UTILITY OF THE REAL-TIME PCR (FLUIDIGM) FOR SEROTYPE-SPECIFIC CHARACTERISATION OF *STREPTOCOCCUS PNEUMONIAE* IN ADULTS HOSPITALISED WITH PNEUMONIA

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Science in Medicine in the field of Clinical Microbiology and Infectious Diseases

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DECLARATION

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

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25th day of June 2022

ABSTRACT

Streptococcus pneumoniae has been identified as the most common cause of bacterial pneumonia; however, the classical tests and sampling methods used to detect and serotype *S. pneumoniae* have limitations. The primary aim of the study was to optimise the Biomark HD real-time qPCR system (Fluidigm) to simultaneously detect and serotype *S. pneumoniae* in urine samples. A secondary objective was to evaluate the carriage prevalence of vaccine-type *S. pneumoniae* in adults hospitalised with pneumonia as diagnosed by the attending physician. Urine samples were collected as part of the "Hospital surveillance for respiratory illness*"* study from April 2018 to August 2019.

The Fluidigm assay was optimised to detect the 13 pneumococcal serotypes included in the 13-valent pneumococcal conjugate vaccine (PCV13) in urine samples spiked with known *S. pneumoniae* control strains. The commercially available BinaxNOW™ *Streptococcus pneumoniae* antigen card and pneumococcal *autolysin* gene (*LytA*) qPCR was used as reference standards for pneumococcal detection.

The efficiency of the reactions ranged from 92% to 105%. Within the linear dynamic range, the correlation coefficients (r^2) of the reactions were >0.95. Further, all assays showed a good analytical sensitivity within their respective primer and probe pairs with the limit of detection (LLD) equivalent to >1 000 copies per PCR for all reactions. The intra-assay variability (repeatability) and inter-assay variability (reproducibility) for all assays were within the acceptable range of ± 0.167 (0-0.15) standard deviation (SD) and (0.02-0.16) SD respectively. The positivity for *S. pneumoniae* detection on urine samples in pneumonia cases was 19% (18/96) using the BinaxNOW™ *Streptococcus pneumoniae* antigen card and 7% (7/96) on *LytA* qPCR. None of the urine samples tested positive on the Fluidigm assay.

In conclusion, Fluidigm was optimised successfully to detect and simultaneously serotype *S. pneumoniae* in spiked urine samples. Nevertheless, the assay failed to detect or serotype *S. pneumoniae* in clinical samples, which could be due to very low bacterial loads. In comparison, testing using the BinaxNOW™ *Streptococcus pneumoniae* antigen card indicated that 19% of the pneumonia cases were

attributable to being associated with *S. pneumoniae.* Thus, BinaxNOW, or alternative methods that rely on antigen testing remain the most effective method for detecting *S. pneumoniae* related pneumonia cases.

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ABBREVIATIONS

% - Percentage

95% CI - 95% confidence interval

ºC - Degrees Celsius

CAP - Community acquired pneumonia

CFU - Colony forming units

COPD - Chronic obstructive pulmonary disease

CSF - Cerebrospinal fluid

Cq – Quantification cycle

DNA - Deoxyribonucleic acid

e - Efficiency

HAP – Hospital-acquired pneumonia

HIC - High income countries

HIV - Human immunodeficiency virus

HREC - Human Research Ethics Committee

ICD - International Statistical Classification of Diseases

IFC - Integrated Fluidic circuits

IPD - Invasive pneumococcal disease

LMIC - Low-middle income countries

LytA - *Autolysin* gene

PCR - Polymerase chain reaction

PCV - Pneumococcal conjugate vaccine

PCV 7 - 7-valent pneumococcal conjugate vaccine

PCV 13 - 13-valent pneumococcal conjugate vaccine

Pyo - Person-year-observation

qPCR - Quantitative polymerase chain reaction

r^2 - Correlation coefficients

RSV - Respiratory syncytial virus

SD - Standard deviation

STA - Specific target amplification

TNA - Total nucleic acid

UAD - Urinary antigen detection

URT - Upper respiratory tract

USA - United States of America

Wits-VIDA - Vaccine and Infectious diseases analytics research unit

VT - Vaccine serotypes

CHAPTER 1: LITERATURE REVIEW

1.1 Pneumonia

Pneumonia is an acute infection of the lungs caused by pathogens including bacteria, viruses, parasites, and fungi (1, 2). Diagnosis of pneumonia is normally based on clinical symptoms and validated through lung consolidations observed on chest x-ray if available (3). Clinical symptoms include cough, fever, chills, fatigue, dyspnea, rigors, pleuritic chest pain, myalgia, and malaise (3). In most cases, pneumonia is easily treated with antibiotics; however, in some patients, it may progress to empyema, effusions, and death (3, 4). Pneumonia can be classified as community-acquired pneumonia (CAP) or hospital-acquired pneumonia (HAP) depending on where the infection was acquired (5). CAP develops outside of a health care facility whereas hospital-acquired pneumonia develops 48 hours after admission to a health care facility (4).

1.2 The burden of pneumonia amongst adults

Globally, the overall case-fatality risk of pneumonia amongst adults ranges between 0.1-28%; Table 1.1 (6-20). Further, higher case fatality ratios have been recorded amongst older adults, particularly those older than 65 years of age; Table 1.1 (10, 13). This variation is likely due to differences in case definitions and geographic location with the majority of adult pneumonia cases have been reported in low to middle-income countries (LMIC) including Sub-Saharan Africa where pneumonia is one of the leading causes of infection-related deaths in younger children and adults, resulting in 390 million deaths annually (21). Furthermore, influenza-like illness and pneumonia were recorded as the fourth leading causes of death accounting for 3.6% (n=15 165/420 442) of all deaths in South Africa in 2018 (22).

High human immunodeficiency virus (HIV) infection, overcrowding, air pollution, malnutrition, and prevalence of other risk factors contribute to the high incidence of pneumonia in LMIC, including Sub-Saharan Africa (23). In Sub-Saharan African adults, the burden of HIV is high, accounting for approximately 70% of the global disease burden (24). In particular, South Africa has a high burden of HIV with an estimated eight million adults living with HIV (25-27). Adults living with HIV have up to

a 25-fold higher risk of pneumonia infection, and a 6-15% higher rate of mortality as compared to their age-matched counterparts without HIV (28).

Table 1.1: Studies investigating the incidence, rate of hospitalisation, and case fatality ratio of pneumonia in people over the age of 15 years

Reference	Country	Study period	Pneumonia case definition	Incidence of pneumonia (per 1 000 person-year observation)	Incidence of pneumonia hospitalisation (per 1 000 pyo)	Case fatality rate (%)
Jones *et al,* 1998 (29)	South Africa	January to October 1996	Patients with *S. pneumoniae* isolated from blood culture	4.2	Participants were hospitalised patients	Overall=(22/129) 17%
Karstaedt *et al,* 2001(30)	South Africa	1986-1987 1996-1997	Patients with bacteraemic pneumonia	Incidence between 1986 and 1987=260 1996-1997=360	Participants were hospitalised patients	Overall case fatality (n=81/414) 19.3%
Corbett et al, 2002 (31)	South Africa	1998	Clinical, radiologic, laboratory, and, if relevant, post-mortem findings (not defined)	No incidence data was reported.	69 amongst HIV positive men and 1.9 amongst HIV negative men	Case fatality amongst HIV infected men = (n=7/82)9%
[a]Cohen *et al,* 2015 (32)	South Africa	2009-2012	(1) Sudden onset of fever (>38°C) or reported fever, (2) cough or sore throat, and (3) shortness of breath, or difficulty breathing.	[b]*Incidence in 2009=591* *2010=617* *2011=389* *2012=325.*	[b]*Incidence in 2009=591* *2010=617* *2011=389* *2012=325.*	Overall case fatality (n=514/7154) 7%.
Scott *et al*, 2000 (7)	Kenya	1994-1996	An illness of 14 days' duration or less that consisted of at least two respiratory symptoms (cough, sputum, breathlessness, chest pain, haemoptysis, or fever) with evidence of pulmonary consolidation (confirmed by a radiologist) on posterioanterior or lateral chest radiographs	No incidence data was reported.	Participants were hospitalised patients	Overall=(n=25/255) 10%
Gilks *et al,* 1996 (8)	Kenya	1989-1992	An acute episode of cough and fever accompanied by new radiographic consolidation.	Incidence of pneumonia amongst HIV seropositive women was 38.6 and the incidence amongst HIV seronegative women was 3.7.	No hospitalisation data	Case fatality amongst HIV seropositive women was (n=40/587) 6.8%
Iliyasu *et al,* 2018 (20)	Nigeria	2009-2011	Adults with features compatible with pneumonia.	No incidence data were reported.	All participants were hospitalised.	Overall case fatality (n=9/117) 7.8%.

Koulla-Shiro *et al,* 1996 (6)	Cameroon	1991-1993	A history and clinical signs consistent with an acute lower respiratory tract infection and a fresh infiltrate on their chest X-ray.	All participants had pneumonia.	All participants were hospitalised.	Overall case fatality rate of (n=7/91) 7.7%.
Heo *et al,* 2018 (10)	Korea	2011-2014	Classified based on clinical and radiological findings	*Overall*=3.1 19-49 years=1 50-69years=3.7 70-79 years=16.8 ≥80 years=48.7	No hospitalisation data reported.	*Overall*=6.2% 19-49 years=(n=1.1/100 000 pyo; 1.1%) 50-69 years=(n=4.5/100 000 pyo; 4.5%) 70-79 years=(n=8.6/100 000 pyo; 8.6%) >80 years=(n=11.4/100 000 pyo; 11.4%)
Choi *et al,* 2017 (33)	South Korea	2009-2013	ICD-10 diagnostic codes were used to define pneumonia.	*Overall*=6.3 19-49 years=1.4-2.3 50-64 years=4.8-6 65-74 years=15.6-18 ≥75 years=36.8-49.4	19-49 years= 1.1-8.1 50-64 years= 3.8-14.3 65-74 years= 15.6-29.9 >75 years= 39-73.6	No case fatality was reported.
Morimoto *et al,* 2015 (17)	Japan	2011-2013	Presence of symptoms compatible with new pulmonary infiltrates on chest X-ray or computed tomography scan consistent with pneumonia.	*Overall*=16.90 15-24 years=7.6 25-34 years=5.8 35-44 years=4.5 45-54 years=6 55-64 years=11.8 65-74 years=24.6 75-84 years=52.9 ≥85 years=79.3	*Overall*=11.8 15-24 years=3.2 25-34 years=1.7 35-44 years=1.4 45-54 years=2.5 55-64 years=6.5 65-74 years=16.9 75-84 years=43.4 ≥85 years=75.4	*Overall*=(0.7 per 1000 pyo; 0.1 %)
Takaki *et al,* 2014 (13)	Japan	2008 to 2010	Presence of respiratory symptoms compatible with pneumonia and showed consolidation on chest X-ray.	*Overall*=9.6 15-64 years=3.4 65-74 years=10.7 ≥75 years=42.9	No hospitalisation data was reported.	*Overall*=(n=6/131; 4.6%) 15-64 years=0% 65-74 years=(n=2/19; 10.5%) ≥75 years=(n=4/77; 5.2%)
Takahashi *et al,* 2013 (16)	Vietnam	2009-2010	ICD-10-coded pneumonia cases as identified from the hospital admission database (34).	*Overall* =0.8 15-29 years= 0.3 30-44 years=0.4 45-64 years=0.8 65-74 years=2.7 ≥75 years=7	No hospitalisation data reported.	*Overall*=(n=17/174; 9.8%)

Ramirez *et al,* 2017 (18)	USA	2014 to 2016	Presence of a new pulmonary infiltrate on chest radiograph and/or chest computed tomography scan and at least one clinical symptom consistent with pneumonia.	*Overall*=6.3 18-64 years=3.3 >65 years =20.9	*Overall*=7.06 18-24 years=0.7 25-34 years=1.2 35-44 years=2.3 45-54 years=3.8 55-59 years=7.2 60-64 years=9 65-74 years=15.1 75-84 years=22.1 ≥85 years=39.5	*Overall*=(n=246/3789; 6.5%)
Jain *et al,* 2015 (35)	USA	2010 to 2012	A chest radiograph consistent with pneumonia and clinical symptoms associated with pneumonia.	*Overall*=2.5 18-49 years=0.7 50-64 years=2.6 65-79 years=6.3 >80 years=16.4	No hospitalisation data was reported.	No case fatality was reported.
Broulette *et al,* 2013 (36)	USA	2008 to 2010	Inpatient CAP diagnosis was identified by a primary inpatient diagnosis for pneumonia.	*Overall*=10.6 *The overall incidence for hospitalised patients*=1.9 *The overall incidence for outpatients*=8.7 18-64 years=8.9 50-64 years=14.1	No hospitalisation data was reported.	No case fatality was reported.
Sun *et al,* 2019 (37)	United Kingdom	2002-2017	Pneumonia was defined using Read codes associated with 'pneumonia'	*Overall*=1.5-2.2	No hospitalisation data was reported.	No case fatality was reported.
Millett *et al,* 2013 (14)	United Kingdom	1997 to 2011	Pneumonia diagnoses using ICD-10 codes (34).	*Overall*= 8 65-69 years=2.8 70-75 years=4.3 75-79 years=6.9 80-84 years=12.1 85-89 years=21.8 >90 years=41.9	No hospitalisation data was reported.	No case fatality reported.
Lopardo *et al,* 2018 (9)	Argentina Uruguay and Paraguay	2012 to 2015	Radiological confirmed pneumonia was defined as new or progressive pulmonary infiltrate(s) on the chest consistent with pneumonia.	**Argentina pneumonia incidences** *Overall*=7 18-49 years= 2.3 50-64 years=11 ≥65 years=29.5 **Uruguay pneumonia incidences** *Overall*=6.3 18-49 years=2.7 50-64 years=6.1 ≥65 years=19.8 **Paraguay pneumonia incidences** *Overall*=1.8 18-49 years=0.5 49-64 years=2.9 ≥65 years=10.9	No hospitalisation data was reported.	*Overall*=12.1% 18-49 years=3.6% 50-64 years=5.1% ≥65 years=18.8%

Fassmer *et al,* 2018 (19)	Germany	2010-2014	A pneumonia ICD code as the main discharge hospital diagnosis	*Overall*=118 65-74 years=113 75-84 years=117 85-94 years=120 ≥95 years =130	No hospitalisation data was reported.	*Overall=(*n=2391/8531*;* 28%)
Rozenbaum *et al,* 2015 (38)	Netherlands	2008 to 2011	Diagnosis codes were used to define pneumonia	*Overall*=3 18-49 years=1.1 50-64 years=2.7 65-74 years=5.8 75-84 years=10.8 ≥85 years =17.2	No hospitalisation data was reported	No case fatality was reported.
Rivero-Calle *et al,* 2016 (39)	Spain	2009-2013	Radiological diagnosis and/or where the diagnosis was reported by another professional in a medical record.	*Overall* =4.6 18-20 years=2 20-25 years=1.9 25-30 years=1.9 30-35 years=2.4 35-40 years=3.3 40-45 years=3.5 45-50 years=3 50-55 years=3.6 55-60 years=4.3 60-65 years=5.5 65-70 years=6.2 70-75 years=8.1 75-80 years=10.5 80-85 years=13.3 85-90 years=16.9 90-110 years=23.7	No hospitalisation data was reported.	No case fatality was reported.
Monge *et al,* 2001 (11)	Spain	1995 to 1996	International Classification of Diseases 9 codes (34).	No incidence data was reported.	*Overall* =1.6 < 65 years=0.9 ≥ 65 years=5.2	*Overall*=7.4% < 65 years=2.7% ≥ 65 years=11.6%
Viegi *et al,* 2006 (12)	Italy	1999-2000	Family practitioner's diagnosis using chest x-ray results and systemic symptoms.	*Overall*=1.7 15-44 years=0.9 45-64 years=1.6 >64 years=3.3	**Overall*=31.8%	*Overall*=6%

*Hospital incidence rate reported as a percentage. [a] Study included participants with severe acute respiratory illness including pneumonia. [b]Incidence reported per 100 000 person-year observation. Pubmed and Scopus were used to search for articles. Abbreviations: ICD- International Classification of Diseases

1.3 Laboratory diagnosis of pneumonia

Despite the high burden of pneumonia, in many cases, the aetiological agent remains unknown (30). This is mainly due to the limitations of the available sampling methods including that sampling the upper respiratory tract (URT) is limited, as the majority of pathogens identified are also common colonisers of the URT thus making it difficult to attribute causality to pneumonia (40). Lung aspirates and pleural fluids are thus the recommended samples for determining the aetiology of pneumonia; however, such sample collections are invasive, require medical expertise and infrastructure, and are therefore not routinely undertaken (41). Sputum samples are tested in adults to identify the pathogens associated with pneumonia, as they are a non-invasive specimen type (42); however, the detection of pathogens in sputum may be affected by prior antibiotic use which inhibits bacterial growth (42). Further, as sputum is expectorated it could also be contaminated with colonisers of the URT which too limits the use of such samples in attributing causality to pneumonia (42). Sterile site samples including blood culture can also be used to determine the bacterial causes of pneumonia, however, only 7-13% of adults with CAP are bacteremic (43, 44), suggesting that sensitivity of blood culture is low (15-40%). Furthermore, prior antibiotic use could decrease the sensitivity of blood culture (45).

Urine is a non-invasive sample that can potentially be used to identify pathogens causing pneumonia (43). Urine samples have been used to identify legionellosis and *S. pneumoniae* using antigen-based methods such as the BinaxNOW™ *Streptococcus pneumoniae* antigen card and urine antigen detection (UAD) method (46, 47). Antigen-based methods are also limited in that a proportion of patients with positive blood or sputum cultures can have negative antigen tests (48). Furthermore, antigens may cross-react with closely related species, and antigens in urine can be detected for weeks after the onset of disease (41, 49, 50). It is thus often recommended that urine antigen tests be used in conjunction with other diagnostic assays (47, 51).

Molecular methods including polymerase chain reaction (PCR) have been developed and are alternatives to traditional culture methods for the identification of pathogens associated with pneumonia (52). This is due to their increased sensitivity (94%-

100%) and throughput compared to standard culture-based methods (53, 54). Further, PCR methods are not as affected by prior antibiotic use (55) and multiple pathogens can be detected within a single run (55, 56). While these methods have successfully identified pathogens associated with pneumonia in sputum and blood samples, there are limited data on the utility of PCR in urine samples (57). To our knowledge, only a single study has used PCR to identify the aetiology of pneumonia in urine samples collected from adults (57). In this study, negative urine spiked with known pathogen concentrations were tested using the PCR assay resulting in 100% sensitivity and 90.91% specificity (57). The study also included clinical urine samples (n=30), and when tested using PCR, *Streptococcus pneumoniae* was detected in 10% of the urine samples that tested negative by both the BinaxNOW™ *Streptococcus pneumoniae* antigen card and standard culture (57). However, the overall sensitivity of the PCR (n=13; 43%) using urine from clinical samples that were collected from pneumonia cases was similar to the BinaxNOW™ *Streptococcus pneumoniae* antigen card (n=12; 40%) (57).

1.4 Aetiology of pneumonia

Studies investigating the aetiology of pneumonia in adults living in LMIC identified *S. pneumoniae* as the most prevalent bacterial pathogen in pneumonia patients using sputum samples (3.4-51.3%) followed by *Klebsiella pneumoniae* (1.7-32.8%), *Haemophilus influenzae* (2.4-28%), and *Staphylococcus aureus* (3-24.1%); Table 1.2 (16, 58-63). The detection of viral pathogens in CAP cases is also increasingly being recognised with approximately one-third of pneumonia in adults attributed to viruses (35, 64, 65), with Influenza viruses A and B (0.7-24.3%) being the most frequently detected viruses; Table 1.2 (7, 66). Respiratory syncytial virus (RSV) has also been detected in adult pneumonia cases (2.6-4%), particularly in older adults (4%) and those that are immunocompromised have a 30-70% increased case fatality risk compared to their age-matched healthy counterparts due to RSV infection (65, 67, 68). Parainfluenza viruses have been detected in 2% of adults with pneumonia, adenovirus (1-7%), and endemic coronaviruses (2.5%) (35, 69, 70) The SARS-CoV-2 virus has been detected in 5% of adults with pneumonia and causing mortality in 19% of those with the SARS-CoV-2 virus related pneumonia (71). Fungi, protozoal and helminthic parasites are the least common groups of pathogens associated with

pneumonia; however, they are important pathogens in high-risk populations particularly, those living with HIV (2, 72, 73).

Table 1.2: Aetiology of community-acquired pneumonia in African and Asian adults

Reference	Country (ies) and study period	Participants	Study design	Sample(s)	Method(s)	Major findings
Aston *et al,* 2019 (66)	Malawi 2013-2015	472 adults	Prospective observational study	Blood, nasopharyngeal aspirations, urine, sputum, and pleural fluid	Culture and urine antigen detection method	*S. pneumoniae* (98/458; 21.4%) and *M. tuberculosis* (75/326; 23.0%) were the most frequently identified pathogens.
Regasa 2014 (61)	Ethiopia 2013	170 people ≥ 15 years.	Cross-sectional study	Sputum	Culture	The common isolates of single bacterial pathogens from sputum specimens were *S. pneumoniae* (20/170; 11.8*%), S. aureus* (15/170; 8.8%), *P. aeruginosa* (10/170; 5.8%), *K. pneumoniae* (8/170; 4.7%), E. coli (4/170; 2.4%), *H. influenzae* (4/170; 2.4%).
Aderaye 1994 (60)	Ethiopia	110 hospitalised adults	Prospective	Sputum, blood, and lung aspirate	Culture and gram staining	*Streptococcus pneumoniae* was identified in (4/60; 6%), *Staphylococcus aureus* in (4/60; 6%), Enterobacteriacae in (3/60; 5%), Pseudomonas, *Klebsiella pneumoniae* and Streptococcus Viridans (1/60; 1.7%) case each. Other causes included *Mycoplasma pneumoniae* in (4/104; 4%), Influenza A in (4/104; 4%), Influenza B in (3/104; 3%) and Psittacosis in (4/104; 4%).
Iroezindu *et al,* 2014(63)	Nigeria 2008-2012	232 adults	Multicentre retrospective observational study	Sputum	Culture	*S. pneumoniae* (90/189; 47.6%) was the most frequent isolate followed by *Klebsiella pneumoniae* (62/189; 32.8%), *S. aureus* (24/189;12.7%), and *S. pyogenes* (13/189; 6.9%).
Scott *et al,* 2000 (7)	Kenya 1994- 1996	281 people≥ 15 years	Prospective	Blood, serum, sputum, urine, and lung aspirates (collected from patients with peripheral consolidation only)	Culture and latex agglutination	*S. pneumoniae* was the most common causative agent, it was found in (129/281; 46%) cases; *Mycobacterium tuberculosis* was found in (26/281; 9%).
Koulla-Shiro *et al,* 1997(6)	Cameroon 1991- 1993	91 hospitalised people ≥15 years.	Prospective	Blood, sputum, and serum	Culture and latex agglutination	*S. pneumoniae* was the commonest causative agent, identified in (22 / 91; 24.2%) patients. *M. pneumoniae* and *Coxiella burnetii* were diagnosed in (6/65; 9.2%) cases

						each, and *Chlamydia pneumoniae* in (3/65; 4.6%) patients.
Feldman *et al,* 1995 (59)	South Africa 1982 -1992	*280 adults with severe CAP.	Retrospective	Sputum and blood	Serology and culture	The commonest organism isolated was *S. pneumoniae (*61/119; 51.3%), and the second commonest organism isolated was *K. pneumoniae*, accounting for (38/119; 31.9%) of the isolates. *S. aureus* (4/119; 3.4%) accounted for most of the remaining isolates.
Takahashi *et al,* 2013 (16)	Vietnam 2009-2010	323 people, ≥15 years admitted to hospital,	Prospective surveillance	Sputum	Culture and PCR	*H. influenzae* (79/286; 28%) and *S. pneumoniae* (65/286; 23%) were the isolated organisms.
Shah *et al,* 2010 (58)	India 1998-2000	100 patients ≥15 years admitted to hospital	Prospective	Sputum and blood	Culture	*P. aeruginosa* was the commonest pathogen (10/29; 34.4%), followed by *S. aureus* (7/29; 24.1%), *E. coli* (6/29; 20.7%), *Klebsiella* spp. (3/29; 10.3%), *Streptococcus pyogenes* (1/29; 3.4%), *S. pneumoniae* (1/29; 3.4%), and *Acinetobacter* spp. (1/29; 3.4%).
Hashemi *et al,* 2010 (74)	Iran 2005-2007	150 adults ≥25 years	Prospective	Sputum	Culture	Overall, the most common organisms specified were *S. pneumoniae (17/150;* 11.3%), *S. aureus (12/150;* 8%), and *M. catarrhalis* (10/150; 6.6%). The most frequently identified pathogen in younger adults *was M. catarrhalis* (8/69; 11.5%), *S. pneumoniae* (7/69; 10.1%), and *S. aureus (7/69*; 10.1%). *S. pneumoniae (*10/81*;* (12.3%), *S. aureus* (5/81; 6.1%), and *P. aeruginosa (*5/81; 6.1%) were the pathogens mostly found in the elderly adults
Song *et al,* 2008 (75)	South Korea; China; Taiwan; Hong Kong; India; Singapore; Vietnam; and The Philippines 2002-2004	955 people, ≥15 years admitted to 14 hospitals in eight Asian countries.	Prospective	Sputum and blood	Culture	*S. pneumoniae* (114/390; 29.2%) was the most common isolate, followed by *K. pneumoniae* (60/390; 15.4%) and *H. influenzae* (59/390; 15.1%). Serological tests were positive for *M. pneumoniae* (61/556; 11.0%) and *C. pneumoniae* (55/411; 13.4%). Only (7/648; 1.1%) was positive for Legionella pneumophila by urinary antigen test.
Endeman *et al,* 2008 (62)	China 2004-2006	201 adults were admitted to the hospital.	Prospective	Blood, urine, and sputum	qPCR, urine antigen test, serological testing, and viral culture	*S. pneumoniae* was identified as the causative microorganism in (60/201; 30%), *H. influenzae* in (14/201; 7%), respiratory viruses in (16/201; 8%), *M. pneumoniae* and Legionella species in (9/201; 4%) each, and *S. aureus* in (6/201; 3%).
Tseng *et al,* 2008 (69)	Taiwan 2002-2003	22 adults	Prospective	Tracheal aspirate, blood, and urine	Culture, urine antigen tests, and serology tests	The isolated pathogens included *S. pneumoniae* (3/22; 14%), *K. pneumoniae* (2/22; 9%), *Enterobacter cloacae* (2/22; 9%), *H influenzae* (1/22; 5%), *Escherichia coli* (1/22; 5%), *S. aureus* (1/22; 5%), *P. aeruginosa* (1/22; 5%), Adenovirus (1/22; 5%) and *C. pneumoniae* (1/22; 5%).

Hu *et al,* 2005 (76)	Taiwan 2001	*169 adults with severe CAP	Retrospective	Blood, transtracheal aspirations, and BAL or pleural effusion	Culture	The most-common pathogens *were K. pneumoniae* (16/75; 21.3%), *P. aeruginosa* (13/75; 17.3%), and *Acinetobacter baumannii* (8/75; 10.7%). *S. pneumoniae* (4/75; 5.3%) and *H. influenza (*5/75; 6.7%) were less frequently seen.
Lauderdale *et al,* 2005 (77)	Taiwan 2001-2002	168 patients ≥16 years old, and who were admitted to the hospital	Prospective	Sputum	Gram staining and culture	The most common etiologic agents were *S. pneumoniae* (40/168; 23.8%), *M. pneumoniae* (24/168; 14.3%), *C. pneumoniae* (12/168; 7.1%), Influenza A virus (11/168; 6.5%), *K. pneumoniae (*8/168; 4.8%), and *H. influenza (*8/168; 4.8%).
Saito *et al,* 2006 (78)	Japan 1999-2000	232 adults	Prospective	Blood, sputum, serum, and transthoracic needle aspiration	Culture, PCR, BinaxNOW™ *Streptococcus pneumoniae* antigen card , and serology	The leading organism was *S. pneumoniae* (57/232; 24.6%), followed by *H. influenzae* (43/232; 18.5%), viruses (38/232; 16.4%), *C. pneumoniae* (15/232; 6.5%), *M. pneumoniae* (12/232; 5.2%), and Legionella spp.(9/232; 3.9%).
Yoshimoto *et al,* (79)	Japan 1995-2002	72 adults were admitted to the hospital.	Retrospective	Blood, sputum, BAL, and pleural effusion	Culture	The most common pathogen was *S. pneumoniae* (10/72; 13.9%) followed by *P. aeruginosa* (6/72; 8.3%) and *K. pneumoniae* (65/72; 6.9%).
Ishida *et al,* 2004 (80)	Japan 2001-2004	349 hospitalised people ≥ 15 years.	Prospective	Blood, urine, and sputum	Culture and BinaxNOW™ *Streptococcus pneumoniae* antigen card .	*S. pneumoniae* was the most frequently isolated pathogen (83/349; 23.8%), followed by *M. pneumoniae* (39/349; 11.2%), *Haemophilus influenzae* (21/349; 6.0%), and *C. pneumoniae* (12/349; 3.4%).
Reechaipichitkul *et al,* 2004 (81)	Thailand 1999-2001	*105 people ≥ 15 with severe CAP	Retrospective	Sputum, blood, and pleural fluid	Culture	The pathogens most isolated were *B. pseudomallei* (20/62; 29.4%), *S. pneumoniae* (14/62; 20.6%), *K. pneumoniae (*13/62; 19.1%), and *H. influenzae* (8/62; 11.8%). Other less common pathogens were E. coli (4/62; 5.9%), *S. aureus (*4/62; 5.9%), *Moraxella pneumoniae (1/62*; 1.5%), *Moraxella catarrhalis* (1/62; 1.5%), *Pseudomonas aeruginosa* (1/62; 1.5%), *Pseudomonas fluorescens* (1/62; 1.5%), and *Strongyloides stercoralis* (1/62; 1.5%).
Wattanathum *et al,* 2003 (82)	Thailand 1998- 2001	245 ≥ 15 years (98 outpatients and 147 inpatients)	Prospective	Sputum, blood, pleural fluid, and urine	Culture, gram staining, and urine antigen test	**INPATIENTS** *S. pneumoniae* (33/147; 22.4%), *C. pneumoniae* (24/147; 16.3%), *K. pneumoniae* (14/147; 9.5%), and *M. pneumoniae* (10/147; 6.8%) were the most frequently detected pathogens amongst inpatients. **OUTPATIENTS** *C. pneumoniae* was the most frequently found microorganism (36/98; 36.7%), followed by *M. pneumoniae* (29/98; 29.6%), *S. pneumoniae* (13/98; 13.3%), and *L. pneumophila* (8/98; 8.2%) amongst outpatients.

* Severe CAP is defined as pneumonia resulting in intensive care unit admission, requiring mechanical ventilation and patients usually develop septic shock. Pubmed and Scopus were used to search for articles. Abbreviations: PCR -polymerase chain reaction, BAL- bronchoalveolar lavage

1.5 *Streptococcus pneumoniae*

S. pneumoniae is an important pathogen amongst humans causing a variety of diseases including otitis media, meningitis, sinusitis, sepsis, empyema, and most importantly pneumonia (83). Further, *S. pneumoniae* has contributed significantly to deaths and according to the World Health Organisation, approximately 1.6 million deaths annually are caused by *S. pneumoniae* with a higher mortality rate in LMIC (~ 20%) compared to high-income countries (HIC) (~7%) (18, 84, 85).

S. pneumoniae was first isolated in 1881 by Louis Pasteur (86). It is a gram-positive bacterium that is lancet-shaped, grows in pairs or chains, is non-motile, and is diplococcus in shape (87). The capsule is conserved across all serotypes of *S. pneumoniae* and to date 100 antigenically and structural different serotypes have been identified (88). The polysaccharide heterogeneity of the capsule determines the virulence potential of the serotypes, with some serotypes having a higher potential to cause disease in the human host (89).

1.5.1 Colonisation by *S. pneumoniae*

Colonisation of the nasopharynx by *S. pneumoniae* is a prerequisite in the pathogenesis of all pneumococcal diseases and also for horizontal transmission amongst individuals (90). The duration of nasopharyngeal colonisation varies greatly from two weeks to as long as four months and also varies with age, and the colonising serotype (90). The *S. pneumoniae* serotypes including serotype 3 that have a higher duration of carriage have thicker capsules - the capsule thickness increases the invasiveness of the different serotypes and they have poor immunogenicity (86, 91). The prevalence of nasopharyngeal colonisation is high in children (57-65%) and they are the main reservoir of *S. pneumoniae*, whereas 5-10% of adults are carriers of *S. pneumoniae*; Table 1.3 (90, 92). Although adults have a low carriage of *S. pneumoniae*, in LMICs the carriage of *S. pneumoniae* is three times higher as compared to HIC thus resulting in more pneumonia cases amongst adults from LMIC compared to HIC (93). Overcrowding, smoke exposure, and high HIV infections in LMIC contribute significantly to the higher carriage of *S. pneumoniae* in LMIC compared to HIC (84). Further, HIV-infected mothers are at higher risk of acquiring *S. pneumoniae* from their children that are more susceptible to

pneumococcal colonisation (94) most often with serotypes 3, 6A, 6B, 9V, 10A, 14, 16, 19F, and 23F which are also common colonisers in children <5 years of age; Table 1.3 (94, 95).

1.5.2 Epidemiology of *S. pneumoniae*

Before the introduction of pneumococcal vaccines, pneumococcal serotypes 1, 3, 4, 6A, 6B, 14,19A, 19F, and 23F were associated with invasive pneumococcal disease (IPD); Table 1.3 (15). Post-pneumococcal conjugate vaccine (PCV)-immunisation there has been a shift in the repertoire of serotypes causing IPD, with non-invasive types including serotypes 8, 9N, 11A, 22F, and 33F causing IPD in adults, along with vaccine types 1, 3, 19A and 19F (96-98). Further, in adults, serotypes 3, 6B, 9N, 11A, 19A, and 19F have been associated with an increased case fatality ratio (91, 99, 100). Further, *S. pneumoniae* serotypes have different antibiotic resistance potential due to their capsule with serotypes 6A, 6B, 7F, 9V, 14, 15A, 19F, 19A, and 23F having an increased antibiotic resistance thus resulting in the reduced effect of antibiotics against these serotypes that are more likely to cause invasive disease (89, 101, 102).

Table 1.3: *Streptococcus pneumoniae* prevalence and serotypes in healthy adults and those with pneumococcal diseases

Reference	Year of study	Country (ies)	Study population	*S*ample type	*S. pneumoniae* detection method	*S. pneumoniae* detection results	*S. pneumoniae* serotyping results
***S. pneumoniae* carriage in adults**							
Harumurti *et al,* 2016 (103)	2012	Indonesia	HIV infected adults.	Nasopharyngeal swab	Culture and multiplex PCR for serotyping	*S. pneumoniae* carriage in adults' was10%.	The most frequently detected serotypes 6A/B (20%), 11A (10%), 15B/C (10%), 19F (5%), 19A (5%), 17F (5%), 23F (5%), 3 (5%), 34 (5%). Untypeable serotypes were (15%).
Farida *et al,* 2014 (104)	February to April 2010	Indonesia	Healthy adults in the community.	Nasopharyngeal swabs	Multiplex-PCR which covers 36 serotypes	*S. pneumoniae* was detected in 11% of adults.	The most frequently detected serotypes were 6A/6B (39%), 15B/C (13%), 15A (10%), 11A (3%), 23F (3%), other serotypes (6%) and those that were untypeable (26%).
Chen *et al,* 2007 (105)	2001-2002	Taiwan	Health-care workers	Cotton swab	Culture	0% colonisation	No serotyping results were reported.
Nunes *et al,* 2013 (94)	2007	South Africa	Black-African, HIV-infected, and HIV-uninfected mothers.	Nasopharyngeal and oropharyngeal swabs	Culture and quelling for serotyping	54.2% of HIV-infected and 61.0% of HIV-uninfected mothers were colonised.	The 9 most common acquired serotypes in HIV-infected mothers in decreasing frequency were 19F (14.4%), 23F (13.5%), 14 (7.7%), 6B and 9V (4.8%), 6A, 3, 10A and 16 (3.9%). In HIV-uninfected mothers, the most common acquired serotypes were 19F (8.8%), 3 (7.0%), 6B and 45 (5.3%), 23F, 14, 19A and 19B (4.4%)
Abdullahi *et al,* 2008 (106)	**Period 1**: March 2004 **Period 2**: 2 June to 3 July 2004.	Kenya	Healthy adults within the community.	Nasopharyngeal swabs	Culture	The colonisation rates in different age groups were: 10-19 years: 9.6% 20-29 years: 7.8% 30-49 years:3.2% ≥ 50 years: 4.7%	Of the 72 pneumococci isolated, 18 (25%) were PCV 7vaccine serotypes, 20 (28%) were serogroups that are immunogenically cross-reactive with VT pneumococci and 34 (47%) were non vaccine types.
***S. pneumoniae* prevalence in adults with pneumococcal disease**							
Li *et al,* 2019 (107)	2015-2017	China	Adults with pneumococcal disease	Blood, sputum, and cerebrospinal fluid	Culture and Multiplex PCR for serotyping	Only *S. pneumoniae* positive isolates were used in this study.	The prevalent serotypes were 19F=20% 3=16% 23F=9.3% 14=8% 19A=5.3%.
Zhou *et al,* 2018 (5)	2010-2012	China	Adults with CAP	Urine	BinaxNOW™ *Streptococcus pneumoniae* antigen card	*S. pneumoniae* was isolated in 3.3% of the adults.	No serotyping results were reported.
Wattal *et al,*	2013-2015	Japan	Adult with	Blood, CSF, pus,	Quelleng	All isolates were *S. pneuminiae* positive	Serotype19A =14%, serotype 8=10%,

2017 (96)			pneumococcal infections	pleural fluids. sputum, endotracheal secretions, and bronchial lavage/washing			serotype19F=8%, serotype 3=6%, and serotype 9N =6% were detected.
Albrich *et al,* 2017 (47)	2005-2007	South Africa	HIV-infected adults with CAP and HIV-infected outpatient controls	Induced sputum, urine, oropharyngeal and nasopharyngeal swabs	Gram staining, culture, UAD for serotyping	<u>CAP cases</u> The UAD assay was more frequently positive (104 [44.3%]) than the composite diagnostic (71 [30.2%]; <u>Community controls</u> 5.1% of asymptomatic carriers were positive for *S. pneumoniae*.	PCV 13 serotypes were positive in 44.3 of cases. 5.1% of PCV 13 serotypes were detected in controls.
Cohen *et al,* 2015 (108)	2003 2008	South Africa	Adults with invasive pneumococcal disease	cerebrospinal fluid blood, pleural, peritoneal or joint fluid	Culture	All cases were *S. pneumoniae* positive	Serotype 1=17%, serotype 19A=11%, serotype 4=7%, serotype 3=7%, serotype 6A=7%, serotype 14= 6%, serotype 23F=6%, serotype 8=5%, serotype 19F=5%, serotype 6B=5%, serotype 12F=5%, serotype 9V=4%, serotype 16=3%, serotype 9N=2%, serotype 7F=2%, serotype 18C=2%. Serotype 25=2%, serotype 22F=2%, serotype 13=2%, serotype 5=1% were detected.
Albrich *et al,* 2011 (109)	2005-2007	South Africa	Adults with acute pneumonia admitted at the hospital and healthy community controls.	blood cultures and induced sputum urine	Biological culture, pneumococcal latex agglutination test, a nested PCR and BinaxNOW™ *Streptococcus pneumoniae* antigen card	<u>CAP cases</u> Culture in CAP patients: 125/278 (44.9%) *LytA* PCR in CAP: 167/266 (62.8%) <u>Community controls</u> Culture in controls: 35/298 (11.7%) *LytA* PCR in controls: 57/288 (19.8%)	No serotyping results were reported.

Abbreviations: HIV-human immunodeficiency virus, CAP -community-acquired pneumonia, UAD-urine antigen detection assay, CSF- cerebrospinal fluid, PCV-pneumococcal conjugate vaccine, *LytA- S. pneumoniae autolysin* gene and VT- vaccine serotypes. Pubmed and Scopus were used to search for articles.

1.6 Methods used to detect and serotype *S. pneumoniae* in adults with pneumonia

1.6.1 Capsular typing

Quellung, a culture-based method is the gold standard method for serotyping *S. pneumoniae* which involves serotype-specific antibodies binding to the capsule of *S. pneumoniae* allowing visualisation under a microscope (110). A positive reaction is defined by the capsule appearing enlarged and opaque (110). The main advantage of this method is that it can serotype the majority of the *S. pneumoniae* serotypes known to date (110). Despite the ubiquitous use of culture-dependent methods in both disease and colonisation studies, the detection of concurrent colonising serotypes with a lower density is often missed (54). Further, traditional culture methods are laborious, require viable organisms, are expensive (111), and are only semi-quantitative (112). While latex sweep serotyping, a culture-based serotyping method has improved our detection of *S. pneumoniae*, it still underestimates the rate of less-abundant concurrent colonising serotypes (113). Nevertheless, it eliminated the need for a microscope, uses less anti-serum which allows for the visible detection of the antigen-antibody binding of the different pneumococcal serotypes, and is a reasonably faster method (110).

1.6.2 Antigen tests

The BinaxNOW™ *Streptococcus pneumoniae* antigen card is a rapid immunochromatographic test that is used for the detection of the *S. pneumoniae* antigen in urine (47). The test detects the pneumococcal cell wall C-polysaccharide that is present in all *S. pneumoniae* serotypes (114). The test is affected by high carriage density thus in children, the test is not used due to low positive predictive value (114). Further, BinaxNOW™ *Streptococcus pneumoniae* antigen card is unable to distinguish different *S. pneumoniae* serotypes (48). Regardless, BinaxNOW™ *Streptococcus pneumoniae* antigen card is highly specific (94-100%), sensitive (81%) (41, 48, 49, 115) and is thought to be unaffected by prior antibiotic use (48). Using the antigen method, *S. pneumoniae* has been detected in 3.7-42% of adults that have been hospitalised with pneumonia; Table 1.4 (116, 117). The huge variability in detection rates amongst hospitalised adults could be due to different detection sensitivity for the different *S. pneumoniae* serotypes, with a study showing

BinaxNOW™ *Streptococcus pneumoniae* antigen card has a low sensitivity for some *S. pneumoniae* serotypes, particularly serotype 3 which persists to cause pneumonia in adults because of its thick capsule (118). However, in a study conducted in New Zealand to determine the aetiology of pneumonia using culture and the BinaxNOW™ *Streptococcus pneumoniae* antigen card, the latter using urine samples was able to identify the likely aetiology of pneumonia in an additional 23% of the adult cases that were missed by blood culture (48). However, 20% of the participants with *S. pneumoniae* isolated using blood culture had negative BinaxNOW™ *Streptococcus pneumoniae* antigen card results (48).

The urinary antigen detection (UAD) method can be used to serotype *S. pneumoniae* PCV 13 vaccine serotypes in urine by detecting serotype-specific capsular polysaccharides (51). Recently, UAD has been clinically validated to detect and serotype an additional 11 *S. pneumoniae* non-vaccine serotypes (2, 8, 9N, 10A, 11A, 12F, 15B, 17F, 20, 22F, and 33F) (97). The UAD method is a multiplex immunoassay that uses Luminex technology that works by capturing the antigen with monoclonal antibodies on unique spectral microspheres thus achieving a high specificity (100%) (51) and sensitivity (97.1%) (47). The method allows for the simultaneous detection of multiple PCV13 serotypes and 11 non-vaccine serotypes in a single sample using a small sample volume thus reducing the amount of time required and the costs (51). However, UAD is unable to detect the majority of the non-vaccine serotypes thus underestimating the prevalence of *S. pneumoniae* (47, 51).

Table 1.4: Prevalence of *S. pneumoniae* in adults hospitalised with pneumonia using the urinary antigen test

Reference	Country	Participants	Period of study	*S. pneumoniae* prevalence using the BinaxNOW™ *Streptococcus pneumoniae* antigen card
Tootla *et al,* 2021 (119)	South Africa	212	2017-2018	42% (n=89/212)
Albrich *et al,* 2017 (47)	South Africa	222	2005 to 2007	23.4% (n=52/222)
Forstner *et al,* 2020 (118)	Germany	786	2012 to 2017	3.3% (n=26/786)
AlBarrak *et al*, 2018 (120)	Hajj Pilgrims	249	2016	14.5% (n=36/249)
Sherwin *et al*, 2013 (121)	USA	708	2010 to 2011	4.8% (n=34/708)
Segonds *et al,* 2010 (122)	France	278	2002 to 2004	11.5% (n=32/278)

Shibli *et al*, 2010 (123)	Israel	127	2006 to 2007	14.2% (n=18/127)
Hohenthal *et al,* 2008 (124)	Finland	333	1999 to 2004	24.3% (n=81/333)
Ishida *et al,* 2004 (80)	Japan	349	2001 to 2004	33% (n=115/349)
Murdoch *et al,* 2001 (48)	New Zealand	420	-	29% (n=120/420)
Molinos *et al,* 2015 (125)	Spain	3874	2005 to 2007	23% (n=871/3874)
Sordé *et al,* 2011 (126)	Spain	474	2007 to 2008	15.8% (n=75/474)
Rosón *et al,* 2004 (127)	Spain	220	2000 to 2002	31% (n=43/220)
Marcos *et al,* 2003 (128)	Spain	398	2001 to 2004	39% (n=154/398)
Gutiérrez *et al,* 2003 (129)	Spain	452	1999 to 2001	23% (n=104/452)

Pubmed and Scopus were used to search for articles.

1.6.3 Molecular based methods

Molecular serotyping of *S. pneumoniae* has several potential advantages over culture-based methods, including allowing multiple serotypes to be detected from a single sample with high sensitivity (54), as well as quantitative PCR (qPCR) methods being able to measure the density and relative proportion of each identified serotype (54). It is thus plausible that qPCR would also be able to assess the severity of pneumococcal pneumonia, as a high genomic load has been associated with a higher likelihood of more severe disease and death (130).

Biomark HD real-time PCR system (Fluidigm) is a nanofluidic automated real-time qPCR that has been utilised to detect and serotype *S. pneumoniae* in nasopharyngeal swab samples (131). It uses microfluidic technology which uses dynamic arrays of Integrated Fluidic Circuits (IFCs) with interconnected channels (132, 133). These dynamic arrays allow for 9216 reactions in a single qPCR run (132). The method has the advantages of a larger number of reactions per plate making it cost-effective and less time-consuming (131). Further, it uses a reduced reaction volume of 10nL scale compared to the 20µl that is commonly used for qPCR with an increased throughput compared to qPCR (133). In a study comparing Fluidigm to standard qPCR and culture, the Fluidigm method because of its high sensitivity was able to detect an additional 39.1% more pneumococcal serotypes in nasopharyngeal swabs collected from children compared to culture and an additional 4.2% pneumococcal serotypes compared to qPCR (133). Therefore, based on the findings of that study (133), the Fluidigm has an increased sensitivity compared to the qPCR.

Our Unit, Vaccines and Infectious Diseases Analytical Research Unit (Wits –VIDA) successfully set up a novel nanofluidic real-time qPCR assay (Fluidigm) that can simultaneously detect 55 pneumococcal serotypes (including PCV13 serotypes and the most dominant non-vaccine serotypes) from nasopharyngeal swabs (133). As the Fluidigm allows for up to 96 reactions to be run simultaneously in the Fluidigm assay we have recently been able to expand our assay to now amplify 93 of the most common pneumococcal serotypes in a single qPCR run (Wits-VIDA, unpublished). The utility of this method in urine samples is however unknown.

1.7 Problem statement

The pneumonia case fatality ratio in adults in LMIC is 20%, which is roughly 3-4 times higher compared to HIC (85). Sub-Saharan Africa has the highest burden of pneumonia with approximately four million cases and 0.2 million deaths attributed to pneumonia annually in adults ≥ 15 years of age (84). Despite the high mortality of pneumonia in this setting, there is limited data on the aetiology of pneumonia (97, 134). Regardless, *S. pneumoniae* has been identified as the most common cause of bacterial CAP in adults (3.4-51%), especially those living with HIV in Sub-Saharan African countries; Table 1.2 (58, 59). The high burden of pneumonia does not only encumber the healthcare system but also has economic implications and despite the indirect benefits from childhood PCV immunisation, a large proportion of adults continue to develop pneumococcal pneumonia (1, 135).

Limitations of classical microbiology tests and specimens available for identifying the serotypes causing pneumococcal diseases, including CAP, have hindered our understanding of the epidemiology of pneumococcal pneumonia in adults (84). Improved diagnostic techniques using alternative samples are needed to monitor the changing epidemiology of pneumococcal pneumonia (57).

1.8 Aim and objectives

1.8.1 Study Aim

The study aimed to optimise the Fluidigm qPCR assays to identify and serotype *S. pneumoniae* in urine samples. Once successfully optimised, the utility of the Fluidigm

assays was screened using urine samples collected from adults hospitalised with pneumonia.

1.8.2 Study objectives

Primary objective

- To optimise the nanofluidic qPCR (Fluidigm) method to serotype *S. pneumoniae* in urine samples.

Secondary objective

Contingent to the success of the Fluidigm method being able to detect and serotype *S. pneumoniae* in urine samples:

- To evaluate the utility of the method in identifying and serotyping pneumococcus causing disease in adults hospitalised with pneumonia.

CHAPTER 2: MATERIALS AND METHODS

2.1 Pneumococcal bacterial control strains

Single culture control strains of the pneumococcal PCV 13 serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F, previously obtained from the National Institute for Communicable Diseases were used to optimise the detection of PCV 13 serotypes in urine samples with the nanofluidic qPCR assay (Fluidigm). The *S. pneumoniae* frozen cultures were thawed and cultured on 5% blood agar plates (Media Mage, South Africa) with gentamycin as shown in Figure 2.1 using the standard streaking methods to obtain single colonies and incubated at 37˚C with 5% carbon dioxide for 24 hours. After 24 hours, at least five single colonies were picked and inoculated into Todd-Hewitt broth with 5% yeast, and these were incubated overnight at 37˚C plus 5% carbon dioxide.

Figure 2.1: *Streptococcus pneumoniae* cultured on blood agar (Image from (136)

2.1.2 Spectrophotometry

After the 12-14 hours incubation, 1ml of the *S. pneumoniae* cultures grown in Todd-Hewitt broth was measured at an optical density of 600nm until an absorbance reading of ~0.1 (corresponds to a late log phase and ~10^7 colony-forming units (CFU)/per mL) was obtained. This was repeated for each of the PCV 13 serotypes.

2.1.3 Microtiter method for quantitative viable counts for bacterial cultures

A 96-well-U–bottom plate as shown in Figure 2.2 was used for a 1:10 serial dilution (10^7-10^1) of the undiluted cultures in phosphate-buffered saline and a 20µL drop of each serial dilution was plated on blood agar plates in triplicates. The plates were incubated overnight in 5% carbon dioxide, and the number of visible colonies that grew per drop was counted. The numbers of CFU/mL were calculated using the equation below and the dilution closest to 10^8 CFU/mL was used for subsequent serial dilutions spiked into urine samples. This was repeated for each of the PCV 13 serotypes with each respective control strain having a minimum concentration of 10^7 CFU/mls.

$$\frac{Colonies\ drop\ \#1 + Colonies\ drop\ \#2 + colonies\ drop\ \#3}{3}$$ X 50 X dilution factor = CFU/ml

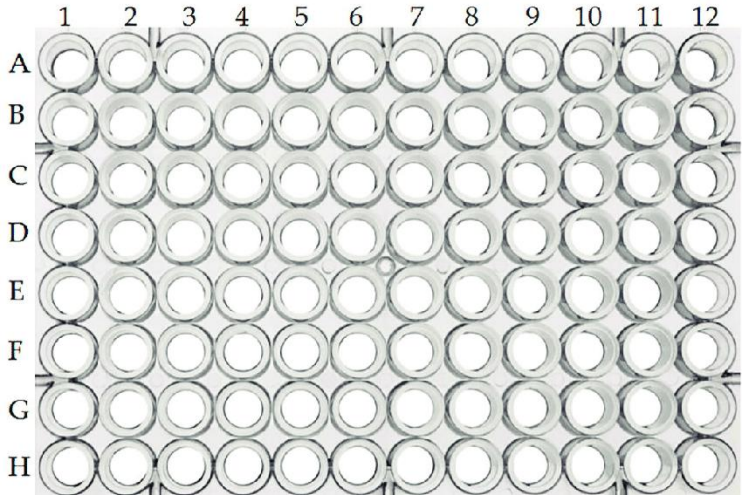

Figure 2.2: U-bottom micro-plate (Image from (137)

2.1.4 Serial dilutions

For the serial dilutions, the 10^8 CFU/mL culture was diluted (1:10) into a urine sample donated by a healthy adult who was BinaxNOW™ *S. pneumoniae* antigen negative and *S. pneumoniae* autolysin gene. This was subsequently diluted (1:10) into urine again to create a 10-fold serial dilution with the linear dynamic range of $10^1 – 10^7$ CFU/mL. The 'spiked' urine samples were processed for total nucleic acid (TNA) extraction using the easyMAG® extraction system (BioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions as described in Section 2.1.5 below. This was repeated for each of the PCV 13 serotypes.

2.1.5 Total nucleic acid extraction of the pneumococcal culture strains

TNA was extracted from 500µL of the "spiked" urine samples in 2ml of lysis buffer (BioMérieux, Marcy l'Etoile, France). A final elution volume of 25 µl was automatically extracted from lysed urine samples with the NucliSENS® easyMAG® extraction system (BioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions using the on-board extraction protocol. The TNA was stored at -20ºC until assayed.

2.2 Nanofluidic qPCR (Fluidigm)

The FAM-dye labelled minor groove binder probes and the oligonucleotide primers (Table 2.1) used in our study are from previously published sequences and have

previously been optimised on the Fluidigm (133, 138-141). Additional, 16S rRNA gene, a common housekeeping gene was used to verify the performance of our extraction (142) and sample quality. Further negative controls were included in every run. Some of the *S. pneumoniae* serotypes have genotypic similarities in the capsule loci, therefore, our assay was unable to identify the individual serotypes within some of the pneumococcal serogroups (i.e. 6A/B/C/D, 7A/F, 9A/V, and 18A/B/C) however, serotypes 1, 3, 4, 5, 14, 19A, 19F, and 23F were identified individually.

2.2.1 Specific target amplification for Fluidigm

Specific target amplification (STA) was done as per the manufacturer's recommendations as the initial step (pre-amplification of deoxyribonucleic acid (143)) for the Biomark HD system (Fluidigm). Briefly, a pool of primers consisting of the 12 assays was prepared for the Real-time qPCR Biomark HD system (Fluidigm). The pools were prepared to make up a final volume of 5 µL, containing 3.75 µL of the pre-amp mixed with 1.25 µL of the TNA. This mixture was loaded onto the Mini Amp Plus thermocycler machine (Applied Biosystems). The cycling conditions used included an initial activation step at 95ºC for 2 minutes followed by 14X cycles consisting of two steps, denaturation at 95ºC for 15 seconds, and the annealing/extension step at 60ºC for 4 minutes. After STA, the amplicons were diluted at a ratio of 1:5 using the dilution reagent.

2.2.2 Real-time qPCR using the Biomark HD system (Flidigm)

The qPCR reactions were carried out on the STA products with the nanofluidic real-time qPCR arrays (Fluidigm Corporation, CA, USA) according to the manufacturer's instructions. The Fluidigm FLEX Six Gene expression IFC plate was used which allows for the testing of 12 assays against 12 samples.

The FLEX Six IFC plate was primed by injecting 150 µl of the control line fluid into the accumulator. After priming the plate, the FLEX Six IFC plate was loaded with the 2.2 µl sample pre-mix and 1.8µl pre-amplified cDNA for each of the sample inlets. An equal volume of 4 µl of the assay was also loaded into the assay inlets. The chip was placed in the Biomark instrument for Fluidigm screening using standard thermal

cycling conditions. The data was analysed using the real-time PCR analysis software in the Biomark instrument (Fluidigm Corporation, CA, USA) using manually defined thresholds and analysed using the real-time PCR analysis software in the Biomark instrument (Fluidigm Corporation, CA, USA). Positive samples were defined as those with Cq (quantification cycle) values ≤35 for the individual qPCR targets.

Table 2.1 Primer and probe sequences for pneumococci and the PCV 13 pneumococcal serotypes

Primer/Probe name	Forward primer	Reverse primer	Probe	Reference
Streptococcus pneumoniae serotype 1	CGTGCGGTAATTGAAGCTATGA	TGTGGCCCCAGCAACTCT	GCTTGCCCTTGTATAGGGT	Azzari *et al,* 2010 (138)
Streptococcus pneumoniae serotype 3	GGTCAGCAGAAAGTATGCATTGG	TCGTTTATCCAGGGTCTGATGA	TATTGGATGTGGTTTATCGTGAAGA	Azzari *et al,* 2010 (138)
Streptococcus pneumoniae serotype 4	TGGGATGACATTTCTACGCACTA	CCGTCGCTGATGCTTTATCA	TCCTATTGGATGGTTAGTTGGTGA	Azzari *et al,* 2010 (138)
Streptococcus pneumoniae serotype 5	TTACGGGAGTATCTTATGTCTTTAATGG	CAGCATTCCAGTAGCCTAAAACTAGA	TTGTCTCAGCAACTCTATTTGGCTGTGGG	Azzari *et al,* 2010 (138)
Streptococcus pneumoniae serogroup 6A/B/C/D	AAGTTTGCACTAGAGTATGGGAAGGT	ACATTATGTCCRTGTCTTCGATACAAG	TGTTCTGCCCTGAGCAACTGG	Azzari *et al,* 2010 (138)
Streptococcus pneumoniae serogroup 7A/F	GATGGCATGTGGCAAACCA	TTTGCCCTCCTTAATCATTTCAC	TTGGCTATCGGCATGGTGGT	Azzari *et al,* 2010 (138)
Streptococcus pneumoniae serogroup 9A/V	AGGTATCCTATATACTGCTTTAGG	CGAATCTGCCAATATCTGAAAG	ACACATTGACAACCGCTACA	Azzari *et al,* 2010 (138)
Streptococcus pneumoniae serotype 14	CGACTGAAATGTCACTAGGAGAAGAT	AATACAGTCCATCAATTACTGCAATACTC	TGTCATTCGTTTGCCAATACTTGATGGTCTC	Azzari *et al,* 2010 (138)
Streptococcus pneumoniae serogroup 18A/B/C/F	CCTGTTGTTATTCACGCCTTACG	TTGCACTTCTCGAATAGCCTTACTC	AACCGTTGGCCCTTGTGGTGGA	Azzari *et al,* 2010 (138)
Streptococcus	TTCGACGACGTATCAGCTTCA	TCATTGAGAGCCTTAACCTCTTCA	ACCCAAAACGGTTGACGCATTATACT	Azzari *et al,* 2010

pneumoniae serotype 19A				(138)
Streptococcus pneumoniae serotype 19F	CGGGGTCAAATATTCAGTGG	CACGAATGAGAACTCGAATAAAAG	TTCGCACTGTCAATTCACCT	(Wits-VIDA, Unpublished)
Streptococcus pneumoniae serotype 23F	TGCTATTTGCGATCCTGTTCAT	AGAGCCTCCGTTGTTTCGTAAA	TTTCTCCGGCATCAAACGTTAAG	Azzari *et al,* 2010 (138)
Streptococcus pneumoniae LytA	TCTTACGCAATCTAGCAGATGAAGC	GCACGAATAACCAACCAAACAAC	CCGCAACTCATCAAGGATTTCTGTTACCA	Carvalho *et al,* 2007 (140)
Streptococcus pneumoniae PiaB	CATTGGTGGCTTAGTAAGTGCAA	TACTAACACAAGTTCCTGATAAGGCAAGT	TGTAAGCGGAAAAGCAGGCCTTACCC	Brown *et al,* 2001 (141)
Streptococcus pneumoniae Ply	GCTTATGGGCGCCAAGTCTA	CAAAGCTTCAAAAGCAGCCTC	CTCAAGTTGGAAACCACGAGTAAGAGTGATGAA	Carvalho *et al,* 2007 (140)

2.3 Standard curves and the optimisation of the *S. pneumoniae* PCV 13 serotypes primers

To evaluate the performance of the Fluidigm to detect *S. pneumoniae* in urine samples, standard curves were determined using serial dilutions (10^1 to 10^7) of the urine spiked with the pneumococcal controls. The correlation coefficients (r^2) were determined, and the amplification efficiency (e) was calculated using $e= 10^{-1/m} - 1$, where m is the slope of the standard curve. The lower limit of detection (LLD) was the minimum copy number within the linear dynamic range that could be detected of the TNA. The analytical specificity of the primers was also determined by cross-checking the amplification of each primer pair against bacterial DNA from all 96 pneumococcal bacterial targets that has been previously validated on the Fluidigm in a single Fluidigm run. Further, intra-assay variability (repeatability) was tested by repeating the *S. pneumoniae* PCV 13 serotypes four times for each sample in the same Fluidigm run and determining the standard deviation for the copy numbers and inter-assay variability (reproducibility) was determined as the standard deviation of the *S. pneumoniae* PCV 13 serotypes copy numbers in different runs. Lastly, accuracy for the different primers was calculated using the ratio of the difference between the observed copy number to the expected copy number. The acceptable ranges for the correlation coefficient, efficiency, lower limit of detection, specificity, accuracy, intra-assay variability, and inter-assay variability are shown in Table 2.2.

Table 2.2 qPCR acceptance ranges according to Minimum information for publication of quantitative real-time polymerase chain reaction experiments guidelines(144)

	Definitions and acceptable ranges for the qPCR
Correlation coefficient	This indicates the width of the spreading of individual data around the linear regression line. A higher r^2 value indicates a more robust assay. The acceptable range is $r^2 \geq 0.98$.
Efficiency	Robust and precise qPCR assays are usually correlated with high PCR efficiency. The acceptable range is 90% to 110%.
The lower limit of detection	It is the DNA concentration that can be detected with reasonable certainty (95% probability) by the assay.

	A theoretically sensitive PCR can detect 3 copies per PCR.
Specificity	Detecting the intended target sequences rather than non-specific targets.
Accuracy	Accuracy is the difference between experimentally measured and actual concentrations, expressed as fold changes or copy number estimates. Accuracy of ±0.1-fold change is acceptable.
Intra-assay variability	It is the precision and robustness of the assay with the same samples repeatedly analysed in the same assay. A standard deviation of <0.167is acceptable.
Inter-assay variability	It is the variation in results between runs expressed as the SD or CV of copy numbers or concentrations. A standard deviation of <0.167 is acceptable.

Abbreviations: PCR-polymerase chain reaction, r^2-correlation coefficients. Acceptable ranges adapted from (144)

2.4 Study cohort

The “Hospital surveillance for respiratory illness” study was conducted at Chris Hani Baragwanath Academic Hospital and Bheki Mlangeni Hospital in Soweto, Johannesburg, South Africa (145, 146). The study enrolled adults hospitalised with CAP that were older than 15 years of age between April 2018 and August 2019. Study participants had to present with at least one of the following four systemic symptoms: fever/feverishness defined as a temperature ≥37.5ºC, headache, myalgia, or malaise, and at least one of the following three respiratory symptoms: cough, sore throat, or shortness of breath. During the study, chest X-rays were done at the discretion of the attending physician; however, the radiology results were unavailable for this study. During admission, patients were administered antibiotics as part of their treatment and the date of commencement of treatment was recorded and some participants were already on antibiotics at the time of sample collection. Urine was collected from the participants enrolled in the study and stored at -80ºC at Wits-VIDA until assayed.

2.4.1 Community-acquired pneumonia case definition

Participants with a physician diagnosis corresponding to the International Statistical Classification of Diseases (ICD) 10 listed in Table 2.3 were classified as pneumonia patients.

Table 2.3: International Statistical Classification of Diseases and Related Health Problems 10 codes assigned to study participants and descriptions

ICD 10 code	Description
J12	Viral pneumonia, not elsewhere classified
J13	Pneumonia due to *Streptococcus pneumoniae*
J14	Pneumonia due to *Haemophilus influenza*
J15	Bacterial pneumonia, not elsewhere classified
J15.2	Pneumonia due to Staphylococcus
J15.4	Pneumonia due to Streptococcus
J16	Pneumonia due to other infectious organisms, not elsewhere classified
J17	Pneumonia in diseases elsewhere classified
J18	Pneumonia, organism unspecified
J18/J91	Pneumonia organism unspecified/ Pleural effusion in conditions classified elsewhere
J18.0	Bronchopneumonia, unspecified
J18.1	Lobar pneumonia, unspecified
J18.9	Pneumonia, unspecified
J18.9/Y95	Pneumonia unspecified, Nosocomial condition

Abbreviation: ICD- International Statistical Classification of Diseases

2.5 Laboratory testing of clinical samples

2.5.1 Real-time qPCR using the Biomark HD system (Fluidigm) on clinical urine samples

TNA extracted from the 96 urine samples was tested using the Fluidigm FLEX Six IFC controller as described for the culture control strains in sections 2.1.5 and 2.2.

2.5.2 *S. pneumoniae* antigen detection using BinaxNOW™ *Streptococcus pneumoniae* antigen test card (Alere, USA)

Before testing, urine was thawed to room temperature (15 to 30ºC), vortexed and a cotton swab was dipped into the urine for testing according to manufacturer's instructions (48). The swab was inserted into the test card and 3 drops of Reagent A (citrate/phosphate buffer with sodium lauryl sulfate, tween 20, and sodium azide) were added to the antigen testing card before sealing it. The results were read after 15 minutes. The results were based on the visibility and presence of coloured lines on the device with two lines indicating the presence of *S. pneumoniae* while one line was denoted as a negative result.

2.5.3 Real-time qPCR testing using standard ABI 7500 system

The ABI 7500 Real-time PCR system (Applied Biosystems, ABI, Foster City, USA) was used to screen for the *S. pneumoniae autolysin (LytA)* pneumococcal gene on the clinical urine samples as previously described (140). Briefly, the qPCR was performed in a 20 µL reaction volume containing 12.5 µL 2xTaqMan gene expression master mix (ABI), 0.313 µL 0.25Mm MGB dye-labelled probe (ABI), mixed with a primer mix, and 5 µL of the extracted total nucleic acid. The cycling conditions included incubation at 50ºC for 2 minutes followed by an initial activation at 95ºC for 10 minutes, then 50 cycles consisting of denaturing at 95ºC for 15 seconds and annealing/extension at 60ºC for 1 minute. Positive samples were defined as those with Cq values ≤40 for the *S. pneumoniae LytA* gene.

2.6 Data analysis

Descriptive statistics were used to compare the demographic and clinical features of the participants. Logistics regression using uni-variate analysis was used to

determine the association between any of the demographic factors with a positive urinary antigen result and those with a positive *S. pneumoniae LytA* qPCR positive result and those with *S. pneumoniae LytA* negative result. The sensitivity and specificity of the qPCR, and Fluidigm to detect *S. pneumoniae* in urine were determined using McNemar's test using the urinary antigen test as the reference standard. *Kappa* statistics were used to evaluate the performance between the qPCR, Fluidigm assay, and the BinaxNOW™ *Streptococcus pneumoniae* antigen card to detect *S. pneumoniae* in the urine. The interpretations of the *Kappa* statistics are given in Table 2.4. All statistical analyses were done using Stata version 13, (Statacorp, Texas, USA).

Table 2.4: *Kappa* statistics and the interpretations for the different ranges

***Kappa* statistic**	**Interpretation**
0.0-0.2	None to slight concordance
0.21-0.4	Fair concordance
0.41-0.6	Moderate concordance
0.61-0.8	Substantial concordance
0.81-1	Almost perfect

2.7 Ethical clearance and approval

Ethical approval was granted by the Human Research Ethics Committee (HREC) of the University of Witwatersrand, approval number M200430. The ethics certificate is included in Appendix 1. This study retrospectively tested samples that were collected from the Hospital surveillance for respiratory illness study that was conducted by Wits-VIDA (HREC M1611115) as a continuation of the hospital-based surveillance for respiratory illnesses study in children and pregnant mothers (145, 146). In the parent study, written informed consent forms were obtained from all study participants including permission for the storage and future testing of their samples.

CHAPTER 3: RESULTS

3.1 Performance of the quantitative nanofluidic polymerase chain reaction assay to detect 13-valent pneumococcal conjugate vaccine serotypes in the urine

All reactions were effective in amplifying their respective targets in the Biomark HD system (Fluidigm), with the efficiency of the reactions ranging from 92% to 105%; Table 3.1. Within the linear dynamic range, the correlation coefficients (r^2) of the reactions were >0.95; Figures 3.1-3.12. Further, all assays showed a good analytical sensitivity within their respective primer and probe pairs with the limit of detection (LLD) equivalent to >1 000 copies per PCR for all reactions, except for primers/probes that detected serotype 19F in which the LLD was 10-fold more sensitive (> 100 copies per PCR); Figure 3.1-3.12, Table 3.1. Additionally, the accuracy ratio for all reactions was within ±0.1-fold change for all reactions which are within the acceptable range; Table 3.1; Figures 3.1-3.12. The intra-assay variability (repeatability) and inter-assay variability (reproducibility) for all assays were within the acceptable range of ± 0.167 (0-0.15) standard deviation (SD) and (0.02-0.16) SD respectively; Table 3.1. All primer/probe pairs were tested with genomic DNA from all PCV13 pneumococcal serotypes, as well as 80 non-vaccine serotype/serogroups, with no cross-reactions between the reactions; Table 3.2.

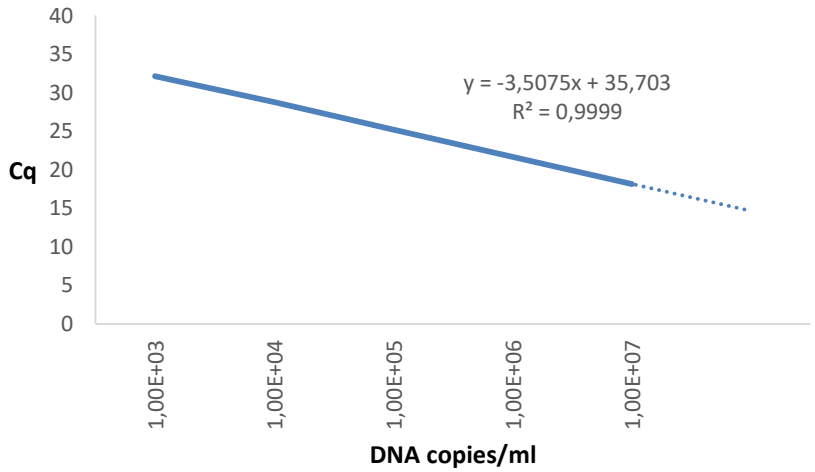

Figure 3.1: Standard curve for *S. pneumoniae* serotype 1

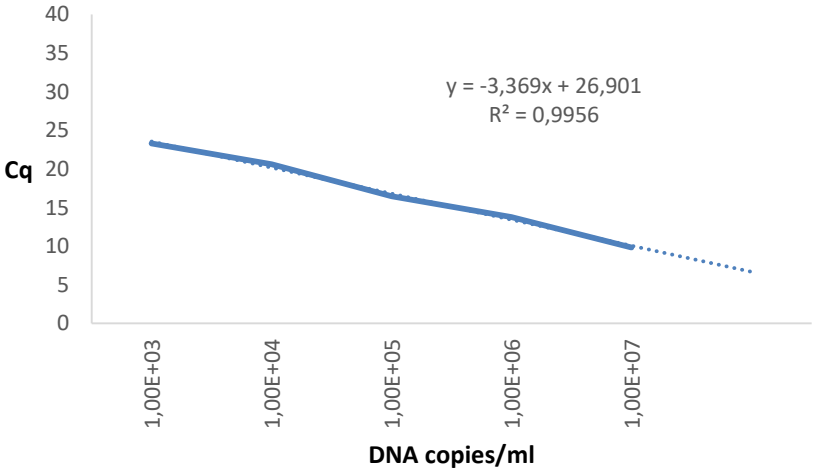

Figure 3.2: Standard curve for *S. pneumoniae* serotype 4

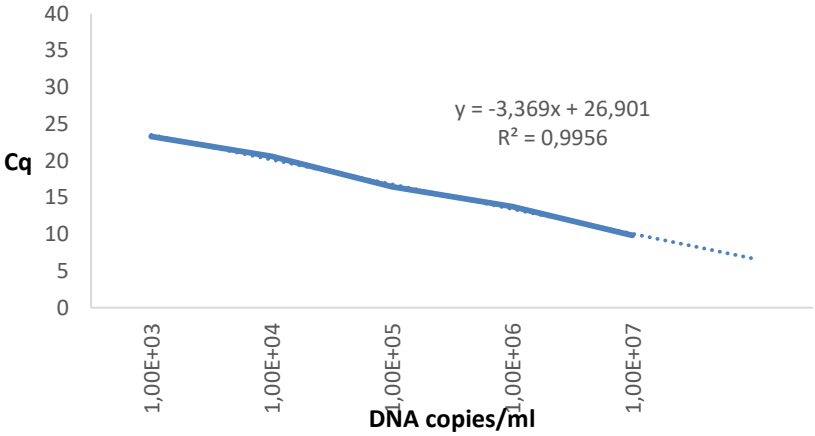

Figure 3.3: Standard curve for *S. pneumoniae* serotype 3

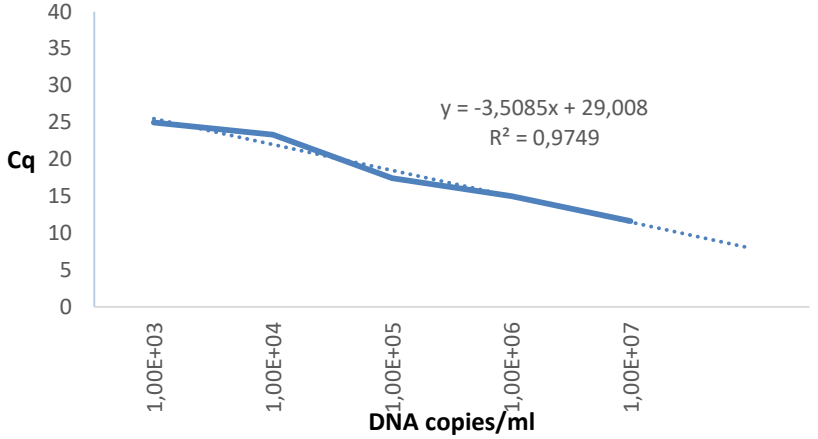

Figure 3.4: Standard curve for *S. pneumoniae* serotype 5

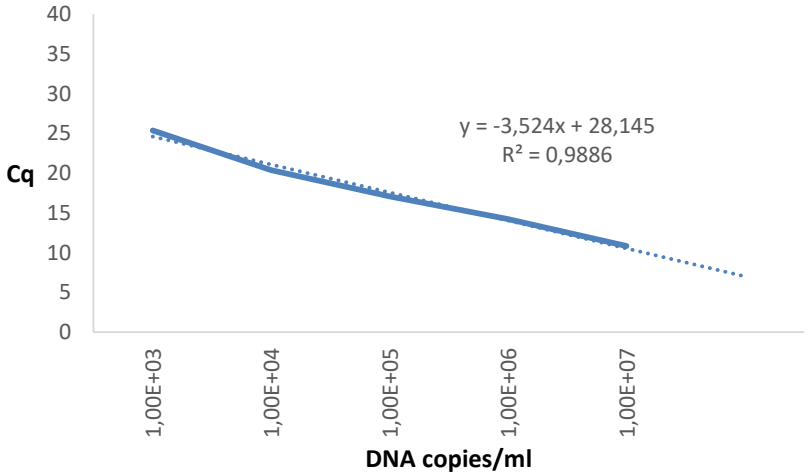

Figure 3.5: Standard curve for *S. pneumoniae* serogroup 6A/B/C/D

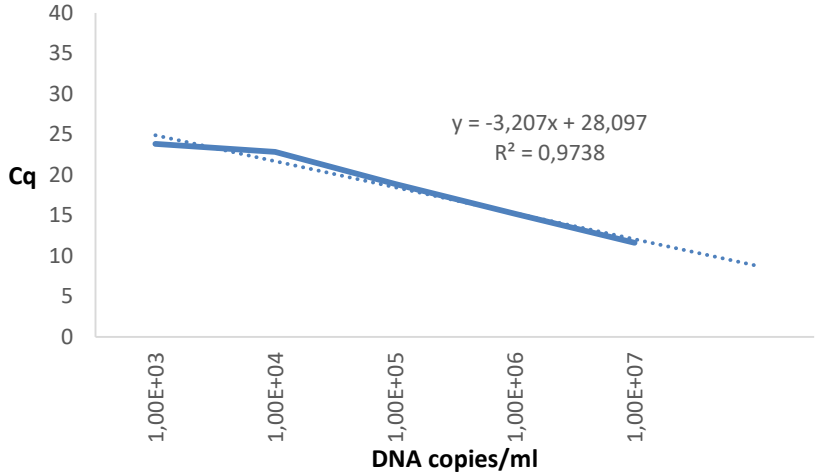

Figure 3.6: Standard curve for *S. pneumoniae* serogroup 9A/V

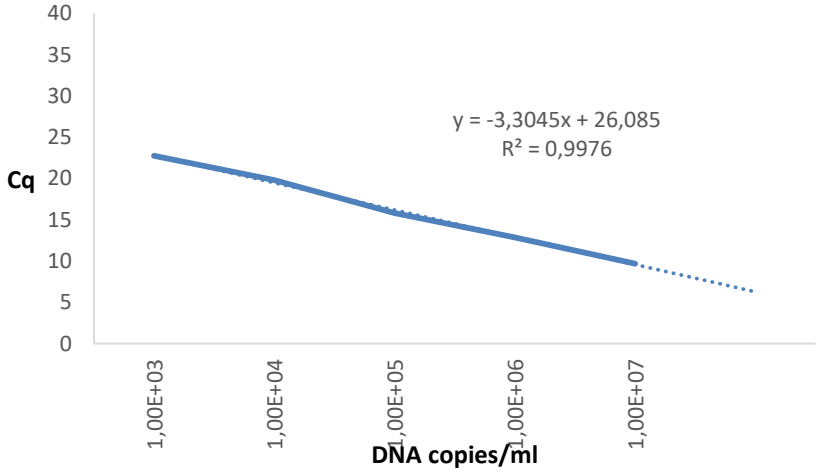

Figure 3.7: Standard curve for *S. pneumoniae* serogroup 7A/F

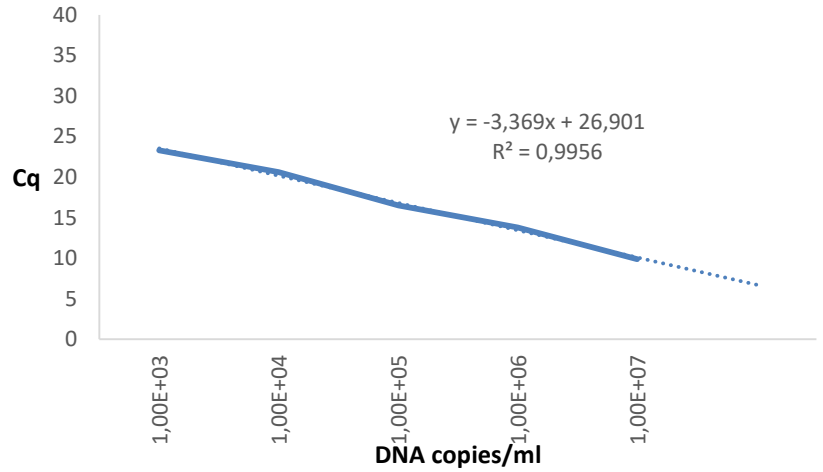

Figure 3.8: Standard curve for *S. pneumoniae* serotype 14

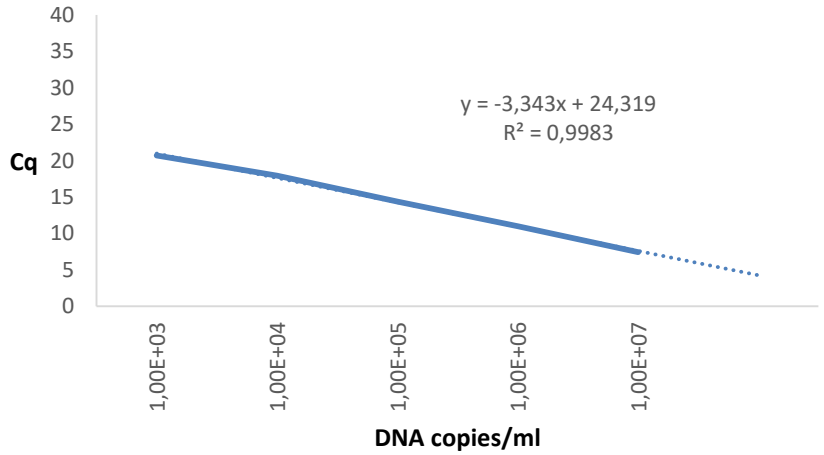

Figure 3.9: Standard curve for *S. pneumoniae* serogroup 18A/B/C

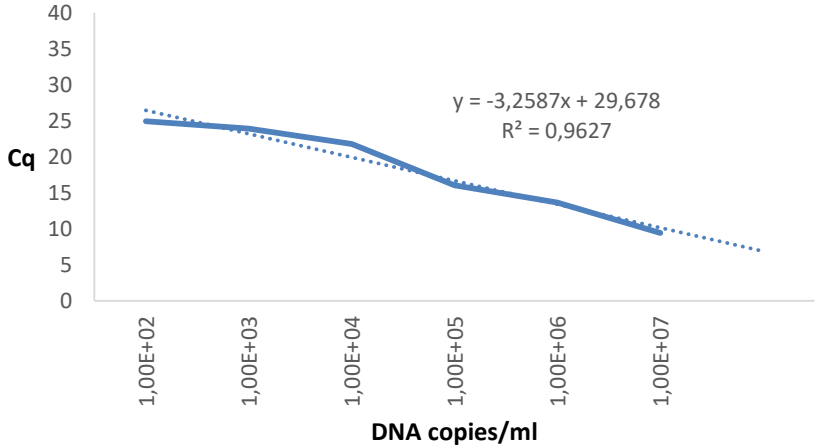

Figure 3.10: Standard curve for *S. pneumoniae* serotype 19F

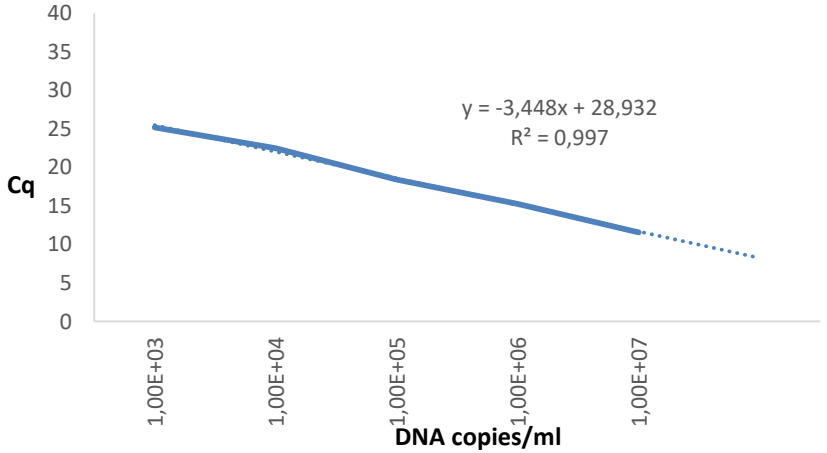

Figure 3.11: Standard curve for *S. pneumoniae* serotype 19A

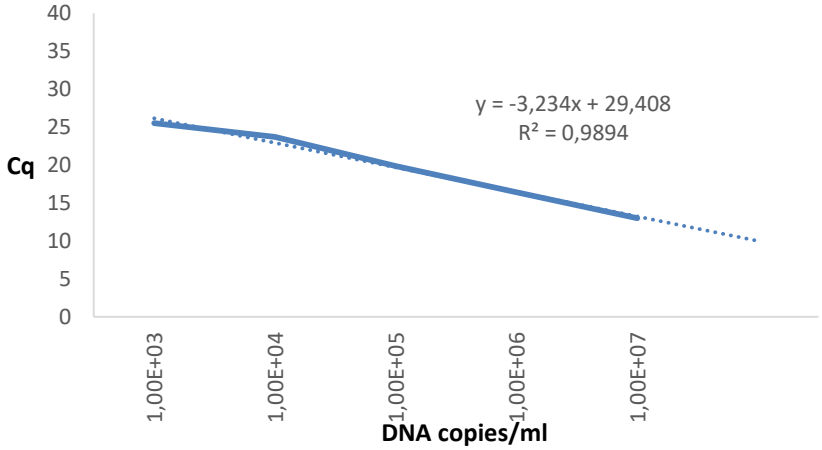

Figure 3.12: Standard curve for *S. pneumoniae* serotype 23F

Table 3.1: The performance of the Fluidigm to detect PCV13 vaccine *S. pneumoniae* serotypes in spiked urine samples

***S. pneumoniae* target**	**Standard curve slope**	**Coefficient correlation (r^2)**	**Efficiency % (e)**	**Limit of detection (LOD)**	**Intra-assay variability (SD)**	**Accuracy**	**Inter-assay variation (SD)**
S. pneumoniae serotype 1	-3.3	0.97	103	1 000	0.02	0.025	0.15
S. pneumoniae serotype 3	-3.4	1	98	1 000	0.08	0.013	0.14
S. pneumoniae serotype 4	-3.2	0.99	105	1 000	0.04	0.068	0.02
S. pneumoniae serotype 5	-3.5	0.97	93	1 000	0.07	0.025	0.03
S. pneumoniae serogroup 6A/B/C/D	-3.5	0.99	92	1 000	0.05	0.006	0.07
S. pneumoniae serogroup 7A/F	-3.3	1	101	1 000	0	0.002	0.04
S. pneumoniae serogroup 9A/V	-3.2	0.97	105	1 000	0.1	0.002	0.14
S. pneumoniae serotype 14	-3.3	0.95	102	1 000	0.12	0.001	0.14
S. pneumoniae serogroup 18A/B/C	-3.3	1	99	1 000	0.15	0.021	0.16
S. pneumoniae serotype 19A	-3.4	1	95	1 000	0.09	0.001	0.04
S. pneumoniae serotype 19F	-3.3	0.96	103	100	0.13	0.003	0.14
S. pneumoniae serotype 23F	-3.2	0.99	104	1 000	0.06	0.004	0.14

Table 3.2: Analytical specificity of the Fluidigm real-time quantitative polymerase chain reaction assay

	S. pneumoniae PCV 13 serotypes targets																		
		Serotype 1	Serotype 3	Serotype 5	Serotype6A	Serotype 6B	Serotype 6C	Serotype 6D	Serotype 7A	Serotype 7F	Serotype 9A	Serotype 9V	Serotype 14	Serotype 18A	Serotype 18B	Serotype 18C	Serotype 19A	Serotype 19F	Serotype 23F
Bacterial control strains	1	X																	
	3		X																
	5			X															
	6A/B/C/D				X	X	X	X											
	6A/C				X		X												
	6C/D						X	X											
	7A/F								X	X									
	9A/L/N/V										X	X							
	14												X						
	18A/B/C													X	X	X			
	18B/C/F														X	X			
	19B/F																	X	
	19F																	X	
	19A																X		
	23F																		X
	23A/B/F																		X

3.2 Study population

Of the 828 adults hospitalised with physician-diagnosed pneumonia, 53% (n=439) were able to provide a urine specimen that was available for testing. We selected a random sample of 96 of the available urine samples to evaluate for detection of *S. pneumoniae* using the Fluidigm, BinaxNOW™ *Streptococcus pneumoniae* antigen card, and *S. pneumoniae LytA* qPCR assays.

The demographics and clinical characteristics of the participants with physician-diagnosed pneumonia stratified by the availability of urine samples and whether or not selected for urine analysis were similar across groups; Table 3.3.

Table 3.3: Demographics and clinical course of patients with community-acquired pneumonia (stratified by those included in the final analysis and those excluded)

Characteristic	Participants with urine samples tested (n=96)	Participants with urine samples not tested (n=343)	Participants with no urine samples (n=389)
Mean age in years, (SD)	45.8 (16.2)	48.2 (18)	48.4 (18)
Female, n (%)	47 (49)	192 (56)	216 (56)
Black race, n (%)	94 (98)	269 (78)	376 (97)
Smoking, n (%)	23 (24)	64 (19)	74 (19)
Alcohol consumption, n (%)	23 (24)	67 (20)	73 (19)
Antibiotic use, n (%)	85 (89)	310 (90)	345 (89)
Clinical features			
Fever, n (%)	59 (61)	195 (57)	172 (44)
Cough, n (%)	93 (97)	333 (97)	350 (90)
Shortness of breath, n (%)	85 (89)	302 (88)	298 (77)
Chest pain, n (%)	70 (73)	250 (73)	215 (55)
Malaise, n (%)	90 (94)	326 (95)	255 (66)
Headache, n (%)	45 (47)	145 (42)	140 (36)

Comorbid conditions			
HIV-infected, n (%)	63 (66)	180 (52)	196 (50)
COPD, n (%)	3 (3)	8 (2)	5 (1)
Asthma, n (%)	3 (3)	14 (4)	14 (4)

Abbreviations - SD: Standard deviation; COPD: Chronic obstructive pulmonary disease; HIV: Human immunodeficiency virus.

3.3 Urinary pneumococcal antigen (BinaxNOW™ *Streptococcus pneumoniae* antigen test card) and *S. pneumoniae* qPCR testing

The BinaxNOW was used as the "gold standard" test and was used to compare the performance of the Fluidigm and the qPCR. Of the urine samples tested in this study, 19% (n=18/96) were positive on the BinaxNOW™ *Streptococcus pneumoniae* antigen assay. Among the BinaxNOW™ *Streptococcus pneumoniae* antigen card positive and negative participants, there were no differences in mean age (mean age: 43.9 vs. 46.1 years) or any other demographic characteristics; Table 3.4. The individuals with a non-reactive BinaxNOW™ test were, however, more likely to have had prior antibiotic use (92%) compared with those with a reactive test (72%) (p= 0.02); Table 3.4.

Table 3.4: Demographics and clinical features of adults with community-acquired pneumonia stratified by pneumococcal urinary antigen status (n=96)

Characteristics	Pneumococcal antigen + (n=18)	Pneumococcal antigen – (n=78)	P- value	OR (95%CI)
Mean age in years, (SD)	43.9 (18)	46.1 (16)	0.61	
Female, n (%)	9 (50)	38 (49)	0.92	1.05 (0.38-2.93)
Black, n (%)	17 (94)	77 (99)	0.25	2.13 (0.51-8.72)
Current smoking, n (%)	5 (28)	18 (23)	0.67	1..28(0.40-4.08)
Alcohol, n (%)	6 (33)	17 (22)	0.30	1.79 (0.58-5.5)
Antibiotic use, n (%)	13 (72)	72 (92)	0.02	0.21 (0.57-0.82)
Days between the collection of urine and the first day of antibiotic use:				
0 days, n (%)	10 (56)	29 (37)		

1 day, n (%)	3 (17)	28 (36)	0.16	0.47 (0.17-1.34)
2 days, n (%)	5 (28)	14 (18)		
3 days, n (%)	0	7 (9)		
Clinical features				
*Fever, n (%)	13 (72)	46 (59)	0.30	1.81 (0.58-5.58)
Cough, n (%)	17 (94)	76 (97)	0.51	0.45 (0.38-5.2)
Shortness of breath, n (%)	16 (89)	69 (88)	0.956	1.04 (0.2-5.3)
Chest pain, n (%)	15 (83)	55 (71)	0.27	2.09 (0.55-7.9)
Malaise, n (%)	17 (94)	73 (94)	0.89	1.16 (0.13-10.6)
Headache, n (%)	9 (50)	36 (46)	0.77	1.16 (0.41-3.25)
Comorbid conditions				
HIV positive, n (%)	14 (78)	49 (63)	0.12	0.99 (0.97-1.01)
COPD, n (%)	0	3 (4)	0.40	ND
Asthma, n (%)	0	3 (4)	0.40	ND
Diabetes mellitus, n (%)	0	7 (9)	0.19	ND

Abbreviations - SD: Standard deviation; COPD: Chronic obstructive pulmonary disease; ND : not done. *Fever was defined as a temperature of more than 37.5ºC. p-values from Chi-squared and Student t-test. ND – Not done - The odds ratio was not calculated for continuous variables or variables with no observations therefore thus no value is recorded for the odds ratio.

Seven percent (n=7/96) of the urine samples had a positive *S. pneumoniae LytA* qPCR result and the demographic or clinical characteristics were compared for those with and those negative for *LytA*; Table 3.5. There was poor concordance between the *LytA* qPCR and BinaxNOW™ reactivity (*kappa*=0.06). Using the BinaxNOW as the reference method, the qPCR method had a low sensitivity for the overall detection of pneumococcus (sensitivity =11%, specificity = 94%); Table 3.6.

Table 3.5 Demographics and clinical features of adults with community-acquired pneumonia stratified by *S. pneumoniae LytA* qPCR results (n=96)

Characteristics	*Lyt A*+ (n=7)	*Lyt A* – (n=89)	OR (95%CI)
Mean age in years, (SD)	48.7 (13)	45.4 (16.6)	
Female, n (%)	4 (57)	43 (48)	1.6 (0.32-7.9)

Black, n (%)	7 (100)	87 (98)	ND
Current smoking, n (%)	0	23 (26)	ND
Alcohol, n (%)	0	22 (26)	ND
Antibiotic use, n (%)	7 (100)	78 (88)	ND
Days between the collection of urine and the first day of antibiotic use:			
0 days, n (%)	5 (71)	34 (38)	
1 day, n (%)	2 (29)	29 (33)	0.13 (0.19-1)
2 days, n (%)	0	19 (21)	
3 days, n (%)	0	7 (8)	
Clinical features			
*Fever, n (%)	5 (71)	54 (61)	1.79 (0.3-10.6)
Cough, n (%)	7 (100)	86 (96)	ND
Shortness of breath, n (%)	5 (71)	80 (90)	0.47 (0.41-5.4)
Chest pain, n (%)	7 (100)	63 (71)	ND
Malaise, n (%)	6 (86)	82 (95)	0.24 (0.21-2.85)
Headache, n (%)	2 (29)	43 (48)	0.5 (0.65-3.83)
Comorbid conditions			
HIV positive, n (%)	5 (71)	58 (66)	0.99 (0.97-1.02)
COPD, n (%)	0	3 (4)	ND
Asthma, n (%)	0	3 (4)	ND
Diabetes mellitus, n (%)	0	6 (7)	11.9 (0.36-399)

Abbreviations - SD: Standard deviation; COPD: Chronic obstructive pulmonary disease, OR: Odds ratio; CI: Confidence interval. ND – Not done - The odds ratio was not calculated for continuous variables or variables with no observations therefore thus no value is recorded for the odds ratio.*Fever was defined as a temperature of more than 37.5ºC.

Table 3.6: Performance of the BinaxNOW compared to the real-time quantitative polymerase chain reaction for *S. pneumoniae autolysin* gene (n=96)

	BinaxNOW (+)	BinaxNOW (-)	Total	p-value	Concordance (*kappa*)	Sensitivity (%)	Specificity (%)

S. pneumoniae LytA qPCR (+)	2 (2)	5 (5)	7 (7)	0.02	0.06	11	94
S. pneumoniae LytA qPCR (-)	16 (17)	73 (76)	89 (93)				
Total	18 (19)	78 (81)	96 (100)				

Numbers are values (%), p-values of ≤0.05 were considered significant. P-value was determined using the McNemar test. Abbreviation: qPCR- quantitative polymerase chain reaction and *LytA-autolysin* gene

3.4 Nanofluidic Fluidigm qPCR

The 16S rRNA gene used as the control was amplified and the there was no amplification for the negative controls. None of the urine samples collected from the adults with CAP were positive for *S. pneumoniae* on the Fluidigm assay.

CHAPTER 4: DISCUSSION

While we were able to optimise the Fluidigm assays for the detection and simultaneous serotyping of *S. pneumoniae* in adult urine samples, we were unable to validate its utility in clinical samples. Whereas the BinaxNOW™ *Streptococcus pneumoniae* antigen card method and the quantitative *S. pneumoniae LytA q*PCR were able to detect *S. pneumoniae* in a limited number of the samples (19% and 7%, respectively). To our knowledge, this was the first study to investigate the utility of a nanofluidic qPCR assay to detect and serotype *S. pneumoniae* in urine samples that were collected from adults hospitalised with clinician-diagnosed pneumonia.

Our failure to validate the Fluidigm in clinical samples could be linked to bacterial loads whereby the *S. pneumoniae* levels in the urine were just too low for the Fluidigm assays (LLD: 100-1000 copies per PCR reactions) to detect. Further, Fluidigm works with nano scale volume of sample input, thus the lower detection as compared to qPCR (7% vs 0%) could be linked to the lower input sample volume of 2.5ul (to test 96 assays) vs.10ul (to test a single assay), respectively. Additionally, previous studies that have detected Legionella DNA in urine using PCR from patients with *legionella* pneumonia found that the bacteria is not excreted continuously (147, 148) which might account for *S. pneumoniae* not being detected by qPCR as DNA excretion in the urine is intermittent.

It is also plausible that the high antibiotic use in our population resulted in very little DNA being excreted in the urine sample as antibiotics inhibit bacterial growth and almost 90% of our population was on antibiotics before sample collection (42). Future studies can also use samples collected from adults without antibiotic exposure and those with prior antibiotic exposure to investigate the effects of antibiotic exposure. Also, a common side effect of pneumonia is dehydration which would increase the urea concentration (149) and might explain why the Fluidigm and qPCR methods failed to detect *S. pneumoniae* as urea inhibits PCR by preventing the annealing of primers (150). Further, the urine samples tested in this study were stored at -80ºC for 2-3 years before testing which could result in the formation of crystals of calcium oxalate, uric acid, and amorphous phosphate that also degrades bacterial DNA (151). Future studies should be done using fresh urine samples and treating the samples before archiving with ethylenediaminetetraacetic acid (Tris-EDTA) as this might reduce the amount of urea and other harmful urine components in the samples and thus protecting the bacterial DNA yield (150). Regardless, neither Fluidigm, nor qPCR method was sufficiently sensitive for the diagnosis of pneumococcal pneumonia using urine samples.

The limited utility of urine for the detection of pneumococcal pneumonia was also noted in a study whereby only 43.3% of urine samples collected from patients with blood culture-confirmed pneumococcal pneumonia were *LytA* PCR positive (57). Further, the study reported that the BinaxNOW™ *Streptococcus pneumoniae* antigen detection method was only able to detect *S. pneumoniae* in 40% of the blood culture-confirmed pneumococcal pneumonia cases which further highlights the limited utility of urine for determining the aetiology of pneumonia. While the BinaxNOW™ *Streptococcus pneumoniae* antigen was found to be more sensitive at detecting *S. pneumoniae* in our study compared to molecular methods (19% versus 7% and 0%) it must also be noted that BinaxNOW has several limitations that might lead to false positives including cross-reacting with closely related *Streptococcus* species, including *Streptococcus constellatus (152)* and *Streptococcus intermedius (153).* Further, the *S. pneumoniae* antigen is shed even weeks after the onset of disease, therefore adults with a previous pneumonia infection may have a false positive antigen test result (49). In addition, a false positive result is possible when using the urine of adults with nasopharyngeal colonisation by *S. pneumoniae* and those with a pneumococcal vaccination history (154). Regardless of these shortcomings, the findings on the BinaxNOW™ in this study yielded similar results (23% positivity) compared with an

earlier study undertaken at the same facility between 2005 and 2007, before the introduction of PCV7 into the childhood immunisation program in South Africa (47).

A major limitation of this study was that even though we were able to report on the overall positivity of *S. pneumoniae* in urine samples collected from hospitalised South African adults using BinaxNOW™ *Streptococcus pneumoniae* antigen card, we were unable to report on the serotype distribution. Other methods such as UAD could be an alternative to serotyping *S. pneumoniae* in urine; however, only the PCV-13 and an additional 11 pneumococcal serotypes can be detected with this method (97). Other study limitations included that we did not have chest X-ray results for the participants which are routinely used to confirm a physician's diagnosis of pneumonia, and thus our study could have potentially included non-pneumonia cases. Moreover, the use of ICD10-codes could have potentially resulted in an overestimation of the burden of pneumonia as hospital-acquired pneumonia could have potentially been included (10).

In conclusion, while the BinaxNOW™ *Streptococcus pneumoniae* antigen card method was the most sensitive of the three methods for the detection of *S. pneumoniae*, all three methods utilised in this study were likely unable to determine the true burden of pneumococcal pneumonia using urine samples. The Fluidigm was unable to detect *S. pneumoniae* in urine samples and future work is needed to identify more suitable samples for the identification of pneumococcal pneumonia using the Fluidigm.

CHAPTER 5: REFERENCES

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APPENDIX 1

HREC certificate

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

R14/49 Ms TR Jeche and Professor M Nunes

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M200430

NAME: (Principal Investigator)	Ms TR Jeche and Professor M Nunes
DEPARTMENT:	School of Pathology Respiratory and Meningeal Pathogens Research Unit/VPD Chris Hani Baragwanath Academic Hospital
PROJECT TITLE:	Utility of real-time PCR (Fluidigm) for serotype specific characterisation of Streptococcus pneumoniae in adults hospitalised with pneumonia
DATE CONSIDERED:	2020/04/24
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Drs V Baillie & C Otwagen; Professor SA Madhi
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2020/10/19

This clearance certificate is valid for 5 years from the date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the 3rd Floor, Philip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.
I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **April** and will therefore reports and re-certification will be due early in the month of **April** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature _____ Date _____