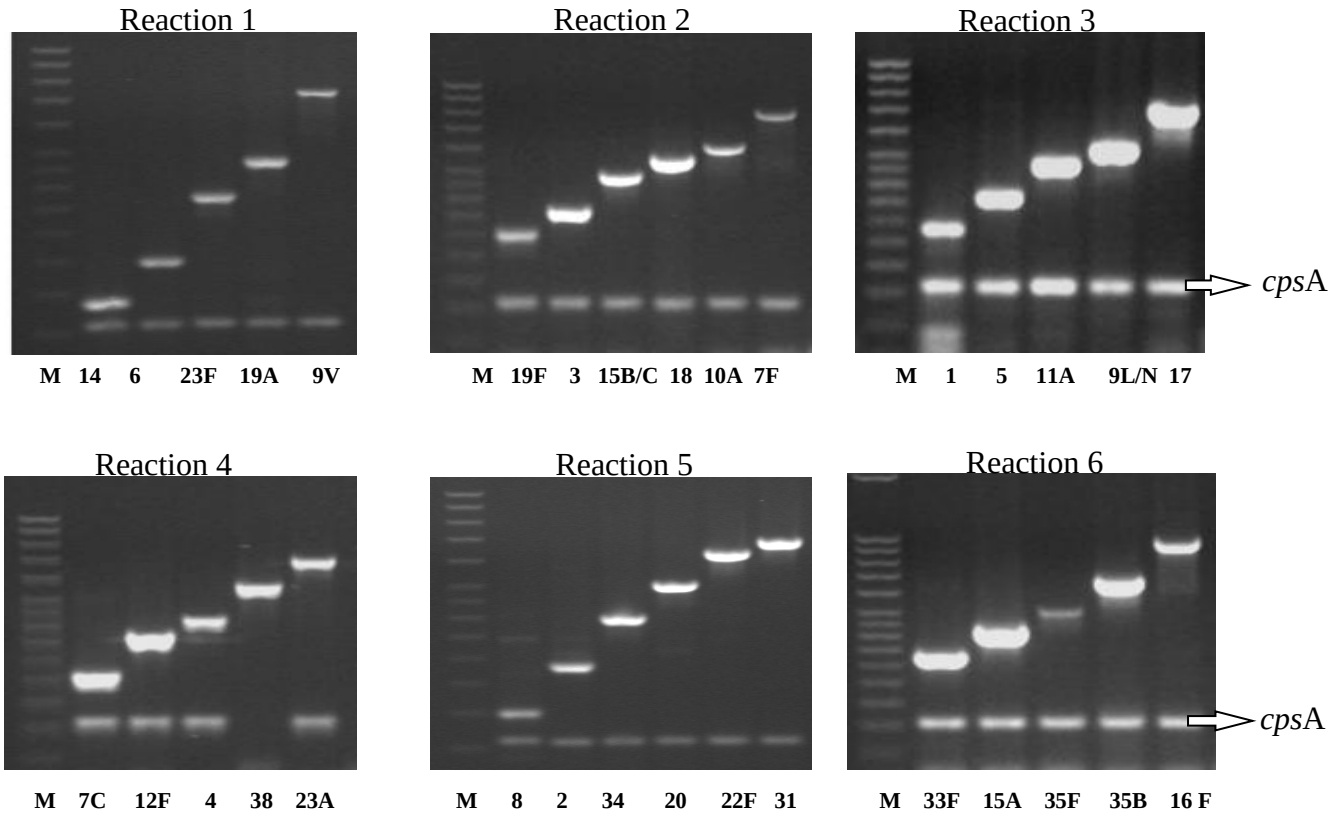


Figure 5. An illustration of agarose gels for the six multiplex reactions showing the serotypes included in each reaction (shown on the bottom of each figure), covering the 33 serotypes detected by the C-PCR assay developed by the CDC. The *cps*A control is pointed out in reaction 3 and 6. M indicates the molecular weight marker used for assigning product sizes [68].

1.6.2 Real-time PCR serotyping assay

The sensitivity of molecular serotyping is increased by the use of RT-PCR, which is more sensitive than C-PCR [33;67]. The advantage of RT-PCR is the speed in obtaining the results. RT-PCR does not require downstream processing of PCR products as in conventional PCR, as products are detected on a real-time basis [69]. It is also useful for detection of low DNA concentration samples, due to its high sensitivity. However, the assay is more expensive than conventional PCR due to the cost of equipments and reagents used e.g. probes. A number of RT-

PCR assays for pneumococcal serotyping have been described [34;70-72]. Tarrago *et al* [70] developed a RT-PCR assay for serotyping of the pneumococcus in culture-negative specimens that are positive for pneumococcal DNA. The assay was validated using isolates of known serotypes from the Quellung reaction, and there was a 100% specificity obtained. The assay targets only sixteen serogroups/types and this selection covered 80.9% of their invasive isolate serotypes in ten reactions (consisting of a combination of singleplex and duplex reactions).

Azzari *et al* [34] developed an assay to cover 21 serotypes and were able to serotype 100% (38/38) of their isolates, and 91% (61/67) of their clinical specimens. The assay consists of 21 singleplex reactions, detecting serogroups/types 1, 3, 4, 5, 6, 7A/F, 8, 9V/A, 10A/B 12A/B/F, 14, 15, 18B/C, 19A, 19B/F, 20, 22A/F, 23F, 33A/F, 35B, and 38. These serotypes, excluding serogroup 38, are part of the 23-valent polysaccharide vaccine and are included in the current PCV formulations i.e. PCV7, PCV10 and PCV13. This assay has been used successfully in Italy where 92% (73/80) of pneumococcal-positive whole blood specimens could be serotyped [73]. An additional multiplex RT-PCR serotyping assay for detecting 13 serotypes in four reactions (three multiplex reactions and 1 singleplex reaction) has been described [71] and 50% (5/10) of the culture-negative specimens were assigned a serotype.

A potential disadvantage of the multiplex RT-PCR assays is loss of sensitivity in the multiplex format, therefore for optimum results, all 21 reactions should be carried out individually or in duplex reactions. The sensitivity of the RT-PCR assays was not reduced in the duplex reactions and multiplex reaction formats developed for detection of respiratory viruses [74;75], and this is needs to be explored further for the pneumococcus.

1.7 Serotype distribution in South Africa

There is a strong association between HIV status and serotype distribution amongst children and adults in SA [76-79]. In 1995, serogroups 6, 23, 14 and 19 were more commonly isolated from HIV-negative children while in HIV-infected children, serogroups 23, 14 and 6 were the prevalent serogroups isolated. Serogroups 6, 14, 19, and 23, commonly known to be paediatric serotypes were isolated from HIV-positive adults in 1993-1995, indicating that immunocompromised adults are more susceptible to infection with paediatric serotypes [78-80]. Serotype 1 is prevalent in HIV-infected and HIV-uninfected adults [79].

In SA, from 1991-1998, an increase in serogroup 19 was observed whereas all the other common serotypes remained stable in children ≤2 years [78]. Serotypes 6A/B remained the leading IPD-causing serotypes in children <5 years. Young adults are at increased risk of IPD due to serotype 3, compared to other age groups [81]. From 2008-2010, annual reports of the Group for Enteric, Respiratory and Meningeal Disease Surveillance in SA (GERMS-SA) showed that the same mentioned serotypes, which were common a decade earlier, were still prevalent in 2010 [82]. The common serotypes isolated in SA (2008-2010) from children <5 years from viable isolates are serotypes 14, 6B, 23F, 6A, 19F, 19A and 1 [82].

1.8 Pneumococcal vaccines

1.8.1 Pneumococcal polysaccharide vaccines

The majority of disease due to *S. pneumoniae* is vaccine preventable. The 23-valent pneumococcal polysaccharide vaccine, (Pneumovax®; Merck & CO, Whitehouse station, NJ, USA and Pnu – immune®, Lederle Laboratories, Pearl river, NY, USA) containing serotypes 1, 2,

3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F,19A, 20, 22F, 23F and 33F was licensed for use in 1983 in the US [18;83;84]. This vaccine replaced the previously licensed 14-valent pneumococcal polysaccharide vaccine licensed in 1977 [85]. The 23 serotypes covered 85-90% of serotypes that were responsible for IPD in US in 1978-1995 for all age groups and that were associated with drug resistance [18;83]. The capsular antigens elicit an immune response of type-specific antibodies enhancing the opsonisation and phagocytosis of pneumococci of these specific serotypes.

The 23-valent polysaccharide vaccine is recommended for individuals >65 years of age as well as individuals from 2 to 64 years of age who are at high risk for acquiring pneumococcal disease e.g. immunocompromised individuals, people living in special environments like old-age homes and prisons [84;86;87]. This vaccine reduced IPD in the vaccinated immunocompetent individuals as well as people with underlying conditions such as diabetes [84]. The vaccine elicits a poor or no immune response in children <2 years of age due to lack of activation of T-cell immunity in young children whose immune systems are immature. Children <2 years of age and immunocompromised individuals of all ages are incapable of mounting a T-cell dependent immune response to the polysaccharide antigens in the vaccine [88]. The vaccine does not provide mucosal immunity and only protects against IPD caused by the serotypes in the vaccine [89].

In SA, Pneumovax® 23 (Merck) available from 1992 has been replaced by Imovax Pneumo 23® (Sanofi Pasteur SA, Lyon, France). Utilization of this vaccine is low in SA compared to other countries [76].

1.8.2 Pneumococcal conjugate vaccines

An immune response to polysaccharides vaccines can be improved by covalently bonding the polysaccharide to a protein carrier such as diphtheria toxin, CRM 197 (cross-reactive protein). The protein carrier activates the T-cell dependent pathway of the immune response thereby enabling young children to elicit an immune response to these vaccines. A disadvantage of polysaccharide conjugate vaccines is that only a limited number of antigens can be linked to a protein carrier.

The 7-valent pneumococcal conjugate vaccine, PCV7 (Wyeth Pharmaceuticals, Inc., Philadelphia), containing serotypes 4, 6B, 9V, 14, 18C, 19F and 23F was licensed for use in children <5 years of age in the US in 2000 [88]. There has subsequently been a significant decline in IPD caused by vaccine and vaccine-related serotypes in the targeted age group [90;91]. Similar trends of the reduction of IPD due to vaccine serotypes were observed in other areas where the vaccine was implemented such as England [92], Spain [93] Canada [94] and Western Australia [95]. It reduces acquisition and carriage of vaccine and vaccine-related serotypes by providing mucosal immunity [96;97].

In addition, PCV7 has caused a significant decrease in IPD and carriage in the unvaccinated community due to indirect protection, known as the “herd effect” [91;96-98]. The herd effect is due to the reduction in carriage of vaccine and vaccine-related serotypes in immunised children, which, in turn causes a reduction in carriage and pneumococcal disease in the elderly who have not been immunised [98]. The reductions in IPD due to vaccine and vaccine-related serotypes were consistent in the US across all sites and in all age groups where the conjugate vaccine was

implemented, supporting the high effectiveness of the vaccine, irrespective of socio-economic status [99]. Similar trends have been observed in other countries where the conjugate vaccine is routinely used [8;92;96;100;101].

Moreover, there was a decline in pneumonia hospitalisation rates subsequent to PCV7 introduction in the US [102-104]. In 2005-2006, rates of pneumococcal pneumonia cases had declined from 25.9 to 13.4 and from 144.9 to 64.7 cases per 100 000 population <1 years and ≥65 years, respectively [103]. These reductions in pneumococcal pneumonia were also observed in unvaccinated age groups due to herd effect.

In countries like Canada, Korea, Argentina, Brazil and some European countries [105-107], PCV7 has been replaced by a 10-valent conjugate vaccine (PHiD-CV10; Synflorix, GSK, Inc., Belgium) based on the results of immunogenicity studies without clinical trials [107]. The serotypes included in PCV10 are PCV7 serotypes and additional serotypes 1, 5 and 7F. New pneumococcal conjugate vaccines can be licensed based on comparable immunogenicity and safety to the already licensed vaccine, as recommended by the World Health Organization (WHO) [108], as in the licensure of PCV10. The 13-valent pneumococcal conjugate vaccine (PCV13; Prevnar 13, Wyeth Pharmaceuticals Inc., Philadelphia, marketed by Pfizer Inc.) was first introduced in Chile in July 2009. The serotypes included in PCV13 are PCV10 serotypes and additional serotypes 3, 6A and 19A. PCV9 (PCV7 serotypes plus serotypes 1 and 5) and PCV11 (PCV9 plus serotypes 3 and 7F) have been used in clinical trials [109;110] and have shown efficacy in reducing pneumococcal diseases [111;112]. Clinical trials in SA and the Gambia showed a significant reduction in radiologically-confirmed pneumonia by 17% and 37% in all

children, respectively, attributed to PCV9 [109;113]. PCV9 was not licensed because of the close similarity to PVC7 and was superseded by PCV13 [114], while PCV11 was not licensed due to reduced immunogenicity response when co-administered with other vaccines in the infant immunisation schedule [115].

PCV13 was introduced in the US in the routine immunisation programme for the same age group in February 2010 to replace PCV7. High immune responses to the PCV13 serotypes were observed in the immunogenicity studies done, except for serotype 1, which elicited poor antibody responses for unknown reasons [116]. Developing countries such as Guyana, Kenya, Yemen, Congo, Sierra Leone have introduced PCV13 in 2011 as part of the GAVI (Global Alliance for Vaccines and Immunisation) global pneumococcal vaccine roll-out [117]. Individuals aged between 2 and 18 years at high risk for pneumococcal infections who had received all doses of the conjugate vaccine/s are recommended to have PPSV23 as a booster dose [15;116]. Moreover, recently in December 2011, PCV13 was approved for people ≥50 years of age in the US to further reduce the burden of pneumococcal diseases in the elderly in addition to the herd effect observed [118].

In SA, PCV7 was introduced (April 2009) into the Expanded Programme on Immunisation (EPI), for all children <2 years of age with a 6- and 14-week dose schedule, followed by a booster at 9 months [119]. PCV13 was introduced into the EPI programme in SA from May 2011 to replace PCV7. The schedule is the same as used for PCV7, with a catch-up dose at 18 months for children who received 2 or 3 doses of PCV7 or all children <2 years. However, by June 2011, only few provinces and health-care facilities had switched to PCV13.

In SA, HIV is endemic and pneumococcal infections are associated with HIV seropositivity in both children and adults [76;113]. Moreover, HIV seropositivity is associated with an increased penicillin and multi-drug resistance in pneumococci [79]. In South African children, there was a 42-fold increase IPD incidence rates in HIV-infected compared to uninfected children from 1997-1999 [120]. The increased risk of IPD in HIV-infected children was sustained even in the era of highly active antiretroviral therapy (HAART) [77]. Similar high incidence rates were observed among Mozambican children; where the incidence rates of pneumonia hospitalisations were between 9.3-19.0-fold higher in HIV-infected children than in HIV-uninfected children [121]. The pneumococcal conjugate vaccines have demonstrated high efficacy against radiologically-confirmed pneumonia and IPD among HIV-infected and uninfected African children in randomised controlled trials conducted in SA and the Gambia using PCV9 [109;122]. In HIV-infected children however, the antibody response was lower than in uninfected children [123;124]. In SA, there was at least a 65% reduction in IPD due to PCV9 in HIV-infected children, while in the HIV-uninfected; IPD was reduced by 83% [109]. There have been studies showing that utilisation of pneumococcal conjugate vaccines can help reduce the fatality and severity of pneumonia and IPD in HIV-infected children [125]. The burden of hospitalisation due to pneumococcal pneumonia in HIV-infected children was also reduced by vaccination [122;126]. Use of the conjugate vaccines in SA have the potential to decrease penicillin and multi-drug resistance amongst pneumococcal strains [78].

1.9 Possible post-vaccine effects

The pneumococcus is naturally transformable and has the ability to take up foreign DNA through homologous recombination [127]. To escape vaccine pressure and as a survival mechanism, the pneumococcus can take up foreign capsular genes and express a different capsule that is not

targeted by the vaccine. This phenomenon is known as capsular switching and the strains are known as vaccine-escape recombinants [128]. The reduction in IPD and carriage due to vaccine serotypes have opened an ecological niche for the serotypes that were not prevalent to fill the gap left by the vaccine serotypes, known as serotype replacement [129]. An increase in non-PCV serotypes in carriage might also be due to "unmasking'' of these serotypes due to eradication of vaccine and vaccine-related serotypes that could not be detected due to serotyping techniques used, as explained by Weinberger *et al* [130]. However, randomised, placebo- controlled clinical trials using PCV9 in SA [131] and the Gambia [132] evidenced that serotype replacement in carriage was due to acquisition of new serotypes (true serotype replacement) in vaccinated children.

Following the introduction of PCV7 and the subsequent reduction in vaccine and vaccine-related serotypes, there has been an increase in non-vaccine serotypes resulting in serotype replacement in areas where the vaccine is routinely used. Serotype replacement has been observed in carriage as well as disease episodes. However, compared to the massive reduction in IPD due to vaccine and vaccine-related serotypes, the burden of disease caused by non-vaccine serotypes remained low in US, although it is statistically significant [133]. An increase in the carriage and incidence rates of IPD caused by non-vaccine serotypes has been observed in countries such as the US, France, England as well as in other countries for both vaccinated and unvaccinated populations [100;128;129;134]. In the United Kingdom (UK), the incidence of IPD after vaccination increased in individuals ≥5 years of age, and remained unchanged in children <5 years of age in 2002-2009 due to the increase in non-PCV7 serotypes [135]. Similarly, in Spain, IPD incidence rates remained unchanged, due to an increase in IPD due to non-vaccine serotypes, especially in

the adult age groups [136-138]. However, the massive benefits offered by PCV7 on reduction of disease due to vaccine and vaccine-related serotypes in those settings, is still substantial [139]. This might be due to the fact that non-PCV serotypes are thought to be less virulent than PCV types.

Prior to meningococcal polysaccharide conjugate vaccine roll-out in routine immunisation programmes, capsular switching was observed in *Neisseria meningitidis* showing that vaccine use is not the only factor contributing to capsular switching and serotype replacement [140;141]. However, the vaccine might be associated with increased rates of these mechanisms. Serotype replacement and capsular switching pose great challenges for current vaccine effectiveness and future vaccine development [100].

1.9.1 Serotype 19A

Serotype 19A is now the leading serotype in carriage, invasive and non-invasive disease in all age groups in the US as well as in certain other countries where PCV7 is used routinely [142-146]. The emergence of 19A was due to the expansion of the pre-existing clone that was circulating before introduction of the conjugate vaccine. However, recent evidence indicates that these emerging global clones are vaccine-escape recombinants [147;148]. IPD caused by serotype 19A increased from 2.6 to 11.1 cases per 100 000 population from 1998-1999 to 2007, for children <5 years in the US [90]. In France in 2007, after PCV7 introduction in 2006, serotype 19A accounted for 63% (110/173) of cases of otitis media in children <5 years [146]. IPD due to serotype 19A has now stabilised in the US with only a small increase observed in adults between 50 and 64 years [149]. The emerging non-vaccine serotype 19A is also more likely to be multidrug resistant [142;145;150;151]. Although the CPS structures of serotypes 19A and 19F are closely related

[44], serotype 19F (PCV serotype) offers little or no cross-protection against serotype 19A IPD [152]. The inclusion of serotype 19A in the recently licensed PCV13 might significantly reduce IPD and carriage due to this serotype.

1.9.2 Serogroup 6

Serogroup 6 serotypes are important in the epidemiology of disease-causing serotypes. There are four serotypes belonging to serogroup 6; namely; 6A, 6B, 6C and 6D. Serotype 6B, historically, was one of the leading paediatric serotypes [153]. There were only 90 serotypes described until 2007, when a 91st serotype belonging to serogroup 6, serotype 6C was discovered through a mouse monoclonal antibodies immunoassay [43;154]. Retrospective studies have revealed that, although unrecognised, this serotype had been in circulation for decades and was misidentified as serotype 6A, due to the serological similarities between the two serotypes. Serotypes 6A and 6B differ by a single nucleotide polymorphism (SNP) in the *wci*P gene of the capsular polysaccharide synthesis locus, due to a single recombinational event leading to a different rhamnose-ribitol linkage. However, in serotype 6C; the gene *wci*N is completely replaced with *wci*N$_{\beta}$ of unknown origin, probably from a non-pneumococcal source [155], resulting in the replacement of galactose residues in serotype 6A by glucose residues in serotype 6C (Figure 6) [154;156]. Polyclonal antisera used for traditional serotyping could not distinguish between the two subtypes [43]. However 6C-specific antiserum has subsequently been developed [157].

6A	⇨2)-**Galactose-**	(1⇨3) Glucose(1⇨3)-	Rhamnose	**(1⇨3)**-Ribitol-(5⇨P⇨
6B	⇨2)-**Galactose**-	(1⇨3) Glucose(1⇨3)-	Rhamnose	**(1⇨4)**- Ribitol-(5⇨P⇨
6C	⇨2)-**Glucose-**	(1⇨3) Glucose(1⇨3)-	Rhamnose	**(1⇨3)**- Ribitol-(5⇨P⇨
6D	⇨2)-**Glucose**-	(1⇨3) Glucose(1⇨3)-	Rhamnose	**(1⇨4)**- Ribitol-(5⇨P⇨

Figure 6. Comparison of serotypes 6A, 6B, 6C and 6D polysaccharide structures with the main differences between the serotypes highlighted in bold [40;43;158]

In the pre-PCV7 era, the prevalence of serotype 6C was low in countries like Brazil and US [159;160]. Similarly, in SA between 2005 and 2006, the prevalence serotype 6C was low, accounting for only 5% (30/606) of isolates initially serotyped as 6A [161]. However, in countries with routine conjugate vaccine use, serotype 6C is the predominant serogroup 6 serotype for both carriage and disease, due to the reduction in disease caused by serotypes 6A and 6B [162-165]. Serotype 6B is included in PCV7 and offers cross-protection to serotype 6A [166]. An increase from 0.22 to 0.58 cases per 100 000 population was observed for IPD caused by serotype 6C alone between 1999 and 2007 in the US [167].

After the discovery of serotype 6C, a new serotype 6D was proposed and constructed *in vitro* from a single homologous recombination event in the serotype 6B *wci*N gene, as had been found in serotypes 6A and 6C [168]. Serotype 6D contains the *wci*P from serotype 6B and *wci*N_{β} from serotype 6C. Subsequently, serotype 6D isolates have been recovered from the nasopharynx of healthy children [40;158]. In 2010-2011, carriage isolates belonging to this new serotype have been reported in various settings in South Korea [169], Japan [170], South America [171] and China [172]. In 2010, the first case of invasive disease due to serotype 6D was reported in Europe [173;174]

In 2011, genetic analysis of the biosynthetic loci of serogroup 6 serotypes revealed that serotypes 6C and 6D did not evolve from serotype 6A and 6B respectively [175]. The genetic analysis showed genetic profiles that were not related to either serotype 6A or 6B for serotype 6C and 6D isolates studied. The *cps* loci of serotypes 6C and 6D are 98.6% identical. Identification of serogroup 6 serotypes is of importance to evaluate whether disease was a result of vaccine failure (serotypes 6A or 6B) and to monitor disease caused by these newly identified serotypes. Recently in 2011, immunological studies showed that in fact, the inclusion of serotype 6A in the 13-valent pneumococcal conjugate vaccine offers cross-protection to disease due to serotype 6C [176]. Thus, constant monitoring of IPD due to serogroup 6 serotypes is of utmost importance for epidemiological analysis.

Other vaccine replacement serotypes that are increasing in the US are serotypes 15A, 23A, 33F, 35B, 22F, and 7F [90;150]. As with serotype 19A, these replacement serotypes are associated with penicillin resistance and multi-drug resistance.

1.9.3 Empyema

While pneumonia hospitalisations and mortality have decreased drastically due to conjugate vaccine utilisation, there has been an increase in pneumonia complicated by empyema [177;178]. Empyema is the accumulation of pus in the normally sterile pleural cavity. The increase during the post-PCV7 era was observed in older children of 2-4 years [179] and was due to *S. pneumoniae.* Non-PCV7 serotypes (serotypes 1, 3, 19A and 7F) [177;180] were associated with this complication, suggesting that empyema might be a replacement disease caused by non-vaccine serotypes [181;182].

1.10 Importance of monitoring serotypes

Determination of serotypes is important in order to monitor the burden of disease caused by specific serotypes and therefore know which serotypes should be included in the conjugate vaccine formulations. The emergence of multidrug-resistant global clones such as 19A, other replacement serotypes and the discovery of new serotypes warrants continuous and efficient monitoring of serotypes. The inclusion of additional serotypes in the new PCV formulations e.g. serotype 1 and 19A in PCV13, further strengthen the ongoing need for continuous surveillance in order to identify any new replacement serotypes that may emerge. The discovery of a new subtype within an already established serotype such as 19A is also of global concern for determining the true burden of disease caused by this serotype. The WHO has initiated the Pneumococcal Global Serotype Project (GSP). One of the objectives of the project was to determine the distribution of invasive disease-causing serotypes and variations between regions, including among GAVI (Global Alliance for Vaccines and Immunisation)-eligible countries [183]. The project committee gave recommendations on which vaccines will be suitable for a specific region and which serotypes are to be included in future vaccine formulations [50;184] by using global serotype distribution data obtained through published and unpublished work. Over ten million cases and around one million deaths worldwide were caused by serotypes included in PCV10 and PCV13 formulations, and the committee recommended that utilisation of this vaccine in Africa and Asia would decrease the high burden of IPD in these areas. It is important to constantly monitor seroepidemiological changes in a fast, less complex and cost effective manner.

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1.11 Aim and Objectives

The aim of this study was to establish and evaluate molecular methods for serotyping of *Streptococcus pneumoniae* from isolates and culture-negative clinical specimens.

This has been achieved through the following objectives:

I. To establish conventional and RT-PCR assays for serotyping of *S. pneumoniae* using pneumococci of known serotypes.
II. To determine the utility of conventional PCR in determining pneumococcal serotypes from culture-positive samples.
III. To determine the utility of conventional PCR in determining pneumococcal serotypes from culture-negative samples positive for pneumococci by *lytA* PCR.
IV. To determine the utility of RT-PCR assays for serotype determination directly from culture-negative samples positive for pneumococci by *lytA* PCR.
V. To determine the serotype distribution in culture-negative samples received as part of SARI and empyema studies.
VI. To compare the distribution of serotypes between culture-positive and culture-negative samples.

2 Materials and Methods

2.1 National surveillance

The Centre for Respiratory Diseases and Meningitis (CRDM) of the National Institute for Communicable Diseases (NICD), a division of the National Health Laboratory Service (NHLS), houses the national reference laboratory for IPD in SA. CRDM conducts laboratory-based nationwide surveillance of IPD. The case definition for IPD is the isolation or identification of *S. pneumoniae* from a normally sterile site (e.g. pleural fluid, blood, joint and cerebrospinal fluid (CSF)) by either a positive culture, *lytA* detection by PCR or positive latex antigen test with a matched Gram stain result. Demographic information and patient details of all cases are stored in the national pneumococcal database for each year of surveillance. IPD cases identified through the NHLS laboratory electronic database but with no isolate submitted to the NICD laboratory are still recorded on the national surveillance database. Approximately 126 NHLS regional laboratories and private laboratories across the nine provinces of SA participate in the national surveillance through a GERMS-SA surveillance network (Group for Enteric, Respiratory and Meningeal Disease Surveillance in South Africa) [82].

The laboratories submit pneumococcal isolates to CRDM on Dorset egg transport media. Some specimens with latex positive and /or Gram stain positive for pneumococci do not yield a positive bacterial growth, and for these cases, the clinical specimens are sent to CRDM for further confirmation of IPD by PCR. Clinical diagnostic specimens without a Gram stain or latex agglutination results from patients with a clinical suspicion of meningitis or septicaemia are also sent to CRDM for urgent detection of pneumococcal DNA by PCR.

2.2 Viable isolates

2.2.1 Source of bacterial cultures

Upon receipt at CRDM, isolates (i.e. culture-positive) from the transport medium were sub-cultured onto 5% horse blood Columbia base agar plates (Oxoid, Hampshire, UK) using a sterile loop. Optochin discs (Mastdisk, Merseyside, UK), were used for identification of *S. pneumoniae*. Optochin (ethyl hydrocuprein) is a chemical agent used for basic screening of pneumococci. The majority of pneumococcal isolates are susceptible to optochin, forming a zone clearance around the discs of ≥11mm, with few exceptions. The blood agar plates were incubated at 37ºC in 5% CO_2 for 18-24 hours. Heavy pure growths of *S. pneumoniae* were stored in 10% skim milk (Oxoid) and stored at -70ºC for further analysis. Optochin-resistant isolates, which were identified as pneumococcus by colony morphology, bile solubility and Gram stain, were confirmed by a positive *lytA* RT-PCR.

Contaminated cultures were sub-cultured on selective media, namely, 5% blood agar plates supplemented with 5 µg/ml gentamycin (Oxoid) to obtain pure growth of the pneumococcus. The pure colonies were sub-cultured on 5% blood plates for heavy growth and stored as previously described. Contaminated cultures from which pneumococcus could not be isolated, were treated as non-viable transport medium samples described below in section 2.3.1.

2.2.2 The Quellung reaction

All pneumococcal isolates were serotyped at CRDM by the Quellung reaction using type-specific antisera (Staten's Serum Institute, Copenhagen, Denmark). The process involved preparing a light suspension of approximately 0.5 McFarland standards by picking not more than five

colonies of the pneumococcus from the 5% blood agar plates and suspending in 1 ml saline solution. Two smears were fixed on the glass slide by air drying. The antiserum to be tested was mixed with ethylene blue on a cover slip and the smears were covered with the cover slip. The slides were viewed on a phase-contrast microscope (Nikon, Melville, USA and Olympus, Centre Valley, PA, USA) to detect swelling of the capsule surrounding the cell wall.

2.2.3 Crude extraction

DNA extraction from pneumococcal cultures was performed using a boiling method. A small loopful of the culture was suspended in 200 µl sterile distilled water (sdH_20) in 0.5 ml tubes (Thermo Scientific, Leicestershire , UK). The pneumococcal suspensions were boiled at 95˚C for 10 min, which bursts open the cell wall and releases the DNA. Suspensions were briefly centrifuged using an Eppendorf Minispin centrifuge (Merck, Whitehouse Station, NJ, USA) at 12000 RPM to pellet the debris. The supernatant was stored at -20˚C until use.

In addition to isolates, CRDM receives culture-negative specimens for confirmation of pneumococcal DNA.

2.3 Non-viable samples

2.3.1 Types of non-viable samples

2.3.1.1 Non-viable transport medium samples

The regional laboratory inoculates the pneumococcus on the Dorset egg transport medium; however, upon receipt at the CRDM, for some, pneumococcal growth was not obtained on 5% blood agar plates. Pneumococcal growth was not detected from the transport medium due to three possible reasons:

I. Incorrect inoculation techniques from the regional laboratory (e.g., not enough organism inoculated, failing to incubate after inoculation or problems with transport media used) or
II. The organism losing viability in transit before reaching CRDM, due to its fastidious nature, or
III. Contamination by another organism that outgrows the pneumococcus.

The following methods were performed to attempt to recover the organism,

I. Sub-culturing on a selective medium (5% blood agar with gentamycin) and incubating at 37°C 5%, CO_2 for longer periods i.e. 48 hours.
II. Adding a nutritious liquid medium, such as BHI (Brain Heart Infusion Broth, Oxoid) and incubating at a 5% CO_2 and 37˚C for 2 hours to enhance the growth of the organism.

These specimens were classified as culture positive at the regional laboratory, but non-viable on receipt at CRDM. 299 NVTMs from year 2010 were available for this study.

2.3.1.2 Clinical diagnostic specimens

Diagnostic specimens for urgent PCR detection of DNA of the three common pathogens causing meningitis i.e. *S. pneumoniae*, *N. meningitidis* and *Haemophilus influenzae* were sent to the CRDM. This is a routine urgent diagnostic assay offered by CRDM since 2005. The clinical specimens used in this study were from normally sterile sites e.g. blood, CSFs, and pleural fluids, with a suspicion of IPD. The specimens were culture negative possibly due to

I. Patients receiving antibiotic therapy before collecting the specimen in the health-care facility.
II. Delays in processing of specimens resulting in non-viability of organism.
III. Specimens kept at unfavourable conditions before being processed or sent to CRDM.

For this study, CSFs (n=40), blood specimens (n=10) and a pleural fluid (n=1) received in 2010 were used.

2.3.1.3 Beep-positive, subculture-negative blood cultures

Culture-negative, beep-positive blood culture bottles, mainly from Chris Hani Baragwanath Hospital, are routinely received by CRDM. These blood cultures were identified by the BacT/ALERT® system as positive for microbial growth at the regional laboratories. The system is rapid and fully automated and is used to detect growth from blood specimens inoculated into vials containing media. Microbial growth is detected by a sensor, which senses the accumulation of carbon dioxide ($C0_2$) in 5-10min intervals. The accumulation of $C0_2$ or consumption of oxygen (0_2) is indicative of microbial metabolism of the media. A “beep-positive” reading indicates the presence of viable microorganism in the vial. For identification, Gram stain and additional microbial identification tests are performed. Presence of the pneumococcus is confirmed in these

specimens by latex agglutination, Gram stain or pneumococcal antigen test such as Binax NOW® [185;186] but no bacterial growth is observed on subculture culture possibly due to:

I. Autolysis of the pneumococcus facilitated by autolysin enzymes present in all pneumococci due to delays in removing the bottles from the automated system.

At our reference laboratory, such samples undergo routine qualitative *lytA* RT-PCR testing to confirm pneumococcal identification. In 2010, 154 beep-positive, subculture-negative blood culture bottles were received.

2.3.1.4 Severe Acute Respiratory Illness (SARI) blood specimens

The SARI surveillance study was established in February 2009 at six hospitals in four provinces of SA, namely, Gauteng (Chris Hani Baragwanath hospital), KwaZulu-Natal (Edendale hospital), Limpopo (Mapulaneng and Matikwana hospitals) and North-West (Klerksdorp and Tshepong hospitals). The aim of the SARI surveillance study was to describe the aetiology of severe pneumonia in SA as well as identify risk factors associated with severe pneumonia. CRDM collects whole blood samples for the quantitative detection of the pneumococcus in order to estimate the prevalence of pneumococcal pneumonia in these patients. Whole blood specimens, from patients meeting the SARI case definition, were collected in EDTA tubes within 24 hours of admission to the hospitals and sent to CRDM for pneumococcal detection using quantitative *lytA* RT-PCR. Vaccination status was obtained from vaccination cards on admission. PCV7 eligible were defined as all children <5 years and born after 15/02/2009 or 3 weeks old by the time of vaccine introduction. Children ≥6 weeks and <2 years were regarded as vaccinated if they had received at least 1 dose of PCV7 at least 14 days before symptoms onset. This was considered sufficient period for the vaccine to have elicited an immune response. 372 of *lytA*-positive specimens from 2009-2010 were used for this study.

2.3.1.5 Pleural fluids collected in an empyema study

CRDM collaborated with medical specialists from Red Cross Children's Hospital (RCCH), Cape Town, SA. The study involved management of pleural empyema with new treatment modalities: fibrinolysis and video-assisted thoracoscopic surgery (VATS) in comparison with the traditional antibiotic treatment (with or without chest drain insertions) for management of this disease. The aim of the study was to determine the aetiological agent and outcome of empyema cases, from children admitted and treated with new methods in comparison with historical controls who never received the new treatment modalities. CRDM detected the presence of pneumococcal DNA in pleural tap specimens using a qualitative RT-PCR assay, which simultaneously detects the presence of *S. pneumoniae, H. influenzae* and *N. meningitidis*. By the end of 2010, 19 *lytA*-positive pleural fluids for this study were available for molecular serotyping.

2.3.2 DNA extraction from culture-negative samples

For clinical specimens, NVTMs and stored isolates that had lost viability during storage, an automated DNA extraction was performed. The MagNA Pure instruments (Roche, Forrenstrasse, Switzerland) extract DNA using magnetic bead technology. The process involves binding of DNA molecules to the magnetic bead particles. After washing of unbound DNA molecules, the bound DNA is then eluted into an elution buffer by magnetic force and the beads are retained. The MagNA Pure LC 2.0 instrument (processing up to 32 samples per run) and MagNA Pure LC DNA Isolation Kit III were used for the extraction of DNA from SARI blood specimens. The MagNA Pure Compact (processing up to 8 samples per run) and MagNA Pure Compact Isolation Kit I (Roche diagnostics, Indianapolis, USA) were used to extract DNA from all clinical specimens and non-viable transport medium samples. DNA extraction was performed according to the manufacturer's instructions from either 200 µl or 100 µl of sample, depending on the

volume of sample available. The DNA was eluted into a 100 µl (for 200 µl extraction volume) or 50 µl (for 100 µl extraction volume) of elution buffer and stored at -20°C until further use.

2.3.3 Molecular detection of pneumococcus from culture-negative samples

2.3.3.1 *lytA* real-time PCR

The *lytA* RT-PCR was performed on the following samples: beep-positive, subculture-negative blood cultures, NVTMs and SARI blood specimens to confirm the presence of pneumococcal DNA. Primers and a FAM-labelled TaqMan minor groove-binding (MGB) probe, (Applied Biosystems, Warrington, UK) were used in the assay at a final concentration of 200 nM. Each 25 µl reaction consisted of 2X TaqMan gene expression mastermix (12.5 µl) (Applied Biosystems), 2.5 µl of DNA, primer pair and probe to the above-mentioned concentrations and sterile distilled water. The primers and probe sequences were; Forward primer (F373) 5'-acgcaatctagcagatgaagca-3', Reverse primer (R424) 5'-tcgtgcgttttaattccagct-3', Probe (Pb400) 5'-tgccgaaaacgcttgatacagggag-3'-FAM [28].

2.3.3.2 HNS real-time PCR

A multiplex RT-PCR assay [187] for the simultaneous detection of *H. influenzae (hpd; protein D)*, *N. meningitidis (ctrA)* and *S. pneumoniae* (*lytA*) (HNS) is a routine diagnostic assay performed at CRDM since 2005. The assay was carried out on all routine diagnostic clinical specimens and pleural fluids for the empyema study. Primers and black hole quencher (BHQs) probes were obtained from the CDC. Each 25 µl reaction consisted of 12.5 µl Invitrogen Platinum PCR super-UDG master mix (Invitrogen, Carlsbad, CA, USA), 2 µl of DNA, primer pairs and probes to the final concentration as listed in Table 10 (appendix D), and sdH_20 to make up the volume.

2.3.4 PCR conditions and interpretation of results

The 7500 Fast real-time PCR instrument (Applied Biosystems) was used. All reactions were carried out in 0.1 ml 96-well reaction plates (Applied Biosystems). Universal cycling conditions were used for both the HNS and *lytA* real-time assays i.e. Stage 1: 50˚C for 2 min; Stage 2: 95˚C for 10 min and Stage 3: 40 cycles of two-staged temperature profile of 95˚C for 15 sec and 60˚C for 1 min. The increase in fluorescent signal of the sample beyond the threshold was recorded as a positive result for the presence of the pneumococcal *lytA* gene. The cycle threshold (Ct-value) value at which the fluorescence crossed the threshold was recorded. If no increase in fluorescent signal above the threshold was observed within 40 cycles for *lytA*, the sample was reported as negative for the pneumococcus. For the HNS assay, the results were considered positive if the C_t-value was <35 and negative if there was no increase in fluorescent signal beyond the threshold within 40 cycles of amplification. For the results with *lytA* C_t-values of ≥35 but ≤40 on HNS, an accompanying latex agglutination positive result for pneumococcus, Gram-positive diplococci, or other clinical information such as septicaemia were needed to confirm the presence of pneumococcus as the disease-causative agent. For the quantitative *lytA* RT-PCR, the threshold was set at 0.05 for all analysis, and results were interpreted as being positive if there was an increase in the fluorescence signal beyond the set threshold within 40 cycles. The DNA copies extrapolated from the standard curve were halved, accounting for the 200 µl sample extraction volume in 100 µl elution volume.

2.4 Establishment of conventional PCR serotyping assay

2.4.1 Selection of isolates for setting up of conventional PCR serotyping assay

Bacterial cultures (n=96) were randomly (www.random.org) selected by serotype from the 2007 surveillance isolates. These isolates were previously serotyped using the Quellung reaction. These isolates represented three isolates each of 32 of 33 serogroups/types detected by C-PCR assay. A serotype 2 control, to make up for the 33 serotypes, was not available amongst the surveillance isolates (2003-2010). Furthermore, isolates representing seven different serotypes (n=21) not detected by the assay but common in SA were selected to confirm the specificity of the assay.

2.4.2 Selection of isolates for determining the utility of conventional PCR serotyping assay

Following setting up of the assay, approximately 12% of isolates were randomly selected (www.random.org/sequences) from the surveillance isolates collected from January 2008 through December 2009 (410/3327 for 2008 and 419/3391 for 2009). All isolates had previously been serotyped using the Quellung reaction. The isolates were retrieved from storage in -70°C as described in section 2.2 and sub-cultured on blood agar plates (Oxoid) overnight. DNA was extracted by the boiling method as described in section 2.2.3. A total of 27 and 49 isolates from 2008 and 2009 respectively, were no longer viable after retrieval from -70°C and therefore DNA was extracted directly from the storage medium i.e. skim milk by automated DNA extraction as described in section 2.3.2

2.4.3 Serotyping assay

Primer pairs are listed in Table 11 (appendix E) The method was adapted from that developed by the Streptococcus Laboratory of the CDC in Atlanta, USA [68]. Primer concentrations and

reagent compositions for each reaction are shown in Table 12 (a-f, appendix E). The serotypes were grouped to allow >50 bp differences between serotypes for greater resolution on an agarose gel. The final volume of the PCR was 25 µl using 2 µl of DNA template.

PCR reactions were tested and compared using two methods:

2.4.3.1 Amplitaq Gold DNA polymerase

Reactions were performed using AmpliTaq® Gold DNA polymerase supplied with GeneAMP 10X PCR Buffer and $MgCl_2$ (Applied Biosystems) using 2.5 units of enzyme per reaction. The final concentration of deoxynucleotide triphosphate (dNTPs) and $MgCl_2$ were 200 µM and 3.5 nM respectively, in a 25 µl reaction. The PCR reactions were carried out in a 2720 Thermal Cycler (Applied Biosystems) using either 0.2 ml thermo–tubes (Thermo Scientific) or 96-well microplates (Axygen Scientific, California, USA).

2.4.3.2 Qiagen Multiplex PCR Mastermix

Reactions were performed using the Qiagen mastermix (containing HotStart DNA polymerase, multiplex PCR buffer with $MgCl_2$ and dNTP mix) (Qiagen, Hilden, Germany).

Cycling conditions included an activation step at 95˚C for 10 min, followed by 35 cycles of 94˚C for 30 sec, 54˚C (59˚C for reaction 4, 5 and 6 when using Amplitaq Gold DNA Polymerase) and a final holding step at 72˚C for 10 min.

2.4.4 Agarose gel electrophoresis

A volume of 10 µl of 2.5% Bromophenol blue solution (Sigma-Aldrich, St Louis, MO USA); loading dye was added to the 25 µl of PCR product. 10 µl of the mixture (PCR product + loading

dye) was loaded on a 2% Seakem LE Agarose gel (Whitehead Scientific, Rockland, USA) containing 10 mg/ml ethidium bromide (Sigma-Aldrich) and electrophoresis was performed in 1X TAE buffer Tris (Sigma-Aldrich and Merck)-Acetate (Merck) –EDTA (Merck) at 100 V (voltage) for 1h30 min. The gels were visualised using ultraviolet light. A Molecular Imager® Gel Doc^{TM} XR System (Bio-Rad Laboratories, Hercules, California, USA) was used to visualise and capture the agarose gel images.

The serotypes of the control isolates were assigned based on band sizes using a 100 base pair (100 bp-1000 bp) HyperLadder IV molecular weight marker (Bioline, Humber Road London, UK). The assigned serotype was then compared with the serotype obtained using the Quellung reaction. Subsequently, serotypes for 2008 and 2009 isolates and non-viable samples were determined by comparing the sizes of products to that of 2007 isolates controls and the molecular weight marker.

2.5 Establishment of real-time PCR serotyping assay

2.5.1 Selection of isolates for setting up of real-time PCR serotyping assay

For RT-PCR, only isolates representing 22 serogroups/types selected were used from 2007 (n=69) isolates used for validation of C-PCR. Serogroup 15 was represented by 3 isolates of serotype 15A and serotype 15B/C each, i.e. serogroup 15 was validated by 6 isolates in total.

2.5.2 Selection of isolates for determining the utility of real-time PCR serotyping assay

To determine the specificity of the RT-PCR serotyping assay, 228 isolates from 2008 surveillance were selected. These isolates were a representation of all 65 serotypes/groups identified by the Quellung reaction in SA for 2008. We aimed to have each serotype represented by four different

isolates. 2008 was used as a reference year, and where four isolates for a specific serotype could not be obtained from 2008 surveillance, isolates from 2009 surveillance were selected to complete the total representation of four isolates per serotype. Where four isolates per serotype could not be obtained from the two years, the total available for the two years was used for that serotype. All four isolates could be obtained for 51 serotypes/groups. Serotypes which had 1-3 isolates due to total number of samples received or culture no longer available for the study years combined were serotypes 27 (1), 29 (3), 42 (1), 43 (1), 10B (1), 12B (3), 17A (1), 19B (3),28 (2), 28A (2), 33B (2), 35A (1), 33A (3) and 9L (1). All isolates were retrieved from storage at -70˚C and sub-cultured overnight on 5% horse blood agar plates and DNA was extracted using the automated extraction method directly from skim milk as described in 2.3.2.

2.5.3 Serotyping assay

The assay used in this study was adapted from Azzari *et al* [34]. The RT-PCR serotyping assay consisted of 21 singleplex reactions initially, later validated to 11 duplex reactions with an additional primers/probe set for serotype 6C/D (Table 13 & 14, appendix F). The primer pairs targeted 11 specific serotypes (serotypes 1, 3, 4, 5, 8, 14, 20, 19A, 23F, 35B and 38) and 11 serogroups (serogroup 6, 6C/D 7A/F, 9A/V, 10A/B, 12A/B/F, 15, 18B/C, 19B/F, 22A/F and 33A/F). Primer pair sequences for serogroups 22A/F and 6C/D were obtained from the CDC. Fluorescent MGB probes (Applied Biosystems) were labelled with either FAM or VIC dye. For FAM-labelled probes, the final concentration for both primers and probes was 200 nM whereas for VIC-labelled probes, both probe and the corresponding primers were used at a final concentration of 400 nM.

All reactions were carried out to a final volume of 25 µl consisting of 12.5 µl 2X TaqMan gene expression mastermix (Applied Biosystems), 2.5 µl of DNA, primer pair and probe to the above-mentioned concentrations for each serotype and sterile distilled water (sdH_2O) to make up the volume. MicroAmp Fast 96-well reaction plates (0.1 ml) were used with a 7500 Fast real-time PCR instrument (Applied Biosystems). A no template control (NTC) was included in all reactions as well as the 10X and 100X dilutions of the controls. The universal cycling conditions, as described were used. Results were interpreted as being negative if no significant increase in fluorescent signal of the sample beyond the threshold was observed within 40 cycles, as previously described.

It was hypothesised that the *lytA* C_t-value and the corresponding serotype C_t-value would not differ significantly from each other. All *lytA*-positive samples were assumed to contain sufficient DNA for a serotype to be detected by RT-PCR. A sample yielding *lytA* positive but serotype-negative results was therefore assumed to be of a serotype/group not detected by any of the serotyping assays. In cases where two serotypes were detected, the serotype with the lower C_t-value (and/or closest to the *lytA* Ct-value) was reported as the disease-causing serotype. A sample was reported as having mixed serotypes if the two serotypes detected were within a range of ±3 C_t-values.

2.6 Establishment of serogroup 6 differentiation assays

2.6.1 Selection of isolates for serogroup 6 differentiation by conventional and real-time PCR assays

Isolates representing serotypes 6A (n=5), 6B (n=5) and 6C (n=5) were randomly selected from 2007 surveillance isolates. Isolates from 2008 (n=62) and 2009 (n=49) were used to further

validate the utility of serogroup 6 differentiation PCR reactions. In addition, 178 isolates from 2010 surveillance isolates that had been serotyped as serotype 6B (n=167) and 6C (n=11) by the Quellung reaction were selected for further validation and to screen for serotype 6D. DNA from 178 isolates received in 2010 was extracted directly from skim milk using an automated extraction technique.

2.6.2 Serogroup 6 differentiation assays

Serogroup 6 differentiation was performed using two PCR assays:

2.6.2.1 The conventional PCR assay

An internal control for confirmation of serogroup 6 that was used in reaction 1 of the C-PCR assay was included in all reactions at a final concentration of 300 nM (product size of 250 bp) [68]. The primers used for serogroup 6 differentiation are listed in Table 15 (appendix G). Initially, the assay consisted of two duplex reactions (Table 16 b & c, appendix G). The first reaction was aimed to target the *wciP* gene of serotype 6B with a product size of 155 bp. The second reaction targets *wciN*$_{beta}$, which amplifies serotype 6C with a product size of 359 bp (*wci*N$_{\beta}$S1 and *wci*NA2, outer primer set) or 308 bp (*wci*N$_{\beta}$S2 and *wci*NA1, inner primer set) but does not amplify serotype 6A's. All isolates that were negative in reaction 2 were assigned serotype 6A. All isolates identified as serotypes 6B from reaction 1 and were positive for the 6C primers in reaction 2, were assigned a serotype 6D.

To limit the number of conventional PCR reactions, the two reactions were combined to a single reaction to differentiate all serogroup 6 serotypes using one multiplex reaction (Table 16a, appendix G). If the internal control band size (250 bp), and both the band for serotype 6B (155 bp) and 6C (309/359 bp) were observed, then the sample was assigned a novel serotype 6D. PCR

products were run on a 2% agarose gel for 1h30min as described in section 2.4.4. For 2007, 2008 and 2009 isolates, both the two duplex reactions and the multiplex reaction were performed, while for 2010 serogroup 6 isolates, only the multiplex reaction was performed.

The final primer concentrations were 500 nM and 700 nM for *wciP* (6B) and *wciN*$_{beta}$ (6C and 6D), respectively. All reactions were performed in a final volume of 25 µl, consisting of 12.5 µl of 2X Qiagen mastermix (Qiagen), primers pairs to the above-mentioned concentration, 2 µl of crude DNA extract and sdH_2O to make up the volume. The PCR reactions were carried out in a 2720 Thermal Cycler (Applied Biosystems) using either 0.2 ml thermo–tubes (Thermo Scientific) or 96-well microplates (Axygen Scientific). The cycling conditions were 95˚C for 10 min, 35 cycles of 94˚C for 30 sec, 62˚C for 60 sec, and 72˚C for 60 sec. The final holding temperature was 72˚C for 10 min and storage at 4˚C.

2.6.2.2 The real-time PCR assay

The primer sequences distinguished serotype 6C/6D from serogroup 6. The assay was duplexed with serogroup 6 primers. Primers and VIC-labelled probes were used at a concentration of 400 nM for serogroup 6, and 200 nM final concentrations were used for primers and FAM-labelled probes for serotype 6C/6D. All reactions and cycling conditions were carried out as described in 2.3.4 for the RT-PCR serotyping assay validation.

Results were interpreted as positive for serotype 6C/6D if an increase in florescent signal was observed within 40 cycles beyond the threshold level in both serogroup 6 primers and serogroup 6C/6D set. If an increase in fluorescent signal was observed before the end of the 40th cycle for serogroup 6 primer set, but no fluorescent signal increase for serotype 6C/6D, the results were

interpreted as being positive for serotype 6A/6B. If no increase in fluorescent signal was observed for both primers/probe sets, then the sample was presumed not to belong to serogroup 6.

Table 1. Summary of samples used in this study

Year	Sample type	Number of samples	Number of viable cultures	Serotyping PCR assay
2007	Stored cultures	132	132	C-PCR[a] and RT-PCR[b]
2008	Stored cultures	411	384[c]	C-PCR
2008-2009	Stored cultures	228	Not sub-cultured[d]	RT PCR
2009	Stored cultures	419	370[e]	C-PCR
2009-10	Empyema pleural fluids	19	-*	RT-PCR
2009	SARI specimens	151	-*	RT-PCR
2010	Stored cultures	178	Not sub-cultured[d]	Serogroup 6 differentiation
2010	SARI specimens	219	-*	RT-PCR
2010	Blood cultures	154	16**	C-PCR and RT-PCR
2010	Cerebrospinal fluids	40	5**	C-PCR and RT-PCR
2010	Blood and pleural fluids	11	6**	C-PCR and RT-PCR
2010	Non-viable transport medium samples	299	66**	C-PCR and RT-PCR

[a] C-PCR–conventional PCR, [b] RT-PCR–real-time PCR, [c]27 cultures lost viability on skim milk storage, [d] not sub-cultured on blood agar plates, DNA extracted directly from skim milk, [e]49 cultures lost viability on skim milk storage
*no culture available, ** Viable isolates retrospectively received.

2.7 Statistical Analysis

Serotyping results for C-PCR and RT-PCR were entered onto a Microsoft Excel database (Microsoft Office professional edition 2003, Microsoft Corporation). For cultures, where Quellung reaction serotypes were available, phenotypic serotypes were used for comparative purposes. Sensitivity and specificity were described as the proportion of true positives and proportion of true negatives, respectively for primers and primers/probe sets. The sensitivity and specificity of the C-PCR assays were calculated using 2008 (411) and 2009 (419) isolates For RT-PCR, sensitivity and specificity were calculated using isolates from 2008 and 2009 (n=228). The sensitivity and specificity of each assay was calculated as follows:

Sensitivity = number of true positives / (number of true positive + false positives).

Specificity = number of true negatives / (number of true negatives + false positives).

Epi Info software version 6 (CDC, Atlanta, GA, USA) was used for all statistical analysis. The Mantel-Haenzel chi-squared test was used to estimate if there was a difference in serotype yield using C-PCR or RT-PCR assays on isolates. The test was also used to compare the sensitivities of C-PCR and RT-PCR on culture-negative samples with low and high bacterial loads. In addition, the test was used to test for significant difference in serotype distribution between viable and non-viable samples. The student's t-test was used to determine if there was significant loss in sensitivity between the singleplex and the duplex reactions of the RT-PCR serotyping assay. For all statistical analysis, $p<0.05$ was regarded as statistically significant. Analysis of data for the SARI surveillance programme was performed using STATA version 11.0 (StataCorp, Texas, USA). The Fisher's exact test and Pearson's chi-squared test were used to analyse trend in *lytA* positivity and PCV7 serotype distribution

2.8 Ethics

Ethics approval for national surveillance (Protocol no.M08117) and ethics clearance for this project (Protocol no. M10364, Appendix H), were obtained from the Human Research Ethics Committee (Medical) at the University of Witwatersrand, Johannesburg, SA.

3 Results

3.1 National IPD surveillance in SA

Using all 2008 (n=3327) and 2009 (n=3391) surveillance serotyping data (by Quellung reaction), it was estimated that the C-PCR assay would have assigned a serotype to at least 96% of all isolates (i.e. culture-positive) received for each year (Tables 2 & 3). The RT-PCR assay would have assigned a serotype to 91% (3045/3327) in 2008 and 90% (3061/3391) in 2009. It was estimated that more serotypes would be assigned to isolates using C-PCR compared to RT-PCR serotyping assay [6460/6718 (96%) for C-PCR vs. 6106/6718 (91%) for RT-PCR, $p<0.001$] for the two years combined.

Overall, 4201 cases were reported in SA in 2010. Of these, 68% (n=2859) were culture positive (isolates), and were serotyped using the Quellung reaction. For 93 *lyt*A-positive culture-negative samples received initially, viable cultures were received at a later stage and hence were included in culture-positive mentioned above. Overall, culture-negative samples accounted for 10% (n=440) of cases and of these, 6% (26/440) were NVTMs presumably inoculated with pneumococcus at the regional laboratories but negative for pneumococcal DNA at CRDM by *lyt*A real-time PCR assay. In addition, 22% (n=902) were cases identified retrospectively on audits of the participating laboratories (Figure 7).

Table 2. Expected serotype coverage of the surveillance isolates using conventional PCR serotyping assay for 2008, South Africa

Reactions	Serotypes	No. detected (N=3327)	Percentage (%)
Reaction 1	14,6A/B/C,23F,19A,9V	1507	45.3
Reaction 2	19F,3,15B/C,18,10A,7A/F	506	15.2
Reaction 3	1,5,11A/D,9N/L,17F	534	16.0
Reaction 4	7C/(7B/40),12F(12A/44/46),4,38/25,23A	350	10.5
Reaction 5	8,2,34,20,22A/F,31	194	5.8
Reaction 6	33F/(33A/37),15A,35F/47,35B,16F	121	3.6
Total detected	33 serogroups/types	3212	96.4
Total not detected	Multiple serotypes	115	3.6

Table 3. Expected serotype coverage of the surveillance isolates using conventional PCR serotyping assay for 2009, South Africa

Reactions	Serotypes	No. detected (N=3391)	Percentage (%)
Reaction 1	14,6A/B/C,23F,19A,9V	1471	43.4
Reaction 2	19F,3,15B/C,18,10A,7A/F	490	14.5
Reaction 3	1,5,11A/D,9N/L,17F	631	18. 6
Reaction 4	7C/(7B/40),12F(12A/44/46),4,38/25,23A	374	11.0
Reaction 5	8,2,34,20,22A/F,31	190	5.6
Reaction 6	33F/(33A/37),15A,35F/47,35B,16F	105	3.0
Total detected	33 serogroups/types	3261	96.2
Total not detected	Multiple serotypes	130	3.8

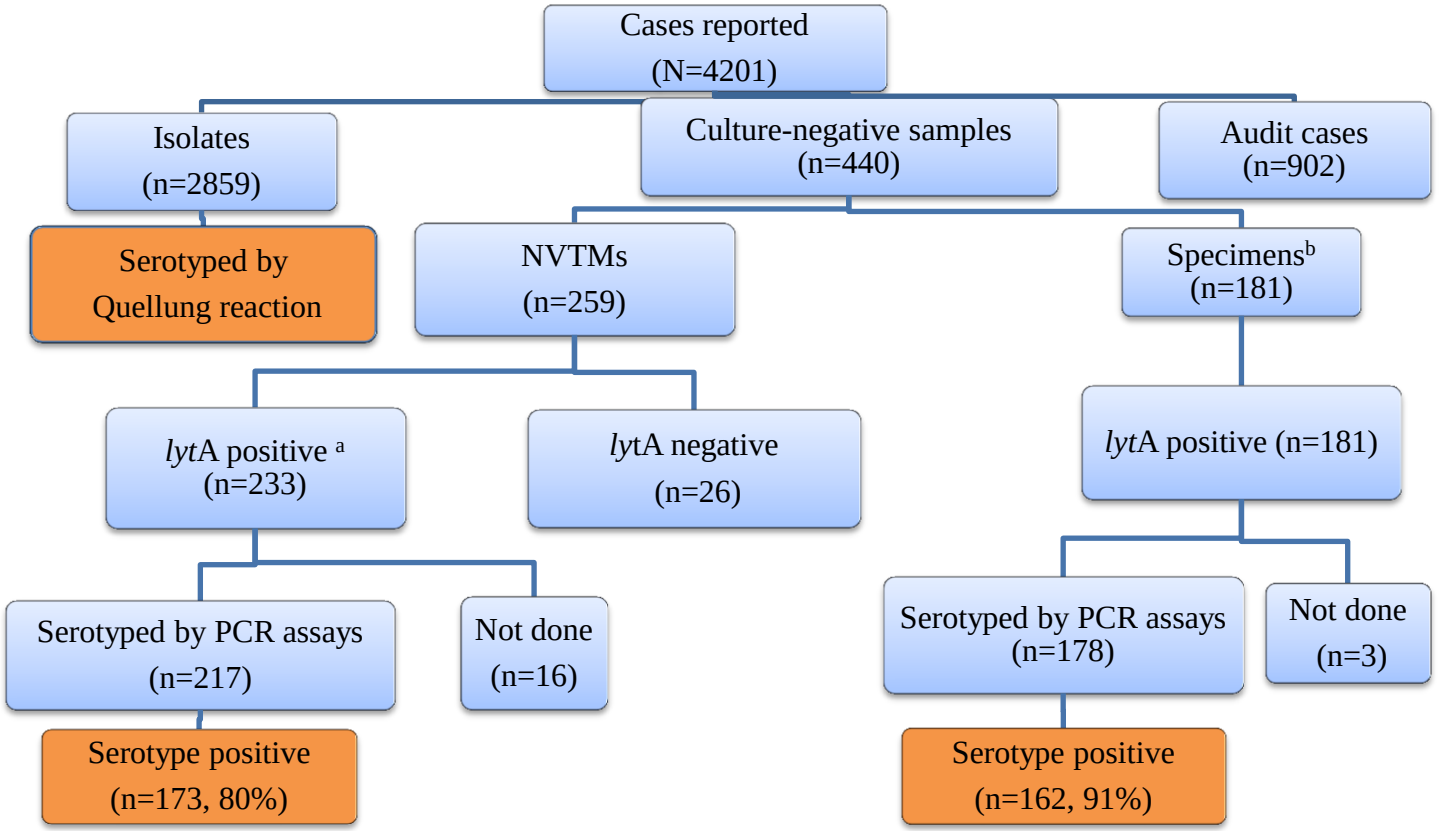


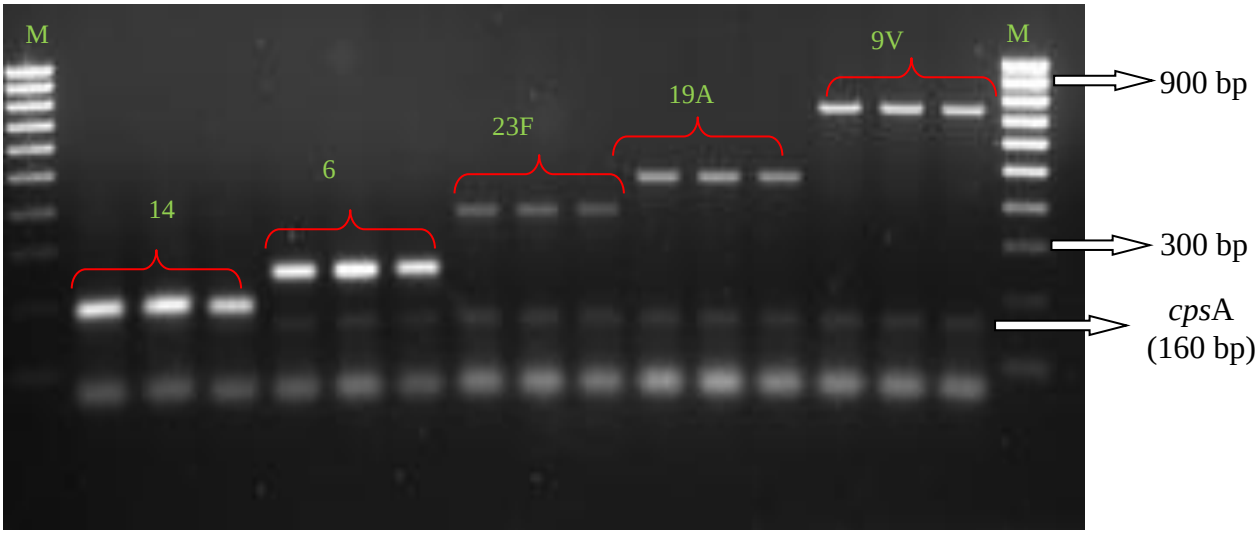
Figure 7. Summary of national invasive pneumococcal disease surveillance for isolates and culture-negative samples received in South Africa, 2010. 19 culture-negative samples were not available for PCR-based serotyping assays. [a] 299 non-viable transport medium samples (NVTMs) initially received, 66 received cultures at a later stage; [b] specimens included blood cultures, CSFs, blood and pleural fluids sent and only *lytA* positive clinical specimens were recorded as a case.

3.2 Validation of conventional sequential multiplex PCR

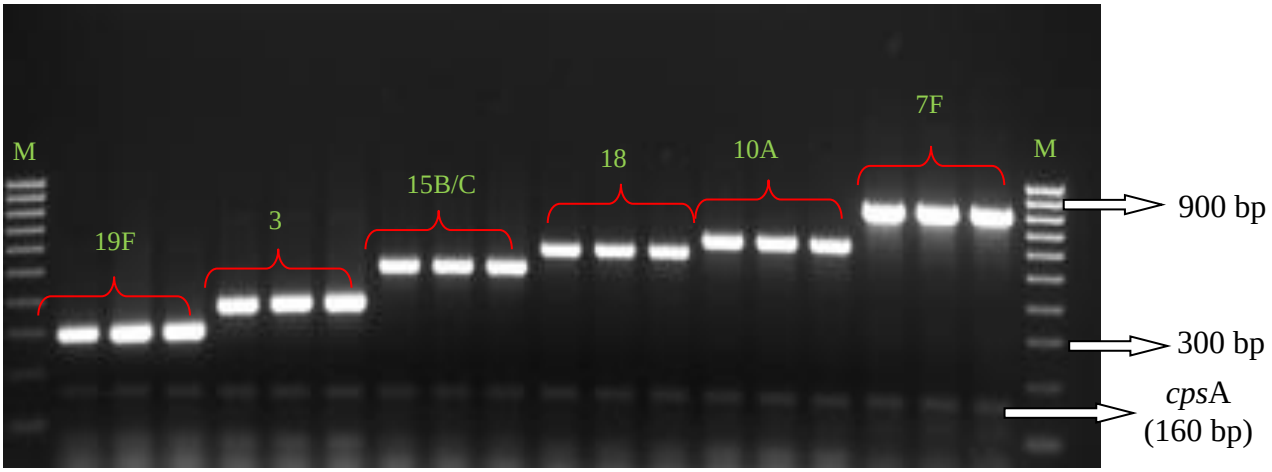
3.2.1 Setting up of the assays using 2007 isolates

A total of 96 isolates selected for validation of the C-PCR were in complete concordance with the Quellung reaction results. All the serotypes detected matched the expected band size in comparison with the molecular weight marker (100 bp-1000 bp) (Figure 8). No serotypes were assigned to the 21 isolates selected for specificity determination. Thus, the primers accurately detected the serotypes which the assay was designed to detect.

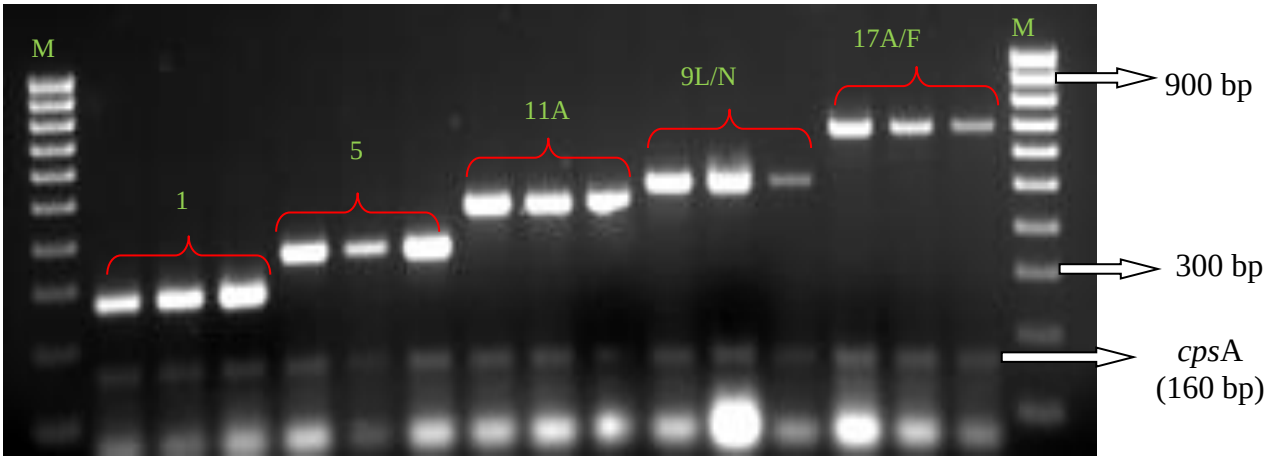
Reaction 1



Reaction 2



Reaction 3



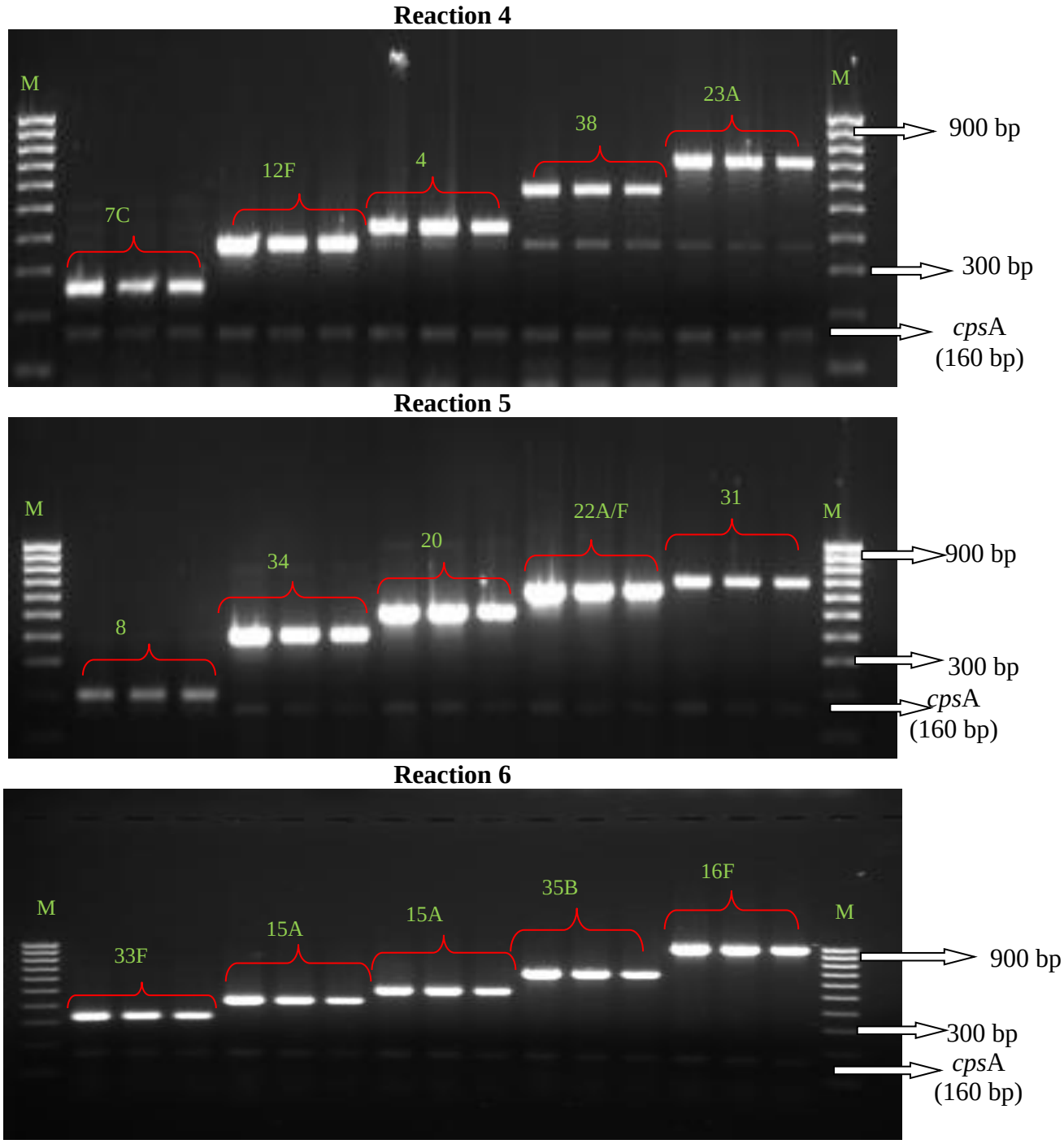


Figure 8. Ethidium bromide stains of an agarose gel under ultraviolet light representing the PCR products detected by six sequential reactions of the conventional PCR assay performed on isolates of known serotypes and used as controls for subsequent reactions (shown in green) in triplicate. All gels are flanked by a 100 bp (100 bp-1000 bp) molecular weight marker.

3.2.2 Determining the utility of the conventional serotyping assay using 2008 and 2009 isolates

The randomly selected isolates represented the majority of serotypes received in 2008 and 2009. All results for 2008 (n=411) and 2009 (n=419) were in concordance with the Quellung reaction results for serotypes included in the assay (Table 4). In some instances where the Quellung reaction had serotyped an isolate to factor sera, the C-PCR assay could only detect the serogroup, as there were no primers to detect to a specific serotype. Table 4 shows the Quellung reaction results, and the serotype detected by the C-PCR assay for 2008 and 2009 surveillance isolates.

In 2008, there was no factor serum for serogroup 16 at CRDM, while the C-PCR primers were specific for serotype 16F. The C-PCR assay assigned all randomly selected 2008 serogroup 16 isolates as serotype 16F. However in 2009, the factor serum for serotype 16F was available, and confirmed that the C-PCR assay had identified the isolates with the correct serotype for both 2008 and 2009 isolates.

PCR results (serotypes 4 and 19F) of two pneumococcal strains from 2008 were initially discrepant to the Quellung reaction results which were 9V and 23F, respectively. The Quellung reaction was repeated on the two discrepant cultures and both were found to contain a mixture of 2 serotypes: 4/9V and 19F/23F. The mixtures were not observed on the initial Quellung reaction performed. The C-PCR confirmed the presence of the other serotype upon repeat.

A total of 27 samples and 49 samples from 2008 and 2009, respectively from the stored skim milk vials did not yield growth upon retrieval. The serotype results obtained from DNA extracts

directly from skim milk were in concordance with the Quellung reaction results originally obtained from the cultures, which had subsequently lost viability.

A total of 97% (397/411) from 2008 and 96% (404/419) from 2009 surveillance isolates could be assigned a serotype using the six sequential multiplex reactions. The majority of serotypes were detected within the first three reactions of the C-PCR assay. By the end of reaction 3, >75% of isolates for 2008 (314/411) and 2009 (315/419) had been assigned a serotype (Figures 9 & 10).

The remaining 3.4% (14/411) and 3.6% (n=15/419) from 2008 and 2009 respectively, for which serotypes could not be assigned using the assay were serotypes that were not included in the assay. The actual serotypes as detected by the Quellung reaction are listed in table 4, but for the C-PCR assay, these samples were recorded as negative for the serotypes tested.

The C-PCR assay was 100% sensitive (801/801) and specific (29/29) using 2008 and 2009 isolates.

Table 4. Serotyping results and percentage coverage of each serotype obtained by Quellung reaction and conventional PCR from randomly selected invasive pneumococcal isolates from 2008 and 2009, South Africa.

Serotypes	2008		2009	
	Isolates detected by Quellung reaction n (%)	Isolates detected by C-PCR n (%)	Isolates detected by Quellung reaction n (%)	Isolates detected by C-PCR n (%)
1	390 (12)	46 (11)	475 (14)	52 (12)
3	108 (3)	11 (3)	112 (3)	19 (4)
4	194 (6)	18.(4)	179 (5)	21 (5)
5	40 (1)	4.(1)	53 (2)	5 (1)
6A	260 (8)	28.(7)	272 (8)	31 (7)
6B	256 (8)	32.(8)	237 (7)	19 (4)
6C	12 (0.4)	2 (05)	5 (0.1)	0
8	112 (3)	16 (4)	100 (3)	12 (3)
14	336 (10)	53 (13)	276 (8)	35 (8)
18A	9 (0.3)	1 (0.2)	13 (0.4)	1 (0.2)
18B	0	0	2 (0.1)	0
18C	85 (3)	8 (2)	76 (2)	16 (4)
18F	7 (0.2)	1 (0.2)	4 (0.1)	0
20	6 (0.2)	2 (0.5)	9 (0.3)	2 (0.5)

Serotypes	Isolates detected by Quellung reaction n (%)	Number detected by C-PCR n (%)	Isolates detected by Quellung reaction n (%)	Number detected by C-PCR n (%)
31	6 (0.2)	1 (0.2)	8 (0.2)	2 (0.5)
34	22 (0.7)	5 (1)	27(0.8)	4 (1)
25/38	28 (0.8)	6 (1)	27 (0.8)	6 (1)
10A	26 (0.8)	1 (0.2)	20 (0.6)	3 (0.7)
11A	16 (0.5)	1 (0.2)	17 (0.5)	3 (0.7)
11D	0	0	0	0
12A	0	0	0	0
12F	96 (3)	7 (2)	122 (4)	15 (4)
15A	26 (0.8)	3 (0.7)	19 (0.6)	4 (1)
15F	10 (0.3)	1 (0.2)	4 (0.1)	0
15B	42 (1)	7 (2)	40 (1)	5 (1)
15C	3 (0.1)	0	3 (0.1)	0
16	48 (1)	4 (1)	63 (2)	5 (1)
17F	31 (0.9)	8 (2)	36 (1)	1 (0.2)
19A	267 (8)	33 (8)	320 (9)	37 (9)
19F	198 (6)	21 (5)	187 (6)	31 (7)

Serotypes	Isolates detected by Quellung reaction n (%)	Number detected by C-PCR n (%)	Isolates detected by Quellung reaction n (%)	Number detected by C-PCR n (%)
22A/A	48 (1)	7 (2)	45 (1)	6 (1)
23A	8 (0.2)	2 (0.5)	17 (0.5)	3 (0.7)
23F	270 (8)	38 (9)	272 (8)	33 (8)
33A	1 (0.03)	0	3 (0.1)	1 (0.2)
33F	12 (0.4)	2 (0.5)	5 (0.1)	1 (0.2)
37	3 (0.1)	1 (0.2)	2 (0.1)	0
35B	17 (0.5)	4 (1)	7 (0.2)	2 (0.5)
35F	6 (0.2)	0	5 (0.1)	0
40	0	0	0	0
44	0	0	0	0
46	0	0	0	0
47F	0	0	0	0
7A	0	0	0	0
7B	2 (0.1)	0	0	0
7C	22 (0.7)	4 (1)	27 (0.8)	3 (0.7)
7F	27 (0.8)	2 (0.5)	34 (1)	4 (1)
9N	55 (2)	4 (1)	49 (1)	5 (1)

9L	1 (0.03)	0	0	0
9V	106 (3)	13 (3)	89 (3)	17 (4)
C-PCR neg.	115[a] (3)	14[b] (3)	130[c] (4)	15[d] (4)
Total	3327 (100)	411 (100)	3391 (100)	419 (100)

[a]Quellung reaction results for 2008 (Serotypes by Quellung reaction (No. of isolates) = 10F (9), 11B (4), 11C (1), 11F (1), 12B (1), 13 (29), 17A (1), 19B (1), 21 (3), 23B (9), 24 (1), 28 (1), 28 (3), 29 (23), 33D (15), 35A (1), 42 (1), 43 (1), 48 (4), NT (7).
[b]C-PCR– Conventional PCR negative for 2008 (Serotypes by Quellung reaction (No. of isolates) = 13 (6), 10F (1), 29 (3), 19B (1), 33D (2), NT (1)
[c]Quellung reaction results for 2009 (Serotypes by Quellung reaction (No. of isolates) = 10F (9), 11B (2), 11C (3), 11F (1), 12B (2), 13 (39), 19B (4), 21 (7), 23B (8), 24 (3), 27 (1), 28 (3), 29 (18), 33D (18), 35C (1), 48 (2), NT (8)
[d]C-PCR– Conventional PCR negative for 2009 (Serotypes by Quellung reaction (No. of isolates) = 13 (7), 10F (2), 23B (2), 35D (3), 35C (1)

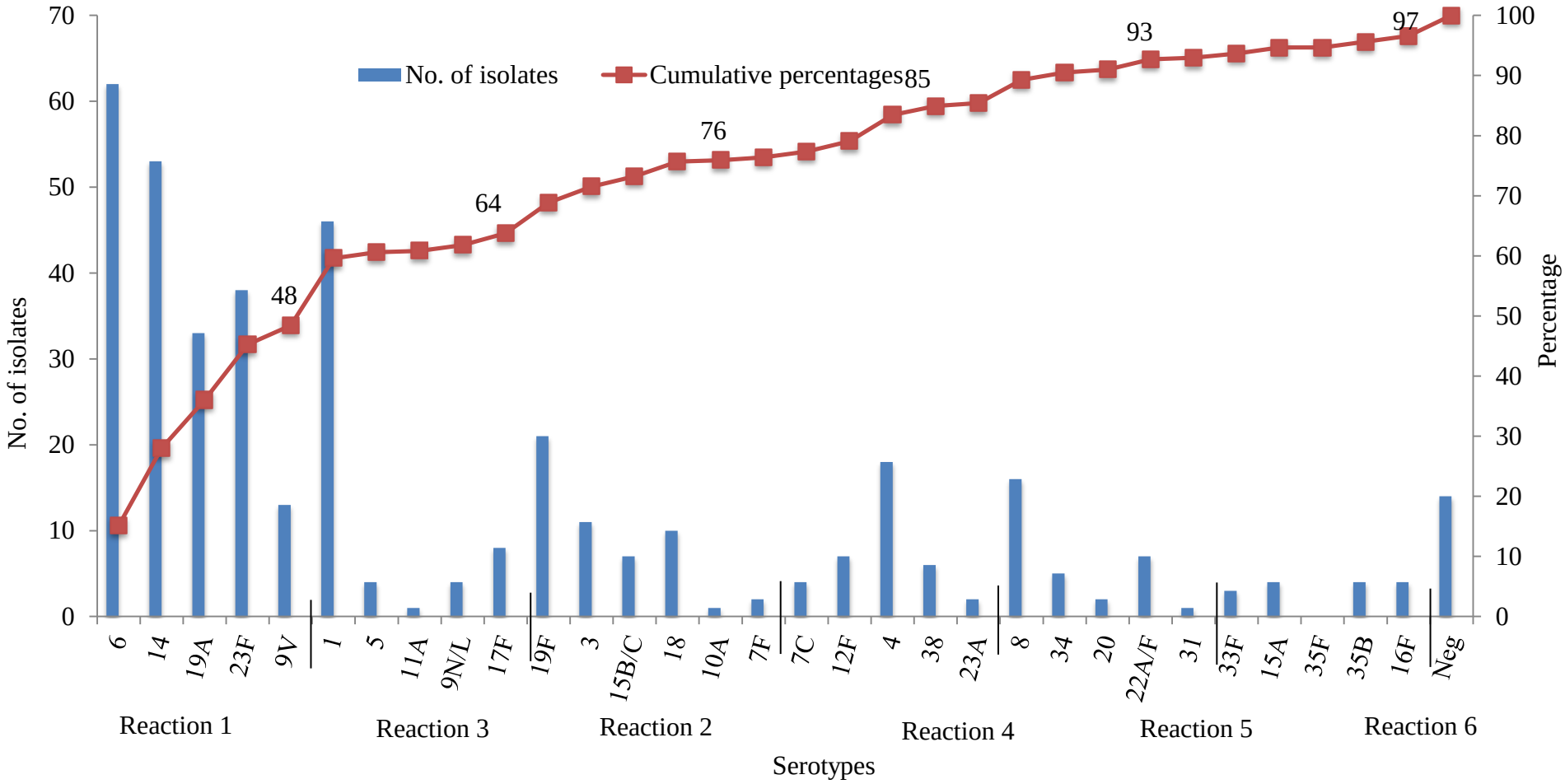


Figure 9. Number of pneumococcal isolates tested by conventional PCR, by serotype and the cumulative percentages detected per reaction for 2008 national invasive pneumococcal disease surveillance isolates, South Africa (n=411)

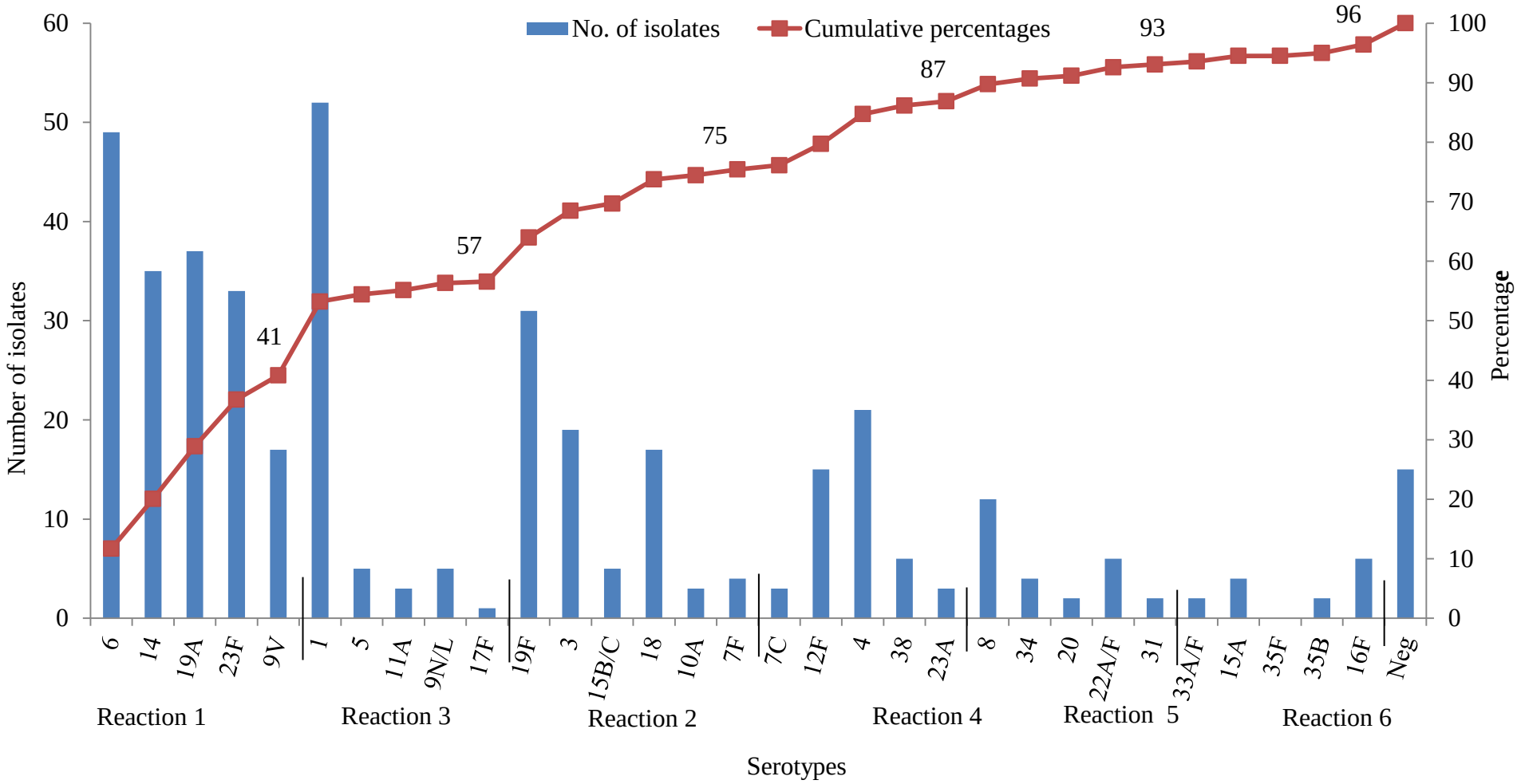


Figure 10. Number of pneumococcal isolates tested by conventional PCR, by serotype and the cumulative percentages detected per reaction for 2009 national invasive pneumococcal disease surveillance isolates, South Africa (n=419)

3.3 Validation of real-time PCR serotyping assay

3.3.1 Setting up of the assay using 2007 isolates

A total of 66 isolates representing 22 serogroups/types targeted by the RT-PCR serotyping assay could be assigned a correct serotype using the assay. The results were 100% concordant with Quellung reaction and the C-PCR assay results. One control belonging to serotype 18F could not be amplified as it is not targeted in the RT-PCR serotyping assay, but it is targeted by the C-PCR as serogroup 18.

The isolates used could be amplified when 10X and 100X diluted and, as expected, there was an increase in C_t-values in the 100X diluted samples compared with the 10X diluted samples. There was no significant difference in the C_t-values of the duplex reactions compared to the singleplex reactions (Table 5).

3.3.2 Validation of the real-time PCR assay using 2008 and 2009 isolates

Among 66 serotypes/groups tested (n=228), 29 serotypes/groups were true negatives upon completion of all 11 duplex reactions. Cross-reactions were observed in 4 serotypes/groups. The primers/probe combination for serotype 38 cross-reacted with serogroup 25; serotype 33A/F cross-reacted with serogroup 37; serotype 18B/C cross-reacted with serotype 18A and serotype 9A/V cross-reacted with serotypes 9N/L. The sensitivity and specificity of the RT-PCR assay was 100% (134/134) and 95% (89/94), respectively.

Table 5. Comparison of the cycle threshold (Ct) values of the singleplex and duplex reactions for the controls used in the validation of the real-time serotyping PCR assays (p=0.06 for 10X dilutions, and p=0.24 for 100X dilutions).

Serogroup/type	Singleplex reactions		Duplex reactions	
	Mean Ct (10X dilution)*	Mean Ct (100X dilution) *	Mean Ct (10X dilution)*	Mean Ct (100X dilution)*
1	20.29	23.42	22.63	25.03
3	20.74	23.92	22.58	26.24
4	20.85	24.01	21.77	24.34
5	19.99	23.19	20.62	24.38
8	19.86	23.16	19.49	22.74
14	20.26	23.66	19.21	22.42
18A/B/C	22.83	26.16	24.19	27.57
20	21.51	25.39	22.42	25.15
38/25	19.97	22.67	21.55	25.89
10A/B	20.67	27.81	21.21	24.81
12A/B/F	18.47	21.71	18.81	22.21
15A	19.57	22.50	20.44	24.03
15B/C	19.76	23.38	20.21	23.44
19A	20.70	23.90	20.33	23.28
19B/F	19.62	22.79	20.37	23.67
22A/F	20.50	23.81	21.44	23.86
23F	20.05	23.49	22.09	25.80
33A/F	20.03	23.24	21.50	24.88
35B	22.36	25.76	26.03	29.59
6A/B/C/D	21.30	26.05	18.80	22.31
6C/D	20.05	22.82	17.42	20.86
7A/F	19.02	22.35	21.36	24.53
9A/V/L/N	18.72	22.12	22.06	24.89

* mean of 3 results for isolates for each serotype tested in triplicate

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3.4 Serogroup 6 differentiation by C-PCR and RT-PCR assays

The results for serogroup 6 differentiation by C-PCR corresponded with the Quellung reaction results for serotypes 6A (n=5), 6B (n=5) and 6C (n=5) surveillance isolates from 2007. Serotype 6B isolates had a 155 bp band size, while for serotype 6A no serotype-specific band was observed as expected. The internal control of 250 bp was visible on all isolates tested. For serotype 6C, the two sets of primer pairs i.e. inner set and outer set, were tested on 5 isolates (6C) from 2007 isolates separately, which gave the product size of 308 bp and 359 bp respectively. Both these primer sets could detect serotype 6C effectively with the difference only in band sizes. Therefore, for all subsequent reactions, only the outer primer pair set; *wci*N$_{\beta}$S1 and *wci*NA2, with a product size of 359 bp was used because of the better band sizes resolution with the 250 bp band for serogroup 6 controls (Figure 11.

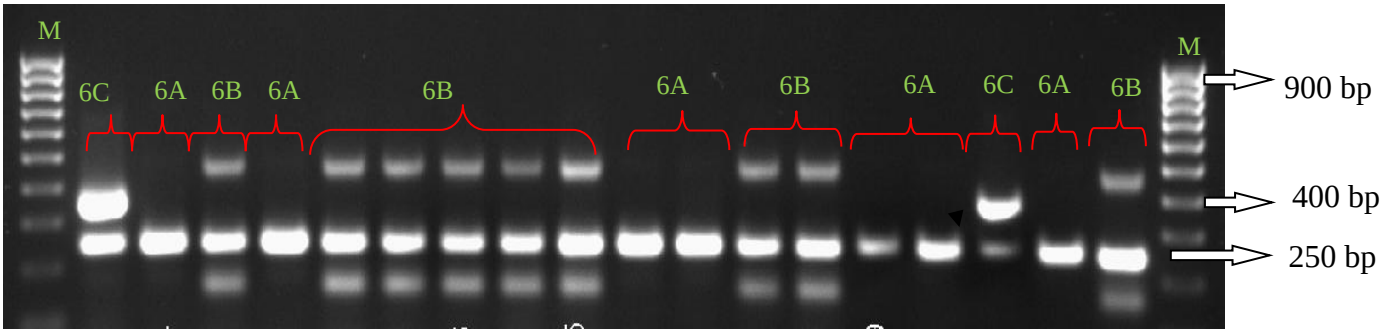


Figure 11. An agarose gel representation of the multiplex reaction for differentiation of serogroup 6 serotypes (M=100 bp molecular marker, 6A=no band, 6B=155 bp and 6C=359 bp) stained with ethidium bromide and viewed under ultraviolet light.

The results obtained for the two duplex reactions and one multiplex reaction for 2008 (n=62) and 2009 (n=49) isolates were 100% concordant with the Quellung reaction results. Of 178 serogroup 6A and 6B isolates from 2010 surveillance, 167 (41%) were assigned serotype 6B, while only 11 (3%) isolates were assigned serotype 6C corresponding to the Quellung reaction results. There were no isolates identified as serotype 6D (Table 6).

The reaction for differentiating serogroup 6 serotypes was included in the 11 duplex reactions of the RT-PCR assay. Using this assay, serotypes 6A/6B could be distinguished from serotypes 6C/6D. Fifteen controls for serotypes 6A (n=5), 6B (n=5) and 6C (n=5) from 2007 used for the initial validation of the reaction were 100% concordant with the Quellung reaction results as serotypes 6A/6B or 6C/6D. The isolates assigned as serogroup 6 from 2008 (n=62) and 2009 (n=49) by the C-PCR and the Quellung reaction, were further confirmed by the RT-PCR assay. Of 408 isolates from 2010 surveillance, 167 and 11 isolates were assigned as serotypes 6B and 6C, respectively, by the Quellung reaction. While screening for serotype 6D on serotype 6B and 6C isolates received in 2010, the real-time PCR assay confirmed serotype 6B isolates as 6A/B, while 6C isolates were assigned as 6C/D (Table 6).

Table 6. Summary of serogroup 6 differentiation results by the Quellung reaction, conventional and real-time PCR serotyping assays, South Africa.

Year	No. of isolates	Quellung reaction	Real-time PCR	Conventional PCR
2007	15	6A (5), 6B (5) 6C (5)	6A (5), 6B (5) 6C (5)	6A/B (10), 6C/D (5)
2008	62	6A (28), 6B(32), 6C (2)	6A (27), 6B (32), 6C (2)	6A/B (59), 6C/D (2)
2009	49	6A (30), 6B (19)	6A (30), 6B (19)	6A/B (49)
2010	178	6B (167), 6C(11)	6B (167), 6C(11)	6A/B (167), 6C/D (11)

3.5 Use of molecular serotyping methods for 2010 national IPD surveillance of culture-negative samples

Identification of pneumococcus by *lytA* RT-PCR was a useful tool in identifying the organism in culture-negative samples. The *lytA* C_t-value and the corresponding serotype C_t-value did not differ from each other by more than 4 C_t-value's. A summary of all results for culture-negative samples is shown in Table 7

3.5.1 Non-viable transport medium samples

Overall, 299 NVTMs were processed. C-PCR and RT-PCR assays were performed on 266 available samples. Of the 33 samples processed using the RT-PCR assay only, 73% (24/33) could be assigned a serotype. The C-PCR assigned a serotype to 32% (85/266). RT-PCR confirmed a serotype for 94% (80/85), however could not confirm serotypes for five samples with serotypes 11A, 17F and 23A as these serotypes were not targeted by the RT-PCR primers/probe sets. RT-PCR added 68% (123/181) serotyping data to the remaining samples that could not be assigned a serotype by C-PCR. Using both C-PCR and RT-PCR assays, 78% (208/266) of NVTMs were assigned a serotype. A *lytA* C_t-value of ≤26 accounted for 82 samples while the remaining 184 samples had a *lytA* C_t-value of >26. C-PCR and RT-PCR assigned serotypes to 72% (59/82) and

90% (74/82), respectively, of samples with *lytA* C_t-values ≤26 [59/82 (72%) vs. 74/82 (90%), p=0.003]. For samples with a *lytA* C_t-value >26, C-PCR and RT-PCR assigned serotypes to 14% (26/184) and 70% (129/184), respectively [26/184 (14%) vs. 129/184 (70%), p<0.001].

Of 299 NVTMs, a culture was received for 22% (n=66) at a later stage. Of these, C-PCR and RT-PCR had assigned 62% (41/66) of cases the same serotypes as the Quellung reaction. 17% of (11/66) samples that were negative on both PCR assays were serotypes presumably detected by both PCR assays as determined by the Quellung reaction. These were serotypes 5, 8, 14, 11A, 15F, 16F, 22F, 23F, 25A, 35F and 7C (*lytA* C_t-value range=26-37, median=33). An additional 6% of samples (4/66) had discordant results, where RT-PCR assigned them as serotype 14, 19A, 23F and 6, while the Quellung reaction assigned these samples as serotypes 35B, 1, 9V and 9V, respectively (*lytA* C_t-value range=24-38, median=32). For these, the Quellung reaction results were used for final analysis. Overall, 15% of samples (10/66) with a *lytA* C_t-value of >26 (range=28-37, median=31) belonged to serotypes not included in RT-PCR assay hence negative for both PCR assays.

3.5.2 Clinical specimens (cerebrospinal fluid, blood and pleural fluid specimens)

40 CSFs were identified as positive for the *lytA* gene and the C-PCR assay assigned a serotype to 55% (n=22) of these samples. RT-PCR confirmed 86% (19/22) of these serotypes. The 3 serotypes that could not be confirmed by RT-PCR were serotypes 11A, 17F and 34 and are not targeted by the RT-PCR primers/probe sets. RT-PCR added serotyping data for meningitis cases for 67% (12/18) of the remaining specimens. Using the two assays, 85% (34/40) of pneumococcal meningitis cases could be assigned a serotype. C-PCR and RT-PCR assigned serotypes to 71% (17/24) and 79% (19/24) (p=0.51), respectively of specimens with *lytA* C_t-value

≤26. For specimens with a *lytA* C_t-values >26, C-PCR assigned a serotype to 31% (5/16), while RT-PCR assigned 75% (12/16) (p=0.01) (Table 7). For five cases for which a "culture-negative" CSF specimen had been received, an isolate was received at a later stage. The Quellung reaction results for cultures were 100% concordant with the C-PCR and RT-PCR assays results for the matched clinical specimen.

Blood (n=10) and a pleural fluid (n=1) specimen had *lytA* C_t-values of <26. The C-PCR was able to assign serotypes to 82% (9/11) of these specimens. No further serotyping results could be added using the real-time PCR, and RT-PCR was not completed on all reactions on 2 specimens, due to shortage of DNA extract. Six blood specimens were also culture positive at a later stage and the Quellung reaction results for cultures were 100% concordant with both PCR serotyping assays results for the matched clinical specimen.

3.5.3 Beep-positive, subculture-negative blood culture bottles

The C_t-values of the *lytA* RT-PCR ranged from 13-26 for beep-positive blood cultures and therefore these samples (n=154) demonstrated high bacterial loads. The majority of these samples could be assigned a serotype (94%, 145/154) using C-PCR. Of these 93%, (135/145) were confirmed by RT-PCR. RT-PCR did not detect 10 serotypes that are not part of its repertoire [serotypes 7C (n=4), 17F (n=4), 16F (n=1) and 35F (n=1)]. The remaining 9 samples that could not be assigned a serotype by either PCR methodology were presumed to be of serotypes not targeted by either of the C-PCR and the RT-PCR serotyping assays. Overall, 94% (145/154) of bacteraemia cases were assigned a serotype using both assays. Five blood cultures showed the presence of mixed infection by simultaneous identification of two different serotypes. While the

C-PCR assays detected only one serotype, the RT-PCR detected the presence of the additional serotype. Co-infection with serotype 19A was found in three samples also assigned as serotypes 1, 5 and 4. The remaining two samples were assigned a serotype 19F and 23F, and were co-infected with serotype 1 and 6A/B respectively. Viable isolates were available retrospectively for 16 blood cultures that were initially identified as culture negative. The Quellung reaction results for cultures were 100% (16/16) concordant with the C-PCR and/or RT-PCR assays results for the matched blood culture sample.

3.6 Sensitivity of PCR assays for culture-negative samples

There was no statistical difference in assigning of serotypes using the C-PCR and the RT-PCR assay for blood cultures [145/154 (94%) vs. 135/154 (88%), p=0.05] and blood/pleural fluid specimens [9/11 (82%) vs. 7/11 (64%), p=0.35]. Among CSFs, RT-PCR was more sensitive than C-PCR [22/40 (55%) serotypes detected by C-PCR vs. 31/40 (76%) by RT-PCR, p=0.03]. Similarly, for NVTMs RT-PCR was more sensitive than C-PCR [85/266 (32%) vs. 203/266 (76%), $p<0.001$] (Figure 12).

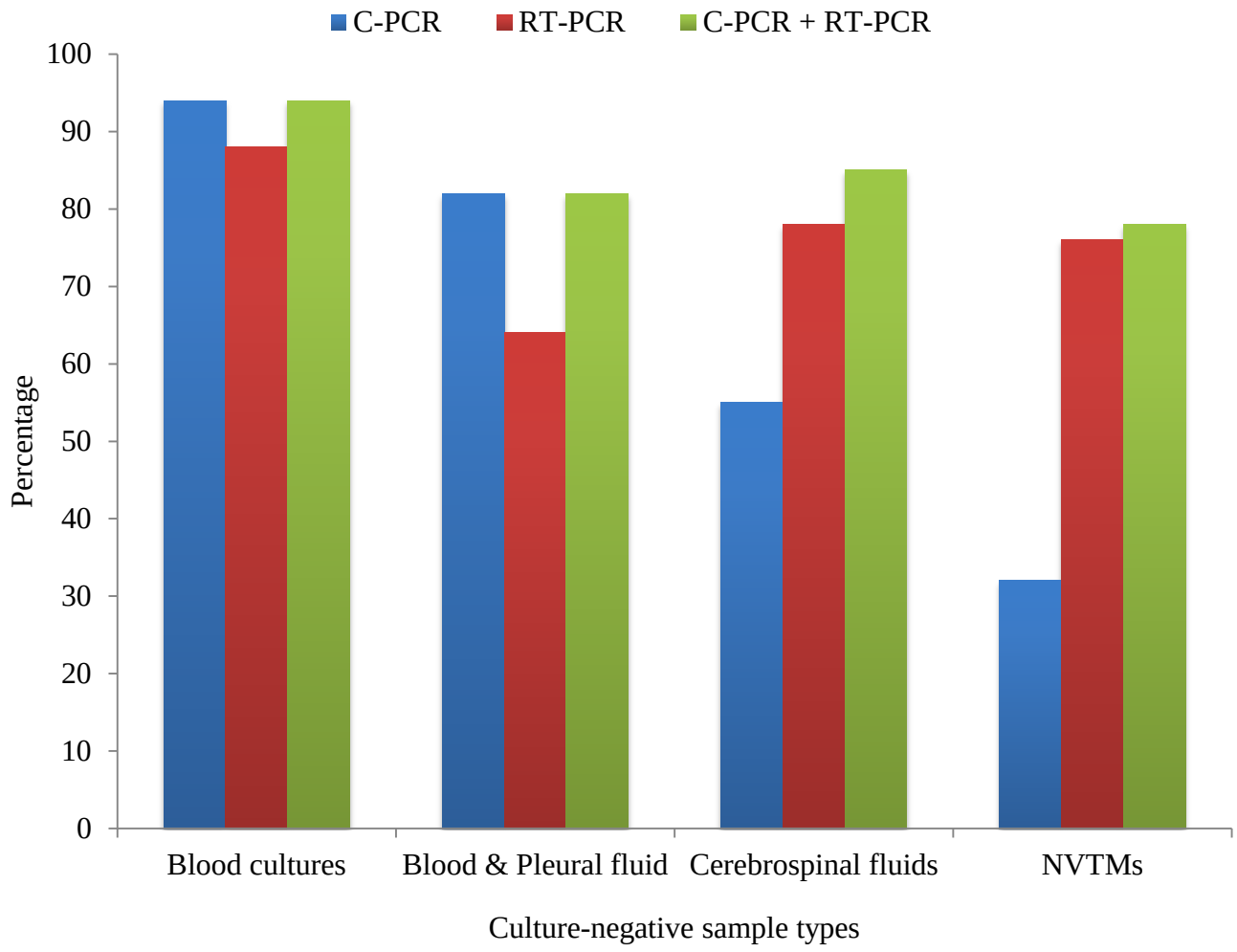


Figure 12. Percentage of serotype yield in culture-negative samples; blood cultures (n=154, median *lytA* C_t-value=24), blood and pleural fluid specimens (n=11, median *lytA* C_t-value =16), CSF specimens (n=40, median *lytA* C_t-value =23) and non-viable transport medium samples (NVTMs, n=266, *lytA* C_t-value median=29) obtained by conventional and real-time PCR serotyping assays, South Africa, 2010.

Table 7. Culture-negative samples received as part of national invasive pneumococcal disease surveillance and serotyped using the conventional multiplex and real-time PCR serotyping assays in South Africa, 2010.

Sample type	Number of samples	Serotyped by C-PCR (%)	Serotyped by RT-PCR (%)	*lytA* C_t-value range (median)	C-PCR pos. (%) (*lytA* ≤26)	C-PCR pos. (%) (*lytA* >26)	RT-PCR pos. (%) (*lytA* ≤26)	RT-PCR pos. (%). (*lytA* >26)
Blood cultures	154	145 (94)	135 (88)	13-26 (24)	145/154 (94)	0 (0)	135/154 (88)	0 (0)
CSFs[a]	40	22 (55)	31 (76)	17-36 (23)	17/24 (71)	5/16 (31)	19/24 (79)	12/16 (75)
Blood/pleural fluids	11	9 (82)	7 (64)	11-23 (16)	9/11 (82)	0 (0)	7/11 (64)	0 (0)
NVTMs[b]	266	85 (32)	203 (76)	13-37 (29)	59/82 (72)	26/184 (14)	74/82 (90)	129/184 (70)

[a]CSFs – Cerebrospinal fluids [b]NVTMs–non-viable transport medium samples

3.7 Use of molecular serotyping assays for special studies

3.7.1 SARI surveillance study

The *lytA* quantitative real-time PCR was performed on 5130 blood specimens from patients hospitalised with severe pneumonia from May 2009 to December 2010. Pneumococcal DNA was detected in 7% (370/5130) of patients of all age groups. The C_t-values of the *lytA* RT-PCR ranged from 22-40 (median 34). Serotypes were determined for 52% (190/368) of specimens by RT-PCR. Two specimens were not available for serotyping. The prevalent serotypes were 19A (n=43, 12%), 1 (n=35, 9%) and 6A/B (n=23, 6%) (Figure 13).

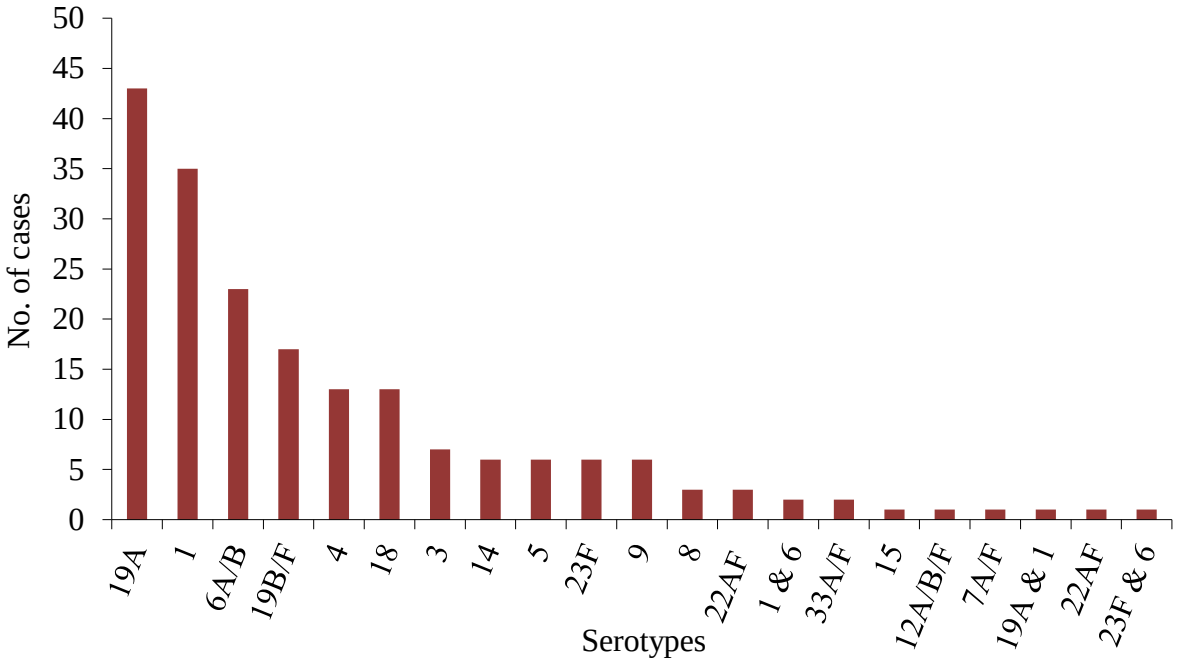


Figure 13. Pneumococcal serotype distribution identified in *lytA*-positive blood specimens from the severe acute respiratory illness (SARI) surveillance study (n=190, median *lytA* C_t-value =34) determined by the real-time PCR assay for all age groups, South Africa, 2009-2010

3.7.2 Empyema study

A total of 42 pleural fluids were received for the study from September 2009 through to May 2011. Pneumococcal DNA was detected in 45% (19/42) of cases. The RT-PCR assay was performed on all 19 specimens and a serotype was determined for 95% (18/19) of empyema cases (*lytA* Ct range=17-37, median Ct=28) using this assay. The C-PCR assay was performed on 14 of these specimens and a serotype was determined for 4/14 (29%) specimens of which all had a *lytA* C_t-value of <26 (range=18-24, median=22). The C-PCR assay assigned serotypes to all pneumococci with a *lytA* C_t-value of ≤26. The RT-PCR assay results for four samples for which both assays were performed were 100% concordant with results previously assigned by the C-PCR assay. Of the 19 *lyt*A positive specimens, serotypes 6A/B (n=8, 42%), 19A (n=3, 16%), 23F (n=3, 16%), 14 (n=2, 11%) and 1 (n=2, 11%) were identified in these specimens.

3.8 Application of molecular-based serotyping techniques to determine serotype distribution

3.8.1 Nationwide invasive pneumococcal diseases surveillance programme

A summary of results for all culture-negative samples received is shown in Table 7. There was no significant difference in the distribution of predominant serotypes between isolates and culture-negative samples. For isolates received in 2010, common serotypes identified were serotypes 1 (369/2859), 19A (301/2859), 6A (232/2859), 23F (208/2859), 14 (168/2859), 4 (166/2859), 6B (166/2859), 19F (143/2859) and 12F (143/2859) accounting for 66% (1896/2859) of all serotypes identified (Figure 14). The same serotypes; serotype 1 (65/395), 19A (36/395), 6A (27/395), 23F (21/395), 14 (26/395), 4 (35/395), 6B (12/395) 19F (12/395) and 12F (16/395) were common in culture-negative samples, accounting for 67% (250/395) of all serotypes identified (1896/2859 vs. 250/395, p=0.23).

There was no significance difference in the distribution of the 11 most prevalent serotypes among individuals <5 years of age between isolates and culture-negative samples (508/647 [78%] in isolates vs. 48/65 [74%] in culture-negative samples, p=0.38). However, serotypes 6A and 6B were commonly identified in cultures, whereas in culture-negative samples, serotypes 14 and 4 were more commonly identified (Figure 15). Similarly, in individuals ≥5 years of age, there was no significant difference in the distribution of the 11 most prevalent serotypes (Figure 16) in cultures and culture-negative samples using the Quellung reaction and the PCR-based assays, respectively (1504/2120 in isolates vs. 225/317 in culture-negative samples, p=0.99). Age was not available for 105/3254 (3%) samples with serotyping results.

PCV7 serotypes accounted for 35% (992/2859) and 28% (112/395) of all serotypes identified in isolates and culture-negative samples, respectively (992/2859 vs. 112/395, p=0.01) (Figure 17). This was also the case when serotype 6A (an important cross-protected serotype in PCV7) was included, accounting for 43% (1222/2859) and 36% (145/395) of all serotypes in culture-positive samples and culture-negative samples, respectively (1222/2859 vs. 145/395, p=0.02).

PCV13 serotypes accounted for 73% (2074/2859) of all serotypes identified from culture-positive samples received in 2010. PCV13 serotypes accounted for 68% (267/395) of serotypes identified in culture-negative samples (2074/2859 vs. 267/395. p=0.04).

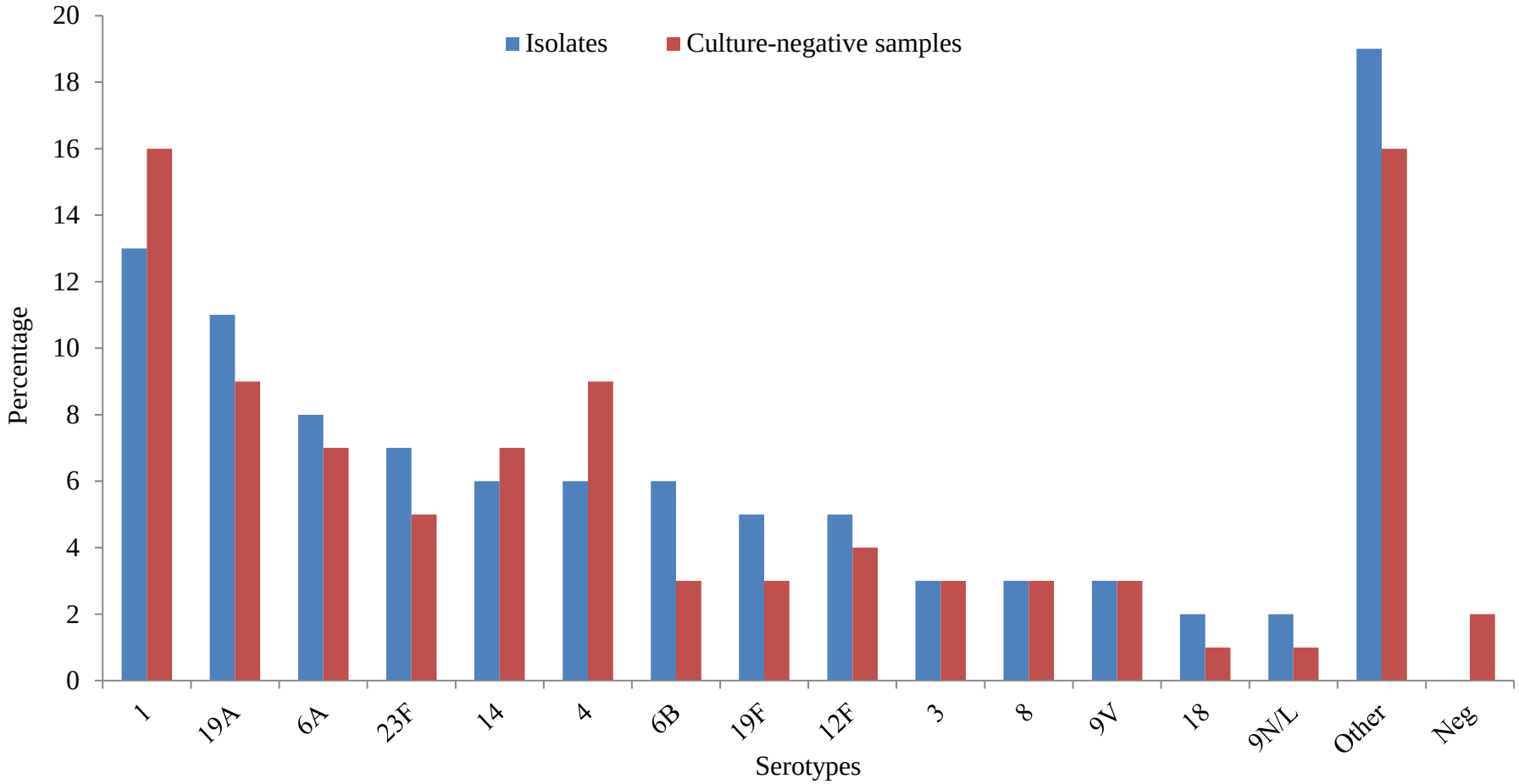


Figure 14. Pneumococcal serotype distribution from isolates (n=2859) and culture-negative samples (n=395) determined by the Quellung reaction and PCR-based serotyping assays respectively, for all age groups, South Africa, 2010

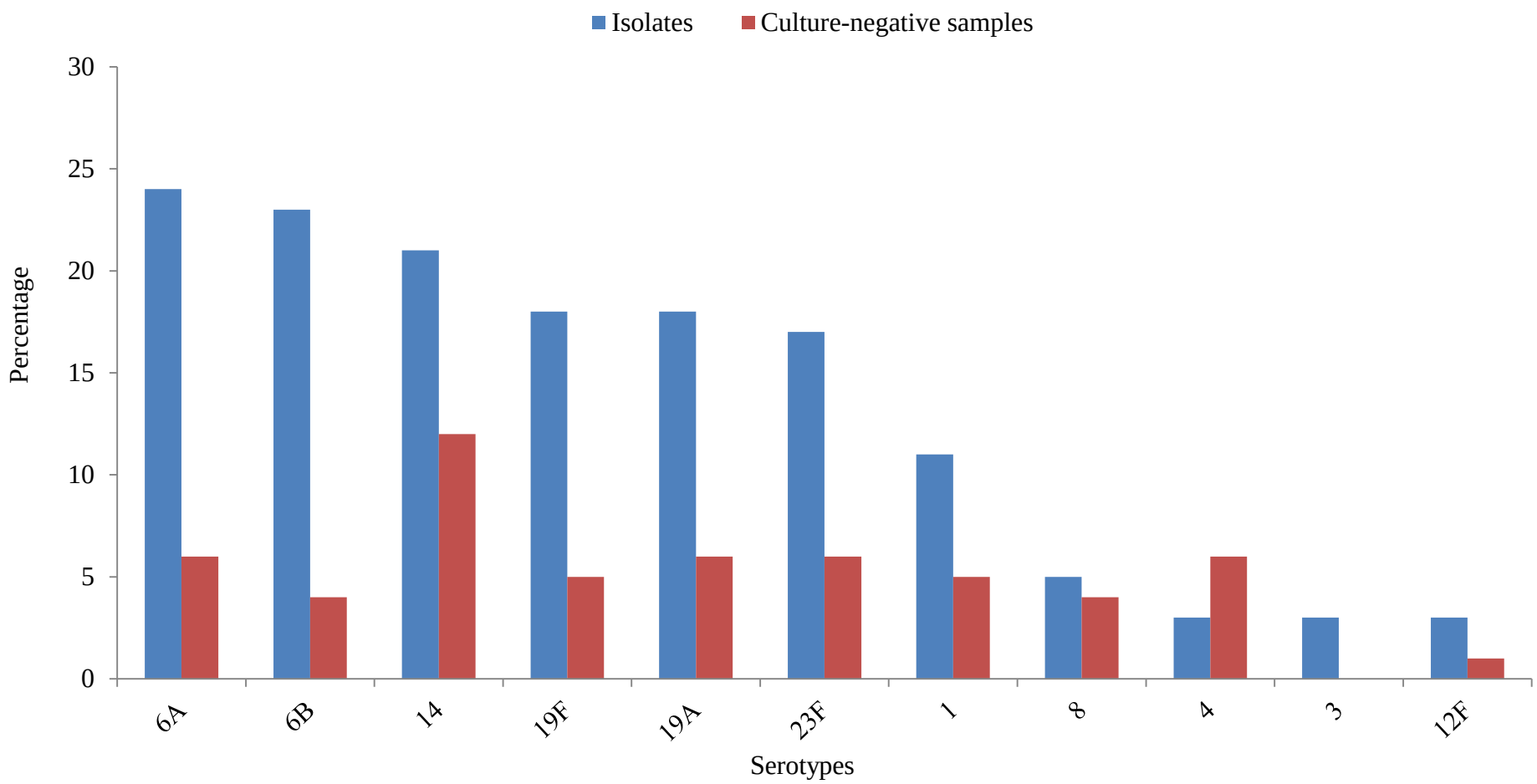


Figure 15. Predominant serotypes identified in invasive pneumococcal disease cases in children <5 years from isolates (n=647) and culture-negative samples (n=65) reported to the national surveillance and serotyped by Quellung reaction and PCR assays respectively, in South Africa, 2010.

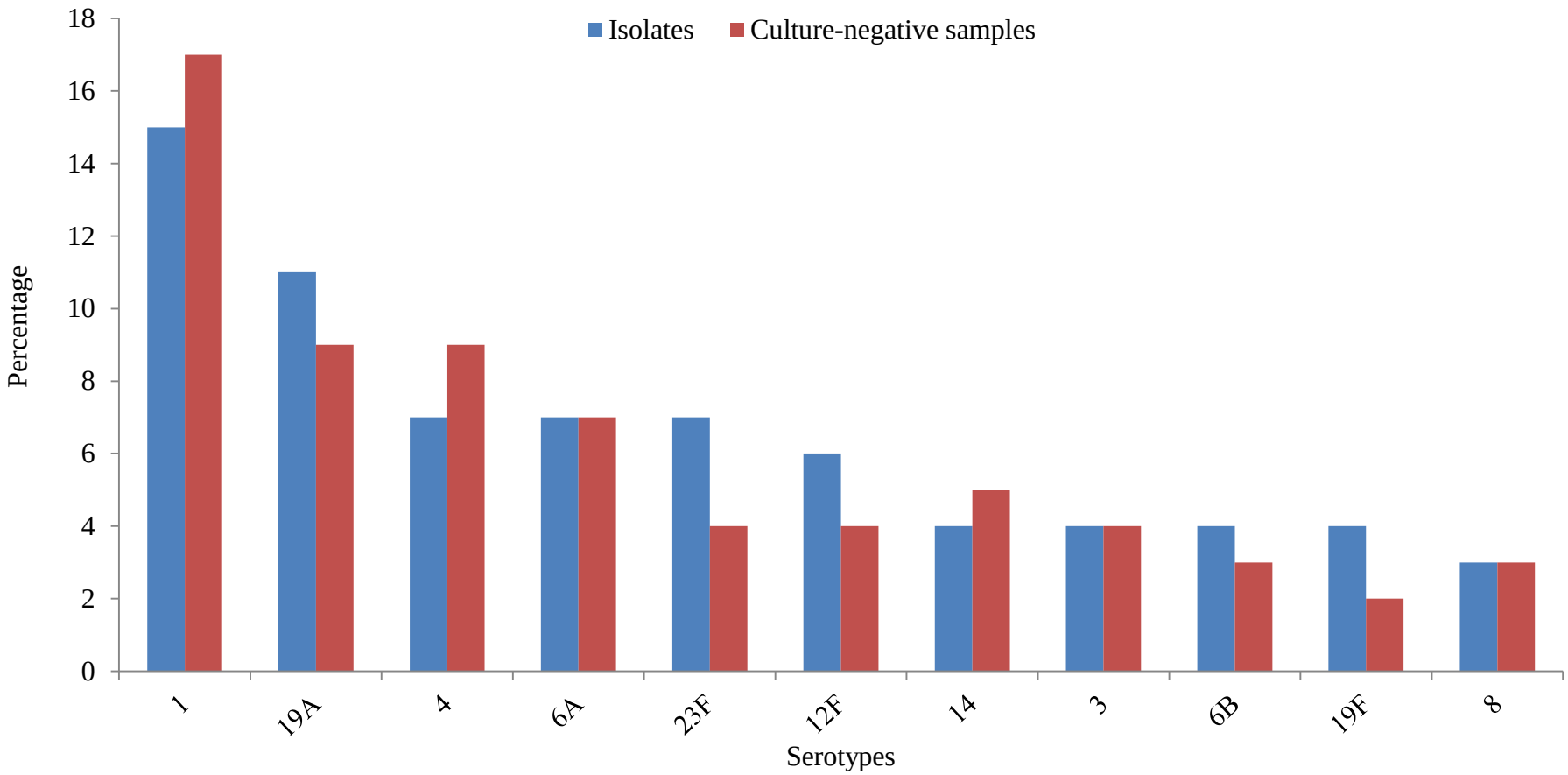


Figure 16. Predominant serotypes identified in invasive pneumococcal disease cases in individuals ≥5 years from isolates (n=2120) and culture-negative samples (n=317) reported to the national surveillance and serotyped by Quellung reaction and PCR assays respectively, in South Africa, 2010.

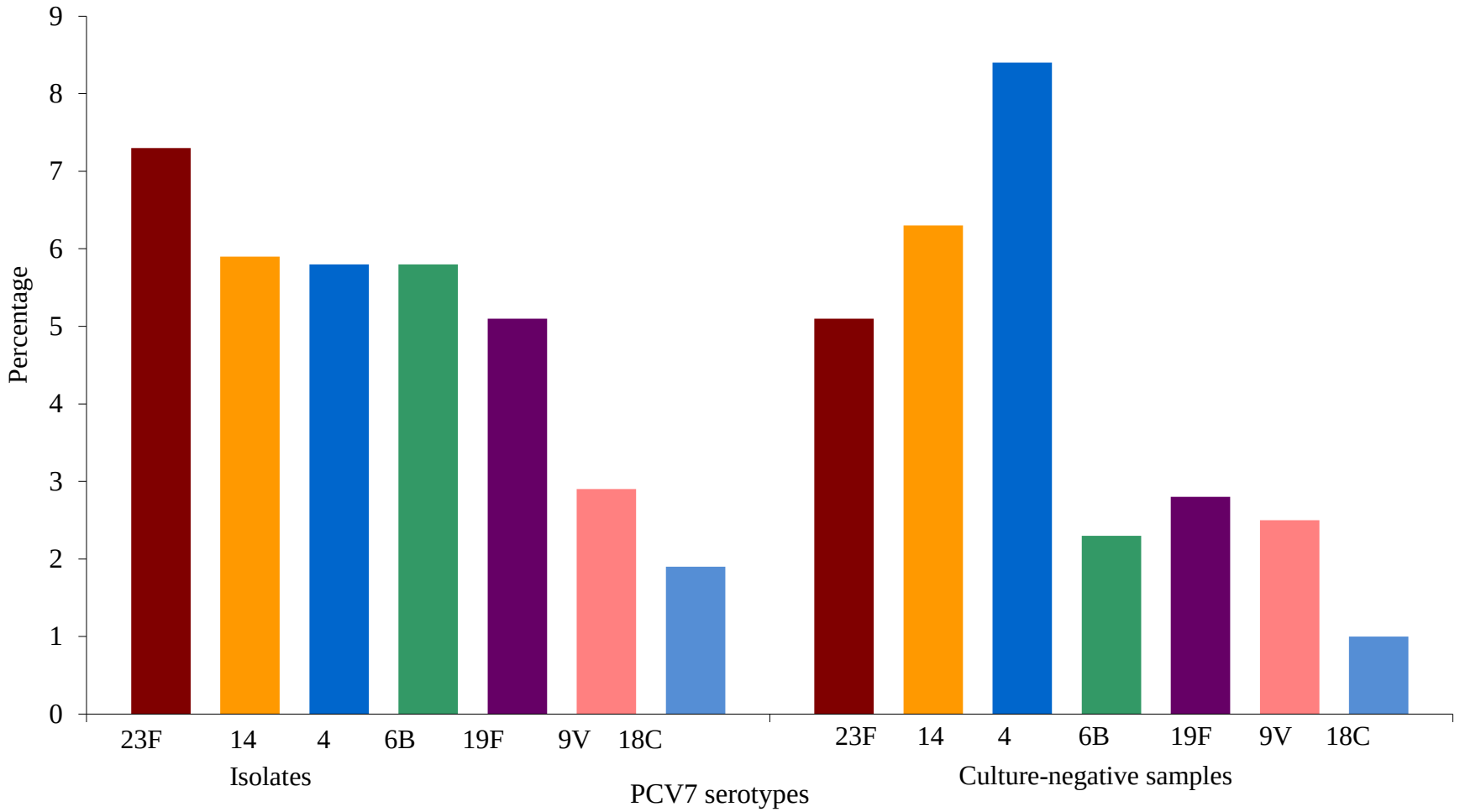


Figure 17. Distribution of PCV7 serotypes among isolates (35%, 992/2859) and culture-negative (28%, 112/395) samples for all age groups in South Africa, 2010.

3.8.2 Severe Acute Respiratory Illness surveillance programme

An overall non-significant decrease (p=0.47) was observed in *lytA* positivity from 8% (151/2005) in 2009 to 7% (219/3125) in 2010 for the SARI surveillance programme. From 2009 to 2010, there was a significant reduction the proportion of *lytA* positivity in children <2 years (n=1696) admitted with severe pneumonia, from 6% (43/723) to 4% (36/973), respectively (p=0.03). PCV7 serotypes accounted for 29% (23/78) of serotypes identified in children <2 years in 2009-2010. There was no significant difference in the proportion of PCV7 serotypes in children <2 years between 2009 and 2010 (14/43 in 2009 vs. 9/35 in 2010, p=0.51). The proportion of PCV7 serotypes differed non-significantly for patients in age groups <2 (23/78), 2-4 (2/18) and ≥5 (61/272) years of age (p=0.19) (Table 8). PCV13 serotypes accounted for 46% (171/368) of all cases in 2009-2010.

Of the 1696 children <2 years, 829 children were eligible to receive PCV7 55% (456), 29% (240) and 16% (133) had received 0, 1 or 2-3 doses, respectively at least 14 days before onset of symptoms of severe pneumonia (Table 9). 40 cases of severe pneumonia due to pneumococcus were identified in all children <2 years eligible for PCV7 from May 2009-December 2010 in SA. In this group, pneumonia due to a PCV7 serotype accounted for 33% (7/21), 20% (3/15) and 0% (0/4) of cases in children who had received 0, 1, or 2-3 doses of PCV7, respectively (p=0.31).

Table 8. *S. pneumoniae lytA* positivity in patients hospitalised with severe pneumonia, May 2009 through December 2010 in South Africa (n=370).

Age in years	*lytA*-positive cases n/N (%)		p-value	Cases with PCV7serotypes n/N (%)		p-value
	2009	2010		2009	2010	
<2	43/721 (6)	36/974 (4)	0.03	14/43 (33)	9/35* (26)	0.51
2-4	12/157 (8)	6/187 (3)	0.06	0/12 (0)	2/6 (33)	0.04
≥5	96/1137 (8)	177/1979 (9)	0.63	25/95* (26)	36/177 (20)	0.26
Total	151/2015 (7)	219/3140(7)	0.48	39/150 (26)	47/218 (21)	0.32

* 2 *lytA* positive samples not available for serotyping.

Table 9. *S. pneumoniae lytA* positivity and 7-valent pneumococcal conjugate vaccine (PCV7) serotypes in children <2 years hospitalised with severe pneumonia, by PCV7 vaccination status, May 2009 through December 2010, South Africa.

No. of PCV7 doses	*lytA* positive cases n/N (%)	Cases with PCV7 types n/N (%)
0 dose	21/456 (5)	7/21 (33)
1 dose	15/240 (6)	3/15 (20)
2 or 3 doses	4/133 (3)	0/4 (0)

4 Discussion

S. pneumoniae serotypes are highly diverse, with 93 serotypes described to date. The high diversity of serotypes and high genetic similarity within serogroups poses a great challenge in assigning a correct serotype using either molecular or immunological assays. Even the traditional immunological reactions used have proven to be ineffective in distinguishing between highly similar serogroups/types. Unlike other pathogens that CRDM conducts national surveillance on, such as *H. influenzae* and *N. meningitidis*, comprising of highly distinguished 6 serotypes and 12 serogroups, respectively, serotyping of the pneumococcus remains a challenge using either method. Therefore any assay, whether molecular or immunological, will be a trade-off between number of serotypes detected versus time, complexity of the assay, technical expertise and cost required to perform the assay.

The present study reports the use of molecular-based serotyping for *S. pneumoniae* in SA. Accurate serotype determination is a crucial factor in the PCV era, as vaccine developments rely on serotyping data. The emergence of vaccine-replacement serotypes post routine usage of PCV7 further emphasises the need for rapid and accurate serotyping data. The Quellung reaction is not feasible in poor-resourced settings, because of the high cost of the assay. Even in well-equipped laboratories, the Quellung reaction remains a challenge because of the technical expertise needed, subjectivity in interpretation of results and the complexity of the assay. An additional major draw-back of the Quellung reaction is that it cannot be used for serotyping of culture-negative samples, as it is culture dependent.

The use of molecular serotyping for culture-negative samples added 8% serotyping data to the national IPD surveillance in SA for 2010. As a result of this study, the C-PCR and RT-PCR serotyping assays were implemented for routine use in South African IPD surveillance for culture-negative samples.

Our study showed that the C-PCR assay is a feasible method that could be applied in SA for accurate serotype determination. The assay showed 100% specificity and sensitivity for isolates. The results were 100% concordant with the Quellung reaction results, with only <1% personal errors that could be easily resolved. For culture-positives, the assay was sufficiently sensitive as cultures can be controlled for sufficient DNA concentrations during crude DNA extraction. The random selection of isolates was an adequate representation of serotypes circulating in SA. The C-PCR assay assigned a serotype to 97% of 2008 and 96% of 2009 isolates randomly selected for serotyping. Based on the Quellung reaction serotypes, we estimate that of all 2008 and 2009 isolates, >96% for each year would be able to be assigned a serotype or serogroup using the C-PCR assay. This finding is important in that this assay independently could assign a serotype or a serogroup to the majority of South African surveillance IPD isolates, without use of the Quellung reaction, although some would not be resolved to specific types. The accuracy, coverage rate and specificity obtained when using the protocol for US isolates was similar to what was obtained elsewhere in Africa [64], Brazil [63] and in Korea [188]. The results showed C-PCR can be used as a reliable alternative or supplement to the Quellung reaction for viable isolate serotyping. This supports the fact that this multiplex C-PCR assay can easily be introduced in many laboratory settings with viable cultures [60].

The most prevalent disease-causing serotypes in SA i.e. serotypes 1, 19A, 6A, 6B, 14, 23F and 19F were detected in the first three reactions. The majority of isolates could be assigned a serotype within the first few reactions, decreasing the number of isolates as the reactions proceed sequentially. As Pai *et al*, [60] recommended, the first three reactions could be performed simultaneously, to allow for rapid detection of serotypes, while the last three reactions could be done separately, with a limited number of isolates remaining. In Korea, a similar finding was observed, where at least 60% of isolates were assigned a serotype by the end of reaction 3 [188].

The grouping of primers and the reactions can be re-arranged to improve the efficiency of serotype yield per region, considering band size resolutions between serotypes in the same reaction. In the current study, no rearrangement of the serotypes within a reaction was necessary. However reaction 3 which contains serotype 1, the leading serotype in SA in adults, was performed sequentially after reaction 1, to allow for more isolates to be serotyped using the first two reactions.

In Spain, modification of the CDC protocol increased serotyping yield from 63% to 73% within the first three reactions [189]. The CDC developed a protocol for African regions (clinical specimens), based on the majority of serotypes reported to be circulating in Africa and Morais *et al* [64] modified the Pai *et al* [60] scheme for African specimens. However, these modified schemes were not used in this study because the majority of serotypes included in the first three reactions are less common in SA than other African regions. The US protocol for isolates from the ABC surveillance data had better serotype coverage in the first three reactions for South African isolates, hence it was used. Similarly, the revised protocol for detecting 40

serotypes/groups (CDC 33 serotypes plus serotypes 31, 21, 24, 39, 10F, 23B, 13), consisting of 8 reactions was not used because the additional reactions detected <1% of serotypes (using 2008 and 2009 surveillance isolates serotypes) while adding two more reactions and therefore increasing the cost and time of the assay. The distribution of serotypes is likely to change due to vaccine pressure, therefore new protocols which cover more serotypes may be useful going forward and new primer sets can be added to the established method.

The C-PCR assay was proven to be less subjective and required less technical skill, compared to the Quellung reaction methodology. The methodology was also suitable for large scale preparation, by allowing for PCR setup of >400 isolates in one reaction and obtaining at least 40% of serotypes in <5 hours from PCR preparation to gel electrophoresis results. C-PCR can be used for carriage isolates with an added advantage of detecting multiple colonisations [190;191] and for other non-invasive pneumococcal infections such as otitis media [192].

Despite the many benefits of the C-PCR assay, it is associated with several disadvantages. The assay currently cannot identify all known pneumococcal serotypes. Although most of the serotypes not included are not historically epidemiologically significant, in the era of replacement serotypes and vaccine-escape recombinants, monitoring of these serotypes may become more important. Recent protocols published [68] have increased serotype coverage, however, not all 93 serotypes are included. In this study, 3% from 2008 and 4% from 2009 of selected isolates belonged to serotypes not included in the assay. Although this percentage is small, concerns of this percentage increasing in SA due to serotype replacement warrants continued serotype surveillance and adaptation to new protocols.

A further disadvantage is that most of the serotypes can only be resolved to genetically related serotypes (e.g. serotypes 18A/B/C/F, 7A/F and 38/25), and cannot be resolved any further as the Quellung reaction would, using serotype-specific factor sera. This poses a challenge in vaccine effectiveness studies, where one serotype in the genetically related group is included in one of the vaccine formulations and the other is not included. For example, for serogroup 18, serotype 18C is in PCV7, while serotypes 18A, 18B and 18F are not.

These limitations may be overcome by designing serotype-specific primers to detect the most prevalent serotypes, instead of detecting a serogroup. This however, might be challenging due to the high genetic similarities between serotypes belonging to the same serogroup or cluster. Training should be focused on DNA extraction procedures, to minimise personal errors such as cross-contamination of stored cultures, as encountered in this study. The accuracy in interpreting the results (band sizes) can be achieved by including serotypes that give clear band resolutions in a reaction i.e. sufficient distance between band sizes for serotypes included in the reaction to be assigned accurately.

Although the C-PCR assay was successfully implemented, advancement due to new technologies that further increase the uses and advantages of PCR assays were considered.

Real-time PCR has advantages over conventional PCR in that it is faster by allowing for results to be analysed in real-time as the product accumulates [69]. In addition, it has increased sensitivity for samples with low DNA concentrations [33;34].

The RT-PCR assay used for serotyping of *S. pneumoniae* isolates demonstrated 100% sensitivity for serotyping of cultures, similar to the C-PCR assay and the Quellung reaction. Bacterial cultures have abundant organism and therefore higher concentrations of DNA. In Italy [34], a similar finding for cultures was observed; both the conventional and the real-time serotyping assays displayed 100% sensitivity for isolates. Due to the time-consuming and laborious nature of the singleplex reactions, the method described by Azzari *et al.* was adapted to duplex reactions. Reactions were performed in both singleplex and duplex formats to determine if there was loss of sensitivity in the duplex assay compared to the singleplex assays. There was no loss in sensitivity when using the duplex assay compared to the singleplex assay. The original [34] 21 singleplex reactions were successfully modified in to 11 duplex reactions, thereby reducing the time and cost required to perform the assay. Based on the Quellung reaction serotypes, we estimate that of all 2008 and 2009 isolates, ≥91% for each year would be able to be assigned a serotype or serogroup using the RT-PCR assay.

For isolates both the C-PCR and RT-PCR assays had high sensitivity; however, there were cross-reactions detected in the RT-PCR assay not reported by Azzari et al [34] between serotypes 25 and 38, 33A/F and 37 and serogroup 18 serotypes (except serotype 18F). In the sequential multiplex PCR from CDC [60;68], these cross reactions were reported. Immunological reactions cannot distinguish between serogroups 38 and 25 [38] and it was not surprising that these serogroups cross-reacted in both PCR assays. Serotype 9A/V primers cross-reacted with serotype 9N and 9L, and this finding was also observed in the RT-PCR developed by Tarrago *et al* [70], although different primer sequences were used in this study. Pai *et al.* recognised and reported the cross-reactivity for C-PCR, however, they were not described in the RT-PCR protocol described

by Azzari *et al* [34]. The cross reaction between serogroup 9 serotypes in RT-PCR assay was taken as false positive in specificity calculations as these serotypes can be distinguished in C-PCR as serotypes 9A and 9N/L. The cross-reactions were detected when the RT-PCR assay was validated as part of this study. Therefore, results in this study were reported accordingly to reflect the inability of the assay to differentiate certain serotypes e.g. serotype 33A/F positive samples are reported as positive for serotypes 33A/33F/37, and serotype 38 positive samples are reported as positive for 38/25. This finding is consistent with what was reported in Italy [73], in a different study where serotype 18B/C was reported as serogroup 18. Considering these findings, the RT-PCR serotyping assay displayed 95% specificity based on the original Azzari protocol [34] and accounting for known cross-reactions. The cross-reacting serotypes are highly similar in their *cps* biosynthetic loci and belong to the same sub-clusters [38]. Thus designing serotype-specific primers is difficult; hence these serotypes cannot currently be differentiated.

A limitation of the RT-PCR assay used is that many of the genetically related serogroups could not be resolved to individual serotypes. While the same scenario was observed for the C-PCR assay (15B/C, 18, 9N/L, 38/25, 22A/F and 3A/F), the number of serogroups that could not be resolved to individual serotypes was increased in the RT-PCR serotyping assay i.e. serogroups 9, 12A/B/F, 15, 6A/B, 6C/D, 7A/F, 10A/B, 18A/B/C, 19B/F, 22A/F and 33A/F. As in the C-PCR assay, the assay could be enhanced by incorporating, where possible, primers and probes targeting specific serotypes. However, this would increase the time and cost of the assay. For isolates however, this is not a limitation as the Quellung reaction can be used to resolve majority of closely-related serotypes.

Although the primers for serogroup 6 differentiation are not included in the C-PCR assay described by Pai *et al.* (only the serotype 6C differentiation reaction is included), an additional reaction can easily be incorporated into the assay. Serogroup 6 differentiation by conventional PCR (original Jin *et al* [156] protocol and the modified protocol) yielded results that were 100% concordant with the Quellung reaction results using factor sera 6b, 6c and 6d for detecting serotypes 6A, 6B and 6C respectively. The factor sera 6b used was the modified Danish antisera [58] developed after discovery of serotype 6C [43;154]. The newly validated factor sera 6d was used for serotype 6C [157]. After the discovery of serotype 6C, all surveillance isolates previously typed as serotype 6A and corrected using the new antisera for 6C [161], were retrospectively serotyped using conventional PCR

The RT-PCR assay used can only distinguish serotypes 6A/B from 6C/D. For a PCV7 effectiveness study, this may be acceptable since serotype 6A is cross-protected by serotype 6B antigens in PCV7 [166]. Several developed countries have reported this cross-protection, however, in an African setting with a high HIV prevalence, this might not hold true and resolving these serotypes might still be crucial. Furthermore, in the era of PCV13, where serotype 6C disease is cross-protected by 6A [176], differentiating serotype 6D from 6C is likely to be of importance. An increase in the prevalence of serotype 6C among vaccinated and unvaccinated populations was observed in countries with routine use of PCV7 [90;159;162-164]. However, in SA the prevalence of serotype 6C is still low. This was similar to what was reported two years earlier (2008) in SA isolates [161]. Serotype 6D was not detected in the 3-year period surveillance isolates screened. For 2010, all serotype 6B and serotype 6C samples were screened, while for 2008-2009, only a randomly selected proportion of culture-positive samples and all

culture-negative samples belonging to serogroup 6 were screened. To date, no serotype 6D has been reported in any African country performing surveillance for IPD. Continued surveillance of serogroup 6 serotypes is needed to screen for an increase in prevalence of serotype 6C, and to screen for an emergence of serotype 6D in SA.

DNA concentration had an impact on yield of serotypes using the C-PCR assay. A higher *lytA* C_t-value was associated with a decrease in serotype yield by C-PCR on all sample types. The higher sensitivity of RT-PCR was particularly useful for the serotyping of NVTMs, with low bacterial loads (average *lytA* C_t-value of >26). The serotype yield increased 2-fold by the use of RT-PCR compared to C-PCR ($p \leq 0.001$). For NVTMs, there was an increase from 14% serotype yield in specimens with high *lytA* C_t-values (>26) compared to >70% serotype yield in specimens with low *lytA* C_t-values of ≤26 using the C-PCR assay. Furthermore, an increase from 32% to 76% in serotype yield was observed from C-PCR to RT-PCR in serotyping of NVTMs, respectively. Azzari *et al* reported an increase from 64% to 91% in serotype yield for culture-negative samples using C-PCR and RT-PCR, respectively [34]. Using RT-PCR alone, 93% of CAP cases in Italian children were assigned a serotype using the Azzari *et al* scheme [73]. Similarly, a serotype could be assigned to 80% of CAP cases from children <5 years using RT-PCR of alternative primers/probe sets in a different region in Italy [193].

Overall, 30% of pneumococcal meningitis cases which the C-PCR assay could not serotype were assigned a serotype using the RT-PCR assay. Independently, the C-PCR and RT- PCR assays could assign a serotype to pneumococcal meningitis cases for 55% and 78%, respectively. In Togo and Burkina Faso, the conventional multiplex PCR assigned a serotype to 90% of

meningitis cases directly from CSFs [66]. However, 64% of the CSFs used had cultures available, and therefore had sufficiently high DNA concentrations for detection by the conventional PCR assay. Similarly, in this study, blood and pleural fluid specimens, had a high serotype yield by both PCR-based serotyping assays, and all had a C_t-value of ≤26. A culture was obtained at a later stage for the majority of blood specimens used in this study, accounting for low C_t-values observed. However, the numbers are small to emphasize the strength of PCR serotyping assays in these specimens.

Both the C-PCR and RT-PCR serotyping assays had high serotype yields on 'beep-positive' blood cultures (≥86%). This is due to the nature of these specimens, in which bacterial growth has occurred, resulting in high DNA concentrations. This is confirmed by the low *lytA* C_t-values (≤26) of these samples. Therefore the lower sensitivity of C-PCR in comparison with RT-PCR was not problematic for these specimens.

The retrospective receipt of cultures for which culture-negative specimens were received provides an opportunity to assess serotyping results for these specimens. Serotyping results from PCR assays on clinical specimens (blood cultures, CSFs, and blood specimens) were 100% concordant with the Quellung reaction results for the corresponding cultures for serotypes included in the PCR assays. However, for NVTMs, only 62% could be assigned a serotype fully concordant with the Quellung reaction results using either or both PCR assays. 21% of the remaining samples (negative on PCR-based serotyping assays) belonged to serotypes detectable by one or both PCR assays, with C_t-values theoretically considered low enough for detection in the RT-PCR serotyping assay. However, it was found that samples with borderline C_t-values of 37-40 might

not have sufficient DNA for detection by the RT-PCR assay. The *lytA* detection and RT-PCR serotyping assays C_t-values differed by ±4 units, thereby resulting in discrepancies between the two PCRs. Another explanation for this discrepancy might be that the original sample sent by the regional laboratory was not the same as the second sample sent for a particular patient. Thus although the NVTMs are *lytA* positive, it might not be the same sample as later received for Quellung reaction. Some laboratories send repeat cultures within a limited time period (<2 weeks), while other laboratories take longer in sending repeat cultures upon request, which increases the chance of error. Regular training on sub-culturing on Dorset media from agar plates at regional laboratories and reviving of NVTMs to obtain culture or sufficient DNA concentration is still warranted in SA. It is also possible that these strains have undergone certain mutational events, altering the primer binding site in the *cps* loci which resulted in no amplification/binding of the PCR primers. This needs to be explored further using gene sequencing.

The predominant serotypes were not different between culture-negative samples and culture-positive samples. Serotypes 14, 19F, 4, 6A/6B, 19A, 23F and 4 were the leading serotypes in children <5 years, while in individuals ≥5 years, both phenotypic and the molecular serotyping assays identified serotypes 1, 19A, 4, 6A, 23F, 12F and 14 as predominant. Thus, there does not seem to be serotypes specifically associated with culture-negative specimens and serotype did not influence the viability of the organism in the specimens in our study.

The most commonly identified paediatric serotypes were serotypes 14, 19F, 4, 6A/6B and 19A. The prevalence of serotype 14 in younger children has been reported, accounting for 23-25% of IPD cases in children compared with 14-17% in adults in Germany between 1992-2008 [153;194]. Serotype 14 was the second most common serotype identified in IPD cases after

serotype 19A in children <5 years, before PCV7 introduction in China [195]. Serotypes 6B, 19F and 23F were found to be significantly associated with paediatric patients compared to the adult age groups in the US and Germany prior PCV7 introduction [153;196].

In individuals ≥5 years, serotype 1 was predominant, followed by serotypes 19A, 4, 6A, 23F and 12F. The high burden of IPD due to serotype 1 in older children is also prevalent in other African regions [51;197;198] and other non-African regions. Serotype 19A, which is the leading serotype in most countries post introduction of PCV7 in many regions [145-147;149], was the second most common serotype in older children and adults.

Although in our study for children <5 years, serotype 1 was the 7th most common serotype identified and serotype 19A was the 4th most common serotype identified, the burden of IPD due to these serotypes is still substantial. These serotypes causing invasive disease are not included in PCV7; however, serotypes 1 and 19A have subsequently been included in the PCV13 formulation. Another PCV13 serotype, serotype 5, which is predominant in other African countries [50;64;197;198], was not found to be common amongst South African invasive pneumococci in both children and adults.

This study was conducted during early introduction of PCV7 and further studies are required to monitor changes which may occur as a result of the vaccine. The difference in serotype distribution by age group supports the fact that paediatric serotypes differ from adult serotype distribution. Year 2010 was a transition year for PCV7 introduction in SA, and comparison between countries with post-vaccine serotype distributions might not provide ideal comparisons

amongst countries. However, previous serotype distribution from IPD surveillance in SA (2007-2009), shows no change in the serotype distribution in all ages in 2010, thus data from pre-vaccine introduction in different countries is more appropriate for comparisons.

Molecular detection and serotyping of pneumococcal disease in paediatric empyema added useful information for estimating the burden of this complication in South African children. The pneumococcus was the leading pathogen detected among the three pathogens studied. The C-PCR assay could assign a serotype to a small proportion (29%) of specimens before it was discontinued for these specimens (due to low yield on specimens with *lytA* C_t-value of >26). The RT-PCR assay increased the serotype yield to 95%.

Pneumonia complicated with empyema has been increasing in countries with routine PCV7 utilisation, with non-vaccine serotypes being predominant [64;177-180;182;197;199]. In the PCV7 era, *S. pneumoniae* remains the leading cause of pleural empyema in children [199]. In this study, pneumococci from pleural fluid specimens were found to be of serotypes 6A/B, 19A, 23F, 1 and 14. All serotypes identified, except 19A and 1 are PCV7 types. However, this study only reflects one hospital in SA (Red Cross Children's Hospital), and one group of children with this syndrome (those who received treatment and were enrolled in the study). The burden and aetiology of empyema in SA needs to be explored further using a larger collection of samples. However, available data suggest that PCV7 serotypes constitute a large proportion, 72% (13/18) of pneumococcal empyema cases in South African children before PCV7 introduction. This study was initiated in September 2009, and some enrolled children were eligible to receive the PCV7, while the majority were not. However, vaccination status was not recorded for this study. The

rollout of PCV7 was slow in some provinces in SA, and it is unlikely that the children who participated in this empyema study were vaccinated. Serotype distribution post vaccine introduction in vaccinated children is yet to be explored. This is the first study to our knowledge in SA on the aetiology and pneumococcal serotypes associated with pleural empyema.

The historically empyema-associated serotypes in children are PCV7 serotypes, and serotypes 1 and 3 [199]. In addition to the non-PCV7 serotypes listed above, serotypes 7A and 7F have emerged as serotypes associated with empyema complication [200]. Serotypes 1 and 19A were also prevalent in paediatric empyema cases identified in other parts of the world [180;181;201;202]. Serotypes 3 and 7F were not identified in SA for the study period. However, the data from this study and other studies described suggest PCV7 would be adequate for the majority of paediatric empyema cases, but PCV13 could further decrease the burden of this disease.

The RT-PCR assay was applied to the serotyping of SARI blood specimens that were PCR-positive for pneumococcal DNA. This is important as it provides information on the pneumococcal serotypes causing severe respiratory illness in SA. A serotype/serogroup was assigned to 53% of the specimens. A limitation of this assay on these specimens was that majority had high C_t-values, some even at the border of the cut-off for *lytA* positivity i.e. 39-40. Hence it is possible serotypes of some specimens with very high *lytA* Ct- values of around 38-40 might have been missed.

The predominant serotypes identified in SA amongst hospitalised pneumonia cases; serotypes 19A, 1, 6A/B and 19BF were common in other countries in children ≤5 years pre PCV usage. In Japan, the most common serotypes identified in hospitalised children <5 years were serotypes 6B, 23F and 19A in both IPD and pneumonia cases [203;204]. Similarly in China, serotypes 19F, 19A and 6B were predominant in hospitalised children <5 years with pneumonia [205]. With routine use of PCV7 and PCV13 in SA, serotype distribution amongst all age group is likely to change due to vaccine pressure, as has been observed in countries with long-term PCV usage [103;206].

Prior to 2010, no molecular based techniques for serotyping of pneumococcal-positive culture-negative samples were available in SA. Although the overall serotype distribution was similar using either serotyping assay for all age groups, a significantly lower proportion of PCV7 serotypes in culture-negative samples (36%) compared to culture-positive samples (43%; p=0.02) was observed. Although overall, no serotype differences were noted among the most common serotypes, the prevalence of PCV7 serotypes was less than in the culture-negative sample and this need to be explored further.

Serotyping data from the SARI study specimens was used to assess the early effectiveness of PCV7 on severe pneumonia. This was possible as vaccine history was recorded as part of the study. A decrease in PCV7 serotypes among children eligible for vaccination was observed from 2009 to 2010. There was a consistent decrease in PCV7 serotypes with an increase in the number of doses received. PCV7 serotypes accounted for 33%, 18% and 11% in children who received 0, 1, or 2-3 doses, respectively (p=0.45). A limitation of this study is that the number of cases was

small, and continued SARI surveillance in the post PCV era will provide additional data on vaccine effectiveness against severe pneumonia cases.

The reduction in the incidence of *lytA* positivity and PCV7 serotypes in vaccinated children cannot be attributed to PCV7 introduction alone. Highly active anti-retroviral therapy (HAART) was scaled up in 2003 in SA. In HIV-seropositive children, a decline in IPD burden, pneumonia hospitilisation and IPD-associated mortality was observed in children <2 years who were on the HAART program [77]. Recent studies show no evidence of reduction of IPD burden in HIV-seropositive adults on HAART [207].

Detection of cases of multiple carriage are important in nasopharyngeal carriage models. In 2006 a *S. pneumoniae* carriage project, known as PneumoCarr, was established to collect data on nasopharyngeal colonisation in children globally. The project aimed to develop guidelines for measuring vaccine efficacy using colonisation rather than disease or hospitalisation episodes as endpoints in clinical trials for future pneumococcal vaccine licensure. This would be less time-consuming and more cost-effective when introducing new pneumococcal vaccines [208] and the preliminary results showed that carriage acquisition can be used as an endpoint in vaccine efficacy studies, using correct models [209]. The PneuCarriage project is a global project established to identify the best method to detect multiple serotypes in colonisation episodes by comparing over 20 different methods to be used in both well-equipped and poor-resourced laboratory settings [210]. Although this study is on-going, preliminary data indicate that the PCR-based serotyping assays were more sensitive for detecting carriage of multiple serotypes when compared with other serotyping techniques [211;212].

Through this study we have been able to determine the early impact of PCV7 on pneumococcal disease in the country. Molecular serotyping will continue to be a useful tool to monitor serotype distribution and changes that may occur as PCV13 usage increases.

5 Conclusion

PCR-based serotyping assays added 8% of serotyping data to the national IPD surveillance, thus enhancing the strength of the national IPD surveillance in SA. This is due to the ability of molecular methods to determine serotypes in specimens for which no bacterial cultures were available and for which serotyping data would not have otherwise been obtained. The ability to determine serotype for culture-negative clinical specimens will enable a more accurate assessment of vaccine effectiveness to be made.

Based on the results of this study, PCR-based serotyping assays are recommended for other developing countries and low resourced settings with a high prevalence of pneumococcal disease. PCR assays are less subjective, less laborious and less time-consuming than the Quellung reaction.

The early impact of PCV7 was assessed using serotyping data from this study. Although the numbers are small, there was evidence from the SARI surveillance study of the 2+1 dose being effective against vaccine-type disease, although more data are needed to test statistical significance. The introduction of PCV13 in SA, which was initiated in May 2011, is expected to have a positive public health impact on IPD. The leading serotypes in 2010 not included in the current PCV7 formulation identified were serotypes 1 and 19A for all age groups. Inclusion of these serotypes in PCV13 is therefore expected to be beneficial in this country. Serotype monitoring in both cultures and culture-negative samples, using low cost, fast methods is of utmost importance to monitor vaccine impact in the targeted age groups and the herd impact in the adult populations.

6 Appendices

6.1 Appendix A: Preparation of reagents for conventional PCR

Preparation of dNTP's from100 mM stock

$c_1v_1=c_2v_2$

100 mM X v_1=5 mM X 1 ml

v_1=0.05 ml

Add 50 µl of each dNTP (dATP, dTTP, dCTP and dGTP) to 800 µl of sterile dH_20 to make up l ml of 5 mM working solution

Preparation of 100 µM primers from 80 nmol stock

Concentration =mol/v

To prepare a 100 µM stock solution:

100 µM= 80 nmol/v

v=800 µl

Add 800 µl of sterile dH_20 to the lyophilised primers to make up a 100 µM stock solution

Preparation of 10 µM primer working solution from 100 µM stock solution (For Amplitaq Gold DNA polymerase)

$c_1v_1=c_2v_2$

100 µM X v_1=10 µM X 1000 µl

v_1=100 µl

Add 100 µl from the stock solution and 900 µl of sterile dH_20

Preparation of 25 µM primer working solution from 100 µM stock solution (for Qiagen Master Mix)

$c_1v_1=c_2v_2$

100 µM X v_1=25 µM X 1000 µl

v_1=250 µl

Add 250 µl from the stock solution to 750 µl of sterile dH_20

Storage of primers = -20˚C

6.2 Appendix B: Preparation for reagents for RT-PCR

Preparation of primers stock solution

Concentration =mol/volume

To prepare a 100 µM stock solution:

100 µM= 80 nmol/v

v=800 µl

Add 800 µl of sterile dH_20 to the lyophilised primers to make up a 100 µM stock solution

Preparation of 10 µM primer working solution from 100 µM stock solution

$c_1v_1=c_2v_2$

100 µM X v_1=10 µM X 1000 µl

v_1=100 µl

Add 100 µl from the stock solution and 900 µl of sterile dH_20

Preparation of 5 µM probe working solution from 100 µM stock solution

$c_1v_1=c_2v_2$

100 µM X v_1=5 µM X 400 µl

v_1=20 µl

Add 20 µl from the stock solution to 380 µl of sterile dH_20 to make up a 400 µl working solution

Storage of primers and probes = -20˚C

6.3 Appendix C: Agarose gel electrophoresis

10X TAE buffer (Tris-acetate-EDTA)

Volume/Mass	Component
96.8 g	Tris base
11.42 ml	Acetic acid (glacial)
14.88 g	EDTA

Adjust volume to 2 L using sterile dH_20

To prepare a running buffer, dilute 10X solution to get a 1X concentration i.e. add 100 ml of 10X TAE buffer to 900 ml of sterile dH_20

0.25% Bromophenol blue

Mass	Component
0.25 g	Bromophenol blue
40 g	Sucrose

Dissolve in 100 ml of sterile dH_20

Ethidium bromide (10 mg/ml)

Volume/mass	Component
1 g	Ethidium bromide
100 ml	Sterile dH_2O

Storage of TAE buffer, ethidium bromide and bromophenol blue = +4˚C

Saekem LE Agarose gel (2%)

Volume/mass	Component
2.6 g	Saekem LE agarose
130 ml	sterile dH_20

Boil the solution until all agarose has dissolved and cool down to 50˚C before adding the ethidium bromide. Pour the agarose solution immediately into the gel casting equipment and insert the appropriate comb while still liquid. After the gel has solidified, remove the combs and add 1X TAE running buffer.

6.4 Appendix D: Identification of *Haemophilus influenzae, Neisseria meningitidis* and *Streptococcus pneumoniae* by real-time PCR assay.

Table 10. Primer and probe sequences and final concentrations used for simultaneous detection of *Haemophilus influenzae, Neisseria meningitidis* and *Streptococcus pneumoniae* by real-time PCR [187]

Organism (target)	Primer	Primer/Probe sequence 5'-3'	Working conc. (µM)	Final conc. (nM)
N. meningitidis (*ctrA*)	Forward	TGTGTTCCGCTATACGCCATT	7.5	300
	Reverse	GCCATATTCACACGATATACC	22.5	900
	Probe	AACCTTGAGCAA"T"CCATTTATCCTGACGTTCT 5'FAM; 3'SpC6; "T" BHQ1	2.5	100
H. influenzae (*hpd*)	Forward	AGATTGGAAAGAAACACAAGAAAAAGA	7.5	300
	Reverse	CACCATCGGCATATTTAACCACT	2.5	100
	Probe	AAACATCCAATCG"T"AATTATAGTTTACCCAATAACCC 5' HEX; 3'SpC6; "T" BHQ1	5	200
S. pneumoniae (*lytA*)	Forward	ACGCAATCTAGCAGATGAAGCA	5	200
	Reverse	TCGTGCGTTTTAATTCCAGCT	5	200
	Probe	TGCCGAAAACGC"T"TGATACAGGGAG 5'Cy5; 3'SpC6; "T" BHQ2	5	200

Note: quencher moved from 3'end to internal position designated as"T".

6.5 Appendix E: Primer sequences and reactions for conventional PCR assay

Table 11. Primers used in the conventional sequential multiplex PCR assay

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	
4-f	CTG TTA CTT GTT CTG GAC TCT CGA TAA TTG G	430
4-r	GCC CAC TCC TGT TAA AAT CCT ACC CGC ATT G	
5-f	ATA CCT ACA CAA CTT CTG ATT ATG CCT TTG TG	362
5-r	GCT CGA TAA ACA TAA TCA ATA TTT GAA AAA GTA TG	
6A/B/C-r	AAT TTG TAT TTT ATT CAT GCC TAT ATC TGG	250
6A/B/C-f	TTA GCG GAG ATA ATT TAA AAT GAT GAC TA	
7F-f	CCT AGC GGA TAT AAA ATT ATT TTT GAG	826
7F-r	CAA ATA CAC CAC TAT AGG CTG TTG AGA CTA AC	
7C(7B/40)-f	CTA TCT CGA TCA TCT ATT GTT AAA GTT TAC GAC GGG GAA CAT	260
7C(7B/40)-r	AGA TGT TGA GAC ATC TTT TGT AAT TTC	
8-f	GAT GCC ATG AAT CAA GCA GTG GCT ATA AAT C	201
8-r	ATC CTC GTG TAT AAT TTC AGG TAT GCC ACC	
9V-f	CTT CGT TAG TTA AAA TTC TAA ATT TTT CTA AG	753
9V-r	GTC CCA ATA CCA GTC CTT GCA ACA CAA G	
9N /L-f	GAA CTG AAT AAG TCA GAT TTA ATC AGC	516
9N/L-r	ACC AAG ATC TGA CGG GCT AAT CAA T	
10A-f	GGT GTA GAT TTA CCA TTA GTG TCG GCA GAC	628
10A-r	GAA TTT CTT CTT TAA GAT TCG GAT ATT TCT C	
11A/(11D)-f	GGA CAT GTT CAG GTG ATT TCC CAA TAT AGT G	463
11A/(11D)-r	GAT TAT GAG TGT AAT TTA TTC CAA CTT CTC CC	
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	
14-f	CTT GGC GCA GGT GTC AGA ATT CCC TCT AC	208
14-r	GCC AA ATA CTGACA AAG CTA GAA TAT AGC C	
15A/(15F)-f	AAT AGT ACA GCT GCT GGA ATA TCT CTT C	436

15A/(15FF)-r	GAT CTAGTG AAC GTA CTA TTC CAA AC	
15B/C-f	TTG GAA TTT TTT AAT TAG TGG CTT ACC TA	496
15B/C-r	CAT CCG CTT ATT AAT TGA AGT AAT CTG AAC C	
16F-f	CTG TTC AGA TAG GCC ATT TAC AGC TTT AAA TC	988
16F-r	CAT TCC TTT TGT ATA TAG TGC TAG TTC ATC C	
17F-f	TTC GTG ATG ATA ATT CCA ATG ATC AAA CAA GAG	693
17F-r	GAT GTA ACA AAT TTG TAG CGA CTA AGG TCT GC	
18-f	TTC GTG ATG ATA ATT CCA ATG ATC AAA CAA GAG	573
18-r	TTA TCT GTA ACA A AC CAT ATC AGC ATC TGA AAC	
19A-f	CTT AGT CCT GTT TTA GAT TTA TTT GGT GAT GT	478
19A-r	GAG CAG TCA ATA AGA TGA GAC GAT AGT TAG	
19F-f	GTT AAG ATT GCT GAT CGA TTA ATT GAT ATC C	304
19F-r	GTA ATA TGT.CTT TAG GGC GTT TAT GGC GAT A	
20-f	GAT CAA GAG TTT TTC ACC TGA CAG CGA GAA G	514
20-r	CTA AAT TCC TGT AAT TTA GCT AAA ACT CTT ATC	
22F/A-f	GAG TAT AGC CAG ATT ATG GCA GTT TTA TTG TC	643
22F/A-r	CTC CAG CAC TTG CGC TGG AAA CAA CAG ACA AC	
23A-f	TAT TCT AGC AAG TGA CGA AGA TGC G	722
23A-r	CCA ACA TGC TTA AAA ACG CTG CTT TAC	
23F-f	GTA ACA GTT GCT GTA GAG GGA ATT GGC TTT TC	384
23F-r	CAC AAC ACC TAA CAC ACG ATG GCT ATA TGA TTC	
31-f	GGA AGT TTT CAA GGA TAT GAT AGT GGT GGT GC	701
31-r	CCG AAT AAT ATA TTC AAT ATA TTC CTA CTC	
33F/(33A/37)-f	GAA GGC AAT CAA TGT GAT TGT GTC GCG	338
33F/(33A/37)-r	CTT CAA AAT GAA GAT TAT AGT ACC CTT CTA C	
34-f	GCT TTT GTA AGA GGA GAT TAT TTT CAC CCA AC	408
34-r	CAA TCC GAC TAA GTC TTC AGT AAA AAA CTT TAC	
35B-f	GCT TTT GTA AGA GGA GAT TAT TTT CAC CCA AC	677
35B-r	CAA TCC GAC TAA GTC TTC AGT AAA AAA CTT TAC	
35F/(47F)-f	GAA CAT AGT CGC TAT TGT ATT TTA TTT AAA GCA A GAC TAG	517
35F/(47F)-r	GAG CAT TAT TCC TAG AGC GAG TAA ACC	
38/(25F)-f	CGT TCT TTT ATC TCA CTG TAT AGT ATC TTT ATG	574
38/(25F)-r	ATG TTT GAA TTA AAG CTA ACG TAA CAA TCC	
*cpc*A-f	GCA GTA CAG CAG TTT GTT GGA CTG ACC	160
*cps*A-r	GAA TAT TTT CAT TAT CAG TCC CAG TC	

Table 12. Composition of the six sequential multiplex reactions of the conventional serotyping PCR assay used in this study.

a)

Reaction 1	**Initial concentration**	**Final concentration**	**Volume (25 µl PCR)**
2X PCR Master mix (Qiagen Multiplex PCR kit)	2X	1X	12.5µl
CPSA-F	25 µM	0.1 µM	0.1 µl
CPSA-R	25 µM	0.1 µM	0.1 µl
14-F	25 µM	0.3 µM	0.3 µl
14-R	25 µM	0.3 µM	0.3 µl
6A/B/C-R	25 µM	0.3 µM	0.3 µl
6A/B/C-F	25 µM	0.3 µM	0.3 µl
23F-F	25 µM	0.5 µM	0.5 µl
23F-R	25 µM	0.5 µM	0.5 µl
19A-F	25 µM	0.3 µM	0.3 µl
19A-R	25 µM	0.3 µM	0.3 µl
9V-F	25 µM	0.5 µM	0.5 µl
9V-R	25 µM	0.5 µM	0.5 µl
DNA			5 µl
dH_2O			6.5 µl

b)

Reaction 2	**Initial Concentration**	**Final Concentration**	**Volume (25 µl PCR)**
2X PCR Master mix (Qiagen Multiplex PCR kit)	2X	1X	12.5µl
CPSA-F	25 µM	0.1 µM	0.1 µl
CPSA-R	25 µM	0.1 µM	0.1 µl
19F-F	25 µM	0.5 µM	0.5 µl
19F-R	25 µM	0.5 µM	0.5 µl
3-F	25 µM	0.3 µM	0.3 µl
3-R	25 µM	0.3 µM	0.3 µl
15B/C-F	25 µM	0.3 µM	0.3 µl
15B/C-R	25 µM	0.3 µM	0.3 µl
18-F	25 µM	0.3 µM	0.3 µl

18-R	25 µM	0.3 µM	0.3 µl
10A-F	25 µM	0.5 µM	0.5 µl
10A-R	25 µM	0.5 µM	0.5 µl
7F-F	25 µM	0.7 µM	0.7 µl
7F-R	25 µM	0.7 µM	0.7 µl
DNA			2 µl
dH_2O			5.1 µl

c)

Reaction 3	**Initial concentration**	**Final concentration**	**Volume (25 µl PCR)**
2X PCR Master mix (Qiagen Multiplex PCR kit)	2X	1X	12.5 µl
CPSA-F	25 µM	0.1 µM	0.1 µl
CPSA-R	25 µM	0.1 µM	0.1 µl
1-F	25 µM	0.3 µM	0.3 µl
1-R	25 µM	0.3 µM	0.3 µl
5-F	25 µM	0.3 µM	0.3 µl
5-R	25 µM	0.3 µM	0.3 µl
11A-F	25 µM	0.3 µM	0.3 µl
11A-R	25 µM	0.3 µM	0.3 µl
9L/N-F	25 µM	0.5 µM	0.5 µl
9L/N-R	25 µM	0.5 µM	0.5 µl
17F-F	25 µM	0.5 µM	0.5 µl
17F-R	25 µM	0.5 µM	0.5 µl
DNA			2 µl
dH_2O			6.5 µl

d)

Reaction 4	Initial concentration	Final concentration	Volume (25 µl PCR)
2X PCR Master mix (Qiagen Multiplex PCR kit)	2X	1X	12.5 µl
CPSA-F	25 µM	0.1 µM	0.1 µl
CPSA-R	25 µM	0.1 µM	0.1 µl
7C-F	25 µM	0.3 µM	0.3 µl
7C-R	25 µM	0.3 µM	0.3 µl
12F-F	25 µM	0.5 µM	0.5 µl
12F-R	25 µM	0.5 µM	0.5 µl
4-F	25 µM	0.3 µM	0.3 µl
4-R	25 µM	0.3 µM	0.3 µl
38-F	25 µM	0.3 µM	0.3 µl
38-R	25 µM	0.3 µM	0.3 µl
23A-F	25 µM	0.5µM	0.5 µl
23A-R	25 µM	0.5 µM	0.5 µl
DNA			2 µl
dH_2O			6.5 µl

e)

Reaction 5	Initial concentration	Final concentration	Volume (25 µl PCR)
2X PCR Master mix (Qiagen Multiplex PCR kit)	2X	1X	12.5 µl
CPSA-F	25 µM	0.1 µM	0.1 µl
CPSA-R	25 µM	0.1 µM	0.1 µl
8	25 µM	0.2 µM	0.2 µl
8	25 µM	0.2 µM	0.2 µl
2-F	25 µM	0.3 µM	0.3 µl
2-R	25 µM	0.3 µM	0.3 µl
34-F	25 µM	0.3 µM	0.3 µl
34-R	25 µM	0.3 µM	0.3 µl
20-F	25 µM	0.3 µM	0.3 µl
20-R	25 µM	0.3 µM	0.3 µl
22F-F	25 µM	0.5 µM	0.5 µl
22F-R	25 µM	0.5 µM	0.5 µl
31-F	25 µM	0.5 µM	0.5 µl

31-R	25 µM	0.5 µM	0.5 µl
DNA			2 µl
dH_2O			6.1 µl

f)

Reaction 6	Initial Concentration	Final Concentration	Volume (25 µl reaction)
2X PCR Master mix (Qiagen Multiplex PCR kit)	2X	1X	12.5 µl
CPSA-F	25 µM	0.1 µM	0.1 µl
CPSA-R	25 µM	0.1 µM	0.1 µl
33F-F	25 µM	0.3 µM	0.3 µl
33F-R	25 µM	0.3 µM	0.3 µl
15A-F	25 µM	0.3 µM	0.3 µl
15A-R	25 µM	0.3 µM	0.3 µl
35F-F	25 µM	0.3 µM	0.3 µl
35F-R	25 µM	0.3 µM	0.3 µl
35B-F	25 µM	0.5 µM	0.5 µl
35B-R	25 µM	0.5 µM	0.5 µl
16F-F	25 µM	0.5 µM	0.5 µl
16F-R	25 µM	0.5 µM	0.5 µl
DNA			2 µl
dH_2O			6.5 µl

6.6 Appendix F: Primer sequences and reactions for the real-time PCR assay

Table 13. Primers used for the determination of 22 serogroup/types by real-time-PCR

Serotype	Primer sequence (5´-3´)	Probe sequence	Dye-label
1-f	CGT GCG GTA ATT GAA GCT ATG A	TGCTTGCCCTTGTATAGGGT	FAM
1-r	TGT GGC CCC AGC AAC TCT		
3-f	GGT CAG CAG AAA GTA TGC ATT GG	TATTGGATGTGGTTTATCGTGAAGA	VIC
3-r	TCG TTT ATC CAG GGT CTG ATG A		
4-f	TGG GAT GAC ATT TCT ACG CAC TA	TCCTATTGGATGGTTAGTTGGTGA	FAM
4-r	CCG TCG CTG ATG CTT TAT CA		
5-f	TTA CGG GAG TAT CTT AT GTC TTT AATGG	TTGTCTCAGCAACTCTATTTGGCTGTGGG	VIC
5-r	CAG CAT TCC AGT AGC CTA AAA CTA GA		
6-f	AAG TTT GCA CTA GAG TAT GGG AAG GT	TGTTCTGCCCTGAGCAACTGG	VIC
6-r	ACA TTA TGT CCR TGT CTT CGA TAC AAG		
6C/D-f	TTG GGA TGA TTG GTC GTA TTA G	CCA CGC AAT TCG CCA TC	FAM
6C/D-r	CTC TTC AAT TAG TTC TTC AGT TCG		
7A/F-f	GAT GGC ATG TGG CAA ACC A	TTGGCTATCGGCATGGTGGT	VIC
7A/F-r	TTT GCC CTC CTT AAT CAT TTC AC		
8-f	CCA CTC ATC AGT TTC CCA TAT GTT T	TGATGGCAGATGGGTTGGGACGAG	VIC
8-r	TCA ATA ATT GAA GAA GCG AAC GTT		
9V/A-f	TGG AAT GGG CAA AGG GTA GTA	TTAATCATGCTAACGGCTCATCGA	FAM
9V/A-r	TCG GTT CCC CAA GAT TTT CTC		
10A/B-f	CCT CTC CTA TCA ACT ATT ACT CAT TAT ACT ACC T	TCATTACAACTCCCTATGTGACACGGGTCTTTT	VIC
10A/B-r	AAT AAC CAT AAG TCC CTA GAT CAT TCA AAG		
12A/B/F-f	GAT TAT TCG CTT GCC TCT TCA TG	ATTTGTAAGCGGACCGTGCGATT	FAM
12A/B/F-r	ATA GCC GAA ATA AGC TTT CCA GAA		
14-f	CGA CTG AAA TGT CAC TAG GAG AAG AT	TGTCATTCGTTTGCCAATACTTGATGGTCTC	VIC

14-r	AAT ACA GTC CAT CAA TTA CTG CAA TAC TC		
15-f	TTG AAT CAG GTA GAT TGA TTT CTG CTA	CTCCGGCTTTTGTCTTCTCTGT	FAM
15-r	CTC TAG GAA TCA AAT ACT GAG TCC TAAT GA		
18B/C-f	CCT GTT GTT ATT CAC GCC TTA CG	AACCGTTGGCCCTTGTGGTGGA	FAM
18B/C-r	TTG CAC TTC TCG AAT AGC CTT ACTC		
19A-f	TTC GAC GAC GTA TCA GCT TCA	ACCCAAAACGGTTGACGCATTATACT	VIC
19A-r	TCA TTG AGA GCC TTA ACC TCT TCA		
19B/F-f	GGT CAT GCG AGA TAC GAC AGA A	ACCTGAAGGAGTAGCTGCTGGAACGTTG	VIC
19B/F-r	TCC TCA TCA GTC CCA ACC AAT T		
20-f	AAA GAT ACT GGC TGA GGA GCT ATC TAT T	AGGATAAGGTCTACTTTGTGGGAGTTC	VIC
20-r	AGT CAA AAG TAC TCA ACC ATT CTG ATA TAT TC		
22A/F-f	CTA TTA AAT AAC CCA TTG GAA TTG AAA CG	TCCGTAATTCGCTTATGGGCACATTCTCCA	VIC
22A/F-r	TCG CAA TTG AAG ACC ACA TAA ACTG		
23F-f	TGC TAT TTG CGA TCC TGT TCA T	TTTCTCCGGCATCAAACGTTAAG	FAM
23F-r	AGA GCC TCC GTT GTT TCG TAA A		
33A/F-f	CGA GAG AGA ATA TGA GGG AAT TGT TA	AGGAAAACTGTGGTCACGGTTCG	FAM
33A/F-r	TCT CAA TCC CCG CAT TTA CTG		
35B-f	GCA TGG AGG TGG AGC ATA CA	CAATTTAAACAATATTAGTAAAGCGCAGGTCAAGCAAA	FAM
35B-r	TGT AAA GAC TGC ACA ACT CGA TAT AAA A		
38-f	GTC TTA CGT AGA ACC TCT CTG GAT GA	TTGCCACAGATTTGGAATATTTTGGTCGG	FAM
38-r	TGG TCC TAC AAG CGA CAT GTG		
*cps*A-f	GCT CCT AAG ACG TCT AAG AAT CAG TCT	TCTATGTTAGTGGAATTGAC	NED
*cps*A-r	CGA CAC CGA ACT AAT AGG ACC AT		

Table 14. Composition of the eleven duplex reactions of the real-time PCR assay

Reactions	Serotypes	Dye label	Final conc. of primers and probe
Reaction 1	19A	VIC	400nM
	18A/B/C	FAM	200nM
Reaction 2	3	VIC	400nM
	1	FAM	200nM
Reaction 3	14	VIC	400nM
	4	FAM	200nM
Reaction 4	6	VIC	400nM
	6C/D	FAM	200nM
Reaction 5	5	VIC	400nM
	23F	FAM	2500nM
Reaction 6	8	VIC	400nM
	12A/B/F	FAM	200nM
Reaction 7	7A/F	VIC	400nM
	15	FAM	200nM
Reaction 8	19B/F	VIC	400nM
	33A/F/37	FAM	200nM
Reaction 9	22A/F	VIC	400nM
	9	FAM	200nM
Reaction 10	10A/B	VIC	400nM
	38/25	FAM	200nM
Reaction 11	20	VIC	400nM
	35B	FAM	200nM

6.7 Appendix G: Primer sequences and reactions for serogroup 6 differentiation by conventional PCR.

Table 15. Primers used in the determination of serogroup 6 serotypes (6A, 6B, 6C and 6D)

Primer pair	Serotype(s) detected	Target gene	Primer sequence (5´-3´)	Product Size
6-F	6A, 6B, 6C, 6D	*wci*P	TTA GCG GAG ATA ATT TAA AAT GAT GAC TA	250 bp
6-R	6A, 6B, 6C, 6D	*wci*P	AAT TTG TAT TTT ATT CAT GCC TAT ATC TGG	
wciNβS1		*wci*N_β	ATC TCT AAA TCT GAA TAT GAA GCG GCT CAA TC	359 bp
wciNβA2	6C, 6D	*wci*N_β	GAA CTG AGC TAA ATA ATC CTC TGG ATT ATC CAC C	
wciNβS2		*wci*N_β	CGG CTC AAT CTT TAA AAA TAC CCC TTA AGA AAT TGA C	308 bp
wciNβA1	6C, 6D	*wci*N_β	CCA CCC ACC CTG TTA TAA AAA ATG AGC TTC G	
wciP584aS		*wci*P	AAG ATT ATT TAT ATA TAG AAA AAC TGT CTC ATG ATA A	155 bp
wciP-r	6B	*wci*P	GCG GAG ATA ATT TAA AAT GAT GAC TAG TTG	

[a] S, sense; A, antisense; r, reverse.

Table 16. Preparation of reaction mixes for differentiating serogroup 6 serotypes using a single multiplex conventional PCR reaction to detect all serogroup 6 serotypes (a) and two duplex reactions for individual detection of serogroup 6 serotypes (b & c).

a)

Reaction 1	**Initial concentration**	**Final concentration**	**25 µl reaction**
2X PCR Master mix (Qiagen Multiplex PCR kit)	2X	1X	12.5 µL
6A/B/C-R	25 µM	0.3 µM	0.3 µl
6A/B/C-F	25 µM	0.3 µM	0.3 µl
6B-F (*wci*P584aS)	25 µM	0.7 µM	0.7 µl
6B-R (*wci*P-r)	25 µM	0.7 µM	0.7 µl
6C/D[*wciN*$_\beta$S1]/ *wciN*$_\beta$S2]	25 µM	0.5 µM	0.5 µl
6C/D *wci*N$_\beta$A2/ *wci*N$_\beta$A1	25 µM	0.5 µM	0.5 µl
DNA			2 µl
dH_2O			7.5 µl

b)

Reaction 1	**Initial concentration**	**Final concentration**	**25 µl reaction**
2X PCR Master mix (Qiagen Multiplex PCR kit)	2X	1X	12.5 µl
6A/B/C-R	25 µM	0.3 µM	0.3 µl
6A/B/C-F	25 µM	0.3 µM	0.3 µl
6B-F	25 µM	0.7 µM	0.7 µl
6B-R	25 µM	0.7 µM	0.7 µl
DNA			2 µl
dH_2O			8.5 µl

c)

Reaction 2	**Initial concentration**	**Final concentration**	**25 µl reaction**
2X PCR Master mix (Qiagen Multiplex PCR kit)	2X	1X	12.5 µl
6A/B/C-R	25 µM	0. 3µM	0.3 µl
6A/B/C-F	25 µM	0.3 µM	0.3 µl
6C/D[*wciN*$_\beta$S1]/ *wciN*$_\beta$S2]	25 µM	0.5 µM	0.5 µl
6C/D [*wci*N$_\beta$A2/ *wci*N$_\beta$A1]	25 µM	0.5 µM	0.5 µl
DNA			2 µl
dH_2O			8.9 µl

6.8 Appendix H: Clearance certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Miss Nandu V Magomani

CLEARANCE CERTIFICATE	**M10364**
PROJECT	Molecular Serotyping of Streptococcus pneumoniae
INVESTIGATORS	Miss Nandu V Magomani.
DEPARTMENT	Virology & Communicable Diseases Surveillance
DATE CONSIDERED	26/03/2010
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 29/03/2010 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Dr A von Gottberg

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**
PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

N.V. Magomani

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