Detection of *Bordetella* species in individuals presenting with severe respiratory illness and influenza-like illness in South Africa, June 2012 – October 2014

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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.

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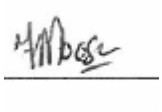
Declaration

I, Fahima Moosa, declare that this dissertation is my own unaided work. The experimental work described was conducted under the supervision of Dr Mignon du Plessis and Dr Anne von Gottberg at the Centre for Respiratory Diseases and Meningitis, National Institute for Communicable Diseases of the 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.



20th day August of 2015

For my parents and my husband

The guiding lights in my life

Presentations

Manuscript – In preparation

Fahima Moosa, Mignon du Plessis, Nicole Wolter, Cheryl Cohen, Sibongile Walaza, Claire von Mollendorf, Maimuna Carrim, Makatisane Papo, Stephano Tempia, Halima Dawood, Ebrahim Variava, and Anne von Gottberg. Detection of *Bordetella pertussis* and other *Bordetella* species in patients with mild and severe respiratory illness in South Africa, June 2012 – October 2014.

Conference presentations

Fahima Moosa, Mignon du Plessis, Nicole Wolter, Maimuna Carrim, Cheryl Cohen, Sibongile Walaza, Halima Dawood, Ebrahim Variava, and Anne von Gottberg. Detection of *Bordetella pertussis* in individuals presenting with severe respiratory illness and influenza-like illness in South Africa, May 2012 – June 2013. Poster presentation. Faculty of Health Sciences Research Day 2014, University of the Witwatersrand, Johannesburg, South Africa, 17th September 2014.

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Stakeholder meetings

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Fahima Moosa, Mignon du Plessis, Nicole Wolter, Maimuna Carrim, Cheryl Cohen, Sibongile Walaza, Halima Dawood, Ebrahim Variava, and Anne von Gottberg. Detection of *Bordetella* species in individuals presenting with severe respiratory and influenza-like illness in South Africa, 2012 – 2013. *Rotavirus and Severe Acute Respiratory illness (SARI) Surveillance Annual Investigators Meeting*. Oral presentation. Sandringham, Johannesburg, South Africa, 12 November 2013.

Fahima Moosa, Mignon du Plessis, Nicole Wolter, Maimuna Carrim, Cheryl Cohen, Sibongile Walaza, Halima Dawood, Ebrahim Variava, and Anne von Gottberg. Detection of *Streptococcus pneumoniae, Haemophilus influenzae* and *Bordetella pertussis* in individuals presenting with severe respiratory and influenza-like illness in South Africa, 2012. *Rotavirus and Severe Acute Respiratory illness (SARI) Surveillance Annual Investigators Meeting*. Oral presentation. Sandringham, Johannesburg, South Africa, 11 December 2012.

Abstract

Pertussis, caused by *Bordetella pertussis*, is a vaccine-preventable disease affecting persons of all ages. Despite vaccination with either the whole-cell or acellular vaccine, the burden of pertussis has increased worldwide. The acellular vaccine was licensed in South Africa in 2009, replacing the whole-cell vaccine; however, due to no active surveillance, pertussis is underestimated in this country. This study describes the burden of disease caused by *B. pertussis* and other *Bordetella* species in patients with severe respiratory illness (SRI), influenza-like illness (ILI) and controls.

Prospective, active surveillance was conducted amongst SRI and ILI patients and controls at two sentinel sites in South Africa. Patients who met the case definitions were enrolled from May 2012 to October 2014. Clinical and demographic data were collected. Induced sputum was collected from SRI patients only and combined nasopharyngeal/oropharyngeal specimens were collected from all patients and controls. Real-time polymerase chain reaction (PCR) was used to target the insertion sequences *IS481*, *pIS1001*, *hIS1001* and pertussis toxin gene *ptxS1*. All data were analysed in Microsoft Excel (Microsoft Corporation). Statistical significance was determined using the chi-squared test and univariate logistic regression at $p < 0.05$ for all parameters.

Of 8569 cases that were enrolled and tested, 118 [1.4%, 118/8569 (95% CI 1.1 – 1.6)] were positive for *B. pertussis* of which 2% [80/3982 (95% CI 1.6 – 2.5)] presented with SRI, 1% [32/3243 (95% CI 0.7 – 1.4)] with ILI and 0.4% [6/1344 (95% CI 0.2 – 1.0)] were asymptomatic. Positive cases were stratified into confirmed pertussis and probable pertussis based on cycle threshold (Ct) value cut-offs generated by real-time PCR for *IS481*. Within the SRI population, there were more probable than confirmed pertussis cases [51/3982, 1.3%

vs. 29/3982, 0.7%; p=0.02] and within the ILI group there were 0.5% confirmed and probable cases, respectively [15/3243, 0.5% vs. 17/3243, 0.5%; p=0.86]. The highest detection rate of pertussis in SRI positive cases was in the ≥65 year olds (2.8%, 6/208) and for the ILI positive cases the highest detection rate was in the 1-4 year olds (1.5%, 9/614). Pertussis disease was observed mainly in the winter and spring months with a 15% increase in disease detected in August 2014. The *B. pertussis* attributable fraction was 67% (95% confidence interval [CI] 18.49 – 86.63) for SRI positive cases. Fifty-eight percent (46/80) of *B. pertussis* positive cases were co-infected with respiratory bacteria (*Streptococcus pneumoniae*, *Haemophilus influenzae*, *Legionella* spp. or *Mycoplasma pneumoniae*) or viruses (influenza, respiratory syncytial virus (RSV), human metapneumovirus or other viruses (adenovirus, enterovirus, parainfluenza or rhinovirus). HIV status and full pertussis vaccination for age did not affect *B. pertussis* positivity.

B. parapertussis was detected in 1% [40/3982 (95% CI 0.7 – 1.4)] of the SRI population, 0.6% [18/3243 (95% CI 0.3 – 0.9)] of the ILI population and in 0.1% [2/1344 (0.02 – 0.5)] of asymptomatic individuals. The highest detection rate for the SRI (1.6%, 8/497) and ILI (1.5%, 9/614) positive cases were in the 1-4 year olds. The *B. parapertussis* attributable fraction was 80% (95% confidence interval [CI] 12.52 – 95.38) for SRI cases. Four cases tested positive for *B. bronchiseptica*, of which one individual was HIV positive.

B. pertussis, *B. parapertussis* and *B. bronchiseptica* were detected despite the case definitions not being ideal for the detection of these pathogens. *Bordetella* spp. was detected in all age groups tested. This study generates baseline data for pertussis in South Africa and surveillance is ongoing.

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Nomenclature

%	–	Percentage
>	–	Greater than
<	–	Less than
±	–	plus/minus
≤	–	Less than or equal to
≥	–	Greater than or equal to
°C	–	Degrees Celsius
µl	–	Microliter
ATCC	–	American Type Culture Collection
BPDNA	–	*Bordetella pertussis* DNA panel
CDC	–	Centers for Disease Control and Prevention
CI	–	Confidence interval
CO_2	–	Carbon dioxide
Ct	–	Cycle threshold
DFA	–	Direct fluorescent antibody
DMP	–	Dignostic Media Products
DNA	–	Deoxyribonucleic acid
DTaP	–	Diphtheria tetanus acelluar pertussis
DTT	–	Dithiothreitol
e.g.	–	Example
EQA	–	External quality assessment
HIV	–	Human immunodeficiency virus
HREC	–	Human Research Ethics Committee
i.e.	–	That is

IgG	–	Immunoglobulin G
ILI	–	Influenza-like illness
IS	–	Induced sputum
LRTI	–	Lower respiratory tract infection
n	–	Number
N	–	Total number
NHLS	–	National Health Laboratory Service
NICD	–	National Institute for Communicable Diseases
NP	–	Nasopharyngeal
OR	–	Odds ratio
PCR	–	Polymerase chain reaction
pH	–	Power of hydrogen
PT	–	Pertussis toxin
QCMD	–	Quality Control for Molecular Diagnostics
rpm	–	Revolutions per minute
RSV	–	Respiratory syncytial virus
RTHC	–	Road-to-Health Card
SARI	–	Severe acute respiratory illness
SD	–	Standard deviation
SOP	–	Standard operating procedure
spp	–	Species
SRI	–	Severe respiratory illness
UTM	–	Universal transport medium
WHO	–	World Health Organization
Wits	–	University of the Witwatersrand

Contents

1. Literature review

1.1. Background

Pneumonia is a severe lower respiratory tract infection that is characterised by pus and fluid build-up in the alveoli of the lungs, making breathing difficult (1). It is most prevalent in sub-Saharan Africa and South Asia (2;3). Pneumonia is differentiated into community-acquired or hospital-acquired infection and is associated with morbidity and mortality in patients of all ages, but is more common in children <5 years of age and the elderly (4). In 2011 approximately 120 million cases of childhood pneumonia were estimated globally (5). The human immunodeficiency virus (HIV) pandemic has resulted in an increased incidence of pneumonia globally (6), and in sub-Saharan Africa where HIV is epidemic, pneumonia is responsible for severe illness, hospitalisation and mortality in HIV-infected children (7). In HIV-endemic areas, pneumonia is the leading cause of hospitalisation; however most cases are only diagnosed HIV positive upon admission (8). Treatment of pneumonia is challenging due to the many respiratory pathogens that may cause this infection, the difficulty in making a clinical and aetiological diagnosis, and the lack of a single antimicrobial drug that is effective against all the pathogens associated with pneumonia (9).

Pneumonia is caused by many respiratory pathogens, both viral and bacterial (10), however, it is most commonly associated with bacterial pathogens (1). There are many diagnostic methods employed to identify these pathogens such as bacterial culture and microscopy, however these techniques only result in approximately 20% to 25% identification of total CAP cases due to limited sensitivity (11). Other methods such as identification of bacterial antigens, e.g. pneumococcal antigen with BinaxNOW® (12), and serology, are either unable to distinguish carriage from disease or provide information retrospectively and are therefore not useful for treatment (11). Of the many techniques available, polymerase chain reaction

(PCR) is a popular method for identification due to its increased sensitivity, being able to detect the minimum number of bacterial cells (13) and has the ability to detect the causative agent after a patient has been on antibiotic treatment (14). The technique is also specific in its ability to detect only the pathogen of interest. Since PCR is rapid, a diagnosis can be made earlier than culture resulting in more timely commencement of treatment (13).

Studies have shown that the most common bacterial pathogens that cause pneumonia are *Streptococcus pneumoniae*, *Haemophilus influenzae, Staphylococcus aureus* (1;10), *Moraxella catarrhalis*, group A streptococci, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae* (1) and *Bordetella pertussis* (15). There have been studies highlighting the bacterial aetiology of pneumonia and describing the burden of disease caused by each of the pathogens; however few studies have focussed on *B. pertussis* as an aetiological agent.

1.2. *B. pertussis*

Pertussis, caused by *B. pertussis*, is a vaccine-preventable respiratory disease affecting persons of all ages (16). The organism belongs to the genus *Bordetellae* and is one of eight other *Bordetella* species namely: *B. parapertussis, B. bronchiseptica, B. holmesii, B. avium, B. trematum, B. hinzii, B. petrii* and *B. ansorpii*. *B. pertussis*, *B. parapertussis*, *B. bronchiseptica* and *B. holmesii* are known to cause disease in humans; however, *B. pertussis* and *B. parapertussis* are the common causative agents of disease.

B. pertussis caused its first well documented outbreak of pertussis in 1578, and in 1679 the disease was named whooping cough (17). The bacterium was discovered in 1900 by Jules Bordet and Octave Gengou after examining sputum from a 6-month-old baby suffering from

whooping cough. *B. pertussis* is a Gram-negative coccobacillus that is catalase positive and oxidises amino acids (17;18). *B. pertussis* is aerobic and requires an optimum temperature between 35-37°C for growth on specialised media without fatty acids, metal ions, sulphides and peroxides. Culture media such as Bordet-Gengou and Regan-Lowe charcoal medium are used for *B. pertussis* isolation with colonies appearing as mercury-like droplets.

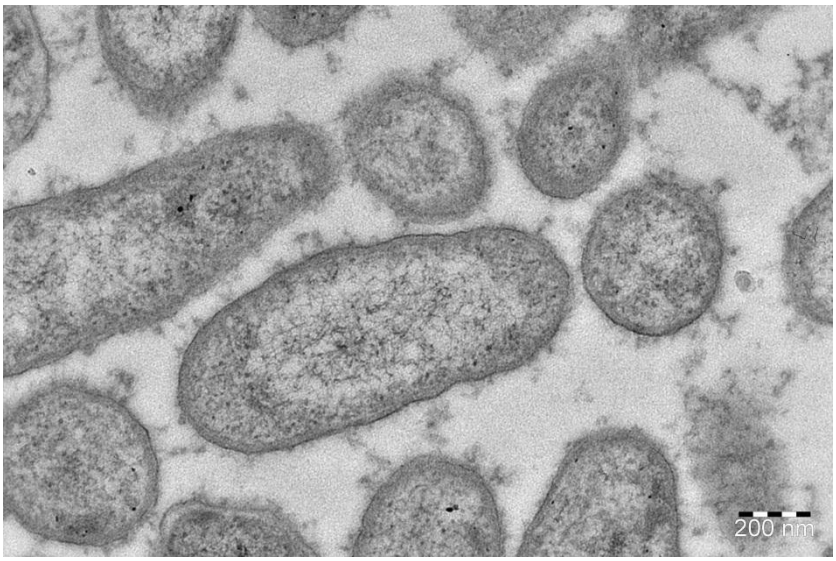

Figure 1: Electron micrograph showing outer structure of *B. pertussis* ATCC 9797.

1.2.1. Pathogenesis and clinical manifestation

B. pertussis is a strict human pathogen, therefore modelling the disease in animals and understanding its pathogenesis is difficult (19). Transmission is from person-to-person via respiratory droplets from an infected person and disease is toxin mediated (20). The disease cycle includes the following process (16;17;19): *B. pertussis* produces filamentous hemagglutinin, pertactin, and 2 fimbrial proteins which aid in attachment of the bacterium to the cilia of the respiratory epithelial cells of the nasopharynx. The organism then replicates and spreads to the ciliated epithelial cells of the trachea and bronchi in the absence of an immune response. *B. pertussis* then produces pertussis toxin, tracheal cytotoxin and adenylate

cyclase toxin which damage the respiratory epithelial cells and alveolar macrophages. This damage results in hyperlymphocytosis and impairment of chemotaxis resulting in the host immune defences being evaded.

The most common systemic manifestation of pertussis are leucocytosis and lymphocytosis (21). Sensitisation to histamine, serotonin and the beta-islet cells of the pancreas has also been observed. The disease manifests in 3 stages (17;18;22;23). The initial catarrhal phase is characterised by symptoms of the common cold which include rhinorrhoea, fever and occasional cough. In this stage the patient is most infectious. The catarrhal stage is followed by the paroxysmal phase where patients have the typical symptoms of pertussis which include whooping cough, paroxysms and posttussive vomiting. This phase is followed by the convalescent phase were disease symptoms are less severe. Disease symptoms in children with pertussis are severe, while adults and adolescents may have asymptomatic/atypical infection (16;24).

1.2.2. Laboratory diagnosis

The ideal specimen type for the diagnosis of pertussis is either a nasopharyngeal aspirate or a posterior nasopharyngeal swab (17). These specimens are ideal as they contain the ciliated epithelial cells to which *B. pertussis* attaches.

Together with clinical history, culture, direct fluorescent antibody (DFA), serology and PCR are used for the diagnosis of pertussis (24). Culture of nasopharyngeal specimens, the gold standard, is highly specific and the most common method of choice. This method is recommended during the catarrhal stage of illness. However, since *B. pertussis* requires between 3 to 10 days of incubation, culture becomes difficult, especially when a rapid

diagnosis is required or if a patient has been previously treated with antibiotics (17). Serology is usually used for the diagnosis of pertussis in older vaccinated children, adolescents and adults and is recommended as a diagnostic tool when the disease has progressed with minimal clinical signs and symptoms (25). A limitation of this method is that the serological tests measure antibodies that could result either from infection or vaccination, making diagnosis inaccurate. DFA can offer rapid diagnosis; however, this technique requires specialised trained staff and has a high false-positive rate (17;23). DFA can be used to screen for pertussis and it is recommended that a DFA result be confirmed by culture or PCR. Due to these limitations, real-time PCR is increasingly used for diagnosis (25-28). Real-time PCR is an ideal diagnostic method during the first three weeks of cough. However, real-time PCR identification of *B. pertussis* is hampered by the lack of availability of validated and ideal gene targets.

The most common gene target for pertussis diagnostics is the *IS481* insertion sequence present in multiple copies (50-238) in the *B. pertussis* genome; however there are problems associated with this target. The *IS481* gene is not species specific and can be detected in *B. bronchiseptica* and *B. holmesii*, making diagnosis difficult (20;29). A qualitative assessment of pertussis diagnostics in the United States revealed that 5% of laboratories reported false positive results in proficiency testing using *IS481* only (30). Another proficiency testing study in Europe found that all laboratories that use only *IS481* for diagnosis reported specimens positive for *B. bronchiseptica* and *B. holmesii* as *B. pertussis* (31). Pseudo-outbreaks linked to patient clinic surfaces contaminated by *IS481*, resulting in contamination of specimens, were reported in the United States (32;33). This phenomenon occurred as a result of using *IS481* only as well as not having cycle threshold (Ct) value cut-offs for the real-time PCR. Outbreaks of respiratory illness in New Hampshire,

Massachusetts, and Tennessee between 2004 and 2006 were falsely attributed to *B. pertussis* due to the use of *IS481* only (34). These issues can be overcome by incorporating the pertussis toxin subunit gene (*ptxS1)* into the real-time PCR assays (26-28). This gene is present as a single copy in the *B. pertussis*, *B. bronchiseptica* and *B. parapertussis* genome and it can help in differentiating *Bordetella* spp. (20). In addition, it is important to have sufficient and stringent control measures in place so that contamination can be minimised and easily detected.

1.2.3. Epidemiology

The whole-cell pertussis vaccine was introduced in the 1940's and was implemented in industrialised countries (16-18). It was later found to be reactogenic and associated with adverse side effects including chronic neurologic damage, sudden infant death syndrome, infantile spasms and hypsarrhythmia (18;35). Due to the side effects the acellular pertussis vaccine was developed and introduced in the 1980's (16). The acellular vaccine is composed of up to 5 purified *B. pertussis* antigens (2 fimbrial antigens, pertactin, filamentous haemagglutinin, and pertussis toxin) in various combinations and concentrations (17).

Despite many countries having high vaccine coverage with either whole-cell or acellular vaccines, the incidence of pertussis has increased during the last 20 years (28;36-41). The marked increase has been attributed to many factors including increased awareness by clinicians, use of more sensitive molecular techniques for diagnosis (28;38), the use of serological markers for identification of infection in adolescents and adults who usually are the asymptomatic carriers of pertussis infection (37), and waning vaccine immunity (40;42). Marked pertussis increases have been noted in the United States (36), Canada (38), Denmark (37) and Tunisia (28) amongst other countries. In addition, recent studies have shown that the

increase could be attributed to the evolution of the *B. pertussis* genome with mutations observed in the virulence-associated genes coding for the pertussis toxin A subunit, pertactin, serotype 2 fimbriae, serotype 3 fimbriae and the promoter for the pertussis toxin (43;44). Studies in the United States have shown that some *B. pertussis* strains do not express the vaccine antigen pertactin (45;46). Pertactin-deficient *B. pertussis* was also observed in France and these isolates were shown to be as virulent as the pertactin-expressing isolates (47). *B. pertussis* isolates analysed from 1998 to 2009 in Europe showed an increased prevalence of isolates that contain the novel pertussis toxin promoter *ptxP3* allele replacing the *ptxP1* allele (48).

The whole-cell vaccine was introduced in South Africa in January 1950 and was later replaced by the diphtheria tetanus acellular pertussis (DTaP) vaccine in April 2009. There are limited data on the prevalence of pertussis in South Africa. Only studies from the Western Cape (41;49;50) and Free State province (51) have been published thus far. These studies had a small sample population and results were not representative of the South African population more broadly. Therefore, there is a need for more systematic pertussis surveillance in South Africa to better understand *B. pertussis*, also considering the high HIV burden.

1.3. Pneumonia surveillance in South Africa

This study was nested within two surveillance platforms, namely, severe respiratory infection (SRI) and influenza-like illness (ILI).

SRI is prospective, hospital-based, sentinel surveillance that was initiated in 2009 and is on-going. The aim of this surveillance is to investigate the aetiology of pneumonia in South Africa. All patients who meet the case definition had specimens taken for laboratory testing. The six sites under surveillance are Chris Hani Baragwanath Hospital (Soweto, Gauteng province), Edendale Hospital (KwaZulu-Natal province), Mapulaneng and Matikwane Hospitals (Mpumalanga province) and the Klerksdorp-Tshepong Hospital complex (North West province). Case investigation forms for the SRI surveillance are listed in Appendix 1. In 2012 the Edendale and the Klerksdorp-Tshepong surveillance sites became enhanced sites.

ILI is a prospective study that began in June 2012 and is aimed to describe the burden and aetiology of mild respiratory disease in South Africa in patients of all ages. In addition a subset of healthy individuals has also been enrolled to determine colonisation of respiratory pathogens. Sites under surveillance are the primary health care clinics, Jouberton that serves the Klerksdorp-Tshepong Hospital complex and Edendale Gateway that serves the Edendale hospital. Case investigation forms for ILI surveillance are listed in Appendix 2.

2. Study Aims and Objectives

2.1. Aim

To use two existing surveillance platforms (SRI and ILI) to determine the prevalence of respiratory disease caused by the bacterial pathogens *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* in paediatric and adult patients presenting with mild or severe respiratory tract infections at selected sentinel sites within South Africa from June 2012 to October 2014.

2.2. Objectives

2.2.1. To implement and validate molecular protocols for the detection of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*

2.2.2. To determine if macroscopic and Bartlett's score evaluation influences real time PCR results for the detection of *B. pertussis* and *B. parapertussis*

2.2.3. To compare the utility of different specimen types i.e. combined nasopharyngeal/oropharyngeal specimens and induced sputum for the detection of *B. pertussis* and *B. parapertussis*

2.2.4. To determine if cases positive for *B. pertussis* differed by demographic characteristics based on Ct-value cut-offs (confirmed vs. probable *B. pertussis* cases)

2.2.5. To determine if there were any co-infections with respiratory bacteria or viruses amongst cases positive for *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*

3. Materials and Methods

3.1. Surveillance population

Patients hospitalised with severe respiratory illness (SRI) were enrolled in prospective, active surveillance conducted at two sentinel sites in South Africa, namely, Edendale hospital in Pietermaritzburg, Kwa-Zulu Natal Province, and Klerksdorp-Tshepong hospital Complex in Klerksdorp, North West Province, from June 2012 to October 2014. Enrolled patients had to meet one of the following criteria: all patients hospitalised with clinical signs and symptoms of lower respiratory tract infection (LRTI) irrespective of duration of symptoms; or any child (2 days to <3 months old) with diagnosis of suspected sepsis or physician-diagnosed LRTI irrespective of signs and symptoms; or any child ≥3 months to <5 years with physician-diagnosed LRTI including bronchiolitis, pneumonia, bronchitis and pleural effusion and any person (≥5 years old) presenting with manifestations of acute lower respiratory infection with sudden onset of fever (>38ºC) and cough or sore throat and shortness of breath, or difficulty breathing with or without clinical or radiographic findings of pneumonia or tachypnea.

Out-patients with influenza-like illness (ILI) and controls were enrolled at two clinics affiliated to the above-mentioned sentinel hospitals during the same study period. A case of ILI was defined as an out-patient presenting with acute fever of >38°C and/or self-reported fever and cough within the last 7 days, or sore throat and the absence of other diagnoses.

Controls were individuals that presented at the clinics with no history of respiratory illness, diarrhoeal illness, or fever in the preceding 14 days. Controls commonly presented to the clinic for visits such as dental procedures, family planning, baby clinics, voluntary HIV counselling and testing or acute care for non-febrile illnesses. Medical and symptoms history were systematically verified by a trained nurse using a structured checklist. One HIV-infected

and one HIV-uninfected control were enrolled every week in each out-patient clinic within each of the following age categories: 0-1, 2-4, 5-14, 15-54 and ≥55 years.

3.2. Demographic and clinical data collection

Demographic and clinical data were collected by surveillance officers through interviews and hospital record reviews. Data collected included socio-demographic factors, presenting symptoms, duration of symptoms and underlying illnesses including HIV and tuberculosis exposure and treatment. For all patients, history of influenza immunisations was recorded. In addition, for children <5 years, routine immunisation history was documented and confirmed from the patients Road-to-Health Card (RTHC).

3.3. Sample collection and transport

Nasopharyngeal (NP) specimens were collected from all enrolled patients and controls whilst induced sputum (IS) was collected from SRI patients only. Nasopharyngeal aspirates were obtained from patients <5 years old and combined nasopharyngeal-oropharyngeal swabs were collected from patients ≥5 years old. For HIV testing, blood specimens were collected from all consenting patients. NP specimens were transported in Universal Transport Medium (UTM) (Copan Italia, Brescia, Italy) on ice packs and IS specimens were transported on dry-ice (Marken Transport, South Africa) to the National Institute for Communicable Diseases (NICD) for testing. IS was initially transported at 4°C (June 2012 to June 2013), however, to improve specimen quality, transport of IS changed to dry ice from July 2013.

3.4. Assessment and processing of induced sputum

All IS specimens were examined macroscopically and were graded as follows: saliva for a clear watery sputum, mucoid for clear sticky sputum, purulent for sputum with pus sometimes mixed with mucus and bloody for sputum with blood sometimes mixed with mucus/pus. Sputum quality was assessed microscopically by using the Bartlett's grading system on a Gram-stained smear (22). This method was based on analysis of both squamous epithelial cells and neutrophils. Good quality sputum was expected to have a higher number of neutrophils which are indicative of inflammation as opposed to squamous epithelial cells which are indicative of saliva (22). A Bartlett's score of 1 indicates the presence of between 10 – 25 neutrophils and a score of 2 indicates the presence of >25 neutrophils. In addition a -1 score indicates the presence of between 10 – 25 epithelial cells and a -2 score indicates the presence of >25 epithelial cells. Good quality sputum was expected to have a positive Bartlett's score and should not be saliva; however, no sputum specimens were rejected based on poor macroscopic or microscopic evaluations.

In addition, specimens were plated onto charcoal agar for *Bordetella* (Diagnostic Media Products, Johannesburg, South Africa) for culture. All plates were incubated at 35-37°C for 10 days before being inspected for possible colonies (52).

IS was then digested and decontaminated with a 1:10 dilution of dithiothreitol (DTT) (Roche Diagnostics, Mannheim, Germany) (53). Sputum volume was measured and an equal volume of DTT was added. The mixture was vortexed for 30 seconds and then incubated at 37°C for 15 minutes. Phosphate buffered saline (Diagnostic Media Products, Johannesburg, South Africa) at a pH of 7.2 was added to remove any excess DTT. Samples were centrifuged at 2000 rpm for 5 minutes and stored at 4°C until DNA extraction.

3.5. DNA extraction

NP specimens in UTM and digested IS specimens underwent an automated DNA extraction using the Roche MagNa Pure 96 instrument (Roche Diagnostics, Mannheim, Germany) and MagNa Pure 96 DNA and Viral NA SV Kit (Roche Diagnostics) and the Pathogen Universal Protocol. DNA was extracted from a 200µl aliquot of sample, eluted into 100µl of elution buffer and stored at -20°C until further testing.

3.6. Real-time PCR

Detection of *B. pertussis*, *B. parapertussis*, *B. holmesii* and *B. bronchiseptica* were performed using previously-published real-time PCR assays (26;27). The multiplex assay detects *IS481* to determine the presence of *Bordetella* spp. (*B. pertussis*, *B. bronchiseptica* and *B. holmesii*), *pIS1001* for *B. parapertussis*, and *hIS1001* for *B. holmesii*. The second assay is a singleplex which confirms *B. pertussis*, *B. bronchiseptica* and *B. parapertussis* by detecting the *ptxS1* toxin gene (Appendix 3). All reactions were carried out in an Applied Biosystems 7500 Fast instrument (Applied Biosystems, Foster City, California, USA) using universal cycling conditions. The reaction volume was 25µl, and consisted of TaqMan Gene Expression master mix, (Applied Biosystems, Foster City, California, USA), 4µl of extracted DNA and primers and probes as previously described (Appendix 3).

A positive PCR result was recorded if a Ct-value of ≤45 for any of the gene targets was obtained. All specimens that tested positive for any gene target were re-extracted and tested in duplicate to confirm the result. A specimen was confirmed as positive if it was positive on 2/3 or 3/3 repeats. For *B. pertussis*, results were interpreted according to the published algorithm (26;27), with minor modifications (Appendix 5): If a specimen tested positive for *IS481* with a Ct-value <35, irrespective of *ptxS1*, then this specimen was recorded as

confirmed positive. If a specimen tested positive for *IS481* with a Ct-value ≥35, irrespective of *ptxS1,* then this specimen was defined as probable positive for *B. pertussis*. For *B. parapertussis*, cases were not classified into confirmed or probable based on Ct-value cut-off's. If a specimen tested positive for *pIS1001* (Ct-value of ≤45), irrespective of the *ptxS1,* then this specimen was defined as positive for *B. parapertussis*.

Detection of the human ribonuclease P (*RNase P*) gene was performed and served as an internal control to identify the presence of potential PCR inhibitors, and/or confirm DNA quality (54). Results of the *RNase P* assay were used to interpret PCR negative results i.e. samples that also tested *RNase P* negative were interpreted as possible false negatives due to the presence of inhibitors or poor DNA quality in the clinical sample.

3.6.1. Validation of real-time PCR

The following steps were followed to validate the PCR assays:
PCR sensitivity and specificity was determined using *Bordetella* spp. controls from the American Type Culture Collection (ATCC) (Appendix 4) diluted to 10^{-3}. Results were interpreted according to the modified algorithm.

PCR robustness was determined by testing two PCR master mixes, namely, TaqMan Gene Expression master mix (Applied Biosystems) and PerfeCTa Multiplex qPCR super mix (Quanta Biosciences, Gaitherburg, MD, US). *B. pertussis* ATCC 9797-D DNA control was serially diluted from 10^{-1} to 10^{-9} and assays were performed in duplicate.

External proficiency testing for the *B. pertussis* assays was performed through Quality Control for Molecular Diagnostics (QCMD) (Glasgow, Scotland) which is proficiency testing

programme that assesses molecular detection methodologies (www.qcmd.org). Each year the panel of interest consists of 5 core and 7 education samples. The 2011 and 2012 panel was given to us by the NHLS Infection Control laboratory. From 2013 onwards we subscribed to this programme (Appendix 6). Each DNA extract from the panel was run in triplicate and results were interpreted according to the modified algorithm. Results from the 2011, 2012, 2013 and 2014 panel were also used to calculate the sensitivity and specificity of the PCR assays.

3.7. Determination of HIV status

HIV results were determined from either the patient's clinical record if available and/or an anonymised linked dried blood spot. For patients <18 months PCR was performed and for patients ≥18 months the enzyme-linked immunosorbent assay was performed. HIV status was determined at the NICD HIV laboratory.

3.8. Co-infections

Additional tests were carried out on all NP, IS and blood specimens that were received at the NICD as part of the SRI and ILI projects. NPs were tested by the CRDM virology laboratory for 10 respiratory viruses (influenza types A and B, adenovirus, enterovirus, rhinovirus, human metapneumovirus, respiratory syncytial virus (RSV) and parainfluenza virus types 1-3); NP and IS specimens were tested for *M. pneumoniae*, *C. pneumoniae* and *Legionella* spp. (54) and blood specimens were tested for *S. pneumoniae* (55) and *H. influenzae* (56) as part of routine testing at CRDM bacteriology laboratory. Only SRI cases were analysed for co-infections.

3.9. Data analysis

Patient demographics, clinical, epidemiological (including vaccine history, age, gender, symptoms, duration of symptoms, HIV status, administration of antibiotics before hospital admission, duration of hospital stay, area of residence) and laboratory results were entered in a Microsoft Access database (Microsoft Corporation, California, USA) in a double-data entry format.

The sensitivity and specificity of the real-time PCR assays were determined 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)

IS specimens that had a macroscopic and/or Bartlett's score results available were evaluated to determine if either of these characteristics influenced the overall result obtained by real-time PCR for the detection of *B. pertussis* and *B. parapertussis*.

To determine which of the specimen types were most ideal for the detection of *B. pertussis* and *B. parapertussis*, patients enrolled into the SRI surveillance population that had both an NP and an IS specimen taken for testing were analysed. Patient characteristics amongst the positive patients were also evaluated to determine if patient characteristics differed by specimen type taken for testing. Analysis was performed on cases that had only 1 of the 2 specimen types test positive on the real-time PCR. Cases that had both specimens types test positive were excluded for this analysis.

Confirmed and probable *B. pertussis* cases as well as *B. parapertussis*-positive and *B. parapertussis*-negative cases were compared amongst the SRI and ILI surveillance populations using patient characteristics. Cases for this analysis were defined as having either or both specimen types test positive on real-time PCR. Ct-values amongst the confirmed and probable *B. pertussis* cases were analysed.

Using the control group as the reference group and controlling for HIV status and age group; the attributable fraction of disease of *B. pertussis* and *B. parapertussis* between cases presenting with SRI, ILI were calculated using the following equation:

Attributable fraction = (odds ratio-1) / (odds ratio*100)

For this analysis a positive case was defined as having either or both specimen types positive by real-time PCR.

SRI cases that tested positive for any *Bordetella* spp. were analysed to determine if they were co-infected with either respiratory bacteria or viruses. A positive co-infected case was defined as having a real-time PCR positive result for any respiratory bacteria or virus mentioned in 3.9.

All data were analysed in Microsoft Excel (Microsoft Corporation). The chi-squared test was used for the analysis of categorical variables and the Kolmogorov-Smirnov test was used for the analysis of continuous variables. Univariate logistic regression was used to determine odds ratios (OR) and 95% confidence intervals .For the analysis of continuous variables statistical significance was determined at $p<0.05$. Statistical significance was obtained using Graph Pad Instat software (version 3.10, California, USA) and Stata® version 12 (Statacorp, College Station, TX).

3.10. Ethics

The SRI protocol (M081042) was approved by the Human Research Ethics Committee-Medical (HREC) of the University of the Witwatersrand (Wits), Johannesburg and includes ethics clearance for the Klerksdorp-Tshepong surveillance sites. An amended SRI and ILI protocol was approved by the HREC of the University of the Witwatersrand which includes approval for work proposed in this study. For the Edendale surveillance site, ethics has been approved by the Kwa-Zulu Natal provincial ethics committee. Ethics for the MSc project was approved by Wits HREC (M130260) (Appendix 7).

4. Results

4.1. Validation of real-time PCR

ATCC controls *B. pertussis*, *B. parapertussis*, *B. bronchiseptica* and *B. holmesii* were correctly identified as such and a negative PCR result was obtained for *B. avium*, *B. hinzii*, and *B. petrii*. PCR sensitivity and specificity were calculated using the results obtained from the ATCC controls and the 2011, 2012, 2013 and 2014 QCMD panels. The PCR was 95% (20/21) sensitive and 100% (31/31) specific for *B. pertussis*. A 100% sensitivity and 100% specificity was obtained for *B. parapertussis* (sensitivity 5/5 and specificity 48/48), *B. bronchiseptica* (sensitivity 9/9 and specificity 44/44) and *B. holmesii* (sensitivity 5/5 and specificity 48/48), respectively.

The mean Ct obtained for each of the dilutions tested for *IS481* using the Taqman Gene Expression master mix and the Quanta super mix are listed in Table 1. PCR performed using the TaqMan Gene Expression master mix (Applied Biosystems) yielded lower Ct-values (±2 Ct difference) for each of the dilutions when compared to the Ct-values obtained using the Quanta super mix although this was not statistically significant (p=0.09).

Table 1: Comparison of Ct-values of *IS481* obtained by performing real-time PCR using the Taqman Gene Expression mastermix and the Quanta super mix.

ATCC 9797-D *B. pertussis* dilution	*IS481* Ct (Taqman Gene Expression master mix)[1]	*IS481* Ct (Quanta super mix)[2]
B. pertussis neat	6	11
B. pertussis 10^{-1}	10	14
B. pertussis 10^{-2}	13	17
B. pertussis 10^{-3}	17	20
B. pertussis 10^{-4}	20	23
B. pertussis 10^{-5}	24	26
B. pertussis 10^{-6}	28	29
B. pertussis 10^{-7}	32	33
B. pertussis 10^{-8}	35	36
B. pertussis 10^{-9}	41	44[3]
B. pertussis 10^{-10}	Negative	Negative
B. pertussis 10^{-11}	Negative	Negative
B. pertussis 10^{-12}	Negative	Negative
B. pertussis 10^{-13}	Negative	Negative
B. pertussis 10^{-14}	Negative	Negative
B. pertussis 10^{-15}	Negative	Negative
p-value	0.09	

[1, 2]: Each dilution of the *B. pertussis* DNA extract was run in duplicate. The mean Ct of each dilution was used in above table.
[3:] Only 1 of the duplicates tested positive at this dilution.

All samples from the 2011, 2013 and 2014 QCMD panels were correctly resulted. For the 2012 panel, 1 DNA extract was a known positive for *B. pertussis* but a negative result was obtained in this study. The specimen was re-tested but the result remained negative. Ct-values for the *B. pertussis* positive samples ranged from 34 to 42, however no Ct-values were sent back from QCMD with result reports so no comparisons could be made.

4.2. Surveillance population

From June 2012 to October 2014, 9684 cases were enrolled into the SRI, ILI and controls surveillance programs. Of the 9684 cases enrolled, 8569 cases had specimens taken for

testing of which 3982, 3243 and 1344 were from SRI, ILI and control individuals, respectively (Figure 2). There were 9684 cases enrolled but only 8569 cases had specimens taken for testing. This difference was due to one of the following reasons: patients enrolled did not give consent to take specimens; patients enrolled within the SRI surveillance were too sick to have specimens taken or could not have both a NP and IS taken; patients were discharged before specimens could be taken; or specimens were lost in transit or were insufficient for testing.

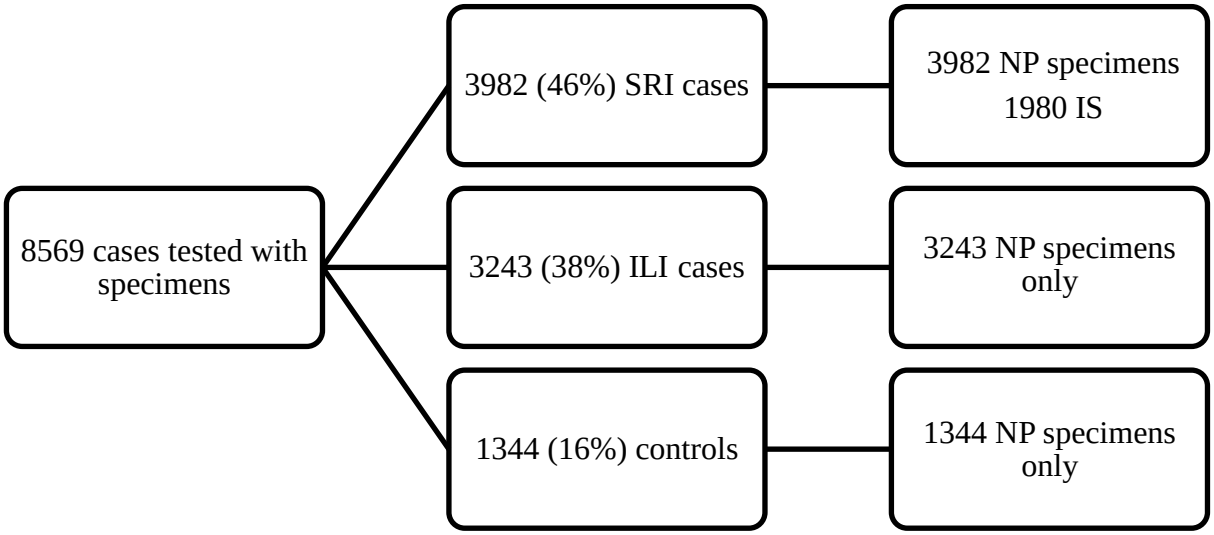

Figure 2: Flow diagram depicting the cases enrolled for SRI, ILI and controls surveillance groups as well as the specimen type received for laboratory testing, South Africa, June 2012 – October 2014.

Specimens from 8569 cases were collected and tested using the *Bordetella* spp. real-time PCR of which 3982 (46%, 3982/8569) were from SRI cases, 3243 (38%, 3243/8569) were from ILI cases and 1344 (16%, 1344/8569) were from controls (Table 2). Of the SRI and ILI cases, 55% (1896/3452) and 30% (855/2809) were HIV positive, respectively. Within the SRI and ILI surveillance groups, the 25-44 year age group had the highest numbers of cases

enrolled and tested [(33%, 1301/3973) and (30%, 971/3242) respectively], and the majority of the population was black.

Table 2: Demographic and clinical characteristics of patients enrolled into the severe respiratory and influenza-like illness surveillance that were tested for *Bordetella* species, South Africa, June 2012 – October 2014 (N=8569).

Characteristic	Surveillance population		
	SRI n/N (%) N=3982	ILI n/N (%) N=3243	Controls n/N (%) N=1344)
Gender			
Male	2013/3975 (51)	1164/3195 (36)	453/1317 (34)
Female	1962/3975 (49)	2031/3195 (64)	864/1317 (66)
Race			
Black	3884/3975 (98)	3192/3195 (100)	1317/1317 (100)
Non-black	91/3975 (2)	3/3195 (0.1)	0
Age group (years)			
<1	880/3973 (22)	303/3242 (9)	169/1343 (13)
1-4	497/3973 (13)	614/3242 (19)	303/1343 (23)
5-14	116/3973 (3)	514/3242 (16)	280/1343 (21)
15-24	208/3973 (5)	453/3242 (14)	93/1343 (7)
25-44	1301/3973 (33)	971/3242 (30)	203/1343 (15)
45-64	761/3973 (19)	333/3242 (10)	209/1343 (16)
≥65	210/3973 (5)	54/3242 (2)	86/1343 (6)
Underlying illness[1]			
No	3536/3973 (89)	3021/3185 (95)	1243/1313 (95)
Yes	437/3973 (11)	164/3185 (5)	70/1313 (5)
HIV status			
Uninfected	1556/3452 (45)	1954/2809 (70)	700/1246 (56)
Infected	1896/3452 (55)	855/2809 (30)	546/1246 (44)
Clinic/Hospital			
Edendale	1770/3982 (44)	N/A	N/A
Edendale Gateway	N/A	2385/3243 (74)	521/1344 (39)
Jouberton Clinic	N/A	858/3243 (26)	823/1344 (61)
KTHC	2212/3982 (56)		N/A

Abbreviations SRI=Severe respiratory illness; ILI=Influenza-like illness; KTHC=Klerksdorp-Tshepong hospital complex; N/A=Not applicable.

[1]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. All percentages rounded off.

4.3. *B. pertussis*

4.3.1. Specimen quality and comparison of specimen types for the detection of *B. pertussis*

Of the 1980 IS specimens collected from SRI patients, the macroscopic evaluation was performed on 1615 (82%, 1615/1980) specimens and the Bartlett's score evaluation was performed on 1088 specimens (55%, 1088/1980) that were tested using the *Bordetella* spp. real-time PCR assays. It was observed that 35% (8/23) of the *B. pertussis* positive cases were mucoid/purulent and 44% (4/9) of the cases had a positive Bartlett's score (Table 3). All 1980 IS specimens were culture negative for *B. pertussis*.

Table 3: Macroscopic (N=1615) and Bartlett's score (N=1088) evaluation of induced sputum collected from SRI patients, by *B. pertussis* PCR result, South Africa, June 2012 – October 2014 (N=2703).

Characteristic	*B. pertussis* positive	*B. pertussis* negative	Total
	n/N (%)	n/N (%)	
Macroscopic evaluation			
Saliva	5/23 (22)	457/1592 (29)	
Mucoid	8/23 (35)	625/1592 (39)	
Purulent	8/23 (35)	347/1592 (22)	
Blood stained	2/23 (9)	163/1592 (10)	
Total	23	1592	1615
Bartlett's score			
Negative	2/9 (22)	343/1079 (32)	
0	3/9 (33)	287/1079 (27)	
Positive	4/9 (44)	449/1079 (42)	
Total	9	1079	1088

Negative (combined -1 and -2 score) = presence of between 10 – 25 (-1) and >25 (-2) epithelial cells.
0= presence of <10 neutrophils.
Positive= (combined +1 and +2 score) = presence of between 10 – 25 (+1) and >25 (+2) neutrophils.
Good quality sputum should have a positive Bartlett's score and should not be saliva.
All percentages rounded off.

There were 1778 SRI cases that had both an NP and an IS specimen taken for testing. The detection rate of *B. pertussis* was lower in NP specimens compared to IS specimens [8/1726, 0.5% vs. 31/1726, 1.8%, p=0.005]. The detection rate in cases with both specimen types was 0.75% (13/1726). Cases positive for *B. pertussis* that had both an NP and IS specimen taken for testing were then stratified by specimen type and patient demographics, however no differences were observed (Table 4).

Table 4: Comparison of nasopharyngeal and induced sputum *B. pertussis* positive specimens from cases presenting with severe respiratory illness in South Africa, South Africa, June 2012 – October 2014 (N=39).

Characteristic	*B. pertussis* PCR result		
	NP *B. pertussis* positive n/N (%)	IS *B. pertussis* positive n/N (%)	OR (95% CI)
Year			
2012	4/5 (80)	1/5 (20)	reference
2013	4/12 (33)	8/12 (68)	8 (0.7 – 97)
2014	0	22/22 (100)	N/A
Positive category			
Confirmed pertussis	2/5 (40)	3/5 (60)	reference
Probable pertussis	6/34 (18)	28/34 (82)	3 (0.4 – 23)
Gender			
Female	3/16 (19)	13/16 (81)	reference
Male	5/23 (22)	18/23 (78)	0.8 (0.2 – 4)
Age group (year)			
<1	0	2/2 (100)	reference
1-4	1/4 (25)	3/4 (75)	N/A
5-14	0	0	N/A
15-24	0	1/1 (100)	N/A
25-44	5/16 (31)	11/16 (69)	N/A
45-64	2/12 (17)	10/12 (83)	N/A
≥65	0	4/4 (100)	N/A
Fever history			
No	6/28 (21)	22/28 (79)	reference
Yes	2/10 (20)	8/10 (80)	1 (0.2 – 7)

HIV status			
Uninfected	3/13 (23)	10/13 (77)	reference
Infected	2/23 (9)	21/23 (91)	3 (0.5 – 22)
HIV treatment			
No	2/9 (22)	7/9 (78)	reference
Yes	3/14 (21)	11/14 (79)	1 (0.1 – 8)
Symptom duration			
<7 days	3/16 (19)	13/16 (81)	reference
7-20 days	3/9 (33)	6/9 (67)	0.5 (0.07 – 3)
≥21 days	1/13 (8)	12/13 (92)	3 (0.3 – 30)
Underlying illness[1]			
No	7/33 (21)	26/33 (79)	reference
Yes	1/6 (17)	5/6 (83)	1 (0.1 – 13)
ICU			
No	8/39 (21)	31/39 (79)	N/A
Yes	0	0	
Antibiotic treatment (24 hours)			
No	8/39 (21)	31/39 (79)	N/A
Yes	0	0	
Hospital duration			
<2 days	1/6 (17)	5/6 (83)	reference
2-4 days	3/14 (21)	11/14 (79)	0.7 (0.06 – 9)
5-7 days	2/8 (25)	6/8 (75)	0.6 (0.04 – 9)
≥8 days	2/11 (18)	9/11 (82)	0.9 (0.06 – 13)
Viral co-infection			
No	6/27 (22)	21/27 (78)	reference
Yes	2/12 (17)	10/12 (83)	1 (0.2 – 8)
Outcome			
Survived	6/32 (19)	26/32 (81)	reference
Died	2/7 (29)	5/7 (71)	0.6 (0.09 – 4)
Vaccination for age[2]			
Full coverage	1/4 (25)	3/4 (75)	N/A
Incomplete coverage	0	2/2 (100)	
Facility			
Edendale	4/12 (33)	8/12 (67)	reference
KTHC	4/27 (15)	23/27 (85)	3 (0.6 – 14)

Abbreviations: NP=nasopharyngeal; IS=induced sputum; KTHC=Klerksdorp-Tshepong hospital complex; N/A=not applicable, OR= Odd ratio; CI=Confidence interval

[1]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.

[2]Only for children ≤5 years of age where vaccine history was available on the road-to-health card.

Confirmed positive=positive for *B. pertussis* if IS481 Ct <35; Probable positive=positive for *B. pertussis* if IS481 Ct ≥35. All percentages rounded off.

4.3.2. Comparison of *B. pertussis* positive cases (confirmed pertussis vs. probable pertussis) by surveillance group

Confirmed and probable *B. pertussis* positives were observed in both NP and IS specimens (Figures 3 and 4). The mean Ct-value (±standard deviation) for confirmed *B. pertussis* positive NP and IS specimens was 34±5 and 25±6 respectively, and the mean Ct for probable *B. pertussis* positive NP and IS specimens was 36±5 and 39±2 respectively.

There were 82 [2%, 82/3982 (95% CI 1.6 – 2.5)] NP specimens that tested positive for *B. pertussis* of which 35 were confirmed and 47 were probable pertussis positives. Of the confirmed pertussis positives, 94% (33/35) were positive for both *IS481* and *ptxS1* and for the probable pertussis cases, only 19% (9/47) were positive on both gene targets.

From 49 [2.5%, 49/1980 (95% CI 1.8 – 3.3)] IS specimens positive for *B. pertussis*, 16 (100%, 16/16) confirmed cases and 12% (4/33) of probable cases were positive on both gene targets, respectively.

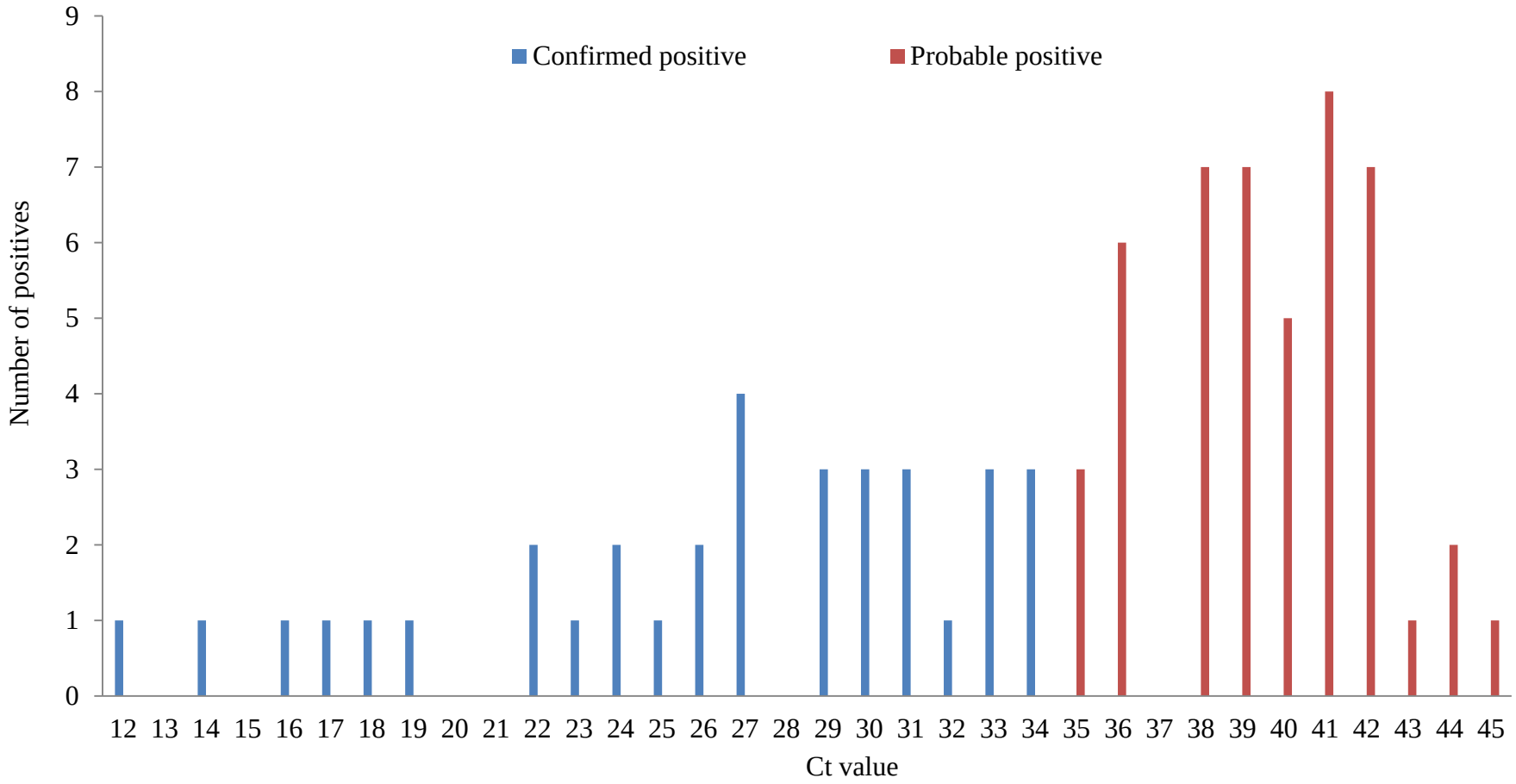

Figure 3: *IS481* Ct-value distribution of *B. pertussis* confirmed (n=35) and probable (n=47) results from nasopharyngeal specimens, South Africa, June 2012 – October 2014 (N=82).

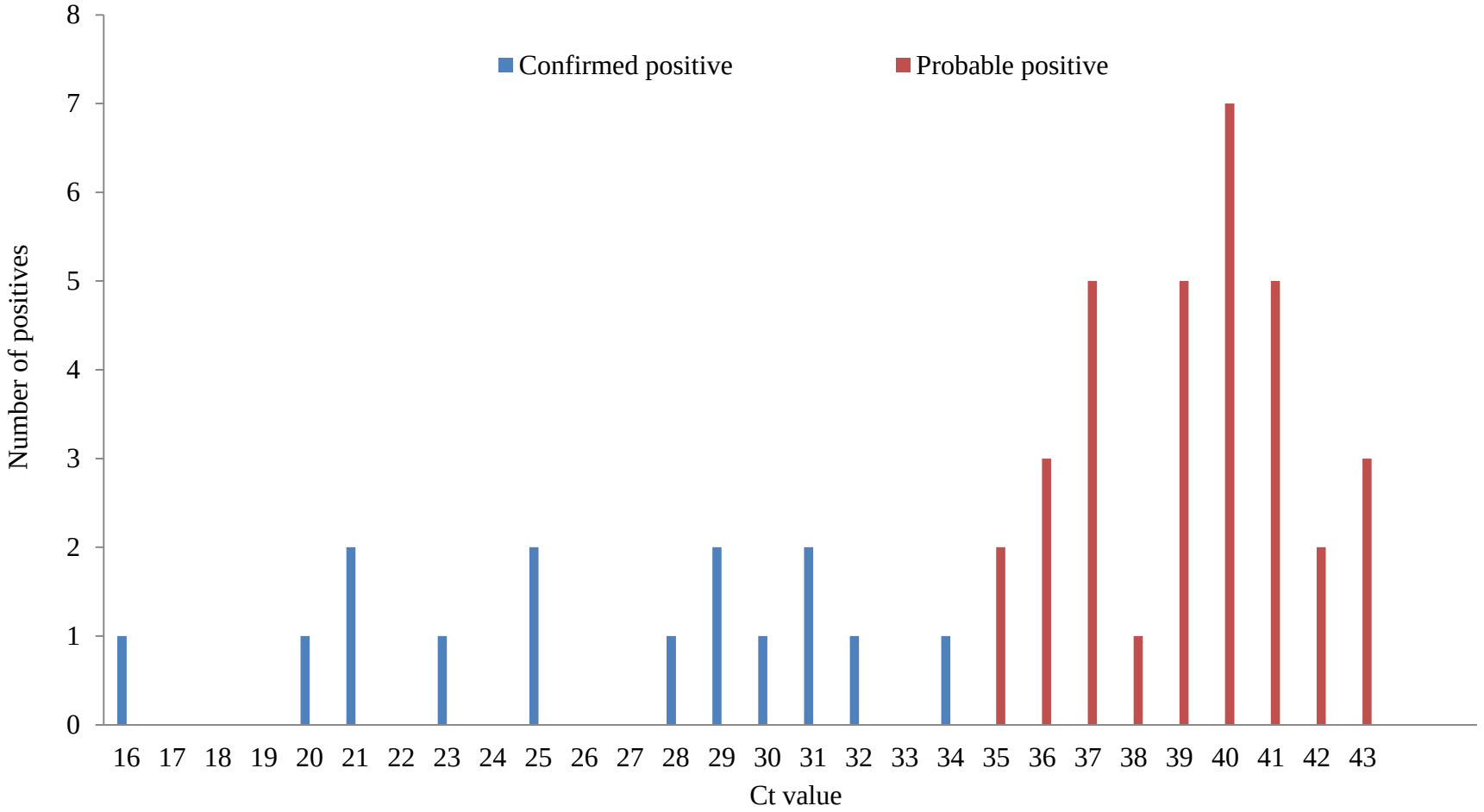

Figure 4: *IS481* Ct-value distribution of *B. pertussis* confirmed (n=16) and probable (n=33) results from induced sputum specimens, South Africa, June 2012 – October 2014 (N=49).

Table 5: Comparison of confirmed and probable *B. pertussis* cases in patients presenting with severe respiratory illness, South Africa, June 2012 – October 2014 (N=3982).

Characteristic	*B. pertussis* PCR result			
	Confirmed n/N (%)	Probable n/N (%)	OR[3] (95% CI)	Negative n/N (%)
Year				
2012	7/18 (39)	11/39 (61)	reference	1152/3902 (30)
2013	9/25 (36)	16/25 (64)	1 (0.3 – 4)	1624/3902 (42)
2014	13/37 (35)	24/37 (65)	1 (0.4 – 4)	1126/3902 (29)
Gender				
Female	19/43 (44)	24/43 (56)	reference	1919/3895 (49)
Male	10/37 (27)	27/37 (73)	2 (0.8 – 5)	1976/3895 (51)
Age group				
<1	10/19 (53)	9/19 (47)	reference	861/3893 (22)
1-4	3/8 (38)	5/8 (63)	2 (0.3 – 10)	489/3893 (13)
5-14	1/1 (100)	0	NA	115/3893 (3)
15-24	0	2/2 (100)	NA	206/3893 (5)
25-44	12/25 (48)	13/25 (52)	1 (0.4 – 4)	1276/3893 (33)
45-64	1/19 (5)	18/19 (95)	**20 (2 – 182)**	742/3893 (19)
≥65	2/6 (33)	4/6 (67)	2 (0.3 – 15)	204/3893 (5)
Fever history				
No	19/52 (37)	33/52 (63)	reference	2095/3870 (54)
Yes	9/27 (33)	18/27 (67)	1 (0.4 – 3)	1775/3870 (46)
HIV status				
Uninfected	8/30 (27)	22/30 (73)	reference	1526/3385 (45)
Infected	13/37 (35)	24/37 (65)	0.7 (0.2 – 2)	1859/3385 (55)
HIV treatment				
No	5/15 (33)	10/15 (67)	reference	703/1588 (44)
Yes	6/20 (30)	14/20 (70)	1 (0.3 – 5)	885/1588 (56)
Symptom duration				
<7 days	19/41 (46)	22/41 (54)	reference	2090/3824 (55)
7-20 days	4/13 (31)	9/13 (69)	2 (0.5 – 7)	818/3824 (21)
≥21 days	6/23 (26)	17/23 (74)	2 (0.8 – 7)	916/3824 (24)
Underlying illness[1]				
No	27/69 (39)	42/69 (61)	reference	3467/3893 (89)
Yes	2/11 (18)	9/11 (82)	3 (0.6 – 14)	426/3893 (11)
ICU				
No	28/77 (36)	49/77 (64)	reference	3755/3811 (99)
Yes	1/2 (50)	1/2 (50)	0.6 (0.03 – 9)	56/3811 (1)

Antibiotic treatment (24 hours)				
No	28/77 (36)	49/77 (64)	reference	3694/3878 (95)
Yes	1/3 (33)	2/3 (67)	1 (0.1 – 13)	184/3878 (5)
Hospital duration				
<2 days	3/11 (27)	8/11 (73)	reference	385/3646 (11)
2-4 days	9/28 (32)	19/28 (68)	0.8 (0.2 – 4)	1073/3646 (29)
5-7 days	2/12 (17)	10/12 (83)	2 (0.2 – 14)	953/3646 (26)
≥8 days	12/26 (46)	14/26 (54)	0.4 (0.09 – 2)	1235/3646 (34)
Viral co-infection				
No	13/46 (28)	33/46 (72)	reference	2120/3872 (55)
Yes	16/34 (47)	18/34 (53)	0.4 (0.2 – 1)	1752/3872 (45)
Outcome				
Survived	26/71 (37)	45/71 (63)	reference	3445/3775 (91)
Died	2/8 (25)	6/8 (75)	2 (0.3 – 9)	330/3775 (9)
Vaccination for age[2]				
Full coverage	3/12 (25)	9/12 (75)	reference	942/1165 (81)
Incomplete	5/9 (56)	4/9 (44)	4 (0.6 – 24)	223/1165 (19)
Facility				
Edendale	13/31 (42)	18/31 (58)	reference	1739/3902 (45)
KTHC	16/49 (33)	33/49 (67)	1 (0.6 – 4)	2163/3902 (55)

Abbreviations: KTHC=Klerksdorp-Tshepong hospital complex, NA – not applicable; OR=Odds ratio; CI=Confidence interval.

[1]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.

[2]only for children ≤5 years of age where vaccine history was available on the road-to-health card

Confirmed positive=positive for *B. pertussis* if IS481 Ct <35; Probable positive=positive for *B. pertussis* if IS481 Ct ≥35. All percentages rounded off. [3]Bold font indicates statistical significance.

B. pertussis confirmed and probable cases differed by age group only (Table 5). The detection rate of probable cases was significantly higher than the detection rate of the confirmed cases in the 45-64 year olds when compared to the <1 year olds [OR=20 (95% CI 2 – 182)]. HIV status and full pertussis vaccination for age did not influence the confirmed pertussis and probable pertussis cases. Overall, the highest detection rate was observed in the ≥65 year age group (2.8%, 6/208) (Figure 5). The detection rate in the <1 year age group was 2.2% (19/880). We stratified this age group into <3 months and ≥4 months. The detection rate in the <3 months age group was 2.1% (8/374) and 2.2% (11/506) in the ≥4 months (p=1.00). None of the infants in this age group died.

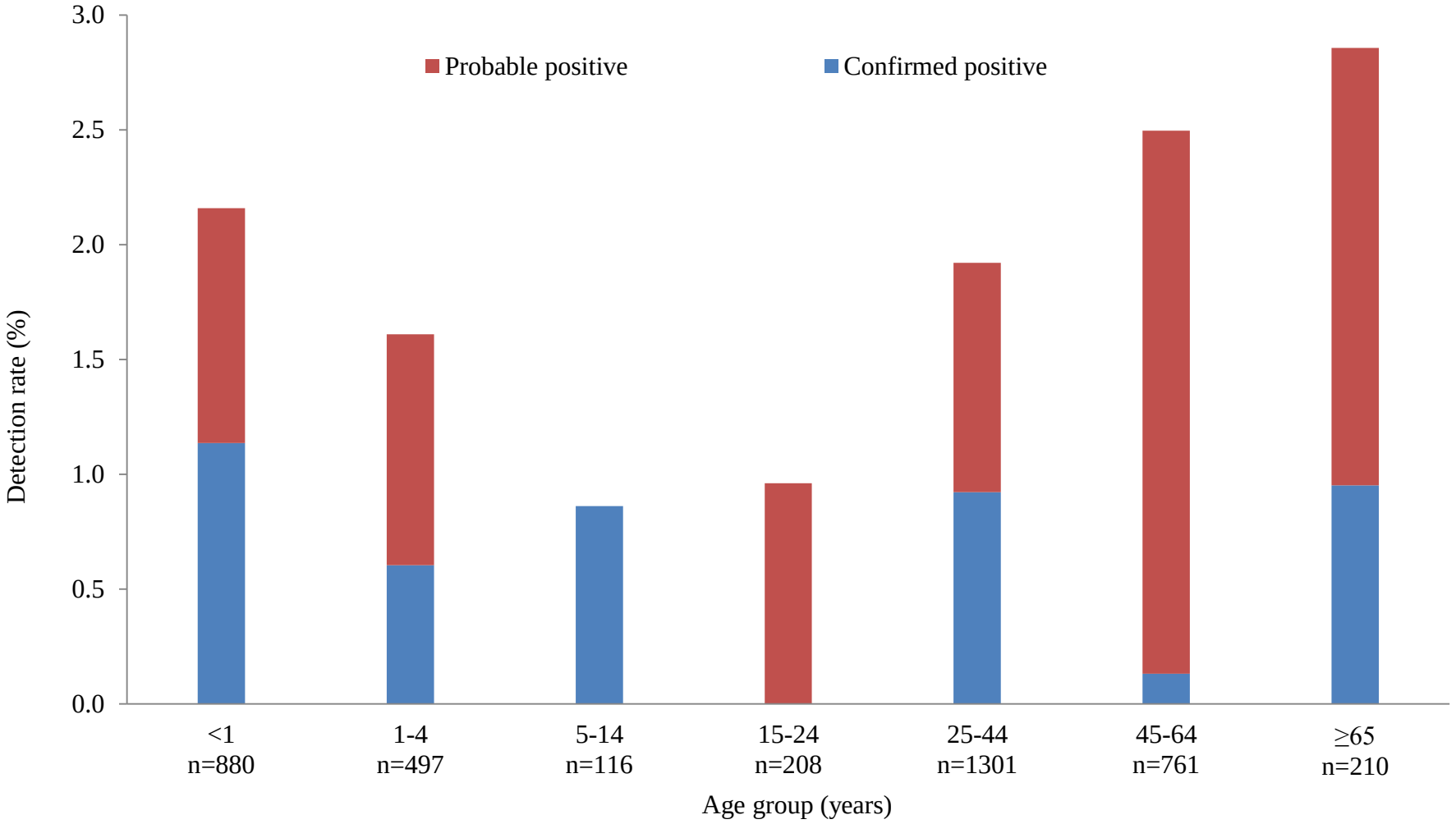

Figure 5: Detection rate of *B. pertussis* (confirmed vs. probable) cases by age group in cases presenting with severe respiratory illness, South Africa, June 2012 – October 2014 (N=3973).

Table 6: Comparison of confirmed and probable *B. pertussis* cases in patients presenting with influenza-like illness, South Africa, June 2012 – October 2014 (N=3243).

Characteristic	*B. pertussis* PCR result ILI cases			
	Confirmed n/N (%)	Probable n/N (%)	OR (95% CI)[3]	Negative n/N (%)
Year				
2012	4/10 (40)	6/10 (60)	reference	960/3211 (30)
2013	3/5 (60)	2/5 (40)	0.4 (0.05 – 4)	851/3211 (27)
2014	8/17 (47)	9/17 (53)	0.8 (0.2 – 4)	1400/3211 (44)
Gender				
Female	10/19 (53)	9/19 (47)	reference	2012/3165 (64)
Male	5/11 (45)	6/11 (55)	1 (0.3 – 6)	1153/3165 (36)
Age group				
<1	1/1 (100)	0	reference	302/3210 (10)
1-4	2/9 (22)	7/9 (78)	N/A	605/3210 (19)
5-14	3/6 (50)	3/6 (50)	N/A	508/3210 (16)
15-24	2/6 (33)	4/6 (67)	N/A	447/3210 (14)
25-44	7/10 (70)	3/10 (30)	N/A	961/3210 (30)
45-64	0	0	N/A	333/3210 (10)
≥65	0	0	N/A	54/3210 (2)
Fever history				
No	1/5 (20)	4/5 (80)	reference	125/3159 (4)
Yes	14/25 (56)	11/25 (44)	0.2 (0.02 – 2)	3034/3159 (96)
HIV status				
Uninfected	4/14 (29)	10/14 (71)	reference	1940/2784 (70)
Infected	7/11 (64)	4/11 (36)	0.2 (0.04 – 1)	844/2784 (30)
HIV treatment				
No	2/5 (40)	3/5 (60)	reference	318/823 (39)
Yes	5/6 (83)	1/6 (17)	0.1 (0.008 – 2)	505/823 (61)
Symptom duration				
<7 days	13/25 (52)	12/25 (48)	reference	177/3080 (6)
7-20 days	2/3 (67)	1/3 (33)	0.5 (0.04 – 7)	66/3080 (2)
≥21 days	0	1/1 (100)	N/A	2837/3080 (92)
Underlying illness[1]				
No	14/28 (50)	14/28 (50)	reference	2993/3155 (95)
Yes	1/2 (50)	1/2 (50)	1 (0.06 – 18)	162/3155 (5)
Viral co-infection				
No	9/20 (45)	11/20 (55)	reference	1692/3194 (53)
Yes	6/10 (60)	4/10 (40)	0.5 (0.1 – 3)	1502/3194 (47)

Vaccination				
Full coverage[2]	2/4 (50)	2/4 (50)	N/A	695/800 (87)
Incomplete	0	2/2 (100)		105/800 (13)
Facility				
Edendale Gateway	10/13 (77)	3/13 (23)	reference	2372/3211 (74)
Jouberton	5/19 (26)	14/19 (74)	**0.1 (0.02 – 0.6)**	839/3211 (26)

Abbreviations: NA=not applicable; OR=Odds ratio; CI=Statistical significance.
[1]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.
[2]only for children ≤5 years of age where vaccine history was available on the road-to-health card
Confirmed positive=positive for *B. pertussis* if IS481 Ct <35; Probable positive=positive for *B. pertussis* if IS481 Ct ≥35.
[3]Bold font indicates statistical significance. All percentages rounded off.

When comparing confirmed to probable pertussis in ILI cases, confirmed cases were significantly less likely to be detected at Jouberton when compared to Edendale Gateway [OR=0.1 (95% CI 0.02 – 0.6)] (Table 6). The highest detection rate for ILI cases positive for *B. pertussis* was in the 1-4 year age group [1.5% (9/614)] (Figure 6). There was only 1 confirmed pertussis case in the <1 year age group and this patient was 3 months of age. There were no fatalities in this age group.

There were 6 control individuals that tested positive for *B. pertussis*. Three of the cases were confirmed pertussis and 3 were probable pertussis. The ages of the positive cases varied between 5 and 44 years. Eighty three percent (5/6) of these individuals were HIV infected.

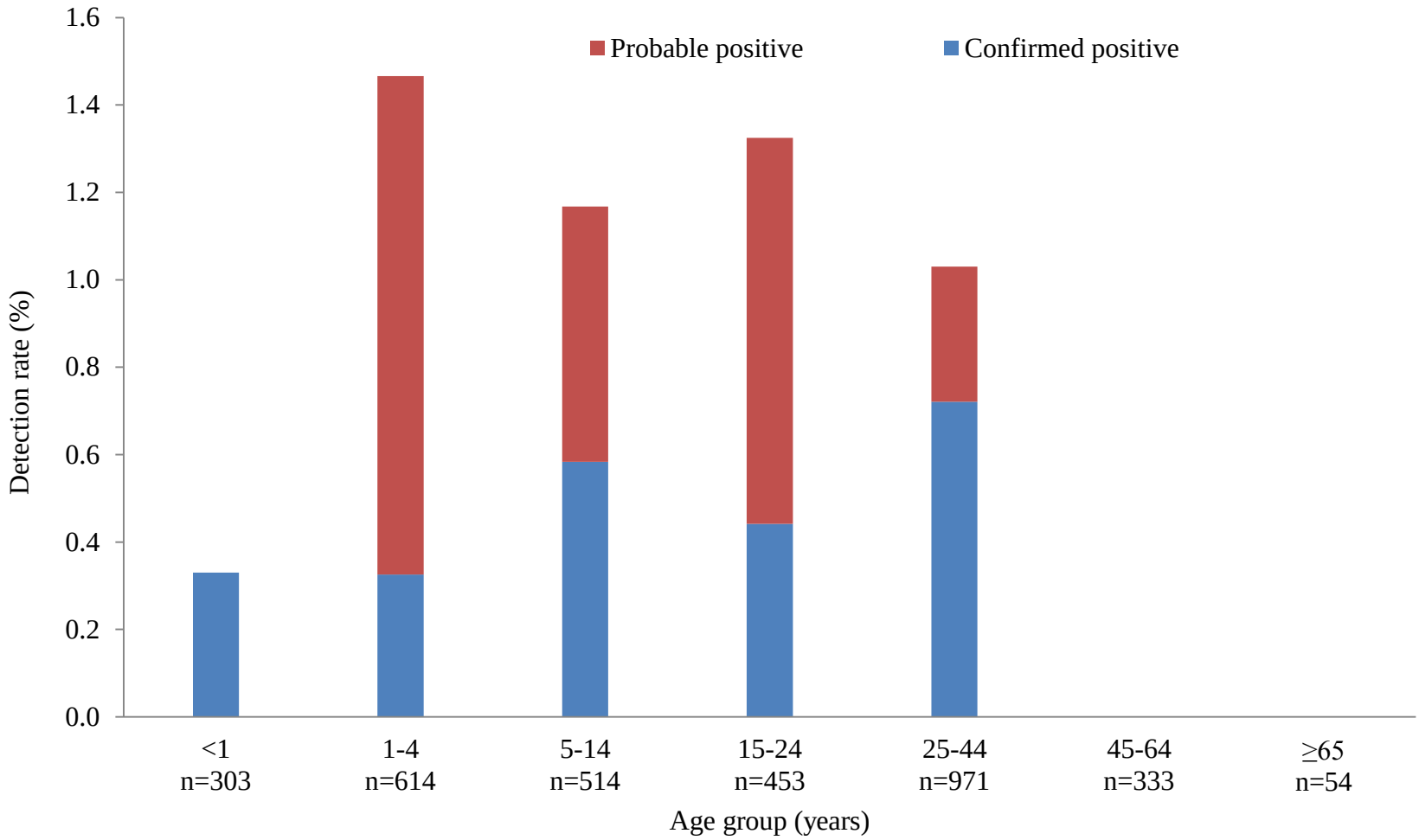

Figure 6: Detection rate of *B. pertussis* (confirmed vs. probable) cases by age group in cases presenting with influenza-like illness, South Africa, June 2012 – October 2014 (N=3242).

4.3.3. Attributable fraction of *B. pertussis* disease

Table 7: Attributable fraction of *B. pertussis* disease in cases with severe respiratory illness and influenza-like illness, South Africa, June 2012 – October 2014 (N=8569).

Surveillance group	Cases tested	Cases positive	Detection rate (%)	Odds ratio (95% CI)	Attributable fraction (95% CI)
SRI – Overall positives[1]	3982	80	2.0%	3.03 (1.23 – 7.48)	66.99 (18.49 – 86.63)
SRI – Confirmed positives[2]	3982	29	0.7%	3.21 (0.85 – 12.14)	68.83 (-17.94 – 91.76)
ILI – Overall positives[1]	3243	32	1.0%	2.11 (0.81 – 5.52)	52.68 (-23.77 – 81.90)
ILI – Confirmed positives[2]	3243	15	0.5%	1.77 (0.44 – 7.06)	43.40 (-126 – 85.84)
Controls	1344	6	0.4%	reference	reference

Abbreviations: SRI=Severe respiratory illness; ILI=Influenza-like illness; CI=Confidence interval.
Attributable fraction calculated for SRI cases using positive nasopharyngeal and induced sputum positive specimens.
[1]Overall positives are all cases that tested positive for *B. pertussis* within the surveillance group.
[2]Confirmed positive=positive for *B. pertussis* if *IS481* Ct <35.

The *B. pertussis* attributable fraction was 67% (95% confidence interval [CI] 18.49 – 86.63) after adjusting for HIV status and age group (Table 7). This result indicates that 67% of *B. pertussis* SRI cases could be attributed to *B. pertussis* infection. When this analysis was restricted to *B. pertussis* confirmed SRI cases the attributable risk was not statistically significant.

4.3.4. Seasonality of *B. pertussis* disease

B. pertussis showed some periodicity during the surveillance period with peaks of disease observed in late winter and early spring (July – September) (Figure 7). The overall detection rate for 2013 was 1.5% (25/1624) and for 2014 this rate increased to 3.2% (37/1126) (p=0.005). The highest detection rate for *B. pertussis* was observed in August 2014 (15.4%, 21/136). This increase in positive cases was observed only at the Jouberton clinic and Tshepong hospital (only data from Tshepong hospital (SRI cases) included in Figure 7). The increase was investigated to determine if it was a true reflection of disease or due to laboratory or environmental contamination. An evaluation of all laboratory control measures and testing of environmental samples from the Jouberton and Tshepong facilities excluded facility and laboratory contamination and indicated a true increase in *B. pertussis* infection. The increase in disease was not sustained and the detection rate of *B. pertussis* decreased from September 2014.

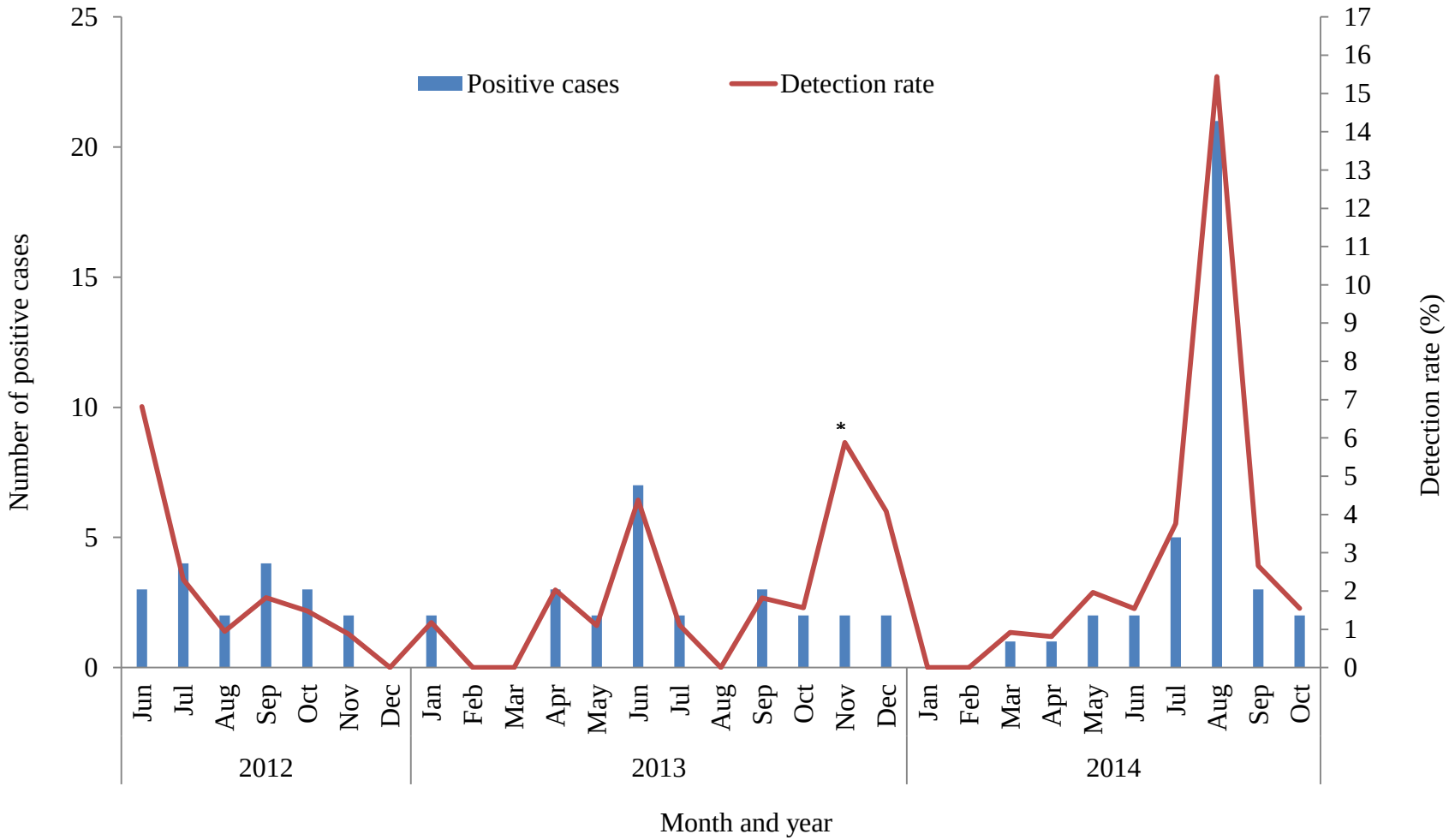

Figure 7: Seasonality of *B. pertussis* in cases presenting with severe respiratory illness, by month and year, South Africa, June 2012 – October 2014 (N=3982). [*Increase in detection rate detected. Not true disease increase. Fewer sample tested due to insufficient sample volumes (November) and fewer samples collected due to festive season (December)].

4.3.5. Co-infections

B. pertussis was detected in 42.5% (34/80) of SRI cases as a single pathogen. For the remainder of *B. pertussis* positive cases (46/80, 57.5%), patients were co-infected with respiratory bacteria or viruses. Co-infections with respiratory bacteria included *S. pneumoniae*, *H. influenzae*, *Legionella* spp. and *M. pneumoniae*. Co-infections with respiratory viruses included influenza, RSV, human metapneumovirus and other viruses (adenovirus, enterovirus, parainfluenza, or rhinovirus).

4.4. *B. parapertussis*

4.4.1. Comparison of specimen types for the detection of *B. parapertussis*

When comparing sputa that were PCR-positive for *B. parapertussis* it was observed that 39% (7/18) of the *B. parapertussis* positive cases were mucoid and 50% (6/12) of the cases had a positive Bartlett's score (Table 8). A similar trend was observed for sputa that was PCR-negative *B. parapertussis*.

Table 8: Macroscopic (N=1615) and Bartlett's score (N=1088) evaluation of induced sputum collected from SRI cases, by *B. parapertussis* PCR result, South Africa, June 2012 – October 2014 (N=2703).

Characteristic	*B. parapertussis* positive	*B. parapertussis* negative	Total
	n/N (%)	n/N (%)	
Macroscopic evaluation			
Saliva	5/18 (28)	457/1597 (29)	
Mucoid	7/18 (39)	626/1597 (39)	
Purulent	5/18 (28)	350/1597 (22)	
Blood stained	1/18 (6)	164/1597 (10)	
Total	18	1597	1615
Bartlett's score			
Negative	1/12 (8)	344/1076 (32)	
0	5/12 (42)	285/1076 (26)	
Positive	6/12 (50)	447/1076 (42)	
Total	12	1076	1088

Negative (combined -1 and -2 score) = presence of between 10 – 25 (-1) and >25 (-2) epithelial cells.
0= presence of <10 neutrophils.
Positive= (combined +1 and +2 score) = presence of between 10 – 25 (+1) and >25 (+2) neutrophils.
Good quality sputum should have a positive Bartlett's score and should not be saliva.
All percentages rounded off.

There was 1 case that tested positive for *B. parapertussis* on NP alone and 12 cases that tested positive on IS alone. No statistical analysis was performed to compare specimen types for the detection of *B. parapertussis* as numbers were too small.

4.4.2. Comparison of *B. parapertussis* positive and negative cases by surveillance group

Table 9: Comparison of *B. parapertussis* positive cases and *B. parapertussis* negative cases in patients presenting with severe respiratory illness, South Africa, June 2012 – October 2014 (N=3982).

Characteristic	*B. parapertussis* PCR result – SRI cases		OR (95% CI)[2]
	B. parapertussis positive n/N (%)	*B. parapertussis* negative n/N (%)	
Year			
2012	15/1170 (1)	1155/1170 (99)	reference
2013	21/1649 (1)	1628/1649 (99)	1 (0.5 – 2)
2014	4/1163 (0.3)	1159/1163 (100)	**0.3 (0.09 – 0.8)**
Gender			
Female	17/1962 (0.9)	1945/1962 (99)	reference
Male	23/2013 (1)	1990/2013 (99)	1 (0.7 – 2)
Age group			
<1	13/880 (1)	867/880 (99)	reference
1-4	8/497 (2)	489/497 (98)	1 (0.4 – 3)
5-14	1/116 (0.9)	115/116 (99)	0.6 (0.08 – 4)
15-24	3/208 (1)	205/208 (99)	1 (0.3 – 3)
25-44	11/1301 (0.9)	1290/1301 (99)	0.6 (0.3 – 1)
45-64	3/761 (0.4)	758/761 (99)	**0.3 (0.07 – 0.9)**
≥65	1/210 (0.5)	209/210 (100)	0.3 (0.04 – 2)
Fever history			
No	29/2338 (1)	2309/2338 (99)	reference
Yes	10/1606 (0.6)	1596/1606 (99)	0.5 (0.2 – 1)
HIV status			
Uninfected	17/1556 (1)	1539/1556 (99)	reference
Infected	17/1896 (0.9)	1879/1896 (99)	0.8 (0.4 – 2)
HIV treatment			
No	6/718 (0.8)	712/718 (99)	reference
Yes	4/905 (0.4)	901/905 (100)	0.5 (0.1 – 2)
Symptom duration			
<7 days	26/2131 (1)	2105/2131 (99)	reference
7-20 days	4/831 (0.5)	827/831 (100)	0.4 (0.1 – 1)
≥21 days	10/939 (1)	929/939 (99)	0.9 (0.4 – 2)
Underlying illness[1]			
No	39/3536 (1)	3497/3536 (99)	reference
Yes	1/437 (0.2)	436/437 (100)	0.2 (0.03 – 2)

ICU			
No	38/3832 (1)	3794/3832 (99)	reference
Yes	2/58 (3)	56/58 (97)	4 (0.8 – 15)
Antibiotic treatment (24 hours)			
No	38/3771 (1)	3733/3771 (99)	reference
Yes	2/187 (1)	185/187 (99)	1 (0.3 – 4)
Hospital duration			
<2 days	7/396 (2)	389/396 (98)	reference
2-4 days	14/1101 (1)	1087/1101 (99)	0.7 (0.2 – 1.7)
5-7 days	10/965 (1)	955/965 (99)	0.5 (0.2 – 1.5)
≥8 days	9/1261 (0.7)	1252/1261 (99)	0.3 (0.1 – 1.0)
Viral co-infection			
No	18/2166 (0.8)	2148/2166 (99)	reference
Yes	21/1786 (1)	1765/1786 (98)	1 (0.8 – 3)
Outcome			
Survived	39/3516 (1)	3477/3516 (99)	reference
Died	1/338 (0.3)	337/338 (100)	0.3 (0.04 – 2)
Facility			
Edendale	18/1770 (1)	1752/1770 (99)	reference
KTHC	22/2212 (1)	2190/2212 (99)	1 (0.5 – 1.8)

Abbreviations: SRI=Severe respiratory illness; KTHC=Klerksdorp-Tshepong hospital complex; N/A=Not applicable; OR=Odds ratio; CI=confidence interval.
[1]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.
All percentages rounded off.
[2]Bold font indicates statistical significance.

When comparing *B. parapertussis* positive cases to negative cases, differences were observed by year of study and age group (Table 9). In 2014, the detection rate of *B. parapertussis* was significantly lower when compared to 2012 [OR=0.3 (95% CI 0.09 – 0.8)]. In addition, positive cases were less likely to be detected in the 45-64 year age group when compared to the <1 year age group [OR=0.3, 95% CI 0.07 – 0.9)].

Table 10: Comparison of *B. parapertussis* positive cases and *B. parapertussis* negative cases in patients presenting with influenza-like illness, South Africa, June 2012 – October 2014 (N=3243).

Characteristic	*B. parapertussis* PCR result – ILI cases		OR (95% CI)[2]
	B. parapertussis positive n/N (%)	*B. parapertussis* negative n/N (%)	
Year			
2012	3/970 (0.3)	967/970 (100)	reference
2013	10/856 (1)	846/856 (99)	**4 (1.04 – 14)**
2014	5/1417 (0.4)	1412/1417 (100)	1.1 (0.2 – 4.7)
Gender			
Female	12/2031 (0.6)	2019/2031 (99)	reference
Male	6/1164 (0.5)	1158/1164 (99)	0.8 (0.3 – 2.3)
Age group			
<1	1/303 (0.3)	302/303 (100)	reference
1-4	9/614 (1)	605/614 (99)	4 (0.6 – 36)
5-14	2/514 (0.4)	512/514 (100)	1 (0.1 – 13)
15-24	1/453 (0.2)	452/453 (100)	0.7 (0.04 – 11)
25-44	3/971 (0.3)	968/971 (100)	0.9 (0.1 – 9)
45-64	2/333 (0.6)	331/333 (99)	2 (0.2 – 20)
≥65	0	54/54 (100)	N/A
Fever history			
No	3/316 (1)	313/3166 (99)	reference
Yes	15/2868 (0.5)	2853/2868 (99)	0.2 (0.1 – 2)
HIV status			
Uninfected	11/1954 (0.6)	1943/1954 (99)	reference
Infected	4/855 (0.5)	851/855 (100)	0.8 (0.3 – 3)
HIV treatment			
No	2/293 (0.7)	291/293 (99)	reference
Yes	2/461 (0.4)	459/461 (100)	0.6 (0.1 – 5)
Symptom duration			
<7 days	14/2862 (0.5)	2848/2862 (100)	reference
7-20 days	1/180 (0.6)	179/180 (99)	1 (0.1 – 9)
≥21 days	2/67 (3)	65/67 (97)	**6 (1.4 – 28)**
Underlying illness[1]			
No	17/3021 (0.6)	3004/3021 (99)	reference
Yes	1/164 (0.6)	163/164 (99)	1 (0.1 – 8)
Viral co-infection			
No	13/1712 (0.8)	1699/1712 (99)	reference
Yes	5/1512 (0.3)	1507/1512 (100)	0.4 (0.2 – 1)

Facility			
Edendale gateway	15/2385 (0.6)	2370/2385 (99)	reference
Jouberton	3/858 (0.4)	855/858 (100)	2 (0.5 – 6)

Abbreviations: ILI=Influenza-like illness; N/A=Not applicable; OR=Odds ratio; CI=Confidence interval.
[1]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.
[2]Bold font signifies statistical significance.
All percentages rounded off.

Positive and negative *B. parapertussis* cases with ILI differed by year of study and symptom duration (Table 10). In 2013 the detection rate of *B. parapertussis* was significantly higher when compared to 2012 [OR=4 (95% CI 1.04 – 14)]. In addition there was a 6-fold increased risk for testing positive for *B. parapertussis* if patients presented with ≥21 days symptom duration when compared to patients presenting with <7 days symptom duration [OR=6 (95% CI 1.4 – 28)].

Two control individuals were PCR-positive for *B. parapertussis*. Both were female and presented at the Edendale Gateway clinic. Individual 1 was a 1 year old and HIV infected whilst individual 2 was 2 years of age and HIV uninfected.

B. parapertussis disease was observed in all age groups for SRI cases (Figure 8). The highest prevalence was observed in the 1-4 year age group (1.6%, 8/497). For ILI cases, the highest prevalence was also observed in the 1-4 year age group (1.5%, 9/614) (Figure 9). No cases were detected in the ≥65 year age group.

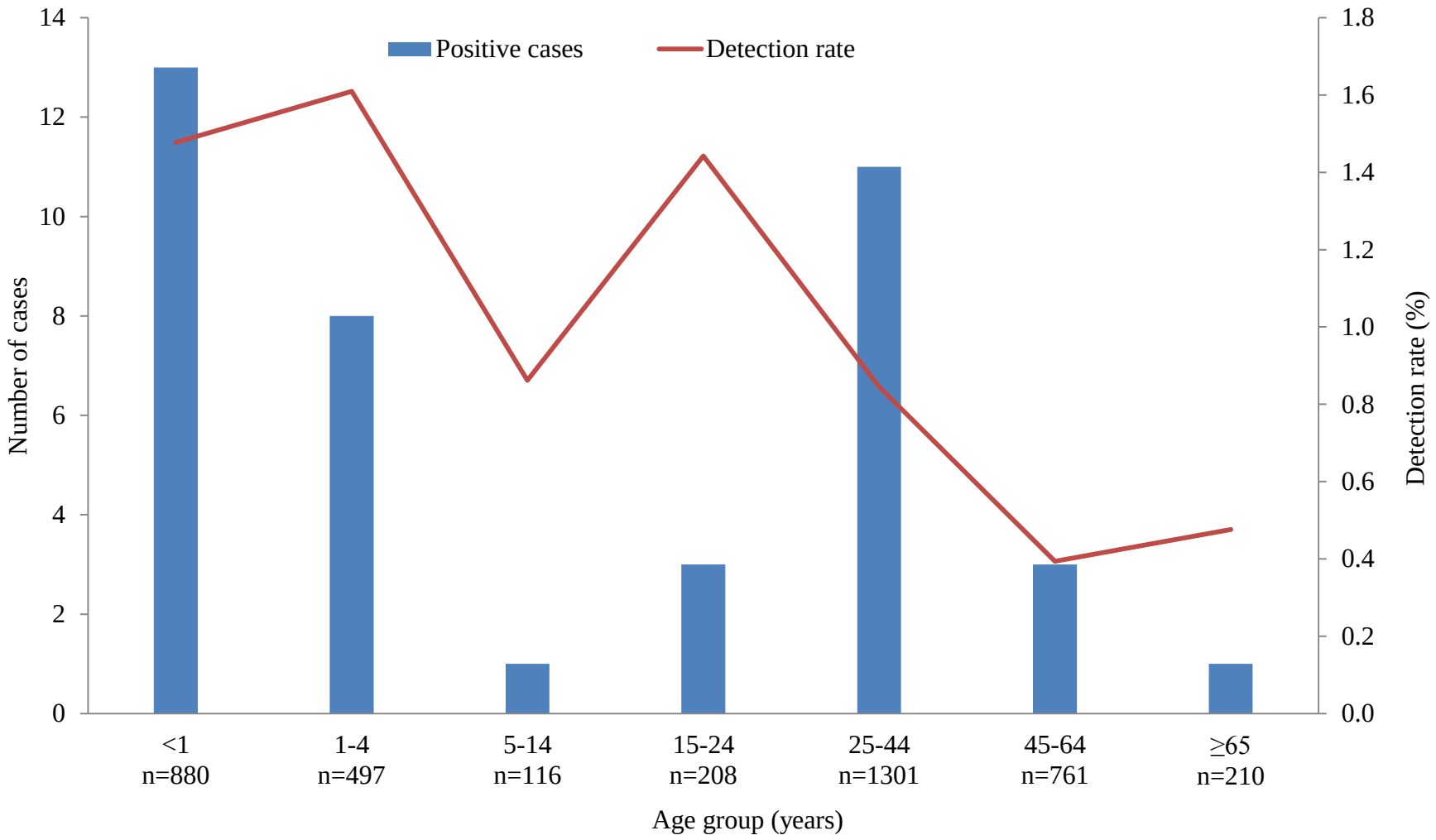

Figure 8: Detection rate of *B. parapertussis* in cases presenting with severe respiratory illness by age group, South Africa, June 2012 – October 2014 (N=3973).

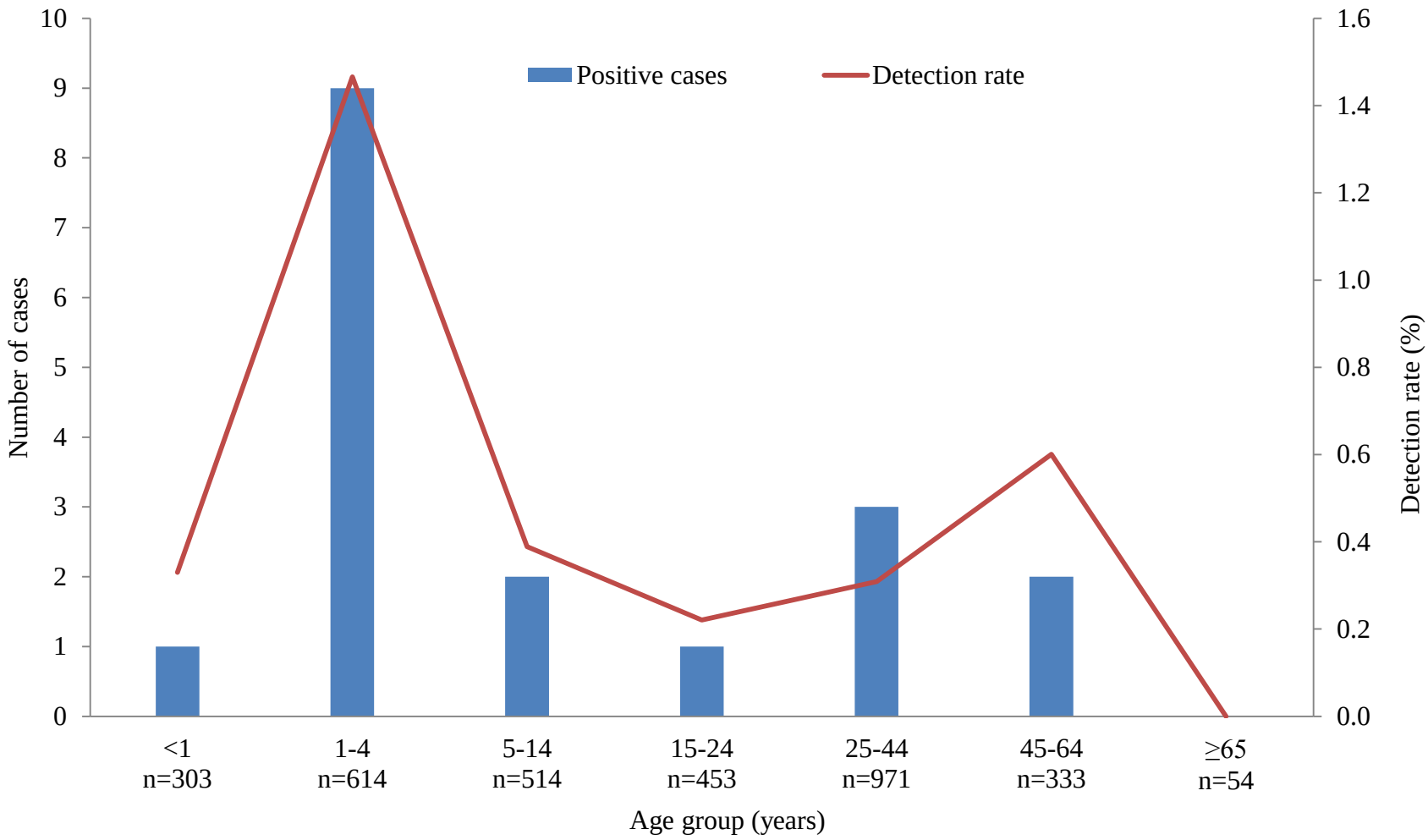

Figure 9: Detection rate of *B. parapertussis* in cases presenting with influenza-like illness by age group, South Africa, June 2012 – October 2014 (N=3242).

Table 11: Attributable fraction of *B. parapertussis* diseases in patients with severe respiratory illness and influenza-like illness, South Africa, June 2012 – October 2014 (N=8569).

Surveillance group	Cases tested	Cases positive	Detection rate (%)	Odds ratio (95% CI)	Attributable fraction (95% CI)
SRI	3982	40	1%	4.97 (1.14 – 21.63)	79.89 (12.52 – 95.38)
ILI	3243	11	0.3%	4.82 (0.97 – 24.03)	79.25 (-3.47 – 95.84)
Controls	1344	2	0.1%	reference	reference

Abbreviations: SRI=Severe respiratory illness; ILI=Influenza-like illness; CI=Confidence interval.
Attributable fraction calculated for SRI cases using positive nasopharyngeal and induced sputum positive specimens.

4.4.3. Attributable fraction of *B. parapertussis* disease

For cases presenting with SRI, the *B. parapertussis* attributable fraction was 80% (95% CI 12.52 – 95.38) after adjusting for HIV status and age group (Table 11). This result indicates that 80% of *B. parapertussis* SRI cases could be attributed to *B. parapertussis* infection. Within the ILI surveillance population there was no attributable fraction of disease.

4.4.4. Seasonality of *B. parapertussis* disease

B. parapertussis disease showed no distinct seasonality (Figure 10). The highest detection rate was observed in August of 2012 (3.8%, 8/212). Only 4 cases positive for *B. parapertussis* were detected in 2014.

4.4.5. Co-infections

B. parapertussis was detected in 35% (14/40) of SRI cases with no other respiratory bacterial or viral pathogen. The other 65% (26/40) of *B. parapertussis* positive cases were co-infected with respiratory bacteria or viruses, *S. pneumoniae* and *M. pneumoniae*, influenza, RSV, human metapneumovirus and other viruses (adenovirus, enterovirus, parainfluenza, and rhinovirus).

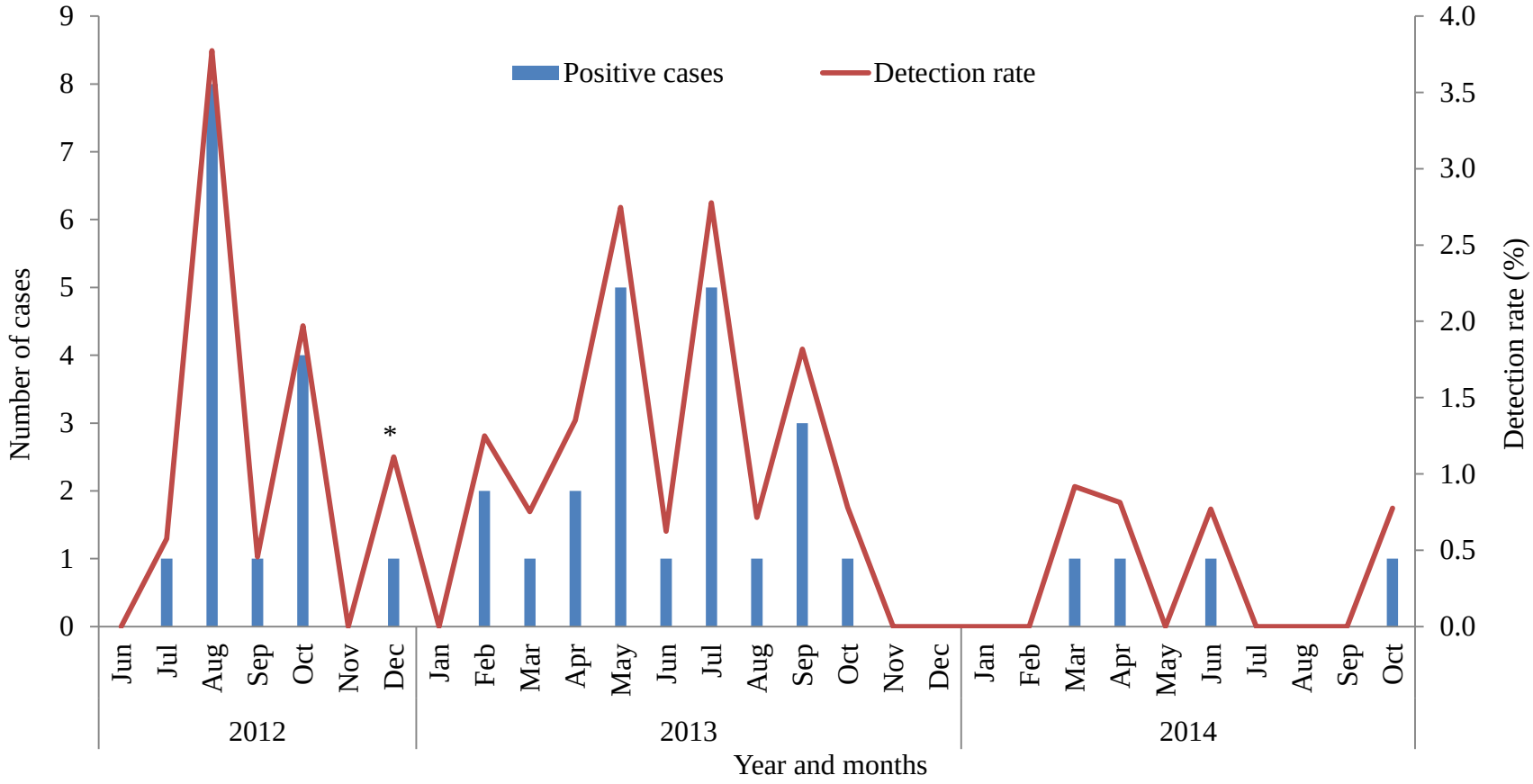

Figure 10: Seasonality of *B. parapertussis* by month and year, South Africa, June 2012 – October 2014 (N=3982).

[*Increase in detection rate detected. Not true disease increase. Fewer samples collected due to festive season (December)].

4.5. *B. bronchiseptica*

During the surveillance period *B. bronchiseptica* was detected in 4 cases only (Table 12). One case tested positive on both specimen types whilst 3 cases tested positive on an NP specimen only. Three cases were co-infected with rhinovirus and 1 case (with no co-infection) was HIV positive.

Table 12: Summary of cases PCR positive for *B. bronchiseptica*, South Africa, June 2012 – October 2014 (N=8569).

Characteristic	Case 1	Case 2	Case 3	Case 4
Gender	Male	Male	Male	Female
Race	Black	Black	Black	Black
Age (years)	63	1	1	45
HIV status	Uninfected	Uninfected	Uninfected	Infected
Underlying illness	Yes	Yes	No	No
Surveillance group	SRI	SARI	ILI	SRI

Abbreviations SRI=Severe respiratory illness; SARI=Severe acute respiratory illness; ILI=Influenza-like illness.

5. Discussion

Following the implementation of the pertussis whole-cell vaccine in South Africa in 1950, there are limited data describing pertussis in South Africa, as well as a lack of standardised molecular methods for pertussis identification. Our study utilised 2 pneumonia surveillance platforms (SRI and ILI) to determine the prevalence of *B. pertussis* at selected sites in South Africa. Real-time multiplex and singleplex PCR assays were validated and implemented to detect *B. pertussis*, *B. parapertussis*, *B. holmesii* and *B. bronchiseptica*. In addition, different specimen types were evaluated for the detection of these *Bordetella* species. Of the 8569 cases enrolled that had specimens taken for testing, 118 [1.4%, 118/8569 (95% CI 1.1 – 1.6)] were positive for *B. pertussis* of which 2% [80/3982 (95% CI 1.6 – 2.5)] were hospitalised, 1% [32/3243 (95% CI 0.7 – 1.4)] were out-patients and 0.4% [6/1344 (95% CI 0.2 – 1.0)] were asymptomatic controls.

Real-time PCR validation

After reviewing the literature two assays were implemented: the first assay is a three-plex which detects the insertion sequences *IS481*, *pIS1001* and *hIS1001* and the second assay is a singleplex which detects the pertussis toxin *ptxS1* (26;27). The multiplex assay detects *IS481* to detect *Bordetella* spp. (*B. pertussis* (50-238 copies per genome), *B. bronchiseptica* (rarely detected in humans) and *B. holmesii* (8-10 copies per genome), *pIS1001* for *B. parapertussis* (20-23 copies per genome), and *hIS1001* for *B. holmesii* (3-5 copies per genome). The second assay is a singleplex that detects *ptxS1* which is a confirmatory target for *B. pertussis*, *B. bronchiseptica* and *B. parapertussis*. An internal validation was performed for these assays and 100% sensitivity and specificity was obtained for the detection of *B. parapertussis*, *B. bronchiseptica* and *B. holmesii*. 95% sensitivity and 100% specificity was obtained for the detection of *B. pertussis*. In addition, all QCMD panels received for 2011, 2013 and 2014

were correct. However for the 2012 panel, one DNA extract was reported as a negative. This may have been due to the DNA yield in the specimen being too low for the real-time PCR assays to detect or due to DNA degradation as the extracts from this panel were stored for over a year before it was given to our laboratory by a second laboratory. In addition, no expected Ct-values were available for any of the QCMD samples so Ct-values could not be compared. With regard to the Ct-values generated for *IS481*, *pIS1001* and *ptxS1*, it was observed that Ct-values generated for *IS481* and *pIS1001* were 5-10 Cts lower than Cts obtained for *ptxS1*. The lower Ct-values is probably due to the fact that there are multiple copies of the insertion sequences (*IS481* and *pIS1001*) in *B. pertussis* and *B. parapertussis*, compared to the single copy *ptxS1*gene (28).

One aspect of PCR robustness was demonstrated by the fact that no differences in Ct-values were detected when using two different master mixes, namely, TaqMan Gene Expression master mix (Applied Biosystems) and the Quanta super mix (Quanta Biosciences). Therefore Taqman gene expression master mix was used as it is cheaper and easier to purchase and was consistent to the published methodology.

B. pertussis

IS481 is a multicopy target (50-238 copies per genome), therefore increasing the risk of laboratory and PCR contamination (31-33). It is advisable to determine reasonable and accurate Ct-value cut-offs when analysing and interpreting PCR data. Many studies published thus far have incorporated Ct-value cut-offs similar to our study when using *IS481* for the detection of *B. pertussis*. In a Tunisian study from 2007 to 2011, *B. pertussis* cases were defined as PCR positive for *IS481* and *ptxS1* with a Ct <45, or as *Bordetella* spp. if they were positive for *IS481* only with a Ct <45 (28). Of the clinically-confirmed *B. pertussis* cases,

82% tested positive for *B. pertussis* and 5% tested positive for *Bordetella* spp by real-time PCR. Another study in Norway from 2011 and 2012, enrolling patients of all ages with respiratory tract infection, used a Ct cut-off <36 and 36-40 for *IS481* (57). Cases positive for *IS481* with Ct <36 were defined as *B. pertussis* positive and cases positive with Ct-values of 36-40 were repeated to confirm results. A study in the United States from 2008 to 2010 utilised two different Ct cut-off algorithms (58). Prior to 2010, specimens were defined as positive for pertussis/parapertussis if they tested positive for *IS481* or *IS1001* with Ct <38 and all specimens with Ct-values of 35-50 were repeated to confirm results. After 2010, interpretation was changed to define positivity as Ct-values <35 for *IS481* or *IS1001*. Samples with Ct-values of 35-40 were repeated to confirm results. This change in interpretation was done to determine if overall positivity would be affected by further Ct cut-offs, however using both interpretation methods the results remained the same. The CDC uses a Ct-value cut-off of 34 for *IS481* and/or a Ct-value cut-off of 39 for *ptxS1* for *B. pertussis* positivity (26;27). A Ct-value of 35-40 for *IS481* and/or a Ct <40 for *ptxS1* are interpreted as indeterminate for *B. pertussis.* Any sample with a Ct ≥40 is interpreted as negative. In trying to determine appropriate Ct-value cut-offs for our study it was observed that many cases that tested positive for *IS481* had Ct-values ranging from 35 to 45. These results would be defined as indeterminate/negative using the CDC published algorithm. The algorithm was therefore modified to interpret all samples with Ct-values <45 as positive and to further define as confirmed pertussis (Ct-values <35) or probable pertussis (Ct-values 35-45) so as not to exclude any cases at this stage (Appendix 5).

In our study, 118 *B. pertussis* cases were detected, 40% were defined as confirmed pertussis and the remaining 60% as probable pertussis. Ideally, in this situation clinical data should be used in conjunction with laboratory results to make a definitive diagnosis, however because

the case definition for enrollment was based on pneumonia symptoms, no clinical data specific for pertussis symptoms were available for any of the positive cases, so this could not aid in diagnosis. Therefore, results were stratified by surveillance group and clinical characteristics to determine if there were any differences between the confirmed and probable pertussis cases. Using univariate analysis, minimal differences were found between confirmed and probable pertussis cases. Amongst the hospitalised pertussis cases, more probable cases were 45-64 year age group (lower bacterial loads) when compared to the <1 year age group. This correlates with a study in Japan from 2007 to 2009 which showed that adults have a significantly lower bacterial load in both early and late stages of disease compared to infants and children (59). For the pertussis cases at the out-patient clinics, more probable pertussis cases were detected at the Jouberton facility compared to the Edendale Gateway facility. Analysis of characteristics of patients presenting at these facilities did not differ and a reason for this could not be determined.

Although there was no clear differentiation between confirmed and probable cases, there were three factors that support the fact that the probable cases were true cases. Firstly, from the 2013 and 2014 QCMD panels, all *B. pertussis* samples were correctly identified as positive using real-time PCR with Ct-values ranging from 34 to 42. In addition, a subset of positive *B. pertussis* patients (probable and confirmed cases) from 2014 was retrospectively interviewed and the majority of these patients reported clinical symptoms consistent with pertussis. Furthermore, because surveillance was not specific for pertussis, some of the cases may have presented late in pertussis infection where bacterial loads are low, resulting in higher PCR Ct-values.

In our study the detection rate of *B. pertussis* in hospitalised and out-patient populations was 2% and 1%, respectively, which is low compared to detection rates observed in other countries. The detection rate observed in our study population could be attributed to the surveillance case definition that is not specific for pertussis. All enrolled cases presented with possible clinical pneumonia and enrollment criteria were based on pneumonia-related clinical symptoms. Studies have shown that pertussis prevalence varies by country; as sample populations, diagnostic tests employed, sample types and vaccination type/status varies between countries and studies (60-62). A population-based study in Toronto from 1993 to 2007 found a 9.4% pertussis detection rate amongst patients of all ages. All patients had clinical symptoms of pertussis and presented to different public health units in the Greater Toronto area (38). Another study conducted in Finland from 1994 to 1997 enrolling out-patients of all ages with paroxysmal coughing found a *B. pertussis* prevalence of 16.3% (63). A serological study, measuring anti-PT IgG levels, was conducted in Denmark from 2006 to 2008 to determine the causative agent in patients with cough of unknown aetiology in all patients aged 8 years and older (37). Three to 11% (depending on the serological cut-off values used) of the population tested positive for *B. pertussis*. A study conducted in Ohio from 2010 and 2011 enrolling patients of all ages, to determine the epidemiological and laboratory features of an outbreak of pertussis-like illness found 29% of the population to be positive for *B. pertussis* (64).

B. pertussis is known to cause severe disease in infants, milder disease in children and asymptomatic infection in adolescents and adults, who are the source of infection for younger children (65). In our study, pertussis was detected in all age groups. Amongst hospitalised patients, the highest detection rate was in the older age groups whereas the detection rate in less severe patients was highest in children <5 years. Other studies have shown a higher

prevalence in children which is contradictory to our study. From 2008 to 2011, approximately 311 laboratory-confirmed *B. pertussis* cases were reported to the South African Department of Health (66). Sixty-seven percent were infants <3 months old and 22% were infants <6 weeks old. A Cape Town study enrolling children ≤7 years of age, from June 1988 to April 1989, using serology, found that 50% of the positive cases were infants <6 months, 22% were in the 6-11 month age group, 24% were in the 12-59 months age group and 3% in the 60 months and older age group (41;50). A serological study in Denmark from 2006 to 2008 to determine the causative agent in patients with cough of unknown aetiology in all patients aged 8 years and older found children between the ages of 8 to 14 years with the highest seroprevalence of pertussis (37). A study conducted in Norway from 2011 and 2012 enrolling patients of all ages with respiratory tract infection, found the highest burden of *B. pertussis* disease in the 13-21 year age group (57). Results from our study highlighted the increased burden of asymptomatic pertussis in the older age groups. The WHO has reported this age group as an important source of transmission of *B. pertussis* to infants too young to be fully vaccinated and due to waning vaccine immunity in the older age groups (16). Another study in Finland, from 1993 to 1994 in patients of all ages, found a large proportion of pertussis cases in the adult age groups, presumably due to decreased protection from *B. pertussis* vaccination with time (63).

Pertussis disease is cyclical with disease peaks observed every two to five years (17;23) and disease has been shown to peak in the summer and autumn months (65). Studies have shown differing pertussis seasonality and it has been stated that pertussis disease is not consistent by place or time (18). In Canada, from 1993 through 2007, pertussis infection peaked in the autumn and winter months (38). In a South African study, from 2008 and 2009, an increase in pertussis disease was detected in the autumn and winter months (51). During the 2010

California epidemic, pertussis disease peaked in summer and autumn (67). In our study *B. pertussis* disease did not follow an obvious seasonal pattern, however disease peaks were observed in the late winter and early spring months (July – September). The highest detection rate was observed in August 2014 (15%). As the increase was only observed at the Jouberton clinic and the Tshepong hospital in the North West province, it was investigated to determine if it was due to *IS481* PCR contamination or a true reflection of disease and possibly an outbreak. Various environmental swabs were collected from the two facilities and a subset of the positive *B. pertussis* patients were interviewed to determine if there was an epidemiological link between the cases. All environmental swabs were PCR negative and an audit of all laboratory processes and testing ruled out contamination. It was thus concluded that the 15% increase was a true reflection of disease; however it was not an outbreak as no epidemiological link could be found between the cases.

B. pertussis was detected in both NP and IS specimens. A lower detection rate (0.5%) was observed in NP specimens compared to the detection rate (1.8%) in IS specimens. This implies that IS may be a better specimen type for the detection of *B. pertussis*. This result contrasts with the recommendation that NP specimens are the preferred specimen type for pertussis diagnostics (17;18;23;52). It has been shown that there is specific binding of *B. pertussis* to the ciliated epithelial cells of the nasopharyx upon attachment to host cells during disease manifestation (68). Only in the absence of an immune response does the bacterium move to the ciliated epithelial cells of the trachea and bronchi (16;17;19). The use of IS for pertussis diagnosis has been suggested by WHO but data supporting this specimen type for diagnostics are lacking (52). In the laboratory, sputum is graded microscopically using the Bartlett's score and macroscopically to determine specimen quality, and specimens that are graded negatively may be rejected for use in diagnostics (69). When comparing the

quality of IS received in our study for testing to the PCR results obtained for these specimens, it was found that the macroscopic and Bartlett's score evaluation did not influence PCR results for the detection of *B. pertussis* and *B. parapertussis.* In the clinical environment IS is difficult to obtain from patients, in particular children. Patients are sometimes too sick to cough up sputum and only patients that have a severe respiratory infection can produce sputum. This may challenge the routine collection of IS for pertussis diagnostics.

In our study, cases positive for *B. pertussis* were, in a few instances, also positive for other respiratory viruses (influenza, RSV, human metapneumovirus or other viruses) or bacteria (*S. pneumoniae, H. influenzae, Legionella* spp. or *M. pneumoniae*). In Brazil in 2013, in infants with suspected pertussis, approximately 5% of *B. pertussis* cases were co-infected with respiratory viruses (RSV, parainfluenza, adenovirus or influenza) (70). Authors concluded that cases that were co-infected had to be further analysed to determine the true causative agent of disease, however the majority of cases did have the classical pertussis symptoms. Another study in Norway in 2011 showed that a proportion of cases positive for *B. pertussis* were also co-infected with either *M. pneumoniae, C. pneumoniae* or influenza virus (57). No conclusions regarding the causative agents of disease were made in the study. When co-infections in patients are detected it is difficult to determine which agent is responsible for causing disease. For this reason and also to rule out possible *B. pertussis* carriage, the attributable risk for *B. pertussis* infection was calculated. Approximately 67% (95% CI 18.49 – 86.63) of *B. pertussis* PCR-positive cases with severe respiratory infection could be attributed to *B. pertussis* infection, whilst for milder cases there was no attributable risk of *B. pertussis* infection.

When analysing data from children positive for *B. pertussis* with an available vaccine status it was found that there were more children with lower bacterial loads that were fully vaccinated for age compared to children who were not fully vaccinated for age. This correlates with a study in Japan that showed that bacterial loads in children vaccinated with four doses of pertussis vaccines were lower and they had milder infection than unvaccinated infants (59). In Germany from 1993 to 1999, children fully vaccinated with the acellular vaccine had milder pertussis symptoms and shorter cough duration compared to unvaccinated children (71). In our study, only children <5 years of age had vaccine records available on RTHC but not all enrolled children had an available RTHC with vaccine records clearly stated. Therefore vaccine status and pertussis disease could not be linked as numbers were too small and records were not available.

The attack rate of *B. pertussis* is higher amongst females than males, however the reason for this is unknown (68). In our study there was a higher proportion of females positive for *B. pertussis*, however this was not statistically significant. Other studies in Canada (38) and South Africa (41;49) have shown a similar result.

Leukocyte and lymphocyte counts of the patients that tested positive for *B. pertussis* were not available. Studies have shown that patients positive for *B. pertussis* have increased leukocyte and lymphocyte counts. A study in Germany from 1992 to 1993 found that cases infected with *B. pertussis* had a mean leukocyte and lymphocyte count of 12500/mm^3 and 7600/mm^3 respectively (72). These counts were significantly higher than the counts found in *B. parapertussis* positive cases. Amongst the infants that tested positive for *B. pertussis* in a Tunisian study from 2007 to 2011, 38% of cases had leukocytosis and 40% of cases had lymphocytosis (28).

Sub-Saharan Africa has a high HIV burden and HIV-infected children are more susceptible to pneumonia than HIV-uninfected children (73). Studies describing *B. pertussis* infection among HIV-infected individuals are lacking. A study in the Free State from 2008 to 2009 highlighted that 28% of PCR-positive *B. pertussis* cases were HIV infected and these patients all required hospitalisation. Three were younger than 10 weeks of age and 2 of these infants required ICU admission (51). No association between HIV and pertussis could be determined in this study as the numbers were too small and not all enrolled cases had an HIV result available.

For culture of *B. pertussis*, NP specimens are recommended and should be taken preferably within the first two weeks of cough onset (52). In addition specimens should be taken at the patient bedside, inoculated into transport medium for pertussis and transported directly to the laboratory within 48 hours at room temperature (52). Due to the fastidious nature of *B. pertussis*, culture is difficult (17). In our study NP specimens were not used for culture as specimens were transported in UTM which is not ideal as it contains antibiotics which impedes culture. Therefore, IS specimens were used; however no positive *B. pertussis* cultures were obtained. This could be due to specimens being stored incorrectly during transportation, delay in specimen transportation to the laboratory, bacterial or fungal contamination of culture plates following incubation at the laboratory (28). In addition, from 2014 all PCR-positive *B. pertussis* patients were retrospectively interviewed and additional NP specimens (transported in the recommended Regan-Lowe transport medium) (Media Mage, Rooderport, Johannesburg) were taken for culture; however this was not ideal as many cases had been treated with antibiotics and some had recovered from infection. Studies using culture and real-time PCR have shown that real-time PCR has increased sensitivity for the detection of *B. pertussis* when compared to culture (74;75). A study in Tunisia from 2007 to

2011, amongst hospitalised infants, also showed the decreased sensitivity of culture (28). During the four-year surveillance period in Tunisia only one sample was culture positive from a total of 606 samples tested.

B. parapertussis

B. parapertussis is closely related to *B. pertussis,* however it causes a milder form of pertussis-like disease in humans as it does not produce pertussis toxin (20;29;76). In our study population, the detection rate of *B. parapertussis* was low in hospitalised and out-patient individuals and this differed to the detection rates showed in other countries. Again, this could be attributed to the broad case definition used for pneumonia surveillance. In Tunisia, the prevalence of *B. parapertussis* in all infants hospitalised from 2007 to 2011 was 7% (28). In Germany, from 1993 to 1999, a high detection rate of 36% of *B. parapertussis* was found in children vaccinated with acellular pertussis vaccine (71).

B. parapertussis was detected in hospitalised patients of all ages; however in the out-patient population, *B. parapertussis* was detected in all age groups except the ≥65 year group. Cases positive for *B. parapertussis* were more likely to present in the younger age groups than the older age groups. The highest detection rate of *B. parapertussis* in the hospitalised and out-patient individuals was in the 1-4-year-olds. This detection rate in the 1-4 year age group is consistent with other studies. Disease caused by *B. parapertussis* is most common during the first five years of life (68). The mean age of children positive for *B. pertussis* and *B. parapertussis* in a German study from 1993 to 1999 were 4.2 years (range 2.2 – 6.0 years) (71). Another study in Germany from 1992 to 1993 found that 86% of *B. parapertussis* cases were younger than 6 years of age (72).

In our study *B. parapertussis* disease showed peaks in August of 2012 and then in May and July of 2014. Only 4 cases of *B. parapertussis* were detected in 2014. No true seasonality could be determined as the time frame for this data analysis was only two years and five months. In the literature there are limited data regarding the seasonality of *B. parapertussis*. There was no seasonal distribution of *B. parapertussis* in the Tunisian study from 2007 to 2011 (28). A clinical trial in Italy between 1992 and 1993 highlighted that *B. parapertussis* and *B. pertussis* disease have a similar seasonal trend (77). Disease caused by both pathogens peaked between spring and summer of the study period.

Studies have shown that the symptom duration in *B. parapertussis* cases is significantly shorter than the symptom duration in *B. pertussis* cases (63;71;72;78). We did not analyse this outcome as one of the criteria for enrollment was symptom duration so this would have biased the result. In addition cases did not present based on classic pertussis/parapertussis symptoms but had lower respiratory tract infection.

None of the hospitalised and out-patient cases positive for *B. parapertussis* were co-infected with *B. pertussis*. This differed to what has been published in the literature as *B. pertussis* and *B. parapertussis* co-infection is common, however, no studies have shown that co-infection with *B. pertussis* and *B. parapertussis* causes an exacerbation of disease (68). Furthermore, infection with one organism does not make the host more susceptible to the second organism. From 1994 to 1997 a study in Finland showed that approximately 7.2% of the total population studied was co-infected with *B. pertussis* and *B. parapertussis* (63). In a Tunisian study from 2007 to 2011, 8% of infants tested were positive for *B. pertussis* and *B. parapertussis* (28).

Similar to *B. pertussis*, the attributable risk for *B. parapertussis* was calculated as cases that were positive for *B. parapertussis* were co-infected with other respiratory bacteria and/or viruses. The attributable risk was 80% (95% CI 12.52 – 95.38) indicating that 80% of cases positive for *B. parapertussis* in the SRI population were sick due to *B. parapertussis*. The remaining 20% of *B. parapertussis* positive cases may be attributed to the co-infecting bacteria or viruses.

B. bronchiseptica

B. bronchiseptica is a commensal of the respiratory tract and a cause of respiratory disease in both wild and domesticated animals (79). This pathogen has been rarely associated with disease in humans (68). Transmission usually occurs from animals to children or animal-caretakers, causing mild respiratory tract infections to pertussis-like symptoms (80).

In our surveillance four cases tested positive for *B. bronchiseptica*. Three patients were hospitalised and 1 presented as an out-patient. No data regarding animal contact were available for any of these patients and 2 of the 4 cases had underlying illnesses. In 1981 Byrd and coworkers described a chronic renal failure patient on dialysis who developed *B. bronchiseptica* peritonitis (81). The patient was in contact with a domesticated dog resulting in contamination of the peritoneal catheter. In 1991 Woolfrey and Moody published a summary of 25 cases positive for *B. bronchiseptica* dating back to 1911 with the majority of cases having a respiratory illness (79). Of the 25 cases, two had respiratory tract infections and an underlying illness; however none had animal contact prior to *B. bronchiseptica* disease onset.

Only one *B. bronchiseptica* patient was HIV infected with no co-infecting bacterial or viral pathogen detected. *B. bronchiseptica* disease has been associated with HIV. A study by Dworkin *et al*., found nine cases positive for *B. bronchiseptica* all of whom were HIV infected (82). All cases had either mild or severe respiratory tract infections. Three of the nine cases had contact with a domestic animal. A case report published in 1994 described an HIV-positive patient suffering with pneumonia caused by *B. bronchiseptica* (83).

Challenges and limitations

The SRI/ILI study is ongoing; however, only data for 2 years and 5 months are presented in this dissertation. The data generated thus far are limited for conclusions to be drawn as the number of positive cases was small and limited statistical analyses could be performed. In addition patients were enrolled only at 2 sentinel sites in South Africa and the data generated is not representative of the entire population. With regard to the interpretation of the PCR data, no definitive conclusions regarding the probable pertussis cases could be made. All positive cases were analysed irrespective of Ct-values as South Africa has limited pertussis data and our study aided in creating baseline data for future studies. In order to make more definitive conclusions surveillance needs to continue. *B. pertussis* could not be cultured therefore no molecular characterisation could be carried out to determine if the strains circulating in South Africa were also lacking the vaccine antigen pertactin as seen quite recently in Europe and the United States following the switch from the whole-cell to the acellular pertussis vaccine.

6. Conclusions and future research

Our study provides some baseline data for *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* in South Africa, even though the case definitions were not ideal for the

detection of these pathogens. In addition, real-time PCR assays were validated and implemented in our laboratory and are now routinely used for both diagnosis and surveillance. No definitive conclusions could be made regarding the probable pertussis cases and whether Ct-value cut-offs of 35 should be used to exclude cases, however based on the findings discussed above we were confident in reporting these cases as true cases. In addition, a subset of positive samples will be sequenced to confirm positive PCR results. Pertussis surveillance will continue using the SRI/ILI platforms and will be expanded to other sites in the country, namely, Gauteng, Mpumalanga and Western Cape provinces, generating additional data more representative of the South African population. More defined case definitions for identification of pertussis disease will be incorporated into the case investigation forms i.e. upon enrollment patients will be asked if they experienced classical pertussis symptoms (whooping cough, posttussive vomiting, and apnoea) prior to enrollment. Furthermore, an additional nasopharyngeal specimen will be taken from all cases that have suspected pertussis upon enrollment and these specimens will be used predominantly for culture. These specimens will inoculated into the transport medium for pertussis and transported within 48 hours to the laboratory to possibly increase the chance of obtaining an isolate. If *B. pertussis* cultures are obtained, the isolates will be used to determine the molecular epidemiology of the circulating isolates.

7. Appendices

Appendix 1: Case investigation form for the SRI study

SARI: Severe Acute Respiratory Infection Surveillance in South Africa
Clinical Case Investigation Form
National Institute for Communicable Diseases: Epidemiology & Surveillance Unit (ESU)
TEL: 011 386 6234 OR 011 555 0353 FAX: 011 386 6077

SO initials: Study number:
Date completed: D D M M Y Y Y Y

1.Patient Hospitalised at: CHBH ☐ Selby ☐ Mapulaneng ☐ Matikwana ☐ Edendale ☐

2. Age group: >2 days< 3months ☐ ≥3 months< 5years ☐ ≥ 5 years ☐

3. Date of birth (DOB): D D M M Y Y Y Y

3.1. If DOB unknown, please enter age: years _____ or months _____ or days _____

4. Admission Diagnosis: Neonatal Sepsis ☐ Bronchiolitis ☐ Bronchopneumonia ☐ Pneumonia ☐ TB ☐
Lower respiratory Tract Infection ☐ Bronchitis ☐ Other ☐
specify:_____

5.Date of admission D D M M Y Y Y Y

6. What is the interviewee's relationship to the participant? Self ☐ Parent/caregiver ☐ Other ☐
Specify_____

7. Gender: Male ☐ Female ☐

8.Race: Asian/Indian ☐ Black ☐ White ☐ Coloured ☐

9. What is your house made of? Bricks ☐ Iron sheeting ☐ Mud ☐ Other ☐
Specify_____

10. Number of rooms used for sleeping? _____

11. Number of people living in house? _____

12. Date of onset of symptoms: D D M M Y Y Y Y

Note: to fit the case definition this should be ≤ 7days from the day of admission

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

13. History of fever? Yes ☐ No ☐ Unknown ☐

14. Maximum recorded temperature within 24 hours of admission _____._____ °C (##.#°C) Temperature not recorded ☐

Note: Record the maximum temperature recorded in the clinical notes or a temperature taken by the surveillance oficer within 24 hours of admission if no temperature in the file

14.1 Date temperature recorded? D D M M Y Y Y Y

15. Respiratory Rate: _____ breaths per minute Not recorded ☐

Note: Complete the following for children < 5years, skip to Q17 if patient ≥5 years

16. 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- 1 year RR>50, 1-5 RR>40)	Y	N	Diarrhoea (>3 loose stool 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

In the 7 Days prior to onset of symptoms have you:

17. Had direct contact with dead/dying birds? Y ☐ N ☐

18. Visited a village/area where there have been unusual deaths of poultry or wild birds? Y ☐ N ☐

19. Had close contact with a person who died of an acute respiratory illness? Y ☐ N ☐
If no skip to question 20, if yes continue with 19.1

19.1 When did this person die? D D M M Y Y Y Y	19.2 How long was this person sick for? Days ☐ Weeks ☐ Months ☐
19.3 Do you know the cause of the illness? Y ☐ N ☐	19.4 If yes what was the cause? _____

20. Do you do any of the following types of work? Veterinarian/animal health worker Laboratory worker ☐ Health care worker

Backyard chicken keeper ☐ Farm worker ☐ Abattoir worker ☐ None ☐

Note: if yes to any of the questions (17-20) notify study doctor who will decide if the patient should be referred to the Department of Health for further investigation for suspected Avian influenza.

Note: Skip to question 23 if child less than 12 years

21. Do you drink alcohol? Y ☐ N ☐	21.1 If yes, how many units per week?_____

22. Do you currently smoke? Y ☐ N ☐
Note: If yes skip to Q 23

22.1 If no have you smoked in the past? Y ☐ N ☐ ***Note: If no skip to Q 23***	22.2 If yes date of stopping? D D M M Y Y Y Y

23. Do you have any underlying illness or condition at the moment? Y ☐ N ☐
Note: check patient notes for details if necessary. If no skip to Question 24

Asthma	Y	N	Other chronic lung diseases e.g bullous 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	Kwashiokor/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					
Does the patient live in an institution/care facility	Y	N	If yes name institution					

24. Has the patient been admitted to hospital in the last 12 months? (prior to this admission) Y ☐ N ☐ Unknown ☐
Note: If no or unknown skip to Q25

24.1What was the date that you were discharged from your last hospital admission? D D M M Y Y Y Y
Note: please fill in 01 for the day if the patient does not know the day(must complete month & year)

24.2 Was the patient admitted more than once in the past year? Y ☐ N ☐ ***Note: If no skip to Q 25***	24.3 If yes how many times? _____

Note: if patient ≥5 skip to 26
25. Did the mother have an HIV test during pregnancy? Y ☐ N ☐ unknown ☐

Note: If no/unknown skip to Q26

25.1 If yes what was the result? Positive ☐ Negative ☐ Unknown ☐
Note: If no/unknown skip to Q26

25.2 What is the source of the results? Verbally from the mother ☐ RTHC ☐ other ☐ specify_____

26.Has the patient been tested for HIV prior to this admission: Yes ☐ No ☐ Unknown ☐

Note: if no/ unknown skip to Q27

26.1 if yes, what was the result? Positive ☐ Negative ☐ Unknown ☐

Note: if negative/ unknown skip to Q27

26.2 if positive, date of diagnosis? D D M M Y Y Y Y

26.3 Currently on ART? Y ☐ N ☐ Unknown ☐	26.3.1 If yes date of initiation of ART D D M M Y Y Y Y
26.4 Bactrim prophylaxis taken currently? Y ☐ N ☐ Unknown ☐	
26.4.1 If yes, how long have you taken Bactrim? _____ Years_____ Months_____ Weeks_____ Days	
27. TB treatment in the last year? Y ☐ N ☐ ***Note: If no skip to question 28***	27.1 if yes date TB treatment initiated D D M M Y Y Y Y
27.2 Are you still taking TB treatment? Y ☐ N ☐ Unknown ☐	27.3 If no, date TB treatment stopped D D M M Y Y Y Y
28. Has the patient been prescribed and taken antibiotics in the 24 hours before this admission? Y ☐ N ☐ Unknown ☐ ***Note: If no/unknown skip to Q 29***	
28.1 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 Cotrimoxazole, VAN vancomycin. If other please specify	

29 **Vaccination history, complete for Children <5 , if aged ≥ 5 years skip to Q 30**

29.1 Was the RTHC seen Yes ☐ No ☐

Note: If no skip to 30

29.2. 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
DTP + HIB vaccine	6 weeks	Y	N		D D M M Y Y Y Y
	10 weeks	Y	N		D D M M Y Y Y Y
	14 week	Y	N		D D M M Y Y Y Y
	18 months	Y	N	N/A	D D M M Y Y Y Y
DTP	18 Months	Y	N	N/A	D D M M Y Y Y Y
***S. pneumoniae* conjugate vaccine(PCV_7/Prevnar)**	6 week	Y	N		D D M M Y Y Y Y
	14 weeks	Y	N		D D M M Y Y Y Y
	9 months	Y	N		D D M M Y Y Y Y
Measles	9 months	Y	N		D D M M Y Y Y Y
	18 months	Y	N		D D M M Y Y Y Y

Rotavirus vaccine	6 weeks	Y	N	D D M M Y Y Y Y
	14 weeks	Y	N	D D M M Y Y Y Y

30. Children aged five and older and adults

Vaccine	**Dose given**			**Date given**	**Date unknown**
Pneumococcal Vaccine (pneumovax)	Y	N	UNK	D D M M Y Y Y Y	☐

31.Did the patient receive an influenza vaccine in the past 12 months ?

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

32. Antibiotics prescribed during this admission Y ☐ N ☐ Unknown ☐
If yes complete the table below, if no End

	PO	IVI	IMI	Date started (enter date prescribed)		PO	IVI	IMI	Date started
Amoxicillin				D D M M Y Y Y Y	Ampicillin				D D M M Y Y Y Y
Augmentin				D D M M Y Y Y Y	Cefotaxime				D D M M Y Y Y Y
Ceftriaxone (Rocephin)				D D M M Y Y Y Y	Cefuroxime (Zinnat)				D D M M Y Y Y Y
Ciprofloxacin (Ciprobay)				D D M M Y Y Y Y	Clindamycin				D D M M Y Y Y Y
Cotrimoxazole (Bactrim)				D D M M Y Y Y Y	Doxycycline				D D M M Y Y Y Y
Erythromycin				D D M M Y Y Y Y	Gentamycin				D D M M Y Y Y Y
Penicillin G				D D M M Y Y Y Y	Vancomycin				D D M M Y Y Y Y
Ampiclox				D D M M Y Y Y Y	Metronidazole				D D M M Y Y Y Y
Other Specify									

QC Performed by	☐☐☐ Date: ☐☐/☐☐/☐☐☐☐

Appendix 2: Case investigation form for the ILI study

NATIONAL HEALTH LABORATORY SERVICE
NATIONAL INSTITUTE FOR COMMUNICABLE DISEASES

Influenza-Like Illness (ILI) 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: ☐☐ ILI/Controls Study ID: _____ Date completed: ☐☐/☐☐/☐☐☐☐ (DD/MM/YYYY)

Patient seen at: Jouberton Clinic ☐ Edendale Gateway Clinic ☐

Note: surveillance oficer to review criteria for all case definitions before making a decision about the case definition met.

ILI ☐ Control ☐

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. Presenting complaint: (Tick all that apply) Sore throat ☐ Runny nose ☐ Headache ☐ Cough ☐ Fever ☐ Myalgia ☐ Other ☐ (Specify) _____
8. Date of onset of symptoms: ☐☐/☐☐/☐☐☐☐

Note: Complete the signs and symptoms at the time of the interview. Some of the signs or symptoms may have resolved by the time you interview the patient.

9. Maximum recorded temperature at time of interview ____.____C° (##.#C°) Temperature not recorded ☐
10. History of fever? Yes ☐ No ☐ Unk ☐ If yes, date of fever onset: ☐☐/☐☐/☐☐☐☐
11. Respiratory Rate: _____breaths per minute Not recorded ☐
12. Patient length (if not recorded, surveillance oficer to measure): _____._____cm Percentile (if patient < 5

years) _____ Unk ☐

12.1 Patient weight (if not recorded, surveillance oficer to measure): _____._____kg Percentile (if patient < 5 years) _____ Unk ☐

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

13. Mental status of the patient (based on AVPU)

Alert ☐ Disorientated ☐ Stuporous ☐ Comatose ☐
Sedated ☐ Unknown ☐
(responds to verbal commands) (responds to painful stimuli)

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

Cough	Y	N	Sore throat	Y	N	Difficulty breathing	Y	N
Headache	Y	N	Rhinorrhoea	Y	N	Myalgia	Y	N
Diarrhoea (>3 loose stool per day)	Y	N	Symptoms presents for ≤3 days	Y	N	Symptoms present for 4-7 days	Y	N

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

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

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

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

17. Do you currently or have you ever worked in a mine before? Yes ☐ No ☐ Unk ☐

Note: If no or unknown, skip to Q18

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

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

Date

Unknown ☐

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

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

18. Do you have any underlying illness or condition at the moment? Yes ☐ No ☐

Note: If no skip to Q 19

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/Emphys	Y	N

						ema		
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:					

Note: Complete for patients < 5 years, if patient ≥ 5 years skip to Q 20.

19. HIV result during pregnancy (mother of patient): Yes ☐ No ☐ Unk ☐

Note: If no or unknown skip to Q 20

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

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

20. Has the patient been tested for HIV prior to this consultation? Yes ☐ No ☐ Unk ☐

Note: If no or unknown skip to Q 21

20.1 If yes, what was the result? Positive ☐ Negative ☐ Unk ☐ Date of test ☐☐/☐☐/☐☐☐☐ date Unk ☐

Note: If result unknown skip to Q 21

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

Note: If negative skip to Q 21

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

20.4 Bactrim (contrimoxazole/trimethoprim) prophylaxis taken currently? Yes ☐ No ☐ Unk ☐

Note: If no or unknown skip to Q 21

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

21. Has the patient been tested for HIV during this consultation? Yes ☐ No ☐

Note: if no to Q 21 skip to Q 21.5
NB:ALL PATIENTS WHO DO NOT HAVE A CONFIRMED CURRENT HIV STATUS SHOULD BE OFERED AN HIV TEST

21.1 If yes, who requested the test? Surveillance oficer ☐ Clinic staff ☐

21.2 Which test was done? Rapid Test ☐ PCR ☐ Dry Blood Spot (DBS) ☐ ELISA ☐

21.3 What was the test result? Positive ☐ Negative ☐ Pending ☐ Unknown ☐

Note: Complete for patients < 5 years, if patient ≥ 5 years skip to Q 21.5.

21.4 If rapid test was done and results are positive, was a DBS collected for testing at NICD? Yes ☐ No ☐

Note: if yes, report that DBS was collected also on the laboratory slip.

21.5 Based on all the information available to you, what is the current HIV status of the patient Positive ☐ Negative ☐ Pending ☐ Unknown ☐

Note: If current HIV status is positive or pending skip to Q 22

21.6 If current HIV status is negative, what was the source of HIV status? RTHC ☐ Laboratory report ☐ Medical records ☐ Verbal ☐ Other ☐ Specify _____

Note: Please ofer an HIV test to all patients >5yrs old with a negative result reported verbally or taken >6 weeks before this consultation.

21.7 If current HIV status unknown, why was the patient not tested at this visit? Refused consent ☐ Other

☐specify_____

22. Have you ever taken TB prophylaxis? Yes ☐ No ☐ Unk ☐ If yes, date TB prophylaxis initiated: ☐☐/☐☐/☐☐☐☐

Note: If no or unknown skip to Q 23

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

Note: If yes skip to Q 23

23. TB treatment in the last 12 months? Yes ☐ No ☐ Unk ☐ If yes, date TB treatment initiated: ☐☐/☐☐/☐☐☐☐

Note: If no or unknown skip to Q 24

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

24. Has the patient been prescribed and taken antibiotics in the 24 hours before this interview? Yes ☐ No ☐ Unk ☐

Note: If no or unknown skip to Q 25

24.1 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 _____

25. Vaccination history. Complete for patients < 5 years, if patient ≥ 5 years skip to Q 26

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

Note: If no skip to Q 25.3

25.2 If yes, has the child ever been vaccinated? Yes ☐ No ☐ Unk ☐

Note: Excluding the vaccines given at birth

25.3 Was the Road to Health Card seen? Yes ☐ No ☐ 25.4 Was a copy of the Road to Health Card made? Yes ☐ No ☐

Note: If no skip to Q 26

25.5 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:

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

If pre-term, record gestational age: _____weeks

25.7 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.pneumoniae	Dose 1 (6 weeks)	Y	N		☐☐/☐☐/☐☐☐☐

conjugate vaccine (PCV$_{7/13}$/Prevenar)		Batch N°_____ Unk ☐		
	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 27

26. Did the patient receive pneumococcal polysaccharide vaccine?

Vaccine	**Dose given**			**Date given**	**Date unknown**
Pneumococcal Vaccine (Pneumovax)	Y	N	UNK	☐☐/☐☐/☐☐☐☐	☐

27. 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** ☐

Outcome of the clinic visit: Discharged ☐ Referred to hospital ☐ Died ☐ Unknown ☐

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

Appendix 3: Primers and probes for real-time detection *Bordetella* species

Gene target	Respiratory pathogen	Sequence 5'-3'
IS481-F[a]	*B. pertussis, B. holmesii, B. bronchiseptica*	CAAGGCCGAACGCTTCAT
IS481-R[a]		GAGTTCTGGTAGGTGTGAGCGTAA
IS481-P[a]		FAM-CAGTCGGCCTTGCGTGAGTGGG-BHQ1
h*IS1001*-F[a]	*B. holmesii*	GGCGACAGCGAGACAGAATC
h*IS1001*-R[a]		GCCGCCTTGGCTCACTT
h*IS1001*-P[a]		LC670-CGTGCAGATAGGCTTTTAGCTTGAGCGC-BBQ
p*IS1001*-F[a]	*B. parapertussis*	TCGAACGCGTGGAATGG
p*IS001*-R[a]		GGCCGTTGGCTTCAAATAGA
p*IS1001*-P[a]		HEX-AGACCCAGGGCGCACGCTGTC-BHQ1
ptxS1-F[a]	*B. pertussis, B. parapertussis, B. bronchiseptica*	CGCCAGCTCGTACTTC
ptxS1-R[a]		GATACGGCCGGCATT
ptxS1-P[a]		FAM-AATACGTCGACACTTATGGCGA-PH
RNase P-F[b]	Human gene present in clinical specimens (determines presence/absence of inhibitors)	AGATTTGGACCTGCGAGCG
RNase P-R[b]		GAGCGGCTGTCTCCACAAGT
RNase P-P[b]		LC610-TTCTGACCTGAAGGCTCTGCGCG-BHQ2

[a] References: (26;27;55), [b] References: (54;56).

Appendix 4: ATCC control strains used for validation of real-time PCR

Microorganism	ATCC number
Bordetella pertussis	9797D-5
Bordetella parapertussis	BAA-587D-5
Bordetella bronchiseptica	BAA-588D-5
Bordetella holmesii	51541
Bordetella avium	35086
Bordetella hinzii	51783
Bordetella petrii	BAA-461

Appendix 5: Modified algorithm used for the multi-target real-time PCR assay for the identification of *Bordetella* spp. (26;27)

PCR targets (Ct <45)				Interpretation
IS481	p*IS1001*	h*IS1001*	*ptx* S1	
Positive (Ct <35)	Negative	Negative	Negative/Positive	*B. pertussis* (confirmed)
Positive (Ct≥35)	Negative	Negative	Negative/Positive	*B. pertussis* (probable)
Negative/Positive	Negative	Positive	Negative	*B. holmesii*
Negative	Positive	Negative	Negative/Positive	*B. parapertussis*
Negative	Negative	Negative	Positive	*B. bronchiseptica*

Appendix 6: Composition of 2011, 2012, 2013 and 2014 QCMD panels

2011 QCMD Panel[1]		2012 QCMD Panel[2]		2013 QCMD Panel[3]		2014 QCMD Panel[3]	
BP1101	*B. pertussis*	BP 1201	*B. bronchiseptica*	BP 1301	*B. bronchiseptica*	BP 1401	Negative
BP1102	*B. bronchiseptica*	BP1202	*B. pertussis*	BP 1302	*B. pertussis*	BP 1402	*B. pertussis*
BP1103	Negative	BP1203	*B. pertussis*	BP 1303	*B. hinzii*	BP 1403	*B. pertussis*
BP1104	*B. bronchiseptica*	BP1204	*B. pertussis*	BP 1304	*B. pertussis*	BP 1404	*B. pertussis*
BP1105	*B. pertussis*	BP1205	*B. pertussis*	BP 1305	*H. influenzae*	BP 1405	*B. pertussis*
BP1106	*B. holmesii*	BP1206	*B. pertussis*	BP 1306	*B. pertussis*	BP 1406	*B. pertussis*
BP1107	No DNA	BP1207	*B. pertussis*	BP 1307	*B. pertussis*	BP 1407	*B. pertussis*
BP1108	*H. influenzae*	BP1208	*B. holmesii*	BP 1308	Negative	BP 1408	*B. parapertussis*
BP1109	No DNA	BP1209	*B. bronchiseptica*	BP 1309	*B. bronchiseptica*	BP 1409	*H. influenzae*
BP1110	*B. pertussis*	BP1210	Negative	BP 1310	*B. pertussis*	BP 1410	*B. bronchiseptica*
BP1111	*B.* hinzii	BP1211	*B. parapertussis*	BP 1311	*B. parapertussis*	BP 1411	*B. holmesii*
BP1112	*B. parapertussis*	BP1212	*H. influenzae*	BP 1312	*B. holmesii*	BP 1412	*B. bronchiseptica*

Abbreviations: QCMD=Quality control for molecular diagnostics.
1, 2: Panel provided by Infection control laboratory.
3, 4: Panel provided by QCMD.

Appendix 7: Ethics clearance certificate

R14/49 Miss Fahima Moosa

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M130260

NAME: (Principal Investigator)	Miss Fahima Moosa
DEPARTMENT:	School of Pathology/Division of Virology National Institute for Communiable Diseases
PROJECT TITLE:	Detection of Streptococcus Peumoniae, Haemoophilus Influenzae and Bordetella pertusis in individuals Presenting with Severe Respiratory and Influenza-Like Illness in South Africa
DATE CONSIDERED:	22/02/2012
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr M du Plessis
APPROVED BY:	Professor PE Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	17/04/2013

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**.

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PRIMARY SOURCES

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3	Gao, F., J. C. Mahoney, E. R. Daly, W. Lamothe, D. Tullo, and C. Bean. "Evaluation	<1%

	of a Multi-Target Real-time PCR Assay for Detection of Bordetella Species During a Pertussis Outbreak in New Hampshire 2011", Journal of Clinical Microbiology, 2013. Publication	
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