

## The Immunogenicity Of Partially Identified Candidates Of Acellular Pertussis Vaccines

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**Abstract:** Since being included in the national immunization program, pertussis whole cell vaccines (WCVs) have been used for protection against pertussis in Egypt and none of the acellular vaccines (ACVs) has been tried. In an attempt to encourage local ACV production and supply it in an available low cost, this study was conducted to optimize production and evaluate the immunogenicity of some partially identified ACVs. Three combinations of *Bordetella pertussis* (*B. pertussis*) antigen candidates were prepared by fractionation of the culture supernatant strain 134, grown in liquid verway medium in presence of cyclodextrin, on a Sephacryl-300 column and evaluated for specific immunogenicity in mice. The media enriched with cyclodextrin enhanced the level of the intact PT yield by ~20 folds more than that without cyclodextrin under the same conditions. The concentrations of the three most immunogenic components, PT, filamentous FHA, and PRN in all the experimental vaccines as well as their protective antibodies in immunized mice were determined by ELISA. The results demonstrated that the ACV containing the highest amount of intact PT induced the highest anti-*B. pertussis* titer in mice, twice higher than that of the reference WCV pointing to the efficacy of the intact toxin, rather than the dissociated toxin, as an important vaccine candidate. It was also demonstrated that the entire *B. pertussis* antigens and / or their dissociated entities seem to act in a concert way that could allow minimizing the intact PT content with only small effect on immunogenicity. The data presented herein could serve as a guide for production and selection of candidate ACVs against pertussis using simple facilities and with a reasonable price making it suitable for mass production in developing countries.

**Keywords:** *B. pertussis* - Pertussis toxin – Vaccines.

## Introduction

Pertussis (whooping cough) is an infectious bacterial disease caused by *Bordetella pertussis* (*B. pertussis*), a gram negative bacillus. Bacterial pneumonia or respiratory distress syndrome is the usual cause of death<sup>1</sup>. The first generation whole cell vaccine (WCV) introduced in the forties, drastically reduced morbidity and mortality due to whooping cough. The side effects associated with the WCV have decreased its use in several countries, with the result of sharp increase in infants mortality caused by the disease. Pertussis is currently an endemic disease with regular epidemic outbreaks<sup>2,3</sup>. Estimates from the World Health Organization (WHO) suggest that about 16 million cases of pertussis occurred worldwide in 2008, 95% of which were in developing countries<sup>4</sup>. *B. pertussis* produces many antigens, among them are pertussis toxin (PT), filamentous hemaagglutinin (FHA), pertactin (PRN), fimbriae, adenylate cyclase and others. Consequently more defined vaccines composed of protective *B. pertussis* antigens in different combinations were proved to be safer, less reactogenic and more reliable to introduce in mass vaccination<sup>5-7</sup>, that made most countries switched to a new generation vaccines "acellular vaccines (ACVs)". ACVs resulted in early immune response in neonates<sup>8</sup> as well as adults<sup>9</sup>. One of the disadvantages of the ACVs is the cost associated with the production of the individual components or multivalent fusion proteins<sup>10</sup>. Partially purified ACVs were prepared from various *B. pertussis* culture strains using simple indigenously available techniques<sup>11,12</sup>. The extracts mainly contained PT and FHA. Cyclodextrin is considered one of the most suitable synthetic media for culture of *B. pertussis*<sup>13</sup>. It was possible to determine a relationship between the presence of cyclodextrin and destabilization of the outer bacterial membrane and the release of proteins. The addition of cyclodextrin to the liquid culture medium ultimately augmented the levels of extracellular proteins like PT and FHA<sup>14</sup>. Several efficacy trials on various ACVs containing one to five components have been reported<sup>15</sup>. Circulating antibodies to PT neutralizes the toxin which in turn confers substantial protection against severe disease. Anti-adhesion antibodies, anti-FHA, anti-PRN and anti-fimbriae prevent the attachment of *B. pertussis* to host cells in the initial stage of infection<sup>16</sup>. Recommended ratio among the three antigens in the vaccine formula is still questionable. Various combinations of antibodies are most likely to correlate with protection<sup>17</sup>. A recent study showed that the efficacy of multi-component ( $\geq 3$ ) vaccines reached ~ 85% in preventing typical whooping cough in contrast to the efficacy of the one- and two- component vaccines that varied from 59-75% against typical whooping cough<sup>7</sup>. The aim of the present study is to optimize the production of partially purified ACVs with respect to efficacy in mice using simple cheap procedures. This could serve to cut down the cost of the vaccine production and encourage local vaccine manufacturing in developing countries. The humoral response of three experimental poorly defined ACVs with different composition and different antigen entities were evaluated and compared.

## 2. Materials and Methods

### 2.1. Bacterial antigens

*B. pertussis* strain 134 and the corresponding culture supernatant were prepared as previously described<sup>12</sup>. Briefly, the bacteria were subcultured in liquid Verwey medium enriched with (1gm/ml) cyclodextrin. For preparation of WCV, the culture was inactivated by heating at 56°C for 30 min, whereas the experimental ACVs were prepared from the culture supernatant as follows: The culture was kept for 40-60 h at 35°C under aeration<sup>18</sup>. After centrifugation the culture separated into culture cells and culture supernatant. The culture supernatant was collected using a cell separator, passed through an EKS-1 Sietz filter for clarification and concentrated by ultrafiltration using a cartridge of 10000 cut-off (Amicon). The concentrated sample formed a precipitate on prolonged storage at 4°C. The precipitate was collected by centrifugation and the clear supernatant was stored at 4°C. Purified PT, FHA and PRN were supplied by the VACSERA authorities.

### 2.2. Fractionation of *B. pertussis* supernatant and preparation of the experimental vaccines

The concentrated *B. pertussis* supernatant was applied onto a Sephacryl S-300 column equilibrated and eluted with PBS 0.15M NaCl in 10 Mm PB, pH7 and the eluate was monitored by measuring the absorbency at 280 nm. The column fractions under selected peaks were collected and their protein content was measured. The Sephacryl fractions (FI, FII and FIII) were inactivated using 0.2% glutaraldehyde<sup>26</sup>, and adsorbed to aluminium phosphate. The final immunogen preparations were adjusted to contain 4 µg of each of the Sephacryl fractions per dose adsorbed to 0.2 ml of aluminum phosphate adjuvant. The antigen protein content was determined according to Bradford (1976)<sup>19</sup>, using bovine serum albumin as a standard protein. The contents of the *B. pertussis* antigens (PT, FHA and PRN) were determined by ELISA and used as immunogens.

### 2.3. Mice immunization

Five groups of Swiss mice (n=5) were used, four groups were immunized at days 1 and 21 with the WCV and the three experimental vaccines (Sephacryl fractions: F I, F II, F III) and the fifth group with the adjuvant alone (control). The mice were bled seven days after the booster dose and the mice sera were collected and quantified, for the anti-*B. pertussis* antigens contents (anti-PT, anti-FHA and anti-PRN) by ELISA. A local reference mouse antiserum to *B. pertussis* comprises a pool of selected high titer mice antisera from mice immunized with the WCV and challenged with virulent *B. pertussis* strains.

### 2.4. Antibodies to *B. pertussis* antigens

Standard sheep anti-PT, anti-FHA and anti-PRN were polyvalent antibodies that were kindly offered by the National Institute of Biological Standards and Controls and stored at -20°C. Antisera to PT, FHA and PRN were raised in rabbits (n=2 for each antigen) according to the following method: New Zealand rabbits (weighing ~2 kg) were primed individually with the antigen doses (from 20-25µg/ 0.5 ml saline) emulsified in 0.5 ml complete Freund's adjuvant. After three weeks, a booster dose of 20 µg in incomplete Freund's adjuvant followed. One week after the booster dose, the animals were bled, sera were separated from blood cells by low speed centrifugation (2000xg) for 5 min at room temperature and stored at 20°C till used.

### 2.5. ELISA

#### 2.5.1. Quantification of *B. pertussis* antigens in the Sephadex-300 fractions

A modified double antibody sandwich ELISA<sup>20</sup> was established for determination of *B. pertussis* antigens (PT, FHA and PRN). Microtiter plates were coated overnight at 4°C with 100 µl of the selected sheep reference antiserum (1:15,000) diluted in 0.05M carbonate buffer pH 9.6 (coating buffer). The plates were washed 3 times with 0.01M PBS pH 7.5 containing 0.1% Tween 20 (PBS-T). The remaining active sites were blocked via incubation for 1 hr at 37°C with 200µl/well PBS containing 2% gelatin. The plates were washed 3 times before adding serial dilutions of the *B. pertussis* test antigens in PBS-T (100µl/well) and incubated at 37°C for 1 h. After washing three times with PBS-T, the wells were filled with 100 µl of the prepared rabbit antisera (anti-PT, anti-FHA and anti-PRN, diluted 1:1000) followed by another wash and then 100 µl of anti-rabbit IgG peroxidase conjugate (Sigma) diluted in PBS-T (1:5000) was added and incubated for 1 hr. The plates were washed thoroughly for 3-4 times with PBS-T buffer before allowing them to react with the enzyme substrate (Sigma fast ortho-phenylene diamine) tablets dissolved in 20 ml distilled water (100 µl/well). Then reaction was allowed to proceed for 30 min at room temp in the dark before the addition of 50 µl 2N Sulfuric acid. The developed optical densities were measured at 490 nm in a Micro ELISA Reader Photometer. The measured optical densities were plotted against the *B. pertussis* antigen concentrations. A preparation of each purified *B. pertussis* antigen was chosen to serve as a reference for the tested antigens. Relative antigen concentrations in ELISA units (EU) were calculated with the aid of the reference antigen.

#### 2.5.2. Quantification of serum antibodies

Microtiter plates were coated with sheep reference antisera, washed and blocked with gelatin using the same reagents and concentrations as in above mentioned ELISA. After washing, 100 µl of either of the purified *B. pertussis* antigens (5µg/ml) in PBS-T was added for 1 hr at 37°C. Then the plates were again washed three times. Serial dilutions of the test mice antisera in PBS-T (100µl/well) were added and incubated at 37°C for 1 hr. After washing with PBS-T, the wells were filled with 100 µl of anti-mouse IgG peroxidase conjugate (Sigma) diluted in PBS-T (1:5000) for 1 hr. The plates were washed thoroughly for 3-4 times with PBS-T buffer before allowing them to react with enzyme substrate (Sigma fast ortho-phenylene diamine) tablets dissolved in 20 ml distilled water (100 µl/well). Then reaction was allowed to proceed and stop as above and the measured optical densities were plotted against mice antibody dilutions. The reciprocal of antibody dilution that gives an optical density value of 0.5 was considered as the end point ELISA titer of the antibody.

### 2.6. Cytotoxicity

The cytotoxicity of PT was determined in a cell culture assay as described by Gillenius *et al.* (1985)<sup>21</sup>. Two fold serial dilutions of the test sample in the culture medium were added to 24 h preformed Chinese hamster ovary (CHO) monolayers and screened for cytotoxicity after 48 h. The logarithm of the reciprocal of

the dilution which induces the least visible clustering was taken as the titer in minimum cytotoxic doses (MCD). The PT content was determined as mg protein using purified PT as a reference.

### 3. Results

#### 3.1. The productivity of PT from *B. pertussis* culture media

The PT content of the culture supernatant *B. pertussis* strain 134 was measured by CHO clustering assay. The PT yield obtained in cyclodextrin-enriched medium was much higher than that corresponding to the same fermentation medium and under the same conditions without cyclodextrin (320 versus 15.9 MCDs respectively) indicating that cyclodextrin increased the intact PT yield by ~ 20 folds.

#### 3.2. Fractionation of *B. pertussis* culture supernatant on Sephacryl S-300 and preparation of the experimental vaccines

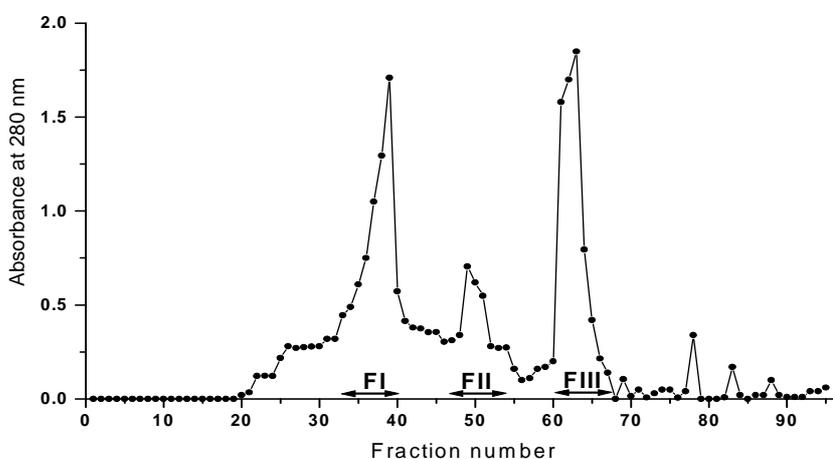
A typical elution profile of *B. pertussis* strain 134 culture supernatant on Sephacryl S-300 column is shown in **Figure (1)**. Measurement of the PT cytotoxicity in the CHO clustering assay revealed that the intact PT content of the Sephacryl fractions I, II and III, were 258.8, 13.94 and 2.09 MCD, respectively, indicating recovery of most of the supernatant cytotoxicity (~80%) in Syphacryl FI. F II and F III retained only 4% and 0.65 %, respectively, of PT cytotoxicity. The fractions were detoxified by glutaldehyde and used as immunogens “experimental vaccines” to induce humoral immunity in mice.

#### 3.3. Evaluation of the main antigenic contents of the experimental vaccines

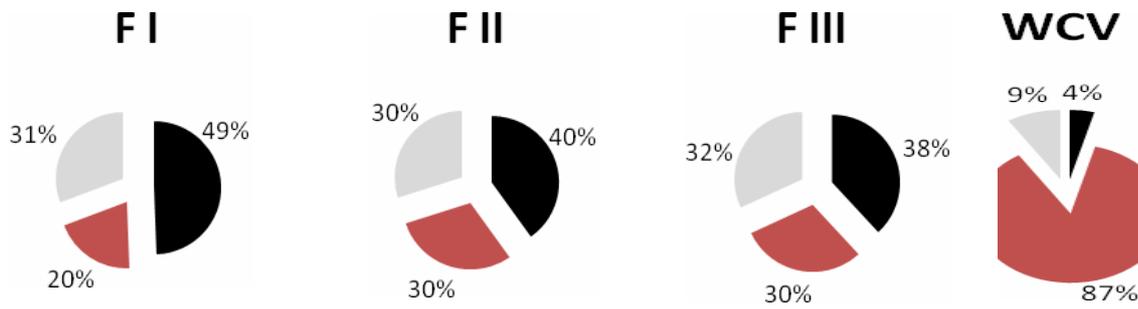
The contents of the three main immunogenic and protective antigens (PT, FHA, and PRN) of the Sephacryl fractions were determined by ELISA and compared with those of the WCV (**Figure 2**). All the three antigens were detected in the various fractions indicating their great dissociation. The results demonstrate that each of various antigenic entities of each immunogen was distributed in all fractions. It is also obvious that the main antigen in the three experimental vaccines is PT while FHA constitutes the major antigen in the WCV.

#### 3.4. Evaluation of humoral response of the experimental vaccines in mice

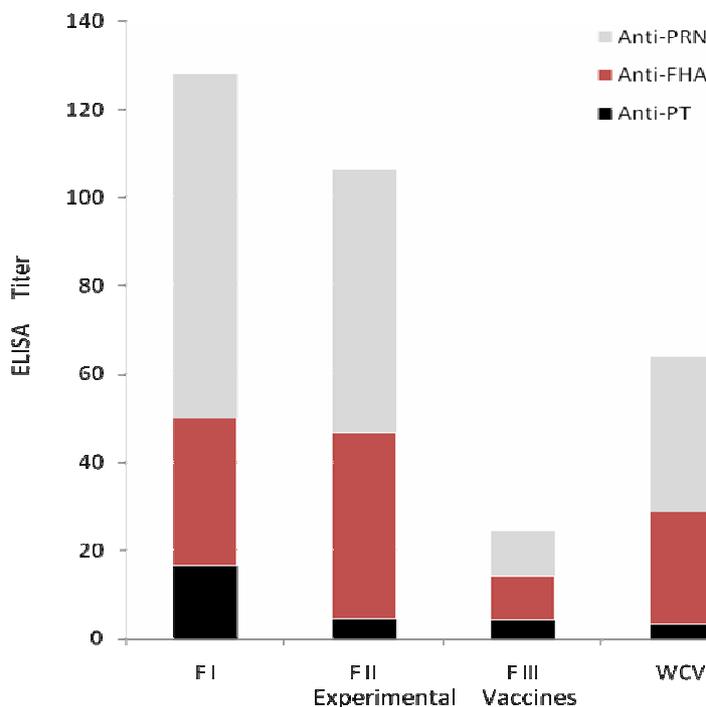
The ELISA titers of the mice antibodies to the main immunogenic components of the experimental vaccines were determined in mice sera and compared to those of the WCV (**Figure 3**). It is obvious that the various combinations of *B. pertussis* antigens were immunogenic, however, F I vaccine induced the highest antibody titer in mice, the highest anti-PT and anti-PRN as well. Whereas F II vaccine registered slightly higher anti-FHA antibody titer (4.2 EU versus 3.3 EU obtained by F I).



**Figure 1.** A typical elution profile of *B. pertussis* strain 134 culture supernatant (CHO titer = ~ 320 MCD) on Sephacryl S-300 column (2.4 x 80 cm) eluted with PBS, pH 7.5 at a flow rate of 25 ml/h. Fractions under each peak were pooled and assigned a number from I to III.



**Figure 2.** The antigenic content of the Sephacryl S-300 fractions (F I, F II and F III) and the WCV as clarified by ELISA.



**Figure 3.** The humoral response of the experimental vaccines and the WCV assessed by measuring the mice antibodies to the three main *B. pertussis* antigens (PT, FHA, and PRN).

**Discussion**

Pertussis ACVs vaccines have proved to be safer and more reliable than WCVs in mass vaccination<sup>7,4</sup>. In the present study three partially defined experimental ACVs have been prepared from strain 134 *B. pertussis* culture supernatant and their efficiency as immunogens was assessed and evaluated in mice. Specific media components were found to increase the PT production<sup>13,14</sup>. Therefore, cyclodextrin was included in the fermentation medium in a successful attempt to increase the yield of PT by ~ 20 folds more than that in cyclodextrin-free medium which could serve to trim down the cost of the vaccine to a great extent. Glutaraldehyde was preferred to be used as a vaccine detoxifying agent rather than formaldehyde as it had a lesser adverse effects on potency than formaldehyde<sup>22</sup>. The column elutes were monitored by ELISA for quantification of the specific *B. pertussis* antigens and also for determination of anti-*B. pertussis* antigens in sera of mice immunized with the experimental vaccines. The three main immunogenic and protective antigens (PT, FHA, and PRN) of *B. pertussis* were detected in all column fractions indicating their great dissociation. Sephacryl FI was especially rich in intact PT where it retained ~ 80% of PT cytotoxicity as indicated by CHO clustering assay. Sephacryl FI vaccine, containing PT: FHA: PRN in a ratio of 49:20:31 respectively, induced the highest anti-*B. pertussis* titer in mice, twice higher than that of the WCV. This result highlights the efficacy

of the intact PT, rather than the dissociated PT entities, as a potent ACV candidate. This result comes in accordance with previous studies that showed that PT is an essential antigen in ACV and that PT mono-component vaccines are quite efficacious<sup>15</sup>. On the other hand, although Sephacryl FII (containing PT: FHA: PRN in a ratio of 40:30:30, respectively) reserved only 4% of the intact PT toxicity as indicated by CHO cytotoxicity assay, yet it induced high levels of anti-*B. pertussis* antibodies in mice, slightly lower than that induced by Sephacryl FI and as one and half folds higher than WCV. This observation is very important pointing to the importance of the total *B. pertussis* antigens and / or their dissociated entities where they act in a concert way that allow minimizing PT content and hence reducing vaccine toxicity with minor effect on immunogenicity. However, further studies have to be accomplished to equilibrate between efficacy and safety of the test vaccines. All the studied pertussis combinations "experimental vaccines" should be considered as partially identified vaccines, containing undefined *B. pertussis* antigens in different concentrations and in different dissociation state. In fact the presence of unknown or poorly defined antigens in the Sephacryl fractions could be a merit. The humoral response to all the three main antigens in the various fractions cannot be linearly correlated to antigen content; higher levels were obtained with lower doses of some antigen components, suggesting a synergetic role of the co-injected undefined *B. pertussis* antigens. The Sephacryl FI stimulated the highest humoral response "levels of antibodies" to PT and PRN antigens, whereas the Sephacryl FII stimulated the highest level of antibodies to FHA antigens. The synergism among all the antigenic contents of the vaccine added to the immunogenicity in mice and allowed minimizing the protein contents of the vaccine. Efficacy studies have not demonstrated a direct correlation between antibody responses measured *in vitro* by serological methods and protection against pertussis disease<sup>27</sup>. However, such antibody studies are useful to compare immune responses elicited by a single vaccine under different conditions and in different studies or to compare among different vaccines under the same conditions. ELISA technique, using purified pertussis factors such as PT or FHA, has extensively been used for epidemiological studies and proved to be reasonably sensitive and specific<sup>23-25</sup>. Nonetheless, *in vivo* efficacy studies are indispensable to measure clinical protection conferred by each pertussis vaccine especially in the last stages of manufacturing. *In vivo* assays still remain the only reliable measure for protection. Therefore more *in vivo* tests have to be established before complete acceptance of the tested vaccines for human use. The present study described a simple procedure for producing pertussis ACVs including the vaccine preparation, formulation, and the assessment of immunogenicity in animals. These laboratory data are of particular importance to serve as supporting documents in guiding production and selection of candidate vaccines for pertussis prevention. Furthermore, the study documented the advantage of the proposed methods in cutting down the cost of the vaccine that make it suitable for mass production especially in developing countries.

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