School of Biomedical Sciences

Live Metabolite-Deficient *Bordetella pertussis*: Determination of Suitability for use as a Vaccine in Humans using the Pertussis Mouse Model

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Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgement has been made. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

ABSTRACT

Bordetella pertussis, the aetiological agent of "whooping cough" an acute upper respiratory tract disease of humans, can infect all age groups with adolescents and adults acting as major source of transmission of this pathogen to infants. Transmission to children is facilitated by the infection in adolescents and adults not exhibiting being either asymptomatic or presenting as a mild but persistent ordinary cold-like illness. It is well established that both antibodies and cell-mediated immune (CMI) responses are crucial for protection against whooping cough, the former being important in the early phase of the disease and the latter being crucial for the long-term protection. Given that protection offered by vaccination with the currently-used acellular pertussis vaccines is due predominantly to antibodies induced by Th2-polarisation of immune response, there is urgent need to develop an alternative vaccine capable of inducing both antibody and CMI responses. One such alternative is the development of live attenuated pertussis vaccines because they mimic natural infections but survive in the host for only a relatively short period of time. Their survival is long enough however to potentially induce an immune response to all the virulence antigens yielding better and longer term protection. One such vaccine is a metabolite-deficient *B pertussis* in which the *aroQ* gene of the common aromatic biosynthetic pathway has been deleted (hereafter referred to as the *aroQ*BP vaccine). The *aroQ*BP vaccine, has been reported to colonise airways of mice efficiently and induce both humoral and cell mediated immunity and protect mice against aerosol challenge with virulent *B pertussis*.

The main goal of this study was to evaluate the potential of the *aroQ*BP vaccine candidate as an alternative vaccine to DTaP for both the primary and secondary vaccination in adolescents and/or adults using a mouse model system. Humoral immune responses were measured by estimating antibody specific immunoglobulin for *B pertussis* in mouse serum using enzyme-linked immunosorbent assay (ELISA). Cell mediated immunity was quantified by measuring INF-γ released from splenocytes stimulated *in vitro* with inactivated whole *aroQ*BP cells and filamentous haemagglutinin (FHA) as a model *B pertussis* protective antigen. The vaccination

schedules used were chosen to mimic, in general terms, the multiple immunisations to which human infants are subjected.

Secondary and tertiary immunisation with the *aroQ*BP vaccine of mice previously vaccinated with three sequential doses of the conventional DTaP vaccine resulted significant immune responses. Of these mice, those receiving two sequential doses of the *aroQ*BP vaccine manifested strong anamnestic responses relative to those mice receiving one vaccination with *aroQ*BP vaccine candidate. Further, a change in the balance of the immune response was observed from Th2 type (humoral) to an enhanced Th1 type (CMI) as assessed by the production of INF-γ.

It was discovered that primary immunisation of mice with *aroQ*BP vaccine candidate did not result in a significant immune response. However such mice displayed an anamnestic response to a secondary immunisation with *aroQ*BP. These mice produced higher levels of *B. pertussis* specific IgG, IgG1, and IgG2a immunoglobulin compared to mice receiving only a primary immunisation with *aroQ*BP vaccine as measured by immunoassay with inactivated *aroQ*BP whole cells (BPWC). These mice also manifested a measurable cell mediated immune response (INF-γ). This result clearly demonstrated the prior sensitisation of the mice with a single dose of *aroQ*BP vaccine.

These results in mice demonstrate that the *aroQ*BP vaccine is capable of eliciting CMI responses when used either alone or in conjunction with the conventional DTaP vaccine. Since DTaP vaccine alone induces only a predominant humoral immune response, *aroQ*BP is an ideal candidate for further genetic detoxification studies to render it suitable for trials in humans. Hence, if substantiated by further research, the *aroQ*BP vaccine will lead to improved vaccination protocols for both children and adults due to its demonstrated potential of complementing the humoral immune response to DTaP by induction of a persistent cell mediated immunity believed to be essential for long-term protection against whooping cough.

A cognate study was undertaken therefore to investigate genetic detoxification mechanisms for adenylate cyclase toxin (ACT), one of the key virulence exotoxins

produced by *B pertussis*. Genetic detoxification of ACT was attempted using two different Bordetella suicide vectors harboring a 1kb *cyaA* gene fragment modified by site-directed mutagenesis. The gene fragment sought encoded methionine in place of lysine at position 58 in the *cyaA* gene of the *B pertussis* genome. The mutant sought was not detected, however a clone showing a random substitution of lysine with asparagine was isolated. The toxicity of this clone with random mutation in the *cyaA* gene of ACT was not assessed due to time constraints. Strategies for overcoming the problems encountered in achieving genetic detoxification of ACT utilising site directed mutagenesis as well as the chances of accomplishment of this goal are discussed in this thesis.

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ABBREVIATION

AC	Adenylate cyclase
ACT	Adenylate cyclase toxin
AEC	Animal ethics committee
agrf	Australian genomic research facility
Amp	Ampicillin
aP	Acellular pertussis vaccine
*aroQ*BP	Metabolitic deficient *aroQ* non-reverting *B pertussis*
BG	Bordet-Gengou agar
bp	Base pair
BPWC	Whole inactivated *B pertussis aroQ*
BrK	*Bordetella* resistance to killing
C	Cytosine
cAMP	Cyclic adenosine monophosphate
CDMEM	Dulbecco's modified Eagle's medium
Ceph	Cephalexin
CL	Cyclodextrin liquid medium
CMI	Cell-mediated immunity
Co1E1	Colicin E1
CR3	Complement receptor type 3
CW	Cohen-Wheeler agar
DHB	Dihydroxybenzoic acid
DNA	Deoxyribonucleic acid
DNT	Dermonecrotic toxin
dNTP	deoxynucleotide triphosphate
DSMO	Dimethyl sulphide oxide
DTaP	Diphtheria and tetanus toxoids and acellular pertussis vaccine
dTaP	Acellular pertussis vaccine with reduced antigen content
EDTA	Ethylenediamine tetraacetate
ELISA	Enzyme linked immunosorbant assay
FBS	Foetal bovine serum
FFBP	Formalin-fixed *B pertussis*

FHA	Filamentous hemagglutinin
FIM	Fimbriae
G	Glycine
GFP	Green fluorescent protein
Gm	Gentamycin
Hly	Hemolysin toxin
HPV	Human papillovirus
i.n.	Intranasally
IBC	Institutional biosafety committee
Ig	Immunoglobulin
IL	Interleukin
INF-γ	Interferon-gamma
IPTG	Isopropyl Thiogalactoside
IU	International units
K	Lysine
kDa	Kilo Dalton
Km	Kanamycin
LA	Lura Britani agar
LB	Lura Britani broth
LPS	Lipopolysaccharide
M	Methionine
MCS	Multiple cloning cite
MPL	Monophosphoryl lipid A
N	Asparagine
NCBI	National center for biotechnology information
NK	Natural killer cells
OD	Optical density
ORF	Open reading frame
OSB	Orthosuccinylbenzoic acid
PABA	Para-aminobenzoic acid
PBS	1% autoclaved phosphate buffer saline
PBST	Tween-20 and PBS
PCR	Polymerase chain reaction

PHB	Para-hydroxybenzoic acid
Phe	Phenylalanine
PLG	Poly-lactide-co-glycolide
PRN	Pertactin
PT	Pertussis toxin
PTxoid	Inactivated pertussis toxin
RGD	Tripeptide sequence arginine, glycine, aspartate
RNase	Ribonuclease
rpm	revolution per minute
rspL	Streptomycin sensitive and gentamycin resistance
s.c.	Subcutaneously
SHD	Standard human dose
Sm	Streptomycin
Spp	Species
SS	Stainer-scholte medium
T	Thymidine
TAE	Tris-acetate EDTA
TCF	Tracheal colonization factor
TCT	Tracheal cytotoxin
Th1	T-helper cells type 1
Th2	T-helper cells type 2
Tris	tris (hydroxymethyl) aminomethane
Try	Tryptophan
Tween-20	Polyoxyethylene (20) sorbitan monolaurate
Tyr	Tyrosine
UV	Ultraviolet
VLA-5	Very late antigen-5
WHO	World Health Organization
wP	Pertussis whole cell vaccine
X-gal	5-bromo-4-chloro-3-indolyl-β-D-glactopyranoside
≈	Approximately

Chapter 1: Literature review

1.1. Introduction

Whooping cough was recognized as an acute respiratory disease of humans in the 16th century and the aetiological agent, *Bordetella pertussis*, was isolated and identified by Bordet and Gengou in 1906 (Cherry 1999). Although all age groups are susceptible to infection, the disease is pronounced in infants due to the severe systemic complications that can accompany the characteristic paroxysmal cough (Kerr and Matthews 2000). The severe and potentially fatal complications include convulsions, bronchopneumonia, and encephalopathy (Olson 1975). Protection, albeit claimed as variable in duration, can be conferred by vaccination with killed whole cell vaccines. As the recommended vaccination schedule does not commence until 2 months of age and the immune system remains immature throughout the first year of an infant's life, children less than 1 year-old being the highest risk group (Kerr and Matthews 2000). Typical pertussis, as defined by the World Health Organization (WHO) (Anonymous 2005) consists of the catarrhal, paroxysmal and convalescent phases. The catarrhal phase manifests as a cold-like illness with coryza and a mild cough with the onset of these symptoms usually 7 to 10 days after exposure. Whooping cough is highly contagious during the catarrhal phase. Proliferation of *B pertussis* and their subsequent abundance in nasopharyngeal secretions provides a means of aerosol transmission via droplet spread or direct contact (Kerr and Matthews 2000). Progression from a mild asymptomatic cough to convulsive or violent attacks (paroxysmal cough) indicates onset of the paroxysmal phase. Excessive mucous production, vomiting and lymphocytosis are additional manifestations that are commonly observed during this period of infection. A feature of the paroxysmal cough is the characteristic 'whoop' sound that occurs as a result of inhalation through a narrow glottis. Coughing attacks can occur frequently and can be so severe that exhaustion and apnoea result. This stage can last for up to 4 weeks, after which time the majority of sufferers enter a convalescent phase that is characterised by a gradual recovery. However, the severe cough can re-emerge in the presence of an opportunistic infection (Olson 1975).

Apart from the classical pertussis syndrome, there are several forms of an atypical infection. Although the cough is the defining symptom of pertussis, it has been reported that culture-positive neonates and infants have presented with symptoms such as apnoea and seizures in the absence of paroxysms (Kerr and Matthews 2000). Adolescents and adults do not exhibit the characteristic cough and infection can be either asymptomatic or manifested as a mild but persistent cold-like illness (Cromer et al. 1993; Birkebaek et al. 1999). *B parapertussis* can also infect humans but the disease state is often milder and far less common than the *B pertussis* aetiology, whereas the remaining *Bordetella* spp, *B bronchiseptica*, *B holmesii*, *B avium*, *B hinzii* and *B trematum* rarely cause infections in humans (Cherry 1999).

1.2. Epidemiology

Whooping cough, caused by *B pertussis*, has the most severe consequences in infants and children and is one of the ten most common causes of death from infectious disease (McIntyre and Wood 2009). Although vaccination with either the whole cell (wP) or acellular pertussis (aP) vaccines, administered in combination with diphtheria and tetanus toxoids (DTwP or DTaP), has reduced morbidity and mortality significantly, pertussis remains an endemic disease. It is the major cause of vaccine-preventable deaths today, with WHO estimates of 40-50 million cases and approximately 297,000 - 409,000 death each year worldwide (Anonymous 2005; Elahi, Holmstrom, and Gerdts 2007; Fennelly et al. 2008), albeit majority of them being in developing countries. The incidence of pertussis was clearly influenced by the advent of effective prophylactic and therapeutic intervention in the mid 1940's. During the pre-vaccine era, pertussis was highly endemic with cyclic epidemics that occurred every 2 to 5 years (Cherry 1999). Although the introduction and wide-spread use of the pertussis vaccines caused a dramatic reduction in the incidence of pertussis, the incidence of whooping cough has risen recently despite high vaccine coverage in developed countries such as Australia, Canada, the United States and the Netherlands despite high levels of immunization rates (Mooi, Van Loo, and King 2001; de Melker et al. 1997). In Australia, pertussis has been endemic since 1993 with notifications rising from 1.8/100,000 population in 1991 to a peak of 127.8/100,000 in 2009 despite a high rate of vaccine coverage (Anonymous 2009).

Recently, incidence of this disease in adolescents and adults has become an area of increasing interest over the last decade because of the obvious consequence of undetected and untreated pertussis, although not often clear, in the mature age groups to transmit pertussis to other adults, and more importantly to highly susceptible infants (Cagney et al. 2008). The recent deaths attributed to pertussis in Australia have been restricted to infants under 2 months to less than 1 year of age (Andrews, Herceg, and Roberts 1997). Although these infants were too young to be vaccinated, it was concluded that a reduction in the level of pertussis within the community, particularly from reservoirs such as asymptomatic adults would have diminished the risk of exposure.

There have been numerous suggestions as to the reasons for the recent and widespread re-emergence of pertussis. Although a number of factors such as increased public awareness, improvement in notification accuracy, decreased or delayed vaccine coverage and a reduction in vaccine quality could be applicable for developing countries, these possibilities have been largely excluded for the industrialised countries (Kolos, Menzies, and McIntyre 2007). The adaptation of *B pertussis* to vaccination has been suggested as a reason for the rise in developed countries. Mooi et al. (2001) DNA fingerprinted Dutch strains collected from 1949 to 1996 and found notable changes between the pre-vaccine and post-vaccine populations of *B pertussis*. This divergence was observed as polymorphisms within the pertussis toxin and pertactin antigens, both of which are dominant immunogens and components of acellular vaccines.

More recently, pertussis has re-emerged even in vaccinated populations confirming that pertussis is not only a childhood disease but is also highly prevalent in adults (Cagney et al. 2008; He and Mertsola 2008), latter being accepted now as the major reservoir of infection for majority of the pertussis cases in infants and young children (Cagney et al. 2008). Several reasons offered to explain the increasing incidence of this disease syndrome in adolescents and adults include better diagnosis, cyclic variation in disease patterns, waning of vaccine-induced immunity in adolescents and adult over time due to the limited protection offered by the currently used vaccines (Mooi, Van Loo, and King 2001; von Konig et al. 2002; Cherry 2003; Mills 2001) and loss of vaccine efficacy due to the emergence of new *B pertussis* strains

overproducing pertussis toxin (Mooi et al. 2009). It is therefore apparent that the immunisation of the adolescent and adult population is one aspect that needs addressing to control the spread of pertussis. The increased risks of potential side effects, excessive cost of expanded vaccination programs particularly in developing countries and the perceived mild nature of the disease in adults are factors that have hindered the notion of vaccination beyond infancy (Cherry 1999; Roduit et al. 2002; Singh and Lingappan 2006).

B pertussis is a complex and highly adapted non-invasive respiratory pathogen, which localises to the tracheobronchial tree and produces a large array of potential virulence factors including toxins, outer membrane proteins and other virulence determinants, which contribute to the disease process and immune system subversion (Fennelly et al. 2008). Clearly, an ideal effective pertussis vaccine should be able to neutralise or abrogate the effects of all the virulence factors associated with the pathogenesis of pertussis as well as induce, considered to be important in imparting long-term protection against whooping cough (Mills 2001; Mahon, Brady, and Mills 2000). However, no such vaccine capable of fulfilling this attribute has been developed and hence the urgency to develop an improved pertussis vaccine capable of delivering this desired outcome.

1.3. Virulence factors

B pertussis produces an array of virulence factors (Table 1), some of which are considered to play a key role in the establishment of *B pertussis* infection. These include filamentous hemagglutinin (FHA), pertactin (P69), pertussis toxin (PT), adenylate cyclase toxin (ACT) (Mills 2001; Weiss and Hewlett 1986), with other factors such as fimbriae, dermonecrotoxin, tracheal cytotoxin, tracheal colonisation factor, serum resistance factor and lipopolysaccharide also playing important roles leading to clinical manifestations of pertussis.

Table 1.3. Accredited virulence factors of *B pertussis.*

Factor	Molecule	Major role	Inclusion in Pa
Pertussis toxin (*ptx)*	A and B subunits	Toxin, ADP ribosylation of host G-proteins and adhesion factor; some disease symptoms	Yes, as inactive toxin
FHA (*fha*)	Large filamentous protein (220DA)	Adhesion factor; predominantly in trachea	Yes
Fimbriea 2 & 3 (*fim2, fim3*)	Filamentous proteins composed of subunits (~23kDa)	Adhesion factor; predominantly in trachea	Yes
Pertactin (*prn*)	69kDa OMP	Adhesion factor	Yes
Adenylate cyclase (*cya*)	Protein toxin	Inhibits phagosytisis	NO
Tracheal cytotoxin (*tct*)	Peptidoglycan derivative	Toxin; paralyses mucociliary clearance	NO
Dermonecrotic toxin (*dnt*)	Heat-labile toxin (140kDa)	Dermal necrosis and	NO
Tracheal colonization factor (*tcfA*)	Proline-rich protein	Cytotoxin; Adhesion factor; predominantly in the trachea	NO
Bordetella resistance to killing factor (*brk*)	OMP (32kDa)	Resistant to classic-complement killing	NO
Lipopolysaccharide		Endotoxin; Resistance to host defence molecules	NO

1.3.1. Filamentous hemagglutinin (FHA)

Filamentous hemagglutinin (FHA), a large 220-kDa outer membrane-associated protein, is one of the earliest protein expressed by *B pertussis* that is detected within few minutes of infection (Scarlato et al. 1991). It binds to the complement receptor type 3 (CR3) on surface of macrophages via conserved a tripeptide sequence RGD (arginine, glycine, aspartate) (Relman et al. 1990) and induces secretion of IL-6 and IL-10. These cytokines down-regulate production of IL-12 from the cells leading to a delayed T-cell response in the lungs of mice (McGuirk et al. 1998). Further, FHA is implicated in suppression of Th1 cell-mediated response by inhibiting IL-12 production upon infection with *B pertussis* potentially leading to waning of host immunity against pertussis (Preston 2005).

The diminished ability of an FHA-deficient mutant to adhere *in vivo* or to *in vitro* cultured epithelial cells, and the ability of anti-FHA antibodies to inhibit the attachment of virulent microorganisms indicates an important role for FHA in the adhesion of *B pertussis* to ciliated epithelia (Urisu, Cowell, and Manclark 1985; Simondon et al. 1998). High titers of anti-FHA antibodies have been detected in human convalescent sera (Simondon et al. 1998) and are elicited to protective levels following immunisation with commercial pertussis vaccines (Mills et al. 1998). Similarly, antibodies generated against purified FHA have been shown to be protective in animal models (Sato and Sato 1984) and in human clinical trials (Edwards et al. 1995).

1.3.2. Pertactin (PRN)

Brennan et al. (1988) identified a 69-kDa membrane-associated protein (P.69 or pertactin) using a library of monoclonal antibodies generated against the surface antigens of *B pertussis*. Unlike FHA-deficient mutants, *B pertussis* mutants deficient in pertactin were not inhibited in attachment to ciliated respiratory epithelial cells (Roberts et al. 1991). However, the identification of an RGD tripeptide, which is present in other bacterial adhesins, including FHA, indicated that pertactin may also have a role in attachment to eukaryotic cells (Leininger et al. 1991).

As with FHA, pertactin is also a potent immunogen of *B pertussis*. Anti-P69 antibodies with bactericidal and opsonic properties have been detected in the serum of patients recovering from infection, whilst high titers of serum antibodies have been observed following immunisation with the wP (Hellwig et al. 2003; Thomas, Redhead, and Lambert 1989). In addition, De Magistris et al. (1988) demonstrated that P69 was recognised by *B pertussis*-specific T cell clones isolated from a convalescent donor. However, thirteen variants of PRN through genotyping have been identified (Mooi et al. 2000), variation being mainly restricted to the N-terminus (Hijnen et al. 2007).

1.3.3. Pertussis toxin (PT)

The term pertussis toxin was coined by Margaret Pittman (Pittman 1979). All *B pertussis, B parapertussis* and *B bronchiseptica* have genes for encoding the toxin but only *B pertussis* is able to produce functional PT (Arico et al. 1987). Other names that have been suggested for the toxin include lymphocytosis-promoting factor, islet-activating factor, and pertussigen (Munoz, Arai, and Cole 1981). Regarded as the central virulence factor during infection, pertussis toxin elicits a number of deleterious consequences including leukocytosis, splenomegaly, histamine sensitisation, hypoglycaemia and hypoproteinemia (Hewlett 1999). PT is an exotoxin composed of five subunits (S1- S5) arranged in a manner that is consistent with the A-B family of toxins (Locht and Antoine 1995). Subunits S2, S3, S4 and S5 present in a 1:1:2:1 ratio forms the B oligomer, which has been demonstrated to provide two functions: receptor-mediated binding to the surface of target cells and translocation of the catalytic domain across the plasma membrane. In addition to upregulating macrophage integrin CR3, which acts as a receptor for FHA, it acts as a toxin by ADP ribosylating G regulatory proteins inhibiting their coupling to receptors of intracellular transduction pathways, and causing blockage of phosphatidyl inositol hydrolysis, arachidonate release and calcium mobilisation (Locht and Antoine 1995; Weiss and Hewlett 1986). *B pertussis* is the only member of the genus that produces the ADP-ribosylating exotoxin which emphasises the importance of pertussis toxin considering that infection with *B parapertussis* often results in a milder or atypical whooping cough syndrome (Linnemann and Perry 1977). The presence of anti-PT antibodies in convalescent sera and the detection of high titers following

immunisation with the wP vaccine provided an indication of the protective potential of PT (Trollfors et al. 1999). The subsequent development and assessment of acellular pertussis vaccines has provided further insight into the immune response and protective efficacy of PT. Phase 1 and 2 clinical trials of a mono-component pertussis toxoid vaccine revealed a statistically significant correlation between toxin neutralising antibodies and protection against pertussis (Taranger et al. 2001). However, the protection observed with the monocomponent vaccine was only partial (Trollfors et al. 1995).

1.3.4. Adenylate cyclase toxin (ACT)

Glaser et al. (1988) identified an 8.7 kb DNA fragment from a *B pertussis* recombinant gene library, which encoded a calmodulin-sensitive adenylate cyclase. Sequence analysis of the cloned DNA revealed an open reading frame consisting of five genes arranged in an operon, denoted as *cya*. The *cyaA* gene encodes an inactive protoxin that exhibits AC activity but is devoid of haemolytic or cell-invasive activity (Sebo et al. 1991). An alcyltransferase encoded by an upstream *cyaC* gene activates the protoxin by palmitolyation of the lysine (K) 983 residue, which confers the invasive and haemolytic activities that are absent in the precursor. Considering the absence of an N-terminal signal sequence in the protoxin, the three accessory genes downstream of *cyaA* (*cyaB*, *cyaD* and *cyaE*) have a postulated role in secretion of the active toxin (Hanski 1989).

The adenylate cyclase-hemolysin toxin (AC-Hly) performs a crucial role in lung colonisation and the establishment of infection. The C-terminal haemolytic domain penetrates mammalian phagocytes (neutrophils, monocytes, NK cells and macrophages) and facilitates translocation of the N-terminal adenylate cyclase across the plasma membrane, which subsequently catalyses the conversion of the energy-deficient cyclic adenosine monophosphate (cAMP) from the high-energy compound adenosine triphosphate (Weiss and Hewlett 1986). A unique feature of AC-Hly is its interaction with the eukaryotic regulatory protein calmodulin, which increases the catalytic activity of the toxin up to a thousand-fold (Weiss and Hewlett 1986). The dramatic reduction of ATP within the infected phagocyte causes a severe impairment of normal cellular functions, arising from an inadequate supply of cellular energy

(Sebo et al. 1991). The resulting inhibition of a phagocyte response is an essential factor in the establishment of infection considering that mutants defective in AC-Hly production have been shown to be avirulent, unable to proliferate, and are rapidly cleared from the lungs (Weiss et al. 1984).

Phagocytosis and killing of *B pertussis* by neutrophils is an effective innate defence mechanism against infection. Lenz et al. (2000) used *B pertussis* labelled with green fluorescent protein (GFP) to demonstrate that up to 99 % of the bacteria phagocytosed by neutrophils are killed following internalisation. In view of the inhibitory effect of AC-Hly on neutrophils, the generation of toxin neutralising antibodies should enhance phagocytosis and thus the clearance of *B pertussis*. High titers of anti-AC-Hly antibodies have been detected in both the sera of convalescent patients and in adults and children immunised with the whole cell vaccine (Farfel et al. 1990). Although the neutralising potential of antibodies present capable of neutralising toxin *in vitro* was not determined, the protective humoral response elicited by immunisation of mice with native and recombinant AC-Hly suggested that neutralising polyclonal antibodies generated *in vivo* are equally effective in enhancing phagocytosis as the *in vitro* assay using monoclonal antibodies (Hormozi, Parton, and Coote 1999).

1.3.5. Fimbriae (Fim)

These are long filamentous protrusions, with helical structure of pentameric repeat units, extend which permit *B pertussis* to bind to their target cells (Steven et al. 1986). *B pertussis* possesses two serotypes fimbriae. The major fimbriae, Fim2 and Fim3, (22.5 and 22 kDa), are encoded by *fim2* and *fim3,* respectively (Locht, Geoffroy, and Renauld 1992; Willems, van der Heide, and Mooi 1992). The major fimbriae helices are bundled to form long filaments with minor subunit FimD on their tip as epitopes (binding site to perspective receptor on host cell). FimD is expressed from *fimD* (Geuijen et al. 1997). FimD is ligand for very late antigen-5 (VLA-5) on monocytes and macrophages and up regulates the expression of CR3 on the cells (Geuijen et al. 1997; Hazenbos et al. 1995). Fim2 and Fim3, have been shown to mediate the binding of non-opsonized *B pertussis* to host cells (Locht, Geoffroy, and Renauld 1992; Willems, van der Heide, and Mooi 1992). FimD has been shown to bind to sulphated

sugars, which is ubiquitous on the surface of respiratory cells and secretions (Geuijen et al. 1997). *FimX* and *fimA* are considered pseudogenes related to major fimbriae subunits (Willems, van der Heide, and Mooi 1992; Pedroni et al. 1988). In *B pertussis* fimX is expressed at a very low level and *FimA* gene is a silent gene (Boschwitz et al. 1997).

The minor fimbriae genes in *B pertussis* include *fimB, fimC* and *fimD* (Locht, Geoffroy, and Renauld 1992; Willems, van der Heide, and Mooi 1992). FimB is transported to the periplasm after cleavage and maturation whereas FimC is involved in anchorage of *pertussis* to its host cells and acts as the molecular syringe for *B pertussis* to inject toxins into neutrophils and macrophages.

Although the complete role of fimbriae in pathogenesis remains unclear, deletion mutants are less persistent than the wild type (Mooi et al. 1992). Fim2 and Fim3 have been incorporated into some licensed acellular vaccines; however their inclusion does not appear to augment the protective efficacy of DTaP (Mills et al. 1998). In contrast, an early clinical trial of a pertussis whole cell vaccine (WCV) in the UK found a correlation between serum agglutinin titers and protection against whooping cough in infants (Anonymous 1956). Of these serum agglutinin titers, antibodies to FIM were found to be main contributors, along with antibodies to pertactin (van den Berg et al. 1999).

1.3.6. Dermonecrotic toxin (DNT)

Identified by Bordet and Gengou in 1909, DNT was one of the first known virulence factors of *B pertussis*. DNT is a heat-labile toxin that when injected intradermally causes necrotic lesions in animals and has been observed to be lethal in mice when given intravenously (Livey and Wardlaw 1984). It is a single polypeptide of 140 kDa that appears to be cytoplasmic rather than secreted (Matsuzawa et al. 2004). It is believed to cause inflammation, vasoconstriction and lesions around the areas of colonisation but the precise mode of action is not well understood. In fibroblastic cell lines, DNT has been reported to inhibit both alkaline phosphatase activity and expression of type-I collagen and to also stimulate DNA and protein synthesis in the

absence of cell division, resulting in polynucleation (Matsuzawa et al. 2004). Unlike other virulence factors, a deficiency of DNT in a mutant strain did not affect its virulence or pathogenicity compared to the parent wild type (Weiss and Goodwin 1989).

1.3.7. BrkA proteins (Bordetella resistance to killing)

The *brk* locus was identified as a source of resistance following the characterisation of a Tn5-induced mutant that showed a dramatic increase in sensitivity to immune serum (Weiss et al. 1983). Subsequent cloning and sequencing of the locus revealed two open reading frames (Fernandez and Weiss 1998), the *brkA* ORF encoding a 103-kDa precursor that is processed at the C-terminal domain to yield the mature 30-kDa BrkA, a putative outer membrane protein, and *BrkB* which encodes a protein predicted to be a cytoplasmic membrane protein. Both BrkA and BrkB proteins have been implicated in this property.

The importance of BrkA to pathogenesis has been demonstrated by two investigations. Weiss & Goodwin (1989) found that a *brkA*-depleted mutant had significantly decreased virulence in mice compared to a wild type strain. It was later demonstrated that neutralising antibody to BrkA enhanced the bactericidal activity of *B pertussis* anti-serum (Oliver and Fernandez 2001).

1.3.8. Tracheal cytotoxin

Tracheal cytotoxin (TCT) is believed to be responsible for destruction of ciliated tracheal epithelial cells, an early and significant feature in the onset of whooping cough (Parton 1999). Although the precise mechanisms of epithelial damage have not yet been determined, the cytopathic effect of *B pertussis* infection has been mimicked *in vitro* by the addition of purified TCT to cultured tracheal epithelial cells (Cookson et al. 1989). Characterisation of TCT revealed that it is a soluble fragment of cell wall peptidoglycan (Rosenthal et al. 1987) composed of *N*-acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramyl-(L)-alanyl-γ-(D)-glutamyl-*meso*-diaminopimelyl-(D) alanine, a small 921Da disaccharide-tetrapeptide monomer (Cookson et al. 1989). Of

interest is the range of biological activities that these muramyl peptides exhibit in addition to cytotoxicity, such as adjuvanticity, pyrogenicity, arthritogenicity and modulation of cell-mediated immune responses (Adam and Lederer 1984).

1.3.9. Tracheal colonization factor (TCF)

This factor is encoded by the *tcfA* gene and is important in the pathogenesis of pertussis as demonstrated by the fact that infection of mice with *B pertussis* lacking this protein strains is decreased 10-fold as compared to the parent strain (Finn and Stevens 1995). Based upon the derived amino acid sequence, TCF is a 64kDa proline-rich protein that contains the RGD motif shows 50% identity with the pertactin precursor. However, *B pertussis* culture supernatants reveal the 60 kDa form of the secreted protein. This protein is unique to *B pertussis* since it is not present in either *B bronchiseptica* and *B parapertussis*.

1.3.10. Lipopolysaccharide

As with the endotoxin of other gram-negative bacteria, the lipopolysaccharide (LPS) of *B pertussis* is pyrogenic, mitogenic and toxic to host cells (Watanabe et al. 1990). *B pertussis* LPS is composed of a lipid A portion linked to a branched oligosaccharide core and a trisaccharide and is structurally unique from other *Bordetella* spp. in that it lacks a repetitive O antigen (Peppler 1984). Allen and Maskell (1996) identified the 12-gene *wlb* locus that was required for the biosynthesis of the trisaccharide component. Mutants with targeted disruptions to these *wlb* genes were shown to be defective in colonisation of the respiratory tract compared to a wild-type strain (Harvill et al. 2000). These mutants were also significantly more sensitive to antibody-mediated killing *in vitro*, which indicated that LPS, in particular the trisaccharide component, may provide the organism with an additional means (other than BrkA) for resistance to the classical complement pathway. LPS-specific IgA and IgM have also been detected in the serum of infected or convalescent children (Harvill, Cotter, and Miller 1999).

1.3.11. Type III secretion

Twenty type 3 secretion protein homologues similar to those in *B bronchiseptica*, *Yersinia* and *Pseudomonas* in select physicochemical properties have been amplified from *B pertussis* but are not expressed (Kerr and Matthews 2000; Preston 2005). The potential role of the protein homologues in virulence is not known.

1.4. History of whooping cough prophylaxis

Whooping cough was responsible for widespread morbidity and mortality during the pre-immunisation era. In response to the high incidence and severity of the disease, a whole cell vaccine for whooping cough was developed, tested and combined the whole-cell pertussis vaccine with diphtheria and tetanus toxoids to generate the first DTwP combination vaccine (Baker and Katz 2004). Many different types of wP vaccine formulations containing varying numbers of microorganisms, killed using different methods and delivered in 2 or 3 doses, have been evaluated with different efficacies claimed or analysed to range from 14-59% with 2 doses to 55-85% for 3 dose vaccination regime in cohort studies (Storsaeter et al. 1998; Fine and Clarkson 1987). In another analysis, absolute efficacy of the whole cell pertussis vaccine was found to vary from 37-92% with the efficacy of the 1 and 2-component acellular pertussis vaccine at 67-70% versus 80-84% for ≥3- component vaccines (Jefferson, Rudin, and DiPietrantonj 2003). To overcome this variability, a standard dose of 4.0 International Units (IU)/standard human dose (SHD) based on potency determined using the Kendrick test has been suggested (World Health Organization 2007).

Although the incidence of pertussis disease quickly diminished following introduction of the DTwP vaccine, a gradual reduction in wP vaccine compliance and coverage due to the perceived risk of severe side effects resulted in the subsequent re-emergence of whooping cough (Frits R. Mooi 2001; Andrews, Herceg, and Roberts 1997; He and Mertsola 2008). Observed side effects, attributed to the wP component of the DTwP vaccine included fever, prolonged crying, irritability, rare neurologic side effects (Donnelly et al. 2001) most likely associated febrile seizures particularly

if the body temperature is not kept under surveillance, hypotonic-hypo-responsive episodes and local reactions at the site of injection (Mills 2001; Poland 1996; Rowe et al. 2005). LPS has been implicated, albeit without any definitive evidence, as a possible cause of the wP-mediated reactogenicity (Rowe et al. 2005).

In response to public uncertainty surrounding the wP vaccine, research was focussed on the development of an alternative and safer prophylactic. Various aP vaccine, which consisted of a combination of the immunogenic antigens filamentous hemagglutinin, pertussis toxoid, pertactin with or incorporation of fimbrial antigens have been evaluated in mice and humans. Favourable results from various human clinical trials led to the acceptance of the aP vaccine, which is delivered in combination with the established tetanus and diphtheria toxoids, to produce the contemporary triple antigen vaccine, referred to as DTaP. The currently marketed vaccines may also contain additional vaccine candidates including hepatitis B, *Haemophilus inflenzae* type B and inactivated poliovirus.

Since the introduction of DTaP, numerous studies have evaluated and compared the efficacy and safety of the acellular versus the whole cell vaccine. The consensus was that aP vaccines conferred a level of protective immunity comparable to that of wP vaccines but with a decrease in the incidence and severity of adverse side reactions (Fine and Clarkson 1987; Novotny et al. 1991; Keitel 1999; Gustafsson et al. 1996). As with many other vaccines, local side reactions were observed in infants following multiple booster doses of the aP vaccine, with reportable adverse events following immunization over a period of 7 years (2000-2006) ranging from 3.8 per 100,000 population (Lawrence et al. 2007). These side effects were reported elsewhere to be due to reactivation of Th2-associated local reactions (Rowe et al. 2005; Decker et al. 1995) in which study large local reactions [≥ 50-mm diameter] were recorded, 24-72 hours after delivery of the DTap booster, in 43% of exclusively DTaP-primed children, in contrast to 6% of those primed with DTwP. There are currently no published data on the incidence of local or systemic reactions in adolescents or adults boosted with dTap (see below). All these studies indicate that side effects do occur but the benefits of vaccination outweigh the risks recorded. It is thus clear research

aimed at developing an entirely side reaction free pertussis vaccine must be carried out.

However, it has been reported that *B pertussis* is capable of infecting individuals previously vaccinated with either the wP or aP. Storsaeter et al. (1998) reported that 33% of individuals that received the licensed five-component acellular vaccine had evidence of post-immunisation infection following exposure to *B pertussis*. In the same study, 82% of individuals immunised with wP were protected. One potential reason for the observed variance may be the composition particularly of the acellular pertussis vaccines containing different and/or variable quantities of the potential protective antigens. Different vaccines evaluated have ranged from one protective antigen viz., chemically inactivated pertussis toxin (PTXoid) to 2 (PTXoid + FHA), 3 (Chemically or genetically inactivated PTXoid + FHA + PRN) or 4-5 potential protective antigens (PTXoid + FHA + PRN + Fim 2 and/or Fim3), which information is available in a communication reported elsewhere (Edwards et al. 1995). Reviews describing the concentrations of the different potential protective antigens in different vaccines in the marketplace, suboptimal vaccines and potential reasons for waning immunity was published recently (Edwards et al. 1995; Berbers, de Greeff, and Mooi 2009).

Although the duration of immunity imparted by both the wP or aP has been claimed not appear to differ markedly (Wendelboe et al. 2005; Guiso et al. 2007), of particular concern has been the recent upward trend in the global incidence of pertussis with a six-fold increase in reported cases over the last two decades (He and Mertsola 2008; Cherry et al. 2005; Tan, Trindade, and Skowronski 2005). The fact that young adults (vaccinated during their childhood) with none or waning immunity against whooping cough may serve as a reservoir for the pathogen and pass it on to their children (He and Mertsola 2008), has stimulated interest in the development of an alternative vaccine which can also be used safely in the adolescent and adult populations. To overcome this problem, acellular pertussis vaccine formulations with reduced antigen content, which was recently reported to induce cellular immunity in adolescents and adults (Meyer 2007), for use in adolescents have been introduced. Although the effectiveness of this vaccine [dTap] in reducing the transmission of pertussis to children is not known, in a clinical trial the vaccine efficacy of the DTwP for transmission of infection to children vaccinated with three doses of the whole cell

vaccine was 85% in comparison with 6%, for the DTaP vaccine indicating the ineffectiveness of the latter (Preziosi and Halloran 2003). Evaluation of the "cocooning" strategy (Berbers, de Greeff, and Mooi 2009; McIntyre and Wood 2009) in which parents and carers of the newborns are recommended to receive dTap vaccine around the time of the birth may help clarify the effectiveness of this approach in reducing pertussis transmission to vulnerable infants.

1.5. Potential mechanisms underpinning Immunity to infection with *B pertussis*

1.5.1. Importance of antibody-mediated immunity against *B pertussis*

Most investigations on the potential mechanisms of protection against infection with *B pertussis* have been carried out using mouse as model system. Both wP and aP vaccines confer protection against virulent *B pertussis* following immunisation (Gustafsson et al. 1996; Cherry et al. 1998). In some clinical trials with DTaP, a direct correlation between a serological antibody response and protective immunity could not be verified (Cherry et al. 1998), whereas in the mouse model the importance of a humoral immune response against pertussis was clearly evident (Sato and Sato 1984). Nevertheless, it is now accepted, and as will become evident from the description in the text below, that wP and aP vaccines elicit different clinical manifestations in humans versus animal models although the immune responses are similar.

Although great emphasis has been placed on the induction of a potent antibody response by traditional pertussis vaccines, there have been contradictory views on the contribution of humoral response to protection against *B pertussis*. Passive or active immunisation experiments, described below, in immunocompetent versus transgenic mice have clearly demonstrated an important role for specific antibodies against pertussis.

Transient protection was conferred against an aerosol or intracerebral challenge of mice by administration of monoclonal antibodies directed against the either the S1

subunit of pertussis toxin (Sato and Sato 1984) or pertactin (Shahin et al. 1990), with anti-FHA being the least potent (Sato and Sato 1984; Bruss and Siber 1999). Granstrom et al. (1991) reported a significant reduction of whoops in infected patients that received a hyperimmune serum preparation raised against a two component acellular vaccine compared to a placebo if the antibodies were administered within 7 days of exposure.

Aside from transient protection imparted by passive immunization with monoclonal or polyclonal antibodies, research involving transgenic mice models revealed that unlike wild-type mice, B cell and antibody deficient ($Ig^{-/-}$) mice developed a chronic infection and failed to clear bacteria from the lungs after experimental infection (Mills et al. 1998; Mahon et al. 1997). Furthermore, immunisation of $Ig^{-/-}$ mice with either whole-cell or acellular pertussis vaccines failed to confer protection against an experimental challenge but protection was restored following the transfer of primed B cells. These observations were supported by another study (Leef et al. 2000) in which intranasal immunisation of $Ig^{-/-}$ mice with formalin-fixed *B pertussis* (FFBP) was reported to provide partial protection against an aerosol challenge compared to complete protection in FFBP-immunised wild-type mice. Once again full protection was restored following transfer of immune B cells.

The effectiveness of the acellular vaccine provides a strong indication of the importance of humoral immunity. Parenteral immunisation of mice with DTaP was reported to induce a Th2-biased response characterised by high titers of antibody with little or no induction of a cell-mediated response (Redhead et al. 1993; Barnard et al. 1996). Furthermore, the Th2-biased response following DTaP immunisation was demonstrated to be clinically efficacious in humans (Gustafsson et al. 1996; Olin 1997). Nevertheless, a theoretical shortcoming of Th2-polarised immune response would be the inability to clear intracellular pathogens as is the case with non-opsonised *B pertussis* phagocytosed by human macrophages *in vitro* (Friedman et al. 1992) or *in vivo* (Hellwig et al. 1999) and neutrophils (Lamberti et al. 2008). In the absence of a memory Th1 response, an infection would have the potential to become persistent despite the absence of acute symptoms in humans or an apparent clearance of bacteria from the respiratory tract of animals. This has been supported by an observation that the co-administration of IL-12 with an acellular vaccine, which

induced a shift in bias from a Th2 to Th1 response, enhanced the rate of clearance following a bacterial challenge (Mahon et al. 1996). However, given the demonstrated absolute need for antibody in successful clearance (Leef et al. 2000) and the discovery that *B pertussis* can also survive and persist within macrophages and neutrophils (Friedman et al. 1992; Hellwig et al. 1999) suggests that both antibody and cell-mediated immune response may be central to long term immunity, as the latter is associated with CMI rather than humoral responses (Mahon et al. 1996; Mills et al. 1993).

1.5.2. Importance of CMI against *B pertussis*

The lack of correlation between protection and antibody levels observed following clinical trials of a two-component acellular pertussis vaccine suggested a lack of insight into the true nature of immunity to *B pertussis* (Cherry et al. 1998). Since then many investigations have been carried out using naive and transgenic mouse models to demonstrate the importance of CMI against infection with *B pertussis*. Redhead et al. (1993) observed variable T cell proliferative responses in mice following convalescent infection or immunisation with whole-cell or acellular vaccines. High levels of IFN-γ and IL-2 but no or low IL-4 or IL-5 were produced from the splenocytes of convalescent mice. The low or undetectable antibody response that accompanied these cytokines indicated a CD4+ Th1 response (Feunou, Bertout, and Locht). Conversely, after two doses of an acellular vaccine, a CD4+ Th2 response characterised by elevated IL-5 secretion from splenocytes and high titers of anti-*B pertussis* IgG was observed. Immunisation of mice with the whole cell vaccine (wP) induced a dichotomous response with evidence of both IFN-γ/IL-2 secretion and serum antibody, and importantly the clearance of bacteria, following an aerosol challenge, was enhanced by the Th1 type response. The demonstration that non-immune wild-type mice developed a prolonged infection that cleared after roughly 35 days, whilst athymic or T-cell deficient mice failed to clear bacteria from the lungs, following aerosol challenge also supports this observation (Mills et al. 1993). Transfer of primed T cells to either athymic or irradiated naïve mice restored the capacity for clearance; however transfer of serum from convalescent mice could not restore the ability to completely eradicate pathogens from the respiratory tract. Finally, the observation of an uncharacteristic disseminated infection in IFN-γR$^{-/-}$ mice

accompanied with a high mortality rate, in contrast to wild-type mice that contained the infection to the respiratory tract with no deaths indicates the importance of CMI in protection against pertussis (Mahon et al. 1997). On the other hand, analysis of human samples from Phase 3 clinical trials were used to demonstrate that natural infection or vaccination with the wP vaccine elicited a predominantly Th1-type cell-mediated response, whereas aP vaccines typically induced a strong humoral response with a Th2-polarised cytokine pattern in infants and children at 4-6 years of age (Ryan et al. 1998; Esposito et al. 2001). However, studies claiming the induction and/or maintenance of CMI after vaccination with Pa vaccines pre- and post-booster to be at least as good as that induced by the Pw vaccines have also been reported (Cassone et al. 1997; Ausiello et al. 1998; Ausiello et al. 1999; Zepp et al. 1996). Although the subjects used in these studies reported no history of infection with *B pertussis*, the potential contribution of subclinical pertussis infections, silent boosters albeit masked or difficult to detect, to induce and/or maintain high levels of CMI should not be over ruled.

1.6. Experimental vaccines

While current acellular and whole cell pertussis vaccines have been found to apparently provide relatively short to medium term protection in neonatal and older children (Wendelboe et al. 2005; Wearing and Rohani 2009), it is also anticipated to do so in adults (Rieber et al. 2008). Regardless, there is a need to develop vaccines that will provide longer-term protection, at least as long as that estimated theoretically for recovery from natural infection estimated at 20 years (Wearing and Rohani 2009). Given the importance of CMI in protection against and possibly transmission of pertussis, there are a number of novel vaccines currently under development, which represent potential alternatives to Pw and Pa vaccines. These include DNA vaccines constructed using genes encoding genetically modified pertussis toxin, filamentous haemagglutinin and pertactin, live attenuated mutants and pertussis antigens encapsulated in biodegradable particles. Potential reasons for development of alternative whooping cough vaccines include:

1) Cost-ineffectiveness of the currently marketed acellular pertussis vaccines particularly in developing countries.

2) Development of a vaccine that can be delivered by a non-invasive route such that it will be side reaction free and also generate both antibody as well as CMI responses for induction of potential long-term protection, and potentially increase in compliance with the recommended vaccination schedules e.g. live attenuated or biodegradable particulate vaccines for delivery by the intranasal route.

3) Development of a vaccine that can be tailored to induce either antibody and/or CMI responses and not require availability of cold chain for storage e.g. DNA vaccines.

1.6.1. DNA vaccines

DNA vaccines may provide a means of generating complete and long-lasting immunity against *B pertussis*, because of their potential to generate both antibody and cell-mediated responses (Donnelly et al. 1997). Genetically-inactivated pertussis toxin pcDNA3.1-based DNA vaccine administered using a gene gun revealed induction of both antibody and CMI responses (Kamachi, Konda, and Arakawa 2003). Li et al. (2006) reported that a recombinant pertussis DNA vaccine expressing the pertussis toxin subunit 1 (PTS1), FHA gene and PRN in pVAX1 were reported to elicit both antibody and CMI responses in NIH strain of mice upon delivery by a parenteral route. This was in contrast to our studies (Fry et al. 2008) in which a genetically inactivated pertussis toxin pcDNA3.1-based DNA vaccine administered by the intramuscular route was found to generate a purely cell-mediated immune response. We have further found that boosting DNA vaccine-primed mice with pertussis toxoid yielded excellent antibody as well as cell-mediated immune responses (Fry 2006). Unfortunately, such a vaccination schedule may not cost-effective particularly in the developing world. It is imperative therefore to investigate effect of different DNA vaccine vectors, with or without co-encoded molecular adjuvants such a cytokines and different modes of delivery that will induce both arms of the immune response.

1.6.2. Biodegradable micro- and nano-particle vaccines

The use of inert carriers such as liposomes and biodegradable particles for delivery of antigens is another potential vaccine development pathway (Guzman et al. 1993; Conway et al. 2001; Sharma et al. 2009). Both oral and parenteral delivery of purified pertussis toxoid and filamentous hemagglutinin encapsulated in poly-lactide-co-glycolide (PLG) polymers were demonstrated to protect mice from an aerosol-induced *B pertussis* infection (Guzman et al. 1993). However, several factors were found have a critical effect on the immunogenicity of the biodegradable formulations, specifically the size of the particles used and the route of delivery. Conway et al. (2001) evaluated the immunogenicity and protective efficacy of systemically and orally delivered pertussis antigens (pertussis toxoid and filamentous haemagglutinin) entrapped in either microparticle PLG or nanoparticle PLG formulations using a murine respiratory challenge model for infection with *B pertussis* and reported a high level of protection against an aerosol challenge. Furthermore protection could be generated with a single parenteral immunization with a combined microparticle and nanoparticle formulation, dependent upon the route of immunization and the size of the particles, which affected the type of T cell response induced. However, nanoparticle formulations were found to favor the induction of Th2 immunity. Regardless, this approach warrants further investigations (Sharma et al. 2009).

1.6.3. Live attenuated vaccines

Roberts et al. (1990) developed an auxotrophic *B pertussis* mutant by insertion of a kanamycin resistance cassette into the *aro*A gene. Mice immunised with the *aro*A mutant via inhalation of aerosols induced an IgM, IgA and IgG response that conferred protection against a subsequent aerosol challenge with the virulent parental strain. However, the mutant strain survived in reasonable numbers in the host for essentially only 4 days [detectable at day 8 in low numbers]. However, the potential induced CMI responses were not investigated. On the other hand, *aroA* mutants of *Salmonella* species were reported to be protective (Hoiseth and Stocker 1981) and induced both antibodies as well as CMI (Hoiseth and Stocker 1981; Mukkur et al. 1987). In contrast, the *aroA* mutants of *Shigella* species were found to be less effective vaccines than the *aroD* deletion mutants of the same species (Verma and

Lindberg 1991) suggesting that the level of attenuation of a given gene may depend upon the bacterial species under investigation thereby suggesting variation between bacterial species.

B pertussis in which the PT gene was deleted reported to be attenuated due to its inability to cause severe infection in mice without affecting the ability of the bacteria to colonize the respiratory tract and protect mice against challenge with the wild type (Mielcarek et al. 1998). More recently, a live attenuated *B pertussis* mutant strain (BPZE1) in which PT was genetically detoxified PT, DNT gene deleted and *B pertussis ampG* replaced by *E coli ampG* to minimize its activity, was reported to induce protection in young mice after a single nasal administration (Mielcarek, Debrie, Raze, Bertout, et al. 2006) and reported to be safe in adult interferon-γ receptor deficient adult mice (Skerry et al. 2009). However, this strain survived in lungs as long as the wild type strain leaving the door open to possibility reversion of back to virulence because no essential gene had been deleted. Dr Mukkur's laboratory while at the University of South Queensland, developed a metabolite deficient *aroQ*BP, non-reverting deletion mutant of *B pertussis* as a live attenuated vaccine candidate. When delivered by the intranasal route, this strain has been found to be safe in mice and survive long enough (about $2/5^{th}$ as long as the wild type parent strain) to induce a significant IgA and IgG responses in both the serum and respiratory secretions (Cornford 2003). The mutant also induces a systemic cell-mediated immune response as indicated by the production of IFN-γ and IL-12. Mice immunised with this mutant vaccine were protected against challenge with sub-lethal or lethal dose infections. The major potential advantage of using metabolite deficient mutants is their inability to revert to virulence in vertebrates because of the lack of availability of essential metabolites required by the pathogen for replication in the host (Hoiseth and Stocker 1981).

The main advantage of live attenuated vaccines is that they offer a non-invasive means of vaccinating infants and children, which closely resembles the natural infectious disease process with potential to provide long-lasting immunity (Locht et al. 2004). However, the concerns with this mode of vaccination include the risk of reversion to virulence, the presence of multiple toxins, and antibiotic resistance

markers used in genetic modification, particularly if the selection markers used in genetic manipulation processes represent the class of antimicrobials to which *B pertussis* is susceptible.

1.6.4. Adjuvants

The only approved adjuvants for use with human vaccines in infants are alum-based (aluminium hydroxide or aluminium phosphate), which promote induction of excellent antibody responses. However, many studies carried out with other killed bacterial vaccines in animal models show that alum-based adjuvants can induce CMI responses, the latter being important for protection against infection with most intracellular pathogens and also *B pertussis*. For example, mice and sheep immunised with killed *S typhimurium* neither elicited any CMI response or were protected against oral challenge infection whereas those vaccinated with live *aroA S typhimurium* stimulated CMI response and were protected against oral challenge infection with the parent strain (Mukkur et al. 1987). Thus the lack of induction of CMI by vaccines absorbed on alum-based adjuvants, which has been known for sometime now, was not a surprise (Gupta 1998). Therefore, the basis of the reported CMI induction in children vaccinated with aP warrants further investigations. Although the mechanism of action of the alum-based is still largely unknown, activation of antigen-presenting cells by an IL-4-dependent mechanism is one possibility (Ulanova et al. 2001). However, the mechanism of induction of both Th2- as well as Th1-polarised immune responses following vaccination of children with the alum-based adjuvanted acellular pertussis vaccine warrants further investigations. In some other countries including Europe, other adjuvants have been approved for use vaccines for adults, for example, M59, a squalene-based adjuvant, and more recently ASO3, an oil-in-water emulsion, and combination adjuvant comprised of monophosphoryl lipid A (MPL) and aluminium hydroxide, named ASO4 for use with hepatitis B (Fendrix) and human papillomavirus (HPV) vaccine (Cervarix) (Tritto, Mosca, and De Gregorio 2009). Clearly, high priority research is warranted to identify an acceptable nontoxic adjuvant that enhances both arms of the immune response.

Chapter 2: Materials and methods

2.1. Bacterial strains, growth conditions and storage

2.1.1. Bacterial strains

Complete list of bacterial strains that were used in this study are listed in Table 2.1.1.

Table 2.1.1. Bacterial strains list used in this study.

Strains	Genotype/Phenotype	Source or Reference
Bordetella pertussis aroQ (*aroQ*BP)	Vaccine candidate: *aroQ*BP $^{-}$, cannot survive in vertebrate, Km^{r}, Sm^{r}	In house; developed in Assoc. Prof. Trilochan Mukkur's lab, University of Southern Queensland
E coli XL10-Gold	Tet^{r} Δ(*mcrA*) 183 Δ (*mcrCB-hsd*SMR-*mrr*) 173 *end*A1 *sup*E44 *thi*-1 *recA1 gyrA96 relA1 lac* The	Stratagene
E coli DH5α	Cloning host, F^{-} ϕ80*lacZ*ΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (r_k^-, m_k^+) *pho*A *sup*E44 λ^{-} *thi*$^{-}$1 *gyr*A96 *rel*A1	Professor Grant Daggard, University of South Queensland
E coli SM10 λ pir	*thi thr leu ton*A *lac*Y *sup*E *rec*A::RP4-2-Tc::Mu Km^{r} *λpir*	Associate Professor Naresh Verma, Australian National University
E coli JM109	Cloning host, *end*A1, *rec*A1, *gyr*A96*, thi,* *hsd*R17 (rk^{-}, mk^{+}), *rel*A1*,* *sup*E44*,* Δ(*lac-pro*AB), [F′, *tra*D36*, pro*AB*,* *lac*^{q}ZΔM15]	Promega

2.1.1.1. *Bordetella pertussis aroQ*

The *aroQ*BP strain is derivative of Tohama I. The *aroQ*BP strain was generated in Dr Mukkur's laboratory, University of South Queensland (Australian Patent Number AU 2002357406 B2). The *aroQ*BP strain was produced by deletion of the *aroQ* gene the common aromatic biosynthesis pathway such that aromatic amino acids including phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Try) and aromatic compounds including para-aminobenzoic acid (PABA), dihydroxybenzoic acid (DHB), and para-hydroxybenzoic acid (PHB) and orthosuccinylbenzoate (OSB) are not synthesised. The *aroQ*BP metabolite-deficient strain was found to be avirulent and survived in the mouse lung from ten to twelve days. In comparison, the virulent parent strain survived more than thirty days post-infection in mice infected by the intranasal route (Mielcarek et al. 2010; Mielcarek, Debrie, Raze, Bertout, et al. 2006).

2.1.1.2. *Escherichia coli* strains

E coli XL10-Gold strain: The XL10-Gold is an *E coli* strain that was provided as component of Quick-Change™ XL Site-Directed Mutagenesis Kit (Stratagene). *E coli* strain XL10-Gold is derived from *E coli* K12 strain XL1-Blue (Stratagene) and has been engineered to repair nicked plasmid DNA efficiently.

E coli DH5α strain: The DH5α strain is a broad host *E coli* cells and suitable for gene bank or the generation of cDNA libraries using plasmid vectors such as pUC19 (invitrogen™). DH5α cells contain ϕ80*lacZ*ΔM15 markers that complement the β-galactosidase gene from pUC19 or similar vectors. When the cells transformed with a vector the transformed cells can be screened based on blue/white colonies on LA plates containing X-gal. According to invitrogen™, efficiency of competent DH5α cells are > 1.0 x10^9 transformants/μg pUC19 with non-saturating amounts (50pg) of DNA. Saturating amounts of pUC19 (25ng) generates > 1.0 x 10^6 ampicillin resistance colonies in a 100μl reaction (invitrogen™). This stain was generously donated by Professor Grant Daggard, University of Southern Queensland, Toowoomba, Queensland.

E coli SM10 λ pir: SM10 λ pir is derivative of *E coli* K12. It is donor strain containing the transfer genes of the broad host range genes IncP-type plasmid RP4 integrated in its genome (Simon 1983). This strain was generously donated by Dr Naresh Verma, Australian National University, Canberra, ACT.

E coli JM109 strain: JM109 is an *E coli* strain which made competent for transformation with transformation efficiency of 1x 10^8 Colony Forming Units (CFU) (Promega). JM109 was provided as component of the pGEM®-T Easy Vector System (Promega).

2.1.2. Media and growth conditions

Handling and manipulation of *B pertussis* strains were carried out under sterilized condition in a Biohazard Class II Cabinet. The identity of the *E coli* strains was confirmed by Gram Staining and use of API20E (BioMerieux). Identification of *aroQ*BP confirmed using oxidase test (positive) and amplification of IS-481 with PCR (Light Cycler 2.0, 48011 PCR Thermocycler).

The *aroQ*BP strain was routinely grown on modified Cohen-Wheeler (CW) agar (Cohen and Wheeler 1946), Bordet-Gengou (BG) agar (Oxoid), supplemented with 15% (v/v) defibrinated horse blood and following aromatic amino acids (aromix) and kanamycin (Km): Phe 40μg/ml, Try 40μg/ml, Tyr 40μg/ml, PHB 10μg/ml, DHB 10μg/ml, PABA 10μg/ml and Km 50μg/ml. The inoculated plates were placed in sealable plastic bag containing autoclaved moist tissues and incubated at 37^0C for four to five days; the growth was used for subsequent study.

For conjugation experiments, the *aroQ*BP strain was grown in Cyclodextrin Liquid medium (CLM) (Imaizumi et al. 1983) (see Appendix A, page 152) and Stainer Scholte medium (SS) broth (Stainer and Scholte 1971) (see Appendix A, page 152) supplemented with the aromix and Km for conjugation. The inoculated plates were placed in sealable plastic bag containing autoclaved moist tissues and incubated at 37^0Cfor four to five days. Broth was incubated at 37^0C on orbital shaker (BOECO-PSU-10i) at 225rpm for two to five days. The growth was used for subsequent study.

CW and BG agar supplemented with the aromix, Km, and with or without streptomycin (Sm) 200μg/ml, ampicillin (Amp, only for use with the pSS1129 plasmid) 100μg/ml and gentamicin 10μg/ml for propagation of the pSS1120 and pJQ200 mp-18 plasmids (Bordetella suicide vector) and for conjugation experiments carried out using nitrocellulose membrane.

Cohen-Wheeler supplemented with the aromix, Km, and cephalexin (Ceph) 40μg/ml was used to culture pulmonary homogenates for detection of *aroQ*BP growth. Cephalexin was used to prevent overgrowth of normal respiratory microflora. Plates were incubated as above.

E coli SM10 λ pir was cultured on Lura Britani (LB) agar or LB broth supplemented with 50μg/ml Km. *E coli* DH5α was cultured on plain LB agar and LB broth. *E coli* strain-harbouring vector(s) were cultured in LB agar/broth with relevant antibiotic(s) (Table 2.11.2) incubated at 37^0C for 16-18 hours and the growth was used for subsequent studies. Inoculated LB broth was incubated at 37^0C on orbital shaker (Ratek) at 225rpm for 16-18 hours and growth was used for subsequent studies.

2.1.3. Preparation and maintenance of bacterial stocks

For the preparation of *aroQ*BP strain stock, the mutant microorganisms were grown on CW or BG supplemented with defibrinated horse blood, aromix, and Km, grown as described in section 2.1.2. Growth from about one and a half plates picked up with sterile disposable 50μl loop (BD) and suspended into CryoCare™ Bacterial preservers tube with beads (Blackaby Diagnostics, Perth, Australia) according to manufacturer's recommendation (Key Scientific Products, USA). The prepared stock tubes were stored at -80^0C. For *E coli*-vector harbouring strains (Table 2.1.1) stocks, the microorganisms were grown on LB agar-Amp or LB agar-Amp/Gm and stocks made and stored at -80^0C as described above.

2.2. Establishment of a growth curve for *aroQ*BP

Suspension of *aroQ*BP was made in about 5ml 1% autoclaved phosphate buffer saline (PBS) from three CW-aromix-(Km 50μg/ml) plates. The plates were incubated for five days as described (Section 2.1.2). The optical density (OD) of the suspension adjusted to 1.303 at 600nm (Shimadzu spectrophotometer UV-120-02). Serial two-fold dilutions were made, their ODs taken as above (Table 2.2). Colony forming units of individual dilutions determined by making further decimal 10-fold dilutions and plating on CW-aromix-(Km 50μg/ml) according to Miles and Mishra (1938). The plates were incubated for five days as described in Section 2.1.2.

Table 2.2. The OD_{600nm} of undiluted and double dilutions of *aroQ*BP used for growth curve establishment.

Dilution factor	Optical density-600nm
1	1.303
1/2	0.679
1/4	0.333
1/8	0.169

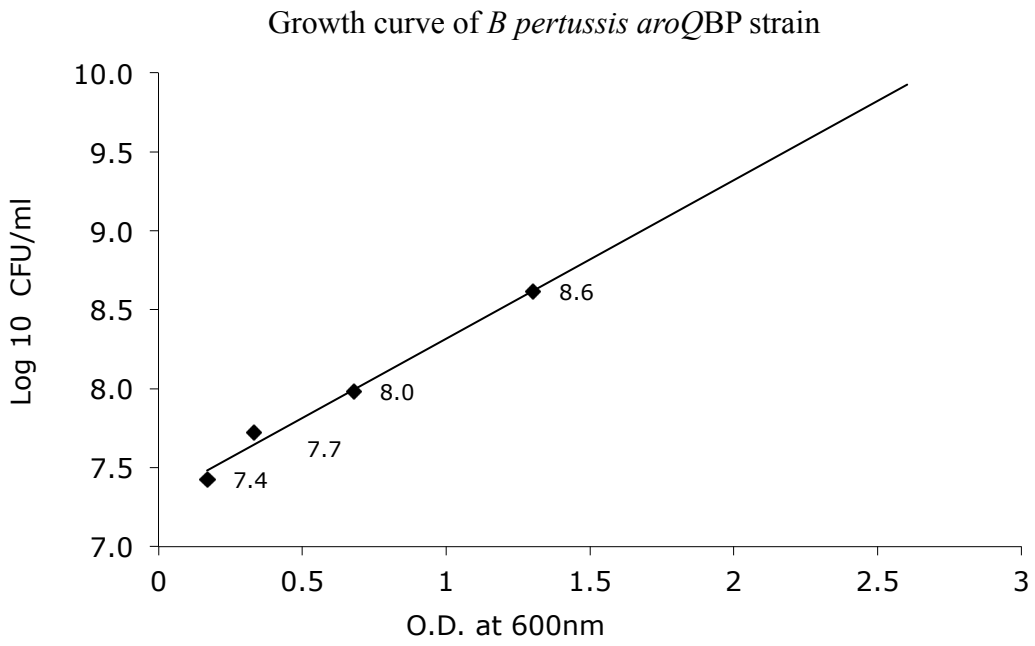

Figure 2.2. Growth profile of aroQ*BP. The diagram drawn is based on CFU of* aroQ*BP serial dilution.*

2.3. Pertussis animal model

Mice were used as a pertussis animal model (Elahi, Holmstrom, and Gerdts 2007) assuminging the fact mice do not cough following infection by the intranasal route either as drops or by aerosolisation (Canthaboo, Xing, et al. 2000). All experiments involving the use of animals were carried out after approval from the Curtin University's Animal Ethics Committee (AEC) and Institutional Biosafety Committee (IBC).

Female specified pathogen free Balb/c mice aged 6-8 weeks were purchased from the Animal Resource Centre, Perth, Australia, and housed in 5mice/cage in the ventilated cabinet in Animal House Facility.

All the mice used in this study were six week-old Balb/c female. Weight of mice ranged from 19-21 grams on first day of the experiment. During duration of the

experiment mice were checked daily for wellness and stress signs such as puffy fur, isolation from group or moribund state.

2.4. Immunological investigation

Reagents used for immunological investigations and their preparation is described below.

2.4.1. Inactivation of *aroQ*BP

Inactivation of *aroQ*BP was carried out using 1% formaldehyde follows: Briefly, growth of bacteria was picked up from three CW-aromix/Km plates and suspended in 8.8ml PBS. To the suspension 0.237ml of 37% formalin was added and incubated at 37^0C for six hours. Then the cells were pelleted by centrifugation at 8,000rpm (7741xg) (BECKMAN Coulter, Avanti® J-E) for twenty minutes. The pellet was washed three times with PBS. Washed pellet was resuspended in PBS at $1x10^9$ CFU per ml using an established standard curve for use in the ELISA assay. A CW-aromix plate was inoculated with 500µl of the suspension and incubated at 37^0C for five days to check for sterility of the culture. The rest of the bacterial suspension was stored at 4^0C.

2.4.2. DTaP

Infanrix (GlaxoSmithKline) Combined Diphtheria, Tetanus, Acellular pertussis and Poliovirus vaccine used in the study. Product code N8343. www.gsk.com.au/infanrix, lot/expiry date: AC20B093DA/ 01-2010.

One dose (0.5ml) of vaccine contained: Diphtheria toxoid (Corynebacterium diphtheriae) ≥ 30lU, Tetanus toxoid (Clostridium tetani) ≥ 40lU, PTxoid 25 microgram, FHA 25 microgram, PRN (Bordetella pertussis) 8 microgram, Poliovirus type 1 (Mahoney) 40 DAgU, Poliovirus type 2 (MEF-1) 8 DAgU, poliovirus type 3 (Saukett) 32 DAgU, Aluminium hydroxide equivalent to 500 micrograms of aluminium (as adjuvant), Phenoxyethanol 2.5mg as preservative, sodium chloride 4.5mg, Medium 199 (M199) 1.15mg including Amino acids 90 microgram,

Polysorbate 80 ≤ 100 micrograms, Formaldehyde ≤ 10micrograms, Glycine ≤ 200 micrograms, Potassium chloride ≤ 40micrograms, Sodium phosphate diabasic dihydrate ≤ 100 micrograms, Potassium phosphate monobasic ≤ 20 micrograms, Neomycin sulfate ≤ 0.05 nanograms, Polymyxin B sulfate ≤ 0.01 nanograms and water for Injections to 0.5ml.

2.4.3. Vaccination

A total of 63 mice were used in this study. Fifteen of these mice were used to produce hyperimmune sera (see Section 2.4.4 for description). The remaining 48 mice were divided into four groups of 10 mice each (A to D; 10 mice/Group) and a fifth group (Group E) comprising 8 mice. Group E mice were used as a sentinel group and were left untreated. Three of the eight mice were subjected to euthanasia at the start of the experiment whereas the remaining five mice were euthanased at the end of the experiment on day 42. To ensure their lack of exposure to the *aro*BP vaccine candidate that was used in different vaccination regimes in different groups (see Table. 2.4.5).

Five mice in Groups A to D were used for vaccination whereas the remaining five mice in each group were sham-vaccinated with PBS as controls. Vaccination mice with the DTaP vaccine involved injection with $1/5^{th}$ of the standard human dose (0.1 ml per mouse) by the subcutaneously (s.c.) route. On the other hand, 1×10^{10} CFU of *aroQ*BP, determined from the growth curve, washed 3 times with PBS, in 1% (w/v) analytical grade casein (Difco) was delivered to mice (10µl/nostril) by intranasally (i.n.) route. The design of this experiment and the specific procedures used for vaccination of different groups of mice is shown in Table 2.4.5 (see Section 2.4.5).

2.4.4. Production of anti-DTaP hyperimmmune sera

High titer antisera (referred to as hyperimmmune sera) against *B pertussis* antigens was produced in Balb/c mice for use as a standard in the ELISAs designed to measure antibody isotypes against different *aroQ*BP antigens. Briefly, ten mice, housed in cages (5 mice per cage) were vaccinated with 100µl of the DTaP ($1/5^{th}$ of the SHD) on day 7 and 14 where as a 3^{rd} group of 3 mice constituted the unvaccinated controls.

On the 23rd day (selected arbitrarily), one mouse from vaccinated group was blood sampled for collection of serum to determine the antibody titer with view to determining the need for additional vaccinations. A 3rd dose of DTaP was given to the remaining 9 mice in the vaccination group on day 28. On day 35, all mice were euthanased and blood samples collected to obtain hyperimmmune sera. The titer of the pooled hyperimmmune sera against whole inactivated BPWC was determined to ensure availability of high titer antisera for use as standard in ELISAs as mentioned above and also for normalisation of data to correct for plate-to-plate variation. The titer of pooled hyperimmune sera against BPWC was determined as described elsewhere (Chen et al., 2006).

2.4.5. Immunization of mice with DTaP and/or *aroQ*BP

In this experiment, mice were immunised with 1, 2 or 3 doses of 100µl of the DTaP (1/5th of the standard human dose) by the s.c. route with or without booster vaccination with 1 to 2 doses of live attenuated *aroQ*BP vaccine candidate by i.n. route (20 µl [µl/nostril] of the stock 1 x 10^{10} CFU/ml). The experimental design used in this study is shown in Table 2.4.5.

Table 2.4.5. Experimental Design: Immunisation and Sampling Regime

GROUP ID					
Day post-immunization	A	B	C	D	E (untreated PBS group)
0	DTaP[s.c.]-5 PBS[s.c.]-5	DTaP[s.c.]-5 PBS[s.c.]-5	*aroQ*BP[i.n.]-5 PBS[i.n.]-5	*aroQ*BP [i.n.]-5 PBS[i.n.]-5	Sample 3 mice 5 mice remain untreated
7	DTaP[s.c.]-5 PBS[s.c.]-5	DTaP[s.c.]-5 PBS[s.c.]-5	No treatment	*aroQ*BP [i.n.]-5 PBS[i.n.]-5	No treatment
14	DTaP[s.c.]-5 PBS[s.c.]-5	DTaP[s.c.]-5 PBS[s.c.]-5	Sample	No treatment	No treatment
21	*aroQ*BP [i.n.]-5 PBS[i.n.]-5	*aroQ*BP [i.n.]-5 PBS[i.n.]-5		Sample	No treatment
28	No treatment	*aroQ*BP [i.n.]-5 PBS[i.n.]-5			No treatment
35	Sample	No treatment			No treatment
42		Sample			Sample 5 mice

On the sampling day, blood and lungs-trachea were collected as sources of serum and pulmonary secretions for determination of serum and mucosal antibody isotypes respectively. Spleens were collected for preparation of splenocytes, which were used for determination of IFN- γ levels, as an indicator of CMI induction, after stimulation with killed BPWC and FHA as a model pertussis antigen. Mice in Group E were left untreated and used as sentinel mice; 3 mice were sampled at day 0 while the remaining 5 mice were samples at day 42 (last day for sampling).

At the day zero, test mice of each group, A, B, C, were individually vaccinated s.c. with 100µl of DTaP. In the same manner, each individual of PBS subjects of respective group was given 100µl of PBS s.c. On same day, test subjects of group D were inoculated with 10µl of 1 x 10^{10} CFU of live *aroQ*BP in 1% (w/v) of analytical grade casein (DIFCO) per nostril. The CFU were pre-determined based on the growth curve of *aroQ*BP (Figure 2.2). The PBS group subjects were inoculated with 10µl of PBS per nostril. On day zero, blood, lungs-trachea, and spleen samples were collected from three of the eight sentinel mice (Group E).

On day 7, five mice from groups A and B were injected with a second dose of DTaP s.c., with the remaining 5 mice in the group receiving sham-vaccination with PBS as at day zero. Likewise, mice of group D were given a second dose of the live *aroQ*BP i.n. The remaining 5 mice in the group received PBS i.n. as at day zero. Mice in group C were left untreated on the day 7.

On day 14, mice of groups of groups A and B were vaccinated with third dose of DTaP as the day zero. On day 14, mice in group C were sampled for blood, lung-trachea, and spleen.

On day 21, mice of groups A and B were inoculated i.n. with 1 x 10^{10} CFU of *aroQ*BP (10 µl/nostril). On same day, blood, lung-trachea, and spleen sampling of mice in group D was carried out.

On day 28, mice of group B were re-inoculated with 1 x 10^{10} CFU of *aroQ*BP vaccine candidate (10 µl/nostril). On days 35 and 42, blood, lung-trachea, and spleen sampling was carried out for groups A and B, respectively. Likewise, on day 42, blood, lung-trachea, and spleen sampling was carried for remaining five sentinel mice (Group E).

2.4.6. Anaesthetization and sampling

Mice were anaesthetized with mixture of ketamine 80mg/kg and xylazine 10mg/kg (Fry et al. 2008). One dose of anaesthetic cocktail contained: 0.8µl of ketamine, 0.5µl

of xylazine, and 0.7µl of PSB in total of 2µl per gram of mice weight. The anaesthetic cocktail was injected intraperitoneally using 26G needle. Anaesthetization of mice was confirmed by pinching the foot if no response (about five minutes) observed then sampling preceded. However, if any foot response observed after five minutes anaesthetisation was repeated with half of the original dose. Upon no foot response, the sampling proceeded as described in the following sections. Sampling was done under aseptic condition.

2.4.6.1. Blood collection

Mice were anaesthetised as described in section 2.4.6 for collection of blood samples, source of serum samples. About 700µl to 800µl blood was collected via incision of the subclavian artery of individual mice. Each individual blood sample was collected on 1.5ml eppendorf tubes and kept on ice for about one hour for a clot to form. The serum separated from red blood cells with 4,000rpm (1,520xg) (Hermle Z 233M) centrifugation for ten minutes. The serum samples were stored at -20^{0}C for subsequent tests. Following collection of blood mice were subjected to cervical dislocation to ensure death.

2.4.6.2. Collection and homogenization of pulmonary (lung-trachea) samples

The pulmonary tissues collected were lungs and trachea. The weight of the tissue in combination ranged from 900-1200mg. The organs were homogenized with glass homogenizers in 1ml PBS and transferred to a 1.5ml eppendorf tube. Total of twelve CW-aromix/Km/Ceph plates inoculated with 20µl of homogenized pulmonary tissues of each mouse for all groups and incubated at 37^{0}C for five days to check for any growth of the *aroQ*BP. To the rest of individual pulmonary tissues 50µl of protease inhibitor cocktail (Sigma-Aldrich) was added per ml of homogenized sample to prevent antibody degradation by protease. The samples were stored at -20^{0}C for subsequent tests.

2.4.6.3 Preparation of splenocytes

All five spleens from each group were collected on 2ml of Dulbecco's modified Eagle's medium (CDMEM, Gibco) without foetal bovine serum (FBS). The spleens were pooled and mash in strainer (BD) with 10ml syringe piston and cells collected into 14ml falcon tube. Collected splenocytes were washed twice with 5ml CDMEM without FBS at 1000rmp (Beckman Coulter Avanti® J-E) for ten minutes and resuspended in 12ml CDMEM with FBS. Viability of the splenocytes was tested using trypan blue as described elsewhere (Fry et al. 2008), and the cell concentration adjusted to 5 x 10^6 cells/ml for use in the antigen stimulation assays.

2.5. Splenocyte stimulation with antigens

Eighteen wells of a twenty-four well tissue culture plate was seeded with 2ml per well of splenocyte suspension for each group in quadruplicate. Three antigens that were used to stimulate splenocytes, included FHA at 2μg/ml, PT at 2μg/ml, BPWC at 5 x 10^6 cfu/ml. ConA a T-cell stimulant 2.5μg/ml was used as a positive control whereas PBS (control group) was used as negative control. The volume of the different stimulants or sham-stimulant added to wells in the plate given in Table 2.5. The plate covered with lose lid and was incubated at 37^0C with 5% CO2 for 72 hours. The plate was checked daily for cloudiness as indicator of contamination and any contaminated wells discarded. At 72 hours post-stimulation, the content of each well was removed and centrifuged at 4,000rpm (1935xg) (Beckman Coulter Avanti® J-E) for ten minutes to remove any sediment. Supernatants were removed individually and stored at -20^0C for subsequent estimation of the levels of cytokine IFN-γ.

Table 2.5. Concentration of different antigens used for splenocyte stimulation.

Stimulant	Volume in ml	Final concentration
FHA	2ml	2μg/well
PT	2ml	2μg/well
BPWC	2ml	$1x10^7$ CFU/well
ConA	2ml	2.5μg/well
PBS	2ml	NA
Untreated	2ml	NA

NA: denotes "not applicable".

2.6. ELISA assay for identification of antibody isotypes and subclasses

Indirect ELISA was used for detection of whole immunoglobulin (IgG) and IgA antibody isotypes. Likewise the assay was employed to detect IgG1 and IgG2a antibody subclasses in the sera and pulmonary tissues of all mice in the experimental groups. In total three antigens including inactivated BPWC, PT, and FHA were used to determine antibodies titers in sera and pulmonary tissues.

2.6.1. Serial dilutions of serum and pulmonary tissues

Individual mouse samples from each group were diluted in 0.05% Tween-20 in PBS (PBST). Dilution for each serum sample in PBST ranged from 1/50 to 1/6400 whereas the serial dilutions of homogenised pulmonary tissues ranged from 1/25 to 1/3200, in duplicate, when inactivated BPWC used as antigen. The range of serial dilutions of sera for estimation of anti-PT and anti-FHA ranged from 1/50 to 1/800 because of the shortage in the availability of serum samples. No anti-PT and ant-FHA titer could be determined in homogenised pulmonary tissues for the same reason; although, anti-whole cell antibody isotypes titers were determined.

2.6.2. ELISA method for antibody detection and measurement

The ELISA was essentially carried out as described elsewhere (Fry et al. 2008). In general for each ELISA assays two wells per plate were used as positive controls

(100µl of 1/100 dilution of pooled hyperimmmune sera) and two well as negative controls (100µl of 1/10 dilution of pooled normal mouse sera). One column in the ELISA plate was not coated with the antigens (Antigen control). A column adjacent to the antigen control column of the plate, no primary antibody of mouse origin was added (Antibody control).

Samples used to detect antibodies were antisera and pulmonary homogenates. Briefly, Three antigens were used as the coating antigen for the estimation of different antibody isotypes or subclass titers. The concentration of antigens used to coat the ELISA plates were as follows:

1) Reconstituted 1µg/ml PT in coating buffer (0.1 mole sodium phosphate, pH 7.0, 0.5 sodium chloride, stock buffer).

2) Reconstituted 1µg/ml FHA in coating buffer (distilled water, stock).

3) Inactivated BPWC 5 x 10^8 cfu/ml (OD_{600}= 0.88) (predetermined from growth curve) in coating buffer.

The antigens were 100µl of 1µg/ml PT or 1µg/ml FHA or inactivated BPWC (OD_{600}=0.88) was added to appropriate wells of a 96-well microtiter plate (Maxisorp Nunc). The plate was sealed with sealing film and incubated at 4^0C for overnight at a moist chamber. The wells were washed manually with PBST 18-24 hours later using 300µl of PBST dispensed into each well and left for one minute then PBST was disposed by inverting and blotting on paper towel. The washing was repeated for second and third time for two and three minutes, respectively. Hundred-fifty microliters of bicarbonate blocking buffer was added to each well of the plate and incubated at room temperature on orbital shaker (BOECO-PSU-10i) at 100rpm for one and a half hour. The plate was washed as above. The primary antibody (antisera and pulmonary tissues homogenate) was serially diluted (Section 2.6.1) in PBST (prepared at same day), and 50µl of each dilution was added to each well and incubated as above for one hour. The plate was washed as above. Hundred microliters of respective goat anti-mouse antibody isotype sera (anti-IgG or anti-IgG1 or anti-

IgG2a conjugated to alkaline phosphatase) diluted 1 in 5000 PBST was added to each well and incubated as above for one hour. The plate washed as above and 100µl of substrate was added to each well (1mg/ml p-Nitrophenyl phosphate in substrate diluent) (Sigma). The plate was covered with aluminium foil and incubated at room temperature. The OD of plate was taken at 405/629nm at ten and twenty minutes time intervals using the Microplate Reader (Labsystem, Multiskan Ascent).

2.6.3. Normalization of ELISA data

ELISA data was normalised for plate-to-plate variation when necessary, as described previously (Chen et al. 2006), with following modifications. In brief, dilutions of hyperimmune sera evaluated for anti-*B pertussis* titers using inactivated BPWC as the antigen. An average±SE was calculated and the ELISA data (OD_{405}) adjusted to the average value calculated for the hyperimmune sera (Section 2.4.4).

2.7. Estimation of antibody titers determined by ELISA

Antibody isotype (IgG, IgG1, IgG2a) titers for sera and pulmonary tissues were determined by linear regression of the data that constituted the linear part of the curve and extrapolation to the x-axis. A single point titration was used for estimation of the serum antibody titers of the IgA and pulmonary homogenate's IgG1, IgG2a, and IgA. The end point titers determined as the reciprocal of the highest dilution multiplied by OD_{405nm}.

2.7.1. Anti -PT antibody titer

Mouse anti-PT IgG titers were determined as follows; a minimum of three dilution points which represented the linear segment of the antibody titration curve were extrapolated to the x-axis in the line graph (reciprocal of the dilution factors plotted) was taken as the antibody titer of individual mice (See Appendix K for line graphs used to calculate the ELISA titers, page 222).

2.7.2. Anti-FHA titer

Mouse anti-FHA IgG antibody titers were determined as described above for anti-PT IgG antibodies (See Appendix L for line graphs used to calculate the ELISA titers, page 237).

2.8. Estimation of IFN-γ levels in the antigen-stimulated splenocyte supernatants

IFNγ levels were estimated in the splenocyte supernatants using the murine IFNγ ELISA kit (abcam) according to the manufacturer's instruction. Each sample was analysed in quadruplicate by ELISA. The absorbances and concentration were read at 405nm on microplate reader (Labsystem, Multiskan Ascent). The final concentration of the cytokine of each sample was determined as follows. A linear standard curve generated by plotting the average absorbance on the y-axis versus the corresponding ml IFNγ standard concentration the on x-axis. The concentration of IFNγ in each sample was determined by extrapolating OD values to ml IFNγ concentration from the standard curve (Figure 2.8) and the value multiplied by appropriate dilution factor.

2.9. Statistical Analysis

Values for antibody isotype titers were compared using two-tailed unequal variance t-test (using Microsoft® Excel® for Mac2011 version 14.4). Results were considered significant at p values ≤ 0.05.

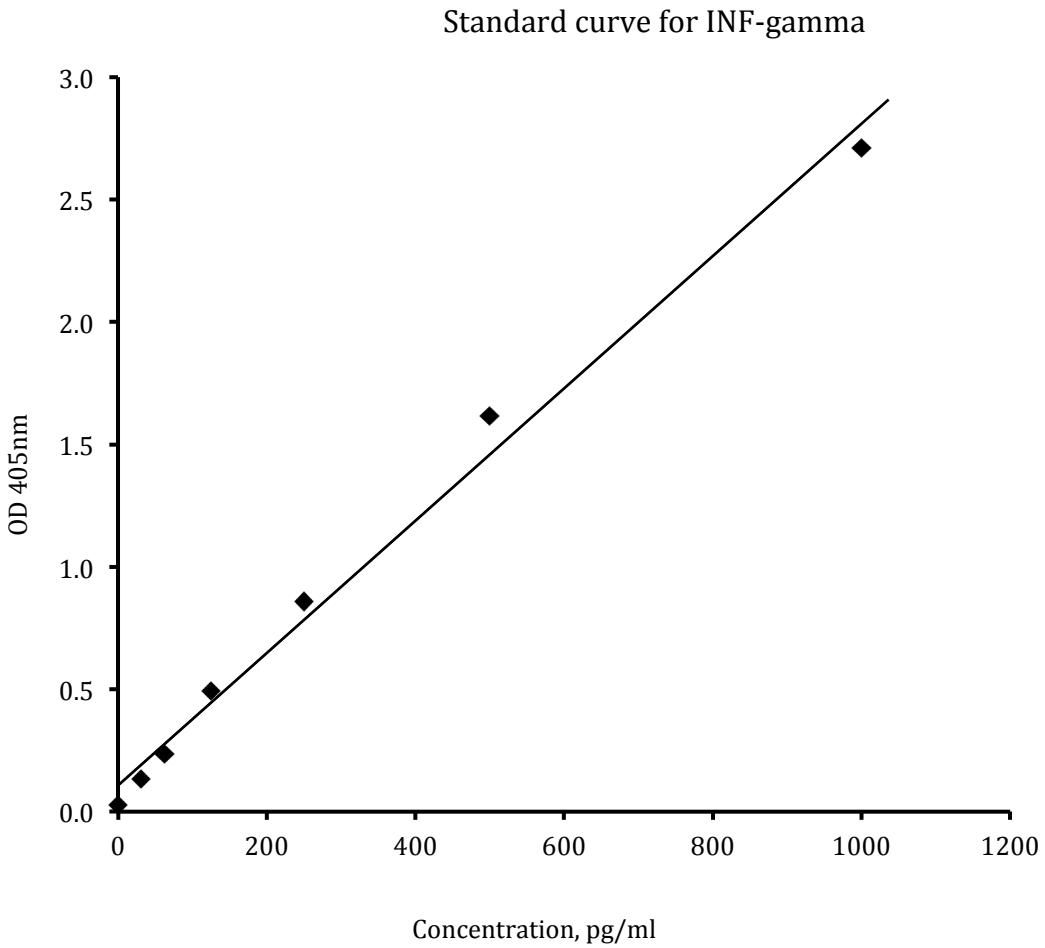

Figure 2.8. Standard curve used to estimate IFN-γ concentration in splenocyte of mice vaccinated with different vaccines. Mice were immunised according to the immunisation regime shown in Table 2.4.5.

2.10. Generation of Adenylate Cyclase (ACT) Mutant of *aroQ*BP

2.10.1. Sub-Cloning of *cyaA* insert into Plasmid Vectors

Plasmid vectors used in the study for sub-cloning of *cyaA* and/or CyaMut insert are listed in Table 2.10.1.

Table 2.10.1. Vectors used for Cya and/or CyaMut sub-cloning.

Vector's Name	Size/Specification	Source/Reference
pUC19	2.686kb/Contains poly-linker for different RE; Amp+	Professor Grant Daggard, University of South, Townsville, Queensland
pGEM®-T Easy Vector	3.015kb/ Contains poly-linker for different RE; Amp+	Promega
pJQ200mp 18-rpsL	4.799kb/Contains poly-linker for different RE; Gm+, Stp+	Dr. Camile Locht, Pasteur institute, lille, France
pSS1129	9.8kb/Specific for *B pertussis* transformation; suicidal; RE: *BamHI, HindIII, EcoRI, SmaI, BstEII, SalI, PvUII*; Stp+, Amp+, Gm+,	Professor Louise Temple Rosebrook

2.10.1.1. pUC19

The vector is small in size, 2.686kb (Appendix M, page 251), which allows for large DNA insert and replicates in a high number (>200 copy/cell) in *E coli* The vector contains parts of pBR322 and M13mp19 (Yanisch-Perron, Vieira, and Messing 1985; Lin-Chao, Chen, and Wong 1992). The vector contains multiple cloning sites (MCS). The MCS is in frame within the α-peptide coding region of the enzyme β-galactosidase (*lacZα*), the enzyme breaks lactose into glucose and glactose. *LacZα* gene is a part of Lac operon. Inactivation of *lacZα* by insert within polyliker cloning site allows recovery of recombinant vector based on colour change (white) when cultured on LA/pGEM/X-gal plate (Promega). The vector also codes for an enzyme that binds and degrades ampicillin. Considering above, *Cya* sequence fragment cloned in pUC19 the cloned vector was multiplied in transformed *E coli* DH5*α*. The *Cya* sequence fragment was excised from pUC19 using *BamHI* and *EcoRI* and subsequently ligated into shuttle vectors, pJQ200mp 18-rspL and pSS1129, for details see section 2.16 and 2.17.

2.10.1.2. pGEM®-T Easy Vector

pGEM®-T Easy Vector (Appendix N, page 252) is constructed specifically for the cloning of PCR products (Promega). The vector is constructed by digestion of pGEM®-T Easy Vector with *EcoRV* and addition of a 3′ terminal thymidine (T) to of both ends. Thymidine overhangs of the vector are compatible to deoxyadenine overhangs of PCR products generated by some thermostable polymerase (Promega). The 3′-T overhangs prevent recirculation of the vector during ligation reaction and improves ligation of PCR products into the pGEM®-T Easy Vector (Promega). The multiple cloning site of the vector contains sequences on either side of the PCR products insert, which are site of restriction for *NotI* and *EcoRI* restriction enzymes. This allows the PCR products to be removed with a single restriction digest using either of these enzymes (Promega).

2.10.1.3. pJQ200mp18-rpsL

pJQ200mp18-rpsL (4799bp) (Appendix O, page 253) is a suicide vector and can be utilized in most of gram negative bacteria gene manipulation. As many other *E coli* plasmid's (ColE plasmid family) derivative vectors; pJQ200mp 18-rspL only replicate in enterobacteria. The vector contains *E coli* rspL (Sm-sensitive [Sm^S]) and Gm-resistance (Gm^R). The vector was developed to study role of iron in *B pertussis* virulence (Stibitz 1994; Pradel et al. 2000).

2.10.1.4. Vector pSS1129

This Bordetella suicide vector/plasmid, pSS1129, (Appendix P, page 254) was generated by Scott Stibitz from pRTP1 (Stibitz 1994). This vector caries a gentamicin-resistance cassette insert. The vector was developed for delineating the role of the virulence determinants of *B pertussis* in whooping cough. This vector allows replacement of a bacterial gene with a genetically modified gene of interest *in situ*. The vector cannot replicate inside this bacterium because the vector belongs to ColE1 (colicin E1) family (Stibitz 1994). In this study this vector, pSS1129, was one of the two vectors used in attempts to genetically-detoxify the ACT (exotoxin).

2.11. Production of different plasmids in *E coli* strains

Processes used for production of different vectors in workable quantities for DNA manipulation are described in sections below.

2.11.1. Generation of competent *E coli* strains

LB broth (10 ml) was inoculated with *E coli* DH5α stock and incubated overnight (16-18 hours) at 37^0C in shaker (Ratek) at 225rpm. This was used to inoculate 100ml of LB medium was inoculated with the 10ml *E coli* DH5α in LB broth and its OD_{600nm} measured. After the OD taken the broth incubated at 37^0C on an orbital shaker (Ratek) at 225rpm and OD_{600nm} measured until it reached 3.2. This broth was transferred into 2 x 50ml polyethylene tubes and incubated in ice for ten minutes. The broths were centrifuged at 4,000rpm (1935xg) (Beckman Coulter, Avanti® J-E) at

4^0C for 10 minutes and the supernatants discarded. Each of the pellets was suspended in 30ml ice-cold 80mM $MgCl_2$-20mM $CaCl_2$ solution followed by re-pelleting by centrifugation as above and discarding the supernatant. The pellets were resuspended in 2ml of ice-cold 0.1M $CaCl_2$ per 50ml of original culture. The suspensions were mixed into one 4ml aliquot and 140µl of dimethyl sulphide oxide (DSMO) added. The suspension was mixed gently with fingertip and the tubes left on ice for fifteen minutes. An additional 140µl of DSMO was added to the mixture and mixed gently as above. The broth was aliquoted in 250µl lots into ice-cold 1.5ml microtubes and snap-frozen on dry ice to which absolute ethanol had been added. The stocks were stored at -80^{0}C.

2.11.2. Transformation of *E. coli* strains with plasmid

The transformation of *E coli* strains with plasmids was accomplished using the heat shock method described by Sambrook et al. (2001).

In general transformation of *E coli* strains, namely; *E coli* DH5α and *E coli* SM10 λ *pir* with different vectors were carried out as follows. The specific antibiotic(s) used to supplement the to media used for cultivation of different transformed clones are shown in Table 2.11.2

The stock of the *E coli* was transferred from -80^{0}C to ice and left on ice for about five minutes to thaw. Fifty microliter of stock *E coli* strain of interest was transferred to each of three labelled chilled 14ml polyethylene tube on ice. One microliter vector with insert (sample tube), 1µl of vector without insert (negative control), and 1µl of pUC19 (5ng/µl) (efficiency tube) were added to test, control and transformation efficiency tubes, respectively. The tubes were incubated on ice for 30 minutes. The samples transferred to water bath at 42^{0}C for 45 seconds and immediately were return on ice for 2 minutes. To each tube 450µl of SOC (for composition see Appendix A, page 152) broth was added and incubated at 37^{0}C on orbital shaker (Ratek) at 225rpm for an hour and half. The transformation efficiency tube was serially diluted on SOC broth as follows 1:10, 1:100 and 1:1000. Fifty microliter of undiluted and 50µl of each diluted broths were spread on LB agar plate supplemented with appropriate antibiotic(s) (Table 2.11.2), total of four plates were inoculated. The test and the

control broths was spread on four LB agar plates (3 plates per dilution) supplemented with appropriate antibiotic(s) (Table 2.11.2.) in volumes of 50, 100, 130 and 150µl and incubated at 37^0C for overnight.

Table 2.11.2. Concentration of different antibiotic(s) in LB media inoculated with *E coli* strains carrying various plasmid vector(s).

Antibiotic	Concentration of antibiotic in LB medium	*E. coli* strain-vector combination
Ampicillin (Amp)	100µg/ml	DH5α-pUC19 DH5α-pUC19Cya (This study) DH5α-pCyaMut (This study)
Gentamicin (Gm)	10µg/ml	DH5α-pJQ200mp 18-rspLCyaMut (This study)
Kanamycin (Km)	50µg/ml	SM10 λ *pir*
Km, Gm	50µg/ml, 10µg/ml	SM10 λ *pir*- pJQ200mp 18-rspLCyaMut (This study)
Amp, Km	100µg/ml, 50µg/ml	SM10 λ *pir*- pUC19CyaMut (This study)
Amp, Km, Gm	100µg/ml, 50µg/ml, 10µg/ml	SM10 λ *pir*-pSS1129Cya SM10 λ *pir*- pSS1129Cya Mut DH5α-pSS1129 DH5α-pSS1129CyaMut

2.12. Bacterial DNA Extraction

Methods for preparation/extraction of plasmids from *E coli* and extraction of chromosomal DNA from *aroQ B pertussis* are described below:

2.12.1. Plasmid Extraction from *E coli* strains

Stocks of *E coli* strains (SM10 λ *pir*, DH5α harbouring the plasmid pJQ200mp 18-rspL or pSS1129) maintained at -80^{0}C were thawed on ice and 10ml of LB broth supplemented with appropriate antibiotic(s) inoculated and incubated at 37^{0}C on orbital shake (Ratek) for 15-17 hours. A loop full of the growth was subcultured on LB agar plate supplemented with appropriate antibiotic(s) and incubated at 37^{0}C for overnight. The broth was transferred into six 1.5ml microtubes and pelleted at 10,000rpm (9,500xg) (HERMLE Z 233M) for five minutes. The plasmid was extracted from cells using Miniprep (QIAprep® Spin Miniprep Kit using a Microcentrifuge) according to manufacturer's recommendations. Briefly, three of the above pellets (from 1.5ml tube) resuspended in 250µl of suspension buffer (P1) and transferred into an autoclaved 1.5ml microcentrifuge tube. To the mixture 250µl of cell lysis buffer (P2) added mixed thoroughly by inverting 6 times and 350µl of neutralisation buffer (N3) added to the tube mixed thoroughly by inverting and immediately centrifuged at 13,500rpm (17320xg) (Hermle Z 2233M) for 10 minutes. The lysate (supernatant) was transferred to a QlAprep spin column, the column put in a 2ml collection tube and centrifuged as above for 1 minute. The flow-through discarded and column washed with 750µl wash buffer (PE) and centrifuged as above for 1 minute. The flow-through discarded and the column was centrifuged as above to remove traces of wash buffer residue. The flow-through discarded. Thirty or 50µl of elution buffer (EB) was added to the centre of the column; the column incubated at room temperature for one minute and centrifuged for 1minute as above. Concentration of eluted plasmid measured by Nano drop and/or by subjecting 2-5µl of the plasmid to (0.8%) gel electrophoresis. The rest of the eluted plasmid was stored at -20^{0}C for subsequent investigations. In general, the above procedure was used for plasmid extraction from all the *E coli* strains used in this study.

2.12.2. Chromosomal DNA extraction from *aroQ*BP

Chromosomal DNA was extracted from *aroQ*BP using DNeasy® Blood & Tissue Handbook kit (QIAGEN). The protocol was followed according to the manufacturer's instructions. Briefly, *aroQ*BP was grown on three BG-aromix-(Km/50mg/ml, Sm/200µg/ml) as described on section 2.1.2. The growth was picked from plates and suspended in 10ml PBS and the optical density adjusted to 1.6nm at 600nm corresponding to $2x10^9$ CFU using the established growth curve for *aroQ*BP (Figure 2.2). The suspension was centrifuged at 8,000rpm (6,090xg) (HERMLE Z 233M) for 10 minutes. The rest of the procedure followed as described in manufacturer's procedure. Briefly, the pellet cells were lysed by addition of 180µl ATL buffer. The proteins in the suspension were inactivated by addition of 20µl proteinase K the mixture incubated on orbital shaker (Ratek) 150rpm at 56^0C for two hours. To the lysate 4µl ribonuclease (RNase) A (100mg/ml) added and solution mixed briefly by vortexing before incubation at room temperature for 2 minutes. The solution vortexed for 15 seconds and DNA was precipitated by adding 200µl buffer AL and 200µl ethanol (100%) to the solution and a brief vortex of the mixture. The DNA was extracted and re-extracted from the mixture as follows: The mixture was pipetted into DNeasy Mini spin column that was placed in a 2ml collection tube and centrifuged at 8,000rpm (6,090xg) (HERMLE Z 233M) for 1 minute. The flow-through from last step discarded the DNeasy Mini spin placed in a new 2ml collection tube and 500µl of AW1 buffer added to the column the tube was centrifuged as above. The column placed in a new 2ml collection tube and 500µl of buffer AW2 added to column and was centrifuged at 13,500rpm (1,7320xg) (HERMLE Z 233M) for 3 minutes. A further centrifugation carried out as above for 1 minute to prevent any residual ethanol carry over to DNA elution step. The DNA was eluted by placing the column in to a 1.5ml microcentrifuge tube and addition of 200µl AE buffer to the column. The solution incubated at room temperature for 1 minute before it was centrifuged at 8,000rpm (6,090xg) (HERMLE Z 233M) for 1 minute. To maximize the DNA yield the elution step was repeated with 100µl AE buffer as above. Two microliter of the eluted chromosomal DNA was subjected to gel (0.8%) electrophoresis to check the quality of the DNA. The DNA did produce a sharp band with minor DNA degradation

(smear) on gel. The suspension was stored at -20^{0}C. The DNA stock was diluted with 200-300µl nuclease free water (Promega) to about 5ng/µl when needed.

2.13. Recombinant DNA techniques

The program described in the National Center for Biotechnology Information database (NCBI) (http://www.ncbi.nlm.nih.gov/gene/2664492) was used to identify the *cyaA,* bifunctional hemolysin-adenylate cyclase precursor (5121bp). For conformation of the *cyaA* sequence accuracy whole *cyaA* sequence was selected and blasted against *B pertussis* using the *B pertussis* genome sequence database [www. sanger.ac.uk/cgi]. The identity of the query sequence (*cyaA* nucleotide sequence) against whole *B pertussis* nucleotide sequence was 100% (see Appendix Q for alignment, page 255).

Standard PCR, cloning and recombination DNA techniques were used according to Sambrook et al. (2001). Restriction and modification enzymes were purchased from Geneworks (Adelaide, Australia) and used according to the manufacturer's recommendations. Oligonucleotide primers (10µM) used described in (Table 2.13a.), and were purchased from Geneworks (Adelaide, Australia). All oligonucleotide were designed from published Genbank (NCBI) sequences of the *cyaA* gene (NCBI reference sequence: NC_002929.2). The primers were designed using PubMed software (http://www.ncbi.nlm.nih.gov/tools/primer).

Lyophilised stocks of primers were reconstituted to 100µM working solutions with nuclease free water (Gibco). Negative control was run along with every PCR but using PCR grade water as a template. When possible a positive internal control was also run along PCR reaction. The positive control compromised of the PCR component except for template. The template included in PCR reaction was known to work under the PCR parameter. PCR reactions were constituted either in 20µl or 50µl aliquot (Table 2.13b.). The cycling condition used for PCR is shown on Table 2.13c. If more than two PCR reaction aliquot were needed a master mix was made. Master mix consisted of PCR grade water, forward and reverse primer, Magic Buffer, and Ezway™ Direct Master Mix. Magic Buffer does improve DNA amplification of template with a high Glycine (G) + Cytosine (C) content and high degree of

secondary structure. The final concentration of the magic buffer did not exceed 25% (v/v) of final PCR volume. Ezway™ Direct Master Mix, is a premixed solution containing *Taq* DNA Polymerase, dNTPs, and direct PCR buffer (Komabiotech 2009).

Table 2.13a. List of primers used for PCRs in this study. Oligonucleotide primers used for PCR and site-directed mutagenesis. Designation is given from 5′ to 3′ direction, and restriction sites are underlined. Nucleotide location for the primers at the (NCBI) *B pertussis* Tohama I genome database is given.

Primer	Sequence	Restriction site	Nucleotide location
FwdCya	GGATCCCATGCAGCAATCGCATCAGGCTG	*BamHI*	776227-776249
RvsCya	GAATTCGTATTCCTTCAGTTGCCCGCGCG	*EcoRI*	777226-777204
FwdMut	GTGGCCACCATGGGATTGGGC	_____	776390-776411
RvsMut	GCCCAATCCCATGGTGGCCAC	_____	776411-776390
FwATG-mutant	GAAGGGGTGGCCACCATG	_____	776384-776402
RvsATG	ATAGTCAAGCCGCTCTTTCG	_____	776384-776593
FwAAA-wild type	GAAGGGGTGGCCACCAAA	_____	776384-776402

Table 2.13b. PCR reaction components and PCR reaction aliquot set up in different volume.

PCR component	23µl reaction aliquot (µl)	50µl reaction aliquot (µl)
PCR grade H_2O	7.5 if liquid form of template used or 6.5 if template was colony touch	12.5
Forward primer	1 (10µM)	1
Reverse primer	1 (10µM)	1
Template	0.5 (5-10ng) or touch of individual colony	0.5 0f (5-10ng)
Magic Buffer	4	10
2x Ezway™ Direct Master Mix	10	25
Total volume	23	50

Table 2.13c. Optimum cycling conditions for *Cya* and *Cya* mutant insert amplification.

Amplification parameters		
Stage 1	1 cycle	95^0C for 5 min
Stage 2	25 cycle	95^0C for 30 sec
		57^0C for 1 min
		72^0C for 10 min
Stage 3	1 cycle	14^0C hold

2.13.1. Agarose gel electrophoresis

Agrose gel electrophoresis was carried our using 0.8% solution of biotechnology grade agarose I (Amresco®) in 50ml or 100ml of Tris Acetate EDTA (TAE) buffer prepared (see Appendix C, page 158) by heating in a microwave for 1 to 3 minutes until dissolved. The gel solution was then cooled to about 55^0C degree before addition of Cyber® safe DNA gel stain (invitrogen) to the with gel solution at ratio of 1:10,000. Twelve or fifty millimetre comb was placed on the casting apparatus and gel solution poured slowly into the apparatus cast with the comb in place. The gel was allowed to solidify before the comb removed from the gel. The gel was removed from casting apparatus and submersed into gel tank (Bio Rad) containing TAE buffer. Samples were prepared by adding the required amount of loading dye (Promega) for visualisation and then loaded into the wells using fine-tipped pipette. The standards used were 1kb DNA Step Ladder (Promega), Lambda DNA/HindIII Markers (Promega), 100 base pair (Fermentas), and HyperLadder I (Fermentas). Samples were electrophoresed by attaching the tank to power pack (Bio Rad, 1000/500) and supplying 100 volts at 100 milliamps for approximately 35 minutes. The power pack was switched off and the gel was transferred to a Bio-Rad Gel Doc System for visualisation and photographing of DNA under UV light. If DNA bands were to be removed from gel then a UV transilluminator (Ultra-Lum, Quantum) was used to visualise and cut out the DNA from the gel with appropriate protective steps against UV light.

2.13.2. Digestion of the inserts and vectors

Digestion of the insert, (wild type and mutant *cyaA* fragment) and respective vectors, pUC19, pJQ200mp 18-rspL, and pSS1129 with or without the insert were followed according to manufacturer's instructions. In general, digestion reaction was carried out in one double-digestion reaction tube since NEB buffer 4 is suitable to use for digestion of DNA with either or both *BamHI* and *EcoRI* restriction enzymes, with 100% activity (New England Biolabs Inc). Any modification to protocol is mentioned in appropriate sections. Digestion reaction was incubated at 37^0C for five-to-seven hours in water bath. The digested inserts and vectors were used for subsequent cloning of the inserts into the respective vectors.

2.13.3. Ligation Condition

Overall procedure that followed for sub-cloning of the *cyaA/cyaMut* insert into pUC19 is presented in Figure 2.13.3. Same procedure was followed for sub-cloning of *CyaMut* insert into vector pJQ200mp-18 rspL and vector pSS1129.

Ligation procedures were followed as manufacturer's protocol, (Promega) and (Sambrook and Russell 2001). The ligation of digested wild type *cyaA* fragment insert into digested pUC19 vector and ligation of mutant *cyaA* fragment insert into, pUC19, pJQ200mp 18-rspL, and pSS1129, was accomplished as follows. In general the ligase reaction was assembled in 15 or 20µl on 0.5ml autoclaved eppendorf tube. The molar ratio of vector: insert for each individual ligation reaction is given below for each individual ligation reaction. To vector DNA (ng as required) insert DNA (ng as required) was added. To the mixture 1µl (Weiss units) of T4 DNA ligase, ligase buffer (Promega) and deionized water to final volume of 15 or 20µl was added. The tube was gently flicked between fingers several times. The tube was centrifuged for a few seconds and incubated at 16^0C for overnight. Ligation reaction between *cyaA*-(*BamHI-EcoRI*) and pUC19-(*BamHI-EcoRI*) was set up with two different vector:insert ratios (1:3 and 1:6) in 15µl at 4^0C and 16^0C incubated for overnight.

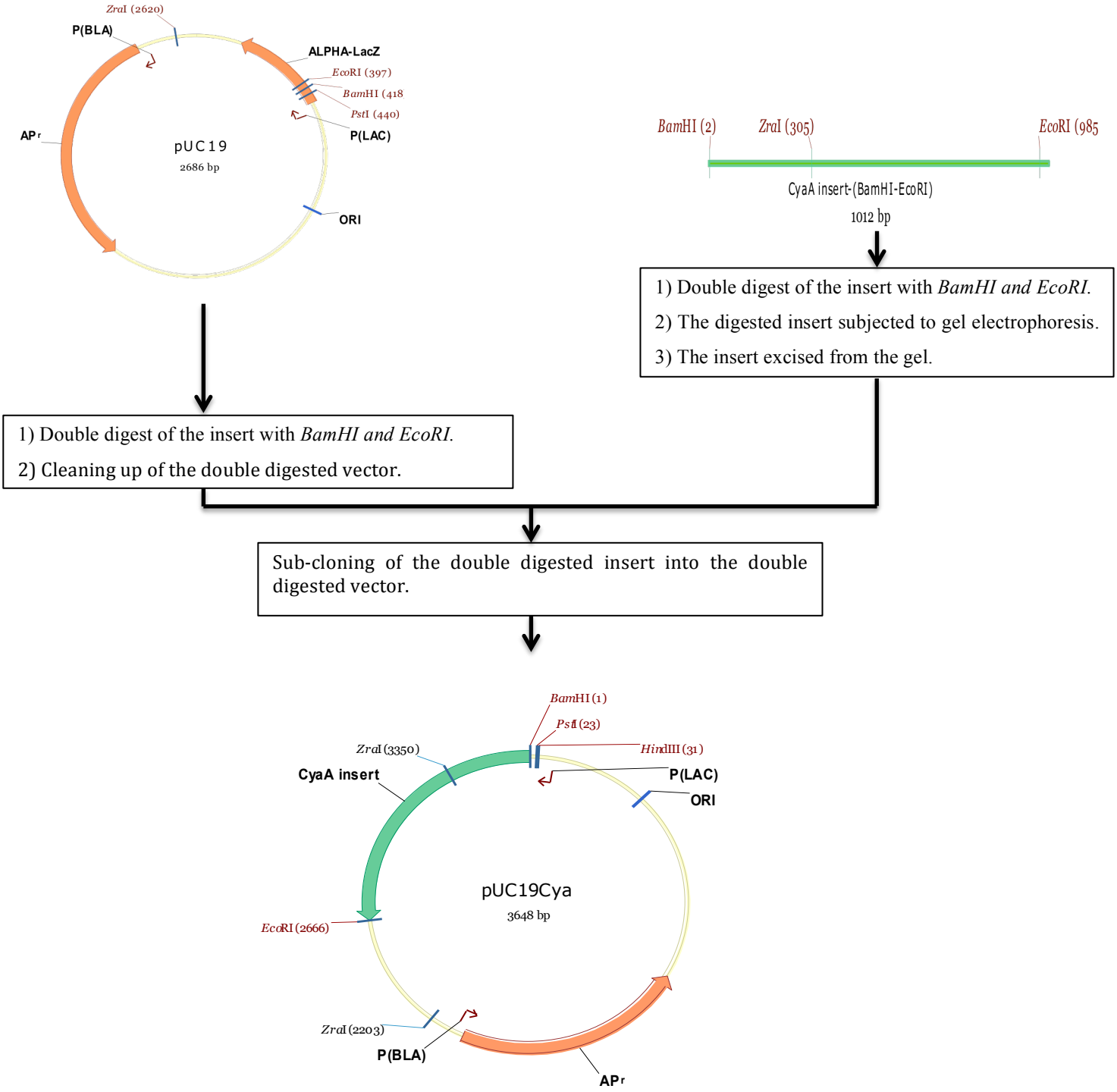

Figure 2.13.3. Schematic drawing of overall procedure for sub-cloning of double digested CyaA/CyaMut DNA fragments into double digested; pUC19, pJQ200mp 18-rspL, and pSS1129 vectors. The insert was double digested with BamHI-EcoRI; the gel-excised double digested insert subsequently sub-cloned into cleaned up double digested pUC19, pJQ200mp 18-rspL, and pSS1129 vectors.

2.13.4 Screen of colonies for vector-insert uptake by phenol-chloroform DNA extraction method

The method followed was as described by Sambrook et al. (2001). Fifteen microliter of 0.5µg/µl RNase A (Sigma) with 1x concentration of methylene blue was dispersed in 1.5ml eppendorf tube. About half colony of growth was resuspended in the solution. To the mixture 15µl of phenol: chloroform: isoamyl alcohol (25: 24: 1) saturated with 10mM Tris, pH 8.0 1mMEDTA was added and centrifuged at 13,500 rpm (17,320xg) (HERMLE Z 233M) for 1 minute. Fifteen microliter of the organic layer (upper aqueous phase) was run on 0.8% gel to check for the uptake of the insert with the plasmid.

2.14. Cloning of *cyaA* insert fragment into pUC19 and pGEM®-T easy vector

About 1000µg of pUC19 vector and 1100µg of *cyaA* fragment insert were digested sequentially with *BamHI* and *EcoRI* in 50µl reaction tube. Digested products of *BamHI,* pUC19-(*BamHI*) and *cyaA*-(*BamHI*) were cleaned in about 50µl according to manufacturer's instruction (QIAquick® PCR Purification Kit, QIAGEN). Briefly, 250µl (5 x of the PCR volume) of neutralisation buffer (PB) was added to the PCR product and the mixture applied to a QIAquick column. The column was placed over a 2ml collection tube and centrifuged at 13,500rpm (17,320xg) (HERMLE Z 233M) for 1 minute. The flow-through was discarded and bound DNA washed with 750µl of wash buffer (PE) followed by centrifugation as above. The column was then placed in an autoclaved 1.5ml microcentrifuge tube. The DNA eluted by adding 50µl of EB buffer; the column incubated at room temperature for one minute and centrifuged as above. Entire 50µl of *cyaA*-(*BamHI*) and 50µl of pUC19-(*BamHI*) digestions product were subjected to second digestion with *EcoRI* (Table 2.14.a-b). The digestion product of pUC19-(*BamHI-EcoRI*) was cleaned up as above (QIAquick® PCR Purification Kit, QIAGEN). Five microliter of pUC19-(*BamHI-EcoRI*) was subjected to electrophoresis on 0.8% gel to verify the purity and concentration of the DNA fragment.

The entire 50µl digestion product of *cyaA*-(*BamHI-EcoRI*) was subjected to electrophoresis on 0.8% gel. The fragment was excised from gel matrix and purified using gel extraction kit (QIAquick® Gel Extraction Kit, QIAGEN). Briefly, The DNA fragment excised from the agarose gel with scalpel. The excised DNA fragment from agarose gel was placed in a pre-weighed autoclaved microcentrifuge tube. The volume of the gel slice was estimated by weighing the tube, assuming one gram being equal to one millilitre. The volume of DNA Purification Kit Binding Buffer (QG) added to the microcentrifuge tube containing the excised gel was calculated using the following formula:

[Volume of the gel] x 3

The maximum amount of gel slice per QIAquick column was 400mg; for gel slice >400mg more than one column were used. The tube was incubated at 50^0C for ten minutes, vortexing every 2 minutes during the incubation. The matrix-bound DNA was pelleted by centrifugation for 1 minute at 13,500rpm (17,320xg) (HERMLE Z 233M). The supernatant was discarded and 500µl DNA purification buffer (QG) added to matrix and centrifuged as above. The DNA complex was pelleted as above, the supernatant discarded and 750µl of Wash Buffer (PE) was added and centrifuged as above. All traces of Wash Buffer (PE) were removed by an additional centrifugation as above. The pellet was resuspended in 30µl of Elution Buffer (EB) and incubated at room temperature for 1 minute. The spin column placed in an autoclaved 1.5ml microcentrifuge tube and centrifuged as above. Five to 10µl of eluted DNA was subjected to electrophoresis on 0.8% gel to verify the purity and concentration of the DNA.

Ligation reaction between *cyaA*-(*BamHI-EcoRI*) and pUC19-(*BamHI-EcoRI*) was set up with two different vector:insert ratios (1:3 and 1:6) in 15µl at 4^0C and 16^0C in two 0.5ml tubes along with negative control, all three tubes were incubated for overnight (Table 2.14c).

Table 2.14a. *cyaA* insert and pUC19 digestion with *BamHI* restriction enzyme.

Cya insert amplicon, volume (µl)		Uncut pUC19 vector, volume (µl)	
d.H_2O	34	d.H_2O	33
Buffer 4	5	Buffer 4	5
Insert	10	vector	11
BamHI	1	*BamHI*	1
Total volume	50	Total volume	50

Table 2.14b. *cyaA-(BamHI)* insert and pUC19-*(BamHI)* digestion with *EcoRI* restriction enzyme.

Cya insert amplicon, volume (µl)		Uncut pUC19 vector, volume (µl)	
d.H_2O	18.5	d.H_2O	23.5
Buffer 4	5	Buffer 4	5
Insert	25	vector	20
EcoRI	1.5	*EcoRI*	1.5
Total volume	50	Total volume	50

Table 2.14c. Ligation condition of *cyaA-(BamHI-EcoRI)* insert into pUC19-*(BamHI-EcoR1)*.

Sample vector:insert, 1:3 molar ratio (µl)		Negative control (µl)	
10xligation buffer	1.5	10xligation buffer	1.5
cyaA-(*BamHI-EcoRI*) insert	1.5 (56ng)	—	—
pUC19-(*BamHI-EcoR1*)	7.1 (50ng)	pUC19-(*BamHI-EcoR1*)	7.1
T4 DNA ligase (3 Weiss units/µl)	1.5	T4 DNA ligase (3 Weiss units/µl	1.5
PCR grade water	3.4	PCR grade water	4.9

2.14.1. Cloning of *cyaA* insert fragment into pGEM®-T easy vector

The procedure for *cyaA* insertion into pGEM-T easy vector was followed as stated in manufacturer's protocol (pGEM®-T Easy Vector System II, Promega), (Figure 2.14.1). The reaction was assembled in 10µl on 0.5ml autoclaved eppendorf tube. To 5µl of 2X Rapid Ligation Buffer, T4 DNA Ligase, 50ng (1µl) pGEM®-T easy vector was added. To the mixture 49.7µg (0.4µl) of gel extracted *cyaA* (QIAquick® Gel Extraction Kit, QIAGEN) and 1µl T4 DNA ligase (3 Weiss units/µl) was added. The reaction volume was adjusted to 10µl with nuclease free water. The reaction mixture was mixed gently 5-6 times with pipetting and incubated at 4°C for overnight. The molar ratio of vector: insert was 1: 3.

One microliter of ligated sample (*cyaA* insert + pGEM®-T easy vector) was transformed into *E coli* JM109 as stated in section 2.11.2. The sample (ligation reaction) was spread on four LB agar plates that supplemented with 100µg per ml of Amp/IPTG/X-Gal. Colonies with white colour indicated of successful transformation and insertion (inactivation of lac*Z* operon). The blue colour colonies on the other hand, indicated that the cell was transformed with the pGEM-T Easy vector without the insert. Total of forty-four white and blue colonies were picked from LB-Amp/IPTG/X-Gal plates and screened for *cyaA* insert in the vector as described in section 2.13.4. Two colonies with *cyaA* transformed colonies were subcultured in two

10ml LB- (100ugAmp/ml) broths and incubated at 37^0C on orbital shaker for 16 to 18 hours. Fifty microliters (40ng/µl) of the plasmid (*cyaA* insert + pGEM®-T easy vector) was extracted from each of two colonies (QIAprep® Spin Miniprep Kit using a Microcentrifuge) as described in section 2.12.1. Five hundred nanograms of each extracted plasmids were double digested with *EcoRI* and *BamHI* endonuclease restriction enzymes, respectively as described in section 2.13.2. Digested plasmids were subjected to electrophoresis on 0.8% gel to confirm the correct size of the *cyaA*-(*BamHI-EcoRI*) insert. Subsequently stock of pGEM-*Cya*Mut in *E coli* JM109 made and stored at -80^0C as describe in section 2.1.3.

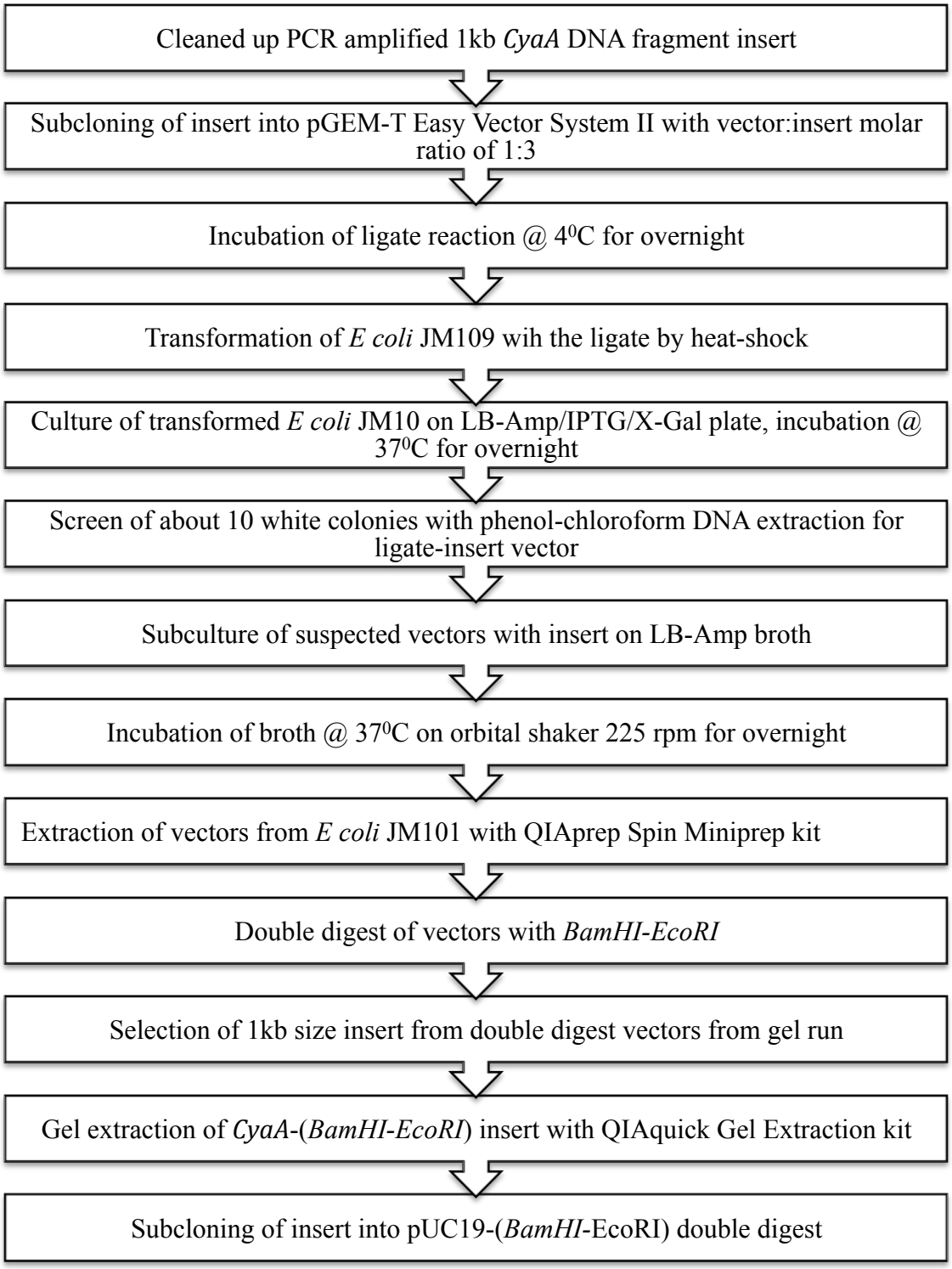

Figure 2.14.1. Summary of overall procedure for insertion of cyaA insert fragments into pGEM-T Easy Vector System II. The clone was double digested with BamHI-EcoRI *subsequently the digested cyaA*-(BamHI-EcoRI) *cloned into pUC19*-(BamHI-EcoRI) *vector.*

2.14.2. Cloning of *cyaA* from pGEM-*cyaA* into pUC19 vector

About 800ng pGEM-*Cya* and 500ng of pUC19 products each were double digested with *EcoRI* and *BamHI*. The whole 50µl of double digested pGEM-*cyaAEcoRI-BamHI* was run on 0.8% gel, and was extracted from gel (QIAquick® Gel Extraction Kit, QIAGEN). Likewise, double digested pUC19-(*EcoRI*-BamHI) was cleaned up (QIAquick® PCR Purification Kit, QIAGEN). Ligation of pEGEM-*cyaAEcoRI-BamHI* into pUC19EcoRI-BamHI was done as stated in section 2.13.3, the molar ratio of vector: insert was 1: 3 (50ng: 55.8ng). The resulting vector was pUC19Cya with size of 3.648kb (Figure 2.13.3).

2.15. Site-directed mutagenesis of cloned *cyaA* insert

The alteration of specific nucleotide(s) in the cloned *cyaA* insert fragment DNA in pUC19Cya vector was carried out using a QuikChange II XL Site-directed mutagenesis kit (Stratagene).

Inactivation of cloned *cyaA* insert involved two point mutations at bases 77640 and 77641 namely adenine to thymine and adenine to glycine with aim of changing lysine codon (AAA) to methionine (M) codon (ATG) (Au, Masure, and Storm 1989) on pUC19Cya vector (Figure 2.13.3). The resulting vector was pUC19CyaMut with size of 3.648kb.

Point mutation of a 4.5kb pWhitescript plasmid (Stratagene) was used as a positive control for mutagenesis protocol. The *lacZ* insert of the plasmid had an in-frame TAA stop codon, which interfered with *β*-galactosidase expression. Subsequent point mutation of the thymidine residue in the stop codon to a cytidine residue using the control oligonucleotide generated a glutamine codon switch. Transformation of *E coli* with the mutated pWhitescript conveyed a blue phenotype on selective LB agar supplemented with IPTG and X-Gal, by virtue of an unimpeded β-galactosidase expression in the mutants.

2.15.1. Detection of mutation in lysine 58 codon by sequencing

In general, when the mutation in manipulated *cyaA* insert or vector was to be checked, the plasmids or the extracted *cyaA* fragment were sent to the Australian Genome Research Facility Ltd (agrf) for sequencing. Products either were cleaned up (QIAquick® PCR Purification Kit, QIAGEN) before sending to agrf or sent as unpurified samples. Between 30-75ng of cleaned PCR products in 9µl with 3µl of 10 pico-mole (pM) of perspective primer (forward or reverse), in total of 12µl were sent to agrf for sequencing. When a large number of PCR samples (about 10) sent for sequencing the PCR reactions were run in 20µl reaction on PCR plate (Axygen PCR-96 M2-HS). The unpurified PCR reaction plate along with 200µl of 3.2µM of perspective primer (forward or reverse) was sent to agrf for sequencing. Vector(s) were pre prepared as purified PCR products before sending for the sequence to agrf.

Before sending any batch samples for sequencing, 5-10 samples were subjected to gel electrophoresis for samples concentration and their quality. In general, concentration of each sample was measured by NanoDrop (Thermo Scientific).

2.16. Sub-cloning of Mutant *cyaA* insert (*CyaMut*) into pJQ200mp 18-rspL

Sub-cloning of CyaMut insert fragment into pJQ200mp 18-rspL was carried out as described in following sections. For overall sub-cloning procedure, see Figure 2.13.3.

2.16.1. Digestion of pJQ200mp18-rpsL

One thousand micrograms in (6µl) of pUC19CyaMut double digested with *BamHI-EcoRI* in 50µl reaction mixture and was subjected to electrophoresis on 0.8% gel. The one kilo-base CyaMut was extracted from the gel using the QIAquick® Gel Extraction Kit (QIAGEN). Also about 1000µg of pJQ200mp18-rpsL (20µl) was double digested as above with *BamHI-EcoRI*, pJQ200mp 18-rspL (*BamHI-EcoRI),* and cleaned up using the QIAquick® PCR Purification Kit (QIAGEN).

2.16.2. Ligation of CyaMut-(*BamHI-EcoRI)* insert and pJQ200mp 18-rspL-(*BamHI-EcoRI)*

Ligation reaction was set up in 15µl reaction mixture with as described in section 2.13.3 with vector: insert ratio of 1:3 (50ng pJQ200mp 18-rspLCyaMut in 5µl: 31ng CyaMut in 6.2µg). The vector size was 4.788kb. The 0.5ml reaction tube was incubated at 4^0C for overnight. The resultant vector named pJQ200mp 18-rspLCyaMut.

2.16.3. Transformation of *E. coli* SM10 *λ pir* with pJQ200mp 18-rspLCyaMut (pJQ200mp 18-rspLCyaMut)

E coli SM10 *λ pir* was transformed with 2µl of pJCyaMut vector as described in section 2.11.2. The transformed *E. coli* SM10 *λ pir* on 500µl SOC broth was spread on 4xLA-(Gm10µg/ml) with 50µl, 100µl, 150µl, and the rest of the broth. All of four plates were incubated at 37^0C for overnight. Twelve colonies were picked up from transformation plate and screened for insert with phenol-chloroform DNA extraction method as described in section 2.13.4.

2.16.4. Transformation of *aroQ*BP with pJ200mp 18-rspLCyaMut

A modified conjugation protocol of (Simon 1983) was followed for transfer of the pJCyaMut to *aroQ*BP strain (Figure 2.16.4). The *aroQ*BP (recipient) was grown on BG-aromix-(Km50µg/ml, Sm 200µg/ml) for three days. A 10ml SS-(Km50µg/ml, Sm-200µg/ml) was inoculated with *aroQ*BP from above plate. The OD_{600nm} of the broth at time zero was 0.020. The broth was incubated at 37^0C on orbital shaker (Ratek) at 225rpm until the broth got slightly turbid (about 58 hours). The OD_{600nm} of broth was taken at different time intervals till OD_{600nm} reached about 0.520 in the log growth phase (Appendix D. page 160).

The pJQ200mp 18-rspLCyaMut transformed *E. coli* SM10 *λ pir* (donor) was grown on LA-(Gm10µg/ml, Km50µg/ml) for 16 to 18 hours. The pJQ200mp 18-rspLCyaMut transformed was grown in 10ml SS medium containing Gm10µg/ml,

Km50µg/ml until the growth curve reached log phase OD_{600nm} of approximate 0.492 (Appendix E, page 161).

Mixture of recipient: donor, 1:1, 10:1, and 100:1 ratio were made in about 1ml of SS-(Km50µg/ml, Sm200µg/ml) medium as follows: Ten fold serial dilutions, 10^{-1}-10^{-3} were made for *aroQ*BP (OD_{600nm}) in 1ml SS-(Km50µg/ml, Sm200µg/ml). Ten microliter of *E coli* SM10 λ *pir* was added to each of the dilutions made above. Each individual recipient:donor mixture was loaded (approximately 200µl) onto nitrocellulose membranes (0.22µm pore size) that had been placed on six CW-aromix-(Km50µg/ml, $MgCl_2$) plates. The plates were incubated with the lid facing upwards at 37^0C for 4 hours. Growth from each plate in the batch was swept with a sterilised dacron swab and suspended in 1ml SS medium. Each individual suspension was plated on 4 CW-aromix-(Km50µg/ml, Sm200µg/ml) plates in different volumes viz., 50µl, 100µl, 200µl and the remaining volume on the 4th plate. The whole twelve plates were incubated at 37^0C in plastic bag with wet tissues for four days. On the fifth day isolated colonies were detected on the plate inoculated with 100µl of the 1:100 recipient:donor ratio. Sixty-three isolated colonies spot-cultured on 3 CW-aromix-(Km50µg/ml, Sm200µg/ml) and 3 CW-aromix-(Km50µg/ml, Sm200µg/ml, Gm10µg/ml) plates were incubated at 37^0C as above for five days.

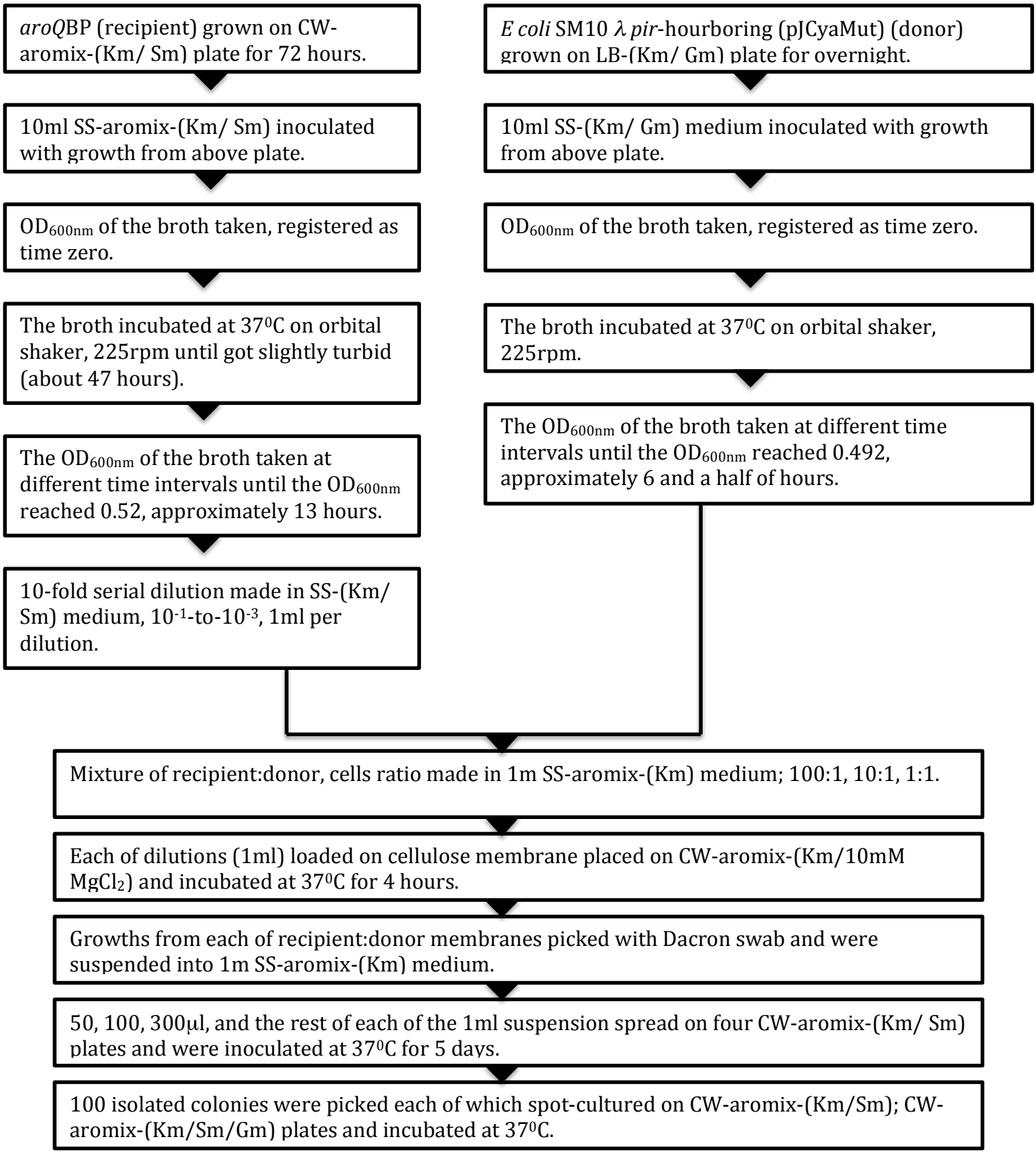

Figure 2.16.4. Diagram describing the protocol followed for transformation of B pertussis aroQ *strain with pJQ200mp 18-rspLCyaMut.*

2.17. Sub-cloning of CyaMut insert into pSS1129 vector

Sub-cloning of CyaMut insert fragment into pSS1129 was carried out as described in following sections. For overall of the sub-cloning procedure see Figure 2.13.3.

2.17.1. Digestion of pSS1129 vector

One thousand (1000) ng pUC19CyaMut vector was digested with *EcoRI* and *BamHI* in 50µl reaction tube as described in section 2.13.2. The whole 50µl of double digested pUC19CyaMut-(*EcoRI-BamHI*) was run on 0.8% gel. The CyaMut-(EcoRI-BamHI) was extracted from gel (QIAquick® Gel Extraction Kit, QIAGEN). Likewise, about 900ng of pSS1129 was double digested with *EcoRI* and *BamHI*. The resultant vector, pSS1129-(*EcoRI-BamHI*) was cleaned up with (QIAquick® PCR Purification Kit, QIAGEN).

2.17.2. Ligation of CyaMut-(*BamHI-EcoRI)* insert and pSS1129-(*BamHI-EcoRI)*

Ligation reaction was set up in 15µl reaction mixture with as described in section 2.13.3 with vector: insert ratio of 1:3 and 1:6, 50ng: 17ng and 50ng: 34ng, in a 0.5ml microtube respectively. The 0.5ml reaction tube was incubated at 4^0C for overnight. The resulting vector pSS1129CyaMut had an estimated size of 10kb. The size of this vector had to be estimated since complete sequence of the pSS1129 vector has not been published.

2.17.3. Transformation of *aroQ*BP with pSS1129CyaMut

A modified conjugation protocol of Stibitz (1994) was followed for transfer of the pSS1129CyaMut to *aroQ*BP strain (Figure 2.17.3). A BG-aromix-(Km50µg/ml, Sm200µg/ml) was inoculated with -80^0C *aroQ*BP strain stock and incubated for three days. Ten ml CL supplemented with aromix, Km 50µg/ml and Sm200µg/ml in twenty ml McCartney tube was inoculated with the *aroQ*BP strain. The OD_{600nm} of the broth was taken at zero time was 0.067. The broth was incubated at 37^0C on orbital shaker

(Ratek) at 225rpm until the broth became slightly turbid (about 48 hours). At the second day, the OD_{600nm} of broth was taken at different time intervals until OD_{600nm} of the broth reached approximately 0.5 in the log phase of the growth curve (Appendix F, page 162).

A LB-(Amp100µg/ml, Km50µg/ml, Gm10µg/ml) agar was inoculated with -80^{0}C stock *E coli* SM10 λ *pir* containing pSS1129CyaMut vector. The plate was incubated at 37^{0}C for 16-18 hours and a 10ml LB-(Amp100µg/ml, Km50µg, Gm10µg/ml) broth was inoculated with *E coli* SM10 λ *pir* harbouring pSS1129CyaMut vector from the plate. The broth was incubated at 37^{0}C on orbital shaker (Ratek) at 225rpm until broth OD_{600nm} reached 5.0 at OD_{600nm}, which took about 6hours.

The *aroQ*BP (approximate OD_{600nm} of 0.5) and *E coli* SM10 λ *pir* (approximate OD_{600} of 0.50) broths were mixed in SS medium at different donor (*E coli* SM10 λ *pir*-pSS1129CyaMut) to recipient (*aroQ*BP) ratios as shown in Table 2.17.3.

Table 2.17.3. Ratio of donor-to-recipient cells number used for conjugation.

Donor: Recipient, Cells Number	*E coli* SM10 λ *pir*-pSS1129CyaMut (µl)	*aroQ*BP (µl)
1:10	10	100
1:25	1	25
1:50	1	50
1:100	1	100
1:500	0.1	50

Each individual conjugation mixture broth was spot cultured on one BG-Aromix-(Km50µg/ml, $MgCl_2$10mM). The plate was incubated at 37^{0}C for fourteen hours. Each growth was suspended in 1ml of SS-aromix-(Km50µg/ml) medium. Each of 1ml broth was spread on 7 BG-aromix-(Km50µg/ml, Sm200µg/ml) plates. A volume 20µl, 30µl, 50µl, 100µl, 200µl, 300µl and remaining mixture were spread plated on individual plates. All the 35 plates were incubated at 37^{0}C in plastic bags with wet

tissues for five days. On second day of incubation, fine growth was detected on all plates. On fifth day, confluent growth was detected on all plates. One plate of each conjugate batch was taken (total of 5 plates) and ten-fold serial dilution was made from each conjugate plates. Suspension was made from each of the five conjugate plates in approximately 1ml SS medium and their OD_{600nm} adjusted to approximately 0.450-0.520. Ten-fold serial dilution in total volume of 500µl for each of the broths from 10^{-1}-10^{-7} dilutions were made. Each of 10^{-5}, 10^{-6}, and 10^{-7} of each of dilution batches was spread plated on 15 BG-aromix-(Km50µg/ml, Sm200µg/ml) plates and incubated at 37^0C in plastic bag with wet tissues for four days. Total of 422 isolated colonies growth were picked up from the plates and spot-streaked on 9 BG-aromix-(Km50µg/ml, Sm200µg/ml), 9 BG-aromix-(Km50µg/ml, Sm200µg/ml, Gm10µg/ml), and 9 BG-aromix-(Amp100µg/ml, Km50µg/ml, Sm200µg/ml, Gm10µg/ml). All 27 plates were incubated at 37^0C in plastic bag with wet tissues for four to five days.

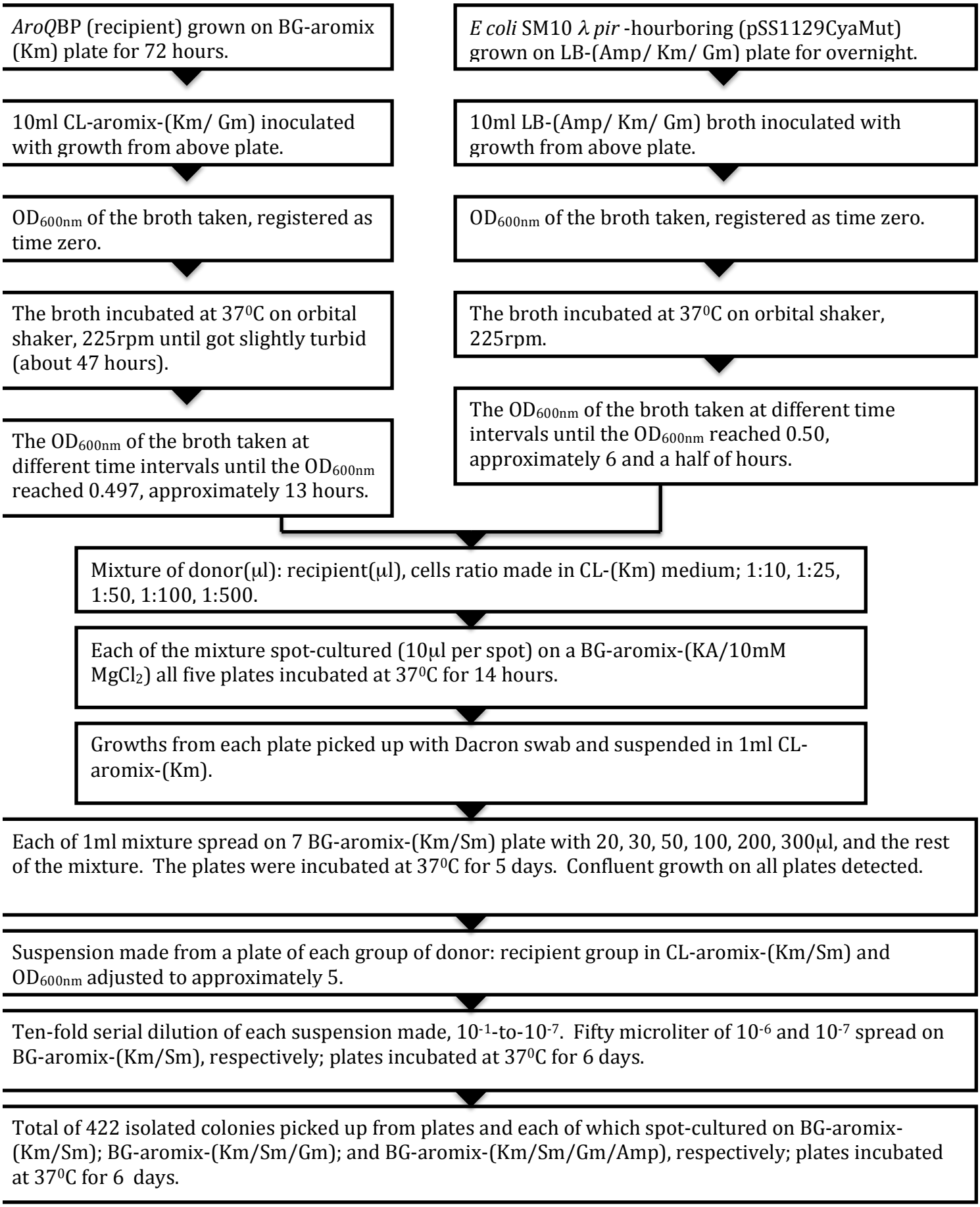

Figure 2.17.3. Diagram describing protocol followed for transformation of B pertussis aroQ *strain with pSS1129CyaMut.*

2.18. Colony screen for lysine mutation using PCR

Two different protocols were followed to detect whether the point mutation had taken place at position 58 of adenylate cyclase toxin. A) Amplification of *cyaA* insert (1kb bases) by PCR using FwdCya and RvsCya primers (Table 2.13a) and isolated colonies from the conjugation plates as template; B) Amplification of 210 bases of *cyaA* (210-base pair sequence) FwATG-mutant, FwAAA-wild type, RvsATG as primers (Table 2.13a) and pooled isolated colonies as template. The *cyaA* insert PCR was designed to amplify first 1kb bases of the ACT, which contains AAA bases, lysine codon. FwATG-mutant primer of the 210-base pair sequence had ATG (methionine codon) at its 3′ end. FwAAA-native primer of the 210-base pair sequence had AAA (lysine codon) at its 3′ end.

2.18.1. Standardization of Cya FwATG-mutant, FwAAA-wild type, RvsATG primers using pUC19CyaMut and pUC19Cya as positive and negative templates respectively

Two sets of PCR were set up to amplify the 210-base pair sequence using FwATG-mutant and FwAAA-wild type primers, respectively. Reverse primer for both PCR sets was RvsATG. Seventeen 20µl PCR reactions aliquot were made. In eight of the aliquot, FwATG-mutant and RvsATG primers included. In the other eight aliquots, FwAAA-wild type and RvsATG primers included. pUC19Cya and pUC19CyaMut were used as template for each four aliquot of mutant PCR batch, respectively. Likewise, each of four aliquot of wild type PCR batches were inoculated with pUC19Cya and pUC19CyaMut templates.

2.18.2. Mutant detection in *aroQ*BP using FwdCya and RvsCya primer

Forty-four colonies growth from BG-aromix-(Km50µg/ml, Sm200µg/ml) were picked and amplified by PCR in 23µl individual PCR reaction aliquot wells. Cycling condition for the amplification of *cya* insert followed as in Table 2.13c. All of the PCRs amplicons were run on gel at 5µl/well. About 17µl of each of the PCR amplicon were sent to agrf.

2.18.3. Mutant detection in *aroQ*BP using Cya FwATG, FwAAA, RvsATG as primer

The whole 422 isolated colonies from BG-aromix-(Km50µg/ml, Sm200µg/ml) were divided into 42 batches (ten colonies per batch) except one batch consisting of 12 colonies. Colonies of each batch was suspended into labelled autoclaved microtubes containing 250µl PCR grade water. Each of individual aliquot was used as template amplification of 210-base pair sequence in 23µl PCR reaction in 42 aliquot wells with FwATG and RvsATG as forward and reverse primers, respectively.

Chapter 3: Results

3a. Immune response of acellular pertussis vaccine (DTaP)-vaccinated mice to booster vaccination with *aroQ*BP:

3a.1. Introduction

The vaccine used in this study was Infanrix (GlaxoSmithKline) vaccine, which is currently used to vaccinate infants in Western Australia against whooping cough. Infanrix contains the following native or modified components from *B pertussis*: PTxoid, FHA, PRN (Bordetella pertussis); Diphtheria toxoid, Tetanus toxoid. The current practice in much of developed world for prevention of whooping cough is vaccination of infants at 2, 4, and 6 months followed two booster vaccination at 12 months and 4-6 years of age with an acellular vaccine pertussis vaccine containing diphtheria toxoid, tetanus toxoid, three to five native, recombinant and chemically or genetically purified potential protective antigens of *B pertussis* including: filamentous haemagglutinin, pertussis toxoid, and pertactin with or without fimbrial antigens 2 and 3/6 (Department of Health and Aging 2008). Depending upon the manufacturer, additional vaccines that may be included in the DTaP formulation are either Hib, or inactivated poliovirus vaccine comprising type 1 and 3. The DTaP replaced the whole cell pertussis vaccine, DTwP, comprising diphtheria toxoid, tetanus toxoid and killed whole cells of *B pertussis*. It has been reported (Rennels et al. 2000; Gold et al. 2003; Jackson et al. 2002) that vaccination with even DTaP results in induction of side-reactions, albeit considered/claimed to be milder and hence one of potential reasons for the compliance with the recommended schedule of vaccination resulting in an increase in incidence of whooping cough in unvaccinated or partially vaccinated populations. Even fully vaccinated populations have been now reported to acquire infection with *B pertussis*, which has been attributed to:

1) PT hyper-producer strains (Frits R. Mooi 2001; de Melker et al. 1997; He and Mertsola 2008; Mooi et al. 2009).

2) Inability of the acellular pertussis to induce CMI, as verified in the mouse model, considered to be necessary for long term protection against whooping cough.

3) Short to midterm immunity offered by the acellular pertussis vaccine (Mills 2001).

There has been an increase in the incidence of pertussis in infants and children (Cherry 2005; Tan et al. 2005) attributed mainly to transmission from infected adults. In Australia, pertussis has reached an epidemic state showing an increase in its incidence in adolescents, adult, and the elderly (Figure 3a.1) (National Notifiable Diseases Surveillance System 2011; Quinn and McIntyre 2007; Simon 1983).

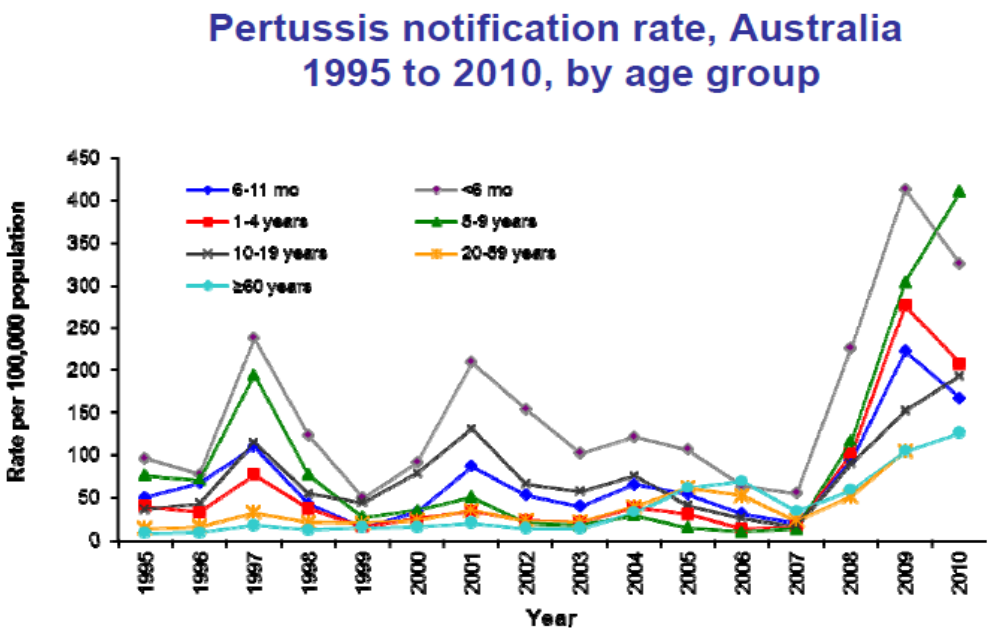

Figure 3a.1. Source for compilation: National Notifiable Diseases Surveillance System. http://www9.health.gov.au/cda/Source/Rpt_5.cfm, (Courtesy of A/Professor Peter Richmond, University of Western Australia)

Whooping cough is also believed to be epidemic in many states of the USA (Centers for Disease Control and Prevention 2011) as exemplified by a recent dramatic increase in the incidence of whooping cough in California (Figure 3a.1b) that has resulted in making it compulsory for parents to vaccinate children with the DTaP.

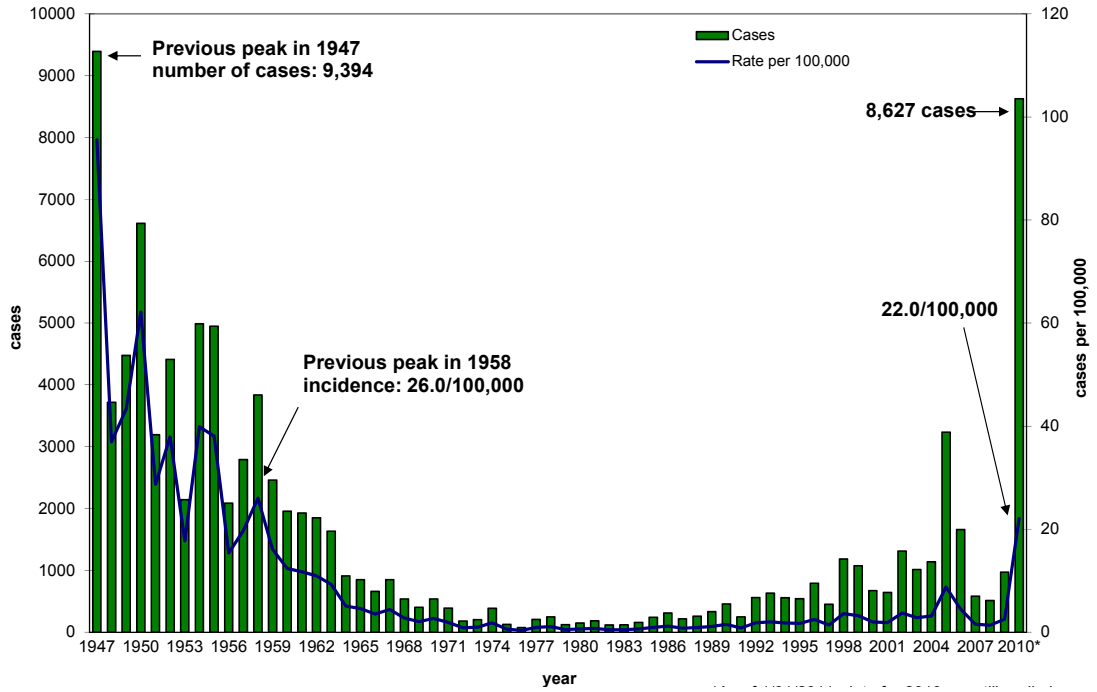

Figure 3a.1b. Number of reported pertussis cases, California 1947-1950, to Centers for Diseases Control and Prevention. http://www.cdc.gov/pertussis/outbreak.html.

Source: Centers for Diseases Control and Prevention.

It has been shown that greater than 70% (Wendelboe et al. 2007) of the pertussis cases encountered infants and children are transmitted from the infected household contacts including adolescents, adults and elderly. Recently, a new vaccine, dTap, containing significantly lower doses of the purified protein antigens was introduced for booster vaccination of adults and elderly (Cherry 2005). Although, reason for reducing the antigens is not known, it may have been due to perceived anticipated side reactions in the target population.

It is well established now that the protection offered by vaccination with acellular pertussis vaccines is due to antibodies induced by Th2-polarisation of immune response (Canthaboo, Williams, et al. 2000). However, it is also generally accepted that induction of CMI induced via Th1-polarisation of immune response is necessary for long-term protection against whooping cough (Redhead et al. 1993; Mills 2001; Mascart et al. 2003).

It has been reported that live attenuated pertussis vaccines may constitute ideal candidates for imparting long-term protection because they mimic natural infection (Locht et al. 2004; Mielcarek et al. 2010) providing the potential of inducing immune response to all virulence antigens. The currently used DTaP vaccines contains only 3-5 of *B pertussis* protective antigens, depending upon the vaccine manufacturer, with only one of the major toxins being covered for neutralisation.

Roberts et al. (1990) reported the development of live attenuated *B pertussis* in which the *aroA* gene of the common aromatic biosynthesis pathway had been insertionally inactivated. Because the *aroA* mutant did not efficiently colonise airways of mice, three doses of the mutant vaccine delivered intranasally were required for induction of high levels of antibodies of different isotypes, (IgM, IgG, and IgA) and protection against challenge. However, no data on either the CMI-indicator cytokines or information on pulmonary secretions was presented.

More recently, development of two other live vaccine candidates has been reported. Mielcarek et al. (2006) reported the development of *B pertussis,* which was attenuated by genetic detoxification of pertussis toxin, deletion of DNT, and replacement of *ampG* gene with the *E coli ampG* gene with a view to attenuating the potential

cytotoxicity of the TCT. However, no essential gene was deleted leaving the door to reversion to virulence of the partially detoxified vaccine candidate open. On the other hand, Mukkur et al. (2005) reported the development of a *B pertussis* vaccine candidate in which the *aroQ* gene of the aromatic biosynthesis pathway has been deleted and insertionally inactivated *aroQ*BP vaccine.

Common aromatic biosynthetic pathway is essential for synthesis of aromatic amino acids in eukaryotes and prokaryotes, and also precursors of aromatic compounds essential for the survival in prokaryotes (Garbe et al. 1991; Bottomley et al. 1996), chorismic acid being the precursor of key essential aromatic compounds including PABA, DHB and OSB (Hoiseth and Stocker 1981). It has been shown that type II dehydroquinase enzymes (Moore et al. 1992) in the aromatic biosynthetic pathway are multifunctional enzymes. The enzymes catalyse a second pathway, conversion of quinic acid to acetyl-CoA by quinate catabolic pathway (Garbe et al. 1991; Lalonde et al. 1994; Bottomley et al. 1996), which catalyse the inter-conversion of 3-dehydroquinate and 3-dehydroshikimate and are related in sequence to the enzyme used in the catabolic pathway (Giles et al. 1985).

The *aroQ*BP vaccine candidate is deficient in synthesis of PABA, DHB, OSB and other aromatic compounds (Rossetti 1997; Cornford 1998). These metabolites are unavailable in the mammalian tissue and hence the avirulence of the *aroQ*BP. It has been reported that *aroQ*BP, in contrast to *aroA*, can colonise airways of mice efficiently and induce both humoral and cell mediated immunity without causing disease (Cornford-Nairn, PhD Thesis [under embargo, University of Southern Queensland]; Mukkur et al. 2005; Mukkur, Personal communication). Mice immunised with a single dose *aroQ*BP were protected against an intranasal challenge with parental *B pertussis* strain. Serum anti-*B pertussis* antibodies of the IgG1 and IgG2 isotypes were induced which were enhanced post-challenge with the virulent parent. Vaccination of mice with two and three of the *aro*QBP vaccine further enhanced IgG1 and IgG2 levels and also induced anti-*B pertussis* pulmonary IgA levels.

Given the recent whooping cough outbreaks reaching an epidemic stage despite extensive coverage with the acellular pertussis vaccine in developed world, it was

important to determine whether the Th2-polarised immune response, induced by the acellular pertussis vaccine (delivered as a combination vaccine, DTaP) could be polarised towards Th1 type by immunisation with the live attenuated *aroQ*BP vaccine candidate. Answer to this question was obtained with the following experimental design using the pertussis mouse model (Table 2.4.5)

In order to evaluate the nature of immune response generated following immunisation with *aroQ*BP vaccine versus DTaP-immunised mice booster-vaccinated with *aroQ*BP vaccine, four groups of 5 mice each were vaccinated with either DTaP with (Groups A and B) or without boosting (Groups C and D), with one or two doses of live *aroQ*BP vaccine candidate. Mice immunised with the *aroQ*BP only (Groups C and D) acted as controls for the booster-vaccinated groups (A and B). Five mice in each group were sham-vaccinated with PBS whereas the 5th group (Group E) constituted the sentinel group that was left untreated.

3a.2. Comparison of immune responses of mice vaccinated with *aroQ*BP vaccine candidate with or without prior vaccination with DTaP

Due to the composition of the DTaP vaccine, which incorporates significant amounts of select *B pertussis* antigens, varying levels of antibody levels to killed whole cells or the selected purified antigens (FHA and PT) were detected using ELISA. Titration was carried out on each individual mouse serum sample. Titration for antibody isotypes of each individual mouse is presented in appendices I-L (page 165-250). However, due to scarcity, antibody, titration of the pulmonary homogenate samples for antibody isotypes were only carried out using killed whole *B pertussis* cells as an antigen. Antibody titers against purified pertussis toxin and filamentous haemagglutinin were not determined.

3a.3. Serum antibody responses

IgG: DTaP-immunised mice that were booster-vaccinated with one dose of the *aroQ*BP vaccine (Group A) developed significantly high antibody levels than those vaccinated with one dose of the *aroQ*BP vaccine candidate (Group C) ($P< 0.00002$). On the other hand, IgG antibody isotype titers in mice vaccinated with two-doses (Group D) of *aroQ*BP vaccine were significantly greater than those in the DTaP-immunised mice booster-vaccinated with one dose of the *aroQ*BP vaccine candidate (Group A) ($P< 0.02$) (Figure 3a.3). However, there was no significant difference between the antibody titers in mice immunised with two-doses of the *aroQ*BP vaccine versus DTaP-vaccinated mice that booster-vaccinated with two-doses of the *aroQ*BP vaccine candidate.

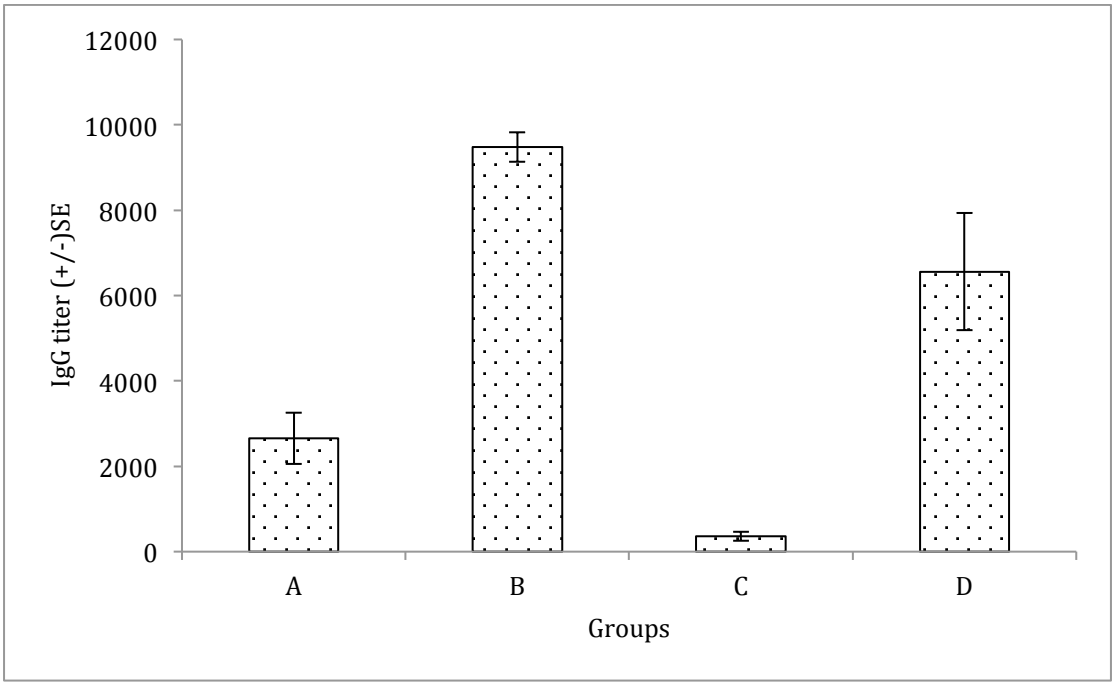

*Figure 3a.3. Serum IgG response of mice vaccinated with the aroQ*BP *vaccine versus its use as a booster in DTaP-vaccinated mice. IgG titers of mice vaccinated with three-doses of DTaP vaccine and one-dose of aroQ*BP *as booster blood sampled two weeks after the last vaccination at day 35 (Group A); IgG titers of mice vaccinated with three-doses of DTaP vaccine and two-doses of aroQ*BP *as booster blood sampled 2 weeks after the last vaccination at day 42 (Group B); IgG titers of mice at day 14, two weeks after the last vaccination (Group C); IgG titers of mice vaccinated with two-doses of aroQ*BP *blood sampled at day 21, two weeks after the last vaccination (Group D). BPWC was used as the coating antigen.*

IgG1: All the groups except the group vaccinated with PBS (Group E) induced serum IgG1 responses against inactivated BPWC *aroQ* strain. IgG1 antibody level produced by Group B mice vaccinated with three-doses of DTaP subcutaneously and booster-vaccinated with two-doses of *aroQ*BP by i.n. route was significantly different from that of group A mice which received three-doses of DTaP subcutaneously and one-dose booster of the *aroQ*BP by the i.n. route ($P < 0.05$; Figure 3a.3a). There was no significant difference between the IgG1 antibody titers of mice booster-vaccinated with one dose of the *aroQ*BP vaccine (Group A) versus those vaccinated with two-doses of the *aroQ*BP vaccine by the intranasal route (Group D) ($P > 0.05$; Figure 3a.3a). However, there was a significant difference ($P < 0.005$) in the IgG1 antibody levels of mice booster-vaccinated with two-doses of the *aroQ*BP vaccine (Group B) versus those immunised with two-doses of the *aroQ*BP vaccine (Group D).

Only one of five mice in Group C, vaccinated intranasally with one-dose of the *aroQ*BP vaccine, produced detectable level of IgG1 to inactivated *aroQ* BPWC strain as the coating antigens (Table 3a.3).

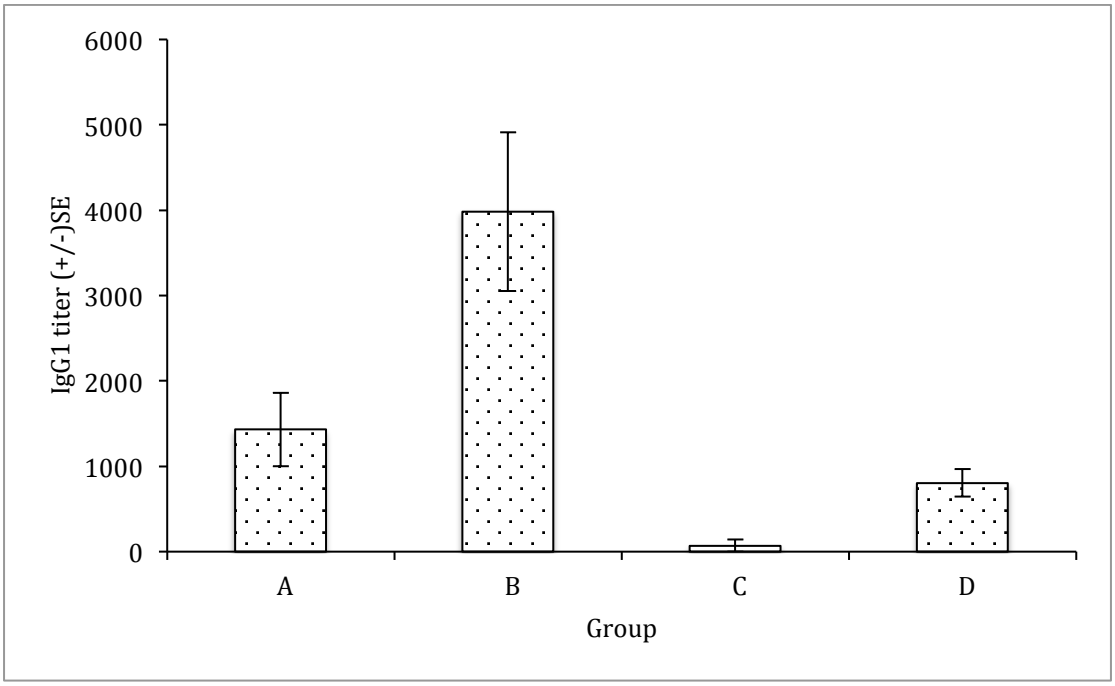

*Figure. 3a.3a. Serum IgG1 response of mice vaccinated with the aroQ*BP *vaccine versus its use as a booster in DTaP-vaccinated mice. IgG1 titers of mice vaccinated with three-doses of DTaP vaccine and one-dose of aroQ*BP *as booster blood sampled two weeks after the last vaccination at day 35 (Group A); IgG1 titers of mice vaccinated with three-doses of DTaP vaccine and two-doses of aroQ*BP *as booster blood sampled 2 weeks after the last vaccination at day 42 (Group B); IgG1 titers of mice at day 14, two weeks after the last vaccination (Group C); IgG1 titers of mice vaccinated with two-doses of aroQ*BP *blood sampled at day 21, two weeks after the last vaccination (Group D). BPWC was used as the coating antigen.*

IgG2a: IgG2a antibody isotype titers of Groups B mice, representing DTaP-immunised mice booster-vaccinated with two-doses of the *aroQ*BP vaccine candidate by the i.n. route were significantly higher than Group A mice vaccinated with one booster dose of the *aroQ*BP vaccine candidate ($p<0.001$). IgG2a response from four mice in Group A fell below the cut-off point (0.1, OD_{405nm}) and they were assigned the IgG2a titer as zero (Figure 3a.3b, Table 3a.3). However, there was no significant difference between the IgG2a antibody titers of Group B versus mice in Group D which were vaccinated with two-doses of *aroQ*BP vaccine ($p>0.05$). The IgG2a titers of 4/5 mice in Group A fell below the cut-off point (0.1 OD_{405nm}) and were assigned the titer as zero (Figure 3a.3b,Table 3a.3).

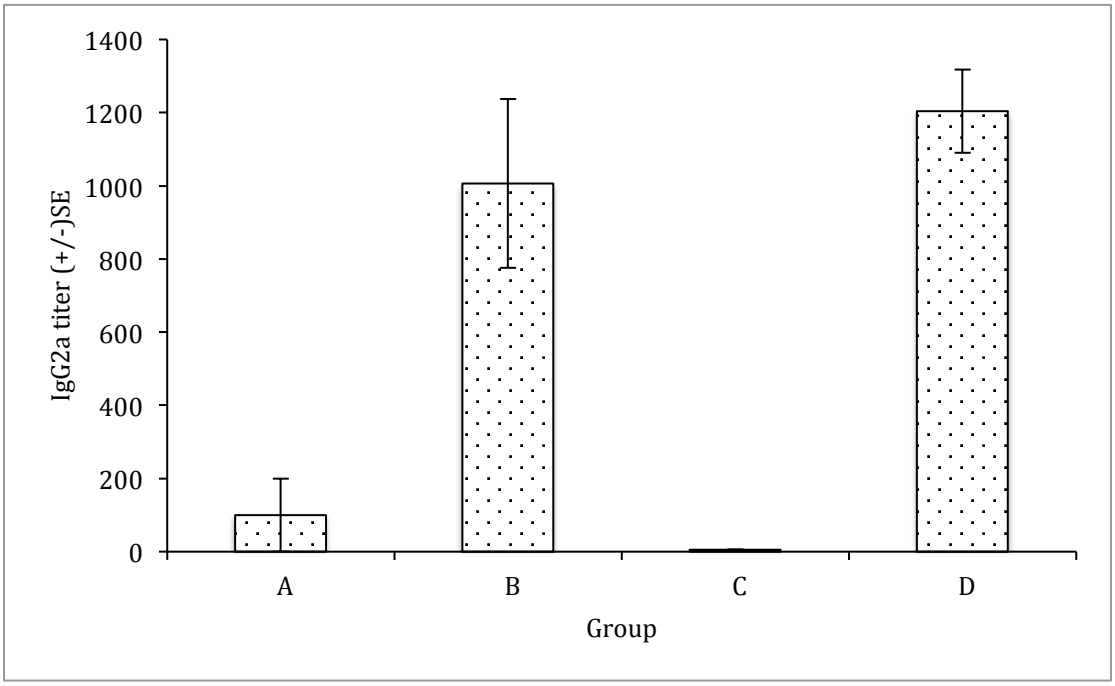

*Figure. 3a.3b. Serum IgG2a response of mice vaccinated with the aroQ*BP *vaccine candidate versus its use as a booster in DTaP-vaccinated mice. IgG2a titers of mice vaccinated with three-doses of DTaP vaccine and one-dose of aroQ*BP *as booster blood sampled two weeks after the last vaccination at day 35 (Group A); IgG2a titers of mice vaccinated with three-doses of DTaP vaccine and two-doses of aroQ*BP *as booster blood sampled 2 weeks after the last vaccination at day 42 (Group B); IgG2a titers of mice at day 14, two weeks after the last vaccination (Group C); IgG2a titers of mice vaccinated with two-doses of aroQ*BP *blood sampled at day 21, two weeks after the last vaccination (Group D). BPWC was used as the coating antigen.*

IgA: All the absorbance values for the serum IgA response of all the groups at the starting dilution (1/100) was below the selected cut-off point (0.1 OD_{405nm}). Hence it was decided to estimate antibody titers using the highest single point value x 100 to represent relative IgA content. All groups of mice, except the sham-vaccinated mice in each group, produced IgA isotype specific antibodies. Mice in Group D that had received two-dose of the *aroQ*BP vaccine showed the highest IgA antibody levels ($P<0.001$) with the remaining groups showing similar IgA antibody levels (Figure 3a.3c).

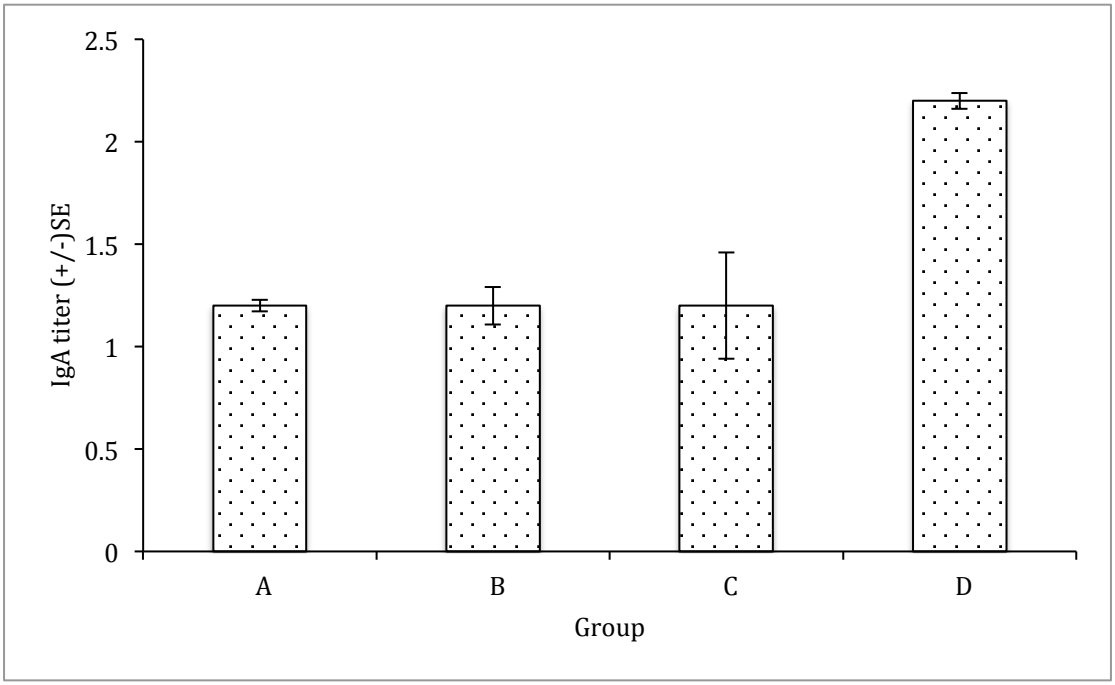

*Figure. 3a.3c. Serum IgA response of mice vaccinated with the aroQ*BP *vaccine versus DTaP-immunised,* aroQ*BP-booster vaccinated mice. IgA titers of mice vaccinated with three-doses of DTaP vaccine and one-dose of aroQ*BP *as booster blood sampled two weeks after the last vaccination at day 35 (Group A); IgA titers of mice vaccinated with three-doses of DTaP vaccine and two-doses of aroQ*BP *as booster blood sampled 2 weeks after the last vaccination at day 42 (Group B); IgA titers of mice at day 14, two weeks after the last vaccination (Group C); IgA titers of mice vaccinated with two-doses of aroQ*BP *blood sampled at day 21, two weeks after the last vaccination (Group D). BPWC was used as the coating antigen.*

Table 3a.3. Serum antibody titers of DTaP vaccine-immunised mice post-intranasal booster vaccination with one or two doses of live attenuated *aroQ*BP vaccine at day 28 post-immunisation.

GROUPS				
Antibody isotype	A 3 DTaP+1 dose *aroQ*BP vaccine	B 3 DTaP+2 doses *aroQ*BP vaccine	C 1 dose *aroQ*BP vaccine	D 2 doses *aroQ*BP vaccine
IgG	2656±606	9,480±347	358±110*	6560±1371
IgG1	1430±429	3983±929	71±71**	806±161
IgG2a	100±100*	1009±231	0***	1204±114
IgA	1.2±0.029	1.2±0.092	1.2±0.026	2.2±0.039

**Value calculated from one mouse; antibodies in other mice were not detectable.*
***Value calculated from one subject; antibodies in other mice were not detectable.*
****OD<0.1 for all five mice.*

3a.4. Pulmonary antibody response to *aroQ*BP

IgG: Vaccinated mice in all the groups (A, B, C, D) except sham vaccinated mice in each group and the sentinel mice (Group E) produced serum IgG responses against inactivated BPWC *aroQ*. Mice in Group D, vaccinated with two-doses of *aroQ*BP by the i.n. route, produced the highest level of IgG (Figure 3a.4). The second highest level of IgG antibody was produced in mice booster-vaccinated with two-doses of the *aroQ*BP vaccine (Group B) (Figure 3a.4, Table 3a.4) that was not significantly different from that of Group D ($P>0.05$). However, there was a significant difference between the IgG antibody titers of DTaP-vaccinated mice booster-vaccinated with one dose of the *aroQ*BP vaccine (Group A) versus those vaccinated with the *aroQ*BP vaccine by the i.n. route (Group D) ($P< 0.003$) which produced higher antibody titers. However, there was no significant difference ($P> 0.05$) in the IgG antibody levels of mice booster-vaccinated with two booster doses of the *aroQ*BP vaccine (Group B) versus those immunised with two doses of the *aroQ*BP vaccine (Group D) as observed with serum antibody responses.

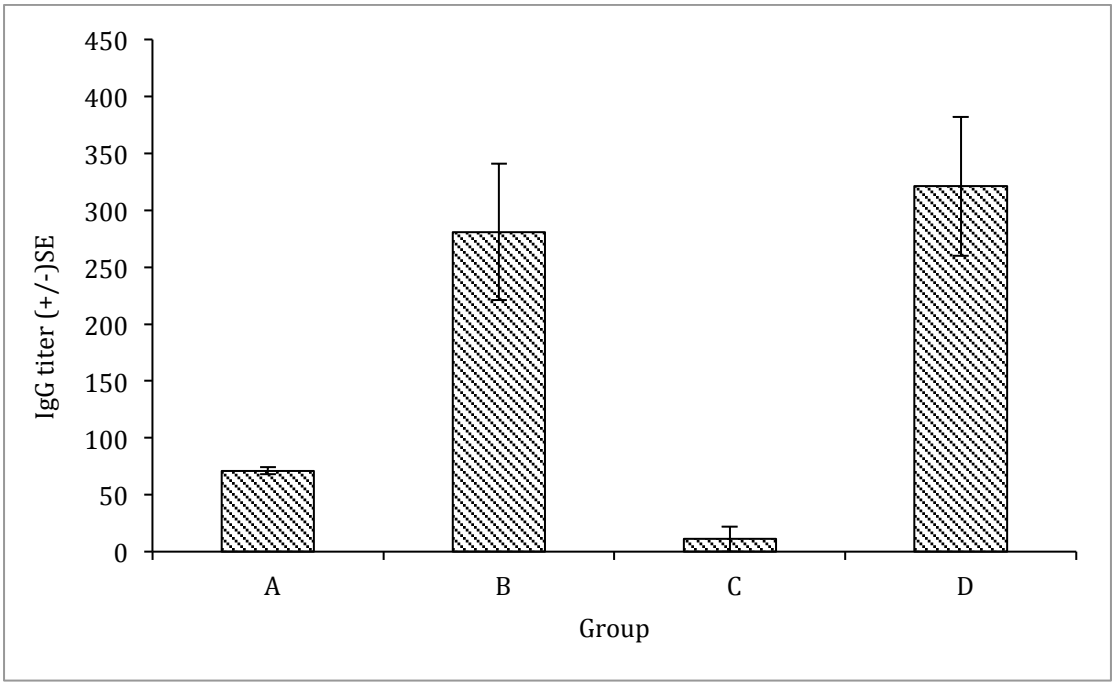

*Figure. 3a.4. Pulmonary IgG response of mice vaccinated with the aroQ*BP *vaccine versus its use as a booster in DTaP-vaccinated mice. IgG titers of mice vaccinated with three-doses of DTaP vaccine and one-dose of aroQ*BP *as booster blood sampled two weeks after the last vaccination at day 35 (Group A); IgG titers of mice vaccinated with three-doses of DTaP vaccine and two-doses of aroQ*BP *as booster blood sampled 2 weeks after the last vaccination at day 42 (Group B); IgG titers of mice at day 14, two weeks after the last vaccination (Group C); IgG titers of mice vaccinated with two-doses of aroQ*BP *blood sampled at day 21, two weeks after the last vaccination (Group D). BPWC was used as the coating antigen.*

IgG1: DTaP-immunised mice booster-vaccinated with two-doses of *aroQ*BP vaccine (Group B) produced the highest IgG1 levels in lung homogenates among all the vaccinated groups of mice (Figure 3a.4a, Table 3a.4), which were significantly different from that produced by mice booster-vaccinated with one dose of the *aroQ*BP vaccine candidate (Group A) (P<0.004) or mice vaccinated two-doses of the *aroQ*BP vaccine (Group D) (P<0.0004). IgG1 antibody titers of DTaP-immunised mice booster-vaccinated with one dose of the aroQBP vaccine (Group A) were also significantly greater (P<0.0009) than that in mice vaccinated with two-doses of the *aroQ*BP vaccine (Group D).

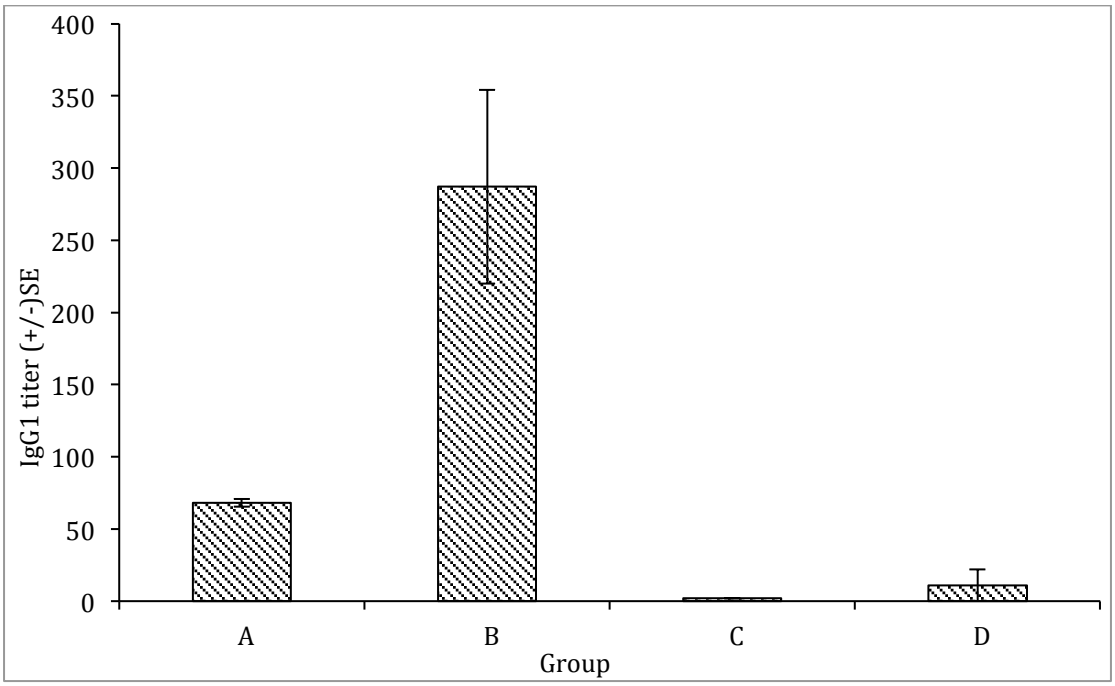

*Figure. 3a.4a. Pulmonary IgG1 response of mice vaccinated with the aroQ*BP *vaccine versus its use as a booster in DTaP-vaccinated mice. IgG1 titers of mice vaccinated with three-doses of DTaP vaccine and one-dose of aroQ*BP *as booster blood sampled two weeks after the last vaccination at day 35 (Group A); IgG1 titers of mice vaccinated with three-doses of DTaP vaccine and two-doses of aroQ*BP *as booster blood sampled 2 weeks after the last vaccination at day 42* (Group B)*; IgG1 titers of mice at day 14, two weeks after the last vaccination (Group C); IgG1 titers of mice vaccinated with two-doses of aroQ*BP *blood sampled at day 21, two weeks after the last vaccination (Group D). BPWC was used as the coating antigen.*

IgG2a: The IgG2a levels, albeit low, were significantly higher in groups B and D representing 2-dose *aroQ*BP booster-vaccinated (Group B) and *aroQ*BP vaccinated mice (Group D) respectively (Figure 3a.4b, Table 3a.4). Essentially no antibody response was detectable in Group C mice that had been vaccinated with one dose of the *aroQ*BP vaccine candidate. The IgG2a isotype titer of mice in Group A that had been booster-vaccinated with only one dose of the *aroQ*BP vaccine candidate was significantly lower than that of mice in Groups B (booster-vaccinated with 2 *aroQ*BP doses) ($P<0.0007$). There was no significant difference ($P> 0.05$) in the IgG antibody levels of mice booster-vaccinated with one-dose of the *aroQ*BP vaccine (Group A) versus those immunised with two doses of the *aroQ*BP vaccine (Group D).

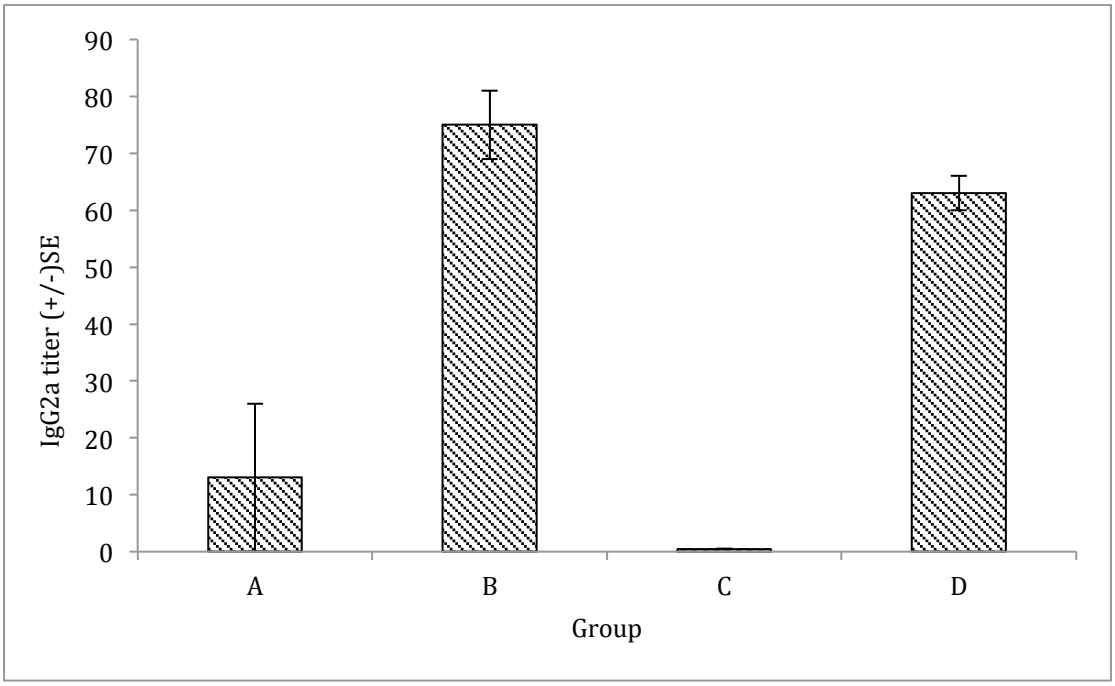

*Figure. 3a.4b. Pulmonary IgG2a response of mice vaccinated with the aroQ*BP *vaccine versus its use as a booster in DTaP-vaccinated mice. IgG2a titers of mice vaccinated with three-doses of DTaP vaccine and one-dose of aroQ*BP *as booster blood sampled two weeks after the last vaccination at day 35 (Group A); IgG2a titers of mice vaccinated with three-doses of DTaP vaccine and two-doses of aroQ*BP *as booster blood sampled 2 weeks after the last vaccination at day 42 (Group B); IgG2a titers of mice at day 14, two weeks after the last vaccination (Group C); IgG2a titers of mice vaccinated with two-doses of aroQ*BP *blood sampled at day 21, two weeks after the last vaccination (Group D). BPWC was used as the coating antigen.*

Secretory IgA (s-IgA): The IgA antibody isotype levels in the lung homogenates were almost as low as those observed in the serum in all the experimental groups. Regardless of the overall low levels of s-IgA, the levels of this isotype in *aroQ*BP vaccinated mice (Group D) or DTaP-immunised mice booster-vaccinated *aroQ*BP vaccine candidate (Group B) were not significantly different ($p>0.05$) (Figure 3a.4c, Table 3a.4).

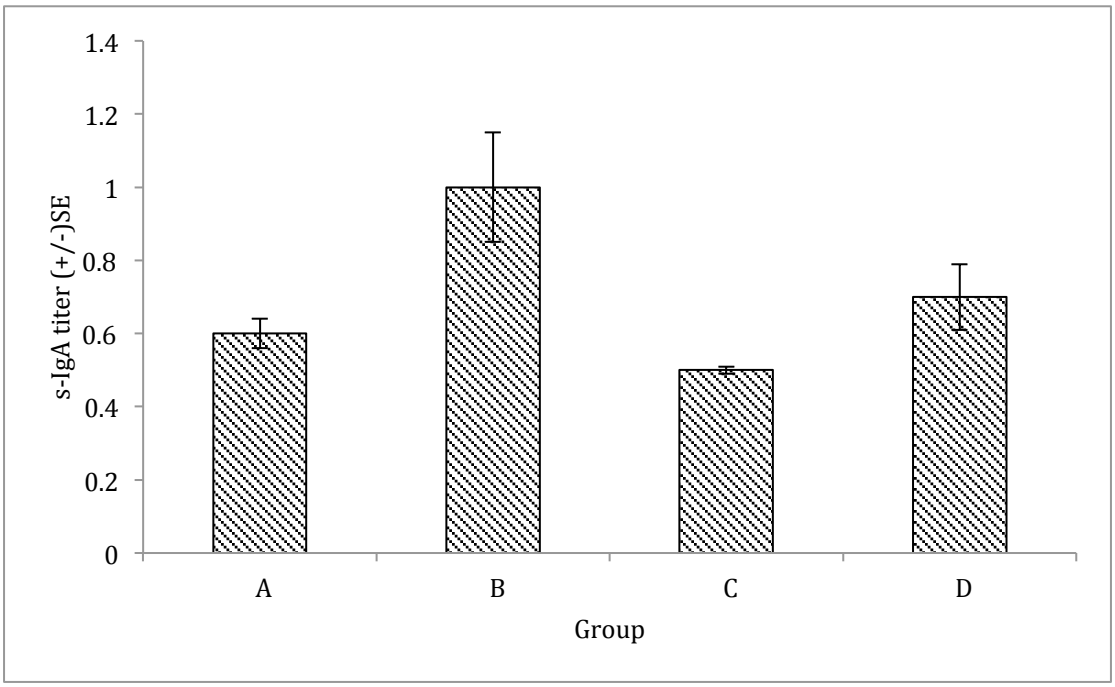

*Figure. 3a.4c. Pulmonary s-IgA response of mice vaccinated with the aroQ*BP *vaccine versus DTaP-vaccinated mice. s-IgA titers of mice vaccinated with three-doses of DTaP vaccine and one-dose of aroQ*BP *as booster blood sampled two weeks after the last vaccination at day 35 (Group A); s-IgA titers of mice vaccinated with three-doses of DTaP vaccine and two-doses of aroQ*BP *as booster blood sampled 2 weeks after the last vaccination at day 42 (Group B); s-IgA titers of mice at day 14, two weeks after the last vaccination (Group C); s-IgA titers of mice vaccinated with two-doses of aroQ*BP *blood sampled at day 21, two weeks after the last vaccination (Group D). BPWC was used as the coating antigen.*

Table 3a.4. Pulmonary homogenate antibody titers of aP vaccine-immunised mice post-intranasal booster vaccinisation with one or two doses of the live attenuated *aroQ*BP vaccine at day 28 post-immunisation.

GROUPS				
Antibody isotype	A 3 DTaP+1 dose *aroQ*BP vaccine	B 3 DTaP+2 doses *aroQ*BP vaccine	C 1 dose - *aroQ*BP vaccine	D 2 doses - *aroQ*BP vaccine
IgG	71±3	281± 60	54±11	321±61
IgG1	68±2.7	287±67	Not detectable	11±11
IgG2a	13±13	75±5.5	Not detectable	63±3.4
IgA	0.6±0.04	1±0.15	0.5±.01	0.7±0.09

3a.5. Serum antibody responses to pertussis toxin

The IgG antibody titers of mice immunised with one (Group C) or two-doses of *aroQ*BP vaccine (Group D) were significantly lower ($P< 0.05$) than those of DTaP-immunised mice booster-vaccinated with *aroQ*BP vaccine candidate (Figure 3a.5) ($P< 0.044$). While there was a significant difference between the antibody levels of mice vaccinated with two versus one booster vaccination with the *aroQ*BP vaccine candidate, there was no significant difference in the IgG1 levels between the two groups (Figure 3a.5a). The IgG2a antibody levels in all the vaccinated groups were quite low (<100) (Figure 3a.5b). There was no significant difference in the IgA antibody isotype titers of mice booster-vaccinated with one or 2 doses of the *aroQ*BP vaccine candidate (Group A versus B) (Figure 3a.5c). IgA antibody was undetectable in groups (Group C and D) that received the one or 2 doses of the *aroQ*BP vaccine intranasally respectively (Figure 3a.5c, Table 3a.5).

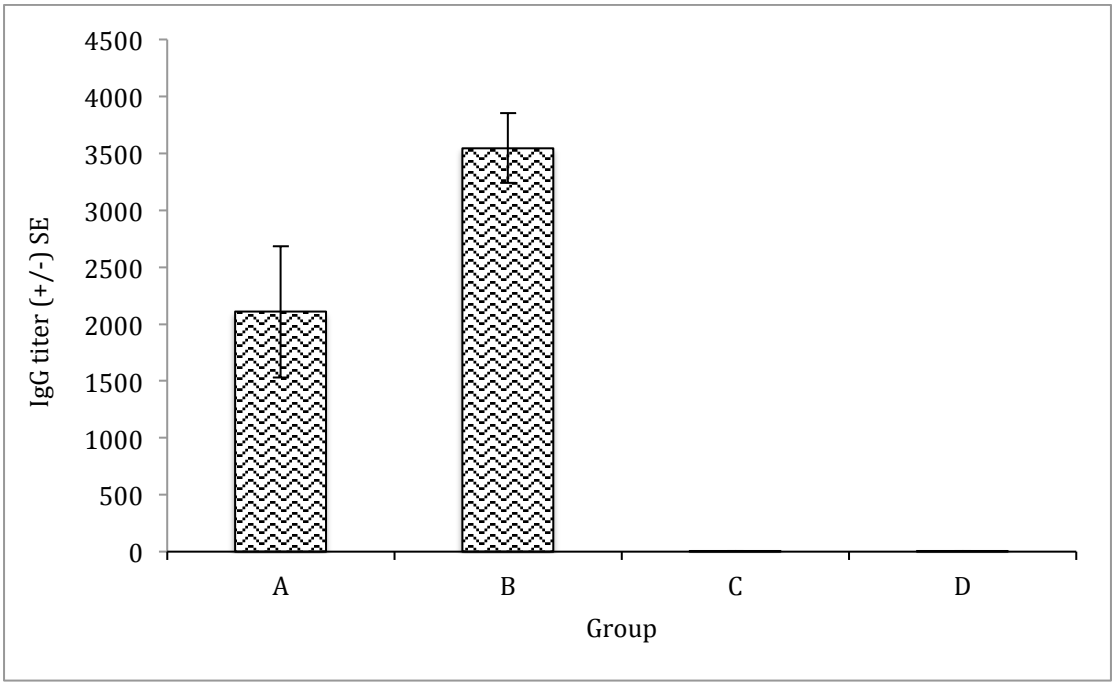

*Figure. 3a.5. Serum IgG antibody isotype response of mice vaccinated with the aroQ*BP *vaccine versus DTaP-immunised aroQ*BP*-booster vaccinated mice to pertussis toxin. IgG titers of mice vaccinated with three-doses of DTaP vaccine and one-dose of aroQ*BP *as booster blood sampled two weeks after the last vaccination at day 35 (Group A); IgG titers of mice vaccinated with three-doses of DTaP vaccine and two-doses of aroQ*BP *as booster blood sampled 2 weeks after the last vaccination at day 42 (Group B); IgG titers of mice at day 14, two weeks after the last vaccination (Group C); IgG titers of mice vaccinated with two-doses of aroQ*BP *blood sampled at day 21, two weeks after the last vaccination (Group D). PT was used as coating antigen.*

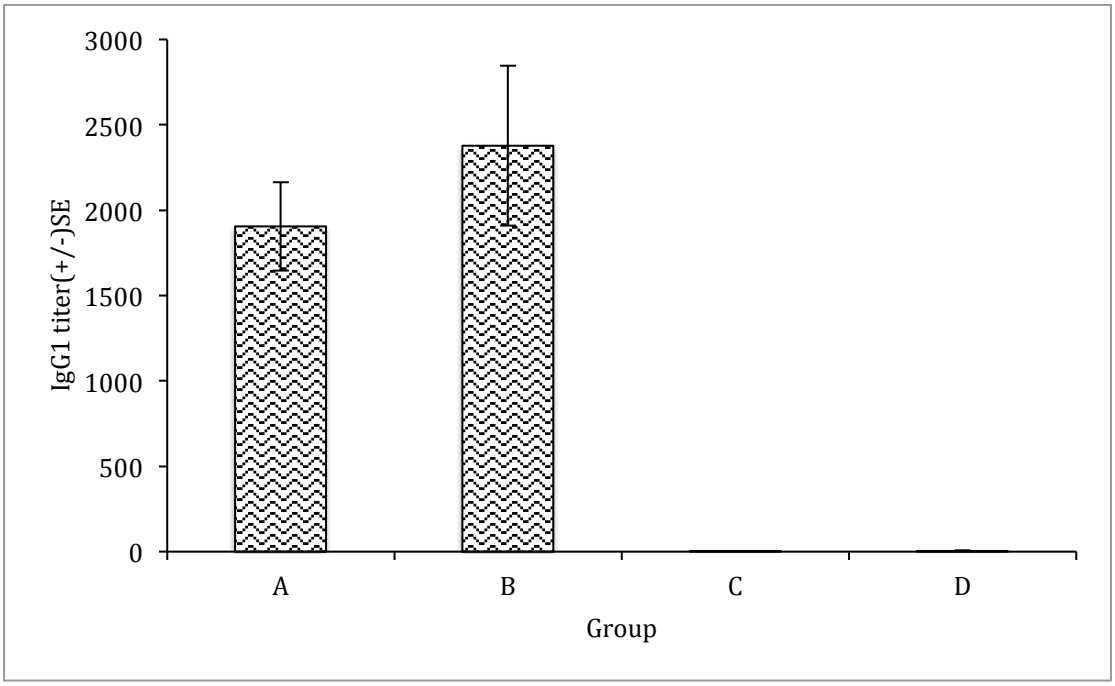

*Figure. 3a. 5a. Serum IgG1 antibody isotype response of mice vaccinated with the aroQ*BP *vaccine versus DTaP-immunised aroQ*BP*-booster vaccinated mice to pertussis toxin. IgG1 titers of mice vaccinated with three-doses of DTaP vaccine and one-dose of aroQ*BP *as booster blood sampled two weeks after the last vaccination at day 35 (Group A); IgG1 titers of mice vaccinated with three-doses of DTaP vaccine and two-doses of aroQ*BP *as booster blood sampled 2 weeks after the last vaccination at day 42 (Group B); IgG1 titers of mice at day 14, two weeks after the last vaccination (Group C); IgG1 titers of mice vaccinated with two-doses of aroQ*BP *blood sampled at day 21, two weeks after the last vaccination (Group D). PT was used as coating antigen.*

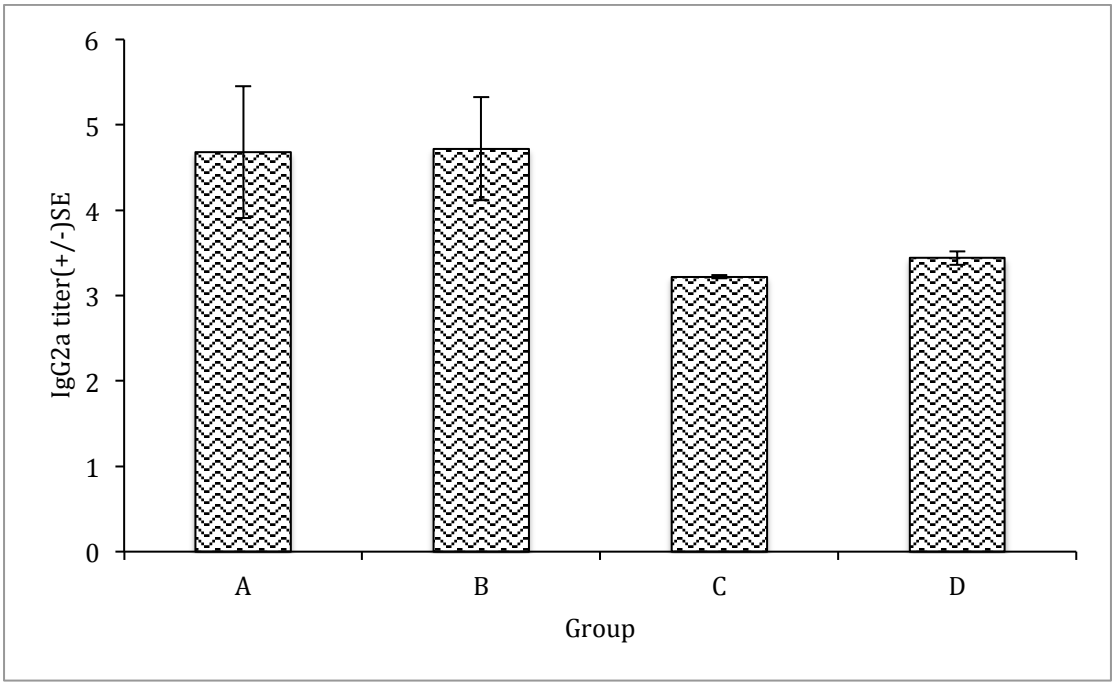

*Figure. 3a. 5b. Serum IgG2a antibody isotype response of mice vaccinated with the aroQ*BP *vaccine versus DTaP-immunised aroQ*BP*-booster vaccinated mice to pertussis toxin. IgG titers of mice vaccinated with three-doses of DTaP vaccine and one-dose of aroQ*BP *as booster blood sampled two weeks after the last vaccination at day 35 (Group A); IgG2a titers of mice vaccinated with three-doses of DTaP vaccine and two-doses of* aroQ*BP as booster blood sampled 2 weeks after the last vaccination at day 42 (Group B); IgG2a titers of mice at day 14, two weeks after the last vaccination (Group C); IgG2a titers of mice vaccinated with two-doses of aroQ*BP *blood sampled at day 21, two weeks after the last vaccination (Group D). PT was used as coating antigen.*

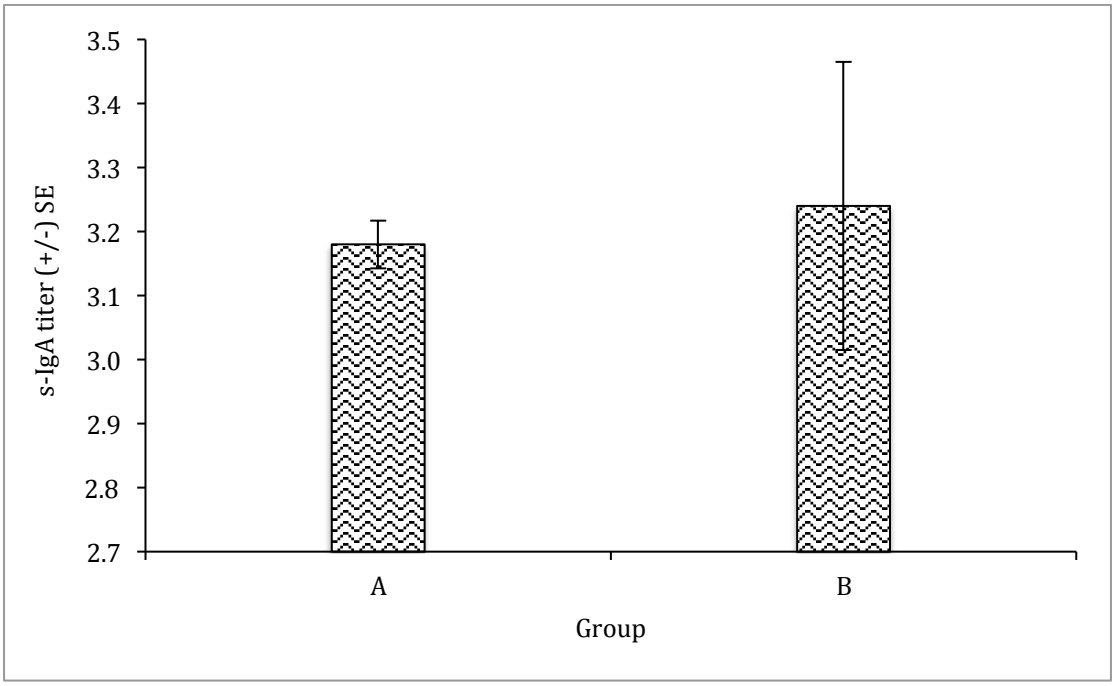

*Figure. 3a.5c. Serum IgA antibody isotype response of mice vaccinated with the aroQ*BP *vaccine versus DTaP-immunised aroQ*BP-*booster vaccinated mice to pertussis toxin. IgG titers of mice vaccinated with three-doses of DTaP vaccine and one-dose of aroQ*BP *as booster blood sampled two weeks after the last vaccination at day 35 (Group A); IgA titers of mice vaccinated with three-doses of DTaP vaccine and two-doses of* aroQ*BP as booster blood sampled 2 weeks after the last vaccination at day 42 (Group B); IgA titers of mice at day 14, two weeks after the last vaccination (Group C); IgA titers of mice vaccinated with two-doses of aroQ*BP *blood sampled at day 21, two weeks after the last vaccination (Group D). PT was used as coating antigen.*

Table 3a.5. Serum antibody titers of aP vaccine-immunised mice post-intranasal immunisation with one or two doses of the live attenuated *aroQ*BP vaccine at day 28 post-immunisation against pertussis toxin.

	GROUPS			
Antibody isotype	A 3 DTaP+1 dose *aroQ*BP	B 3 DTaP+2 doses *aroQ*BP	C 1 dose *aroQ*BP	D 2 doses *aroQ*BP
IgG	2108±578	3547±309	<100	<100
IgG1	1905±259	2378±468	<100	<100
IgG2a	<100	<100	<100	<100
s-IgA	3.18±0.04	3.24±0.22	ND	ND

3a.6. Serum antibody response to filamentous hemagglutinin

IgG: Mice immunised with one or two doses of the *aroQ*BP vaccine candidate produced very low levels of IgG detectable by ELISA. On the other hand, significant levels of IgG antibody were produced by DTaP immunised mice booster-vaccinated with one (Group A) or two (Group B) doses of the *aroQ*BP vaccine. However, there was no significant difference observed in the IgG titers of Groups A and B (Figure 3a.6, Table 3a.6).

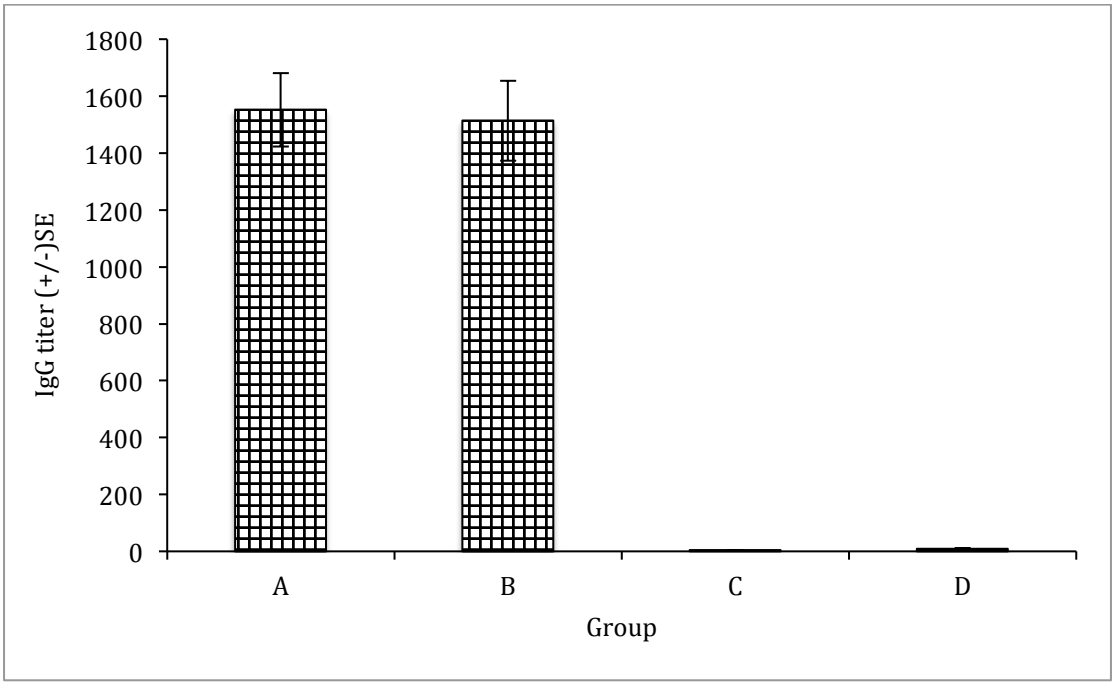

*Figure. 3a.6. Serum IgG antibody isotype response of mice vaccinated with the aroQ*BP *vaccine versus DTaP-immunised aroQ*BP*-booster vaccinated mice to filamentous hemagglutinin. IgG titers of mice vaccinated with three-doses of DTaP vaccine and one-dose of aroQ*BP *as booster blood sampled two weeks after the last vaccination at day 35 (Group A); IgG titers of mice vaccinated with three-doses of DTaP vaccine and two-doses of* aroQ*BP as booster blood sampled 2 weeks after the last vaccination at day 42 (Group B); IgG titers of mice at day 14, two weeks after the last vaccination (Group C); IgG titers of mice vaccinated with two-doses of aroQ*BP *blood sampled at day 21, two weeks after the last vaccination (Group D). FHA was used as coating antigen.*

IgG1: IgG1 was the predominant isotype in DTaP-immunised mice booster vaccinated with one or two doses of *aroQ*BP vaccine candidate (Figure 3a.6a, Table 3a.6). However, there was no significant difference between the two vaccination regimes.

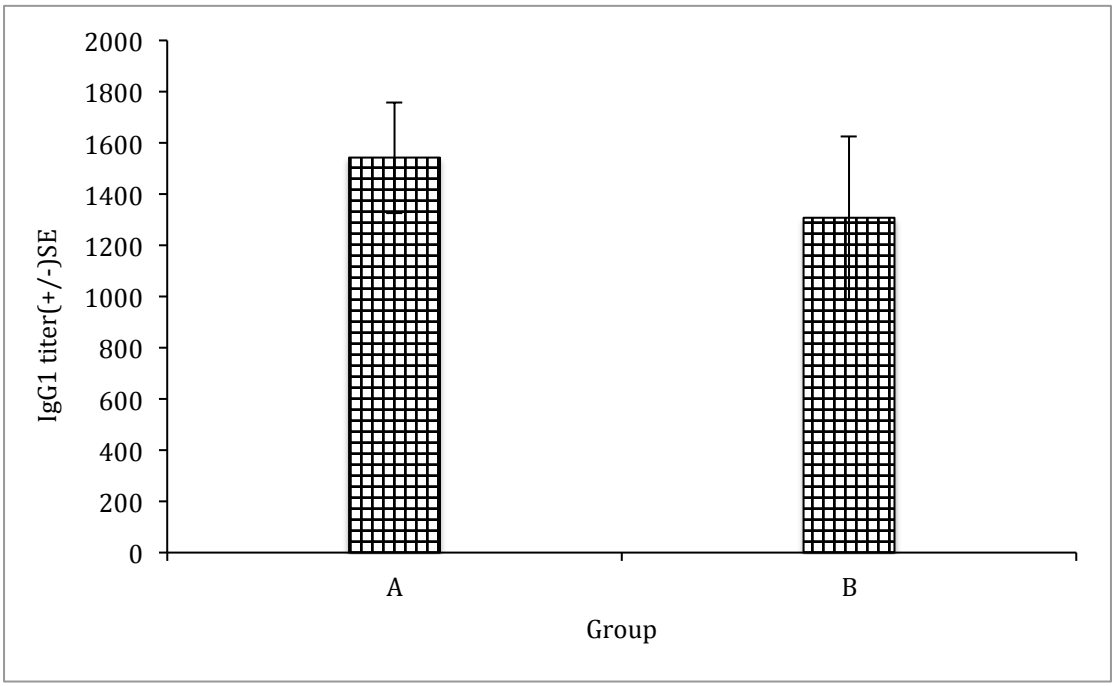

*Figure. 3a. 6a. Serum IgG1 antibody isotype response of mice vaccinated with the aroQ*BP *vaccine versus DTaP-immunised aroQ*BP*-booster vaccinated mice to filamentous hemagglutinin. IgG titers of mice vaccinated with three-doses of DTaP vaccine and one-dose of aroQ*BP *as booster blood sampled two weeks after the last vaccination at day 35 (Group A); IgG1 titers of mice vaccinated with three-doses of DTaP vaccine and two-doses of aroQ*BP *as booster blood sampled 2 weeks after the last vaccination at day 42 (Group B); IgG1 titers of mice at day 14, two weeks after the last vaccination (Group C); IgG1 titers of mice vaccinated with two-doses of aroQ*BP *blood sampled at day 21, two weeks after the last vaccination (Group D). FHA was used as coating antigen.*

IgG2a: Presence of anti-FHA IgG2a was either not detectable (Group A, C, D) or was limited to one of five mice (Group B) (Table 3a.6).

IgA: No anti-FHA IgA antibody isotype was detectable in any of vaccinated groups (Table 3a.6).

No pulmonary antibody response to FHA could be determined because of scarcity of lung homogenates.

Table 3a.6. Serum antibody titres of aP vaccine-immunised mice post-intranasal immunisation with one or two doses of the live attenuated *aroQ*BP vaccine at day 28 post-immunisation against FHA.

GROUPS				
Antibody isotype	A 3 DTaP+1 dose *aroQ*BP	B 3 DTaP+2 doses *aroQ*BP	C 1 dose *aroQ*BP	D 2 doses *aroQ*BP
IgG	1552±129	1514±140	<100	<100
IgG1	1542±216	1307±319	ND	ND
IgG2a	ND	595	ND	<100
s-IgA	ND	ND	ND	ND

ND: Not detectable

3a.7. Evaluation of CMI response in mice immunised with the *aroQ*BP vaccine candidate versus DTaP-immunised mice booster-vaccinated with the *aroQ*BP vaccine candidate using interferon-gamma as an indirect indicator

Induction of CMI immunity in mice subjected to the different immunisation regimes was assessed using IFN-γ as an indirect indicator. One complex antigen comprising killed BPWC and one purified antigen, FHA as model antigen, were selected for stimulation of splenocytes from immunised mice for assessment of CMI. Production of IFN-γ was determined at day 28 post-immunisation.

3a.7.1. IFN-γ levels in BPWC-stimulated splenocyte supernatant

Mice in Group D that had received two-doses of the *aroQ*BP vaccine candidate by the i.n. route were found to produce the highest level of IFN-γ compared to the other groups (Figure 3a. 7.1, Table 3a.7). However, there was no substantial difference between the levels of IFN-γ produced by DTaP-immunised *aroQ*BP booster-vaccinated groups of mice that had received either one (Group A) or two doses (Group B) of the *aroQ*BP booster. Mice in Group C that had received one-dose of *B pertussis aroQ* by the i.n. route produced the lowest level IFN-γ. No IFN-γ was produced by the antigen-stimulated splenocytes of sham-vaccinated mice. For this experiment, splenocytes in each group were pooled to ensure availability of adequate number of cells for antigen stimulation, thereby precluding calculation of standard error estimates.

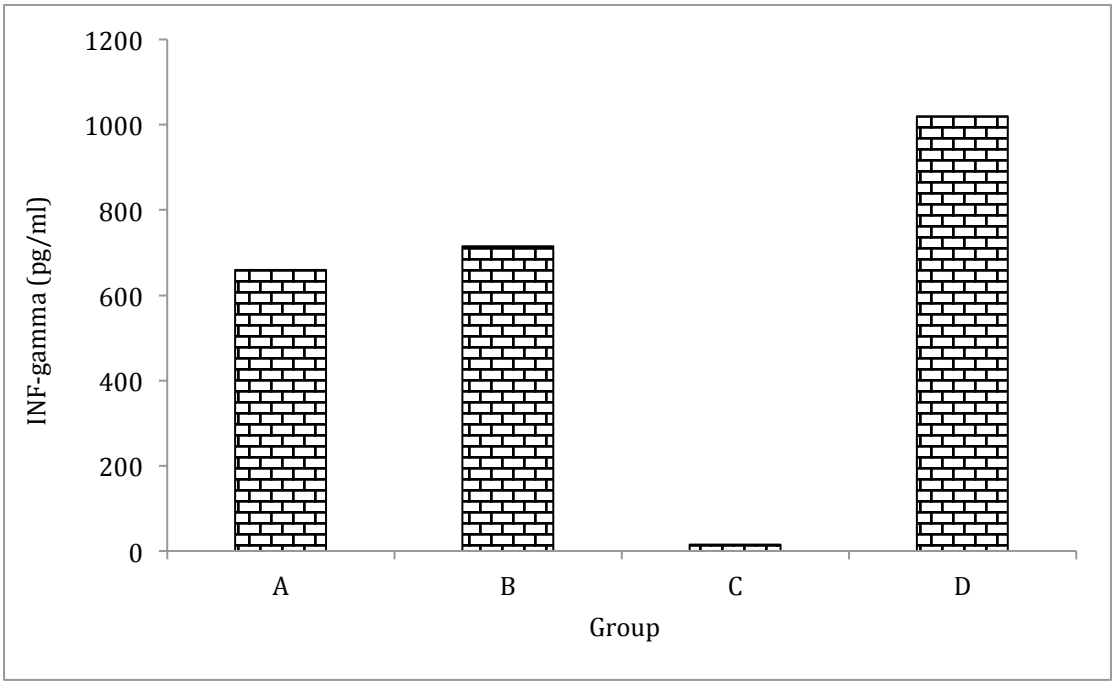

*Figure. 3a.7.1. Concentration of IFN-γ produced by BPWC- stimulated splenocytes of mice immunised using different vaccination regimes. Group A mice were vaccinated with 3 doses of DTaP followed by booster vaccination with one i.n. dose of the aroQ*BP *vaccine candidate whereas mice in Group B were given 2 i.n. doses of the aroQ*BP *vaccine. Group C had received one i.n. dose of the* aroQ*BP vaccine candidate whereas mice in Group D were administered 2 doses of the* aroQ*BP vaccine candidate by the i.n. route.*

Table 3a.7. Average IFN-γ levels of DTaP-immunised mice post-intranasal immunisation with one or two doses of the live attenuated *aroQ*BP vaccine at day 28 post-immunisation.

Groups	A 3 DTaP+1 dose *aroQ*BP	B 3 DTaP+2 doses *aroQ*BP	C 1 dose *aroQ*BP	D 2 doses *aroQ*BP	E PBS
IFN-γ (pg/ml)	660	700	25	1,020	<1

3a.7.2. IFN-γ levels in FHA-stimulated splenocyte supernatants

Group D mice that had received two-doses of the *aroQ*BP vaccine by the intranasal route were found to produce highest level of IFN-γ compared to the other groups (Figure 3a.7.2, Table 3a.7.2). Group C mice that had received one-dose of *B pertussis aroQ*BP by intranasal route induced second highest IFN-γ production. On the other hand booster vaccination of the DTaP-immunised mice with one or two doses of the *aroQ*BP vaccine candidate produced comparable amounts of IFN-γ levels (Figure 3a.7.2, Table 3a.7.2).

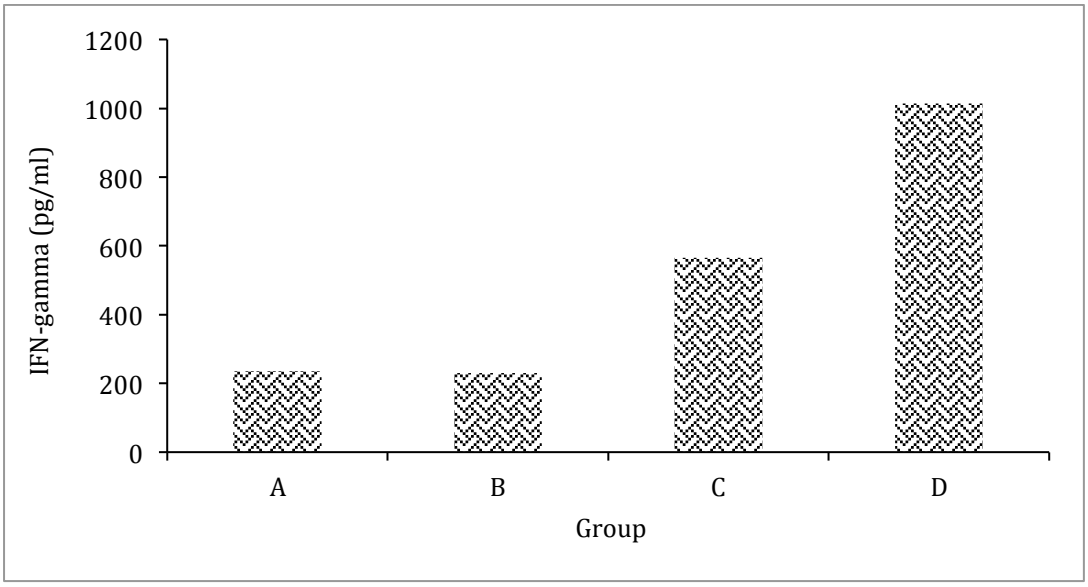

*Figure. 3a.7.2. Concentration of IFN-γ produced by FHA-stimulated splenocytes of mice immunised using different vaccination regimes. Group A mice were vaccinated with 3 doses of DTaP followed by booster vaccination with one i.n. dose of the aroQ*BP *vaccine candidate whereas mice in Group B were given 2 i.n. doses of the aroQ*BP *vaccine. Group C had received one i.n. dose of the aroQ*BP *vaccine candidate whereas mice in Group D were administered 2 doses of the aroQ*BP *vaccine candidate by the i.n. route.*

Table 3a.7.2. IFN-γ levels in FHA-stimulated splenocyte supernatants of *aroQ*BP vaccinated mice versus DTaP-immunised mice subjected to one or two doses of i.n. booster vaccination with *aroQ*BP at day 28 post-immunisation.

Groups	A 3 DTaP+1 dose *aroQ*BP	B 3 DTaP+2 doses *aroQ*BP	C 1 dose *aroQ*BP	D 2 doses *aroQ*BP	E PBS
IFN-γ (pg/mL)	235	230	565	1,015	<1

3a.8. Discussion

A study carried out previously at the University of Southern Queensland (Cornford 2003) revealed that immunisation of mice with three-doses of DTaP induced a high level of IgG, IgG1, and essentially no IgG2a but no production of IL-12 or IFN-γ. Therefore, the DTaP group was not included in this study due to the animal ethics regulations requiring application of the "Reduce, Refine and Replacement" Strategy (National Health and Medical Research Council 2004). It was clear that booster vaccination of mice, previously vaccinated with acellular pertussis components-containing vaccine (DTaP), with the *aroQ*BP vaccine candidate resulted in polarisation of immune response from Th2 type to Th1 type as judged by the production of IFN-γ as an indirect indicator of CMI as well production of IgG2a, an isotype associated with induction of CMI. Mice booster-immunised with two-doses of the *aroQ*BP vaccine yielded significantly higher levels of IgG, IgG1, and IgG2a as compared with the one-dose booster. Immunisation of mice with two-doses of the *aroQ*BP vaccine as the primary vaccine also yielded high levels of anti-BPWC antibodies as observed with the DTaP-*aroQ*BP booster vaccinated mice. However, the serum and mucosal IgA responses against BPWC were low. On the other hand, whereas anti-whole cells *B pertussis* IgG2a was not detectable in the lung homogenates of mice vaccinated with one of the *aroQ*BP *B pertussis* vaccine candidate, low levels of this antibody isotype were detectable in mice either immunised with two-doses of the *aroQ*BP *B pertussis* vaccine or in DTaP-immunised mice subjected to booster vaccination with one or two doses of the *aroQ*BP vaccine. Low levels of IgA observed in the lung homogenates of immunised mice regardless of the immunisation regime were consistent with low serum IgA levels. Previous studies, presented as an abstract at the ASM meeting (2005), reported induction of detectable moderate levels of anti-FHA and anti-PT antibodies of the isotypes IgG1 and IgG2a in mice vaccinated with *aroQ*BP that were measured using dot blotting rather than an ELISA assay used in this investigation. This disparity appears to be due to the potential lower sensitivity of the ELISA as compared to the dot-blotting assay (Gerthoffer and Ba; Lin-Chao, Chen, and Wong 1992).

Induction of high levels of IFN-γ by BPWC stimulated splenocyte of DTaP-vaccinated mice following booster-vaccination with either one or two doses of live attenuated *aroQ*BP vaccine candidate clearly indicated polarisation of immune response to Th1-type. Similar was the case when FHA of *B pertussis* was used as the model antigen to stimulate splenocytes. The production of IFN-γ by stimulation of splenocyte with other virulence antigens such as pertussis toxoid and pertactin was not carried out because of shortage in the availability of sufficient number of splenocytes. This experiment needs to be carried out but could not be accomplished because of time constraints. Subject to confirmation, the polarisation of immune response to Th1-type by booster vaccination of DTaP-immunised mice raises the possibility of evaluating the efficacy of the *aroQ*BP vaccine candidate in adolescent and adults previously immunised with acellular pertussis vaccines. However, it will be important to detoxify the *aroQ*BP vaccine candidate, particularly with respect to the immunosuppressive toxins particularly adenylate cyclase and pertussis toxin (Paccani et al. 2011; Carbonetti et al. 2005), prior to execution of confirmatory studies, hence the next phase attempted in this investigation describing the different strategies evaluated for detoxification of adenylate cyclase, which was selected because a method for the detoxification of pertussis toxin has already been reported (Mielcarek, Debrie, Raze, Quatannens, et al. 2006).

3b. Evaluation of different strategies for genetic detoxification of ACT

3b.1. Introduction

Mechanisms of ACT function have been the subject of many investigations due to the fundamental role of this enzyme in cellular signalling. It has been established that adenylate cyclase is an important virulence factor in the pathogenicity of *B pertussis* (Goodwin and Weiss 1990; Ohnishi et al. 2008). ACT enters eukaryote host cells resulting in an abnormal increase in cAMP synthesis in the host, that in turn perturbs normal cellular functions (Confer and Eaton 1982; Friedman, Farfel, and Hanski 1987). Intracellular ACT is activated by binding to calmodulin, a eukaryotic specific regulatory protein (Masure, Shattuck, and Storm 1987; Glaser et al. 1988; Wolff et al. 1980). Moreover, a study carried out by Weiss et al. (1984) has established that ACT deficient *B pertussis* is avirulent. Hence ACT deficient *B pertussis* is an interesting potential candidate for a vaccine.

In this study site directed mutagenesis of the *cyaA* gene in *aroQ*BP was attempted to generate a double mutant variant of *aroQ*BP that retained broad antigenicity and would be less likely to revert to wild type. It is known that the lysine 58 residue encoded by *cyaA* is critical for ACT to catalyse the synthesis of cAMP in eukaryotic cells (Au, Masure, and Storm 1989). This function is achieved via the positively charged amino group of lysine 58 binding to the negatively charged phosphate group of adenosine triphosphate (ATP) (Fry, Kuby, and Mildvan 1985), thereby resulting in elevated cAMP in the eukaryotic cell (Gerlt, Coderre, and Wolin 1980).

The following strategy was followed.

1) To achieve the aim the first 1000 bases of the *cyaA* gene of *B pertussis* Tohama I (NCBI reference sequence NC_0022929.2 bases 776227-777240) was PCR amplified with a *BamH1* and *EcoRI* cleavage sites at the flanking 5′ and 3′ ends, respectively (Figure 3b.1).

2) Site directed mutagenesis was then undertaken to mutate the AAA codon for lysine 58 to an ATG codon for methionine in the *cyaA* DNA fragment.
3) Mutated sequences were confirmed by sequencing as follows. The mutated *cyaA* oligonucleotides were cloned into the following vectors, pJQ200mp 18-rspL, and the pSS1129 *B pertussis* suicide vector. Detection of mutation was initially performed by PCR amplification of a 210-base pair amplicon of the *cyaA* insert using putative mutant clones lifted from the conjugation plates as template. Finally, the sequence of the 1kb *cyaA* insert fragment was determined from clones also lifted from the conjugation plates.

Figure 3b.1. Sequence (5'-3') of the 1kb fragment of the B pertussis Tohama I cyaA locus subjected to site directed mutagenesis. BamHI and EcoRI cleavage sites are underlined. The first base of the sequence is shown (776227 from NC_002929.2). Yellow highlighted nucleotides show the AAA codon (for lysine 58, sequence 173-175). Arrows 1 and 2 show primers used for amplification of the 1kb cyaA fragment. Arrows 3 and 4 identify primers used for site directed mutagenesis (ATG nucleotides were substituted for the AAA nucleotides). Two different primers were used to screen for mutants as shown by arrow 5 (FwATG-mutant and FwAAA-wild type) and one reverse primer shown by arrow 6.

3b.2. Amplification and cloning of *cyaA* insert fragment into different vectors

The *cyaA* insert sequence was amplified using primers as described in the methodology section (Table 2.13a). PCRs were performed for the *cyaA* insert at temperatures between 56^0C and 67^0C. A product of the expected size (1kbp) was produced at all temperatures tested. An annealing temperature of 57^0C was chosen for subsequent work since less nonspecific amplification occurred as shown by gel electrophoresis.

3b.2.1. Digestion and cloning of *cyaA* insert into pUC19

The *cyaA* insert, flanked by 5' *BamHI* and 3' *EcoRI* cleavage sites, was amplified, double digested with *BamHI* and *EcoRI* and cloned into similarly digested pUC19 plasmid. Ligation of excised *cyaA*-(*BamHI-EcoRI)* fragment with the pUC19-(*BamHI-EcoRI)* fragment was attempted with varying insert:vector molar ratios and ligation temperatures. Forty-six colonies picked from ligation plates were screened, most of which exhibited small (pin point) morphology (Section 2.14). Only two of the larger colonies contained the vector with the insert sequence. The two positive colonies were grown in 2 x 10ml LB-(100μl/ml Amp) as described in section 2.1.2. The pUC19 vectors were extracted from *E coli* DH5α and subjected to double and single digestion with *BamHI-EcoRI* and *BamHI*. Neither vector contained the desired *cyaA* insert sequence; only digest fragments of size ≈1.5kb and ≈0.7kb, were observed (Figure 3b.2.1a-b).

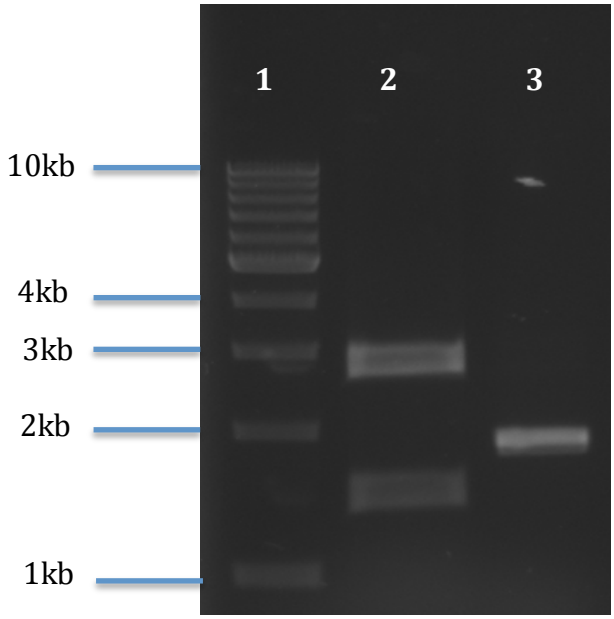

Figure. 3b.2.1a. Agarose gel electrophoresis (0.8%) showing double and single digestion of a ligated fragment (pUC19 plus cyaA fragment) extracted from E coli DH5α. Lane 1, 1kb marker. Lane 2, double digestion of the ligated plasmid with BamHI and EcoRI showing two fragments: A fragment with expected size of pUC19 (2.686kb), and a ≈1.5kb fragment. Lane 3, undigested pUC19.

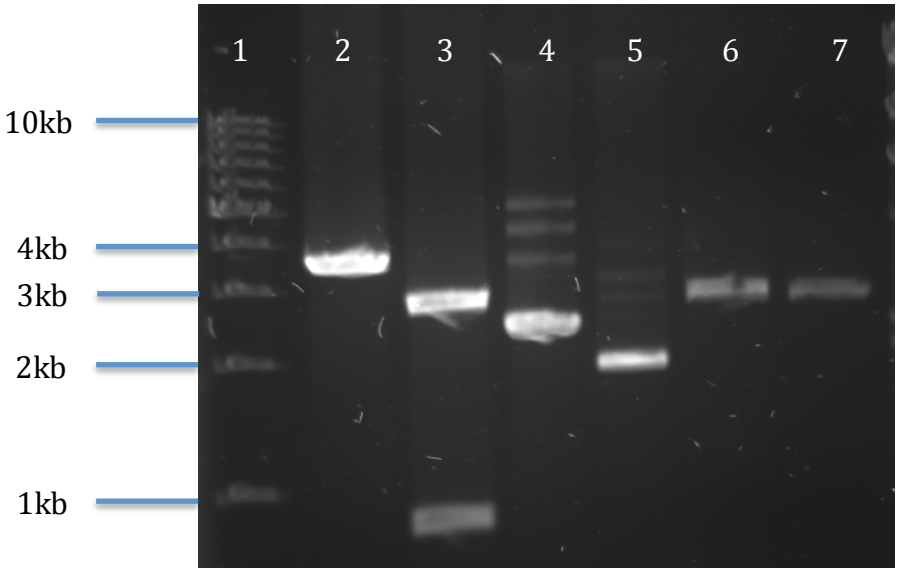

Figure. 3b.2.1b. Gel electrophoresis showing double and single digestion of a ligated fragment with insert (ligation of pUC19 and the cyaA fragment insert) extracted from E coli DH5α. Lane 1, 1kb marker. Lane 2, single digestion of the ligate with BamHI. Lane3, double digestion of the ligate with BamHI and EcoRI showing two fragments: A fragment with expected size of pUC19, 2.686kb, and a fragment with size of ≈0.8kb. Lane 4, undigested ligate. Lane 5, undigested pUC19. Lane 6, single digestion of pUC19 with BamHI. Lane 7, double digestion of pUC19 with BamHI and EcoRI.

3b.2.2. Digestion and cloning of *cyaA* insert into pGEM-T Easy Vector System and subsequent subcloning of *cyaA* insert into pUC19

Difficulties experienced in cloning the *cyaA* insert directly into pUC19 led to use of the pGEM-T Easy Vector System II. The PCR amplified *cyaA* insert was double digested with *BamHI* and *EcoRI*. The excised *cyaA* fragment was isolated by gel electrophoresis purified as described previously, prior to cloning into the pGEM®-T Easy Vector using the manufacturer's instructions (Figure 2.14.1). The pGEM vector with its 1kb *cyaA* inserted fragment was again subjected to double digestion with *BamHI* and *EcoRI* and the *cyaA* insert fragment isolated and purified as previously described. The purified *cyaA* fragment was once again cloned into double digested pUC19 (*BamHI* and *EcoRI*). The pUC19 vector with its successfully incorporated *cyaA* inserted fragment is identified hereafter as pUC19Cya (Figure 3b.2.2 and Figure 3b.2.2b).

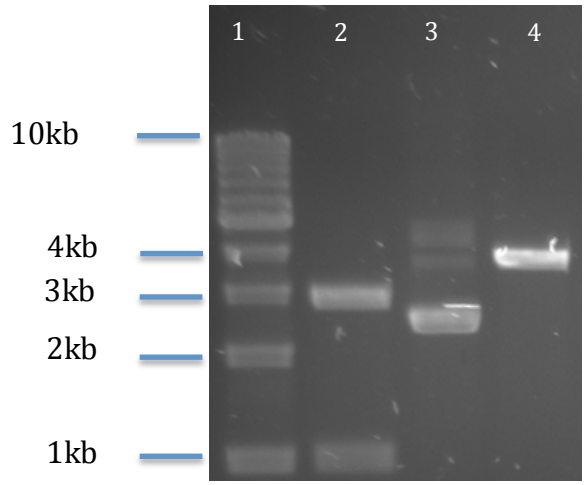

Figure. 3b.2.2. Incorporation of the 1kb CyaA fragment from the pGEM vector into the pUC19 vector. Lane 1. 1kb marker. Lane 2, double digest of pUC19Cya with BamHI and EcoRI. Showing two fragments-the 1kb Cya insert and the pUC19 vector (2.648kb). Lane 3, undigested pUC19Cya vector. Lane 4, the expected ≈ 3.7kb fragment following digestion of the vector plus inserted fragment with EcoR1.

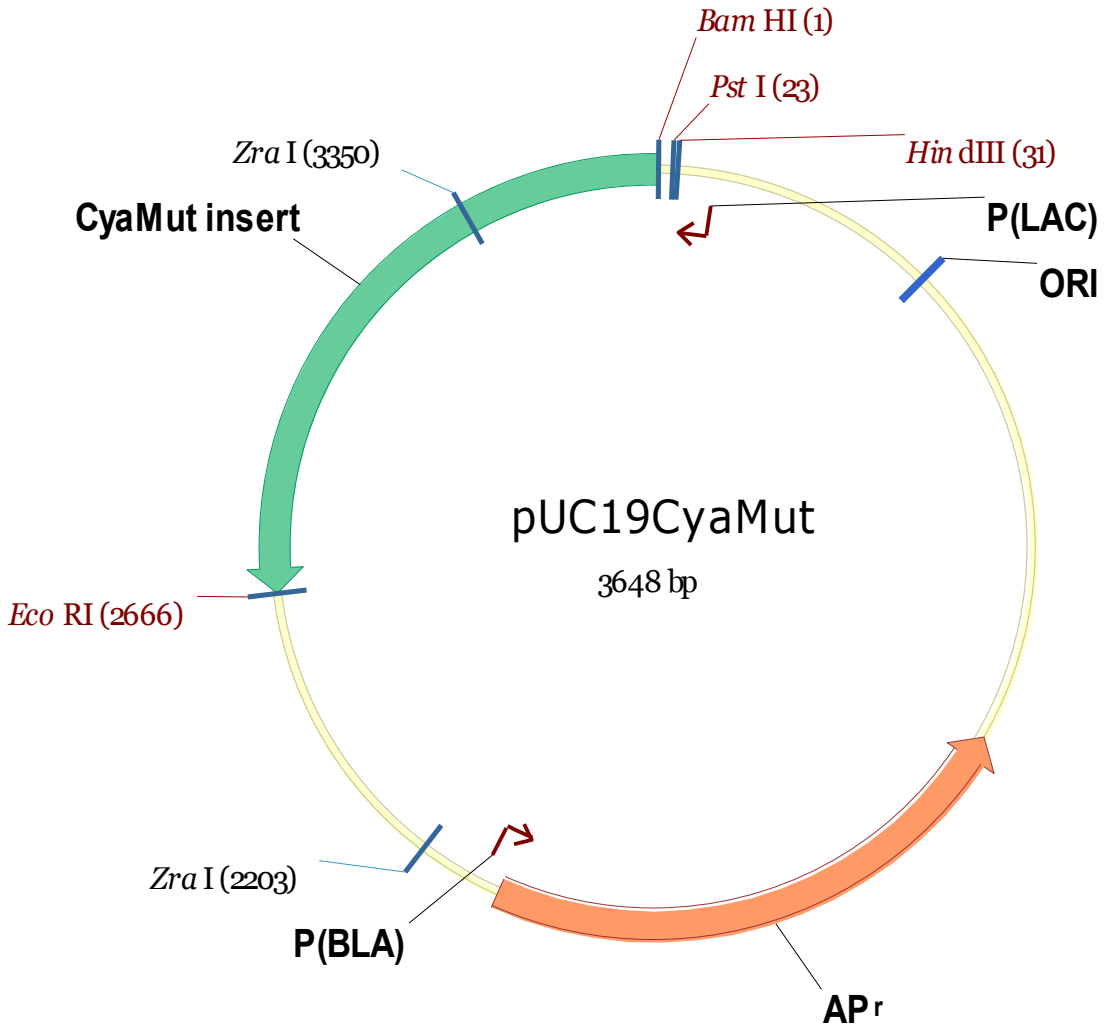

Figure. 3b.2.2b. Schematic drawing of vector pUC19CyaMut which was generated in this study.

Following of unsuccessful direct cloning of CyaMut fragment into pUC19, the insert initially was cloned into the pGEM vector. The vector was subsequently cloned into pUC19 vector after its transformation to E coli DH5α, vector extraction, and excision of the insert from pGEM vector by BamHI and EcoRI restriction enzymes. Double digested CyaMut-(BamHI-EcoRI) and pUC19-(BamHI-EcoRI) were ligated the resulted pUC19CyaMut was used for subsequent experiments.

3b.3. Mutation of lysine 58 to Methionine (AAA-to-ATG) in the pUC19Cya vector

The purified pUC19Cya vector was subjected to site directed mutagenesis in an attempt to induce the desired K -> M mutation using the same procedures as described previously (Section 2.15). Twenty five colonies picked from the nutrient plate following the site directed mutagenesis procedure were screened (Section 2.13.4) for the pUC19Cya mutated vector following transformation of XL10-Gold Ultracompetent Cells. All of the colonies tested contained the vector (Figure 3b.3). A colony of the transformed XL10-Gold Ultracompetent Cells was grown over night and the vector extracted. *E coli* DH5α was transformed with the extracted pUC19Cya mutant vector as a means of preserving the plasmid and for future use.

The pUC19CyaMut vector was extracted from the *E coli* DH5α and double digestion with *BamHI* and *EcoRI*. The product was isolated and purified as described previously and sequenced. Sequencing of both the original 1kb Cya fragment and the pUC19Cya mutant vector confirmed that the desired mutation at lysine 58 codon (AAA-to-ATG) was present. The pUC19 vector harbouring the mutated *cyaA* fragment is referred to hereafter as pUC19CyaMut. This vector was then used to transform *E coli* SM10 *λ pir* for use in conjugation with *B pertussis aroQ* strains as described briefly below and more fully in chapter 2 in sections 16.4 and 17.3.

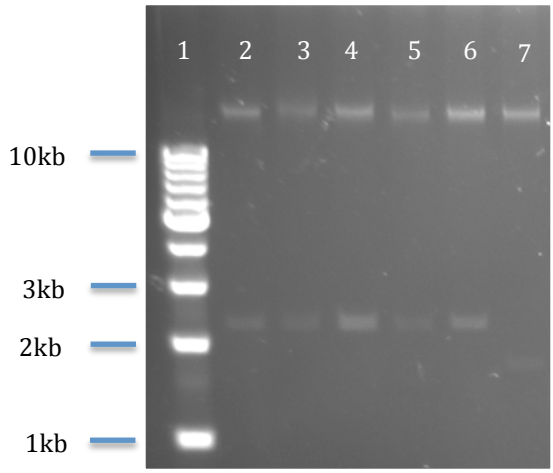

Figure 3b.3. Gel electrophoresis showing transformation of XL10-Gold Ultracompetent Cells with the pUC19CyaMut vector. Lane 1, 1 kb marker; Lanes 2-6 showing the pUC19CyaMut vector (bands between 2kb and 3kb) and chromosomal DNA (bands above 10kb marker) extracted from six distinct transformed XL10-Gold Ultracompetent Cells colonies picked from site directed mutagenesis nutrient plates; Lane 7, pUC19 vector used as a control for transformation (pUC18 has a lower molecular weight than pUC19CyaMut).

3b.4. Discrimination between wild type and mutant *B pertussis aroQ* at the lysine 58 codon by PCR

Three individual primers were designed to detect the mutated ATG codon in *B pertussis aroQ* mutant colonies following conjugation with the *E coli* SM10 λ *pir* harbouring the suicide vectors pJQ200mp 18-rspLCyaMut or pSS1129CyaMut vectors. Two oligonucleotides, (wild type 5'-3' AAA, and mutant 5'-3' ATG - identical except for the two nucleotides at their 3′ ends), were used as forward primers. The reverse primer (RvsATG) was used in conjunction with either of the two forward primers to discriminate between the wild type and mutant Cya fragment (Table 2 13a, Figure 3b.1). The amplicon produced by these two PCR reactions were expected to be 210-bp for the mutated *cyaA* fragment and no amplicon product using the forward primer incorporating the AAA codon at its 3' end.

3b.4.1. Standardization of mutation detection by PCR

The wild type and mutant discriminatory primers described above (Table 2.13a) were also employed to detect the lysine (K-AAA) to methionine (M-ATG) mutation in conjugated variants of the *aroQ*BP strain. At annealing temperatures ranging from 57^0C to 64^0C the wild type forward primer did amplify the expected 210-base pair sequence (see Table 2.13c for cycling condition). However, PCR amplification with the mutant forward primer (FwATG) was greatly reduced or non-existent. These results are shown in Figure 3b.4.1.

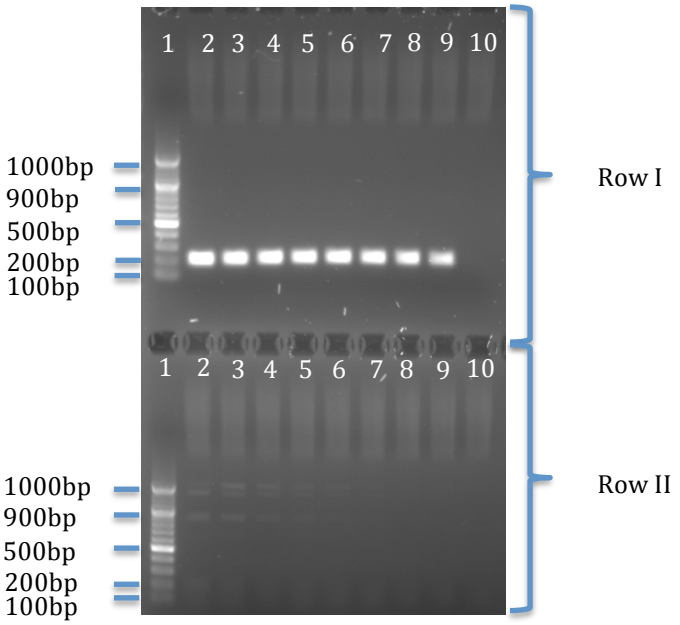

Figure.3b.4.1. Detection of the lysine to methionine mutation in the CyaA fragment in aroQBP using PCR with the discriminatory primers. Each well contained 5µl of amplicon.

Row I. Lane 1, 100bp marker. Lanes 2-9, Gel electrophoresis showing the 210-base pair sequence from aroQBP strain with the native primer at annealing temperatures from 57-64^{0}C, respectively. Lane 10, negative control.

Row II. Lane 1, 100bp marker. Lane 2-9 Absence of the expected 210-base pair sequence at annealing temperatures from 57-64^{0}C with the mutant primer; Lane 10, negative control.

3b.4.2. Detection of the lysine to methionine mutation in vectors harbouring the non mutated and mutated Cya fragments.

To confirm that the PCR reaction can discriminate between wild type and mutated Cya fragments within vectors, amplifications were performed on PSS1129CyaMut and pCU19Cya vectors (see Table 3b.4.1 for templates and primers). The results of this experiment are shown in Figure 3b.4.1 PCR of PSS1129 with the mutant specific primers showed the expected 210-base pair amplicons. However PCR with the wild type primers still resulted in 210-base pair amplicons but in much reduced concentrations. PCR amplifications using the pUC19Cya template showed strong amplification with the wild type primers and still present but in reduced concentrations with the mutant primer pair. It can be seen also that the PCRs were not sensitive to the annealing temperatures used. Although these PCRs were clearly not specific for the mutated versus the wild type *Cya* fragment, the differential concentrations of amplicon produced were considered useful as an internal control. Reasons for the lack of specificity are included in the discussion.

Table 3b.4.1. Templates and primers used to amplify the 210-base pair sequence.

Template	pSS1129CyaMut	pSS1129CyaMut	pUC19Cya	pUC19Cya
Forward primer	Mutant, FwATG	Wild type, FwAAA	Mutant, FwATG	Wild type, FwAAA
Reverse primer	RvsATG	RvsATG	RvsATG	RvsATG

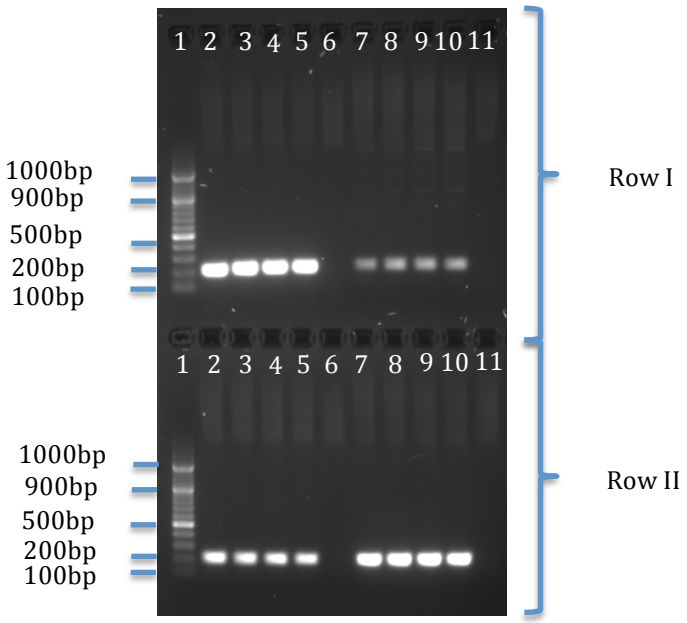

Figure 3b.4.2 PCR amplification of mutated and wild type Cya fragments with native and mutant designed primer pairs.

Row I. Amplification of the PSS1129CyaMut vector. Lane 1, 100bp marker. Lane, 2-6 PCR amplification of pSS1129CyaMut using the mutant primers; with annealing temperature ranging from 57^0C-60^0C. The expected 210-base pair band is present. Lane 6, negative control. Lane 8-10, amplification of pSS1129CyaMut with wild type primers using the same range of annealing temperatures. The 210-base pair band is still present but at reduced concentrations. Lane 11, negative control.

Row II. Amplification of the pUC19Cya vector. Lane 1, 100bp marker. Lane, 2-6 PCR amplification of pUC19Cya using the mutant primer; with annealing temperature ranging from 57^0C-60^0C. The 210-base pair band is still present but at reduced concentrations. Lane 6, negative control. Lane 8-10, amplification of pUC1Cya with wild primers using the same range of annealing temperatures. The 210-base pair band is present. Lane 11, negative control. Each well contained 5µl of amplicon.

3b.5. Attempts to produce *B pertussis aroQ variants by conjugation using two suicide vectors incorporating CyaMut*

Attempts to introduce the lysine to methionine mutation into the *cyaA* gene of *aroQ*BP were performed by conjugation with *E coli* SM10 *λ pir* harbouring two suicide vectors. Two conjugation partners were used

1) *E coli* SM10 *λ pir* harbouring pJQ200mp 18-rspLCyaMut.
2) *E coli* SM10 *λ pir* harbouring pSS1129CyaMut.

3b.5.1. Ligation of *cyaA* mutant insert into pJQ200mp 18-rspL, Bordetella suicide vector, and transfer into *aroQ*BP by conjugation

Use of pJQ200mp 18-rspL Bordetella suicide vector represents the first strategy aimed at generating a *B pertussis aroQ* variant in which the adenylate cyclase gene has been altered so that its product is non-toxic. The pUC19CyaMut vector was extracted from an overnight culture of *E coli* DH5α in LB-(Amp100μg/ml) broth as described in Chapter 2, Section 11.12. Following double digestion (BamH1, EcoRI), isolation by gel electrophoresis and purification the CyaMut 1kbp fragment was cloned into double digested pJQ200mp 18-rspL with *BamHI-EcoRI* (Figure 3b.5.1a), (see Chapter 2, Section 16 for methodology). Successful cloning of CyaMut insert into pJQ200mp 18-rspL was confirmed by gel electrophoresis of a *BamHI* and *EcoRI* double digest as shown in Figure 3b.5.1.

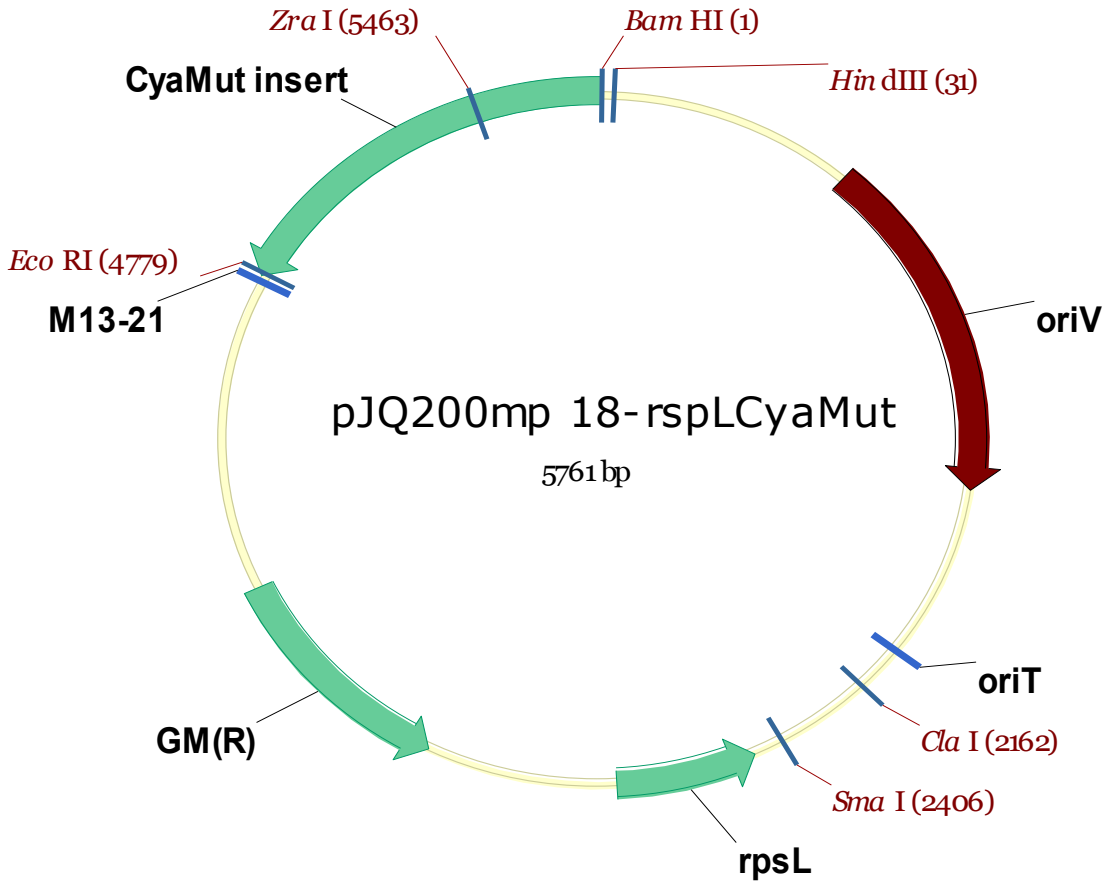

Figure 3b.5.1a. Schematic drawing of vector pJQ200mp 18-rspLCyaMut which was generated in this study.

The CyaMut fragment was excised pUC19CyaMut extracted from E coli DH5α by BamHI and EcoRI restriction enzymes. The CyaMut-(BamHI-EcoRI) insert and double digested pJQ200mp 18-rspL-(BamHI-EcoRI) vector were ligated. The resulted vector, pJQ200mp 18-rspLCyaMut, was exploited for introduction the mutant base (M) into aroQBP chromosome by conjugation.

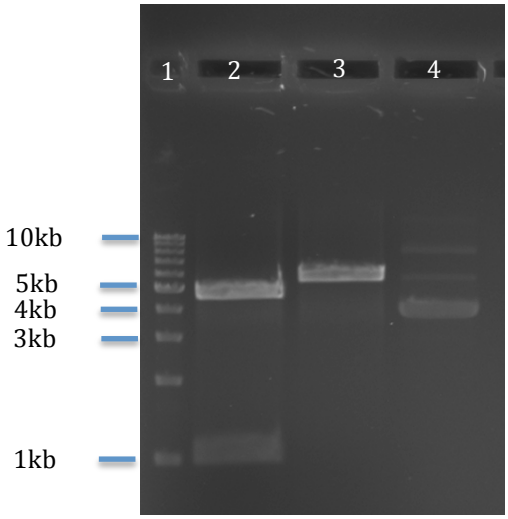

Figure 3b.5.1. Gel electrophoresis of the composite pJQ200mp 18-rspLCyaMut vector (≈5.8kb) after double and single digestion with BamHI *and* EcoRI. *Lane 1, 1kb marker. Lane 2, double digested pJQ200mp 18-rspLCyaMut showing two expected fragments, the 1kb CyaMut fragment and the original pJQ200mp 18-rspL vector size 4.799kb (pJQ200mp 18-rspL vector). Lane 3, single digestion of pJQ200mp 18-rspLCyaMut with* BamHI *with expected size of ≈ 5.8kb. Lane 4, undigested pJQ200mp 18-rspLCyaMut vector.*

The constructed pJQ200mp 18-rspLCyaMut vector was transferred into *E coli* SM10 *λ pir* (Chapter 2, Section 16.4). Conjugation between the *E coli* SM10 *λ pir* harbouring pJQ200mp 18-rspLCyaMut and the *aroQ*BP strain was then performed in the expectation of creating an *aroQ*BP variant carrying a chromosomal copy of the *cyaA* mutated fragment (Chapter 2, Section 16.4). Successful conjugation was identified by the differential growth of colonies on CW-aromix media with or without gentamicin. Only *B pertussis* containing the constructed pJQ200CyaMut plasmid will grow in the presence of gentamicin on CW-aromix-(Km50μg/ml, Sm200μg/ml, Gm10μg/ml). No such colonies were observed after five days of incubation. However, growth was present by day two for cultures on CW-aromix-(Km50μg/ml, Sm200μg/ml) without gentamicin. This result indicates that the conjugation probably occurred and that recombination may have taken place and the vector has been lost.

Thirty-three isolates colonies were picked up from CW-aromix-(Km50μg/ml, Sm200μg/ml) plates and used as templates for amplification of the 1kb *cyaA* insert by

the PCR using primer pair as discussed above (FwdCya and RvsCya). Sequencing of the amplified Cya insert did not reveal the expected mutation at the Lysine 58 codon (AAA to ATG).

3b.5.2. Ligation of *cyaA* mutant insert into pSS1129, Bordetella suicide vector, and transfer into *aroQBP* by conjugation

Use of pSS1129 Bordetella suicide vector represents the second strategy aimed at generating *aroQ*BP strain in which adenylate cyclase toxin has been detoxified. This plasmid has been used successfully to inactivate multiplicity of *aroQ* BP genes (Gross et al. 1992; Walker and Weiss 1994; Bannan et al. 1993). The pUC19CyaMut vector was extracted from an overnight culture of *E coli* DH5α of in LB-(Amp100μg/ml) broth as described in Chapter 2, Section 11.2. Following double digestion (BamH1, EcoRI), isolation by gel electrophoresis and purification the CyaMut 1kbp fragment was cloned into double digested pSS1129 with *BamHI-EcoRI* (see Chapter 2, Section 17 for methodology). Successful cloning of CyaMut insert into pSS1129 was confirmed by gel electrophoresis of a *BamHI* and *EcoRI* double digest (≈10kb) as shown in Figure 3b.5.2. For reassurance, the composite vector was also extracted from cultures of E. coli SM10 λ *pir* with identical results as those obtained with *E coli* DH5α. Successful conjugation was identified by the differential growth of colonies on BG-aromix media with or without gentamicin. Only *B pertussis* containing with the constructed plasmid will grow in the presence of gentamicin on BG-aromix-(Km50μg/ml, Sm200μg/ml, Gm10μg/ml) and ampicillin. No such colonies were observed after five days of incubation. However, growth was present by day two for cultures on BG-aromix-(Km50μg/ml, Sm200μg/ml) without gentamicin or ampicillin. This result indicates that the conjugation probably occurred and that recombination has taken place and the vector has been lost. Four hundred twenty two isolated colonies were detected on BG-aromix-(Km50μg/ml, Sm200μg/ml).

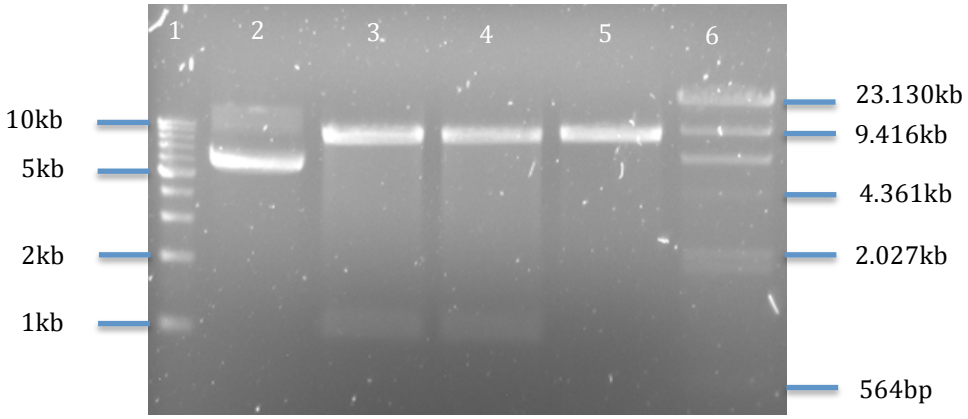

Figure 3b.5.2. Gel electrophoresis of the constructed pSS1129CyaMut vector (≈10kb) after double and single digestion with BamHI and EcoRI. Lane 1, 1kb marker. Lane 2, undigested vector. Lanes 3 and 4, double digested pSS1129CyaMut (BamHI and EcoRI) extracted from E coli DH544 and E coli SM10 respectively; bands at 1kb are CyaMut inserts. Lane 5, double digest of pSS1129 with BamHI and EcoRI. Lane 6, lambda HindIII marker.

3b.5.2.1. Detection of the mutation in the lysine 58 codon of *B pertussis aroQ* strain

Two methods (Chapter 2, Section 18.2-18.3) were employed to detect whether mutation has introduced into lysine 58 codon (AAA-to-ATG) of *aroQ*BP. These are PCR amplification using mutant specific primer pairs and direct sequencing of the 1kb *cyaA* fragment.

All of the 422 isolated clones from BG-aromix-(Km50ug/ml, Sm200ug/ml) conjugation plates were picked and consolidated into in 42 groups with ten colonies per group, except one group which had eleven colonies. An aliquot of each group was suspended in 250µl PCR grade water and used as template for subsequent PCRs in conjunction with mutant FwATG and RvsATG primers (Chapter 2, Section 18.3). A 210-base pair product was amplified in 12 of the 42 groups (Figure 3b.5.3). One of the 12 batches that showed the clearest 210-base pair target was selected for further investigation. Each of the ten individual clones comprising this batch was amplified and the product identified by gel electrophoresis (Figure 3b.5.3a). Nine of the ten clones produced 210-base pair amplicons (Figure 3b.5.3a). Three randomly selected positive clones producing the 210-base pair sequence were amplified, together with one clone from which the 210-base pair sequence was not be amplified. All four clones were then used as templates for PCR amplification of 1kb *cyaA* insert with the

FwdCya and RvsCya primers set (Table 2.13a). The expected 1kb Cya amplicon was obtained from all four PCRs. These four fragments were then sequenced. The expected mutation in the lysine 58 codon was not observed in these sequences. However one of the four sequences manifested a mutation at the lysine 58 codon resulting in a non-synonymous substitution of asparagine; the AAA codon being replaced by AAT. The expected ATG mutation described by Douglas et al. (1989) was unfortunately not found. Stocks of the clone was made and stored at -80^{0}C for subsequent characterisation.

As a final endeavour, 1kb *cyaA* fragments were amplified from forty-four individual isolated clones picked from the BG-aromix-(Km50gμ/ml; Sm200μg/ml) conjugate plates. Successful amplication was confirmed by gel electrophoresis and the products submitted for sequencing. None of the 44 fragments manifested the desired mutation for the lysine 58 position as shown by a multiple sequence alignment. It is possible that other putative mutations may also have occurred but pursuing this question is outside the scope of this project.

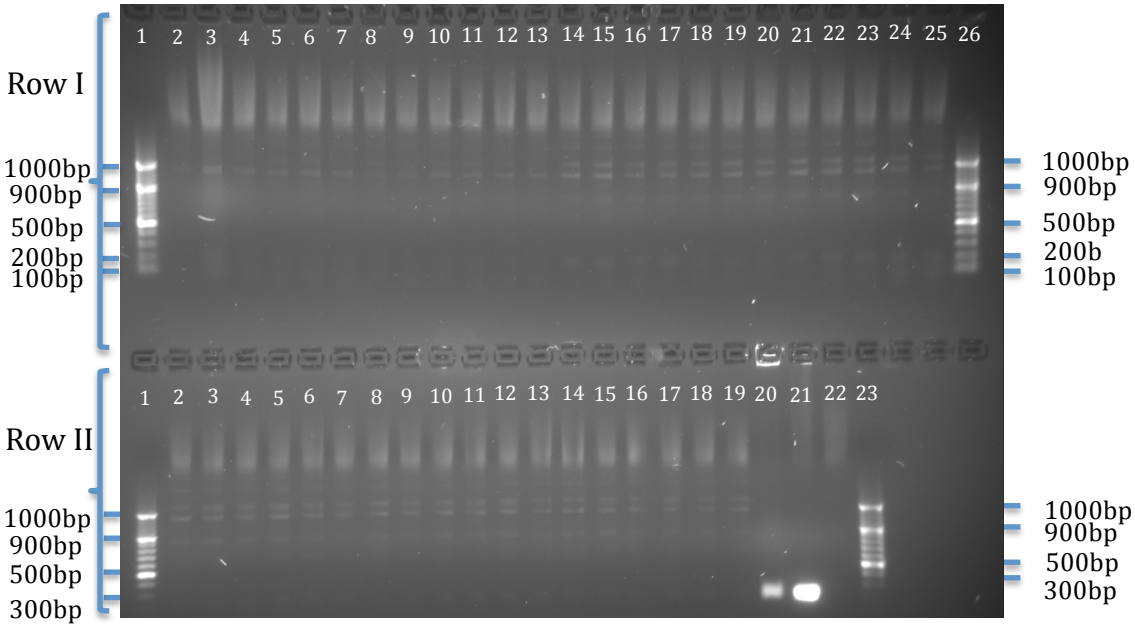

Figure 3b.5.3. Electrophoresis of 42 pooled samples from the conjugation plate. Row I, lane 2-25 and Row II, lane 2-19, showing attempts to amplify the 210-base pair target following PCR with the mutant primer pair (FwATG) in putative aroQBP mutants from the conjugation plates. Only 12 of the 42 samples gave an expected 210-base pair band, albeit very weakly. Row I, lane 1 and 26; Row II, lane 1 and 23 100bp marker. Row II, lane 20, amplification of the target sequence from pSS1129CyaMut in conjunction with the native (AAA) primer. Lane 21, amplification of the target sequence from pSS1129CyaMut in conjunction with the mutant (ATG) primer. Row II Lane 23, negative control. Bands other than the 210-base pair bands are due to non- specific amplification.

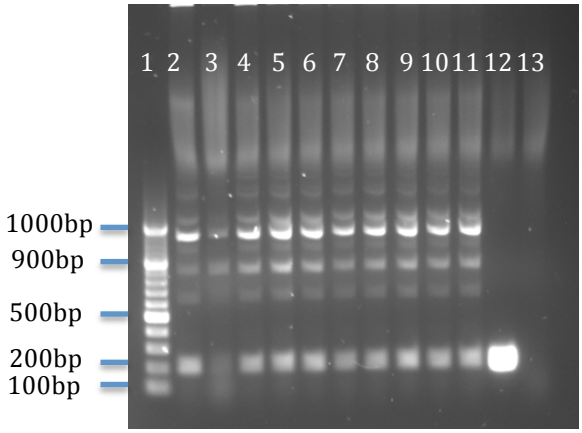

Figure 3b.5.3a. Gel electrophoresis of the 10 individual colonies comprising batch 23 from the conjugation plate. Lane 1, 100bp marker. Lane 2-11, showing gel electrophoresis of the PCR attempts to amplify the 210-base pair sequence from ten isolates in conjunction with wild type primer. The ten isolates were from the batch the 210-base pair sequence could not be amplified by PCR in conjunction with the mutant primer (see text for details). Lane 3, the 210-base pair sequence was not amplified from this isolate (which was subsequently shown to have the lysine to asparagine mutation). Lane 12, amplification of the 210-base pair sequence from pSS1129CyaMut vector in conjunction with the mutant primer. Lane 13, negative control. Each well contained 5µl of amplicon. Bands other than the 210-base pair bands are due to non-specific amplification.

3b.6. Discussion

Bordetella pertussis produces four exotoxins including PT, ACT, DNT and an endotoxin, LPS (Mills 2001; Marzouqi et al. 2010; Weiss et al. 1984; Roberts et al. 1990). While the role of PT in the virulence of *B pertussis in vivo* has been well documented to induce lymphocytosis, histamine sensitivity and immunosuppression (Carbonetti et al. 2005) that of ACT has emerged only over the last decade. Production of ACT is now considered to be one of the major virulence determinants of *B pertussis* acting to promote immune evasion or suppression and hence subsequent colonisation of the host, albeit later than PT (Paccani et al. 2011; Mills 2001; Ross et al. 2004; Boyd et al. 2005). Adenylate cyclase is a calmodulin-sensitive, which enters animal cells and increases intracellular cAMP thereby contributing to the clinical manifestations of the disease.

Gross et al. (1992) demonstrated that a mutant of *B pertussis* in which lysine at position 58 (bases 174-175, AAA) in the *cyaA* gene replaces methionine (ATG) abolished adenylate cyclase catalytic activity and decreased pathogenicity by over 1000-fold relative to the wild type in newborn mice, clearly demonstrating the importance of this exotoxin for the virulence of *B pertussis*. More recently, Carbonetti et al. (2005) showed that failure to produce ACT by mutant strains of *B pertussis* in which the *cyaA* gene had been deleted resulted in a significant colonization defect in the mouse model. On the other hand, this defect was apparent two days earlier relative to *B pertussis* with in-frame-deleted PT mutations.

One of the aims of the "Vaccine Development Laboratory" at Curtin University is to genetically modify the exotoxins produced by *aroQ*BP vaccine candidate such that the toxicity of the vaccine candidate is eliminated or substantially reduced while retaining immunogenicity. Given the immunosuppression potentially mediated via apoptosis of macrophages and disengagement of immune response via binding of the ACT to T cells (Khelef, Zychlinsky, and Guiso 1993; Paccani et al. 2011) and the polarisation toward of immune response to Th2 type by these toxins (Boyd et al. 2005; Ross et al. 2004), the importance of genetically detoxifying ACT is obvious in the development of any live attenuated vaccine that retains immunogenicity.

The key question is whether any non-reverting live attenuated *B pertussis* vaccine candidate will be suitable as a primary replacement vaccine for the aP component of DTaP, or as a booster for infants previously immunised with 1, 2 or 3 doses of the DTaP. Because the non-reverting *aroQ*BP vaccine candidate has not yet been detoxified, its potential as booster for mice already vaccinated with DTaP was investigated to determine if the Th2-polarised immune response induced by DTaP could be polarized to a Th1 mediated CMI resulting in more prolonged protection than achieved with DTaP alone.

The rational underpinning execution of the next phase of this study aimed at genetic detoxification of the *aroQ*BP vaccine candidate were as follows:

1) The clear demonstration in this study that booster vaccination of mice, previously vaccinated with DTaP, with the non-reverting *aroQ*BP vaccine candidate result polarisation of the immune response from Th2 to Th1 type, and
2) the recently reported property of the ACT to disengage T cell function by binding to T cells (Carbonetti et al. 2005; Paccani et al. 2011).

Because the genetic modification of PT had already been reported (Mielcarek et al. 2006), it was decided to attempt to genetically modify ACT by site directed mutagenesis of the *cyaA* gene. This chapter reports the results of inactivation of *cyaA* gene using site directed mutagenesis directed to replace lysine with methionine at position 58 of the gene as originally described by Gross et al. (1992). This study focussed on a 1kb fragment of the *cyaA* gene containing lysine 58 rather than the entire *cyaA* gene. This fragment was cloned into the pUC19 vector and subjected to the site directed mutagenesis protocol. Presence of the desired mutation in the *cyaA* fragment was confirmed by direct sequencing. The mutated 1kb *cyaA* insert was excised from pUC19 and cloned into two different *B pertussis* suicide vectors, pJQ200mp-18 and pSS1129, to allow for conjugation and allelic exchange between the *E coli* SM10 *λ pir* harbouring these vectors with *aroQ*BP chromosomal DNA.

The creation of the original *aroQ*BP mutant was achieved by partial deletion of the *aroQ* gene, followed by insertional inactivation of the *aroQ* gene, using selection

based on a Km resistance gene similar to the way the *aroA* gene was generated (Roberts et al. 1990). In the present study, the use of a selection marker was not possible due to the requirement to abrogate toxicity of ACT while retaining immunogenicity. This objective required that both the mutated *cyaA* gene fragment and the corresponding wild type fragment could both be cloned and sequenced.

To circumvent the lack of a selection marker protocol, two alternative procedures were used to detect the AAA (K) → ATG (M) mutation in the *cyaA* fragment. In first method, the entire 1kb *cyaA* insert was PCR-amplified using FwdCya and RvsCya primers and sequenced. In the second method, a 210-base pair sequence within the *cyaA* gene fragment was amplified using two sets of primers such that only one amplicon would be present if the desired ATG mutation had not taken place.

Although the experimental procedures reported in this chapter did generate plasmids containing the AAA (K) → ATG (M) mutation in the 1kb *cyaA* gene fragment as expected, however introduction of this mutation into the aroQBP chromosome by conjugation was not successful. Reason for this failure was not investigated due to time constraints. However a quite unexpected non synonymous AAA (K) to AAT (N) mutation was observed. The event could be attributed to random mutation, which is known to occur in bacterial populations with frequency of the order of $10^{-4} – 10^{-5}$ per base per generation (Cairns, Overbaugh, and Miller 1988; Jeremy W. D and Simon F. P 2004).

It will be interesting to determine whether this mutation (K to N) affects the activity of the resulting adenylate cyclase toxin. Due to time constraints this question could not be addressed in this project. It will be resolved at a later time in Dr Mukkur's laboratory.

With the advantages of hindsight, it is interesting to reflect on the reasons why lysine to methionine mutated *cyaA* gene fragment was not isolated. Probable reasons include the following.

1) Conjugation between *E coli* SM10 λ *pir* and *aroQ B pertussis* may not have taken place despite the fact that the amp^R and Gm^R selection markers were not present in the vectors of the Bordetella suicide vectors. Clearly, this procedure requires further effort.

2) Expected recombination may not have taken place between the homologous sequences (1kb *cyaA* insert) of the vector(s) and *aroQ B pertussis* chromosomal DNA at the lysine codon. This may be coincidental or explained by sequence attributes at this locus.

3) The flanking homology region (at 173bp corresponding to the AAA codon) was not long enough for the mutated sequence to cross from the vector(s) to the chromosomal DNA of *cyaA* of *B pertussis aroQ*. Support for this possibility has been suggested by Stibitz (Stibitz 1994) who reported that 500bp of homologous flanking region was sufficient for mutant sequence to cross over to incorporate a desired mutation from the suicide vector to the bacterial chromosomal DNA. However, a lower limit for homologous recombination was not determined. Regardless, it will be interesting to determine the effect of the random substitution of the lysine residue with asparagine on the toxicity of adenylate cyclase, notwithstanding the need to repeat the experiment but using the site-directed mutagenesis on longer segments of the *cyaA* gene.

In conclusion, the work reported herein provides information and experience that will facilitate the generation of an ACT deficient aroQBP that retains adenylate cyclase antigenicity, whether this is due to the K to M mutation or indeed the fortuitous K to N mutation described above. The availability of a double mutated *B pertussis* variant with broad antigenicity will undoubtedly lead to a new generation of whooping cough vaccines eliciting protracted humoral and cell mediated immunity while being safe for use in humans.

Chapter 4: Conclusions and future directions

Even though the currently marketed DTaP whooping cough vaccine has been claimed to induce long-term protection against infection with *B pertussis* in the population, children vaccinated with DTaP become susceptible to the disease within 4-12 years (Cherry 2005; Tan et al. 2005) notwithstanding the contribution made by lack of compliance with the recommended vaccination schedule to the recent increases in incidence in many countries due either to side reactions (Jackson et al. 2002; Gold et al. 2003; Rennels et al. 2000; Rowe et al. 2005). Even fully vaccinated populations have now been reported and accepted to acquire infection with *B pertussis* (Mooi, Van Loo, and King 2001; He and Mertsola 2008; Mooi et al. 2009; de Melker et al. 1997). As a result, there is need for development of side reaction-free and more effective vaccines capable of inducing long-term protection against whooping cough. However, for this objective to be realised, an ideal future pertussis vaccine must also induce CMI, latter being considered to be necessary for induction of long-term immunity (Redhead et al. 1993; Mills 2001; Mascart et al. 2003). Protection offered by vaccination with acellular pertussis vaccines is now accepted to be due predominantly to antibodies induced by Th2-polarisation of immune response (Canthaboo, Williams, et al. 2000). However, it is also generally accepted that induction of CMI induced via Th1-polarisation of immune response is necessary for long-term protection against whooping cough (Redhead et al. 1993; Mills 2001; Mascart et al. 2003).

DNA vaccines against pertussis have yielded controversial results with one laboratory claiming induction of both antibodies and CMI prepared using pcDNA3.1 eukaryotic plasmid (Kamachi, Konda, and Arakawa 2003) as compared to another laboratory reporting induction of only CMI using a construct prepared using the same plasmid (Fry et al. 2008). While a 3rd laboratory (Li et al. 2006) claimed induction of both antibodies and CMI with a DNA vaccine using a different plasmid (pVAX1), research in a different laboratory showed that boosting of mice with recombinant proteins was necessary for induction of high antibody responses and induction of protection against pertussis (Fry 2006).

Enthusiasm for DNA vaccination in humans is tempered by the concern on the possibility vaccine's DNA integrating into host chromosomes potentially leading to

turning on of the oncogenes or turning off the tumor suppressor genes. This is not withstanding the concern of provoking chronic inflammation or autoantibody production due to continued immunostimulation (http://virology-online.com/general/typesofvaccines.htm.).

It has been reported that live attenuated pertussis vaccines may constitute ideal candidates for imparting long-term protection because they mimic natural infection (Locht et al. 2004; Mielcarek et al. 2010) providing the potential of inducing immune responses to all the virulence antigens. The currently used DTaP vaccines contain only 3-5 of *B pertussis* protective antigens, depending upon the vaccine manufacturer, with only one of the major toxins, pertussis toxin, being covered for neutralisation.

Mukkur et al. (2005) has reported the development of a *B pertussis* vaccine candidate in which the *aroQ* gene of the aromatic biosynthesis pathway had been deleted and insertionally inactivated. It has been reported *aroQ*BP can colonise airways of mice efficiently and induce immunity both humoral and cell mediated immunity without causing disease (Cornford-Nairn, PhD Thesis [under embargo, University of Southern Queensland]; Mukkur et al, 2005; Mukkur, Personal communication). Mice immunised with a single dose *aroQ*BP were protected against an intranasal challenge with parent virulent *B pertussis*. Serum anti-*B pertussis* antibodies of the IgG1 and IgG2 isotypes were induced which were enhanced post-challenge with the virulent parent strain. Vaccination of mice with two and three of the *aro*QBP vaccine further enhanced IgG1 and IgG2 levels and also induced anti-*B pertussis* pulmonary IgA levels.

Given the recent outbreaks despite extensive coverage with the acellular pertussis vaccine in developed world, it was important to determine whether the Th2-polarised immune response induced by the acellular pertussis vaccine could be polarised towards Th1 type using *aroQ*BP vaccine as booster with a view to prolonging the duration of immunity originally induced by the acellular pertussis vaccines thus eliminating or reducing the need for repeated boosters.

In this study it was established that booster vaccination of mice, previously vaccinated with DTaP, with the *aroQ*BP vaccine candidate resulted in polarisation of immune

response from Th2 type to Th1 type as judged by the production of IgG2a and IFN-γ used as an indirect indicator of CMI. Mice booster-immunised with two-doses of the *aroQ*BP vaccine yielded significantly higher levels of IgG, IgG1, and IgG2a as compared with the one-dose booster. Immunisation of mice with two-doses of the *aroQ*BP vaccine as the primary vaccine also yielded high levels of anti-BPWC antibodies as observed with the DTaP-*aroQ*BP booster vaccinated mice. However, the serum and mucosal IgA responses against BPWC were low. On the other hand, whereas anti-whole cells *B pertussis* IgG2a was not detectable in the lung homogenates of mice vaccinated with the *aroQ*BP vaccine candidate, low levels of this antibody isotype were detectable in mice either immunised with two-doses of the *aroQ*BP vaccine or in DTaP-immunised mice subjected to booster vaccination with one or two doses of the *aroQ*BP vaccine. Low levels of IgA observed in the lung homogenates of immunised mice regardless of the immunisation regime were consistent with low serum IgA levels.

Induction of high levels of IFN-γ by BPWC and FHA, used as a purified *B pertussis* model antigen, stimulated splenocyte of DTaP-vaccinated mice following booster-vaccination with either one or two doses of live attenuated *aroQ*BP vaccine candidate clearly indicated polarisation of immune response to Th1-type.

Given the ability of the *aroQ*BP vaccine candidate to polarise the immune response from Th2 type to Th1 type laid the foundation for further attenuation of this non-reverting vaccine candidate particularly with respect to the immunosuppressive toxins particularly adenylate cyclase and pertussis toxin in the first instance. Genetic detoxification of the adenylate toxin was given priority because a method for genetic detoxification of pertussis toxin had already been reported (Mielcarek, Debrie, Raze, Quatannens, et al. 2006). Genetic detoxification of ACT was attempted using 2 different Bordetella suicide vectors harboring site-directed mutagenized 1kb *cyaA* gene fragment by introducing methionine in place of lysine base at position 58 in the *cyaA* of bacterium genome. Unfortunately, the desired mutation was not achieved; instead, one clone in which a mutation at position 174 (A-to-T) which resulted in replacement of lysine to asparagine (AAT codon) was isolated. This clone is now ready to be tested on its toxic properties to determine if this unexpected random

substitution has resulted in detoxification. Unfortunately, answer to this question has not been obtained in this study due to time constraints.

In this study, it was proven that Th2 immune response in mice previously immunised with an acellular pertussis vaccine such as the DTaP can be polarised to Th1 type immune response by one or two booster of doses of the live metabolite-deficient *aroQ*BP delivered by the intranasal route providing an opportunity for the development of vaccine ideal for induction of long-term immunity.

Considering the findings of this study, future studies aimed at genetically detoxifying adenylate cyclase toxin need to be carried out by allelic exchange using at least 500bp upstream of ATG (lysine 58) of *cyaA* gene sub-cloned in the Bordetella suicide vectors for successful homologous recombination leading to the generation of an inconsequentially toxic *aroQ*BP vaccine candidate. If successful, the next step should be genetic detoxification of pertussis toxin as reported for a different live attenuated, albeit potentially reverting strain (Mielcarek, Debrie, Raze, Quatannens, et al. 2006) prior to the characterisation of its safety and immunogenicity in immunocompetent and in immunodeficient mice.

Since innate immunity is critical to the development of acquired antibody and cell-mediated immune responses, future studies should also include identification of dendritic cell subpopulations including other antigen-presenting cells and their ability to drive the vaccine-induced Th2-mediated and Th1-mediated responses. This could be facilitated by quantification of the broad spectrum of proinflammatory and regulatory cytokines, such as IL-4, IL-10 produced including analysis of regulatory T cell responses measured by estimation of IL-17 produced by Th17 lymphocytes, as reported previously for a different live attenuated *B pertussis* vaccine candidate with potential to revert back to virulence. These studies should not only be carried out in the pertussis mouse model, the most commonly used model for assessing the potency of different pertussis vaccines (see section 1.5.1) but also using an ex-vivo model using human monocyte derived dendritic cells (Fedele et al. 2011) or using human peripheral blood mononuclear cells (Rowe et al. 2005).

References

Adam, A., and E. Lederer. 1984. Muramyl peptides: immunomodulators, sleep factors, and vitamins. *Medicinal Research Reviews* 4 (2):111-52.

Allen, A., and D. Maskell. 1996. The identification, cloning and mutagenesis of a genetic locus required for lipopolysaccharide biosynthesis in Bordetella pertussis. *Molecular Microbiology* 19 (1):37-52.

Andrews, R., A. Herceg, and C. Roberts. 1997. Pertussis notifications in Australia, 1991 to 1997. *Communicable Diseases Intelligence* 21 (11):145-8.

Anonymous. 1956. Vaccination against whooping-cough; relation between protection in children and results of laboratory tests; a report to the Whooping-cough Immunization Committee of the Medical Research Council and to the medical officers of health for Cardiff, Leeds, Leyton, Manchester, Middlesex, Oxford, Poole, Tottenham, Walthamstow, and Wembley. *British Medical Journal* 2 (4990):454-62.

Anonymous. 2005. Pertussis vaccines. In *WHO Position paper. Weekly epidemiological Records*: http://www.who.int/wer.

Anonymous. 2009. *National notifiable diseases surveillance system* Australian government department of health and aging. http://www.health.gov.au/cda

Arico, B., R. Gross, J. Smida, and R. Rappuoli. 1987. Evolutionary relationships in the genus Bordetella. *Molecular Microbiology* 1 (3):301-8.

Au, Douglas C., H. Robert Masure, and Daniel R. Storm. 1989. Site-directed mutagenesis of lysine 58 in a putative ATP-binding domain of the calmodulin-sensitive adenylate cyclase from Bordetella pertussis abolishes catalytic activity. *Biochemistry* 28 (7):2772-2776.

Ausiello, C. M., R. Lande, A. la Sala, F. Urbani, and A. Cassone. 1998. Cell-mediated immune response of healthy adults to Bordetella pertussis vaccine antigens. *Journal of Infectious Diseases* 178 (2):466-70.

Ausiello, Clara M., Roberto Lande, Francesca Urbani, Andrea la Sala, Paola Stefanelli, Stefania Salmaso, Paola Mastrantonio, and Antonio Cassone. 1999. Cell-Mediated Immune Responses in Four-Year-Old Children after Primary Immunization with Acellular Pertussis Vaccines. *Infection and Immunity* 67 (8):4064-4071.

Baker, J. P., and S. L. Katz. 2004. Childhood vaccine development: An overview. *Pediatric Research* 55 (2):347-356.

Bannan, J. D., M. J. Moran, J. I. MacInnes, G. A. Soltes, and R. L. Friedman. 1993. Cloning and characterization of btr, a Bordetella pertussis gene encoding an FNR-like transcriptional regulator. *Journal of Bacteriology* 175 (22):7228-35.

Barnard, A., B. P. Mahon, J. Watkins, K. Redhead, and K. H. Mills. 1996. Th1/Th2 cell dichotomy in acquired immunity to Bordetella pertussis: variables in the in vivo priming and in vitro cytokine detection techniques affect the classification of T-cell subsets as Th1, Th2 or Th0. *Immunology* 87 (3):372-80.

Berbers, Guy A. M., Sabine de Greeff, and Frits Mooi. 2009. Improving pertussis vaccination. *Human Vaccines* 5 (7):497-503.

Birkebaek, N. H., M. Kristiansen, T. Seefeldt, J. Degn, A. Moller, I. Heron, P. L. Andersen, J. K. Moller, and L. Ostergrd. 1999. Bordetella pertussis and chronic cough in adults. *Clinical Infectious Diseases* 29 (5):1239-42.

Boschwitz, J. S., H. G. van der Heide, F. R. Mooi, and D. A. Relman. 1997. Bordetella bronchiseptica expresses the fimbrial structural subunit gene fimA. *Journal of Bacteriology* 179 (24):7882-5.

Bottomley, J. R., C. L. Clayton, P. A. Chalk, and C. Kleanthous. 1996. Cloning, sequencing, expression, purification and preliminary characterization of a type II dehydroquinase from Helicobacter pylori. *Biochemical Journal* 319 (Pt 2):559-65.

Boyd, Aoife P., Padraig J. Ross, Helen Conroy, Nicola Mahon, Ed C. Lavelle, and Kingston H. G. Mills. 2005. Bordetella pertussis adenylate cyclase toxin modulates innate and adaptive immune responses: distinct roles for acylation and enzymatic activity in immunomodulation and cell death. *Journal of Immunology* 175 (2):730-8.

Brennan, M. J., Z. M. Li, J. L. Cowell, M. E. Bisher, A. C. Steven, P. Novotny, and C. R. Manclark. 1988. Identification of a 69-kilodalton nonfimbrial protein as an agglutinogen of Bordetella Pertussis. *Infection and Immunity* 56 (12):3189-3195.

Bruss, J. B., and G. R. Siber. 1999. Protective effects of pertussis immunoglobulin (P-IGIV) in the aerosol challenge model. *Clinical and Diagnostic Laboratory Immunonolgy* 6 (4):464-70.

Cagney, M., Peter B. McIntyre, Leon Heron, Anna Giammanco, and C. Raina MacIntyre. 2008. The relationship between pertussis symptomatology, incidence and serology in adolescents. *Vaccine* 26 (44):5547-5553.

Cairns, J., J. Overbaugh, and S. Miller. 1988. The origin of mutants. *Nature* 335 (6186):142-5.

Canthaboo, C., L. Williams, D. K. Xing, and M. J. Corbel. 2000. Investigation of cellular and humoral immune responses to whole cell and acellular pertussis vaccines. *Vaccine* 19 (6):637-43.

Canthaboo, C., D. Xing, A. Douglas, and M. Corbel. 2000. Investigation of an aerosol challenge model as alternative to the intracerebral mouse protection test for potency assay of whole cell pertussis vaccines. *Biologicals* 28 (4):241-6.

Carbonetti, N. H., Galina V. Artamonova, Charlotte Andreasen, and Nicholas Bushar. 2005. Pertussis toxin and adenylate cyclase toxin provide a one-two punch for establishment of Bordetella pertussis infection of the respiratory tract. *Infection and Immunity* 73 (5):2698-703.

Cassone, A., C. M. Ausiello, F. Urbani, R. Lande, M. Giuliano, A. La Sala, A. Piscitelli, and S. Salmaso. 1997. Cell-mediated and antibody responses to Bordetella pertussis antigens in children vaccinated with acellular or whole-cell pertussis vaccines. The Progetto Pertosse-CMI Working Group. *Archives of Pediatrics and Adolescent Medicine* 151 (3):283-9.

Centers for Disease Control and Prevention. *Pertussis*. Centers for Dieases Control and Prevention 2011 [cited March 3/2011]. Available from http://www.cdc.gov/pertussis/outbreaks.html.

Chen, A. Y., Scott R. Fry, Judy Forbes-Faulkner, Grant Daggard, and T. K. S. Mukkur. 2006. Evaluation of the immunogenicity of the P97R1 adhesin of Mycoplasma hyopneumoniae as a mucosal vaccine in mice. *Journal of Medical Microbiology* 55 (Pt 7):923-9.

Cherry, J. D. 1999. Epidemiological, clinical, and laboratory aspects of pertussis in adults. *Clinical Infectious Diseases* 28 Suppl 2:S112-7.

Cherry, J. D. 2003. The science and fiction of the "resurgence" of pertussis. *Pediatrics* 112 (2):405-6.

Cherry, J. D. 2005. Pertussis vaccines for adolescents and adults. *Pediatrics* 116 (3):755-6.

Cherry, J. D., Jeffrey Gornbein, Ulrich Heininger, and Klemens Stehr. 1998. A search for serologic correlates of immunity to Bordetella pertussis cough illnesses. *Vaccine* 16 (20):1901-1906.

Cherry, J. D., Emmanuel Grimprel, Nicole Guiso, Ulrich Heininger, and Jussi Mertsola. 2005. Defining pertussis epidemiology: clinical, microbiologic and serologic perspectives. *Pediatric Infectious Disease Journal* 24 (5 Suppl):S25-34.

Cohen, S. M., and M.W. Wheeler. 1946. Pertussis vaccine prepared from Phase I cultures grown in fluid medium. *American Journal of Public Health* 36:371-382.

Confer, D. L., and J. W. Eaton. 1982. Phagocyte impotence caused by an invasive bacterial adenylate cyclase. *Science* 217 (4563):948-50.

Conway, M. A., L. Madrigal-Estebas, S. McClean, D. J. Brayden, and K. H. Mills. 2001. Protection against Bordetella pertussis infection following parenteral or oral immunization with antigens entrapped in biodegradable particles: effect of formulation and route of immunization on induction of Th1 and Th2 cells. *Vaccine* 19 (15-16):1940-50.

Cookson, B. T., H. L. Cho, L. A. Herwaldt, and W. E. Goldman. 1989. Biological activities and chemical composition of purified tracheal cytotoxin of Bordetella pertussis. *Infection and Immunity* 57 (7):2223-9.

Cornford, R. A. 1998. Charicterisation of the aroD and/or aroQ mutants of Bordetella pertussis (thesis), University of Southern Queensland, Toowoomba.

Cornford, R. A. 2003. A novel, genetically engineered, intranasal whooping cough vaccine (thesis), University of Southern Queensland, Toowoomba.

Cromer, B. A., J. Goydos, J. Hackell, J. Mezzatesta, C. Dekker, and E. A. Mortimer. 1993. Unrecognized pertussis infection in adolescents. *American Journal of Diseases of Children* 147 (5):575-7.

De Magistris, M. T., M. Romano, S. Nuti, R. Rappuoli, and A. Tagliabue. 1988. Dissecting human T cell responses against Bordetella species. *Journal of Experimental Medicine* 168 (4):1351-62.

de Melker, H. E., M. A. Conyn-van Spaendonck, H. C. Rumke, J. K. van Wijngaarden, F. R. Mooi, and J. F. Schellekens. 1997. Pertussis in The Netherlands: an outbreak despite high levels of immunization with whole-cell vaccine. *Emerging Infectious Diseases* 3:175 - 178.

Decker, M. D., K. M. Edwards, M. C. Steinhoff, M. B. Rennels, M. E. Pichichero, J. A. Englund, E. L. Anderson, M. A. Deloria, and G. F. Reed. 1995. Comparison of 13 acellular pertussis vaccines: adverse reactions. *Pediatrics* 96 (3 Pt 2):557-66.

Department of Health and Aging. 2008 *The Australian Immunisation Handbook* (9) [Website]. http://www.health.gov.au/internet/immunise/publishing.nsf/content/handbook 2008 [cited December 16/2011].

Donnelly, J. J., Jeffrey B. Ulmer, John W. Shiver, and Margaret A. Liu. 1997. DNA Vaccines. *Annual Review of Immunology* 15 (1):617-648.

Donnelly, S., C. E. Loscher, M. A. Lynch, and K. H. Mills. 2001. Whole-cell but not acellular pertussis vaccines induce convulsive activity in mice: evidence of a role for toxin-induced interleukin-1beta in a new murine model for analysis of neuronal side effects of vaccination. *Infection and Immunity* 69 (7):4217-23.

Edwards, K. M., B. D. Meade, M. D. Decker, G. F. Reed, M. B. Rennels, M. C. Steinhoff, E. L. Anderson, J. A. Englund, M. E. Pichichero, and M. A.

Deloria. 1995. Comparison of 13 acellular pertussis vaccines: overview and serologic response. *Pediatrics* 96 (3 pt 2):548-57.

Elahi, S., James Holmstrom, and Volker Gerdts. 2007. The benefits of using diverse animal models for studying pertussis. *Trends Microbiology* 15 (10):462-468.

Esposito, S., Tiziano Agliardi, Anna Giammanco, Giacomo Faldella, Antonio Cascio, Samantha Bosis, Ornella Friscia, Mario Clerici, and Nicola Principi. 2001. Long-Term Pertussis-Specific Immunity after Primary Vaccination with a Combined Diphtheria, Tetanus, Tricomponent Acellular Pertussis, and Hepatitis B Vaccine in Comparison with That after Natural Infection. *Infection and Immunity* 69 (7):4516-4520.

Farfel, Z., S. Konen, E. Wiertz, R. Klapmuts, P. A. Addy, and E. Hanski. 1990. Antibodies to Bordetella pertussis adenylate cyclase are produced in man during pertussis infection and after vaccination. *Journal of Medical Microbiology* 32 (3):173-7.

Fedele, G., Manuela Bianco, Anne-Sophie Debrie, Camille Locht, and Clara Maria Ausiello. 2011. Attenuated Bordetella pertussis vaccine candidate BPZE1 promotes human dendritic cell CCL21-induced migration and drives a Th1/Th17 response. *Journal of Immunology* 186 (9):5388-96.

Fennelly, N. K., Federico Sisti, Sarah C. Higgins, Padraig J. Ross, Han van der Heide, Frits R. Mooi, Aoife Boyd, and Kingston H. G. Mills. 2008. Bordetella pertussis Expresses a Functional Type III Secretion System That Subverts Protective Innate and Adaptive Immune Responses. *Infection and Immunity* 76 (3):1257-1266.

Fernandez, R. C., and A. A. Weiss. 1998. Serum resistance in bvg-regulated mutants of Bordetella pertussis. *FEMS Microbiology Letters* 163 (1):57-63.

Feunou, Pascal Feunou, Julie Bertout, and Camille Locht. 2010. T- and B-cell-mediated protection induced by novel, live attenuated pertussis vaccine in mice. Cross protection against parapertussis. *PLoS ONE [Electronic Resource]* 5 (4):e10178.

Fine, P. E., and J. A. Clarkson. 1987. Reflections on the efficacy of pertussis vaccines. *Reviews of Infectious Diseases* 9 (5):866-83.

Finn, T. M., and L. A. Stevens. 1995. Tracheal colonization factor: a Bordetella pertussis secreted virulence determinant. *Molecular Microbiology* 16 (4):625-34.

Friedman, E., Z. Farfel, and E. Hanski. 1987. The invasive adenylate cyclase of Bordetella pertussis. Properties and penetration kinetics. *Biochemical Journal* 243 (1):145-51.

Friedman, R. L., K. Nordensson, L. Wilson, E. T. Akporiaye, and D. E. Yocum. 1992. Uptake and intracellular survival of Bordetella pertussis in human macrophages. *Infection and Immunity* 60 (11):4578-85.

Fry, D. C., S. A. Kuby, and A. S. Mildvan. 1985. NMR studies of the MgATP binding site of adenylate kinase and of a 45-residue peptide fragment of the enzyme. *Biochemistry* 24 (17):4680-94.

Fry, Scott R. 2012. *The development and evaluation of DNA vaccines against whooping cough using a murine respiratory model of infection. [Thesis (_PhD/Research)] (Unpublished)*. University of Southern Queensland 2006 [cited January 1/2012]. Available from http://eprints.usq.edu.au/4787/.

Fry, Scott R., Austen Y. Chen, Grant Daggard, and Trilochan K. S. Mukkur. 2008. Parenteral immunization of mice with a genetically inactivated pertussis toxin DNA vaccine induces cell-mediated immunity and protection. *Journal of Medical Microbiology* 57 (1):28-35.

Garbe, T., S. Servos, A. Hawkins, G. Dimitriadis, D. Young, G. Dougan, and I. Charles. 1991. The Mycobacterium tuberculosis shikimate pathway genes: evolutionary relationship between biosynthetic and catabolic 3-dehydroquinases. *Molecular and General Genetics* 228 (3):385-92.

Gerlt, J. A., J. A. Coderre, and M. S. Wolin. 1980. Mechanism of the adenylate cyclase reaction. Stereochemistry of the reaction catalyzed by the enzyme from Brevibacterium liquefaciens. *Journal of Biological Chemistry* 255 (2):331-4.

Gerthoffer, W.T., and Mariam. Ba. Comparative Antibody Titers Using A Microtiter Plate Assay Versus Dot Blotting on Nitrocellulose http://biosupport.licor.com/docs/Gerthoffer.pdf.

Geuijen, C. A., R. J. Willems, M. Bongaerts, J. Top, H. Gielen, and F. R. Mooi. 1997. Role of the Bordetella pertussis minor fimbrial subunit, FimD, in colonization of the mouse respiratory tract. *Infection and Immunity* 65 (10):4222-8.

Giles, N. H., M. E. Case, J. Baum, R. Geever, L. Huiet, V. Patel, and B. Tyler. 1985. Gene organization and regulation in the qa (quinic acid) gene cluster of Neurospora crassa. *Microbiological Reviews* 49 (3):338-58.

Glaser, P., D. Ladant, O. Sezer, F. Pichot, A. Ullmann, and A. Danchin. 1988. The calmodulin-sensitive adenylate cyclase of Bordetella pertussis: cloning and expression in Escherichia coli. *Molecular Microbiology* 2 (1):19-30.

Gold, M. S., Sara Noonan, Maggi Osbourn, Stella Precepa, and Ann E. Kempe. 2003. Local reactions after the fourth dose of acellular pertussis vaccine in South Australia. *Medical Journal of Australia* 179 (4):191-4.

Goodwin, M. S., and A. A. Weiss. 1990. Adenylate cyclase toxin is critical for colonization and pertussis toxin is critical for lethal infection by Bordetella pertussis in infant mice. *Infection and Immunity* 58 (10):3445-3447.

Graeff-Wohlleben, H., S. Killat, A. Banemann, N. Guiso, and R. Gross. 1997. Cloning and characterization of an Mn-containing superoxide dismutase (SodA) of Bordetella pertussis. *Journal of Bacteriology* 179 (7):2194-201.

Granstrom, M., A. M. Olinder-Nielsen, P. Holmblad, A. Mark, and K. Hanngren. 1991. Specific immunoglobulin for treatment of whooping cough. *Lancet* 338 (8777):1230-3.

Gross, M. K., D. C. Au, A. L. Smith, and D. R. Storm. 1992. Targeted mutations that ablate either the adenylate cyclase or hemolysin function of the bifunctional cyaA toxin of Bordetella pertussis abolish virulence. *Proceedings of the National Academy of Sciences of the United States of America* 89 (11):4898-902.

Guiso, N., E. Njamkepo, F. Vie le Sage, F. Zepp, C. U. Meyer, V. Abitbol, N. Clyti, and S. Chevallier. 2007. Long-term humoral and cell-mediated immunity after acellular pertussis vaccination compares favourably with whole-cell vaccines 6 years after booster vaccination in the second year of life. *Vaccine* 25 (8):1390-1397.

Gupta, R. K. 1998. Aluminum compounds as vaccine adjuvants. *Advanced Drug Delivery Reviews* 32 (3):155-172.

Gustafsson, L., H. O. Hallander, P. Olin, E. Reizenstein, and J. Storsaeter. 1996. A controlled trial of a two-component acellular, a five-component acellular, and a whole-cell pertussis vaccine. *New England Journal Medicine* 334 (6):349-55.

Guzman, C. A., G. Molinari, M. W. Fountain, M. Rohde, K. N. Timmis, and M. J. Walker. 1993. Antibody responses in the serum and respiratory tract of mice following oral vaccination with liposomes coated with filamentous hemagglutinin and pertussis toxoid. *Infection and Immunity* 61 (2):573-9.

Hanski, E. 1989. Invasive adenylate cyclase toxin of Bordetella pertussis. *Trends in Biochemical Sciences* 14 (11):459-63.

Harvill, E. T., P. A. Cotter, and J. F. Miller. 1999. Pregenomic comparative analysis between bordetella bronchiseptica RB50 and Bordetella pertussis tohama I in murine models of respiratory tract infection. *Infection and Immunity* 67 (11):6109-18.

Harvill, E. T., A. Preston, P. A. Cotter, A. G. Allen, D. J. Maskell, and J. F. Miller. 2000. Multiple roles for Bordetella lipopolysaccharide molecules during respiratory tract infection. *Infection and Immunity* 68 (12):6720-8.

Hazenbos, W. L., B. M. van den Berg, C. W. Geuijen, F. R. Mooi, and R. van Furth. 1995. Binding of FimD on Bordetella pertussis to very late antigen-5 on monocytes activates complement receptor type 3 via protein tyrosine kinases. *Journal of Immunology* 155 (8):3972-8.

He, Q., and Jussi Mertsola. 2008. Factors contributing to pertussis resurgence. *Future Microbiology* 3:329-39.

Hellwig, S. M., W. L. Hazenbos, J. G. van de Winkel, and F. R. Mooi. 1999. Evidence for an intracellular niche for Bordetella pertussis in broncho-alveolar lavage cells of mice. *Immunology and Medical Microbiology* 26 (3-4):203-7.

Hellwig, S. M. M., Maria E. Rodriguez, Guy A. M. Berbers, Jan G. J. van de Winkel, and Frits R. Mooi. 2003. Crucial role of antibodies to pertactin in Bordetella pertussis immunity. *Journal of Infectious Diseases* 188 (5):738-42.

Hewlett, E. L. 1999. A commentary on the pathogenesis of pertussis. *Clinical Infectious Diseases* 28 Suppl 2:S94-8.

Hijnen, M., Dirk J. van Zoelen, Cristina Chamorro, Pieter van Gageldonk, Frits R. Mooi, Guy Berbers, and Rob M. J. Liskamp. 2007. A novel strategy to mimic discontinuous protective epitopes using a synthetic scaffold. *Vaccine* 25 (37-38):6807-6817.

Hoiseth, S. K., and B. A. Stocker. 1981. Aromatic-dependent Salmonella typhimurium are non-virulent and effective as live vaccines. *Nature* 291 (5812):238-9.

Hormozi, K., R. Parton, and J. Coote. 1999. Adjuvant and protective properties of native and recombinant Bordetella pertussis adenylate cyclase toxin preparations in mice. *Immunology and Medical Microbiology* 23 (4):273-82.

http://virology-online.com/general/typesofvaccines.htm. *Different Types of Vaccine*. Wong's Virology [cited January 2/2012].

Imaizumi, A., Y. Suzuki, S. Ono, H. Sato, and Y. Sato. 1983. Heptakis(2,6-O-dimethyl)beta-cyclodextrin: a novel growth stimulant for Bordetella pertussis phase I. *Journal of Clinical Microbiology* 17 (5):781-6.

Jackson, L. A., Barbara A. Carste, Darren Malais, and James Froeschle. 2002. Retrospective population-based assessment of medically attended injection site reactions, seizures, allergic responses and febrile episodes after acellular pertussis vaccine combined with diphtheria and tetanus toxoids. *Pediatric Infectious Disease Journal* 21 (8):781-6.

Jefferson, T., Melanie Rudin, and Carlo DiPietrantonj. 2003. Systematic review of the effects of pertussis vaccines in children. *Vaccine* 21 (17-18):2003-2014.

Jeremy W. D, and Simon F. P. 2004. *Molecular Genetics of Bacteria*. Vol. 4. Original edition, John Wiley & Sons Ltd, The Atrium, Southern Gate, Chichester, West Sussex PO19 8SQ, England.

Kamachi, K., Toshifumi Konda, and Yoshichika Arakawa. 2003. DNA vaccine encoding pertussis toxin S1 subunit induces protection against Bordetella pertussis in mice. *Vaccine* 21 (31):4609-4615.

Keitel, W. A. 1999. Cellular and Acellular Pertussis Vaccines in Adults. *Clinical Infectious Diseases* 28 (s2):S118-S123.

Kerr, J. R., and R. C. Matthews. 2000. Bordetella pertussis infection: Pathogenesis, diagnosis, management, and the role of protective immunity. *European Journal of Clinical Microbiology* 19 (2):77-88.

Khelef, N., A. Zychlinsky, and N. Guiso. 1993. Bordetella pertussis induces apoptosis in macrophages: role of adenylate cyclase-hemolysin. *Infection and Immunity* 61 (10):4064-71.

Kolos, V., Robert Menzies, and Peter McIntyre. 2007. Higher pertussis hospitalization rates in indigenous Australian infants, and delayed vaccination. *Vaccine* 25 (4):588-90.

Komabiotech. *EzWay Direct Taq MasterMix*. Koma Biotech Inc. 2009. Available from koma@komabiotech.com.

Lalonde, G., P. D. O'Hanley, B. A. D. Stacker, and K. T. Denich. 1994. Characterization of a 3-dehydroquinase gene from Actinobacillus pleuropneumoniae with homology to the eukaryotic genes qa-2 and QUTE. *Molecular Microbiology* 11 (2):273-280.

Lamberti, Y., Maria Laura Perez Vidakovics, Ludo- W. van der Pol, and Maria Eugenia Rodriguez. 2008. Cholesterol-rich domains are involved in Bordetella pertussis phagocytosis and intracellular survival in neutrophils. *Microbial Pathogenesis* 44 (6):501-11.

Lawrence, G. L., Padmasiri E. Aratchige, Ian Boyd, Peter B. McIntyre, and Michael S. Gold. 2007. Annual report on surveillance of adverse events following immunisation in Australia, 2006. *Communicable Diseases Intelligence* 31 (3):269-82.

Leef, M., K. L. Elkins, J. Barbic, and R. D. Shahin. 2000. Protective immunity to Bordetella pertussis requires both B cells and CD4(+) T cells for key functions other than specific antibody production. *Journal of Experimental Medicine* 191 (11):1841-52.

Leininger, E., M. Roberts, J. G. Kenimer, I. G. Charles, N. Fairweather, P. Novotny, and M. J. Brennan. 1991. Pertactin, an Arg-Gly-Asp-containing Bordetella pertussis surface protein that promotes adherence of mammalian cells. *PNAS USA* 88 (2):345-9.

Lenz, D. H., C. L. Weingart, and A. A. Weiss. 2000. Phagocytosed Bordetella pertussis fails to survive in human neutrophils. *Infection and Immunity* 68 (2):956-9.

Li, Q., Yongzhang Zhu, Jiayou Chu, Yaying Wang, Yinghua Xu, Qiming Hou, Shumin Zhang, and Xiaokui Guo. 2006. Protective immunity against Bordetella pertussis by a recombinant DNA vaccine and the effect of coinjection with a granulocyte-macrophage colony stimulating factor gene. *Microbiology and Immunology* 50 (12):929-36.

Lin-Chao, S., W. T. Chen, and T. T. Wong. 1992. High copy number of the pUC plasmid results from a Rom/Rop-suppressible point mutation in RNA II. *Molecular Microbiology* 6 (22):3385-93.

Linnemann, C. C., and E. B. Perry. 1977. Bordetella parapertussis. Recent experience and a review of the literature. *American Journal of Diseases of Children* 131 (5):560-3.

Livey, I., and A. C. Wardlaw. 1984. Production and properties of Bordetella pertussis heat-labile toxin. *Journal of Medical Microbiology* 17 (1):91-103.

Locht, C., and R. Antoine. 1995. A proposed mechanism of ADP-ribosylation catalyzed by the pertussis toxin S1 subunit. *Biochimie* 77 (5):333-40.

Locht, C., M. C. Geoffroy, and G. Renauld. 1992. Common accessory genes for the Bordetella pertussis filamentous hemagglutinin and fimbriae share sequence similarities with the papC and papD gene families. *EMBO Journal* 11 (9):3175-83.

Locht, C., Rudy Antoine, Dominique Raze, Nathalie Mielcarek, David Hot, Yves Lemoine, and Francoise Mascart. 2004. Bordetella pertussis: from functional genomics to intranasal vaccination. *International Journal of Medical Microbiology* 293 (7-8):583-588.

Mahon, B. P., M. T. Brady, and K. H. Mills. 2000. Protection against Bordetella pertussis in mice in the absence of detectable circulating antibody: implications for long-term immunity in children. *Journal of Infectious Diseases* 181 (6):2087-91.

Mahon, B. P., M. S. Ryan, F. Griffin, and K. H. Mills. 1996. Interleukin-12 is produced by macrophages in response to live or killed Bordetella pertussis and enhances the efficacy of an acellular pertussis vaccine by promoting induction of Th1 cells. *Infection and Immunity* 64 (12):5295-301.

Mahon, B. P., B. J. Sheahan, F. Griffin, G. Murphy, and K. H. Mills. 1997. Atypical disease after Bordetella pertussis respiratory infection of mice with targeted disruptions of interferon-gamma receptor or immunoglobulin mu chain genes. *Journal of Experimental Medicine* 186 (11):1843-51.

Marzouqi, I., Peter Richmond, Scott Fry, John Wetherall, and Trilochan Mukkur. 2010. Development of improved vaccines against whooping cough: current status. *Human Vaccines* 6 (7):543-53.

Mascart, F., Virginie Verscheure, Anne Malfroot, Marc Hainaut, Denis Pierard, Stephane Temerman, Alexandra Peltier, Anne-Sophie Debrie, Jack Levy, Giuseppe Del Giudice, and Camille Locht. 2003. Bordetella pertussis infection in 2-month-old infants promotes type 1 T cell responses. *Journal of Immunology* 170 (3):1504-9.

Masure, H. R., R. L. Shattuck, and D. R. Storm. 1987. Mechanisms of bacterial pathogenicity that involve production of calmodulin-sensitive adenylate cyclases. *Microbiological Reviews* 51 (1):60-5.

Matsuzawa, T., A. Fukui, T. Kashimoto, K. Nagao, K. Oka, M. Miyake, and Y. Horiguchi. 2004. Bordetella dermonecrotic toxin undergoes proteolytic processing to be translocated from a dynamin-related endosome into the cytoplasm in an acidification-independent manner. *Journal of Biological Chemistry* 279 (4):2866-2872.

McGuirk, P., B. P. Mahon, F. Griffin, and K. H. Mills. 1998. Compartmentalization of T cell responses following respiratory infection with Bordetella pertussis: hyporesponsiveness of lung T cells is associated with modulated expression of the co-stimulatory molecule CD28. *European Journal of Immunology* 28 (1):153-63.

McIntyre, P., and Nicholas Wood. 2009. Pertussis in early infancy: disease burden and preventive strategies. *Current Opinion in Infectious Diseases* 22 (3):215-23.

Meyer, C. 2007. Cellular immunity in adolescents and adults following acellular pertussis vaccine administration. *Clinical and Vaccine Immunology* 14 (3):288-292.

Mielcarek, N., G. Riveau, F. Remoue, R. Antoine, A. Capron, and C. Locht. 1998. Homologous and heterologous protection after single intranasal administration of live attenuated recombinant Bordetella pertussis. *Nature Biotechnology* 16 (5):454-7.

Mielcarek, N., Anne-Sophie Debrie, Severine Mahieux, and Camille Locht. 2010. Dose response of attenuated Bordetella pertussis BPZE1-induced protection in mice. *Clinical and Vaccine Immunology: CVI* 17 (3):317-24.

Mielcarek, N., Anne-Sophie Debrie, Dominique Raze, Julie Bertout, Carine Rouanet, Amena Ben Younes, Colette Creusy, Jacquelyn Engle, William E. Goldman, and Camille Locht. 2006. Live attenuated B. pertussis as a single-dose nasal vaccine against whooping cough. *PLoS Pathogens* 2 (7):e65.

Mielcarek, N., Anne-Sophie Debrie, Dominique Raze, Julie Quatannens, Jacquelyn Engle, William E. Goldman, and Camille Locht. 2006. Attenuated Bordetella

pertussis: new live vaccines for intranasal immunisation. *Vaccine* 24 (Supplement 2):S54-S55.

Miles, A.A., and S.S. Mirsa. 1938. The estimation of the bacterial power of the blood. *Journal of Hygiene* 38:732.

Mills, K. H., A. Barnard, J. Watkins, and K. Redhead. 1993. Cell-mediated immunity to Bordetella pertussis: role of Th1 cells in bacterial clearance in a murine respiratory infection model. *Infection and Immunity* 61 (2):399-410.

Mills, K. H., M. Ryan, E. Ryan, and B. P. Mahon. 1998. A murine model in which protection correlates with pertussis vaccine efficacy in children reveals complementary roles for humoral and cell-mediated immunity in protection against Bordetella pertussis. *Infection and Immunity* 66 (2):594-602.

Mills, K. H. G. 2001. Immunity to Bordetella pertussis. *Microbes and Infection* 3 (8):655-677.

Mooi, F. R., H. Hallander, C. H. Wirsing von Konig, B. Hoet, and N. Guiso. 2000. Epidemiological typing of Bordetella pertussis isolates: recommendations for a standard methodology. *European Journal of Clinical Microbiology and Infectious Diseases* 19 (3):174-81.

Mooi, F. R., W. H. Jansen, H. Brunings, H. Gielen, H. G. van der Heide, H. C. Walvoort, and P. A. Guinee. 1992. Construction and analysis of Bordetella pertussis mutants defective in the production of fimbriae. *Microbial Pathogenesis* 12 (2):127-35.

Mooi, F. R., I. H. Van Loo, and A. J. King. 2001. Adaptation of Bordetella pertussis to vaccination: a cause for its reemergence? *Emerging Infectious Diseases* 7:526 - 528.

Mooi, F. R., Loo Van, IHM., Gent M. van, Q. He, MJ. Bart, KJ. Heuvelman, SC. de Greef, D. Diavatopoulos, P. Teunis, N. Nagelkerke, and J. Mertsola. 2009. Bordetella pertussis Strains with Increased Toxin Production Associated with Pertussis Resurgence. *Emerging Infectious Diseases* 15:1206-1213.

Moore, J. D., H. K. Lamb, T. Garbe, S. Servos, G. Dougan, I. G. Charles, and A. R. Hawkins. 1992. Inducible overproduction of the Aspergillus nidulans pentafunctional AROM protein and the type-I and -II 3-dehydroquinases from Salmonella typhi and Mycobacterium tuberculosis. *Biochemical Journal* 287 (Pt 1):173-81.

Mukkur, T. K., R.A. Cornford, T.R. Rossetti, Kershaw, Scott R. Fry, Xu. Youhong, and G. Daggard. 2005. A metabolite-deficient mutant of Bordetella pertussis that induces secretory IgA and cell-mediated immunity in mice vaccinated by the intranasal route. In *105th American Society for Microbiology: General Meeting*. Atlanta, Georgia: F.C.Fang.

Mukkur, T. K., G. H. McDowell, B. A. Stocker, and A. K. Lascelles. 1987. Protection against experimental salmonellosis in mice and sheep by immunisation with aromatic-dependent Salmonella typhimurium. *Journal of Medical Microbiology* 24 (1):11-19.

Munoz, J. J., H. Arai, and R. L. Cole. 1981. Mouse-protecting and histamine-sensitizing activities of pertussigen and fimbrial hemagglutinin from Bordetella pertussis. *Infection and Immunity* 32 (1):243-50.

National Health and Medical Research Council. 2004. *Australian code of practice for the care and use of animals for scientific purposes 7th Edition.* http://www.nhmrc.gov.au/_files_nhmrc/publications/attachments/ea16.pdf 2004.

National Notifiable Diseases Surveillance System. 2011. *Notifications of communicable diseases* 2011 [cited March 4/2011. Available from http://www9.health.gov.au/cda/Source/Rpt_5.cfm.

Novotny, P., A. P. Chubb, K. Cownley, and I. G. Charles. 1991. A novel bivalent acellular pertussis vaccine based on the 69 kDa protein and FHA. *Developments in Biological Standardization* 73:243-9.

Ohnishi, H., Masami Miyake, Shigeki Kamitani, and Yasuhiko Horiguchi. 2008. The morphological changes in cultured cells caused by Bordetella pertussis adenylate cyclase toxin. *FEMS Microbiology Letters* 279 (2):174-179.

Olin, P. 1997. Commentary: The best acellular pertussis vaccines are multicomponent. *Pediatric Infectious Disease Journal* 16 (5):517-519.

Oliver, D. C., and R. C. Fernandez. 2001. Antibodies to BrkA augment killing of Bordetella pertussis. *Vaccine* 20 (1-2):235-241.

Olson, L. C. 1975. Pertussis. *Medicine* 54 (6):427-69.

Paccani, S. R., Francesca Finetti, Marilyne Davi, Laura Patrussi, Mario M. D'Elios, Daniel Ladant, and Cosima T. Baldari. 2011. The Bordetella pertussis adenylate cyclase toxin binds to T cells via LFA-1 and induces its disengagement from the immune synapse. *Journal of Experimental Medicine* 208 (6):1317-30.

Parton, R. 1999. Review of the Biology of Bordetella pertussis. *Biologicals* 27 (2):71-76.

Pedroni, P., B. Riboli, F. de Ferra, G. Grandi, S. Toma, B. Arico, and R. Rappuoli. 1988. Cloning of a novel pilin-like gene from Bordetella pertussis: homology to the fim2 gene. *Molecular Microbiology* 2 (4):539-43.

Peppler, M. S. 1984. Two physically and serologically distinct lipopolysaccharide profiles in strains of Bordetella pertussis and their phenotype variants. *Infection and Immunity* 43 (1):224-32.

Pittman, M. M. 1979. Pertussis toxin: the cause of the harmful effects and prolonged immunity of whooping cough. A hypothesis. *Reviews of Infectious Diseases* 1 (3):401-12.

Poland, G. A. 1996. Acellular pertussis vaccines: new vaccines for an old disease. *The Lancet* 347 (8996):209-210.

Pradel, E., N. Guiso, F. D. Menozzi, and C. Locht. 2000. Bordetella pertussis TonB, a Bvg-independent virulence determinant. *Infection and Immunity* 68 (4):1919-27.

Preston, A. 2005. Bordetella pertussis: the intersection of genomics and pathobiology. *Canadian Medical Association Journal* 173 (1).

Preziosi, M-P., and M. Elizabeth Halloran. 2003. Effects of pertussis vaccination on transmission: vaccine efficacy for infectiousness. *Vaccine* 21 (17-18):1853-1861.

Quinn, H. E., and Peter B. McIntyre. 2007. Pertussis epidemiology in Australia over the decade 1995-2005--trends by region and age group. *Communicable Diseases Intelligence* 31 (2):205-15.

Redhead, K., J. Watkins, A. Barnard, and K. H. Mills. 1993. Effective immunization against Bordetella pertussis respiratory infection in mice is dependent on induction of cell-mediated immunity. *Infection and Immunity* 61 (8):3190-8.

Relman, D., E. Tuomanen, S. Falkow, D. T. Golenbock, K. Saukkonen, and S. D. Wright. 1990. Recognition of a bacterial adhesion by an integrin: macrophage CR3 (alpha M beta 2, CD11b/CD18) binds filamentous hemagglutinin of Bordetella pertussis. *Cell Host and Microbe* 61 (7):1375-82.

Rennels, M. B., M. A. Deloria, M. E. Pichichero, G. A. Losonsky, J. A. Englund, B. D. Meade, E. L. Anderson, M. C. Steinhoff, and K. M. Edwards. 2000. Extensive swelling after booster doses of acellular pertussis-tetanus-diphtheria vaccines. *Pediatrics* 105 (1):e12.

Rieber, N., Anna Graf, Bernd H. Belohradsky, Dominik Hartl, Simon Urschel, Marion Riffelmann, Carl-Heinz Wirsing von Kˆnig, and Johannes Liese. 2008. Differences of humoral and cellular immune response to an acellular pertussis booster in adolescents with a whole cell or acellular primary vaccination. *Vaccine* 26 (52):6929-6935.

Roberts, M., N. F. Fairweather, E. Leininger, D. Pickard, E. L. Hewlett, A. Robinson, C. Hayward, G. Dougan, and I. G. Charles. 1991. Construction and characterization of Bordetella pertussis mutants lacking the vir-regulated P.69 outer membrane protein. *Molecular Microbiology* 5 (6):1393-404.

Roberts, M., D. Maskell, P. Novotny, and G. Dougan. 1990. Construction and characterization in vivo of Bordetella pertussis aroA mutants. *Infection and Immunity* 58 (3):732-739.

Roduit, C., Paola Bozzotti, Nathalie Mielcarek, Paul-Henri Lambert, Giuseppe del Giudice, Camille Locht, and Claire-Anne Siegrist. 2002. Immunogenicity and protective efficacy of neonatal vaccination against Bordetella pertussis in a murine model: evidence for early control of pertussis. *Infection and Immunity* 70 (7):3521-8.

Rosenthal, R. S., W. Nogami, B. T. Cookson, W. E. Goldman, and W. J. Folkening. 1987. Major fragment of soluble peptidoglycan released from growing Bordetella pertussis is tracheal cytotoxin. *Infection and Immunity* 55 (9):2117-20.

Ross, P. J., Ed C. Lavelle, Kingston H. G. Mills, and Aoife P. Boyd. 2004. Adenylate cyclase toxin from Bordetella pertussis synergizes with lipopolysaccharide to promote innate interleukin-10 production and enhances the induction of Th2 and regulatory T cells. *Infection and Immunity* 72 (3):1568-79.

Rossetti, T. R. 1997. Cloning the aroD gene of Bordetella pertussis. Thesis, University of Southern Queensland., Toowoomba.

Rowe, J., Stephanie T. Yerkovich, Peter Richmond, Devinda Suriyaarachchi, Elizabeth Fisher, Leonie Feddema, Richard Loh, Peter D. Sly, and Patrick G. Holt. 2005. Th2-associated local reactions to the acellular diphtheria-tetanus-pertussis vaccine in 4- to 6-year-old children. *Infection and Immunity* 73 (12):8130-5.

Ryan, M., G. Murphy, E. Ryan, L. Nilsson, F. Shackley, L. Gothefors, K. Oymar, E. Miller, J. Storsaeter, and K. H. G. Mills. 1998. Distinct T-cell subtypes induced with whole cell and acellular pertussis vaccines in children. *Immunology* 93 (1):1-10.

Sambrook, J., and David W. Russell. 2001. *Molecular Cloning A Lboratory Manual*. 3 vols. Vol. 1. New York: Cold Spring Harbor Laboratory Press.

Sato, H., and Y. Sato. 1984. Bordetella pertussis infection in mice: correlation of specific antibodies against two antigens, pertussis toxin, and filamentous hemagglutinin with mouse protectivity in an intracerebral or aerosol challenge system. *Infection and Immunity* 46 (2):415-21.

Scarlato, V., B. Arico, A. Prugnola, and R. Rappuoli. 1991. Sequential activation and environmental regulation of virulence genes in Bordetella pertussis. *EMBO Journal* 10 (12):3971-5.

Sebo, P., P. Glaser, H. Sakamoto, and A. Ullmann. 1991. High-level synthesis of active adenylate cyclase toxin of Bordetella pertussis in a reconstructed Escherichia coli system. *Gene* 104 (1):19-24.

Shahin, R. D., M. J. Brennan, Z. M. Li, B. D. Meade, and C. R. Manclark. 1990. Characterization of the protective capacity and immunogenicity of the 69-kD outer membrane protein of Bordetella pertussis. *Journal of Experimental Medicine* 171 (1):63-73.

Sharma, S., T. K. S. Mukkur, Heather A. E. Benson, and Yan Chen. 2009. Pharmaceutical aspects of intranasal delivery of vaccines using particulate systems. *Journal of Pharmaceutical Sciences-US* 98 (3):812-43.

Simon, R. 1983. A Broad Host Range Mobilization System for In Vivo Genetic Engineering: Transposon Mutagenesis in Gram Negative Bacteria. *Bio/Technology* 1 (9):784-791.

Simondon, F., I. Iteman, M. P. Preziosi, A. Yam, and N. Guiso. 1998. Evaluation of an immunoglobulin G enzyme-linked immunosorbent assay for pertussis toxin and filamentous hemagglutinin in diagnosis of pertussis in Senegal. *Clinical and Diagnostic Laboratory Immunology* 5 (2):130-4.

Singh, M., and Krithika Lingappan. 2006. Whooping cough: the current scene. *Chest* 130 (5):1547-53.

Skerry, C. M., J. P. Cassidy, K. English, P. Feunou-Feunou, C. Locht, and B. P. Mahon. 2009. A Live Attenuated Bordetella pertussis Candidate Vaccine Does Not Cause Disseminating Infection in Gamma Interferon Receptor Knockout Mice. *Clinical and Vaccine Immunology* 16 (9):1344-1351.

Stainer, D. W., and M. J. Scholte. 1971. A simple chemically defined medium for the production of phase I Bordetella pertussis. *Journal of General Microbiology* 63 (2):211-20.

Steven, A. C., M. E. Bisher, B. L. Trus, D. Thomas, J. M. Zhang, and J. L. Cowell. 1986. Helical structure of Bordetella pertussis fimbriae. *Journal of Bacteriology* 167 (3):968-74.

Stibitz, S. 1994. Use of Conditionally Counterselectable Suicide Vectors for Allelic Exchange. *Methods in Enzymology* 235:458-465.

Storsaeter, J., H. O. Hallander, L. Gustafsson, and P. Olin. 1998. Levels of anti-pertussis antibodies related to protection after household exposure to Bordetella pertussis. *Vaccine* 16 (20):1907-16.

Tan, T., Scott Halperin, James D. Cherry, Kathryn Edwards, Janet A. Englund, Paul Glezen, David Greenberg, Edward Rothstein, and Danuta Skowronski. 2005. Pertussis immunization in the global pertussis initiative North American region: recommended strategies and implementation considerations. *Pediatric Infectious Disease Journal* 24 (5 Suppl):S83-6.

Tan, T., Evelinda Trindade, and Danuta Skowronski. 2005. Epidemiology of pertussis. *Pediatric Infectious Disease Journal* 24 (5 Suppl):S10-8.

Taranger, J., B. Trollfors, E. Bergfors, N. Knutsson, V. Sundh, T. Lagergard, L. Lind-Brandberg, G. Zackrisson, J. White, H. Cicirello, J. Fusco, and J. B. Robbins. 2001. Mass vaccination of children with pertussis toxoid--decreased incidence

in both vaccinated and nonvaccinated persons. *Clinical Infectious Diseases* 33 (7):1004-10.

Thomas, M. G., K. Redhead, and H. P. Lambert. 1989. Human serum antibody responses to Bordetella pertussis infection and pertussis vaccination. *Journal of Infectious Diseases* 159 (2):211-8.

Tritto, E., Flaviana Mosca, and Ennio De Gregorio. 2009. Mechanism of action of licensed vaccine adjuvants. *Vaccine* 27 (25-26):3331-3334.

Trollfors, B., J. Taranger, T. Lagergard, L. Lind, V. Sundh, G. Zackrisson, C. U. Lowe, W. Blackwelder, and J. B. Robbins. 1995. A placebo-controlled trial of a pertussis-toxoid vaccine. *New England Journal of Medicine* 333 (16):1045-50.

Trollfors, B., J. Taranger, T. Lagergard, V. Sundh, D. A. Bryla, R. Schneerson, and J. B. Robbins. 1999. Serum IgG antibody responses to pertussis toxin and filamentous hemagglutinin in nonvaccinated and vaccinated children and adults with pertussis. *Clinical Infectious Diseases* 28 (3):552-9.

Ulanova, M., A. Tarkowski, M. Hahn-Zoric, and L. A. Hanson. 2001. The common vaccine adjuvant aluminum hydroxide up-regulates accessory properties of human monocytes via an interleukin-4-dependent mechanism. *Infection and Immunity* 69 (2):1151-1159.

Urisu, A., J. L. Cowell, and C. R. Manclark. 1985. Involvement of filamentous hemagglutinin in the adherence of Bordetella pertussis to human WiDr cell cultures. *Developments in Biological Standardization* 61:205-14.

Van den Berg, B. M., H. Beekhuizen, F. R. Mooi, and R. van Furth. 1999. Role of antibodies against Bordetella pertussis virulence factors in adherence of Bordetella pertussis and Bordetella parapertussis to human bronchial epithelial cells. *Infection and Immunity* 67 (3):1050-5.

Verma, N. K., and Alf A. Lindberg. 1991. Construction of aromatic dependent Shigella flexneri 2a live vaccine candidate strains: deletion mutations in the aroA and the aroD genes. *Vaccine* 9 (1):6-9.

Von Konig, C. H. Wirsing, S. Halperin, M. Riffelmann, and N. Guiso. 2002. Pertussis of adults and infants. *Lancet Infectious Diseases* 2 (12):744-750.

Walker, K. E., and A. A. Weiss. 1994. Characterization of the dermonecrotic toxin in members of the genus Bordetella. *Infection and Immunity* 62 (9):3817-28.

Watanabe, M., H. Takimoto, Y. Kumazawa, and K. Amano. 1990. Biological properties of lipopolysaccharides from Bordetella species. *Journal of General Microbiology* 136 (3):489-93.

Wearing, H. J., and P. Rohani. 2009. Estimating the Duration of Pertussis Immunity Using Epidemiological Signatures. *PLoS Pathogens* 5 (10).

Weiss, A. A., and M. S. Goodwin. 1989. Lethal infection by Bordetella pertussis mutants in the infant mouse model. *Infection and Immunity* 57 (12):3757-64.

Weiss, A. A., and E. L. Hewlett. 1986. Virulence factors of Bordetella pertussis. *Annual Review of Microbiology* 40:661-86.

Weiss, A. A., E. L. Hewlett, G. A. Myers, and S. Falkow. 1983. Tn5-induced mutations affecting virulence factors of Bordetella pertussis. *Infection and Immunity* 42 (1):33-41.

Weiss, A. A., E. L. Hewlett, G. A. Myers, and S. Falkow. 1984. Pertussis toxin and extracytoplasmic adenylate cyclase as virulence factors of Bordetella pertussis. *Journal of Infectious Diseases* 150 (2):219-222.

Wendelboe, A. M., Elisabeth Njamkepo, Antoine Bourillon, D. Daniel Floret, Joel Gaudelus, Michael Gerber, Emmanuel Grimprel, David Greenberg, Scott Halperin, Johannes Liese, Flor Munoz-Rivas, Remy Teyssou, Nicole Guiso, Annelies Van Rie, and Group Infant Pertussis Study. 2007. Transmission of Bordetella pertussis to young infants. *Pediatric Infectious Disease Journal* 26 (4):293-9.

Wendelboe, A. M., Annelies Van Rie, Stefania Salmaso, and Janet A. Englund. 2005. Duration of immunity against pertussis after natural infection or vaccination. *Pediatric Infectious Disease Journal* 24 (5 Suppl):S58-61.

Willems, R. J., H. G. van der Heide, and F. R. Mooi. 1992. Characterization of a Bordetella pertussis fimbrial gene cluster which is located directly downstream of the filamentous haemagglutinin gene. *Molecular Microbiology* 6 (18):2661-71.

Wolff, J., G. H. Cook, A. R. Goldhammer, and S. A. Berkowitz. 1980. Calmodulin activates prokaryotic adenylate cyclase. *Proceedings of the National Academy of Sciences of the United States of America* 77 (7):3841-4.

World Health Organization. 2007. Recomemendation for whole-cell pertussis vaccine. In *(WHO Technical Report Series No 941, Annex 6) : 301-333.*

Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* 33 (1):103-19.

Zepp, F., M. Knuf, P. Habermehl, J. H. Schmitt, C. Rebsch, P. Schmidtke, R. Clemens, and M. Slaoui. 1996. Pertussis-specific cell-mediated immunity in infants after vaccination with a tricomponent acellular pertussis vaccine. *Infection and Immunity* 64 (10):4078-84.

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Appendix A: Media

Stainer-Scholte Medium (SS) without supplement: 200ml

$CaCl_2$	2mg
KCl	40mg
KH_2PO_4	100mg
$MgCl_2$	20mg
NaCl	500mg
Tris base	304mg
L-Glutamic acid monosodium salt	2142mg
L-Proline	48mg
$d.H_2O$	194ml

The pH of medium adjusted to 7.5 with 1N HCl; the medium aliquoted in 10mls in McCartney bottles and was autoclaved. Upon cooling the bottles' cap sealed with paraffin and were stored at -20^0C for up to six months.

Stock Solution: 10ml

Ascorbic acid	20mg
Nicotinic acid	4mg
Glutathione	100mg
$FeSO_4.H_2O$	10mg
L-cysteine	40mg
$d.H_2O$	10ml

The solution dissolved on stirring plate and was filter sterilised (FS) by 0.2μm filter. The stock stored at -20^0C.

Cyclodextrin Stock (1mg/ml): 10ml

10mg of Cyclodextrin dissolved in 10ml distilled water and filtered sterilised and was stored at -20^{0}C.

To make SS medium, SS without supplement, and the stocks (above) thawed and 100µl of the stock solution and 200µl of Cyclodextrin stock were added to SS medium.

Cyclodextrin Liquid (CL) Medium without supplement: 200ml

$CaCl_2$	4mg
KCl	40mg
Na_2HPO4 (Dibasic sodium phosphate)	100mg
$MgCl_2$	20mg
NaCl	500mg
Tris base	1220mg
L-Glutamic acid monosodium salt	2140mg
L-Proline	48mg
Casamino acids	2000mg
Cyclodextrin	50mg
d.H_2O	182ml

The pH of medium adjusted to 7.5 with 1M HCl; the medium aliquoted in 10mls in McCartney bottles and was autoclaved. Upon cooling the bottles' cap sealed with paraffin and were stored at -20^{0}C for up to six months.

To make CL, the stock medium without supplement and stock solution (same stock solution as for the SS medium) thawed and 200µl of stock solution was added to CL without supplement.

Bordet Gengou (BG) agar:

Bordet Gengou agar was made according to manufacturer's instruction except in that the medium contained 15% (v/v) sheep blood instead of 12% (v/v). The medium was supplemented with aromix and antibiotic(s) accordingly (see Section 2.1.2).

Cohen Wheeler (CW) agar: 1L

Casamino acid	10g
NaCl	2.5g
Na_2HPO_4 (Dibasic sodium phosphate)	0.50g
$MgCl_2.6H_2O$	0.40g
Starch	1.50g
$CaCl_2$ 1% (see blow)	1ml
$FeSO_4.5H_2O$ 0.5% (see below)	2ml
$CuSO_4.5H_2O$ 0.5% (see below)	1ml
L-Cysteine 1% (see below)	2.5ml
Yeast extract	5
Bacteriological agar	10
d.H_2O	800ml

The PH of the medium adjusted to 7.3 with 1M NaOH and the ingredient dissolved on hot stirring plate. The medium aliquoted into 170mls in McCartney bottles and was autoclaved. Cap of the bottles containing the agar were sealed with paraffin and stored at 4^0 C degree. When needed the medium melted in steam boiler and upon cooling to 50-55^0 C, supplemented with 15% (v/v) blood, aromix, and antibiotic(s) accordingly (Section 2.1.2.)

1% $CaCl_2$: 10ml

0.10g of $CaCl_2$ dissolved in 10ml d.H_2O filter sterilised with 0.2μm filter and was stored at 4^0C.

0.5% $FeSO_4.7H_2O$ (Ferrous sulfate): 100ml

0.50g of $FeSO_4.7H_2O$ dissolved in 100ml $d.H_2O$ filter sterilised with 0.2µm filter and was stored at 4^0 C.

0.05% copper sulfate ($CuSO_4.5H_2O$): 100ml

0.05g of $CuSO_4.5H_2O$ dissolved in 100ml $d.H_2O$ filter sterilised with 0.2µm filter and was stored at 4^0 C.

1% L-Cysteine: 10ml

0.10g of L-Cysteine dissolved in 10ml $d.H_2O$ filter sterilised with 0.2µm filter and was stored at 4^0 C.

Luria Bertani (LB) 1% Agar: 1L

Trypton	10g
Yeast extract	5.0g
Sodium chloride	10g
Bacteriological agar	10g
$d.H2O_2$	1000ml

The medium kept on stirring hot plate and brought to boiling point. The medium cooled down to 60-50^0C and aliquoted into 200ml or 100ml in McCartney bottles; the medium were autoclaved. Upon solidification of medium the bottles' cap sealed with paraffin and stored at 4^0C. When needed stock medium melted in steam boiler and antibiotic(s) was added to the medium at temperature between 45-50^0C. The medium was poured into petri dishes, 12-15ml per petri dish.

LB broth:

Composition of LB broth was as same as LB agar except it did not contain agar.

LB agar-(Amp/IPTG/X-Gal): 200ml

LB agar supplemented with 0.5mM Isopropyl Thiogalactoside (IPTG) and 80µg/ml 5-bromo-4-chloro-3-indolyl-β-D-glactopyranoside (X-Gal)

IPTG 0.1M	1ml
X-Gal [50mg/ml]	320µl

The reagents were added to LB medium at temperature about 50^0 C to 200ml final volume.

SOB Medium: 200ml

Trypan	4g
Yeast extract (auto lysed)	1g
Sodium chloride	0.1g
d.H_2O	190ml

The ingredient dissolved on stirring plate and 1ml of 250mM solution of KCl was added to the broth. The pH adjusted to 7.0 with 1M NaOH. The volume made up to 200ml with distilled water. The broth was aliquoted into 5mls in McCartney bottles and autoclaved. Upon cooling the bottles' cap were sealed with paraffin film and stored at 4^0C.

SOC Medium: 5ml

SOC is identical to SOB except contains 20mM glucose and 50mM $MgCl_2$.
When SOC medium 100µl of 1M glucose (FS) and 25µl 2M $MgCl_2$ were added to 5ml SOB stock at room temperature.

Appendix B: Aromix stock solutions

Phenylalanine	10mg/ml in d.H_2O
Tyrosine	5mg/ml in 50:50 in d.H_2O:1M NaOH
Tryptophan	4mg/ml in d.H_2O
Para-aminobenzoic acid	4mg/ml in d.H_2O
Dihydroxybenzoic acid	4mg/ml in d.H_2O
Para-hydroxybenzoic acid	4mg/ml in d.H_2O

The ingredients were dissolved on hot stirring plate with additional of about 200µl of 1M NaOH to all solutions except for tyrosine. Upon cooling 500µl chloroform added to each stock and stored at 4^0C.

Appendix C: Buffers and Reagents

10X PBS, 0.1M, pH 7.2: 1L

Na2HPO4	10.9g
NaH2PO4	3.2g
NaCl	90g
d.H_2O	800ml

The solution left on stirring plate until the solutes dissolved; PH adjusted to 7.2 and volume was made up to 1L with distilled water. 1%PBS prepared in distilled water when needed from the stock and autoclaved.

50X TAE (Tris-acetate-EDTA) buffer: 1L

Tris bas	242g
Glacial acetic acid	57.1ml
EDTA (0.5M) (see below)	100ml

The volume made to 1L with d.H_2O. 1X TAE was made from the stock by adding 20ml of 50X TAE stock to 980ml d.H_2O.

0.5M EDTA (Ethylenediamine tetraacetic acid): 500ml

Disodium ethylene diamine tetra-acetate.2H_2O	93.5g
d.H_2O	400ml

The solution mixed on stirring plate and pH was adjusted to 8.0 with about 10g of NaOH pellets. The volume of buffer adjusted to 500ml with d.H_2O and was autoclaved.

250mM KCl: 100ml

1.86g of KCl dissolved in 10ml d.H_2O autoclaved. and was stored at 4^0C.

1M Glucose: 10ml

1802mg of D-glucose dissolved in 10ml d.H_2O filter sterilised with 0.2μm filter and was stored at 4^0C.

2M $MgCl_2$: 50ml

9.52g of $MgCl_2$ dissolved in 10ml d.H_2O autoclaved and was stored at 4^0C.

0.1M $CaCl_2$: 100ml

1110mg of $CaCl_2$ dissolved in 10ml d.H_2O filter sterilised with 0.2μm filter and was stored at 4^0C.

80mM $MgCl_2$, 20mM $CaCl_2$: 100ml

762mg of $MgCl_2$ dissolved in 100ml d.H_2O and 22mg of $CaCl_2$ added to the solvent on stirring plate. The solution filter sterilised with 0.4μm filter and was stored at 4^0C.

Appendix D: Optical Density measurement of *aroQ*BP in SS-(Km-50µg/ml, Sm-200µg/ml) medium at different time intervals for *aroQ*BP- *E coli* SM10 *λ pir* harbouring pJQ200mp 18-rspLCyaMut conjugation

Growth of *aroQ*BP in SS-(Km-50µg/ml, Sm-200µg/ml) medium until the growth curve approximately reached log phase.

Time interval in hour	OD_{600nm}
0	0.020
47	0.188
48	0.190
49	0.220
50	0.227
52	0.321
53	0.352
55	0.373
57	0.415
59	0.460
60	0.520

Appendix E: Optical Density measurement of *E coli* SM10 λ *pir* harbouring pJQ200mp 18-rspLCyaMut growth in SS -(Gm10µg/ml, Km 50µg/ml) and SM10 λ *pir* harbouring pJQ200mp 18-rspLCyaMut growth in SS -(Amp100 µg/ml Gm10µg/ml, Km 50µg/ml) broth

E coli SM10 λ *pir* harbouring pJQ200mp 18-rspLCyaMut growth in 10ml SS -(Gm10µg/ml, Km 50µg/ml) SM10 λ *pir* harbouring pJQ200mp 18-rspLCyaMut growth in SS -(Amp100 µg/ml Gm10µg/ml, Km 50µg/ml) broth until the growth reaches log phase.

Time interval in Minute	OD_{600nm}
0	0.030
180	0.084
210	0.114
235	0.186
300	0.330
310	0.382
320	0.416
330	0.488
*340	0.50
380	0.440
385	0.457
390	0.492

*At 340minutes about 3ml SS medium was added to the growth broth.

Appendix F: Optical Density measurement of *aroQ*BP in CL-(Km50µg/ml, Sm200µg/ml) medium at different time intervals for *aroQ*BP- *E coli* SM10 *λ pir* harbouring pSS1129CyaMut conjugation

Growth of *aroQ*BP in CL-(Km50µg/ml, Sm200µg/ml) broth until the growth of bacterium got to log phase.

Time interval in hour	OD_{600nm}
0	0.067
38	0.106
39	0.122
41.5	0.182
42.5	0.208
44.5	0.308
45.5	0.333
46.5	0.363
47	0.385
47.5	0.405
48	0.417
49	0.460
50	0.497

Appendix G: ELISA Reagents

Carbonate bicarbonate Coating Buffer (CB): 1L

Na_2CO_3	1.59g
$NaHCO_3$	2.94g
d.H_2O	900ml

PH adjusted to 9.6 with 1M NaOH and volume was made to 1L. The buffer was stored at 4^0C.

Blocking Buffer (BB) stock: 1L

Tris base	1.21g
NaCl	8.50g
Tween-20	0.50ml
1%PBS	999.5ml

The stock stored at 4^0C. BB with 5% skimmed milk prepared fresh by dissolving 1g of skimmed milk into 20ml BB stock by sprinkling so no clomps were formed.

Diethanolamine Buffer (Substrate Buffer): 1L

Diethanolamine	106g
$MgSO_4$ .7H_2O	0.249g
d.H_2O	800ml

PH adjusted to 10 and the volume made up to 1L with d.H_2O. The stock stored at 4^0C.

Appendix H: Dulbecco's Modified Eagle Medium (CDMEM): 100ml

HEPES (1M)	0.25ml
2 mercaptoethanol	0.90ml
Polymixin B	1.00ml
Penicillin/streptomycin/glutamase	2.00ml
DMEM	96.66ml

For culture Fetal Calf Serum (FBS) was added to a concentration of 10%.

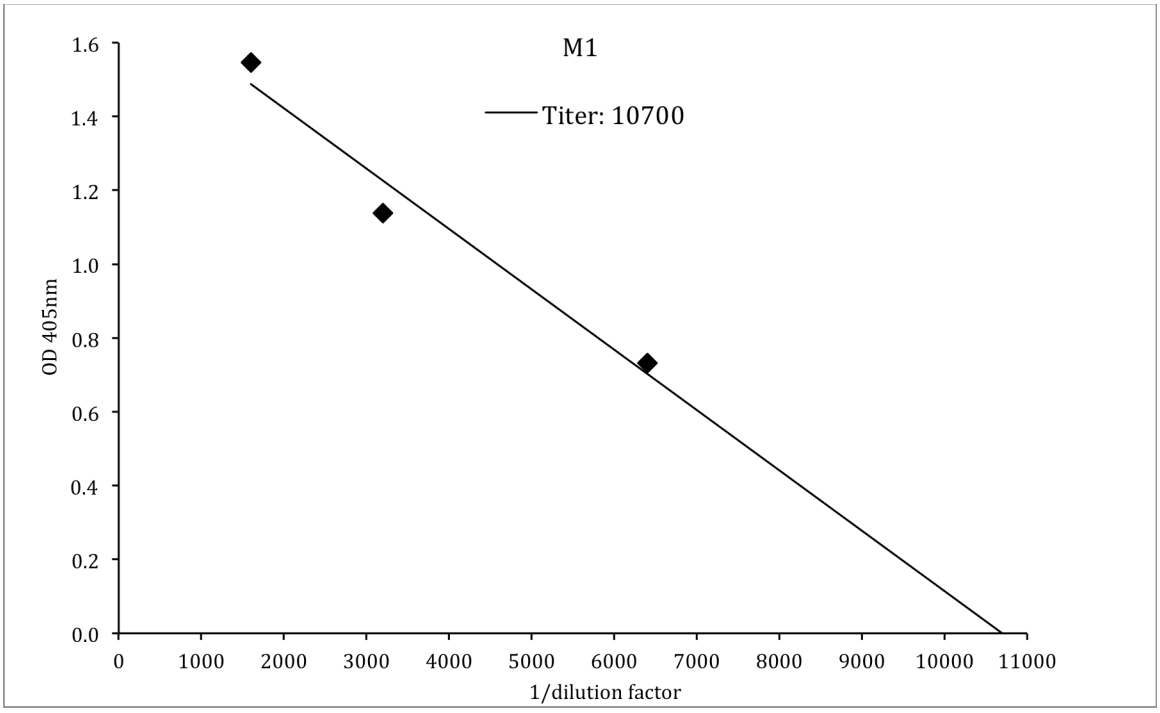
Appendix I: Serum antibody isotypes against inactivated *aroQ*BP

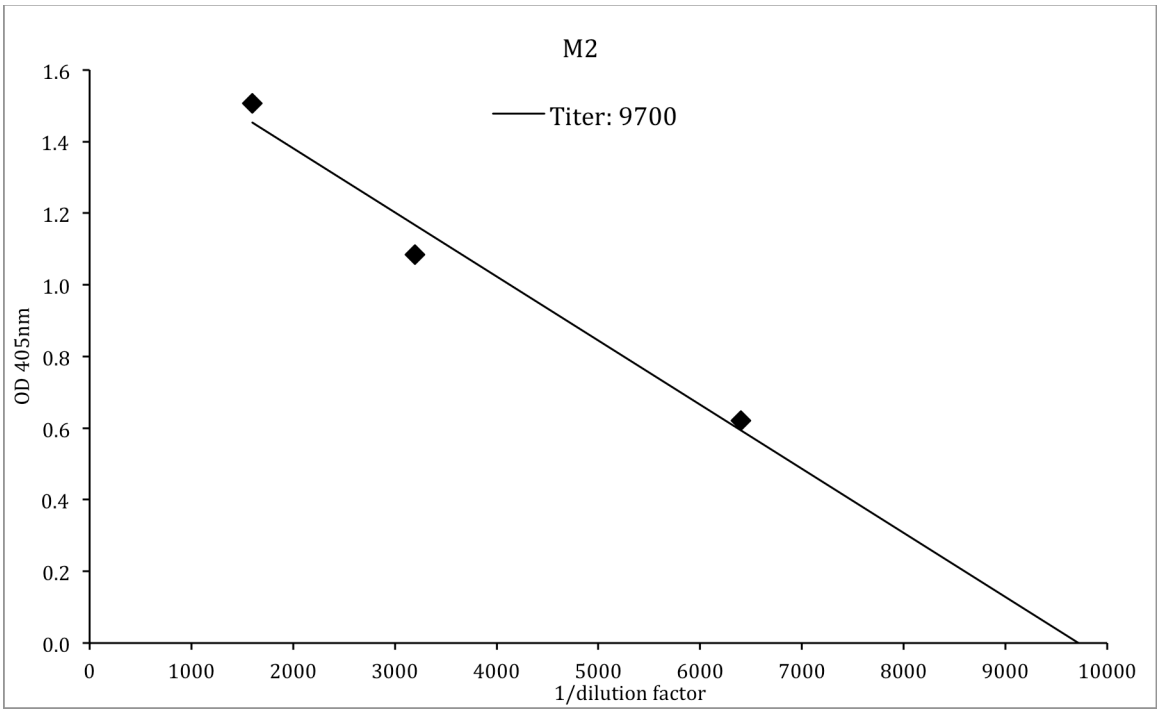
Antigen-specific immune response of mice vaccinated with three doses of DTaP and two booster doses of live *aroQ*BP.

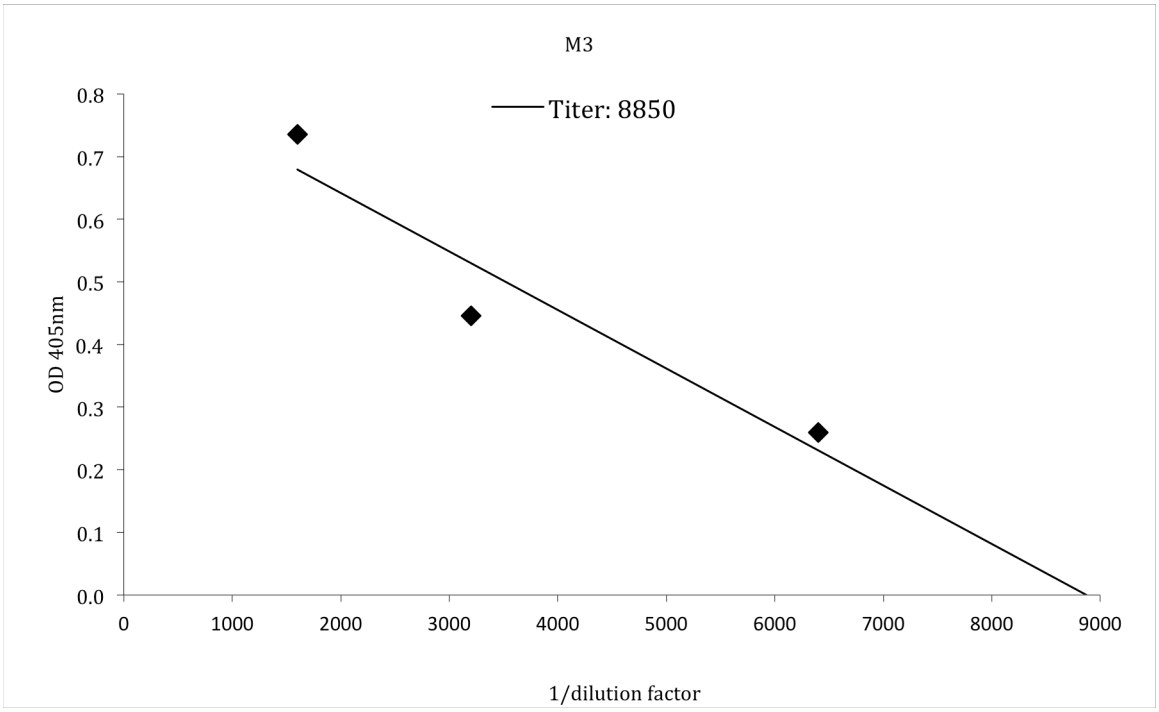
PBS sham-vaccinated Group: Titers for the PBS control groups were zero.

IgG titers

Mice Number	Serum IgG titers
M1	10700
M2	9700
M3	8850
M4	8800
M5	9350




M2
Titer: 9700
OD 405nm
1/dilution factor
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
0
1000
2000
3000
4000
5000
6000
7000
8000
9000
10000


M3
Titer: 8850
OD 405nm
1/dilution factor
0.0
0.1
0.2
0.3
0.4
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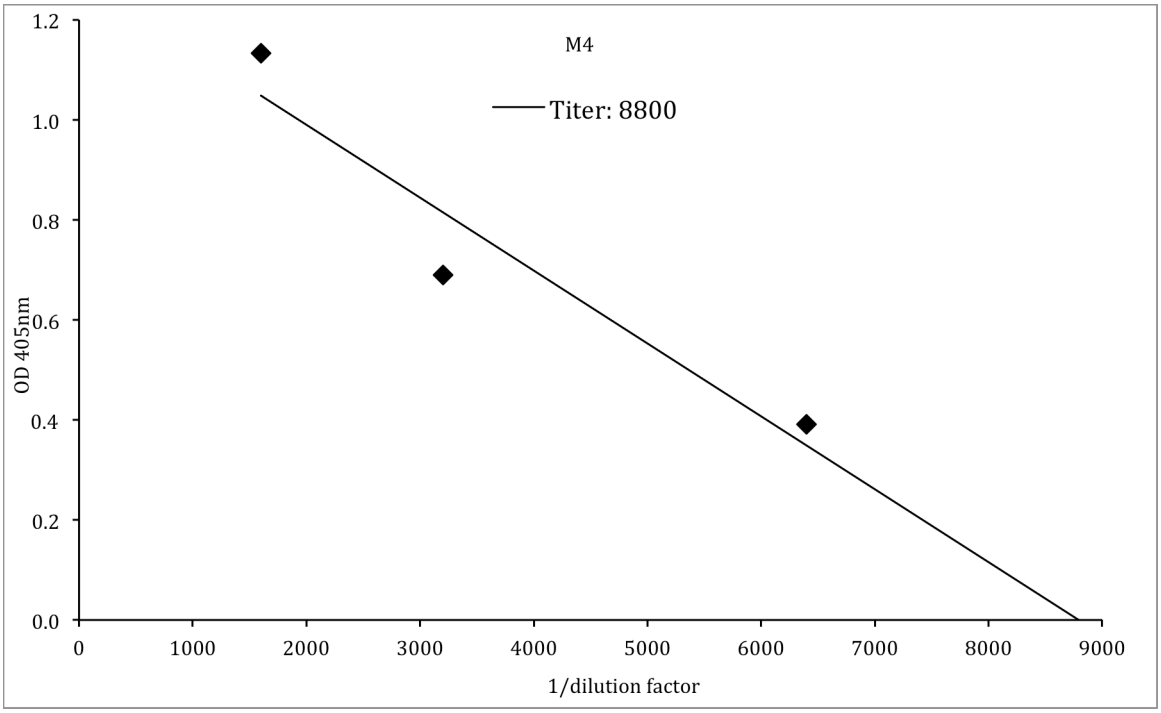
0.6
0.7
0.8
0
1000
2000
3000
4000
5000
6000
7000
8000
9000


M4
Titer: 8800
OD 405nm
1/dilution factor
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0.2
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0.8
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1.2
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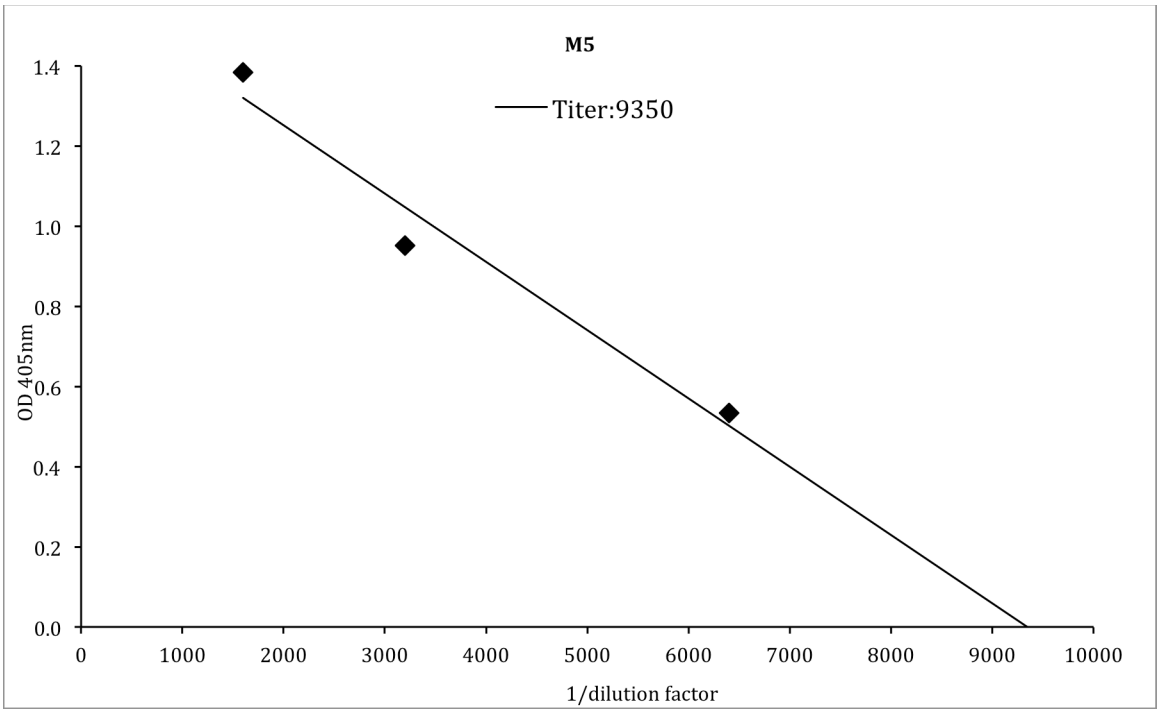
4000
5000
6000
7000
8000
9000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Titer:9350
OD 405nm
1/dilution factor
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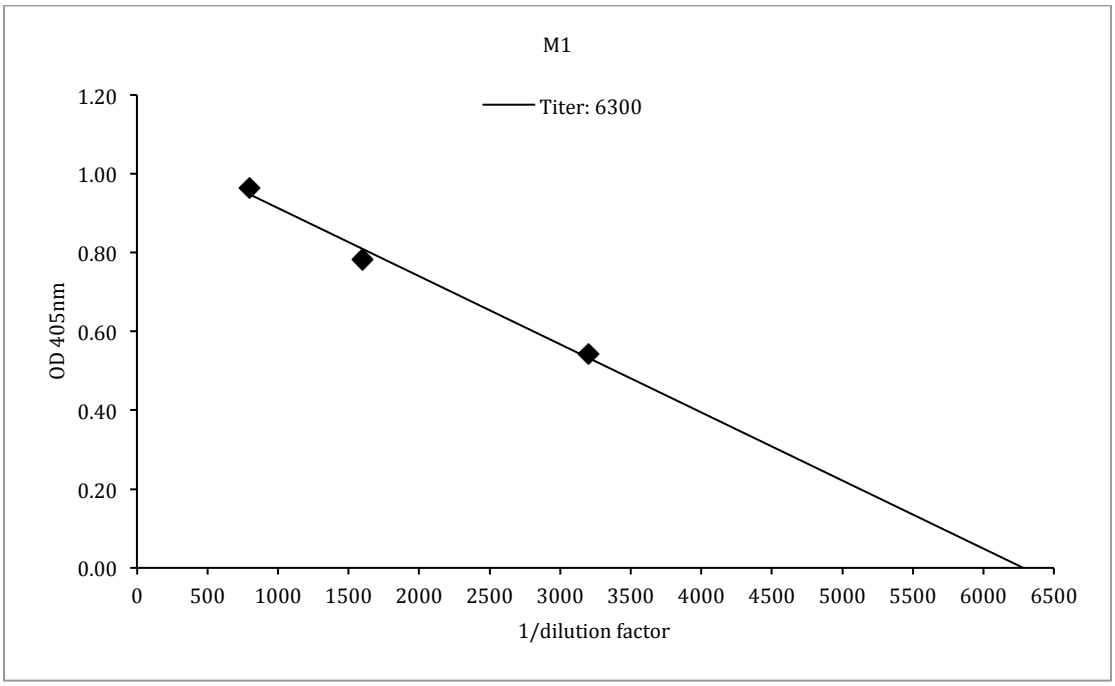
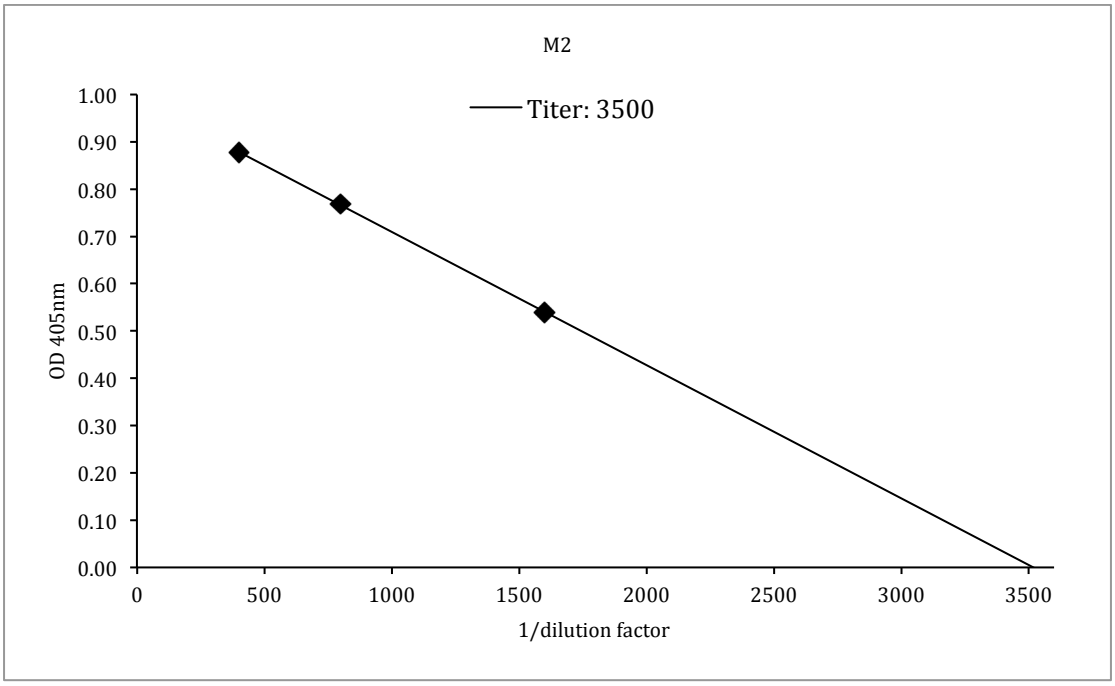
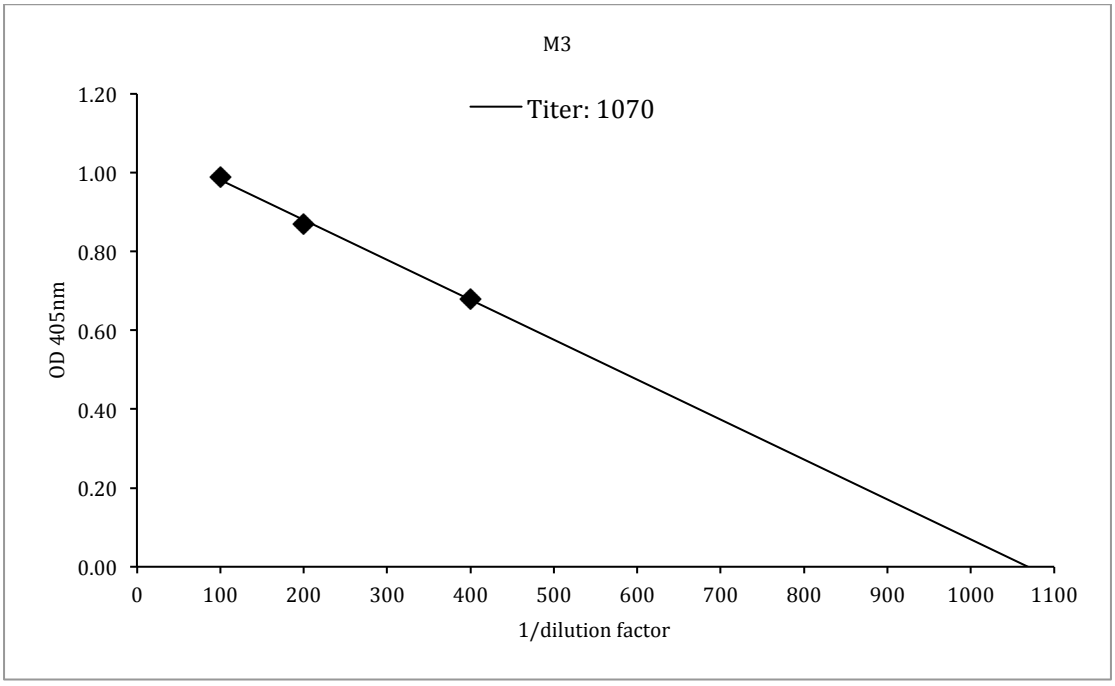
0.2
0.4
0.6
0.8
1.0
1.2
1.4
0
1000
2000
3000
4000
5000
6000
7000
8000
9000
10000

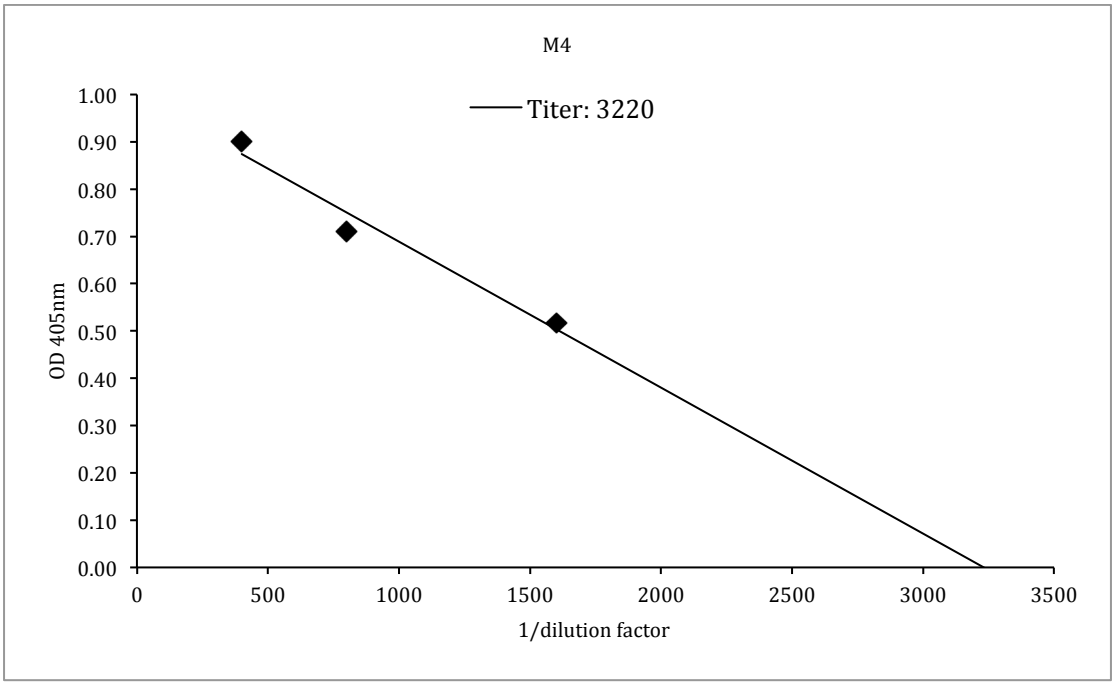
IgG1 titers

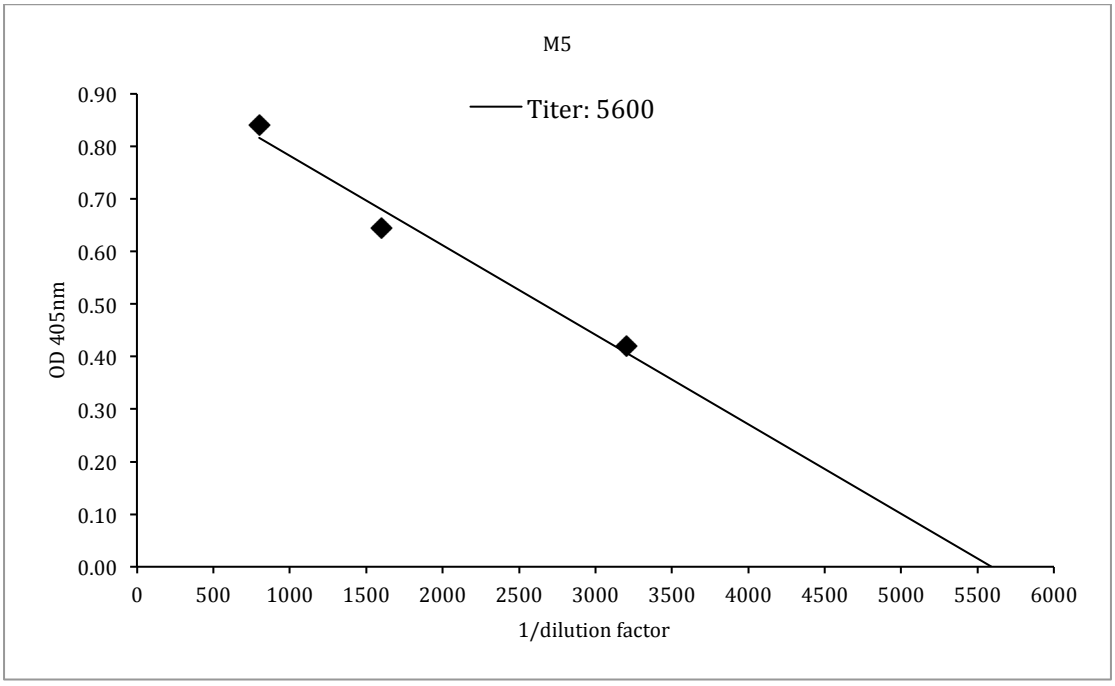
Mice Number	Serum IgG1 titers
M1	6300
M2	3500
M3	1070
M4	3220
M5	5600




M2
Titer: 3500
OD 405nm
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
500
1000
1500
2000
2500
3000
3500
1/dilution factor

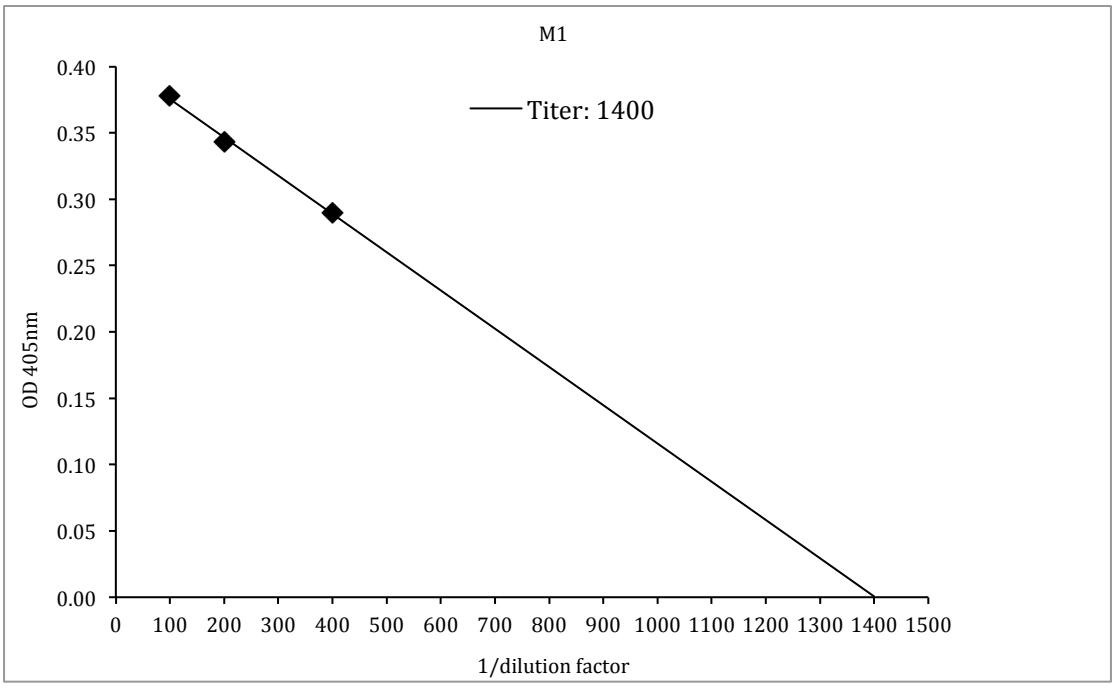

M3
Titer: 1070
OD 405nm
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0
100
200
300
400
500
600
700
800
900
1000
1100
1/dilution factor

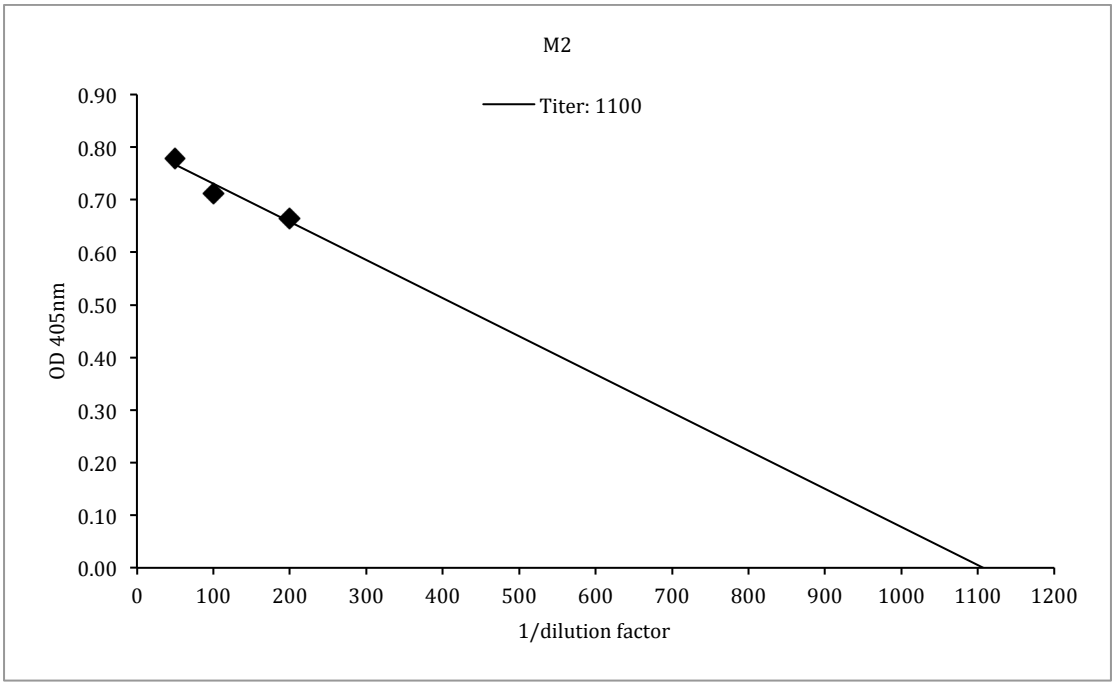

M4
Titer: 3220
OD 405nm
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
500
1000
1500
2000
2500
3000
3500
1/dilution factor

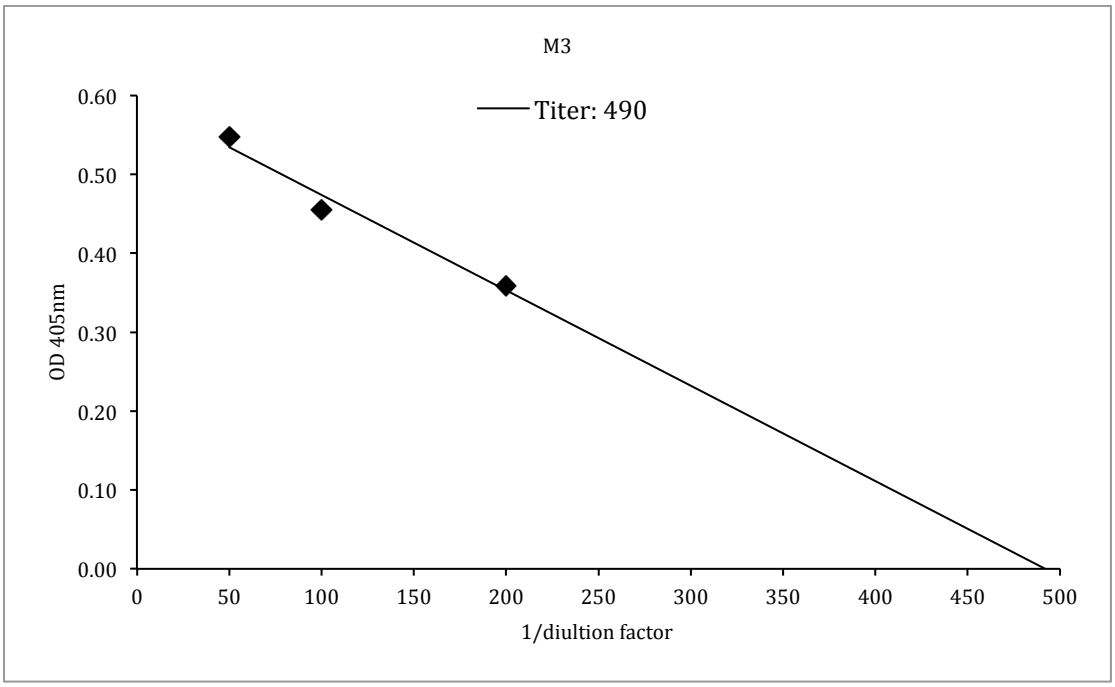

M5
Titer: 5600
OD 405nm
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
5500
6000
1/dilution factor

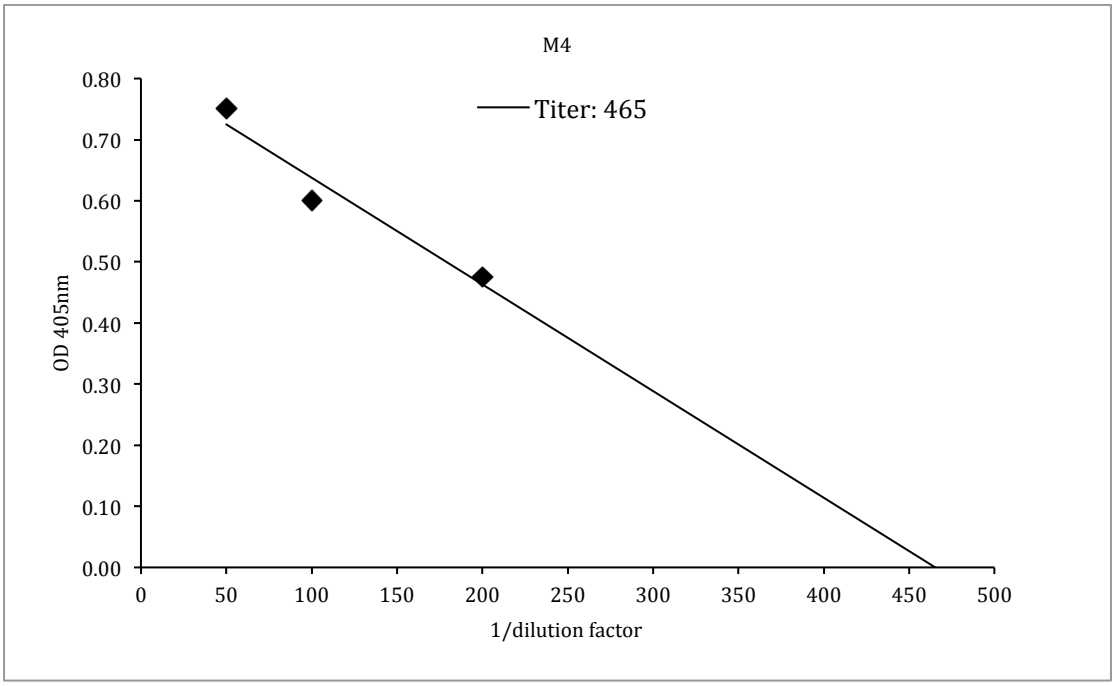
IgG2a titers

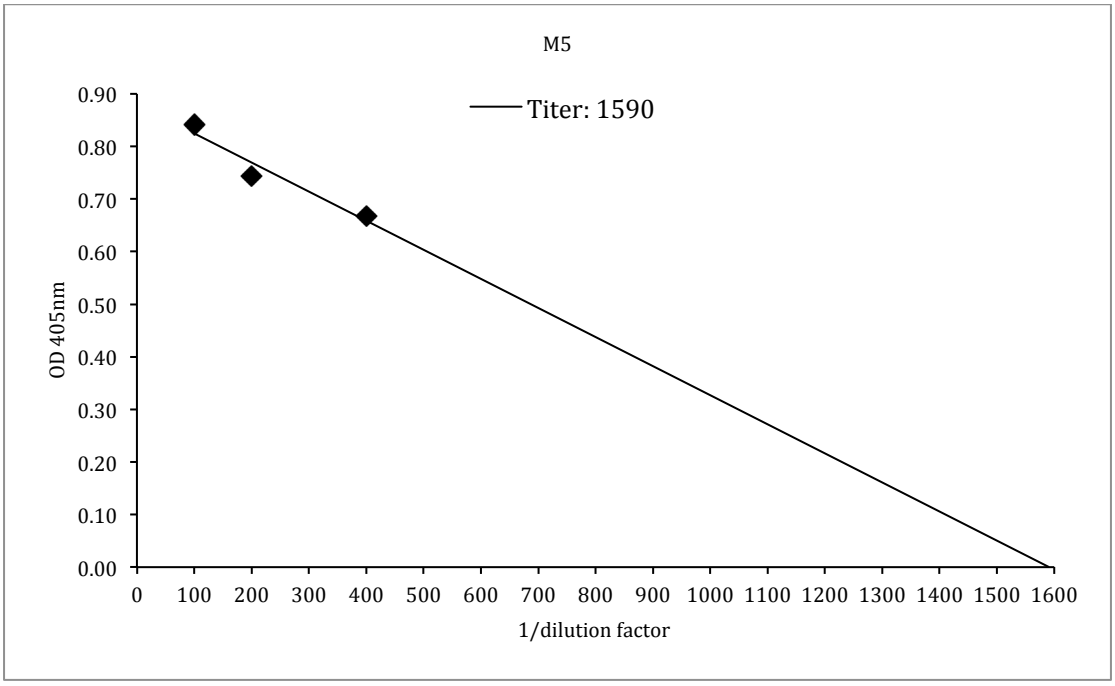
Mice Number	Serum IgG2a titers
M1	1400
M2	1100
M3	490
M4	465
M5	1590




M2
Titer: 1100
OD 405nm
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1/dilution factor


M3
Titer: 490
OD 405nm
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
50
100
150
200
250
300
350
400
450
500
1/diultion factor


M4
Titer: 465
OD 405nm
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
50
100
150
200
250
300
350
400
450
500
1/dilution factor


M5
Titer: 1590
OD 405nm
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
1600
1/dilution factor

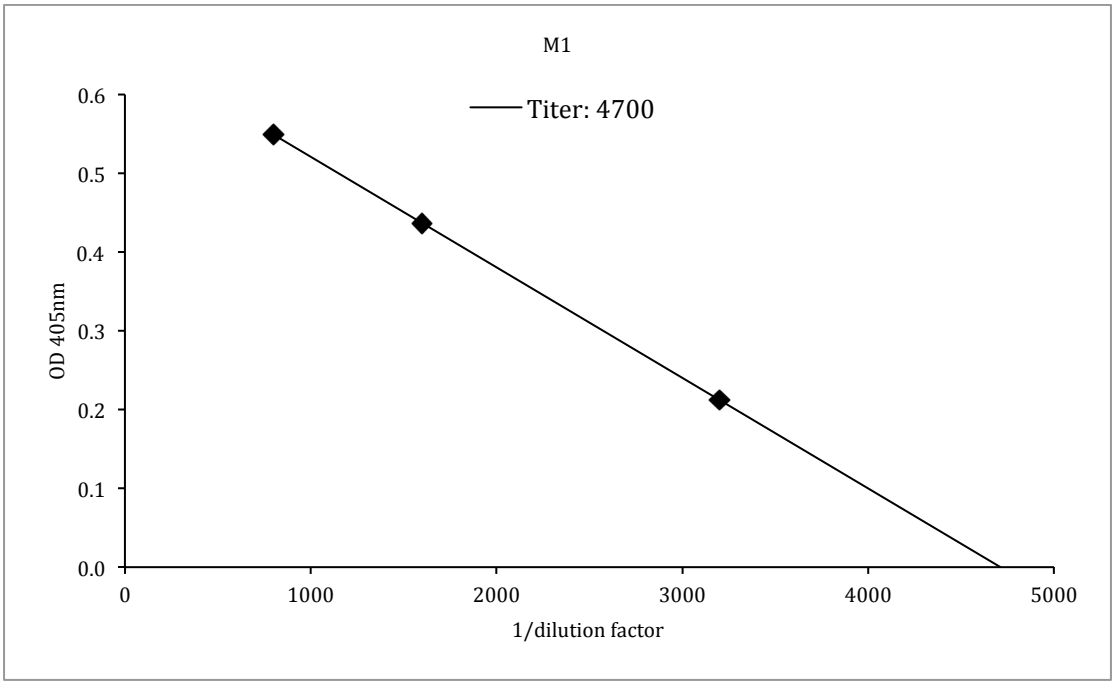
IgA [Single point titers]

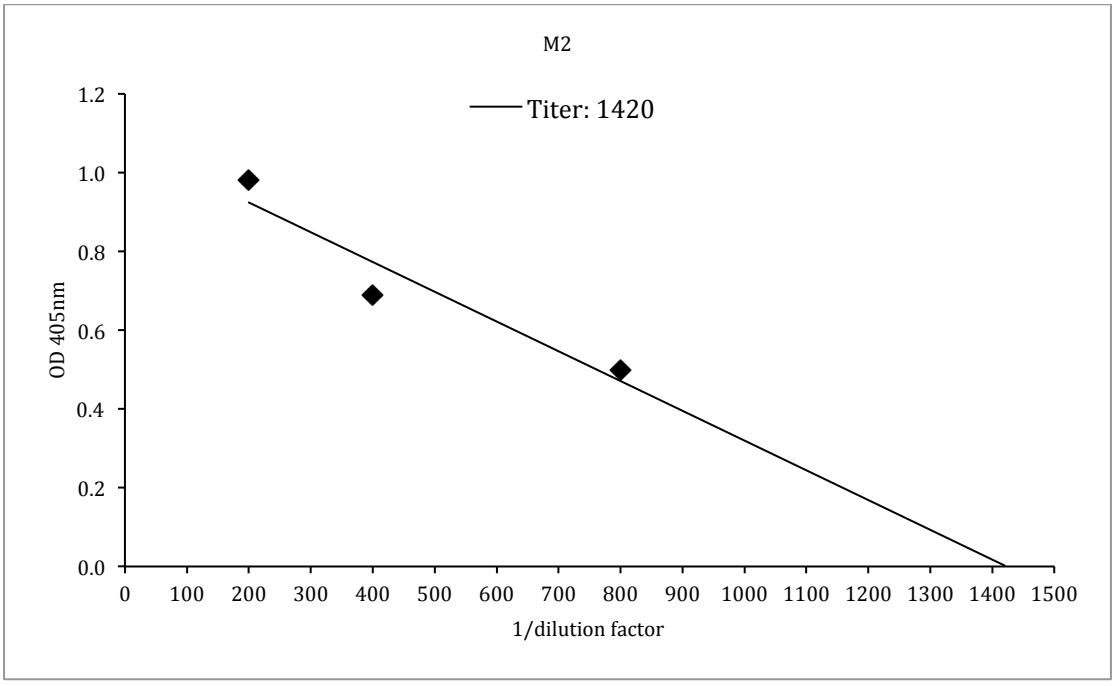
Mice Number	Serum IgA titers
M1	1.3
M2	1.5
M3	1.2
M4	1.1
M5	1

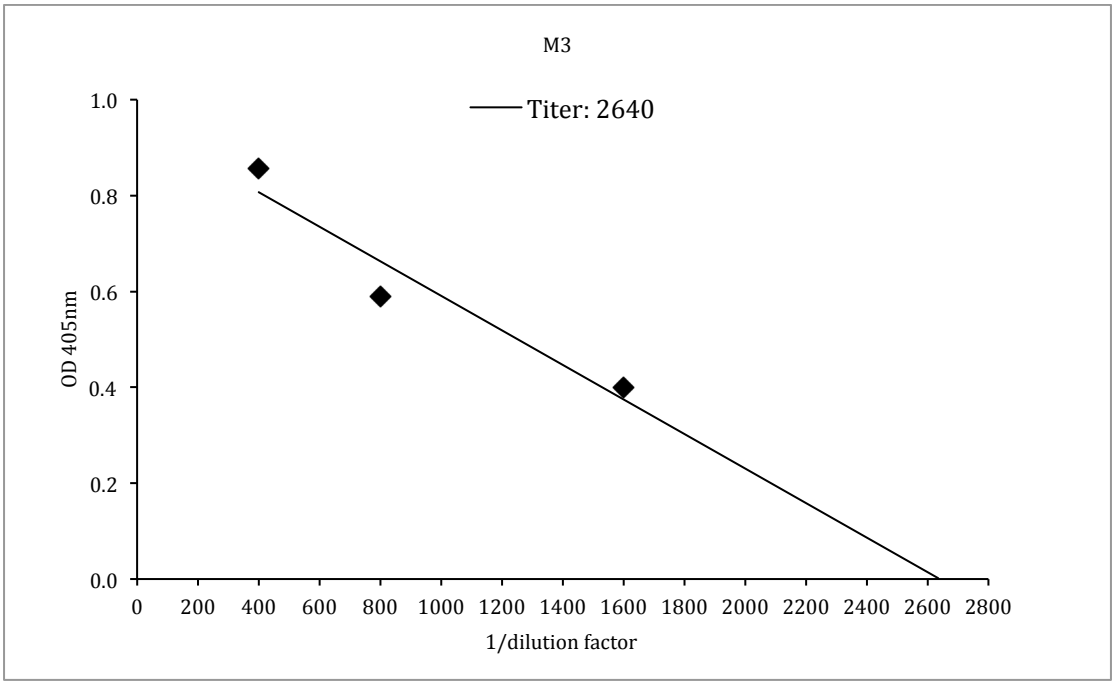
Antigen-specific immune response of mice vaccinated with three doses of DTaP and one booster dose of live *aroQ*BP.

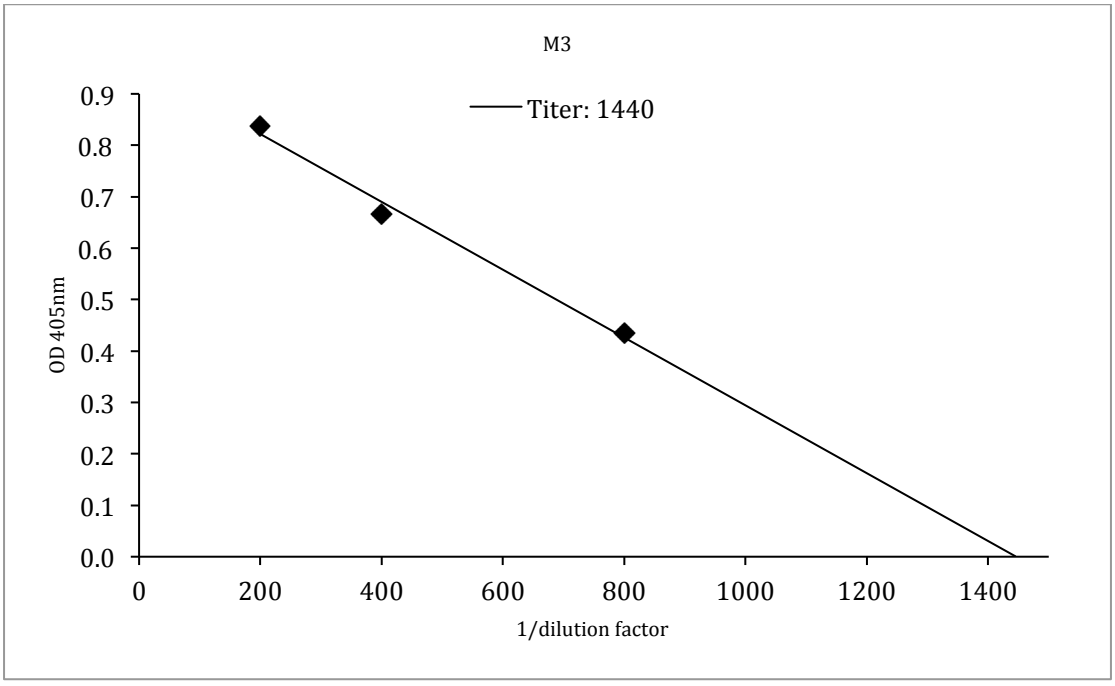
IgG titers

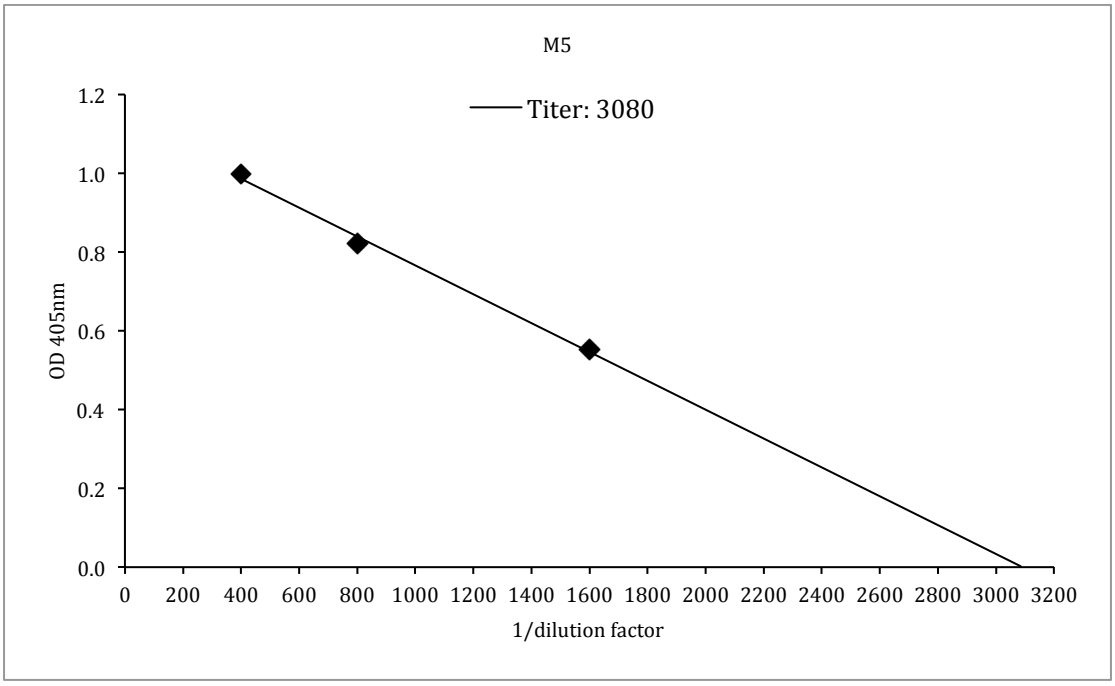
Mice Number	Serum IgG titers
M1	4700
M2	1420
M3	2640
M4	1440
M5	3080


M1
Titer: 4700
OD 405nm
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
1000
2000
3000
4000
5000
1/dilution factor


M2
Titer: 1420
OD 405nm
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
1/dilution factor

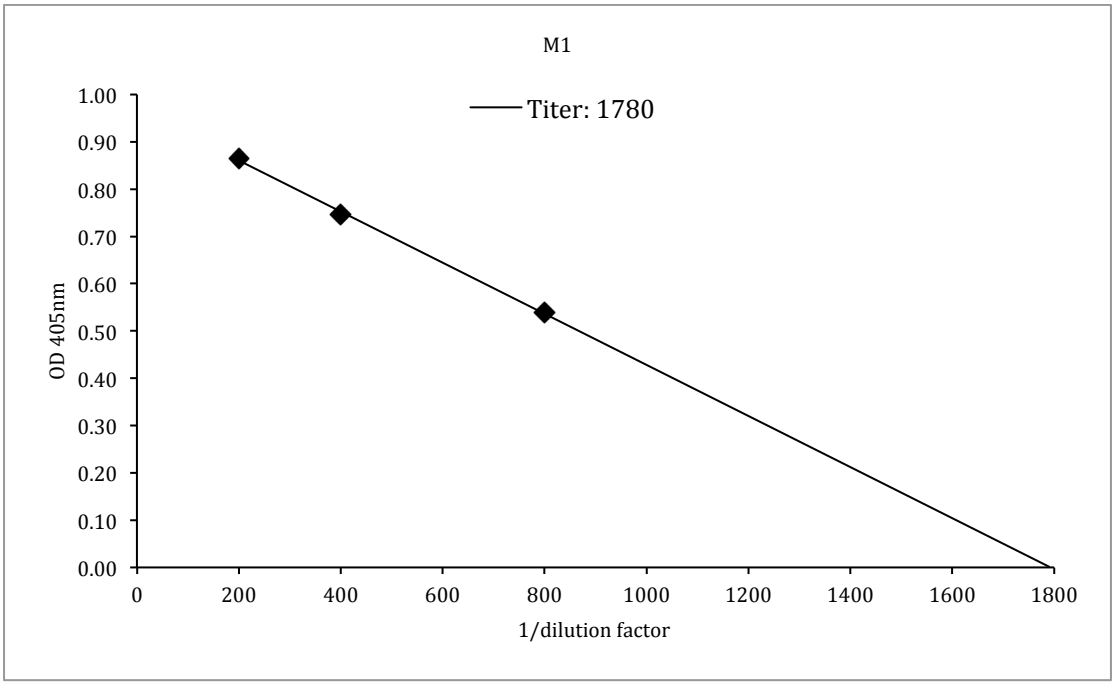

M3
Titer: 2640
OD 405nm
1.0
0.8
0.6
0.4
0.2
0.0
0
200
400
600
800
1000
1200
1400
1600
1800
2000
2200
2400
2600
2800
1/dilution factor

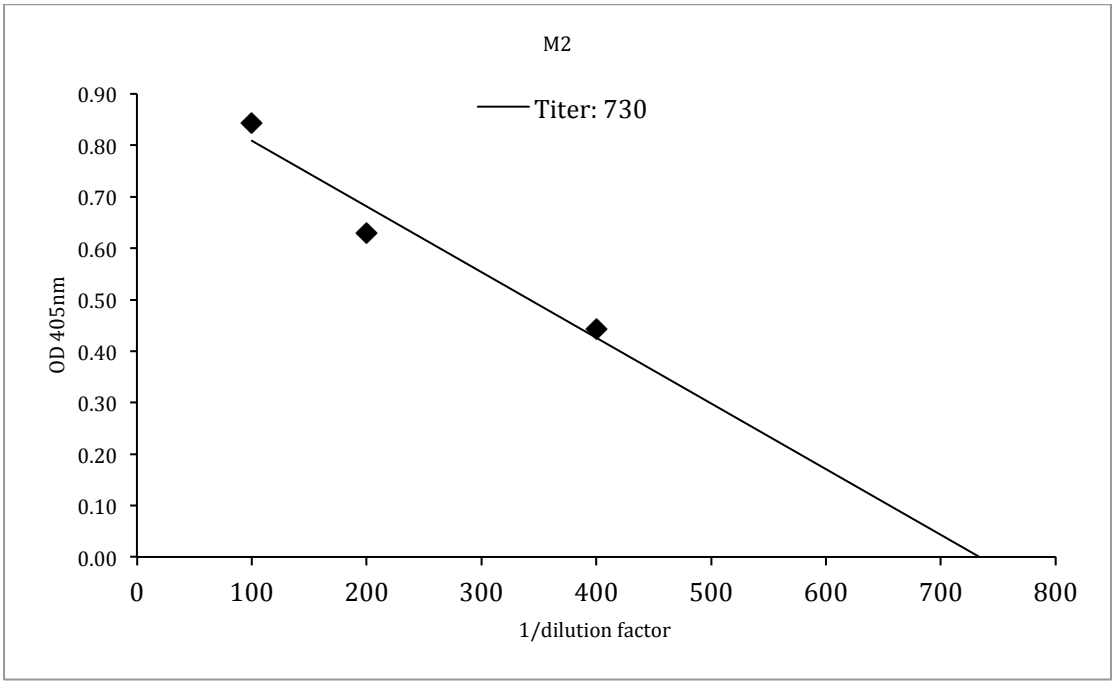

M3
Titer: 1440
OD 405nm
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
200
400
600
800
1000
1200
1400
1/dilution factor

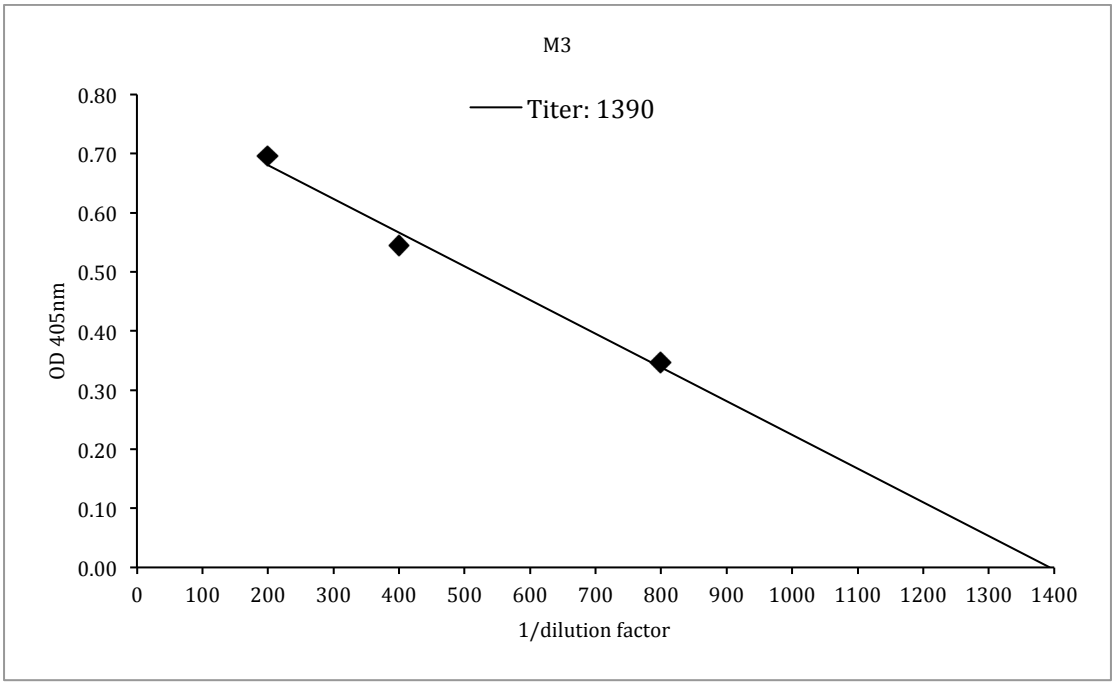


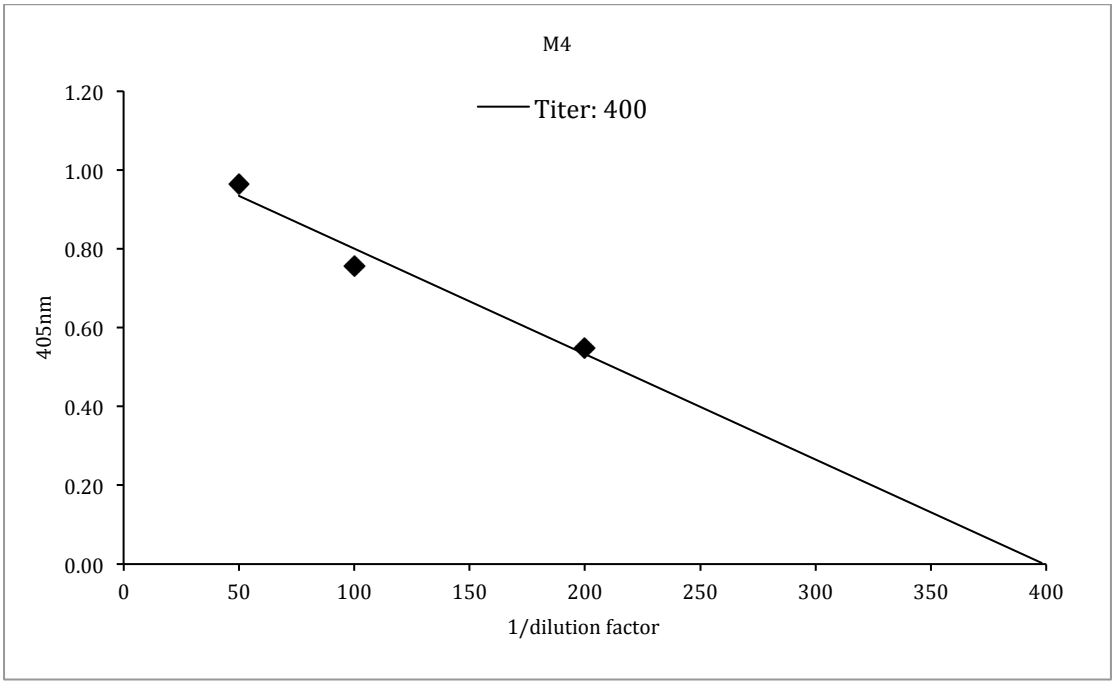
IgG1 titers

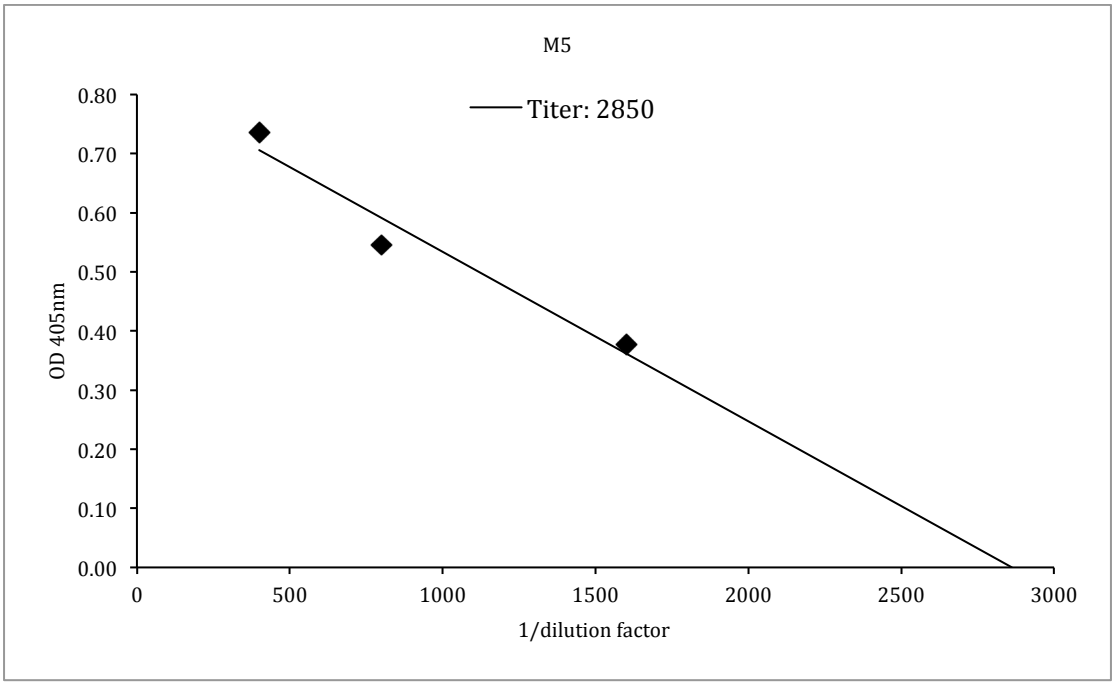
Mice Number	Serum IgG1 titers
M1	1780
M2	730
M3	1390
M4	400
M5	2850


M1
Titer: 1780
OD 405nm
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
200
400
600
800
1000
1200
1400
1600
1800
1/dilution factor


M2
Titer: 730
OD 405nm
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
100
200
300
400
500
600
700
800
1/dilution factor

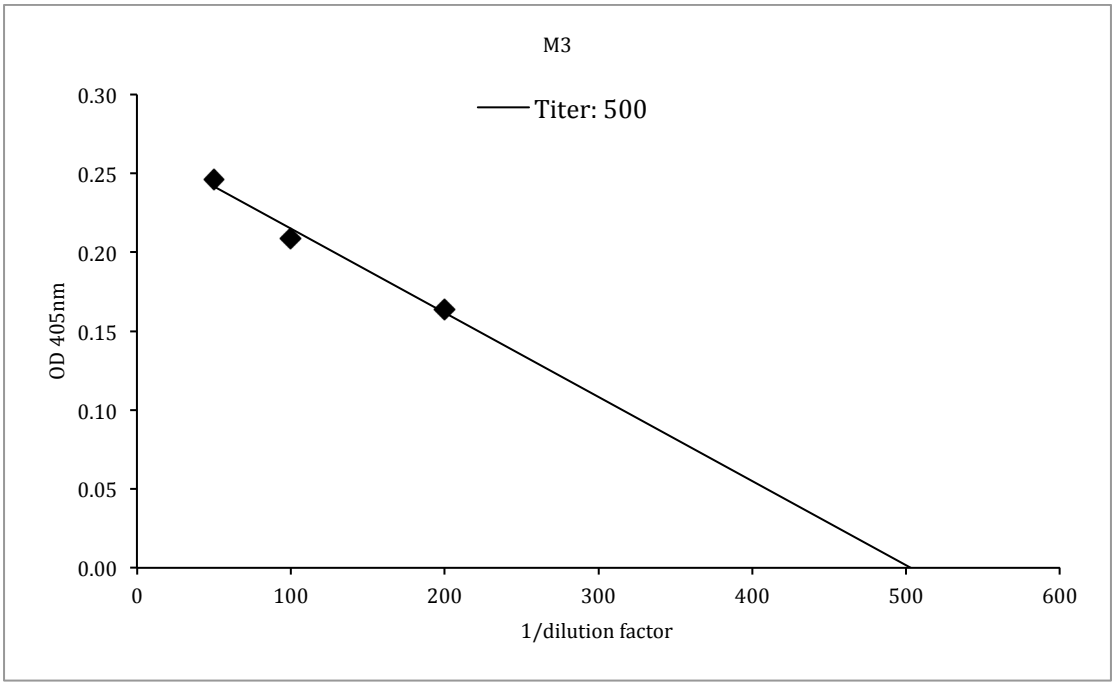

M3
Titer: 1390
OD 405nm
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1/dilution factor


M4
Titer: 400
405nm
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0
50
100
150
200
250
300
350
400
1/dilution factor



IgG2a titers

Mice Number	Serum IgG2a titers
M1	N/D
M2	N/D
M3	500
M4	N/D
M5	N/D



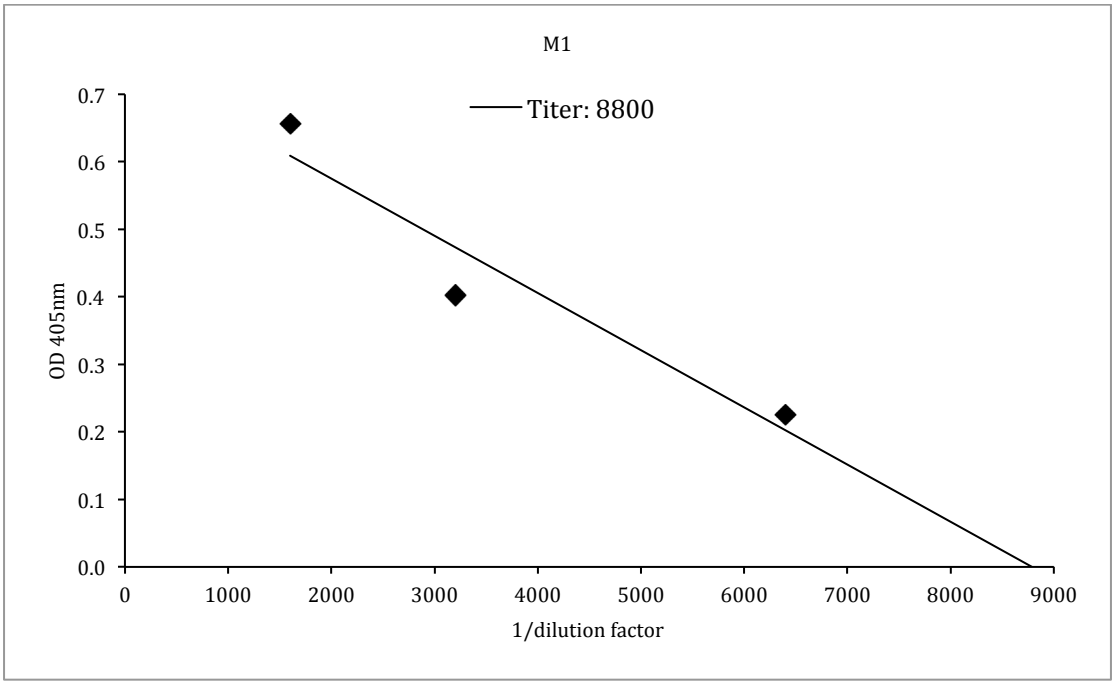
IgA [Single point titers]

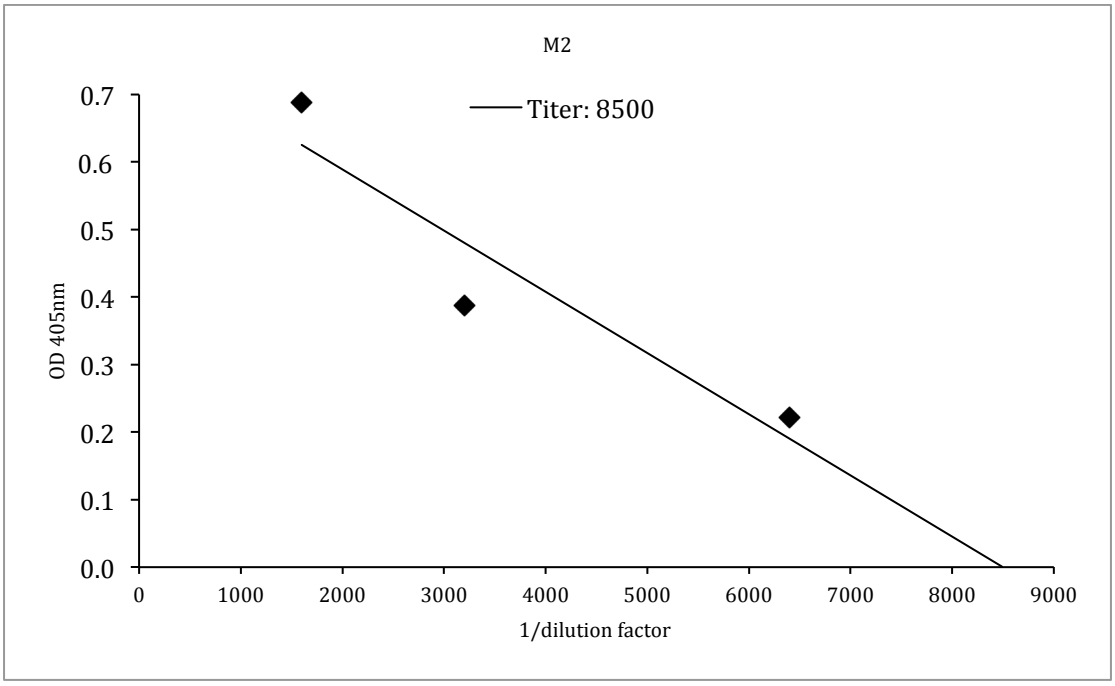
Mice Number	Serum IgA titers
M1	1.3
M2	1.2
M3	1.2
M4	1.1
M5	1.2

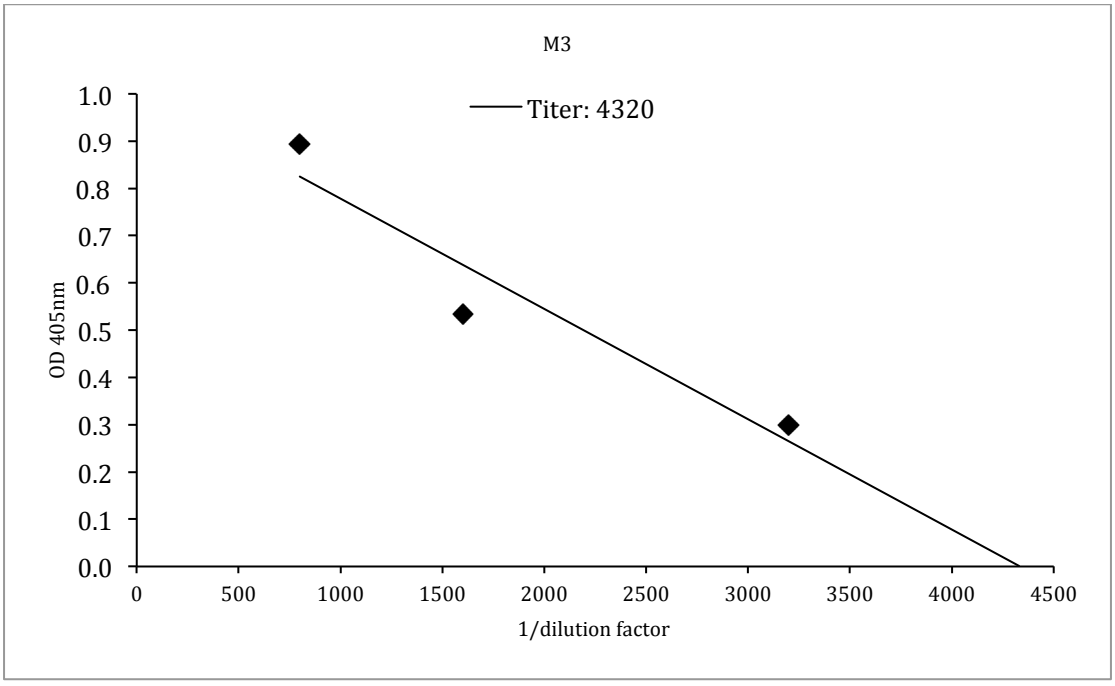
Antigen-specific immune response of mice vaccinated with two doses of live *aroQ*BP.

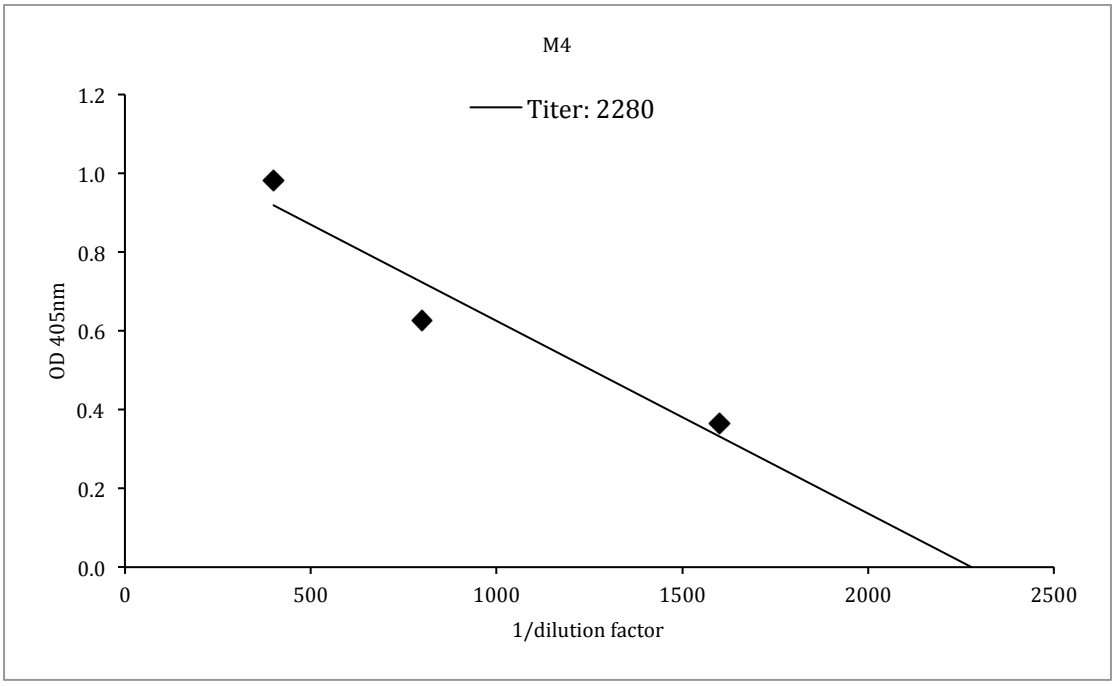
IgG titers

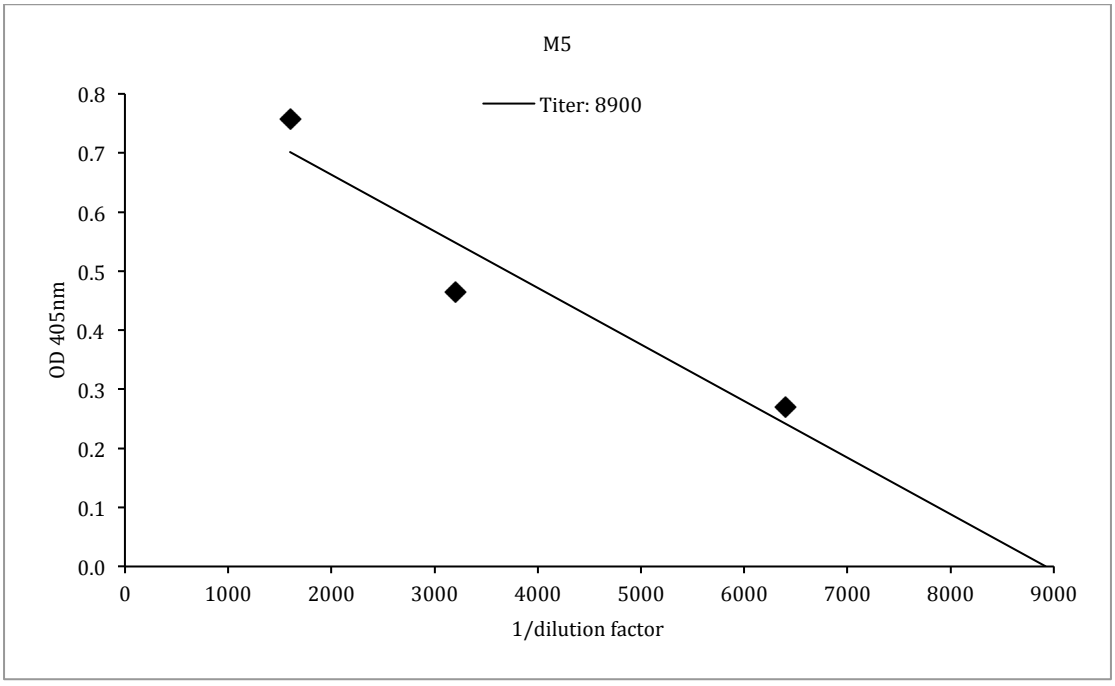
Mice Number	Serum IgG titers
M1	8800
M2	8500
M3	4320
M4	2280
M5	8900




M2
Titer: 8500
OD 405nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
1000
2000
3000
4000
5000
6000
7000
8000
9000
1/dilution factor

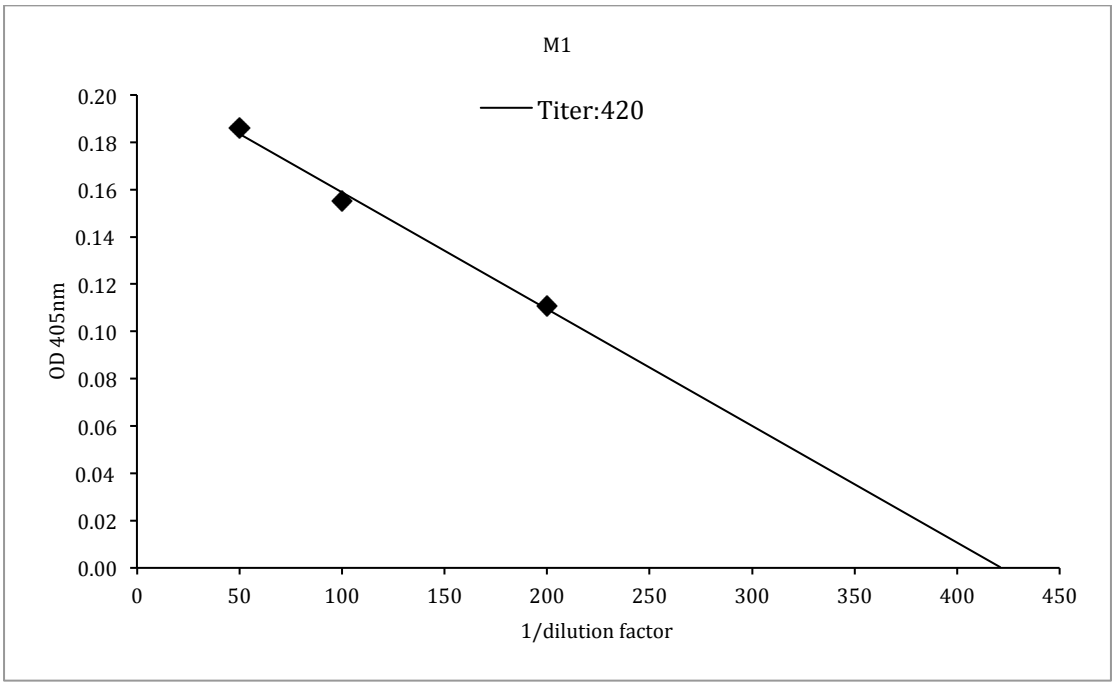

M3
Titer: 4320
OD 405nm
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
500
1000
1500
2000
2500
3000
3500
4000
4500
1/dilution factor

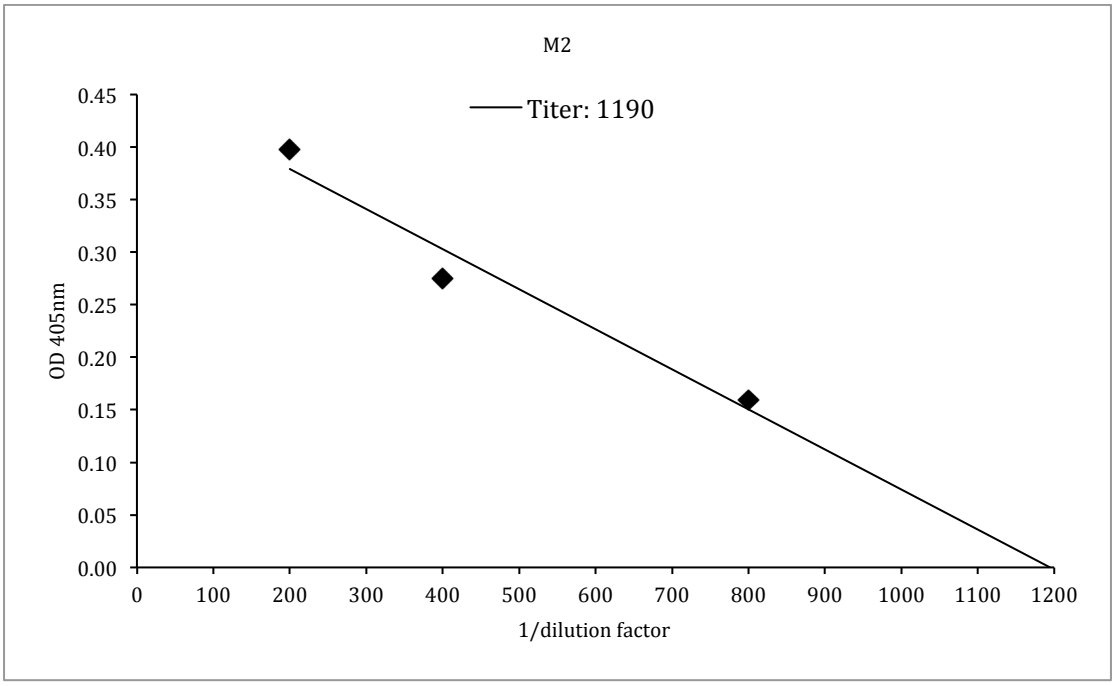

M4
Titer: 2280
OD 405nm
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
500
1000
1500
2000
2500
1/dilution factor

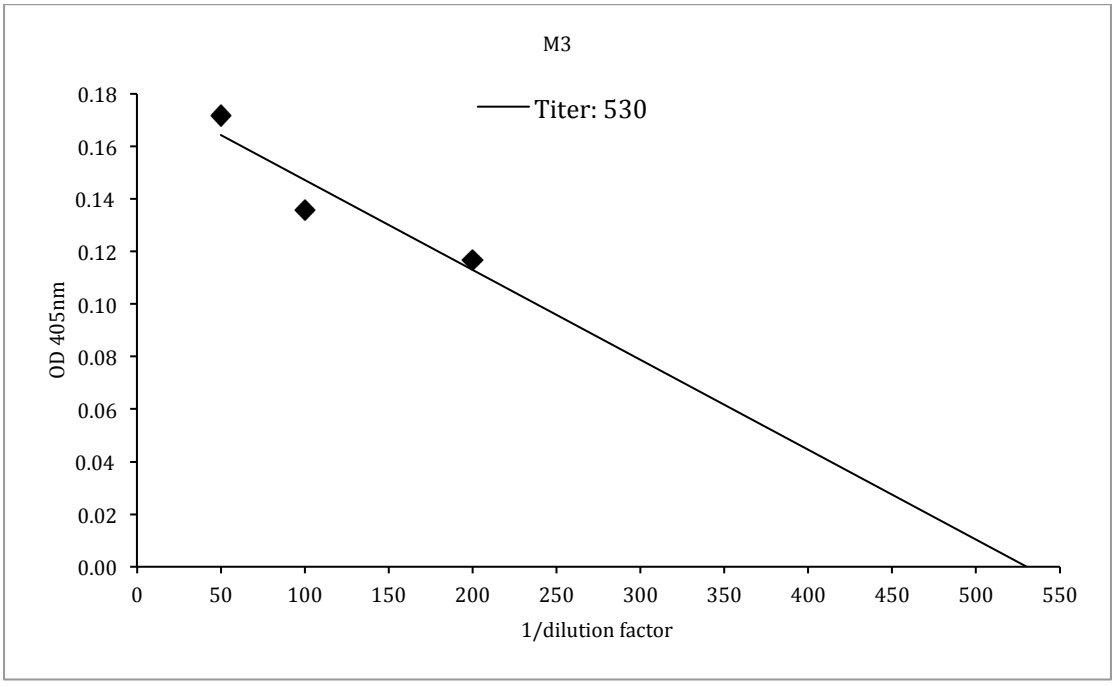

M5
Titer: 8900
OD 405nm
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
1000
2000
3000
4000
5000
6000
7000
8000
9000
1/dilution factor

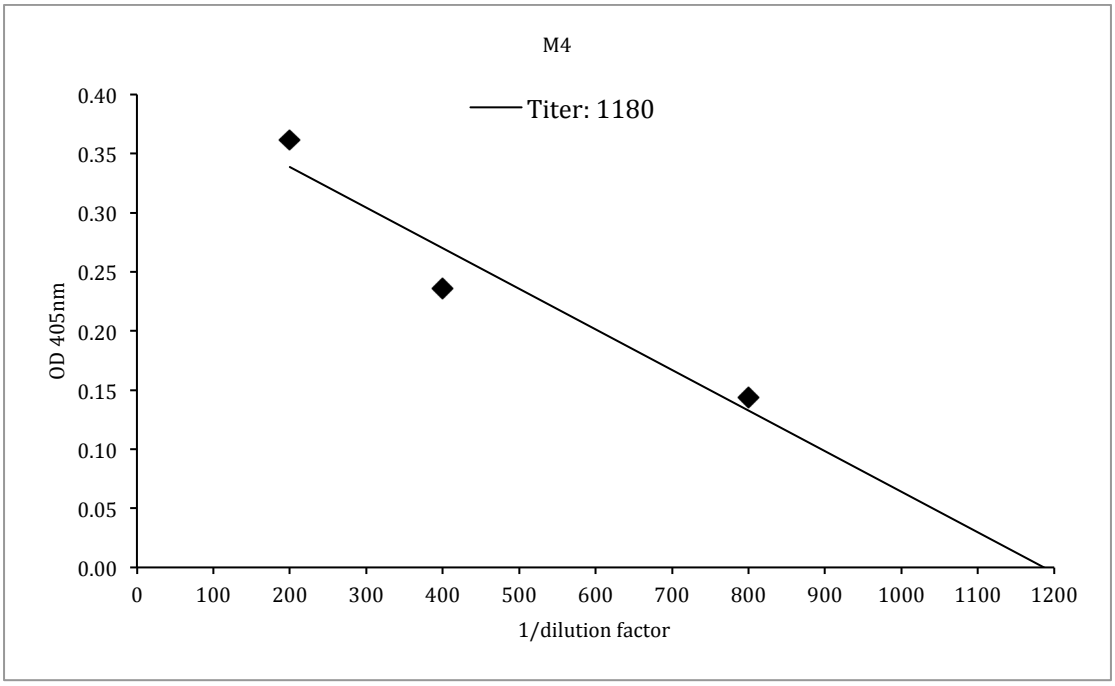
IgG1 titers

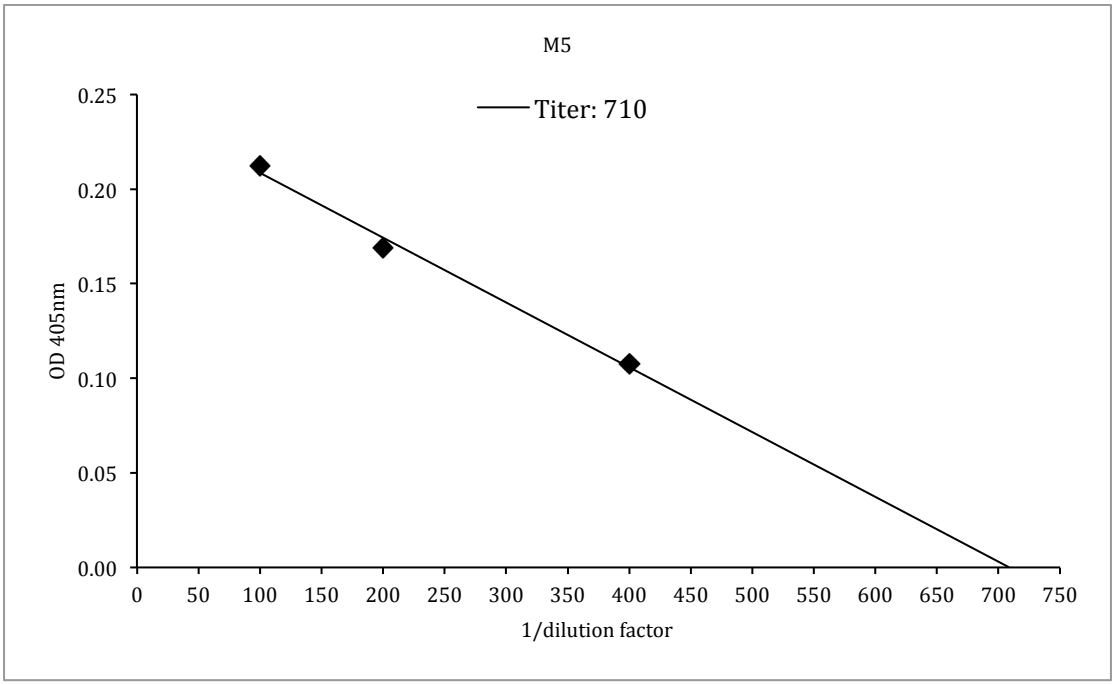
Mice Number	Serum IgG titers
M1	420
M2	1190
M3	530
M4	1180
M5	710




M2
Titer: 1190
OD 405nm
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1/dilution factor

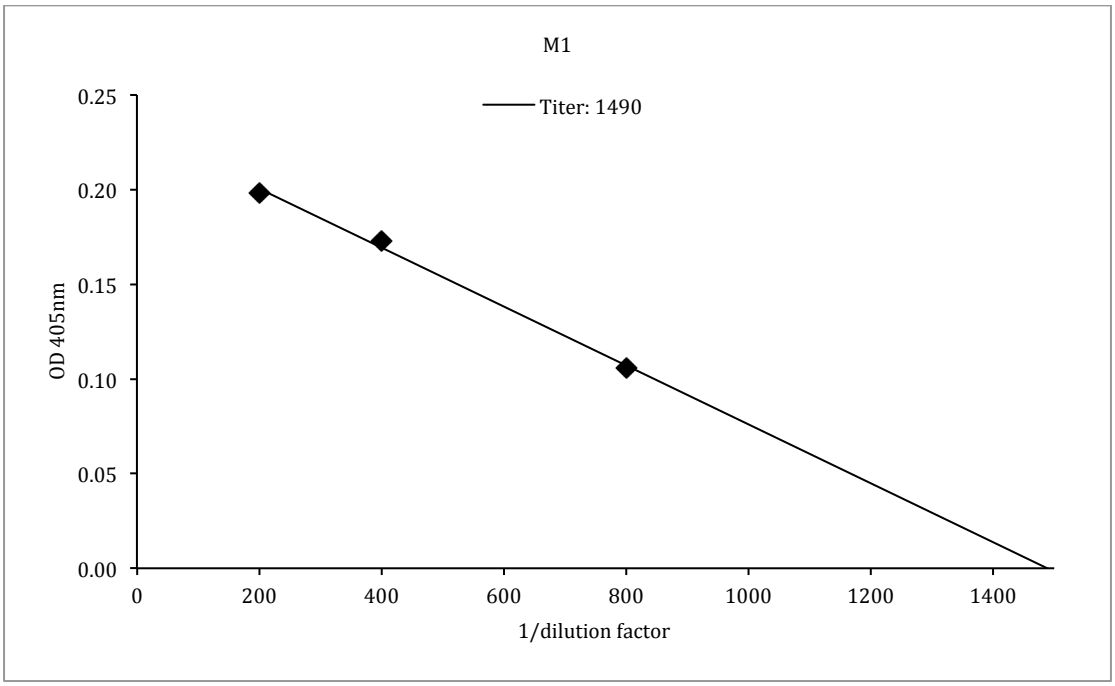

M3
Titer: 530
OD 405nm
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
50
100
150
200
250
300
350
400
450
500
550
1/dilution factor

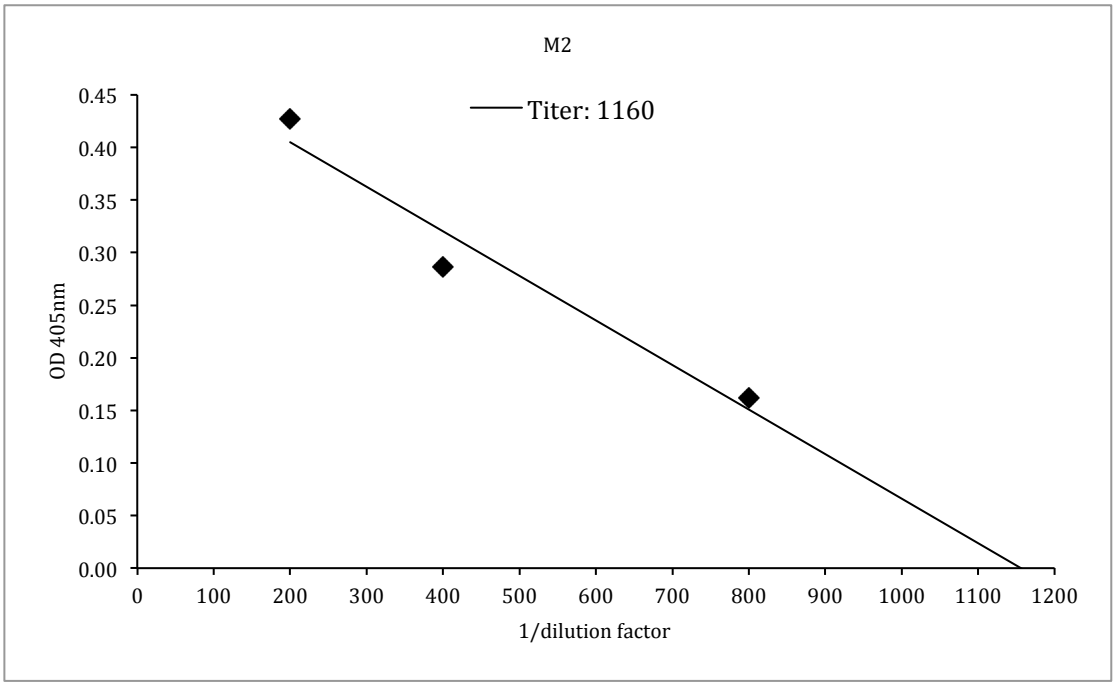

M4
Titer: 1180
OD 405nm
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1/dilution factor

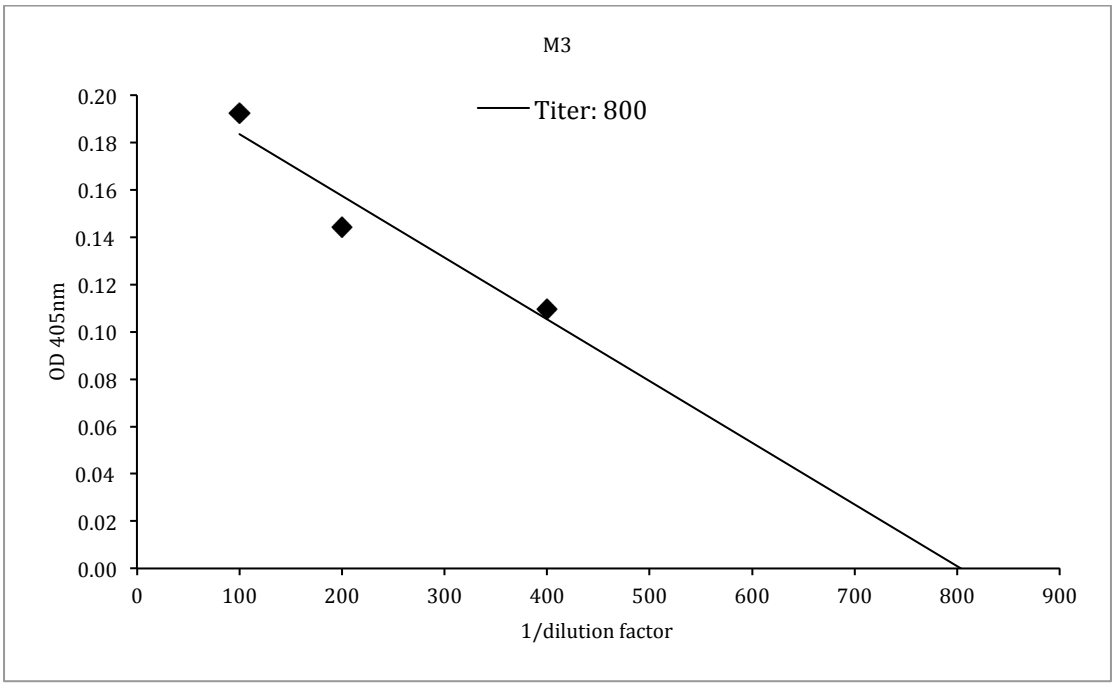

M5
Titer: 710
OD 405nm
0.25
0.20
0.15
0.10
0.05
0.00
0
50
100
150
200
250
300
350
400
450
500
550
600
650
700
750
1/dilution factor

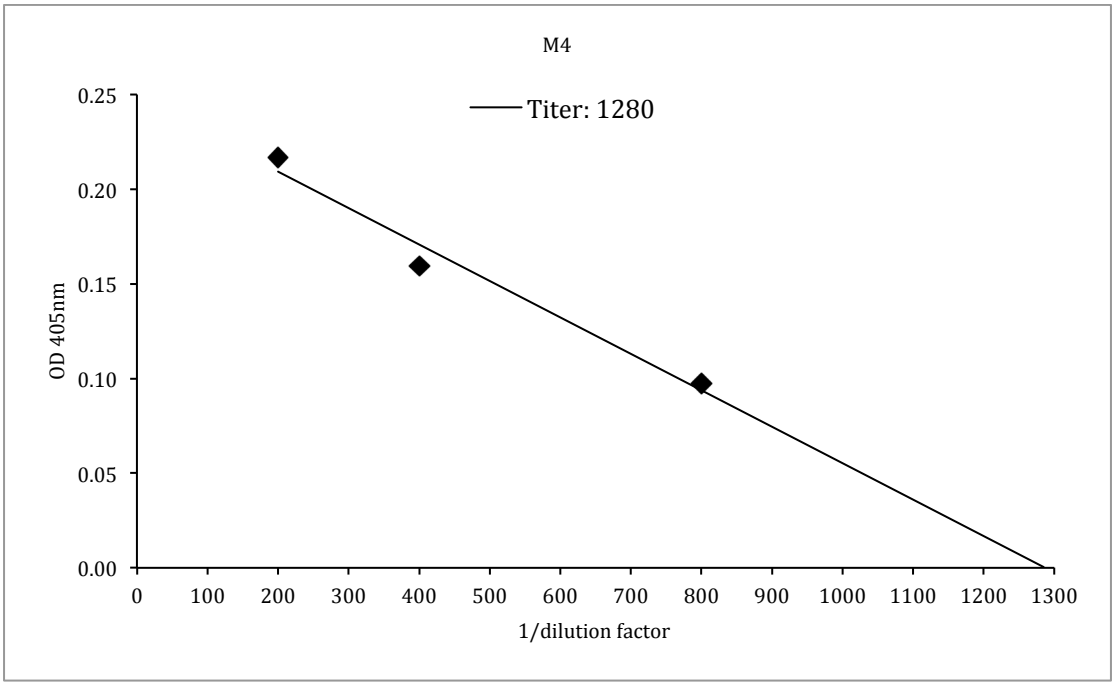
IgG2a titers

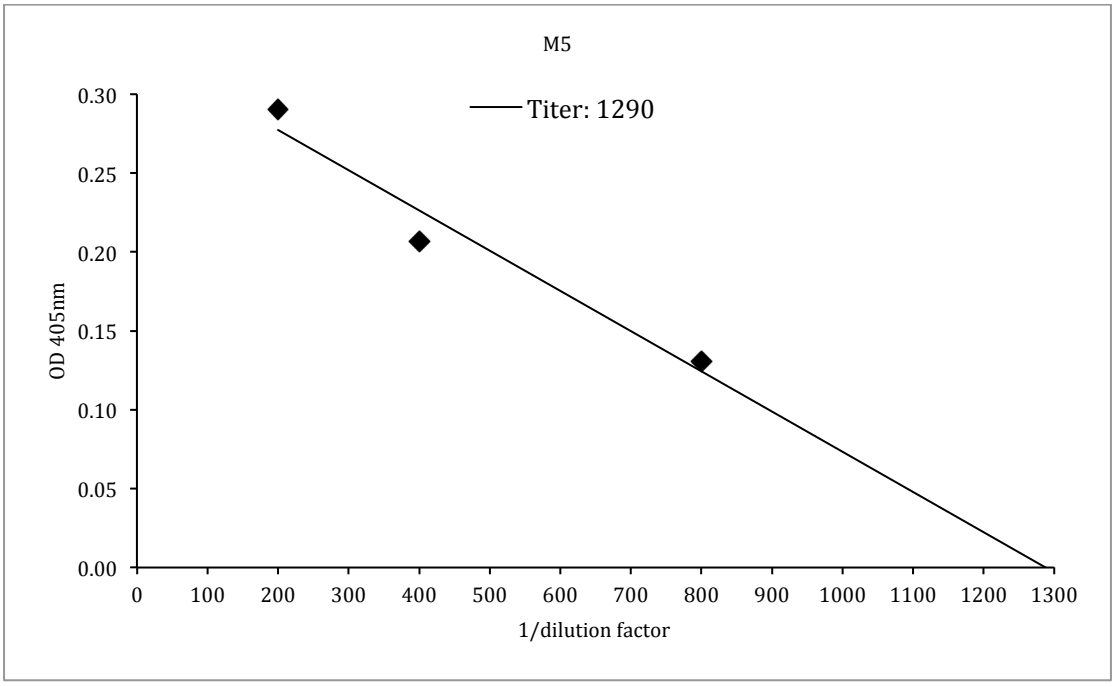
Mice Number	Serum IgG titers
M1	1490
M2	1160
M3	800
M4	1280
M5	1290




M2
Titer: 1160
OD 405nm
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1/dilution factor


M3
Titer: 800
OD 405nm
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
100
200
300
400
500
600
700
800
900
1/dilution factor


M4
Titer: 1280
OD 405nm
0.25
0.20
0.15
0.10
0.05
0.00
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1/dilution factor


M5
Titer: 1290
OD 405nm
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1/dilution factor

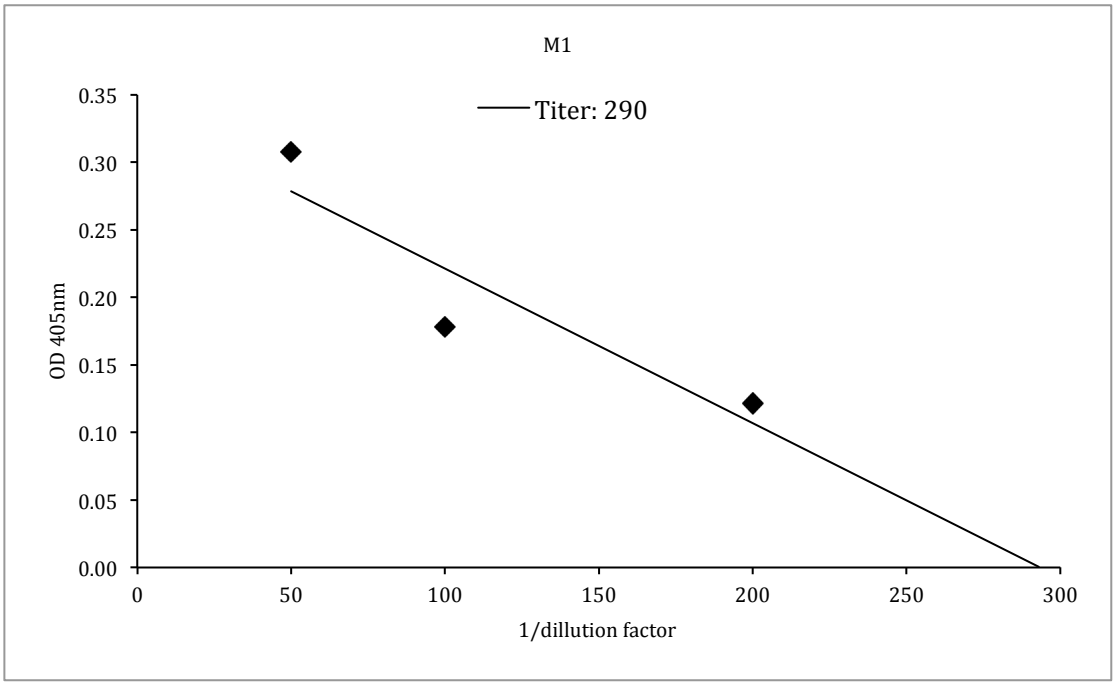
IgA titers

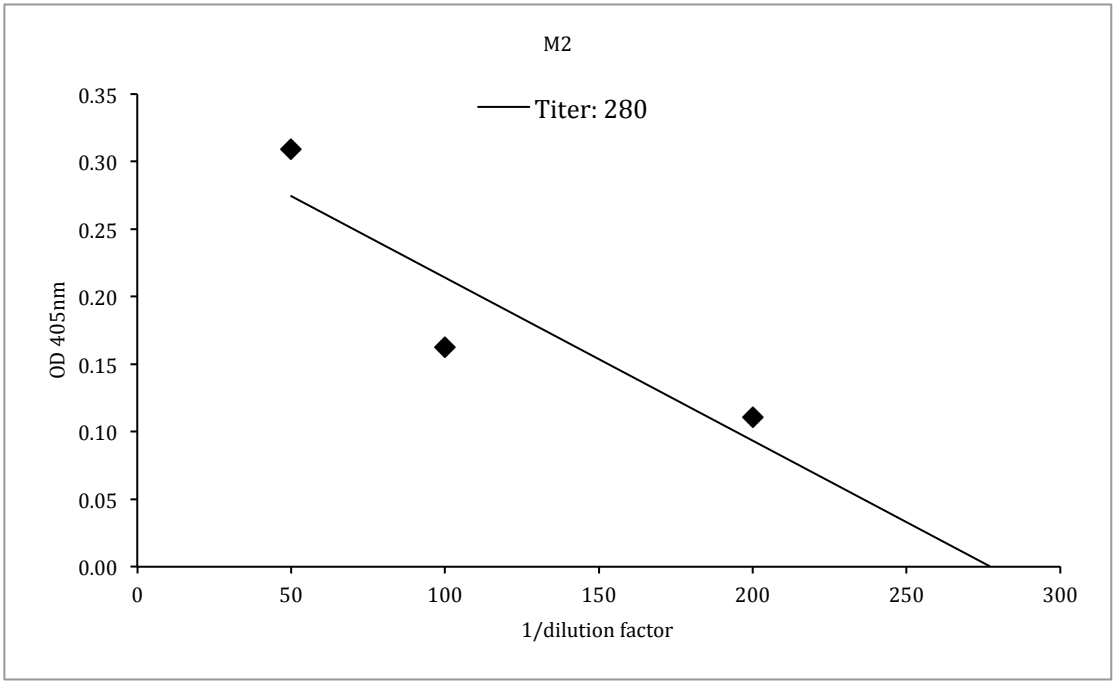
Mice Number	Serum IgA titers
M1	2.02
M2	2.21
M3	2.23
M4	2.09
M5	2.14

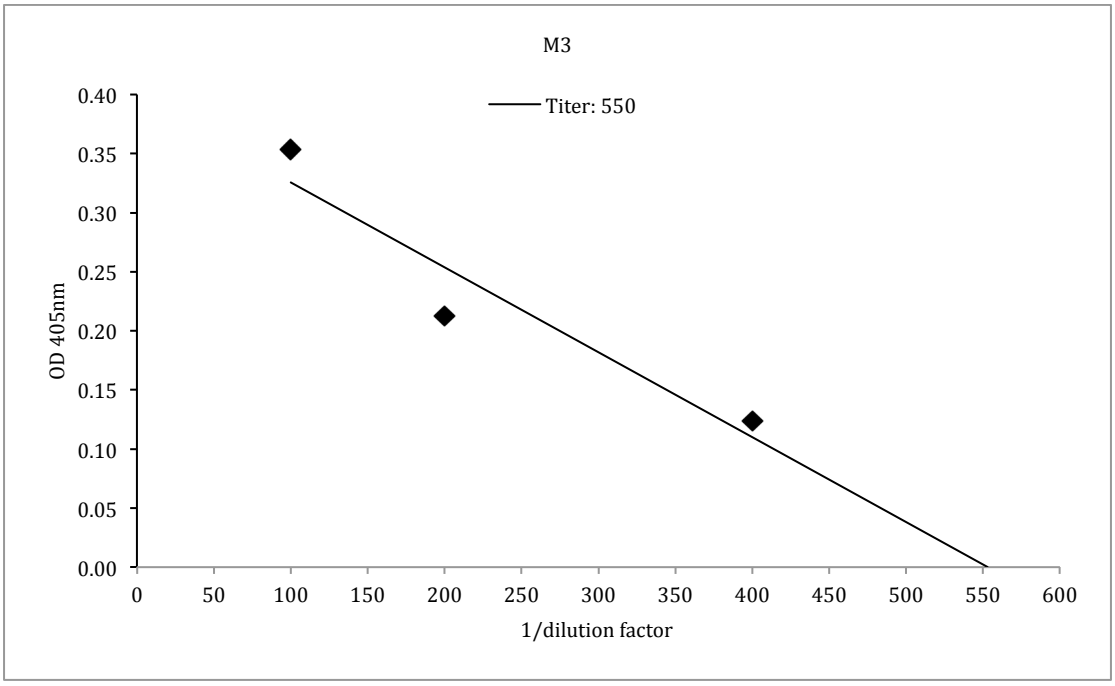
Antigen-specific immune response of mice vaccinated with one dose of live *aroQ*BP.

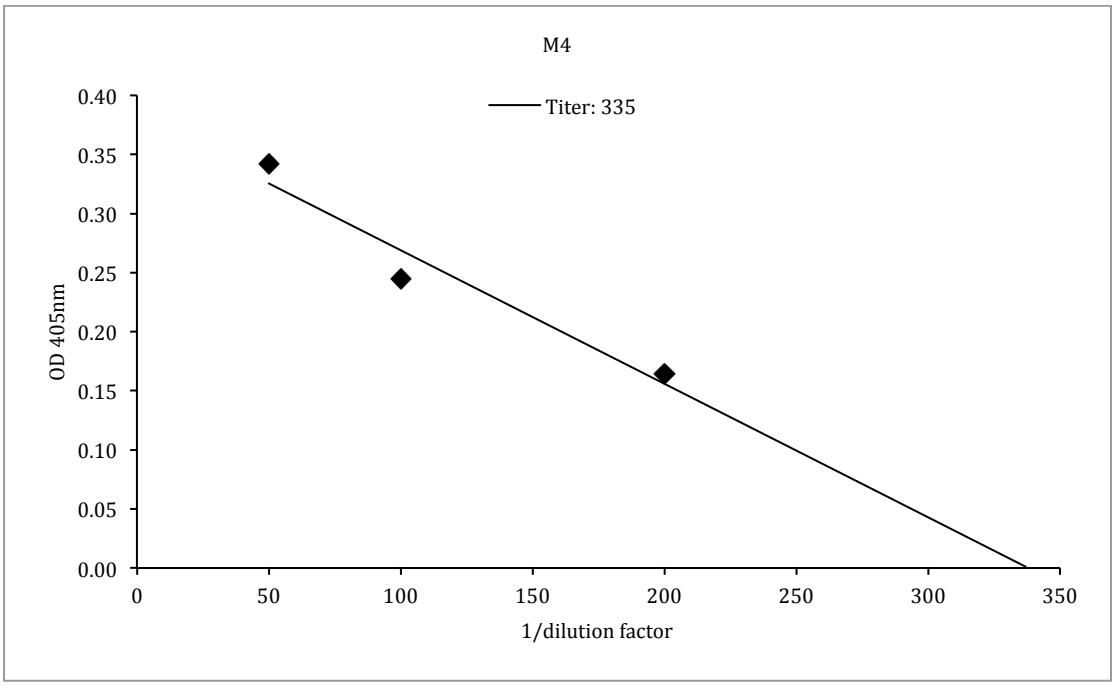
IgG titers

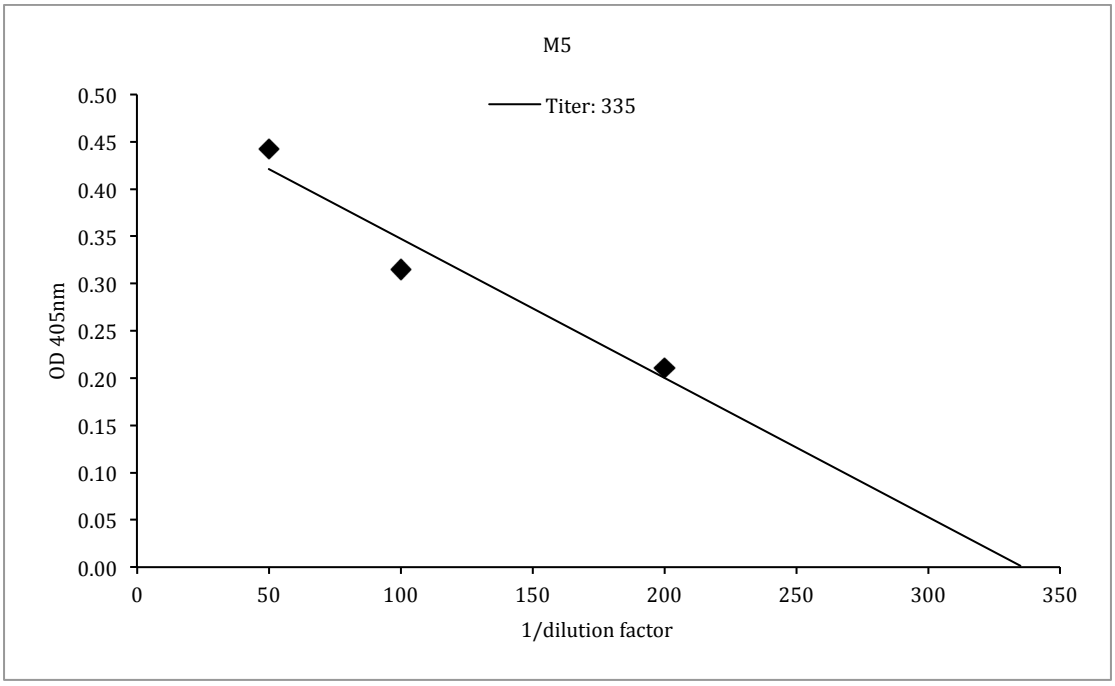
Mice Number	Serum IgG titers
M1	290
M2	280
M3	550
M4	335
M5	335


M1
Titer: 290
OD 405nm
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
50
100
150
200
250
300
1/dillution factor


M2
Titer: 280
OD 405nm
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
50
100
150
200
250
300
1/dilution factor

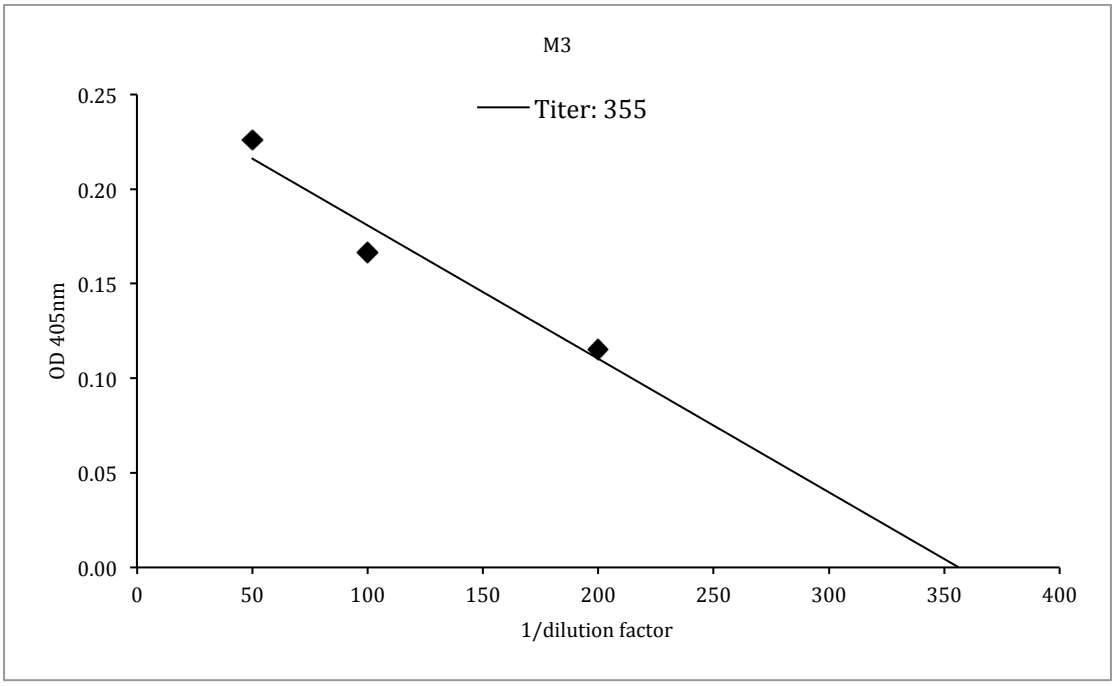

M3
Titer: 550
OD 405nm
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
50
100
150
200
250
300
350
400
450
500
550
600
1/dilution factor


M4
Titer: 335
OD 405nm
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
50
100
150
200
250
300
350
1/dilution factor



IgG1 titers

Mice Number	Serum IgG1 titers
M1	N/D
M2	N/D
M3	355
M4	N/D
M5	N/D



IgG2a titers

Mice Number	Serum IgG2a titers
M1	N/D
M2	N/D
M3	N/D
M4	N/D
M5	N/D

IgA [Single point titers]

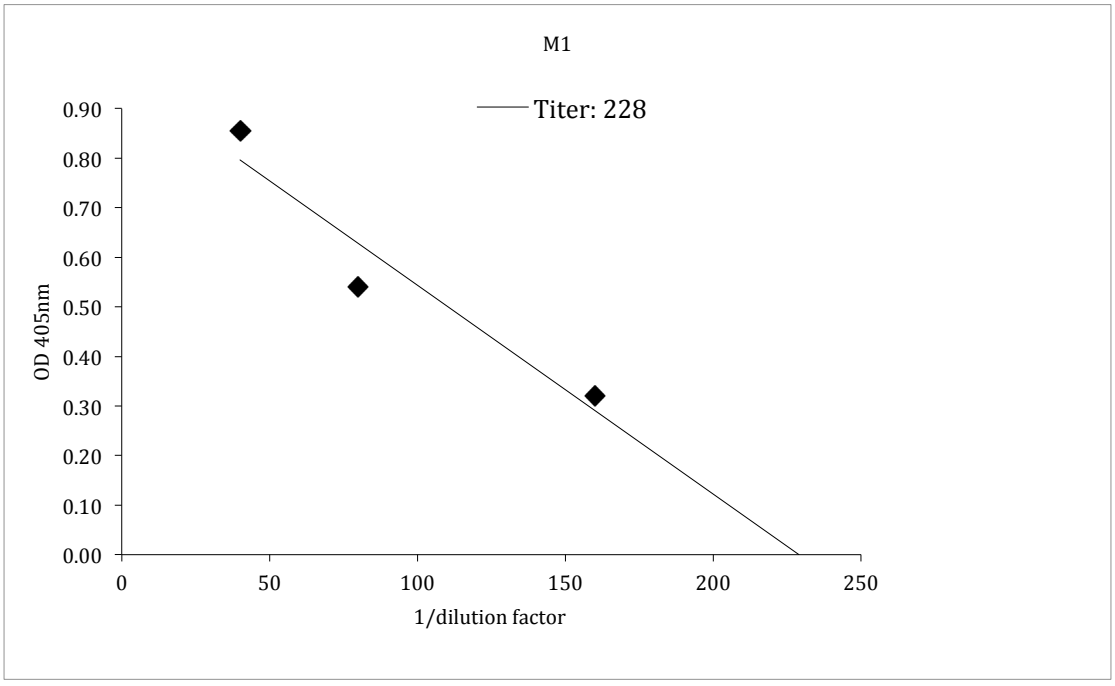
Mice Number	Serum IgA titers
M1	1.2
M2	1.3
M3	1.2
M4	1.1
M5	1.1

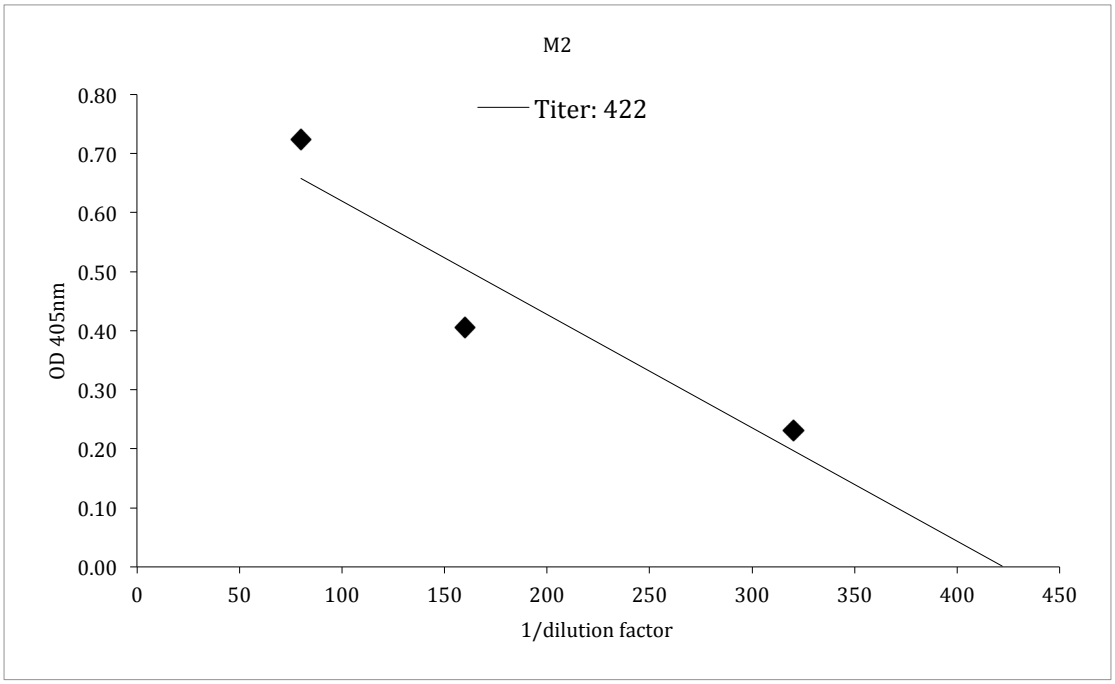
Appendix J: Lungs antibody isotypes against inactivated *aroQ*BP

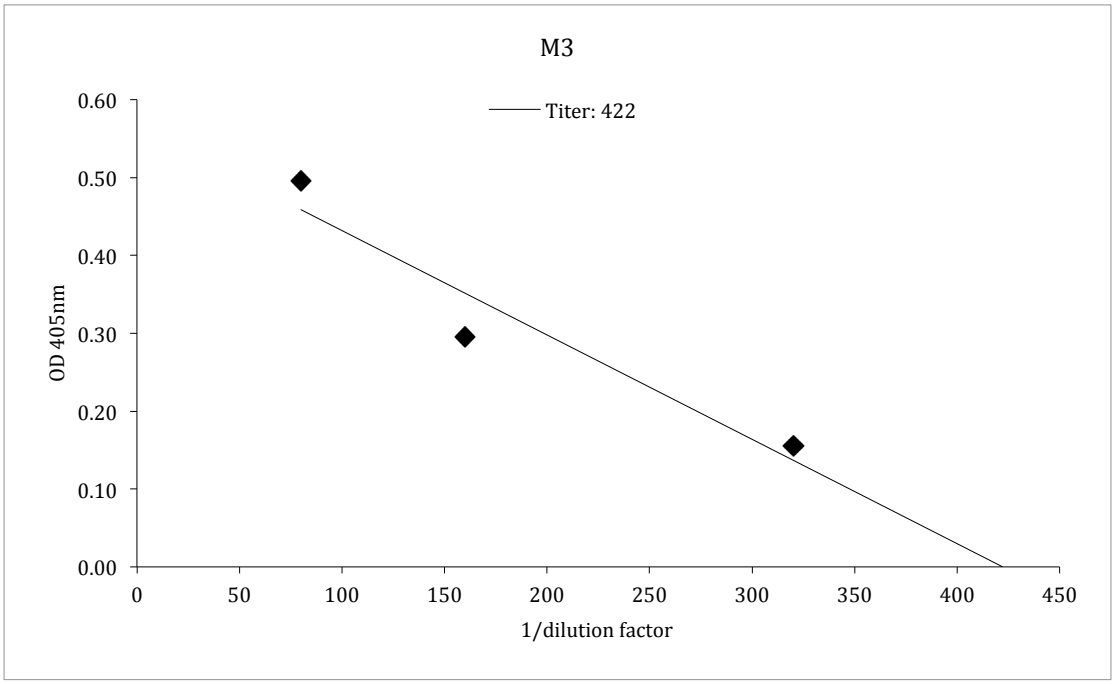
Antigen-specific immune response of mice vaccinated with three doses of DTaP and two booster doses of live *aroQ*BP.

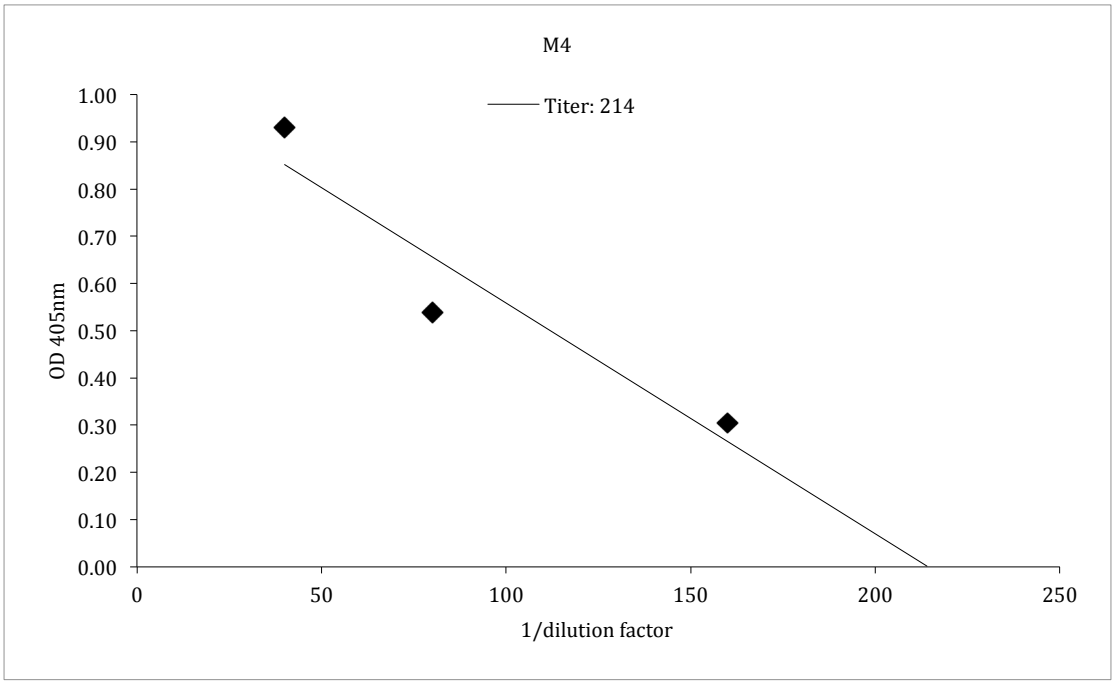
IgG titers

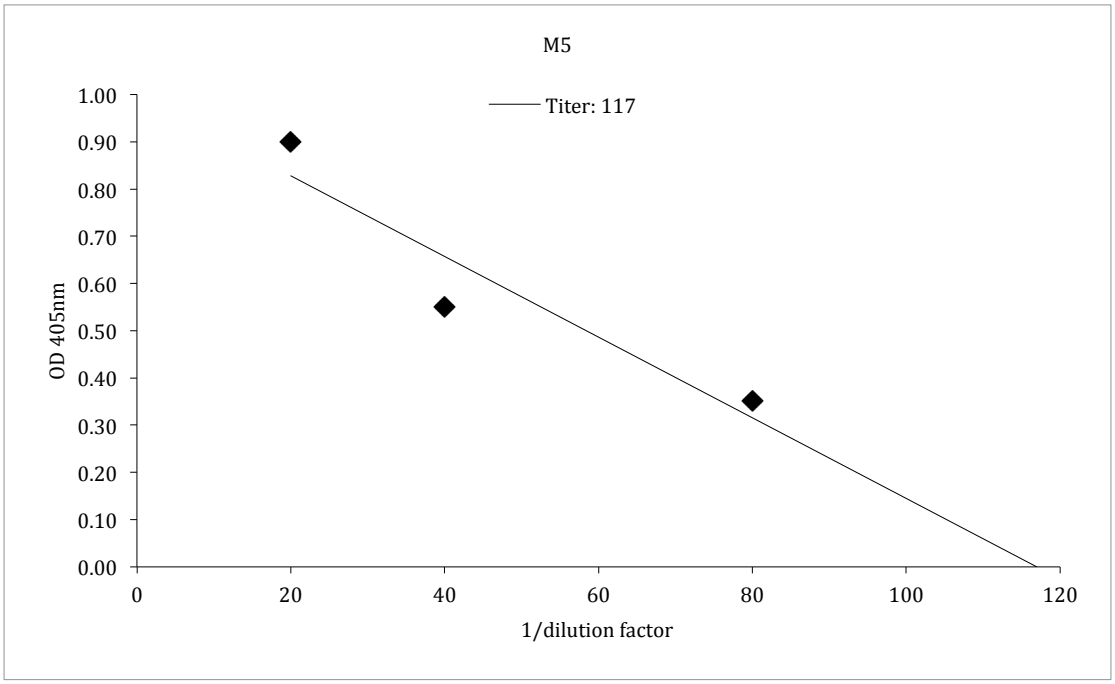
Mice Number	Lungs IgG titers
M1	228
M2	422
M3	422
M4	214
M5	117




M2
Titer: 422
OD 405nm
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
50
100
150
200
250
300
350
400
450
1/dilution factor

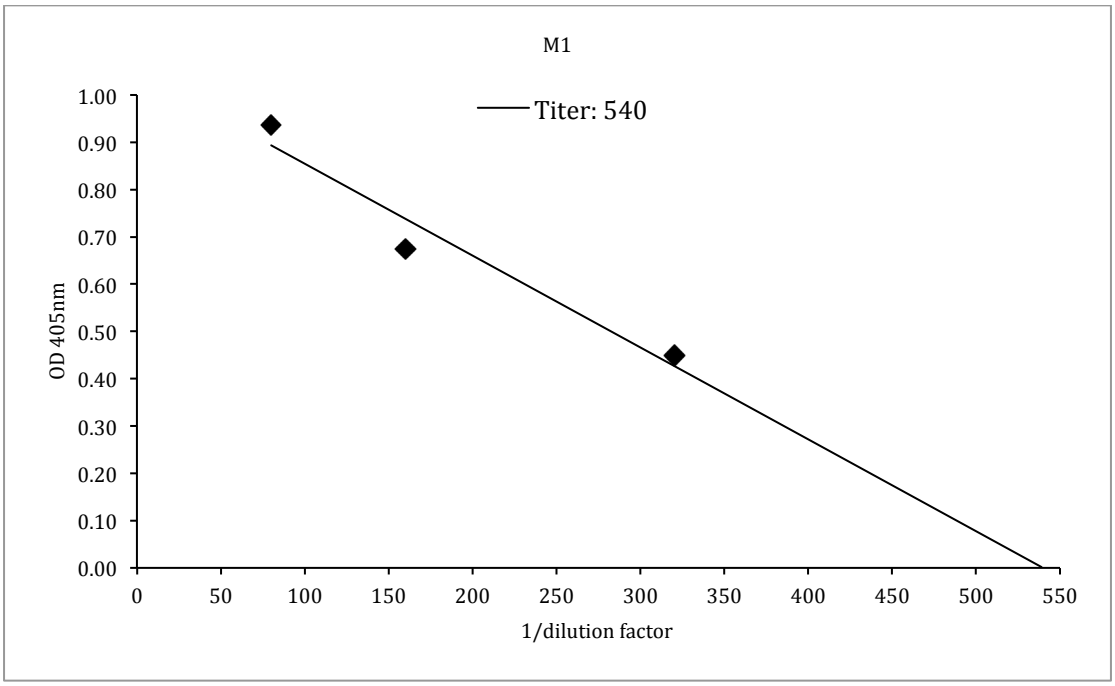

M3
Titer: 422
OD 405nm
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
50
100
150
200
250
300
350
400
450
1/dilution factor

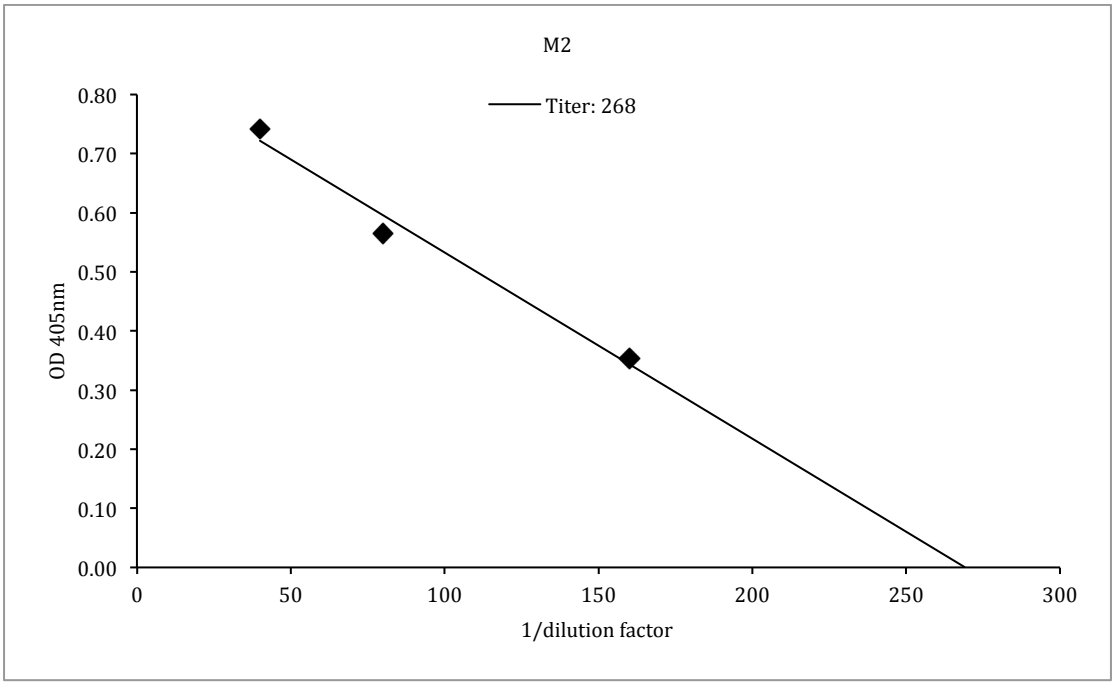

M4
Titer: 214
OD 405nm
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
50
100
150
200
250
1/dilution factor

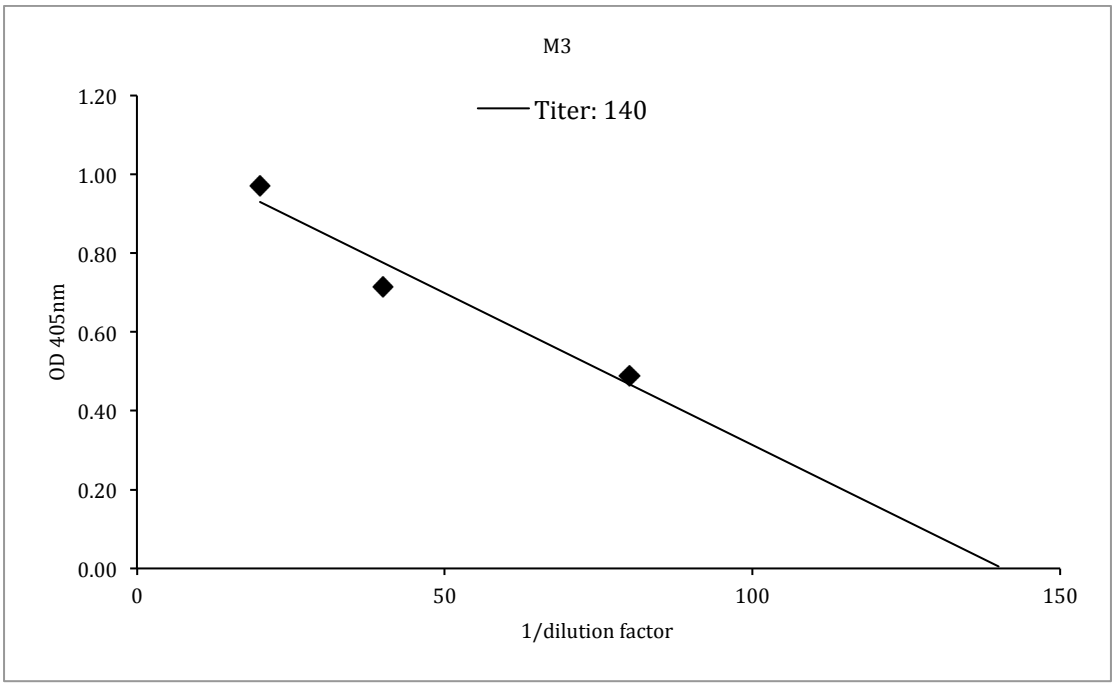

M5
Titer: 117
OD 405nm
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
20
40
60
80
100
120
1/dilution factor

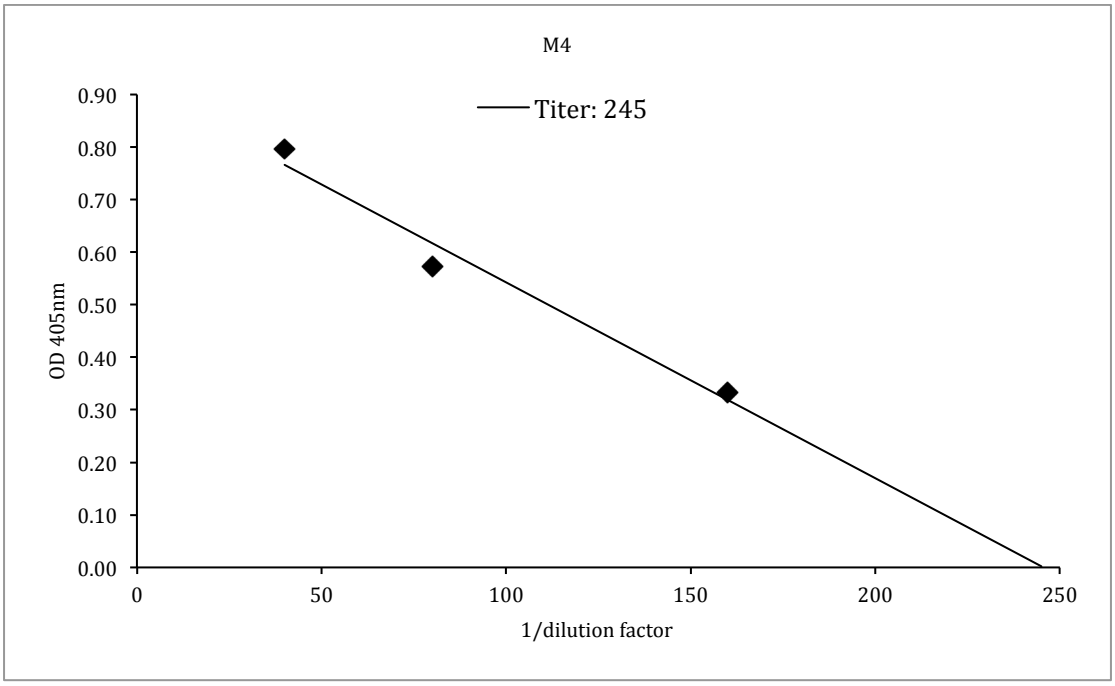
IgG1 titers

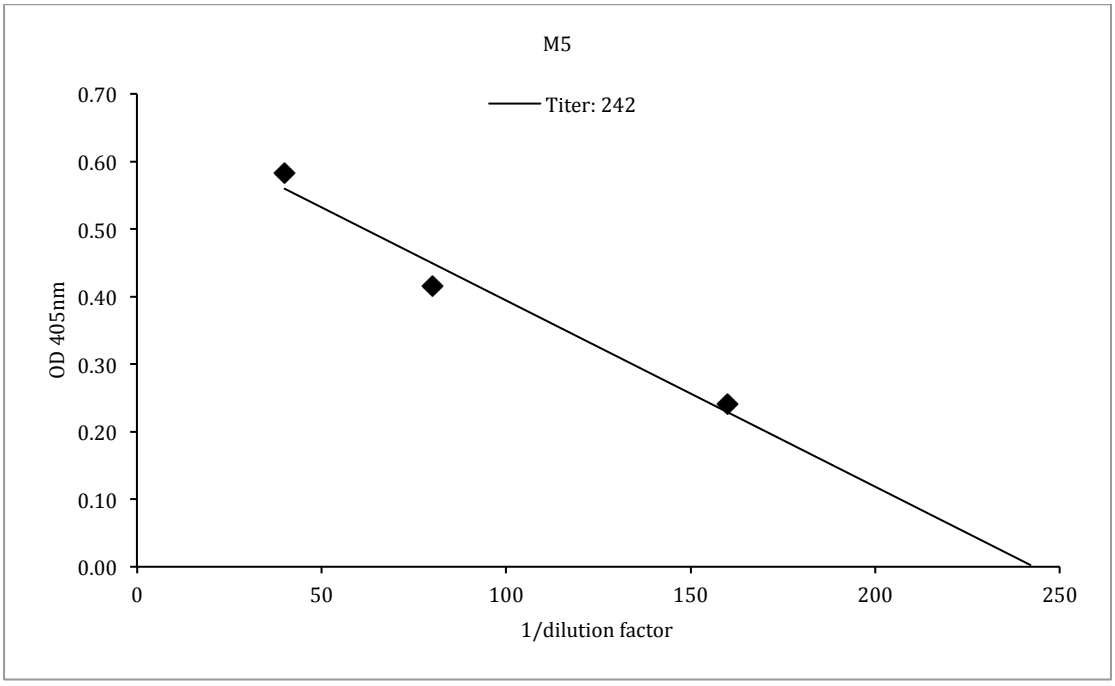
Mice Number	Lungs IgG1 titers
M1	540
M2	268
M3	140
M4	245
M5	242




M2
Titer: 268
OD 405nm
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
50
100
150
200
250
300
1/dilution factor

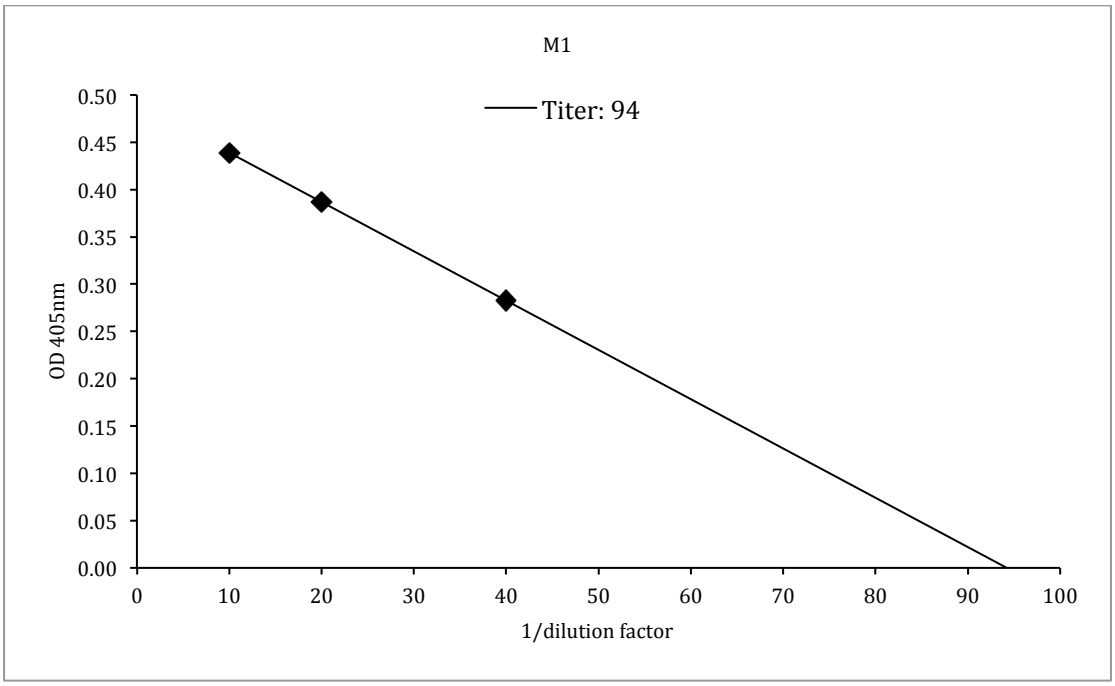

M3
Titer: 140
OD 405nm
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0
50
100
150
1/dilution factor

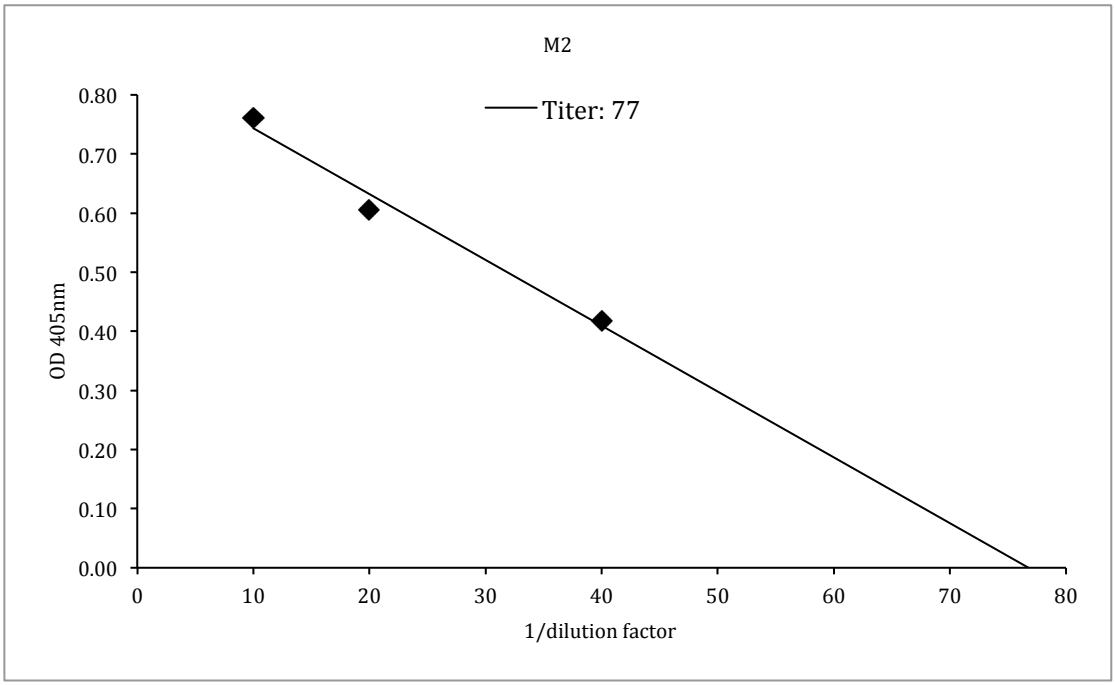

M4
Titer: 245
OD 405nm
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
50
100
150
200
250
1/dilution factor

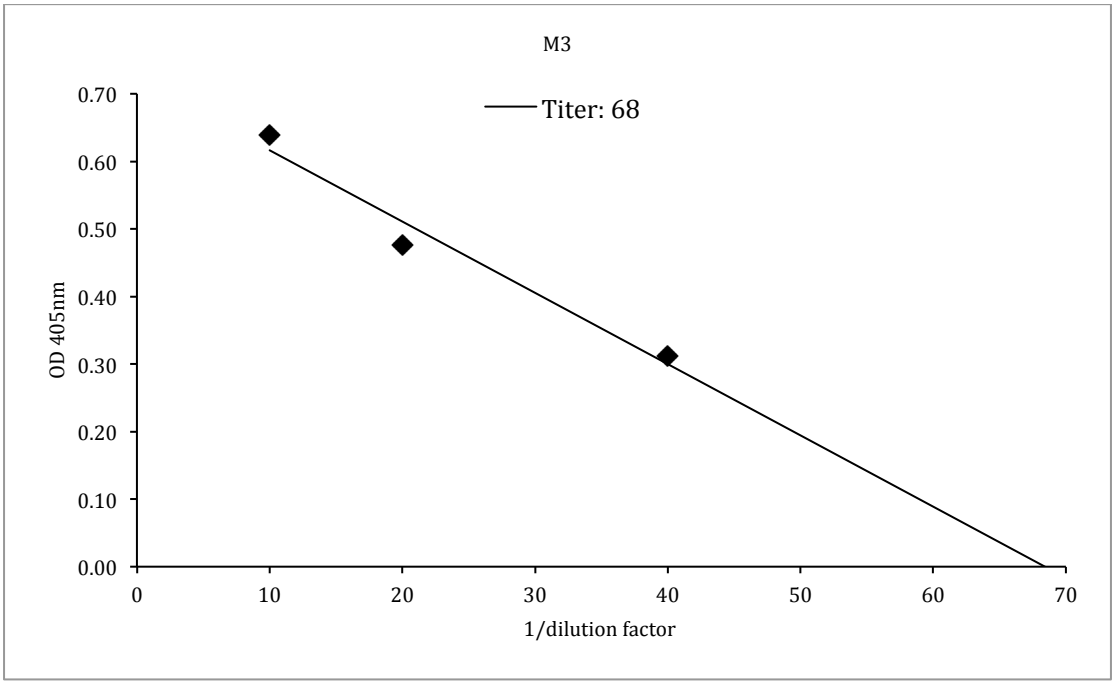

M5
Titer: 242
OD 405nm
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
50
100
150
200
250
1/dilution factor

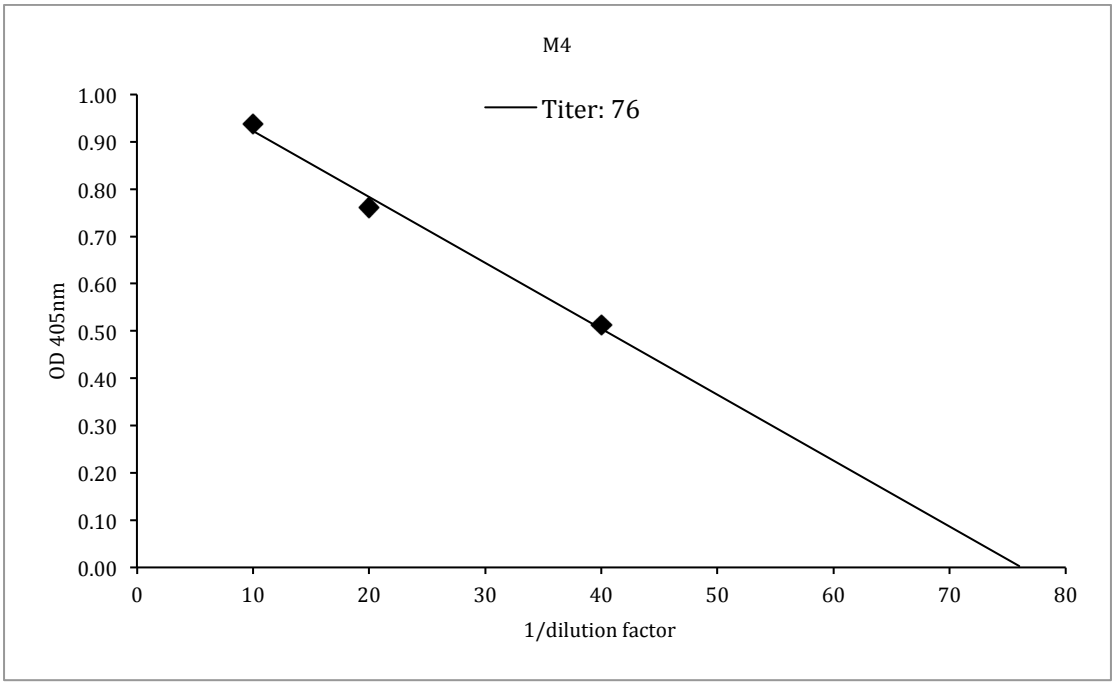
IgG2a titers

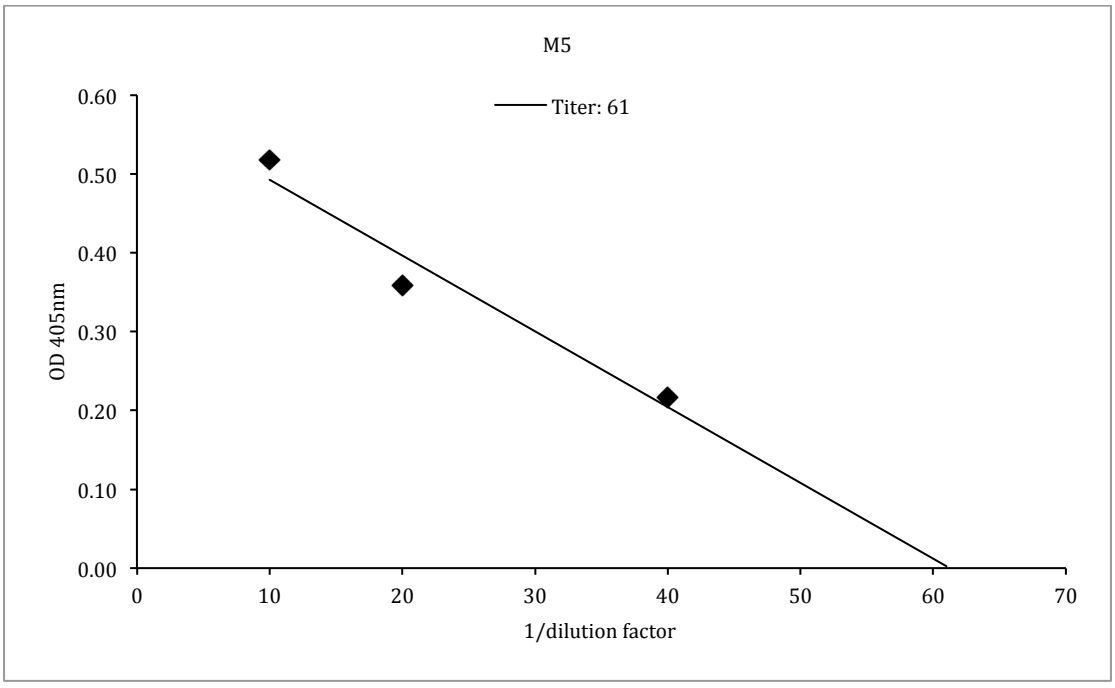
Mice Number	Lungs IgG2a titers
M1	94
M2	77
M3	68
M4	76
M5	61




M2
Titer: 77
OD 405nm
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
10
20
30
40
50
60
70
80
1/dilution factor


M3
Titer: 68
OD 405nm
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
10
20
30
40
50
60
70
1/dilution factor


M4
Titer: 76
OD 405nm
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
10
20
30
40
50
60
70
80
1/dilution factor


M5
Titer: 61
OD 405nm
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
10
20
30
40
50
60
70
1/dilution factor

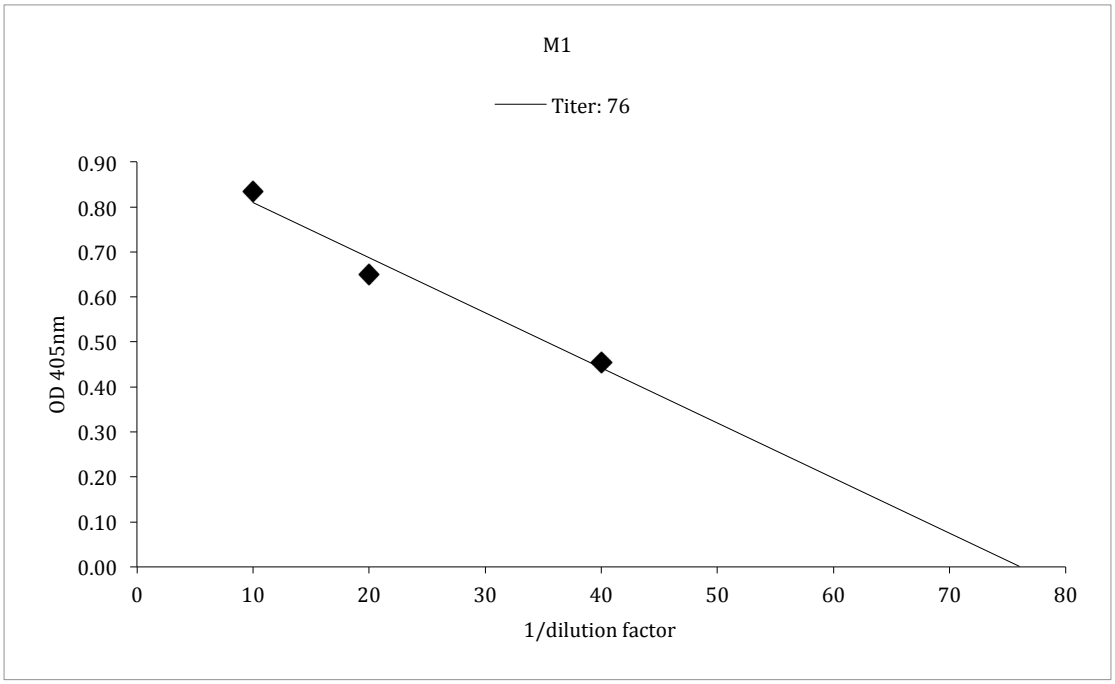
IgA [Single point titers]

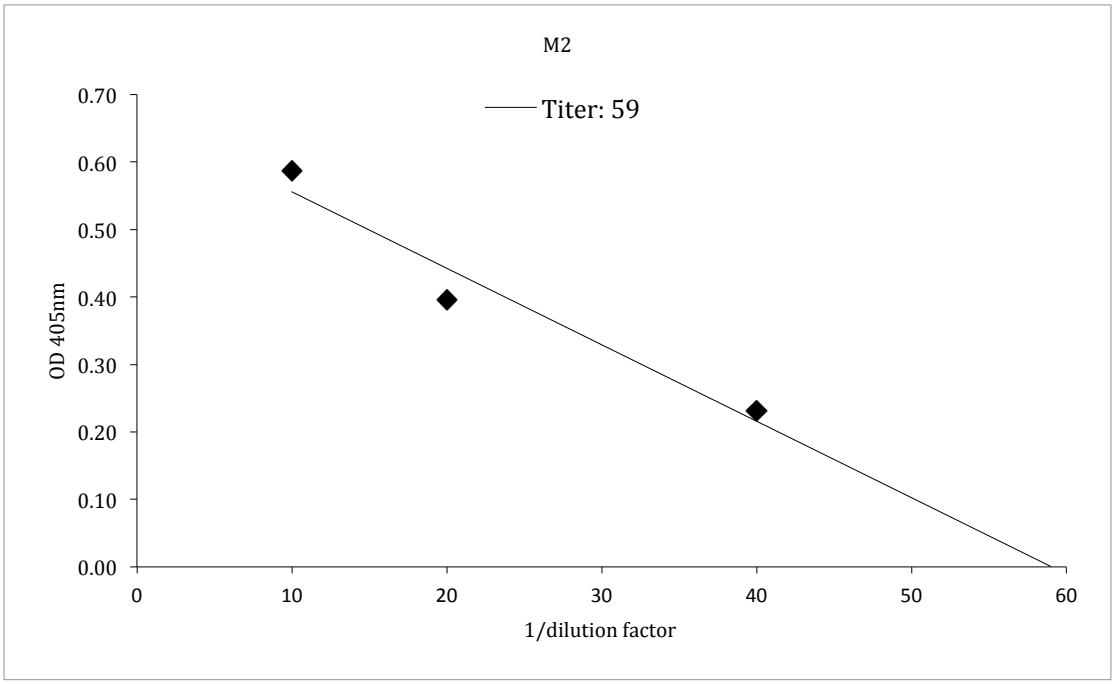
Mice Number	Lungs IgA titers
M1	1.3
M2	1.3
M3	0.6
M4	0.6
M5	1.1

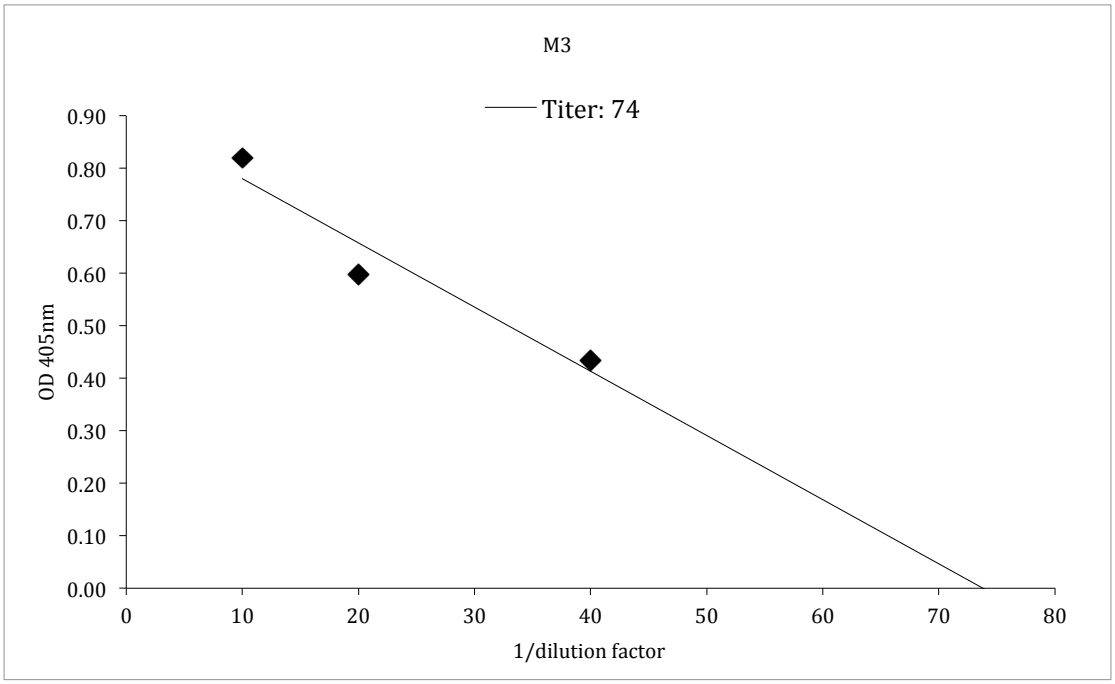
Antigen-specific immune response of mice vaccinated with three doses of DTaP and one booster dose of live *aroQ*BP.

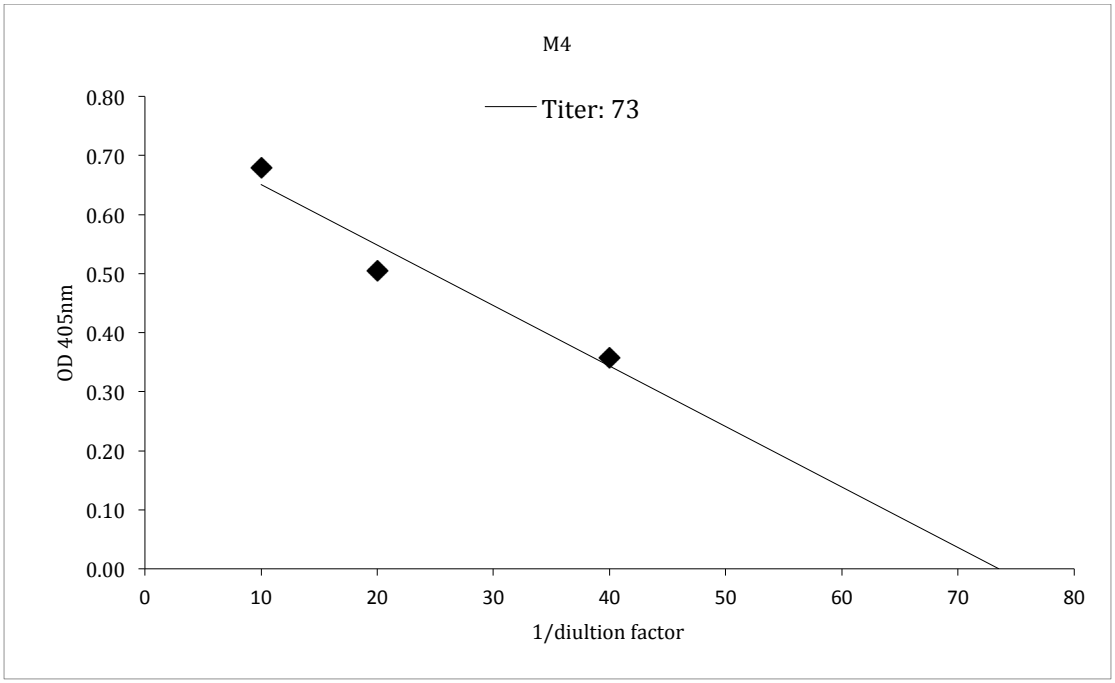
IgG titers

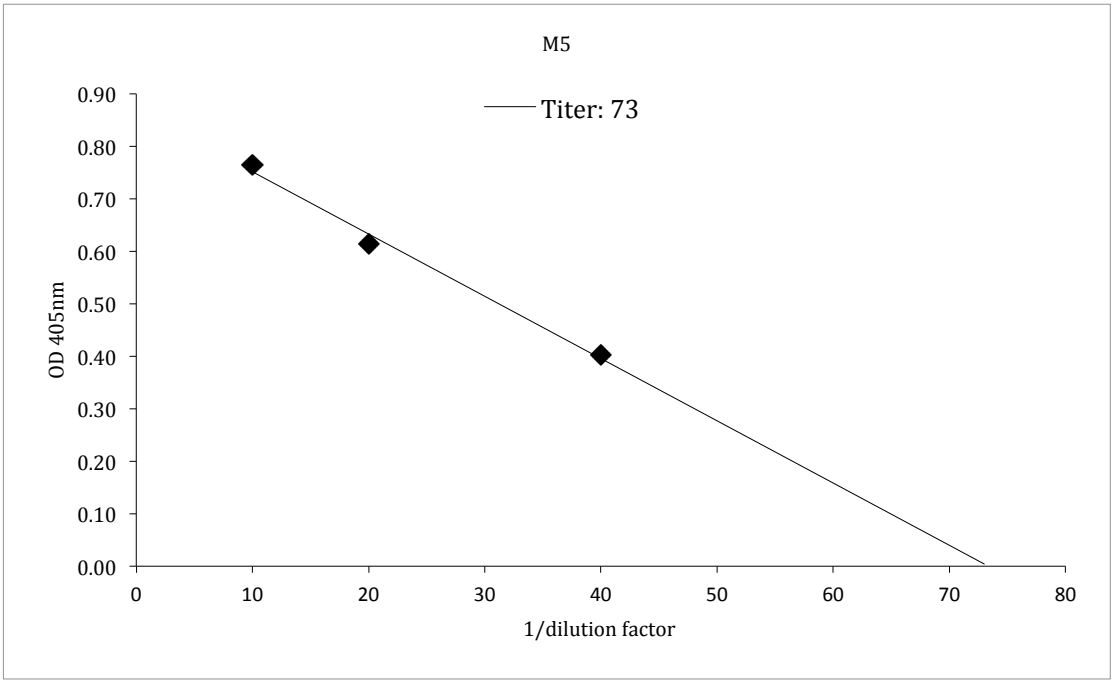
Mice Number	Lungs IgG titers
M1	76
M2	59
M3	74
M4	73
M5	73


M1
Titer: 76
OD 405nm
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
10
20
30
40
50
60
70
80
1/dilution factor


M2
Titer: 59
OD 405nm
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
10
20
30
40
50
60
1/dilution factor

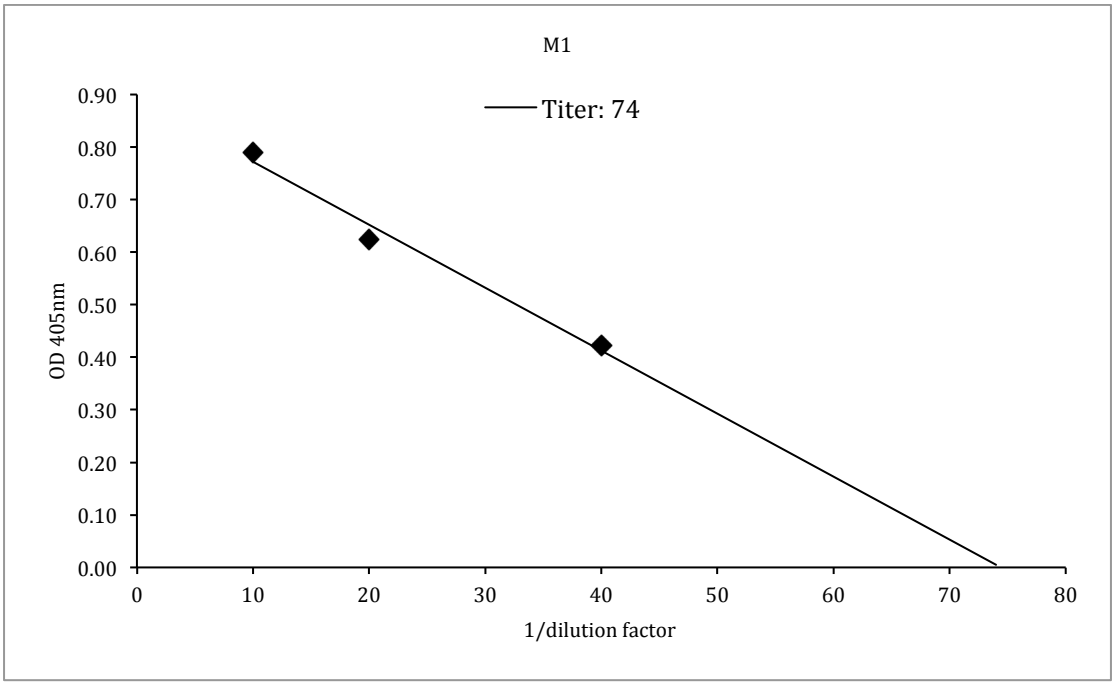

M3
Titer: 74
OD 405nm
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
10
20
30
40
50
60
70
80
1/dilution factor

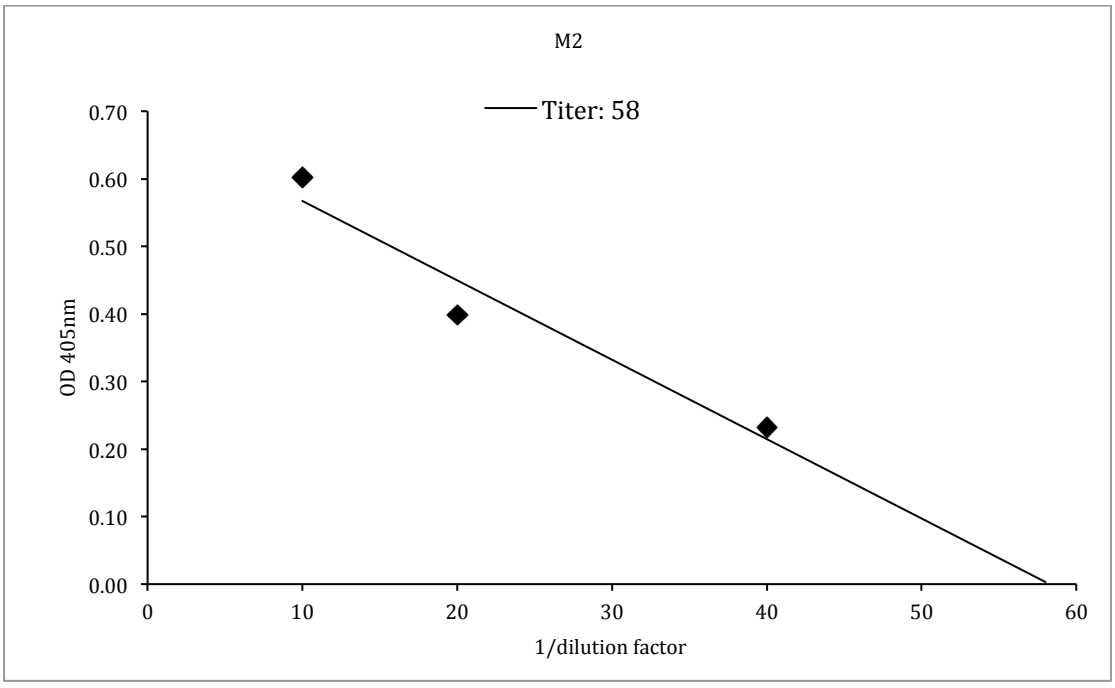

M4
Titer: 73
OD 405nm
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
10
20
30
40
50
60
70
80
1/diultion factor

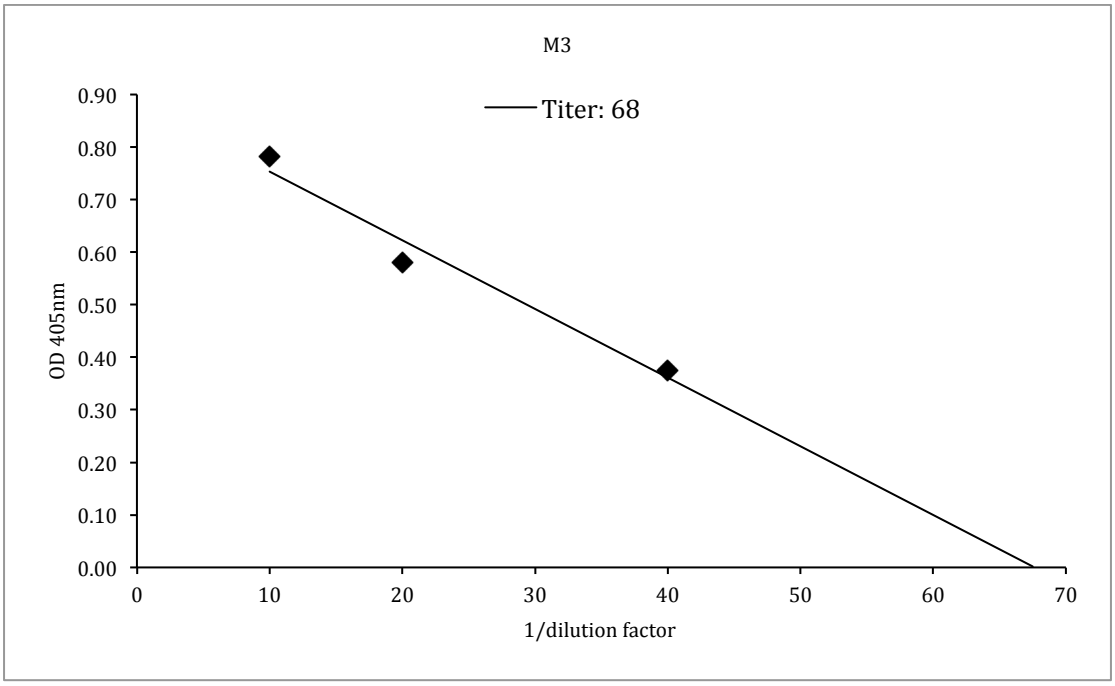


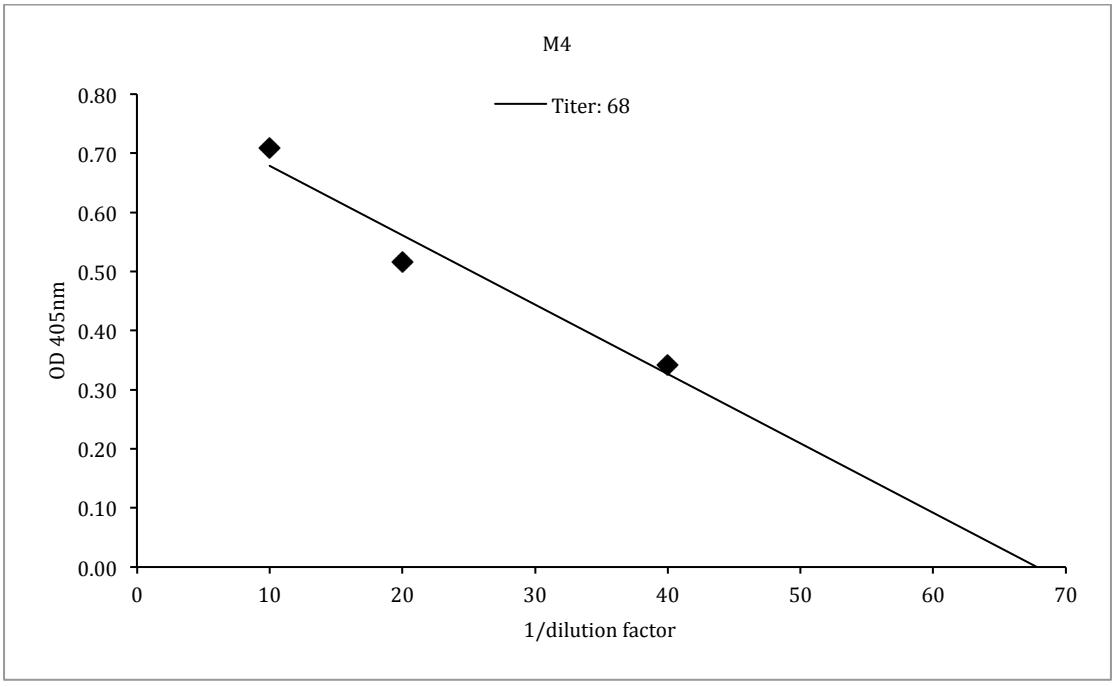
IgG1 titers

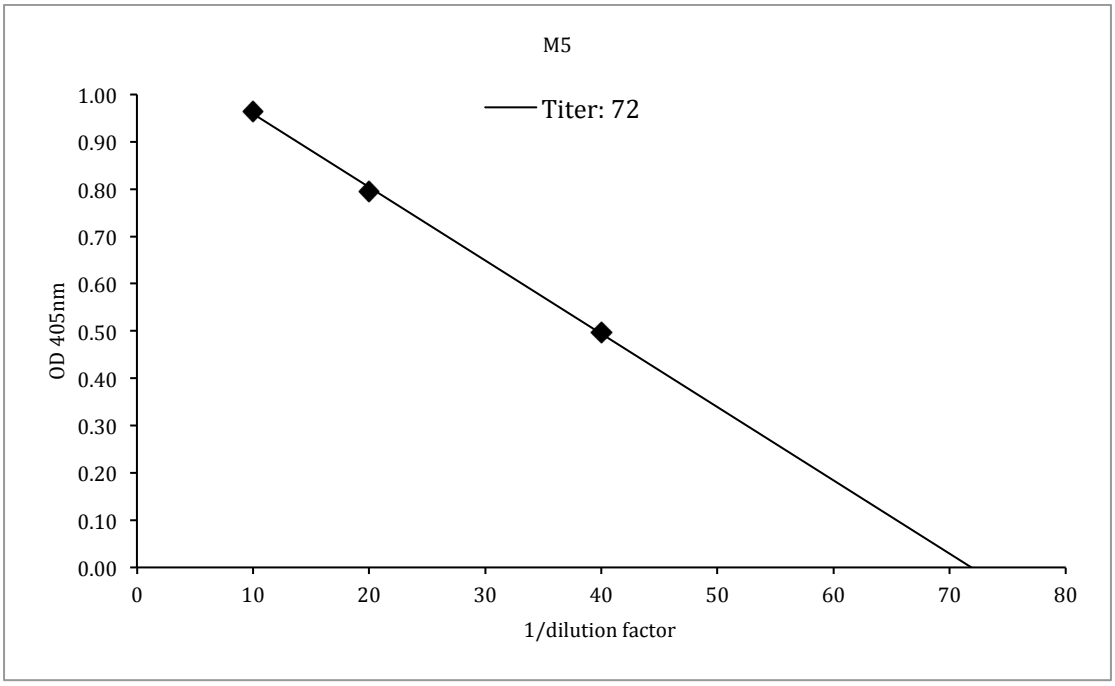
Mice Number	Lungs IgG1 titers
M1	74
M2	58
M3	68
M4	68
M5	72


M1
Titer: 74
OD 405nm
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
10
20
30
40
50
60
70
80
1/dilution factor


M2
Titer: 58
OD 405nm
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
10
20
30
40
50
60
1/dilution factor

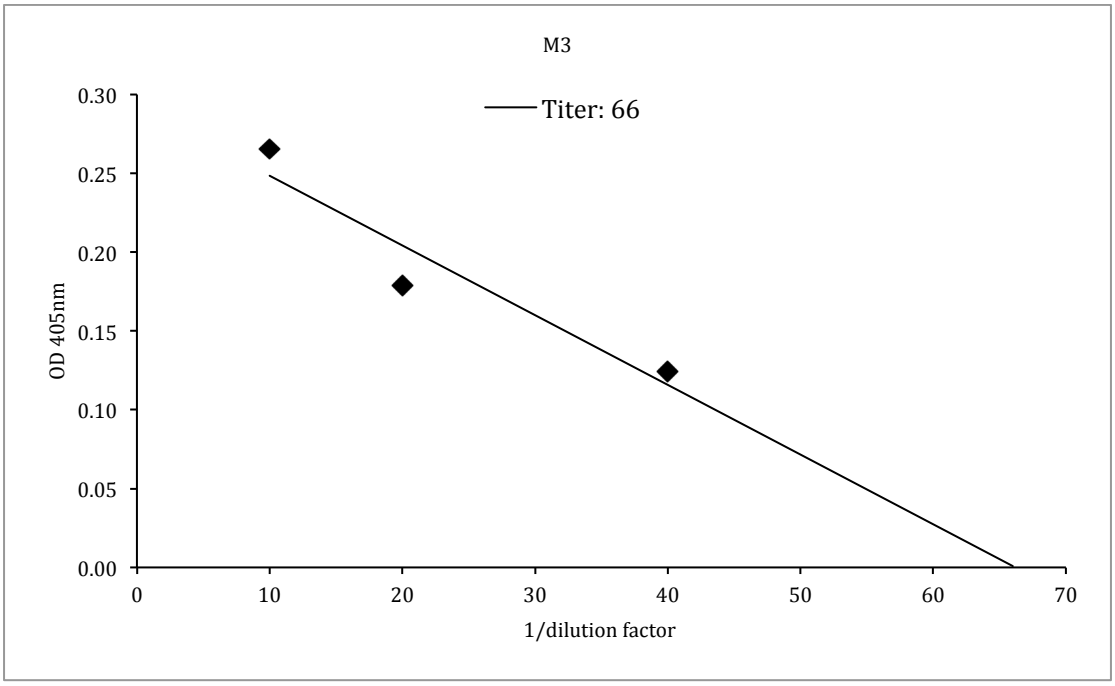

M3
Titer: 68
OD 405nm
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
10
20
30
40
50
60
70
1/dilution factor


M4
Titer: 68
OD 405nm
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
10
20
30
40
50
60
70
1/dilution factor



IgG2a [Single point titers except for M3 titer, three-point extrapolation]

Mice Number	Lungs IgG2a titers
M1	1.1
M2	0.6
M3	66*
M4	1.3
M5	0.7



IgA [Single point titeration]

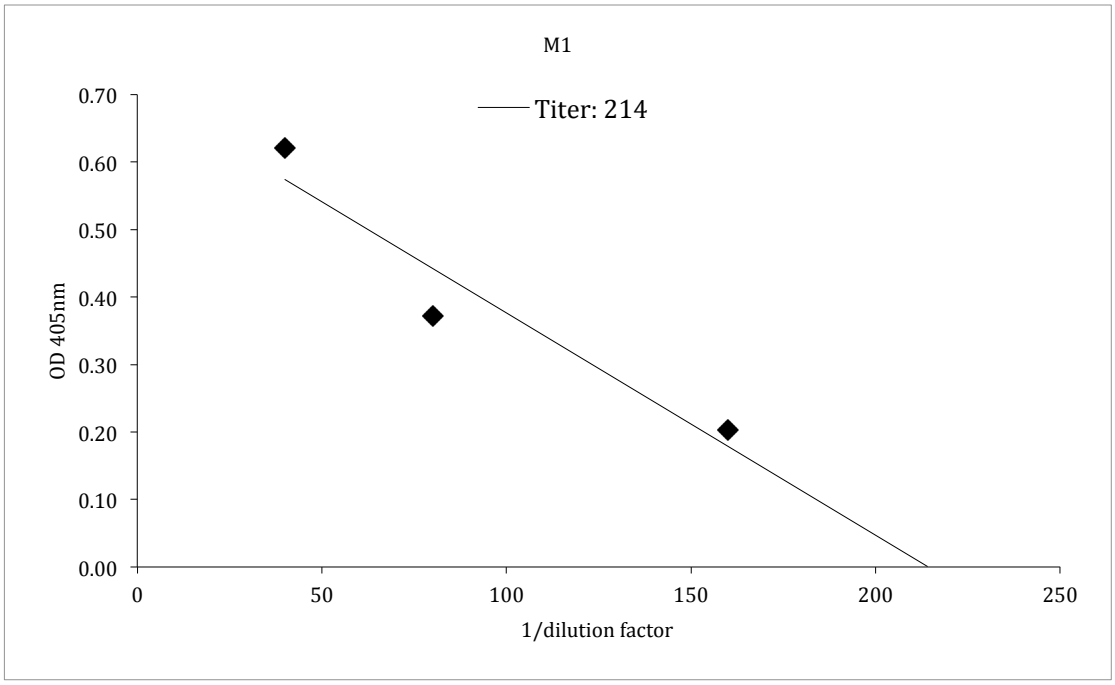
Mice Number	Lungs IgA titers
M1	0.6
M2	0.5
M3	0.7
M4	0.6
M5	0.5

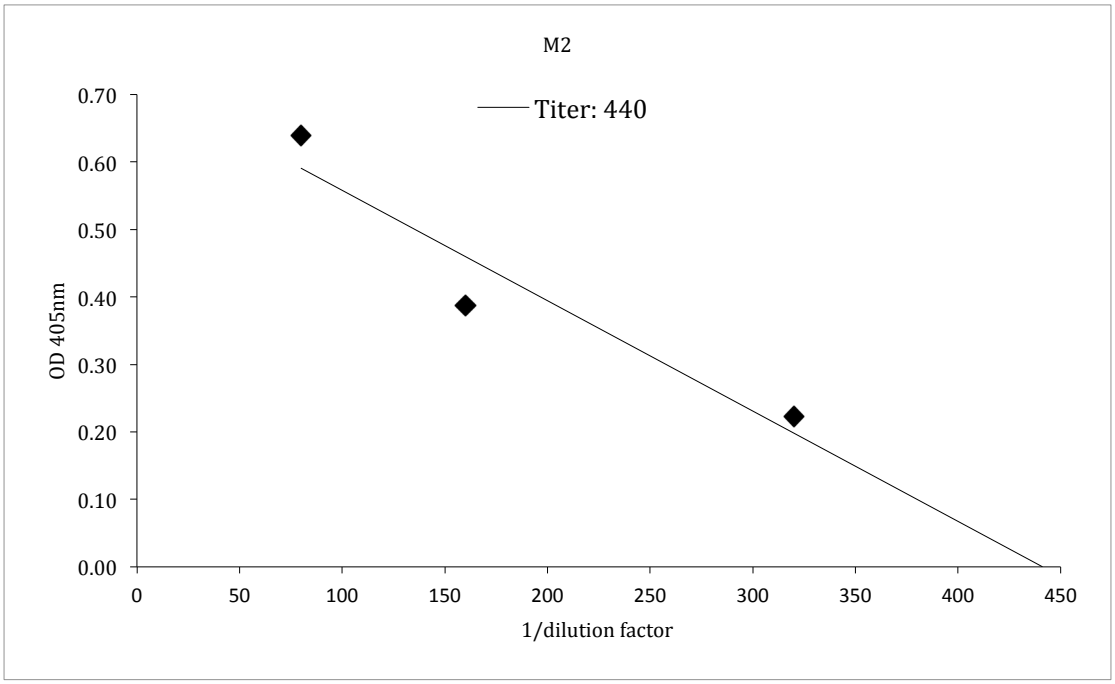
Antigen-specific immune response of mice vaccinated with two doses of live *aroQ*BP.

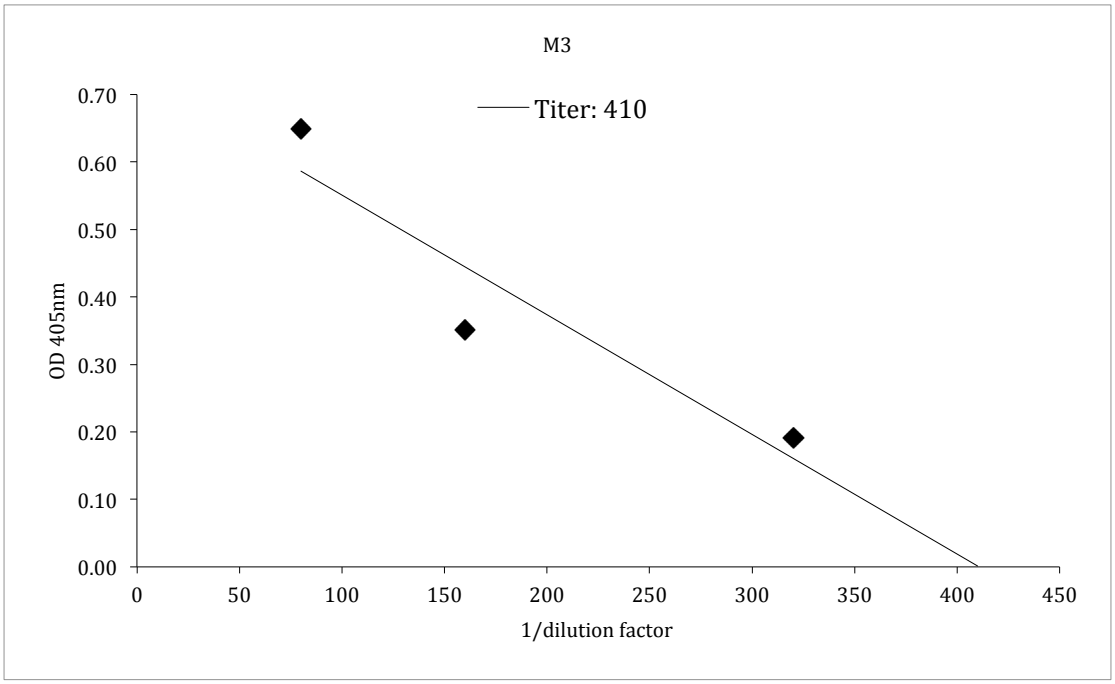
IgG titers

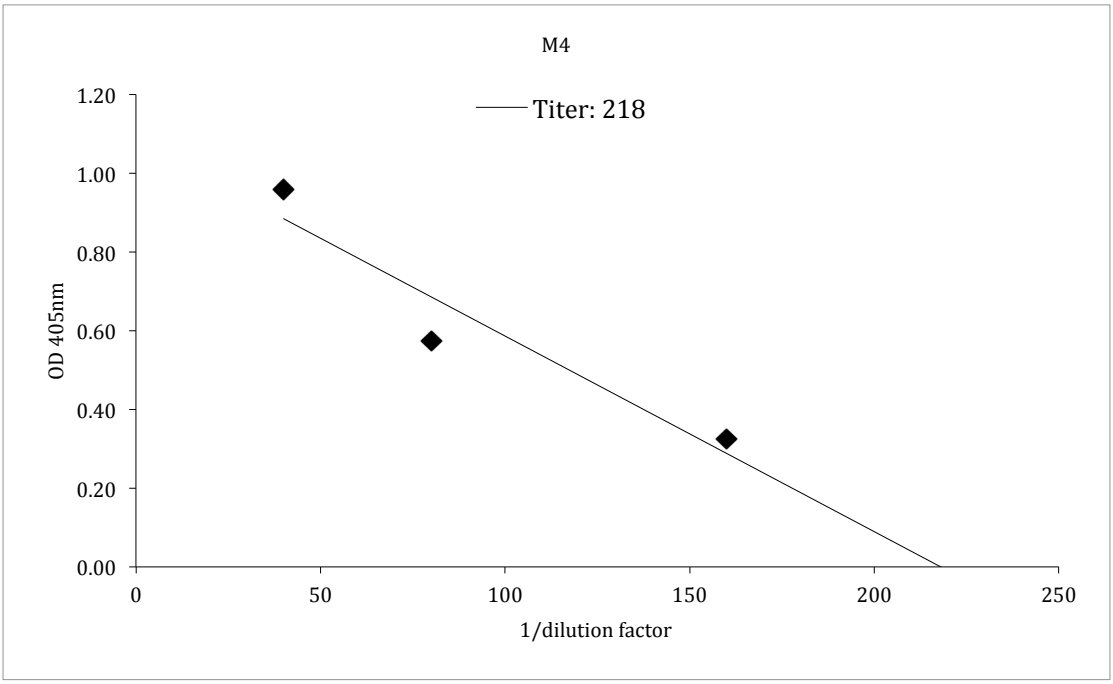
Mice Number	Lungs IgG titers
M1	214
M2	440
M3	410
M4	218
M5	ND

ND denotes not determined due to lack of availability of lung homogenate.



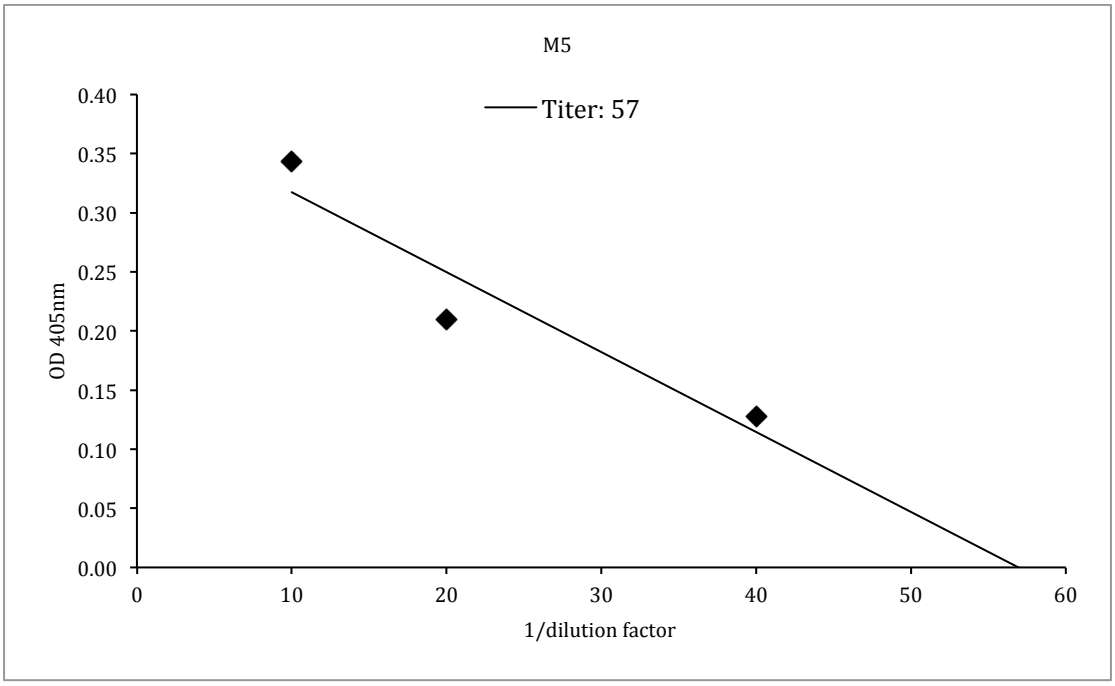

M2
Titer: 440
OD 405nm
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
50
100
150
200
250
300
350
400
450
1/dilution factor


M3
Titer: 410
OD 405nm
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
50
100
150
200
250
300
350
400
450
1/dilution factor



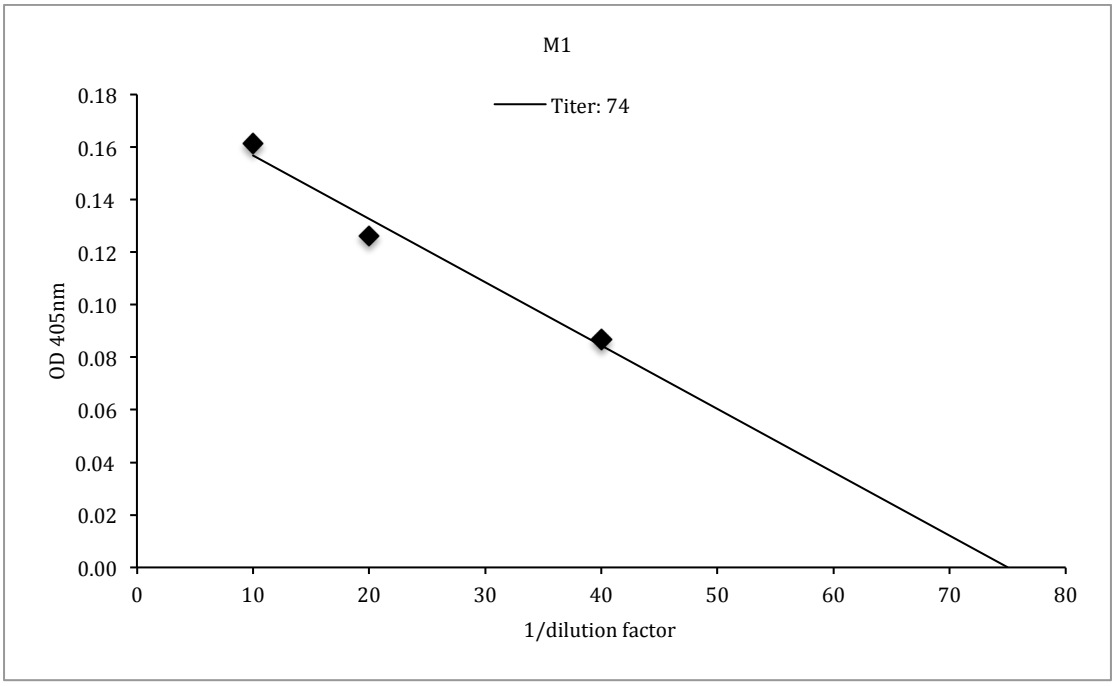
IgG1 [Single point titers except for M5 titer, three-points extrapolation]

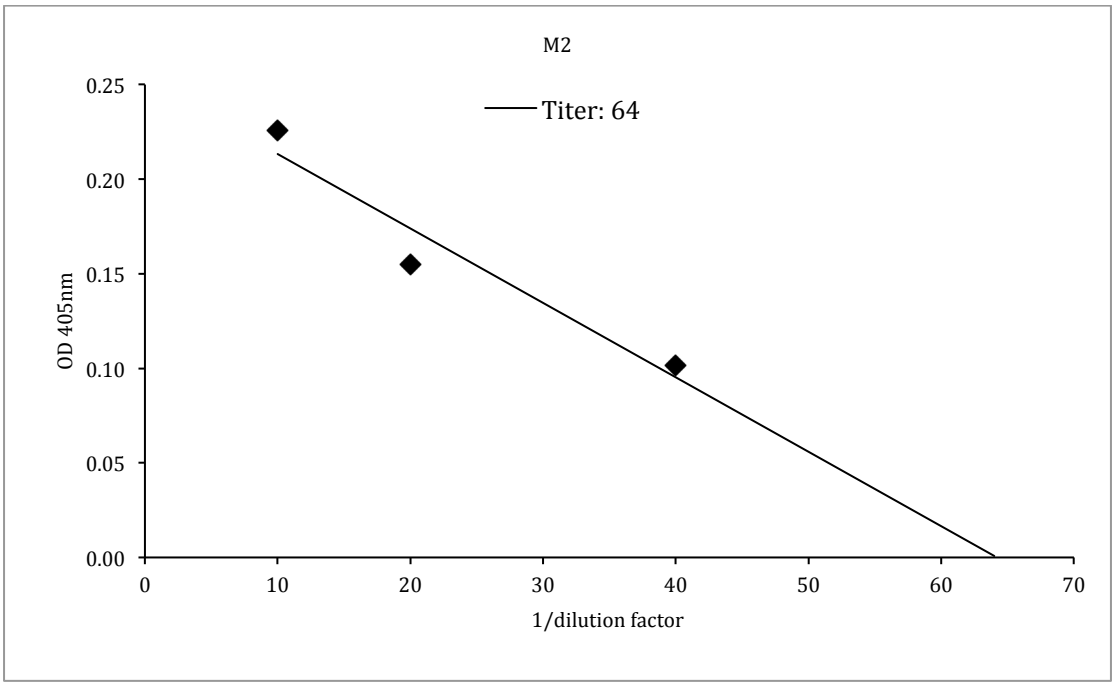
Mice Number	Lungs IgG1 titers
M1	1.1
M2	1.7
M3	1.2
M4	1.2
M5	57*

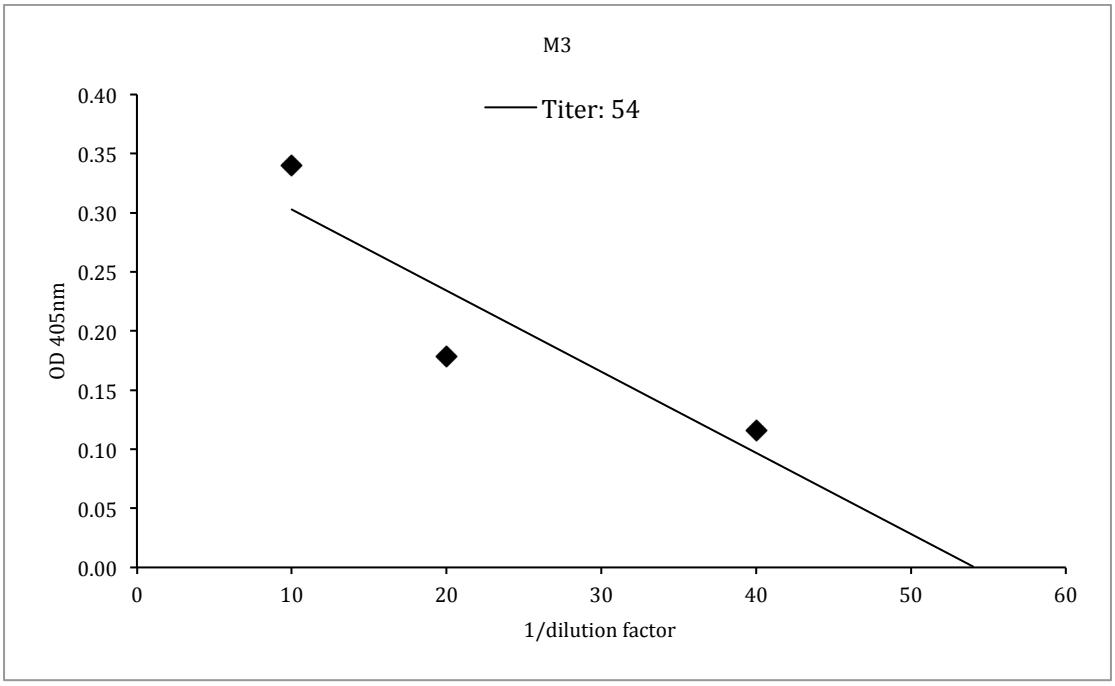


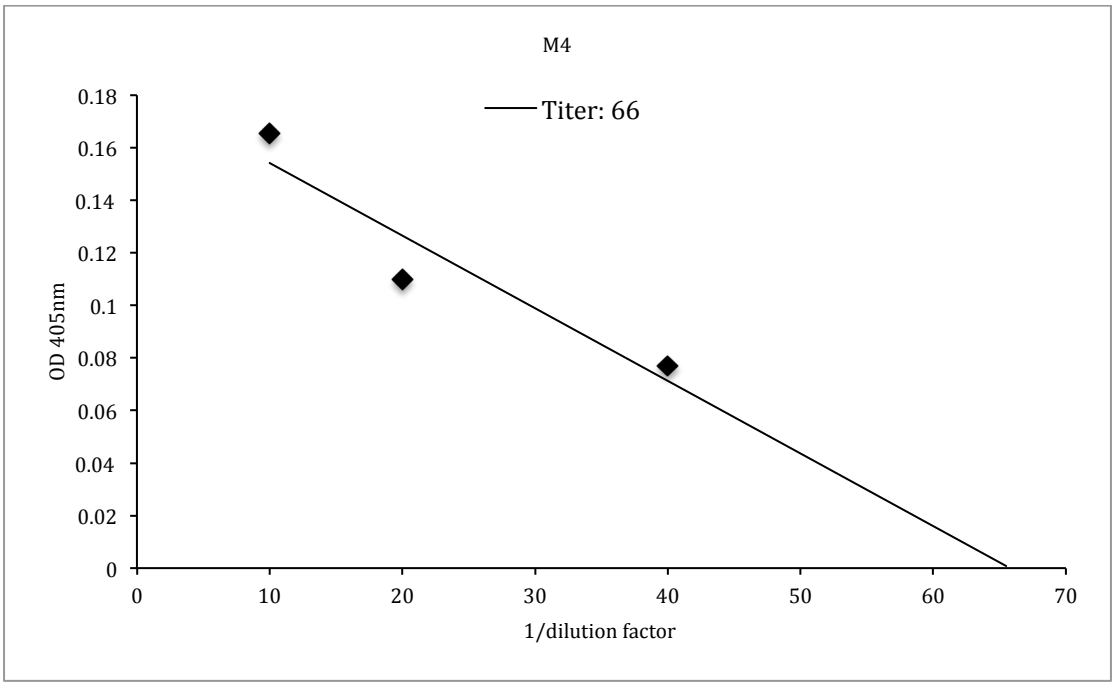
IgG2a titers

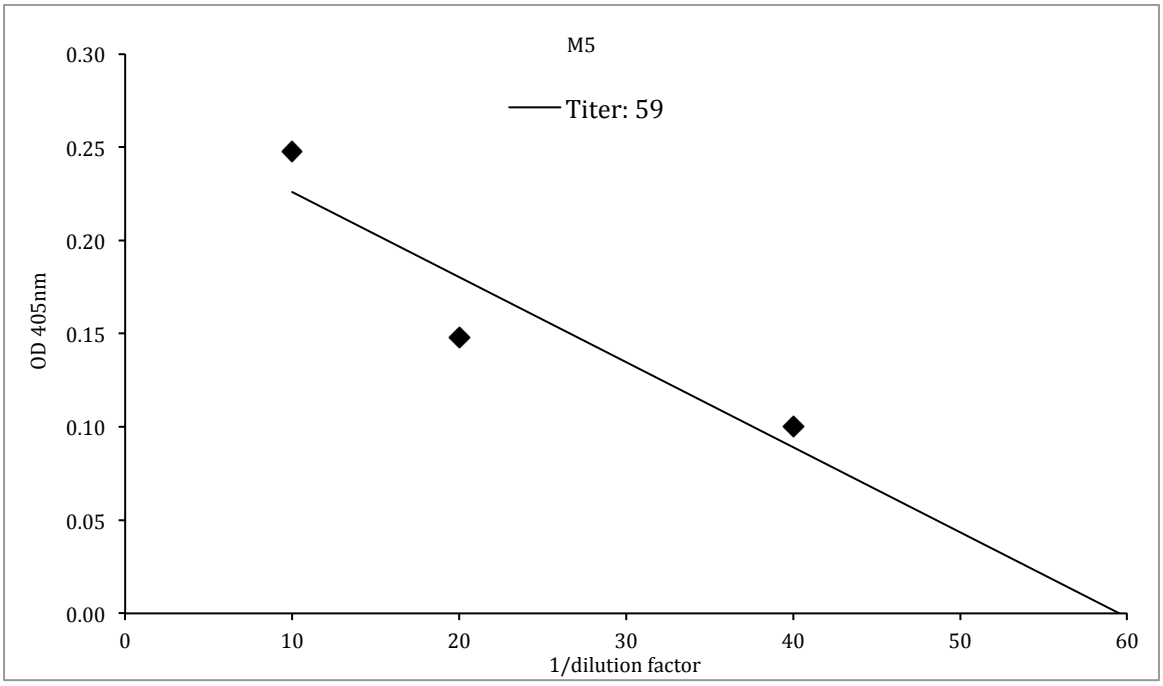
Mice Number	Lungs IgG2a titers
M1	74
M2	64
M3	54
M4	66
M5	59


M1
Titer: 74
OD 405nm
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
10
20
30
40
50
60
70
80
1/dilution factor


M2
Titer: 64
OD 405nm
0.25
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
50
60
70
1/dilution factor


M3
Titer: 54
OD 405nm
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
50
60
1/dilution factor


M4
Titer: 66
OD 405nm
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
10
20
30
40
50
60
70
1/dilution factor



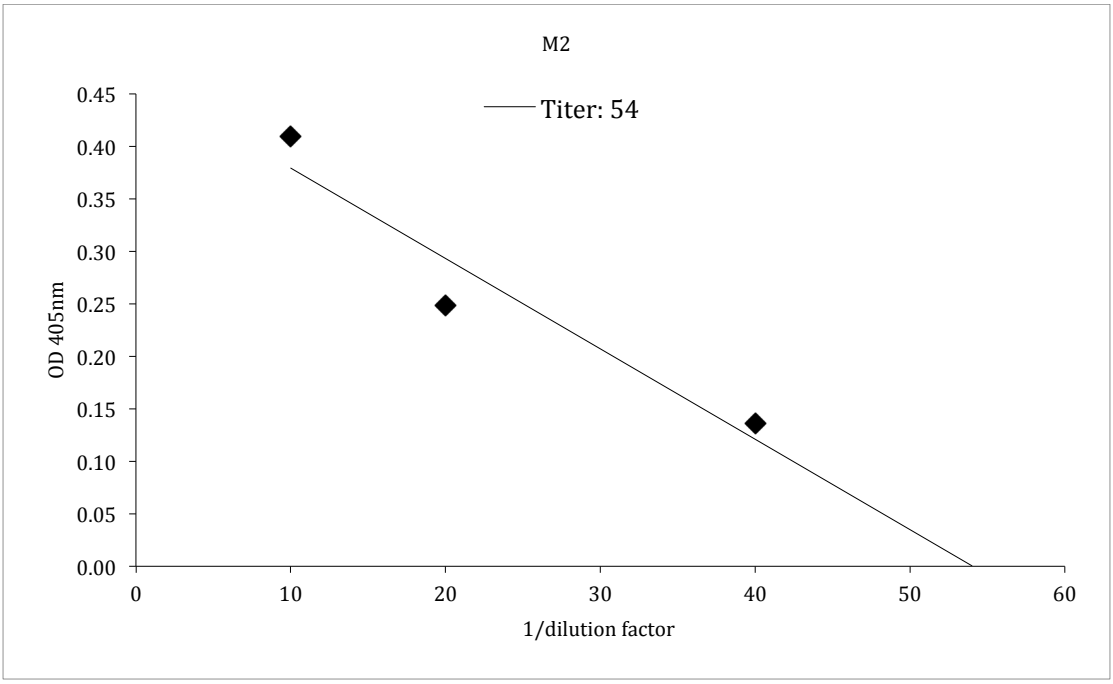
IgA titers

Mice Number	Lungs IgA titers
M1	0.7
M2	0.6
M3	1.1
M4	0.6
M5	0.6

Antigen-specific immune response of mice vaccinated with one dose of live *aroQ*BP.

IgG [single point titers except for M2 titer determined by the 3-points extrapolation]

Mice Number	Lungs IgG titers
M1	1.4
M2	54
M3	1.5
M4	2.3
M5	1.0



IgG1 [Single point titers]

Mice Number	Lungs IgG1 titers
M1	0.5
M2	0.9
M3	0.5
M4	0.6
M5	0.6

IgG2a titers

Mice Number	Lungs IgG2a titers
M1	0.5
M2	0.8
M3	0.6
M4	0.7
M5	0.6

IgA titers

Mice Number	Lungs IgA titers
M1	0.5
M2	0.5
M3	0.5
M4	0.5
M5	0.4

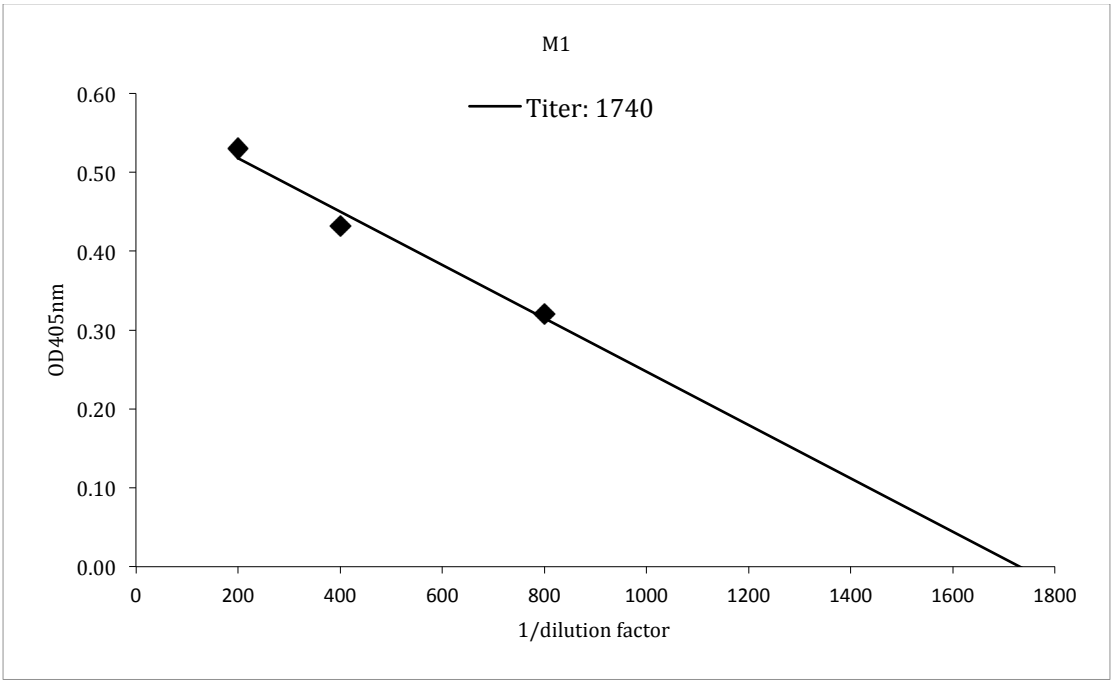
Appendix K: Serum antibody isotypes against purified pertussis toxin

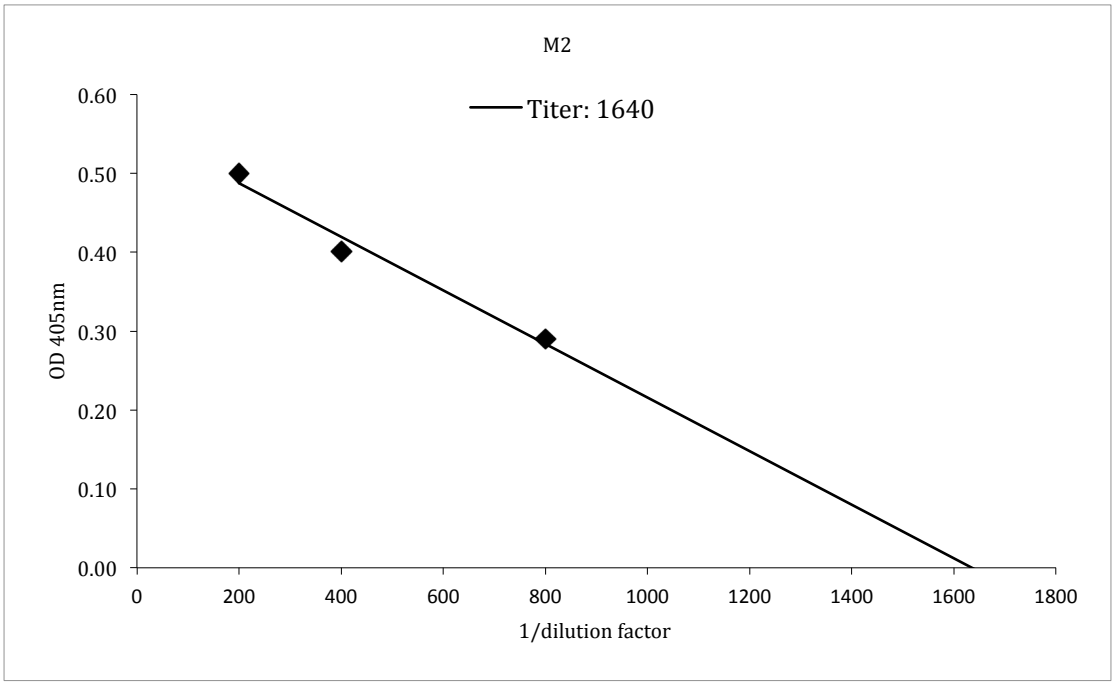
Antigen-specific immune response of three doses DTaP and one dose booster of live *aroQ*BP.

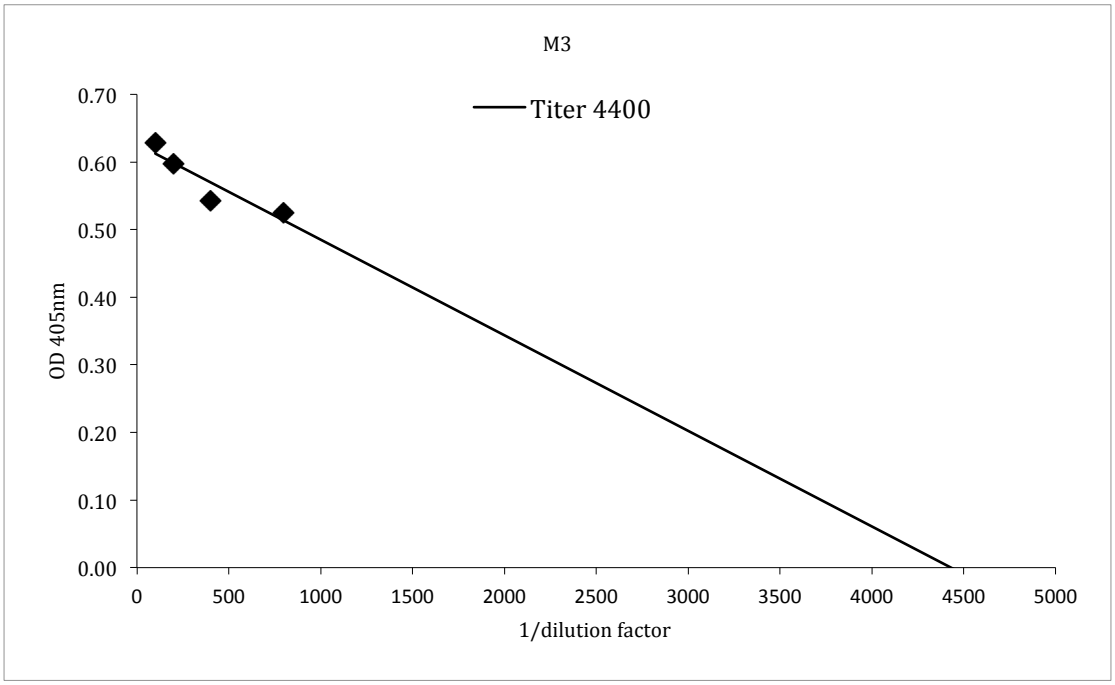
PBS sham-vaccinated Group: Titers for the PBS control groups were zero.

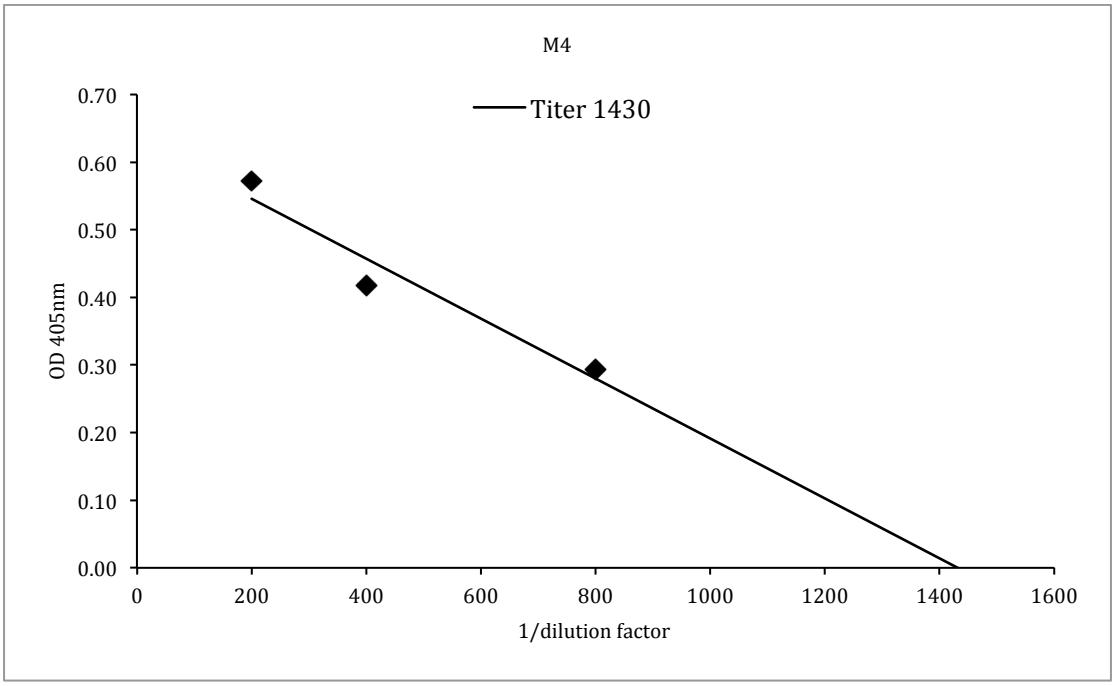
IgG titers

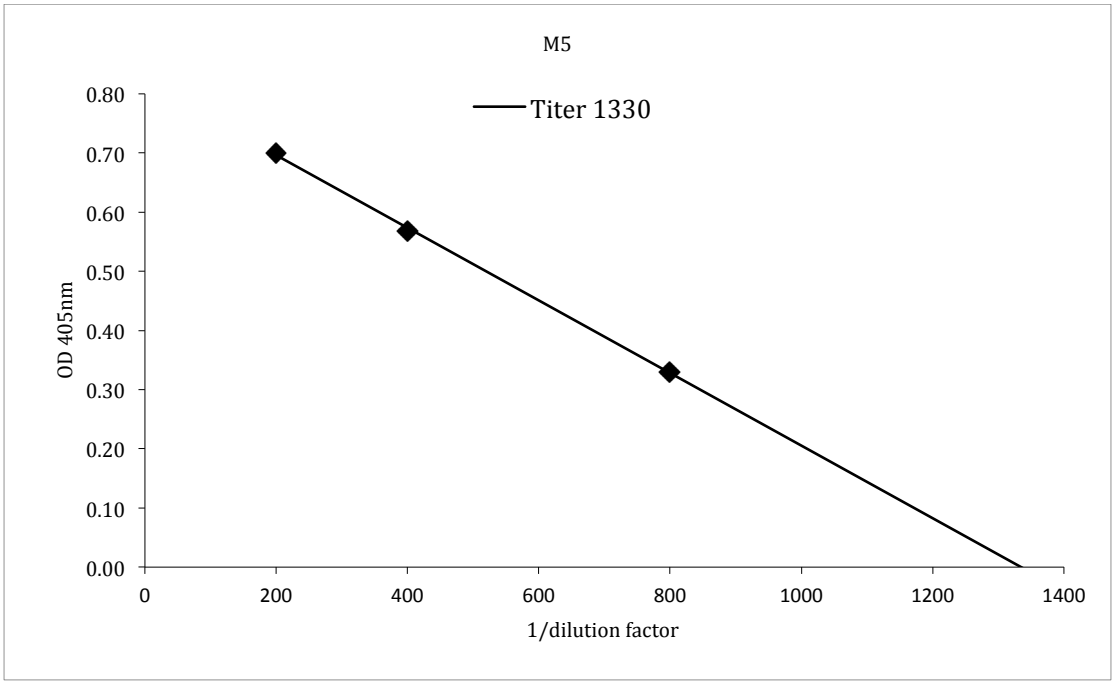
Mice Number	Serum IgG titers
M1	1740
M2	1640
M3	4400
M4	1430
M5	1330




M2
Titer: 1640
OD 405nm
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
200
400
600
800
1000
1200
1400
1600
1800
1/dilution factor

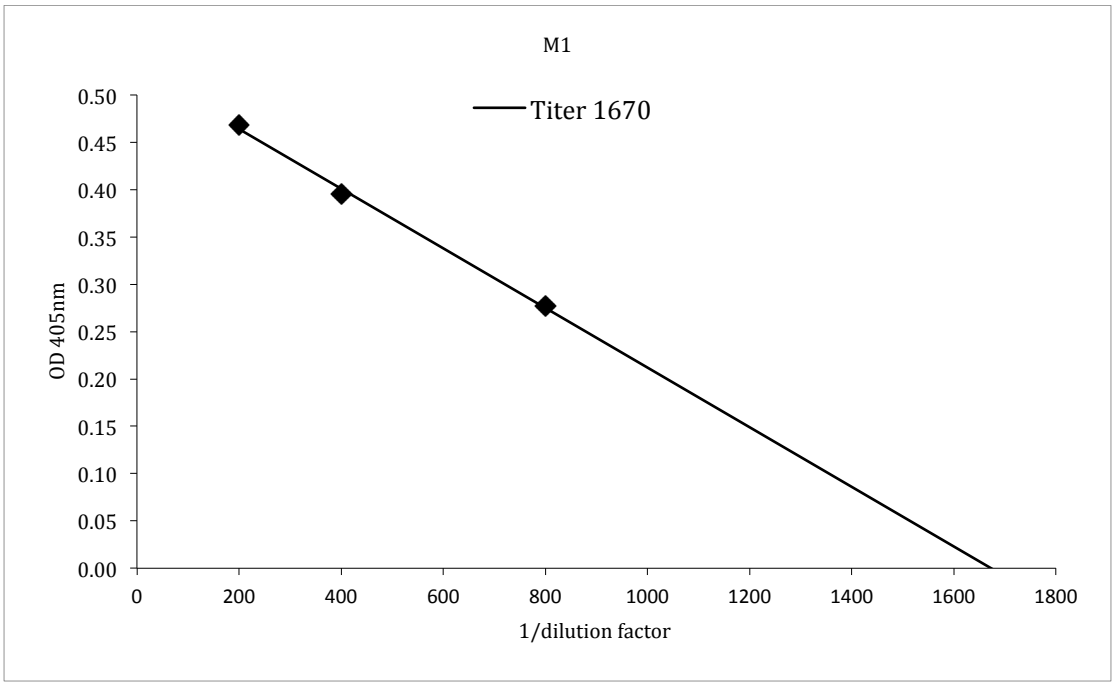

M3
Titer 4400
OD 405nm
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
1/dilution factor

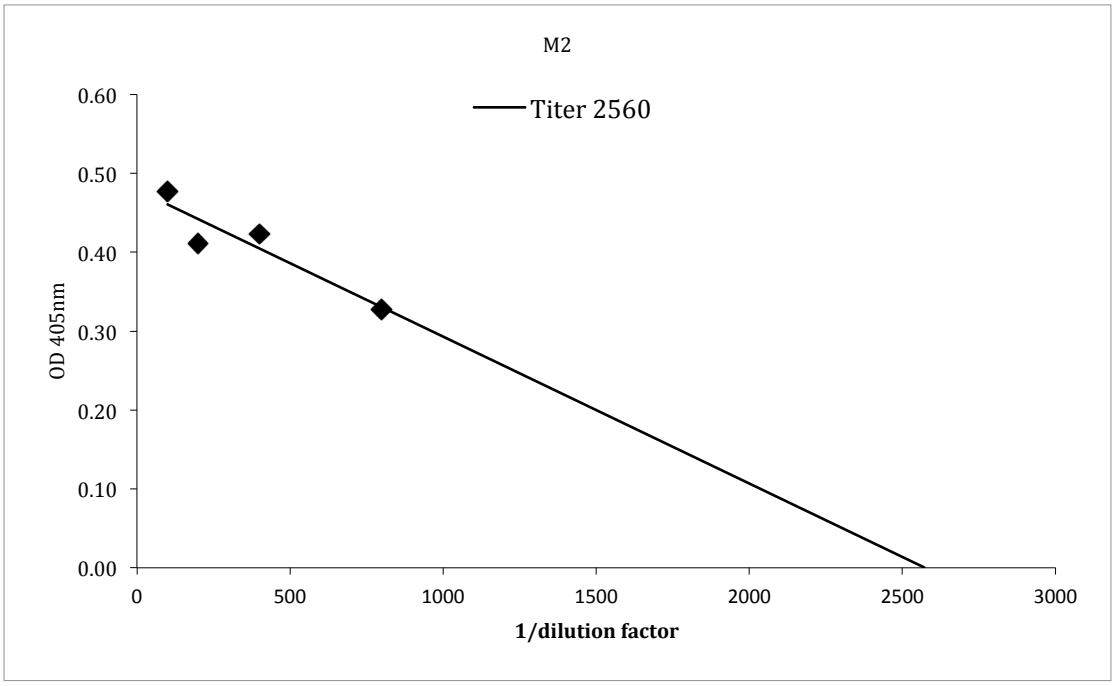

M4
Titer 1430
OD 405nm
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
200
400
600
800
1000
1200
1400
1600
1/dilution factor

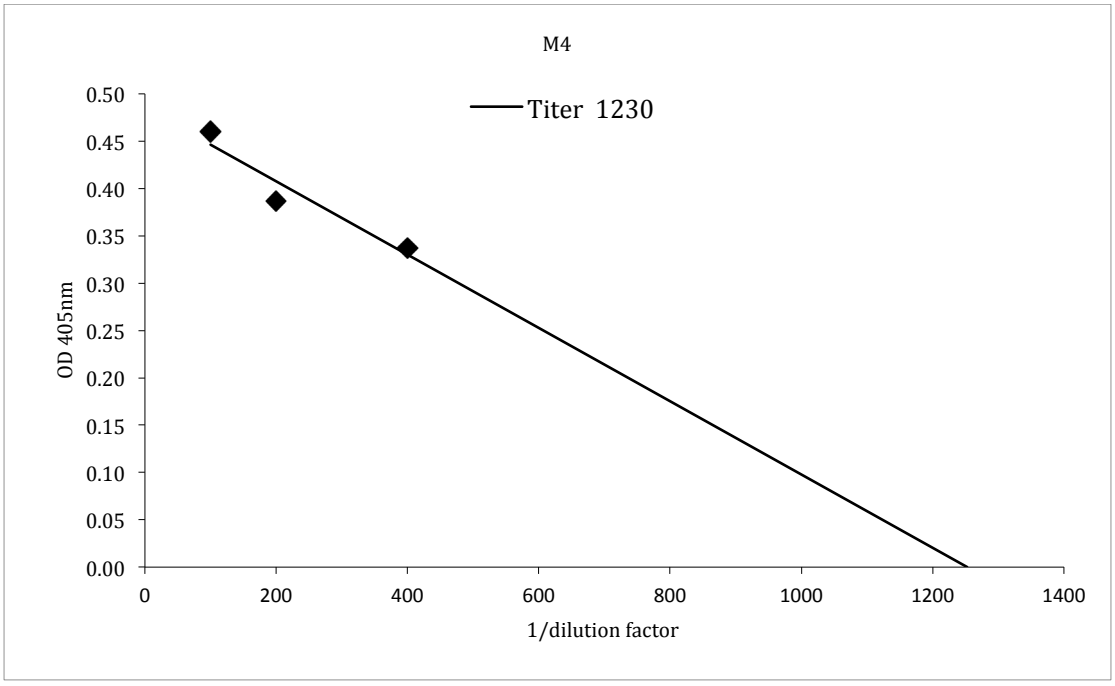

M5
Titer 1330
OD 405nm
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
200
400
600
800
1000
1200
1400
1/dilution factor

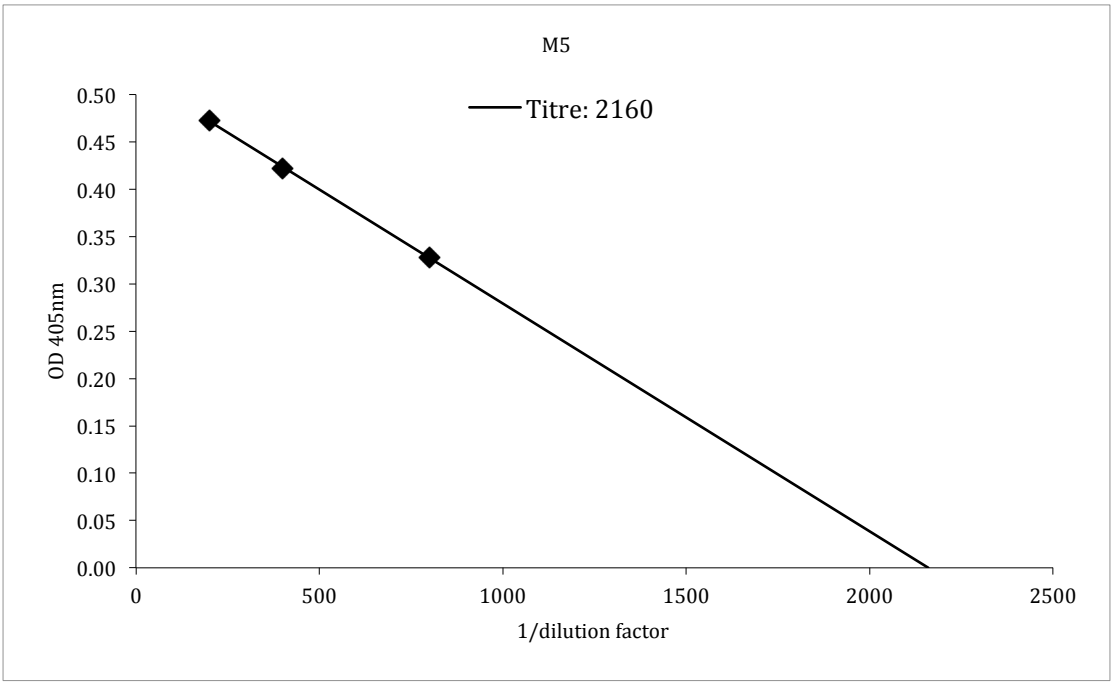
IgG1 titers

Mice Number	Serum IgG1 titers
M1	1670
M2	2560 (4 point titer)
M3	Taken out of calculation
M4	1230
M5	1080




M2
Titer 2560
0.60
0.50
0.40
0.30
0.20
0.10
0.00
OD 405nm
0
500
1000
1500
2000
2500
3000
1/dilution factor


M4
Titer 1230
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
OD 405nm
0
200
400
600
800
1000
1200
1400
1/dilution factor



IgG2a [Single point titers]

Mice Number	Serum IgG2a titers
M1	3.7
M2	7.7
M3	4.1
M4	4.4
M5	3.5

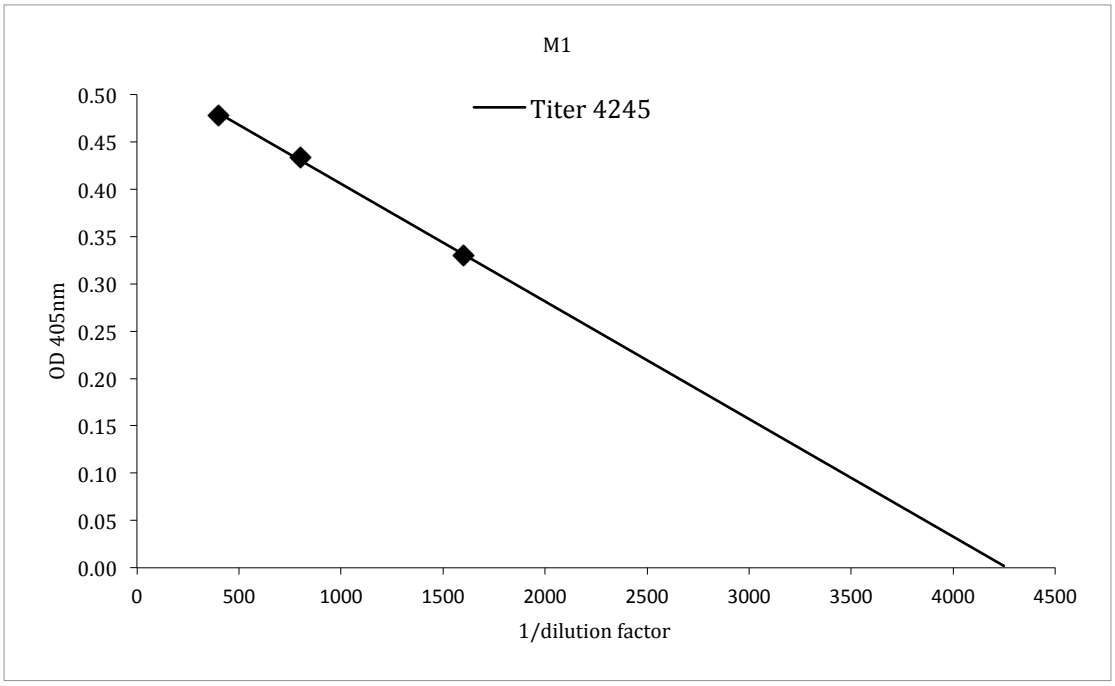
IgA [Single point titers]

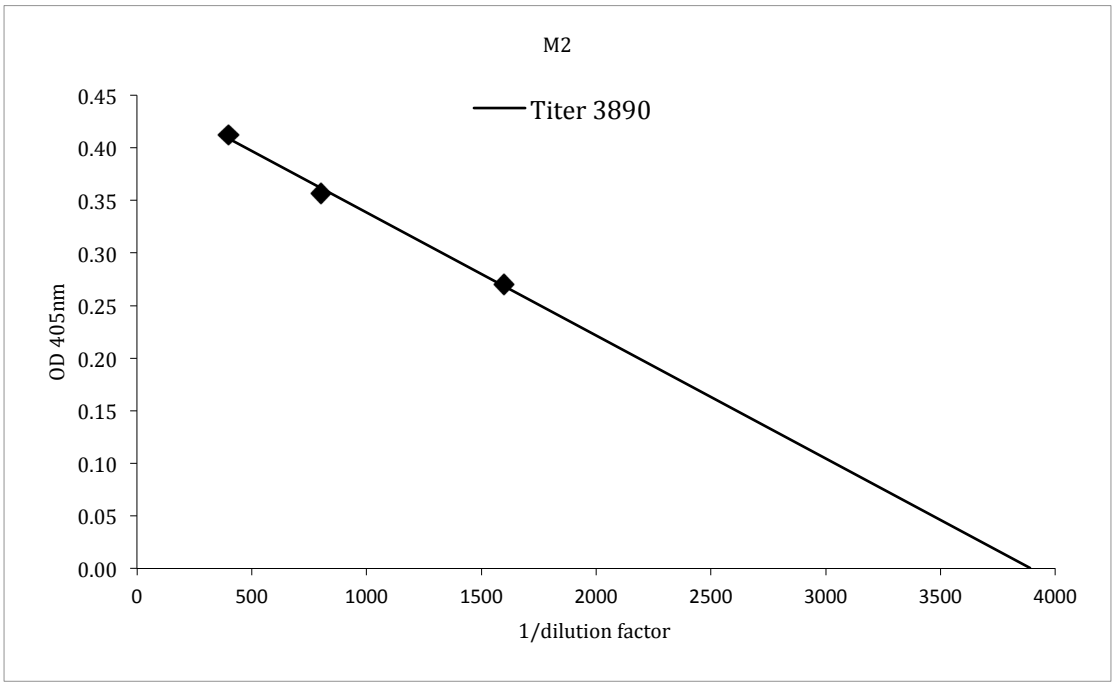
Mice Number	Serum IgA titers
M1	3.3
M2	3.2
M3	3.1
M4	3.1
M5	3.2

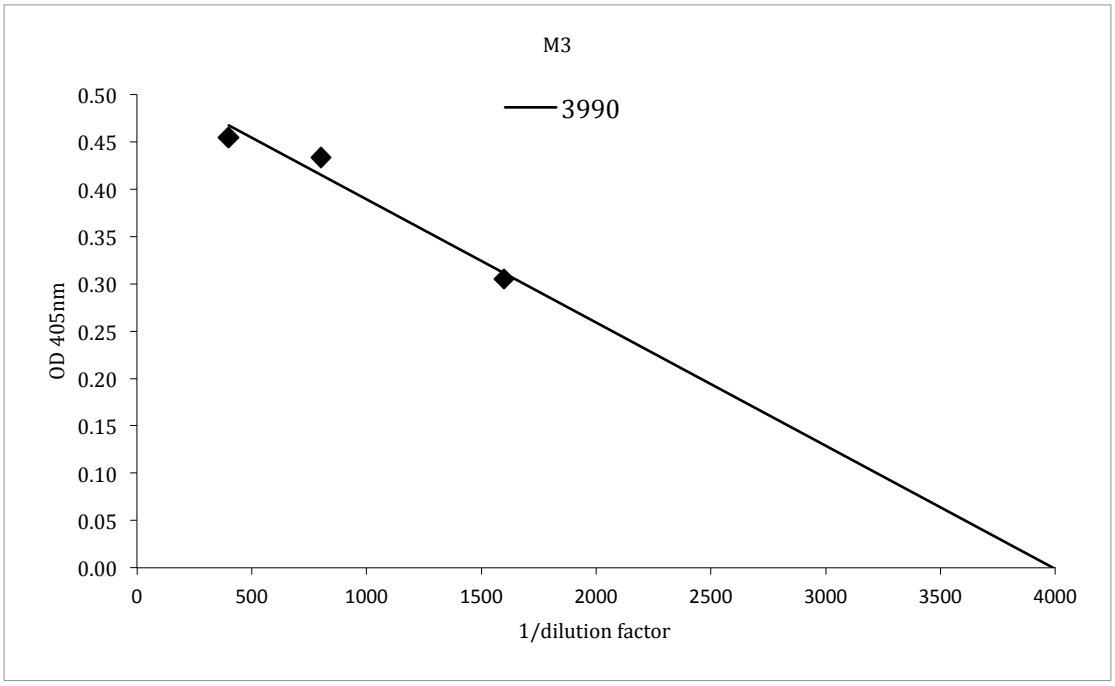
Antigen-specific immune response of mice vaccinated with three doses DTaP and two booster doses of live *aroQ*BP.

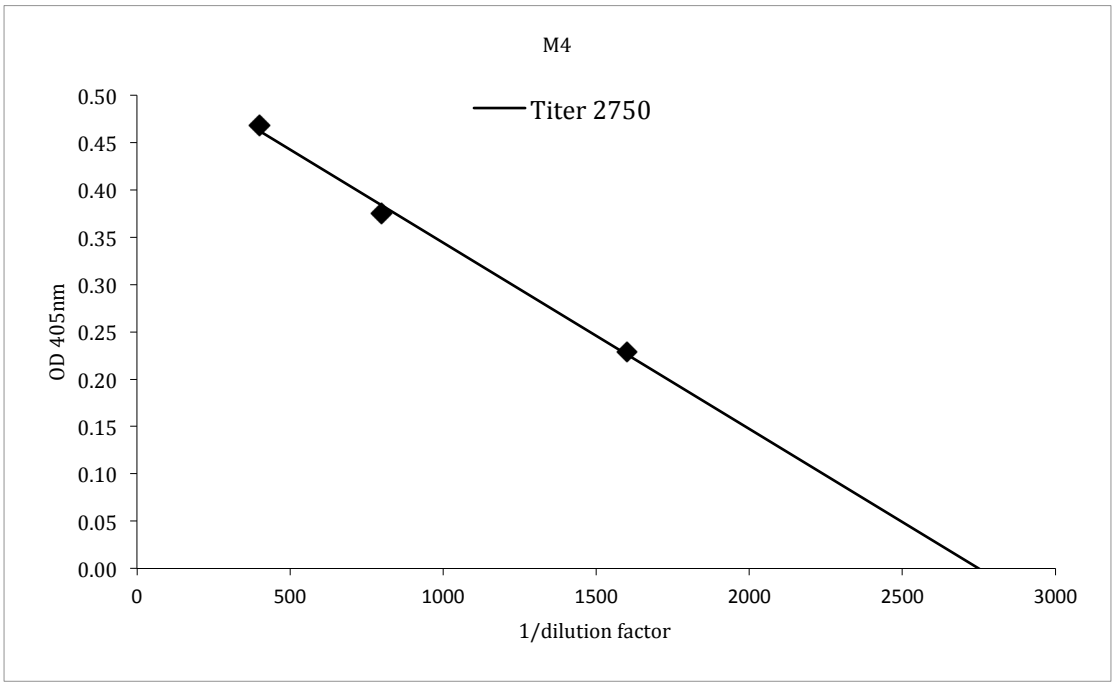
IgG titers

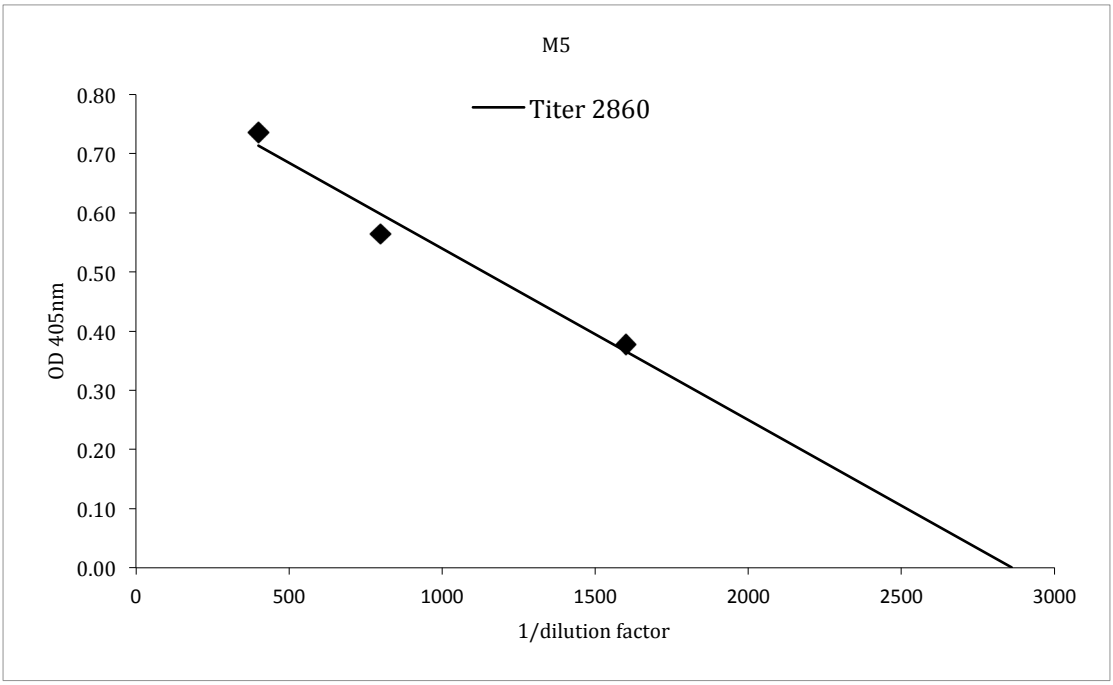
Mice Number	Serum IgG titers
M1	4245
M2	3890
M3	3990
M4	2750
M5	2860


M1
Titer 4245
OD 405nm
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
500
1000
1500
2000
2500
3000
3500
4000
4500
1/dilution factor


M2
Titer 3890
OD 405nm
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
500
1000
1500
2000
2500
3000
3500
4000
1/dilution factor

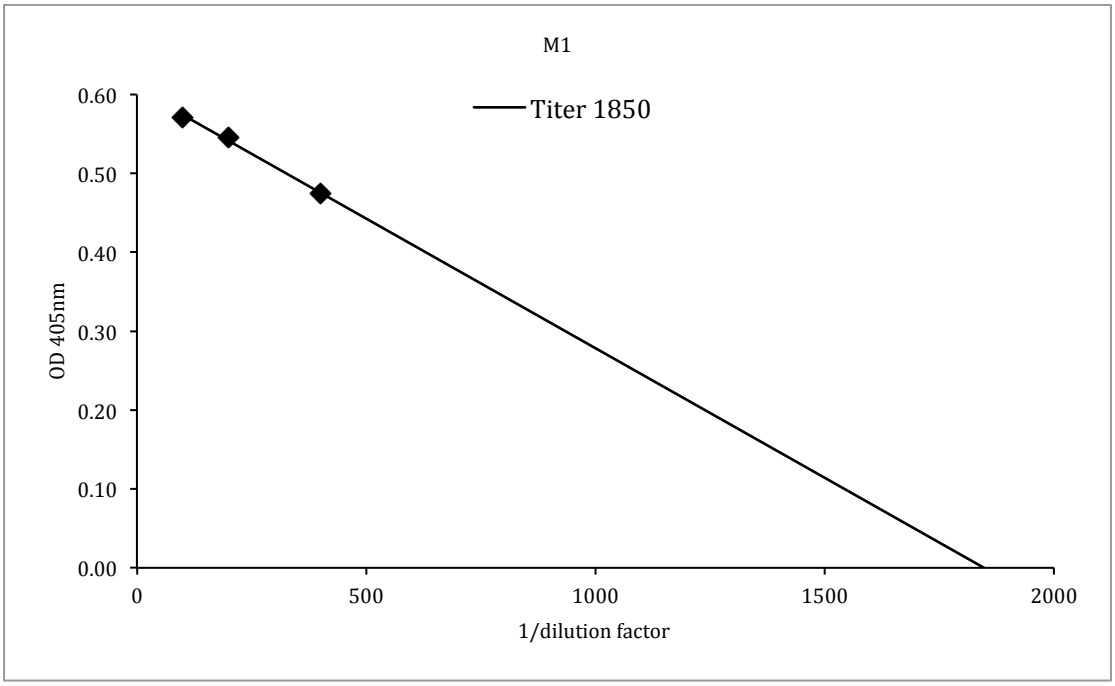

M3
3990
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
OD 405nm
0
500
1000
1500
2000
2500
3000
3500
4000
1/dilution factor

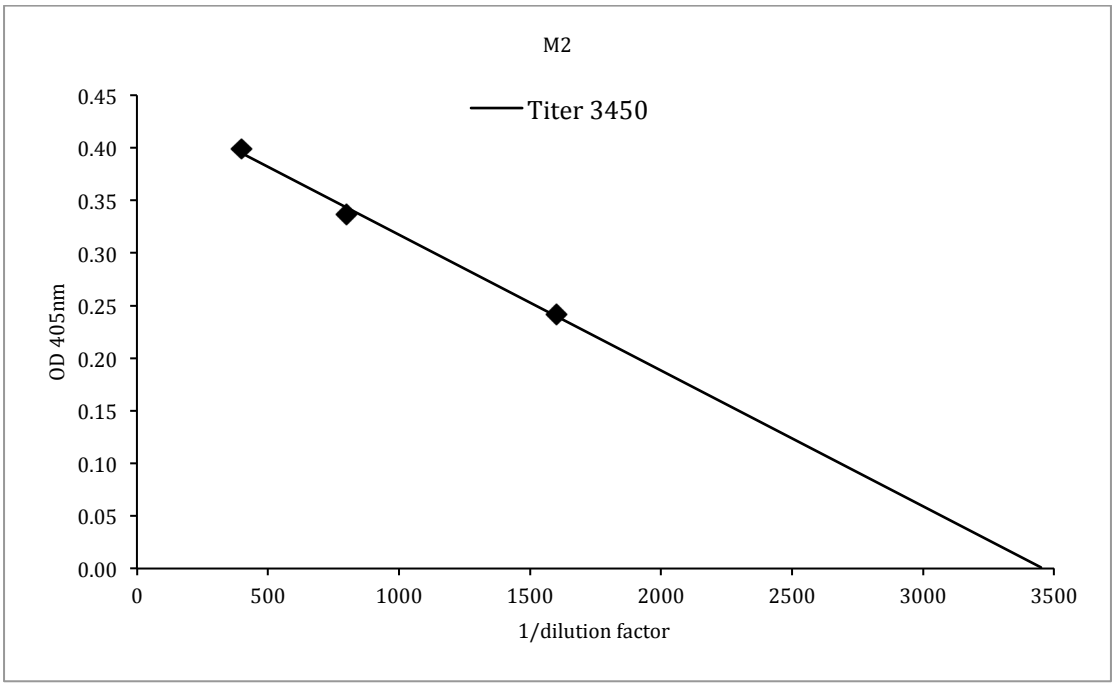

M4
Titer 2750
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
OD 405nm
0
500
1000
1500
2000
2500
3000
1/dilution factor

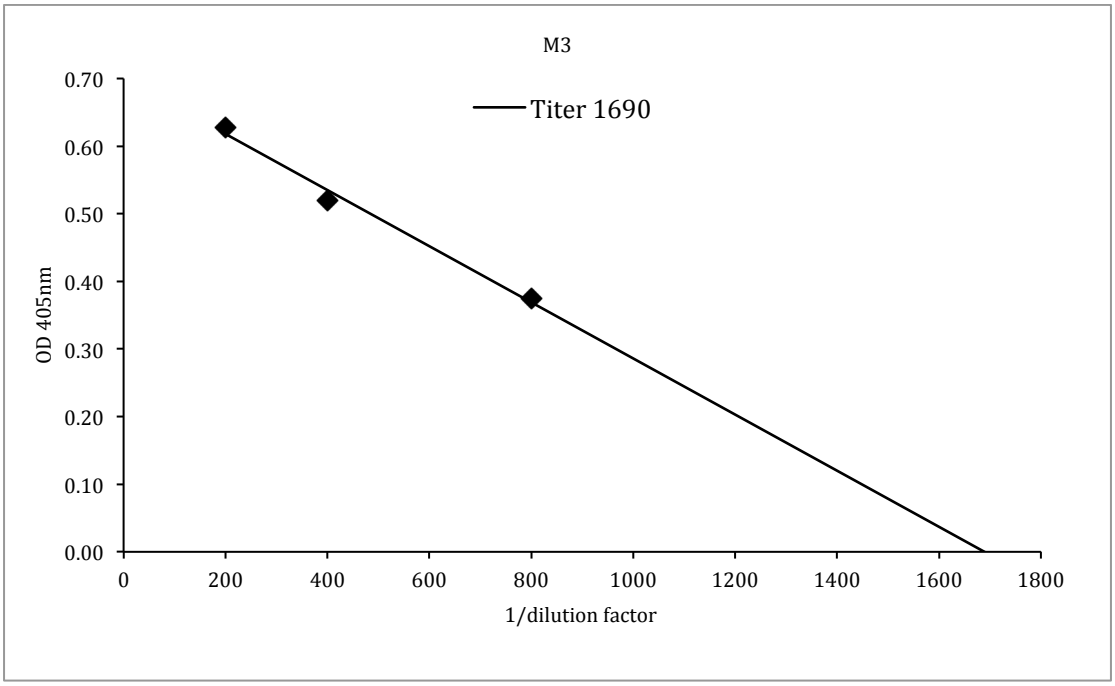


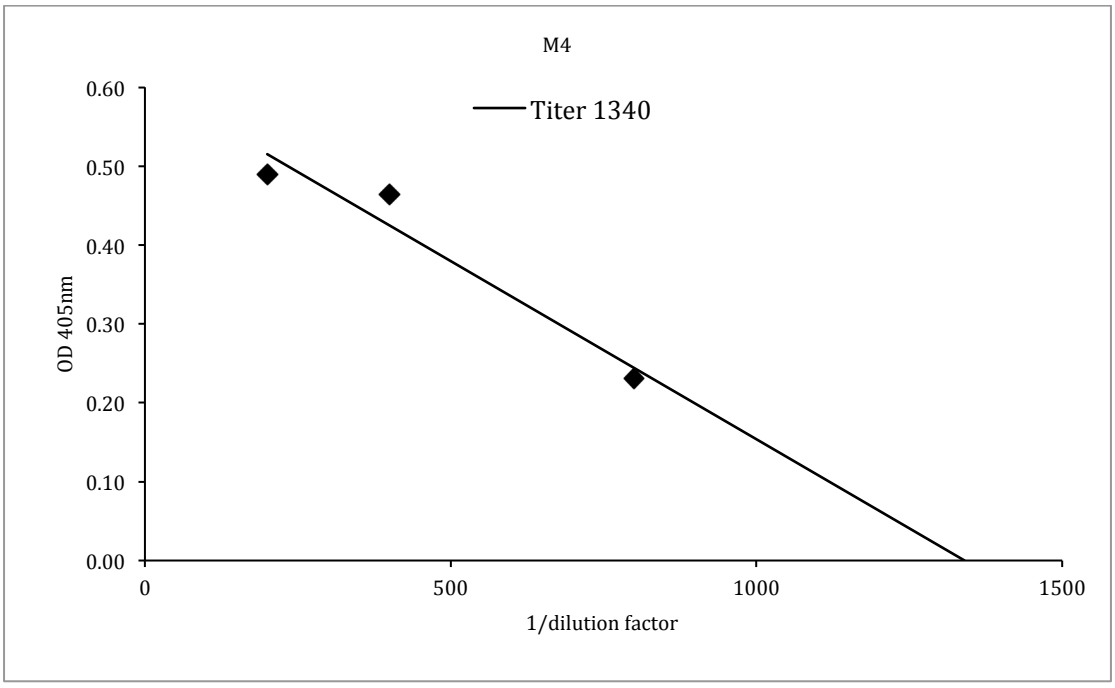
IgG1 titers

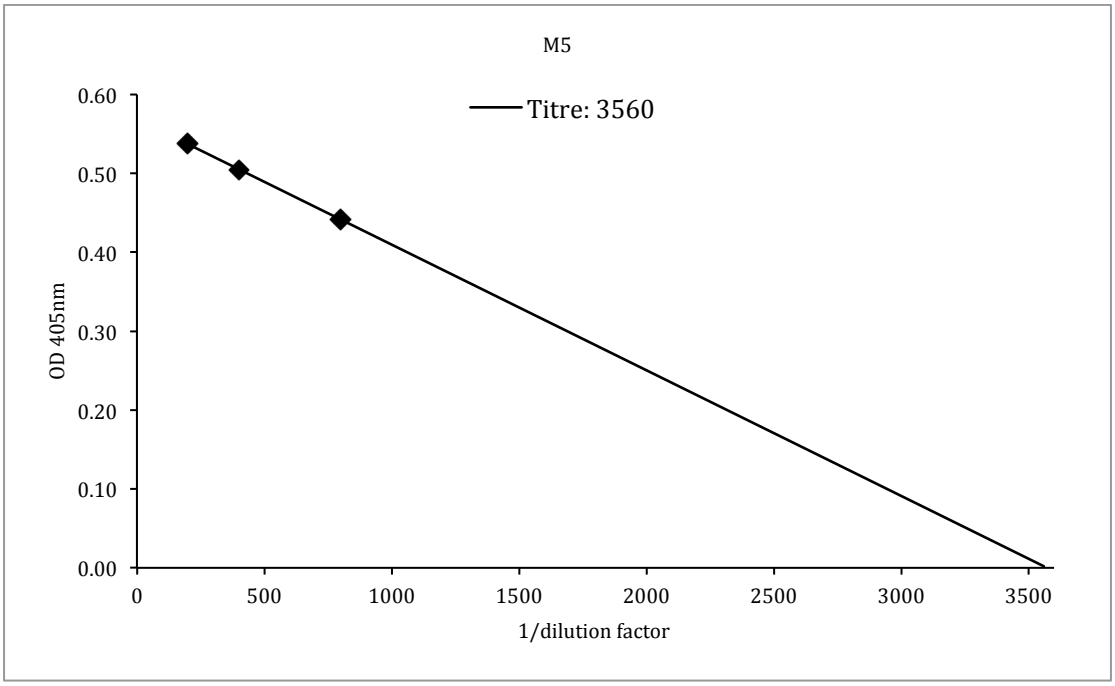
Mice Number	Serum IgG1 titers
M1	1850
M2	3450
M3	1690
M4	1340
M5	3560


M1
Titer 1850
0.60
0.50
0.40
0.30
0.20
0.10
0.00
OD 405nm
0
500
1000
1500
2000
1/dilution factor


M2
Titer 3450
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
OD 405nm
0
500
1000
1500
2000
2500
3000
3500
1/dilution factor


M3
Titer 1690
OD 405nm
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
200
400
600
800
1000
1200
1400
1600
1800
1/dilution factor


M4
Titer 1340
OD 405nm
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
500
1000
1500
1/dilution factor



IgG2a [Single point titers]

Mice Number	Serum IgG2a titers
M1	4.2
M2	3.7
M3	5.9
M4	3.4
M5	6.4

IgA [Single point titers]

Mice Number	Serum IgA titers
M1	4.1
M2	3.2
M3	3.0
M4	3.1
M5	2.8

Antigen-specific immune response mice vaccinated with two doses of live *aroQ*BP.

IgG [Single point titers]

Mice Number	Serum IgG titers
M1	3.8
M2	4.3
M3	3.7
M4	10.0
M5	3.7

IgG1 [Single point titers]

Mice Number	Serum IgG1 titers
M1	3.2
M2	4.7
M3	3.2
M4	9.2
M5	3.3

IgG2a [Single point titers]

Mice Number	Serum IgG2a titers
M1	3.5
M2	3.4
M3	3.4
M4	3.7
M5	3.2

*No IgA isotype was detected.

Antigen-specific immune response of mice vaccinated with one dose of live *aroQ*BP.

IgG [Single point titers]

Mice Number	Serum IgG titers
M1	3.4
M2	3.3
M3	3.3
M4	3.4
M5	3.3

IgG1 [Single point titers]

Mice Number	Serum IgG1 titers
M1	3.4
M2	3.3
M3	3.3
M4	3.3
M5	3.3

IgG2a [Single point titers]

Mice Number	Serum IgG2a titers
M1	3.3
M2	3.2
M3	3.2
M4	3.2
M5	3.2

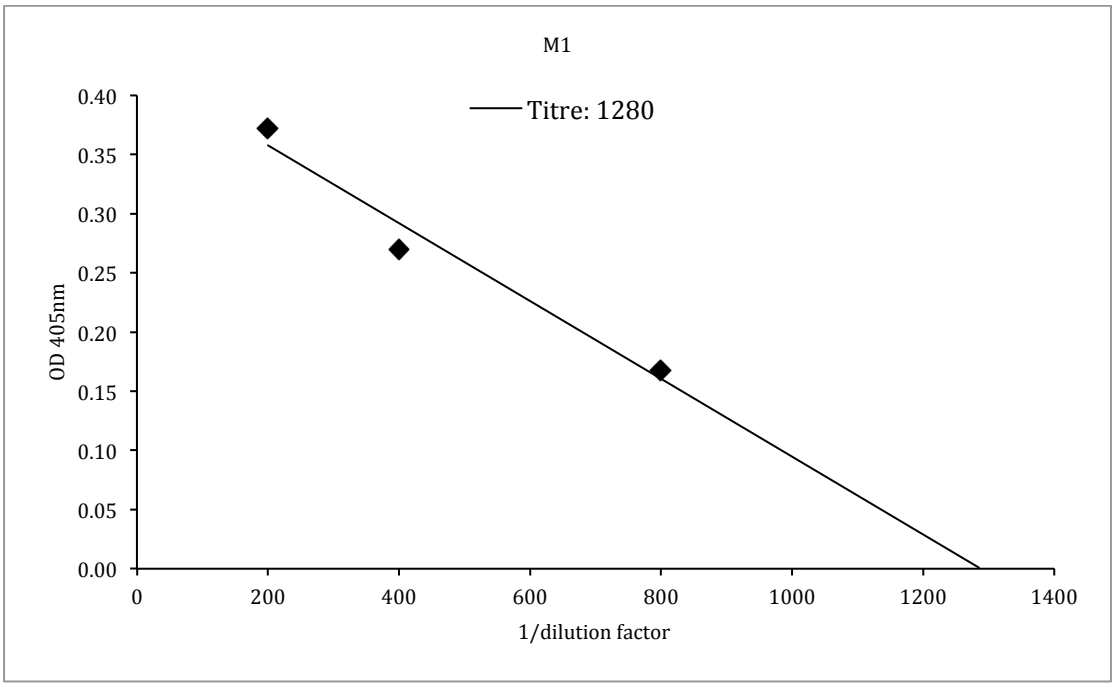
Appendix L: Serum antibody isotypes against purified filamentous haemagglutinin

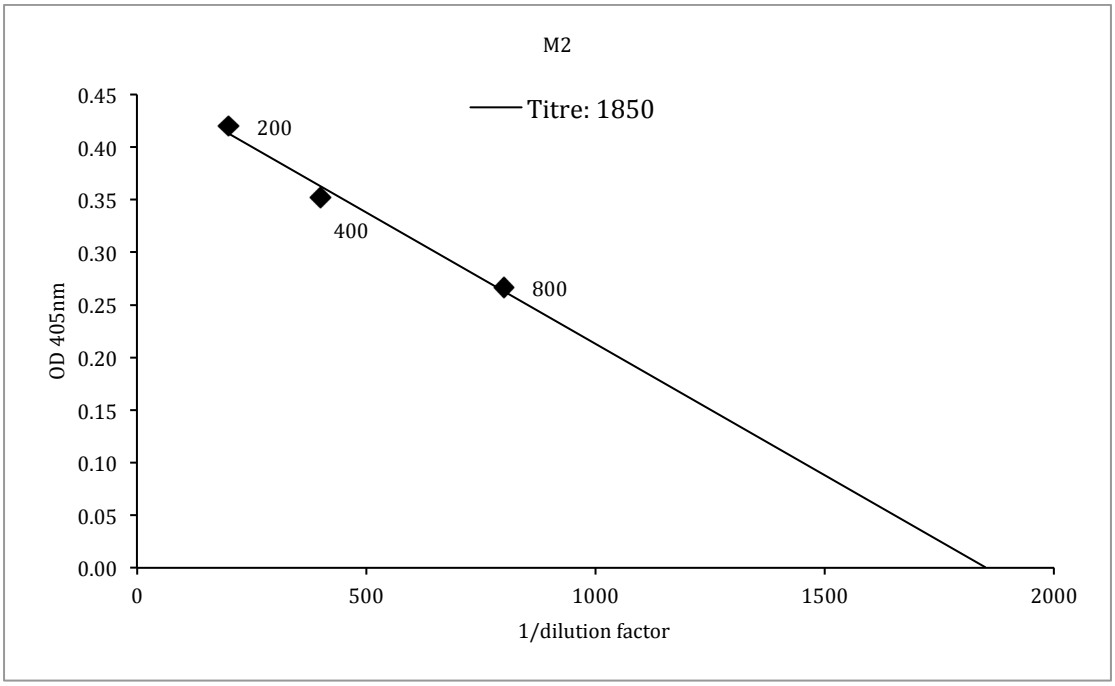
Antigen-specific immune response of mice vaccinated with three doses of DTaP and one booster dose of live *aroQ*BP.

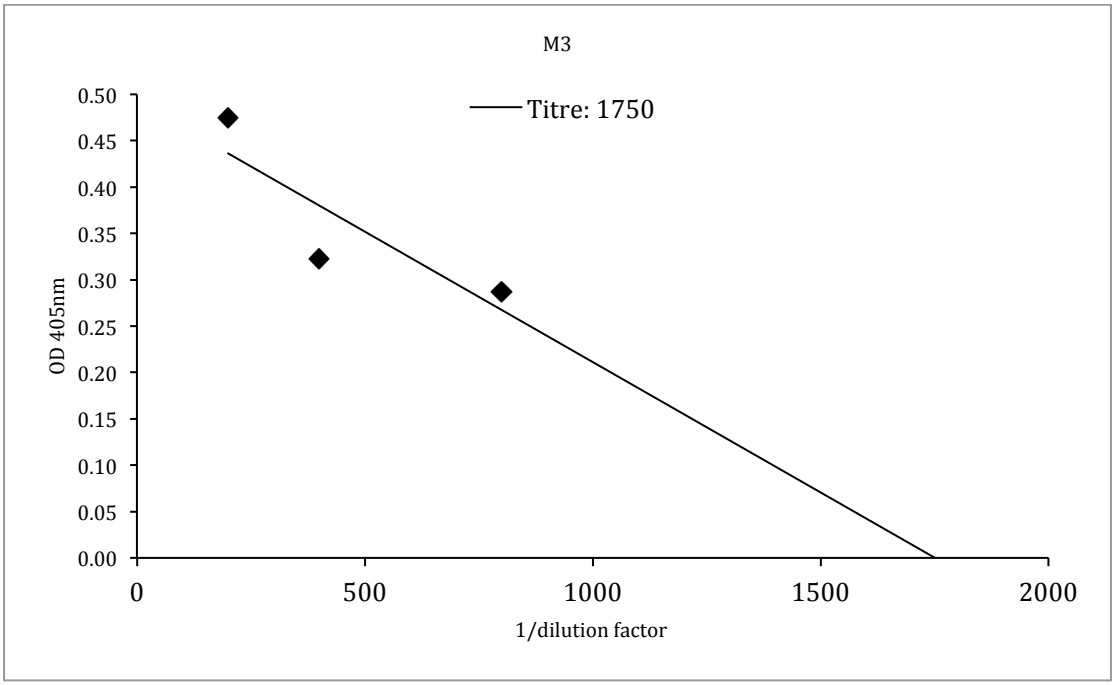
PBS sham-vaccinated Group: Titers for the PBS control groups were zero.

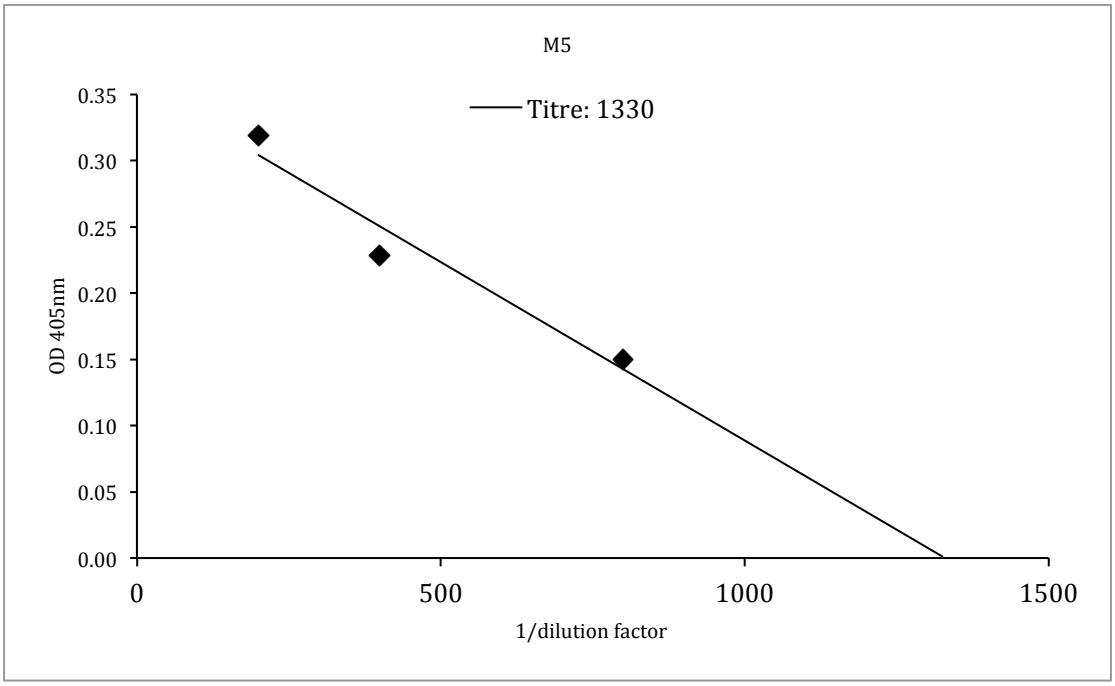
IgG titers

Mice Number	Serum IgG titers
M1	1280
M2	1850
M3	1750
M4	Taken out
M5	1330



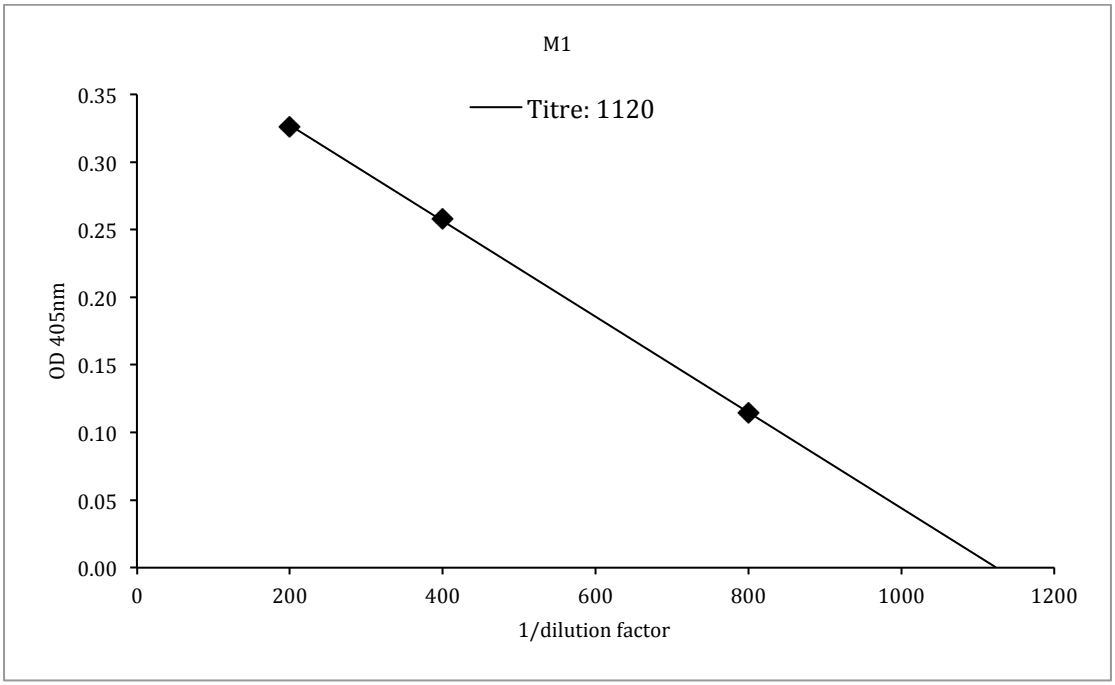

M2
Titre: 1850
200
400
800
OD 405nm
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
500
1000
1500
2000
1/dilution factor

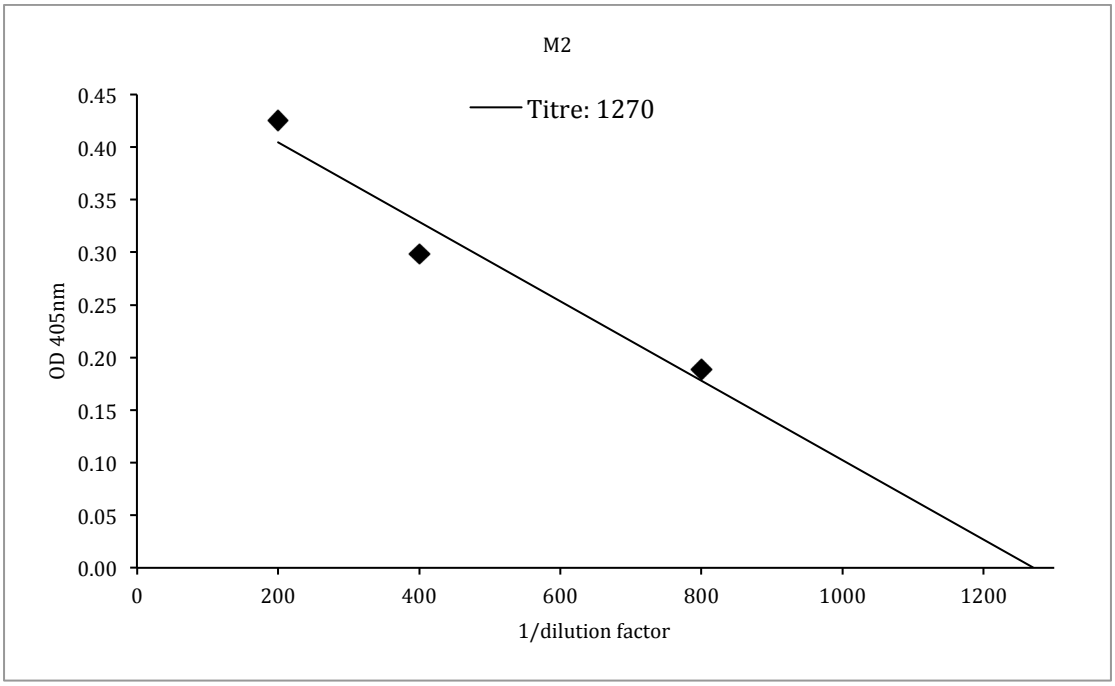

M3
Titre: 1750
OD 405nm
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
500
1000
1500
2000
1/dilution factor

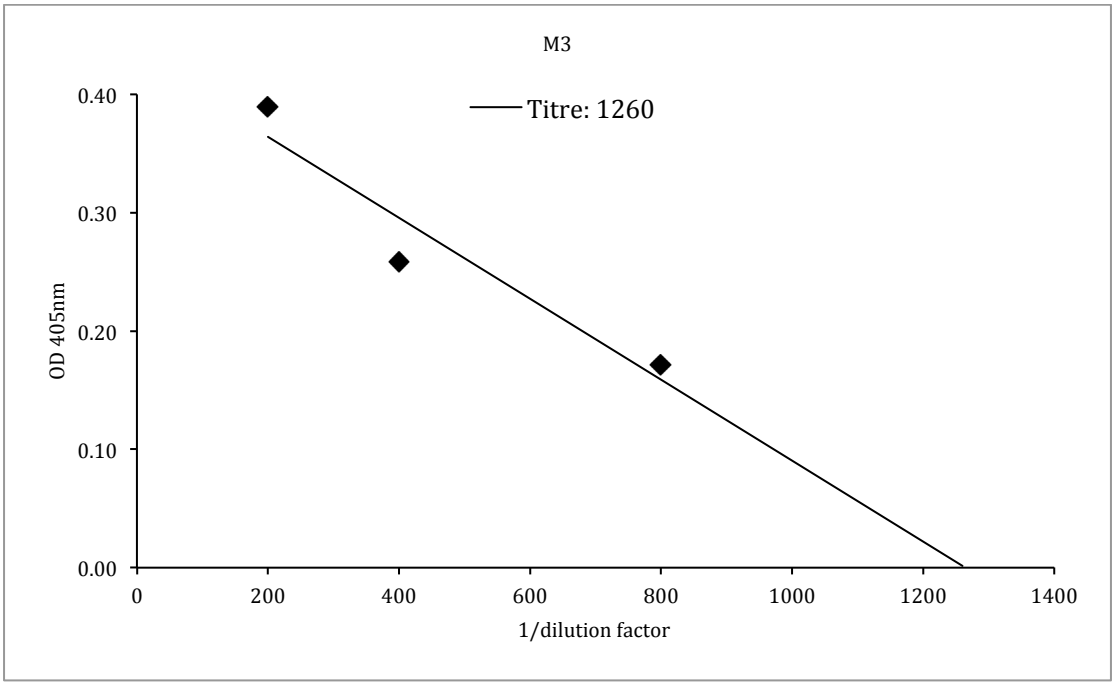

M5
Titre: 1330
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
OD 405nm
0
500
1000
1500
1/dilution factor

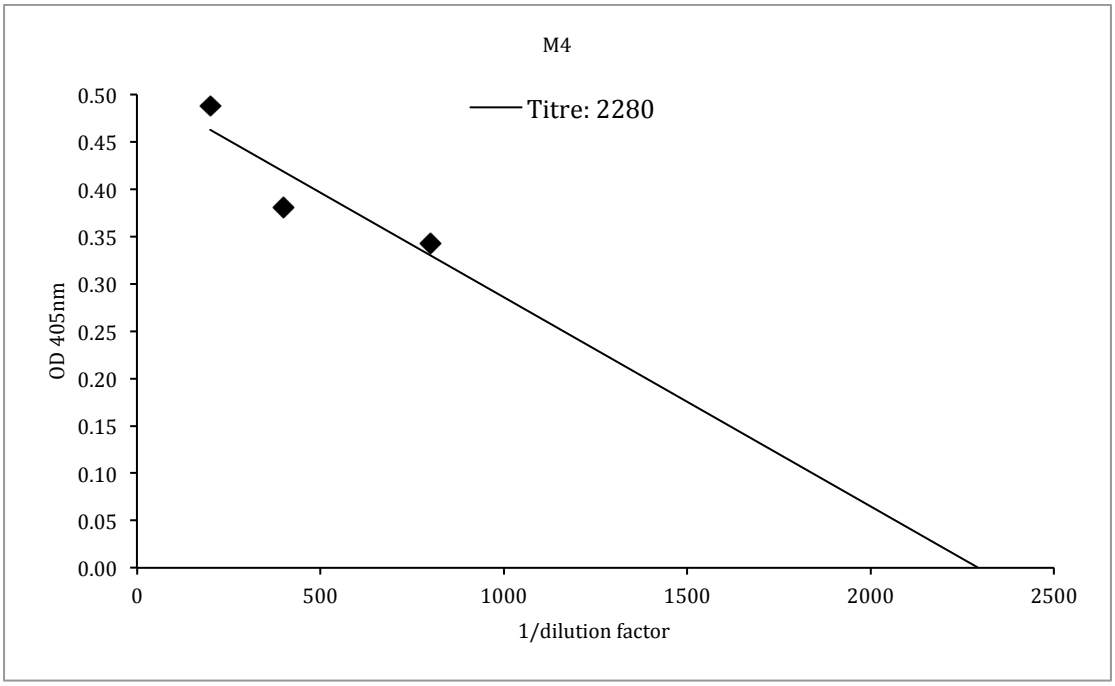
IgG1 titers

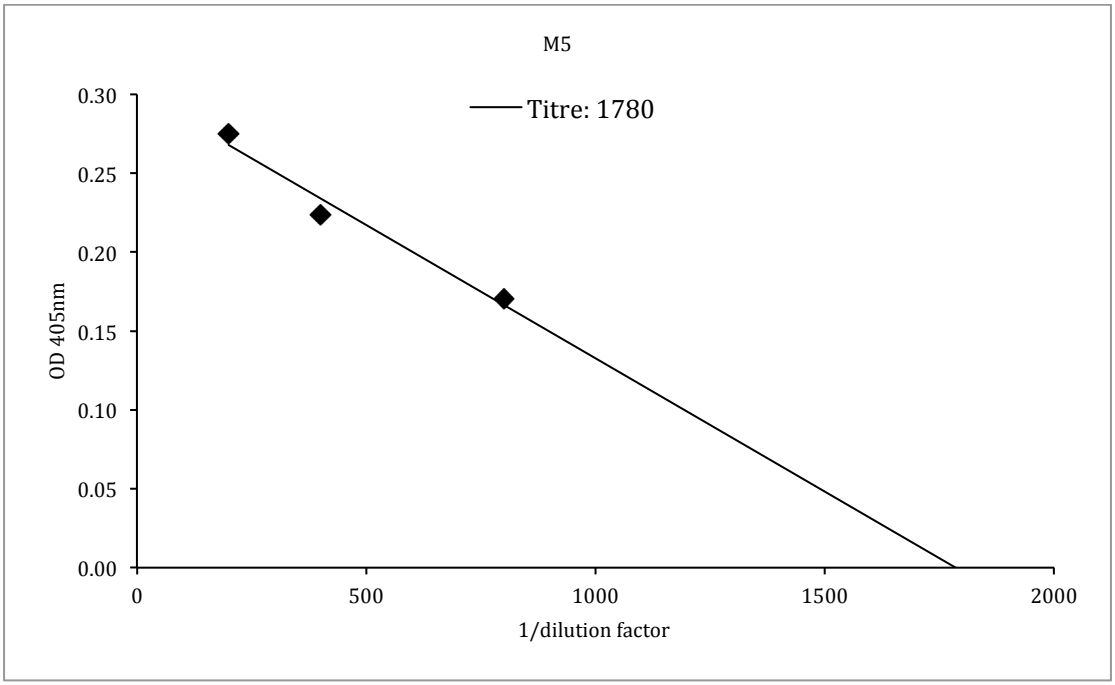
Mice Number	Serum IgG1 titers
M1	1120
M2	1270
M3	1260
M4	2280
M5	1780




M2
Titre: 1270
OD 405nm
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
200
400
600
800
1000
1200
1/dilution factor


M3
Titre: 1260
OD 405nm
0.40
0.30
0.20
0.10
0.00
0
200
400
600
800
1000
1200
1400
1/dilution factor



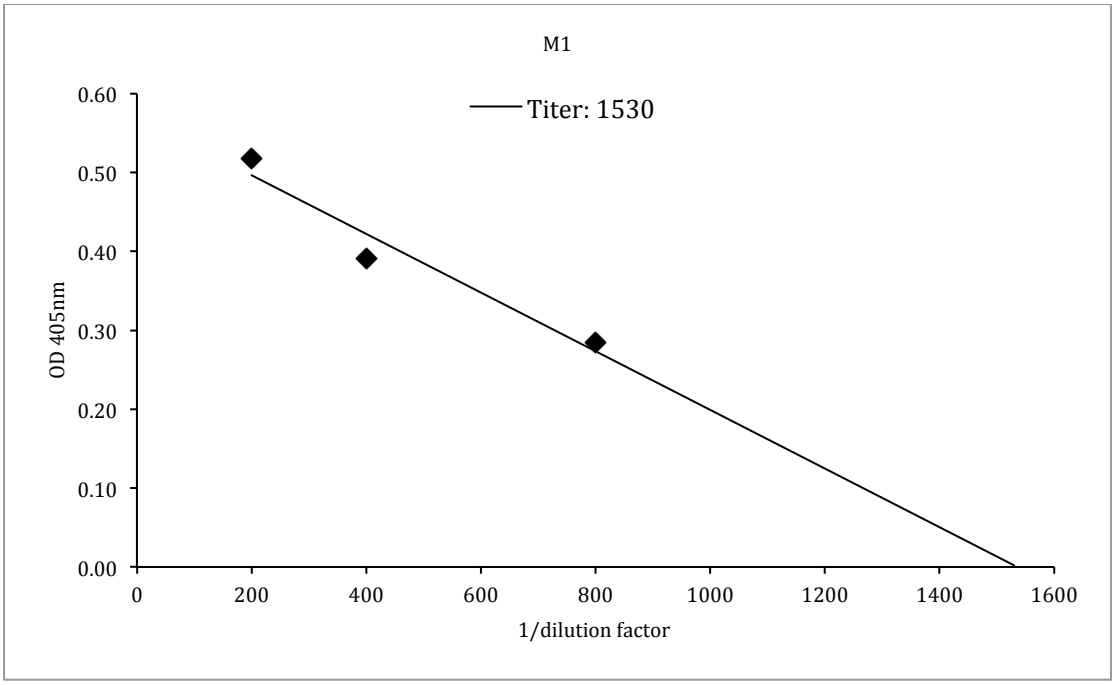


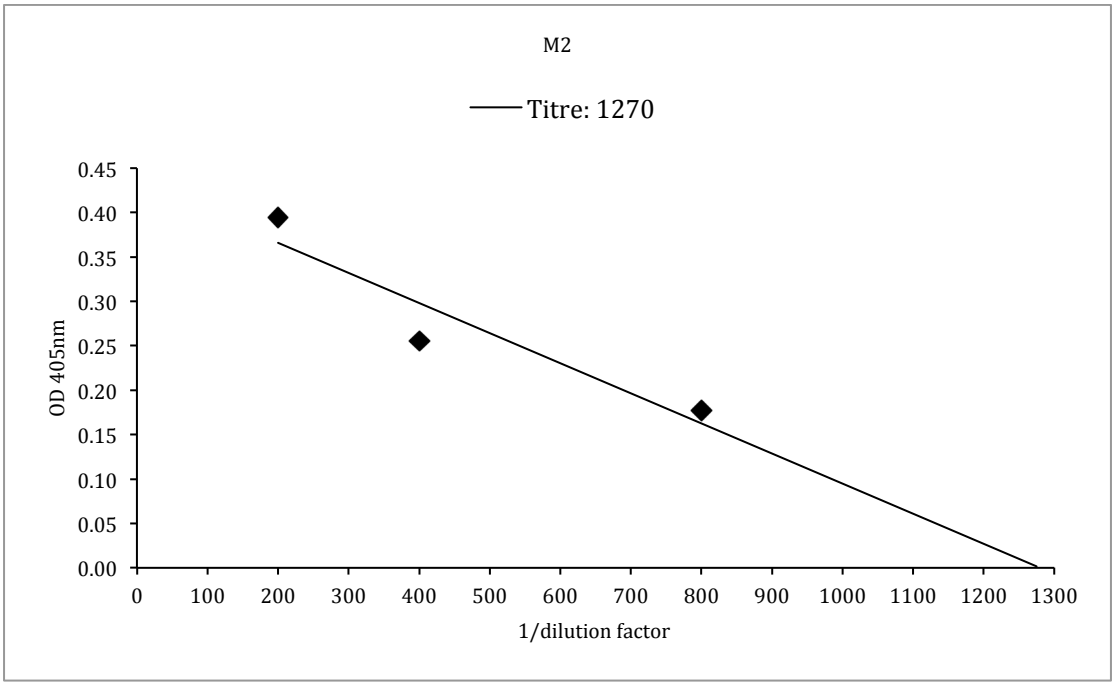
*No IgG2a and IgA isotypes detected.

Antigen-specific immune response of mice vaccinated with three doses DTaP and two booster doses of live *aroQ*BP.

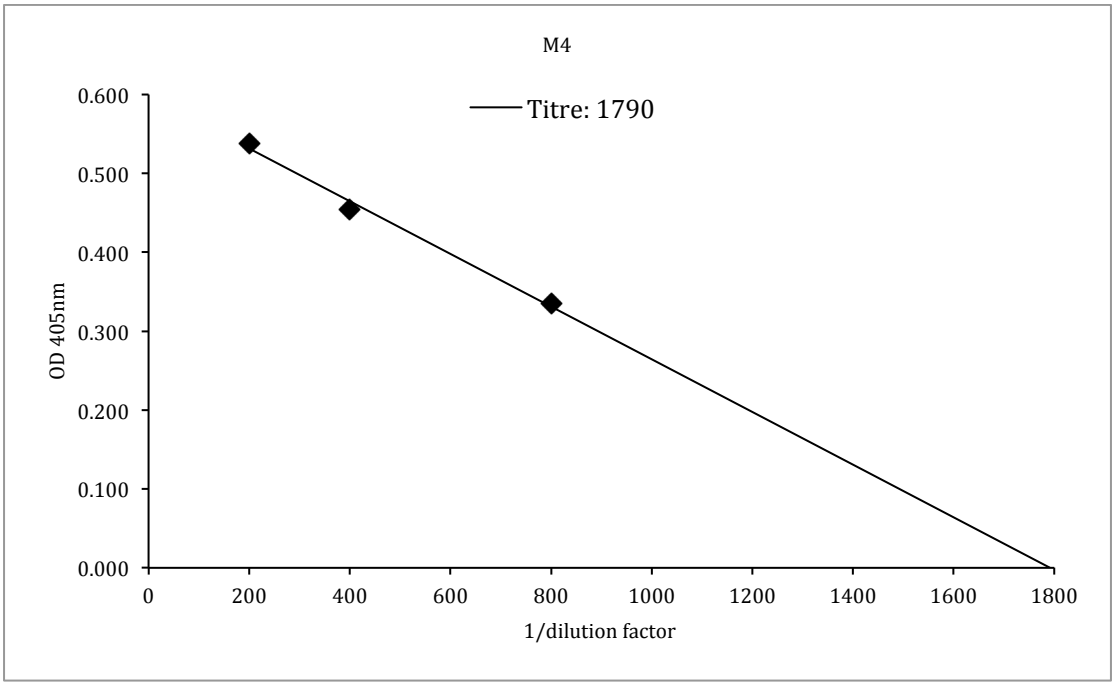
IgG titers

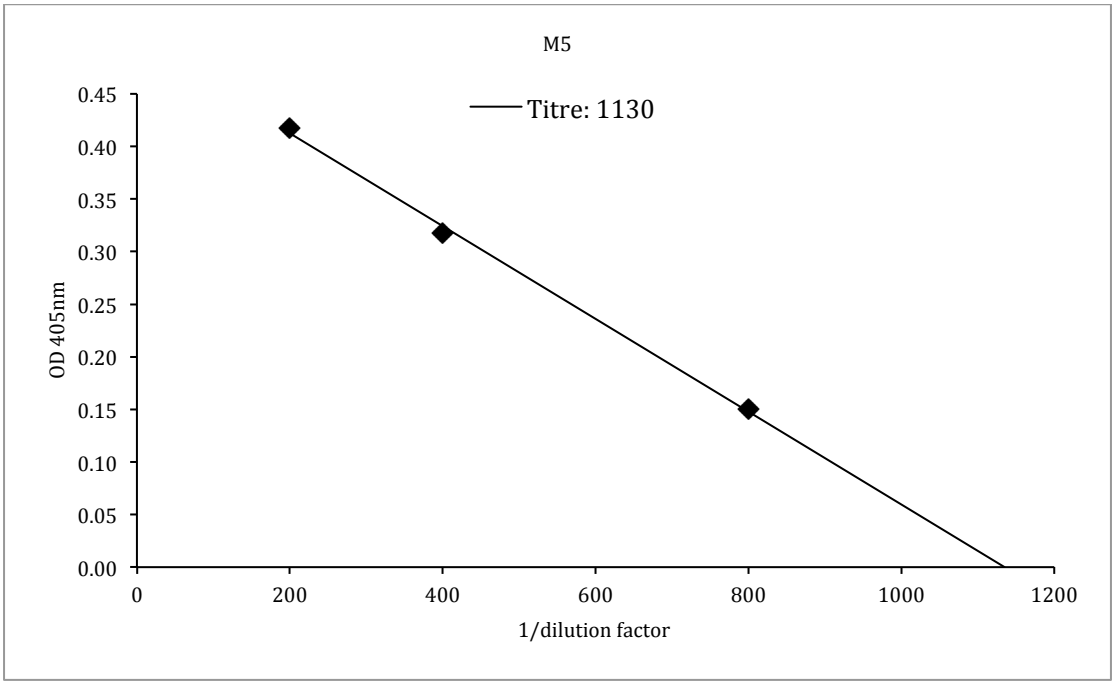
Mice Number	Serum IgG titers
M1	1530
M2	1270
M3	1850
M4	1790
M5	1130




M2
Titre: 1270
OD 405nm
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1/dilution factor

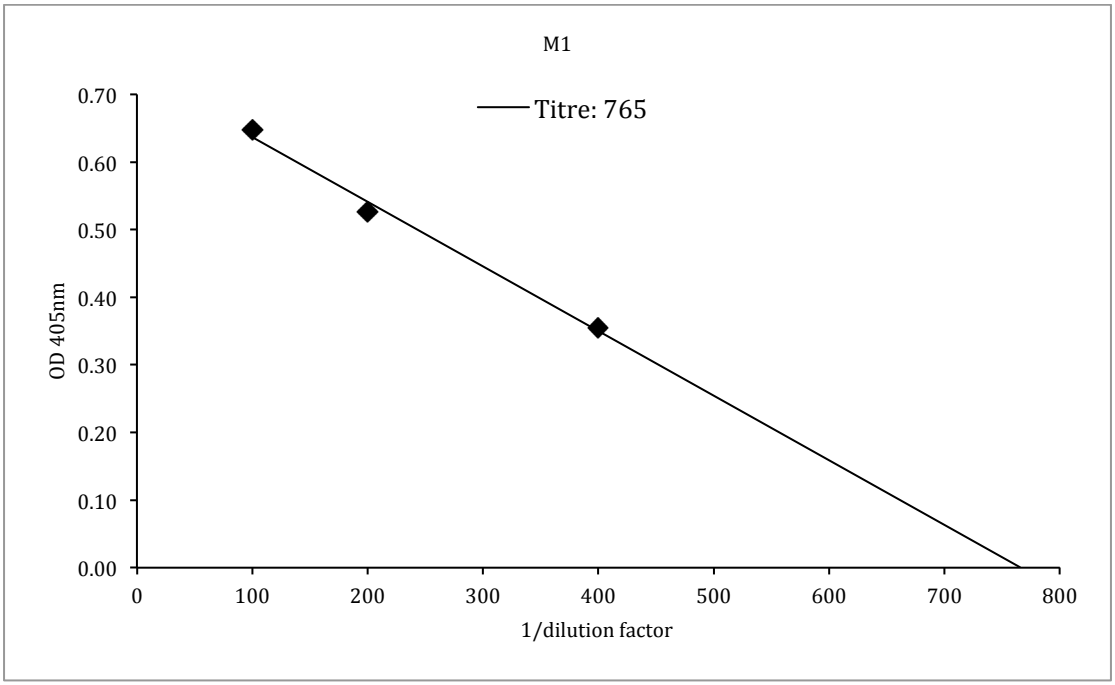

M3
Titer: 1850
OD 405nm
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
200
400
600
800
1000
1200
1400
1600
1800
2000
1/dilution factor

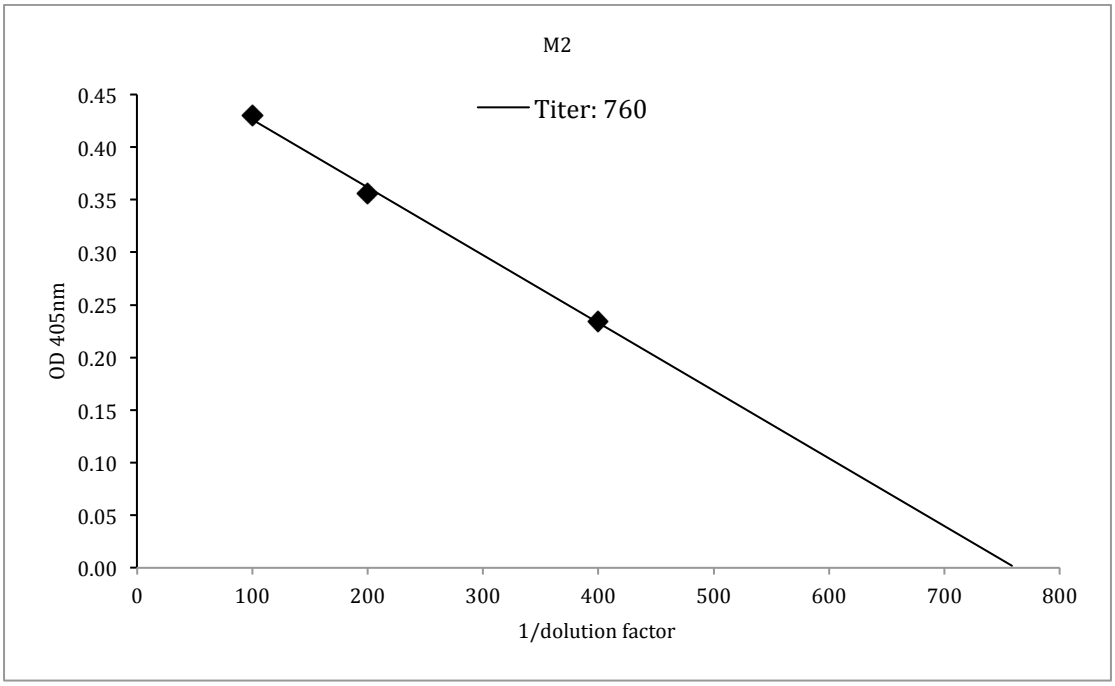

M4
Titre: 1790
0.600
0.500
0.400
0.300
0.200
0.100
0.000
OD 405nm
0
200
400
600
800
1000
1200
1400
1600
1800
1/dilution factor

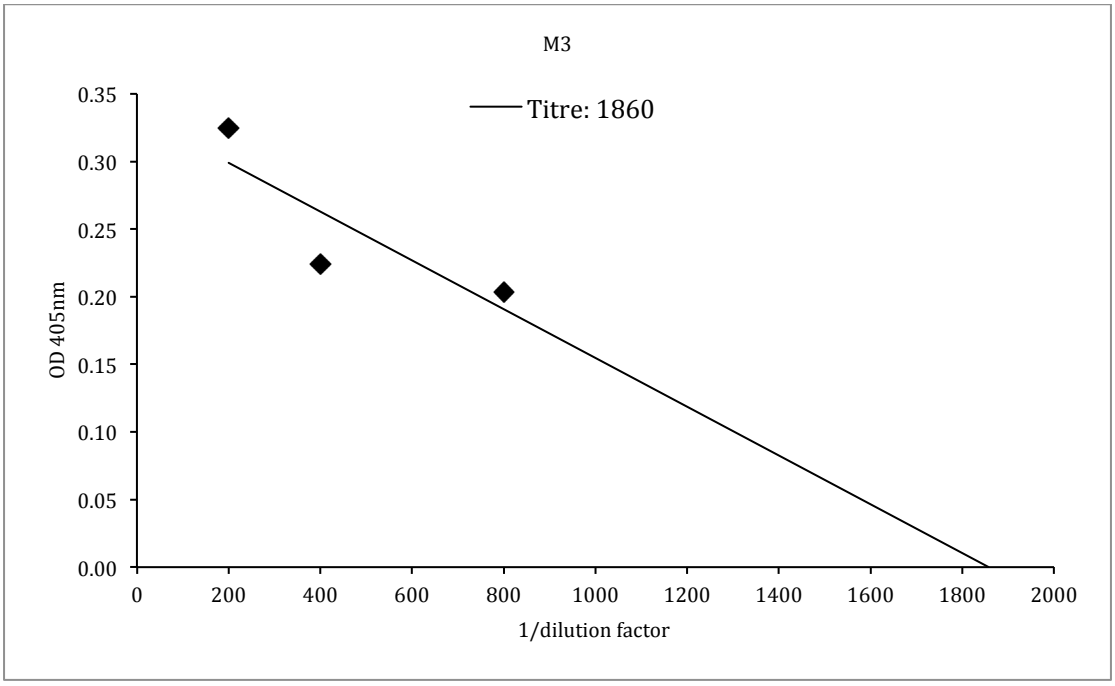

M5
Titre: 1130
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
OD 405nm
0
200
400
600
800
1000
1200
1/dilution factor

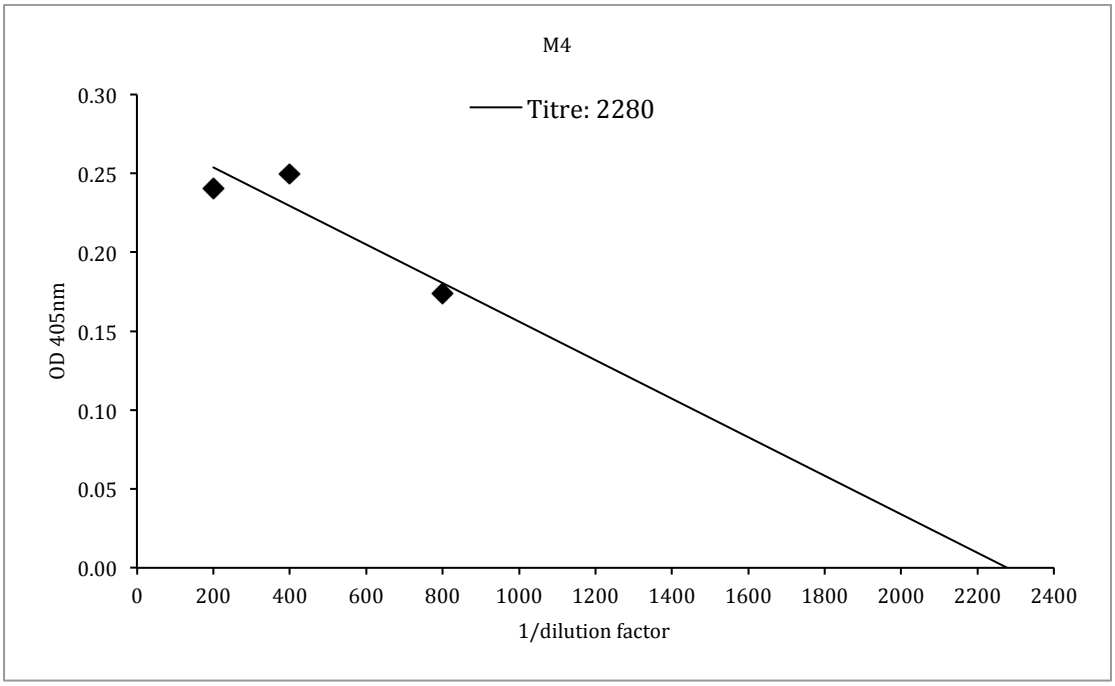
IgG1 titers

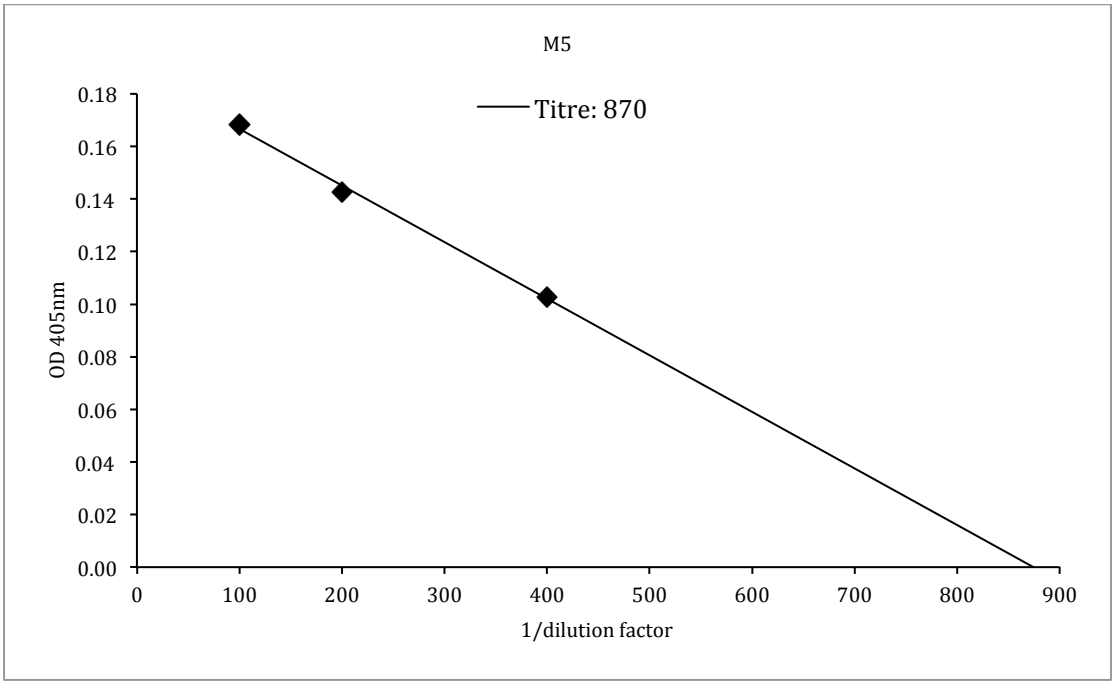
Mice Number	Serum IgG1 titers
M1	765
M2	760
M3	1860
M4	2280
M5	870




M2
Titer: 760
OD 405nm
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
100
200
300
400
500
600
700
800
1/dolution factor


M3
Titre: 1860
OD 405nm
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
200
400
600
800
1000
1200
1400
1600
1800
2000
1/dilution factor


M4
Titre: 2280
OD 405nm
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
200
400
600
800
1000
1200
1400
1600
1800
2000
2200
2400
1/dilution factor


M5
Titre: 870
OD 405nm
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
100
200
300
400
500
600
700
800
900
1/dilution factor

IgG2a titers

Mice Number	Serum IgG2a titers
M1	595
M2	N/D
M3	N/D
M4	N/D
M5	N/D

*No IgA isotype detected.

Antigen-specific immune response of vaccinated with two doses of live *aroQ*BP.

IgG [Single point titers]

Mice Number	Serum IgG titers
M1	11.2
M2	15.3
M3	6.5
M4	8.6
M5	7.9

*No IgG1, IgG2a, and IgA isotypes detected.

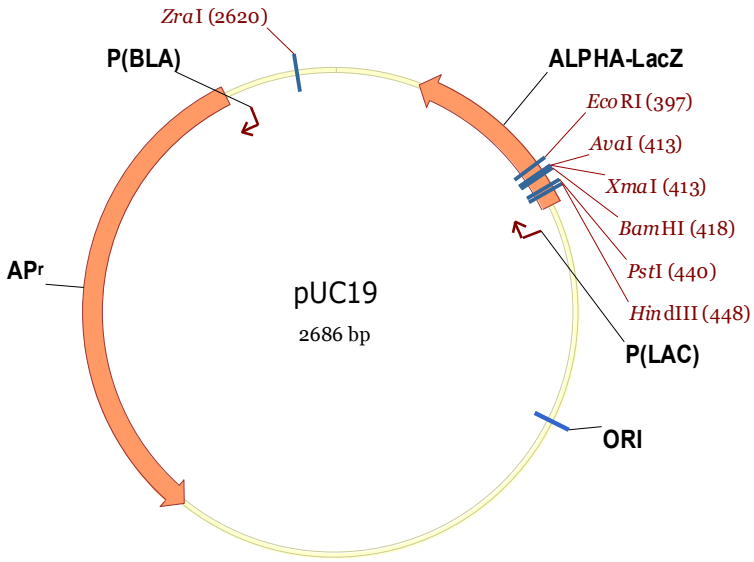
Antigen-specific immune response of mice vaccinated with one dose of live *aroQ*BP.

IgG [Single point titers]

Mice Number	Serum IgG titers
M1	4.4
M2	4.8
M3	7.3
M4	3.8
M5	3.5

*No IgG1, IgG2a, and IgA isotypes detected.

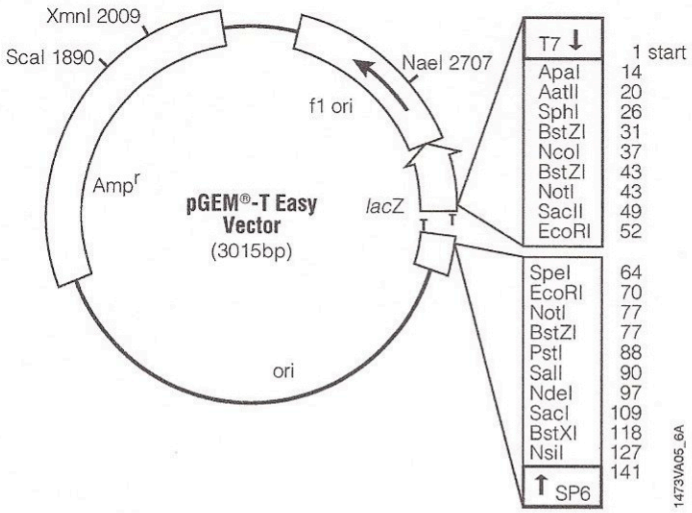
Appendix M: Schematic drawing of vector pUC19



Schematic drawing of vector pUC19. The vector is a small, high-copy number E coli plasmid cloning vector. The vector contains lacZα gene within frame with multiple cloning site and allows screening for insert utilizing blue/white colony color. Restriction sites, BamHI and EcoRI, used to clone the Cya sequence fragment into the vector.

Source: Invitrogen, Vector NTI.

Appendix N: Schematic drawing of pGEM®-T Easy Vector



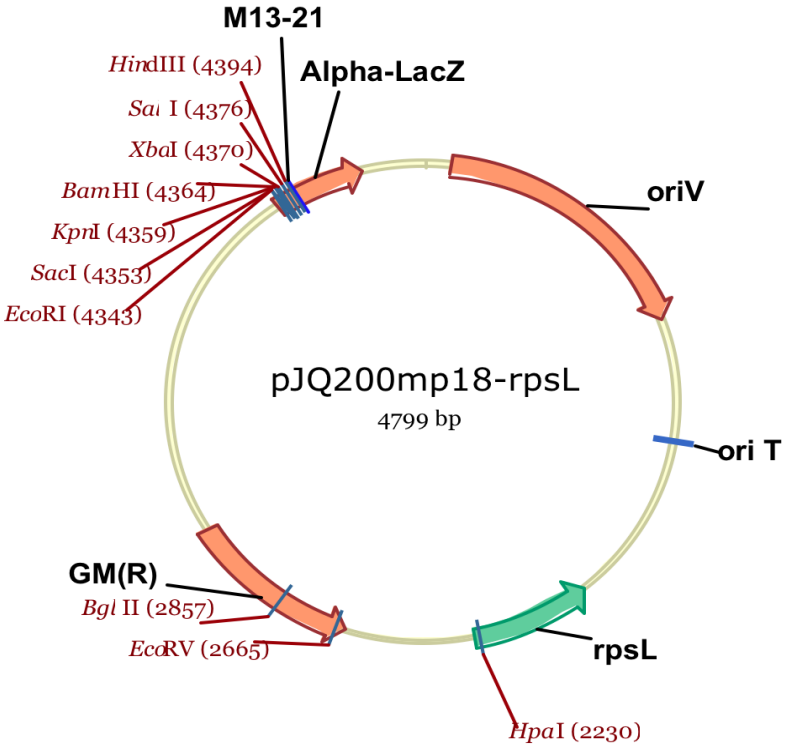
pGEM®-T Easy Vector sequence reference points:

T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–128
SP6 RNA polymerase promoter (–17 to +3)	139–158
SP6 RNA polymerase transcription initiation site	141
pUC/M13 Reverse Sequencing Primer binding site	176–197
lacZ start codon	180
lac operator	200–216
β-lactamase coding region	1337–2197
phage f1 region	2380–2835
lac operon sequences	2836–2996, 166–395
pUC/M13 Forward Sequencing Primer binding site	2949–2972
T7 RNA polymerase promoter (–17 to +3)	2999–3

Schematic drawing of pGEM®-T Easy Vector. The vector is constructed by digestion of pGEM®-T Easy Vector with EcoRV and addition of a 3′ terminal thymidine (T) to of both ends. Thymidine overhangs of the vector are compatible to deoxyadenine overhangs of PCR products generated by thermostable polymerase (Promega). The 3′-T overhangs prevent recirculation of the vector during ligation reaction and improves ligation of PCR products into the pGEM®-T Easy Vector (Promega).

Source: Promega Technical Manual No. 042.

Appendix O: Schematic drawing of pJQ200mp 18-rspL Vector



Schematic drawing of vector pJQ200mp 18-rspL. The vector is a *B pertussis* suicide vector it is one of the vectors used to introduce the mutated lysine codon, ATG, into *aroQ*BP. The vector belongs to ColE1 plasmid family with inserted gentamicin cassette.

Source: Courtesy of Camille Locht, Pasteur institute, lille, France.

Appendix P: Schematic drawing of pSS1129

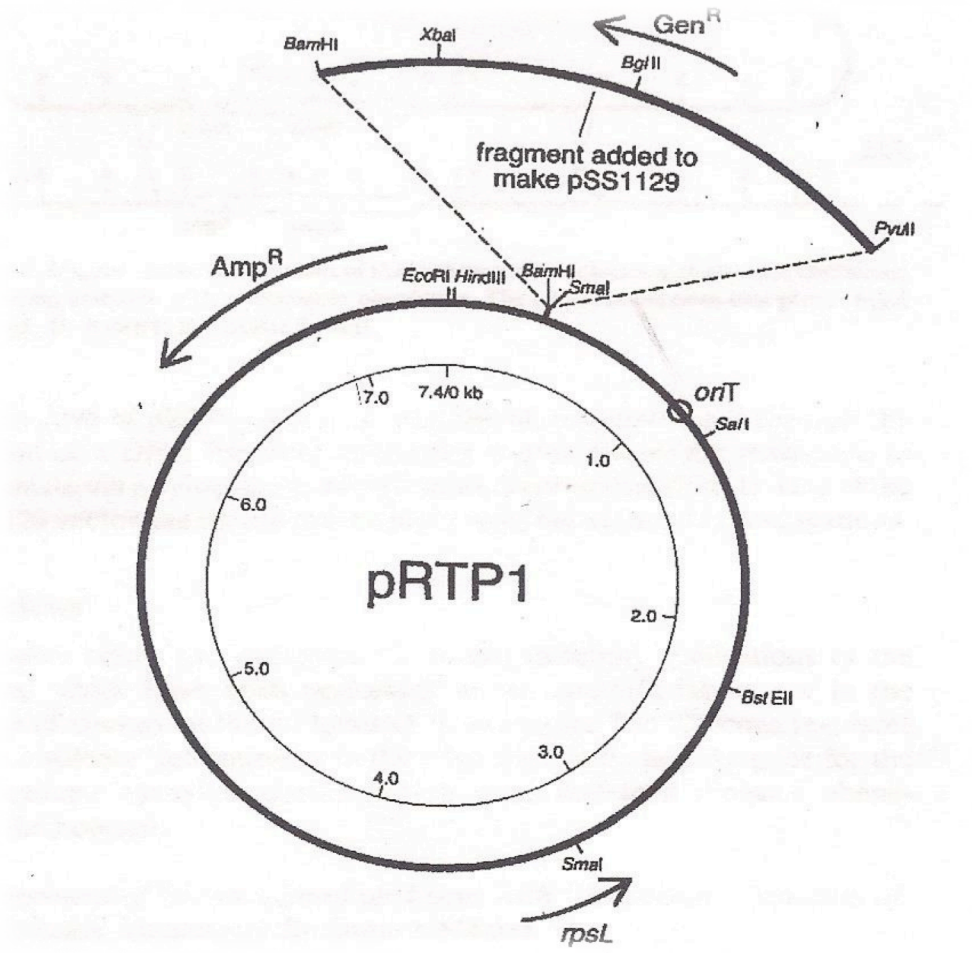
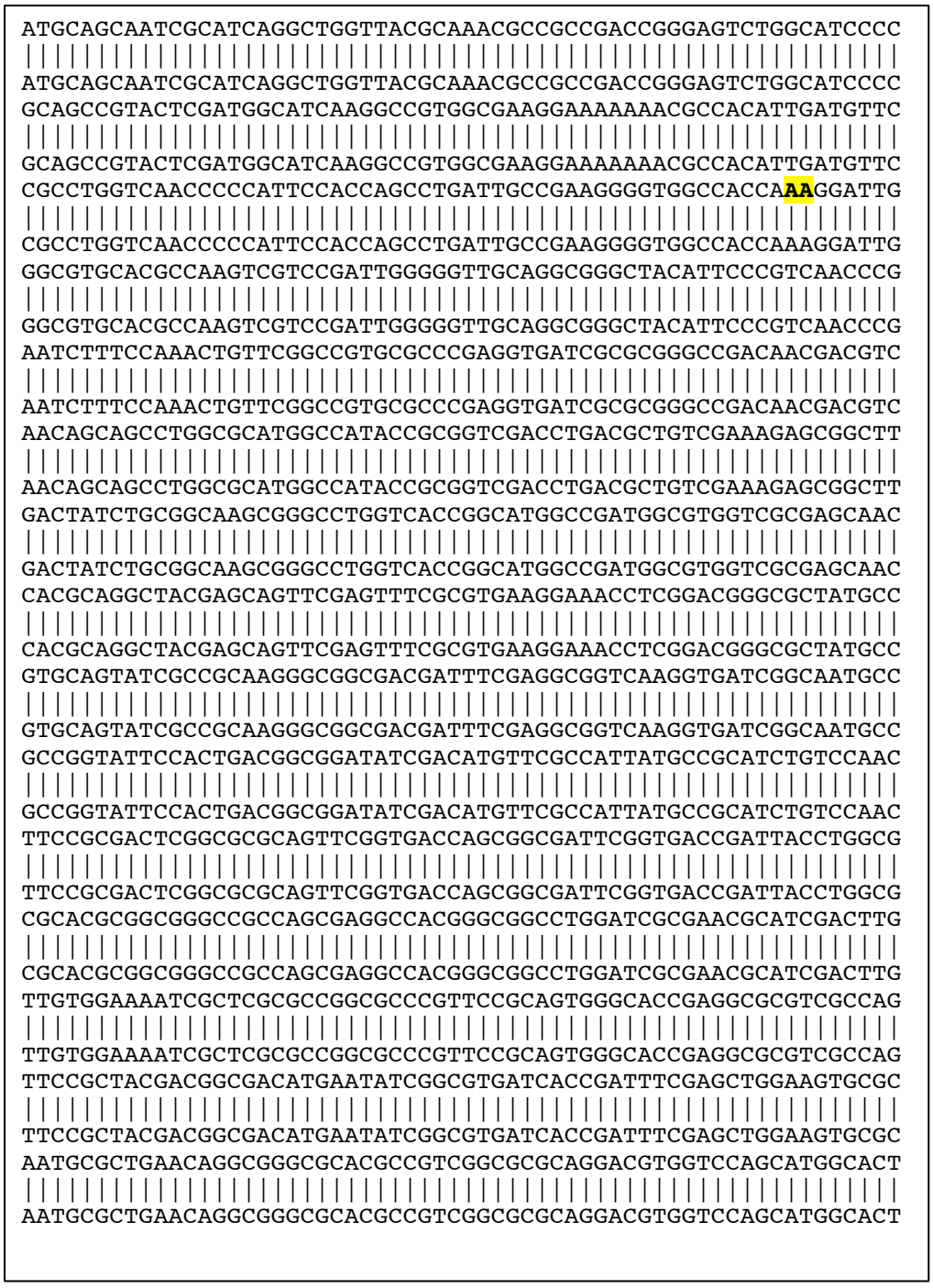


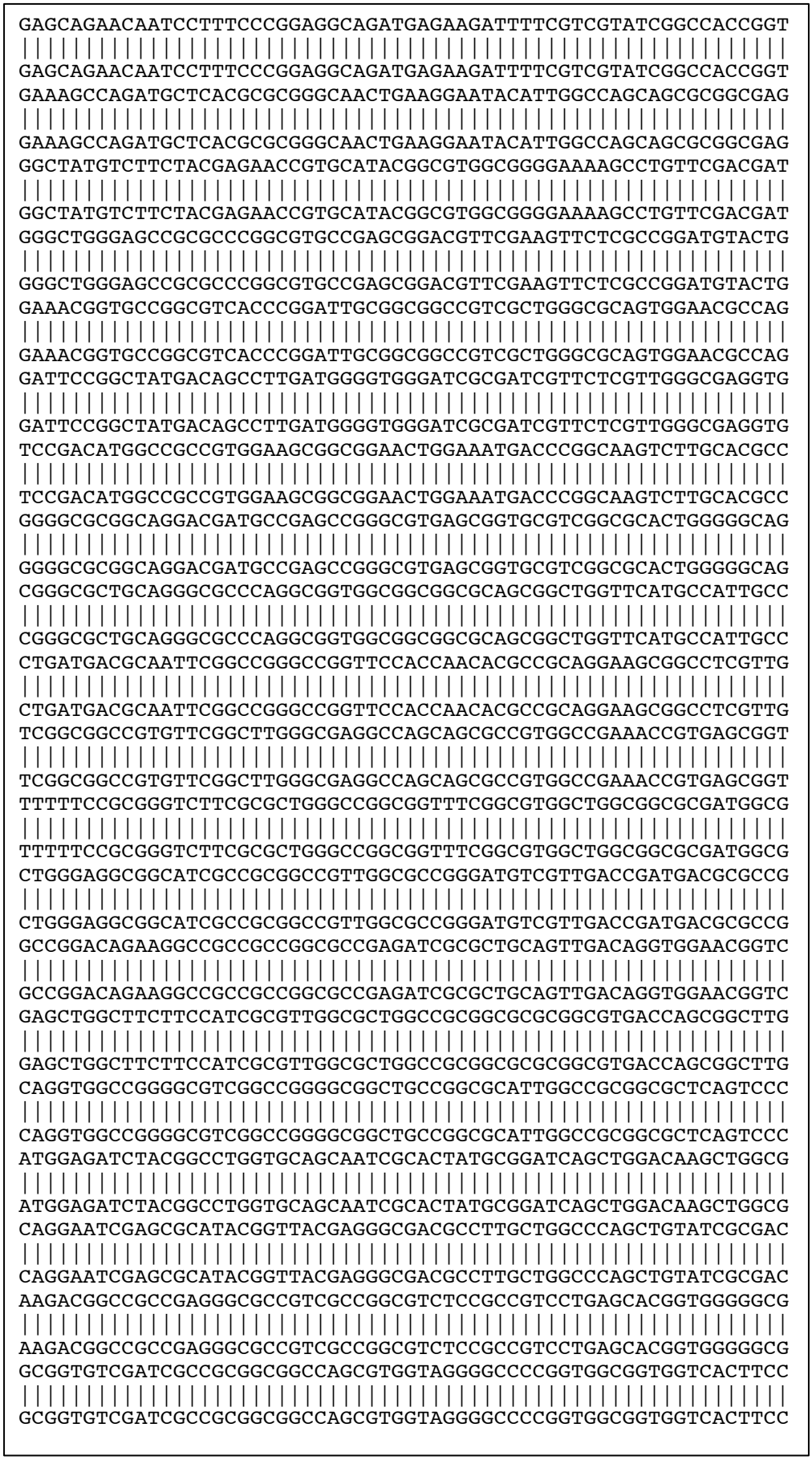
Figure 2.10.1.4. Schematic drawing of Vector pSS1129. The vector is a B pertussis *suicide vector it carries a gentamicin-resistance cassette insert. The vector is the second vector that was used in the study to introduce the mutated lysine codon, ATG, into* aroQ*BP.*

Source: Stibitz, Scott (1994).

Appendix Q: Blast analysis of *B pertussis* Tohama I *cyaA*

Blast analysis of *Bordetella Tohama I cyaA* sequence (5121bp) (http://www.ncbi.nlm.nih.gov) against *Bordetella pertussis* (776228-781384) (www.sanger.ac.uk/cgi). Highlighted nucleotides (174-175) were changed to TG using site directed mutagenesis.

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GAGCAGAACAATCCTTTCCCGGAGGCAGATGAGAAGATTTTCGTCGTATCGGCCACCGGT
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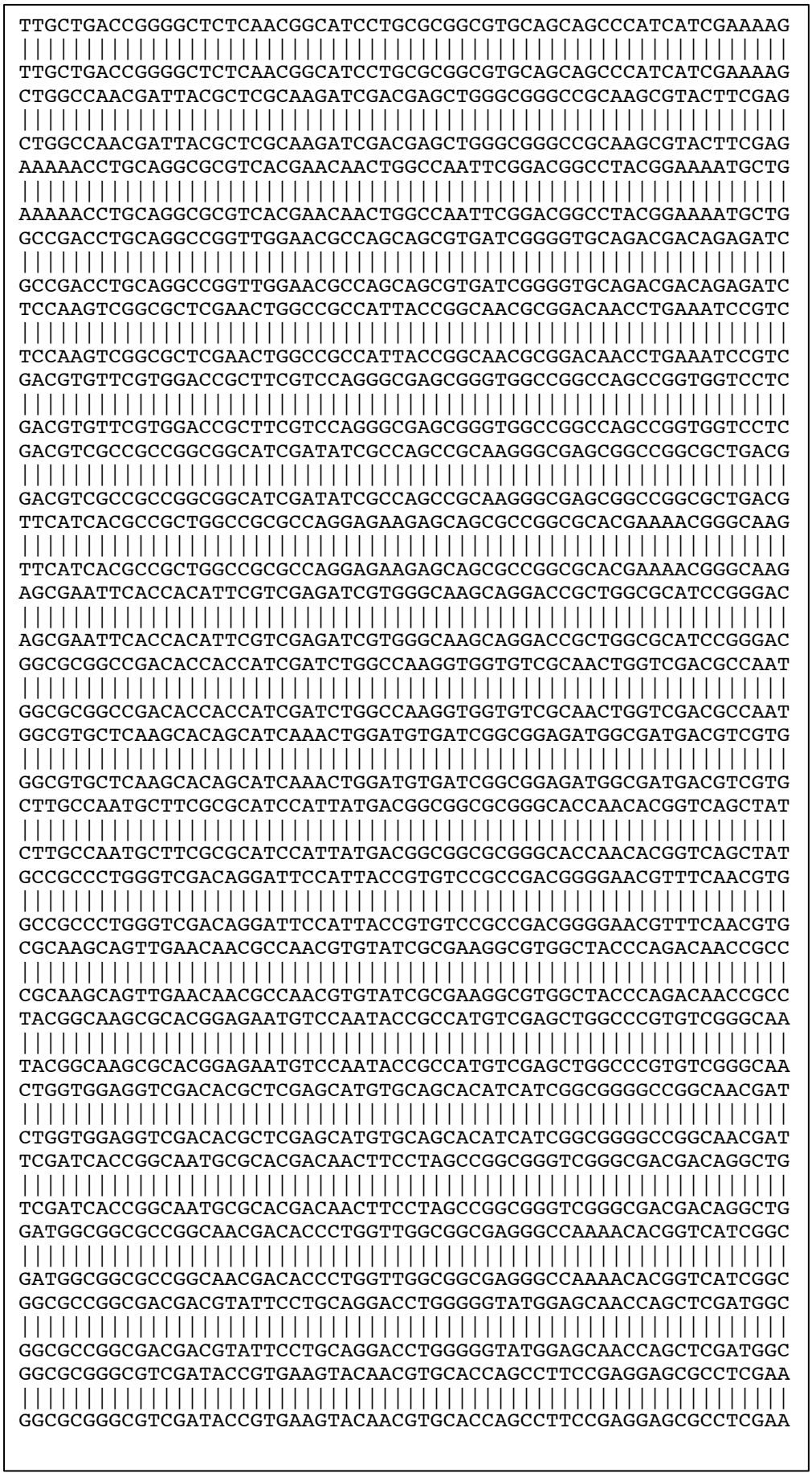
GAAAGCCAGATGCTCACGCGCGGGCAACTGAAGGAATACATTGGCCAGCAGCGCGGCGAG
GGCTATGTCTTCTACGAGAACCGTGCATACGGCGTGGCGGGGAAAAGCCTGTTCGACGAT
GGCTATGTCTTCTACGAGAACCGTGCATACGGCGTGGCGGGGAAAAGCCTGTTCGACGAT
GGGCTGGGAGCCGCGCCCGGCGTGCCGAGCGGACGTTCGAAGTTCTCGCCGGATGTACTG
GGGCTGGGAGCCGCGCCCGGCGTGCCGAGCGGACGTTCGAAGTTCTCGCCGGATGTACTG
GAAACGGTGCCGGCGTCACCCGGATTGCGGCGGCCGTCGCTGGGCGCAGTGGAACGCCAG
GAAACGGTGCCGGCGTCACCCGGATTGCGGCGGCCGTCGCTGGGCGCAGTGGAACGCCAG
GATTCCGGCTATGACAGCCTTGATGGGGTGGGATCGCGATCGTTCTCGTTGGGCGAGGTG
GATTCCGGCTATGACAGCCTTGATGGGGTGGGATCGCGATCGTTCTCGTTGGGCGAGGTG
TCCGACATGGCCGCCGTGGAAGCGGCGGAACTGGAAATGACCCGGCAAGTCTTGCACGCC
TCCGACATGGCCGCCGTGGAAGCGGCGGAACTGGAAATGACCCGGCAAGTCTTGCACGCC
GGGGCGCGGCAGGACGATGCCGAGCCGGGCGTGAGCGGTGCGTCGGCGCACTGGGGGCAG
GGGGCGCGGCAGGACGATGCCGAGCCGGGCGTGAGCGGTGCGTCGGCGCACTGGGGGCAG
CGGGCGCTGCAGGGCGCCCAGGCGGTGGCGGCGGCGCAGCGGCTGGTTCATGCCATTGCC
CGGGCGCTGCAGGGCGCCCAGGCGGTGGCGGCGGCGCAGCGGCTGGTTCATGCCATTGCC
CTGATGACGCAATTCGGCCGGGCCGGTTCCACCAACACGCCGCAGGAAGCGGCCTCGTTG
CTGATGACGCAATTCGGCCGGGCCGGTTCCACCAACACGCCGCAGGAAGCGGCCTCGTTG
TCGGCGGCCGTGTTCGGCTTGGGCGAGGCCAGCAGCGCCGTGGCCGAAACCGTGAGCGGT
TCGGCGGCCGTGTTCGGCTTGGGCGAGGCCAGCAGCGCCGTGGCCGAAACCGTGAGCGGT
TTTTTCCGCGGGTCTTCGCGCTGGGCCGGCGGTTTCGGCGTGGCTGGCGGCGCGATGGCG
TTTTTCCGCGGGTCTTCGCGCTGGGCCGGCGGTTTCGGCGTGGCTGGCGGCGCGATGGCG
CTGGGAGGCGGCATCGCCGCGGCCGTTGGCGCCGGGATGTCGTTGACCGATGACGCGCCG
CTGGGAGGCGGCATCGCCGCGGCCGTTGGCGCCGGGATGTCGTTGACCGATGACGCGCCG
GCCGGACAGAAGGCCGCCGCCGGCGCCGAGATCGCGCTGCAGTTGACAGGTGGAACGGTC
GCCGGACAGAAGGCCGCCGCCGGCGCCGAGATCGCGCTGCAGTTGACAGGTGGAACGGTC
GAGCTGGCTTCTTCCATCGCGTTGGCGCTGGCCGCGGCGCGCGGCGTGACCAGCGGCTTG
GAGCTGGCTTCTTCCATCGCGTTGGCGCTGGCCGCGGCGCGCGGCGTGACCAGCGGCTTG
CAGGTGGCCGGGGCGTCGGCCGGGGCGGCTGCCGGCGCATTGGCCGCGGCGCTCAGTCCC
CAGGTGGCCGGGGCGTCGGCCGGGGCGGCTGCCGGCGCATTGGCCGCGGCGCTCAGTCCC
ATGGAGATCTACGGCCTGGTGCAGCAATCGCACTATGCGGATCAGCTGGACAAGCTGGCG
ATGGAGATCTACGGCCTGGTGCAGCAATCGCACTATGCGGATCAGCTGGACAAGCTGGCG
CAGGAATCGAGCGCATACGGTTACGAGGGCGACGCCTTGCTGGCCCAGCTGTATCGCGAC
CAGGAATCGAGCGCATACGGTTACGAGGGCGACGCCTTGCTGGCCCAGCTGTATCGCGAC
AAGACGGCCGCCGAGGGCGCCGTCGCCGGCGTCTCCGCCGTCCTGAGCACGGTGGGGGCG
AAGACGGCCGCCGAGGGCGCCGTCGCCGGCGTCTCCGCCGTCCTGAGCACGGTGGGGGCG
GCGGTGTCGATCGCCGCGGCGGCCAGCGTGGTAGGGGCCCCGGTGGCGGTGGTCACTTCC
GCGGTGTCGATCGCCGCGGCGGCCAGCGTGGTAGGGGCCCCGGTGGCGGTGGTCACTTCC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TTGCTGACCGGGGCTCTCAACGGCATCCTGCGCGGCGTGCAGCAGCCCATCATCGAAAAG
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CTGGCCAACGATTACGCTCGCAAGATCGACGAGCTGGGCGGGCCGCAAGCGTACTTCGAG
AAAAACCTGCAGGCGCGTCACGAACAACTGGCCAATTCGGACGGCCTACGGAAAATGCTG
AAAAACCTGCAGGCGCGTCACGAACAACTGGCCAATTCGGACGGCCTACGGAAAATGCTG
GCCGACCTGCAGGCCGGTTGGAACGCCAGCAGCGTGATCGGGGTGCAGACGACAGAGATC
GCCGACCTGCAGGCCGGTTGGAACGCCAGCAGCGTGATCGGGGTGCAGACGACAGAGATC
TCCAAGTCGGCGCTCGAACTGGCCGCCATTACCGGCAACGCGGACAACCTGAAATCCGTC
TCCAAGTCGGCGCTCGAACTGGCCGCCATTACCGGCAACGCGGACAACCTGAAATCCGTC
GACGTGTTCGTGGACCGCTTCGTCCAGGGCGAGCGGGTGGCCGGCCAGCCGGTGGTCCTC
GACGTGTTCGTGGACCGCTTCGTCCAGGGCGAGCGGGTGGCCGGCCAGCCGGTGGTCCTC
GACGTCGCCGCCGGCGGCATCGATATCGCCAGCCGCAAGGGCGAGCGGCCGGCGCTGACG
GACGTCGCCGCCGGCGGCATCGATATCGCCAGCCGCAAGGGCGAGCGGCCGGCGCTGACG
TTCATCACGCCGCTGGCCGCGCCAGGAGAAGAGCAGCGCCGGCGCACGAAAACGGGCAAG
TTCATCACGCCGCTGGCCGCGCCAGGAGAAGAGCAGCGCCGGCGCACGAAAACGGGCAAG
AGCGAATTCACCACATTCGTCGAGATCGTGGGCAAGCAGGACCGCTGGCGCATCCGGGAC
AGCGAATTCACCACATTCGTCGAGATCGTGGGCAAGCAGGACCGCTGGCGCATCCGGGAC
GGCGCGGCCGACACCACCATCGATCTGGCCAAGGTGGTGTCGCAACTGGTCGACGCCAAT
GGCGCGGCCGACACCACCATCGATCTGGCCAAGGTGGTGTCGCAACTGGTCGACGCCAAT
GGCGTGCTCAAGCACAGCATCAAACTGGATGTGATCGGCGGAGATGGCGATGACGTCGTG
GGCGTGCTCAAGCACAGCATCAAACTGGATGTGATCGGCGGAGATGGCGATGACGTCGTG
CTTGCCAATGCTTCGCGCATCCATTATGACGGCGGCGCGGGCACCAACACGGTCAGCTAT
CTTGCCAATGCTTCGCGCATCCATTATGACGGCGGCGCGGGCACCAACACGGTCAGCTAT
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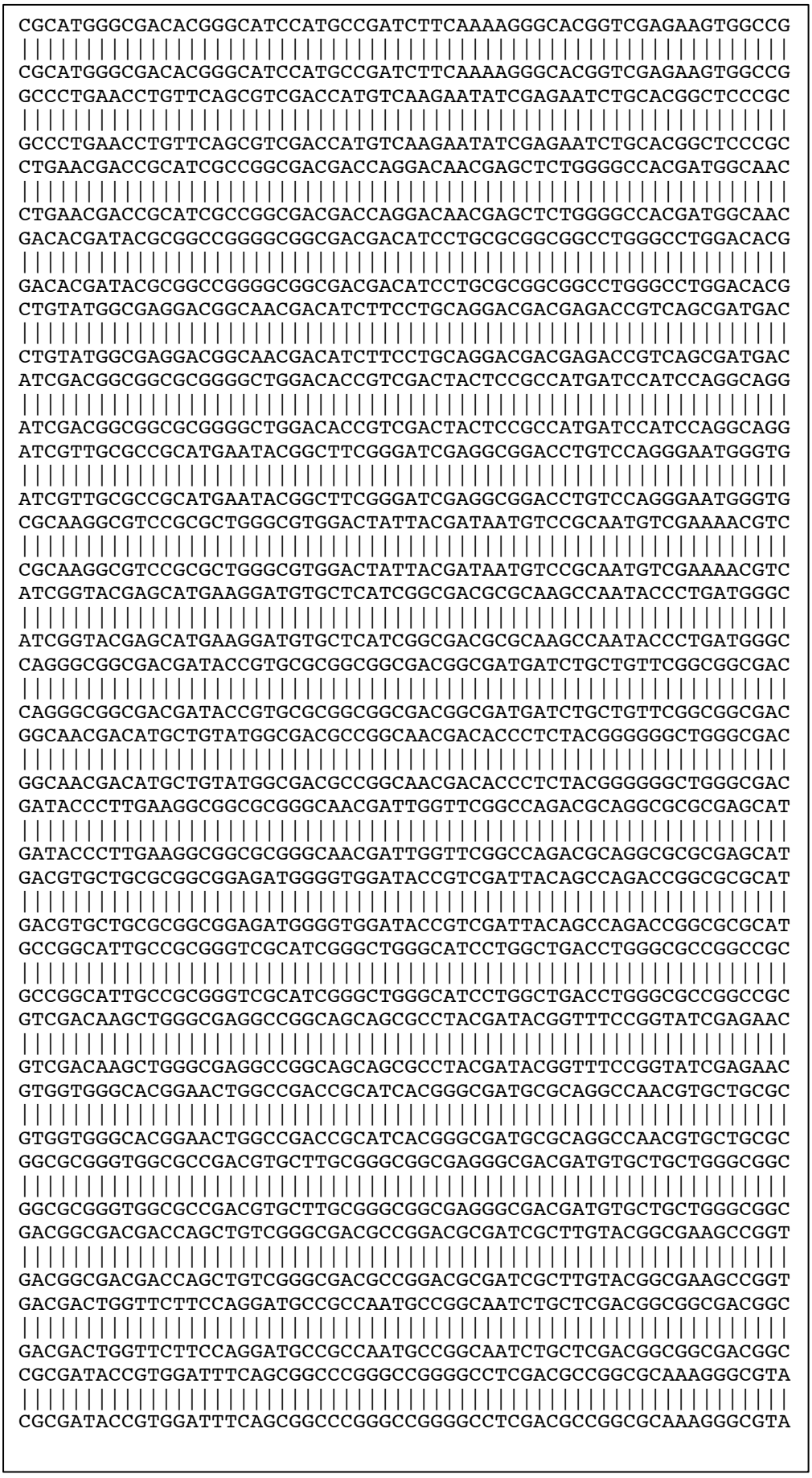
GCCGCCCTGGGTCGACAGGATTCCATTACCGTGTCCGCCGACGGGGAACGTTTCAACGTG
CGCAAGCAGTTGAACAACGCCAACGTGTATCGCGAAGGCGTGGCTACCCAGACAACCGCC
CGCAAGCAGTTGAACAACGCCAACGTGTATCGCGAAGGCGTGGCTACCCAGACAACCGCC
TACGGCAAGCGCACGGAGAATGTCCAATACCGCCATGTCGAGCTGGCCCGTGTCGGGCAA
TACGGCAAGCGCACGGAGAATGTCCAATACCGCCATGTCGAGCTGGCCCGTGTCGGGCAA
CTGGTGGAGGTCGACACGCTCGAGCATGTGCAGCACATCATCGGCGGGGCCGGCAACGAT
CTGGTGGAGGTCGACACGCTCGAGCATGTGCAGCACATCATCGGCGGGGCCGGCAACGAT
TCGATCACCGGCAATGCGCACGACAACTTCCTAGCCGGCGGGTCGGGCGACGACAGGCTG
TCGATCACCGGCAATGCGCACGACAACTTCCTAGCCGGCGGGTCGGGCGACGACAGGCTG
GATGGCGGCGCCGGCAACGACACCCTGGTTGGCGGCGAGGGCCAAAACACGGTCATCGGC
GATGGCGGCGCCGGCAACGACACCCTGGTTGGCGGCGAGGGCCAAAACACGGTCATCGGC
GGCGCCGGCGACGACGTATTCCTGCAGGACCTGGGGGGTATGGAGCAACCAGCTCGATGGC
GGCGCCGGCGACGACGTATTCCTGCAGGACCTGGGGGGTATGGAGCAACCAGCTCGATGGC
GGCGCGGGCGTCGATACCGTGAAGTACAACGTGCACCAGCCTTCCGAGGAGCGCCTCGAA
GGCGCGGGCGTCGATACCGTGAAGTACAACGTGCACCAGCCTTCCGAGGAGCGCCTCGAA


CGCATGGGCGACACGGGCATCCATGCCGATCTTCAAAAGGGCACGGTCGAGAAGTGGCCG
CGCATGGGCGACACGGGCATCCATGCCGATCTTCAAAAGGGCACGGTCGAGAAGTGGCCG
GCCCTGAACCTGTTCAGCGTCGACCATGTCAAGAATATCGAGAATCTGCACGGCTCCCGC
GCCCTGAACCTGTTCAGCGTCGACCATGTCAAGAATATCGAGAATCTGCACGGCTCCCGC
CTGAACGACCGCATCGCCGGCGACGACCAGGACAACGAGCTCTGGGGCCACGATGGCAAC
CTGAACGACCGCATCGCCGGCGACGACCAGGACAACGAGCTCTGGGGCCACGATGGCAAC
GACACGATACGCGGCCGGGGCGGCGACGACATCCTGCGCGGCGGCCTGGGCCTGGACACG
GACACGATACGCGGCCGGGGCGGCGACGACATCCTGCGCGGCGGCCTGGGCCTGGACACG
CTGTATGGCGAGGACGGCAACGACATCTTCCTGCAGGACGACGAGACCGTCAGCGATGAC
CTGTATGGCGAGGACGGCAACGACATCTTCCTGCAGGACGACGAGACCGTCAGCGATGAC
ATCGACGGCGGCGCGGGGCTGGACACCGTCGACTACTCCGCCATGATCCATCCAGGCAGG
ATCGACGGCGGCGCGGGGCTGGACACCGTCGACTACTCCGCCATGATCCATCCAGGCAGG
ATCGTTGCGCCGCATGAATACGGCTTCGGGATCGAGGCGGACCTGTCCAGGGAATGGGTG
ATCGTTGCGCCGCATGAATACGGCTTCGGGATCGAGGCGGACCTGTCCAGGGAATGGGTG
CGCAAGGCGTCCGCGCTGGGCGTGGACTATTACGATAATGTCCGCAATGTCGAAAACGTC
CGCAAGGCGTCCGCGCTGGGCGTGGACTATTACGATAATGTCCGCAATGTCGAAAACGTC
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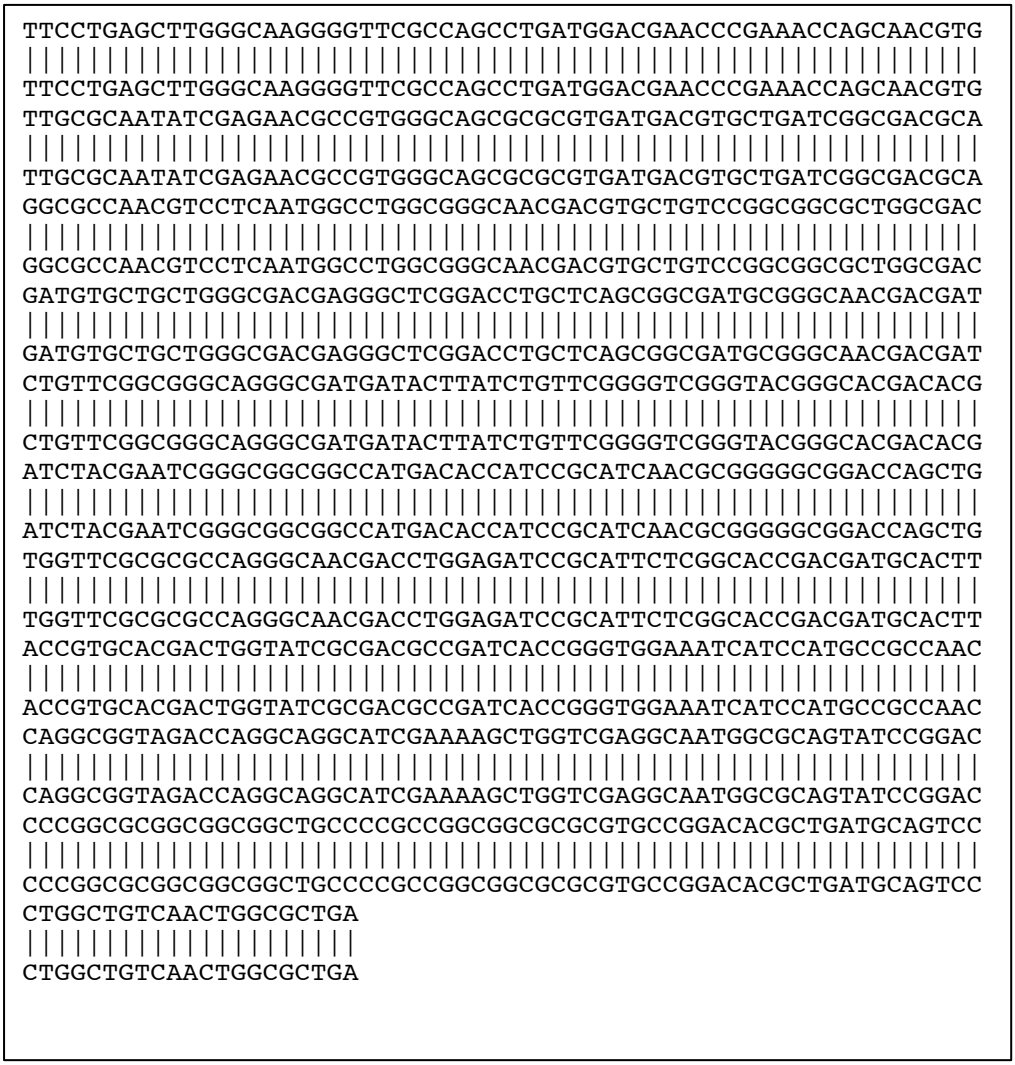
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Appendix R: List of media, reagent and disposables

Media/Reagents/disposable	Company
Bordet-Gengou agar	Becton Dickinson (BD)
Casein digest (Enzymatic digest)	"
Skim milk	"
Bacteriological agar	Acorn Biological (Oxoid)
Trypton	"
Yeast extract (hydrolysate)	"
Luria broth	"
Casamino acids (Technical grade)	"
Sheep blood	Animal Biological Collection and Delivery Enterprises
D-glucose Ar grade	Thermo fisher scientific
Sodium chloride	Sigma-Aldrich
Potassium chloride	"
Potassium hydroxide	"
Monopotassium phosphate (KH_2PO_4)	"
Magnesium chloride hexhydrate ($MgCl_2.6H_2O$)	"
Magnesium sulphate	"
Ferrous sulfate hexhydrate ($FeSO_4.7H_2O$)	"
Copper sulfate ($CuSO_4$)	"
Calcium chloride anhydrase	"
Malic Acid	"
Glycerine	"
Starch	"
Gelatine	"
BCIP/NBT liquid substrate system	"
Sodium bicarbonate ($NaHCO_{3)}$	"
Sodium citrate	"
Sodium sulphate	"
Cysteine hydrochloride	"
Monosodium glutamate	"
Nicotonic Acid	"
2,6-O-methyl-ß-cyclodextrin	"
L-Cysteine	"
L-Glutathione reduced	"
L-Glutamic Acid	"
L-Proline	"
Dihydroxybenzoic acid	"
Para-aminobenzoic acid	"
Para-hydroxybenzoic acid	"
L-Phenylalanine	"
L-Tryptophan	"
L-Tyrosine	"

Tween-20	"
Ampicillin sodium salt	"
Kanamycin sulfate	"
Streptomycin sulfate salt	"
Phenol: chloroform: Isoamyl Alcohol 25:24:1 Saturated with 10mM Tris, PH-8.0, 1mM EDTA-for molecular biology	"
Para-nitrophenyl phosphate disodium salt hexahydrate	"
Protease inhibitor cocktail	"
Albumin from bovin serum	"
Diethanolamine (Bioultra)	"
Polymyxin B solution	"
Trypan blue	"
Gentamycin sulfate	"
Jackson alkaline phosphatase affinipure goat anti-mouse IgG subclass1	Astral Scientific
Agarose biotechnology grade I	"
Jackson alkaline phosphatase affinipure goat anti-mouse IgG subclass 2a	Abacus Als
Jackson alkaline phosphatase affinipure goat anti-mouse IgG subclass 2b	"
Nuclease free water	
1kb DNA step ladder	Promega
Lambda DNA/HindIII marker	"
100bp DNA ladder (low)	"
HyperLadder I	"
pGEM-Teasy vector system II	Fermentas
X-Gal (5-bromo-4-chloro-3-indoyl-ß-D-galactopyranoside)	Promega
IPTG (isopropyl-ß-D-thioglactopyranoside), Dioxane free	"
T4 DNA ligase	"
T4 DNA ligase buffer	"
BamHI-HF (100,000 U/ml)	"
EcoRI-HF (100,000 U/ml)	Genesearch
ZraI (10,000 U/ml)	"
pUC19 DNA	"
Komabiotec Ezway Direct *Taq* PCR mastermix	"
Quikchange II XL Site-Directed mutagenesis kit	Fisher biotec
Dneasy Blood and Tissue kit	Integrated Sciences
QIAquick PCR purification kit	QIAGEN
QIAquick Gel Extraction	"
QIAprep Spin miniprep	"
RNase A	"
Pertussis toxin salt free	"
Filamentous haemagglutinin	Sapphire Bioscience

Murine IFN-gamma ELISA kit (abcam)	"
DMEM medium	"
2-mercapto ethanol (2ME)	Invitrogen
Hepes buffer solution	"
Fetal bovine serum	"
Ultra pure Dnase/Rnase-free distilled water	"
15ml ultra series disposable tissue grinder	"
14ml BD falcon polypropylene round-bottom tube	Quantum Scientific
Disposable inoculating loops, 10µl	"
Dacron Swab	BD
Cell strainer, 40µl, Nylon	"
Microtiter plate F96 maxisorp (Nunc)	"
CryoCare preserver	Thermo Scientific
Nitrocellulose membrane 0.45µm	Blackabyd Diagnostics
Acrodisc 32mm syringe filter with 0.2µm super membrane non-pyrogenic	Bio Rad
Acrodisc 32mm syringe filter with 0.45um super membrane non-pyrogenic	Row Scientific
Tissue culture 24-well flat bottom with lid	"
Axygen 96 well PCR amplification plate with a single notch, half skirt	Sarstedt Australia
Axygen cycler sealer PCR sealing film	Fisher Biotec
Semi micro cuvettes, 1.5ml	"
	Interpath