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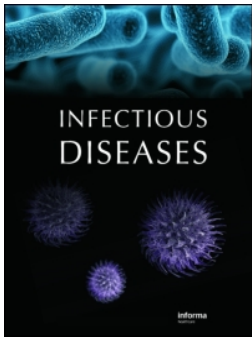
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ORIGINAL ARTICLE

Restricted antibody response to *Bordetella pertussis* filamentous hemagglutinin induced by whole-cell and acellular pertussis vaccines

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ABSTRACT

Background: Filamentous hemagglutinin (FHA) is a principal virulence factor, an important immunogenic antigen of *Bordetella pertussis*, and a major component of many acellular pertussis vaccines. In the present study, the human antibody response to different regions of FHA was determined in healthy children and adults vaccinated with either whole-cell or acellular pertussis vaccines. **Methods:** To define the immunodominant regions of FHA, four overlapping recombinant fragments were expressed and produced in *Escherichia coli* and then purified by His-tagged based affinity chromatography. Two groups comprising healthy preschool children ($n = 50$) and adults ($n = 26$) were vaccinated with a single dose of commercial whole-cell and acellular DTaP vaccines, respectively. An antigen-based ELISA was applied to measure serum levels of anti-FHA antibody to both native and recombinant proteins in vaccinated volunteers. **Results:** In both groups of vaccinated individuals, the anti-FHA antibody response was mainly directed against epitopes located within a fragment of FHA spanning amino acid residues 1877–2250 of the mature FHA molecule ($p < 0.001$). No or little antibody was detected against the other recombinant segments of FHA. **Conclusion:** Our results suggest that the human antibody response to FHA is directed to an immunodominant region located within residues 1877–2250 of the FHA molecule. Characterization and epitope mapping of the major components of acellular pertussis vaccine and future modifications in vaccine formulation may improve its efficacy and protectivity.

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Introduction

Childhood vaccination with inactivated whole-cell *Bordetella pertussis* bacteria in the mid-1940s resulted in a dramatic decrease of whooping cough incidence worldwide [1]. Many years later the World Health Organization (WHO) approved inclusion of triple diphtheria, tetanus toxoid, and whole-cell pertussis (DTwP) vaccine in the Expanded Program of Immunization (EPI) of all countries [2]. Problems in vaccine standardization together with severe side effects and reactogenicity of the whole-cell pertussis vaccines were the main reasons to shift to the acellular vaccines, which contain some immunodominant antigens instead of whole bacteria [3,4]. The triple diphtheria-tetanus-acellular pertussis vaccines (DTaP) are produced by various manufacturers with minor differences in their formulations. Filamentous hemagglutinin (FHA) together with pertussis toxoid and pertactin are the major pertussis bacterial components of most acellular vaccines [3,5,6]. Bivalent and monovalent vaccines formulated with pertussis

toxoid plus FHA or pertussis toxoid alone have also been employed successfully [7].

In the last two decades, pertussis resurgence has been reported in many geographical regions and became a main health problem in both industrial and developing countries [5,8]. Several factors, such as increasing awareness and improving diagnostic methods, waning immunity, and pathogen adaptation, as well as shifting from traditional whole-cell vaccines to acellular vaccines are proposed as possible causes of pertussis resurgence [9,10]. This proposition has initiated extensive investigations of the acellular vaccine components, formulations, their immunogenicity, and their interactions with the host immune system. Initial case-control double-blinded trials confirmed efficacy of the acellular vaccines [11,12]. Other studies, however, demonstrated that the antibody response to most acellular pertussis vaccines is weak and the level of specific antibody diminishes within a short period, but the immunized subjects remain protected against severe disease [13–16]. Further studies on the protective role of antibody have

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shown that acellular or whole-cell vaccines failed to develop a protective immunity against pertussis and clear bacteria from the respiratory system in immunoglobulin-defective ($Ig^{-/-}$) mice [6,13,17]. Thus, both the humoral and cellular arms of the host immune system are involved in protection against pertussis. These findings indicate the need for more immunogenic and effective vaccines for the control of pertussis.

Most recently, it has been shown in baboons that immunization with acellular pertussis vaccine could protect the animals from the disease, but colonization of the bacteria in the epithelium of bronchial airways was unaffected. Immunized animals exposed to *B. pertussis* were able to disseminate the infection to unimmunized animals [18]. Although the latter study was conducted on two pairs of animals and the results are too limited and preliminary to draw firm conclusions, these findings imply the need for more immunogenic and effective vaccines for the control of pertussis.

FHA, a 220 kDa secreted and membrane-bound protein, is a crucial receptor-binding virulence factor of *B. pertussis* that plays a key role in bacterial pathogenesis by adhesion to human ciliated respiratory epithelial cells [6,13]. Previous reports have indicated that the C-terminal part displays the most immunodominant epitopes of FHA [19–22].

In the present study, human antibody response to four C-terminal overlapping recombinant fragments of FHA was evaluated in sera obtained from healthy individuals who received commercial whole-cell or acellular pertussis vaccines to identify the most immunogenic region of FHA. Our results indicate that a small region of this large molecule encompassing residues 1877–2250 displays the major immunodominant epitopes for the induction of the antibody response to FHA following vaccination with both cellular and acellular vaccines.

Material and Methods

Study population

Two groups comprising healthy children and adults, respectively, were enrolled in this study. The first group consisted of 50 preschool children (20 males and 30 females) aged 4–6 years who had received four doses of DTWP vaccine at ages of 2, 4, 6, and 18 months according to the national vaccination program of Iran. These preschool children were later vaccinated with a single booster dose of a commercial DTWP vaccine (Aventis Sanofi Pasteur, Lyon, France) as part of the national vaccination program [23]. The second group of vaccinees consisted of 26 healthy adults (15 males and 11 females) aged 25–35 years who had been vaccinated with five doses of DTWP vaccine in their childhood according to the national vaccination program. These adults voluntarily received a single dose of a commercial acellular DTaP vaccine (Infanrix; GlaxoSmithKline Biologicals, Rixensart, Belgium), containing pertussis toxin, FHA, and pertactin. All participants or their parents signed a consent letter and the Ethical Committees of Tehran University of Medical Sciences and Avicenna Research Institute approved the study. Peripheral blood samples were obtained from both groups of subjects 1 month after vaccination. Pre-vaccination blood samples were also collected from adult vaccinees. Pre-vaccination specimens were not taken from

preschool children due to the ethical restrictions. Serum was recovered from all blood samples and was frozen at -20°C until performance of the serological assays.

Construction and purification of the recombinant FHA fragments

Four overlapping regions of FHA coding sequence designated as rFHA1–4 were selected and amplified from genomic DNA of the Tohama I strain of *B. pertussis* (ATCC BAA-589, Manassas, VA, USA). Amplification, cloning, production, and purification of three recombinant fragments of FHA (rFHA1–3) have been reported elsewhere [24]. Briefly, amplification of rFHA1–4 was performed by PCR using specific primers containing *EcoRI* and *HindIII* restriction sites in both ends (shown as bold sequences): 5'-**GAATTCTCGTCCGCAGATCACCGACGCGGT**-3' as sense and 5'-**AAGCTTATCGTGGCCTGCCTTCAGGCTGC**-3' as anti-sense for rFHA1 (amplicon size 1152 bp; aa 1200–1584), 5'-**GAATTCTGACATCGTCATCAAGACGGAACAG**-3' as sense and 5'-**AAGCTTCTTGAATACTCCATGGCGGACAC**-3' as anti-sense for rFHA2 (amplicon size 1119 bp; aa 1544–1917), 5'-**GAATTCTGACGAACATCGCATCTGCTCAAT**-3' as sense and 5'-**AAGCTTGCCGTCGGCAAGGATGTCTGAG**-3' as anti-sense for rFHA3 (amplicon size 1119 bp; aa 1877–2250), 5'-**GAATTCTGGCATCATCCAGGAGTTCCGGC**C-3' as sense and 5'-**AAGCTTCTGCTCGCGACCAGCGTGGTAT**-3' as anti-sense for rFHA4 (amplicon size 1029 bp; aa 2067–2410). Amplified products were purified from agarose gel (Fermentas, Moscow, Russia), directly cloned in pGEMT-easy cloning vector (Promega, Madison, WI, USA) and transformed into *E. coli* JM109 competent cells (Novagen, Merck KGaA, Darmstadt, Germany). After confirmation of the selected colonies by sequencing, all inserts were digested with restriction endonucleases *EcoRI* and *HindIII* (Fermentas) and then ligated in pET22b(+) (rFHA1–3) (Merck Millipore, Darmstadt, Germany) or pET28b(+) (rFHA4) expression vector. *E. coli* BL21 (DE3) (Novagen, Merck KGaA) competent cells were transformed with all constructs and positive colonies were selected by colony PCR. After confirmation by nucleotide sequencing, transformed cells were grown in Luria Bertani (LB) broth containing 100 $\mu\text{g/ml}$ ampicillin (rFHA1–3) or 50 $\mu\text{g/ml}$ kanamycin (Sigma, St Louis, MO, USA) (rFHA4). Finally, 1 mM IPTG was added for induction when OD_{600 nm} reached 0.6. After incubation for 3–4 h at 37°C , cells were harvested using centrifugation at 2000 *g* for 30 min at 4°C .

The expressed recombinant proteins were purified using nickel-nitrilotriacetic acid (Ni-NTA) agarose gel (Qiagen, Germantown, MD, USA) in a denaturing condition and renatured based on the manufacturer's recommendation. Purity and specificity of all recombinant proteins were confirmed by SDS-PAGE and Western blotting analyses as described previously [24,25].

Polyacrylamide gel electrophoresis and immunoblotting

All purified recombinant fragments (15 μl) were mixed with 5 μl non-reducing sample dye (192 mM Tris-HCl, pH 6.8, 5.2% SDS, 25% glycerol); 0.1% bromophenol blue solution was used

as tracking dye. The mixture was heated at 80–100 °C for 5 min and loaded onto the 12% acrylamide gel using the Bio-Rad system (Bio-Rad Laboratories, Munich, Germany). Finally, gels were stained with Coomassie brilliant blue R 250 (Merck). The molecular weight of the recombinant fragments was confirmed according to protein size markers (Fermentas, Waltham, MA, USA). To further characterize the recombinant proteins, immunoblotting was performed as described elsewhere [19]. Briefly, the proteins were electroblotted from the gel to polyvinylidene fluoride membrane (Merck). Blotted membrane was then blocked in 3% skimmed milk (Merck) and incubated with rabbit anti-FHA1–4 polyclonal antibodies produced in our laboratory. The membrane was finally treated with peroxidase-conjugated sheep anti-rabbit immunoglobulin (Sina Biotech, Tehran, Iran) and developed with enhanced chemiluminescence (ECL) (GE Healthcare, Uppsala, Sweden).

Measurement of anti-FHA antibody by enzyme-linked immunosorbent assay (ELISA)

Serum levels of antibody against rFHA1–4 fragments or native FHA protein (NIBSC, Hertfordshire, UK) were determined using an indirect ELISA. Briefly, 2.5 µg/ml of FHA proteins dissolved in phosphate-buffered saline (PBS; 0.15 M, pH 7.2) were coated separately on a 96-well ELISA plate (Nunc Maxisorp, Roskilde, Denmark) for 90 min at 37 °C. After blocking with 3% skim milk, different dilutions (1:100, 1:250, and 1:1000) of all sera were added to the plates. After incubation at 37 °C for 90 min, plates were washed three times with PBS containing 0.05% Tween 20 (PBS/T; Sigma). Peroxidase-conjugated sheep anti-human antibody (1:1000) (prepared in our laboratory) was applied to all wells and the plates were incubated for a further 90 min at 37 °C. All plates were then developed using tetramethyl benzidine substrate (TMB) followed by addition of 1 N HCl as stopping solution. Optical densities (ODs) were finally recorded at 450 nm.

Statistical analysis

Normal distribution and equality of variances of data for both groups of subjects were first tested using Kolmogorov-Smirnov and Levene's tests, respectively. The non-parametric data sets were first normalized using a square root transformation and then analyzed with a generalized linear model (GLM) followed by a Tukey test. Non-parametric paired data were assessed by

Wilcoxon test using the SPSS 21 program (SPSS, Chicago, IL, USA). Probability values <0.05 were considered statistically significant.

Results

Construction, expression, and purification of the recombinant FHA1–4 fragments

Recombinant FHA1–3 fragments were expressed and purified as described previously [24]. In addition to rFHA1–3 proteins, rFHA4 was constructed and purified in this study to cover the whole C-terminal part of the FHA molecule (Figure 1). SDS-PAGE analysis of the final products showed highly purified preparations of all rFHA1–4 fragments (Figure 2a). The reactivity and identity of the recombinant proteins were confirmed by immunoblot assays using different commercial and lab-made detector antibodies (Figure 2b) as described previously [24].

Antibody response to recombinant FHA fragments in adults vaccinated with acellular pertussis vaccine

Serum levels of antibody to all four overlapping recombinant fragments as well as native FHA were measured in adults vaccinated with a commercial acellular pertussis vaccine before and 1 month after vaccination. No substantial levels of specific antibody were detected against either of the four rFHA fragments or nFHA in prevaccinated samples. However, after vaccination the antibody response was mainly directed against rFHA3 segment spanning amino acids 1877–2250 of the FHA molecule ($p < 0.001$) (Figure 3). No significant difference was observed for the levels of antibody specific for rFHA1, 2, and 4 fragments between pre- and post-vaccination samples.

Antibody response to recombinant FHA fragments in children vaccinated with whole-cell pertussis vaccine

Similar to adults vaccinated with DTaP vaccine, the antibody response to FHA induced by DTwP vaccine in children was restricted to rFHA3 segment ($p < 0.001$) (Figure 4). We could not obtain prevaccination samples from the children due to ethical restrictions. No statistical differences were observed between the antibody levels induced to the other recombinant fragments of FHA.



Figure 1. Schematic representation of precursor filamentous hemagglutinin (FHA), mature native FHA, and the location of the four overlapping recombinant FHA fragments (rFHA1–4) employed in this study. The amino acid lengths and positions of proteins are shown in parentheses.

Discussion

Mature FHA is a high molecular weight molecule with 220 kDa and 2440 amino acids, which is produced and secreted by

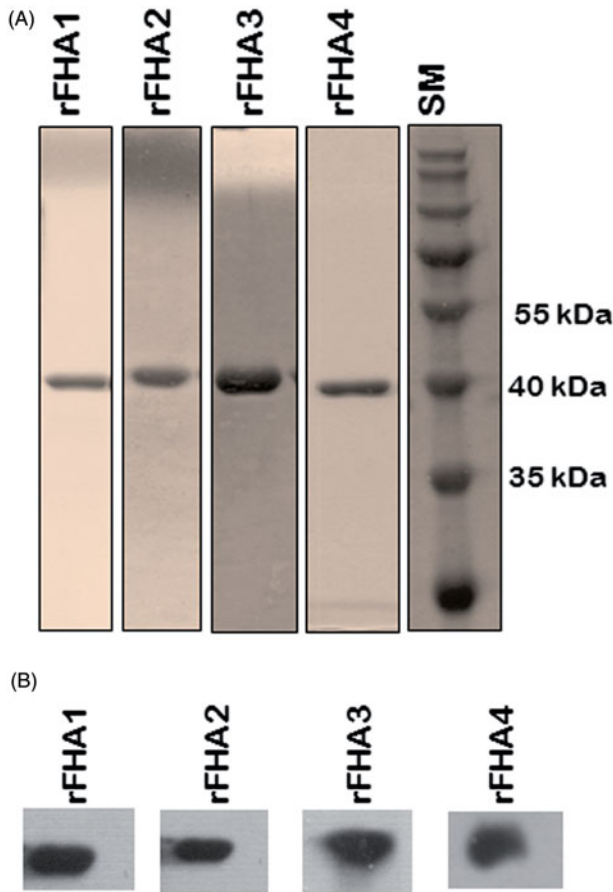


Figure 2. Analysis of the purified recombinant filamentous hemagglutinin (FHA) fragments by SDS-PAGE and immunoblotting. All recombinant fragments were purified by nickel-nitrilotriacetic acid (Ni-NTA) chromatography. Final products were run on 12% polyacrylamide gel followed by Coomassie blue staining (a). For immunoblot analysis, all proteins were transferred from polyacrylamide gel to PVDF membranes. Blocked membranes were then incubated with rabbit anti-FHA1–4 polyclonal antibodies and developed by enhanced chemiluminescence (ECL) (b). SM, protein size marker.

processing of a large 370 kDa membrane-bound precursor [3]. It is one of the major adhesion molecules and virulence factors of *B. pertussis* [26]. Nearly all acellular pertussis vaccines from various manufacturers contain FHA in their formulations and thus immunogenic analysis and detailed characterization of this large protein may improve the efficacy of acellular vaccines. Some reports in the literature have demonstrated the C-terminal fragment of FHA as the most immunodominant region [19–22]. The presence of at least four antibody-recognizing epitopes in the carboxy-terminal part of FHA, within amino acids 1200–2300, was first identified by Delisse-Gathoye et al. [22]. In a comprehensive study, Leininger and colleagues used both FHA-specific monoclonal antibodies and human sera following vaccination or pertussis infection to define the immunodominant parts of FHA [21]. They showed that there are two major immunodominant regions in FHA designated as domain types I and II located in regions spanning amino acids 1653–2111 and 1–1073, respectively [21]. Investigation of sera collected from pertussis-infected or vaccinated individuals indicated that human natural infection

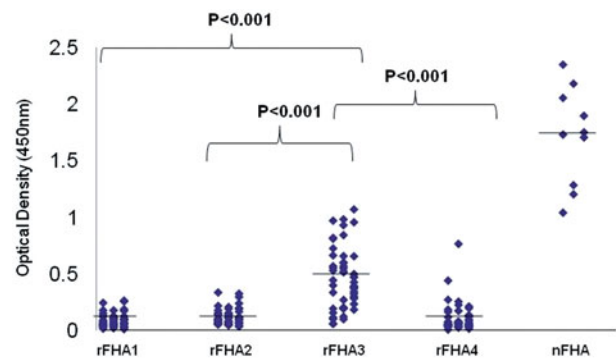


Figure 4. Antibody response to four overlapping recombinant recombinant filamentous hemagglutinin (FHA) fragments in healthy preschool children vaccinated with a whole-cell pertussis vaccine. Fifty children were vaccinated with a commercial whole-cell pertussis vaccine and antibody titers against recombinant FHA (rFHA) and native FHA (nFHA) proteins were measured in their post-immunization serum samples by ELISA. Only 10 samples were randomly tested for nFHA specific antibody due to shortage of antigen. The results represent optical densities obtained at 450 nm. A 1:250 dilution of the serum is shown.

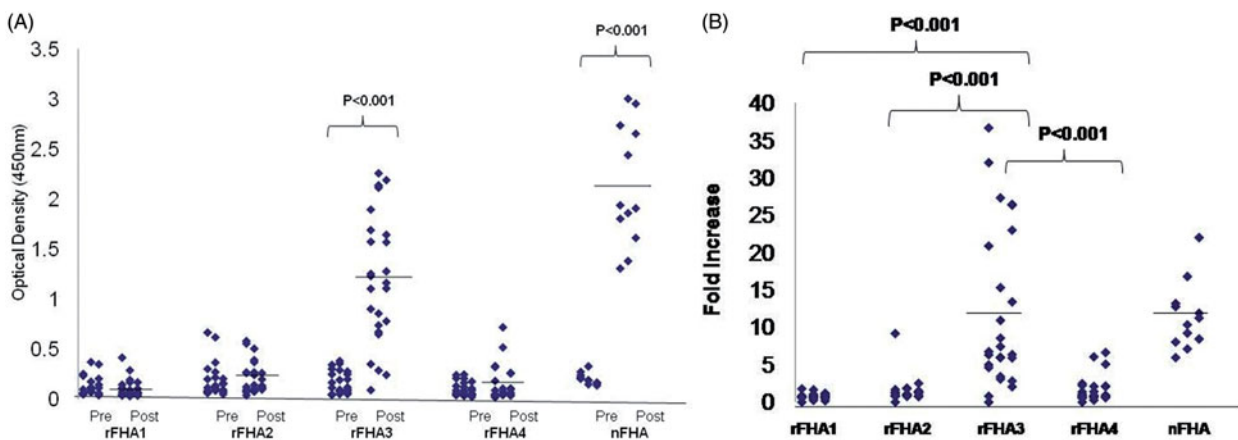


Figure 3. Antibody response to four overlapping recombinant filamentous hemagglutinin (FHA) fragments in normal volunteers vaccinated with acellular pertussis vaccine. Twenty-six normal individuals were vaccinated with a single dose of commercial acellular pertussis vaccine and antibody titers against recombinant FHA (rFHA) and native FHA (nFHA) proteins were measured in their pre-immunization and 1 month post-immunization sera by ELISA. Only 12 samples were randomly tested for nFHA specific antibody due to shortage of antigen. The results represent optical densities obtained at 450 nm for pre- and post-vaccination samples (a) and the fold increase of optical densities in post-vaccination compared to pre-vaccination serum samples (b). A 1:250 dilution of the serum is shown.

or immunization with whole-cell pertussis vaccine elicit a similar humoral immune response pattern mainly directed to the type I domain, which is located in the C-terminal region of mature FHA [21]. Later the high immunogenicity of the C-terminal region was confirmed using different FHA peptides displayed by a phage library and anti-FHA polyclonal antibodies [20]. In this study a peptide derived from residues 1929–2019 of FHA was introduced as the most immunogenic fragment containing immunodominant linear epitopes [20]. Finally, in another study the reactivity of different recombinant fragments covering the whole 220 kDa FHA molecule was studied with sera from patients who had recovered from whooping cough. This study showed a region in the C-terminal of FHA between residues 1927 and 2266, which displays the most immunogenic B cell epitopes [19]. However, the N-terminal region of FHA contains some repetitive motifs, which could render this region less immunogenic [21,22].

Since adherence of FHA to ciliated epithelial respiratory cells is mediated by its C-terminal sequence, thus induction of antibody against this region is very crucial for protection against pertussis [26]. Recent findings regarding the inefficiency of the acellular pertussis vaccine to inhibit adherence of *B. pertussis* to bronchial epithelial cells and their dissemination to unimmunized baboons [18], imply that the antibody response to the FHA component of the acellular vaccine might be defective or the antibody response to FHA might be directed to epitopes different from those implicated in the cellular pertussis vaccines. This prompted us to investigate the anti-FHA antibody response to four overlapping FHA fragments covering the C-terminal region of FHA in healthy individuals vaccinated with either cellular or acellular pertussis vaccines.

Measurement of antibody levels in prevaccinated sera from acellular pertussis-vaccinated adults showed no or negligible response to all four recombinant fragments, as well as the native FHA (Figure 3a), indicating lack of previous antibody response to natural infection or childhood vaccination with DTWP vaccines. The incidence of pertussis in Iranian adults is not well known but it is well understood that the humoral immune response induced by either whole-cell or acellular pertussis vaccines is diminished within 1–5 years post-vaccination [13]. On the other hand, the antibody response in post-vaccinated sera from both whole-cell and acellular vaccinated subjects was mainly directed to rFHA3 fragment spanning amino acids 1877–2250. We also checked the reactivity of three polyclonal anti-native FHA antibodies produced in sheep, rabbit, and mouse. Interestingly, all three antibodies reacted strongly with rFHA3, but not other recombinant fragments of FHA (data not shown). These results indicate cross-species immunodominancy of this region for the induction of humoral immune response. It seems that epitopes within an extended rFHA3 region are involved in induction of the T-cell response to FHA. Cloning of FHA specific T lymphocytes demonstrated that linear epitopes within a region between residues 1653 and 2347 are predominantly recognized by T cells [27]. Since low efficiency of acellular vaccines is proposed as a major cause of recent pertussis hygiene problems, so it seems that the current formulation of acellular vaccine needs to be reconsidered. Epitope mapping analysis and immunological characterization

of vaccine components followed by possible modifications in its composition may enable recognition of more immunoprotective epitopes that might be masked or encrypted in the native conformation of FHA by B or T lymphocytes. This may lead to induction of a more efficient and protective immune response resulting in inhibition of adherence and colonization of bacteria to bronchial epithelial cells.

In summary, our results showed the recombinant FHA3 fragment, corresponding to amino acid residues 1877–2250, to be the most immunodominant region of FHA for the induction of antibody response following immunization with either whole-cell or acellular pertussis vaccines. We are now embarking on evaluation of the protective capacity of this recombinant fragment in a mouse protection model against natural pertussis infection, which would extend the present data.

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Declaration of interest

The authors report no conflict of interests. The authors alone are responsible for the content and writing of the paper.

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