Determining sequence types of circulating *Bordetella pertussis* **strains isolated from South African infants**

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

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Declaration

I, Bronwan Margaret Tamzen Smith, declare that this dissertation is my own unaided work. It is being submitted for the Degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

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Bronwan Margaret Tamzen Smith

_____day of_____20_____in_____

Presentations arising from this masters research dissertation

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Abstract

There is paucity of pertussis disease data from Africa. This study aimed to molecularly characterise *B. pertussis* strains infecting South African infants to gain knowledge on strains' antigenic and genetic patterns.

Western Blot analysis was done to determine the protein expression of important *B. pertussis* antigens, namely: pertactin, fimbriae serotypes 2/3 and pertussis toxin, in strains isolated in 2015 from hospitalised infants. The 6 clinical isolates tested had expression for all tested proteins.

Additionally, a novel high-throughput single nucleotide polymorphism (SNP) genotyping assay targeting different pertussis toxin promoters (ptxP) was developed and optimised in the Biomark-HD system. We were able to successfully detect 35 recently published SNPs compiled from worldwide whole genome sequencing data. All reactions were effective in amplifying their respective targets in the system. Fifty-two clinical samples positive for *B. pertussis* by PCR collected during two prospective surveillance studies were retrospectively analysed. The surveillance Babies of Soweto study explored pathogen specific etiology associated with lower respiratory tract hospitalisations in children from the Soweto region while PERCH is a multi-site public health research study on the aetiology and risk factors of pneumonia involving children from South Africa. The expansion of strains containing the more virulent ptxP3 allele was observed from the earlier period of2011-2013 (38.1%) to 2014-2015(65.6%); combined with a decrease in the frequency of the less virulent ptxP1 allele (24.1% in2011-2013 vs.14.7% in 2014-2015). Furthermore, 50% of variability of *B. pertussis* strains compared to the reference strain was found in unknown genes.

In conclusion, we observed no deficiencies in the proteins targeted in most acellular vaccines and that the ptxP3 allele is spreading in this population, as described globally in other studies. A novel SNP typing method was established and this tool will be useful for future studies.

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Contents

Nomenclature

% - Percentage

°C- degrees Celcius

ROX - 6-carboxy-X-rhodamine

aP - Acellular pertussis vaccine

ASP - Allele specific primer

ATCC - American Type Culture Collection

BLAST - Basic Local Alignment Search Tool

BoSS - Babies of Soweto Study

C -Cytosine

CHBAH - Chris Hani Baragwanath Academic Hospital

CLF - Control line fluid

cm - Centimetre

CO_2- Carbon dioxide

dH_2O- Distilled water

DNA - Deoxyribonucleic acid

EPI - Expanded Programme on Immunisation

FHA - Filamentous haemagglutinin

fim 2 - Fimbriae serotype 2

fim 3 - Fimbriae serotype 3

G -Guanine

h*IS*1001 – *Bordetella holmesii* insertion sequence 1001

HIV - Human immunodeficiency virus

HREC - Human Research Ethics Committee

HRP - Horseradish peroxidase

IFC - Integrated fluidic circuit

IS - Induced sputum

IS481 - Insertion sequence 481

kDa -Kilodalton

LMICs - Low and middle-income countries

LSP - Locus specific primer

mA -Milliamp

ml - Millilitre

MLVA - Multiple-locus variable-number tandem repeat analysis

MTA-Seq - Multiplex PCR targeted amplicon sequencing

nL -Nanoliter

NP - Nasopharyngeal

OD - Optical density

PBS - Phosphate buffered saline

PCR - Polymerase chain reaction

PCR-RFLP - Polymerase chain reaction-restriction fragment length polymorphism

PERCH - Pneumonia etiology research for child health

PFGE - Pulsed-field gel electrophoresis

pH - Per hydrogen

p*IS*1001 –*Bordetella parapertussis* insertion sequence 1001

PRN - Pertactin

PT - Pertussis toxin

ptxP - Pertussis toxin promoter

ptxP1 - Pertussis toxin promoter P1

ptxP15 - Pertussis toxin promoter 15

ptxP18 - Pertussis toxin promoter 18

ptxP4 - Pertussis toxin promoter 4

ptxP6 - Pertussis toxin promoter 6

ptxS1 - Pertussis toxin subunit 1

PVDF - Polyvinylidene difluoride

qPCR - Quantitative polymerase chain reaction

RIPA - Radioimmunoprecipitation assay

RMPRU - Respiratory and Meningeal Pathogens Research Unit

rpm - Revolutions per minute

RSV - Respiratory syncytical virus

S - Subunit

SDS - Sodium dodecyl sulfate

SDS-PAGE - Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SNP - Single nucleotide polymorphism

STA - Specific Target Amplification

STGG - Skim-milk, tryptone, glucose and glycerine broth

TEMED - Tetramethylethylenediamine

UTM - Universal transport media

V - Volt

wP - Whole cell pertussis

µL - Microliter

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CHAPTER 1: INTRODUCTION

1.1 Pertussis

Bordetella pertussis (B. pertussis) is a Gram-negative encapsulated coccobacillus that causes a highly contagious acute respiratory infection known as whooping cough (pertussis) in humans(1). *B. pertussis* is transmitted via aerosolized droplets from an infected person coughing and/or sneezing (2). Pertussis disease is characterised by three distinct phases, namely: the catarrhal phase, which lasts for 1-2 weeks; the paroxysmal phase, which lasts for several weeks and the convalescent phase, which lasts for 2-3 weeks (3). The catarrhal phase is the most contagious phase and presents with symptoms that are indistinguishable from minor respiratory tract infection such as coughing, fever and runny nose (4), while the paroxysmal phase includes the characteristic cough or intermitted bursts of cough with a 'whoop' on inspiration. During the convalescent phase, paroxysms of cough wane in number and severity (4), however, the severity of symptoms can be worsened by co-infections with other respiratory pathogens such as Respiratory syncytical virus (RSV) and influenza virus during this final stage of pertussis infection (5). Although pertussis infection can be acquired at any age (6), people who are not immune to the bacterium through pervious vaccination or infection are at increased risk(7). Infants <6 months of age are most likely to develop complications (6), this is because of their developing immune systems which causes these infants to be more vulnerable to infectious diseases (8). Furthermore, the clinical presentation of severe pertussis in young infants includes presenting with apnea, seizures and acute pneumonia while pertussis disease in older children and adults is atypical. Other individuals at increased risk of disease include unvaccinated or partially vaccinated children (9, 10) as well as previously vaccinated older children, adolescents and adults whose immunity has waned (7).

1.2 Burden of pertussis disease in Africa

It has been estimated that in 2014 there were 24.1 million pertussis cases(11)it has also been estimated that there were 54 000 deaths (uncertainty range: 53 000-60 000) in 2015 in children aged 1-59 months (12). The African region was the largest contributor with 7.8 million pertussis cases and 92 500 deaths (11). In low and middle-income countries (LMICs), especially Africa, surveillance for pertussis disease is poor and thus the epidemiology of the disease has been difficult to characterise (13). Table 1 shows a summary of studies from 1984-2015 describing the burden of pertussis disease in different African countries including

pertussis detection rate in a certain study population, case fertility rate and HIV status of pertussis positive cases. In South Africa the true burden of pertussis infection is still unclear as many cases are not recognised or reported.

Table 1.1: Studies assessing the burden of pertussis disease in Africa

Country	Study date	Study population	Type of clinical sample	Patients' age	Pertussis detection rate	Vaccination status	Case fatality rate	HIV-infection status of pertussis positive cases
South Africa (Bloemfontein) Reference: Hallbauer *et al*, 2016 (14)	April 2008 – March 2015	1259 hospitalised children with suspected pertussis	Nasal and nasopharyngeal swabs	Patients diagnosed with pertussis: 0 - 28 days 9 (4.9), 29 - 42 days 21 (11.5), 43 - 70 days 37 (20.2), 71 - 98 days 27 (14.8),99 days	183 / 1259 (14.5 %)	NR[1]	5.2 %	HIV infected = 1.3% (2 / 154, patients with available data)
South Africa (Johannesburg) Reference: Soofie *et al*, 2016 (15)	January 2015 – December 2015	1839 infants hospitalised with lower respiratory tract infection	Nasopharyngeal swabs	<12 months of age	42 / 1839 (2.3%)	Of the cases: DTaP[2]dose 1 at 6 weeks n = 18 DTaP dose 2 at 10 weeks n = 6 DTaP dose 1 at 14 weeks n = 2	5% in the <6 months old	HIV exposed = 16 / 1839; (0.9%)

[1]NR: Not reported

[2] Pentavalent vaccine containing diphtheria toxoid, tetanus toxoid, acellular pertussis, trivalent inactivated polio vaccine, and Haemophilus influenza type b-conjugate vaccine

Table 1.1: *continued*

Country	Study date	Study population	Type of clinical sample	Patients' age	Pertussis detection rate	Vaccination status	Case fatality rate	HIV-infection status of pertussis positive cases
South Africa (Cape Town) Reference: Muloiwa *et al*, 2016 (16)	September 2012 – September 2013	460 children hospitalised with lower respiratory tract infection	Nasopharyngeal swabs and induced sputum samples	<13 years of age	17 / 460 (3.7%)	369 / 460 (80.2%) received full schedule of acellular pertussis vaccine	None	HIV infected = 19, (15.8%) positive for *B. pertussis*
Algeria Reference: Benamrouche *et al*, 2016 (17)	February 2012 – September 2013	248 patients with clinically suspected pertussis	Nasopharyngeal aspirates and nasopharyngeal swabs	Median age of patients with pertussis – 3 months	134 / 248 (54%)	104 / 134 (77.6%) patients positive for *B. pertussis* were unvaccinated	NR	NR
Zambia Reference: Gill *et al*, 2016 (18)	March 2015 – November 2015	1981 infants with clinically suspected pertussis	Nasopharyngeal swabs	Median age of patients with pertussis – 6 months	10 / 1981 (0.5%)	4 / 10 (40%) infants positive for *B. pertussis* were unvaccinated	None	NR

[1]NR: Not reported

[2] Pentavalent vaccine containing diphtheria toxoid, tetanus toxoid, acellular pertussis, trivalent inactivated polio vaccine, and *Haemophilus influenza* type b-conjugate vaccine

Table 1.1: *continued*

Country	Study date	Study population	Type of clinical sample	Patients' age	Pertussis detection rate	Vaccination status	Case fatality rate	HIV-infection status of pertussis positive cases
Niger Reference: Juso *et al*, 2014 (19)	December 2010 – April 2011	342 children with respiratory symptoms	Nasopharyngeal aspirates	<5 years	4 / 342 (1.17%)	201 / 342 (58.8%) vaccinated with 3 doses of DTP[2]	NR	NR
Uganda Reference: Kayina *et al*, 2015 (20)	July 2013 – December 2013	449 children with persistent cough ≥14 days enrolled and evaluated for pertussis	Whole blood and nasopharyngeal swabs	3 months – 12 years	68 / 449 (15%)	61 / 449 (13.6%) received full schedule of acellular pertussis vaccine	NR	NR
Gambia Reference: Scott *et al*, 2015 (21)	2008	1067 individuals included in a hepatitis B virus serosurvey (retrospective analysis of serosurvey samples available)	Serum samples	≥ 2.2 years – 90 years	NR	NR	2.8%	NR

[1]NR: Not reported

[2] Pentavalent vaccine containing diphtheria toxoid, tetanus toxoid, acellular pertussis, trivalent inactivated polio vaccine, and *Haemophilus influenza* type b-conjugate vaccine

Table 1.1: *continued*

Country	Study date	Study population	Type of clinical sample	Patients' age	Pertussis detection rate	Vaccination status	Case fatality rate	HIV-infection status of pertussis positive cases
Senegal Reference: Préziosi *et al*, 2002(22)	January 1984 – December 1996	31 797 children living in Niakhar village southeast of Senegal	NR	<15 years of age	First outbreak (1986): 127.3 incidence per 1000 person years at risk Second outbreak (1990): 93.5 incidence per 1000 person years at risk Third outbreak (1993): 68.9 incidence per 1000 person years at risk	13 264 / 31 797 (41.7%) received at least one pertussis vaccine dose 9202 / 31 797 (28.9%) received at least 3 pertussis vaccine doses 127 / 31 797 (0.4%) received more than 3 pertussis vaccine doses	Infants <6 months of age: 1.7 per 1000 person years in 1993	NR

[1]NR: Not reported

[2] Pentavalent vaccine containing diphtheria toxoid, tetanus toxoid, acellular pertussis, trivalent inactivated polio vaccine, and *Haemophilus influenza* type b-conjugate vaccine

1.3 Structure of *B. pertussis*

The genome of *B. pertussis* consists of a circular chromosome with 4 086 189 base pairs and an average guanine (G) and cytosine (C) content of 67.3% (23). The surface of *B. pertussis* is composed of three main components, namely: the outer membrane, the inner membrane and the periplasmic space with a thin peptidoglycan layer in between; Figure 1 (24). The outer membrane contains lipopolysaccharide (LPS) endotoxins that are unlike those from other Gram-negative bacteria in that they contain two forms of LPS differing in its phosphate composition of the lipid portion(25). *B. pertussis* genome also contains a number of virulence factor genes, with particular importance to this study examples of virulence factors are: pertussis toxin (PT), pertactin (PRN) and fimbriae serotypes 2 and 3 (fim2 and fim3); Table 1.2. Due to their role in pathogenesis and induction of immunity, PT, PRN, fim2/fim3 and filamentous haemagglutinin (FHA) constitute candidate antigens that are included in most acellular pertussis (aP) vaccines (26).

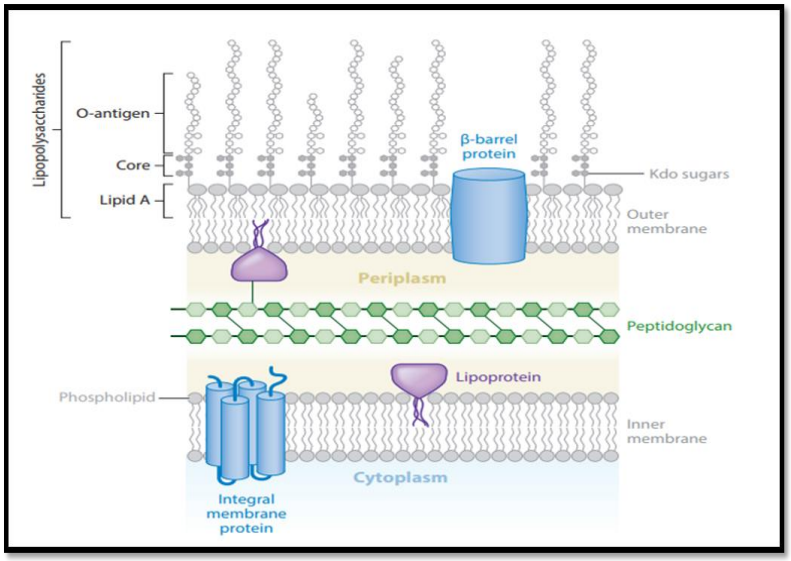


Figure 1.1: Surface of Gram-negative bacteria (from Henderson, *et al* (24))

Table 1.2: *Bordetella pertussis* antigens included in acellular pertussis vaccines

Virulence factor	Molecular weight (kDa)	Function
Pertussis toxin (subunits S1-S5)	S1: 26 S2: 21 S3: 21 S4: 12 S5: 11 Reference: (27)	Suppression of host immune and inflammatory responses Reference: (28, 29)
Pertactin	69 Reference: (7)	Resistance of neutrophil clearance Reference: (30, 31)
Fimbriae	Fim2: 22.5 Reference: (32) Fim3: 22 Reference: (33)	Allows bacteria to adhere to host tissue Reference: (34)
Filamentous haemagglutinin	220 Reference: (35)	Adhesion to host respiratory epithelium cells Reference: (35)

1.3.1 Pertussis toxin

The most complex virulence factor expressed by *B. pertussis* is PT (36). Pertussis toxin consists of five different subunits (S1-S5) and two functional moieties (A and B) (36, 37). The B moiety is responsible for the binding of the toxin to receptors of eukaryotic cells (37), thereafter the enzymatically active S1 can enter eukaryotic cells (38, 39). It has been shown that the toxin can bind to proteins of 43kDa (40) as well as 165 kDa (41); in effect any mammalian cell is sensitive to PT (42). This cytoplasmic protein is actively secreted through the cell envelope by a secretion system called Pt1 (43). One crucial role of PT is the modulation of host immune responses (36). When PT is not expressed due to mutations for example (44), patients experience milder pertussis symptoms such as a lower frequency of coughing spasms (45, 46). A commonly used target for deoxyribonucleic acid (DNA) based pertussis diagnosis is the pertussis toxin promoter (*ptxP*) region (47). Multiple *ptxP* alleles have been identified globally (48). The *ptxP1* allele was the most prevalent allele in *B. pertussis* strains until the 1990s, after which the *ptxP3* strains gradually began to replace the *ptxP1* strains (49). Strains with the *ptxP3* allele have been shown to have increased PT production (48, 50-52).

1.3.2 Pertactin

Pertactin is an autotransporter produced by *B. pertussis* (31) and is a surface protein that allows the bacteria to attach to the host respiratory epithelial cells (36) and confers resistance to neutrophil mediated clearance (31). Pertactin consists of proline-rich regions as well as leucine rich repeats which makes the binding to eukaryotic cell possible (53). In regions where PRN is a vaccine immunogen, molecular analysis of circulating *B. pertussis* strains revealed a range of mutations in the *prn* gene resulting in strains lacking PRN expression (54-62). In a study conducted in 8 states in the United States from May 2011- February 2013 where the vaccine formulation used includes PRN, 640/753 (85%) of strains isolated were PRN deficient; the study also found a 2-4 fold greater odds of fully vaccinated patients being infected with a PRN deficient strain compared to unvaccinated or not completely vaccinated patients (63).

1.3.3 Fimbriae

Fimbriae is an attachment pilus composed of subunit 2 or 3 (36), both containing heparin binding activity enabling attachment to the extracellular matrix of epithelial host cells (64). These adhesins are found on the surface of the bacterium enabling the bacterium to adhere to host cells (65).

1.3.4 Filamentous haemagglutinin

Filamentous haemagglutinin (FHA) is a large surface exposed and secreted protein composed of beta helices, this protein is highly immunogenic (66-68). FHA plays a role in adhesin to airway mucosa during the early stages of infection (69) as well as influencing the development of an immune response mounted by a pertussis infection (70).

1.4 Mechanism of infection

B. pertussis infects the human host by attaching to the cilia of the cells lining the upper respiratory tract system(71). Once anchored the bacterium releases PT and adenylate cyclase toxins which paralyse the cilia (72, 73). Adenylate cyclase toxin affects human cells that come into contact with the bacterium as this toxin is a bacterial surface protein (71). When neutrophils and monocytes are exposed to adenylate cyclase toxin, processes such as chemotaxis, phagocytosis and microbial killing are inhibited (74).

1.5 Human immunodeficiency virus infection and *B. pertussis*

The burden of human immunodeficiency virus (HIV) infection in South Africa is high, with an estimated 7.1 million people being infected in 2017 and of those, 21.2% being women of child bearing age (75). Data of the impact of HIV infection on pertussis illness is lacking. To our knowledge only 3 pertussis studies, all from South Africa, included HIV-infected patients. The first of these studies was conducted in Cape Town, and *B. pertussis* was identified by PCR in 7% of children <13 years of age hospitalised with lower respiratory tract infection (LRTI).

In this study identification of *B. pertussis* in children hospitalised for LRTI was slightly more common among HIV-infected (15.8%) and HIV-exposed but uninfected infants (10.9%) than HIV-unexposed infants (5.4%) children (16).

A study conducted in Soweto involving children <12 months of age hospitalised with signs of respiratory illness from January to December 2015, estimated that the incidence for hospitalisation of pertussis-associated disease was marginally higher in HIV-exposed children [2.9 cases/1000 children (95%CI: 1.8–4.5)] compared to the HIV-unexposed group [1.9 cases/1000 children (95%CI: 1.3–2.6)]; albeit not significant. The two pertussis-associated deaths in this study were HIV-exposed children (15). In another cohort study in Soweto which enrolled 188 HIV-exposed and 1028 HIV-unexposed infants who were followed for any respiratory illness during the first 24 weeks of life, the incidence of pertussis associated respiratory illness were7.4 and 5.5 episodes per 1000infant-months (p=0.47), respectively(76).

1.6 Prevention and treatment of *B. pertussis*

1.6.1 Antibiotics

The antibiotic of choice for the treatment and post-exposure prophylaxis of pertussis is erythromycin (77). Despite the effectiveness of antibiotics in reducing the infectious period of *B. pertussis*, they are unlikely to alter the course of the illness unless taken in the early stages of the disease (78). Erythromycin has been shown to reduce pertussis disease duration and severity if given during the catarrhal phase (79, 80). Even though studies demonstrating antibiotic resistance by *B. pertussis* isolates are rare (81, 82), the possibility of antimicrobial resistance combined with the ineffective timing of antibiotic treatment has led to an interest in

preventive strategies such as vaccination to optimise the health of infants as well as other individuals at increased risk of developing complications from pertussis infections (83).

1.6.2 Whole cell pertussis vaccines

Whole cell pertussis (wP) vaccines were the first pertussis vaccines to be developed and were licensed for use in the African region in 1964 (84) and as early as 1914 in the United States (85)(South Africa switched to the acellular vaccine in 2009). These whole cell vaccines consist of heat killed or formalin inactivated *B. pertussis* organisms (86) with the immune response thus being directed against a large number of *B. pertussis* antigens (87). Since the introduction of wP vaccines, there was a notable decrease in pertussis cases, as seen in the United States in Figure 1.2 as well as in South Africa (Gauteng) after aP vaccination; Figure 1.3.

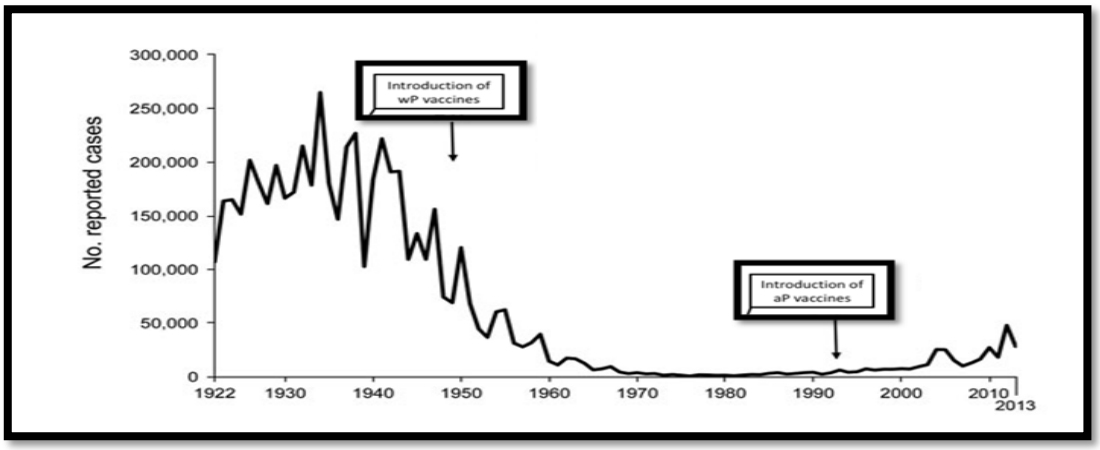


Figure 1.2: Reported pertussis cases from the United States of America from 1922-2013 after the introduction of whole cell and acellular pertussis vaccines (adapted from Skoff e*t al* (88))

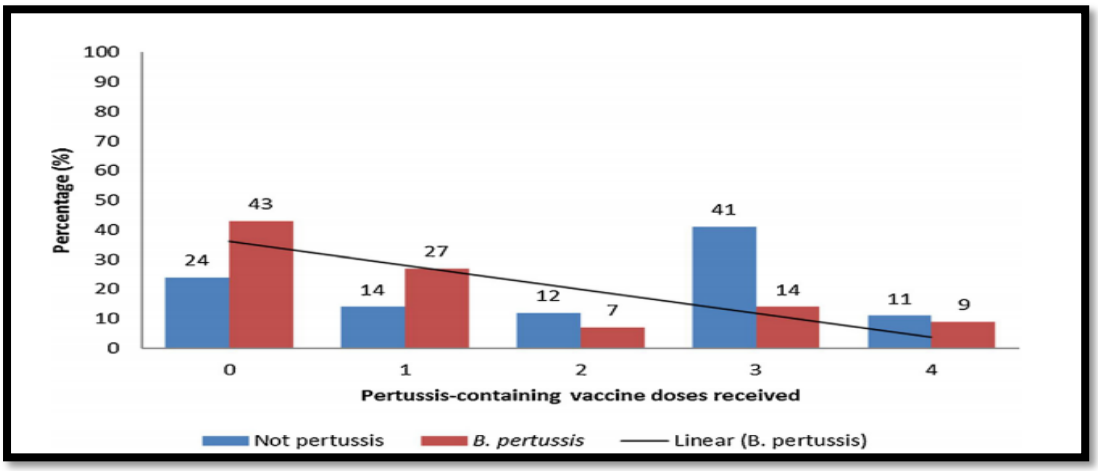


Figure 1.3: Vaccine dose percentage received in non-pertussis and pertussis groups as well as the decline in *B. pertussis* cases to acellular pertussis vaccine doses (from du Plessis *et al* (89))

While these vaccines are easy to produce at low cost and are effective in preventing whooping cough in infants as young as 6 weeks old (90), wP vaccines may have several adverse effects including effects at the injection site leading to limb swelling, abnormal continual crying as well as seizures (91, 92). Due to the adverse effects of the wP vaccines, aP vaccines were then developed (93).

1.6.3 Acellular pertussis vaccines

Acellular vaccines consist of one to five purified proteins namely: FHA, fim (2 or 3), PRN and/or PT in different combinations (94, 95), and thus, immunity is induced only against these selected antigens (96). Table 1.3 shows examples of the different aP vaccine components used in different parts of the world.

Table 1.3: Candidate antigens included in acellular pertussis vaccines around the world (adapted from Halperin1996, (97) unless otherwise stated)

Antigen included in acellular vaccine production	**Pertussis toxin**	**Filamentous haemagglutinin**	**Pertactin**	**Fimbriae**
Region				
Denmark Manufacturer: Statens Serum Institut Reference: (98)	✓			
Germany (Erlangen) Manufacturer: Wyeth-Lederle	✓	✓	✓	✓
Sweden (Stockholm) Manufacturer: Connaught Ltd	✓	✓	✓	✓
Italy Manufacturer: Chiron-Biocine	✓	✓	✓	
South Africa Manufacturer: Sanofi Pasteur Reference: (99)	✓	✓		
West Africa (Senegal) Manufacturer: Pasteur-Merieux	✓	✓		
United States of America(Wisconsin) Manufacturer: GlaxoSmithKline Reference: (100)	✓	✓	✓	
Australia Manufacturer: Boostrix Reference: (101)	✓	✓	✓	

Even though aP vaccines have significantly less adverse effects, studies have shown that protection with aP vaccine is less enduring compared to the wP vaccine (102) also, age-related waning of protective immunity has been observed with the aP vaccines (103-106).

South Africa transitioned to the aP vaccine in 2009. Even though vaccines are available for more than half a century, pertussis remains a health problem throughout the world (107).

1.6.4 Waning immunity

Higher levels of memory B cells have been observed in older age groups after accumulating exposures to circulating *B. pertussis* strains and/or greater number of vaccine doses (108). High levels of T cells, memory B cells and antibodies are raised shortly after natural exposure to *B. pertussis* or vaccination which provides effective immune protection (109). Immunological memory is, however, short lived regardless of pertussis infection or vaccination (109), with aP vaccines inducing an even shorter immunological memory (110-112).

Due to waning immunity, booster vaccinations with aP vaccines have been introduced in several countries in order to improve vaccine effectiveness and to decrease pertussis morbidity (113, 114).

1.7 Pathogen evolution

It has been hypothesised that the change from wP to aP vaccines is a major driver for *B. pertussis* evolution (115). In several countries with high aP vaccination coverage, circulating *B. pertussis* strains have lost the ability to produce certain antigens included in the vaccines used in those regions(116). In countries where PRN is included in the aP vaccines, PRN deficient strains have been isolated; these countries include: France (62), United States of America (117), the Netherlands and Norway (118), Japan (56) and Australia (119). The emergence of these strains may be the result of natural evolution or adaptation to the host immunological milieu induced by aP vaccination (116). Also, these strains are thought to be less susceptible to vaccine induced immunity in comparison to strains circulating during the wP vaccine era (114, 120). It has been hypothesised that the change from wP to aP vaccines is a major driver for *B. pertussis* evolution (115).

B. pertussis has undergone structural evolution including gene deletions and gene rearrangement causing circulating strains to be highly structurally diverse (121). The

differences observed in circulating strains are thought to be associated with adaptation to the mammalian host and immune pressure (122).

This evolution raises concern about the ability of current aP vaccines to adequately control pertussis disease (115). Discrete changes in circulating strains are also evident at the level of single nucleotide polymorphisms (SNPs) (121).

1.8 Detection of *B. pertussis*

1.8.1 Culture for *B. pertussis*

Culture of nasopharyngeal (NP) specimens or respiratory secretions is the gold standard for *B. pertussis* detection and is useful for uncovering emerging antigenic variants and resistant strains(95); however, the sensitivity of the culture methods is affected by several factors including antibiotic treatment, patient age and/or immunisation status(123, 124).

Also, culture is not suitable for diagnosis due to the long time needed to obtain results (125). Further, clinical samples taken three weeks or longer after the onset of infection or in the paroxysmal phase are rarely positive and may yield false negatives (4).

1.8.2 Polymerase chain reaction

Polymerase chain reaction (PCR) assays have recently been developed to detect *B. pertussis* directly from clinical specimens in order to overcome some of the shortcomings of culture and have become an increasingly important diagnostic tool in which multiple respiratory organisms can be detected from a single clinical specimen, with an increased sensitivity compared to standard culture methods (126). Several studies have illustrated the usefulness of PCR for *B. pertussis* detection (127-129), the accuracy of the PCR method is however, also dependent on the timing of specimen collection and the gene targets used for detection (130).

1.9 Detection of *B. pertussis* virulence factors: Western Blot analysis

Western Blot analysis has been used to detect *B. pertussis* proteins directly from clinical specimens by separating them according to their molecular weight through gel electrophoresis

(131) and is thus a commonly used tool for studying clinical isolates for *B. pertussis* virulence factor production (132).

Bordetella pertussis, the bacterium responsible for the highly infectious vaccine preventable disease known as whooping cough (pertussis) (133), expresses a range of virulence factors that have immunomodulating properties (96). The different virulence factors are important in order for the bacterium to establish effective infection within the human host (134). The main virulence factors that are commonly included in the current pertussis vaccines are: pertussis toxin (PT), pertactin (PRN), fimbriae serotype 2 or 3 (fim2/3) and filamentous haemagglutinin (FHA) (26). The main pertussis vaccines used in South Africa during the study period were Pentaxim and Hexaxim (Sanofi Pasteur) that include PT and FHA as pertussis antigens in their composition. Bacterial genetic adaptation in the loci of these proteins may introduce mutations and cause interruptions in protein expression (135). Monitoring antigenic mutations in circulating *B. pertussis* strains is important, as alterations in protein expression may affect bacterial virulence and pathogenesis, alter the severity of the disease and modify bacterial fitness (60, 135). This has been observed with the increased production of pertussis toxin due to the replacement of pertussis toxin promoter P1 (*ptxP1)* by *ptxP3* (136).

Protein deletion mutants have also been observed, especially for PRN. A study conducted in the United States of America (where the acellular pertussis vaccine formulations contain PRN) from May 2011 to February 2013 analysed 753 respiratory specimens and found that there was a strong association between vaccination and PRN deficiency: pertussis vaccinated patients had a greater likelihood (adjusted odds ratio: 2.2) of being infected with PRN deficient *B. pertussis* strains compared to unvaccinated patients in the same population (63). In terms of fimbriae, an insertion mutation in the *fimD* gene has the ability to abolish the expression of both fim2 and fim3 (137).

1.10 Single nucleotide polymorphisms

Single nucleotide polymorphism (SNP) refers to a nucleotide base change that occurs at a specific position in the genome, whereby one oligonucleotide is substituted for another (138).

SNPs have become genetic markers of choice for disease loci mapping (139), and SNP genotyping is used extensively to study the evolution of bacterial pathogens by measuring the

genetic variations between members of the same species (140). SNPs also have the ability to change gene expression (141).

A commonly used reference strain to compare the gene content of different *B. pertussis* strains is the well characterised TohamaI strain (142), which was originally isolated from a whooping cough case in Japan in the 1950s (143) and has since been used for various genetic studies as it is fully sequenced and well annotated (144). Recently, sequencing of different *B. pertussis* strains has identified various SNPs (145-147) which allows for genetic relationships between strains to be established (148, 149).

For example, SNP analysis showed that strains with SNP profiles associated with the PT promoter P3 (ptxP3) (148) and the PRN allele 2 (prn2) are now predominating worldwide (149), with one study involving 19 countries from 6 continents, identifying 5 414 SNPs when comparing wild-type strains to the TohamaI reference strain (150).The authors suggest that these SNPs may reflect *B. pertussis* physiological changes (150).

SNPs can occur approximately once every 100-300 base pairs and can be detected by a number of different techniques including: polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (151) and multiplex PCR targeted amplicon sequencing (MTA-Seq) (152), however, these methods are expensive, time consuming, labour intensive and require computational resources and skilled personal to analyse and interpret the data (141). Multi-locus sequence typing is a common typing method used to study bacterial pathogens, however; *B. pertussis* is a homogenous species and finding polymorphic sites required for multi-locus sequence typing is difficult (153) Due to limitations of the above mentioned techniques, there is a need for high throughput genotyping platforms (154)able to detect *B. pertussis* SNPs from a broad range of sample types such as cultured isolates, or directly from clinical samples.

1.11 Fluidigm

Fluidigm is a nanofluidic automated PCR system that relies on microfluidic technology using dynamic arrays of integrated fluidic circuits (IFCs). These IFCs contain thousands of controlled valves and interconnected channels in which biological samples and reagents can be automatically mixed in a variety of grid like matrices.

The instrument uses dynamic arrays for PCR, in which a FLEXSix chip allows for 864 PCR reactions (6 portion format: 12 samples X 12 assays) in a single PCR run

(www.fluidigm.co.za). Fluidigm has several advantages over current SNP detection methods in that it allows for a larger number of reactions per plate, thus making it more cost effective and less time consuming. Further, IFCs not only reduces the reaction volume from 10 µl – 20µl down to 10 nl scale, but the technology also allows for increased parallelism and throughput of PCR reactions.

1.12 Study rationale

Data on pertussis disease burden and on the strains circulating in LMICs is lacking due to lack of good surveillance and under-reporting (13) which makes it difficult to review and amend pertussis control policies which is vital for monitoring the disease (155).

While aP vaccines have been introduced in some LMICs as part of the national Expanded Programme on Immunization (EPI) schedules (156), the effectiveness of the vaccine is uncertain due to paucity of robust epidemiological studies(157, 158).

Although extensive characterisation of circulating *B. pertussis* strains has been done in several countries, there is paucity of data from Africa, Muloiwa *et al*, 2018, stated that in Africa 'pertussis is more prevalent among young infants'(13). The burden of pertussis disease in Africa as shown in Table 1.1 shows a range of pertussis detection rates ranging from 0.5% (in Zambia) to 54% (in Algeria). The EPI was launched in South Africa in 1974 and South Africa switched from the wP to the aP vaccine in 2009 (159).

While *B. pertussis* strains lacking expression of protein antigens such as pertactin have been observed in Europe (118), pertactin and pertussis toxin deficiencies have been isolated in France (62) and the United States of America (117). Filamentous haemagglutinin deficient strains have also been isolated in France (55) and fim deficient strains have been isolated in the United States of America (135). These studies were conducted in high income countries; the presence of such mutant strains has not been investigated in South Africa (an upper middle income country) where the EPI program transitioned from using wP to aP vaccines since 2009. In this study, Western Blot analyses were done on *B. pertussis* strains cultured from children who presented with respiratory illness to analyse the protein expression of circulating *B. pertussis*. The *B. pertussis* strains were retrospectively analysed for antigenic and SNP divergence, and analysed in relation to year of isolation, HIV-exposure and vaccination history of the children. To our knowledge, this is the first study to use Fluidigm technology to analyse SNPs in *B. pertussis*.

1.2 Study Objectives

A convenience sampling of available positive Bordetella pertussis NP and IS samples were used to:

1. Characterise the protein expression and antigen profiles by Western Blot of *B. pertussis* strains isolated from hospitalised infants participating in a pertussis surveillance study.

2.Establish a novel nanofluidic based SNP typing method for *B. pertussis* in order to determine the antigenic divergence of South African *B. pertussis* strains to stratify the results according to period of collection, infants' HIV exposure status and vaccination history.

1.3 Ethics

The project entitled: 'Determining sequence type of circulating *Bordetella Pertussis* strains isolated from South African infants' was approved by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand, Johannesburg, South Africa (Reference: M160961).

CHAPTER 2: PROTEIN EXPRESSION IN *BORDETELLA PERTUSSIS* ISOLATES

In molecular biology, Western Blot is a technique that enables researchers to identify a specific protein from a mixture of proteins in cells (131). A specific protein is identified based upon its molecular weight and subsequent interaction with specific antibody on the membrane used for blotting (131). This protein/antibody interaction creates a band on the membrane and can be visualised using an imager (131). Western Blot cell preparation is heavily dependent on: proper handling of specimens and the usage of acceptable reagents such as lysis buffer for example(160). While good quality Western Blot results are dependent on excellent sample preparation, attention to technique, correction usage of detection methods and software for subsequent analysis (160).

In order to separate proteins present in specific biological samples, gel electrophoresis is carried out (161). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a technique used for estimating the molecular weights of proteins (162). Proteins can be characterised into their constituent polypeptide chains by sodium dodecyl sulfate (SDS) (162). Within the gel, proteins have distinctive electrophoretic mobility; this mobility within the gel is used to deduce the molecular weight of each protein (162). After Western Blot analysis these proteins are blotted onto a membrane followed by immunodetection of an antigen / antigens of choice due to an antigen-antibody interaction using the molecular weight of the antigen as a reference (161).

This technique has allowed researches to identify immunogenic *B. pertussis* proteins (163-165).Some of the proteins(for example pertussis toxin (28)) produced by *B. pertussis* have been correlated with virulence (166). Recent isolation of circulating *B. pertussis* strains deficient in vaccine antigens highlights the need for a revision of vaccines (167). In this chapter, we present on the protein expression by *B. pertussis s*trains isolated in 2015 from hospitalised South African infants. We investigated the expression of four proteins, namely: PRN, Fim2, Fim3 and PT by Western Blot.

2.1 Materials and Methods

2.1.1 Study population

Archived nasopharyngeal swab (NP) and induced sputum (IS) samples positive for *B. pertussis* infection confirmed by PCR as well as culture (n=10) collected from hospitalised

infants enrolled in a prospective respiratory disease surveillance study named: Babies of Soweto Study (BoSS) were used in this project (168). The BoSS study was nested in a study aimed to determine the pathogen specific etiology associated with lower respiratory tract hospitalisations in children (168). Legal guardians of infants with symptoms of respiratory illness such as cough, cyanosis or features suggestive of respiratory infection were approached for participation (168).

Enrolled infants had at least one nasopharyngeal swab and one other respiratory specimen (nasopharyngeal aspirate or induced sputum) collected (168).

Detailed demographic as well as clinical information was collected from hospital records and interviews from infant caregivers (168). The BoSS parent study began in November 2014 and is ongoing at the Chris Hani Baragwanath Academic Hospital (CHBAH).

2.1.2 Collection of participant demographics and clinical data

All clinical and demographic data was collected by research nurses through interviews, questionnaires and medical records review. Collected variables included: gender, race, clinical presentation (symptoms), Road to Health Chart (which includes vaccination history), and HIV status of the mother while pregnant.

2.1.3 Collection and transport of samples

Nasopharyngeal swabs (NP) were collected using nylon flocked swabs which were placed in Universal Transport Media (UTM) (Media Mage, South Africa), induced sputum (IS) samples were collected from a sub-set of infants enrolled in BoSS (168). All samples were tested for *B. pertussis* by PCR (168) within 72 hours of collection and were kept at -70°C for long-term storage at the Respiratory and Meningeal Pathogens Research Unit (RMPRU) at CHBAH.

For detection of Bordetella species by culture, IS specimens were plated on charcoal agar (Regan-Lowe agar, Media Mage, South Africa) and an additional NP swab was collected on Regan-Lowe transport media. All of the *B. pertussis* strains were isolated by culturing clinical specimens collected during 2015 and were previously screened for confirmation of *B. pertussis* infection by quantitative PCR (qPCR) for insertion sequence element 481 (IS481) and pertussis toxin subunit 1 (ptxS1), (168); see Appendix B, Tables 1 and 2 for primer sequences (169).

2.1.4 Culture of control strains and clinical isolates

Bacterial culture is used in proteomic studies to obtain enough material to study specific proteins (170, 171). Two types of plates were used for routine culture in this study: charcoal agar plates to successfully isolate Bordetella spp. (172) and blood agar plates to differentiate the alpha haemolytic properties of Bordetella spp.(173).

The American Type Culture Collection (ATCC) 9797 sample, designation HE965805, was used as positive control for *Bordetella pertussis*.

The ATCC provides useful representative strains that can be used as positive controls and provides documentation on these strains (174). All cultures of *B. pertussis* isolates and controls were done in a class II biosafety fume hood.

2.1.4.1 *B. pertussis* control strain

A vial containing 6 lyophilised pellets of ATCC 9797 (*B. pertussis*), a control strain initially isolated from an American man who tested positive for whooping cough in 1946, was purchased from Davies Diagnostics (South Africa) and stored at 4°C. Before use, the vial was brought up to room temperature and sterile forceps were used to remove one pellet. The pellet was then placed in a 1.5 ml micro-centrifuge tube containing 0.5 ml phosphate buffered saline (PBS) (Appendix C, Table 3), pH 7.4, and crushed with a sterile swab. The pellet-PBS homogenised solution was then plated out using a standard streaking method on charcoal agar (Media Mage, South Africa) and 5% blood agar (Media Mage, South Africa), plates were dried in ambient incubator at 37°C for approximately 15 minutes before use. After plating the plates were incubated for 5-7 days in a humid (aerobic) CO_2 incubator at 37°C. *B. pertussis* colonies were grey, shiny, small, round high-domed, convex and smooth on the charcoal agar plates (appearance of colonies was reported on a result sheet, Appendix A). Blood plates were used to test for alpha haemolysis of *B. pertussis* colonies (175).

2.1.4.2 *B. pertussis* clinical isolates

One charcoal plate (Media Mage, South Africa) and one 5% blood plate (Media Mage, South Africa) per clinical isolate were dried in ambient incubator for approximately 15 minutes.

A loopful of *B. pertussis* colonies previously isolated from NP or IS samples and stored in skim-milk, tryptone, glucose and glycerine (STGG) broth was taken and streaked on each of the dried plates.

Plates were incubated for 5-7 days and analysed for colonies with appearance reported on a result sheet; Appendix A. Positive *B. pertussis* colonies were grey, shiny, small, round high-domed, convex and smooth on charcoal agar plates.

2.2 Spectrophotometry

B. pertussis colonies were scraped in 1 ml PBS and heat killed for 30 minutes at 56°C. The bacteria were then diluted in PBS to an OD_{600} of approximately 1.25-1.35 in order to ensure adequate protein expression for Western Blot analysis.

2.2.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

RIPA buffer (ThermoFisher Scientific, United States of America) was used for cell lysis of *B. pertussis* isolates as per manufacturer's instructions. Reducing agent beta-mercaptoethanol (FlukaBiochemika, Germany) was added to 2X Laemmli loading buffer (Bio-Rad, South Africa) at a ratio of 50 µl beta mercaptoethanol per 950 µl loading buffer.

Loading buffer containing beta-mercaptoethanol was added to the lysed protein samples at a ratio of 1:1 (15 µl sample:15 µl loading buffer) for a final loading volume of 30 µl.

For SDS-PAGE analyses, the Mini-PROTEAN Terta Vertical Electrophoresis System (Bio-Rad, South Africa) was used. The Bio-Rad casting stand and frame were assembled as follows: short glass plates (Bio-Rad, South Africa) and spacer plates (Bio-Rad, South Africa) were combined and clamped in a casting frame on a flat surface creating a glass sandwich. The running gel (10% for pertactin and pertussis toxin; 12.5% for fim2/3) and stacking gels (4.5% for all antibodies) were prepared by combining the reagents described in Appendix C, Tables 4 and 5. As soon as Tetramethylethylenediamine (TEMED) (Sigma Aldrich, China) was added to the running gel solution, it was poured into the glass sandwich and allowed to polymerize for approximately 30 minutes. After polymerization of the running gel, TEMED was added to the stacking solution and immediately poured on top of the running gel.

A 10 well gel comb was placed in between the glass sandwich into the stacking gel and the gel was allowed to polymerise for approximately 30 minutes; after polymerisation, the comb was removed.

The loading buffer-protein solutions (30 µl) of each clinical isolate and control were incubated at 95°C for 5 minutes, in parallel 5 µl of Precision PlusProtein Dual Colour Standard marker (Bio-Rad, South Africa) was added to 25 µl loading buffer for a total loading volume of 30 µl. The electrophoresis tank was filled with running buffer (Appendix C; Table 3) to the '2 gel' mark on the tank and 30µl of the marker-loading buffer solution was loaded into the first lane of the gel. The next 9 lanes were each loaded with 30 µl of loading buffer-protein sample from the different isolates and control. A constant voltage of 100V was applied and the protein samples were allowed to move through the gel for approximately 2 hours until the dye front reached the bottom of the gel.

For transfer of proteins from the gel to the polyvinylidene difluoride (PVDF) membrane, the Mini-Trans-Blot Module (Bio-Rad, South Africa) was used. For the blotting procedure, 1X transfer buffer (Appendix C; Table 3) was first chilled to 4°C. The gel holder cassette was then assembled with fibre pads and extra thick blot filter paper sized 7 x 8.4 cm (Bio-Rad, South Africa) being pre-soaked in chilled transfer buffer. First, a fibre pad was placed on the black side of the gel holder cassette. Next pre-soaked filter paper was placed on top of the fibre pad followed by the gel.

Meanwhile a 7 x 8.4 cm PVDF membrane (Bio-Rad, South Africa) was first wet in 100% methanol for 15 seconds and equilibrated in transfer buffer for 20 minutes. After the membrane was placed on top of the gel and pre-soaked filter paper and a second pre-soaked fibre pad were placed on top of the membrane. The sandwich was clamped in between the holder cassette and placed in the blot module, Figure 2.1.

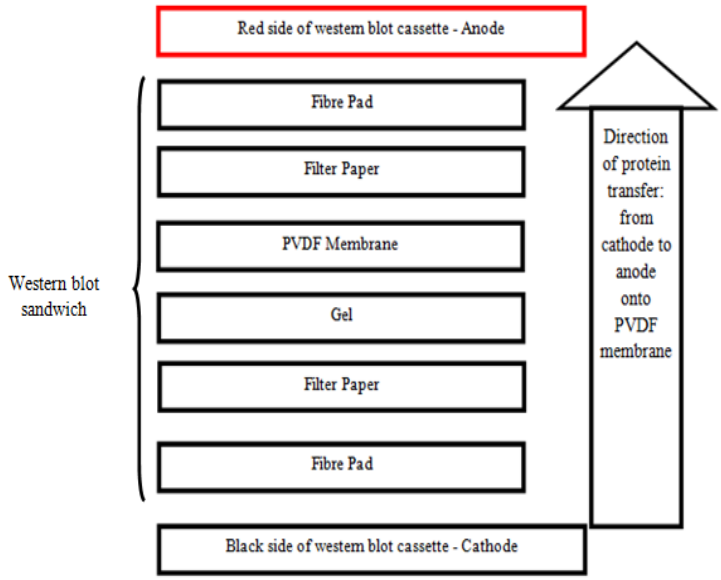


Figure 2.1: Western Blot sandwich inside cassette

The blot module was placed in the electrophoresis tank and a Bio-Ice cooling unit was placed in the tank in order to disperse heat generated from the Western Blot procedure. The tank was filled with transfer buffer (Appendix C; Table 3) to the 'Blotting line' mark. A constant voltage of 35mA was applied and the transfer was allowed to run overnight at 4°C. Following the transfer of proteins to the PVDF membrane, the membrane was washed twice in distilled water (dH_2O).

For visual confirmation of the transfer of proteins from the gel onto the PVDF membrane after Western Blot, the membrane was stained with Ponceau S (Sigma Aldrich, South Africa) solution (Appendix C, Table 3) for approximately 1 minute. Ponceau S staining was removed by rinsing again the membrane in dH_2O.

The membrane was then placed in a tray containing PBS overnight at 4°C, after which was placed in blocking buffer (Appendix C, Table 3) for 1 hour at room temperature on a shaking platform.

2.2.2 Binding of Antibodies

2.2.2.1 Horseradish peroxidase conjugated antibodies

Horseradish Peroxidase (HRP) conjugated antibodies were procured for PRN (Abbexa, United Kingdom), Fim2 (Cusabio, United States of America) and Fim3 (Cusabio, United States of America). Dilutions of 1:10 000, 1:5000, 1:1000, 1:500 and 1:200 were tested for optimisation of antibody dilution. The 1:500 dilution was selected as the optimal dilution for the 3 conjugate antibodies. The conjugated antibodies diluted (1:500) in 5 ml blocking buffer were poured over the membrane for a 2 hour incubation at room temperature. Thereafter the membrane was washed 3 times (5 minutes each) in washing solution (Appendix C, Table 3) on a shaker at room temperature.

2.2.2.2 Unconjugated antibody

The antibody for PT (Abcam, United States of America) was unconjugated. Dilutions of 1:10 000, 1:5000, 1:1000 and 1:500 were tested for optimisation of antibody dilution. The unconjugated antibody was diluted (1:500) in 5 ml blocking buffer and poured over the membrane for 2 hour incubation at room temperature. Thereafter the membrane was washed 3 times (5 minutes each) in washing solution on a shaker at room temperature. The STAR121P HRP-conjugated secondary antibody (Bio-rad, United States of America) was then used for detection. Upon optimisation of secondary antibody dilution (1:10 000, 1:5000 and 1:1000), the antibody was used at 1:10 000 in 10 ml blocking buffer. The secondary antibody solution was poured over the membrane and incubated at room temperature for 1 hour. Thereafter the membrane was washed 3 times (5 minutes each) in washing solution.

2.2.2 Visualisation of protein of interest

Two milliliters of ClarityMax Western ECL substrate (Bio-rad, South Africa) composed of 1 ml Clarity Max Western Peroxide Reagent and 1 ml Clarity Max Luminol Enhancer Reagent was poured on the membrane after the final wash. The membrane was then incubated at room temperature in the dark for approximately 1 minute, and protein signals were analysed using the Bio Rad ChemDoc XRS+Imager System. This imager system utilises a charge coupled device camera to capture chemiluminescent reactions on a PVDF membrane (Gel Doc XR+ and Chem Doc XRS+ Imaging systems with Image Lab Software Instrument Guide, version 6) produced by the ClarityMax Western ECL substrate.

2.3 Chapter two study rationale and aim

Western blot analysis of South African B. pertussis isolates is lacking; South African isolates deficient in *B. pertussis* proteins may not be identified and published which is important to understand the evolution of the pathogen (in a South African context), therefore, the aim of Chapter 2 was to characterise the protein expression and antigen profiles of *B. pertussis* strains isolated from hospitalised infants.

2.4 Results

2.4.1 Culture

Six *B. pertussis* isolates previously recovered from clinical specimens collected during 2015 (168) were used in this study, we were unable to successfully test the other four isolates. These four isolates may have yielded a low protein concentration due to an unsuccessful lysis step. Table 2.1 shows infant demographics from which isolates were used for Western Blot analysis.

Table 2.1: List of samples collected in 2015 from infants enrolled in the pertussis surveillance study: Babies of Soweto used for Western Blot analysis (cultured isolates previously tested positive for *B. pertussis* by PCR)

Patient ID	**Infant age at hospitalisation, in days**	**HIV exposure**	**Number of acellular pertussis doses received by infant**
PET1022	22	Unexposed	0
PET0802	35	Exposed	0
PET1237	49	Exposed	0
PET1651	73	Unexposed	0
PET1650	78	Exposed	2
PET1213	100	Exposed	2

Briefly, all clinical isolates confirmed positive for *B. pertussis* as well as the ATCC 9797 control strain were grey, shiny, small, round high-domed, convex and smooth on the charcoal agar plates; Figure 2.2 and had an OD_{600} of 1.25-1.35; Table 2.2.

Figure 2.2: Growth of positive *Bordetella pertussis* colonies

Table 2.2: OD_{600} readings for cultured clinical isolates used for Western Blot analysis

Clinical isolate	**OD_{600}**
Bordetella pertussis positive control	1.367
PET1213	1.317
PET0802	1.292
PET1237	1.270
PET1022	1.362
PET1651	1.364
PET1650	1.368

2.5 Western Blot membranes

Table 2.3 shows the order of isolates tested by Western Blot for pertactin (Figure 2.3), fimbriae serotype 2 (Figure 2.4) and fimbriae serotype 3 (Figure 2.5) and pertussis toxin (Figure 2.6). Western blot experiments were done at least twice for each antibody.

Table 2.3: Clinical isolates used for Western Blot protein expression analysis

Lane	**Clinical isolate**
1	Molecular weight marker
2	*Bordetella pertussis* positive control
3	PET1650
4	PET1213
5	PET1651
6	PET1237
7	PET1022
8	PET0802

Pertactin is a 69 kDa auto-transporter. The horseradish peroxidase conjugated pertactin antibody, after optimisation, was used at a final dilution of 1:500, all tested isolates showed pertactin protein expression; Figure 2.3.

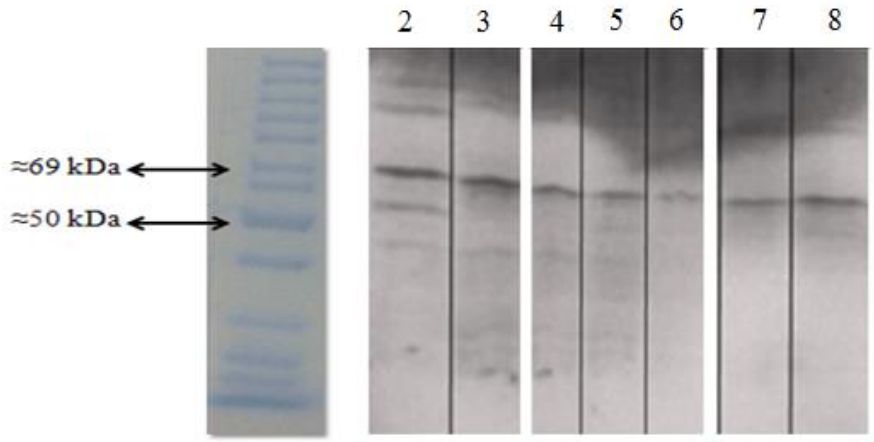


Figure 2.3: Western Blot of pertactin expression in *Bordetella pertussis* control strain and clinical isolates listed in Table 2.3

Fimbriae serotype 2 is a 22.5 kDa adhesin. The horseradish peroxidase conjugated fimbraie serotype 2 antibody after optimisation, was used at a final dilution of 1:500, all tested isolates showed fimbriae serotype 2 protein expression; Figure 2.4.

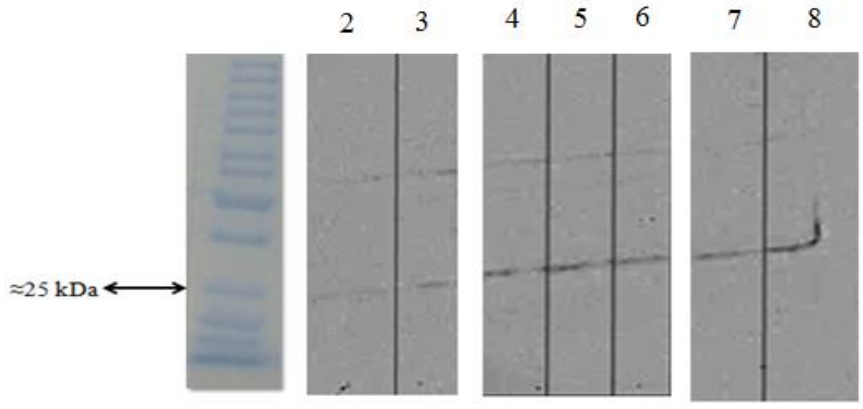


Figure 2.4: Western Blot of fimbriae serotype 2 expression in *Bordetella pertussis* control strain and clinical isolates listed in Table 2.3

Fimbriae serotype 3 is a 22kDa adhesin. The horseradish peroxidase conjugated fimbriae serotype 3, after optimisation was used at a final dilution of 1:500, all tested isolates showed fimbriae serotype 3 protein expression; Figure 2.5.

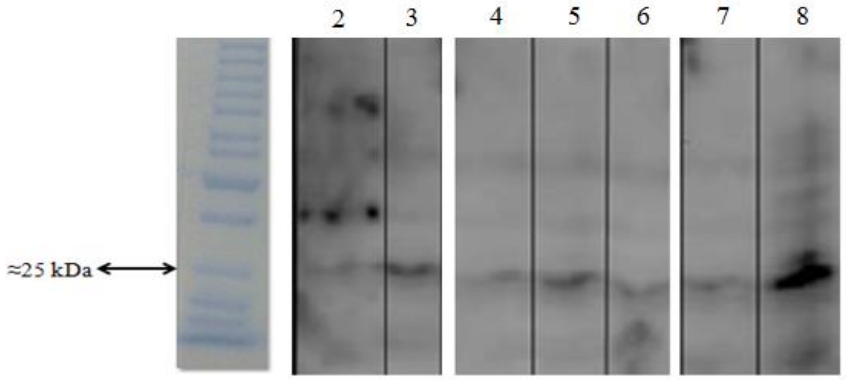


Figure 2.5: Western Blot of fimbriae serotype 3expression in *Bordetella pertussis* control strain and clinical isolates listed in Table 2.3

Table 2.3 shows the order of isolates tested by Western Blot for pertussis toxin.

Pertussis toxin is a 5 subunit toxin. Subunit 1 (S1) is 25 kDa, subunits 2 and 3 (S2 and S3) are 21 kDa and subunits 4 and 5 (S4 and S5) are 12 kDa.

After optimisation, the unconjugated pertussis toxin antibody was used at a dilution of 1:500, while the secondary antibody was used at a dilution of 1:10 000. All tested isolates showed pertussis protein expression of all 5 subunits; Figure 2.6

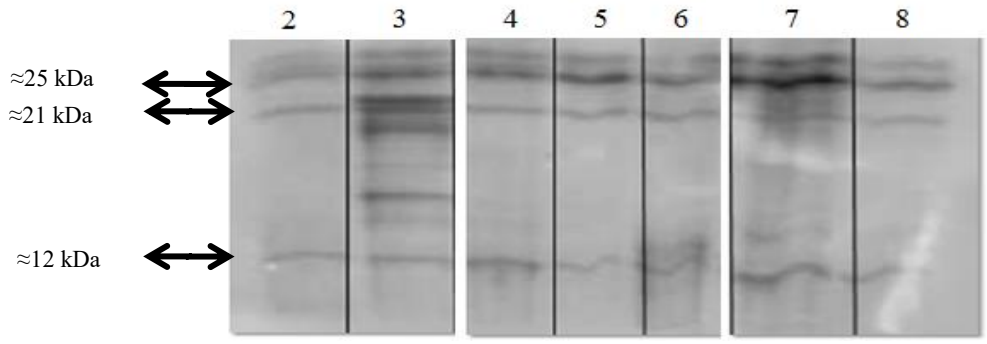


Figure 2.6: Western Blot of pertussis toxin expression in *Bordetella pertussis* control strain and clinical isolates listed in Table 2.3

Table 2.4: Summary of the Western Blot protein expression results from clinical isolates

Patient ID	Pertactin expression	Fimbriae serotype 2 expression	Fimbrae serotype 3 expression	Pertussis Toxin expression
PET1213	+	+	+	Subunit 1: + Subunit 2: + Subunit 3: + Subunit 4: + Subunit 5: +
PET0802	+	+	+	Subunit 1: + Subunit 2: + Subunit 3: + Subunit 4: + Subunit 5: +
PET1237	+	+	+	Subunit 1: + Subunit 2: + Subunit 3: + Subunit 4: + Subunit 5: +
PET1022	+	+	+	Subunit 1: + Subunit 2: + Subunit 3: + Subunit 4: + Subunit 5: +
PET1651	+	+	+	Subunit 1: + Subunit 2: + Subunit 3: + Subunit 4: + Subunit 5: +
PET1650	+	+	+	Subunit 1: + Subunit 2: + Subunit 3: + Subunit 4: + Subunit 5: +

+: isolate expresses antigen following Western Blot

2.6 Discussion

South Africa started using the aP vaccine in 2009. It has been proposed that a transition from wP to aP vaccines might be an important driver to change the epidemiology of *B. pertussis* (176). As discussed in Chapter 1; Table 1.3, there are different aP vaccines formulations, containing varied number of antigens as well as antigen concentrations (177). Here we analysed the expression of PRN, fim2, fim3 and PT proteins by *B. pertussis* isolates recovered from clinical specimens in 2015 from a South African cohort.

From the 6 isolates tested, PRN, fim2, fim3 and all 5 subunits of PT were detected (Table 2.5). A study by Hegerle and colleagues reported on French isolates under aP vaccine pressure collected from 2000-2011 and found 40 isolates deficient in at least one virulence factor (55). The authors found 2/40 (5%) PT deficient isolates, 37/40 (92.5%)

PRN deficient strains and one isolate (2.5%) deficient in both PRN and FHA (55). Isolates with variations in these proteins or even lacking PRN expression have been described in different countries. *B. pertussis* isolates with variation in PRN have been first reported in the Netherlands (178). Since the introduction of aP vaccines, *B. pertussis* strains lacking PRN have been reported in different European countries, the USA, Japan and Australia (56, 57, 118, 119, 179, 180). These PRN-negative isolates were fully virulent. Animal models have shown that antigenic divergence between vaccine strains and resurgent strains reduces vaccine efficacy (181, 182), therefore inclusion of pertactin in aP vaccines warrants further research.

The proportion of pertussis strains in the USA lacking PRN sharply increased from 14% in 2010 to 85% in 2012 (58, 60, 63). Pertactin, fim and PT are components of all aP vaccines currently used in the USA. For example, a study conducted by the USA CDC during the 2010 California pertussis outbreak analysed the protein expression by Western Blot of 6 strains isolated from infants less than 3 months of age (135); the authors found PRN deficiency in 3/6 (50%) of the isolates including also 1 isolate with fim2 deficiency 1/6 (16.7%) (135). No deficiencies were observed in any of the 6 strains for PT (135). In 2016, Williams *et al* reported on an 11 month old unvaccinated infant from New York who had a *B. pertussis* strain deficient in both PT and PRN (117). Another study conducted in Philadelphia from 2007-2014 found an overall PRN deficiency of 68% (41/60) in respiratory specimens from children with a median age of 95 days where 95% of the children received less than 1 aP vaccine dose (183).

The PRN deficiency in Japan is interesting because prior to November 2012, Japan used 5 brands of aP vaccine, 3 of which did contain PRN (Diphtheria-tetanus-acellular pertussis vaccine; DTaP) while the other two did contain PRN (DTaP-inactivated poliovirus; DTaP-IPV)(184). From the period of 2005-2007, 16/39 isolates (41%) were classified as PRN deficient and from the period of 2008-2010 fewer, 15/43 (35%) were PRN deficient (185). After November 2012, only two brands of aP vaccines were available, both of which did not contain PRN (185), and the number of PRN deficient isolates further decreased: from the period of 2011-2013, 24/97 isolates (25%) were PRN deficient and from the period of 2014-2016, 4/453 (8%) were PRN deficient (185).

Multiple PRN deficiency mutations have been described. Vodzak and colleagues (study conducted from 2007 to 2014 in Philadelphia) described stop codons at positions 760 and 1273 interrupting the *prn* gene, the authors also describe an IS481 insertion at positions 245, 1613 and 2728causing PRN deficiency (183). Hiramatsu and colleagues studied PRN

deficiencies from a period of 2005-2016 in Japan and found an 84 base pair deletion in the *prn* gene as well as an IS481 insertion at position 1598 (185).

It has been proposed that the emergence of *B. pertussis* strains that vary in the expression of antigens contained in the aP vaccine of that region is due to the evolution of *B. pertussis* toward vaccine escape (186). In South Africa the most widely used aP vaccine consists of PT and FHA and although our sample size was very small it is encouraging to find that in this study the isolates were not deficient in any of the tested proteins. Continued surveillance for new mutant strains is therefore needed.

CHAPTER 3: ESTABLISHMENT OF A NOVEL *BORDETELLA PERTUSSIS*FLUIDIGM-BASED SINGLE NUCLEOTIDE POLYMORPHISM TYPING METHOD

Due to the monomorphic nature of *B. pertussis,* typing of strains based on SNPs is not commonly used(122). Recent studies sequencing the genomes of different *B. pertussis* strains have however identified various SNPs which could potentially be used as genotyping tools to investigate genetic variations in circulating *B. pertussis* strains (145-147). While most studies have used methods such as restriction fragment length polymorphism (PCR-RFLP) (151) and multiplex PCR targeted amplicon sequencing (MTA-Seq) (152) to detect *B. pertussis* SNPs, these methods are limited in that they are expensive, time consuming, labour intensive and require computational resources and skilled personnel to analyse and interpret the data (141). There is thus a need for high throughput genotyping platforms (154) able to detect *B. pertussis* SNPs from a broad range of sample types including cultured isolates, or directly from clinical samples.

The aim of the study was to establish a novel nanofluidicbased SNP typing method for *B. pertussis* in order to detect 38 previously described pertussis virulence-associated SNPs (187).

3.1 Methods

3.1.2 Bacterial control strains

ATCC 9797 control strain (Davies Diagnostics) was used to optimise all SNP reactions. The lyophilised pellet was re-suspended and cultured as described in Chapter 2, section 2.4.1,*B. pertussis* colonies grown on charcoal agar plates (Media Mage, South Africa) were collected using a sterile inoculation loop and placed in PBS and stored at 4ºC until further use.

3.1.3 Total nucleic acid extraction

Total nucleic acids were extracted from the ATCC reference strains (positive control strains) colonies stored in PBS with the NucliSens easyMAG (BioMerieux, Marcy l'Etoile, France) extraction system according to manufactures' instructions. Extracted DNA from the reference strain was stored at -20ºC.

3.1.4 Fluidigm single nucleotide polymorphism typing method

3.1.4.1 In silico primer design using the Fluidigm D3 System

SNP genotyping assays for 38 predefined *B. pertussis* SNPs targets (187) were designed using the Fluidigm D3 platform (https://d3.fluidigm.com/account/login) according to manufactures instructions. The reference strain; TohamaI NC_002929, was used to design all respective primers. These SNPs have been described by Zeddeman *et al* and were chosen from the whole-genome sequencing data of 74 *B. pertussis* strains that represent a diverse collection of commonly studied pertussis virulence-associated alleles (187). The strains used in the original work were collected between 1949 and 2010 from Africa, Europe, North and South America (150).

The Fluidigm D3 platform was used to design assay specific primers for each individual SNP by using 2 allele specific forward primers and 1 reverse primer; Figure 3.1. Briefly, SNP target primers were designed by selecting 200 base pairs upstream and 200 base pairs downstream a specific location on TohamaI as shown by Zeddeman *et al* (187) using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch),and entering them into the D3 platform to be reviewed by the Fluidigm team (*in silico* for specificity) before synthesis. As an example Figure 3.2 shows 200 base pairs upstream and 200 base pairs downstream position at TohamaI: 3650318 for SNP3.

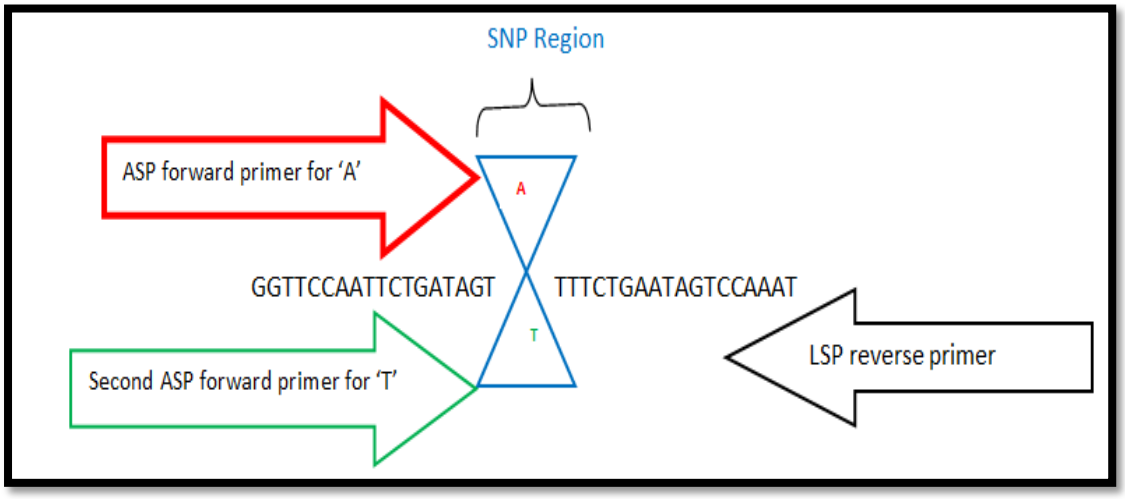


Figure 3.1: Two Allele Specific forward primers and 1 Locus Specific reverse primer allows for allelic discrimination during SNP analysis

Figure 3.2: Resultant primer sequences for TohamaI position: 3650318 using the Basic Local Alignment Search Tool program for SNP3.Square brackets are placed around single nucleotide polymorphism for SNP3

From the 38 selected targets, 37 could be successfully designed and synthesised; the SNP target at position 0860069 of the TohamaI reference strain did not have specificity *in silico*, had a high GC content and was thus excluded.

The Fluidigm system supports specific fluorophores detection: the SNPType-FAM and SNPType-HEX probes were used, i.e. SNPType assay probes were labelled with FAM and HEX fluorophores. Allele Specific Primer 1 (ASP1), which corresponds to the X allele of the primer, yields a FAM-labelled allele-specific amplicon, while Allele Specific Primer 2 (ASP2), which corresponds to the Y allele of the primer, yields a HEX-labelled allele-specific amplicon (Figure 3.1). ROX dye was used as a passive reference.

For this project primers were designed to allow the SNPType-FAM probe to hybridise to the TohamaI reference strain 'X' allele of the primer yielding red clusters. The SNPType-HEX probe hybridizes to the variant SNP 'Y' allele of the primer yielding green clusters. Non-template controls shouldn't hybridise any probes and are represented by black clusters. Information on SNP targets used in this study is summarised in Table 3.1, for additional information on primer sequences see Appendix D; Tables 7, 8 and 9.

Table 3.1: Single nucleotide polymorphism targets for *Bordetella pertussis* used in the Fluidigm assay (adapted from Zeddeman *et al* (187))

Single nucleotide polymorphism (SNP)	Gene in which SNP is located	Pertussis toxin promoter allele associated with SNP	Position in TohamaI	Gene[a]	Nucleotide of TohamaI at SNP	Variant SNP	Primer sequences (reference: this study)
SNP1	*B. pertussis* autotransporter protein C (*bapC)*	*ptxP4*	T2905672	BP2738	A	G	ASP1: GCGACACCTCGGACTGA ASP2: GCGACACCTCGGACTGG LSP: GTTGCACGACGGCTGG
SNP2	-	*ptxP6*	T2678045	BP2526	G	A	ASP1: CCACCATCGTCTCTCCCTC ASP2: CCACCATCGTCTCTCCCTT LSP: GCTTCGGGAAATGCCTGAT
SNP3	Dermonecrotic toxin *(dnt)*	*ptxP2*	T3650318	BP3439	G	A	ASP1: CCACCTGCTCAGCCTGG ASP2: CCACCTGCTCAGCCTGA LSP: GGCGAAACGGCCTCCA
SNP4	-	*ptxP1*	T0824268	Intergenic	C	T	ASP1: GGGGTCGGGTCAAACCTG ASP2: GGGGTCGGGTCAAACCTA LSP: CCCTGGTAACGGCGCA
SNP5	Glutathione reductase *(gor)*	-	T2244171	BP2120	G	A	ASP1: CGACCTCATCCCCACCG ASP2: CGACCTCATCCCCACCA LSP: AGGCCGACCGTGCCG
SNP6	-	*ptxP1*	T2166028	Intergenic	C	T	ASP1: CGTTTATGCGGCGGCG ASP2: TCGTTTATGCGGCGGCA LSP: AGATAAAAGCGGCCCGGTCA
SNP7	-	*ptxP1*	T2277180	BP2156	G	A	ASP1: GGCATGGTGCACGGG ASP2: CGGCATGGTGCACGGA LSP: GCGGATCGTGCGCCAC
SNP8	-	-	T0835998	Intergenic	G	A	ASP1: GGAGGAAAGATACAAGACACCTGC ASP2: GGAGGAAAGATACAAGACACCTGT LSP: GCTATGAGGTTGCCACCAG
SNP9	-	*ptxP1*	T0063037	BP0063	C	T	ASP1: CCATaGTGCCGTTGCTG ASP2: AGATATCCATaGTGCCGTTGCTA LSP: CAGTGGTGAGTTCGCACGC

- unknown

[a]Gene in which SNP is located

ASP: Allele Specific Primer (forward primer)

LSP: Locus Specific Primer (reverse primer)

Table 3.1: *continued*

Single nucleotide polymorphism (SNP)	Gene in which SNP is located	Pertussis toxin promoter allele associated with SNP	Position in TohamaI	Gene[a]	Nucleotide of TohamaI at SNP	Variant SNP	Primer sequences (reference: this study)
SNP10	*ptxA*	-	T3988941	BP3783	G	A	ASP1: GGCACATTGGTGCGCATG ASP2: GGCACATTGGTGCGCATA LSP: GCTTTCGGCCTGCCGC
SNP11	-	-	T1931433	Intergenic	A	G	ASP1: GCAACTTTAAGTGCTAGAATTTCCCA ASP2: GCAACTTTAAGTGCTAGAATTTCCCG LSP: GCCAGACGAATCACCAGCAT
SNP12	-	*ptxP1*	T3686708	BP3474	G	A	ASP1: CCTGCGCTACGGTCTGC ASP2: CCTGCGCTACGGTCTGT LSP: GCGATAGGTGCGCAGCC
SNP13	*risS*	*ptxP1*	T3764370	BP3553	G	A	ASP1: TCCATCAGCTGCCCGATG ASP2: GTATTCCATCAGCTGCCCGATA LSP: GCCCGCCAGGCCATC
SNP14	-	-	T0514171	Intergenic	G	A	ASP1: CCTCGAACGCCTCGCC ASP2: CCTCGAACGCCTCGCT LSP: GGACCTCAGCCACGACATCA
SNP15	-	-	T0511992	Intergenic	A	G	ASP1: CCGGGGAGGTCAGCATAT ASP2: CCGGGGAGGTCAGCATAC LSP: GGAACATGGCATCTCCTGAGC
SNP16	-	-	T1362787	BP1291	G	A	ASP1: GCGGGCGCTACAACAG ASP2: GCGGGCGCTACAACAA LSP: ACGAGGCGTACACCACGG
SNP17	-	-	T2587815	Intergenic	T	C	ASP1: TGCAGGAAGTTCGATATGAGCA ASP2: TGCAGGAAGTTCGATATGAGCG LSP: AGCCTCACCGTCAGGCG
SNP18	-	-	T1107425	BP1062	C	T	ASP1: CGGCCAAGGTCGAGAAAATG ASP2: GCGGCCAAGGTCGAGAAAATA LSP: CGCGCAGATCGCCAGG

- unknown

[a]Gene in which SNP is located

ASP: Allele Specific Primer (forward primer)

LSP: Locus Specific Primer (reverse primer)

Table 3.1: *continued*

Single nucleotide polymorphism (SNP)	Gene in which SNP is located	Pertussis toxin promoter allele associated with SNP	Position in TohamaI	Gene[a]	Nucleotide of TohamaI at SNP	Variant SNP	Primer sequences (reference: this study)
SNP19	-	-	T2615461	Intergenic	G	A	ASP1: GCAGGAAAAAAACACGGGCG ASP2: GCAGGAAAAAAACACGGGCA LSP: CCAAGGCCGCCGCAT
SNP20	Bordetellalegiolysin *(bllY)*	*ptxP1*	T3240976	BP3040	G	A	ASP1: AGGAAGGCACCGAAGAGAAG ASP2: GAGGAAGGCACCGAAGAGAAA LSP: CGCGGTACAGGTCCAGGT
SNP21	High temperature protein G *(htpG)*	*ptxP1*	T0072405	BP0074	C	A	ASP1: CGGATATTACCGACGAGCAGTATC ASP2: TCGGATATTACCGACGAGCAGTATA LSP: ACGCCAGCGGATCGTCA
SNP22	-	-	T1702741	BP1619	C	T	ASP1: GCGCCATGCATTCGAGC ASP2: AGCGCCATGCATTCGAGT LSP: CGACAACCGCGCCGAA
SNP23	-	-	T3085804	Intergenic	G	T	ASP1: CGCCAGCATGGATTCCTATG ASP2: CCGCCAGCATGGATTCCTATT LSP: GGCGAATACAGAAGCTCCGC
SNP24	-	-	T3568653	BP3346	G	A	ASP1: CGATGTCGTTGGTGAAGCG ASP2: TCGATGTCGTTGGTGAAGCA LSP: GCCTGTTCGCGGCCAT
SNP25	*ptxP*	-	T3988168	BP3783P	G	A	ASP1: CGCAAAGTCGCGCGATG ASP2: CGCAAAGTCGCGCGATA LSP: CCGGACGGTGACCGGTA
SNP26	*ptxC*	*ptxP3 ptxP15 ptxP18*	T3991376	BP3787	C	T	ASP1: CGGCAGCGTCGATATGC ASP2: CCGGCAGCGTCGATATGT LSP: GGACAGGCGAACAGATCCGA
SNP27	-	*ptxP3*	T3161156	BP2974	G	A	ASP1: GCCAGAAGAACAGCGAGATGAG ASP2: GCCAGAAGAACAGCGAGATGAA LSP: TGGCGCTGGCCACCTA

- unknown

[a]Gene in which SNP is located

ASP: Allele Specific Primer (forward primer)

LSP: Locus Specific Primer (reverse primer)

Table 3.1: *continued*

Single nucleotide polymorphism (SNP)	Gene in which SNP is located	Pertussis toxin promoter allele associated with SNP	Position in TohamaI	Gene[a]	Nucleotide of TohamaI at SNP	Variant SNP	Primer sequences (reference: this study)
SNP28	-	*ptxP3 ptxP15 ptxP18*	T1547488	BP1471	A	G	ASP1: CCGGATCACCGACCTATGAA ASP2: CCGGATCACCGACCTATGAG LSP: CGGCGATGGAGGCGAAA
SNP29	Ribosomal protein *(rplV)*	*ptxP3*	T3833330	BP3618	C	A	ASP1: CATCCTCACCTTCTCGCCC ASP2: CATCCTCACCTTCTCGCCA LSP: AGCCGACTCGACAGCCTTC
SNP30	Type III secretion protein *(bscI)*	*ptxP3 ptxP15 ptxP18*	T2374322	BP2249	T	C	ASP1: AGCCGACTCGACAGCCTTC ASP2: ACAGGCATCCGTGGAGTG LSP: CAGCGTATCGACGTTTTGG
SNP31	-	*ptxP3*	T0667028	BP0658	G	A	ASP1: CGAGCACCTGACTGGGC ASP2: CGAGCACCTGACTGGGT LSP: CAGCACGATGTGAGGCACT
SNP32	-	*ptxP3*	T0835998	BP0814	G	A	ASP1: GGGCAGCAGGGTCTCG ASP2: GGGCAGCAGGGTCTCA LSP: GAACGCCTGCAGGGCAC
SNP33	-	*ptxP3*	T2681216	BP2528A	G	A	ASP1: GCTTGGGACAGCACAGC ASP2: CGCTTGGGACAGCACAGT LSP: GCCGCCATGAACGCCG
SNP34	*fim3*	*ptxP3 ptxP15 ptxP18*	T1647861	BP1568	C	A	ASP1: AGGAATGCCCCCAGGC ASP2: GAAGGAATGCCCCCAGGA LSP: GTGATGCCGGGCTCGAAA
SNP35	Phosphoprotein phosphatase *(prpB)*	*ptxP3 ptxP15 ptxP18*	T2505238	BP2366	T	C	ASP1: CGGTGATGTTGGCCAGCA ASP2: GGTGATGTTGGCCAGCG LSP: CCTGCCTACCTACGATCGCTT

- unknown

[a]Gene in which SNP is located

ASP: Allele Specific Primer (forward primer)

LSP: Locus Specific Primer (reverse primer)

3.1.4.2 Specific target amplification

High-throughput PCR reactions require highly concentrated DNA (188) thus Specific target amplification (STA) is a prerequisite for each Fluidigm run. STA was done as per manufacturers recommendations as the initial step (pre-amplification of DNA) for the Biomark HD system (Fluidigm). Briefly a primer pool containing SNP assays was prepared by mixing 2 µl of each ASP forward primer and 2 µl of each LSP reverse primer to give a final concentration of 500 nM for each primer. PCR reactions were carried out for the STA pool in 5 µl volumes each containing 2.5 µl Qiagen 2X multiplex PCR maser mix (Biotium, United States of America), 0.5 µl pooled assay and 1.25 µl of extracted DNA. Reactions were amplified with the T100 Thermal Cycler (Bio-Rad, United States of America) and cycling conditions included an initial activation at 95ºC for 10 minutes followed by a 14 two-step cycles: denaturation at 95ºC for 15 seconds and annealing/extension at 60ºC for 4 minutes; Appendix D, Table 12. The STA products were then diluted 1:100 in DNA suspension buffer (Thermofisher Scientific, South Africa) and stored at -20°C until further use in the Fluidigm experiments; Appendix D, Table 12.

3.1.5 Single nucleotide polymorphism PCR using the Biomark HD system (Fluidigm)

For SNP genotyping, the FLEXsix Genotyping Reagent Kit (Lasec, United States of America) was used. The IFC Controller HX machine was used to pressurise samples and reagents into the valves within the IFC plate while the Biomark HD system carries out the SNP PCR genotyping reactions. The SNP typing was carried out with FLEXsix integrated fluidic chip (IFC) plates (Fluidigm Corporation, United States of America) according to manufacturer's instructions. Briefly 10X assay and sample pre-mixes were prepared. Each 10X assay mixture contained 1.2 µl SNPType assay mix, 3 µl 2X assay loading reagent as well as 1.8 µl PCR certified water. The final concentration (at 10X) for each the forward and reverse primers was 500 µM. Sample premix for 24 samples was made by combining 54 µl Biotium fast probe master mix (Analytical Technology, South Africa), 5.4 µl 20X SNPType sample loading reagent (Lasec, United States of America), 1.8 µl 60X SNPType reagent (Lasec, United States of America), 0.648 µl ROX reference dye (Thermofisher Scientific, South Africa) and 1.15 µl PCR certified water.

IFC controller (Fluidigm Corporation, United States of America) was used to prime the FLEXsix IFC Chip (Fluidigm) with control line fluid. Five microliters of each assay and 6 µl of sample was then transferred into the appropriate inlets of the primed IFC chip and loaded into the IFC controller machine.

After loading, the chip was placed in the Biomark instrument for PCR with thermal cycle conditions described in Table 3.2. Data were analysed with Fluidigm SNP genotyping Analysis program in the Biomark instrument (Fluidigim Corporation, United States of America).

Table 3.2: SNP Type FLEXSix thermal cycle protocol

Type			**Temperature**	**Time**
Thermal Mix			25 °C	30 minutes
			70 °C	60 minutes
Hot Start			95 °C	5 minutes
PCR Cycle	1 Cycle	Denaturation	95 °C	15 seconds
		Annealing	64 °C	45 seconds
		Extension	72 °C	15 seconds
PCR Cycle	1 Cycle	Denaturation	95 °C	15 seconds
		Annealing	63 °C	45 seconds
		Extension	72 °C	15 seconds
PCR Cycle	1 Cycle	Denaturation	95 °C	15 seconds
		Annealing	62 °C	45 seconds
		Extension	72 °C	15 seconds
PCR Cycle	1 Cycle	Denaturation	95 °C	15 seconds
		Annealing	61 °C	45 seconds
		Extension	72 °C	15 seconds
PCR Cycle	34 Cycles	Denaturation	95 °C	15 seconds
		Annealing	60 °C	45 seconds
		Extension	72 °C	15 seconds
End Point		Capture	20 °C	30 seconds

Ramp Rate: Normal 2° C/s

After loading the samples and assays into their respective inlets on the IFC plate and immediately after the thermal cycle was finished the IFC plate was removed from the Biomark machine and placed back into the IFC controller machine for a post run. In order to analyse the Fludigim chip experiment according to SNP calls, the 'Fluidigm SNP Genotyping Analysis' program was used. Figure 3.3 shows a summary of the Fluidigm technique workflow.

Figure 3.3: Fluidigm technique workflow (http://www.incodom.kr/Fluidigm)

3.2 Results

3.2.1 Primer optimisation: Cartesian display scatter plots of the control strain

As described, ATCC 9797 control strain was used to optimise all SNP reactions.

Upon testing of the resultant 37 assays against the ATCC control strain, several reactions gave a high non-template control intensity and did not yield distinct XX clusters shown in Figure 3.4, panel A. The PCR certified water that was used during these runs was replaced and the assay master mix was re-made. Replacing the PCR certified water and remaking assay master mixes resulted in assay runs that yielded a XX distinct cluster as shown in Figure 3.4, panel B. For all future runs, the assay master mix was remade every three months before being used for sample and control screening.

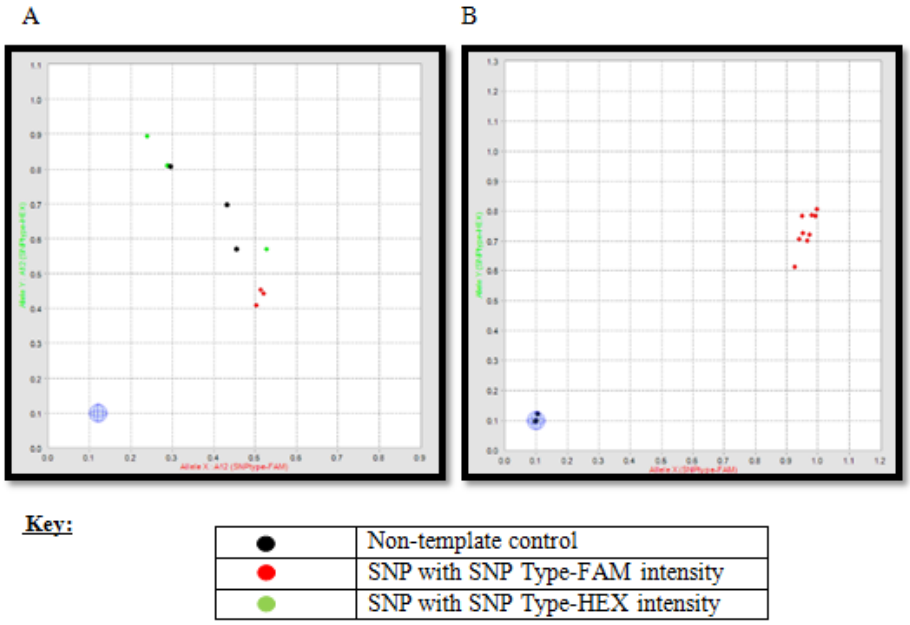


Figure 3.4: Cartesian display scatter plot comparisons of control run against SNP9 TohamaI position: 0063037, using assay master mix more than 3 months old (A) and less than 3 months old (B)

After the above mentioned conditions were changed, 4 assays still yielded unexpected results. Two assays, T2905672 (SNP1 in this study) and T3686708 (SNP12 in this study), gave unexpected SNPType-HEX intensity (YY cluster) instead of an SNP Type-FAM intensity (XX cluster) in multiple runs. The primer design (as seen in Figure 3.2) was then tested *in silico* against the ATCC 9797 control strain used to test the respective targets, and it was found that the ATCC 9797 control strain had a different SNP type from the TohamaI

reference strain used to design the respective reactions and thus these primers targeted the reverse strain. The previously unexpected SNPType-HEX intensity was thus found to be correct and these two assays were then included in further analysis.

The remaining 2 assays that yielded unexpected results, namely, T1774569 and T0220937 were further investigated. Figure 3.5 shows a diagram of resultant runs for SNP analysis against T1774569 and T0220937 after a series of optimisation techniques such as remaking master mixes, redoing pre-amplification of control strains and replacing PCR certified water for non-template controls; Figure 3.5, Panel A shows an initial run which did not yield a distinct XX or YY cluster while panel B shows a second run with newly prepared master mixes and control strain pre-amplification redone; amplification again was unsuccessful. These targets were thus excluded from further analysis.

A B

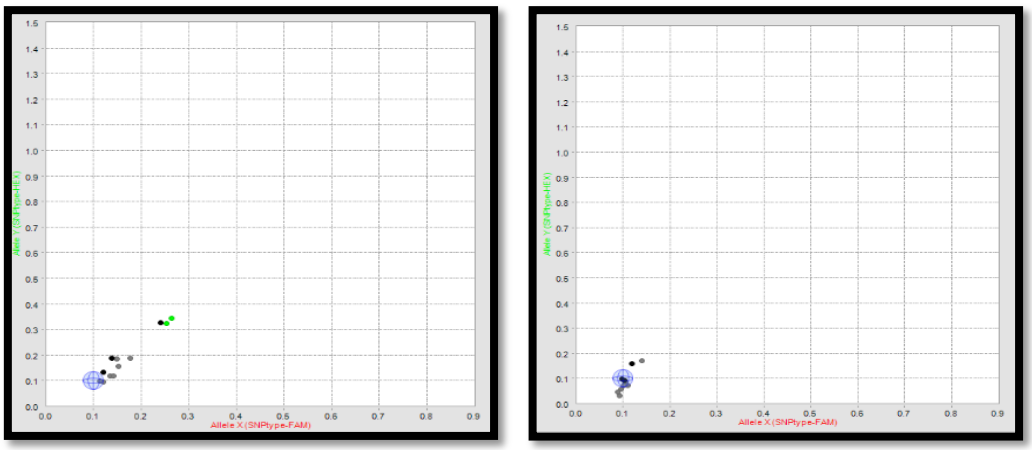


Figure 3.5: Cartesian display scatter plots showing unsuccessful amplification of *Bordetellapertussis* control strain for SNPs targeted against TohamaI position: 1774569 (A) and a second run in (B)

In Figure 3.6, panels A and B shows a control run against TohamaI position 220937. Multiple control samples were used, yielding multiple points that yielded both mixed FAM and HEX intensities instead of a distinct FAM cluster even after newly prepared master mixes and control strain pre-amplification redone. The amplification of both FAM and HEX resulted in this assay against TohamaI position 220937 being excluded from further analysis.

A B

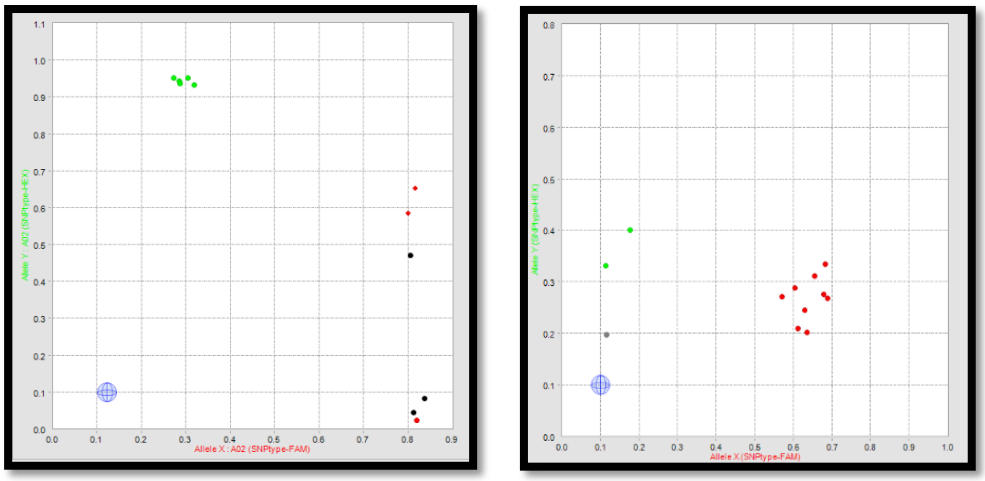


Figure 3.6: Cartesian display scatter plots of *Bordetella pertussis* control strain unsuccessful amplification of SNP targeted against TohamaI position: 220937. Both FAM and HEX intensities were detected even after newly prepared master mixes and control strain pre-amplification redone (B)

The assay master mix was kept at -20ºC, however; while testing the control strain using this mix it was found that the assay mix had to be remade every three months because after 3 months at -20ºC, low quality Cartesian scatter plots were seen. SNP assays T1774569 and T0220937 were excluded because even after remaking master mixes, redoing pre-amplification of control strains and replacing PCR certified water for non-template controls the assays did not successfully amplify the targeted SNP. After changing conditions for the control strain samples and non-templates controls, T1774569 did not yield distinct XX clusters and T0220937 in addition to not yielding distinct XX clusters consistently detected signal in non-template controls.

A final total of 35 out of the 38 (91.1%) designed assays successfully amplified their respective targets and were thus included for subsequent analysis of clinical samples.

Examples of successfully optimised experiments with control strain yielding SNPType-FAM clusters and non-template controls not amplifying (black clusters) are shown in Figure 3.7.

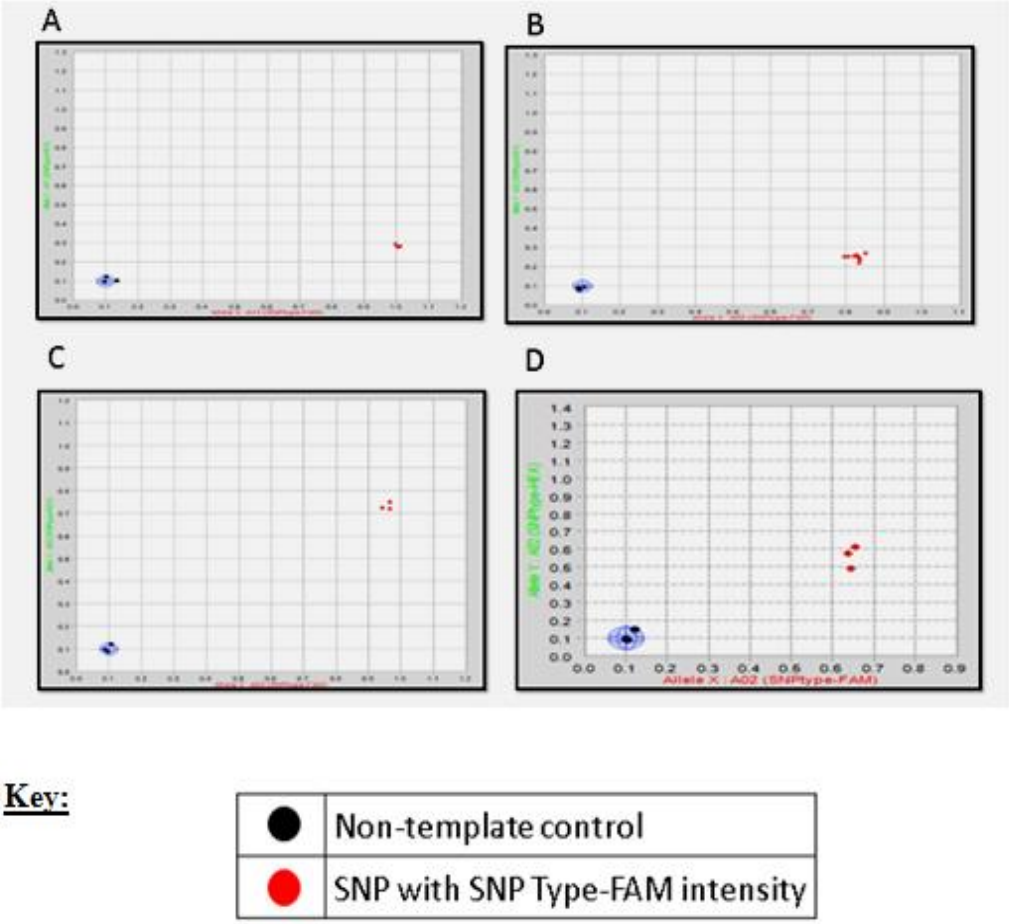


Figure 3.7: Cartesian display scatter plots of *Bordetella pertussis* control strain amplification of Dermonecrotic toxin (SNP3) (A), Glutathione reductase (SNP5) (B), Bordetella legiolysin (SNP20) (C) and Type III secretion protein (SNP30) (D) single nucleotide polymorphisms

3.3 Discussion

In this study a reliable novel nanofluidic SNP typing method was successfully developed to detect 35 previously described pertussis virulence-associated SNPs.

Any given bacterium contains several SNP sites which may correlate with phylogeny, protein expression, survival fitness and ultimately disease pathogenesis. A reliable SNP typing method can thus be useful to characterise and relate different *B. pertussis* strains (189) to the TohamaI reference strain for example. While several SNP genotyping technologies have been developed they are lacking in that the majority use junction probes (190), apurinic/apyrimidinic based probes (191) or enzyme-mediated detection methods (192) that require strict thermodynamic conditions (189). Furthermore, PCR-RFLP and MTA-seq methods that are commonly used to detect *B. pertussis* SNPs are limited in that they are expensive, time consuming, labour intensive and require computational resources and skilled personal to analyse and interpret the data (141). The Fluidigm method was thus able to overcome some of these shortcomings, including enabling a larger number of reactions per plate, thus making it more cost effective, less labour intensive and thus less time consuming. Further the IFCs not only reduces the reaction volume from 10µl – 20 µl down to a 10 nl scale, but this technology also allows for validations as well as increased parallelism and throughput of SNP reactions.

SNPType assays were designed using the Fluidigm D3 platform (https://d3.fluidigm.com/account/login). These allele specific assays allowed for rapid screening of control strain SNPs by PCR (D3 Assay Design User Guide PN 100-6812). After a series of optimisation runs, 35 assays out of 38 assays that were designed to target SNPs within the TohamaI (NC_002929) strain gave expected fluorescent clusters with non-template controls yielding no fluorescent intensities (as seen in Figure 3.6). Three of the published assays namely assays at positions: T1774569 (Locus gene ID: BP1690), T0220937 (Locus gene ID: BP0215) and T0860069 (Locus gene ID: Integenic) were excluded because either the primers could not be synthesised (T0860069) or the assays did not effectively amplified their respective targets in the Biomark-HD system (T1774569 and T0220937).

The Biomark HD system is a new instrument that has not been used to detect *B. pertussis* virulence-associated SNPs before. While the platform was able to successfully amplify 35 known virulence associated SNPs, the assay did not incorporate all known virulence-associated SNPs and thus characterisation of circulating *Bordetella pertussis* strains is limited to the SNPs included in the assay.

The platform can, however, incorporate up to 96 targets and thus our method can be expanded on to include additional SNPs in future studies. Further; from the 38 previously described pertussis virulence-associated SNPs we were only able to successfully design SNP reactions for 35 SNP reactions. The three assays were excluded as they did not meet high sensitivity and specificity criteria and thus careful design of future SNP targets is needed. Fluidigm is sensitive to contamination, air bubbles and expiration of reagents. Careful monitoring and optimisation of primer design is needed for future studies, especially if more virulence associated SNPs are to be included in the assay to minimise exclusion of assays. Lastly, we were not able to test our assays against the samples used by Zeddeman *et al* (187) therefore a direct comparison could not be made.

In conclusion we developed a novel nanofluidic SNP typing assay able to detect 35 previously described pertussis virulence-associated SNPs. Improved methods of detection of these SNPs will help to determine the antigenic divergence of circulating *B. pertussis* strains and improve our understanding the evolution of *B. pertussis* from a genetic point of view. This method is useful for identifying pertussis toxin subunits and specifically the expansion of ptxP3 in a certain region

Following optimisation, 52 positive *B. pertussis* samples were tested using these assays to detect the 35 SNPs as described in chapter 4.

CHAPTER 4: CHARACTERISATION OF SINGLE NUCLEOTIDE POLYMORPHISMS TARGETING PERTUSSIS TOXIN PROMOTERS IN SOUTH AFRICAN *BORDETELLA PERTUSSIS* STRAINS USING FLUIDIGM TECHNOLOGY

There is evidence that *B. pertussis* has undergone structural evolution including gene deletions and gene rearrangement causing circulating strains to be highly structurally diverse (121). The differences observed in circulating strains are thought to be associated with adaptation to the mammalian host and immune pressure (122). This evolution raises concern about the ability of current aP vaccines to control pertussis disease (115). Discrete changes in circulating strains are also evident at the level of single nucleotide polymorphisms (SNPs) (121). Recent studies sequencing different *B. pertussis* strains have identified various SNPs (145-147)that can be useful for genetic relationships between strains to be established (148, 149). Although extensive research on the characterisation of circulating *Bordetella pertussis* strains has been done worldwide (193), there is a paucity of genotypic data on pertussis strains from Africa, including South Africa, which transitioned from using whole cell pertussis vaccines to acellular pertussis vaccines in 2009. The acellular vaccines that contain pertussis toxin have been considered most protective in animal models (194).Several different alleles of pertussis toxin promoter (ptxP) have been observed worldwide(49)with pertussis toxin promoters 1 and 3 (ptxP1 and ptxP3 respectively) being of particular importance due to their dominance (195). While ptxP1 predominated prior to the 1990s, ptxP3 is gradually replacing ptxP1 (49). Bacterial strain fitness is influenced by ptxP3 due to the fact that ptxP3 strains have increased pertussis toxin production (48).

The aim of chapter 4 was to use the high-throughput SNP genotyping assays established in Chapter 3 to detect 35 known SNPs in circulating *B. pertussis* strains collected from South African infants in order to gain a better understanding on antigenic and SNP divergence. The SNP targets used in this study target a range of pertussis toxin promoters.

4.1 Methods

4.1.2 Study samples and population

Archived nucleic acid extracts collected from nasopharyngeal swabs (NP) and induced sputum (IS) samples (n=24) confirmed to be positive for *B. pertussis* by PCR as well as isolates (n=10) recovered from cultured samples (as described in Chapter 2; four isolates unsuccessful in Western Blot analysis included here) collected from hospitalised infants

enrolled in a prospective respiratory disease surveillance study (BoSS) [Reference: (168)] were used for this study; Figure 4.1. Further, archived nucleic acid extracts collected from children participating in the Pneumonia Etiology Research for Child Health (PERCH) study (HREC number M101129) from 2011-2013 were also retrospectively analysed. Briefly, PERCH was a multi-site public health research study on the aetiology and risk factors of pneumonia involving children from seven low and middle income countries (196). Samples from children 1-59 months of age hospitalised with severe or very severe pneumonia as defined by the World Health Organisation underwent clinical evaluation of nasopharyngeal and induced sputum for *B. pertussis* by PCR, pertussis-associated findings were recorded(197). Eighteen samples collected from South African children, previously recognised to be positive for *B. pertussis* by PCR were used from the PERCH study(197); Figure 4.1, panel C. The total number of *B. pertussis* samples tested in this study for SNP analysis using Fluidigm technology was 52; Figure 4.1; panel D.

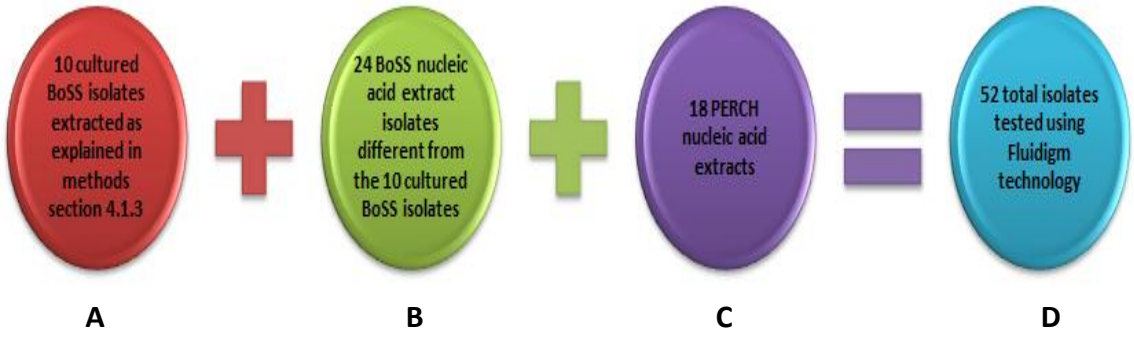


Figure 4.1: Flow diagram showing *B. pertussis* samples tested for 35 virulence-associated SNPs using the Fluidigm platform

4.1.3 DNA extraction

Total nucleic acids were automatically extracted from 10 cultured *B. pertussis* isolates from the BoSS study (Figure 4.1; panel A) and from the *B. pertussis* positive control (ATCC 9797) reference strain with the NucliSens easyMAG extraction system (BioMérieux, Marcy l'Etoile, France) according to manufactures instructions.

DNA from the remaining BoSS and PERCH samples (n=42) were previously extracted during their respective parent studies and therefore DNA was not extracted again in current study.

4.1.4 SNP typing using the Biomark HD System (Fluidigm)

DNA extracts were tested for 35 SNP reactions with the nanofludic real-time PCR system as described in Chapter 3, sections 3.1.4.2 and 3.1.5. Briefly, specific target amplification (STA) was done per manufactures recommendations as the initial step (pre-amplification of DNA) for the Biomark HD system (Fluidigm). SNP typing was then carried out on the STA products with FLEXsix dynamic array (Fluidigm Corporation, CA, United States of America) according to manufactures instructions. Data was analysed with Real-Time PCR Fluidigm SNP Genotyping Analysis Software in the Biomark instrument (Fluidigm Corporation, United States of America).

The SNP results were analysed according to pertussis toxin promoters as well as known and unknown genes as described by Zeddeman *et al* (187). This study stratified the divergence of alleles found in these known and unknown genes according to year of sample isolation, HIV exposure and aP vaccination status. During analysis, an SNP allele was treated as a 'no genotypic call' if neither an SNPType-FAM nor a SNPType-HEX intensity was detected for that allele. An allele was treated as 'inconclusive' if the resultant SNP allele was an intermediate i.e. both SNPType-FAM and SNPType-HEX probes had intensity. Targets that had no genotypic calls or inconclusive allele results were excluded from further analysis. Allele intensities refer to end point fluorescence intensities from each fluorophore i.e. FAM and HEX which correspond to positive calls (198).

4.2 Results

4.2.1. Participant demographics and clinical data

Nucleic acids extracted from NP and IS samples collected from infants enrolled in a pertussis surveillance study (BoSS) or in the pneumonia etiology research for child health (PERCH) project that tested positive for *B. pertussis* by PCR were retrospectively analysed using the novel nanofluidic based SNP-typing method established in Chapter 3. The demographics of the infants are shown in Table 4.1. Briefly, the majority of infants from both the BoSS and PERCH studies were 0-5 months old at the time of hospitalisation. PERCH samples were collected from 2011 to 2013 with 78% of the samples collected in 2011, while BoSS samples were collected during 2014 and 2015, but the majority (94%) of the samples were collected from 2015. While the majority of infants enrolled in BoSS received no aP vaccine dose, the majority of infants from the PERCH cohort received at least one aP vaccine dose because the infants from this study were older and thus were eligible for the aP dose.

Table 4.1: Demographic characteristics of the infants enrolled in BoSS and PERCH whose samples were analysed in the current study

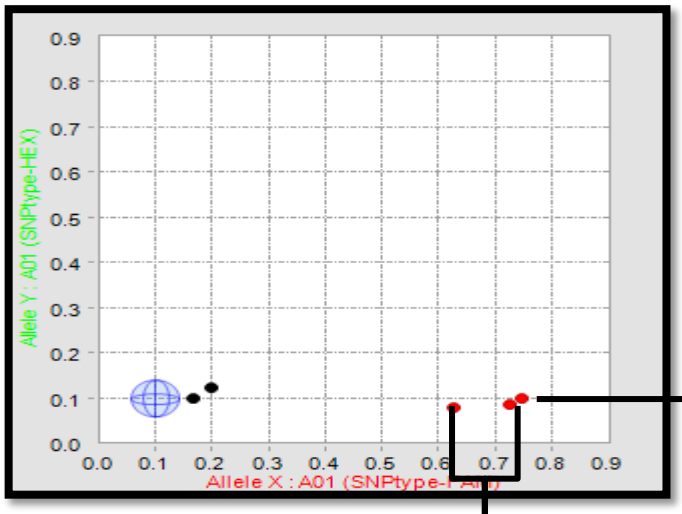
	No. participants (%)	
Study and number of participants		
BoSS PERCH	34 (65.8) 18(34.6)	
Gender	**BoSS**	**PERCH**
Female	19 (55.9)	6(33.3)
Male	15(44.1)	12 (66.7)
Age(overall mean age = 94.63 days)		
0-5 months	32(94.1)	15(83.3)
6-11 months	2(5.9)	2(11.1)
12-23 months	0	1(5.6)
Mean age in days	68.12	144.72
Year of Admission		
2011	0	14 (77.8)
2012	0	3 (16.7)
2013	0	1 (5.6)
2014	2 (5.9)	0
2015	32(94.1)	0
HIV exposure		
Exposure	14 (41.2)	6 (33.3)
Unexposed	20 (58.8)	10 (55.6)
Unknown exposure	0	2 (11.1)
AP Vaccination dosage		
0 doses	16 (47.1)	5 (27.8)
1 dose	7 (20.6)	6 (33.3)
2 doses	5(14.7)	4(22.2)
3 doses	1(2.9)	2(11.1)
Unknown	5(14.7)	1(5.6)

Numbers in parenthesis are percentages

4.3.1 Cultured isolates vs. nucleic acid extract samples

After screening 10 nucleic acids obtained from BoSS cultured samples using Fluidigm (as shown for control strain in Chapter 3, sections 3.1.4.2 and 3.1.5) nucleic acids extracted from swabs in UTM collected in parallel from the same patients as the specimens used for culture were tested for all respective SNP targets. This analysis was done in order to assess if similar Fluidigm SNP results were obtained from samples in UTM and cultured isolates. If UTM samples were proven suitable for SNP testing, this would mean that nucleic acids extracted directly from clinical samples could be used in this assay without the need for a culture step. Figure 4.2 is a Cartesian display example showing results obtained from nucleic acids extracted from the two types of samples collected from the same infant screened against

SNP4, TohamaI position: 0824268. No differences in the results between extracts from cultured samples and UTM samples were observed for all 35 SNPs tested and thus for the remaining samples (which did not have a parallel culture), DNA was extracted directly from clinical samples in UTM.



B. pertussis positive control

PET0802 culture and nucleic acid extract isolates

●	Non-template control
●	SNP with SNP Type-FAM intensity

Isolate	Allele	Allele Intensity
PET0802 culture isolate	XX	0.73
PET0802clinical sample	XX	0.63
B. pertussis positive control	XX	0.75

Figure 4.2: Cartesian display scatter plot showing signals from nucleic acids extracted from a culture isolate or extracted directly from a clinical sample and *B. pertussis* control strain and negative controls for SNP4, position at TohamaI: 3650318

4.3.2 Frequency of pertussis toxin promoters

Different pertussis toxin promoter (*ptxP*) alleles are targeted by the SNP targets used in this study; the overall frequency of the different *ptxP* for 52 *B. pertussis* positive samples is shown in Figure 4.3. Briefly, the different SNP targets for this study targeted the following pertussis toxin promoter alleles: SNP1(ptxP4), SNP7(ptxP1), SNP12(ptxP1), SNP13(ptxP1), SNP21(ptxP1), SNP26(ptxP3), SNP28(ptxP3), SNP29(ptxP3), SNP30(ptxP3), SNP31(ptxP3), SNP32(ptxP3), SNP34(ptxP3) and SNP35(ptxP3); Figure 4.3. As shown in Chapter 3, Table 3.1 the ptxP3 promoter is either reported alone as ptxP3 or in a group with ptxP15 and ptxP18, for analysis in Chapter 4, this ‘ptxP3 ptxP15 ptxP18’ group is discussed as ptxP3 as

only 1/218 (0.46%) of the strains analysed by Zeddeman *et al* (187) had the ptxP15 and ptxP18 allele while 217/218 (99.54%) of the strains had the ptxP3 allele.

Overall, the samples tested positive for a wide range of pertussis toxin promoters, with ptxP4 having the highest frequency (100%; 52/52) and SNP32 associated with ptxP3 being detected at the lowest frequency (1.9%; 1/52).

As shown in Figure 4.3, all samples contain the ptxP4 allele (SNP1) as well as another ptxP type; this is because strains that contain multiple ptxP alleles is due to different genetic backgrounds and thus form distinct lineages(142). Furthermore, strain gene content correlates with the specific ptxP types of those specific strains (142). SNP12 had the highest ptxP1 frequency at 34.62% while SNP28 had the highest ptxP3 frequency at 84.62%; Figure 4.3.

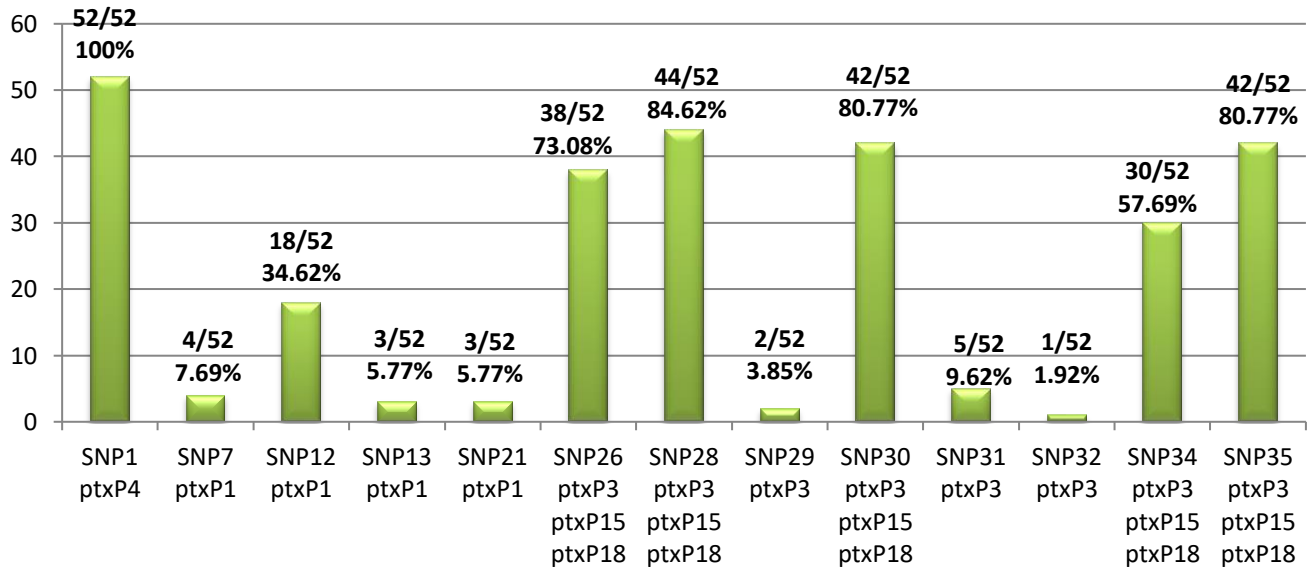


Figure 4.3: Overall frequency of the single nucleotide polymorphisms that targeted pertussis toxin promoters for 52 *B. pertussis* positive samples collected from 2011-2015

The frequencies of the dominating pertussis toxin promoter alleles,ptxP1 and ptxP3, is shown in Figure 4.4for the two study periods. SNPs associated with ptxP1 ranged from 0% (SNP7) to 44% (SNP12) and 0% (SNP13)to12% (SNP7) for study periods 2011-2013 and 2014-2015 respectively, while SNPs associated withptxP3 ranged from 0% (SNP34)to72% (SNP30) in 2011-2013compared to 0% (SNP32)to100% (SNP35) for the study period of 2014-2015; Figure 4.4.

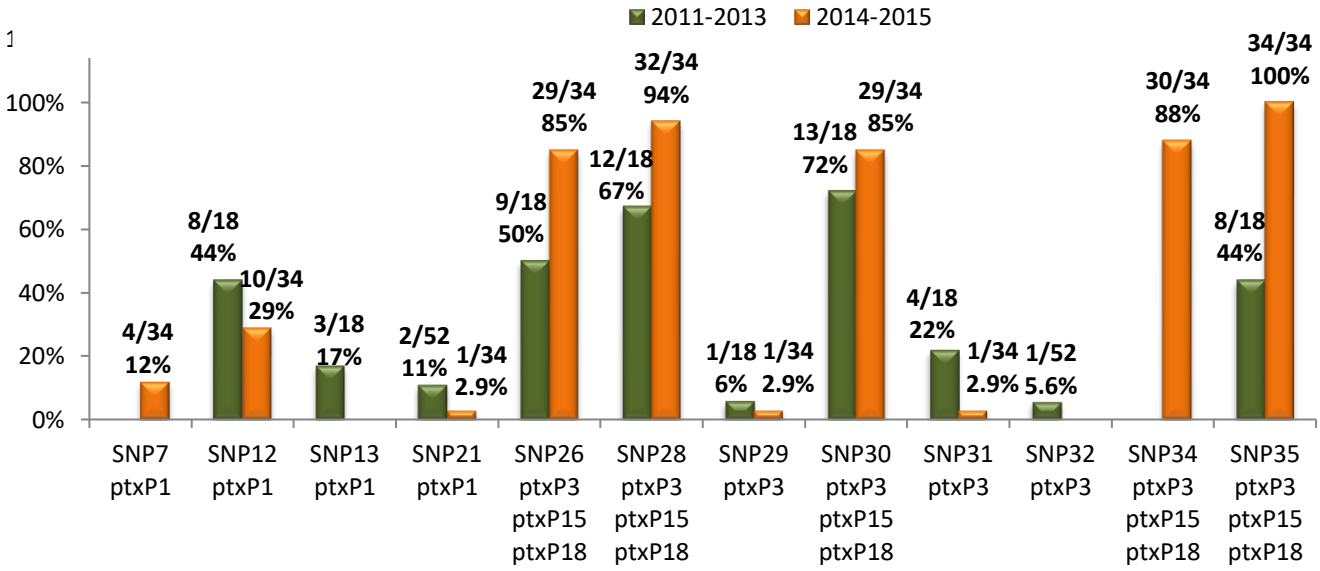


Figure 4.4: Frequency of pertussis toxin promoter 1 (ptxP1) and pertussis promoter 3 (ptxP3) stratified by year of collection: 2011-2013 (n=18) and 2014-2015 (n=34)

A number of no genotypic calls and inconclusive alleles were observed. For the BoSS samples, at least one no genotypic call was found in 80% (28/35) of all tested assays and at least one inconclusive call was found in 25.7% (9/35) tested assays; Table 4.2.For the PERCH samples, at least one no genotypic call was found in 77.1% (27/35) tested assays and at least one inconclusive call was found in 20% (7/35) tested assays; Table 4.2. For the BoSS cohort, SNP19 and SNP32 yielded the highest no genotypic call frequencies at42.86% (15/35) while SNP7 yielded the highest inconclusive frequency at 85.71% (30/35); Table 4.2. For the PERCH cohort, SNP34 yielded the highest no call frequency 100% (18/18) while SNPs 6,7 and 8 yielded the highest inconclusive frequencies at 100% (18/18); Table 4.2.

Table 4.2: Number of no genotypic calls and inconclusive alleles results for infants from the Babies of Soweto study and the Pneumonia etiology research for child project (PERCH)

	BoSS (N=34)		PERCH (N= 18)	
	No Call (%)	**Inconclusive (%)**	**No Call (%)**	**Inconclusive (%)**
SNP1	0	0	0	0
SNP2	7 (20.6%)	2 (5.9%)	1 (5.6%)	2 (11.1%)
SNP3	2 (5.9%)	0	1 (5.6%)	0
SNP4	4 (11.8%)	0	5 (27.8%)	0
SNP5	1 (2.9%)	0	2 (11.1%)	0
SNP6	0	20 (58.8%)	0	18 (100%)
SNP7	0	30 (88.2%)	0	18 (100%)
SNP8	0	19 (55.9%)	0	18 (100%)
SNP9	4 (11.8%)	0	4 (22.2%)	0
SNP10	3 (8.8%)	0	0	0
SNP11	5 (14.7%)	0	4 (22.2%)	0
SNP12	4 (11.8%)	4 (11.8%)	0	4 (22.2%)
SNP13	0	24 (70.6%)	0	14 (77.8%)
SNP14	3 (8.8%)	4 (11.8%)	0	6 (33.3%)
SNP15	6 (17.7%)	0	4 (22.2%)	0
SNP16	6 (17.7%)	0	4 (22.2%)	0
SNP17	5 (14.7%)	0	2 (11.1%)	0
SNP18	8 (23.5%)	1 (2.9%)	7 (38.9%)	0
SNP19	15 (44.1%)	7 (20.6%)	12 (66.7%)	0
SNP20	6 (17.7%)	0	4 (22.2%)	0
SNP21	3 (8.8%)	0	1 (5.6%)	0
SNP22	7 (20.6%)	0	5 (27.8%)	0
SNP23	5 (14.7%)	0	4 (22.2%)	0
SNP24	3 (8.8%)	0	3 (16.7%)	0
SNP25	0	0	3 (16.7%)	0
SNP26	4 (11.8%)	0	8 (44.4%)	0
SNP27	5 (14.8%)	0	4 (22.2%)	0
SNP28	2 (5.9%)	0	6 (33.3%)	0
SNP29	3 (8.8%)	0	3 (16.7%)	0
SNP30	5 (14.7%)	0	4 (22.2%)	0
SNP31	2 (5.9%)	0	2 (11.1%)	0
SNP32	15 (44.1%)	0	11 (61.1%)	0
SNP33	10 (29.4%)	0	6 (33.3%)	0
SNP34	4 (11.8%)	0	18 (100%)	0
SNP35	0	0	10 (55.6%)	0

4.3.2.1Frequency of pertussis toxin promoters stratified by time of sample collection

The frequency of SNP alleles associated with different pertussis toxin promoters in the 52 *B. pertussis* positive samples collected from children admitted during the period of 2011-2013 (n=18) and 2014-2015 (n=34) is shown in Figure 4.5. There was no difference in circulating *B. pertussis* strains containing pertussis toxin promoter 4 for the two study periods. In the period of 2011-2013, ptxP1 ranged in frequency from 11.11% (SNP21) – 44.44% (SNP12); in the period of 2014-2015, ptxP1 ranged in frequency from 2.94% (SNP21) – 29.41% (SNP12). The range in ptxP3 frequencies was higher in both periods: in the period of 2011-2013, ptxP3 ranged in frequency from 5.56% (SNP29 and SNP32) – 50% (SNP26); in the period of 2014-2015, ptxP3 ranged in frequency from 2.94% (SNP31) – 100% (SNP35).

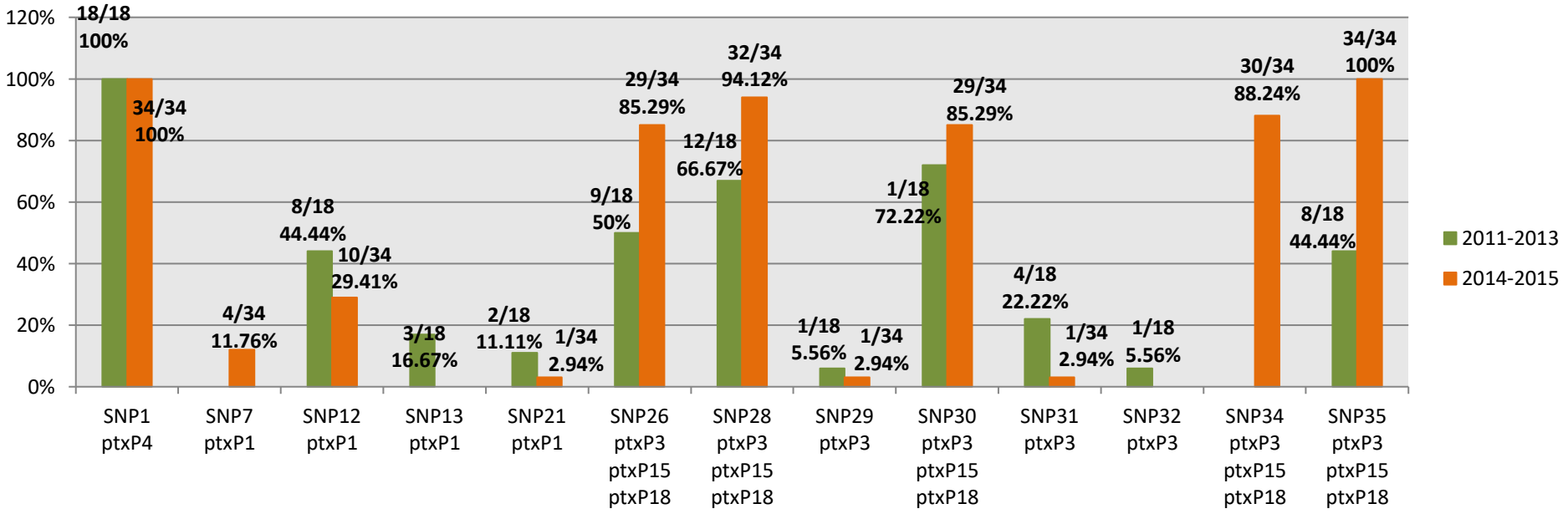


Figure 4.5: Graph showing frequency of pertussis toxin promoter alleles for the periods of 2011-2013 and 2014-2015

4.3.2.2Frequency of pertussis toxin promoters stratified by HIV status

The frequency of pertussis toxin promoter alleles for the 52 samples according to HIV exposure [HIV exposed (n=20), HIV unexposed (n=30)] is shown in Figure 4.6. Two infants had unknown HIV exposure information and were thus excluded from the current analysis. There was no difference in the frequency of ptxP4 between HIV-exposed and HIV-unexposed infants. The frequency ranges of ptxP1 and ptxP3 were also similar between HIV-exposed (5% [SNP13] - 30% [SNP12] and 0% [SNP29 and SNP32] - 85% [SNP35], respectively) and HIV-unexposed (0% [SNP21] - 36.7% [SNP12] and 3.3% [SNP32] - 90% [SNP28 and SNP30] respectively).

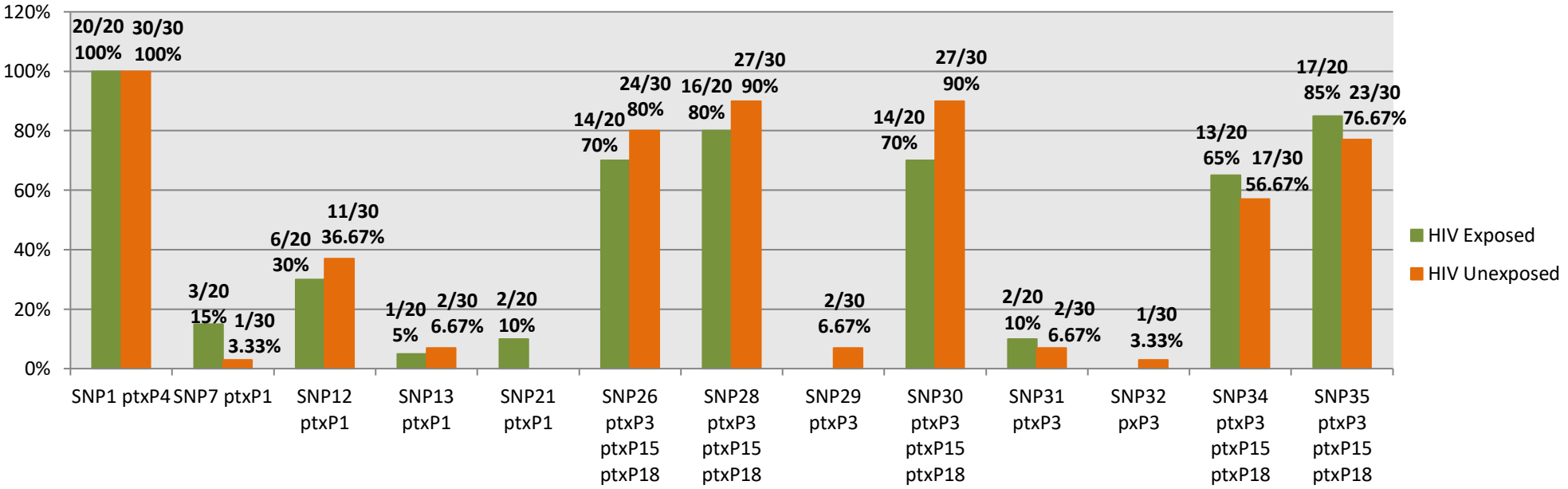


Figure 4.6: Graph showing frequency of pertussis toxin promoter frequency of alleles for HIV exposed and HIV unexposed infants

4.3.2.3Frequency of pertussis toxin promoters stratified by aP vaccination status

The frequency of pertussis toxin promoter alleles for the 52 samples according to aP vaccination doses received by the infant is shown in Figure 4.7. Thirty four infants received less than 2 doses while12 infants received 2 or more doses. Six infants had unknown vaccination information and were thus excluded from the current analysis. All samples had the SNP1 (ptxP4) allele. The ptxP3 tended to have higher frequencies than ptxP1 in infants who received ≥2 doses(75% vs. 41.67%) and <2 doses (91.18% vs. 29.41%).

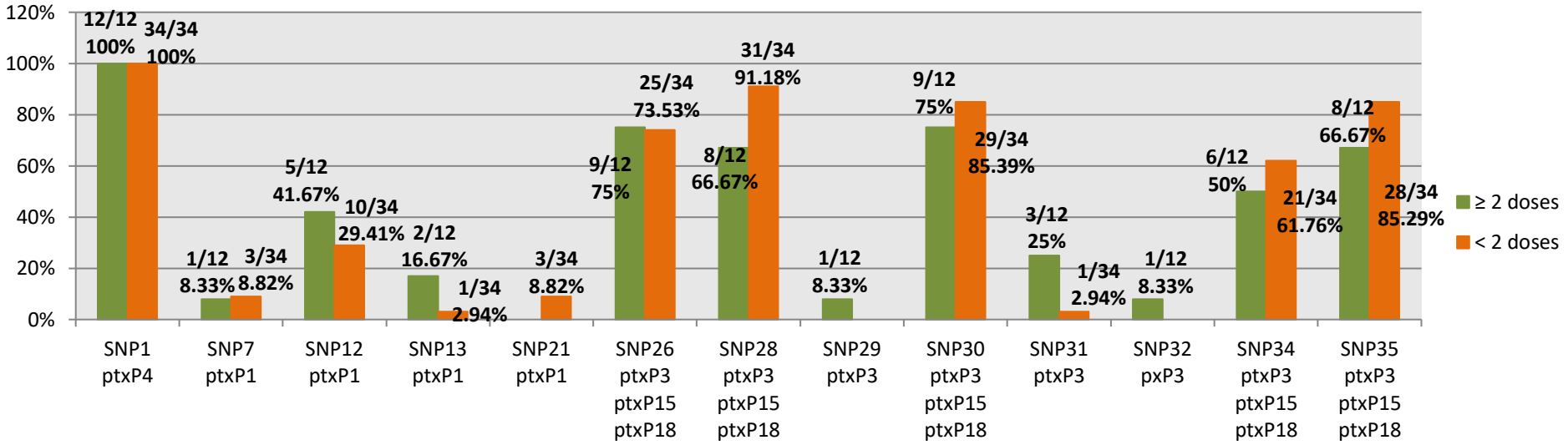


Figure 4.7: Graph showing frequency of pertussis toxin promoter frequency of alleles for infants who received less than 2 acellular pertussis vaccine doses as well as infants who received more than 2 acellular pertussis vaccine doses

4.4 Single nucleotide polymorphism variation

It has been suggested that genetic variance in unknown genes is due to bacterial adaptation to the host (150). This section explores the frequency of SNP variation in known and unknown genes (Table 4.3) and is further stratified by year of isolation, HIV exposure and vaccination dosage.

Table 4.3: List of known and unknown genes where the single nucleotide polymorphisms are located according to Zeddeman *et al* (187)

	SNP annotation in this study	**Gene in which SNP is located**
<u>Known genes</u> n=13	SNP1	*B. pertussis* autotransporter protein C (*bapC)*
	SNP3	Dermonecrotic toxin *(dnt)*
	SNP5	Glutathione reductase *(gor)*
	SNP10	*ptxA*
	SNP13	*risS*
	SNP20	Bordetella legiolysin *(bllY)*
	SNP21	High temperature protein G *(htpG)*
	SNP25	*ptxP*
	SNP26	*ptxC*
	SNP29	Ribosomal protein *(rplV)*
	SNP30	Type III secretion protein *(bscI)*
	SNP34	*fim3*
	SNP35	Phosphoprotein phosphatase *(prpB)*
<u>Unknown genes</u> n=22	SNP2	N/ A
	SNP4	
	SNP6	
	SNP7-SNP9	
	SNP11-SNP12	
	SNP14-SNP19	
	SNP22-SNP24	
	SNP27-SNP28	
	SNP31-SNP33	

Overall genetic variation in unknown genes was 11/22 (50%); Figure 4.8. Isolates with allelic divergence from the reference strain (TahomaI) was observed for several known genes namely: *bapC*(SNP1) 100%, *gor*(SNP5) 79.6%, *ptxA*(SNP10) 100%, *risS*(SNP13) 21.4%, *htpG*(SNP21) 6.3%, *ptxP*(SNP25) 100%, *ptxC*(SNP26) 95%, *rpIV*(SNP29) 4.4%, *bscI*(SNP30) 97.7%, *fim3*(SNP34): 100% and *prpB*(SNP35): 100%; Figure 4.9 (no genotypic call and inconclusive alleles were excluded from the analysis).

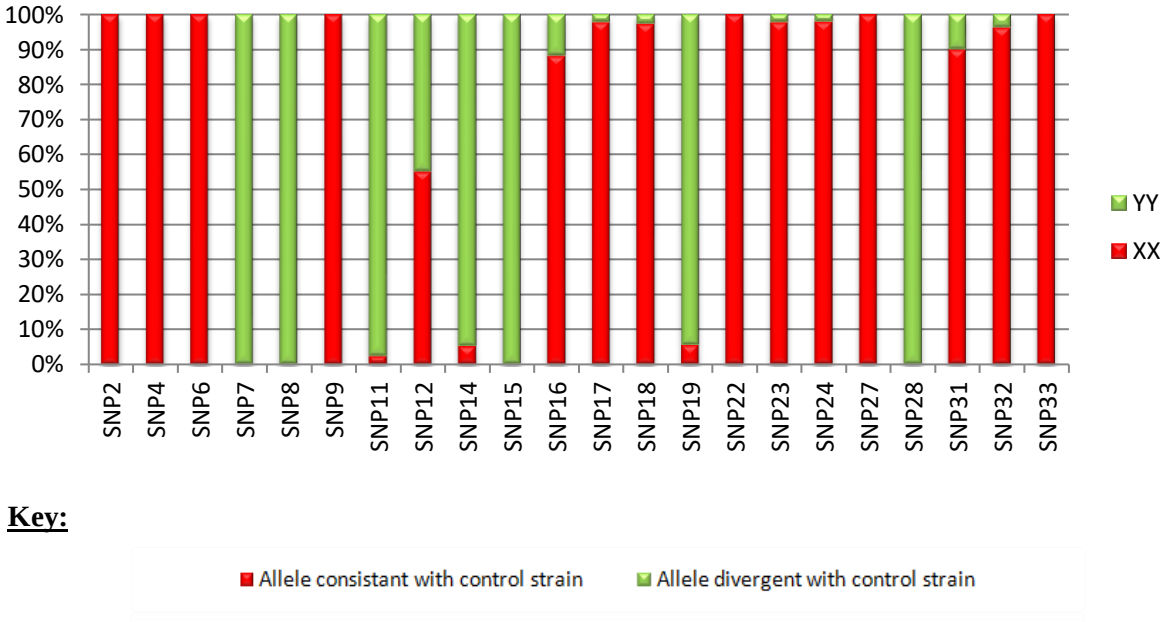


Figure 4.8: Overall frequency of genetic variation in SNPs targeting unknown genes for clinical samples positive for *B. pertussis*

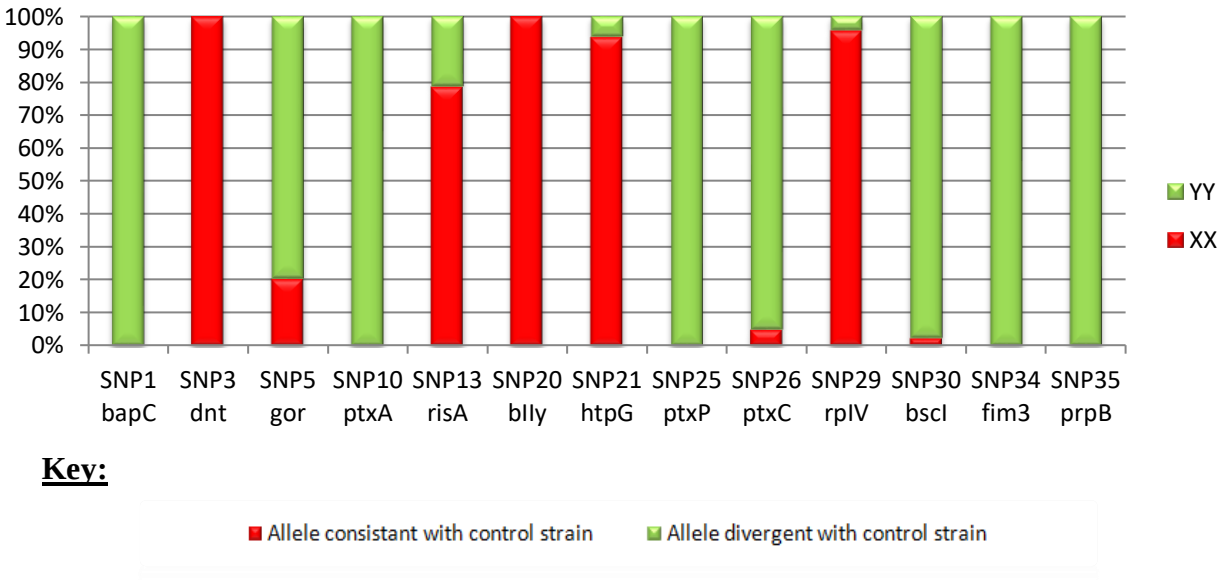


Figure 4.9: Overall frequency of genetic variation in SNPs targeting known genes for clinical samples positive for *B. pertussis*

4.4.1 Single nucleotide polymorphism variation according to period of sample collection

Eighteen *B. pertussis* positive samples collected during 2011-2013 and thirty-four collected during 2014-2015 were retrospectively tested for the 35 SNP targets.

Divergence from the TohamaI reference strain in assays were found at 40% (14/35) and 31.4% (11/35) for 2011-2013 and 2014-2015 respectively. Of the divergent strains 64.3% (9/14) and 54.6% (6/11) were in genes with unknown location for study periods 2011-2013 and 2014-2015, respectively; Figure 4.10. The divergence from the TohamaI reference strain for known genes tended to be lower in samples collected during 2011-2013 [35.7% (5/14)] than in 2014-2015[45.5% (5/11)].

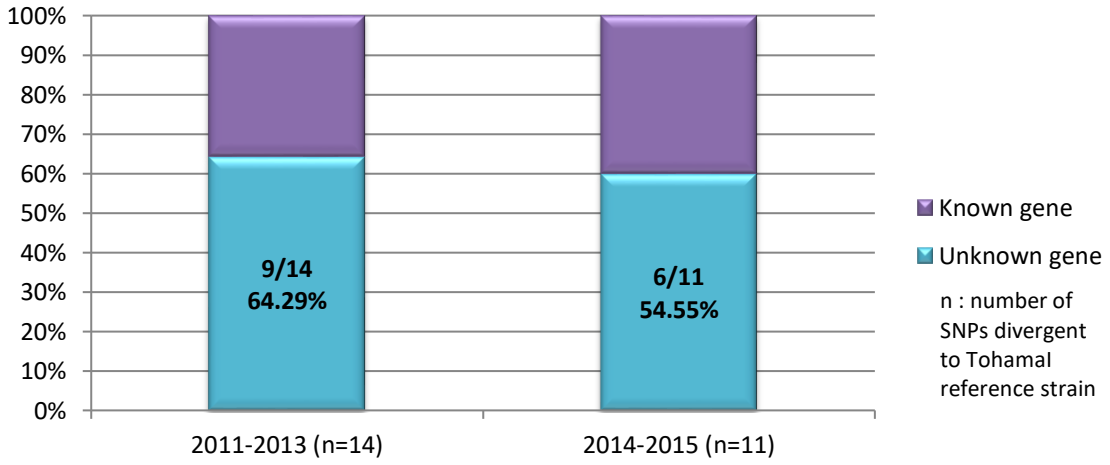


Figure 4.10: Single nucleotide polymorphisms that were divergent to TohamaI reference strain for known and unknown genes according to year

4.4.2 Single nucleotide polymorphism variation according to HIV-exposure status

Twenty HIV exposed infants and30 HIV-unexposed infants were collected. Two infants had unknown HIV exposure status and were thus excluded from this analysis. Divergences from the TohamaI reference strain were found in 42.9% (15/35) and 34.3% (12/35) of the SNP reactions for HIV exposed and HIV unexposed infants, respectively. Of those divergent strains 60% (9/15) and 50% (6/12) were in genes with unknown location for HIV exposed and HIV unexposed infants respectively; Figure 4.11.

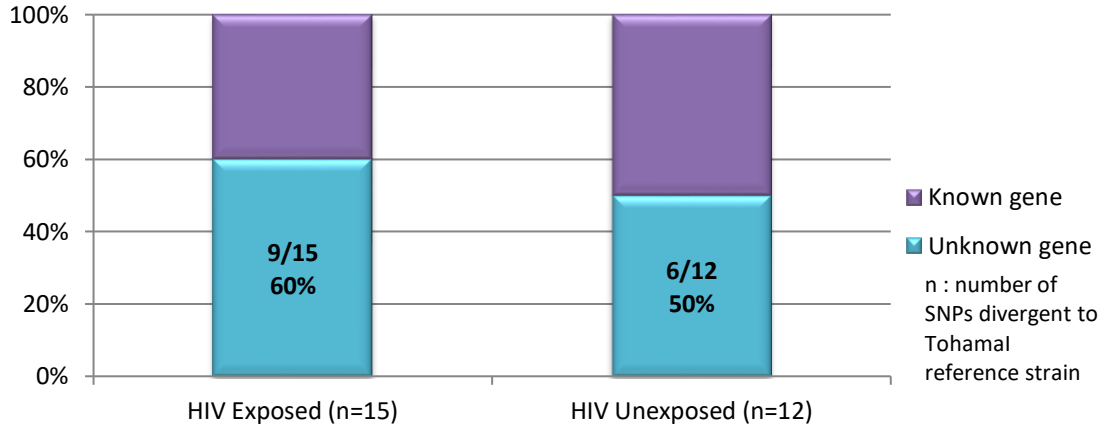


Figure 4.11: Single nucleotide polymorphisms that were divergent to TohamaI reference strain for known and unknown genes according to HIV exposure of infant

4.4.3 Single nucleotide polymorphism variation according to vaccination status

Since South Africa transitioned to the aP in 2009, the aP vaccine is scheduled to be given at 6,10 and 14 weeks of age (15). Table 4.4 shows the number of vaccine doses that the children included in this analysis received accordingly to their age. Six children with unknown vaccination history were excluded from the analysis.

Table 4.4: Number of vaccine doses received by the study participants

Age	**N of participants**	**Number of aP vaccine doses**	**Vaccination status**
<6 weeks	15	N/A	N/A
6 - <10 weeks	16	0 doses - 7	Unvaccinated – 7
		1 dose – 9	Fully vaccinated - 9
		2 doses – N/A	N/A
		3 doses – N/A	N/A
10 - <14 weeks	8	0 dose – 1	Unvaccinated – 1
		1 dose – 1	Partially vaccinated – 1
		2 doses – 6	Fully vaccinated – 6
		3 doses – N/A	N/A

N/A: Not applicable

Table 4.4: *continued*

Age	**N of participants**	**Number of aP vaccine doses**	**Vaccination status**
≥14 weeks	7	0 doses – 1	Unvaccinated – 1
		1 dose – 0	Partially vaccinated – 0
		2 doses – 3	Partially vaccinated – 3
		3 doses - 3	Fully vaccinated – 3

N/A: Not applicable

Divergence from the TohamaI reference strain were found in 8.6% (3/35) of the assays for unvaccinated infants, 2.9% (1/35) for partially vaccinated infants and increased to 25.7% (9/35) for fully vaccinated infants according to their age. Of the divergent strains 33.3% (1/3) and 66.7% (6/9) were in genes with unknown location for unvaccinated and fully vaccinated infants respectively; Figure 4.12. For partially vaccinated infants, divergence was found only in a known gene: Glutathione reductase (SNP5 in this study). The divergence from the TohamaI reference strain for known genes was higher for known genes for unvaccinated infants: 66.7% (2/3) than fully vaccinated infants: 33.3% (3/9).

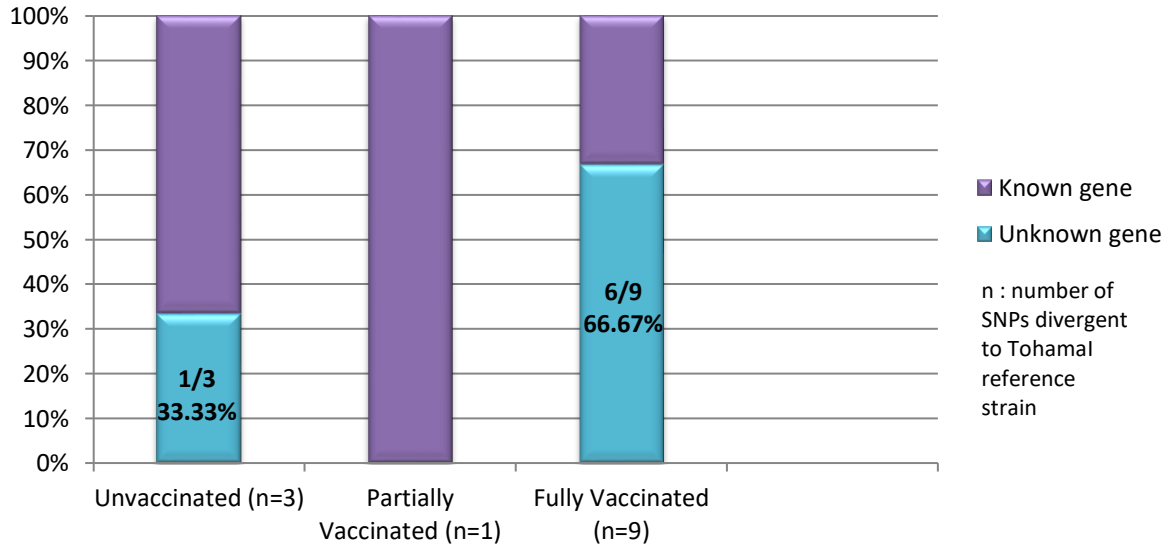


Figure 4.12: Single nucleotide polymorphisms that were divergent to TohamaI reference strain for known and unknown genes according to vaccination history of infant

Strains that carry the ptxP3 promoter allele may produce more virulence factors than the strains carrying the ptxP1 promoter allele (185), this includes Type III secretion toxin virulence factor (199), SNP30 in this study. Figure 4.13 shows the frequency of Type III secretion toxin from 2011-2013: 72.2% and a steady increase to 85.3% in the period of 2014-2015

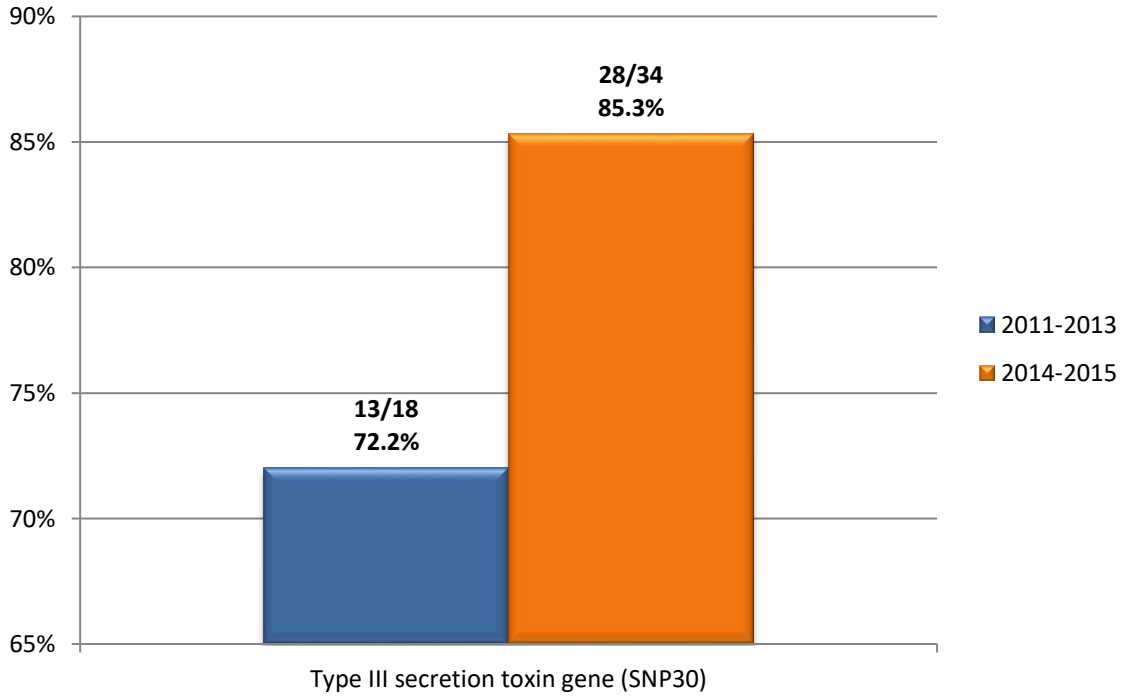


Figure 4.13: Frequency of Type III Secretion Toxin Gene (SNP30) from the periods 2011-2013 and 2014-2015

4.5 Discussion

Pertussis disease continues to cause a significant burden in LMICs (200) and the variable vaccination coverage in LMICs has led to heterogeneity in pertussis disease burden (201).

Strains carrying the ptxP3 allele have been observed in both pre and post vaccination (145). The main findings of this chapter was the expansion of ptxP3 strains in this cohort as seen worldwide. It has been suggested that strains having the ptxP3 promoter have an increased capacity to spread within populations (48) and thus strains carrying the ptxP3 allele have expanded worldwide (48, 52, 148, 185, 202). Furthermore, ptxP3 strains have gradually replaced

ptxP1 strains (49). The results from our study although not conclusive tend to support this observation.

Pertussis disease in relation to HIV status is poorly described (89) however, a study conducted in South Africa from 1 August 2013 to 31 October 2015 assessing pertussis disease in 1268 hospitalised children less than 10 years of age found no association between HIV exposure and pertussis infection (p=0.90) (89). Since no literature exists about pertussis toxin promoter alleles and HIV exposure our study aimed to explore HIV exposure in relation to pertussis toxin promoter alleles.

This study shows that the frequency ranges for ptxP1 and ptxP3 between HIV exposed and unexposed infants were similar. The frequencies of ptxP3 alleles were, however higher compared to the frequencies ptxP1 in both HIV exposed and HIV unexposed. Frequency range of 5 - 30% for ptxP1 for HIV exposed infants and a frequency range of 0 - 85% for ptxP3 for HIV exposed infants was observed. For HIV unexposed infants a ptxP1 range was 0 - 36.7% was observed while the ptxP3 range for HIV unexposed infants was 3.3 - 90%; Figure 4.6.

B. pertussis strains carrying the ptxP3 allele may have the ability to evade aP vaccine induced protection (149). From the infants enrolled in BoSS study, 32.4% (11/34) received ≥2 aP doses and similarly, 38.9% (7/18) of the infants enrolled in PERCH received ≥2 aP doses. In the analysis of aP vaccine doses, the ptxP1 allele frequency range tended to be higher in infants who received ≥2 doses (0-41.7%) compared to those who received <2 doses (2.9-29.4%). Furthermore, infants who received ≥2 doses had a ptxP3 allele frequency slightly lower than the ptxP3 allele frequency (8.3-75%) of infants who received <2 doses (0-91.2%). Overall, ptx3 were observed at higher frequencies than ptxP1, Figure 4.7.

It has also been suggested that strains that carry the ptxP3 promoter allele produce more virulence factors than the strains carrying the ptxP1 promoter allele (185), this includes the Type III secretion toxin (199). Interestingly, the Type III secretion toxin gene associated SNP (SNP30 in this study) was found in 72.2% of isolates collected from 2011-2013 and increased as the frequency of ptxP3 increased to 85.3% of isolates collected from 2014-2015 (Figure 4.13)supporting the hypothesis that ptxP3 strains produce more of the Type III secretion toxin virulence factor (199).

Sequences with important functionality tend to be less variable (203) and SNPs in these regions are useful markers for evolutionary studies of pathogens (140). In a study by Xu *et al*., the authors analysed 29 Chinese and 11 Finnish *B. pertussis* isolates from 1956-2008 and identified 120 SNPs (204). Of these50.8%, (61/120) were SNPs located at non-coding regions or at genes of unknown function (204). SNPs in known or unknown genes may relate to their importance in the adaptation of *B. pertussis* (150).

In this study similar divergence was found in known and unknown genes: divergence from the TohamaI reference strain in alleles for unknown genes was 50% (11/22); Figure 4.8.

For known genes, 46.2% variability was found (Figure 4.9). When stratifying for year of isolation, divergence in unknown genes was found in 64.29% in 2011-2013 and 54.55% in 2014-2015 (Figure 4.10) and 60%in HIV exposed infants (Figure 4.11). In terms of vaccination, only fully vaccinated infants had a higher divergence in unknown genes (66.67%) compared to known genes (33.33%); Figure 4.12. Unvaccinated infants had a higher divergence in known genes (66.67%) compared to unknown genes (33.33%) while partially vaccinated infants had variability only in a known gene: Glutathione reductase (SNP5).

SNP alleles were treated as 'no genotypic call' if neither an SNPType-FAM nor a SNPType-HEX intensity was detected and inconclusive if the resultant SNP allele was an intermediate, i.e. both SNPType-FAM and SNPType-HEX probes were intensified. No genotypic call alleles were tested at least twice to rule out an error that may have occurred during sample loading. For some SNPs the frequency of no call or inconclusive results was as high (as high as 88.2% for inconclusive alleles and 100% no call alleles as seen in the PERCH cohort; Table 4.2). The isolates from the PERCH cohort was isolated in 2011-2013; it should be taken into consideration that factors such as poor DNA quality(205)or presence of secondary SNPs (206) could prevent amplification of target SNP causing no call SNPs. Even so, it can't be ruled out that perhaps novel SNPs have been generated at these regions. Identifying uncharacterised SNPs may offer a better understanding to disease pathogenesis (207).With no call SNPs as well as inconclusive data from this study there is room for future projects to expand on this technique to study *B. pertussis* SNPs. Future studies may confirm novel SNPs (from no call or inconclusive allele results for example) by sequencing.

In conclusion, in this cohort the ptxP3 allele seems to be expanding and is most likely gradually replacing the ptxP1 allele in agreement with studies conducted globally on the ptxP3 allele.

CHAPTER 5: INTEGRATED DISCUSSION AND CONCLUSION

The estimates of the burden of pertussis disease in LMICs are based on sparse data and there is no information on the circulating strains (208). The aims of the studies reported in chapters 2 and 4 were to provide data on pertussis cases from the Soweto region in South Africa, albeit small number of cases. In chapter 3, I present on the development of a new tool for the analysis of pertussis single nucleotide polymorphisms (SNPs). With the development of novel laboratory tools for genetic analysis, the evolution of B. *pertussis* can be better documented. This technique will be useful for the documentation of the expansion of ptxP3 strains in certain regions. Tracking ptxP3 strains is important as these strains have been observed to be more virulent (48).

This study was conducted in the Soweto region of South Africa and found no protein deficiencies from isolates analysed by Western Blot analysis for PRN, fim2, fim3 and PT.

In terms of SNP analysis, genes with important functionality yielded uniform alleles, namely, *B. pertussis* autotransporter protein C (SNP1 in this study), Dermonecrotic toxin (SNP3 in this study) as well as Pertussis toxin subunit 1 (SNP10 in this study) for example yielded uniform alleles (excluding no genotypic call and inconclusive alleles). The majority of variable alleles observed were located in unknown genes. The study also provides a new tool to investigate *B. pertussis* genetics and has shown that ptxP3 may be replacing ptxP1 strains in South African, as seen globally.

This study had limitations. Firstly, being a retrospective study, the quality of DNA used for SNP analysis could have been compromised. Secondly, because no other study (to our knowledge) has used Fluidigm technology to analyse virulence-associated SNPs published by Zeddeman *et al* (187) in a South African cohort, a comparison could not be made. Also, Fluidigm technology could not detect novel SNPs.

Our study however shed some light on protein expression and on SNPs carried by *B. pertussis* strains from South African hospitalised infants. The tool developed during this project will be useful for future studies to identify pertussis toxin alleles in strains.

CHAPTER 6: REFERENCES

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CHAPTER 7: APPENDICES

Appendix A: Culture: *B. pertussis* control strain

B. pertussis culture results

Subject ID

Indicate sample type cultured:

Nasopharyngeal flocked swab in transport media	Induced sputum	Other specify:

Date & Time received: D D M M M 2 0 1 Y H H : M M

Date & Time plated in laboratory: D D M M M 2 0 1 Y H H : M M

Primary media – Charcoal agar:

Time incubated in CO2	No Growth	< 25 colonies in quadrant 1	≥ 25 colonies in quadrant 1 & < 25 colonies in quadrant 2	≥ 25 colonies in quadrant 2 & < 25 colonies in quadrant 3	≥ 25 colonies in quadrant 3 & < 25 colonies in quadrant 4	≥ 25 colonies in quadrant 4	Done By:
		Scant	1+	2+	3+	4+	
24hrs							
48hrs							
72hrs							
96hrs							
120hrs							
144hrs							
168hrs							
Day 10							

(Quantify Pertussis-like growth only which is typically small, round, high domed convex, grey, shiny, mercury-like colonies with a smooth texture)

Contaminating bacteria seen – Comment & Quantify:

Appendix B: Real time Polymerase Chain Reaction (PCR) assay algorithms[1]

Table 1: Algorithm to interpret the real time PCR resultsfor pertussis testing as described by Centers for Disease Control and prevention (CDC)

PCR Targets				Interpretation
IS481	p*IS*1001	h*IS*1001	ptxS1	
Positive	Negative	Negative	Positive	*B. pertussis*
Positive*	Negative	Negative	Negative	*B. pertussis*
Positive	Negative	Positive	Negative	*B. holmesii*
Negative	Positive	Negative	Positive	*B. parapertussis*
Negative	Positive	Negative	Negative	*B. parapertussis*
Negative	Negative	Negative	Positive	*B. bronchiseptica*

*If cycle-threshold (CT) for IS481 is 35-40 cycles and is ptx S1 negative, specimen will be considered 'intermediate'

Table 2: Primers and probes sequences utilized in real time PCR

Gene Name	Sequence 5'-3'
IS481	Forward primer:CAAGGCCGAACGCTTCAT
	Reverse primer: GAGTTCTGGTAGGTGTGAGCGTAA
	Probe: 5'-NED-CAGTCGGCCTTGCGTGAGTGGG-MGB-3'
ptx S1	Forward primer: CGCCAGCTCGTACTTC
	Reverse primer: GATACGGCCGGCATT
	Probe: 5'-VIC-AATACGTCGACACTTATGGCGA-MBG-3'
h*IS*1001	Forward primer: GGCGACAGCGAGACAGAATC
	Reverse primer: GCCGCCTTGGCTCACTT
	Probe: 5'-VIC-CGTGCAGATAGGCTTTTAGCTTGAGCGC-MGB-3'
p*IS*1001	Forward primer: TCGAACGCGTGGAATGG
	Reverse primer: GGCCGTTGGCTTCAAATAGA
	Probe: 5'-FAM-AGACCCAGGGCGCACGTGTC-MGB-3'

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Appendix C: Western Blot working solutions

Table 3: Working solutions for western Blot

Solution	**Reagents**	**Volume**
4 x Lower Buffer	Tris Base (1.5M) 20% SDS (0.4%) V_{tot}425 ml with dH_2O adjust pH to 8.8 V_{tot} to 500 ml	90.85 g 10 ml
4 x Upper Buffer	Tris Base (0.5M) 20% SDS (0.4%) V_{tot}425 ml with dH_2O adjust pH to 6.8 V_{tot} to 500 ml	30.3 g 10 ml
10 x Running Buffer	Tris Base (250 mM) Glycine (1.92 M) SDS V_{tot} 5 000 ml with dH_2O	151.4 g 720.7 g 50 g
10 x Blotting (transfer) Buffer	Tris Base (250 mM) Glycine (1.92 M) V_{tot} 5 000 ml with dH_2O	151.4 g 720.7 g
1 x Blotting Buffer	10 x Blotting Buffer Methanol dH_2O	100 ml 200 ml 700 ml
Ponceau S	Glacial acetic acid Ponceau S V_{tot}100 ml with dH_2O	1 ml 0.5 g
Phosphate Buffered Saline	NaCl KCl Na_2HPO_4 KH_2PO_4 dH_2O	4 g 0.1 g 0.55 g 0.15 g 498.5 ml
Wash Buffer	1L PBS/0.1%Tween	
Blocking Buffer	50ml PBS/0.1%Tween/2% Skim milk/1% BSA	

Table 4: Components for two 0.75 mm acrylamide gels (running gel)

Gel Percentage	Reagents	Volume (ml)
10% (For Pertactin and pertussis toxin)	dH_2O	4.11
	30% Bis-acrylamide	3.33
	Lower Buffer	2.50
	10% APS	0.05
	TEMED	0.01
	Total	10
12.5 % (For Fim2/3)	dH_2O	3.27
	30% Bis-acrylamide	4.17
	Lower Buffer	2.50
	10% APS	0.05
	TEMED	0.01
	Total	10

Table 5: Components for two 0.75 mm acrylamide gels (stacking gel)

Gel Percentage	Reagents	Volume (ml)
4.5% (For Pertactin, Fim2/3 and pertussis toxin)	dH_2O	2.94
	30% Bis-acrylamide	0.75
	Upper Buffer	1.25
	10% APS	0.05
	TEMED	0.02
	Total	5

Appendix D: High Throughput Single Nucleotide Polymorphism Genotyping using Fluidigm Technology

Table 7: Fluidigm SNP Sequence Information for Fluidigm Assay Project 7074FSTP16O1

SNP	SNP Sequence	AMP GC
SNP1	GCCCGGCCACCGCTCAGGTCGGTGCGCAGCCCGTACCCGTTGGTGCGAACGGTCGTGACGCCTTTGAATTCCTGCAGCCAGCTCAGGGTGGCATAGGGCTGGATCACG CGGTCCTTGCCCAGGTCGATGCGTCGCCCGGCCGCCAAGCCCAGGCGCAGCACGGCGGAGGTGCCGCCTTCGTCCTTGACCGACAGGTTGTTCGCGGCACGGTAGGTT CCGCCGCTGGCATGGAACAGCGACACCTCGGACTG**[A/G]**GGTTCGACGAACCAGCCGTCGTGCAACGTGAAACGTTTGCCGGCCTCCAAGGTGGCGCCTACCCCATTG GCCCGGTACTTGCCCCGTACGGAAACGGC	0.64
SNP2	CCAGGCACGACAGGCGCGCATGCACCCTGCCCACTCGCCAGTTGGCGATGCGTAACGCGCATTGCGCGCCGCGCCCGATGGTCACCGGCGCGGCCAGCCGCATGCGT CGGCGGGCGCCATCCTCGAAATGCAGCGCCAGTTCGAGAACGTTCATGGGGCGCCCTCCTGTGCGTCGGCCCAAAGCGGCCGGCGCCATTGCGAACCCGCGCCCCCC GGACGGCTCCAGTCGGCGGACTGGTCGGCCGCCGCGCCCGTGCCCAGCCGCGGGGCTTCGGGAAATGCCTGATCCAGCTGCATCTGCCCCAGCAGCGCGCGGCGTTG CAAGAATGAATCC**[G/A]**AGGGAGAGACGATGGTGGGCGTCACGAAAATGGCCAGCTCGGTTTCGCGCAGGGCGGCACGGCGCGCGCCGAACAAGGCGCCGATCAAG GGCAGCTCGCTCAGCCACGGCAGGCCTTTTTCCTCGTGCGATCGTTCACGCGACAGGAAACCGCCCAACAC	0.6
SNP3	ATCCGCACGCGTAATCTGGTCGCCCTTGCCGCCGAGCAGGCCGCGCCGATGCGCCGCCTGCTCAACCAGGCGAGGCGTGTGGCGCTCAGGCATATCGATACCTGCAGG AGCAGGCTTGCGTTGCCGCGCGCTGAATCCGATATGGACGCGGCGATCCGGATTTTCTTCGGAGAGCCGGACGCCGGCCTTCGCCAGCGCATCGGGCGACGCCTGCAG GAGGTCAGGGCCTATATCGGCGATCTGAGTCCGGTCAATGACGTGCTGTACCGGGCGGGATATGACCTCGACGATGTCGCAACGCTGTTTAACGCAGTGGACCGGAA CACGTCGCTGGGCAGGCAGGCTCGGATGGAGTTGTATCTGGATGCCATTGTCGATTTGCATGCCAGGCTCGGCTATGAAAATGCGCGTTTTGTCGACCTGATGGCGTT CCACCTGCTCAGCCTG**[G/A]**GCCATGCCGCGACGGCCAGTGAGGTCGTGGAGGCCGTTTCGCCCCGGC	0.72
SNP4	GGGTCTGGCCCTTGCTGGAAAACGGCACCCTCAAGCCGGTGGTGCACGCCACGCTGCCGCTGGAGCAGGCCGCCGCGGCCCATGCCATGATGGAAGCCGGGGAAAAC ATCGGCAAGATCATCCTGACCGTATAGCGCCAGCCGGACGGCGGGCGCGCCGGATCGCCCGGTGCGCCCGGATTTTCTTGCCCTGGTAACGGCGCATCGGCCGGCAA CAGGATACAATCTn**[C/T]**AGGTTTGACCCGACCCCGGGTCAGGCCTTTTGACTCTTTTTTCTGCGCCATGACCACAGCTGAGAATCGCGCCCGCCTGGTGCTGGGCAACT GGAAGATGCACGGCAACCTGGCCGAGAACGCCGCCTTGCTGGCCGAGTTGCGCGCCGCCGATGCCGCCGCCCATTGCGAAATGGGGGTGTGCGTGCCGTTTCCGTAC CTGGCCCAGACCGCTGCCGCCTTGCAGGGCAGCGCCATCGGCTGGGGCGCGCAGGATGTCAGC	0.61
SNP5	CCTGGGCGCGCAGACCGTGCAGGTCTACCGGCGCGAACTGTTCCTGCGCGGCTTCGACGGCAGCGTGCGCGAGCACCTGCGCGACGAACTGGTCAAGAAAGGCCTGG ACCTGCGCTTCAATACCGACGTCGAACGCATCGACAAGCGCGCCGATGGCGTGCTGGCGGTCACGCTGTCCGACGGCTCGGTGCTCGAAACCGATTGCGTCTTCTACG CCACCGGCCGGCGCGCCATGCTCGACGACCTGGGCCTGGAGAACACCGGCGTGCGGCTGGCCGAGTCCGGCTACATCGAGGTGGACGACGAATACCGCACCAGCGAG CCGTCCATCCTCGCCATCGGCGACGTGATCGGCCGCGTCCGCTCACCCCCGTGGCGCTGGCCGAAGGCATGGCCGTGGCGCGCCGCCTGTTCCGCCCGCAGGAGTAC CGCAAGGTCGACTACGACCTCATCCCCACC**[G/A]**CCGTCTTCAGCCTGCCCAACATCGGCACGGTCGGC	0.69
SNP6	AGCAGGTCGAACTGTCCCTGGTGCAGGCCGGCGGCGCGAAACAGCGGCTCGATGCGCCGCAGCGCGGCGGTATTGAACCGGAAAATACGCGCCAGCACGGCCATGG ACGAGACGTCCAGGTCGGGGCGTTCGTGCGCCCATTGGGACAGCACCAGGTCGACGTGGTCTTTCATTATTGTCTTGACGTAAAGTATCTTTATATCAAGATAAAAGC GGCCCGGTCAAAT**[C/T]**GCCGCCGCATAAACGAAGAGCCCGCCGGTGCGGCGGGCTCTGCGGTTTGACCTGAGACGTCAGGCGCTTACTGCGCGGCGTCCTTCAGCTT CTTCAGGGGACGAACCTTGACCTTCACCGAGGCGGGCTTGGCCGGGAACCAGCGCTCTTCGCCGGTGAACGGGTCCTTGCCGAAGCGCTTGGCCTTGGCGG	0.56

Red allele: allele corresponding to reference allele
Green allele: allele divergent from reference allele
AMP GC: Amplicon GC content

Table 7: *continued*

SNP	SNP Sequence	AMP GC
SNP7	CGCCCTGCTCGCCCAGGCCGTGGCCGAGGGCGCGCGCCAGTCCGGCGCCGCCGCCACGCTGCTGTTTCTCGACGACTACATCGCGGGCTTCCTGACGGACGAGAAAG AGGCCGATGCGCGGCGCCCCGACGACCGCTACGGCGAACTGTTCTTCGACCACTTCCTGCCCGCGCGCGCCGTGGTGCTCTGCACGCCGGTGTACTGGTACGGCATGT CGGCCCAGGCCAAGGCCTTCTTCGACCGCTCTTTCACCTATTACTCGGCCAGCCATCCCCGCCACGCCGAGGTGCTGCGGCGCATGACGGGCAAGCGGCTGGCGCTGG CCGTATCCTCCGAGGAAACCTACCCCGGCGCGGCCCTGGGCATCGTGCACCAGTTGCAGGAGTTCACGCGTTACACCCACTCGCAGTTCGTCGGCATGGTGCACGG**[G/A]**GCGGGCAACCGCCGAGGCGAAGTGGCGCACGATCCGCGCCGTCCGCTGCAAGCCGCGCACCGGCTG	0.75
SNP8	CGATGAGGCCGGCGTGGCCGGCTACGATGTGCAGACCTGGTACGGCATCCATGCGCCGGCGGGCACGCCGGCGGCGGTCGTGGATCGGCTCAACCAGGCCCTGGTGG CCGTCCTGTCCAACGCCCAGGTGCGCGCGGCGCTGGTGGGGCAGGGCTATGAGGTTGCCACCAGCACCCCCGGCGAGTTTTCCCGGATGGTGCGCGACGACGTGGCG AAGTGGCGCAAGGTCGTGAAGGAGGCCAAGGTCAAGGTCGACTGAGCCGGCGC**[G/A]**CAGGTGTCTTGTATCTTTCCTCCGGTGGTGATTAGCTATCACCACCGCACG CCGATGACCGCGAGTTGGTGCCCAAAGCGAGGACTTTGTGGGACAGATAGCGGCTCGGCAGCGCACTGCTGGGCCGGCGAGACACTGGACGGTATCGTAGGGCGAG GACCCTGTATGTACAAGGCAAGTGGGCGCAGTGGCTGACAGCAGTTTGTTATCTGCAGGAGGCCA	0.63
SNP9	CATCGATATAACATCGAGAATGCCAATGGACACCTGCCCGGACAGCAGCTCGGTGATTGCCTCGCTGCCACCCTTGTATGGAATGTGCAGCATTTTCATGCCTGCCAA CTCCTGTATTTTCTGCCCGGCGAGATGGGGTGGAGAGGCGACGCCAAAGGTAGCAAAAGAAATACTATCTTCATTTTGTTTTGCCGCTGCGACAAGATCTGAAATCGA GTTCAGTGGTGAGTTCGCACGCGTTACCAC**[C/T]**AGCAACGGCACCATGGATATCTGCGTTATTGCAGTAAAGTCCTTGATGGGGTGAAAGCTGAGATCTGGAAAAAT GGCGGGAGCGATGGCAAATGGAGCGGATGTCATTAATAGTGTATAGCCATCATTATCCGCACGCGCCACGGAAGCCGCCCCAATATTACCGGCAGCTCCACTCCGATT TACCACAATGACCGGCTGCTTTAACTCCCTGCCTAACTGTTCTGCAAGGGCGCGGCCCATG	0.6
SNP10	CCATCTGACCGGACGTTCCTGCCAGGTCGGCAGCAGCAACAGCGCTTTCGTCTCCACCAGCAGCAGCCGGCGCTATACCGAGGTCTATCTCGAACATCGCATGCAGGA AGCGGTCGAGGCCGAACGCGCCGGCAGGGGCACCGGCCACTTCATCGGCTACATCTACGAAGTCCGCGCCGACAACAATTTCTACGGCGCCGCCAGCTCGTACTTCG AATACGTCGACACTTATGGCGACAATGCCGGCCGTATCCTCGCCGGCGCGCTGGCCACCTACCAGAGCGAATATCTGGCACACCGGCGCATTCCGCCCGAAAACATCC GCAGGGTAACGCGGGTCTATCACAACGGCATCACCGGCGAGACCACGACCACGGAGTATTCCAACGCTCGCTACGTCAGCCAGCAGACTCGCGCCAATCCCAACCCC TACACATCGCGAAGGTCCGTAGCGTCGATCGTCGGCACATTGGTGCGCAT**[G/A]**GCGCCGGTGATAGGC	0.68
SNP11	ATGGAAAGCTCCTGTCGATGCGATGAAACAGCGGGCGGTGTATGCTTGCATACCTGCCGGGCCGGCTGCCTTTGCAGGCAGGCCGGCGCGCCGCTTGCGAGGGGCCTT CGGGCCGCCCGCGTGGCCGCCCCCCGATTGCGTCACGCAAGCATACGCCTGTGGCGCGGGCGGCGTTCAGCGCAGTTGGGCATTCGTCCCATTTTTTTGTCTGTCCATA GGGGAAAAACGCAACTTCAGTTGCCACCAGGCAACTTTAAGTGCTAGAATTTCCC**[A/G]**TTTTTCACAACCAATTCAAGGGATTACCTATGCTGGTGATTCGTCTGGCC CGCGGTGGCTCGAAGAAGCGTCCGTTTTACAACCTGGTAGCTACCGATTCGCGCAATCGTCGCGATGGCCGTTTCGTCGAGCGCGTTGGCTTTTACAACCCGGTTGCCG CTGAAGGCACCGAGAATCTGCGCATCGCCCTGGACCGCGTGCAATACTGGACCGGCAA	0.41
SNP12	CGTGCGCGAGCTGGTCGAACGCTTCCTGGGCCTGCGGCGTCAGGCCGTCGAGCCGGGCGCCGATCCAGCCCACCGCGAACCAGGCCTGCGGGTGCGCCTGCAGCGCC GCCTGGTAGTAATCGCGCGCCACCGCCAGATCGTTGATCTCGCCCAGCAGGTCCTGCACGCGGGCCAGTTGCTTGCGATAGGTGCGCAGCCGGGCTGCCGGCAGCAG GGATTCGGCGAAC**[G/A]**CAGACCGTAGCGCAGGCGCTTGACCCGTTTGCGCAGCTCGTGGCGCGCCTCCAGCTCCAGCGCGCCGAAGCGCAGGCCTTCGTCGATGACC TTGAGATGCCAGCGGCGCAGCCGGCCGGTAAGGCGCTTGCGCAGGGCGGGCGGTGCGGCCTCGGCGGGCGGCGCCAGCGGAATGATGGTGGGGCTGGCCGCCGCGG CCTCGGCCGTGGCGGCCGGGGCGGGCGGGGGCGGCACGTCCAGGCTCCAGGCCAGCAGCTCCAG	0.71

Red allele: allele corresponding to reference allele
Green allele: allele divergent from reference allele
AMP GC: Amplicon GC content

Table: 7: *continued*

SNP	SNP Sequence	AMP GC
SNP13	CGCCCTGGCGCGGCAGCATGCGCAGCGAGCCGCCCACGTGCTTGAGCAGGCGTTCGACGATCGCCAGGCCTAGCCCCGCGCCGCTGACGCCGGTGCGGGCCGCTTCT CCACGCGAAAACGGACGCAGCAGGCGGTCGACTTCCTCGGGGGCGATGCCGGGGCCGCGGTCGGCCACTTCGATCGCCAGGATATTGCCTTCCGAATGCACGCTGAT GGCCAGGTGGGCCAGTCCGTCGGACGAGCGGCCGTAGCGGCGCGCGTTCTCGATCAGATTGCTGACGATGCGCTTGAGATCCAGCGCCGTGATGCGCGCCCGCAGGC CCGGATCGATCGAGGCTTCCAGCTCGCCGCCCAGCGAGGCGGTATGGCTGCGCTCGCGCTCATACAGCTCGGCCAGCACCGACGAAATGTCGGTGGCGAGCTGGGGC AGGGTTCCGGCCGGGCGCGCGTATTCCATCAGCTGCCCGAT**[G/A]**CTGTGGTCGATCTGGCCGAGGTCCTC	0.68
SNP14	GCACTGAACAGCGCCAGCCGGATCCGCAAGGTGGACGGGTTTGCCGCCGGCGACGGCGCCCAGGCGGCGGCCGGCCTGGCGGCACAAATGGCCTTGGTGGGAACGG GCGCGGACCTCAGCCACGACATCAAGATCACGGCGCTGAATCTACGCGCATGAC**[G/A]**GCGAGGCGTTCGAGGCGAGGCGCTCAGCCGCTGCGCCGGAACGCCTCGG CCTCGTCGGCCGGCGCGCTCTGGTCGTACAGGGGAATCCAGAACACGGCGCACAATCCGATCCCGCCCTGGGCGTTGGGCTCGCCGTCGCGCAATTCGATATCGCCAT CGTTGCGCCGCGCATAGGCACGGGCAATCGACAGCCCCAGCCCCGATCCCGACGACGACGCATGGCGGTCGCCCGCCATGCGGTCGAACCGGGCAAACGCCC	0.61
SNP15	CTCGCATCCGATATGGACCGCCCATTGCTCATCGATGGCCAGGGAGGTGACCGGCTCGTCGGCGCCAACGGGCAGCCCCAGCAGGTGGCGCAGGTTGCGGATGAGCA AAGGGAACATGGCATCTCCTGAGCATACGTACGGATGGGGTCAC**[A/G]**TATGCTGACCTCCCCGGCGCCCGGCCCAAATCAGGCAAACGCTGCCCGCCATGGGGGAA AGCGGCCTAACGAGCACGGACTTCTCCGCGCCACGCGGGACGCGAAGAACGCCGGACAGGCCGCGGTTACCAACATGGCATCCGGCGGGCTGGCTGTCGCCCGCGCC GCATCCGGCCATCGCCGCCCCAACCACGGAGGACCCGCACATGTTGAGCAACAACGTCAATCCGGTCGTCGGCCTGTCGTACCGCCCCCTGCCCGAGACGCC	0.58
SNP16	GCGGGCTGTCGCCGCGCCTGCTCGACGACGCGGCCGATTACCTGCAAGTGCCCAAGTCCGAGATCATGGCCATCACCGAGGTGAAGGCAGCCTCGCTGTCGCGCTGG ATGCGCGACGGGCAGGCGCTGCCCCTGGGCGAGTCGGACCGCCTGGCGCGGGTGGCGCGCGTGACCAAGGTGGCGCGCCAGGTGCTGGGCGGCGACGCCGAAGCGG TGCAATGGCTCAATACGCCGGTGCCCGCCCTGGGCAACGTCAAGCCGCTGTCGCTGCTGACCTCGGACGCCAGCAGCCGCATCGTCGAGGATACCCTGGCCCGCGCCG CCGCCGGGGTCTACGCTTGAGCCATGTATCGCTGTGGCGCATCGCCACCACGGCGGGCAAGTACCGCGCCGACGACCTGAGCGGCGCCGGCGCCGGCGCGGCGGCGG TGGGCGGGCGCTACAACA**[G/A]**TCCGGGCACCGCCGTGGTGTACGCCTCGTCCTGCGTGGCGCTGGCGGTG	0.71
SNP17	GCAGAAGCGGCTGCCCGGATATCGGGTGGCCGGTGCGGCTGCTGCCCTGCCCCGTCCCGCCCTCGACCGCCCAGCGCGCCAGGCCCGCCTCGCCGCCAGACTGCTCGG GCAGCCCCTCCATGAAGCGCGCAAGCCGCATCAGAGGAATCGAGCCCTCCAGCCGCTTGGCCTGCCGCGCAAACATGAACGCATCAATGTGGCTTTGTGCATTTTGCG CGTCCGTCCCGCTACCCATAGCCTCACCGTCAGGCGCGCCCTGCGTAG**[T/C]**GCTCATATCGAACTTCCTGCAAAAATGCCGCAAAATATTGGATAATACCGTGGCTTA CTGAACATCGTCAAATAACGCATGCCCGCCATTCCTCCAAAGCTGATCCTCGCATCGAGCTCGCGCTATCGCCGCGAGCTGCTGTCCCGCCTGCGCCTGCCGTTTACCG CCATTTCGCCCGATGTGGACGAAACGCCACAGCCTGGCGAAGCGCCCGCCGACCTGGCG	0.61
SNP18	CTTCGGGCATCGCGCTGGCGCGCGATTGACGCCGGGCCGGCGCCAGCCGGCCCGCGCCGCCGCCTACAGCAGCTTCTCGATGCGGGCCACGGCTTCCTGCAGGCGGTC CAGCCCGGTGGCGTACGAGAAGCGCATGGTGCGTTCGCCGTGGGCGGGGCCGAAATCCAGCCCCGGCACCGCGGCCACGCCGCCCTCGTGCAGCAGGCGATGCGAGA ACGCCATGCTGGTCGTGCCCAGCGCCGCGATGTCCGCATAGATGTAGAACGCGCCGTCCGGGCGCACCGGCACCGGGATGCCCAGGCGCTCGAACTCCGGCAGCAGG TAGTCGCGCCGCTGCTTGAAGGCCTCGCGGCGGTGCTCGAATATCTTCAGCGCGTCGGGCGTGAAGCAGGCCAGCGCGGCATGCTGGGCCAGCGTGGGCGCGCAGAT CGCCAGGCTGGCGGC**[C/T]**ATTTTCTCGACCTTGGCCGCCATGCTTTCGGGCACGATCATCCAGCCCAGG	0.68

Red allele: allele corresponding to reference allele

Green allele: allele divergent from reference allele

AMP GC: Amplicon GC content

Table 7: *continued*

SNP	SNP Sequence	AMP GC
SNP19	TCATCGAATGCGGCCGCCCGGAGTCGAACGCGACGCGCAGCGGTTTGTCCGGCCCCAGCAGATGCGACTGCCCGACCACATCGGACAGCGTACGGGGCCGCAGGCGT TCGGCCAGCGGCACATAGGGCCGATGGGCCGGATCGGAGGCGAACAGGTCGTTGCTCATGGACAGCAGGAAAAAAACACGGGC**[G/A]**nGATCCAACGCGCATTACAC CATGCGGCGGCCTTGGCCCGCAGGATTGCCCTGCGCGCGACGCAGAGCGTCGCGGCCCTACATCTTCACCACGTCCACTCCCTGAGGCGGGGTGAACTGGAATTCGGA GGCCGGCACGGCGCCGGGCAGCAGGTTGGACAGCTCGACGCGCGTGGTCTGCCCGAAGGCGTCGACCAGTTCGATGCGCGCGGGCTGGTTGTCGCGCAAGCCGATAT CGACCTGCGAGAAACCCGCGTCGGCGTTGCGCGGCTTGGCGCGCAGCCATTGCAGGCCGTCGCGG
SNP20	CTGACGCACAACGTACACAAGGGCCGCATGGCCGAGTGGTCCGAGTTCTACGAGCGCCTGTTCAATTTCCGCGAGATCCGCTACTTCGACATCGAAGGCAAGGTGACC GGCGTGAAGTCGAAGGCCATGACCTCGCCCTGCGGCAATATCCGTATCCCGATCAACGAGGAAGGCACCGAAGAGAA**[G/A]**nGGGCAGATCCAGGAATACCTGGACC TGTACCGCGGCGAAGGCATCCAGCACATCGCGATGGCCACCGACGATATCTACCAGACGGTGGAAGCGCTGCGCCGCAACGGCGTGGTGTTCCTGGATACGCCGGAC ACCTACTACGAATTGCTGGACCGCCGCCTTCC
SNP21	GGTACGGATGTGGTGCTGCACTTGCGCGCCGACGAGGACGAGTTGCTCAATGGTTGGAAGCTGCGCGAGATCCTGCGCCGCTATTCGGACCATATTTCGCTGCCGATC CGCATGGCCAAGGAGGATTGGGACGCGGAGAAGGGCGAGCAGGTCAAGGGCGATGAGCTGGAGACGGTGAACCAGGCGAATGCGCTGTGGACGCGCAACAAATCG GATATTACCGACGAGCAGTAT**[C/A]**nGCGAGTTCTACAAAACGGTGTCGCACGATTATGACGATCCGCTGGCGTGGACGCATAACCGGGTGGAGGGCCGCAGCGAGTA TACGCAGCTGCTGTATGTGCCCAAGCATGCGCCGTTCGATCTGTGGGACCGCGATGCGCGCCGCGGGGTGAAGCTGTATGTGAAGCGGGTGTTCATCATGGACGACGC GGAGCAGTTGCTGCCGTCGTATCTGCGTTTCGTGCGCGGGGTGATCGATTCGGCGGATTTGCCGC
SNP22	GGCACGAACCTATTGTTGAAATAATCACGGGTCAATACTCCTTCTTAACAGCAGGGAAAAAGAGGCCCCGCGCCGTGCCCGCCGCGCGGGCGCCGTCGTGCTGGACG GGCTTTTGCCTTGGGCCGGTCCGGCGCGACGCATGGCGGGGCTCGGGGCTGGCATGGCTTACGACACCTTCAGGTGCAGCAATTGCGGTTCGGCGCGCCGGCCGAGTA TGTGCGGCTGCGGCGTGGCGGCCTGCAGCAGCAGGGCGCCGATGGCCTCGTCGTAGTCGTGCCAGGGCAGATCGCGCTGCACGCTTTCGCGCGCGCGCTTGCCCAGCT CGCCGGCCTCGCCCAGCACCTGCTGCAGGTGCGCGGCGGTTTCGGCGGCGGGCGCGTTCAGGTCGATCGGCACGCCGTTGCGGCCAGCCTCCACATGGGCGGCGTAG GCGGGCGCCGGGCCGACGGCGGGACACACGCCCAGCGCCATGCATTCGAG**[C/T]**AGGGCCGCGGCCGAC
SNP23	GGTACGGCGATTCGTCGCGGCCGTGGTGCTCGCGCACCGCGTGCGCCATCGACAGGCGATCGCCGAAGCGCGCCTTCAAGGCGTCGAGACAGGCGGGCGGCACCGGA CGGCGCAACGCTTCGGCAGGCTGGGGAGCGTTCATGGCATCTACACAATTGAGGAAGCAAGTGCCGCCATTTTACGCCCACCTTCCTACTGAGCCCACGACACTCGCT GCGGACATGCCCCGGCCGTCAGGGCAGGATCCTATGCACCGCCAGCATGGATTCCTAT**[G/T]**TTCGCCAGCCGCCCCGGACCACTATCGTATGCGGAGCTTCTGTATTC GCCTTCCGGCTGTATGAACAAGCATTGCTTCAAACTGGTCCATTCCCCAGCCCTCGGCATGCTGGTCCCCGTCCACGAACACCGTACCGGCCGCCCGCTGCGCGGCGC GCGGCGGGCAATGGCCGTGACGCTGGCCGTGCTCGCGCCGGCCGGCGCGGCGGCGGCGGGC
SNP24	GCTGGAACGCAATTCGTCCATGACTTTCACCACCGACTCGTACGGCACCTTGCCGTCGGCGGCGATCACCACCGGCGTTTCGGCGGTAATGCGCGAACGGACCTGGTT GACCAGCTCGGTGCGCGCGATGTCCTGGGGCGTGGCGCCCGGCTCGCGCATGCGCAAGGCAATCTTGCCGTCTTCCGAAATCTGCACTTCCAGCGGCTTGGCCGGCAC ATCGGCGGCCTGCCCCACCGAGGGCAGCTCGATCAGCCCCGGCGTGATCAGGGGCGCGGTGACCATGAAGATCACCAGCAGCACCAGCATGACGTCGATATAGGGCA CGACGTTGATGTCGGCCTTCATGCGGCGGCCGGCCCGGCCGTTGGAGCGTACCGAAGGCATTAGCGCACCTGCCGCTGCAGAATGTTCAGGAACTCGTCGACGAAGCT GTCGAACCGGATCGACAGGCGGTCGATGTCGTTGGTGAAGC**[G/A]**GTTATAGGCCACCACGGCGGGGA

Red allele: allele corresponding to reference allele
Green allele: allele divergent from reference allele
AMP GC: Amplicon GC content

Table 7: *continued*

SNP	SNP Sequence	AMP GC
SNP25	GTCGTCGGATCGCTCGACGCATTTCCCGGCCGTACCCACCATGGCGGAGCTGGGATATCCCGAATTCGTCGCCTCGCCCTGGTTCGCCGTCATGGCCCCCAAGGGAAC CGACCCCAAGATAATCGTCCTGCTCAACCGCCACATCAACGAGGCGCTGCAGTCCAAGGCGGTCGTCGAGGCCTTTGCCGCCCAAGGCGCCACGCCGGTCATCGCCAC GCCGGATCAGACCCGCGGCTTCATCGCAGACGAGATCCAGCGCTGGGCCGGCGTCGTGCGCGAAACCGGCGCCAAGCTGAAGTAGCAGCGCAGCCCTCCAACGCGCC ATCCCCGTCCGGCCGGCACCATCCCGCATACGTGTTGGCAACCGCCAACGCGCATGCGTGCAGATTCGTCGTACAAAACCCTCGATTCTTCCGTACATCCCGCTACTGC AATCCAACACGGCATGAACGCTCCTTCGGCGCAAAGTCGCGCGAT**[G/A]**GTACCGGTCACCGTCCGG	0.69
SNP26	GACGCTGCCCGAACGGAACCCGCGCCTTGACCGTGGCCGAACTGCGCGGCAACGCCGAATTGCAGACGTATTTGCGCCAGATAACGCCCGGCTGGTCCATATACGGT CTCTATGACGGTACGTACCTGGGCCAGGCGTACGGCGGCATCATCAAGGACGCGCCGCCAGGCGCGGGGTTCATTTATCGCGAAACTTTCTGCATCACGACCATATAC AAGACCGGGCAACCGGCTGCGGATCACTACTACAGCAAGGTCACGGCCACGCGCCTGCTCGCCAGCACCAACAGCAGGCTGTGCGCGGTATTCGTCAGGGACGGGCA ATCGGTCATCGGAGCCTGCGCCAGCCCGTATGAAGGCAGGTACAGAGACATGTACGACGCGCTGCGGCGCCTGCTGTACATGATCTATATGTCCGGCCTTGCCGTACG CGTCCACGTCAGCAAGGAAGAGCAGTATTACGACTACGAGGACGCCACATTCCAGACCTATGCCCTCACC	0.67
SNP27	AGCGACAGGATCAGCGTGCGTTCGGCAACCAGGGCGGTGGCCACGCCGGCGGCTTTGGCGCTGATGCCGGGCGCGACGCCGATGACATAGTCGGACGACAGAGCCAT GCCCTGCCCGGCGATGGCGATGGCACAGGCCGCGCCCAGCGGCGGCAGGCCGGCGGCGATGGCCACGGGCAGCAGGATGGCGGCCACCAGGGGCACGGCCGGCGTG GGCCAGAAGAACAGCGAGATGA**[G/A]**ATAGGTGGCCAGCGCCAGCACGACGAATGCGATATGGCCGTTGGTCATGACGGCCCGGAAGGGCTGCACCATGCGCACGTC CGAGCCCAGCGTCTTGAGCGCGTTGAGCAGGGCGGTCATGAATGCGATGACCAGGAATATGTTGAAAAGCTCCTTGGCCGCCATGAAGCTGGCGTTGAACACGGCCG TCACGCCGCTGACGATGCTGCCGCCGAAGGCGATGCCGACCAGCAACGTACCCAGCAGGGATGGAA	0.59
SNP28	ACCTGTCCCGAGAACGCCATCACGCTGGTGCCGCGCCTGCTGCTTGCGCCGCGGCGCCGCGAAGCCGTGGTCCTCAACGAAAGCGCGCCGTTCCATTGTGTGCGCTGC AACAAGGCTTTCGGCACCCAGAAGGGGGTCGAAGCCATGCTGGGCCGCCTGGGCGGCCACGCCATGTTCCAGGGCGCGGCGCTGGAACGGCTGAAAATGTGCGGCG ACTGCCGCGTGATCGATCTGTATTCCGCCGAAAACGAAACCCGGATCACCGACCTATGA**[A/G]**CGTATCCGTCACTTTCGCCTCCATCGCCGCCCCGGGCTTCGACGAA GAGGTCGCCCGCGCCGAGATCTACGGGCTGCTGGCGCAGCTGTACTATGCGCCGCCGCCGTCCGACCTGCTTGCGCAGCTGCGCGCCGCGCCCAGGCAGGCGCCCGA CCAGGGCGGCTTTCTCGAGGCCTCGTGGCAGGCGCTGGTTGCCTGCGCGGCCGAGATGGGCGATGACGC	0.59
SNP29	TTCATCGGCCTGACGATTGCCGTCCACAACGGCCGCCAGCACGTTCCCATTTACATCAACGAGAACATGGTCGGTCACAAGCTGGGCGAGTTCGCGCTGACCCGTACG TTCAAGGGTCACGCCGCGGACAAGAAGTCGAAGAGGTAAGCGATGGAAACTACTGCCATTATCCGTGGTGTTCACATCTCGGCTCAGAAGACCCGTCTGGTTGCGGA CCTGATCCGCGGCAAGTCGGTGGCTCAAGCCCTGAACATCCTCACCTTCTCGCC**[C/A]**AAGAAGGCCGCCGTCATCCTGAAGAAGGCTGTCGAGTCGGCTATCGCCAA CGCCGAGCACAACGACGGCGCCGATATCGACGAGTTGAAGGTCACCACCATTTTTGTGGACAAGGCTCAATCGATGAAGCGTTTCTCGGCTCGCGCCAAGGGCCGCG GTAACCGTATCGAGAAGCAGACCTGCCATATCACGGTCAAGGTCGGAGCTTAAGGAGTCACGA	0.6
SNP30	AAACCGCGTAGCCGGCCTTGCCGGACTGCTTCTGCGCAGCGATGCCTGCCTCGAGCAGCGCCGCCAATACTTCGTTGGCTTCGTTCTCGGGCGCCGCGCCCAACAGCT CGACGCGGGCACCGCAGCCGGCCAGCAGCGCCAGGGCGAGCACCAGGGCCGCCCATCCCGCGCCGCGCCGATACCGTTGGATCGCCCCGATGGCGTTCATGACATTC TCGCCAGCGTATCGACGTTTTGGGTGGCGCGCCCTATTGCCTTGCCCACCAACTCG**[T/C]**ACTCCACGGATGCCTGTAGCAACCTGGCCTGCAGATCGAGCATACCCAC CACGGTAGGCGCCTGGCTCACCTCGGCCAAGCCCGTCTGCACCGCCCGCCATTTTCCTGCCACATCGGCCAGGCCGCCGCGCAACTGTCCAAGAATGCGACGGCCCAG CGGCTCGTCGAGCGCGACCGGCGCCAGGGCGATCGCCCCGTTCGGGGCCGATGCGCCGGGC	0.6

Red allele: allele corresponding to reference allele
Green allele: allele divergent from reference allele
AMP GC: Amplicon GC content

Table 7: *continued*

SNP	SNP Sequence	AMP GC
SNP31	TCTCCACCGTAAAGCCGTACCTGCCGCCATTGCGCTCGGCGCGCACCACTTCCACGCCGGTACGGATGGGCGCATTGAACTTGCGCGCATAAGCCTCCAGGTAGTCGG CCACGGCGTCCTTGGACGCGAAACCGTCCGGATCGAGCCCTTCGAATTCCAGGCCGGGGAAGCGATCGTGCCAGGCGGGGCCGTTCGCCACCAGGGAGTCCCACCTC CCGGTTCGCCAGCGCTGCGCGATGCGGTCACGCTCCAGCACGATGTGAGGCACTCCCT**[G/A]**CCCAGTCAGGTGCTCGCTCATCGCGATGCCCGCCTGGCCACCGCCG ACGACGAGGGTGTCGGTCAAAATGGGGCTATCAACAGTCACGTCTTCTCCGTAATCGACGTCAAGGGTTTCATGCCGTGGGCGCGCGGCGAATGGGAGACCGCGCAA CGTCGCCATCGGCATCGGGGTGCCACAACGTGCACCCGCTGGCTTGGCAGCATTACAGCGAAAAAAGC	0.63
SNP32	TCGTCCAGCATGCGCATGTCGGGCGCGAGCAGGTCGATGCTGCGCGGGGTGACGCCCAGTCCGGCGCGGATCGCCGCCTTGATGCCGACCAGGTCGGGCACGATGCG CGTGACCGTCCAGCGTATATGGTGCTGCTCGAGCGCCGCCAGGGCCATTTCGCGATAGATGCTGGAGCCGTCGGCCAGGATCAGCGGCAAGGCCTTGTCGGGGCGGT GCACATAGTCCGCGGCGCATAGCCAGACCACCGGCGAGCGTTTCAGGATCAGCCCCTCGAATTCCTGGTGAAAGCGGGCCGAGATGGCCATGTCGATTTCGCCGCGCT GCAGCAGCTCGAACAGCCGCGGCGCGCTGTCTATGCAGACGTCGATGTTCAACTGCGGCAAGGCCTGGCTGGCCTGCCGCAGGATGGAGGGCAGCAGGGTCTC**[G/A]** ACGGCGTCGTGCGGCGAGCCGATGCGCAGCGTGCCCTGCAGGCGTTCCTGGAAAACGCGGAAGACCTCGT	0.75
SNP33	CATCGGCCGATGCCATATCGGCCCAGGTCCGCAGCCAGTCCTGCCCGGCGTCGCGCAGGCCCCAGGCAGCCTCGACCGGACGCACGCGCCGCCGCTCGGTGCGCAAG CTGCCAGCCGGCGACCGACCGCGCCAGCCTGTCGTTGCCCCCCCTGGCGTCCGAGGCGTGCCCGGGGCCGATGGCAAGCACCGTGACGCCCCTCAGGACGGCGGTAA TCGCGCCTTCGCGCAGCAGTCCGCCCGGCATGGCCGCGGGCTCGGCGGCTTGCGCACGCAGCAGGCGCGTGTCGACCTGCAGCCAGTCGCGCGCCAGCAAGGCAGGC ATGCCATCGGCAGCCGTGCGCCCGGCAACGCCGGCCTGCCGCGCCAAGATGGCGGTGCGCGGGGCAGACGGCGCCGCCGCGGCCGCCATGAACGCCGCGACCCG**[G/ A]**CTGTGCTGTCCCAAGCGCAGCGCCTGGTGGCGCAACGTACCCAACCATGCCACGGCGGCCCAGGCCAACA	0.72
SNP34	ACGGAGGCCATTTTCATTGCGCGAAGCCGCCCGCCGATCTGGCGCGATTACCGGCAAATTCCCACACAACCATCAGCCCCCCCCCCCCCGGACCTGATATTCTGATGC CGACGCCAAGCACATGACGGCACCCCTCAGTATCAGAATCACCATGTCCAAGTTTTCATACCCTGCCTTGCGCGCCGCGCTTATCCTTGCCGCCTCGCCCGTACTGCCA GCGCTGGCCAACGACGGCACCATCGTCATCACCGGCAGCATCTCCGACCAGACCTGCGTCATCGAAGAGCCCAGCACCCTCAACCATATCAAGGTCGTGCAACTGCCC AAGATTTCCAAGAACGCGCTCAGGAACGACGGCGACACCGCCGGCGCCACGCCCTTCGACATCAAGCTGAAGGAATGCCCCCAGG**[C/A]**GCTGGGCGCGCTCAAGCT GTATTTCGAGCCCGGCATCACCACCAACTACGACACGGGCGATCTGATTGCCTACAAGCAGACCTAC	0.65
SNP35	GAGGAGATGGCCGACCGCGTCAAGGCCGCGGCCGACGCCCGCACCGACTCCGATTTCTACCTGATCGCGCGTACCGACGCCATCGCCTCGCACGGCGTGGACGCCGC CATCGAGCGCGCCATTGCCTGCGTGGAAGCAGGCGCCGACGCCATCTTCGCCGAGGCGGCCTACGACCTGCCTACCTACGATCGCTTCGTCAAGGCGGTCAAGGTGCC GG**[T/C]**GCTGGCCAACATCACCGAATTCGGCAAGACGCCGCTGTTCTCGGTGGAAGAGCTCAAAAGCGTGGGCGTGGGCATGGTGCTGTACCCGCTGTCGGCATTCCG CGCCATGAACAAGGCTGCCGAAACCGTCTACCAGGCCATCCGCCGCGATGGCCACCAGAAGAACGTGGTGGACCTGATGCAGACGCGCGACGAACTGTACGACCGCA TCGGCTATCACGAATTCGAATCGCAGCTGGACCAGCTGTTCCAGCAAGGCAAATCGCAATAAT	0.63

Red allele: allele corresponding to reference allele

Green allele: allele divergent from reference allele

AMP GC: Amplicon GC content

Table 8: Fluidigm SNP Allele Specific Primer (ASP) Information for Fluidigm Assay Project 7074FSTP16O1

SNP	Allele	ASP 1 Name	ASP 2 Name	ASP 1 Sequence	ASP 2 Sequence
SNP1	AG	A	G	GCGACACCTCGGACTGA	GCGACACCTCGGACTGG
SNP2	GA	G	A	CCACCATCGTCTCTCCCTC	CCACCATCGTCTCTCCCTT
SNP3	GA	G	A	CCACCTGCTCAGCCTGG	CCACCTGCTCAGCCTGA
SNP4	CT	C	T	GGGGTCGGGTCAAACCTG	GGGGTCGGGTCAAACCTA
SNP5	GA	G	A	CGACCTCATCCCCACCG	CGACCTCATCCCCACCA
SNP6	CT	C	T	CGTTTATGCGGCGGCG	TCGTTTATGCGGCGGCA
SNP7	GA	G	A	GGCATGGTGCACGGG	CGGCATGGTGCACGGA
SNP8	GA	G	A	GGAGGAAAGATACAAGACACCTGC	GGAGGAAAGATACAAGACACCTGT
SNP9	CT	C	T	CCATaGTGCCGTTGCTG	AGATATCCATaGTGCCGTTGCTA
SNP10	GA	G	A	GGCACATTGGTGCGCATG	GGCACATTGGTGCGCATA
SNP11	AG	A	G	GCAACTTTAAGTGCTAGAATTTCCCA	GCAACTTTAAGTGCTAGAATTTCCCG
SNP12	GA	G	A	CCTGCGCTACGGTCTGC	CCTGCGCTACGGTCTGT
SNP13	GA	G	A	TCCATCAGCTGCCCGATG	GTATTCCATCAGCTGCCCGATA
SNP14	GA	G	A	CCTCGAACGCCTCGCC	CCTCGAACGCCTCGCT
SNP15	AG	A	G	CCGGGGAGGTCAGCATAT	CCGGGGAGGTCAGCATAC
SNP16	GA	G	A	GCGGGCGCTACAACAG	GCGGGCGCTACAACAA
SNP17	TC	T	C	TGCAGGAAGTTCGATATGAGCA	TGCAGGAAGTTCGATATGAGCG
SNP18	CT	C	T	CGGCCAAGGTCGAGAAAATG	GCGGCCAAGGTCGAGAAAATA
SNP19	GA	G	A	GCAGGAAAAAAACACGGGCG	GCAGGAAAAAAACACGGGCA
SNP20	GA	G	A	AGGAAGGCACCGAAGAGAAG	GAGGAAGGCACCGAAGAGAAA
SNP21	CA	C	A	CGGATATTACCGACGAGCAGTATC	TCGGATATTACCGACGAGCAGTATA
SNP22	CT	C	T	GCGCCATGCATTCGAGC	AGCGCCATGCATTCGAGT
SNP23	GT	G	T	CGCCAGCATGGATTCCTATG	CCGCCAGCATGGATTCCTATT
SNP24	GA	G	A	CGATGTCGTTGGTGAAGCG	TCGATGTCGTTGGTGAAGCA
SNP25	GA	G	A	CGCAAAGTCGCGCGATG	CGCAAAGTCGCGCGATA
SNP26	CT	C	T	CGGCAGCGTCGATATGC	CCGGCAGCGTCGATATGT
SNP27	GA	G	A	GCCAGAAGAACAGCGAGATGAG	GCCAGAAGAACAGCGAGATGAA
SNP28	AG	A	G	CCGGATCACCGACCTATGAA	CCGGATCACCGACCTATGAG
SNP29	CA	C	A	CATCCTCACCTTCTCGCCC	CATCCTCACCTTCTCGCCA
SNP30	TC	T	C	CTACAGGCATCCGTGGAGTA	ACAGGCATCCGTGGAGTG
SNP31	GA	G	A	CGAGCACCTGACTGGGC	CGAGCACCTGACTGGGT
SNP32	GA	G	A	GGGCAGCAGGGTCTCG	GGGCAGCAGGGTCTCA
SNP33	GA	G	A	GCTTGGGACAGCACAGC	CGCTTGGGACAGCACAGT
SNP34	CA	C	A	AGGAATGCCCCCAGGC	GAAGGAATGCCCCCAGGA
SNP35	TC	T	C	CGGTGATGTTGGCCAGCA	GGTGATGTTGGCCAGCG

Table 9: Fluidigm SNP Locus Specific Primer (LSP) and Specific Target Amplification (STA) Information for Fluidigm Assay Project 7074FSTP16O1

SNP	LSP Sequence	STA Sequence
SNP1	GTTGCACGACGGCTGG	CCGCTGGCATGGAACA
SNP2	GCTTCGGGAAATGCCTGAT	GGCCATTTTCGTGACGCC
SNP3	GGCGAAACGGCCTCCA	GTCGACCTGATGGCGTTC
SNP4	CCCTGGTAACGGCGCA	GAGTCAAAAGGCCTGACCC
SNP5	AGGCCGACCGTGCCG	CGCAGGAGTACCGCAAG
SNP6	AGATAAAAGCGGCCCGGTCA	GTCAAACCGCAGAGCCC
SNP7	GCGGATCGTGCGCCAC	ACCCACTCGCAGTTCGT
SNP8	GCTATGAGGTTGCCACCAG	TATCTGTCCCACAAAGTCCTCG
SNP9	CAGTGGTGAGTTCGCACGC	TTCACCCCATCAAGGACTTT
SNP10	GCTTTCGGCCTGCCGC	GTCCGTAGCGTCGATCGT
SNP11	GCCAGACGAATCACCAGCAT	CAACTTCAGTTGCCACCAGG
SNP12	GCGATAGGTGCGCAGCC	GCGCAAACGGGTCAAGC
SNP13	GCCCGCCAGGCCATC	GGGCGCGCGTATTCC
SNP14	GGACCTCAGCCACGACATCA	GATTCCCCTGTACGACCAGA
SNP15	GGAACATGGCATCTCCTGAGC	TTGCCTGATTTGGGCCG
SNP16	ACGAGGCGTACACCACGG	CCGACGACCTGAGCGG
SNP17	AGCCTCACCGTCAGGCG	GGTATTATCCAATATTTTGCGGCATT
SNP18	CGCGCAGATCGCCAGG	GGATGATCGTGCCCGAAAG
SNP19	CCAAGGCCGCCGCAT	AGGTCGTTGCTCATGGACA
SNP20	CGCGGTACAGGTCCAGGT	AATATCCGTATCCCGATCAACGA
SNP21	ACGCCAGCGGATCGTCA	TGTGGACGCGCAACAAATC
SNP22	CGACAACCGCGCCGAA	CGGGACACACGCCCA
SNP23	GGCGAATACAGAAGCTCCGC	GGCAGGATCCTATGCACC
SNP24	GCCTGTTCGCGGCCAT	CCGGATCGACAGGCGG
SNP25	CCGGACGGTGACCGGTA	CGCTACTGCAATCCAACACG
SNP26	GGACAGGCGAACAGATCCGA	CGGCATTTCCCTCTGCAAC
SNP27	TGGCGCTGGCCACCTA	ATGGCGGCCACCAGG
SNP28	CGGCGATGGAGGCGAAA	TCCGCCGAAAACGAAACC
SNP29	AGCCGACTCGACAGCCTTC	GATGGAAACTACTGCCATTATCCGTG
SNP30	CAGCGTATCGACGTTTTGG	GTGGTGGGTATGCTCGATCT
SNP31	CAGCACGATGTGAGGCACT	GTTGATAGCCCCATTTTGACC
SNP32	GAACGCCTGCAGGGCAC	CCTGCCGCAGGATGGA
SNP33	GCCGCCATGAACGCCG	GTTGGGTACGTTGCGCC
SNP34	GTGATGCCGGGCTCGAAA	CCACGCCCTTCGACATCA
SNP35	CCTGCCTACCTACGATCGCTT	CGGCGTCTTGCCGAATT

Table 10: Components for primer pool of 35 assays

Component	**Volume (µl)**	**Final Concentration (nM)**
100 µM SNPtype Assay STA Primer	2 x 35 = 70 in total	500
100 µM SNPtype Assay LSP	2 x 35 = 70 in total	500
DNA Suspension Buffer	260	N / A
Total	**400**	N / A

Table 11: Components for STA pre-Mix

Component	**Volume (µl) per sample**
Qiagen 2X Multiplex PCR Master Mix (Qiagen, Product Number: 206143)	2.5
10X SNPType™ STA Primer Pool	0.5
PCR-Certified Water	0.75
Genomic DNA	1.25
Total	**5**

Table 12: Thermal cycle protocol for STA of genomic DNA Samples

	Hold	14 Cycles	
Temperature	95°C	95°C	60°C
Time	15 minutes	15 seconds	4 minutes

Table 12: Dilution of STA products

STA product (µL)	**DNA suspension buffer (µL)**
4	396

Table 13: SNPType™ assay mixes

Component	**Volume (µl) for each primer**	**Final Concentration (µM)**
SNPType™ Assay ASP	3	7.5
SNPType™ Assay LSP	8	20
DNA Suspension Buffer	29	-
Total	**40**	-

Table 14: 10X assay

Component	**Volume (µl) per inlet**
2X Assay Loading Reagent PN: 100-7611/ 85000736	3
PCR Purified Water	1.8
SNPType™ Assay Mix	1.2 (individual SNPType Assay mix)
Total	**6**

Table 15: Sample pre-mix

Component	**Volume for 12 samples with overage µl**
Biotium Fast Probe Master Mix 2X PN: 31005	54
20X SNPType™ Sample Loading Reagent PN: 100-7608	5.4
60X SNPType™ Reagent PN: 100-7607	1.8
ROX reference dye PN: 12223-012	0.648
PCR certified water	1.152
Total	**63**

Appendix E: Ethics

R14/49 Miss Bronwan Smith et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M160961

NAME: (Principal Investigator)	Miss Bronwan Smith et al
DEPARTMENT:	Pathology Respiratory and Meningeal Pathogens Research Unit
PROJECT TITLE:	Determining Sequence type of Circulating Bordetella Pertussis Strains Isolated from South African Infants
DATE CONSIDERED:	30/09/2016
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Prof Shabir Madhi
APPROVED BY:	Professor P Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	28/10/2016

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 Research Office Secretary in Room 301, Third Floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. 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**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in September and will therefore be due in the month of September each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Proposal number:	**CRA# 034**
Proposal title:	**Determining Sequence type of Circulating *Bordetella pertussis* Strains Isolated from South African Infants**
Date of EC review:	**21 July 2016**
EC review decision:	**Approved**

Reviewer comments:

- **Strengths**:
  - Straightforward project with clear objectives and part of a Masters project.
  - Findings will have relevance to pertussis vaccination program in S. Africa
  - New and highly relevant surveillance methodology established in RSA
  - Provides valuable information on evolution of *B. pertussis* strains with regard to virulence factors and RSA aP vaccine formulation.
  - Will help inform factors contributing to the evolving pertussis emergence.
- **Weaknesses:**
  - Samples may need re-extraction as the quality of stored extracts may have deteriorated.
  - Data on non-PERCH samples is lacking.
  - Unclear if Fluidigm method has been established, and, if not, whether there are any anticipated obstacles.
  - Requesting archived nucleic acid when *B. pertussis isolates* (where available) would suffice.
  - Analytic plan could be more specific and developed.
- **Overall score** = Excellent; very strong with some minor weaknesses

Additional EC comments:

- The increasing rate of pertussis globally has been attributed to the use of acellular vaccine; however, there are also concerns that changes in strain virulence may be contributory. Information from this sub-study on local prevalence of SNPs on virulence-associated alleles in the African setting would contribute to this hypothesis.
- An analytic plan is not included. Are there strains collected prior to introduction of DTaP for comparison? It would be interesting to include strains isolated in other African PERCH sites where DTwcP continues to be used for comparison.