JOURNAL
OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

© The Korean Society for Microbiology and Biotechnology

Construction and Preliminary Immunobiological Characterization of a Novel, Non-Reverting, Intranasal Live Attenuated Whooping Cough Vaccine Candidate

Cornford-Nairns, R.[1], G. Daggard[1], and T. Mukkur[1,2]*

[1]*Department of Biological and Physical Sciences, University of Southern Queensland, Toowoomba, 4350 Queensland, Australia*
[2]*School of Biomedical Sciences, Curtin Health Innovation Research Institute, Curtin University, Bentley Campus, Perth 6102, Western Australia, Australia*

We describe the construction and immunobiological properties of a novel whooping cough vaccine candidate, in which the *aroQ* gene, encoding 3-dehydroquinase, was deleted by insertional inactivation using the kanamycin resistance gene cassette and allelic exchange using a *Bordetella* suicide vector. The *aroQ B. pertussis* mutant required supplementation of media to grow but failed to grow on an unsupplemented medium. The *aroQ B. pertussis* mutant was undetectable in the trachea and lungs of mice at days 6 and 12 post-infection, respectively. Antigen-specific antibody isotypes IgG1 and IgG2a, were produced, and cell-mediated immunity [CMI], using interleukin-2 and interferon-gamma as indirect indicators, was induced in mice vaccinated with the *aroQ B. pertussis* vaccine candidate, which were substantially enhanced upon second exposure to virulent *B. pertussis*. Interleukin-12 was also produced in the *aroQ B. pertussis*-vaccinated mice. On the other hand, neither IgG2a nor CMI-indicator cytokines were produced in DTaP-vaccinated mice, although the CMI-indicator cytokines became detectable post-challenge with virulent *B. pertussis*. Intranasal immunization with one dose of the *aroQ B. pertussis* mutant protected vaccinated mice against an intranasal challenge infection, with no pathogen being detected in the lungs of immunized mice by day 7 post-challenge. *B. pertussis aroQ* thus constitutes a safe, non-reverting, metabolite-deficient vaccine candidate that induces both humoral and cell-mediated immune responses with potential for use as a single-dose vaccine in adolescents and adults, in the first instance, with a view to disrupting the transmission cycle of whooping cough to infants and the community.

*Corresponding author
Phone: +61 (08) 92667520; Fax: +61 (08) 92662342;
E-mail: T.Mukkur@curtin.edu.au

Whooping cough is the major cause of vaccine-preventable deaths today, with WHO estimates of 40–50 million cases and approximately 297,000–409,000 deaths each year worldwide [1, 7, 8, 15], the majority of them being in developing countries although some countries are currently recording increases in the incidence of whooping cough despite high vaccine coverage [39]. A killed whole-cell pertussis vaccine, generally given in combination with diphtheria and tetanus toxoids, has been available in many countries for over 40 years, and although its use seems to have controlled pertussis epidemics, concerns over the reactogenicity, ranging from high fever, persistent crying, pain at the site of injection, and possible, albeit rare, neuropathic manifestations [12, 20, 30, 33, 36], steered the research towards development of the currently marketed acellular pertussis vaccines given in combination with tetanus and diphtheria toxoids (DTaP). Although the introduction and widespread use of the pertussis vaccines caused a dramatic reduction in the incidence of whooping cough, it has risen recently despite high vaccine coverage in developed countries such as Australia, The Netherlands, and the United States despite high levels of immunization rates [http://cdc.gov/pertussis/outbreaks.html; 14]. In Australia, pertussis has been endemic since 1993 with notications rising from 1.8/100,000 in 1991 to a peak of 156.9/100,000 in 2010 despite a high rate of vaccine coverage [29].

Children under the age of 2 and up to the age of 5 years are highly susceptible to whooping cough. More recently, however, pertussis has re-emerged even in vaccinated populations, confirming that pertussis is not only a childhood

disease but is also highly prevalent in adults [39, 46], the latter being accepted now as the major reservoir of infection for the majority of the pertussis cases in infants and young children. This has stimulated interest in the development of an alternative vaccine that can also be used safely in the adolescent and adult population. 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 [24], loss of vaccine efficacy due to the emergence of new *B. pertussis* strains [25] producing high levels of pertussis toxin (a key virulence factor) [45], and lack of compliance with the recommended vaccination schedule because of the fear of side reactions [12, 19, 33, 34, 36]. Therefore, it is important not only to characterize the newly emerging strains with respect to overproduction of all significant virulence antigens but also to develop alternative vaccine candidates that can be delivered by non-invasive routes (*e.g.*, by oral, intranasal, or epidermal/cutaneous routes) and with potential to impart longer term protection than that offered by the currently marketed acellular pertussis vaccines, an approach divergent from the practices/formulations currently being promoted by most of the major global vaccine manufacturing enterprises.

It is apparent now that the protective efficacy of the acellular vaccine formulations is short to medium, given the recent introduction of new combined acellular vaccine formulations containing reduced antigen content, dTap or Tdap depending upon the vaccine manufacturer, for use in adolescents [26]. To overcome potential side reactions ranging from extensive swelling of the injected limb, which may occur in a concerning percentage of children vaccines receiving booster vaccinations, particularly post-primary immunization with three doses of DTaP, one alternative suggested has been either to reduce the number of booster shots with ensuing reduced levels of immunity or to find a replacement adjuvant, which unlike alum favors the induction of Th1 responses that have been proposed to be responsible for long-term protection against whooping cough [24]. However, as compared with the DTaP, DTwP has been reported to be more protective despite inducing lower antibody titers to pertussis toxoid, as judged by intracerebral and lung clearance experiments, leading to the conclusion that cell-mediated immunity may play a crucial role in eliminating bacteria that escape the onslaught of the humoral defence mechanisms in the early phase of infection [2, 17].

An alternative vaccine development approach involving intranasal or parenteral administration of biodegradable particle-encapsulated *B. pertussis* antigens (PTxoid, FHA, PRN) to mice either induced CMI (Th1) or antibody (Th2) responses that yielded protection that was no better than that induced by the antigens administered in solution [4, 40]. Given the relatively recent communication suggesting Th1/Th17-mediated host immunity against pertussis [28], it is apparent that the exact nature of immunity against infection with *B. pertussis* is still not completely understood and requires further elucidation.

Mice vaccinated with PT-deficient live *B. pertussis* were reported to be protected against challenge with virulent *B. pertussis* [22]. In another study, an aromatic-dependent mutant (*aroA*) of *B. pertussis* [35] was reported to persist in the lungs of mice for a short period of time (4 days at reasonable numbers), thus casting doubt on its ability to stimulate the cell-mediated immunity (CMI) required for long-lasting protection. This result was unexpected, given previous reports regarding the success of the *aroA* mutant of *Salmonella* species [13, 27] as a successful vaccine. On the other hand, the *aroA* mutants of *Shigella* species were found to be poorer vaccines than the *aroD* deletion mutants of the same species [43]. It was suggested that the development of live attenuated whooping cough vaccine delivered by the nasal route, so as to mimic the natural route of infection, leading to induction of long-lasting immunity, represented an ideal solution that deserved serious attention [18]. More recently, a live attenuated *B. pertussis* mutant strain, in which PT was genetically detoxified, the dermonecrotoxin (DNT) gene deleted, and *B. pertussis* ampG replaced by *E. coli* ampG to reduce its toxic activity, was reported to induce protection in young mice after a single nasal administration [23] and reported to be safe in adult interferon-γ receptor deficient adult mice, although the vaccine candidate survived in lungs of vaccinated mice for as long as the wild-type strain. We report the development of an aromatic-dependent (*aroQ*) *B. pertussis* mutant that was found not only to induce *B. pertussis*-specific antibody response and cell-mediated immunity in mice immunized with a single dose of this vaccine candidate by the intranasal route, but was also protective against challenge infection with virulent *B. pertussis*. Upon challenge infection of immunized mice with virulent *B. pertussis*, both antibody and CMI responses were significantly or substantially enhanced, respectively, imparting protection against the challenge infection.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Media, and Growth Conditions

The bacterial strains used in this study were virulent *Bordetella pertussis*, ATCC 9340 (Microdiagnostics, Brisbane, Australia), Streptomycin-resistant [Sm^R] *B. pertussis* (obtained through courtesy of Dr. A. Weiss, University of Cincinnati, OH, USA), *aroD E. coli* mutant (obtained through courtesy of Dr. Naresh Verma, Australian National University, Canberra, Australia), *E coli* DH5α [F$^-$ φ80d*lacZ*ΔM15 Δ(*lacZYA-arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r$_k^-$,m$_k^+$) *pho*A *sup*E44 λ$^-$ *thi*-1 *gyr*A96 *rel*A1; Invitrogen Life Technologies],

E. coli JM101 [F' traD36 proA⁺B⁺ lacq Δ(lacZ)M15/Δ(lac-proAB) glnV thi; New England Biolabs, Inc], and *E. coli* Sm10λpir (Mobilising strain, *thi thr leu tonA lacY supE recA:*:RP4-2-Tc::Mu Km λpir, obtained through the courtesy of Dr. Roy Curtiss, Washington University in St. Louis, MO, now at Arizona State University, Tempe, USA). The plasmids used in this study included pNEB193 (Cloning vector, Ap^R, *LacZ*; New England Biolab, Inc.,), pUK (Pharmacia Biotech), and pRTP1 (Ap^R, *rpsL, oriT, cos*; obtained through the courtesy of Dr. Scott Stibitiz, Centre for Biologics Evaluation and Research, Bethesda, MD, USA).

The *E. coli* strains were routinely cultured in LB broth or on LB agar overnight at 37°C. *B. pertussis* was routinely grown on Bordet–Gengou (BG) solid medium containing 15% sterile-defibrinated sheep blood for 3–5 days at 35–37°C. Cohen–Wheeler (CW) agar [3] containing 10% sterile-defibrinated sheep blood was used for *B. pertussis* growth for conjugation experiments only.

Modified cyclodextrin solid media (CSM) as described elsewhere [10] together with M9 minimal agar (Difco), with or without aromatic compounds (aromix), was used to test for the aro⁻ phenotype of *E. coli* or *B. pertussis* mutants. The final concentrations of aromix consisted of 40 μg/ml each of tryptophan, tyrosine, and phenylalanine; and 10 μg/ml each of dihydroxybenzoic acid (DHB) and *para*-aminobenzoic acid (PABA). The antibiotic concentrations used for selection were as follows: kanamycin 50 μg/ml, streptomycin 200 μg/ml, and ampicillin 100 μg/ml.

Preparation and Manipulation of DNA

Plasmid DNA was extracted from *E. coli* strains and agarose gel using a Prep-A-Gene DNA Purification Kit (BioRad). Genomic DNA (gDNA) was purified from *B. pertussis* using a BioRad Genomic DNA Isolation Kit. All DNA manipulations were carried out using the protocols in Sambrook *et al.* [38]. Restriction endonucleases, T4 DNA ligase, and alkaline phosphatase were supplied by either MBI Fermentas, New England Biolabs, or Amersham Pharmacia Biotech and were used according to the manufacturers; recommendations. The PCR kit used was purchased from Fisher Biotec.

Identification of *aroQ* Gene of *B. pertussis*

Construction of *aroQ B. pertussis* (*aroQ*BP) mutants was underpinned by the discovery that the *Sau*3A digest of the chromosomal DNA of *B. pertussis* cloned in pNEB193 (pUSQBord1 fragment), while complementing the *aroD* gene of *E. coli* strain 583/90, had a sequence that was not similar to the published consensus sequence of the *aroD* gene of *E. coli* (GenBank Accession No. X59503). As the cloned *B. pertussis* fragment was too large (1,587 bp) to fully sequence at the time of this discovery, only approximately 500 bp from the 5' and 3' ends of the fragment was available for analysis. A BLAST search (ANGIS) using the partial sequences showed that the isolated *B. pertussis* DNA fragment contained at least part of a gene sequence that possessed high homology (77%) with the *aroQ* gene, which exists in many microorganisms, including *Pseudomonas aeruginosa, Actinobacillus pleuropneumoniae,* and *Corynebacterium pseudotuberculosis,* but is not known to be present in *E. coli* [38, Unpublished]. However, there is a body of evidence, including complementation of the *aroD* mutants and an inability of *B. pertussis* to survive on quinic acid, to suggest that despite the apparent type II nature, the *aroQ* gene of *B. pertussis,* and similar genes of other prokaryotes, function in the aromatic biosynthetic pathway [16].

DNA Sequencing

DNA sequencing was carried out by the Australian Genome Research Facility (Ritchie Laboratories, University of Queensland). Sequences were then analyzed using the ANGIS (Australian National Genome Information Service) program.

Bacterial Conjugation

Conjugal transfer of DNA from *E. coli* to *B. pertussis* was carried out using the generic methodology as described elsewhere [24]. Briefly, after 72 h growth, *B. pertussis* BP304 was swept from BG agar specifically using a sterile dacron swab and resuspended in phosphate-buffered saline (PBS). The optical density of the suspension at 630 nm was determined and adjusted to an absorbance of 0.4. This procedure was repeated using *E. coli* SM10λpir containing the cloned insertionally inactivated *aroQ* gene, pUSQBord10 (see results section) except that growth was swept from LB agar supplemented with kanamycin. Transconjugants displaying both Km^R and Sm^R were verified using CW agar plates with and without aromix. *B. pertussis aroQ* mutant colonies grown on medium with aromix were found to have a slower growth rate than that of the wild type. Mutant clones failed to grow without supplementation of the media with aromix.

Animal Experiments

Five- to six-week-old female BALB/c mice were used throughout this investigation and all animal experiments were carried out at the University of Southern Queensland after gaining approval from the Animal Ethics Committee in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Determination of the MMD₅₀ of *B. pertussis* Tohama I

In order to examine the rate of clearance of infection in vaccinated versus control mice after challenge with virulent Tohama I, it was necessary to determine the Minimum Moribund Dose at which 50% of the mice inoculated by the intranasal route (MMD_{50}) became moribund. Moribund was defined as "being in a sick or dying state characterized by dorsal recumbency requiring immediate euthanasia." It was of great importance that mice did not die during the course of the experiment. A total of 32 mice were used in this experiment. Groups of 3–6 mice were intranasally challenged with different doses of virulent *B. pertussis* Tohama I (1×10^7 CFU: 5 mice; 1×10^8 CFU: 6 mice; 5×10^8 CFU: 3 mice; 1×10^9 CFU: 6 mice; 5×10^9 CFU: 3 mice; 1×10^{10} CFU: 3 mice; 5×10^{10} CFU: 3 mice; 1×10^{11} CFU: 3 mice). MMD_{50} was found to be 2.1×10^8 CFU (data not shown).

Determination of Safety of *aroQ B. pertussis*

A total of 15 mice (5 mice per group) were inoculated with 10^8, 10^9, or 10^{10} CFUs of *aroQ B. pertussis* by the intranasal route and observed over a period of 3 weeks for any morbidity or mortality. Lung homogenates were collected from these mice at 3 weeks post-immunization and plated out on CW agar plates supplemented with the aromix for detection of the challenge *B. pertussis* strain.

Determination of Persistence and Immunogenicity of *aroQ B. pertussis*

Three sets of animal experiments were carried out as follows:
Experiment 1 was aimed at determining the persistence of the *aroQ B. pertussis* mutant in the lungs of mice. A total of 67 mice were used in this experiment. Seven to eight mice [7, 8] per interval were

vaccinated by the intranasal route, with one dose consisting of 1.5×10^9 CFU of the *aroQ B. pertussis* per mouse, to determine the persistence of the mutant strain in the lungs at days 2, 4, 6, 7, 8, 9, 10, 11, 12, and 14 post-inoculation. Mice were euthanized at each interval, and serum samples and lungs were homogenized for bacterial counts using CW agar plates supplemented with aromix.

Experiment 2 was aimed at determining the tracheal bacterial clearance profile of the *aroQ B. pertussis* mutant. A total of 20 mice were used for this experiment. Four [4] mice per group were used for vaccination with the *aroQ B. pertussis* mutant by the intranasal route followed by euthanasia at days 1, 3, 7, 14, and 21 post-vaccination to determine its clearance profile from the trachea of infected mice. Tracheal homogenates were homogenized and plated out on BG media containing 40 µg/ml of cephalexin.

Experiment 3 was aimed at determining the immune response of mice (20 mice) immunized with a single dose of *aroQ B. pertussis* (1×10^9 CFU per mouse) delivered by the intranasal route in 20 µl PBS (Group 1) versus one dose of DTaP vaccine, 1/5th of the standard human dose (20 mice; Group 2) administered subcutaneously, using sham-immunized (PBS) mice (20 mice; Group 3) as controls. Four mice from each group were euthanized at 5, 7, 14, and 21 days post-immunization for each interval. The remaining 4 mice in each group were challenged with the virulent *B. pertussis* at day 21. The challenge dose, administered by the intranasal route, was 2.1×10^8 CFU (0.5 MMD_{50}). At day 28 post-challenge, sera and lungs were collected for determination of IgG isotype and/or subclass antibody titers using FHA, PTd, and *Bordetella pertussis* killed whole cells (BPWC) as antigens. At day 14 post-vaccination, splenocyte transformation assays for determination of IL-2 and IFN-γ at 72 h post-stimulation, with PTd and FHA as antigens, were also carried out. The splenocytes from 4 mice in each group were pooled to ensure the availability of an adequate number of cells for stimulation with antigens in triplicate permitting determination of average quantity of cytokines produced. Lung homogenates were plated out on the BG-aromix plate for detection of the challenge strain.

Determination of Protective Potential of *aroQ B. pertussis* in Mice

A total of 10 mice were used in this experiment. Mice immunized with either a single dose of *aroQ B. pertussis* or PBS (sham-immunized) were challenged by the intranasal route with the parent virulent *B. pertussis* (ATCC9340) at day 21 post-immunization with a 0.5 MMD_{50} dose (2.1×10^8 CFU per mouse delivered in 20 µl of PBS). The number of mice exhibiting moribund state post-challenge was recorded and lung homogenates were plated out on the BG-aromix plate for detection of the challenge strain.

Detection of Production of FHA, PT, and Pertactin by *aroQ B. pertussis*

Determination of the production of FHA, PT, and pertactin by the mutant strain was carried out using specific monoclonal antibodies by Western blotting essentially as described by Towbin *et al.* [42]. Briefly the *aroQ* mutant of *B. pertussis* was grown in Stainer Scholtes medium [41] for a period of 48−72 h, and subjected to centrifugation in a Beckman high-speed centrifuge at 2,500 ×*g*. The supernatant was concentrated by pervaporation and the lysed bacterial sediment was subjected to SDS-PAGE followed by Western blotting. The blot was developed using monoclonal antibodies, obtained through the courtesy of CSL Ltd, Parkville, against FHA, PT, or pertactin.

Measurement of Antibody Responses

Sera from experimental mice were collected for determination of antibody titers using indirect dot blotting as described elsewhere [9]. The antigens used to adsorb to the nitrocellulose membranes (BioRad) were formalin-killed whole cells of *B. pertussis* (BPWC), FHA, or PT. The secondary antibody used was either goat anti-mouse IgG, IgG1, or IgG2a−alkaline phosphate conjugate. Substrate comprising 5-bromo-4-chloro-3-indoryl phosphate (BCIP)/Nitroblue Tetrazolium (NBT) (Sigma-Aldrich) was used to stain the dot blots.

Cytokine Quantifications

Quantification of IL-12 in pooled mouse sera, and IL-2 and IFN-γ in antigen-stimulated splenocyte supernatants were determined using commercial sandwich ELISA kits (Biosource). For measurement of IL-2 and IFN-γ, splenocytes from each group were pooled to ensure availability of adequate number of cells for the experiment to be carried out in triplicate and resuspended in Dulbecco's Modified Eagle's Medium (Gibco) containing 100 µg penicillin ml^{-1}, 100 µg streptomycin ml^{-1}, 10% fetal bovine serum, and 50 µM 2-mercaptoethanol and seeded at 6×10^5 cells per well. Cells were stimulated *in vitro* with either 5 µg of PTd or FHA. Supernatants were removed after 24 h incubation at 37ºC with 5% CO_2 to determine IL-2 production and at 72 h for interferon-γ measurements.

Statistical Analysis

Statistical analysis was carried out using ANOVA using SPSS for Windows version 13.0 (SPSS, Chicago, IL, USA). The $P < 0.05$ was considered to be statistically signicant.

RESULTS

As only a part of the *aroQ* gene sequence was identified owing to insufficient sequencing, the fragment was reduced from 1,587 bp to 1,019 bp by digestion with *Eco*RI and then subcloned into *Eco*RI-cut pNEB193 (labeled as pUSQBord4). Further sequencing revealed the *aroQ* gene of *B. pertussis* to comprise 435 bp [5] encoding 145 amino acids and to be homologous to that predicted in the entire *B. pertussis* Tohama I genome sequence published in 2003 [31].

Insertional Inactivation of the *aroQ* Gene

The *aroQ* containing 1,019 bp fragment cloned in pNEB193 was extracted from *E. coli* JM101, the host strain, using a plasmid miniprep kit. A large section [303 bp] at the 3' end of the cloned *aroQ* gene and two additional fragments (719 bp and 44 bp) were removed by double digesting the plasmid with the restriction enzymes *Ngo*MIV and *Bss*HII.

A kanamycin resistance gene cassette containing its own promoters derived from pUC4K was amplified by the polymerase chain reaction (PCR) using primers with *Ngo*MIV and *Bss*HII restriction ends to create a fragment size of approximately 940 bp (see Table 1 for FORKAN/BACKAN primers).

This *Ngo*MIV-*Bss*HII cassette was then ligated into the modified pUSQBord4 plasmid, from which the *Ngo*MIV-

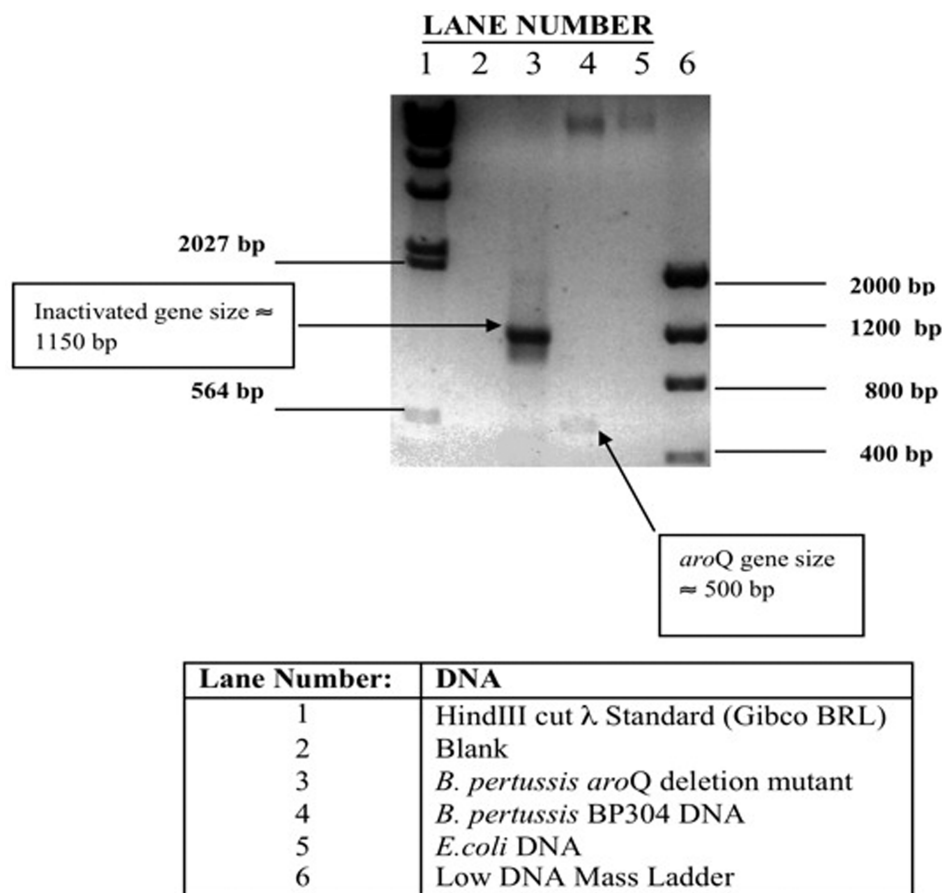**Table 1.** Primers used for the amplification of kanamycin or the *aroQ* gene.

Primer name	5'→ 3' Primer sequence
FORKAN	GTGCCGGCGTGAATCTCTGATGTTACATTG
BACKAN	GGGCGCGCACTAGTGTTACAACCAATTAAC
FORQ	ATGGCGCAACGCATTCTTGT
BACQ	GTTTTGAGTTTTCGGAGGTC

*Bss*HII fragment had been removed, in the same orientation as the *aroQ* gene, to produce a new plasmid labeled as pUSQBord7. The 719 bp *Bss*HII-*Bss*HII fragment was then religated back into the alkaline phosphatased *Bss*HI-cut plasmid, pUSQBord7, in its original orientation providing a total of 730 bp homology at the 3' end, subsequently renamed pUSQBord8.

In order to transfer the inactivated *aroQ* gene into *B. pertussis*, the plasmid pUSQBord8 was digested with *Eco*RI and *Bam*HI and the fragment containing the modified *aroQ* gene and kanamycin gene was ligated

directly into pRTP1 to produce another new plasmid, labeled as pUSQBord9. In order to increase the chances of a recombination event occurring, a scaffold sequence from the original 1.5 kb fragment upstream of the inactivated *aroQ* locus (located in pUSQBord1) was designed to increase the 5' noncoding region from 43 bp to 689 bp. A triple ligation involving inserts from pUSQBord9 and pUSQBord1 ligated together was inserted into the pRTP1 to create a new shuttle vector, pUSQBord10, ready for conjugation. This plasmid was transformed into *E. coli* SM10*lpir* followed by conjugation with the Sm^R *B. pertussis* on CW agar and transconjugants were selected on CW agar containing kanamycin and streptomycin.

As pRTP1 is a suicide vector, it was assumed that *B. pertussis* transconjugants exhibiting Kan^R had also incorporated the modified inactivated *aroQ* gene into their genome by homologous recombination. To confirm this assumption, PCR (FORQ/BACQ2) was used to amplify a fragment containing the *aroQ* gene in the parental strain. The FORQ primer was designed for that start of the *aroQ* gene and the BACQ primer was designed for the 3' non-coding region close to the end of the *aroQ* gene. This

LANE NUMBER

2027 bp

Inactivated gene size ≈ 1150 bp

564 bp

2000 bp
1200 bp
800 bp
400 bp

*aro*Q gene size ≈ 500 bp

Lane Number:	DNA
1	HindIII cut λ Standard (Gibco BRL)
2	Blank
3	*B. pertussis aroQ* deletion mutant
4	*B. pertussis* BP304 DNA
5	*E.coli* DNA
6	Low DNA Mass Ladder

Fig. 1. PCR analysis using primers from the *aroQ* gene of *B. pertussis*.
The fragment of ≈1,150 bp corresponds to ≈500 bp for the *aroQ* gene minus 303 bp deletion plus 940 bp for the Kan^R cassette.

resulted in a fragment of approximately 1,150 bp (Fig. 1). This corresponded to the size of the *aroQ* fragment (500 bp) minus the 303 bp deletion plus the insertion of the 940 bp Kan^R cassette.

To confirm this result, the 1,150 bp fragment was extracted from an agarose gel, and PCR with Kan^R gene primers was found to amplify the Kan^R gene (results not shown).

Screening for Production of FHA, PT, and the 69 kD Pertactin in *aroQ B. pertussis* by Western Dot Blotting Using Specific Monoclonal Antibodies

Evaluation of the *aroQ B. pertussis* strain for production of the major virulence antigens currently used in either modified or native state in the currently marketed acellular pertussis vaccines revealed that FHA was present both in the supernatant as well as associated with the bacterial pellet, PT was present in the supernatant, and pertactin was cell associated, as expected (Table 2).

Persistence of *aroQ B. pertussis* Mutant in Mice

The *aroQ B. pertussis* mutant was undetectable in the trachea of vaccinated mice at day 6 post-immunization (Fig. 2). However, it was detectable in the lungs of mice up to day 11 but undetectable at day 12 post-administration by the intranasal route (Fig. 2), which was longer than that reported for the *aroA B. pertussis* mutant [24]. The challenge strain, however, after an initial drop in growth, continued to replicate (Fig. 2).

Safety of *aroQ B. pertussis* Mutant in Mice

No mortality or morbidity was observed in mice exposed to 10^8, 10^9, or 10^{10} CFUs of *aroQ B. pertussis* by the intranasal route, indicating the safety of the *aroQ B. pertussis* mutant *in vivo* in mice.

Protective Potential of *aroQ B. pertussis* in Mice

All mice vaccinated with a single dose of *aroQ B. pertussis* or DTaP were protected, as judged by the absence of moribund state against challenge infection with virulent *B. pertussis* strain, whereas 3/5 unvaccinated mice had to be euthanized by day 7 post-challenge because of their moribund state (data not shown). No *B. pertussis* was isolated from the lung homogenates of mice vaccinated with either DTaP or *aroQ B. pertussis* vaccine candidate at day 7 post-challenge, whereas up to 10^{10} CFU of virulent *B*
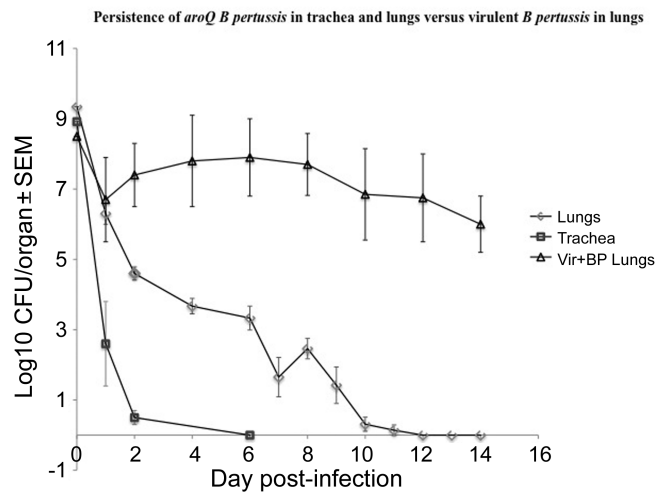
Fig. 2. Persistence of *aroQ B. pertussis* in trachea and lungs versus virulent *B. pertussis* in lungs of vaccinated mice.
The *aroQ B. pertussis* mutant was cleared from the trachea and lungs within 7 and 12 days, respectively, whereas the virulent strain continued to replicate until day 7 and persist in the lungs of infected mice.

pertussis per lung was isolated from mice euthanized in the moribund state (Mouse #1: 1.4×10^9 CFUs; Mouse #2: 5×10^9 CFUs; Mouse #3: 1.8×10^{10} CFUs; Mouse # 4: 7.4×10^5 CFUs; Mouse #5: 4.8×10^4 CFUs).

Antibody Response of Mice Vaccinated with a Single Dose of *aroQ B. pertussis*

IgG antibodies against FHA and BPWC in the sera of *aroQ B. pertussis* and DTaP-vaccinated mice were detectable against all the antigens used in this study at all days post-vaccination (Fig. 3A-3C); however, anti-PT antibodies were detectable from day 7 post-immunization onwards with the *aroQ* BP vaccine. Whereas the serum anti-FHA antibody titers of DTaP-vaccinated mice were significantly higher ($p<0.05$) than those of *aroQ*BP-vaccinated mice at all days post-immunization [5, 7, 14, 21], anti-PT antibody titers of mice were significantly higher ($p<0.05$) at day 14, with anti-BPWC titers being significantly higher ($p<0.05$) at days 14 and 21 post-vaccination.

Upon challenge of the *aroQ*BP-vaccinated group with the parent *B. pertussis*, there was an enhancement of antibody response, with IgG1 isotype antibody activity against FHA, PT, and BPWC being significantly higher ($p<0.05$ to $p<0.01$) in DTaP-vaccinated mice than those of *aroQ*BP-vaccinated mice (Fig. 4A–4C). No IgG2a antibody was produced in DTaP-vaccinated mice pre-challenge in contrast to the *aroQ*BP-vaccinated mice in which IgG2a antibody was induced and detectable. Although low levels of the IgG2a against FHA, PT, and BPWC were produced in DTaP-vaccinated mice post-challenge, they were significantly lower ($p<0.05$) than those produced by challenged *aroQ*BP-vaccinated mice (Fig. 4A-4C).

Table 2. Detection of virulence antigens of *aroQ B. pertussis* by dot blotting using specific monoclonal antibodies.

Fraction tested	Supernatant	Bacterial pellet
FHA	+	+
PT	+	-
PRN	-	+

A. Anti-FHA IgG

B. Anti-PT IgG
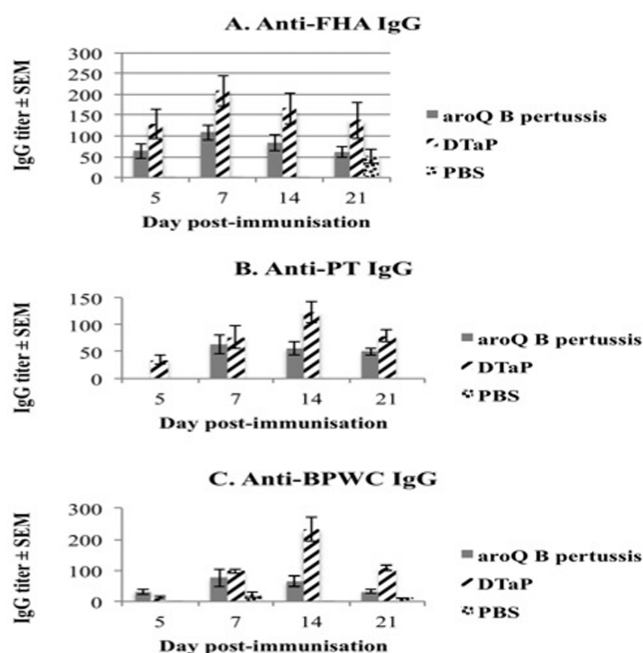

C. Anti-BPWC IgG

Fig. 3. Kinetics of IgG response against filamentous hemagglutinin (FHA), pertussis toxin (PT), and killed *B. pertussis* whole cells (BPWC) post-immunization with *aroQ B. pertussis* (*aroQ*BP). Mice immunized with *aroQ B. pertussis* produced antibodies against the *B. pertussis* immunogens tested. Statistical analysis: (**A**) Anti-FHA response [DTaP vs *aroQ B. pertussis* significantly greater at days 5, 14, 21 ($p<0.05$), day 14 ($p<0.01$)], (**B**) Anti-PT response [DTaP vs *aroQ B. pertussis* at day 14 ($p<0.05$), (**C**) Anti-BPWC [DTaP vs *aroQ B. pertussis* at days 14, 21 ($p<0.01$).

Cytokine Analysis Mice Vaccinated with a Single Dose of the *aroQ B. pertussis*

Analysis of sera from Experiment 3 revealed production of a substantial level for IL-12 levels at all days post-immunization with the *aroQ*BP vaccine candidate (Fig. 5), whereas mice vaccinated with 3 doses of the DTaP (1/10th of the standard human dose) produced no IL-12 (data not shown).

Mice vaccinated with *aroQ*BP produced high levels of IL-2 against FHA and pertussis toxin on day 21 post-immunization, whereas those vaccinated with DTaP produced no IL-2 (Fig. 6A and 6B). Whereas there was a substantial increase in the level of IL-2 produced by *aroQ*BP-vaccinated mice, substantially lower levels were produced in DTaP-immunized mice (Fig. 6A and 6B). Although a low level of IFN-γ post-stimulation of splenocytes with FHA and pertussis toxoid of *B. pertussis* pre-challenge were recorded (Fig. 6A and 6B) at day 21 post-vaccination, they were substantially enhanced at 28 days post-challenge with virulent *B. pertussis* (Fig. 6A and 6B). Although there was slight decrease in the level of IL-2 produced by antigen-stimulated pooled splenocytes post-challenge versus the pre-challenge level, it does not appear to be substantial. On the other hand, neither IL-2 nor IFN-γ were detectable in DTaP-vaccinated mice pre-challenge,
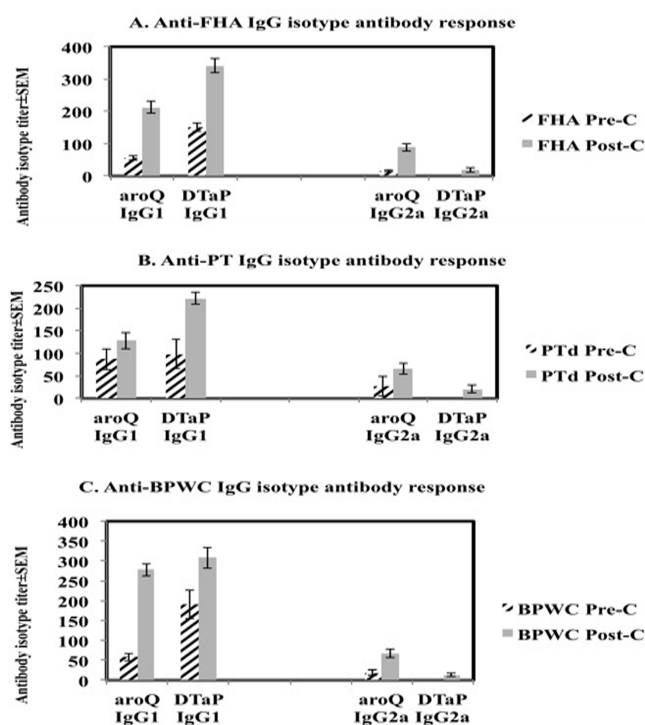
A. Anti-FHA IgG isotype antibody response

B. Anti-PT IgG isotype antibody response

C. Anti-BPWC IgG isotype antibody response

Fig. 4. Antibody isotype response of mice immunized with *aroQ B. pertussis* versus DTaP, pre- versus post-challenge.

Mice vaccinated with *aroQ B. pertussis* generated both IgG1 and IgG2a isotypes, whereas mice vaccinated with DTaP only produced IgG1. Abbreviations: aroQ denotes *aroQ B. pertussis*; FHA Pre-C denotes pre-challenge antibody isotype titers against FHA; FHA Post-C denotes post-challenge antibody isotype titers against FHA. Statistical analysis: (**A**) Anti-FHA IgG isotype response: IgG1 [DTaP vs *aroQ B. pertussis* (aroQ IgG1) significantly greater at day 21 pre-challenge (PreC) and post-challenge at day 28 ($p<0.05$); IgG2a: Anti-FHA Pre-C at day 21 versus Post-C day 28 ($p<0.01$)]. (**B**) Anti-FHA IgG isotype response: IgG1 [DTaP versus *aroQ B. pertussis* (aroQ IgG1) significantly greater at day 21 pre-challenge (PreC) versus post-challenge at day 28 ($p<0.01$); IgG2a: *aroQ B. pertussis* vs DTaP significantly greater anti-PT response Pre-C at day 21 versus Post-C at day 28 (significantly greater at $p<0.05$)]. (**C**) Anti-BPWC IgG isotype response: IgG1 [DTaP vs *aroQ B. pertussis* (aroQ IgG1), significantly greater at day 21 pre-challenge (PreC) and post-challenge at day 28 ($p<0.01$); IgG2a [*aroQ B. pertussis* vs DTaP]: significantly greater post-challenge (Post-C) at day 28 ($p<0.05$)].

but they were detectable post-challenge with virulent *B. pertussis* (Fig. 6A and 6B).

DISCUSSION

The incidence of whooping cough, caused by *Bordetella pertussis*, in infants has surged in epidemic proportions in Australia as well as worldwide despite high coverage with the currently marketed pertussis vaccines [14, 25, 29]. The focus of the vaccine manufacturing pharmaceutical industries to prevent this disease, understandably, has been the promotion of further booster vaccinations with a lower antigen content vaccine formulation of the currently used
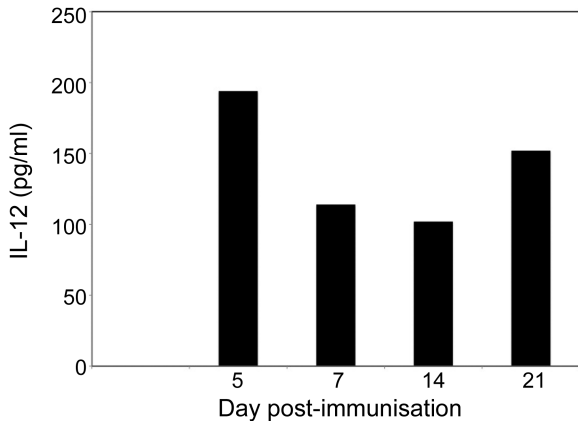
Fig. 5. Serum IL-12 levels post-immunization with *aroQ B. pertussis* at different days post-immunization.

Mice immunized with *aroQ B. pertussis* induced substantial production of IL-12 detectable at days 5 to 21 post-immunization by the intranasal route.

aP vaccines, given that there are no better alternative vaccines available at the present time.

There are a number of potential reasons for the lack of compliance with currently marketed pertussis vaccines, the major one being the cost of these vaccines, albeit free for infants in some developed countries, particularly for parents in the low socioeconomic groups. It is desirable therefore to develop a cost-effective, non-invasive, long-lasting whooping cough vaccine, which can impart long-term immunity when used in one or two doses at best.

Fig. 6. Quantification of IL-2 and IFN-γ induced in mice immunized with a single dose of *aroQ B. pertussis* post-stimulation of splenocytes with filamentous hemagglutinin (FHA) and pertussis toxoid (PTd).

B. pertussis aroQ induced production of both IL-2 and IFN-γ pre- and post-challenge with virulent *B. pertussis*; DTaP, on the other hand, produced low levels of these cytokines only after challenge with virulent *B. pertussis*.

Other than the cost-ineffectiveness of the currently used pertussis vaccine formulations, particularly for the developing nations, other major problems associated with the adoption of currently marketed aP vaccines are listed below:

(i) Short to medium duration of protection, at best, imparted against pertussis infection attributed to waning of antibody-mediated immunity, mandating frequent booster vaccinations, (ii) induction of low level, if any, of cell-mediated immunity considered to be important for long-term protection against whooping cough, (iii) limited protection against the major exotoxins, and (iv) the lack of compliance with the recommended vaccination schedule due to fear of potential side reactions as already mentioned in the text.

The aP vaccines consist of two native virulence antigens [filamentous hemagglutinin (FHA) and pertactin (PRN)] and one chemically or genetically inactivated exotoxin (PTd)], with or without the two fimbrial antigens (Fim 2 and Fim 3/6), depending upon the vaccine manufacturing enterprise, and only one of the three major toxins, pertussis toxin, included as a toxoid. This vaccine was introduced a decade ago in the developed world because of the perceived fear of potential side reactions reported for the killed whole-cell pertussis vaccine, the latter still in use in most developing countries.

Alternatives to regular boosters with current aP vaccines suggested are to discover safe, CMI-inducing replacement adjuvants for alum-based compounds that favor the induction of Th1 responses, the latter being considered as important for long-term protection against whooping cough [17, 20]. Given that both DNA and microparticle-based vaccines have yielded variable results thus far [reviewed in 21], development of an alternative non-invasive, safe, intranasal pertussis vaccine capable of inducing both arms of the immune response with a view to imparting long-term immunity is highly desirable.

The short duration of persistence of live attenuated *aroA B. pertussis* [35] in the host appears to have precipitated relative uncertainty on their ability to stimulate CMI responses required for long-lasting protection. This result was unexpected given previous reports regarding the success of the *aroA* mutant of *Salmonella* species [13, 27] as a successful vaccine. On the other hand, the *aroA* mutants of *Shigella* species were found to be poorer vaccines than the *aroD* deletion mutants of the same species [43]. It was therefore of interest to determine whether the *aroQ B. pertussis* may be more effective as a live attenuated vaccine strain because of the induction of CMI.

PT-deficient live *B. pertussis* had been reported to offer protection against challenge with virulent *B. pertussis* [22] leading to the suggestion that a live attenuated whooping cough vaccine delivered by the nasal route, so as to mimic the natural route of infection, may lead to the induction of long-lasting immunity [18]. A more recent report of the
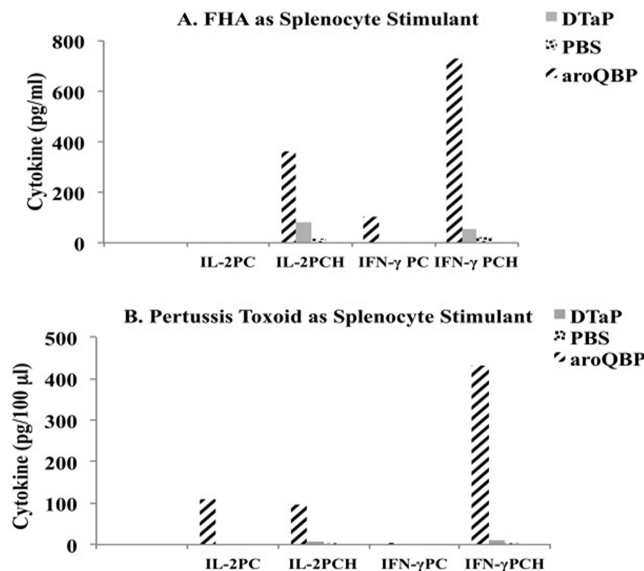
immunogenicity and protective potential of genetically detoxified *B. pertussis* with production of low levels of tracheal cytotoxin production and no dermonecrotoxin was reported to be immunogenic in young (3-week-old) and IFN-γ receptor knockout mice [23]. Given that this genetically modified vaccine candidate multiplies in mice essentially to the same degree as the virulent *B. pertussis* in mice, and no essential gene in the *B. pertussis* vaccine candidate has been deleted, further studies to ascertain its non-reversion to virulence and its toxicity profile are warranted. Regardless, based on the estimated duration of pertussis immunity [44], it should be possible to achieve a protection for a significantly longer period than that obtained with the acellular pertussis vaccine formulations if a non-reverting, non- or inconsequentially toxic rationally attenuated metabolite-deficient *B. pertussis* vaccine candidate was available for immunization by the intranasal route the natural route of infection.

This study reports the generation of a non-reverting, potentially side-reaction-free *B. pertussis* vaccine candidate, *aroQ B. pertussis*, which multiplies in the host only for a short period of time but induces both antibody and cell-mediated immune responses to protective antigens tested thus far and which are enhanced upon exposure to a challenge infection with the pathogen. Interestingly, challenge infection of DTaP-vaccinated mice also induced production of CMI-indicator antibody isotype as well the cytokines, which provides support to the possibility that CMI reported after vaccination of adolescents with the reduced antigen pertussis booster or DTaP vaccine (with or without added IPV) may be attributable to "silent pertussis booster infections" [47].

Given that FHA and PTd are incorporated in the currently marketed acellular pertussis vaccines, and the *aroQ B. pertussis* vaccine candidate induces both antibody and CMI responses to these antigens, makes this vaccine candidate an attractive option for further development as a non-reverting live attenuated whooping cough vaccine. Further studies on the safety and immunizing efficacy of the novel *aroQ B. pertussis* vaccine candidate are clearly warranted.

Acknowledgments

Abbreviations

IFN-γ, interferon-gamma; IL-2, interleukin-2; Th1, T-helper cell Type 1; Th2, T-helper cell Type 2

References

1. Andrews, R., A. Herceg, and C. Roberts. 1997. Pertussis notifications in Australia, 1991 to 1997. *Commun. Dis. Intell.* **21:** 145–148.
2. 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:** 637–643.
3. Cohen, S. M. and M. W. Wheeler. 1946. Pertussis vaccine prepared with phase-I cultures grown in fluid medium. *Am. J. Pub. Health* **36:** 371–376.
4. 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 immunisation with antigens entrapped in biodegradable particles: Effect of formulation and route of immunization on induction of Th1 and Th2 cells. *Vaccine* **19:** 1940–1950.
5. Cornford, R. A. 1998. Characterisation of the *aroD* and/or *aroQ* gene of *Bordetella pertussis*. BSc. Hons. Thesis, University of Southern Queensland, Toowoomba, Queensland, Australia.
6. Cornford, R. A. 2003. A novel, genetically engineered intranasal whooping cough vaccine. PhD Thesis, University of Southern Queensland, Toowoomba, Queensland, Australia.
7. de Melker, H. E., M. A. Conyn-van Spaendonck, H. C. Rumke, J. K. van Wijngaarden, F. R. Mooi, and J. F. P. Schellekens. 1997. Pertussis in The Netherlands: An outbreak despite high levels of immunization with whole-cell vaccine. *Emerg. Infect. Dis.* **3:** 175–178.
8. Elahi, S., J. Holmstro, and V. Gerdts. 2007. The benefits of using diverse animal models for studying pertussis. *Trends Microbiol.* **15:** 462–468.
9. Ermens, A. A. M., A. J. M. Bayens, A. C. M. van Gemert, and J. L. P. van Duijnhoven. 1997. Simple dot-blot method evaluated for detection of antibodies against extractable nuclear antigens. *Clin. Chem.* **43:** 2420–2422.
10. Frohlich, B. T., E. R. De Bernardez Clark, G. R. Siber, and R. W. Swartz. 1995. Improved pertussis toxin production by *Bordetella pertussis* through adjusting the growth medium's ionic composition. *J. Biotech.* **39:** 205–219.
11. Fry, S. R., A. Y. Chen, G. Daggard, and T. K. Mukkur. 2008. Parenteral immunization of mice with a genetically inactivated pertussis toxin DNA vaccine induces cell-mediated immunity and protection. *J. Med. Microbiol.* **57:** 28–35.
12. Gold, M. S., S. Noonan, M. Osbourn, S. Precepa, and A. E. Kempe. 2003. Local reactions at the fourth dose of acellular pertussis vaccine in South Australia. *Med. J. Aust.* **179:** 191–194.
13. Hoiseth, S. K. and B. A. D. Stocker. 1981. Aromatic-dependent *Salmonella Typhimurium* are non-virulent and effective as live vaccines. *Nature* **291:** 238–239.
14. He, Q. and J. Mertsola. 2008. Factors contributing to pertussis resurgence. *Future Microbiol.* **3:** 329–339.
15. Kolos, V., R. Menzies, and P. McIntyre. 2007. Higher pertussis hospitalization rates in indigenous Australian infants, and delayed

vaccination. *Vaccine* **25**: 588−590.

16. Lalonde, G., P. D. O'Hanley, B. A. D. Stocker, and K. T. Denich. 1994. Characterisation of a 3-dehydroquinase gene from *Actinobacillus pleuropneumoniae* with homology to the eukaryotic genes qa-2 and QUTE. *Mol. Microbiol.* **11**: 273−280.

17. Leef, M., K. L. Elkin, 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. *J. Exp. Med.* **191**: 1841−1852.

18. Locht, C., R. Antoine, D. Raze, N. Mielcarek, D. Hot, Y. Lemoine, and Mascart F. 2004. *Bordetella pertussis*: From functional genomics to intranasal vaccination. *Int. J. Med. Microbiol.* **293**: 583−588.

19. 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. *J. Infect. Dis.* **181**: 2087−2091.

20. Marshall, H. S., M. S. Gold, R. Gent, P. J. Quinn, L. Piotto, M. F. Clarke, and D. M. Roberton. 2006. Ultrasound examination of extensive limb swelling reactions after diphtheria-tetanus-acellular pertussis or reduced-antigen content diphtheria-tetanus-acellular pertussis immunization in preschool-aged children. *Pediatrics* **118**: 1501−1509.

21. Marzouqi, I., P. Richmond, S. Fry, J. Wetherall, and T. Mukkur. 2010. Development of improved vaccines against whooping cough: Current status. *Hum. Vaccin.* **6**: 1−11.

22. 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*. *Nat. Biotechnol.* **16**: 454−457.

23. Mielcarek, N., A.-S. Debrie, D. Raze, J. Bertout, C. Rouanet, A. B. Younes, *et al*. 2006. Live attenuated *B. pertussis* as a single-dose nasal vaccine against whooping cough. *PLOS Pathog.* **2**: e65.

24. Mills, K. H. G. 2001. Immunity to *Bordetella pertussis*. *Microbes Infect.* **3**: 655−677.

25. Mooi, F., I. H. Van Loo, M. van Gent, Q. He, M. J. Bart, K. J. Heuvelman, *et al*. 2009. *Bordetella pertussis* strains with increased toxin production associated with pertussis resurgence. *Emerg. Infect. Dis.* **15**: 1206−1213.

26. Morbidity and Mortality Weekly Report. 2011. *General Recommendations on Immunization*. Recommendations of the Advisory Committee on Immunization Practices (ACIP) Recommendations and Reports. Vol. 60, No. 2.

27. Mukkur, T. K., G. H. McDowell, B. A. D. Stocker, and A. K. Lascelles. 1987. Protection against experimental salmonellosis in mice and sheep by immunisation with aromatic-dependent *Salmonella* Typhimurium. *J. Med. Microbiol.* **24**: 11−19.

28. Nasso, M., G. Fedele, F. Spencieri, R. Palazzo, P. Costantino, R. Rappuoli, *et al*. 2011. Genetically detoxified pertussis induces Th1/Th17 immune response through MAPKs and IL-10-dependent mechanisms. *J. Immunol.* **183**: 1892−1899.

29. National Notifiable Diseases Surveillance System. 2011. Available at http://www9.health.gov.au/cda/Source/Rpt_3.cfm, accessed 4/3/2011.

30. Olson, L. C. 1975. Pertussis. *Medicine (Baltimore)* **54**: 427−469.

31. Parkhill, J., M. Sebaihia, A. Preston, L. D. Murphy, N. Thomson, D. E. Harris, *et al*. 2003. Comparative analysis of the genome sequences of *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica*. *Nat. Genet.* **35**: 32−40.

32. Pertussis [whooping cough]: Outbreaks. http://www.cdc.gov/pertussis/outbreaks.html. Accessed 14 June 2011.

33. Quinn, P., M. Gold, J. Royle, J. Buttery, J. P. Richmond, P. McIntyre, *et al.* 2011. Recurrence of extensive injection site reactions following DTPa or dTpa vaccine in children 406 years old. *Vaccine* **29**: 4230−4237.

34. Rennels, M. B., M. A. Deloria, M. E. Pichichero, G. A. Losonsky, J. A. Englund, B. D. Meade, *et al*. 2000. Extensive swelling after booster doses of acellular pertussis-tetanus-diphtheria vaccines. *Pediatrics* **1**: e1−e12.

35. Roberts, M., D. Maskell, P. Novotny, and G. Dougan. 1990. Construction and characterization *in vivo* of *Bordetella pertussis aroA* mutants. *Infect. Immun.* **58**: 732−739.

36. Rowe, J., S. T. Yerkovich, P. Richmond, D. Suriyaarachchi, E. Fisher, L. Feddema, *et al*. 2005. Th2-associated local reactions to the acellular diphtheria-tetanus-pertussis vaccine in 4- to 6-year-old children. *Infect. Immun.* **73**: 8130-8135.

37. Rossetti, T. R. 1997. Cloning the *aroD* gene of *Bordetella pertussis*. BSc. Hons. Thesis, University of Southern Queensland, Toowoomba, Queensland, Australia.

38. Sambrook, J., E. F. Fritsch, and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

39. Schellekens, J., C. H. von Konig, and P. Gardner. 2005. Pertussis sources of infection and routes of transmission in the vaccination era. *Pediatr. Infect. Dis. J.* **24**: 721−728.

40. Shahin, R., M. Leef, J. Eldridge, M. Hudson, and R. Gilley. 1995. Adjuvanticity and protective immunity elicited by *Bordetella pertussis* antigens encapsulated in poly[DL-lactide-co-glycolide] microspheres. *Infect. Immun.* **63**: 1195−1200.

41. Stainer, D. W. and M. J. Scholte. 1971. A simple and chemically defined medium for the production of phase I *Bordetella pertussis*. *J. Gen. Microbiol.* **34**: 778−784.

42. Towbin, H., T. Stachelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**: 4350−4354.

43. Verma, N. K. and A. 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**: 6−9.

44. Wearing, H. J. and P. Rohani. 2009. Estimating the duration of pertussis immunity using epidemiological signatures. *PLoS Pathog.* **5**: e1000647.

45. Weiss, A. A. and E. L. Hewlett. 1986. Virulence factors of *Bordetella pertussis*. *Annu. Rev. Microbiol.* **40**: 661−686.

46. Wendelboe, A. M., E. Njamkepo, A. Bourillon, D. D. Floret, J. Gaudelus, M. Gerber, *et al*. 2007. Infant Pertussis Study Group. Transmission of *Bordetella pertussis* to young infants. *Pediatr. Infect. Dis. J.* **26**: 293−299.

47. Rieber, N., A. Graf, D. Hartl, S. Urschel, B. H. Belohradsky, and J. Liese. 2011. Acellular pertussis booster in adolescents induces Th1 and memory CD8+ T cell immune response. *PLoS ONE* **6**: e17271.