Validation of a sandwich ELISA for the quantification of the main toxin (Pt) of Bordetella pertussis

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TECHNIQUE

ABSTRACT

Bordetella pertussis bacterium causes a highly contagious respiratory disease named Pertussis or whooping cough, which represents a major threat to public health. Pt is the main toxin of *B. pertussis* and one of the most important virulence factors. The inactivated toxin is considered the major protective antigen in all the new acellular vaccines available today. In this work, a previously established sandwich capture ELISA test for a genetically detoxified Pertussis toxin (Ptg) was validated, with the aim of monitoring the expression of Ptg in bacterial cell culture, and to quantify the antigen throughout the purification process. The validation of this method is a requirement of the pharmaceutical industry; therefore, extensive experiments were performed in order to employ the assay as an analytical tool. The intra- and inter-assay precision was tested and proved a robust starting platform for the assay. The repeatability for the assay was determined using a calibration curve and spiked samples. The assay had a coefficient of variation (CV) of less than 10 %. The inter-assay precision (CV) was lower than 20 % for the calibration curve and lower than 10 % for control samples. Dilution linearity and parallelism were demonstrated. Accuracy (spike recovery) for all concentrations was shown in the range 80-120 %. This assay is highly accurate and reproducible in determining the levels of Ptg in spiked samples, fulfilling the most stringent acceptance criteria and being adequate for the intended analytical purpose. It is currently being applied as an analytical tool in production process development of pertussis antigens for vaccine candidates.

Keywords: ELISA, validation, Pertussis, Ptg

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RESUMEN

Validación de un ensayo de ELISA sandwich para la cuantificación de la toxina principal (Pt) de *Bordetella pertussis***.** La bacteria *Bordetella pertussis* causa una enfermedad respiratoria altamente contagiosa llamada Pertusis o tos ferina, la cual es una gran amenaza para la salud pública. Su principal toxina, la Pt, es uno de los factores virulentos más relevantes de esta bacteria, e inactivada se considera como uno de los antígenos que confiere mayor protección en las nuevas vacunas acelulares disponibles. En este trabajo se validó un ensayo previamente establecido de tipo ELISA sandwich de captura para la cuantificación de una variante de Pt genéticamente detoxificada (Ptg), pues su disponibilidad como herramienta analítica depende de su validación para satisfacer los requerimientos de la industria farmacéutica. La precisión intra- e inter-ensayo fue evaluada y se demostró la robustez de la prueba. La repetibilidad del ensayo se determinó mediante una curva de calibración y las muestras dopadas, con un coeficiente de variación (CV) menor del 10 %. La precisión inter-ensayo (CV) fue menor del 20 % para la curva de calibración y menor del 10 % para las muestras controles, y se demostró la linealidad y el paralelismo del ensayo. La exactitud se mantuvo en un intervalo entre el 80 y el 120 % para todas las concentraciones evaluadas. Este ensayo es altamente exacto y reproducible para la detección de los niveles de Ptg en muestras dopadas, y reúne los criterios de aceptación más estrictos. Se emplea actualmente como una herramienta analítica en el desarrollo del proceso productivo de los antígenos de pertussis para la obtención de candidatos vacunales.

Palabras clave: ELISA, validación, Pertussis, Ptg

*I*ntroduction

Bordetella pertussis, a Gram negative bacillus, was first described by Bordet and Gengou in 1906 [1]. The bacterium causes a highly contagious respiratory disease named pertussis or whooping cough, which actually represents a major threat to public health. 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 less than five years old [2]. The bacterium is transmitted directly from human to human, probably via aerosolized respiratory droplets. Severe pertussis can cause apnea, pneumonia, pulmonary hypertension, hypoxemic respiratory failure, and lead ultimately to death [3].

In the late 1990s, concerns about whole-cell pertussis vaccine (wP) safety led to the development of more purified (acellular) pertussis vaccines (aP), with successful clinical trials and associated with a lower frequency of adverse reactions [4-7]. New formulations of acellular *B. pertusis* vaccines containing protective antigens in different combinations have been proved to be safer, less reactogenic and more reliable to introduce in mass vaccination [8-11]. Moreover, replacing the wP vaccine with the aP vaccines increased the pertussis vaccination coverage among newborns and infants. Although vaccination is the most effective preventive control measure, both natural and artificial immunity wane over time, and

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thus the protection offered by current vaccines is not long-lasting. For that reason, pertussis still remains a challenging public health problem and resurgence has occurred in some countries, the highest frequency in developed countries, with well-established vaccination programs, particularly among adolescents and young adults [12].

The pertussis toxin (Pt) is the main toxin of *B. pertussis* and one of the most important virulence factors [13]. The protein is globular, with a molecular weight of 117 kDa. Pt has several biological activities and is composed of five subunits, S1 to S5, which assemble into two functionally distinct moieties [13]. The S1 subunit is responsible for the enzymatic activity, while subunits S2 to S5 are involved in binding to the target cell [14]. Pt induces high antibody levels after infection or vaccination with classical whole cell vaccines, and the toxin provides full protection against challenge in mouse models. For these reasons, inactivated Pt is considered the major protective antigen in all the new acellular vaccines available today [15, 16].

Recently, we started the development of a process to obtain an acellular pertussis vaccine [17]. The new vaccine components are obtained from a *B. pertussis* strain genetically modified to produce a genetically detoxified pertussis toxin (Ptg). It was also engineered to express the Pertactin 2 protein (Prn2), a variant most frequently found in clinical isolates [18].

This work was aimed to the validation of a pre-established Ptg-capture ELISA [17], which is intended to be used to monitor the expression of Ptg during bacterial cell growth and for antigen quantification throughout the isolation process. This assay is a modification of a previously reported Fetuin-capture ELISA tests [19], developed to determine antibodies against Pt. The validation of this method is a requirement of the pharmaceutical industry to guarantee the results reliability [20, 21]. The sandwich ELISA was validated for the control process of the production of Ptg, allowing the assessment of antigen recovery based on concentration values, with precision and accuracy complying with current international regulations.

Materials and methods

Bacterial culture

B.pertussis strain BpCNIC0311 [22] was grown for 18 h in the defined THIJS medium described by Thalen *et al*. [23], modified by the addition of amino acids and 2,6-di-o-dimethyl-beta-cyclodextrin. The cultures were maintained at pH 7.3, 35 °C, under 20 ± 5 % oxygen atmosphere and agitation at 100-150 rpm.

Biological reagents

The Reference Material (RM) was composed of *B. pertussis* culture supernatant at 6 µg/mL concentration (code PER-01-1111), prepared and supplied by the Stability and Reference Materials Group, Quality Control Department, Center for Genetic Engineering and Biotechnology (CIGB, Havana, Cuba). The Ptg concentration in the culture supernatant was previously verified against a NIBSC Pt reference protein (JNIH 90/518) [17].

The purified Ptg protein was obtained using an experimental procedure based on the protocol reported by Özcengiz *et al*. [13]. The protein was isolated from NaCl-washed *B. pertussis* cells and clarified culture supernatant. The Ptg was further purified by sequential filtration and ion exchange chromatography [22]. Samples of the active pharmaceutical ingredient (API) were used to prepare Quality Control samples (QCs) used for test validation.

The horseradish peroxidase-MAb conjugate against Ptg (2G3-peroxidase) was produced and supplied by the Center for Genetic Engineering and Biotechnology of Sancti Spíritus (CIGBSS). Fetuin (Cat. # F3004) was purchased from Sigma-Aldrich, USA.

Sodium dodecyl sulphate-polyacrylamide electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis was performed as described by Laemmli [24] on a 15 % gel. Culture supernatant samples were previously concentrated 10 to 18-fold by using a Centricon YM-10 (Millipore, USA). Samples were boiled at 100 °C for 10 min in the sample buffer containing 2 % of 2-mercaptoethanol. The gel was visualized by zinc-imidazole reverse staining technique [25]. Protein expression levels were estimated by densitometric analysis of SDS-PAGE. SDS-PAGE Molecular Weight Standards Broad Range (Cat # 161-0317, Biorad, USA) was used for molecular weight estimation.

Protein quantification

Total protein quantification was determined with the BCA protein assay kit (Pierce/ThermoFisher, Rockford, USA), using bovine serum albumin (BSA) as the calibrator. The linear regression coefficient (r^2) was above 0.99. The amounts of Ptg in bacterial cell culture supernatants were estimated combining SDS-PAGE densitometric analysis and total protein quantification by the BCA colorimetric method and UV absorbance at 260 and 260 nm [26], by the formula: Protein (mg/mL) = $1.55\ A_{280} - 0.76\ A_{260}$.

Established Ptg-capture ELISA procedure

Ptg-capture ELISA is a modification of Fetuin-capture method first described by Wong and Skelton in 1988 [19]. Costar 3590 plates were coated with 4 µg of Fetuin per well in coating buffer (0.1 M carbonate/bicarbonate buffer; pH 9.6). The plates were incubated at 37 °C for 1 h. 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, the coated plates were blocked with 200 µL per well of blocking solution (PBS, 1 % BSA w/v, 0.1 % Tween 20) for 1 h at 37 °C. A calibration curve was prepared from a reference material (RM) at a certified concentration, at concentrations of 60, 30, 15, 7.5, 3.75 and 1.875 ng/mL in sample buffer (SB; PBS, 0.1 % w/v BSA, 0.1 % Tween 20), to cover established working range, with duplicates of each concentration. Blocking solution was discarded by tapping the plate and 100 µL per well of the calibration curve, controls and test samples at desired dilutions in SB, were added and further incubated for 1 h at 37 °C. Blanks contained only SB. After a washing step, the bound antigen was detected by adding 100 µL per well of 2G3-peroxidase conjugate diluted in SB. After a final wash, 100 µL per well of substrate buffer (10 µg/mL of

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3,3',5,5'-Tetramethylbenzidine, Cat. # Sigma-Aldrich, USA) in phosphate-citrate buffer (0.2 M phosphate, 0.1 M citrate, pH 5.0) and 0.006 % hydrogen peroxide was added followed by incubation 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.

Analytical results were analyzed as follows. For antigen identification, it was considered present if its absorbance was equal or higher than 2 standard deviations (SD) of the average absorbance of the blanks, considered as the limit of detection (LD) of the test. Calculations for antigen quantification were done using the Microsoft Excel software. The mean for each duplicate point of the standard curve was computed and the obtained values were fit to a straight line by means of linear regression (r^2). The concentration of the samples was calculated by interpolation of the absorbance obtained for each sample, multiplied by its dilution factor.

The test was considered valid when the following conditions were sufficed: i) Background absorbance less than 0.1; ii) Regression coefficient (r^2) equal or above 0.98; iii) Intra-assay variation coefficient less than 10 %; and iv) Positive control value in the estimated range.

Validation method

The ELISA was validated according to the National Regulatory Agency, the Center for State Control of Drugs, Equipment and Medical Devices (Cecmed), Regulation Nr. 41-2007 [20] and ICH Q2/R1 [21] and evaluated parameters are included in "Methods of content or potency".

Linearity and working range

The RM concentration was evaluated in a wide range (0.78 to 200 ng/mL). Statistical analysis was made using a Microsoft Excel sheet and Parallelism calculations by ParLin v4.2 software (Quality Control Department, CIGB). The linearity was obtained using the Minimum Square Method employing the model equation: $y = mx + b$, in which the relationship among the response (absorbance at 450 nm) and sample concentration was evaluated. The best adjustment equation was determined and r^2 should be equal or above 0.98 for a good fit of the linear regression. The F-observed and parallelism values provided by the ParLin v4.2 software should be above 0.05. The slope and intercept of each calibration curve were also analyzed.

To establish the working range, a calibration curves were performed with the RM in the following concentrations: 60, 30, 15, 7.5, 3.75, 1.875 and 0.9375 ng/mL, each point with duplicates and 5 independent assays were performed, as minimum. The working range was established between the upper and lower limits established for the precision (coefficient of variation, CV, lower than 20 %) and accuracy (recovery in the range 80-120 %) parameters.

Specificity

The specificity of the assay was determined by the analysis of the negative control of the test (SB) and the following solutions used during the production process [22] such as: elution buffer or EB (20 mM sodium phosphate, 20 mM NaCL, 2 M Urea; pH 7.5) and API conservation buffer (IB; 20 mM sodium phosphate, 300 mM NaCl; pH 8). The experiment was performed using 6 duplicates of the working dilution (1/100) of the SB reconstituted in the solution evaluated. For both solutions the upper limit of the confidence intervals (CI) was analyzed and the acceptance criteria was set that the calculated upper limit absorbance value of each buffer were below the detection limit of the test absorbance. Absorbance results were statistically analyzed by a paired Student's t-Test, the differences considered as significant for $p < 0.05$.

Accuracy

The accuracy of the test was assessed by the recovery (spiking) and parallelism methods. The first method consisted in the addition of knows concentrations of Ptg representing high, medium and low ranges (40, 20 and 8 ng/mL respectively), previously determined by UV absorbance, to the matrixes employed through protein isolation process [22]. The recovery percentages of triplicated spiked samples were evaluated for each Ptg concentration for values in the range 80-120 %. The Student's t Test (n-1 degrees of liberty) with a confidence level of 95 % and the CIs were determined for each sample. Parallelism was performed by preparing calibration curves with the RM in EB and IB buffers (concentrations from 60 to 0.9375 ng/mL) and compared to the calibration curve prepared in SB. Samples were prepared in triplicates. Parallelism values were provided by the ParLin v.42 software and significance values for linearity and parallelism should be above 0.05. The slope and intercept of each calibration curve were also analyzed.

Intra- and inter-assay precision

The precision of the Ptg calibration curve and different QCs employed as positive controls of the test were evaluated by repeatability (intra-assay precision) or intermediate precision (inter-assay and inter-analyst precision).

The intra-assay precision of the calibration curve was evaluated using the standard curves (n = 3) in the same assay prepared by the independent-way. Inter-assay and inter-analyst precision (3 runs each) were performed in different days, with one or two different analysts respectively.

The repeatability of the samples (n = 7) was assessed using QCs (high, medium and low range), in the same plate by the same analyst. The inter-assay precision of the samples was studied by one (5 runs) or two analysts (3 runs each) in different days.

In all cases, the average (arithmetic mean), SD and CV were determined. The coefficient of variation was considered acceptable for values below 10 % for repeatability, except at the lower limit of quantitation (LLOQ) where 20 % is acceptable and of 20 % for intermediate precision.

Applicability of Ptg-capture ELISA for monitoring Ptg expression and protein recovery during the experimental production process

To assess the practical functionality of validated Ptg-capture ELISA test, Ptg expression levels analysis

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from 7 experimental cultures of *B. pertussis*, grown under similar conditions, were performed. Samples from time points (0, 3, 6, 9, 12, 15 and 18 h) were taken to evaluate the antigen expression. The protein recoveries (%) from three experimental purification processes carried out up to the elution step with similar procedures. Samples analyzed were: washed cells (WC), clarified culture supernatant (CLS), concentrated culture supernatant (CS), concentrated supernatant containing urea (CSU), concentrated supernatant containing urea and pH adjusted to pH 6 (CSUpH), protein eluate (PE) and dialyzed protein eluate or API. EB and IB matrixes corresponded to PE and API steps, respectively. CSU and CSUpH steps matrixes [21] were not subjects of the validation study.

Results

Linearity

A wide range of the RM calibration curve (0. 78 to 200 ng/mL) was analyzed to determine the linearity of the test (Figure 1A). The calibration curve (Figure 1B) shows the average of seven calibration curves to determine the linear range of the test (0.937, 1.875, 3.75, 7.5, 15, 30 and 60 ng/mL). The resulting linear equation was $y = 0.01986\ x + 0.08506$, with a coefficient of regression (r^2) above 0.99. Linearity and parallelism analysis by the ParLin v4.2 software showed significance values of 0.47 and 0.663, respectively (Figure 1C), both parameters evidenced for significances above 0.05. The test showed lower limit of qualification (LLOQ) values of 3.75 ng/mL, with precision and accuracy (CV 12.9 % and recovery 94.7 %) and lower limit of detection (LLOD) was 0.937 ng/mL. Background values were below 0.08.

Specificity

The specificity of the assay was determined by the analysis of the negative control of the test (SB) and the solutions used during the production process. Since RM is composed of *B. pertussis* culture supernatant, there was no need to study the interference of the growth milieu. As shown in Table 1, the upper limits of the confidence intervals were analyzed and their values were below the limit of detection (LD) of the test's absorbance; hence, it can be concluded that the buffers studied did not interfere in the ELISA test.

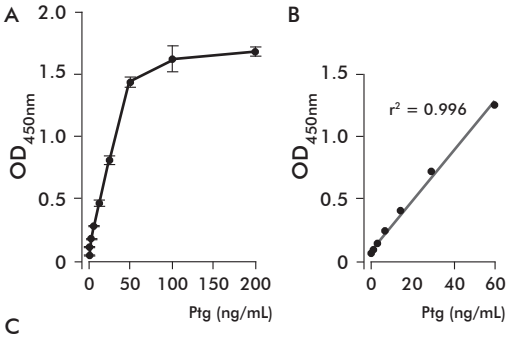
Accuracy

In the accuracy study, the recovery percentages obtained were in the range 80-120 % for the spiked samples (Table 2) and the calculated Students' t-test value was below the tabulated value.

Parallelism analysis (Table 3) showed linearity and parallelism significance values above 0.05 and quantification of the Ptg present in the RM calibration curves prepared with the IB and EB buffers showed an accuracy value above 93 % for both buffers.

Intra- and inter-assay precision

Precision data generated from the validation experiments are summarized in Table 4. Precision over the dynamic range was found to have acceptable tolerances, with intra-assay precision (CV) for calibration curves ranging 1.2-8.4 % (acceptance lower than 10 %).



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ANOVA

Source	d.f.	SS	MS	F	Signif.
Preparation	7	0.145	0.024	2.038	0.164
Regression	1	3.462	3.462	1143.72	0
Paralelism	7	0.004	0.001	0.194	0.663
Linearity	40	0.006	0	0.980	0.470
Between doses	55	3.611	3.611	104.863	0
Within doses	0	0	0		

Figure 1. Linearity of a sandwich ELISA for the quantification of a genetically-detoxified variant of the main toxin of *Bordetella pertussis* toxin (Ptg). A) Wide range of reference material (RM) at a serial two-fold dilution in the range 0.78-200 ng/mL. The error bar stands for the means of triplicates. B) Linear regression showing the average of seven calibration curves. The linear range for Ptg detection was 60 to 0.937 ng/mL. C) Analysis by the ParLin v4.2 software (CIGB), confirming parallelism and linearity for Ptg calibration curves (significance above 0.05). d.f.: degree of freedom; SS: sum of squares; MS: Mean squares; F: Fisher's F distribution; Signif.: statistical significance (probability value).

Table 1. Specificity of a sandwich ELISA for the quantification of a genetically-detoxified variant of the main toxin of *Bordetella pertussis* (Ptg)*

Assay	Sample	Absorbance 450 nm Mean	p	ULD
1	EB	0.067	0.024	0.164
	LLD	0.148		0
2	IB	0.060	0.024	0.164
	LLD	0.135		0

* The absorbance of samples EB and IB were below the absorbance obtained for the LLD of the test (0.9375 ng/mL)
EB: elution buffer; 20 mM sodium phosphate, 20 mM NaCL, 2 M Urea, pH 7.5.
IB: Active pharmaceutical ingredient conservation buffer; 20 mM sodium phosphate, 300 mM NaCl, pH 8.0.
LLD: lower limit of detection of the test.
ULD: upper limit of detection of the test.

Table 2. Accuracy (spiking) of a sandwich ELISA for the quantification of a genetically-detoxified variant of the main toxin of *Bordetella pertussis* (Ptg)*

Matrix	Calibration curve range	Recovery (%)	CI (%)	t-Test
EB	high	102.2	99.7-104.6	3.83
	medium	102.1	96.8-107.4	1.73
	low	94.4	81.6-107.3	–0.15
IB	high	102.7	93.0-112.4	1.21
	medium	107.2	99.7-114.8	4.12
	low	105.8	93.2-118.5	1.99

* Known concentrations of Ptg in the high, medium and low range of the curve (40, 20 and 8 ng/mL, respectively) were added to the sample buffer and the matrixes employed in the protein isolation process. In all studied cases, the calculated t-Test value was below the tabulated value: 4.3 (0.05; 2degrees of freedom).
EB: elution buffer; 20 mM sodium phosphate, 20 mM NaCL, 2 M Urea, pH 7.5.
IB: Active pharmaceutical ingredient conservation buffer; 20 mM sodium phosphate, 300 mM NaCl, pH 8.0.
LLD: lower limit of detection of the test.
ULD: upper limit of detection of the test.

Meanwhile, the intermediate precision evaluated by either one or two analyst (CV acceptance lower than 20 %), was 4.3-8.7 % and 7 to 12 %, respectively. On the other hand, repeatability for control samples value ranged 2.2-7.5 %, with intermediate precision evaluated by one or two analyst of 3.6-15.1 % and 6.8-9.7 % respectively.

Intermediate precision results showed that the analyst factor did not contribute to assay variability, as reported by Welfringer *et al*. [27], since CVs for this parameter were below 12 % for calibration curves and less than 10 % (repeatability acceptance criteria) for control samples. This confirmed the satisfactory performance of the test related to variability.

Monitoring Ptg expression and protein recovery with the Ptg capture ELISA throughout the production process

Preliminary results of using the ELISA test to evaluate the Ptg isolation process are shown in Figure 2, including the antigen expression kinetics of seven experimental cultures of *B. pertussis*, grown under similar conditions (Figure 2A). Pertussis antigen exhibited similar expression kinetics among batches, and the ELISA test was capable of detecting the Ptg expression from the starting fermentation time point. At the end of fermentation, Ptg reached 8.5 mg/L concentration in average. Similar results were observed when comparing the protein recovery percentages from three experimental purification processes (Figure 2B). All the ELISA values were accurate, with the exception of CSU and CSUpH steps, in which there was an underestimation of Ptg amounts by ELISA (approximately 90 % less of the expected value) as compared to previous CS step, what was corroborated by SDS-PAGE (Figure 3) and Ptg amount estimations (data not shown). PE and API values obtained by ELISA were accurate, as ascertained by protein quantification, and the average Ptg recovery as determined by ELISA was around 36 % at the end of the purification processes, with more than 90 % pure Ptg antigen (Figure 3).

Table 3. Parallelism analysis of a sandwich ELISA for the quantification of a genetically-detoxified variant of the main toxin of *Bordetella pertussis* (Ptg)*

Calibration curve	Matrix	Linearity	Parallelism	Ptg (µg/mL)		Accuracy (%)
				Expected	Observed	
1	EB	0.470	0.470	6	5.631	93.8
2	IB	0.425	0.425	6	5.559	93.2

* The RM calibration curves were prepared in the matrixes employed through protein isolation process and compared to the standard curve prepared in sample buffer. Significance values for linearity and parallelism should be above 0.05.
EB: elution buffer; 20 mM sodium phosphate, 20 mM NaCL, 2 M Urea, pH 7.5.
IB: Active pharmaceutical ingredient conservation buffer; 20 mM sodium phosphate, 300 mM NaCl, pH 8.0.

Table 4. Repeatability and intermediate precision of a sandwich ELISA for the quantification of a genetically-detoxified variant of the main toxin of *Bordetella pertussis* (Ptg)

Matrix	Repeatability		Intermediate precision		Inter-analyst precision	
	n	CV (%)	n	CV (%)	n	CV (%)
Calibration curves	3	1.2-8.4	3	4.3- 8.7	6 runs 2 analysts	7-12
QCs	7	2.2-7.5	5	3.6-15.1	6 runs 2 analysts	6.8-9.7

n: replicates for repeatability and runs for intermediate precision.
QCs- control samples.
CV: the range of the coefficient of variation obtained for each parameter.

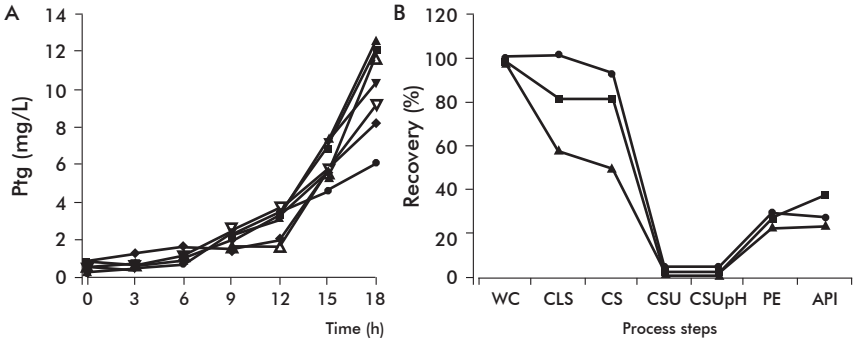

Figure 2. Monitoring of a genetically-detoxified variant of the main toxin of *Bordetella pertussis* (Ptg) during the fermentation and purification experimental processes. A) Kinetics of seven fermentation processes. B) Ptg recovery percentages obtained from 3 experimental purifications processes. WC: washed cells; CLS: clarified supernatant; CS: Concentrated supernatant; CSU: Concentrated supernatant containing urea; CSUpH: Concentrated supernatant containing urea and adjusted to pH 6.0; PE: protein eluate and active pharmaceutical ingredient (API)-dialyzed protein eluate. The values represent the average of triplicate determinations.

Discussion

Bio-analytical validation methods are essential for the performance and robustness of bioprocesses, being addressed in scientific meetings and publications [28-31]. Therefore, in our lab, a pre-established Ptg-capture ELISA test [17], which is a modification of previously reported Fetuin-capture ELISA test [19] was validated according to current regulations [20, 21].

In fact, the benchmark definition of a validation method has been presented by the International Organization for Standardization as 'the confirmation by examination and the provision of objective evidence that the particular requirements for a specific intended use are fulfilled' [21].

Validation, as Cummings refers [32], ..."validation should progress down two parallel tracks which eventually converge: one experimental, the other operational. The first is aimed to establish the purpose of the method and agree upon outcomes, target values or acceptance limits, whereas the second is to characterize the performance of the assay by experimentation"; hence, validation is a continuous process as long as the assay is in use.

Therefore, our purpose was to develop a specific, accurate and sensitive ELISA method, capable of detecting and quantifying the smallest amount of *B. pertussis* antigen present in bacterial culture and throughout the purification process of the antigen. The results obtained by the ELISA test, strengthened by methods like SDS-PAGE and total protein quantification, altogether, is a necessary and powerful analytical tool required for vaccine production.

Additionally, the selection of a standard or calibration model that accurately fits the concentration-response relationship for standard samples should be determined prior to generating results for independently prepared validation samples [28]. In our study, a calibration curve was prepared from serial dilutions of Ptg and its linearity ranged 0.937 to 60 ng/mL, with LLOQ and LLOD values of 3.75 and 0.937 ng/mL, respectively.

Regarding the specificity of the assay, non-interference in Ptg detection was observed using the studied buffers during the process (Table 1).

Regarding the precision and accuracy of the method, these are performance characteristics describing the magnitude of random errors (variation) and

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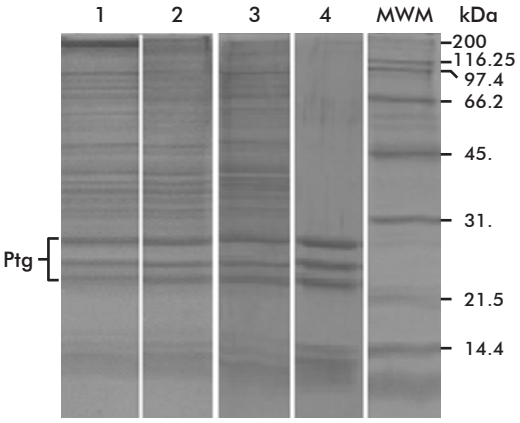

Figure 3. SDS-PAGE analysis of a genetically-detoxified variant of the main toxin of *Bordetella pertussis* (Ptg). Purification steps were pooled from three experimental processes on a 15 % gel. Lanes: 1, concentrated culture supernatant (CS); 2, concentrated supernatant containing urea (CSU); 3, concentrated supernatant containing urea adjusted to pH 6.0 (CSUpH); 4, active pharmaceutical ingredient (API)-dialyzed protein eluate; MWM: molecular weight marker (Unstained SDS-PAGE Standard, broad range; Bio-Rad, Cat. No. 1610317). Samples were loaded at equivalent concentrations for all the purification steps, except the API sample which was concentrated approximately 14 times.

systematic error (mean bias) associated with repeated measurements of the same homogeneous (spiked) sample under specified conditions [33, 34]. Analytical accuracy is dependent on the total error in the method and takes account of all relevant sources of variation: for example, day, analyst, analytical platform or batch [33]. Moreover, parallelism is the performance characteristic that is typically evaluated during in-study validation. It is conceptually similar to dilution linearity except that it is assessed with multiple dilutions of actual study samples or samples that represent the combination of the same matrix and the analyte as those that will be generated during a study [32].

Since RM consisted of bacterial culture supernatant, the accuracy of the method was determined by analyzing two matrixes employed in the process, spiked with Ptg at three concentration levels, covering high, medium and low ranges of the calibration curve. The mean recoveries with 95 % CI given in Table 2, with values ranged between 94.4 to 107.2 %. As the theoretical value of 100 % was included in the confidence interval, the test procedure could be considered accurate in the range studied.

The repeatability and intermediate precision of this assay fell within the 20 % (less than 10 % for repeatability), in agreement with the acceptance criteria described in current regulations [20, 21], also corroborating what is expected for macromolecule analysis and ELISA. Values were even below the 25 % threshold, which was proposed as acceptance limit for immunoassays [28], since they should be less precise than chromatographic assays and, therefore, criteria for accuracy (mean bias) and precision should be more permissive than for chromatographic assays [28, 33].

A validated assay needs constant monitoring and maintenance to retain that designation. Once the assay is put into routine use, internal quality control is accomplished by consistently monitoring the assay for assessment of repeatability and accuracy, and fit for purpose they were designed for, all supported by statistical analysis [35, 36].

In our case, the purification process to obtain the Ptg antigen is still under technological development. Therefore, the validity of the assay was preliminary assessed by performing experimental bacterial cultures and protein purifications. ELISA results showed that Ptg exhibited similar expression kinetics among batches and the test was capable to detect the expression of Ptg since the initial fermentation points. After 18 h of culture, Ptg expression levels reached an average of 8.5 mg per liter by ELISA.

Similar results were obtained by comparing the protein recovery from the experimental purification processes (Figure 2B). All values given by ELISA were accurate, with the exception of CSU and CSUpH steps, in which Ptg values showed substantial underestimation. This result is probably due to a transient conformational change of the protein due to the immediate addition of urea and pH adjustment to 6.0 by adding orthophosforic acid, as a part of the purification process [22]. These may affect Mabs recognition of the antigen. Nevertheless, protein eluate (same as CSU buffer composition, but at pH 7.5) and API quantification values by ELISA were accurate.

Altogether, our results point out the consistency of *B. pertussis* fermentation and purifications processes analysis performed by Ptg-capture ELISA so far, and the possibility of accurately and precisely follow up Ptg through the entire pertussis antigen obtaining process by the ELISA test, with the exception of above mentioned steps.

In summary, we have validated a Ptg-capture ELISA which is currently applied as analytical tool for the development of pertussis antigens production, and as such has been regarded as complying with the expected specifications. This sandwich ELISA fulfills the most stringent acceptance criteria, supporting the assessment of antigen recovery based on concentration values, with precision and accuracy according to current international regulations for vaccine production.

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