The Pneumococcus Urinary Antigen Test Kit: Use in the laboratory for the presumptive diagnosis of pneumococcal bacteraemia

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Contents:

Declaration page:

I, Hafsah Deepa Tootla, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Abstract:

Introduction:

Culture remains the 'gold standard' for diagnosis of *Streptococcus pneumoniae* bacteraemia. Time to definitive identification using culture is 24–48 hours, and prior antibiotic therapy, the ability of *S. pneumoniae* to self-autolyse and its fastidious nature can yield no growth on culture. Novel detection methods for invasive pneumococcal disease include PCR and antigen tests. We evaluated using a urine antigen test directly on selected blood cultures with appropriate Gram stain results, immediately after signalling positive for the rapid identification of *S. pneumoniae* bacteraemia.

Method:

We collected 212 blood cultures that had signalled positive with an automated blood culture system, and then yielded gram-positive cocci in pairs/chains or cocci with uncertain morphological arrangement. The BinaxNOW *Streptococcus pneumoniae* urinary antigen test, routine culture with optochin and real time *lyt*A PCR was performed on all samples. Diagnostic accuracy analysis (sensitivity and specificity) of the antigen test and Gram stain with gram-positive cocci in pairs was each compared to culture positivity for *S. pneumoniae*, PCR positivity and the composite of culture or PCR positivity for *S. pneumoniae* as the reference standards.

Results:

S. pneumoniae (Spn) was cultured in 55 samples, gram-positive organisms other than *S. pneumoniae* (NSpn) in 140 samples and 17 samples had no growth (NG). Gram-positive cocci in pairs was predominant on Gram stain in the Spn/NG groups whilst the minority in the NSpn group.

In the Spn group, all except 1 sample which was antigen positive but PCR negative, were antigen and PCR positive. In the NSpn group, antigen and PCR was negative in 123 samples, antigen and PCR positive in 1 sample and antigen positive but PCR negative in the remaining 16 samples. In the NG group, antigen and PCR were positive in 16 samples and antigen positive but PCR negative in 1 sample.

Sensitivity of the antigen test compared to culture, PCR or the composite of culture or PCR was 100%. Specificity was 87-88% but increased to 93-96% when used in subsets with gram-positive cocci in pairs or clinical history compatible with respiratory illness or meningitis. Sensitivity and specificity of the antigen test when compared to Gram stain using gram-positive cocci in pairs (69%-75% and 81% respectively) were both higher.

Discussion and Conclusion:

Accurate and rapid diagnosis of *S. pneumoniae* bacteraemia is challenging with current diagnostic tools. Specificity of the antigen test is mostly limited by cross-reactivity with viridans streptococci, coagulase negative staphylococci and enterococcus species, but this can be overcome if Gram stain morphology and clinical history is available. Sensitivity and specificity of Gram stain alone in predicting *S. pneumoniae* bacteraemia is poor and is increased with use of the antigen test.

The antigen test is a useful adjunctive tool improving diagnosis and turnaround time of *S. pneumoniae* bacteraemia. In settings like ours, where high-level resistance, defined as minimum inhibitory concentration ≥2μg/mL to penicillin is still relatively low (~7%), rapid de-escalation to penicillin in the appropriate clinical setting would be possible with the introduction of such test and could also potentially be a suitable alternative to molecular testing for *S. pneumoniae* identification in samples with no growth on culture.

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Abbreviations:

CAP: Community-acquired pneumonia

CDC: Centre for Disease Control and Prevention

CoNS: Coagulase negative staphylococci

CSF: Cerebrospinal fluid

CT: Cycle threshold

HIV: Human Immunodeficiency Virus

ICT: Immunochromatographic antigen detection test

IPD: Invasive pneumococcal disease

MIC: Minimum inhibitory concentration

NHLS: National Health Laboratory Service

NHLSRT: National Health Laboratory Service Research Trust

NICD: National Institute for Communicable Diseases

PCR: Polymerase chain reaction

PCV: Pneumococcal conjugate vaccine

ROC: Receiver operating characteristic

SOP: Standard operating procedure

WHO: World Health Organisation

Chapter 1: Introduction and Literature review

S. pneumoniae can cause serious invasive disease resulting in meningitis, bacteraemia or infection of other normally sterile sites(1, 2). The World Health Organisation (WHO), in 2005, estimated that 1.6 million people die from invasive pneumococcal disease (IPD) annually and that children under the age of 5 and those from developing countries are most affected(3). Non-invasive manifestations are more common, less severe and include pneumonia without bacteraemia, otitis media, sinusitis and bronchitis(3).

National surveillance is central in evaluating IPD burden in countries like South Africa where multiple preventative strategies such as the introduction of pneumococcal conjugate vaccines for children and high-risk individuals, and improved access to HIV care are occurring. Accurate diagnosis of IPD is fundamental in determining true disease burden, accurate antimicrobial resistance profiles (falsely identifying other streptococcal species as *S. pneumoniae* incorrectly drives up the rates of resistance, as many of these organisms tend to be penicillin and multidrug resistant) and the efficacy of interventions to prevent IPD(2, 4).

South Africa has an active laboratory-based surveillance program for pathogens of public health importance and includes an extensive database on *S. pneumoniae* IPD(5). Isolates from microbiology laboratories nationally, in both the public and private sector, are sent to GERMS-SA, a group under the Division of Public Health, Surveillance and Response in the National Institute for Communicable Diseases (NICD), South Africa, for serotyping and characterisation(5). Most isolates are recovered from public sector laboratories that service over 80% of the country's population(6). In 2009, a 7-valent pneumococcal conjugate vaccine (PCV) was introduced into the routine infant immunization program in South Africa and was replaced with the 13- valent pneumococcal conjugate vaccine in 2011(7).

Surveillance data from GERMS-SA between 2005 and 2012 showed that overall IPD incidence rates decreased from 9.4 to 5.7 cases per 100 000 person years, with an even more substantial decrease in children less than 2 years of age where cases decreased from 54.8 to 17.0 cases per 100 000 person years (7). However, in HIV uninfected children the incidence of nonvaccine serotype IPD increased by 33%(7). Current GERMS-SA surveillance data from 2017, report that the IPD incidence

nationally was 4.32 per 100 000 person years but rates varied by province, ranging from 1.28 per 100 000 person years in Limpopo to 10.37 per 100 000 person years in the Western Cape(8). The above is consistent with data from other countries demonstrating a reduction in the number of cases and deaths with the introduction of PCV, however despite this reduction, IPD is still a cause of significant morbidity and mortality(9). In South Africa, the majority of IPD episodes in 2017 were from blood cultures (61%) and cerebrospinal fluid (32%). Samples from other sterile sites accounted for the remaining 7%(8).

For decades, diagnosis of IPD has relied on culture and this has been the reference method in routine diagnostic laboratories. Culture is limited by many factors including prior antibiotic administration to sample collection, autolysin production by the organism which causes the organism to self-autolyse and die and the fastidious nature of *S. pneumoniae* imposing requirements for enriched media and specialised incubation conditions(10-13). Additional factors not specific to *S. pneumoniae* like low levels of the organism in the sample, delays in transporting specimens to the laboratory, delays in sub-culturing specimens onto appropriate media and delays in incubation of media in the appropriate culture environment also affect growth and limit the utility of culture. Upon growth of the organism, *S. pneumoniae* is differentiated from other streptococci based on colony morphology, alpha haemolysis on blood agar and susceptibility to optochin (ethylhydrocupreine) which can also be problematic(10, 14, 15). One study reported that approximately 10% of their *S. pneumoniae* isolates were resistant to optochin(14). In such cases, identification was usually confirmed by performing the bile solubility test, which is based on autolysis of the organism in the presence of sodium deoxycholate (10, 14, 15). Isolates of *S. pneumoniae* that are bile insoluble have also been described but the prevalence is low (~1%)(14). Despite the numerous complexities around culture, it persists as the method of choice as no perfect alternative for diagnoses of IPD has yet been described.

Newer laboratory detection methods for *S. pneumoniae* infection include molecular methods (such as PCR) and immunochromatographic antigen detection tests (ICT)(10). PCR techniques are usually highly sensitive because they are able to detect small amounts of target nucleic acid in clinical specimens and are also able to detect non-viable organisms (10, 16, 17). PCR is also less influenced by prior antimicrobial therapy and results can be provided within a short time frame if PCR is easily

accessible(10, 13). Conversely, molecular techniques are limited by cost, availability in routine clinical laboratories and a delay in obtaining the results when the test is not easily available(10, 13, 18). Multiple gene targets (*ply*, *lyt*A, *psa*A, *sod*A, and *pbp*) for the identification of *S. pneumoniae* have been assessed with variable diagnostic accuracy parameters being reported(17). Two commonly used targets are the *ply* and *lyt*A gene(10). The WHO recommends that the *lyt*A PCR, developed by the Centre for Disease Control and Prevention (CDC) in Atlanta, be used as a non-culture-based method to identify *S. pneumoniae*(16, 17). Most of these pneumococcal gene targets used for PCR thus far demonstrate compromised specificity due to cross-reactivity with other closely related streptococcal species such as *S. mitis* and *S. oralis*(17) . A study looking at relatedness between *S. pneumoniae* and other streptococcal species by analysing the whole genome sequences of the strains tested, found that ~48% of the pneumococcal genome (998 057 base pairs) could be aligned against other streptococcal species (19). Given the genetic relatedness between *S. pneumoniae* and other members of the streptococcus mitis group, as well as its ability to acquire genetic material from other streptococcal species in its environment through transformation and recombination, it has been difficult to use a single gene in isolation to accurately discriminate *S. pneumoniae* from other streptococcal species (19, 20). Recently, the novel “Xisco” gene has been identified as a potential gene target that has demonstrated improved sensitivity and specificity for the identification of *S. pneumoniae* in silico and in vitro (100%) and may in the future supercede culture and *lyt*A PCR as the recommended diagnostic tool for IPD (21). But for now, the overall poor specificity of PCR has led researchers to look at other molecular methods such as 16s RNA analyses, PCR followed by restriction fragment length polymorphism analysis of the *lytA* gene, multilocus sequence typing analysis and whole genome sequencing for accurate identification (17). Whilst these tests may be highly discriminatory, their place in routine diagnostic laboratories especially in the developing world will not be feasible due to the complexity of testing, interpretation of results and the associated cost.

ICT is being used more commonly as a complementary test to diagnose *S. pneumoniae* infection. Advantages of ICT include being easy and rapid to perform with a short turnaround time to providing a result (~15 minutes), being culture independent, being unaffected by prior antibiotic therapy and being cheaper than molecular

methods(10, 13). The BinaxNOW *Streptococcus pneumoniae* Antigen Card test is an ICT test based on the detection of the C-polysaccharide cell wall protein which is common to all *S. pneumoniae* serotypes, by using a sandwich assay on a nitrocellulose strip(12, 18, 22, 23). It has been validated for use on urine samples to assist in the diagnosis of community-acquired pneumonia (CAP), and for use on cerebrospinal fluid (CSF) to assist in the diagnosis of pneumococcal meningitis(11, 12, 24). A systematic review and meta-analysis concluded that the pooled sensitivity and specificity of the test when used on urine for the diagnosis of community-acquired pneumonia in adult hospitalized patients is 74% and 97% respectively(22). It's use in children for diagnosis of CAP is limited because of higher levels of *S. pneumoniae* colonisation in the nasopharynx yielding false positive results in healthy carriers (18, 25, 26). With regards to CSF, the sensitivity and specificity is higher in patients who presented with symptoms of meningitis. A 2001 study reported a sensitivity and specificity of 100%, but was limited by the small sample size and the exclusion of children.(23). A study in 2002 with a much larger sample size and including both children and adults, reported a sensitivity of 95% and a specificity of 100%(18). Although not validated for use on other samples, the BinaxNOW *Streptococcus pneumoniae* Antigen Card test has aided in the diagnosis of IPD in other sterile sites such as pleural and pericardial fluid infection(13, 27).

This led to the consideration of the BinaxNOW *Streptococcus pneumoniae* Antigen Card test as an alternative for the diagnosis of *S. pneumoniae* bacteraemia, particularly in culture-negative specimens. Petti and colleagues tested broth from blood culture bottles that had cultured *S. pneumoniae* and showed that ICT remained positive after isolates became non-viable on culture by testing blood culture broth on day 2 and day 30 after signalling positive(12). Baggett and colleagues, tested 182 blood culture negative specimens that had signalled positive and reported that the antigen test was positive in 43 (24%) specimens, potentially increasing the yield of *S. pneumoniae* diagnosis(11). However, no confirmatory tests were performed, and false positives results could not be excluded(11). In the study by Saha and colleagues, 10 culture-negative specimens that were brown or 'chocolate' coloured in appearance were tested using ICT and confirmatory *ply* PCR. *S. pneumonia*e was detected in eight of these samples with a positive ICT and positive PCR result. The remaining 2 samples were both negative on ICT and PCR(28). A further study compared the BinaxNOW

Streptococcus pneumoniae Antigen Card test with three different latex agglutination kits(29). All four tests were performed directly on blood culture broth that had flagged positive with gram-positive cocci in pairs or chains on microscopy(29) . Compared to the other kits, the BinaxNOW *Streptococcus pneumoniae* Antigen Card test had the best sensitivity (100% vs 99.6% in each of the other methods) but the worst specificity (64% vs 82 – 89%) as it cross reacted with other streptococcal species, mostly *S. mitis* and *S. oralis*, and enterococcal species(29). Most recently Moisi and colleagues compared the BinaxNOW *Streptococcus pneumoniae* antigen test and *lyt*A PCR directly on signal positive blood cultures in Mali and Thailand(30). Compared to routine culture both ICT and *lyt*A PCR had limited sensitivity (ICT 87-100% and *lyt*A PCR 90-100%) and specificity (ICT 96-100% and *lyt*A PCR 98-100%) with *lyt*A PCR outperforming ICT (30).

It is clear that there is no perfect test for the laboratory detection of *S. pneumoniae* despite numerous variations of multiple test methods becoming available. The BinaxNOW *Streptococcus pneumoniae* Antigen Card test and *lyt*A PCR have been used to diagnose *S. pneumoniae* bacteraemia directly on blood culture broth(11, 12, 28, 30). The sensitivity of both methods has been good but limitations in specificity have been described for both and has been attributed to relatedness and horizontal gene transfer between streptococcal species(19). These features are unlikely to change in the organism itself and currently it would appear, given the test methods available to the routine diagnostic laboratory, that accurate diagnosis of *S. pneumoniae* infection may sometimes be complicated and more advanced molecular tools, like whole genome sequencing, may be necessary to differentiate between *S. pneumoniae* and its closely related relatives. Despite the unavailability of the perfect test, the reality is that practical strategies are required for rapid and accurate identification of *S. pneumoniae* from clinical specimens especially in developing countries where the burden of disease is highest, and resources limited.

The focus of this study will be to determine the suitability of the BinaxNOW *Streptococcus pneumoniae* Antigen Card test for the identification of *S. pneumoniae* from blood culture samples **immediately** when Gram stain is suggestive of pneumococcus as being the causative organism of infection. In our setting, if the test is found to be accurate, this would prevent the typical time delay (between 24 – 48 hours) for identification by phenotypic methods (colony morphology, alpha haemolysis

on blood agar and susceptibility to optochin). If the organism is not viable a further delay would occur as molecular testing is not available on site and the sample would be sent to a reference laboratory, where tests are done in batches and results are not available in real time, having no impact on clinical management. We hypothesis that in these culture-negative cases, ICT testing will be a suitable alternative to molecular testing for the identification of *S. pneumoniae* bacteraemia. If true, this will be beneficial to other laboratories where access to molecular diagnostic tools is challenging. We hope to show that despite its reported limitations with specificity, a positive ICT result from blood culture with a suggestive Gram stain, in combination with a common compatible clinical manifestation of *S. pneumoniae* IPD, aids in the rapid identification of the organism with the potential to have an impact on patient care and antibiotic stewardship in settings like ours, where high level penicillin resistance, defined as a minimum inhibitory concentration (MIC) ≥2 µg/mL, is still relatively low (~ 7%)(8).

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Chapter 2: Methods

A prospective study was carried out at the National Health Laboratory Service, Groote Schuur Microbiology Laboratory, Cape Town, South Africa, between June 2017 and March 2018. The laboratory provides diagnostic microbiology services to seven hospitals, as well as to surrounding clinics, that drain an approximate population of 1.85 million people from predominantly low socio-economic backgrounds. The laboratory receives approximately 40 000 blood cultures annually and uses the BacT/ALERT automated blood culture system for bacterial culture and enrichment (bioMererieux Inc., Durham, NC, USA). These systems signal positive after detecting a change in signal fluorescence in the blood culture bottles, indicating possible growth of an organism. Aliquots from blood culture bottles which signal positive within 5 days (120 hours) of incubation are Gram stained by laboratory staff and examined with light microscopy to detect organisms present. Aliquots of blood culture broth are inoculated onto routine culture media and incubated overnight. Clinically significant blood culture Gram stain results are relayed telephonically to clinicians as soon as possible and clinical details including clinical presentation, admitting hospital and antibiotic administration are collected by microbiology pathologists-in-training as part of the routine service provided.

For this study, aliquots of blood culture broth were collected by the investigator from samples where the Gram stain morphology was suggestive of *S. pneumoniae*, i.e. gram-positive cocci in pairs or chains, as well as gram-positive cocci where it was difficult to determine whether the cocci were forming clusters, pairs or chains. BinaxNOW *Streptococcus pneumoniae* Antigen Card Test (Alere) (ICT) and PCR were performed on the aliquots as described below. Clinical information collected by microbiology pathologists-in-training was collated. Dates and times of incubation in the automated blood culture system, signal detection by the automated blood culture system and authorisation of final sample reports were collected from the BacT/ALERT automated blood culture instrument and the NHLS-LABTRAK laboratory information system respectively. Time interval from incubation to signalling positive was determined to see if there was any difference in growth patterns between samples after grouping by culture results. Time interval from incubation to authorisation was calculated and used as a proxy to determine if there were any differences in

turnaround time for identification between samples after grouping by culture results. The presence of autolysis was determined based on the colour of the blood culture broth. All samples that had an obviously chocolate or brown colour appearance were classified as having undergone autolysis and this was determined by the investigator prior to performing ICT.

Culture:

Blood culture broth was processed using the laboratory standard operating procedure (SOP), by inoculating broth onto 2% horse blood agar and 5% sheep blood agar. The sheep blood agar had a 5µg optochin disc placed on the inoculum. Both plates were incubated in 5% CO2 and checked at 24 and 48 hrs for growth of alpha haemolytic streptococci and the presence of a zone of inhibition around the optochin disk. If the zone of inhibition was ≥ 14 mm around the optochin disk, the organism was identified as *S. pneumoniae*(31). If the zone of inhibition was < 14 mm, the isolate was further identified using the Vitek 2 GP-ID card according to the manufacturer's instructions.

BinaxNOW *Streptococcus pneumoniae* Antigen Card Test (ICT):

Aliquots of blood culture broth were tested by the investigator. Samples were selected based on Gram stain result after microscopy was performed independently by laboratory staff. The investigator was blinded to the clinical details obtained and performed the antigen test on the day the blood culture flagged positive. Briefly, the test is a lateral flow assay that has an internal control indicator line and an antigen detection indicator line. The antigen test detects the C-polysaccharide cell wall protein antigen which is common to *S. pneumoniae* serotypes(12, 18, 22, 23). Swabs provided by the manufacturer were submerged into a 1ml aliquot of blood culture broth and inserted into the test card. Reagent provided by the manufacturer was added and the test card securely sealed. Results were read 15 minutes after sealing the test card (looking for visualisation of the control and antigen indicator lines) and interpreted according to the package insert(24). The manufacturer considers any visible line, including faint lines, to be reactive. Faint reactive lines were recorded, since reports of cross reactivity of the test with other streptococcal species has previously been observed (11).

DNA extraction:

DNA extraction of blood culture broth was performed using the QIAsymphony SP automated extraction instrument with the QIAsymphony Virus/Bacteria mini kit, according to the manufacturer's instructions (Qiagen, Hilden, Germany). DNA extracted from 200 µl of blood culture broth was eluted into 60 µl of elution buffer and stored at -70° C.

Real time *lyt*A PCR:

PCR assays were batched and performed using the Rotor-Gene 6000 (Corbett Research) real time analyser. Each 25µl reaction contained 5µl of extracted DNA (at a 1:10 dilution to reduce the effect of co-purified inhibitors from the blood culture broth), 0.5µl (200 nM) each of the forward primer, reverse primer and probe, 12.5µl of LightCycler® 480 Probes Master (Roche Life Science) master mix and 6µl of PCR grade water (16). A no template control and a *S. pneumoniae* positive DNA control (*S. pneumoniae* ATCC 49619) were included in every PCR run. DNA was amplified using the following cycling conditions: 95°C for 5 minutes (activation) followed by 40 cycles of 95°C for 10 seconds (denaturation) and 60°C for 1 minute (amplification and detection). Results were analysed using the instrument's software (Rotor-Gene 6000 Series Software 1.7). We used a cycle threshold (CT) cut-off value of ≤ 22 to determine positivity. This value was chosen based on review of the CT values in samples with software-detected signal (Figure 2) and receiver operating characteristic (ROC) curve analysis of these samples. For ROC curve analysis we considered *S. pneumoniae* culture positive samples as true positives, and culture positive samples with organisms other than *S. pneumoniae* as true negatives. ROC curve analysis suggested that the optimal CT cut-off value for determining a positive PCR result was between 17 and 22 (AUC 0.99), and the upper interval was selected as our CT cut-off value for positivity.

Reference Methods:

Culture was selected as a reference method as it is the current 'gold standard' for the diagnosis of pneumococcal disease. PCR was selected as an additional reference method as it is well-recognised and recommended by the CDC as a culture-independent method for diagnosing pneumococcal disease and is especially useful if samples do not yield growth on culture and Gram stain results are suggestive of pneumococcal disease(16). The combination of culture positivity for *S. pneumoniae* or

PCR positivity was chosen as a composite reference method to create an improved reference standard that would also allow for the inclusion of culture-negative samples. ICT was compared to culture positivity for *S. pneumoniae,* PCR positivity and the composite reference of culture positivity for *S. pneumoniae* or PCR positivity. Gram stain with gram-positive cocci in pairs was also compared against culture positivity for *S. pneumoniae,* PCR positivity and the composite reference of culture positivity for *S. pneumoniae* or PCR positivity. This analysis was performed to determine if, by adding ICT to Gram stain as an adjunctive test, one could increase the yield of pneumococcal disease that could be identified immediately after blood culture signals positive and Gram stain is performed, in our setting

Sample size:

An online sample size calculator and an additional manual calculation method were used to estimate the sample size needed to power the study using the following parameters: prevalence of *S. pneumoniae* from blood cultures in our laboratory with gram-positive cocci in pairs, chains or where cocci could not be distinguished from those in clusters = 20%, hypothesised sensitivity and specificity of the ICT using blood culture broth after signalling positive = 99% and 82% respectively (based on performance on blood culture broth in previous studies)(11, 12, 29, 30), desired precision = 95%, and desired confidence interval = 90%(32-34). To meet these criteria a sample size ≥ 201 was required.

Definitions:

Definitions of community or hospital-acquired infection were modifications of those suggested by Friedman *et al*.(35). A positive blood culture was classified as community-acquired if it was taken within 48 hours of admission and the patient had not been admitted to hospital in the previous 3 months. A positive blood culture was classified as hospital-acquired if it was taken after 48 hours of admission or if the patient had been admitted to hospital in the previous 3 months.

Ethics:

The study was approved by the Human Research Ethics Committee at the University of Cape Town (HREC REF Number 729/2015).

Statistical analysis:

Data was analysed using Stata version 14.2 (Stata Corp, College Station, TX) and Microsoft Excel. Summary statistics were used to describe clinical, laboratory and epidemiological characteristics. Categorical variables were compared between groups using the chi-square test. Time between events was compared between groups using the Wilcoxon rank sum test for non-parametric data. Receiving operator characteristic (ROC) curves were generated to determine the optimum real-time PCR CT cut-off value for positivity. Diagnostic accuracy (sensitivity and specificity with 95% confidence intervals) was calculated using the DIAGTEST Stata module for each, Gram stain appearance with gram-positive cocci in pairs and ICT, versus culture positivity for *S. pneumoniae*, PCR positivity or the combination of culture positivity for *S. pneumoniae* or PCR positivity as a composite reference method. (36).

Chapter 3: Results

Sample collection:

We tested 212 blood culture samples that had signalled positive with gram-positive cocci in pairs, chains, or indeterminate morphology on microscopy. Samples were obtained from 193 patients and duplicate samples from the same patient were either collected on a different day, on the same day at a different time or on the same day at the same time but from different lumens of a central venous catheter.

Clinical, laboratory and epidemiological characteristics of samples:

Based on culture results, we grouped samples into the *S. pneumoniae* group (Spn), the Non-*S. pneumoniae* group (NSpn) (growth of gram-positive organisms other than *S. pneumoniae*) and the no growth (NG) group and the characteristics of each are summarised in Table 1. Several significant differences were noted between the Spn/NG groups and the NSpn group. Most samples from the Spn and NG groups were community-acquired infections and most of these samples were referred from primary and secondary level care facilities, whereas the converse applied to the NSpn group ($P<0.001$). The most common clinical syndromes in the Spn and NG groups were respiratory illness and meningitis ($P<0.001$) whilst other illnesses predominated in the NSpn group. There was limited data available for all the groups regarding antibiotic exposure prior to culture and no conclusions could be drawn as a result. Significant differences in the proportions of samples demonstrating autolysis were noted between each of the three groups with 94% in the NG group, 56% in the Spn group, and 16% in the NSpn group demonstrating autolysis($P<0.001$). Significant differences in Gram stain morphology between all groups where found except when comparing the Spn group to the NG group. Gram-positive cocci in pairs was the predominant morphology on Gram stain for the Spn (69%) and NG groups (88%), whereas for the NSpn group only 19% of isolates had gram-positive cocci in pairs on Gram stain.

	Spn group N = 55		NSpn group N = 140		NG group N = 17		Spn vs NSpn vs NG	Spn vs NSpn	Spn vs NG
	N	**%**	**N**	**%**	**N**	**%**			
Hospital of origin									
Tertiary hospital/Groote Schuur Hospital	12	22	66	47	2	12			
Non-tertiary facility / Other	43	78	74	53	15	88			
							P=<0.001*	**P=0.001***	P=0.360
Community versus Nosocomial infection									
Community	37	67	52	37	14	82			
Nosocomial	17	31	88	63	3	18			
No data	1	2	0	0	0	0			
							P=<0.001*	**P=<0.001***	P=0.461
Clinical									
Respiratory illness	34	62	22	16	9	53			
Meningitis	7	13	4	3	2	12			
Other	2	4	86	61	1	6			
No data	12	22	28	20	5	9			
							P=<0.001*	**P=<0.001***	P=0.884
Gram Stain									
Pairs	38	69	27	19	15	88			
Chains	16	29	63	45	1	6			
Cocci	1	2	50	36	1	6			
							P<0.001*	**P<0.001***	P=0.112
Autolysis									
Yes	31	56	22	16	16	94			
No	24	44	118	84	1	6			
							P=<0.001*	**P=<0.001***	P=0.004

*** Significant differences between groups (defined as P ≤ 0.001)**

Spn: Growth of *S. pneumoniae* on culture

NSpn: Growth of an organism other than *S. pneumoniae* on culture

NG: No growth of any organism on culture

Table 1: Clinical, laboratory and epidemiological characteristics of samples after being grouped based on culture positivity for *S. pneumoniae*.

Median time intervals between incubation and signal detection of growth by the automated blood culture system and between incubation and authorisation of the Spn, NSpn and NG groups have been summarised in Table 2, for all but 3 samples from the NSpn group, where the data was not available. Median time intervals between incubation and signal detection of growth by the automated blood culture system was shorter in the Spn (10.2 hours, IQR 8.1-12.2 hours) group than in the NSpn (13.8 hours, IQR 10.7-18.7) group and this finding was significant (P<0.001). However, there was no significant difference in the time interval between incubation and authorisation between these 2 groups. Time intervals from incubation to authorisation in the Spn (69.8 hours, IQR 62.6-88.4 hours) group was shorter than in the NG (102.2 hours, IQR 76.3-212.1 hours) group and this finding was significant (P<0.001).

Table 2: Median time intervals between incubation and signal detection by the automated blood culture system, and incubation to authorisation of blood culture samples, after being grouped according to culture positivity for *S. pneumoniae*.

	Spn group N = 55			**NSpn group N = 140**			**NG group N = 17**			**Spn Vs NSpn**	**Spn Vs NG**
	Hrs	**IQR**	**Nr**	**Hrs**	**IQR**	**Nr**	**Hrs**	**IQR**	**Nr**		
Incubation to signal detection	10.2	8.1–12.2		13.8	10.7-18.7		10.2	8.8–10.8			
			55			137[#]			17	**P<0.001***	P=0.080
Incubation to authorisation	69.8	62.6–88.4		69.7	59.5–92.2		102.2	76.3–212.1			
			55			137[#]			17	P=0.486	**P<0.001***

[#] Data not available for 3 samples in the NSpn group

*** Significant differences between groups (defined as P ≤ 0.001)**

Hrs = hours, IQR = Interquartile range, Nr = Sample number

Spn: Growth of *S. pneumoniae* on culture

NSpn: Growth of an organism other than *S. pneumoniae* on culture

NG: No growth of any organism on culture

Test results and diagnostic accuracy analysis:

On Gram stain, 80/212 (38%) samples had gram-positive cocci in pairs, of which 38/80 (47%) were culture positive for *S. pneumoniae*, 27/80 (34%) were culture-positive for organisms other than *S. pneumoniae* and 15/80 (19%) were culture-negative. Gram-

positive cocci in chains were found in 80/212 (38%) samples and of these16/80 (20%) were culture positive for *S. pneumoniae*, 63/80 (79%) were culture-positive for organisms other than *S. pneumoniae* and 1/80 (1%) was culture-negative. The remaining 52/212 (24%) samples were classified as gram-positive cocci, where it was unclear on microscopy if the cocci were in clusters, pairs or chains. Of these samples, 1/52 (2%) was culture positive for *S. pneumoniae*, 50/52 (96%) were culture-positive for organisms other than *S. pneumoniae* and 1/52 (2%) was culture-negative.

S. pneumoniae was isolated from 55/212 (26%) samples. Of these *S. pneumoniae* culture-positive samples, Gram staining revealed that 38/55 (69%) had gram-positive cocci in pairs, 16/55 (29%) had gram-positive cocci in chains, and 1/55 (2%) was described as gram-positive cocci. Gram-positive organisms other than *S. pneumoniae* (non-*S. pneumoniae*) were cultured in 140/212 (66%) samples. On Gram stain of these samples, 27/140 (19%) had gram-positive cocci in pairs, 63/140 (45%) had gram-positive cocci in chains, and 50/140 (36%) had gram-positive cocci. The remaining 17/212 samples (8%) did not yield growth of any organism on culture and 15/17 (88%) had gram-positive cocci in pairs, 1/17 (6%) had gram-positive cocci in chains, and 1/17 (6%) had gram-positive cocci on Gram stain. Gram stain results classified according to culture positivity for *S. pneumoniae* can be found in Figure 1.

Pneumococcal antigen was detected using the BinaxNOW *Streptococcus pneumoniae* Antigen Card Test (ICT). ICT was positive in 89/212 (42%) samples. *S. pneumoniae* was cultured in 55/89 (62%) of these samples, and the remaining ICT positive samples either had no growth on culture (n=17) (19%) or grew gram-positive organisms other than *S. pneumoniae* (n=17) (19%). *S. pneumoniae* was not cultured from any of the 123/212 (58%) ICT negative samples, and other gram-positive organisms were cultured from all these samples. ICT results classified according to culture positivity for *S. pneumoniae* can be found in Figure 1.

Distribution of PCR CT values in signal positive isolates based on culture results has been displayed in Figure 2. PCR was classified as positive if the CT value of the detected signal in the sample was ≤ 22. PCR was classified as negative if there was no detected signal in the sample, or if the CT value of the detected signal was > 22. Using this CT value cut-off, PCR was positive in 71/212 (33%) samples, of which *S. pneumoniae* was cultured in 54/71 (76%) of these samples, gram-positive organisms other that *S. pneumoniae* in 1/71 (1%) of these samples, and 16/71 (23%) of these

samples did not yield growth of any organism on culture. There were 141/212 (67%) PCR negative samples, and these yielded gram-positive organisms other that *S. pneumoniae* on culture in 139/141 (98%) of these samples, *S. pneumoniae* in 1/141 (1%) of these samples, and 1/141 (1%) of these samples did not yield growth of any organism on culture. PCR results classified according to culture positivity for *S. pneumoniae* can be found in Figure 1.

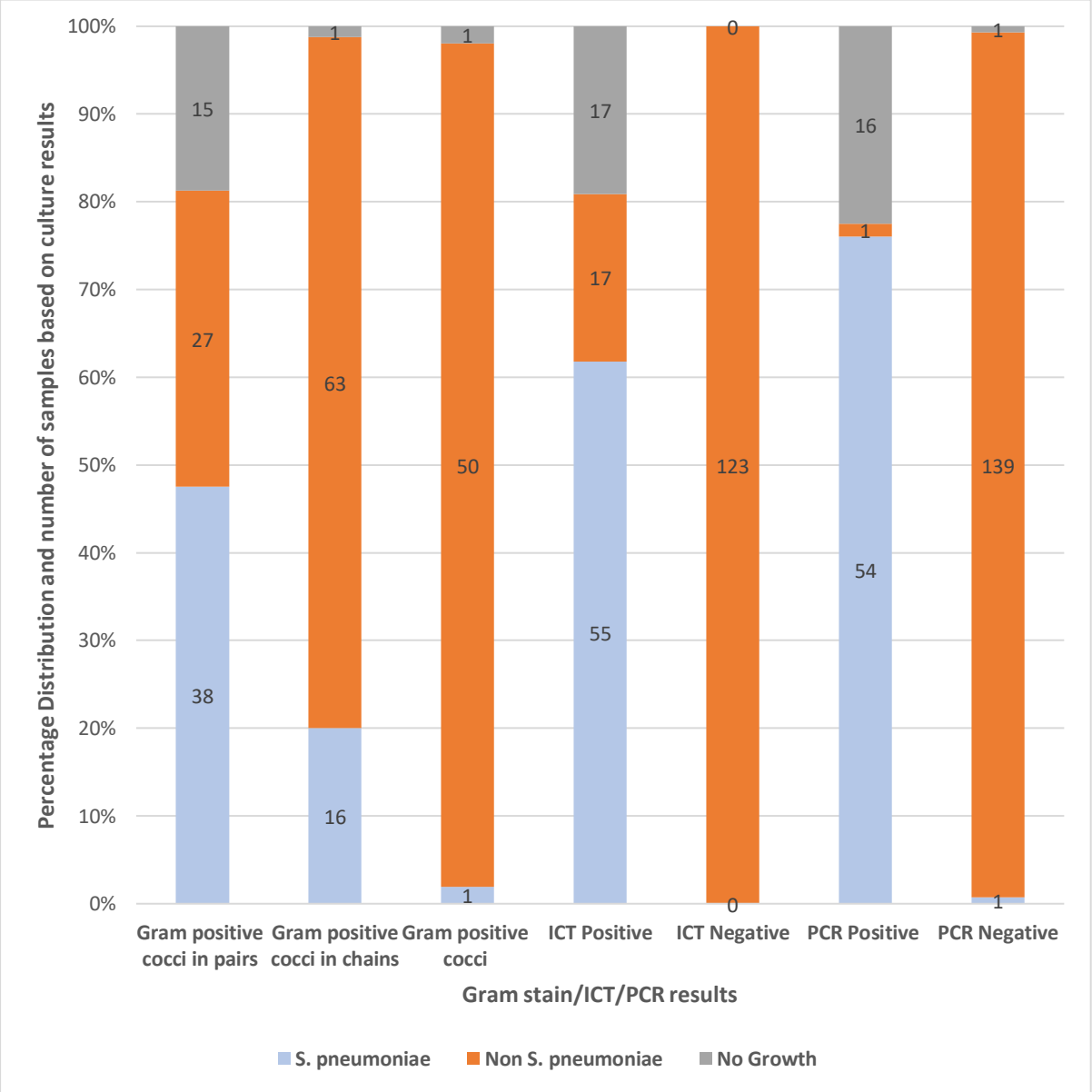

Figure 1: Percentage distribution and number of samples for Gram stain, ICT and PCR results based on culture positivity for *S. pneumoniae*.

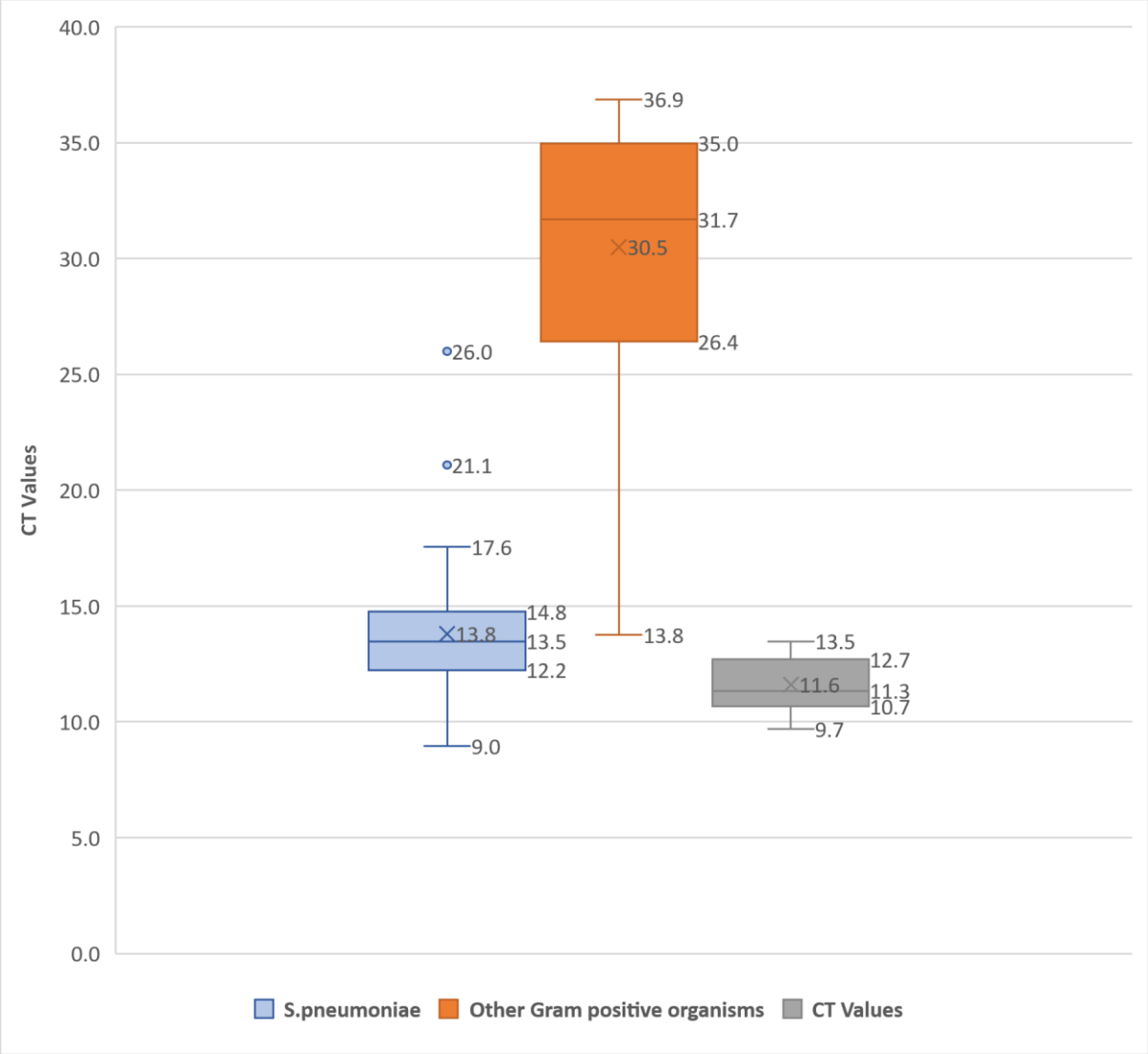

Figure 2: Distribution of PCR cycle threshold (CT) values in signal positive samples based on culture positivity for *S. pneumoniae*.

In summary, culture yielded *S. pneumoniae* in 55/212 (26%) samples, ICT was positive in 89/212 (42%) samples, and PCR was positive in 71/212 (33%) samples. Analysis of concordance between ICT and PCR with culture was performed and is summarised in Figure 3. For 54/55 (98%) *S. pneumoniae* culture-positive samples, both ICT and PCR results were positive. The 1/55 (2%) remaining *S. pneumoniae* cultured isolate was ICT positive and was detected on *lyt*A PCR but classified as PCR-negative, based on CT value = 26, above the established cut-off for our study. For the 140/212 (66%) samples which cultured gram-positive organisms other than *S.*

pneumoniae, 123/140 (88%) samples were both ICT and PCR negative, 1/123 (1%) sample was both ICT and PCR positive, and for the remaining 16/140 (11%) samples ICT was positive but PCR was negative. ICT and PCR were positive for 16/17 (94%) culture-negative samples and ICT positive but PCR negative on 1 (6%) sample. All ICT and PCR results which were discordant with culture or each other were repeated with no change in results. Discrepant results are summarised in Table 3.

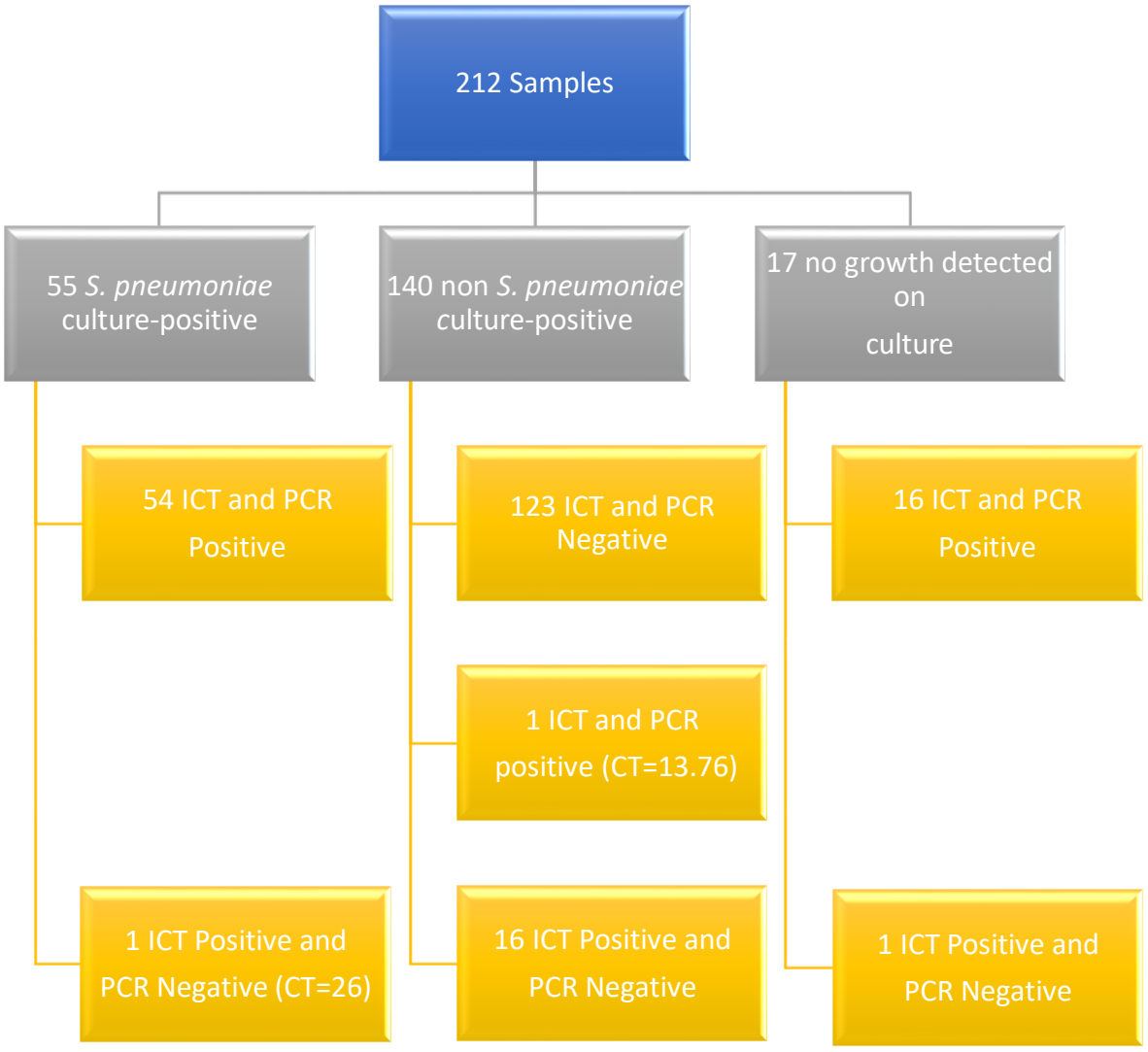

Figure 3: Concordance of ICT and PCR results with culture results

Table 3: Discordant Antigen (ICT)/PCR results when compared with culture

Culture Results	Antigen (ICT)	PCR	CT value
Culture and antigen positive for *S. pneumoniae* but PCR negative			
S. pneumoniae + coagulase negative staphylococcus	Positive	Negative	26.00
Culture negative for *S. pneumoniae* but antigen and PCR positive for *S. pneumoniae*			
Coagulase negative staphylococcus	Positive	Positive	13.76
Culture and PCR negative for *S. pneumoniae* but antigen positive			
E. faecium + coagulase negative staphylococcus	Weak positive	Negative	
S. agalactiae	Weak positive	Negative	
S. anginosus	Weak positive	Negative	
E. faecalis + coagulase negative staphylococcus	Weak positive	Negative	
Viridans streptococcus	Positive	Negative	
Viridans streptococcus*	Positive	Negative	25.04
*S. mitis**	Positive	Negative	26.49
*S. mitis**	Positive	Negative	26.32
S. mitis	Positive	Negative	25.69
S. mitis	Positive	Negative	
S. mitis + coagulase negative staphylococcus	Positive	Negative	
S. alactolyticus	Positive	Negative	
Coagulase negative staphylococcus	Positive	Negative	
Skin flora~	Positive	Negative	29.22
Skin flora~	Positive	Negative	
Skin flora~	Positive	Negative	
No growth on culture and PCR negative but antigen positive			
No growth	Positive	Negative	
*** Isolates from the same patient** **~ Skin flora were mixed viridans streptococci +/- coagulase negative staphylococcus**			

We determined the sensitivity and specificity of ICT compared to culture positivity with *S. pneumoniae*, PCR positivity and the composite of culture or PCR positivity for *S. pneumoniae* as reference methods, for all samples, as well as for subsets based on Gram stain morphology, or based on a clinical history compatible with pneumococcal disease (i.e. respiratory illness or meningitis). Results are summarised in Table 4. Analysis of samples after exclusion of duplicate samples did not alter estimates of sensitivity and specificity significantly and results can be found in Table 6 in the appendix. The sensitivity of ICT was 100% (95% CI 100 - 100%) compared to culture, PCR and the composite of culture or PCR positivity. Whilst the specificity of ICT was 87 - 88% (95% CI 83 - 92%), this increased to 93 – 96% (95% CI 92-100%) when

used in the subset of samples with gram-positive cocci in pairs on Gram stain or if the clinical history was suggestive of respiratory illness or meningitis using the same reference standards.

Table 4: Diagnostic accuracy of ICT when compared to culture positivity for *S. pneumoniae*, PCR positivity and the composite of culture or PCR positivity for *S. pneumoniae*.

	Diagnostic accuracy of ICT when compared with culture positivity for *S.pneumoniae*		Diagnostic accuracy of ICT when compared to PCR positivity		Diagnostic accuracy of ICT when compared to culture or PCR positivity for *S.pneumoniae*	
	Sensitivity (95% CI)	**Specificity (95% CI)**	**Sensitivity (95% CI)**	**Specificity (95% CI)**	**Sensitivity (95% CI)**	**Specificity (95% CI)**
All isolates (n=212)	100 (100-100%)	88 (83-92%)	100 (100-100%)	87 (83-92%)	100 (100-100%)	88 (83-92%)
Gram positive cocci in pairs (n=80)	100 (100-100%)	96 (92-100%)	100 (100-100%)	96 (92-100%)	100 (100-100%)	96 (92-100%)
Gram positive cocci in chains (n=80)	100 (100-100%)	78 (69-87%)	100 (100-100%)	77 (67-86%)	100 (100-100%)	77 (67-86%)
Gram positive cocci (n=52)	100 (100-100%)	96 (90-100%)	100 (100-100%)	96 (91-100%)	100 (100-100%)	98 (94-100%)
Clinical history compatible with pneumococcal disease (n=78)	100 (100-100%)	96 (92-100%)	100 (100-100%)	93 (92-100%)	100 (100-100%)	96 (92-100%)
Clinical history not clearly compatible with pneumococcal disease (n=134)	100 (100-100%)	86 (80-92%)	100 (100-100%)	86 (80-92%)	100 (100-100%)	86 (80-92%)

In order to assess incremental yield of ICT over usage of Gram stain morphology with gram-positive cocci in pairs to diagnose pneumococcal disease, diagnostic accuracy analysis was performed, comparing each to culture positivity for *S. pneumoniae*, PCR positivity and the composite of culture or PCR positivity for *S. pneumoniae* as the reference standards. The sensitivity and specificity of Gram stain with gram-positive cocci in pairs alone was 69 - 75% (95% CI 63-81%) and 81% (95% CI 75-86%) respectively which is too low to use as a diagnostic test for the identification of pneumococcal disease (Table 5). However, sensitivity and specificity of ICT was both

higher that Gram stain morphology of gram-positive cocci in pairs and was 100% (95% CI 100 – 100%) and 87 - 88% (95% CI 83 - 92%) respectively (Table 4) suggesting a role for its use in combination with Gram stain for the rapid identification of *S. pneumoniae* bacteraemia. These estimates of sensitivity and specificity did not change significantly after duplicate samples were excluded and these results can be found in Table 7 in the appendix. A similar increase in diagnostic accuracy yield was also identified in the subset of samples that had a clinical history of meningitis or respiratory illness.

Table 5: Diagnostic accuracy of gram-positive cocci in pairs when compared to culture positivity for *S. pneumoniae*, PCR positivity and the composite of culture or PCR positivity for *S. pneumoniae*.

	Diagnostic accuracy of gram-positive cocci in pairs compared to culture positivity for *S. pneumoniae*		Diagnostic accuracy of gram-positive cocci in pairs compared to PCR positivity		Diagnostic accuracy of gram-positive cocci in pairs compared to culture or PCR positivity for *S. pneumoniae*	
	Sensitivity (95% CI)	**Specificity (95% CI)**	**Sensitivity (95% CI)**	**Specificity (95% CI)**	**Sensitivity (95% CI)**	**Specificity (95% CI)**
All isolates (n=212)	69 (63-76%)	81 (75-86%)	75 (69-81%)	81 (76-86%)	74 (68-80%)	81 (75-86%)
Clinical history compatible with pneumococcal disease (n=78) *	66 (55-77%)	81 (71-90%)	71 (60-81%)	81 (73-90%)	69 (59-79%)	81 (72-90%)

Of the 17 (8%) samples with no growth, 15/17 (88%) had gram-positive cocci in pairs and were both ICT and PCR positive. For the remaining 2/17 (12%) samples, 1/17 (6%) was classified as Gram positive cocci and was both ICT and PCR positive, and the other isolate had Gram positive cocci in chains and was ICT positive but PCR negative.

Chapter 4: Discussion and Conclusions

S. pneumoniae is a significant cause of mortality and morbidity in both the developed and developing world and no ideal gold standard test for the diagnosis of invasive pneumococcal disease (IPD) is available. We wanted to determine if the BinaxNOW *Streptococcus pneumoniae* Antigen Card test (ICT) would enable us to rapidly identify *S. pneumoniae* from blood culture samples **immediately** when Gram stain is suggestive of pneumococcus as being the causative organism of infection, as well as being an alternative method to PCR for identification of *S. pneumoniae* in culture-negative samples.

Based on the clinical, laboratory and epidemiological information that we collected with our samples, we observed that most isolates from the *S. pneumoniae* (n=55)(Spn) / no-growth (n=17)(NG) groups had gram-positive cocci in pairs (53/72, 74%)(P<0.001), and had autolysed (47/72, 65%)(P<0.001) when compared to the Non-*S. pneumoniae* group (n=140)(NSpn), and that these differences were statistically significant. Also statistically significant was that respiratory illness and meningitis were the most common clinical syndromes in both the Spn and NG groups (P<0.001), and most of these were community acquired (P<0.001), when compared to the NSpn group. These similarities between the Spn and NG groups further add to the suggestion that culture-negative samples are most likely *S. pneumoniae* that have autolysed and would benefit from an easier more rapid presumptive identification tool of IPD such as ICT. Minimal data was available regarding antibiotic exposure prior to cultures being collected and no conclusions could be drawn from this.

ICT was positive in all samples from the Spn (n=55) and NG (n=17) groups. One isolate from the NG group was PCR negative, with no signal detected, and the rest were PCR positive. This negative PCR result was confirmed as negative, by our reference laboratory using a different *lyt*A PCR and CT cut-off value of ≤ 39.9 to determine positivity. This sample, even though it exhibited gram-positive cocci in chains was considered to be an ICT false positive result, as the patient presented with meningitis, but CSF investigations were in keeping with viral meningitis and CSF PCR was positive for enterovirus. CSF microscopy and bacterial culture were both negative. In the Spn group (n=55), only 1 sample was ICT positive but PCR negative, and this PCR negative result was based on a CT value of 26. Culture results of this sample indicated it was mixed and included growth of both *S. pneumoniae* and coagulase

negative staphylococcus (CoNS). This false negative PCR result is the result of our experimentally determined CT cut-off value, and the reduced yield of *S. pneumoniae* DNA, as indicated by the higher CT value.

The NSpn group (n=140) had 17 samples which were ICT positive. However, only 1 of these 17 samples was PCR positive, with a CT value of 13.76. Gram positive cocci were observed on Gram stain of this isolate and culture only yielded growth of CoNS. It is unlikely that both ICT and PCR were falsely positive, especially with the low PCR CT value. It is extremely likely that *S. pneumoniae* was present in the sample, but was non-viable, or that the quantity of CoNS was much higher than *S. pneumoniae* and outcompeted it on culture. The remaining 16 isolates were all PCR negative. Only 5/16 of these had signal detected on PCR with CT values that ranged between 25.04 – 29.22. It is possible that *S. pneumoniae* was present in these 5 samples but were non-viable or outcompeted by growth of the other organisms. Positive reactions with ICT, in the above 16 samples, was observed with growth of viridans streptococci, *E. faecalis*, *E. faecium,* CoNS, mixed skin flora, *S. alactolyticus* and *S. agalactiae* in our study (Table 3). The cross-reactivity of ICT with viridans streptococci, enterococcus species and *S. agalactiae* has also been observed in other studies(17, 29, 30).

The benefit of the BinaxNOW *Streptococcus pneumoniae* Antigen Card test as an adjunct for the diagnosis of *S. pneumoniae* bacteraemia, particularly in culture-negative specimens has been demonstrated by others. Petti and colleagues showed that ICT performed on blood culture broth that yielded growth of *S. pneumoniae* on culture remains positive even after these isolates became non-viable on culture, by testing blood culture broth on day 30 after signalling positive(12). Baggett and colleagues, demonstrated that ICT was positive in 43/182 (24%) signal positive blood culture samples that did not yield growth of an organism on culture, potentially increasing the diagnostic yield of *S. pneumoniae* disease, however, false positives results could not be excluded as no confirmatory tests were performed for IPD(11). In the study by Saha and colleagues, 8/10 culture-negative specimens that were brown or ‘chocolate’ coloured in appearance were ICT positive and confirmed to have IPD with *ply* PCR, increasing the diagnostic yield of IPD. Altun and colleagues compared ICT and three latex agglutination kits with culture for the diagnosis of IPD(29). They performed each test directly on blood culture broth that had flagged positive with gram-positive cocci in pairs or chains on microscopy, which is the most similar to what we

had done(29). They reported that, ICT had the best sensitivity (100% vs 99.6% in each of the other methods) but the worst specificity (64.1% vs 82.6 – 89.1%) as it cross reacted with other streptococcal and enterococcal species. Specificity in this study, was potentially underreported, as no confirmatory molecular testing was performed for IPD, and they had 33/92 (36%) samples that had growth of other gram-positive organisms that were ICT positive, and the presence of *S. pneumoniae* that had either autolysed or were outcompeted in growth on culture could not be excluded(29). Moisi and colleagues also performed ICT directly on signal positive blood cultures in Mali and Thailand, but this was done irrespective of Gram stain results. These samples had *lyt*A PCR performed but they used a CT cut-off value of < 35 to determine positivity(30). This study had country specific inclusion criteria and patient populations were different between the 2 sites. Both ICT and PCR were compared to routine culture. Sensitivity of ICT and PCR was 87-100% and 90-100% respectively. Specificity of ICT and PCR was 96-100% and 98-100% respectively with PCR outperforming ICT(30). In our study, sensitivity of ICT when compared to culture, PCR or the composite of culture or PCR positivity for *S. pneumoniae*, was 100% and is similar to what Altun and colleagues and Moisi and colleagues had reported. Specificity of ICT in our study was 87-88% when compared to culture, PCR or the composite of culture or PCR positivity for *S. pneumoniae* and was compromised mostly by cross-reactivity with viridans streptococci, CoNS and enterococcus species. Specificity of ICT increased to 93 – 96% if used in the subset of samples that had clinical histories compatible with meningitis or respiratory disease (n=78) or had gram-positive cocci in pairs on Gram stain (n=80). Specificity of ICT in our study was higher than what Altun and colleagues (64%) had reported and lower than what Moisi and colleagues (96-100%) had reported. However, the availability or the distribution of Gram stain results and clinical details of patient samples were not available in either and may have affected the performance of the test.

Pragmatically, when blood culture systems detect potential growth in a sample, they emit a signal alerting laboratory staff, and Gram stain is an easily accessible test available to laboratory staff with immediate results. The sensitivity and specificity of Gram stain with gram-positive cocci in pairs for presumptively identifying pneumococcal disease when compared to culture, PCR or the composite of culture or PCR positivity for *S. pneumoniae* was 69-75% and 80-81% respectively. With ICT,

sensitivity and specificity were both higher when compared to using Gram stain with gram-positive cocci in pairs for identifying IPD and were 100% and 87-88% respectively, even after duplicate samples were removed from analysis, clearly indicating a role for ICT as an adjunctive test in the preliminary identification of *S. pneumoniae* bacteraemia especially in a setting like ours where clinical history and Gram stain morphology is readily available.

Turnaround time is often used as a quantifiable key performance indicator in busy diagnostic laboratories to measure performance levels. The difference in turnaround time measured from incubation in the automated blood culture system to authorisation between the *S. pneumoniae* (69.8 hours) versus the no-growth group (102.2 hours) was significant (P<0.001). This time delay in providing results could potentially influence clinical management and appropriate antibiotic de-escalation to a narrower spectrum antibiotic in the appropriate clinical context. ICT could improve turnaround time for identification as this result would be available within an hour of the blood culture system signalling positive.

Although the number of culture-negative samples (n=17) were small, PCR was concordant with ICT in all but 1 sample, advocating that ICT be a potential alternative to PCR for the preliminary diagnosis of IPD, in culture-negative samples. The main advantage would be the improvement in turnaround times in settings where PCR is not readily available.

A major limiting factor in our study is the lack of an appropriate reference standard to adequately assess the diagnostic accuracy of ICT. We used culture positivity for *S. pneumonia*e, PCR positivity and the composite of culture or PCR positivity for *S. pneumoniae* as our reference methods, but both culture and PCR are flawed. In our study, PCR positivity was determined by using a CT cut off value of ≤ 22. This value was chosen based on review of the CT values in specimens with detected signal (Figure 2) and ROC curve analysis. For ROC curve analysis we considered *S. pneumoniae* culture-positive samples as true positives and culture-positive samples with organisms other than *S. pneumoniae* as true negatives. ROC curve analysis suggested that the optimal CT cut-off value to determine positivity was between 17 and 22 (AUC 0.99). These values are considerably lower than the conventional diagnostic PCR CT cut-off value (~37), but our samples were blood culture broth and hence classified as enriched media allowing for good growth of organisms and a lower

CT cut-off value was expected. The mean CT value for *S. pneumoniae* culture-positive samples was 13.8 (range 9.0 – 26.0), 30.5 (range 13.8 – 36.9) for specimens that grew organisms other than *S. pneumoniae* and 11.6 (range 9.7-13.5) for specimens that were culture-negative. Using our CT cut-off value resulted in one false negative PCR result. Additionally, in the NSpn group, there were 5 samples that were ICT positive and had signal detected on PCR with CT values that ranged between 25.04 – 29.22. It is possible that *S. pneumoniae* was present in these samples at low levels, and were truly PCR positive, but were non-viable or out-competed by growth of other organisms, resulting in the ICT being incorrectly labelled as false positive and hence affecting the specificity of the test. A further limitation of the study was to follow the ICT manufacturer's instruction that weak positive results should be reported as positive for *S. pneumoniae.* We only had 4 samples that were weak positive but all of these samples had growth of gram-positive organisms other than *S.pneumoniae* on culture and all were PCR negative with no signal detected. Whilst these numbers were small, we would recommend against reporting these weak positive results as *S. pneumoniae* invasive disease.

In conclusion, accurate diagnosis of IPD is challenging with current diagnostics. ICT is a useful adjunctive diagnostic tool that improves turnaround time in the identification of IPD but is limited by cross-reactivity with other streptococcal and enterococcal species. In a setting like ours where Gram stain morphology and clinical history is available, the test still offers added value, especially in the midst of antimicrobial resistance and a shift toward antibiotic stewardship programs targeting appropriate antibiotic use and rapid de-escalation of broad-spectrum antibiotics to narrow spectrum antibiotics, if appropriate. In our setting, ICT has the potential to impact patient care and antibiotic stewardship, as high-level penicillin resistance, defined as MIC ≥ 2µg/mL is still relatively low (~ 7%) and rapid de-escalation to penicillin is possible, in the appropriate clinical setting. ICT is potentially a suitable alternative to molecular testing for the identification of *S. pneumoniae* bacteraemia in culture-negative specimens, however due to small numbers of culture-negative samples (n=17) in our study, this should be further validated with a larger sample number.

Appendices:

Table 6: Diagnostic accuracy of ICT when compared to culture positivity for *S. pneumoniae*, PCR positivity and the composite of culture or PCR positivity for *S. pneumoniae* after exclusion of duplicate samples.

	Diagnostic accuracy of ICT when compared with culture positivity for *S.pneumoniae*		Diagnostic accuracy of ICT when compared to PCR positivity		Diagnostic accuracy of ICT when compared to culture or PCR positivity for *S.pneumonaie*	
	Sensitivity (95% CI)	**Specificity (95% CI)**	**Sensitivity (95% CI)**	**Specificity (95% CI)**	**Sensitivity (95% CI)**	**Specificity (95% CI)**
All isolates after removal of duplicate samples (n=193)	100 (100-100%)	88 (83-93%)	100 (100-100%)	87 (83-92%)	100 (100-100%)	88 (83-92%)
Gram positive cocci in pairs after removal of duplicate samples (n=76)	100 (100-100%)	96 (91-100%)	100 (100-100%)	96 (92-100%)	100 (100-100%)	96 (91-100%)
Gram positive cocci in chains after removal of duplicate samples (n=69)	100 (100-100%)	77 (67-87%)	100 (100-100%)	75 (65-86%)	100 (100-100%)	75 (65-86%)
Gram positive cocci after removal of duplicate samples (n=48)	100 (100-100%)	96 (90 -100%)	100 (100-100%)	96 (90-100%)	100 (100-100%)	98 (94-100%)
Clinical history compatible with pneumococcal disease after removal of duplicate samples (n=76)	100 (100-100%)	96 (91-100%)	100 (100-100%)	92 (86-198%)	100 (100-100%)	96 (92-100%)
Clinical history not clearly compatible with pneumococcal disease after removal of duplicate samples (n=117)	100 (100-100%)	86 (79-92%)	100 (100-100%)	86 (79-92%)	100 (100-100%)	86 (79-92%)

Table 7: Diagnostic accuracy of gram-positive cocci in pairs when compared to culture positivity for *S. pneumoniae*, PCR positivity and the composite of culture or PCR positivity for *S. pneumoniae* after exclusion of duplicate samples

	Diagnostic accuracy of gram-positive cocci in pairs compared to culture positivity for *S. pneumoniae*		Diagnostic accuracy of gram-positive cocci in pairs compared to PCR positivity		Diagnostic accuracy of gram-positive cocci in pairs compared to culture or PCR positivity for *S. pneumoniae*	
	Sensitivity (95% CI)	**Specificity (95% CI)**	**Sensitivity (95% CI)**	**Specificity (95% CI)**	**Sensitivity (95% CI)**	**Specificity (95% CI)**
All isolates after removal of duplicate samples (n=193)	69 (62-75%)	80 (74-86%)	74 (68-80%)	80 (75-86%)	73 (67-79%)	80 (75-86%)
Clinical history compatible with pneumococcal disease after removal of duplicate samples (n=76)	65 (53-77%)	80 (70-90%)	70 (60-80%)	81 (72-90%)	69 (59-79%)	80 (71-89%)