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Sanet Steyn Date

Evaluating the Sensitivity and Specificity of Dried Blood Spots by Real-Time PCR for the Common Protein Gene, *lytA*, of *Streptococcus pneumoniae* in Diagnosing Pneumococcal Disease

By

Sanet Steyn
Master of Science

Graduate Division of Biological and Biomedical Sciences
Microbiology and Molecular Genetics

_____
George M. Carlone, Ph.D.
Advisor/ P.I.

_____
Darrell R. Stokes, Ph.D.
Program Director and Committee Member

_____
Amanda I. Starnes, Ph.D.
Committee Member

_____
Kathleen L. Campbell, Ph.D.
Committee Member

Accepted:

_____
Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____
Date

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An abstract of
A thesis submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of
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Microbiology and Molecular Genetics
2011

Abstract

Evaluating the Sensitivity and Specificity of Dried Blood Spots by Real-Time PCR for the Common Protein Gene, *lytA*, of *Streptococcus pneumoniae* in Diagnosing Pneumococcal Disease

Sanet Steyn

Acute Respiratory Infection (ARI), acute infection of the upper and/or lower respiratory tract, is the leading cause of infectious death worldwide. Approximately 70% of ARI cases are in developing countries (6), which do not have the facilities and resources for proper and potentially life saving diagnosis. Pneumonia is the most severe form of ARI and is the leading cause of death in children under the age of five worldwide, killing an approximated 1.6 million children every year (11). The most common cause of bacterial pneumonia is *Streptococcus pneumoniae* (74).

Diagnosis of pneumonia is a considerable obstacle to proper treatment, epidemiological studies, and estimation of disease burden (6, 87, 86, 72); it is especially difficult in developing countries where pneumonia is most common because of the basic problem of acquiring a specimen for culture and analysis. Preliminary diagnosis in children is done ideally in developed countries by chest X-ray followed if possible, by further molecular testing (49). Real-time PCR may currently be the best method for identifying the microbial cause of pneumonia from a blood specimen (72), but the facilities for any of these tests are often not available in rural areas of developing countries.

Dried blood spots (DBS) have shown success in studies diagnosing HIV infection in infants safely and with ease (83,75). Whole blood can be spotted directly onto filter cards, which are chemically activated to bind DNA and inactivate pathogens (63). DBS are easier to collect, transport, store, and safer to handle than liquid samples (5).

This study proposes to use DBS to collect blood samples from patients with severe acute respiratory infection (SARI) in South Africa and ship them to the facilities at CDC to be tested for the presence of the common pneumococcal protein gene, *lytA*. By comparing two kinds of filter paper and two extraction techniques it was found that the Neonatal screening cards are more sensitive to detecting *Streptococcus pneumoniae* infected patients. The use of DBS will provide a simple and effective method of sample collection and preservation that can be used in developing and resource poor countries for improved diagnosis of *Streptococcus pneumoniae*.

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Finally, I would like to thank my family. My father for stimulating my lust for knowledge, my mother for being the most wonderful, loving person I know, and my step father; your passion has become mine, thank you for sharing such an important part of your life with me.

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Chapter 1: *Streptococcus pneumoniae*

1.1. Background

Streptococcus pneumoniae is a Gram-positive, alpha-hemolytic bacterium that is a normal component of the commensal microflora in the human nasopharynx and is endemic to all people worldwide. It is generally harmless and carried asymptomatically, but on occasion can migrate into various body sites, such as the ears, upper airways, or, more seriously, the lungs or blood (83) and become pathogenic (64). *S. pneumoniae* is the most commonly identified cause of community-acquired pneumonia (22, 63), bacterial meningitis (75), acute otitis media (9, 72), acute bacterial sinusitis (86, 74, 11), and a multitude of other diseases. It is the leading cause of serious bacterial infection worldwide and produces substantial morbidity and mortality (36). In the developing world the pneumococcus has the largest impact, causing over 2 million cases of pneumonia, mostly in young children under 5 years of age (83, 47).

As a lethal pathogen *Streptococcus pneumoniae* has demanded much attention and study, and as a consequence its study has lead to many beneficial scientific findings. Since its simultaneous discovery in 1881 by two independent scientists, namely George Sternberg and Louis Pasteur (83,87) *S. pneumoniae* has helped science take giant steps forward. Because nonvirulent pneumococcal strains have the ability to transform into a virulent strain when a live, harmless pneumococcus is co-inoculated in an animal model alongside a heat killed virulent pneumococcus (as was done by Frederick Griffith in 1928), it was proposed and later proven that the genetic material responsible for this transformation is comprised of DNA (83, 6). Studies on the pneumococcus have primed the foundation for the study of immunology. Experiments such as those done by the

Klemperer brothers, who observed immunity in rabbits injected with serum of patients after infectious crisis (83), have lead to advancements in vaccine design and also antibiotic drugs. Pneumococcal studies are responsible for the development of polyvalent polysaccharide vaccines and later studies of *Haemophilis influenzae* type b led to the creation of polyvalent polysaccharide-protein conjugate vaccines from which analogous vaccines for the pneumococcus were made (71, 8). Development of these conjugate vaccines is probably one of the most important contributions to the eradication of pneumonia and pneumococcal disease although better epidemiological and population based burden of disease data are needed to accurately determine where these vaccines are most urgently needed. (98).

1.2. Carriage and infection

The human oro- and nasopharynx is the principle ecological niche for the heterogeneous population of *S. pneumonaie* (the pneumococcus) (88), which exists as over 90 different capsular types or serotypes (35). Upper respiratory colonization can happen as early as the day of birth (28, 4) and a person can carry several serotypes at one time (29). Acquisition is the first step of nasopharyngeal colonization and occurs by transmission of aerosolized respiratory droplets containing the bacterium from one human to another (57). Transmission is usually increased during the course of other respiratory infections, causing coughing, sneezing, or secretions (64). If the person is susceptible, this may result in establishment of the bacterium in the new host's nasopharynx. Individuals at risk of acquiring *S. pneumoniae* are those exposed

to crowded areas (78), those with colonized children or siblings (10), and those below the age of 5 or above the age of 65 (83, 80). At this point the pneumococcus is usually carried asymptomatically (21), the duration of carriage depending on various factors, such as serotype and host immune system (83). If it effectively evades the host's defensive mechanisms it is able cause disease.

Acquisition commonly occurs earlier in life, especially in the young of developing countries (64). Some studies have reported nearly 100% carriage in 1 year old children (63) which suggests that children at their peak age of pneumococcal carriage (i.e., 2 to 5 years) are a main source of pneumococcal transmission (25). Data from infants show risk for progression to invasive disease to be greatest soon after exposure and acquisition of the organism (of a new serotype) in the nasopharynx (26), but disease may also develop after months of colonization (74, 64). Median duration of carriage of each serotype varies from 6 to 22 weeks (79). However; carriage of a single serotype over a period of 3 years in an adult in the absence of illness has also been observed (5). The increase and subsequent decrease in pneumococcal carriage between 0 and 5 years of age is consistent with acquired immunity playing a role in reducing carriage (83, 51).

Colonization can either be terminated or, in the case of host perturbation, it may lead to illness. The mammalian lower respiratory tract is highly resistant to infection and pneumococcal invasion is usually cleared rapidly (83), but when there is visible evidence of pulmonary injury or edema, pneumococcal infection is more likely to develop (32). Influenza infection has also been shown to cause lung epithelial damage and increase the adherence of *Streptococcus pneumoniae* to exposed respiratory tract receptors (20, 68).

Thus there usually exists an exacerbating condition that gives the potential pathogen the opportunity for infection.

Risks of acquisition leading to invasive infection are chronic medical conditions, such as chronic bronchitis (16); underdeveloped (immature) immune response (103, 14) or compromised immune response, such as children and persons with [sickle cell disease leading to] functional/anatomic asplenia (2) or HIV (52, 46, 48); preceding or coincident infection, such as the development of acute otitis media within days after onset of upper respiratory tract infection (33); and the association between influenza A and pneumococcal disease (20, 76). The increased risk of disease among children younger than 2 years of age is likely related to immature immunological response to the pneumococcal polysaccharide capsule and high prevalence of colonization (83). Thus host immunity is a very important factor contributing to the risk of invasive infection by *Streptococcus pneumoniae* (83, 24). However, living in a developing or resource poor country may be the most important risk factor because of the exposure and prevalence of the aforementioned conditions (83, 45, 43). Disease results in only a small number of colonized individuals, but the ubiquity of *S. pneumoniae* in the human population results in a substantial burden of disease (99).

Changes in the proportions of *Streptococcus pneumoniae* serotypes in the upper respiratory tract may occur during pneumococcal infection. Real-time PCR detection of some common protein genes, such as *lytA* and *psaA*, may be used as a method to measure this variation in diagnosis of the disease (27), though this is not a very accurate approach. Real-time PCR done directly on typically sterile sites such as blood or cerebrospinal fluid (CSF) gives a less ambiguous clinical prognosis; if *S. pneumoniae* is present in the blood

it is most likely the causative agent of disease (83). Thus real-time PCR is more specific and does not pick up innocuous nasopharyngeal colonization (73).

1.3. Pathobiology

Without colonization pneumococcal disease is unlikely to naturally occur, thus it is the first stage leading to pathogenesis of all pneumococcal infections (83). When the organism moves from the site of colonization into an area not normally colonized, such as the Eustachian tube, paranasal sinus, or bronchioles invasive disease can occur if host defensive mechanisms fail to clear the organism (83). This can lead to otitis media, sinusitis, and several other types of infection (17). If the pneumococcus is allowed to proliferate, virulence factors trigger a powerful inflammatory response, which is in itself the cause of the disease in the respiratory tract (83); inflammatory response of the lungs to these bacterial products is the cause of symptoms of pneumococcal pneumonia (50). Pneumococci may also directly invade the blood, causing bacteremia. Incursion directly from the site of colonization with no focal source is considered to be primary bacteremia (83, 3). Secondary bacteremia arises from a site of established infection (38), which occurs in adults typically as a complication of pneumonia (83).

I. Otitis Media, Sinusitis, and Meningitis

Otitis media is the most common form of ear infection and the bacterial agent is generally *Streptococcus pneumoniae* (18). As the bacterium moves into the Eustachian tube it is normally cleared by ciliary action. If a coexisting infection or allergy causes

edema the movement is obstructed and the pneumococcus cannot be removed (83, 33) causing inflammation and fluid secretions.

Sinusitus has a similar progression to otitis media, congestion of the mucosal membranes resulting in obstruction of the osteomeatal complex being an important factor. Fluid accumulates in the paranasal sinuses, supplying the organism with a medium for growth (30). If opsonizing antibody is not present, the bacterium may multiply and infection result (83).

Bacterial meningitis in adults can be caused by *S. pneumoniae* (69) and is one of the most severe infectious diseases (66). Hematogeneous infection results from the movement of the bacterium across vascular endothelium and into the blood, causing high-grade bacteremia. This can lead to central nervous system invasion causing higher permeability of the blood brain barrier and movement of proteins and neutrophils into the subarachnoid space causing inflammation of the meninges (82).

II. Pneumonia

Acute respiratory infections (ARI) are acute infections of the upper and lower respiratory tract ranging from self limiting colds to fatal pneumonia. ARIs are the foremost cause of death among children under 5 years of age, causing as many as 2.2 million fatalities worldwide, especially in developing countries (47). In 2000, 1.9 million children died from ARI, 70% of them in Africa and Southeast Asia (106). Pneumonia, a lower respiratory tract infection (LRTI), accounts for the majority of these deaths, killing more than AIDS, malaria and tuberculosis combined (107)! Microbial causes of

pneumonia include viruses, fungi, and bacteria (107). The most common cause of bacterial pneumonia is *Streptococcus pneumoniae*.

Pneumococcal pneumonia results when the organism gains access to the alveoli and replicate (83). Carriage of the bacteria along the alveolar septa activates a complement immune response, causing inflammation and accumulation of exudative fluid and white blood cells. This fluid filling of the alveoli defines the presence of pneumonia (85).

Diagnosis of pneumonia is a considerable obstacle to proper treatment and estimation of disease burden. Data are difficult to obtain, as the lung itself is not easily amenable to culture and ARI is often associated with other illnesses, such as tuberculosis (TB) and measles (106). Currently hospitals rely on radiographic finding to be suggestive of pneumococcal disease. Chest X ray or computed tomography are used to examine the presence of lobar consolidation, though this identifies pleural effusion (41) and does not recognize the biological origin (58). Thus, most patients who present for clinical care are treated empirically (83).

The means to gain the appropriate population based disease data are especially difficult in developing countries, where ARI is most common, and many lack national estimates of disease burden (65, 105, 84, 77). This may in part be attributed to the difficulty of acquiring specimens for culture and testing because of the general lack of equipped facilities in these rural areas. It may also be ascribed to the methodology in clinical definitions of pneumonia and the suboptimal sensitivity of current diagnostic tests in defining the etiology of disease. (47, 91). Thus, local and global policy on

effective intervention, such as the pneumococcal conjugate vaccine, of incidence languish because of inadequate population based disease burden data (65).

1.4. Identification and diagnosis of pneumococcal disease

Detecting the presence of pneumococcal infection historically relied on a convoluted method of isolating the bacteria. First an animal model would be inoculated with the nasopharyngeal secretions or sputum from an infected or colonized person, then samples from the animal would be cultured and evaluated for evidence of pneumococcal infection (5). This process was extremely slow, laborious and expensive. Later it was determined that isolation can be achieved just as effectively by directly culturing the secretions onto blood agar containing gentamicin (34), and in 2003 there was finally a concentric method of isolation generated by the World Health Organization on the "gold standard" of pneumococcal isolation and identification of colonization (64). The requirements specify the samples to be collected through deep nasopharyngeal swab, with a calcium alginate or Dacron polyester tip to avoid potential inhibitory properties of the cotton swabs, and inoculate the specimen into skim milk-tryptone-glucose-glycerine (STGG) for transport and storage. A small portion of the mixture is then plated onto a blood agar (BA) plate, testing it for optochin susceptibility during its growth of 24h-48h. If susceptible, further tests can be done on the grown bacteria, such as bile solubility and agglutination with antipneumococcal polysaccharide capsule antibodies (83, 31). Identification has relied heavily on these, and on methods such as colony morphology and hemolytic activity on BA plates (15). A combination of these may be helpful in

identifying *Streptococcus pneumoniae*, but these are not without limitations and shortcomings; atypical pneumococci may be optochin resistant (40), bile insoluble (88), and other organisms that occupy the same space may have morphological and characteristic similarities to *S. pneumoniae* (88).

The culture itself has proved to be an unreliable method for identifying pneumococcal disease. Nasopharyngeal cultures represent an array of organisms that may present problems to the identification of the pneumococcus (88). The amount of bacterial growth also depends upon the density of the organism at the site of sampling; below a certain concentration the bacterium will not be detected. Thus an under or overrepresentation of the organism colonizing the individual may be present in culture (83). This type of culture from a nonsterile site is not reliable for confirmation of disease (104).

Blood, cerebrospinal fluid, or pleural fluid will give the cleanest samples for the organisms likely to be associated with the presented disease (83). But cultures from these sites are still unreliable. Culture growth is stunted by the administration of antibiotics and prone to give false negative results (70). Cultures give positive results for only a small number of disease cases, as low as 15%.(83, 44, 27) and even with proper sampling and without the use of antibiotics it has a sensitivity of 86% in clinical cases (60, 1, 7). This most likely has lead to an underestimation in the number of pneumococcal infections in recent years (83). Thus, these difficulties with culture limit its usefulness for positive identification of the etiology of pneumococcal disease diagnosis, although it is relied upon in molecular epidemiologic typing, testing sensitivity to antibiotics, detecting

previously unknown serotypes, and identifying new mechanisms of antimicrobial resistance (64)

Molecular techniques, such as DNA based assays, have shown promise as useful diagnostic tools. Possibly the preeminent current technique being explored being real-time PCR (31, 55), which allows the organism to be detected and quantified from very small amounts of genetic material without the need of time consuming and arduous culturing steps (64). Real-time PCR is highly sensitive and simultaneously detects the presence and measures the relative amount of bacteria. It has increased the sensitivity and decreased the time for identification of the pathogenic agent and thus allows more rapid treatment.

1.5. Current needs in pneumococcal disease research

The first step in treating pneumococcal disease is through accurately and definitively identifying the causative agent, *S. pneumoniae* (96). This is difficult because of the lack of effective diagnostic methodologies (47) and because the highest prevalence of disease is in rural areas that do not have facilities able to perform molecular testing. Currently, international epidemiological field studies require difficult to obtain preservation conditions for samples, such as significant and costly amounts of dry ice and packaging (95). Transporting whole blood or other fluid samples carry the risk of being lost or mishandled and potentially infecting those coming in contact with the fluid. Thus a safe and cost effective method of collecting, transporting, storing, and testing clinical blood samples of potential pneumococcal disease patients is lacking (64).

Chapter 2. Molecular Diagnosis

Chapter 2. Molecular Diagnosis

2.1. Pneumococcal genetics, the *lytA gene*

Important in the understanding of pathogenesis and subsequent treatment of pneumococcal disease is the role of certain virulence factors that may be targeted in molecular research and diagnostics. The *Streptococcus pneumoniae* peptidoglycan cell wall consists of the disaccharide polymer, *N*-acetylmuramic acid-(1-4)-*N*-acetylglucosamine (MurNAc-GlcNAc), and is important in the study of this organism (61). Autolysin is an N-acetylmuramoyl-L-alanine amidase, which cleaves the bond between the lactyl group of MurNAc and the alpha-amino group of L-alanine (the first amino acid of the stem peptide of the cell wall) (83). This degradation causes the cell to lyse, releasing pneumococcal cytoplasmic content and virulence factors into the surrounding environment (54). Autolysin is encoded for by the *lytA gene,* which represents an effective molecular target because of its limited genetic variability between serotypes (89).

2.2. Real-time PCR

Real-time polymerase chain reaction is a highly sensitive and precise technique based on the quantitative amplification of specific genetic material, such as the *lytA gene*, directly from small amounts of clinical sample. It can distinguish between specific nucleotide sequences from a convoluted mixture of DNA (100). Thus it can be used for the accurate and rapid detection of *S. pneumoniae* without the laborious steps required of other diagnostic methods (27).

I. Fluorescent probes

Real-time PCR relies on the use of fluorescent dyes or profluorescent oligonucleotide probes. Each fluorogenic labeled probe (42) emits a unique wavelength of light when cleavage transpires and the 5' fluorophore is separated from its adjoining 3' quencher (39). This is achieved by the 5' to 3' exonuclease activity of the DNA polymerase as extension of the genetic material occurs (93). If the gene target is present the probe hybridizes to a specific sequence between the forward and reverse primers (92). As amplification occurs fluorescence increases with the accumulation of the amplicon. This increase in fluorescence only occurs if the probe is complementary to the target sequence and is allowed to anneal, thus nonspecific amplification is not detected (39).

The real-time polymerase chain reaction consists of several stages. For reactions requiring the activation of the polymerase enzyme, such as the AmpliTaq Gold® DNA polymerase (Roche Molecular Systems, Inc.), the reaction mixture (containing the enzyme, dNTPs, primers, probe, and optimized buffer components) is heated to 95°C (39). Breaking of the nucleotide hydrogen bonds and separation of the DNA allowing the probe and primers to bind follow this step. The temperature is then lowered to permit elongation of the DNA target sequence. Denaturation and elongation are then repeated a number of times and define the PCR cycles. During the initial cycles little change in fluorescence signal occurs, delineating the baseline of fluorescence detection. Sequence Detection System (SDS) instruments and specialized computer software detect amplification beyond that threshold. The point at which the signal is perceived is the threshold cycle (C_T) (39), this number can be used in the identification and quantification of the pathogen present in the sample.

2.3. Dried Blood Spots

Chemically activated filter papers, such as QIAcard FTA (Whatman®, QIAGEN®, Valencia, CA) (**Figure 1.**) filter cards can be used to collect blood samples from diseased patients and have shown potential in newborn screening (19), HIV detection(13), and other studies as an alternative to working with aliquoted whole blood specimens (53, 37). Whole blood can be spotted directly onto the filter cards impregnated with chemicals that lyse cell membranes, denature proteins, and intercalate the nucleic acids with the cellulose fiber matrix of the card, immobilizing and stabilizing the sample. This process protects the DNA from nucleases, oxidation, and UV damage allowing the cards to be stored at room temperature with little difficulty (98). These cards use little space, are economical and may theoretically be kept for years. The card chemistry also inactivates disease-causing agents, making them safer to handle than whole blood specimens (67). Multi-barrier pouches can be used to transport the dried blood spots (DBS) without the need of expensive packaging requirements, making them useful in field studies where samples may otherwise be difficult to collect and ship (98). The collected sample may then be extracted by several different techniques (98, 102) to yield purified liquid or on-card DNA for molecular analysis, such as real-time PCR.

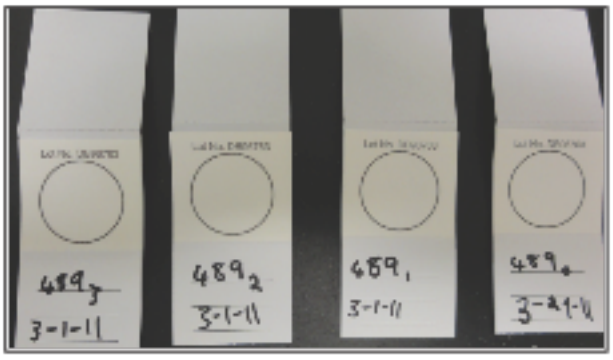

Figure 1. QIAcard FTA filters. One-inch diameter circle indicates area where blood or other sample fluid is spotted for collection, transport, and storage.

Chapter 3.
Evaluating Dried Blood Spots by Real-Time PCR for *Streptococcus pneumoniae* in Diagnosing Pneumococcal Disease

Chapter 3.
Evaluating Dried Blood Spots by Real-Time PCR for *Streptococcus pneumoniae* in Diagnosing Pneumococcal Disease

3.1. Purpose/objectives:

To develop, optimize, and evaluate the sensitivity and specificity of neonatal screening grade filter dried blood spots as a method of collecting, transporting, storing, and testing whole blood samples of potentially pneumococcal pneumonia infected individuals using real-time PCR. Also, to secondarily compare the sensitivity and specificity of QIAcard FTA dried blood spots to the Neonatal DBS.

3.2. Description:

This study will involve the cooperation of two sites. The Respiratory and Meningeal Pathogens Research Unit in Johannesburg, South Africa collected blood samples from patients with suspected pneumococcal disease over the course of two years as part of routine, ongoing pneumococcal research. Real-time PCR assays had been done on all samples to test for the presence of the *Streptococcus pneumoniae* gene, lytA, and the remainder of the blood was stored. A collection of these bloods was simultaneously spotted onto Whatman QIAcard FTA cards and Neonatal Screening grade filter cards in duplicate for this study. Those dried blood spots were sent back to the Meningitis and Vaccine Preventable Diseases Branch at the CDC for testing.

3.3. Impact

This study will equip doctors in rural and resource poor areas that lack the proper lab facilities for molecular testing with a simplified inexpensive method of sample collection and storage that preserves the sample for subsequent molecular testing; it will also facilitate large study collection of samples for obtaining population-based burden of disease data on pneumococcal pneumonia. Collectively this will provide the means to ensure a proper diagnosis to patients in order to more accurately treat their diseases, potentially saving many lives.

3.4. Participating Sites

Atlanta, Georgia
United States of America

- Centers for Disease Control and Prevention (CDC), National Center for Immunizations and Respiratory Diseases, (NCIRD), Meningitis and Vaccine Preventable Diseases Branch (MVPDB)
- Emory University

Johannesburg, Gauteng
South Africa

- National Health Laboratory Service (NHLS)
- University of the Witswatersrand (WITS)
- Respiratory and Meningeal Pathogens Research Unit (RMPRU), National Institute for Communicable Diseases (NICD)

Soweto,
South Africa

- Chris Hani Baragwanath Hospital (CHB)

Manchester,
United Kingdom

- University of Manchester

I. IRB approval/ exemption

A collective Ethics and IRB approval for all South African sites was applied for and received prior to shipment and testing of the samples. (**Appendix B. III.**)

CDC IRB and Emory eIRB was also applied for and documents of non-engagement granted, since no direct interactions with the patients were made, and all information was coded and anonymized. (**Appendix B. I. & II.**)

Chapter 4. South Africa: RMPRU, WITS, and CHB

Chapter 4. South Africa: RMPRU, WITS, and CHB

All of the clinical samples used in this study came from laboratories in South Africa who are part of a continuing study assessing quantitative real-time PCR as a method of identifying and quantifying *Streptococcus pneumoniae* in blood specimens, based on the detection of the *lytA* gene.

4.1. Study purpose and participant selection

Surveillance officers identified hospitalized patients who met the Severe Acute Respiratory Infection (SARI) case definition[*]. These patients were approached for inclusion in a prospective hospital-based sentinel surveillance program in place at several participating hospitals in the Gauteng Province: Klerksdorp Hospital, two Mpumalanga (Mapulaneng and Matikwane) hospitals, Edendale Hospital, Province Hospitals, and Chris Hani Baragwanath Hospital. Chris Hani Baragwanath in Soweto received the largest number of cases. Patients who met the criteria were asked to consent to giving a whole blood sample for real-time PCR diagnosis of pneumococcal bacteremia.

All information was completely coded. The South African laboratory sites had no direct contact with any of the patients nor would they be able to ascertain the identity of the subjects with the given information. Only the blood sample with SARI study number was received, all other patient information was sent to the Epidemiology and Surveillance Unit to be entered into a centralized database.

[**] Many of these patients were likely to have been on oral antibiotics on suspicion or preemptive diagnosis of pneumonia, which could affect the outcome of cultures.

4.2. Materials and methods

All collected materials were handled according to the standard operating procedures for detection of *Streptococcus pneumoniae* from blood specimens of patients with severe acute respiratory infections in South Africa according to RMPRU protocol. CDC did not have any information on the patients or results from the study. The study was completely blinded; no definitive numbers of positive and negative results were given to the CDC site before or at the time of testing. All samples were only used for this and no other purpose or study and will be destroyed or discarded at the conclusion of this project.

I. Sample selection

Equal subsets of real-time PCR *lytA* positive and negative specimens from the Chris Hani Baragwanath Hospital for the years 2009 and 2010 were randomly, by use of randomization tables, selected for use in this study.

The whole blood samples were removed from the -70 °C freezers, where they had been placed in storage after testing. Those samples selected by the South African collaborators were then spotted in duplicate onto both Neonatal and FTA filter cards.

A total of 197 samples were selected and each was arbitrarily assigned an alphanumeric code (A1-A53, B1-B39, B51-B54, C1-C53, D1-D39, and D51-D59) prior to being shipped to the CDC.

2009

A total of 96 samples were selected from the 2009 group of whole blood samples (**Figure 2.**). Positive and negative specimens were randomly selected and coded A1-53, B1-39, and B51-54.

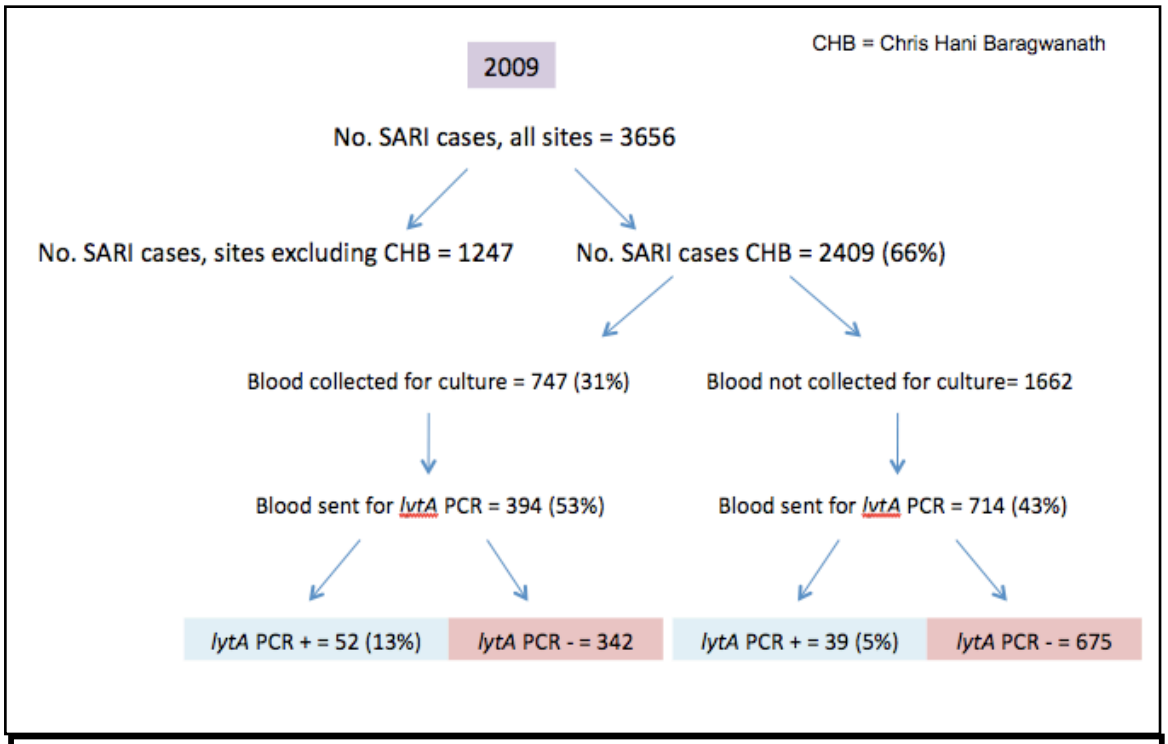

Figure 2. Original prospective study diagram showing the paths taken by assembled blood samples after collection in 2009 and their quantitative real-time PCR results. Samples shown in blue represent the positive assay results. Samples in red indicate negative results.

2010

A total of 101 samples were selected from the 2010 group of whole blood samples (**Figure 3.**). Positive and negative samples were randomly selected and coded C1-53, D1-39, and D51-59.

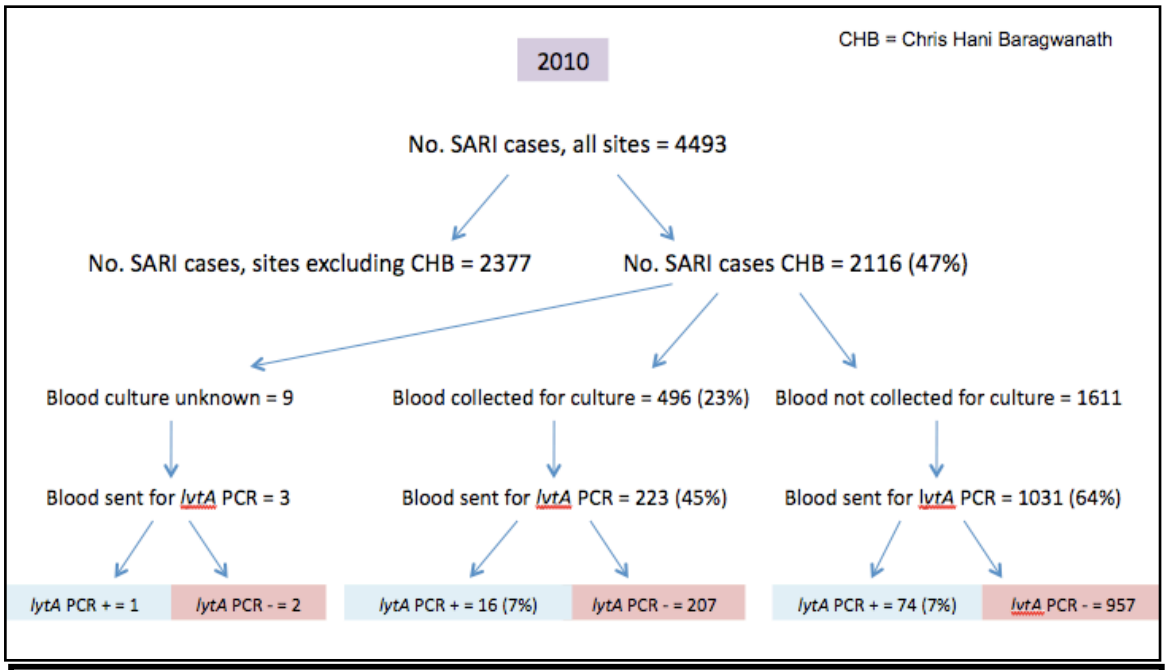

Figure 3. Original prospective study diagram showing paths taken by assembled blood samples after collection in 2010 and their quantitative real-time PCR results. Samples shown in blue represent the positive assay results. Samples in red indicate negative results.

Exclusion of samples:

If a sample contained an insufficient volume i.e., less than 200 µL, it was excluded from the study.*

* A small subsidiary of the samples had been tested for blood culture at the hospital. These results were not observed or taken directly by participants of this study, merely noted and reported to the SARI database by hospital staff. Blood cultures are only performed at the discretion of the hospital staff so are not available for most cases.

II. Specimen collection, transport, and storage

Surveillance officers at sentinel sites that recognized a potential clinical case of SARI approached the patient for enrollment in the study. Nasopharyngeal swabs (NPS) and throat swabs (TS) were taken from all consenting patients over the age of 5. With children under 5 years of age, a nasopharyngeal aspirate (NPA) was collected. Blood samples were acquired from all patients using EDTA-vacutainer tubes within 24 hours of admission to the study. The blood was kept refrigerated at 4°C and moved to RMPRU lab facilities within a week of collection where the blood was assigned an isolate number. DNA extraction was done within a week after arrival.

After initial testing the specimens were stored at -70°C (67) until DBS spotting was done in January 2011. 50 µL of each specimen was applied to unique circles in duplicate to both the FTA and Neonatal filter cards (**Figure 4.)**. The cards were left to dry at room temperature for at least one hour then stored in the appropriate FTA envelopes.

Figure 4. Neonatal and FTA DBS. Whole blood was dotted directly onto the filters.

The DBS specimens were then labeled and sent to the CDC following the "standard precautions" for packaging and handling samples to be shipped. This procedure meets the basic "triple packaging system" requirements (64) i.e., blood absorbed into paper, a fold-over flap or inner envelope containing a desiccant material, and an outer envelope of high quality paper.

III. DNA extraction

DNA was extracted directly from a 200 µL aliquot of whole blood using the automated Roche MagNA Pure LC 2.0 instrument and the Roche MagNA Pure LC DNA Isolation Kit III (for bacteria). The extraction process consisted first, of the preparation of the samples by addition of a lysis buffer and proteinase K to disrupt the cells and digest proteins, followed by addition of binding buffer and magnetic glass particles (MGPs) to bind the nucleic acids to the beads. The nucleic acid-bead complex was then magnetically separated and removed from the solution followed by three wash buffers to remove PCR inhibitors, salts, proteins, and other extraneous cellular debris and materials. Again the complex was magnetically separated from the solution before the DNA was eluted and the MGPs removed (101). DNA extracts were then moved to -20 °C storage until further use.

This method is used routinely by the RMPRU to extract DNA from clinical specimens for high DNA yield.

IV. Quantitative real-time PCR

Single-target real-time PCR for the common pneumococcal gene, *lytA*, was done using the protocol as described previously by Cavalho et al. (12). The Applied Biosystems 7500 real-time PCR instrument was used to perform the assays.

Primer and sequences used by the RMPRU were designed based on *lytA* amplification (**Table 1.**).

lytA Primer/ Probe	Sequence
Forward primer (F373)	5'-ACGCAATCTAGCAGATGAAGCA-3'
Reverse primer (R424)	5'-TCGTGCGTTTTAATTCCAGCT-3'
Probe (Pb400)	5'-FAM -TGCCGAAAACGCTTGATACAGGGAG-BHQ1-3'

Table 1. Oligonucleotide sequence of *lytA* primers and probe. Sequences were designed for the amplification of the *S. pneumoniae* autolysin gene. The probe sequence was modified by the addition of a 5' 6-carboxyfluorescein (FAM) fluorophore and a 3' black hole quencher 1 (BHQ1).

The qPCR reaction mixture was set up as follows (**Table 2.**) for a 25 µL final volume:

Reagents	Working concentration	1 Reaction (µl)	Final concentration
TaqMan gene expression master mix	2x	12.5	1x
Forward primer	10 µM	0.5	200 nM
Reverse primer	10 µM	0.5	200 nM
Probe	10 µM	0.5	200 nM
MilliQ H_20 (Sterile)	-	8.5	-
Template DNA	-	2.5	-
TOTAL		25	

Table 2. RMPRU Gene Expression Master Mix kit setup. Mix was set up according to a modified Applied Biosystems manufacturer instructions as described by Cavalho et al. (12) based on a 200 nM primer and probe reaction.

Universal Cycling conditions were set up as follows (**Table 3.**):

Stage	Temperature	Time
Stage 1	50°C	2 minutes
Stage 2	95°C	10 minutes
Stage 3 (40 cycles)	95°C	15 seconds
	60°C	1 minute

Table 3. Parameters for RMPRU real-time PCR cycling conditions. A 2 minute 50°C incubation period required for optimal UNG enzyme activity (88), is followed by a 10 minute incubation at 95°C needed to activate the AmpliTaq Gold enzyme in the TaqMan Master Mix. Temperatures are kept above 55°C to prevent amplicon degradation.

Controls

DNA extracted form *S. pneumoniae* ATCC 49619 was used as a positive control.

Sterile distilled water was added in place of DNA as a negative control.

***RNAse P* control**

60 samples were randomly selected for *RNAse P* real-time PCR detection, to detect the presence of PCR inhibitors.

V. Standard Curve

Serial dilutions of a spectrophotometrically confirmed quantity of the positive control DNA extracts were made. These were assayed to construct a standard curve of cycle threshold (C_T) vs. bacterial load (DNA copies/mL), which was used to calculate the bacterial loads of *S. pneumoniae* from the C_T values obtained for the study specimens. Standard curves were done in triplicate for each run.

VI. Collection of data

All real-time PCR results, along with all other test results, were sent weekly to the Epidemiology and Surveillance Unit for entry into the centralized database at the National Institute for Communicable Diseases (NCID).

Results of the samples selected for DBS were initially recorded as positive and negative and sent to the CDC after they completed the first two real-time PCR analyses on all samples. The tests were repeated for samples that did not match results with their South African counterparts. Finally RMPRU sent the unblinded information on the samples for CDC final comparison.

Chapter 5. Materials and Methods

Chapter 5. Materials and Methods

The study was done in two phases, an experimental phase in which the protocols were tested and developed and a clinical sample phase in which the prepared methods were used.

5.1. Preparation of protocols

Before testing of clinical samples began, definitive extraction protocols were established in order to obtain the most efficient and effective method for DNA purification. Trials were first done with samples spiked at the CDC site and later run on samples prepared and sent from the University of Manchester with the optimized protocols.

I. Culturing bacteria and spiking filter cards

Ten known *Streptococcus pneumoniae* positive samples (coded: 510, 94, 502, 501, 503, 504, 86, 90, 95, and 99) suspended in skim milk were plated onto blood agar (BA) plates and left to grow in 5% CO_2 for 24 hours. A single colony was lifted from each plate with a plastic loop and used to inoculate 5.0 mL of phosphate buffered saline (PBS) to mimic the base concentration at which the bacterium may be found in blood of infected patients. 90 µL of each solution was dotted onto CDC Whatman 903 Neonatal Screening Cards and left to dry for a minimum of one hour.

II. Removing discs from filter cards

The Harris Cutting Mat™ and Harris Uni-Core™ Micro Punches were first sterilized with 10% bleach, followed by 75% ethanol, and distilled water. Clean, unused filter paper was punched out using 1.2 mm, 2.0mm, and 3.0 mm sized hole punches, then varying numbers of circular disks were removed from the dried filter cards of each sample using each micro punch and collected in 1.5 mL microcentrifuge tubes. The mat and punches were sterilized again after each sample and blank filters punched to avoid carryover between samples. The blank disks were also collected and used as negative controls and to check for contamination in the punching process.

III. Acquisition of spiked samples

A collaborator at the University of Manchester, United Kingdom spiked PBS buffer and blood with *S. pneumoniae* of known concentration derived from CFU counts. The samples were collected on both Whatman 903 Neonatal Screening Cards and Whatman QIAcard FTA Cards, inserted into multi-barrier pouches along with desiccant packets to protect against environmental effects, placed in envelopes, and shipped to the CDC for testing in intervals of several weeks.

IV. Determining lower limits of detection (LLD)

Serial dilutions of the cultured bacteria were done and plated onto blood agar plates to count the number of *S. pneumoniae* colony forming units in order to estimate the lower limits of detection for *lytA* amplification from Neonatal filter cards.

A single colony was selected from each of the plates of cultured bacteria and suspended in 5.0 mL of PBS buffer. Five serial 10-fold dilutions of these solutions were made: 100 µL of the inoculated fluid was transferred into a 1.5 mL centrifuge tube containing 900 µL PBS and the process repeated until five dilutions were prepared. 100 µL of each dilution was then plated onto BA plates to determine the number of colony forming units (CFU). The plates were incubated for 24 hours and CFUs counted. If the number of colonies was above 50, counting the colonies in a single quadrant of the plate and multiplying the resulting number by four achieved estimations. Numbers above 1000 were recorded as "full", indicating bacterial growth fully covered the surface of the plate.

A colony forming unit theoretically grows from a single bacterium, thus the number of CFUs represent the amount of bacterium found in 100 µL of each dilution factor. Thus the number of bacteria present in the original solution (i.e. one colony from the BA plate) can be determined as CFU/mL.

Dilution of colonies per filter were used to perform a standard curve in the early stages of the study and compared to the previous standard curve published by Cavalho et al. (12). Results were found to be similar i.e. C_T values were equivalent. These data were used when working with the clinical blood samples

Calculations of dilution factor

$$\frac{\text{Volume of original 5.0 mL sample}}{\text{Volume of original sample + volume of PBS buffer}} = 10^{-X}$$

Calculations of CFU/mL

$$\frac{\text{(Number of colony forming units per quadrant x 4)}}{\text{(Volume plated x dilution factor)}} = \text{CFU/mL}$$

Spiked filters

90 µL of each aliquot was also dotted onto Neonatal cards in duplicate and left to dry. Punches of 1.2 mm, 2.0 mm, and 3.0 mm were taken and extracted using the two protocols mentioned above. The purified DNA and filters were then tested by real-time PCR.

A single colony suspended in 130 µL PBS was dotted, extracted, and assayed with all other samples as a positive control.

Blank filters, sterile water, PBS buffer, and TE buffer (10mM Tris-HCl, 0.1mM EDTA, pH 8.0) filter spots were all used as negative controls.

V. Optimizing the FTA purification reagent extraction

QIAcard FTA Purification Reagent and TE buffer can be used on FTA filter cards for on-card DNA purification and direct analysis from the disks with real-time PCR. This method was explored because of the decreased time required and overall reduced cost to run extractions on samples.

The Whatman protocol for DNA extraction with the FTA purification reagent (98) recommendations require the use of 200 µL FTA purification reagent and TE buffer per wash per disk. In order to minimize time and maximize efficiency of DNA collection, several trials were done using different sized punched spiked filter disks and varying the amounts of reagents used in the washing process (**Table 4.**).

Trial	Disk size	Number of disks	FTA and TE reagents (µL)
Trial 1	1.2 mm	2	200
Trial 2	2.0 mm	1	200
Trial 3	3.0 mm	1	200
Trial 4	3.0 mm	4	400
Trial 5	3.0 mm	8	400
Trial 6	3.0 mm	12	400

Table 4. Specifications of the FTA protocol preparation trials. Size and number of filters disks were increased with minimal increases in FTA purification reagent used, attempting to maximize the number of disks used per extraction.

FTA purification protocol

FTA purification reagent was added to the 1.5 mL microcentifuge tubes containing the punched disks and vortexed for 10 seconds every minute until the 5 minute incubation time was complete. Fresh, clean pipette tips were used to remove the supernatant before more reagent was added. This was repeated three more times. TE buffer was then added to the tubes containing the disks and vortexed again for 10 seconds every minute until the 5 minute incubation time was complete. The supernatant was removed and more TE buffer added to repeat the process once more. After the final wash all remaining fluid was removed from the tube. The disks were allowed to dry within the fume hood, or immediately stored at -20 °C. If the disks were allowed to dry at room temperature, they were used for real-time PCR analysis within the same day as extraction to avoid [observed] degradation of the DNA sample.

VI. Optimizing the Qiagen QIAamp DNA Mini Kit extraction protocol

The Qiagen QIAamp DNA mini kit can be used on Neonatal filter cards[**] for DNA purification and direct analysis with real-time PCR. This method was explored because of the reduced cost of Neonatal filter papers.

The QIAamp DNA Mini Kit extraction protocol (102) for blood spots recommends the use of three 3 mm punches per spin column. In order to minimize time and maximize efficiency of DNA collection, several trials were done using varying numbers of punched spiked filter disks and adjusting the amounts of reagents used in the extraction process

[**] FTA cards were also initially run in the Mini Kit extraction trials, but results were found to be unreliable, thus was abandoned during the early stages of clinical testing.

(**Table 5.**).

Trial	Disk size	Number of disks	Pre-kit lysis solution (µL)	Buffer AL (µL)	Proteinase K (µL)	Ethanol (µL)	AE buffer (µL)
Trial 1	3.0 mm	3	100	200	20	260	100
Trial 2	3.0 mm	6	100	200	20	260	100
Trial 3	3.0 mm	9	200	400	40	520	100
Trial 4	3.0 mm	12 (2x 6)	300 (2x 150)	600 (2x300)	60 (2x 30)	780 (2x 390)	100
Trial 5	3.0 mm	16 (2x 8)	350* (2x 175)	600 (2x300)	60 (2x 30)	780 (2x 390)	100
Trial 6	3.0 mm	16 (2x 8)	400 (2x 200)	800 (2x400)	80 (2x 40)	1040 (2x 520)	100
Trial 7	3.0 mm	16 (2x 8)	350* (2x 175)	600 (2x300)	60 (2x 30)	780 (2x 390)	75

Table 5. Specifications of the Mini Kit protocol preparation trials. Numbers of disks were increased in order to maximize the total DNA retrieved form each extraction with minimal increase in protocol reagents.

For trials using more than 9 FTA filter punches, the disks were divided into two separate microcentrifuge tubes for the first four reagent additions. The Qiagen extraction protocol (102) states that increasing the amount of sample does not constitute an increase in the amount of AW1 and AW2 wash buffer used, thus 500 µL of both was used in all trials. It also states that volume of AE buffer need not change with quantity of sample, though in the final trial increasing the DNA concentration through decreasing the volume of AE buffer was tested.

* Enough pre-kit lysis solution was added to submerge all disks.

Note: Sample contamination

During initial stages of this study there were several instances of contamination. Extractions were done on all reagents used as well as directly added to the PCR mix to check for the source of the contamination. When the reagents were tested alone, no contamination occurred. When spiked samples were tested alongside the reagents contamination occurred in all reagents. The source of the contamination was found to be the ventilation within the hood, thus for all work done inside the hood, the shield remained down but the ventilation was turned off.

5.2. Clinical Samples

Flow chart of methods

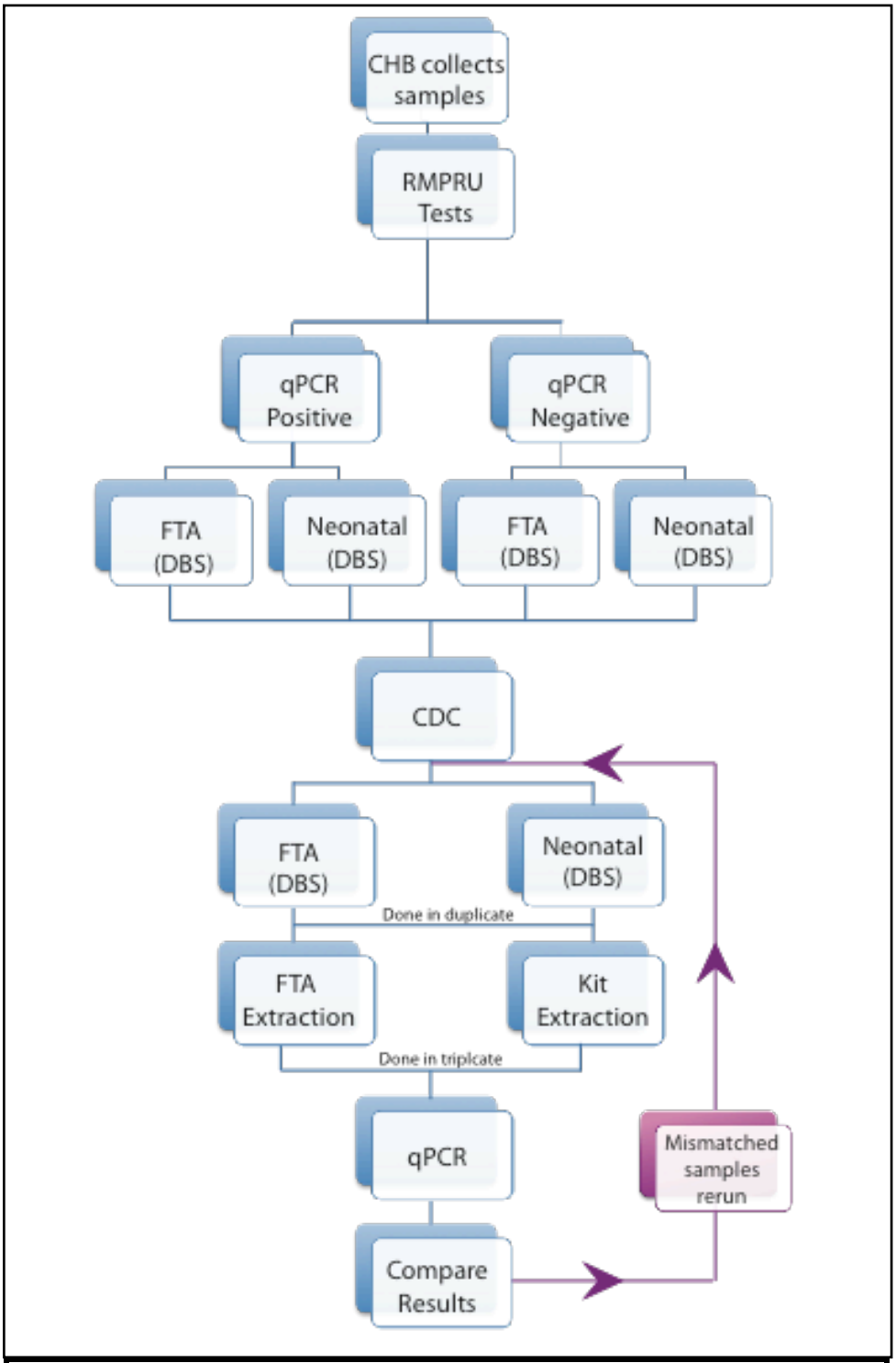

Figure 5. Flow chart of methods showing the blood specimens progression throughout this study.
*CHB: Chris Hani Baragwanath Hospital; DBS: Dried Blood Spots; CDC: Centers for Disease Control and Prevention; qPCR: Quantitative PCR (real-time PCR)

I. Acquisition and storage of samples

After arrival at the CDC, the samples were cataloged and placed back into the multi-barrier pouches and packaging envelopes. Samples were kept in the lab at ambient temperature until needed for testing; no further storage parameters were required.

5.3. DNA extraction

All Neonatal filter samples were extracted twice (once for each DBS) using the optimized Qiagen DNA Mini Kit protocol and all on-card DNA FTA filter purifications were done twice using the optimized FTA Purification Reagent protocol.

I. FTA purification reagent protocol

Six 3.0 mm disks were punched and expelled into a 1.5 mL microcentrifuge tube. Three washes with 400 µL FTA purification reagent was done, each followed by 10 seconds vortexing every minute for 5 minutes and removal of the supernatant. During the final FTA wash, if the filter still had a significant amount of color, it was continuously vortexed until only slight pink could be seen. This was followed by two washes with 400 µL TE buffer, each followed by 10 seconds vortexing every minute for 5 minutes and removal of the supernatant. After removing all the remaining liquid from the centrifuge tube, the disks were placed into -20 °C storage until needed for real-time PCR.

Blank filter disks, taken between DBS samples used as negative controls and *Streptococcus pneumoniae* spiked filters used as positive controls were included in the

FTA extraction process and subsequent assays.

II. QIAGEN DNA mini kit protocol

An adaptation of the modified QIAamp DNA Mini Kit protocol (102, 97) was used to do 10 DBS specimen and 3 control extractions per set. Approximately 4600 µL pre-kit lysis buffer was freshly prepared before each set by suspending 112.5 µL of 75U/mL mutanolysin and 0.180 g of 0.04g/mL lysozyme in 4500 µL TE buffer. The 16 filter disks from each sample were divided into two centrifuge tubes. 175 µL of the pre-kit lysis buffer was added to each tube, vortexed briefly to ensure all disks were wet and submerged, and placed into 37 °C heating blocks for 1 hour.

After initial incubation 30 µL proteinase K and 300 µL Buffer AL was added per tube, vortexed, and incubated a in a heating block again for 30 minutes at 56 °C. 390µL ethanol was then added to each tube and pulse-vortexed for 15 seconds before briefly being centrifuged to gather all liquid and disks to the bottom of the tube. The liquid mixture was then drawn off, 600 µL at a time, from both tubes and added to the QIAmp spin columns inside the 2 mL collection tube. Each sample was centrifuged at 8000 rpm for 1 minute three times, discarding eluate and placing the spin column into a fresh collection tube between each, to ensure all liquid was used. The remaining filters were then pooled and added to the spin column and centrifuged again at 8000 rpm for 1 minute. 500 µL AW1 wash buffer was added to the column and centrifuged at 8000 rpm for 1 minute, eluate was discarded and the column added to a new collection tube. At this point the filters appeared clean and were removed by inverting and lightly tapping the side of the clean collection tube

containing the column. 500 µL AW2 buffer was added to the column and centrifuged at 14000 rpm for 3 minutes. The collection tube was swapped out for a sterile 1.5mL microcentrifuge tube and 75µL Buffer AE added directly onto the spin column membrane. After a 5 minute incubation period at room temperature, the column was centrifuged a final time for 1 minute at 8000 rpm. The collected eluate (purified DNA) was stored at -20 °C until needed.

Water and blank filter disks taken between DBS samples used as negative controls and *Streptococcus pneumoniae* spiked filters used as positive controls were included in the Mini Kit extraction process and subsequent assays.

5.4. Quantitative real-time PCR

Single-target real-time PCR for the common pneumococcal gene, *lytA*, was done using a modified adaptation of the method previously described by Cavalho et al. (12). The assays were preformed on a Stratagene Mx3005P (Stratagene, La Jolla, CA) real-time QPCR instrument.

Oligonucleotide primer and profluorescent-labeled probe sequences used were designed using Primer Express software (Applied Biosystems, AB, Foster City, CA.) based on *lytA* and *RNAse P* amplification (**Table 6**). Both probe nucleotide sequences were modified by the addition of a 6-carboxyfluorescein (FAM) and black hole quencher 1 (BHQ1) on the 5' and 3' ends, respectively.

Primer/ Probe	Sequence
lytA-CDC	
forward primer	5'-ACGCAATCTAGCAGATGAAGCA-3'
reverse primer	5'-TCGTGCGTTTTAATTCCAGCT-3'
probe	5'-FAM -TGCCGAAAACGCTTGATACAGGGAG-BHQ1-3'
RNAse P	
forward primer	5'-CCAAGTGTGAGGGCTGAAAAG-3'
reverse primer	5'-TGTTGTGGCTGATGAACTATAAAAGG-3'
probe	5'-FAM-CCCCAGTCTCTGTCAGCACTCCCTTC-BHQ1-3'

Table 6. Oligonucleotide sequences of the *lytA* and *RNAse P* primers and probes. Sequences were designed for the amplification of the *S. pneumoniae* autolysin gene and the Ribonuclease P RNA gene. Both probe sequences were modified by the addition of a 5' 6-carboxyfluorescein (FAM) fluorophore and a 3' black hole quencher 1 (BHQ1) for real-time PCR fluorescence detection.

The PCR reaction mixture was set up using the TaqMan® Universal Master Mix kit (Applied Biosystems) as follows (**Table 7.**) for a 25 µL final volume:

Reagents	Working concentration	Volume (µl)	Final concentration
TaqMan Unversal master mix	2x	7.25	1x
Forward primer	10 µM	1.25	500 nM
Reverse primer	10 µM	1.25	500 nM
Probe	10 µM	0.25	100 nM
Sterile Water	-	12.5	-
Template DNA	-	2.5	-
TOTAL		25	

Table 7. CDC Universal Master Mix kit setup. Mix was set up according to modified Applied Biosystems manufacturer instructions based on a 500 nM primer and 100 nM probe reaction.

Universal Cycling conditions were set up as follows (**Table 8.**):

Stage	Temperature	Time
Stage 1*	95°C	10 minutes
Stage 2 (40 cycles)	95°C	15 seconds
	60°C	1 minute

Table 8. Parameters for CDC real-time PCR cycling conditions. A 10 minute incubation period at 95°C is needed to activate the AmpliTaq Gold enzyme in the TaqMan Master Mix. Temperatures are kept above 55°C to prevent amplicon degradation (88).

Controls

Filter papers spiked with known *lytA* positive *S. pneumoniae* samples were punched out and extracted with each set of DBS (Kit and FTA) extractions and the DNA or disks used as positive controls in each assay of that set.

Blank disks punched out between DBS sample punches were extracted alongside each set of DBS (Kit and FTA) extractions and the resulting DNA or disks, used alongside sterile water as negative controls in each assay of that set.

***RNAse P* control**

60 DBS extracted DNA samples were randomly selected, including specimens both positive and negative for *lytA*, after assays had been completed for use in *RNAse P* real-time PCR detection, to detect the presence of PCR inhibitors.

* The TaqMan Universal master mix used by the CDC does not contain the UNG enzyme found in the TaqMan gene expression master mix used by the RMPRU, thus does not require a 2 minute 50°C incubation stage.

5.6. Collection of data and statistical analysis

Sample data were compiled into lists of positive and negative samples, which were exchanged between the CDC and the RMPRU. Both sites reviewed the data and reran assays on all non-matched results. Bacterial load data were also determined by the RMPRU (**Appendix C.**) and analysis done by the CDC to estimate the sensitivity of assays above a certain DNA/mL concentration.

Percentage match was calculated by:

$$\frac{\text{Number of Matched Positive and Negative Samples}}{\text{Total Number of Samples}} \times 100 = \%\ \text{Match}$$

Chi-Square

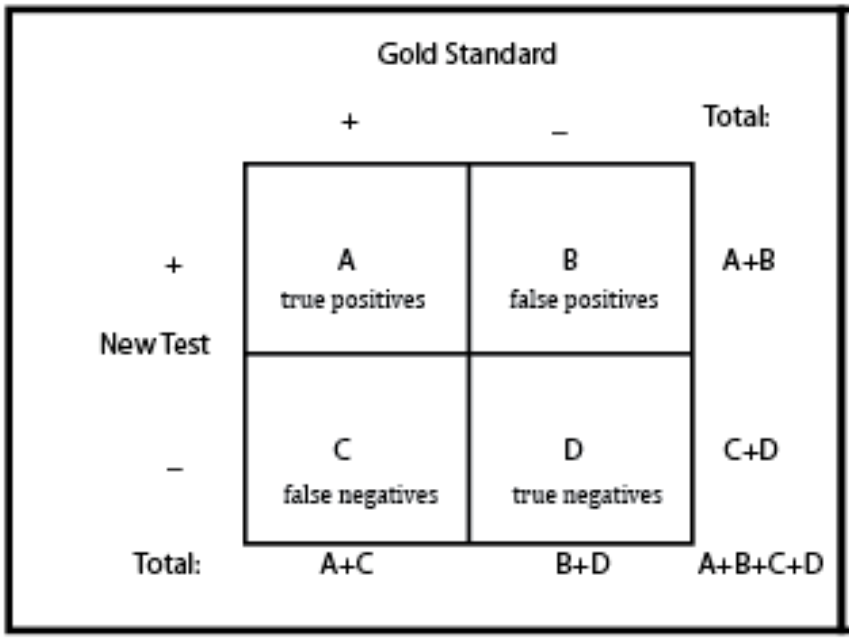

Figure 6. Chi Square.
Results that matched between the RMPRU and CDC are designated as "true"
Results that did not match are designated as "false"
"A" represents the number of true positives; (+ +)*
"B" represents the number of false positives; (- +)*
"C" represents the number of false negatives; (+ -)*
"D" represents the number of true negatives; (- -)*

*As shown in results tables for both assays below.

Chi-square tests (**Figure 6.**) using *Epi Info* version 3.5.1 and SAS® Software were used for statistical analysis at the conclusion of the study. The RMPRU whole blood *lytA*

qPCR results were considered to be the gold standard to which the CDC's new test of DBS *lytA* qPCR was evaluated. Compared proportions with a p-value < 0.05 were regarded as statistically significant.

Using the Chi-square a confidence interval was also calculated for each assay.

Sensitivity and specificity celculations

Sensitivity of the new test was calculated by:

$$\frac{\text{Number of True Positives}}{(\text{Number of False Negatives} + \text{Number of True Positives})} \times 100 = \% \text{ Sensitivity}$$

Specificity of the new test was calculated by:

$$\frac{\text{Number of True Negatives}}{(\text{Number of True Negatives} + \text{Number of False Negatives})} \times 100 = \% \text{ Specificity}$$

Chapter 6. Results

Chapter 6. Results

6.1. Preparation of protocols

Results from the first phase of this study were used to determine the best methods to use in the extraction process in preparation for working with clinical samples. The methods used in the "Clinical samples" section of Chapter 5 were those protocols that yielded the lowest overall cycle threshold (C_T) values for all samples.

I. Colony counts and lower limits of detection

Sample code	Number of colony forming units Dilution factor 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	CFU/mL at lowest CFU count dilution 10^(X)
510	Full	192	36	3	0	3 X 10^{-1} (4)
94	Full	900	152	68	3	3 X 10 (5)
502	Full	192	22	0	0	2.2 X 10^{-1} (3)
501	Full	212	23	0	0	2.3 X 10^{-1} (3)
503	Full	232	64	13	4	4 X 10 (5)
504	Full	900	140	60	4	4 X 10 (5)
86	Full	996	64	7	0	7 X 10^{-1} (4)
90	Full	Full	212	140	12	1.2 X 10^{1} (5)
95	Full	300	48	5	0	5 X 10^{-1} (4)
99	Full	992	92	11	2	2 X 10 (5)

Table 9. Colony counts at differing dilutions of *S. pneumoniae*. The numbers of CFUs were counted and the concentration of CFU/mL calculated at the lowest dilution factor with growth.

A plate count was done for each sample at each dilution (**Table 9.**) and real-time PCR ran on spiked filters of each dilution to determine the LLD. No amplification was detected at a dilution factor of 10^{-5} for any of the filters. Thus the lowest concentration of original sample from which *Streptococcus pneumoniae* could be detected was found for sample 502 at 2.2 X 10^2 CFU/mL of the original PBS mixture, where the 10^{-4} dilution gave a $C_T < 39.00$ when no bacteria was present on the BA plate.

All 10^{-4} dilutions of cultured bacteria spiked filters were amplified with a $C_T < 39.00$, thus the sensitivity of using Neonatal filter cards for the detection of *Streptococcus pneumoniae* was 100% for samples with ~2.2 X 10^2 CFU/mL.

6.2. Clinical dried blood spots

Comparison of both the Neonatal DBS and FTA DBS showed that Neonatal cards had an overall greater match to the RMPRU and greater sensitivity, though less specificity than FTA cards in detecting *S. pneumoniae* in the blood of infected patients (**Table 10.**).

Number of samples	**RMPRU**	**CDC Neonatal**		**CDC FTA**	
		True	**False**	**True**	**False**
Positive	**94**	**80**	**8**	**67**	**3**
Negative	**103**	**95**	**14**	**100**	**27**
Total	**197**	**175**	**22**	**167**	**30**

Table 10. Real-time PCR results from the RMPRU and the CDC. Number of RMPRU positives and negatives, compared to results of true (A result is designated as "true" if it matched with results from the RMPRU data and "false" if it did not.) and false positives and negatives from both CDC assays. The sum of true and false positives for each assay add to equal the total number of RMPRU positives, the same applies to negative results. Sensitivity calculations are done by dividing the number of true positives by the number of RMPRU positives. Specificity is calculated by number of true negatives divided by RMPRU negatives.

I. CDC *lytA* Neonatal DBS and RMPRU *lytA* whole blood real-time PCR

Table 11. Neonatal DBS matched with RMPRU results. Negative and positive results of all specimens with C_T < 39.00. "YES" indicated that results from both sites agreed. "NO" indicated there was a difference in the conclusion of the results.

Specimen number	RMPRU results	CDC Neonatal results	MATCH
A1	+	+	YES
A2	+	+	YES
A3	+	+	YES
A4	+	+	YES
A5	+	–	NO
A6	+	+	YES
A7	–	–	YES
A8	+	+	YES
A9	+	+	YES
A10	+	+	YES
A11	+	+	YES
A12	+	+	YES
A13	+	+	YES
A14	+	+	YES
A15	+	+	YES
A16	+	+	YES
A17	+	–	NO
A18	–	–	YES
A19	+	+	YES

A20	+	+	YES
A21	_	_	YES
A22	+	+	YES
A23	+	_	NO
A24	+	+	YES
A25	_	_	YES
A26	+	+	YES
A27	+	+	YES
A28	+	_	NO
A29	+	+	YES
A30	_	_	YES
A31	_	_	YES
A32	+	+	YES
A33	+	+	YES
A34	+	+	YES
A35	_	+	NO
A36	_	+	NO
A37	_	_	YES
A38	+	+	YES
A39	+	+	YES
A40	_	_	YES
A41	+	+	YES
A42	_	_	YES
A43	+	+	YES

A44	+	+	YES
A45	+	+	YES
A46	+	+	YES
A47	+	+	YES
A48	+	+	YES
A49	+	+	YES
A50	+	+	YES
A51	+	+	YES
A52	+	+	YES
A53	_	_	YES
B1	_	+	NO
B2	_	_	YES
B3	_	_	YES
B4	_	_	YES
B5	_	_	YES
B6	_	_	YES
B7	_	_	YES
B8	_	_	YES
B9	_	_	YES
B10	_	_	YES
B11	_	_	YES
B12	_	_	YES
B13	_	_	YES

B14	_	_	YES
B15	_	_	YES
B16	_	_	YES
B17	_	_	YES
B18	_	_	YES
B19	_	_	YES
B20	_	_	YES
B21	_	_	YES
B22	_	_	YES
B23	_	_	YES
B24	_	_	YES
B25	_	_	YES
B26	_	_	YES
B27	_	_	YES
B28	_	_	YES
B29	_	_	YES
B30	_	_	YES
B31	_	_	YES
B32	_	_	YES
B33	_	_	YES
B34	_	_	YES
B35	_	_	YES
B36	_	_	YES
B37	_	_	YES

B38	_	_	YES
B39	_	_	YES
B51	_	_	YES
B52	_	_	YES
B53	_	_	YES
B54	_	+	NO
C1	+	+	YES
C2	+	+	YES
C3	+	+	YES
C4	+	+	YES
C5	+	+	YES
C6	+	+	YES
C7	+	+	YES
C8	+	+	YES
C9	+	+	YES
C10	+	+	YES
C11	+	+	YES
C12	+	+	YES
C13	+	_	NO
C14	+	+	YES
C15	+	+	YES
C16	+	+	YES
C17	+	+	YES

C18	+	_	NO
C19	+	+	YES
C20	+	+	YES
C21	+	_	NO
C22	+	+	YES
C23	+	+	YES
C24	+	+	YES
C25	+	+	YES
C26	+	+	YES
C27	+	+	YES
C28	+	+	YES
C29	+	+	YES
C30	+	+	YES
C31	+	+	YES
C32	+	+	YES
C33	+	+	YES
C34	+	+	YES
C35	+	_	NO
C36	+	_	NO
C37	+	+	YES
C38	+	_	NO
C39	+	_	NO
C40	+	_	NO
C41	+	+	YES

C42	+	_	NO
C43	+	+	YES
C44	+	_	NO
C45	+	+	YES
C46	+	+	YES
C47	+	+	YES
C48	+	+	YES
C49	+	+	YES
C50	+	+	YES
C51	+	+	YES
C52	+	+	YES
C53	+	+	YES
D1	_	_	YES
D2	_	_	YES
D3	_	_	YES
D4	_	_	YES
D5	_	_	YES
D6	_	_	YES
D7	_	_	YES
D8	_	_	YES
D9	_	_	YES
D10	_	_	YES
D11	_	_	YES

D12	_	_	YES
D13	_	_	YES
D14	_	_	YES
D15	_	_	YES
D16	_	_	YES
D17	_	_	YES
D18	_	_	YES
D19	_	_	YES
D20	_	_	YES
D21	_	_	YES
D22	_	_	YES
D23	_	_	YES
D24	_	_	YES
D25	_	_	YES
D26	_	_	YES
D27	_	_	YES
D28	_	_	YES
D29	_	_	YES
D30	_	_	YES
D31	_	_	YES
D32	_	_	YES
D33	_	_	YES
D34	_	_	YES
D35	_	_	YES

D36	_	_	YES
D37	_	_	YES
D38	_	_	YES
D39	_	_	YES
D51	_	+	NO
D52	_	+	NO
D53	_	_	YES
D54	_	+	NO
D55	_	+	NO
D56	_	_	YES
D57	_	_	YES
D58	_	_	YES
D59	_	_	YES

A. Statistical analyses

There was an 89% match between the RMPRU whole blood and the CDC Neonatal DBS true positive and true negative results (**Table 11.**) for all samples. Chi-square analysis (**Figure 7.**) showed the proportion of matched results between the two assays was statistically significant ($\chi^2 = 118.9, \mathrm{DF} = 1, P < 0.001$).

The sensitivity of the Neonatal DBS real-time PCR analysis for detecting true positive results for *S. pneumoniae* was calculated to be 85 % (with a 95% confidence interval (CI) that the results fall between 78% and 92%) by comparing the number of true positives detected by the CDC assay to the total number of positives obtained by real-

time PCR (80/94).

The specificity of the CDC Neonatal DBS assay for detecting true negative results was calculated to be 92% (with a 95% confidence interval that the results fall between 87% and 97%) by comparing the number of true negatives to the total number of negatives obtained by real-time PCR (95/103).

Chi-square test

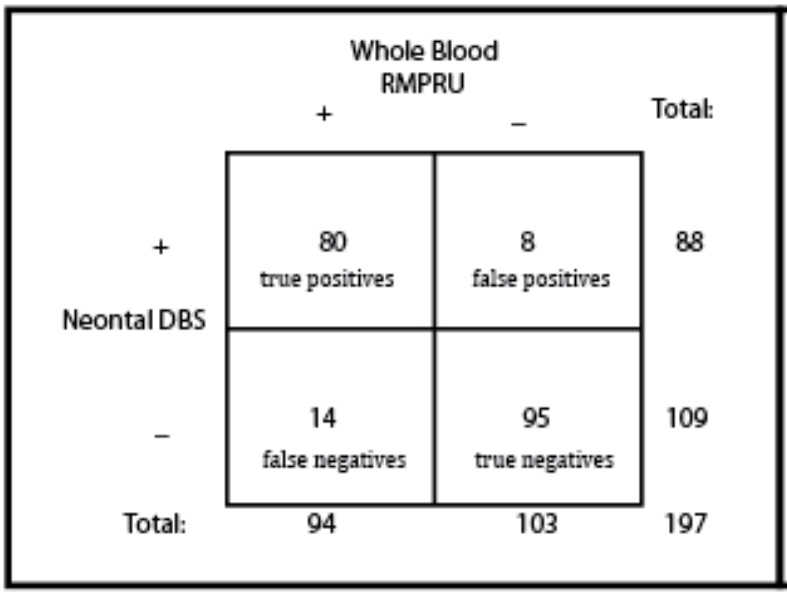

Figure 7. Chi-square results from Neonatal DBS comparison with RMPRU results. Whole blood RMPRU real-time PCR results were considered to be the "gold standard to which the CDC Neonatal DBS real-time PCR was compared. Values calculated by software: ($\chi^2 = 118.9$, DF = 1, $P < 0.001$).

Compared proportions were found to be statistically significant.

B. Bacterial load

Bacteremic patients who die from their infections and those who are HIV infected have higher *Streptococcus pneumoniae* bacterial loads (DNA copies/ mL) than those who do not die or are HIV uninfected (RMPRU, unpublished data). The CDC Neonatal DBS had 100% sensitivity above a bacterial load of 8700 copies/ mL.

6.3. Secondary comparisons of results with FTA DBS

I. CDC *lytA* FTA DBS and CDC *lytA* Neonatal DBS qPCR

Results were compared within the CDC analyses of FTA DBS and Neonatal DBS (**Appendix D.I.)** in their ability to detect the *lytA* gene amplification in the real-time PCR process. There was a 93% match between the assays, the difference being solely in the reduction in ability of the FTA cards to yield positive results.

II. CDC *lytA* FTA DBS and RMPRU *lytA* whole blood qPCR

Table 12. FTA DBS matched with RMPRU results. Negative and positive results of all specimens with C_T < 39.00. "YES" indicated that results from both sites agreed. "NO" indicated there was a difference in the conclusion of the results.

Specimen number	RMPRU lytA result	FTA Protocol	lytA MATCH
A1	+	_	NO
A2	+	+	YES
A3	+	_	NO
A4	+	+	YES
A5	+	_	NO
A6	+	+	YES
A7	_	_	YES
A8	+	+	YES
A9	+	+	YES

A10	+	+	YES
A11	+	+	YES
A12	+	+	YES
A13	+	+	YES
A14	+	+	YES
A15	+	+	YES
A16	+	+	YES
A17	+	_	NO
A18	_	_	YES
A19	+	_	NO
A20	+	_	NO
A21	_	_	YES
A22	+	+	YES
A23	+	_	NO
A24	+	+	YES
A25	_	_	YES
A26	+	+	YES
A27	+	_	NO
A28	+	_	NO
A29	+	+	YES
A30	_	_	YES
A31	_	_	YES
A32	+	+	YES
A33	+	+	YES

A34	+	+	YES
A35	_	+	NO
A36	_	+	NO
A37	_	_	YES
A38	+	+	YES
A39	+	+	YES
A40	_	_	YES
A41	+	+	YES
A42	_	_	YES
A43	+	+	YES
A44	+	+	YES
A45	+	+	YES
A46	+	+	YES
A47	+	_	NO
A48	+	_	NO
A49	+	+	YES
A50	+	+	YES
A51	+	+	YES
A52	+	+	YES
A53	_	_	YES
B1	_	_	YES
B2	_	_	YES
B3	_	_	YES
B4	_	_	YES

B5	_	_	YES
B6	_	_	YES
B7	_	_	YES
B8	_	_	YES
B9	_	_	YES
B10	_	_	YES
B11	_	_	YES
B12	_	_	YES
B13	_	_	YES
B14	_	_	YES
B15	_	_	YES
B16	_	_	YES
B17	_	_	YES
B18	_	_	YES
B19	_	_	YES
B20	_	_	YES
B21	_	_	YES
B22	_	_	YES
B23	_	_	YES
B24	_	_	YES
B25	_	_	YES
B26	_	_	YES
B27	_	_	YES
B28	_	_	YES

B29	_	_	YES
B30	_	_	YES
B31	_	_	YES
B32	_	_	YES
B33	_	_	YES
B34	_	_	YES
B35	_	_	YES
B36	_	_	YES
B37	_	_	YES
B38	_	_	YES
B39	_	_	YES
B51	_	_	YES
B52	_	_	YES
B53	_	_	YES
B54	_	_	YES
C1	+	+	YES
C2	+	+	YES
C3	+	+	YES
C4	+	+	YES
C5	+	+	YES
C6	+	+	YES
C7	+	+	YES
C8	+	+	YES

C9	+	+	YES
C10	+	_	NO
C11	+	+	YES
C12	+	+	YES
C13	+	_	NO
C14	+	+	YES
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C16	+	+	YES
C17	+	+	YES
C18	+	_	NO
C19	+	+	YES
C20	+	+	YES
C21	+	_	NO
C22	+	+	YES
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C24	+	+	YES
C25	+	_	NO
C26	+	_	NO
C27	+	+	YES
C28	+	_	NO
C29	+	+	YES
C30	+	+	YES
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C32	+	+	YES

C33	+	+	YES
C34	+	+	YES
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C37	+	+	YES
C38	+	_	NO
C39	+	_	NO
C40	+	_	NO
C41	+	+	YES
C42	+	_	NO
C43	+	+	YES
C44	+	_	NO
C45	+	_	NO
C46	+	+	YES
C47	+	+	YES
C48	+	+	YES
C49	+	+	YES
C50	+	+	YES
C51	+	+	YES
C52	+	+	YES
C53	+	+	YES
D1	_	_	YES
D2	_	_	YES
D3	_	_	YES

D4	_	_	YES
D5	_	_	YES
D6	_	_	YES
D7	_	_	YES
D8	_	_	YES
D9	_	_	YES
D10	_	_	YES
D11	_	_	YES
D12	_	_	YES
D13	_	_	YES
D14	_	_	YES
D15	_	_	YES
D16	_	_	YES
D17	_	_	YES
D18	_	_	YES
D19	_	_	YES
D20	_	_	YES
D21	_	_	YES
D22	_	_	YES
D23	_	_	YES
D24	_	_	YES
D25	_	_	YES
D26	_	_	YES
D27	_	_	YES

D28	_	_	YES
D29	_	_	YES
D30	_	_	YES
D31	_	_	YES
D32	_	_	YES
D33	_	_	YES
D34	_	_	YES
D35	_	_	YES
D36	_	_	YES
D37	_	_	YES
D38	_	_	YES
D39	_	_	YES
D51	_	+	NO
D52	_	_	YES
D53	_	_	YES
D54	_	_	YES
D55	_	_	YES
D56	_	_	YES
D57	_	_	YES
D58	_	_	YES
D59	_	_	YES

A. Statistical analysis

Comparison of the CDC FTA DBS and the RMPRU whole blood real-time PCR results (**Table 13.**) showed an 85 % match between true positive and true negative results for all samples. Chi-square analysis (**Figure 8.)** showed the proportion of matched results between the two assays was statistically significant ($\chi^2 = 100.2, \mathrm{DF} = 1, P < 0.001$).

The sensitivity of the FTA DBS on-card real-time PCR analysis for detecting true positive results for *S. pneumoniae* was calculated to be 71 % (with a 95% confidence interval that the results fall between 62% and 80%) by comparing the number of true positives detected by the CDC assay to the total number of positives obtained by real-time PCR (67/94).

The specificity of the FTA DBS on-card assay for detecting true negative results was calculated to be 97% (with a 95% confidence interval that the results fall between 92% and 99%) by comparing the number of true negatives to the total number of negatives obtained by real-time PCR (100/103).

Chi-Square

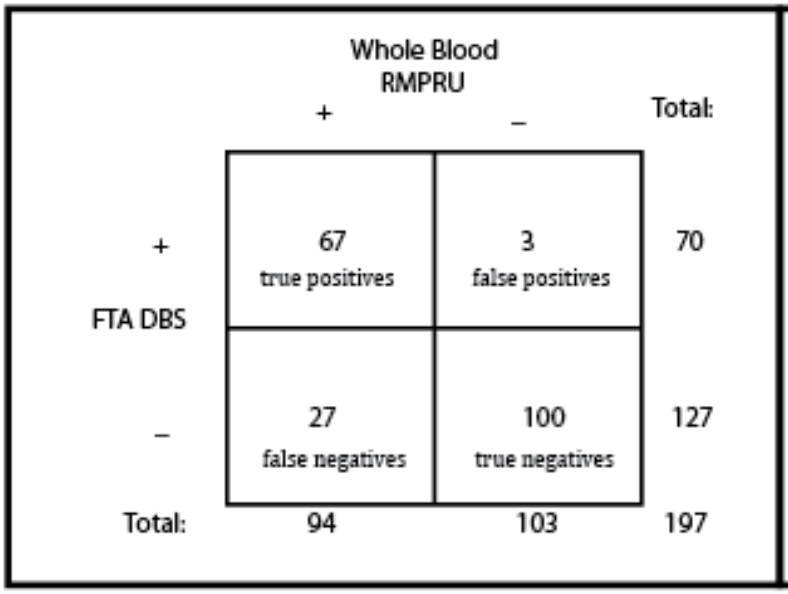

Figure 8. Chi-square results from FTA DBS comparison with RMPRU results.
Whole blood RMPRU real-time PCR results were considered to be the "gold standard to which the CDC FTA DBS real-time PCR was compared.
Values calculated by software: ($\chi^2 = 100.2$, DF = 1, $P < 0.001$).

Compared proportions were found to be statistically significant.

RNAse P controls

None of the 60 samples chosen for *RNAse P* real-time PCR analysis were negative, thus it was assumed that none of the bloods contained any PCR inhibitors.

Chapter 7. Discussion

Chapter 7. Discussion

Real-time PCR is already being evaluated as a potential replacement for the current "gold standard" in identifying pneumococcal disease (12, 27). This molecular technique has shown to be significantly more accurate than blood or sputum cultures in the detection of *Streptococcus pneumoniae* in bacteremic patients (72, 73). But a considerable barrier to achieving an etiological diagnosis of pneumococcal pneumonia is the lack of resources and facilities to implement this assay in rural areas where this disease is most common. This study proposed to test blood collected from patients with severe acute respiratory infections that was collected transported and stored as dried blood spots for the presence of *S. pneumoniae*. The purpose was to evaluate the sensitivity and specificity of using real-time PCR assays to detect the presence of *S. pneumoniae* when these blood cards were used for specimen collection. Neonatal filter cards spiked with *S. pneumoniae* were used to develop, and optimize current protocols for DNA extraction from DBS.

Lowest limit of detection was assessed using the 10-fold serially diluted spiked filters to evaluate the ability of real-time PCR to detect *S. pneumoniae* and to approximate the lowest concentration detectable by DBS real-time PCR. This assay showed 100% sensitivity for spiked filters > 2.2 X 10^2 CFU/mL. Using spiked filters likely did not reflect accurately the lowest number of CFU/mL present in clinical blood samples, since samples directly from culture are shown to have a higher sensitivity than clinical samples (12), but were appropriate for general estimation of real-world behavior.

The foremost aim of this study was to compare the use of DNA extracted from

Neonatal DBS to DNA extracted from whole blood in real-time PCR. An 89 % match between the assays along with 85% sensitivity and 92% specificity was calculated. Loss of sensitivity and specificity was expected since the volume of blood used was roughly one-fourth the amount used in whole blood extraction. C_T below 39.00 was considered to be indicative of a positive result. The range of differences in C_T values for matched positive between the CDC and the RMPRU results was as $\pm$ 5, but averaged an increase of less than 1 C_T count*.

The Neonatal DBS may be useful in detection of fatally septicemic and HIV infected pneumococcal pneumonia patients, as shown by the correlation between bacterial loads >8700 DNA copies/ mL and increased sensitivity (100%) of the assay. Neonatal DBS may be useful in future research as a tool for recognizing severe forms of pneumococcal disease with high bacterial loads.

Dried blood spots taken on FTA filter paper were also tested in this study to evaluate its ability to detect *Streptococcus pneumoniae* by *lytA* amplification in clinical samples. Real-time PCR values recorded as positive (C_T <39.00) and negative from both CDC assays were compared to obtain a 93% match between the two kinds of DBS. The results from the FTA DBS assay was then compared to the RMPRU whole blood results and showed an 85% match between the assays along with a sensitivity of 76% and a specificity of 93%. Thus the FTA DBS had a reduced number of true and false positive results along with an increase in the number of true and false negative results. This is not surprising since significantly less blood is used in the FTA on-card test (approximately 3 - 5µL).

* Increase in C_T count was estimated by calculating the differences between the lowest C_T values from both research sites.

The cost of the Neonatal cards was significantly less than the FTA cards, but extracting from the FTA card was less expensive. Extracting 100 samples of Neonatal DBS cost roughly 30% more than on-card DNA purification of 100 samples. The FTA purification reagent required 1/5th the time for 20 extractions than did the Neonatal Mini Kit protocol. In theory filters from the FTA on-card real-time PCR analysis can be reused for subsequent analyses, since the DNA remains on the card while the amplification product is left in the PCR well (98), though it was not done in this study. Liquid samples are easier to handle and only require a single extraction for use, but use more disks during the extraction.

The Neonatal cards showed a significantly higher sensitivity with smaller confidence interval but lower specificity than the FTA cards in detecting pneumococcal infected blood, according to the results from the whole blood analysis. Thus Neonatal cards have more promise to be of value for the future use of DBS in rural areas for clinical testing of pneumococcal pneumonia.

A small portion {19} of the whole blood samples was cultured by CHB hospital and the results for culture sent with the RMPRU whole blood results for analysis in this study. RMPRU had 5/19 positive results for the whole blood, CDC FTA DBS was positive for whole blood DBS (not from culture) 6/19, and CDC Neonatal DBS was positive for 10/19 of the whole blood DBS specimens that were also culture positive. Thus the ability of the CDC to pick up clinical culture positives from whole blood DBS results was comparable or better than the whole blood RMPRU. Disparity may be attributed to the different extraction methods.

It is hoped that results from this study will provide insight into the use of dried

blood spots as a method of collection, storage, and sample platform in molecular testing of pneumococcal disease in regions that may not have access to proper facilities. DBS also have the possibility to be used for epidemiological studies in collecting population-based data on the burden of disease in these areas. Having simple inexpensive methods of sample collection, storage, and preservation will enhance diagnostics and provide doctors with the accurate information needed to establish the proper course for treatment; and will lead to improved ability to execute studies on the prevalence and incidence of pneumococcal pneumonia potentially saving millions of lives by making the case for introduction of pneumococcal conjugate vaccines.

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APPENDIX

Abbreviations

ARI, Acute respiratory infection

BA, Blood agar

BHQ1, black hole quencher

CDC, Centers for Disease Control and Prevention

CFU, Colony forming units

CHB, Chris Hani Baragwanath Hospital

CI, Confidence interval

CO_2, Carbon Dioxide

CSF, Cerebrospinal fluid

CT, Computer tomography

C_T, Threshold cycle

DBS, Dried Blood Spot

DF, Degrees of freedom

DNA, Deoxyribonucleic acid

dNTP, Deoxyribonucleotide triphosphate

EDTA, Ethylenediaminetetraacetic acid

FAM, 6-carboxyfluorescein

FTA, Flinders Technology Associates

IRB, Institutional Review Board

LLD, Lower limits of detection

LytA, Autolysin, N-acetylmuramoyl-L-alanine amidase (encoded by *lytA gene*)

MGPs , magnetic glass particles

mL, Mililiter

mM, Milimolar

MurNAc-GlcNAc, *N*-acetylmuramic acid-(1-4)-*N*-acetylglucosamine

MVPDB, Meningitis and Vaccine Preventable Diseases Branch

NCID, National Institute for Communicable Diseases

NCIRD, National Center for Immunization and Respiratory Diseases

NHLS, National Health Laboratory Service

NPA, Nasopharyngeal aspirate

NPS, Nasopharyngeal swabs

P (value), Probability

PBS, Phosphate buffered saline

PCR, Polymerase Chain Reaction

qPCR, quantitative PCR

RMPRU, Respiratory and Meningeal Pathogens Research Unit

RNAse P, Ribonuclease P

SARI, severe acute respiratory infections

SAS, Statistical Analysis System

SDS, Sequence Detection System

TE buffer, Tris-HCl, EDTA

TS, Throat swab

UNG, Uracil-N-Glycosylase

WHO, World health organization

WITS, University of the Witswatersrand

μL, Microliter

c^2 ,Chi-square

Appendix B

I. Emory University

EMORY UNIVERSITY

Institutional Review Board

TO: Sanet Steyn
Principal Investigator

DATE: February 25, 2011

RE: Notification of Submission Determination: No IRB Review Required
Evaluating Blood Spots using Real-Time PCR for lyta and psaA genes [S. pneumoniae] in Diagnosing Pneumococcal Disease

The above-referenced study has been vetted by the Institutional Review Board (IRB), and it was determined that it does not require IRB review. Emory's involvement does not meet the criteria for "Engagement" in accordance with the OHRP Guidance on Engagement of Institutions in Human Subjects Research III B(7)(b).

B. Institutions are Not Engaged in Human Subjects Research if
(7) Institutions whose employees or agents:
(b) are unable to readily ascertain the identity of the subjects to whom the coded information or specimens pertain.

Please note that any changes to the protocol could conceivably alter the status of this research under the federal regulations cited above. Accordingly, any substantive changes in the protocol should be presented to the IRB for consideration prior to their implementation in the research.

Sincerely,

Carol Corkran, MPH, CIP
Senior Research Protocol Analyst
This letter has been digitally signed

Emory University
1599 Clifton Road, 5th Floor - Atlanta, Georgia 30322
Tel: 404.712.0720 - Fax: 404.727.1358 - Email: irb@emory.edu - Web: http://www.emory.edu/irb

II. Centers for Disease Control and Prevention

Based on the information provided below, the planned project titled "Evaluating Blood Spots using Real-Time PCR for lytA and psaA genes in Diagnosing pneumococcal disease" can be determined:

RESEARCH INVOLVING HUMAN PARTICIPANTS - CDC NOT ENGAGED

Conditions of non-engagement are as follows:
-The protocol be adhered to, whereby CDC will not have access to identifiable, private information for the purposes of their proposed research.
- IRB approval be maintained by the collaborator for the course of the study and must remain in compliance with human subjects protections in accordance with federal regulation for the protection of human subjects in research with documentation of renewals and amendments.

Save the determination form as documentation of the original determination.

Micah H. Milton, MPH, CIP
NCIRD Human Subjects Advisor
Associate Director for Science Office
National Center for Immunization and Respiratory Diseases| CDC |
Office: 404.639.8814| Mobile: 404.509.0759

Determination of Applicability of Human Subjects Regulations

For Any Activities/Projects When Human Information/Specimens Will Be Collected

Project Title: Evaluating Blood Spots using Real-Time PCR for lytA and psaA genes in Diagnosing pneumococcal disease

Date to Begin: 01/01/2011 **End:** 12/31/2011 ☑ **New Project** or ☐ **Changes to Existing Project**

Primary Contact: Sanet Steyn **Phone:** 639-7914

Division/Branch: DBD/MVPD **Supervisor's Name:** Edwin Ades

Below describe the nature of the activity or project, considering the intended purpose and all aspects that are planned to date. This form should be completed by the CDC scientist, project officer, or other staff responsible for the project. Attach a description of the activity or project (i.e. protocol, concept paper, précis, etc).

I. PUBLIC HEALTH NON-RESEARCH: Mark all that apply.

The activities/project is not intended to include research, but to:

☐ Identify, control or prevent disease, illness, disability, or death in response to an immediate public health threat

☐ Assess the implementation, performance, coverage, and/or satisfaction with an existing public health program, service, function, intervention or recommendation

☐ Routinely monitor indicators of the public's health and known risk factors

☐ Provide public health services, interventions, education, etc.

II. RESEARCH-NO HUMAN SUBJECTS: Mark all that apply.

The activities/project is not intended to involve human subjects. CDC will obtain:

☐ Data in the aggregate only or about groups, organizations, etc. No individual level data will be collected

☐ Data/specimens from or about deceased persons

☐ Data/specimens from animal subjects

☐ Microbiological isolates only without the ability to link to individuals' data/specimens

Data/specimens:

☐ Not collected specifically for the currently proposed research through interaction or intervention with human subjects;

☐ Never collected with individually identifiable private information about human subjects or the key or linkages to such information was removed or destroyed by the holders of the data/specimen;

☐ Individually identifiable private information was collected but the holders of the data/specimens are prohibited from releasing the identifying link due to conditions of IRB approval or non-disclosure agreement.

III. HUMAN SUBJECTS RESEARCH: The activities/project is human subjects research. However, the following is being requested with respect to review for human subjects protections:

☐ **CDC IRB Review Requested –** by completing the CDC form 1250 and other required forms along with the study materials (i.e. Protocols, consent forms, data collection forms, recruitment fliers, collaborator IRB approvals)

☐ **Reliance on a Non-CDC IRB –** to have an outside non-CDC IRB review for human subjects protections review in lieu of CDC IRB.

☐ **Exemption from IRB Review at CDC –** as we believe the study meets one of the criteria for exemption.

☑ **CDC Non-Engagement** - CDC will not be engaged. Mark all that apply.

- ☑ CDC employees (FTE or contractors) will not have contact with human research subjects;
- ☐ CDC employees will not obtain nor access any individual level data/specimens (included coded) for this study;
- ☐ CDC involvement is limited to providing assistance and guidance with technical aspects of the research such as study design, methodology, analytic plan, interpretation of results, and training.
- ☑ All collaborating institution(s) conducting human research or receiving federal funds for research will have appropriate review for human research protections and hold a valid Federal-wide Assurance (FWA).

Note: Non-engagement requests are considered on a case-by-case basis. If non-engagement status is granted then CDC scientists cannot, at any point, have access to data/specimens or research participants for the purposes of this study.

Next Page

Other Considerations: Mark all that apply.

- ☐ FDA review is required under IND, IDE, or EUA.
- ☐ Clinical, pharmacological, or therapeutic intervention will be involved.
- ☐ Involves greater than minimal risk to participants.
- ☐ Results may be of clinical relevance for individuals and/or their family members.
- ☐ Involves potentially controversial, sensitive, or high profile issues, populations or testing.
- ☑ Informed consent will be sought.
- ☐ CDC will fund the study through grant, cooperative agreement, or contract mechanisms.
- ☑ Findings will be submitted for publication in the peer reviewed literature.

Approvals and Determinations- This section to be completed by reviewers only. Clearance requirements will depend on the NC, division, and branch specific policies and procedures. Please indicate all that provided review and comment.

The proposed project has been reviewed by the following:

☑ Branch Chief Nancy Messonier

☑ Division ADS Gina Mootrey

☑ NC Human Subjects Contact Micah Milton

☐ NC ADS _____

Determination of Applicability of Human Subjects Regulations and Review Requirements

The proposed project was determined to be: Human Research

☐ **No further review required at this time.** If changes to the project/activities are considered, re-review is required before implementing the changes.

☑ **Further action and review is required. Please complete the forms and submit them division clearance:**

- ☐ HR Exemption from IRB Review - Include Form(s) 1250X _____
- ☐ HR Review by Non-CDC IRB for Reliance - Include Form(s) 1250, 1370, 1371 _____
- ☐ HR Review by CDC IRB - Include Form(s) 1250 _____
- ☑ HR Oversight of Activities Not Reviewed by CDC HRPO Expressed in IRB approved protocol pg 3
- ☐ NR Non-Disclosure Requirements _____
- ☐ Public Health Non-Research: Monitoring Human Participation in CDC Public Health Activities

Comments/Rationale:

The purpose of the research is to develop, optimize, and evaluate the use of Dried Blood Spots (DBS) as a method of collecting, transporting, storing, and testing whole blood samples of potentially pneumococcal infected individuals using real-time PCR. The Univ of Witwatersrand, South Africa will be collecting blood samples and providing CDC with de-identified samples per the protocol for the purposes of the original study. CDC will not be engaged as there will be no ability to identify participants directly or indirectly.

Tracking System ID Number: RD 2011 6019

Final Determination Made by (print name): Micah Milton

Signature: Micah Milton Digitally signed by Micah Milton DN: cn=Micah Milton, o=CDC, ou=NCIRD, email=mmilton@cdc.gov, c=US Date: 2011.01.11 16:54:17 -05'00'

Date: January 11, 2011

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III. South Africa, University of the Witwatersrand

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

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Dr Mignon du Plessis

CLEARANCE CERTIFICATE	**M10M101162**
PROJECT	Evaluating Blood spots using Real-Time PCR for lytA and psaA genes (Streptococcus pneumoniae) in Diagnosing Pneumococcal Disease
INVESTIGATORS	Dr Mignon du Plessis.
DEPARTMENT	School of Pathology
DATE CONSIDERED	26/11/2010
DECISION OF THE COMMITTEE*	Approved unconditionally
	26/11/2010

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

DATE 26/11/2010 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Dr M du Plessis

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...

C. Positive results comparison with bacterial load

Table 13. Neonatal & RMPRU results including bacterial load data. The correlation of positive and negative CDC Neonatal DBP results and RMPRU bacterial load data are shown by the arrangement below. RMPRU positive real-time PCR bacterial load data are arranged in descending order according to "YES" and "NO" matches with CDC results.

Specimen number	RMPRU results	CDC Neonatal results	MATCH	Bacterial Load
C53	+	+	YES	2134698
C48	+	+	YES	560148
C34	+	+	YES	445542
C20	+	+	YES	382774
C19	+	+	YES	287100
C50	+	+	YES	258995
A43	+	+	YES	256142
A13	+	+	YES	252865
C8	+	+	YES	200880
C5	+	+	YES	158851
C1	+	+	YES	114510
A45	+	+	YES	85703
A8	+	+	YES	84725
C6	+	+	YES	79437
A2	+	+	YES	68477
C15	+	+	YES	68245
C52	+	+	YES	59710

C11	+	+	YES	57224
A51	+	+	YES	56349
C7	+	+	YES	39624
C24	+	+	YES	38653
A39	+	+	YES	35645
A44	+	+	YES	28009
C46	+	+	YES	24970
A26	+	+	YES	23260
A22	+	+	YES	20565
C2	+	+	YES	19370
C22	+	+	YES	17988
A20	+	+	YES	16683
A9	+	+	YES	15820
C49	+	+	YES	15727
C9	+	+	YES	8053
A11	+	+	YES	7128
A33	+	+	YES	6626
C12	+	+	YES	4233
A15	+	+	YES	4119
C23	+	+	YES	3645
C41	+	+	YES	3637
C3	+	+	YES	3604
A50	+	+	YES	3212
C29	+	+	YES	3129

A14	+	+	YES	2899
A29	+	+	YES	2748
A49	+	+	YES	2385
C51	+	+	YES	1889
A1	+	+	YES	1703
A10	+	+	YES	1579
C45	+	+	YES	1513
C4	+	+	YES	1500
A32	+	+	YES	1477
C43	+	+	YES	1441
C32	+	+	YES	1363
A27	+	+	YES	1356
C17	+	+	YES	1304
A46	+	+	YES	1217
A6	+	+	YES	1083
C16	+	+	YES	1002
A3	+	+	YES	959
A52	+	+	YES	897
C27	+	+	YES	875
A47	+	+	YES	610
C30	+	+	YES	551
A24	+	+	YES	536
A12	+	+	YES	527
A48	+	+	YES	495

A41	+	+	YES		446
A34	+	+	YES		369
C26	+	+	YES		359
C33	+	+	YES		348
A16	+	+	YES		301
C37	+	+	YES		265
C31	+	+	YES		262
C14	+	+	YES		197
A4	+	+	YES		192
C25	+	+	YES		150
A19	+	+	YES		105
C47	+	+	YES		97
C28	+	+	YES		86
A38	+	+	YES		74
C10	+	+	YES		17.12
C40	+	_		NO	8653
C21	+	_		NO	2355
C38	+	_		NO	2006
C39	+	_		NO	1905
C42	+	_		NO	748
C35	+	_		NO	646
C18	+	_		NO	552
C13	+	_		NO	466
A5	+	_		NO	279

A28	+	_		NO	268
C36	+	_		NO	202
A17	+	_		NO	92
A23	+	_		NO	65
C44	+	_		NO	17.92
A35	_	+		NO	
A36	_	+		NO	
B1	_	+		NO	
B54	_	+		NO	
D51	_	+		NO	
D52	_	+		NO	
D54	_	+		NO	
D55	_	+		NO	
A7	_	_	YES		
A18	_	_	YES		
A21	_	_	YES		
A25	_	_	YES		
A30	_	_	YES		
A31	_	_	YES		
A37	_	_	YES		
A40	_	_	YES		
A42	_	_	YES		
A53	_	_	YES		
B2	_	_	YES		

B3	_	_	YES
B4	_	_	YES
B5	_	_	YES
B6	_	_	YES
B7	_	_	YES
B8	_	_	YES
B9	_	_	YES
B10	_	_	YES
B11	_	_	YES
B12	_	_	YES
B13	_	_	YES
B14	_	_	YES
B15	_	_	YES
B16	_	_	YES
B17	_	_	YES
B18	_	_	YES
B19	_	_	YES
B20	_	_	YES
B21	_	_	YES
B22	_	_	YES
B23	_	_	YES
B24	_	_	YES
B25	_	_	YES
B26	_	_	YES

B27	_	_	YES
B28	_	_	YES
B29	_	_	YES
B30	_	_	YES
B31	_	_	YES
B32	_	_	YES
B33	_	_	YES
B34	_	_	YES
B35	_	_	YES
B36	_	_	YES
B37	_	_	YES
B38	_	_	YES
B39	_	_	YES
B51	_	_	YES
B52	_	_	YES
B53	_	_	YES
D1	_	_	YES
D2	_	_	YES
D3	_	_	YES
D4	_	_	YES
D5	_	_	YES
D6	_	_	YES
D7	_	_	YES
D8	_	_	YES

D9	_	_	YES
D10	_	_	YES
D11	_	_	YES
D12	_	_	YES
D13	_	_	YES
D14	_	_	YES
D15	_	_	YES
D16	_	_	YES
D17	_	_	YES
D18	_	_	YES
D19	_	_	YES
D20	_	_	YES
D21	_	_	YES
D22	_	_	YES
D23	_	_	YES
D24	_	_	YES
D25	_	_	YES
D26	_	_	YES
D27	_	_	YES
D28	_	_	YES
D29	_	_	YES
D30	_	_	YES
D31	_	_	YES
D32	_	_	YES

D33	_	_	YES
D34	_	_	YES
D35	_	_	YES
D36	_	_	YES
D37	_	_	YES
D38	_	_	YES
D39	_	_	YES
D53	_	_	YES
D56	_	_	YES
D57	_	_	YES
D58	_	_	YES
D59	_	_	YES

D. Results tables for secondary FTA protocol testing

I. CDC neonatal and CDC FTA

Table 14. Neonatal DBS matched with FTA DBS results. Negative and positive results of all specimens with C_T < 39.00. "YES" indicated that results from both assays agreed. "NO" indicated there was a difference in the conclusion of the results.

Specimen number	CDC lytA result	FTA Protocol	lytA MATCH
A1	+	_	NO
A2	+	+	YES
A3	+	_	NO
A4	+	+	YES
A5	_	_	YES
A6	+	+	YES
A7	_	_	YES
A8	+	+	YES
A9	+	+	YES
A10	+	+	YES
A11	+	+	YES
A12	+	+	YES
A13	+	+	YES
A14	+	+	YES
A15	+	+	YES
A16	+	+	YES
A17	_	_	YES
A18	_	_	YES

A19	+	_	NO
A20	+	_	NO
A21	_	_	YES
A22	+	+	YES
A23	_	_	YES
A24	+	+	YES
A25	_	_	YES
A26	+	+	YES
A27	+	_	NO
A28	_	_	YES
A29	+	+	YES
A30	_	_	YES
A31	_	_	YES
A32	+	+	YES
A33	+	+	YES
A34	+	+	YES
A35	+	+	YES
A36	+	+	YES
A37	_	_	YES
A38	+	+	YES
A39	+	+	YES
A40	_	_	YES
A41	+	+	YES
A42	_	_	YES

A43	+	+	YES
A44	+	+	YES
A45	+	+	YES
A46	+	+	YES
A47	+	_	NO
A48	+	_	NO
A49	+	+	YES
A50	+	+	YES
A51	+	+	YES
A52	+	+	YES
A53	_	_	YES
B1	+	_	NO
B2	_	_	YES
B3	_	_	YES
B4	_	_	YES
B5	_	_	YES
B6	_	_	YES
B7	_	_	YES
B8	_	_	YES
B9	_	_	YES
B10	_	_	YES
B11	_	_	YES
B12	_	_	YES

B13	_	_	YES
B14	_	_	YES
B15	_	_	YES
B16	_	_	YES
B17	_	_	YES
B18	_	_	YES
B19	_	_	YES
B20	_	_	YES
B21	_	_	YES
B22	_	_	YES
B23	_	_	YES
B24	_	_	YES
B25	_	_	YES
B26	_	_	YES
B27	_	_	YES
B28	_	_	YES
B29	_	_	YES
B30	_	_	YES
B31	_	_	YES
B32	_	_	YES
B33	_	_	YES
B34	_	_	YES
B35	_	_	YES
B36	_	_	YES

B37	_	_	YES
B38	_	_	YES
B39	_	_	YES
B51	_	_	YES
B52	_	_	YES
B53	_	_	YES
B54	+	_	NO
C1	+	+	YES
C2	+	+	YES
C3	+	+	YES
C4	+	+	YES
C5	+	+	YES
C6	+	+	YES
C7	+	+	YES
C8	+	+	YES
C9	+	+	YES
C10	+	_	NO
C11	+	+	YES
C12	+	+	YES
C13	_	_	YES
C14	+	+	YES
C15	+	+	YES
C16	+	+	YES

C17	+	+	YES
C18	_	_	YES
C19	+	+	YES
C20	+	+	YES
C21	_	_	YES
C22	+	+	YES
C23	+	+	YES
C24	+	+	YES
C25	+	_	YES
C26	+	_	YES
C27	+	+	YES
C28	+	_	YES
C29	+	+	YES
C30	+	+	YES
C31	+	_	YES
C32	+	+	YES
C33	+	+	YES
C34	+	+	YES
C35	_	_	YES
C36	_	_	YES
C37	+	+	YES
C38	_	_	YES
C39	_	_	YES
C40	_	_	YES

C41	+	+	YES
C42	_	_	YES
C43	+	+	YES
C44	_	_	YES
C45	+	_	NO
C46	+	+	YES
C47	+	+	YES
C48	+	+	YES
C49	+	+	YES
C50	+	+	YES
C51	+	+	YES
C52	+	+	YES
C53	+	+	YES
D1	_	_	YES
D2	_	_	YES
D3	_	_	YES
D4	_	_	YES
D5	_	_	YES
D6	_	_	YES
D7	_	_	YES
D8	_	_	YES
D9	_	_	YES
D10	_	_	YES

D11	_	_	YES
D12	_	_	YES
D13	_	_	YES
D14	_	_	YES
D15	_	_	YES
D16	_	_	YES
D17	_	_	YES
D18	_	_	YES
D19	_	_	YES
D20	_	_	YES
D21	_	_	YES
D22	_	_	YES
D23	_	_	YES
D24	_	_	YES
D25	_	_	YES
D26	_	_	YES
D27	_	_	YES
D28	_	_	YES
D29	_	_	YES
D30	_	_	YES
D31	_	_	YES
D32	_	_	YES
D33	_	_	YES
D34	_	_	YES

D35	_	_	YES
D36	_	_	YES
D37	_	_	YES
D38	_	_	YES
D39	_	_	YES
D51	+	+	YES
D52	+	_	NO
D53	_	_	YES
D54	+	_	NO
D55	+	_	NO
D56	_	_	YES
D57	_	_	YES
D58	_	_	YES
D59	_	_	YES