

Development of sandwich ELISA-based assays for screening *Bordetella pertussis* antigens expressed in the bacterial cell culture

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TECHNIQUE

ABSTRACT

Pertussis is a highly contagious respiratory disease caused by *Bordetella pertussis*, a Gram negative bacterium. Concerns about whole cell vaccine safety led to the development of acellular pertussis vaccines with a lower frequency of adverse reactions. But they require a quick and reliable method for antigen detection, its quantification, or both. For this aim, a sensitive capture (sandwich) enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies was established, to identify and quantification the acellular pertussis vaccine components: detoxified Pertussis toxin (Ptg), Pertactin 2 (Prn2) and filamentous hemagglutinin (FHA). The Ptg-capture ELISA uses Fetuin in the solid phase, and Mabs 2G3 and 6D4 against Ptg were evaluated as detection reagents, the best results attained with Mab 2G3. The linearity range of the ELISA was 0.78-50 ng/mL. The Prn capture ELISA was established by evaluating Mabs 9C6 and 6B3 indistinctly as capture or detection reagents. The best combination was Mab 9C6 for capture and 6B3 for detection, with a linearity range 0.15-10 ng/mL. For the evaluation of FHA, monoclonal antibodies 11E8, 437 and 5E12 were evaluated indistinctly as capture or detection reagents, the optimal combination using Mab 11E8 as capture and Mab 437 for detection, with a linearity range 3.25-200 ng/mL. Under optimized ELISA tests conditions, the limit of quantification were 0.78, 0.07 ng/mL and 3.25 ng/mL for the Ptg, Prn2 and FHA proteins, respectively. Given its performance, the ELISA tests developed could be efficaciously implemented for process control during the production of these three *B. pertussis* vaccine antigens.

Keywords: ELISA, *Bordetella pertussis*, pertussis toxin, Pertactin 2, filamentous hemagglutinin, vaccine antigen

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RESUMEN

Desarrollo de un ensayo ELISA sandwich para el pesquijaje de antígenos de *Bordetella pertussis* expresados en cultivos de células bacterianas. La pertusis o tosferina es una enfermedad respiratoria altamente contagiosa causada por la bacteria Gram negativa *Bordetella pertussis*. Cuestionamientos sobre la seguridad de las vacunas basadas en células completas han conducido al desarrollo de vacunas acelulares que generan reacciones adversas con menor frecuencia, pero que requieren de métodos rápidos y confiables para la detección de los antígenos vacunales, su cuantificación, o ambas. Con tal propósito, se establecieron ensayos ELISA de tipo sándwich con el uso de anticuerpos monoclonales (Mabs) para la detección de los componentes de una vacuna acelular: la toxina pertúsica detoxificada (Ptg), la pertactina 2 (Prn2) y la hemaglutinina filamentosa (FHA). El ELISA de captura contra Ptg emplea la fetuina en fase sólida, y se estableció el Mab 2G3 contra Ptg como reactivo de detección con mejores resultados. La linealidad del ensayo se mantuvo en el rango 0.78-50 ng/mL. Para el ELISA de captura de la Prn2 se obtuvo como mejor combinación la que empleó el Mab 9C6 para la captura y Mab 6B3 para la detección, con una linealidad en el rango 0.15-10 ng/mL. Para la FHA, la mejor combinación se obtuvo con el Mab 11E8 para la captura y el Mab 437 para la detección, con un rango de linealidad 3.25-200 ng/mL. Los límites de detección en las condiciones óptimas de los ensayos fueron: Ptg 0.78 ng/mL, Prn2 0.07 ng/mL y FHA 3.25 ng/mL. Estos ensayos ELISA se pueden implementar eficazmente para el control de procesos durante la producción de los tres antígenos vacunales de *B. pertussis*.

Palabras clave: ELISA, *Bordetella pertussis*, toxina pertúsica, Pertactina 2, hemaglutinina filamentosa, antígeno vacunal

Introduction

Pertussis or whooping cough is a highly contagious respiratory disease caused by *Bordetella pertussis*, a Gram negative bacillus first described by Bordet and Gengou in 1906 [1]. The disease still represents a major threat to public health. Estimates suggest that, in 2008, about 16 million cases of pertussis occurred worldwide and that about 195 000 children died from the disease, making pertussis the fourth largest cause

of vaccine-preventable death in children under five years of age [2]. The bacterium is transmitted directly from human to human, probably via aerosolized respiratory droplets. Severe pertussis can lead to apnea, pneumonia, pulmonary hypertension, hypoxic respiratory failure, and ultimately death [3].

Whole-cell pertussis vaccine (wP), which is composed of a suspension of formalin-inactivated

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B. pertussis cells, was used widely in clinical practice by the mid-1940s, resulting in a rapid reduction in both the incidence of pertussis and the number of deaths caused by the infection [4]. The vaccine is still in use in most developing countries, as in Cuba.

In the late 1990s, concerns about safety led to the development of more pure (i.e., acellular) pertussis vaccines (aP), with successful clinical trials showing lower frequencies of adverse reactions [5-8].

Among the antigens considered, pertussis toxin or Pt, filamentous hemagglutinin (FHA), pertactin (Prn or P69k) and fimbriae induce antibodies contributing to protection in an animal model, and the natural infection with *B. pertussis* in humans induces antibodies to each of these antigens [9]. New formulations of acellular *B. pertussis* vaccines containing protective antigens in different combinations have been proved to be safer, less reactogenic and more reliable to introduce in mass vaccination campaigns [10-13]. Also replacing the wP vaccine with aP vaccines has resulted in an increase in pertussis vaccination coverage in newborns and infants.

Nevertheless, the number of pertussis cases has increased and epidemics returned in the acellular vaccine era, especially in adolescents and adults, in spite of the effectiveness attained with pertussis vaccination [2, 14]. It is unknown whether the re-emergence of pertussis is due to the waning of vaccine-induced immunity and/or pathogen adaptation [15], or to fundamental differences in the nature of the immune responses induced by aP vaccines as compared with wP vaccines or with natural infection [16].

For these reasons, vaccination strategies have changed during the past years and booster doses have been implemented in national vaccination programs. The development of new vaccines is aimed to improve control by increasing population coverage, allowing more frequent boosters by the use of acellular pertussis-only vaccines, or making the boosters unnecessary by inducing a more protective or long-lasting immune response, for a better match to circulating strains, and/or allowing the immunization of neonates.

Considering the antigens used in acellular vaccines, Pt is the main toxin of *B. pertussis* and one of the most important virulence factors [17]. This ADP-ribosylating protein is a globular protein with a molecular weight of 117 kDa. Pt has several biological activities and it is composed of five subunits, S1 to S5, which assemble into two functionally distinct moieties [18]. The S1 subunit is responsible for the enzymatic activity, while subunits S2 to S5 are involved in binding to the target cell [19]. Previous experiments using monoclonal antibodies against Pt have demonstrated that the antibody specific for the S1 subunit neutralized the leukocyte-promoting and islet-activating activities of Pt and protected against *B. pertussis* infection [20-22]. Pt induces high levels of antibody after infection or vaccination with classical whole cell vaccine, and the toxin provides full protection against challenge in mouse models. For this reason, inactivated Pt is considered the major protective antigen in all the new acellular vaccines available today [23, 24].

Regarding Prn, this is an auto-transporter protein that undergoes auto-proteolytic processing [25]. The

protein is processed from a 93 kDa large precursor to a 69 kDa and 22 kDa proteins, located at the cell-surface and in the outer membrane, respectively [26]. The 69 kDa product is used in aP vaccines. Prn contains an Arg-Gly-Asp (RGD) motif involved in mediated attachment of *B. pertussis* to mammalian cells [27], is a polymorphic protein and 13 variants (P.69 Prn1 to P.69 Prn13) have been identified so far [28]. Variation is mainly limited to two regions, designated region 1 and two, which are comprised of Gly-Gly-X-X-Pro (r1 repeat) and Pro-Gln-Pro (r2 repeat) repeats, respectively, more variation found in region 1 near to the N-terminus and flanking the RGD sequence. Region 2 is located towards the C-terminus. It is reported that the r1 repeat induces type-specific antibodies which show little cross-reactivity between P.69 Prn1 and P.69 Prn2 [29].

In the case of FHA, this is a large and complex molecule [30, 31] that is synthesized as a 367-kDa precursor (FHAB), translocated to the periplasm and exported through the outer membrane [32-34]. N-terminal processing [33] and cleavage of the C-terminal third of FHAB yield the 220-kDa mature FHA molecule [32, 34]. FHA is a multifunctional adhesin that is both cell-associated and secreted into the external milieu. FHA improves vaccine efficacy when included in multicomponent aP vaccines and in animal models, FHA alone elicits protective immunity [35, 36].

The variety of aP vaccines formulations approved for human use, which is a distinctive feature with respect to all other bacterial vaccines, should not be expected to confer equal levels of efficacy and long-term protection. Formulations with two (Pt and FHA) to five (Pt, FHA, pertactin, and fimbriae 1 and 2) immunizing antigens have been licensed for human use [13, 37, 38].

Specifically in Cuba, efforts are being made to reduce the reactogenicity attained with the whole-cell pertussis vaccine available, for use in booster doses in children aged above five years and in pregnant women after the third month, included in the National Immunization Program [39]. A new vaccine under development is an acellular vaccine composed of antigens from a *B. pertussis* strain genetically modified to produce a detoxified pertussis toxin (P_{tg}) and to express the Prn2 protein as the most frequent variant in clinical isolates. Antigens P_{tg} and FHA are isolated from the supernatant of the bacterial culture medium and Prn2 is obtained from heat inactivated cells precipitate.

Hence, this work was aimed to establish sensitive and simple capture (sandwich) enzyme-linked immunosorbent assays (ELISA) for the screening of pertussis antigens and readily transferable to process development units during the bioproduction scaling process. The tests developed were effectively used to identify and quantify the P_{tg}, Prn2 and FHA antigens, expressed by *B. pertussis* bacterium as acellular pertussis vaccine components.

Materials and methods

B. pertussis culture

B. pertussis strain BpCNIC0311 is a derivative of Bp165 strain, genetically modified to produce the

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pertussis toxoid PTg (*ptxA2*, 9K/129G) under the regulation of the *ptxP1* promoter and the pertactin 2 protein (*prn2*). The *fhaB* gene encoding filamentous hemagglutinin (FHA) remained as a wild type [40]. Bacteria was grown for 18 h in the defined THJS medium as described by Thalen *et al.* [41] and modified by the addition of aminoacids and 2,6-di-o-dimethyl-beta-cyclodextrin (CDX). For monitoring the antigens expression by ELISA tests, three experimental batches were produced in an INFORS HT bioreactor. Cultures were maintained at 35 °C with agitation from 100 to 150 rpm, at pH 7.3 and 20 ± 5 % oxygen. Samples were taken at 0, 3, 6, 9, 12, 15 and 18 h of culture.

Proteins and antibodies

Antigens were purified as previously described by Quintana-Vazquez *et al.* [40]. Pt and FHA were isolated from NaCl-washed cells and clarified culture medium supernatant. Prn was obtained from heat-inactivated and precipitated bacterial cells by Tris/NaCl extraction. Pertussis antigens were purified by sequential filtration, salt-precipitation and/or extraction, ultrafiltration and ion exchange chromatography [40].

Mabs and peroxidase-conjugates against pertussis antigens were supplied by the Center for Genetic Engineering and Biotechnology of Sancti Spiritus (CIG-BSS). The specificities of the Mabs are the following: anti-Ptg: Mabs 6D4 and 2G3; anti-Prn2: Mabs 9C6 and 6B3; anti-FHA: Mabs 11E8, 5E12 and 437.

Mabs were biotinylated using EZ-Link® Sulfo-NHS-LC-Biotin (Cat. # 21335, Pierce Biotechnology, USA), according to manufacturer's specifications. Fetuin (Cat. # F3004) and streptavidin-peroxidase were purchased from Sigma-Aldrich, USA.

B. pertussis proteins reference standards

NIBSC reference proteins were used as standards for the development of the ELISA tests: Pt (JN1H 90/518), Prn (Prn1, NIBSC 90/520) and FHA (NIBSC 90/654). Ampoules were completely dissolved in 500 µL of distilled water (40 µg/mL protein final concentration). Aliquots were stored at -70 °C until use.

Protein quantification

Total protein quantification was determined with the BCA protein assay kit (Pierce/ThermoFisher, Rockford, USA), using bovine serum albumin (BSA) as calibrator. The regression coefficient (r^2) was above 0.99. The concentration of purified antigens was determined by the BCA and UV absorbance method [42] using the formula: Protein (mg/mL) = $1.55 A_{280} - 0.76 A_{260}$.

Sandwich ELISA procedure

ELISA tests were established using a general sandwich-type procedure. Costar 3590 plates were coated with 100 µL of specific Mab or fetuin (this last for Pt-capture ELISA) in coating buffer (0.1 M carbonate/bicarbonate buffer, pH 9.6). A modification of Fetuin-capture method first described by Wong and Skelton for Pt capture ELISA [43]. Plates were coated with 4 µg of Fetuin per well, and incubated for 1 h at 37 °C. After washing three times with PBS (2.68 mM KCl, 1.47 mM KH_2PO_4 , 136.89 mM NaCl, 8.1 mM

Na_2HPO_4) and 0.05 % Tween 20, plates were blocked with 200 µL per well of blocking solution (PBS, 1 % BSA w/v, and 0.1 % Tween 20) for 1 h at 37 °C. Blocking solution was discarded by tapping the plate and 100 µL of test sample were added per well at the desired dilution in Sample solution (PBS, 0.1 % BSA w/v and 0.1 % Tween 20), and further incubated for 1 h at 37 °C. Calibration curves were prepared with purified proteins or NIBSC reference reagents in Sample solution at the suitable concentrations for each test. Baseline controls contained only Sample solution. After the washing step, the bound antigen was detected using 100 µL per well of a specific Mab, biotinylated or conjugated to peroxidase in Sample solution.

For ELISA setting up, tested Mabs used for detection were biotinylated and used at 10 µg/mL. Once chosen the best detection Mab for each antigen, they were conjugated and used as Mab-peroxidase conjugate. When Mab-biotin was used, after a washing step, a 1/10000 diluted streptavidin-peroxidase (streptavidin-HRP) was added followed by incubation for 30 min. After a final wash, 100 µL of Substrate solution (10 µg/mL of TMB- 3,3',5,5'-Tetramethylbenzidine, Cat. # Sigma-Aldrich, USA) was added per well in phosphate-citrate buffer (0.2 M phosphate, 0.1 M citrate, pH 5.0), followed by 0.006 % hydrogen peroxide and further incubated at room temperature for 10 min. The reaction was stopped by adding Stop solution (2 M H_2SO_4). Microtiter plates were read at a wavelength of 450 nm in a MultiScan microplate reader (Senselident Scan, Sweden).

Antigen was identified by considering the antigen present if absorbance was equal to or higher than 2 standard deviations (SD) of the average absorbance of baseline controls, regarded as the limit of detection (LD) of the test.

Antigen quantification was analyzed by using the GraphPad Prism 6.1 software. The mean for each duplicate or triplicate point of the calibration curve was computed and the obtained values fit to a straight line using linear regression. The best adjustment equation was determined and the regression coefficient (r^2) should be equal or above 0.98. The concentration of samples was calculated by interpolation of the absorbance obtained for each sample, and further multiplied by their dilution factor.

Indirect Ptg ELISA procedure

Costar 3591 plates were coated with 100 µL of 10 µg/mL of purified Ptg in coating buffer. The plates were incubated for 1 h at 37 °C. After washing three times with PBS and 0.05 % Tween 20, Mabs or Mab-conjugates were added at desired concentrations or dilutions, respectively, in Sample solution and were incubated for 1 h at 37 °C. After a washing step, in the case of non-conjugated Mabs, they were detected using 100 µL per well of a secondary antibody (1/10 000 dilution of mouse, Fc specific, goat peroxidase-conjugated (Cat. # A2554, Sigma-Aldrich, USA), for 30 min at 37 °C. In both cases, after the final washing step, 100 µL per well of Substrate solution (TMB and hydrogen peroxide) was added, followed by incubation at room temperature for 10 min. The reaction was stopped by adding Stop solution. Microtiter plates were read at 450 nm. All solutions

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were the similar to the previously described sandwich ELISA procedure.

SDS-PAGE

Electrophoresis was performed as described [44], on 15 % gels. Culture supernatant samples were previously concentrated 10- to 18-fold by Centricon YM-10 (Millipore, USA). Samples were boiled at 100 °C for 10 min in the Sample buffer containing 2 % of 2-mercaptoethanol. Gels were visualized by the zinc-imidazole reverse staining technique [45]. Protein expression levels were estimated by densitometric analysis of images from SDS-PAGE stained gels.

Results

Ptg-capture ELISA

Mabs 2G3 and 6D4 against Ptg were evaluated as detection reagents. In a previous western blot analysis carried out under denaturing conditions, Mab 2G3 showed reactivity against the S1 Pt subunit, whereas Mab 6D4 showed not reactivity at all (data not shown). In indirect ELISA (Ptg-coated plates), Mab 6D4 exhibited more sensitivity in ELISA (Figure 1 A and B) by using non-conjugated or peroxidase-conjugated Mab. On the other hand, when Mabs and Mab-conjugates were tested in the sandwich ELISA format (fetuin-coated plates), the outcome was completely different, with Mab 2G3 showing the best performance as detection reagent (Figure 1 C and D). Therefore, the 2G3-HRP conjugate was further used for Ptg-capture ELISA, and the titer was set up in the as 1:10 000 (results not shown).

LD of the Pt antigen using the modified Ptg-capture ELISA was determined with two-fold serial dilutions of Pt reference protein (NIBSC) at an initial concentration of 100 ng/mL (Figure 2). Under optimized ELISA conditions, the LD of the Ptg protein was 0.78 ng/mL (Figure 2A), and the linearity range of the Ptg-capture ELISA was obtained at the concentration range 0.78 to 50 ng/mL. The resulting linear equation was $Y = 0.02528 X + 0.1742$, with a coefficient of regression (r^2) above 0.99. The ELISA to identify and quantify the presence of Ptg in the supernatant of *B. pertussis* culture was performed using two-fold serial dilutions of Pt reference protein (NIBSC) as calibration curve. The minimum supernatant dilution (100×) having optical density (OD) value within the dynamic range was quantified as 0.055 µg/mL. The LD of the test was established as a 1/6400 dilution of the concentrated supernatant with a 0.17 OD_{450nm} value corresponding to 0.8 ng/mL (0.052 µg/mL × 100/6400) of the Ptg protein. Furthermore, the linear formula was similar to that of Ptg (Figure 2C).

Prn-capture ELISA

To establish the Prn capture ELISA, Mabs 9C6 and 6B3 were tested as capture or detection (biotin-conjugated) reagents. To determine the best antibodies combination, a two-fold diluted Prn1 (NIBSC reference protein) was used from 100 to 1.6 ng/mL, the best result achieved by combining Mab 9C6 as capture and 6B3 as detection antibodies (Figure 3). Mab 6B3 was conjugated to peroxidase and the optimal titer determined in ELISA as 1:10 000 (data not

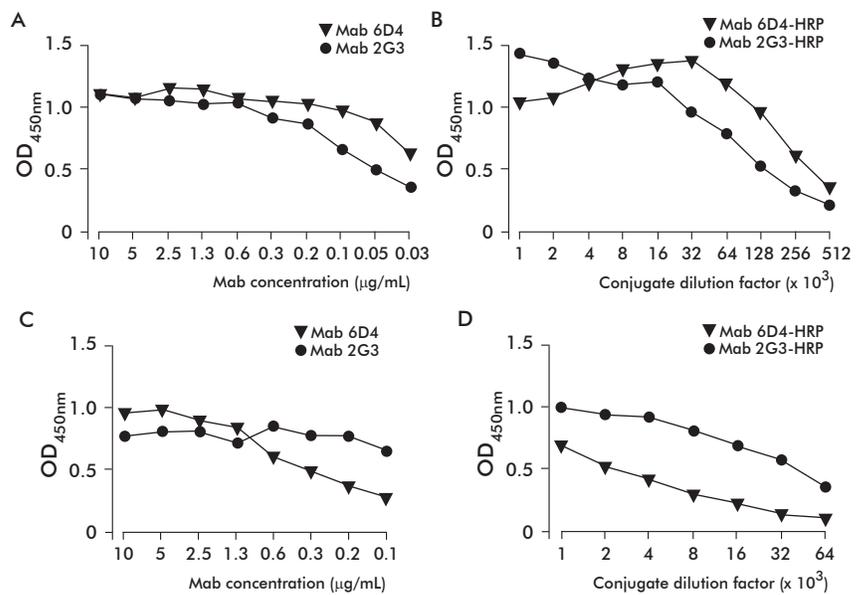


Figure 1. Evaluation of Mabs 2G3 and 6D4 against Pertussis toxin in ELISA. A and B) Indirect ELISA; plates were coated with 10 µg/mL of detoxified Pertussis toxin (Ptg) purified protein. C and D) Sandwich ELISA format; plates were coated with 40 µg/mL of Fetuin and the Ptg protein was used at 30 ng/mL. The values represent the average of triplicate determinations.

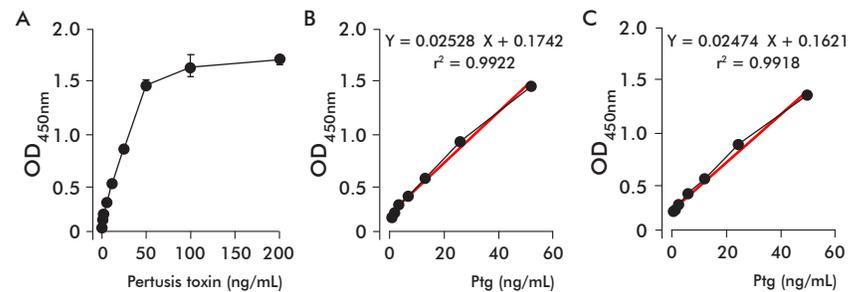


Figure 2. Sensitivity of detoxified *Bordetella pertussis* toxin (Ptg) capture ELISA assay. A) Different concentrations of the NIBSC reference Pertussis toxin (Pt) protein with a serial twofold dilution from 200 ng/mL to 0.78 ng/mL were tested. B) Linearity range of detection for Ptg was 50-0.78 ng/mL. C) Serially twofold-diluted *B. pertussis* supernatant was quantified with the aid of the Ptg-capture ELISA. The amount of Ptg in the minimum dilution (1:100) of bacterial cell culture supernatant with the OD value in the dynamic range was calculated using the linear equation of NIBSC Pt reference protein. There was also a good linear correlation from 1:100 to 1:6400 dilutions of *B. pertussis* supernatant. The error bar shows the means of triplicates. Linear regression is showed in black. Best adjustment curves are shown in red.

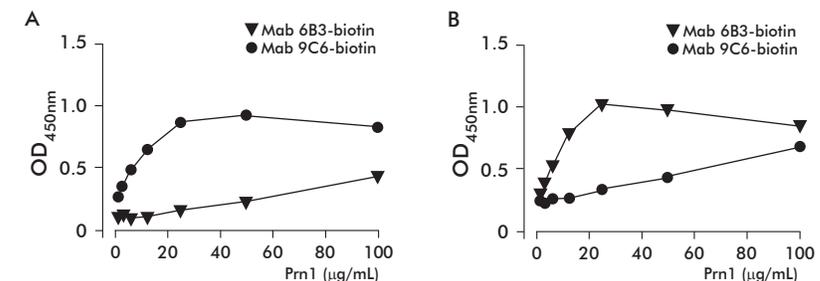


Figure 3. Evaluation of monoclonal antibodies (Mabs) against pertactin 2 in ELISA. A) Plates coated with Mab 9C6. B) Plates coated with Mab 6B3. Plates were Mab-coated at 10 µg/mL, in carbonate/bicarbonate buffer. Mabs were combined in a sandwich ELISA format, as a capture or biotin-conjugated detection reagents, respectively. Prn reference antigen (NIBSC) was twofold-diluted, from 100 ng/mL to 1.6 ng/mL in PBS, 0.1 % Tween 20 and 0.1 % BSA. Biotinylated Mabs were used at 5 µg/mL and streptavidin peroxidase at a 1:10 000 dilution. The values represent the average of triplicate determinations.

shown). When the recognition of Prn1 NIBSC reference protein was compared against purified Prn2 by the established Prn-capture ELISA (Figure 4), the test showed higher sensitivity for capturing Prn2 antigen, with the NIBSC antigen less recognized by the ELISA test. Then, to avoid inaccurate results, the purified Prn2 antigen was further used to prepare the calibration curve.

Under optimized Prn-capture ELISA conditions, the minimum detection of Prn2 protein was 0.07 ng/mL and the linearity range of the ELISA was obtained at concentrations 0.15 to 10 ng/mL. The resulting linear equation was $Y = 0.1583 X + 0.08701$, with a linear regression coefficient (r^2) above 0.99 (Figure 5A). To identify and quantify the presence of Prn2 in the *B. pertussis* culture, the material extracted from the heat-inactivated bacterial cell precipitate was diluted from 1:200 to 1:102 400 (Figure 5B). The lowest dilution of the extracted material (3200 \times) having an OD value within the dynamic range was established at 7.5 ng/mL. The LD was a 1/102 400 dilution of the concentrated extraction material with a 0.121 OD_{450nm} value corresponding to 0.23 ng/mL (7.5 ng/mL \times 3200/102 400) of the Prn2 protein. Although the majority of Prn protein is bacterial cell surface-associated, the presence of free Prn2 was also monitored in the culture supernatant during bacterial growth (Figure 5C). Samples obtained at different time points during bacterial fermentation were evaluated by Prn-capture ELISA. At the end of the fermentation process (18 h), the free Prn2 concentration in the supernatant was quantified as 150 ng/mL. These results indicate that Prn-capture ELISA is sensitive enough for Prn2 detection in *B. pertussis* cultures, to monitor the expression of this protein in the culture supernatant as well as in the bacterial cell precipitate-extracted material.

FHA-capture ELISA

Monoclonal antibodies 11E8, 437 and 5E12 against FHA were evaluated as capture or detection reagents (peroxidase-conjugated) to establish an FHA-capture ELISA (Figure 6). The best antibodies combination was determined by using a twofold serial dilution of purified FHA in the range 200 to 3.125 ng/mL. Since FHA protein (NIBSC reference) was not available at the time of setting up the ELISA, purified FHA protein was used instead. The best ELISA results were

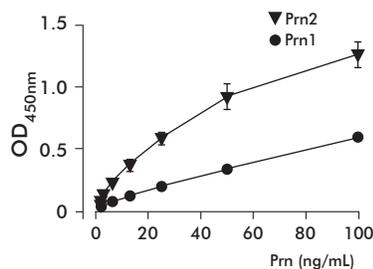


Figure 4. Comparison of the recognition of pertactins (Prn) 1 and 2 antigens in a Prn-capture ELISA. Plates were coated with 10 μ g/mL of Mab 9C6 in carbonate/bicarbonate buffer. Prn antigens were twofold-diluted, from 10 ng/mL to 0.2 ng/mL, in 0.1% PBS 0.1 % Tween 20 and BSA. Mab 6B3 conjugated to peroxidase was used at 1:10,000 dilution. The error bar shows the average of triplicate determinations.

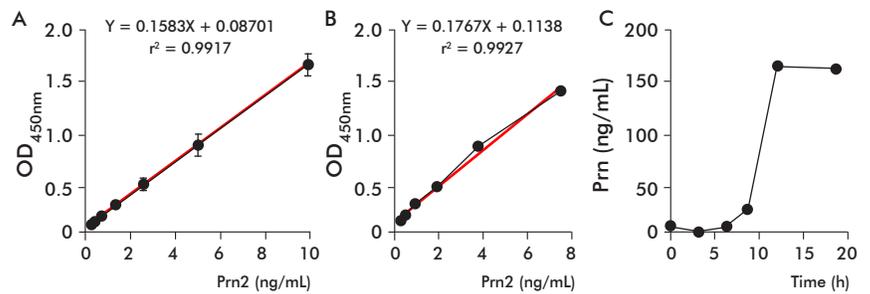


Figure 5. Capture ELISA against the Pertactin 2 (Prn 2) antigen of *Bordetella pertussis*, using the Mab 6B3 conjugated to peroxidase was used at 1:10,000 dilution. Plates were coated with Mab 9C6 in carbonate/bicarbonate buffer. A) Capture ELISA against the purified Prn2 antigen, twofold-diluted from 10 ng/mL to 0.2 ng/mL, in PBS, 0.1 % Tween 20 and 0.1 % BSA. B) Capture ELISA against Prn2 in *B. pertussis* extracted material from cultured bacterial cells. The amount of Prn in the minimum bacterial cell precipitate extraction dilution (1:3200) with OD value in the dynamic range was calculated, by using the linear equation of Prn2 NIBSC reference protein. A good linear correlation is shown in the dilution range 1:3200 to 1: 102 400 of the extracted material. C) Kinetics of free Prn2 protein in *B. pertussis* culture during fermentation process. Error bars represent the means of triplicates. Linear regression is shown in red.

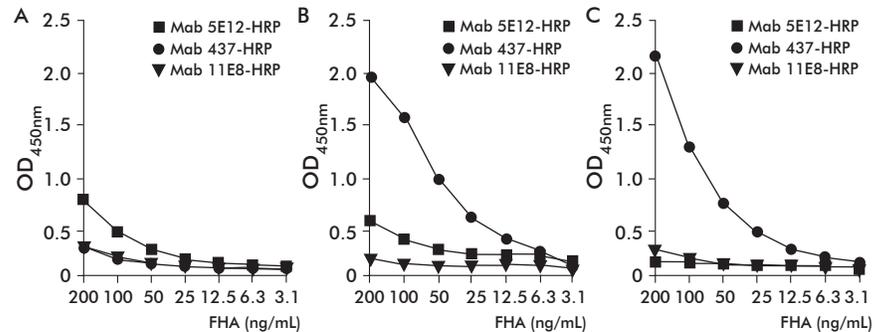


Figure 6. Evaluation different Mabs for the development of a sandwich ELISA assay against filamentous hemagglutinin (FHA) of *Bordetella pertussis*. Coating with: A) Mab 437; B) Mab 5E12; C) Mab 11E8. Plates were coated with 10 μ g/mL of each Mab in carbonate/bicarbonate buffer. Purified FHA antigen was twofold-diluted, from 200 ng/mL to 3.125 ng/mL, in PBS plus 0.1 % Tween 20 and 0.1 % BSA. Mabs conjugated to horse-radish peroxidase (HRP) were used at a 1:10 000. The values represent the average of triplicate determinations.

obtained with the combination of Mab 11E8 as capture and Mab 437 peroxidase-conjugated as detection reagents (Figure 6B), with an optimal conjugate titer of 1:8000 (data not shown).

When comparing the recognition of NIBSC reference protein (FHA) and purified FHA by the established FHA-capture ELISA (Figure 7), the NIBSC antigen was poorly recognized with an average absorbance ratio (OD_{FHA}/OD_{FHA} NIBSC) of 5.1. Therefore, and to avoid inaccurate results, the purified FHA antigen was further used to prepare the calibration curve.

Under optimized FHA-capture ELISA conditions, the minimum detection of FHA protein was 3.25 ng/mL and the linearity range of the ELISA was obtained in the range 3.25 to 200 ng/mL. The resulting linear equation was $Y = 0.006726 X + 0.05578$, with a coefficient of regression (r^2) above 0.99 (Figure 8A). To identify and quantify the presence of FHA in the *B. pertussis* culture, the supernatant was evaluated from non-diluted and a serial twofold dilution up to 1:128. The non-diluted supernatant showed OD values within the dynamic range of the assay and was quantified as 121 ng/mL. The LD was a 1/128 dilution of culture supernatant, with a 0.06 OD_{450nm} value corresponding to 0.94 ng/mL (121 ng/mL/128) of the FHA protein.

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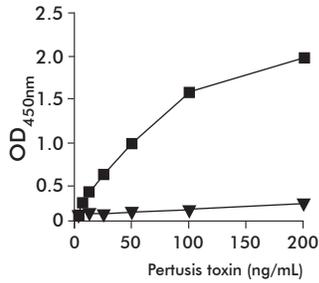


Figure 7. Comparison of filamentous hemagglutinin (FHA) of *Bordetella pertussis* antigens recognition in FHA-capture ELISA. Plates were coated with 10 $\mu\text{g/mL}$ of Mab 11E8 in carbonate/bicarbonate buffer. FHA antigens were twofold-diluted, from 200 ng/mL to 1.5 ng/mL, in PBS, 0.1 % Tween 20 and 0.1 % BSA. Mab 437-HRP was used at 1:10 000 dilution. The absorbance relationship average ($\text{OD}_{\text{FHA}}/\text{OD}_{\text{FHA NIBSC}}$) of 5.1 was obtained. The values represent the average of triplicate determinations.

Monitoring of *B. pertussis* antigens expression by ELISA

To assess the practical functionality of developed ELISA tests, three experimental cultures of *B. pertussis* were performed and samples were taken at three-hours intervals (0, 3, 6, 9, 12, 15 and 18 h) for monitoring the expression of Ptg, Prn2 and FHA bacterial antigens. The results (average) obtained in the three processes is shown in Figure 9. After 18 h, Ptg was detected by ELISA at 6.7 mg/L (Figure 9A). On the other hand, FHA exhibited values of 0.07 mg/L, not corresponding with the amount of protein estimated by SDS-PAGE (Figure 10, lane 1). This result suggested the underestimation of FHA by the ELISA test. The presence of aggregates was suspected and confirmed later on when a denaturing agent such as urea (2 M, final concentration) was added to the culture supernatant, the sample being further evaluated by FHA-capture ELISA. The urea-treated supernatant exhibited FHA values of approximately 8 mg/L in *B. pertussis* culture supernatants as determined by ELISA.

In the case of the Prn-capture ELISA, it was capable of detecting detached Prn2 in culture supernatants at a 0.15 mg/L concentration. Additionally, Prn was also quantified in extracted material since most of Prn2 was present in cells precipitate fraction (Figure 10, lane 2), the total amount of Prn2 in *B. pertussis* culture being estimated as 3 mg/L by ELISA.

Discussion

The possibility of monitoring the expression of *B. pertussis* antigens during bacterial growths or to measure their quantity through purification steps, allows improving and making necessary modifications to the process to obtain vaccine antigens and to achieve more accurate recovery values. So, it is important to count with simple, specific and sensitive ELISA methods, capable of detecting and quantifying small amounts of proteins present in bacterial cell cultures and their results, which strengthened by methods like SDS-PAGE and total protein quantification, altogether, are powerful analytical tools required for any vaccine production process.

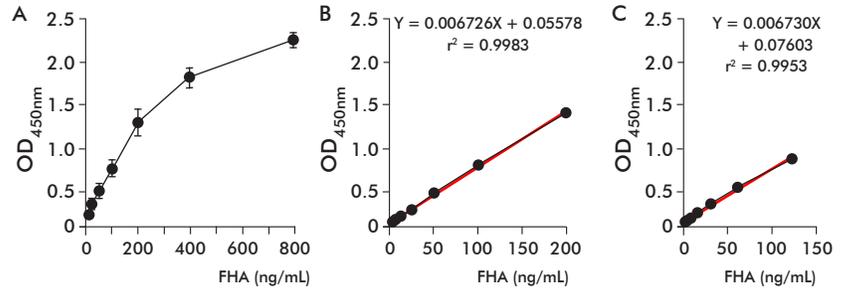


Figure 8. Capture ELISA against the Pertactin 2 (Prn 2) antigen of *Bordetella pertussis*, using the Mab 6B3 conjugated to peroxidase was used at 1:10,000 dilution. Plates were coated with Mab 9C6 in carbonate/bicarbonate buffer. A) Capture ELISA against the purified Prn2 antigen, twofold-diluted from 10 ng/mL to 0.2 ng/mL, in PBS, 0.1 % Tween 20 and 0.1 % BSA. B) Capture ELISA against Prn2 in *B. pertussis* extracted material from cultured bacterial cells. The amount of Prn in the minimum bacterial cell precipitate extraction dilution (1:3200) with OD value in the dynamic range was calculated, by using the linear equation of Prn2 NIBSC reference protein. A good linear correlation is shown in the dilution range 1:3200 to 1:102 400 of the extracted material. C) Kinetics of free Prn2 protein in *B. pertussis* culture during fermentation process. Error bars represent the means of triplicates. Linear regression is shown in red.

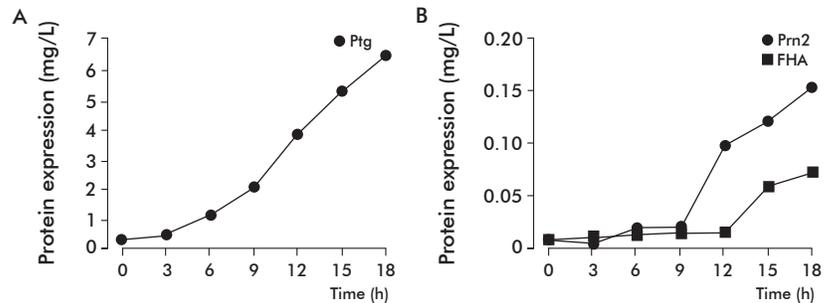


Figure 9. Quantification of the *Bordetella pertussis* antigens detoxified pertussis toxin (Ptg), filamentous hemagglutinin (FHA) and Pertactin 2 (Prn2), in experimental fermentation processes' culture supernatants by the established ELISA tests. Graphs show the average of values obtained in the three processes. A) Ptg quantification after 18 hours, showing a value of 6.7 mg/L in ELISA. B) Determination of detached Prn2 and FHA by capture ELISA of detached Prn2 and FHA in culture supernatants (0.15 mg/L and 0.07 mg/L, respectively). Values represent the average of triplicate determinations.

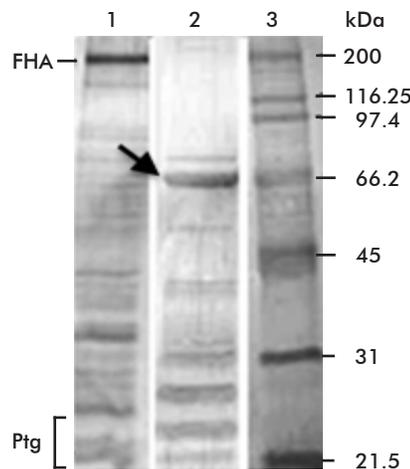


Figure 10. SDS-PAGE analysis of *B. pertussis* culture for the production of vaccine antigens for an acellular vaccine, in 12.5 % gels. Lanes: 1, supernatant; 2, extracted material from cell precipitate on 12.5 % gel; 3, 6.5–200 kDa molecular weight marker (Bio-Rad Laboratories). Culture supernatant and extracted materials pooled from fermentation processes were 10- and 18-fold concentrated and boiled at 100 °C for 10 min. The gel was revealed by the zinc-imidazole reverse staining method. The arrow indicates the Prn2 antigen protein band.

A previously reported Fetuin-capture ELISA test was modified [43] and a sandwich ELISA test developed, to identify and quantify Prn and FHA proteins from *B. pertussis*. In fact, ELISA-based methods have been reported for rapid screening of different antigens produced by *B. pertussis*, such as PT and FHA [46] or Prn and Fim [46-49]. Those methods are reliable, fast and cheap, but their results (expressed as OD) are not reproducible from culture to culture, and no antigen quantification is really made.

Ptg-capture ELISA showed good sensitivity and results, indicating that Ptg quantification in *B. pertussis* supernatants was consistent to Pt reference protein (NIBSC) quantification. FHA and Prn2 capture ELISA tests showed also good sensitivity, but results did not correlate with NIBSC reference antigens, most probably due to Mabs affinity, which is higher for BpCNIC0311 strain expressed antigens. Besides, the Prn expressed antigen is a pertactin type 2 variant whereas the Prn reference protein (NIBSC) is a pertactin 1. Moreover, Pt is a secreted protein, whereas Prn and FHA are surface-associated proteins [46].

Our results showed that the presence of Ptg and Prn2 in a chemically defined medium did not affect their quantification by ELISA. Moreover, the free Prn2 was quantified in the supernatant and also successfully extracted from precipitated cells without affecting its recognition by ELISA.

Nevertheless, FHA expression in the supernatant was underestimated by ELISA. In spite of putting CDX in the culture medium, FHA is a highly hydrophobic protein. Therefore, it is unstable in low salt solution and forms aggregates easily; therefore, hindering of Mabs specific epitopes is probably due to FHA-FHA or FHA-bacterial surface interactions [50] or to the conformation adopted by the expressed protein when bacteria is cultivated *in vitro* with chemically defined medium, or both. All these could cause blocking of the epitopes recognized by the Mab(s) used in the test. The possible presence of aggregates was corroborated when 2 M urea was added to the culture supernatant, and the sample was further evaluated by FHA-capture ELISA. At this urea molarity, the protein is not denatured and its stability and recovery in the purification steps is improved [14, 51]. So, accurate quantification of FHA in the supernatant by the established ELISA was only possible when the protein was disaggregated. Therefore, the ELISA should be used only for FHA identification in culture supernatants unless supernatants will be pre-treated with urea.

Conclusions

Easy, accurate and sensitive sandwich ELISA tests were developed, capable of identifying, quantifying or both the Pt, Prn and FHA antigens from *B. pertussis* in bacterial cultures. Their use in other steps throughout the vaccine antigen production process is under study.

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