Development of an enzyme-linked immunosorbent assay using recombinant protein antigen for the diagnosis of Chikungunya virus

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ABSTRACT

We describe here the development of an in-house enzyme linked immunosorbent assay (ELISA) for the diagnostic of Chikungunya virus (CHIKV) infections using a recombinant protein from CHIKV. The recombinant protein gene was designed based on 154 sequences and we used computational methods to predict its structure and antigenic potential. To confirm predictions, the gene coding for the recombinant CHIKV protein (rCHIKVp) was synthesized and expressed in prokaryotic system. Subsequently, the protein was purified by affinity chromatography and used as antigen in an indirect ELISA. We present data regarding the optimization of the recombinant antigen production and preparation of the ELISA to detect IgG against CHIKV in human sera.

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1. Data

We design a synthetic gene coding for a recombinant Chikungunya virus protein (rCHIKp) to be used as an antigen in serologic assays. In silico analysis revealed that rCHIKp secondary structure has predominant coils and identified B lymphocyte epitopes in regions of structural disorder, which are advantageous for antigenic recognition. The overall antigenic prediction score was 0.53 (which suggests a probable efficient antigen). The gene was expressed in E. coli cells and purified by affinity chromatography. Coomassie Brilliant Blue staining showed the presence of rCHIKp with the predicted molecular mass (~42 kDa). Next, we developed an In House Enzyme-Linked Immunosorbent Assay (ELISA) for the detection of immunoglobulin G (IgG) anti-CHIKV using the rCHIKp as the antigenic solid phase. The rCHIKVp-based ELISA showed a sensitivity value of 95% and specificity of 96% (Table 1). No cross-reactivity was found against sera from Zika- (ZIKV) and Dengue- (DENV) positive patients (Fig. 1). The developed ELISA was used in the assessment of human patients suspected of arboviral infections in Minas Gerais State, Brazil [1].

2. Experimental design, materials, and methods

2.1. Design of the antigen gene

CHIKV genome sequences deposited at GenBank were aligned using MEGA7 software [2]. The selection criteria were: complete annotation of the genome and absence of indefinite nucleotides in the sequence. Brazilian (n = 42) and foreign samples belonging to the genotypes circulating in Brazil were used. Sequences belonged to the Asian genotype (n = 112) and East-Central South African (ECSA)
genotype (n = 274). A consensus sequence was generated for each group (Group 1: Brazilian samples, Group 2: Asian genotype samples and Group 3: ECSA genotype samples). These consensual sequences were, then, compared to each other to generate a unique nucleotide sequence.

The gene sequence was codon-optimized for expression in *E. coli* by OptimumGene™ - Codon Optimization software (Genescript). Transmembrane and hydrophobicity analyzes were performed using TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) [3] and Protscale -Expasy (http://web.expasy.org/Protscale/) [4]. From these predictions, a cut-off point for the generation of the protein without its transmembrane domain was determined. The nucleotide sequence of the truncated protein-coding gene was commercially synthesized and subcloned into the pET-21 expression vector, which included a histidine tag to the construction.

2.2. In silico analysis

To evaluate the potential of our rCHIKVp as a diagnostic tool, the protein’s predicted amino acid sequence was submitted to BepiPred (http://www.cbs.dtu.dk/services/BepiPred/) for the prediction of linear B cell epitopes [5] and IUPred (http://iupred.enzim.hu/), for the prediction of intrinsic structural disorder, indicating absence of secondary structure and, consequently, regions of possible interaction with antibodies [6]. The antigenicity of the designed protein was evaluated by the Vaxijen V2.0 online server (http://www.ddphpharmfac.net/vaxijen/Vaxijen/Vaxijen.html) [7].

![ROC curve](image)

**Fig. 1.** Seroreactivity of the rCHIKVp using the developed indirect ELISA against Chikungunya-positive sera (CHIKV+), virus-negative sera (CHIKV-/DENV-/ZIKV-). Potential cross reactivity with sera samples that were positive for co-circulating arboviruses were also tested: Dengue positive (DENV+) and Zika positive (ZIKV+) sera. A cut-off value of 0.279 was obtained, according to the Roc curve (inset).

| Table 1 | Relative sensitivity and specificity of the rCHIKVp-based in house-Anti-CHIKV ELISA in comparison to a commercial kit (Chikungunya IgG ELISA Euroimmun, Germany). |
|---------|-----------------------------------------------------------------------------------|
| In house-Anti-CHIKV ELISA | No. of pos. | No. of neg. | Parameters |
| Commercial kit | Pos. (n = 71) | 68 | 3 | Sensitivity: 96% |
| | Neg. (n = 76) | 74 | 2 | Specificity: 97% |
2.3. Recombinant protein production

The pET-21 vector containing the gene of interest was used to transform E. coli BL21(DE3) strain by heat shock. Plasmid-positive clones were induced by IPTG and expression of the recombinant protein was optimized and analyzed by SDS-PAGE. The antigen was purified by affinity chromatography using nickel columns in an AKTAprime plus system (GE Healthcare, USA).

2.4. In house Anti-Chikungunya virus ELISA

The seroreactivity of the rCHIKVp was evaluated using a panel of sera samples from human patients, CHIKV seropositive or not, by an in-house indirect IgG ELISA. Strips of polystyrene microwells (Costar, USA) were coated overnight at 4 °C with 100 µl per well of rCHIKVp diluted in carbonate buffer (0.05 M, pH 9.6). Wells were washed five times with phosphate-buffered saline (PBS) containing 0.1% Tween20 (PBS-T) and blocked with 1% bovine serum albumin (BSA, SIGMA,USA) for 2 h at 25 °C. Then 100 µl of serum diluted in PBS-T was added and incubated for 1 hour at 37 °C. After five washes, we added 100 µl of horseradish peroxidase (HRP)-conjugated anti-human IgG goat immunoglobulin (Fapon, China), diluted at 1:100,000 in stabilizing diluent (MOSS, USA). Plates were incubated for 30 min at 37 °C, washed five times, and 100 µl of TMB (3,3′, 5,5′-tetramethylbenzidine, MOSS, USA) were added and incubated for further 15 min. Then, 100 µl of H2SO4, 0.5 M solution was added to the wells to stop the reaction. The plates were analyzed in a Microplate Reader at an optical density (O.D.) of 450 nm.

The optimal concentration of the recombinant rCHIKVp per plate well was determined based on a clear distinction of anti-CHIKV antibodies using positive and negative samples. We tested a range of 25–800 ng/well of rCHIKVp and sera dilutions ranging from 1:25 to 1:3200 (Fig. 2). The cut-off value was determined by ROC curve analysis, and an index (I) of each absorbance value of the patient sample over the value of cut-off was calculated, according to the equation:

\[ I = \frac{a}{c}, \]

where \( a \) is the absorbance of the patient sample and \( c \) is the cut-off value (0.279).

The data is classified as follows:

- \( I < 0.9 \): negative
- \( 0.9 \leq I < 1.1 \): borderline
- \( I \geq 1.1 \): positive

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Conflict of interest

None of the authors have any conflict of interests.

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