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Development and characterisation of a novel vaccine for *Bordetella pertussis*

(whooping cough)

A thesis submitted for the degree of

Doctor of Philosophy



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ABSTRACT

Bordetella pertussis is the causative agent of pertussis (whooping cough) which is a respiratory infection, particularly dangerous for babies and young infants. Protection against pertussis relies on the induction of potent T-cell and antibody responses. Current acellular pertussis (aP) vaccines induce strong antibody responses and T-cell helper type 2 (Th2) response but are less effective at producing Th1-type immunity necessary for complete and fast bacterial clearance. Although, aP vaccines protect against severe disease, the vaccine-induced protection wanes over time, and despite high aP vaccination coverage in many developed countries, pertussis is a re-emerging disease.

In this thesis, I explored the use of replication-deficient adenovirus (Ad) and modified vaccinia virus Ankara (MVA) vectors as platforms for development of novel pertussis vaccines. Pertussis toxin (PT), fimbriae adhesins (Fim2, Fim3 and FimD), iron-binding proteins (IRP1-3 and AfuA) and adenylate cyclase toxin (ACT) were selected as vaccine candidate antigens. Our aim was to enhance Th1-type responses against *B. pertussis* and induce strong antigen-specific and neutralising antibodies that prevent lung (and nasal) colonisation of *B. pertussis*. I therefore evaluated the vaccines' different immunisation regimens and their potential to mediate such immune responses in mice.

A single intramuscular immunisation with a number of the Ad vaccines induced partial protection in mice. These constructs encoded the full length of the catalytic S1-235 subunit of PT, Fim3, AC and RTX domains of ACT. In particular, the Ad RTX vaccine may have the potential to prevent pertussis infection as 60% of mice immunised with one dose of the vaccine were fully protected against lung infection. In addition, the Ad S1-235 vaccine generated a PT-specific Th1-type response. The results indicate the potential of viral vectored vaccines to prevent pertussis infection either on their own or as future studies may reveal, using them in combination with different types of pertussis vaccines.

(300 words)

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List of Abbreviations

AC	Adenylate Cyclase (enzyme)
ACT	Adenylate Cyclase Toxin
ABTS	2,2'-Azinobis[3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt)
Ad	Adenoviral
AfuA	Iron-binding protein
aP	Acellular pertussis (vaccine)
3aP	Three-component acellular pertussis (vaccine)
5aP	Five-component acellular pertussis (vaccine)
ATP	Adenosine 5'-triphosphate
B. pertussis	*Bordetella pertussis*
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CD	Cell differentiation antigen
CFU	Colony Forming Units
CHO	Chinese Hamster Ovary (cells)
DCs	Dendritic cells
DPBS	Dulbecco′s Phosphate Buffered Saline
ELISA	Enzyme-linked Immunosorbent Assay
EU	ELISA Units
FHA	Filamentous Haemagglutinin
Fim	Fimbriae
HRP	Horseradish Peroxidase (conjugate)
IFN-γ	Interferon gamma
IL	Interleukin
IRP1-3	Iron-binding protein
IU	Infectious Units
MCD	Mature C-terminal domain
MFI	Median Fluorescence Intensity
OD	Optical Density
PRN	Pertactin
PT	Pertussis Toxin
rpm	Revolutions Per Minute
RTX	Repeats in Toxin (family of toxins)
SLA	Surface-labelling Assay
Th	T-helper (cell)
TLR	Toll-like receptor
TMB	3,3',5,5'-Tetramethylbenzidine (substrate)
WHO	World Health Organisation
wP	Whole-cell pertussis (vaccine)

Chapter 1: General Introduction

1.1 Pertussis disease

Pertussis (whooping cough) is a highly contagious, respiratory and vaccine-preventable disease caused by the bacterium *Bordetella pertussis* (1). The disease is transmitted from an infected person via aerosolised droplets through coughing and sneezing (2). The incubation period for pertussis is 7 – 10 days (3). Clinical presentations depend on many factors such as vaccination status, age, previous pertussis exposure, presence of *B. pertussis*-specific serum antibodies, genetics and early treatment intervention with antibiotics (4, 5). The symptoms are characterised by three phases: catarrhal, paroxysmal and convalescent. Non-specific symptoms such as coughing, sneezing, fatigue and rhinorrhoea are typical for the catarrhal phase that is followed by the paroxysmal phase one to two weeks from the onset of the disease. The paroxysmal phase is characterised by uncontrollable coughing attacks with a characteristic whooping sound and difficulty breathing. The symptoms slowly improve in the convalescent phase, but it can take a few months until they fully subside (1, 5). Older children, adolescents and adults usually have milder disease after acquiring pertussis infection (6). The mild symptoms often include prolonged non-distinctive cough, sleep disturbance, pharyngeal symptoms, weight loss, sneezing, sinus pain, sweating and headaches (7). Unvaccinated babies and infants who have not completed their full immunisation programmes are susceptible to serious and sometimes life-threatening complications such as pulmonary hypertension and respiratory failure (1, 8).

In addition to the characteristic symptoms mentioned above, pertussis infection can be diagnosed by serologic testing of *B. pertussis*-specific antibodies in nasopharyngeal secretions and PCR-based diagnostic tools (5, 9). Treatment with antimicrobial agents such as erythromycin or azithromycin have limited effectiveness unless administered at the early stage of the infection. The optimal time to reduce symptoms and prevent pertussis from progressing into severe disease is within the first three weeks of the infection (2). It is not fully understood why the treatment is most effective at the early stage of the infection (10). Therefore vaccination is the most effective approach to prevent severe disease and control transmission of the infection (2, 11).

1.2 Pathogenesis of *B. pertussis* during natural infection

The pathogenesis of *B. pertussis* has the following stages: Bacterial attachment, expression and production of virulence factors, evasion of host's immune responses, local tissue damage and induction of the systemic symptoms of the disease (4, 12). At the early stage of pertussis disease, the infection is mediated through respiratory secretion droplets containing the infectious *B. pertussis* bacteria that attach to ciliated-epithelial cells located in the host's upper respiratory tract (4, 13). Upon inhalation of contaminated aerosolised droplets, bacteria adhere to the nasopharynx and trachea via their adhesive surface structures such as filamentous haemagglutinin (FHA), fimbriae serotype 2 and 3 (Fim2, Fim3) (Figure 1.1). Following the attachment, bacteria start to multiply in the local tissues and initiate dysregulation of the host's immune responses. The recruitment of innate immune cells to the site of infection, removal of bacteria from the airways and synthesis of antimicrobial peptides are all disrupted leading to local tissue damage and induction of systemic symptoms of the

disease (2). The combined expression of the virulence factors inhibits bacterial clearance from the respiratory tract and promotes bacterial multiplication and potential dissemination to the lungs (13). The spread of bacteria to the lower respiratory tract is particularly dangerous for infants and young children as they can suffer from necrotising bronchiolitis, intra-alveolar haemorrhage and fibrinous oedema. In severe cases, the infection may lead to lymphocytosis, pulmonary hypertension, respiratory failure and the combination of these symptoms can even result in death (2). It has been estimated that up to 6% of non-immunised children suffer from bronchopneumonia as a result of pertussis infection in developed countries and this rate is even higher in young infants. In developing countries, the average case-fatality ratio in infants younger than 12 months has been estimated to be 4% and in children aged one to four years the ratio has been 1% (14).

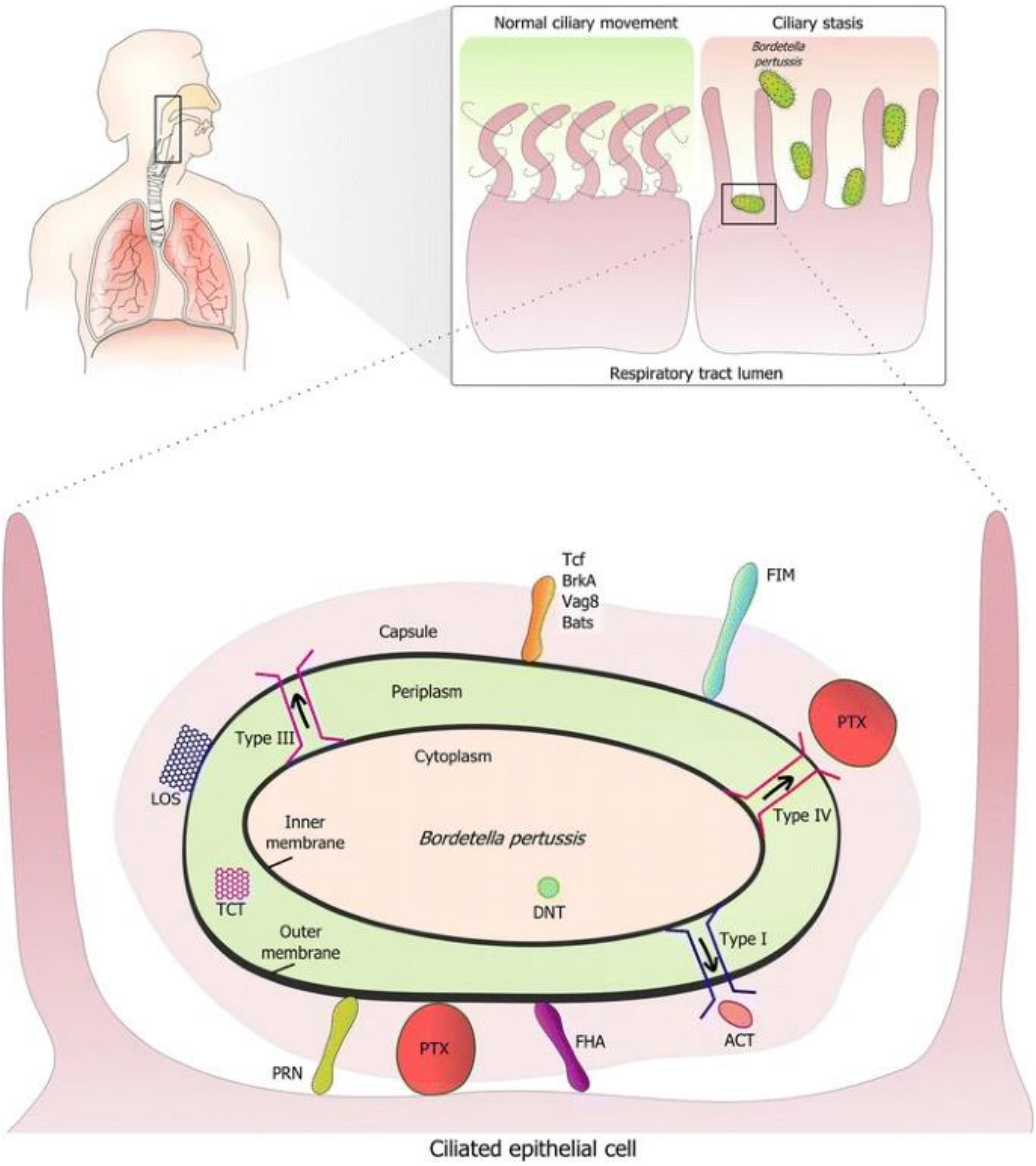


Figure 1.1. Invasion of the respiratory mucosal epithelium by *B. pertussis*. *B. pertussis* binds ciliated epithelial cells in the respiratory tract. *B. pertussis* has many virulent factors and some of them are involved in adhesion to the host cells such as pertactin (PRN), pertussis toxin (PT), FHA, tracheal colonization factor A, BrkA protein, the autotransporter Vag8 and other transporters of *B. pertussis*. PT and adenylate cyclase toxin (ACT) are toxic for host cells including phagocytes. Dermonecrotic toxin (DNT) stimulates vasoconstriction *in vitro*. *B. pertussis* also expresses endotoxin in its outer membrane. Type I, III, and IV secretion systems are indicated in red, blue, and pink, respectively. Ciliostasis is induced by tracheal cytotoxin (TCT) and some researchers hypothesise that the TCT contributes to severe coughing to remove mucus. Taken from Jongerius *et al.* (2015) (15).

Host environmental factors, such as a low temperature, and/or chemicals e.g. magnesium sulphate ($MgSO_4$) or nicotinic acid influence virulent phenotype of *B. pertussis* (1). This regulation of the expression of virulence factors affects the pathogenesis of *B. pertussis* (16). In response to the environmental stimuli, *B. pertussis* can undergo phase variation meaning that the bacteria regulate the expression of genes, encoding the virulence factors (16, 17). In addition to the role of toxins and adhesins in the pathogenesis of pertussis disease, *B. pertussis* acquires iron that is used for bacterial survival once the bacteria enter a host's iron-depleted environment (16). *B. pertussis* acquires iron using its haeme uptake system or the bacteria produce alcaligin siderophore to scavenge iron from the host and bring it to the bacterial surface (18). Understanding the mechanisms of natural pertussis infection, how the disease develops in the hosts, infection-induced and vaccine-induced immune responses are important factors for developing more effective vaccines and treatment against the disease.

1.3 Immune responses to pertussis infection

Understanding natural immunity to *B. pertussis* may allow the design of improved pertussis vaccines that prevent infection, transmission and provide long-lasting immunity against the disease. During colonisation of the respiratory tract, *B. pertussis* attachment to ciliated epithelial cells of the upper respiratory tract elicits strong humoral and cell-mediated immune responses. The ciliated epithelial cells act as the first line of defence and goblet cells produce mucin to protect the lungs against *B. pertussis* (19). Although *B. pertussis* is an extracellular pathogen, studies have demonstrated that the bacteria can survive inside pulmonary alveolar macrophages in mouse and rabbit models of infection. Moreover, it has been demonstrated that the

bacteria can survive inside alveolar macrophages of human subjects (20). Intracellular survival of *B. pertussis* suggests that T-cell responses may be important in the clearance of the infection and protective immunity (21). The role of T-cells, specifically T helper 1 type (Th1) $CD4^+$ T cells, in the clearance of primary pertussis infection was first indicated by Mills *et al.* in 1993. The study, which used mouse respiratory infection model, demonstrated that adoptive transfer of IFN-γ- and IL-2-secreting $CD4^+$ T-cells from convalescent mice induced bacterial clearance and protective responses in mice lacking *B. pertussis*-specific antibodies (22). The priming of Th1-type T-cell responses was also demonstrated in children with acute pertussis infection and in children who have recovered from a recent pertussis infection. Pertussis-infected children had high levels of IFN-γ and IL-2 upon stimulation of peripheral blood mononuclear cells (PBMCs) with PT, FHA, PRN or killed *B. pertussis,* suggesting a stimulation of Th1-type CD4+ T-cells during the infection. *B.* pertussis-specific Th1-type responses were also detected in healthy adults who have not been vaccinated against *B. pertussis* and have no recollection of acquiring the infection. These healthy adults might have been exposed to *B. pertussis* and experience subclinical pertussis or mild non-specific symptoms. The data suggest that naturally acquired infection induces Th1-type cell-mediated immunity in humans (23).

Using mouse respiratory challenge model of pertussis, Ross and colleagues showed that Th1-type and Th17-type T-cells play an important role in protection against *B. pertussis.* Their study showed that transfer of Th1- and Th17-type T-cells to naïve mice, before challenge, resulted in reduced bacterial load in the lungs of infected mice (24). In addition to mouse models, the stimulation of Th1- and Th17-type $CD4^+$ T-cells has been observed in humans recovered from pertussis infection (25).

The role of serum *B. pertussis*-specific antibodies is essential but not enough for long-term protection against the infection (21). In addition, no correlation between serum antibody levels and clinical protection against pertussis has been established (26). However, PT-specific antibodies effectively protect against severe pertussis symptoms and are also used to monitor immune responses induced by either pertussis vaccine or natural infection (27). Studies involving household contacts also showed that patients who were exposed to pertussis infection and had high levels of serum PT-specific and PRN-specific antibodies displayed lower incidence of severe symptoms (28). Natural infection with *B. pertussis* is cleared more efficiently and faster in individuals who have been re-infected in comparison to people who have been vaccinated with either whole-cell pertussis or acellular pertussis vaccines (29). However, immunity to pertussis acquired from natural exposure to *B. pertussis* is not lifelong and does not protect against secondary infection (30).

1.4 Existing pertussis vaccines, their mode of action and limitations

Two types of pertussis vaccines have been developed and licensed to prevent pertussis disease: Whole-cell pertussis (wP) and acellular pertussis (aP) vaccines. The use of wP vaccines, first introduced in the 1940s, led to a decrease in pertussis outbreaks and mortality in young children globally (1). Although, wP vaccines are efficacious, they have been replaced by less reactogenic and better tolerated aP vaccines in many developed countries since 1990s due to alleged reactogenicity issues associated with wP vaccines (31).

1.4.1 Whole-cell pertussis (wP) vaccines

The wP vaccines, made of heat-killed or chemically inactivated whole bacterial cells, were developed in the USA in the 1930s (32, 33). The wP vaccines mediate antibody responses to a wide range of antigens as these vaccines contain whole inactivated bacterial cells, although the concentration of specific antigens is lower than in antigen-purified aP vaccines (34, 35). The wP vaccines have been available, in combination with diphtheria and tetanus toxoids (DTwP), for routine childhood immunisation in the USA since 1943 (33, 36). Many other developed countries started introducing DTwP vaccines into their national immunisation programmes in the 1950s, which led to a significant decrease in morbidity and mortality of pertussis cases (37). However, the manufacturing of wP vaccines has not been standardised, therefore wP vaccines have a wide range of efficacies (38, 39). A serological correlation between immunisation with wP vaccines and protection has not been established. However World Health Organisation (WHO) has published suggested correlates of protection for serum IgG antibodies to PT, PRN, Fim and FHA (Table 1.1) (40).

Table 1.1. Suggested correlates of protection for serum IgG antibodies to PT, PRN, Fim and FHA (41).

Antibody type	Study type	Vaccine type	Correlation
Agglutinins (anti-Fim)	Vaccine trial	wP	High titres protect
Agglutinins (anti-Fim)	Household contact	wP	High titres protect
Anti-PRN	Household contact	wP	High titres protect
Anti-PRN	Vaccine trial	aP	High titres protect
Anti-PRN	Vaccine trial	aP	High titres protect
Anti-PT	Household contact	aP	Low titres make susceptible
Anti-PT	Household study	aP	High titres protect
Anti-Fim	Household contact	aP	High titres protect
Anti-FHA	Cohort study	wP	High titres protect

The inactivated bacterial cells contain lipo-oligosaccharide that has a strong adjuvant activity and promotes Th1-type immune response (35). Studies using mouse models demonstrated that wP vaccines elicit Th1- and Th17-type responses. The importance of Th1-type response in vaccine-induced immunity was also highlighted in a study using mice with a selective impairment of IFN-γ signalling. IFN-γ-receptor deficient mice immunised with wP vaccines failed to induce a protective response against *B. pertussis* challenge, highlighting the importance of secretion of IFN-γ by immune cells such as natural killer cells and antigen-specific $CD4^+$ T-cells (19, 24).

Studies suggest that the antibody responses induced against many surface antigens inhibit bacterial attachment to the host epithelial cells in the respiratory tract. It has also been hypothesised that wP vaccines induce a high level of opsonising antibodies and Th17-type immune responses that enhance recruitment of neutrophils to the site of infection and help to remove the bacteria. Warfel *et al.* (2016) showed that even a single immunisation of baboons with wP vaccine reduced nasopharyngeal colonisation and prevented transmission of the infection to non-immunised baboons. Although wP vaccines do not prevent pertussis infection, a reduced level of bacterial burden helps to reduce paroxysmal coughing and decreases the chance of bacterial transmission (34).

In the 1970s, reports about the possible reactogenicity of wP vaccines started to emerge. The administration of the wP vaccine was associated with local and systemic adverse reactions and neurological complications seen in immunised babies (42). Although correlation between wP immunisation and neurological side effects was not proven, public concerns about safety and systemic reactions led to reduced vaccine

acceptance and a fall in the rate of pertussis vaccination in many developed countries. As a result, a new generation of pertussis vaccines with improved safety profiles were developed (42-44).

1.4.2 Acellular pertussis vaccines

The development of aP vaccines was triggered by the concerns over safety profile of wP vaccines (45, 46). These vaccines replaced wP vaccines in Japan in the 1980s, whereas the USA and many countries in Europe switched from wP to aP vaccines throughout the late 1990s (37). However, some European countries such as Serbia and Poland, and developing countries in Africa, Middle East, Asia and South America still use DTwP vaccines for routine immunisations (8).

The aP vaccines are composed of between one and five purified and inactivated antigens of *B. pertussis* (46). A monocomponent aP vaccine only contains highly purified, genetically or chemically inactivated forms of PT (45, 47). Multicomponent aP vaccines always contain PT and one or more of the other *B. pertussis* components: PRN, FHA and Fim2/Fim3. However, the role and contribution of the additional bacterial antigens PRN, FHA and Fim2/3 in the vaccine efficacy have been debated (48). In addition, the concentration of each pertussis vaccine antigen in aP vaccines (Table 1.2) and purification as well as detoxification techniques of PT vary among manufacturers (45, 49).

Table 1.2. Acellular pertussis vaccine composition. Taken from Locht and Mielcarek (2012) (45).

Vaccine/ trade name	FHA (µg)	PT (µg)	PRN (µg)	Fim2/3 (µg)	Targeted population/ age
DTaP/ Daptacel	5	10	3	5	Babies and children (6 weeks - 6 years old)
DTaP/ Infanrix	25	25	8	8	Babies and children (6 weeks - 7 years old)
DTaP/ Tripedia	23.4	23.4	-	-	Babies and children (6 weeks - 7 years old)
DTaP/Hib/ TriHIBit	23.4	23.4	-	-	Children (15 - 18 months old)
DTaP/polio/ Kinrix	25	25	8	-	Children (4 - 6 years old)
DTaP/polio/Hib/ Pentacel	20	20	3	5	Babies and children (6 weeks - 4 years old)
DTaP/polio/ hepatitis B/Pediarix	25	25	8	-	Babies and children (6 weeks - 6 years old)
Tdap/ Adacel	5	2.5	3	5	Adolescents and adults (11 - 64 years old)
Tdap/ Boostrix	8	8	2	-	Individuals (10+ years old)

Due to a shortage of wP vaccine in 1999 – 2001, a three-component aP (3aP) vaccine (Infanrix®IPV+Hib), which contains PT, FHA and PRN, was used in childhood primary immunisation programme in the UK. The 3aP vaccine protected the vaccinated children up to the age when they were eligible for pre-school booster (50). The five-

component aP (5aP) vaccine (Pediacel®) was introduced into the primary immunisation programme in the UK in 2004 and the vaccine provided the same or even better protection against pertussis disease than the wP vaccine (50). The Repevax® (dTaP-IPV) and Boostrix®-IPV vaccines have been used as booster aP vaccines for adolescents and adults, including pregnant women, in the UK (51). According to WHO, three primary doses of aP vaccine should be administered to babies, with a first primary dose at six weeks. The recommended interval between each primary dose is at least four weeks (49). The interval between the three primary doses varies among different countries. For instance, some countries administer aP primary vaccine to babies at the age of two, three and four months. Other countries use eight-week vaccination interval or administer the three doses of aP vaccine to babies who are three, five and 11 months old (8). One or two booster immunisations with aP vaccines are also advised by the WHO guidelines for children who are four to nine years old (8, 49).

Moreover, aP vaccines have been introduced into maternal immunisation programmes in many countries. Adults and older children, especially family members, can be reservoirs of pertussis infection and therefore, young infants are at risk of acquiring the infection. This led to concerns about pertussis transmission to the unimmunised babies, and as a consequence, immunisation with pertussis vaccines was introduced during pregnancy, allowing placental transfer of pertussis-specific antibodies to newborn babies. The aim is to protect the babies from severe disease, health complications and death in the first months of life (52-54). In the UK, DTaP vaccines were implemented into the maternal immunisation programme in October 2012 as a response to the national outbreak of pertussis cases. The latest data show relatively

high pertussis vaccination coverage, in pregnant women in England, which was around 70% in 2020 (Figure 1.2).

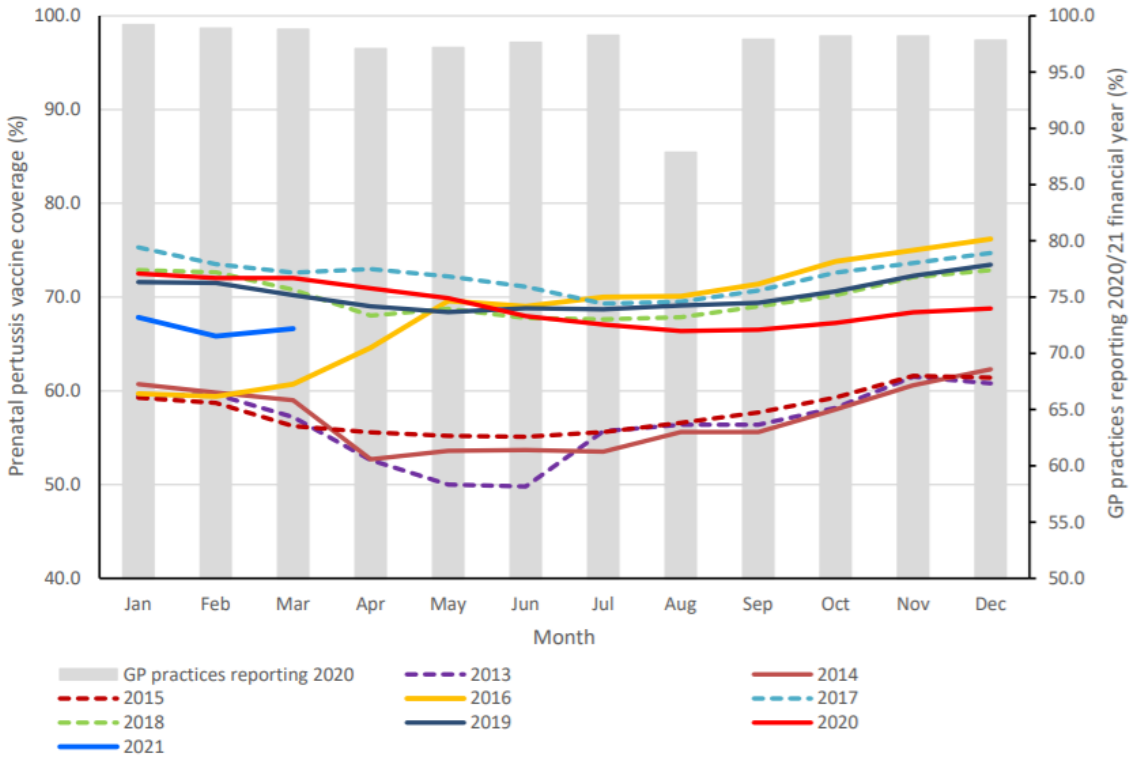


Figure 1.2. Pertussis vaccine coverage in pregnant women with commercial aP vaccine in England between 2013 – 2021. Taken from Public Health England. Pertussis vaccination programme for pregnant women update: Vaccine coverage in England, January to March 2021 and 2020 to 2021 annual coverage. Health Protection Report. Volume 15. Number 9.

1.5 *B. pertussis* antigens included in the current protein subunit vaccines

B. pertussis has many virulence factors but only a small number of them have been selected for use in current aP vaccines, which are licensed for paediatric and adult

immunisations. The antigenic selection was based on their pathogenic, immunogenic, and protective properties. The components are described below.

1.5.1 Pertussis toxin

Pertussis toxin (PT) plays a key role in the pathogenesis of pertussis (55). PT is a six-component protein complex, with a molecular weight of 105 kDa, that belongs to the AB_5 toxins family and consists of two functionally unique domains: The ADP-ribosyltransferase catalytic subunit, S1, also known as A-protomer, and the host cell binding B oligomer, made of S2, S3, two S4 and one S5 subunits (56-58). The B subunit enables binding of the toxin to the TLR-4 and other glycoconjugates, mainly oligosaccharide chains with sialic acid, on the host target cells (Figure 1.3A) (58, 59). An analysis of the PT crystal structure has found at least two carbohydrate binding sites on both S2 and S3 subunits of the B oligomer. These two subunits are 70% homologous and their N-terminal domains are involved in receptor binding to the target cells (60).

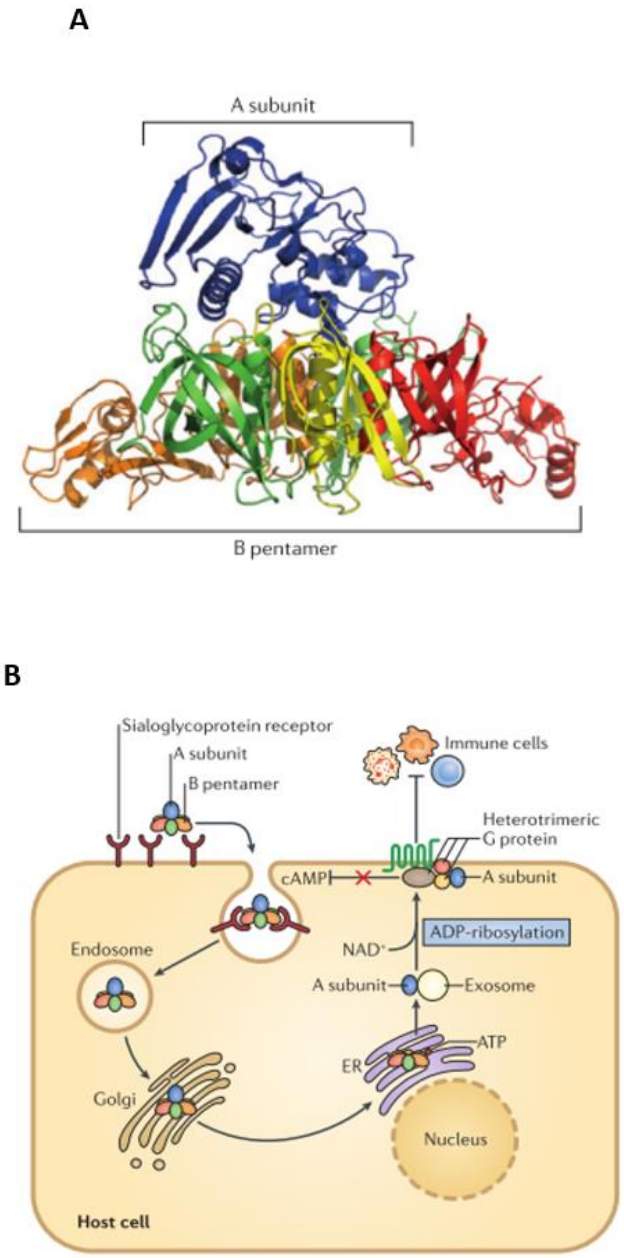


Figure 1.3. Pertussis toxin and its binding to host cells. (A) Structural organisation of PT. The schematic shows catalytic subunit (the A subunit) and membrane-binding subunit (the B pentamer). **(B) Schematic diagram of binding of PT to a sialoglycoprotein host cell receptor and of the ADP-ribosylation of α subunit of heterotrimeric $G_{i/o}$ protein by PT.** The pentameric B-subunit initiates binding and uptake of PT to host cells leading to alterations in cAMP levels and further disruption of cell signalling pathways. Taken from Melvin *et al.* (2014) (1).

PT is secreted as an exotoxin and upon binding to the host cells, the toxin is translocated into cytoplasm by receptor-mediated endocytosis (1, 55). The S1 subunit then dissociates from the B oligomer in the endoplasmic reticulum upon binding to adenosine triphosphate (ATP) which leads to the inactivation of the inhibitory effects of the host's heterotrimeric G proteins and disruption of cell signalling pathways (1, 60, 61). As a result, PT suppresses and modulates the host's inflammatory and immune responses leading to promotion of the infection. Studies in mouse models showed that the toxin is capable of reducing neutrophil and macrophage migration to the site of the infection and reducing synthesis of pro-inflammatory cytokines and chemokines. It also contributes to an increase in bacterial load during the early stages of pertussis infection (Figure 1.3B) (1).

PT plays an important role in pertussis infection as it promotes bacterial colonisation, hinders host innate immunity at the initial stage of the infection and provokes systemic symptoms of the disease. *B. pertussis* mutant strains that do not express functional PT are less pathogenic than wild type bacteria expressing PT, demonstrating PT's role in pathogenesis. Currently, it is not clear what causes severe coughing during the infection, however, it has been hypothesised that PT worsens cough responses by preventing the desensitisation of bradykinin receptors (62).

The importance of PT as a protective antigen has been shown by data obtained from mouse protection studies and clinical trials using purified pertussis toxoid (63). Sato and Sato (1990) demonstrated that the catalytic subunit of PT, S1, contains protective epitopes. In their study, mice passively transferred with 20 µg of IgG antibodies raised against different regions of PT were exposed to an aerosol challenge with *B. pertussis.*

The most protective antibodies were IgG binding to 1B7, 1D7, 3F11 and 10D6 epitopes on the S1 subunit of PT which suggest that the most protective epitopes are located at this region (63). In addition, two monoclonal antibodies, 1B7 and 11E6, which recognise epitopes on PT, inhibit catalytic and cell-binding activities, respectively (64).

The significance of PT as a vaccine antigen was also demonstrated by clinical trials, in Denmark. The pertussis toxoid as a monocomponent aP vaccine has 71% efficacy in vaccinated children and reduced transmission of the infection among people in the same household (48, 59). Since PT is a major virulence factor of *B. pertussis* and due to its protective capacity, all aP vaccine formulations contain detoxified PT as vaccine antigen (65).

Measurement of serum PT-specific IgG antibodies is used for diagnosis of pertussis infection and although correlates of protection have not been established, serum PT-specific IgG levels above 5 IU/ml has been deemed potentially protective (3, 66). The native PT is toxic, therefore it has to be detoxified to ensure vaccine safety. Different manufacturers use either genetic or chemical deactivation of PT. The chemical deactivation includes treatment of the toxin with either formaldehyde, glutaraldehyde, hydrogen peroxide or with a combination of formaldehyde and glutaraldehyde (67). Different modification procedures, especially chemical toxin detoxification, can diminish stimulation of neutralising antibodies against PT protective epitopes (56). However, in Denmark, hydrogen peroxide has been used to inactivate PT used in the aP vaccine that is used for paediatric immunisations. The hydrogen peroxide detoxification method preserves the structure of antigen epitopes more than toxin

inactivation by formaldehyde and can lead to development of more efficient immune responses (59).

PT has been shown to be a protective antigen and antibody responses against PT are induced after pertussis infection in humans, therefore it is an attractive candidate for development of novel pertussis vaccines.

1.5.2 Pertactin

Pertactin (PRN) is a bacterial surface protein containing an arginine-glycine-aspartate (RGD) tripeptide motif which enables bacteria to attach to integrin binding sites of eukaryotic cells (Figure 1.4.) (59, 68). The role of PRN in *B. pertussis* pathogenicity has been studied using mouse models which revealed that PRN inhibits neutrophil-mediated clearance in the lower respiratory tract leading to inflammatory responses (68). Many multicomponent aP vaccines contain PRN in their formulations, however, PRN-deficient strains, which have not lost their virulent properties, have been reported in some European countries, the United States, Japan and Australia (59).

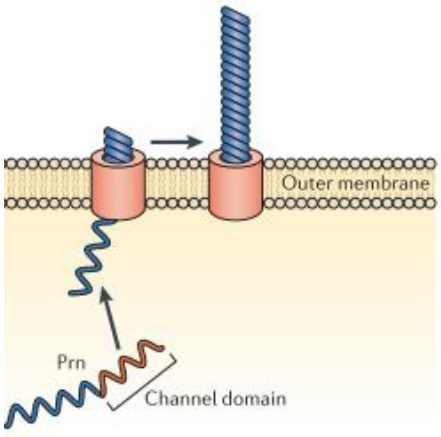


Figure 1.4. Structure of pertactin. The C-terminal ~30 kDa domain (orange) creates a channel in the outer membrane, which allows translocation of the ~70 kDa β-helical passenger domain (blue) to the cell surface. Taken from Melvin *et al*. (2014) (1).

1.5.3 Filamentous haemagglutinin

Filamentous haemagglutinin (FHA) is a major adhesin of *B. pertussis*. FHA is first synthesised as a large 367 KDa FhaB precursor that is processed into a 220 KDa mature cell-surface associated protein (16, 69). As shown in Figure 1.5, the carboxy-terminal pro-domain (yellow) is cleaved from the FhaB protein to synthesise the mature FHA protein. Mature C-terminal domain (MCD) of FHA is necessary for adherence to host's ciliated epithelial cells and establishment of pertussis infection. It has been hypothesised that the MCD domain of FHA may directly or indirectly influence host immune responses (1).

FHA can serve as both surface-associated protein and secreted protein (70). Both, N-terminal and C-terminal domains of the adhesin have been found to be highly immunogenic (59). According to *in vitro* studies, FHA has an important role in the initial stage of pertussis infection as it mediates adhesion to the respiratory epithelial cells and macrophages through three binding sites (16). A heparin-binding domain mediates attachment to sulphated polysaccharides, a carbohydrate-recognition

domain promotes binding to ciliated respiratory epithelial cells and macrophages, and RGD triplet engages with monocytes/macrophages, suggesting that FHA binds the same sites as PRN (71). The binding of FHA to monocytes/macrophages results in the inhibition of antigen-dependent $CD4^+$ T-cell proliferation. Purified FHA has been shown to reduce the activation of mouse macrophages and dendritic cells (DCs) by downregulating secretion of cytokines such as IL-10 and IL-12 (71). In addition, *B. pertussis* deficient in FHA display reduced colonisation and induce powerful host's immune responses which leads to more efficient bacterial clearance in comparison to wild type *B. pertussis*, expressing FHA (70). The data together suggest that FHA may have a significant role in modulation of host immune responses during pertussis infection, bacterial colonisation and persistence of the infection (70, 71). Although, current multicomponent aP vaccines contain FHA in their formulations, more studies are needed to determine FHA's ability to induce protective immunity in *B. pertussis*-infected hosts (59).

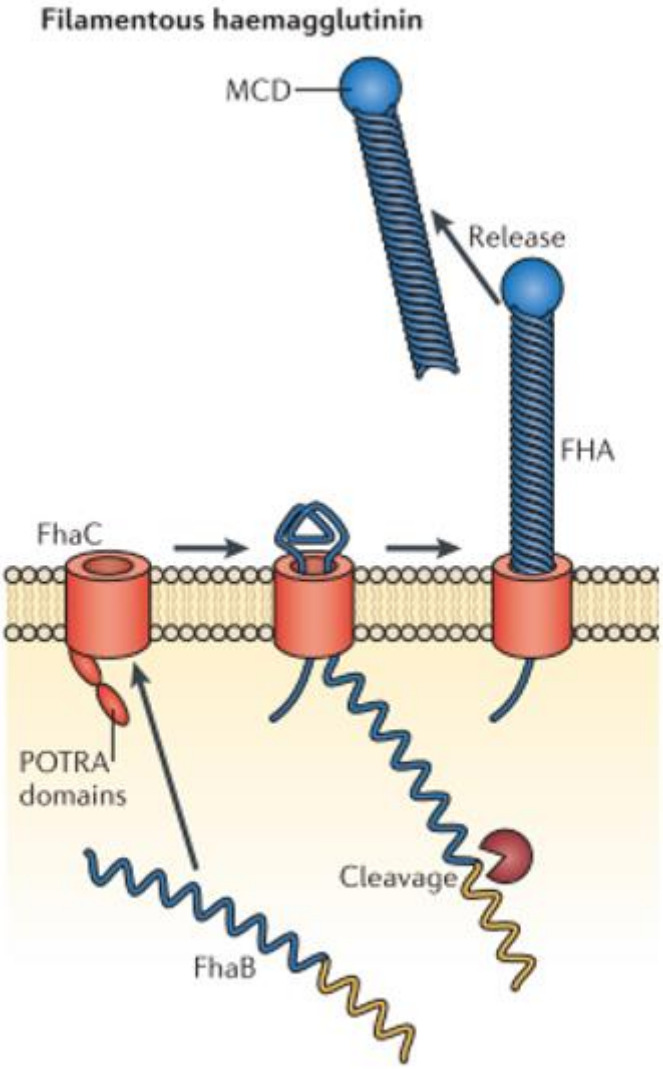


Figure 1.5. Processing of FHA. Several steps are undertaken before mature FHA is produced. FhaB protein is secreted first and its carboxy-terminal pro-domain (yellow) is cleaved involving several proteases. This results in a mature ~250 kDa FHA protein which has been assumed to be the biologically active form of the protein. Taken from Melvin *et al.* (2014) (1).

1.5.4 Fimbriae

All *Bordetella* species produce long filamentous structures known as fimbriae. Fimbriae are adhesins expressed on the bacterial surface and are divided into two major and one minor protein subunits. Serological typing revealed that major subunits of fimbriae are classified into Fim2 and Fim3 and different *B. pertussis* strains express Fim2, Fim3 or both Fim2/3 serotypes. The protein subunits of Fim2 and Fim3 are 22 kDa and 22.5 KDa, respectively. (72). The bacteria also produce a minor fimbrial subunit, FimD, with a molecular weight of 32.7 KDa (Figure 1.6). FimD has a role in enabling bacterial adhesion to the host cells as it binds cell surface receptors via very late activation antigen-5 (73, 74).

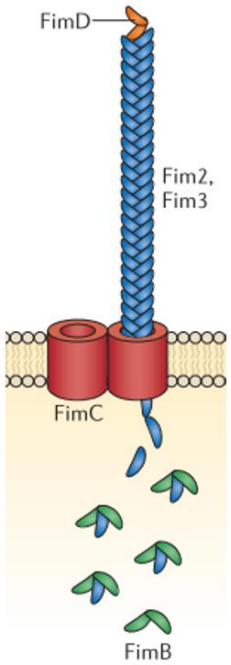


Figure 1.6. Structure of *Bordetella* fimbriae. FimB works as a chaperone protein trafficking Fim2 and Fim3 to the outer membrane usher protein known as FimC. Fim2 and Fim3 are the major pilin subunits, whereas FimD is the fimbrial tip protein. Taken from Melvin *et al.* (2014) (1).

Both Fim2 and Fim3 expressing strains are included in wP vaccines as well as in five-component aP vaccines. While developing aP vaccines, fimbriae were deemed to be important vaccine antigens due to their role in bacterial attachment. Stimulation of Fim-specific antibodies could prevent the binding of the bacteria and eventually lead to inhibition of the early stage of the infection (73). Rodríguez *at al.* showed that Fim-specific antibodies obtained from wP- and aP-immunised children play a role in the inhibition of bacterial attachment to human alveolar epithelial cell line. Further analysis found that both Fim-specific IgA and IgG antibodies are responsible for the decreased bacterial attachment (75).

However, the role of fimbriae in protective immunity against pertussis infection has been questioned. Xu *et al.* produced recombinant Fim2 (rFim2) and Fim3 (rFim3) and investigated their ability to induce protective immunity in immunised mice. rFim2 and rFim3 protein immunisation did not mediate clearance of the bacteria from mouse lungs following intranasal challenge model with *B. pertussis* 18-323 strain. However, the study showed that both rFim2 and rFim3 are immunogenic as they stimulated high Fim-specific antibody responses and cell-mediated responses, specifically production of IL-2 cytokine (76). Clinical trials, which focused on humoral immune responses, also failed to find a correlation between Fim-specific antibodies and protection against pertussis infection (77). On the other hand, a recent study by Queenan *et al.* demonstrated the importance of fimbriae in the reduction of bacterial colonisation. The study found that increasing quantity of Fim2/3 antigens (up to 50 µg in paediatric dose) in aP vaccines decreased bacterial colonisation of mouse lungs following intranasal challenge with *B. pertussis* 18-323 strain. Moreover, the higher amount of Fim2/3

antigens enhanced vaccine efficacy against PRN-deficient *B. pertussis* in dose-dependent manner (78).

Due to bacterial evolution, *B. pertussis* changes its virulence factors to survive in the host environment. The change in the expression of fimbriae has been noticed in different countries. There is an evidence that Fim2-dominant strains switched to increased expression of Fim3 antigen and became Fim3-dominant in countries like Australia and Netherlands (74). The reason behind the shift in fimbriae serotype expression is not obvious. Gorringe and Vaughan suggested that it is either driven by pertussis vaccination or by bacterial predominant genotype (73). On the other hand, Heikkinen *et al.* proposed that many *B. pertussis* strains expressing Fim2 prevail in non-immunised individuals, whereas Fim3-expressing isolates have been detected in wP-immunised individuals despite wP vaccine containing both Fim2 and Fim3 antigens (72).

In the early post-vaccination era (1963 – 1967) in the UK, there were 16% and 38% of *B. pertussis* strains expressing Fim2 and Fim2/3, respectively. Following decreased vaccine acceptance in the UK throughout the mid-1970s, the shift from Fim3 to Fim2 strains was observed. In Sweden and Australia, switch from Fim2 to Fim3-dominant strains was observed despite the use of two- or three-component aP vaccines, which do not contain fimbriae antigens. This suggests that the antigenic switch is not necessarily driven by using pertussis vaccines but it is associated with successful outgrowth of new lineages (73).

1.6 Immunogenicity and efficacy mediated by pertussis vaccines

Both wP and aP vaccines contribute to a reduction of pertussis cases globally. Although serological correlates of protection for pertussis have not been established, pre-clinical studies showed that PT-specific antibodies are capable of preventing severe disease. For example, passive transfer of PT-specific antibodies to baboons lead to protection against severe symptoms (79). A monocomponent aP vaccine containing PT alone has been shown to be efficacious (~80%) in immunised children (68), and correlation between titres of PT-specific antibodies and protection against severe pertussis disease was demonstrated in other clinical studies (79).

The aP vaccines generate high levels of IgG antibodies against the antigens contained in the vaccines such as PRN, Fim2/3, FHA and PT. Warfel and colleagues analysed sera of aP- and wP-immunised baboons as well as *B. pertussis* infected baboons. They showed that serum IgG responses against the aP vaccine antigens were comparable or even greater than IgG responses mediated by natural infection or immunisation with wP vaccine. The ability of aP vaccine to induce potent antibody responses specific for the vaccine antigens may contribute to the prevention of severe clinical symptoms but may not be enough for bacterial clearance from the respiratory tract, thus allowing subsequent transmission of the infection (80). Baboons immunised with aP vaccines displayed no symptoms of pertussis, despite a high number of bacteria in their lungs, however, they were able to transmit the infection to non-immunised baboons. Furthermore, the rate of bacterial clearance following aerosol challenge with infectious *B. pertussis* was lower in aP-immunised baboons as compared with wP-immunised baboons (80, 81). Based on these observations, it was

proposed that aP vaccines are ineffective at preventing bacterial colonisation, and asymptomatic individuals can, therefore, serve as a reservoir for further infection (81).

Despite effective national immunisation programmes and high vaccination rates in developed countries, there are reports of increased pertussis cases not only in unimmunised or partially immunised children but also in adolescents and adults. Epidemiological data suggest that aP vaccine-induced immunity typically wanes around adolescent age. For instance, a U.S. study reported pertussis cases among 8-12-years old teenagers who were vaccinated either with a 3aP or a 5aP vaccine in infancy, suggesting waning of the immunity following immunisation with aP vaccine (80, 82). Cohort and case studies found that five years after administration of the fifth dose of aP vaccine, children are up to 15-fold more likely to acquire pertussis infection than one year after the fifth dose (80). In comparison with the aP vaccines, natural pertussis infection can trigger protection for 7 – 10 years or even up to 20 years, although the protection can be as short as 3.5 years in some cases (83). A 10-year study in the UK, which assessed the duration of protection induced by wP vaccine, showed that wP vaccines are efficacious in 85% of children four years after the completion of the immunisation schedule, but the wP vaccine efficacy was reduced to 50% in the following three years (83). Furthermore, epidemiological data indicate that priming of newborns with either wP or aP vaccine influences the humoral and cell-mediated immune responses to additional aP booster until the preadolescent age. Van der Lee *et al.* (2018) showed that six-year old children primed with aP vaccine had higher levels of antigen-specific antibodies two years after a pre-school booster when compared with wP-primed children. However, by the age of nine years, the antibody levels decreased in both aP- and wP-primed children. When these children were

boosted with a pre-adolescent aP booster, lower antigen-specific antibody responses, as well as memory B-cells, were detected in aP-primed children in comparison to children primed with wP vaccine (84). Data from studies performed in the U.S. and Australia also support the hypothesis that the pre-school and adolescent aP boosters protect against severe pertussis symptoms for a few years and that priming of babies with aP vaccine leads to a faster waning of immunity against *B. pertussis* than priming with wP vaccine (84).

Another explanation for the differences in duration of protection could be a difference in helper T-cell responses mediated by wP and aP vaccines. The prime-boost with aP vaccines induces Th2-type response, whereas prime-boost with wP and aP vaccine, respectively, induces Th1/Th2-type response; the Th1-type response is associated with stimulation of opsonising antibodies, bacterial uptake and intracellular killing of bacteria (23). Pre-clinical models of pertussis using mouse and baboon models also demonstrated that aP vaccines induce mainly Th2-type or a mixed Th1/Th2-type cell-mediated responses, whereas, wP vaccine induces a strong Th1-type response similar to natural infection (77, 85).

The preparation of aP vaccines can vary from one manufacturer to another. For instance, the vaccine manufacturers use different antigen concentrations in the vaccine formulations (Table 1.2), purification techniques and detoxification media for deactivation of the proteins. Different countries use different vaccination schedules, which adds to the complexity to establish accurate duration of the pertussis vaccine-induced immunity (8). A comparison of the differences in immune response of wP and aP vaccines can be seen in Table 1.3.

Table 1.3. Summary of differences between wP and aP vaccines.

	wP vaccine	**aP vaccine**
Vaccine formulation	Inactivated whole bacterial cells containing LPS, TLR ligands, antigens in their native conformation	Contains up to five chemically or genetically detoxified antigens (PT, PRN, FHA, Fim2/3)
Immune responses induce by the vaccine	Efficiently activates Th1 T-cells that generate IFN-γ	Stimulates predominantly Th2 T-cells towards specific antigens
Serological correlation of protection	No accepted serological correlate of protection after vaccination with wP vaccine has been established	No accepted serological correlate of protection after vaccination with aP vaccine has been established
Reactogenicity	More reactogenic than aP vaccines due to LPS content in on the whole bacterial cells included in the formulation	Less reactogenic than wP vaccine as it contains purified pertussis antigens without LPS content

1.7 Epidemiology and re-emergence of pertussis

Prior to the introduction of pertussis vaccination, the disease used to play a key role in the morbidity of new-born babies and young children, often leading to severe and life-threatening complications (86). In the early 1900s, it was acknowledged that pertussis also affects adults, however, with milder clinical manifestations. In addition, it was discovered that the infected adults can pass the infection to naïve children (87). The development of pertussis vaccines and routine pertussis immunisations contributed to low incidence of pertussis cases in the vaccination era (87). Recent figures reported by the WHO show that pertussis vaccine coverage with three doses of DTaP vaccine has been about 86% worldwide between 2010 – 2018 (88).

The estimation of the global burden of pertussis is complex primarily due to poor surveillance data, testing, diagnostics, clinical identification of pertussis and reporting of pertussis cases in low-income and middle-income countries (2). Despite the limitations with surveillance data, WHO estimates that 20 to 40 million pertussis cases and 300,000 pertussis-related deaths occur each year worldwide, of which a majority takes place in low-income and middle-income countries (89).

Despite reduced pertussis cases and relatively high pertussis vaccination coverage, pertussis remains a major public health problem, with an increasing incidence of cases in the countries with high aP vaccination rates (90, 91). The epidemiological data show that recent pertussis cases are still high despite high vaccination rates in developed countries (Figure 1.7)

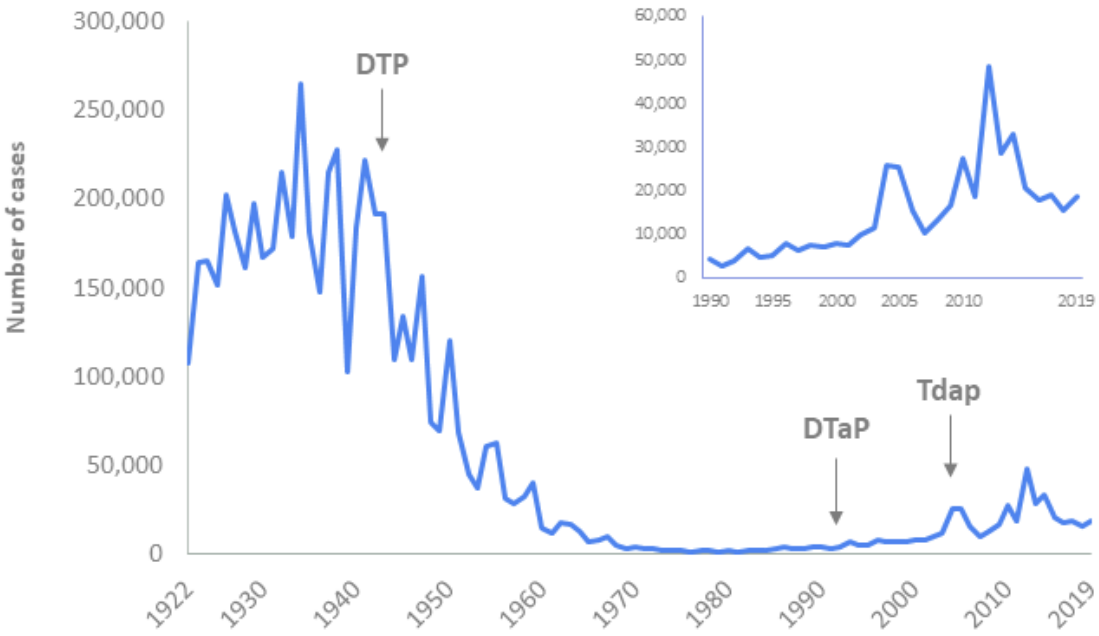


Figure 1.7. Reported pertussis cases from 1922 – 2018. The graph shows the number of pertussis cases in the U.S. reported to the Centres for Disease Control and Prevention (CDC) from 1922 to 2019. After the introduction of wP vaccines in the 1940s, the pertussis cases declined significantly by 1965. However, during the 1980s, the reports of pertussis cases started to increase and by 2019 more than 18,000 cases were recorded nationwide. Taken from CDC https://www.cdc.gov/pertussis/surv-reporting.html.

The epidemiological data suggest that waning of aP vaccine-induced immunity contributes to pertussis resurgence. However, there are other factors that play a role in the rise of pertussis cases (Figure 1.8A). The link between waning of aP-vaccine induced immunity and pertussis outbreaks was initially observed in 7-10 years old children who received aP vaccines in the U.S. Other countries such as UK, Australia, Canada, Spain, Belgium, and the Czech Republic also experienced the same increase in the infection rates after replacing wP vaccines with aP vaccines. (92). In 2012, over 9,300 laboratory-confirmed cases were reported in England (Figure 1.8B) and in the

same year, Public Health England declared national pertussis outbreak in the UK. Since the outbreak, the number of pertussis cases has remained high in England, albeit lower than in 2012 (Figure 1.8B) (93). In the same year, public health authorities recorded 48,277 pertussis cases in the U.S., which was the highest number of pertussis cases since 1955 (79, 86, 94, 95).

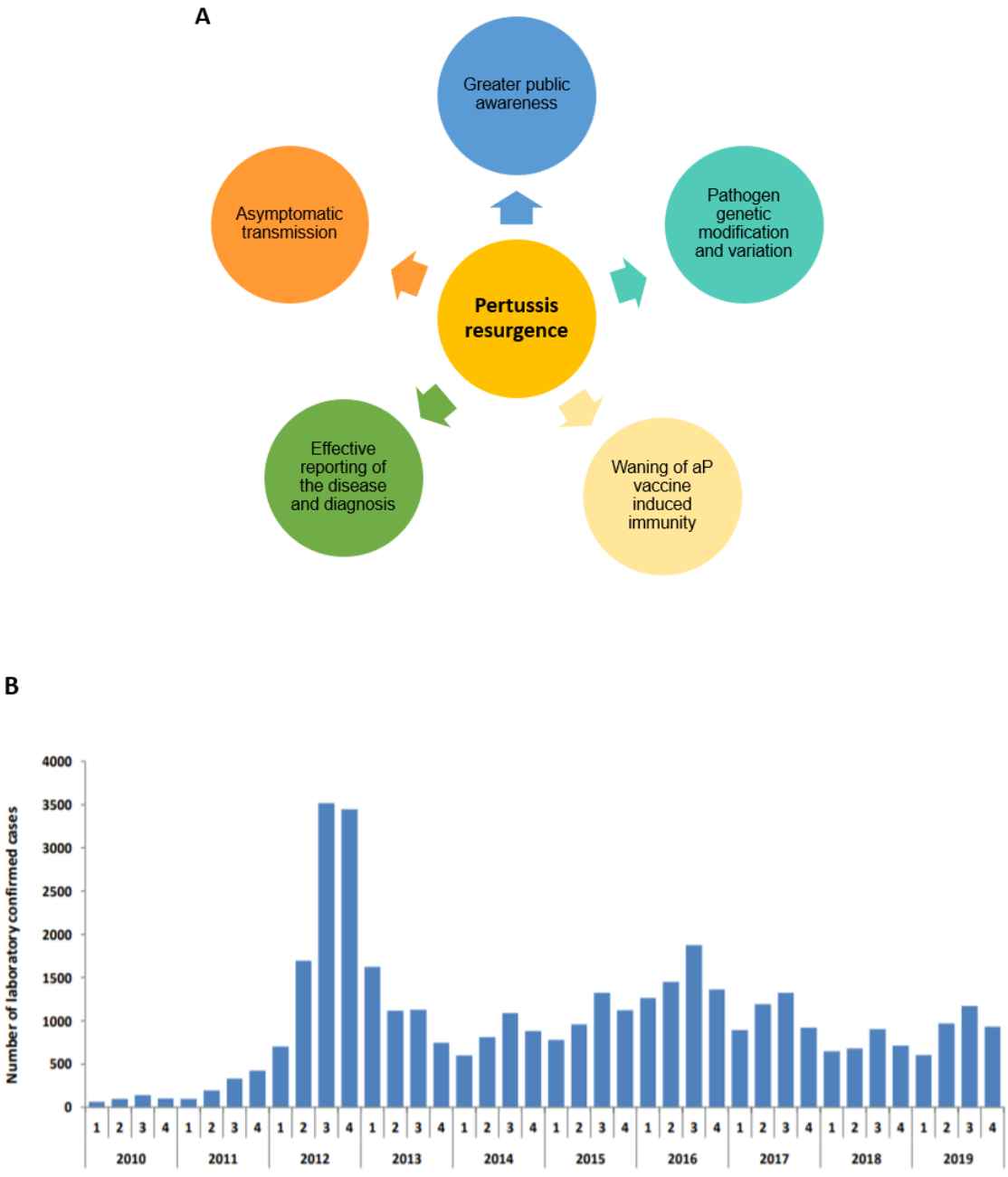


Figure 1.8. Pertussis resurgence. (A) A summary of contributing factors to pertussis resurgence. Many factors contribute to pertussis resurgence such as pathogen genetic modification and adaptation to vaccination, asymptomatic transmission of the infection, effective reporting of the disease, greater public awareness and reduced aP vaccine efficacy. **(B) Laboratory confirmed pertussis cases in England (2010-2019).** According to Public Health England, approximately 9,300 pertussis cases were reported in England in 2012.

Naturally acquired and vaccine-induced immunity are not life-long, which contributes to the prevalence of pertussis in the older population (3). Moreover, the increased incidence of pertussis cases might be associated with asymptomatic infection and transmission of the infection by aP vaccinated individuals (96). Many studies showed that asymptomatic individuals or infected individuals with mild symptoms can transmit pertussis to susceptible individuals. Warfel and colleagues studied *B. pertussis* colonisation and transmission of pertussis in baboons immunised with either wP or aP vaccines, and in baboons naturally exposed to pertussis infection. The study found that aP vaccines protect against severe pertussis disease but are unable to prevent colonisation of the respiratory tract in baboons and contribute to transmission of the infection from aP-immunised to naïve baboons. In addition, based on serological and PCR data, observational studies also propose that sub-clinical pertussis infection can exist in vaccinated children and adults (80). Further evidence of asymptomatic transmission from infected persons has been described in a recent study using human subjects infected with a wild-type *B. pertussis,* suggesting that sub-clinical pertussis infection may contribute to transmission in the population (97).

1.8 A summary of different approaches to prevent resurgence of pertussis by developing novel vaccines

The pre-clinical and clinical studies suggest that development of a new pertussis vaccine promoting a strong Th1-type response in addition to Th2-type response might be beneficial for stimulation of long-lasting protective immunity and effective bacterial clearance (77). To develop such a novel vaccine or enhance the immune responses triggered by the current vaccine, the following approaches have been taken across pertussis field:

1) Use of different adjuvants as opposed to alum adjuvant. Some researchers have proposed to substitute the alum adjuvant for other adjuvants which would improve aP vaccine efficacy by enhancing Th1-/Th17-type responses rather than Th2-type response. For example, Dunne *et al.* (2015) investigated the adjuvant potential of novel TLR2-activating lipoproteins from *B. pertussis* and created a synthetic lipopeptide LP1569 mimicking the bacterial lipoproteins. It was found that synthetic lipopeptide is a potent adjuvant enhancing stimulation of Th1-, Th17-type responses and IgG2a subclass antibodies in mice immunised with purified *B. pertussis* antigens (98). Auderset e*t al.* (2019) used a model of adoptive transfer and showed that 5aP alum-based booster containing genetically modified PT complemented with TLR9 agonist enhances antigen-specific IgG2a responses in aP-primed mice. In addition, the aP booster complemented with the TLR9 enhanced protection of mice against *B. pertussis* (99).

2) New vaccine formulation, such as a vaccine candidate based on outer membrane vesicles (OMVs) has been developed as another strategy. The OMV-based vaccine formulation is made of membrane components of *B. pertussis* enabling the antigens to be presented in their native conformation, along with pathogen-associated

molecular patterns that act as immunostimulatory molecules. This type of vaccine protected mice against PRN-deficient isolate of *B. pertussis* and also induced antigen-specific INF-γ and IL17-secreting tissue resident memory T-cells that are necessary for bacterial clearance from the respiratory tract (100). For example, Carriquiriborde *et al.* (2021) showed that OMV derived from *B. pertussis* biofilm was more immunogenic in mice than OMV derived from planktonic life. In addition, the OMV biofilm-based vaccine was more protective against PRN-deficient *B. pertussis* and *B. pertussis* expressing PRN than OMV planktonic life-based vaccine. These findings suggest that the biofilm-based OMV vaccine has a potential to mediate protective response against PRN-deficient *B. pertussis* strains (101).

3) Addition of novel antigens of *B. pertussis*, in the new vaccine formulations, which are not currently included in aP vaccines. Examples of a few new potential antigen vaccine candidates are adenylate cyclase toxin, biofilm protein *Bordetella* intermediate protein A (BipA) and antigens expressed on the bacterial surface during iron starvation in a host, such as IRP1-3 and AfuA (46, 102-106).

4) Other examples of new vaccine development strategies include exploring different routes of vaccine administration and the introduction of live attenuated pertussis vaccines (45).

5) The pertussis resurgence has accelerated consideration to use new vaccine platforms, distinct from classical vaccines, to develop a new generation of pertussis vaccines. In this study, relatively novel technology using viral vectors has been used to develop new pertussis vaccines. Herein, the viral vectors have been used as vaccine delivery platform against pertussis for the first time. The aim is to explore if this new vaccine technology is able to enhance protective responses and long-term

duration of the immune response against *B. pertussis* in mouse models, through improvement of antigen-specific T-cell responses.

1.9 Recombinant viral vectors as vaccine delivery platform for *B. pertussis*

Recombinant viral vectored vaccines are genetically modified viruses such as adenoviruses or poxviruses that have been developed to express and deliver vaccine antigens. This approach has been used for development of novel vaccines against many infectious diseases such as for malaria or tuberculosis (107). Administration of viral vectored vaccine via intramuscular route leads to infection of muscle cells and expression of the vaccine antigen within 24 hours. The nucleic acid of the viral vector activates pathogen recognition receptors leading to stimulation of innate immune responses, and the expressed antigens go through degradation process in a proteasome followed by presentation to $CD8^+$ T-cells via MHC class I molecules. The expressed antigens can also be secreted and taken up by antigen presenting cells (APCs) which process the antigens, and after migration to draining lymph nodes they present the processed antigens to activated lymphocytes such as $CD4^+$ or $CD8^+$ T-cells and B-cells (Figure 1.9) (108).

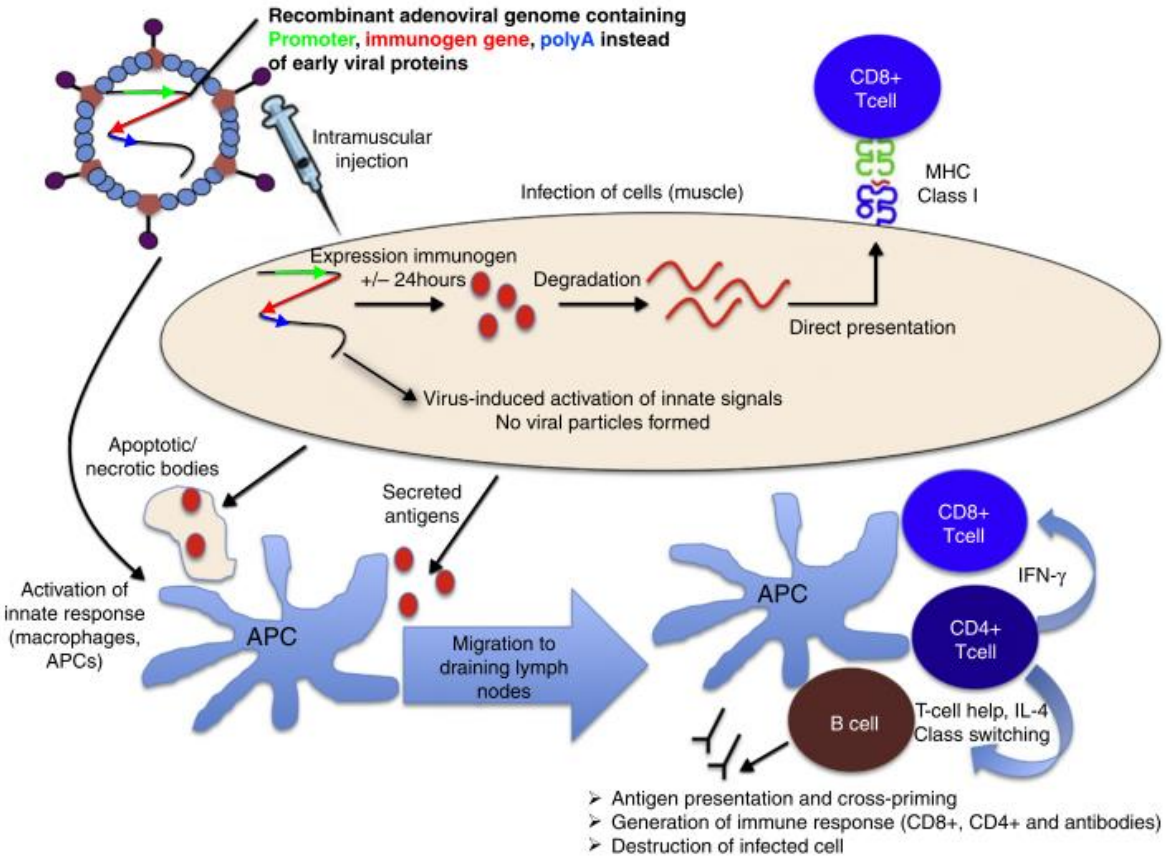


Figure 1.9. Mechanism of antigen presentation after viral vectored vaccine administration. Process of stimulation of antibody and cell-mediated responses against an antigen expressed in host cells by replication-defective viral vectored vaccines. Taken from Ewer *et al.* (2016) (108).

1.9.1 Non-replicating adenoviral vector-based vaccines

Adenoviruses are double-stranded DNA viruses with a small genome of ~ 34 – 43 kb, amenable for genetic manipulation (109). Adenoviral vectors can be genetically engineered to be replication-defective by deletion of E1A, E1B and/or E3 genes responsible for viral DNA synthesis in the infected cells (Figure 1.10). Those deleted genes can be replaced by transgenes encoding the antigen of choice for the target disease (109, 110). The gene deletion prevents the virus generating further copies of its genome after delivery to host cells and instead the antigen of interest is synthesised. In comparison with other vaccine technologies such as mRNA vaccine, adenoviral vector-based vaccines are more cost-effective and have better thermostability (111).

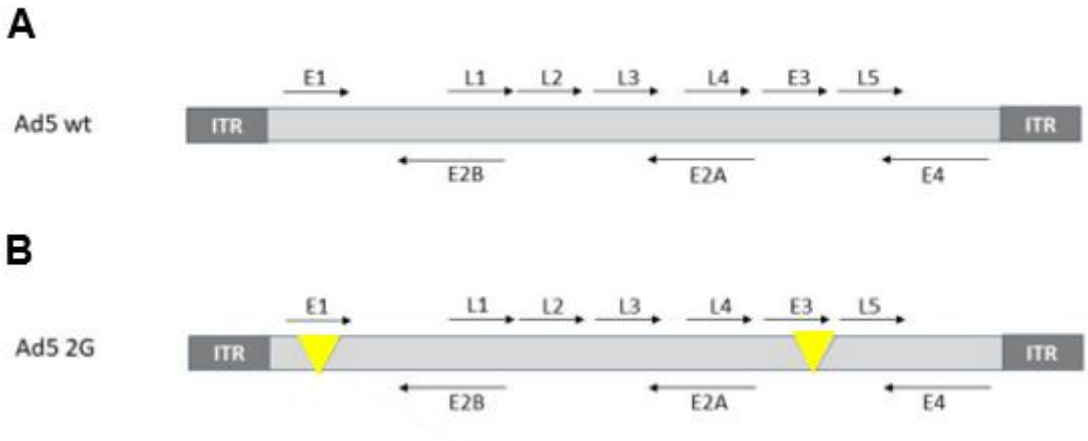


Figure 1.10. A schematic structure of the adenoviral genome. (A) Adenovirus type 5 wild type (Ad5 wt). **(B)** Second generation Ad5 vector with deletions of the E1 and E3 early genes which are enabling replication of the adenovirus (Ad5 2G). The transgene encoding antigen of interest is inserted in place of the E1 and E3 deleted genes (highlighted in yellow upside-down triangle). ITR: Inverted terminal repeats; E1, E2, E3, E4: early adenoviral genes, L1, L2, L3, L4: late adenoviral genes. Taken from Lundstrom (2021) (112).

A Middle East respiratory syndrome coronavirus (MERS-CoV) outbreak in 2012 prompted the Jenner Institute to develop vaccine delivery platform for MERS-CoV vaccine using adenoviral vector technology. When the SARS-CoV-2 outbreak started, the well-established technology and protocols to design viral vector vaccines were used to develop the Oxford/AstraZeneca ChAdOx1-nCoV vaccine (111). Currently, the ChAdOx1 nCoV-19 vaccine is one of the most licensed vaccines for SARS-CoV-2 and the vaccine has proven to be safe and immunogenic in pre-clinical as well as human clinical trials (112). Human Adenovirus Type-5 (HuAd5) was often used as vaccine platform, however, its high seroprevalence and pre-existing immunity in humans can lead to reduced immunogenicity. To overcome this limitation, rare serotypes of human adenoviruses with a low seroprevalence such as HuAd26, HuAd35 or non-human primate adenoviruses have been used for vaccine development for human use (113, 114).

1.9.2 Recombinant modified vaccinia Ankara virus

Poxviruses are large enveloped viruses that contain a linear double-stranded DNA encoding approximately 250 genes. Although poxviruses are DNA viruses, they solely replicate in the cytoplasm of infected cells (115).

Modified vaccinia Ankara (MVA) virus is an attenuated poxvirus used as a viral vector backbone for the development of vaccines against infectious diseases such as malaria, Ebola or HIV. MVA vector-based vaccines induce a high level of transgene expression and both innate and adaptive immune responses (116, 117). As opposed to adenovirus, MVA has a potential to uptake large heterologous DNA (up to 30 kb) into its genome (118). It can express either one transgene or combination of various

genes making MVA a multivalent vaccine. The non-replicating capacity of MVA has been studied and verified in many *in vivo* models, including immunodeficient animals, suggesting it has an excellent safety profile (119). MVA vectors have immunostimulatory capacity, inducing both humoral and cell-mediated responses, especially $CD8^+$ T-cells against heterologous immunogens with long-lasting immunity (117). MVA is often used as a booster vaccine or repeated administration of the MVA vaccine is necessary to stimulate effective immune response (118). In addition, MVA viral particles are sensed by TLR on innate immune cells leading to induction of host innate immune response with adjuvant effect (110). Adenoviral and MVA vector-based vaccines are mostly used in prime-boost strategies to induce antibody and T-cell responses against the vaccine antigens. Adenoviral vector primes a strong antigen-specific T-cell response whereas MVA broadens and enhances memory T-cell responses. Many studies demonstrated Ad-MVA prime-boost as an effective vaccination strategy in pre-clinical as well as clinical studies (120). Currently, there is Ad26/MVA prime-boost vaccine against Ebola virus disease licensed in Europe (121).

As indicated earlier, a vaccine inducing not only antigen-specific antibodies, but also T-cell responses can contribute to better protection against pertussis infection. Viral vectors were originally developed to stimulate T-cell responses against intracellular pathogens (122). However, they are also able to induce antibody responses (108). Therefore, in this project the viral vectors have been explored as vaccine delivery platform to enhance both humoral and cellular immune responses against *B. pertussis* antigens. In this study, adenovirus, and poxvirus, specifically MVA, were used to create vaccine candidates that express different *B. pertussis* antigens. The candidate vaccines' ability to induce humoral and cellular immune responses as well as

protection against *B. pertussis* was assessed in well-established aerosol challenge study.

1.10 Research objectives and aims

The overall aim of the research project is to develop and characterise new generation of pertussis vaccines that promote both Th1-type responses and induce the production of protective antibodies which provide long-lasting protection against *B. pertussis.*

Several studies demonstrated that current aP vaccines may induce only short-term protection and do not mediate Th1-type responses. In addition, the aP vaccines do not prevent subclinical infection, thus allowing transmission to susceptible individuals. The waning of vaccine-induced immunity and recent pertussis resurgence suggest that new improved pertussis vaccine formulations are needed. Therefore, in collaboration with the Preclinical and Novel Vaccine Development team at the Oxford Vaccine Group (OVG), we developed recombinant Ad and MVA viral vectored vaccines encoding PT, Fim, iron-binding proteins (AfuA and IRP1-3) and ACT antigens.

Chapter 2: Selection of other virulence factors of *B. pertussis* for inclusion into viral vector-based vaccines. In this chapter, based on literature search, I selected new antigen candidates for the development of viral vectors against *B. pertussis* and highlighted their potential for the development of the novel vaccine.

Chapter 3: Development and evaluation of viral vectored vaccines expressing the S1 subunit of PT. In chapter 3, I characterised the immune responses induced by Ad and MVA vectored vaccines expressing a truncated version of the S1 subunit (PT S1-220) and a full length of the S1 subunit (PT S1-235), and combination thereof in mouse

models, and further investigated their protective capacity. This was achieved through the following objectives:

- Assessment of immunogenicity and functional antibodies

  The potential of vectors, expressing PT S1-220 and PT S1-235, to induce PT-specific IgG antibodies and functional antibodies, e.g., neutralising antibodies against PT, was assessed by enzyme-linked immunosorbent assay (ELISA) and CHO-cell based PT neutralisation assay, respectively

- Assessment of Th1-type responses
  - The vaccines' ability to mediate PT specific Th1-type response was assessed by measuring cytokine profiles through enzyme-linked immunospot (ELISPOT) assay
- Protection study
  - The protective efficacy of the vaccines was assessed in mouse aerosol challenge model and the vaccines efficacy was compared to the responses mediated by current aP vaccines

Chapter 4: *B. pertussis* iron-binding proteins as antigen candidates for novel viral vectored-based vaccines against pertussis. I developed the vectors and the tools to evaluate the immunogenicity and protective efficacy in mice of vectored vaccines encoding iron binding proteins, including:

- Cloning of the iron-binding proteins (IRP1-3 and AfuA) into viral vectors and explore their potential to induce immune and protective responses in mouse models

- Producing recombinant IRP1-3 and AfuA proteins which can be used as coating antigens in immunogenicity assays to assess antibody responses in mice immunised with vectors expressing the proteins
- Assessing vaccine's ability to induce antibody responses by ELISA
- Examining protective efficacy of the vaccines using mouse aerosol challenge model

Chapter 5: Preclinical development of adenoviral based vaccine candidates expressing fimbriae antigens. This chapter describes development of a model of infection that can differentiate the impact of vaccines on colonisation as well as on infection. This was achieved through the following objectives:

- A recent clinical isolate of *B. pertussis*, B1917 strain, was selected for the optimisation of the colonisation and infection model in mice
- Different challenge doses of *B. pertussis* B1917 strain were selected for the pilot study, based on literature search, to find out an optimal challenge dose that colonises nasal cavity and lungs of mice. After establishing the optimal challenge dose with B1917 strain, the protective capacity of adenoviral vectored vaccine expressing Fim3 (Ad Fim3) can be assessed in aerosol challenge model using the B1917 strain
- I used the challenge model to assess the protective capacity of Ad Fim3 (developed at OVG) in immunised mice.

Chapter 6: Adenylate cyclase toxin as antigen candidate for novel viral vectored-based vaccines against pertussis. In this chapter, I characterised the immune responses induced by adenoviral vectored vaccine expressing fragments of ACT

antigen in mouse models, and further investigated the vaccine's protective capacity. This was achieved through the following objectives:

- Optimisation of ELISA assay prior immunogenicity study
- Assessing vaccine's ability to induce antibody responses by ELISA
- Examining protective efficacy of the vaccines using mouse aerosol challenge model

Chapter 7: Materials & Methods

Chapter 8: General discussion

Chapter 2: Selection of other virulence factors of *B. pertussis* for inclusion into viral vector-based vaccines

2.1 Introduction

Due to the re-emergence of pertussis cases, it has become evident that the development of a novel pertussis vaccine is important. The current aP vaccines are effective at protecting vaccinated children from severe symptoms, however the vaccine-induced immunity wanes over time. In addition, the aP vaccines do not prevent bacterial colonisation and therefore contribute to asymptomatic transmission (80) and promote *B. pertussis* circulation in the population. Isolates that do not express some *B. pertussis* antigens e.g. PRN, FHA and Fim have been identified which is an additional concern (59). Therefore there is a need for novel *B. pertussis* antigens to be included in the new generation of pertussis vaccines.

Here, based on the non-systematic literature search, we proposed a few specific *B. pertussis* antigenic candidates for cloning into viral vectored vaccines. The antigen selection was based on each antigen's immunogenic and protective properties demonstrated in pre-clinical studies. This chapter also describes antigens that had a great potential to be included in vaccine constructs but were eventually excluded. The excluded antigenic candidates were either too big to be cloned into the vectors or published data provided very small evidence about their protective capacities.

The PubMed and Google Scholar databases were used for peer-reviewed and research articles with the terms novel/new *B. pertussis* antigens, immunogenic and protective antigens of *B. pertussis*, novel pertussis vaccine and/or *B. pertussis* vaccine antigenic candidates. All the *B. pertussis* antigenic candidates are summarised in Table 2.1

Table 2.1. Selection of antigenic candidates for cloning into viral vector vaccines

Vaccine antigenic candidate	Brief description of the antigen	Immunogenicity and efficacy in animal models	References
***Bordetella* resistance to killing (BrkA) protein**	A 73 KDa surface-exposed protein. BrkA inhibits bactericidal activity of the complement system resulting in decreased bacterial clearance, opsonisation, killing of the pathogenic bacteria and reduced inflammatory responses in the hosts.	Human BrkA-specific antibodies were shown to have bactericidal activity. Mice immunised with recombinant BrkA protein were not protected against *B. pertussis* infection. Addition of BrkA antigen to 3aP vaccine mediated protective responses against *B. pertussis* 18-323 and Tohama I strains, however it did not induce such immune responses which would outperform those induced by the current aP vaccines.	(123-125)
Tracheal cytotoxin (TCT)	A low molecular weight (925 Da) muramyl peptide expressed at high levels by all *Bordetella s*pp. TCT contributes to nitric oxide production which is toxic to the host cells. It results in damaged ciliated cells.	Data on TCT immunogenicity and protective capacity not available to this date	(126)
Virulence associated gene 8	It is a 95 KDa complement evasion	Vag8-specific antibodies have been	(127)

(Vag8)	molecule that is highly present on the outer membrane vessicles of *B. pertussis* as well as outer membrane of *B. pertussis*. Vag8 inhibits both classical and lectin pathway of the complement system	detected in pertussis patients suggesting that the antigen is synthesised by *B. pertussis* during infection. Data on Vag8 protective capacity not available	
S2 and S3 subunits of PT	S2 and S3 subunits of the cell-binding B oligomer of PT. The B oligomer subunit binds to the ciliated epithelial cells	Immunogenic in mice. Anti-S2 and anti-S3 monoclonal antibodies protective against aerosol challenge in mice	(63)
***Bordetella* intermediate protein A (BipA)**	A surface-exposed polypeptide and the most abundant protein of *B. pertussis* biofilm. It has been hypothesised that the biofilm protein may play a role in pertussis infection, specifically during transmission of the infection	BipA-specific antibodies were found to efficiently opsonise *B. pertussis.* BipA-specific antibodies detected in sera of individuals infected with *B. pertussis*. Immunisation with rBipA protein reduced CFUs in mouse lungs following intranasal challenge	(104, 128)
Adenylate cyclase toxin (ACT)	A 177 KDa secreted protein, consisting of catalytic and receptor	ACT-specific antibodies found in sera of patients recovering	(129-131)

	binding domains. It has an important role in bacterial colonisation at the early stage of the infection. The toxin inhibits bactericidal function of phagocytic cells	from pertussis infection. Non-enzymatic ACT is a potent protective antigen and adjuvant that boosts immunogenic properties of co-administered *B. pertussis* antigens and enhances potency of aP vaccines in mice	
Iron-binding proteins (IRP1-3 and AfuA)	Surface exposed proteins with relatively small molecular weight (IRP1-3 = 19 KDa, AfuA = 36 KDa). Two novel antigens of *B. pertussis* involved in iron acquisition during host colonisation	Both recombinant IRP1-3 and AfuA proteins were found to be immunogenic in mice. The recombinant iron-binding proteins were partially protective in mice against *B. pertussis* intranasal challenge	(105, 106)

One of the potential antigens that could be included in such a vaccine is Virulence associated gene 8 (Vag8). Immunisation of mice with Vag8 has been shown to give rise to antibodies which protect mice from pertussis infection (128). Anti-Vag8 IgG antibodies have been detected in people infected with *B. pertussis* suggesting that Vag8 is produced in humans during pertussis infection (132). Other novel pertussis vaccines currently in development such as the outer-membrane vesicle (OMV)-based pertussis vaccine and the live attenuated BPZE1 vaccine also contain Vag8 antigen in their formulation. Vag8 is highly abundant on OMVs of *B. pertussis* and can also be found on the bacterial membrane of the bacteria (127).

B. pertussis has a capacity to form a biofilm that plays an important role in infection. Biofilm enables the bacteria to remain in the human hosts and promote transmission of the infection and circulation of the strains in the population (59). *B. pertussis* antigens that are expressed during formation of the biofilm might represent potential targets for inclusion into the novel vaccines. *Bordetella* intermediate protein A (BipA) is a biofilm-associated protein expressed on the bacterial surface and it is possibly involved in interactions between the pathogen and a host (104, 133).

BipA has the potential to be an antigenic candidate for the development of novel pertussis viral vectored vaccines. Recombinant BipA protein is immunogenic and protective in mice. BipA was found to reduce bacterial load in lungs of immunised mice following pertussis infection and serum BipA-specific antibodies were able to opsonise bacteria. Moreover, Gouw e*t al.* (2014) demonstrated that BipA-specific antibodies were found in serum samples of individuals recovering from pertussis infection (104). Stockbauer *et al.* (2001) hypothesised that BipA may be able to enhance high-affinity binding of the bacteria to the host's mucosal surfaces during the initial stage of the

infection (134). These pre-clinical studies have provided overall encouraging results in terms of immunogenicity and protective capacity of BipA protein and provide support for the inclusion of the protein in the viral vectored vaccines. However, we were unable to make viral vector construct encoding the BipA transgene cassette. During the cloning process, we experienced difficulties to ligate the BipA gene into the vector, possibly due to its high molecular weight which is 137 KDa.

Bordetella resistance to killing protein (BrkA) is a 73-kDa surface-exposed protein produced by *B. pertussis*. The protein is involved in complement evasion as it inhibits bactericidal activity of the complement system resulting in decreased bacterial clearance, opsonisation, killing of the pathogenic bacteria and reduced inflammatory responses in the hosts (125). Human BrkA-specific antibodies were shown to have bactericidal activity and a 3aP vaccine containing BrkA, PT and FHA antigens induced protective response in mice. However, mice immunised with recombinant BrkA protein were not protected against *B. pertussis* infection (123). Current aP vaccines do not contain BrkA protein as vaccine antigen, however, its role in protection of *B. pertussis* against host's immune responses suggests that BrkA might be a potent protective antigen that could be included in newly developed pertussis vaccine. Eliciting vaccine-induced immune responses against BrkA might lead to a greater bactericidal activity and decreased bacterial load in the respiratory tract of the host, and potentially reduced transmission of the infection (135). Marr and colleagues analysed the protective activity of BrkA protein in mouse intranasal respiratory challenge model. This study found that the 3aP vaccine containing BrkA protein, PT and FHA antigens was as efficacious as the commercial 3aP vaccine (124). Addition of BrkA antigen to the aP vaccine mediated protective responses against *B. pertussis* 18-323 and Tohama I strains, however it did not induce such immune responses which would

outperform those induced by the current aP vaccines. Combinations of BrkA protein with different *B. pertussis* antigens or using higher doses of vaccine containing BrkA might contribute to enhanced protection. Based on the published data, we decided not to include BrkA in the viral vector constructs as it may not induce effective protection against pertussis infection in mice.

Tracheal cytotoxin (TCT) is *B. pertussis* antigen that is involved in destruction of ciliated epithelial cells (126). Targeting TCT by antibodies may lead to prevention of the cellular damage, therefore we suggested that the potential inclusion of this novel antigen in viral vector vaccines could avoid TCT's cytotoxicity and therefore enable the host ciliated epithelial cells to clear the pathogen more effectively. However, TCT's immunogenic and protective potential has not been researched extensively and for that reason, we excluded its cloning into the viral vector constructs.

The B-oligomer of PT is composed of one S2, one S3, two S4 and one S5 subunits. Sato and Sato (1990) studied protective properties of five monoclonal antibodies (mAbs) against PT. The mAbs included anti-S1 and anti-S2, anti-S3, and anti-S4 antibodies. The mAb binding to both S2 and S3 subunits, known as 11E6 mAb, protected mice against B. pertussis aerosol challenge. In fact, the highest protectivity was shown by four anti-S1 mAbs (1B7, 1D7, 3F11, and 1OD6) and anti-S2/S3 antibodies (11E6, lOB5, and 1OC9) which indicates that some protective epitopes are localised in the S1 and S2/S3 subunits. They also found that a combination of anti-S1 and anti-S2/S3 mAbs, showed higher specific activity in PT neutralization assays than polyclonal antibodies against PT. A combination of 10 µg of antiS1 mAb, 1B7, and anti-S2/S3 mAb, 11E6, induced almost complete protection against pertussis in mouse

aerosol challenge study (63). Although, the above mentioned subunits had a great potential to mediate effective protective response in mice, we decided not to clone these three subunits into the viral vectors due to technical challenges. In addition, it was uncertain how these subunits would express and/or fold in the mammalian cells as the other subunits would not have been present and there is strong evidence that the S4 subunit of the B-oligomer plays a role between the S1 subunit interaction with the B-oligomer. Also we may face challenges during cloning. The molecular weight of the PT is 105 KDa which may be difficult to ligate into the vector.

Beside the above mentioned potential antigens, here we describe those attractive novel candidate antigens that have been successfully selected and cloned into the viral vectors.

Adenylate cyclase toxin (ACT) is synthesised as an inactive precursor by *B. pertussis.* Its activation is initiated by palmitoylation of K983. It allows binding to the host cell receptors and forming a cationic pore in the plasma membrane of the target cells (16). The toxin is a 177-KDa protein made of two functionally different domains: A C-terminal haemolytic domain and a N-terminal catalytic domain. The C-terminal domain, which is made of approximately 1,300 amino acids, belongs to repeat-in-toxin (RTX) family. The RTX domain of the toxin has a role in binding to αMϐ2 integrins receptors on phagocytic leukocytes such as macrophages and neutrophils (Figure 2.1). In addition to binding to target cells, another role of the C-terminal domain is formation of a cationic pore in plasma membrane of the target cells. The N-terminal domain, which is made of 400 amino acids, has adenylate cyclase (AC) enzymatic activity which is activated once inside the cytoplasm of eukaryotic cells having been transported across the plasma membrane (130, 136). The catalytic domain binds

calmodulin inside the cytoplasm and catalyses conversion of cytosolic ATP to cyclic adenosine monophosphate (cAMP) (137). High levels of cAMP are toxic for the cells and lead to weakening of host innate immune responses and inhibiting the activation of macrophages, neutrophils and dendritic cells (DCs). The inhibited immune cells are unable to phagocyte opsonised bacteria. Furthermore, the impaired cell signalling pathways have an impact on the DCs as they are unable to induce adaptive immune responses by presenting processed antigenic peptides to T-cells (138).

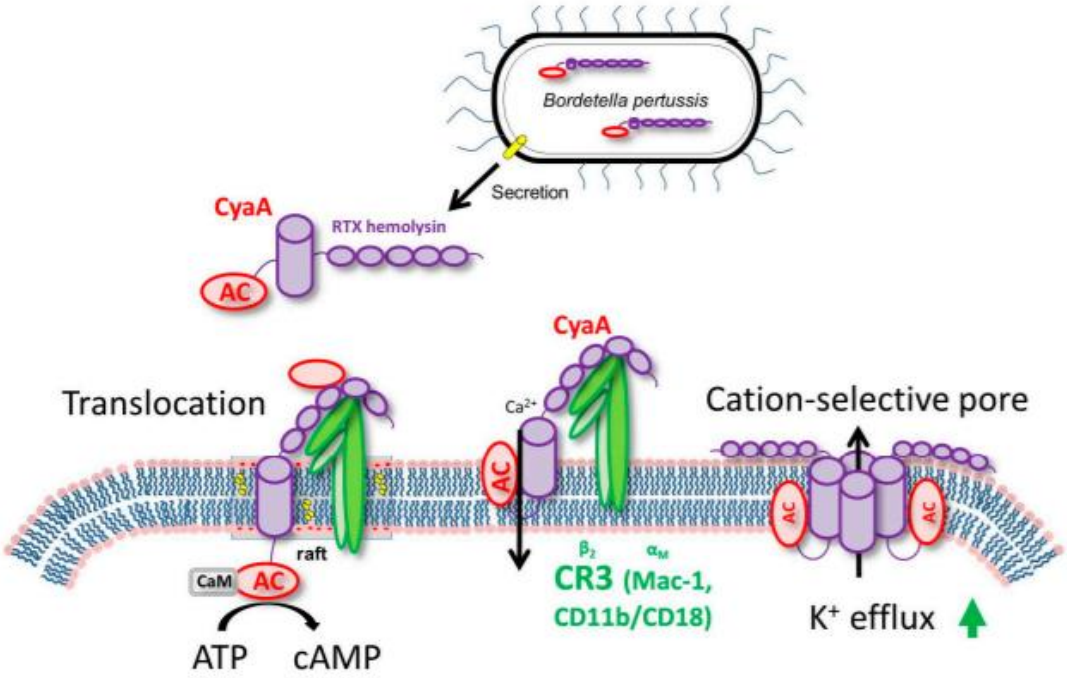


Figure 2.1. Mode of action of ACT on a membrane of phagocytic immune cell. *B. pertussis* secrets ACT into calcium-containing fluid on mucosal surfaces. ACT binds Ca^{2+}, folds into its shape and then binds the complement receptor 3 (CR3), known as the αMβ2 integrin (CD11b/CD18 or Mac-1), on the surface of myeloid phagocytic cells. This is followed by the toxin insertion into the lipid bilayer of cellular membrane. The catalytic AC domain then translocates across the cell membrane. The AC enzyme binds cytosolic calmodulin and catalyses conversion of ATP to cAMP. The RTX domain of ACT is functionally independent of the AC domain and the pore-forming precursors create cation-selective pores that permeabilise cell membrane for outflow of K^+ ions from the cytosol. Taken from Fedele *et al.* (2017) (139).

ACT plays a critical role in bacterial colonisation at the early stage of the infection. *Bordetella* strains which lack the expression of ACT have impaired colonisation and a high rate of bacterial clearance in mouse lungs (140). Wang *et al.* (2015) have shown that the RTX domain, located on the C-terminal domain of ACT, stimulates production of neutralising antibodies in mice, proposing that the antibodies prevent binding of the toxin to the host cell receptors (130). Both active and passive immunisation of mice with ACT purified from *B. pertussis* and polyclonal anti-ACT antibodies, respectively, provided protection against *B. pertussis* in mouse challenge model suggesting protective efficacy of ACT (129). Although ACT is a promising vaccine candidate, currently, the toxin is not included in pertussis vaccine formulations as it is susceptible to mis-folding, formation of protein aggregates and degradation when recombinantly expressed in *E. coli* (130). Recent preclinical research demonstrated immunogenic and protective characteristics of ACT, therefore it has been selected as antigen candidate for inclusion into the viral vectored vaccine.

One of the most important nutrients for bacterial survival in a host is iron. In humans, the level of free iron is very low due to iron-sequestering proteins (141). Bacterial survival under iron-poor conditions is difficult, therefore, bacteria developed mechanisms to acquire extracellular iron and use it for their growth and existence (142). The mechanisms include low-molecular-weight iron chelators (siderophores), and bacteria also use their cell surface receptors to extract iron from host proteins. Several *B. pertussis* proteins expressed under iron depletion conditions have been identified (105). These iron-binding surface-associated proteins also enable attachment of *B. pertussis* to the host's epithelial respiratory cells, and moreover, they are immunogenic in mice and humans during infection and may therefore be suitable as antigens in pertussis vaccines (141). Alvarez-Hayes and colleagues identified two

such iron-binding proteins of *B. pertussis*, IRP1-3 (BP1152) and AfuA (BP1605) (105, 106).

IRP1-3 is a dimeric outer membrane protein, and its bacterial surface exposure is enhanced during iron starvation; the protein is expressed irrespective of the bacterial virulence phase on clinical isolates of *B. pertussis.* Moreover, IRP1-3 protein was shown to bind to human IgG serum samples obtained from patients having pertussis infection, demonstrating that the protein is immunogenic in humans and naturally presented to the immune system during the infection (105).

Another iron binding protein known as AfuA (BP1605) is involved in using the main iron sources, namely transferrin and lactoferrin, in an *in vivo* environment for bacterial survival. Antibodies raised against recombinant (rAfuA) in mice bound to heat inactivated and live iron-starved whole-cell *B. pertussis* bacteria, suggesting that the protein is exposed on the bacterial surface (106). Alvarez-Hayes *et al.* (2013) demonstrated that 2 µg of rAfuA emulsified in complete Freund's adjuvant was immunogenic in mice following active immunisation. Immunisation with rAfuA was able to induce both Th1- and Th2-type responses and bacterial load was significantly reduced in mice actively immunised with rAfuA as opposed to mice that received adjuvant only (negative control) following intranasal challenge.

Chapter 3: Development and evaluation of viral vectored vaccines expressing the S1 subunit of pertussis toxin

3.1 Introduction

Pertussis toxin (PT) is one of the most important virulence factors of *B. pertussis.* PT is a 105 KDa secreted exotoxin synthesised exclusively by *B. pertussis*. The toxin is composed of six subunits, the catalytic A (S1) subunit and the cell-binding B oligomer, which is made of S2, S3, two S4 and S5 subunits (55, 58, 60, 143). Following the toxin's attachment to host cells, the toxin undergoes endocytosis, retrograde transport to the endoplasmic reticulum where the catalytic A (S1) subunit gets translocated to the cytosol. The role of the S1 subunit is to disrupt cell signalling pathways which leads to an increased level of intracellular cAMP. The increased cAMP level results in immunosuppression of innate immune responses, such as recruitment and migration of neutrophils and macrophages to the site of the infection (143).

At an early stage of pertussis infection, PT inhibits host immune responses which promotes *B. pertussis* growth in the respiratory tract. However, at the peak of the infection, studies demonstrated that PT correlates with increased inflammatory responses in the airways of mice (144). Although there is no direct link between PT and severe coughing, baboon models of pertussis suggest that PT may be responsible for the coughing episodes, probably due to promotion of the inflammation in the airways at the peak of the infection (144, 145).

The role of PT as a vaccine antigen was supported in mouse and human studies. A study, which assessed anti-PT monoclonal antibody prophylaxis in mice before aerosol challenge, found that the antibody treatment was effective at reducing pertussis symptoms. Household studies conducted in Sweden and Germany indicated

that PT-specific antibodies correlate with protection against *B. pertussis.* It has been hypothesised that the protection is multifactorial and probably associated with both humoral and cell-mediated immune responses, following natural pertussis infection, in local mucosal tissues as well as in the systemic immunity (146).

Those results encouraged the inclusion of PT in the aP vaccine that was first developed and introduced in Japan in 1981, and PT has become the main antigenic component of all aP vaccine formulations (61, 147). The important role of PT as the protective antigen was demonstrated in a study using a monocomponent PT aP vaccine. The vaccine was shown to be 80% efficacious in fully immunised infants in Denmark, and its efficacy was similar to efficacy of the three-component (3aP) and five-component (5aP) aP vaccines. Similar data were observed in the study conducted in Sweden, where the monocomponent PT vaccine was 71% efficacious (68).

Since PT is able to induce protective response as a single antigen in mice, it is a promising antigen candidate for development of viral vectored vaccines. From literature search, we found out that the catalytic S1 subunit of PT contains protective epitope binding to 1B7 monoclonal antibody (mAb) which can potently neutralise PT's activity in mice (143). Humanised 1B7 (hu1B7) antibody, which binds the same protective epitope on the S1 subunit, was also able to significantly reduce colonisation of mouse lungs by *B. pertussis* through toxin inactivation (148). Therefore, using the S1 subunit only, as opposed to the entire PT, has the potential to induce protective antibodies and was selected as our candidate antigen for viral vectors. In addition, due to PT's complex structure and size, cloning all PT subunits into viral vectors would be technically difficult. The S2/S3 subunits were also considered as vaccine antigen candidates. The 11E6 mAb binding to an epitope spanning S2/S3 subunits on the B

oligomer has ability to inhibit toxin-receptor binding and protect mice against *B. pertussis* (64). However, we decided not to include those subunits as previous studies showed that both S2 and S3 subunits are more stable in the presence of the S4 subunit (149).

Different types of viruses can be genetically modified and used as vaccine delivery platforms for cancer, viral and parasitic antigens by inducing potent antigen-specific humoral and cellular immune responses (108, 150, 151), activating the innate immune responses through their pathogen-associated molecular patterns, while remaining safe (108). In particular, replication-defective recombinant adenovirus (Ad) and poxviruses such as modified vaccinia virus Ankara (MVA) induce Th1-type responses, desired in the context of pertussis vaccination (122). Thus, we created a series of viral vectored vaccines expressing truncated version and full length of the S1 subunit, consisting of amino acid residues 1-220 and 1-235, respectively, to allow their insertion into viral vectors. Truncated version of the S1 subunit (PT S1-220) was chosen because of better expression of the antigen in *E. coli.* Unlike PT S1-235, the truncated S1-220 subunit does not contain a long hydrophobic tail anchored into the B subunit which could potentially affect the antigen expression (143). Here, we explore the ability of viral vectored vaccines to induce immunogenic as well as protective response to two different versions of the S1 subunit in mouse models of pertussis.

3.2 Materials and Methods

3.2.1 Cloning process of adenoviral vectored vaccines expressing PT S1-220 and PT S1-235

The cloning process of the viral vectored vaccines is described in detail in Chapter 7 (General Materials and Methods, section 7.1, subsection 7.1.1).

3.2.2 Immunisation of mice with the viral vectored vaccines expressing PT S1-220 and S1-235

Six to eight week-old female BALB/c mice (n = 6) were immunised intramuscularly with one dose of adenoviral vectored vaccine or MVA vaccine expressing either S1-220 or S1-235 subunit of PT. Some groups of mice received prime-boost immunisation with Ad, MVA S1-235; MVA, Ad S1-220 or MVA, Ad S1-235 at eight-week interval. Positive control groups were immunised with either two doses of wP or aP vaccines at eight-week interval. Naïve mice were immunised with sterile DPBS. Ad and MVA vaccine dose per mouse were 1 x 10^9 IU/100 µl and 1 x 10^7 IU/100 µl, respectively (Table 3.1). Blood was drawn from the tail to collect serum six, 10 and 14 weeks after the primary immunisation. Cardiac puncture was performed to collect serum 20 weeks after primary immunisation.

Table 3.1. Mouse immunisations with viral vectored vaccines expressing PT S1-220 or PT S1-235.

Group	Prime vaccine (DAY 0)	Boost vaccine (DAY 56)	Immunisation route
1	Ad S1-235 (1×10^9 IU/100 µl)	-	IM
2	Ad S1-235 (1×10^9 IU/100 µl)	MVA S1-235 (1×10^7 IU/100 µl)	IM
3	Ad S1-220 (1×10^9 IU/100 µl)	MVA S1-220 (1×10^7 IU/100 µl)	IM
4	MVA S1-235 (1×10^7 IU/100 µl)	Ad S1-235 (1×10^9 IU/100 µl)	IM
5	MVA S1-220 (1×10^7 IU/100 µl)	Ad S1-220 (1×10^9 IU/100 µl)	IM
6	DPBS	DPBS	IM
7	WHO International Standard Bordetella pertussis Vaccine (Whole Cell) NIBSC code:94/532 0.4IU/dose	WHO International Standard Bordetella pertussis Vaccine (Whole Cell) NIBSC code:94/532 0.4IU/dose	IM
8	aP vaccine ($1/10^{th}$ human dose)	aP vaccine ($1/10^{th}$ human dose)	IM

3.2.3 Determination of serum anti-PT IgG, IgG1 and IgG2a isotypes in mice by ELISA

Serum PT-specific IgG, IgG1 and IgG2a antibodies titres were measured in immunised and naïve mice by indirect enzyme-linked immunosorbent assay (ELISA) ('General Materials and Methods' section, Chapter 7, sections 7.4 and 7.5). Total PT-specific IgG responses were analysed in sera obtained from tail bleeds of mice immunised with Ad and MVA vaccines encoding PT S1-220 or PT S1-235. The tail bleeds were obtained six, 10, 14 and 20 weeks after the primary immunisation. PT-specific IgG1 and IgG2a isotypes were analysed in sera obtained from terminal bleeds of the immunised mice 20 weeks after the primary immunisation. The immunisation schedules are shown above in Table 3.1. Control groups were immunised with two doses of either 0.4 IU/dose of wP vaccine or 1/10th human dose of aP vaccine at eight-week interval.

3.2.4 Mouse aerosol challenge study

Two studies were conducted in which protective capacity of viral vectored vaccines expressing either S1-220 or S1-235 subunits of PT was assessed. In both studies, six to eight week-old female BALB/c mice (n = 10/group) were immunised intramuscularly as shown in Table 3.2. The Ad vaccine and MVA vaccine were administered at dose of 1 x 10^9 IU/50 µl and 1 x 10^7 IU/50 µl, respectively. The positive control group in the first challenge study received two-dose immunisation with 1/10th human dose of 5aP vaccine. The positive control group in the follow-up challenge study received two-dose immunisation with 1/10th human dose of 3aP vaccine. The interval between prime-boost immunisation was eight weeks. Naïve mice (negative control group) were immunised with sterile PBS (Figure 3.1).

Table 3.2. Mouse immunisations with Ad and MVA vectored vaccines encoding either PT S1-220 or PT S1-235 before aerosol challenge study.

Group	Prime vaccine (Day 0)	Booster (Day 56, week 8)	Dose	Immunisation route
1	Ad	-	1 x 10^9 IU/100 µl	IM
2	Ad	MVA	1 x 10^9 IU/100 µl 1 x 10^7 IU/100 µl	IM
3	MVA	Ad	1 x 10^7 IU/100 µl 1 x 10^9 IU/100 µl	IM
4	aP vaccine	aP vaccine	1/10th human dose	IM
5 (naïve)	PBS	PBS	-	IM

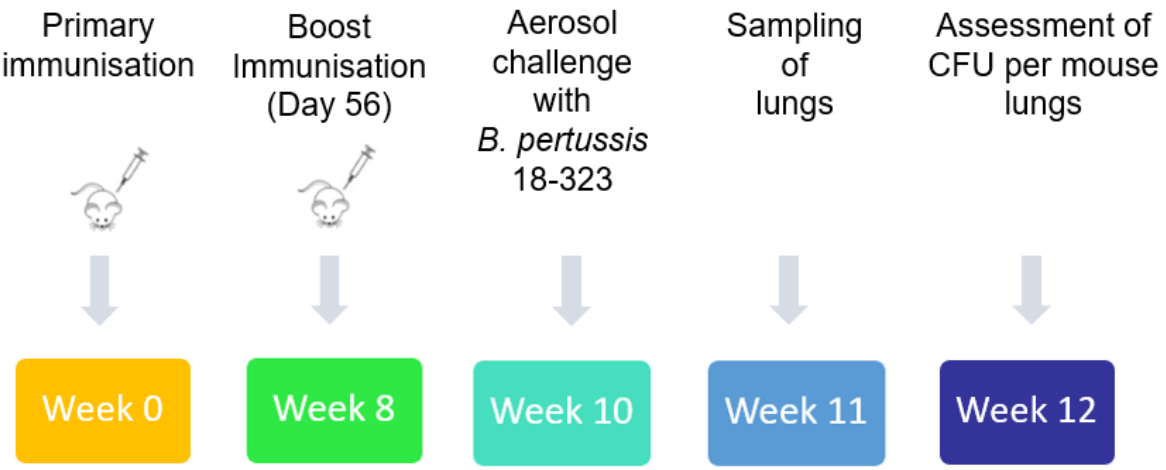


Figure 3.1. Timeline of the challenge following immunisation of mice. Mice were immunised with one dose of adenoviral vectored vaccine, or they were prime-boosted with Ad, MVA or MVA, Ad vaccine at eight-week interval. Two weeks after the booster, mice were challenged with *B. pertussis* 18-323 strain. One week after challenge, mouse lungs were harvested and homogenised in sterile PBS to assess bacterial colonies in the lungs and determine the vaccine's efficacy.

3.2.5 Aerosol challenge of mice with virulent *B. pertussis* 18-323 strain

The aerosol challenge is described in Chapter 7 (General Materials and Methods), section 7.2 (subsections 7.2.1 – 7.2.3).

3.2.6 Pertussis toxin neutralisation assay

Serum samples were obtained from mice immunised with Ad and MVA vaccines encoding either PT S1-220 or PT S1-235 (immunogenicity study, sub-section 3.2.2). Tail bleeds were obtained six and ten weeks after primary immunisation. Sera were serially diluted two-fold (starting at 1/8 dilution) in a sterile 96-well plate in Ham's F-12K Kaighn's medium (Gibco). The WHO reference serum (NIBSC code 97/642) was used as a positive control. Each vial of the freeze-dried serum was dissolved in 0.5 ml of distilled water and the solution was kept cool at 4°C prior to use. 25 µl of the reference serum was added to well number one (row G in 96-well plate) and other 25 µl of the serum was added to 25 ul of Ham's F12-K Kaighn's medium (Gibco) in well number two (row G in 96-well plate). This is then diluted across the plate in two-fold dilutions. Blank wells, which served as negative control, contained only 25 µl/well of Ham's F-12K Kaighn's medium (Gibco). A concentration of 4 ng/ml of PT was added to the diluted test sera and the positive control. The samples were incubated at 37°C for three hours. Following the incubation, confluent Chinese Hamster Ovary (CHO) cells were trypsinised with 0.25% trypsin-EDTA (Gibco) and their concentration (cells/ml) was determined using a haemocytometer. CHO cell suspension was diluted to a concentration of 2 x 10^4 cells/ml with Ham's F-12K Kaighn's medium, 10% foetal calf serum (FCS), 2 mM L-glutamine and streptomycin/penicillin. Sterile 96-well plates were seeded with 200 µl of CHO cell suspension and incubated at 37ºC with 5% CO_2 for 48 hours. Cell morphology was examined using a light microscope at four-fold

magnification. CHO cell morphology was examined in each well. Active PT alters the CHO cell morphology and causes them to form clusters in the absence of PT neutralising antibodies (nAbs) in serum samples. CHO cell neutralisation titre is determined by calculating the highest dilution of serum that prevents clustering of CHO cells. Due to plate to plate variation, the neutralisation titres of the test sera were determined as a ratio of test sera to reference sera neutralisation titres.

3.2.7 Assessment of PT-specific IFN-γ, IL-5 and IL-17 by *ex vivo* FluroSpot assay in mice immunised with Ad S1-220 and Ad S1-235 vaccine

Six to eight weeks old female BALB/c mice (n = 5/group) were immunised intramuscularly with either Ad S1-220 or Ad S1-235 vaccine. Positive control group was immunised with two doses of the aP vaccine (1/10th of a human dose) at three-week interval. Naïve mice were immunised with 50 µl of sterile DPBS at three-week interval (Table 3.3). Spleens and terminal bleeds were obtained from aP- and DPBS-immunised mice two weeks after the second dose (week five), and from Ad S1-220- and Ad S1-235-immunised mice two weeks after the first dose.

Table 3.3. Immunisation of mice with Ad S1-220 and Ad S1-235 for the assessment of PT-specific T-cell responses.

Vaccine	First immunisation (Day 0)	Second immunisation (Day 21)	Dose	Spleen harvest and cardiac bleeds (Day 35)
Ad S1-235	-	✓	1 x 10^9 IU/µl	✓
Ad S1-220	-	✓	1 x 10^9 IU/µl	✓
3aP vaccine	✓	✓	1/10^{th} human dose (50 µl)	✓
Naïve mice	✓	✓	50 µl of DPBS	✓

Mouse spleens from immunised and naive mice were removed, homogenised, and depleted of erythrocytes by treatment with Red Blood Cell Lysis Solution (QIAGEN, U.S.). FluroSpot assays were performed on frozen splenocytes extracted from spleens of mice 14 days after immunisation with a single dose of Ad S1-235 or Ad S1-220, and with two 1/10^{th} human doses of 3aP vaccine. The splenocytes were stimulated with a single pool of 65 individual 15-mers peptides, overlapping by 11 amino acids, and covering the entire S1 subunit of PT (ProImmune, UK). Prior the splenocyte stimulation, the peptides were dissolved in sterile Dimethyl sulfoxide (DMSO) and pooled to get a final concentration of 1 mg/ml.

Sterile IPVH-membrane plates (Millipore) were coated with 15 µg/ml of anti-IFN-γ monoclonal antibody (mAb), 10 µg/ml of anti-IL-17 mAb and 15 µg/ml of anti-IL-5 mAb (100 µl/well). Following overnight incubation at 4°C, the wells were washed five times with sterile PBS, dried and blocked with Dulbecco's Modified Eagle's Medium (DMEM)

supplemented with 10% FCS and 1% Penicillin/Streptomycin for 30 minutes at 37°C. Appropriate wells were coated with 6 µg/ml of PT S1 peptide, 25 µg/ml of Concanavalin A (Sigma-Aldrich) and 0.6 % DMSO in DMEM, which were used as positive and negative controls, respectively. Then, 4 x 10^6 cells/ml were seeded onto 96-well plates and incubated for 18 hours at 37°C in the presence of 5% CO_2. After the incubation, the cells were removed, and the plates were washed five times with PBS (1X). Anti-IFN-γ, anti-IL-17A and anti-IL-5 biotinylated detection mAbs (Mabtech) were diluted 1/200, 1/50 and 1/500, respectively, in PBS containing 0.1% BSA. The prepared detection mAbs were then added to appropriate wells (100 µl/well) and the plates were incubated for two hours at room temperature. Afterwards, plates were washed five times with PBS (1X) and dried. Fluorophore-conjugates were diluted to 1/200 in PBS containing 0.1% BSA and added to each well (100 µl/well). The plates were incubated at room temperature for one hour in a dark box to prevent exposure to light. Afterwards, plates were washed five times with PBS (1X) again and dried. Fluorescence enhancer was added to each well (50 µl/well) and was left to incubate for maximum of 15 minutes in the dark at room temperature. After the incubation, the enhancer was removed, and plates were left to dry in the dark at room temperature. Spots were visualised by using the AID Elispot 7.0iSpot reader (AID GmbH, Germany).

3.3 Results

3.3.1 A single dose of Ad S1-220 and Ad S1-235, and heterologous prime-boost regimen with Ad and MVA candidates induced PT-specific IgG responses in mice

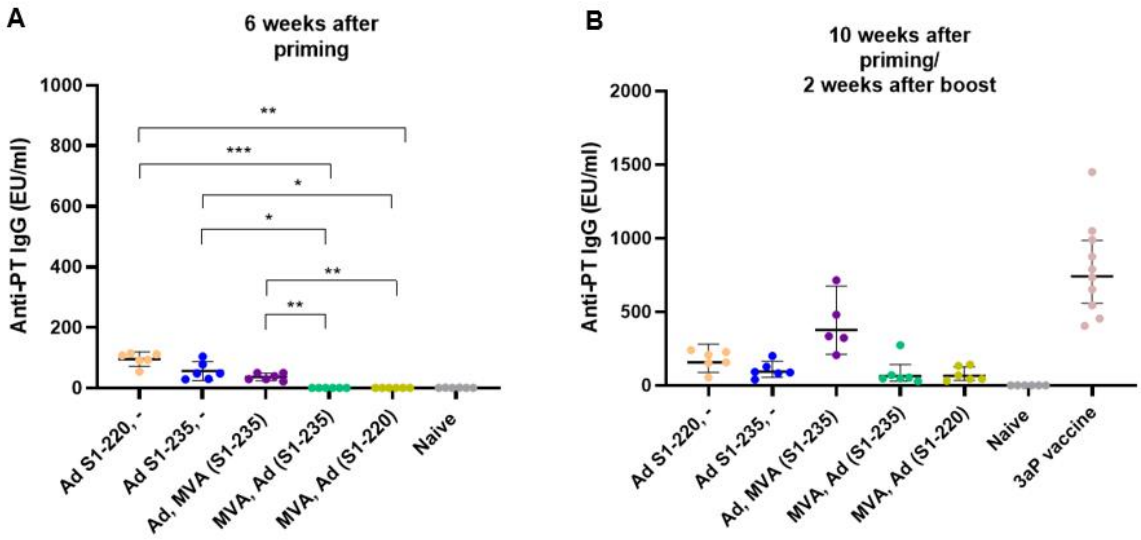
The first objective was to evaluate whether immunisation with the viral vectored vaccine candidates was able to induce a PT-specific IgG antibody response in mice. To assess the immunogenicity of the vaccines, groups of mice were immunised intramuscularly with either one dose of Ad vaccine or a prime boost regimen (Ad, MVA or MVA, Ad) at eight-week interval. A single dose immunisation with either Ad vaccine expressing S1-220 or S1-235 region of PT was able to induce anti-PT IgG antibody responses in mice six weeks after the vaccination. Ad S1-220 vaccine induced significantly higher anti-PT IgG antibodies than Ad S1-235 vaccine ($p = 0.0032$) (Figure 3.2A). However, a single dose immunisation with MVA S1-220 or MVA S1-235 failed to induce anti-PT antibody responses at week six (Figure 3.2A).

As shown in Figure 3.2B, booster immunisation with MVA S1-235 at week eight induced strong anti-PT IgG antibody responses in mice primed with Ad S1-235 two weeks after the boost. These responses were higher than those induced by a single dose immunisation with Ad S1-220 or Ad S1-235 ($p = 0.0023$ and $p < 0.0001$, respectively). Prime-boost regimen with Ad and MVA S1-235 also induced significantly higher anti-PT IgG antibodies in mice two weeks after the boost than prime-boost regimen with MVA followed by Ad S1-220 or Ad S1-235 ($p < 0.0001$) (Figure 3.2B).

As shown in Figure 3.2C, 14 weeks after primary immunisation (six weeks after the boost), anti-PT IgG responses were detected in all immunisation regimens. However, the prime-boost regimen with MVA, Ad S1-235 elicited higher anti-PT IgG antibodies than one dose immunisation with Ad S1-235 ($p = 0.0103$).

Ad S1-220 vaccine induced higher anti-PT IgG responses than Ad S1-235 20 weeks after primary immunisation (p = 0.0276, Figure 3.2D). The prime-boost regimen with MVA and Ad S1-220 induced higher anti-PT IgG antibodies than prime-boost regimen with MVA followed by Ad S1-235 and a single dose immunisation with Ad S1-235 (p = 0.0033 and p = 0.0004, respectively).

Prime-boost immunisation with aP vaccine (positive control group), at eight-week interval, induced PT-specific IgG responses in mice 10 and 14 weeks after the primary immunisation (Figure 3.2B and 3.2C). The data for the positive control group were obtained at different time than data from the viral vector immunisations.



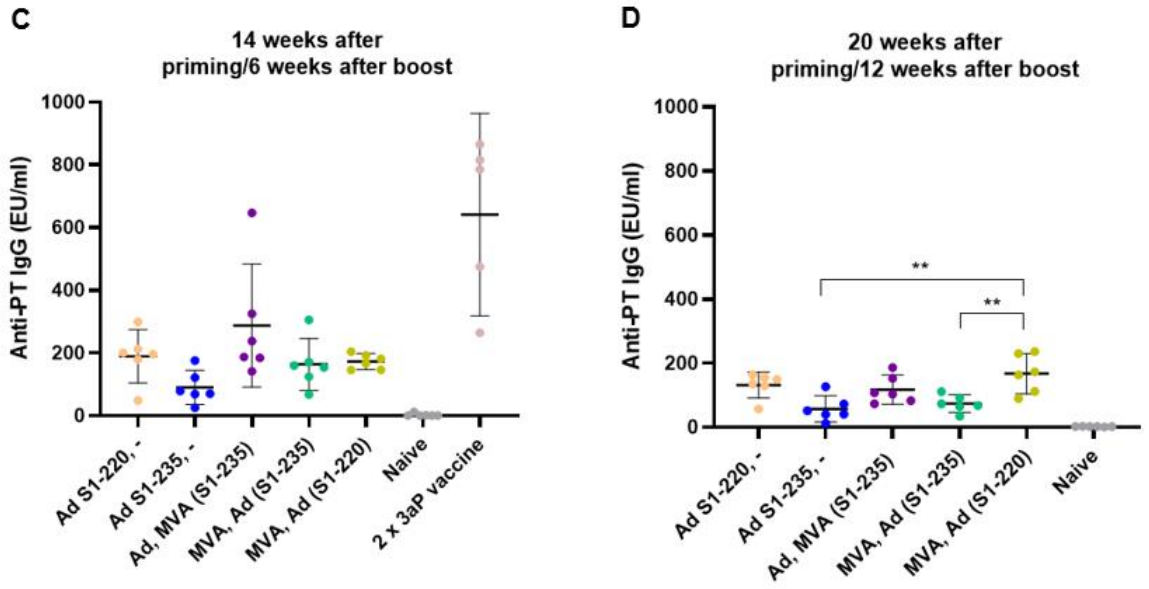


Figure 3.2. Anti-PT IgG antibody responses in mouse sera following vaccination with viral vectored vaccines expressing either PT S1-220 or PT S1-235 subunits, and 3aP vaccines at different timepoints. A) Six weeks after primary immunisation, **B)** 10 weeks after primary immunisation (two weeks after boost), **C)** 14 weeks after primary immunisation (six weeks after boost), **D)** 20 weeks after primary immunisation (12 weeks after boost). Data from prime-boost immunisation with 3aP vaccine, at eight-week interval, were obtained at different time as the immunisations with viral vectored vaccines. Each dot represents an individual mouse. For each group (n = 6 mice), horizontal black lines represent geometric mean titres for each group with error bars indicating ±95% confidence intervals. The statistical significance was analysed by one-way ANOVA followed by Tukey's multiple comparison tests (α = 0.05, *p < 0.05, **p < 0.01, ***p < 0.001, **** p < 0.0001, not significant p > 0.05).

3.3.2 Immunisation with the Ad and MVA vectored vaccines induced IgG2a antibody isotypes in mice

Production of IgG1 and IgG2a antibodies suggests induction of T-cell response. We evaluated PT-specific IgG2a and IgG1 antibodies in mice immunised with different viral vectored vaccines to determine the vaccines' potential to induce the Th1- and Th2-type responses. PT-specific IgG2a and IgG1 antibodies were analysed by ELISA in sera obtained from cardiac bleeds 20 weeks following the first immunisation. Our data show that Ad prime and prime-boost with Ad, MVA or MVA, Ad expressing S1-

220 or S1-235 induced PT-specific IgG2a antibodies (Figure 3.3A). Ad, MVA S1-220 prime-boost immunisation induced significantly higher PT-specific IgG2a antibodies than a single dose immunisation with Ad S1-235 or Ad S1-220 (Ad S1-235 vs. Ad, MVA S1-220, $p<0.0001$; Ad S1-220 vs. Ad, MVA S1-220, $p<0.0001$). Prime-boost with Ad, MVA S1-220 also induced significantly higher PT-specific IgG2a antibodies than Ad, MVA or MVA, Ad expressing S1-235 (Ad, MVA S1-235 vs. Ad, MVA S1-220, $p<0.0001$; Ad, MVA S1-220 vs. MVA, Ad S1-235, $p<0.0001$). Prime-boost regimen with Ad, MVA S1-220 and MVA, Ad S1-220 induced significantly higher PT-specific IgG2a responses than two dose immunisations with 3aP vaccine (Ad, MVA S1-220 and MVA, Ad S1-220 vs. 3aP vaccine, $p<0.0001$ and $p<0.0001$, respectively) (Figure 3.3A).

The viral vector vaccines failed to induce strong PT-specific IgG1 antibody response. Two-dose immunisation with the 3aP vaccine, however, induced a strong IgG1 response against PT which was significantly higher than immunisations with the viral vector vaccines ($p<0.0001$ compared with a single dose of adenoviral vectored vaccines and prime-boost viral vector immunisations) (Figure 3.3B).

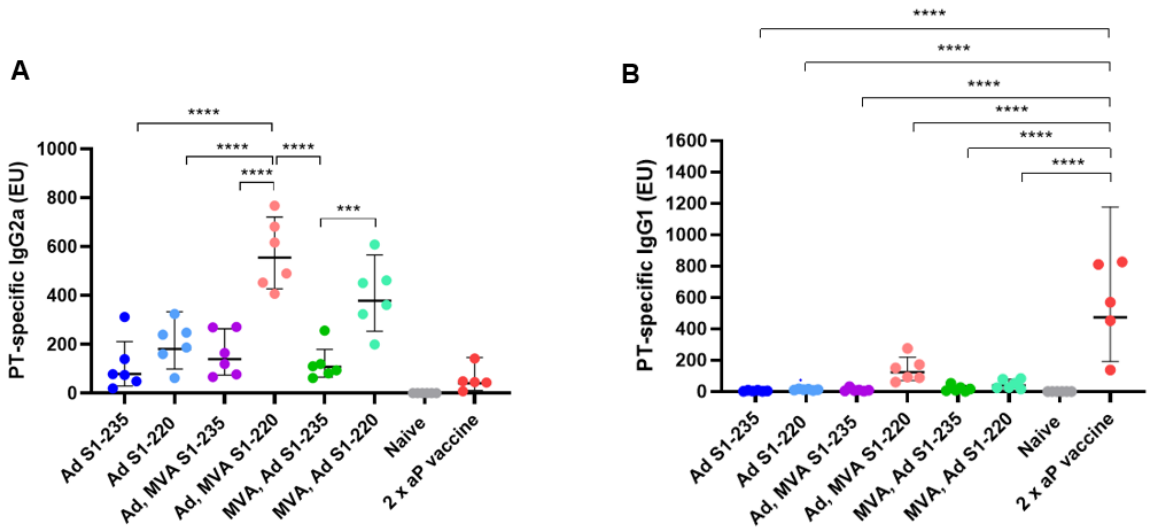


Figure 3.3. Analysis of PT-specific IgG2a and IgG1 isotypes in mice following vaccination with Ad and MVA vectored vaccines expressing PT S1-220 and PT S1-235. Mice received either a single dose or two-dose immunisation at eight-week interval via intramuscular route. Sera were analysed by ELISA 20 weeks after the first dose. Data represent individual mice and geometric mean with ± 95% confidence interval (n = 6 mice/group). **A)** PT-specific IgG2a isotypes and **B)** PT-specific IgG1 isotypes were compared between each group by one-way ANOVA, followed by Tukey's multiple comparison test.

3.3.3 Ad S1-235 vaccine induced partial protection in mice after aerosol challenge

Protective efficacy of Ad and MVA vectors expressing S1-220 and S1-235 was assessed using an aerosol challenge model with a virulent *B. pertussis* 18-323 strain (1×10^8 CFU/ml) via aerosolised droplets. Mice immunised with PBS served as a negative control group and mice immunised with a commercial aP vaccine served as positive controls.

Mice immunised with 5aP vaccine had significantly lower bacterial counts in their lungs than the negative control group and mice vaccinated with the viral vectored vaccines on day seven post challenge ($p < 0.0001$, comparison with control group on day seven, mice primed with Ad S1-220, prime-boosted with MVA, Ad S1-220 and those prime-boosted with Ad, MVA S1-220). Bacterial counts in the lungs of mice vaccinated with a single dose of Ad S1-220 or two doses with Ad, MVA or MVA, Ad S1-220 were similar to those found in naive mice on day seven (Figure 3.4A).

Two-dose immunisation with 3aP vaccine resulted in complete bacterial clearance in mouse lungs on day seven post challenge ($p <0.0001$, as compared with the control group on day seven). Partial reduction in bacterial colonies was observed in mice vaccinated with a single dose of Ad S1-235 and two dose immunisation with Ad, MVA S1-235 on day seven post challenge ($p = 0.0038$ and $p = 0.0014$, respectively, as compared with the control group on day seven) (Figure 3.4B).

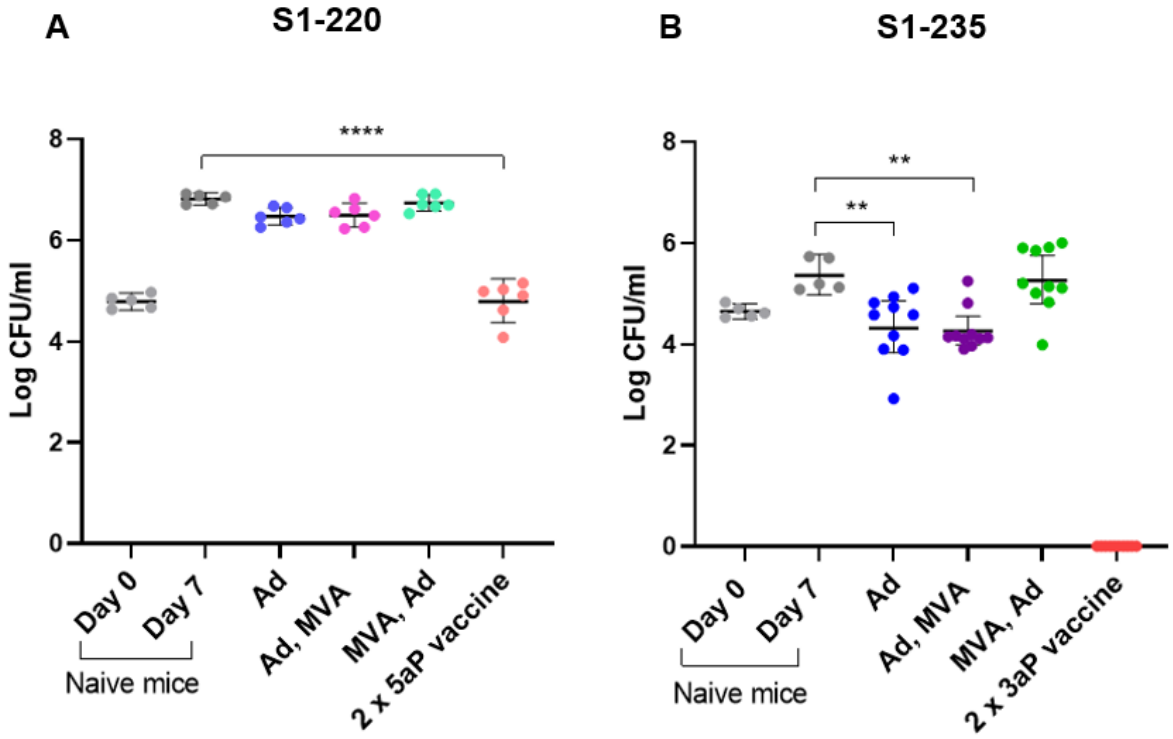


Figure 3.4. Protective efficacy of Ad and MVA vectored vaccines expressing PT S1-220 and PT S1-235 in immunised mice. A) Mice were immunised with one or two doses of viral vectored vaccines expressing S1-220 or **B)** S1-235 at eight-week interval. Challenge was performed two weeks after the booster immunisation (week 10). Five mice in the control group, at day zero, were sacrificed three hours after aerosol challenge with the virulent bacteria to establish the successful infection by analysing bacterial counts in their lungs. The remaining five mice were sacrificed on day seven along other groups to determine viable counts in the mouse lungs. Results for each group are shown as individual counts and geometric mean ± 95% confidence interval (n = 6 mice). One-way ANOVA followed by Tukey's multiple comparison test was used to compare data and determine different differences between each group. Only significant differences between the groups are indicated in the graph.

3.3.4 Pertussis viral vectored vaccines do not induce a significant level of PT neutralising antibodies

To assess if nAbs were responsible for the partial protection observed in mice immunised with Ad S1-235 and Ad, MVA S1-235, we analysed mouse sera using PT neutralisation assay. Neutralisation titre was analysed by determining the highest dilution of serum that prevents clustering of CHO cells in the presence of 4 ng/ml of PT that normally has toxic effects on the CHO cells. A single dose of Ad S1-235 and Ad S1-220 induced PT nAbs in some individual mice six weeks after the immunisation. Immunisation with 5aP vaccine, which was used as a positive control, mediated PT nAbs in all mice within the group (Figure 3.5A). A single dose of MVA S1-235 or MVA S1-220 failed to induced PT nAbs (Figure 3.5A). Prime-boost immunisation with Ad vaccines at eight-week interval did not induced PT nAbs, except one or two mice immunised with Ad, MVA or MVA, Ad expressing S1-235 and MVA, Ad S1-220. Prime-boost immunisation with 5aP stimulated PT nAbs in all mice within the group (Figure 3.5B).

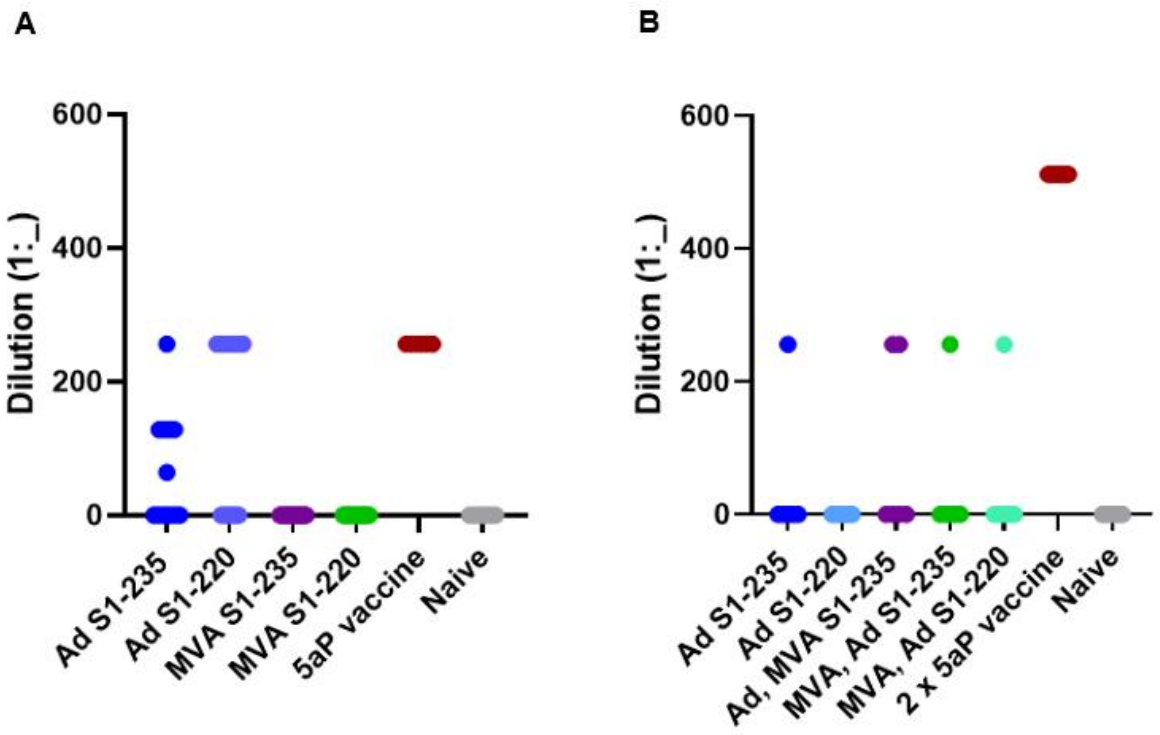


Figure 3.5. Neutralisation antibody titre in sera of mice immunised with Ad and MVA vaccines expressing PT S1-220 or PT S1-235. A) Neutralisation antibody titre six weeks after primary immunisation. **B)** Neutralisation antibody titre 10 weeks after primary immunisation**.** Groups of 12 mice were immunised with viral vector vaccines as indicated in the X-axis. Neutralisation titre which represents the highest dilution of sera, containing nAbs against PT, and prevents CHO cell clustering was determined for each mouse within each group.

3.3.5 Ad S1-220 and Ad S1-235 vaccines induce a strong Th1-type response against PT

We evaluated the T-cell response and its helper profile to find out if the partial protection observed in mice immunised with Ad S1-235 vaccine was associated with an induction of cell-mediated immunity. *Ex vivo* T-cell FluroSpot assay was performed two weeks after vaccination as that is the peak of the T-cell response for adenoviral vectors. *Ex vivo* release of IFN-γ, IL-17A and IL-5 cytokines by PT-specific T-cells was assessed by FluroSpot assay following overnight stimulation of splenocytes with 65 pools (peptide libraries) of overlapping peptides spanning the entire S1 subunit of PT. The numbers of IFN-γ, IL-17A and IL-5 secreting T-cells were assessed as these cytokines represent

Th1-, Th17- and Th2-type responses, respectively. IFN-γ response, but not IL-17A and IL-5, was seen in mice immunised with one dose of Ad S1-235 or Ad S1-220 vaccine and the response was significantly higher than mice immunised with 1/10th human dose of 3aP vaccine (Ad S1-235 vs. 2 x 3aP vaccine, $p<0.000$; Ad S1-220 vs. 2 x 3 aP vaccine, $p<0.000$) (Figure 3.6A-C). Moreover, two doses of 3aP vaccine at three-week interval did not mediate IFN-γ response two weeks after the second dose. These data show that, as expected, adenoviral vectored vaccine elicits Th1-type response.

PT-specific IgG, IgG2a and IgG1 responses were also assessed by ELISA. As shown in Figure 3.6D, Ad S1-235 or Ad S1-220 vaccine did not induce strong PT-specific IgG antibodies two weeks after immunisation. On the other hand, two-dose immunisation with 3aP vaccine mediated strong IgG responses against PT antigen two weeks after the last immunisation (3aP vaccine vs. Ad S1-235, $p <0.0001$; 3aP vaccine vs. Ad S1-220, $p <0.0001$). Ad S1-235 and Ad S1-220 vaccine mediated PT-specific IgG2a but failed to induce IgG1 subclass, suggesting that these vaccines stimulate Th1-type response (Figure 3.6E). The 3aP vaccine mediated PT-specific IgG1 subclass antibodies but failed to induce PT-specific IgG2a antibodies, promoting the stimulation of Th2-type response (Figure 3.6E).

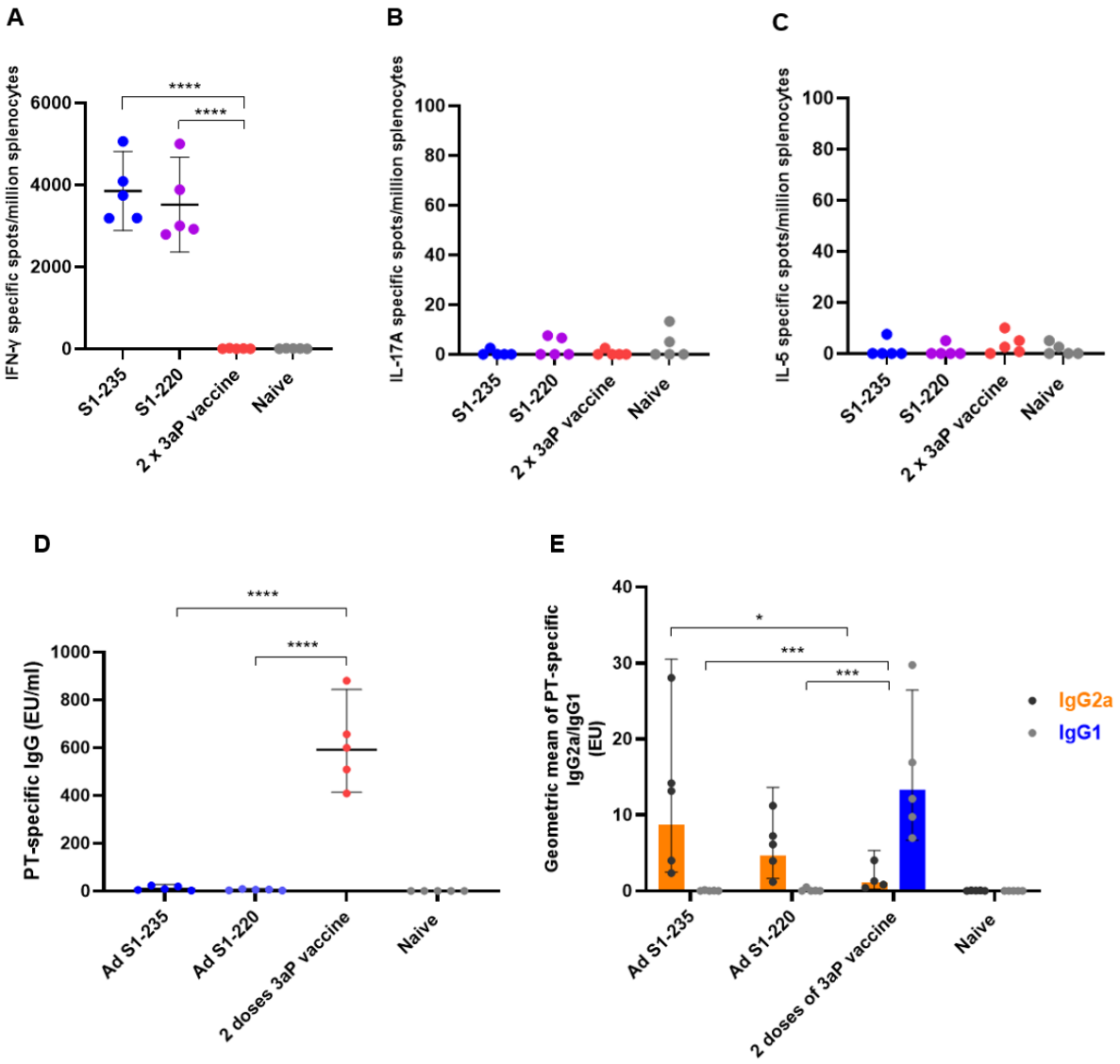


Figure 3.6. Assessment of PT S1-specific *ex vivo* IFN-γ, IL-17A and IL-5 ELISpot responses and PT S1-specific IgG, IgG2a and IgG1 responses by ELISA in mice immunised with Ad S1-235, Ad S1-220 and 3aP vaccine. A) IFN-γ, **B)** IL-17A and **C)** IL-5 secretion levels following *in vitro* stimulation, with the PT S1 peptide pool, of mouse splenocytes were detected using *ex vivo* T-cell FluroSpot assay. Depicted are the numbers of cytokine-specific spots/1 x 10^6 cells, which represents the normalised data after subtracting the DMSO negative control using the FluroSpot assay (n = 5). **D)** Total serum PT-specific IgG and **B)** IgG2a and IgG1 titres. Mice (n =5) were immunised with two 1/10^{th} human doses of 3aP vaccine at day zero and 21 or with either one dose of Ad S1-235 or Ad S1-220 vaccine on day 21. Cardiac bleeds were obtained two weeks after last immunisation (Day 35) and were processed to obtain serum and to measure anti-PT IgG as well as IgG2a and IgG1 antibody titres. Data are represented as geometric mean ± 95% confidence interval. One-way ANOVA followed by Tukey's multiple comparison test was used to compare data and determine different differences between each group. Only significant differences between the groups are indicated in the graph.

3.4 Discussion

The purpose of this study was to develop novel pertussis vaccines that can induce long-term and more effective immune responses than those induced by the subunit pertussis vaccines. Mills *et al.* (1998) suggested that protective immunity against *B. pertussis* depends on the generation of Th2- and Th1-type immune responses, characterised by the secretion of IFN-γ from antigen-specific T-cells. Those immune responses provide helper function for generation of pertussis-specific antibody responses, and Th1 cytokines play an additional role in stimulation of cell-mediated immunity against intracellular bacteria in the mouse respiratory tract and therefore enhance the bacterial clearance (29). Mouse as well as baboon models of pertussis showed that wP vaccine, which predominantly induces Th1-type response, contributes to faster bacterial clearance from the lungs than the aP vaccines. The aP vaccines, which induce mainly Th2-type response leading to production of antigen-specific antibodies, prevent severe disease but are unable to inhibit bacterial colonisation (152).

In this study, we examined two strategies to induce PT-specific antibodies, Th1-type cellular immunity and protective responses against *B. pertussis* infection: 1) we cloned the full length (amino acid residues 1-235) as well as truncated version (amino acid residues 1-220) of the PT S1 into the viral vectors, and 2) we compared Ad and MVA priming strategy with heterologous prime-boost regimen. Here, the results showed that adenoviral vaccines expressing the S1 subunit are immunogenic after a single dose, whereas MVA vaccine expressing the S1 subunit failed to induce antibody responses after primary vaccination (Figure 3.2A). However, the antibody responses elicited by Ad S1-235 were improved upon boosting with MVA S1-235 vaccine two weeks after

the boost (Figure 3.2B). Moreover Ad, MVA S1-235 regimen was more immunogenic than MVA, Ad S1-220 or MVA, Ad S1-235, thus suggesting that MVA is able to induce antibody response, particularly when used as a boosting vector (Figure 3.2B). MVA vectored vaccines are known to have excellent capacity to boost both humoral and cell-mediated immunity in combination with DNA-based or adenoviral vectored vaccines (153).

Our results are consistent with other studies which suggest that adenoviral vaccines are highly immunogenic and are effective at inducing immune response after administration of a single dose alone or in heterologous prime-boost regimen followed by a second dose of MVA vaccine (109). The adenoviral vectors induce potent B-cell responses and the prime-boost (Ad, MVA) immunisation strategy stimulates potent B-cell and T-cell responses (122). Unlike the adenoviral vaccines, MVA vectored vaccines are often used as a booster in the heterologous prime-boost strategy to boost B-cell responses (122). When used for booster immunisation, the MVA vaccines have been shown to broaden immunogenic responses and protective properties in other infectious bacterial diseases such as tuberculosis (154). In addition, Kou *et al.* (2018) showed that vaccination regimen containing adenoviral vectored vaccine expressing one of *Mycobacterium tuberculosis* antigens may have a higher capacity to stimulate mice humoral and cell-mediated immune responses against tuberculosis than vaccination with a live attenuated BCG vaccine or MVA vaccine alone (155). Clinical trials also demonstrated that heterologous prime-boost immunisation with simian adenoviral vector and MVA expressing malaria antigens mediated strong antibody responses that were higher than those mediated by a single vector (156).

A previous study reported technical difficulties in expressing full length S1-235 subunit in *E. coli* and, a better yield of the S1-220 subunit was achieved following expression in the bacterial cells (143). We decided to clone both S1-235 and S1-220 subunits into the viral vectors in case there was an issue with the expression of the S1-235 subunit in mammalian cells. In addition, the S1-235 subunit contains a hydrophobic tail which anchors the S1 subunit into the bacterial cell membrane (143) and this could lead to a potential issue with protein folding and stability when the S1-235 subunit is expressed in mammalian cells. Moreover, a C-terminally truncated S1 subunit has the same catalytic activity as the full length S1 subunit. The hydrophobic tail is not involved in membrane translocation and thus the truncated S1-220 subunit can be involved in intracellular trafficking mechanism which is important for the toxin's cytotoxic effects (157). We then examined if the S1-220 and S1-235 subunits, inserted into the viral vectors, had an impact in the immunogenic responses in mice. Since the length of amino acid residues for each subunit differs, the exposure of immunogenic epitopes following expression can vary, leading to different immune responses. The S1-235 subunit contains a greater number of B-cell epitopes which are sporadic epitopes made of amino acid residues found on different regions of the protein and connected together by folding of the chain (158). Therefore, the additional 15 amino acid residues might affect the immunogenicity of the vaccine expressing S1-235. Furthermore, the S1-235 subunit might be more protective as the additional amino acid residues can influence folding of the expressed protein and availability of the conformational protective epitopes that are necessary for the protective immune responses. Here, the results show that MVA, Ad S1-220 induced higher antibody responses than MVA, Ad S1-235 regimen and single-dose immunisation with Ad S1-235 12 weeks after the boost (Figure 3.2D).

We further explored if the vaccine regimens can induce protection against *B. pertussis* and whether the protective response is better than protection induced by current aP vaccines. Although, priming with Ad S1-220 and prime-boost regimen with Ad, MVA S1-220 or MVA, Ad S1-220 was shown to be immunogenic, these vaccination strategies did not induce protection against *B. pertussis* in mice (Figure 3.4A). Surprisingly, priming with Ad S1-235 and prime-boost with Ad, MVA S1-235 induced similar level of partial protection (Figure 3.4B), suggesting that MVA S1-235 did not have boosting effect on the protective response in Ad S1-235-primed mice. This is in contrast with studies that found heterologous prime-boost immunisations with viral vectors were more promising than homologous prime-boost or viral vector immunisation alone. Although the MVA vaccine is an effective booster, studies in the tuberculosis field showed that Ad, MVA regimen is more protective when administered by the intranasal route rather than the systemic route in mice (159). A likely explanation for the lack of enhanced protection after MVA boosting is that the intranasal route of Ad, MVA administration could be more effective at stimulating protective response in mice. The intranasal route of vaccine administration might elicit local immune responses in the respiratory tract and therefore be more protective. However, this hypothesis would have to be tested in further experiments.

Our next objective was to evaluate if PT nAbs and/or T-cell responses were responsible for the partial protection induced by the Ad S1-235 and Ad, MVA S1-235 vaccines. Examination of PT nAbs at the time of aerosol challenge (10 weeks after priming) showed the absence of nAbs in mice immunised with the vaccines (Figure 3.5B), suggesting that partial protection was not associated with PT nAbs. Although, there are several possibilities why nAbs against PT were not elicited by the vectored vaccines or they were not detected in the PT neutralisation assay:

1. The conformation of epitopes expressed by the viral vectors were not identical to the conformational protective epitopes located on the native PT. The conformation of the S1 subunit might not have been relevant without the B oligomer. However, the S1 subunit does not require the B oligomer for its activity and expression in mammalian cells (160). On the other hand, Sutherland *et al.* proposed that binding of the 1B7 mAb to protective epitopes may involve the presence of the S4 subunit found on the B subunit of PT. The study hypothesised that the antibody may recognize one epitope influenced predominantly by the S1 subunit, but it might be amplified by a weak B-oligomer interaction, or, the S1 subunit is stabilised by interacting with the B oligomer. However, the study was unable to test these hypotheses due to complications with S4 subunit recombinant expression (143). Also differences between bacterial and mammalian post-translational modifications could account for different protein folding, conformation of the antigen and the loss or accessibility of protective epitopes. The S1 disulphide bond comprised by Cys 41-Cys200 require for function and holotoxin assembly, may not be formed or maintained in the reducing intracellular environment of the mammalian cell (161). Despite these potential limitations, viral vectors are able to deliver bacterial antigens into mammalian cells where they induce functional antibodies (Marsay *et al.*, in submission).
2. The antibody affinity and their specific binding to PT epitopes may play an important role in the antibody potency to neutralise the toxin (146). We therefore hypothesised that the nAbs induced by the vectors may bind the correct protective epitopes on the S1 subunit of PT to block the toxic activities, but their binding affinity was too weak relative to binding affinity of the interaction between the toxin and target cells.

We then further investigated T-cell responses, and PT-specific IgG2a and IgG1 antibodies to find out if the partial protection against pertussis infection induced by the vaccines was associated with a Th1-type response that is important for bacterial clearance in the lungs. The different IgG subclasses have different biological roles. Mouse IgG1 subclass antibodies, which are indicative of Th2-type responses, support toxin neutralisation and inhibit adherence of bacteria to host cells, whereas IgG2a antibodies, indicative of Th1-type response, are complement-fixing antibodies associated with bacterial opsonisation in mice (162). Moreover, IFN-γ, which is associated with Th1-type response, stimulates IgG2a subclass and IL-4, which is associated with Th2-type response, stimulates IgG1 subclass in mice (162). This study showed that a single intramuscular vaccination with Ad S1-220 as well as Ad S1-235 induced a strong $CD4^+$ IFN-γ T-cell response (Figure 3.6A) indicating generation of Th1-type response. Interestingly, it was only a one dose vaccination with Ad S1-235 which led to the protective responses in mice, and not the Ad S1-220 in the challenge study. Therefore, correlation of the partial protection with the induction of Th1-type response in case of the Ad S1-235 vaccine is not clear. One possible explanation is that Ad S1-235 vaccine contributed to the partial protection against pertussis infection by inducing strong Th1-type response as well as PT nAbs that were not detected in the CHO cell-based PT neutralisation assay that only estimates the ability of nAbs to bind to PT and preventing the toxin's binding to the CHO cells (146). The IgG subclasses were also assessed, and the results showed that the viral vectored vaccines induced a Th1-type immune response as all immunisation regimens induced IgG2a subclass against PT (Figure 3.6E) (Interestingly, none of the vaccination strategies induced IgG1 subclass antibodies and therefore the low IgG1 antibodies may explain poor neutralisation of PT).

3.5 Conclusion

In summary, this study provided an insight into the immunogenic and protective properties induced by the viral vectored vaccines expressing PT S1-220 or PT S1-235. Data obtained in this study show that primary immunisation with Ad S1-235 as well as heterologous prime-boost immunisation with Ad, MVA S1-235 mediated partial protection against pertussis infection in mice. According to our data, the partial protection does not correlate with production of PT nAbs as immunisation with Ad S1-235 and Ad, MVA S1-235 failed to induce substantial amount of nAbs against PT in mice. In addition, the protection was not enhanced by MVA S1-235 booster in Ad S1-235-primed mice which suggests that the protection was due to the effect of Ad S1-235 vaccine. As hypothesised above, the partially protective vaccine (Ad S1-235) could have induced PT nAbs with low avidity and these nAbs were unable to compete with the PT binding in CHO cell assay. The potential induction of the nAbs in combination with the Th1-type response could have accounted for the partial protection against *B. pertussis.* These results suggest that the Ad S1-235, but not the AdS1-220 vaccine is a promising candidate to be considered for further studies in which it would be worth investigating if the vaccine combined with other protective antigens of *B. pertussis*, cloned into viral vectors, could lead to better protection against the pertussis infection than current aP vaccines.

Chapter 4: *B. pertussis* iron-binding proteins as antigen candidates for novel viral vectored-based vaccines against pertussis

4.1 Introduction

Environmental factors and nutrients determine bacterial phenotype. One of the most important nutrients for bacterial survival in a host is iron. In humans, the level of free iron is very low due to iron-sequestering proteins (141). Bacterial survival under iron-poor conditions is difficult, therefore, bacteria developed mechanisms to acquire extracellular iron and use it for their growth and existence (142). The mechanisms include low-molecular-weight iron chelators (siderophores), and bacteria also use their cell surface receptors to extract iron from host proteins. Several *B. pertussis* proteins expressed under iron depletion conditions have been identified (105). These iron-binding surface-associated proteins also enable attachment of *B. pertussis* to the host's epithelial respiratory cells, and moreover, they are immunogenic in mice and humans during infection and may therefore be suitable as antigens in pertussis vaccines (141).

Alvarez-Hayes and colleagues identified two iron-binding proteins of *B. pertussis*, IRP1-3 (BP1152) and AfuA (BP1605) (105, 106). IRP1-3 is a dimeric outer membrane protein, and its bacterial surface exposure is enhanced during iron starvation; the protein is expressed irrespective of the bacterial virulence phase on clinical isolates of *B. pertussis.* Moreover, IRP1-3 protein was shown to bind to human IgG serum samples obtained from patients having pertussis infection, demonstrating that the protein is immunogenic in humans and naturally presented to the immune system during the infection (105).

Another iron binding protein known as AfuA (BP1605) is involved in using the main iron sources, namely transferrin and lactoferrin, in an *in vivo* environment for bacterial survival. Antibodies raised against rAfuA in mice bound to heat inactivated and live iron-starved whole-cell *B. pertussis* bacteria, suggesting that the protein is exposed on the bacterial surface (106). Alvarez-Hayes *et al.* (2013) demonstrated that 2 µg of recombinant AfuA (rAfuA) emulsified in complete Freund's adjuvant was immunogenic in mice following active immunisation. Immunisation with rAfuA was able to induce both Th1- and Th2-type responses and bacterial load was significantly reduced in mice actively immunised with rAfuA as opposed to mice that received adjuvant only (negative control) following intranasal challenge.

To improve the immunogenicity and efficacy of existing pertussis vaccines, the IRP1-3 and AfuA proteins were selected as antigenic candidates for development of novel viral vectored vaccine components based on the following criteria:

- The iron-binding proteins of *B. pertussis* are able to mediate antigen-specific antibody responses in humans after natural infection (106)
- The proteins can mediate protective responses in mouse models upon challenge with virulent *B. pertussis* (106)
- Both, IRP1-3 and AfuA proteins are expressed by *B. pertussis* while colonising human hosts as a response to iron starvation (106)

In this chapter, we assessed the immunogenic and protective properties of the Ad IRP1-3 and Ad AfuA vaccines. In addition, recombinant IRP1-3 and AfuA proteins were expressed in *E. coli*, purified and used as direct vaccine comparators against the viral vector vaccines. In one of our protection studies (chapter 6), we observed a partial

protection with adenoviral vector vaccine expressing AC domain of the RTX antigen. Therefore, we decided to explore if immunisation of mice with Ad AfuA + Ad AC provide better protection together as we should be able to see if there was synergism with this combination.

4.2 Materials and Methods (Cloning)

4.2.1 Plasmid design and production of adenoviral vectors expressing iron-binding proteins

Please refer to 'General Materials and Methods' in Chapter 7, section 7.1, sub-section 7.1.1.

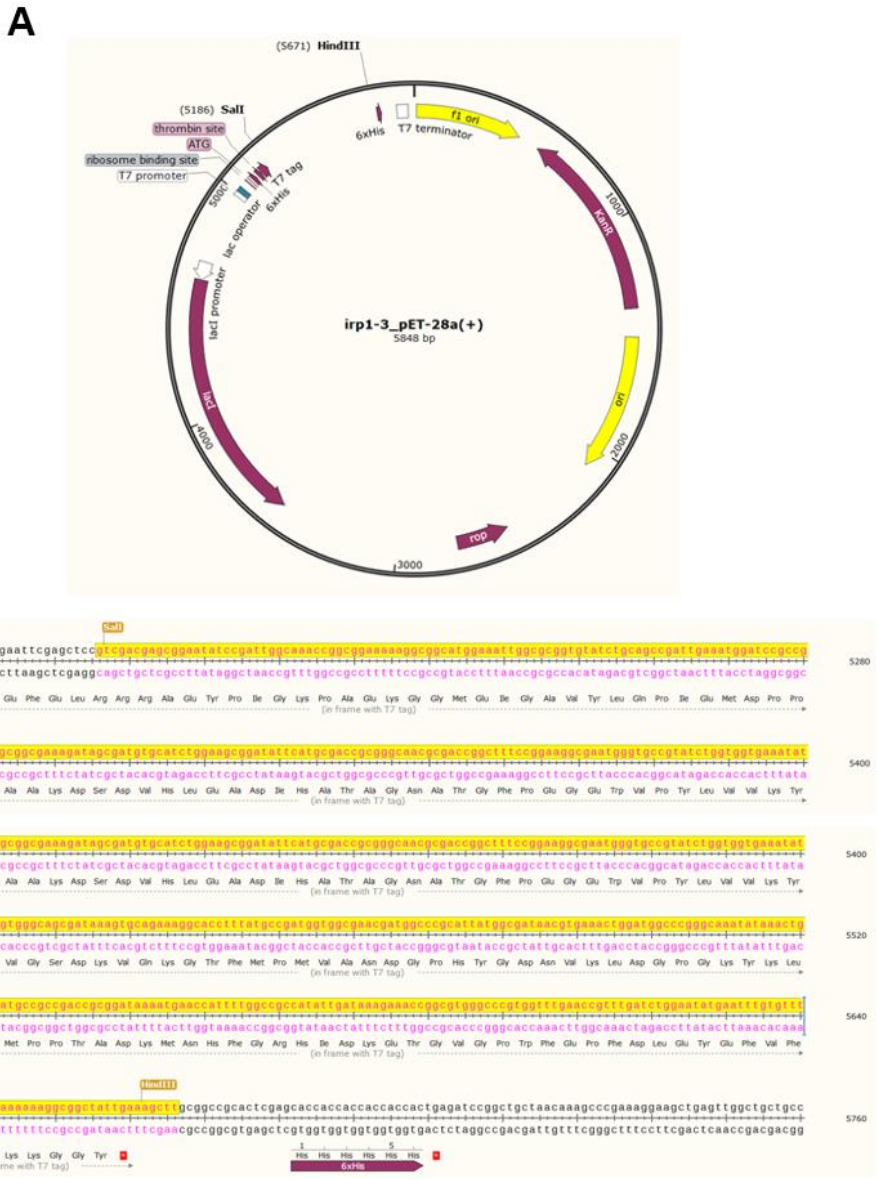
4.2.2 Recombinant protein production

4.2.2.1 Cloning of IRP1-3 and AfuA genes into pET28a+ expression vectors

DNA sequence of the IRP1-3 gene, also known as BP1152, (UniProt accession number: Q7VYZ7) lacking the N-terminal signal sequence (amino acids 1–20) was codon optimised for expression in *Escherichia coli*, synthesised and cloned into a pET28a+ expression vector (Novagen) by GenScript. The signal sequence was removed to enable *E. coli* cytoplasmic expression.

A six-histidine sequence (His-tag) was added to the N-terminus of IRP1-3 to allow for easy purification by metal affinity chromatography (Figure 4.1A). Commercial gene synthesis of AfuA sequence from *B. pertussis* strain Tohama I (UniProt accession number: Q7VXW9) with an additional N-terminal six His-tag was also performed by GenScript (Piscataway, NJ, USA) (Figure 4.1B).

The sequences were provided as lyophilised plasmid constructs cloned into pET28a+ expression vectors. The plasmids were reconstituted in 20 µl of de-ionised water to dissolve the DNA. Plasmid DNA was amplified in the *E. coli* strain DH5α and extracted using a QIAprep Spin Miniprep Kit (Qiagen). The extracted plasmid DNA was used for transformation into competent cells (T7 Express lysY/Iq Competent *E. coli*).



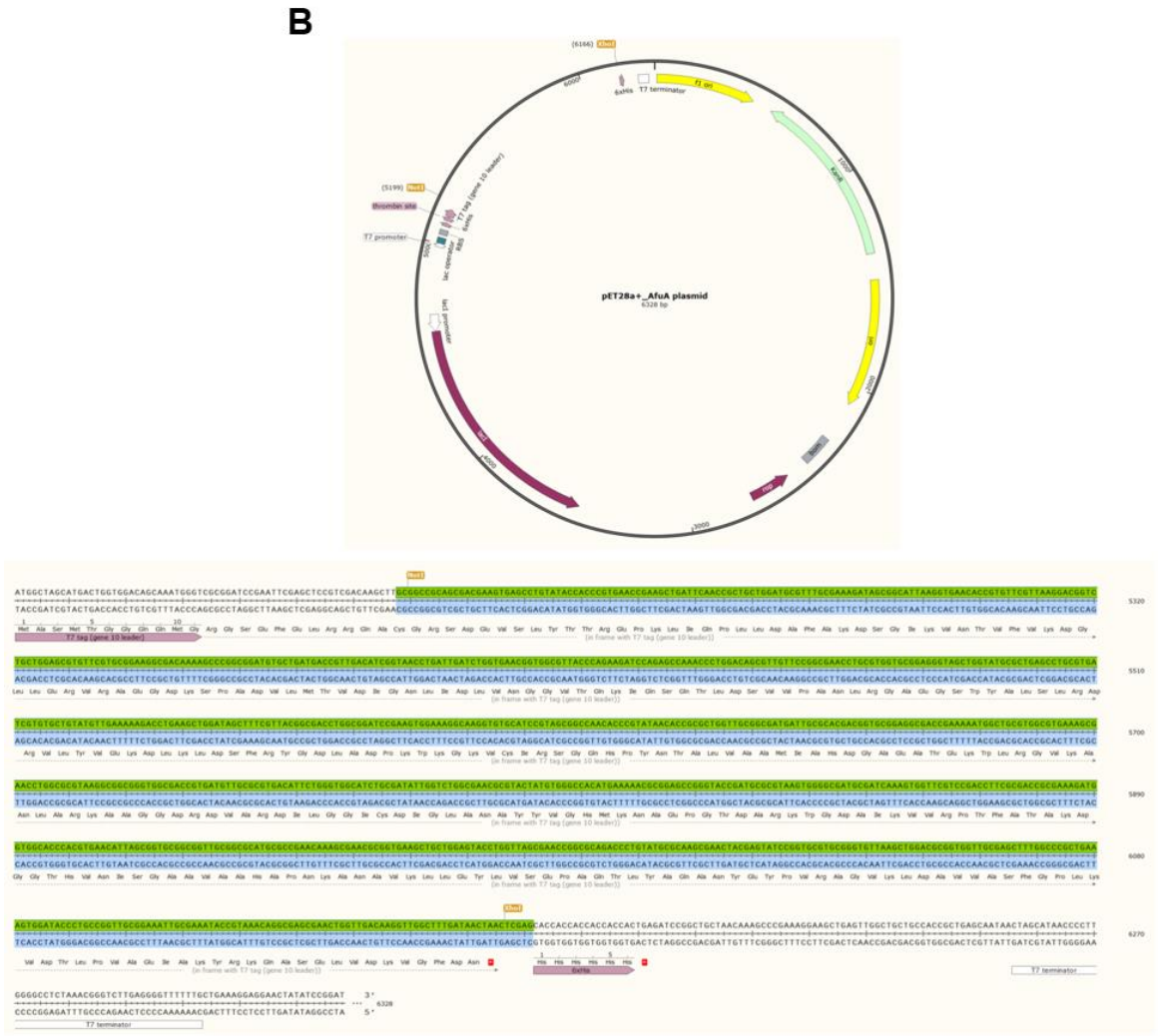


Figure 4.1. Map of pET 28a (+) expression vector and multiple cloning site region. (A) IRP1-3 protein nucleic acid sequence with N-terminal His-tag was commercially cloned into SalI and HindIII digested pET28a (+) vector through restriction and ligation. **(B)** AfuA protein nucleic acid sequence with N-terminal His-tag was commercially cloned into XhoI and NotI digested pET 28a (+) vector through restriction and ligation. The plasmids were designed using SnapGene software (version 4.3.5).

4.2.2.2 Chemical transformation of pET28a+ expression vector into competent *E. coli* DH5α

Commercially produced chemically competent *E. coli* DH5α (New England Biolabs) were used for initial transformation of the expression vector. Following transformation of the plasmid into *E. coli* DH5α, transformants were selected on Luria-Bertani (LB) agar plates supplemented with 50 µg/ml kanamycin at 37°C incubation overnight. Two to three colonies were picked from a freshly streaked LB/kanamycin plate and used to inoculate 10 ml sterile LB broth containing 50 µl/ml kanamycin. The culture was incubated overnight (12 – 16 hours) at 37°C with vigorous shaking at 250 rpm.

4.2.2.3 Isolation of plasmid DNA

Following the ~ 14-hour incubation, the cell culture was centrifuged at 10,000 rpm for three minutes at room temperature. Supernatant was removed and the cell pellet was kept for plasmid DNA isolation which was based on alkaline lysis with a Qiagen DNA Mini-Prep kit (Qiagen) according to the manufacturer's instructions. Briefly, the pellet was resuspended in 250 µl of resuspension buffer. The bacteria were then lysed by addition of an alkaline lysis buffer to release plasmid DNA without release of chromosomal DNA. The reaction mixture was then neutralised and adjusted to a high salt concentration. Isolated DNA was quantified using a two wavelength spectrophotometric method on Nanodrop spectrophotometer (ThermoFisher Scientific) and was stored at -20°C until transformation into the expression system. Plasmid DNA was digested with HindIII and SalI (or XhoI and NotI) restriction enzymes at 37°C for 15 minutes, and the digested products were separated by agarose gel electrophoresis to verify their size. The gel electrophoresis was performed for one hour at 100 V with 1.2% agarose gel electrophoresis in Tris-borate-EDTA (TBE) buffer

(0.5%) and the gel was visualised using a Gel Doc System (Syngene PXi). Plasmid identities were confirmed with Sanger sequencing (Source Bioscience) using the forward T7 primer (TAA TAC GAC TCA CTA TAG GG) and reverse T7 primers (GCT AGT TAT TGC TCA GCG G).

4.2.2.4 Chemical transformation of purified plasmid DNA into *E. coli* protein expression system

The purified plasmid DNA was transformed into T7 Express lysY/Iq Competent *E. coli* strain (New England Biolabs) for expression with the N-terminal 6x His-tag. Briefly, 50 µl competent cells were thawed on ice and 5 µl purified plasmid DNA was added to the cells. The mixture was placed on ice for 30 minutes and then was heat-shocked at 42°C for 10 seconds before being returned to ice for five minutes. Following the incubation, 950 µl SOC medium (Invitrogen) was added to the mixture which was incubated for one hour at 37°C at 250 rpm. Several 10-fold dilutions were prepared and 50 µl of each dilution was plated on LB agar plates containing kanamycin selection. The plates were incubated at 37°C overnight.

4.2.2.5 Small scale expression trials of the recombinant IRP1-3 and AfuA proteins

The successfully transformed constructs (from T7 LysY glycerol stocks) were grown overnight in LB (50 µg/ml kanamycin) plates. Three to four colonies were isolated and transferred to Falcon tubes containing 10 ml LB medium supplemented with 50 µg/ml kanamycin. The culture was incubated overnight at 37°C, 250 rpm. Following day, LB broth (50 ml) supplemented with 50 µg/ml kanamycin was inoculated with 1 ml of overnight bacterial culture (1:50 ml dilution) and incubated at 37°C, 250 rpm, until the culture reached OD 0.4 – 0.8 (A_{600}). Recombinant protein expression was induced

with 0.4 mM isopropyl-β-D-thiogalactoside (IPTG) at 37°C for four hours with shaking at 250 rpm. The cultures were harvested at 10,000 g for 10 minutes at 4°C, supernatant was discarded and pellets, containing the expressed protein of interest with N-terminal His-tag, were stored at -20°C. Uninduced cells were treated the same way as IPTG-induced cells and were used as a negative control for Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western blot analysis.

Following small scale protein expression trials, uninduced cells (negative control), induced whole-cell lysate, soluble, and insoluble cell fractions were analysed by SDS-PAGE gel with the purpose of determining that induction and expression of the His-tagged protein occurred. Western blot was performed to confirm detection of the IRP1-3 protein expression only.

4.2.2.6 SDS-PAGE

SDS-PAGE Novex Tris-Glycine Gels (Invitrogen) with 16% polyacrylamide concentration were assembled into XCell SureLock Mini-Cell Electrophoresis System (ThermoFisher Scientific). Samples were heated at 85°C for 5 minutes and loaded into the wells of the gel, along with 8 µl SeeBlue™ Plus2 Pre-stained Protein Standard (Invitrogen). Gels were run at 180 V for one hour and were stained with Coomassie Blue stain and de-stained using a destain solution. Following analysis of the pre-run gel for the protein expression, the gel was prepared for immunodetection.

4.2.2.7 Western blot and immunodetection of blots

By using dry blotting method proteins that have been separated by gel electrophoresis were transferred to a nitrocellulose membrane and afterwards detected by incubation with specific antibody. Protein transfer was performed for seven minutes at 20 V (1.3 A current).

Following the protein transfer, nitrocellulose membrane was blocked in blocking solution (5% skimmed milk in phosphate-buffered saline-0.1%Tween-20 (PBST)) for one hour at room temperature on a shaker. The membrane was then probed with a horseradish peroxidase (HRP)-conjugated 6x-His Epitope Tag monoclonal antibody (Invitrogen) at a dilution of 1:1,000 overnight at 4°C on a rocking platform and was washed four times with PBST for five minutes. The specific antigen-bound antibody was visualised with Pierce™ Enhanced Chemiluminescence (ECL) Western Blotting Substrate (Thermo Scientific). Briefly, ECL detection reagent 1 and detection reagent 2 were mixed (1:1 ratio) and the blot was then imaged to visualise proteins using GeneSys image software (Syngene, United States).

4.2.2.8 Large scale expression trials of the recombinant proteins

The T7 Express lysY/Iq Competent *E. coli* strain (New England Biolabs), transformed with pET28a expression plasmids, were cultured on LB/kanamycin (50 µg/ml) plates overnight at 37°C. A single colony was selected and added to LB broth supplemented with 30 µg/ml kanamycin. The culture was incubated for four hours at 37°C at 250 rpm followed by sub-culturing into 300 ml of TB Overnight Express autoinduction media (Merck) supplemented with 30 µg/ml kanamycin in one litre baffled flask. The culture was incubated at 37°C overnight, shaking at 180 rpm. Following day, the OD of the

culture was measured at A_{600}, until the OD reached stationary phase. A sample of 1 ml culture was collected and kept for SDS-PAGE analysis to verify the protein expression. Cells were harvested at 4,000 rpm at 4°C for 40 minutes. Supernatants were removed and pellets were stored at -20°C.

4.2.2.9 Immobilized metal affinity chromatography

To enable the isolation of His-tagged IRP1-3 protein from cell lysate, the cell pellet was thawed and treated with lysis buffer containing BugBuster® HT 1X (Merck), 1 mg/ml lysozyme (Sigma-Aldrich), 50 mM Tris-HCl pH 8.0 and 500 mM NaCl for 30 minutes at room temperature with rotation, followed by 30-minute incubation at 37°C to allow full activity of lysozyme and DNase. The lysate was centrifuged at 14,000 rpm for 10 minutes to remove aggregates.

Proteins were purified using the HisTrap™ High Performance 1 ml column (GE Healthcare). The column was attached to ÄKTA start protein purification system (Amersham Pharmacia, USA) and used for purification of His-tagged IRP1-3 by immobilized metal ion affinity chromatography (IMAC). After removal of aggregates, the lysate was applied to the His-tagged column. Wash buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 20 mM Imidazole) equilibrated the chromatography system and elution buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 250 mM Imidazole) eluted purified proteins from the column. Eluted fractions were then analysed by SDS-PAGE and Coomassie stain.

4.2.2.10 Isolation of rIRP1-3 with size exclusion chromatography (Gel filtration chromatography)

Pooled eluted fractions of His-tagged purified recombinant IRP1-3 (rIRP1-3) were subjected to further purification with size exclusion chromatography (SEC). Briefly, HiPrep 16/60 Sephacryl S-200 HR column (GE Healthcare, UK) was attached to ÄKTA start protein purification system. The column was washed with 240 ml of 0.2 µm filter-sterilised dH_2O, followed by equilibration with a size exclusion buffer (20 mM Tris-saline buffer 10 mM NaCl, pH 7.4). The eluted protein fractions were analysed by SDS-PAGE to verify the molecular weight of the eluted protein.

4.2.2.11 Dialysis and protein quantification

The eluted recombinant protein was dialysed against a buffer containing 20 mM Tris/ HCl, pH 8.0, 150 mM NaCl. Dialysis was performed overnight at 4°C using a 10 KDa cut-off Slide-A-Lyzer Dialysis Cassettes (Thermo Fisher Scientific, UK). Dialyzed solution was concentrated in Amicon Ultra-15 Centrifugal Filter unit (Millipore Ltd, Watford, UK) and protein concentration was measured with the bicinchoninic acid (BCA) protein assay (Pierce™ Thermo Scientific™, UK), using 2 mg/ml albumin standard.

4.2.2.12 Bacterial endotoxin test: Limulus Amoebocyte Lysate (LAL) Gelation

To quantify the endotoxin levels in the recombinant protein samples obtained after affinity chromatography and size exclusion purification procedures, the Limulus Amoebocyte Lysate (LAL) Gelation test was used (Pyrogens Team at NIBSC assessing). Please refer to 'General Materials and Methods' in Chapter 7, section 7.3.

4.2.2.13 Refolding by dialysis of recombinant AfuA from inclusion bodies

The rAfuA protein was dialysed in a buffer containing 4.0 M urea + 0.5 M NaCl for eight hours and then refolded by dialysing overnight at 4°C against 20 mM Tris HCl pH7.5, 0.5 M NaCl. The following day the buffer was changed, and dialysis continued for a day.

4.3 Results

4.3.1 IRP1-3 and AfuA protein cloning, expression and purification

4.3.1.1 Restriction digest of plasmid DNA by agarose gel electrophoresis

Prior to transformation of bacterial T7 LysY expression strain with plasmid DNA, the plasmid DNA was extracted and eluted IRP1-3 and AfuA DNA were digested with restriction enzymes. The IRP1-3 and AfuA fragments were run on an agarose gel to verify their size. The bands on top of the gel correspond to a digested fragment of the pET28a vector (5369 bp) and the arrow points to the bands that correspond to the size of IRP1-3 gene insert (491 bp) (Figure 4.2A) and AfuA gene insert (974 bp) (Figure 4.2B).

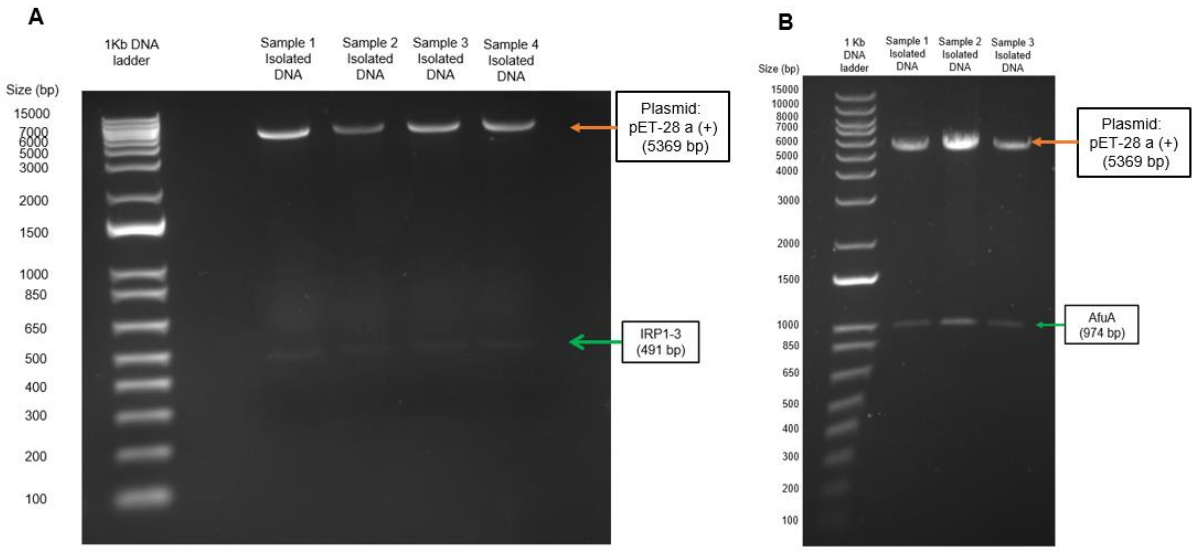


Figure 4.2. Analysis of the restriction digest of plasmid DNA encoding IRP1-3 and AfuA genes, along with a marker, by agarose gel electrophoresis. A) IRP1-3 fragment. Lane 1: 1 Kb Plus DNA Ladder. **Lane 2:** Sample 1. **Lane 3:** Sample 2. **Lane 4:** Sample 3. **Lane 5:** Sample 4. The size of band corresponding IRP1-3 gene insert was estimated by visual comparison with the 1Kb DNA ladder. **B) AfuA fragment. Lane 1:** 1 Kb Plus DNA Ladder. **Lane 2:** Sample 1 of isolated DNA. **Lane 3:** Sample 2 of isolated DNA. **Lane 4:** Sample 3 of isolated DNA. Both images show the expected size of the digested plasmid to be ~5369 bp.

4.3.1.2 Small-scale IRP1-3 and AfuA protein expression trails

Both recombinant IRP1-3 and AfuA proteins from *B. pertussis* were successfully expressed in the T7 Express LysY *E. coli* expression system with an N-terminal His-tag. Following the small expression protein trials, whole-cell, soluble and insoluble lysate fractions of IPTG-induced and uninduced *E. coli* were analysed by SDS-PAGE electrophoresis (Figure 4.3A). Figure 4.3B shows an image of SDS-PAGE depicting expression of recombinant AfuA in IPTG-induced colonies. Both IPTG-induced soluble and insoluble fractions show expression of AfuA protein (highlighted in yellow in the figures below).

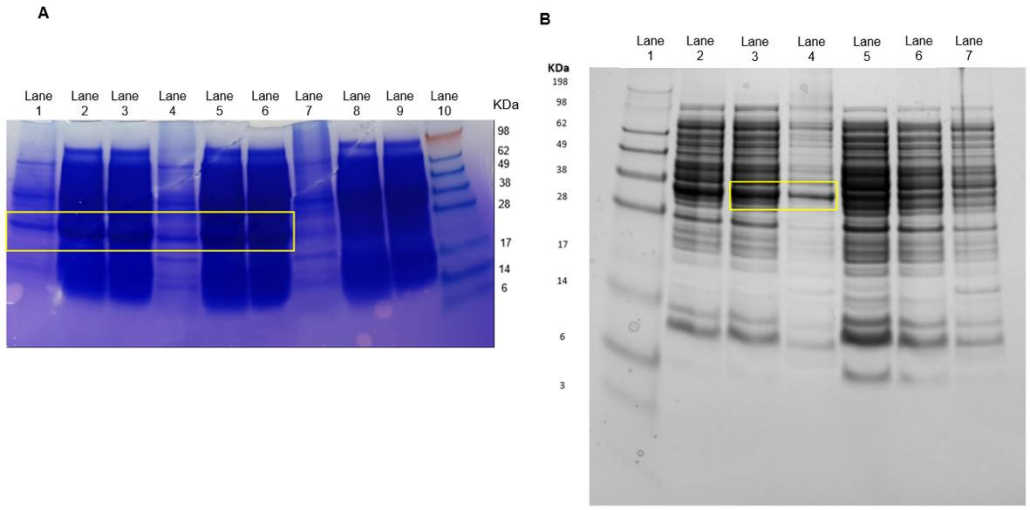


Figure 4.3. SDS-PAGE analysis of small-scale IRP1-3 and AfuA protein expression trials. A) IRP1-3 protein expression. Lane 1 represents IPTG-induced lysates of insoluble fraction, lane 2 = soluble fraction and lane 3 = whole-cell lysates induced with 0.4 mM IPTG. The lysates were obtained from selected bacterial colony 2. Lane 4 = insoluble fraction, lane 5 = soluble fraction and lane 6 = whole-cell lysates of IPTG-induced bacteria obtained from bacterial colony 1. IRP1-3 protein expression is highlighted in the figure and the molecular weight corresponds to ~ 19 KDa. Lanes 7, 8, 9 represent insoluble, soluble, and whole-cell lysates of uninduced bacteria, respectively. In the uninduced lysates, there is no indication of the protein expression. Lane 10 = molecular marker. **B)** AfuA protein expression. Lane 1 = molecular marker, lane 2, 3 and 4 = whole-cell, soluble and insoluble fractions of bacteria induced with 0.4 M IPTG at 16°C overnight. Lanes 5, 6 and 7 = whole-cell, soluble and insoluble fractions of uninduced bacteria. The AfuA protein expression is highlighted in the figure (yellow).

4.3.1.3 Western blot analysis of fractions after small-scale IRP1-3 protein expression trials

Expression of IRP1-3 protein was confirmed by Western blot analysis and was observed in the soluble fractions and whole-cell lysates obtained from IPTG-induced colony one and two (Figure 4.4, highlighted in yellow).

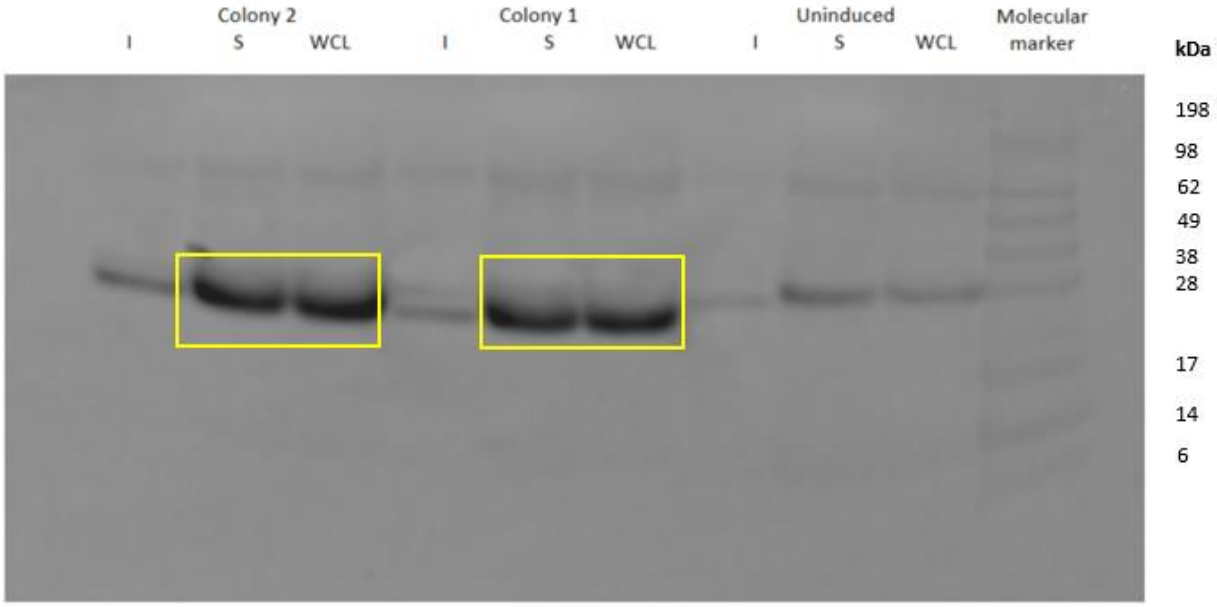


Figure 4.4. Western blot analysis of the expression of rIRP1-3 protein in *E. coli.* Induced proteins from *E. coli* were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with the HRP-conjugated 6x-His Epitope Tag monoclonal antibody. The reaction was developed with enhanced-chemiluminescence detection system (Pierce™ ECL Western Blotting Substrate). WCL = Whole-cell lysate, S = Soluble fraction, I = Insoluble fraction. The His-tagged IRP1-3 protein expression was detected by the monoclonal antibody as highlighted in yellow.

4.3.1.4 Large-scale protein expression trials

After successful small-scale protein expression trials, large scale expression was performed. As shown in Figure 4.5A, rIRP1-3 was highly expressed in whole-cell lysate and soluble protein fractions. The rAfuA was expressed in the insoluble fraction (Figure 4.5B).

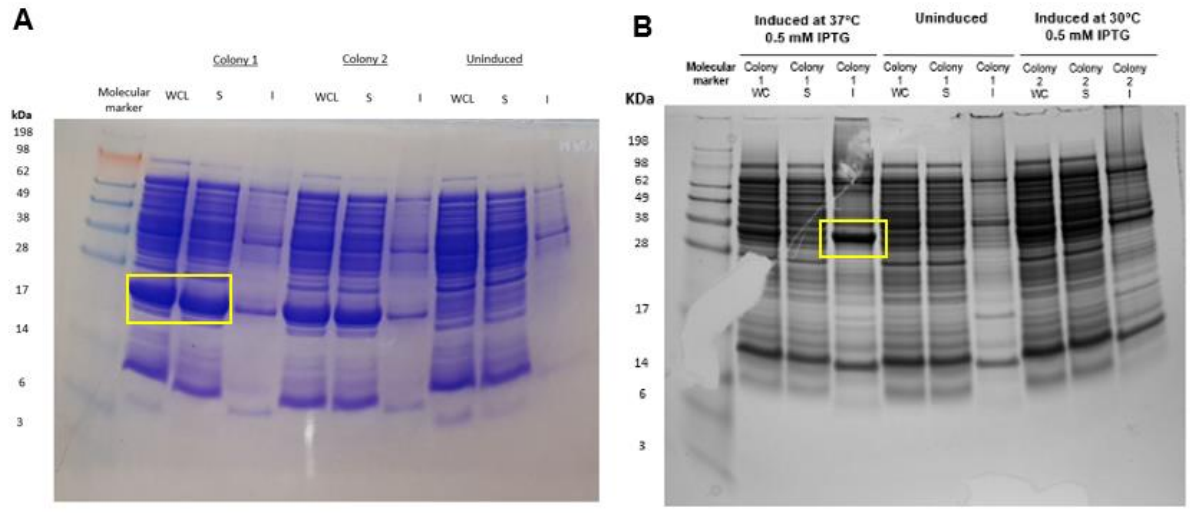


Figure 4.5. SDS-PAGE analysis of whole-cell, soluble and insoluble fractions obtained from large-scale IRP1-3 and AfuA expression trials. A) IRP1-3 protein expression. Lane 1: Molecular ladder (SeeBlue™ Plus2 Pre-stained Protein Standard). **Lane 2, 3 and 4:** IPTG-induced WCL = Whole-cell lysate, S = Soluble fraction, I = Insoluble fraction of colony one. **Lane 5, 6 and 7:** IPTG-induced WCL, S and I fractions of colony two. **Lane 8, 9**, **10:** Uninduced WCL, S and I fractions of *E. coli* T7 LysY lysate control. Expected molecular weight of expressed rIRP1-3 is ~19 kDa. Whole-cell and soluble fractions of both colonies one and two show high level of rIRP1-3 expression. **B) AfuA protein expression. Lane 1:** Molecular ladder (SeeBlue™ Plus2 Pre-stained Protein Standard). **Lane 2, 3 and 4:** WCL, S and I fractions induced by 0.5 mM IPTG at 37°C. **Lane 5, 6 and 7:** Uninduced WCL, S and I fractions. **Lane 8, 9 and 10:** WCL, S and I fractions induced by 0.5 mM IPTG at 30°C. Insoluble fraction, colony one that was induced by 0.5 mM IPTG at 37°C shows expression rAfuA protein.

4.3.1.5 Immobilised metal affinity chromatography

An automated liquid chromatography system was used for purifying rIRP1-3 protein. Eluted protein fractions are shown as distinct UV peak on chromatogram (Figure 4.6A). The protein fractions were collected and analysed by SDS-PAGE and the gel indicates that the fractions contain rIRP1-3 of which molecular weight corresponds to ~19 kDa (Figure 4.6B).

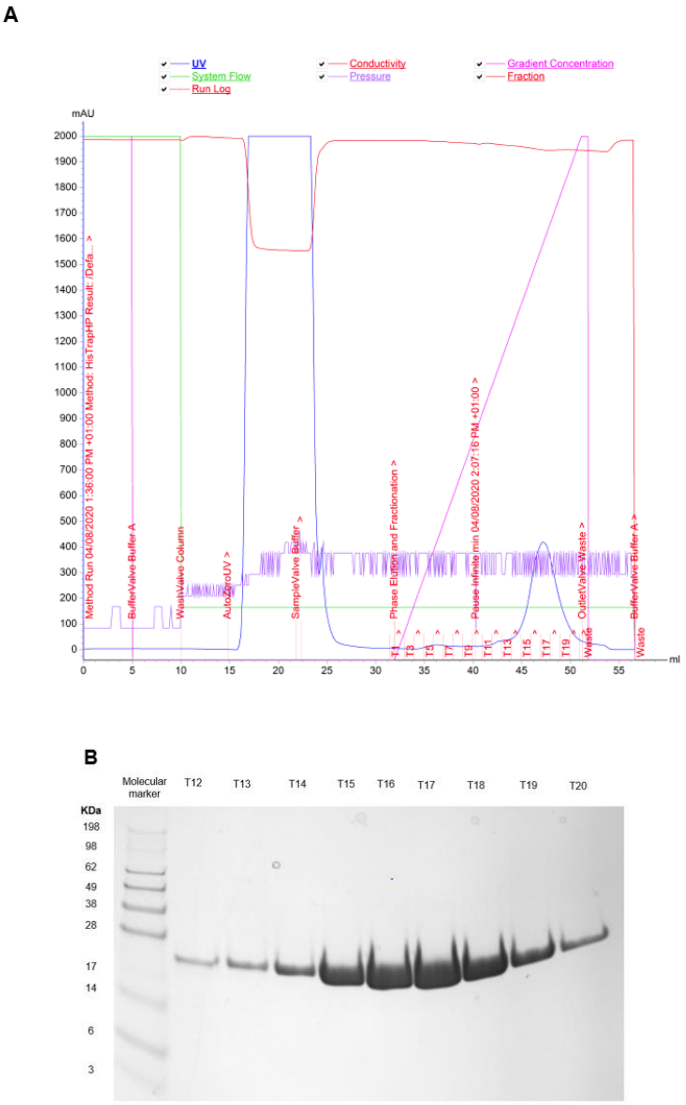


Figure 4.6. rIRP1-3 protein purification. A) Chromatogram of nickel His-tagged purification of rIRP1-3 protein. UV absorbance (A_{280}) and conductivity are shown for eluted fractions T1 – T23. The UV absorbance peak on the chromatogram indicates protein purification (T15 – T19). **B) Coomassie Blue stained analytical SDS-PAGE of eluted fractions (T12 – T20), after protein purification, showing IPTG induction of rIRP1-3**

expression in *E. coli* T7 LysY strain. Eluted fractions T12 – T20 were collected and analysed by SDS-PAGE to verify the protein's molecular weight which corresponds to ~19 kDa.

4.3.1.6 Analysis of IRP1-3 protein concentration and endotoxin levels after protein purification

Eluted fractions containing the purified protein (T15 – T18) were pooled to concentrate and purify the protein. The IRP1-3 protein concentration was 744.4 µg/ml as measured by BCA assay. The removal of bacterial endotoxins from the purified protein sample was essential to prevent reactogenicity when administered to mice. The endotoxin content was 30 - 60 IU/ml endotoxin, however, it has not been established what the acceptable limit of LPS content in the protein sample should be. To minimise adverse reactions in mice, a minimal amount of LPS in the sample is preferable. Therefore, the rIRP1-3 protein was further purified by size exclusion chromatography (Figure 4.7A) to remove endotoxin and was then analysed by SDS-PAGE (Figure 4.7B). The results in Figure 4.7A show distinct UV peak which represents eluted fractions containing the purified protein. The gel in Figure 4.7B shows that the eluted protein fractions (F27 – F29 and F1 – F6) contain the purified rIRP1-3 protein with a corresponding size of ~ 19 KDa. The endotoxin concentration in the purified protein sample, determined by the LAL assay, was then 0.06 – 0.12 IU/ml. We considered these LPS levels low and therefore the use of purified rIRP1-3 protein for mouse immunisations was deemed safe under the license that we hold.

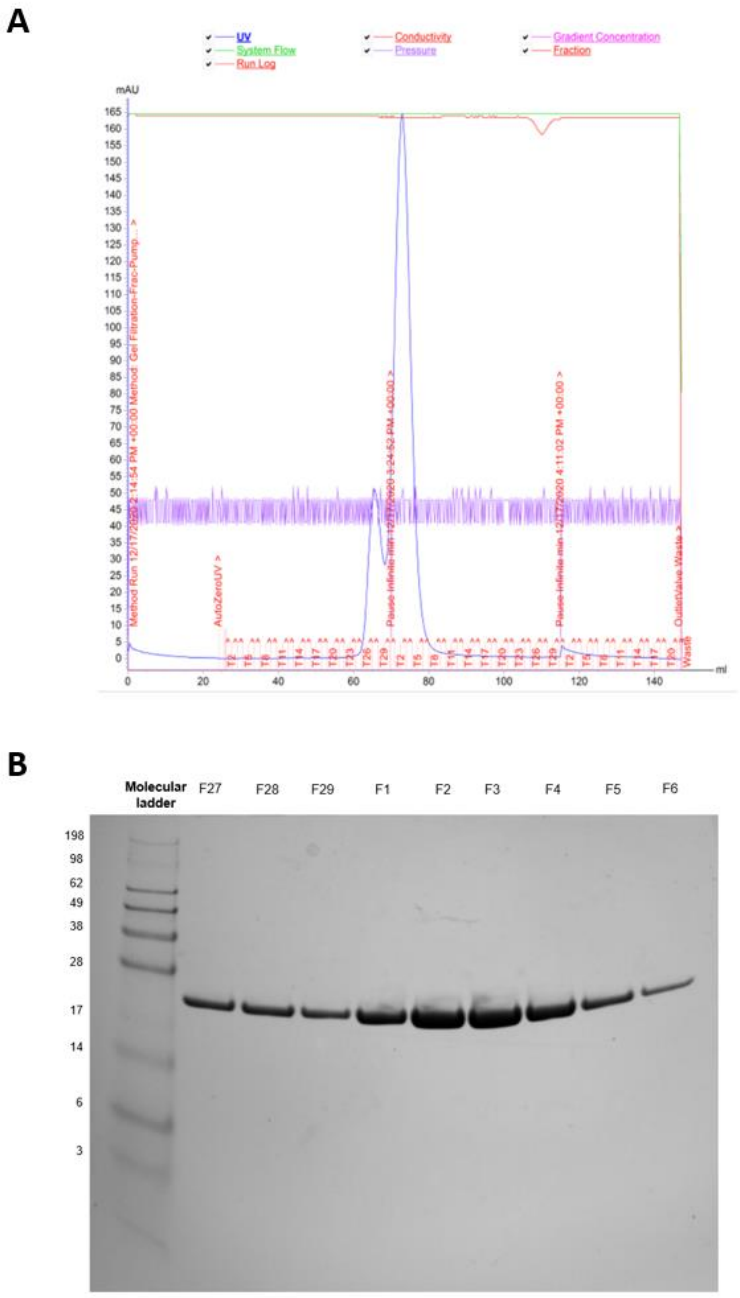


Figure 4.7. Purification of IRP1-3 recombinant protein and associated eluted fractions analysed by SDS-PAGE. A) Size exclusion chromatogram of rIRP1-3 protein fractioned by HiPrep 16/60 Sephacryl S-200 HR column on ÄKTA start purification system. The first and second blue peaks, representing UV absorbance readouts are associated with the eluted rIRP1-3 protein which is a dimer. **B)** Eluted fraction from the chromatography, were run on the gel to assess size of the eluted protein. SDS-PAGE gel shows resulting eluted protein fractions (T27 – T29 and T1 - T6), the corresponding size of ~ 19 KDa indicates purified rIRP1-3 protein.

4.3.1.7 Verification of IRP1-3 protein sequence by mass spectrometry after purification procedures

To confirm the identity and purity of the rIRP1-3, tandem mass spectrometry (MS) was performed by the Proteomics Team at NIBSC. The original amino acid sequence for IRP1-3 protein was obtained from UniProt protein sequence database and was compared against amino acid sequence of the IRP1-3 sample analysed by the tandem MS. The results showed that the amino acid sequence of the recombinant IRP1-3 protein 100% matched the sequence provided (Appendices). The corresponding protein molecular weight was confirmed by SDS-PAGE.

4.3.1.8 Purification of rAfuA protein using affinity chromatography and size exclusion chromatography

The His-tagged AfuA protein was subjected to affinity chromatography purification and SDS-PAGE analysis. The results show a sharp UV peak which indicates purification of rAfuA protein (Figure 4.8A) with the expected molecular weight of ~ 36 KDa (Figure 4.8B). Tandem MS revealed that the His-tagged purified rAfuA matched its protein sequence (UniProt), and the purity of the samples was 99% (Appendices). After refolding of the protein structure, SDS-PAGE analysis was performed again to analyse the protein size. SDS-PAGE image indicates that refolded AfuA protein has been refolded into two different protein species. Prominent bands were observed at approximately ~37 kDa and ~60 kDa (Figure 4.8C).

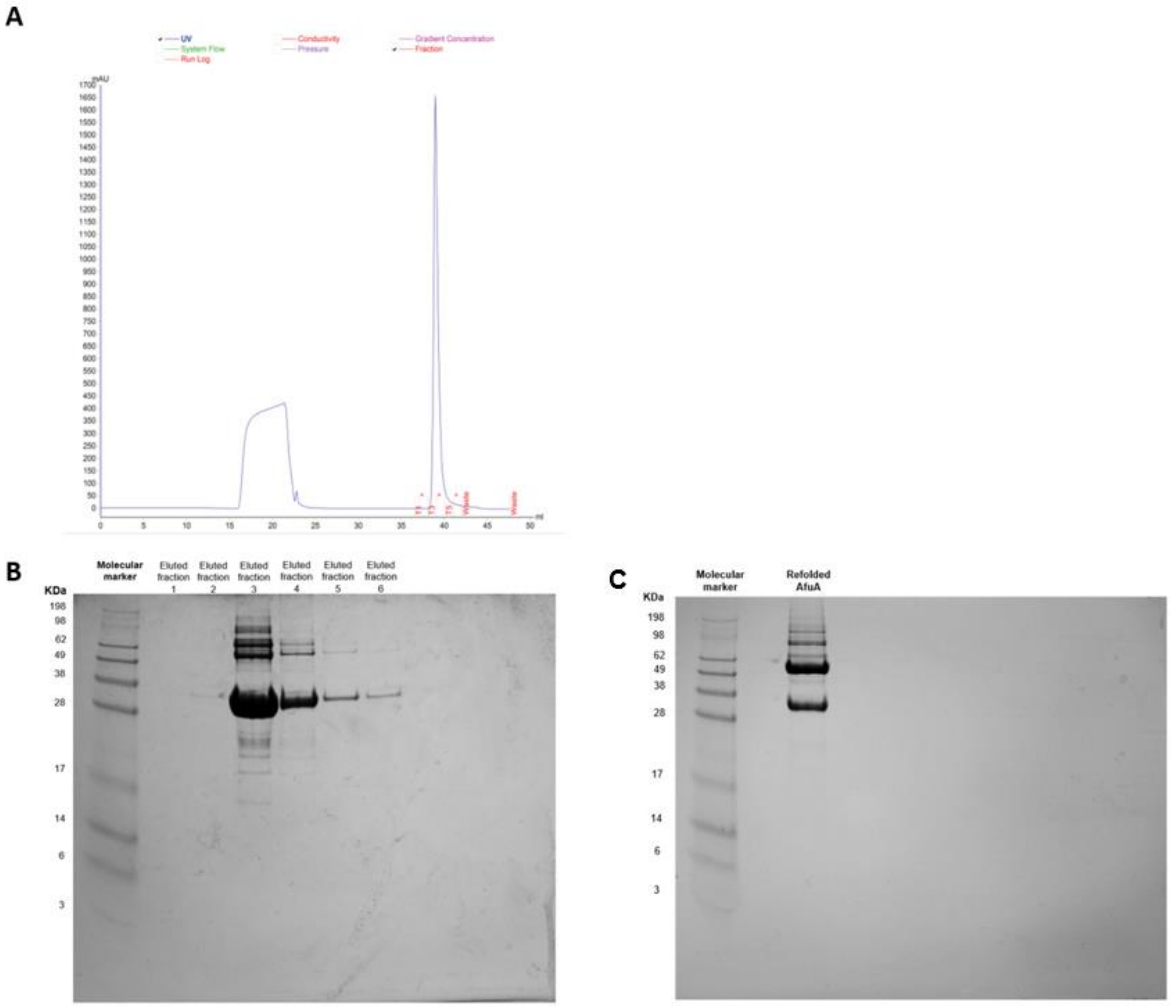


Figure 4.8. Purification of rAfuA protein. A) IMAC of urea-denatured His-tagged AfuA protein from inclusion bodies. The x-axis shows the volume in ml during elution of a 1 ml-HisTrap column. The left y-axis shows arbitrary UV absorbance units at 280 nm. Absorbance is shown in a blue solid line, one distinct peak represents the eluted His-tagged protein. **B) Coomassie stained SDS-PAGE gel showing samples obtained from the IMAC purification.** Following the purification procedure of AfuA protein, six eluted samples were loaded onto 16% Tris-Glycine gel. SDS-PAGE gel shows resulting six eluted protein fractions. Bands in fractions 3, 4, 5 and 6 (~ 36 KDa) indicate purified His-tagged AfuA protein. **C) Coomassie stained SDS-PAGE gel image showing refolded AfuA protein.** IMAC purified and refolded AfuA protein, in 20 mM Tris-HCl pH 7.5, 0.5 M NaCl, was separated on SDS-PAGE electrophoresis.

4.4 Materials and Methods

4.4.1 Immunogenicity studies with adenoviral vectored vaccine expressing IRP1-3 and AfuA

4.4.1.1 Immunisation of mice with Ad IRP1-3 and Ad AfuA vaccines, rIRP1-3 and rAfuA proteins

Female BALB/c mice (six to eight weeks old) were divided into 6 groups of five mice to assess and compare immunogenicity of the Ad IRP1-3 vaccine and rIRP1-3 protein. An additional group, consisting of eight mice, was used to raise polyclonal antibodies against rIRP1-3 antigen. This group received three doses of 10 µg/50 µl of rIRP1-3 (Table 4.1). Mice were immunised intramuscularly with 2.5 µg, 5.0 µg and 10 µg/50 µl of rIRP1-3 antigen adsorbed to alum adjuvant (0.350 mg/dose), or 1 x 10^9 IU/50 µl of Ad IRP1-3 vaccine on day 0. As shown in Table 4.1, some groups were administered with a second dose of the vaccine on day 14 to compare one dose versus two doses of adenoviral vectored vaccines. Naïve mice were non-immunised.

Three groups of six mice were obtained to assess immunogenicity of the Ad AfuA vaccine in endpoint titration ELISA. One group of mice was immunised with two doses of Ad AfuA (1 x 10^9 IU/50 µl) at days zero and 14, another group of mice was immunised with 0.4 IU/dose of wP vaccine, which represents 1/10^{th} of human dose of wP vaccine (positive control). Sera obtained from naïve, non-immunised, mice served as negative control (Table 4.2) All mice were immunised via intramuscular route.

Table 4.1. Immunisation of mice with Ad IRP1-3 vaccine and rIRP1-3 protein.

Group	Vaccine	Dose/ mouse	First dose (Day 0)	Second dose (Day 14)	Third dose (Day 28)
1	rIRP1-3	2.5 µg/ 50 µl	✔	✔	-
2	rIRP1-3	5 µg/ 50 µl	✔	✔	-
3	rIRP1-3	10 µg/ 50 µl	✔	✔	-
4	rIRP1-3	10 µg/ 50 µl	✔	✔	✔
5	Ad IRP1-3	1×10^9 IU/50 µl	✔	-	-
6	Ad IRP1-3	1×10^9 IU/50 µl	✔	✔	-
7	Naive	-	-	-	-

Table 4.2 Immunisation of mice with Ad AfuA vaccine and wP vaccine

Group	Vaccine	Dose/ mouse	First dose (Day 0)	Second dose (Day 14)
1	Ad AfuA	1×10^9 IU/50 µl	✔	✔
2	wP vaccine	0.4 IU/dose	✔	-
3	Naive	-	-	-

Tail bleeds were collected two, four and six weeks after the immunisation. Bleeding schedules for the experiment are shown in Figure 4.9.

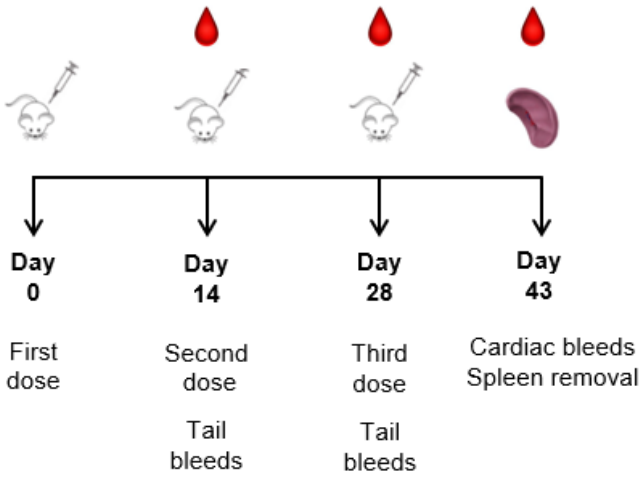


Figure 4.9. Experimental design for mouse immunisation schedule. The timeline shows that groups of mice were immunised with two doses of rIRP1-3 antigen at day 0 and 14, and with either a single dose or two doses of Ad IRP1-3 vaccine. One group of mice received three doses of 10 µg rIRP1-3 antigen to induce anti-IRP1-3 polyclonal IgG antibodies. Two doses of Ad AfuA vaccine were administered to six mice and one dose of wP vaccine was administered to other group of six mice on day 0.

4.4.2 Optimisation of Enzyme-linked immunosorbent assay (ELISA) for analysis of IRP1-3-specific IgG antibody response in immunised mice

4.4.2.1 Determination of optimal IRP1-3 coating antigen by ELISA

A microtitre 96-well plate was coated with 100 µl of 0.5 µg, 1.0 µg, 1.5 µg, 2.0 µg, 2.5 µg, 3.0 µg/ ml of IRP1-3 antigen in carbonate coating buffer, pH 9.6 (in duplicates, column 1-12). The plate was incubated overnight at 4°C, washed four times with PBST (PBS containing 0.05% (v/v) Tween 20), and then blocked with 200 µl of blocking solution made of 1% Bovine Serum Albumin in Phosphate Buffered Saline (BSA/PBS) at 37°C for two hours. Positive serum samples obtained from group seven described above (Table 4.1) were diluted two-fold in 1% BSA/PBS down the plate. Wells in row H were used as blank containing only 100 µl of 1% BSA/PBS. The plate was then

incubated at room temperature for one hour and half and washed four times with PBST. Goat anti-mouse IgG HRP conjugate (Sigma-Aldrich), diluted 1:1,000, was added to the plate (100 µl/well) and incubated for one hour and a half at room temperature. After washing with PBST, 100 µl of 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate (Sigma Aldrich) was added to each well and the plate was incubated for 10 minutes at room temperature. The enzymatic reaction was stopped by adding 50 µl of 1 M of sulphuric acid. The plates were read at absorbance of 450 nm using a Molecular Devices (Vmax model) plate reader and Soft Max Pro software (Version 7.1). The criterium for selection of the optimal coating antigen concentration was as follows: if no further increase in antigen concentration did not result in increased antibody binding (when OD readouts plateau), it was deemed to be the optimal antigen coating concentration.

4.4.2.2 Determination of optimal IgG secondary antibody concentration by ELISA

A microtitre 96-well plate was coated with the optimal protein concentration determined from the experiment above, 1.0 µg/ml of IRP1-3 antigen (100 µl/well) in carbonate coating buffer, pH 9.6, overnight at 4°C. The plate was washed four times with PBST, blocked with 100 µl/well of 1% BSA/PBS at 37°C for two hours, and washed four times with PBST. To establish the optimal IgG secondary antibody concentration, goat anti-mouse IgG (Fc specific)-peroxidase antibody was diluted 1/40,000 – 1/65,000 in 1% BSA/PBS buffer and each dilution was added to rows A – G (columns 1 -12) in duplicates. Positive serum sample was added to row A at dilution of 1/3000 and was diluted two-fold up to row G. Row H contained 200 µl of 1% BSA/PBS which served as negative control (blank). The plate was incubated at room

temperature for one hour and half, then washed four times with PBST. The enzyme reaction was initiated with the addition of TMB substrate (100 µl/well). The reaction was stopped with 50 µl/well of 1 M sulphuric acid. The plate was read at absorbance of 450 nm using a Molecular Devices (Vmax model) plate reader and Soft Max Pro software (Version 7.1).

4.4.3 Detection of IRP1-3-specific IgG antibody titres in mouse sera by ELISA

The optimal conditions identified as described above were used to determine individual antibody titres. Microtiter 96-well plates were coated with 1.0 µg/ml of IRP1-3 antigen (100 µl/well) in carbonate coating buffer, pH 9.6, overnight at 4°C. Plates were washed four times with PBST, blocked with 100 µl/well of 1% BSA/PBS at 37°C for two hours, and washed four times with PBST. Sera from immunised mice were obtained two, four and six weeks after the immunisation as shown above in Figure 4.2. Sera were serially diluted two-fold starting from 1/100 dilution. Quality control (QC) samples (low, medium and high), prepared from mice immunised with 10 µg of rIRP1-3 protein, were plated in duplicates, and 100 µl/well of 1% BSA/PBS was added to two wells which served as blank. The QC sample readouts must hit the top, middle and low part of the linear part of the standard curve before it plateaus at the top and bottom.

The reference standard was made from pooled sera of mice immunised with the rIRP1-3 protein and was diluted 1:10 in 1% BSA/PBS. The serum samples, reference sera and controls were incubated for one hour and 45 minutes and were washed prior to incubation with goat anti-mouse IgG (Fc specific)-peroxidase antibody (diluted 1:60,000 in 1% BSA/PBS). The plates were then washed and 100 µl/well of TMB substrate (Sigma Aldrich) was added to all wells and incubated for 15 minutes to detect

secondary antibodies. Stop solution (1.0 M sulphuric acid, 50 µl/well) was added to each well to stop the enzymatic reaction. The plates were read at absorbance of 450 nm using a Molecular Devices (Vmax model) plate reader and Soft Max Pro software (Version 7.1). ELISA units were calculated for each serum sample using the reference standard parameters which was assigned arbitrary values of 250 – 1.95 ELISA units (EU) for the respective dilutions (1/1,000 – 1/128,000).

4.4.4 Optimisation of endpoint titration ELISA for analysis of AfuA-specific IgG antibody response in immunised mice

4.4.4.1 Determination of optimal AfuA coating antigen by ELISA

A microtiter 96-well plate was coated with 100 µl of 0.5 µg, 1.0 µg, 1.5 µg, 2.0 µg, 2.5 µg, 3.0 µg/ ml of rAfuA antigen in carbonate coating buffer, pH 9.6 (in duplicates, column 1-12). The plate was incubated overnight at 4°C, washed four times with PBST, and then blocked with 200 µl of blocking solution made of 1% BSA/PBS at 37°C for two hours. Serum samples obtained from mice immunised with one dose of Ad AfuA vaccine, as shown in Table 4.1, were diluted two-fold in 1% BSA/PBS down the plate. Wells in row H were used as blank containing only 100 µl of 1% BSA/PBS. The plate was then incubated at room temperature for one hour and half and washed four times with PBST. Goat anti-mouse IgG HRP conjugate (Sigma-Aldrich), diluted 1:1,000, was added to the plate (100 µl/well) and incubated for one hour and a half at room temperature. After washing with PBST, 100 µl of TMB substrate was added to each well and the plate was incubated for 10 minutes at room temperature. The enzymatic reaction was stopped by adding 50 µl of 1 M of sulphuric acid. The plate was read at absorbance of 450 nm using a Molecular Devices (Vmax model) plate reader and Soft Max Pro software (Version 7.1).

4.4.4.2 Determination of optimal IgG secondary antibody concentration by ELISA

A microtiter 96-well plate was coated with the optimal protein concentration, determined from the experiment above, 1.0 µg/ml of IRP1-3 antigen (100 µl/well) in carbonate coating buffer, pH 9.6, overnight at 4°C. The plate was washed four times with PBST, blocked with 100 µl/well of 1% BSA/PBS at 37°C for two hours, and washed four times with PBST. To establish the optimal IgG secondary antibody concentration, goat anti-mouse IgG (Fc specific)-peroxidase antibody was diluted 1/40,000 – 1/65,000 in 1% BSA/PBS buffer and each dilution was added to rows A – G (columns 1-12) in duplicates. Positive serum sample was added to row A at dilution of 1/50 and was diluted two-fold up to row G. Row H contained 200 µl of 1% BSA/PBS which served as negative control (blank). The plate was incubated at room temperature for one hour and half, then washed four times with PBST. The enzyme reaction was initiated with TMB substrate (100 µl/well). The reaction was stopped with 50 µl/well of 1 M sulphuric acid. The plate was read at absorbance of 450 nm using a Molecular Devices (Vmax model) plate reader and Soft Max Pro software (Version 7.1).

4.4.5 Detection of AfuA-specific IgG antibody titres in mouse sera by endpoint titration ELISA

The endpoint titration ELISA selects a cut-off absorbance readout above the background noise. The highest dilution that is above the selected cut-off point is the endpoint titre. Microtiter 96-well plates were coated with 1.0 µg/ml of AfuA antigen (100 µl/well) in carbonate coating buffer, pH 9.6, overnight at 4°C. The plates were washed four times with PBST, blocked with 200 µl/well of 1% BSA/PBS at 37°C for two hours, and washed four times with PBST. Serum samples from non-immunised

mice and mice immunised with Ad AfuA vaccine and were serially diluted two-fold, starting from 1/100 dilution. A sample's dilution was plotted against its OD value and fitted to a sigmoidal curve. The endpoint titre was determined by the last diluted sample that gives positive results on the ELISA and its OD readout is over of OD value of the naïve mice.

4.4.6 Efficacy studies of the Ad IRP1-3 and Ad AfuA vaccine candidates

Female BALB/c mice (n = 10/group) were immunised via intramuscular route with either one dose or two doses of Ad IRP1-3 (1 x 10^9 IU/50 µl), or two doses of 5 µg of rIRP1-3 protein adsorbed to alum adjuvant (0.350 mg/dose), and wP vaccine (NIBSC code 94/532). Groups of mice were also immunised with either one dose of Ad AfuA vaccine (1 x 10^9 IU/50 µl), two doses of 4 µg of rAfuA protein adsorbed to aluminium phosphate gel adjuvant, Adju-Phost® (InvivoGen), (0.350 mg/dose), mixture of Ad AfuA + Ad AC vaccine (5 x 10^8 IU/50 µl, each vaccine) and 1/10^{th} human dose of wP vaccine (NIBSC code 94/532). Negative control groups of mice were added (non-immunised) (Table 4.3 and Table 4.4). Mice were challenged with *B. pertussis* 18-323 (1 x 10^8 bacteria/ml) two weeks after the second dose and lung samples were obtained one week after challenge (Figure 4.10).

Table 4.3. Mouse immunisations for the efficacy studies of the Ad IRP1-3.

Group	Vaccine	Dose/mouse	First dose (DAY 0)	Second dose (DAY 14)
1	Ad IRP1-3	1×10^9 IU/50 µl	✔	-
2	Ad IRP1-3	1×10^9 IU/50 µl	✔	✔
3	rIRP1-3	5.0 µg/50 µl	✔	✔
4	wP vaccine	0.4 IU/dose	✔	-
5	naïve	-	-	-

Table 4.4. Mouse immunisations for the efficacy studies of the Ad AfuA vaccines.

Group	Vaccine	Dose/ mouse	First dose (DAY 0)	Second dose (DAY 14)
1	Ad AfuA	1×10^9 IU/50 µl	✔	✔
2	rAfuA	4.0 µg/50 µl	✔	✔
3	Ad AfuA + Ad AC	5×10^8 IU/50 µl (each vaccine)	✔	✔
4	wP vaccine	1/10th human dose	✔	-
5	Naïve	-	-	-

B. pertussis suspension for aerosol challenge was prepared as described in the 'General Materials and Methods' Chapter 7, section 7.2 (subsection 7.2.2). Briefly, mice were challenged with *B. pertussis* 18-323 strain (1 x 10^8 bacteria/ml) on day 28. Mice were exposed to aerosolised bacterial suspension after placing their noses into the nose parts of the aerosol apparatus for five minutes. One week after challenge (Day 35), lungs were removed, and homogenised and viable bacterial counts were established by automated colony counter (Synbiosis Synoptics analyser Protocol 3).

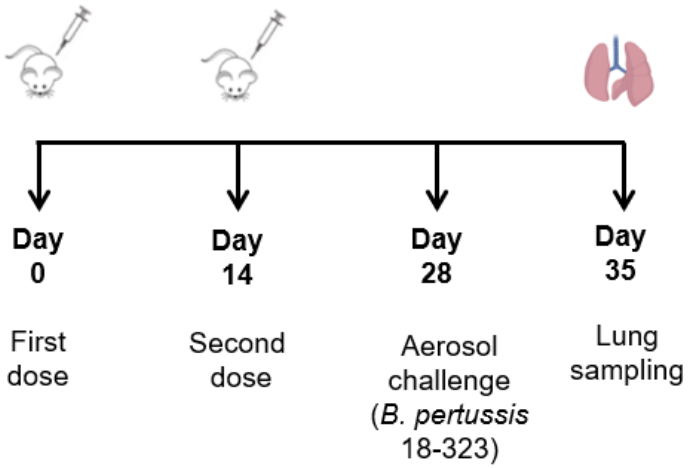


Figure 4.10. Experimental design for aerosol challenge study. The timeline shows that groups of mice were immunised with two doses of rIRP1-3 and rAfuA antigens, a single and two doses of Ad IRP1-3, two doses of Ad AfuA and a mixture of Ad AfuA + Ad AC vaccines at day 0 and 14. Mice were challenged with *B. pertussis* 18-323 on day 28 and lungs were harvested on day 35.

4.5 Results

4.5.1 Assessment of immunogenic and protective properties of Ad IRP1-3 vaccine)

4.5.1.1 Optimisation of IRP1-3 coating antigen concentration and IgG HRP conjugate dilution for IRP1-3-specific IgG ELISA

The ELISA conditions were optimised to ensure reproducibility and accuracy of results. The OD read-outs of the serum dilutions (1/1,000 – 1/64,000) were plotted against different IRP1-3 antigen coating concentrations. The optimal IRP1-3 coating antigen concentration was determined to be 1 µg/ml (Figure 4.11A). The optimal IRP1-3 coating antigen concentration was selected when further increase in antigen concentration did not result in increased serum antibody binding.

The OD values of the serum dilutions (1/1,000 – 1/106,000) were plotted against conjugate antibody dilutions (1/40,000 – 1/65,000). A curve generated for the detection antibody dilution which gave an OD ~0.10 and a top OD of ~2.00 was chosen. The optimal IgG HRP-conjugated antibody dilution was determined to be 1/55,000 or 1/60,000 (Figure 4.11B). Estimation of the optimal antibody dilution was based on the upper and lower tail of the sigmoidal curve covering the top and bottom dilution corresponding to OD ~2.00 and ~0.10, respectively.

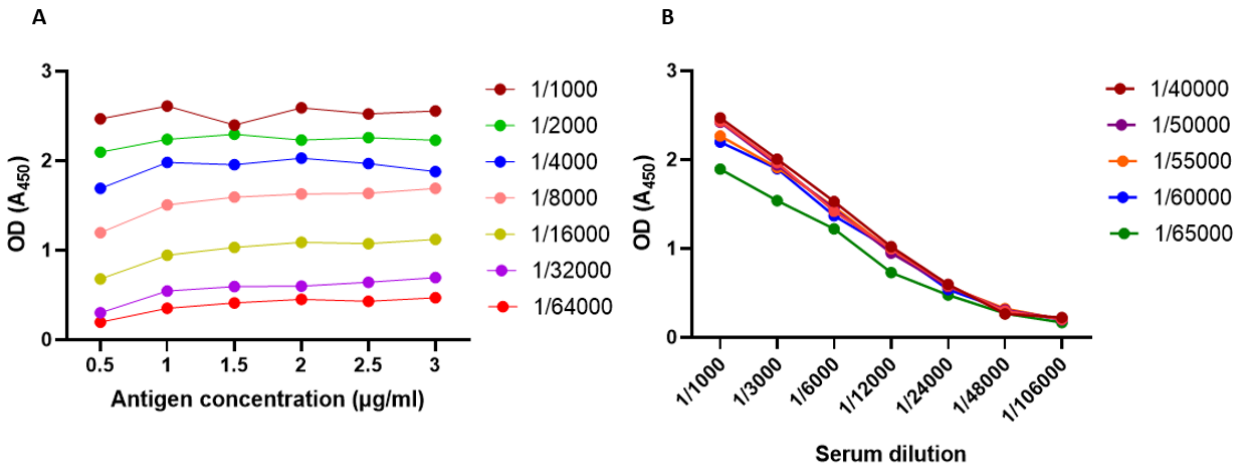


Figure 4.11. Optimisation of ELISA for assessing IRP1-3-specific IgG antibody response in immunised mice. A) Optimisation of IRP1-3 antigen coating concentration. 0.5 – 3 µg/ml of rIRP1-3 protein concentrations were assessed. Sera were diluted two-fold down the plate starting from 1/1,000 dilution. **B) Optimisation of dilution of IgG secondary antibody conjugated to HRP.** Based on the manufacturer's recommendation, the following range of dilutions was selected for the IgG secondary antibody: 1/40,000 – 1/65,000. Sera were diluted three-fold down the plate starting from 1/1,000 dilution.

4.5.1.2 Optimisation of the reference standard and quality controls for IRP1-3-specific IgG ELISA

Following optimisation of the IRP1-3 coating antigen and IgG conjugate secondary antibody, sera obtained from four mice (mice 1 – 4) and from another four mice (mice 5 – 8) immunised with 10 µg/ml of rIRP1-3 protein were pooled to prepare the standard reference and QC samples (low, medium and high), respectively. The reference standard and QC samples were serially diluted (two-fold) in 1% BSA/PBS down the plate, starting from 1/1,000 dilution and ending with 1/32768000 dilution. The top dilution (1/1,000) for the reference standard and for the QC had OD readings of 2.0 and 2.1 (A_{450}), respectively (Figure 4.12A). The results show that both calibration curves (one for the reference standard and one for the QC) have similar OD readouts which cover the OD range ~0.1 - 2.0, before the standard curves plateau.

Both QC samples (QC 1 and QC 2) are in the linear part of the standard curve (Figure 4.12B) and will be run in each subsequent assay to provide confidence that the results obtained from ELISA assays are correct.

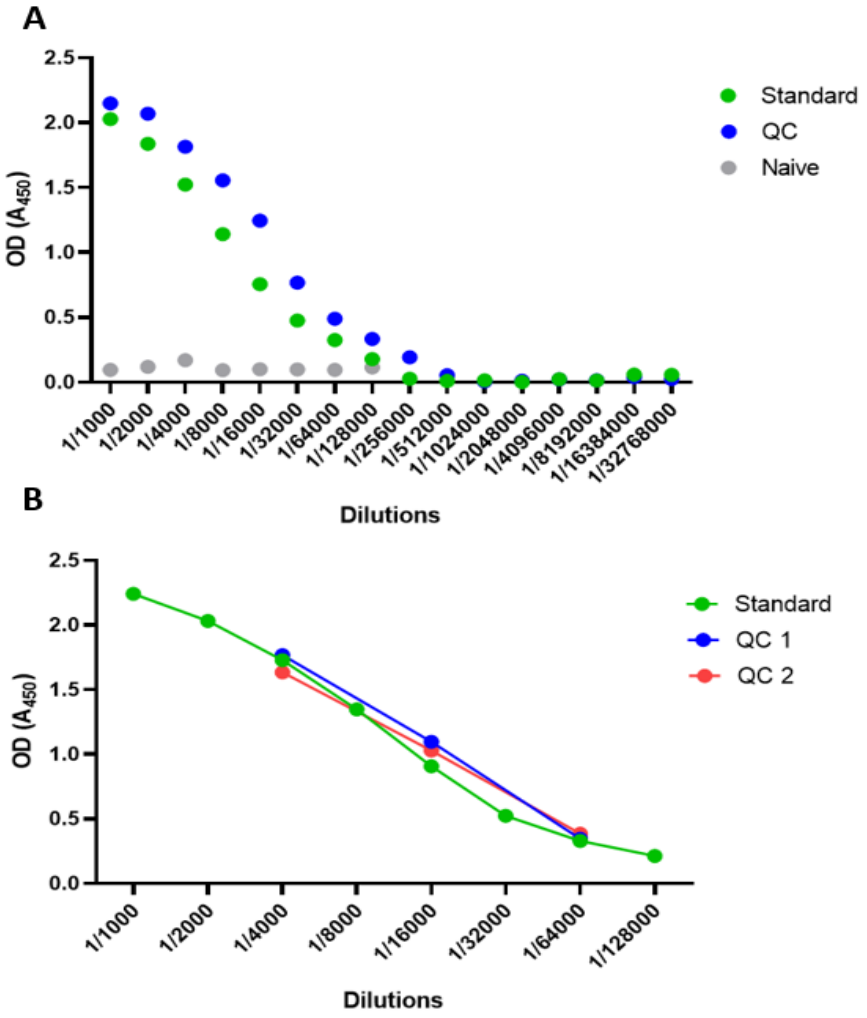


Figure 4.12. Optimisation of standard curve and establishment of QC samples for IRP1-3-specific IgG ELISA. A) First step in optimisation of the reference standard and QC. The top dilution for both, the standard and QC has given OD readout of ~2.00. **B) Establishing QC samples.** QC values that fall at the top, middle and bottom of the standard curve (linear dilution curve) are high, middle, and low QC samples that will be used for all the subsequent assays to ensure that data obtained from the assay are reliable.

4.5.1.3 Ad IRP1-3 vaccine is immunogenic in mice

To analyse the immunogenicity of Ad IRP1-3 vaccine, mice were immunised, via intramuscular route, with either one dose or two doses of Ad IRP1-3 at a two-week interval. Immunisation of mice with two doses of rIRP1-3 protein in a dose-dependent manner served as a direct recombinant protein-based vaccine comparator to the Ad IRP1-3 vaccine. To evaluate the humoral immune response induced by the vaccines, IRP1-3-specific IgG antibody levels in mouse sera were measured by ELISA developed and standardised as described above.

Immunisation with a single dose of Ad IRP1-3 vaccine elicited high anti-IRP1-3 IgG antibodies two weeks after immunisation (Figure 4.13A). The antibody response in mice induced by one dose of Ad IRP1-3 vaccine was significantly higher than immunisation with a single dose of 2.5 µg of rIRP1-3, 5 µg of rIRP1-3 or 10 µg of rIRP1-3 ($\alpha = 0.05$; ****, $p < 0.0001$ ***, $p = 0.0009$) (Figure 4.13A). Two immunisations with Ad IRP1-3 vaccine induced the same level of IRP1-3-specific IgG response as one dose and two dose immunisations with 2.5 µg of rIRP1-3, 5 µg of rIRP1-3 and 10 µg of rIRP1-3, two weeks after the second dose (or four weeks after prime) (Figure 4.13B). Anti-IRP1-3-specific IgG titres remained similar for all immunisation schedules four weeks after immunisation with the second dose (six weeks after a single dose immunisation), except two-dose immunisation with Ad IRP1-3 (Figure 4.13C). Administration of two doses of Ad IRP1-3 vaccine induced significantly higher IRP1-3-specific IgG antibody responses than the two doses of rIRP1-3 protein and one dose of Ad IRP1-3 vaccine (****, $p < 0.0001$) (Figure 4.13C).

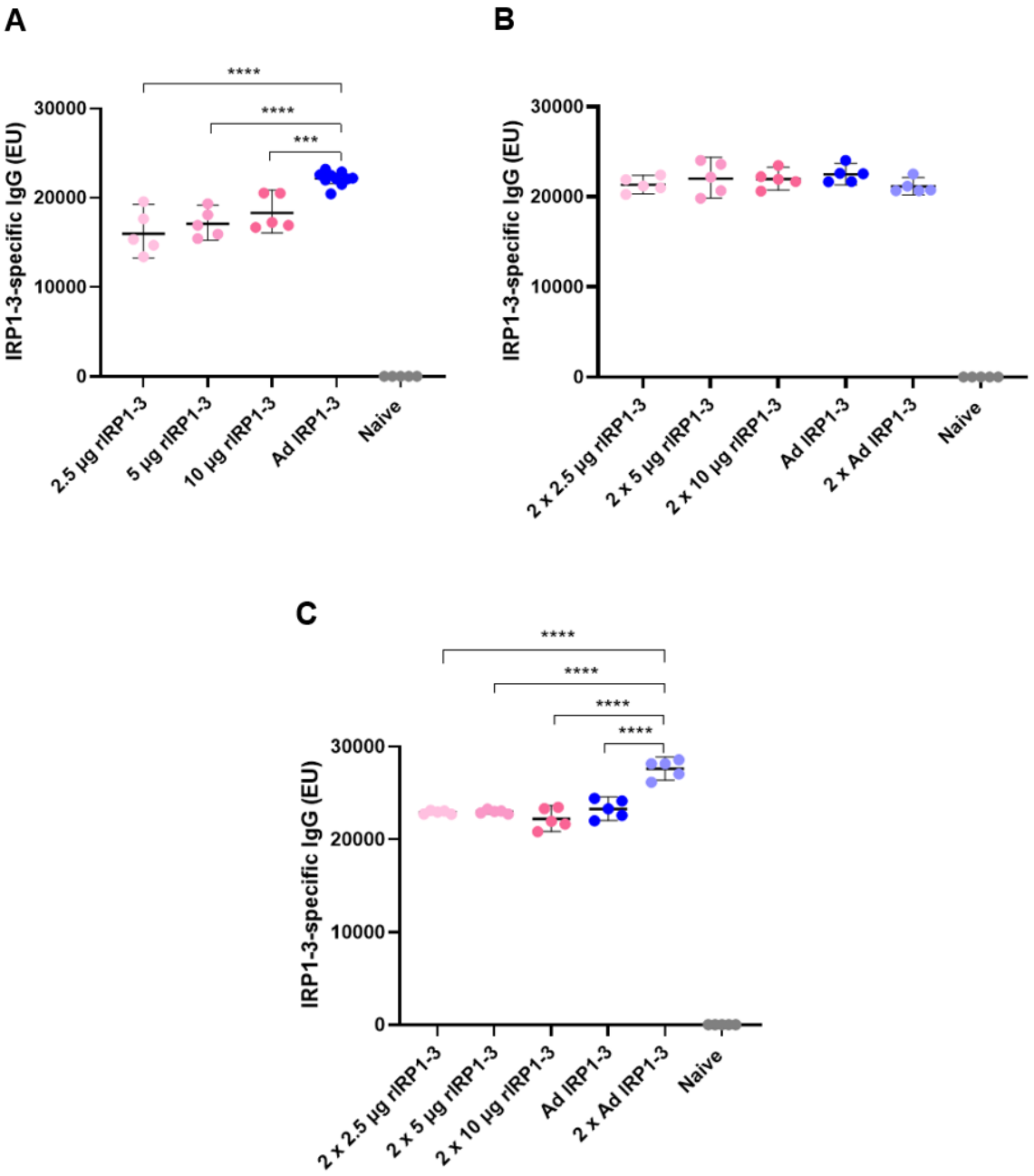


Figure 4.13. Analysis of IRP1-3-specific IgG antibody titres following immunisation with Ad IRP1-3 vectored vaccine and different doses of rIRP1-3 protein-based vaccine in mice. A) Two weeks after primary immunisation. **B)** Four weeks after priming (two weeks after boost) and **C)** Six weeks after priming (four weeks after boost). Vaccine-induced IRP1-3-specific IgG responses were measured in mice vaccinated with Ad vectored vaccine expressing IRP1-3 antigen and rIRP1-3 protein vaccine. The antibody responses are depicted on the X-axis at different time points. Data represent geometric mean of anti-IRP1-3 IgG titres obtained from individual mice in each group (n = 5/group). Error bars indicate ± 95% confidence intervals.

4.5.1.4 Evaluation of protective capacity of Ad IRP1-3 vaccine in mice using a pertussis aerosol challenge model

Groups of BALB/c mice were immunised with one or two doses of Ad IRP1-3 vaccine at two-week interval. Two doses of 5 µg/dose of rIRP1-3 protein vaccine served as a protein vaccine comparator against the adenoviral vectored vaccine. Two weeks after the second dose, mice were challenged with aerosolised 1 x 10^8 CFU/ml of *B. pertussis* suspension and lungs were harvested one week later for CFU enumeration. Bacterial load in the lungs of mice immunised with single dose of Ad IRP1-3 and two doses of Ad IRP1-3 was comparable to naïve mice (Figure 4.14), suggesting that the adenoviral vectored vaccines did not elicit protection against *B. pertussis* 18-323 strain. Two-dose immunisation with the rIRP1-3 also did not mediate protection against pertussis infection, however, the wP vaccine induced partial protection and, in some mice, even full protection against *B. pertussis* (Figure 4.14).

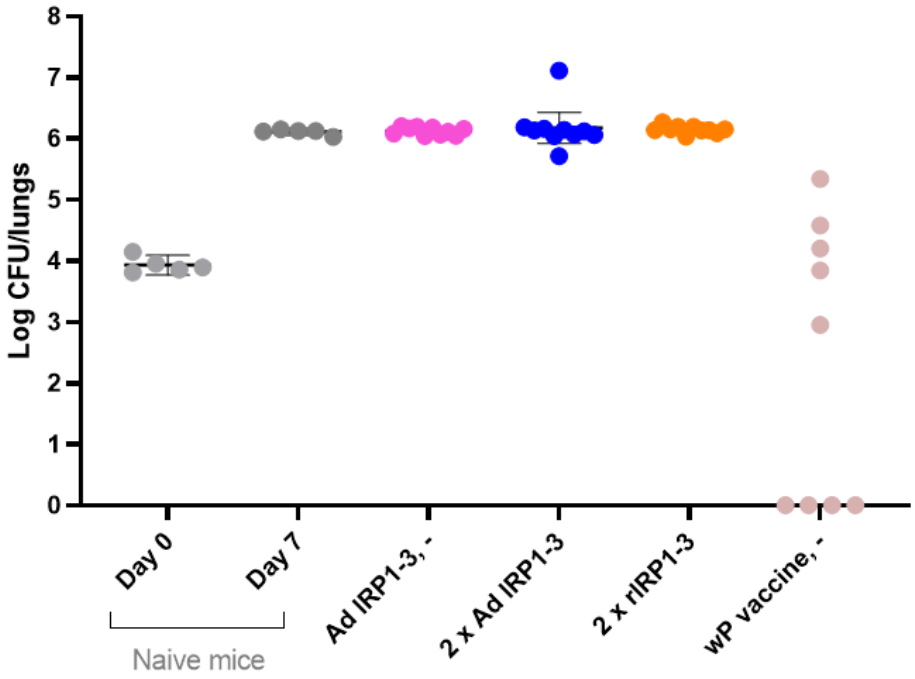


Figure 4.14. Protective efficacy of adenoviral vectored vaccine expressing IRP1-3 in immunised mice. Mice were immunised with one or two doses of Ad IRP1-3 vaccine at two-week interval. Challenge was performed four weeks after the single immunisation (or two weeks after the second immunisation). Five naïve mice in the control group, at day zero, were sacrificed three hours after aerosol challenge with the virulent bacteria to establish the successful infection by analysing bacterial counts in their lungs. The remaining five mice were sacrificed on day seven along other groups to determine viable counts in the mouse lungs. Results for each group are shown as individual counts and geometric mean ± 95% confidence interval (n = 10 mice).

4.6.2 Assessment of immunogenic and protective properties of Ad AfuA vaccine

4.6.2.1 A single dose of Ad AfuA vaccine was immunogenic in mice

Mouse sera collected two, four and six weeks after single dose immunisation with Ad AfuA and negative sera from non-immunised mice were analysed in endpoint titration ELISA to assess immunogenic potential of the vaccine. The serum samples were diluted to 1/100 – 1/12,800 by two-fold dilution. The immunisation with a single dose of Ad AfuA vaccine induced AfuA-specific IgG antibodies in mice two, four and six weeks following vaccination (Figure 4.15).

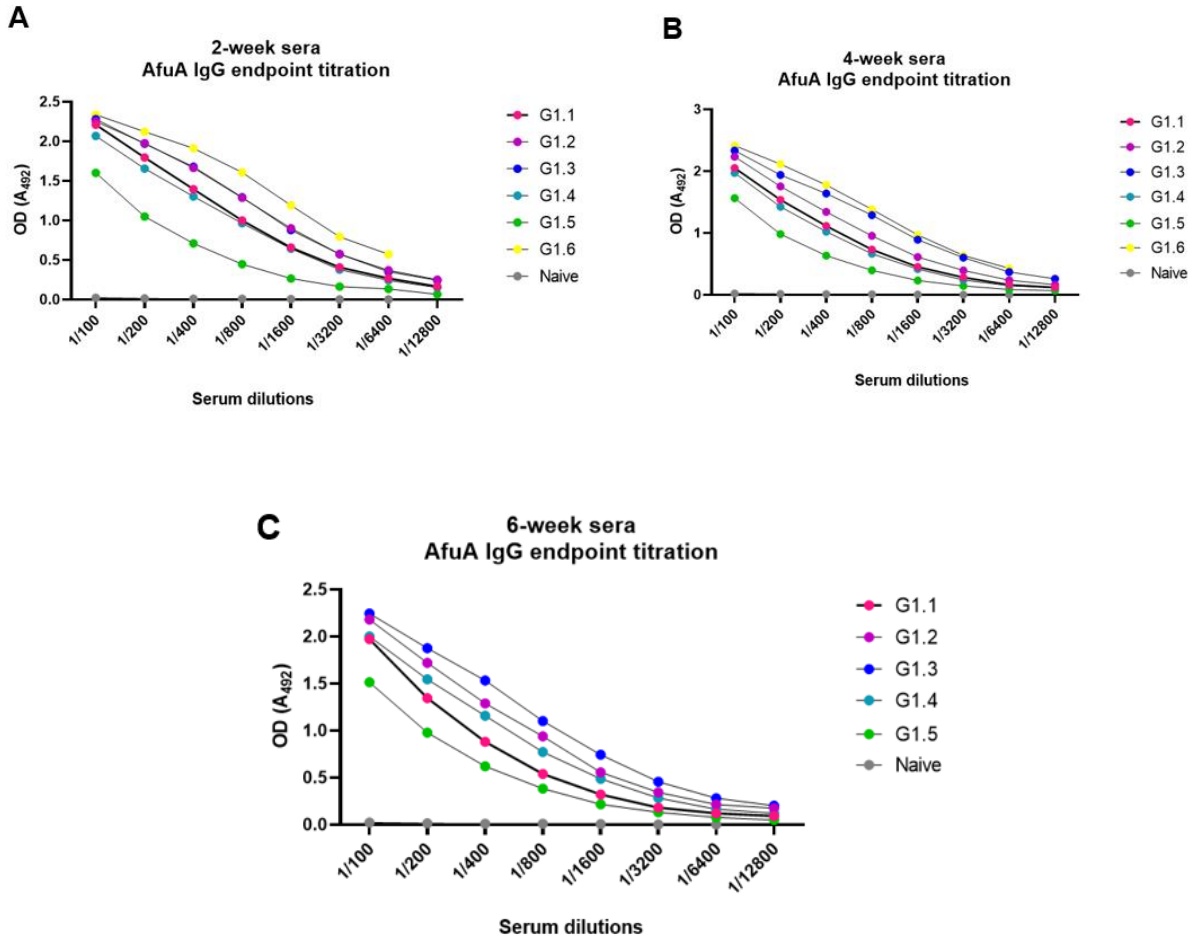


Figure 4.15. Assessment of Ad AfuA vaccine's immunogenic properties by endpoint titration ELISA. AfuA-specific IgG ELISA endpoint titres for individual mouse **A)** at week two, **B)** at week four and **C)** at week six after single dose immunisation with the vaccine. Non-immunised mice represent naïve group as negative control.

4.6.2.2 Evaluation of protective capacity of Ad AfuA vaccine in mice using a pertussis aerosol challenge model

To evaluate the protective efficacy of Ad AfuA vaccine, an aerosol challenge experiment was set up. Groups of mice received two doses of Ad AfuA vaccine, recombinant AfuA protein and a mixture of Ad AfuA + Ad AC vaccines at two-week interval via intramuscular route. Mice were challenged with *B. pertussis* 18-323 two weeks after the second dose (or four weeks after the first dose). Lungs were harvested one week later for CFU enumeration. Results show that mice immunised with two doses of Ad AfuA vaccine or rAfuA protein had the same level of bacterial load in their lungs than non-immunised mice, seven days after the challenge. Mice that were immunised with two doses of the mixed vaccine (Ad AfuA + Ad AC) had significantly lower number of bacteria in their lungs than non-immunised mice (Log 4.5 CFU for Ad AfuA + Ad AC-immunised mice vs Log 7 CFU for non-immunised mice). Mice immunised with one dose of wP vaccine (positive control group) were fully protected against pertussis lung infection, except two mice that were partially protective (Figure 4.16).

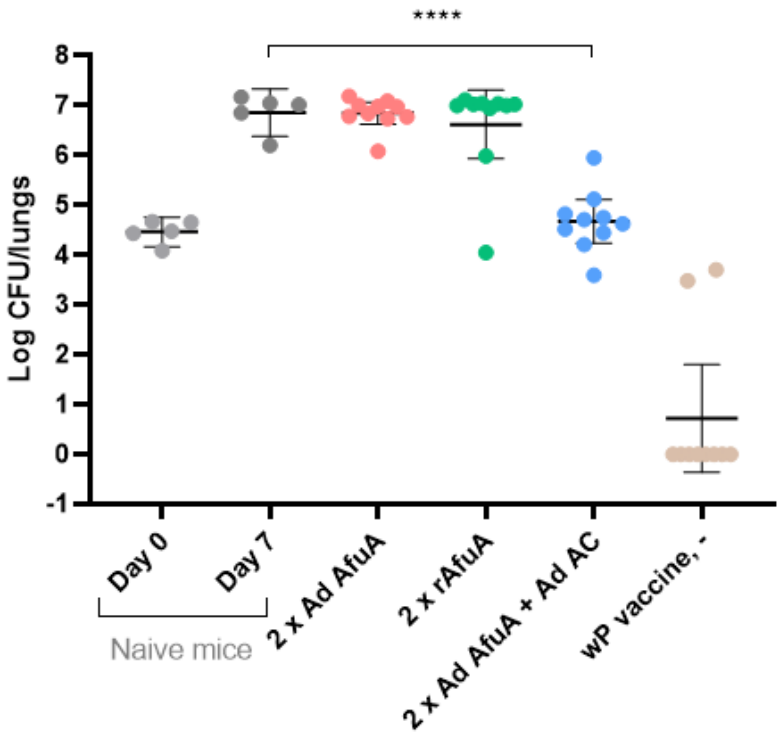


Figure 4.16. Assessing the protective potential of Ad AfuA vaccine alone and as a combination with partially protective Ad AC vaccine. Data show lung CFU from naïve and immunised mice one week after aerosol challenge with *B. pertussis* 18-323. wP-immunised mice served as positive control. Results for each group are shown as individual counts and geometric mean ± 95% confidence interval (n = 10 mice).

4.7 Discussion

Different pertussis vaccines are being developed due to ineffective protective immunity induced by aP vaccines and significant reactogenicity issues associated with wP vaccines. The exploration of adenoviral vector for pertussis immunisation has not been reported before. Therefore, replication-deficient adenoviral vector was selected for development of such novel pertussis vaccines in this study. Here, we evaluated a single dose and two-dose immunisation strategies with the Ad IRP1-3 and Ad AfuA vaccine, exploring if two-dose immunisation can be more effective than the single dose of Ad IRP1-3 or Ad AfuA vaccine.

To assess the immunogenic potential of the adenoviral vectored vaccines expressing the iron-binding proteins, it was necessary to produce rIRP1-3 and rAfuA proteins. The recombinant proteins served as coating antigens in ELISAs and direct protein vaccine comparators against the adenoviral vectored vaccines in immunogenicity and efficacy studies. They were also used to induce antigen-specific polyclonal antibodies in mice from which sera were obtained and used for establishment of QC samples and the reference in ELISA optimisation steps. The rIRP1-3 protein was successfully expressed in *E. coli*, extracted and purified as a soluble protein , whereas AfuA protein expression and purification as soluble protein was more difficult. Despite using different conditions and optimisation methods for AfuA expression in *E. coli*, the protein was expressed as an insoluble protein which had to be denatured and refolded during purification. The formation of insoluble proteins often occurs when the rate of a protein expression is higher than the ability of the host cell to control protein post-translational modification and folding (163), which could be the potential reason why the rAfuA was not expressed as a soluble protein. Further purification of AfuA protein resulted in

folding into two different protein species which did not make this protein ideal for mouse immunisations. The under-folded protein conformation or partially folded protein species are missing a three-dimensional structure (164) which may affect the exposure of essential protective epitopes and diminish synthesis of the protective antibodies. *B. pertussis* AfuA antigen is an outer membrane protein, therefore a yeast expression system may have been more effective than bacterial system for expression of proteins that form insoluble inclusions in bacteria and for the synthesis of membrane-bound proteins (165).

Although, clinical trials with a longer interval (12 weeks) between the first and the second dose of ChAdOX1-nCoV mediated higher antibody response in humans (111), we selected two-week interval between first and second dose of Ad IRP1-3 vaccine. Recent pre-clinical trials, evaluating the development of the COVID-19 (ChAdOx1 nCoV-19) vaccine using a replication-deficient adenoviral vector derived from chimpanzees, showed that one dose of the vaccine induced antibodies against the vaccine antigen and these responses were significantly enhanced after a second dose in mice and to a greater extent in pigs (166). In this study, intramuscular immunisation with one dose of the Ad IRP1-3 vaccine induced higher IRP1-3-specific IgG antibodies in mice than 2.5 µg, 5 µg and 10 µg of rIRP1-3 protein, as early as two weeks after the immunisation (Figure 4.13A). The early immunogenic response was also observed in a study with rhesus macaques for another vaccine, which showed that a single dose of ChAdOx1 nCoV-19 vaccine elicited Spike-specific IgG antibodies as early as 14 days after vaccination (167). In addition, ChAdOx1 nCoV-19 vaccine elicited significantly increased Spike-specific IgG antibody responses after the second immunisation (167). In the current study, homologous prime-boost immunisation with

Ad IRP1-3 induced significantly higher IRP1-3-specific antibody response compared with single immunisation four weeks after the boost (Figure 4.13C). Similar findings were observed in human clinical trials which showed that one dose of the ChAdOx1 nCoV-19 vaccine induced Spike-specific IgG antibodies four weeks after immunisation in adults (aged over 55 years) and the antibodies decreased eight weeks after the immunisation. Homologous prime-boost with ChAdOx1 nCoV-19 vaccine (four-week interval) elicited similar level of IgG antibodies in the immunised individuals and these responses were significantly higher on day 56 than those induced by single immunisation (168). These data suggest that two-dose immunisation with the same vectored vaccine can be highly immunogenic despite a potential induction of anti-vector immunity that can inhibit the boosting effect of the second dose (169). Pre-clinical studies, which assessed homologous prime-boost immunisation with Ad vaccine expressing a *Mycobacterium tuberculosis* antigen, in non-human primates, showed that the homologous Ad-Ad prime-boost strategy was not as effective as heterologous Ad-MVA regimen (170). However, another pre-clinical study in mice demonstrated that homologous Ad-Ad prime-boost regimen induced strong antibody responses against a *M. tuberculosis* antigen and the humoral responses were similar to those induced by Ad-MVA heterologous prime-boost regimen (155). Different immune responses mediated by viral vectored vaccines across different studies depend on the selection and use of various serotypes of adenoviral vectors, antigen specificity and expression, the persistence of antigen, animal models, vaccine dose, route of administration and interval between multiple vaccine administrations (155, 171).

Here, we demonstrated that single and two-dose intramuscular immunisations with Ad IRP1-3 was immunogenic, stimulating systemic IgG antibodies against IRP1-3

antigen. However, the aerosol challenge study revealed that the vaccine and 5 µg of rIRP1-3 did not protect against virulent *B. pertussis* in immunised mice (Figure 4.14). This absence of protection contrasts with published results in which the intraperitoneal administration of 4 µg of rIRP1-3 adsorbed to Freund's adjuvant led to partial reduction of bacteria in lungs of immunised mice when compared to naïve mice (105). We also demonstrated that adenoviral vector encoding AfuA antigen induce systemic IgG antibodies in mice after a single dose but failed to protect mice from aerosol challenge with *B. pertussis* 18-323 strain. We observed partial protection with Ad vaccine encoding AC domain of ACT antigen, in other challenge study (chapter 6). Therefore, we explored if a combination of Ad AfuA and Ad AC vaccines provides better protection together as we should be able to see if there was synergism with this combination. We also included the rAfuA protein in the immunisations (adsorbed to aluminum phosphate adjuvant) to confirm that the protein is immunogenic on its own as a potential vaccine antigen in our study, comparing to the literature. Our data show partial protection against *B. pertussis* in mice immunised with the combined Ad AfuA + Ad AC vaccines. Since two-dose immunisation with Ad AfuA vaccine was not protective, the partial protection could be associated with the Ad AC vaccine. In our other challenge study, a single-dose immunisation with Ad AC vaccine mounted a similar level of partial protection in mice, indicating that this vaccine contributed to the partial protection observed in this study.

Below are described differences between our study and that of Alvarez-Hayes *et al.* that could account for different protective responses observed in our study:

- The previous study immunised mice with rIRP1-3 or rAfuA adsorbed to Freund's adjuvant via intraperitoneal route and the immunised mice were challenged intranasally with the iron restricted virulent BP536 strain that is streptomycin-resistant strain derived from the Tohama 1 strain (172), at a dose of 1.5 x 10^6 bacteria/ml

- In this study, mice were immunised via intramuscular route with the adenoviral vectored vaccines, rIRP1-3 and rAfuA proteins adsorbed to aluminium hydroxide or aluminium phosphate adjuvants, respectively. The mice were then challenged via aerosol route with *B. pertussis* 18-323 strain, at a dose of 1 x 10^8 bacteria/ml which is higher dose than the one used in previous study. *B. pertussis* 18-323 challenge strain was not prepared in iron restricted conditions as it was done in the original study. In humans, *B. pertussis* uses mechanisms such as high affinity iron uptake systems via specific bacterial cell surface receptors to obtain iron from the host cells and survive (105).

- The use of a different strain under iron-restricted conditions as well as the lower challenge dose could have contributed to protective responses seen in the previous study. We hypothesised that growing bacteria under the iron-restricted conditions could induce expression of the iron-binding proteins that are available at the time of challenge. Due to not culturing *B. pertussis* 18-323 strain in iron-restricted conditions, the expression of iron-binding proteins could have been reduced and this could have enabled the bacteria initially establish the infection in mouse lungs.

- In addition, the type and role of vaccine adjuvant has an effect on the stimulation of humoral antibody response (173). Aluminium hydroxide is often used as adjuvant in human vaccine formulations. One of the reasons why the adjuvant was selected in our studies is that we wanted to mimic immunisation strategy for human use. Moreover, the use of Freund's vaccine adjuvant, in other animal experiments, led to adverse reactions and therefore due to concerns for animal welfare and safety, the use of the Freund's adjuvant was not selected. Although, aluminum-based adjuvants are more effective at stimulating antibody response when used in booster immunisations rather than primary immunisation (173), our data showed that the rIRP1-3 and rAfuA were immunogenic as early as two weeks after the first dose

- Previous study showed that 4 µg of rIRP1-3 and 2 ug of rAfuA on its own, and rAfuA protein combined with 1/5th human dose of 3aP vaccine led to partial protection in mice. In addition, the protective response was greater in mice immunised with the aP vaccine combined with both recombinant antigens, IRP1-3 and AfuA, suggesting that these antigens have potential to reduce pertussis infection in mice (106). A possible explanation why Ad IRP1-3 and Ad AfuA vaccines did not elicit protective responses is that the proteins, when expressed in mammalian cells, may not exhibit their native conformational structures that would generate protective antibodies. Post-translation modifications also play an important role in the formation of mature protein and these modifications could affect the generation of AfuA and IRP1-3 proteins in mice following immunisation with adenoviral vector vaccine.

4.8 Conclusion

The results presented here show that both Ad IRP1-3 and Ad AfuA are immunogenic in mice, and despite the potential anti-vector immunity, homologous prime-boost with Ad IRP1-3 stimulated significantly higher antibody responses than a single dose immunisation. However, these vaccines did not induce protection against *B. pertussis* due to the potential reasons described above.

Both IRP1-3 and AfuA proteins are expressed on the bacterial surface, which makes these proteins attractive candidates for development of new-generation of pertussis vaccines. The surface-associated proteins are able to stimulate opsonic antibodies enhancing bacterial phagocytic clearance (3). In addition, both Th1 and Th2 cytokines assist in the antibody productions but Th1-type immunity also enables clearance of intercellular *B. pertussis* in the lungs (77). For that reason, further studies will be necessary to find out if the vaccines have potential to induce such functional antibodies as well as cell-mediated immune responses.

The immunisation regimen in this study served as a proof-of-concept, and our goal was to assess systemic immune responses. However, due to the vaccines' immunogenic potential, exploring different vaccine doses, interval between two doses and/or routes of vaccine administrations could be the next step to find out if the Ad IRP1-3 and Ad AfuA vaccines could have an impact on the formation of conformational epitopes, induction of protective antibodies and vaccines' efficacy. Chasaide and Mills (2021) strongly suggest that a role of local mucosal immunity is essential for protection against nasal colonisation with *B. pertussis.* In particular, stimulation of IL-17 and tissue resident memory cell responses in the respiratory tract may provide better

protection than systemic immune response (174). It would be interesting to assess if the adenoviral vectored vaccines, delivered via mucosal or intranasal route, are suitable for the induction of such responses against the IRP1-3 and AfuA proteins in the local respiratory environment and if those responses lead to protection.

Chapter 5 Preclinical development of adenoviral based vaccine candidates expressing fimbriae antigens

5.1 Introduction

B. pertussis expresses long filamentous surface-associated structures known as fimbriae (Fim). Fimbriae are crucial factors for the virulence of *B. pertussis* by mediating adhesion to host epithelial cells during pertussis infection (175). There are two major serologically distinct fimbriae subunits, Fim2 or Fim3 (22.5 and 22.0 kDa, respectively), and the minor subunit FimD that is likely located at the fimbrial tip (73, 175). *B. pertussis* can express either Fim2 or Fim3 only, or both Fim2 and Fim3 at the same time.

A variety of animal models, including mice and baboons, have been used to demonstrate the role of fimbriae in the bacterial colonisation. *B. pertussis* mutants deficient in fimbriae were strongly compromised in their ability to adhere to the mouse and baboon respiratory tract (1, 73, 78, 176). It was also shown that Fim-specific antibodies, obtained from individuals prime-boosted with wP and a 5aP vaccine, decrease attachment of *B. pertussis* to respiratory epithelial cells, suggesting their role in the inhibition of bacterial adherence (75).

A recent study, using a mouse intranasal challenge model, demonstrated that adding up to 5 µg of Fim2/3 per dose to aP vaccine increased the vaccine efficacy suggesting that the fimbriae contribute to protection against pertussis infection (78). In addition, PRN-deficient *B. pertussis* isolates have been detected in France, Australia and U.S., and less commonly, other antigens such as FHA and PT, have been reported to be deficient on some *B. pertussis* isolates (177, 178). Therefore, strong and long-lasting

vaccine-induced immunity against the other surface exposed vaccine antigens, such as fimbriae is increasingly important (177). Although, *B. pertussis* isolates that lack expression of Fim2 and Fim3 have been observed in Norway, Japan and Canada (178).

Developing vaccines that would effectively prevent bacterial attachment is important in the prevention of asymptomatic transmission by reducing the nasal carriage of *B. pertussis*. Therefore, adenoviral vectored vaccines expressing Fim2, Fim3 and FimD were successfully developed at the Oxford Vaccine Group. The aim was to evaluate whether the fimbriae expressed through this platform would be better than purified fimbriae included in aP vaccine and whether the vaccines could inhibit nasal colonisation as well as lung infection.

Following the production of the Ad Fim2, Ad Fim3 and Ad FimD vaccines, the next step was to assess if the vaccines are able to induce Fim-specific antibody responses in mice. A study, conducted at the Oxford Vaccine Group and Public Health England, assessed such responses using a surface-labelling assay (SLA) which analysed Fim-specific antibodies binding to different *B. pertussis* strains (179). SLA has an advantage over ELISA as the SLA detects antigen-specific antibodies which bind to epitopes expressed on the surface of bacteria in their natural conformation. The SLA study showed that Ad Fim3 was the most immunogenic out of the three constructs, as one dose of Ad Fim3 vaccine induced Fim3-specific IgG antibodies that bound to *B. pertussis* B1917 strain, which only expresses Fim3, with the highest median fluorescence intensity (MFI). In addition, the Fim3-specific IgG antibody responses

induced by Ad Fim3 immunisation were comparable to those responses induced by wP and aP vaccines. Immunisation of mice with Ad Fim2 induced the lowest MFI on *B. pertussis* UK22 strain expressing Fim2 and FimD, and Ad FimD vaccine also induced low MFI on *B. pertussis* B1917 expressing Fim3 and FimD (179).

Due to the strong antigen binding antibodies induced by Ad Fim3, I proceeded to explore if a single dose of Ad Fim3 vaccine can mediate a protective response against *B. pertussis* challenge in mice. Since the immunogenicity study analysed Fim3-specific IgG responses against the B1917 strain, which is a clinical isolate of *B. pertussis* expressing Fim3 (isolated from a patient in Netherland in 2000) and not Fim2, an aerosol challenge study was designed using this strain (127, 180). The efficacy of Ad Fim3 vaccine was also assessed against 18-323 strain (isolated in the USA in 1947) expressing both Fim2 and Fim3 antigens (181). I aimed to evaluate if Ad Fim3 has different efficacy against the two different strains as those strains might express different level of Fim3 antigen and therefore affect the efficacy.

To study the impact of the Ad Fim3 vaccine on prevention of the bacterial attachment and infection, a mouse nasal colonisation and lung infection model was designed. Initially, the colonisation model focused on evaluating an optimal aerosol challenge dose with B1917 strain which is an important step to assess the vaccine's protective capacity. If the challenge is too severe, the vaccine's protection may be masked or if the challenge is too mild, it can overestimate the vaccine's potential. Therefore, based on a published challenge study and our previous aerosol challenge studies with 18-323 strain, it was decided to use three different challenge doses in our pilot aerosol

challenge study with the B1917 strain (182). It was vital to assess if the B1917 strain is able to colonise mouse nasal cavities as well as infect mouse lungs after the aerosol challenge. It is also important to note that the establishment of nasal colonisation with B1917 strain was analysed through mouse aerosol challenge rather than intranasal challenge as the former route mimics the route of natural infection, since humans contract pertussis via aerosolised droplets disseminated through coughing and sneezing by infected individuals.

5.2 Materials and Methods

5.2.1 Cloning of adenoviral vectored vaccines expressing FimD/2/3

The cloning process of the viral vectored based vaccines is described in detail in Chapter 7 (General Materials and Methods, section 7.1, subsection 7.1.1 - 7.1.2).

5.2.2 Establishing challenge dose for *B. pertussis* B1917 strain using a calibration curve

A standard curve was generated to establish challenge dose for *B. pertussis* B1917. Bacteria were resuspended in 1% casein in saline and bacterial suspensions were measured at absorbance of 625 nm using a spectrophotometer (Cecil CE1011). The spectrophotometer was calibrated with 3 ml of 1% casein in saline before measuring six absorbance readings, in a range from 0.05 – 1.5. Each suspension was further diluted by three 100-folds and two 10-fold dilutions in sterile 1% casein in saline to final dilutions of 10^{-2}, 10^{-4}, 10^{-6}, 10^{-7} and 10^{-8}. Four 20 µl droplets from each dilution were plated on charcoal agar plate containing 10% (v/v) defibrinated horse blood and incubated at 37ºC for five to seven days. Average number of colonies was determined on each plate and multiplied by five to obtain colonies per 0.1 ml. The average number

of colonies/0.1 ml was then multiplied by 10 to obtain number of colonies/ml for each OD reading, while taking into consideration the dilution factor. A calibration curve was prepared by plotting CFU/ml against corresponding readings from 0.05 – 1.5, measured at A_{625}.

5.2.3 Optimisation of the lung infection and nasal colonisation model with B1917 strain

Naïve mice were randomly allocated to three groups (*n* = 12/group) varying by challenge doses (Table 5.1). The three groups were exposed to aerosol with the following concentrations: 1 x 10^7, 1 x 10^8 and 1 x 10^9 CFU/ml. Mice were exposed to aerosolised droplets inside the aerosol apparatus for five minutes. Four mice per each group were sacrificed three hours (day zero), three days, and seven days after the challenge to remove lungs and noses. The lungs and noses were homogenised in 1 ml and 0.2 ml of 1% casein in saline, respectively, using a mini-beadbeater (Biospec Products, Doylestown, PA, USA) with 2.5 – 3.5 mm glass balls (BDH, Poole, UK) for 10 seconds at 4,600 rpm at room temperature. The homogenates were then serially diluted (10-fold) and plated out on charcoal agar plates supplemented with 10% (v/v) horse blood and incubated for seven days at 37°C. Viable bacterial colonies were then counted by the Synbiosis™ ProtoCOL 3 Automatic Colony Counting System (Version 1.3.6.0). Viable counts per lungs and noses from four mice exposed to the specific challenge dose, at specific timepoint, were performed in four replicates and the geometric mean of the replicates was taken as colony forming units (CFU) per mouse lung or nose.

Table 5.1. Experiment design for testing different concentrations of bacterial inoculums for the aerosol challenge with *B. pertussis* B1917.

Group (n = 12)	Challenge dose of *B. pertussis* B1917 strain	Sampling of lungs and noses on specific day (4 mice/group/day)
1	**10^7** CFU/ml	Days 0, 3 and 7
2	**10^8** CFU/ml	Days 0,3 and 7
3	**10^9** CFU/ml	Days 0,3 and 7

5.2.4. Aerosol challenge of Ad Fim3-immunised mice with *B. pertussis* 18-323 and B1917 strains

Mice were randomly allocated to eight different groups (*n* = 10/group) and each mouse was immunised via intramuscular route as shown in Table 5.2. Vaccine dilutions were performed using DPBS. Six weeks following immunisation, four groups of mice were subjected to aerosol challenge with *B. pertussis* 18-323 strain, at challenge dose of 1 x 10^8 bacteria/ml. The other four groups of mice were subjected to aerosol challenge with *B. pertussis* B1917 strain, at challenge dose of 1 x 10^9 bacteria/ml. Lungs and noses were removed and processed seven days after the challenge (day 49). More details of the standard procedure for aerosol challenge are described in the Chapter 7 (General Materials & Methods, section 7.2, subsections 7.2.1 – 7.2.3).

Table 5.2. Immunisation of mice with Ad Fim3 and pertussis vaccines prior challenge studies with *B. pertussis* 18-323 and B1917 strains.

Group	Vaccine	Dose/mouse	One dose (DAY 0)	Challenge (DAY 42)
1	Ad Fim3	1 x 10^9 IU/50 µl	✔	*B. pertussis* 18-323
2	Commercial 5aP vaccine containing PT, PRN, FHA, Fim2/3	1/10th human dose	✔	*B. pertussis* 18-323
3	wP vaccine (NIBSC code 94/532)	1/10th human dose	✔	*B. pertussis* 18-323
4	Naïve (Non-immunised)	-	-	*B. pertussis* 18-323
5	Ad Fim3	1 x 10^9 IU/50 µl	✔	*B. pertussis* B1917
6	Commercial 5aP vaccine containing PT, PRN, FHA, Fim2/3	1/10th human dose	✔	*B. pertussis* B1917
7	wP vaccine (NIBSC code 94/532)	1/10th human dose	✔	*B. pertussis* B1917
8	Naïve (Non-immunised)	-	-	*B. pertussis* B1917

5.2.5 Serotyping of *B. pertussis* by indirect whole-cell ELISA using monoclonal antibodies to Fim2 and Fim3 antigens

In the protection study, Ad Fim3 vaccine was partially protective against the B1917 strain, but the vaccine was not protective against the 18-323 strain. For that reason, we assessed the expression of Fim3 antigen on the two strains. The aim was to find out if the differences in Fim3 expression could have contributed to the different protective responses in immunised mice. The following bacterial strains were analysed by indirect whole-cell ELISA: 18-323 and Welcome 28 (W28) strains expressing both Fim2 and Fim3, B1917 and 134 strains expressing Fim3, GL353 strain lacking fimbriae and 36E strain expressing Fim2. A smooth suspension of each strain grown for 48 hours on charcoal agar plates containing 10% (v/v) defibrinated horse blood was prepared using sterile PBS (pH 7.4) and was heat inactivated at 56°C for one hour. The inactivated bacterial suspension was cooled and stored at 4°C until ready for testing. The inactivated *B. pertussis* bacteria were diluted in sterile PBS to give an OD of 0.1 at 620 nm and 100 µl/well of each bacterial suspension was added to Nunc Maxisorp 96-well flat-bottomed Immuno microtiter plates (Nalge Nunc International, Rochester, NY) and incubated overnight at 37°C. The plates with coated bacteria were washed three times with 0.9% saline containing 0.05% (v/v) Tween 20 (saline-Tween). The plates were then blocked with 2% BSA/PBS (300 µl/well) and incubated for 37°C for one hour and half. The plates were washed again three times with saline-Tween. Anti-Fim2 (NIBSC code 06/124) and anti-Fim3 (NIBSC code 06/128) monoclonal antibodies, diluted to a range of 1/100, 1/500, 1/1000 and 1/4000, were added to the bacteria (100 µl/well) and the plates were incubated at 37°C for one hour and half. The plates were washed with saline-Tween three times and HRP conjugated goat anti-mouse IgG antibody was diluted to 1:5,000 and added to the plates (100 µl/well) to detect binding of the monoclonal antibodies. The plates were incubated at 37°C for half an hour, washed with saline-Tween three times and 2,2'-Azinobis[3-

ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) ABTS HRP substrate was added to the plates (100 µl/well). Following half an hour incubation at room temperature on a shaker, the plates were read at 405 nm. A Fim2-positive control strain (36E strain) and a Fim3-positive control strain (134 strain) were used in the assay.

5.3 Results

5.3.1 Estimation of optical densities corresponding to different *B. pertussis* B1917 concentrations

Adenoviral vectored vaccines expressing fimbriae were developed with the aim of reducing the nasal carriage and prevent the spread of the infection. To study the vaccines' potential to prevent the bacterial colonisation, a mouse model of *B. pertussis* nasal colonisation and lung infection was developed. The aim of the colonisation model was to allow understanding of the optimal bacterial challenge dose in mice that would be safe. First, we selected the following bacterial concentrations (1×10^7, 1×10^8 and 1×10^9 CFU/ml of *B. pertussis* B1917) to be assessed in the lung infection model. To prepare bacterial suspension with the specific concentrations, we then examined optical density corresponding to those different concentrations using a calibration curve. As shown in Figure 5.1, OD 0.340 corresponds to 1×10^9 CFU/ml.

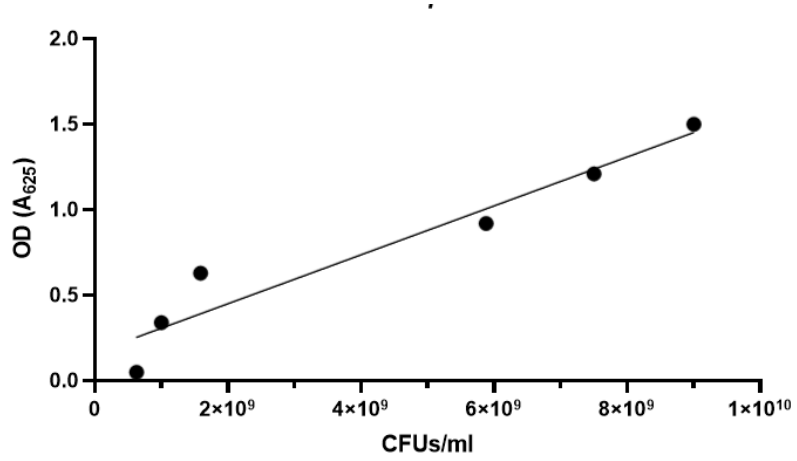


Figure 5.1. Calibration curve for *B. pertussis* B1917 strain. Different bacterial concentrations were measured with a spectrophotometer at absorbance of 625 nm.

5.3.2 Optimisation of the lung infection model with *B. pertussis* B1917

Following selection of the three challenge doses, naïve BALB/c mice were infected via the aerosol route with three doses of *B. pertussis* strain B1917. Lungs were obtained from each mouse to establish CFU counts three hours (day 0), three and seven days after the infection. Three hours after the challenge (day 0), all three doses infected mouse lungs as expected (Figure 5.2). The B1917 strain induced lung infection at log 4 CFU/lungs which is typically seen in the challenge studies with 18-323 strain on day 0. On day three, bacterial load remained the same for all three challenge doses as on day zero (log 4 CFU/lungs). Seven days after challenge, only the highest challenge dose (1 x 10^9 bacteria/ml) resulted in log ~5 CFU/lungs which is one log higher than lung CFU observed with the lower doses. Therefore, the optimal dose for lung infection, in this study, was established to be 1 x 10^9 CFU/ml, seven days following challenge, as we aimed to have higher CFU/lungs at day seven than at day zero (Figure 5.2). In addition, there was low variability in viable bacterial counts in the

mouse lungs and all mice survived the exposure to aerosol challenge of *B. pertussis* B1917.

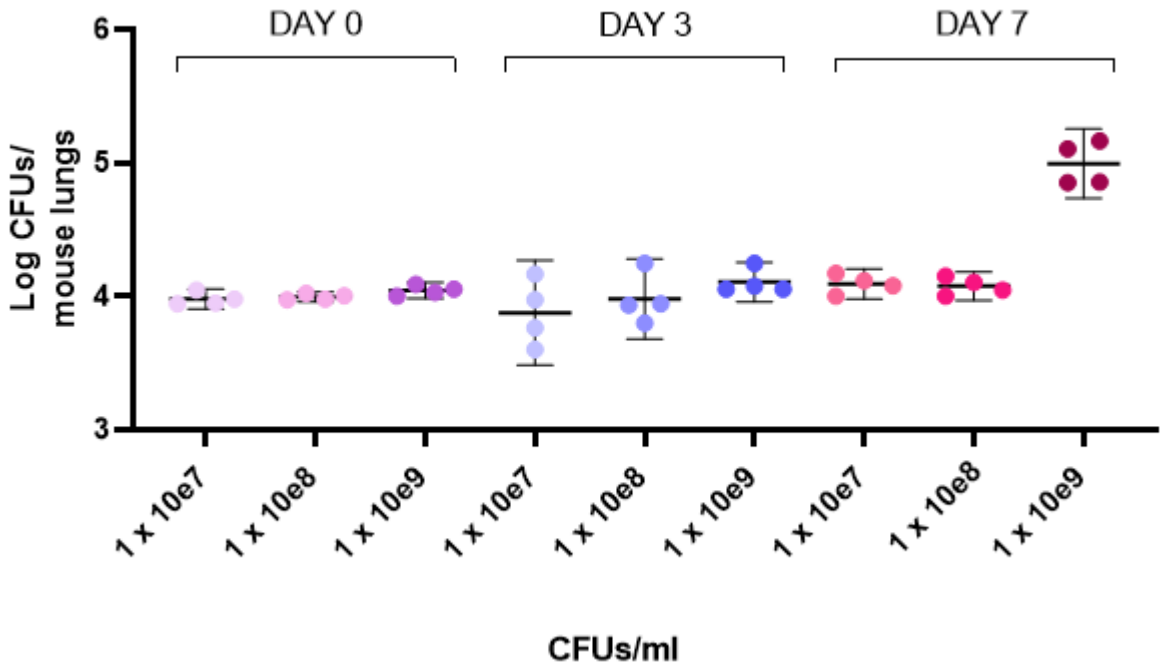


Figure 5.2. CFU counts in mouse lungs at day zero, three and seven days after aerosol challenge with B1917 strain. Naïve mice were infected via the aerosol route with *B. pertussis* B1917 strain at the following doses: 1 x 10^7, 1 x 10^8 and 1 x 10^9 CFU/ml. Bacterial load in mouse lungs was assessed three hours, and then three and seven days after the challenge with respective bacterial doses. Each dot represents lungs sampled from one mouse. Horizontal lines represent geometric mean ± 95% confidence interval.

5.3.3 Optimisation of the nasal colonisation model with *B. pertussis* B1917

Noses were removed along with lungs from each mouse as described above. Attempts were made to determine the pertussis bacterial load in homogenised noses however it was not possible to do so due to high contamination of mouse nasal cavities with various other microorganisms that colonise mice (Figure 5.3). Three days after the challenge with different bacterial challenge doses, the level of contamination was much lower, especially with challenge dose of 1×10^7 and 1×10^9 CFU/ml (Figure 5.3). The bacterial load per 0.1 ml in a mouse nose after the challenge with different bacterial suspensions is depicted in Table 5.3. Data suggest that the contamination was most severe with all challenge doses on day zero. However, the level of contamination was reduced on day three and seven, and the lowest level of contamination in mouse noses was observed with the highest challenge dose (1×10^9 CFU/ml). It appears that the highest challenge dose and longer interval between the challenge and nose sampling seem to prevent the contamination or the growth of contaminants on the charcoal agar plates.

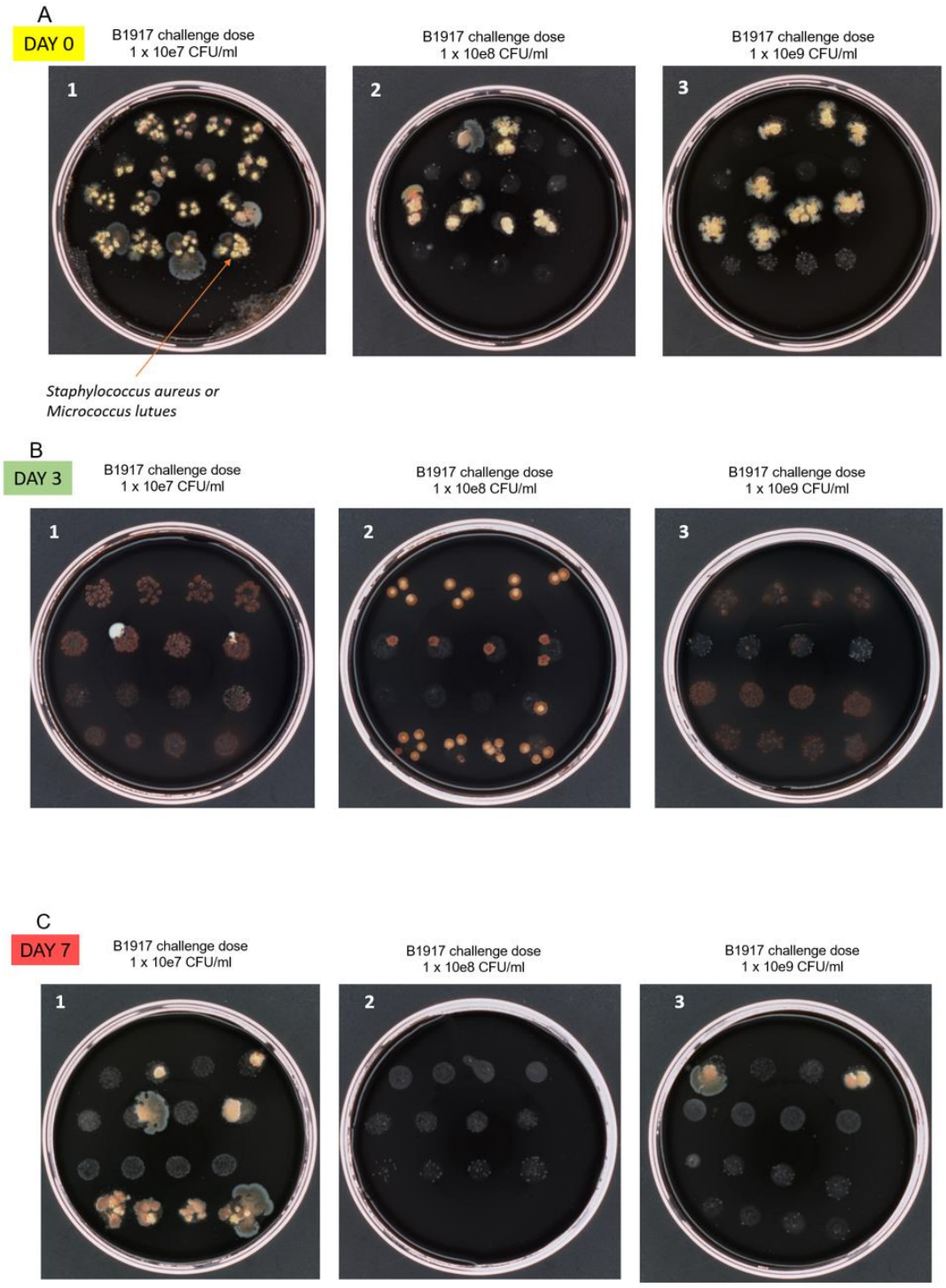


Figure 5.3. Pictures of *B. pertussis* colonies obtained from homogenised noses, from challenged mice, grown on charcoal agar plates. Naïve mice were challenged with 1 x 10^7, 1 x 10^8 and 1 x 10^9 CFU/ml of *B. pertussis* B1917 strain and noses were sampled from each mouse **A)** three hours, **B)** three days and **C)** seven days after the challenge. The noses were homogenised and four drops of 25 µl of homogenised tissue were dropped on the charcoal agar plate containing 10% (v/v) defibrinated horse blood per each mouse. One row of four drops represents bacterial colonies in homogenised noses per 0.1 ml from one mouse.

Table 5.3. CFU counts in mouse nose samples after challenge with three different doses of *B. pertussis* B1917 strain, three and seven days after sampling.

		Challenge dose		
		1×10^7 CFU/ml	1×10^8 CFU/ml	1×10^9 CFU/ml
	Mouse	**CFU/0.1 ml homogenised nose**	**CFU/0.1 ml homogenised nose**	**CFU/0.1 ml homogenised nose**
DAY 3	1	71	6	110
	2	49	34	185
	3	-	51	68
DAY 7	1	-	93	54
	2	166	67	61
	3	-	-	38

5.3.4 Assessment of Ad Fim3 efficacy in aerosol challenge studies with *B. pertussis* B1917 and 18-323 strains

To analyse the efficacy of Ad Fim3 vaccine against pertussis infection, two strains of *B. pertussis* were selected: B1917 (expressing Fim3 antigen) and 18-323 (expressing Fim2/3 antigens). A two-log reduction in CFU in the lungs of mice immunised with a single dose of the Ad Fim3 vaccine and challenged with B1917 strain was observed compared to naïve mice on day seven, ($p < 0.0001$, Figure 5.4A). This data thus suggest that a partial protection was elicited by immunisation with Ad Fim3 against the B1917 strain in mice. Mice immunised with a single dose of Ad Fim3 and challenged with 18-323 strain had the same level of bacterial load in their lungs as naïve mice on day seven which shows that the vaccine was not protective against 18-323 strain in mice (Figure 5.4B). Two-dose immunisation with a 5aP vaccine containing Fim3, and

one-dose immunisation with wP vaccine both induced partial protection (and in some mice even full protection) against both *B. pertussis* strains (compared with naïve mice, day seven, p <0.0001, Figures 5.4A and 5.4B).

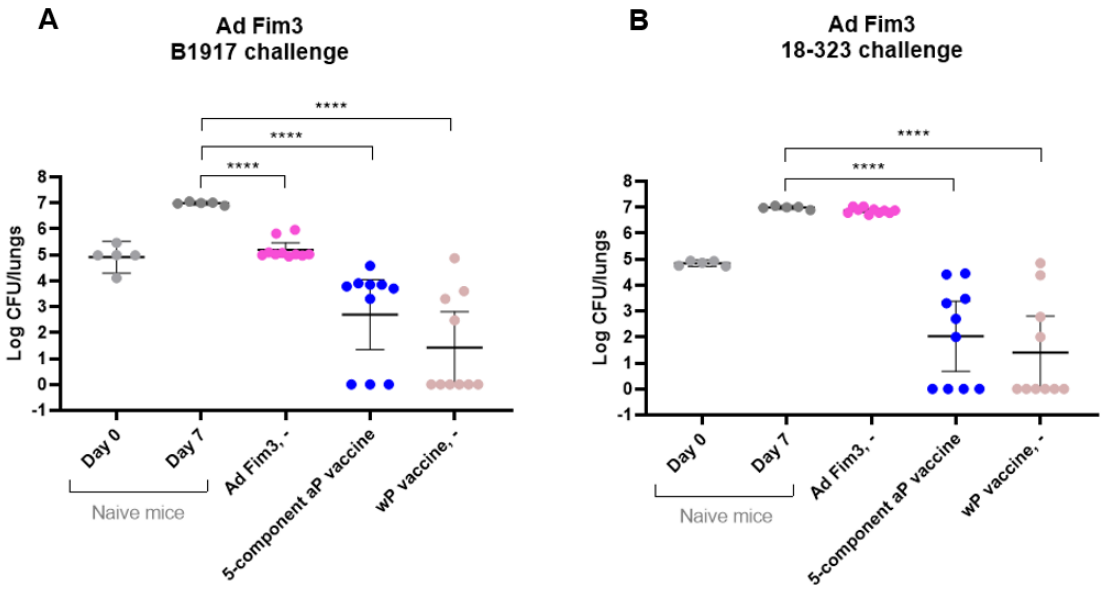


Figure 5.4. Assessment of protective capacity of Ad Fim3 vaccine against *B. pertussis* B1917 and 18-323 strains in mice. Mice (n = 10/group) were immunised intramuscularly once with Ad Fim3 vaccine, aP and wP vaccine. Non-immunised mice served as negative control. Six weeks after the vaccination, mice were challenged via aerosol route with either **A)** 1×10^9 bacteria/ml of B1917 strain, or **B)** 1×10^8 bacteria/ml of 18-323 strain. Lungs were removed one week after the challenge, homogenised and CFU counts were established five to seven days after plating the homogenised lungs on charcoal agar plates containing 10% (v/v) defibrinated horse blood. Data in each graph were analysed with a one-way ANOVA test followed by Dunn's multiple comparisons test to compare differences between vaccination groups and naïve mice on day seven (negative control group), ****; $p<0.0001$.

5.3.5 Fimbriae serotyping of *B. pertussis* showed reduced expression of Fim2 and Fim3 antigens on *B. pertussis* 18-323 strain

To evaluate and compare whether *B. pertussis* 18-323 and B1917 strains express different levels of Fim2 and Fim3, serotyping by indirect whole-cell ELISA was performed. We also wanted to find out if 18-323 strain expresses Fim3 antigen as Ad Fim3 vaccine was not protective against this strain. Fim3 antigen expression was detected on *B. pertussis* B1917 strain, while Fim2 and Fim3 antigens were not detected on 18-323 strain (Figure 5.5). Both Fim2 and Fim3 antigens were detected on W28 strain, which was used as a positive control. No expression of Fim2 and Fim3 was detected on GL353 strain, as expected, as this strain does not express these antigens (negative control, Figure 5.5).

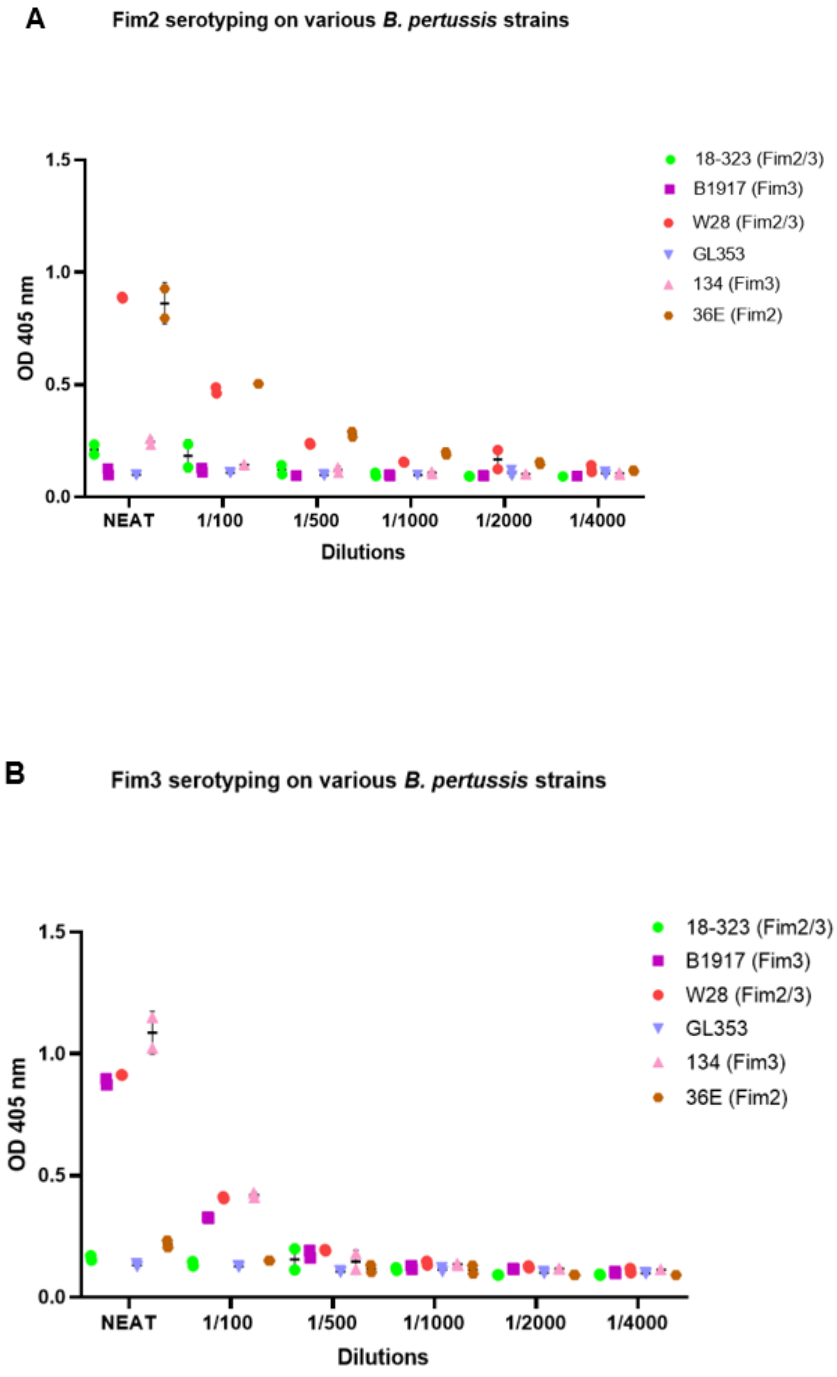


Figure 5.5. Comparison of Fim3 expression on *B. pertussis* B1917 and 18-323 strains. A) Fim2 serotyping and **B)** Fim3 serotyping on *B. pertussis* 18-323 and B1917 strains. The expected serotype of each strain is given in parenthesis. W28 strain served as a positive control, expressing both Fim2 and Fim3 antigens. GL353 strain served as negative control as this strain expresses Fim1 antigen only. 134 strain expresses Fim3 antigen alone and 36E strain expresses Fim2 antigen alone.

5.4 Discussion

Fimbriae have been selected as candidate antigens for developing new and effective viral vectored pertussis vaccines due to their role in facilitating attachment to the host cells. The aim was to use viral vectors as vaccine delivery platform that may be useful for the delivery of the fimbriae vaccine antigens to the host cells, expression and presentation of the antigens and stimulation of T-cell responses as well as enhancement of fimbriae-specific antibody responses.

A previous study showed that only Ad Fim3 vaccine was able to induce Fim3-specific antibodies against B1917 strain, and antibody responses were similar to those induced by the wP vaccine and the 5aP vaccine (data not shown). However, Ad Fim2 and Ad FimD vaccines failed to induce Fim2- and FimD-specific antibodies binding to the strains expressing those antigens. The reason why Ad Fim2 and Ad FimD vaccines failed to elicit antigen-specific antibody responses may be associated with a misfolding of protein following gene expression or lack of protein post-translational modifications. The post-translational modifications are necessary for the assembly of the multimeric fimbriae in mice, as they would normally occur in bacteria (183) and therefore protective epitopes may not have formed. As the Ad Fim 3 vector was immunogenic and partially protective, we could rule out this hypothesis. There is no evidence that multimers were formed from the adenoviral vector expressing Fim3 protein, but linear T-cell epitopes are predicted for the expressed polypeptide which may have augmented the response to any conformational or linear epitopes present on the single subunit (184). We hypothesised that these were of sufficient quality to partially block the adhesion of fully-formed fimbriae to mouse lung cells There is also the possibility that the vectors may not have transduced the host cells, or the antigen may have not

been expressed at all. These issues could be investigated by Luciferase Transduction Assay to profile the transduction efficiency of adenoviral vector in mammalian cells *in vitro* (185). To measure gene expression, there are several options. We could assess RNA levels by RNA sequencing, Quantitative reverse transcription PCR (RT-qPCR), Reverse transcription PCR (RT-PCR), Northern blotting, DNA microarrays, or *in situ* hybridization. To check gene expression at the protein level, we could perform either mass spectrometry, Western blotting or ELISA.

After demonstrating that a single dose of Ad Fim3 vaccine induces Fim3-specific antibody responses in mice, we wanted to assess if the vaccine can impede nasal colonisation and lung infection in mice. In the present study, we selected three challenge doses of B1917 strain to find out the optimal dose for mouse aerosol challenge. Data obtained from the optimisation study showed that a challenge dose of 1×10^9 CFU/ml of B1917 strain induced approximately one log higher bacterial load in mouse lungs, seven days after the infection, in comparison to the lower challenge doses (1×10^7 and 1×10^8 CFU/ml on day 0) (Figure 5.2). Three days after the infection of mice, none of the three challenge doses induced higher bacterial load in the lungs in comparison to day 0 (Figure 5.2). This suggests that the B1917 strain takes at least seven days to establish the lung infection, at dose of 1×10^9 CFU/ml. In addition, there was 100% mouse survival and low variability of lung CFU among infected mice. Although dose of the inoculum prepared from the B1917 strain was higher (1×10^9 CFU/ml) than dose of the inoculum prepared from the 18-323 strain (1×10^8 CFU/ml), the number of viable bacteria in the lungs of naïve mice challenged with the B1917 strain was lower (log 5 CFU/lungs) than in mice challenged with 18-323 strain (log 7 CFU/lungs) on day seven (Figure 5.2). Based on these observations, we hypothesised that the two different *B. pertussis* strains and possibly their antigenic composition may

influence the ability to induce infection and therefore the number of bacterial colonies in the lungs of infected mice. It has been suggested that due to the nature of *B. pertussis* 18-323, this strain is an outlier in *B. pertussis* taxonomy, and it is not certain how serial passage and variations of *B. pertussis* 18-323 strain compares to the current isolates (186). In addition, the 18-323 strain has long been used as a lab strain and may have become better adapted to murine lungs over time.

Establishment of the bacterial nasal colonisation was more complicated as there were high levels of contamination with other bacteria and/or fungi masking the *B. pertussis* colonies, especially on day 0. The CFU/0.1 ml in mouse nasal cavities could be detected for each challenge dose in only two out of four mice on day three and one out of four mice on day seven (Figure 5.3). However, exploration of nasal colonisation by *B. pertussis* did not give promising results due to the methodological procedure using nose samples obtained from non-sterile environment. It was not possible to compare the CFU with day 0 due to the high level of contamination. Therefore, it was decided not to proceed with the nasal colonisation study. The contamination was more likely with murine nasal microbiota such as *Staphylococcus aureus* (187), although there are other dominant families in the nasal tissue of mice such as *Streptococcaceae* and *Enterococcaceae* microbiota (187). The orange-yellow colonies with rough, uneven margins are typical of *Staphylococcus aureus* or *Micrococcus luteus* (188), however, the contaminants could also have fungal origin. As mouse nasal cavity is not a sterile environment the use of antibiotics, such as penicillin, in media to prevent the growth of Gram-positive organisms, or amphotericin B to prevent fungal growth, could have been solutions to prevent growth of the most common microorganisms found in murine nasal microbiota.

The 1 x 10^9 CFU/ml challenge dose of B1917 strain was then used in the aerosol challenge study in addition to the conventional challenge strain, 18-323. The aim was to analyse the ability of Ad Fim3 to induce protective response in mice against the clinical isolate, B1917, and strain 18-323 and to compare if there is any difference in protection using these two different strains. Data obtained from the protection study show that a single dose of Ad Fim3 vaccine was partially protective against B1917, however the vaccine was not effective against 18-323 (Figure 5.4). The fimbriae serotyping assay showed that 18-323 strain does not express Fim2 and Fim3 antigens (Figure 5.5). Strain 18-323 is an old strain, which has been passaged many times and has also been considered as an outlier in *B. pertussis* taxonomy (186). High serial passage of 18-323 strain could have caused genotypic and phenotypic changes of the bacteria and therefore influence the expression of Fim2 and Fim3 antigens. The strain probably lost its ability to synthesise fimbriae antigens and/or it can produce these antigens in different hosts and environments. That could be an explanation why Ad Fim3 was protective against B1917 but not against 18-323. Weiss and Goodwin (1989) showed that fimbrial expression is variable and *B. pertussis* has the ability to switch fimbrial expression on and off (189). In addition, *fim2*, *fim3*, and *fimX* genes are affected by transcriptional control, known as phase variation. The phase variation is a defence mechanism of *B. pertussis* which prevents the host's immune system from recognising and eliminating the pathogen (190). Therefore, the 18-323 strain could have undergone a fimbrial phase variation which means that the bacteria randomly switch between low and high expression of a specific fimbriae gene leading to the serotype switch (191). The expression of fimbriae is also regulated by small mutations such as insertions or deletions within a stretch of a cytosine-rich promoter region, and these mutations contribute to the fimbrial phase variation (59).

5.5 Conclusion

Different adenoviral vectored vaccines expressing either Fim2, Fim3 or FimD were developed and their immunogenicity was compared with commercial wP and aP vaccines. Only Ad Fim3 was immunogenic six weeks after a single-dose immunisation as observed by SLA. The Fim3-specific IgG responses induced by the vaccine were comparable to those responses mediated by wP vaccine (single dose) and aP vaccine (two doses). In addition, Ad Fim3 induced a significant reduction in bacterial lung colonisation as compared with naïve mice, suggesting the vaccine's partial protection against *B. pertussis* B1917 strain. Based on these data, we hypothesised that Ad Fim3 vaccine in combination with aP vaccine or with another viral vectored vaccine expressing a protective antigen may lead to enhanced or even complete protection against pertussis infection, and this might be elucidated in future work.

Chapter 6. Adenylate cyclase toxin as antigen candidate for novel viral vectored-based vaccines against pertussis.

6.1 Introduction

B. pertussis has many sophisticated virulence factors to evade host immunity at the early stage of infection. One such virulence factor is the immunosuppressive adenylate cyclase toxin (ACT). ACT is a 1,706 amino acid polypeptide. At its N-terminus, ACT consists of the adenylate cyclase (AC) enzyme domain that is linked to a pore-forming repeats in toxin (RTX) haemolysin moiety (Figure 6.1).

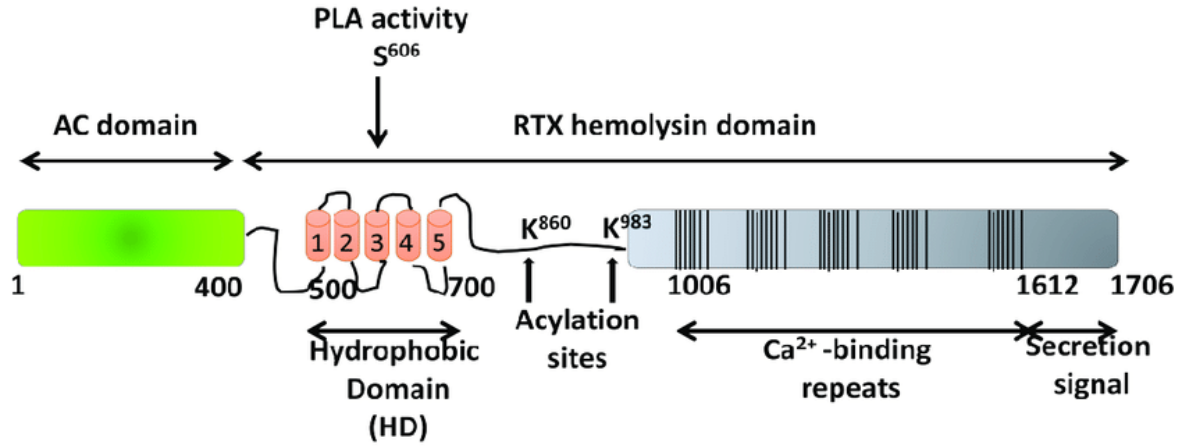


Figure 6.1. Schematic representation of the ACT. In green, the adenylate cyclase domain is made of 400 amino acid residues. Residues 500 – 1706 represent the RTX haemolysin domain which contains of the following: the hydrophobic sub-domain (HD), two acylation sites (K860 and K983) that are essential for activation by palmitoylation, and five blocks formed by low affinity Ca^{2+}-binding repeats. The section between 1638 –1706 amino acid residues represents the C-terminal secretion signal. Taken from Ostolaza *et al*. (2017) (192).

ACT functions through a haemolysin moiety that binds to the host CD11b subunit of the CD11b/CD18 integrin. This integrin acts as complement receptor on myeloid phagocytic cells. After the binding, the haemolysing moiety is embedded in the cell membrane which results in translocation of the AC domain into the cytosol. The AC domain then catalyses the conversion of cytosolic adenosine 5'-triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) (Figure 6.2) (193). The increased levels of cAMP leads to the disruption of the bactericidal functions of the innate phagocytic cells; inhibition of opsonophagocytic uptake of bacteria and oxidative burst of neutrophils which prevents destroying of bacteria by reactive oxygen species (194, 195). In addition, the toxin not only inhibits the innate immune responses but is also able to suppress recruitment and activation of T-cell adaptive immune responses (129).

ACT has several properties that make the toxin an interesting vaccine candidate. In the early stage of aP vaccine development, there was a general agreement that pertussis disease is mainly driven by the pertussis toxin. At that time, inclusion of ACT in aP vaccines was not considered as there was insufficient knowledge about its molecular characteristics, immunogenicity and stability (129). However, as research in the pertussis field progressed, ACT was found to have an important role in the pathogenesis of *B. pertussis*. Mutant strains of *B. pertussis* not expressing ACT were unable to infect mice, demonstrating ACT's role in pertussis infection (196). Other studies showed that immunisation with purified ACT protects mice against virulent *B. pertussis* (55). Wang and Maynard (2014) showed that the RTX domain of ACT contains two, non-overlapping neutralising epitopes and antibodies binding to those epitopes inhibited binding of ACT to macrophages and soluble $\alpha_M\beta_2$ integrin receptor,

indicating that the antibodies block ACT's interaction with the host cell receptor. The RTX domain also elicited similar level of ACT-specific neutralising antibodies in mice than the full-length ACT (130). Therefore, a vaccine-induced ACT-specific antibody responses may be able to neutralise the toxin and thus contribute to protection against the bacterial colonisation and establishment of the infection.

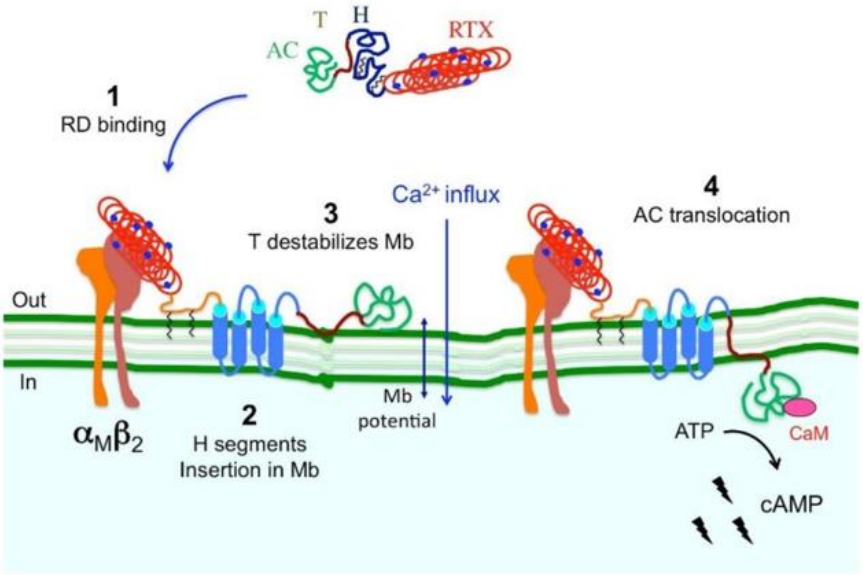


Figure 6.2. Schematic of ACT intoxication of host cells. (1) The ACT RTX domain binds to CD11b/CD18 receptor at the host cell surface, (2) Hydrophobic region of ACT gets inserted into the plasma membrane of cells, (3) Destabilisation of the plasma membrane by the translocation domain T (4) AC domain of ACT gets translocated across the plasma membrane of target cells. ACT interacts with calmodulin, via its low affinity Ca^{2+} repeating sequences, in the cytosol of eukaryotic host cells which results in stimulation of its catalytic activity to produce cAMP. H, hydrophobic domain; Mb, membrane, $\alpha_M\beta_2$, integrin receptor; CaM, calmodulin. Taken from Chenal and Ladant (2018) (196).

The preclinical team at the Oxford Vaccine Group designed and produced two adenoviral vectors expressing the AC domain and the RTX domain. The AC domain was genetically detoxified by adding Leu-Gln between amino acid 188 and 189, and the RTX domain construct includes amino acids 985 to 1680. The bacterial secretion signal at the C-terminal sequence (1680 to 1706 amino acid residues) was deleted, as it is not immunogenic (131).

In the present study, we evaluated the immunogenicity and protective capacity of the adenoviral vectors expressing the AC or the RTX domain in mice either as a single or two-dose immunisation, and we also compared the immunogenic properties of the adenoviral constructs with recombinant ACT (rACT) protein.

6.2 Materials and Methods

6.2.1 Immunisation of mice

Female BALB/c mice (six to eight weeks old) were divided into nine groups of five mice to assess and compare immunogenicity of the Ad AC, Ad RTX vaccines, and rACT protein which was provided by Prof. Peter Sebo (Laboratory of Molecular Biology of Bacterial Pathogens, Czech Republic). Group nine, consisting of eight mice, was used to raise polyclonal antibodies against rACT antigen and the mice received three doses of 10 µg/50 µl of rACT (Table 6.1). Mice were immunised intramuscularly with 2.5 µg, 5.0 µg and 10 µg of rACT antigen adsorbed to alum adjuvant (0.350 mg/dose), or 1 x 10^9 IU/50 µl of Ad AC or Ad RTX vaccine on day 0. For some groups, a second dose was given after two weeks (on day 14). Naïve mice were immunised with 50 µl of

sterile DPBS. Blood samples were taken from the tail vein two and four weeks after the first immunisation. Cardiac bleeds were obtained six weeks after the immunisation.

Table 6.4. Vaccine dose and immunisations.

Group	Vaccine	Dose/ mouse	First dose (Day 0)	Second dose (Day 14)	Third dose (Day 28)
1	rACT	2.5 µg rACT/ 50 µl	✔	✔	-
2	rACT	5 µg rACT/ 50 µl	✔	✔	-
3	rACT	10 µg of rACT/ 50 µl	✔	✔	-
4	Ad AC	1×10^9 IU/ 50 µl	✔	-	-
5	Ad RTX	1×10^9 IU/ 50 µl	✔	-	-
6	Ad AC	1×10^9 IU/ 50 µl	✔	✔	-
7	Ad RTX	1×10^9 IU/ 50 µl	✔	✔	-
8	DPBS	50 µl	✔	✔	-
9	rACT	10 µg rACT/ 50 µl	✔	✔	✔

6.2.2 Determination of optimal ACT coating antigen by ELISA

A microtiter 96-well plate was coated with 100 µl of 0.5 µg, 1.0 µg, 1.5 µg, 2.0 µg, 2.5 µg, 3.0 µg/ml of rACT antigen in carbonate coating buffer, pH 9.6 (in duplicates, column 1-12). The plate was incubated overnight at 4°C, washed four times with PBST, and then blocked with 200 µl of blocking solution made of 1% BSA/PBS at 37°C for two hours. Serum samples obtained from mice immunised with 10 µg of rACT were diluted two-fold in 1% BSA/PBS down the plate starting from 1:1,000 dilution. Wells in row H were used as blank containing only 100 µl of 1% BSA/PBS. The plate was then incubated at room temperature for one hour and half and washed four times with PBST. Goat anti-mouse IgG horseradish peroxidase conjugate (Sigma-Aldrich), diluted 1:10,000, was added to the plate (100 µl/well) and incubated for 1.5 hours at

room temperature. After washing with PBST, 100 µl of TMB substrate was added to each well and the plate was incubated for 10 minutes at room temperature. The enzymatic reaction was stopped by adding 50 µl/well of 1.0 M sulphuric acid. The plates were read at absorbance of 450 nm using a Molecular Devices (Vmax model) plate reader and Soft Max Pro software (Version 7.1). The criterium for selection of the optimal coating antigen concentration was as follows: The optimal antigen concentration was determined based on when the OD readouts plateaued, despite increasing antigen concentration suggesting maximum of antibody binding.

6.2.3 Determination of optimal IgG secondary antibody concentration by ELISA

A microtitre 96-well plate was coated with the optimal protein concentration determined from the experiment above, 1.5 µg/ml of rACT antigen (100 µl/well) in carbonate coating buffer, pH 9.6, overnight at 4°C. The plate was washed four times with PBST, blocked with 100 µl/well of 1% BSA/PBS at 37°C for two hours, and washed four times with PBST. To establish the optimal IgG secondary antibody concentration, goat anti-mouse IgG (Fc specific)-peroxidase antibody was diluted 1/40,000 – 1/65,000 in 1% BSA/PBS buffer and each dilution was added to rows A – G (columns 1 -12) in duplicates. Positive serum sample was added to row A at dilution of 1/4000 and was diluted two-fold up to row G. Row H contained 200 µl of 1% BSA/PBS which served as negative control (blank). The plate was incubated at room temperature for 1.5 hours, then washed four times with PBST. The enzyme reaction was initiated with TMB substrate (100 µl). The reaction was stopped with 50 µl/well of 1.0 M sulphuric acid. The plate was read at absorbance of 450 nm using a Molecular Devices (Vmax model) plate reader and Soft Max Pro software (Version 7.1).

6.2.4 Detection of ACT-specific IgG antibody titres in mouse sera by ELISA

The optimal conditions identified as described above were used to determine individual antibody titres from mice immunised according to the schedule in Table 6.1. Microtiter 96-well plates were coated with 1.5 µg/ml of rACT antigen (100 µl/well) in carbonate coating buffer, pH 9.6, overnight at 4°C. Plates were washed four times with PBST, blocked with 100 µl/well of 1% BSA/PBS at 37°C for two hours, and washed four times with PBST. Sera from immunised mice were serially diluted two-fold starting from 1/1,000 dilution. Quality control samples, prepared from mice immunised with 10 µg of rACT protein, were plated in duplicates, and 100 µl/well of 1% BSA/PBS was added to two wells which served as blank. The reference standard was made from pooled sera of mice immunised with the rACT protein and was diluted 1:2000 in 1% BSA/PBS. The serum samples, reference sera and controls were incubated for one hour and 45 minutes and were washed prior to incubation with goat anti-mouse IgG (Fc specific)-peroxidase antibody (diluted 1:10,000 in 1% BSA/PBS). The plates were then washed and 100 µl of TMB substrate (Sigma Aldrich) was added to all wells and incubated for 15 minutes to detect secondary antibodies. Stop solution (1.0 M sulphuric acid, 50 µl/well) was added to each well to stop the enzymatic reaction. The plates were read at absorbance of 450 nm using a Molecular Devices (Vmax model) plate reader and Soft Max Pro software (Version 7.1). ELISA units (EU) were assigned for the range of dilutions of the reference standard to calculate a concentration of ACT-specific antibodies in serum samples. The reference standard parameters were assigned arbitrary values of 250 – 1.95 EU for the respective dilutions (1/2,000 – 1/256,000).

6.2.5 Aerosol challenge study

Female BALB/c mice (*n* = 10/group) were immunised via intramuscular route with either one dose of Ad AC or Ad RTX vaccine (1 x 10^9 IU/50 µl), one dose of wP vaccine (NIBSC code 94/532) or two doses of the 3aP vaccine. A negative control group (group 5) of mice was added (non-immunised) (Table 6.2).

Table 6.5. Immunisation of mice with Ad AC and Ad RTX vaccines for assessment in the efficacy study.

Group	Vaccine	Dose/ mouse	First dose (DAY 0)	Second dose (DAY 14)
1	Ad AC	1 x 10^9 IU/50 µl	✓	-
2	Ad RTX	1 x 10^9 IU/50 µl	✓	-
3	3aP vaccine	1/10^{th} human dose	✓	✓
4	wP vaccine	1/10^{th} human dose	✓	-
5	Naïve	-	-	-

Preparation of *B. pertussis* suspension for aerosol challenge and the challenge are described in detail in Chapter 7 (General Materials and Methods, section 7.2, subsections 7.2.1 – 7.2.4). Briefly, 14 days after administration of the second vaccine dose (Day 28), mice were exposed to aerosolised *B. pertussis* suspension (1 x 10^8 bacteria/ml) for five minutes. One week after challenge (on day 35), lungs were removed, homogenised and viable bacterial counts were established by using an automated colony counter (Synbiosis Synoptics analyser Protocol 3).

6.3 Results

6.3.1 Optimisation of ACT coating antigen concentration and IgG HRP conjugate dilution for ACT-specific IgG ELISA

The ELISA conditions were optimised to ensure reproducibility and accuracy of results. The OD read-outs of the serum dilutions (1/1,000 – 1/64,000) were plotted against different ACT antigen coating concentrations. The optimal ACT coating antigen concentration was determined to be 1.5 µg/ml (Figure 6.3), as further increases in antigen concentration did not result in increased serum antibody binding. Initially, the conjugate antibody dilution used was 1/1000, however OD read-outs were around 3.0 (data not shown), therefore 1/10,000 dilution of IgG conjugate was selected as 1/1000 dilution might have been the cause of antibody saturation. ACT-specific IgG antibodies were detected in sera that were diluted from 1/1000 dilution on a plate coated with 1.5 µg/ml of rACT and detected by secondary antibody at dilution of 1/10,000 (Figure 6.3).

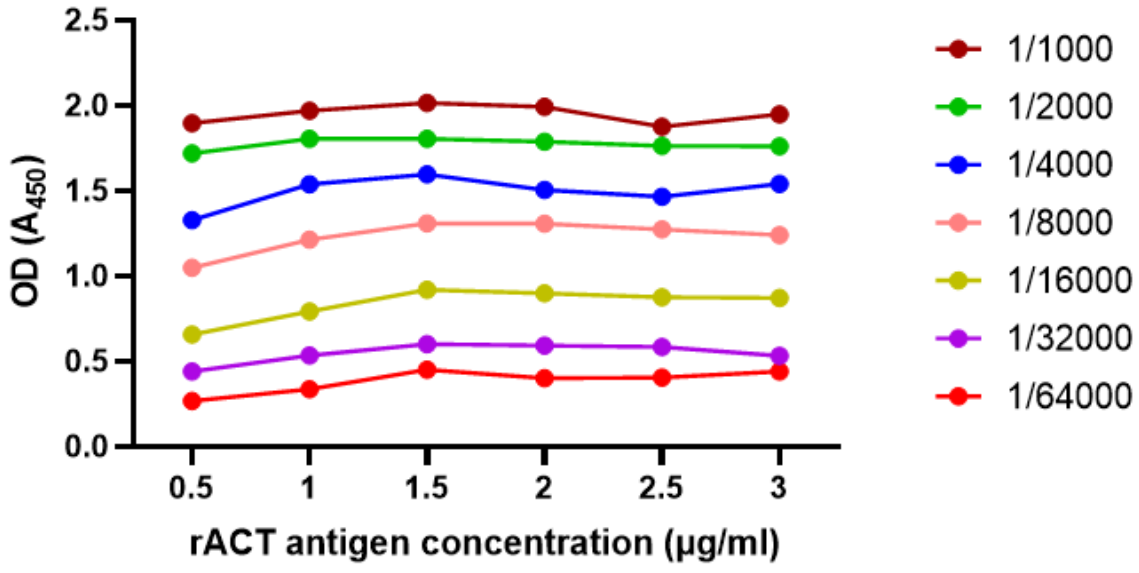


Figure 6.3. Optimisation of ELISA for assessing ACT-specific IgG antibody response in immunised mice. 0.5 – 3 µg/ml of rACT protein concentrations were assessed. Sera were diluted two-fold down the plate starting from 1/1,000 dilution. Based on the manufacturer's recommendation and data obtained from the optimisation steps, 1/10,000 dilution of the IgG secondary antibody was selected.

6.3.2 Optimisation of the reference standard and quality controls for ACT-specific IgG ELISA

Following optimisation of the ACT coating antigen and IgG conjugate secondary antibody, sera obtained from four mice (mice 1 – 4) and from another four mice (mice 5 – 8) immunised with 10 µg/ml of rACT protein were pooled to prepare the standard reference and three levels of quality control (QC) samples (low, medium and high), respectively. The QC sample readouts aim to fall in the top, middle and bottom of the linear part of the curve before it plateaus at the top and bottom. The reference standard and QC samples were serially diluted (two-fold) in 1% BSA/PBS down the plate, starting from 1/1,000 dilution and ending with 1/32768000 dilution.

The top dilution (1/1,000) for the reference standard and for the QC had OD readings of 2.4 and 2.1 (A_{450}), respectively (Figure 6.4A). The results show that both calibration curves (one for the reference standard and one for the QC) have similar OD readouts which cover the OD range ~0.1 - 2.0, before the standard curves plateau. Sera from mouse G9.3, G9.4, G9.5 and G9.6 were pooled and used for QC 1 sample and sera from mouse G9.1, G9.2, G9.7 and G9.8 were pooled and used for QC 2 sample. Both QC samples (QC 1 and QC 2) are in the linear part of the standard curve and will be run in each subsequent assay to provide confidence that the results obtained from ELISA assays are correct (Figure 6.4B).

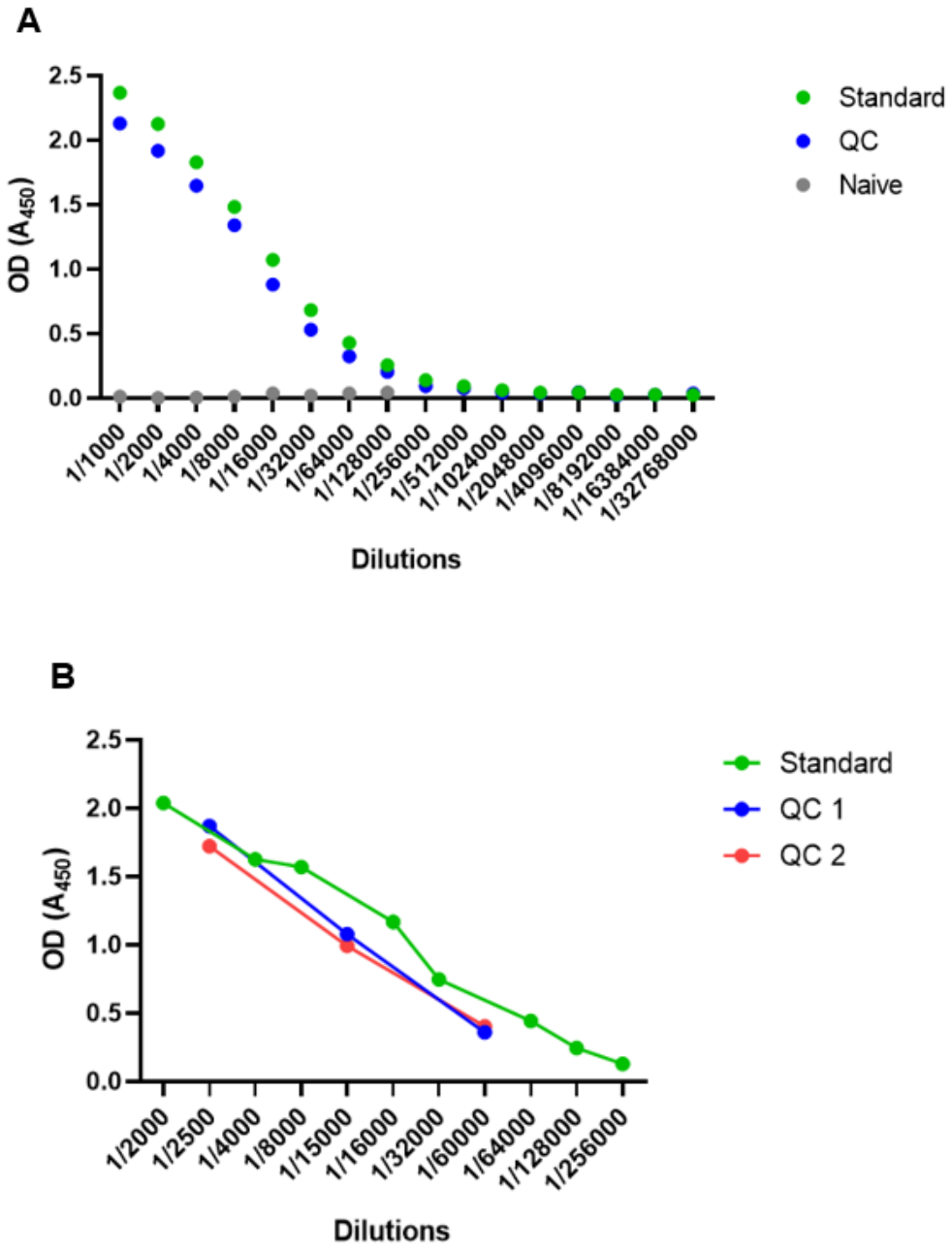


Figure 6.4. Optimisation of standard curve and establishment of QC samples for ACT-specific IgG ELISA. (A) First step in optimisation of the reference standard and QC. The top dilution for both, the standard and QC has given OD readout of ~2.00. **(B) Establishing QC samples.** QC values that fall at the top, middle and bottom of the standard curve (linear dilution curve) are high, middle, and low QC samples that will be used for all the subsequent assays to ensure that data obtained from the assay are reliable.

6.3.3 Ad AC and Ad RTX vaccines mediate high ACT-specific IgG antibody responses in mice

To assess whether Ad AC and Ad RTX induce immunogenicity in mice, groups of BALB/c mice were immunised intramuscularly with the vaccines and ACT-specific IgG antibody responses were compared with the antibody responses induced by different doses of rACT adsorbed to alum adjuvant. One dose of Ad AC vaccine mediated higher ACT-specific IgG antibody responses two weeks after the immunisation than 2.5 µg , 5 µg rACT or 10 µg rACT ($p < 0.0001$, $p = 0.0032$ and $p = 0.0389$, respectively, Figure 6.5A). One dose of Ad RTX also induced higher ACT-specific IgG antibody responses two weeks after the immunisation than 2.5 µg rACT ($p = 0.0142$; Figure 6.5A).

Two-dose immunisation with Ad AC induced higher ACT-specific IgG antibodies than two-dose immunisation with 10 µg rACT two weeks after the second dose (or four weeks after the first dose was administered) ($p = 0.0391$) (Figure 6.5B). The other immunisation strategies induced similar level of ACT-specific IgG antibodies (Figure 6.5B).

Four weeks following the second dose, the levels of ACT-specific IgG antibodies were higher in mice immunised with Ad RTX than in mice immunised with Ad AC, 2.5 µg and 5 µg rACT ($p = 0.0426$, $p = 0.0027$ and $p = 0.0115$, respectively) (Figure 6.5C).

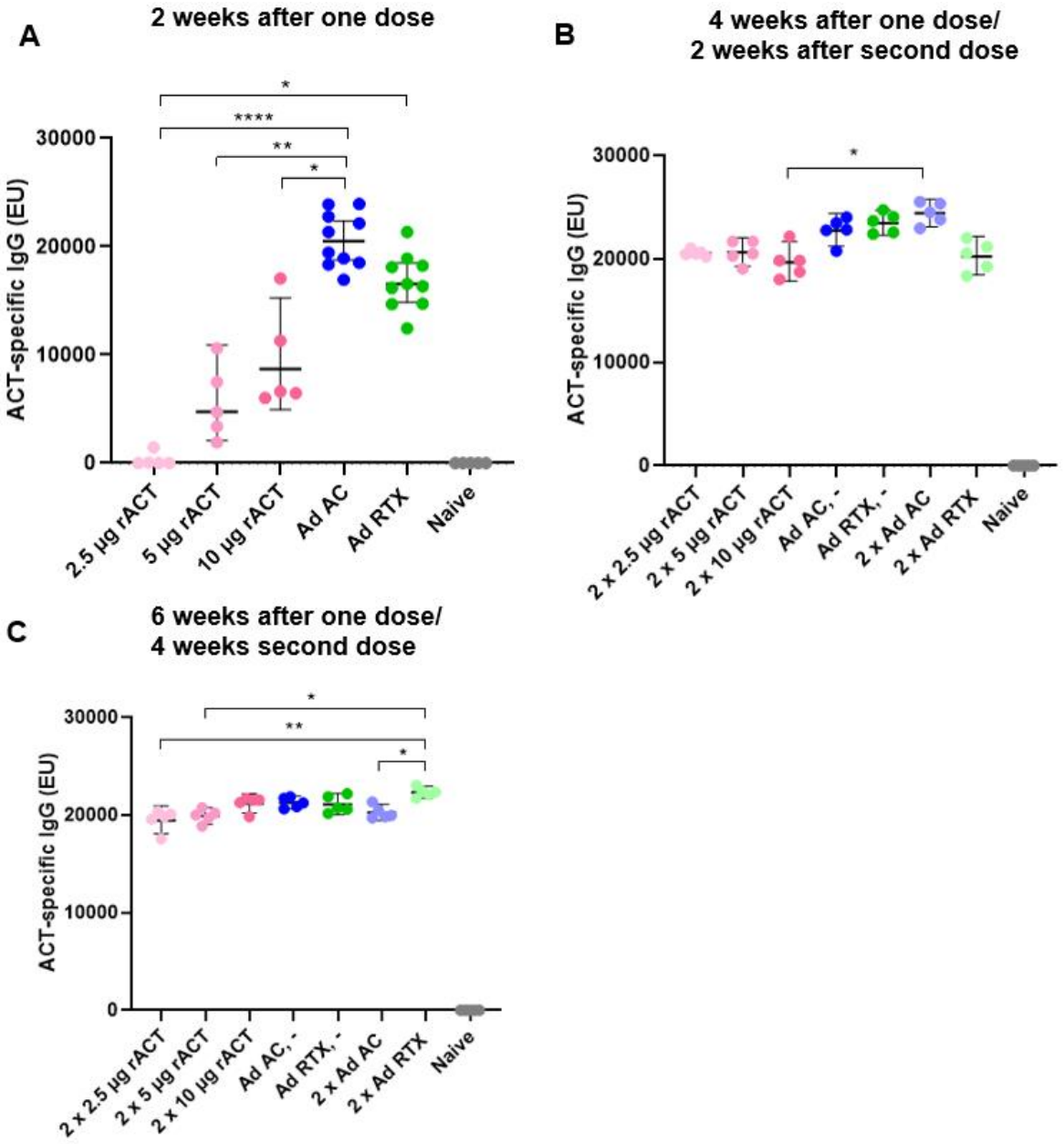


Figure 6.5. Analysis of immunogenic responses of Ad AC and Ad RTX vaccines in immunised mice. ELISA was used to determine ACT-specific IgG responses in serum of immunised mice **(A)** two weeks after the first dose **(B)** four weeks after the first dose (or two weeks after the second dose) **(C)** six weeks after the first dose (or four weeks after the second dose). The antibody titres were compared by one-way ANOVA followed by Tukey's multiple comparison tests. Results plotted on the graphs represent geometric mean with ± 95% confidence intervals (n = 5 mice/group), $p < 0.05$,

6.3.4 A single dose of adenoviral vectored vaccine expressing AC and RTX domains induced protective responses in mice

The protective effect of a single intramuscular immunisation with Ad AC and Ad RTX vaccine was assessed in the mouse aerosol model. To determine the efficacy of the vaccines, viable bacterial counts were enumerated in lungs of mice one week after the aerosol challenge with *B. pertussis* 18-323 strain (Figure 6.6). Immunisation with Ad AC or Ad RTX reduced bacterial colonies in the lungs in comparison with non-immunised mice on day seven ($p<0.0001$). There was a two-log decrease in CFU in lungs of mice immunised with single dose of Ad AC vaccine when compared with naïve mice on day seven. No bacterial colonies were detected in the lungs of six mice that were immunised with single dose of Ad RTX vaccine, suggesting complete protection against *B. pertussis*. In addition, a two-log reduction in bacterial load was observed in four mice immunised with Ad RTX vaccine when compared with the naïve mice, indicating partial protection against *B. pertussis*. Two-dose immunisation with the three-component aP vaccine provided complete protection of mice against pertussis infection. One dose of wP vaccine was also fully protective, except for two mice that were partially protected against *B. pertussis*. These results suggest that adenoviral vaccine encoding RTX domain mounted more effective protection against pertussis infection than Ad AC vaccine, likely due to induction of antibodies recognising protective epitopes on ACT antigen.

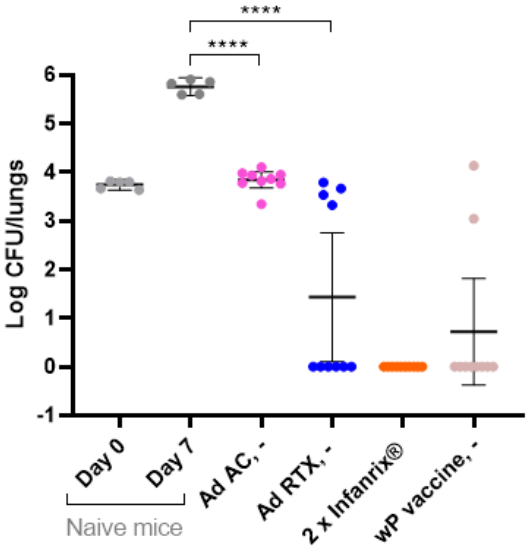


Figure 6.6. Assessment of the protective capacity of Ad AC and Ad RTX vaccines five weeks after the vaccination. BALB/c mice were immunised intramuscularly with one dose of Ad AC and Ad RTX vaccine on day zero and were then challenged with virulent *B. pertussis* 18-323 strain four weeks after the immunisation. Mice were sacrificed one week later and lung CFU were enumerated. Data represent geometric mean with ± 95% confidence intervals (n = 10 mice/group). Statistical significance was determined using one-way ANOVA.

6.4 Discussion

In this study, ACT was selected for the development of the new vectored-based vaccine candidates, as it is a key virulence factor of *B. pertussis*. Due to its large molecular weight (180 KDa), truncated forms of the ACT, specifically the AC and RTX domains, were cloned into the adenoviral vectors.

ACT is synthesised as an inactive precursor that is converted to the active toxin by post-translational modification. CyaC acyltransferase links fatty-acyl residues to the ε-amino groups of the internal lysine residues K860 and K983 which is essential process for the correct toxin conformation, receptor binding and subsequent translocation of the catalytic AC domain across the plasma membrane (131, 195, 197). The adenoviral construct expressing RTX domain does not contain residues K860 or K983. These residues were omitted due to the post-translational modifications being restricted to bacterial cells. It was uncertain how the lack of post-translation activation would affect conformation, activity and stability of the protein when expressed in eukaryotic cells following immunisation with the adenoviral vectored vaccine. However, the receptor binding site on the RTX domain is likely to include residues 1166 – 1287 (131) that are included in the RTX transgene inserted by the adenoviral vectored vaccine. Therefore, the Ad RTX vaccine may induce ACT-specific antibodies binding to the receptor binding site residues which may prevent ACT binding to the host's cells.

Here, we evaluated the immunogenic characteristics of Ad AC and Ad RTX vaccines by assessing ACT-specific antibody responses in mice. The immunogenicity data show that a single dose of Ad AC and Ad RTX vaccine is immunogenic as early as

two weeks after first immunisation and the humoral immune response remained elevated up to six weeks after the immunisation (Figure 6.5). These findings are similar with a study which found that a single intramuscular immunisation of BALB/c mice with Ad5-nCoV vaccine induced serum Spike-specific IgG antibodies two weeks after the immunisation and the antibodies peaked at day 28 and then slightly decreased six and eight weeks later (198).

In the case of homologous prime-boost immunisation with the same viral vectored vaccine, there is a dogma that the homologous regimen has a potential to initiate anti-vector immunity that could impact the effectiveness of the second dose (150). Despite that assumption, a recent clinical trial demonstrated that homologous prime-boost with ChAdOx1 nCoV-19 vaccine induced Spike-specific antibodies that peaked at day 28 and remained high up to day 56 (150). Our data also showed that immunisation of mice with two doses of Ad AC or Ad RTX vaccine was as immunogenic as immunisation with one dose of Ad AC or Ad RTX vaccine. Two-dose immunisation with Ad RTX induced a significantly greater level of anti-ACT IgG antibodies in mice than two doses of Ad AC vaccine four weeks after the second immunisation (Figure 6.5C). Although, the difference between the antibody levels was small it was found to be statistically significant. This suggests that two-dose immunisation with Ad AC vaccine is also effective at mediating antibodies against ACT. The data are consistent with other studies which showed that ACT is immunogenic antigen in mice. Wang *et al.* showed that full ACT antigen or RTX domain, consisting of 985 amino acid residues, both mediated high ACT-specific IgG antibodies in mice four weeks after a first dose and the antibody responses were higher after the second and third dose (administered at week six and eight) (130).

The protection study showed that both Ad AC and Ad RTX represent a promising avenue as a potential component of novel pertussis vaccines. There was complete clearance of *B. pertussis* in lungs of six mice immunised with a single dose of Ad RTX and the partial bacterial clearance from lungs of mice immunised with single dose of Ad AC. The protective effect may have been due to neutralising antibodies. Wang *et al.* (2015) demonstrated that the RTX domain induces ACT neutralising antibodies in mice following immunisation (130). So far only one antibody preventing translocation step of the AC domain of ACT has been characterised to this date. The 3D1 antibody binds a conformational epitope between residues 373 and 399, which are adjacent to the catalytic AC domain, inhibiting complete delivery of the catalytic domain to the host cytosol (130). The partial protection of the Ad AC vaccine could be associated with protective antibodies binding to the region between 373 and 399 residues. The vast majority of ACT-specific antibodies obtained from phage libraries recognise the RTX domain of ACT. Two antibodies blocking the binding of ACT to its receptor were characterised (130), suggesting that more effective protective role of the RTX domain over the AC domain as seen in our experiment. A limitation of this study is that the functionality of serum IgG antibodies was not characterised *in vitro* and therefore it may be worth to explore if the protective responses are associated with the toxin neutralisation or whether other aspects of the vaccine-induced immune response was involved. The Ad vectored vaccine platform is capable of inducing strong $CD4^+$ and $CD8^+$ T cell responses (111) therefore cell-mediated immunity may play a role in the partial protection observed with the Ad AC and Ad RTX vaccines in this study.

Scientists at the Public Health England assessed binding of vaccine-induced ACT-specific antibodies on the surface of heat-killed *B. pertussis* by a surface labelling

assay (SLA) (179). The SLA has advantages over the ELISA as it examines antibody binding to the antigens expressed during natural infection, whereas some epitopes can be hidden or lost in the ELISA format. Mouse sera, which were collected six weeks after a single immunisation with Ad AC and Ad RTX vaccines, were analysed for binding of ACT-specific IgG antibodies to different *B. pertussis* strains by the SLA. The results showed that sera from mice vaccinated with Ad AC or Ad RTX vaccine did not stimulate IgG binding on the *B. pertussis* strains, which could be a sign of the absence of ACT-specific antibodies in mouse sera and/or the antibodies were not able to bind to the selected bacterial strains. However, it is important to mention that ACT is synthesised and acylated inside bacterial cells and then secreted across *B. pertussis* cell membrane into the extracellular environment where it binds calcium and folds into its native conformation (199). For that reason, it would be more effective to assess if the Ad AC and Ad RTX vaccines are able to induce neutralising antibodies against ACT, rather than binding of the antibodies to ACT that is secreted and not exposed on the bacterial cell surface.

Due to partial protection mediated by Ad AC and Ad RTX vaccine following single-dose immunisation, it would be interesting to study the mixing of Ad AC and Ad RTX vaccines, and their relative doses. It would be useful to explore if the higher dose of the vaccines and their combination are effective to prevent pertussis infection in mouse lungs. If the Ad RTX vaccine proves to be immunogenic as it was shown in our first immunogenicity study, then we could evaluate if the combination of Ad AC + Ad RTX vaccine can induce better protection than Ad AC or Ad RTX alone.

6.5 Conclusion

In conclusion, the data show that ACT is a promising antigen to be included in viral vectored vaccines. Previous mouse studies demonstrated that the RTX domain of ACT is immunodominant and stimulates neutralising antibodies against ACT (200). This study also showed that Ad RTX vaccine is immunogenic in mice and can induce partial as well as complete protection against pertussis infection in some mice. This study also demonstrated that Ad AC vaccine is immunogenic as well as partially protective in mice. The cell-invasive AC domain of ACT inserts itself into host cell membrane which is followed by translocation into the cell cytosol where it catalyses conversion of ATP to cAMP. The AC domain of ACT enables *B. pertussis* to avoid host innate immune response and survive within the host environment (193, 200). For that reason, having an effective vaccine targeting the AC domain of ACT could block its translocation to host cell cytosol, prevent damaging of cell-signalling pathways which would lead to induction of innate immunity against *B. pertussis*.

Chapter 7. General Materials and Methods

7.1 Selection of *B. pertussis* antigens for the development of pertussis viral vectored vaccines

B. pertussis antigens that are involved in pathogenesis of the disease were identified from the literature and selected for development of viral vectored vaccines based on the following characteristics:

• Antigens are expressed during natural infection and have pathogenic function

• Antigens can induce protective immune responses and are immunogenic in animals and/or humans

7.1.1 Cloning process of adenoviral vectored vaccines expressing *B. pertussis* antigens

The gene sequences of the S1 subunit of PT, Fim2/3/D, IRP1-3 (BP1152), AfuA (BP1605) and ACT proteins of *B. pertussis* were obtained either from the National Center for Biotechnology Information (NCBI) or UniProt databases as shown in Table 7.1.

Table 7.1. Gene sequences of the selected vaccine antigenic candidates.

Viral vectored constructs	Selected B. *pertussis* antigen	Amino acid residues	Gene sequence obtained from / Reference sequence
Adenoviral vectored vaccine expressing S1 subunit of PT (full-length)	Pertussis toxin S1-235 subunit	35 - 235	UniProt database / NP_882282.1
Adenoviral vectored vaccine expressing S1 subunit of PT (truncated version)	Pertussis toxin S1-220 subunit	35 - 220	
Adenoviral vectored vaccine expressing Fim2	Fimbriae serotype 2	27 - 207	NCBI / CAE41417.1
Adenoviral vectored vaccine expressing Fim3	Fimbriae serotype 3	26 - 204	NCBI / CAE41857.1
Adenoviral vectored vaccine expressing FimD	Fimbriae serotype D	1 - 365	NCBI / CAE42166.1
Adenoviral vectored vaccine expressing AC domain of ACT	Adenylate cyclase	1 - 400	NCBI / NC_002929.2
Adenoviral vectored vaccine expressing RTX domain of ACT	Repeat-in-toxin domain	985 - 1680	
Adenoviral vectored vaccine expressing iron-binding protein IRP1-3	IRP1-3	21-178	UniProt database / NP_879928.1
Adenoviral vectored vaccine expressing iron-binding protein AfuA	AfuA	31-349	NCBI / NP_880337.1

The cloning process was performed in a containment level 2 laboratory. Figure 7.1 shows an overview of the cloning process which is described in detail below.

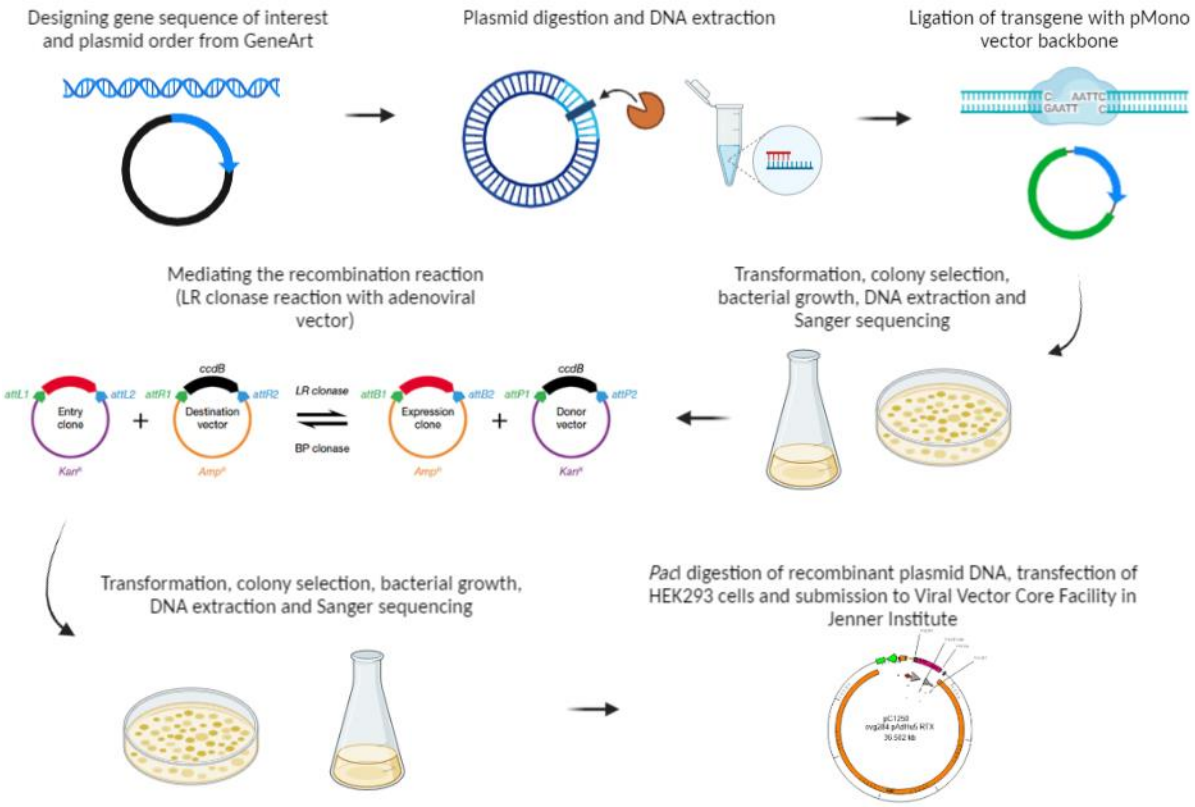


Figure 7.1. A schematic of cloning of pertussis antigens into viral vectors. The vector, named human adenoviral serotype 5, expresses one of the selected *B. pertussis* antigens.

The genes were synthesised with HindIII and NotI restriction enzymes (New England Biolabs) at the restriction sites, for digestion and ligation, and overhangs to stabilise the DNA. Sequences were codon optimised to enhance their efficient expression in mammalian cells. The gene sequences were ordered as DNA strings or depending on the gene size as plasmid DNA (GeneArt, Thermo Fisher Scientific). Lyophilised DNA was reconstituted in 50 µl H2O. To cut the transgene from the plasmid backbone, 50 µl restriction digest reaction was set up as described in Table 7.2.

Table 7.2. Restriction digest of plasmid DNA

Reagent	Volume (µl)
DNA	20
HindIII	1.5
NotI	1.5
CutSmart® Buffer (10X)	5
Nuclease free H_2O	22

Restriction digest reaction was also prepared for pMono mammalian expression vector (Oxford University, Oxford) as shown in Table 7.3.

Table 7.3. Restriction digest of pMono mammalian expression vector

Reagent	Volume (µl)
pMono plasmid	8
HindIII	1.5
NotI	1.5
CutSmart® Buffer (10X)	5
Nuclease free H_2O	34

The restriction digest reactions were run for four hours at 37°C. To visualise the results of the reactions, gel electrophoresis was performed. DNA samples were run on a 1% agarose gel at 100 V for one hour. The digested DNA fragments were visualised under UV light, cut out and extracted using the QIAquick Gel Extraction Kit (Qiagen). Concentrations of the extracted DNA were quantified by using NanoDrop2000 Software. Then the DNA encoding each specific protein was ligated with the digested backbone of the pMono expression vector, carrying kanamycin resistance gene, using T4 DNA ligase enzyme and T4 DNA Ligase Reaction Buffer (New England Biolabs) for at least 16 hours.

Once the ligation reaction was completed, each pMono vector expressing the specific antigenic DNA was transformed into chemically competent *E. coli* DH5α cells suitable for high efficiency transformation (New England Biolabs). Ligated DNA (containing the antigen DNA within the pMono plasmid) was incubated with competent *E. coli* cells on ice for 30 minutes and then heat-shocked at 42°C for 30 seconds. The DNA/competent cells mixture was then placed on ice for two minutes, following addition of 250 µl of pre-warmed Super Optimal Broth (SOC) (ThermoFisher Scientific Invitrogen) outgrowth medium to the bacteria which were then incubated by shaking at 37°C, 220 rpm, for one hour. Following the incubation, 100 µl of transformed bacterial suspension was spread on LB agar plates, supplemented with 50 µg/ml of kanamycin (Sigma-Aldrich) for selection. The bacteria were incubated at 37°C overnight. To allow plasmid extraction, three to four different single *E. coli* colonies were picked from the LB agar plates and placed in 50 ml of LB broth supplemented with 50 µg/ml kanamycin. The bacterial colonies were incubated by shaking at 37°C, 220 rpm, overnight. Bacterial suspensions were then centrifuged at 4,000 g, at 4°C, for 10 minutes and DNA sample was extracted according to the manufacturer's instructions using a Plasmid Plus Midi Kit (Qiagen). The concentration of DNA was measured using NanoDrop2000 Software and the clones that had inserts were further analysed by restriction endonuclease digestions as described above. The digested DNA fragments were run on 1% agarose gel at 100 V for one hour. The bands with the correct size of insert were sent for sequencing to SourceBioscience (Cambridge, UK) using forward and reverse primers for the 3' cytomegalovirus (CMV) promoter and bovine growth hormone (bGH) polyA region sequences, respectively, to confirm that the inserted sequences match the expected antigen sequences. If the sequencing confirmed that the insert was 100% match to the antigen sequences, the purified plasmids were resuspended with 1X TE

buffer (Invitrogen) to obtain 150 ng of DNA that was incubated with 150 ng of HuAd5 entry vector plasmid containing ampicillin resistant cassette and LR Clonase (Gateway® LR Clonase® II enzyme mix, Invitrogen) at 25°C for 16 hours leading to *in vitro* recombination between the DNA sample and AdHu5 vector. In the following step, the recombinant DNA sample was digested with 1 µl of Proteinase K for 10 minutes at 37°C. The highly competent *E. coli* DH5α cells were transformed with the recombinant DNA and the transformants were plated on plates made from LB agar supplemented with 100 µg/ml of ampicillin (Sigma-Aldrich). DNA plasmids were again extracted and sequenced as described previously. Samples that 100% matched the antigen sequences were subjected to linearisation of the recombinant adenoviral vector DNA, which was cleaved with PacI enzyme (New England Biolabs), to remove the ampicillin resistant cassette, and transfected into a packaging cell line (HEK293 cells) (201). The final process of viral vectored vaccine production was performed at the viral vector core facility (VVCF) at the Jenner Institute, Oxford, UK. The VVCF has been specialised in production of recombinant viral vectors required for pre-clinical vaccine studies (202).

7.2 Animal experiments

7.2.1 Ethics statement for animal procedures

All animal procedures were approved by NIBSC's Animal Welfare and Ethical Review Body (AWERB) and were conducted according to the UK Home Office regulations.

7.2.2 Preparation of *B. pertussis* 18-323 and B1917 strain suspensions for mouse aerosol challenge studies

Bacterial colonies of *B. pertussis* 18-323, used as the challenge strain for most assays, and B1917 strain were grown on charcoal agar plates supplemented with 10% (v/v) defibrinated horse blood (Thermo Scientific Oxoid) at 37°C for 18 - 24 hours. The bacteria were resuspended in sterile 6 ml of 0.9% saline containing 1% casein. The density of bacterial suspension of 18-323 strain was adjusted to $OD_{0.26}$ at 625 nm, corresponding to 1 x 10^8 bacteria/ml, and for B1917 strain, the suspension was adjusted to $OD_{0.1663}$, $OD_{0.1792}$ and $OD_{0.340}$ at 625 nm, which correspond to 1 x 10^7, 1 x 10^8 and 1 x 10^9 bacteria/ml, respectively. These suspensions were kept on ice until used for aerosol challenge. The corresponding viable counts of the suspensions were validated by plating out serial dilutions on the charcoal agar plates and colonies were counted after seven days of incubation at 37 °C.

7.2.3 Aerosol challenge of mice with *B. pertussis* (Standard procedure)

Female BALB/c mice (Charles Rivers, UK) entered experiments at six to eight weeks of age. Bacterial suspensions (1 x 10^8 bacteria/ml for 18-323 strain and 1 x10^9 bacteria/ml for B1917 strain) for aerosol challenge were prepared as described above one to two hours before the challenge and were kept on ice. The mice were placed inside an aerosol apparatus, their noses were fitted into the nose ports of the

apparatus and were then exposed to bacterial suspensions administered as aerosol, using a nebuliser, for five minutes. Two to three hours after the challenge, five out of 10 non-immunised mice, which serve as control, were anaesthetised and sacrificed to remove lungs and/or nasopharynxes for colony counting and an assessment if the challenged mice were infected after the procedure. The lungs and/or nasopharynxes from the remaining five non-immunised mice and immunised mice were harvested seven days after the challenge. The lungs and nasopharynxes were homogenised in 1 ml and 0.2 ml of sterile 1% casein in saline, respectively, and serially diluted to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} of the original culture in sterile 1% casein in saline. Four drops (25 µl / drop) of each diluted homogenate was plated out on charcoal agar plate supplemented with 10% (vol/vol) defibrinated horse blood (TCS Biosciences, UK) and the corresponding viable counts for each mouse were determined after incubation at 37°C for five to seven days (203, 204).

7.2.4 Collection of mouse sera from tail bleeds or cardiac bleeds

Blood samples were obtained by tail bleeding or cardiac puncture for terminal bleeds. Blood was allowed to clot at 4°C for 18 hours. The blood samples were centrifuged at 4,000 rpm for 10 minutes to separate sera from the pellets into new vials which were stored at -20°C until use.

7.3 Bacterial endotoxin test: Limulus Amoebocyte Lysate (LAL) Gelation test

To quantify the endotoxin levels in the recombinant protein samples obtained after affinity chromatography and size exclusion purification procedures, the Limulus Amoebocyte Lysate (LAL) Gelation test was used and performed by the Pyrogens Laboratory at NIBSC. The current WHO International Standard (NIBSC code 10/178)

for endotoxin was used for quantitation. Samples were assayed using Endosafe (Charles River Laboratories, France). Endotoxin standard dilutions were prepared covering a range of 0.008 – 0.125 IU/ml (this is to cover 2x two-fold concentration each side of the lysate label claim of 0.03 IU/ml). Each endotoxin standard concentration was tested in duplicate in a petri dish by adding 10 µl of lysate on the test plate followed by adding 10 µl of standard to its allocated lysate spot. LAL reagent water was used as a negative control. The plate was incubated at 37˚C for one hour in a designated humidified incubator. Using a Hamilton syringe, 1 µl of 0.2% new methylene blue in 70% ethanol was added to each droplet (lysate + test solution) on the plate and signs of gelation were observed for each droplet. To calculate endotoxin content, the lysate label claim was multiplied by the dilution factor of the most dilute preparation that gives a positive result to give a value ≥ the endotoxin content (lower limit). The lysate label claim was multiplied by the dilution factor of the most concentrated preparation that gave a negative result to give a value ≤ the endotoxin content (upper limit).

7.4 Detection of IgG antibody titres by Enzyme-linked immunosorbent assay (ELISA)

A 96-well microtiter plates (Thermo Scientific) were coated with PT (2 µg/ml, Sanofi Pasteur) and incubated at room temperature for 18-22 hours. The plates were then washed with PBS/0.05% Tween-20 (PBST) and blocked with 10% FCS + PBST (diluted 1:10) at room temperature for 30 minutes. The 1st WHO Reference Reagent pertussis mouse antiserum (NIBSC code 97/642) served as a reference serum (diluted 1:100 in FCS/PBST) and positive control (diluted 1:10 in FCS/PBST). Serum samples were pre-diluted 1:100. The serum samples, reference sera and control were 3-fold diluted and incubated for 1.5 hours and washed prior to incubation with goat anti-

mouse IgG (Fc specific)-peroxidase antibody (diluted 1:1,000 in FCS/PBST) and washed. To detect secondary antibodies, 100 µl of tetramethylbenzidine (TMB) substrate (Sigma Aldrich), was added to all wells and incubated for 15 minutes. Stop solution (1.0 M sulphuric acid, 50 µl/well) was added to each well to stop the enzymatic reaction. The plates were read at absorbance of 450 nm using a Molecular Devices (Vmax model) plate reader and Soft Max Pro software (Version 6.5.1) (205).

7.4.1 Data analysis and statistics

PT-specific IgG antibody titres (EU/ml) were determined against a reference (NIBSC code 97/642), with a potency unit of 34 EU/ml. Raw data were analysed by eight-point dose scheme (sigmoidal curve) using CombiStats software v 5.0. The anti-PT IgG concentration was obtained (EU/ml) for each mouse and the geometric mean for each group was calculated. Data are expressed as geometric mean ± 95% confidence intervals. Statistical analysis was preformed using One-way ANOVA followed by a Tukey multiple comparison test. Data were analysed using GraphPad Prism software (v 8.1.0). A p-value < 0.05 was considered statistically significant.

7.5 Detection of IgG2a and IgG1 isotypes by ELISA

Microtiter plates were coated with 2 µg/ml of PT (NIBSC code 10/212) and were incubated for 18–22 hours. Following the incubation, the plates were blocked with FCS/PBST (1:10 dilution) for 30 minutes and washed. Serum samples for IgG2a ELISA were pre-diluted 1:200 and the reference serum (NIBSC code 97/642) were pre-diluted 1:10 in FCS/PBST. Serum samples for IgG1 ELISA were pre-diluted to 1:1,000 and the reference was also pre-diluted to 1:1,000 in FCS/PBST. Following

pre-dilutions, serum samples and the reference serum were serially diluted (3-fold) and incubated at room temperature for 1.5 hours and washed. Biotin-conjugated rat anti-mouse IgG2a or IgG1 antibody (BD Pharmingen), diluted 1:1,000 in FCS/PBST, was used as detection antibody and was added to all wells and incubated for 1.5 hours. The plates were washed and 100 µl of Streptavidin-Horseradish Peroxidase (diluted 1:1,000 in FCS/PBST) was added to each well and incubated for one hour. The plates were washed with PBST and 100 µl of TMB substrate was added to all wells and incubated at room temperature for 15 minutes. Stop solution (50 µl/well of 1.0 M sulphuric acid) was added to all plates to stop the enzymatic reaction. The optical density was read at absorbance of 450 nm using a Molecular Devices microtiter plate reader (Vmax model) and Soft Max Pro software (Version 6.5.1) (162).

7.5.1 Data analysis and statistics

IgG2a and IgG1 anti-PT titres (U/ml) were measured against reference mouse sera (NIBSC code 97/642) with an arbitrary unit of 1 U/ml. Raw data were analysed by eight-point dose scheme (sigmoidal curve) using CombiStats software v 5.0. Antibody titres were calculated in U/ml relative to the reference for each mouse and the mean was determined for each group of mice. Data are expressed as geometric mean ± 95% confidence intervals. Statistical analysis was preformed using One-way ANOVA followed by a Tukey multiple comparison test. Data were analysed using GraphPad Prism software (v 8.1.0). A p-value < 0.05 was considered statistically significant.

Chapter 8: General Discussion

The introduction of wP and aP vaccines in the paediatric immunisation programmes led to decreased pertussis cases (83). However, the use of wP vaccines in paediatric immunisation has reduced, especially in developed countries, due to local and systemic reactions associated with the wP vaccine (42-44). Although, the aP vaccines are able to prevent severe disease, they offer short-term protection when compared to the natural infection or wP vaccines, and also contribute to asymptomatic transmission of the infection (80). The previously mentioned factors have led to an increase in pertussis cases in all countries that use aP vaccines. For example, a resurgence of pertussis cases was also reported in U.K in 2012 (206). Thus, there is a need to develop new pertussis vaccination strategies.

Viral vectors have been used for development of vaccines against many infectious diseases due to their capacity to induce potent antibody responses and cell-mediated immunity (207). Studies in the pertussis field suggest that in addition to humoral immunity, cell-mediated immunity is also essential to stimulate long-lived protection against pertussis disease (208). For that reason, the use of viral vectored vaccines is a promising strategy for stimulation of strong antibody as well as T-cell responses against pertussis antigens. ACT, Fim, the S1 subunit of the major protective antigen, PT, and iron-binding proteins were selected as antigen candidates for viral vectored vaccines. The selection of the candidate antigens was based on their immunogenic and efficacious properties observed in mouse studies conducted by other researchers. This thesis describes our exploration of viral vectors, particularly replication-deficient Ad and MVA, as vaccine platforms against *B. pertussis*. We investigated if these

vaccines have immunogenic effect against the above-mentioned antigens and if the vaccines have protective effect against *B. pertussis* in mouse models.

A single-dose immunisation with AdHu5 vector encoding a *Plasmodium* protein was shown to be immunogenic and efficacious against sporozoite challenge in a malaria study (209). Therefore, immunogenicity following a single immunisation with an adenoviral vector expressing S1-220 or S1-235 of PT was investigated. One-dose immunisation with Ad S1-220 or S1-235 was able to induce a strong humoral response as early as six weeks after vaccination (Figure 3.2A). We also investigated one-dose immunisation with MVA vaccine expressing S1-220 or S1-235. However, humoral immune response to the MVA prime was low which suggests that priming with the MVA expressing S1-220 or S1-235 is not suitable for induction of humoral immune response in mice (Figure 3.2A).

The heterologous prime-boost with the Ad vector followed by MVA increases the T-cell response while avoiding potential difficulties associated with vector-specific immune responses aimed at the prime vaccine. This is because the immune response against the vector has the potential to decrease the infection of target cells by subsequent administration of the vector and thus decrease the expression of a transgene (210, 211). The Ad, MVA vaccination regimen was found protective in many infectious diseases such as malaria, and this approach was more successful than prime-boost with MVA, Ad (207). The prime-boost approach using Ad, MVA and MVA, Ad regimens was also assessed to find out how the order of administration of adenovirus and MVA vaccines impacts the immune responses in mice. Results show that the prime-boost with Ad, MVA S1-235 induced stronger humoral response than

MVA, Ad prime-boost two weeks after the boost (Figure 3.2B). However, both Ad, MVA and MVA, Ad regimens induced strong humoral immune responses 12 weeks after the boost administration (Figure 3.2D). The data in this study show that the viral vectors are able to elicit PT-specific antibody responses that last up to 12 weeks in mice.

In this study, we also examined the protective effects of priming with Ad expressing S1-220 or S1-235, and prime-boost immunisation with Ad, MVA expressing S1-220 or S1-235 in the mouse aerosol challenge model. Interestingly, a partial protection against pertussis infection was observed in mice immunised with Ad S1-235 and Ad, MVA S1-235 vaccines, but not with the vaccines encoding the truncated version of the S1 subunit (S1-220) (Figure 3.4). One possible explanation for different protective responses observed in the immunised mice is a potential of the S1-220 and S1-235 subunits to adopt different conformations from each other and therefore hinder exposure of some protective epitopes when expressed in host cells. The differences between the truncated and full-length subunit structures may also affect the protein conformation following expression in host cells. The carboxyl (C-) terminus of the S1 subunit enters a pore in the centre of the B subunit and ends within the pore. Stein *et al.* (1994) found that ten residues (211-220) of the S1 subunit, near the C-terminus, had poor density and could not be modelled. Therefore, it remains unknown how these specific residues, close to the C-terminus, effect conformation of the S1 subunit (58). However, Sutherland and Maynard (2009) showed that following recombinant protein expression, there was a low production of the full length S1-235 subunit in *E. coli* when the C-terminus was retained, possibly due to a long hydrophobic tail being anchored into the B subunit of PT. On the other hand, the truncated version of the S1 subunit (S1-220) was expressed and purified effectively (143). This could suggest how the C-

terminus could have an impact not only on production of the protein but also on protein conformation.

Anti-S1 1B7 mAb has been shown to neutralise PT and protect mice against aerosol challenge with *B. pertussis* (63). Interestingly, Sutherland and Maynard (2009) showed reduced binding affinity of the S1 subunit-specific 1B7 mAb to the protective epitopes on commercially prepared S1-235 subunit as well as recombinant S1-220. They hypothesised that the 1B7 mAb may recognise an epitope located on the PT S1 and their antigen-antibody binding might be supported by a weak B-oligomer interaction. Also, the B-oligomer may have an effect on the stability of PT S1 (143). Nevertheless, the partial, weak protection observed with the use of Ad S1-235 and Ad, MVA S1-235 vaccines may be considered as an encouraging result.

Neutralising antibodies targeting PT antigen have been associated with protection against severe pertussis disease (65). In this study, the functionality of antibodies induced by the viral vectored vaccines expressing PT S1 was examined through the ability to neutralise PT. The results from PT neutralisation assay showed that primarily single-dose immunisation with Ad S1-220 and Ad S1-235 vaccine elicits PT-specific nAbs six weeks after the immunisation in 50% of immunised mice (Figure 3.5). These vaccines, however, did not induce PT nAbs 10 weeks after the immunisation, and prime-boost with Ad, MVA or MVA, Ad also did not induce PT nAbs. The lack of PT nAbs after immunisation with the viral vectored vaccines encoding PT S1 could be potentially associated with insufficient exposure of protective epitope(s) located on the PT S1 and/or folding of the expressed protein in mammalian cells. If the S1 subunit of PT is not folded in a way that resembles native PT structure and some protective

epitopes are hidden, then this could explain why the viral vectored vaccines did not induce strong PT nAbs.

The importance of the antigen structure and correct conformation of its amino acid residues was also highlighted in a study that assessed recombinant human IgG1 antibodies binding to PT. The study found that residue R76 is only partially exposed in the holotoxin, therefore its specific A8 antibody was unable to neutralise the toxin (212). Furthermore, a high concentration of low-affinity antibodies or a low concentration of high-affinity antibodies, interacting with the three-dimensional structure of the S1 subunit, could have contributed to the vaccine's inability to induce high level of PT nAbs. T-cells are also important for somatic hypermutation to produce high affinity IgG antibodies and for the production of B-cell memory cells (213), therefore whether T-cell responses did not support the generation of high affinity nAbs antibodies remains unknown. Studies that analysed PT nAbs in vaccinated children found that the nAbs increased significantly after a second dose of aP vaccine when compared with one-dose immunisation or to pre-vaccination status. Surprisingly, the neutralisation titre did not increase after the first dose of aP vaccine when compared with the pre-vaccination status (146). In this study, data suggest the opposite effect. The heterologous prime-boost did not have a huge impact on the induction of PT nAbs 10 weeks after vaccination, whereas one-dose immunisation with adenoviral vectored vaccine stimulated higher level of neutralisation antibodies, especially six weeks after immunisation. However, these nAbs were not detected 10 weeks after the single-dose immunisation which could also be related to the stability and/or affinity of PT nAbs. A single dose of Ad vector is able to induce strong T-cell and B-cell responses (214), and Ad, MVA prime-boost should have induced affinity maturation. Here, our data

show that stimulation of functional antibodies did not occur, and it was therefore hypothesised that it was due to a wrong protein conformation.

In general, T-cell responses after one-dose immunisation with adenoviral vectors peak two weeks after the immunisation (211). Here, we found that the levels of Th1-related cytokine, IFN-γ, in the Ad S1-220- and Ad S1-235-immunised mice were the same two weeks after priming (Figure 3.6A). However, it was the Ad S1-235 vaccine that was partially protective in the mouse aerosol challenge model. The Th1-type response was measured two weeks after immunisation with Ad S1-220 and Ad S1-235 vaccine and at this timepoint, our data showed that both Ad S1-220 and Ad S1-235 vaccines induced PT-specific IgG2a antibodies suggesting the stimulation of Th1-type response. IgG2a antibodies are associated with production of complement-fixing antibodies associated with bacterial opsonisation. However, it is unlikely that PT is able to act as a bactericidal target since PT is a secreted antigen (55, 215, 216). It is the surface-bound antigens, such as fimbriae, or capsular polysaccharide in the *Meningococcus*, for example, that are able to mediate the complement fixing function of antibodies and opsonisation of bacterial cells (217).

We also assessed the immunogenicity and protective properties of adenoviral vectored vaccines expressing IRP1-3, AfuA, Fim and, AC and RTX domains of ACT. The IRP1-3 and AfuA candidate protein vaccines were found to be immunogenic and partially protective against pertussis infection in previous studies (105, 106, 141). In this study, the Ad IRP1-3 and Ad AfuA vaccines were also found to be immunogenic in mice (Figures 4.13 and 4.15, respectively), however, they were not protective against pertussis infection (Figures 4.14 and 4.16), and nor were the rIRP1-3 and

rAfuA proteins (Figures 4.14 and 4.16) produced in this study as protein vaccine comparators. This is in contrast with previous work in which both rIRP1-3 and rAfuA proteins induced partial protection against *B. pertussis* in mice (105, 106). The rIRP1-3 and rAfuA proteins were adsorbed to Freund's adjuvant in the previous study, however, we did not use Freund's adjuvant, in our study, due to the adjuvant reactogenicity observed in mice. Because of the high positive charge of AfuA (electrostatic point is 8.6) (218), we would not expect any binding of aluminium hydroxide to AfuA. With the use of aluminium phosphate, a negatively charged adjuvant, we could expect medium binding (due to fact that aluminium phosphate comparatively binds with lower affinity). For that reason, we selected aluminium phosphate adjuvant for immunisations with the rAfuA protein. The electrostatic point of IRP1-3 protein is not known therefore we used the most commonly used adjuvant, aluminum hydroxide, for immunisations with the rIRP1-3 protein. The vaccine adjuvants can affect protein conformation. For instance, adsorption to alum adjuvant may promote unfolding of a protein by setting up electrostatic and coordination bonds with the amino acid side chains and peptide bonds (219). The charge and the pH of alum supports electrostatic interactions with different antigens. At neutral pH, the positive surface charge of aluminum hydroxide and negative surface charge of aluminum phosphate facilitates electrostatic adsorption of negatively and positively charged antigens, respectively (220). Although, the aluminium phosphate adjuvant was used for rAfuA protein, based on the antigen's electrostatic point, the adjuvant may not have an effect on the exposure of protective epitopes in this study. We hypothesised that the same may apply to the aluminum hydroxide adjuvant adsorbed to rIRP1-3 protein.

One of the limitations of the protection study in which the Ad IRP1-3 and Ad AfuA vaccines were investigated, is that the *B. pertussis* 18-323 strain was different to the challenge strain used in previous published studies. In addition, we did not use iron-rich media to culture the challenge strain which could have contributed to poor expression of the iron-binding proteins on the bacterial cell surface and therefore the Ad IRP1-3 and Ad AfuA vaccines were not protective in the challenged mice. In addition, the Ad IRP1-3 and Ad AfuA vaccines were administered via the intramuscular route, whereas in the original study, the recombinant IRP1-3 and AfuA proteins were administered via the intraperitoneal route. Our aim was to assess the systemic immune responses and if the vaccines were taken to clinical trials, the intramuscular route would be used for human use. Although, the intraperitoneal route is often used in animal studies, we aimed to reflect the vaccine application in humans. Also, pharmacokinetics of vaccine administration via the intraperitoneal route resembles oral administration of the vaccine as the main route of absorption is into the mesenteric vessels, which drain into the portal vein and pass through the liver. Consequently, there is a possibility that the vaccine administered via the intraperitoneal route may be metabolised in liver before getting into the systemic circulation (221), however it is not always the case.

Fimbriae are involved in adherence to the host's ciliated epithelial cells and are another important virulence factor of *B. pertussis* (222). Although, the inclusion of fimbriae in aP vaccine formulations has been questioned, most clinical studies have demonstrated their importance in pertussis vaccines. The protective role of fimbriae was initially demonstrated in a study that compared 14 different batches of wP vaccines. The study found correlation between wP vaccine's protection in children and

agglutinating antibodies directed against fimbriae (216). Subsequent clinical studies in Sweden and Germany have shown that serum anti-Fim antibodies correlate with protection against pertussis in young children. Interestingly, children that were primed with 5aP vaccines, containing Fim2/3 antigens, had a strong antibody response against the antigens after infection, whereas children primed with 2aP vaccine, containing just PT and FHA, had less anti-Fim2 and anti-Fim3 antibodies than the 5aP-vaccinated group after infection (223). Cherry *et al.* (2010), also suggested that primary vaccination directs the immune responses against the vaccine's antigens including Fim2/3 and several antigens should be included in the primary vaccine formulation (224).

Fimbriae genes (Fim2, Fim3 and FimD) were inserted into the adenoviral vectored vaccines for the purpose of inducing stronger antibody responses against fimbriae, and cell-mediated immunity, than the current pertussis subunit vaccines. The T-cells could promote generation of functional antibodies that may inhibit the adhesin function of the fimbriae and therefore prevent or reduce *B. pertussis* attachment to the host cells. The Ad Fim2 and Ad FimD vaccines induced specific IgG antibodies able to bind *B. pertussis* strains producing Fim2 and FimD antigens, however, the binding were not as strong as with the Ad Fim3 vaccine. Only immunisation with Ad Fim3 vaccine was effective in eliciting antibodies binding to *B. pertussis* strain B1917 (strain expressing Fim3 only). This suggests that Fim3 was probably folded in a structure that is similar or identical to the native Fim3 antigen.

The Ad Fim3 construct was then assessed for protective efficacy in an aerosol challenge model because the vaccine induced better immune response than Ad Fim2

and Ad FimD constructs. The vaccine was partially protective against the B1917 bacterial strain (Figure 5.4A). Similar results were obtained in a study with a recently developed live attenuated BPZE1 vaccine which contains Fim3 and Fim2. The BPZE1 vaccine protected mice against lung colonisation by *B. pertussis* expressing Fim3 only. The protection against lung colonisation with the BPZE1 vaccine containing both Fim3 and Fim2 antigens was better than with BPZE1 vaccine containing only Fim2. This indicates the importance of Fim3 in the vaccine formulations and that this antigen may have more significant role against *B. pertussis* strains expressing Fim3 (225). The lower level of Fim2- and Fim-D IgG binding against the relevant bacterial strains could be explained by a decreased level of target epitopes on the bacterial surface, as compared with the amount of Fim3 expressed in strain B1917.

The limitation of this study is that we did not evaluate the T-cell responses in mice immunised with Ad Fim3 to find out if the vaccine induced Th1-type response and whether it correlates with the partial protection against *B. pertussis* B1917 strain. In a previous study, primary vaccination of babies with 5aP vaccine induced T-cell responses against PT and PRN but very low responses to fimbriae. However, one month after a boost with the aP vaccine, there was an increased production of IFN-γ cytokine against fimbriae in the vaccinated infants (226). It would be interesting to know if a two-dose immunisation regimen with Ad Fim3 vaccine is more protective in mice than the one dose, and if two doses of Ad Fim3 vaccine could elicit stronger Th1-type response, which is essential for clearance of pertussis infection, than the single dose.

ACT is an immunosuppressive virulence factor of *B. pertussis* which plays an important role in the initial stage of pertussis infection by inhibiting the host immune responses and promoting bacterial survival (193). ACT is made of the AC and RTX domains which complement each other's role to enable the toxin to assist in the establishment of pertussis infection. The RTX domain is immunodominant and induces strong neutralising antibodies in mice, indicating it could be a target for next-generation pertussis vaccines (130). Moreover, the RTX domain's protective potential was demonstrated in another study in which a mixture of a low-dose 3aP vaccine ($1/80^{th}$ human dose) and 5.6 µg of the RTX domain, followed by booster immunisation with the same vaccine 21 days later, improved protection against lung infection by *B. pertussis* UT25Sm1 strain in mice (102). We also selected the catalytic AC domain as a candidate antigen for viral vectored vaccines with the aim to inhibit delivery of the catalytic domain into the cytosol of target cells.

In this study, a single dose of Ad AC and Ad RTX vaccine was immunogenic and the immune responses were not increased by the second dose of the vaccine (Figure 6.5B and 6.5C). Both Ad AC and Ad RTX vaccines induced partial protection against lung infection in mice (Figure 6.6), however, some mice immunised with the Ad RTX vaccine were fully protected against the infection. These results indicate that the Ad RTX vaccine is a promising candidate to prevent pertussis infection. In addition, the protective response might be associated with the induction of ACT-specific nAbs as the functional antibodies are able to prevent binding to the $\alpha_M\beta_2$ integrin receptor (200).

8.1 Overall conclusions

The recent resurgence of pertussis cases has encouraged the development of new pertussis vaccines. This is the first study which has explored the use of Ad and MVA vectored vaccines against *B. pertussis.* The capacity of adenoviral vectored vaccines, even after a single dose, to elicit cell-mediated immunity as well as humoral immunity was clearly demonstrated in mice immunised with adenovirus vectors expressing regions of PT (Ad S1-220 and Ad S1-235). In addition, the Ad S1-235 vaccine was effective at stimulating partial protection against colonisation of lungs by pertussis in mice. The adenoviral vectored vaccines expressing Fim3, iron-binding proteins and AC and RTX domains of ACT were also immunogenic. The Ad Fim3 and Ad AC vaccines induced partial protection against pertussis infection in mice. Moreover, a single intramuscular immunisation with Ad RTX vaccine induced full protection against pertussis infection in 60% of immunised mice and partial protection in the remaining mice within the group. Perhaps, a combination of those partially protective vaccines together or maybe mixing of a commercial aP vaccine with each of these adenoviral vectored vaccines, would provide better, even full, protection against pertussis infection. The vaccine candidate antigens, especially those involved in partial protection, could also be further explored in other platforms like mRNA based vaccines. The mRNA-based vaccines have a larger range in a size of insert than Ad vectored vaccine and they can encode multiple antigens in a single formulation, thus targeting multiple antigens and enhancing immune responses (227). A recent head-to-head comparison of mRNA-based vaccine (the Pfizer-BioNTech BNT162b2) versus adenoviral vector vaccine (Oxford-AstraZeneca's ChAdOx1) against the Coronavirus 2019 (Covid-19) disease was conducted. The study showed that the mRNA-based vaccine is more protective than the adenoviral vectored vaccine (228). Another study

also showed that immunisation with the mRNA-based vaccine mediated higher levels of circulating binding and neutralising antibodies against the Spike protein than the adenoviral vectored vaccine five months after the immunisation. On the other hand, the study did not find significant differences between B-cell and T-cell responses induced by those vaccines (229). Furthermore, immunisation with non-viral delivered nucleic acid-based vaccines simulates immunisation with live-attenuated vaccines or infection, thus induces potent T-follicular helper and germinal centre B-cell immune responses (230).

Further investigations could also explore the efficiency of expression of the bacterial proteins following immunisation with the viral vectors. Post-translation modifications of bacterial proteins, and complex formation and membrane interactions influence many cellular processes such as protein synthesis, persistence, and toxin activity or virulence (231). Protein phosphorylation is an important post-translational modification in *B. pertussis* and the phosphorylation affects many cellular functions such as cell division, metabolism, stress response and virulence of the bacteria (232). Although vital vectors can deliver codon-optimised bacterial antigens to the eukaryotic host cells, the expression of the bacterial antigens may result in poor protein expression and/or incorrectly folded proteins due to the differences between prokaryotic and eukaryotic expression systems. By analysing the bacterial protein expression in mammalian cells, we might be able to learn the quality and level of the protein expression as well as folding of the protein into its correct conformation that would reveal the main protective epitopes. The expression level of a gene can be analysed by measuring the transcribed mRNA using northern blot method. The expressed protein can be also analysed by Western Blot or by directly staining the expressed

protein or mRNA while it is still in the cell. We could also use *in silico* prediction methods to map B- and T-cell epitopes in the antigens and explore how the epitope location and composition impact stimulation of B- and T-cells. Examples of the T-cell epitope prediction tools available online include EpiDOCK, MotifScan, PREDIVAC and many others. CEP, PEPITO and SEPPA are among many prediction methods that predict conformational B-cell epitopes (233).

Appendices

Recombinant IRP1-3 Protein Sequencing Using Tandem Mass Spectrometry

One sample was provided by Martina Kristof and a unique laboratory reference number was assigned to the sample:

- MS2020_128: 88 µg/mL, approx. 300 µL

For sample Preparation, LC-MS/MS analysis & data analysis, please see the report dated 20200920_MK_Report.

Database Preparation

The custom database used for searching including:

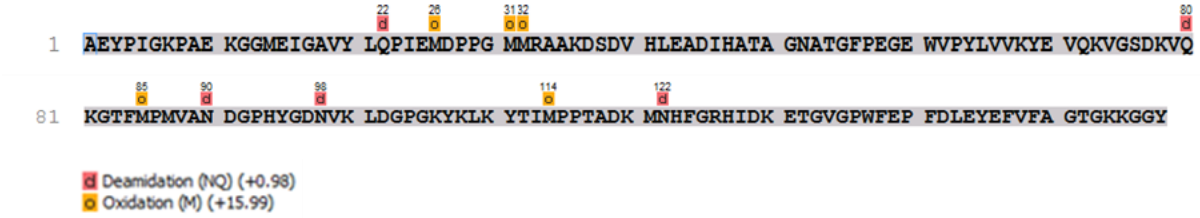
1. Amino acid sequence of recombinant IRP1-3 protein is provided by MK
2. UniProt *E. coli* database

AEYPIGKPAE KGGMEIGAVY LQPIEMDPPG MMRAAKDSDV HLEADIHATA GNATGFPEGE WVPYLVVKYE VQKVGSDKVQ

GTFMPMVAN DGPHYGDNVK LDGPGKYKLK YTIMPPTADK MNHFGRHIDK ETGVGPWFEP FDLEYEFVFA GTGKKGGY

Results

1. Amino acid sequence of the recombinant IRP1-3 protein is 100% matched to the sequence provided (see Figure below and details of identified peptides and peptide overlapping can be found in Appendix):



2. No other *E. coli* proteins were identified in the given sample.

20210407_Recombinant AfuA Protein Sequencing Using Tandem Mass Spectrometry

One sample (rAfuA after affinity chromatography purification) was provided by Martina Kristof on 24/03/2021 and a unique laboratory reference number was assigned to the sample:

- MS2021_011: 60 µg/mL, approx. 1000 µL

For sample Preparation, LC-MS/MS analysis & data analysis, please see the report dated 20200920_MK_Report.

Database Preparation

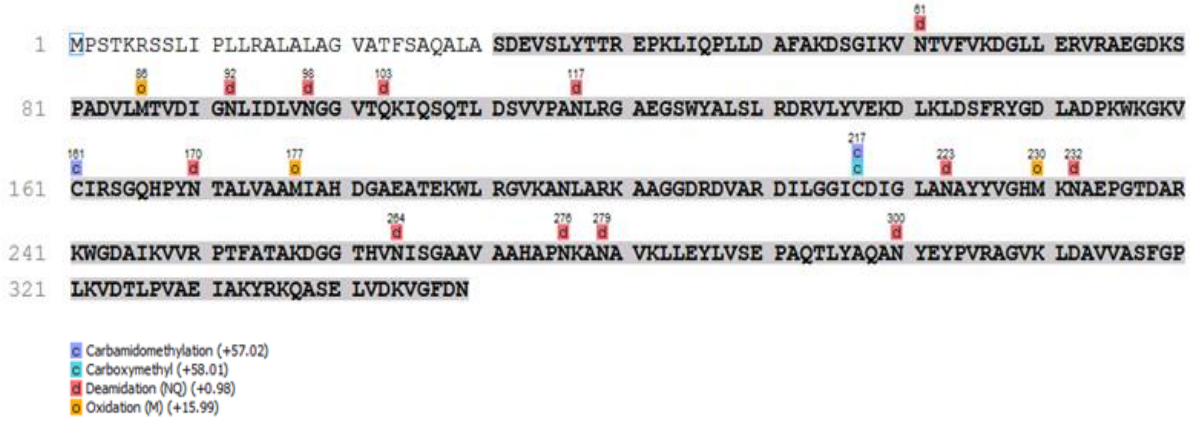
The custom database used for searching including:

3. Amino acid sequence of recombinant protein is provided by MK (highlighted is the signal sequence of the recombinant protein)
4. UniProt *E. coli* database as background

MPSTKRSSLIPLLRALALAGVATFSAQALASDEVSLYTTREPKLIQPLLDAFAKDSGI
KVNTVFVKDGLLERVRAEGDKSPADVLMTVDIGNLIDLVNGGVTQKIQSQTLDSVVP
ANLRGAEGSWYALSLRDRVLYVEKDLKLDSFRYGDLADPKWKGKVCIRSGQHPYN
TALVAAMIAHDGAEATEKWLRGVKANLARKAAGGDRDVARDILGGICDIGLANAYY
VGHMKNAEPGTDARKWGDAIKVVRPTFATAKDGGTHVNISGAAVAAHAPNKANAV
KLLEYLVSEPAQTLYAQANYEYPVRAGVKLDAVVASFGPLKVDTLPVAEIAKYRKQA
SELVDKVGFDN

Results

3. **Amino acid sequence of** the recombinant protein is 100% matched to the sequence provided excluding the message signal (see Figure below and details of identified peptides and peptide overlapping can be found in Appendix:



4. **Purity of sample** is 99.32%, *i.e.,* only 0.68% of proteins are from *E. coli*.

20210426_Recombinant Protein Sequencing Using Tandem Mass Spectrometry

Three samples (rAfuA after size exclusion chromatography and re-folding) were provided by Martina Kristof on 07/04/2021 and a unique laboratory reference number was assigned to the sample:

- MS2021_013_Sample26: 60 µg/mL, approx. 300 µL
- MS2021_014_Sample27: 60 µg/mL, approx. 300 µL
- MS2021_015_Sample28: 60 µg/mL, approx. 300 µL

For sample Preparation, LC-MS/MS analysis & data analysis, please see the report dated 20200920_MK_Report.

Database Preparation

The custom database used for searching including:

5. Amino acid sequence of recombinant protein is provided by MK
6. UniProt *E. coli* database as background

SDEVSLYTTREPKLIQPLLDAFAKDSGIKVNTVFVKDGLLERVRAEGDKSPADVLMT
VDIGNLIDLVNGGVTQKIQSQTLDSVVPANLRGAEGSWYALSLRDRVLYVEKDLKLD
SFRYGDLADPKWKGKVCIRSGQHPYNTALVAAMIAHDGAEATEKWLRGVKANLAR
KAAGGDRDVARDILGGICDIGLANAYYVGHMKNAEPGTDARKWGDAIKVVRPTFAT
AKDGGTHVNISGAAVAAHAPNKANAVKLLEYLVSEPAQTLYAQANYEYPVRAGVKL
DAVVASFGPLKVDTLPVAEIAKYRKQASELVDKVGFDN

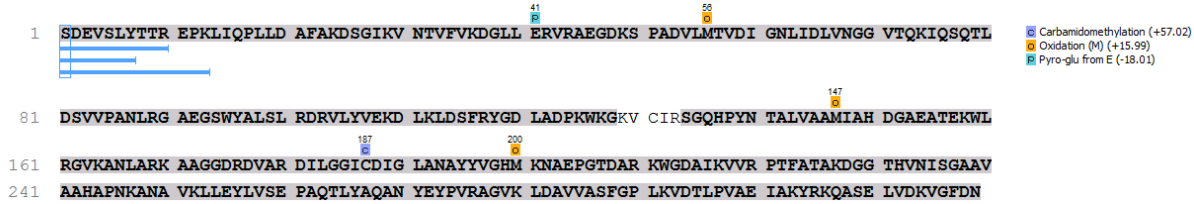
Results

1. **Amino acid sequence coverage**

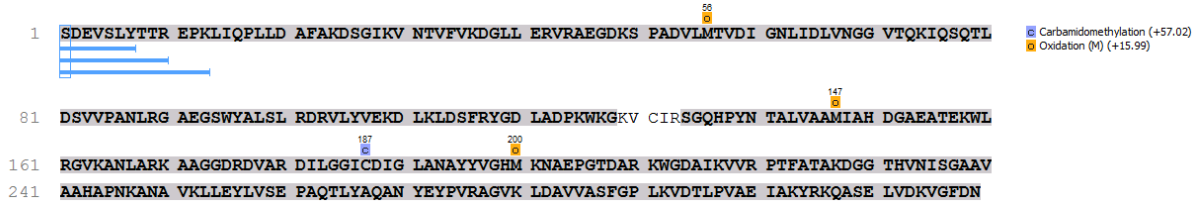
   Amino acid sequence of the recombinant protein is 98% matched to the sequence for all three samples provided except the sequence KVCIR (amino acid 129 – 133)* (see Figures below and details of identified peptides and peptide overlapping can be found in Appendixes).

   * For some unknown reason, Cysteine in the sequence KVCIR is not carbamidomethylated, hence KVCIR is missing from LC-MS/MS analysis.

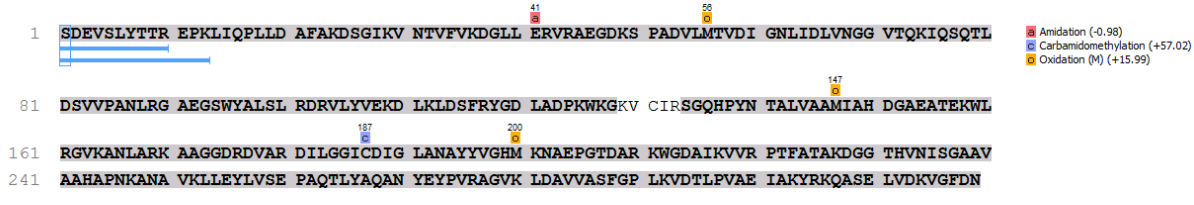
- ***MS2021_013_Sample26 amino acid sequence coverage is 98%***



- ***MS2021_014_Sample27 amino acid sequence coverage is 98%***



- ***MS2021_015_Sample28 amino acid sequence coverage is 98%***



2. Purity of sample

	MS2021_013_Sample26	MS2021_014_Sample27	MS2021_015_Sample28
% Sample	98.46	99.86	99.74
% E.coli	1.54	0.14	0.26

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