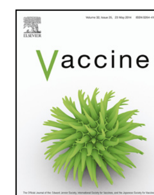
Vaccine 32 (2014) 6251–6258

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine



Identification of linear B epitopes of pertactin of *Bordetella pertussis* induced by immunization with whole and acellular vaccine

Flavio R. da Silva [a,b], Paloma Napoleão-Pego [a], Salvatore G. De-Simone [a,b,c,*]

[a] Centro de Desenvolvimento Tecnológico em Saúde (CDTS)/Instituto Nacional de Ciência e Tecnologia de Inovação em Doenças Negligenciadas (INCT-IDN), Rio de Janeiro, RJ, Brazil

[b] Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil

[c] Departamento de Biologia Celular e Molecular, Universidade Federal Fluminense, Instituto de Biologia, Niterói, Rio de Janeiro, RJ, Brazil

ARTICLE INFO

Article history:
Received 1 September 2013
Received in revised form 13 August 2014
Accepted 8 September 2014
Available online 22 September 2014

Keywords:
Bordetela pertussis
Pertactin
B-epitope
Peptide array
SPOT-synthesis
Synthetic peptides

ABSTRACT

Pertussis is a serious infectious disease of the respiratory tract caused by the gram-negative bacteria *Bordetella pertussis*. There has been a reemergence of this disease within the population of several countries that have well established vaccination programs. Analyzes of clinical isolates suggest an antigenic divergence between the vaccine-based strains to the circulating strains. Although antibodies against P.69 are involved in the observed protective immunity, the sequences recognized as antigenic determinants in P.133, the precursor for P.69, P.3.4 and P.30, have not be determined. Here, the precise mapping of linear B-cell epitopes within the predicted P.133 pertactin sequences was accomplished using the SPOT-synthesis of peptide arrays onto cellulose membranes and screening with murine sera generated by vaccination with either the Pertussis cellular (miPc) or Pertussis acellular (miPa) vaccine. A total of 23 major epitopes were identified by sera from miPc vaccinated mice, while thirteen were identified by sera from miPa vaccinated mice. Of these epitopes, 12 epitopes were specifically identified by antibodies produced in response to the miPc vaccine and two were specific to the miPa vaccine. These epitopes were distributed throughout the pertactin sequence but a significant number were concentrated to the P.30 Prn segment. An analysis of the epitope correlation homologies indicated that the variations from the observed mutations in pertactin would not constitute a problem using these vaccines. In addition, the mapping of epitopes demonstrated a higher number of linear B-cell epitopes immunized with the Pc vaccine than the Pa vaccine.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

Pertussis, or whooping cough, is an acute infectious disease of the bacterium *Bordetella pertussis* that is especially dangerous in young children. There has been resurgence over the past 15 years of infections in countries with a high compliance for vaccine administration [1]. Several explanations have been suggested for the reemergence of *B. pertussis* including improvements in diagnosis, a waning of immunity from vaccinations and an adaptation of the circulating *B. pertussis* population to induced immunity [2]. Genetic comparisons of clinical isolates to the strains used for vaccine production revealed sequence divergence [3–15], which suggests that pathogen adaptation and innate resistance are the most probable causes for the increased occurrence of infection [5,16,17].

Of particular interest is the protein pertactin (P.69 Prn), which is a key component of vaccines since antibody titers against P.69 Prn correlate with protective immunity [18,19] and clinically observed protection [20–22]. Multiple polymorphisms have been described defining 13 variants [2,23,24,19] with variations limited to two regions comprised of the repeats of Gly-Gly-X-X-Pro or Pro-Gln-Pro that are designated as the r1 (aa 276–280) and r2 (aa 575–590/594–598) repeat, respectively. Greater variation has been observed within r1, which is located proximal to the N-terminus and flanks an Arg-Gly-Asp (RGD) motif implicated in the ligand-receptor interactions in eukaryotes [25,26].

Despite a number of studies in both animals and humans demonstrating that P.69 Prn can elicit protective antibodies [19,27,28], information about the sequences and locations of the

Abbreviations: DTP, diphtheria–tetanus–pertussis vaccine; DTPa, diphtheria–tetanus–pertussis acellular vaccine; FHA, filamentous hemagglutinin; miPa, mice immunized with Pertussis acellular; miPc, mice immunized with Pertussis cellular; mAbs, monoclonal antibodies; Prn, pertactin Pertussis acellular; Pc, Pertussis cellular; Pt, Pertussis toxin.

* Corresponding author at: Centro de Desenvolvimento Tecnológico em Saúde, Fundação Oswaldo Cruz, IOC-FIOCRUZ, 21040900 Rio de Janeiro, RJ, Brazil.
Tel.: +55 21 38658183; fax: +55 21 38658240.
E-mail address: dsimone@ioc.fiocruz.br (S.G. De-Simone).

http://dx.doi.org/10.1016/j.vaccine.2014.09.019
0264-410X/© 2014 Elsevier Ltd. All rights reserved.

B-linear epitopes involved is limited to a few segments [29]. Most of the continuous and discontinuous epitopes identified to date were obtained using various methodologies and sera from different hosts [30]. While these studies addressed the involvement of important segments or domain in the toxins, to our knowledge the studies did not compare the immune response elicited by the two types of vaccines at the epitope level.

The analysis of the immune response obtained from vaccination with the two forms of vaccines, Pertussis cellular (Pc) and Pertussis acellular (Pa), indicate that a robust protection is induced independent of the process of antigen preparation [31]. The Pc vaccine is prepared using heat-killed bacteria, a physical process that denatures proteins and destroys most of the discontinuous epitopes. Pa vaccines, on the other hand, are prepared containing proteins that are purified and fixed with formaldehyde, which preserves most epitopes [32]. However, the commonality between the two vaccines is linear B-cell epitopes, which suggests that their presence is key to understanding the mechanisms of protection induced by vaccination and its loss over time.

Our results confirm and refine those from previous studies by identifying the linear B-cell epitopes recognized in P.133 Prn (the P.69 Prn precursor) by antibodies generated through immunization with the two types of available vaccines. The identity of the epitopes allowed an extensive comparison between the immunological response against the cellular and acellular generated vaccines to address the hypothesis that genetic drift was contributing to the resurgence of pertussis in the world's population.

2. Material and methods

2.1. Materials

Cellulose membranes were from Intavis Bioanalytical Instruments (Germany). Sheep anti-mouse immunoglobulin was obtained from Promega Biosciences (CA, USA) and the SuperSignal West Pico chemiluminescent substrate from Pierce (IL, USA). All other reagents and chemicals including amino acids were from Merck–Calbiochem (Darmstadt, Germany). The DTP vaccine containing inactivated whole-cell bacterium of *B. pertussis* combined with formaldehyde inactivated diphtheria and tetanus toxoid was obtained from Bio-Manguinhos (FIOCRUZ, RJ, Brazil). The DTPa vaccine (Prn, pertussis toxin, filamentous hemagglutinin and fimbrial serotypes 2 & 3) was from GlaxoSmithKline. Both the DTP and DTPa vaccine containe aluminum hydroxide as an adjuvant.

2.2. Immunization of mice

NIH Swiss mice (12–16 g) were immunized with either DTP or DTPa vaccines reconstituted with saline and 2 IU (defined by the Brazilian National Immunization Program) administered in 0.5 ml with an interval of 21 days. For the DTPa vaccine, 2 IU contains 25 μg PT, 25 μg FHA and 8 μg pertactin adsorbed to aluminum salts. Groups of 15 mice received injections from one of three different lots of the DTP or one of three different lots of DTPa vaccine. Sera was collected one week after the last inoculation, stored at −20 °C and pooled for each vaccine before use.

2.3. Ethical aspects

All experiments involving animals were approved by ethical committees and the approvals are acknowledged within the manuscript.

2.4. Synthesis of the cellulose–membrane-bound peptide array

A peptide array was synthesized onto amino-PEG_{500}-UC_{540} cellulose membranes according to standard SPOT-synthesis protocols [33] using an Auto-Spot Robot ASP222 (Intavis). The array was comprised of 179 peptides that covered the entire coding region of the P.69 Prn precursor protein (Fig. 1). Each peptide was a 14-mer and its sequence was offset from the previous by 5 amino acids. After synthesis, membranes were either probed immediately or stored at −20 °C until needed. The negative control was an area without peptide and the positive control was the peptide IHLVNNESSEVIVHK from *Clostridium tetani* precursor.

2.5. Measurement of spot signal intensities

Spot signal intensity was measured as described previously [34]. Chemiluminescent signals were detected on a MF-ChemiBis 3.2 (DNR Bio-Imaging Systems, USA) at a resolution of 5 MP and the image analyzed with Total Lab Software (Nonlinear Dynamics, USA) to quantify signal intensities and to define the empirical probability that the intensity from a spot was distinct from that of the background signal using algorithms that compared the intensities between background, spot area and negative controls.

2.6. Bioinformatics and in silicon analysis model

The complete sequence of *B. pertussis* pertactin (A4UTL5) was used for epitope mapping and the other strains of *B. pertussis* (Q03035, K4QEP0, K4TPW9, Q9L4E2, Q6U896, K4TCV0, Q9AIX8, K0MTC8, J7RKB0, A4UTL5, F4LFL2, P14283) were retrieved from the National Center for Biotechnology Information, USA (http://www.ncbi.nlm.nih.gov). Multiple sequence alignments were performed using the programs ClustalW (http://www.ebi.ac.uk/clustalw) and BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit. html).

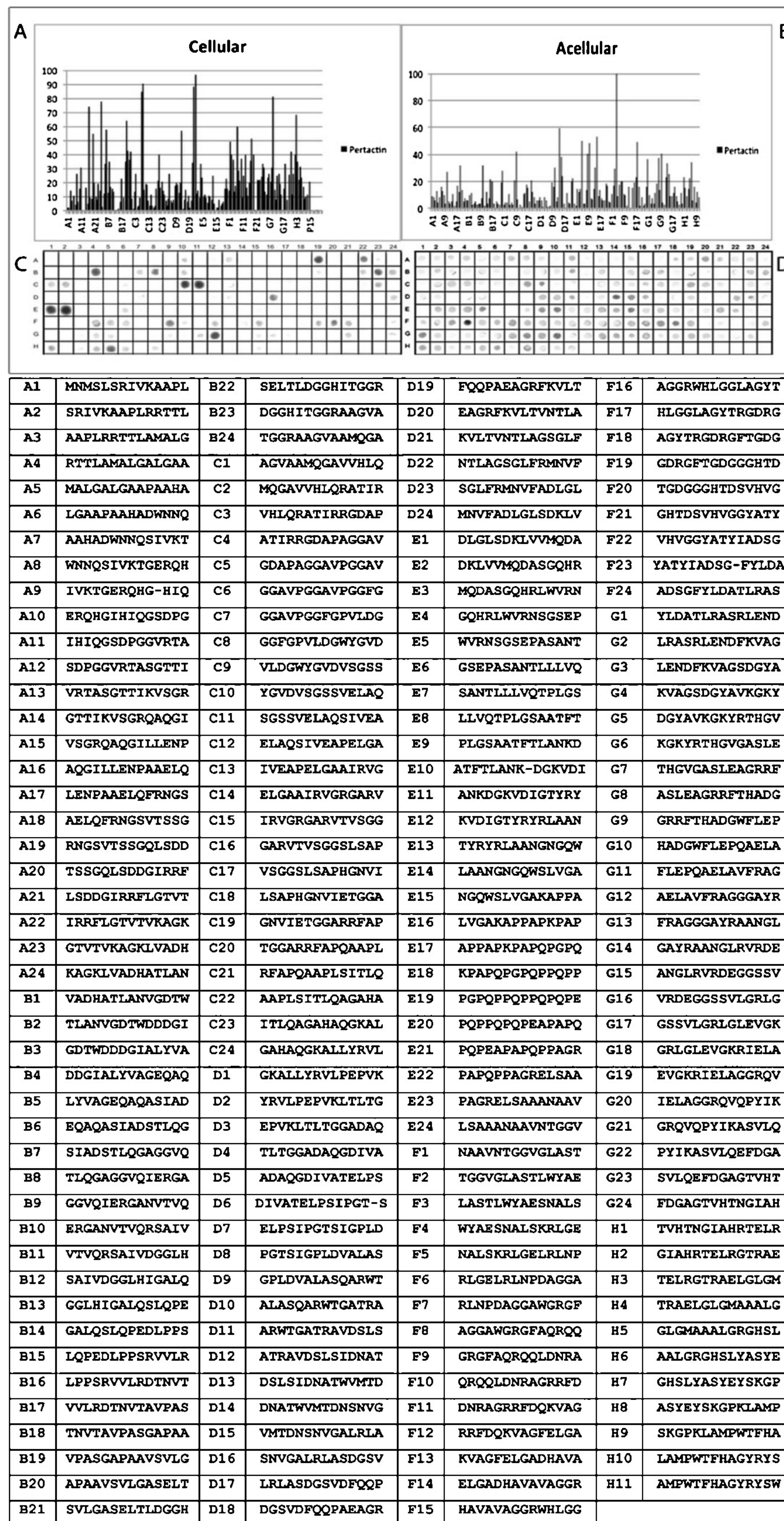
The alignment of the P.69 Prn from the three major species, *Bordetella spp*. (*B. pertussis* (Tahoma I), *Bordetella parapertussis* (12822) and *Bordetella bronchiseptica* (RB50), was performed using the program Kodon (Applied Maths, Belgium). The predicted structural model of P.133 pertactin was obtained by submission of the sequence (A4UTL5) to the I-Tasser server [35,36].

3. Results

3.1. Epitope mapping

Epitopes in the pertactin precursor protein (900 aa) were identified based on recognition of peptides in a synthesized library by murine antibodies immunized with a *B. pertussis* vaccine, either Pc or Pa (see Materials and Methods). Fig. 1, Panels A and C present the position of each peptide and the measured intensity from the chemiluminescent detection of mouse IgG antibodies in sera pooled from mice vaccinated with the Pc vaccine. The intensities were normalized using 100% as defined by the positive control (data not shown). Panels B and D show the results from the reactivity of IgG antibodies in pooled murine sera vaccinated with the Pa vaccine. A list of the peptides synthesized and their positions on the membranes is presented in Panel E.

The pattern of reactivity for the antibodies generated in mice immunized with the Pc vaccine demonstrated that a greater number of peptides were recognized than in mice immunized with the Pa vaccine (Fig. 1A). An analysis of the sequences constituting the peptides synthesized in reactive regions defined 23 epitopes generated by the miPc vaccine while only 13 epitopes were created by the miPa vaccine (Table 1). Twelve epitopes (Prn-1, Prn-2, Prn-3, Prn-4, Prn-7, Prn-10, Prn-20, Prn-21, Prn-22, Prn-23, Prn-24, Prn-25) were uniquely specified by the miPc vaccine and two epitopes (Prn-14



A1	MNMSLSRIVKAAPL	B22	SELTLDGGHITGGR	D19	FQQPAEAGRFKVLT	F16	AGGRWHLGGLAGYT
A2	SRIVKAAPLRRTTL	B23	DGGHITGGRAAGVA	D20	EAGRFKVLTVNTLA	F17	HLGGLAGYTRGDRG
A3	AAPLRRTTLAMALG	B24	TGGRAAGVAAMQGA	D21	KVLTVNTLAGSGLF	F18	AGYTRGDRGFTGDG
A4	RTTLAMALGALGAA	C1	AGVAAMQGAVVHLQ	D22	NTLAGSGLFRMNVF	F19	GDRGFTGDGGGHTD
A5	MALGALGAAPAAHA	C2	MQGAVVHLQRATIR	D23	SGLFRMNVFADLGL	F20	TGDGGGHTDSVHVG
A6	LGAAPAAHADWNNQ	C3	VHLQRATIRRGDAP	D24	MNVFADLGLSDKLV	F21	GHTDSVHVGGYATY
A7	AAHADWNNQSIVKT	C4	ATIRRGDAPAGGAV	E1	DLGLSDKLVVMQDA	F22	VHVGGYATYIADSG
A8	WNNQSIVKTGERQH	C5	GDAPAGGAVPGGAV	E2	DKLVVMQDASGQHR	F23	YATYIADSG-FYLDA
A9	IVKTGERQHG-HIQ	C6	GGAVPGGAVPGGFG	E3	MQDASGQHRLWVRN	F24	ADSGFYLDATLRAS
A10	ERQHGIHIQGSDPG	C7	GGAVPGGFGPVLDG	E4	GQHRLWVRNSGSEP	G1	YLDATLRASRLEND
A11	IHIQGSDPGGVRTA	C8	GGFGPVLDGWYGVD	E5	WVRNSGSEPASANT	G2	LRASRLENDFKVAG
A12	SDPGGVRTASGTTI	C9	VLDGWYGVDVSGSS	E6	GSEPASANTLLLVQ	G3	LENDFKVAGSDGYA
A13	VRTASGTTIKVSGR	C10	YGVDVSGSSVELAQ	E7	SANTLLLVQTPLGS	G4	KVAGSDGYAVKGKY
A14	GTTIKVSGRQAQGI	C11	SGSSVELAQSIVEA	E8	LLVQTPLGSAATFT	G5	DGYAVKGKYRTHGV
A15	VSGRQAQGILLENP	C12	ELAQSIVEAPELGA	E9	PLGSAATFTLANKD	G6	KGKYRTHGVGASLE
A16	AQGILLENPAAELQ	C13	IVEAPELGAAIRVG	E10	ATFTLANK-DGKVDI	G7	THGVGASLEAGRRF
A17	LENPAAELQFRNGS	C14	ELGAAIRVGRGARV	E11	ANKDGKVDIGTYRY	G8	ASLEAGRRFTHADG
A18	AELQFRNGSVTSSG	C15	IRVGRGARVTVSGG	E12	KVDIGTYRYRLAAN	G9	GRRFTHADGWFLEP
A19	RNGSVTSSGQLSDD	C16	GARVTVSGGSLSAP	E13	TYRYRLAANGNGQW	G10	HADGWFLEPQAELA
A20	TSSGQLSDDGIRRF	C17	VSGGSLSAPHGNVI	E14	LAANGNGQWSLVGA	G11	FLEPQAELAVFRAG
A21	LSDDGIRRFLGTVT	C18	LSAPHGNVIETGGA	E15	NGQWSLVGAKAPPA	G12	AELAVFRAGGGAYR
A22	IRRFLGTVTVKAGK	C19	GNVIETGGARRFAP	E16	LVGAKAPPAPKPAP	G13	FRAGGGAYRAANGL
A23	GTVTVKAGKLVADH	C20	TGGARRFAPQAAPL	E17	APPAPKPAPQPGPQ	G14	GAYRAANGLRVRDE
A24	KAGKLVADHATLAN	C21	RFAPQAAPLSITLQ	E18	KPAPQPGPQPPQPP	G15	ANGLRVRDEGGSSV
B1	VADHATLANVGDTW	C22	AAPLSITLQAGAHA	E19	PGPQPPQPPQPQPE	G16	VRDEGGSSVLGRLG
B2	TLANVGDTWDDDGI	C23	ITLQAGAHAQGKAL	E20	PQPPQPQPEAPAPQ	G17	GSSVLGRLGLEVGK
B3	GDTWDDDGIALYVA	C24	GAHAQGKALLYRVL	E21	PQPEAPAPQPPAGR	G18	GRLGLEVGKRIELA
B4	DDGIALYVAGEQAQ	D1	GKALLYRVLPEPVK	E22	PAPQPPAGRELSAA	G19	EVGKRIELAGGRQV
B5	LYVAGEQAQASIAD	D2	YRVLPEPVKLTLTG	E23	PAGRELSAAANAAV	G20	IELAGGRQVQPYIK
B6	EQAQASIADSTLQG	D3	EPVKLTLTGGADAQ	E24	LSAAANAAVNTGGV	G21	GRQVQPYIKASVLQ
B7	SIADSTLQGAGGVQ	D4	TLTGGADAQGDIVA	F1	NAAVNTGGVGLAST	G22	PYIKASVLQEFDGA
B8	TLQGAGGVQIERGA	D5	ADAQGDIVATELPS	F2	TGGVGLASTLWYAE	G23	SVLQEFDGAGTVHT
B9	GGVQIERGANVTVQ	D6	DIVATELPSIPGT-S	F3	LASTLWYAESNALS	G24	FDGAGTVHTNGIAH
B10	ERGANVTVQRSAIV	D7	ELPSIPGTSIGPLD	F4	WYAESNALSKRLGE	H1	TVHTNGIAHRTELR
B11	VTVQRSAIVDGGLH	D8	PGTSIGPLDVALAS	F5	NALSKRLGELRLNP	H2	GIAHRTELRGTRAE
B12	SAIVDGGLHIGALQ	D9	GPLDVALASQARWT	F6	RLGELRLNPDAGGA	H3	TELRGTRAELGLGM
B13	GGLHIGALQSLQPE	D10	ALASQARWTGATRA	F7	RLNPDAGGAWGRGF	H4	TRAELGLGMAAALG
B14	GALQSLQPEDLPPS	D11	ARWTGATRAVDSLS	F8	AGGAWGRGFAQRQQ	H5	GLGMAAALGRGHSL
B15	LQPEDLPPSRVVLR	D12	ATRAVDSLSIDNAT	F9	GRGFAQRQQLDNRA	H6	AALGRGHSLYASYE
B16	LPPSRVVLRDTNVT	D13	DSLSIDNATWVMTD	F10	QRQQLDNRAGRRFD	H7	GHSLYASYEYSKGP
B17	VVLRDTNVTAVPAS	D14	DNATWVMTDNSNVG	F11	DNRAGRRFDQKVAG	H8	ASYEYSKGPKLAMP
B18	TNVTAVPASGAPAA	D15	VMTDNSNVGALRLA	F12	RRFDQKVAGFELGA	H9	SKGPKLAMPWTFHA
B19	VPASGAPAAVSVLG	D16	SNVGALRLASDGSV	F13	KVAGFELGADHAVA	H10	LAMPWTFHAGYRYS
B20	APAAVSVLGASELT	D17	LRLASDGSVDFQQP	F14	ELGADHAVAVAGGR	H11	AMPWTFHAGYRYSW
B21	SVLGASELTLDGGH	D18	DGSVDFQQPAEAGR	F15	HAVAVAGGRWHLGG		

Fig. 1. Fine mapping of epitopes by sera from mice immunized with DTP ((A) and (C)) and DTPa ((B) and (D)) with the library (E) of the pertactin *Bordetella pertussis* protein synthetic 15-mer peptides. Binding of antibody to individual peptides was detected by incubating the membrane with alkaline phosphatase goat anti-mouse immunoglobulin. The intensity of signal ((A) and (B)) was obtained by scanning of the membranes ((C) and (D)) probed with the antisera.

Table 1
Epitopes of *Bordetella pertussis* P.133 Prn protein identified in this study by SPOT-synthesis analysis.

Epitope	Cellular vaccine	Acellular vaccine	Murine antibodies [39]
Prn-1	46–59		45–49
Prn-2	61–74		65–70
Prn-3	80–84		78–83
Prn-4	104–119		105–111
Prn-5	126–139	135–149	133–137
Prn-6	191–199	181–194	190–195
Prn-7	221–234		224–228
Prn-8	245–264	257–265	256–285
Prn-9	281–289	286–294	256–285
Prn-10	351–359		350–358/362–367
Prn-11	426–440	426–444	425–482
Prn-12	471–484	471–479	468–473
Prn-13	491–499	495–504	
Prn-14		526–534	527–533
Prn-15		551–564	
Prn-16	611–624	616–629	620–627
Prn-17	641–654	641–654	651–655
Prn-18	682–699	686–699	681–688
Prn-19	776–779	761–779	
Prn-20	791–799		
Prn-21	811–824		
Prn-22	831–839		
Prn-23	851–854		
Prn-24	866–871		
Prn-25	876–889		

and Prn-15) by the sera of miPa vaccine (Table 1). The remaining epitopes were common between the two vaccines (Table 1). Overall, the epitopes were designated as Prn-1 to Prn-25 for this study.

3.2. Localization of the B-epitopes within the P.133-Prn protein

The 25 linear B-epitopes identified by the SPOT-synthesis analysis were distributed throughout the pertactin protein (Fig. 2). The P.133 pertactin protein gene contains three well defined segments: a signaling N-terminal extension (aa 1–34), P.69 Prn (aa 35–566) and the P.30 segment (aa 607–900) [19].

No reactivity was observed against the signal peptide in the first thirty-five amino acids by either vaccine. Thirteen epitopes were identified in the P.69 Prn segment by the miPc vaccine sera (Prn-1 to Prn-13) and 10 epitopes (Prn-5, Prn-6, Prn-8, Prn-9, Prn-11 to Prn-15) by the miPa vaccine sera (Table 1). Neither the variable region r1 (aa 276–280) nor r2 (aa 575–590/594–598) displayed epitope.

The major response of the Pa vaccine was found within sequences between regions 1 and 2. Most of the identified epitopes were located in loop/coil structures, which were present on the protein surface and accessible to the solvent (Fig. 2).

The P.30 segment (aa 607–900) contained 10 epitopes (Prn-16 to Prn-25) recognized by miPc vaccine sera. Only four epitopes (Prn-16 to Prn-19) were reactive to miPa vaccine sera (Table 1).

One sequence [300-GGXXP-304] is located directly after the RGD motif that could mediate interactions with epithelial cells [37]. While the RGD motif (aa 260–272) from region 1 is part of an epitope (Prn-8) recognized by sera of miPc (aa 245–265) and miPa (aa 257–265) vaccine sera, the adjacent sequence involved in the epithelial adhesion is not present in this epitope (Table 1).

3.3. Spatial location of the P.133 reactive epitopes

The tridimensional structure of mature P.69 pertactin has been determined to a resolution of 2.5 Å by X-ray diffraction, but no structure exists for the precursor, P.133 Prn. In our study, a predicted structural model of the entire P.133 Prn was obtained (Fig. 2) with a confidence score equal to −2.16 and a TM-score of 0.47 ± 0.15 (borderline quality) and displays the spatial localization of the most reactive epitopes identified by the SPOT-synthesis array experiments. Most of the linear epitopes were located in coil/loop structures in the P.133 protein structure. The hydropathy plot of the protein also suggested that all of the epitopes were present on the surface of the proteins (data not shown).

3.4. Cross-immunity conferred by strains of B. pertussis

To investigate the cross-immunity conferred by the *B. pertussis* pertactin protein, 12 sequences deposited in NCBI data bank were aligned to compare the sequences of the epitopes. This analysis showed that, in totality, all of the epitopes identified by sera from immunized mice were identical between the different strain sequences (Fig. 3). The high conservation of the structure of the epitopes suggests that a strong cross-immunity is induced by immunization.

The epitopes identified in this work by the SPOT-synthesis approach compared to those identified in the literature by other methodologies and murine immune sera [37,38] or monoclonal antibodies [37] were 100% correlative. Our epitope mapping revealed nine (Prn-13, Prn-15, Prn-19 to Prn-25) new murine epitopes (Table 1).

3.5. Correlation of pertactin within Bordetella sp

As shown in Fig. 4, the pertactin proteins shares a high level of structural similarity and therefore all of linear epitopes identified with other *Bordetella sp* adhesins proteins.

4. Discussion

Studies in animals and humans have indicated that immunity against pertussis was highly dependent on the inclusion of pertactin in vaccines through correlations between anti-pertactin antibodies and clinical protection. Pertactin belongs to a family of secreted proteins, which is proteolytically processed at its N- and C-termini to produce P.69 and P.30 located at the cell surface and in the outer membrane, respectively [38]. A major difference between the two available Pertussis vaccines is that P.69 pertactin is only included in the Pa vaccine while both proteins segments are present in the Pc vaccine.

In practice, vaccination with either vaccine is effective at eliciting a protective immune response. The mapping results show that the immune response in mouse to each, while comparable, was not identical (Fig. 1). Sera from mice immunized with the Pc vaccine recognized 23 epitopes and with the Pa vaccine, 11 epitopes were identified (Fig. 1 and Table 1). With regards pertactin, the most robust immune responses from the Pc vaccine were directed against both the N-terminus (P.69 Prn) and C-terminus (P.30 Prn). For the Pa vaccine, reactivity was directed against the segment spanning regions 1 and 2.

Immunizing mice with the Pc or Pa vaccine induced distinct and specific antibodies that, along with the decreasing antigenicity of P.69 Prn by the Pa vaccine, could be explained by the effects of chemical treatments necessary for detoxification of preparations. Although the modifications from formaldehyde treatment are not known, it was previously shown that treatment of Prn greatly reduced the epitope binding activity by a panel of murine mAbs and induced antibodies that bound Prn, but was not bactericidal [39]. Conversely, the association between antibody responses to site accessibility is a frequently reported phenomenon for various antigens [40] and most likely reflects the exposure of the antigen and maturation of the immune system.

Most Pc vaccines are composed of 1, 2 or 3 different strains, while Pa vaccines by 3–4 components. The Pa vaccine used in our

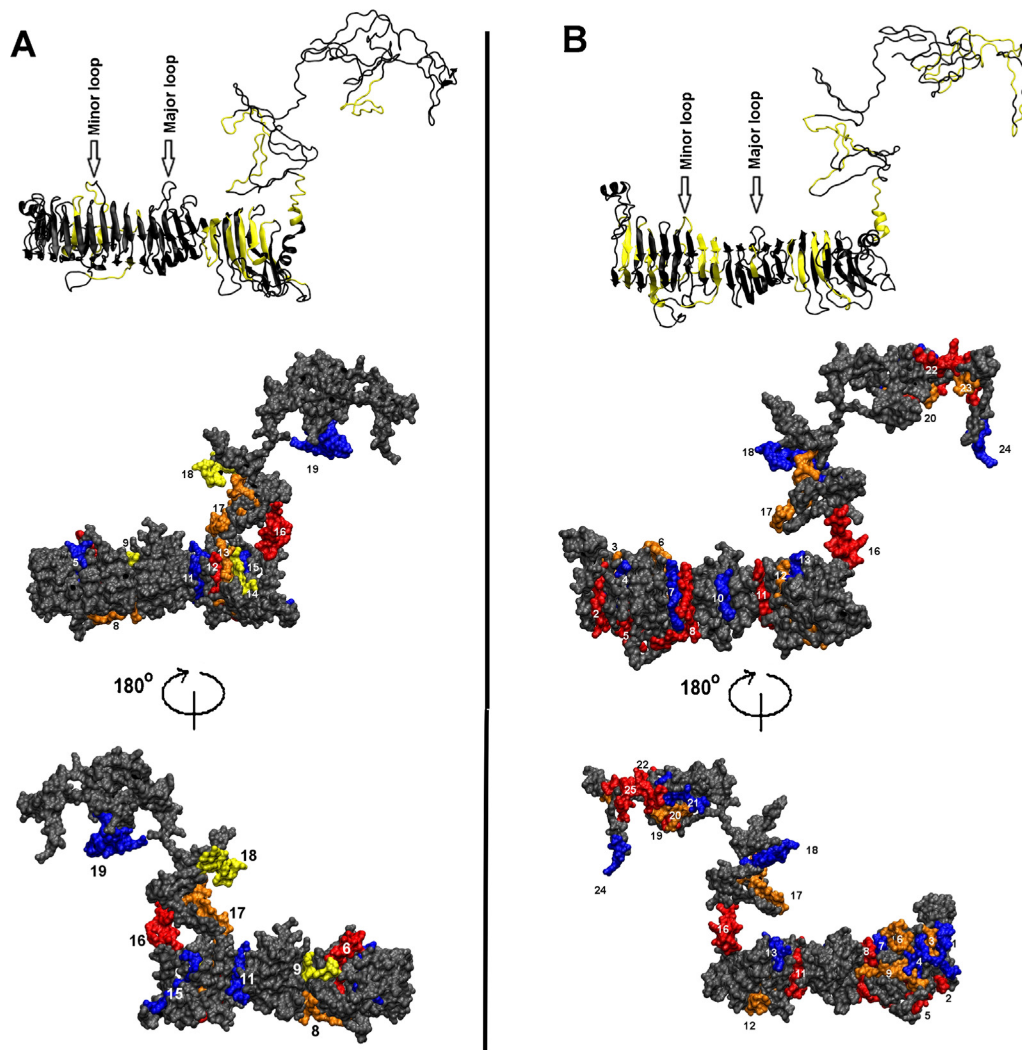


Fig. 2. Spatial localization of the linear epitopes recognized by sera from mice immunized with acellular (A) and cellular (B) *Bordetella pertussis* vaccine in three dimensional structures of P.133 Prn toxin. The epitopes numbers (Prn-1-25) are highlighted in different colors to facilitate identification. The P.133 Prn structure was obtained from protein data bank (http://www.pdb.org) and drawn using PyMol. The C-terminal amino acid residues 600–887, are not part of the resolved structure (1DAB.pdb) and was obtained by molecular modeling. Arrows indicate the positions of the minor loop (224–233aa) and major loop (433–441aa). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

study included four components; the PT, FHA, fimbrial and Prn protein. Since both are protective, the common epitopes in the P.69 Prn become the more important targets of the immune response for neutralization. However, the protective importance of the epitopes present in the P.30 segment should not be ignored since there was a robust response induced specially by the Pc vaccine. Nonetheless, the reactivity of the Pa vaccine suggests the presence of some P.30 in the Pa vaccine. The industrial process of Pa vaccine preparation includes the use of *B. pertussis* culture filtrates, which are subjected to ultrafiltration on a 30 kDa membrane that could retain P.30.

In relation to the structure-function of the toxin, pertactin possesses two important regions, designated regions 1 (aa 276–280) and 2 (aa 575–590 and 594–598), which are comprised of repeats known to create polymorphisms that could contribute to immune escape [41] from their role in conferring immunity in rabbit [29]. However, our analysis showed that these regions were not immunogenic in mice after immunization by either vaccine (Fig. 2). Contiguous regions contain the amino acid triplet RGD, which has been shown to be involved in adherence to host cells [25]. Both RGD site 1 [aa 257-–265] and 2 [aa 691–693] were discovered within epitopes Prn-8 (aa 245–264/257–265) and Prn-18 (aa 682–699/686–699) recognized by sera from the Pc and Pa vaccine, which should contribute to neutralization by interfering with attachment of the bacterium to human cells.

The C-terminal region of the P.69 Prn contains another important motif loop [37] that appears to represent the major human immunoprotective epitope [aa 567–573]. Here, this epitope was not recognized by either sera obtained after vaccination (Table 1). Likewise, the human immunodominant regions composed of repeating units of 5 (GGXXP) or 3 (PQP) amino acids [8], were not recognized after vaccination by both the Pa and Pc. However, in general, there was good correlation between epitopes identified in this study and those previously described using murine antibodies (Table 1).

Different factors can influence antigenicity of an antigen such as host, strain, adjuvant, antigen concentration and number of injections for immunization. These factors were similar for both vaccines in our study. Therefore, the observed differences of antigenicity against the pertactin within miPa and miPc can be attributed to antigen preparation. For pertactin, the cellular vaccine has primarily membrane-inserted protein whereas it is purified in the acellular vaccine. However, the presence of more lipids could increase antigenicity of the proteins in the cellular vaccine suggesting

Fig. 3. Cross-reactivity of epitopes in the P.133 Prn protein for strains of *Bordetella pertussis* contained or not contained within vaccine preparations [(1) Q03035, (2) K4QEP0, (3) K4TPW9, (4) Q9L4E2, (5) Q6U896, (6) K4TCV0, (7) Q9AIX8, (8) K0MTC8, (9) J7RKB0, (10) A4UTL5, (11) F4LFL2, (12) P14283] deposited in data bank. The boxed sequences represent the 25 IgG epitopes identified in this work by SPOT-synthesis.

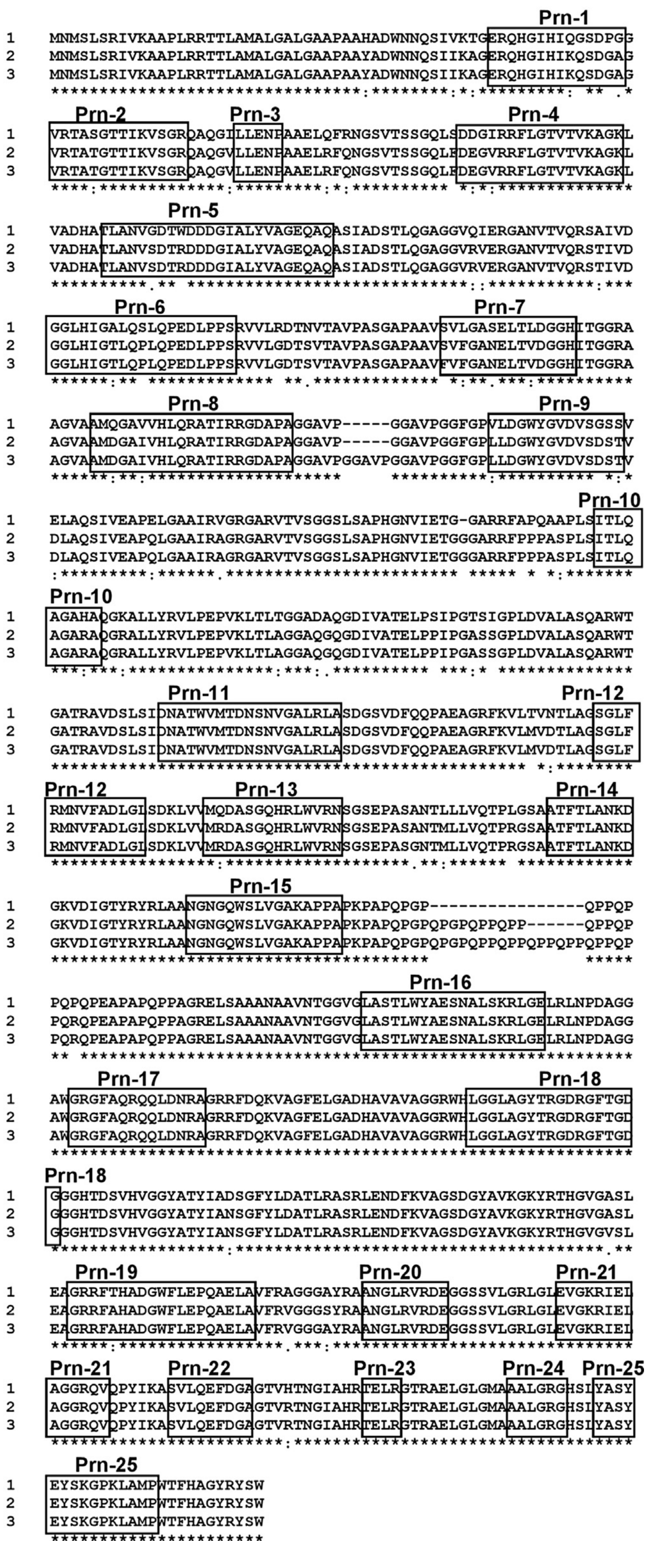


Fig. 4. Cross reactivity of the P.133 Prn within *Bordetella sp*. (1) *Bordetella pertussis* (access number, A4UTL5); (2) *B. parapertussis* (access number, P24328); (3) *B. bronchiseptica* (access number, Q9L4E2). The boxed sequences represent the 25 pertactin IgG epitopes identified in this work by mouse sera vaccinated with Pc and Pa vaccine.

that immunogenicity of the acellular vaccine could be improved by incorporating a stronger adjuvant than aluminum salts into the preparation to stimulate Th1 responses. Extensive literature exist reporting adjuvants that improve antibody responses to pertussis antigens [42–44], including a recent report for oligosaccharides derived from a *B. pertussis* lipopolysaccharide that, when conjugated with a protein induced bactericidal antibodies [45].

Vaccination with Pc induces a broad immune response against hundreds of bacterial proteins while the Pa vaccine-induced immune response targets the virulence of *B. pertussis* and not of *B. parapertussis* [33], another causative agent. These species are, in general, immunologically distinct [29], but this study shows that all of the epitopes induced by Pc vaccine and Pa vaccine shared common sites within *Bordetella sp* that includes *B. parapertussis* and *B. bronchiseptica* suggesting that the use of Prn as an immunogen generates a broad reactivity that covers all strains and species that express and secrete the toxin. However, some isolates are not affected by vaccination, including their virulence. Therefore, the absence or decrease in protection afforded by vaccination with both Pc and Pa does appear not to related to the generation of neutralizing antibodies against the Prn *B. parapertussis* protein. Rather, the results suggest that other virulent factors are present in the circulating strains and species of *Bordetella*, which are not addressed by the current Pc and Pa vaccines.

In conclusion, the peptide arrays formed directly onto cellulose membranes allowed the identification of the major antigenic determinants in the P.133 recognized by mouse sera after being immunized by either the Pc or Pa vaccine. The epitopes common and specific to each vaccine epitopes were determined and located within a structural model. This study also provides evidence that antibodies against P.30 are induced by the Pc vaccine and could be effective in neutralizing the toxin effect.

Mouse is the most frequently used animal in the literature for investigating immune responses against *B. pertussis*. Although humans are the only natural host for this bacterium, mice can be infected with *B. pertussis*, and despite the absence of cough after infection, they present other reactions resembling the human response and therefore are acceptable as a model for studies on immunity against this pathogen [46].

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgements

This work was supported by the CNPq (400151-2011-1), FAPERJ and CAPES (143). The results are part of a DrSci Thesis (IPEC, FIOCRUZ) of FSA. We thank L.P. Gomes for help in establishing the modeling figures, DWPJr for helpful comments and revision of the manuscript.

References

[1] Kerr JR, Matthews RC. *B. pertussis* infection: pathogenesis, diagnosis, management, and the role of protective immunity. Eur J Clin Microbiol Infect Dis 2000;19:77–88.

[2] Mooi FR, Vander Maas NA, De Melker HE. Pertussis resurgence: waning immunity and pathogen adaptation—two sides of the same coin. Epidemiol Infect 2013;13:1–10.

[3] de Melker HE, Schellekens JF, Neppelenbroek SE, Mooi FR, Rumke HC, Conyn-van Spaendonck MA. Reemergence of *pertussis* in the highly vaccinated population of the Netherlands: observations on surveillance data. Emerg Infect Dis 2000;6:348–57.

[4] de Melker HE, Conyn-van Spaendonck MA, Rumke HC, van Wijngaarden JK, Mooi FR, Schellekens JF. Pertussis in The Netherlands: an outbreak despite high levels of immunization with whole-cell vaccine. Emerg Infect Dis 1997;3:175–8.

[5] King AJ, van der Lee S, Mohangoo A, van Gent M, van der Ark A, van de Waterbeemd B. Genome-wide gene expression analysis of *B. pertussis* isolates associated with a resurgence in pertussis: elucidation of factors involved in the increased fitness of epidemic strains. PLoS One 2013;8:e66150.

[6] Mastrantonio P, Spigaglia P, van Oirschot H, van der Heide HG, Heuvelman K, Stefanelli P, et al. Antigenic variants in *B. pertussis* strains isolated from vaccinated and unvaccinated children. Microbiology 1999;145:2069–75.
[7] Mooi FR, He Q, van Oirschot H, Mertsola J. Variation in the *B. pertussis* virulence factors *pertussis* toxin and pertactin in vaccine strains and clinical isolates in Finland. Infect Immun 1999;67:3133–4.
[8] Charles IG, Li JL, Roberts M, Beesley K, Romanos M, et al. Identification and characterization of a protective immunodominant B cell epitopes of pertactin (P.69) from *Bordetella pertussis*. Eur J Immunol 1991;21:1147–53.
[9] Cassiday P, Sanden G, Heuvelman K, Mooi F, Bisgard KM, Popovic T. Polymorphism in *B. pertussis* pertactin and *pertussis* toxin virulence factors in the United States, 1935–1999. J Infect Dis 2000;182:1402–8.
[10] Gzyl A, Augustynowicz E, Gniadek G, Rabczenko D, Dulny G, Slusarczyk J. Sequence variation in *pertussis* S1 subunit toxin and *pertussis* genes in *B. pertussis* strains used for the whole-cell *pertussis* vaccine produced in Poland since 1960: efficiency of the DTwP vaccine-induced immunity against currently circulating *B. pertussis* isolates. Vaccine 2004;22:2122–8.
[11] Weber C, Boursaux-Eude C, Coralie G, Caro V, Guiso N. Polymorphism of *B. pertussis* isolates circulating for the last 10 years in France, where a single effective whole-cell vaccine has been used for more than 30 years. J Clin Microbiol 2001;39:4396–403.
[12] Poynten M, McIntyre PB, Mooi FR, Heuvelman KJ, Gilbert GL. Temporal trends in circulating *B. pertussis* strains in Australia. Epidemiol Infect 2004;132:185–93.
[13] Gzyl A, Augustynowicz E, Loo I, Slusarczyk J. Temporal nucleotide changes in pertactin and *pertussis* toxin genes in *B. pertussis* strains isolated from clinical cases in Poland. Vaccine 2001;20:299–303.
[14] Kourova N, Caro V, Weber C, Thiberge S, Chuprinina R, Tseneva G, et al. Comparison of the *B. pertussis* and *B. parapertussis* isolates circulating in Saint Petersburg between 1998 and 2000 with Russian vaccine strains. J Clin Microbiol 2003;41:3706–11.
[15] Guiso N, Boursaux-Eude C, Weber C, Hausman SZ, Sato H, Iwak M, et al. Analysis of *B. pertussis* isolates collected in Japan before and after introduction of acellular *pertussis* vaccines. Vaccine 2001;19:3248–52.
[16] Zurita E, Moreno G, Errea A, Ormazabal M, Rumbo M, Hozbor D. The stimulated innate resistance event in *B. pertussis* infection is dependent on reactive oxygen species production. Infect Immun 2013;81:2371–8.
[17] Mooi FR, van Loo IH, King AJ. Adaptation of *B. pertussis* to vaccination: a cause for its reemergence. Emerg Infect Dis 2001;7:526–8.
[18] Kobisch M, Novotny P. Identification of a 68-kilodalton outer membrane protein as the major protective antigen of *B. bronchiseptica* by using specific-pathogen-free piglets. Infect Immun 1990;58:352–7.
[19] King AJ, Berbers G, van Oirschot HF, Hoogerhout P, Knipping K, Mooi FR. Role of the polymorphic region 1 of the *B. pertussis* protein pertactin in immunity. Microbiology 2001;147:2885–95.
[20] Miller E. Overview of recent clinical trials of acellular *pertussis* vaccines. Biologicals 1999;27:79–86.
[21] Gotto JW, Eckhardt T, Reilly PA, Scott JV, Cowell JL, Metcalf TN, et al. Biochemical and immunological properties of two forms of pertactin, the 69,000-molecular-weight outer membrane protein of *B. pertussis*. Infect Immun 1993;61:2211–5.
[22] Hellwig SM, Rodriguez ME, Berbers GA, VanDeWinkel JG, Mooi FR. Crucial role of antibodies to pertactin in *B. pertussis* immunity. J Infect Dis 2003;188:738–42.
[23] van Loo IH, van der Heide HG, Nagelkerke NJ, Verhoef J, Mooi FR. Temporal trends in the population structure of *B. pertussis* during 1949–1996 in a highly vaccinated population. J Infect Dis 1999;179:915–23.
[24] He Q, Makinen J, Berbers G, Mooi FR, Viljanen MK, Arvilommi H, et al. *B. pertussis* protein pertactin induces type-specific antibodies: one possible explanation for the emergence of antigenic variants. J Infect Dis 2003;187:1200–5.
[25] Leininger E, Ewanowich CA, Bhargava A, Peppler MS, Kenimer JG, Brennan MJ. Comparative roles of the Arg-Gly-Asp sequence present in the *B. pertussis* adhesins pertactin and filamentous hemagglutinin. Infect Immun 1992;6:2380–5.
[26] Leininger E, Roberts M, Kenimer JG, Charles IG, Fairweather N, Novotny P, et al. Pertactin, an Arg-Gly-Asp-containing *B. pertussis* surface protein that promotes adherence of mammalian cells. Proc Natl Acad Sci USA 1991;88:345–9.
[27] Cherry JD, Gornbein J, Heininger U, Stehr K. A search for serologic correlates of immunity to *B. pertussis* coughs illnesses. Vaccine 1998;16:1901–6.
[28] Storsaeter J, Hallander HO, Gustafsson L, Olin P. Levels of anti *pertussis* antibodies related to protection after household exposure to *B. pertussis*. Vaccine 1998;16:1907–16.
[29] Khelef N, Danve B, Quentin-Millet MJ, Guiso N. *B. pertussis* and *B. parapertussis*: two immunologically distinct species. Infect Immun 1993;61:486–90.
[30] Hijnen M, Voer R, Mooi FR, Schepp R, Moret EE, van Gageldonk P, et al. The role of peptide loops of the *B. pertussis* protein P.69 pertactin in antibody recognition. Vaccine 2007;25:5902–14.
[31] Canthaboo C, Williams L, Xing DK, Corbel MJ. Investigation of cellular and humoral immune responses to whole cell and acellular *pertussis* vaccines. Vaccine 2000;19:637–43.
[32] Gustafsson L, Hallander HO, Olin P, Reizenstein E, Storsaeter J. A controlled trial of a two-component acellular, a five-component acellular, and a whole-cell *pertussis* vaccine. N Engl J Med 1996;334:349–55.
[33] Frank R. The SPOT-synthesis technique. Synthetic peptide arrays on membrane supports-principles and applications. J Immunol Methods 2002;267:13–26.
[34] De-Simone SG, Napoleão-Pego P, Teixeira-Pinto LAL, Santos JDL, De-Simone TS, Melgarejo AR, et al. Linear B-cell epitopes in BthTX-1, BthTX-II and BthA-1, phospholipase A2's from *Bothrops jararacussu* snake venom, recognized by therapeutically neutralizing commercial horse antivenom. Toxicon 2013;72:90–101.
[35] Zhang Y. I-TASSER server for protein 3D structure prediction. BMC Bioinformatics 2008;9:40.
[36] Roy A, Kucukural A, Zhang Y. I-TASSER: a unified platform for automated protein structure and function prediction. Nat Protoc 2010;5:725–38.
[37] Hijnen M, Mooi FR, van Gageldonk PG, Hoogerhout P, King AJ, Berbers GA. Epitope structure of the *B. pertussis* protein P.69 pertactin, a major vaccine component and protective antigen. Infect Immun 2004;72:3716–23.
[38] Henderson IR, Navarro-Garcia F, Nataro JP. The great escape: structure and function of the autotransporter proteins. Trends Microbiol 1998;6:370–8.
[39] Ibsen PH. The effect of formaldehyde, hydrogen peroxide and genetic detoxification of pertussis toxin on epitope recognition by murine monoclonal antibodies. Vaccine 1996;14:359–68.
[40] Trollfors B. Factors influencing antibody responses to acellular pertussis vaccines. Dev Biol Stand 1997;89:279–82.
[41] Sutherland JN, Chang C, Yoder SM, Rock MT, Maynard JA. Antibodies recognizing protective pertussis toxin epitopes are preferentially elicited by natural infection versus acellular immunization. Clin Vaccine Immunol 2011;18:954–62.
[42] Hijnen M, He Q, Schepp R, Van Gageldonk P, Mertsola J, Mooi FR, et al. Antibody responses to defined regions of the *B. pertussis* virulence factor pertactin. Scand J Infect Dis 2008;40:94–104.
[43] Moyer MW. New adjuvants aim to give whooping cough vaccine a boost. Nat Med 2012;18:991.
[44] Asokanathan C, Corbel M, Xing D. A CpG-containing oligodeoxynucleotide adjuvant for acellular pertussis vaccine improves the protective response against *B. pertussis*. Hum Vaccine Immunother 2013;9:325–31.
[45] Kubler-Kielb J, Vinogradov E, Lagergard T, et al. Oligosaccharide conjugates of *B. pertussis* and bronchiseptica induce bactericidal antibodies, an addition to pertussis vaccine. Proc Natl Acad Sci USA 2011;108:4087–92.
[46] Shahin R, Leef M, Eldridge J, Hudson M, Gilley R. Adjuvanticity and protective immunity elicited by *B. pertussis* antigens encapsulated in poly(DL-lactide-*co*-glyolide) microspheres. Infect Immun 1995;63:1195–200.