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Article

Epitope mapping of the diphtheria toxin and development of an ELISA-specific diagnostic assay

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Abstract: (1) Background: The diphtheria toxoid antigen is a major component in pediatric and booster combination vaccines and is known to raise a protective humoral immune response upon vaccination. Although antibodies are considered critical for diphtheria protection, little is known about the antigenic determinants that maintain humoral immunity. (2) **Methods:** One hundred twelve 15-mer peptides covering the entire sequence of DTx protein were prepared by Spot-synthesis. Membrane-bound peptides immunoreactivity with sera from mice immunized with triple DTP vaccine allowed mapping of continuous B-cell epitopes**,** topological studies, MAPs synthesis, and ELISA development. **(3) Results**: Twenty epitopes were identified, being 2 in the signal peptide, 5 in the CD, 7 in the HBFT domain, and 5 in the RBD. Two 17-mer (CB/Tx-2/12 and CB/DTx-4-13) derived bi-epitope peptides linked by a Gly-Gly spacer were chemically synthesized. The peptides were used as antigens to coat ELISA plates and assayed with human (huVS) and mice vaccined sera (miVS) for in vitro diagnosis of diphtheria. The assay proved to be highly sensitive (99.96%) and specific (100%) either for huVS and miVS and when compared with a commercial ELISA test, demonstrate high performance. **(4) Conclusions:** Our work displayed the complete picture of the linear B cell IgG response epitope of the DTx responsible for the protective effect and demonstrated the specificity and eligibility to enter phase IIB studies of some epitopes to develop new and fast diagnostic assays.

Keywords: Diphtheria toxin; B epitopes; epitope mapping; bi-specific peptides; ELISA; diagnostic performance

1. Introduction

Diphtheria is once again a growing concern, vaccine-preventable, bacterial disease caused by toxin-producing strains of *C. diphtheria*. Is endemic in Asia (India, Indonesia, Iran, Nepal, Pakistan, etc.), Africa (Ghana), and some countries of South America [1-3] but well-controlled in countries with high vaccination coverage. However, in the last few years is has been re-emergent in part of Europe [4], Venezuela [5], and imported cases in other countries of Europe [6-9], the United States of America [10], and Brazil [5].

Not only *C. diphtheriae,* but also *C. ulcerans* and *C. pseudotuberculosis* have the potential to produce diphtheria toxin (DTx) and hence can cause classic respiratory diphtheria (spread by droplets) as well as cutaneous diphtheria (direct contact) [11].

Due to the potential local or systemic spread of DTx, classical diphtheria may give rise to severe life-threatening respiratory symptoms characterized by the development of an adherent pseudomembrane in the upper respiratory tract as well as myocarditis and polyneuritis with a fatality rate between 5 to 30% [6]. In contrast, cutaneous diphtheria has typically characterized by well-demarcated ulcers that might have a membrane. The

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lesions are slow healing and might act as a reservoir from which bacteria can be transmitted to susceptible contacts, potentially resulting in cutaneous or respiratory disease [12].

Disease severity is mediated by successful bacterial secretion of the potent DTx that inhibits protein synthesis, in eukaryotic cells, by disrupting elongation factor 2 (EF2) function causing cell death [13]. DTx consists of a single protein with a disulfide bond linking two fragments processed by trypsin [14,15]. A fragment A contains the catalytic domain (aa 1-188) [15], and fragment B with the translocation (PFMT, aa 200-378) and receptor-binding domain (RBD, aa 387-535) [16].

Vaccination with diphtheria toxoid-containing vaccine might not prevent cutaneous colonization or infection with *C. diphtheriae* [2,12]. Non-toxin-producing strains of *C. diphtheriae* can also cause disease that is generally less severe, although invasive associated with non-toxin-producing strains has been reported [17]. As the mortality of infections caused by the three corynebacteria species mentioned above is almost entirely due to DTx [17, 18-28], the availability of rapid methods for the identification of the species as well as for the detection of DTx or the DT tox gene is of primary importance.

DTxs produced by the different corynebacteria are structurally very similar [29-32] and the identification and characterization of corynebacteria are conducted by phenotypic methods [11,33,34], polymerase chain reaction (PCR) [35-37], and mass spectrometry (MALDI-TOFF) [38]. The immunological tests developed for the disease diagnostic were the microcell culture [39], fluorescence immunoassay [40], and duple antigen [41] or sandwich-ELISA [42].

The diphtheria toxoid antigen is a major component in pediatric and booster combination vaccines is known to raise a protective humoral immune response upon vaccination. However, a structurally resolved analysis of TxD epitopes with underlying molecular mechanisms of antibody neutralization is scarce or has not yet been revealed.

Two neutralizing anti-DTx monoclonal antibodies (mAbs) recognizing conformational epitopes blocking the heparin-binding epidermal grow factor (HBEGF) binding site have been identified by mass spectrometry/interferometry and used to develop an ELISA [43]. Another approaches used for identifying epitopes/blocking antibodies was the phage display assay [44,45], targeting of the specific domain [46], and human mAbs isolated from vaccined volunteers [47], however, in most of the epitopes were not identified or longer peptides sequences have been proposed. A recent study, using dynamic simulation and temperature variation also predicted some conformation B-cell epitopes in DTX [48].

In this work, using a more refined methodology of epitope synthesis, we report the mapping of B-cell continuous epitopes of DTx, the topological characterization of the epitopes, and the production by chemical synthesis of DTx-bi-epitope and its use as antigen for diphtheria diagnosis. The printable Spot-synthesis is an accessible and very stretchy technique for microarray production of peptides on membrane supports. Furthermore, it allows rapid and low-cost access to a large number of peptides for systematic epitope analysis [49]. The bi-epitope MAP4 used as coating antigen in ELISA accurately distinguishes high sensitivity and specificity sera of immunized mice and humans, from healthy animals and individuals.

2. Materials and Methods

2.1 Materials and antibodies

Amino acids, reagents for peptide synthesis, casein, goat anti-human IgG-biotin, rabbit anti-mouse IgG-biotin, peroxidase labeled-neutravidin and TMB (3,3′,5, 5′tetramethyl benzidine) were from Sigma-Merck (St Louis, EUA). Cellulose membranes of the ester type were from Intavis (Koeln, Germany). Alkaline phosphatase labeled sheep anti-mouse IgG (H+L) was obtained from KPL (CA, USA). HRP labeled neutravidin, and SuperSignal West Pico chemiluminescent substrate was from Thermo (IL, USA). Immulon 2HB flat-bottom 96-well microtiter plates were from Corning. All other reagents were of analytical grade. The triple DTP-vaccine used during the vaccination of children/infants containing

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inactivated whole-cell bacterium of *B. pertussis* combined with formaldehyde-inactivated diphtheria and tetanus toxoid was produced by Bio-Manguinhos (FIOCRUZ, RJ, Brazil).

2.2. Human, horse, and mice sera

Ninety children, aged 6-16 years (median age 7.5 years) vaccinated with the whole DTP with no evidence of acute infection or known history of whooping cough and diphtheria were enrolled in this study. One hundred sera samples from healthy blood bank donators (HEMORIO) were included in the study.

To obtain mice sera, thirty-eight NIH Swiss mice (12–16 g) were immunized as described previously [50] with wDTP vaccine reconstituted with saline and 2 IU (defined by the Brazilian National Immunization Program) administered in 0.5 ml with an interval of 21 days. Sera were collected one week after the last inoculation and stored at -20 ◦C.

The commercial horse therapeutic (hoThe) sera was obtained from Butantan Institute, São Paulo, SP, Brazil.

2.3. Synthesis of the cellulose-membrane-bound peptide array

The entire sequence of the DTx (Q6NK15, strain ATCC 700971/NCTC 13129/Biotype gravis, 560 aa) was covered with the synthesis of 15-residues-long peptides with overlapping of 10 residues, automatically prepared on cellulose membranes according to standard SPOT synthesis protocol using an Auto-Spot Robot ASP-222 (Intavis Bioanalytical Instruments AG, Köln, Germany).

The **GSGSG** sequence was used as a spacer at the beginning (spot A1) and end (spot E16) of the protein sequence and the programming was done with the Multipep software (Intavis). Negative [**QEVRKYFCV,** Vaccinia virus spot F9 and F17] and positive [**KEVPALTAVETGATN** (Poliovirus, spots F3 and F11)/ **GYPKDGNAFNNDRI** (*Clostridium tetani,* spot F5 and F13)/ **YDYDVPDYAGYP YDV** (*H. influenza* virus hemagglutinin, spot F7, and F15)] controls were included. The entire library contained 112 peptides plus 6 positive and 2 negative control peptides.

The coupling reactions were followed by acetylation with acetic anhydride (4%, v/v) in N, N-dimethylformamide to render the peptides N-reactive during subsequent steps. After acetylation, the F-moc protecting groups were removed by the addition of piperidine to make the nascent peptides reactive. The remaining amino acids were added by this same coupling, blocking, and deprotection process until the desired peptide was generated. After the addition of the last amino acid, the side chains of the amino acids were deprotected using a solution of dichloromethane-trifluoracetic acid-triisopropyl silane (1: 1: 0.05, v/v/v) and washed with ethanol as described previously [51]. Membranes containing the synthetic peptides were probed immediately.

2.4. Screening of SPOT membranes

SPOT membranes were washed for 10 min with TBS-T (50 mM Tris,136 mM NaCl, 2 mM KCl and 0.05 Tween, pH 7.4) and then blocked with TBS-T (containing 1.5% BSA) for 90 min at 8°C under agitation. After extensive washing with TBS-T, membranes were incubated for 12h with a pool (n=30) mice vaccined sera diluted (1:150 for IgG detection) in TBS-T+0.75% BSA and then washed again with TBS-T. After that, membranes were incubated with sheep anti-mouse IgG alkaline phosphatase labeled (diluted 1:5000), prepared in TBS-T+0.75% BSA for 1h, and then washed with TBS-T and CBS (50 mM citrate-buffer saline). The chemiluminescent substrate Nitro-Block II was added to complete the reaction.

2.5. Scanning and quantification of spot signal intensities

Chemiluminescent signals were detected on an Odyssey FC (LI-COR Bioscience) using the same conditions described previously [50] with minor modifications. Briefly, a digital image file was generated at a resolution of 5 MP and the signal intensities quantified using the TotalLab TL100 (v 2009, Nonlinear Dynamics, USA) software. This program

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has an automatic grid search for 384 spots but does not offer the automatic identification of possible epitope sequences. Due to this, obtained data were analyzed with the aid of a Microsoft Excel program, and to be considered an epitope the sequences of two or more positive contiguous spots should present signal intensity (SI) greater than or equal to 30% of the highest value obtained from the set of spots on the respective membrane. The signal intensity (SI) used as a background was a set of negative control spotted in each membrane.

2.6. Preparation of the bi-specific-antigen peptides

The single peptide epitope CB/Tx2 (G**SFVMENFSS)** and CB/Tx12 (**VDIGF**) and the peptides CB/Tx4 (**KGFYSTDNKY)** and CB/Tx13 (**SPGHK)** were synthesized in tandem containing GG interpeptides as a spacer (Top-Peptide Bio Co., Pudong, Shanghai, China). The peptides were purified by HPLC and their identity was checked by MS (MALDI-TOF or electrospray).

For preparation of the dendrimeric multi antigen peptide (MAP), a standard solid phase synthesis protocol was followed using the tetrameric Fmoc4-Lys2-Lys1-B-Ala Wang resin and the sequence of the bi-epitope described above. The construct was prepared in an automated peptide synthesizer (PSS8-model, Shimadzu, Kyoto, Japan) as described previously [52].

2.7 Enzyme-linked immunosorbent assay (ELISA)

The in house-ELISA was realized as described previously with minor modifications [51]. Briefly, the plates of ELISA were coated with 100 µl (25 µg/ml) of each MAP4 (CB/Tx2-12 and CB/Tx4-13) prepared on coating buffer (Na_2CO3–$NaHCO_3$, pH 9.6) overnight at 4°C. After each incubation step, the plates were washed three times using PBS-T washing buffer (PBS with 0.1% Tween 20 adjusted to pH 7.2) and blocked (200 µl) with 2% defatted milk (2 h at 37°C). Either the huVS as the miVS were dilutes (1:100) in coating buffer and 100 µl were applied onto Immulon 2HB flat-bottom 96-well microtiter plates and incubated for 2 h at 37°C. Following several washes with PBS-T, the plates were incubated with 100 µl of goat IgG anti-human IgG (1: 5000) or rabbit IgG anti-mice IgG conjugated to biotin (1:2000) for 2 h followed by HRP labeled neutravidin (1:8000 for human sera and 1: 5.000 for mice sera). The plates were developed with TMB (3,3′,5, 5′tetramethyl benzidine) as substrate. The absorbance values at 405 nm were read using an ELX800 Microplate Reader (Bio-Tek Instruments Inc) and the immune response was defined as elevated significantly when the reactivity index (RI; O.D.450 value of Target - O.D.450 values of cut-off)) was more than 0.05m.

Analysis using the commercial diphtheria IgG-ELISA Kit was conducted as described by the manufacturer (Serion, Brazil)

2.8. Bioinformatics tools

The data bank search for DTx from *C. diphtheriae* (Q6NK15), *C. ulcerans* (A0A1Y0HBB0), and *C. pseudotuberculosis* (WP_014654963.1) were carried in the database UniProtKB [http://www. uniprot. org/] and NCBI. The alignment of the sequences was performed using the T-Coffee server (HTTP:// tcoffee.vital-it.ch/cgi-bin/Tcoffee/tcoffeecgi/index.cgi).

The prediction of the secondary structure of the protein was performed by the PSIPRED servers [http://bioinf.cs.ucl.ac.uk/psipred/] and CDM [http://gor.bb.iastate.edu/cdm/]. Some physicochemi cal parameters related to the peptides used in this study (molecular weight, theoretical pI, and hydrophobicity) were calculated with the ProtParam tool (http://web.expasy.org/ program/). The prediction of transmembrane domains was obtained using TopCons (https://topcons.net/pred).

2.9. Structural localization of the IgG epitopes

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The orientation of epitopes in the protein crystallographic structure of DTx protein (PDB:1xdt) was performed using PyMOL, Molecular Graphics System, Version 2.0 Schrödinger, LLC. For the amino acid sequence of epitopes and transmembrane topology visualization, the platform Protter was used (http://wlab.ethz.ch/protter/).

2.10. Statistical analysis

Statistical analysis was performed using Graph Pad Prism version 5.0. The statistical difference using a t-test was considered if p-value _ 0.05.

3. Results

3.1. Identification of the immunodominant IgG epitopes in diphtheria toxin

The epitopes in the DTx (560 aa) were identified based on recognition of peptides in a synthesized library by mice antibodies immunized with DTx (see Materials and Methods). **Figure 1, panels A and B** present the position of each peptide and the measured intensity, respectively, from the chemiluminescent detection of mice IgG antibodies in sera pooled from mice vaccinated. The intensities were normalized using 100% as defined by the positive control (data not shown). The list of the synthetic peptides and their positions on the membranes is presented in a **supplementary Table S1**.

The pattern of reactivity for the antibodies generated in mice immunized with DTx demonstrated that a greater number of peptides were recognized **(Fig. 1).** An analysis of the sequences constituting the peptides synthesized in reactive regions defined 20 IgG epitopes generated by the DTx vaccine **(Table 1).**

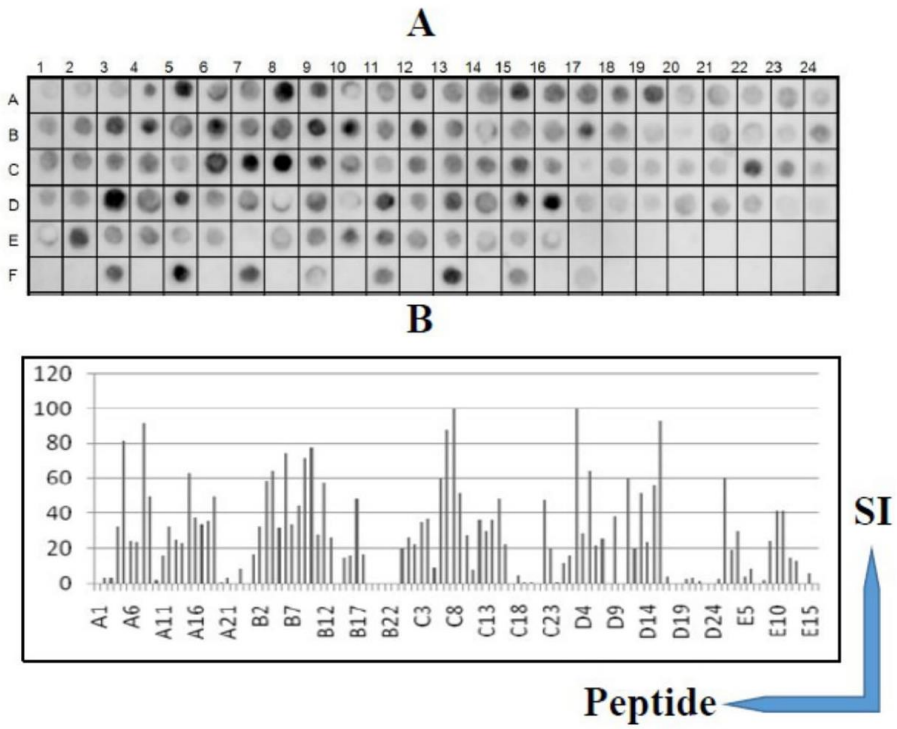

Figure 1. B-cell linear epitopes mapping along the primary sequence (560aa, 112 peptides) of the diphtheria toxin (DTx, Q6NK15) protein in the SPOT synthesis array (15 residues with overlapping of 10) with the sera (n=15) of DTP vaccined mice. IgG-reactive peptides (A); signal intensity (SI) (B). The peptides (listed in **Table S2, supplementary**) represented the coding region of DTx protein. Each peptide was identified by the Spot-synthesis membrane position numbering. Spot intensities below 20% were considered as background.

Table 1. List of the identified CB/TxD IgG epitopes.

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Name of epitope	Squence	Name of epitope	Sequence
CB/DTX-1	^{16}GIGAPPSAHA^{25}	CB/DTx-11	^{280}LEHPEL^{285}
CB/DTx-2	^{31}VDSSK^{35}	CB/DTx-12	^{301}ANYAAW^{306}
CB/DTx-3	^{46}HGTKPGY^{52}	CB/DTx-13	^{315}DSETAD^{320}
CB/DTx-4	^{76}KGFYSTDNKY^{85}	CB/DTx-14	^{342}ADGAVHHNT^{350}
CB/DTx-5	^{91}SVDNENPLSGKAGGV^{105}	CB/DTx-15	^{376}VDIGF^{380}
CB/DTx-6	^{132}GLSL^{135}	CB/DTx-16	^{406}SPGHKTQPFLHDGY^{419}
CB/DTx-7	^{153}GDGASR^{158}	CB/DTX-17	^{434}GFQGESGHDIKI^{445}
CB/DTx-8	^{163}LPFAEGSSS^{171}	CB/DTx-18	^{477}VNGRKIR^{483}
CB/DTx-9	^{196}GKRGQ^{200}	CB/DTx-19	^{490}DGDVTFCRPKS^{500}
CB/DTx-10	^{247}EHGPIKNKMSESPN^{260}	CB/DTx-20	^{526} SNEISSDS^{533}

3.2. Identification of the IgG epitopes in SF23 peptide sequence

The SF23 peptide sequence (**aa533-555**) corresponds to the receptor bind fragment of DTx and forms a beta harping structure [53], which bind to the cellular receptor HBGF. DTx hoThe antibodies are capable to block the infection [54,55]. Thus, in this assay a library of 24 peptides covering all the sequence (**SIGVLGYQKTVDHTKVNSKLSLF**) of the SF23 was produced (**Fig 2**) and probed with sera huVS, miVS, and hoTS. The results are presented in the **Figure 2** and shown that this segment display the antigenic sequence **SIGVLGYQ (aa533-540)** recognized by either huVS (**Fig 2A**), miVS **(Fig 2B**) or hoTS (**Fig 2C**). The identified sequence is only part of the CB/Tx-20/miG epitope (**Table 1**).

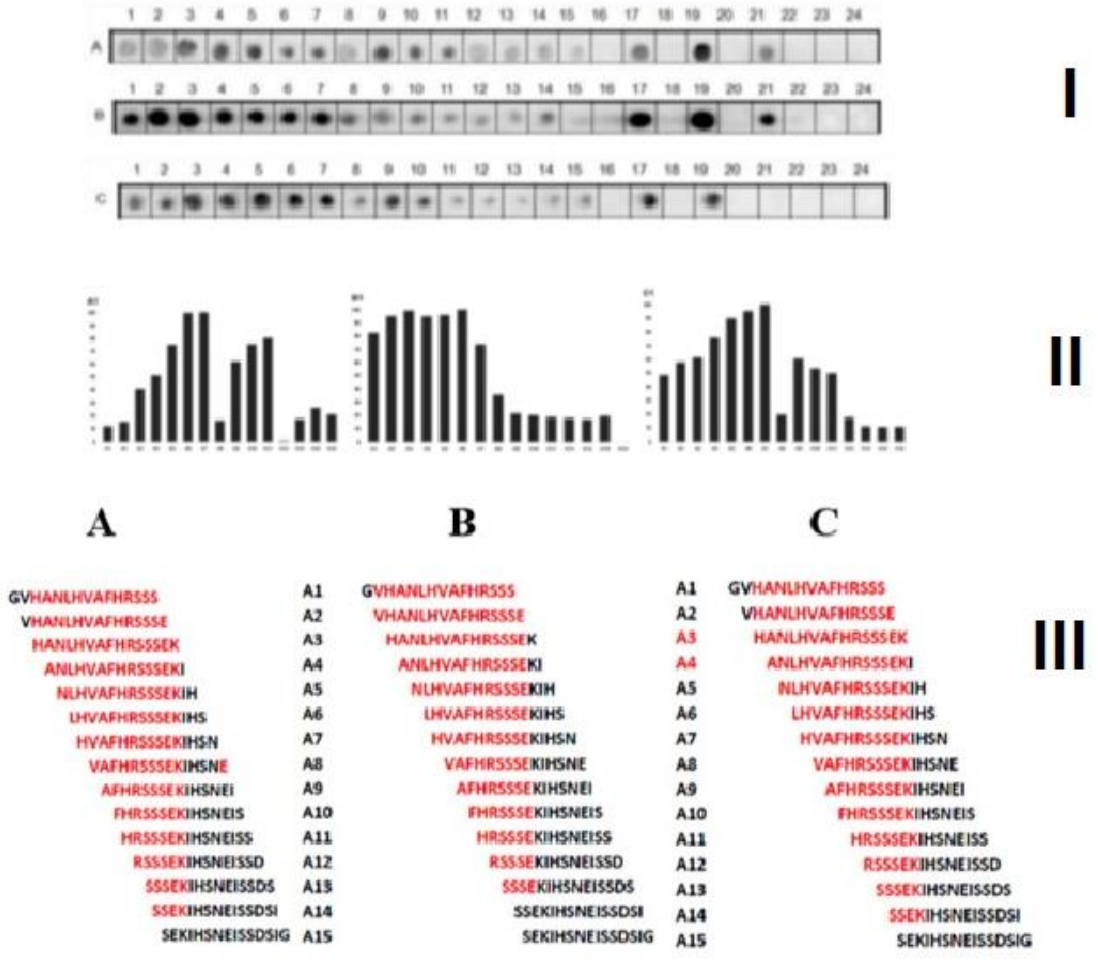

Figure 2. Reanalysis of the DTx sequence (aa 511-525) by Spot-synthesis (15 aa with overlapping of 14 residues) using huVS (A, 1:150), miVS (B, 1:150) and hoTS (C, 1:200). I, Spot membrane; II, measurement of the signal intensity; III,

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overlapping of positive spots, in red. Spots, 17,19,21 positive controls respectively [**KEVPAL TAVETGATN** (Poliovirus), **GYPKDGNAFNNDRI** (*Clostridium tetani*) and **YDYDVPDYAGYP YDV** (*H. influenza* virus hemagglutinin).

3.3. Cross-immune IgG epitopes

To investigate the cross-immunity conferred by the DTx protein, the *C. diphtheria* (Q6NK15), *C. ulcerans* (AQA1Y0HBB0, 97.5% similarity) and *C. pseudotuberculosis* (WP_014654963.1, 93.93% similarity) deposited in NCBI data bank were aligned to compare the epitope sequences. The results shown that 100% of the epitopes were common indicating that the immunity is genera specific (**Table S1).** In other analysis, the epitopes of the DTx protein were compared to the putative toxins sequences of *C. botulinum* and *C, tetani* but in this case no cross reactivity was detected **(data not shown).**

3.4. Epitope reactivity by ELISA

The serological cross-reactivity of bacterial proteins is well known. The impetus for identifying the linear B-cell epitopes in DTx was to improve on their use as antigens for diagnostic ELISA assays. To prove their utility, two bi-epitope peptides were synthesized from sequences derived from the potential **CB/DTx-2/miG (SFVMENFSSY) and CB/DTx-12/miG (VDIGF) epitopes and CB/DTx-4/miG (KGFYSTDNKY) and CB/DTx-13/miG (SPGHK)** (**Table 1),** from the major antigenic regions in DTx by solid phase synthesis using the F-moc strategy. The two 17 mer peptides were synthesized either as single peptides as 4 branch MAP (DTx2/12-MAP4 and DTx4/13-MAP4) contain the dipeptide GG inter peptides purified by HPLC-gel filtration and analysed by MASS-spectometry.

To verify the diagnostic performance of the peptides, sera from ninety-two individual DTP vaccined were tested on an ELISA-peptide based assay. All 90 DTP-positive human sera and 32 mice sera reacted with the highly conserved DTx2-12 and DTx4-13 peptides but did not react with the peptides EP7 [56] from ***T. cruzi*** (PPGEDMHTRDGPRE) considered by a scale value as a good test. Both CB/DTx2-12 and CB/DTx4-13 antigens demonstrated 100% and 99.96% sensitivity, respectively and 100% specificity (**Figure 3 C and D**). Similar results were obtained using either single or MAP4.

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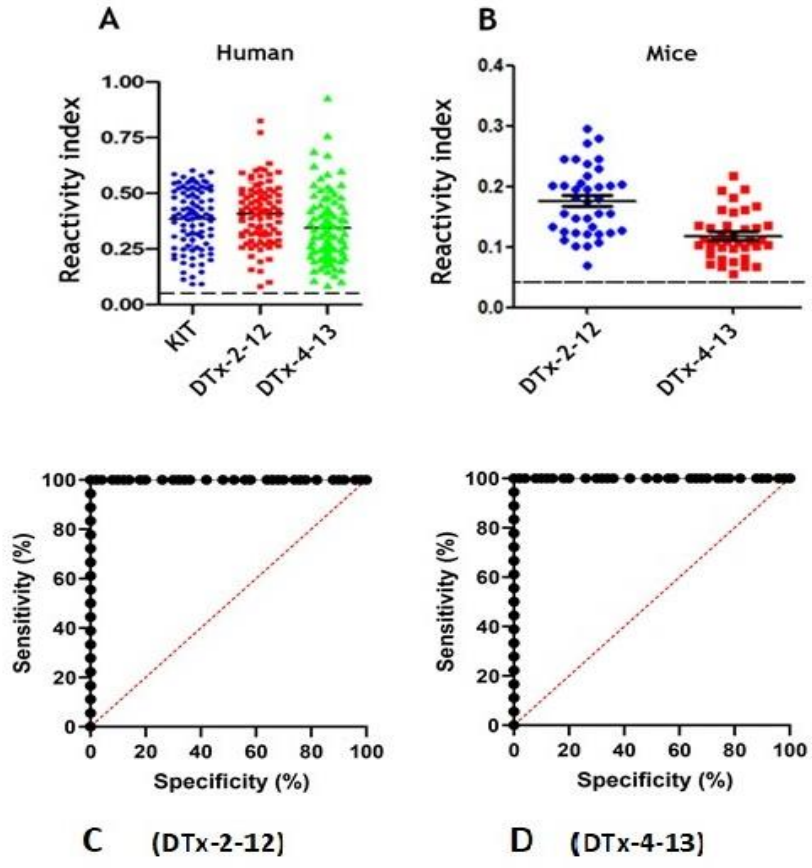

Figure 3. Reactivity of huVS (A, n= 90), and miVS (B, n=38) with synthetic bi-specific spanning epitopes MAP4 in an ELISA compared with a commercial Kit (A, n=90). The y-axis shows the mean reactivity (positive/mean negative) of sera from vaccined patients and mice. The ROC analysis (C and D) using huVS showed that the sensitivity of the in-house ELISA-peptide was 100% for DTx-2-12 and 99.96% for DTx4-13 in a range similar to the commercial kit and specificity of 100%.

3.5. The spatial location of the enterotoxins A, B, and P IgG reactive epitopes

To analyze the localization of epitopes in DTx, the crystallographic structure available in PDB (PDB: 1xdt) was used **(Figure 4),** it displays the spatial location of the 20 reactive epitopes identified by the SPOT-synthesis array experiments **(Table 1**). Most of the identified epitopes were in loop/coil structures, which were present on the protein surface and accessible to the solvent (data not shown).

In the **Figure 5** is presented a linear model showing the single transmembrane domains of the protein and the distribution intra and extracellular of the epitopes.

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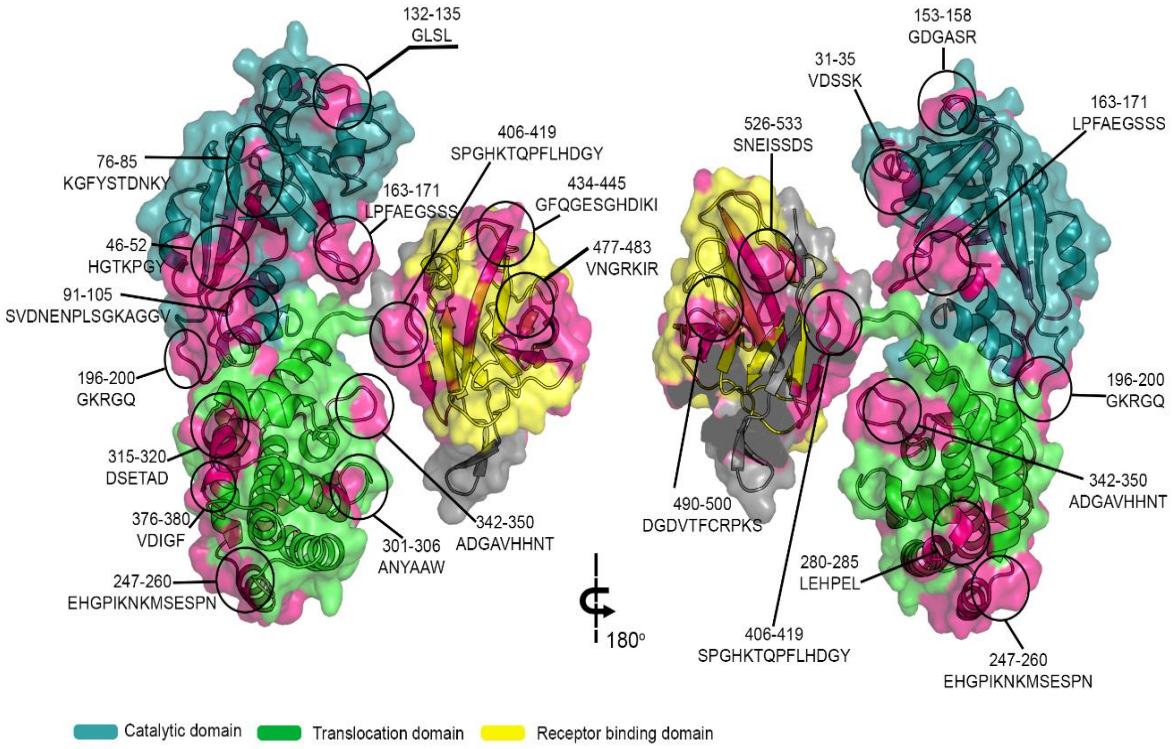

Figure 4. Three-dimensional structure of DTx (Q6NK15) with the signal peptide and position of the epitopes identified by Spot-synthesis. Molecular model overlay of neutralizing epitopes within the DTx protein. Image constructed using crystal structure of toxin (PDB:1xdt), showing catalytic (cyan), translocation (green) and receptor binding (yellow) domain. Identified epitope sequences were highlighted in magenta. Image was created using PyMOL.

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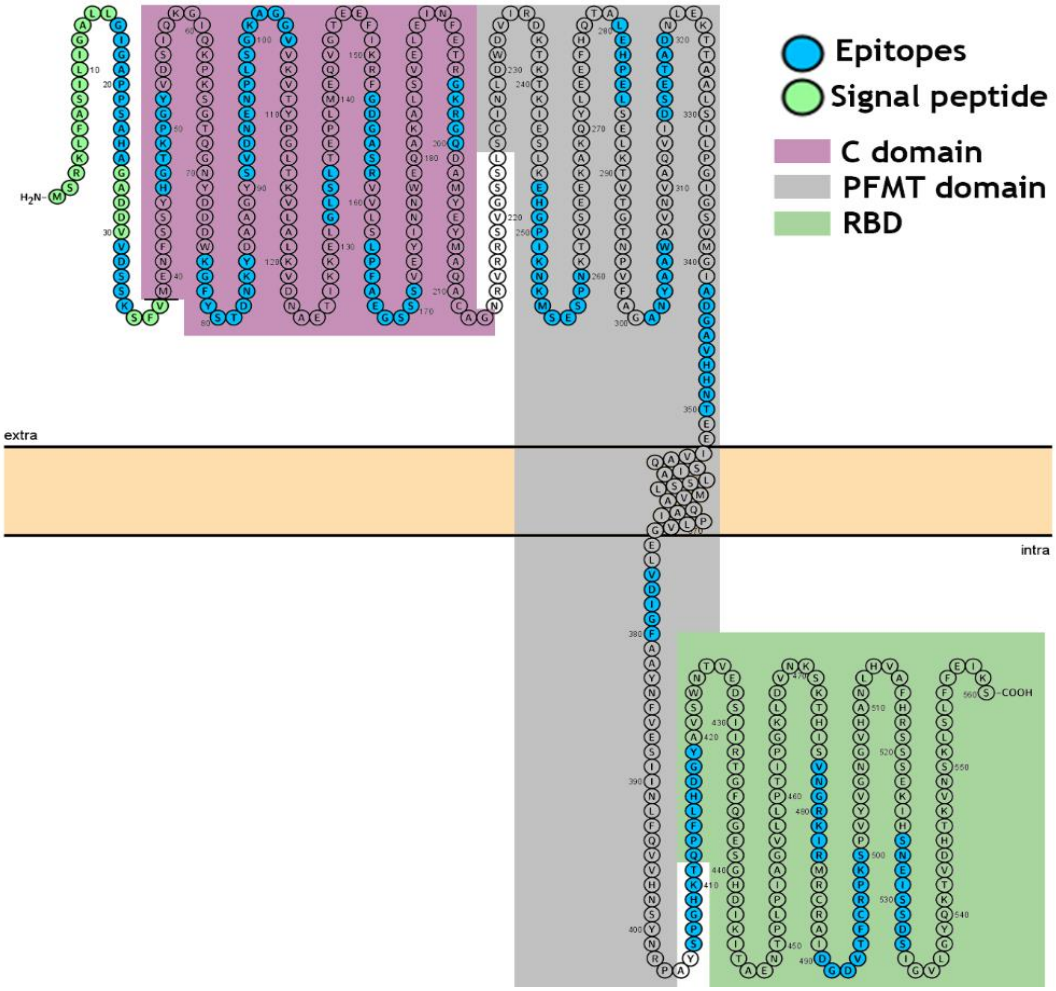

Figure 5. Modeling of DTx of *C. diphtheria* as a single-pass transmembrane protein. The model is based on the predictive results obtained using TopCons (https://topcons.net/pred), and layout was generated using Protter (http://wlab.ethz.ch/protter/). The model with the TM in the pore-forming membrane domain (PFMT), reveals all 20 identified epitopes. 1-38 signal peptide; aa39-aa188, C domain; aa200-aa378, PFMT domain and aa-387-aa535 RBD.

4. Discussion

The high-throughput immune-profiling peptide array formed directly onto cellulose membranes allowed the identification of the major antigenic determinants of the DTx recognized by sera of mice orally vaccinated with a single dose of the DTP. Twenty epitopes covering the full extent of the bacterial protein were identified **(Table 1).** This toxin is secreted by Corynebacterium and causes disease in humans by inhibiting protein synthesis. It possesses a molecular weight of 62 kDa and can exist as a monomer (when secreted) or dimer, with two sub-unities linked by SH bridges. Three well-defined domains were identified based on trypsin hydrolysis [16,17]. Two epitopes (CB/DTx-1-2) were positioned in the signal peptide (aa 1-aa38), seven epitopes (CB/DTx2-8) in the catalytic domain (CD, aa 39-188), six in the pore-forming membrane-translocation (PFMT) domain (CB/DTx-10-15, aa200-378), and five in the receptor bind domain (RDB, aa 387-535) (CB/DTx-16-20).

The spatial folding of proteins plays a decisive role in determining their antigenic specificity, and it has been useful to distinguish between sequential and conformational determinants. The current production process for DTx vaccines is largely based on the traditional methods first used to detoxify DTx [57]. Recently Metzel et al., [58] reveled the chemical modification of the DTx induced by formaldehyde treatment. The detoxification used for vaccine preparation typically resulted in a combination of intramolecular cross-links and formaldehyde-glycine attachments. In this process, both the NADþ-binding cavity and the receptor-binding site of diphtheria toxin are chemically modified.

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Functionally the process affected to some extension CD4þ T-cell epitopes but universal CD4þ Tcell epitope remained almost completely unaltered [58]. In our study, we can conclude that apparently the detoxification process does not interfere in the recognition of linear B epitopes, since antibodies present in the sera of mice immunized recognized almost of all linear epitopes of the DTx.

In a synthetic model, we have shown that all the twenty B-linear epitopes identified are positioned on the surface of the molecule, thus exposed to the immune system. Thus, based on the function of the three-domain either apparently, the entire 20 identified epitopes can present important functions as the blocking of the enzymatic activity of the DTx (**CB/DTx2-8),** or the intracellular translocation **(CB/DTx-10-15, aa200-378)** and sterically impairment of DTx receptor bind (**CB/Tx-16-20**). To our knowledge, there are only a few studies concerning the identification of DTx key epitopes responsible for vaccine protection. Studies conducted by others produced antibodies in guinea pigs against a synthetic peptide covering the sequence, aa186-201 [53]. This epitope corresponds to the epitope CB/DTx-9 (aa **196GKRGQ200).** Another study established a humAb that binds to the RDB domain and physically blocks the toxin from binding to the putative receptor, heparin-binding epidermal growth factor-like growth factor [52].

Another important motif of the DTx studied is the RDB. A beta-hairpin synthetic peptide (SF23, aa 526-548; **SIGVLGYQRTVDHTKVNSKLSLF)** has been considered as a promising candidate for the development of a synthetic vaccine [52]. This peptide binds to antibodies from the sera of persons infected by toxigenic *C. diphtheria,* those immunized by diphtheria toxoid, hoThe sera and block the interactions with HBEGF cellular receptor [52]. This beta-hairpin sequence encompasses only part of the CB/DTx-20 epitope (aa526-540) identified by us using mice and children immunized sera **(Table 1).** Due to the biological importance of this peptide sequence, a new round of Spot-Synthesis analysis was performed **(Fig. 3).** Now a small library containing mutated amino acids was synthesized and reacted with both human and mice vaccine sera and hoThe sera. This study demonstrated that a central peptide amino acid sequence is recognized by sera from miVc, huVac, and hoThe, but small N and C terminus aa variation recognition occurred depending on the vaccinated species **(Fig. 1B**).

In all clinical cases, the primary therapeutic option is still treated with equine hyperimmune sera anti-DTx. However, the production of therapeutic antibodies in horses raises ethical issues surrounding the use of animals, especially by substandard housing and veterinary care of the horses. Besides, the hoThe is of limited supply and often unavailable to patients due to interrupted production in several countries [59]. Hence is urgent to find alternatives to replace the equine sera anti-DTx.

One of these approaches has been the use of phage display to obtain neutralizing recombinant human antibodies [45]. However, the success of this methodology seems low, since repetitive epitopes with different sizes and longer sequences, encompassing at least six of our identified epitopes have been recently described [45]. On the other hand, a potent toxin neutralization would require blockage of several points or domains of the molecular surface, which could not be covered by a single antibody. Therefore, our epitope mapping on the planar surface provides superior information describing with greater coverage the set of IgG epitopes responsible for the neutralization of the DTx.

The epitopes identified in this study can serve as a basis for the construction of a recombinant polyprotein that can replace the DTx in vaccine preparations since were identified for antibodies from human, mice, and hoThe sera. The improvement of the vaccine would allow avoiding the detoxification stage, a fact that causes side effects, besides introducing in the process cost reduction with greater efficiency [57].

Another interesting finding in our study was the identification of 2 epitopes (CB/DTx 1-2) present in the signal peptide using serum from mice vaccinated with the DTP vaccine. The presence of human antibodies to one of these epitopes was also hardly detected by ELISA using sera from vaccinated infants **(Fig 4).** This fact means that a reasonable amount of diphtheria toxoid used in vaccination processes contains the signal peptide since it was able to stimulate the immune system. Although this fact is important in terms

of quality control of the production of immunizers, in terms of vaccination it should not represent any counterpoint or impediment. However, it should be noted that a significant amount of proteins did not mature during cell processing of vaccine manufacture.

Diphtheria is caused by toxin-producing Corynebacteria. Not only *C. diphtheria*e, but also *C. ulcerans* and *C. pseudotuberculosis* have the potential to produce DTx and hence can cause classic respiratory diphtheria as well as cutaneous diphtheria [11]. It is noteworthy that, in recent years, *C. ulcerans* infections recrudescence have been reported worldwide and fatal infections have occurred [25,28]. *C. diphtheriae* strains, whether toxigenic or nontoxigenic, have been found to cause severe and often fatal systemic infections also in immunized subjects [25-27,60,61]. DTx produced by the different corynebacteria (*C. diphtheriae, C. ulcerans*, and *C. pseudotuberculosis)* differ at the nucleotide and amino acid levels but are immunologically identical as shown in this work **(Figure S1).** Other similar toxins from *C. tetani* and *C, botulinum* were also analyzed and although some cross-reactivity against the SF23 peptide has been described [52], in our study no common epitope with those described for DTx was identified (data not shown).

Concerning the diagnosis, the ELISA results using the MAP4 bi-epitope peptides revealed that all epitopes appropriately discriminated between negative and positive samples ($p<0.0001$). No cross-reactivity was observed as anticipated from the BLAST analysis criteria. In comparison to the commercial kit, the use of the bi-epitope improved the performance of our in-house ELISA by generating a higher signal that showed a greater difference between positive and negative samples. From the ROC analysis, the sensitivity of the in-house ELISA-peptides was 99.96-100% similar to the commercial kit. This increase in signal most likely reflects the performance of the selected epitopes. Another benefit of the bi-epitope protein design was to increase the specific activity of the molecule to bind antibodies.

Phase IIA comprises studies designed to estimate the accuracy (sensitivity and specificity) of the index test in discriminating between diseased and non-diseased people in a clinically relevant population [62]. For diphtheria, the scope of the likelihood score of the ROC analysis was obtained since a much larger and diverse panel of negative controls was available to support our conclusion that the MAPs have 100% specificity. The performance of selected epitopes strongly supports continued using these epitopes now in a chimeric multi-epitope protein [63] as a target in more sensitive and fast diagnostic tests and suggest that the antigen may be eligible to enter phase IIB studies [60].

5. Conclusions

In this work, we have provided new insights describing with greater coverage the set of IgG linear B epitopes responsible for the neutralization of DTx and demonstrated the specificity and eligibility to enter phase IIB studies of some epitopes to develop new and fast diagnostic assays.

Supplementary Materials: The following are available online at **www.mdpi.com/xxx/s1,** Table S1: List of DTx peptide sequences (supplementary) A1-C24. (F3,F4,F5, F11,F12,F13 positive controls and F9,F17 negative controls. Figure S1: Alignment of the primary structure of DTx [(1-*C. diphtheria* (Q6NK15), 2-*C. ulcerans* (AQA1Y0 HBB0) and 3-*C. pseudotuberculosis* (WP_014654963.1)] showing in grey the epitopes (CB/DTx1-20) identified by Spot-synthesis.

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