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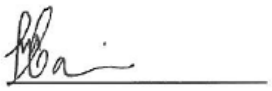
Maimuna Carrim

Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg in fulfillment of the requirements for the degree of Master of Science in Medicine.

Johannesburg, 2015

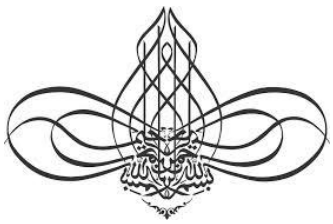
DECLARATION

I, Maimuna Carrim, declare that this dissertation is my own work. Experiments described were conducted under the supervision of Dr Nicole Wolter and Dr Anne von Gottberg at the Centre for Respiratory Diseases and Meningitis – Bacteriology Unit, National Institute for Communicable Diseases, National Health Laboratory Service, Johannesburg. It is being submitted for the degree of Master of Science in Medicine to the Faculty of Health Sciences at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination to this or any other university.



16th day of September 2015

DEDICATION



For mum and dad,

My guiding lights

Who helped me soar to great heights

My well-wishers, my protectors

My pillars of strength

On whom I completely depend

My rocks, my greatest fans

I owe it all to you

Thank you!

PUBLICATIONS IN PREPARATION

M. Carrim, A. J. Benitez, N. Wolter, M. du Plessis, S. Walaza, F. Moosa, M. Diaz, B. Wolff, M. Papo, H. Dawood, E. Variava, C. Cohen, J. M. Winchell and A. von Gottberg. Molecular identification and characterisation of *Mycoplasma pneumoniae* in South Africa, 2012 – 2013. (Article in preparation).

N. Wolter, M. Carrim, C. Cohen, S. Tempia, S. Walaza, P. Sahr, I. Kennedy, L. de Gouveia, F. Treurnicht, O. Hellferscee, A.L. Cohen, A. J. Benitez, M. Papo, H. Dawood, E. Variava, J. M. Winchell, and A. von Gottberg. Legionnaires' disease in South Africa, 2012 – 2014. (Article in preparation).

PRESENTATIONS

M. Carrim. Identification and prevalence of bacteria causing atypical pneumonia in patients with severe respiratory and influenza-like illness in South Africa, 2012. *Rotavirus and Severe Acute Respiratory Illness (SARI) Surveillance Annual Investigators' Meeting 2012*, James Gear Auditorium, NICD, Sandringham, Johannesburg, South Africa, 11 December 2012.

M. Carrim, N. Wolter, M. du Plessis, L. de Gouveia, S. Walaza, F. Moosa, M. Venter, H. Dawood, E. Variava, C. Cohen, and A. von Gottberg. Prevalence of *Legionella* species in patients with severe respiratory illness and influenza-like illness in South Africa, 2012 – 2013. *5th Congress of the Federation of Infectious Disease Societies of Southern Africa,* Drakensburg, South Africa, 10 – 12 October 2013.

M. Carrim. Prevalence of atypical pneumonia-causing bacteria at SARI enhanced sites in South Africa, 2012 – 2013. *Rotavirus and Severe Acute Respiratory Illness (SARI) Surveillance Annual Investigators' Meeting 2013*, James Gear Auditorium, NICD, Sandringham, Johannesburg, South Africa, 12 November 2013.

M. Carrim. *Mycoplasma pneumoniae* among patients with severe respiratory and influenza-like illness in South Africa, 2012 – 2013.*NICD Research Forum*. James Gear Auditorium, NICD, Sandringham, Johannesburg, South Africa, 26 February 2014.

M. Carrim, N. Wolter, M. du Plessis, L. de Gouveia, S. Walaza, F. Moosa, H. Dawood, E. Variava, C. Cohen and A. von Gottberg. Molecular detection of *Mycoplasma pneumoniae* among patients with severe respiratory and influenza-like illness in South Africa, 2012 – 2013. *16th International Conference for Infectious Diseases,* Cape Town, South Africa, 3 – 5 April 2014.

M. Carrim, N. Wolter, M. du Plessis, L. de Gouveia, S. Walaza, F. Moosa, H. Dawood, E. Variava, C. Cohen and A. von Gottberg. Molecular detection of *Mycoplasma pneumoniae* among patients with severe respiratory and influenza-like illness in South Africa, 2012 – 2013. University of the Witwatersrand, *Faculty of Health Sciences, Biennial Research Day and Postgraduate Expo*. Johannesburg, South Africa, 17 September 2014.

ABSTRACT

Atypical pneumonia-causing bacteria contribute considerably to community-acquired pneumonia (CAP), however this contribution is largely underestimated due to the difficulty in identifying these organisms and limited studies targeting them. Rapid diagnostic tools such as real-time polymerase chain reaction (PCR) help to overcome the difficulty in identifying atypical pneumonia-causing bacteria. Our aim was to establish a real-time PCR assay for identification of atypical pneumonia-causing bacteria and determine the prevalence of atypical pneumonia-causing bacteria (*Mycoplasma pneumoniae, Legionella* spp. and *Chlamydia* (*Chlamydophila*) *pneumoniae*) in South Africa.

We enrolled severe respiratory illness (SRI) patients, influenza-like illness (ILI) patients and controls prospectively from June 2012 through December 2013 at two hospitals and two out-patient clinics located in two provinces of South Africa. Demographic, clinical and in-hospital outcome data were collected. Naso-oropharyngeal specimens were collected from all patients and induced sputa were collected from SRI patients only. Total nucleic acids were extracted from clinical specimens and were tested for *M. pneumoniae, C. pneumoniae, Legionella* spp. and human *RNAseP* by a multiplex real-time PCR assay. Macrolide susceptibility testing using high-resolution melt-curve analysis (HRM) and multiple-locus variable-number tandem-repeat analysis (MLVA) was performed on 94% (75/80) of *M. pneumoniae*-positive specimens. *M. pneumoniae*-positive specimens were cultured and P1 typing was performed on culture-positive specimens using HRM. We described trends in disease by age-group, time, gender, HIV prevalence, symptom duration, underlying conditions and clinical outcome. For the comparative analysis the $\chi 2$ test and univariate

logistic regression was performed. Statistical significance was assessed at P<0.05 for all parameters.

From June 2012 through December 2013, 6122 patients were enrolled. Real-time PCR for the identification of the atypical pneumonia-causing bacteria was performed on 85% (5210/6122) of cases which included 86% (2793/3239) of SRI patients, 86% (1670/1940) of ILI patients and 79% (747/943) of controls. The percentage of patients tested by age group was 15% (783/5183), 15% (779/5183), 10% (536/5183), 9% (466/5183), 31% (1603/5183), 16% (827/5183), 4% (189/5183) amongst those aged <1 years, 1-4 years, 5-14 years, 15-24 years, 25-44 years, 45-64 years and ≥65 years, respectively. A total of 4534 of 5210 patients (87%) had a known HIV status of which 2148/4534 (47%) were HIV infected.

The prevalence of atypical pneumonia-causing bacteria among SRI patients in two hospitals in South Africa was 3.3% (91/2793), with a prevalence of 1.5% (25/1670) and 1.3% (10/747) amongst patients with ILI and asymptomatic controls, respectively.

The prevalence of *M. pneumoniae* was 2.1% (59/2793) among SRI patients, 1% (16/1670) among ILI patients and 0.4% (3/747) among controls. *M. pneumoniae* detection was significantly higher amongst individuals aged <5 years (2.6%; 41/1563) compared to individuals ≥5 years (1%; 37/3624) (P<0.001). *M. pneumoniae* cases were detected throughout the study period. The overall attributable fraction of *M. pneumoniae* for patients with SRI, was 89.0% (95% confidence interval [CI] 48.7 – 97.5), after adjusting for age and HIV status. A culture was obtained for 11/75 (15%) of the *M. pneumoniae*-positive specimens. The isolates were distributed into 4/11 (36%), 4/11 (36%) and 3/11 (27%) for P1 type 1, type 2 and a variant of type 2, respectively. Analysis of the combination of tandem

repeats at the four MLVA loci, revealed three distinct MLVA types namely 3562 (43%; 16/37), 3662 (41%; 15/37) and 4572 (16%; 6/37). In *M. pneumoniae*-positive specimens with susceptibility profiles available (43%; 32/75), the mutation conferring macrolide resistance was absent in all (100%; 32/32) cases.

When comparing specimen types for the detection of *Legionella* spp. a significant difference was observed [16/16 (100%) induced sputum-positive vs. 0/16 (0%) nasopharyngeal specimens-positive; P<0.001] between the specimen types. *Legionella* spp. were only detected in SRI patients, with a prevalence of 0.8% (21/2793). Furthermore, among SRI patients, the organism was only detected in patients 15 to 64 years old [2% (3/155) in 15-24 year, 1.1% (10/939) in 25-44 year and 1.5% (8/530) in 45-64 year age groups]. There was no difference in prevalence of *Legionella* spp. between the two SRI sites [6/1312 (0.5% at Edendale hospital vs. 15/1481 (1%) at Klerksdorp-Tshepong hospital; P=0.14]. *Legionella* spp.-positive patients were more likely to have chronic symptom duration >7 days [15/19 (79%) *Legionella* positive vs. 1125/2728 (41%) *Legionella* negative, P=0.003]. A species was only identified for 1 of the 21 cases, and the species was identified as *L. pneumophila* serogroup 1.

The prevalence of *C. pneumoniae* was 0.4% (11/2793) among patients with SRI, 0.5% (9/1670) among patients with ILI and 1% (7/747) among asymptomatic controls. There was no statistically significant difference identified in the detection rate of *C. pneumoniae* between patients with SRI or ILI and controls as the attributable fraction was calculated to be -31% (95% CI –276.75 – 54.28) for SRI cases and -111% (95% CI -629.2 – 38.7) for ILI cases compared to controls.

Our study presented the utility of a multiplex real-time PCR assay for the identification of atypical pneumonia-causing bacteria. This study helps bridge the gap of limited data in South Africa and provides baseline data that can be used for future surveillance programs in the hope of better understanding atypical pneumonia-causing bacteria.

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NOMENCLATURE

<	–	Less than
>	–	Greater than
±	–	Plus-minus
≤	–	Less than or equal to
≥	–	Greater than or equal to
°C	–	Degree Celsius
µl	–	Microliters
ADP	–	Adenosine diphosphate
ATCC	–	American type culture collection
BCYE	–	Buffered charcoal yeast extract
bp	–	Base-pair
BREC	–	Biomedical Research Ethics Committee
CAP	–	Community-acquired pneumonia
CARDS	–	Community-acquired respiratory distress syndrome
CDC	–	Centers for Disease Control and Prevention
CO_2	–	Carbon dioxide
CRDM	–	Centre for Respiratory Disease and Meningitis
CRP	–	C-reactive protein
Ct	–	Cycle threshold
DNA	–	Deoxyribonucleic acid
dNTP	–	Deoxyribonucleotide triphosphate
DTT	–	Dithiothreitol
EB	–	Elementary body

ELISA	–	Enzyme-linked immunosorbent assay
EWGLINET	–	European Surveillance Scheme for Travel Associated Legionnaires' disease
HiDi	–	Highly deionized
HIV	–	Human immunodeficiency virus
HREC	–	Human Research Ethics Committee
HRM	–	High-resolution melt-curve analysis
IgG	–	Immunoglobulin G
IgM	–	Immunoglobulin M
ILI	–	Influenza-like illness
kDa	–	Kilodalton
LD	–	Legionnaires' disease
LRTI	–	Lower respiratory tract infection
$MgCl_2$	–	Magnesium chloride
MIF	–	Microimmunofluorescence
ml	–	Milliliters
MLVA	–	Multiple-locus variable-number tandem repeat analysis
mM	–	Millimolar
MOMP	–	Major outer membrane protein
n	–	Number
N	–	Total number
NA	–	Nucleic acid
ng	–	Nano-grams
ng/µl	–	Nano-grams per microliter
NICD	–	National Institute for Communicable Diseases

nM	–	Nanomolar
NP	–	Nasopharyngeal
P	–	Probability
PBS	–	Phosphate buffered saline
PCR	–	Polymerase chain reaction
PFGE	–	Pulse-field gel electrophoresis
pH	–	Power of hydrogen
PHC	–	Public health care
QCMD	–	Quality Control for Molecular Diagnostics
RB	–	Reticulate body
RFLP	–	Restriction fragment length polymorphism
rpm	–	Revolutions per minute
rRNA	–	Ribosomal ribonucleic acid
RSV	–	Respiratory syncytial virus
SARI	–	Severe acute respiratory illness
SG 1	–	Serogroup 1
SRI	–	Severe respiratory illness
SV	–	Small volume
TB	–	Tuberculosis
U	–	International Units
USA	–	United States of America
UTM	–	Universal transport media
UV	–	Ultraviolet
VNTR	–	Variable number tandem repeat

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1 Literature Review

1.1 Pneumonia – burden of disease and risk factors

Pneumonia, an inflammation of the lung tissue, causes acute illness in adults and children. Patients commonly present with a cough productive of purulent sputum, pyrexia and dyspnoea [1]. Lower respiratory tract infections (LRTI) were the fourth leading cause of death, accounting for 5.5% of deaths in all age groups, globally and is the leading cause of death in low-income countries causing 91 deaths per 100 000 population in 2012 [2]. Worldwide, in children younger than five years of age, approximately 18% of deaths were attributed to pneumonia in 2008 [3]. Acute pneumonia can be subdivided into nosocomial pneumonia and community-acquired pneumonia (CAP).

Nosocomial pneumonia is a pneumonia that is caused by an infection with pathogens acquired in a hospital whereas, CAP is defined as a pneumonia that is caused by pathogens, most often bacteria, acquired outside of a hospital setting which results in lung infiltrates which are visible by chest radiographs [4]. The incidence of CAP differs between countries ranging from 1.6 to 11 per 1000 adults per year [5]. In a multicentre study from 2010 to 2012 in the USA, 21% of children with CAP required intensive care and 3% died [6]. In South African children, up to 40% of hospital admissions between the periods 1992 to 1997 were due to CAP [7;8].

With the advent of HIV there has been an increase in the incidence of childhood pneumonia [7]. In sub-Saharan African, 11% to 45% of children hospitalised with pneumonia are also HIV positive [8-10]. Results from a study in South Africa in 2000 indicated that the case-fatality ratio is approximately 6-fold higher amongst HIV-infected children compared to

HIV-uninfected children [9]. In a study conducted in Kwa-Zulu Natal, South Africa in 2007, HIV-infected children were more likely to develop severe pneumonia and have a higher mortality rate than HIV-uninfected children [10]. Furthermore, it was shown that children infected with more than one respiratory pathogen have a higher risk of mortality than children infected with a single pathogen [10].

1.2 Diagnosis of pneumonia and identification of aetiology

Diagnosis of CAP is based on clinical evaluations [11]. Even though there are numerous methods to identify the causative agents of CAP, the exact incidence of specific pathogens remains unknown.

Current diagnosis of CAP is achieved using clinical manifestations confirmed by chest radiography. Chest radiographs only facilitates the clinical diagnosis and is unable to identify the microbial aetiology of pneumonia as the observations on radiographs may be similar for different respiratory organisms [12]. Furthermore, the ability to diagnose patients with pneumonia using chest radiographs differs by age in that it is more difficult in children compared to adults [13]. Diagnosis using chest radiography in adults and children suspected of having CAP does not improve the clinical outcome of the patient [13].

Blood and sputum culture may determine aetiology however, they are time-consuming, potentially taking weeks for growth and an aetiological agent is only identified in 20% to 25% of CAP cases [14;15].

Serology has been used for the identification of the aetiological agent; however there are drawbacks to this method. Serology usually requires both acute and convalescent sera resulting in a retrospective diagnosis which has limited utility for treatment [16]. Additionally, serology has low sensitivity in acute illness attributable to false-negative results obtained in the early onset of illness as antibodies may only appear after two weeks of infection [17].

Molecular techniques such as polymerase chain reaction (PCR) have been shown to overcome the aforementioned limitations. Consequently, using PCR could assist in determining the microorganisms responsible for causing CAP [17]. Recent studies comparing culture, serology and PCR have shown that instead of culture, PCR and serology should be used to identify these organisms [18]. Multiplex real-time PCR assays have been developed to detect an increased number of pathogens thereby increasing the speed of detection of bacterial pathogens [18;19]. Rapid diagnosis of the aetiology of CAP is of importance as quick identification will assist in the use of appropriate antibiotics [18].

1.3 Causes of disease

CAP can be caused by both viral and bacterial pathogens, however it is frequently associated with bacterial pathogens [20]. Bacterial causes of CAP can be divided into typical and atypical bacteria based on the therapeutics required to treat patients [16;21].

'Typical' respiratory bacterial pathogens include *Streptococcus pneumoniae, Haemophilus influenzae, Staphylococcus aureus* and *Moraxella catarrhalis* to name a few. *S. pneumoniae* is the most common bacterial cause of CAP. Global rates reported in 2000 indicated that

approximately 13.8 million cases of pneumonia in children less than 5 years of age were due to the pneumococcus [21].

Although *Mycoplasma pneumoniae*, *Legionella* species and *Chlamydia* (*Chlamydophila*) *pneumoniae* cause pneumonia which cannot be clinically distinguished from other aetiological causes of pneumonia, they have been termed collectively as atypical pneumonia-causing bacteria as the empiric treatment used for pneumonia which is β-lactam antibiotics are ineffective against them. Atypical pneumonia-causing bacteria account for approximately 10% to 20% of CAP worldwide [22]. A study performed in 1994, in South Africa using serology found that approximately 35% of pneumonia cases in hospitalised adults were due to atypical bacteria [23]. In other South African studies reviewed between 1983 and 2001, it was found that the prevalence differs by geographical location, the type of patients investigated and whether the study was performed in-hospital or in the community [24]. However, little data are available for the prevalence of atypical bacteria responsible for CAP in South Africa. This is particularly important in a high HIV-prevalence setting as HIV-infected individuals are more susceptible to a wider range of pathogens not commonly associated with HIV-negative individuals [25].

1.4 Treatment

The first-line treatment for patients infected with typical bacterial pathogens are β-lactam antibiotics, however in patients infected with atypical bacteria these antibiotics are ineffective. The inactivity of β-lactam antibiotics against atypical pneumonia-causing bacteria is due to either the absence of a cell wall, the absence of peptidoglycan or due to poor intracellular penetration among atypical pathogens [16]. Hence, macrolides, ketolides and

fluoroquinolones are used to treat patients infected with atypical pneumonia-causing bacteria [26].

1.5 Atypical pneumonia-causing bacteria

1.5.1 Methods of identification

Atypical pneumonia-causing bacteria can be identified with culture, serology, PCR and immunohistochemistry. Although culture may be the gold standard for identification, *M. pneumoniae*, *Legionella* spp. and *C. pneumoniae* are fastidious, slow-growing organisms requiring specialised media for growth [18;27]. Furthermore, culturing requires experienced, well-trained personnel and lacks inter-laboratory consistency [28]. Serology can be used as it is more sensitive than culture; however, serology results are often delayed due to false negatives observed in early stages of illness [18]. Research has focused on the development of rapid and accurate methods for detection. Molecular methods for detection offer a rapid and sensitive alternative for organism identification.

Mycoplasma pneumoniae

Studies comparing serology to PCR have shown that during early infection, PCR has superior sensitivity and is more rapid than serology for the diagnosis of *M. pneumoniae* [17]. Real-time PCR assays using different gene targets have been used. Targets include the P1 adhesin gene, 16S rRNA and the ATPase operon. PCR using these targets have been shown to be more sensitive than culture [29] and results comparing serology to PCR have correlated [30]. Sensitivities and specificities of real-time PCR assays differ depending on the targets used. PCR assays using the 16S rRNA gene have been found to be more sensitive than assays using P1 adhesin gene. Hence, the 16S rRNA gene is more suitable for the diagnosis of *M.*

pneumoniae infections in clinical samples compared to the P1 adhesin gene [31;32]. Evaluation of three PCR targets for the identification of *M. pneumoniae* was done by Winchell *et al*. in which the use of two ATPase targets (*MP3* and *MP7*) and the CARDS toxin gene target (*MP181*) were compared [33]. The *MP181* target was more sensitive and has been used to identify outbreak and sporadic cases [33].

Chlamydia (Chlamydophila) pneumoniae

Immunohistochemistry can be used to identify *C. pneumoniae* in tissue specimens. However, it is difficult to differentiate between true positive and negative results [28]. The wide variety of pre-treatment and staining methods further hinders inter-laboratory comparisons. Microimmunofluorescence (MIF) serological assays are the gold standard for the diagnosis of a *C. pneumoniae* infection. MIF assay is an indirect fluorescent antibody technique that detects the binding of an antigen to an antibody (IgG or IgM) which is detected by a secondary fluorescein [34]. The fluorescein is conjugated to an anti-globulin corresponding to the antibody molecule. The MIF assay however has drawbacks as trained personnel are required for interpretation of results and analyses of results are subjective [28]. In a study comparing MIF assays with real-time PCR it was observed that real-time PCR is better for rapid and accurate detection, and more useful for the identification of *C. pneumoniae* in outbreaks [35]. PCR has been found to be more sensitive than culture for the identification of *C. pneumoniae* in sputum, nasopharyngeal and throat swabs, bronchoalveolar lavages and oral washes. However, the lack of a 'gold standard' makes assessing the specificity difficult. A variety of gene targets have been investigated for *C. pneumoniae* identification such as rRNA sequences, a 60kDa cysteine-rich outer membrane protein [36], the major outer membrane protein (MOMP) [37], the arginine repressor protein [19], 53kDa protein and the 16S-23S spacer rRNA [29]. Depending on the target used, real-time PCR assays have varying

sensitivities and specificities. An assay targeting the 16S rRNA gene has been reported to have a sensitivity of 88.5% and a specificity of 99.3% [38]. Research has indicated that the arginine repressor protein has 100% efficiency in detecting *C. pneumoniae* in a multiplex PCR reaction compared to 93% efficiency in a singleplex reaction. The increased efficiency observed in a multiplex reaction compared to the singleplex reaction could be attributed to improvements in reagents used specifically for multiplex real-time PCR [19].

***Legionella* spp.**

Urinary antigen tests, which are rapid and low in cost, have been developed for the diagnosis of Legionnaires' disease however, they are only able to identify *Legionella pneumophila* serogroup 1 (Sg 1) infection, resulting in an infection with non-*Legionella pneumophila* and other serogroups of *Legionella pneumophila* to be undiagnosed and is one of the reasons the burden of disease caused by *Legionella* spp. is underestimated [16].

PCR based tests are considered to be the test of choice for identification of *Legionella* spp. in lower respiratory tract specimens [30]. Targets used for PCR identification of *Legionella* spp. are the 16S rRNA gene [39], 5S rRNA gene, *mip* gene responsible for allowing the bacteria to infect host macrophages [40] and the *ssrA* gene encoding for a binding protein for stable association with ribosomes and have been found to be as sensitive as culture [29]. Diederen *et al.* evaluated the use of PCR assays targeting the 16S rRNA gene, 5S rRNA and the *mip* gene. All three assays were 100% specific but differed in the sensitivities. The limit of detection for assays targeting the 16S rRNA gene, 5S rRNA and the *mip* gene were 10fg, 100fg and 1000fg, respectively [41;42]. Optimisation of a multiplex PCR performed by Thurman *et al*. indicated that the *ssrA* gene was able to detect 48 *Legionella* species, with an efficiency of 97% and the limit of detection of the assay was 50 fg [19;43].

1.5.2 *Mycoplasma pneumoniae*

a. Microbiology

M. pneumoniae is a common atypical pneumonia-causing pathogen that affects both upper and lower respiratory tracts of children and adults and is spread by respiratory droplets produced by coughing [44-46]. The incubation period of *M. pneumoniae* is two to three weeks [44]. *M. pneumoniae* is an extracellular organism and the smallest known bacterium both in terms of its phenotypic size as well as its genome size [44]. *M. pneumoniae* is known as a short rod-shaped bacterium however, due to the absence of a cell wall it can be found to be pleomorphic [44]. It is a fastidious organism that takes approximately one to two weeks to grow *in vitro*. Due to the absence of a cell wall, β-lactam antibiotics are ineffective against *M. pneumoniae* and identification using Gram-staining is unsuccessful [18;44]. At one end of the bacterium there is an attachment organelle which allows *M. pneumoniae* to attach to respiratory epithelial cells [18;44]. The specialized end has a core made of a rod-like filament surrounded by a transparent space enclosed by the cell membrane. The tip comprises of adhesins, interactive proteins and adherence proteins. One such adhesion protein is the P1 adhesin which is a 170kDa protein found at the tip of the terminal organelle [45].

b. Clinical manifestations

M. pneumoniae is transmitted easily to household contacts resulting in a broad range of clinical manifestations. The progression of disease varies amongst individuals with the worst outcome being death [47]. *M. pneumoniae* has a cyclical nature of transmission, with epidemics lasting 1 to 2 years and no clear association with seasons [48].

Symptoms of disease include a cough, malaise, myalgia and a headache. Lung infiltrates are visible on radiographs [49]. Patients can have pneumonia, exacerbation of wheezing, tracheobronchitis and pharyngitis [45].

M. pneumoniae adherence to the respiratory epithelium is a pre-requisite for disease [45]. Clinical characteristics of *M. pneumoniae* differ with age in that fever persists for a longer period and there is a higher level of C-reactive protein (CRP) with severe pulmonary lesions in children ≥6 years. Lymphocyte differentials and platelet counts are found to be lower in children ≥6 years [45].

Extrapulmonary manifestations may occur with *M. pneumoniae* infections since *M. pneumoniae* adheres to and alters red blood cells causing acute and chronic blood infections [50].

The severity of disease is dependent on the biological properties of individual strains and concentration of CARDS (community-acquired respiratory distress syndrome) toxin [51]. The CARDS toxin is an ADP-ribosylating and vacuolating cytotoxin and is responsible for *M. pneumoniae* induced pulmonary inflammation. Furthermore, CARDS toxin concentration in bronchoalveolar lavage is directly related to the ability of specific *M. pneumoniae* strains to colonise, replicate and persist [51].

c. **Epidemiology**

The incidence of *M. pneumoniae* infection is unknown due to the lack of reliable tests and clinicians rarely requesting testing as *M. pneumoniae* are predominantly treated empirically.

Furthermore, due to the fastidious nature of the bacterium identification of *M. pneumoniae* is difficult [18;44]. Nevertheless, globally *M. pneumoniae* is suggested to be responsible for approximately 15% to 20% of cases of CAP with varying incidence depending on the method of identification [45]. Over an 8-year period (1996 to 2004), in 4133 adult patients in 21 countries using serology, PCR and culture, *M. pneumoniae* was found to be the most common atypical bacterium, responsible for 12% of atypical CAP [26]. In another study, conducted in South Africa among hospitalised adults using serology for identification, it was found that *M. pneumoniae* was responsible for 1% of atypical CAP cases [23].

d. **Treatment**

Similar to all other atypical pneumonia-causing bacteria, treatment of pneumonia caused by *M. pneumoniae* involves the use of macrolide or azalide antibiotics [44;52]. Furthermore, azithromycin prophylaxis administered in institutional outbreaks of *M. pneumoniae* was found to reduce secondary transmission of *M. pneumoniae* [52;53]. However, resistance to macrolides has been documented in many countries [45;54-59] including USA [54;59] and Japan [56]. In 1995, Lucier *et al.* described that macrolide-lincosamide-streptogramin (MLS) mutation can occur due to a point mutation in the 23S rRNA gene of *M. pneumoniae* which reduces the affinity of these antibiotics for the ribosome thus conferring resistance to macrolides [55]. Macrolide resistance can be identified by sequencing domain V of the 23S RNA or use of a PCR targeting this region [59].

e. Molecular characterisation

P1 typing

M. pneumoniae has a highly uniform genome. It can be characterised due to variation of the P1 gene. The P1 gene is an important virulence factor and helps in the adherence of *M. pneumoniae* to respiratory epithelial cells [60]. *M. pneumoniae* is characterised as type 1 or type 2 and variants thereof, based on the P1 gene. Real-time PCR followed by high-resolution melt-curve analysis (HRM) has been successful in distinguishing the two *M. pneumoniae* types, as type 2 isolates have a lower melting temperature compared to type 1 isolates [60]. Deviations in melting temperature from the type 1 and type 2 indicate the presence of a variant. Differentiating isolates based on the P1 gene is important in monitoring epidemiological trends of outbreaks and can be used to identify type switching which may occur due to population immune pressure [60].

Multiple-locus variable number tandem repeat analysis (MLVA)

Bacterial genomes naturally contain a number of tandem repeated DNA sequences. MLVA is a molecular technique that exploits this characteristic of the genome as it is based on the copy number of these tandem repeats in specific loci. Analysis is based on the size of the fragment generated by PCR of specific loci. The size of the DNA fragment corresponds to the amount of tandem repeats present. Variation in the amount of tandem repeats in the specific loci may differ amongst isolates hence isolates can be differentiated [61].

The use of MLVA typing for *M. pneumoniae* is important to differentiate strains of *M. pneumoniae*. In 2009, MLVA was developed to differentiate 26 variable number tandem repeat (VNTR) types by determining the variation in the copy number of tandemly repeated

sequences within five different loci within the genome [62]. MLVA has been shown to have a higher discriminatory power for *M. pneumoniae* compared to restriction fragment length polymorphism (RFLP) and pulse-field gel electrophoresis (PFGE) [62].

1.5.3 *Legionella* species

Legionnaire's disease is caused by bacteria of the genus *Legionella* [43]. There are currently 48 different species of *Legionella* comprising of 70 serogroups. In the United States, *Legionella pneumophila* is of clinical importance as approximately 90% of cases of Legionellosis are due to this species [63] whereas in Australasia *L. longbeachae* is more prevalent [64]. *L. pneumophila* was first identified by Brenner, Steigerwalt and McDade in 1979 due to an outbreak occurring at a hotel in Philadelphia in 1976. The patients in the outbreak were former military veterans attending a legion conference thus the bacterium was named *Legionella* [65].

a. Microbiology

Legionella spp. are small Gram-negative, opportunistic bacilli mostly found in warm water environments such as swimming pools, cooling towers, air-coolers, freshwater ponds and fountains. It is spread by inhalation of mist droplets containing bacteria. *L. longbeachae* is an exception to this as it is a soil-borne pathogen [63]. Transmission of *Legionella* spp. occurs by exposure to an environmental source while person-to-person transmission has not been documented [44]. Prevention of a *Legionella* infection involves adequate chlorination, temperature control and proper sealing of water [1]. *Legionella* spp. are fastidious organism that are grown on buffered charcoal yeast extract (BCYE) agar with or without antibiotics as they require cysteine. *Legionella* spp. are unable to produce urease, nitrate reductase but

produce catalase and β-lactamase. Some species of Legionella fluoresce under UV light. Fluorescence may be blue-white as in the case of *L. bozemanii* or red as in *L. erythra*.

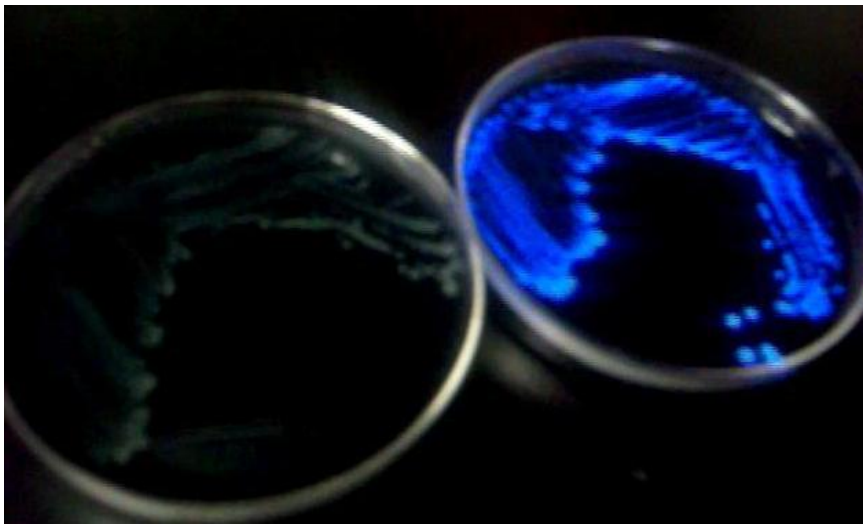


Figure 1: Culture of *Legionella* species indicating non-fluorescent *Legionella* species (left) and blue-white fluorescent *Legionella* species (right)

b. **Clinical manifestations**

Patients with Legionellosis may present with Pontiac fever or Legionnaires' disease (LD). Pontiac fever is a mild, self-limiting fever without pneumonia and is often undiagnosed as patients do not seek care [1;44]. It is most often diagnosed in an outbreak setting. Patients may present with fever, myalgia, asthenia and headaches lasting for three to five days.
LD causes an acute pneumonia. Patients with LD present with a dry cough that becomes purulent, malaise, myalgia, headaches, confusion, hyponatremia, hypophosphatemia and fevers of up to 40°C with rigors. Vomiting, diarrhoea and abdominal pain can occur in some

patients [1;44]. However, presentation of disease is nonspecific making clinical diagnosis difficult. Therefore, treatment is often empirical.

c. Epidemiology

LD may occur in community outbreaks, nosocomial outbreaks and can be associated with travel [44].

Travel-associated Legionnaires' disease

The incidence of travel-associated LD is underestimated mainly because many people from different countries may be affected and there is a lack of inter-country reporting of cases. However, in Europe, travel-associated LD surveillance exists which began in 1987. In 2010, the European Surveillance Scheme for Travel Associated Legionnaires' Disease (EWGLINET) reported an increase of 6% of travel-associated LD cases (confirmed by serology, culture, urinary antigen tests and PCR) in 19 of the 35 collaborating countries and two non-European countries, compared to cases confirmed in 2009 [66;67].

Community-acquired Legionnaires' disease

From 1996 to 2004, community-acquired LD was found to be responsible for 5% of community-acquired atypical pneumonia cases, globally, with 4% and 9% of cases occurring in North America and Europe, respectively [26].

In the United States of America, incidence rates increased from 0.39 cases per 100 000 persons in 2000 to 1.15 cases per 100 000 persons in 2009. Furthermore, it was found that the majority of the *Legionella* cases (99.5%) had LD [68]. Surveillance data from 33 countries in

Europe, in 2007, reported an incidence rate of 11.3 cases per million population for LD. In the following year, in 34 countries the incidence rate of LD was 11.8 per million population using urinary antigen tests and culture for diagnosis [69].

In adults hospitalised with pneumonia in South Africa from 1987 to 1988, using serology, *L. pneumophila* was found to be responsible for approximately 9% of pneumonia cases [23]. In a study published by Yu *et al*. in 2002, speciating 509 cases of culture-confirmed, community-acquired *Legionella* from the United States, Italy, Switzerland, Australia and New Zealand, it was found that *L. pneumophila* caused 91%, *L. longbeachae* caused approximately 4% and *L. bozemanii* caused 2.4% of *Legionella* infections followed by a combined prevalence of 2.2% of *L. micdadei, L. feeleii, L. dumofii, L. wadworthii* and *L. anisa* [70]. Limited data exist for the incidence of LD in South Africa. Concerted efforts for active surveillance are required to accurately ascertain incidence of Legionellosis in South Africa.

1.5.4 *Chlamydia* (*Chlamydophila*) *pneumoniae*

C. pneumoniae was first isolated in 1965 from the eye of a child enrolled in a trachoma vaccine study in Taiwan. However, the first isolate from the respiratory tract was obtained in 1983 from a university student [44].

a. **Microbiology**

C. pneumoniae is a Gram-negative, obligate intracellular respiratory pathogen [26]. However, it has a biphasic life cycle. It exists in an extracellular form called an elementary body (EB) and an intracellular replicative form called a reticulate body (RB). Elementary bodies are

phagocytosed into the host cell. Inside the phagosome, the EB are transformed into RB and undergo replication using the host cell energy resources. Thereafter, the RB transforms into an EB and lyses out of the host cell. *C. pneumoniae* can be cultured using human cell lines such as HeLa 229 cells with dextran, HL cells, HeP-2 cells or NCI-H 292 cells [71].

b. **Clinical manifestations**

C. pneumoniae typically causes mild disease such as bronchitis and sinusitis [44]. Clinical symptoms closely resemble symptoms of *M. pneumoniae* infections. Patients with a *C. pneumoniae* infection may experience central nervous system syndromes (such as headaches and confusion), shortness of breath and increase in white blood count. However, a decrease in pulse rate is not associated with *C. pneumoniae*. Patients with a *C. pneumoniae* infection are more likely to have a cough on admission to a healthcare facility than patients with a *M. pneumoniae* infection [72]. *C. pneumoniae* is commonly associated with gastrointestinal tract syndromes such as vomiting and diarrhoea.

c. **Epidemiology**

The prevalence of *C. pneumoniae* causing CAP varies among studies due to the different methods used for identification, the population studied, the location of the study and the season in which the study was performed. However, it was reported that in 1996 through 2004, *C. pneumoniae* was responsible for 7% of atypical pneumonia cases, globally [26]. In South African hospitalised adults from 1987 to 1988, using serology, *C. pneumoniae* was the most common atypical pneumonia-causing bacteria detected, responsible for 21% of atypical CAP cases, compared to *L. pneumophila, M. pneumoniae, C. psittaci* and *C. burnetii* [23].

1.5.5 Importance of monitoring atypical pneumonia-causing bacteria

Atypical pneumonia-causing bacteria contribute considerably to CAP, however this contribution is largely underestimated due to the difficulty in identifying these organisms and limited studies targeting them. In South Africa, the prevalence of CAP attributable to atypical pneumonia-causing bacteria is unknown. With advances in molecular techniques, the aetiology of CAP and the burden that atypical bacteria contribute to CAP can be more accurately determined. Establishment of a rapid diagnostic tool such as PCR for identification of atypical bacteria-causing pneumonia is important as current tools used are inadequate and have drawbacks hindering effective diagnosis. In addition, as multiple pathogens, including viral pathogens, are detected, co-infections and interactions between different respiratory pathogens can be investigated. Characterisation of the pathogens responsible for CAP in South Africa will assist in the improvement of prevention and treatment strategies.

2 Objectives

i. To utilise a multiplex real-time PCR assay for the detection of the atypical pneumonia-causing bacteria: *M. pneumoniae, Legionella* spp. and *C. pneumoniae*

ii. To investigate the influence of the type and quality of specimen on the detection of *M. pneumoniae, Legionella* spp. and *C. pneumoniae*

iii. To describe and compare the prevalence and characteristics of patients with severe respiratory illness, mild respiratory illness and asymptomatic controls that are infected with *M. pneumoniae, Legionella* spp. or *C. pneumoniae*

iv. To genotypically characterise and determine macrolide susceptibility of *M. pneumoniae* strains

3 Materials and Methods

3.1 Surveillance programmes

Patients of all ages presenting with severe respiratory illness (SRI), influenza-like illness (ILI) and a sample of controls from two sentinel surveillance sites (Edendale hospital and Klerksdorp-Tshepong hospital complex) in two provinces (Kwa-Zulu Natal and North-West province) of South Africa were investigated in this study. SRI surveillance started in 2009, and was enhanced to include surveillance for atypical pneumonia-causing bacteria in June 2012. ILI surveillance was initiated in 2012. Surveillance programmes are on-going and this study includes cases enrolled from June 2012 through December 2013.

Cases were enrolled as part of two surveillance studies being conducted by the Centre for Respiratory Disease and Meningitis (CRDM) of the National Institute for Communicable Diseases (NICD):

3.1.1 Severe respiratory illness (SRI) surveillance programme

SRI surveillance is prospective, hospital-based surveillance at two sentinel sites (Edendale hospital and Klerksdorp-Tshepong hospital complex) in two provinces of South Africa. The programme aims to describe the aetiology of, and risk factors for, severe respiratory illness in South Africa. Patients enrolled in the SRI study include patients hospitalised with clinical signs and symptoms of lower respiratory tract infection (LRTI) irrespective of duration of symptoms; any child (2 days to <3 months old) with a diagnosis of suspected sepsis, children ≥3 months to <5 years with physician-diagnosed acute LRTI including bronchiolitis, pneumonia, bronchitis and pleural effusion, and any person ≥5 years old with sudden onset or a history of fever and cough or sore throat and shortness of breath, difficulty breathing with

or without clinical radiographic findings of pneumonia or tachypnea [73]. Clinical specimens, as well as demographic and clinical information, are collected from all enrolled patients.

3.1.2 Influenza-like illness (ILI) programme

The ILI surveillance study is a prospective study aimed to describe the burden and aetiology of mild respiratory disease in South Africa. Patients of all ages who fit the clinical case definition of ILI are enrolled and clinical and demographic information together with clinical specimens are collected. Patients and controls are enrolled at primary health care (PHC) clinics (Jouberton and Edendale Gateway clinics) that serve the two sentinel sites. Patients are regarded as having ILI if they have an acute fever of >38°C or a self-reported fever within the last 7 days, and either a cough or sore throat and the absence of other diagnoses.

Controls were systematically selected if they presented at PHC clinics with no history of respiratory illness, diarrhoeal illness or fever in the preceding 14 days. They presented at the clinic for visits such as dental procedures, family planning, well-baby clinics, voluntary HIV counselling and testing or acute care for non-febrile illnesses. Individuals' medical and symptom history was systematically verified by a trained nurse using a structured checklist. This information was obtained through a medical chart review and interview with the patient or legal guardian for children less than 15 years old. One HIV-infected and one HIV-uninfected control were enrolled every week in each ILI clinic within each of the following age categories: 0-1, 2-4, 5-14, 15-54 and ≥55 years.

3.1.3 Demographic and clinical data collection

Demographic and clinical data were collected through a structured interview and hospital record review by trained surveillance officers. Data were collected and recorded on a case investigation form, hospital result form and final outcome form (Appendix 1, Appendix 2 and Appendix 3). Data collected included socio-demographic factors, presenting symptoms, duration of symptoms and underlying illnesses including HIV and tuberculosis (TB) exposure and treatment. For all patients, history of influenza immunisation was recorded. In addition, for children <5 years, routine immunisation history was documented, and confirmed from the patients Road-to-Health card.

3.1.4 Sample collection and transport

For SRI cases, induced sputum and nasopharyngeal specimens were collected. For ILI cases and controls, only nasopharyngeal specimens were collected. Blood specimens were collected from all consenting individuals for HIV testing. Nasopharyngeal specimens included combined oropharyngeal and nasopharyngeal flocked swabs (Copan Italia, Brescia, Italy) from ≥5 year old patients and nasopharyngeal aspirates from <5 year old patients. Nasopharyngeal specimens in Universal Transport Media (UTM) (Copan Italia, Brescia, Italy), and induced sputa were transported at refrigeration temperature (4°C-8°C) to the NICD, Johannesburg. From July 2013 induced sputum specimens were stored at -20˚C after collection, and transported to the NICD on dry-ice.

3.2 Processing of specimens

3.2.1 Sputum assessment

Prior to processing, induced sputum specimens were thawed completely and assessed macroscopically and microscopically to ascertain the quality of the sputum, and cultured. For the macroscopic evaluation, the induced sputum was classified as saliva for clear, watery sputum, as mucoid for clear and sticky sputum, purulent for sputum with varying amounts of pus, sometimes mixed with mucus, or blood-stained for sputum with varying amounts of blood, sometimes mixed with mucus and/or pus. The Bartlett score was used for the microscopic evaluation as follows: a smear of the sputum was prepared on a slide, thereafter Gram staining was performed and an assessment of the smear was made [74]. The Bartlett score evaluation was achieved by assessing the amount of neutrophils and epithelial cells present on the smear. A positive Bartlett score implies the sputum is of good quality indicative of a lower respiratory tract sample whereas a negative Bartlett score implies the sputum is of poor quality. The induced sputum was cultured on 5% horse-blood agar and buffered charcoal yeast extract agar (BCYE) with and without antibiotics (Diagnostic Media Products, Johannesburg, South Africa) (for the growth of *Legionella* spp.). The agar plates were incubated at 37°C in 5% CO_2 for 7 days. Predominant growth of one organism on the 5% horse-blood agar with a positive Bartlett score is indicative of good quality sputum whereas growth of a multitude of organisms with a negative Bartlett score is indicative of normal flora, hence a poor quality sputum specimen. All sputum specimens were further tested irrespective of the quality of the specimen.

3.2.2 Liquefication and decontamination of induced sputum specimens

Induced sputum was liquefied using dithiolthreitol (DTT) (Roche Diagnostics, Mannheim, Germany), by adding an equal amount of DTT to the volume of sputum. The induced sputum and DTT solution were thereafter vortexed for approximately 30 seconds followed by incubation at 37°C for 15 minutes. Phosphate buffered solution (PBS) (Diagnostic Media Products) at a pH of 7.2 was used to remove the DTT once digestion had been achieved. This was done by adding PBS to the digested sputum to a final volume of 14ml and centrifuging at 2000 rpm for 5 minutes. The liquefied sputum was stored at 4°C.

3.2.3 Total nucleic acid extraction

Total nucleic acids were extracted from 200µl of nasopharyngeal specimens or digested sputum, and eluted into 100µl of elution buffer using an automated instrument based on magnetic bead technology, namely the MagNA Pure 96 instrument (Roche Diagnostics) with the MagNA Pure 96 DNA and Viral NA SV kit (Roche Diagnostics) using the Pathogen Universal protocol. Extracted nucleic acids were stored at -20°C.

3.3 Detection of *M. pneumoniae, Legionella* spp. and *C. pneumoniae*

A multiplex real-time PCR was performed for the detection of *M. pneumoniae, Legionella* spp. and *C. pneumoniae* with the inclusion of the human ribonuclease P gene (*RNaseP*) gene as an internal control [19]. The genes targeted in the assay are CARDs Tx for *M. pneumoniae*, *argR* for *C. pneumoniae*, *ssrA* for *Legionella* spp. and *RNaseP* as a PCR inhibition control to confirm true negative results. The real-time PCR assays were performed on an Applied Biosystems 7500 Fast instrument (Life Technologies, Foster City, California, USA) or the Applied Biosystems ViiA7 instrument with a 96-well plate block (Life

Technologies).The reaction mix comprised of 12.5µl of PerfeCTa™ Multiplex qPCR Super Mix (Quanta Biosciences, Gaithersburg, USA), 6.5µl of template DNA and primers and probes as shown in Appendix 6, made to a 25µl total reaction volume with PCR-grade water (Promega Corporation, Wisconsin,USA). The cycling conditions were: 95°C for 10 minutes, 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. When a positive result was obtained for *M. pneumoniae* (CARDs TX gene), *C. pneumoniae* (*argR* gene) or *Legionella* spp. (*ssrA* gene), with a cycle threshold (Ct) value ≤45, the original specimen was re-extracted as described above and testing was repeated in duplicate. A specimen was considered PCR-positive for the organism if the specimen tested positive in at least two of the three repeats. An *RNaseP* negative result indicates the possible presence of PCR inhibitors and therefore potential false-negative results. Thus, a specimen that tested negative for all three pathogens and the *RNaseP* gene was recorded as inconclusive.

A positive atypical pneumonia case was defined as a patient having a positive PCR result for *M. pneumoniae*, *C. pneumoniae* and/or *Legionella* spp. on either the naso-oropharyngeal specimen or induced sputum or both specimen types.

3.3.1 Validation of the real-time PCR assay for atypical pneumonia-causing bacteria

The sensitivity and specificity of the assay was investigated *in silico* and *in vitro.* To investigate specificity, *in silico* analysis was done by aligning primer pairs to assembled genomes of prokaryotes using the Basic Local Alignment Search Tool (BLAST) function of the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/, accessed May 2012).

In vitro analysis was performed using reference strains (see Appendix 4 for a list of strains used) and Quality Control for Molecular Diagnostic (QCMD) specimens. The QCMD program is an external quality assessment program for real-time PCR assays (Glasgow, Scotland). The programme aims to assess molecular detection methodologies of different laboratories, globally. The panel subscribed to was the *C. pneumoniae-M. pneumoniae* panel and the *L. pneumophila* panel. In 2013, for the *C. pneumoniae-M. pneumoniae* panel a total of 12 samples were received comprising of three core samples and nine educational samples for the detection of *M. pneumoniae* and five core samples and seven educational samples for the detection of *C. pneumoniae*. For the *L. pneumophila* panel, a total of seven core and three educational samples were received. Sensitivity and specificity were described as the measure of the proportion of true positive and the proportion of true negatives using the following equations:

Sensitivity = number of true positives / (number of true positives + number of false negatives

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

Repeatability of the assay was determined by the measurement of the intra-assay precision. For this, the triplicates of reference strains ATCC 29342D, ATCC 53592D and ATCC 33152D-5, were tested twice under the same conditions performed by one scientist on different days.

Reproducibility was measured by inter-assay precision. Duplicates of the three ATCC strains and 16 clinical specimens were tested by two different scientists on different days.

Robustness of the assay was assessed using different real-time PCR instruments. This was achieved by comparing results obtained on the ABI 7500 Fast real-time PCR instrument (Life Technologies) with the ABI ViiA7 real-time PCR instrument (Life Technologies) using reference strains ATCC 29342D, ATCC 53592D and ATCC 33152D-5 as positive controls for *M. pneumoniae, C. pneumoniae* and *Legionella* spp., respectively.

An inter-laboratory comparison of the assay was carried out with the Division of Bacterial Diseases, Respiratory Diseases Branch of the CDC, in which specimens were exchanged between the laboratories. A proficiency test panel of six specimens was obtained from the CDC, Atlanta, USA. Furthermore, a subset of 120 specimens testing positive on the assay at NICD was sent to the CDC and was used for inter-laboratory comparisons.

3.3.2 Speciation of *Legionella* species positive specimens

Specimens identified as positive for *Legionella* spp., were tested using two additional real-time PCR assays which identify common species of *Legionella*. The first was a singleplex assay for the identification of *L. longbeachae*. This assay targeted LLO 1129, a hypothetical protein specific to *L. longbeachae* (see Appendix 6 for sequences). The reaction mix comprised of 12.5µl of PerfeCTa™ Multiplex qPCR Super Mix (Quanta Biosciences), 5µl of template DNA, 25µM primers and 5µM probe made to a final volume of 25µl. The cycling conditions were as follows: 95°C for 10 minutes, 50 cycles of 95°C for 15 seconds and 60°C for 1 minute.

The second assay was a multiplex assay for the detection of *Legionella pneumophila* serogroup 1 and non-serogroup 1 species [75]. The assay targeted the *ssrA* gene for

Legionella spp., the *mip* gene for the identification of *Legionella pneumophila*, the *wzm* gene for *Legionella pneumophila* serogroup 1 as well as the *RNAseP* gene as an inhibition control. The reaction mix comprised of 12.5µl of PerfeCTa™ Multiplex qPCR Super Mix (Quanta Biosciences), 6.5µl of template DNA, 500nM primers or 125nM for the *RNAseP* target and100nM probes or 25nM for the *RNAseP* target as in Appendix 5, made to a final volume of 25µl. The cycling conditions were 95°C for 10 minutes, 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. A specimen was regarded as positive according to the algorithm in Table 1.

Table 1: Algorithm used for analysis of results for the multiplex *Legionella pneumophila* real-time PCR assay

Target gene				Result
ssrA	*mip*	*wzm*	*RNAseP*	
Positive	Negative	Negative	Positive	*Legionella* spp.
Positive	Positive	Negative	Positive	*L. pneumophila*
Positive	Positive	Positive	Positive	*L. pneumophila* serogroup1

3.4 Detection of other respiratory pathogens

As part of the SRI and ILI surveillance studies, cases were tested for additional respiratory pathogens using real-time PCR. Nasopharyngeal specimens were tested for 10 respiratory viruses (influenza types A and B, adenovirus, enterovirus, rhinovirus, human metapneumovirus, respiratory syncytial virus (RSV) and parainfluenza virus types 1-3) were tested by a multiplex reverse-transcription real-time PCR assay at the virology laboratory of the CRDM [76]. Nasopharyngeal and induced sputum specimens were tested for *Bordetella*

pertussis, *Bordetella parapertussis*, *Bordetella bronchiseptica and Bordetella holmesii* [77]. Blood specimens were tested for *Streptococcus pneumoniae* [78] and *Haemophilus influenzae* [79] as previously described.

3.5 HIV testing

HIV status was determined for consenting individuals as part of standard-of-care or by anonymised linked dried blood spot testing at the NICD, by PCR for children <18 months old and by enzyme-linked immunosorbent assay (ELISA) for persons ≥18 months old.

3.6 Culture and molecular characterisation of *M. pneumoniae*

3.6.1 Culture of *M. pneumoniae*

M. pneumoniae PCR-positive specimens were cultured at the Division of Bacterial Diseases, Respiratory Diseases Branch of the CDC in SP4 media (Thermo Fisher Scientific, Kansas, USA), a specialised media for the isolation, differentiation and maintenance of *M. pneumoniae*. SP4 media contains casein, gelatine, foetal bovine serum and yeast extract with penicillin, polymixin B and amphotericin B as well as phenol red as a pH indicator. Three tubes of 2 ml each of SP4 media were prepared. The first contained 200µl of the original nasopharyngeal specimen in UTM or digested induced sputum. In the second and third tubes, a dilution series was made using 1ml of the solution prepared in the first tube with 2ml of SP4 media. The three tubes were incubated at 37°C in 5% CO_2 for 7 to 10 days. After the 7 to 10 days incubation period, the samples were then passaged and re-incubated. This was repeated four times. Growth was observed as a colour change from red to orange, without turbidity, with each passage.

3.6.2 P1 genotyping

P1 genotyping was performed at the Division of Bacterial Diseases Respiratory Diseases Branch of the CDC, on *M. pneumoniae* culture-positive isolates using a real-time PCR assay targeting the 1900bp region of the P1 gene, which is variable between type 1 and type 2, followed by HRM analysis performed using the Rotor-Gene Q 6000 system (Qiagen) [60].

Real-time PCR primers were designed (see Appendix 6 for sequences) to target the 1900bp region which also contains a portion of the repetitive region, RepMP2/3. The reaction mix contained 12.5µl of 2X Universal SYBR GreenER qPCR kit (Life Technologies) with 250nM of both forward and reverse primers, 5µl of 1 ng/µl of DNA measured using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, USA). The mixture was further supplemented with 1µl of 10mM dNTP nucleotide mix (Promega) and 0.25µl Platinum *Taq* polymerase (Life Technologies) and made to a final volume of 25µl. The cycling conditions were 95°C for 2 minutes followed by 35 cycles of 95°C for 30 seconds, 55°C for 15 seconds and 68°C for 90 seconds. HRM was performed between 84°C and 90°C at a rate of 0.05°C per second. The pre-melt and post-melt baseline regions were between 85.25°C and 85.5°C and 88.25°C and 88.5°C respectively. *M. pneumoniae* M129 strain was used as a reference type 1 strain and *M. pneumoniae* FH (ATCC 15531) was used as a reference type 2 strain. Therefore, an isolate sharing the same melt-curve profile as M129 was regarded as being a P1 type 1 isolate while an isolate sharing the same melt-curve profile as FH were regarded as being a P1 type 2 isolate. Isolates that deviated slightly from the control strains were regarded as variants of P1 type 1 and type 2.

3.6.3 Multiple-locus variable-number tandem repeat analysis (MLVA) typing

MLVA typing was performed using five variable number tandem repeats (VNTR) loci [62]. Two multiplex PCR assays were performed at the Respiratory Diseases Branch of the CDC, one amplifying loci Mpn 1, Mpn 14 and Mpn 16 and the other amplifying loci Mpn13 and Mpn 15 of *M. pneumoniae* (see Appendix 5 for sequences). The reactions contained 1X Qiagen PCR buffer (Qiagen) supplemented with 0.2mM dNTPs, 0.5µM primers, 1.25U Hotstart *Taq* polymerase (Life Technologies) with either 2mM $MgCl_2$ for the triplex assay or 3mM $MgCl_2$ for the duplex assay. Forward or reverse primers were labelled with fluorescent dyes, FAM, HEX or NED (Appendix 6); 1µ1 of DNA template was added to each reaction mixture. The cycling conditions were as follows: 95°C for 15 minutes followed by 45 cycles of 95°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute followed by 72°C for 10 minutes. Following PCR, the PCR product was diluted 1:50 in distilled water and 1µl of the diluted samples were added to 10µl HiDi formamide (Life Technologies) and 0.5µl of GeneScan 600 LIZ dye size standard. Thereafter, the solution was denatured for 5 minutes at 95°C followed by rapid cooling on ice for 1 minute. An ABI 3130 genetic analyser (Life Technologies) was used to separate the fragments and was electrophoresed at 15000V for 20 minutes at 60°C. Sizing analysis was performed using GeneMapper software (version 4.0; Life Technologies), with the number of repeats rounded off to the nearest integer.

3.6.4 Macrolide susceptibility analysis

M. pneumoniae-positive specimens were tested for their susceptibility to macrolide antibiotics using a real-time PCR assay followed by high-resolution melt-curve (HRM) analysis using the Rotor-Gene Q 6000 system (Qiagen, Hilden, Germany) as previously

described [59]. Macrolide susceptibility testing was performed at the Respiratory Diseases Branch of the CDC.

In brief, the reaction mixture consisted of 12.5 µl of 2X Platinum Quantitative PCR Super Mix-UDG kit (Life Technologies), 125 nM forward and reverse primers (see Appendix 5 for sequences) targeting domain V of the 23S rRNA gene, 0.25 µl of Platinum *Taq* polymerase (Life Technologies) and 5µl of DNA made to a final volume of 25 µl with PCR-grade water (Promega, WI, USA). Real-time PCR was performed using the following cycling conditions: 1 cycle of 95°C for 2 minutes followed by 45 cycles of 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds. Following amplification, HRM was performed between 79°C to 80°C and data was collected at 0.02°C per second. For analysis, the normalisation region was between 81°C and 81.5°C for the pre-melt baseline region, while the post-melt baseline region was between 83.5°C and 84°C. *M. pneumoniae* M129 (ATCC 29342) was used as a reference strain for a susceptible genotype. Any isolates that had a HRM-curve that deviated from the reference genotype were considered to be resistant to macrolides (Appendix 7).

3.6.5 Sequencing of isolates to confirm macrolide susceptibility

Sequencing of isolates was performed at the Respiratory Diseases Branch of the CDC. Sequencing of domain V of the 23S rRNA gene was performed on *M. pneumoniae*-positive cases using a previously described method [59]. In brief, primers were designed to amplify a 200bp region of the domain V. The forwards primer sequence was 5'-AACTATAACGGTCCTAAGGTAGCG-3' and reverse primer sequence was 5'-GCTCCTACCTATTCTCTACATGAT-3'. The PCR was prepared using 12.5 µl Platinum

Quantitative PCR Super Mix-UDG kit (Life Technologies) supplemented with 0.25 µl of Platinum Taq polymerase with 100nM of each primer, made to a final volume of 25 µl in the DNA Engine Dyad Peltier thermocycler (Bio-Rad, California, USA) using the following cycling conditions: 1 cycle of 95°C for 2 minutes, followed by 45 cycles of 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 60 seconds. Thereafter, the PCR product was purified using the Geneclean Turbo kit (qBiogene, California, USA). Sequencing was performed using BigDye Terminator Cycle Sequencing Kit V3.1 (Life Technologies) and the cycling conditions were as follows: 1 cycle of 96°C for 1 minutes, followed by 35 cycles of 96°C for 10 seconds, 55°C for 10 seconds and 60°C for 3 minutes. Purification was thereafter performed using CentriSep 8 spin columns (Princeton Separations, New Jersey, USA) and sequencing was performed using the ABI 3130XL instrument (Life Technologies). Sequence analysis was done using DNAStar Lasergene SeqMan Pro Software and was aligned to a known macrolide susceptible strain (1005) and a known resistant strain (1006) using the ClustalW software.

3.7 Data analysis

Demographic, clinical and laboratory data were entered on a Microsoft Access database (Microsoft Corporation, California, USA), using double data entry. SRI cases were categorised as acute if their duration of symptoms was ≤7 days and chronic if their duration of symptoms was >7 days.

For the comparison of specimen types for the detection of *M. pneumoniae*, *Legionella* spp. and *C. pneumoniae,* a subgroup of SRI patients from which both specimen types were collected were selected for analysis. To determine whether there was an association between

the quality of sputum and the detection of *M. pneumoniae* and *Legionella* spp., only SRI cases were selected from whom induced sputum had been collected and macroscopic and microscopic quality data were available.

Determination and comparison of the prevalence of atypical pneumonia-causing bacteria between study groups was achieved by analysis of the results for nasopharyngeal specimens only, as this was the only specimen type that was collected from all study groups. We performed a univariate logistic regression analysis to determine the association of *M. pneumoniae* and *C. pneumoniae* infection with SRI and ILI patients compare to controls. This was followed by estimating the attributable fraction which measures the proportion of disease in the study population which is caused by the organism detected [80], using the formula:

$$\text{Attributable Fraction} = (\text{odds ratio} - 1) / \text{odds ratio} \times 100$$

To determine seasonal patterns of atypical pneumonia-causing bacteria, SRI cases only were selected to determine the seasonal distribution of disease caused by *M. pneumoniae* and *Legionella* spp. Seasonality of *C. pneumoniae* was determined for all patients enrolled in all three surveillance groups to determine the overall prevalence over the study period since *C. pneumoniae* is known to cause asymptomatic infection.

To identify factors associated with severe disease caused by *M. pneumoniae* or *Legionella* spp. SRI patients positive for *M. pneumoniae* or *Legionella* spp. were compared to patients negative for *M. pneumoniae* or *Legionella* spp. using univariate logistic regression analysis.

For the analysis of co-infections, a case was defined as a patient presenting with SRI, with *M. pneumoniae* or *Legionella* spp., detected in their nasopharyngeal specimens and/or induced

sputum specimens. A co-infecting virus was defined as a real-time PCR positive result for any of the 10 viruses tested in the nasopharyngeal specimen collected from a patient with SRI. Co-detected bacteria were defined as a real-time PCR positive result for *B. pertussis, B. parapertussis, B. bronchiseptica, B. holmesii* and/or *C. pneumoniae,* in either induced sputum and/or nasopharyngeal specimen and for *S. pneumoniae* and *H. influenzae* in blood specimens only.

A descriptive analysis of the results was done using Microsoft Excel (Microsoft Corporation) and for the comparative analysis GraphPad Instat analysis, version 3.10 (GraphPad Software Inc., California, USA) and STATA version 12.1 (STATA Corporation, Texas, USA) were used. Statistical significance was assessed at $P<0.05$ for all parameters.

3.8 Ethics

The SRI protocols (M081042) and ILI protocol (M120133), as well as amendments to include additional testing of specimens in this study, were approved by the Human Research Ethics Committee-Medical (HREC) of the University of the Witwatersrand, Johannesburg. Ethics approval (BF081/12) has been received from the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal. The Klerksdorp-Tshepong surveillance site ethics is included in the ethics for the SRI protocol hence separate ethics was not required. For the Edendale surveillance site ethics has been approved by the hospital and provincial ethics committees. Ethical approval was obtained from the University of the Witwatersrand HREC by Maimuna Carrim for this study (M130211) (Appendix 8).

4 Results

4.1 Validation of the multiplex real-time PCR assay for the detection of *M. pneumoniae, Legionella* spp. and *C. pneumoniae*

In silico analysis of primer pairs targeting *M. pneumoniae, Legionella* spp. and *C. pneumoniae* specific genes revealed 100% identity match to *M. pneumoniae, Legionella* spp. and *C. pneumoniae,* respectively. Individual primer pairs did not significantly align to any other organism sequences in the NCBI database.

In vitro analysis revealed that amplification of the CARDS toxin gene was 100% sensitive (2/2) and 100% (22/22) specific for the detection of *M. pneumoniae,* the arginine repressor gene was 100% (4/4) sensitive and 100% (22/22) specific for the amplification of *C. pneumoniae* and the pan-*Legionella* target (*ssrA* gene) was 100% (9/9) sensitive and 100% (21/21) specific for the detection of *Legionella* spp.

Results of the 2013 QCMD panels revealed a 100% concordance in results with the core samples for both the *C. pneumoniae-M. pneumoniae* panel and 100% concordance in results with the core and educational samples for the *L. pneumophila* panel. However, for the educational samples of the *M. pneumoniae* and *C. pneumoniae* panel, 67% (6/9) and 71% (5/7) were correctly identified, respectively. Discrepancy in the results i.e. results that were expected to be positive but were negative in our laboratory, had Ct-values of >35 thus the inability to detect the organism was likely due to low copy numbers of the genes present in the specimen which were below the limit of detection of the assay.

The results between repeats to assess the intra-assay precision correlated. Furthermore, there was no statistical significance observed between mean Ct-values (Table 2).

Reproducibility of the assay performed by different scientists on different days revealed 100% (16/16) concordance and no statistical significance between mean Ct-values amongst specimens (Table 2).

Table 2: Analysis of the precision of the real-time PCR assay for the detection of atypical-pneumonia causing bacteria

Target	Inter-assay precision			Intra-assay precision		
	Mean Ct-value 1	**Mean Ct- value 2**	**P-value**	**Mean Ct- value 1**	**Mean Ct- value 2**	**P-value**
CARDS toxin	27.0	28.6	0.4	29.4	29.4	>0.99
Arginine repressor gene	25.0	25.6	0.2	30.1	31.4	0.23
ssrA gene	27.3	26.0	0.3	27.5	29.3	0.42
RNAseP gene	27.0	27.0	0.9	24.6	25.7	0.41

*Bold font denotes statistical significance (P<0.05)

Robustness assessment performed on ATCC controls for *M. pneumoniae, Legionella* spp. and *C. pneumoniae* using the two real-time instruments revealed a 100% concordance in qualitative results for all targets between the ABI 7500 Fast instrument and the ABI ViiA7 instrument. The semi-quantitative results indicated that the mean (±standard deviation) cycle threshold (Ct) value for the *M. pneumoniae*-positive control was 28.0 ± 1.1 on the ABI 7500 instrument and 27.0 ± 1.8 on the ABI ViiA7 instrument (P=0.1). For the *C. pneumoniae*-positive control the mean Ct-value was 25.0 ± 0.6 on the ABI 7500 instrument and 27.0 ± 2.1on the ABI ViiA7 instrument (P=0.01). Results for the pan-Legionella target, revealed a

mean Ct-value of 27.0 ± 1.0 on the ABI 7500 instrument and a mean Ct-value of 26.0 ± 0.5 on the ABI ViiA7 real-time PCR instrument (P=0.002).

Furthermore, inter-laboratory comparisons indicated a 100% (120/120) correlation in positive and negative results with the reference laboratory, and 100% (6/6) correlation in results for the proficiency test panel received.

4.2 Study population

From June 2012 through December 2013, 6122 patients were enrolled of which 3239 (53%), 1940 (32%), 943 (15%) and were SRI patients, ILI patients and controls, respectively. Real-time PCR for the identification of the atypical pneumonia-causing bacteria was performed on 85% (5210/6122) of cases including 86% (2793/3239) of SRI patients, 86% (1670/1940) of ILI patients and 79% (747/943) of controls, with a summary of the clinical and demographic characteristics of patients shown in Table 3. Not all patients enrolled were tested due to some patients being too sick for specimens to be collected, participants not giving consent for testing, participants being discharged before a specimen could be collected, specimens being lost in transit or specimens being of insufficient volume for testing. A total of 4534 of 5210 patients (87%) had a known HIV status of which 2148/4534 (47%) were HIV infected. The HIV prevalence amongst SRI patients was 56% (1360/2414), amongst ILI patients it was 32% (464/1429) and amongst controls it was 47% (324/691).

There was a difference observed in age distribution, gender, HIV prevalence, symptom duration and underlying conditions of patients presenting with severe disease compare to patients with influenza-like illness (Table 3).

Table 3: Characteristics of patients tested for atypical pneumonia-causing bacteria by surveillance group, South Africa, June 2012 – December 2013 (N=5210)

Characteristics	Cases		P-value*	Controls	Total
	SRI	ILI			
	n (%)	n (%)		n (%)	n (%)
	N=2793	N=1670		N=747	N=5210
Age Group (years)	N=2770	N=1669	**<0.001**	N=744	N=5183
<1	571 (21)	137 (8)	-	75 (10)	783 (15)
1-4	355 (13)	266 (16)	**<0.001**	158 (21)	779 (15)
5-14	92 (3)	269 (16)	**<0.001**	175 (24)	536 (10)
15-24	154 (6)	265 (16)	**<0.001**	47 (6)	466 (9)
25-44	939 (34)	554 (33)	**<0.001**	110 (15)	1603 (31)
45-64	530 (19)	161 (10)	**<0.001**	136 (18)	827 (16)
≥65	129 (5)	17 (1)	0.22	43 (6)	189 (4)
Gender	N=2790	N=1669		N=742	N=5201
Male	1383 (50)	619 (37)	-	266 (36)	2268 (44)
Female	1407 (50)	1050 (63)	**<0.001**	476 (64)	2933 (56)
Race	N=2785	N=1665		N=743	N=5193
Black African	2715 (97)	1665 (100)	-	743 (100)	5123 (99)
Non-Black	70 (3)	0	UD	0	70 (1)
HIV Status	N=2414	N=1429		N=691	N=4534
Uninfected	1054 (44)	965 (68)	-	367 (53)	2386 (53)
Infected	1360 (56)	464 (32)	**<0.001**	324 (47)	2148 (47)
Symptom duration	N=2747	N=1669		N=253	N=4669
≤7 days	1607 (59)	1668 (100)	-	220 (87)	3495 (75)
>7 days	1140 (41)	1 (0.1)	**<0.001**	33 (13)	1174 (25)

Underlying conditions**	N=2789	N=1668		N=744	N=5201
No	2474 (89)	1582 (95)	-	698 (94)	4754 (91)
Yes	315 (11)	86 (5)	**<0.001**	46 (6)	447 (9)
Outcome	N=2696	N=1534		N=656	N=4886
Death	244 (9)	0	-	0	244 (5)
Survived	2452 (91)	1534 (100)	UD	656 (100)	4642 (95)

Abbreviations: ILI=Influenza-like illness; SRI=Severe Respiratory illness; N/A=not applicable; UD=Undetermined. *P-value calculated for SRI vs. ILI cases. Bold font denotes statistical significance (P<0.05)

**Underlying conditions defined as patients with previously diagnosed chronic conditions including asthma, chronic lung diseases, cirrhosis/liver failure, chronic renal failure, heart failure, valvular heart disease, coronary heart disease, immunosuppressive therapy, splenectomy, diabetes, burns, kwashiorkor/marasmus, nephrotic syndrome, spinal cord injury, seizure disorder, emphysema or cancer.

4.3 *Mycoplasma pneumoniae*

4.3.1 Comparison of specimen types for detection of *M. pneumoniae*

Both induced sputum and nasopharyngeal specimens were collected from 40% (1124/2793) of SRI cases. Three percent (34/1124) of cases were positive for *M. pneumoniae.* There was no significant difference observed between the detection rate of *M. pneumoniae* in induced sputum (1.4%; 16/1124) compared nasopharyngeal specimens (0.8%; 9/1124) and patients in which both specimen types were positive (0.8%; 9/1124) (P=0.23). The mean (± standard deviation) Ct-value for nasopharyngeal specimens, positive for *M. pneumoniae* was 34.67 ± 5.13 and for induced sputum it was 31.93 ± 5.52 (P=0.11). Comparing cases positive only on one type of specimen, either induced sputum or nasopharyngeal specimens, there was no statistically significant difference observed in age group, gender, HIV status, symptom duration, hospital duration, whether the patient had an underlying illness or outcome of patients (Table 4).

Of the 1290 SRI cases that had induced sputum specimens collected, Bartlett scores were performed on 59% (766/1290) of specimens and macroscopic evaluations were performed on 95% (1225/1290) of specimens (Table 5). It was observed that more *M. pneumoniae*-positive induced sputum specimens had a positive Bartlett score than *M. pneumoniae*-negative specimens and purulent sputum was more often obtained from *M. pneumoniae*-negative patients than *M. pneumoniae*-positive patients, although both observations were not statistically significant. There was no significant difference observed in the detection rate of *M. pneumoniae* with respect to the specimen Bartlett score or the macroscopic evaluation.

Table 4: Characteristics of *M. pneumoniae*-positive severe respiratory illness patients by specimen type, South Africa, June 2012 – December 2013 (N=25)

Characteristics	Induced sputum	NP specimens	P-value*
	n/N (%)	n/N (%)	
Age Group			
<5	5/16 (31)	5/9 (56)	-
≥5	11/16 (69)	4/9 (44)	0.24
Gender			
Male	6/16 (38)	2/9 (22)	-
Female	10/16 (62)	7/9 (78)	0.44
HIV Status			
Uninfected	4/15 (27)	5/8 (63)	-
Infected	11/15 (73)	3/8 (38)	0.10

Symptom duration			
≤7 days	10/15 (67)	7/9 (78)	-
>7 days	5/15 (33)	2/9 (22)	0.56
Hospital duration			
≤7 days	8/15 (53)	6/9 (67)	-
>7 days	7/15 (47)	3/9 (33)	0.52
Underlying illness**			
No	14/16 (88)	8/9 (89)	-
Yes	2/16 (13)	1/9 (11)	0.92
Outcome			
Death	0/16	0/9	-
Survived	16/16 (100)	9/9 (100)	UD

Abbreviations: NP=nasopharyngeal; UD=Undetermined. *Bold font denotes statistical significance (P<0.05). Table excludes patients in which both specimen types were obtained.

**Underlying conditions defined as patients with previously diagnosed chronic conditions including asthma, chronic lung diseases, cirrhosis/liver failure, chronic renal failure, heart failure, valvular heart disease, coronary heart disease, immunosuppressive therapy, splenectomy, diabetes, burns, kwashiorkor/marasmus, nephrotic syndrome, spinal cord injury, seizure disorder, emphysema or cancer.

Table 5: Comparison of *M. pneumoniae* detection by quality of the sputum specimen, South Africa, June 2012 – December 2013

Induced sputum evaluation method	*M. pneumoniae* negative	*M. pneumoniae* positive	P-value*
	n (%)	n (%)	
Macroscopic category	N=1197	N=28	0.43
Saliva	353 (29)	11 (39)	-
Mucoid	452 (38)	10 (36)	0.44
Purulent	253 (21)	3 (11)	0.14
Blood stained	139 (12)	4 (14)	0.89
Bartlett Score	N=755	N=11	0.71
Negative	283 (37)	3 (27)	-
0	150 (20)	2 (18)	0.80
Positive	322 (43)	6 (55)	0.43

Abbreviations: 0=equal number of neutrophils and epithelial cells. *Bold font denotes statistical significance (P<0.05).

4.3.2 Detection and comparison of the prevalence of *M. pneumoniae* by surveillance group

Of 5210 cases that were tested for *M. pneumoniae*, 5048 (97%) had nasopharyngeal specimens collected and tested. The overall detection rate amongst these cases was 1.1% (58/5048), with the highest detection rate amongst patients with severe respiratory illness (1.5%; 39/2631) compared to ILI patients (1%; 16/1670) (P=0.17) and controls (0.4%; 3/747)

(P=0.03). The overall *M. pneumoniae* attributable fraction for patients with SRI, using nasopharyngeal specimens only, was 89.0% (95% confidence interval [CI] 48.7 – 97.5), after adjusting for age and HIV status. The attributable fraction for patients with ILI was 59.0% (95% CI -83.9 – 91.0). When comparing the characteristics of *M. pneumoniae*-positive cases of SRI patients with ILI patients, there was a significantly higher detection rate in SRI patients <5 years (72%; 28/39) compared to ILI patients (38%; 6/16) (P=0.02) (Table 6). Although not statistically significant, the prevalence of HIV was higher among *M. pneumoniae* cases identified among SRI patients (33%; 11/33) than ILI patients (8%; 1/12).

Table 6: Characteristics of *M. pneumoniae*-positive cases by study group, South Africa, June 2012 – December 2013 (N=58)

Characteristics	Case		P-value*	Control
	SRI	ILI		
	n (%)	n (%)		n (%)
Age Group (years)	N=39	N=16		N=3
<5	28 (72)	6 (38)	-	1 (33)
≥5	11 (28)	10 (63)	**0.02**	2 (67)
Gender	N=39	N=16		N=3
Male	19 (49)	7 (44)	-	2 (67)
Female	20 (51)	9 (56)	0.74	1 (33)
HIV Status	N=33	N=12		N=3
Uninfected	22 (67)	11 (92)	-	3 (100)
Infected	11 (33)	1 (8)	0.12	0

Symptom duration	N=38	N=16		N=3
≤7 days	28 (74)	16 (100)	-	2 (67)
>7 days	10 (26)	N/A	UD	1 (33)
Underlying illness**	N=39	N=16		N=3
No	35 (90)	15 (94)	-	3 (100)
Yes	4 (10)	1 (6)	0.60	0
Outcome	N=39	N=15		N=3
Survived	37 (95)	15 (100)	-	3 (100)
Death	2 (5)	0	UD	0

Abbreviations: ILI=influenza-like illness; SRI=Severe respiratory illness; UD=undetermined; N/A=not applicable. *Bold font denotes statistical significance (P<0.05).

**Underlying conditions defined as patients with previously diagnosed chronic conditions including asthma, chronic lung diseases, cirrhosis/liver failure, chronic renal failure, heart failure, valvular heart disease, coronary heart disease, immunosuppressive therapy, splenectomy, diabetes, burns, kwashiorkor/marasmus, nephrotic syndrome, spinal cord injury, seizure disorder, emphysema or cancer.

4.3.3 Prevalence of and factors associated with severe disease caused by *M. pneumoniae*

The prevalence of *M. pneumoniae* was 2.1% (59/2793) among SRI patients. Univariate logistic regression analysis of SRI cases (N=2793) revealed that younger age (<5 years) was associated with *M. pneumoniae* infection (34/59 (58%) *M. pneumoniae*-positive vs. 892/2713 (33%) *M. pneumoniae*-negative, P<0.001) (Table 7). HIV-infection was not associated with *M. pneumoniae* disease (25/52 (48%) *M. pneumoniae*-positive vs. 1336/2363 (57%) *M. pneumoniae*-negative, P=0.22). SRI patients with asthma did not have a greater risk of being infected with *M. pneumoniae* (2/59 (3%) *M. pneumoniae*-positive patients vs. 83/2732 (3%)

M. pneumoniae-negative, P=0.87). The detection rate was significantly higher at Edendale hospital (47/1313, 3.6%) than at Klerksdorp-Tshepong hospital (12/1480, 0.8%), P<0.001.

Table 7: Univariate analysis of factors associated with severe disease due to *M. pneumoniae*, South Africa, June 2012 – December 2013 (N=2793)

Characteristic	*M. pneumoniae* positive n (%)	*M. pneumoniae* negative n (%)	P-value*
Age Group	N=59	N=2713	
<5	34 (58)	892 (33)	-
≥5	25 (42)	1821 (67)	**<0.001**
Gender	N=59	N=2733	
Male	27 (46)	1357 (50)	-
Female	32 (54)	1376 (50)	0.55
HIV Status	N=52	N=2363	
Uninfected	27 (52)	1027 (43)	-
Infected	25 (48)	1336 (57)	0.22
Facility	N=59	N=2734	
Edendale	47 (80)	1266 (46)	-
Klerksdorp-Tshepong	12 (20)	1468 (54)	**<0.001**
Symptom duration	N=57	N=2645	
≤7 days	39 (68)	1567 (59)	-
>7 days	18 (32)	1078 (41)	0.12

Hospital duration	N=56	N=2563	
≤7 days	37 (66)	1676 (65)	-
>7 days	19 (34)	887 (35)	0.92
Underlying illness**	N=59	N=2732	
No	53 (90)	2423 (89)	-
Yes	6 (10)	309 (11)	0.79
Asthma	N=59	N=2732	
No	57 (97)	2649 (97)	-
Yes	2 (3)	83 (3)	0.87
Outcome	N=59	N=2635	
Survived	57 (97)	2393 (91)	-
Death	2 (3)	242 (9)	0.14

*Bold font denotes statistical significance (P<0.05).

**Underlying conditions defined as patients with previously diagnosed chronic conditions including asthma, chronic lung diseases, cirrhosis/liver failure, chronic renal failure, heart failure, valvular heart disease, coronary heart disease, immunosuppressive therapy, splenectomy, diabetes, burns, kwashiorkor/marasmus, nephrotic syndrome, spinal cord injury, seizure disorder, emphysema or cancer.

4.3.4 Seasonality of disease caused by *M. pneumoniae*

Amongst 2793 SRI cases tested during the study period, 59 (2.1%) were infected with *M. pneumoniae.* Cases were detected throughout the study period with two peaks detected during the late autumn month of May 2013 (6.1%; 11/179) and late spring month of November 2013 (6.1%; 2/33) (Figure 2).

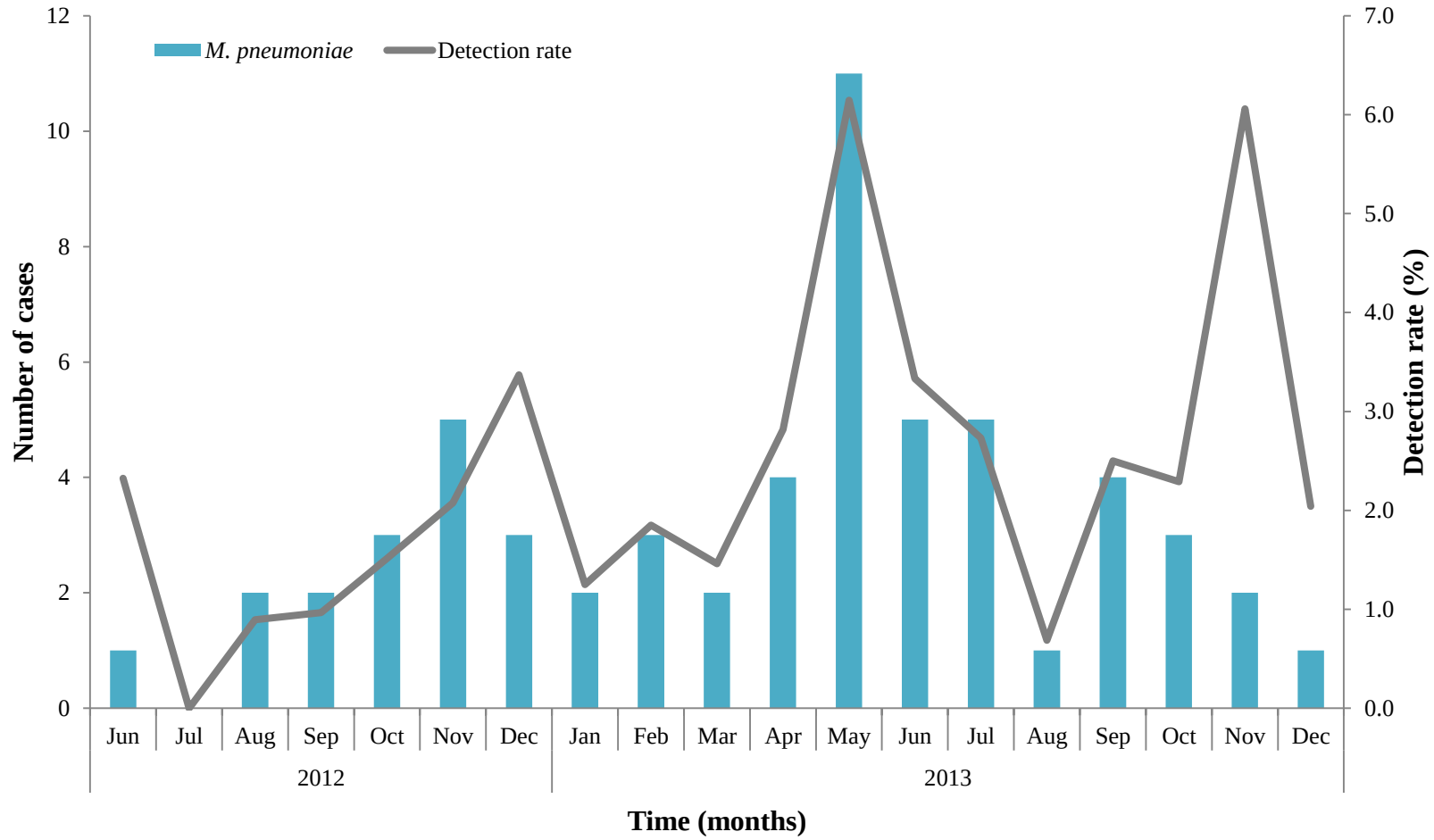


Figure 2: Monthly distribution of severe disease caused by *M. pneumoniae*, South Africa, June 2012 – December 2013 (N=2793)

4.3.5 *M. pneumoniae* and co-infection in patients with severe respiratory illness

Among patients with severe respiratory illness, *M. pneumoniae* was the only pathogen identified in 25% (15/59) of cases. *M. pneumoniae* was co-detected with a respiratory virus in 63% (37/59) of cases, with co-infecting bacteria in 8% (5/59) and with both a co-infecting bacteria and virus in 3% (2/59) of cases. *M. pneumoniae* was co-detected with one virus in 46% (17/37) of cases and with >1 virus in 54% (20/37) of cases. Of the cases with virus co-infection detected, *M. pneumoniae* was identified with RSV in 27% (10/37), influenza virus in 8% (3/37), human metapneumovirus in 3% (1/37) and other respiratory viruses (adenovirus, rhinovirus, parainfluenza virus) in 81% (30/37). Of the cases with bacterial co-infection, *M. pneumoniae* was identified with *S. pneumoniae* in 40% (2/5), with *H. influenzae* in 20% (1/5), *B. pertussis* in 20% (1/5) and *B. parapertussis* in 20% (1/5).

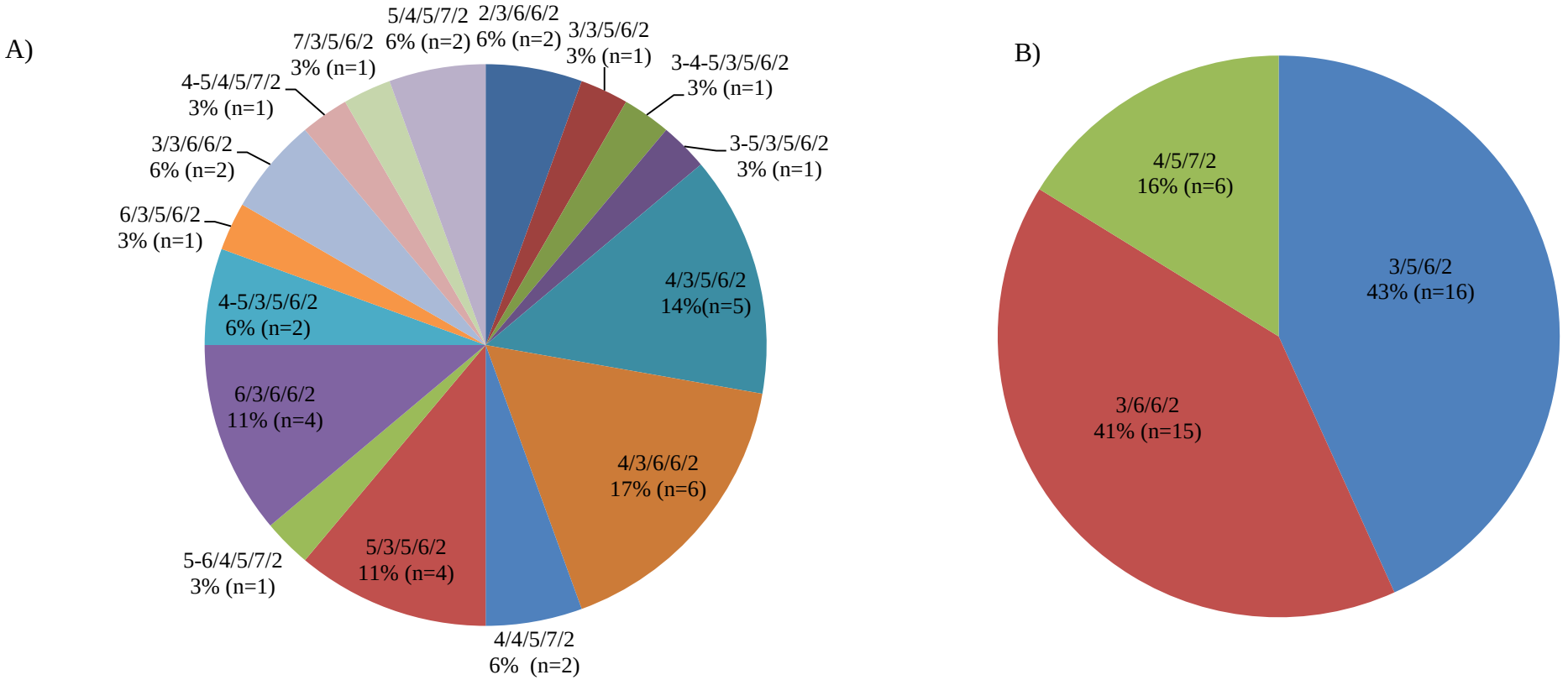
4.4 Culture and molecular characterisation of *M. pneumoniae*

a. Culture and P1 genotyping of *M. pneumoniae*

Culture was performed on specimens obtained from 75 of 80 (94%) positive *M. pneumoniae* cases, the remaining five cases had insufficient specimen for culture and further molecular testing could not be done. A culture was obtained for 11/75 (15%) of the *M. pneumoniae* cases. The mean Ct-value (29.64 ± 5.18) of the culture-positive *M. pneumoniae* specimens was significantly lower than the mean Ct-value (35.00 ± 4.30) of the culture-negative *M. pneumoniae* specimens (P=0.001). The isolates were distributed into 4/11 (36%), 4/11 (36%) and 3/11 (27%) for P1 type 1, type 2 and a variant of type 2, respectively.

b. **Multiple-locus variable-number tandem repeat analysis (MLVA) typing**

MLVA types were identified for 45% (36/80) of *M. pneumoniae*-positive cases. For 49% (39/80) of cases, the samples had insufficient quantities of nucleic acid thus the fragment size of one or more of the five-VNTR loci could not be determined. For five cases there was insufficient sample available for MLVA analysis. Analysis revealed 16 distinct types using the five-loci nomenclature (Figure 3A). Five specimens revealed mixed MLVA types; each mixed type was regarded as being a different MLVA type. Mixed MLVA types were due to the variability observed in the Mpn1 locus. In comparison, analysis of the combination of tandem repeats at the four-loci, omitting the Mpn1 locus from analysis as previously proposed [81], revealed three distinct MLVA types (Figure 3B). One of the 39 samples with insufficient quantities of nucleic acid could not be characterised using the 5-loci nomenclature due to the inability to attain a fragment size for the Mpn 1 locus, however classification according to the 4-loci nomenclature was successful and a MLVA type of 3/6/6/2 was identified.



*Fragment size not determined for 1 or more loci in (A) 39/75 cases and in (B) 38/75 cases

Figure 3: Distribution of *M. pneumoniae* MLVA types based on five-loci (A) (N=36) or four-loci (B) (N=37), in South Africa, June 2012 – December 2013

c. **Macrolide susceptibility analysis**

Five out of 80 cases had insufficient sample available for macrolide susceptibility analysis. HRM macrolide susceptibility profiles were available for 43% (32/75) of *M. pneumoniae* samples. The mean Ct-value of *M. pneumoniae*-positive specimens with a macrolide susceptibility profile was significantly lower compared to *M. pneumoniae* specimens for which macrolide susceptibility was not able to be determined (32.03 ± 4.92 vs. 35.36 ± 4.24; P=0.001). Of the *M. pneumoniae*-positive specimens with susceptibility profiles available, all (32/32) did not have the mutation conferring resistance.

d. **Sequencing of *M. pneumoniae* to confirm macrolide susceptibility**

Sequencing of domain V of the 23S rRNA region was performed on 13% (4/32) of the samples with available HRM macrolide profiles. Sequences of the samples aligned with strain 1005 the control macrolide susceptible strain, and not strain 1006, as the mutation A2063G was not observed (Figure 4).



Figure shows the 4 samples (6, 7, 18 and 19), the control macrolide susceptible strain (1005) and the control macrolide resistant strain (1006). Base position 2063 is highlighted in yellow. An asterisks (*) denotes an identical base and a dot (.) denotes where a difference in a base has occurred.

Figure 4: Sequence and alignment data of the 200bp region of domain V of the 23S rRNA gene for 4 *M. pneumoniae*-positive cases

4.5 *Legionella* species

4.5.1 Comparison of specimen types for detection of *Legionella* species

Induced sputum and nasopharyngeal specimens were collected from 40% (1124/2793) of SRI cases, of which 1.4% (16/1124) were positive for *Legionella* spp. When comparing specimen types for the detection of *Legionella* spp. a significant difference was observed (16/16 induced sputum-positive vs. 0/16 nasopharyngeal specimens-positive; P<0.001) between the specimen types. The mean Ct-value of the *Legionella* spp. target in the induced sputum specimens was 38.50 ± 2.68.

Bartlett scores were performed on 59% (766/1290) and macroscopic evaluations were performed on 95% (1225/1290) of induced sputum specimens from patients with SRI. There was no significant difference observed in the detection rate of *Legionella* spp. with respect to the specimen Bartlett score (4/286 (1.4%) negative Bartlett score vs. 5/152 (3.3%) Bartlett score of 0 vs. 5/328 (1.5%) positive Bartlett score; P=0.37) or the macroscopic evaluation (12/364 (3%) saliva vs. 7/462 (1.5%) mucoid vs. 0/265 (0%) purulent vs. 1/143 (0.7%) blood stained; P=0.09) (Table 8).

Table 8: Comparison of *Legionella* species-positive and negative cases by Bartlett Scores and macroscopic evaluation of induced sputum, South Africa, June 2012 – December 2013

Induced sputum evaluation method	*Legionella* species negative n (%)	*Legionella* species positive n (%)	P-value*
Macroscopic	N=1205	N=20	0.09
Saliva	352 (29)	12 (60)	-
Mucoid	455 (38)	7 (35)	0.09
Purulent	256 (21)	0	UD
Blood stained	142 (12)	1 (5)	0.13
Bartlett Score	N=752	N=14	0.37
Negative	282 (38)	4 (29)	-
0	147 (20)	5 (36)	0.20
Positive	323 (43)	5 (36)	0.90

Abbreviations: 0=equal number of neutrophils and epithelial cells; UD=Undetermined. *Bold font denotes statistical significance (P<0.05).

4.5.2 Prevalence of and factors associated with severe disease caused by *Legionella* species

Legionella spp. was only detected in SRI patients, with a prevalence of 0.8% (21/2793). Furthermore, in SRI patients, it was only detected in patients 15 to 64 years old [2% (3/155) in 15-24 year, 1.1% (10/939) in 25-44 year and 1.5% (8/530) in 45-64 year age groups]. Cases of *Legionella* spp. were detected at the Edendale (0.5%, 6/1312) and Klerksdorp-

Tshepong hospitals (1%, 15/1481), where SRI patients were enrolled. There was no difference in prevalence of *Legionella* spp. between the two sites (P=0.14). We attempted to speciate all (n=21) *Legionella* spp.-positive specimens. However, a species was only identified for 1 of the 21 cases, and the species was identified as *L. pneumophila* SG1. This case was identified following improvements in transport conditions of induced sputum. Of the *Legionella* spp.-positive cases, 95% (20/21) were detected in induced sputum and 5% (1/21) were detected in nasopharyngeal specimens. Culture was performed on all (1292/1292) induced sputa received, however we were not able to culture *Legionella* spp. from any of these specimens.

Analysis of SRI cases (N=2793) indicated that severe disease caused by *Legionella* spp. was associated with older patients, aged ≥5 years (21/21 (100%) *Legionella* spp.-positive vs. 1823/2749 (66%) *Legionella* spp.-negative) (P=0.0006) (Table 9). In addition, *Legionella* spp.-positive patients were more likely to have chronic symptom duration >7 days (15/19 (79%) *Legionella* spp.- positive vs. 1125/2728 (41%) *Legionella* spp.-negative, P=0.003). The prevalence of HIV was higher amongst *Legionella* spp. cases than negative cases (14/19 (74%) *Legionella* positive vs. 1346/2395 (56%) *Legionella* negative, P=0.14), although not significant. No difference in the case fatality rate could be determined with the cases identified so far (15% (3/20) *Legionella* spp.-positive cases vs. 9% (241/2676) *Legionella* spp.-negative cases, P=0.36).

Table 9: Comparison of *Legionella* species-positive and *Legionella* species-negative SRI cases, South Africa, June 2012 – December 2013 (N=2793)

Characteristic	*Legionella spp.* negative n (%)	*Legionella spp.* positive n (%)	P-value*
Age Group (years)	N=2749	N=21	
<5	926 (34)	0	-
≥5	1823 (66)	21 (100)	UD
Gender	N=2769	N=21	
Male	1372 (50)	11 (52)	-
Female	1397 (50)	10 (48)	0.80
HIV Status	N=2395	N=19	
Uninfected	1049 (44)	5 (26)	-
Infected	1346 (56)	14 (74)	0.14
Facility	N=2772	N=21	
Edendale	1306 (47)	6 (29)	-
Klerksdorp-Tshepong	1466 (53)	15 (71)	0.09
Symptom duration	N=2728	N=19	
≤7 days	1603 (59)	4 (21)	-
>7 days	1125 (41)	15 (79)	**0.003**
Hospital duration	N=2584	N=20	
≤7 days	1686 (65)	17 (85)	-
>7 days	898 (35)	3 (15)	0.07

Underlying illness**	N=2768	N=21	
No	2453 (89)	21 (100)	-
Yes	315 (11)	0	UD
Outcome	N=2676	N=20	
Survived	2435 (91)	17 (85)	-
Death	241 (9)	3 (15)	0.36

Abbreviations UD=Undetermined *Bold font denotes statistical significance (P<0.05).

**Underlying conditions defined as patients with previously diagnosed chronic conditions including asthma, chronic lung diseases, cirrhosis/liver failure, chronic renal failure, heart failure, valvular heart disease, coronary heart disease, immunosuppressive therapy, splenectomy, diabetes, burns, kwashiorkor/marasmus, nephrotic syndrome, spinal cord injury, seizure disorder, emphysema or cancer.

4.5.3 Seasonality of disease caused by *Legionella* species

During the study period, 2793 SRI patients were tested for *Legionella* spp. of which 0.8% (21/2793) was positive. Detection of *Legionella* spp. did not show a seasonal pattern during the study period. There was a cluster of cases observed between June and December 2012 (16/21, 76%), and a small cluster of cases from May to July 2013 (Figure 5).

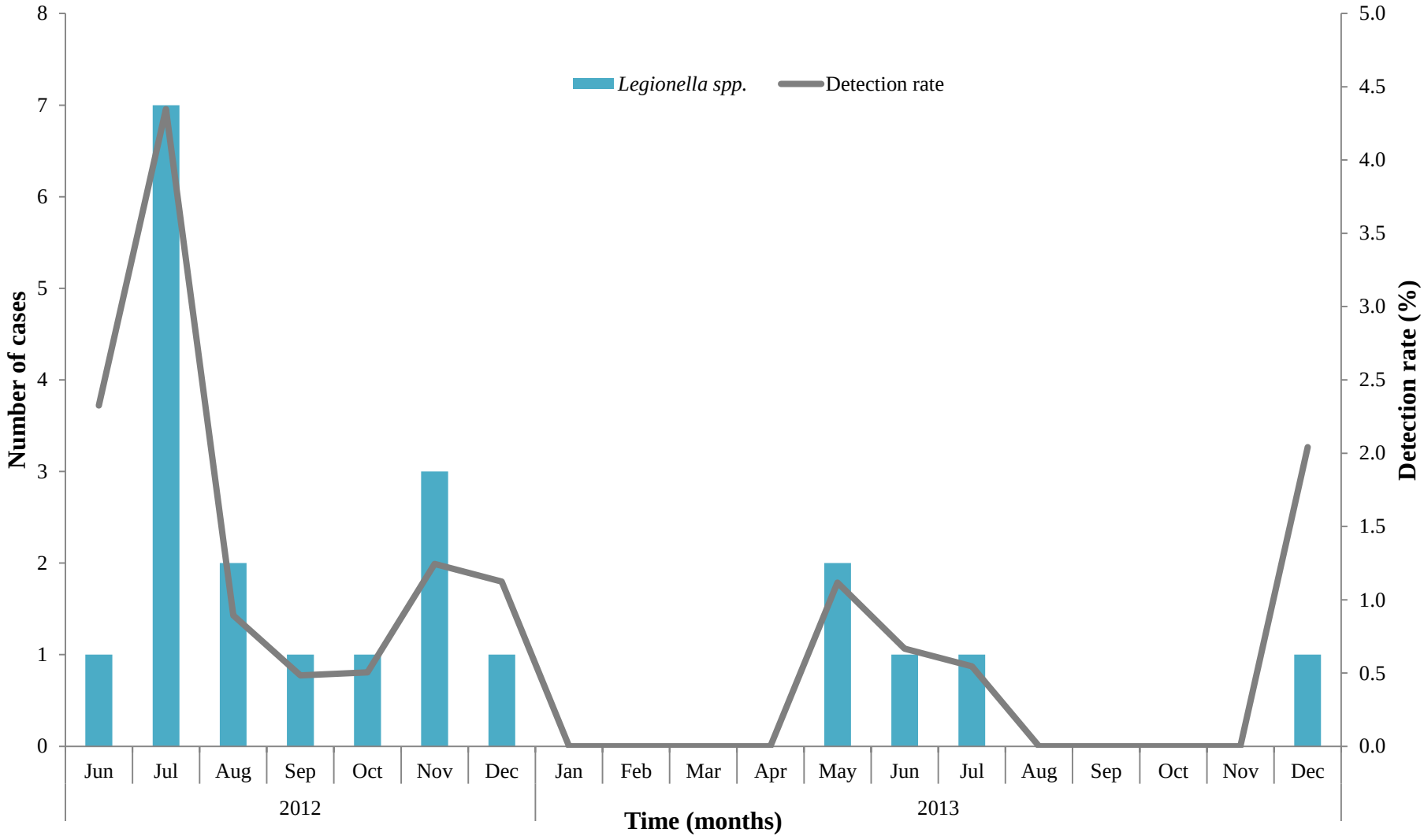


Figure 5: Monthly distribution of severe disease caused by *Legionella* species, South Africa, June 2012 – December 2013 (N=2793)

4.5.4 *Legionella* species and co-infection

Of all *Legionella* spp.-positive cases, *Legionella* spp. was the only pathogen identified in 38% (8/21) of cases. *Legionella* spp. was co-detected with a respiratory virus in 52% (11/21) of cases. Of the viruses, *Legionella* spp. was found to be co-detected with RSV in 27% (3/11) of cases and other respiratory viruses (adenovirus and rhinovirus) in 73% (8/11) of cases. Ten percent (2/21) of cases of *Legionella* spp. were co-detected with bacteria, of which one case was co-detected with *S. pneumoniae* and *C. pneumoniae,* and one case was co-detected with *B. pertussis.*

4.6 *Chlamydia (Chlamydophila) pneumoniae*

4.6.1 Comparison of specimen types for *C. pneumoniae*

Six cases (6/11, 55%) of *C. pneumoniae* were detected in both the nasopharyngeal specimen and induced sputum of SRI cases, 5 (5/11, 45%) cases had only a nasopharyngeal specimen positive and no cases were positive on induced sputum only and therefore an analysis of characteristics associated with specimen type could not be performed. For *C. pneumoniae*-positive nasopharyngeal specimens the mean Ct-value was 35.95 ± 5.34 whereas for induced sputum it was 32.17 ± 4.21. There was no significant difference between the mean Ct-values of the specimen types (P=0.22).

4.6.2 Prevalence of *C. pneumoniae*

The prevalence of *C. pneumoniae* was 0.4% (11/2793) in patients with SRI, 0.5% (9/1670) in patients with ILI and 1% (7/747) in controls. In patients with SRI, *C. pneumoniae* was detected in patients in the age groups <1 through 5-14 years and in the 25-44 year age group (0.4%, 2/570 in <1 year age group; 1.7%, 6/357 in 1-4 year age group; 2.2%, 2/92 in 5-14

year age group; 0.1%, 1/939 in 25-44 year age group). In patients with mild disease, *C. pneumoniae* was detected in patients in the age groups <1 through 45-64 years. The highest prevalence was 1.3% (4/301) in children aged 5-14 years. Among controls, *C. pneumoniae* was only detected in patients in the <5 and 5-14 year age groups with a prevalence of 1.8% (4/221) and 2% (3/152), respectively.

4.6.3 Comparison of characteristics of *C. pneumoniae* infection by surveillance group

The overall prevalence amongst 97% (5048/5183) of cases that had a nasopharyngeal specimen collected and tested for *C. pneumoniae* was 0.5% (26/5048), and the highest prevalence was observed among controls (1%; 7/747) compared to ILI (0.5%, 9/1670) (P=0.40) and SRI patients (0.4%, 10/2631) (P=0.11). When comparing characteristics of SRI and ILI patients positive for *C. pneumoniae* using univariate logistic regression, no significant differences were observed (Table 10). However, there was a higher detection rate among ILI patients ≥5 years (67%; 6/9) compared to SRI patients (20%; 2/10). The *C. pneumoniae* attributable fraction for SRI and ILI were -31% (95% CI –276.75 – 54.28) and -111% (95% CI -629.2 – 38.7), respectively, implying that there was no statistically significant difference in the detection of *C. pneumoniae* between patients with SRI or ILI and control individuals.

Table 10: Comparison of characteristics of all study participants positive for *C. pneumoniae*, South Africa, June 2012 – December 2013 (N=26)

Categories	Case		P-value*	Control
	SRI	ILI		
	n (%)	n (%)		n (%)
Age Group (years)	N=10	N=9		N=7
<5	8 (80)	3 (33)	-	4 (57)
≥5	2 (20)	6 (67)	0.05	3 (43)
Gender	N=10	N=9		N=7
Male	2 (20)	4 (44)	-	4 (57)
Female	8 (80)	5 (56)	0.26	3 (43)
HIV Status	N=10	N=5		N=7
Uninfected	9 (90)	4 (80)	-	2 (29)
Infected	1 (10)	1 (20)	0.60	5 (71)
Symptom duration	N=10	N=9		N=4
≤7 days	7 (70)	9 (100)	-	4 (100)
>7 days	3 (30)	0	UD	0
Underlying illness**	N=10	N=9	UD	N=7
No	10 (100)	9 (100)	-	7 (100)
Yes	0	0	UD	0
Outcome	N=10	N=9	UD	N=6
Death	0	0	-	0
Survived	10 (100)	9 (100)	UD	6 (100)

Abbreviations ILI=influenza-like illness; SRI=Severe respiratory illness; UD= undetermined. *SRI compared to ILI, Bold font denotes statistical significance (P<0.05).

**Underlying conditions defined as patients with previously diagnosed chronic conditions including asthma, chronic lung diseases, cirrhosis/liver failure, chronic renal failure, heart failure, valvular heart disease, coronary

heart disease, immunosuppressive therapy, splenectomy, diabetes, burns, kwashiorkor/marasmus, nephrotic syndrome, spinal cord injury, seizure disorder, emphysema or cancer.

4.6.4 Seasonality of *C. pneumoniae* infection

Seasonality of *C. pneumoniae* was determined, analysing 5210 cases which comprised of 2793 SRI cases, 1670 ILI cases and 747 controls. Over the study period, a distinct seasonal pattern of *C. pneumoniae* was not observed. The highest detection rate occurred in July 2013 (1.6%; 4/243) (Figure 6).

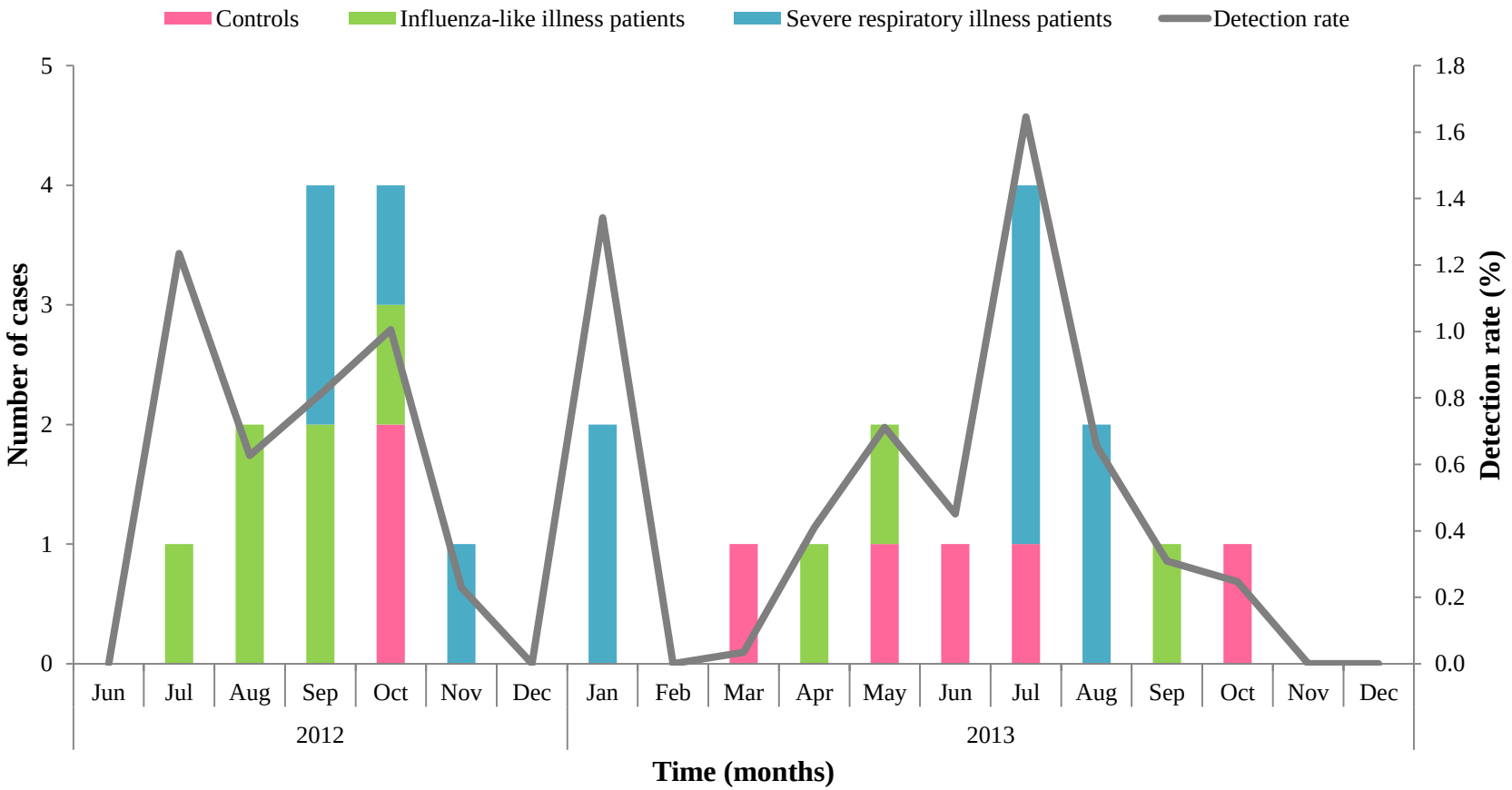


Figure 6: Seasonal distribution of *C. pneumoniae*-positive cases by surveillance group, South Africa, June 2012 – December 2013 (N=5210)

5 Discussion

The prevalence of atypical pneumonia-causing bacteria among SRI patients in two hospitals in South Africa was 3.3%, with a prevalence of 1.5% and 1.3% amongst patients with ILI and controls, respectively.

A paper published in 2011, in which a multiplex real-time PCR assay was validated, indicated an improvement in the diagnosis of *M. pneumoniae, Legionella* spp. and *C. pneumoniae* in terms of sensitivity and speed with use of this assay [19]. This multiplex PCR assay was validated for use in this study. The performance of the PCR in our laboratory was as had been previously described.

For the educational samples in the QCMD panel, bacterial load varied, ranging from a bacterial load frequently detected (low Ct-value) to a bacterial load infrequently detected (high Ct-value). The samples that had discordant results (lack of detection in our laboratory), observed in the *M. pneumoniae-C. pneumoniae* panel were reported by QCMD, to have high Ct-values. This difference in the results was most likely attributed to the low bacterial load in the samples that was below the limit of detection of the assay. We found that the assay was repeatable, reproducible and robust. Thereafter, we used this assay to identify the prevalence of *M. pneumoniae, Legionella* spp. and *C. pneumoniae* in South Africa.

5.1 Study population

We observed that there was a significant difference in many of the characteristics of patients presenting with severe disease compared to patients with mild disease. This difference was expected as patients that present to hospitals are different to those at clinics. Young children

and elderly individuals, HIV-infected individuals and individuals with underlying illness are more likely to present with severe disease than their counterparts [7;9;10;25;82]. The differences in the characteristics of patients tested in the SRI and ILI surveillance groups needs to be taken into consideration when interpreting the results as comparisons between these groups may be biased. We also found that the HIV prevalence was higher in patients presenting with SRI compared to patients presenting with ILI. This is not surprising as HIV-infected individuals are more likely to develop severe pneumonia than HIV-uninfected individuals [9;10;82].

5.2 *Mycoplasma pneumoniae*

The quality of induced sputum, based on the Bartlett scores and the macroscopic evaluations, had no influence on the detection of *M. pneumoniae* since no significance difference in the detection rate was identified. We found that the characteristics of *M. pneumoniae* cases did not differ between those identified with nasopharyngeal specimens (i.e. combined naso-oropharyngeal swabs) compared to induced sputum specimens. There are conflicting reports on the optimal specimen type for the detection of *M. pneumoniae*. Honda *et al.* detected the highest prevalence of *M. pneumoniae* in throat swabs compared to sputa and bronchoalveolar lavages, however Dorigo-Zetsma *et al.* have reported that sputum is the preferred specimen for the identification of *M. pneumoniae* by PCR compared to nasopharyngeal specimens, throat swabs, bronchoalveolar lavages and bronchial aspirates [83;84]. This finding is further reinforced by Räty *et al*. in which sputum was found to be superior to nasopharyngeal specimens and throat swabs for the detection of *M. pneumoniae* by PCR [85]. Even though, there was no significant difference between the detection of *M. pneumoniae* in induced sputum and nasopharyngeal specimens in our study, overall there was a higher detection rate

in induced sputum. Moreover, the mean Ct-values of *M. pneumoniae* were lower in induced sputum specimens than nasopharyngeal specimens suggesting a higher bacterial load in induced sputum. This indicates that induced sputum might be a better specimen type for the detection of *M. pneumoniae*, however induced sputum is difficult to obtain from young patients, patients with severe pneumonia may be too sick to collect induced sputum, and from patients with mild disease induced sputum may not be obtainable. Therefore, in patients where induced sputum cannot be obtained, nasopharyngeal specimens should be obtained.

M. pneumoniae was detected in 2.1% of SRI patients, 1% of ILI patients and 0.4% of controls. Detection rates of *M. pneumoniae* have been reported in France, in which over a 5-year period the prevalence ranged from 2% to 10% in out-patients [48]. Higher detection rates have been reported elsewhere. In Finland in 2004, using serology for diagnosis, a detection rate of 30% was observed in children under 15 years of age with definite or suspected pneumonia [86]. In another study conducted in children in the USA the prevalence of *M. pneumoniae* was 27% detected by culture, serology and PCR [87]. In addition, Foy *et al.* observed infection rates of 2% in endemic years to 35% in epidemic years [88]. In Japan in hospitalized adults enrolled from 2008 to 2009 *M. pneumoniae* prevalence of approximately 60% was observed in patients in whom an etiological diagnosis was obtained [89]. The variability of detection rates could be attributed to whether the study was performed during an endemic or epidemic year, or whether children were predominantly enrolled [88]. Further underestimations of cases in our study, may have been because patients may not have sought care at hospitals and clinics and were not enrolled which could result in cases being missed hence, lower detection rates. The attributable fraction of *M. pneumoniae* was calculated for our study, and *M. pneumoniae* was associated with disease in 89% of SRI patients and 59% of ILI patients.

In our study, the highest detection rate of *M. pneumoniae* was found among SRI patients <5 years old. The majority of studies have shown variability in detection rates amongst different age groups, especially in endemic areas where *M. pneumoniae* has been shown to occur predominantly in children <5 years old [90]. This variability is evident as previous studies by Foy *et al*. using serology and culture to identify *M. pneumoniae,* have reported higher rates amongst children between 5 to 15 years old than <5 years [90-92].

In a study conducted in India from 2004 to 2007, it was found that there was a higher prevalence of *M. pneumoniae* infection in patients infected with HIV compared to HIV-uninfected patients [93]. *M. pneumoniae* has not been described as an opportunistic pathogen. Our study did not reveal an association between HIV-infection and *M. pneumoniae* infection.

M. pneumoniae has been previously shown to play a role in the exacerbation of asthma [94]. Furthermore, *M. pneumoniae* prevalence has been found to be higher in patients with asthma compared to healthy individuals with no history of respiratory tract infection or lung disease [95;96]. However, we did not find an association of *M. pneumoniae* infection with asthma amongst the SRI patients. This difference, however, could be due to the low numbers of patients with asthma enrolled in the study, and therefore the sample size was insufficient to determine whether an association between asthma and *M. pneumoniae* infection exists.

Over the 18-month study period no seasonality for *M. pneumoniae* was observed. *M. pneumoniae* was detected throughout the study period with the highest detection rate observed in May 2013 (late autumn), followed by a peak in November (late spring) of the same year. We concluded that the peak in disease caused by *M. pneumoniae* in November

2013 was not a true increase in disease as we noted that patients with more severe disease were more likely to be enrolled and tested during this period which could have biased the results. Layani-Milon *et al*. reported that over a 5-year period (1993 to 1997), rates of *M. pneumoniae* disease varied monthly and yearly and suggested that *M. pneumoniae* occurs in cycles of epidemics. This study also reported a high incidence of *M. pneumoniae* in late autumn for three of the five years [48]. In a serological study of *M. pneumoniae* infection in the Witwatersrand, South Africa from 1969 to 1975, the periodicity of *M. pneumoniae* was described to peak in 3-year intervals. Our study included data collected over an 18-month period which may not be sufficient for periodicity and seasonality to be attained as *M. pneumoniae* occurs in cycles of three to five years. Therefore, surveillance over a longer period is required to identify potential trends.

Investigation of co-infections and *M. pneumoniae* revealed that among SRI patients *M. pneumoniae* was the only pathogen identified in 25% of the positive cases. Similarly to previously published data by Lieberman *et al*. who found in a population of adult patients hospitalised with CAP, *M. pneumoniae* was the only agent identified in 36% of 101 positive *M. pneumoniae* cases [97]. They further showed that a virus was co-detected with *M. pneumoniae* in 10% of the cases. In contrast, we found that 63% of cases with *M. pneumoniae* were positive for a respiratory virus and the most common virus was found to be RSV. Therefore it is difficult to determine the causative agent for lower respiratory tract infection in these cases.

A culture was obtained for 15% of *M. pneumoniae* PCR-positive specimens in our study. The mean Ct-value of culture-positive *M. pneumoniae* specimens was lower than the mean Ct-value of the culture-negative specimens which confirms that a high *M. pneumoniae* load in a

specimen increases the chances of obtaining a culture. Culture is important to ascertain the P1 types and to determine the molecular epidemiology of *M. pneumoniae.*

Differentiating isolates based on the P1 gene is important for monitoring epidemiological trends, to detect outbreaks and can be used to identify type switching which may occur due to population immune pressure [60]. Analysis of P1 typing of *M. pneumoniae* in our study revealed that both type 1 and type 2 isolates circulated in the population at equal frequencies. Likewise, in the USA, over an 8-year period (2006 to 2013) both P1 types were identified to be co-circulating [98].

MLVA typing for *M. pneumoniae* was developed to differentiate 26 variable number tandem repeat (VNTR) types by determining the variation in the copy number of tandemly repeated sequences at five different loci within the genome [62]. The variability of the Mpn 1 locus previously described was demonstrated in our study [81;99;100]. Using the 4-loci MLVA typing scheme and nomenclature, three distinct MLVA types were identified (3/6/6/2, 3/5/6/2, and 4/5/7/2) which were also found circulating in the USA, Kenya, Guatemala, Egypt, Denmark and Canada [98-100]. The majority of the *M. pneumoniae* samples in our study were MLVA type 3/5/6/2. This predominance of MLVA type 3/5/6/2 was different to what has been previously described elsewhere in the world. Degrange *et al*. using samples from Germany, Denmark, Japan, France, Spain, Tunisia and Belgium showed the most common MLVA type to be 4/5/7/2 [62] which was also observed by Benitez *et al*. using samples from Kenya, Guatemala, Egypt, Denmark and Canada from 1962 to 2012 and Diaz *et al*. using American isolates from 2006 to 2013 [98;99].

In our study, analysis of the domain V of the 23S rRNA gene to identify macrolide resistance mutations revealed that all positive *M. pneumoniae* samples did not have the mutation commonly known to confer macrolide resistance in this organism. Macrolide resistance in *M. pneumoniae* has been documented in some countries. In Japan, in 2000 to 2003, it was observed that 17% of *M. pneumoniae* clinical isolates were resistant to macrolides [56]. In our study, sequence analysis further confirmed that the resistance-conferring mutation was not present in the specimens tested. Using these data, we can conclude that macrolide treatment would be effective against *M. pneumoniae* in our setting. However, excessive macrolide usage should be discouraged, as it has been shown in a study conducted in Japan using specimens from paediatric out-patients, that over use of macrolides increases the chances of the organism developing mutations in the 23S rRNA gene [57].

5.3 *Legionella* species

Furthermore, the data analysis used in the study had limitations. The attributable fraction was calculated for *M. pneumoniae* and *C. pneumoniae* however this approach has limitations in that it is a mathematical model thus does not always depict what occurs in nature and is reliant on the choice of control or comparator population [80]. Another limitation was that the numbers were too small thus limited statistical analysis could be performed.Over the 18-month period from June 2012 through December 2013, 0.8% of SRI cases were positive for *Legionella* spp., which was similar to the prevalence reported in Asia (1.1%) in 2001 to 2002, Japan (1%) in 1998 to 2000, Argentina (1.2%) in 1997 to 1998 and Canada (0.7%) in 1991 to 1994 in hospitalised adults [101].

We were unable to obtain a culture and ascertain a species for majority of the *Legionella* spp. PCR-positive cases. The delay in collecting a specimen from a patient to the time it is inoculated on agar may have hindered the ability to obtain a successful culture. In order to improve the success rate of culturing *Legionella* spp., specimens should be transported immediately to the lab and processed timeously. The inability to obtain a species using species-specific PCR may be related to the low bacterial loads attributed to inappropriate shipping conditions as transport of the specimens from the sites to the NICD was a challenge. In July 2013, transport of specimens was changed so that induced sputum specimens were shipped on dry ice in order to prevent DNA degradation. This was an effective change as the additional positive case identified after July 2013 was successfully speciated as *L. pneumophila* SG1.

Increasing age has been described as a risk factor for Legionnaires' disease [43]. In our study all cases of *Legionella* spp. were detected in adults only, in the age groups ranging from 15 to 65 years with the majority of cases occurring in patients aged 15-24 years contrary to the literature in which the majority of *Legionella* cases occurred in patients >50 years old [102]. The higher detection rate of *Legionella* spp. among males has been reported elsewhere [43;102], however this was not observed in our study. Previously it has been shown that patients with disease caused by *Legionella* spp. have symptoms of disease of 2 to 10 days [102], which was different to our population as patients positive for *Legionella* spp. were more likely to have had a symptom duration of >7 days. This difference in age, gender distribution and symptom duration could be as a result of the high HIV burden in our adult population as *Legionella* has been described as an opportunistic pathogen in HIV-infected individuals [103].

It has been previously reported that disease caused by *Legionella* spp. peaks in the summer season [102;104]. We did not observe a seasonal pattern and the positive cases were assumed to be sporadic cases. However, in hindsight it is thought that the cases obtained in 2012 were a cluster of cases. These cases were identified retrospectively hence environmental sampling was not done. The lack of seasonality may also be attributed to the short period of surveillance conducted.

In over one third of positive cases, *Legionella* spp. was the only pathogen identified, and was identified with a respiratory virus in more than half of the positive cases and 10% of cases were co-infected with bacteria. This was higher than the rates reported in previous studies in which co-infection was reported to range between 2% to 10% in patients with Legionnaires' disease [63]. This difference may be attributed to the sensitivity of the PCR employed in our study. Previous studies have also shown that *L. pneumophila* has been associated with an influenza infection, however in our study *Legionella* spp. was not found to be co-detected with influenza virus [105]. In addition, viruses were assessed in controls from 2012 to 2015 by Pretorius *et al*. there was a prevalence of 36% of viruses in controls. The most common virus being rhinovirus (21%) followed by adenovirus (12%) (Unpublished data).

5.4 *Chlamydia* (*Chlamydophila*) *pneumoniae*

There are limited data comparing specimen types, specifically induced sputum to nasopharyngeal specimens for the detection of *C. pneumoniae*. One study has shown that when comparing induced sputum to nasopharyngeal specimens there was no difference in the detection rate of *C. pneumoniae* [96]. In our analysis, nasopharyngeal specimens (i.e.

combined naso-oropharyngeal swabs) seemed to be a better specimen type as no cases were detected in induced sputum specimens alone. On analysis of Ct-values, however not significant, nasopharyngeal specimens had higher Ct-values compared to induced sputum specimens implying a higher bacterial load of *C. pneumoniae* in induced sputum contradicting our previous assumption. Hence, the appropriate specimen type for the detection of *C. pneumoniae* could not be determined.

There are limited data regarding the prevalence of *C. pneumoniae* in South Africa. Over the 18-month period, 1% of control individuals were found to be positive for *C. pneumoniae* which was higher than the prevalence found in out-patients (0.5%) and patients with severe disease (0.4%). In addition, there was no statistical attributable risk of *C. pneumoniae* in patients presenting with disease compared to controls. These findings suggest that patients may asymptomatically carry *C. pneumoniae,* which has been reported in other studies in which *C. pneumoniae* was found to be carried in healthy individuals [106-108].

Higher incidences of *C. pneumoniae* have been previously reported, ranging from 2% to 50% globally, differing by age, geographical location of participants and methods of diagnosis [96;106-108]. *C. pneumoniae* has been found to be prevalent in school-aged children [109]. Thus, studies in which children were only enrolled have shown higher prevalence of *C. pneumoniae* [95;96;108]. In our study, a significantly higher detection rate was observed among children ≥5 years, in particular among school-aged children, compared to younger patients. This increase amongst school-age children infers that social interactions may contribute to transmission of *C. pneumoniae*. This is consistent with previous reports in which *C. pneumoniae* was most often detected in children 5-14 years old [108-110]. Normann *et al*. identified *C. pneumoniae* in 22.7% of their study population in which healthy

children attending day care were enrolled [108]. Hence, the differences in the overall detection rate in our study compared to previous studies could be attributed to the age of study participants as in our study patients were enrolled from all age groups.

Kumar and Hammerschlag (2007) noted that studies in which PCR was used for identification reported lower detection rates than serological assays for the detection of *C. pneumoniae* amongst patients with lower respiratory illness [111]. Thus, this could also explain the lower detection rate in our study. Another plausible reason for the low detection rate could be that *C. pneumoniae* infections occur in cycles of three to four years [108]; thus our study may reveal a nadir of *C. pneumoniae* disease. However, on-going surveillance is required for corroboration. *C. pneumoniae* is found to be more prevalent in tropical areas [109], which differs to the enrolment sites in our study which were located in temperate and sub-tropical regions thus this could also explain the lower prevalence detected in our study.

Over the 18-month study period, no seasonal pattern in *C. pneumoniae* infection was seen. Additional research for *C. pneumoniae* needs to be conducted for the seasonal pattern to be identified.

5.5 Limitations

There are various limitations to the study that need to be considered. Enrollment of participants occurred only during the week and not over weekends and the number of patients enrolled decreased over the festive season as surveillance officers were on vacation during this time and enrollment was performed by fewer surveillance officers. This may bias results if more severe patients are enrolled at surveillance sites over the festive season. Moreover,

during the festive period patients may only present at sites if they have severe disease thus more severe patients will be enrolled which can influence the results. The prevalence could be underestimated if patients did not seek medical care or if they presented at a hospital or clinic outside of the catchment area. The data may be an underestimation of patients with severe disease and who died, as severely ill patients were less likely to be able to consent to be included in the study or may die before or shortly after hospital admission. In addition, if they were enrolled they were less likely to have specimens collected for testing as they were too ill. Additionally, control individuals were not enrolled from the community but rather from clinics and this may differ from the general population in terms of their risk factor status.

Not all patients enrolled were tested since some patients were too sick for specimens to be collected, participants did not give consent for testing or some participants were discharged before a specimen could be collected.

Furthermore, the data analysis used in the study had limitations. The attributable fraction was calculated for *M. pneumoniae* and *C. pneumoniae* however this approach has limitations in that it is a mathematical model thus does not always depict what occurs in nature and is reliant on the choice of control or comparator population [80]. Another limitation was that the numbers were too small thus limited statistical analysis could be performed.

M. pneumoniae and *C. pneumoniae* are known to occur in cyclical epidemics of between three to seven years. Our surveillance only occurred over an 18-month period thus long-term data and the true burden of disease could not be determined. Our findings of *M. pneumoniae,*

Legionella spp. and *C. pneumoniae* were at two sentinel sites which are not representative of the entire South African population.

In children, consent to obtain induced sputum was less likely thus induced sputum was collected more frequently from adult patients, therefore the detection of atypical pneumonia-causing bacteria in induced sputum in children and overall could be underestimated.

Culture was unsuccessful for all cases, and speciation data was only available for one of the positive *Legionella* cases. In hindsight, there appeared to be a cluster of cases detected in 2012; however we were not able to speciate or obtain strain typing data for the *Legionella* spp.-positive specimens which was a limitation of our study. Species data and strain typing data is crucial in investigating clusters of disease caused by *Legionella* spp. as well as determining the source of the cluster therefore, sequencing of the *mip* gene will be attempted on all *Legionella* spp.-positive cases without species data.

6 Conclusions

The identification of atypical pneumonia-causing bacteria is important for guiding empirical treatment, however it remains a challenge. Molecular techniques overcome many disadvantages associated with other diagnostic methods as it is a fast, specific and sensitive method. Our study presented the utility of a multiplex real-time PCR assay for the identification of atypical pneumonia-causing bacteria amongst patients with mild and severe respiratory illness.

Even though the prevalence was low among SRI patients (3.3%), *M. pneumoniae, Legionella* spp. and *C. pneumoniae* were detected in patients with severe disease reiterating the need for testing and identification of atypical pneumonia-causing bacteria in patients with CAP in South Africa, especially amongst patients with a higher risk for atypical infections, which would result in an earlier diagnosis and improved management of these patients.

M. pneumoniae and *Legionella* spp. were found to be co-detected with other organisms in a subset of patients presenting with SRI, inferring that the pathogens may contribute independently or may augment disease. In our study, *C. pneumoniae* was found in control individuals at a higher prevalence than patients with disease suggesting that *C. pneumoniae* is a commensal and healthy individuals may act as a reservoir for *C. pneumoniae*.

Surveillance for *M. pneumoniae, Legionella* spp. and *C. pneumoniae* will be on-going and will entail the enrolment of patients at additional sites in the Western Cape, Gauteng and Mpumalanga provinces in order for the surveillance to be more representative of the South African population. The turn-around-time of testing and reporting of results will be improved to identify and respond to outbreaks rapidly as well as carry out epidemiological investigations timeously. Induced sputum specimens will continue to be shipped at appropriate conditions and on-going culture of *Legionella* spp. will be attempted in order to obtain speciation and molecular typing data for *Legionella* spp. in South Africa. Furthermore, urine will be collected from adults and urinary antigen tests will be performed to identify *L. pneumophila* SG1 to aid in the diagnosis of Legionnaire's disease.

Routine diagnostics and surveillance of atypical pneumonia-causing bacteria is pivotal for determining the aetiology of CAP and to determine the burden that atypical bacteria

contribute to CAP. Our study helps bridge the gap of limited data in South Africa and provides baseline data that can be used for future surveillance programmes in the hope of better understanding atypical pneumonia-causing bacteria in South Africa.

7 Appendices

Appendix 1: Severe Respiratory Illness (SRI) Surveillance Case Investigation Form (CIF)

NATIONAL HEALTH LABORATORY SERVICE
NATIONAL INSTITUTE FOR COMMUNICABLE DISEASES

Severe Acute Respiratory Illness (SARI)

Surveillance

Case Investigation Form (CIF)

Centre for Respiratory Diseases and Meningitis (CRDM)

TEL: 011 386 6410 or 011 386 6434

FAX: 086 723 3569

SO Initials: ☐☐☐

Date completed: ☐☐/☐☐/☐☐☐☐ (DD/MM/YYYY)

SARI Study ID:

TSAP Study ID:

Note: For EdendaleHospital

Patient Hospitalised at: CHBH ☐ Selby ☐ Mapulaneng ☐ Matikwana ☐ Edendale ☐ Klerksdorp ☐ Tshepong ☐

Note: surveillance officer to review criteria for all case definitions before making a decision about the case definition/s met

SARI* ☐ SRI** (severe respiratory illness not SARI) ☐ Febrile illness*** (enrolled on TSAP) ☐

**SARI with duration of symptoms ≤ 7 days.*
*** SARI with duration of symptoms > 7 days, SARI/TB or TB (for Edendale and Klerksdorp-TshepongHospital Complex only).*
****If patient co-enrolled in the TSAP study (for EdendaleHospital only).*

NB: For Edendale and Klerksdorp-TshepongHospital Complex only

Enrolled in shedding study Yes ☐ No ☐

1. Date of birth (DOB): ☐☐/☐☐/☐☐☐☐ If DOB unknown, please enter age:_____Years ☐ Months ☐ Days ☐
2. Gender: Male ☐ Female ☐
3. Race: Asian/Indian ☐ Black ☐ Coloured ☐ White ☐ Other ☐ (Specify) _____
4. What is your house made of: Bricks ☐ Iron sheeting ☐ Mud ☐ Other ☐ (Specify) _____
5. Number of rooms used for sleeping? _____ 5.1 Number of people living in the house? _____
6. What is the interviewee's relationship to the participant? Self ☐ Parent/Caregiver ☐ Other ☐ (Specify) _____
7. Date of admission: ☐☐/☐☐/☐☐☐☐
8. Admission diagnosis: (Tick all that apply) Neonatal Sepsis ☐ Bronchiolitis ☐ Bronchopneumonia/Pneumonia/Lower Respiratory Tract Infection ☐ TB ☐ Bronchitis ☐ Diarrhoea ☐ Febrile seizures ☐ Meningitis ☐ Sepsis (not neonatal) ☐ Other ☐ (Specify) _____
9. Date of onset of ☐☐/☐☐/☐☐☐☐

symptoms: _____

9.1 Duration of symptoms: 0-7days ☐ 8-14days ☐ >14days

Note: Complete the signs and symptoms at the time of admission, some of the signs or symptoms may have resolved by the time you interview the patient. This information should be available in the patient records.

10. Maximum recorded temperature within 24hours of admission ____.____C° (##.#C°)
Note: Record the maximum temperature recorded in the clinical notes or a temperature taken by the surveillance officer within 24 hours of admission if no temperature in the file.

11. Date of maximum temperature recorded: ☐☐/☐☐/☐☐☐☐ Temperature not recorded ☐ ☐

12. History of fever? Yes ☐ No ☐Unk ☐ If yes, date of fever onset: ☐☐/☐☐/☐☐☐☐

13. Respiratory Rate: _____breaths per minute Not recorded ☐

14. Oxygen saturation (room air): _____% Date of O_2 Saturation: ☐☐/☐☐/☐☐☐☐
Recorded on file ☐ Measure by Surveillance Officer within 24h of admission ☐
Not recorded on file and not measured by Surveillance Officer within 24h of admission ☐

15. Patient length (if not recorded, surveillance officer to measure): _____._____cm Percentile (if patient < 5 years) _____Unk☐

15.1 Patient weight (if not recorded, surveillance officer to measure): _____._____kg Percentile (if patient < 5 years) _____Unk☐

15.2 Mid upper arm circumference (MUAC) (for patients < 5 years of age only) _____ cm

16. Blood pressure on day of admission: _____/_____ Not recorded ☐

17. Mental status of the patient (based on AVPU), within 24 hours of admission
Alert ☐Disorientated☐Stuporous☐Comatose☐ Sedated☐Unknown☐
(responds to verbal commands) (responds to painful stimuli)

Note: If patient is alert report 15/15.

18. Glasgow Coma Score (GCS) _____/15 GCS from medical records: Yes ☐ No ☐
GCS Unk☐(if patient not alert and GCS not recorded on medical records)

Note: Complete for patients ≥ 12 years, if patient < 12 years skip to Q 20.

19. Patient oriented to:
Person: Yes ☐ No ☐Unk☐ Place: Yes ☐ No ☐Unk☐ Time: Yes ☐ No ☐Unk☐

Note: Complete the table below for children < 5 years, if patient ≥ 5 years skip to Q 21.

20. Were any of the following signs and symptoms present?

Cough	Y	N	Difficulty breathing	Y	N	Chest in drawing (retraction)	Y	N
Stridor in a calm child	Y	N	Tachypnoea/respiratory distress (2mnth-1yr RR>50, 1-5 RR>40)	Y	N	Diarrhoea (>3 loose stools per day)	Y	N
Unable to drink or breast feed	Y	N	Vomits everything	Y	N	Convulsions	Y	N
Lethargy	Y	N	Unconsciousness	Y	N	Any vomiting	Y	N
Wheezing	Y	N						

Note: Complete the table below for patients ≥ 5 years, if patient < 5 years skip to Q 22.

21. Were any of the following signs and symptoms present?

Sore throat	Y	N	Cough	Y	N	Chest pain	Y	N
Shortness of breath/difficulty breathing	Y	N	Diarrhoea (>3 loose stools per day)	Y	N	Wheezing	Y	N

Note: Complete the table below for all patients.

22. Were any of the following signs and symptoms present?

Chronic cough for 2 weeks or more	Y	N	Fever for more than 2 weeks (14 days)	Y	N	Cough up blood (Hemoptysis)	Y	N
Loss of appetite	Y	N	Night sweats	Y	N	Loss of weight	Y	N
Painless enlarged lymph glands	Y	N	History of close contact with a person with recently active TB (diagnosis ≤ 12 months ago)	Y	N	If yes, is the patient staying in the same house with the TB contact	Y	N

Note: Complete the following questions for patients ≥ 18 years, if patient < 18 years skip to Q 26.

23. Do you drink alcohol? Yes ☐ No ☐ If yes, how many units per week? _____

24. Do you currently smoke? Yes ☐ No ☐ If yes, how many cigarettes do you smoke per day? _____

24.1 If no, have you smoked in the past? Yes ☐ No ☐ If yes, date stopped smoking: ☐☐/☐☐/☐☐☐☐

25. Do you currently or have you ever worked in a mine before? Yes ☐ No ☐ Unk ☐
Note: If no or unknown, skip to Q 26)

25.1 If yes, date started working in the mine: ☐☐/☐☐/☐☐☐☐ Date unknown ☐

25.2 If working in a mine or worked in a mine before, when did you stop working in the mine? ☐☐/☐☐/☐☐☐☐ Ongoing ☐

Date Unknown ☐

25.3 If currently or worked in mine before, what type of mine/s? (tick all that apply)

Gold ☐ Coal ☐ Platinum ☐ Asbestos ☐ Other ☐ (Specify) _____

26. Do you have any underlying illness or condition at the moment? Yes ☐ No ☐
Note: Check patient's notes for details if necessary. If no skip to Q 27

Asthma	Y	N	Other chronic lung disease	Y	N	CVA/Stroke	Y	N
Cirrhosis/Liver failure	Y	N	Chronic renal failure	Y	N	Heart failure	Y	N
Valvular heart disease	Y	N	Coronary artery disease (except H/T)	Y	N	Pregnancy	Y	N
Organ transplant	Y	N	Any immunosuppressive therapy, cortisone, chemotherapy, radiation therapy	Y	N	Sickle cell	Y	N
Splenectomy	Y	N	Diabetes	Y	N	Burns	Y	N
Immunoglobulin deficiency	Y	N	Autoimmune disease, SLE	Y	N	Kwashiorkor/ Marasmus	Y	N
Nephrotic syndrome	Y	N	Spinal cord injury	Y	N	Seizure disorder	Y	N
Prematurity	Y	N	Obesity / BMI >=30	Y	N	COPD/Emphysema	Y	N
Malignancy/Cancer	Y	N	If yes, specify:					
Other	Y	N	If yes, specify:					
Does the patient live in an institution/care facility	Y	N	If yes, name of institution:					

27. Has the patient been admitted to hospital in the last 12months? (prior to this admission) Yes ☐ No ☐ Unk ☐
Note: If no or unknown, skip to Q 28.

27.1 If yes, what was the date that you were discharged from your last hospital admission? ☐☐/☐☐/☐☐☐☐

Date Unk☐

27.2 Was the patient admitted more than once in the past year? Yes ☐ No ☐Unk ☐ If yes, how many times? _____

Note: Complete for patients < 5 years, if patient ≥ 5 years skip to Q 29.

28. HIV result during pregnancy (mother of patient): Yes ☐ No ☐Unk☐
Note: If no or unk skip to Q 29

28.1 If yes, what was the result? Positive ☐ Negative ☐

28.2 What was the source of the results? RTHC ☐ Laboratory report ☐ Medical records ☐ Verbal ☐ Other ☐ Specify _____

29. Has the patient been tested for HIV prior to this admission? Yes ☐ No ☐Unk☐
Note: If no or unknown skip to Q 30

29.1 If yes, what was the result? Positive ☐ Negative ☐Unk☐
Note: If negative or unknown skip to Q 30

29.2 Currently on ART? Yes ☐ No ☐ Unknown ☐ If yes, date of initiation of ART: ☐☐/☐☐/☐☐☐☐

29.3 Bactrim (contrimoxazole/trimethoprim) prophylaxis taken currently? Yes ☐ No ☐Unk☐
Note: If no or unknown skip to Q 29.5

29.4 If yes, how long have you taken Bactrim? _____Years _____Months_____Weeks _____Days

29.5 What is the patient's clinical HIV stage according to WHO criteria (refer to WHO clinical staging information and HIV staging tick box document)
1 ☐ 2 ☐ 3 ☐ 4 ☐

30. Have you ever taken TB prophylaxis? Yes ☐ No ☐Unk☐ If yes, date TB prophylaxis initiated: ☐☐/☐☐/☐☐☐☐
Note: If no or unknown skip to Q 31

30.1 Are you still taking TB prophylaxis? Yes ☐ No ☐Unk☐ If no, date TB prophylaxis stopped: ☐☐/☐☐/☐☐☐☐
Note: If yes skip to Q 32

31. TB treatment in the last 12 months? Yes ☐ No ☐Unk☐ If yes, date TB treatment initiated: ☐☐/☐☐/☐☐☐☐
Note: If no or unknown skip to Q 32

31.1 Are you still taking TB treatment? Yes ☐ No ☐Unk☐ If no, date TB treatment stopped: ☐☐/☐☐/☐☐☐☐

Note: Ask patients and check medical records for PCP diagnosis and/or treatment

32. Have you ever been diagnosed with PCP before this admission? (check from medical records) Yes ☐ No ☐Unk☐
Note: If no or unknown skip to Q 33

32.1 If yes, date last treatment started: ☐☐/☐☐/☐☐☐☐ Date Unk☐

32.2 If yes, date last treatment stopped: ☐☐/☐☐/☐☐☐☐ Date Unk☐Ongoing☐

32.3 If yes, treatment started with steroid therapy? Yes ☐ No ☐Unk☐

33. Has the patient been prescribed and taken antibiotics in the 24 hours before this admission? Yes ☐ No ☐Unk☐
Note: If no or unknown skip to Q 34

33.1 If yes, what is the name of the antibiotic? 1. _____ 2. _____ 3. _____

AMO Amoxicillin; AMP Ampicillin; AUG Augmentin; CEF Cefuroxime, CIP Ciprofloxacin; CLI Clindamycin; CTX Ceftriaxone; DOX Doxycycline; ERY Erythromycin, PEN Penicillin, TMX/SMX Contrimoxazole, VAN Vancomycin. If other, specify _____

34. Vaccination history. Complete for patients < 5 years, if patient ≥ 5 years skip to Q 35

34.1 Is the person being interviewed the primary caregiver of the child? Yes ☐ No ☐

Note: If no skip to Q 34.3

34.2 If yes, has the child ever been vaccinated? Yes ☐ No ☐ Unk ☐
Note: Excluding the vaccines given at birth

34.3 Was the Road to Health Card seen? Yes ☐ No ☐

34.4 Was a copy of the Road to Health Card made? Yes ☐ No ☐
Note: If no skip to Q 35

34.5 If copy was not made, state reason: Mother refused ☐ Other ☐ (specify) _____

If Road to Health Card seen, please copy the following information from the card:

34.6 What is patients' gestational age: Term ☐ Pre-term ☐ Not recorded on Road to Health Card ☐

If pre-term, record gestational age: _____weeks

34.7 If Road to Health Card seen, please complete the details on the following vaccines for all children < 5 years old

Note: Tick no for vaccines that are not yet due according to the schedule. At 18 months if only the DTP was given tick DTP only and N/A for the combined DTP/HIB, if a combined DTP/HIB was given tick yes under combined and N/A under DTP only

Vaccine	Dose due	Given			Date given
BCG	Birth	Y	N		☐☐/☐☐/☐☐☐☐
DTP + HIB vaccine	Dose 1 (6 weeks)	Y	N		☐☐/☐☐/☐☐☐☐
	Dose 2 (10 weeks)	Y	N		☐☐/☐☐/☐☐☐☐
	Dose 3 (14 weeks)	Y	N		☐☐/☐☐/☐☐☐☐
	Dose 4 (18 months)	Y	N	N/A	☐☐/☐☐/☐☐☐☐
DTP only (tick N/A if DTP+HIB)	Dose 4 (18 months)	Y	N	N/A	☐☐/☐☐/☐☐☐☐
S.pneumoniaeconjugatevaccine ($PCV_{7/13}$/Prevenar)	Dose 1 (6 weeks)	Y Batch N°_____ Unk ☐	N		☐☐/☐☐/☐☐☐☐
	Dose 2 (14 weeks)	Y Batch N°_____ Unk ☐	N		☐☐/☐☐/☐☐☐☐
	Dose 3 (9 months)	Y Batch N°_____ Unk ☐	N		☐☐/☐☐/☐☐☐☐
	Catch up	Y Batch N°_____ Unk ☐	N		☐☐/☐☐/☐☐☐☐
Measles	Dose 1 (9 months)	Y	N		☐☐/☐☐/☐☐☐☐
	Dose 2 (18 months)	Y	N		☐☐/☐☐/☐☐☐☐
Hepatitis B	Dose 1 (6 weeks)	Y	N		☐☐/☐☐/☐☐☐☐
	Dose 2 (10 weeks)	Y	N		☐☐/☐☐/☐☐☐☐
	Dose 3 (14 weeks)	Y	N		☐☐/☐☐/☐☐☐☐
Rotavirus	Dose 1 (6 weeks)	Y	N		☐☐/☐☐/☐☐☐☐
	Dose 2 (14 weeks)	Y	N		☐☐/☐☐/☐☐☐☐

Note: Complete for patients ≥ 5 years, if patient < 5 years skip to Q 36

35. Did the patient receive pneumococcal polysaccharide vaccine?

Vaccine	Dose given	Date given	Date unknown ☐

Pneumococcal Vaccine (Pneumovax)		Y	N	UNK	☐☐/☐☐/☐☐☐☐	

36. Did the patient receive an influenza vaccine in the past 12 months (For all patients)

Vaccine		**Dose given**			**Date given**	
Influenza vaccine	Dose 1	Y	N	UNK	☐☐/☐☐/☐☐☐☐	**Date unknown** ☐
	Dose 2	Y	N	UNK	☐☐/☐☐/☐☐☐☐	**Date unknown** ☐

QC Performed by: Initials:☐☐☐ Date:☐☐/☐☐/☐☐☐☐

Appendix 2: Severe Respiratory Illness (SRI) Hospital Results Form (HRF)

SO Initials: ☐☐☐ Date completed:☐☐/☐☐/☐☐☐☐ (DD/MM/YYYY) SARI Study ☐ TSAP Study ☐				*Note: For Edendale Hospital only if patient co-enrolled in TSAP*
Please record these results from the laboratory if they are available. *Note: Only results from this admission except for Q 4, 5 and 8 where the most recent available result should be entered and Q 7 where results before admission must be recorded.*				
Test			Date of test	Result
1. CRP on admission	Done ☐	Not done ☐	☐☐/☐☐/☐☐☐☐	_____mg/l
2. ESR on admission	Done ☐	Not done ☐	☐☐/☐☐/☐☐☐☐	_____mm/Hr
3. Urea on admission	Done ☐	Not done ☐	☐☐/☐☐/☐☐☐☐	_____mmol/l
4. Documented HIV Antibody Result (ELISA or rapid)	Yes ☐ No ☐		☐☐/☐☐/☐☐☐☐ *Note:Enter date of most recent result available*	Reactive ☐ Non reactive ☐ *Note: Enter most recent result available*
5. Documented HIV PCR Result	Yes ☐ No ☐		☐☐/☐☐/☐☐☐☐ *Note:Enter date of most recent result available*	Positive ☐ Negative ☐ *Note: Enter most recent result available*
6. CD4 count on	Done ☐	Not	☐☐/☐☐/☐☐☐☐	_____ Absolute CD4

admission	done ☐		____._____%
7. CD4 count collected before this admission	Done ☐ Not done ☐	☐☐/☐☐/☐☐☐☐	_____ Absolute CD4 ____._____%
8. HIV viral load	Done ☐ Not done ☐	☐☐/☐☐/☐☐☐☐ *Note: Enter date of most recent result available*	_____ HIV RNA copies/ml ____._____ Viral load log *Note: Enter most recent result available*
9. Was a CXR done on the patient? Yes ☐ No ☐ *Note :If not done go to Q 10* 9.1. If X-ray done record X- ray number here: _____		*Note: For Klerksdorp Tshepong Hospital Complex only* 9.2 Copy of X-ray downloaded? Yes ☐ No ☐ 9.3 if yes, date X-ray copy downloaded ☐☐/☐☐/☐☐☐☐	

10. Bacterial Culture, done within 48 hours of admission? Yes ☐ No ☐

Note: If yes, complete the table below, if no skip to Q11

Date of culture	Site			Organism Isolated	If other (99), specify
	Blood	CSF	Pleural fluid		
☐☐/☐☐/☐☐☐☐					

□□/□□/□□□□					
□□/□□/□□□□					
□□/□□/□□□□					

Organism codes:

01 = No growth	02 = *Streptococcuspneumoniae*	03 = *Haemophilusinfluenzae*	04 = *Klebsiella pneumoniae*
05 = *Staphylococcus aureus / MRSA*	06 = *Neiserria meningitides*	07 = *Coagulase negative staphylococcus / Staphylococcus epidermidis*	08 = *Bacillus Species*
09 = *Corynebacteriumspecies*	11 = *Cryptococcus neoformans*	12 = *Escherichia coli*	13 = *Salmonella species*
14 = *Streptococcus agalactiae / Group B Streptococcus*	15 = *Acinetobacter baumanii*	16 = *Pseudomonas aeruginosa*	17 = *Enterococcus faecalis*
18 = *Streptococcus viridans*	19 = *Micrococcus species*	20 = *Salmonella enterica_ Non-Typhodial* (NTS)	*21 = Salmonella enterica- Typhi*
22 = Salmonella	99 = Other		

parathyphi				
10.1 Blood Specimen and Culture taken by	☐SO ☐ Clinician			
10.2. Blood Culture Specimen requested by clinician	☐Yes ☐ No			
11. TB testing done during this admission? Yes ☐ No ☐				
Date of Test	☐☐/☐☐/☐☐☐☐	☐☐/☐☐/☐☐☐☐	☐☐/☐☐/☐☐☐☐	☐☐/☐☐/☐☐☐☐
Specimen*				
AFB's present (Yes/No/Not Applicable)	Y N N/	Y N N/	Y N N/	Y N N/
Culture Done (Yes/No)	Y N	Y N	Y N	Y N
Culture Results (Positive/Negative/ Contaminated)	PO NE CO Date of final results report ☐☐/☐☐/☐☐☐☐	PO NE CO Date of final results report ☐☐/☐☐/☐☐☐☐	PO NE CO Date of final results report ☐☐/☐☐/☐☐☐☐	PO NE CO Date of final results report ☐☐/☐☐/☐☐☐☐
GeneXpert Done	Y N	Y N	Y N	Y N
GeneXpert Results	PO NE INC	PO NE INC	PO NE INC	PO NE INC

(Positive/ Negative/ Inconclusive)				

*Specimen codes:

1 = Sputum 2 = Gastric Washing 3 = Bone Marrow 4 = Pleural Aspirate 5 = Lymph Node 6 = Other (if other, write in full)

Note: Culture results will be reviewed at a later date.

Do not delay submission of CIF and Result Form while waiting for TB culture results.

Note: complete the table below for TSAP (For Edendale Hospital KZN Only):

12. Was a blood count done during this admission? Yes ☐ No ☐

Note: If not done end; if yes, complete table below

WBC ☐☐☐.☐ (1000/µl)	Platelets ☐☐☐.☐ (1000/µl) ☐ Not recorded
RBC ☐☐.☐ (10^6/ µl)	Neutrophils ☐☐.☐ (%WBC) ☐ Not recorded
Hb ☐☐.☐ (g/dl)	
Hct ☐☐.☐ (%)	Monocytes ☐☐.☐ (%WBC) ☐ Not recorded
MCV ☐☐☐.☐ (fl)	Lympocytes ☐☐.☐ (%WBC) ☐ Not recorded
MCH ☐☐☐.☐ (pg)	Eosinophils ☐☐.☐ (%WBC) ☐ Not recorded

MCHC	☐☐.☐ (g/dl)	Basophils	☐☐.☐ (%WBC)	☐ Not recorded
QC perfomed by: Initials ☐☐☐		Date:☐☐/☐☐/☐☐☐☐		

Appendix 3: Severe Respiratory Illness (SRI) Surveillance Final Outcome Form (FOF)

NATIONAL HEALTH LABORATORY SERVICE
NATIONAL INSTITUTE FOR COMMUNICABLE DISEASES

Severe Acute Respiratory Illness (SARI)

Surveillance

Final Outcome Form (FOF)

Centre for Respiratory Diseases and Meningitis (CRDM)

TEL: 011 386 6410 or 011 386 6434

FAX: 086 723 3569

SO Initials: ☐☐☐	SARI Study ID:	Date of admission: ☐☐/☐☐/☐☐☐☐ (DD/MM/YYYY)

Did any of the following occur during admission?

1.	Was TB therapy started?	Yes ☐ No ☐
2.	Did the patient receive oxygen?	Yes ☐ No ☐
3.	Did the patient receive mechanical ventilation?	Yes ☐ No ☐
4.	Did the patient have a cardiac arrest?	Yes ☐ No ☐
5.	Was the patient admitted to ICU?	Yes ☐ No ☐
6.	Did the patient go into shock (Systolic BP < 90mmHg) or require inotropes?	Yes ☐ No ☐

37. Antibiotics prescribed during this admission: Yes ☐ No ☐Unk☐
Note: If yes, complete the table below. If no/unknown, skip to Q 8

	PO	IVI	IMI	Date started (enter date prescribed)		PO	IVI	IMI	Date started (enter date prescribed)
Amoxicillin				☐☐/☐☐/☐☐☐☐	**Ampicillin**				☐☐/☐☐/☐☐☐☐
Augmentin				☐☐/☐☐/☐☐☐☐	**Cefotaxime**				☐☐/☐☐/☐☐☐☐
Ceftriaxone (Rocephin)				☐☐/☐☐/☐☐☐☐	**Cefuroxime (Zinnat)**				☐☐/☐☐/☐☐☐☐
Ciprofloxacin (Ciprobay)				☐☐/☐☐/☐☐☐☐	**Clindamycin**				☐☐/☐☐/☐☐☐☐
Contrimoxazole (Bactrim)				☐☐/☐☐/☐☐☐☐	**Doxycycline**				☐☐/☐☐/☐☐☐☐
Erythromycin				☐☐/☐☐/☐☐☐☐	**Gentamycin**				☐☐/☐☐/☐☐☐☐
Penicillin G				☐☐/☐☐/☐☐☐☐	**Vancomycin**				☐☐/☐☐/☐☐☐☐

Ampiclox				☐☐/☐☐/☐☐☐☐	**Metronidazole**				☐☐/☐☐/☐☐☐☐
Other				☐☐/☐☐/☐☐☐☐	**Specify**				

38. Was Oseltamivir (Tamiflu®) prescribed during this admission? Yes ☐ No ☐Unk☐

38.1 If yes, date prescribed: ☐☐/☐☐/☐☐☐☐

39. Was *Pneumocystis jirovecii* pneumonia (PCP) treatment prescribed during this admission? Yes ☐ No ☐Unk☐
Note: If yes, complete table below, if no or unknown skip to Final Outcome of Patient

	Dose (Specify quantity and unit)	**Oral**	**IMI**	**IVI**	**Frequency**	**Date started (enter date prescribed)**	**Total number of days**
Cotrimoxazole						☐☐/☐☐/☐☐☐☐	
Dapsone						☐☐/☐☐/☐☐☐☐	
Prednisone						☐☐/☐☐/☐☐☐☐	
Hydrocortisone						☐☐/☐☐/☐☐☐☐	
Other (specify) _____						☐☐/☐☐/☐☐☐☐	

Final outcomes of patient (for this admission)

What was the final outcome (for this admission)?

Discharge ☐ Death ☐ Refused hospital treatment/absconded ☐

Referred to step down ☐ Name of facility: _____

Transferred ☐ Name of facility: _____

Date of final outcome: ☐☐/☐☐/☐☐☐☐

Discharge/final diagnosis or diagnosis on transfer/referral: Neonatal Sepsis ☐ Bronchiolitis ☐ Bronchopneumonia/Pneumonia/Lower Respiratory Tract Infection ☐ Suspected TB ☐ Confirmed TB ☐Bronchitis ☐ Diarrhoea ☐ Febrile seizures ☐ Meningitis ☐ Sepsis (not neonatal) ☐Not recorded☐Other☐(Specify) _____

Outcome follow up ***(for all SARI and SRI cases enrolled at Edendale, Klerksdorp and TshepongHospitals only)***

Date of follow up: ☐☐/☐☐/☐☐☐☐Not done☐

What was the follow up outcome? Death ☐If died, date of death☐☐/☐☐/☐☐☐☐Date Unk☐
Alive ☐Unknown (not possible to determine)☐

QC performed by: Initials: ☐☐☐ Date: ☐☐/☐☐/☐☐☐☐

Appendix 4: List of reference strains used for the validation of the atypical multiplex real-time PCR assay

Name of Organism	Number
Mycoplasma pneumoniae	ATCC 29342D
Mycoplasma hominis	DSM 19104
Mycoplasma genitalium	DSM 19775
Legionella pneumophila	ATCC 33152D-5
Legionella longbeachae	ATCC 33462D-5
Chlamydia.(*Chlamydophila*) *pneumoniae*	ATCC 53592D
Chlamydia trachomatis	ATCC VR-879D
Chlamydia psittaci	ATCC VR125D
Staphylococcus aureus	ATCC 29213
Staphylococcus aureus	ATCC 25923
Haemophilus influenzae	ATCC 49247
Haemophilus parainfluenzae	CDC 3438
Bordetella pertussis	ATCC 9797
Streptococcus viridans	ATCC 9811
Streptococcus pneumoniae	ATCC 49619
Neisseria meningitidis	EMGM 6
Enterococcus faecalis	ATCC 29212
Morraxella cattarhalis	ATCC 25238
Escherichia coli	ATCC 35218
Klebsiella pneumoniae	ATCC 700603
Pseudomonas aeruginosa	ATCC 27853

Abbreviations: ATCC=American Type Culture Collection; CDC=Centers for Disease Control and Prevention; EMGM=European Monitoring Group for Meningococci; DSM=German Collection of Microorganisms and Cell Cultures

Appendix 5: Primers and probes for the detection of *M. pneumoniae, Legionella* species, *C. pneumoniae* and human *RNaseP*

Primer/Probe name	Sequence (5'→3')	Gene Target	Final Concentration of primer/probe (nM)
MP 181-F	TTTGGTAGCTGGTTACGGGAAT	*M. pneumoniae* CARDS Tx	250
MP 181-R	GGTCGGCACGAATTTCATATAAG		250
MP 181-Probe	HEX-TGTACCAGAGCACCCCAGAAGGGCT-BHQ1		100
CP-Arg-F	CGTGGTGCTCGTTATTCTTTACC	*C. pneumoniae argR*	250
CP-Arg-R	TGGCGAATAGAGAGCACCAA		250
CP-Arg-Probe	Cy5-CTTCAACAGAGAAGACCACGACCCGTCA-BHQ3		50
Pan-Leg-F	GGCGACCTGGCTTC	*Legionella* spp. *ssrA*	125
Pan-Leg-R	GGTCATCGTTTGCATTTATATTTA		125
Pan-Leg-Probe	6FAM-ACGTGGGTTGC-MGBNFQ		25
RNaseP-F	AGATTTGGACCTGCGAGCG	Human *RNAseP*	250
RNaseP-R	GAGCGGCTGTCTCCACAAGT		250
RNaseP-Probe	LC610-TTCTGACCTGAAGGCTCTGCGCG-BHQ2		50

Appendix 6: List of primer and probes used for *Legionella* speciation real-time PCR, *M. pneumoniae* macrolide susceptibility testing, P1 typing and MLVA typing for molecular characterisation of *M. pneumoniae*

Primer/Probe name	Sequence (5'→3')
Primer and probes sequences for *L. longbeachae* PCR	
LLB-F	TGGTTTTCGAAATCATCAGTATGC
LLB-R	CTGTCTAAAACACTTCTCTCCCGATA
LLB-Probe	Quasar670-TTTAATTTAGTTCCCACCAGCAAGGATGGC-BHQ3
Primer and probes sequences for *Legionella* speciation PCR	
ssrA-F	GGCGACCTGGCTTC
ssrA-R	GGTCATCGTTTGCATTTATATTTA
ssrA-Probe	6FAM-ACGTGGGTTGC-MGBNFQ
mip-F	TTGTCTTATAGCATTGGTGCCG
mip-R	CCAATTGAGCGCCACTCATAG
mip-Probe	Quasar670-CGGAAGCAATGGCTAAAGGCATGCA-BHQ3
wzm-F	TGCCTCTGGCTTTGCAGTTA
wzm-R	CACACAGGCACAGCAGAAACA
wzm-Probe	VIC-TTTATTACTCCACTCCAGCGAT-MGBNFQ
RNAseP-F	AGATTTGGACCTGCGAGCG
RNAseP-R	GAGCGGCTGTCTCCACAAGT
RNAseP-Probe	CalRd610-TTCTGACCTGAAGGCTCTGCGCG-BHQ2

Primer sequences for macrolide susceptibility testing

Forward Primer[1]	gacagtcTGGTGTAACCATCTCTTGAACTG"t"C
Reverse Primer	GCTCCTACCTATTCTCTACATGAT

Primer sequences for P1 typing

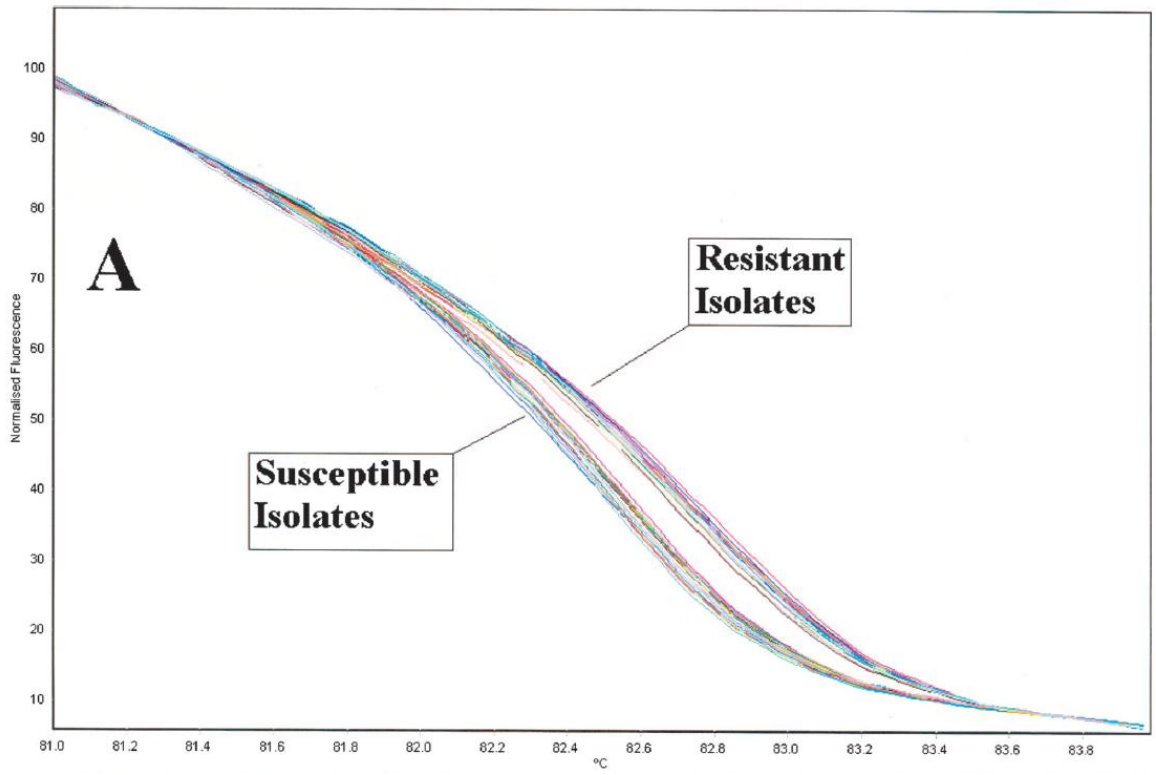
Mpt-F	TTAGCAGCTCTTCCCGACAA
Mpt-R	ACATCGTCATTCATCTTTGCGGC

Primer sequences for MLVA typing

Mpn 1-F	GTTGAAGTTATGCCGGTAGC
Mpn 1-R	HEX-CGCGATAGAAGGCATACTGC
Mpn13-F	GACCAGCATTAGATTGCTATG
Mpn13-R	NED-AACAAATTAAGCAGCTCACG
Mpn14-F	CTCAGGGCGAAACCTTAAAG
Mpn14-R	6-FAM-GCAATGGCTTTCAGCACAAC
Mpn15-F	HEX-CAACAGCACCACATCTTTAG
Mpn15-R	GCTAATCTTGCAAACGCTGC
Mpn16-F	NED-GACGCGTTCGCTAAAAGAG
Mpn16-R	GAGCGGCTGTCTCCACAAGT

[1]6-Carboxyfluorescein is attached to the "t" on the 3' end of the forward primer. The lower cased bases on the 5' end are added for quenching and hairpin formation when the primer is in the unbound state

Appendix 7:



Real-time high resolution melt curves depicting the difference between macrolide resistant and susceptible strains [59].

Appendix 8: Ethics clearance certificate

R14/49 Miss Maimuna Carrim

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M130211

NAME: (Principal Investigator)	Miss Maimuna Carrim
DEPARTMENT:	School of Pathology/Division of Virology National Institute for Communicable Diseases
PROJECT TITLE:	Identification and Prevalence of Bacteria Causing Atypical Pneumonia in Patients with Severe Respiratory Illness and Influenza-like Illness in South Africa, 2012
DATE CONSIDERED:	22/02/2013
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr Nicole Wolter
APPROVED BY:	Professor PE Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 22/02/2013

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

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

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 resubmit the application to the Committee. **I agree to submit a yearly progress report**

Principal Investigator Signature

Date 2-4-13

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix 9: Plagiarism report generated by Turnitin software



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ORIGINALITY REPORT

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PRIMARY SOURCES

1	Wolter, N., C. Cohen, S. Tempia, S. A. Madhi, M. Venter, J. Moyes, S. Walaza, B. M. Kgokong, M. Groome, M. du Plessis, M. Pretorius, H. Dawood, K. Kahn, E. Variava, K. P. Klugman, and A. von Gottberg. "HIV and Influenza Infection Are Associated with Increased Blood Pneumococcal Load, South Africa, 2009-2011", The Journal of Infectious Diseases, 2013. Publication	1%
2	B. J. Wolff. "Detection of Macrolide Resistance in Mycoplasma pneumoniae by Real-Time PCR and High-Resolution Melt Analysis", Antimicrobial Agents and Chemotherapy, 08/11/2008 Publication	1%
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4	"Posters", Clinical Microbiology and Infection, 5/2008 Publication	1%
5	S. B. Schwartz. "Genotyping of *Mycoplasma*	

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