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Article

Fine epitope mapping of the *Vibrio cholera* toxins A, B, and P and an ELISA assay

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Abstract: Oral immunization with the choleric toxin (CT) elicits a high level of protection against its enterotoxin activities and can control cholera in endemic settings. However, the complete B-cell epitope map of the CT responsible for protection remains to be clarified. Here, we have mapped the B-cell linear epitopes of the three chains of the CT protein (CTP) prepared by Spot synthesis. The immunoreactivity of sera from mice immunized with an oral, inactivated vaccine (Schankol†™) was measured against membrane-bound peptides for mapping. **Results:** Eighteen IgG epitopes were identified; eight in the CTA, three in the CTB, and seven in the protein P. Three epitopes, TQTGFVRHDDGYVST (aa 66-77, Vc/TxA-3), KNGAIFQVE VPGSQN (aa 64-78, Vc/TxB-11), and LNDEHK (aa 90-95, Vc/TxP-16), were chosen to synthesize a multiple antigen peptide that was used to coat ELISA plates to screen immunized mouse sera as a test for an *in vitro* diagnostics for cholera. **Conclusion:** Vaccination with inactive CT-generated antibodies against multiple linear epitopes and the selected epitopes can be used to construct immunological reagents for rapid serological diagnosis of cholera with high sensitivity and specificity.

Keywords: Cholera toxin, B-cell epitope, peptide microarray, synthetic peptide, MAPs, ELISA.

1. Introduction

The gram-negative bacterium *Vibrio cholera* causes cholera, a life-threatening diarrheal disease that is spread by ingesting contaminated food or water. This pathogen continues to be important globally, with epidemics occurring largely in developing countries that lack sufficient infrastructure to treat sewage and provide clean water, such as Southeast Asia, Latin America, and parts of Africa. Worldwide, 1.3-4 million cases of cholera and 20,000-140,000 cholera-related deaths are recorded yearly, with ~50% children aged five

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years or younger [1,2]. In addition, climate change and warming temperatures have spread *V. cholerae* to new geographical areas highlighting the urgent need for further research to improve our understanding of this pathogen [3,4].

The strains of *V. cholerae* known to cause disease, and pandemics, are categorized in the O1 or O139 serogroups. While most *V. cholerae* are "non-O1/ O139" environmental strains, they may or may not cause some form of gastroenteritis [5]. The disease is highly contagious and characterized by symptoms such as the classical profuse-watery diarrhea, known as "rice-water stool," which often leads to extreme dehydration, shock, and eventual death [6]. This watery diarrhea is flecked with mucus, intestinal epithelial cells, and bacteria. A normal functioning mucus gel layer covering intestinal epithelial cells serves many functions, including a dynamic defensive barrier against both resident and foreign microbes [7]. The secretion of this mucus gel layer is the primary function of a specialized group of cells lining mucosal tissue, called goblet cells [8]. Alteration of their mucin production is hypothesized to occur in one of two ways: 1) by microbial factors that modulate mucin either its synthesis, secretion, or altering its chemical composition, or 2) by host factors that are released by local epithelial cells or immune cells in response to intestinal microbes [9,10]. One consequence of a *V. cholerae* infection is the large secretions of mucus partially attributable to the effects of cholerae toxin (CT) that induces the release of massive amounts of mucin via a cAMP-dependent mechanism [11, 12]. *V. cholerae* has also been shown to penetrate mucus layers using its flagellum, while non-motile *V. sp* are significantly less efficient at colonization or even avirulent [13]. According to WHO reports, more than 80% of cholera patients can be successfully treated via oral rehydration therapy [14].

The toxin is an 84 kDa protein that belongs to the AB class of bacterial toxins. The heterodimeric holotoxin A subunit (CTA) has a molecular weight of 27.2 kDa and is responsible for the toxicity of the bacteria, while the homopentameric B (five identical 11.6 kDa polypeptides) subunit (CTB5) has a molecular weight of 85.2 kDa. It binds the holotoxin to the eukaryotic cell receptor to transport the bacteria into the cell. The A subunit has a specific enzymatic function that acts intracellularly [15]. The intracellular target of CT is adenyl cyclase, one of the most important regulatory systems in eukaryotic cells. This enzyme mediates the transformation of ATP into cyclic AMP (cAMP), a crucial intracellular messenger in various cellular processes. In addition, CT catalyzes ADP-ribose transfer from NAD (Nicotinamide Adenine Dinucleotide) to a specific arginine residue in the target (Gsa) protein.

Consequently, this results in the activation of adenyl cyclase and, therefore, an increase in the intracellular level of cAMP that activates cAMP-dependent protein kinase, which leads to protein phosphorylation, altered ion transport, and diarrhea with its massive fluid loss [16]. Therefore, public health interventions are critical to limit cholera dissemination that can easily become epidemics, particularly in areas without proper sanitation and clean water. Importantly, many cholera survivors are immune to a second infection showing that immunity can be acquired [17].

Immunization with cholera vaccines is an important strategy to control and prevent cholera epidemics or pandemics. *V. cholerae* virulence factors ranging from CT [18], toxin-coregulated pili [19], lipopolysaccharide (LPS) [20] to outer membrane proteins (Omps)

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[21-23] are ideal candidates for designing a cholera vaccine. Today, two types of oral vaccines are available; attenuated oral cholera vaccines (aOCVs) and killed oral vaccines (kOCVs). The aOCVs consist of attenuated, whole *V. cholerae* cells that display both direct and indirect (herd) efficacy in protecting populations in endemic regions [24,25]. These vaccines were recently deployed during outbreaks in non-endemic areas as part of "reactive" vaccination programs to block the spread of cholera [26,27]. kOCVs are effective in inducing heard protection for at least three years and are effective tools for cholera control. In addition, one dose provides at least short-term protection, which has important implications for outbreak management. However, the optimal efficacy of kOCVs requires two doses administered 14 days apart and needs refrigeration [28]. These features may limit the capacity of kOCVs to rapidly constrain ongoing outbreaks in destabilized or resource-limited settings. Nevertheless, single-dose, live aOCVs have shown satisfactory efficacy in challenge studies [29] and early-phase clinical trials in endemic regions [30]. The design of reactive vaccination programs with a live aOCV may have the best chance of decreasing the incidence of cholera during outbreaks [31,32].

Oral cholera vaccination is a rapid approach to preventing outbreaks in at-risk settings and controlling cholera in endemic settings [33-35]. However, the benefits of vaccine-derived herd immunity may be short-lived due to human mobility combined with waning vaccine efficacy. As the supply and utilization of oral cholera vaccines expand, critical questions must be addressed concerning the coverage and vaccine application schedule necessary to generate herd immunity [36]. Serological diagnostic assays have the potential to be employed for large population studies to provide information on the duration of the immune response that could be improved by the identification of epitopes in CT that are recognized by the antibodies produced by a vaccine, which would advance our understanding of response continuance and herd protection.

An efficient, high-content screening method for epitope mapping involves screening peptide libraries that represent target coding sequences. By synthesizing peptides directly onto cellulose membrane, it is possible to generate refined maps of the complete epitome in viruses and the hundreds of epitopes possible in a large protein [37,38]. Commonly referred to as SPOT synthesis analysis, this strategy was applied here to identify the linear B-cell epitopes in three toxins from *V. cholerae*, enterotoxins A (8), B (3), and P (7). A total of 18 linear B epitopes were identified. Three were chosen to perform peptide ELISAs and evaluated using sera from mice vaccinated with oral cholera. Two specifically reactive sequences were observed, further validating the systematic definition of pathogen epitomes.

2. Results

2.1. Identification of the immunodominant IgG epitopes in cholera toxin

Epitopes in the three components of CT, CTA (258aa), CTB (124aa), and CTP (221 aa), were identified based on the recognition of representative peptides, synthesized as a library by mice antibodies immunized with oral *V. cholera* vaccine (see Materials and Methods). The data in **Figure 1A** and **Figure 1B** present the position of each peptide and the measured intensity, respectively, from the chemiluminescent detection of human IgG antibodies present in sera pooled from mice vaccinated with aOCV. Intensities were normalized by defining 100% using a positive control (data not shown). The list of the synthetic

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peptides and their positions on the membrane is presented in supplementary **Table 1**. The sequences constituting the reactive peptides defined 18 IgG epitopes generated by vaccination with aOCV (**Table 2**).

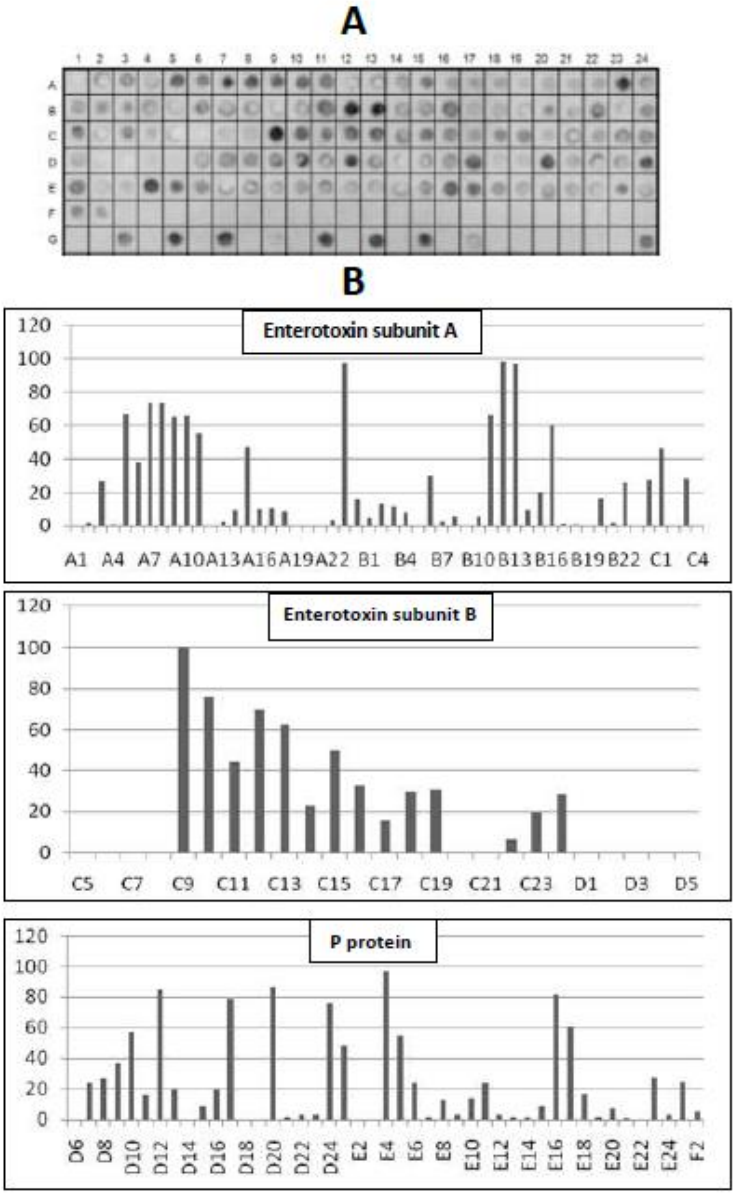

Figure 1: Fine epitope mapping of *Vibrio cholerae* toxin A (P01555), B (P01556), and P (P29485). A membrane-bound peptide library representing the three chains of the toxin was probed with a pool of vaccinated mouse sera (n=15), and reactivity was detected by sheep anti-mouse IgG alkaline phosphatase labeled secondary and chemiluminescence substrate. Panel A- Image of the peptide array showing reactivity as dark circles. Panels B graphically presents the percent signal based on normalizing the positive and negative controls. The sequences of peptides in each position are listed in **Supplementary Figure 1**.

2.2. Localization of the major B-epitopes within the three enterotoxins

The 18 linear B-epitopes identified by the SPOT-synthesis analysis were distributed throughout Vc/TxA, Vc/TxB, and Vc/TxP (**Figure 2).** The enterotoxin chain A1 contains six epitopes (Vc/TxA-1 to Vc/TxA-6), the chain A2 houses two epitopes (Vc/TxA-7 and

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Vc/TxA-8), the enterotoxin B has three (Vc/TxB-9 to Vc/TxB-11), and seven epitopes are present in enterotoxin P seven (Vc/TxP-12 to Vc/TxP-18) **(Table 1).**

Table 1. List of identified miIgG epitopes in the TxA, TxB, and P.

Protein Code	aa	Sequence	2nd Structure*	Code	Peptide search**
P01555	26-30	ADSRP	C	Vc/TxA-1/miG	Various bacteria
	37-45	SGGLMPRGQ	C	Vc/TxA-2/miG	Sp
	66-77	TQTGFVRHDDGY	C	Vc/TxA-3/miG	Sp
	108-115	TAPNMFNV	C	Vc/TxA-4/miG	*E. coli*
	176-180	AADGY	C	Vc/TxA-5/miG	Various bacteria
	191-204	AWREEPWIHHAPPG	C	Vc/TxA-6/miG	Sp
	244-245	YQSD	C	Vc/TxA-7/miG	Various bacteria
	250-258	THNRIKDEL	C	Vc/TxA-8/miG	Sp
P01556	20-25	HGTDQN	C	Vc/TxB-9/miG	Sp
	53-56	AGKR	C	Vc/TxB-10/miG	Sp
	64-77	KNGAIFQVEVPGSQ	C+S+C	Vc/TxB-11/miG	Sp
P29485	17-21	NECTN	C	Vc/TxP-12/miG	Various bacteria, virus
	26-40	AQDPMKPERLIGTPS	C+S+C	Vc/TxP-13/miG	Sp
	53-59	YHPAPCP	C	Vc/TxP-14/miG	Sp
	68-77	WPHGFISSESL	C + H	Vc/TxP-15/miG	Sp
	90-95	NDEHKT	C	Vc/TxP-16/miG	Sp
	112-121	VIVSENVVDE	S + C	Vc/TxP-17/miG	Sp
	174-177	GITH	C	Vc/TxP-18/miG	Various bacteria

Sp, specific epitopes; C, coil; H, helix; S, strand; *based on an I-TASSER analysis; **UNIPROT

2.3. Specific and cross-immune IgG epitopes

To investigate possible cross-immunity conferred by the CT proteins, the sequences of CTA, CTB and CTP were used as templates in a multi-peptide match (**Error! Hyperlink reference not valid.**) that searched for peptides deposited in the UniProtKB data bank. Search criteria were for four or more identical and consecutive amino acids. This analysis suggested that 12 epitopes from *V. cholerae* are expected to be specific **(Table 1)**, while five (**Vc/TxA-1, Vc/TxA-5, Vc/TxA-7, Vc/TxP-12, Vc/TxP-18**) were expected to display cross-reactivity with various bacteria**.**

2.4. Epitope reactivity by ELISA

Serological cross-reactivity with bacterial proteins is well known. Therefore, the impetus for identifying the linear B-cell epitopes in cholerae enterotoxins was to improve diagnostic ELISA assays by using them as antigens to capture reactive antibodies. To that

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end, three epitopes were chosen, one from the major antigenic region of chain A1 (aa 37-45; Vc/TxA-2 epitope), one from the chain B (aa 20-25; Vc/TxB-9), and one from the protein P (aa 68-77; Vc/TxP-15), to generate multi-antigen peptides (MAPs) by solid-phase synthesis using the F-moc strategy.

To confirm the diagnostic performance of the peptides, sera from twelve mice vaccinated with oral cholera vaccine and twelve healthy mouse sera were screened by peptide-based ELISA assays of each epitope sequence. Sera from each immunized mouse reacted with all three synthetic peptides derived from *V. cholera* without reacting to the negative control of the peptide QEVRKYFCV (*Vaccinia virus;* Figure 1A-G9, G17). No reactivity was observed with the sera obtained from pre-vaccination animals. The results are shown in **Figure 2** and were calculated to be significant ($p < 0.001$). From a ROC curve analysis, the best values associated with the epitope Vc/TxB-11 were considered satisfactory (Figure 2,2). Vc/TxA-2 and Vc/TxB-9 epitopes demonstrated 100% sensitivity and specificity. As the reactivity to Vc/TxP-15 peptide was weak, a ROC curve was not determined.

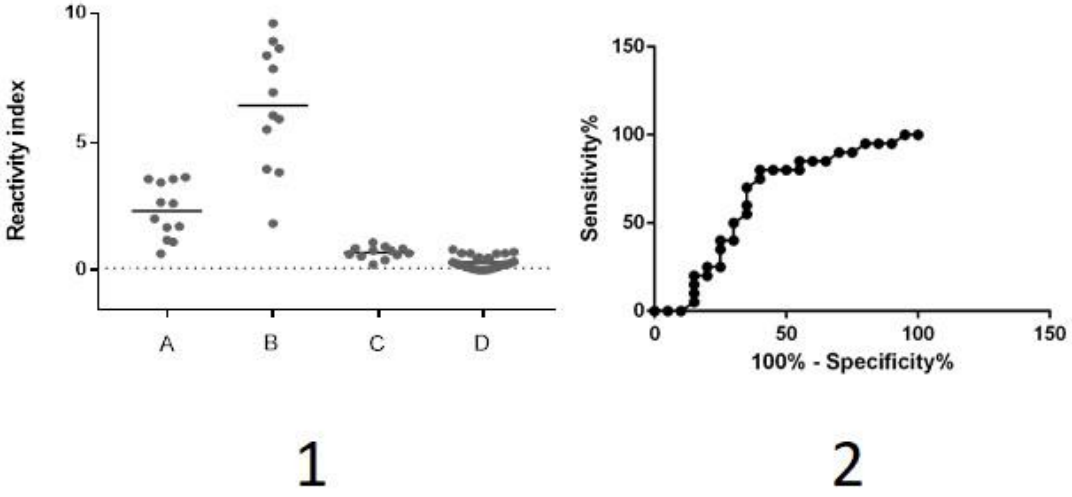

Figure 2. Reactivity of vaccinated mice sera (n=12) against the MAP-4 peptide (A) Vc/TxA-3, (B) Vc/TxB-11, and (C) Vc/TxP-15 by ELISA. As a control, sera from non-vaccinated mouse sera (n=30) was tested against Vc/TxB-11 (D) (1). The reactivity index was calculated using the cut-off of each MAP to normalize the measured value from the sera of 12 vaccinated mice. The ROC curve was used to determine the cut-off of the peptide B (Vc/TxB-11; 0.053), reactivity (100%), specificity (100%), and AUC (0.9987) (2).

2.5. Spatial location of the enterotoxins A, B, and P IgG reactive epitopes

The structures of the enterotoxin A, B, and P proteins, were obtained from the protein data bank (PDB) or predicted using AlphaFold DB to access the location of the epitopes. Alternatively, the tertiary structure was performed using the I-TASSER server (http://zhanglab.ccmb). Predicted structural models of the enterotoxins A, B, and P proteins were obtained (**Figure 3A-C**) and displayed the possible spatial localization of the eight reactive epitopes in CTA, the 3 in CTB, and the seven epitopes in CTP. Most of the

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identified epitopes were in loop/coil structures, which were present on the protein surface and accessible to the solvent (**Figure 3**).

The eighteen linear B-epitopes identified by the Spot-synthesis analysis were distributed throughout the *V. cholera* toxin proteins (Figure 3). The *V. cholera* protein gene contains three well-defined segments: a short signal peptide in the N-terminal extension (aa 1-18), the A1 subunit chain segment (aa 19-212), the A2 subunit chain segment (aa 213-258), the B subunit (aa 1-124) and the P protein (aa 1-221). Sequence alignments were performed to evaluate the specificity of discovered epitopes. In CTA, four epitopes were highly specific (SGGLMPRGQ, TQTGFVRHDDGY, AWREEPWIHHAPPG, THNRIKDEL), all three epitopes identified for CTB were specific as well as five sequences observed in CTP (AQDPMKPERLIGTPS, YHPAPCP, WPHGFISSESL, NDEHKT, VIVSENVVDE).

3. Discussion

Vaccine production is usually a laborious and costly experimental process. Nevertheless, these immunobiological molecules are the most potent countermeasure to prevent infectious diseases. Today, two different types of cholera vaccines exist that are commercially available or under development: inactivated [such as Dukoral® (Crucell, Netherlands, 1990, denatured vCh-), mORCAX™ (VaBiotech, Vietnam) and Sanchol™ (Shantha, Biotechnics-Sanofi Pasteur, India) and live attenuated (like CVD103-HgR and Peru-15 or CholeraGarde) [39,40]. Although it has been proven that both types are protective for cholera, the immunological mechanisms of the protective effect are unknown. It is recognized that the most important stimulators of innate immunity and the subsequent adaptive immune response are the LPS O antigen and CT [6,17], which activate the NF-κB and IL-1 systems, critical factors for promoting long-term mucosal protection [8,13,40,41]. However, less is known about the immunodominant epitopes that interact with the antibodies generated in response to vaccination.

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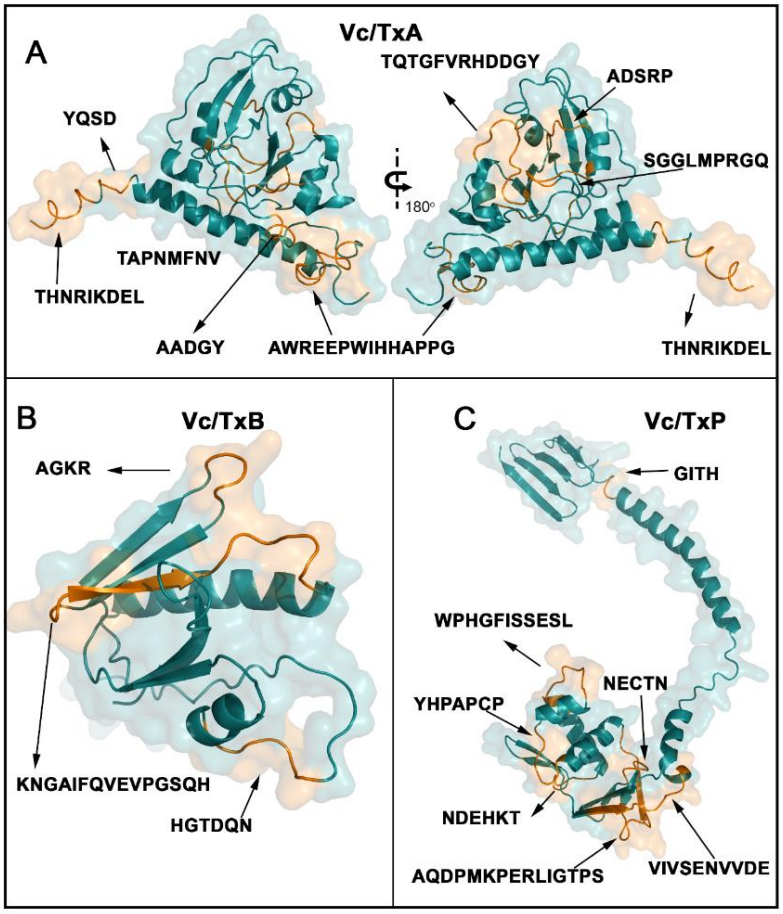

Figure 3: Three-dimensional structure of *Vibrio cholerae* toxins A, B, and P. Image constructed using the crystal structure of toxin A (PDB: 1xtc), and protein structure prediction from AlphaFoldDB of toxin B and P. Identified epitopes in toxins 3D structures were highlighted in orange. Images were created using PyMOL.

In this work, we experimentally identified all the linear B epitopes in CT recognized by IgG antibodies from mice vaccinated with the inactivated Sanchol™ vaccine by a peptide microarray analysis. CT is the primary virulence factor of *V. cholerae*, and ingestion of as little as 5 g produces the symptoms of cholera [42]. The reactivity of the serum from vaccinated animals against a subset of regions in CT showed that the vaccine has a high antigenic potential with only a single dose. Eight B linear epitopes were readily identified in CTA, three in CTB, and seven in protein CTP. All eighteen epitopes were exposed on the molecular surface and accessible to the immune system **(Figure 3).**

The CTA subunit is synthesized as a single polypeptide with a signal peptide (aa 1-18). Enzymatic activity is initiated after proteolytic cleavage, "nicking," at residue Arg-192, which produces two functional domains, A1 (~21.8 kDa, aa19-204) and A2 (~5.4 kDa, aa206-258). The two fragments remain covalently associated by a disulfide bond. The B subunit contains five chains that form a pentameric ring around a central pore structure. CTA1 is responsible for the ADP-ribosyltransferase activity, and the alpha-helical CTA2 has an α–helix tail that moors the CTA1 and CTB5 subunits together [43–46]. CTB is a non-toxic subunit that produces target cells blocking antibodies and is a potent mucosal immunostimulatory adjuvant that can incite potent mucosal and system immune responses [47]. Because of the strong adjuvanticity of CTB, it is applied in different peptide and DNA

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vaccines such as microbial and cancer [48,49]. In addition to playing a role as an immunostimulatory adjuvant, CTB is also a powerful protective antigen.

CTA and CTB seem to have the greatest protective importance of the three chains that constitute the cholera toxin. Initially, CTA was not considered to have immunological importance, despite having the toxic portion of the molecule. However, this assumption has been recently revised by recent studies that showed antibodies anti-CTA capable of toxin neutralization at very low concentrations in persons living in areas where cholera is endemic. This ability has been attributed to CTA epitopes cross-reactive with the enterotoxigenic *Escherichia coli* (ETEC) heat-labile toxin (LT) [50] together with the frequent exposure of people living in endemic areas to ETEC LT toxin [51]. Our study mapped three epitopes in CTA that present extensive cross-reactivity potential with bacterial toxins (Table 1), more than the other subunits.

Studies describing the identification of CT epitopes are rare in the literature. Nevertheless, previous cross-reaction studies, using polyclonal rabbit sera and B-toxin synthetic peptides neutralization, had identified the presence of three immunogenic peptides, aa 8-20, aa 50-64 (anti-CP3) [52], and aa 69-85 in the CTB [53]. However, our studies also identified similar immunogenic peptides by sera from mice orally vaccinated (Table 1). They were identified as Vc/TxB-9miG (aa20-25), Vc/TxB-10/miG (aa53-56) and Vc/TxB-11/miG (aa64-77). In another study in mice, it was demonstrated that the immune response anti-CTB specific was dominant and protective [54]. The observation supports this hypothesis that circulating levels of CTB-IgG antibodies and CTB-specific memory B cells wane within months after natural infection [55,56] and in human challenge studies [47,57]. Together, the CTB provides only a short-term immunity boost [55]. More concerning, the overall protection with the CTB-containing vaccine appeared to drop to lower levels than the non-CTB-containing vaccine within two years after vaccination [47]. Therefore, the three epitopes (Vc/TxB-9/miG, Vc/TxB-10/miG, and Vc/TxB-11/miG) identified in the B subunit should be of interest for vaccine development despite descriptions against lasting protection conferred by the B subunit. Concerning CTP, no detailed studies on its immunogenic importance were found. However, six of the seven identified epitopes are unique and can potentially be used in developing chimeric polyproteins for diagnostic purposes or pave the way to developing safer vaccines.

Concerning serological diagnosis, the ELISA results using the MAP4 bi-epitope peptides revealed that all epitopes appropriately discriminate between negative and positive samples ($p < 0.0001$). Further, no cross-reactivity was observed, as anticipated from the BLAST analysis criteria. Another benefit of the bi-epitope MAP design was to increase the specific activity of the molecule to bind antibodies, an important step to phase IIA 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 [58]. The performance of selected epitopes strongly supports the continued use of these epitopes with a chimeric multi-epitope protein [59] as a target in more sensitive and fast diagnostic tests and suggests that the antigen may be eligible to enter phase IIB studies [60].

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4. Materials and Methods

4.1. Immunization of mice

Thirty Balb C mice (15-21g) were immunized orally (20 µl) with whole attenuated Schancol ™ cholera vaccine (lot SCN021A15) produced by Shantha Biotechnics Ltd (Hyderabad**,** India**)**. Fifteen days after vaccination, the serum of 15 animals was obtained a full bleed was followed at 30 days. In addition, serum from 30 healthy animals was collected for control. Each group's serum was collected separately, distributed in aliquots of 0.5 ml Eppendorf tubes, and stored at -20 °C.

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

The entire sequence of the cholera enterotoxin A (P01555, 258 aa), enterotoxin B (P01556, 124aa), and Toxin coregulated a library of 15-aa peptides represented pilus biosynthesis protein P (P29485, 221aa) serotype O1 with a ten residue overlap that was automatically synthesized on a cellulose membrane according to standard SPOT synthesis protocols using an Auto-Spot Robot ASP-222 (Intavis Bioanalytical Instruments AG, Köln, Germany) [61]. A **GSGSG** sequence was used as a spacer at the beginning and end of each protein sequence (spots A1, D5, D6, C4, C5, F2). Library construction and program execution were conducted with the Multipep program (Intavis). Negative [**QEVRKYFCV,** *Vaccinia virus* spots G9 and G17] and positive [**KEVPALTAVETGATN** (Poliovirus, spots G3 and G11)/**GYPKDGNAFNNLDRI** (*Clostridium tetani,* spots G5 and G13)/**YDYDVP DYA GYPYDV** (*Influenza virus* hemaglutinin, spots G7, G15, and G24)] were included as controls, respectively. The entire library contained 122 peptides plus nine 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 adding piperidine to make the nascent peptides reactive. This same coupling, blocking, and deprotection process added the remaining amino acids until the desired peptide was generated. After adding 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 [62]. Membranes containing the synthetic peptides were probed immediately. Negative and positive controls were included in each assay.

4.3. Screening of SPOT membranes

SPOT membranes were washed for 10 min with TBS-T (50 mM Tris,136 mM NaCl, two 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=10) of vaccinated mouse 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 1 h, and then washed with TBS-T and CBS (50 mM citrate-buffer saline). Finally, the chemiluminescent substrate Nitro-Block II was added to complete the reaction.

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4.4. Scanning and measurement of spot signal intensities

Chemiluminescent signals were detected on an Odyssey FC (LI-COR Bioscience) using the same conditions described previously [63] with minor modifications. Briefly, a digital image file was generated at a resolution of 5 MP, and the signal intensities were quantified using the TotalLab TL100 (v 2009, Nonlinear Dynamics, USA) software. This program 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 the Microsoft Excel program. To be considered an epitope, the sequences of two or three 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 identification was made visually when three or more contiguous spots were positive. The Spot-synthesis and ELISA selected the singular spots with SI greater than or equal to 30% of the highest signal value obtained for a new round of analysis. The negative control spotted in each membrane was the SI used as a background.

4.5. Preparation of the multi-antigen peptides

For the preparation of the dendrimer multi-antigen peptides (MAPs), a solid phase synthesis protocol was performed with tetrameric Fmoc4-Lys2-Lys-B-Gly Wang resin as described previously [64]. The peptides possessed the sequence: A) Vc/TxA-3 (*GG*TQTGFVRHDDGY*G*), (B) Vc/TxB-11 (*G*KNGAIFQVEVPGSQ), (C) Vc/TxP-13 (*GGGG*NDEHK T*GGGGG*). The constructs were prepared in an automated peptide synthesizer (MultiPep-1 CEM, Corp, Charlotte, NC, USA), and the side chains of tetrafunctional Fmoc-amino acids were protected with TFA-labile protecting groups as required. Once sequence assembly was completed, the Fmoc groups were removed, and the peptide-resin was cleaved and fully deprotected with TFA/H2O/EDT/TIS (94/2.5/2.5/1.0 v/v, 90 min). The peptides were precipitated by adding chilled diethyl ether, centrifuged for 3 × 10 min at 4 °C, and the pellet was dissolved in aqueous AcOH (10% v/v) dried and stored as a lyophilized powder. When necessary, the MAP was dissolved in water, centrifuged (10.000g, 60 min, 15°C) and the supernatant filtered by a Centricon™ ten filter. Purification of the MAPs was done on an RP-HPLC using a Schimadzu C18 (21.2 × 250 mm, 10 μm) column coupled to an HPLC system (model 7a; Schimadzu, Kyoto, Japan) at a flow rate of 1.2 ml min^{-1} using a gradient (7-40%) of solvent B into A [A and B: 0.07% TFA (v/v) in water and acetonitrile, respectively]. UV detection at 220 nm was used to identify fractions of good purity (⩾95%) that were pooled and lyophilized with their identity confirmed by MS (MALDI-TOF or electrospray).

4.6. ELISA

Peptide-based ELISA assays were performed as described previously [65]. Briefly, wells of 4HB NUNC plates for ELISAs were coated with 50 ng of each peptide in 100 μl of coating buffer ($Na2CO3–NaHCO_3$, pH 9.6) overnight at 4 °C. Next, 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 2.5% BSA over 2h at 37 °C. Next, mouse sera were diluted (1:100) in coating buffer and 100 μl applied and incubated for 2h at 37 °C. Following several washes with PBS-T, plates were incubated with 100 μl with goat, anti-mouse IgG conjugated to

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alkaline phosphatase (1:5000 dilutions at blocking buffer; Sigma Merck, Saint Louis, MO, USA) for 2h, washed, and exposed to PNPP substrate (Sigma, Saint Louis, MO, USA). Absorbance at 405 nm was measured on a FlexStation 3 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

4.7. Data interpretation and statistical analysis

The best sera dilution to segregate the IgG reactivity of vaccinated mouse sera was initially established along the titration curve, considering the highest difference between the median OD for positive and negative samples (delta median - Δ) **(Figure 2).** After that, the sera dilution shown to have a higher performance by constructing the receiver operating characteristic curve (ROC) using the Medcalc statistical program (Broekstraat, Mariakerke, Belgium) was selected and identified as the most useful condition to discriminate vaccinated mice from non-vaccinated. Finally, the selected conditions were used to evaluate the IgG reactivity of sera samples from vaccinated mice with a control peptide (Figure 2D). The sensitivity and specificity intersection point (θ_0) cut-off provides equal test indices.

4.8. Structural localization of the IgG epitopes and bioinformatics tools

To ascertain the location of the epitope within the 3D molecular structure of the enterotoxin A and B and toxin coregulated pilus biosynthesis protein P (Toxin P) from *V. cholerae*, we performed *in silico* protein models using the I-TASSER server [http://zhanglab.ccmb]. Models were chosen according to best C-score and TM-score (topological evaluation value) [66]. The resulting 3D structural models were also analyzed in the AlphaFold database [67]. Data bank searches for *V. cholerae* sequence homologies were performed using previously identified sequences and annotated proteins in other organisms in the database UniProt [http://www.uniprot.org/] and multiple peptide match [https://research.bioinfor matics.udel.edu/ peptide match/index.jsp].

5. Conclusions

A high-throughput immune-profiling using peptide arrays generated directly on cellulose membranes facilitated the identification of the major antigenic determinants in the enterotoxin A, B, and P recognized by sera of mice orally vaccinated with a single dose of the aOCV. Eighteen IgG epitopes were identified, covering the full extent of the bacterial CT proteins. Six epitopes were distributed in chain A1 from the enterotoxin, two in the A2, and three were identified in the enterotoxin B. In contrast, seven were in the enterotoxin P. Six cross-reactive epitopes, twelve specific epitopes were defined, and their locations were found in structural models by bioinformatics. The specificity of three distinct epitopes (Vc/TxA-3, Vc/TxB-, and Vc/TxP-15**)** was confirmed by an indirect ELISA. The molecular characterization of the IgG epitopes of the cholera enterotoxins exposes potential benefits, both from an applied perspective and a basic research perspective, and confirms that the oral vaccine is effective in inducing long-lasting specific neutralizing antibodies. In addition, several of the identified epitopes can be used in the construction of a chimeric polyprotein to be used in the diagnosis of cholera.

Supplementary Materials: The following are available online at https://zenodo.org/record/6857643#.YtWe3nbMJvw10.5281/zenodo.6857643. Supplementary Figure 1- List of *Vibrio cholerae* [toxin A (P01555), B (P01556), and P (P29485)] synthetic peptides and position in the cellulose membrane of Spot synthesis.

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Institutional Review Board Statement: The study was approved by the UNIGRANRIO (CEUA 052/2021) study center ethics committee and conducted under good clinical practice and all applicable regulatory requirements, including the Declaration of Helsinki.
Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author

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References

1. Ali, M.; Nelson, A.R.; Lopez, A.L.; Sack, D.A, Updated global burden of cholera in endemic countries. *PLOS Negl Trop Dis* **2015**, *9*, e0003832. doi: 10.1371/journal.pntd.0003832
2. Yuki, Y.; Nojima., M.; Hosono, O.; Tanaka, H.; Kimura, Y.; Satoh, T.; Imoto, S.; Uematsu, S.; Kurokawa, S.; Kashima, K.; Mejima, M.; Nakahashi-Ouchida, R.; Uchida, Y.; Marui, T.; Yoshikawa, N.; Nagamura, F.; Fujihashi, K.; Kiyono, H. Oral MucoRice-CTB vaccine for safety and microbiota-dependent immunogenicity in humans: a phase 1 randomized trial. *Lancet Microbe* **2021**, *2*, e429-e440. doi: 10.1016/S2666-5247(20)30196-8
3. Vezzulli, L.; Grande, C.; Reid, P.C.; Hélaouët, P.; Edwards, M.; Höfle, M.G.; Brettar, I.; Colwell, R.R.; Pruzzo, C. Climate influence on Vibrio and associated human diseases during the past half-century in the coastal North Atlantic. *Proc. Natl. Acad. Sci.* **2016**, *113*, E5062–E5071. doi: 10.1073/pnas.1609157113
4. Deeb, R., Tufford, D., Scott, G.I., Moore, J.G., Dow, K. Impact of climate change on *Vibrio vulnificus* abundance and exposure risk. *Estuaries Coasts* **2018**, *41*, 2289–2303. doi: 10.1007/s12237-018-0424-5
5. Baker-Austin, C., Oliver, J. D., Alam, M., Ali, A., Waldor, M. K., Qadri, F.; Martinez-Urtaza, J. Vibrio spp. Infections. *Nat Rev Dis Primers* **2018**, *4*, 1–19. doi: 10.1038/ s41572-018-0005-8
6. Legros, D.; Partners of the global task force on cholera control. Global cholera epidemiology: opportunities to reduce the burden of cholera by 2030. *J Infect Dis* **2018**, 218, S137-S140. doi: 10.1093/infdis/jiy486. Erratum in: *J Infect Dis.* **2019**, *219*,509
7. Gaskins, H.R. Immunological aspects of host/microbiota interactions at the intestinal epithelium. *Gastrointest Microbiol* **1997**, *2*, 537–587. doi: 10.1007/ 978-1-4757-0322-1_14

8. Birchenough, G.M., Johansson, M.E., Gustafsson, J.K., Bergström, J.H.; Hansson, G.C. New developments in goblet cell mucus secretion and function. *Mucosal Immunol* **2015**, 8712–8719. doi: 10.1038/mi.2015.32
9. Belley, A., Keller, K., Göttke, M., Chadee, K.; Göettke, M. Intestinal mucins in colonization and host defense against pathogens. *Am J Trop Med Hyg* **1990**, *60*, 10–15. doi: 10.4269/ajtmh.1999.60.10
10. Zheng, D.; Liwinski, T.; Elinav, E. Interaction between microbiota and immunity in health and disease. *Cell Res.* **2020**, *30*, 492-506. doi: 10.1038/s41422-020-0332-7
11. Yardley, J.H.; Bayless, T.M.; Luebbers, E.H.; Halsted, C.H.; Hendrix, T.R. Goblet cell mucus in the small intestine. Findings after net fluid production due to cholera toxin and hypertonic solutions. *Johns Hopkins Med J* **1972**, *131*, 1–10
12. Lencer, W. I.; Reinhart, F. D.; Neutra, M. R. Interaction of cholera toxin with cloned human goblet cells in monolayer culture. *Am J Physiology-Gastrointest Liver Physiol* **1990,** *258*, G96–G102. doi: 10.1152/ ajpgi.1990. 258.1.G96
13. Jones, G. W. The attachment of bacteria to the surfaces of animal cells," in Microbial Interactions (Boston, MA: Springer) 1977, 139–176.
14. WHO. Cholera. *World Health Organization*, **2017** (accessed 05 February 2021).
15. Merritt, E.A.; Sarfaty, S.; Chang T.; Palmer, L.M.; Jobling, M.G.; Holmes, R.K.; Hol, W. G. Surprising leads for a cholera toxin receptor-binding antagonist: crystallographic studies of CTB mutants. *Structure* **1995**, *3*, 561–570. doi: 10.1016/s0969-2126(01)00190-3
16. Clemens, J.D.; Nair, G.B.; Ahmed, T.; Qadri, F.; Holmgren, J. Cholera. *Lancet* **2017**, *390*, 1539-1549. doi: 10.1016/S0140-6736(17)30559-7
17. Levine, M.M.; Pierce, N.F. Immunity and vaccine development. *In* Barua D, and Greenough WB, III (eds), Plenum Press, New York, **1992**, p 285-327.
18. Sánchez, J.; Holmgren, J. Cholera toxin-a foe & a friend. *Indian J Med Res* **2011**, *133*, 153.
19. Taylor, R.K.; Miller, V.L.; Furlong, D.B.; Mekalanos, J.J. Use of phoA gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. *Proc Natl Acad Sci* **1987**, *84*, 2833–2837. doi: 10.1073/pnas.84.9.2833
20. Waldor, M.K.; Colwell, R.; Mekalanos, J.J. The *Vibrio cholerae* O139 serogroup antigen includes an O-antigen capsule and lipopolysaccharide virulence determinants. *Proc. Natl. Acad. Sci.* **1994,** *91*, 11388–11392. doi: 10.1073/pnas.91.24.11388.
21. Sperandio, V.; Giron, J.A.; Silveira, W.D.; Kaper, J.B. The OmpU outer membrane protein, a potential adherence factor of *Vibrio cholerae*. *Infect Immun* **1995**, *63*, 4433–4438. doi: 10.1128/iai.63.11.4433-4438.1995
22. Sengupta, D.K.; Sengupta, T.K.; Ghose, A.C. Major outer membrane proteins of *Vibrio cholerae* and their role in induction of protective immunity through inhibition of intestinal colonization. *Infect Immun* **1992**, *60*, 4848–4855. doi: 10.1128/iai.60.11.4848-4855.1992.
23. Nandi, B.; Nandy, R.K.; Sarkar, A.; Ghose, A.C. Structural features, properties and regulation of the outer-membrane protein W (OmpW) of *Vibrio cholerae*. *Microbiology* **2005**, *151*, 2975–2986. doi: 10.1099/mic.0.27995-0.
24. Qadri, F.; Ali, M.; Chowdhury, F.; Khan, A.I.; Saha, A.; Khan, I.A.; Begum, Y.A.; Bhuiyan, T.R.; Chowdhury, M.I.; Uddin, M.J.; Khan, J.A.M.; Chowdhury, A.I.; Rahman, A.; Siddique, S.A.; Asaduzzaman, M.; Akter, A.; Khan, A.; Ae You, Y.; Siddik, A.U.; Saha, N.C.; Kabir, A.; Riaz, B.K.; Biswas, S.K.; Begum, F.; Unicomb, L.; Luby, S.P.; Cravioto, A.; Clemens, J.D. Feasibility and effectiveness of oral cholera vaccine in an urban endemic setting in Bangladesh: A cluster randomized open-label trial. *Lancet* **2015**, *386*, 1362–1371. doi: 10.1016/S0140-6736(15)61140-0.
25. Bi, Q.; Ferreras, E.; Pezzoli, L.; Legros, D.; Ivers, L.C.; Date, K.; Qadri, F.; Digilio, L.; Sack, D.A.; Ali, M.; Lessler, J.; Luquero, F.J.; Azman, A.S. Protection against cholera from killed whole-cell oral cholera vaccines: a systematic review and meta-analysis. Oral cholera vaccine working group of the global task force on cholera control. *Lancet Infect. Dis.* **2017**, *17*, 1080-1088. doi: 10.1016/S1473-3099(17)30359-6.
26. Peak, C.M.; Reilly, A.L.; Azman, A.S.; Buckee, C.O. Prolonging herd immunity to cholera via vaccination: Accounting for human mobility and waning vaccine effects. *PLoS Negl. Trop. Dis*. **2018**, 12, e0006257. doi: 10.1371/journal.pntd.0006257.

27. Royal, J.M.; Reeves, M.A.; Matoba, N. Repeated oral administration of a KDEL-tagged recombinant cholera toxin B subunit effectively mitigates DSS colitis despite a robust immunogenic response. *Toxins* (Basel) **2019**, *11*, 678. doi: 10.3390/toxins11120678
28. Kabir, S. Critical analysis of compositions and protective efficacies of oral killed cholera vaccines. *Clin. Vaccine Immunol*. **2014**, *21*, 1195-1205. doi: 10.1128/CVI.00378-14
29. Chen, W.H.; Cohen, M.B.; Kirkpatrick, B.D.; Brady, R.C.; Galloway, D.; Gurwith, M.; Hall, R.H.; Kessler, R.A.; Lock, M.; Haney, D.; Lyon, C.E.; Pasetti, M.F.; Simon, J.K.; Szabo, F.; Tennant, S.; Levine, M.M. Single-dose live oral cholera vaccine CVD 103-HgR protects against human experimental infection with *Vibrio cholerae* El Tor. *Clin Infect Dis* **2016,** *62*, 1329-1335. doi:10.1093/cid/ciw145.
30. Holmgren, J.J. Modern history of cholera vaccines and the pivotal role of icddr. *Infect Dis* **2021,** *224*, S742-S748. doi: 10.1093/infdis/jiab423.
31. Wierzba, T.F. Oral cholera vaccines and their impact on the global burden of disease. *Hum Vaccin Immunother* **2019**, *15*, 1294-1301. doi: 10.1080/21645515.2018.1504155
32. Qadri, F.; Chowdhury, M.I.; Faruque, S.M.; Salam, M.A.; Ahmed, T.; Begum, Y.A.; Saha, A.; Al Tarique, A.; Seidlein, L.V.; Park, E.; Killeen, K.P.; Mekalanos, J.J.; Clemens, J.D.; Sack, D.A. PXV Study Group, Peru-15, a live attenuated oral cholera vaccine, is safe and immunogenic in Bangladeshi toddlers and infants. *Vaccine* **2007**, *25*, 231-238. doi: 10.1016/j.vaccine.2006.08.031
33. Calain, P.; Chaine, J.P.; Johnson, E.; Hawley, M.L.; O'Leary, M.J.; Oshitani, H.; Chaignat, C.L. Can oral cholera vaccination play a role in controlling a cholera outbreak? *Vaccine* **2004**, *22*, 2444–2451. doi: 10.1016/j.vaccine.2003.11.070.
34. Song, K.R.; Lim, J.K.; Park, S.E.; Saluja, T.; Cho, S.I.; Wartel, T.A.; Lynch, J.; Oral cholera vaccine efficacy and effectiveness. *Vaccines* (Basel) **2021**, *9*,1482. doi: 10.3390/vaccines9121482
35. Khatib, A.M.; Ali, M.; von Seidlein, L.; Kim, D.R.; Hashim, R.; Reyburn, R.; Ley, B.; Thriemer, K.; Enwere, G.; Hutubessy, R.; Aguado, M.T.; Kieny, M.P.; Lopez, A.L.; Wierzba, T.F.; Ali, S.M.; Saleh, A.A.; Mukhopadhyay, A.K.; Clemens, J.; Jiddawi, M.S.; Deen, J. Effectiveness of an oral cholera vaccine in Zanzibar: findings from a mass vaccination campaign and observational cohort study. *Lancet Infect Dis* **2012**, *1*, 837-844. doi: 10.1016/S1473-3099(12)70196-2
36. Ali, M.; Sur, D.; You, Y.A.; Kanungo, S.; Sah, B.; Manna, B.; Puri, M.; Wierzba, T.F.; Donner, A.; Nair, G.B.; Bhattacharya, S.K.; Dhingra, M.S.; Deen, J.L.; Lopez, A.L.; Clemens, J. Herd protection by a bivalent killed whole-cell oral cholera vaccine in the slums of Kolkata, India. *Clin Infect Dis* **2013**, *56*, 1123-31. doi: 10.1093/cid/cit009
37. De Groot, A.S.; Moise, L.; McMurry, J.A.; Martin, W. Epitope-based immunome-derived vaccines: a strategy for improved design and safety. *Clin Appl Immun*. Springer, **2009**; pp. 39–69.
38. Winkler, D.F.H. SPOT Synthesis: The solid-phase peptide synthesis on planar surfaces. *Methods Mol Biol* **2020,** *2103*, 151–173.
39. Pastor, M.; Pedraz, J.; Esquisabel, A. The state-of-the-art of approved and under-development cholera vaccines. *Vaccine* **2013**, *31*, 4069–4078. doi: 10.1016/j.vaccine.2013.06.096.
40. Chiappelli, F.; Khakshooy, A.; Balenton, N. Clinical immunology of Cholera - Current trends and directions for future advancement. *Bioinformation* **2017**,*13*, 352-355. doi: 10.6026/97320630013352
41. Holmgren, J. An update on cholera immunity and current and future cholera vaccines. *Trop Med Infect Dis* **2021**, *6*, 64. doi: 10.3390/tropicalmed6020064
42. Pinkhasov, J.; Alvarez, M.L.; Pathangey, L.B.; Tinder, T.L.; Mason, H.S.; Walmsley, A.M.; Gendler, S.J.; Mukherjee, P. Analysis of a cholera toxin B subunit (CTB) and human mucin 1 (MUC1) conjugate protein in a MUC1-tolerant mouse model. *Cancer Immunol Immunother* **2010,** *59*, 1801–1811. doi: 10.1007/s00262-010-0906-1
43. Mekalanos, J.; Collier, R.; Romig, W. Enzymic activity of cholera toxin. II. Relationships to proteolytic processing, disulfide bond reduction, and subunit composition. *J Biol Chem* **1979**, *254*, 5855–5861. doi:10.1016/S0021-9258(18)50491-9
44. Sánchez, J.; Holmgren, J. Cholera toxin structure, gene regulation, and pathophysiological and immunological aspects. *Cell. Mol. Life Sci* **2008**, *65*, 1347–1360. doi: 10.1007/s00018-008-7496-5
45. Bharati, K.; Ganguly, N.K. Cholera toxin: A paradigm of a multifunctional protein. *Indian J Med Res* **2011,** *133*, 179.

46. Sikora, A.E. Proteins secreted via the type II secretion system: Smart strategies of Vibrio cholerae to maintain fitness in different ecological niches. *PLoS Pathog* **2013**, *9*, e1003126. doi: 10.1371/journal.ppat.1003126.
47. Mayo, S.; Royo, F.; Hau, J. Correlation between adjuvanticity and immunogenicity of cholera toxin B subunit in orally immunized young chickens. *APMIS* **2005,** *113*, 284-287. doi: 10.1111/j.1600-0463.2005.apm_07.x
48. Kim, M.S.; Yi, E.J., Kim, Y.I.; Kim, S.H.; Jung, Y.S.; Kim, S.R.; Iwawaki, T.; Ko, H.J.; Chang, S.Y. ERdj5 in innate immune cells is a crucial factor for the mucosal adjuvanticity of cholera toxin. *Front Immunol* **2019,** *10,* 1249. doi: 10.3389/fimmu.2019.01249
49. Hou, J.; Liu, Y.; Tao, R.; Hsi, J.; Shao, Y.; Wang, H. Cholera Toxin B subunit acts as a potent systemic adjuvant for HIV-1 DNA vaccination intramuscularly in mice. *Hum Vaccines Immunother* **2014**, *10,* 1274-1283. doi: 10.4161/hv.28371. doi: 10.4161/hv.28371.
50. Price, G.A.; Holmes, R.K. Evaluation of TcpF-A2-CTB chimera and evidence of additive protective efficacy of immunizing with TcpF and CTB in the suckling mouse model of cholera. *PLoS One* **2012**, *7*, e42434. doi: 10.1371/journal.pone.0042434
51. Zareitaher, T.; Sadat, T.; Seyed, A.S.; Gargari, L.M. Immunogenic efficacy of DNA and protein-based vaccine from a chimeric gene consisting OmpW, TcpA and CtxB, of *Vibrio cholerae*. *Immunobiology* **2022,** *227,* 152190. doi:10.1016/j.imbio.2022.152190
52. Jacob, C.O.; Harari, I.; Arnon, R.; Sela, M. Antibodies to cholera toxin synthetic peptides of increasing size and their reactivity with related toxins. *Vaccine* **1986**, *4,* 95-98. doi: 10.1016/0264-410x(86)90045-9
53. Jacob, C.O.; Sela, M.; Arnon, R. Antibodies against synthetic peptides of the B subunit of cholera toxin: cross-reaction and neutralization of the toxin. *Proc Natl Acad Sci U S A.* **1983,** *80,* 7611-7615. doi: 10.1073/pnas.80.24.7611
54. Sela, M.; Arnon, R.; Jacob, C.O. Synthetic peptides with antigenic specificity for bacterial toxins. *Ciba Found Symp* **1986**,*119*,184-99. doi: 10.1002/9780470513286.ch11
55. Provenzano, D.; Kovác, P.; Wade, W.F. The ABCS (antibody, B cells, and carbohydrate epitopes) of cholera immunity: considerations for an improved vaccine. *Microbiol Immunol* **2006**, 50, 899–927. doi: 10.1111/j.1348-0421.2006.tb03866.x.
56. Ali, M.; Lopez, A.L.; You, Y.A.; Kim, Y.E.; Sah, B.; Maskery, B.; Clemens, J. The global burden of cholera. *Bull WHO* **2012**, *90*, 209-218. doi: 10.2471/BLT.11.093427
57. Jeon, S.; Kelly, M.; Yun, J.; Lee, B.; Park, M.; Whang, Y.; Lee, C.; Halvorsen, Y.D.; Verma, S.; Charles, R.C.; Harris, J.B.; Calderwood, S.B.; Leung, D.T.; Bhuiyan, T.R.; Qadri, F.; Kamruzzaman, M.; Cho, S.; Vann, W.F.; Xu, P.; Kováč, P.; Ganapathy, R.; Lynch, J.; Ryan, E.T. Scalable production and immunogenicity of a cholera conjugate vaccine. *Vaccine* **2021**, *39*, 6936-6946. doi: 10.1016/j.vaccine.2021.10.005
58. Glenny, A.T.; Hopkins, B.E. Diphtheria toxoid as an immunizing agent. *Br J Exp Pathol* **1923,** *4*, 283
59. Napoleão-Pêgo, P.; Carneiro, F.R.G.; Durans, A.M.; Gomes, L.R.; Morel, C.M.; Provance-Jr, D.W.; De-Simone, S.G. Performance assessment of multi-epitope chimeric antigen for serodiagnosis of acute phase of Mayaro fever. *Scient Rep* **2021**, *11,* 15374. 10.1038/s41598-021-94817-x
60. Colli, A.; Fraquelli, M.; Casazza, G.; Conte, D.; Nikolova, D.; Duca, P.; Thorlund, K.; Gluud, C. The Architecture of diagnostic research: From bench to bedside-research guidelines using liver stiffness as an example. *Hepatology* **2014**, *60,* 408–418.
61. Gray, G.; Buchbinder, S.; Duerr, A. Overview of STEP and Phambili trial results: two-phase IIb test-of-concept studies investigating the efficacy of MRK adenovirus type 5 gag/pol/nef subtype B HIV vaccine. *Curr Op HIV AIDS* 2010, *5,* 357-361. doi:10.1097/COH.0b013e32833d2d2b
62. De-Simone, S.G.; Gomes, L.R.; Napoleão-Pêgo, P.; Lechuga, G.C.; Pina, J.C.; Silva, F.R. Identification of linear B epitopes liable for the protective immunity of diphtheria toxin. *Vaccines* (Basel) **2021,** 9, 313. doi:10.3390/vaccines9040313
63. Silva, F.R.; Napoleão-Pêgo, P.; De-Simone, S.G**.** Identification of linear B epitopes of pertactin of *Bordetella pertussis* induced by immunization with whole and acellular vaccine. *Vaccine* **2014**, *32 32*, 6251-6258**.** doi: 10.1016/j.vaccine.2014.09.019
64. De-Simone, S.; Souza, A.L.A.; Melgarejo, A.R.; Aguiar, A.S.; Provance-Jr, D.W. Development of elisa assay to detect specific human IgE anti-therapeutic horse sera. *TOXICON* **2017,** *138,* 37-42. doi: 10.1016/j.toxicon.2017.08.012.
65. Bottino, C.G.; Gomes, L.P.; Zauza, P.L.; Pereira, J.B.; Coura, J.R.; De-Simone, S.G. Chagas disease-specific antigens: characterization of CRA/FRA epitopes by synthetic peptide mapping and evaluation on ELISA-peptide assay. *BMC Infect. Dis.* **2013**, *13*, 568-578. doi: 10.1186/1471-2334-13-568

66. Roy, A.; Kucukural, A.; Zhang, Y. I-TASSER: a unified platform for automated *protein structure and function prediction. Nat Protoc* **2010**, *5*, 725–738. doi: 10.1038/nprot.2010.5

67. Jumper, J.; Evansm, R.; Pritzel, A.; Green, T.; Figurnov, M.; Ronneberger, O.; Tunyasuvunakool, K.; Bates, R.; Žídek, A.; Potapenko, A.; Bridgland, A.; Meyer, C.; Kohl, S.A.A.; Ballard, A.J.; Cowie, A.; Romera-Paredes, B.; Nikolov, S.; Jain, R.; Adler, J.; Back, T.; Petersen, S.; Reiman, D.; Clancy, E.; Zielinski, M.; Steinegger, M.; Pacholska, M.; Berghammer, T.; Bodenstein, S.; Silver, D.; Vinyals, O.; Senior, A.W.; Kavukcuoglu, K.; Kohli, P.; Hassabis, D. Highly accurate protein structure prediction with AlphaFold. *Nature* **2021**, *596*, 583-589. doi: 10.1038/s41586-021-03819-2.