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Research Article

Quantitative Dot Blot Assays to determine *Vibrio cholerae* O1 Lipopolysaccharide concentration using Monoclonal Antibody

Abstract

Introduction: Rapid diagnosis is fundamental for epidemiological control of Cholera disease. Contradictorily the gold standard test (stool culture) takes several days. Finlay Vaccine Institute obtained an Immunoagglutination test for rapid diagnosis of Cholera (FCIT), based on a monoclonal antibody anti-LPS O1 coupled to latex particles. FCIT, includes a positive control (LPS O1 Ogawa), which quantification is mandatory to obtain the registration of the test.

The **objective** of the work was to develop a quantitative Dot Blot to determine the LPS O1 concentration in FCIT Positive control, using a peroxidase-conjugated mAb.

Materials and Methods: Conjugation of mAb anti LPS O1 to peroxidase enzyme was carry out by periodate method. Quantitation of LPS O1 was accomplish by quantity Dot Blot. For capture *Vibrio cholerae* 569B Lipopolysaccharides from Sigma, was used as standard of the curve (40 µg/mL to 0.6 µg/mL) and five lots of FCIT positive control were applied as sample. For detection conjugated mAb-HRP was employed at dilution 1:5000. The development of the reaction was carried out using SIGMAFAST™ DAB Tablet. The images were captured using the GS-800 densitometer and the spots density (Int/mm²) were calculated using the ImageJ software. The LPS concentration in positive control lots was calculated employed Ascent Software.

Results: The mAb was conjugated to the HRP efficiently with working dilutions range from 1:2500 to 1:10,000. A four-parameter fit model curve was obtained with R² of 0.99. Of the five FCIT positive control lots evaluated, four complied with 30% of the expected concentration, for an 80% effectiveness of the technique.

Conclusions: These results suggest that the quantitative Dot Blot using the mAbC anti *Vibrio cholerae* LPS O1, can be employed for the quantification of LPS in batches of the FCIT positive control, from the purification and production stages, as well as for the stability evaluation.

Introduction

Cholera is an acute diarrheal disease (ADD) caused by ingestion of food or water contaminated with bacillus *Vibrio cholerae*. Although, more than 200 serogroups have been identified to date, only serogroups O1 and O139 are responsible for the epidemics that have occurred [1,2]. From the clinical point of view Cholera is characterized by secretory diarrhea, from moderate to severe, leading to rapid dehydration, that cause a considerable number of deaths [3,4]. The torpid evolution of the disease, together with its similarity with other ADDs and the high cost involved to implement epidemiological control measures, makes the rapid diagnosis

of the disease, a fundamental pillar in the control of the illness [5,6]. Contradictorily, stool culture is the gold standard for confirmation and characterization of the outbreak strain, but it is time-consuming, requires trained technicians and a functional laboratory. So a rapid diagnostic test is a promising tool to detect cholera outbreaks in areas without laboratory infrastructure [7].

Several methods have been developed for the rapid diagnosis of *V. cholerae* O1 and O139. Some methods are based on polyclonal antibodies that allow identifying the bacteria directly from the primary isolation in culture medium [7-9]. The most recent methods are based on monoclonal antibodies

(mAbs), coupled to: membranes (immunochromatography or test strips) or to latex particles (immunoagglutination). Both systems have been used either individually or simultaneously and have revolutionized the diagnosis of the disease [10-12].

In 2010, with the re-emergence of Cholera in Haiti, Cuba revitalized epidemiological surveillance for the timely diagnosis of this infection [13]. At that time, the Finlay Vaccine Institute had already obtained a monoclonal antibody against LPS of *V. cholerae* O1, which coupled to latex particles, allowed to obtain an Immunoagglutination test for the rapid diagnosis of the disease, so-called for us as Finlay Cholera Immunoagglutination Test (FCIT) [14,15]. The FCIT performance was evaluated in 210 samples of patients with a presumptive diagnosis of Cholera by the reference laboratory of the Institute of Tropical Medicine Pedro Kouri (IPK), showing sensitivity and specificity higher than 90%.

Once the performance was demonstrated, the next step was to obtain the sanitary registration of the FCIT by the Cuban health regulatory authority, CECMED. To obtain the registration of this type of test is mandatory the qualitative and quantitative analysis of all its components to guarantee a good assay performance [16].

The FCIT, includes in addition to the latex reagent, a positive control, constituted by LPS O1 Ogawa, obtained, purified and drying under GMP conditions at the Finlay Vaccine Institute [15]. Currently, the concentration of LPS, as positive control, have been carried out by dry weight determination, after which the sample is diluted to a concentration of 20 µg/mL (final concentration in FCIT). Even when the dry weight method is accepted and widely used, the main disadvantage of the method is that it does not allow the quantification of LPS in the final sample, and therefore, track the stability of the product over the time.

Taking into account our experience in the quantification of several vaccines antigens by Dot Blot and ELISAs using mAbs [17,18], we proposed in this work the use of Quantitative Dot Blot to determine the concentration of LPS O1 in FCIT Positive control, using the peroxidase-conjugated anti-LPS O1 mAb.

Materials and methods

Conjugation of anti-LPS O1 mAb to Peroxidase enzyme

For the conjugation of mAb to Peroxidase enzyme, the modified periodate method according to Gavilondo et al 1995 was used [19].

mAb preparation: The mAb is stored in PBS, for changing buffer from PBS to Sodium Carbonate Solution (SCS) 10 mM, pH 9.5, a Size Exclusion Chromatography (SEC) using Sephadex G-25M (General Electric's Healthcare) as matrix, was carried out. The desalted specific fraction (Def) was collected and concentrated to 1 mL using Amicon® Ultra 50 kDa Filter (Milipore). The mAb concentration was adjusted to 8 mg/mL in SCS.

Enzyme Preparation and Oxidation: Four milligrams of Sigma Horseradish Peroxidase enzyme (HRP) type VI-A was dissolved in 1 mL of distilled water. Freshly prepared 200 µL of

Sodium Meta-Periodate (NaIO₄) 100mM, pH 4.4, was added to HRP under agitation. The mixture was incubated 20 min in the dark. After this time, NaIO₄ was removed by SEC similar to the one described above but in this case Sodium Acetate Solution (SAS) 1 mM pH 4.4 was used as mobile phase. The first peak corresponding to oxidized HRP was collected and concentrated to a final volume of 1 mL using Amicon® Ultra 10 kDa Filter (Milipore).

Conjugation of mAb to HRP: The oxidized HRP, was activated adding 20 µL of Carbonate/Bicarbonate Solution (SCB) at 200 mM pH 9.5, to be consecutively added to the mAb, drop to drop, under slow agitation. The mixture was incubated in darkness 2h at room temperature, keeping agitation. After this time, the conjugation reaction was stabilized by adding 100 µL of Sodium Borohydride (NaBH₄) 0.4% freshly prepared and incubating 2h at 4°C without stirring. Excess of reactants was removed by SEC using PBS as buffer. The first peak was collected.

Purification of conjugated mAb: Purification of conjugated mAb was performed by SEC using Sephacryl S-200 in order to separate the conjugate mAb (mAbC) from free MAb (mAbF) and free HRP (HRPf). Different fractions from peaks were collected and measured at 280nm and 403nm, to calculate the Reinheitszahl Factor (RZ). All fractions where the RZ values were between 0.3-0.6 were selected as peaks of interest. Finally, purified mAbC was concentrated to 1mL using Amicon® Ultra 100 kDa Filter (Milipore). For preservation, BSA 1%, Tiomersal 0.01% and Glycerol 85% v/v was used and stored at -20°C.

Selection of working dilution of the mAbC

Selection of working dilution was carried out using Direct Dot Blot technique. Minifold II one S&S equipment (Germany) coupled to a vacuum pump and Nitrocellulose membrane (MNC) 0.2 µm from Sartorius were used. For capture, *Vibrio cholerae* 569B Lipopolysaccharides (LPS-O1p) from Sigma was used as standard of the two-fold serial dilutions curve, with range of 40 µg/mL to 0.6 µg/mL and application volume per well of 100 µL. The curve was applied by triplicate, to be faced each one, to different concentrations of the mAbC. After 1 min vacuum to ensure membrane drying, the MNC was removed from the equipment and the remaining active sites were blocked with PBS/Skim Milk (SM) at 3% and incubated for 30 min at 37°C. Three washings of 5 min were performed with PBS/Tween 20 (0.05%). The MNC was cut into three strips and each one were incubated 1h at 37°C with 10 mL of the different dilutions of the mAbC evaluated (1:2500, 1:5000 and 1:10000). After five washing steps the development of the reaction was carried out using SIGMAFAST™ DAB Tablet for 15 min in the dark. The reaction was stopped by several changes of distilled water. The images were captured using the GS-800 densitometer (Bio-Rad, USA). The maximum dilution at which signal was observed at all points on the LPS-O1p curve was selected as the optimum working dilution.

Quantification of LPS O1 in positive control lots of FCIT

Quantitation of LPS O1 was evaluated in five lots of FCIT Positive Control by quantity Dot Blot. The procedure used was

similar to described in II.2. In this case, in lane one (L1) LPS-O1p was applied as curve similar to the II.2 Dot Blot. In lane two (L2) five lots of FCIT positive control (100 μ L/well) were applied without prior dilution. In lane three (L3) 100 μ L/well of the quality controls (QCs) was applied. QCs consisted of LPS-O1p dissolved in PBS and prepared to a final concentration of 15 μ g/mL (w/v). Curve and QCs were stored as single ready-to-use aliquots at -20°C . The mAbC was used at selected working dilution of 1: 5000 in PBS. The remainder of the procedures were performed as described above. The images were captured using the GS-800 densitometer (Bio-Rad, USA) and the signal density (Int/mm²) of each point of the curve and samples was calculated using the ImageJ software. Then using the Ascent Software Version 2.6 a four-parameter logistic curve was constructed by plotting the Int/mm² value of each point of the LPS-O1p vs the LPS concentration. With the four-parameter logistic equation (4PL) obtained, the LPS concentration in the five lots of positive control evaluated, was calculated by interpolating the Int/mm² of each samples in the LPS curve obtained. This Dot Blot was repeated three times to corroborate the results.

Results and Discussion

Obtaining of anti-LPS O1 mAb conjugated to HRP enzyme

Conjugation of enzymes to antibodies involves the formation of a stable, covalent linkage between enzyme and antigen-specific monoclonal or polyclonal antibody, in which neither the antigen-combining site of the antibody nor the active site of the enzyme is functionally altered [19-21].

Many procedures have been described for enzyme-antibody conjugation, and their efficiencies differ widely.²¹ Most of them using different reagents such as sodium periodate or glutaraldehyde and several enzymes e.g. HRP, urease, or alkaline phosphatase [22]. HRP is the most widely used, it is relatively small (44 kDa), stable, and has a broad specificity that allows it to be measured by absorption, fluorescence, and luminescence. Also the enzyme is cheap and many chromogenic substrates for it are also available [23,24]. In this work, HRP was used as enzyme and the modified PM was used to obtain the anti LPS O1 mAbC.

The PM used here was separate in four main stages: 1) preparation of mAbs, 2) preparation of the HRP enzyme, 3) Conjugation mAb-HRP and 4) Purification of mAbC.

The **preparation of the mAb** has as main objective that the mAb is in the indicated buffer and at the concentration suitable for the conjugation. The indicated buffer was SCS at pH 9.5, condition in which the amino groups are in non-protonated form (NH₂⁺), available for coupling. The adequate concentration was 8 mg/mL to achieve a mAb-HRP ratio (m/m) of 1:2.

Post SEC using Sephadex G-25M as matrix and SCS pH 9.5 as mobile phase, a total of 25 mL of desalting mAb was obtained and concentrated to 1 mL by Amicon-50 kDa, pore size three times smaller than the molecular size of IgG, 150

kDa. The final protein concentration was 9.3 mg/mL, adjusted at 8 mg/mL. Figure 1 shows the chromatogram obtained from the SEC, where a single peak can be observed, typical profile of the desalting processes.

The **preparation of the HRP** enzyme consisted mainly in the oxidation with sodium periodate. The method exploits the glycoprotein nature of the enzyme, where the saccharide (mannose residues) are oxidized with NaIO₄ to produce aldehyde groups (from vicinal hydroxyl group of sugar moieties of HRP). The aldehyde groups react with the non-protonated amino groups of the IgG molecule producing a Schiff bases. This reaction was performed in the dark to prevent periodate breakdown and in a short period of time (20 min) to avoid loss of enzymatic activity by overoxidation [23-25].

To eliminate the NaIO₄, SEC-Sephadex G-25M was employed using SAS pH 4.4. With this pH auto-conjugation of HRP molecules is prevented because its amine groups are protonated. In figure 2A can be seen the peak obtained from the SEC. A single peak corresponding to the enzyme oxidized and now diluted product to the desalting process. Therefore, a concentration step was carry out using Amicon-10kDa (taking into account that molecular size of the HRP is 44 kDa) to concentrated the enzyme at 4 mg/mL and thus maintain the mAb-HRP ratio (m/m) of 1:2, optimal for the conjugation process.

The **conjugation process** consisted of four steps: 1) activation of the enzyme (previously oxidized) with SCB pH 9.5, 2) Schiff base formation between activated peroxidase and amino-groups of the mAb, 3) Stabilization of the reaction by addition of NaBH₄ and 4) Elimination of borohydride.

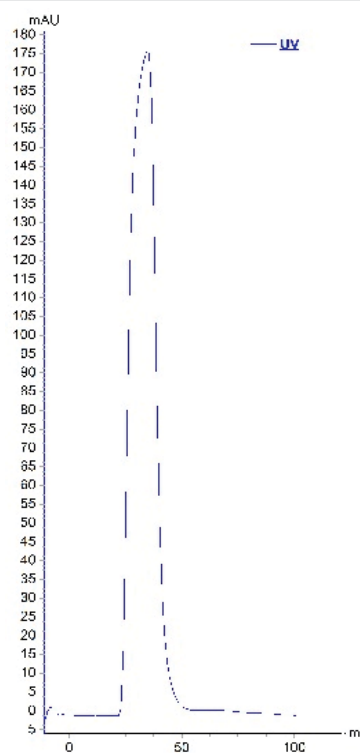


Figure 1: mAb's desalting. SEC using Sephadex G-25M as matrix and SCS pH 9.5 as mobile phase. 15 mL of the mAbs were applied at Fr of 5 mL/min.

The third step is very important because Schiff bases are relatively labile and NaBH_4 stabilizes the reaction by the reduction to a secondary amine linkage (Reductive animation), this reduction of the Schiff base forms a more stable conjugate [24,25]. Then the borohydride was removed by SEC-Sephadex G-25M (Figure 2B). In the chromatogram two peaks are observed, the first peak corresponds to the mAbC, being the majority fraction, and the second peak could be related with free mAb, that elutes very close to the mAbC because the difference between their molecular sizes is small (no greater than 44 to 50 kDa taking into account the size of the HRP). On the other hand, the matrix used (Sephadex G-25) is well established for desalting and buffer exchange, objective pursued with this SEC, but is not recommended for separation of molecules by size, especially molecules above their exclusion limit M_r 5000, such as IgG.

Precisely with the purpose of **purify the conjugated mAb** and separate it from the free mAb and the free peroxidase, a SEC was performed using Sephacryl S-200 (Figure 2C).

Sephacryl size exclusion chromatography allow fast and reproducible purification of proteins, polysaccharides, and other macromolecules. Sephacryl is a highly versatile gel filtration resins is a cross-linked copolymer of allyl dextran and N,N' -methylene bisacrylamide that offers a wide range of fractionation capabilities and this crosslinking gives good rigidity and chemical stability. The hydrophilic nature of the resins minimizes nonspecific adsorption and maximizes recovery.

Specifically, Sephacryl S-200 is designed for purifying antibodies, serum proteins and mid-size proteins with a fractionation range between 5 kDa and 250 kDa [26], optimal range to separate mAbC (190 kDa) from free peroxidase (44 kDa), Reason for which this was the selected matrix.

As can be observed in the chromatogram 2C, three peaks were obtained, the first one (1) correspond to the mAbC, which elutes first to have a larger size (around 190 kDa), very close to this peak, is the second (2) that correspond to the free mAb (about 150 kDa) and separated from these, a third peak that could be related with free peroxidase (about 44 kDa). These results are in correspondence with what the literature suggests about this type of chromatography, where molecules with size within the range of the particles that make up the matrix, penetrate the particles and are delayed by the stationary phase, to a greater extent, the smaller their size. [26] Therefore, the molecules elute in descending order of molecular size, as it is observed in the chromatogram C, first the mAbC, later the free one and finally the enzyme.

To confirm the results of the chromatogram, the RZ was calculate to the three peaks (Table 1).

Based on the RZs observed in table 1, the first peak corresponds to the mAbC with a RZ between 0.3-0.6, while the second peak corresponds to the free mAb and the third RZ of the HRP that according to manufacturer reports is between 2.5-4.0. These results demonstrate the efficiency of the separation method employed and the conjugation method performed.

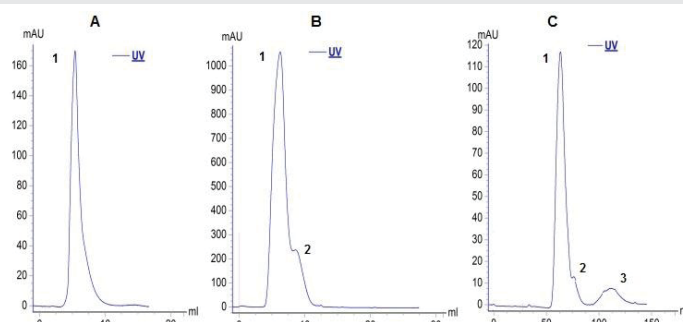


Figure 2: Chromatograms obtained during the conjugation process of mAb to HRP enzyme. A) SEC-Sephadex G-25M to eliminate the NaIO_4 , B) SEC-Sephadex G-25M to eliminate the NaBH_4 and C), SEC-Sephacryl S-200 to separate the conjugated mAb (1) from free mAb (2) and free peroxidase (3).

Table 1: Absorbance at 280/403 nm and RZ of CGF peaks.

Sample	Abs 280 nm	Abs 403 nm	RZ
Peak 1	0.733	0.396	0.54
Peak 2	0.103	0.068	0.66
Peak 3	0.2	0.733	3.6

Selection of the working dilution of the mAbC

As the objective of obtaining the conjugated MAb is to be applied in the quantification of *V. cholerae* LPS O1 by the immunoenzymatic assay Dot Blot, it was decided to evaluate the working dilution of the mAbC using Dot Blot.

For capture LPS-O1p was applied as standard curve from 40 $\mu\text{g}/\text{mL}$ to 0.3 $\mu\text{g}/\text{mL}$ and for detection three dilutions (1:2500, 1:5000 and 1:10000) of the conjugate mAb was evaluated (Figure 3).

The working dilution selected as the optimum for mAbC was 1:5000 being the highest dilution at which signal is observed in at least six LPS concentrations, number of points required for a calibration curve.

In immunoenzymatic assays such as Dot Blot, where there is a biomolecule capture step, determining the optimum concentration thereof to immobilize (in this case LPS) is a paramount factor. So is the selection of optimal concentrations of the reactant molecule (the conjugate mAb in this case). These parameters influence the formation of Ag-Ac complexes, where the concentrations of both molecules have to be in the range of the equivalence zone [27].

The yield or working dilution of the conjugates dependent on numerous factors such as antibody affinity, type of immunoenzymatic assay and quality of antigen. In general, the working dilutions range from HRP conjugated mAb are 1:100 to 1:10,000 [24,25].

Calibration curve construction

Using LPS-O1p as standard a calibration curve was obtaining (Figure 4). A four-parameter fit model was applied to obtain the function describing a sigmoid model. Coefficient of determination (R^2) ≥ 0.993 was obtained.

Calibration curves for Immunoassays are generally characterized by a nonlinear relationship between the mean response and the analyte concentration, so it is not recommended linear and log-log linear model. Even though the R^2 of the log-log linear model be ≥ 0.99 , it has been seen that when quantifying in samples, this model is not as effective [27]. Typically, the response between Ag-Ac (ligand binding Assay) exhibits a sigmoidal relationship with concentration. The currently accepted reference model for these calibration curves is the 4-parameter logistic (4-PL) model, which optimizes accuracy and precision over the maximum usable calibration range [28]. In immunoassays the response is the result of the interaction between Ag-Ac, which in turn depends on the specificity and affinity of the Ac for the Ag, while colorimetric chemical reactions are generally directly related to the amount of substance in the detector [28].

Quantification of LPS O1 in positive control lots

The four-parameter logistic equation (4PL) obtained from the LPS-O1p curve, was used to calculate the concentration of LPS in five lots of FCIT positive control (Figure 5).

The FCIT Positive Control lots have a theoretical amount of 20 $\mu\text{g}/\text{dose}$ of LPS O1. The common specified range is $\pm 30\%$ of the expected amount per mL [29,30]. Considering this guideline, the positive control lots should have between 14 and 26 $\mu\text{g}/\text{mL}$ of LPS O1. Values from Table of Figure 5C indicate that of five lots evaluated, four of them had the LPS concentrations into the accepted range ($\pm 30\%$), with CVs under the 15% accepted for immunoassays. Also the QC that should have a concentration of 15 $\mu\text{g}/\text{mL}$, quantified 14.11, concentration that is within the established parameters.

Diagnostic Reagents (DR) are a fundamental element for the diagnosis, treatment and monitoring of diseases.[31] The

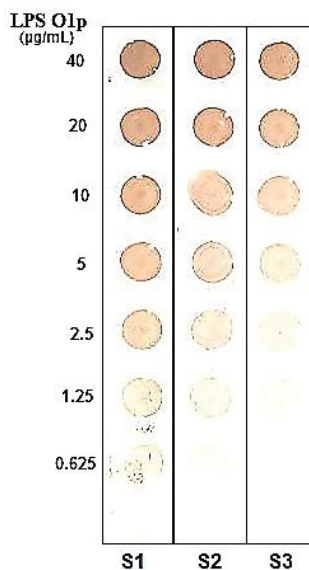


Figure 3: Dot Blot to select the working dilution of the conjugate mAb. For capture LPS-O1p was applied as standard curve from 40 $\mu\text{g}/\text{mL}$ to 0.6 $\mu\text{g}/\text{mL}$ and for detection three dilutions 1:2500 (S1), 1:5000 (S2) and 1:10000 (S3) of the conjugate mAb was evaluated. The images were captured using the GS-800 densitometer (Bio-Rad, USA).

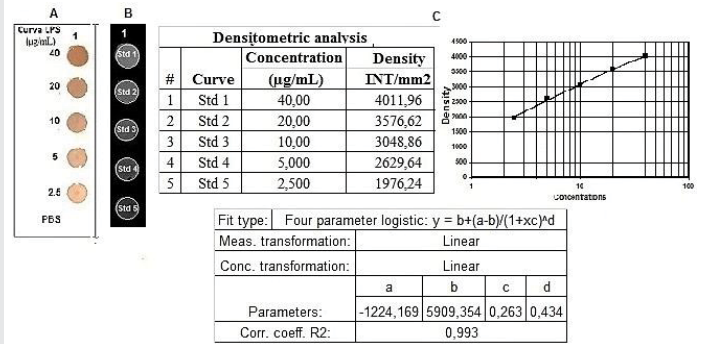


Figure 4: Calibration Curve Construction. (A) Dot blot, (B) Densitometric analysis using ImageJ software and (C) Calibration Curve using the Ascent Software.

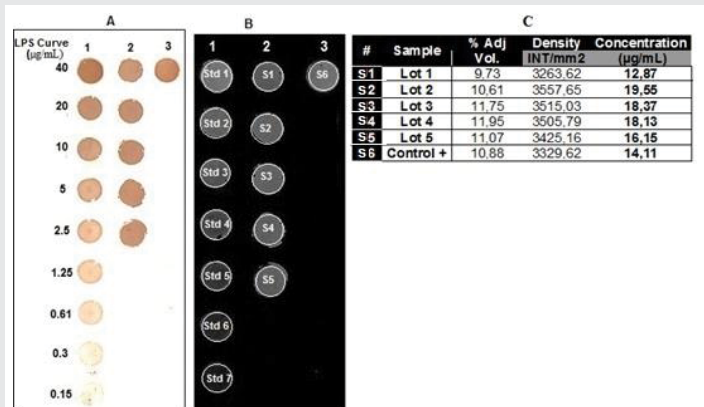


Figure 5: Quantification of LPS O1 in positive control lots by Dot Blot with Densitometric analysis. (A) Dot blot, (B) Densitometric analysis using ImageJ software version 1.5. (C) LPS concentration in lots using the Ascent Software.

FCIT was obtained at the Finlay Vaccine Institute, in response to the re-emergence of Cholera in Cuba, a disease where the rapid and accurate diagnosis saves many lives and allows the containment of the transmissibility of the disease. Failure in the function of a DR involves a risk to the health of the individual, which can have consequences ranging from almost insignificant to very serious, including death. Therefore, the DR must be effective, safe and with a quality according to the state of the art of the different medical specialties. Hence, the evaluation and registration of DR is a regulatory process that contributes to guarantee the quality of these products [31,32].

The Regulation no. 8-2001 of MINSAP [33], provides the necessary requirements and procedures for the Health Register of the DR, being inside them the qualitative and quantitative composition of all DR components, especially of those which have a definite and irreplaceable function for the execution of the test, as is the positive control of the FCIT.

Several methods can be used for the quantification of LPS in samples. The determination of KDO (keto-3-deoxy-d-mannose-oculosonic acid) has been widely used. This method is not advisable for the quantification of *V. cholerae* LPS, because in its native structure the KDO molecule is very internal and not reactive, therefore it is necessary to apply strong hydrolysis methods that destroy the molecule and allow exposure of the KDO [34,35]. Another method for the quantification of LPS is the use of the Limulus Amebocytes Lysate (LAL) assay, but

this assay is costly, labor intensive and requires skilled and trained personnel, usually in analytical units [34]. Likewise, quantification by Western Blot with densitometric analysis, where first you have to perform the SDS-PAGE, transfer to an MNC to perform the WB and then the densitometric analysis [36,37].

However, when referring to the antigen (LPS) in the pure state, several authors report the quantification of LPS, by dry weight [35,38]. This is an accepted variant, and in fact, is the one used until now for the initial quantification of LPS in the positive control of the FCIT. The main problem of the dry weight technique is that does not allow the quantification of the LPS in the final product already in its liquid form so is not effective in evaluating the stability of the positive control reagent. For this, the colorimetric and immunoenzymatic techniques are very useful. Within the immunoenzymatic techniques, the Dot Blot is a fast technique (from 4 to 5 hours) and easy to perform, which together with the use of a mAb of high specificity, affinity and sensitivity, makes the technique be very attractive. In fact, of the five FCIT positive control lots evaluated, four and the QC complied with 30% of the expected concentration, for an 80% effectiveness of the technique.

These results suggest that the quantitative Dot Blot using the mAbC anti *Vibrio cholerae* LPS O1, can be employed for the quantification of LPS in batches of the FCIT positive control, from the purification and production stages, as well as for the stability evaluation.

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